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Isolation and Characterization of Alkaline Protease-Deficient Mutants of *Pseudomonas aeruginosa* In Vitro and in a Mouse Eye Model

TIMOTHY R. HOWE AND BARBARA H. IGLEWSKI*

Department of Microbiology and Immunology, Oregon Health Sciences University, Portland, Oregon 97201

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Mutants of *Pseudomonas aeruginosa* are described which are markedly deficient in alkaline protease production. Characterization of these mutants in vitro suggests that the mutations in two of these strains are specific for alkaline protease production. Examination of these mutants in a mouse eye model demonstrates that alkaline protease is required for the establishment of corneal infections with *P. aeruginosa* PA103. Mutants deficient in alkaline protease production could not colonize traumatized cornea and did not produce the corneal damage characteristic of infection by the parental strain. Addition of subdamaging amounts of alkaline protease to eyes infected with the protease-deficient mutants resulted in infections which were indistinguishable from infections caused by the parental strain.

Infection of traumatized cornea by *Pseudomonas aeruginosa* is usually rapid and self-limiting and often results in impaired vision due to corneal scarring (22). The hallmark of this infection is a liquefactive necrosis associated with extensive ulceration and corneal perforation (1).

Years ago, Fisher and Allen (7) suggested that the characteristic damage observed with *P. aeruginosa* eye infections may be attributable to the action of bacterial proteases on the corneal proteoglycan. Several groups, including Kawaharajo et al. (16), Hirao and Homma (12), Kreger and Gray (20) and Kreger and Griffin (21) have since provided data to support this hypothesis. Recently, Ohman et al. (27) initiated a genetic study to examine the role of bacterial toxin and protease in corneal infection. They examined the virulence of an elastolysis-deficient mutant of *P. aeruginosa* and concluded that elastolysis was not required to establish an active infection. They also concluded that if extracellular protease is required for virulence in *Pseudomonas* eye infections, then alkaline protease produced by the mutant strain they examined may have been sufficient to cause corneal damage.

Isolation of mutants specifically deficient in the production of alkaline protease would facilitate definitive studies addressing the role of alkaline protease in *P. aeruginosa* infections. This approach has been complicated by the fact that most strains of *P. aeruginosa* produce at least two distinct extracellular proteases (25) and that alkaline protease does not possess a unique or stringent substrate specificity (26). We have been unable to specifically inhibit the activity of interfering protease (elastase) to facilitate detection of alkaline protease mutants on skim milk agar plates (unpublished data). To circumvent these problems, we chose to isolate alkaline protease mutants in strain PA103, which produces alkaline protease (4) but no detectable elastase (28). Despite the fact that strain PA103 is serum sensitive (41), it is virulent in a mouse eye model of corneal infection (17, 27). We have isolated alkaline protease-deficient mutants of strain PA103 after ethyl methanesulfonate (EMS) mutagenesis and screening of mutagenized colonies on skim milk agar plates. After in vitro characterization of these mutants, mutants specifically deficient in alkaline protease production were identified, and the virulence of these mutants was compared with that of the wild-type strain in a mouse eye model.

* Corresponding author.

MATERIALS AND METHODS

Organism. *P. aeruginosa* PA103, isolated by Liu (23), was selected for this study. This strain is naturally elastase deficient (28). We found that all protease activity in culture supernatants of strain PA103 was neutralized by specific alkaline protease antisera, demonstrating that the only extracellular protease produced by this strain is alkaline protease. This strain and derived mutants were maintained on nutrient agar plates and stored in 10% skim milk at −70°C. Where indicated, spontaneous mutants resistant to streptomycin and rifampin were selected by growth on 250 μg of streptomycin (streptomycin sulfate; Sigma Chemical Co., St. Louis, Mo.) per ml, followed by growth on 50 μg of rifampin (Sigma) per ml.

Isolation of mutants. EMS (methanesulfonic acid ethyl ester; Sigma) was employed by the method of Carlton and Brown (2). A solution of Vogel-Bonner minimal medium (36) containing 4% EMS was equilibrated at 37°C. To this solution was added an equal volume of a late-exponential-phase culture of *P. aeruginosa* PA103 grown in nutrient broth. This mixture was incubated with shaking for 90 min at 37°C, and then cells were washed with phosphate-buffered saline (PBS), grown overnight in nutrient broth, and used to inoculate skim milk plates at dilutions sufficient to result in ca. 100 colonies per plate. The conditions of EMS mutagenesis employed resulted in ca. 90% lethality. After incubation of these plates at 37°C for 48 h, colonies were examined for zones of clearing indicative of alkaline protease production. Colonies which did not produce zones of clearing after 48 h were chosen for further study.

Preliminary characterization of mutants. Mutants were examined for prototrophy by growth on minimal agar medium (36). Pigment production was determined by growth on agar plates prepared by the method of King et al. (19). Motility was determined by growth in 0.4% nutrient agar and by microscopic examination of broth cultures by the hanging drop method. Generation time was determined by growth in

nutrient broth as described by Miller (24). Serotype and pyocin type were determined as described previously (6, 28). Total protease was determined by growth on skim milk agar plates as previously described (33). Hemolysin production was determined by growth on heart infusion agar supplemented with sheep blood (28). Lipase production was demonstrated by clearing of tributyrin agar (15). Esterase production resulted in a blue coloration of colonies grown on brain heart infusion agar supplemented with 0.1% indoxyl acetate (13). Toxin A activity was determined by an ADP-ribosyl transferase assay as previously described (28). The reaction mixtures for toxin A determinations were incubated at 25°C for 10 min.

Liquid culture techniques. For quantitative determination of extracellular alkaline protease and toxin A, cells were grown in tryptic soy broth which had been dialyzed, deferrated by chelex (Chelex-100; Biorad Laboratories, Richmond, Calif.) treatment, and supplemented with monosodium glutamate and glycerol (TSB-DC) (28). This medium is optimal for toxin A production (28) and supports the production of alkaline protease by strain PA103 (4). This medium was also used to prepare inocula for animal studies, employing conditions previously demonstrated as optimal for enhancing the virulence of *P. aeruginosa* for corneal infections (38). Davis minimal medium (5) supplemented with tryptose, yeast extract, and glucose (MTYG) (41) was also used to obtain supernatants for the quantitation of alkaline protease activity as described previously (4).

Determination of alkaline protease activity. *P. aeruginosa* PA103 produces low levels of alkaline protease (4). Therefore, a modification (35) of the sensitive protease assay described by Rinderknecht et al. (30) was used for its quantitation. To 50 mg of hide azure blue powder substrate (Sigma) in a polycarbonate tube (17 by 25 mm) was added 1.5 ml of buffer (20 mM Tris-hydrochloride, 1 mM $CaCl_2$ [pH 8.0]) and 0.5 ml of culture supernatant or cell extract. This mixture was incubated at 37°C for 1 h with constant rotation, and then tubes were chilled on ice and centrifuged at 4,000 × *g* for 5 min in a clinical table top centrifuge. The absorbance of the reaction mixture was then determined at 595 nm (A_{595}). Preliminary studies with purified alkaline protease (Nagase Chemical Co., Tokyo, Japan) demonstrated that this assay was capable of detecting 100 ng of alkaline protease per ml of culture supernatant. Protease activity was expressed in terms of protease units per milliliter (PU/ml), where one unit is equivalent to an increase in A_{595} of 1.0 per h at 37°C.

Preparation of anti-alkaline protease antisera. A toxoid of alkaline protease was prepared by Formalin-lysine treatment of purified alkaline protease (Nagase Chemical) as described by Homma et al. (14). The purified alkaline protease gave a single band on sodium dodecyl sulfate-polyacrylamide gels after electrophoresis. The toxoid was used to immunize adult New Zealand white rabbits. Rabbits were injected intramuscularly with 1 mg of toxoid in complete Freund adjuvant. At 2-week intervals, rabbits were boosted with 1 mg of toxoid in incomplete Freund adjuvant, and blood samples were collected for determination of the anti-alkaline protease titer. The titer was determined against 1 μg of pure alkaline protease by a standard immunodiffusion assay (29). Animals were exsanguinated 4 days after the fourth injection of toxoid. The immunoglobulin G fraction of this antiserum was prepared by differential ammonium sulfate precipitation (11), dialyzed against PBS, and stored at −20°C. This antiserum had a precipitin titer of 1:512.

Quantitation of alkaline protease antigen. An enzyme-linked immunosorbent assay (ELISA) was developed to quantitate alkaline protease antigen in culture supernatants and cell lysates. A conjugate of alkaline phosphatase and the immunoglobulin G fraction of antiserum raised against alkaline protease was prepared as described previously (18). This conjugate was used in the ELISA as described previously (9). Coating buffer, PBS, PBS with Tween, PBS with 1% bovine serum albumin, and diethanolamine buffer were prepared as described previously (37). The limit of detection of this assay was 100 pg of alkaline protease per ml.

Extraction of intracellular or cell-associated protease. TSB-DC grown cells were pelleted by centrifugation, washed twice with an equal volume of PBS, then ruptured in a French pressure cell press (American Instrument Co., Inc., Silver Spring, Md.) as described previously (31), except that the cells were suspended in PBS, and nucleases and $MgCl_2$ were not added.

Mouse model of corneal infection. A modification of the model previously described by other investigators (8, 10, 17, 27) was used. Bacteria were grown to mid-exponential phase in TSB-DC at 32°C. When the culture reached an A_{540} of 0.23, cells were removed by centrifugation, washed twice with PBS, then suspended in a 0.1 volume of PBS to obtain 2 × 10^9 CFU/ml. Viable numbers of bacteria were confirmed by plating serial dilutions on nutrient agar to obtain the CFU.

Female Swiss-Webster mice weighing ca. 20 g were obtained from Simonson, Hayword, Calif. Mice were anesthetized by inhalation of metofane (methoxyfluorane; Pitman-Moore, Inc., Washington Crossing, N.J.) and then subjected to three 1-mm corneal incisions with a sterile 27-gauge needle using a stereoscopic microscope to avoid penetration of the anterior chamber. Dilutions of bacteria suspended in 5 μl were applied to the traumatized cornea. Where indicated, an inoculum containing bacteria and 5 μg of purified alkaline protease or 0.5 μg of purified elastase was used.

The progress of infection was determined by examination of infected eyes with a 50× stereoscopic microscope. All mice were anesthetized (metofane) before examination. The extent of damage was estimated by a previously established corneal damage index (CDI) (27). As previously defined (27), a CDI of 1.0 corresponds to light or partial opacity, whereas a CDI of 4.0 indicates necrosis and perforation of the cornea. To assess colonization and persistence of bacteria in infected eyes, eyes were periodically swabbed with sterile saline-soaked swabs, and the swabs were used to inoculate King B plates (19). Representative mice with culture-negative eyes were sacrificed by cervical dislocation, and their eyes were enucleated (8), macerated, and suspended in PBS, and dilutions were plated on King B plates. This was done to address the possibility of intra-stromal persistence of bacteria that would not be evident by culturing of the corneal surface.

RESULTS

Isolation of alkaline protease-deficient mutants. Alkaline protease mutants were identified by their failure to produce a zone of clearing on skim milk plates when incubated at 37°C for 48 h. After three independent EMS mutageneses, a total of 21,500 colonies were examined. Colonies with no zones of clearing were subcultured for heavy growth on skim milk agar and incubated at 37°C for 48 h. A total of 19 presumptive alkaline protease mutants, which produced no zones of clearing under either condition, were subcloned. These presumptive mutants were grown in liquid TSB-DC, and alkaline protease activity was determined. Although the 19

TABLE 1. Extracellular products of *P. aeruginosa* PA103 and alkaline protease mutants

Strain	Protease activity (PU/ml)[a]	Toxin activity (cpm)	Fluorescein (+/−)	Sheep hemolysin (zone, mm)	Esterase (+/−)	Lipase (zone, mm)
PA103	4.3	2,580	+	0.5	+	2.0
PA103-AP1	0.0	2,642	+	0.5	+	2.0
PA103-AP2	0.054	2,455	+	0.5	+	2.0
PA103-AP3	0.075	2,010	+	0.5	+	2.0
PA103-AP4	0.02	640	+	0.5	+	2.0
PA103-AP5	0.61	2,520	+	0.5	+	2.0

[a] PU, Protease units in hide powder blue assay. One unit = increase in A_{595} of 1.0 per h at 37°C. Assays were done in duplicate in each of two separate experiments, and the results were averaged.

presumptive mutants were indistinguishable on skim milk agar plates, the amount of alkaline protease produced by these mutants was variable and ranged from 0 to 60% parental level. Of the original 19 presumptive mutants, 5 mutants produced less than 15% parental levels of protease activity and were chosen for further examination.

Characterization of protease-deficient mutants. Previous studies have demonstrated that mutants isolated on the basis of protease deficiency are often deficient in the production of other extracellular products which may be a consequence of coregulation, common transport mechanisms, or secondary mutations (40). For this reason, the production of several extracellular products was examined (Table 1). The mutant strains were all indistinguishable from the parental strain PA103 with respect to the production of fluorescein, hemolysin, esterase, and lipase. However, two strains, designated PA103-AP3 and PA103-AP4, produced decreased levels of toxin A and were not examined further. The mutant strain PA103-AP5 produced at least 10% of the parental levels of protease and was therefore eliminated from future studies. Two mutants, designated PA103-AP1 and PA103-AP2, produced consistently low or undetectable levels of extracellular protease but were otherwise indistinguishable from strain PA103. These mutants were chosen for further characterization. No differences in serotype, pyocin type, or motility were noted in these mutants (data not shown). To determine whether protease deficiency in these two mutants was due to altered growth rate in vitro, growth rates in TSB-DC at 32°C were determined. Parent and mutant strains were not different in generation time (about 40 min during early-exponential phase).

Quantitation of protease activity and antigen in mutant strains. To address the possibility that the mutants produced an altered protein which was proteolytically inactive but antigenically related to alkaline protease, supernatants were examined for the presence of alkaline protease antigen. Mutants were cultivated in TSB-DC as described previously (4). Total protease activity of culture supernatants was determined, and supernatants were examined for the presence of protein antigenically related to alkaline protease by an ELISA specific for alkaline protease. Both mutants examined produced markedly decreased or undetectable levels of protease activity and correspondingly low levels of alkaline protease antigen (Table 2). Previous studies have demonstrated repression of alkaline protease production in complex medium (25). To address the possibility that protease production of these mutants was adversely affected by the growth medium, these strains were grown in MTYG, which has been shown to support a high ratio of alkaline protease to total protein (4). Both mutants produced either low or undetectable levels of protease in this medium (Table 2).

To determine whether the mutations in these strains resulted in impaired secretion of alkaline protease, cells were grown in TSB-DC or MTYG, and whole cell lysates were prepared. Examination of these lysates for alkaline protease antigen failed to demonstrate the accumulation of protease or material antigenically related to alkaline protease in a cell-associated form (Table 2).

To determine whether alkaline protease deficiency in mutant strains was due to a temperature-sensitive defect in protease production, mutant and parental strains were grown on skim milk agar plates at 25, 32, 37, and 43°C. Strain PA103 grew and produced zones of clearing at every temperature examined. The mutant strains grew well at all temperatures but produced no zones of clearing at any temperature (data not shown).

Examination of mutant strains in the mouse eye model. Previous studies (27) have demonstrated that strain PA103 establishes an active corneal infection in mice with inocula of 10^6 to 10^8 bacteria per eye.

In the current study, the inoculation of 10^6 to 10^8 CFU of PA103 produced an active, necrotic infection associated with marked opacity and ulceration. Average corneal damage in these mice ranged from 3.0 to 3.5, and permanent eye damage was evident in the majority of these mice (Fig. 1). In

TABLE 2. Quantitation of extracellular and cell-associated alkaline protease of parental strain PA103 and alkaline protease mutants[a]

Strain	Medium	Supernatant		Whole cell lysate
		Protease activity[b] (PU/ml) (% parent)[c]	Antigen[d] (μg/ml) (% parent)	Antigen[d] (μg/ml)
PA103	TSB-DC	4.6 (100)	0.9 (100)	0.05
	MTYG	4.0 (100)	0.75 (100)	0.02
PA103-AP1	TSB-DC	0.0 (0)	ND[e] (0)	ND
	MTYG	0.0 (0)	ND (0)	ND
PA103-AP2	TSB-DC	0.06 (1.3)	0.02 (2.2)	ND
	MTYG	0.046 (1.2)	0.01 (1.3)	ND

[a] Cells were grown in TSB-DC or MTYG as indicated to an equivalent cell density. These experiments were repeated twice with comparable results.

[b] Determined by hide powder blue assay (see Table 1).

[c] Numbers in parentheses represent percent yields of protease as compared with the parent strain PA103.

[d] Determined by an ELISA specific for alkaline protease.

[e] ND, Not detectable (less than 100 pg of alkaline protease per ml).

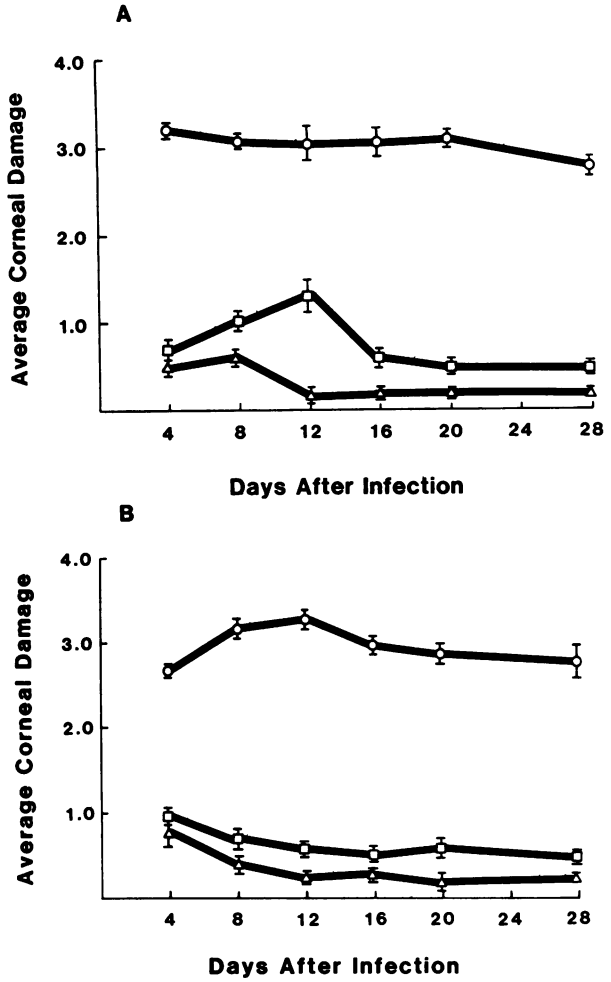

FIG. 1. Average corneal damage ± standard error of the mean resulting from the inoculation of 10^6 (A) and 10^7 (B) CFU of *P. aeruginosa* PA103 (○), the alkaline protease mutant strain PA103-AP1 (△), or the alkaline protease mutant strain PA103-AP2 (□).

contrast, neither of the mutant strains deficient in protease production (PA103-AP1 and PA103-AP2) was able to induce pathological changes beyond minimal corneal opacity and neovascularization, with average corneal damage in these groups of mice ranging from 0 to 1.5 early in infection and resolving rapidly (Fig. 1). Infection with either of these mutant strains resulted in essentially no permanent damage.

Recovery of bacteria from infected mice. With an initial inocula of 10^6 to 10^8 organisms, strain PA103 could be cultured from greater than 50% of infected eyes 8 days after inoculation, whereas infection with either mutant strains was considerably more transitory (Fig. 2). To examine the possibility that organisms were present intrastromally, in a form which would not be demonstrated by superficial culturing, enucleated eyes were examined for the presence of viable organisms. Enucleated eyes were uniformly culture negative by day 8 after infection with alkaline protease mutants, indicating that the mutant strains were not persisting in low numbers within the infected corneas. Culture results obtained throughout the course of infection with the mutant strains revealed that the mutants were unable to persist in the cornea, and in 50% of the mice, the mutants failed to establish even a transitory infection (Fig. 2).

Persistence in mixed infection and stability of mutants in vivo. The mutations in the alkaline protease-deficient strains appear to affect alkaline protease specifically. Still, there existed the possibility that an undetected second mutation of pleiotropic effect of the initial mutation resulted in the inability of mutants to adhere to traumatized cornea, rendered them unable to grow in vivo, or caused them to become unusually sensitive to the host defenses. If the defect in the mutant strains which results in decreased colonization is due to their lack of alkaline protease production, then the effect of these mutations should be complemented in vivo by the production of extracellular protease produced by another strain. A derivative of mutant strain PA103-AP1 which was resistant to streptomycin and rifampin was prepared. This strain, designated PA103-AP101, was also avirulent and in this regard indistinguishable from strain PA103-AP1. Mutant strain PA103-AP101 was employed in a mixed infection in which the inocula contained equal numbers of PA103-AP101 and the original alkaline protease positive, antibiotic-sensitive parental strain PA103, with a total inoculum of 10^7 CFU per eye. The mutant strain PA103-AP101 was recoverable from infected eyes as long as was the parental strain PA103. Bacteria resistant to streptomycin and rifampin were recovered from mixed infections as late as day 8 after infection (Table 3). The antibiotic-resistant strains recovered from the mixed infections were uniformly deficient in alkaline protease activity, demonstrating in vivo stability of the alkaline protease mutation.

Complementation of mutants with exogeneous protease. To confirm the correlation of alkaline protease deficiency in mutant strains and avirulence in the mouse eye model, we examined the effect of exogenous protease on establishment of corneal infection and associated corneal pathology. The addition of 20 μg of alkaline protease to uninfected, incised cornea resulted in demonstrable corneal damage. The addition of 10 μg of alkaline protease produced no evident corneal damage, even when 10 μg of alkaline protease was instilled daily for 8 days. Mice were infected with 10^7 CFU of the mutant strain PA103-AP1 alone or with 5 μg of alkaline protease. At daily intervals for 8 days, mice in the second group were treated with 5 μg of alkaline protease. Uninfected control mice received 5 μg of alkaline protease daily for 8 days. The control mice treated with protease alone did not show any sign of corneal damage. Mice infected with the

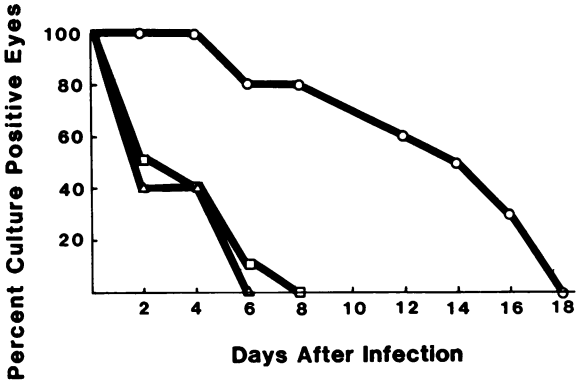

FIG. 2. Recovery of *P. aeruginosa* parental strain PA103 (○) or alkaline protease-deficient strain PA103-AP1 (△) or PA103-AP2 (□) from infected mouse eyes. A minimum of 10 mice were infected with each strain at two different inocula (10^7 and 10^8), and each point represents a composite of the data obtained with both.

protease-deficient mutant PA103-AP1 alone had minimal corneal damage (CDI, 0.0 to 1.0). Extensive corneal damage was observed in mice treated with alkaline protease and infected with the protease-deficient mutant strain PA103-AP1; corneal damage in these mice was comparable to that obtained with the parental strain PA103 (CDI, ca. 3.0) (Fig. 3). Similarly, when mice infected with the mutant strain PA103-AP2 were treated with 5 μg of alkaline protease for 8 days, they had extensive damage (CDI, ca. 3.0) (data not shown). Either mutant strain (PA103-AP1 or PA103-AP2) inoculated along with alkaline protease persisted in infected eyes longer than did the mutant inoculated in buffer, indicating that the exogenously added alkaline protease facilitated colonization of traumatized cornea with either of these mutant strains (Table 3).

We also examined the effect of exogeneous *Pseudomonas* elastase on corneal infection caused by protease-deficient mutants to determine whether alkaline protease specifically is required or whether another protease might complement the mutants in vivo. The addition of 1 μg of purified elastase did not cause detectable corneal damage. Mice infected with the protease-deficient mutant PA103-AP1 and treated with 0.5 μg of elastase daily for 8 days had extensive corneal damage which was comparable to that observed with infection by the parental strain PA103 (data not shown).

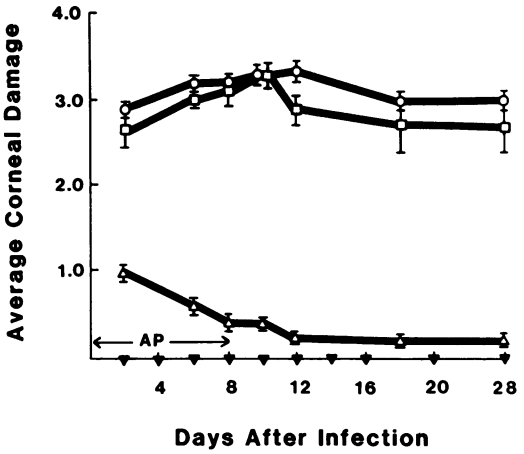

FIG. 3. Effect of exogenous alkaline protease on corneal infections by the alkaline protease-deficient strain PA103-AP1. Ten mice were infected with 10^7 CFU of strain PA103-AP1 suspended in buffer (PBS) and were treated daily for 8 consecutive days with PBS (△). Ten mice were infected with 10^7 CFU of strain PA103-AP1 suspended in PBS containing 5 μg of alkaline protease (□). Ten mice were infected with the parental strain PA103 (○) and five uninfected mice had their corneas incised and were treated with 5 μg of alkaline protease daily for 8 consecutive days (▼). Data shown are the averages of corneal damage ± standard error of the mean. The period during which exogenous alkaline protease was instilled in the mouse eyes is represented by ← AP →.

DISCUSSION

The data presented show that at least low levels of extracellular protease are essential for maximum virulence of *P. aeruginosa* in a mouse eye model. The strain chosen for this study, PA103, produces no detectable elastase and such low levels of alkaline protease that it is considered by other investigators to be protease deficient (17, 32). Our study and others (17, 27) have shown that this strain is virulent in a mouse eye model. We found, however, that alkaline protease-deficient mutants derived from strain PA103 were avirulent in the mouse eye model. Virulence of these mutants could be restored if subdamaging amounts of purified *Pseudomonas* alkaline protease were added at the time of infection and for a total of 8 consecutive days, indicating that the loss of virulence in these mutant strains was indeed due to a specific mutation affecting alkaline protease and not some undetected secondary or pleiotropic effect of the mutagen. Interestingly, we found that subdamaging amounts of *Pseudomonas* elastase could substitute for alkaline protease and restore the virulence of the alkaline protease mutants. These data indicate that although an extracellular protease is required for maximum virulence of *P. aeruginosa* in this model, a single specific *Pseudomonas* protease is not required.

Previous studies employing purified *Pseudomonas* proteases suggested a role for them in *Pseudomonas* keratitis as tissue-damaging agents (16, 20, 21). Our results indicate that alkaline protease may, in addition, function as a colonization factor in mouse eye infections, since mutants deficient in alkaline protease were less efficient in colonizing the wounded mouse cornea and cleared much more readily than the parental strain. Several studies (3, 34, 39) have described mechanisms which could explain the role of alkaline protease in this colonization capacity. Stern et al. (34) have demonstrated the importance in epithelial cell damage in predisposing the cornea to adhesion of *P. aeruginosa*, and Woods et al. (39) have demonstrated a role for proteases in enhancing adherence of *P. aeruginosa* to buccal epithelium. Alternatively, alkaline protease may interfere with host clearance mechanisms, allowing both colonization and persistence in the eye. Finally, protease production and associated tissue damage may provide essential nutrients for *P. aeruginosa*. This role for *Pseudomonas* proteases has been suggested previously for burn infections in which protease-producing strains of *P. aeruginosa* are associated with better growth (lower generation time) in burned skin extract than are protease-deficient strains (3).

Although our studies indicate an important role for bacterial proteases in *Pseudomonas* keratitis, it is clear that protease production itself is insufficient for virulence. Ohman et al. (27) showed that toxin A-deficient mutants derived from either strain PA103 or PAO were less virulent in a mouse eye model than their toxinogenic parental strains. These (27) and our current findings underscore the multifactorial nature of virulence in *P. aeruginosa*.

TABLE 3. Recovery of *P. aeruginosa* parental strain PA103 or alkaline protease-deficient mutant strains from infected mouse eyes 4 and 8 days after infection[a]

Inocula	Day 4 (%)	Day 8 (%)
PA103	100	70
PA103-AP1	40	0
PA103-AP101 + PA103[b] (mixed infection)	100	60
PA103-AP1 + alkaline protease	80	60
PA103-AP2	50	0
PA103-AP2 + alkaline protease	100	60
Alkaline protease	0	0

[a] Data are presented as the percentage of mouse eyes that yielded positive culture for the indicated *P. aeruginosa* strain. A minimum of 10 mice were infected with 10^7 CFU per eye on day 0.

[b] Both strains PA103 and PA103-AP101 were recovered from all mice which were culture positive on day 4 or 8.

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