GENETIC STUDIES ON THE CONTRIBUTION OF TOXIN A AND ELASTASE TO THE VIRULENCE OF *Pseudomonas aeruginosa*

by

Dennis Edward Ohman

A THESIS

Presented to the Department of Microbiology and Immunology
and the Graduate Division
of the
University of Oregon Health Sciences Center
in partial fulfillment of
the requirements for the degree of

Doctor of Philosophy
January 1980

APPROVED:

(Professor in Charge of Thesis)

(Chairman, Graduate Council)

TABLE OF CONTENTS

LIST OF TABLES

Paper 1

Paper 2

Paper 3

Paper 4

Appendix A

Appendix B

LIST OF FIGURES

Appendix A

Figure

I. Introduction and Statement of the Problem

Pseudomonas aeruginosa is ubiquitous; it is found in soil, fresh water, and virtually anywhere in the human habitat. One very significant ecologic niche is the hospital. *P. aeruginosa* is unique among human pathogens in that it infects not only other vertebrates, both warm and cold blooded, but lower animals, both terrestrial and aquatic, as well as insects and plants. *P. aeruginosa* can multiply on numerous and varied organic compounds. Given this ubiquity and versatility, it is fortunate that *P. aeruginosa* seldom if ever successfully attacks a normal individual. However, in individuals whose defenses are compromised, *Pseudomonas* can produce severe and often life-threatening infections.

P. aeruguinosa produces a variety of extracellular products which may play a significant role in its pathogenesis. Toxin A and extracellular proteases are likely candidates as major virulence factors of *P. aeruginosa*. However, the role and interaction of these factors in the pathogenesis of *Pseudomonas* infections has not yet been clarified.

The objective of this study is to use genetic techniques to evaluate the contribution of toxin A and elastase (protease II) to the virulence of *P. aeruginosa*. The specific aims of this research are:

i. Develop methods for the isolation of mutants deficient in toxin A and elastase.

ii. Isolate toxin A and elastase mutants of two virulent strains which posses the phenotypes (toxin A^+ $elastase^+$) and (toxin A^+ $elastase^-$).

iii. Characterize the mutants in vitro in an effort to determine the nature of the mutations.

iv. Determine the effects of mutations that are apparently specific for toxin A or elastase on the virulence of P. aeruginosa in:

a. The mouse corneal infection model

b. The mouse burn infection model

II. Literature Review

A. Morphology and Physiology of P. aeruginosa

The cells of P. aeruginosa are typically straight rods which vary in length from 1 to 3 μm and from 0.5 to 1 μm in width. P. aeruginosa is motile due to the presence of a single polar flagella, and pili are inserted in a polar fashion (103). P. aeruginosa, like most other gram-negative organisms, has a cell-envelope structure consisting of an outer membrane, a peptidoglycan layer, and an inner cytoplasmic membrane. The proteins and lipopolysaccharide (LPS) components of the outer membrane complex have been isolated and characterized (118). Although Pseudomonas LPS has many properties in common with the LPS from members of the Enterobacteriaceae, important differences do exist as is evident by the fact that Pseudomonas LPS is considerably less toxic (27, 52, 53, 75).

Pseudomonas has an enormous metabolic potential. P. aeruginosa can grow on at least 80 different organic compounds, and it can even multiply in water with atmospheric CO_2 as its source of carbon (30). Pseudomonas is known to utilize carbohydrates, amino acids, alcohols, amines, amides and fatty acids (103), and has been found to grow in dilute solutions of antiseptics and disinfectants (30).

While most human pathogens are facultative anaerobes, P. aeruginosa is aerobic with a tricarboxylic acid cycle and electron transport system (19). However, in the absence of oxygen, nitrates can be utilized as the terminal electron acceptor which results in the formation of N_2 gas (19). Glucose and other hexoses are utilized

by the Entner-Douderoff pathway instead of the Embden-Myerhoff pathway (20).

To determine the epidemiological origin of P. aeruginosa strains associated with disease, a number of typing methods have been developed. Serotyping schemes which are based on antisera directed at the somatic "O" LPS antigens have been described by Habs (37), Verder and Evans (138), and Fisher et al. (35). Farmer and Herman (32) have developed a pyocin (bacteriocin) typing scheme. Bacteriocins are antibiotic-like substances produced by many bacteria, and pyocins are the bacteriocins produced by P. aeruginosa (31, 32, 48). In this procedure, standard P. aeruginosa indicator strains are tested for their sensitivity (inhibition of growth) to pyocins produced by the P. aeruginosa test strain. Bacteriophage typing schemes have been developed (9), but are still in the experimental stages and not ready for routine use (30). Other epidemiological markers for P. aeruginosa strains include the antibiotic susceptibility pattern (antibiogram) and biotyping. Biotyping includes the examination of an isolate's colony type, hemolysis reactions, pigment production, reactions in biochemical tests, growth on selective media, and utilization of certain carbon sources that are known to produce reactions which vary from strain to strain (9).

B. Genetic Organization of P. aeruginosa

The genome is the sum of the genetic information. In a bacterium, this is usually divided into two major structural entities, the chromosome and plasmids. Plasmids may be thought of as additional

smaller chromosomes that are often not essential to the replicative survival of the bacterium. Bacteriophages may also be considered as components of the bacterial genome or as genetic elements which interact with the bacterial genome. Conjugation and transduction, processes of chromosomal transfer, are found in P. aeruginosa and both have had a role in determining the genetic organization of this organism.

The chromosome of P. aeruginosa has been shown to be a single, circular, double-stranded DNA molecule with a molecular mass of 2.1×10^9 daltons (110). Such a molecule is large enough to code for approximately 2500 proteins. In terms of investigating the genetic constitution of the chromosome, conjugation is the most useful. It has been found that 15 to 30% of hospital isolates of P. aeruginosa carry plasmids that have chromosome mobilization ability (Cma). These plasmids are referred to as FP plasmids (50). The first plasmid used for mapping and the basic genetic analysis of P. aeruginosa was the plasmid FP2 (50). Techniques of recombinant analysis and interrupted mating were shown to be effective in locating the relative position of chromosomal genes (40). Strain PAO (50) has been used more extensively for genetic work than any other strain of this genus. Plasmid FP2 has only one origin of transfer (designated 0 min) on the PAO chromosome. Interrupted matings have mapped the region 0 to 40 min, but for more-distal markers (40 to 100 min), too few recombinants are obtained for accurate mapping (50).

The inability to map the late region of the P. aeruginosa PAO chromosome with FP2 stimulated the search for other plasmids with

Cma. Unfortunately, most of the Cma plasmids that were isolated have the same site of origin for chromosome transfer as FP2 (50). Plasmid FP5 (87), another widely used Cma plasmid, also has an origin of 0 min. Plasmid FP39 (111) may have a site of origin about 10 min proximal to that of FP2, but the absence of markers between the two sites of origin has prevented proof of this. Only one plasmid has been isolated, FP110, which shows a major site of origin at 25 min and transfers the chromosome in the opposite direction to FP2, FP5 and FP39 (50). But this has enabled the mapping of only one new marker on the chromosome at a few minutes proximal to the FP2 origin.

Over 100 plasmids which carry genes coding for resistance to certain antibiotics (R plasmids) have been described for P. aeruginosa, and these were classified into 10 incompatibility groups (60). Members of group 1 (or IncP-1) have a wide host range as well as the ability to promote chromosome transfer (45). In strain PAO, IncP-1 plasmids generally do not promote chromosome transfer, but a variant of plasmid R68 was found, R68.45 (38, 39), which did have this property. This plasmid has a higher frequency of chromosome transfer than FP2, and unlike FP plasmids, has a multiplicity of transfer origins.

Plasmid R68.45 contains the full R68 genome together with an additional segment of DNA about 1,800 base pairs long (13, 59). This additional segment, denoted ISP (i.e., insertion sequence - Pseudomonas) probably acts as a recognition site for some chromosomal DNA sequence, and this interaction initiates chromosome transfer (50). Recombinants of R68.45 mediated matings inherit only short donor chromosome fragments which are usually less than 10 min long. But

R68.45 has multiple sites of origin, and these short fragments come from all regions of the donor chromosome. The relative locations of a variety of markers have been determined with R68.45, FP2 and FP5; however, map distances in the distal region of the chromosome beyond 60 min still cannot be determined quantitatively.

Plasmids range in size from 2×10^6 to over 200×10^6 daltons (44). They can contribute a range of phenotypic properties to bacteria. Plasmids in Pseudomonas are of several types including FP plasmids, R plasmids which may confer multiple antibiotic resistance, degredative plasmids which code for the breakdown and/or utilization of certain organic compounds, and cryptic plasmids which have no known function but may play a role in plasmid evolution (18, 44). Relating the presence of a plasmid to a particular phenotypic character is first indicated by transmissibility of the character at conjugation, and secondly, when plasmid-curing techniques (mitomycin C treament or growth at high temperatures) result in loss of the particular character (46). However, very few plasmids in P. aeruginosa have ever been successfully cured (46).

Bacteriophages which propagate in P. aeruginosa are very common, and all strains are probably lysogenic for at least one phage (49). Phage E79 is virulent for strains which carry FP2, and this phage has been used successfully as a contraselective agent in interrupted matings (111). Transduction systems for genetic analysis of P. aeruginosa have utilized phages F116 (47, 71) and G101 (51). Both are generalized transducing phages of 41 and 38 megadaltons, respectively. These phages transduce markers at a frequency of 1×10^{-7} to

5×10^{-7} per plaque-forming unit (49). The size of the segment of bacterial chromosome transferred is about 1%. Transduction has been used extensively to establish the order of closely linked markers (29, 90, 112). It is of interest that the F116 prophage is not integrated into the bacterial chromosome but is located extrachromosomally, very much like a plasmid (46). Specialized transducing phages have not been reported for P. aeruginosa even though some prophages have a chromosomal location (16, 17, 72).

In some cases the presence of a prophage can confer new phenotypic aspects upon the bacterium which carries it (5). This process, known as lysogenic conversion, has been observed in P. aeruginosa by changes in the cell wall (46) and other phenotypic effects (82).

In P. aeruginosa strain PAO, plasmid transformation of Ca^{2+} treated recipient cells has been reported (121, 130), but all attempts to transform chromosomal markers have thus far failed (50). It is probable that, as with Escherichia coli (24, 102), incoming linear DNA is degraded by an endonuclease.

Studies on the regulation of gene expression have shown that the structural genes involved in the sequential steps in the biosynthesis of amino acids, purines, and pyrimidines in P. aeruginosa (29) do not show the clustering characteristic of such pathways in E. coli and Salmonella typhimurium (4, 120). For example, the histidine genes form a contiguous sequence of 9 structural genes in enteric bacteria, whereas, at least 5 unlinked loci are involved in P. aeruginosa (91).

A detailed analysis of the arginine pathway has revealed that the mechanisms for the regulation of specific enzymes are different in P. aeruginosa and that repression is less important as a regulatory mechanism than in the Enterobacteriaceae (40).

C. Potential Virulence Factors of P. aeruginosa

P. aeruginosa produces a variety of extracellular products that may make a significant contribution to the pathogenesis of Pseudomonas infections (84, 85). A number of toxic substances produced by P. aeruginosa have been purified and examined for biological activity. Colonization of the host may require yet other factors possessed by the organism.

1. Endotoxin. Since P. aeruginosa is a gram-negative rod, its pathogenicity had generally been attributed to endotoxin. Although the cell wall contains lipopolysaccharide, which has been implicated as a cause of shock and other sequelae of sepsis, the evidence indicates that Pseudomonas endotoxin is less active than that produced by other gram-negative organisms (27, 52). It takes 2 to 3 mg to kill a 20 g mouse. The equivalent amount required to fatally poison a 20 Kg child would be 2 to 3 g. Furthermore, killed Pseudomonas cells injected into experimental animals produced no significant toxic response (85), unlike the cells of many other gram-negative organisms.

2. Surface slime. The slime of P. aeruginosa is a loosely defined polysaccharide layer on the surface of the cell and may function like the antiphagocytic capsules of many gram-negative bacteria (84, 129). Purified preparations of these polysaccharides are not

toxic to animals (1, 2). Strains isolated from cystic fibrosis patients generally produce a great deal of slime and are termed mucoid. Such mucoid strains are almost exclusively confined to cystic patients (15). In this infection, slime production may lead to increased obstruction of the small airways.

3. Pigments. P. aeruginosa is known to produce a number of pigments. The best known are pyocyanine (a blue-green phenazine) and fluorescein (greenish-yellow) (36). Injections of these pigments into animals do not cause apparent deleterious effects, but a phenazine pigment of P. aeruginosa has been shown to act as an antibiotic (127). In fact, a complex extract of P. aeruginosa called "pyocyanase" was used in many parts of Europe in the early 20th century to treat some bacterial infections (54). The significance of pigments in pathogenicity may be in the suppression of other bacterial flora and their replacement by P. aeruginosa, a common event in long standing infection such as burns (84).

4. Hemolysins. At least two distinct hemolysins of P. aeruginosa have been described. One is a heat-labile phospholipase C (lecithinase) which liberates phosphorylcholine from lecithin (28). It should be noted that the lethal toxin of Clostridium perfringens, alpha toxin, is also a phospholipase. The other hemolysin is a heat-resistant glycolipid (61). Both are produced in vitro in a media that is high in carbohydrates and low in phosphate content (79).

The glycolipid is not very toxic; 5 mg are necessary to kill a mouse (61). It seems to act primarily as a detergent to

solubilize phospholipids, thus enhancing the activity of the phospholipase C (74). An alkaline phosphatase is usually produced along with these two hemolysins, and this enzyme liberates inorganic phosphate from the phosphorylcholine released when phospholipase C acts upon lecithin.

A crude preparation of P. aeruginosa phospholipase C injected into the skin of animals resulted in a central abscess that was surrounded by an area of redness and induration within 24 h. This resembles the skin lesions that result from injections of live cultures of bacteria. When injected intraperitoneally, the phospholipase C preparation caused hepatic necrosis and pulmonary edema (80).

Phospholipase C may play a significant role in the pathogenesis of Pseudomonas pneumonia. Alveolar membranes are covered with surfactant to reduce surface tension and prevent atelectasis (84). The main component of this surfactant is lecithin. Production of phospholipase C during a Pseudomonas lung infection may result in the destruction of pulmonary surfactant with enhanced destruction due to synthesis of the glycolipid hemolysin (84). It is unknown whether hemolysin production is stimulated by growth in pulmonary surfactant. Lecithinase activity was detected in vitro from greater than 70% of clinical strains of P. aeruginosa (141).

5. Staphylolytic enzyme. Burke et al. (12) have reported that an enzyme produced by most clinical isolates of P. aeruginosa will rapidly lyse viable cells of Staphylococcus aureus and other gram-positive species. The enzyme, an endopeptidase, cleaves the crossbridges of the cell wall peptidoglycan, but it has no protease

activity (12). This factor may play a role expecially at sites of burn-wound infections where P. aeruginosa must compete with pathogenic S. aureus to become the predominant organism.

6. Enterotoxin. P. aeruginosa has been associated with diarrheal conditions described as five-day fever or Shanghai fever (84). An enterotoxin was shown to cause an outpouring of fluid and electrolytes into the lumen of the intestine (73). The enterotoxin was not characterized, but was probably protein in nature because it was heat-labile and readily destroyed by proteolytic enzymes. This enterotoxin may actually be Pseudomonas proteases because rabbits injected with purified proteases from P. aeruginosa showed fluid accumulation in ileal loops (34).

7. Leucocidin. This protein was shown by Scarmann (122-126) to destroy leucocytes of man, cattle, sheep, dog, rabbit, guinea pig and mouse, but was nonhemolytic towards erythrocytes from the same species. Also damaged were lymphocytes and various tissue culture cells. Leucocidin was not released to the culture supernatant but is bound to cellular components. Release of the enzyme for assay required autolysis or treatment of cells with trypsin or P. aeruginosa elastase. Leucocidin was purified from cell lysates and has a molecular weight of 27,000 daltons (125, 126). The minimum lethal dose for mice was 1 µg. The importance of this toxin in infection has not been determined. Only 4 out of 110 strains of human and animal origin were shown to produce the toxin, which suggests that the toxin is of minor importance as a virulence factor.

8. Proteases. Morihara (96) was the first to characterize the proteases found in culture filtrates of P. aeruginosa. An alkaline protease (96) and a neutral protease (elastase or protease II) (99) were isolated, purified and characterized. Wretlind et al. (142) and Kreger et al. (70) have also purified these two proteases. Some reports indicated that a third protease may also be produced at low levels (96, 142).

Injections of alkaline protease or elastase into the skin of animals induce hemorrhagic lesions within minutes (65, 80). When alkaline protease or elastase were injected by various routes into mice in microgram quantities, hemorrhagic lesions of the lungs and intestinal tracts were seen upon autopsy (64, 92). Human patients with P. aeruginosa septicemia commonly have edematous, hemorrhagic lungs and hemorrhagic lesions in the gastrointestinal tract and kidney (33).

Elastase has a relatively low toxicity; the minimum lethal dose for mice was 0.1 to 0.5 mg depending upon the route of inoculation (142). The purest preparations of elastase failed to show significant membrane-damaging effects on HeLa cells and fibroblasts. Wretlind et al. (142) speculated that the tissue damage caused by P. aeruginosa elastase is probably due to its effect on the ground substance (i.e. elastin) of connective tissue, and not to cytotoxic effects. A chemopathogenic function for Pseudomonas elastase has also been suggested (128). Elastase was shown to destroy the chemotactic fragment of complement C5 and also to reduce the ability of leucocytes to

phagocytize sensitized P. aeruginosa due to inactivation of cell-bound C3 (128).

Purified alkaline protease, as the name implies, is most active at a slightly alkaline pH, and it does not hydrolyze elastin (96, 97). Its molecular weight has been reported at both 48,000 (98) and 20,000 (70, 142), and its isoelectric point is at pH 4.1 (98). This enzyme is inactivated by various metal chelators but not by the other usual protease inhibitors, such as ethylenediaminetetraacetate (EDTA) (96). The enzyme is inactivated by o-phenanthroline in the presence of a high concentration of Ca ion, suggesting that the enzymatic activity is related not to Ca ion but to heavy metal ions (98). Among various metal ions tested, Co^{2+} was effective in promoting hydrolysis (98). Using oxidized insulin B chain as substrate, the specificity of the enzyme appeared to be very broad (98).

The elastase is most active in the neutral pH range (97). Its molecular weight has been reported as 39,500 (98) and 20,000 (70, 142), and its isoelectric point is at pH 5.9 (99). Although alkaline protease and elastase have similar molecular weights, their difference in isoelectric points allows these enzymes to be separated by diethylaminoethyl(DEAE)-cellulose column chromatography (97). The elastase is a typical metal chelator-sensitive neutral protease which requires Zn^{2+} for activity (98).

9. Toxin A. This heat-labile protein was originally described by Liu (81). Toxin A is the most toxic substance known to be produced by P. aeruginosa with an LD_{50} for mice of 140 ng (15). At

very low doses, it produced hypotensive shock in dogs and monkeys (3). In separate studies, Bjorn et al. (8) and Pollack et al. (114) showed that approximately 90% of all clinical isolates produced toxin A. Also, the toxin appears to be produced in vivo during P. aeruginosa infection. Pollack et al. (113) showed that patients who have recovered from P. aeruginosa infections have significantly higher titers of antitoxin antibodies than control groups.

Toxin A has a molecular weight of 73,000 and can be readily purified using either a variety of classical biochemical techniques (15, 57, 81, 86, 137) or affinity chromatography using insolubilized antitoxin immunoglobulin (135). Toxin A is synthesized as a single polypeptide chain (22, 78, 136) with an isoelectric point of 5.1, and it contains 4 disulfide bridges (77).

Toxin A inhibits eucaryotic protein synthesis in the same manner as diphtheria toxin (56-58, 109). The mechanism of action is to catalyze the transfer of the adenosine diphosphate ribose moiety (ADPR) of NAD onto elongation factor 2 (EF-2). This eventually inactivates the cell's content of EF-2 and thus stops protein synthesis (58). Toxin A has been shown to inhibit protein synthesis in the liver, kidney and spleen of mice following injection. Toxin A also inhibits protein synthesis in mammalian cell cultures (93, 94, 104, 105) and in cell free systems (56-58).

Toxin A, like diphtheria toxin, is a proenzyme which must be activated for the ADPR-transferase activity to be expressed. In routine assay for the toxin's activity, the toxin is activated by

denaturation with 4 M urea and reduction of a disulfide bond with 1% dithiothreitol. Apparently, a similar denaturation-reduction takes place as the toxin passes through the mammalian cell membrane to cause the activation of its enzymatic activity (78, 136). However, once the toxin is activated *in vitro*, it is no longer toxic (78, 136). Toxin A apparently consists of 2 components, one (designated A for active) catalyzes the intracellular reaction and the other (termed B for binding) is a carrier component which makes contact with the surface of susceptible cells (22, 136). The A fragment has been isolated, but the B fragment has only been tentatively identified (22, 136).

Toxin A is not produced constitutively by *P. aeruginosa*. Liu (83) reported that toxin A production was greatly enhanced by growth with aeration, glycerol, L-glutamate and incubation at 32° C. Bjorn *et al.* (6, 7) showed that the concentration of iron in the culture medium has an inverse effect on the final yield of toxin A. Toxin A yields are poor in simple minimal media (glucose plus salts), but a defined medium for toxin A production has been reported (25). Thus, it is likely that specific regulatory systems control the synthesis and secretion of toxin A in a manner that is distinct from the regulation of bulk protein synthesis.

D. Corneal Infections by *P. aeruginosa*

P. aeruginosa can produce severe corneal infections (*Pseudomonas* keratitis) that usually result in ulceration and loss of vision in the infected eye (76). Currently, *P. aeruginosa* causes 15 to 20% of all bacterial corneal ulcers in the United States, and the trend

has been for an increasing role of Pseudomonas since the 1950s (117). Such infections usually originate as a result of corneal wounds (11). Other predisposing factors include improper use of contact lenses, irritation by foreign objects, use of eyedrops containing corticosteroids, contaminated eye solutions, preexistant herpetic keratitis (68), and corneal trauma due to cataract surgery (11). Persons are also at risk when local or systemic defenses are compromised, such as premature infants (14), burn patients (95), and semicomatose tracheostomized patients (68). The cornea, having no direct blood supply, is itself an immunologically weak tissue (68).

Typically, patients with Pseudomonas corneal ulcers seek medical attention about 2 to 3 days following removal of a superficial ocular foreign body (11). The rapid course of corneal destruction causes pain, greatly diminished visual acuity, and drainage. The corneal ulceration is usually centrally located and frequently terminates in corneal rupture or panophthalmitis if untreated (11). Thus, treatment must begin as early in the course of infections as possible, usually before results of cultures are available. Gentamicin and carbenicillin are the drugs of choice. However, animal studies showed that neither systemic or topical routes of administration alone will produce adequate corneal tissue or aqueous humor levels of the antibiotics to eradicate the Pseudomonas in corneal infections (68). Currently, the recommended treatment for a severe case of Pseudomonas keratitis includes intravenous antibiotics, subconjunctival injections of antibiotics twice-daily, and antibiotic

eye drops every 15 min. The carbenicillin and gentamicin are generally injected at separate sites (68). In very severe cases, such as Pseudomonas endophthalmitis, intravitreal injections of gentamicin may be required to obtain vitreous levels of 2 μg/ml, although retinal degeneration and cataract formation may develop as a result of the high concentration of gentamicin (68).

Experimental Pseudomonas keratitis animal models have been developed; the bacteria are applied to traumatized corneas or are injected intracorneally. Using a guinea pig Pseudomonas keratitis model, polymorphonuclear leukocytes (PMNs) were shown to play a major role in limiting bacterial multiplication (23). Also, PMNs keep the P. aeruginosa infection localized. When mice were made leukopenic by treatment with cyclophosphamide before challenge with P. aeruginosa, the typically localized infection progressed to a Pseudomonas septicemia that resulted in death within 48 h (41). Although PMNs have been shown to be important in the defense of the eye against bacterial infection, other studies indicate that PMN-derived enzymes, capable of degrading both collagen and proteoglycans, may also contribute to corneal destruction. The intrastromal injection of heat-killed P. aeruginosa produced an extensive PMN infiltration which progressed to ulceration, whereas, no ulcers occurred if eyes were pretreated with corticosteroids (67).

The marked virulence of P. aeruginosa for the cornea has largely been attributed to the production of extracellular proteases by the organism. Pseudomonas protease preparations injected into animal corneas have produced gross corneal damage which appeared

similar to that observed during severe experimental and human keratitis produced by P. aeruginosa (10, 62, 69, 70). A preparation of Pseudomonas proteases was shown to degrade isolated rabbit corneal proteoglycan ground substance (10) and to degrade and solubilize stromal proteoglycan when incubated with suspensions of finely ground rabbit stromas (66). When independently isolated strains of P. aeruginosa were compared, two low-protease producing strains were reported to cause a less severe experimental corneal disease than strongly proteolytic strains (63).

Recently, a comparison was made between the structural alterations to the eye during experimental P. aeruginosa keratitis in rabbits and the intracorneal injection of Pseudomonas proteases (69, 70). The purified protease preparation was a mixture of alkaline protease and elastase. Light and electron microscopic examination of rabbit corneal lesions were made 4 to 6 h after the injection of submicrogram amounts of the proteases. These observations showed: (i) degeneration of epithelium, endothelium, and keratocytes; and (ii) degradation of corneal and stromal proteoglycan ground substance with the dispersal of collagen fibrils. The collagen fibrils, which usually have an ordered structure, were not actually degraded (69). These structural alterations due to the intracorneal injection of proteases were very similar to those observed during experimental Pseudomonas keratitis (70). This supports the idea that P. aeruginosa extracellular proteases may be at least partly responsible for the necrosis characteristic of Pseudomonas keratitis.

Kawaharajo et al. (62) demonstrated the individual effects of alkaline protease and elastase on corneal tissue. The corneas of mice were incised, purified alkaline protease or elastase were topically applied once, and the course of damage was followed. Opacity in the wounded area of the cornea resulted from 0.8 to 2 μg of the enzymes, and opacity with ulcers was observed with 4 to 50 μg. Alkaline protease and elastase reportedly caused the same histological changes in the cornea. Epithelium and endothelium degeneration, inflammatory cellular infiltration, and stromal collagen fiber pattern degeneration followed the application of either alkaline protease or elastase (62).

Only one report currently exists in the literature which indicates that toxin A may play a role in the pathogenesis of Pseudomonas keratitis. Iglewski et al. (55) reported that the injection of purified toxin A (0.1 and 2.5 μg) into the corneas of rabbits produced opacity and abscess at the injection site. Histopathologic examination showed that toxin A killed the epithelial, endothelial and stromal cells of the cornea within 24 h. Loss of endothelium caused the cornea to become edematous and cloudy; loss of epithelium and keratocytes promoted later ulceration. PMN infiltration was also apparent. Control eyes were injected with toxin A neutralized by antitoxin, and these showed only slight stromal opacity. This study indicated that toxin A, as well as the proteases, should be considered as a corneal damaging enzyme capable of playing a role in the pathogenesis of Pseudomonas keratitis.

E. Burn-Wound Infections by P. aeruginosa

Before the early 1940s, group A Streptococcus was a significant pathogen for burned patients. Following the introduction of penicillin, Staphylococcus was the predominant life-threatening infectious agent in burn patients until the development of semisynthetic penicillins. In the late 1950s, P. aeruginosa became the bacterial species most often associated with fatal infection of the burn wound (115). P. aeruginosa is seldom encountered on burn wounds during the first 24 h postburn, but by 48 h about one-half of all patients are culture-positive for Pseudomonas (117). Sources of infecting strains include sinks, water pitchers, flowers, attending personnel to the patient, and organisms from previous patients. These routes of spread are now under greater control, but the patient's own gastrointestinal tract may provide a source of the organisms (134).

The nonviable eschar of the burn wound provides an environment for rapid bacterial proliferation (101). The predisposition to infection by P. aeruginosa (and other opportunistic bacteria) reflects the impairment of host defense capacity caused by the burn injury. The burn itself is a destruction of the cutaneous mechanical barrier, but the trauma has also been shown to cause a decline in immunoglobulin levels (100), T lymphocyte levels (117), reticuloendothelial system functions, neutrophil functions (140), and granulocyte bactericidal activity (88, 89). P. aeruginosa infected burn wounds often show degeneration and hemorrhage into the subcutaneous tissue, dark-green discoloration due to the pyocyanine produced by P.

aeruginosa, and a dark-brown neoeschar (117). If Pseudomonas proliferates to 10^5 or more organisms per gram of tissue, then viable unburned tissue is invaded along hair follicles, sweat glands, small vessels and nerves (117). P. aeruginosa shows a propensity to invade vascular walls and the associated vasculitis leads to Pseudomonas bacteremia (101). Metastic lesions may also occur in unburned tissue. Such lesions of the skin are known as ecthyma gangrenosa, and hematogenous dissemination to the lungs often results in pneumonia (115, 117). The generalized invasion of the organisms frequently leads to the death of the patient.

The avascular nature of the eschar makes systemically administered antibiotics ineffective in controlling burn wound infection. But the recent use of topical antimicrobial agents has considerably reduced the incidence of Pseudomonas burn wound sepsis. Preventing the initial wound infection is the key to current burn care (117). However, topical anti-Pseudomonas therapy has only reduced but not eliminated invasive Pseudomonas burn wound sepsis. None of the agents sterilize the burn wound, but merely inhibit further growth of the organisms in the burn site (117). P. aeruginosa can escape therapeutic control if the host defense capacity is particularly disturbed (117). Vaccines (117) and passively administered gamma-globulins (133) directed against Pseudomonas cellular components have not shown definitive efficacy as immunotherapy for the burn patient.

In an effort to determine which factors possessed by P. aeruginosa are responsible for its virulence in burn patients, experimental

animal infection models were developed. The burned mouse model of Stieritz and Holder (132) was shown to be clinically relevant; the animal host was rendered susceptible to infection by a controlled thermal injury which was rarely in itself fatal. The enhanced susceptibility to infection appeared to be restricted to P. aeruginosa. The result was a rapidly fatal sepsis after injection of low numbers of P. aeruginosa.

Saelinger et al. (119) showed in the burned mouse model that P. aeruginosa multiplying at the burned site produce toxin A. The evidence was (i) an increase of ADPR-transferase activity in serum and the extracts of burned infected animals, and (ii) the level of active elongation factor 2 (EF-2) in the livers of infected mice was reduced significantly after infection. This suggested that toxin A, released by bacteria at the burn site, enters the circulation and is disseminated to different organs where it acts by depletion of EF-2 to reduce host protein synthesis (119).

Pavlovskis et al. (108) showed that intravenously administered antitoxin had a protective effect in the Pseudomonas infected burned mouse. Survival after infection with a toxin A producing, low-protease producing strain (PA103) was enhanced in antitoxin-treated mice compared to infected controls. Infections with toxigenic, high-protease producing strains and antitoxin treatment resulted in a prolonged time of survival, but there was no change in the ultimate lethality of the infections. In contrast, antitoxin had no protective effect in mice challenged with a nontoxigenic strain. A closer examination showed

that there were fewer viable bacteria in the blood and liver of antitoxin-treated mice than controls after infection with toxin A producing organisms. Antitoxin had no effect on infections with the nontoxigenic strain. This suggested that toxin A contributes to lethality in burn infections (108). Further studies showed that the passive administration of antitoxin before infection with a toxigenic strain (PA103) would prevent the inactivation of liver EF-2 (107). These data suggested that antitoxin has immunoprophylactic potential.

The recent study of Walker et al. (139) using the burned rat model showed conflicting results with those above. This approach has some advantages over the burned mouse model: (i) the P. aeruginosa cells are topically seeded at the burn site rather tha injected, and (ii) death is caused by hematogenous spread of the infection to various organs, whereas, inflammatory lesions are not seen in the burned mouse model. Their results showed that a toxoid of toxin A failed to protect burned rats infected with P. aeruginosa (139). These authors suggested that the rat is an infectious model for P. aeruginosa and the mouse may be a toxic model. The problem with the rat model is that this animal is unusually insensitive to toxin A, actually 8-fold less sensitive to toxin A on a body weight basis than the mouse (139). Furthermore, the rat model requires extremely large inocula (10^7). Although neither model may perfectly mimic the human disease, both may be necessary at this time to examine all aspects of P. aeruginosa burn sepsis.

Evidence has also been presented which suggests that Pseudomonas proteases and toxin A are virulence factors. Snell et al. (131)

showed that in burned mice challenged with a toxigenic, low-protease producing strain (PA103), the mean time to death was shortened when the challenge inoculum was supplemented with purified alkaline protease (10 μg injected subcutaneously at 0, 8, and 24 h post-infection). The LD_{50} for strain PA103 was reduced by a factor of 10^3 by this regimen of protease injection. Also, the reduction in liver EF-2 normally seen only with large inocula of PA103 was obtained with smaller inocula when alkaline protease was added (131). These data suggest a synergistic effect between toxin A and protease is required for full virulence in burn infection by P. aeruginosa.

Holder et al. (42) later showed that alkaline protease, elastase, or even a purified Bacillus protease, would show an enhancement of mortality when a low-protease producing strain (PA103) was used to challenge burned mice. No mortality-enhancing effect was observed when other organisms were substituted for P. aeruginosa, indicating that the proteases did not simply lower host resistance non-specifically. When α_2-macroglobulin, a serum protein which inhibits protease activity, was injected into burned mice infected with a high-protease producing strain (M2), an increased survival time was observed. These authors (42) concluded that toxin A may be a prime requisite for virulence of the organism, but that proteolytic enzymes are virulence factors which contribute to the mortality of Pseudomonas burn infections.

Further studies have suggested that the role of the proteases is to permit an enhanced growth rate of the organisms at the burn site (21). The rate of growth of a low-protease producing strain (PA103)

was slow compared to a highly-proteolytic strain (M2) in burned skin extracts. The addition of alkaline protease allowed PA103 to grow as rapidly as M2. Thus, the protease may make nutrients from burned tissue available to the organism. Interestingly, strain PA103 and M2 grew rapidly and at the same rate in normal skin extracts (121).

F. Genetic Approaches to the Study of Pathogenesis

A genetic approach to determining the role of a potential virulence factor was described by Holmes (43). This begins with the isolation of mutants (from a virulent strain) which lack the one factor in question. This requires the development of sensitive and specific screening techniques to detect these mutants. Because virulence factors like toxins and proteases are not required for growth in vitro, no methods have yet been developed to select for these mutants. Generally, mutagenic agents are used to increase the rate of mutation. The mutants are extensively characterized in vitro and compared to the parental strain to determine whether any other properties besides the phenotype selected have been altered by the mutation. Epidemiological markers show that the mutants were derived from the parental strain.

Mutants lacking only one potential virulence character can be compared to the parental strain in an experimental animal infection which mimics the disease in humans. A decrease in virulence with the mutant directly indicates that the factor has a role in that disease. Convincing evidence of the factor's contribution to virulence would be an increase in virulence by the mutant when the factor is returned

(either by repair of the mutation or exogenous addition of the purified factor).

A genetic study to evaluate the contribution of protease production to the virulence of P. aeruginosa was reported by Pavlovskis and Wretlind (106). Protease deficient mutants of P. aeruginosa PAKS were isolated on the basis of reduced casein digestion (143). However, this is not a specific screening procedure since both alkaline protease and elastase hydrolyze casein. Thus, it was not surprising that all mutants isolated had general extracellular product deficiencies (143). When tested in the burned mouse model, the mutants were less virulent than the parental strain (106). However, the altered virulence could not be attributed to only a single deficiency. Thus, to evaluate the contribution of the extracellular products of P. aeruginosa to virulence, mutants with single specific deficiencies are needed, and mutant screening techniques capable of detecting such mutations are required.

References

1. Alms, T.H. and Bass, J.A. 1967. Immunization against Pseudomonas aeruginosa. I. Induction of protection by an alcohol-precipitated fraction from the slime layer. J. Infect. Dis. 117: 249-256.

2. Alms, T.H. and Bass, J.A. 1967. Immunization against Pseudomonas aeruginosa. II. Purification and characterization of the protective factor from the alcohol-precipitated fraction. J. Infect. Dis. 117:257-264.

3. Atik, M., LIu, P.V., Hanson, B.A., Amini, S. and Rosenberg, C.F. 1968. Pseudomonas exotoxin shock. J. Am. Med. Assoc. 205: 84-90.

4. Bachmann, B.J., Low, K.B. and Taylor, A.L. 1976. Recalibrated linkage map of Escherichia coli K-12. Bacteriol. Rev. 40: 116-167.

5. Barksdale, L. and Arden, S.A. 1974. Persisting bacteriophage infections, lysogeny, and phage conversions. Annu. Rev. Microbiol. 28:265-299.

6. Bjorn, M.J., Iglewski, B.H., Ives, S.K., Sadoff, J.C. and Vasil, M.L. 1978. Effect of iron on yields of exotoxin A in cultures of Pseudomonas aeruginosa PA-103. Infect. Immun. 19:785-791.

7. Bjorn, M.J., Sokol, P.A. and Iglewski, B.H. 1979. Influence of iron on yields of extracellular products in Pseudomonas aeruginosa cultures. J. Bacteriol. 138:193-200.

8. Bjorn, M.J., Vasil, M.L., Sadoff, J.C. and Iglewski, B.H. 1977. Incidence of exotoxin production by Pseudomonas species. Infect. Immun. 16:362-366.

9. Brokopp, C.D. and Farmer, J.J. III. 1979. Typing methods for Pseudomonas aeruginosa, p. 90-131. In R.G Doggett (ed.), Pseudomonas aeruginosa, clinical manifestations of infection and current therapy. Academic Press, New York.

10. Brown, S.I., BLoomfield, S.E. and Tom, W.I. 1974. The cornea-destroying enzyme of Pseudomonas aeruginosa. Invest. Ophthal. 13:174-180.

11. Bohigian, G.M. and Escapini, H. 1971. Corneal ulcer due to Pseudomonas aeruginosa. Arch. Ophthalmol. 85:405-409.

12. Burke, M.E. and Pattee, P.A. 1967. Purification and characterization of a staphylolytic enzyme from Pseudomonas aeruginosa. J. Bacteriol. 93:860-865.

13. Burkhardt, H.J., Riess, G. and Puhler, A. 1978. Molecular relationship between R plasmids RP1, RP4, RP8, RK2, R68 and R68.45 revealed by electron microscopical techniques. Hoppe-Seyler's Z. Physiol. Chem. 359:1068.

14. Burns, R.P. and Rhodes, D.H. 1961. Pseudomonas eye infections as a cause of death in premature infants. Arch. Ophthalmol. 65:517-525.

15. Callahan, L.T. III. 1976. Pseudomonas aeruginosa exotoxin: purification by preparative polyacrylamide gel electrophoresis and the development of a highly specific antitoxin serum. Infect. Immun. 14:55-61.

16. Carey, K.E. and Krishnapillai, V. 1974. Location of prophage H90 on the chromosome of Pseudomonas aeruginosa strain PAO. Genet. Res. 23:155-169.

17. Carey, K.E. and Krishnapillai, V. 1975. Chromosomal location of prophage J51 in Pseudomonas aeruginosa strain PAO. Genet. Res. 25:179-187.

18. Chakrabarty, A.M. 1976. Plasmids in Pseudomonas. Annu. Rev. Genet. 10:7-30.

19. Clark, P.H. and Ornston, L.N. 1975. Metabolic pathways and regulation: I., p. 191-261. In P.H. Clarke and M.H. Richmond (eds.), Genetics and Biochemistry of Pseudomonas. John Wiley and Sons, New York.

20. Clark, P.H. and Ornston, L.N. 1975. Metabolic pathways and regulation: II., p. 263-340. In P.H. Clarke and M.H. Richmond (eds.), Genetics and Biochemistry of Pseudomonas. John Wiley and Sons, New York.

21. Cicmanec, J.F. and Holder, I.A. 1979. Growth of Pseudomonas aeruginosa in normal and burned skin extract: role of extracellular proteases. Infect. Immun. 25:477-483.

22. Chung, D.W. and Collier, R.J. 1977. Enzymatically active peptide from the adenosine diphosphate-ribosylating toxin of Pseudomonas aeruginosa. Infect. Immun. 16:832-841.

23. Chusid, M.J. and Davis, S.D. 1979. Experimental bacterial keratitis in neutropenic guinea pigs: polymorphonuclear leukocytes in corneal host defense. Infect. Immun. 24:948-952.

24. Cosloy, S.D. and Oishi, M. 1973. The nature of the transformation process in Escherichia coli K12. Mol. Gen. Genet. 124: 1-10.

25. DeBell, R.M. 1979. Production of exotoxin A by Pseudomonas aeruginosa in a chemically defined medium. Infect. Immun. 24: 24:132-138.

26. Dogget, R.G. 1969. Incidence of mucoid Pseudomonas aeruginosa from clinical sources. Appl. Microbiol. 18:936-937.

27. Dyke, J.W. and Berk, R.S. 1973. Comparative studies on Pseudomonas aeruginosa endotoxin. Z. Allg. Mikrobiol. 13:307-313.

28. Esselmann, M. and Liu, P.V. 1961. Lecithinase production by gram-negative bacteria. J. Bacteriol. 81:939-945.

29. Fargie, B. and Holloway, B.W. 1965. Absence of clustering of functionally related genes in Pseudomonas aeruginosa. Genet. Res. 6:284-299.

30. Farmer, J.J. III. 1976. Pseudomonas in the hospital. Hosp. Practice 10:63-70.

31. Farmer, J.J. III and Herman, L.G. 1969. Epidemiological fingerprinting of Pseudomonas aeruginosa by the production of and sensitivity to pyocin and bacteriophage. Appl. Microbiol. 18: 760-765.

32. Farmer, J.J. III and Herman, L.G. 1974. Pyocin typing of Pseudomonas aeruginosa. J. Infect. Dis. 130:s43-s46.

33. Fetzer, A.E., Werner, A.S. and Hagstrom, J.W.C. 1967. Pathologic features of Pseudomonas pneumonia. Am. Rev. Respir. Dis. 96:1121-1130.

34. Finkelstein, R.A. 1976. Progress in the study of cholera and related enterotoxins, p. 53-84. In A.W. Bernheimer (ed.), Mechanisms in Bacterial Toxinology. John Wiley and Sons, New York.

35. Fisher, M.W., Devlin, H.B. and Gnabasik, F.J. 1969. New immunotype schema for Pseudomonas aeruginosa based on protective antigens. J. Bacteriol. 98:835-836.

36. Garibaldi, J.A. 1967. Media for the enhancement of fluorescent pigment production of Pseudomonas aeruginosa. J. Bacteriol. 94: 1296-1299.

37. Habs, J. 1957. Untersuchungen uber die O-antigene von Pseudomonas aeruginosa. Z. Hyg. Infektionskr. 144:218-228.

38. Haas, D. and Holloway, B.W. 1976. R factor variants with enhanced six factor activity in Pseudomonas aeruginosa. Mol. Gen. Genet. 144:243-251.

39. Haas, D. and Holloway, B.W. 1978. Chromosome mobilization by the R plasmid R68.45: a tool in Pseudomonas genetics. Mol. Gen. Genet. 158:229-237.

40. Haas, D., Holloway, B.W., Schomböck, A. and Leisinger, T. 1977. The genetic organization of arginine biosynthesis in Pseudomonas aeruginosa. Mol. Gen. Genet. 154:7-22.

41. Hazlett, L.D., Rosen, D.D. and Berk. R.S. 1977. Pseudomonas eye infections in cyclophosphamide-treated mice. Invest. Ophthalmol. Visual Sci. 16:649-652.

42. Holder, I.A. and Haidaris, C.G. 1979. Experimental studies of the pathogenesis of infections due to Pseudomonas aeruginosa: extracellular protease and elastase as in vivo virulence factors. Can. J. Microbiol. 25:593-599.

43. Holmes, R.K. 1975. Genetic aspects of toxinogenesis in bacteria, p. 296-301. In D. Schlessinger (ed.) Microbiology 1975. American Society for Microbiology, Washington, D.C.

44. Holloway, B.W. 1975. Genetic organization of Pseudomonas, p. 133-162. In P.H. Clarke and M.H. Richmond (eds.), Genetics and Biochemistry of Pseudomonas. John Wiley and Sons, New York.

45. Holloway, B.W. 1979. Plasmids that mobilize bacterial chromosome. Plasmid 2:1-19.

46. Holloway, B.W. 1979. Role of formal genetics in medical microbiology, p. 9-35. In R.G. Doggett (ed.), Pseudomonas aeruginosa, clinical manifestations of infection and current therapy. Academic Press, New York.

47. Holloway, B.W., Egan, J.B. and Monk, M. 1960. Lysogeny in Pseudomonas aeruginosa. Aust. J. Exp. Biol. 38:321-330.

48. Holloway, B.W. and Krishnapillai, V. 1975. Bacteriophages and bacteriocins, p. 99-132. In P.H. Clarke and M.H. Richmond (eds.), Genetics and Biochemistry of Pseudomonas. John Wiley and Sons, New York.

49. Holloway, B.W. and Krishnapillai, V. 1975. Bacteriophages and bacteriocins, p. 99-132. In P.H. Clarke and M.H. Richmond (eds.), Genetics and Biochemistry of Pseudomonas. John Wiley and Sons, London.

50. Holloway, B.W., Krishnapillai, V. and Morgan, A.F. 1979. Chromosomal genetics of Pseudomonas. Microbiol. Rev. 43:73-102.

51. Holloway, B.W. and van de Putte, P. 1968. Lysogeny and bacterial recombination, p. 175-183. In W.J. Peacock and R.D. Brock (eds.), Replicationand Recombination of Genetic Material. Australian Academy of Sciences, Canberra.

52. Homma, J.Y. 1968. The protein moiety of endotoxin of Pseudomonas aeruginosa. Z. Allg. Mikrobiol. 8:277-281.

53. Homma, J.Y. and Suzuki, N. 1964. Cell-wall protein A of Pseudomonas aeruginosa and its relationship to original endotoxin protein. J. Bacteriol. 87:630-640.

54. Honl, J. and Bukivsky, J. 1899. Therapie der Schenkelegeschwure mit Bakterienproteinen. Zentralbl. Bakteriol. Abt. I. 26:20.

55. Iglewski, B.H., Burns, R.P. and Gipson, I.K. 1977. Pathogenesis of corneal damage from Pseudomonas exotoxin A. Invest. Ophthal. 16:73-75.

56. Iglewski, B.H., Elwell, L.P., Liu, P.V. and Kabat, D. 1976. ADP-ribosylation of elongation factor 2 by Pseudomonas aeruginosa exotoxin A and by diphtheria toxin, p. 150-155. In S. Shaltiel (ed.), Proceedings of the 4th International Symposium on the Metabolic Interconversion of Enzymes. Springer-Verlag, Berlin.

57. Iglewski, B.H. and Kabat. D. 1975. NAD-dependent inhibition of protein synthesis by Pseudomonas aeruginosa toxin. Proc. Natl. Acad. Sci. USA 72:2284-2288.

58. Iglewski, B.H., Liu, P.V. and Kabat, D. 1977. Mechanism of action of Pseudomonas aeruginosa exotoxin A: adenosine diphosphate-ribosylation of mammalian elongation factor 2 in vitro and in vivo. Infect. Immun. 15:138-144.

59. Jacob, A.E., Cresswell, J.M. and Hedges, R.W. 1977. Molecular characterization of the P group plasmid R68 and variants with enhanced chromosome mobilizing ability. FEMS Microbiol. Lett. 1:71-74.

60. Jacoby, G.A. and Shapiro, J.A. 1977. Plasmids studied in Pseudomonas aeruginosa and other pseudomonads, p. 639-656. In A.I. Bukhari, J.A. Shapiro, and S.L. Adhya (eds.), DNA Insertion Elements, Plasmids, and Episomes. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

61. Jarvis, F.G. and Johnson, M.J. 1974. A glycolipid produced by Pseudomonas aeruginosa. J. Am. Chem. Soc. 71:4124-4126.

62. Kawaharajo, K., Abe, C., Homma, J.Y., Kawano, M., Gotoh, E., Tanaka, N. and Morihara, K. 1974. Corneal ulcers caused by protease and elastase from Pseudomonas aeruginosa. Jpn. J. Exp. 44:435-442.

63. Kawaharajo, K. and Homma, J.Y. 1975. Pathogenesis of the mouse keratitis produced with Pseudomonas aeruginosa. Jpn. J. Exp. Med. 45:515-524.

64. Kawaharajo, K., Homma, J.Y., Aoyama, Y. and Morihara, K. 1975. In vivo studies on protease and elastase from Pseudomonas aeruginosa. Jpn. J. Exp. Med. 45:89-100.

65. Kawaharajo, K., Homma, J.Y., Aoyama, Y., Okadao, K. and Morihara, I. 1975. Effects of protease and elastase from Pseudomonas aeruginosa on skin. Jpn. J. Exp. Med. 45:79-88.

66. Kessler, E., Kennah, H.E. and Brown, S.I. 1977. Pseudomonas protease. Purification, partial characterization, and its effect on collagen, proteoglycan, and rabbit corneas. Invest. Ophthalmol. Visual Sci. 16:488-497.

67. Kessler, E., Mondino, B.J. and Brown, S.I. 1977. The corneal response to Pseudomonas aeruginosa. Invest. Ophthalmol. Visual Sci. 16:116-125.

68. Kohler, R.B. and White, A. 1979. Miscellaneous Pseudomonas disease, p. 446-486. In R.G. Doggett (ed.), Pseudomonas aeruginosa, Clinical Manifestations of Infection and Current Therapy. Academic Press, New York.

69. Kreger, A.S. and Gray, L.D. 1978. Purification of Pseudomonas aeruginosa proteases and microscopic characterization of pseudomonal protease-induced rabbit corneal damage. Infect. Immun. 19:630-648.

70. Kreger, A.S. and Griffen, G.K. 1974. Physiochemical fractionation of extracellular cornea-damaging proteases of Pseudomonas aeruginosa. Infect. Immun. 9:828-834.

71. Krishnapillai, V. 1971. A novel transducing phage. Its role in recognition of a possible new host-controlled modification system in Pseudomonas aeruginosa. Mol. Gen. Genet. 114:134-143.

72. Krishnapillai, V. and Carey, K.E. 1972. Chromosomal location of a prophage in Pseudomonas aeruginosa strain PAO. Genet. Res. 20:137-140.

73. Kubota, Y. and Liu, P.V. 1971. An enterotoxin of Pseudomonas aeruginosa. J. Infect. Dis. 123:97-98.

74. Kurioka, S. and Liu, P.V. 1967. Effect of the hemolysin of Pseudomonas aeruginosa on phosphatides and on phospholipase C activity. J. Bacteriol. 93:670-674.

75. Laborde, H.F. and Tajardo, C.L. 1965. Pseudomonas vaccine. J. Bacteriol. 90:290-291.

76. Laibson, P.R. 1972. Annual review. Cornea and sclera. Arch. Ophthalmol. 88:553-574.

77. Leppla, S.H. 1976. Large-scale purification and characterization of the exotoxin of Pseudomonas aeruginosa. Infect. Immun. 14:1077-1086.

78. Leppla, S.H., Martin, O.C. and Muehl, L.A. 1978. The exotoxin of Pseudomonas aeruginosa: a proenzyme having an unusual mode of activation. Biochem. Biophys. Res. Commun. 81:532-538.

79. Liu, P.V. 1964. Factors that influence toxigenicity to Pseudomonas aeruginosa. J. Bacteriol. 88:1421-1427.

80. Liu, P.V. 1966. The roles of various fractions of Pseudomonas aeruginosa in its pathogenesis. II. Effects of leicthinase and protease. J. Infect. Dis. 116:112-116.

81. Liu, P.V. 1966. The roles of various fractions of Pseudomonas aeruginosa in its pathogenesis. III. Identity of the lethal

toxin produced in vitro and in vivo. J. Infect. Dis. 116: 481-489.

82. Liu, P.V. 1969. Changes in somatic antigens of Pseudomonas aeruginosa induced by bacteriophages. J. Infect. Dis. 119: 237-246.

83. Liu, P.V. 1973. Exotoxins of Pseudomonas aeruginosa. I. Factors that influence the production of exotoxin A. J. Infect. Dis. 128:506-513.

84. Liu, P.V. 1974. Extracellular toxins of Pseudomonas aeruginosa. J. Infect. Dis. 130:S94-S99.

85. Liu, P.V. 1976. Biology of Pseudomonas aeruginosa. Hosp. Practice 10:139-147.

86. Liu, P.V., Abe, Y. and Bates, L.J. 1961. The roles of various fractions of Pseudomonas aeruginosa in its pathogenesis. J. Infect. Dis. 108:218-228.

87. Matsumoto, H. and Tazaki, T. 1973. FP5 factor, an undescribed sex factor of Pseudomonas aeruginosa. Jpn. J. Microbiol. 17: 409-417.

88. McEuen, D.D., Blair, P., Delbene, V.E. and Eurenius, K. 1976. Correlation between pseudomonas burn wound infection and granulocyte antibacterial activity. Infect. Immun. 13:1360-1362.

89. McEuen, D.D., Gerber, G.C., Blair, P. and Eurenius, K. 1976. Granulocyte function and pseudomonas burn wound infection. Infect. Immun. 14:399-402.

90. Mee, B.J. and Lee, B.T.O. 1967. An analysis of histidine requiring mutants in Pseudomonas aeruginosa. Genetics 55:709-722.

91. Mee, B.J. and Lee, B.T.O. 1969. A map order for HISI, one of the genetic regions controlling histidine biosynthesis in Pseudomonas aeruginosa, using the transducing phage F116. Genetics 62:687-696.

92. Meinke, G., Barum, J., Rosenberg, B. and Berke, R.S. 1970. In vivo studies with the partially purified protease (elastase) from Pseudomonas aeruginosa. Infect. Immun. 2:583-589.

93. Middlebrook, J.L. and Dorland, R.B. 1977. Response of cultured mammalian cells to the exotoxins of Pseudomonas aeruginosa and Corynebacterium diphtheriae: differential cytotoxicity. Can. J. Microbiol. 23:183-189.

94. Middlebrook, J.L. and Dorland, R.B. 1977. Serum effects on the response of mammalian cells to the exotoxins of Pseudomonas aeruginosa and Corynebacterium diphtheriae. Can. J. Microbiol. 23:175-182.

95. Mitchell, W.H., Parson, B.J. and Weiner, L.J. 1976. Pseudomonas ulceration of the cornea following major total body burn: a clinical study. J. Trauma 16:317-319.

96. Morihara, K. 1963. Pseudomonas aeruginosa proteinase. I. Purification and general properties. Biochim. Biophys. Acta 73: 113-124.

97. Morihara, K. 1964. Production of elastase and proteinase by Pseudomonas aeruginosa. J. Bacteriol. 88:745-757.

98. Morihara, K. and Tsuzuki, H. 1977. Production of protease and elastase by Pseudomonas aeruginosa strains isolated from patients. Infect. Immun. 15:679-685.

99. Morihara, K., Tsuzuki, H., Oka, T., Inoue, H. and Ebata, M. 1965. Pseudomonas aeruginosa elastase. Isolation, crystallization. J. Biol. Chem. 240:3295-3304.

100. Munster, A.M., Hoagland, H.C. and Pruitt, B.A., Jr. 1970. The effect of thermal injury on serum immunoglobulins. Ann. Surg. 172:965-969.

101. Order, S.E., Mason, A.D., Jr., Walker, H.L., Lindberg, R.B., Switzer, W.C. and Moncrief, J.A. 1965. The pathogenesis of second and third degree burns and conversion to full thickness injury. Surg. Gynec. Obstst. 120:983-991.

102. Oishi, M. and Cosloy. 1972. The genetic and biochemical basis of the transformability of Escherichia coli K12. Biochem. Biophys. Res. Commun. 49:1568-1572.

103. Palleroni, N.J. 1975. General properties of the genus Pseudomonas, p. 1-36. In P.H. Clarke and M.H. Richmond (eds.), Genetics and Biochemistry of Pseudomonas. John Wiley and Sons, New York.

104. Pavlovskis, O.R., Callahan, L.T. III, and Pollack, M. 1975. Pseudomonas aeruginosa exotoxin, p. 252-256. In D. Schlessinger (ed.), Microbiology 1975. American Society for Microbiology, Washington, D.C.

105. Pavlovskis, O.R. and Gordon, F.B. 1972. Pseudomonas aeruginosa exotoxin: effect on cell cultures. J. Infect. Dis. 125:631-636.

106. Pavlovskis, O.R. and Wretlind, B. 1979. Assessment of protease (elastase) as a Pseudomonas aeruginosa virulence factor in experimental mouse burn infection. Infect. Immun. 24:181-187.

107. Pavlovskis, O.R., Iglewski, B.H. and Pollack, M. 1978. Mechanism of action of Pseudomonas aeruginosa exotoxin A in experimental mouse infections: adenosine diphosphate ribosylation of elongation factor 2. Infect. Immun. 19:29-33.

108. Pavlovskis, O.R., Pollack, M., Callahan, L.T. III and Iglewski, B.H. 1977. Passive protection by antitoxin in experimental Pseudomonas aeruginosa burn infections. Infect. Immun. 18: 596-602.

109. Pavlovskis, O.R. and Schackelford, A.H. 1974. Pseudomonas aeruginosa exotoxin in mice: localization and effect on protein synthesis. Infect. Immun. 9:540-546.

110. Pemberton, J.M. 1974. Size of the chromosome of Pseudomonas aeruginosa PAO. J. Bacteriol. 119:748-752.

111. Pemberton, J.M. and Holloway, B.W. 1972. Chromosome mapping in Pseudomonas aeruginosa. Genet. Res. 19:251-260.

112. Phibbs, P.V., Jr., McCowen, S.M., Feary, T.W. and Blevins. W.T. 1978. Mannitol and fructose catabolic pathways of Pseudomonas aeruginosa carbohydrate-negative mutants and pleiotropic effects of certain enzyme deficiencies. J. Bacteriol. 133:717-728.

113. Pollack, M., Callahan, L.T. III and Taylor, N.S. 1976. Neutralizing antibody to Pseudomonas aeruginosa exotoxin in human sera: evidence for in vivo toxin production during infections. Infect. Immun. 14:942-947.

114. Pollack, M., Taylor, N.S. and Callahan, L.T. III. 1977. Exotoxin production by clinical isolates of Pseudomonas aeruginosa. Infect. Immun. 15:776-780.

115. Pruitt, B.A., Jr. 1974. Infections caused by Pseudomonas species in patients with burns and in other surgical patients. J. Infect. Dis. 130:S8-S13.

116. Pruitt, B.A., Jr. and Curreri, P.W. 1971. The burn wound and its care. Arch. Surg. 100:461-468.

117. Pruitt, B.A., Jr. and Lindberg, R.B. 1979. Pseudomonas aeruginosa infections in burn patients, p. 339-365. In R.G. Doggett (ed.), Pseudomonas aeruginosa, Clinical Manifestations of Infection and Current Therapy. Academic Press, New York.

118. Sadoff, J.C. 1974. Cell-wall structure of P. aeruginosa with immunologic significance: a brief review. J. Infect. Dis. 130:S61-S64.

119. Saelinger, C.B., Snell, K. and Holder, I.A. 1977. Experimental studies on the pathogeneisis of infections due to Pseudomonas aeruginosa: direct evidence for toxin production during Pseudomonas infection of burned infected tissues. J. Infect. Dis. 136:555-561.

120. Sanderson, K.E. and Hartman, P.E. 1978. Linkage map of Salmonella typhimurium, edition V. Microbiol. Rev. 42:471-519.

121. Sano, Y. and Kageyama, M. 1977. Transformation of Pseudomonas aeruginosa by plasmid DNA. J. Gen. Appl. Microbiol. 23:183-186.

122. Scharman, W. 1976. Cytotoxic effects of leukocidin from Pseudomonas aeruginosa on polymorphonuclear leukocytes from cattle. Infect. Immun. 13:836-843.

123. Scharman, W. 1976. Interaction of purified leukocidin from Pseudomonas aeruginosa with bovine polymorphonuclear leukocytes. Infect. Immun. 13:1046-1053.

124. Scharman, W. 1976. Formation and isolation of leukocidin from Pseudomonas aeruginosa. J. Gen. Microbiol. 93:283-291.

125. Scharman, W. 1976. Purification and characterization of leukocidin from Pseudomonas aeruginosa. J. Gen. Microbiol. 93:292-302.

126. Scharman, W., Jacob, F. and Porstendorfer, J. 1976. The cytotoxic effect of leukocidin from Pseudomonas aeruginosa on human polymorphonuclear leukocytes. J. Gen. Microbiol. 93:303-308.

127. Schoental, R. 1941. The nature of the antibacterial agents present in Pseudomonas pyocyanea cultures. Br. J. Exp. Pathol. 22:137-147.

128. Schultz, D.R. and Miller, K.D. 1974. Elastase of Pseudomonas aeruginosa: inactivation of complement components and complement-derived chemotactic and phagocytic factors. Infect. Immun. 10:128-132.

129. Sensakovic, J.W. and Bartell, P.F. 1974. The slime of Pseudomonas aeruginosa and possible role in experimental infection. J. Infect. Dis. 129:101-109.

130. Sinclair, M.I. and Morgan, A.F. 1978. Transformation of Pseudomonas aeruginosa strain PAO with bacteriophage and plasmid DNA. Aust. J. Biol. Sci. 31:679-688.

131. Snell, K., Holder, I.A., Leppla, S.A. and Saelinger, C.B. 1978. Role of exotoxin and protease as possible virulence factors in

experimental infections with Pseudomonas aeruginosa. Infect. Immun. 19:839-845.

132. Steritz, D.P. and Holder, I.A. 1975. Experimental studies of the pathogenesis of infections due to Pseudomonas aeruginosa: description of a burned mouse model. J. Infect. Dis. 131: 688-691.

133. Stone, H.H., Braber, C.D., Martin, J.D., Jr. and Kolb, L. 1965. Evaluation of gamma globulin for prophylaxis against burn sepsis. Surgery 58:810-824.

134. Sutter, V.L. and Hurst, V. 1966. Sources of Pseudomonas aeruginosa infection in burns: study of wound and rectal cultures with phage typing. Ann. Surg. 163:597-602.

135. Taylor, N.S. and Pollack, M. 1978. Purification of Pseudomonas aeruginosa exotoxin by affinity chromatography. Infect. Immun. 19:66-70.

136. Vasil, M.L., Kabat, D. and Iglewski, B.H. 1977. Structure-activity relationship of an exotoxin of Pseudomonas aeruginosa. Infect. Immun. 16:353-361.

137. Vasil, M.L., Liu, P.V. and Iglewski, B.H. 1976. Temperature-dependent inactivating factor of Pseudomonas aeruginosa exotoxin A. Infect. Immun. 13:1467-1472.

138. Verder, E. and Evans, J. 1961. A proposed antigenic schema for the identification of strains of Pseudomonas aeruginosa. J. Infect. Dis. 109:183-193.

139. Walker, H.L., McLeod, G.C., Jr., Leppla, S.H. and Mason, A.D., Jr. 1979. Evaluation of Pseudomonas aeruginosa toxin A in experimental rat burn wound sepsis. Infect. Immun. 25:828-830.

140. Warden, G.D., Mason, A.D., Jr., and Pruitt, B.A., Jr. 1974. Evaluation of leukocyte chemotaxis in vitro in thermally injured patients. J. Clin. Invest. 54:1001-1004.

141. Wretlind, B., Heden, L., Sjoberg, L. and Wadstrom, T. 1973. Production of enzymes and toxins by hospital strains of Pseudomonas aeruginosa in relation to serotype and phage-typing pattern. J. Med. Microbiol. 6:91-100.

142. Wretlind, B. and Wadstrom, T. 1977. Purification and properties of a protease with elastase activity from Pseudomonas aeruginosa. J. Gen. Microbiol. 103:319-327.

143. Wretlind, B., Sjoberg, J. and Wadstrom, T. 1977. Protease-deficient mutants of Pseudomonas aeruginosa: pleiotropic changes in the activity of other extracellular enzymes. J. Gen. Microbiol. 103:329-336.

Paper 1.

Toxin A deficient mutants of Pseudomonas aeruginosa strain PA103: Isolation and characterization

Abstract

An immunological assay utilizing double diffusion principles was developed for identification of Pseudomonas aeruginosa mutants deficient in toxin A. Mutations were chemically induced and mutants were isolated from P. aeruginosa strain PA103. Quantitative assays, both enzymatic and immunologic, indicated that five mutants produced toxin A in vitro at levels of 0.3% or less of parental strain levels. Characterization indicated that the mutants fell into four classes and suggested that multiple genes are involved in the regulation of toxin A yields. Classes 1-3 produced less than 1% parental levels of extracellular toxin A. Class 1 mutants are apparently specific for toxin A. Class 2 mutants are pleotropic and produced toxin A, protease and other extracellular proteins at reduced yields. Class 3 mutantsare pleiotropic and in addition have relatively high levels of cell bound toxin A. Class 4 mutants produce toxin A at levels greater than 1% of parental yields. Out of 16 toxin A deficient mutants examined, only one was a class 1 mutant. This mutant (PA103-29) was shown to be identical to the parental strain in all respects tested except for its marked deficiency in toxin A. The suitability of this class 1 mutant for use in virulence studies is discussed.

Introduction

Pseudomonas aeruginosa produces a variety of extracellular products which may play a significant role in the pathogenesis of infections in experimental animal models and in humans (42). Toxin A is

the most toxic substance known to be produced by P. aeruginosa (42, 46). This toxin has been found to inhibit intracellular protein synthesis by the same mechanism as diphtheria toxin (31, 32). Toxin A is produced in vitro by most clinical isolates of P. aeruginosa (6) and appears to be produced in humans during P. aeruginosa infections (49). With some strains of P. aeruginosa, specific anti-toxin A has a protective effect in experimental burn infections of mice (47). These observations suggest that toxin A is a virulence factor.

The pathogenic role of an individual characteristic of an organism can be analyzed by using mutants deficient in that characteristic (25). A mutant which lacks only one potential virulence factor can be compared to the parental strain in animal infection models which mimic the disease in humans. A decrease in virulence with the mutant indicates that the virulence factor has a role in determining the course of the disease. This study was undertaken to isolate toxin A deficient mutants of P. aeruginosa strain PA103 and thoroughly characterize them to assess their potential value in virulence tests.

Materials and Methods

Organisms. Pseudomonas aeruginosa strain PA103, isolated by Liu (40), was selected for this study because it is highly toxigenic and well characterized. This strain is virulent in some experimental animal infections (35, 46) despite its relatively low proteolytic activity (40, 58). Staphylococcus aureus strain Seattle was used in

the staphylolytic enzyme assay. All cultures were stored in sterile, 10% (w/v) skim milk (Difco) at -70° C.

Toxin A culture medium. Trypticase soy dialysate (41) treated with chelex-100 (TSB-DC) was used for the production of toxin A. To 30 g trypticase soy broth (BBL) in 90 ml water, 10 g sodium chelex-100 (minus 400 mesh, Bio-Rad) was added as a deferrating agent. This mixture was stirred for 6 h at room temperature then dialyzed against 1 L of deionized water at 5° C for 15 h. The dialysate was supplemented with 0.05 M monosodium glutamate (Sigma), and 1% glycerol. This TSB-DC had an iron concentration of approximately 0.08 µg/ml (44).

Preparation and purification of antitoxin. P. aeruginosa strain PA103 was used as a source of toxin A which was produced and purified as previously described (33). The purified toxin had a mouse median lethal dose of 0.15 µg/22 g mouse when injected intraperitoneally and migrated as a single band in sodium dodecyl sulfate polyacrylamide gels. Specific antitoxin was obtained by immunization of a sheep as previously described (33). Antitoxin A immunoglobulin was obtained from sheep immune serum as previously described (55). The immunoglobulin fraction was further purified using a solid-phase immunoabsorbant (Sepharose 4B). Purified toxin A was covalently coupled to Sepharose 4B utilizing a modification (33) of the method of March et al. (43). The immunoabsorbant purified antitoxin A immunoglobulin contained 0.5 mg protein/ml.

Isolation of mutants. Log phase cultures of P. aeruginosa PA103 grown in nutrient broth with 0.5% yeast extract (Difco) NYB) were

mutagenized with 100 µg/ml N-methyl-N'-nitro-N-nitrosoguanidine (NTG) (Sigma) at 37° C for 60 min according to the method of Finkelstein et al. (22). The cells were washed twice in NYB, resuspended in NYB, and incubated with shaking at 37° C overnight. Mutagenesis with ethyl methane sulfonate (EMS) (Sigma) was performed on log phase cultures in NYB with exposure to 0.5% (v/v) EMS at 37° C for from 1.5 to 5 h. The cultures were then diluted 1/30 in NYB and incubated at 37° C with shaking overnight.

To identify toxin A deficient mutants, an agar-well assay was developed. NYB was inoculated with the mutagenized overnight cultures and incubated at 37° C with shaking until reaching a cell density of 3×10^8 cells/ml. These cultures were then diluted in saline and 0.1 ml was spread onto each petri plate containing 10 ml of freshly poured TSB-DC with 1.5% Noble agar (Difco) yielding approximately 50 colonies per plate. The plates were dried and incubated 24-36 h in a humid 32° C incubator. Small wells were made in the media approximately 5 mm from each colony with a sterile Pasteur pipet attached to a vacuum source. Each well was filled with approximately 2 µl of filter-sterilized sheep antitoxin and the plates incubated at 32° C for 18 h. Colonies which produced sufficient amounts of toxin A had a distinct band of precipitation between the colony and an adjacent antiserum well (Fig. 1). The intensity of the precipitin bands was accentuated by substituting a partially purified gamma globulin (55) for the whole antisera. Each colony which did not produce a detectable precipitin band was streaked for an isolated

colony and retested for toxin A production by the agar-well assay using a 1 cm streak of overnight growth on a TSB-DC agar plate instead of an isolated colony. Those isolates which were negative in the agar-well test were subcultured and stored in sterile skim milk at -70° C.

Cultivation for toxin A production. Overnight cultures in TSB-DC (0.1 ml) were used to inoculate 10 ml of TSB-DC in 250 ml Erlenmeyer flasks. The flasks were incubated at 32° C with maximum aeration in a reciprocating shaker (Lab-Line Inst.) for 18 or 22 h. Cell growth was monitored at optical density 540 nm (OD_{540}) with a Beckman Spectrophotometer (Model 35). Culture supernatants were obtained by centrifugation at 10,000 x g for 15 min and sterilized via membrane filtration (0.45 μ, Millipore). These supernatants were either stored at -70° C or dialyzed against 0.05 M Tris-HCl (Sigma) pH 8.0 at 5° C and concentrated 10-fold in Minicon-B15 cells (Amicon Corp.) with minimal (<5%) loss of toxin A.

Toxin A yields were also measured in a defined medium for toxin A production (T8S). Medium T8S was medium T8A (18) modified (DeBell, personal communication) to include succinate (0.021 M), a 7-fold increase in the trace salts (0.6 ml TS2), and the omission of glucose. Nitrilotriacetate and $FeCl_3$ were also omitted. This medium had an iron concentration of 0.03 μg/ml. Standard sterile solutions of $FeSO_4 \cdot 7H_2O$ were added at various concentrations. One ml of log phase T8S cultures with an OD_{540} of 0.5 were used to inoculate 9.0 ml of medium T8S in 125 ml Erlenmeyer flasks (nitric acid cleaned). The

flasks were incubated at 32° C with maximum aeration for 18 h, and the supernatants obtained by centrifugation.

Extraction of cell associated toxin A. Cells cultured 22 h in TSB-DC as described above were washed three times in phosphate buffered saline (PBS) pH 7.4, resuspended in one-half the original culture volume in PBS and disrupted by sonication with fifteen 20 s bursts (50% maximal output) using a Biosonik IV (Bronwill) sonicator. After each burst, the sample was swirled in an ice-water bath for 1 min. Fixed and stained samples examined by light microscopy showed that this procedure resulted in the destruction of greater than 95% of the cells. This cellular debris was removed by centrifugation at 12,000 x g for 30 min. The intracellular extract was stored at -70° C.

ADPR-transferase activity. Aminoacyl transferase-containing enzymes were prepared from crude extracts of wheat germ as previously described (15). Extracellular toxin A samples were activated by incubation with an equal volume of 8 M urea, 2% dithiothreitol (DTT) (Sigma) for 15 min at 25° C as previously described (57). The adenosine 5'-diphosphate-ribose (ADPR) transferase activity of toxin A was measured according to the previously described modification (6) of the procedure of Collier and Kandell (16). The reaction was allowed to proceed at 25° C for 60 min. The amount of toxin A present in a given sample was calculated from a standard curve obtained daily using activated purified toxin A (33).

Antiserum neutralization of toxin A. Enzyme neutralization by antibody was determined by assaying the ADPR-transferase activity of

toxin A after incubation of urea-DTT treated samples with equal volumes of antitoxin A or normal sheep serum for 15 min at 37° C as previously described (31).

Reversed passive hemagglutination (RPHA) assay. The RPHA assay for toxin A was developed using modifications of the procedure of Holmes et al. (26). A stock solution of bis-diazotized benzidine (BDB) was prepared and stored exactly as described by Butler (13). Formalinized sheep erythrocytes were prepared and stored at 4° C for up to two months (24). The sensitizing mixture varied from batch to batch of BDB and had to be determined empirically. A representative sensitizing mixture consisted of: 1.8 ml buffer A (82 mM NaCl, 43 mM $Na_2HPO_4 \cdot 2H_2O$, 10.7 mM KH_2PO_4, pH 7.4), 0.24 ml of 10% formalinized sheep erythrocytes, 0.1 ml of purified anti-A immunoglobulin and 0.24 ml of a 1:10 dilution of stock BDB solution in buffer A. The cells were sensitized by incubation of the above mixture for 10 min at 37° C with gentle agitation. The sensitized erythrocytes were washed as described by Holmes et al. (26) and suspended to 0.2% in diluent (buffer A containing 1% crystalline bovine serum albumin and 0.01% merthiolate). The sensitized erythrocytes were used on the same day they were prepared.

RPHA assays were performed in plastic microtiter trays with V-bottom wells (Linbro Chemical Co.) as described by Holmes et al. (26). Serial two-fold dilutions of purified toxin A served as a positive control for passive hemagglutination and were used to

determine the endpoint. This assay detected as little as 2 ng toxin A/ml and did not vary more than one serial dilution between experiments.

Biochemical characterization. The ability to assimilate various organic compounds as sole carbon sources was determined by individually adding to a final concentration of 0.03 M the following filter sterilized carbon sources to minimal media (9): glucose, acetate, propionate, succinate, fumarate, adipate, malate, lactate, citrate, pyruvate, glycine, alanine, arginine, aspartate, glutamate, lysine and acetamide. Each medium was inoculated from an overnight culture on a NYB agar slant and incubated up to 8 days at 37° C before growth was considered negative.

The ability to oxidize various carbohydrates was determined in OF basal media (Difco). The following filter sterilized carbohydrate solutions were added to individual tubes of melted base medium to 1% (w/v) final concentration: glucose, fructose, mannose, galactose, arabinose, xylose, cellobiose, lactose, maltose, sucrose, glycogen, dextrin, erythritol, glycerol, and mannitol. The reactions were read daily during a 5 day incubation at 37° C.

Other tests were performed as previously described and included: cytochrome oxidase test (20); gluconate oxidation (23); arginine dihydrolase (54); indole test (59); lysine decarboxylase (59), citrate utilization (59); urease activity (59); nitrate reduction (59), and anaerobic fermentation of glucose (59).

Also tested was the ability to grow on cetrimide agar (10), deoxycholate citrate agar (Difco), endo agar (Difco), MacConkey agar

(Difco), and SS agar (Difco). These media were lightly inoculated and growth was ascertained after 24 h at 37° C. Growth at 42° C was determined after 24 h incubation of inoculated tubes of brain heart infusion broth (BHI) (Difco).

Assays for extracellular products. The ability to produce various extracellular products was determined on the following plate tests. Unless otherwise stated the zone of activity around a 15 mm streak of growth was recorded after incubation at 37° C for 48 h. Protease production was determined on agar plates containing 1.5% (w/v) skimmed milk (52). Elastase production was detected on nutrient agar (Difco) plates at pH 7.5 containing 0.3% (w/v) elastin (Sigma); lipase production on tributyrin agar (29); and esterase production was detected as dark blue colonies on brain heart infusion agar plates at pH 7.6 containing 0.1% indoxyl acetate (Sigma) (28). Hyaluronidase production was determined using nutrient agar plates at pH 6.8 containing umbilical cord hyaluronic acid (Sigma) using the procedure described by Smith (51). DNase and RNase activities were determined on nutrient agar to which filter sterilized DNA or RNA solutions were added to a final concentration of 3 mg/ml(34). Hemolysin production by isolated colonies was observed on media containing 0.4% (w/v) heart infusion broth (Difco), 1.5% (v/v) bacto agar and 6% (v/v) human or sheep blood. Production of pyocyanin and fluorescin were determined on agar media slants as described by King et al. (36). Gelatinase was determined on a medium of 23 g nutrient agar, 0.5 g KH_2PO_4, 4.0 g gelatin, 0.05 g glucose, 1 liter water, pH 7.0, 20 ml per plate. Cultures were streaked onto plates,

incubated at 37° C for 24 h then flooded with a solution of 15% (w/v) $HgCl_2$ in 20% (v/v) HCl. Clear zones formed around gelatin liquefying colonies (17).

Cultivation and assay conditions for lecithinase production were according to Stinson and Hayden (53) except that phosphate concentrations were determined according to Ames (1). Staphylolytic activity was determined according to Burke and Pattee (12) using an overnight NYB culture of Staphylococcus aureus strain Seattle as substrate. Production of alkaline phosphatase was determined using the medium and culture conditions described by Cheng et al. (14) and assayed according to Torriani (56). Production of leukocidin activity utilized the culture conditions and cell autolysis method described by Scharmann (50). Leukocidin activity was determined by a modification of the procedure previously described (50) whereby 0.2 ml of autolysis sample was incubated with 0.2 ml of isolated human granulocytes at 5×10^6/ml in phosphate buffered saline. The granulocytes were isolated from heparinized, peripheral venous blood via hypaque-ficoll separation and 3% dextran sedimentation followed by erythrocyte lysis with hypotonic saline (7). Viability of the granulocytes was determined by their exclusion of trypan blue (Gibco).

Serotyping and pyocin typing. Serotypes were determined by slide agglutination using seven immunotype sera kindly provided by Dr. M. Fisher (Parke, Davis and Co.). Typing for the production of pyocins was by the method of Farmer and Herman (21) using 18 indicator strains kindly supplied by Dr. B. Minshew (Univ. of Washington, Seattle, Wa.).

Motility and growth curves. Motility was observed microscopically by the hanging-drop method. Growth of the parent and mutant strains were compared in vitro. Fifty ml of TSB-DC per 1 liter Erlenmeyer flask was inoculated with 0.1 ml of an overnight culture and incubated with maximum aeration in a 32° C shaking water bath. Samples were withdrawn hourly and the OD_{540} was measured. Growth curves were constructed and the generation time was calculated according to Miller (44).

Antibiotic sensitivity. Sensitivity to ampicillin, cephalothin, carbenicillin, tetracycline, chloramphenicol, kanamycin, amikacin, sulfamethizole, nitrofurantoin, tobramycin, and trimethopsulfa was determined by the Kirby and Bauer (3) quantitative disc diffusion method.

Polyacrylamide gel electrophoresis. The sodium dodecyl sulfate (SDS)-polyacryalmide gel electrophoresis system (SDS-PAGE) was that described by Laemmli (38). Gels contained 10% acrylamide with a stacking gel of 4.5% acrylamide. All samples of culture supernatant preparations were diluted in an equal volume of sample buffer containing 0.0312 M Tris-hydrochloride (pH 6.8), 10% 2-mercaptoethanol, 20% glycerol and 4% SDS. For slab gel electrohporesis, a 10 µl sample containing 7-20 µg of protein was heated in sample buffer to 100° C for 5 min prior to loading. The following proteins (with corresponding molecular weights) were used as standards: phosphorylase B, 93,000; bovine serum albumin, 68,000; pyruvate kinase, 57,000; ovalbumin, 43,000; lactate dehydrogenase, 36,000; carbonic anhydrase,

30,000; soybean trypsin inhibitor, 21,000; and lysozyme, 14,000. Electrophoresis was performed at room temperature using a constant power of 1 watt. Gels were stained at room temperature for 12-18 h in a solution of 25% isopropanol, 10% acetic acid, and 0.05% Coomassie blue. Gels were then decolorized with 10% acetic acid.

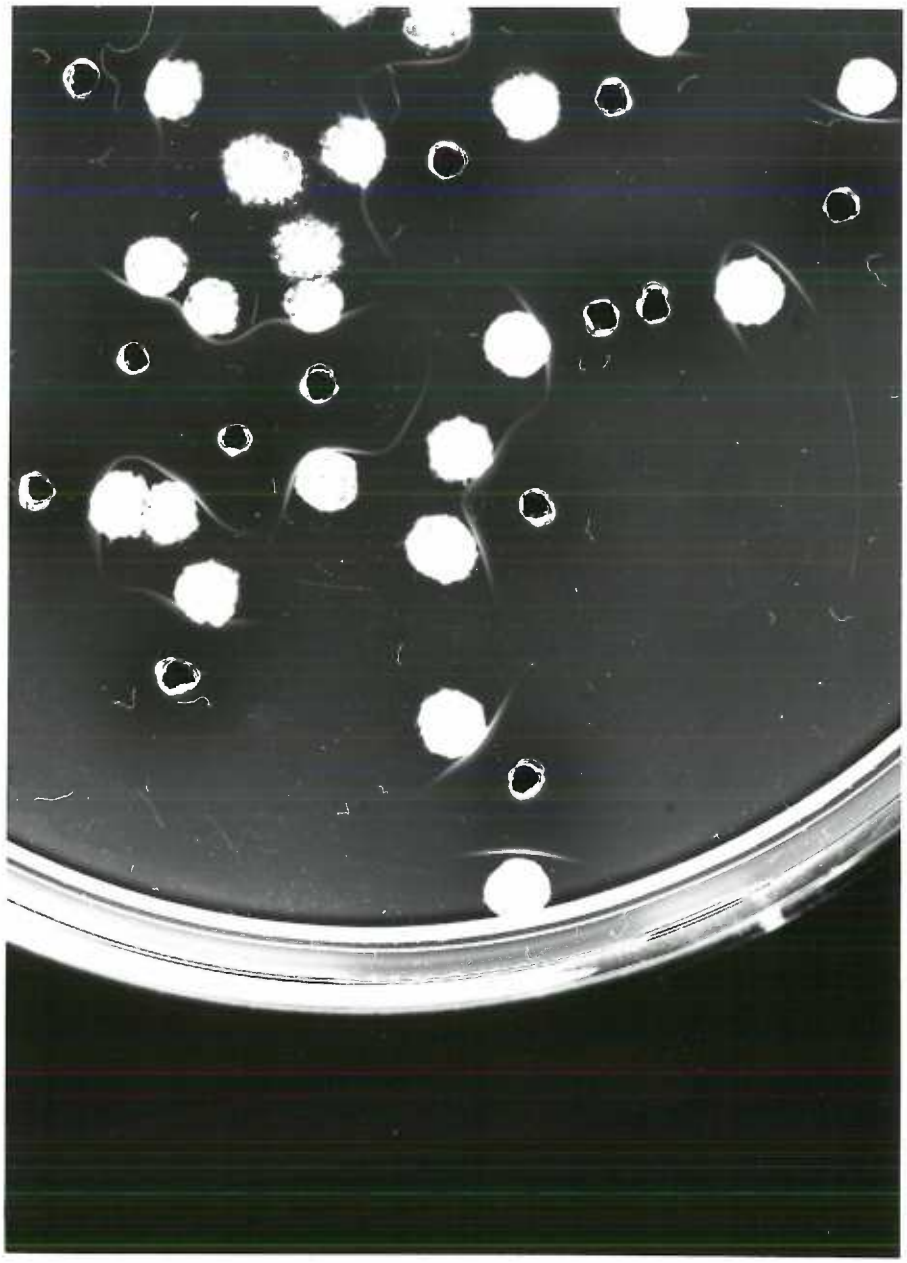
Cylindrical gels (5.5 cm x 0.6 cm) were prepared, electrophoresed and stained as described above. Samples (50 µl) were applied to these gels without prior heating. A duplicate gel of each sample was electrophoresed and immediately sliced (approximately 1.1 mm/fraction). Each fraction was crushed and eluted at 5° C for 24 h with 150 µl of elution buffer (0.125 M Tris-hydrochloride, pH 7.0 and 0.1 M dithiothreitol). The ADPR-transferase activity of 2.5 µl of the eluate from each gel fraction was assayed as described above.

Protein determination. Protein was determined by the method of Bradford (8) modified by using a commercial reagent, Bio-Rad Protein Assay Dye Reagent Concentrate, purchased from Bio-Rad Laboratories (Richmond, Ca.). Ovalbumin was used as the standard.

Results

Isolation of toxin A deficient mutants. An agar-well assay was developed to identify toxin A mutants (Fig. 1). Two mutagenic agents, EMS and NTG, were tested for their ability to induce toxin A mutants. The use of EMS for the induction of toxin A deficient mutants was found to be ineffective. After incubation with 0.5% EMS, all of approximately 6,000 colonies tested were positive in the agar well

Figure 1. Agar-well assay for the detection of toxin A production by *P. aeruginosa*.



assay. Following NTG mutagenesis, approximately 15,000 scorable colonies were examined, and colonies were found which were negative in the agar-well assay. These were recloned, subcultured and cultivated for toxin A production in TSB-DC broth. The relative amount of toxin each mutant produced was determined in the ADPR-transferase assay. The ADPR-transferase activities of the mutant culture supernatants were compared to the reactions of 10-fold dilutions of the parental strain supernatant (Fig. 2). A total of 16 toxin A deficient mutants was isolated at a frequency of approximately 10^{-3}. The toxin A enzymatic activities of these 16 mutants ranged from <1 to 40% of that produced by the parental strain, designated PA103-0 (Fig. 2).

Quantitation of toxin A production by PA103 mutants. Only five of the 16 mutants, PA103-8, 15, 16, 19, and 29 produced toxin A at 0.5% or less than the parent (Fig. 2). The amount of extracellular toxin A produced by each of these five mutants and the parent was determined by assaying aliquots of 10-fold concentrated supernatants in the ADPR-transferase assay and in the RPHA assay (Table 1). The parental strain produced 25-30 μg toxin A/ml of culture supernatant, and the five mutants produced less than 0.1 μg of toxin A/ml in both assays. Thus, these five mutants produced less than 0.33% of the extracellular toxin A produced by the parent. When the samples were preincubated with specific toxin A antiserum, their ADPR-transferase activity was totally inactivated. Preincubation with normal sheep serum had no effect on the ADPR-transferase activity of the samples (data not shown).

Figure 2. Comparison of the amount of ADPR transferase activity in culture supernatants of the parent to those of the mutants. Ten-fold dilutions of parental strain PA103-0 supernatant (■), and toxin A mutant supernatants (○). The numerical strain designation of the mutants is indicated.

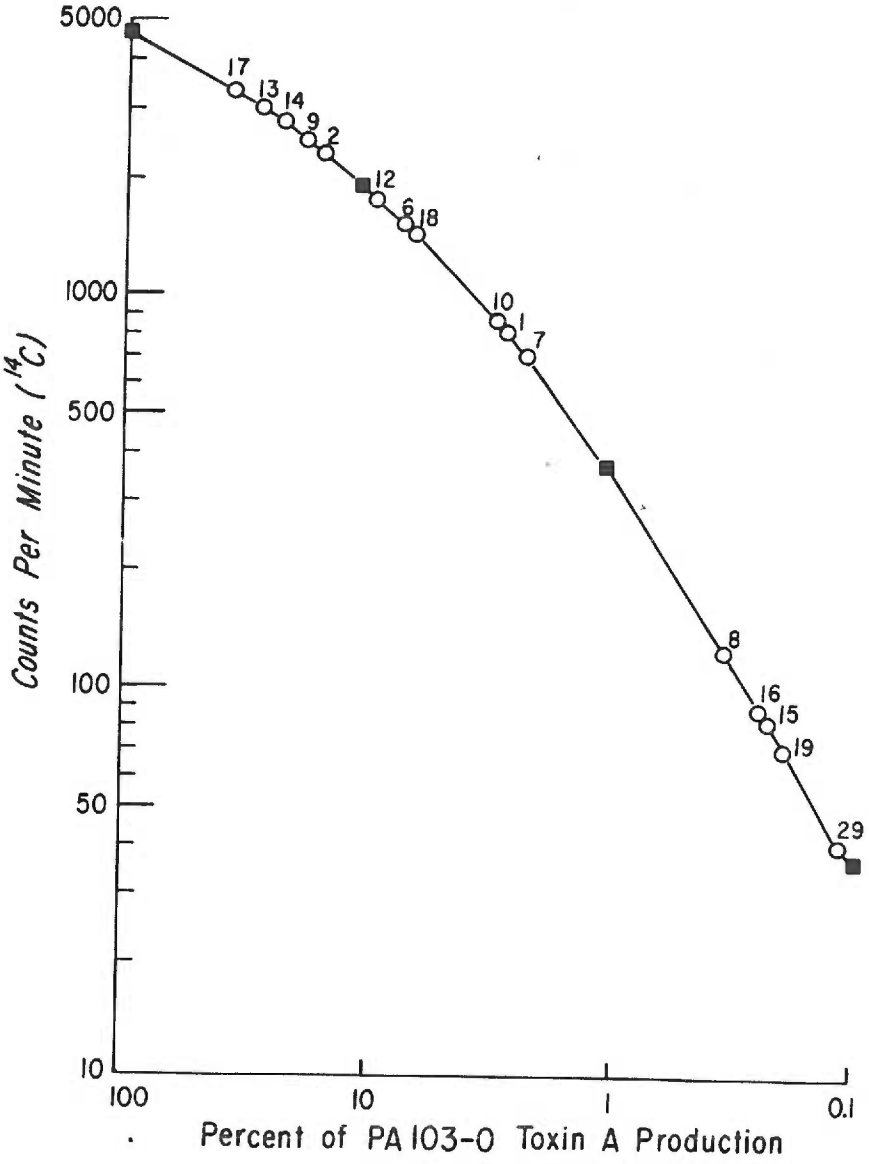

5000
1000
500
100
50
10
Counts Per Minute (^{14}C)
17
13
14
9
2
12
6
18
10
1
7
8
16
15
19
29
100
10
1
0.1
Percent of PA 103-0 Toxin A Production

Table 1. Quantitation of toxin A in the extracellular supernatants of parental strain PA103-0 and toxin A mutants using ADPR transferase assay and a reverse passive hemagglutination assay.

	ADPR-transferase activity			RPHA		
Strain	CPM[a]	µg/ml[c]	% Rel. act.	End pt.[d]	µg/ml[c]	% Rel. act.
PA103-0	144,000[b]	30	100	51,200	25.6	100
PA103-8	4,120	0.10	0.33	64	0.032	0.13
PA103-15	1,394	0.04	0.13	64	0.032	0.13
PA103-16	2,011	0.06	0.20	64	0.032	0.13
PA103-19	1,878	0.06	0.20	8	0.004	0.02
PA103-29	2,136	0.06	0.20	32	0.016	0.06

[a]Values obtained with 10X concentrated supernatants. The background has been subtrated.

[b]Extrapolated from 1 x 10^{-3} dilution of 10X concentrated supernatant.

[c]Values corrected to correspond to 1X supernatants.

[d]Values represent reciprocol of highest dilution giving total agglutination using 10X concentrated culture supernatants.

The amount of cell associated toxin in cell lysates of the parent and mutants was quantitated in the ADPR-transferase assay and the RPHA assay (Table 2). The lysates from the parental cells (PA103-0) contained about 0.64 μg toxin A/ml. Most of the cell extracts from the mutants did not contain detectable levels of toxin A. However, the cell extract from PA103-19, while not detectable in the ADPR-transferase assay, did give a positive RPHA reaction (Table 2). Based on the RPHA assay, mutant PA103-19 had 25% as much cell associated toxin A as the parent.

Characterization of toxin A deficient mutants. Strain PA103-0 and mutants PA103-8, 15, 16, 19, and 29 showed the same pattern of reactions when tested for the ability to utilize 17 organic compounds as sole carbon sources and to oxidize 15 carbohydrates (Table 3). The parental and mutant strains were able to grow on the selective media: cetrimide agar, deoxycholate citrate agar, endo agar, MacConkey agar, SS agar, and in BHI broth at elevated temperatures (42° C). No fermentation of glucose was evident in sealed tubes. Positive reactions were observed with all strains in Simmons' citrate, malonate, arginine dihydrolase, oxidase, nitrate to gas, and gluconate tests. Lysine and ornithine decarboxylase, indole, and urea reactions were negative. The parent and five mutants were motile and had the identical pyocin production pattern, serotype, and antibiotic resistance pattern.

Deficiencies among the mutants in toxin A production could not be attributed to a mutation affecting growth in vitro. In TSB-DC

Table 2. Measurement of toxin A in cell-associated preparations of parental strain and toxin A mutants

	ADPR-transferase activity		RPHA		
Strain	CPM[a]	% Rel. act.	End pt.	µg/ml	% Rel. act.
PA103-0	3,800[b]	100	128	0.64	100
PA103-8	20	0.5	BD[c]	--	--
PA103-15	17	0.4	BD	--	--
PA103-16	19	0.5	BD	--	--
PA103-19	26	0.7	32	0.16	25
PA103-29	6	0.2	BD	--	--

[a]The background has been subtracted.

[b]Extrapolated from 1 x 10^{-2} dilution of cell-associated preparation.

[c]BD, below detection.

Table 3. Biochemical characterization of *P. aeruginosa* strain PA103-0 and toxin A mutants

Carbon Assimilation		Carbohydrate Oxidation		Growth	
Carbohydrate		Monosaccharides		Cetrimide Agar	+
Glucose	+	D-Glucose	+	Deoxycholate Citrate	+
Fatty Acids		D-Fructose	+	Endo Agar	+
Acetate	+	D-Mannose	+	MacConkey Agar	+
Propionate	+	D-Galactose	+	SS Agar	+
Dicarboxylic Acids		L-Arabinose	+	BHI broth @ 42°C	+
Succinate	+	D-Xylose	+	Glucose sealed OF	-
Fumarate	-	Disaccharides			
Adipate	-	Cellobiose	-	Other Tests	
Hydroxyacids		Lactose	-	Motility	+
DL-malate	-	Maltose	-	Simmons' Citrate	+
DL-lactate	+	Sucrose	-	Malonate	+
Organic Acids		Polysaccharides		Arg. Dihydrolase	+
Citrate	+	Glycogen	-	Oxidase	+
Pyruvate	+	Dextrin	-	Nitrate to Gas	+

Table 3. (Continued)

Carbon Assimilation		Carbohydrate Oxidation		Other Tests	
Amino Acids		Alcohols		Gluconate	+
Glycine	+	Erythritol	-	Lys. Decarboxylase	-
L-alanine	+	Glycerol	+	Orn. Decarboxylase	-
L-arginine	+	Mannitol	+	Indole	-
DL-aspartate	+			Urea	-
L-glutamate	+			Pyocin Pattern	611,231
L-lysine	+			Serotype	2
Nitrogenous Cmpd.				Gen. time (TSB-DC)	31 min.
Acetamide	+				

[a]The parental strain and mutants PA103-8, 15, 16, 19 and 29 all showed the same reactions indicated.

at 32° C the growth curves constructed over a 24 h period were found to be identical within experimental error (data not shown). No deviation from the parental strain generation time of 31 min during early log phase could be measured in the five mutant strain cultures.

None of the five mutants appear to have a temperature-dependent conditional mutation affecting toxin A production. When the parent and toxin mutants were cultivated for toxin A production in TSB-DC at 24°, 28°, 37° and 42° C, no increase in ADPR-transferase activity could be detected by the mutants at temperatures below or above the optimal (32° C) temperature (data not shown).

The formation of extracellular products was estimated using agar and broth methods (Table 4). The cultivation of the mutants and parental strain in the different media indicated that the production of gelatinase, esterase, lipase, alkaline phosphatase, sheep erythrocyte hemolysin, and fluorescin was not altered. None of the strains produced elastase, human erythrocyte hemolysin, pyocyanin, hyaluronidase, DNase, RNase, staphylolytic enzyme or leukocidin activities. However, protease activity varied from that of the parental strain in all but one of the mutants. Three mutants (PA103-15, 16, and 19) showed marked protease deficiencies, and, PA103-8 produced a clearing zone that was about 0.5 mm smaller than the parent (PA103-0). Mutant PA103-29 produced an identical protease reaction to the parental strain.

The effect of iron on yields of toxin A by PA103 mutants. The presence of excess iron in the culture medium has been shown to

Table 4. Extracellular products of *P. aeruginosa* strain PA103-0 and toxin A mutants

Extracellular Product	Assay Type	PA103-0	PA103-8	PA103-15	PA103-16	PA103-19	PA103-29
Protease	Zone(mm)	1.5	1.0	0.1	0.1	0	1.5
Gelatinase	Zone(mm)	3.0	3.0	3.0	3.0	3.0	3.0
Lipase	Zone(mm)	2.0	2.0	2.0	2.0	2.0	2.0
Alkaline Phosphatase	% Rel. Act.	100	100	100	100	100	100
Esterase	Plate	+	+	+	+	+	+
Hemolysin:							
Sheep	Plate	+	+	+	+	+	+
Fluorescin	Slant	+	+	+	+	+	+

[a]None of the strains produced detectable levels of elastase, human erythrocyte, hemolysin, pyocyanin, hyaluronidase, DNase, RNase, staphylolytic enzyme, lecithinase, exoenzyme S, or leukocidin activities.

inhibit yields of toxin A (4). It was possible that some of the toxin A mutants had an altered sensitivity to iron which could account for their decreased toxin A yields. The yields of toxin A by each mutant were measured at various iron concentrations. A defined medium designed for toxin A production, medium T8S, permitted the use of very low iron concentrations. At the optimal concentration of iron (0.08 μg/ml) for toxin A yields by the parent (PA103-0) the mutant strains produced less than 0.1% of parental levels of toxin A (Table 5). When iron was added to the culture media at two suboptimal concentrations (0.03 and 0.05 μg/ml), toxin A yields decline in the mutant cultures as well as the parental cultures. Thus, toxin A production by the mutants was no more sensitive to iron than the parental strain. It was also possible that some of the mutants required more iron for toxin A production. However a concentration of iron that was 0.05 μg/ml higher than the optimum for toxin A yields from the parent (0.13 μg/ml) did not increase toxin A synthesis by the mutants (Table 5).

The effect of iron on yields of total extracellular protein. Excess iron in the culture medium has also been shown to reduce the yield of total extracellular protein produced by P. aeruginosa (5). Iron regulation in the toxin A mutant strains was compared to the parental strain in TSB-DC medium at 32° C. Cultures were uniformly inoculated and reached very similar cell densities at 18 h. The optical density at 540 nm (OD_{540}) of each culture was approximately 9.0 with low-iron media and 23.0 in high-iron media. The amount of protein was expressed in terms of micrograms of extracellular protein

Table 5. Effect of iron concentration on yields of extracellular toxin A concentrations of P. aeruginosa mutant strains[a]

Strain	Relative ADPR transferase activity (CPM)[d]			
	0.03 Fe[b]	0.05 Fe	0.08 Fe	0.13 Fe
PA103-0[c]	6,550	9,750	17,000	8,580
PA103-8	84	79	130	109
PA103-15	38	61	78	69
PA103-16	23	52	124	128
PA103-19	0	0	14	0
PA103-29	34	68	168	142

[a]All cultures (T8S medium) at each iron concentration were at approximately the same cell density when supernatants were obtained by centrifugation at 18 h post-inoculation.

[b]Indicates μg/ml final concentration of iron (Fe) in T8S defined medium.

[c]CPM of PA103-0 were extrapolated from 10^{-1} dilution of supernatant.

[d]CPM of sample treated with urea, DTT (57).

per milliliter of culture supernatant and also as a function of bacterial growth (micrograms of extracellular protein per milliliter per unit of OD_{540}) to compensate for the differences in growth between cultures grown in high- and low-iron media (Table 6).

The yields of extracellular protein from mutant PA103-29 low-iron cultures were consistently about 20-30 µg/ml. This can be accounted for by a decrease of approximately 30 µg/ml of toxin A by PA103-29. The cultures of mutants PA103-8, 15, and 16 had yields of extracellular protein that were reduced by about one-half to two-thirds (200-310 µg/ml) compared to yields of the parent. Mutant PA103-19 produced extracellular protein in low-iron media at less than one-half (150 µg/ml) the parental supernatant level. The markedly reduced yields of extracellular protein in cultures of PA103-8, 15, 16 and 19 could not be accounted for simply by their decreased toxin A yields and suggest that yields of other extracellular proteins are also reduced. When cultured in medium containing an increased iron concentration (5.0 µg/ml), the amount of extracellular protein per milliliter per OD_{540} unit was dramatically decreased for all five mutant strains as well as the parent (Table 6). Thus, as the iron concentration of the TSB-DC growth medium increased, the yields of other extracellular proteins besides toxin A decreased in the cultures of the five mutants and the parent.

PAGE of extracellular products from PA103 mutants. The TSB-DC supernatants from 18 h cultures of PA103-0 contained 32 visible bands upon SDS-PAGE (Fig. 3). Protein bands of interest or reference are

Table 6. Effect of iron on yields of extracellular protein in TSB-DC cultures of *P. aeruginosa* mutant strains[a]

	Low-iron[d]		High-iron[d]	
Strain	µg/ml[b]	µg/OD_{540}[c]	µg/ml[b]	µg/OD_{540}[c]
PA103-0	400	47.6	140	6.3
PA103-8	200	22.2	140	6.2
PA103-15	250	27.8	110	4.6
PA103-16	310	34.4	130	5.4
PA103-19	150	16.7	110	4.6
PA103-29	380	42.2	210	8.7

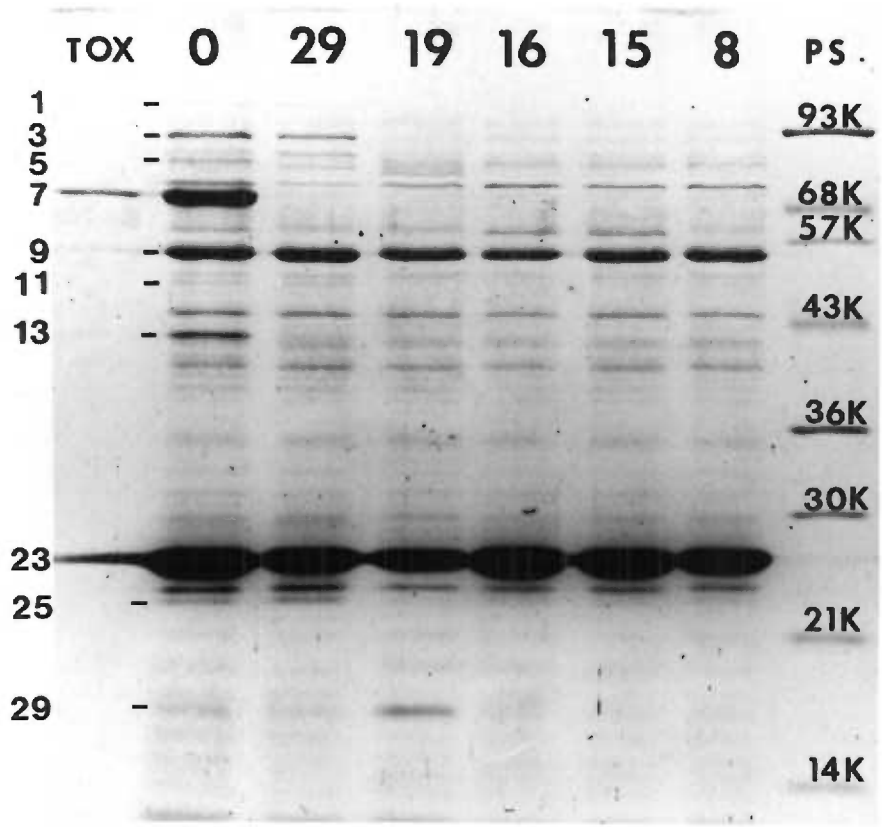
[a]All cultures of each iron concentration group were at approximately the same cell density when supernatants were removed by centrifugation at 18 h post-inoculation.

[b]Indicated µg extracellular protein per ml in dialyzed culture supernatant.

[c]Indicates µg extracellular protein per ml per OD_{540} unit.

[d]Low-iron (0.08 µg/ml) medium; High-iron (5.0 µg/ml) medium.

Fig. 3. SDS-PAGE of extracellular proteins produced by P. aeruginosa. 0, PA103-0 toxin A producing parental strain; 29, 19, 16, 15, 8, PA103 toxin A deficient mutants. Cells were grown in TSB-DC for 18 h to a cell density of approximately OD_{540} 9.0. Supernatants were concentrated 5-fold and 10 µl of each was applied to the gel. Toxin A (Tox) and protein standards (PS) were used as molecular weight markers.


TOX
0
29
19
16
15
8
PS.
1
3
5
7
9
11
13
23
25
29
93K
68K
57K
43K
36K
30K
21K
14K

labeled (calculated molecular weights are given below). In all five of the mutant strain supernatants, two proteins were lost, protein 7 (73,000) and protein 13 (42,500). The ADPR-transferase activity profiles of SDS-PAGE of parental strain supernatants showed ADPR-transferase activity associated with both protein 7 and protein 13 (data not shown). The enzymatic activity associated with both of these proteins was totally inactivated by pre-incubation with toxin A antiserum but not normal sheep serum. Protein 7 was identified as toxin A on the basis of its enzymatic activity and its comigration on SDS-PAGE with purified toxin A. Protein 13 produced by the parental strain but not by the mutants appears to be a fragment of toxin A.

Based on the SDS-PAGE profiles of the culture supernatants the mutants had three distinct patterns. First, the pattern of mutant PA103-29 supernatant proteins was the same as the parental extracellular protein pattern except for the loss of the toxin A band (protein 7) and the toxin A fragment band (protein 13). Mutant PA103-29 was the only mutant examined whose extracellular protein profile was apparently identical to that of the parental strain except for the loss of toxin A material. The supernatants of PA103-8, 15 and 16 showed a second pattern with SDS-PAGE. Compared to the parent, the supernatants of these mutant strains lacked proteins 1 (100,500), 7 (toxin A), 13 (toxin A fragment) and had reduced concentrations of proteins 3 (93,000), 11 (50,000) and 25 (23,500). A third SDS-PAGE pattern was obtained with the supernatant of mutant 19. Supernatants of mutant 19 lacked proteins 1, 7, 11 and 13; had reduced

concentrations of proteins 3, 11, 23 and 25; had an increased concentration of protein 29 (17,500); and contained two additional bands, one immediately above and one immediately below protein 5 (96,500) (Fig. 3).

Discussion

The agar well assay, which was developed to identify toxin A deficient mutants, was found to have several advantages over modified Elek plates (4). The agar well assay was more rapid, allowing the examination of a larger number of colonies, and was more economical, requiring much less antiserum and fewer petri plates than the Elek technique.

Toxin A deficient mutants of P. aeruginosa strain PA103 were isolated and their phenotypic properties were compared to the parental strain. While none of the mutants completely lacked extracellular toxin A, five mutants were isolated whose extracellular yields of toxin A were <0.33% of the parent (Table 1). An extensive characterization showed that the parental and mutant strains all had identical growth rate, pyocin production pattern, serotype, antibiotic resistance pattern, and biochemical reaction pattern. Growth on minimal media containing single carbon sources showed that none of the mutants had developed any auxotrophic requirements. The results of these tests (Table 3) indicated that the mutants were derived from the parental strain and were not contaminants. Furthermore, these tests acted as a probe of the genome to show that no secondary mutation

affecting the above characteristics had occurred.

Toxin A is not formed constitutively, and the regulation of its production by P. aeruginosa has not yet been elucidated. Excess iron in the culture medium has been shown to inhibit yields of toxin A (4) and other extracellular products (5). The molecular mechanism(s) underlying iron regulation of toxin A yields remains unknown. None of the five PA103 toxin A mutants characterized could be classified as having mutations involved with regulation of toxin A by iron (Table 5 and 6).

Toxin A production is also regulated by the growth temperature. The optimal temperature for toxin A yields in vitro is 32° C (41). None of the mutant strains characterized appeared to have conditional temperature-sensitive mutations which permit increased toxin A production at a temperature below or above the optimum.

Wretlind et al. (61) reported that protease deficient mutants of P. aeruginosa had pleiotropic changes in the activities of other extracellular enzymes. We have found that chemically induced toxin A deficient mutants often show reduced yields and/or lower activities of other extracellular proteins. Four of the five mutants which were severely toxin A deficient (PA103-8, 15, 16 and 19) had deficiencies in protease activity, total extracellular protein concentrations were considerably lower, and specific proteins were found to be missing or reduced in concentration when culture supernatants were examined by SDS-PAGE. Our data and that of Wretlind et al. (61) suggests that a common regulatory mechanism(s) probably exists for the synthesis,

processing and/or release of toxin A, protease and other extracellular proteins.

Among the five mutants characterized, four produced toxin A intracellularly at relative concentrations that were less than 1% of the parental strain (PA103-8, 15, 16 and 29). This would indicate that these four are not permease mutants, but either have defects in their ability to synthesize toxin A, or possibly an increased rate of intracellular degradation of toxin A. The markedly pleiotropic mutant PA103-19 had a relatively high level of intracellular enzymatically inactive toxin A: 25% of parental levels was found in the RPHA assay, but <1% in the ADPR-transferase assay (Table 2). This may indicate a defect in the intracellular processing or secretion of toxin A by this mutant.

Mutant PA103-29 was found to be identical to the parental strain except for its markedly reduced toxin A yields. This mutant is the most likely among the five mutants to have a specific toxin A (tox) gene mutation. SDS-PAGE patterns showed that every protein band in the parent culture supernatant was present in PA103-29 supernatants except for two bands associated with the tox structural gene product: toxin A itself (73,000 daltons) and an enzymatically active fragment found at 43,500 daltons (Fig. 3). An enzymatically active fragment of approximately this molecular weight with ADPR-transferase activity has been previously observed (S. Lory and R.J. Collier, Abstr. Annu. Meet. Am. Soc. Microbiol. 1979, B6). Firm support for a structural gene mutation would be the presence of toxin A cross reacting material (CRM) in culture supernatant of mutant PA103-29. However, it is the

nature of our agar well screening technique to detect mutants which produce no extracellular toxin A antigens. Thus, our initial mutant selection process prohibits the identification of a CRM producing mutant. However, the mutation in PA103-29 could be very early in the structural gene sequence such that no detectable CRM product was formed. The production of toxin A by PA103-29 at 0.2% of the parental strain (Table 1) could be due to occasional read-throughs of a nonsense mutation early in the tox structural gene sequence. Alternatively, the specificity of this mutation for toxin A could be due to mutation in a tox specific regulatory gene. Since the locus and fine structure of tox genes are unknown, we cannot distinguish between these two possibilities.

In an effort to categorize the various types of mutations which result in toxin A deficiencies, we have established a broad classification system. Class 1 toxin A mutants, such as PA103-29, have mutations which are apparently specific for toxin A. Other cell functions appear to be normal. The genotype of this mutant is referred to as tox-1. Class 2 mutants are also markedly deficient in toxin A production, but in addition are deficient in the production of protease and other extracellular proteins. Mutants PA103-8, 15 and 16 fall into this group. Class 3 mutants have pleiotropic deficiencies in the production of multiple extracellular proteins including toxin A but have relatively high levels of intracellular toxin A antigen. Mutant PA103-19 was placed in this category. Such mutants may have defects in the transport of extracellular products through the cell

membrane. Class 4 mutants are those which produce toxin A at levels greater than 1% of parental yields. The wide range of toxin A yields observed in this group (Fig. 2) suggests that there are several subgroups in this class.

Investigators have studied the purified toxic products of P. aeruginosa and their effects when injected into animals (30, 37). Others have compared different strains of P. aeruginosa possessing various combinations of pathogenic properties in animal infections in an effort to correlate a suspected virulence trait of the organism with pathogenicity (47). Such a correlation is difficult because independently isolated P. aeruginosa strains are so heterogeneous with respect to the production of potential virulence products (60). Genetic techniques have been used to understand the role of colonization factors, invasiveness, toxins and other virulence factors of other bacterial pathogens (2, 11, 19, 27, 39, 48). A decrease in virulence with a mutant deficient in only a single characteristic, when compared to the parental strain in a suitable animal model, indicates that this characteristic has a role in the disease (25).

The long term goal of my studies is to determine the effect of a specific tox mutation on the virulence of P. aeruginosa. The current study is the initial step in using this approach to study virulence in P. aeruginosa, the isolation of a specific tox mutant. Mutant PA103-29 appears to be identical to the parental strain, except for a specific mutation affecting yields of toxin A (tox). The wide range of tests performed not only indicated that no other alterations are associated with this mutation, but also layed a groundwork

of characterization for strain PA103 in virulence tests. Thus, PA103-29 is well suited for comparisons with its toxigenic parental strain in experimental animal infection models. Comparisons between these strains should make it possible to determine the relative contribution of toxin A as a virulence factor of *P. aeruginosa*.

References

1. Ames, B.N. 1966. Assay of inorganic phosphate, total phosphate and phosphatases. Methods in Enzymol. 8:115-118.

2. Barksdale, L. and Arden, S.B. 1974. Persisting bacteriophage infections, lysogeny, and phage conversions. Ann. Rev. Microbiol. 28:265-299.

3. Bauer, A.W., Kirby, W.M.M., Sherris, J.C. and Turck, M. 1966. Antibiotic susceptibility testing by a standardized single disc method. Amer. J. Clin. Path. 45:493-496.

4. Bjorn, M.J., Iglewski, B.H., Ives, S.K., Sadoff, J.C. and Vasil, M.L. 1978. Effect of iron on yields of exotoxin A in cultures of Pseudomonas aeruginosa PA-103. Infect. Immun. 19:785-791.

5. Bjorn, M.J., Sokol, P.A. and Iglewski, B.H. 1979. Influence of iron on yields of extracellular products in cultures of Pseudomonas aeruginosa. J. Bacteriol. 138:193-200.

6. Bjorn, M.J., Vasil, M.L., Sadoff, J.C. and Iglewski, B.H. 1977. Incidence of exotoxin production by Pseudomonas species. Infect. Immun. 16:362-366.

7. Böyum, A. 1968. Isolation of mononuclear cells and granulocytes from human blood. Scand. J. Clin. Lab. Invest. 21 (Suppl. 97):77-89.

8. Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72:248-254.

9. Brammar, W.J. and Clarke, P.H. 1964. Induction and repression of Pseudomonas aeruginosa amidase. J. Gen Microbiol. 37:307-319.

10. Brown, V.I. and Lowbury, J.L. 1965. Use of an improved cetrimide agar medium and toher culture methods for Pseudomonas aeruginosa. J. Clin. Pathol. 18:752-756.

11. Brubaker, R.R. 1972. The genus Yersinia: biochemistry and genetics of virulence. Current Topics in Microbiol. 57:111-158.

12. Burke, M.E. and Pattee, P.A. 1967. Purification and characterization of a staphylolytic enzyme from Pseudomonas aeruginosa. J. Bacteriol. 93:860-865.

13. Butler, W.T. 1963. Hemagglutination studies with formalized erythrocytes. Effect of bis-diazo-benzidine and tannic acid treatment on sensitization by soluble antigen. J. Immunol. 90:663-671.

14. Cheng, K.J., Ingram, J.M. and Costerton, J.W. 1970. Release of alkaline phosphatase from cells of Pseudomonas aeruginosa by manipulation of cation concentration and of pH. J. Bacteriol. 104:748-753.

15. Chung, D.W. and Collier, R.J. 1977. Enzymatically active peptide from the adenosine diphosphate-ribosylating toxin of Pseudomonas aeruginosa. Infect. Immun. 16:832-841.

16. Collier, R.J. and Kandel, J. 1971. Structure and activity of diphtheria toxin. I. Thiol-dependent dissociation of a fraction of toxin into enzymatically active and inactive fragments. J. Biol. Chem. 246:1496-1503.

17. Cruickshank, R., Duguid, J.P., Marmion, B.P. and Swain, R.H.A. 1975. Tests for identification of bacteria, p. 170-189. In Medical Microbiology, Vol. II, Churchill-Livingstone, New York.

18. DeBell, R.M. 1979. Production of exotoxin A by Pseudomonas aeruginosa in a chemically defined medium. Infect. Immun. 24: 132-138.

19. Evans, D.G., Silver, R.P., Evans, D.J., Chase, D.G. and Gorbach, S.L. 1975. Plasmid-controlled colonization factor associated with virulence in Escherichia coli enterotoxigenic for humans. Infect. Immun. 12:656-667.

20. Ewing. W.H. and Johnson, J.G. 1960. The differentiation of Aeromonas and C27 cultures from Enterobacteriaceae. Intern. Bull. Bacteriol. Nomencl. Taxon. 10:223-230.

21. Farmer III, J.J. and Herman, L.G. 1979. Epidemiological fingerprinting of Pseudomonas aeruginosa by the production and sensitivity to pyocin and bacteriophage. Appl. Microbiol. 18: 760-765.

22. Finkelstein, R.A., Vasil, M.L. and Holmes, R.K. 1974. Studies on toxigenesis in Vibrio cholerae. I. Isolation of mutants with altered toxinogenicity. J. Infect. Dis. 129:117-123.

23. Gaby, W.L. and Free, E. 1958. Differential diagnosis of Pseudomonas-like microorganisms in the clinical laboratory. J. Bacteriol. 76:442-444.

24. Galazka, A. and Abgarowicz, A. 1967. Assays of diphtheria and tetanus antibodies by the passive hemagglutination method. Epidmiol. Rev. 21:237-252.

25. Holmes, R.K. 1975. Genetic aspects of toxinogenesis in bacteria, p. 296-301. In D. Schlessinger (ed.), Microbiology 1975. American Society for Microbiology, Washington, D.C.

26. Holmes, R.K., Baine, W.B. and Vasil, M.L. 1978. Quantitative measurements of cholera enterotoxin in cultures of toxinogenic wild-type and nontoxinogenic mutant strains of Vibrio cholerae by using a sensitive and specific reversed passive hemagglutination assay for cholera enterotoxin. Infect. Immun. 19:101-106.

27. Holmes, R.K., Vasil, M.L. and Finkelstein, R.A. 1975. Studies on toxinogenic mutants in vitro and in experimental animals. J. Clin. Invest. 55:551-560.

28. Holt, R.J. 1971. The detection of bacterial esterases and lipases. Med. Lab. Technol. 28:208-210.

29. Hugo, W.B. and Beveridge, E.G. 1962. A quantitative and qualitative study of the lipolytic activity of single strains of seven bacterial species. J. Appl. Bacteriol. 25:72-82.

30. Iglewski, B.H., Burns, R.P. and Gipson, I.K. 1977. Pathogenesis of corneal damage from Pseudomonas exotoxin A. Invest. Opth. Visual Sci. 16:73-76.

31. Iglewski, B.H. and Kabat, D. 1975. NAD-dependent inhibition of protein synthesis by Pseudomonas aeruginosa toxin. Proc. Soc. Natl. Acad. Sci. USA 72:2284-2288.

32. Iglewski, B.H., Liu, P.V. and Kabat, D. 1977. Mechanism of action of Pseudomonas aeruginosa exotoxin A: ADP-ribosylation of mammalian elongation factor 2 in vitro and in vivo. Infect. Immun. 15:138-144.

33. Iglewski, B.H. and Sadoff, J.C. 1979. Toxin inhibitors of protein synthesis: production, purification and assay of Pseudomonas aeruginosa toxin A. Methods in Enzymol. 60:780-793.

34. Jeffries, C.D., Holtman, D.F. and Guse, D.G. 1957. Rapid method for determining the activity of microorganisms on nucleic acids. J. Bacteriol. 73:590-591.

35. Kawaharajo, K. and Homma, J.Y. 1975. Pathogenesis of the mouse keratitis produced with Pseudomonas aeruginosa. Japan. J. Exp. Med. 45:515-524.

36. King, E.O., Ward, M.K. and Raney, D.E. 1954. Two simple media for the demonstration of pyocyanin and fluorescin. J. Lab. Clin. Med. 44:301-307.

37. Kreger, A.S. and Gray, L.D. 1978. Purification of Pseudomonas aeruginosa proteases and microscopic characterization of pseudomonal protease-induced rabbit corneal damage. Infect. Immun. 19:630-648.

38. Laemli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) 227: 680-685.

39. Levine, M.M., Dupont, M.L., Formal, S.B., Hornick, R.B., Takeuchi, A., Gangarosa, E.J., Snyder, M.J. and Libonate, J.P. 1973. Pathogenesis of Shigella dysenteriae 1 (Shiga) dysentery. J. Infect. Dis. 127:261-270.

40. Liu, P.V. 1966. The role of various fractions of Pseudomonas aeruginosa in its pathogenesis. III. Identity of the lethal toxins produced in vitro and in vivo. J. Infect. Dis. 116:481-489.

41. Liu. P.V. 1973. Exotoxins of Pseudomonas aeruginosa. I. Factors that influence the production of exotoxin A. J. Infect. Dis. 128:506-513.

42. Liu, P.V. 1974. Extracellular toxins of Pseudomonas aeruginosa. J. Infect. Dis. 130:S94-S99.

43. March, S., Parikh, I. and Cuatrecasas, P. 1974. A simplified method for cyanogen bromide activation of agarose for affinity chromotography. Anal. Biochem. 60:149-152.

44. Miller, J.H. 1972. Determination of viable cell counts: bacterial growth curves, p. 32. In Experiments in Molecular Genetics. Cold Springs Harbor Laboratory, Cold Springs Harbor, New York.

45. Mueller, J.H. and Miller, P.A. 1941. Production of diphtheria toxin of high potency (100LF) on a reproducible medium. J. Immunol. 40:21-32.

46. Pavlovskis, O.R., Callahan III, L.T. and Pollack, M. 1975. Pseudomonas aeruginosa exotoxin, p. 252-256. In D. Schlessinger (ed.), Microbiology 1975. American Society for Microbiology, Washington, D.C.

47. Pavolvskis, O.R., Pollack, M., Callahan III, L.T. and Iglewski, B.H. 1977. Passive protection by antitoxin in experimental Pseudomonas aeruginosa burn infections. Infect. Immun. 18:596-602.

48. Pavlovskis, O.R. and Wretlind, B. 1979. Assessment of protease (elastase) as a Pseudomonas aeruginosa virulence factor in experimental mouse burn infection. Infect. Immun. 24:181-187.

49. Pollack, M., Callahan III, L.T. and Taylor, N.S. 1976. Neutralizing antibody to Pseudomonas aeruginosa exotoxin in human sera: evidence for in vivo toxin production during infections. Infect. Immun. 14:942-947.

50. Scharmann, W. 1976. Formation and isolation of leucocidin from Pseudomonas aeruginosa. J. Gen. Microbiol. 93:283-291.

51. Smith, R.F. and Willett, N.P. 1968. Rapid plate method for screening hyaluronidase and chondroitin sulfatase-producing microorganisms. Appl. Microbiol. 16:1434-1436.

52. Sokol, P.A., Ohman, D.E. and Iglewski, B.H. 1979. A more sensitive plate assay for detection of protease production by Pseudomonas aeruginosa. J. Clin. Microbiol. 9:538-540.

53. Stinson, M.W. and Hayden, C. 1979. Secretions of phospholipase C by Pseudomonas aeruginosa. Infect. Immun. 25:558-564.

54. Sutter, V.L. 1968. Identification of Pseudomonas species isolated from hospital environment and human sources. Appl. Microbiol. 16:1532-1538.

55. Taylor, N.S. and Pollack, M. 1978. Purification of Pseudomonas aeruginosa exotoxin by affinity chromatography. Infect. Immun. 19:66-70.

56. Torriani, A. 1968. Alkaline phosphatase of Escherichia coli. Methods in Enzymol. 12B:212-218.

57. Vasil, M.L., Kabat, D. and Iglewski, B.H. 1977. Structure-activity relationships of an exotoxin of Pseudomonas aeruginosa. Infect. Immun. 16:353-361.

58. Vasil, M.L., Liu, P.V. and Iglewski, B.H. 1976. Temperature-dependent inactivating factor of Pseudomonas aeruginosa exotoxin A. Infect. Immun. 13:1467-1472.

59. Vera, H.D. and Dumoff, M. 1974. Culture media, p. 881-929. In Lennette, E.H., Spaulding, E.H. and Truant, J.P. (eds.), Manual of Clinical Microbiology, American Society for Microbiology, Washington, D.C.

60. Wretlind, B., Heden, L., Sjöberg, L. and Wadström, T. 1973. Production of enzymes and toxins by hospital strains of Pseudomonas aeruginosa in relation to serotypes and phage-typing pattern. J. Med. Microbiol. 6:91-100.

61. Wretlind, B., Sjöberg, L. and T. Wadström. 1977. Protease-deficient mutants of Pseudomonas aeruginosa: Pleiotropic changes in activity of other extracellular enzymes. J. Gem Microbiol. 103:329-336.

Paper 2.

Characterization of a Toxin A Mutant of *Pseudomonas aeruginosa* PAO

Abstract

Pseudomonas aeruginosa strain PAO was mutagenized with nitrosoguanidine and 25 toxin A deficient mutants were identified in an agar-well assay. One of these mutants (PAO-T1) was found to be identical to the parental strain except for its toxin A deficiency.

Manuscript (Note Format)

Pseudomonas aeruginosa toxin A is the most toxic protein known to be produced by this organism (13, 17). Toxin A is produced in vitro by approximately 90% of clinical isolates (3, 19). Toxin A inhibits mammalian protein synthesis by catalyzing the transfer of the adenosine 5'-diphosphate-ribose (ADPR) from NAD^+ (nicotinamide adenine dinucleotide) onto mammalian elongation factor 2 (EF-2) (10, 11). Toxin A is not formed constitutively (1, 12), but the mechanisms by which specific regulatory systems control its synthesis are unknown.

This report describes the isolation and characterization of a mutant in strain PAO with an apparently specific deficiency in the production of toxin A. This is an initial step in the characterization of the genetic basis of toxin A, the regulation of its production, and its contribution to the virulence of P. aeruginosa.

An agar-well assay (16) was used to identify toxin A mutants. Following 9 separate nitrosoguanidine mutagenesis treatments (15) approximately 22,000 clones were examined. A total of 25 mutants were identified by the lack of a precipitin band in the presence of specific antitoxin. The 25 toxin A mutants were tested for production of elastase (15) and total proteolytic activity (12, 20). Eight mutants

produced zones of elastin-clearing and casein-clearing that were equivalent to that of the parental strain (PA01). These 8 mutants were cultivated for toxin A production in a Trypticase soy dialysate (TSBD) (15), and the toxin A yields were assayed in the ADPR-transferase assay (15) (Table 1). Four mutants, PAO-T1, T2, T8, and T21 had less than 1% of the toxin A yields of the parent, and they were selected for further studies.

Mutant characterization was performed in the same manner as recently described (15). These studies included tests for carbon assimilation, growth rate, biochemical tests, motility, serotype, pyocin pattern and extracellular products (Table 2). Mutant PAO-T8 was unable to utilize 6 of 24 carbon sources that serve as sole carbon source for the parental strain. PAO-T8 was also negative in the Simmons' citrate and malonate tests. Mutants PAO-T2 and PAO-T21 produced an altered colony morphology on some of the minimal media for carbon source utilization testing. In addition, PAO-T21 produced an altered reaction from the parental strain in the test for fluorescein production. Except for its deficiency in toxin A yields, only one mutant, PAO-T1, produced an identical reaction to the parental strain in all tests. This mutant (PAO-T1) was further characterized. Assays for 13 extracellular products (15) produced the same results for PAO-T1 as the parental strain (PA01). Sensitive radioimmunoassays (RIA) for alkaline protease (4) and elastase (15) showed that the yields of these two enzymes by PAO-T1 were the same as that of PA01 (Table 3).

The amount of toxin A produced by PAO-T1 was quantitated in the ADPR-transferase assay (15) using standard curves obtained daily with

Table 1. Relative ADPR transferase activity in culture supernatants of PA01 and toxin A mutants

Strain	CPM[a]	% of Parent
PA01	6,450[b]	
PAO-T1	0	0
PAO-T2	11	0.2
PAO-T8	0	0
PAO-T9	114	1.8
PAO-T10	194	3.0
PAO-T16	199	3.1
PAO-T21	41	0.6
PAO-T23	111	1.7

[a]Values represent one of three comparable experiments utilizing a 60 min ADPR transferase assay system. The background was subtracted.

[b]Extrapolated from 10^{-1} dilution.

Table 2. Characterization of P. aeruginosa strains PA01, PAO-T1, PAO-T2, PAO-T8 and PAO-T21[a]

Tests	Strains				
Carbon Assimilation	PA01	PAO-T1	PAO-T2	PAO-T8	PAO-T21
Adipate	+	+	+	-	+
Alanine	+	+	+[b]	+	+
Citrate	+	+	+	±[d]	+
Fumarate	+	+	+[b]	+	+[b]
Glutamate	+	+	+	-	+
Glycine	+	+	+	-	+
Lactate	+	+	+[b]	+	+
Lysine	+	+	+	-	+
Malate	+	+	+[b]	+	+
Pyruvate	+	+	+	-	+
Succinate	+	+	+	-	+
Other Tests					
Malonate	+	+	+	-	+
Fluorescin	+	+	+	+	+[c]
Simmons' citrate	+	+	+	-	+

[a]Using methods described previously (15), mutants PAO-T1, T2, T8 and T21 were tested as shown above and were also found to be the same as the parental strain (PA01) in the following properties: carbon assimilation from acetate, acetamide, arabinose, arginine, aspartate, fructose, galactose, glucose, mannitol, mannose, propionate, ribose, and xylose; growth on cetrimide, deoxycholate-citrate, endo, MacConkey, and SS agars; pyocin pattern; the biochemical tests arginine dihydrolase, gluconate and nitrate to gas; and the extracellular products elastase, total protease, gelatinase, hemolysin (human and

sheep), esterase, and pyocyanine. Mutant PAO-T1 was also identical to PA01 in generation time (TSBD), motility, serotype, and in the production of alkaline phosphatase, lipase and lecithinase.

[b]Colony morphology was altered.

[c]Produced fluorescin (yellow) and pyocyanine (green) in this test, wherease, PA01 only produced fluorescin.

[d]Weak growth (±) occurred after prolonged incubation.

Table 3. Quantitation of toxin A, elastase and alkaline protease produced by *P. aeruginosa* strains PAO1 and PAO-T1

Strain	Toxin A (µg/ml)[a]			Elastase[b]	Alkaline Protease[c]
	supernatants		cell-lysates	(µg/ml)	(µg/ml)
	(ADPR)	(RIA)	(ADPR)	(RIA)	(RIA)
PAO1	3.0	1.6	0.40	147	2.7
PAO-T1	<0.005[d]	<0.0001[d]	0.02	144	2.8

[a]Toxin A from TSBD cultural supernatants (15) and cell-lysates obtained by sonication (16) was measured in a 60 min ADPR transferase assay standardized with purified toxin A and in a radioimmunoassay (RIA) for toxin A. Cell-lysate values represent µg toxin A bound to washed cells in one ml of culture.

[b]Elastase was measured in the supernatants of cultures (5% Bacto-peptone and 0.25% Trypticase soy broth) incubated at 37° C for 15 h (15).

[c]Alkaline protease was measured in the supernatants of cultures (TSBD) incubated at 32° C for 20 h (15).

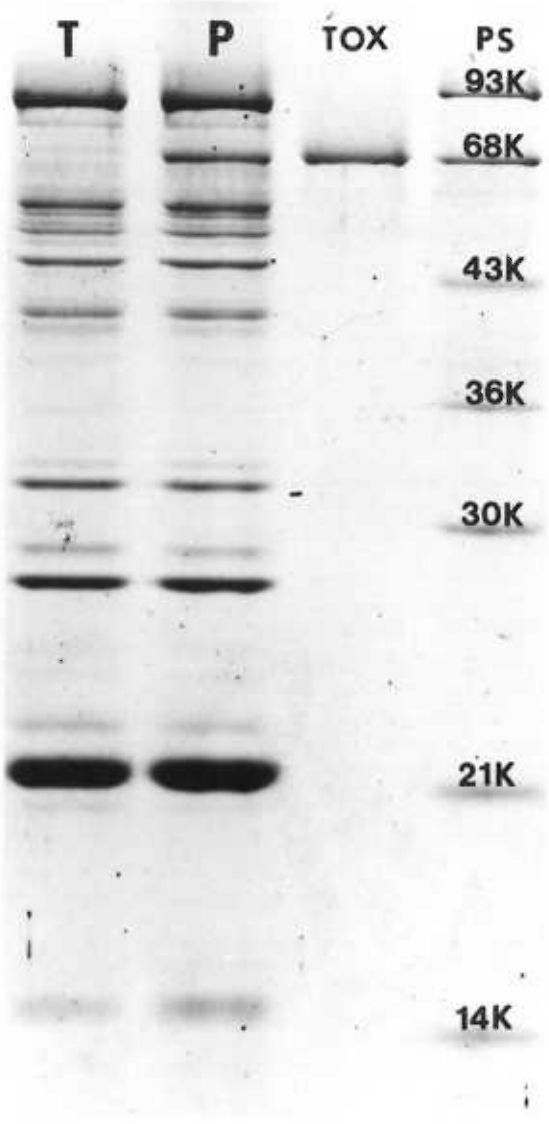
[d]Below detection with 10X concentrated supernatant.

pure toxin A, and in an RIA for toxin A (S. Cryz and B. Iglewski, manuscript in preparation) (Table 3). No extracellular toxin A could be detected in supernatants (1X or 10X) of PAO-T1 cultures (TSBD) when it was grown under optimal conditions for toxin A production by PA01 (15). Furthermore, the cell-lysate of PAO-T1 had ADPR-transferase activity that was barely detectable (0.02 μg/ml). Since PAO-T1 does not accumulate toxin A intracellularly, it seems unlikely that this mutant has a toxin A transport defect.

The culture supernatant (TSBD) of PA01 contained 25 visible protein bands upon sodium dodecyl sulfate-10% polyacrylamide slab-gel electrophoresis (SDS-PAGE) (15, 16) (Fig. 1). The extracellular protein profile obtained with supernatants of PAO-T1 was identical to that of the parental strain except for the loss of a single protein which comigrated with purified toxin A. Thus, the only detectable extracellular protein deficiency of PAO-T1 detected was toxin A (Table 3 and Fig. 1).

The mutation in PAO-T1 could be at the beginning of the toxin A structural gene sequence such that no detectable cross-reacting material (CRM) of toxin A is produced, or the mutation could be in a regulatory gene for toxin A. In an effort to determine the nature of the toxin A mutation in PAO-T1, two factors (temperature and iron concentration) which regulate toxin A yields were examined. Yields of toxin A are maximum when cultures are grown at 32° C with maximum aeration (12). When PAO-T1 was cultured at 24°, 37° and 42° C, no toxin A could be detected. This indicates that PAO-T1 did not have a temperature-sensitive mutation affecting toxin A yields.

Fig. 1. SDS-PAGE of extracellular proteins produced by P. aeruginosa PAO; parental strain PAO1 (P) and toxin A mutant PAO-T1 (T). Cells were grown in TSBD for 20 h, supernatants were concentrated 10-fold, and 10 µl of each was applied to the gel. Toxin A (Tox) and the following protein standards (PS) were used as molecular weight markers: phosphorylase B, 93,000; bovine serum albumin, 68,000; ovalbumin, 43,000; lactate dehydrogenase, 36,000; carbonic anhydrase, 30,000; soybean trypsin inhibitor, 21,000; and lysozyme, 14,000.


T
P
TOX
PS
93K
68K
43K
36K
30K
21K
14K

The concentration of iron has also been shown to regulate toxin A yields (2, 16). The mutant PAO-T1 could have an altered sensitivity or requirement for iron that would account for its decreased toxin A yields. PAO1 and PAO-T1 were cultured in a defined medium for toxin A production, medium T8S (15) which was modified to contain 0.042 M succinate. The iron concentrations tested were: 0.03, 0.05, 0.08, and 0.13 µg Fe/ml. PAO-T1 grew to the same cell density as PAO1 at each iron concentration which suggests that this mutant did not have a defect in the transport of iron into the cell (data not shown). The mutant PAO-T1 did not produce any detectable toxin A activity at any of the iron concentrations tested, whereas, the parental strain produced 1.4 µg toxin A/ml in T8S (0.08 µg Fe/ml). However, the total extracellular protein yield (µg/ml per OD_{540} unit) was decreased 80% with the parental and mutant strains when the iron content of the medium (TSBD) was increased from 0.08 to 5.0 µg/ml (data not shown). This indicates that the inhibitory effect of excess iron on extracellular protein yields (2, 16) was not altered in PAO-T1. These data suggest that the toxin A mutation in PAO-T1 is not associated with iron regulation.

Genetic mapping of the mutation in PAO-T1 conferring the toxin A deficient (Tox^-) phenotype was attempted (Table 5) using the PAO strains listed in Table 4. This mutation is referred to as *tox*-1. Mutagenesis with ethyl methane sulfonate and enrichment of auxotrophs was as previously described (22). The strain construction techniques of Haas *et al*. (7) were used to place one marker in PAO-T1 (PAO0136, Table 4). Transduction was performed according to Haas *et al*. (6)

Table 4. *P. aeruginosa* strains

Strain	Genetic characteristics[a]	Source or origin
PA01	wild type, prototrophic	D. Haas (8)
PA025	*argF*10, *leu*10	D. Haas (5)
PA0505	*met*9011, *ami*200	D. Haas
PA0519	*car*161, *thi*1, *str*57, FP2$^+$	D. Haas
PA0-T1	*tox*1	PA01 (NTG)[b]
PA00107	*tox*1, *argH*1	PA0-T1 (EMS)[c]
PA00135	*tox*1, *argF*1	PA0-T1 (EMS)
PA00136	*tox*1, *leu*10	Arg+ recombinant of PA00135 x PA00912 (R68.45)
PA00141	*tox*1, *catA*2	PA0-T1 (EMS)
PA00905	His-1, *str*1	PA0-T1 (EMS)
PA00912	*leu*10	Arg+ transductant of PA025 x F116.PA01

[a]Genotype symbols: biosynthesis of arginine (*arg*), leucine (*leu*), methionine (*met*), histidine (His), and thiamine (*thi*); car = uracil and arginine requirement; *catA* = benzoate utilization (14); *ami* = acetamide utilization as carbon and nitrogen source; *str* = streptomycin resistance; *tox* = toxin A production. Marker *argF* identified by utilization of citruline and ornithine (6). Marker *argH* identified by non-utilization of citruline or ornithine, and close linkage to His$^-$ markers (6).

[b]NTG, Nitrosoguanidine mutation.

[c]EMS, Ethyl methane sulfonate mutation.

Table 5. Segregation of *tox*-1 marker in conjugal chromosome transfer[a]

Recipient	Donor	Selected marker	Recombinants/ cross	Chromosome[b] mobilized	Tox^+/total[c] recombinants
PA00107	x PA025 (FP2)	*argH*	324	0-20'	0/324
PA00135	x PA0519 (FP2)	*argF*	38,205	0-55'	0/362
"	x PA0505 (FP2)	"	119		
PA00141	x PA025 (FP2)	catA	6	0-65'	0/104
"	x PA0519 (FP2)	"	19		
"	x PA00905 (FP2)	"	79		
PA00107	x PA00905 (R68.45)	*argH*	200	15-25'	0/200
PA00135	x PA00905 (R68.45)	*argF*	200,1186	50-60'	0/2386
PA00136	x PA00905 (R68.45)	*leu*-10	38,162	55-65'	0/200
PA00141	x PA00905 (R68.45)	*catA*	59,86 284,118	65-75'	0/547

[a]Plate matings were carried out as previously described (21). Selection of prototrophic recombinants was made on minimal media.

[b]Indicates the approximate region of the chromosome mobilized to obtain recombinants with the given selected marker.

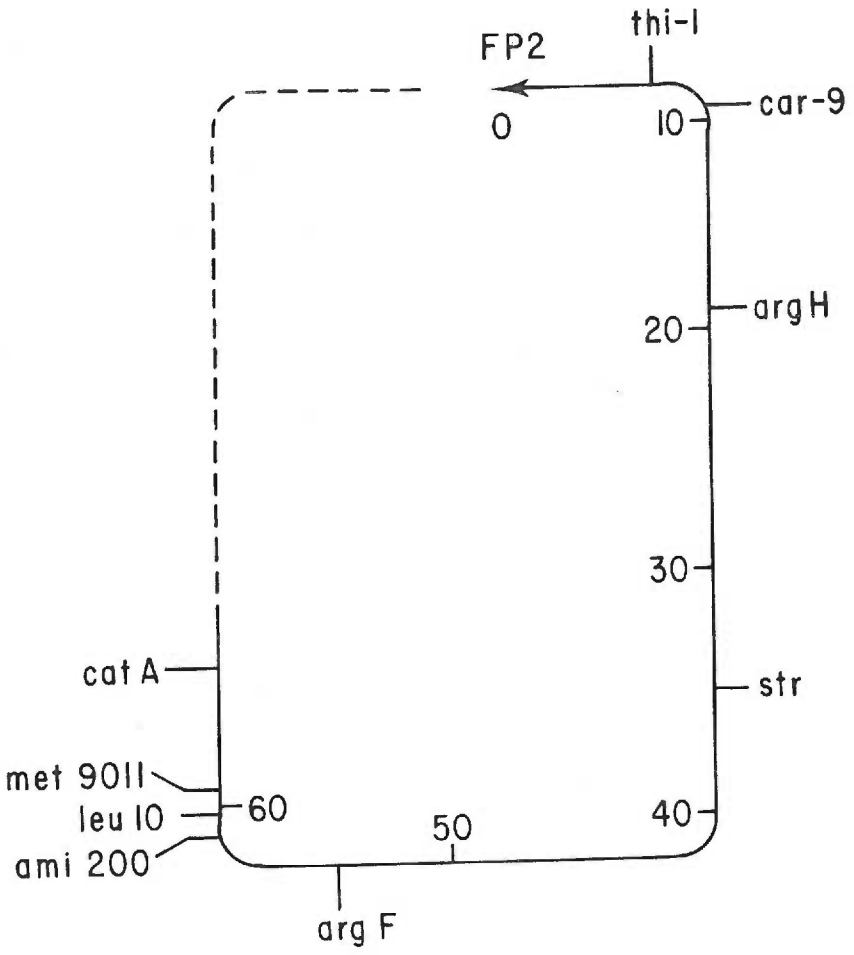
[c]Coinheritance of the unselected *tox*$^+$ marker was determined in the agar-well assay (16).

using the generalized transducing phage F116 . Matings on the plate were performed according to Stanisich et al. (21) using the conjugative plasmids FP2 for polarized chromosome transfer and R68.45 for non-polarized transfer. In R68.45 mediated matings, occasional reversion of the donor strain (PA00905) to prototrophy was detected by the str-1 marker. Recombinants were purified (21) and their Tox phenotype tested in the agar-well assay (16).

Over 3,000 recombinants of PAO-T1 auxotrophs were obtained by the transfer of approximately the first 75 min of chromosome (Fig. 2), but all retained the Tox^- phenotype. The size of the region between leu-10 and the FP2 origin is unknown (9), but the entire chromosome has been approximated to be 80 to 110 min long (18). These data indicate that if tox-1 is a chromosomal marker, then it probably resides in this late region where no markers have yet been accurately mapped.

I have recently reported an apparently specific toxin A mutant (PA103-29) of the highly toxigenic strain PA103 (16). Strains PA103 and PA01 represent two different categories of clinical isolates, $elastase^-$ and $elastase^+$, respectively. Both of these strains and their tox mutants will be useful in studies to determine the relative contribution of toxin A to the virulence of P. aeruginosa.

Fig. 2. Chromosome map of Pseudomonas aeruginosa PAO. Markers used in this study are positioned as previously described (9, 14). Genetic symbols are those given in footnote a of Table 1. The arrow indicates the chromosome transfer mediated by Plasmid FP2. The dotted line shows the segment of the chromosome not examined in this study.


thi-1
FP2
0
10
car-9
20
arg H
30
str
40
50
60
cat A
met 9011
leu 10
ami 200
arg F

References

1. Bjorn, M.J., Iglewski, B.H., Ives, S.K., Sadoff, J.C. and Vasil, M.L. 1978. Effect of iron on yields of exotoxin A in cultures of Pseudomonas aeruginosa PA-103. Infect. Immun. 19:785-791.

2. Bjorn, M.J., Sokol, P.A. and Iglewski, B.H. 1979. Influence of iron on yields of extracellular products in Pseudomonas aeruginosa cultures. J. Bacteriol. 138:193-200.

3. Bjorn, M.J., Vasil, M.L., Sadoff, J.C. and Iglewski, B.H. 1977. Incidence of exotoxin production by Pseudomonas species. Infect. Immun. 16:362-366.

4. Cryz, S.J. and Iglewski, B.H. 1980. A specific and sensitive assay for Pseudomonas aeruginosa alkaline protease. Infect. Immun. submitted.

5. Haas, D. and B.W. Holloway. 1976. R factor variants with enhanced sex factor activity in Pseudomonas aeruginosa. Molec. gen. Genet. 144:243-251.

6. Haas, D. and Holloway, B.W. 1977. The genetic organization of arginine biosynthesis in Pseudomonas aeruginosa. Molec. gen. Genet. 154:7-22.

7. Haas, D. and Holloway, B.W. 1978. Chromosome mobilization by the R plasmid R68.45: a tool in Pseudomonas genetics. Molec. gen. Genet. 158:229-237.

8. Holloway, B.W. 1969. Genetics of Pseudomonas. Bact. Rev. 33:419-433.

9. Holloway, B.W., Krishnapillai, V. and Morgan, A.F. 1979. Chromosomal genetics of Pseudomonas. Microbiol. Rev. 43:73-102.

10. Iglewski, B.H. and Kabat, D. 1975. NAD-dependent inhibition of protein synthesis by Pseudomonas aeruginosa toxin. Proc. Soc. Natl. Acad. Sci. USA 72:2284-2288.

11. Iglewski, B.H., Liu, P.V. and Kabat, D. 1977. Mechanism of action of Pseudomonas aeruginosa exotoxin A: ADP-ribosylation of mammalian elongation factor 2 in vitro and in vivo. Infect. Immun. 15:138-144.

12. Liu, P.V. 1973. Exotoxins of Pseudomonas aeruginosa. I. Factors that influence the production of exotoxin A. J. Infect. Dis. 128:506-513.

13. Liu, P.V. 1974. Extracellular toxins of Pseudomonas aeruginosa. J. Infect. Dis. 130:S94-S99.

14. Matsumoto, H., Shin, O., Kobayashi, R. and Terawaki, Y. 1978. Chromosomal location of genes participating in the degradation of purines in Pseudomonas aeruginosa. Molec. gen. Genet. 167: 165-176.

15. Ohman, D.E., Cryz, S.J. and Iglewski, B.H. 1980. Isolation and characterization of a mutant of Pseudomonas aeruginosa PAO producing an altered elastase. J. Bacteriol. submitted.

16. Ohman, D.E., Sadoff, J.C. and Iglewski, B.H. 1980. Toxin A deficient mutants of Pseudomonas aeruginosa strain PA103: Isolation and characterization. Infect. Immun. Submitted.

17. Pavlovskis, O.R., Callahan, L.T. III and Pollack, M. 1975. Pseudomonas aeruginosa exotoxin, p. 252-256. In D. Schlessinger (ed.), Microbiology 1975. American Society for Microbiology, Washington, D.C.

18. Pemberton, J.M. 1974. Size of the chromosome of Pseudomonas aeruginosa PAO. J. Bacteriol. 119:748-752.

19. Pollack, M., Taylor, N.S. and Callahan, L.T. III. 1977. Exotoxin production by clinical isolates of Pseudomonas aeruginosa. Infect. Immun. 15:776-780.

20. Sokol, P.A., Ohman, D.E. and Iglewski, B.H. 1979. A more sensitive plate assay for detection of protease production by Pseudomonas aeruginosa. J. Clin. Microbiol. 9:538-540.

21. Stanisich, V.A. and Holloway, B.W. 1972. A mutant sex factor of Pseudomonas aeruginosa. Genet. Res. 19:91-108.

22. Watson, J.M. and Holloway, B.W. 1978. Chromosome mapping in Pseudomonas aeruginosa PAT. J. Bacteriol. 133:1113-1125.

Paper 3

Isolation and Characterization of a Mutant of *Pseudomonas aeruginosa* PAO Producing an Altered Elastase

Abstract

Pseudomonas aeruginosa PAO mutants defective in elastase were isolated by plate assays of nitrosoguanidine mutagenized clones. One mutant (PAO-E64) was apparently identical to the parental strain except for its deficiency in elastase activity. This mutant produced an enzyme which was antigenically indistinguishable from parental elastase. Equal levels of elastase antigen were produced by this mutant and its parental strain. Mutant PAO-E64 is presumed to have a mutation in the structural gene for elastase.

Introduction

Pseudomonas aeruginosa is an opportunistic pathogen which is associated with a variety of severe and often fatal infections (16). The organism produces a number of extracellular substances including proteases which may play a role in pathogenesis (16). The proteolytic activity of P. aeruginosa was first characterized by Morihara (19). Two distinct P. aeruginosa proteases have been isolated and purified to homogeneity (18, 24). These enzymes, alkaline protease (18) and elastase (protease II) (20, 22) are classic metallo-proteases although their substrate specificities and pH optima are different. (23). P. aeruginosa elastase is of particular interest due to its ability to produce corneal ulcers (10, 13), nectrotic skin lesions (11, 17), pulmonary hemorrhage (13), and to inactivate human plasma α_1-proteinase inhibitor (23).

This report describes the identification and characterization of a mutation in the structural gene for elastase.

Materials and Methods

Bacterial strain. Pseudomonas aeruginosa strain PA01 isolated by B.W. Holloway (8), was used in this study. Strain PA01 was chosen for this study as it produces toxin A, elastase and alkaline protease. Additionally, it is virulent in several animal models, and its genome is well characterized (8).

Isolation of mutants. Log phase cultures of P. aeruginosa PA01 grown in nutrient broth with 0.5% yeast extract (Difco) (NYB) were mutagenized with 50 µg/ml N-methyl-N'-nitrosoguanidine (Sigma) at 37° C for 30 min according to the method of Finkelstein et al. (6). The cells were washed twice in NYB, resuspended in NYB, and incubated with shaking at 37° C overnight. NYB was inoculated with the mutagenized overnight cultures and incubated at 37° C with shaking until the cell density was approximately 3×10^8 cells/ml. The cultures were diluted in sterile saline such that 0.1 ml yielded approximately 50 colonies per plate of nutrient agar containing 0.3% elastin (elastin-NA) (25). These elastin-NA plates were incubated at 37° C for 48 h. The zone of clearing was seen surrounding colonies producing elastase. Those colonies which showed no zone of clearing were recloned, tested again on elastin-NA plates and stored in sterile skim milk at -70° C.

Culture conditions for toxin A production. The culture medium for the production of toxin A consisted of deferrated Trypticase soy broth dialysate, 1% glycerol, and 0.05 M monosodium glutamate (TSBD), as previously described (1). One ml of log phase cultures [optical

density at 540 nm (OD_{540})] was used to inoculate 9.0 ml of medium in 125 ml Erlenmeyer flasks (nitric acid cleaned). The flasks were incubated at 32° C with maximum aeration for 20 h. Bacterial growth was measured as the turbidity at OD_{540}. The supernatants were obtained by centrifugation, dialyzed against 0.05 M Tris-hydrochloride buffer (pH 8.0) at 5° C for approximately 18 h, and sterilized by membrane filtration (0.45 μ, Millipore). Portions of these supernatants were concentrated 10-fold in Minicon-B15 cells (Amicon Corp.) prior to storage at -70° C.

Adenosine 5'-diphosphate ribose (ADPR) transferase activity. Partially purified EF-2 was prepared from extracts of wheat germ as described by Chung and Collier (3). The ADPR transferase activity of activated (urea and dithiothreitol-treated) supernatants was measured as previously described (27). Toxin A was quantitated by comparing its enzymatic activity to standard curves obtained daily with pure toxin. The amount of toxin A present in a crude supernatant was then calculated from the standard curve (9).

Culture conditions for elastase production. The liquid medium employed for elastase production consisted of 5% Bacto-peptone and 0.25% Trypticase soy broth (PTSB) (5). Two ml of log phase cultures (OD_{540} of 0.5) were used to inoculate 18.0 ml of medium in 250 ml Erlenmeyer flasks. The flasks were incubated with maximum aeration at 37° C for 16 h or at 21° C for 36 h. Supernatants were obtained by centrifugation, sterilized by membrane filtration and stored at -70° C. Other liquid media tested for elastase production were TSBD,

MTYG medium developed by Wretlind et al. (29), Bacto-nutrient broth (NB), and nutrient broth with 0.5% yeast extract (Difco) (NYB).

Elastase-activity assays. Elastase activity was quantitated by a modification of the procedure previously described (2). To 15-ml glass tubes were added 10 mg elastin-Congo red (Sigma) and 2.0 ml reaction buffer (0.1 M Tris-maleate buffer, pH 7.0, and 1 mM $CaCl_2$). Following preincubation to the reaction temperature, 1.0 ml of PTSB culture supernatant was added. The tubes were capped and incubated horizontally with rapid shaking at room temperature (21° C) for 3 h or at 37° C for 2 h. The reaction was terminated by the addition of 2.0 ml sodium phosphate buffer (0.7 M, pH 6.0) and placed in an ice bath. The substrate was removed by membrane filtration (0.45 μ, Millipore). Culture supernatant was replaced with reaction buffer in the blank. The absorbance of the filtrates was read at 495 nm (Beckman spectrophotometer, model 35). The background absorbance at 495 nm was obtained by mixing culture supernatants with the assay buffers in the proportions described above but omitting the substrate. The background absorbance was subtracted from enzyme samples to obtain final values. Elastase activity (EA) was expressed as A_{495nm}/h/ml and the specific activity calculated as (EA/μg elastase per ml) x 10^2. The elastase was quantitated in a radioimmunoassay (RIA) as described below.

Elastase activity was also quantitated using elastin-NA plates. Elastase production was measured as the zone of clearing extending from the edge of a 1.5 cm streak of growth.

Elastase antigens were detected in an agar-well assay (25) adapted for elastase. Cultures (1.5 cm streaks) were incubated on a nutrient agar plate at 37° C for 48 h. Small wells were placed between cultures approximately 5 mm from the end of the streaks of growth and filled with elastase antiserum. Following overnight incubation at 10° C, a precipitin band formed between the antiserum wells and strains that produced elastase.

Radioimmunoassay for Elastase. The following procedures were used to prepare radiolabeled preparations of elastase for use in radioimmunoassays. Highly purified elastase was the kind gift of Dr. Morihara. One mCi of carrier free NA ^{125}I (Amersham Corporation, Arlington Heights, Illinois) was added to 37.5 µg of elastase and 10 µg of lactoperoxidase in 1 ml of phosphate buffered saline, pH 7.2 (PBS). The enzymatic iodination reaction was initiated by the addition of 25 µl aliquot of 0.03% H_2O_2 was added and the reaction continued for 5 min. The labeled preparation was dialyzed for 18 h at 4° C against 1 L of PBS. The dialyzed sample was applied at 4° C to a 1.2 x 30 cm Sephadex G-100 column (bed Volume 88 ml) equilibrated with PBS containing 1 mg/ml bovine serum albumin (BSA) and eluted with the same buffer. Fractions containing the radiolabeled elastase were pooled; sodium azide added to a final concentration of 0.02%; and the samples stored at 4° C. Samples possessed specific activities of at least 1 mCi/µg of elastase protein and were greater than 85% immunoprecipitable in the presence of excess elastase antiserum (see below).

IgG Sorb (Enzyme Center, Boston, Mass.), a formalin fixed preparation of a protein A bearing strain of Staphylococcus aureus, was used as a particulate immunoadsorbent for immune complexes containing IgG (12, 15). Lyophilized samples were reconstituted to 10% (v/v) in NET buffer (150 mM NaCl, 5 mM EDTA and 50 mM Tris, pH 7.4) and stored at 4° C. Immediately prior to use in radioimmunoassays, cells were pelleted by centrifugation at 4000 x g for 15 min at 4° C. Cells were resuspended to 10% (v/v) in NET buffer containing 0.5% Nonidet P-40 (NP-40) (Particle Data Laboratories, Elmhurst, Ill.) and incubated for 10 min at 22° C. The cells were then washed once with NET buffer containing 0.05% NP-40 and finally resuspended to 10% (v/v) in assay buffer (NET buffer containing 0.05% NP-40 and 1 mg/ml BSA).

Specific anti-elastase antiserum was the kind gift of Dr. B. Wretlind, Naval Medical Research Institute, Bethesda, Maryland. This antiserum contained no anti-alkaline protease activity nor was it able to precipitate alkaline protease.

Radioimmunoassays were carried out in 0.5 ml final volume in 10 x 75 mm polystyrene test tubes. Assay buffer was used as diluent for all reagents. Reagents were added in the following sequence: 1) assay buffer; 2) approximately 10,000 CPM of [^{125}I] elastase in 10 µl; 3) non-radioactive antigen (either purified elastase or culture supernatants sterilized by membrane filtration) 5-100 µl/assay; and 4) 0.010 µl of elastase antiserum, sufficient to immunoprecipitate 50-60% of the total counts in the absence of competing antigen. Assay mixtures were incubated for 15 min at 22° C, and then 25 µl of prepared IgG Sorb was added. The samples were incubated an additional

10 min at 22° C. Immune complexes were collected by centrifugation at 4000 x g for 15 min at 4° C; washed twice with 1 ml of assay buffer and the final pellets counted in a Beckman Biogamma counter.

Biochemical characterization. The tests, media and procedures for the determination of biochemical activities, serotype, pyocin type, motility and generation time were as previously described (25).

Extracellular product characterization. The production of the following extracellular products was determined as previously described (25): protease, esterase, hyaluronidase, DNase, RNase, hemolysins, pyocyanin, fluorescin, gelatinase, lecithinase, staphylolytic activity, and alkaline phosphatase. Lipase production in TSBD culture supernatants was determined according to Wretlind et al. (29) using p-nitrophenyl caprylate (Sigma) as the substrate. The production of alkaline protease in TSBD culture supernatants was determined by radioimmunoassay as previously described (4).

SDS-polyacrylamide gel electrophoresis. TSBD culture supernatants were dialyzed and concentrated as described above. Extracellular proteins were analyzed by electrophoresis on sodium dodecyl sulfate-10% polyacrylamide slab-gels (SDS-PAGE) as previously described (25).

Genetic techniques. Mutagenesis with ethyl methane sulfonate and enrichment of auxotrophs was as previously described (28). The marker argF (55 min) was identified by utilization of citruline and ornithine (7). The donor strain PA0505 met9011, ami200, $FP2^{+}$ was the kind gift of Dr. D. Haas, Zurich, Switzerland. Matings on the plate

were performed as previously described (26). Recombinants were purified on selective media, and coinheritance of the elastase positive phenotype was tested on elastin-NA plates incubated at 37° C.

Results

Mutant isolation. Elastase mutants were identified by their failure to produce a zone of clearing on elastin-NA plates when incubated at 37° C for 48 h. A total of approximately 43,000 clones were examined following 12 independent nitrosoguanidine mutagenesis. Seventy-five mutants were identified by their reduced elastase activity.

Toxin A production by elastase mutants. Among the 75 elastase mutants identified, 31 were found to produce toxin A as determined by the agar-well assay (25). The relative amount of toxin A produced by the 31 mutants was determined in the ADPR transferase assay. Only two mutants (PAO-E23 and PAO-E64) from the original 75 produced parental levels (3 μg/ml) of toxin A. These two mutants were selected for further study.

Biochemical characterization. Elastase mutants PAO-E23 and PAO-E64 were tested for the ability to utilize 24 organic compounds which were found to serve as sole carbon sources for the parental strain PAO1 (Table 1). PAO-E64 utilized each of the 24 compounds, but PAO-E23 was unable to utilize glutamate. Both elastase mutants were able to grow on five selective media, and their generation time (36 min) was the same as that of the parent. Five biochemical tests

(arginine dihydrolase, gluconate, malonate, nitrate to gas, and Simmons' citrate) were positive for the parental strain and the two elastase mutants. The pyocin pattern and serotype of the 2 mutants were identical to the parent (Table 1). The lack of diffusion of growth through motility-agar indicated that the motility of PAO-E23 was altered.

Production of extracellular products. The release of extracellular products by PA01, PAO-E23 and PAO-E64 was determined using agar plate and liquid culture assays (Table 2). The production of the following extracellular products was not detectably altered as a result of the mutations affecting elastase production in PAO-E23 and PAO-E64: gelatinase, human and sheep hemolysin, esterase, pyocyanine, fluorescin and toxin A. In addition, PA01 and PAO-E64 produced the same levels of alkaline phosphatase, lipase, lecithinase and alkaline protease. However, PAO-E23 demonstrated decreased alkaline phosphatase activity.

The above tests indicated that PAO-E23 did not have a single mutation which affected only the activity of elastase. The elastase mutant PAO-E64 was identical to the parental strain by all of the above criteria, except for its lack of elastase activity. Therefore, only PAO-E64 was further characterized.

Extracellular protein profiles on SDS-PAGE. The culture supernatants of PA01 or PAO-E64 each contained 25 visible bands on SDS-PAGE (Fig. 1). These gel profiles were indistinguishable. Despite its lack of elastase activity, no bands corresponding to elastase

Table 1. Biochemical characterization of P. aeruginosa strains PA01, PA0-E23 and PA0-E64

Tests	Strains		
	PA01	PA0-E23	PA0-E64
Carbon Assimilation			
Acetate	+	+	+
Acetamide	+	+	+
Adipate	+	+	+
Alanine	+	+	+
Arabinose	+	+	+
Arginine	+	+	+
Aspartate	+	+	+
Citrate	+	+	+
Fructose	+	+	+
Fumarate	+	+	+
Galactose	+	+	+
Glucose	+	+	+
Glutamate	+	-	+
Glycine	+	+	+
Lactate	+	+	+
Lysine	+	+	+
Malate	+	+	+
Mannitol	+	+	+
Mannose	+	+	+
Propionate	+	+	+
Pyruvate	+	+	+
Ribose	+	+	+
Succinate	+	+	+
Xylose	+	+	+

Table 1 (Continued)

Tests	Strains		
	PA01	PAO-E23	PAO-E64
Growth			
Cetrimide agar	+	+	+
Deoxycholate citrate agar	+	+	+
Endo agar	+	+	+
MacConkey agar	+	+	+
SS agar	+	+	+
Generation time, min (TSBD)	36	36	36
Other tests			
Arg dihydrolase	+	+	+
Gluconate	+	+	+
Malonate	+	+	+
Motility	+	-	+
Nitrate to gas	+	+	+
Simmons' citrate	+	+	+
Serotype	3,7	3,7	3,7
Pyocin pattern	(111,424)	(111,424)	(111,424)

Fig. 1. SDS-PAGE of extracellular proteins produced by P. aeruginosa PAO; parental strain PAO1 (P) and elastase mutant PAO-E64 (E). Cells were grown in TSBD for 20 h, supernatants were concentrated 10-fold, and 10 µl of each was applied to the gel. Toxin A (Tox) and the following protein standards (PS) were used as molecular weight markers: phosphorylase B, 93,000; bovine serum albumin, 68,000; pyruvate kinase, 57,000; ovalbumin, 43,000; lactate dehydrogenase, 36,000; carbonic anhydrase, 30,000; soybean trypsin inhibitor, 21,000; and lysozyme, 14,000.

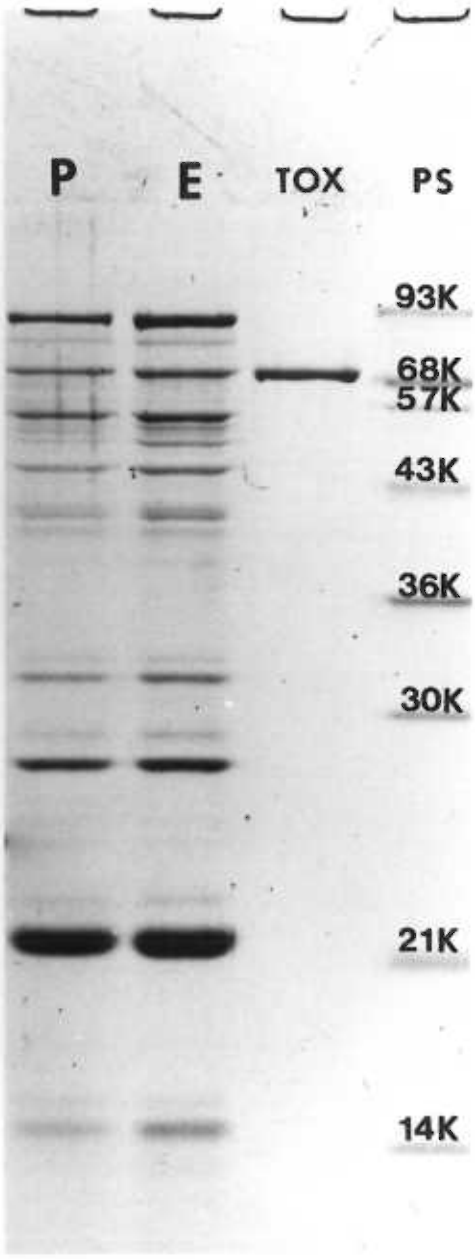

P
E
TOX
PS
93K
68K
57K
43K
36K
30K
21K
14K

Table 2. Extracellular products of P. aeruginosa strains PA01, PAO-E23 and PAO-E64

Extracellular product[a]	Assay units	Strains		
		PA01	PAO-E23	PAO-E64
Elastase	Zone (mm)	4.5	0.5	0
Protease (casein)	Zone (mm)	6.0	6.0	6.0
Gelatinase	Zone (mm)	9.0	9.0	9.0
Hemolysis (human)	Zone (mm)	2.0	2.0	2.0
Hemolysis (sheep)	Zone (mm)	0.5	0.5	0.5
Esterase	Agar	+	+	+
Pyocyanine	Agar	+	+	+
Fluorescin	Agar	+	+	+
Alk. Phosphatase	Units/ml	3.9	1.8	3.8
Lipase[b]	Units/ml	16	ND[d]	16
Lecithinase[c]	Units/ml	0.18	ND	0.18
Alkaline Protease[b]	µg/ml	2.7	ND	3.4
Toxin A	µg/ml	3.0	3.0	3.0

[a]None of the strains produced detectable levels of exoenzyme S, RNase, DNase, staphylolytic enzyme or hyaluronidase. Zones of activity for protease and gelatinase were measured after 24 h, hemolysis after 48 h, and elastase after 72 h of incubation at 37° C.

[b]Activity determined in TSBD culture supernatants.

[c]One lecithinase unit of activity was defined as the liberation of 1 µM water-soluble organic phosphorous from phosphatidyl choline (from egg yolk, Sigma IXE).

[d]ND, not done.

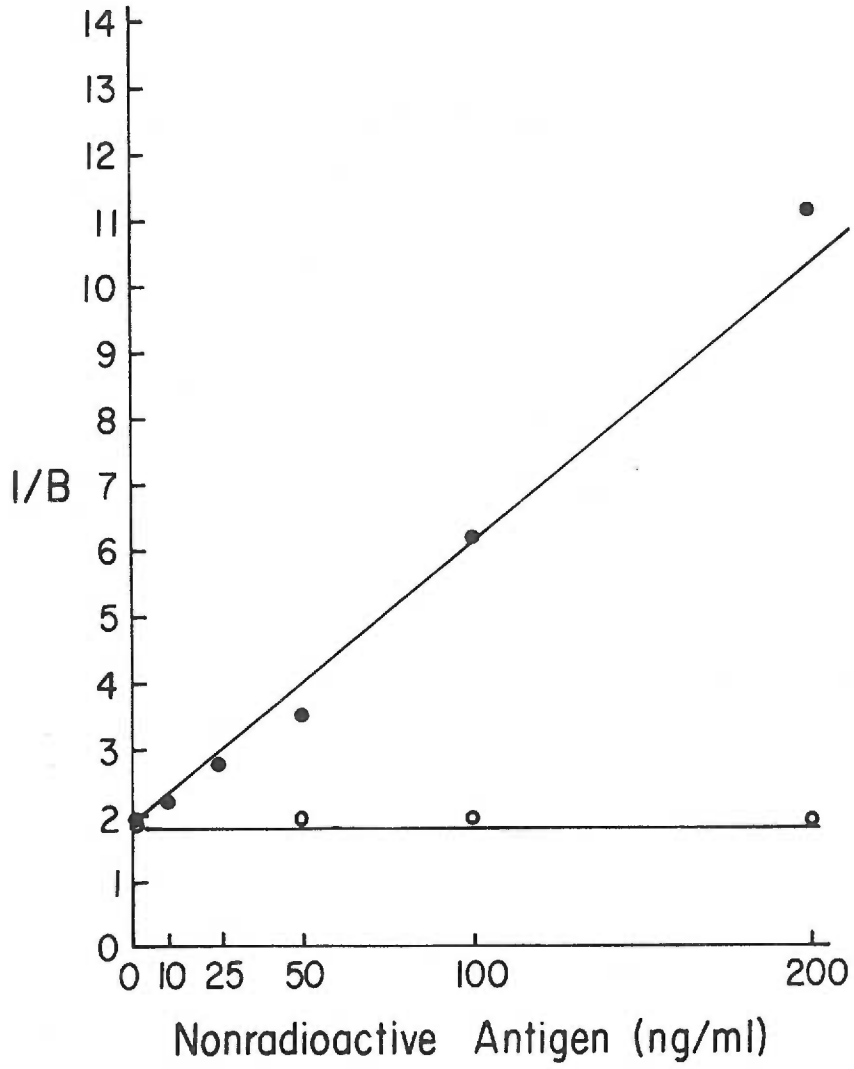
[molecular weight 23,000 (14, 30) or 39,000 (24)] were missing or reduced in the protein profile of the mutant PAO-E64 (Fig. 1).

Production of elastase in liquid medium by PAO1. Medium PTSB was found to be optimal for elastase production by strain PAO1 compared to other media tested. Culture supernatant of PTSB medium was found to contain at least 4-fold more elastase activity than that in culture supernatants of TSBD, MTYG (29), NB or NYB (data not shown). Therefore, PTSB was used for all studies on elastase production.

Elastase activity, as measured in the elastin-Congo red assay at 37° C, was determined as a function of both time of incubation and bacterial cell density. When grown at 37° C, maximum elastase activity and maximum cell density (OD_{540} of approximately 10) was observed 15 h after inoculation. When PTSB cultures were incubated at 21° C, maximum growth and elastase activity were seen 36 h after inoculation (data not shown).

Production of elastase antigen. In order to accurately measure the production of elastase protein by PAO1 and PAO-E64, a highly sensitive liquid phase radioimmunoassay (RIA) specific for P. aeruginosa elastase was developed. The standard curve for the elastase RIA is linear when the concentration of elastase was between 5 and 400 µg/ml. Purified alkaline protease exhibited no cross-reactivity with elastase (Fig 2). The addition of PTSB growth medium produced no displacement of labeled antigen from precipitated immune complexes (data not shown). The limit of detection with this assay was 5 ng of elastase/ml.

Fig. 2. Radioimmunoassay for P. aeruginosa elastase. 1/B, where B represents the percent of total labeled antigen immunoprecipitated. (●) purified elastase, (○) purified alkaline protease.


14
13
12
11
10
9
8
I/B 7
6
5
4
3
2
1
0
0 10 25 50 100 200
Nonradioactive Antigen (ng/ml)

The RIA for elastase showed that PA01 and PAO-E64 produced the same amount of elastase antigen when cultured at 37° C (approximately 150 μg/ml), and when cultured at 21° C (approximately 200 μg/ml) (Table 3). The higher levels of elastase antigen obtained with 21° C culture supernatants may be due to increased production or increased stability of this protein at the lower temperature. In addition, the RIA for elastase could not detect any loss of antigenic determinants on the PAO-E64 elastase protein. These observations were confirmed with the agar-well assay (25) which was adapted for elastase detection. A precipitin band of complete identity and uniform intensity formed between PA01 and PAO-E64 and wells containing elastase antiserum (data not shown).

Effect of assay and growth temperature on elastase activity. The observation that PAO-E64 had parental yields of elastase antigen, but did not produce a detectable zone of clearing on elastin-NA plates (at 37° C), suggested it may be producing an altered enzyme. This possibility was examined by comparing the specific activities of the PAO-E64 elastase with that of PA01 (Table 4). The specific activity of the mutant enzyme was much lower (66% reduced at 37° C) than that of the parental enzyme. Furthermore, when the assay temperature was increased from 21° C to 37° C the specific activity of the parental elastase markedly increased whereas the specific activity of the mutant elastase increased only slightly. Increasing the growth temperature from 21° C to 37° C had no appreciable effect on the specific activity of the mutant or parental enzymes (Table 4).

Table 3. Production of elastase antigen as determined by radioimmunoassay[a]

	Elastase Ag (µg/ml) at growth temperatures	
Strain	21° C	37° C
PA01	218	147
PA0-E64	216	168

[a]Strains were cultured in PTSB at 21° C for 36 h or at 37° C for 15 h.

Table 4. Effect of growth and assay temperature on elastase specific activity

Growth temperature	Strains	Elastase specific activity[b] 21° C	37° C
21° C	PAO1	.043	.100
	PAO-E64	.028	.032
37° C	PAO1	.066	.098
	PAO-E64	.028	.033

[a]Strains were grown in PTSB at 21° C for 36 h or at 37° C for 15 h.

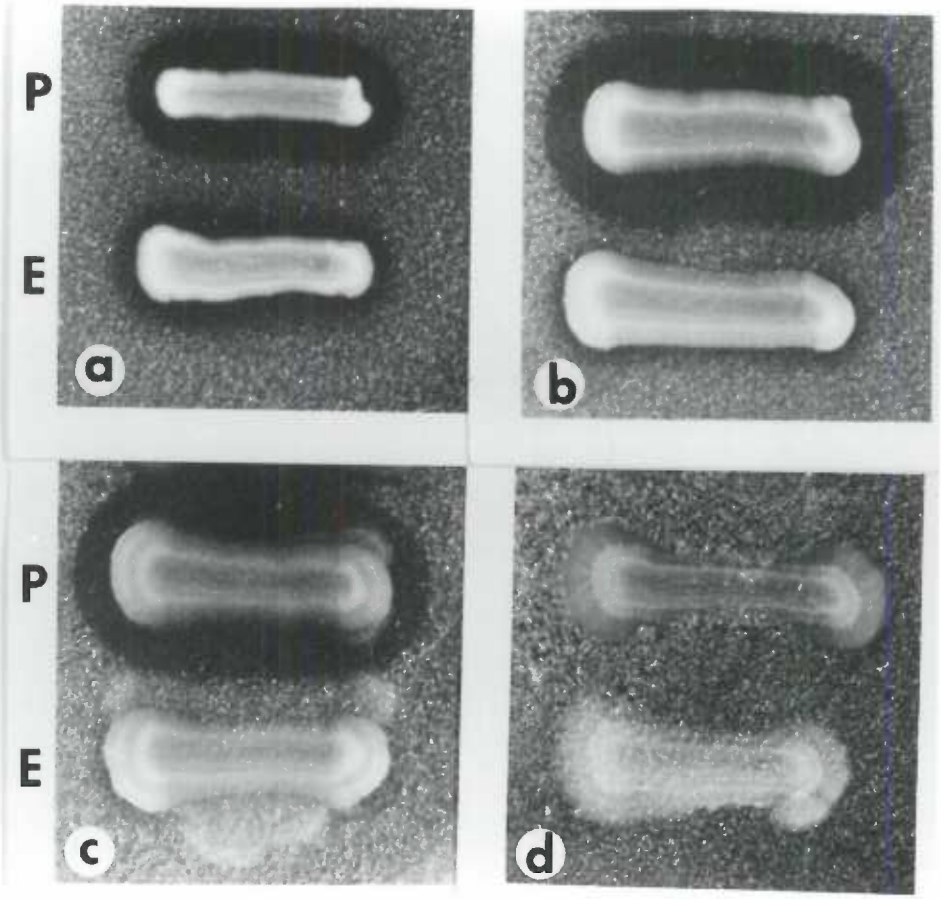
[b]Elastase specific activity was defined as $[(\Delta A_{495\ nm}/h$ per ml)/µg elastase per ml] x 10^2. This experiment was repeated twice with comparable results.

The reduced specific activity of the mutant elastase was not due to the synthesis of an inhibitor by the mixture since the activity of mixtures of mutant and parental supernatants gave additive results (data not shown).

The observation that the specific activity of the mutant PAO-E64 elastase did not increase when the temperature of assay increased suggested that this mutant may have a temperature-dependent conditional mutation affecting elastase activity. This possibility was explored by incubating cultures of PAO1 and PAO-E64 on elastin-NA plates at various temperatures (Fig. 3). Following 72 h of incubation, PAO1 produced zones of clearing at 21° (3.0 mm), 32° (4.0 mm), and 37° C (4.5 mm), but not at 32° C. No elastin-clearing was seen with PAO-E64 at 37° and 42° C. However, at 32° C, PAO-E64 produced detectable elastase activity. The zone of clearing surrounding PAO-E64 increased to 1.0 mm when the plate was incubated for 72 h at 21° C (Fig. 3). At 21° C in this assay, the mutant produced approximately one-third the elastase activity of the parent. In addition, an elastin-NA plate inoculated with PAO-E64 and incubated at 37° C for 48 h showed no elastin-clearing but subsequent incubation of that plate at 10° C overnight produced a zone of clearing around PAO-E64 (data not shown).

Preliminary mapping of lasA. The genotype of mutant PAO-E64 was designated lasA-1. An argF derivative of PAO-E64 (PAO0418) was identified following the isolation of Arg^- auxotrophs and analysis of their utilization of arginine biosynthesis intermediates. From

Fig. 3. Comparison of the zones of elastase activity produced by P. aeruginosa strain PA01 (P) and elastase mutant PAO-E64 (E) following 3 days incubation on elastin-NA plates at (a) 21° C, (b) 32° C, (c) 37° C, and (d) 42° C.


P
E
a
b
P
E
c
d

matings between the lasA recipient strain PA00418 and an elastase-positive donor strain (PA0505 $FP2^+$), Arg^+ recombinants were obtained through the transfer of the first 55 min of the chromosome. None of the 85 recombinants obtained produced elastin-clearing at 37° C on elastin-NA plates.

Discussion

Morihara et al. (21) observed that P. aeruginosa produces two major extracellular proteases termed alkaline protease and elastase (or protease II). These enzymes have been implicated in the pathogenesis of a variety of P. aeruginosa infections (10, 11, 13, 17, 23). Both of these enzymes hydrolyze casein, but only the elastase degrades elastin (18, 19). Previously protease-deficient mutants of P. aeruginosa have been isolated on the basis of reduced casein digestion (29). As one might expect, all of these mutants have been pleiotropic (29). However, to evaluate the contribution of each of these proteases to virulence, mutants with specific elastase or alkaline protease deficiencies are needed. The use of elastin-NA plates enabled us to isolate a mutant (PAO-E64) with a specific elastase deficiency (Table 1). This mutant produces parental levels of alkaline protease and other extracellular proteins (Table 2 and Fig. 1). The isolation of this elastase-deficient, apparently non-pleiotropic mutant was obtained at a frequency of 2.3×10^{-5}. We have designated the genotype of the mutant PAO-E64 as lasA-1. Preliminary genetic mapping studies failed to locate the lasA gene within the first 55

min of the chromosome. Due to the lack of late markers and sex-plasmids which transfer the late region of the chromosome (8), I have been unable to accurately map much farther beyond this point in strain PAO.

Despite the fact that mutant PAO-E64 was isolated as elastase deficient, no protein identifiable as elastase was absent in PAO-E64 culture supernatants (Fig. 1). Furthermore, PAO-E64 was found to produce the same amount of elastase antigen as the parental strain (PAO1) (Table 3). The mutant enzyme was immunologically indistinguishable from the native elastase in RIA and in an immunodiffusion assay.

These data indicate that this mutation in PAO-E64 does not affect the production of elastase. Rather, they suggest that the mutant elastase either has a reduced activity due to a structural alteration of the enzyme, or that the mutant produces an elastase inhibitor. Since the activity of mixtures of mutant and parental supernatants gave additive results, it seems unlikely that PAO-E64 produces an inhibitor of elastase. Elastase in PAO-E64 culture supernatants had a lower specific activity than parental elastase at both temperatures tested. Typical of many enzyme catalyzed reactions, the specific activity of the parental elastase increased markedly as the temperature of the reaction was increased from 21° to 37° C. In contrast, the specific activity of the mutant enzyme increased only slightly when the reaction temperatures was increased (Table 4). The failure of the specific activity of the mutant enzyme to increase

appreciably with increasing reaction temperature suggests it might be thermally unstable. Presumably its instability was partially offset by its increased rate of reaction at higher temperatures.

The possibility that the mutant elastase was thermally unstable was examined by culturing the mutant on elastin-NA plates at various temperatures. The mutant produced detectable elastase activity at 21° C but not at 37° C. Again, the parental strain expressed maximal activity at 37° C (Fig. 3). When PAO-E64 was allowed to grow on an elastin-NA plate at 37° C for 48 h, no evidence of elastin-clearing was detected. However, when this plate was then incubated overnight at 10° C, a prominant zone of clearing formed around the PAO-E64 growth. This indicates that the elastase produced by PAO-E64 is not irreversibly inactivated at 37° C.

My data (Table 3, 4 and Fig. 2) suggest that PAO-E64 has a mutation in the structural gene for elastase which results in the formationof an altered enzyme. A single base-pair change in the structural gene, resulting in one amino acid alteration, could be sufficient to completely alter the kinetic properties of the enzyme. However, I cannot discount the possibility that a proenzyme elastase failed to be converted to an active form due to a defect in post-translational processing systems. Definitive data on the nature of the alteration and thermal stability of the mutant elastase requires the purification of the mutant enzyme.

References

1. Bjorn, M.J., Iglewski, B.H., Ives, S.K., Sadoff, J.C. and Vasil, M.L. 1978. Effect of iron on yields of exotoxin A in cultures of Pseudomonas aeruginosa PA-103. Infect. Immun. 19: 785-791.

2. Bjorn, M.J., Sokol, P.A. and Iglewski, B.H. 1979. Influence of iron on yields of extracellular products in cultures of Pseudomonas aeruginosa. J. Bacteriol. 138:193-200.

3. Chung, D.W. and Collier, R.J. 1977. Enzymatically active peptide from the adenosine and diphosphate-ribosylating toxin of Pseudomonas aeruginosa. Infect. Immun. 16:832-841.

4. Cryz, S.J. and Iglewski, B.H. 1980. A specific and sensitive assay for Pseudomonas aeruginosa alkaline protease. Infect. Immun. submitted.

5. Diener, B., Carrick, L. Jr. and Berk, R.S. 1973. In vivo studies with collagenase from Pseudomonas aeruginosa. Infect. Immun. 7:212-217.

6. Finkelstein, R.A., Vasil, M.L. and Holmes, R.K. 1974. Studies on toxigenesis in Vibrio cholerae. I. Isolation of mutants with altered toxinogenicity. J. Infect. Dis. 129:117-123.

7. Haas, D., Holloway, B.W., Schambock, A. and Leisinger, T. 1977. The genetic organization of arginine biosynthesis in Pseudomonas aeruginosa. Molec. gen. Genet. 154:7-22.

8. Holloway, B.W., Krishnapillai, V. and Morgan, A.F. 1979. Chromosomal genetics of Pseudomonas. Microbiol. Rev. 43:73-102.

9. Iglewski, B.H. and Sadoff, J.C. 1979. Toxin inhibitors of protins synthesis: production, purification and assay of Pseudomonas aeruginosa toxin A. Methods in Enzymol. 60:780-793.

10. Kawaharajo, K., Abe, C., Homma, J.Y., Kawano, M., Botoh, E., Tanaka, N. and Morihara, K. 1974. Corneal ulcers caused by protease and elastase from Pseudomonas aeruginosa. Jpn. J. Exp. Med. 44:435-442.

11. Kawaharajo, K., Homma, J.Y., Aoyama, Y., Okada, K. and Morihara, K. 1975. Effects of protease and elastase from Pseudomonas aeruginosa on skin. Jpn. J. Exp. Med. 45:79-88.

12. Kessler, S.W. 1975. Rapid isolation of antigens from cells with a staphylococcal protein A-antibody absorbent: parameters of the interaction of antibody-antigen complexes with protein A. J. Immun. 115:1617-1624.

13. Kreger, A.S. and Gray, L.D. 1978. Purification of Pseudomonas aeruginosa protease and microscopic characterization of pseudomonal protease-induced rabbit corneal damage. Infect. Immun. 19:630-648.

14. Kreger, A.S. and Griffin, O.K. 1974. Physiochemical fractionation of extracellular cornea-damaging proteases of Pseudomonas aeruginosa. Infect. Immun. 9:828-834.

15. Kronvall, G., Seal, U.S., Finstad, J. and Williams, R.C. 1970. Phylogenetic insight into evolution of mammalian F_c fragment of gamma G-globulin using staphylcoccal protein A. J. Immunol. 104:140-147.

16. Liu, P.V. 1974. Extracellular toxins of Pseudomonas aeruginosa. J. Infect. Dis. 130:S94-S99.

17. Meinke, G., Barum, J., Rosenberg, B. and Berk. R. 1970. In vivo studies with the partially purified protease (elastase) from Pseudomonas aeruginosa. Infect. Immun. 2:583-589.

18. Morihara, K. 1963. Pseudomonas aeruginosa proteinase. I. Purification and general properties. Biochim. Biophys. Acta 73: 113-124.

19. Morihara, K. 1964. Production of elastase and proteinase by Pseudomonas aeruginosa. J. Bacteriol. 88:745-757.

20. Morihara, K. and Tsuzuki, H. 1975. Pseudomonas aeruginosa elastase: Affinity chromatography and some properties as a metallo-neutral proteinase. Agr. Biol. Chem. (Tokyo) 39:1123-1128.

21. Morihara, K. and Tsuzuki, H. 1977. Production of protease and elastase by Pseudomonas aeruginosa strains isolated from patients. Infect. Immun. 15:679-685.

22. Morihara, K. and Tsuzuki, H. 1978. Phosphoramidon as an inhibitor of elastase from Pseudomonas aeruginosa. Jpn. J. Exp. Med. 48:81-84.

23. Morihara, K., Tsuzuki, H. and Oda, K. 1979. Protease and elastase of Pseudomonas aeruginosa: inactivation of human plasma α_1-proteinase inhibitor. Infect. Immun. 24:188-193.

24. Morihara, K., Tsuzuki, H., Ika, T., Inoue, H. and Ebata, M. 1965. Pseudomonas aeruginosa elastase. Isolation, crystallization, and preliminary characterization. J. Biol. Chem. 240:3295-3304.

25. Ohman, D.E., Sadoff, J.C. and Iglewski, B.H. 1980. Toxin A deficient mutants of Pseudomonas aeruginosa strain PA103: Isolation and characterization. Infect. Immun. submitted

26. Stanish, V.A. and Holloway, G.W. 1972. A mutant sex factor of Pseudomonas aeruginosa. Genet. Res. 19:91-108.

27. Vasil, M.L., Kabat, D. and Iglewski, B.H. 1977. Structure-activity relationships of an exotoxin of Pseudomonas aeruginosa. Infect. Immun. 16:353-361.

28. Watson, J.M. and Holloway, B.W. 1978. Chromosome mapping in Pseudomonas aeruginosa PAT. J. Bacteriol. 133:1113-1125.

29. Wretlind, B., Sjöberg, L. and Wadström, T. 1977. Protease-deficient mutants of Pseudomonas aeruginosa: Pleiotropic changes in activity of other extracellular enzymes. J. Gen. Microbiol. 103:329-336.

30. Wretlind, B. and Wadström, T. 1977. Purification and properties of a protease with elastase activity from Pseudomonas aeruginosa. J. Gen. Microbiol. 103:319-327.

31. Yukitaka, M., Vaitukaitis, J.L., Neischlag, E. and Lipsett, M.B. 1972. Enzymatic radioiodination of gonadotropins. J. Clin. Endocr. 34:23-28.

Paper 4

Corneal Infections in Mice with Toxin A and Elastase Mutants of *Pseudomonas aeruginosa*

Abstract

The data presented indicates that in experimental infections of the mouse cornea, Pseudomonas aeruginosa toxin A contributes to its pathogenicity while active elastase may not be required. Following traumatization, corneas were infected with wild type parental toxin A producing strains, toxin A deficient mutants, or an elastase mutant. The infections produced by both toxin A deficient mutants were less severe than infections produced by the parental strains. Furthermore, the toxin A deficient mutants were not able to persist in the eyes as long as the parental strains. Addition of subdamaging doses of exogenous toxin A to eyes infected with the toxin A deficient mutant PA103-29 significantly increased its virulence. The course of infection and the resulting corneal damage produced by the elastase mutant was indistinguishable from that of its parental strain.

Introduction

Pseudomonas aeruginosa is one of the leading bacterial causes of central ulcers of the human cornea (19). Following trauma to the cornea, these infections proceed on a rapid and destructive course which usually result in permanent corneal damage or loss of vision in the infected eye (5). Because P. aeruginosa is often resistant to most antimicrobial agents, these infections are difficult to treat.

The mechanisms by which P. aeruginosa produces corneal disease have not been clarified. Extracellular products produced by this organism are believed to contribute to its pathogenicity (21). Two

distinct proteases produced by P. aeruginosa, alkaline protease (23) and elastase (24), may be responsible for some of the gross corneal damage seen in Pseudomonas keratitis. Kreger and Gray (18) showed that total extracellular protease preparations purified from P. aeruginosa culture supernatants caused rapid and extensive damage to rabbit corneas following intracorneal injection. These proteases also degraded rabbit corneal proteoglycan ground substance. Kawaharajo et al. (15) reported that opacity and ulceration occurred when purified alkaline protease or elastase were dropped onto wounded corneas of mice.

Toxin A, the most toxic extracellular product of P. aeruginosa (16, 28), may also contribute to corneal damage during P. aeruginosa ocular infections. Intracorneal injections of microgram quantities of toxin A led to the rapid death of endothelial, epithelial, and stromal cells and resulted in necrosis (11). Toxin A, which has no proteolytic activity, inhibits cellular protein synthesis by catalyzing the transfer of the adenosine 5'-diphosphate ribose (ADPR) portion of nicotinamide adenine dinucleotide (NAD) to mammalian elongation factor 2 (EF-2) (12, 13).

This report describes a genetic approach to the determination of the relative importance of toxin A and elastase in P. aeruginosa infections of the cornea. Mutant strains have recently been isolated and characterized which are specifically deficient in the production or activity of these enzymes (25, 26, 27). Mutant strains were compared to their virulent parental strains in experimental mouse corneal infections.

Materials and Methods

Bacterial strains. P. aeruginosa strain PA01 (9) was kindly provided by D. Haas, Eidgenossische Technische Hochschule, Zurich, Switzerland; PA103 (15) was provided by P. V. Liu, University of Louisville School of Medicine, Louisville, KY; WR4, WR9, WR27, and WR35 (2) were provided by J. Sadoff, Walter Reed Army Institute of Research, Washington, D.C.; and strains Davis and Clark (32) were provided by E. Zigler, University of California at San Diego. Also utilized were recently described PA01 mutants, PAO-T1 (27) which is toxin A deficient, and PAO-E64 (25) which produces an altered elastase with reduced enzymatic activity. The toxin A mutant PA103-29 (26) derived from strain PA103-0 (wild-type, parental strain designation) was also used in this study. These three mutants have been extensively characterized and each was apparently identical to its parental strain except for the specific toxin A or elastase deficiency described (25-27). All cultures were stored in sterile 10% (w/v) skim milk (Difco) at -70° C.

Toxin A production, quantitation and purification. The culture medium for toxin A production consisted of deferrated Trypticase soy broth dialysate, 1% glycerol, and 0.05 M monosodium glutamate (TSBD), as previously described (1). The culture conditions (32° C) were as previously described (25). Supernatants were obtained following centrifugation, and the ADPR transferase activity of toxin A in activated (urea and dithiothreitol-treated) supernatants measured as previously described (1). Standard curves were obtained daily with activated

pure toxin, and the amount of toxin A present in crude supernatants was calculated from the standard curves (14). Toxin A was purified as previously described (14).

Production of toxin A was also detected in an agar-well plate assay using antitoxin A sera as previously described (26).

Production and quantitation of proteases. The liquid culture medium for elastase production consisted of 5% Bacto-peptone and 0.25% Trypticase soy broth (PTSB) (25). Elastase activity in PTSB culture supernatants was measured at 37° C in an assay previously described (25) using elastin-Congo red (Sigma) as substrate. The production of elastase activity was also measured as the zone of clearing extending from the edge of a 1.5 cm streak of bacterial growth following incubation at 37° C on nutrient agar plates containing 0.3% elastin (Sigma) (elastin-NA) (26).

Total proteolytic activity was measured as the zone of clearing extending from the edge of a 1.5 cm streak of growth on D-BHI milk plates (30) following 48 h incubation at 37° C.

Mouse corneal infection model. The mouse corneal infection model described by other investigators (6, 7, 8, 16) was used with some modification. Bacterial strains at mid log-phase in TSBD were used to inoculate 50 ml TSBD in a 500 ml Erlenmeyer flask to achieve an optical density at 540 nm (OD_{540}) of 0.05. The flask was incubated at 32° C with maximum aeration until the culture reached an OD_{540} of 0.40. The organisms were washed twice with sterile phosphate-buffered saline (PBS) pH 7.4 to remove extracellular products and then

suspended in PBS. Viable numbers of bacteria were obtained by plating dilutions on nutrient agar.

Female Swiss-Webster mice, weighing 20 ± 2 g, were anesthetized with methoxyflurane (Pitman-Moore, Inc., Washington Crossing, N.J.) and subjected to three 1 mm corneal incisions with a 27_{ga} needle while viewed through a 50X power stereoscopic microscope. Care was taken not to penetrate the anterior chamber. Bacteria suspended in 5 µl of PBS at the desired cell concentrations (1×10^3 to 1×10^8) were immediately applied onto the traumatized corneas. Control eyes were traumatized and received PBS or were not traumatized and received bacterial suspensions. Experiments were terminated after 32 days.

Where indicated, purified toxin A (0.15 µg/5 µl) was applied onto infected eyes once daily from day 3 through day 12. Toxin A was diluted to this concentration in dilution buffer (50 mM sodium-phosphate buffer, pH 7.0, 50 mM NaCl, and 20% glycerol) and stored in small aliquots at -70° C for a single use. This concentration of toxin A was chosen because preliminary studies showed that applying toxin A once daily for 10 days to traumatized, uninfected eyes at a concentration of 1.5 µg/5 µl resulted in extensive opacity, whereas, a concentration of 0.15 µg/5 µl or dilution buffer alone had no visible effect (data not shown).

Strain recovery from infected corneas. Bacteria were recovered during the course of infection by gently streaking across the eye with a sterile swab soaked in saline. The swabs then were streaked onto the agar medium of King et al. (17) which enhances fluorescin

production, and P. aeruginosa identified as yellow-green colonies after 37° C incubation. When P. aeruginosa could not be recovered from eyes on 2 consecutive days, they were considered culture negative. Isolates of each strain cultured from all diseased corneas were tested for toxin A, elastase, and total extracellular protease in the plate assays described above.

Microscopic observations of corneas. Infected and control eyes were examined with a 50X-power stereomicroscope at specific intervals during each 32 day experiment. The level of corneal damage was quantitated according to a corneal damage index (CDI) (as defined in Table 1) to evaluate the progression of corneal pathology. Advanced corneal disease (CDI $\geq$2.5) also included neovascularization, edema, hemorrhage, puss, and central granuloma. A perforated ulcer (CDI 4.0) resulting in rupture and dislodgement of the cornea was a very rare event and may represent primarily a complication of physical trauma to a weakened infected cornea. The pathology observed in stereomicroscopic observations of live mice was verified by histological examinations. The eyes of mice exhibiting representative pathology were enucleated, fixed, sectioned, stained and examined microscopically as previously described (6).

Results

Selection of virulent strains. Eight strains of P. aeruginosa were characterized with respect to relative yields of toxin A and elastase (Table 2). One strain (PA103) was known to elastase-negative

Table 1. Corneal damage index

Grade	Description
0	Normal
0.5	Small area of opacity or neovascularization
1.0	Light or partial opacity
1.5	Extensive opacity with iris visible
2.0	Extensive opacity
2.5	Extensive opacity with dense central opacity or with globe of irregular shape
3.0	Central necrosis and opacity
3.5	Necrotic area >50% of cornea
4.0	Perforation of cornea

Table 2. Characteristics of P. aeruginosa strains

Strain	Extracellular products		Mouse corneal infection	
	Toxin A[a] relative activity (CPM)	Elastase[b] 48 h zone of clearing (mm)	Established[c] infections (day 2)	Average[d] corneal damage (day 9)
PA103	2844	0	5/5	3.3
WR9	949	2.5	0/5	0
WR35	518	4.0	2/5	0.4
PA01	457	2.0	5/5	2.6
Davis	383	1.0	0/5	0
WR27	337	2.5	5/5	0.7
WR4	160	5.0	5/5	0.2
Clarke	76	4.0	2/5	0

[a]Strains were cultured (TSBD) at 32° C as previously described (25). ADP-ribosyl transferase activity was measured in a 15 min enzyme assay sytem.

[b]Zone of clearing on elastin-NA plates following incubation at 37° C for 48 h.

[c]The fraction of mice which showed any sign of corneal damage on day 2.

[d]According to the corneal damage index (Table 1).

and highly toxigenic (20). The 7 elastase-positive strains had a wide range of toxin A yields, from almost 1000 CPM (WR9) to less than 100 CPM (Clarke). The levels of elastase produced also varied considerably among these strains from a zone of 1.0 mm (Davis) to 5.0 mm (WR4).

These eight strains were tested in the mouse corneal infection model. Each strain was applied to the incised corneas of five mice with an inoculum of 10^6 organisms. Strain PA103 has previously been shown to be virulent in mouse corneal infections (16) and served as a positive control. Three of the elastase-positive strains were able to establish infections with corneal pathology in all five mice (PA01, WR27, and WR4). However, only one elastase-positive strain (PA01) produced significant corneal damage (CDI > 1.5) which persisted for at least 9 days.

Strains PA01 (toxin A^+elastase$^+$) and strain PA103 (toxin A^+elastase$^-$), were used as virulent representatives of two phenotypes for the following corneal infection studies. In addition, the recently characterized mutant derivatives of these strains were used: PAO-T1 (27), PA103-29 (26), and PAO-E64 (25). Mutants PAO-T1 and PA103-29 are toxin A deficient, and mutant PAO-E64 produces an altered elastase with reduced enzymatic activity. Each mutant produced parental levels of alkaline protease (Table 3). These mutants appeared to be identical to their parental strain in all characteristics except for the specific deficiency indicated (25-27, Table 3).

Corneal damage by PAO and PA103 strains. When the 3 PAO strains (PA01, PAO-E64 and PAO-T1) were applied to incised corneas at an

Table 3. Extracellular products of P. aeruginosa strains and mutants

Strain	Toxin A[a] (µg/ml)	Elastase		Alkaline[d] protease (µg/ml)
		Plate[b]	Broth[c]	
PA01	3.0	3.5	19.9	2.7
PAO-T1	<0.001	3.5	20.0	2.8
PAO-E64	3.0	0[e]	5.5	3.4
PA103-0[f]	30.0	0	0	1.0
PA103-29	0.06	0	0	1.0

[a]Determined in a 60 min ADPR transferase system standardized with purified toxin A (14).

[b]Zone of clearing seen on elastin-NA plates incubated at 37° C for 4 days.

[c]Activity in PTSB culture supernatants determined in an elastin-Congo red assay at 37° C (25).

[d]Determined by a radioimmunoassay for alkaline protease (4).

[e]No elastin-clearing seen after 7 days of incubation at 37° C.

[f]Designation (-0) for parental, wild-type strain.

inoculum size of 10^5 organisms, corneal disease was observed in two-thirds or more of the mice (Table 4), but all infected eyes were normal by day 8 (data not shown). However, when the inocula sizes were increased to 10^6 and 10^7 organisms per eye, corneal infections by strain PAO1 produced pronounced pathological changes by day 2 in all infected mice (Table 4), and the average corneal damage on day 2 was extensive opacity (CDI 2.3 and 2.6) (Fig. 1). The average corneal damage showed gradual improvement after day 8 but was still significant (CDI 1.5) on day 32. Corneal infections with the elastase mutant (PAO-E64) were very similar to those of the parental strain (PAO1). Although the average corneal damage with PAO-E64 was consistently slightly lower than that produced by the parental strain, the statistical difference was not significant.

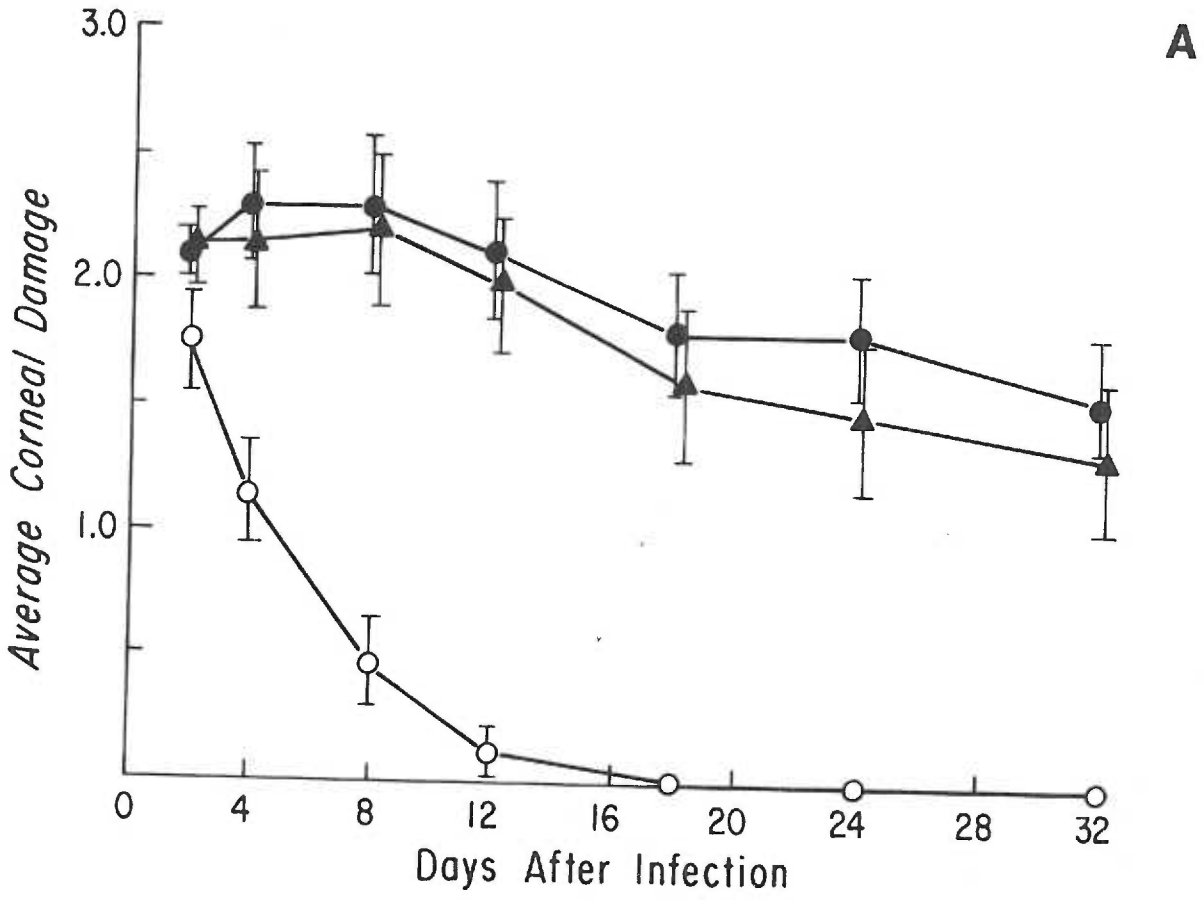
The progress of corneal damage by the toxin A mutant (PAO-T1) was very different from that produced by the parental strain (Fig. 1). Similar to strain PAO1 infections, corneal disease was seen in 94% of the PAO-T1 infected eyes (inoculated with 10^6 and 10^7) (Table 4), and the average damage on day 2 was considerable opacity (CDI 1.7 and 1.8) (Fig. 1). However, in contrast to infections by PAO1, the condition of the eyes infected with the toxin A mutant rapidly improved. By day 8, the average damage was barely detectable (CDI 0.4) and almost every PAO-T1 infected eye was normal by day 18 (Fig. 1).

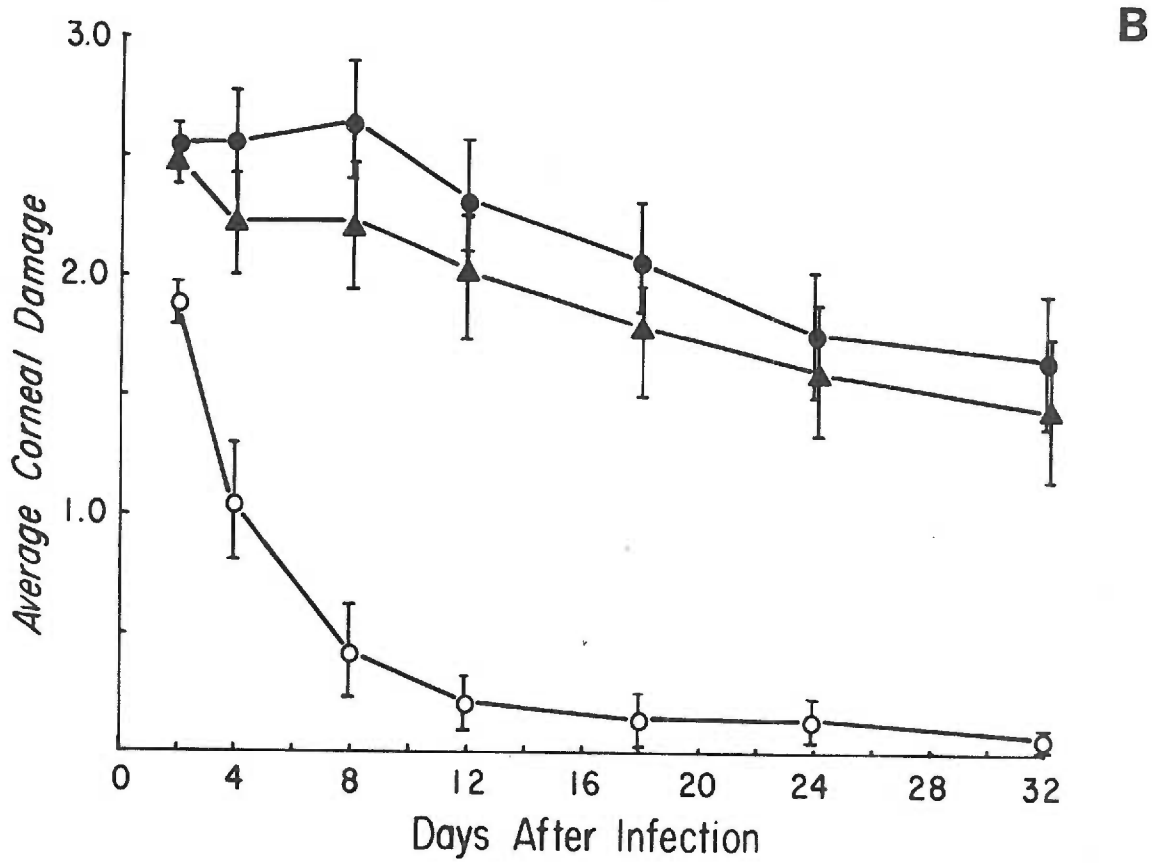
Similar results were obtained when the parental strain (PA103-0) was compared to its toxin A deficient mutant (PA103-29). With an inoculum of 10^5 viable bacteria, 4 out of 5 of the PA103-0 infected

Table 4. Proportion of mice inoculated with PAO strains that showed corneal disease on day 2

	Number of organisms inoculated			
Strain	10^7	10^6	10^5	10^4
PAO1	21/21	21/21	11/12	0/12
PAO-T1	17/17	15/17	11/12	0/12
PAO-E64	18/18	18/18	8/12	0/12

Fig. 1. Average corneal damage ± standard error resulting from the inoculation of (A) 10^6 and (B) 10^7 organisms of PAO1 (●), PAO-E64 (▲), and PAO-T1 (○). Female Swiss mice were anesthesized and corneas were incised prior to inoculation. Corneal damage was measured according to Table 1.


A
3.0
2.0
1.0
Average Corneal Damage
0
4
8
12
16
20
24
28
32
Days After Infection


B
3.0
2.0
1.0
Average Corneal Damage
0
4
8
12
16
20
24
28
32
Days After Infection

mice had corneal disease on day 4, whereas, corneal disease was observed in only one of 5 mice infected with the toxin A mutant (PA103-29) on day 4 (Table 5). The average corneal damage caused by 10^5 parental strain bacteria was higher than that caused by the mutant only during the early stages of the infection (Fig. 2a). The inoculation of 10^6 to 10^8 viable PA103-0 bacteria per eye produced a necrotic infection (CDI 3.0) in all mice which improved very little over the 32 day period (Fig. 2b, c and Table 5). The toxin A mutant (PA103-29) also produced corneal disease in all infected eyes at these inoculum sizes (Table 5). The inoculation of 10^6 PA103-29 organisms produced markedly less damage than did 10^6 PA103-0 organisms (Fig. 2b). The PA103-29 infected eyes showed moderate opacity (CDI 1.7) on day 4 and then markedly improved from day 12 to day 32 (CDI 0.8). Similarly, inoculation of 10^7 and 10^8 PA103-29 organisms produced initial disease (day 4) with considerable corneal pathology, but the damage was transitory, and by day 32 the average corneal damage was only a mild opacity (CDI 1.0).

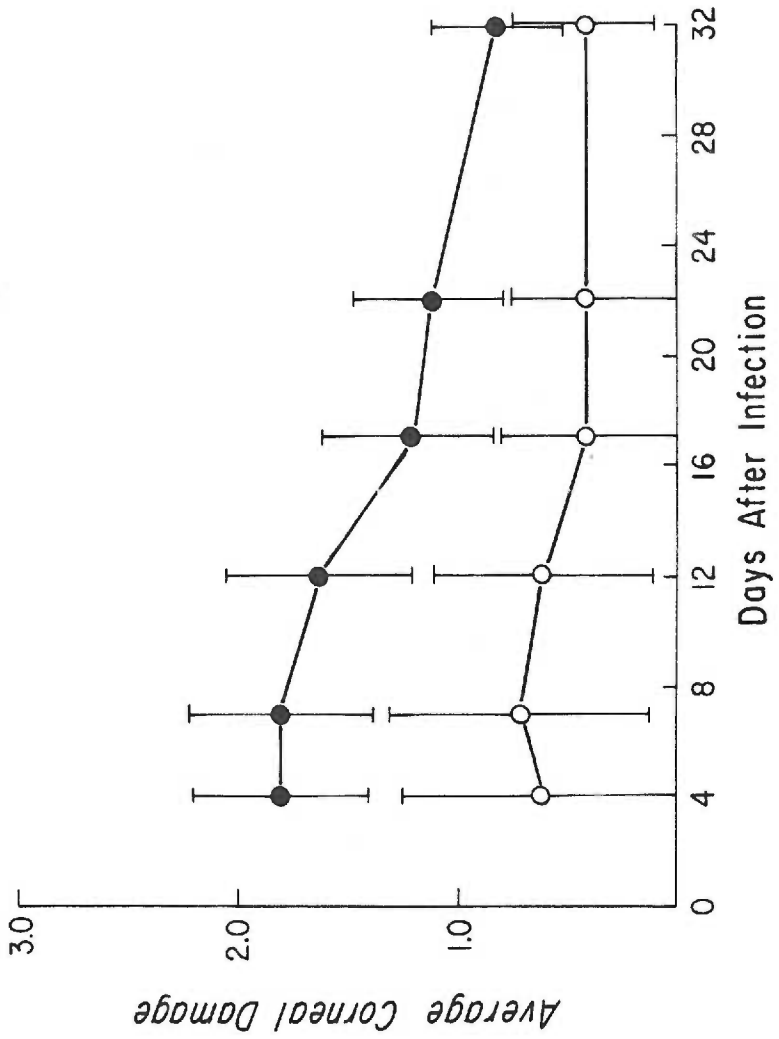
These data indicate that corneal infections with the 2 toxin A deficient mutants (PAO-T1 and PA103-29) resulted in far less permanent corneal damage than that observed with their parental strains.

Effect of exogenous toxin A on PA103-29 infections. In order to confirm the conclusion that the toxin A mutants were less virulent in mouse corneal infections because of their toxin A deficiency, we examined the effect of exogenous toxin A on corneal infections by toxin A mutant PA103-29. Mice were infected with 10^7 PA103-29 bacteria, and on day 3 those with well established infections (average CDI 2.7)

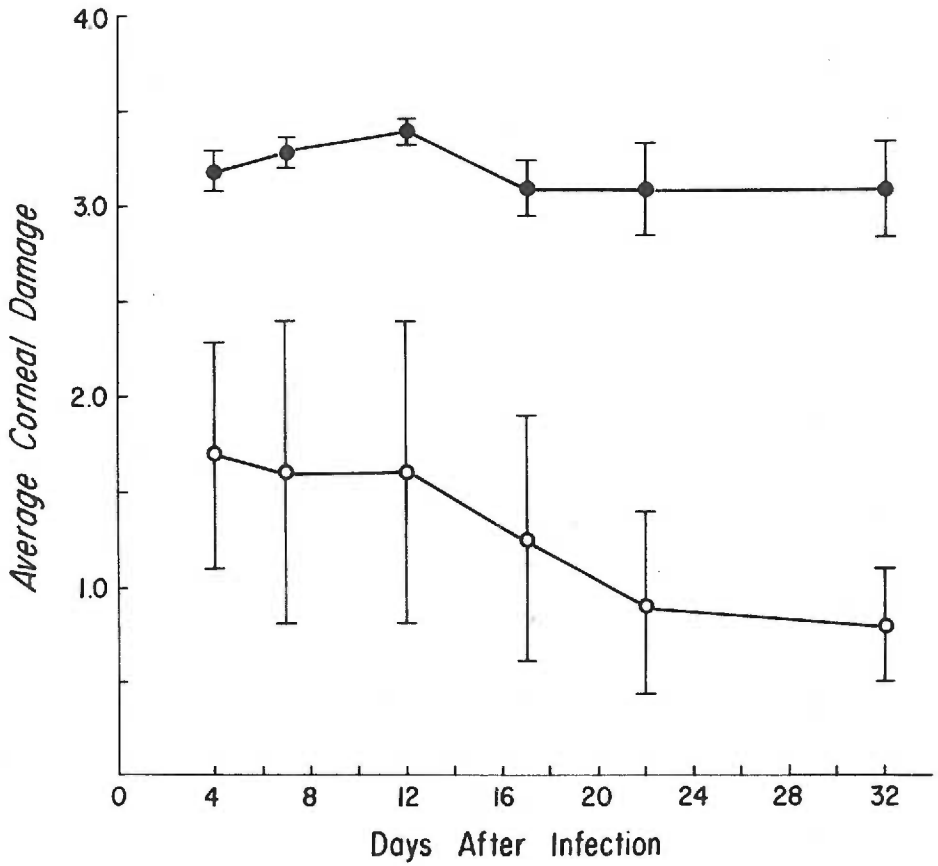
Table 5. Proportion of mice inoculated with PA103 strains that showed corneal disease on day 4

	Number of organisms inoculated					
Strain	10^8	10^7	10^6	10^5	10^4	10^3
PA103-0	5/5	5/5	5/5	4/5	1/5	0/5
PA103-29	5/5	5/5	5/5	1/5	0/5	0/5

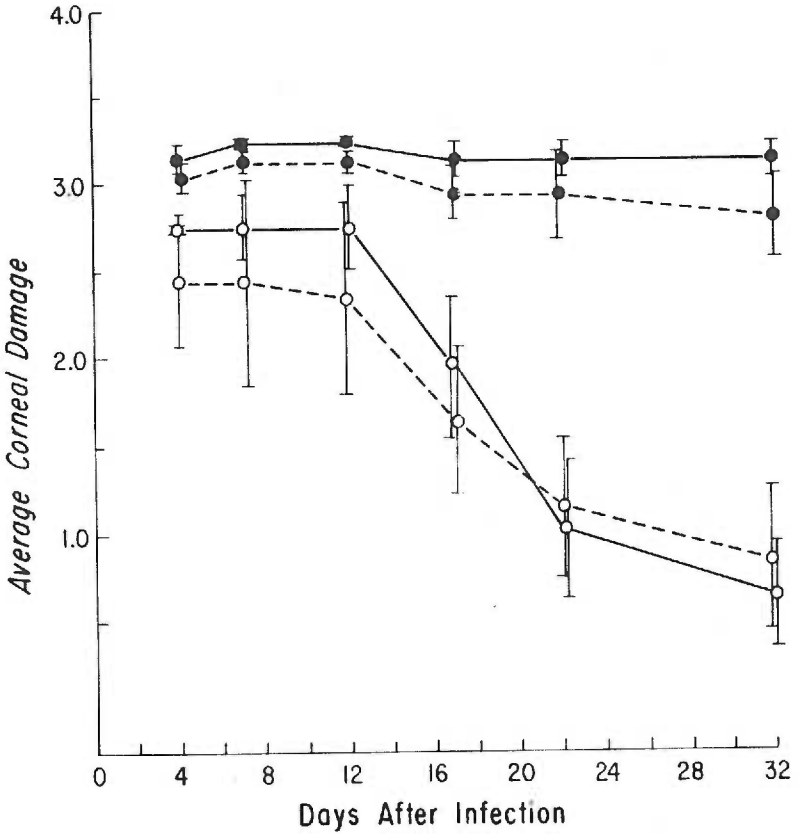
Fig. 2. Average corneal damage ± standard error resulting from the inoculation of (A) 10^5, (B) 10^6, (C) 10^7 (- - -) and 10^8 (———) organisms of PA103-0 (●) and PA103-29 (○). Female Swiss mice were anesthetized and corneas were incised prior to inoculation. Corneal damage was measured according to Table 1.


A
Average Corneal Damage
3.0
2.0
1.0
0
4
8
12
16
20
24
28
32
Days After Infection

B


4.0
3.0
2.0
1.0
Average Corneal Damage
0
4
8
12
16
20
24
28
32
Days After Infection

C

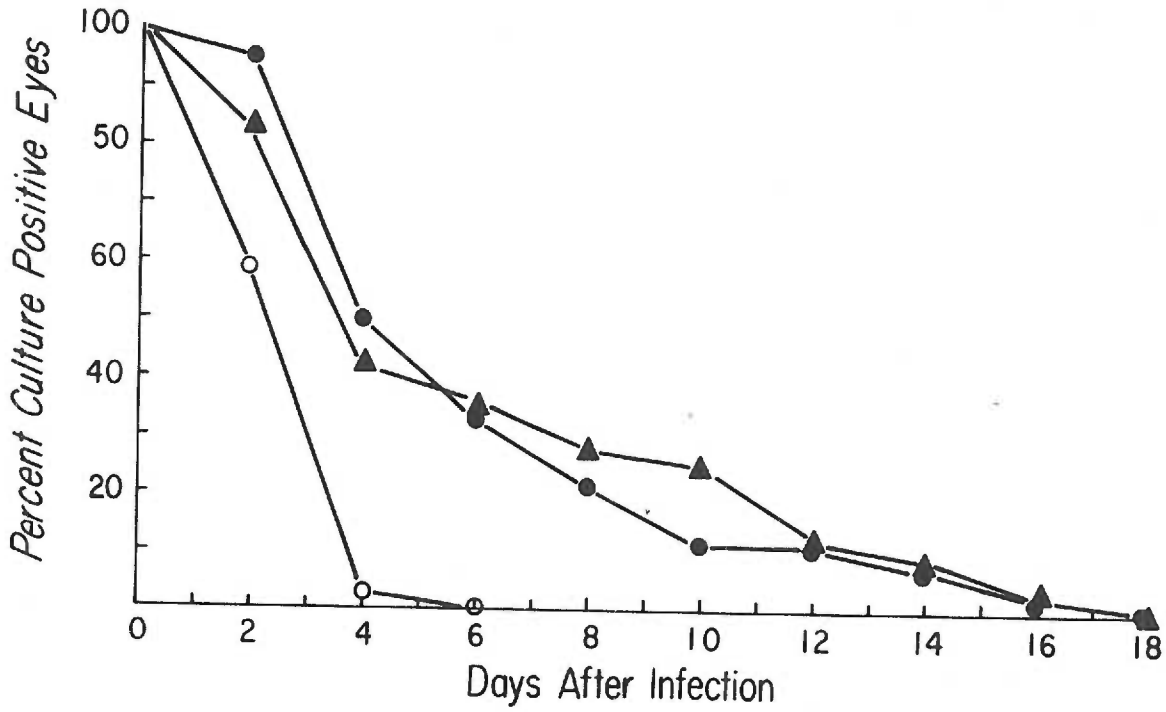

4.0
3.0
2.0
1.0
Average Corneal Damage
0
4
8
12
16
20
24
28
32
Days After Infection

were placed into two identical groups of 10 mice each. The infected eyes of one group received toxin A and the other group received buffer (Fig. 3). The PA103-29 infected group which received buffer daily had a necrotic infection (CDI 3.3) until day 7; these eyes resolved rapidly and by day 32 the average corneal damage was only a moderate opacity (CDI 1.3). The PA103-29 infected group treated with toxin A (0.15 μg/day) had extensive corneal damage (CDI 3.3) throughout the period of toxin A application (days 3-12), and even after 32 days these animals exhibited extensive corneal opacity (CDI 2.2). The uninfected mice that received toxin A (0.15 μg/day) for 10 days did not show any sign of corneal damage. The mice infected with the parental strain (PA103-0) exhibited a very necrotic corneal infection (CDI >3.0) which changed little over the 32 day examination period (Fig. 3).

Recovery of P. aeruginosa from infected eyes. Since the toxin A deficient mutants produced milder infections for mouse corneas than their parental strains, we examined the possibility that these mutants may not be able to persist as long as the parental strains in the infected eyes. After 2 days, we were unable to isolate the 3 P. aeruginosa PAO strains from eyes infected with 10^4 bacteria. With an inoculum of 10^5 organisms, the PAO strains could be isolated from 50% of the infected eyes on day 2, but by day 4 these eyes were also negative for P. aeruginosa. With higher inocula (10^6-10^7), the parental strain (PAO1) could be recovered from 95% of the eyes on day 2 and from 35% of the eyes by day 4 (Fig. 4). The parental strain (PAO1) could not be isolated by day 18. The elastase mutant

Fig. 3. Effect of exogenous toxin A on PA103-29 corneal infections. Mice were infected with 10^7 organisms of P. aeruginosa. Twenty mice with established infections by strain PA103-29 were divided into two groups identical with respect to corneal damage. These two groups were treated daily during the period (T) of days 3 through 12 with 0.15 µg of toxin A (◑) or the buffer solution (○). Ten mice were infected with strain PA103-0 (●), and five mice had corneas incised on day 3 and received 0.15 µg of toxin A daily (▲) during period T. Data shown are the averages of corneal damage ± standard error.

Fig. 4. Percent of eyes from which could be cultured PAO1 (●), PAO-E64 (▲), and PAO-T1 (○) following the inoculation of 10^6 and 10^7 organisms.


Percent Culture Positive Eyes
100
50
60
40
20
0
2
4
6
8
10
12
14
16
18
Days After Infection

(PAO-E64) was recovered from eyes infected with 10^6 and 10^7 organisms at a rate and frequency very similar to that of the parental strain (PAO1) (Fig. 4). However, even at these high inocula (10^6-10^7) the toxin A mutant (PAO-T1) was more rapidly cleared than the parental strain. None of the PAO-T1 infected eyes were culture-positive after day 4 (Fig. 4).

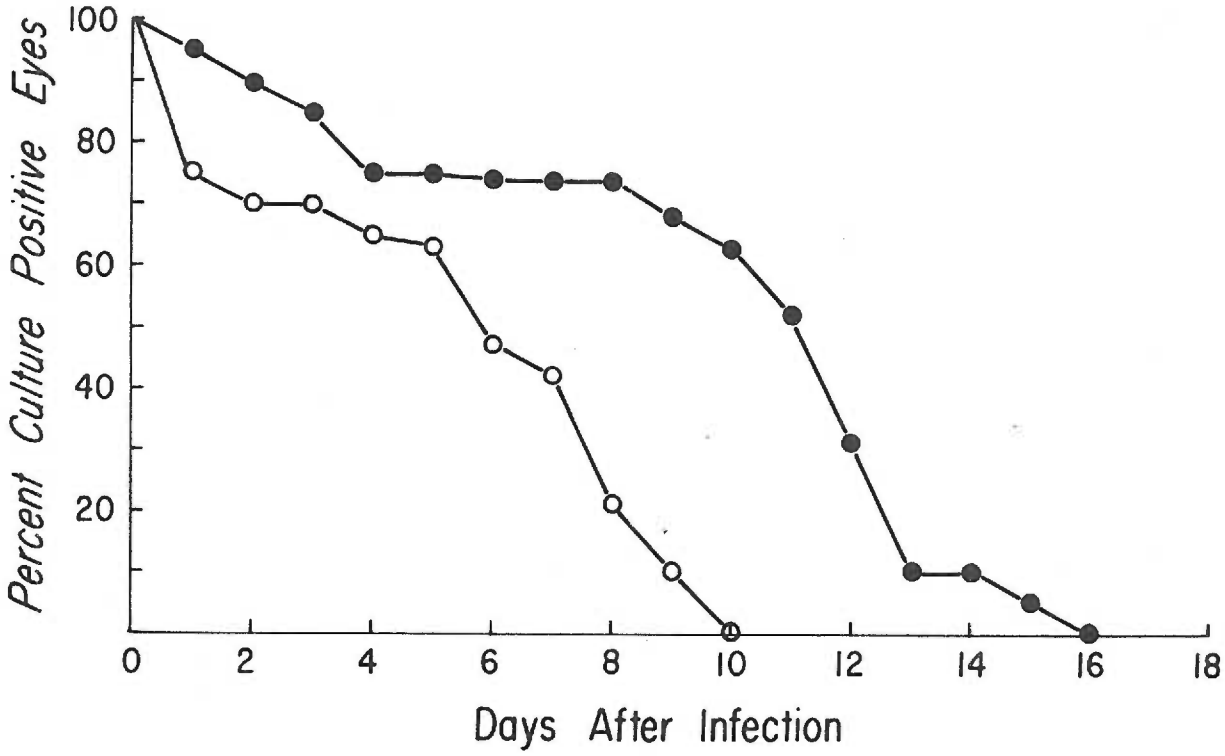
The other parental strain (PA103-0) could be cultured on day 2 from 90% of all eyes infected with 10^5-10^8 organisms, and from over 50% of the eyes through day 11 (Fig. 5). In contrast, infections with the toxin A mutant (PA103-29) were more transitory than those with PA103-0. PA103-29 could not be isolated from any eyes after day 9 (Fig. 5).

Stability of the mutations in vivo. Plate assays indicated that the toxin A and elastase mutations used in this corneal infection study were stable in vivo. All PAO-T1 and PA103-29 isolates from infected eyes retained their toxin A deficiency. Similarly, all PAO-E64 isolates were deficient in elastase activity at 37° C. Parental strain isolates (PAO1 and PA103-0) gave the typical reactions for toxin A, elastase and total extracellular protease (data not shown).

Discussion

Toxin A and elastase are thought to contribute to the pathogenesis of Pseudomonas aeruginosa infections. Toxin A is a potent inhibitor of protein synthesis (12, 13) and caused rapid necrosis when injected into the corneas of rabbits (11). Elastase is one of two distinct

Fig. 5. Percent of eyes from which could be cultured PA103-0 (●), and PA103-29 (○) following the inoculation of 10^5 - 10^8 organisms.


Percent Culture Positive Eyes
100
80
60
40
20
0
2
4
6
8
10
12
14
16
18
Days After Infection

P. aeruginosa proteases (24), both of which have been shown to cause opacity and rapid necrosis when applied to traumatized mouse eyes (15).

To correlate a suspected virulence trait of P. aeruginosa with pathogenicity, investigators have compared different strains possessing various combinations of potentially pathogenic properties in experimental corneal infections (16). Conclusions from such experiments are complicated because independently isolated P. aeruginosa strains are heterogeneous with respect to the production of potential virulence products (31). This indicates that such strains of P. aeruginosa must have genetic differences. It is not surprising that our initial strain selection procedure (Table 2) which compared several independently isolated strains showed no correlation of virulence with the quantitative yields of toxin A and/or elastase. Presumably, these strains had other differences that reduced their ability to produce eye disease.

Genetic techniques were used to determine whether toxin A or elastase has a role in the pathogenesis of P. aeruginosa. This study was based on the principle that a decrease in virulence with a mutant lacking a single factor is a direct indication that the factor has a role in determining the course of the disease (10). Therefore, I utilized recently isolated mutants with specific deficiencies in toxin A (25, 26) or elastase activity (25), and compared them to their parental strains in the mouse corneal infection model.

Virulent strains of two phenotypes, PAO1 (toxin A^+ elastase$^+$) and PA103 (toxin A^+ elastase$^-$), were used in these corneal infection

studies, as well as their toxin A mutants, PAO-T1 (27) and PA103-29 (26), and an elastase mutant, PAO-E64 (25) (Table 3). These mutants were well suited for comparisons with their virulent parental strains in experimental animal infection models because they are congenic and thus have all the known characteristics of the parental strain except for their specific toxin A or elastase deficiency (25-27).

The mouse corneal infection model has been used by other investigators (6, 7, 8, 16). This model infection mimics human Pseudomonas keratitis in that it required trauma to the cornea and topical inoculation of the organisms is sufficient to cause infection (6). The comparisons of mutants with parental strains in this experimental corneal infection allowed us to evaluate the effect of toxin A and/or active elastase on three parameters of P. aeruginosa virulence: establishment of infection, maintenance of infection, and corneal tissue destruction.

Elastase activity does not appear to be essential for P. aeruginosa corneal disease since strain PA103-0 (elastase$^-$) showed considerable virulence in the mouse corneal infection (Fig. 2). Also, I could not show that elastase activity contributes significantly to the pathogenesis of strain PAO corneal infections (Fig. 1 and 4). These data suggest that if proteolytic activity is required for corneal damage, then it is supplied by the alkaline protease which is produced by the PAO and PA103 parental and mutant strains.

My results indicated that toxin A is not involved in the establishment of corneal infections; both toxin A mutants (PAO-T1 and

PA103-29) used in this study were able to establish infection and initiate corneal disease (Fig. 4 and 5). The toxin A mutants could still be recovered from most infected eyes one to 2 days after infection (Fig. 5 and 5). Furthermore, both toxin A mutants produced initial levels of corneal damage in a similar percentage of animals as the parental strain (Tables 4 and 5).

My results indicated that toxin A does play a significant role in the maintenance of infection. The length of time that the toxigenic parental strain persisted at the site of infection, as measured by the ability to culture the strains from the eyes, was considerably greater than that of the toxin A mutant strains (Fig. 4 and 5). This suggests that toxin A plays a role in inhibiting host bacterial-clearance systems. Toxin A was also shown to be responsible for much of the corneal tissue destruction (Fig. 1 and 2). Corneal damage was much greater with infections by the toxigenic parental strains compared to that accompanying infections by toxin A mutants. The permanent damage to toxin A mutant infected eyes was considerably reduced as a result of the toxin A deficiency. Furthermore, when small quantities of toxin A (0.15 μg) were applied only once daily to toxin A mutant (PA103-29) infected corneas, a high level of corneal damage was sustained, and for a longer period of time, than was seen in the toxin A mutant eyes receiving no exogenous toxin A (Fig. 3). In addition, the permanent corneal damage following this toxin A treatment was significantly higher than the mutant infected control group. The concentration of toxin A applied was equivalent to that in the culture supernatant of the parental strain (30 μg/ml, Table 3) and

did not detectably damage incised, uninfected eyes. These data support the conclusion that the decreased virulence of the toxin A mutant PA103-29 was due to it toxin A deficiency.

My results indicate that toxin A has at least two roles in corneal infections, inhibition of the host's bacterial clearance mechanisms and destruction of corneal tissue. Previous results have shown that toxin A has a cytotoxic effect (22, 29), and this may lead to a breakdown of normal anatomical defense barriers. A role of toxin A in corneal tissue destruction has also been shown directly by its killing of epithelial and stromal cells upon injection into rabbit corneas (11). Toxin A may also have an effect on the large numbers of damaged polymorphonucleocytes (PMN) which have been observed in the lesions of Pseudomonas keratitis (18) such that host-derived enzymes enhance the damage elicited by toxin A and Pseudomonas proteases. Brown et al. (3) has reported that PMN lysosomes contain an enzyme or enzymes capable of degrading corneal proteoglycan at neutral pH in vitro.

It is clear that a number of pathogenic factors are involved in P. aeruginosa corneal infections. The mechanisms which allow the organisms to establish infections in the wounded cornea, which may be absent in certain strains of P. aeruginosa (Table 2), are not yet defined but apparently do not include toxin A or elastase. Previous studies suggest that the production of proteolytic activity may be part of the destructive potential of this organism (15, 18). However, this genetic study using the mouse corneal infection model directly

indicates that toxin A plays a key role in the pathogenesis of *Pseudomonas* keratitis.

References

1. Bjorn, M.J., Iglewski, B.H., Ives, S.K., Sadoff, J.C. and Vasil, M.L. 1978. Effect of iron on yields of exotoxin A in cultures of Pseudomonas aeruginosa PA-103. Infect. Immun. 19:785-791.

2. Bjorn, M.J., Sokol, P.A. and Iglewski, B.H. 1979. Influence of iron on yields of extracellular products in cultures of Pseudomonas aeruginosa. J. Bacteriol. 138:193-200.

3. Brown, S.I., Hook, C.W. and Tragakis, M.P. 1972. Presence, significance, and inhibition of lysosomal proteoglycanase. Invest. Ophthalmol. 11:149-152.

4. Cryz, S.J. and Iglewski, B.H. 1980. A specific and sensitive assay for Pseudomonas aeruginosa alkaline protease. Infect. Immun. submitted.

5. Duke-Elder, S. and Leigh, A.G. 1965. Systems of Ophthalmology, Vol. 8, The C.V. Mosby Co., St. Louis, pp. 782-784.

6. Gerke, J.R. and Magliocco, M.V. 1971. Experimental Pseudomonas aeruginosa infection of the mouse cornea. Infect. Immun. 3: 209-216.

7. Hazlett, L.D., Rosen, D.D. and Berk, R.S. 1976. Experimental eye infections caused by Pseudomonas aeruginosa. Ophthalmic Res. 8:311-318.

8. Hazlett, L.D., Rosen, D.D. and Berk, R.S. 1978. Age-related susceptibility to Pseudomonas aeruginosa ocular infections in mice. Infect. Immun. 20:25-29.

9. Holloway, B.W., Krishnapillai, V. and Morgan, A.F. 1979. Chromosomal genetics of Pseudomonas. Microbiol. Rev. 43:73-102.

10. Holmes, R.K. 1975. Genetic aspects of toxinogenesis in bacteria, p. 296-301. In D. Schlessinger (ed.), Microbiology-1975. American Society for Microbiology, Washington, D.C.

11. Iglewski, B.H., Burns, R.P. and Gipson, I.K. 1977. Pathogenesis of corneal damage from Pseudomonas exotoxin A. Invest. Ophthalmol. Visual Sci. 16:73-76.

12. Iglewski, B.H., Liu, P.V. and Kabat, D. 1977. Mechanism of action of Pseudomonas aeruginosa exotoxin A: adenosine diphosphate-ribosylation of mammalian elongation factor 2 in vitro and in vivo. Infect. Immun. 15:138-144.

13. Iglewski, B.H., Liu, P.V. and Kabat, D. 1977. NAD-dependent inhibition of protein synthesis by Pseudomonas aeruginosa toxin. Proc. Natl. Acad. Sci. USA 72:2284-2288.

14. Iglewski, B.H. and Sadoff, J.C. 1979. Toxin inhibitors of protein synthesis: production, purification and assay of Pseudomonas aeruginosa toxin A. Methods in Enzymol. 60:780-793.

15. Kawaharajo, K., Abe, C., Homma, U.Y., Kawano, M., Gotoh, E., Tanaka, N. and Morihara, K. 1974. Corneal ulcers caused by protease and elastase from Pseudomonas aeruginosa. Jpn. J. Exp. Med. 44:435-442.

16. Kawaharajo, K. and Homma, J.Y. 1975. Pathogenesis of the mouse keratitis produced with Pseudomonas aeruginosa. Jpn. J. Exp. Med. 45:515-524.

17. King, E.O., Ward, M.K. and Raney, D.E. 1954. Two simple media for the demonstration of pyocyanin and fluorescin. J. Lab. Clin. Med. 44:301-307.

18. Kreger, A.S. and Gray, L.D. 1978. Purification of Pseudomonas aeruginosa protease and microscopic characterization of Pseudomonal protease-induced rabbit corneal damage. Infect. Immun. 19:630.

19. Laibson, P.R. 1972. Annual review. Cornea and sclera. Arch. Ophthalmol. 88:553-574.

20. Liu, P.V. 1966. The roles of various fractions of Pseudomonas aeruginosa in its pathogenesis. III. Identity of lethal toxins produced in vitro and in vivo. J. Infect. Dis. 116:481-489.

21. Liu, P.V. 1974. Extracellular toxins of Pseudomonas aeruginosa. J. Infect. Dis. 130:S94-S99.

22. Middlebrook, J.L. and Dorland, R.B. 1977. Response of cultured mammalian cells to exotoxins of Pseudomonas aeruginosa and Corynebacterium diphtheria: differential cytotoxicity. Can. J. Microbiol. 23:183-189.

23. Morihara, K. 1963. Pseudomonas aeruginosa proteinase I. I. Purification and general properties. Biochim. Biophys. Acta 73:113-124.

24. Morihara, K., Tsuzuki, H., Ika, T., Inoue, H. and Ebata, M. 1965. Pseudomonas aeruginosa elastase. Isolation, crystallization, and preliminary characterization. J. Biol. Chem. 240: 3295-3304.

25. Ohman, D.E., Cryz, S.J. and Iglewski, B.H. 1980. Isolation and characterization of a mutant of Pseudomonas aeruginosa PAO producing an altered elastase. J. Bacteriol. submitted.

26. Ohman, D.E., Sadoff, J.C. and Iglewski, B.H. 1980. Toxin A deficient mutants of Pseudomonas aeruginosa strain PA103: isolation and characterization. Infect. Immun. submitted.

27. Ohman, D.E. and Iglewski, B.H. 1980. Characterization of a toxin A mutant of Pseudomonas aeruginosa strain PAO. J. Bacteriol. submitted.

28. Pavlovskis, O.R., Callahan, L.T. III and Pollack, M. 1975. Pseudomonas aeruginosa exotoxin, p. 252-256. In D. Schlessinger (ed.), Microbiology-1975. American Society for Microbiology, Washington, D.C.

29. Pavlovskis, O.R. and Gordon, F.B. 1972. Pseudomonas aeruginosa exotoxin: effect on cell culture. J. Infect. Dis. 125:631-636.

30. Sokol, P.A., Ohman, D.E. and Iglewski, B.H. 1979. A more sensitive plate assay for detection of protease production by Pseudomonas aeruginosa. J. Clin. Microbiol. 9:538-540.

31. Wretlind, B., Heden, L., Sjöberg, L. and Wadström, T. 1973. Production of enzymes and toxins by hospital strains of Pseudomonas aeruginosa in relation to serotypes and phage-typing pattern. J. Med. Microbiol. 6:91-100.

32. Ziegler, E.J. and Douglas, H. 1979. Pseudomonas aeruginosa vasculitis and bacteremia following conjunctivitis: a simple model of fatal Pseudomonas infection in neutropenia. J. Infect. Dis. 139:288-296.

Appendix A. Reduced Virulence of Toxin A and Elastase Mutants of *P. aeruginosa* in Experimental Burn Infections.

Pseudomonas aeruginosa, an opportunitistic pathogen, causes fatal infections in burn patients and other compromised hosts (1, 6). Despite recent advances in topical chemotherapy, these infections too often escape therapeutic control.

P. aeruginosa produces a large number of extracellular products, some of which may play a role in disease (5). An experimental burn-wound infection model was developed by Stieritz and Holder (11). This has permitted investigators to study *P. aeruginosa* extracellular products during the course of burn infections in mice. It has been reported that *P. aeruginosa* toxin A (4, 8, 10), protease and elastase (3, 9, 10) may contribute to its pathogenesis in such burn infections.

This study used a genetic approach in an effort to directly determine the relative importance of toxin A and elastase in *P. aeruginosa* burn-wound infections. Mutant strains have been isolated which appear to be specifically deficient in the production or activity of these enzymes. Strain PAO-T1 (Paper 2) is toxin A deficient, strain PAO-E64 (Paper 3) produces an altered elastase with reduced enzymatic activity, and both are mutants of strain PAO1 (toxin A^+ elastase$^-$). Strain PA103-29 (Paper 1) is a toxin A mutant derived from strain PA103-0 (toxin A^+ elastase$^-$). All of these strains produced alkaline protease (Paper 4).

The burned-mouse model (11) was used with the modifications described previously (8). Anesthetized, Swiss white female mice

(strain NIH-NMRI CV), weighing 20 ± 2 g were subjected to a 10-s, 2.5 by 2.5 cm ethanol burn. Washed, log phase bacteria suspended in 0.5 ml PBS at the desired concentration were immediately injected subcutaneously in the burn site. Viable numbers of bacteria were determined by spreading dilutions on nutrient agar.

The inoculation of these PAO and PA103 strains in burn sites produced infections with bacteremia, systemic invasion and mortality. However, each of the 5 challenge strains was lethal in a dose characteristic for the individual strain. The LD_{50} of the wild type strain PAO1 was approximately 1 log lower than that of the toxin A mutant (PAO-T1) and the elastase mutant (PAO-E64) (Table 1). The LD_{50} of the wild type strain PA103-0 ($10^{4.1}$) was approximately 1.6 log lower than that of the toxin A mutant PA103-29 ($10^{5.7}$) (Fig. 1). These data would indicate that both toxin A and elastase contribute to the mortality of P. aeruginosa burn-wound infections.

The LD_{50} for PAO-T1 was very similar to that of PAO-E64 (Table 1). This would suggest that both extracellular products, toxin A and elastase, have comparable roles in the mortality associated with Pseudomonas burn-wound infections by strain PAO. Furthermore, the relative contribution of toxin A to virulence varies with the strain used. Thus, toxin A appears to make a larger contribution to virulence of strain PA103 than to PAO (Table 1 and Fig. 1).

Elongation factor-2 (EF-2) in the livers of infected mice was extracted and quantitated as a measure of in vivo toxin A production. Mice were infected with a lethal dose of bacteria (3.0×10^4 for

Fig. 1. Lethal dose response of burned mice infected with PA103-0 and PA103-29.

MORTALITY OF INFECTED BURNED MICE

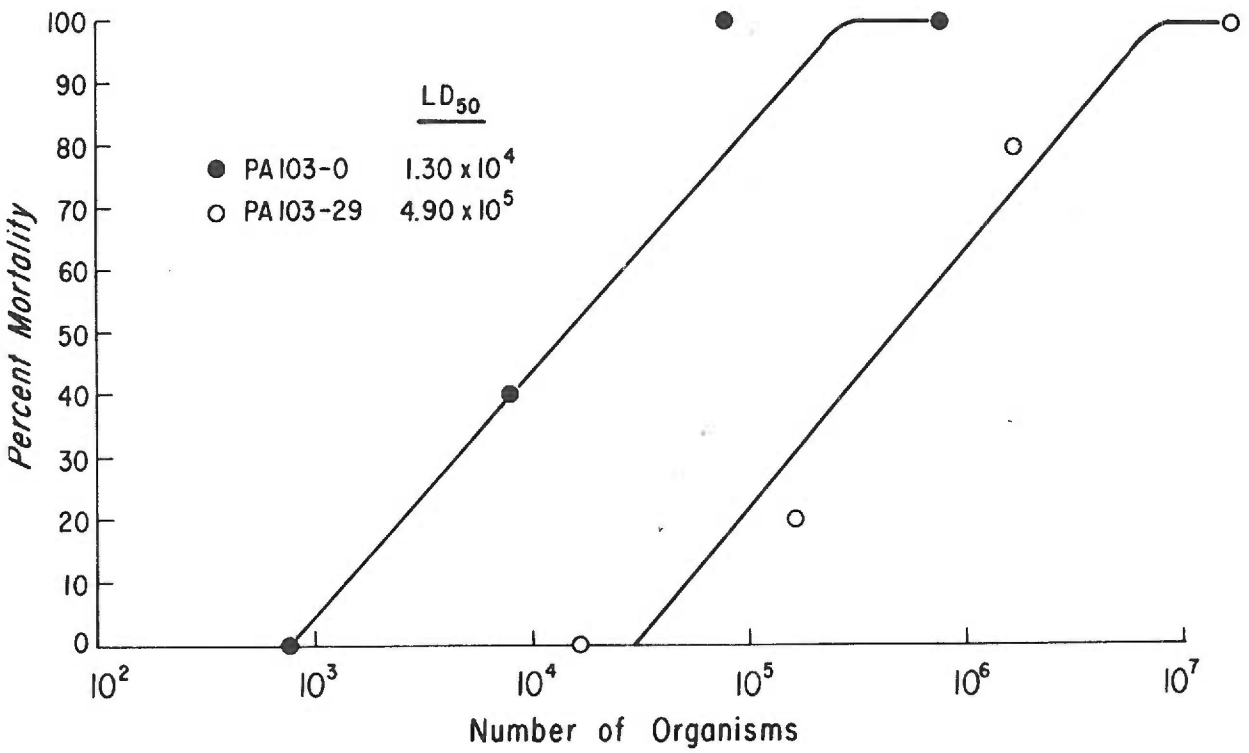


Table 1. LD_{50} of P. aeruginosa PAO strains in burned mice[a]

Strain	LD_{50} (log_{10}) Expt. no. 1	2	3	4	Average ± SEM
PAO1	4.5	4.0	3.6	3.6	3.9 ± 0.2
PAO-T1	5.1	4.9	4.3	ND	4.8 ± 0.2
PAO-E64	4.9	5.1	ND[c]	4.6	4.9 ± 0.1

[a]Serial 10-fold dilutions of bacterial cells were used. Each dilution was injected into five mice.

[b]SEM, Standard error of the mean.

[c]ND, not done.

PA103-0; 1.2 x 10^7 for PA103-29). At 20, 27, 42 and 50 h after infection, mice were sacrificed by cervical dislocation, livers were excised, rinsed and stored at -70° C until tested. EF-2 was extracted from liver homogenates as previously described (4). EF-2 was measured as previously described (4) by incubation with diphtheria toxin fragment A (provided by R.J. Collier, University of California at Los Angeles) and [^{14}C] NAD; the radiolabeled adenosine diphosphate-ribosyl (ADPR)-EF-2 formed was precipitated with trichloroacetic acid and counted. All assay were done in duplicate and averaged. The difference between the counts with and without diphtheria toxin fragment A represented the ADPR-EF-2 formed. The active EF-2 content of each tissue was calculated as previously described (2).

Pavlovskis et al. (7) have shown that the organ with the most significant decrease in EF-2 activity due to toxin A is the liver. The levels of active EF-2 in liver tissue during infections with PA103-0 and PA103-29 were compared. As early as 20 h after infection with strain PA103-0, the liver contained approximately one-half the EF-2 activity measured in normal mice (Table 2). In contrast, the liver EF-2 activity in mice infected with the toxin A mutant (PA103-29) was not reduced even 50 h after infection (Table 2). The liver EF-2 levels in PA103-29 infected mice were actually higher than the control level. It has been observed that the EF-2 levels of burned-noninfected mice are also higher than those obtained with normal, nontraumatized mice (O.R. Pavlovskis, personal communication). The data indicate that strain PA103-29 is toxin A deficient both in vivo and in vitro. Furthermore, isolates of PA103-29 recovered from the blood of

Table 2. Active EF-2 content of livers of burned mice infected with P. aeruginosa PA103-0 and PA103-29[a]

Strain	nMoles EF-2/g liver at hours after infection[a]			
	20	27	42	50
PA103-0	0.89 ± 0.10 (52)	0.97 ± 0.25 (56)	0.92 ± 0.25 (53)	0.98 ± 0.20 (57)
PA103-29	1.96 ± 0.13 (114)	2.11 ± 0.10 (123)	2.13 ± 0.21 (124)	2.09 ± 0.09 (122)

[a]EF-2 values (nanomoles/g of tissue ± standard error of mean) represent the mean of 6 mice infected with 3.0×10^4 bacteria of PA103-0 or 1.2×10^7 bacteria of PA103-29.

[b]Parentheses indicate per cent of EF-2 content in the livers of normal mice (1.72 ± 0.10).

infected animals at 42 h retained the toxin A deficient phenotype when reexamined *in vitro* which confirmed that its toxin A mutation was stable. These data also confirmed that the reduced EF-2 activity which accompanied infection with strain PA103-0 was due to toxin A and not some other bacterial product produced *in vivo* during the infection (7).

Previous studies indicated that toxin A is probably not the sole virulence factor of *P. aeruginosa* since specific antitoxin did not offer complete protection in the burned mouse model infection (8). This study provides direct evidence that toxin A and elastase are virulence factors expressed in burned animals.

References

1. Feigen, R.D. and Shearer, W.T. 1975. Opportunistic infection in children. II. In the compromised host. J. Pediatr. 87:677-694.

2. Gill, D.M. and Dinius, L.L. 1973. The elongation factor 2 content of mammalian cells. Assay method and relation to ribosome number. J. Biol. Chem. 248:654-658.

3. Holder, I.A. and Haidaris, C.G. 1979. Experimental studies of the pathogenesis of infections due to Pseudomonas aeruginosa: extracellular protease and elastase as in vivo virulence factors. Can. J. Microbiol. 25:593-599.

4. Iglewski, B.H., Liu, P.V. and Kabat, D. 1977. Mechanism of action of Pseudomonas aeruginosa: adenosine diphosphate-ribosylation of mammalian elongation factor 2 in vitro and in vivo. Infect. Immun. 15:138-144.

5. Liu, P.V. 1974. Extracellular toxins of Pseudomonas aeruginosa. J. Infect. Dis. 130:S94-S99.

6. Nathan, P., Holder, I.A. and MacMillan, B.G. 1973. Burn wounds: microbiology, local host defenses and current therapy. Crit. Rev. Clin. Lab. Sci. 4:61-100.

7. Pavlovskis, O.R., Iglewski, B.H. and Pollack, M. 1978. Mechanism of action of Pseudomonas aeruginosa exotoxin A in experimental mouse infections: adenosine diphosphate ribosylation of elongation factor 2. Infect. Immun. 19:29-33.

8. Pavlovskis, O.R., Pollack, M., Callahan, L.T., III and Iglewski, B.H. 1977. Passive protection by antitoxin in experimental Pseudomonas aeruginosa burn infections. Infect. Immun. 18: 596-602.

9. Pavlovskis, O.R. and Wretlind, B. 1979. Assessment of protease (elastase) as a Pseudomonas aeruginosa virulence factor in experimental mouse burn infection. Infect. Immun. 24:181-187.

10. Snell, K., Holder, I.A., Leppla, S.A. and Saelinger, C.B. 1978. Role of exotoxin and protease as possible virulence factors in experimental infections with Pseudomonas aeruginosa. Infect. Immun. 19:839-845.

11. Stiertz, D.D. and Holder, I.A. 1975. Experimental studies of the pathogenesis of infections due to Pseudomonas aeruginosa: description of a burned mouse model. J. Infect. Dis. 131: 688-691.

Appendix B: Construction and Initial Characterization of a Defined Medium for the Production of Toxin A.

Toxin A is not produced constitutively by P. aeruginosa, and its synthesis is apparently distinct from bulk protein synthesis. It is likely that specific regulatory systems control the synthesis of toxin A, but little is known about its regulation. To obtain toxin A, P. aeruginosa is typically cultured in a dialysate of Trypticase soy broth, and Liu (7) showed that toxin A production was greatly enhanced by growth at 32° C with maximum aeration and the addition of glycerol and L-glutamate (TSBD). Bjorn et al. (1, 2) showed that increasing iron concentrations inhibit toxin A yields.

To study the factors involved in the induction and repression of a gene product, like toxin A, it is preferable to study the organisms under defined conditions. Medium TSBD has been the only medium known for toxin A cultivation, but its use prevents defined conditions. Thus, it is advantageous to develop a medium which is chemically defined and permits high yields of toxin A by P. aeruginosa.

I began the construction of a defined medium with the observation that P. aeruginosa PA103 grown in the minimal medium of Vogel and Bonner (8) would produce toxin A if glycerol and L-glutamate were present. Toxin A yields were measured in the ADPR-transferase assay (Paper 1). This minimal medium was used for mapping studies (Papers 2 and 3) and contains only 5 original components (citrate, glucose, $NaNH_4HPO_4$, K_2HPO_4 and $MgSO_4$). The $NaNH_4HPO_4$ was replaced with

NaH_2PO_4 and NH_4Cl so that the effects of PO_4 and NH_4 ions could be tested separately. Each component was tested at varying concentrations for maximum toxin A yields by strain PA103. The first medium constructed, TMM1, contained 208 mM citrate; however, toxin A yields were improved by substituting 110 mM succinate. This medium, TMM2, is detailed in Table 1. When strain PA103 was used to compare TMM2 to TSBD, relative toxin A concentrations in culture supernants of the 2 media were usually very similar. (See Paper 1 for toxin A production by PA103 in TSBD).

Alterations in TMM2 with respect to the carbon sources greatly influenced toxin A yields. Marked increases in toxin A yields were seen when the glycerol concentration was increased stepwise from 1 to 8%. Toxin A yields increased by 50% when the succinate concentration was increased from 20 to 110 mM. When succinate (110 mM) was replaced with glucose (50 and 100 mM), toxin A yields fell by 50% or more. Pseudomonas is dependent upon the tricarboxylic acid cycle for energy (3); thus carbon sources like succinate, citrate and glycerol are easily metabolized and promote a rapid growth rate. Glucose is known to be a poor growth substrate for Pseudomonas (3). Thus, toxin A production appears to be associated with a rapid rate of growth.

Glutamate was the only amino acid tested, but it produced high yields of toxin A and is also part of the formulation of TSBD (7). Toxin A yields were not significantly different when glutamate concentrations ranged from 2 to 9 mM; however, above and below these values toxin A yields decreased. Toxin A production was only reduced by 25% when glutamate was the only nitrogen source in the medium, but

Table 1. Defined media, TMM2 and T8S, for the production of toxin A by P. aeruginosa PA103.[a]

TMM2		T8S	
Component	Molarity ($\times 10^{-3}$)	Component	Molarity ($\times 10^{-3}$)
	Basal Salts		
$NaH_2PO_4 \cdot H_2O$	3.7	Na_2HPO_4	1.4
K_2HPO_4	10.3	K_2HPO_4	14.0
NH_4Cl	93.4	NaCl	42.7
		KCl	33.5
	Metals		
$MgSO_4 \cdot 7H_2O$	0.4	$MgSO_4 \cdot 7H_2O$	2.9
$FeSO_4 \cdot 7H_2O$	(0.08 µgFe/ml)	$MnCl_2 \cdot 4H_2O$	0.2
		$FeSO_4 \cdot 7H_2O$	(0.05 µgFe/ml)
	Amino Acids		
L-glutamate	5.0	L-arginine	16.0
		L-aspartate	13.0
		L-alanine	6.0
	Carbon Sources		
Succinate	110.0	Succinate	21.0
Glycerol	(8.0% v/v)	Glycerol	(1.0% v/v)

[a]Components without $FeSO_4$ were adjusted to pH 6.5 and sterilized by membrane filtration (0.45 µ, Millipore). $FeSO_4$ was added from sterile stock solutions made daily.

was reduced at least 90% when NH_4Cl was the only nitrogen source. Thus, an organic source of nitrogen appears to be important to toxin A synthesis.

A phosphate buffer (NaH_2PO_4 and K_2HPO_4) at pH 6.5 was used as the PO_4 source, and toxin A yields were not significantly different between concentrations of 15 to 400 mM. A low phosphate concentration was chosen (15 mM) because iron is bound by PO_4 which could interfere with future iron regulation studies.

The effect of iron on toxin A yields by strain PA103 was tested in TMM2. Relative toxin A yields were found to increase as iron was added at low concentrations: 0.01 µg Fe/ml (273 CPM), 0.02 µg Fe/ml (439 CPM), and 0.05 µg Fe/ml (1,200 CPM). Relative toxin A values (CPM) were obtained in a 5 min ADPR transferase assay. Later studies indicated that 0.08 µg Fe/ml added to the medium produced a peak of toxin A activity. Further increases in the concentration of iron to 5.0 µg Fe/ml had no significant effect on toxin A yields. This result was opposed to the studies of Bjorn et al. (1, 2) which showed that when the iron concentration of TSBD was only increased from 0.05 to 0.15 µg Fe/ml, toxin A yields by strain PA103 were greatly reduced.

It was possible that the absence of the iron inhibitory effect on toxin A production in TMM2 was due to a reduced uptake of iron in this medium. However, this seems unlikely because the cell densities (OD_{540}) of stationary phase cultures increased with added iron: no added iron (OD_{540} 4.8), 0.08 µg Fe/ml (OD_{540} 8.0), and 5.0 µg Fe/ml (12.0). A recent study (6) showed that transport into the cell of amino acids was enhanced by glucose, malate and Na^+. However, when

these and K^+ were added to TMM2, separately or in combination, excess iron was still not inhibitory to toxin A yields.

Concurrent to these studies, another defined medium was independently constructed by DeBell (4) which used glucose and glycerol as major carbon sources. He later modified this formulation, replacing glucose with succinate, to enhance toxin A yields (R. Debell, personal communication). This latter medium, T8S (Table 1), does show the inhibitory effect of iron on toxin A production (Table 2) as is seen in TSBD (1, 2). T8S was used in iron regulation studies in Papers 1 and 2 of this thesis.

The media TMM2 and T8S have many similarities. Both contain basic salts, metals, amino acids, succinate and glycerol. Most importantly, both contain a combination of components that induced P. aeruginosa to synthesize toxin A. However, repression of the synthesis of toxin A by iron was observed when the bacteria were grown in T8S (as well as TSBD), but not in TMM2. Thus, under defined conditions, induction and repression of toxin A synthesis have been separated. While a definition of the factors necessary for the repression of toxin A by iron is still not clear, it must lie in the differences between the 2 media. As stated above, Na^+ and K^+, which are found at higher concentrations in T8S, do not affect iron repression in TMM2. The presence of Mn^{++} and several other metals also had no effect on iron inhibition of toxin A yields.

Two differences between TMM2 and T8S remain to be tested for their influence on the regulation of toxin A by iron. First, the choice of amino acids in T8S, or simply the higher concentration of

Table 2. Effect of iron on toxin A yields in TMM2 and T8S cultures of P. aeruginosa PA103

	Relative toxin A (cpm)[a]	
Medium	Low iron[b]	High iron[b]
TMM2	1,903	2,200
T8S	1,569	270

[a]Toxin A measured in a 5 min ADPR transferase system (Paper 1).

[b]Both media contain approximately 0.03 µg Fe/ml to which was added 0.08 µg Fe/ml (TMM2) or 0.05 µg Fe/ml (T8S) for low iron media; 5.0 µg Fe/ml was added to both for high iron.

organic nitrogen, may be important in the regulation of toxin A by iron. Second, the carbon sources (succinate and glycerol) are at a much higher concentration in TMM2 than T8S. This may be significant in that substrates, like succinate which support a rapid growth rate in Pseudomonas are known to suppress intracellular levels of 3',5'-cyclic adenosine monophosphate (c-AMP), and c-AMP levels are known to regulate inducible enzymes (5, 9). Thus, a certain level of c-AMP, which may be found in T8S but not in TMM2 grown P. aeruginosa may be required to interact with excess iron to repress toxin A synthesis. These possibilities can be experimentally tested and the results may help clarify the regulation of toxin A production by iron.

References

1. Bjorn, M.J., Iglewski, B.H., Ives, S.K., Sadoff, J.C. and Vasil, M.L. 1978. Effect of iron on yields of exotoxin A in cultures of Pseudomonas aeruginosa PA103. Infect. Immun. 19:785-791.

2. Bjorn, M.J., Sokol., P.A. and Iglewski, B.H. 1979. Influence of iron on yields of extracellular products in Pseudomonas aeruginosa cultures. J. Bacteriol. 138:193-200.

3. Clarke, P.H. and Ornston, L.N. 1975. Metabolic pathways and regulation: I., p. 191-261. In P.H. Clarke and M.H. Richmond (eds.), Genetics and Biochemistry of Pseudomonas. John Wiley and Sons, New York.

4. DeBell, R.M. 1979. Production of exotoxin A by Pseudomonas aeruginosa in a chemically defined medium. Infect. Immun. 24: 132-138.

5. Emmer, M., DeChrombrugghe, B., Pastan, I. and Perlman, R. 1970. The cyclic AMP receptor protein of E. coli: Its role in the synthesis of inducible enzymes. Proc. Natl. Acad. Sci. USA 66: 480-487.

6. Hoshino, T. and Kageyama, M. 1979. Sodium-dependent transport of L-leucine in membrane vesicles prepared from Pseudomonas aeruginosa. J. Bacteriol. 137:73-81.

7. Liu, P.V. 1973. Exotoxins of Pseudomonas aeruginosa. I. Factors that influence the production of exotoxin A. J. Infect. Dis. 128:506-513.

8. Vogel, H.J. and Bonner, D.M. 1956. Acetylornithinase of Escherichia coli: partial purification and some properties. J. Biol. Chem. 218:97-106.

9. Zubzy, G., Schwartz, D. and Beckwith, J. 1970. Mechanism of activation of catabolite-sensitive genes: a positive control system. Proc. Natl. Acad. Sci. USA 66:104-110.

V. Discussion and Summary

The disease manifestations of P. aeruginosa infection are extremely varied and depend upon the site of infection and underlying disease state of the host (3). P. aeruginosa is an opportunistic pathogen which attacks individuals whose defenses are compromised. Following a corneal wound, P. aeruginosa infection of the human cornea has a rapid course of destruction which usually results in loss of vision. To the patient with extensive burns, P. aeruginosa infection frequently results in bacteremia which has a 70 to 80% mortality rate (3). The therapeutic control of both diseases is currently very difficult because of the antibiotic resistance of P. aeruginosa strains. The best clinical approach to the prevention and control of these P. aeruginosa infections will probably depend upon an understanding of this organism's pathogenic properties.

P. aeruginosa produces a variety of extracellular products which may play a significant role in the pathogenesis of Pseudomonas infections (9). Among these factors, toxin A and the proteases are likely candidates to be major virulence factors of P. aeruginosa (9). The biological activities of these products have been determined. Toxin A inhibits protein synthesis in susceptible eucaryotic cells by catalyzing the transfer of the adenosine diphosphate ribosyl (ADPR) moiety of nicotinamide adenine dinucleotide (NAD) onto elongation factor 2 (EF-2) (5, 6). Toxin A is lethal to experimental animals (9) and cytotoxic to a variety of tissue culture cells (10, 13). The proteases of P. aeruginosa, alkaline protease and elastase,

had a low toxicity (15) but following injection into animals they caused rapid dermonecrosis (7) and ulceration of the cornea (8), depending upon the site of injection. P. aeruginosa purified proteases also enhanced the rate of multiplication in burn skin extracts (2). Other studies indicated a possible synergistic effect upon toxin A by purified Pseudomonas proteases (12). However, the role and interaction of these factors when produced by the organisms in vivo have not been clarified.

A great deal of study has gone into the analysis of the potential virulence factors of P. aeruginosa to determine their biological properties both in vitro and in vivo. More recently, investigators have compared independently isolated strains (which possess various combinations of pathogenic properties) in experimental infections in an effort to correlate a property of the organism with virulence. However, such a correlation is complicated by the fact that P. aeruginosa clinical isolates are very heterogeneous with respect to the production of many potential virulence products. This reflects that fact that independently isolated strains of P. aeruginosa must have genetic differences.

Although genetics and pathogenesis are both recognized as important disciplines in microbiology, there are few systems in which genetic techniques have been used to investigate the pathogenic mechanisms of bacteria. When genetic techniques are used to complement other experimental methods for the study of pathogenesis, the role of a potential virulence factor in disease can be evaluated

(4). This thesis describes a system where genetically related strains are compared in actual infections which mimic human Pseudomonas diseases in order to directly determine the contribution of toxin A and elastase to the pathogenesis of P. aeruginosa infection.

This study of the genetics of toxin A and elastase was based on the fact that the ability of a bacterium to produce a specific product can be changed by mutation. Mutations were chemically induced in two virulent strains: PAO1 (toxin A^+ elastase$^+$) and PA103 (toxin A^+ elastase$^-$). The assay developed to identify toxin A mutants (from both strains) detected their failure to show precipitation with specific antibodies (Papers 1 and 2). The assay developed for elastase mutants (from strain PAO1) detected loss of enzymatic activity (Paper 3).

The production of toxin A and elastase is subject to regulatory mechanisms, and maximal yields of each were produced only under specific conditions (Papers 1, 2, and 3; appendix B). It was important, therefore, to distinguish between mutations which specifically affect toxin A or elastase and those which affect extracellular products in general. These studies showed that several classes of toxin A mutants exist, both specific and nonspecific for toxin A. Likewise, mutations could affect elastase specifically or show pleiotropic changes in various phenotypic properties including the activities of other extracellular proteins. The data in this thesis on the characterization of the mutants indicated that common regulatory mechanisms exist for the synthesis and/or release of toxin A, proteases and other extracellular proteins. Among the mutants

isolated, 95% (15/16) of the PA103 toxin A mutants were detectably protease deficient, 64% (16/25) of the PAO toxin A mutants were elastase deficient, and 97% (73/75) of the PAO elastase mutants were toxin A deficient. It was very unlikely that such a high frequency of pleiotropic changes was due to multiple-hit mutations. Furthermore, I showed that at least 4 classes of toxin A mutants exist (Paper 1). This indicated that a number of genes coregulate toxin A and protease synthesis and/or release. Future studies may utilize these mutants in the identification of the regulatory steps in toxin A and elastase production.

The preliminary characterization of a defined medium (TMM2) for toxin A production may contribute to the elucidation of the factors that induce and repress toxin A synthesis (appendix B). The ability to induce P. aeruginosa to produce high yields of toxin A in this medium, without the repression associated with high-iron concentrations, indicates that the regulation of the expression of the tox structural gene is more complex (and perhaps more intriguing) than previously realized. Further characterization of medium TMM2 may clarify the environmental factors necessary for the induction and repression of toxin A synthesis. This information may suggest the isolation of mutant types that could be used to elucidate the regulatory scheme of toxin A synthesis. Since antibiotics are too often ineffective in controlling P. aeruginosa infections, understanding the regulation of toxin A synthesis may lead to the discovery of selective metabolic inhibitors which prevent the synthesis and/or release of toxin A in vivo.

The extensive characterization of the toxin A and elastase mutants, in relation to the parental strains, examined properties which included metabolic pattern, growth rate, epidemiological markers, extracellular enzyme reactions and the profile of all extracellular proteins. The results indicated that mutants PAO-T1 and PA103-29 were identical to their parental strains except for a marked deficiency in the production of toxin A (Papers 1 and 2), and mutant PAO-E64 was identical to parental strain PAO1 except that it produced an altered elastase with markedly reduced enzymatic activity.

Mapping studies could not locate the toxin A (tox-1) and elastase (lasA-1) mutations in strain PAO within approximately the first half of the chromosome (Papers 2 and 3). The technology to examine the remainder of the chromosome is not yet available. But it should be possible to construct an FP plasmid which is capable of transferring the region of the chromosome beyond 55 min. This might be accomplished by inserting into an FP plasmid a DNA segment (e.g., transposon) which has homology with a late region segment of the chromosome and would thus initiate chromosomal transfer from that site. It is also possible that tox-1 and/or lasA-1 are located on a plasmid or bacteriophage instead of the chromosome. These latter possibilities seem unlikely because (i) random transfer of these markers did not occur during conjugation (Papers 2 and 3), (ii) both toxin A (1) and elastase (14) are produced by the vast majority of P. aeruginosa strains (iii) there are no reports of a P. aeruginosa strain being cured of these characteristics, and (iv) a strain

which contains no detectable plasmids produces toxin A and elastase (Iglewski, B.H., unpublished observations).

The 3 mutants which showed a deficiency in only toxin A or elastase were compared to the parental strains in the mouse corneal infection model which mimics the disease seen in humans (Paper 4). Both toxin A mutants showed a decrease in virulence which indicates that toxinA is a virulence factor of P. aeruginosa in corneal infections. Toxin A mutants produced infections that were less severe and were not able to persist in the eyes as long as the parental strains. However, toxin A mutants were able to establish transient infections.

Thus, the roles of toxin A in Pseudomonas corneal disease include (i) damaging corneal tissue, and (ii) prolonging the time in which the organisms can multiply in the presence of host's bacterial clearance mechanisms. This latter property of toxin A may be the most important in terms of P. aeruginosa pathogenesis. The interference of host defense by toxin A would allow the organisms to produce tissue damaging enzymes (e.g., toxin A, proteases, hemolysins, etc.) for a longer period of time, and also implies that bacterial cell densities at the site of infection may be higher than with nontoxigenic mutants. Thus, all extracellular (toxic) products and LPS probably reach much higher concentrations in vivo when toxin A is produced. The mechanism by which toxin A affected host defense was not determined. However, the high level of cytotoxicity observed with toxin A in vitro (10, 13) suggests that both mechanical barriers and cellular phagocytic defenses could be affected.

The corneal infection produced by the elastase mutant (PAO-E64) was not significantly different from that of the parental strain indicating that the parental level of elastase activity was not required for virulence (Paper 4). However, this mutant continues to produce alkaline protease which may play a role in corneal infection. To determine if protease activity contributes to the virulence of P. aeruginosa, alkaline protease mutants could be isolated from elastase mutant PAO-E64 and from strain PA103-0, and then tested in the corneal infection model for loss of virulence compared to the parental strains. A possibility that I cannot discount is that the low level of enzymatic activity possessed by the altered elastase of PAO-E64 is sufficient for the normal disease process.

Because the mutation in PAO-E64 appears to be in the structural gene for elastase, this mutant may become very important in the study of the genetics and molecular biology of Pseudomonas elastase. Purification and characterization of the mutant elastase may provide information as to structure-function relationships of this enzyme.

Neither elastase or toxin A appeared to have a role in the colonization of the traumatized corneas. Thus, toxin A and elastase are not colonization factors. The factors necessary for the establishment of corneal infections are unknown. A number of independently isolated strains that possessed high levels of toxin A and protease activity were deficient in establishing corneal disease (Paper 4). Thus, such colonization factors may be possessed by only certain strains of P. aeruginosa.

Initial burn-wound infection studies of the toxin A mutants (PA103-29 and PAO-T1) and elastase mutant (PAO-E64) indicate that both factors contribute to the pathogenesis of Pseudomonas burn infections (appendix A). As measured by the incidence of mortality in burned mice, both toxin A and elastase appear to be equally important. This study also confirmed that the reduced EF-2 levels found in the livers of P. aeruginosa infected burned mice were due to toxin A and not some other bacterial product produced in vivo during infection (11). These studies will be continued to determine the effect of liver EF-2 levels following infections with mutants PAO-T1 and PAO-E64.

This study utilizing genetic techniques has offered convincing evidence for contribution to virulence by toxin A in corneal and burn infections and elastase in burn infections. The mutants which have been described could be utilized in other experimental models of disease such as Pseudomonas pneumonia, cystic fibrosis, vasculitis, and infections of the immunodeficient. The methods developed here could be adapted to study mutations affecting other potential virulence factors such as alkaline protease and hemolysins. As this research has shown, a genetic approach to understanding pathogenic mechanisms not only offers evidence for the contribution of a given bacterial product to virulence, but can suggest the probable role(s) of a factor during disease.

References

1. Bjorn, M.J., VAsil, M.L., Sadoff, J.C. and Iglewski, B.H. 1977. Incidence of exotoxin production by Pseudomonas species. Infect. Immun. 16:362-366.

2. Cicmanec, J.F. and Holder, I.A. 1979. Growth of Pseudomonas aeruginosa in normal and burned skin extract: role of extracellular proteases. Infect. Immun. 25:477-483.

3. Doggett, R.G. (ed.). 1979. Pseudomonas aeruginosa: clinical manifestations of infection and current therapy. Academic Press, New York.

4. Holmes, R.K. 1975. Genetic aspects of toxinogenesis in bacteria. p. 296-301. In D. Schlessinger (ed.), Microbiology-1975. American Society for Microbiology, Washington, D.C.

5. Iglewski, B.H. and Kabat, D. 1975. NAD-dependent inhibition of protein synthesis by Pseudomonas aeruginosa toxin. Proc. Natl. Acad. Sci. USA 72:2284-2288.

6. Iglewski, B.H., Liu, P.V. and Kabat, D. 1977. Mechanism of action of Pseudomonas aeruginosa exotoxin A: adenosine diphosphate-ribosylation of mammalian elongation factor 2 in vitro and in vivo. Infect. Immun. 15:138-144.

7. Kawaharajo, K., Homma, J.Y., Aoyama, Y., Okadao, K. and Morihara, K. 1975. Effects of protease and elastase from Pseudomonas aeruginosa on skin. Jpn. J. Exp. Med. 45:79-88.

8. Kreger, A.S. and Gray, L.D. 1978. Purification of Pseudomonas aeruginosa proteases and microscopic characterization of

pseudomonal protease-induced rabbit corneal damage. Infect. Immun. 19:630-648.

9. Liu, P.V. 1973. Exotoxins of Pseudomonas aeruginosa. I. Factors that influence the production of exotoxin A. J. Infect. Dis. 128:506-513.

10. Middlebrook, J.L. and Dorland, R.B. 1977. Reponse of cultured mammalian cells to the exotoxins of Pseudomonas aeruginosa and Corynebacterium diphtheriae: differential cytotoxicity. Can. J. Microbiol. 23:183-189.

11. Pavlovskis, O.R., Iglewski, B.H. and Pollack, M. 1978. Mechanism of action of Pseudomonas aeruginosa exotoxin A in experimental mouse infections: adenosine diphosphate ribosylation of elongation factor 2. Infect. Immun. 19:29-33.

12. Snell, K., Holder, I.A., Leppla, S.A. and Saelinger, C.B. 1978. Role of exotoxin and protease as possible virulence factors in experimental infections with Pseudomonas aeruginosa. Infect. Immun. 19:839-845.

13. Vasil, M.L. and Iglewski, B.H. 1978. Comparative toxicities of diphtherial toxin and Pseudomonas aeruginosa toxin A: evidence for different cell receptors. J. Clin. Microbiol. 108: 333-337.

14. Wretlind, B., Heden, L., Sjöberg, L. and Wadström, T. 1973. Production of enzymes and toxins by hospital strains of Pseudomonas aeruginosa in relation to serotype and phage-typing pattern. J. Med. Microbiol. 6:91-100.

15. Wretlind, B. and Wadström, T. 1977. Purification and properties of a protease with elastase activity from Pseudomonas aeruginosa. J. Gen. Microbiol. 103:319-327.

VII. Acknowledgements

Many thanks go to my major advisor, Dr. Barbara Iglewski, whose scientific insight was surpassed only by tremendous patience.

I thank the members of my thesis committee, Drs. B. Iglewski, Hallick, Leslie and Bigley for their helpful advice.

I would like to thank the members of the laboratory during my graduate studies for their friendship, help and counsel: Drs. Michael Bjorn, Stanley Cryz, Richard Freidman, Michael Vasil and Michael Thompson, as well as Pam Sokol, Tim Howe, John Bradley, Jack Lile, David Oldenburg and Sophia Chung-Fegan.

Lonnie Prather is thanked for her expert typing.

I would like to give my wife, Marianne, very special thanks for her sacrifices, support, interest and contributions which helped make this work possible.