GENETIC ANALYSIS OF

PSEUDOMONAS AERUGINOSA VIRULENCE FACTORS

by

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I. Introduction and Statement of the Problem

P.aeruginosa is a ubiquitous microorganism, which can be isolated from the soil, water, plants, and in every part of the human habitat. P.aeruginosa has emerged as a major pathogen, in part due to the widespread use of antibiotics. An opportunist, this pathogen can establish severe, life-threatening infections in patients compromised by anergy or immunosuppression, neoplasia, thermal injury, or chronic pulmonary disease such as cystic fibrosis.

Renewed interest in P.aeruginosa has led to the identification of several potential determinants of virulence (virulence factors), including exotoxin A, elastase, alkaline protease, hemolysin, and alginate. These potential virulence factors have been the subject of intensive investigation by several groups, but the precise role of these factors in the various P.aeruginosa infectious processes remains unclear.

One approach to determine the role of various factors in disease involves the isolation and characterization of mutants which are altered in the production or activity of individual factors of interest. By examining these mutants in appropriate animal models, and comparing their virulence to the virulence of the wild-type organisms from which they were derived one can determine the role of individual factors in disease.

The objective of this study is to undertake a genetic analysis of the toxin and the two major proteases produced by P.aeruginosa, elastase and alkaline protease. The specific aims of this research are:

i. Isolate mutants deficient in alkaline protease and characterize these mutants *in vitro*.

ii. Examine alkaline protease mutants in the mouse eye model to determine directly the role of *P.aeruginosa* proteases in this disease state.

iii. Determine the genetic locus of the elastase structural gene.

iv. Genetically characterize the toxin-deficient mutant PAO-T1.

II. Literature Review

A. General Characteristics of P.aeruginosa

P.aeruginosa is an obligate aerobe, although in the absence of oxygen it can respire with nitrate as the terminal electron acceptor (35). This feature distinguishes Pseudomonas from the majority of human pathogens, which tend to be facultative anaerobes (35). A typical pseudomonad, P.aeruginosa has an enormous metabolic potential and can utilize at least eighty organic compounds, including carbohydrates, amino acids, alcohols, amides, amines, and fatty acids (61). The tricarboxylic acid cycle is of primary importance in the catabolic and biosynthetic activities of P.aeruginosa (35). The respiratory dissimilation of glucose and other hexoses is accomplished almost exclusively through the Entner-Doudoroff pathway (36). The metabolic diversity, characteristic of pseudomonads, is attributable to the possession by P.aeruginosa of many specialized pathways, most of which converge on the tricarboxylic acid cycle (35).

The ultrastructure of Pseudomonas is similar to most gram-negative organisms, and consists of an inner membrane, a thin peptidoglycan layer, and an outer membrane containing lipopolysaccharide (233). The comparatively low endotoxic activity of Pseudomonas suggests that there may be significant differences in the lipopolysaccharide of this organism compared to other gram-negative bacteria (57). In addition, the characteristic antibiotic resistance of this organism (18), its sensitivity to EDTA and polymyxin (276), and its ability to secrete a variety of extracellular products (161,268) indicate that other differences exist between the envelope of this and other gram-negative bacteria.

Most strains of P.aeruginosa are motile, possessing a single polar flagellum (202). Genetic studies on flagellation have involved the isolation and characterization of flagellum-deficient mutants and multi-trichous variants (259). Genetic characterization of these mutants, involving FP5 and R68.45 plasmid-mediated conjugation, bacteriophage G101 transduction, and plasmid prime complementation revealed two distinct clusters of genes involved in flagellation, both of which are located at approximately 85' on the strain PAO chromosome (265,266).

In addition to a single flagellum, most strains of P.aeruginosa possess additional surface appendages in the form of pili or fimbrae (69). These pili may function as bacteriophage receptors (16), and may mediate adherence of P.aeruginosa to mucosal surfaces, a property discussed more thoroughly in the next section of this review.

Pigmentation is a common characteristic of strains of P.aeruginosa, most of which exhibit some pigmentation on agar media designed for optimal pigment production (137). At least three pigments have been described, including pyocyanine (a phenazine pigment), fluorescein, and pyomelanin (a melanin-like brown pigment) (157).

Antibiotic resistance is another widely accepted characteristic of P.aeruginosa. Resistance to antibiotics generally occurs through three mechanisms, acting singly or in concert. These mechanisms include alteration of the permeability barrier of the cell envelope, modification of the specific target of the antibiotic, or enzymatic inactivation of the antibiotic (17,18,58,225). Antibiotic-inactivating enzymes may be encoded by extrachromosomal elements (plasmids), certain of which have a wide host range and are readily transferred between strains of

P.aeruginosa and among many genera of pathogenic bacteria. These factors encode numerous antibiotic-modifying or inactivating enzymes, conferring resistance to antibiotics such as streptomycin, chloramphenicol, carbenicillin, tobramycin, amikacin, gentamicin, kanamycin, and neomycin (19,21,116,141). Certain of these resistance plasmids may also mediate changes in outer membrane permeability, conferring resistance to antibiotics in the absence of demonstrable antibiotic-inactivating enzymes (43).

That R plasmids are responsible for the general antibiotic resistance of P.aeruginosa is an attractive and widely held hypothesis, but this hypothesis appears untenable when the low frequency of distribution of R plasmids among clinical isolates is considered. R plasmids were demonstrable in only two percent of clinical isolates in Australia (45). In another study, plasmid-mediated resistance to gentamicin was examined at the University of Alberta. In that study, R factor-mediated resistance to gentamycin was observed in less than one percent of isolates examined (20). This latter study, involving the mechanism of resistance to one of the more clinically relevant antibiotics, demonstrated that alteration in the permeability of over 30% of these strains resulted in high level resistance to gentamicin. This indicates that exclusion is an effective mechanism of resistance. Studies in Japan have provided similar results (114,133), suggesting that this exclusion mechanism is a more important mechanism of resistance than is R plasmid-mediated antibiotic resistance in P.aeruginosa .

B. Genetics of P.aeruginosa

The metabolic diversity and medical importance of P.aeruginosa has stimulated considerable research on the genetics of this organism. The genome of P.aeruginosa consists of its chromosome and one or more extrachromosomal elements. The chromosome is a double-stranded molecule of DNA with a molecular weight of 2.1×10^9 (208). Circularity of the chromosome was recently established by demonstrating linkage of late and early markers in genetic exchange mediated by the conjugative plasmid R68.45 (230). Common extrachromosomal elements (plasmids) range in mass from $10\text{-}312 \times 10^6$ daltons, and include sex factors (FP, fertility plasmids), degradative plasmids, resistance (R) plasmids, and plasmids of unknown function (cryptic plasmids)(7,92). The fertility plasmids and certain of the R plasmids confer limited chromosome mobilizing ability (cma) (31,251).

Pseudomonas plasmids have been divided into ten incompatability groups (118). Plasmids of the Inc-P2 group are most frequently found in Pseudomonas, and have been detected in isolates from around the world (117). Plasmids of this group have a restricted host range, and have been associated with inhibition of bacteriophage B3, D3, E79, and G101 replication, inhibition of pyocin production, and with resistance to carbenicillin, gentamicin, tobramycin, and amikacin (32,117,118). The presence of these plasmids in clinical isolates could conceivably interfere with the epidemiological typing of strains based upon pyocin or bacteriophage typing, and may preclude the production in these strains of G101 bacteriophage suitable for generalized transduction. Similarly, the presence of Inc-P6 plasmids renders strains insusceptible to infection with the generalized transducing phage F116 (117).

Several sex factors have been identified in Pseudomonas, only three of which are routinely used in genetic analysis (64,99). In addition to conferring chromosome mobilizing ability, sex factors often confer resistance to ultraviolet light, and to chemical agents such as methylmethane sulfonate and nitrosoguanidine which produce single-strand breaks in DNA (145,255). The presence of these plasmids is also associated with an increased frequency of spontaneous and induced mutation. These observations suggest that these plasmids encode an auxilliary error-prone repair pathway, or in some way regulate the error-prone repair properties encoded on the bacterial chromosome (145,255).

Sex factors in Pseudomonas are not subject to restriction (97,101,250). This indicates that special structural features exist which render them resistant to the restriction enzymes, or that like several Pseudomonas bacteriophage (92,227) they do not acquire the host specificity of the strain in which they are grown. While the sex factors themselves are not restricted, the DNA which they introduce from the donor strain to the recipient strain is subject to restriction. Growth at 43°C for as few as five generations, however, results in an alteration of the host-controlled modification properties of the recipient. Once established, this alteration results in impairment of restriction and modification processes for up to sixty generations (228). The nature of this thermosensitive process is not understood, but can be exploited to promote successful interstrain recombination, enabling the genetic analysis of unrelated strains of P.aeruginosa.

The sex factors FP2 and FP39 have been isolated by ethidium bromide-cesium chloride gradient centrifugation and characterized

physically (209). Both plasmids are present at one to two copies per cell. The DNA of these plasmids differs significantly from Pseudomonas chromosomal DNA in terms of GC content (58 to 60 compared to 67% GC, respectively) . This suggests that these plasmids did not originate in P.aeruginosa, and may explain the limited origins of transfer in this organism. The sex factor FP2 belongs to incompatability group 8 (118), has a molecular weight of 59 X 10^6 and confers resistance to mercury and tellurite in addition to cma (252). The plasmid FP2 mediates polar chromosomal transfer in a clockwise direction from a single site, arbitrarily designated 0' on the strain PAO chromosome. Mapping studies in P.aeruginosa have traditionally involved the use of this sex factor. Polarity of transfer associated with the cma of this plasmid enables one to conduct interrupted mating experiments, facilitating the determination of gene location by the technique of gradient of transmission analysis (99). The major limitation of this sex factor is its single origin of transfer and its consequent inability to promote repair of late markers, presumably due to an inherrent instability of the FP2-encoded conjugal bridge. The sex factor FP39, of unknown incompatibility (118), confers cma from a position approximately 10' to the left of 0'(210). This plasmid shares the limitations discussed for FP2, but can mobilize very late genes. An additional limitation of FP39 is its lack of antibiotic or metal resistance determinants (210). This requires that selection for inheritance of this plasmid be based on complementation of mutations in genes involved in leucine synthesis, as this plasmid encodes as yet uncharacterized genes involved in leucine biosynthesis or regulation(210).

The sex factor FP110 confers cma from a position at 27' on the strain PAO chromosome (99). Like FP39, this plasmid confers no selectable resistance characteristics. Royle and Holloway have overcome this limitation by introducing the transposon Tn*1* encoding carbenicillin resistance, into FP110 (231). Using this plasmid derivative, they presented evidence that FP110 is related to plasmids of the Inc-P1 group, a group of plasmids of broad host range (231). This evolutionary relationship between the sex factor FP110 and the broad host range R plasmids is intriguing in light of the prevalence of FP-like plasmids in hospital isolates (46). This relationship has led to the suggestion that R factors may have evolved from FP factors, but there is no direct evidence to support that hypothesis.

The inability of any of the characterized sex factors to mediate transfer of distal markers has led to considerable research on other mechanisms of DNA transfer. Certain of the R plasmids, notably R68 and R91, promote chromosome transfer in strain PAT (251), but not in the genetically characterized strain PAO used for most genetic studies (94). The plasmid R68 is of incompatability group 1, and has recently been shown to be identical to the plasmids RP1, RP4, and RK2 (23). Variants of this plasmid were isolated which could promote chromosome mobilization in strain PAO. One variant, designated R68.45, was isolated following repair of an arginine mutation in strain PAO. Exconjugants isolated at the *argB* locus maintained plasmids of improved cma, while plasmids isolated from exconjugants resulting from repair at other sites retained the characteristic low level cma of R68 (78). It was subsequently established that plasmids with improved cma were larger than the original plasmid R68 (78). The prototype variant R68.45, for example, contains a

2.1 kilobase insert of DNA, originally designated IsP (insertion sequence Pseudomonas). It was initially assumed that the insertion sequence in R68.45 originated in the P.aeruginosa PAO chromosome near the argB locus, and that improved cma resulted from site-specific recombination between this insertion sequence on the plasmid and other insertion sequences distributed about the PAO chromosome. This model predicted a finite number of specific sites for R68.45-mediated chromosome transfer, a prediction which is inconsistant with the observed random nature of exchange mediated by this plasmid. This discrepancy led to a more thorough molecular characterization of R68.45, revealing that the additional DNA present in R68.45 resulted from a tandem duplication of a 1.8-2.1 kilobase fragment already present on the plasmid R68. This tandem duplication resulted in the construction in plasmid R68.45 of a functional transposable element, since designated Is21. The molecular basis for cma associated with the plasmid R68.45 is now thought to involve cointegrate formation and resolution during transposition of Is21 between the plasmid R68.45 and the P.aeruginosa chromosome (226).

The plasmid R68.45 mediates the non-polar transfer of DNA from virtually any site on the PAO chromosome. Chromosome mobilization occurs at a frequency of about 10^{-5} and typically involves the transfer of approximately 10' of chromosome (79). These features amply demonstrate the broad utility of this plasmid for genetic studies in P.aeruginosa. However, the random, non-polar nature of genetic exchange, and the inability to conduct interrupted matings with this plasmid makes the precise determination of gene location difficult, necessitating the determination of co-inheritance frequencies with several markers near the gene of interest.

No high frequency recombination (Hfr) systems exist for genetic exchange in P.aeruginosa (99). For this reason, a related system based upon transposon-facilitated recombination (Tfr) has been developed. Transposon-facilitated recombination is comparable to high frequency recombination in its polarity and efficiency of genetic exhange, and, in theory, provides for genetic exchange in any chosen direction from any position on the chromosome. Tfr systems utilize transposable elements as portable regions of homology between the chromosome and conjugative plasmid. They provide a means of constructing versatile, efficient donors from a given position by introducing transposons into that region of the chromosome. A tfr system has been used to construct improved genetic donors in V.cholerae (127), and was instrumental in mapping toxin regulatory mutations in that organism (172). Haas recently developed a tfr system in P.aeruginosa using the transposon Tnl (80). By allowing a temperature-sensitive derivative of the plasmid RP1 to insert into the trpA,B locus of the PAO chromosome, an hfr-like donor was generated which promoted efficient genetic exchange from trpA,B at 27'. Polar genetic exchange in a clockwise direction was observed. Derivatives of this strain were isolated in which the majority of the original plasmid was deleted, leaving the transposon Tnl and an undetermined amount of plasmid material behind. By introducing the plasmid RP1 into these strains, he obtained efficient, polar chromosome mobilization (80). Krishnapillai, et. al. (146) have since introduced the transposon Tnl in opposite orientations into several independent sites in the late region of the strain PAO chromosome. They further expanded the utility of this tfr system by including the plasmid R91-5. This plasmid contains a Tnl insert in an orientation opposite to that of RP1 (146). When introduced

into strains containing chromosomal Tn*1* inserts, the plasmids RP1 and R91-5 promote chromosome mobilization in opposite directions. The Tn*1* inserts constructed by Krishnapillai appear to be stable, and may be readily transferred from one strain to another by F116 bacteriophage-mediated transduction and selection for carbenicillin resistance. By choosing the appropriate bacteriophage lysate and conjugative plasmid, tfr donors can be constructed for efficient analysis of the late region of the strain PAO chromosome.

Another mechanism of genetic exchange in *P.aeruginosa* is transduction. Lysogeny is a common characteristic of *P.aeruginosa* (26,90,96,203) and several temperate bacteriophage have a demonstrated ability to promote genetic exchange (98). A few of these bacteriophage have been characterized, including B, B3, B110, F116, and G101 (100,102,162,163). All of these are generalized transducing phage: No evidence for specialized transduction exists for any strain of *P.aeruginosa*. These generalized transducing phage characteristically exhibit transducing frequencies of up to 10^{-5} per recipient (91,98). The phage F116 and G101 are most commonly used for recombinational analysis. These phage transduce up to 1-2% of the bacterial chromosome, corresponding to a DNA content for these phage of 40-50 X 10^6 daltons (102,162,163,244). The general properties of these phage and the techniques for their use are the same as with generalized transducing phage of *S.typhimurium* and *E.coli* (91). Their use in genetic studies in *Pseudomonas* is limited primarily by the availability of selectable markers for co-transduction. Another consideration regarding genetic studies which employ transducing phage involves the phenomenon of phage or lysogenic conversion (5,95). As with phenotypic changes which

accompany R plasmid content, lysogeny following transduction may result in considerable cell surface alterations and other unpredictable effects (95,155). The potential complication of phage conversion may be overcome by employing clear plaque variants of transducing phage which do not lysogenize the recipient, or by scoring for lysogeny prior to characterization of transductants (272).

A third mechanism of genetic exchange in P.aeruginosa is transformation. Transformation is the process whereby isolated DNA from one bacterium is taken up and incorporated into the genome of another bacterium. This process was first demonstrated in S.pneumonia by Griffith, and has since been observed in a variety of bacterial genera. This procedure may be used for mapping studies, and is most useful in studies involving recombinant DNA. In 1967, Kahn and Sen (128) reported transformation of P.aeruginosa with isolated chromosomal DNA. Competent recipients were obtained by cultivation to late exponential phase at 25-30°C. Transformation to gelatin-liquefaction (protease production) or the ability to synthesize isoleucine-valine (conversion of ilv-deficient mutants to prototrophy) was observed at a frequency of up to 4.8%. Many groups have since tried to repeat this work, but have been unsuccessful, presumably due to nuclease digestion of transforming DNA or lack of competence of recipients (94). Several groups, including Olsen and Hansen (199), Sano and Kageyama (234), and Sinclair and Morgan (243) have successfully transformed P.aeruginosa with plasmid DNA.

Genetic complementation studies in P.aeruginosa have been attempted using plasmid primes. Plasmid primes are plasmids which contain a section of the bacterial chromosome and and are stably maintained episomally. Plasmid primes may be considered in vivo cloned genes, and

as such are a potentially powerful tool in microbial genetics. Plasmid primes are necessary for transdominance testing of regulatory genes and provide an efficient means of mobilizing and manipulating genes of interest. Plasmid primes were instrumental in the development of the E.coli genetic system, and so their construction has been attempted in P.aeruginosa.

In P.aeruginosa, plasmid primes have been made almost exclusively with R plasmids, particularily with the plasmid R68.45 and the related plasmid R68.44. R prime plasmids have been constructed by three distinct strategies; intergeneric transfer to E.coli (86), interspecific transfer to P.putida (174), or by transfer to recombination-deficient strains of P.aeruginosa (93) . These strategies have inherent limitations which have precluded the use of plasmid primes in many genetic studies of P.aeruginosa. The strengths and limitations of these techniques should be more clear after a review of previous work.

Hedges, Jacob, and Crawford (86) isolated a derivative of the plasmid R68.44 which contained the trpA,B genes of P.aeruginosa and which were expressed in E.coli. In this study, plasmid primes containing the tryptophan biosynthetic genes were obtained by selecting for complementation of tryptophan auxotrophy in an E.coli recipient. The use of E.coli as a recipient in these studies was advantageous in circumventing problems associated with gene dosage, but was associated with a very low recovery of prime plasmids (10^{-11} /donor). To attempt to improve the frequency of isolation of plasmid primes, Holloway examined plasmid prime construction in recombination-deficient strains of P.aeruginosa. By mating P.aeruginosa containing the plasmid R68.45 with P.aeruginosa recipients containing rec-102 (a recA-like mutation),

plasmid primes were recovered at a considerably higher frequency than was reported with intergeneric construction (10^{-8}/donor cell) (93). Plasmid primes were constructed in this way by patch plate mating under non-selective conditions, followed by selection and maintainance on selective media. Transfer of the resultant plasmid primes to recombination positive recipients, or maintainance under non-selective conditions resulted in loss of chromosomal determinants and retention of plasmid markers. Transfer of plasmid primes to other recombination-deficient strains was very efficient (2×10^{-3}/donor). This strategy of plasmid prime construction is presently limited by the paucity of recombination-deficient strains of P.aeruginosa bearing selectable markers. Also, merodiploids in P.aeruginosa have been associated with severely impaired growth rates, rendering them unsuitable for most genetic studies.

Recently, plasmid primes have been isolated following interspecific mating. Morgan (174) mated P.aeruginosa donors containing the plasmid R68.45 with P.putida auxotrophic recipients, and selected for plasmid primes on minimal medium containing carbenicillin. Plasmid primes were isolated at a frequency of about 1×10^{-8}/donor, and contained approximately 4.5' of chromosomal material. The resultant plasmid primes were unstable even when transferred to recombination-deficient strains of P.aeruginosa , suggesting that impaired growth rates or gene dosage effects may be prohibitive with merodiploids containing certain parts of the P.aeruginosa chromosome. This strategy of plasmid prime construction is also limited by the paucity of P.putida recipients containing selectable markers.

Royle and Holloway (231) recently constructed plasmid primes by conjugation of P.aeruginosa donors containing the sex factor FP110 marked with Tnl with P.aeruginosa recombination deficient recipients. One of the plasmid primes constructed in this way was stable in a recombination proficient background, greatly increasing the potential utility of this approach.

To date, only the flagellation studies of Tsuda, et.al. (265) have exploited plasmid primes for genetic mapping and complementation studies. The limitations discussed previously are presently too severe to provide for efficient utilization of this technique in most genetic studies of P.aeruginosa products.

Cloning of P.aeruginosa genes has been attempted by several groups. Attempts to clone P.aeruginosa genes into E.coli have met with mixed success. Potential explanations for problems which have been encountered are not exclusive, and include differential GC content and associated problems with AT-rich E.coli promoters, lack of auxilliary metablolic pathways, improper processing or secretion of P.aeruginosa gene products in E.coli, or inherent instability of Pseudomonas genes in E.coli. A consideration of the experimental design of successful cloning endeavors suggests that the first and third possiblilities are likely.

Hedges, et.al. ,(86) cloned the Pseudomonas tryptophan synthase genes in E.coli by construction of plasmid primes. A recombinant plasmid was isolated from one of these prime plasmids which contained about 75 megadaltons of P.aeruginosa DNA . This plasmid was unstable, and expression of tryptophan genes in E.coli was very low. Stable

variants were obtained, however, which did support high expression of these enzymes (86).

Nakezawa, et.al. (191) utilized the technique of integrative suppression to construct recombinant plasmids containing toluate degrading genes. By constructing strains containing thermosensitive toluate degrading determinants on one plasmid, and non-thermosensitive drug resistance determinants on a second plasmid, they were able to isolate recombinants by selection for toluate utilization at the non-permissive temperature. These recombinants arose at a frequency of 4 X 10^{-8}/ donor, and conferred non-thermosensitive, non-inducible expression of toluate-degrading genes in Pseudomonas. Transfer of a typical recombinant to E.coli did not support the growth of E.coli on toluate agar, apparently due to low expression of these genes in E.coli. This speculative explanation is supported by the elegant experiments of Benson and Shapiro (7), in which tol plasmids were tagged with the carbenicillin resistance determinant Tn401 Tol plasmids containing Tn401 were transferred to E.coli and P.aeruginosa at a frequency of 4 X 10^{-7} and 4 X 10^{-5}, respectively. While both the tol and resistance determinants were expressed in P.aeruginosa, only the carbenicillin resistance determinant was expressed in E.coli. The authors demonstrated that the toluate-deficient phenotype of E.coli containing the recombinant plasmid was not due to a metabolic defect in the recipient strain, plasmid loss or deletion, or toxicity of m-toluate to E.coli, and concluded that P.aeruginosa tol genes were expressed poorly in E.coli (7). Apparently, this Pseudomonas promoter site was recognized poorly by the RNA polymerase of E.coli.

The nature of the heterologous expression of Pseudomonas genes in E.coli was recently addressed by Buckel and Zehelein (22). They employed a cosmid cloning system and an immunological detection method to isolate E.coli clones expressing galactose dehydrogenase (gal-dh) of P. fluorescens. As predicted, all E.coli clones expressed gal-dh at very low levels, approximately equivalent to expression of gal-dh in the uninduced state in P.fluorescens. By in vitro mutagenesis of the recombinant plasmid containing gal-dh, variants were obtained which expressed one hundred-fold elevated levels of gal-dh in E.coli, suggesting that an alteration in the cloned gal-dh gene rendered it more compatible with high level expression in E.coli. A logical extension of this study, which unfortunately was not reported, would be to transfer the variants to a Pseudomonas background, and determine the effect of the mutation on expression in Pseudomonas.

At least one potential virulence factor of P.aeruginosa has been successfully cloned. Phospholipase C genes, encoding heat-labile hemolysin, have been cloned by three independent laboratories. First, Vasil, et.al. (268) cloned a 4.1 Mdal BamH1 fragment encoding phospholipase C into the high copy plasmid pBR322, and selected recombinants in E.coli by isolation of hemolytic colonies on blood agar plates. After several days, a slow growing clone was detected which had cell-associated hemolysin activity (the hemolysin activity in Pseudomonas is extracellular). From the plasmid isolated from this clone was obtained the fragment encoding phospholipase. This fragment was subcloned into the vector pMW79 and transferred to P.aeruginosa. In Pseudomonas, in contrast to E.coli, the phospholipase activity was extracellular. In both systems, constitutive phospholipase activity

which was not phosphate repressible was observed, indicating that synthesis was directed from the promoter on the vector plasmids rather than from the promoter for phospholipase found normally on the P.aeruginosa chromosome. In addition to phospholipase, five other proteins were expressed, three of which correspond in molecular weight to three Pseudomonas proteins which had previously been shown to be regulated by phosphate. The possibility of a phosphate regulon in Pseudomonas was postulated by these investigators.

Coleman, et.al. (38) cloned the phospholipase C gene of Pseudomonas on a 10-15 kb Sau3a fragment into the lambda replacement vector w174.1. This vector contains the strong pΩ promoter of phage lambda, enables the detection of recombinant clones by virtue of the Spi- phenotype of recombinants in the P2 lysogenic recipient, and leads to the lysis and release of products of cloned genes in E.coli. Hemolytic plaques on blood agar were detected at a frequency of one in twenty five hundred recombinants. A 9.5 kb fragment from one of these clones was subcloned into the hindIII site of the vector pHC79, resulting in cell-associated synthesis of a 78,000 D peptide. Fractionation of cells expressing phospholipase C established that the enzyme activity was located in the soluble fraction (the cytoplasm). The authors reported that previous attempts to clone Pseudomonas genes using a cosmid system were unsuccessful, apparently due to the instability of large DNA inserts in the cosmids.

More recently, Lory and Tai (161) cloned a 4.9 kb Sau3a fragment of P.aeruginosa DNA into the BamH1 site of the plasmid pBR322. They observed constitutive, cell-associated synthesis of phospholipase as reported by the other groups. They (161) determined that the

phospholipase activity of their recombinants was associated with the outer membrane rather than the cytosol.

C. Potential Virulence Determinants of P.aeruginosa

Several properties of P.aeruginosa may contribute to its virulence. These properties include the biological activity of Pseudomonas LPS (endotoxin), the ability to adhere to mammalian cells mediated by pili, and the production of several extracellular substances which have been implicated in the pathogenesis of P.aeruginosa. These extracellular products include glycolipoprotein slime, alginate polysaccharide, pigments, exotoxin A, exoenzyme S, heat-stable hemolysin, phospholipase C, elastase, and alkaline protease. The biochemical and biological properties of these potential virulence determinants will be discussed briefly, followed by a more detailed review of the properties of exotoxin A, elastase, and alkaline protease, which are the subjects of investigation in this thesis.

1. Endotoxin

Purified lipopolysaccharide (LPS,endotoxin) of P.aeruginosa is considerably less toxic than is the endotoxin of other gram-negative pathogens, suggesting that significant differences in the composition of the lipopolysaccharide of these orgamisms exist, and that endotoxin alone is not responsible for the morbidity and mortality of patients infected with P.aeruginosa (57,103). A major difference in the LPS of this organism is the high degree of phosphorylation of the core sugars of the LPS, associated with the extreme sensitivity of this organism to EDTA (276). Injection of heat-killed organisms into laboratory animals does not induce marked pathology (158). Endotoxin is not thought to

contribute to the virulence of P.aeruginosa to the same extent that it does to the virulence of other gram negative pathogens. P.aeruginosa endotoxin has, however been implicated in shock and other sequelae associated with Pseudomonas sepsis (286).

2. Exopolysaccharides

Pseudomonas has a loosely defined slime polysaccharide layer, believed to be anti-phagocytic (238-240). This glycolipoprotein isolated from various strains is antigenically distinct but is not associated with the classical serotypes (48,66).

P.aeruginosa isolated from cystic fibrosis patients characteristically produces copious amounts of alginate slime, a polysaccharide composed of polymers of guluronic and galuronic acid (27,60). This slime is distinct from the glycolipoprotein slime, and is observed only upon primary isolation from cystic fibrosis patients (50,53,89,224). Alginate may function as an anti-phagocytic barrier (149), and may increase obstruction in the lungs, leading to impaired mucociliary clearance (J.R.W. Govan, personal communication).

Synthesis of alginate appears to be highly regulated, and is stimulated by surfactants in vivo and in vitro (73). The alginate positive (mucoid) phenotype is unstable, and in vitro quickly reverts to the stable alginate negative phenotype (288). Genes involved in alginate synthesis have been characterized by Ohman and Chakrabarty (196), who have isolated a stable mucoid variant.

3. Pigments

Injection of purified pyocyanine or fluorescein, the two major pigments of P.aeruginosa, does not produce obvious detrimental effects in laboratory animals (41). Pyocyanine has anti-bacterial activity, and

may suppress the normal flora of infected hosts, leading to the emergence of P.aeruginosa as the major organism isolated from infected burn patients (157).

4. Pili

Bacterial adherence is an obligatory step in the colonization and infection of mucosal surfaces (220). Pili mediate adherence in several systems, and as such may be considered a determinant of virulence. P.aeruginosa adheres preferentially to injured tracheal cells, a phenomenon which has been defined as opportunistic adherence (220). Buccal cells from colonized patients attach more P.aeruginosa than do cells obtained from normal controls, suggesting that injury or alteration of these cells renders them susceptible to colonization and infection (220). Similarily, the requisite trauma associated with Pseudomonas corneal infections is thought to expose epithelial cells to which the invading organisms adhere (253). Woods, et.al. (280) demonstrated that loss of fibronectin from the cell surface is associated with increased adherence of P.aeruginosa to buccal cells. They further established that treatment with either P.aeruginosa protease or host-derived protease enhances adherence of P.aeruginosa to these buccal epithelial cells.

Adherence to buccal epithelial cells, a prototype model of Pseudomonas adherence, is mediated by pili (281). It was demonstrated that removing pili rendered the de-piliated bacteria non-adherent. The involvement of pili in adhesion was confirmed by blocking adhesion by pretreatment of piliated bacteria with purified pili from homologous, but not heterologous strains of P.aeruginosa.

5. Exoenzyme S

Exoenzyme S is an ADP-ribosyl transferase distinct from exotoxin A (111). This enzyme, produced by approximately 38% of clinical isolates, differs from exotoxin A in its substrate specificity (111). Exoenzyme S ribosylates several proteins in crude extracts of eucaryotic cells, but does not ribosylate or inactivate EF-2 (111). Also, exoenzyme S shares no demonstrable immunological relationship with toxin A, is heat stable, and is destroyed by treatment with urea and dithiothreitol, whereas toxin A is heat labile and its enzymatic activity is potentiated by treatment with urea and dithiothreitol (111). Exoenzyme S is produced in media similar to that used for toxin A production, except that addition of a metal chelator such as nitrilotriacetic acid is required for optimal exoenzyme S production (111,263). In vivo production of exoenzyme S has been demonstrated in the burned mouse model (10), however there is no clear evidence that exoenzyme S contributes to the virulence of P.aeruginosa.

6. Hemolysin

P.aeruginosa produces two distinct hemolysins, a heat stable rhamnolipid and a heat-labile protein (phospholipase C) (59,120,125). Heat stable hemolysin is of low toxicity, with a median lethal dose of 5 milligram for a mouse (120). This hemolysin has detergent-like activity, solubilizing phospholipids (147). As such, this hemolysin may act in concert with phospholipase C to cause tissue damage and liberate nutrients such as phosphate (129). Production of both factors is regulated by phosphate (152).

Phospholipase C catalyzes the hydrolysis of phosphatidyl choline (lecithin) to phosphoryl choline and diacyl glycerol (59). This

hemolysin is produced by nearly 70% of all clinical isolates (282). As lecithin is the major surfactant of the lung, it is not surprising that phospholipase is thought to enhance colonization of lung tissue and so promote the establishment of Pseudomonas lung infections (159,247). High levels of phospholipase are found in 80% of the nonmucoid strains of P.aeruginosa isolated early in chronic lung infections of cystic fibrosis patients, but is demonstrable in only 20% of the mucoid strains isolated later in this type of infection (8). This observation is consistant with the hypothesis that phospholipase is involved in the establishment of primary Pseudomonas lung infections, but raises questions regarding the later emergence of hemolysin-deficient isolates. High levels of phospholipase production are also typical of urinary tract isolates, suggesting a role for phospholipase in the establishment of urinary tract infections by P.aeruginosa (8).

Injection of purified phospholipase C into skin produces redness and induration characteristic of experimental skin infections caused by Pseudomonas (153). Intraperitoneal injection causes hepatic necrosis and pulmonary edema (153).

A regulatory gene affecting the synthesis of phospholipase C and alkaline phosphatase has been characterized by Gray and Vasil (76). This gene, designated plcA,B, maps at approximately 20' on the strain PAO chromosome.

7. Exotoxin A

Exotoxin A is a heat-labile protein with a molecular weight of approximately 66,000(270). First isolated by Liu (154), this enzyme is the most toxic protein produced by P.aeruginosa, with a median lethal dose of 100-200 nanograms for a mouse (25).

The enzymatic activity of exotoxin A is identical to that of diptheria toxin, catalyzing the ADP-ribosylation of elongation factor 2 (EF-2) (108,110). This reaction inactivates EF-2, thereby inhibiting protein synthesis (108,109). Toxin A has been shown to inhibit protein synthesis *in vivo* and *in vitro* (33,109,110,207,270). Additionally, examination of the livers of burned mice following infection with toxinogenic strains of *P.aeruginosa* has revealed decreased levels of EF-2 (110).

Toxin A is cytotoxic for cultured mammalian cells (173,205), and is toxic for a variety of animal species (4,204). Injection of toxin A into mice leads to inhibition of protein synthesis in the liver, spleen, and kidneys (110). Injection of toxin A into the cornea of rabbits results in the death of epithelial, endothelial, and stromal cells, and ultimately, necrosis (107).

90% of all clinical isolates produce toxin A (12). Despite production by the majority of strains, overall levels of toxin production are strain dependant and highly regulated (9,156,217). Production of toxin *in vitro* is regulated by iron as well as other factors, as indicated by poor production of toxin in most minimal and complex media, and in any medium which contains appreciable amounts of free iron (9,156).

A variety of toxin mutants have been isolated and characterized. Ohman, *et.al.* isolated toxin deficient mutants of *P.aeruginosa* strains PA103 (198) and PAO1 (194). The mutations in these strains result in toxin deficiency and appear to be regulatory in nature, as most of the mutants produce decreased but detectable levels of toxin A. Several of these mutants are also altered in the production of other

factors such as protease (198). Whether these additional alterations are a consequence of a mutation affecting general regulatory or transport processes or secondary mutations is not clear.

Gray and Vasil (77) isolated toxin deficient mutants in P.aeruginosa strain PAO, and determined the genetic loci corresponding to the mutations in two of these strains. The mutation in one of these strains, designated tox-1, is located at approximately 35' on the strain PAO chromosome (77). The mutation in this strain appears to confer toxin deficiency specifically, and is similar to the class I toxin mutants described by Ohman ,et.al.(198). Gray and Vasil also described a second mutant, which produced decreased levels of protease as well as toxin. This phenotype is similar to the class 2 mutants described by Ohman, et.al.(198), and may correspond to the xcp-2 type mutants described by Wretlind, et.al. (284). The genetic locus of the mutation in this strain, tox-2, maps at or near the xcp-2 locus, at approximately 38' on the strain PAO chromosome (77).

Cryz, et.al.(42) isolated a mutant of P.aeruginosa PAO that produces enzymatically inactive, immunologically cross-reactive (CRM) protein. The mutation resulting in this phenotype, designated tox-A1, presumably arose within the toxin A structural gene. Hanne, et.al. (81) mapped the tox-A1 mutation to determine the genetic locus of the toxin A structural gene. R68.45 plasmid-mediated conjugation and linkage analysis demonstrated a position for tox-A1 at approximately 85' on the strain PAO chromosome.

Sokol, et.al. (246) isolated mutants of P.aeruginosa in which toxin production was no longer regulated by iron. One of these mutants, designated PAO-Fe18, results in the synthesis of toxin A in the presence

of concentrations of iron which repress toxin synthesis by the parental strain. Other products such as protease are still regulated by iron, indicating that the mutation in this strain specifically affects toxin production.

Several observations suggest a role for toxin A in virulence. Antitoxin antibodies are demonstrable in the convalescent sera of patients recovering from P.aeruginosa infections, demonstrating that toxin A is produced in vivo (138,216). Additionally, survival in bacteremic patients has been correlated with high titres of anti-toxin antibody, and death from P.aeruginosa bacteremia has been associated with toxin production by infecting strains in the absence of anti-toxin antibodies (216). Furthermore, antitoxin protects mice from subsequent challenge with some toxinogenic strains of P.aeruginosa in the burned mouse model (206).

The role of P.aeruginosa toxin A in virulence has been studied directly by comparing toxin-deficient mutants and a mutant which produces a non-toxic CRM protein to their toxinogenic parental strains in several animal models. In the mouse corneal infection model mutants altered in the production or activity of toxin A were able to initiate an infection, but were cleared more rapidly than their toxinogenic parental strains (195). Corneal damage was also greater with the toxinogenic parents, indicating that toxin A contributes to the virulence of P.aeruginosa in the mouse eye model, perhaps through inhibition of the host response as well as toxin-mediated corneal damage (195). In the chronic lung infection model described by Cash ( ), the toxin mutants were able to initiate an infection and persist in the lungs, but were unable to elicit the extensive pathologic changes observed upon infection with the

toxinogenic parental strain (279). In this system toxin does not seem to interfere with host clearance mechanisms, but is involved in the pathogenesis of this type of lung infection. In the mouse burn model the toxin-deficient mutant PAO-T1 was less virulent than the toxinogenic parental strain, demonstrating a role for toxin in this septic burn model (194). In contrast, the acute guinea pig model of fatal pneumonia the toxin mutants were as virulent as the parental strain (13). This finding suggests that toxin may be more important in certain infectious processes than in others. However, the possibility that these differences reflect differences in the susceptibility of various animal species to P.aeruginosa toxin A needs to be clarified.

8. Proteases

Most strains of P.aeruginosa produce two extracellular proteases, elastase and alkaline protease (177). These enzymes have been characterized biochemically, and have been associated with marked pathologic effects in animal models and in human infection. The biochemical and biological properties of these enzymes will be discussed in detail in the following sections.

A third protease (protease III) has been detected by several groups. Morihara (177) identified protease III as a protease with an alkaline isoelectric point by chromatography on DEAE-Sephadex. Later, Kreger and Griffin (143) and Wretlind and Wadstrom (284) detected this third protease by isoelectric focusing. This protease has an isoelectric point of 8.5-8.8, considerably higher than that of elastase and alkaline protease (177). This protease also differs from the two major proteases in its lack of enzyme inhibition by metal chelators such as EDTA. This protease is generally produced in lower yields than the

major proteases, amounting to less than 4% of total protease activity (177). This enzyme has not been further characterized.

In addition to these proteases, several groups (29,47,236) have described a collagenase. Other groups (143,182,284) have not been able to detect this enzyme. While this discrepancy may reflect differences in assay procedures or production of collagenase by only a few strains of P.aeruginosa , the striking similarities in reported properties of collagenase and elastase, and the demonstration that Pseudomonas elastase can degrade the non-helical portions of collagen would suggest that the reported collagenase corresponds to Pseudomonas elastase (143).

D. Biochemistry of P.aeruginosa Elastase and Alkaline Protease

P.aeruginosa elastase was first purified by Morihara in 1965 (185). Since that time, several purification procedures have been reported, most of which employ molecular sieve and ion-exchange chromatography (135,185). Purification by isoelectric focusing (143,284) and affinity chromatography (181,192) have also been described.

The molecular weight of elastase has been reported as 20,000-39,000 (142,143,185,284). The isoelectric point is between 5.7-6.6 (135,143,257,284). Differences in molecular weight have been attributed to differences in methodology or to the detection of monomeric and multimeric forms of the enzyme. Differences in isoelectric point are thought to reflect the presence of tightly bound contaminants or result from post-synthetic modification such as autodigestion.

Elastase is considered a typical metallo-neutral protease (181). Elastase contains 1 molecule zinc/mole enzyme, which is essential for enzymatic activity (181). Enzymatic activity of elastase is inhibited

by metal chelators such as EDTA or o-phenanthroline, heavy metal ions, and reducing agents (182). The enzymatic activity is unaffected by serine protease inhibitors such as diisofluorophosphate, but is inhibited by phosphoramidon and by plasma alpha-two macroglobulin (88,182,183).

Elastase is active against casein, hemoglobin, elastin, fibrin, and other proteins which have been previously denatured (177,185,284). Elastolytic activity is inhibited by high salt concentrations, consistant with the hypothesis that elastase binds to elastin through a non-specific, hydrophobic interation (72).

Elastase is stable over the pH range of 6.0-10.0 at temperatures up to 70°C (185). Its specific proteolytic activity is 50 mPU/mg, determined against casein (177). The substrate specificity, determined against the oxidized B chain of insulin, is broad (179). Elastase is specific for bulky or hydrophobic amino acids at imido-side of the splitting point (179,192).

Elastase is thought to be synthesized as a cell-associated proenzyme, which is activated in vivo by limited proteolysis by itself or by alkaline protease (122). Microheterogeneity with respect to the molecular weight and isoelectric point of elastase has been reported (257).

Elastase production is influenced by growth phase, growth temperature, and medium composition, but not to the same extent as is observed for toxin A (11,121,177,274). Repression of elastase synthesis by iron (11) and by glucose (M. Bjelefeld, pers. comm.) has been observed. Recently, Whooley, et.al. (274) demonstrated that production of elastase and alkaline protease by P.aeruginosa is regulated by growth rate. An inverse relationship between growth rate and protease

production was observed, indicating that protease production was depressed by available energy sources and was de-repressed by any nutrient limitation. They further demonstrated that this repression is related to the proton motive force, in that delta pH and ATP synthesis are reflections of the availability of energy sources, and an artificial alteration of delta pH results in alteration of protease synthesis (275).

Alkaline protease was first purified by Morihara in 1957 (175). This enzyme ,may be purified by the same methods used for elastase purification, but production of alkaline protease appears to be more dependant upon medium composition (175,177,284). The molecular weight and isoelectric point of alkaline protease is 48,400 and 4.1-5.0, respectively (113). Calcium or cobalt are required for maximal activity (178,180). Enzymatic activity is inhibited by metal chelators, particularily at low pH (178). The enzyme is stable at pH 5-9, and at temperatures up to 60°C (176).

The specific activity of alkaline protease as determined against casein is 5 mPU/mg or one tenth that of elastase (178). The substrate specificity of this enzyme is broad (179,186). As is the case with elastase, a broad range of denatured proteins are degraded, however, this enzyme is not active against elastin.

E. Biologic Effects of Proteases

70-85% of all clinical isolates characteristically produce elastase, while elastase production is rare in environmental isolates (85,119,187). This observation suggests that elastase production confers a selective advantage in infection. Additionally, elastase production is two to

three times more prevalent in systemic isolates than in isolates from local infections, suggesting that it contributes to invasiveness (119).

Mull and Callahan (187) first suggested that elastase was responsible for the characteristic destruction of arterial elastic laminae observed in vasculitis due to P.aeruginosa infection. Infected mice which had been compromised by treatment with 6-mercaptopurine were infected by subcutaneous injection of viable P.aeruginosa. Acute, purulent inflammation and vascular lesions were noted, and selective vasculitis was observed. In addition, necrosis of vessel walls and loss of elastic laminae was observed. This group also noted that, in contrast to pancreatic elastase, Pseudomonas elastase was not inhibited by normal serum. More recently, Ziegler and Douglas (287) compared elastase proficient and elastase-deficient isolates of P.aeruginosa in the neutropenic rabbit model and observed selective vasculitis and necrosis similar to that observed by Mull and Callahan. They found no correlation between elastase production and the formation of vascular lesions, implying that elastase is not responsible for selective vasculitis and loss of elastic laminae in Pseudomonas bacteremia. In this study, they also examined the ability of E.coli, K.pneumonia, and Serratia marcescens to induce vascular lesions and found that S.marcescens was capable of inducing such lesions. Comparative studies on the properties of P.aeruginosa and S.marcescens might help elucidate the etiology of this characteristic lesion.

Liu (153) injected rabbits with a crude preparation of elastase and alkaline protease intracutaneously, and observed immediate hemorrhage which became necrotic over time. Intraperitoneal injection of protease led to extensive hemorrhage of the wall of the peritoneal cavity, while

intravenous injection led to lung hemorrhage. Pulmonary injection produced hemorrhage and necrosis of alveolar septal cells. Hemorrhage of lung tissue was attributed by this investigator to the proteolytic degradation of clotting factors such as fibrin.

Johnson,et.al. (126) injected mice with 50 micrograms (a lethal dose) of purified protease I (alkaline protease). Autopsy of the mice revealed pitted areas in the liver and abdominal hemmorhage. Injection of 100-300 microgram protease resulted in massive abdominal hemmorhage, open lesions in the small and large intestine, and confluent, blanched and necrotic pinpoint lesions in the liver. Autopsies of human patients who died of P.aeruginosa bacteremia have revealed hemmorhagic lesions in the gastrointestinal tract, liver, and kidney (62). This type of damage has also been observed in lung tissue specimens from Pseudomonas pneumonia patients (62). However, similar effects have also been noted upon injection of mice with purified P.aeruginosa toxin A (110), implying that these effects may be a reflection of toxicity and cell death.

Gray and Kreger (75) subjected rabbits to intrapulmonary injection of purified preparations of both proteases and observed lung hemmorhage and necrosis of alveolar septal cells. Progressive injury and necrosis of type I epithelial cells and capillary endothelial cells was observed, along with accumulation of erythrocytes, fibrin and other plasma proteins, and macrophages. Later, interstitial pneumonitis, a progressive infiltration of alveolar septal cells by monocytes, was observed (75). These investigators did not observe any alterations of connective tissue or alveolar structure, and no damage to pulmonary arterioles or venules.

Several groups (65,129,135,143) have examined the effects of Pseudomonas protease on the cornea of experimental animals. Both elastase and alkaline protease are capable of degrading corneal proteoglycan: Collagen is not degraded, but is dispersed in the absence of the proteoglycan ground substance (143). Intracorneal injection of either elastase or alkaline protease seems to reproduce the liquefactive necrosis observed in severe cases of Pseudomonas keratitis (143).

Elastase has the potential to affect several host defense mechanisms. This chemopathogenic activity includes degradation of 7 of 9 classical complement components and complement-derived chemotactic factors (237), proteolysis of IgG (55), inactivation of human alpha-one proteinase inhibitor (184), and degradation of clotting factors such as fibrin (235). Elastase also elicits agglutination and vacuolation of rabbit alveolar macrophages in vitro (150). The in vivo significance of these effects is unclear.

Patients recovering from pneumonia or chronic lung infections have increased titres of antibody to both Pseudomonas elastase and alkaline protease (105,138). Vaccines containing toxoids of both of these proteases plus original endotoxin protein (OEP) protect mink from hemmorhagic pneumonia (104). Similarily, vaccines containing elastase and alkaline protease toxoids protect mice from experimental burn infection (131). Vaccines based upon toxoids to elastase and alkaline protease protected mice to the same extent as did the multi-component vaccine containing OEP as well as protease toxoids, and protected mice to a greater extent than did vaccination with OEP alone. However, the possibility that these protease toxoid preparations were contaminated with lipopolysaccharide was not ruled out.

Protease-deficient strains of P.aeruginosa are less virulent than protease producing strains in the mouse eye model (130). In experimental septicemia models conflicting results have been obtained with respect to the importance of protease production. Liu and Hsieh (160) reported that protease producing strains were less virulent than were strains which did not produce protease. In contrast, Musxynski and Kedzia (169) reported that protease production enhanced virulence, while Kabayashi found no correlation between protease production and virulence. This considerable discrepancy may be attributed to the use of unrelated strains in different animal models. Furthermore, if proteases are required to initiate an infection and reach the bloodstream, this step is bypassed by certain inoculation regimens.

Cicmanec and Holder (34) have suggested that elastase and alkaline protease provide nutrients for P.aeruginosa growing in burned skin. They observed that protease producing strains of P.aeruginosa grew much better than protease-deficient strains in minimal media supplemented with burned skin extract.

Direct evidence for the participation of elastolytic activity in virulence was provided by examination of an elastase mutant in the guinea pig model of acute pneumonia. The mutant PAO-E64, which produces normal levels of alkaline protease and an elastase which is markedly decreased in elastolytic activity (197), was less virulent than the parental strain PAO, demonstrating that elastolytic activity, per se contributes to the virulence of P.aeruginosa PAO in acute pneumonia in guinea pigs (13). No direct evidence for the participation of alkaline protease in virulence exists, due to the lack of mutants specifically deficient in that enzyme.

F. Disease States

P.aeruginosa may produce a variety of distinct infectious processes, including local and generalized infections. Local infections include infections of skin, wounds, the urinary tract, the respiratory tract, the intestinal tract, the eye, and the ear. Generalized infection (bacteremia) generally results from primary local infection in burn patients, patients with acute pneumonia, and immunocompromised patients. The pathogenesis of Pseudomonas corneal infection, pneumonia, and bacteremia have been extensively studied and are described in detail below.

1. Corneal Infection

Considered the most fulminant and destructive bacterial infection of the cornea, Pseudomonas keratitis is associated with an acute, ulcerative corneal lesion accompanied by hypopyon and liquefation of the cornea (14). Permanent visual damage often results from perforation of the cornea and extensive scarring (56,148). Up to 15% of bacterial keratitis cases are attributed to P.aeruginosa, making it the most common cause of bacterial corneal ulceration (140). Usually an acute, self-limiting disease, recurrence or chronicity of Pseudomonas keratitis has been noted (82). At risk are individuals who recently suffered corneal trauma due to improper use of contact lenses, irritation from a foreign body, or post-operative cataract surgery (14,144).

Various animal models of Pseudomonas keratitis have been described, involving either corneal injection or inoculation of traumatized mouse, rabbit, or guinea pig cornea with viable P.aeruginosa (70,125,267,268). The course of infection in these animal models is

similar, and appears to reproduce the pathology observed in humans with Pseudomonas keratitis.

The cornea is considered an immunologically weak tissue, since it has no direct blood supply (70). Host defense mechanisms include anatomical barriers (untraumatized cornea) and secretory IgA (253). In addition, serum immunoglobulins and polymorphonuclear leukocytes (PMNs) appear soon after corneal damage occurs (37).

The first step in establishing Pseudomonas keratitis is thought to involve adherence of bacteria to traumatized cornea. Ramphal, et.al.(194) determined that corneal injury is required for adherence of P.aeruginosa to the cornea. Stern, et.al. (253) extended these findings by demonstrating that P.aeruginosa adheres preferentially to epithelial cells exposed by trauma, rather than to stromal cells.

Various bacterial factors are thought to contribute to the pathogenesis of Pseudomonas keratitis. Kessler, et.al. (136) examined the effect of intrastromal injection of heat-killed Pseudomonas in rabbits. They noted a massive infiltration of PMNs and ulceration of the cornea, which they attributed to the degranulation of PMNs and release of host-derived proteolytic enzymes.

Bacterial strain related differences in the outcome of Pseudomonas keratitis were first described by Bohigian and Escapini (14), where strains of various pyocin types differed in virulence in a rabbit corneal model. While no attempt was made in that study to correlate the outcome of infection with particular bacterial virulence determinants, a study by Kawaharajo and Homma (130) associated protease production with the ability to establish an active corneal infection in mice. Related studies demonstrated protection against eye infection in mice by prior

immunization with toxoids of elastase, alkaline protease, and original endotoxin protein (OEP)(87).

Several investigators have suggested that Pseudomonas proteases are responsible for the ulceration and liquefactive necrosis observed in Pseudomonas eye infections. Indirect support for this hypothesis was obtained by examining the effect of Pseudomonas proteases on animal eyes. Fisher and Allen (65) inoculated rabbit eyes intracorneally with crude preparations of elastase and alkaline protease, resulting in extensive corneal damage. Addition of sufficient elastase or alkaline protease to incised mouse cornea produces opacity and ulceration similar to that observed in Pseudomonas eye infections (129,142). The pathologic effects of both proteases on mouse or rabbit cornea are qualitatively similar to the effects observed in experimental corneal infection, suggesting that Pseudomonas proteases are responsible for much of the characteristic damage associated with Pseudomonas keratitis.

Pseudomonas toxin A also appears to contribute to corneal damage. Injection of toxin A into rabbit eyes leads to death of epithelial, endothelial, and stromal cells, resulting in corneal necrosis (83,107). The effect of toxin alone on rabbit eyes does not totally reproduce the clinical observations accompanying infection, as there is a considerably higher infiltration of PMNs in active infection than is observed with toxin alone (83). Evidence for the participation of toxin A in Pseudomonas keratitis was obtained by Ohman, et.al.(195), who compared the virulence of toxin-deficient mutants and toxinogenic parental strains in a mouse eye model. They reported that the toxin-deficient mutants could establish, but could not maintain an active corneal infection, and that infection with toxin-deficient mutants

resulted in less corneal pathology than was observed with infection with the parental organisms from which the mutants were derived.

Johnson and Allen (124) examined the role of heat-stable hemolysin in Pseudomonas eye infections. Intracorneal injection of mice with purified hemolysin resulted in corneal opacity and an extensive influx of PMNs. Ulceration of the cornea was not observed, even in the presence of marked inflammation. While this suggested to the authors that ulceration and corneal liquefaction is not attributable to host-derived proteases, they made no attempt to demonstrate degranulation and release of host-derived proteases in that study.

In addition to Pseudomonas keratitis, Pseudomonas has been associated with conjunctivitis in premature infants (24). This rare syndrome is more common and decidedly more serious than is gonoccal opthalmia, in that it is associated with a high mortality rate. In contrast to the typically local infection of Pseudomonas keratitis in adults, Pseudomonas conjunctivitis of the newborn is a rapidly invasive infection, leading to septicemia and death within days after the onset of infection. Animal models using newborn mice or cyclophosphamide-treated mice have been described which appear to reproduce this type of infection (84,288). In these models, Pseudomonas eye infections rapidly progress to septicemia and death, demonstrating the critical importance of a fully functional and mature immune response in keeping Pseudomonas eye infections localized.

2. Bacteremia

P.aeruginosa is not the most frequently isolated organism associated with bacteremia, but the fatality rate associated with

Pseudomonas sepsis is higher than for any other bacteria (224). The leading cause of death in leukemia and lymphoma patients continues to be gram-negative infection, and P.aeruginosa is a significant cause of death in these immunocompromised patients (67,224,260). Similarily, bacterial infection, particularly with P.aeruginosa, remains the leading cause of death in burn patients (3,218). P.aeruginosa rarely infects healthy individuals; immunocompromised patients and burn patients are particularly predisposed to bacteremia with P.aeruginosa (277).

Immunocompromised patients and burn patients encounter P.aeruginosa from a variety of diverse sources in the hospital environment, including sinks, reservoirs, flowers and salads, hospital personnel, other patients, and even the patients' own gastrointestinal tract (165,258). Exposure of compromised patients to P.aeruginosa apparently leads to focal colonization, from which bacteremia develops. The respiratory tract and the urinary tract are common sites of colonization prior to the onset of bacteremia (6,273).

In burn patients, the burn wound eschar provides an ideal environment for P.aeruginosa, such that some studies report that half of all burn patients are colonized within 48 hours after thermal injury (219). Trauma, resulting from extensive thermal injury, predisposes the patient to infection through several potential mechanisms, including decrease in circulating immunoglobulin, T lymphocytes, neutrophils, granulocytes, and reticuloendothelial system function (168,188,200).

Independent of the predisposing factors and primary sites of infection, bacteremia develops as a result of the considerable invasive capacity of P.aeruginosa. Of fundamental importance to the invasiveness of this organism is thought to be its propensity to invade vascular

walls. Invasion of the vascular system leads to hematogeneous dissemination and bacteremia, resulting in death due to septic shock (200,201). Invasion of the vascular walls and resultant dissemination may be accompanied by hemorrhagic necrosis at metastases in the skin, particularily on the extremities (189). Ecthyma gangrenosum is the most extensively described of these cutaneous manifestations of sepsis (68,166,189). Presenting as discrete, round lesions, central vesicles (bullae) develop, and necrosis is evident by 24-48 hours after the onset of the primary lesion. Less commonly, Pseudomonas sepsis is accompanied by the presence of bullous and multiform lesions, and, with deeper necrosis, progression to nodular or gangrenous cellulitis. This syndrome, resembling a decubitis ulcer, involves very deep necrosis, and has been observed only in association with P.aeruginosa bacteremia (169,261). A common observation in these tissue manifestations of sepsis is focal necrosis and bacterial proliferation with a negligible inflammatory response (54,189).

Antibiotic therapy and passive gamma-globulin therapy have not significantly altered the outcome of P.aeruginosa bacteremia (67,219,256). To develop more effective immunotherapeutic regimens requires a critical analysis of the microbial factors involved in this type of infection. Several animal models have been developed to facilitate this type of analysis. A model which attempts to duplicate the vasculitis and bacteremia observed in neutropenic patients was developed by Ziegler and Douglas (287). This model involves inoculation of P.aeruginosa strains into the conjunctival sac of agranulocytic rabbits. Virulent strains invade the eyelid, then are disseminated

through the bloodstream, resulting in death in one to six days after inoculation.

Stiertiz and Holder (254) have described a burned mouse model. A burn over 10-30% of the body surface is inoculated by subcutaneous injection, leading to a rapidly fatal bacteremia. This model is considered to be clinically relevant, in that the mice are compromised by thermal injury alone, are infected with low numbers of bacteria, and the progression of disease appears to mimic the situation observed with infected human burn patients.

Various rat burn models have also been described (151,271). Burns are induced by ethanol flame, by immersion in boiling water, or contact with hot metal, to cover 20-30% of the body surface. These burns are then seeded topically with viable P.aeruginosa. These rat models differ from the mouse model described above in that organisms are inoculated rather than injected. Also, rats are innately more resistant to toxin A than are mice (271). While the rat models have been considered models of infection or invasiveness, and the mouse a model of toxicity, no direct comparisons of these types of models have been undertaken, rendering this distinction debatable. In the burned rat model, lesions have been observed which are similar to those described as secondary manifestations of sepsis in humans. In these lesions, as with ecthyma gangrenosum, bullous lesions, and gangrenous cellulitis, no inflammatory response was observed (54,261,271).

3. Pulmonary Infections

P.aeruginosa is one of the leading gram-negative pathogens associated with hospital-aquired pneumonia (229). At risk are patients with serious underlying disease such as neoplasia or chronic heart or

lung disease (106,264). Clinical observations include involvement of one or both lower lobes of the lung, with alveolar infiltration and large abscess formation (62). The typical histologic observation includes necrotizing pneumonia with hemorrhage and microabscess formation. Acute inflammation, alveolar septal cell necrosis, and coagulative necrosis have been reported (264). This type of pneumonia is often accompanied by bacteremia, a complication with a high (greater than 50%) mortality rate (106,214,229). The complication of sepsis is probably related to underlying neutropenia in many of the patients who are predisposed to this type of pneumonia (106). While prior antimicrobial therapy and exposure to contaminated inhalation equipment have been implicated (221), a study by Johanson, et.al.(123) suggests that the severity of underlying disease is correlated with risk of pharyngeal (and presumably lung) colonization.

Several animal models of Pseudomonas pneumonia have been described, involving neutropenic dogs (44,134), mice (241), rats (30), rabbits (222) and normal or immunocompromised guinea pigs (211,212).

Acute, hemorrhagic pneumonia is a naturally occuring disease in minks (193,242). In the guinea pig model described by Pennington (211), certain strains of P.aeruginosa produce a non-fatal pneumonia in normal guinea pigs, but establish a rapidly fatal infection in animals compromised by treatment with cyclophosphamide or cortisone (212). Mortality in compromised animals was correlated with a diminished PMN response, consistant with current concepts regarding the role of PMNs in pulmonary defense. With other strains of P.aeruginosa, an acute, rapidly fatal pneumonia occurs in the absence of immunodeficiency (213). This strain-related difference suggests that differences in bacterial

virulence factors may contribute to the outcome of this type of infection. With regard to potential virulence determinants, several studies have implicated proteases in the pathogenesis of Pseudomonas pneumonia. Elevated antibody titres to Pseudomonas proteases have been detected in the sera of patients with Pseudomonas lung infections (138), and Homma, et.al. (104) demonstrated partial protection of minks by prior immunization with toxoids of Pseudomonas proteases. Also, intranasal or intratracheal instillation of crude or purified preparations of Pseudomonas proteases reportedly reproduces the characteristic hemmorhagic lung lesions observed in humans (75,153). For example, Gray and Kreger (75) examined the effect of purified elastase and alkaline protease on rabbits and noted intra-alveolar hemorrhage, injury and necrosis of alveolar septal cells, and infiltration of monocytes, identical to the microscopic observations of lung tissue taken from patients who died of Pseudomonas lung infections. Additionally, rapid and extensive pulmonary hemorrhage, and capillary endothelial cell damage were noted in the absence of structural alterations of pulmonary venules or arterioles. While none of the animals treated with protease died, death and alveolar septal cell necrosis resulted from intratracheal instillation of purified Pseudomonas toxin A. Treatment with toxin A was not associated with intra-alveolar hemmorhage and monocyte infiltration, as was observed with protease treatment or in an active infection.

Pennington, et.al. (211,213) utilized the guinea pig model to evaluate the efficacy of Pseudomonas vaccines in preventing Pseudomonas pulmonary infection. This model involves intratracheal instillation of P.aeruginosa into anesthetized guinea pigs, and results in an extensive

bilateral hemorrhagic pneumonia accompanied by intraalveolar hemorrhage, alveolar septal wass edema, inflammation, and death. Vaccines employing Pseudomonas lipopolysaccharide or cell extracts elicited elevated serum hemagluttinin titres, which were correlated with improved clearance of bacteria and improved survival of the experimental animals. Interestingly, increased levels of opsonic antibody were associated with the presence of fewer phagocytes, suggesting to these investigators that this regimen might result in improved survival in neutropenic patients and in less PMN-derived tissue damage.

Recently, P.aeruginosa strain PAO, an exotoxin A-deficient mutant (PAO-T1), a mutant producing enzymatically inactive exotoxin (PAO-PR1), and a mutant producing an elastase with markedly decreased elastolytic activity (PAO-E64) were examined in this guinea pig model. Active elastase, but not toxin, was shown to be necessary to produce acute, fatal pneumonia in guinea pigs (13).

The major cause of death in cystic fibrosis (CF) patients is progressive pulmonary infection (49,89,278), and P.aeruginosa is the predominant organism isolated from these infected patients (49). This type of pulmonary infection differs considerably from the acute pulmonary infection observed in patients without cystic fibrosis. Lung infections in CF patients are chronic, and may exist for many years. Patients typically undergo long periods of infection and remission, with pulmonary colonization that may exist over the course of 15-20 years. Also, in contrast to the acute, necrotizing pneumonia described earlier, lung infections in CF patients remains localized and never progresses to bacteremia. While several potential predisposing factors have been identified, this infectious process is still poorly understood.

Cystic fibrosis is an inborn error in metabolism which results in exocrine anomalies. Exocrine dysfunction results in pancreatic insufficiency, elevated levels of sweat electrolytes, and recurrent pulmonary disease (49). Several studies have noted host defects which could contribute to the predilection of these patients for chronic Pseudomonas lung infections. One finding in the serum of cystic fibrosis patients is the presence of a ciliotoxic factor which induces dyskinesis in tracheal explants and mussel cell cilia (39). A cationic protein of 4-10 Mdaltons, this factor is found non-covalently associated with serum beta 2 macroglobulin and with the constant regions of IgG1 and IgG2 heavy chains (39). While impairment of mucociliary clearance could predispose an individual to pulmonary infection, the in vivo relevance of this factor is controversial, as blind studies have failed to unequivocally demonstrate the presence of this factor (285). For this reason, this defect by itself is not considered responsible for the propensity of these patients towards Pseudomonas colonization and infection.

Pseudomonas pulmonary infection in cystic fibrosis patients does not typically lead to bacteremia (215), and infected CF patients have normal or elevated levels of circulating antibody to many Pseudomonas antigens (49,215,50). These patients also have apparently normal cell-mediated immunity (215). Potential immune defects in these patients have, however,been noted. Thommason, et.al. (262) have identified a circulating factor in the serum of cystic fibrosis patients which inhibits phagocytosis by alveolar macrophages. The intrinsic activity of the phagocytes appeared normal. This defect appears to be selective and specific for Pseudomonas (15). The possibility of a related humoral

defect was raised by the observation that hypogammaglobulinemic patients fared better than did patients with normal or elevated immunoglobulin levels (215). Recently, Fick, et.al. (63) demonstrated that defective opsonins are present in CF serum which are unable to bind to the macrophage membrane Fc receptors, resulting in impaired phagocytosis.

Another potential immune response defect involves progressive lymphocyte unresponsiveness, resulting in decreased macrophage activation (248,249). One study (248) noted a specific incapacity to respond to P.aeruginosa in cystic fibrosis patients with advanced disease.

The variety of potential host defects identified by different investigators and the lack of agreement between different groups regarding the presence of these defects is confusing. However, these patients are infected over the course of many years and so may represent several distinct populations which manifest different stages of this disease. This has not been taken into account in these various studies. It is also difficult to differentiate the effects of the disease itself from effects which may be induced or exacerbated by the chronic pulmonary infections which accompany cystic fibrosis.

The lung of cystic fibrosis patients appears to represent a unique ecological niche. Mucoid strains of P.aeruginosa are rarely observed in environmental isolates, or in strains isolated from several types of human infection, but are particularily common in cystic fibrosis patients with chronic P.aeruginosa pulmonary infection (49,170,288). Indeed, mucoidy has gained diagnostic significance when such strains are isolated from seemingly normal adults with chronic pulmonary infection (223). In general, infection of CF patients is initiated with rough strains of P.aeruginosa, followed by the emergence of mucoid strains later in

infection (52). Mucoid strains corresponding to Homma serogroup 8 appear to be particularily prevalent in CF lung infections (288).

Mucoidy in smooth strains of P.aeruginosa reflects the synthesis by those strains of alginate slime. This material is thought to impair mucociliary clearance (J.R.W. Govan, pers. comm.) and may lead to decreased opsonization and a resultant decreased phagocytosis (149).

An animal model has been described which reproduces a chronic P.aeruginosa pulmonary infection similar in some respects to that observed in cystic fibrosis patients. This model involves the intratracheal instillation of agar beads containing viable P.aeruginosa in rats (30). Histologic examination of these infected lungs reveals goblet cell hyperplasia, focal necrosis, and acute and chronic infiltrate, similar to the findings reported for human patients. Also, the chronicity of the infection, and the lack of progression to bacteremia are similar to that observed in the chronic lung infections of CF patients. This model is of particular value in assessing the role of various P.aeruginosa virulence factors in acute P.aeruginosa lung infections. Mutants altered in the production or activity of toxin A and elastase have been examined in this model of infection. In this system, both active toxin and elastase were required for maximum virulence of P.aeruginosa (279).

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III. Manuscripts

Paper 1.

ALKALINE PROTEASE DEFICIENT MUTANTS OF *PSEUDOMONAS AERUGINOSA*: ISOLATION AND CHARACTERIZATION *IN VITRO* AND IN A MOUSE EYE MODEL

Abstract

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

Introduction

Infection of traumatized cornea by P.aeruginosa is usually rapid and self-limiting, and often results in impaired vision due to corneal scarring (21). The hallmark of this infection is a liquefactive necrosis associated with extensive ulceration and corneal perforation (1).

Years ago, Fisher and Allen (7) suggested that the characteristic damage observed with P.aeruginosa eye infections may be attributable to the action of bacterial proteases on the corneal proteoglycan. Several groups, including Kawaharajo, et.al.(16), Hirao and Homma (11), and

Kreger and Gray (19) have since provided data to support this hypothesis. Recently, Ohman et.al. (26) initiated a genetic study to examine the role of bacterial toxin and protease in corneal infection. They examined the virulence of an elastolysis-deficient mutant of P.aeruginosa and concluded that elastolysis was not required to establish an active infection. They also concluded that if extracellular protease is required for virulence in Pseudomonas eye infections, then alkaline protease produced by the mutant strain they examined may have been sufficient to cause corneal damage.

Isolation of mutants specifically deficient in the production of alkaline protease would facilitate definitive studies addressing the role of alkaline protease in P.aeruginosa infections. This approach has been complicated by the fact that most strains of P.aeruginosa produce at least two distinct extracellular proteases (24), and that alkaline protease does not possess a unique or stringent substrate specificity(25). We have been unable to specifically inhibit the activity of interfering protease (elastase) to facilitate detection of alkaline protease mutants on skim milk agar plates (unpublished observations). To circumvent these problems, we chose to isolate alkaline protease mutants in strain PA103,which produces alkaline protease (4), but no detectable elastase (27). Despite the fact that strain PA103 is serum sensitive (40) it is virulent in a mouse eye model of corneal infection (15,26). We have isolated alkaline protease-deficient mutants of strain PA103 following ethylmethane sulfonate mutagenesis and screening of mutagenized colonies on skim milk agar plates. After in vitro characterization of these mutants, mutants specifically deficient in alkaline protease

production were identified, and the virulence of these mutants compared to that of the wild-type strain in a mouse eye model.

Materials and Methods

Organism. P.aeruginosa strain PA103, isolated by Liu (22) was selected for this study. This strain is naturally elastase deficient (27). We found that all protease activity in culture supernatants of strain PA103 was neutralized by specific alkaline protease antisera, demonstrating that the only extracellular protease produced by this strain is alkaline protease. This strain and derived mutants were maintained on nutrient agar plates and stored in 10% skim milk at -70°C. Where indicated, isolation of spontaneous mutants resistant to streptomycin and rifampicin were selected by growth on 250 microgram/ml streptomycin (streptomycin sulfate, Sigma Chemical Co, St.Louis) followed by growth on 50 microgram/ml rifampicin (Sigma).

Isolation of mutants. Ethylmethane sulfonate (EMS, methanesulfonic acid ethyl ester, Sigma Chemical Co., St. Louis, Mo.) was employed according to the procedure of Carlton and Brown (2). A solution of Vogel-Bonner minimal medium (35) containing 4% EMS was equilibrated at 37°C. To this solution was added an equal volume of a late exponential phase culture of P.aeruginosa PA103 grown in nutrient broth. This mixture was incubated with shaking for 90 minutes at 37°C, then cells were washed with phosphate buffered saline, grown overnight in nutrient broth, and used to inoculate skim milk plates at dilutions sufficient to result in approximately 100 colonies per plate. The conditions of EMS

mutagenesis employed resulted in approximately 90% lethality. After incubation of these plates at 37°C for 48 hours colonies were examined for zones of clearing indicative of alkaline protease production. Colonies which did not produce zones of clearing after 48 hours were chosen for further study.

Preliminary characterization of mutants. Mutants were examined for prototrophy by growth on minimal agar medium (35). Pigment production was determined by growth on agar plates prepared according to King (18). Motility was determined by growth in 0.4% nutrient agar, and by microscopic examination of broth cultures by the hanging drop method. Generation time was determined by growth in nutrient broth, as described by Miller (23). Serotype and pyocin type were determined as described previously (6,27). Total protease was determined by growth on skim milk agar plates (31). Hemolysin production was determined by growth on heart infusion agar supplemented with sheep blood (27). Lipase production was demonstrated by clearing of tributyrin agar (14). Esterase production resulted in a blue coloration of colonies grown on brain heart infusion agar supplemented with 0.1% indoxyl acetate (12). Toxin A activity was determined in an ADP-ribosyl transferase assay as previously described (27). The reaction mixture was incubated at 25°C for 10 minutes.

Liquid culture techniques. For quantitative determination of extracellular alkaline protease and toxin A, cells were grown in trypticase soy broth which had been dialyzed, deferrated by chelex (Chelex-100, Biorad Laboratories, Richmond, Ca.) treatment, and supplemented with monosodium glutamate and glycerol (TSB-DC)(27), This medium is optimal for toxin A production (27) and supports the production

of alkaline protease by strain PA103 (4). This medium was also used to prepare inocula for animal studies, employing conditions previously demonstrated as optimal for enhancing the virulence of P.aeruginosa for corneal infections (37). Davis minimal medium (5) supplemented with tryptose, yeast extract, and glucose (MTYG;40) was also used to obtain supernatants for the quantitation of alkaline protease activity, as described (4).

Determination of alkaline protease activity. P.aeruginosa PA013 produces low levels of alkaline protease (4). Therefore, a modification (34) of the sensitive protease assay described by Rinderknecht (29) was used for its quantitation. To 50 mg hide azure blue powder substrate (Sigma) in a 17 by 25 mm polycarbonate tube was added 1.5ml buffer (20mM tris-HCL, 1mM $CaCl_2$), pH 8.0) and 0.5 ml culture supernatant or cell extract. This mixture was incubated at 37°C for 1 hour with constant rotation, then tubes were chilled on ice and centrifuged at 4000 x g for 5 minutes in a clinical table top centrifuge. The absorbance of the reaction mixture was then determined at 595 nm. Preliminary studies using purified alkaline protease (Nagase Chemical Company, Tokyo, Japan) demonstrated that this assay was capable of detecting 100 nanogram alkaline protease per ml culture supernatant. Protease activity was expressed in terms of protease units/ml (pU/ml), where one unit is equivalent to an increase in A_{595} of 1.0/hr at 37°C.

Preparation of anti-alkaline protease antisera. A toxoid of alkaline protease was prepared by formalin-lysine treatment of purified alkaline protease as described by Homma, et.al. (13). This toxoid was used to immunize adult New Zealand white rabbits. Rabbits were injected intramuscularly with 1 mg toxoid in complete Freund's adjuvant. At two

week intervals, rabbits were boosted with 1 mg toxoid in incomplete Freund's adjuvant, and blood samples collected for determination of anti-alkaline protease titre. Titre was determined against 1 microgram pure alkaline protease, employing a standard immunodiffusion assay (29). After the fourth boost, the animals were rested for four days, then exsanguinated. The IgG fraction of this antisera was prepared by differential ammonium sulfate precipitation (10), then dialyzed against phosphate-buffered saline and stored at -20°C. This antiserum had a precipitin titre of 1:512.

Quantitation of alkaline protease antigen. An enzyme linked immunosorbant assay (ELISA) was developed to quantitate alkaline protease antigen in culture supernatants and cell lystates. A conjugate of alkaline phosphatase and the IgG fraction of antiserum raised against alkaline protease was prepared as described (17). Coating buffer, phosphate buffered saline (PBS), PBS with Tween (PBS-Tween), PBS with 1% bovine serum albumin (PBS-BSA), and diethanolamine buffer were prepared as described (17). Microelisa plates (Dyntatech Laboratories, Inc.) were coated overnight with rabbit anti-alkaline protease (20 microgram protein/ml) in a humid chamber, then plates were rinsed four times with PBS-Tween, followed by four rinses with PBS. Supernatant samples, and alkaline protease standards (Nagase Chemical Company, Japan) diluted in PBS-BSA were added to microtitre wells in a total volume of 100 microlitres, and the plates incubated in a humid chamber for 2 hours at 25°C. Plates were then rinsed as before, then 100 microlitres of the alkaline phosphatase-conjugated anti-alkaline protease was added to each well and the plates incubated for 2 hours as before. Plates were then rinsed, and 100 microlitres of the substrate p-nitrophenyl phosphate

(Sigma, 1 mg/ml in diethanolamine buffer) was added to each well, and the plates incubated for 2 hours at 37°C. The reaction was terminated by the addition of 50 microlitres of 60 mM ethylenediamine tetraacetic acid (EDTA) to each well, and the absorbance at 405 nanometers determined on a microelisa reader (Dyntatech). The limit of detection of this assay was 100 picogram alkaline protease/ml.

Extraction of intracellular or cell-associated protease. TSB-DC grown cells were pelleted by centrifugation, washed twice with an equal volume of PBS, then ruptured in a French pressure cell press (American Instrument Company) as described (30), except that the cells were suspended in PBS, and nucleases and $MgCl_2$ were not added.

Mouse model of corneal infection. A modification of the model described by other investigators (8,9,15,26) was used. Bacteria were grown to mid-exponential phase in TSB-DC at 32°C. When the culture reached an A_{540} of 0.23, cells were removed by centrifugation, washed twice with PBS, then suspended in 0.1 volume PBS to obtain ca. 2×10^9 cfu/ml. Viable numbers of bacteria were confirmed by plating serial dilutions on nutrient agar to obtain colony forming units (cfu).

Female Swiss Webster mice weighing ca. 20g were obtained from Simonson (Hayword, Ca). Mice were anesthetized by inhalation of metofane (methoxyfluorane; Pitman-Moore, Inc., Washington Crossing, New Jersey) then subjected to three 1-mm corneal incisions with a sterile 27-guage needle, avoiding penetration of the anterior chamber. Dilutions of bacteria suspended in 5 microlitres were applied to the traumatized cornea. Where indicated, an inoculum of bacteria suspended in PBS containing 5 microgram of purified alkaline protease or 0.5 microgram of purified elastase was used.

The progress of infection was determined by examination of infected eyes with a 50x stereoscopic microscope. Extent of damage was estimated according to a previously established corneal damage index (26). As previously defined (26), a CDI of 1.0 corresponds to light or partial opacity, wheras a CDI of 4.0 indicates necrosis and perforation of the cornea. To assess colonization and persistance of bacteria in infected eyes, eyes were periodically swabbed with sterile saline-soaked swabs, and the swabs used to inoculate King B plates (18) . Representative mice with culture negative eyes were sacrificied by cervical dislocation and their eyes enucleated (8), macerated and suspended in PBS, and dilutions plated on King B plates. This was done to address the possibility of intra-stromal persistance of bacteria that would not be evident by superficial culturing.

Results

Isolation of alkaline protease-deficient mutants. Alkaline protease mutants were identified by their failure to produce a zone of clearing on skim milk plates when incubated at 37°C for 48 hours. Following 3 independant EMS mutageneses a total of 21,500 colonies were examined. Colonies with no zones of clearing were subcultured for heavy growth on skim milk agar, and incubated at 37 for 48 hours. A total of 19 presumptive alkaline protease mutants, which produced no zones of clearing under either condition, were subcloned. These presumptive mutants were grown in liquid TSB-DC, and alkaline protease activity was determined. While the 19 presumptive mutants were indistinguishable on skim milk agar plates, the amount of alkaline protease produced by these mutants was variable, and ranged from 0-60% parental levels. Of the original 19 presumptive mutants, 5 mutants produced less than 15% parental levels of protease activity and were chosen for further examination.

Characterization of protease-deficient mutants . Previous studies have demonstrated that mutants isolated on the basis of protease deficiency are often deficient in the production of other extracellular products which may be a consequence of co-regulation, common transport mechanisms, or secondary mutations (39). For this reason, the production of several extracellular products was examined (Table 1). The mutant strains were all indistinguishable from the parental strain PA103 with respect to the production of fluorescein, hemolysin, esterase, and lipase. However, two strains, designated PA103-AP3 and PA103-AP4, produced decreased levels of toxin A, and were not examined further.

The mutant strain PA103-AP5 produced at least 10% of the parental levels of protease and was therefore eliminated from future studies. Two mutants, designated PA103-AP1 and PA103-AP2 produced consistantly low or undetectable levels of extracellular protease, but were otherwise indistinguishable from strain PA103. These mutants were chosen for further characterization. No differences in serotype, pyocin type, or motility was noted in these mutants (data not shown). To determine whether protease deficiency in these two mutants was due to altered growth rate in vitro, growth rates in TSB-DC at 32° C were determined. Parent and mutant strains were not different in generation time (about 40' during early exponential phase).

Quantitation of protease activity and antigen in mutant strains. To address the possibility that the mutants produced an altered protein which was proteolytically inactive but antigenically related to alkaline protease, supernatants were examined for the presence of alkaline protease antigen. Mutants were cultivated in TSB-DC as described (4). Total protease activity of culture supernatants was determined, and supernatants were examined for the presence of protein antigenically related to alkaline protease by an ELISA method specific for alkaline protease. Both mutants examined produced markedly decreased or undetectable levels of protease activity and correspondingly low levels of alkaline protease antigen (Table 2). Previous studies have demonstrated repression of alkaline protease production in complex medium (24). To address the possibility that protease production of these mutants was adversely affected by the growth medium, these strains were grown in MTYG, which has been shown to support a high ratio of alkaline

protease to total protein (4). Both mutants produced either low or undetectable levels of protease in this medium (Table 2).

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

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

Examination of mutant strains in the mouse eye model. Previous studies (26) have demonstrated that strain PA103 establishes an active corneal infection in mice with inocula of 10^6 to 10^8 bacteria/eye.

In the current study, the inoculation of 10^6-10^8 colony-forming units (cfu) of PA103 produced an active, necrotic infection associated with marked opacity and ulceration. Average corneal damage in these mice ranged from 3.0-3.5 and permanent eye damage was evident in the majority of these mice (Figure 2). In contrast, neither of the mutant strains deficient in protease production (PA103-AP1 and PA103-AP2) were able to induce pathologic changes beyond minimal corneal opacity and neovascularization, with average corneal damage in these groups of mice ranging from 0-1.5 early in infection and resolving rapidly (Figure 2).

Infection with either of these mutant strains resulted in essentially no permanent damage.

Recovery of bacteria from infected mice. With an initial inocula of 10^6-10^8 organisms strain PA103 could be cultured from greater than 50% of infected eyes eight days after inoculation, while infection with either mutant strains was considerably more transitory (Figure 3). To examine the possibility that organisms were present intrastromaly, in a form which would not be demonstrated by superficial culturing, enucleated eyes were examined for the presence of viable organisms. Enucleated eyes were uniformly culture negative by day 8 following infection with alkaline protease mutants , indicating that the mutant strains were not persisting in low numbers within the infected corneas. Culture results obtained throughout the course of infection with the mutant strains revealed that the mutants were unable to persist in the cornea and in 50% of the mice the mutants failed to establish even a transitory infection (Figure 3).

Persistence in mixed infection and stability of mutants in vivo.

The mutations in the alkaline protease-deficient strains appear to affect alkaline protease specifically. Still, there existed the possibility that an undetected second mutation or pleiotropic effect of the initial mutation resulted in inability of mutants to adhere to traumatized cornea, were unable to grow in vivo, or were unusually sensitive to the host defences. If the defect in the mutant strains which results in decreased colonization is due to their lack of alkaline protease production, then the effect of these mutations should be complemented in vivo by the production of extracellular protease produced by another strain. A derivative of mutant strain PA103-AP1 was prepared

which was resistant to streptomycin and rifampicin. This strain, designated PA103-AP101 , was also avirulent and in this regard indistinguishable from strain PAO-AP1. Mutant strain PA103-AP101 was employed in a mixed infection where the inocula contained equal numbers of PA103-AP101 and the original alkaline protease positive, antibiotic sensitive parental strain PA103, using a total inoculum of 10^7 cfu/eye. The mutant strain PA103-AP101 was recoverable from infected eyes as long as was the parental strain PA103. Bacteria resistant to streptomycin and rifampicin were recovered from mixed infections as late as day 12 following infection (Table 3). The antibiotic resistant strains recovered from the mixed infections were uniformly deficient in alkaline protease activity, demonstrating in vivo stability of the alkaline protease mutation.

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

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

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

Discussion

The data presented shows that at least low levels of extracellular protease are essential for maximum virulence of P.aeruginosa in a mouse eye model. The strain chosen for this study, PA103, produces no detectable elastase and such low levels of alkaline protease that it is considered by other investigators to be protease deficient (15,32). Our study and others (15,26) have shown that this strain is virulent in a mouse eye model. We found, however, that alkaline protease-deficient mutants derived from strain PA103 were avirulent in the mouse eye model. Virulence of these mutants could be restored if subdamaging amounts of purified Pseudomonas alkaline protease were added at the time of infection and for a total of 8 consecutive days, indicating that the loss of virulence in these mutant strains was indeed due to a mutation affecting alkaline protease and not some undetected secondary or pleiotropic effect of the mutagen. Interestingly, we found that subdamaging amounts of Pseudomonas elastase could substitute for alkaline protease and restore the virulence of the alkaline protease mutants. These data indicate that while an extracellular protease is required for maximum virulence of P.aeruginosa in this model, a single specific Pseudomonas protease is not required.

Previous studies employing purified Pseudomonas proteases suggested a role for them in Pseudomonas keratitis as tissue damaging agents (16,19,20). Our results indicate that alkaline protease may, in addition, function as a colonization factor in mouse eye infections since mutants deficient in alkaline protease were less efficient in colonizing

the wounded mouse cornea and they were cleared much more readily than the parental strain. Several studies (3,33,38) have described mechanisms which could explain the role of alkaline protease in this colonization capacity. Stern, et.al. (33) have demonstrated the importance in epithelial cell damage in predisposing the cornea to adhesion of P.aeruginosa, and Woods, et.al. (38) have demonstrated a role for proteases in enhancing adherence of P.aeruginosa to buccal epithelium. Alternatively, alkaline protease may interfere with host clearance mechanisms allowing both colonization and persistance in the eye. Finally, protease production and associated tissue damage may provide essential nutrients for P.aeruginosa. This role for Pseudomonas proteases has been suggested previously for burn infections where protease-producing strains of P.aeruginosa are associated with better growth (lower generation time) in burned skin extract than are protease deficient strains (3).

While our studies indicate an important role for bacterial proteases in Pseudomonas keratitis, it is clear that protease production itself is insufficient for virulence. Ohman, et.al. (26) showed that toxin A deficient mutants derived from either strain PA103 or PAO were less virulent in a mouse eye model than their toxinogenic parental strains. These (26) and our current findings underscore the multifactorial nature of virulence in P.aeruginosa.

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TABLE 1. Extracellular Products of *P. aeruginosa* PA103 and Alkaline Protease Mutants

STRAIN	PROTEASE ACTIVITY PU/ml[a]	TOXIN ACTIVITY (CPM)[b]	FLUORESCEIN (+/-)	SHEEP HEMOLYSIN	ESTERASE (+/-)	LIPASE (zone,mm)
PA103	4.3	2580	+	0.5	+	2.0
PA103-AP1	0.0	2642	+	0.5	+	2.0
PA103-AP2	0.054	2455	+	0.5	+	2.0
PA103-AP3	0.075	2010	+	0.5	+	2.0
PA103-AP4	0.02	640	+	0.5	+	2.0
PA103-AP5	0.61	2520	+	0.5	+	2.0

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

b. CPM (counts per minute).

Table 2. Quantitation of Extracellular and Cell Associated Alkaline Protease of Parental Strain PA103 and Alkaline Protease Mutants[a]

Strain, medium	SUPERNATANT Protease Activity[b] PU/ml	% parent	Antigen[c] (Microgram/ml)	% parent	WHOLE CELL LYSATE Antigen[c]
PA103, TSB-DC	4.6	---	0.9	---	0.05
MTYG	4.0	---	0.75	---	0.02
PA103-AP1, TSB-DC	0.0	0	ND[d]	0	ND
MTYG	0.0	0	ND	0	ND
PA103-AP2, TSB-DC	0.06	1.3	0.02	2.2	ND
MTYG	0.046	1.2	0.01	1.3	ND

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

b. Determined by hide powder blue assay, see Table 1.

c. Determined by an elisa specific for alkaline protease.

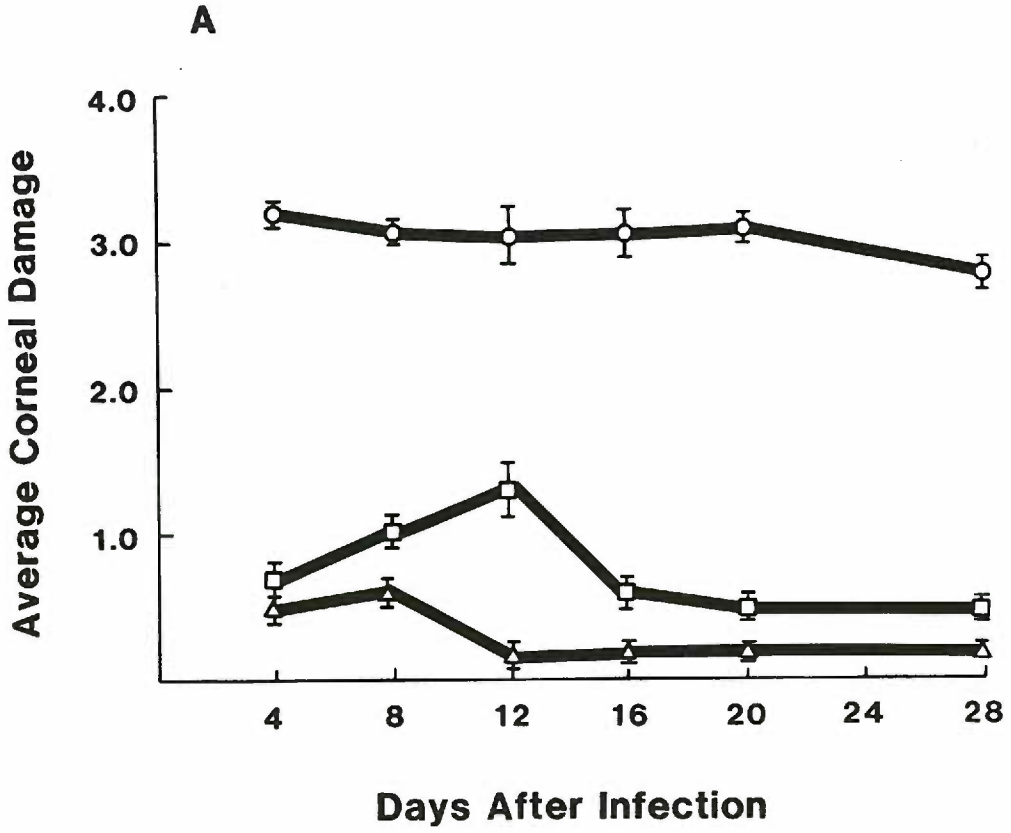
d. ND, not detectable (less than 100 picogram alkaline protease/ml).

Table 3. Recovery of P. aeruginosa parental strain (PA103) or Alkaline Protease Deficient Mutant Strains from Infected Mouse Eyes 4 and 8 days after Infection[a]

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

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

Figure 1. Average corneal damage ± standard error of the mean resulting from the inoculation of (A) 10^6 and (B) 10^7 colony-forming units of P.aeruginosa strain PA103 (O–O), the alkaline protease mutant strain PA103-AP1 (△–△) or the alkaline proteaese mutant strain PA103-AP2 (□–□).


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

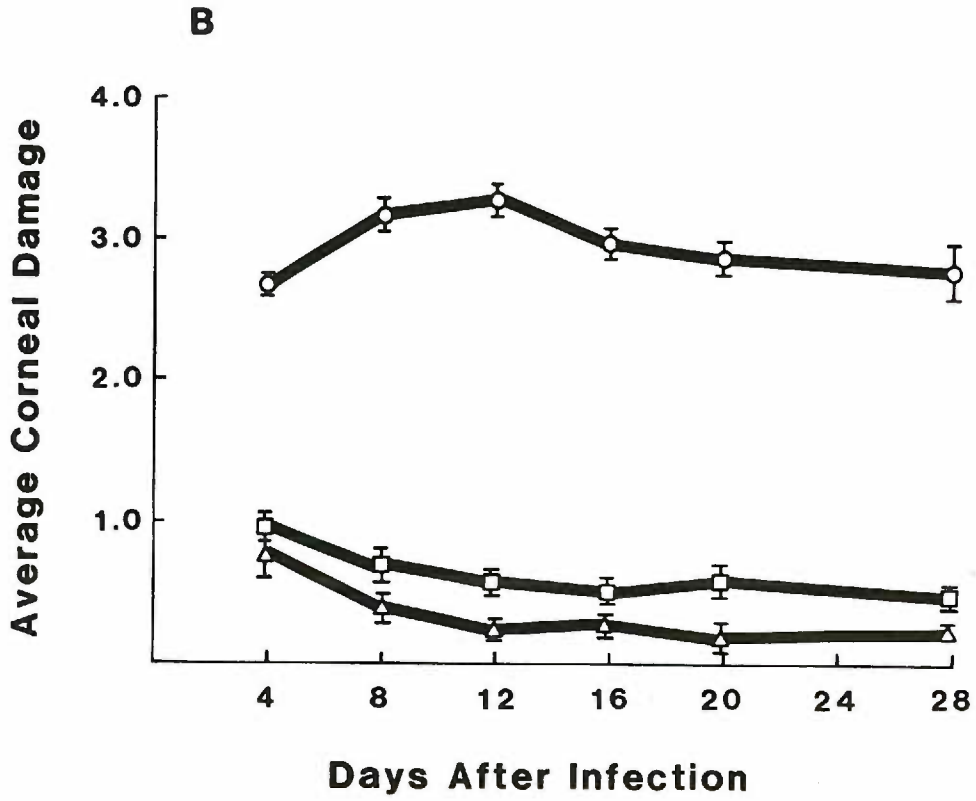

B
Average Corneal Damage
4.0
3.0
2.0
1.0
4
8
12
16
20
24
28
Days After Infection

Figure 2. Recovery of P.aeruginosa parental strain PA103 (o), or alkaline protease deficient strains, PA103-AP1 (Δ) or PA103-AP2 (■) from infected mouse eyes. A minimum of 10 mice were infected with each strain at two different inocula (10^7 and 10^8).

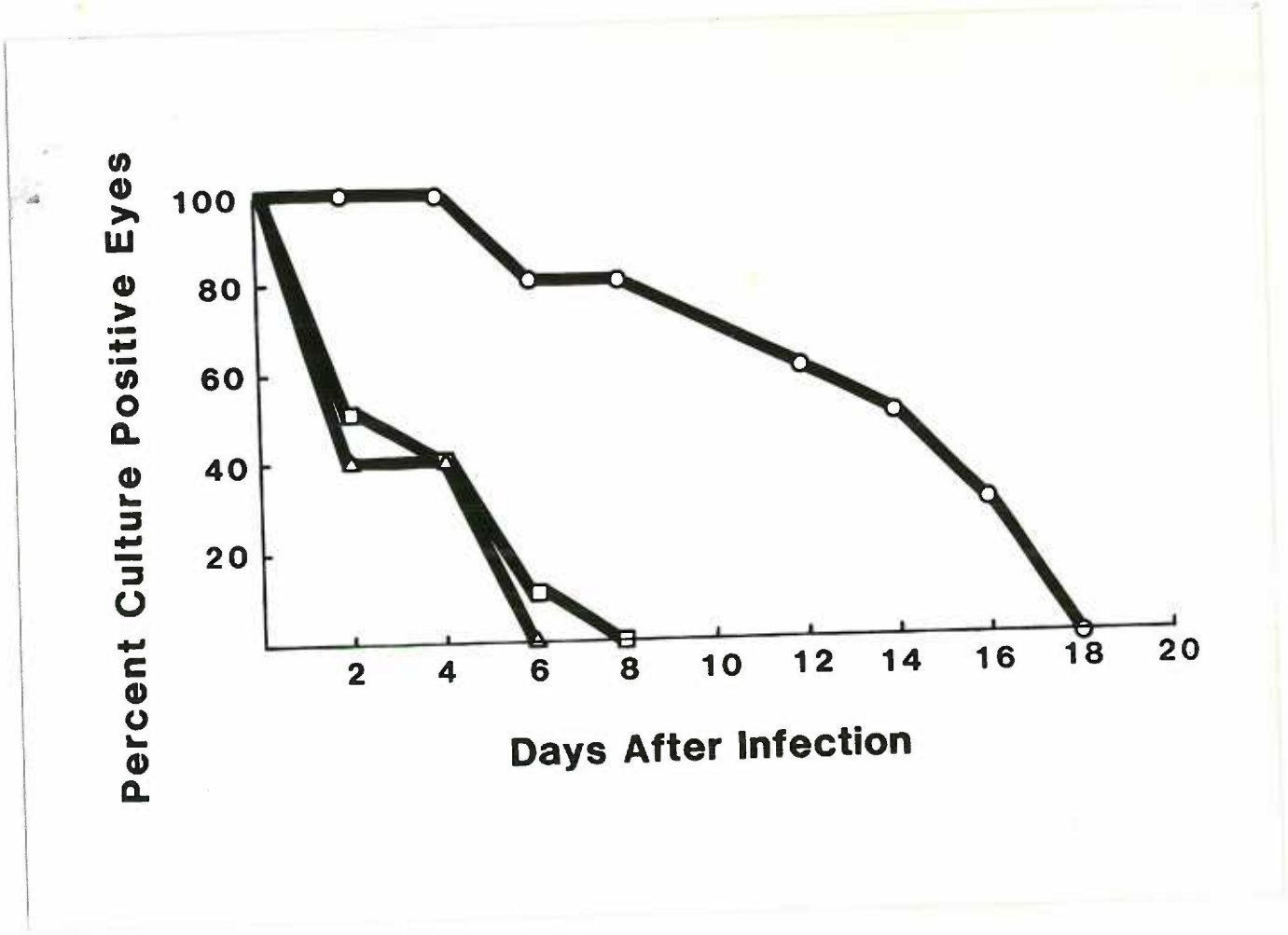
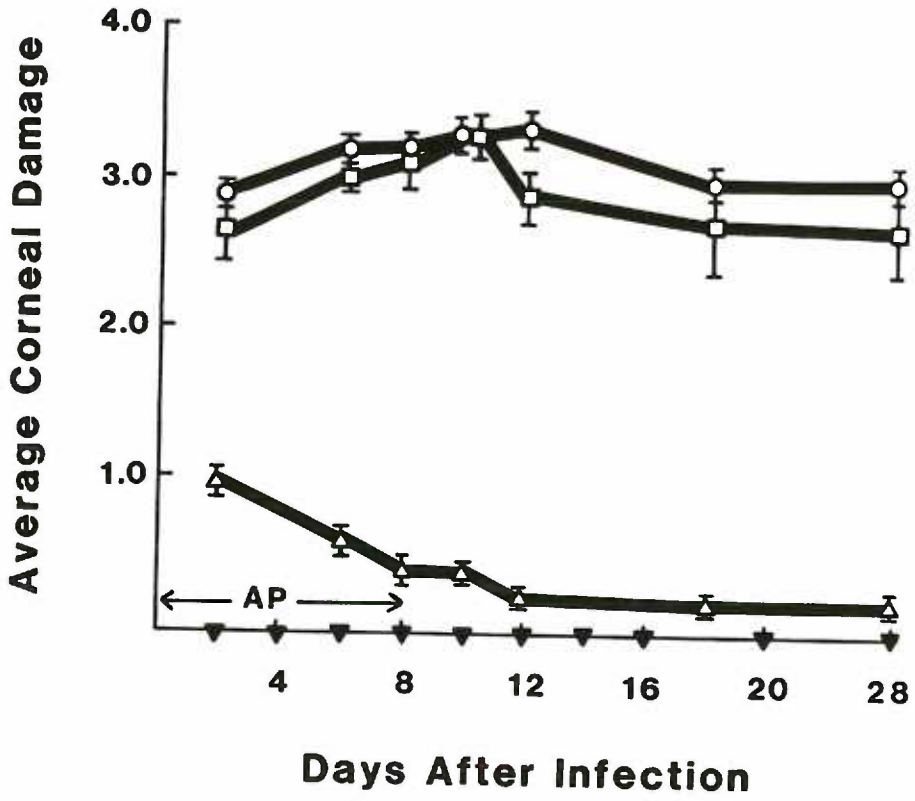

Percent Culture Positive Eyes
100
80
60
40
20
2
4
6
8
10
12
14
16
18
20
Days After Infection

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


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

Paper 2.

COMPARISON OF TWO METHODS OF GENETIC EXCHANGE USED TO DETERMINE THE GENETIC LOCUS OF THE *PSEUDOMONAS AERUGINOSA* ELASTASE STRUCTURAL GENE

Abstract

This laboratory has previously isolated and described a mutant of Pseudomonas aeruginosa which synthesizes an enzymatically inactive, but immunologically cross-reactive (CRM) elastase protein. The mutation resulting in this phenotype presumably arose within the structural gene for elastase (26).

The location of the mutation in this strain, designated las A1, was determined by R68.45 plasmid mediated conjugation and by transposon-facilitated recombination with elastase-producing recipients bearing selectable nutritional markers. Las A1 is located between tyu-9009 and nar-9011, corresponding to a map location at approximately 75' on the P.aeruginosa strain PAO chromosome.

Introduction

Pseudomonas aeruginosa produces up to three proteases, which differ in substrate specificity, molecular weight, and isoelectric point (19-21). Protease 2 (elastase) is a metallo-neutral protease with specificity for bulky or aromatic amino acids in hydrophobic proteins such as elastin (22,24). Elastase is produced by 74-98% of clinical isolates examined (7,10,25). In contrast, elastase production is rarely demonstrable by environmental isolates (10). Injection of purified elastase into animals produces necrotic skin lesions (13,18), pulmonary hemmorrhage (14), and corneal ulcers (12,14), depending upon the site and route of injection. Additionally, elastase degrades fibrin, fibrinogen, and other clotting factors (28), and in vitro, it inactivates the

complement components C1, C3, C5, C8, and C9 (29). Elastase also degrades alpha 1-proteinase inhibitor (23), one of the major serine proteinase inhibitors of human serum.

While these features suggest a role for elastase in the pathogenesis of P. aeruginosa, its relative contribution to virulence remains unclear. Our limited understanding of the genetics of P. aeruginosa proteases has precluded the construction of genetically characterized strains for appropriate virulence studies in animal models. Prior to the isolation and characterization of the mutant PAO-E64, the only protease deficient mutants previously described were pleiotropic, deficient in alkaline protease as well as elastase (33). The nature and location of genes specific for the synthesis and regulation of individual Pseudomonas proteases is obscure.

We have used a transposon-facilitated recombination system similar to the one recently described by Haas, et.al. (4) to prepare improved genetic donors in the PAO-E64 mutant strain. Similar Hfr-like donors were used successfully by Johnson and Romig (11) and by Mekalanos, et.al., (17) to facilitate mapping studies in Vibrio cholerae. These transposon- facilitated donors and R68.45 plasmid-mediated donors were mated with elastase producing auxotrophic recipients to determine the co-inheritance of the las A1 mutation with nutritional markers. Our results demonstrate that the structural gene for elastase is located between tyu-9009 and nar-9011, at approximately 75 minutes on the strain PAO chromosome. In addition, this study directly compares two methods of genetic exchange; the R68.45 plasmid-mediated system, and the Hfr-like system, involving transposon-facilitated recombination.

Materials and Methods

Strains. The bacterial strains, plasmids, and bacteriphage used in this study are shown in Table 1. All bacterial strains were derived from the prototroph PAO (27).

Media. Vogel Bonner minimal media (VBMM) (31) was used for all bacterial crosses involving selection for recombinants at catabolic markers. VBMM without citrate was used for selection of exconjugants at anabolic markers. Exconjugants were purified by cultivation on the same media used for selection of prototrophs. Elastin-nutrient agar (26) was used to differentiate elastase producing recipients from las Al donors and recipients which inherited the elastase negative (las Al) phenotype.

Recipients were maintained on nutrient agar. Donors were maintained on nutrient agar supplemented with carbenicillin (Geopen, Pfizer) or kanamycin (Kanamycin sulfate, Sigma) at 500 μg/ml. One mg/ml streptomycin (Streptomycin sulfate, Pfizer) was incorporated into minimal media to select against donors in matings employing transposon-facilitated genetic exchange.

Construction of R68.45 Plasmid Mediated las Al Donors. Auxotrophs of PAO-E64 were isolated following ethylmethane sulfonate (EMS) mutagenesis (32). The conjugative plasmid R68.45, with chromosome mobilizing ability, was introduced into PAO-E64 auxotrophs by direct plate mating (30) with the R68.45 plasmid donor PAO25 argf leu10 (R68.45).

Construction of las A1 Donors by Transposon-Facilitated-Recombination. Bacteriophage f116 lysates of P. aeruginosa strains PAO1001 trpA,B::Tn1 and PAO436 ser3 bla436 were prepared by conventional methods (15). These lysates were used to introduce the transposon Tn1 into strain PAO-E64. The conjugative plasmids RP1 (2) or R91-5 (16) were then introduced into the E64::Tn1 strains by membrane filter mating (1) under non-selective conditions, then plating to select the E64 recipients. Inheritance of the plasmid RP1 was determined by kanamycin resistance. Inheritance of R91-5 was not selectable, but was confirmed by sensitivity to the male-specific bacteriophage PRD1 (9).

Bacterial Matings. R68.45 plasmid-mediated conjugation was performed by direct plate mating (30). Transposon-facilitated genetic exchange was performed by membrane filter mating (1). Exponontial phase broth cultures of donor and recipient were mixed, then filtered through a .45 μm filter. The filter was placed on nutrient agar for two hours at 37°C, then placed in a test tube containing two mls of .85% sodium chloride (saline) to harvest exconjugants. Exconjugants were then plated on minimal agar plates with appropriate supplements and containing one mg/ml streptomycin to select against donors.

Assay for las A1 Phenotype. The mutation in strain PAO-E64 results in the synthesis of an elastase which is unable to degrade elastin at 37°C (26). Elastin nutrient agar plates were prepared as described (26). Exconjugants were picked onto these plates, and the plates incubated at 37°C for 48 hours. Colonies were examined for clear zones of elastin hydrolysis and scored as elastase negative (las A1) or elastase positive. Plates containing exconjugants which had apparently inherited las A1 were then transferred to 32°C for 24 hours. Expression

of elastase activity under these conditions confirmed inheritance of the las A1 mutation.

Results

To define the general location of the elastase structural gene, (las A1), strain PAO-E64(R68.45) was mated with various recipients containing selectable nutritional markers located at 5-10 minute intervals around the chromosome. High coinheritance with cys-54 (60%) indicated that las A1 is located in the late region of the chromosome. No linkage of las A1 to pur-67 was observed and only low (2%) linkage to catA was observed (Table 2). Since the plasmid R68.45 characteristically mediates the transfer of at least 10 minutes of genetic material, these data indicated a map position for las A1 between 70 and 80 minutes on the strain PAO chromosome.

Recipients with selectable markers in the 60-90 minute (late) region of the strain PAO chromosome were then used in R68.45 plasmid-mediated and transposon-facilitated genetic exchange. Donor strain PAO-E641, containing the plasmid R68.45, and donor strain PAO-E64 las A1 trpA,B::Tn1, containing the plasmid RP1, were mated with elastase producing auxotrophic recipients. The results of these experiments, shown in Table 2, confirm that las A1 is located near cys-54 and the tyrosine utilization gene tyu-9009 (high co-inheritance of las A1 with these markers and low co-inheritance of las A1 to markers located outside this 70-80 minute region of the strain PAO chromosome). Analysis of tyu+ exconjugants with respect to unselected inheritance of nar-9011 and las A1 [donor strain PAO-E64 las A1 trpA,B::Tn1 (RP1),Table 3] indicate that las A1 is located between tyu-9009 and nar-9011. An f116

bacteriophage lysate of strain PAO-E64 las A1 was used to transduce recipient PAO2376 to tyu+ or nar+. Tyu-9009 and nar-9011 were not co-transducible, and las A1 was not co-transducible with either of these markers, suggesting that las A1 is located at least one minute from each of markers (data not shown). These data indicate that the genetic locus of the structural gene for elastase is at approximately 75 minutes on the strain PAO chromosome.

To more precisely define the map location of las A1 we prepared donors by introducing the Tn1 insert bla 436 by transduction, then introduced the plasmids RP1 or R91-5 by conjugation. The strain from which the bacteriophage lysate was derived contains a Tn1 insert in the clockwise orientation at approximately 75 minutes on the strain PAO chromosome (15). We assumed that the Tn1 element introduced into strain PAO-E64 is in the same location and orientation as in the strain from which the bacteriophage lysate was prepared. The validity of this assumption was confirmed by examination of the results of the experiment employing these donors (Table 4). The polarity and efficiency of gene transfer associated with these donors was as would be predicted by the assumed orientation and location of the Tn1 insertion. The donor strain PAO-E64::bla 436(RP1) promoted polar chromosome mobilization in a clockwise direction, transferring cys-54 as the first selectable marker. Donor strain PAOE64::bla 436(R91-5) promoted polar chromosome mobilization in a counterclockwise direction, transferring tyu-9009 as the first selectable marker. These donors were mated with elastase producing recipients auxotrophic for cys-54 and tyu-9009, respectively. The results, shown in table 4, demonstrate high coinheritance of las A1 with tyu-9009. These data indicate that las A1 is located

counterclockwise to the Tn*1* insertion bla 436, and confirm the map order *tyu-9009*, *las A1*, (bla 436), *nar-9011*, *cys-54*.

Discussion

Genetic studies in Pseudomonas have traditionally been limited by the paucity of selectable markers in the late region of the chromosome, and the lack of high frequency recombination (Hfr) donors to promote efficient chromosome mobilization of material in this late region. However, the PAO chromosome has recently been shown to be circular, and additional selectable nutritional markers have been identified in the late region of the chromosome (27). Further, transposon facilitated recombination has been used to generate Hfr-like genetic donors in Pseudomonas (4). We have employed these advances to determine that the genetic locus of the structural gene for *Pseudomonas* elastase is at approximately 75' on the chromosome of strain PAO.

We have directly compared two systems of genetic exchange, the R68.45 plasmid-mediated system , and a transposon-facilitated recombination system similar to the one recently described in *P.aeruginosa* by Haas, *et.al.*(4). Our results suggest that, while the linkage data are qualitatively similar with both mechanisms of chromosome mobilization, the donors constructed by transposon-facilitated recombination are associated with higher overall co-inheritance values for the markers examined (Table 2). The polar nature of transposon-facilitated genetic exchange, and the improved efficiency of transfer of genes located near the site of the chromosomal Tn*1* insertion underscore the utility of this system for genetic mapping. Also, the

donors constructed by transposon-facilitated recombination seem to promote co-transfer of genes separated by distances beyond the typical range of the R68.45 plasmid-mediated system (Table2). However, the fixed origin of transfer (the site of the chromosomal Tn*1* insertion) provides for inefficient transfer of genes located distal to the site of the chromosomal Tn*1* insertion. Much higher co-inheritance of *las A1* with *tyu-9009* is observed in crosses involving the Tn*1* insert at 75'(Table 4) than in crosses involving the Tn*1* insert at 25' (Table 2). Therefore, transposon-facilitated genetic exchange is most useful when a general location of the gene of interest is known, so that the optimal chromosomal Tn*1* insert and conjugative plasmid can be chosen for construction of the most efficient donor.

The polarity of chromosome mobilization associated with donors constructed by transposon-facilitated recombination has not yet been fully exploited. Polar chromosome mobilization should facilitate the rapid determination of gene location by interupted mating and analysis of gradient of transmission (8), while mapping involving the plasmid R68.45 necessitates a measure of co-inheritance frequencies (6). Donors constructed by transposon-facilitated recombination, when employed in interupted mating experiments, should more accurately define gene locations in the late region of the strain PAO chromosome.

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Table 1
P. aeruginosa strains, plasmids, and bacteriophage

Strain	Genotype	Reference or Source
PAO-E64	las A1	Ohman, et.al.(26)
PAO-E641	las A1, his	This study[2]
PAO-E645	las A1,arg	This study[2]
PAO12[3]	leu-8, pur-136,chl-3	Royle, et.al.(27)
PAO18[3]	pro-64, pur-66, chl-3	" " " "
PAO25[3]	argF, leu-10	" " " "
PAO166[3]	pyrF63, leu-17	" " " "
PAO236[3]	his-4, ilvB, lys-12 trp-6, met-28, proA82	Haas and Holloway (3)
PAO436	ser-3, bla 436	Krishnapillai, et.al. (16)
PAO944[3]	thr-9001, cys-54, pur-67	Royle, et.al. (27)
PAO9441[3]	thr-9001, cys-54, pur-67 strI	spontaneous derivative of PAO944
PAO949[3]	thr-9001, cys-59, pur-67	Royle, et.al. (27)
PAO9491[3]	thr-9001, cys-59, pur-67, strI	spontaneous derivative of PAO949
PAO1001	trpA,B::Tn1 his-301 strI	Haas et.al. (4)
PAO2249[3]	catA1, met-9011, puuE	Matsumoto collection
PAO2368[3]	catA1, cnu-9001, met-9020, nar-9011, puuE8	Royle, et.al. (27)
PAO2376[3]	catA1, met-9020, nar-9011, tyu-9030, mtu-9002	" " " "
PAO23761[3]	catA1, met-9020, nar-9011, tyu-9030, mtu-9002, strI	spontaneous derivative of PAO2376

Table 1, continued

Plasmid[4]		
R68.45	Cb Km Tc Tra Cma	Haas and Holloway (3)
RP1	Cb Km Tc Phi(G101) Dps (PRR1, Pf3, PRD1, PR3, PR4) Tra IncP-1	Grinsted, et.al. (2)
R91-5	Cb Phi(G101) Dps (PRD1, PR3,PR4) Tra IncP-10	Jacoby and Shapiro (9)

Bacteriophage	Characteristics	
F116L[5]	General transducing phage	Krishapillai (15)
PRD1[6]	Donor specific phage for RP1 and R91-5	Jacoby and Shapiro (9)

1. The genotype symbols are according to Holloway, et. al. (8) except for mtu = inability to use mannitol as sole carbon source and bla = resistance to carbenicillin. Plasmid symbols are according to Jacoby and Shapiro (9) except for cma = host chromosome mobilizing ability.

2. Auxotrophs of strain PAO364 were obtained following EMS mutagenesis, using the procedure described by Watson and Holloway (32).

3. Elastase positive recipients, all derived from prototroph PAO.

4. Used to construct las A1 donors.

5. Used for generalized transduction, and to effect Tn1 insertion.

6. Used to confirm presence of the plasmid R91-5.

Table 2
Co-inheritance of Las Al and Selected Nutritional Markers
Comparison of R68.45 plasmid-mediated and tfr-mediated systems[1]

Recipient	Selected Marker[1]	Map Position[2]	Co-inheritance Mediated by R68.45[3]	Co-inheritance Mediated by Tfr[3]
PAO166	pyrF63	58[1]	0%	2%
PAO2249	catAl	64[1]	2%	10%
PAO2376	mtu-9002	69[1]	11%	43%
PAO2376	tyu-9030	74[1]	45%	69%
PAO944	cys-54	79[1]	65%	86%
PAO2368	cnu-9001	81[1]	43%	34%
PAO944	pur-67	89[1]	0%	12%

1. See Table 1 for genotype symbols. Donors construced by transposon-facilitated recombination contain a Tnl insertion at TrpA,B.

2. Based on recalibrated PAO genetic map (27). Positions at markers between 60-95' are approximate.

3. Greater than 100 excongugants were scored for each selected marker.

Table 3

Co-inheritance of *las A1* with *tyu-9009* and *nar-9011* [1,2]

	tyu^+ nar^+	tyu^+ nar^-	*las A1*
tyu^+	180(46%)	209(54%)	29(75%)
tyu^+ nar^+	-	-	155(86%)
tyu^+ nar^-	-	-	136(65%)

1. This cross involved conjugation of the donor PAO-E64 *trpA,B*::Tn*1*(RP1) with the elastase-producing recipient PAO23761 *tyu-9009*, *mtu-9002*, *catA1*, *nar-9011*, *met-9009*, *strI*.

2. A total of 389 *Tyu+* exconjugants were selected, then these tyu^+ exconjugants were examined for unselected inheritance of *las A1* with *tyu-9009* and *nar-9011*.

Table 4

Demonstration of *las A1* Location by Transposon-Facilitated Recombination

Donor	Cma[1]	Recipient	Selected Marker	% Unselected Co-Inheritance of Las A1
E64::Tn*1* (436)RP1	C[1]	PA09441	cys-54	1
E64::Tn*1* (436)R91-5	CC[1]	PA023761	tyu-9009	99[3]

1. Cma=chromosome mobilizing ability. C = chromosome mobilization in a clockwise direction , CC =chromosome mobilization in a counterclockwise direction.

2. Greater than 100 exconjugates were examined at each selected marker.

3. R91-5 has chromosome mobilizing ability at a low frequency from sites other than the Tn*1* insert.

Paper 3.

GENETIC CHARACTERIZATION OF A TOXIN-DEFICIENT MUTANT OF *PSEUDOMONAS AERUGINOSA* PAO

Abstract

The location of a mutation conferring toxin A deficiency has been determined by R68.45 plasmid-mediated conjugation and linkage analysis. The mutation, designated tox-3, results in a complete lack of detectable toxin A activity. Examination of supernatants and whole cell lysates of this mutant with an enzyme linked immunosorbant assay specific for toxin A reveals that this mutant synthesizes no immunologically related toxin A protein in either an extracellular or cell-associated form. The tox-3 mutation was linked most strongly to the markers cys-54, at ca. 80', and pur-67, at ca. 90'. Tox-3 is transferred most efficiently when selection for co-repair of the markers cys-54 and pur-67 is made, indicating a position for tox-3 between these markers, at ca. 85' on the chromosome of P.aeruginosa PAO. This places tox-3 at or near the structural gene locus, tox A1.

Introduction

Mutants have been described previously which are deficient in the production (3,10,12,14) or activity (2) of toxin A. One such mutant, designated PAO-T1, produces no detectable toxin activity in either an extracellular or cell-associated form (10), but is otherwise indistinguishable from the parental strain PAO. This feature distinguishes this mutant from the hypotoxinogenic mutants described previously which produce markedly decreased, but detectable, levels of toxin A activity (3,12). The mutant PAO-T1 has been preliminarily

characterized and found to be of decreased virulence in several animals, suggesting a role for toxin A in virulence (10,11).

Gray and Vasil isolated mutants of P.aeruginosa PAO which produce greatly decreased levels of toxin A (3). One of these mutants, PAO-T20, produces 100-fold decreased levels of toxin A, but is otherwise indistinguishable from the parental strain PAO. The mutation in this strain, designated tox-2, is located near trpC,D,E at ca. 35' on the PAO chromosome (3). They also described a second mutant, designated PAO-T10, which produces decreased levels of toxin A and total protease. The mutation in this strain, designated tox-1, is located at ca. 38' on the strain PAO chromosome (3). More recently, Hanne, et.al.(5) determined that the genetic locus of the P.aeruginosa toxin A structural gene, tox A1, is at ca. 85' on the PAO chromosome. This study was initiated to genetically characterize the mutant strain PAO-T1 to attempt to discern the relationship between the mutation in this strain, designated tox-3, and the three toxin loci which have been previously been characterized.

Materials and Methods

Bacterial strains. All strains were derived from the prototroph P.aeruginosa PAO (13). The toxin-deficient mutant PAO-T1 has been described (10). The distinguishing characteristics of this strain and the wild-type strain PAO are shown in table 1. All genetic strains used were derived from PAO, and are toxinogenic. Their phenotypes are listed in table 2.

Media. Vogel Bonner minimal medium (VBMM) (17) was used for all bacterial crosses. Exconjugants were grown in trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) which had been chelex-treated, dialyzed, and supplemented with 50 mm monosodium glutamate and 1% glycerol (TSB-DC) (1). When the recipient strains PAO944 or PAO949 were used, this medium was further supplemented with 0.01% adenine (Sigma Chemical Co., St. Louis, Mo.) (5).

Recipients were maintained on nutrient agar. Donors were maintained on nutrient agar containing carbenicillin (Geopen, Pfizer, Inc., New York) or kanamycin (Kanamycin sulfate; Sigma) at 500 microgram/ml.

Construction of tox-3 donors. Auxotrophs of P.aeruginosa strain PAO-T1 were obtained following ethylmethane sulfonate (EMS) mutagenesis (19) and carbenicillin enrichment (20). The plasmid R68.45, with chromosome mobilizing ability (4), was introduced into auxotrophs of PAO-T1 by direct plate mating (15) with the strain PAO25 argF leu-10 (R68.45) and selecting for carbenicillin resistance. The presence of

the plasmid R68.45 was confirmed and maintained by growth on nutrient agar containing kanamycin at 500 microgram/ml.

Bacterial matings. R68.45 plasmid-mediated conjugation was performed by direct plate mating (15). Briefly, mid-exponential phase cultures of donors and recipients were centrifuged, washed with phosphate-buffered saline (PBS), and resuspended in PBS to 0.5 volume. Next, 0.1 ml aliquots of donor and recipient were combined and added to VBMM plates, which were spread and then incubated at 37°C for 48 hours. Exconjugants were picked onto the same selective medium to purify them, then onto TSB-DC plates and incubated at 32 C prior to examination of their toxin A phenotype.

Preparation of anti-toxin. Exotoxin A was purified from P.aeruginosa strain PA103 as described previously (8). This purified toxin was used to immunize sheep as previously described(8). Anti-toxin A immunoglobulin fraction G (anti-A IgG) was obtained from sheep immune serum as described (16).

Agar well assay for toxin production. An agar well assay has been described which differentiates toxin A deficient and proficient colonies (12). Exconjugants were picked onto TSB-DC plates and incubated at 32°C for 24 hours. Next, small wells were made between colonies using a sterile pasteur pipette attached to a vacuum source, and each well filled with 5 microlitres of sterile anti-toxin. After incubation of these plates for 18 hours at 32°C, toxin production by exconjugants resulted in

a visible precipitin band between exconjugant colonies and anti-serum wells.

Enzyme-linked immunosorbant assay (ELISA) for toxin A. Toxin production was quantitated using an ELISA. Coating buffer, phosphate-buffered saline (PBS), PBS-Tween, PBS with 1% bovine serum albumin (Sigma) and diethanolamine buffer were prepared as described by Voller, et.al.(18). A conjugate of alkaline phosphatase and sheep anti-toxin A was prepared essentially as described by Kearney, et.al. (9). Briefly, 0.5 mg sheep anti-toxin A IgG was added to 1.5 mg alkaline phosphatase (intestinal type VII; Sigma). PBS was added to a final volume of 0.5 ml, and the mixture dialyzed against PBS for 18 hours. To this material was added 4 microlitres 25% glutaraldehyde, and the mixture was incubated at 25°C for 4 hours. This material was then dialyzed extensively with PBS, then diluted to 20 ml with 0.5 M tris-Hcl, 0.001 M $MgCl_2$ 0.04% azide, 5% BSA, pH 8.0. This conjugate was stored in aliquots at -20 C. This conjugate was used in the elisa assay as described (5).

Results

Preliminary characterization of the mutant PAO-T1 demonstrated that this mutant was devoid of extracellular toxin A activity (10). To determine whether this mutant produced toxin A in an immunologically related, but inactive form, or accumulated a proenzyme of toxin A in a cell-associated form supernatants and whole cell lysates of PAO-T1 were prepared and examined in a sensitive ELISA assay. No toxin-related antigen was detected in concentrated supernatants or cell lysates from

PAO-T1, indicating that this mutant does not synthesize detectable quantities of toxin A (Table 1).

To determine the genetic locus for tox-3, auxotrophs of P.aeruginosa PAO-T1 containing the plasmid R68.45 were mated with toxin A-producing recipients, and the resultant exconjugants examined for toxin production. Toxinogenic recipient strains containing selectable markers in the 0-60' region of the chromosome produced sharp precipitin bands in an agar well immunoassay for toxin A, while toxin-deficient donor strains derived from PAO-T1 produced no precipitin band in this assay. Therefore, inheritance of the tox-3 mutation resulted in loss of the ability to produce a precipitin band in the agar well assay. Exconjugants resulting from repair of markers in the 0-60' region of the chromosome uniformly produced sharp precipitin bands in the agar well assay, indicating that the tox-3 mutation is not located in this region of the chromosome (table 3). Next, tox-3 donors derived from PAO-T1 were mated with toxinogenic recipient strains with selectable markers in the 60-95' region of the chromosome, and the resultant exconjugants examined for inheritance of tox-3. The tox-3 mutation was linked to the markers cys-54, at ca. 80', and pur-67, at ca. 90', resulting in lack of a precipitin band in a fraction of the exconjugants selected by repair of these late markers. However, the recipient strains which contain markers in this region of the chromosome, i.e. PAO944 and PAO949, are relatively poor toxin producers, such that they can not be conveniently scored for toxin production using the relatively insensitive agar well assay. For this reason, a more sensitive enzyme linked immunoassay (ELISA) for toxin A production was developed. Exconjugants of PAO944 and PAO949 obtained from matings with PAO-T1 (R68.45) were grown in

liquid TSB-DC in microtitre wells, and the toxin A levels in supernatants determined using an ELISA for toxin A. The results of this analysis, shown in table 4, demonstrate linkage of tox-3 to cys-54, located at ca. 80', and to pur-67, located at ca. 90' on the strain PAO chromosome. The tox-3 mutation was linked to both of these markers by ca. 20%: lower linkage to markers outside of this region of the chromosome was observed. Further, exconjugants which were selected for co-repair of cys-54 and pur-67 were almost uniformly tox-3, while exconjugants which resulted from co-repair of pur-67 and cys-59 (located at ca. 95') show considerably less linkage of tox-3. These results indicate a position for tox-3 between cys-54 and pur-67, in the 80-90' region of the P.aeruginosa PAO chromosome. The relatively low linkage of tox-3 to each of these markers indicates that tox-3 is located at a considerable distance from each of these markers. For this reason, transduction was not attempted.

Discussion

A genetic analysis of P.aeruginosa virulence factors has recently been made feasable by demonstration of chromosomal cirularity (13) and development of a conjugative plasmid which can mobilize the chromosome from any position (4). These advances have facilitated the determination of the chromosomal location of the structural genes for elastase (7) and toxin A (5), as well as the location of genes which regulate the synthesis or secretion of several extracellular products (3,21).

Toxin production by P.aeruginosa is strain dependant (11) and is influenced by culture conditions and composition of the growth medium (1). Iron concentration of the growth medium is particularily important (14). These observations indicate that toxin synthesis in P.aeruginosa is highly regulated. Several distinct classes of toxin mutants have been described, including a toxin-deficient mutant (10), hypo-toxinogenic mutants (3,12), a toxin A structural gene mutant (2), and an iron-deregulated mutant (14). These observations, and the demonstration of at least two distinct genetic loci which govern toxin production (3) indicate that regulation of toxin production is complex. A thorough genetic characterization of mutants altered in toxin production should elucidate the mechanism(s) by which toxin production is regulated.

In this study, the location of a mutation, tox-3, which confers toxin-deficiency has been determined. It is of interest that the tox-3 mutation, which appears to be specific for toxin A (10), maps at or near the location of the toxin A structural gene. This is in contrast to the

observed map location of the tox-2 mutation described by Gray and Vasil (3), for which a map position at ca. 35' was determined. The mutation in PAO-T1 may reside within the promoter region or within the toxin A structural gene, such that stable product is not synthesized. Alternatively, the tox-3 mutation may affect processing or secretion of toxin. Were this the case, one would anticipate the detection of low levels of toxin, or enzymatically inactive toxin in a pro-enzyme form. When the complete lack of detectable toxin antigen in PAO-T1 is considered in the context of the observed map location of the tox-3 mutation, the first possibility appears more likely. The toxin-deficient phenotype and observed specificity of the mutation in this strain suggests that this mutant should be an ideal candidate for use as a recipient in recombinant DNA experiments designed to clone the P.aeruginosa toxin A structural gene. Successful cloning of the toxin gene, and molecular characterization of the tox-3 mutation are necessary to definatively address the molecular nature of this mutant.

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Table 1. Toxin production by the parental strain PAO and the mutant strain PAO-T1.[1]

Strain	Exotoxin A[2]
PAO	0.3µg/ml
PAO-T1	ND[3]

1. Concentration of exotoxin A determined on supernatants from organisms grown in TSB-DC in microtiter plates.
2. Determined by an ELISA specific for exotoxin A.
3. ND, not detectable (less than 1 ng per ml).

Table 2. Bacterial Strains[1]

STRAIN	GENOTYPE or PHENOTYPE	REFERENCE or SOURCE
PAO	prototroph, chl-2	Holloway, et.al. (6)
PAO12	leu-8, pur-136	Royle, et. al. (13)
PAO18	proB64, pur-66, chl-3	" "
PAO25	argF10, leu-10	" "
PAO236	met-28, trp-6, lys-12, his-4, ilvB226, proA82, nal-2	Haas and Holloway (4)
PAO944	thr-9001 cys-54, pur-67	Royle, et. al. (13)
PAO949	thr-9001, pur-67, cys-59,	" "
PAO2249	catA1, met-9020, puuE8	Matsumoto Collection
PAOT1	prototroph, tox-3	Ohman Thesis (10)
PAOT103	His_, tox-3	EMS[2]mutagenesis of PAOT1
PAOT105	argF, tox-3	" "

1. All strains were derived from PAO. Genotype symbols are according Holloway, et. al.(6).
2. EMS, ethyl methane sulfonate mutagenesis as described (19).

Table 3. Linkage of *tox-3* to Selected Markers[1]

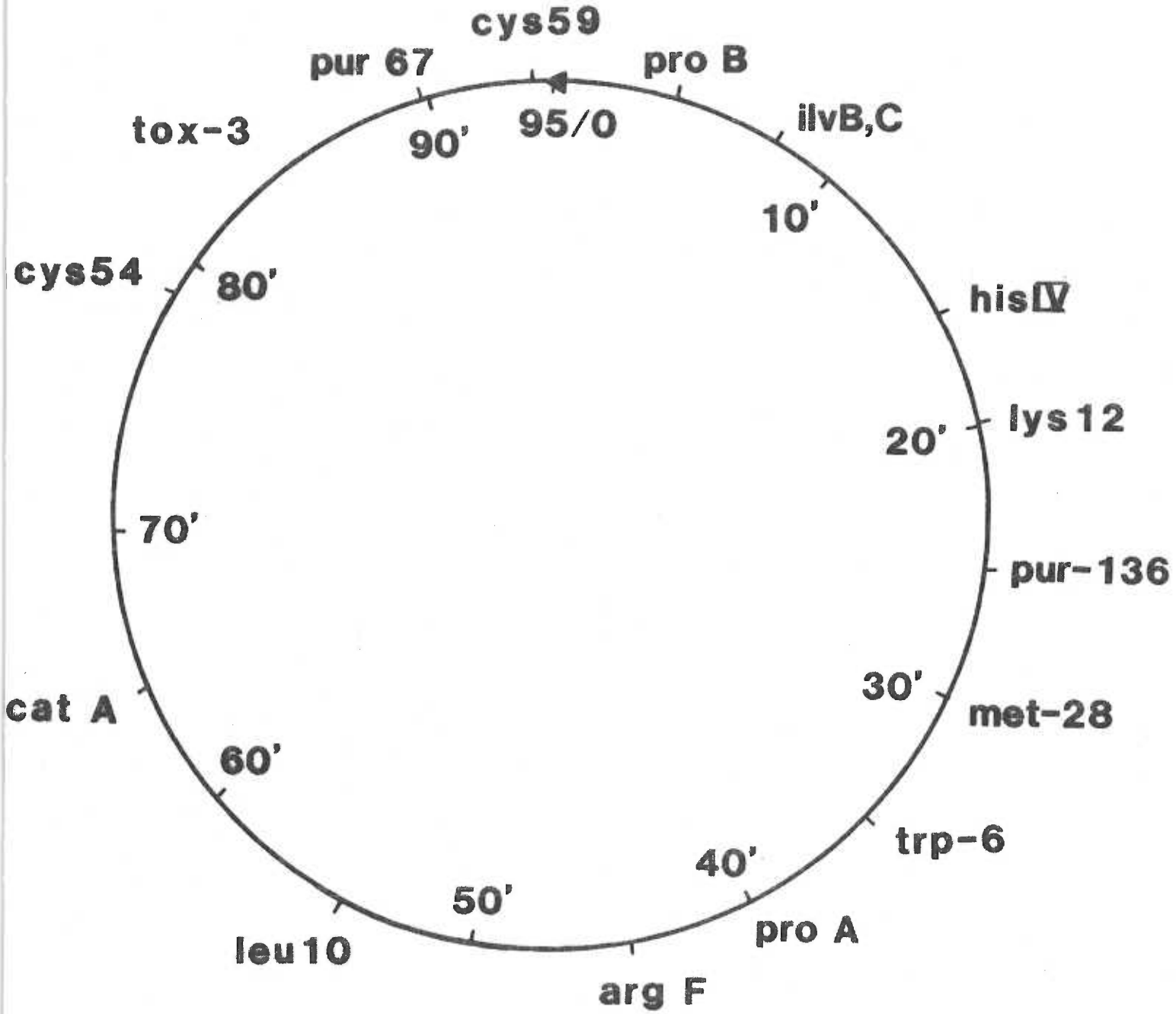
Recipient	Selected Marker	Map Position[2]	%Co-Inheritance of tox-3[3]
PAO18	*proB64*	4'	0/100 (0)
PAO236	*ilvB226*	8'	0/100 (0)
PAO236	*his4*	16'	0/100 (0)
PAO236	lys-12	20'	0/100 (0)
PAO12	*pur-136*	25'	0/100 (0)
PAO236	*met-28*	30'	0/100 (0)
PAO236	*trp-6*	35'	0/100 (0)
PAO236	*proA82*	40'	0/100 (0)
PAO25	*argF*	45'	0/100 (0)
PAO25	*leu-10*	55'	0/100 (0)
PAO2249	*catA1*	65'	1/100 (1%)
PAO944	*cys-54*	80'	19/80 (20%)
PAO944	*cys-54-pur-67*	80'-90'	45/48 (94%)
PAO949	*pur-67*	90'	11/40 (28%)
PAO949	pur-67-cys-59	90-95'	6/40 (15%)
PAO949	*cys-59*	95'	7/40 (15%)

1. Exconjugants at selected markers were purified and inheritance on *tox-3* scored as an unselected marker.

2. Locations of markers from 60-95' are approximate. All map locations are according to Royle, *et. al.* (13).

3. Linkage of *tox-3* to markers from 0-65' were determined by the agar well assay. Linkage of *tox-3* to markers from 80-95' were determined by the ELISA assay.

Figure 1. Genetic map of Pseudomonas aeruginosa PAO containing the map location of tox-3 . Markers used in this study are positioned as previously described (6).


cys59
pro B
ilvB,C
10'
hisIV
lys 12
20'
pur-136
30'
met-28
trp-6
40'
pro A
arg F
50'
leu 10
60'
cat A
70'
cys54
80'
tox-3
pur 67
90'
95/0

IV. Discussion and Summary

P.aeruginosa is an organism of remarkable diversity. The metabolic potential of this organism has long been appreciated. This organism also has a diverse host range, infecting man and other vertebrates, terrestrial and aquatic invertebrates, and plants (4). Within a single host such as man, P.aeruginosa can produce a variety of distinct infectious processes, depending upon underlying host factors and characteristics of the infecting strain. For example, P.aeruginosa produces a chronic, localized pneumonia in infected cystic fibrosis patients (18), whereas pulmonary infections in immunologically compromised patients may progress rapidly to septicemia, and are associated with a high mortality rate (8). Similarily, eye infections in healthy adults compromised by corneal trauma are localized and self-limiting (9), while eye infections in premature infants and immunocompromised adults are not localized, and may lead to dissemination and death (2). Strain related differences also contribute to the outcome of infection. In the guinea pig model of acute lung infection, certain strains of P.aeruginosa produce considerable lung damage but low mortality, while other strains of P.aeruginosa are rapidly fatal in this experimental system (17).

Strain related differences in the outcome of infection may be attributable to differences in virulence determinants possessed by different strains of P.aeruginosa. Several extracellular products have been identified which may enhance the virulence of P.aeruginosa. Among these are exotoxin A, elastase, alkaline protease, heat-stable hemolysin, phospholipase C, enterotoxin, pigments, and exopolysaccharides. Diversity

of virulence factors and diversity in infectious processes underscore the complexity of the pathogenesis of P.aeruginosa.

Several studies have attempted to correlate the properties of virulent but unrelated strains of P.aeruginosa with virulence in animal models. We now know that such an approach is inadequate to adequately assess the role of individual factors in disease. Often, such an approach has failed to correlate any individual product with virulence and such studies have provided conflicting information regarding the relative importance of individual factors. For example, in studies examining the role of protease in bacteremia, Liu and Hsieh (10) reported that protease-producing strains of P.aeruginosa were less virulent than protease-deficient strains, while Muszynski and Kedzia (12) observed that protease enhanced virulence of strains in this type of infection. These confusing results arise from attempts to compare strains so vastly dissimilar as to preclude any valid association of individual factors with virulence.

A genetic approach has been described which circumvents many of the variables which complicate studies of bacterial pathogenesis (7). By isolating mutants specifically deficient in individual virulence factors, and examining these mutants in animal models, alterations in virulence of the mutant are correlated with the individual factor which corresponds to the mutant phenotype. This approach has recently been applied to the study of the pathogenesis of P.aeruginosa infections. Ohman, et.al. isolated and characterized mutants of P.aeruginosa altered in the production or activity of exotoxin A (13,15) and elastase (14). Examination of these mutants in several animal models has demonstrated a role for toxin A in corneal infections (16), chronic pulmonary infections

(19), and burn infections (13), and a role for elastolysis in acute and chronic lung infections (1,19). These studies, which for the first time demonstrate directly the role of exotoxin A and elastase in different types of infection, illustrate the contribution such genetic studies can make to our understanding of bacterial pathogenesis.

In the current study, a genetic approach has been undertaken to address the role of alkaline protease in the virulence of P.aeruginosa (paper 1). The first step in such an approach involves the isolation of mutants specifically deficient in the production or activity of alkaline protease. The majority of strains examined, including the genetically characterized strain PAO, produce two extracellular proteases, elastase and alkaline protease. Both proteases are active against casein, while elastase is also capable of degrading elastin. Therefore, specific alkaline protease mutants cannot be isolated from such strains on the basis of inability to degrade casein, since this approach would result in the isolation of mutants deficient in elastase as well as alkaline protease. Certain inhibitors of protease activity such as phosphoramidon and ethlyene diaminetetraacetic acid (EDTA) inhibit elastase activity specifically under defined conditions. Unfortunately, such inhibitors adversely affect cell growth at functional concentrations, such that selective inhibition of elastase is not presently a viable approach to isolate specific alkaline protease mutants. Immunological methods of mutant detection, based upon antibody raised against alkaline protease may be employed in agar well type assays or enzyme linked immunosorbant assays (elisa) to identify mutants deficient in alkaline protease. However, such assays are extremely labor intensive, and can not identify mutants which produce enzymatically

inactive, immunologically cross reactive (CRM) protein. For these reasons, I chose to isolate alkaline protease mutants in a strain which produces only a single extracellular protease, alkaline protease. This enabled me to identify mutants following mutagenesis with the relatively mild mutagen EMS by screening colonies on skim milk agar plates. In this study, mutants were identified which were specifically deficient in the production of alkaline protease. A hypoprotease producing mutant and an alkaline protease deficient mutant were examined in the mouse eye model to address the role of alkaline protease in Pseudomonas keratitis. Neither alkaline protease mutant could establish an infection and they were cleared from infected eyes much more rapidly than was the parental protease-producing strain PA103. This role of alkaline protease in establishing an eye infection was confirmed by in vivo complementation studes which demonstrated that the persistance of alkaline protease-deficient mutants was enhanced in the presence of alkaline protease produced by another strain in a mixed infection, or by the effect of exogeneous protease added daily to infected eyes. The results of this study demonstrate a role for alkaline protease in eye infections caused by P.aeruginosa . In this study, the effect of exogenous elastase on eye infections caused by the mutant strains was also addressed. Addition of sub-damaging amounts of elastase to eyes infected with protease-deficient mutants also restored the ability of these mutants to establish an infection and cause corneal damage. I observed no marked difference in the outcome of infections complemented by alkaline protease or elastase, indicating that there is a requirement for bacterial protease in this type of infection, and that this requirement may be met by either of the proteases produced by

P.aeruginosa. The results of this study extend the findings of Ohman, et.al. (16) who demonstrated a role for toxin A in Pseudomonas eye infections, and illustrate the multi-factorial nature of the pathogenesis of P.aeruginosa keratitis.

The above work illustrates one aspect of a genetic analysis of virulence. Another aspect involves the genetic characterization of mutant strains. Genetic mapping studies are important with respect to virulence studies since one inherrent limitation of studies which employ chemical mutagens is the potential for secondary mutations which alter the characteristics of the mutant strain. This potential for secondary mutations, which often go undetected, is particularily likely with studies which employ nitrosoguanidine (NTG). NTG often induces multiple mutations, and, additionally, it induces prophages and aeruginocins, resulting in alterations in the mutant cell envelope (6). Even after extensive characterization of NTG-induced mutants prior to examination in animal models, the potential presence of undetected secondary mutations complicates the interpretation of animal studies involving these mutants. Identification of the location of the gene of interest allows one to move the desired mutated gene from the mutagenized strain into a pristine strain which can then be used in animal studies. Knowledge of map location of genes of interest may also facilitate localized mutagenesis, resulting in the isolation of several mutants with mutations in the gene of interest. Availability of such mutants would contribute to our understanding of the genetic organization of genes encoding the virulence factor under investigation.

The requirements for genetic characterization have been outlined by Holloway (6). First, mutants must be available which have scorable

phenotypes corresponding to alterations in their virulence properties. Second, a system of genetic exchange must be available, as well as multiply marked strains to be used as recipients in genetic exchange. The location of the mutation in the strain of interest can be determined by examination of the segregation of the mutation with selected markers in the recipient strain, that is, by examining the linkage of the mutation with markers whose map location is known. Genetic mapping studies enable one to ask the following questions. First, is the gene which has been mutationally altered on the chromosome? Second, are mutants of identical phenotype genetically the same or different? Demonstration of different map locations for the mutations in two mutants with similar phenotype would argue that they correspond to mutations in separate genes, while demonstration of similar map locations would argue that the mutant strains result from mutations in the same or functionally related genes. Third, do genes affecting related functions show any special spacial relationship on the chromosome? While gene clustering is not as evident in P.aeruginosa as has been described for E.coli , this may be a reflection of our limited knowledge of gene locations in Pseudomonas. Recent studies (11) have in fact demonstrated clustering of utilization genes, whose products enable Pseudomonas to utilize purines as sources of carbon.

Attempts to genetically characterize the alkaline protease mutants obtained in by study were unsuccessful. While no pyocin or related bacterial antagonism was observed, conjugation between alkaline protease mutants of strain PA103 containing the plasmid R68.45 and auxotrophic recipients derived from the genetically characterized strain PAO were unsuccessful, even after cultivation of the recipients at 43°C to

inactivate their restriction and modification processes. Other investigators have experienced similar problems regarding conjugation between unrelated strains of P.aeruginosa (D. Ohman, pers. comm.). It is evident from these studies that the so-called 43°C effect does not always provide for successful conjugation between distinct strains of P.aeruginosa.

In theory, it may be possible to transfer the mutated alkaline protease genes from the mutants of P.aeruginosa strain PA103 to strain PAO by transduction, then conjugally transfer the mutated genes into auxotrophic recipients derived from strain PAO. This approach to mapping alkaline protease genes is not feasable, as it would be necessary to select for inheritance of the mutated alkaline protease genes following transduction into strain PAO, and such selection is not possible. Another approach towards genetic characterization of alkaline protease genes would be to isolate alkaline protease mutants in strain PAO. The inability to design a convenient bioassay for detecting alkaline protease mutants in a strain such as PAO which produces elastase as well as alkaline protease has been discussed. However, mutants of strain PAO deficient in alkaline protease production could be identified by assays employing antibody to alkaline protease. Alternatively, the elastase activity of PAO could be mutationally inactivated, allowing the detection of alkaline protease mutants on skim milk agar plates. In this approach, transposon mutagenesis of alkaline protease genes would be particularily convenient, as the resultant alkaline protease mutations could be mapped by conjugation with auxotrophic recipients and scoring for inheritance of the drug resistance marker specified by the transposon.

All of the requirements for a genetic characterization of toxin A and elastase determinants have recently been met. Mutants deficient in the production (3) or activity (13) of toxin A have been isolated in strain PAO and characterized. A mutant resulting from a mutation within the structural gene for elastase has also been identified (14). A conjugal system of genetic exchange which promotes the efficient transfer of approximately 10' of DNA from virtually any site on the strain pAO chromosome exists, and a transposon-facilitated recombination system has recently been developed. While by no means complete, a circular map of the PAO chromosome exists which includes selectable markers which encompass the entire chromosome. Since the aforementioned toxin A and elastase mutants were all derived from this genetically characterized strain, mapping studies are not complicated by inter-strain restriction.

The genetic characterization of the elastase mutant PAO-E64 (paper 2) and the toxin-deficient mutant PAO-T1 (paper 3) are described. The elastase mutant PAO-E64 produces cross-reactive protein, suggesting that the mutation within this strain arose within the elastase structural gene (14). Mapping studies involving the plasmid R68.45 and a transposon-facilitated recombination system (paper 2) indicate that the structural gene for elastase, las A1, is located between the markers tyu-9009 and nar-9011. at approximately 75' on the strain PAO chromosome. This study also directly compared the transposon-facilitated recombination system with the R68.45 plasmid system of genetic exchange. Both systems of exchange provide qualitatively similar linkage values with individual markers. However, the transposon-facilitated recombination system provides for transfer of larger pieces of DNA, as indicated by linkage of las A1 with markers located up to 20' away.

Also, the transposon-facilitated recombination system is up to 100 times more efficient than the R68.45 plasmid-mediated system when selected markers are near the origin of transfer of the transposon-facilitated recombination system. The transposon-facilitated recombination system is presently limited by the number of characterized transposon insertions which serve as origins of transfer in this system. Also, transfer of markers distal to the origin of transfer seems to be as inefficient in this system as in exchange mediated by the classical sex factors such as FP2. This indicates that mapping studies which employ transposon-facilitated recombination must employ several (at least three) distinct tfr donors, or must be used in conjunction with conjugation mediated by FP or R68.45 plasmids.

Genetic characterization of the toxin-deficient mutant PAO-T1 was accomplished by R68.45 plasmid-mediated conjugation and linkage analysis. The mutation in this strain, designated tox-3, is located between the markers cys-54, at 80', and pur-67, at 90'. This map location is similar to the one recently determined for the toxin structural gene (tox A1), suggesting that the mutation in strain PAO-T1 strain arose within a gene specific for toxin A synthesis. The nature of the mutation in this strain is not known, but the phenotype of the mutant strain (13), and the observed map location (paper 3) is consistant with a hypothesis that the mutation in this strain is located within the promoter region or within the structural gene for toxin A, such that stable product is not synthesized.

The results of these mapping studies (papers 2 and 3), and previous studies which have mapped the chromosomal location of the toxin A structural gene (5) contribute to our understanding of the genetics of

virulence factors of P.aeruginosa. Previous studies have suggested that the toxin and elastase are encoded by chromosomal rather than extra-chromosomal determinants. The determination of the location of these genes demonstrates directly that they are chromosomally encoded. Also, the location of these genes in the late region of the chromosome contributes to the number of markers available for other mapping studies in that poorly characterized region. The paucity of markers in the late region of the P.aeruginosa chromosome, and the observation that the toxin and elastase genes examined are not co-transducable with any available markers makes it unfeasable to employ localized mutagenesis to obtain more mutants in these genes. However, our knowledge of the location of these genes will facilitate future studies involving construction of strains with altered virulence phenotypes, and as the chromosomal map of P.aeruginosa becomes more detailed markers will be identified which should allow for co-transduction and localized mutagenesis of these genes. Additionally, our knowledge of the location of these genes provides a framework for future genetic studies involving the characterization of additional toxin and elastase mutants.

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APPENDIX

Statistical Analysis of Corneal Damage Indices

In paper 1 of this thesis, the virulence of alkaline-protease deficient mutants of P. aeruginosa was compared to that of the wild-type organism from which the mutants were derived in a mouse eye model of infectious keratitis. The extent of damage associated with infection was expressed according to a previously described corneal damage index. To determine the significance of the differences in virulence which were observed, a statistical analysis was performed on the corneal damage indices obtained in this study. An analysis of variance (ANOVA[1]) was performed on the data from the three groups. This was significant, $F = 822.35$, $df = 2,225$ ($P \leq .01$) indicating that the means from the three groups differ significantly. A Newman-Keuls[1] test was then performed to determine exactly which groups were different. The results of this analysis indicated that each of the means was significantly different from the other two.

ANOVA Summary Table

Source	SS	df	MS	F
Total	422.92	257		
Between	366.15	2	183.07	822.35
Within	56.77	255	.22	

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