Elastase Deficiency Phenotype of *Pseudomonas aeruginosa* Canine Otitis Externa Isolates

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Received 28 August 2000/Returned for modification 25 January 2001/Accepted 14 February 2001

*Pseudomonas aeruginosa* veterinary isolates were assayed for elastase and total matrix protease activity. The elastase activity of canine ear isolates was much less than that of strain PAO1 and that of all other veterinary isolates (*P* < 0.0001). The results indicate that canine ear isolates have a distinct elastase phenotype.

*Pseudomonas aeruginosa* secretes several toxins and enzymes that enhance its virulence. Among the enzymes are three well-characterized proteases: two elastases (LasA and LasB) and an alkaline protease (AprA) (for reviews, see references 18 and 23). Two additional proteases have been reported: LasD (21) and protease IV (6). Each of these proteases has broad substrate specificity; in addition, the proteases often act synergistically to cleave connective tissues and immune system components. Connective tissues degraded by *P. aeruginosa* proteases include elastin, mediated by LasA and LasB, and collagen, mediated by alkaline protease and elastases (18, 23). Proteases, either individually or synergistically, mediate Hageman factor activation (11), immunoglobulin and complement degradation (5, 9, 13, 25), cytokine inactivation (22), and host protease activation (32).

The contribution of *P. aeruginosa* proteases to the pathogenesis of acute infections is well documented (for a review, see reference 19). In particular, Tang et al. (31) found that a genetically defined, protease-deficient strain was virtually avirulent compared to the parental strain in a mouse model of acute pneumonia. Interestingly, the protease-deficient strain and the parental strain colonized similar numbers of mice in this study (31).

The contribution of proteases to chronic infection is more controversial. The role of *P. aeruginosa* proteases in chronic infection is best studied in cystic fibrosis (for reviews, see references 3, 4, 10, 16, and 30). *P. aeruginosa* proteases and *lasB* and *lasA* mRNA have been detected in cystic fibrosis lung sputa (14, 27); however, other studies implicate host neutrophil elastase over bacterial elastases in cystic fibrosis lung pathology (2, 33).

Reflecting the relative contribution of proteases to virulence, *P. aeruginosa* strains express levels of proteases that vary with isolation site and disease (34). The mucoid strains that characterize cystic fibrosis isolates are known to secrete less elastase than nonmucoid strains (20, 34). Woods et al. (34) found that the frequency of protease production from cystic fibrosis isolates was significantly lower than that from isolates from other sites. In contrast, the levels of protease activity from blood isolates and elastase from acute pneumonia sputum isolates were significantly higher than levels from other infection sites (34).

Most of the research regarding *P. aeruginosa* virulence factor production in disease has focused on human serology, isolates, or samples. In contrast, little is known about the virulence phenotypes of animal isolates. In this study, we surveyed animal intestinal and fecal *P. aeruginosa* isolates for protease activity. In addition, we sought to determine if *P. aeruginosa* associated with acute or chronic animal diseases displayed protease phenotypes comparable to those displayed by *P. aeruginosa* associated with acute or chronic human diseases. We used colorimetric assays to detect in vitro elastase and total matrix protease activities semiquantitatively and included well-characterized human wound isolate PAO1 (12) as an internal control. Interestingly, while total matrix protease activity among animal isolates was comparable to that of *P. aeruginosa* PAO1, we found that *P. aeruginosa* isolates from canine ear infections exhibited significantly lower elastase activity when cultured in vitro than strain PAO1 or isolates from all other animal sources.

*P. aeruginosa* isolates were collected at the North Dakota Veterinary Diagnostic Laboratories over the course of 4 years. Isolates were presumptively identified as *P. aeruginosa* based on colony morphology, odor, and reactions (k/k) on triple sugar iron agar slants. Suspect colonies were inoculated onto King B agar and grown overnight at 37°C. Isolates displaying the typical fluorescence of *P. aeruginosa* were positively identified using Sensititre technology (Accumed International, Inc., Westlake, Ohio) with AP80-VET Gram-ID plates (Trek Diagnostic Systems, Inc., Westlake, Ohio). All isolates were typed with 98% or greater probability.

Forty-four isolates were assayed for protease and elastase activity; of these, 16 were from canine chronic ear infections. The hosts and tissue sources of the noncanine isolates are given in Table 1. About 30% (13 of 44) were characterized as normal flora of the gastrointestinal tract unrelated to the diagnosis, 27% (12 of 44) were characterized as causative agents or secondary pathogens of acute infection, and 43% (19 of 44) were characterized as causative agents or secondary pathogens of chronic infection. Of the isolates from chronic infections, 84% (16 of 19) were from canine ear infections of otherwise healthy pets.
**Total matrix protease activity.** *P. aeruginosa* PAO1 and veterinary isolates were cultured in 10 ml of Luria-Bertani broth at 37°C overnight and subcultured by inoculating fresh, prewarmed broth to an optical density at 640 nm of 0.001. Culture densities from early-stationary-phase secondary cultures were read at 640 nm to ensure comparable levels of growth between isolates and *P. aeruginosa* PAO1. The culture was harvested, and the supernatant was clarified by centrifugation at 20,800 g for 5 min. Two microliters of culture supernatant was added to triplicate tubes of 20 mg of hide powder azure (Sigma Chemical Co., St. Louis, Mo.) suspended in 2 ml of 10 mM sodium-HEPES (pH 7.5)–0.5 mM CaCl₂ and rotated for 2 h at 37°C. An assay tube without culture supernatant was rotated to subtract background absorbance. Insoluble substrate was pelleted by centrifugation at 150 × g for 15 min. Absorbance of the assay supernatant was read at 595 nm. The average and sample standard deviation were calculated and expressed as a fraction of the triplicate assay average of the internal control, *P. aeruginosa* PAO1. Strains exhibiting low levels or an absence of matrix protease activity were typically cultured for activity twice (six or more replicate assays).

The results of matrix protease activity assays of canine otitis externa isolates are shown in Fig. 1. An absorbance of less than half that of *P. aeruginosa* PAO1 in the hide powder azure assay corresponds to less than 10% of the activity of strain PAO1, based on assays of a serial dilution of PAO1 supernatant. In all, 25% (4 of 16) of the canine ear isolates exhibited less than half the assay absorbance of *P. aeruginosa* PAO1 in the hide powder azure assay, compared to only 3.5% (1 of 29) of isolates from other sources showing low matrix protease activity. Fisher’s exact test (1) yields a two-sided *P* value of 0.0468, leading us to marginally reject the null hypothesis of equal proportions of isolates with low matrix protease activity at the 95% confidence level. Thus, a higher proportion of canine ear isolates than of isolates from other sources may have low matrix protease activity, but this difference in proportions was not significant.

**TABLE 1.** *P. aeruginosa* noncanine animal isolates used in this study

| Isolate | Host | Source | Infection type* |
|---------|------|--------|----------------|
| 273 Lg  | Bovine | Lung   | A              |
| 313 I   | Bovine | Intestine | N              |
| 325     | Bovine | Liver   | A              |
| 334 Ab  | Bovine | Abomasum | A              |
| 406 L   | Bovine | Milk    | C              |
| 461     | Bison  | Lymph node, nose | A              |
| 469 ISm | Bovine | Small intestine | N            |
| 469 LSmI| Bovine | Lung    | A              |
| 1590    | Bovine | Feces   | N              |
| 2341    | Bovine | Intestine and feces | N             |
| 2714    | Bovine | Feces   | N              |
| 2732    | Bovine | Intestine | N              |
| 2733    | Bovine | Intestine | N              |
| 2738    | Equine | Placenta | A              |
| 2803    | Bovine | Intestine | N              |
| 2836    | Bovine | Lung    | A              |
| 2875    | Bovine | Brain, liver, lymph node | A            |
| 2905    | Bovine | Intestine | N              |
| 4143    | Bovine | Feces   | N              |
| 4299    | Equine | Feces   | N              |
| 4987    | Bovine | Lung    | A              |
| 7555    | Bovine | Milk (possible fecal origin) | N or C       |
| 7563    | Canine | Skin swab | C              |
| 7936    | Canine | Pustule swab | C            |
| 8001    | Mink   | Lung    | A              |
| 8003SWS | Raccoon | Lung, mesenteric lymph node | A           |
| 8003LGS | Raccoon | Lung, mesenteric lymph node | A           |

* C, chronic, localized; A, acute opportunistic, primary or secondary pathogen; N, normal flora.
The distributions of matrix protease activity for canine ear isolates and isolates from other sources are represented by box plots (Fig. 1A) and scatter plots (Fig. 1B). This figure suggests that the matrix protease activity for isolates from canine sources is more variable than that for isolates from other sources but that the average activity levels for isolates from the two sources are equivalent. We compared mean matrix protease activity between isolates from canine sources and from other sources using Student’s t tests (26). Hypothesis tests confirm these impressions, as we reject the null hypothesis of equal variances between isolates from the two sources (F* = 5.32; P < 0.0001) but we do not reject the null hypothesis of equal means (t = 0.32; P = 0.7538).

Elastase activity. *P. aeruginosa* PAO1 and veterinary isolates were cultured and supernatant was clarified as described above. Two microliters of culture supernatant was added to triplicate tubes of 20 mg of elastin-Congo red (Elastin Products, Inc., Owensville, Mo.) suspended in 2 ml of 10 mM sodium phosphate buffer, pH 7.0, and rotating the mixture overnight at 37°C (24). The absorbance of the assay supernatant was read at 495 nm after subtracting background absorbance. Due to the prolonged incubation of this assay mixture, the comparisons with *P. aeruginosa* PAO1 elastase activity are not intended to reflect a linear relationship but merely relative absorbance readings of the assay. Canine ear isolates were assayed twice or more in independent experiments. The highest absorbance reading of each isolate relative to *P. aeruginosa* PAO1 was used for statistical analysis.

In all, 75% (12 of 16) of the canine ear isolates exhibited less than half the assay absorbance of *P. aeruginosa* PAO1, compared to only 10.3% (3 of 29) of the isolates from other sources showing low elastase activity (Fig. 2). We used Fisher’s exact test (1) to test the null hypothesis of equal proportions of canine isolates versus other isolates having low elastase activity (where low elastase activity is defined as less than one-half the assay absorbance of *P. aeruginosa* PAO1). Fisher’s exact test yields a two-sided P value of 0.00002, leading us to strongly reject the null hypothesis of equal proportions of isolates with low elastase activity at the 95% confidence level.

The distributions of elastase activity for isolates from canine and other sources are represented by box plots (Fig. 2A) and scatter plots (Fig. 2B). In contrast to the total matrix protease results, the elastase activities for isolates from canine and other sources show approximately the same amounts of variability but the average activity levels for isolates from the canine source appear to be somewhat lower than those for isolates from other sources. We compared mean elastase activities between isolates from canine and other sources using Student’s t tests (26). Hypothesis tests confirm these observations as we do not reject the null hypothesis of equal variances between the two sources (F* = 1.10; P = 0.8029) but we do reject the null hypothesis of equal means (t = 5.68; P < 0.0001). In addition to having a lower mean than isolates from other sources, canine ear isolates cluster at high and low levels in a bimodal fashion (Fig. 2B). This clustering indicates that a subpopulation of canine ear isolates has typical levels of elastase activity, while the majority of isolates exhibit low activity levels and lower the mean for canine ear isolates as a population.

Twelve isolates that gave elastin-Congo red readings of <0.1 absorbance units at 495 nm were further characterized for an elastase-negative phenotype. Isolates were inoculated onto elastin nutrient agar, a more sensitive but qualitative assay for elastase activity (24). The zones of elastolysis were compared to those for strain PAO1 and strain PAO-R1 (an elastase-negative strain [7]). Lack of zones indicated an elastase-negative phenotype. Only 3 of the 12 isolates, 2 of them canine ear isolates, were elastase negative (data not shown). One canine ear isolate was elastase negative yet exhibited low levels of
matrix protease activity, while the other two elastase-negative isolates were also negative for matrix protease activity.

The elastase-negative isolates may have null mutations in the structural coding region or promoter or in regulatory or processing genes. Alternatively, the elastases may be produced but not active on the elastin-Congo red substrate. Likewise, several possibilities for low elastase activity exist. These isolates may produce or secrete elastase at suboptimal levels due to an alteration in one of the elastase regulatory or secretion pathways. An alteration in an elastase pathway as opposed to an alkaline protease pathway may account for the phenotypic difference between canine ear isolates and other animal isolates. Alternatively, the elastase-deficient isolates may produce a less active elastase enzyme(s). While mucoidy has been inversely correlated with protease production (17, 20), it is worth noting that none of the canine ear isolates exhibited a mucoid phenotype in vitro.

These possibilities raise the question of why canine ear isolates would harbor an elastase phenotype distinct from that of most other isolates. First, host factors may provide the stimuli to induce or repress expression of the various virulence factors from a population of diverse, pluripotent P. aeruginosa strains. Adaptation to the infection site may affect virulence factor production from early in vitro cultures. In particular, Hamood et al. (8) found that elevated exoenzyme S production from wound and urinary tract infection isolates was subsequently reduced by continuous in vitro subculturing. Second, as suggested by Sundström et al. (29), a subset of P. aeruginosa strains may colonize particular infection sites: strains isolated from human external otitis exhibited distinctive biochemical profiles and pigmentation compared to those isolated from varicose ulcers and urinary tract infections. In addition, Sundström et al. (28) found that the adhesion of P. aeruginosa external otitis isolates to guinea pig epithelial cells was significantly increased compared to that of isolates from leg ulcers or urinary tract infections. The authors conclude that P. aeruginosa strains causing human external otitis can be considered as having a particular phenotype. Third, strain selection may be at play. Strain selection would harbor an elastase phenotype distinct from that of most other isolates. First, host factors may provide the stimuli to induce or repress expression of the various virulence factors from a population of diverse, pluripotent P. aeruginosa strains. Adaptation to the infection site may affect virulence factor production from early in vitro cultures. In particular, Hamood et al. (8) found that elevated exoenzyme S production from wound and urinary tract infection isolates was subsequently reduced by continuous in vitro subculturing. Second, as suggested by Sundström et al. (29), a subset of P. aeruginosa strains may colonize particular infection sites: strains isolated from human external otitis exhibited distinctive biochemical profiles and pigmentation compared to those isolated from varicose ulcers and urinary tract infections. In addition, Sundström et al. (28) found that the adhesion of P. aeruginosa external otitis isolates to guinea pig epithelial cells was significantly increased compared to that of isolates from leg ulcers or urinary tract infections. The authors conclude that P. aeruginosa strains causing human external otitis can be considered as having a particular phenotype. Third, strain selection may be at play.

We thank the North Dakota Veterinary Diagnostic Laboratory, especially Darlene Krogh, Ronda DeVold, and Lynn Schaan, for P. aeruginosa isolation and preliminary identification and for instruction on the use of the Sensititre. We also thank Heather Hertz, Rusty Rybolt, Karen Lone Fight, and Troy Wegman for protease assays and record retrieval.

This work was funded by the North Dakota Agricultural Experiment Station and NIH grant 1 R15 AI46506-01.

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