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Phenotypic Comparison of *Pseudomonas aeruginosa* Strains Isolated from a Variety of Clinical Sites

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***Pseudomonas aeruginosa* elaborates a number of extracellular products which have been shown to play a role in the pathogenesis of disease caused by this organism. In this study, we showed that the host environment markedly affects the levels of exoproducts produced. We compared the phenotypes of a number of *P. aeruginosa* strains obtained from a variety of clinical sources, including burn wounds, skin wounds, urine, cystic fibrosis sputum, acute pneumonia sputum, and blood. The clinical isolates were examined quantitatively for levels of total protease, elastase, phospholipase C, exotoxin A, and exoenzyme S produced in vitro under defined conditions. The exoproduct levels varied significantly, depending on the site of isolation. Elevated levels of elastase were demonstrated in strains isolated from acute lung infections, phospholipase C levels were elevated in urinary tract and blood isolates, exotoxin A levels were elevated in blood isolates, and exoenzyme S levels were increased in acute pneumonia isolates. Isolates from cystic fibrosis sputum produced low amounts of virtually all of the tested exoproducts, particularly as compared with sputum isolates from acute *P. aeruginosa* lung infections.**

As with a number of bacterial pathogens, the virulence of *Pseudomonas aeruginosa* is multifactorial and is the product of many interacting variables, involving both the bacterium and the host. These multiple interactions no doubt contribute to the broad spectrum of disease caused by *P. aeruginosa*, which ranges from acute infections in burn victims and neutropenic patients to chronic infections in the lungs of cystic fibrosis (CF) patients (28).

Damage to host tissue may occur directly through the action of bacterial products or indirectly through the induction of an overexuberant host response to these products. *P. aeruginosa* may injure host tissue through either or both of these mechanisms, depending on the underlying disease of the host and the anatomic location of the infection. With respect to its marked necrotizing activity, *P. aeruginosa* has been shown to produce a variety of potential tissue-damaging factors; however, the exact role they play in disease is only beginning to be understood.

In addition to cell-associated factors, *P. aeruginosa* elaborates a number of exoenzymes, including exotoxin A, proteolytic enzymes, phospholipase C, and exoenzyme S, each of which has been implicated as a pathogenic determinant in infections caused by this organism. Information obtained from recent studies employing techniques of microbial genetics, immunology, and analytical biochemistry increasingly points to the significance of these exoenzymes as virulence factors in *P. aeruginosa* infections (23, 30).

Exotoxin A has been shown to be a pathogenic determinant in experimental infections of the eye (11, 20) and burn wounds (22, 23, 25) and in chronic lung infections caused by *P. aeruginosa* (29). The presence of antibody to exotoxin A has been demonstrated in human sera (13, 16), and initial high titers of antibody to exotoxin A and lipopolysaccharide have been correlated with an improved prognosis in bacteremic patients (8). Approximately 80 to 90% of all *P. aeruginosa* clinical isolates produce exotoxin A in vitro (5), compared with only 27% of *P. aeruginosa* environmental isolates (24).

Protease activity among most *P. aeruginosa* strains is well established (15). Morihara was the first to characterize the proteases found in *P. aeruginosa* culture filtrates when he identified an alkaline protease and an elastase (18). Recent studies showed that alkaline protease and elastase are associated with virulence in *P. aeruginosa* infections in a burned mouse model (25). Elastase has been shown to contribute to pathogenesis in acute and chronic lung infections (6, 29) but not in mouse corneal infections (21), presumably owing to the absence of elastin in the mouse cornea. In the latter case, it is presumed that alkaline protease plays a significant role (21). The intratracheal administration of purified proteases in rabbits elicits extensive, grossly observable lung damage consisting of epithelial and endothelial cell necrosis with a pronounced inflammatory reaction (10).

Phospholipase C is a lecithinase which liberates phosphorylcholine from lecithin (2). It has been reported that blood culture isolates produce greater quantities of phospholipase C than do nonbacteremic isolates (1). However, a correlation was not demonstrated between in vitro quantities of phospholipase C and patient prognosis. The study suggested a local rather than systemic role for phospholipase C in *P. aeruginosa* pathogenesis (1). This was confirmed by Granström et al. (9), who showed that phospholipase C plays a role in the colonization of airways in CF patients. It has also been speculated that a lecithinase in the lungs of *P. aeruginosa* pneumonia patients has the potential to be a virulence factor, because the normal surfactant lining the lungs and preventing atelectasis is lecithin (2).

In addition to toxin A, *P. aeruginosa* produces a second ADP ribosyl (ADPR) transferase enzyme, termed exoenzyme S (12). Exoenzyme S differs from toxin A in that it

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does not ADP ribosylate elongation factor-2 (EF-2) but rather modifies one or more different proteins present in eucaryotic cell extracts. The role of exoenzyme S in human infections has not been determined. An increased mortality rate has been shown to be associated with patients infected with *P. aeruginosa* strains which produce both exoenzyme S and exotoxin A, as compared with strains producing only exoenzyme S or exotoxin A (26). Exoenzyme S was shown to be produced in vivo in a burned mouse model (3) and more recently was shown to play a significant role in burn infections caused by *P. aeruginosa* in the mouse model (19). Using transposon-induced mutants singly deficient in exoenzyme production, we recently showed that exoenzyme S may be required for maximum virulence in *P. aeruginosa* chronic lung infections (31). The extensive pathology and increased mortality rate seen with exoenzyme S-producing strains indicate that exoenzyme S plays a prominent role in the progressive pulmonary pathology associated with chronic *P. aeruginosa* lung infection.

A number of environmental factors have been shown to influence the production of *P. aeruginosa* exoproducts. The production of exotoxin A, elastase, and alkaline protease is independently regulated by iron (4). P_i, cations, and the nature of the carbon source have been found to affect the production of phospholipase C (2). The purpose of the present study was to determine the effects of the host environment on the production of exoenzymes by *P. aeruginosa*. *P. aeruginosa* strains from a variety of clinical isolation sites were examined quantitatively for levels of total protease, elastase, phospholipase C, exotoxin A, and exoenzyme S produced in vitro under defined conditions.

MATERIALS AND METHODS

Twenty-two *P. aeruginosa* strains isolated from each of the following infection sites, for a total of 132 strains, were used in this study: burn wounds, skin wounds, urine, CF sputum, acute pneumonia sputum, and blood. The strains were obtained directly from the primary isolation media used for culture at the Foothills Hospital Clinical Microbiology Laboratory, Calgary, Alberta, Canada, which in most cases was MacConkey agar. Smooth-colony types of the organisms were transferred directly into the media used for the assay of extracellular products.

Proteolytic activity was measured in cell culture supernatants of organisms grown for 18 h at 37°C in brain heart infusion broth (Difco Laboratories, Detroit, Mich.). The A_{540} was measured for each culture and adjusted to 2.0 by using sterile culture medium. Elastase activity was measured by using elastin-congo red (Sigma Chemical Co., St. Louis, Mo.) as substrate (4). Total proteolytic activity was quantitated by using Hide powder azure (Sigma) as substrate. Culture supernatants were diluted in 10 mM Tris (pH 7.5). Samples (3 ml) of the supernatants were incubated with 15 mg of Hide powder azure at 37°C for 1 h while being shaken vigorously. The undissolved substrate was removed by centrifugation (3,000 × *g*, 10 min). Protease activity was determined by reading the A_{595} of the supernatants. The blank containing Tris buffer and Hide powder azure was processed identically.

Phospholipase C activity was measured by the method of Berka and Vasil (2). Isolated colonies from primary media were used to inoculate 2 ml of tryptose minimal medium in capped test tubes (16 by 150 mm). The tubes were shaken vigorously at 32°C for 24 h, and the A_{540} of each culture was measured and adjusted to 2.0 by using sterile culture medium. Cells were removed by centrifugation (10,000 × *g*, 20 min), and to 1 ml of culture supernatant 10 mg of decolorizing carbon was added to remove interfering pigment. After centrifugation (5,000 × *g*, 10 min), 10 μl of the clear supernatant fluid was added to 90 μl of a solution containing 250 mM Tris buffer (pH 7.2), 60% glycerol, 1.0 μM $ZnCl_2$, and 10 mM *p*-nitrophenylphosphorylcholine (Sigma) in individual wells of a microtiter test plate (96 wells per plate). The plates were incubated at 37°C for 1 h, and the A_{405} was measured spectrophotometrically in a MicroElisa Autoreader (Dynatech Industries, Inc., McLean, Va.).

ADPR transferase activity was measured in the supernatants of organisms grown for 18 h at 32°C. The medium used for the measurement of toxin A was Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) dialysate which had been treated with Chelex (Bio-Rad Laboratories, Richmond, Calif.) to remove iron; the medium was supplemented with 1% glycerol and 100 mM monosodium glutamate (4). For exoenzyme S production, organisms were grown in M9 mineral salts medium supplemented with 5 mM EDTA (disodium salt), 100 mM sodium succinate, 1% glycerol, and 100 mM monosodium glutamate (S medium; 31). The A_{540} for 10-ml cultures of each of the strains in each of the above media was adjusted after incubation to 2.0 by using sterile media, and the cells were removed by centrifugation (10,000 × *g*, 20 min). Exoenzyme S activity was measured by using wheat germ extract as substrate (26). Reaction mixtures consisted of 10 μl of supernatant, 25 μl of buffer (50 mM Tris, [pH 7.2], 0.1 mM EDTA, 40 mM dithiothreitol), 25 μl of wheat germ extract, and 5 μl of [^{14}C]NAD (specific activity, 280 mCi/mM; 25 μCi/ml; Amersham Corp., Arlington Heights, Ill.). The mixtures were incubated at 25°C for 30 min, and the reaction was terminated by the addition of 10% trichloroacetic acid. The precipitates were collected by filtration and counted as previously described (7). Exotoxin A activity was measured as described for exoenzyme S, except that supernatants were activated with 4 mM urea and 1% dithiothreitol for 20 min at 24°C before assay (3). The results reported for exoenzyme S activity represent the remaining counts after reaction with anti-toxin A antiserum developed in rabbits (29). The results reported for exotoxin A activity represent the remaining counts after reaction with anti-exoenzyme S monoclonal antibody (D. E. Woods, Abstr. Annu. Meet. Am. Soc. Microbiol. 1985, B150, p. 43).

To determine the incidence of exoenzyme S and exotoxin A antigen production by each of the strains from various clinical isolation sites, an immunoblot assay procedure was used (27). Culture supernatants were electrophoresed on 12% sodium dodecyl sulfate-polyacrylamide by the method of Laemmli (17). After electrophoretic transfer to nitrocellulose paper, samples were reacted with 100 μl of a 1:100 dilution of antibody to either exotoxin A or exoenzyme S for 1 h at 37°C. After thorough washing, the immunoblots were reacted with protein A conjugated with horseradish peroxidase (1:100 dilution, 1 h at 37°C; Sigma) and again washed thoroughly, and the immunoblots were developed with *o*-dianisidine (Sigma).

RESULTS AND DISCUSSION

The frequencies of production of phospholipase C, exotoxin A, and exoenzyme S by *P. aeruginosa* strains isolated from burns, urine, CF patient sputum, acute pneumonia sputum, and blood were not significantly different when compared on the basis of isolation site (chi-square

TABLE 1. Comparison of the in vitro production of total protease, elastase, phospholipase C, exotoxin A, and exoenzyme S by *P. aeruginosa* isolates obtained from burns, wounds, urine, CF sputum, acute pneumonia sputum, and blood

Isolation source	Exoproduct[a]				
	Total protease[b]	Elastase[c]	Phospholipase C[d]	Exotoxin A[e]	Exoenzyme S[f]
Burns	0.104 ± 0.037	0.024 ± 0.008	0.075 ± 0.007	304 ± 34	472[g] ± 82
Wounds	0.029[g] ± 0.006	0.022 ± 0.007	0.085 ± 0.010	353 ± 37	2,055 ± 690
Urine	0.117 ± 0.047	0.029 ± 0.008	0.120[g] ± 0.019	288 ± 43	1,346 ± 460
CF sputum	0.067 ± 0.030	0.017 ± 0.007	0.062 ± 0.006	317 ± 30	2,066 ± 927
Acute pneumonia sputum	0.071 ± 0.029	0.053[g] ± 0.021	0.057 ± 0.008	357 ± 40	4,352[g] ± 1,319
Blood	0.214[g] ± 0.046	0.014 ± 0.003	0.102[g] ± 0.011	527[g] ± 98	1,913 ± 551

[a] Values represent means ± standard error of the mean.

[b] Total protease measured by Hide powder azure assay. Values given are milligrams of protease per milliliter of culture supernatant and were derived from a standard curve constructed from values obtained by adding known amounts of *P. aeruginosa* alkaline protease (Nagase Biochemicals, Ltd., Tokyo, Japan) to the assay.

[c] Elastase measured by elastin-congo red assay (4). Values given are units of elastase activity per milliliter of culture supernatant. One unit is defined as 1.0 A_{495} unit; the data represent relative values for elastase activity among *P. aeruginosa* isolates.

[d] Phospholipase C activity measured by *p*-nitrophenylphosphorylcholine assay (2). Values given are units of phospholipase C activity per milliliter of culture supernatant. One unit is defined as 1.0 A_{495} unit, and the data represent relative values for phospholipase C activity among *P. aeruginosa* isolates.

[e] Exotoxin A measured by ADPR transferase assay (4). Values given are dpm/10 μl of culture supernatant and represent relative values for exotoxin A activity among *P. aeruginosa* isolates. dpm represents those remaining counts following reaction with anti-exoenzyme S monoclonal activity.

[f] Exoenzyme S activity measured by ADPR transferase assay (24). Values given are dpm/10 μl of culture supernatant and represent relative values for exoenzyme S activity among *P. aeruginosa* isolates. dpm represents those remaining counts after reaction with anti-exotoxin A antiserum developed in rabbits.

[g] Significantly different from grand mean (analysis of variance, one-way).

analysis), nor were the frequencies different from previously published results (1, 5, 26). In agreement with previously published results, the incidence of exotoxin A production determined by assay of ADPR transferase activity was identical to that obtained by imunoassay (5). Similarly, the incidence of exoenzyme S production determined by assay of ADPR transferase activity was in agreement with previous studies (26). In the present studies, we examined the incidence of exoenzyme S production by an immunoblot assay and found a significantly increased value for the frequency of exoenzyme S production. The value obtained by enzyme assay was 44%, whereas immunoblot assay for exoenzyme S incidence in clinical isolates gave a value of 90.9% ($P < 0.01$, chi-square).

The frequency of protease production was significantly lower in the groups of strains isolated from CF sputum when compared with the frequency of protease production by strains isolated from other sites ($P < 0.001$, chi-square), regardless of whether measured as total protease or elastase activity. The incidence of protease production in strains isolated from CF sputum was approximately 41%, whereas in strains isolated from other infection sites the incidence exceeded 90% in all cases.

The levels (mean ± standard error of the mean) of total protease, elastase, phospholipase C, exotoxin A, and exoenzyme S produced in vitro by *P. aeruginosa* strains isolated from burns, wounds, urine, CF patient sputum, acute pneumonia sputum, and blood are shown in Table 1. Statistical analysis of the data (analysis of variance, one-way) revealed significant differences in the mean levels of exoproducts produced by *P. aeruginosa* strains isolated from different infection sites. Significantly elevated levels of total protease activity were produced by strains isolated from blood cultures (0.214 mg/ml, $P < 0.001$), and significantly decreased levels of total protease activity were produced by strains isolated from wound cultures (0.029 μg/ml, $P < 0.001$). Strains isolated from sputum cultures of patients with acute *P. aeruginosa* pneumonia produced significantly higher levels of elastase (0.053 U/ml, $P < 0.01$) than strains isolated from the other infection sites. Elevated levels of phospholipase C were produced by strains isolated from urine (0.120 U/ml, $P < 0.01$) and blood (0.102 U/ml, $P < 0.01$). *P. aeruginosa* strains isolated from blood cultures produced significantly higher levels of exotoxin A in vitro (527 dpm/10 μl, $P < 0.01$) than strains isolated from any of the other isolation sites examined. Exoenzyme S activity was significantly elevated in strains isolated from sputum cultures of patients with acute *P. aeruginosa* pneumonia (4,352 dpm/10 μl, $P < 0.01$) and significantly decreased in strains of *P. aeruginosa* isolated from burn infections (472 dpm/10 μl, $P < 0.001$).

Serotyping of *P. aeruginosa* isolates was performed by using commercial antisera obtained from Difco. No correlation between serotype and site of isolation or between serotype and exoproduct levels could be demonstrated. Regression analysis (two-tailed, critical r value = 0.4217) did reveal, however, a positive correlation between exotoxin A and exoenzyme S production in strains isolated from burn infections ($r = 0.6329$). This most likely reflects the low levels of each of these exoproducts produced by *P. aeruginosa* strains isolated from burn infections.

A positive correlation was also demonstrated between elastase and exotoxin A production by strains isolated from CF patient sputum ($r = 0.6165$) and between exotoxin A and exoenzyme S production in these same strains ($r = 0.4336$). Again, these results most likely reflect the low levels of these exoproducts produced by *P. aeruginosa* strains isolated from this infection site, and this might explain the chronic nature of the infection in CF patients. The heterogeneity seen in protease production by CF patient sputum isolates of *P. aeruginosa* could possibly be explained by a concomitant heterogeneity in the clinical status of the CF patients from whom the strains were isolated. Recent studies in our laboratory have attempted to correlate in vitro levels of exoproducts released by *P. aeruginosa* with clinical status in CF patients. Preliminary results demonstrated a direct correlation between diminished clinical status and infection with *P. aeruginosa* strains which produce elevated levels of exotoxin A and exoenzyme S in vitro. Studies to correlate in vitro protease and phospholipase C levels with clinical status in CF patients are ongoing.

The elevated levels of total protease, phospholipase C,

and exotoxin A produced in vitro by *P. aeruginosa* strains isolated from blood cultures argue for a role for those exoproducts in promoting bacteremia. These results are in agreement with those obtained by Baltch et al. (1) and Janda et al. (14), who found that *P. aeruginosa* isolates producing elevated exoproduct levels are more capable of invading tissues locally and result in bacteremia more commonly. Similarly, the elevated levels of elastase and exoenzyme S produced in vitro by *P. aeruginosa* strains isolated from sputum cultures of patients with acute pneumonia indicate that these exoproducts may play a role in the pathogenesis of lung infection caused by *P. aeruginosa*. These findings confirmed the observations made from a number of animal model studies which indicated that these exoproducts may play a significant role in lung infections (6, 29, 31).

The finding of significantly low levels of exoenzyme S production by *P. aeruginosa* strains isolated from burn infections conflicts with previously published results from animal model studies which predicted that exoenzyme S is an important virulence factor in burn infections caused by *P. aeruginosa* (19). These studies were performed with a single parental strain and an isogenic mutant deficient in exoenzyme S production in a mouse burn model. It appears that further studies with additional strains and different animal species are necessary to determine the role of exoenzyme S in burn infections.

In summary, the present studies confirmed previous observations that the virulence of *P. aeruginosa* is multifactorial; however, the results indicate that certain exoproducts may play a more important role in certain types of infection. In particular, the indications are that proteolytic activity promotes bacteremia and is important in lung infections, exotoxin A promotes bacteremia, phospholipase C also promotes bacteremia and may play a role in urinary tract infections, and exoenzyme S is a significant pathogenic determinant in pulmonary infections caused by *P. aeruginosa*.

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