Pseudomonas Keratitis
The Role of an Uncharacterized Exoprotein, Protease IV, in Corneal Virulence

Richard J. O'Callaghan,*† Lee S. Engel,* Jeffery A. Hobden,*† Michelle C. Callegan,* Linda C. Green,*† and James M. Hill*†

Purpose. The role of exoproteins in the pathogenesis of Pseudomonas aeruginosa keratitis was investigated in three animal models by assessing the relationship between corneal virulence and the activities of exotoxin A, elastase, alkaline protease, and an uncharacterized protease, protease IV.

Methods. The four Pseudomonas strains tested included a prototype strain (ATCC 27853) producing exotoxin A, elastase, and alkaline protease; a parent strain (PA103) producing only exotoxin A and protease IV; a mutant (PA103-29) producing only protease IV; and a mutant (PA103-AP1) producing exotoxin A and having only approximately 5% of the protease IV activity of its parent. Corneal virulence was evaluated in the mouse scratch, rabbit scratch, and rabbit intrastromal models in terms of clinical signs (slit lamp examination, slit lamp examination), and viable bacteria.

Results. Protease IV, the only protease produced by PA103 and PA103-29, was found to produce a unique band on zymograms (120 kDa) and to react distinctively with a synthetic substrate. Evidence for the role of protease IV in corneal virulence included two findings: PA103-29, which produced protease IV but not the other exoproteins, caused infections that were as severe as those caused by the prototype strain (ATCC 27853) in all three models (P > 0.24); and PA103-AP1, the strain deficient in 95% of the parent protease IV activity, mediated infections characterized by slit lamp examination scores significantly lower than those of infections caused by the parent (PA103) or the prototype strain (ATCC 27853) in the rabbit and mouse scratch models (P < 0.02).

Conclusions. Protease IV was found to be a novel Pseudomonas protease contributing to corneal virulence in rabbits and mice when infections were initiated at the corneal surface. Furthermore, production of protease IV in low quantities was sufficient for virulence when the topical stages of keratitis were bypassed by an intrastromal injection of Pseudomonas. Invest Ophthalmol Vis Sci. 1996; 37:534–543.

Bacterial products released during Pseudomonas aeruginosa keratitis may damage the cornea directly and induce host reactions that contribute to corneal scarring and eventual reduction in visual acuity.1,2 Inflammation contributes to corneal damage, especially the migration of polymorphonuclear leukocytes into the cornea and the subsequent release from these cells of damaging lysosomal enzymes and oxidative molecules.3,4 Antibiotic and antiinflammatory therapy can reduce, but not prevent, corneal damage and scarring.5,6 The future development of therapies capable of preventing corneal damage is dependent on the identification and inhibition of those bacterial products that directly damage the host and/or elicit damaging inflammatory reactions.

The role of specific Pseudomonas exoproteins in corneal damage has been difficult to determine. Application of pure exoproteins, such as exotoxin A, elastase, and alkaline protease, to scarified corneas demonstrated the potential of several such molecules to damage the cornea.7–20 These findings show that Pseu-
Pseudomonas products can induce damage, but they fail to demonstrate that the exoproteins are produced in quantities sufficient to induce tissue damage during keratitis. Kernacki et al demonstrated the production of alkaline protease in corneas of infected mice, evidencing a role for this enzyme in corneal virulence. Further studies are needed to clarify the relationship between protease production and corneal virulence. Previous studies of this relationship have focused on PA103 because this Pseudomonas strain produces relatively few exoproteins. However, there are discrepancies regarding the protease production of this strain. PA103 has been described by some investigators as protease free and by others as a producer of alkaline protease. Howe and Iglewski attributed the protease activity of PA103 to alkaline protease and reported that a mutant deficient in most of this protease activity was less virulent than its proteolytic parent in a mouse model of keratitis. Guzzo et al and Jaffer-Bandjee et al also detected protease activity in PA103 but could not attribute this activity to any known Pseudomonas protease, including elastase and alkaline protease. Gambello and Iglewski reported the absence of a transcriptional activator needed for expression of elastase and alkaline protease in PA103. Toder and Gambello could not detect mRNA specific for elastase or alkaline protease in PA103 and ascribed the protease activity of this organism to an uncharacterized enzyme, designated protease IV.

The current study correlates the relationship of Pseudomonas corneal virulence with exoprotein production and analyzes an uncharacterized protease of PA103. Four strains, selected for their reported exoprotein differences, were characterized as to their exoprotein production and their virulence in three animal models of Pseudomonas keratitis. Our results demonstrate the existence of Pseudomonas protease IV and correlate the activity of this protease with significant ocular damage in Pseudomonas keratitis when infections are initiated at the corneal surface. Findings also indicate that protease IV production in small quantities is sufficient to yield extensive corneal damage when keratitis is initiated by an intrastromal inoculation. Furthermore, the results describe the electrophoretic mobility (i.e., molecular weight) and substrate specificity of this previously uncharacterized protease.

METHODS

Bacterial Strains

Strain ATCC 27853 (American Type Culture Collection, Rockville, MD) has been used as a standard for antibiotic susceptibility testing and for studies of Pseudomonas keratitis. Strains PA103, PA103-29, and PA103-AP1 were kindly provided by Dr. Barbara H. Iglewski, University of Rochester (Rochester, NY). Strain PA103, originally described by Liu, is reported to be deficient in elastase and alkaline protease. PA103-29 has been reported to be deficient in elastase and alkaline protease as well as in exotoxin A production. PA103-AP1 is a mutant of PA103 that is reported to express low protease activity.

Cultural Characterization of Strains

All strains were characterized biochemically by inoculation of Oxi/Ferm tubes (Roche Diagnostic Systems, Nutley, NJ). Strains also were evaluated for pyocyanin and fluorescein production on Pseudomonas Agar(s) P and F (Difco Laboratories, Detroit, MI), and hemolysin production was evaluated on 5% sheep blood agar (Adams Scientific, Sulfur, LA). The Oxi/Ferm tubes and Pseudomonas agar plates were examined for positive reactions after incubation at 37°C for 24 and 48 hours. Strains PA103, PA103-29, and PA103-AP1 produced nitrogen gas and pyocyanin. Strain ATCC 27853 did not produce nitrogen gas but did produce both fluorescein and pyocyanin. All strains were hemolytic on sheep blood agar. The growth rate of all strains in tryptic soy broth (TSB, Difco) at 37°C was essentially identical throughout log phase as measured by the change in optical density at 650 nm.

Exoprotein Assays

Exotoxin A activity was measured in supernatants of cultures grown in TSB that was dialyzed, chelated, and supplemented with 0.11 M glycerol and 0.05 M monosodium glutamate (TSB-DC). Exotoxin A production in culture supernatants was quantified using the ADP-ribosylation assay described by Chung and Collier. Elastase production was determined by the proteolysis of elastin on tryptic soy agar (TSA, Difco) plates containing 2% elastin congo-red (Sigma Chemical, St. Louis, MO). The caseinase activity of each strain was determined by observing the zone of proteolysis around isolated colonies on skim milk agar. To quantitate the relative caseinase activity of each strain, cultures were grown to log phase in brain heart infusion broth (BHI, Difco) that was dialyzed, chelated, and mixed with cetrimide (100 µg/ml, Sigma). These bacteria were mixed with an equal volume of the same medium containing 1.5% agar. The bacteria in molten agar (50°C) were plated into wells (6-mm diameter) of a skim milk agar plate. The plates were incubated at 37°C for the development of zones of proteolysis. Diameters of these zones were compared as a means of quantitating the relative caseinase activity.

All strains were assayed for production of elastase, alkaline protease, and exotoxin A using specific polyclonal antisera to these exoproteins. Rabbit antiserum to P. aeruginosa elastase (Nagase Biochemicals, Fukushima, Japan), alkaline protease (Nagase),
TABLE 1. Exoproteins of *Pseudomonas aeruginosa* Strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Exotoxin A*</th>
<th>Elastase†</th>
<th>Caseinase‡</th>
<th>Alkaline Protease§</th>
<th>Protease IV¶</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATCC 27853</td>
<td>Positive</td>
<td>Positive</td>
<td>++++</td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>PA103</td>
<td>Positive</td>
<td>Negative</td>
<td>++</td>
<td>Negative</td>
<td>Intermediate</td>
</tr>
<tr>
<td>PA103-29</td>
<td>Negative</td>
<td>Negative</td>
<td>++++</td>
<td>Negative</td>
<td>Maximal</td>
</tr>
<tr>
<td>PA103-AP1</td>
<td>Positive</td>
<td>Negative</td>
<td>+/−</td>
<td>Negative</td>
<td>Minimal††</td>
</tr>
</tbody>
</table>

* Exotoxin A production was determined by a ribosylation assay and by precipitation in agar with antibody to exotoxin A; both assays are described in the text. Strains ATCC 27853, PA103, and PA103-AP1 had ribosylation activities of ≥20-fold that of background, whereas strain PA103-29 had a ribosylation activity equivalent to background.
† Elastase production was determined by digestion of elastin around isolated colonies on skim milk agar after 24 hours of incubation of 37°C and confirmed by inoculating 10⁶ CFU log phase bacteria into wells on a skim milk agar (Fig. 3).
‡ Caseinase activity was determined by precipitation in agar with antibody to alkaline protease, as described in the text, and by zymography as shown in Figure 1.
§ Alkaline protease production was determined by precipitation in agar with antibody to alkaline protease, as described in the text, and by zymography as shown in Figure 1 and as has been reported by Toder and Gambello and Gambello.
¶ Protease IV activity was determined by zymography using gelatin as substrate as shown in Figure 1 and by a colorimetric reaction with a synthetic substrate (Fig. 3). PA103-AP1 produced approximately 5% of the protease IV activity of its parent, PA103.

** PA103-AP1 produced minimal protease activity as evidenced by the hydrolysis of a synthetic substrate (Fig. 3) and as reported by Ohman et al. **

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and exotoxin A (Berna Products, Coral Gables, FL) were produced as previously described.

Bacteria grown on TSB-DC medium solidified with 1.5% noble agar (Difco) were used for detection of exotoxin A and elastase by immunoprecipitation. For detection of alkaline protease by immunoprecipitation, bacteria were grown on dialyzed brain heart infusion medium (BHI-D). BHI-D was prepared by dialyzing brain heart infusion broth (Difco; prepared 10X) against 4.1 of distilled water and adding 1.5% noble agar to the material outside of the dialysis bag before sterilization. TSB-DC and BHI-D plates were streaked in a line (6 cm long) using an inoculum from an overnight broth culture and incubated in a humidified chamber at 34°C for 24 to 36 hours. After a line of growth appeared, wells (5-mm diameter) were aseptically cut in the agar 5 mm from the streak of bacterial growth and filled with antisera. A second well was cut 2 mm from the first well and filled with antigen to serve as positive control. Plates were incubated at 34°C in a humidified chamber until precipitin lines were clearly visible.

The protease activity produced by each strain was assessed by zymography, as previously described.

The supernatant of log phase cultures grown in TSB-DC medium was concentrated approximately 500-fold by ultrafiltration using a 30,000-dalton porosity filter. The supernatants were subjected to zymography using 7.5% sodium dodecyl sulfate polyacrylamide gels with 5% Coomassie blue (0.5% in acetic acid:isopropyl alcohol:water [1:3:6]), and destained in water. Purified *Pseudomonas* alkaline protease (50 kDa), elastase (163 kDa), beta-galactosidase (116 kDa), phosphorylase B (97.4 kDa), fructose-6-phosphate kinase (84 kDa), and bovine serum albumin (66 kDa) were included in the zymograms as standards.

The protease IV activity of each strain was quantitated after the concentration of TSB-DC culture supernatant by ultrafiltration. The protein content was determined using a bicinchoninic acid test kit (Sigma). Supernatants of all strains were adjusted to contain 1.2 mg/ml of total protein, and then 10 μl of each supernatant was mixed in a microtiter plate with 20 μl of Chromozym PL substrate (2 mg/ml, Boehringer Mannheim, Indianapolis, IN) and 90 μl of phosphate buffer. The reaction mixture was immediately placed into a microtiter plate reader (model MR5000; Dynatec Laboratories, Chantilly, VA), the optical density at 410 nm was read at 2-minute intervals for 30 minutes, and the change in optical density (ΔA) per minute was determined. One unit of protease IV activity was defined as:

\[
\text{ΔA/min} \times \frac{\text{Total Assay Volume}}{\text{Sample Volume} \times E \times \text{Light Path (cm)}}
\]

where the total volume of the assay was 120 μl, the sample volume was 10 μl, the extinction coefficient (E) of the product (para-nitroaniline) at 410 nm was 9.75, and the light path was 0.53 cm.

**Rabbit Intrastromal Injection Model of Keratitis**

New Zealand white rabbits (1 to 3 kg) were anesthetized with a 1:5 mixture of xylazine (100 mg/ml; Miles, Shawnee, KS) and ketamine hydrochloride (100 mg/ml; Aveco, Fort Dodge, IA). Topical corneal anesthesia was achieved with proparacaine hydrochloride...
Pseudomonas Protease IV and Corneal Virulence

Female ND4 Swiss Webster mice (18 to 22 g; Harlan Sprague-Dawley, Indianapolis, IN) were anesthetized with an intraperitoneal injection (0.15 ml per mouse) of a 1:10 dilution in water of a mixture (1:10 vol/vol) of xylazine and ketamine (100 mg/ml solutions). Corneas were scarified centrally with a 27-gauge needle by making three parallel lesions approximately 1 mm in length and 0.5 mm apart. P. aeruginosa strains were grown in TSB at 34°C to an OD_{660} of 0.8. Scarified corneas were inoculated with 5 μl of culture (~10^7 CFU). The eyes were then gently massaged with the lids closed. A total of 16 eyes were infected per strain of P. aeruginosa.

Mouse Topical Inoculation (Scratch) Model of Keratitis

Female ND4 Swiss Webster mice (18 to 22 g; Harlan Sprague-Dawley, Indianapolis, IN) were anesthetized with an intraperitoneal injection (0.15 ml per mouse) of a 1:10 dilution in water of a mixture (1:10 vol/vol) of xylazine and ketamine (100 mg/ml solutions). Corneas were scarified centrally with a 27-gauge needle by making three parallel lesions approximately 1 mm in length and 0.5 mm apart. P. aeruginosa strains were grown in TSB at 34°C to an OD_{660} of 0.8. Scarified corneas were inoculated with 5 μl of culture (~10^7 CFU). The eyes were then gently massaged with the lids closed. A total of 16 eyes were infected per strain of P. aeruginosa.

Evaluation of Ocular Virulence

The inflammatory changes in rabbit eyes were assessed by two or more observers in a masked fashion using a Topcon SL-5D slit lamp biomicroscope (Kogaku Kikai K.K., Tokyo, Japan). For the rabbit intrastromal model, seven parameters (conjunctival injection, conjunctival chemosis, iritis, fibrin in the anterior chamber, hypopyon, stromal infiltrate, and stromal edema) were scored on a scale of 0 (absent) to 4 (severe), and the scores from each of the parameters were added to produce a total SLE score. The scoring system for the rabbit scratch model has been described by Brockman et al. The scoring system for mouse corneas has been described by Gerke and Magliocco.

Rabbits were sacrificed and corneas were harvested for enumeration of viable bacteria as previously described. Intact mouse eyes were harvested, placed into 0.5 ml sterile phosphate-buffered saline at 4°C, and homogenized with an Ultra Turrax tissue mixer (Tekmar, Cincinnati, OH). Aliquots (0.1 ml) of homogenate were plated on TSA or Pseudomonas Isolation Agar (Difco). Plates were incubated at 37°C for 24 to 48 hours to determine the presence of viable bacteria.

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Rabbit Topical Inoculation (Scratch) Model of Keratitis

Contact lenses (Acuvue, Johnson and Johnson, Jacksonville, FL) were incubated with 1.0% bovine serum albumin (Sigma) at 4°C for 18 hours. The lenses were then incubated with 10^7 CFU of log phase P. aeruginosa, as previously described. Ten eyes were infected per strain of P. aeruginosa. All animals were maintained according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Mouse Topical Inoculation (Scratch) Model of Keratitis

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RESULTS

Exoprotein Production

All strains were analyzed for activity or production of specific exoproteins by biochemical assays, zymography, and immunoprecipitation (Table 1; Figs. 1, 2). The prototype strain, ATCC 27853, produced exotoxin A, elastase, and a small amount of alkaline protease. Strain PA103 produced a protease, designated protease IV, that had a molecular weight of approximately 120 kDa (Fig. 1). A protease of this molecular size has not been reported previously for *Pseudomonas aeruginosa*. Strain PA103-29 produced the 120-kDa band for protease IV and a diffuse region of protease activity near the top of the zymogram (Fig. 1), and it was deficient in exotoxin A, elastase, and alkaline protease (Table 1). Strain PA103-AP1 produced exotoxin A but not elastase or alkaline protease (Table 1). This strain expressed reduced amounts of protease activity as evidenced by its failure to produce proteolytic zones when grown on skim milk agar (Fig. 2), to produce a band of precipitation with antisera specific for alkaline protease or elastase (Table 1), or to demonstrate a proteolytic band on a zymogram (Fig. 1).

Concentrated culture supernatants of all four strains were adjusted to contain 1.2 mg/ml of protein and then reacted with Chromozym PL. The reactivity of each supernatant with the synthetic protease substrate, expressed in enzyme units per milligram total protein, was determined over a 30-minute interval (Fig. 3). The protease activities of strains PA103, PA103-29, and PA103-AP1, as measured by Chromozym PL cleavage, were proportional to the relative amount of caseinase activity determined from the size of the proteolytic zone produced by 10⁸ log phase bacteria inoculated into wells on skim milk agar plates (Fig. 2). The diffuse proteolytic material observed near the top of zymograms of PA103-29 (Fig. 1) was extracted from the gel and found to react with Chromozym PL.

Statistical Analysis of Data

Statistical analysis was carried out using the Statistical Analysis System (SAS) software program for personal computers. Analysis of variance was performed on the CFU per cornea. Where a significant analysis of variance was found, *t*-tests between the least square means from each strain were performed. Nonparametric one-way analysis of variance (Kruskal–Wallis test) was used for SLE scores. Wilcoxon scores were used for comparison among groups in this analysis. *P* < 0.05 was considered significantly different.

### TABLE 2. Virulence of *Pseudomonas* Strains in the Rabbit Intrastromal Injection Model of Keratitis

<table>
<thead>
<tr>
<th>Strain*</th>
<th>Exoprotein(s) Produced</th>
<th>SLE†</th>
<th>16 Hours</th>
<th>22 Hours</th>
<th>27 Hours</th>
<th>CFU‡</th>
<th>27 Hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATCC 27853</td>
<td>Elastase, alkaline, protease, and exotoxin A</td>
<td>7.4 ± 1.99</td>
<td>10.22 ± 1.55</td>
<td>13.94 ± 4.90</td>
<td>7.37 ± 0.73</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PA103</td>
<td>Exotoxin A and protease IV</td>
<td>7.50 ± 3.60§</td>
<td>10.10 ± 3.44§</td>
<td>13.00 ± 4.28§</td>
<td>7.38 ± 0.25§</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PA103-29</td>
<td>Protease IV</td>
<td>8.0 ± 2.09¶</td>
<td>9.19 ± 1.99¶</td>
<td>14.19 ± 4.74¶</td>
<td>7.66 ± 0.38¶</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PA103-AP1</td>
<td>Exotoxin A¶</td>
<td>6.50 ± 1.45#</td>
<td>9.88 ± 2.24#</td>
<td>14.13 ± 3.26#</td>
<td>7.58 ± 0.57#</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Rabbit eyes were intrastromally injected with 10⁶ CFU.
† Mean slit lamp examination score (±SD); n = 10 eyes per strain.
‡ Mean colony forming units at 27 hours postinfection per cornea expressed as log10 (±SD).
§ Not significantly different from ATCC 27853 (*P* > 0.06).
¶ Not significantly different from ATCC 27853 (*P* > 0.24) and PA103 (*P* > 0.06).
# Produces 5% of parent PA103 protease IV activity.
* Not significantly different from ATCC 27853 (*P* > 0.07) and PA103 (*P* > 0.10).
TABLE 3. Virulence of *Pseudomonas* Strains in the Rabbit Scratch Model of Keratitis

<table>
<thead>
<tr>
<th>Strain*</th>
<th>Exoprotein(s) Produced</th>
<th>Protease IV Activity</th>
<th>CFU at 24 Hours</th>
<th>SLE at 30 Hours</th>
<th>CFU at 42 Hours</th>
<th>SLE at 42 Hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATCC 27853</td>
<td>Elastase, alkaline protease, and exotoxin A</td>
<td>None</td>
<td>4.15 ± 0.78†</td>
<td>12.03 ± 2.04†</td>
<td>7.10 ± 0.60§</td>
<td>18.88 ± 4.42</td>
</tr>
<tr>
<td>PA103</td>
<td>Exotoxin A and protease IV</td>
<td>Intermediate</td>
<td>5.41 ± 0.90†</td>
<td>10.25 ± 1.12†</td>
<td>4.13 ± 2.28§</td>
<td>11.0 ± 1.14#</td>
</tr>
<tr>
<td>PA103-29</td>
<td>Protease IV</td>
<td>Maximal</td>
<td>4.94 ± 0.84†</td>
<td>12.34 ± 2.64†</td>
<td>7.15 ± 0.46§</td>
<td>17.25 ± 4.38#</td>
</tr>
<tr>
<td>PA103-AP1</td>
<td>Exotoxin A**</td>
<td>Minimal</td>
<td>5.06 ± 1.22†</td>
<td>6.44 ± 2.24†</td>
<td>2.86 ± 0.60§</td>
<td>5.44 ± 2.60</td>
</tr>
</tbody>
</table>

* Rabbit corneas (four eyes per strain) were scarified with a diamond knife and inoculated with 6.28 ± 2.28 CFU bacteria adhering to contact lenses.
† No significant differences (P > 0.06) among all groups in CFU per cornea at 24 hours postinfection.
‡ No significant differences in SLE scores among strains ATCC 27853, PA103, and PA103-29 at 30 hours postinfection (P > 0.15).
§ ATCC 27853 and PA103-29 were not significantly different from each other (P = 0.87) in CFU per cornea at 42 hours postinfection, but were different from PA103 and PA103-AP1 (P < 0.0001).
∥ ATCC 27853 and PA103-29 were not significantly different from one another (P = 0.39) in SLE scores at 42 hours, but were significantly different from PA103 and PA103-AP1 (P < 0.006).
¶ PA103 and PA103-AP1 were significantly different from each other (P = 0.006) in CFU per cornea at 42 hours postinfection.
# PA103 and PA103-AP1 were significantly different from one another (P = 0.002) in SLE scores at 42 hours postinfection.
** Produces 5% of parent PA103 protease IV activity.
†† PA103-AP1 was significantly different from all other strains in SLE scores at 30 hours postinfection (P < 0.02).

mozym PL (data not shown). Strain ATCC 27853 had ample caseinase activity, as evidenced by a large proteolytic zone around the well on skim milk agar (Fig. 2), but cleaved the Chromozym PL substrate at a relatively inefficient rate compared with PA103 and PA103-29 (Fig. 3). Zymography and immunoprecipitation results showed that strain ATCC 27853 produced elastase and alkaline protease (Table 1, Fig. 1). Purified alkaline protease produced virtually no reaction with this substrate. Purified elastase cleaved the substrate inefficiently (Fig. 3) and probably accounts for the low level of Chromozym PL cleavage mediated by ATCC 27853 supernatants.

**Corneal Virulence**

**Rabbit Intrastromal Model of Keratitis.** In the intrastromal injection model of keratitis, the CFU per cornea (27 hours after infection) and SLE scores (16, 22, and 27 hours after infection) produced by the four strains were not significantly different (P > 0.06; Table 2). Fifty colonies of each strain obtained from infected eyes were tested for elastase and caseinase activities, and the results confirmed that the bacteria recovered had the properties typical of the strain inoculated.

**Rabbit Scratch Model.** At 24 hours after application of bacteria-coated contact lenses, the numbers of CFU per cornea of the four strains were not significantly different (P > 0.06; Table 3). At 30 hours after infection, the SLE scores of strains ATCC 27853, PA103, and PA103-29 were not significantly different (P > 0.15); however, the protease-deficient strain, PA103-AP1, produced a significantly lower SLE score than the three other strains (P < 0.02).

At 42 hours after infection, the SLE score for strain PA103-AP1 continued to be significantly lower than those of the three other strains tested (P < 0.002). Infection with PA103-29, which produces only protease IV, resulted in SLE scores that were not sig-

TABLE 4. Virulence of *Pseudomonas* Strains in the Mouse Scratch Model of Keratitis

<table>
<thead>
<tr>
<th>Strain*</th>
<th>Exoprotein(s) Produced</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 4</th>
<th>Day 6</th>
<th>Day 2</th>
<th>Day 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATCC 27853</td>
<td>Elastase, Alkaline Protease, and Exotoxin A</td>
<td>3.38 ± 2.40</td>
<td>4.63 ± 5.12</td>
<td>6.33 ± 3.96</td>
<td>5.63 ± 4.00</td>
<td>8/8</td>
<td>4/8</td>
</tr>
<tr>
<td>PA103</td>
<td>Exotoxin A and Protease IV</td>
<td>4.25 ± 1.96†</td>
<td>4.5 ± 2.92†</td>
<td>5.25 ± 4.88§</td>
<td>5.38 ± 5.12†</td>
<td>8/8</td>
<td>5/8</td>
</tr>
<tr>
<td>PA103-29</td>
<td>Protease IV</td>
<td>1.88 ± 1.92§</td>
<td>4.25 ± 1.92§</td>
<td>6.88 ± 6.00</td>
<td></td>
<td></td>
<td>7.13 ± 5.44</td>
</tr>
<tr>
<td>PA103-AP1</td>
<td>Exotoxin A††</td>
<td>1.50 ± 0.76§</td>
<td>1.88 ± 1.20†</td>
<td>2.00 ± 0.76‡</td>
<td>1.0 ± 0.00#</td>
<td>8/8</td>
<td>7/8</td>
</tr>
</tbody>
</table>

* Mouse eyes were scarified with a needle and inoculated with a drop of bacteria (10⁵ colony-forming units). Eyes were homogenized and cultured on TSA or *Pseudomonas* Isolation Agar for detection of viable bacteria.
† SLE = mean slit lamp examination score (±SD); n = 16 eyes per strain.
‡ Not significantly different from ATCC 27853 (P > 0.11).
§ Significantly different from ATCC 27853 (P = 0.016) and PA103 (P = 0.0006).
|| Not significantly different from ATCC 27853 (P < 0.72) and PA103 (P > 0.06).
# Produces 5% of parent PA103 protease IV activity.
†† Significantly different from ATCC 27853 (P < 0.004) and PA103 (P < 0.008).
SLE = slit lamp examination, TSA = tryptic soy agar.
significantly different from those caused by infection with the prototype strain, ATCC 27853 (P = 0.39). Strain PA103, which had less protease activity than PA103-29 or ATCC 27853, produced significantly lower SLE scores than either of these two strains (P < 0.006; Fig. 2, Table 3), but these scores were not as low as those produced by PA103-AP1.

The growth of Pseudomonas in the rabbit cornea correlated with the protease production of the strain used. Eyes infected with PA103-AP1 for 42 hours contained significantly fewer CFU per cornea, compared with eyes infected with any of the other strains, including PA103 (P < 0.006). Also, eyes infected with strain PA103 contained significantly fewer CFU per cornea than eyes infected with either ATCC 27853 or PA103-29 (P < 0.0001). Fifty colonies of each strain isolated from infected eyes at 24 and 42 hours after infection were tested for elastase and caseinase activities, and the results confirmed that the bacteria recovered had the properties typical of the strain inoculated.

**Mouse Scratch Model of Keratitis.** Topical inoculation of the mouse eye with the mutant deficient in protease IV, strain PA103-AP1, produced significantly lower SLE scores at all time points (from day 1 through day 6), compared with both its parent strain, PA103, and the prototype strain, ATCC 27853 (P < 0.008; Table 4). The SLE scores produced by infection with strain PA103-29 on days 2 through 6 were not significantly different from those produced by PA103 (parent) or ATCC 27853 (prototype; P > 0.06). The SLE scores for eyes infected with parent strain PA103 were not significantly different from those of eyes infected with the prototype strain, ATCC 27853 (P > 0.11; Table 4). Viable bacteria could be recovered from half or more of the infected corneas on days 2 and 6, regardless of the strain. Bacteria isolated from the eyes of each group were tested, and the expected phenotype of the inoculated strain was confirmed.

**DISCUSSION**

The purpose of this study was to determine the relationship between key exoproteins produced by Pseudomonas aeruginosa and corneal virulence. Four selected bacterial strains were analyzed for their exoproteins with special emphasis given to the role of a previously uncharacterized enzyme, protease IV. Corneal virulence was determined for these strains, which differed in exoprotein production, in three models of keratitis.

One major result of the current study is the initial characterization of protease IV, which has a molecular size and substrate specificity distinct from those of other Pseudomonas enzymes. Protease IV produced a band at 120 kDa and some larger aggregates (Fig. 1). The relationship between the 120-kDa protease and the 50-kDa and 70-kDa proteases reported for PA103 by Twining et al is unknown. Protease IV was found to react with Chromozym PL, a substrate cleaved by serine proteases but not efficiently by Pseudomonas metalloproteases, elastase, and alkaline protease. Protease IV is the major protease expressed in strains PA103 and PA103-29. These findings confirm the work of Toder and Gambello and Guzzo et al who reported that the protease of PA103 was an enzyme other than alkaline protease or elastase. Additional molecular and enzymatic properties of protease IV are now under investigation.

Another major finding of this study is evidence showing that protease IV contributes to corneal virulence. The direct relationship between protease production and corneal virulence was seen in the rabbit topical inoculation model in which the protease activity of each strain corresponded with the extent of infection. Differences in the number of bacteria per rabbit cornea after topical inoculation (Table 3) occurred despite the similarity in the growth rates of these strains both in vitro and in the rabbit cornea after intrastromal inoculation (Table 2). These findings support the earlier studies of Kawaharajo et al and Howe and Iglewski, who found that protease-deficient Pseudomonas had reduced virulence in topically infected corneas. Our findings also support the work of Steuhl et al who found that production of protease at high concentrations was not critical for virulence in a rabbit intrastromal model of infection. Furthermore, our observations of undetectable protease activity in unconcentrated supernatants of PA103 could explain why Hazlett et al did not observe protease in this strain, which led them to suggest that protease-deficient Pseudomonas is fully virulent in the mouse scratch model of keratitis.

Pertinent to the understanding of corneal virulence is the finding that the route of corneal inoculation can determine the extent of bacterial replication and corneal damage mediated by a particular strain. The protease IV-deficient mutant strain (PA103-AP1) had markedly reduced virulence in both the rabbit and mouse scratch models of infection compared with the protease-producing strains (PA103-29 and ATCC 27853). Hazlett et al demonstrated that the binding of Pseudomonas to the cornea is stimulated by protease digestion of the corneal surface. Protease digestion could unmask receptors or induce synthesis of additional receptors. The findings of Hazlett et al and our findings strongly suggest that protease digestion of the corneal surface could be one factor required for infections resulting in extensive corneal damage.

In the rabbit intrastromal injection model of keratitis, there were no significant differences in SLE scores between the protease IV-deficient mutant PA103-AP1 and the protease-producing strains, PA103, PA103-29, and ATCC 27853. This finding suggests that small amounts of protease activity could be
sufficient to allow full virulence in the intrastromal injection model of keratitis where the mechanisms required for adherence to and penetration of an epithelial barrier are not needed. Another consideration is that only in the intrastromal model do the bacterial exoproteins remain tightly confined in the stroma and could accumulate at the site of infection. A strain fully deficient in *Pseudomonas* proteases is needed to determine whether the presence of protease IV, even at low concentrations, significantly contributes to virulence in the intrastromal injection model of keratitis. The loss of protease IV activity from PA103-29 could cause extensive attenuation of its corneal virulence. If so, such an attenuated mutant would confirm the role of protease IV in virulence and provide a strain that could serve as the host for plasmids coding for a single *Pseudomonas* exoprotein; this genetic system would allow a measurement of the contribution of specific proteins to corneal virulence.

The significance of protease IV as a mediator of virulence relates to the development of therapy designed to prevent corneal damage. There have been multiple reports describing the therapeutic use of protease inhibitors to limit corneal damage caused by *Pseudomonas* keratitis. The discovery of protease IV as a *Pseudomonas* virulence factor with specific enzymatic activity could, in part, explain why such inhibitors previously failed to minimize corneal damage. Furthermore, the results provide a target for the development of new inhibitors which, in conjunction with antibiotics, could reduce or eliminate corneal damage.

**Key Words**
aminal model, keratitis, protease, *Pseudomonas*, virulence

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