INFECTION AND IMMUNITY, Aug. 1997, p. 3086–3090
0019-9567/97/$04.00+0
Copyright © 1997, American Society for Microbiology
Vol. 65, No. 8

Contribution of Proteases and LasR to the Virulence of *Pseudomonas aeruginosa* during Corneal Infections

MICHAEL J. PRESTON,[1]* PATRICK C. SEED,[2] DEBBIE S. TODER,[3] BARBARA H. IGLEWSKI,[2] DENNIS E. OHMAN,[4] JEAN K. GUSTIN,[4] JOANNA B. GOLDBERG,[1] AND GERALD B. PIER[1]

Channing Laboratory, Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts 02115[1]; Department of Microbiology, University of Rochester, Rochester, New York 14610[2]; Department of Pediatrics, Wayne State University School of Medicine, Detroit, Michigan 48201[3]; and Department of Microbiology and Immunology, University of Tennessee and Veterans Affairs Medical Center, Memphis, Tennessee 38163[4]

Received 22 August 1996/Returned for modification 18 November 1996/Accepted 25 April 1997

The roles of the *Pseudomonas aeruginosa* proteases LasB (elastase) and LasA and the transcriptional activator LasR, which regulates the expression of these proteases, were evaluated in a murine model of *P. aeruginosa* corneal infection. In scarified corneas, *P. aeruginosa* PAO-A1 (LasA negative) or PAO-B1A1 (LasB and LasA negative) at a dose of 10^8 CFU per eye caused very mild or no disease following infection; however, the defect in PAO-A1 could not be complemented by supplying a functional copy of *lasA* either on a plasmid or inserted into the chromosome. In contrast, PAO-B1 (LasB negative) colonized the cornea and caused disease equal in severity to disease caused by the parental strain, PAO1-I. Although LasR is a known regulator of *lasA* expression, PAO-R1, a *lasR*-negative derivative of PAO1-I, was as virulent as the parental strain during corneal infection. When transcriptional fusion plasmids were used to quantify the expression of the *lasB* and *lasA* genes in *P. aeruginosa* PAO1-I and PAO-R1, the *lasB::lacZ* fusion in PAO-R1 showed only 3.5% as much activity as it did in PAO1-I, while the activity of the *lasA::lacZ* fusion in PAO-R1 was 27.8% of that in PAO1-I. Coadministration of 5 μg of purified LasA protease with PAO-A1 did not reconstitute a wild-type infection. This treatment produced an acute toxic reaction leading to prolonged eyelid closure without inflammatory destruction of the cornea that was similar to that observed when LasA was administered alone. These results indicate that insertional inactivation of *lasA* renders *P. aeruginosa* avirulent in a murine model of keratitis and that neither LasR nor elastase production is required for the establishment and maintenance of corneal infection. However, the lack of virulence of the LasA-deficient strains cannot be ascribed with certainty to the deficiency of LasA from the available data.

Previous studies have suggested that corneal pathology in *Pseudomonas aeruginosa*-induced keratitis results mostly from the development of a local inflammatory reaction. The inflammation is a response to both cell-associated and secreted bacterial products (28, 34). *P. aeruginosa* produces many extracellular products, such as proteases and toxins that are candidates for important virulence factors in keratitis. These products include exotoxin A, phospholipase C, LasB (elastase), alkaline protease, and the LasA protease. We previously described a modified version of the murine model of corneal keratitis and found that neither hemolytic nor nonhemolytic phospholipase C is an important virulence factor in this model (24). Using a related murine model, others have shown that *P. aeruginosa* mutants deficient in exotoxin A produced less corneal damage than toxin-producing strains did (20).

Although studies with experimental infection models have delineated the roles of factors such as phospholipase C and exotoxin A in corneal infections, they have not fully established a requirement for *P. aeruginosa* proteases in corneal damage. The roles of the *P. aeruginosa* proteases in the progression of corneal keratitis have been assessed through several approaches. Direct intrastromal injection of semipurified (3, 7) or purified (10, 12) proteases has resulted in histopathologically detectable damage to eye tissues. These studies, however, have yielded neither insights into the cooperative interactions of the proteases with other *P. aeruginosa* factors in corneal pathology nor an understanding of the contribution of proteases to the overall progression of keratic disease. Some studies evaluating *P. aeruginosa* isolates with altered profiles of protease production in eye infection models have suggested a correlation between protease production and virulence (3, 8, 32), but these studies have not used isogenic strains in the infectious models.

Other studies with isogenic strains producing and not producing proteases have used chemical mutagenesis to affect protease production. Ohman et al. found that PAO-E64, a nitrosoguanidine-generated strain with a temperature-sensitive deficiency in LasA activity, was as virulent as the parental strain in eye infection models (20). More recent studies have shown that strain PAO-E64 produces low levels of LasA (11) and may have pleiotropic mutations resulting in the decreased expression of *lasA* and *lasB* (30). Additional undefined mutations may also confound the interpretation of data obtained with chemically derived strains. The present studies sought to define further the role that proteases play in the pathogenesis of corneal infections with *P. aeruginosa* by using isogenic strains with defined mutations in the structural genes for two proteases, LasA and LasB, as well as in *lasR*, whose product is involved in the transcriptional activation of the *P. aeruginosa* *lasA* and *lasB* genes (4, 5, 31).

* Corresponding author. Mailing address: Channing Laboratory, 181 Longwood Ave., Boston, MA 02115. Phone: (617) 525-2152. Fax: (617) 731-1541. E-mail: mpreston@channing.harvard.edu.

Downloaded from http://iai.asm.org/ on September 25, 2020 by guest

TABLE 1. Bacterial strains used in the study of *P. aeruginosa* virulence factors in corneal infections

P. aeruginosa strain	Description	Phenotype	Reference or source
PAO1-I	Parental strain	Wild-type serogroup O5	B. Iglewski, Rochester, N.Y.
PAO-R1	Str^r, Ω*lasR*::*tet* of PAO1-I	No LasR protein	5
PAO-B1	*lasB*::Ω of PAO1-I	No LasB protease (elastase)	34
PAO-A1	*lasA*::Tet^r of PAO1-I	No LasA protease	33
PAO-B1A1	*lasA*::Tet^r of PAO-B1	No LasA or LasB protease	33

MATERIALS AND METHODS

Bacterial strains and inocula. The strains of *P. aeruginosa* used in the present study are listed in Table 1. Bacteria were stored and prepared for infection as described previously (24).

Construction of a *lasA* merodiploid strain. A *Sal*I-*Sph*I restriction fragment (2.1 kb) containing *lasA* from *P. aeruginosa* FRD was cloned into *Sal*I-*Sph*I-digested pUC19. This clone was designated pJKG152. An *Eco*RI-*Sal*I fragment containing a *mob* (*oriT*) cassette (1.5 kb) was cloned into *Eco*RI-*Sal*I-digested pJKG152. The resulting clone was designated pJKG152*mob*. This plasmid (in *Escherichia coli* JM109) was conjugally transferred to PAO-A1 via triparental matings with the helper plasmid pRK2013 (in *E. coli* HB101). Mating mixtures were plated on one-half-strength *Pseudomonas* isolation agar (Difco, Detroit, Mich.) supplemented with carbenicillin (300 μg/ml). Since pJKG152*mob* is a suicide plasmid in *P. aeruginosa*, the resulting carbenicillin-resistant clones represented crossovers of the plasmid onto the PAO-A1 chromosome at the *lasA* locus. Since the resulting clones were also tetracycline resistant, the recombinants were single crossovers. Several such clones were selected for analysis. Overnight cultures of PAO1-I, PAO-A1, and three PAO-A1::pJKG152*mob* isolates were diluted 1:100 in fresh medium without antibiotics and grown with incubation and shaking at 37°C for 19 h. One-milliliter samples were taken from each culture, the cells were removed by centrifugation, and the supernatants were assayed for staphylolytic (LasA protease) activity as described previously (36). PAO-A1 was devoid of staphylolytic activity, while PAO1-I and the three PAO-A1::pJKG152*mob* isolates all had identical high levels of activity. Thus, pJKG152*mob* had complemented the *lasA* mutation in PAO-A1, restoring *lasA* expression to levels equivalent to that of PAO1-I.

Construction of transcriptional *lacZ* fusions and measurement of β-galactosidase activity in *P. aeruginosa*. A series of multicopy *lacZ* transcriptional fusion plasmids were constructed to monitor the expression of the *lasB* and *lasA* genes in *P. aeruginosa* PAO1-I and PAO-R1. For the construction of a *lacZ* transcriptional fusion vector capable of replicating in *P. aeruginosa*, a 1.4-kb *Eco*RI-*Eco*RV fragment was removed from pTL61T (14) and ligated into the same sites in pSW205 (22). This step created a vector, pLP170, with a *P. aeruginosa* origin of replication, multiple cloning sites for the insertion of gene promoters, and a *lacZ* structural gene complete with its own ribosome-binding site and translational start site. An important feature of this vector is an RNase III splice sequence positioned between the multiple cloning sites and *lacZ*. After transcription of an operon fusion, the mRNA transcript is spliced by RNase III, removing the multiple cloning site, allowing for the independent translation of *lacZ* transcripts, and thus giving a more accurate representation of any transcriptional events (14).

To construct the *lasB*::*lacZ* fusion, an *Eco*RI-*Nae*I fragment of approximately 400 bp containing a portion of the *lasB* gene was isolated from pRB1804 (22) and ligated into pLP170 digested with *Eco*RI and *Sma*I. A *lasA*::*lacZ* transcriptional fusion was created by ligation of a *Bam*HI-*Sal*I fragment of approximately 900 bp of the *lasA* gene from pSFJ6280 into pLP170 digested with *Xho*I and *Bam*HI. PAO1-I and PAO-R1 were transformed with these constructs by electroporation as has been described previously (27).

For the measurement of β-galactosidase activity, strains containing the reporter plasmids were grown overnight at 32°C at 250 rpm in PTSB medium (21) supplemented with carbenicillin (200 μg/ml). Strains were subcultured 1:100 in the same medium to an approximate absorbance at 540 nm of 0.05. Each strain was grown for 18 h at 32°C with shaking and assayed for β-galactosidase activity as described by Miller (17).

Infection of mice. C3H/HeN female mice (5 to 6 weeks old) were obtained from Charles River Breeding Laboratories, Wilmington, Mass. The mice were infected in the eye as described previously (24).

Grading of corneal infection. Infected eyes were observed macroscopically, and infection was graded every 24 to 48 h after inoculation by an investigator unaware of the challenge strain given or amount of bacterial inoculum administered. The following grading scheme, which has been described previously (1), was used: grade 0, eye macroscopically identical to the uninfected contralateral control eye; grade 1, faint opacity partially covering the pupil; grade 2, dense opacity covering the pupil; grade 3, dense opacity covering the entire anterior segment; and grade 4, perforation of the cornea and/or phthisis bulbi (shrinkage of the eyeball following inflammatory disease). For calculation of the dose of bacteria required to establish infection in 50% of the mice challenged (ID_{50}), a grade of ≥2 was used to signify infection.

Viable counts of bacteria remaining in the eye after inoculation. To determine the number of bacteria remaining in the eye 24 h after inoculation, the *P. aeruginosa* strains were inoculated as described above onto scratched mouse eyes at a dose of 2.0×10^7 CFU per eye. Twenty-four hours after inoculation, five mice that had received each *P. aeruginosa* strain were killed by an overdose of carbon dioxide; the cornea was dissected from each eye with a sterile scalpel blade. A coronal incision was made at the limbus extending to approximately the middle of the eye. The cornea was then removed by finishing the cut to the opposite limbus with sterile microdissecting scissors. The cornea was homogenized in 1 ml of sterile 1% proteose peptone, serially diluted, and plated in 0.1-ml aliquots onto tryptic soy agar plates for bacterial enumeration by colony counts.

Purification of LasA protease and use in experimental eye infections. Cultures of *P. aeruginosa* FRD2128, which bears a plasmid (pJKG107) allowing high-level expression of *lasA*, were initiated by inoculation of 4 liters of L broth at a 1:100 dilution from an overnight culture. Cells were incubated at 37°C with rapid aeration for 12 h, at which time 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG; Sigma, St. Louis, Mo.) was added. The cultures were then incubated for an additional 6 h, after which cells were removed by centrifugation (10,000 × *g*). Total supernatant proteins were precipitated with ammonium sulfate (80% saturation) overnight at 4°C, collected by centrifugation (20,000 × *g* for 60 min), dissolved in and dialyzed against buffer A (20 mM Tris-HCl [pH 7.5]), and separated on a DEAE-cellulose column (2 by 20 cm; flow rate, 2 ml of buffer A per min) with a protein purification system (model 650E; Waters, Milford, Mass.). Flowthrough fractions that showed LasA protease activity were concentrated in dialysis bags (10-kDa molecular size cutoff; Baxter, McGraw Park, Ill.) against solid sucrose (Baxter). Further fractionation was performed with a sulfopropyl column (8 by 75 mm; Waters Protein-Pak SP5PW; 1 ml of buffer A/min). LasA protease (detected by its staphylolytic activity) was eluted early in a linear salt gradient (0 to 1.6 M NaCl over 30 min). Active fractions were again concentrated in dialysis bags against solid sucrose (Baxter) and then dialyzed against buffer A. Aliquots were frozen at −80°C. Mice were anesthetized, and their corneas were scarified as described above, after which either purified LasA alone or purified LasA and *P. aeruginosa* strains were applied to the eyes. Animals were evaluated as described above for evidence of corneal destruction.

Statistical analysis. ID_{50}s and 95% confidence intervals (CIs) were calculated by probit analysis with the Systat (Evanston, Ill.) statistical program (release 5.2.1). When a single ID_{50} result is reported for animals observed over the course of 2 to 3 weeks, the ID_{50} calculated is taken from the day when the pathologic scores were maximal. Data on the bacterial counts in infected eyes were log transformed, and analysis of variance was used to determine whether differences between *P. aeruginosa* strains were significant at the 95% level; the Statview statistical program (Abacus Concepts, Berkeley, Calif.) was used.

RESULTS

Corneal infections of mice. To evaluate the effects of inactivation of genes involved in the regulation and production of *P. aeruginosa* proteases on corneal infections, we infected mice with *P. aeruginosa* PAO1-I and isogenic derivatives defective in the production of LasR (PAO-R1), elastase (PAO-B1), LasA protease (PAO-A1), or both elastase and LasA proteases (PAO-B1A1) at doses ranging from 10^3 to 10^8 CFU per eye. After an evaluation of the corneal damage, the minimal ID_{50} was determined for each strain. The ID_{50} for the parent strain, PAO1-I, had previously been determined in the corneal infection model to be 2.5×10^6 CFU (24) and is provided here for reference (Table 2). The ID_{50}s for strains PAO-R1 and

TABLE 2. ID_{50} of wild-type and mutant strains of *P. aeruginosa* in the murine corneal-scratch infection model

Strain	ID_{50} (CFU)	95% CI
PAO1-I	2.5×10^6	2.5×10^5–1.6×10^7
PAO-R1	7.5×10^4	1.3×10^4–4.3×10^6
PAO-B1	1.9×10^6	1.6×10^5–2.4×10^7
PAO-A1	$>10^8$	NA[a]
PAO-B1A1	$>10^8$	NA

[a] NA, not applicable.

Downloaded from http://iai.asm.org/ on September 25, 2020 by guest

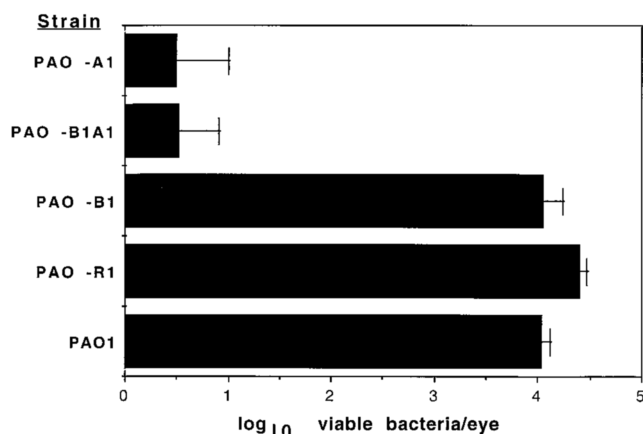

FIG. 1. Levels of viable bacteria recovered from the corneas of mice infected for 24 h with *P. aeruginosa* PAO1-I (PAO1), PAO-R1, PAO-B1, PAO-B1A1, or PAO-A1.

PAO-B1 were not significantly different from that for PAO1-I at a *P* value of $\leq$0.05 (overlapping 95% CIs of ID_{50}s) (Table 2). However, neither of the two strains defective in the production of the LasA protease (PAO-A1 and PAO-B1A1) established infections that were significant enough, even at a dose of 10^8 CFU per eye, for the calculation of an ID_{50} (Table 2). Given the lack of virulence of the LasA-deficient strains, we first attempted to complement the LasA deficiency by supplying a functional copy of wild-type *lasA* on a plasmid. Challenge of mice with 10^8 CFU of PAO-A1 containing pRB1822SF, a multicopy plasmid with *lasA* (30), per eye did not result in apparent infections in any of the mice. To control for the possibility that the plasmid was not maintained in vivo in the absence of antibiotic selection, a merodiploid strain with a wild-type copy of *lasA* inserted into the chromosome was created in PAO-A1 as described in Materials and Methods. Challenge of mice with 10^8 CFU of the merodiploid strain PAO-A1::pJKG152*mob* per eye did not result in apparent eye infections in any of the mice, indicating an inability to complement the *lasA* defect.

Quantitative microbiology of infected corneas. We sought to evaluate whether observed corneal infection scores were due to differences in the ability of the strains to colonize the cornea and induce inflammation, the pathologic process suggested by the studies of Twining et al. as the cause of tissue damage (32). Therefore, the levels of viable bacteria in the corneas were determined 24 h after inoculation with the *P. aeruginosa* strains producing variable amounts of proteases. The numbers of viable bacteria of strains PAO1-I (parental strain), PAO-R1, and PAO-B1 recovered from the corneas were similar (Fig. 1). However, fewer than 10 viable organisms of PAO-A1 and PAO-B1A1, which have the *lasA* gene interrupted, were recovered from four of five corneas ($P < 0.05$); this figure represents the lower limit of detection.

Transcriptional fusion studies in PAO-R1. The initial data reported above for the LasA-deficient strains do not clearly indicate whether LasA is a virulence factor in corneal pathogenesis. Since previous studies with nonquantitative Northern (RNA) blot analysis had shown that *lasA* transcription, like *lasB* transcription, is dependent on the transcriptional regulator LasR (5, 31), the maintenance of the full virulence potential of PAO-R1 suggested that LasA itself was not critical for producing *P. aeruginosa* keratitis and that the loss of virulence in PAO-A1 and PAO-B1A1 was due to an effect on other factors. To explore this in more detail, we compared the dependencies of the *lasA* and *lasB* genes on LasR for expression. As shown in Table 3, the transcription of *lasB* was greatly reduced in the absence of LasR; strain PAO-R1 expressed only 3% of parental levels of the *lasB* transcript. In PAO-R1, *lasA* expression was measured at 27.8% of parental levels. These data confirm that elastase is not a virulence factor in *P. aeruginosa* keratitis, but since PAO-R1 transcribes *lasA* at a low level, it was not clear whether the reduced level of LasA protease contributes to the virulence of *P. aeruginosa* in eye infections or the nonvirulent strains with an interrupted *lasA* gene have become nonvirulent due to other effects.

Topical application of purified LasA and coadministration with LasA-deficient *P. aeruginosa* to scarified mouse corneas. To determine whether topical administration of purified LasA protease alone could damage corneas, we administered two doses of LasA protease, 5 and 0.5 μg, to groups of five anesthetized mice after corneal scarification. The mice were observed daily for 10 days. Mice that received 0.5 μg of purified LasA protease had no macroscopically evident eye damage at any time after administration. However, the eyelids of the mice that received 5 μg of the purified LasA protease were sealed shut within 24 h of application and remained that way for the entire observation period. When the lids were manually opened after 10 days, the entire eyes of three of the five mice were severely wasted and shrunken, while the eyes of the remaining two mice appeared normal. There was no visible sign of cellular inflammation in any of these eyes. Subsequently, both eyes of an additional six mice were scarified. One eye was treated with 5 μg of LasA protease, while the contralateral eye received phosphate-buffered saline alone. Half of the mice were sacrificed at 24 h and the other half were killed at 48 h, and paraffin-embedded sections of the LasA protease-treated and untreated eyes were examined microscopically. At these time points, the LasA protease-treated corneas appeared normal and were indistinguishable from the corneas of untreated control eyes (data not shown). These results indicate that purified LasA protease at high doses produces a toxic reaction leading to eye damage without eliciting cellular inflammation.

We also sought to determine whether the administration of either 5 or 0.5 μg of LasA protease along with 10^7 CFU of either strain PAO-B1A1 (LasB and LasA negative) or strain PAO-A1 (LasA negative) could restore the ability of these strains to cause corneal disease. The results of these experiments were similar to the results obtained after administration of LasA protease alone: mice receiving 5 μg of LasA protease and either bacterial strain had sealed eyelids for a 5-day observation period, while the eyes of the mice receiving 0.5 μg of LasA protease and either bacterial strain appeared normal for a 10-day observation period. When the eyes of the mice receiving 5 μg of LasA protease and either LasA-deficient strain of *P. aeruginosa* were manually opened after 5 days, the cor-

TABLE 3. Relative expression of *lasB*::*lacZ* and *lasA*::*lacZ* in *P. aeruginosa* strains PAO1-I and PAO-R1

Plasmid construct	Fusion tested	β-Galactosidase activity (Miller units)[a]		Expression in PAO-R1 (%)
		PAO1-I	PAO-R1	
pLPLB	*lasB*::*lacZ*	41,142 ± 2,964	1,447 ± 171	3.52
pLPLA	*lasA*::*lacZ*	24,519 ± 326	6,821 ± 801	27.8
pLP170	*lacZ*::vector	399 ± 70	590 ± 98	147

[a] Data represent the means ± standard deviations of three independent cultures.

Downloaded from http://iai.asm.org/ on September 25, 2020 by guest

neas had no macroscopic signs of inflammation beyond the mild inflammation observed in the corneas of mice that had received the bacteria alone.

DISCUSSION

In this study, we evaluated the role of the *P. aeruginosa* proteases LasB and LasA and the transcriptional regulator LasR in an experimental model of corneal disease. The requirement of an intact *lasA* gene for full virulence of *P. aeruginosa* in this model was suggested by the inability of two different strains (PAO-A1 and PAO-B1A1) with an insertion in *lasA* to produce infections, even at high challenge doses. However, we could not confirm, by use of complementation studies, that the loss of virulence was specifically due to the loss of LasA production. In contrast, LasB elastase did not appear to be a critical factor in corneal disease. Using strain PAO-R1, we found that the absence of the LasR protein, known to regulate the expression of a variety of potential virulence factors, did not affect the pathogenesis of *P. aeruginosa* eye disease. Since strain PAO-R1 transcribed the *lasA* gene at approximately one-quarter the level documented for the parental strain, PAO1-I, this lower level of expression either apparently suffices to ensure the full virulence of strain PAO-R1 in the eye infection model or indicates that the loss of LasA protease is not the determinant of the loss of virulence in the *lasA* insertional mutants. These findings are consistent with those previously reported by Ohman et al., who showed that nonelastolytic *P. aeruginosa* PA103 is highly virulent in a corneal infection model (20). Gambello and Iglewski demonstrated that PA103 is a phenotypically LasR-null strain (4). The complementation of the strain with *lasR* resulted in the restoration of elastolysis. Thus, both the naturally occurring LasR deficiency in strain PA103 and the genetically defined deficiency in strain PAO-R1 result in the same phenotype in the corneal infection model.

Additional studies examining the role of LasA in infection suggested that the lack of virulence of the LasA-deficient strains may be due to factors other than LasA. First, restoration of full LasA production by genetic complementation does not restore virulence. Second, adding LasA to the inoculum did not affect virulence. Howe and Iglewski demonstrated that adding subdamaging amounts of alkaline protease could restore virulence in the eye infection model to an alkaline protease-deficient *P. aeruginosa* strain (6), suggesting that this is a valid strategy for complementation of protease mutants in vivo. Third, in studies not reported above, we attempted to immunize mice with purified LasA protease in adjuvant and thereby to diminish the infectious process and determine if LasA was significantly contributing to virulence. Although high titers of serum-neutralizing antibody to LasA were produced, animals challenged with 10^7 CFU of strain PAO1-I per eye were not protected against corneal disease (data not shown). Previous studies using active or passive immunization with proteases have also met with limited success in protecting against corneal damage (13, 28). However, these results may be explained, in part, by the failure in recent studies to detect elastase (LasB) in corneas infected with *P. aeruginosa* 19660 (9).

Another study with the eye infection model suggested that the LasA protease is not important for the virulence of *P. aeruginosa*. Ohman et al. found that *P. aeruginosa* PAO-E64, a chemical mutant with reduced, temperature-sensitive production of LasA, was fully virulent in a murine corneal infection model (20). When later examined in immunoblots, this strain was shown to produce small but detectable amounts of LasA (11). In studies using *lacZ* transcriptional reporter plasmids, strain PAO-E64 has recently been shown to have altered expression of *lasA* and *lasB*; this finding suggests an additional defect in a regulatory gene (30). Since elastase was shown not to be a critical virulence factor in the present study, these data further support the conclusion that LasA may not be a critical factor affecting virulence in the murine corneal infection model. Taken together, the failure to complement the *lasA* defect by genetic complementation or supplying the protein with the inoculum, the lack of protection after immunization with LasA despite high serum antibody levels, and the studies of Ohman et al. (20) suggest that the defect in strains lacking functional *lasA* may be more complicated than the lack of production of LasA protease alone. There may be other genes in the *lasA* locus that were affected by the mutation but which are unknown at this time. It is certain that whatever gene(s) was affected had a significant impact on the virulence of *P. aeruginosa* in the murine model of corneal infection and should be investigated in the future.

The expression of a number of *P. aeruginosa* exoproducts is controlled by the transcriptional activator protein LasR. These include LasB elastase, LasA protease, and alkaline protease (4, 5, 31). In addition, LasR is required for transcription of *lasI*, which is needed for production of the *P. aeruginosa* autoinducer PAI (22, 26). In the regulatory process termed autoinduction, a complex of the LasR protein and the PAI molecule has been proposed to interact with specific LasR-responsive operator elements upstream of a gene, promoting expression of the target gene (23). However, genes responsive to autoinduction are not equally dependent on LasR and PAI for expression. The *lasI* gene, for instance, requires a 10-fold-lower concentration of PAI for half-maximal expression than does *lasB*; this difference supports the notion of a hierarchy of autoinduction (26). Using sensitive and quantitative *lacZ* transcriptional fusions, we confirmed that *lasB* is minimally expressed in the absence of LasR. Specifically, we documented 3.5% as much expression without LasR as with LasR (25). However, *lasA* is transcribed in strain PAO-R1, albeit at lower levels than in strain PAO1-I (28%). Gambello et al., using a *toxA*::*lacZ* expression plasmid, demonstrated that PAO-R1 expressed the *toxA* gene at 60% of the level found for strain PAO1-I (5). These results suggest the following order of dependence on transcriptional regulation by LasR-PAI: *lasI* > *lasB* > *lasA* > *toxA*. LasR, therefore, may simply enhance the expression of some genes in vivo while being required for the expression of others. In contrast to a previous study in which *lasA* mRNA was not detected by Northern blot analysis (5), we have demonstrated by use of transcriptional fusions that the *lasA* promoter is active in PAO-R1, albeit at diminished levels compared to PAO1-I. The discrepancy in these findings is likely due to differences in the methods used, i.e., measuring promoter activity versus direct detection of mRNA, which may be unstable in vitro.

LasR may serve as a tissue-specific regulator of *P. aeruginosa* virulence determinants. While this report shows that LasR is not required for the virulence of *P. aeruginosa* in the cornea, Tang et al. recently reported that strain PAO-R1 was less virulent than strain PAO1-I in a neonatal mouse model of acute pneumonia (29). Compared with strain PAO1-I, strain PAO-R1 was associated with lower mortality and also with fewer bacteremias and lower bacterial burdens in infected lungs. Tissue-specific requirements for various virulence factors of *P. aeruginosa* may explain the differences between the results of these studies. In support of this explanation, strain PAO-E64 has been demonstrated to have reduced virulence in experimental lung infection models (2, 21, 35) but not in a murine corneal infection model (20).

Downloaded from http://iai.asm.org/ on September 25, 2020 by guest

Although previous studies have implicated proteases in the pathogenesis of *P. aeruginosa* corneal infection, none of these studies used isogenic strains with insertional or other specific inactivation of protease genes to evaluate pathogenesis (6, 18, 20, 32). Thus, some of the effects on virulence reported in these other studies could be due to virulence factors other than proteases that were affected by chemical mutagenesis. Currently, little is known about the specific biologic effects of the proteases during infection. *P. aeruginosa* elastase has been shown to activate host matrix metalloproteases (MMPs) via limited proteolysis (15, 16, 33). Corneal MMPs have been suggested to contribute to the degradation of corneal proteins during *P. aeruginosa* keratitis (33). It is likely that other proteases, either host derived (19) or bacterial, could activate these MMPs in the absence of elastase. However, these processes need to be evaluated during an in vivo infection before the roles they might play in pathogenesis can be elucidated. What is clear from the present study is that the *lasA* gene needs to be intact for the full virulence of *P. aeruginosa* in corneal infections but that the loss of the LasA protease itself is not the outcome of the interruption of *lasA* that is likely to be the critical factor for causing tissue damage during infection.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grants from the National Institutes of Health (AI22535 to G.B.P. and EY06426 to M.J.P.). P.C.S. was supported by a National Institutes of Health predoctoral training grant in microbial pathogenesis. D.S.T. was supported by a Physician Scientist Award from the Cystic Fibrosis Foundation.

REFERENCES

1. **Beisel, K. W., L. D. Hazlett, and R. S. Berk.** 1983. Dominant susceptibility effect on the murine corneal response to *Pseudomonas aeruginosa*. Proc. Soc. Exp. Biol. Med. **172:**488–491.
2. **Elsheikh, L. E., T. Kronevi, B. Wretlind, S. Abaas, and B. H. Iglewski.** 1987. Assessment of elastase as a *Pseudomonas aeruginosa* virulence factor in experimental lung infection in mink. Vet. Microbiol. **13:**281–289.
3. **Fisher, E., and J. H. Allen.** 1958. Corneal ulcers produced by cell free extracts of *Pseudomonas aeruginosa*. Am. J. Ophthalmol. **46:**21–27.
4. **Gambello, M. J., and B. H. Iglewski.** 1991. Cloning and characterization of the *Pseudomonas aeruginosa lasR* gene, a transcriptional activator of elastase expression. J. Bacteriol. **173:**3000–3009.
5. **Gambello, M. J., S. Kaye, and B. H. Iglewski.** 1993. LasR of *Pseudomonas aeruginosa* is a transcriptional activator of the alkaline protease gene (*apr*) and an enhancer of exotoxin A expression. Infect. Immun. **61:**1180–1184.
6. **Howe, T. R., and B. H. Iglewski.** 1984. Isolation and characterization of alkaline protease-deficient mutants of *Pseudomonas aeruginosa* in vitro and in a mouse eye model. Infect. Immun. **43:**1058–1063.
7. **Kawaharajo, K., C. Abe, J. Y. Homma, M. Kawano, E. Gotoh, N. Tanaka, and K. Morihara.** 1974. Corneal ulcers produced by protease and elastase from *Pseudomonas aeruginosa*. Jpn. J. Exp. Med. **44:**435–442.
8. **Kawaharajo, K., and J. Y. Homma.** 1975. Pathogenesis of the mouse keratitis produced with *Pseudomonas aeruginosa*. Jpn. J. Exp. Med. **45:**515–524.
9. **Kernacki, K. A., J. A. Hobden, L. D. Hazlett, R. Fridman, and R. S. Berk.** 1995. In vivo bacterial protease production during *Pseudomonas aeruginosa* corneal infection. Investig. Ophthalmol. Vis. Sci. **36:**1371–1378.
10. **Kessler, E., H. E. Kennah, and S. I. Brown.** 1977. *Pseudomonas* protease. Purification, partial characterization, and its effect on collagen, proteoglycan, and rabbit corneas. Investig. Ophthalmol. Vis. Sci. **16:**488–497.
11. **Kessler, E., M. Safrin, J. C. Olson, and D. E. Ohman.** 1993. Secreted LasA of *Pseudomonas aeruginosa* is a staphylolytic protease. J. Biol. Chem. **268:** 7503–7508.
12. **Kreger, A. S., and L. D. Grey.** 1978. Purification of *Pseudomonas aeruginosa* proteases and microscopic characterization of pseudomonal protease-induced rabbit corneal damage. Infect. Immun. **19:**630–648.
13. **Kreger, A. S., D. M. Lyerly, L. D. Hazlett, and R. S. Berk.** 1986. Immunization against experimental *Pseudomonas aeruginosa* and *Serratia marcescens* keratitis. Vaccination with lipopolysaccharide endotoxins and proteases. Investig. Ophthalmol. Vis. Sci. **27:**932–939.
14. **Linn, T., and R. St. Pierre.** 1990. Improved vector system for constructing transcriptional fusions that ensures independent translation for *lacZ*. J. Bacteriol. **172:**1077–1084.
15. **Matsumoto, K., N. B. Shams, L. A. Hanninen, and K. R. Kenyon.** 1993. Cleavage and activation of corneal matrix metalloproteases by *Pseudomonas aeruginosa* proteases. Investig. Ophthalmol. Vis. Sci. **34:**1945–1953.
16. **Matsumoto, K., N. B. Shams, L. A. Hanninen, and K. R. Kenyon.** 1992. Proteolytic activation of corneal matrix metalloproteinase by *Pseudomonas aeruginosa* elastase. Curr. Eye Res. **11:**1105–1109.
17. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
18. **O'Callaghan, R. J., L. S. Engel, J. A. Hobden, M. C. Callegan, L. C. Green, and J. M. Hill.** 1996. *Pseudomonas* keratitis: the role of an uncharacterized exoprotein, protease IV, in corneal virulence. Investig. Ophthalmol. Vis. Sci. **37:**534–543.
19. **Ogata, Y., J. J. Enghild, and H. Nagase.** 1992. Matrix metalloproteinase 3 (stromelysin) activates the precursor for the human matrix metalloproteinase 9. J. Biol. Chem. **267:**3581–3584.
20. **Ohman, D. E., R. P. Burns, and B. H. Iglewski.** 1980. Corneal infections in mice with toxin A and elastase mutants of *Pseudomonas aeruginosa*. J. Infect. Dis. **142:**547–555.
21. **Ohman, D. E., S. J. Cryz, Jr., and B. H. Iglewski.** 1980. Isolation and characterization of a *Pseudomonas aeruginosa* PAO mutant that produces altered elastase. J. Bacteriol. **142:**836–842.
22. **Passador, L., J. M. Cook, M. J. Gambello, L. Rust, and B. H. Iglewski.** 1993. Expression of *Pseudomonas aeruginosa* virulence genes requires cell-to-cell communication. Science **260:**1127–1130.
23. **Passador, L., and B. H. Iglewski.** 1995. Quorum sensing and virulence gene regulation in *Pseudomonas aeruginosa*, p. 65–78. *In* J. A. Roth, C. A. Bolin, K. A. Brogden, F. C. Minion, and M. J. Wannemuehler (ed.), Virulence mechanisms of bacterial pathogens, 2nd ed. ASM Press, Washington, D.C.
24. **Preston, M. J., S. M. Fleiszig, T. S. Zaidi, J. B. Goldberg, V. D. Shortridge, M. L. Vasil, and G. B. Pier.** 1995. Rapid and sensitive method for evaluating *Pseudomonas aeruginosa* virulence factors during corneal infections in mice. Infect. Immun. **63:**3497–3501.
25. **Rust, L., E. C. Pesci, and B. H. Iglewski.** 1996. Analysis of the *Pseudomonas aeruginosa* elastase (*lasB*) regulatory region. J. Bacteriol. **178:**1134–1140.
26. **Seed, P. C., L. Passador, and B. H. Iglewski.** 1995. Activation of the *Pseudomonas aeruginosa lasI* gene by LasR and the *Pseudomonas* autoinducer PAI: an autoinduction regulatory hierarchy. J. Bacteriol. **177:**654–659.
27. **Smith, A. W., and B. H. Iglewski.** 1989. Transformation of *Pseudomonas aeruginosa* by electroporation. Nucleic Acids Res. **17:**10509.
28. **Steuhl, K.-P., G. Döring, A. Henni, H.-J. Thiel, and K. Botzenhart.** 1987. Relevance of host-derived and bacterial factors in *Pseudomonas aeruginosa* corneal infections. Investig. Ophthalmol. Vis. Sci. **28:**1559–1568.
29. **Tang, H. B., E. DiMango, R. Bryan, M. Gambello, B. H. Iglewski, J. B. Goldberg, and A. Prince.** 1996. Contribution of specific *Pseudomonas aeruginosa* virulence factors to pathogenesis of pneumonia in a neonatal mouse model of infection. Infect. Immun. **64:**37–43.
30. **Toder, D. S., S. J. Ferrel, J. L. Nezezon, L. Rust, and B. H. Iglewski.** 1994. *lasA* and *lasB* gene products of *Pseudomonas aeruginosa*: analysis of transcription and gene product activity. Infect. Immun. **62:**1320–1327.
31. **Toder, D. S., M. J. Gambello, and B. H. Iglewski.** 1991. *Pseudomonas aeruginosa* LasA: a second elastase under the transcriptional control of *lasR*. Mol. Microbiol. **5:**2003–2010.
32. **Twining, S. S., S. D. Davis, and R. A. Hyndiuk.** 1986. Relationship between proteases and descemetocele formation in experimental *Pseudomonas* keratitis. Curr. Eye Res. **5:**503–510.
33. **Twining, S. S., S. E. Kirschner, L. A. Mahnke, and D. W. Frank.** 1993. Effect of *Pseudomonas aeruginosa* elastase, alkaline protease, and exotoxin A on corneal proteinases and proteins. Investig. Ophthalmol. Vis. Sci. **34:**2699–2712.
34. **Twining, S. S., K. M. Lohr, and J. M. Moulder.** 1986. The immune system in experimental *Pseudomonas* keratitis: model and early effects. Investig. Ophthalmol. Vis. Sci. **27:**507–515.
35. **Woods, D., S. J. Cryz, R. L. Friedman, and B. H. Iglewski.** 1982. Contribution of toxin A and elastase to virulence of *Pseudomonas aeruginosa* in chronic lung infection of rats. Infect. Immun. **36:**1223–1228.
36. **Wretlind, B., L. Sjoberg, and T. Wadstrom.** 1977. Protease-deficient mutants of *Pseudomonas aeruginosa*: pleiotropic changes in activity of other extracellular enzymes. J. Gen. Microbiol. **103:**329–336.

Editor: A. O'Brien

Downloaded from http://iai.asm.org/ on September 25, 2020 by guest