Molecular biology of Pseudomonas Aeruginosa elastase genes : a dissertation

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1988

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ACKNOWLEDGEMENTS

I would like to thank my mentor, Barbara Iglewski, for all her support and suggestions throughout my doctorate.

I would also like to thank my examining committee Dr.'s Crosa, Landfear, Litt, and Richardson for their suggestions in improving this dissertation.

Most especially I would like to thank my wife Pamela for all her tremendous support and love through this whole process.

To my mother who always said "You can be whatever you want to be, just do it", who can now say its all over. Maybe?

Lastly, I like to thank the immortal scientist Sir Sidney Fudd for the discoveries which lead to the formulation for his first law of opposition, and its derivation [for without them this dissertation would not have been possible] and are stated thusly, "If you push something hard enough it will fall over" and "it comes in, it must go out!".

I. Introduction and statement of the problem.

P. aeruginosa is widely distributed in nature, readily acquires resistance to antimicrobial agents, and is highly adaptable to its environment. It is ubiqitous in nature so that controlling patient exposure is difficult. *Pseudomonas aeruginosa* is one of the most common opportunistic pathogens in hospitals. *P. aeruginosa* can cause life-threatening infections in patients with immunocompromised conditions such as: burns, cystic fibrosis, trauma, diabetes, and post-surgical infections. Emergence of this organism as a major opportunistic pathogen has stimulated research which has led to the identification of several virulence factors. One of these factors, elastase, is the major protease produced by 75% to 99% of all *P. aeruginosa* strains examined. Elastase is able to degrade a striking variety of biologically active compounds including complement, elastin, collagen, immunoglobulin G, fibrin, and α_1-proteinase inhibitor. Antibodies have been found in patients recovering from infections with *P. aeruginosa.* Additionally, toxoids made from elastase protect animals in experimental *P. aeruginosa* infections.

Little is known about the gene(s) controlling elastase production, processing or excretion. Several mutants and environmental strains have been characterized as lacking elastolysis. One way to study elastase production, processing, and regulation in *P. aeruginosa* is to create a gene bank and attempt to complement strains deficient in elastolysis. Characterization of these genes would lead to a better understanding of elastolysis in *P. aeruginosa.*

The objective of this study is to examine on a molecular level, genes involved in the production of elastase. The specific aims are:

1. Characterize mutants that hyperproduce elastase when they are grown in high salt or high iron media.

2. Clone, characterize, and sequence the *lasA* gene that complements the elastase (*lasA*) defective mutant PAO-E64.

3. Characterize the *lasA* gene product produced in *E. coli* and begin to study its role in elastase processing or activation.

II LITERATURE REVIEW

A. The Organism.

The genus *Pseudomonas* was first given its name in 1894 by Migula. He derived it from the word "pseudomonad" commonly used at the time to designate rod-shaped, gram negative, nonsporulating, polar flagellated bacteria (108). *Pseudomonas* are gram negative rods, typically flagellated, with a size range of greater than 1μm to less than 5μm in length. Superficial examination of some species may lead to the mistaken identification of spores because of accumulation of poly-β-hydroxybutyrate under nitrogen limitation. These granules are highly refractive which may have lead Migula to include this phenotype in his original description of the genus in 1894. In 1895 Migula named the first type species of this group as *Pseudomonas pyocanea*, but renamed it *Pseudomonas aeruginosa* in 1900 (109, 110). *Pseudomonas aeruginosa* is ubiquitous in nature and is frequently found in soil, water, on plants, foods and as an opportunistic pathogen in plants, insects, and animals including humans. In contrast to many human pathogens which tend to be facultative aerobes, *Pseudomonas* strains are absolute aerobes but can use nitrate as an electron acceptor under limiting oxygen conditions. *Pseudomonas* can utilize a wide variety of compounds as energy sources with hexose sugars being metabolized through the Entner-Doudoroff pathway. *Pseudomonas* has been shown to utilize low molecular weight carbon compounds (with the exception of C_1 compounds such as CO_2), along with a striking variety of organic compounds. These organic compounds can include hydrocarbons, amines, amides, aliphatic acids, unusual carbohydrates,

aromatic compounds, and alcohols. When catabolized, these organic compounds usually converge at the tricarboxylic acid cycle (17, 158).

B. Virulence factors.

P. aeruginosa produces a variety of potential virulence factors. These factors allow the organism to gain a foothold in the host, persist, acquire nutrients, and in some instances cause death. Virulence factors include components of the organism itself such as the outer membrane, endotoxin, exopolysaccharides, pili, and flagella, along with excreted products such as exotoxin A, exoenzyme S, hemolysins, leukocidin, phospholipase C, and proteases (55, 97, 122, 158).

1. Outer membrane.

The outer membrane of *P. aeruginosa* may be considered a virulence factor due to its high level of impermeability to some antibiotics and physiological inhibitors. The major outer membrane protein of *P. aeruginosa* is porin F which accounts for 15% of the total outer membrane proteins (6). Porin F was shown to have a larger pore size than the enteric bacteria. It therefore should have increased permeability, allowing larger hydrophilic substances to penetrate its outer membrane (6). This was not found to be the case, however. It was subsequently thought that this impermeability to antibiotics and other inhibitors was due either to the porin F forming closed channels that could be selectively opened at certain times, or to only a small portion of channels being open at any one time in the membrane. There is now evidence that a large fraction of the porin F channels are smaller than those of enterics, although not completely closed. Nevertheless, they would be essentially impermeable to large hydrophilic compounds such as antibiotics (177).

2. Endotoxin (LPS).

The lipopolysaccharide of *P. aeruginosa* is similar to that of the enteric bacteria. It contains a hydrophobic lipid-A core with O-polysaccharide side chains. It has been estimated that only 10% to 25% of the core molecules are capped with the O-polysaccharide side chains (46). The major difference between the LPS of enteric bacteria and the LPS of *P. aeruginosa* is its higher degree of phosphorylation of the core sugars linked together by magnesium ions, accounting for the high degree of sensitivity to EDTA and other chelating agents of the latter (46). The O-polysaccharide side chain of *P. aeruginosa* has been found to frequently contain unusual sugars (25, 87). More importantly these structural differences in the O-polysaccharide side chain correlate well with the serological classification of Lanyi and that of Meadow and his colleagues (16, 88, 93). The LPS of *P. aeruginosa* has been found not to be as toxic as the LPS from other gram-negative organisms (31, 58). However, the LPS of *P. aeruginosa* has been implicated in shock and sequellae associated with septic infections in humans (185).

3. Exopolysaccharide.

P. aeruginosa has been described as producing two exopolysaccharides, one called slime or glycolipoprotein, and the second called alginate. The chemical composition of "slime" and "alginate" appear different with slight variation in chemical composition among the "slime" producing strains (2, 153). The glycolipoprotein or slime appears to be anti-phagocytic, and antibody to this product is protective (161, 162, 186).

Alginate is produced in copious amounts by *P. aeruginosa* strains isolated from the lungs of cystic fibrosis patients (26, 54, 141). Alginate production appears in primary isolates from cystic fibrosis patients and is quickly lost upon sub-culturing. Alginate appears to be anti-phagocytic in the host (131). Its synthesis

appears to be highly regulated in *P. aeruginosa* involving many genes (127, 129). Several alginate genes have recently been cloned and characterized from *P. aeruginosa* (21, 22, 39, 40, 41, 144).

4. Pili and Flagella.

Pili mediate adherence in *P. aeruginosa.* Pili have been shown to allow adherence to injured tracheal cells, termed "opportunistic adherence" by Ramphal *et al.* (140). Woods has demonstrated that when fibronectin is lost from the buccal cell surface, increased adherence of *P. aeruginosa* is observed (181). It was also established that treatment of buccal cells with *P. aeruginosa* proteases enhances the adherence of piliated *P. aeruginosa* to buccal cells. Moreover, it has been demonstrated that de-piliated *P. aeruginosa* are rendered non-adherent, and that piliated *P. aeruginosa* are less adherent to buccal cells pretreated with purified *P. aeruginosa* pili (180).

Most strains of *Pseudomonas* are polarly flagellated. Although not toxic, flagella may give the organism an advantage in some infections and hence contribute to its virulence. Since flagella allow motility of the organism, they may allow the organism to spread to surrounding tissues or move against the flow in the host. One study, using the burned mouse model, showed that a flagella negative strain of *P. aeruginosa* was less virulent than its parent strain (111). The flagellar genes of *P. aeruginosa* have been found to be organized into two sections (region I and region II) located in the late region of the chromosome at approximately 85 min. (170).

5. Hemolysins.

Two hemolysins are produced by *P. aeruginosa.* One, a heat stable hemolysin called rhamnolipid, acts by solubilizing phospholipids due to its detergent like quality. Rhamnolipid has a low toxicity in mice with an LD_{50} of 5 mg (70). It has been implicated in corneal infections and in lung infections but there is still no

direct evidence for its pathogenic involvement (76, 96). The second hemolysin produced by *P. aeruginosa* is a heat-labile hemolysin called phospholipase C (33). Phospholipase C acts by hydrolyzing phosphatidylcholine (lecithin) to phosphorylcholine and diacylglycerol (33). The gene for Phospholipase C has been cloned by several investigators (18, 100, 171). It has been mapped on the chromosome at 80 min (95) and Phospholipase C is produced by 70% to 100% of the strains tested depending on the detection method used (8, 182). Injections of Phospholipase C into the skin causes induration and redness characteristic of skin infections of *Pseudomonas* while intraperitoneal injections cause hepatic necrosis and pulmonary edema (98). Antibody to Phospholipase C has been found in cystic fibrosis patients colonized with *P. aeruginosa*, suggesting a role in pathogenesis (43).

6. Exotoxin A.

Exotoxin A is the most toxic of exoproducts produced by *P. aeruginosa* (97). It is a heat labile protein with a molecular weight of 66,000 daltons (14, 65, 94, 99). Exotoxin A is an ADP-ribosyltransferase enzyme which catalyzes the transfer of ADP-ribose from NAD to elongation factor-2, required for eukaryotic protein synthesis (63, 64). The mechanism of this transfer has been found to be identical to that of diphtheria toxin. Interestingly, exotoxin A and diphtheria toxin share no homology either at the DNA or protein level and yet catalyze the identical reaction (145). Exotoxin A has a typical A-B toxin like structure in which the A fragment is the active (enzymatic) portion and the B fragment is the binding fragment which interacts with a eukaryotic cell receptor which has yet to be identified (172). The structural gene has been mapped and found to be at 85 min on the *P. aeruginosa* chromosome (47).

A potential role for exotoxin A in infections is suggested by its extreme toxicity (97), its production by 90% of clinical isolates (11, 136), the decreased

virulence of exotoxin A deficient mutants in experimental animal models (122, 126, 179), and detection of anti-exotoxin A antibodies in human and experimental animals (7, 15, 19, 20, 28, 69, 85, 86, 118, 135). The precise role of exotoxin A in chronic pulmonary infections is as yet unclear. Current data indicate that exotoxin A causes damage in lungs indirectly by formation of immune complexes with anti-exotoxin A antibodies which in turn elicit tissue destructive host immune responses, such as the excretion of proteases by PMN leukocytes (28). For example, significantly higher levels of anti-exotoxin A antibodies were found in sera and sputum of cystic fibrosis patients than in control groups of non-pulmonary *P. aeruginosa* infected patients (20, 69, 86, 118). In addition, patients colonized by *P. aeruginosa* who died had higher levels of IgG antibodies to exotoxin A than survivors (118). However, the opposite is seen in septic infections where increased antibody titers to exotoxin A correlates with patient survival.

7. Exoenzyme S.

This excreted exoprotein is also an ADP-ribosyltransferase. However, unlike exotoxin A, exoenzyme S transfers the ADP-ribose from NAD to a variety of eukaryotic proteins, but not to elongation factor-2 (66). To date, no one specific eukaryotic protein has been identified as a substrate for exoenzyme S. The active portion of exoenzyme S was found to be a 49,000 dalton excreted protein, with an immunologically related 53,000 dalton, enzymatically inactive, protein appearing on western blots and co-purifying in immunoaffinity purification (Lile and Iglewski, unpublished observation). Exoenzyme S shows no immunological relationship with exotoxin A although both are produced in the same culture conditions. Optimal exoenzyme S production occurs, however, when a metal chelator such as nitrilotriacetic acid is added to the media (66, 166). Recently, 90% of clinical and environmental strains have been shown to produce exoenzyme S (Frank and Iglewski,

unpublished observation). High levels of anti-exoenzyme S antibody have been found in the sera of recovering bacteremia patients infected with exoenzyme S producing strains of *P. aeruginosa* (Lile and Iglewski, unpublished observation). When a transposon induced exoenzyme S deficient mutant was tested in the burned mouse model, it was found to be 2,000 times less virulent than its isogenic parent strain (121). Furthermore, exoenzyme S deficient mutants were much less virulent in causing lung damage in the chronic rat lung model (9, 122). In these studies it was observed that the exoenzyme S deficient mutant was not inhibited in its ability to colonizing the site of infection although, it was necessary to cause a disseminated infection in the burned mouse model (122, 123)

8. Leukocidin and or Cytotoxin

There is currently some confusion as to whether leukocidin and what is currently being described as cytotoxin are one and the same. Leukocidin has been described as a cell associated cytotoxin that causes the lysis of lymphocytes and granulocytes. It was first described by Scharmann (147, 148) and has more recently been called cytotoxin by Lutz (102). It has been shown to have a mean lethal dose of 1μg when injected intravenously into the mouse (148). It has been reported to have a molecular weight of 44,700 by Hirayama et al. (52). Recently Baltch *et al.* (3) reported on the isolation of cytotoxin in clinical isolates of *P. aeruginosa.* Using immunological analysis, they determined that it has a biologically active size of 29,000 daltons with immunologically related larger proteins of 50,000 and 52,000 daltons, and a smaller protein of 17,000 daltons (3). This cytotoxin was purified to homogeneity by column chromatography. Only the 29,000 dalton protein was found to have biological activity. Lutz (101) discussed the differences between the two cytotoxins in terms of their different molecular weights, marked variation in amino acid composition, and lack of antibody to "leukocidin" reacting to the 29,000 dalton

cytotoxin. It appears from the data presented in these papers that *P. aeruginosa* may be producing two cytotoxins or leukocidins.

The relationship of these cytotoxins to exoenzyme S is still unclear. For example, in the paper by Hirayama *et al.* on leukocidin, the active fragment is close to the size of the reported active fragment of exoenzyme S, and only lymphocyte toxicity was examined. In the latest paper by Baltch *et al.* on cytotoxin, they state, "that there are two immunologically related larger proteins (50,000 and 52,000 Da) and one smaller protein (17,000 Da) than the biologically active 29,000 Da purified cytotoxin". The authors hypothesize that these larger proteins may be precursors and find it interesting that exoenzyme S is a similar molecular weight protein. Until these discrepancies are clarified it remains uncertain whether *P. aeruginosa* produces one or two distinct cytotoxins.

9. Proteases.

P. aeruginosa produces three proteases which have been described by Morihara as neutral, semialkaline and alkaline proteinase, according to their pH optimum (117).

Little work has been done on neutral protease, also referred to as "protease fraction I" by Wretlind and Wadstrom (184). Elliot and Cohen reported on the isolation and characterization of a lysine specific protease of *P. aeruginosa* that has the characteristics of "neutral protease" and/or "protease fraction 1" (32). This protease, named "Ps-1" and/or "Endoproteinase Lys-C" has a molecular weight of 30,000 daltons as determined by SDS-PAGE. Its pI was determined to be 8.5 to 8.8 (94, 184). Ps-1 has a pH optimum of 6.5-7.0 and is not inhibited by thiol, carboxyl, or metalloprotease inhibitors. Partial inactivation is seen with serine reactive inhibitors suggesting that this protease may have an unusual conformation. Further analysis by these authors revealed that this protease exhibits one of the most

restricted specificities known for an endoprotease in that it only hydrolyzes the carbonyl group of lysine. This enzyme cleaves both fibrinogen (168) and myosin (176) *in vitro*. Although this protease accounts for only a small fraction of the total proteolytic activity in *P. aeruginosa in vitro* (94), it may aid *P. aeruginosa* in certain local environments when infecting the human host.

Semialkaline protease, also referred to as protease II (183, 184) or protease 1 (74), is currently referred to as elastase. Elastase is a metalloprotease and can account for 75 to 95% of extracellular protease activity of *P. aeruginosa* (74, 183, 184). A more detailed discussion of elastase and its role in virulence of *P. aeruginosa* will follow in later sections.

Alkaline protease, which has been referred to as protease I (183, 184) or protease 2 (74), accounts for most of the proteolytic activity not attributed to elastase. Alkaline protease has a molecular weight of 52,700 daltons as determined by SDS-PAGE (74), or 48,400 as determined by analytical ultracentrifugation and has a pI of 4.1 (67) to 4.5 (184). Alkaline protease is considered a metalloprotease and is inhibited by EDTA. Alkaline protease contains one atom of calcium per molecule which was shown not to be essential for enzymatic activity. Since cobalt (Co) increased its activity sevenfold (115), it may be more appropriate to consider alkaline protease as an apo-enzyme (55). Purified alkaline protease has been shown to be lethal in mice with an LD_{50} of 63 μg to 300 μg per mouse depending on the route of administration. Mutants deficient in alkaline protease have been shown to be less virulent than their parent strains in a mouse eye model and a burn mouse model (60, 122).

C. Physical characteristics and genetics of P. aeruginosa elastase.

Elastase was first reported by Mandl *et al.* in fermentation broth of *P. aeruginosa* in 1962 (103). In 1964, Morihara described it as one of three proteases generated by *P. aeruginosa* (112). Elastase is the most active protease produced by *P. aeruginosa* (74, 184) and it is produced by 75% to 99% of the strains tested (42, 51, 71, 119, 120, 149, 182). The molecular weight of excreted elastase has been reported to be 20,000 to 23,000 by column chromatography (90, 154, 184), 33,000 to 37,000 by SDS-PAGE (32, 82, 89), and 39,500 by ultracentrifugation (117). Explanations for this variability in molecular weight include the possibility that elastase dimerizes, thus accounting for the larger ultracentrifugation molecular weight. However, SDS-PAGE studies are run under reducing conditions with detergent which should break up any dimer molecules formed. It is more likely that elastase is interacting with the column chromatography matrix which would slow down its migration through the column, making it appear to have a smaller molecular weight than it actually does. The isoelectric point has been determined by several investigators and found to be between 5.7 and 6.6 (154, 164, 184). This range in pI has been attributed to the presence of contaminants and autodegradation.

Elastase is a metalloprotease and has been found to contain 1 atom of Zn per molecule which is essential for enzymatic activity (114). Elastase is inhibited by metal chelators such as EDTA and *o*-phenanthroline, by reducing agents, and by heavy metals including Zn (114). Synthetic metal chelating peptides have also been found to inhibit elastase (80) along with N-α-Phosphoryl Dipeptides (139, 160). Natural inhibitors of elastase include plasma α2-macroglobulin (53) and phosphoramidon (113).

Elastase was originally purified by $(NH_4)_2SO_4$ precipitation and ion-exchange chromatography by Morihara (117). More recent methods have included isoelectric

were not necessary for elastase production, and that free amino acids did not repress elastase induction as previously believed (72).

A mutant of *P. aeruginosa* PAO1 (PAO-E64) was found to be devoid of elastolytic activity at 37°C. This mutant was normal in all other aspects examined including the production of parental levels of extracellular elastase antigen (128). When elastase of this strain was purified it had only 33% of the parental *P. aeruginosa* elastase activity (J. Lyle, unpublished observation). It was therefore concluded that this mutation was in the structural gene. This lesion, *lasA1*, was mapped by Howe *et al.* (61) and found to be at 75 min on the *P. aeruginosa* PAO1 chromosome. It has since been determined that this mutation is not in the structural gene but in a gene encoding a presumed membrane associated elastase processing protein (manuscripts 2 and 3 of this dissertation). The cloning of the *lasA* gene along with the elastase structural gene (*lasB*) is discussed in manuscript 2 (146). Sequencing of the *las*A gene and characterization of the *lasA* protein is discussed in manuscript 3.

Whether elastase is processed from a larger proelastase or inhibited when cell-associated has been somewhat vague until recently. Jenson *et al.* (74) were the first to suggest that a cell-associated inactive precursor of elastase was present in *P. aeruginosa.* They reported that this inactive precursor was in the periplasm. Jensen *et al.* stated that SDS-PAGE indicated only a slight change in molecular weight was possible. They hypothesized that elastase was processed in several steps before excretion. Fecycz and Campbell (34) reported on the mechanisms of activation and secretion of a cell-associated elastase precursor. They discovered that several mild non-enzymatic procedures were effective in activating pre-elastase. They stated that the precursor and mature protease have identical molecular weight, that a significant change in hydrophobicity is seen in the active elastase indicating a change in

conformation, and that these mild activation procedures may be removing a non-covalently associated inhibitor. In addition, they found that outer membranes of a mutant found to be deficient in the excretion of elastase lacked a 25,000 dalton outer membrane protein found in the parent strain. Kessler and Safrin (82) recently reported the purification and characterization of the inactive elastase precursor found in the periplasm of *P. aeruginosa.* They have found that immunoaffinity-chromatography with elastase specific antibodies actually leads to the copurification of two proteins with the molecular weights of 36,000 daltons and 20,000 daltons. They demonstrated that this 20,000 dalton protein does not react with anti-elastase antisera by itself but is associated with the larger 36,000 dalton precursor elastase which does react with the elastase antibody. They further demonstrated that the precursor elastase is larger than active elastase when run without SDS in non-reducing PAGE conditions, but only by about 500 to 1,000 daltons. The authors do not expand on the role of the 20,000 dalton protein, but merely state that this may be a large leader sequence of a larger pre-proelastase. They do not speculate that this may be another protein necessary for excretion, or a periplasmic inhibitor as eluded to by Fecycz and Campbell.

D. Biological effects of elastase and its relation to disease.

Elastase inactivates a variety of biologically important components. These compounds include, complement, α_1-proteinase inhibitor, IgG, IgA, elastin, and collagen. Elastase was found by Schultz and Miller to be highly destructive for the fluid and cell bound phase of C1 and C3. It was equally destructive for the fluid phase of C5, C8, and C9. Inactivation of the other complement components varied from 0 to 67% (152). It was concluded from this work that elastase is the agent responsible for the characteristic non-inflammatory *Pseudomonas* vasculitis.

Inactivation of blood plasma α_1-proteinase inhibitor was also demonstrated (152). Elastase was shown to almost completely inhibit human α_1-proteinase inhibitor within 1 h at 25°C. The α_1-proteinase inhibitor is one of two protease inhibitors in plasma used for controlling endogenous serine proteases. It has been suggested that tissue destruction could be caused by the host leukocyte elastase released during pseudomonas-induced diseases (116). Genetic deficiencies in α_1-proteinase inhibitor are also associated with the early onset of emphysema in humans. It was reported that purified *Pseudomonas* elastase was able to inhibit the bronchial mucous inhibitor (BMI), which is known to inhibit PMN elastase in the upper airways and protect them from proteolysis (75). It has since been shown that *Pseudomonas* elastase is not responsible for *in vivo* inactivation of BMI when leukocyte elastase is present. Tournier *et al.* has shown that *in vivo*, *Pseudomonas* elastase is not produced in sufficient quantities to inhibit BMI due to the high affinity of BMI for leukocyte elastase (169). *Pseudomonas* elastase has also been shown to degrade human IgG. This was confirmed in studies by Doring *et al* (29), and Holder and Wheeler (56). They found that protective IgG was depleted in burned mice infected with protease and exotoxin A producing strains of *P. aeruginosa.* Non-infected mice showed a slight decrease in IgG levels after burning but they rapidly returned to normal. They concluded that elastase was the prime component responsible for the IgG decline since animals infected with non-elastase, exotoxin A producing strains did not show a decrease in IgG levels (29, 56). They also demonstrated that when α_2-macroglobulin, an inhibitor of *Pseudomonas* elastase, was injected at the site of infection, it prevented the drop in IgG observed in the untreated infected animals (56).

The ability of *Pseudomonas* elastase to degrade collagen has been somewhat controversial. Some studies report that elastase does degrade collagen, while other

studies report that it does not (23, 81, 151, 173). Recently, a study by Heck *et al.* (50) reported that purified elastase does degrade human type III and IV collagens with the formation of specific cleavage products. They also observed that type I collagen was also degraded but much more slowly. Type III collagen is an interstitial collagen found in abundance in the dermis, lung vessels, blood vessel walls, and stroma of the spleen and liver. Type IV collagen is primarily found in the basement membranes and is a major component of vascular tissue (110a). The controversy about the production of collagenase activity appears to be related to interpretation of the collagen assays and types and source of collagen used.

P. aeruginosa produces a variety of infections. Localized infections include wounds, skin, intestinal, urinary, reproductive tract, and infections of the eye and ear. Systemic types of infections tend to occur in burn victims, pneumonia, and immunocompromised patients.

Diarrhea has been attributed to *P. aeruginosa.* Patients suffering from *P. aeruginosa* enteritis infection have a muco-sanguineous or mucopurulent-sanguineous diarrhea. Investigations as to the importance of elastase in this type of infection were done by Okada *et al.* (130). They found that addition of whole organisms or purified elastase caused atrophied villi, submucosal blood vessel hemorrhaging (less prominent when purified elastase was used) and flattening of the mucosa. These findings are consistent with the idea that *Pseudomonas* elastase is involved with *P. aeruginosa* enteritis.

Pseudomonas has been cited as one of the protease producing organisms, found in the reproductive tract, that may lead to complications in pregnancy. Protease production by these cervical/vaginal organisms may alter or inactivate proteins important in host defense and structural integrity. These structural membranes are rich in elastin and collagens. These host tissues may also be made

more susceptible to other non-protease producing local flora. A recent report by McGregor *et al.* (105) found that 100% of *P. aeruginosa* isolates from the reproductive tract of females produced protease, while 50% were found to have elastolytic and collagenase activity. In a second report, McGregor *et al.* (104) reported that *in vitro* exposure to collagenases or elastases significantly reduced the strength and elasticity of human amniochorion in a dose-dependent fashion. Non-elastase producing strains did not alter the the strength of the membrane.

Corneal infections may lead to a severe, ulcerative corneal lesion accompanied by liquification of the cornea (13). It was found that by inducing corneal damage along with the addition of purified elastase or alkaline protease from *P. aeruginosa*, opacity and ulcers formed. The magnitude of damage was dose dependent and both alkaline protease and elastase caused the same type of damage. However, more alkaline protease was required to produce the same degree of damage to the eye than the elastase treated eyes (74). The virulence of non-elastase producing strains were tested in the mouse cornea. It was found that the elastase producing strains produced ulcers over the damaged cornea whereas a non-elastase producing strain caused uveitis but not ulcers (74). Ohman *et al.* (126) showed that exotoxin A appeared to be a major factor in the pathology of these infections. They determined that traumatized corneas were damaged by the exotoxin A producing strains and not by non-exotoxin A strains, whereas the extracellular elastin negative, antigen positive mutant, PAO-E64, produced the same amount of corneal damage as its parent strain PAO. The authors did not consider, however that PAO-E64 produces parental levels of total protease but is elastolytically negative at 37°C. Since the eye is probably not at 37°C but slightly lower, and since PAO-E64 has elastolytic activity below 37°C, this report was inconclusive as to whether elastase is necessary for corneal infections. It was later determined by Howe and Iglewski (60) that once the cornea was

damaged, a protease was required for initial infection. Once the infection was established the protease was not required. It was further determined that both elastase and or alkaline protease together were not required for maximum virulence; either one alone was sufficient (60). Since the cornea has no direct blood supply, it is an immunologically weak tissue and established infections are difficult to control. These infections have a rapid onset leading to additional problems in control and management. Topical treatment with non-specific elastase inhibitors was not entirely effective in preventing corneal perforations (1, 4, 81). One study by Kessler *et al.* looked at the use of specific inhibitor of *P. aeruginosa* elastase. These inhibitory peptides were found to completely prevent corneal perforation. They found that frequent topical applications of the inhibitor completely inhibited corneal liquification and ulcers. It was suggested that these inhibitors might be helpful in the management of *P. aeruginosa* corneal infections (84, 160).

Bacteremia associated with *P. aeruginosa* has a fatality rate higher than that caused by other gram negative bacteria (156). Immunocompromised patients and burn patients are those who most frequently develop bacteremia with *Pseudomonas* (36, 137, 156, 165). In burn victims, the wound provides an ideal location for colonization and it has been reported that *P. aeruginosa* usually has colonized the site within 48 h after injury (138). Several studies have been done to determine the virulence of *Pseudomonas* using the mouse animal model. One study by Stieritz and Holder determined that the LD_{50} was 10 organisms in the burned mouse as opposed to 10^6 for the control group. No other organism was shown to have this dramatic an effect (163). To determine if elastase was involved, Snell *et al.* (157) demonstrated that elastase negative and exotoxin A negative strains required several log units more bacteria than the wild type strain that did produce elastase and exotoxin A. Using a similar approach Pavlovskis and Wretlind (132) found that the LD_{50} of elastase

negative or an elastase and alkaline protease negative mutant were at least one log unit higher than required to cause death. A study by Howe and Iglewski (60) using a mutant that was deficient in production of both proteases but that did produce exotoxin A, was found to be 16 times less virulent than the parent strain. These results suggest elastase is required for maximum pathogenesis in the burn model.

Pseudomonas is a leading cause of hospital acquired pneumonias (142). This pneumonia is usually followed by bacteremia with a high mortality rate (62, 134, 142). Additionally, a hemorragic pneumonia is a frequent disease in minks (125, 154). Strain-related differences in animal models suggesting certain virulence factors may play key roles in the disease pathology (62, 167). It was found that antibody titers to *Pseudomonas* proteases were present in patients recovering from acute lung infections (85) and that immunization with protease toxoids of *P. aeruginosa* gave partial protection in minks (59). It has also been reported that intratracheal instillation of purified or crude proteases from *P. aeruginosa* caused hemorrhagic lung lesions like those seen in human pneumonia (45, 98). In a study by Blackwood *et al.* (12) using guinea pigs in a pneumonia model, it was found that a mutant deficient in extracellular elastase was less virulent and more easily cleared from the lung. Another major type of lung infection in humans with *P. aeruginosa* is that demonstrated by the cystic fibrosis patient. This disease is a progressive lung infection (24, 27, 49, 178) but unlike the hemorrhagic pneumonia, the infections remain localized, do not progress to bacteremia, and it persist for years. An animal model was developed by Woods *et al.* (179) to study chronic lung infections in rats by *P. aeruginosa.* Using this model it was found that elastase deficient strains were less virulent than elastase producing strains (179).

E. Genetics of P. aeruginosa.

P. aeruginosa has been found to contain a circular chromosome. The molecular weight of the double stranded DNA is approximately 2.1 x 10^9 (133, 143). Pseudomonads frequently contain extrachromosomal elements (plasmids) which have been found to encode for a variety of functions e.g., fertility factors, antibiotic resistance, degradative metabolic functions, as well as cryptic plasmids for which no function(s) has yet been identified (5, 57). The plasmids found in *Pseudomonas* have been divided into 10 incompatibility groups (68). Mechanisms of genetic exchange are difficult but not insurmountable in Pseudomonads. Conjugation is possible with the use of a particular conjugative plasmid called R68.45. This plasmid can mobilize all regions of the chromosome effectively and is one of the best characterized plasmids of this type (48). R68.45 can carry as much as 10 min of the chromosome and is frequently used like a large generalized transducing phage. Transduction is also used for mapping and other genetic studies of *P. aeruginosa.* Fine structured mapping has been done using the phage F116 or its variant, F116L. Additionally, this phage has the ability to transduce Pseudomonads of different restriction modification types (30, 92). A third form of genetic manipulation in *Pseudomonas* is transformation of linear or plasmid DNA. Linear double stranded DNA, with transformation frequencies of 10^{-4}, can be obtained using 10 kilobases or larger (106, 107). Transformation of plasmid DNA is also possible with the induction of competent cells using methods similar to those used for *E. coli* (155). This method is especially useful in manipulating recombinant plasmids in *P. aeruginosa* for molecular studies.

Unfortunately, plasmids of the ColE1 compatibility group that are frequently found and used for molecular studies in *E. coli* cannot survive in *Pseudomonas.* This incompatibility has lead to some difficulties in molecular analysis and cloning in

Pseudomonads. The disadvantage of using these vectors to clone *Pseudomonas* genes in *E. coli* is that expression of many *Pseudomonas* genes is restricted in *E. coli*. It appears that this restriction is due to transcriptional rather than translational inefficiencies. Cloning strategy therefore depends on whether the desired gene can be detected in *E. coli* or the recombinant plasmid can be shuttled back into a *Pseudomonas* strain lacking the specific trait of interest. For example, many biosynthetic genes may be directly selected for in *E. coli* by complementation, if a similar pathway exists in *E. coli* and mutant strains are available. Those biodegradative and biosynthetic genes that do not share a similar pathway in *E. coli* must be shuttled back into *Pseudomonas*. This has required that vector systems be developed for *Pseudomonas*. These vector systems require use of broad host range plasmids. One problem with these broad host range plasmids is that they have more genes required for maintenance and these genes tend to be scattered around the plasmid, making it difficult to construct small broad host range cloning vectors. Shuttle systems that exist for *Pseudomonas* have been reviewed by Mermod *et al.* (158). Among the most useful shuttle vectors is the broad host range cosmid pLAFR (37). This vector allows the generation of genomic libraries, packaging by the λ phage system, and propagation in *E. coli* as large plasmids. This cosmid vector is not self-transmissible and is mobilized by the use of a helper plasmid such as pRK2013 (35). This type of transfer that requires a donor, helper, and recipient strain is frequently referred to as a "triparental cross" or "triparental mating". It is the primary way of returning plasmids to *P. aeruginosa* when direct transformation is not possible or advisable. The work in this dissertation utilizes the pLAFR cosmid vector in the generation of a *P. aeruginosa* gene bank along with the helper plasmid required in the triparental mating (manuscript 2).

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III. MANUSCRIPTS

Manuscript 1

Genetic Mapping and Characterization of *Pseudomonas aeruginosa* Mutants that Hyperproduce Exoproteins

ABSTRACT

We isolated two mutants of *Pseudomonas aeruginosa* PAO with defective iron uptake. In contrast to the wild-type strain, the mutants produced extracellular protease activity in media containing high concentrations of salts or iron and hyperproduced elastase, staphylolytic enzyme, and exotoxin A in ordinary media (*Xch* mutants). The mutations were located in the 55 min region of the chromosome, between the markers *met-9011* and *pyrD*.

NOTE

Most strains of *Pseudomonas aeruginosa* produce at least two extracellular proteases, elastase and alkaline protease, and a toxin, exotoxin A (19, 23, 24). Liu and Hsieh (17) found that ammonium sulfate inhibits protease formation by *P. aeruginosa* and some other microorganisms. Kessler and Safrin recently reported that sodium ions have a similar effect (14). Thus, this phenomenon was not due to specific end product inhibition of protease production, but to a nonspecific effect caused by the high ionic strength. Bjorn *et al.* (3) demonstrated that high concentrations of iron in the growth medium inhibit the formation of several exoproteins, e.g., elastase, alkaline protease, and exotoxin A. In this report we present data which indicate that the inhibitory effects on exoprotein production caused by high ionic strength and by iron are related phenomena. Mutants which produced protease activity in media with a high salt concentration were also found to hyperproduce certain exoproteins in ordinary growth media and in media with high concentrations of iron.

The bacterial strains, plasmid, and bacteriophage used are listed in Table 1. *P. aeruginosa* strains were derived from strain PAO1. Its chromosomal map has been published (10, 25). The strains were kindly supplied by B. W. Holloway, Monash University, Clayton, Victoria, Australia, and D. Haas, Eidgenossische Technische Hochschule, Zurich, Switzerland. The mutants (PAO7501 and PAO7502) were isolated after treatment of *P. aeruginosa* PAO1 with a mutagen (ethyl methanesulfonate) (25, 26) as protease-positive colonies on tryptic soy agar plates containing 15% skim milk and 0.3 M ammonium sulfate. The wild-type strain showed no proteolytic activity under these conditions, but the mutants showed a zone of casein precipitation after 1 to 2 days of incubation at 37°C. Two proteolytic

mutants (strains PAO7501 and PAO7502) were found after screening ca. 20,000 colonies. The phenotype was designated *Xch* (*e*x*t*ra*c*ellular protein *h*yperproduction), and the corresponding genotypes were designated *xch*-1 and *xch*-2, respectively.

Proteolytic activity was scored on tryptic soy agar plates containing 15% skim milk. Ammonium or sodium salts were added at a concentration of 0.3 M for testing salt-resistant formation of proteolytic activity. The following broth media were used for quantitative determinations of exoproteins: (i) MYG medium (minimal medium [8] supplemented with 2 g of yeast extract and 10 g of glucose per liter [25]) for alkaline protease; (ii) MCYG medium (MYG medium supplemented with 10 g of Casamino Acids per liter) for elastase; (iii) MCG broth (minimal medium with Casamino Acids [10 g/liter] and glucose [10 g/liter] for pyocyanin; (iv) defined medium (13) for elastase and staphylolytic enzyme; (v) TSBD-NTA medium (dialyzed tryptic soy broth with 10 ml of glycerol per liter and 5 mM nitrilotriacetic acid) for exotoxin A (4); (vi) phosphate-deficient medium for alkaline phosphatase and phospholipase C (2); and (vii) CAA medium (0.5% Casamino Acids supplemented with 0.02 mM $MgCl_2$) for assaying ferripyochelin uptake. The strains were grown in baffled Erlenmeyer flasks at 30°C for TSBD-NTA and phosphate-deficient media and at 37°C for the other media.

Protease activity was tested by the azocasein method described by Kreger and Griffin (15). The azocasein was used at a final concentration of 1.7 g/liter. One unit of enzyme activity was defined as that which caused an increase in absorbancy at 440 nm of 1.0 in 10 min at 37°C. Since alkaline protease was not produced in defined or MCYG medium, the proteolytic activity in these media was considered to be due to the elastase alone (18, 19). Alkaline protease and exotoxin A were determined by rocket immunoelectrophoresis (16) against specific rabbit antisera. The supernatants were concentrated 10 times with Amicon filters for the exotoxin assays. Staphylolytic enzyme was determined by the degree of lysis of a suspension

of heat-killed cells of *Staphylococcus aureus* Copenhagen at 25°C (25, 26). One unit of enzyme activity was defined as that which caused a decrease in absorbancy at 650 nm of 1.0 per 1 min. Alkaline phosphatase was determined with *p*-nitrophenyl phosphate, and phospholipase C was determined with *p*-nitrophenyl phosphorylcholine as the substrate (2). These reactions were carried out at 25°C.

For assay of iron uptake, bacteria were grown to an OD_{540} of 0.25 in TSBD-NTA medium at 32°C in a shaking water bath. Iron uptake assays in these cultures were initiated by adding $FeCl_3$ containing 50% ^{59}Fe (1.3 $\mu Ci/\mu g$) to a final concentration of 50 ng/ml of Fe^{3+}. At 10 min intervals, 1 ml samples of the cultures were removed and filtered through membrane filters. The filters were washed with 10 ml of 0.5% thioglycolate and counted in a Beckman Biogamma Counter (Beckman Instruments, Inc.) (21).

For assay of ferric pyochelin uptake, pyochelin was purified as described previously (5, 7). Bacteria were grown overnight in CAA medium at 37°C. The cells were harvested by centrifugation and washed three times in MOPS (morpholinepropanesulfonate) buffer (10 mM MOPS, 1mM $MgCl_2$, pH 7.4). The cells were then suspended in MOPS buffer to an OD_{540} of 0.25 and kept on ice. Before use, CAA medium was added to a final concentration of 0.4% Casamino Acids, and the cells were preincubated at 37°C for 10 min. Ferric pyochelin was made by mixing 46nM $[^{59}Fe]Cl_3$ (1.3 $\mu C/\mu g$ of Fe), and 50 nM pyochelin, samples were taken every minute for 4 min. The suspension was filtered through Millipore filters, washed with 10 ml of distilled water, and placed in a gamma counter.

The iron concentration in broth media was determined by the method of Ichida et al. (12). The amount of pyocyanin produced in broth was determined as the absorbancy at 320 nm in chloroform extracts of cultures (1). Pyochelin was extracted from acidified spent culture media by methylene chloride and measured by

high-pressure liquid chromatography (5, 6). Pyoverdin was measured by fluorescence emission at 460 nm; the samples were excited at 400 nm (20, 21).

Conjugation experiments were performed by a membrane filter mating technique (25). The bacteria were plated on selective medium containing streptomycin to eliminate the donor cells. The transduction experiments were done as described previously (25). The chi-square test was used for statistical analysis.

P. aeruginosa alkaline protease and elastase were obtained from Nagase Biochemicals, Fukuchiyama, Kyoto, Japan; exotoxin A was from Swiss Serum and Vaccine Institute Berne, Berne, Switzerland; ethyl methanesulfonate, MOPS, thioglycolate, streptomycin, azocasein, nitrilotriacetic acid, *p*-nitrophenyl phosphate, and *p*-nitrophenyl phosphorylcholine were from Sigma Chemical Co., St Louis, Mo.; tryptic soy broth TSB, Casamino Acids, and peptone were from Difco Laboratories, Detroit, Mich.; and $[^{59}Fe]Cl_3$ was from Amersham Corp., Arlington Heights, Ill.

The two *P. aeruginosa* PAO1 mutants resistant to ammonium sulfate inhibition of protease formation (strains PAO7501 and PAO7502) were tested for protease production on tryptic soy agar plated with skim milk and a high salt concentration to determine whether the inhibitory effect was due to the ammonium ions per se or to the ionic strength. Ammonium sulfate, ammonium chloride, sodium sulfate, and sodium chloride (0.3 M) caused a similar decrease in protease production on agar plated by the wild-type strain and a smaller decrease in production by the mutants.

Growth rates and final cell dry weight of the *xch* mutants in the broth media tested were virtually identical to those of the wild-type strain. The *xch* mutants hyperproduced elastase, staphylolytic enzyme, and exotoxin A, but not alkaline protease (Table 2). Addition of Fe^{3+} (90 μM) caused a decrease in the formation of elastase, staphylolytic enzyme, alkaline protease, and exotoxin A by the wild-type strain. The mutants were less susceptible to the effect of iron than was the wild-

type strain PAO1. Assay of iron uptake demonstrated that strains PAO7501 and PAO7502 had a much slower rate of uptake of $^{59}Fe^{3+}$ and ferric pyochelin than strain PAO1 (Fig. 1) The *xch* mutants behaved in this assay as did strain Fe10, which is a mutant with defective iron uptake (21).

As expected from previous results (25), there was no difference in the production of the phosphate-regulated exoenzymes alkaline phosphatase and phospholipase C between the wild-type strain and the *xch* mutants.

Strains PAO7501 and PAO7502 produced siderophores (pyochelin and pyoverdin) in amounts comparable to those produced by the wild-type strain. We observed that the mutants produced more pigments than the wild type on agar plates. In MCG broth with 9 μM Fe^{3+}, the three strains produced similar amounts of pyocyanin. The addition of iron (final concentration, 165 μM) caused a 56% decrease in pyocyanin formation by strain PAO1. However, the mutants increased their production by 41 to 86%.

Genetic mapping experiments with R plasmid variant R68.45-mediated conjugation (9) demonstrated that the *xch* mutations were located close to the *leu-10* (strain PAO7625) marker in the 55 min region of the chromosome (10). Transduction experiments with phage F116Lc4 showed that the *xch* mutations were contransducible with the markers *met-9011* (strain PAO503) and *pyrD48* (strain PAO7626), with the gene order *met-9011-xch-1-xch-2-pyrD* (Fig. 2). Statistical analysis of the linkage data demonstrated that *xch-1* and *xch-2* probably were located in different genes ($P < 0.001$).

It is concluded that the *xch* mutants are specifically affected in the synthesis or release of elastase, staphylolytic enzyme, and exotoxin A but not of alkaline protease, alkaline phosphatase, or phospholipase C. The *xch* mutants are defective in their ability to take up iron from the medium, which probably leads to derepression of iron-regulated exoproteins (3). There are probably two adjacent *xch* genes. Work

is in progress to determine their relationship to the nearby genes for the release of exoproteins (25).

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TABLE 1. *P. aeruginosa* strains, plasmids, and phage used.

Strain, plasmid, or phage	Genotype or phenotype[1]	Source or reference
P. aeruginosa		
PAO1	Prototroph, *chl-2*	11
PAO25	*argF10 leu10*	9
PAO114	*pyrD48*	Holloway collection
PAO503	*met-9001*	Holloway collection
PAO7625[2]	*argF10 leu10* Sm^r	This study
PAO7626[3]	*argF10 pyrD48*	This study
FE10	*toxC*	21
PAO7501	*xch-1*	This study
PAO7502	*xch-2*	This study
Plasmid		
R68.45	Cb^r Nm^r Km^r Tc^r Cma IncP1	9
Phage		
F116Lc4	General transducing phage	22

[1] Symbols: *arg*, arginine requirement; *chl*, resistance to chloramphenicol; *leu*, leucine requirement; *met*, methionine requirement; *pyr*, uracil requirement; *toxC*, iron-resistant production of exotoxin A. Resistance phenotypes: Cb, carbenicillin; Nm, neomycin; Km, kanamycin; Sm, streptomycin; Tc, tetracycline. Phenotypes: Cma, chromosome-mobilizing ability; Tra, plasmid conjugil transfer.

[2] Spontaneous streptomycin-resistant mutant of strain PAO25.

[3] Recombinant of cross between PAO25 and PAO114(R68.45).

TABLE 2. Production of exoproteins[1] by *P. aeruginosa* PAO1 and mutants PAO7501 and PAO7502 in broth with and without 90 μM Fe^{3+}

Strain	Elastase (U/mg)		Staphylolytic enzyme (U/mg)		Alkaline protease (μg/mg)		Exotoxin A (ng/mg)	
	No Fe	With Fe	No Fe	With Fe	No Fe	With Fe	No Fe	With Fe
PAO1	0.05	0.004	0.03	0.01	7.8	4.3	8	0
PAO7501	0.09	0.05	1.7	1.3	7.5	5.9	18	10
PAO7502	0.08	0.05	1.3	1.5	8.7	6.2	17	9

[1] Defined medium (13) was used for determination of elastase and staphylolytic enzyme, MYG broth (25) for alkaline protease, and TSBD-NTA medium (4) for exotoxin A. All values are for milligrams (dry weight) of cells.

FIG. 1 Uptake of $^{59}Fe^{3+}$ (A) and ferripiochelin (B) by strains PAO1 (○), Fe10 (●), PAO7501 (□), and PAO7502 (■). The curves represent average values from assays run in triplicate.

FIGURE 1.

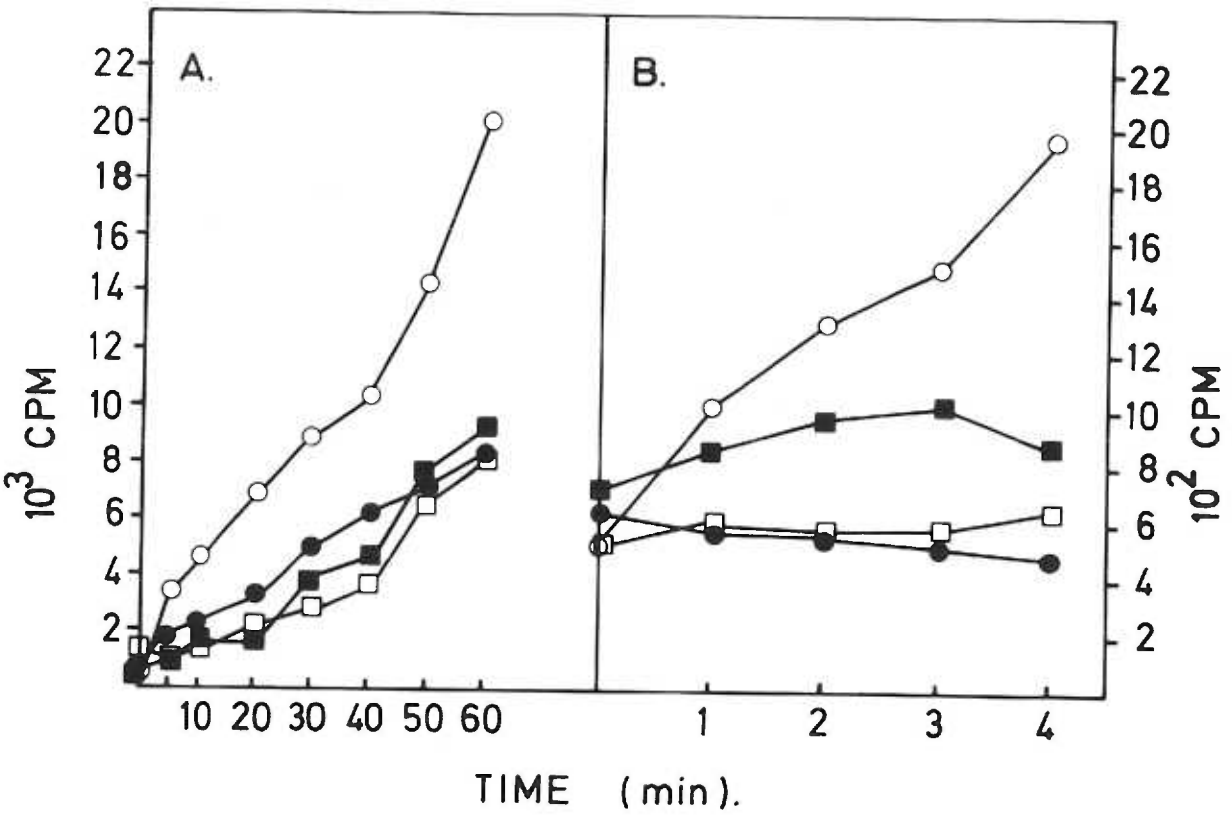
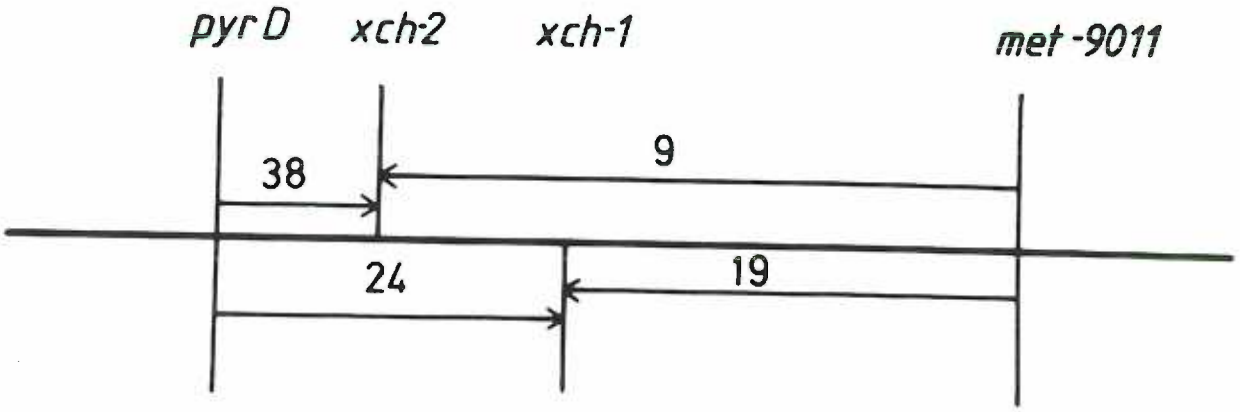

A.
B.
22
20
18
16
14
12
10
8
6
4
2
10^3 CPM
10^2 CPM
10 20 30 40 50 60
1 2 3 4
TIME (min).

FIG. 2 Percent transductional linkage between markers in the 55 min area. Donor strains were PAO7501 (*xch-1*) and PAO7502 (*xch-2*), and the recipient strains were PAO503 (*met-9011*) and PAO7626 (*pyrD48*). The transducing phage was F116Lc4. More than 200 recombinants were examined in each cross.

FIGURE 2



Manuscript 2

Cloning and Characterization of Elastase Genes from *Pseudomonas aeruginosa*.

ABSTRACT

A gene bank was constructed from *Pseudomonas aeruginosa* PAO1 and used to complement three *P. aeruginosa* elastase deficient strains. One clone, pRF1, contained a gene which restored elastase production in two *P. aeruginosa* isolates deficient in elastase production (PA-E15 and PAO-E105). This gene also coded for production of elastase antigen and activity in *E. coli*, and is the structural gene for *Pseudomonas* elastase. A second clone, pHN13, contained a 20 kilobase *Eco*RI insert which was not related to the 8 kilobase *Eco*RI insert of pRF1 as determined by restriction analysis and DNA hybridization. A 2.2 kilobase *Sal*I-*Hin*dIII fragment from pHN3 was subcloned into pUC18 forming pRB1822-1. Plasmid pRB1822-1 restored normal elastolytic activity to PAO-E64, a mutant for elastase activity. Clones derived from pHN13 failed to elicit elastase antigen or enzymatic activity in *E. coli*.

INTRODUCTION

Pseudomonas aeruginosa produces a number of extracellular proteins, including two proteases. One of these proteases, elastase, is able to degrade or inactivate a variety of biologically important proteins, including elastin (29), complement components (40), human IgG (6), fibrin (29), and serum α1-proteinase inhibitor (33). Furthermore, there is evidence that elastase plays a role in the pathogenesis of *P. aeruginosa* infection (16, 23, 26, 30, 35, 44). Elastase has been purified and characterized by a number of investigators (21, 24, 30-32, 34, 37, 45). However, very little is known about the regulation and structure of elastase genes.

Previously, we have reported the isolation of a mutant strain of *P. aeruginosa* PAO1 altered in elastolytic activity (38). This mutant, PAO-E64, produces parental levels of elastase antigen but has no elastolytic activity at 37°C. The PAO-E64 mutation, named *lasA1*, was mapped at 75 min on the chromosome of PAO1 (17). Naturally occurring strains of *P. aeruginosa* which produce little or no elastase have also been reported (18, 36).

This paper describes the cloning of two genes from *P. aeruginosa* involved in elastase production and activity. One of the cloned genes restores normal elastase activity to mutant PAO-E64, but it is not the structural gene. A second cloned gene restores elastase production in *P. aeruginosa* isolates deficient in elastase production. This second gene codes for the production of elastase antigen and activity in *E. coli*, and is the structural gene for *Pseudomonas* elastase.

MATERIALS AND METHODS

Growth media. L-broth was 1% tryptone 0.5% yeast extract (Difco Labs, Detroit, Mi.), and 0.5% NaCl, (pH 7.2). Minimal media (VBMM) was described by Vogel and Bonner (42). Elastin nutrient agar was prepared as previously described (38). Reversed elastin plates were prepared by applying 5 ml overlays containing 2% Noble agar (Difco) to which 8 g per liter nutrient broth base (Difco) and 2.5% of bovine neck elastin (Sigma Chemical Co., St. Louis, Mo.) was added, to prewarmed nutrient agar plates (8 g nutrient broth per liter and 2% Noble agar pH 7.5). Antibiotics and mercuric chloride were used at the following concentrations: tetracycline 25 μg/ml for *E. coli* and 200 μg/ml for *P. aeruginosa*; kanamycin 25 μg/ml for *E. coli*; ampicillin 100 μg/ml for *E. coli*; carbenicillin 500 μg/ml for *P. aeruginosa*; and $HgCl_2$ 10 μg/ml for *E. coli* or *P. aeruginosa*. Antibiotics and mercuric chloride were obtained from Sigma, with the exception of carbenicillin which was obtained from Roerig, New York, N.Y.

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are shown in Table 1. *E. coli* strains produce no elastolytic activity or antigen. *P. aeruginosa* PAO1 produced approximately 150 μg/ml of elastase antigen and gave a zone of clearing of >5 mm on elastin plates after 48 h of incubation at 37°C. The mutant strain PAO-E64 has no elastolytic activity at 37°C but produces parental levels of extracellular elastase antigen (38). Strain PA-E15 produces very low levels (clearing zone of <0.5 mm) of elastase activity after 48 h at 37°C (36). An elastase-negative mutant was generated by transposon mutagenesis. Using the suicide vector pUW942::Tn*501* (43), PAO1 Tn*501* insertion mutants were selected on VBMM supplemented with mercuric chloride. Approximately 3,000 nonauxotrophic

PAO1::Tn501 mutants were assayed on reverse elastin plates for their ability to produce zones of elastin hydrolysis. One mutant, PAO-E105, lacked the ability to produce clearing on reverse elastin plates. PAO-E105 was further characterized for total proteolytic activity, elastolytic activity, and production of elastase antigen by Hide powder azure digestion, elastin Congo red digestion, and Western immunoblot analysis (described below). Analysis showed low levels of proteolytic activity, no elastolytic activity, and no elastase antigen in culture supernatants or cell lysates.

Cell lysates and protein quantitation. *E. coli* cultures were grown for 18 h at 37°C in L broth supplemented with ampicillin or tetracycline. All further manipulations were done at 4°C. Cultures were centrifuged, and the culture supernatants were filter sterilized and immediately frozen at -70°C. Cell lysates were prepared by washing bacterial cells twice in PBS, resuspending in $1/5^{th}$ the original volume in 10 mM $NaHPO_4$ (pH 7.0) containing 1mM phenylmethylsulfonyl fluoride (Sigma), and passed twice through a French pressure cell (American Inst. Co., Silver Spring, Md.) at 15,000 lb/in^2. The resulting lysed cell suspension was centrifuged at 10,000 X g for 15 min, filter sterilized and stored frozen (-70°C). Protein quantitations were performed by the Lowry method (27).

Proteolytic assays. Total proteolytic activity was determined by a modification of the method described by Wretlind and Wadstrom (45). Approximately, 2.5 μg of cellular protein extract or 0.1 ml culture supernatants (or dilutions) was added to 50 mg of Hide powder azure (Sigma) suspended in 2 ml of 10 mM $NaHPO_4$ buffer (pH 7.0). Tubes were rotated at 37°C for 1 hr. The reaction was stopped by the addition of EDTA to 10 mM. Tubes were centrifuged and color development in the supernatant was read. Commercially available *Pseudomonas* elastase (Nagase

Biochemicals Ltd., Fukuchiyama Japan) was used as the standard and gave an A_{595} of approximately 0.111/ ng elastase per h.

Elastolytic activity was determined by a modification of the method described by Bjorn et al. (4). Approximately 50 μg of cellular protein extract or 0.1 ml culture supernatants was added to 20 mg of elastin Congo red (Sigma) suspended in 1 ml of 10 mM $NaHPO_4$ buffer (pH 7.0). Tubes were rotated for 6 to 18 hr at 37°C. The reaction was stopped by the addition of EDTA to 10 mM. *Pseudomonas* elastase standard gave an A_{495} of approximately 0.207/ μg elastase per h.

Polyacrylamide gel electrophoresis and immunoblotting. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was done by the method of Laemmli (25). Stacking gels consisted of 5% acrylamide, and separating gels consisted of 7, 10, or 12% acrylamide (acrylamide to bis 30:8).

Protein transfer and immunoblotting were described in detail by Hindahl and Iglewski (13). Polyclonal anti-pseudomonas elastase serum was prepared in rabbits as previously described (38) and was used at a 1:1000 dilution.

Construction of P. aeruginosa PAO1 gene bank and mobilization of recombinant plasmids. Isolation of *P. aeruginosa* genomic DNA from PAO1, packaging by the cosmid vector pLAFR (10), and propagation in *E. coli* HB101 were carried out as described by Goldberg and Ohman (11). Competent cells were prepared as described by Hedstrom et al. (12). DNA transformation was carried out as described by Maniatis et al. (28). The conjugative properties of pRK2013 were used to transfer recombinant plasmids from *E. coli* to *P. aeruginosa* (8). For crosses into PAO-E64, elastin nutrient agar containing 100 μg of tetracycline per ml was used. Elastin hydrolysis was scored after 48 h at 37°C. For other crosses, VBMM containing 200 μg of tetracycline or 400 μg of carbinicillin per ml was used as a selective medium.

P. aeruginosa strains containing the recombinant plasmids were maintained on L broth with tetracycline, and elastolytic activity was scored on reverse elastin plates without tetracycline since we found that tetracycline inhibited production of elastase, probably because of its high iron levels.

Isolation and analysis of plasmid DNA. Small-scale purifications (1.5-5.0 ml culture volumes) of plasmid DNA from *E. coli* were performed by the method of Holmes and Quigley (15) and for *P. aeruginosa* by the method of Kado and Liu (22). Large-scale preparations were done by the alkaline lysis method of Birnboim and Doly (3) as modified by Bethesda Research Laboratories, Gaithersburg, Md. (1) and were purified by cesium chloride-ethidium bromide (Sigma) isopycnic centrifugation.

Restriction enzymes and T4 DNA ligase were purchased from New England Bio Labs Inc., Beverly, MA., Bethesda Research Laboratories, and International Biotechnologies Inc., New Haven, Ct.

Agarose gel electrophoresis and DNA fragment isolation. Electrophoresis was carried out as described by Maniatis et al. (28) with 0.7% agarose in Tris-borate-EDTA buffer. DNA restriction fragments were separated by agarose gel electrophoresis and purified on NA-45 DEAE paper (Schleicher and Schuell, Keene, N.H.), as described by the manufacturer.

Amino acid sequencing and oligonucleotide synthesis. As the commercial *P. aeruginosa* elastase contained autodegradative peptides, it was further purified before amino acid analysis. Elastase (10 mg) was applied over a Bio-Gel P-30 (100-200 mesh) column, 1.5 cm x 25 cm in 250 mM ammonium acetate, 5 mM EDTA (pH 7.0). One ml fractions were collected and protein concentrations were determined. The main peak was lyophilized, resuspended in 0.5 ml of 50 mM tris, pH 8.0, and 30

μl aliquots were removed for SDS-PAGE. The remaining sample was lyophilized, resuspended in 2 ml of 0.1% trifluroacetic acid in water, and separated on reverse phase high pressure liquid chromatography (RP 304 Bio Rad). A gradient containing 0.1% trifluroacetic acid in 0 to 60% acetonitrile in water was used. SDS-PAGE revealed the main protein peak from high pressure liquid chromatography purification was free of contaminating autodegradative peptides. Amino acid analysis was carried out from the amino terminus to amino acid 40 on two separate preparations of purified elastase.

Oligonucleotide synthesis and purification was carried out as described by Finn et al. (9) and a septadecamer probe pool containing 32 probes was made against the amino acid sequence of least the theoretical codon permutations. The amino acid sequence and probe region is shown in Figure 1.

DNA hybridization. Nick translation of the 2.2 kilobase *Hin*dIII-*Sal*I, the 1.75 *Eco*RI-*Sma*I fragment and λ DNA was carried out as recomended by the manufacturer (nick translation kit, Amersham, Arlington Heights, Il.) with [α-^{35}S]dCTP, (1000 Ci/mM). DNA was purified from unincorporated isotope with a Nensorb 20 column (New England Nuclear Research Products, Boston, Ma.). End labeling of the synthetic DNA oligonucleotide pool was carried out as described by the instructions of Finn et al. (9) with [γ-^{32}P]ATP, (3,000 Ci/mM Amersham). DNA was purified from unincorporated isotope as stated above. Southern transfer of DNA (41) to Hybond-N membrane was carried out as recomended by the manufacture (Amersham). The Hybond-N membranes were prehybridized for 2 h at room temperature, hybridized overnight at 37°C with the synthetic oligonucleotides, and washed for 1 h at 37°C in each of the following; 2X, 1X, and 0.5X SSPE (28) containing 10 mM sodium pyprophosphate, and 0.2% sodium dodecyl sulfate. When the nick translated probes were used, prehybridization was for 2 h at 37°C,

hybridization was overnight at 65°C, and the membranes were washed as stated above but at 65°C. Filters were dried and exposed to X-ray film (Kodak, X-Omat AR).

RESULTS

Selection of recombinant plasmids complementing the elastase deficient P. aeruginosa strains, PAO-E64 and PA-E15. Approximately 10,000 PAO-E64 exconjugants containing the pLAFR-PAO1 recombinant plasmids were examined for elastase activity in PAO-E64. Of the exconjugants 49 showed some elastin hydrolysis at 37°C and 12 clones were chosen for further characterization. Plasmids from these were purified and used to transform *E. coli* HB101. *Eco*RI restriction analysis of these plasmids isolated from *E. coli* showed two populations of inserts. One group contained a 20 kilobase fragment and the second contained a 20 kilobase fragment and a 5 kilobase fragment (data not shown). One clone from each group was chosen for further study: pHN13 which had a 20 kilobase insert and pHN3 which had a 25 kilobase insert (fig.2, 3A).

The same gene bank was transferred to PA-E15, an environmental isolate with low elastolytic activity (36). Resultant colonies were screened for enhanced zones of proteolysis on skim milk plates. Of 1,000 colonies screened, one produced large zones of casein hydrolysis (>8 mm). This clone, pRF1, also hydrolyzed elastin on elastin nutrient agar. Plasmid DNA from this clone was isolated and used to transform *E. coli* HB101. Plasmid pRF1 was found to contain an 8 kilobase *Eco*RI insert which was purified and ligated into pUC18 to form pRB1808 (fig. 3C).

Complementation of P. aeruginosa elastase deficient mutants by recombinant plasmids. Plasmids pRF1 and pHN13, were transferred to parent strain PAO1 and to mutant strains PAO-E64 and PAO-E105. Plasmid pRF1 significantly increased the proteolytic and elastolytic activities of the parent strain PAO1 and complemented the

Tn*501* lesion in PAO-E105 (Table 2). Plasmid pRF1 in PAO-E64 did not complement the mutation to wild type levels of proteolytic or elastolytic activity. Plasmid pHN13 complemented PAO-E64, restoring wild type elastolytic activity at 37°C. In contrast, this plasmid (pHN13) did not complement the Tn*501* induced lesion in PAO-E105, nor did it increase elastolytic or proteolytic activity of the parent strain PAO1.

Subcloning of pHN3 and pHN13. Figure 2 shows a restriction map of the 20 kilobase fragment insert in pHN13 and the 25 kilobase fragment insert in pHN3. Deletions were generated by partial restriction digestion and religation. Only one deletion derivative, pPS4, gave normal elastolytic activity. This plasmid contained a 7.5 kilobase *Eco*RI-*Hin*dIII fragment. To facilitate its manipulation and analysis, the 7.5 kilobase *Eco*RI-*Hin*dIII fragment was isolated and ligated into the vector pUC18 to form pPS1875. A restriction map of the 7.5 kilobase *Eco*RI-*Hin*dIII subcloned fragment insert in pPS1875 is shown (Fig 3B). The 7.5 kilobase *Eco*RI-*Hin*dIII fragment was digested and subclones were generated in pUC18. The pUC18 vector is under the ColE1 replicon and cannot be maintained in *P. aeruginosa.* Therefore, these recombinant plasmids were linearized and ligated into the *Eco*RI restriction site of the broad host range plasmid pLAFR and used to transform *E. coli* HB101. Plasmids from these transformants were crossed into PAO-E64. When the elastase activity of PAO-E64 containing the subclones was examined, only one subclone, pRB1822-1, gave a zone of elastin hydrolysis at 37°C. This plasmid contained a 2.2 kilobase *Sal*I-*Hin*dIII fragment (Fig. 3B).

Localization of the elastase structural gene. Synthetic DNA septadecamer oligonucleotide probes corresponding to the amino acid sequence of the amino terminus of purified elastase were used in DNA hybridization analysis of pHN3 and

pRB1808. The synthetic oligonucleotides did not hybridize with any portion of pHN3. A 2.7 kilobase *Eco*RI-*Kpn*I fragment and a 1.7 kilobase *Eco*RI-*Sma*I fragment in pRB1808 hybridized with the probes (Fig. 4). This suggested that the structural gene for elastase was on the pRB1808 plasmid and not on pHN3.

Restriction analysis further indicated that the 8 kilobase *Eco*RI fragment of pRB1808 was not related to the 20 kilobase fragment of pHN13 or the 25 kilobase fragment of pHN3 (fig. 3A and C). A 1.75 kilobase *Eco*RI-*Sma*I fragment from pRB1808 was purified, nick translated, and hybridized to *Eco*RI digested PAO1 chromosome. The 1.75 kilobase fragment hybridized to an 8 kilobase *Eco*RI restriction fragment of chromosome (fig. 4). The septadecamer synthetic probes hybridized to this same region of the PAO1 chromosome. This 2.2 kilobase *Sal*I-*Hin*dIII fragment from pRB1822, which had been shown to complement PAO-E64 was purified, nick translated, and used to probe the PAO1 chromosome. This 2.2 kilobase fragment hybridized to a 20 kilobase *Eco*RI restriction fragment of PAO1 chromosome. The synthetic probes did not hybridize to this region of PAO1 chromosome. Southern blots and restriction analyses confirmed that the elastase structural gene and the gene which complements the nonelastolytic phenotype of PAO-E64 are two different genes.

Detection of elastase antigen and activity in E. coli TB1. Cell lysates and culture supernatants of *E. coli* TB1 containing recombinant plasmids were examined for their ability to produce elastase antigen and proteolytic activity. No proteolytic or elastolytic activity was detected in culture supernatants from *E. coli* TB1 containing any of the recombinant plasmids. Significant proteolytic and elastolytic activity was found in cell lysates of TB1 pRB1808. Lysates of TB1 containing pRB1822 or pUC18 showed no detectable proteolytic or elastolytic activity (Table 3). The TB1 cell lysates were analyzed by immunobloting with elastase specific antiserum.

Elastase antigen was detected in cell lysates of only TB1 pRB1808. This elastase produced in TB1 had the same apparent molecular weight as the elastase standard purified from *P. aeruginosa* culture supernatants (Fig. 5).

DISCUSSION

We cloned two genes from *P. aeruginosa* PAO1 that are involved in elastase production. These genes complement different mutations in *P. aeruginosa*. A gene encoded by pHN13 and pHN3 complements the *lasA1* mutation of the mutant PAO-E64. This gene does not complement the elastase null mutant PAO-E105. In contrast, a gene encoded by pRF1 complements the mutation in PAO-E105 but not that of PAO-E64. The inserts containing these genes had different restriction maps. Furthermore, probes isolated from both genes hybridize to themselves but not to each other, and they each hybridize to DNA fragments of different sizes in Southern blots of PAO1 chromosomal DNA. These data indicate that these two elastase genes are different, and they support the conclusion that more than one gene is involved in elastase production in *P. aeruginosa*.

The elastase gene encoded by pRF1 is the structural gene encoding for *P. aeruginosa* elastase. It hybridizes with structural-gene specific oligonucleotide probes. When it is expressed in *E. coli* active *P. aeruginosa* elastase is produced. When it is introduced into the parent strain PAO1 the level of elastase is increased consistent with a gene dosage effect. The fact that this gene complements PAO-105 suggests that this mutant has a lesion in the elastase structural gene. We propose that the structural gene mutation of PAO-E105 be designated *lasB*. This null mutant (PAO-E105) should prove useful as a host for expressing altered elastase genes to elucidate the structural-functional domains of the elastase protein.

We observed that *P. aeruginosa* elastase produced in *E. coli* is cell associated. It is, however, both proteolytic and elastolytic and it has the same apparent molecular weight as purified extracellular elastase produced by *P. aeruginosa*. Previous studies have shown that in *P. aeruginosa*, cell associated elastase was

enzymatically inactive; located in the periplasm of *P. aeruginosa* and of the same apparent molecular weight as the active, extracellular elastase (20). It was concluded that, upon transport across the outer membrane of *P. aeruginosa* the proelastase is processed to active elastase, and Jensen et al. suggest that this may involve separation of elastase from an inhibitor substance (7, 20). Our data suggest that *E. coli* is capable of processing elastase to its active form while it is still cell associated. Perhaps the putative inhibitor associated with proelastase in *P. aeruginosa* is not present in *E. coli*.

Interestingly, the structural gene encoding elastase does not complement the *lasA1* mutation in PAO-E64. Since this mutant produces a cross reacting material (CRM) elastase protein, we had previously concluded that it had a mutation in the elastase structural gene (17, 38). Clearly, this is not so. To our knowledge, PAO-E64 is the first example of a mutant with a CRM phenotype for which the mutation is not in the structural gene. The mutation in PAO-E64 (*lasA1*) is complemented by a gene encoded by a 2.2 Kilobase *Sal*I-*Hin*dIII restriction fragment in the plasmids pHN13, pHN3, pPS4, and pRB1822-1. We do not know the function of the *las*A1 gene or its product. Based on the phenotype of the mutant PAO-E64, it is unlikely that the *lasA1* gene is a positive or negative regulator of elastase production. The mutant PAO-E64 produces parental levels of elastase antigen which is excreted and which is the same size as native *P. aeruginosa* elastase. The CRM elastase produced by PAO-E64 is, however, devoid of elastase activity at 37°C. Perhaps the *lasA1* gene functions in altering conversion of proelastase to active elastase. It has been suggested that processing involves more than one step (7, 19, 20). We are currently sequencing the *lasA1* gene to clarify its role in elastase production.

In summary. two different genes involved in *P. aeruginosa* elastase production have been isolated and characterized. One gene is the structural gene. The function of the second gene remains unknown.

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Genotype or phenotype	Source
E. coli		
HB101	general cloning host	5
MM294	general cloning host	39
TB1	cloning host for pUC vectors	2
P. aeruginosa		
PAO1	Prototroph	14
PAO-E64	*lasA1* lesion of PAO1	38
PAO-E105	Tn*501* mutation of PAO1, elastase null	This study
PA-E15	environmental isolate, elastase deficient	36
Plasmids		
pLAFR	IncP1, Tc^r, λ cos^+	10
pRK2013	ColE1, Kn^r, Tra^+(RK2)	8
pUW942	pAS8Rep-1 *zxx*::Tn*501*	43
pUC18	ColE1, Ap^r, *laci* ϕ80d*lacZ*	46
pHN3	25 kb *Eco*RI insert in pLAFR, *lasA1*	This study
pHN13	20 kb *Eco*RI insert in pLAFR, *lasA1*	This study
pPS4	*Hin*dIII deletion of pHN3, *lasA1*	This study
pPS1875	pPS4 7.5 kb *Eco*RI-*Hin*dIII fragment ligated to pUC18, *lasA1*	This study
pRB1822	pPS1875 2.2 kb *Sal*I-*Hin*dIII fragment ligated to pUC18, *lasA1*	This study
pRB1822-1	pRB1822 ligated to pLAFR, *lasA1*	This study
pRF1	8 kb *Eco*RI insert in pLAFR, *lasB*	This study
pRB1808	pRF1 8 kb *Eco*RI insert ligated to pUC18, *lasB*	This study

TABLE 2. Protease and elastase assays of culture supernatants of *P. aeruginosa* strains.

Strain(plasmid)	Mean(sd) μg of protease/mg of total protein[1] Hide powder azure		Elastin Congo red	
PAO1(pLAFR1)	28	(1.2)	32	(1.5)
PAO1(pRF1)[2]	99	(9.3)	104	(5.2)
PAO1(pHN13)	21	(2.1)	23	(2.4)
PAO-E105(pLAFR)	0.17	(0.14)	<5	
PAO-E105(pRF1)	42	(17)	62	(23)
PAO-E105(pHN13)	0.16	(0.14)	<5	
PAO-E64(pLAFR)	0.21	(0.12)	<5	
PAO-E64(pRF1)	0.53	(0.3)	14	(9.2)
PAO-E64(pHN13)	27	(9.9)	49	(21)

[1] From four independent samples. Data was extrapolated from a standard curve by using commercial *P. aeruginosa* elastase (see materials and methods). Numbers were converted to microgram protease per mg of total protein. The limit of detection was >0.1 μg of elastase/mg total protein with Hide powder azure and >5 μg of elastase/mg total protein with Elastin Congo red.

[2] PAO(pRF1) was significantly more active then PAO(pLAFR) in both assays (Student t test).

TABLE 3. Proteolytic activity of cell lysates of *E. coli*.

Strain(plasmid)	Mean (sd) μg protease/mg total protein[1] Hide powder azure	Elastin Congo red
TB1(pUC)	<0.1	<5
TB1(pRB1808)	20.4 (14)	17.3 (13)
TB1(pRB1822)	<0.1	<5

[1] From three independent samples. The standard curve and limit of assay detection were the same as for table 2.

Fig.1 *P. aeruginosa* elastase amino acid sequence from the amino terminus. Permutations of the nucleotide sequence are shown below the amino acids. Dotted line above amino acid 32 to 37 indicate where the septadecamer probe pool was made.

FIGURE 1

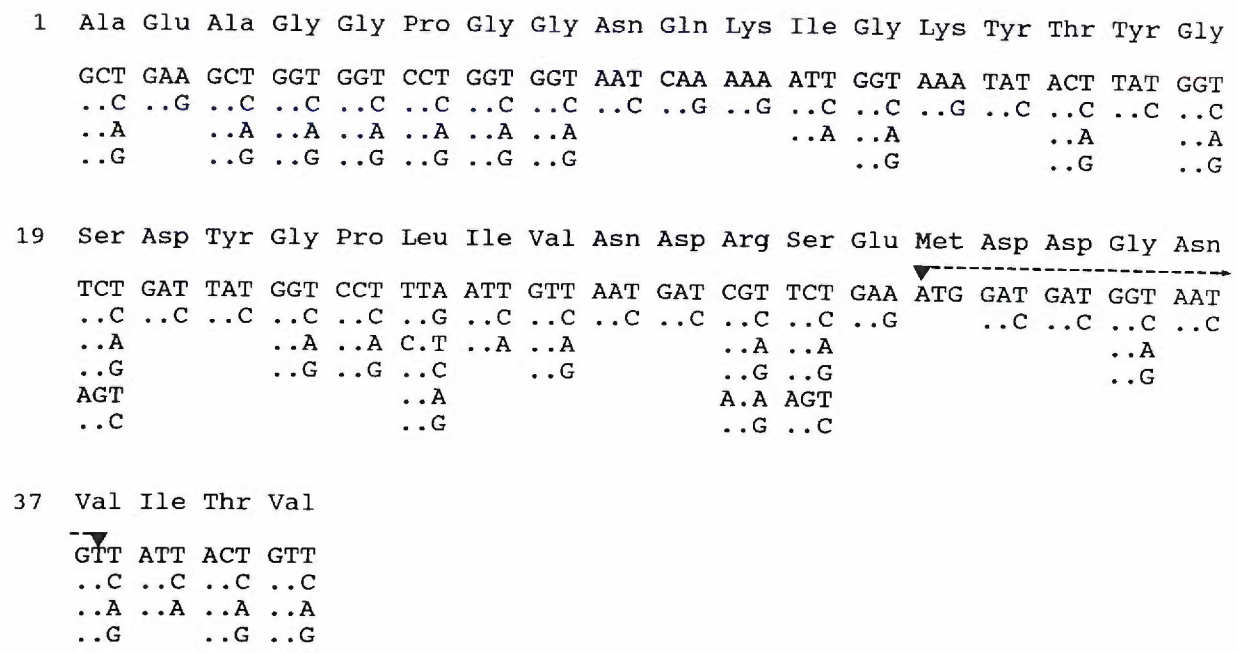

1 Ala Glu Ala Gly Gly Pro Gly Gly Asn Gln Lys Ile Gly Lys Tyr Thr Tyr Gly
GCT GAA GCT GGT GGT CCT GGT GGT AAT CAA AAA ATT GGT AAA TAT ACT TAT GGT
..C ..G ..C ..C ..C ..C ..C ..C ..C ..G ..G ..C ..C ..G ..C ..C ..C ..C
..A ..A ..A ..A ..A ..A ..A ..A ..A ..A ..A ..A
..G ..G ..G ..G ..G ..G ..G ..G ..G ..G
19 Ser Asp Tyr Gly Pro Leu Ile Val Asn Asp Arg Ser Glu Met Asp Asp Gly Asn
TCT GAT TAT GGT CCT TTA ATT GTT AAT GAT CGT TCT GAA ATG GAT GAT GGT AAT
..C ..C ..C ..C ..C ..G ..C ..C ..C ..C ..C ..C ..G ..C ..C ..C ..C
..A ..A ..A C.T ..A ..A ..A ..A ..A ..A ..A
..G ..G ..G ..C ..G ..G ..G ..G ..G
AGT ..A A.A AGT
..C ..G ..G ..C
37 Val Ile Thr Val
GTT ATT ACT GTT
..C ..C ..C ..C
..A ..A ..A ..A
..G ..G ..G

Fig 2. Deletions were made from pHN13 and pHN3. Plasmids pPS1, pPS2, and pPS3 were deletions of pHN13 using the restriction enzymes; *Bam*HI, *Pst*I, and *Xho*I respectively. Plasmid pPS4 was a *Hin*dIII deletion of pHN3. Plasmids were scored for their ability to complement PAO-E64 on reverse elastin plates. Dotted line indicates deleted DNA. Abbreviations: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; P, *Pst*I; X, *Xho*I.

FIGURE 2

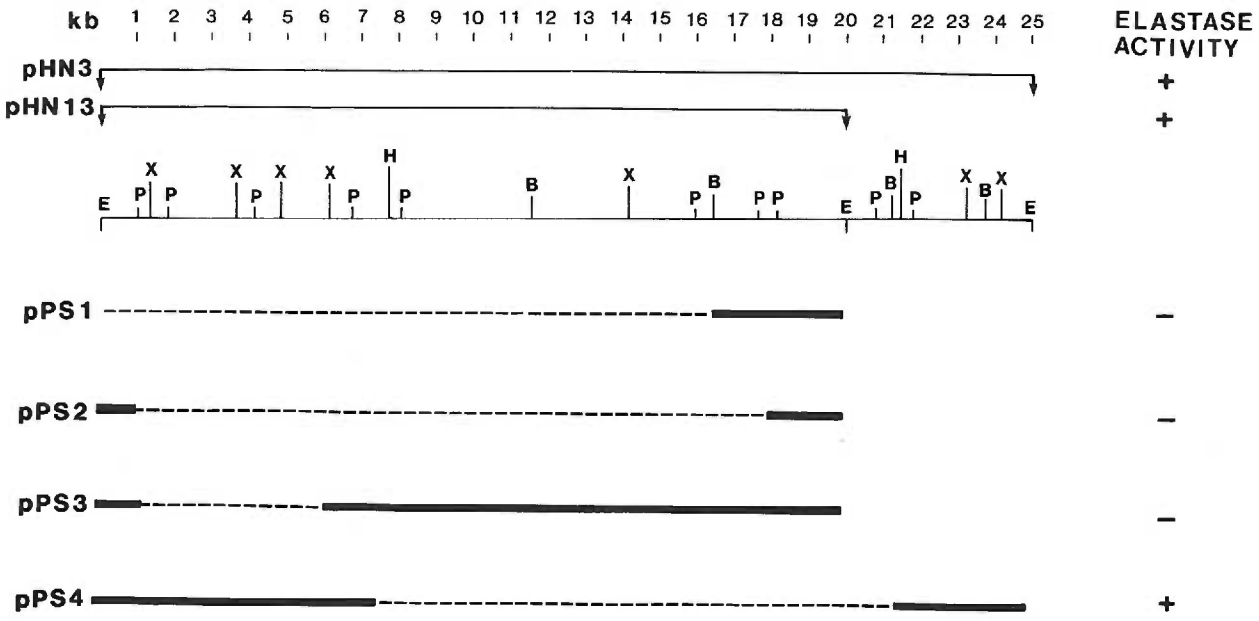

kb 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25
ELASTASE ACTIVITY
pHN3
pHN13
+
+
E P X P P X X P X P H P B X P B P P E P B H P X B X E
pPS1
pPS2
pPS3
pPS4
–
–
–
+

FIG.3 (A) Restriction map of 20 and 25 kilobase *Eco*RI cloned insert in the vector pLAFR forming pHN13 and pHN3. Lines with small arrows indicate the DNA contained within each clone. The additional 5 kilobase *Eco*RI insert of pHN3 may not be contiguous in the PAO-1 chromosome. (**B**) Restriction map of pPS1875 and pRB1822 cloned insert. The bold line indicates smallest restriction fragment (2.2 kilobase *Sal*I-*Hin*dIII fragment) that complements the *lasA1* lesion in PAO-E64. (**C**) Restriction map of the 8 kilobase *Eco*RI cloned insert in pUC18 forming pRB1808. The map shows the insert in relation to the *lac* promoter and the multicloning sites (MCS). The dotted line indicates the smallest restriction fragment that hybridizes with the structural gene oligonucleotide probes. The bold lines indicate vector sequences. Abbreviations: B, *Bam*HI; C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; M, *Sma*I; S, *Sal*I; T, *Sst*I; X, *Xho*I.

FIGURE 3.

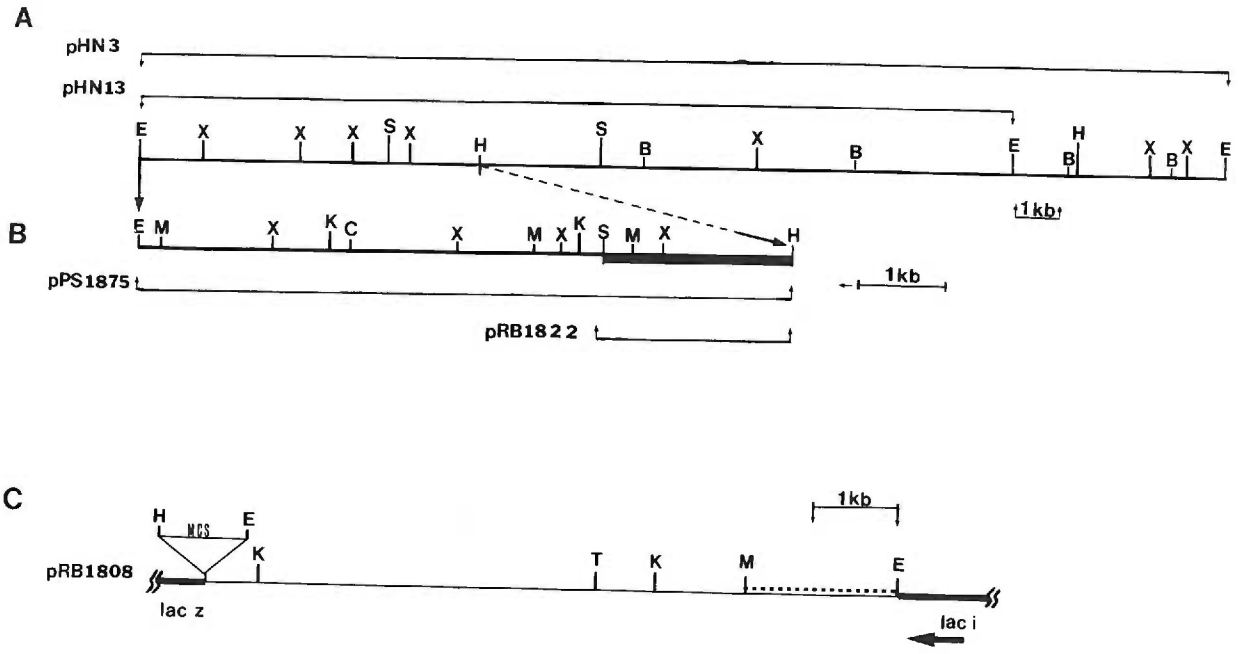

A
pHN3
pHN13
E X X X S X H S B X B E H B X B X E
1kb
B
E M X K C X M X K S M X H
1kb
pPS1875
pRB1822
C
1kb
H MCS E
K
pRB1808
T K M E
lac z
lac i

FIG. 4 Restriction analysis and DNA hybridization of plasmid DNAs and PAO1 *Eco*RI chromosome digests. Lanes 1 and 5, λ *Hin*dIII DNA standard; 2, pRB1808 cut with *Eco*RI and *Sma*I; 3, pRB1808 cut with *Eco*RI and *Kpn*I; 4, pHN3 cut with *Eco*RI and *Pst*I. 1' and 5' are λ *Hin*dIII DNA standard; Lanes 2',3',and 4' hybridization of synthetic oligonucleotide probes, specific to *P. aeruginosa* elastase; 6, 7, and 8, *Eco*RI digested PAO1 chromosome hybridized to the 2.2 kilobase *Sal*I-*Hin*dIII probe (*lasA1*), 1.7 kilobase *Eco*RI-*Sma*I probe (*lasB*) and the synthetic oligonucleotide probes, respectively.

FIGURE 4.

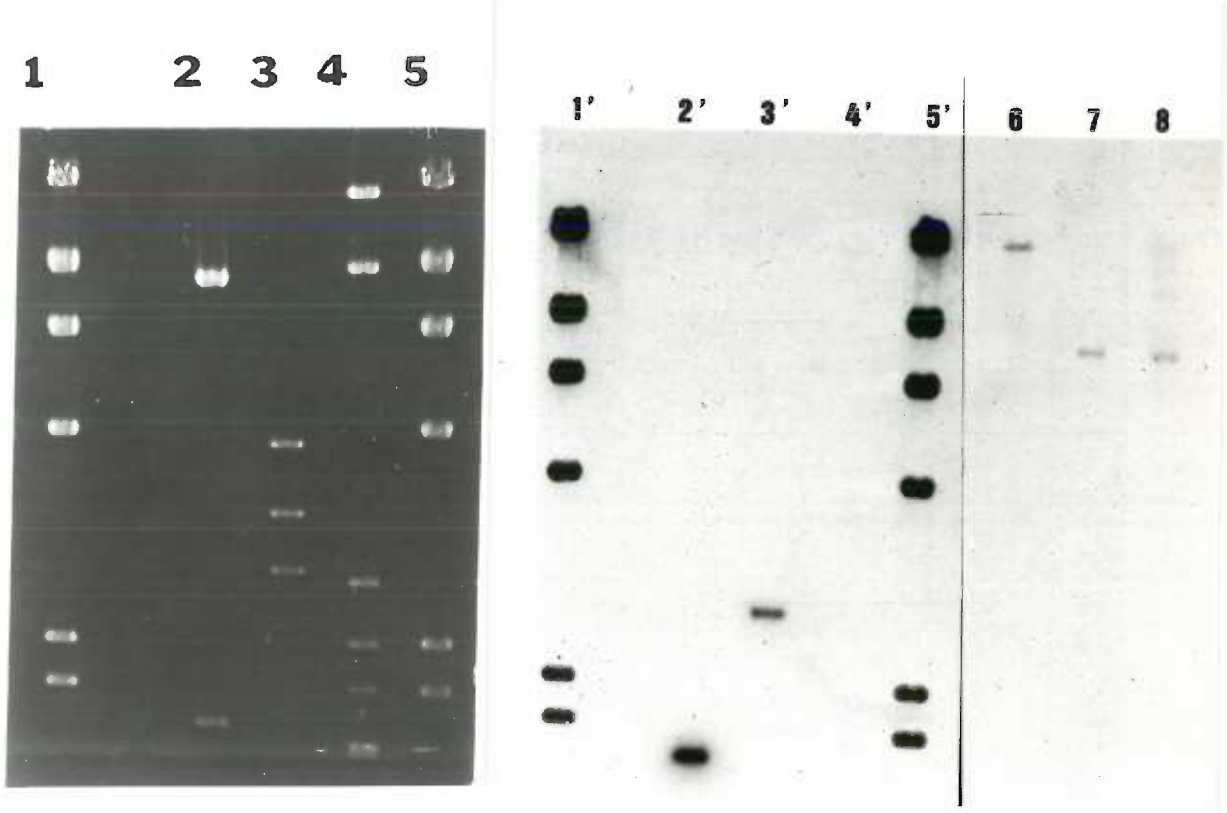
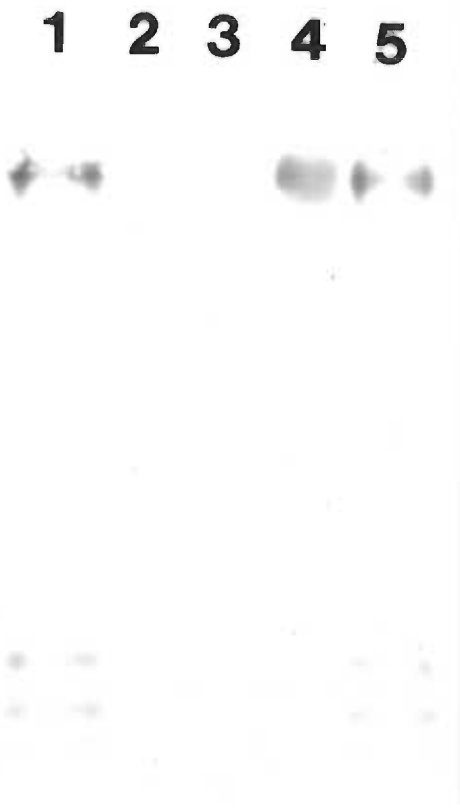


FIG. 5 Western immunoblot of *E. coli* TB1 lysates containing the recombinant plasmids. Lanes 1 and 5 contain 100 ng commercial *P. aeruginosa* elastase. Lanes 2-4 contain 10 μg of total protein. **2.** TB1(pUC18); **3.** TB1(pRB1822); **4.** TB1(pRB1808).

FIGURE 5.



Manuscript 3

Nucleotide Sequence and Expression in *Escherichia coli* of the *Pseudomonas aeruginosa lasA* Gene.

ABSTRACT

Pseudomonas aeruginosa PAO-E64 is a mutant which produces parental levels of elastase antigen but has no elastolytic activity at 37°C. The lesion (*lasA1*) in PAO-E64 is not a mutation in the structural gene for *P. aeruginosa* elastase (P. A. Schad et al. J. Bacteriol. 169:2691-2696, 1987). A 1.7 kilobase segment of DNA that complements the *lasA1* lesion was sequenced. Computer analysis of the DNA sequence showed that it contained an open reading frame which encoded a 41,111 dalton protein. The *lasA* gene was expressed under an inducible PT-7 promoter, and a 40,000 dalton protein was detected in *E. coli* lysates. The *lasA* protein was localized in the outer membrane fraction of *E. coli*. This *lasA* protein produced in *E. coli* activated the extracellular elastase produced by the *P. aeruginosa* mutant, PAO-E64.

INTRODUCTION

Pseudomonas aeruginosa produces two extracellular proteases believed to be involved in virulence. Elastase, the most active protease (22, 42), is produced by 75 to 95% of *P. aeruginosa* strains tested (32). This protease inactivates a variety of biologically active compounds including IgG, complement, elastin, collagen, and α_1-proteinase inhibitor (8, 31, 33, 39). In addition to the *in vitro* inactivation of these compounds, there is evidence that elastase plays a role in the pathogenesis of *P. aeruginosa* infections (18, 23, 27, 32, 34).

Although elastase is recognized as an important factor in the pathogenesis of *P. aeruginosa* infections, little is known about its regulation, processing, and secretion. The elastase found in the periplasm of *P. aeruginosa* is elastolytically inactive (9, 22). Presumably, elastase is activated upon transport across the outer membrane (9, 22). The inactive periplasmic elastase is slightly larger than the excreted (active) form by approximately 500 to 1,000 daltons (da) (24). Previously, Ohman et al. (35) reported the isolation of a *P. aeruginosa* PAO1 mutant (PAO-E64) that produced parental levels of elastase antigen and was proteolytic but devoid of elastolytic activity at 37°C (35). The mutation in PAO-E64, *lasA1*, was mapped at 75 min on the *P. aeruginosa* PAO1 chromosome (19). It was previously shown that the *lasA* gene is not the elastase structural gene (38). However, the *lasA* gene product may be involved in processing or secretion of elastase (11, 12, 38). In this study, we have analyzed the DNA sequence of the *lasA* gene, its expression and location in *E. coli*, and its possible role in elastase processing and activation.

MATERIALS AND METHODS

Bacterial strains, plasmids, phage, and growth conditions. Bacterial strains, plasmids, and phage are shown in Table 1. *E. coli* and *P. aeruginosa* were grown in LB broth containing 1% bacto tryptone, 0.5% yeast extract (Difco Labs, Detroit, Mi.) and 1% NaCl (pH 7.2) at 37°C unless stated otherwise. M9 minimal salts are described by Maniatis et al. (29). Antibiotics used for *E. coli* were ampicillin (100 μg/ml), kanamycin (50 μg/ml), chloramphenicol (100 μg/ml), and tetracycline (25 μg/ml) (Sigma Chemical Co., St. Louis, Mo.).

Gel electrophoresis. Agarose gel electrophoresis was carried out as described by Maniatis et al. (29), using Tris-borate-EDTA buffer. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out by the method of Laemmli (26). Stacking gels were 5% acrylamide with 10% separating gels (acrylamide:bis, 30:1). SDS-PAGE reagents were purchased from Bio-Rad Laboratories (Richmond, Ca.). The [^{14}C]-methylated protein standards were from Amersham Corp. (Arlington Heights, Il.).

DNA purification and subcloning. DNA was purified from *E. coli* as described by Holmes and Quigley for small volumes (17). Large scale preparations of plasmid DNA were described previously (38). DNA fragments used in subcloning were purified by agarose gel electrophoresis and eluted on an IBI Electroeluter as recommended by the manufacturer (International Biotechnologies Inc. (IBI), New Haven, Ct.). Deletion subclones were generated by digestion with the appropriate restriction enzymes. Blunt end ligation of incompatible ends was done using Klenow fragment followed by ligation with T4 DNA ligase. Restriction enzymes were

purchased from Boehringer Mannheim Biochemicals (Indianapolis, In.), BRL (Bethesda Research Laboratories, Gaithersburg, Md.), IBI, New England Biolabs Inc., (Beverly, Ma.) and Promega Biotec (Madison, Wi.). T4 DNA ligase and Klenow fragment were from Bethesda Research Laboratories and Boehringer Mannheim Biochemicals.

DNA sequencing. A modification of the Sanger dideoxy-DNA sequencing method was used for clones prepared in the M13 vectors (37, 43). This modification was the substitution of dGTP with 7-deaza-dGTP (1, 30). Subclones for sequencing were generated based on the restriction map shown in Fig. 1A. Additional overlapping clones were generated by the method of Dale as described in the IBI cycloning kit (6). Deoxynucleotides and dideoxynucleotides were purchased from Pharmacia, Inc. (Piscataway, N.J.). The nucleotide 7-Deaza-dGTP was purchased from Boehringer Mannheim Biochemicals. The 17mer primer was from New England Biolabs. The isotope, [α-^{35}S]dCTP (1,000 Ci/mM) was from Amersham Corp. Sequence data were analyzed with IBI sequencing software and the University of Wisconsin Genetics Computer Group software (version 5.0) (7). The codon usage algorithm of Gribskov et al. (13) was used with a codon usage table compiled from published *P. aeruginosa* sequence data (S. West, and B. Iglewski manuscript in preparation).

Promoter studies of lasA and expression in E. coli. The promoterless chloramphenicol acetyltransferase gene block cartridge (CAT) (Pharmacia, Inc.) was inserted into the *Xho*I site of pRB1822, pPS1816, and pPS1916, and the orientation of the gene cartridge was determined by restriction analysis (4).

The T7 RNA polymerase-promoter expression system (pT7-7) was used to express the *lasA* gene in *E. coli* K38 (41). The 1.7 kilobase (kb) *Sma*I-*Hin*dIII fragment of pPS1816 was inserted into the *Sma*I-*Hin*dIII sites of pT7-7 (pT7-17).

The proteins expressed were labeled by using a modification of the method described by Tabor and Richardson (personal communication). Briefly, cells containing both plasmids (pGP1-2 and pT7-17) were grown in LB medium with appropriate antibiotics to an A_{595} of 0.5. The cells (0.2 ml) were washed in M9 salts and suspended in M9 salts containing 0.01% of 19 of the 20 amino acids (minus leucine) supplemented with 20 μg of thiamine per ml. The cells were incubated for 2 h at 30°C to deplete free leucine, followed by an additional incubation at 42°C for 15 min. Rifampicin (200 μg/ml, final concentration) was added, and the cells were incubated an additional 10 min at 42°C. The temperature was shifted to 30°C for 20 min and after addition of 10 μCi of [^{3}H]leucine (50 Ci/mM) (Amersham), the cells were incubated for an additional 15 min at 30°C. Cells were pelleted and resuspended in 75 μl of cracking buffer (60 mM Tris-HCl (pH 6.8), 1% SDS, 1% 2-mercaptoethanol, 10% glycerol, and 0.01% bromophenol blue). The samples were heated for 5 min at 100°C, and 25 μl was loaded onto a 10% SDS-PAGE gel.

E. coli fractionation. *E. coli* K38 cells (15 ml) were grown and the T7 RNA polymerase was induced as described above. The labeling was done at 37°C with 200 μCi of [^{3}H]leucine (50 Ci/mM) for 45 min. Labeled cells were fractionated by a modification of the method of Koshland and Botstein (25). Cells were washed twice in M9 salts and resuspended in 0.5 ml of sucrose buffer (20% sucrose, 10mM Tris (pH 7.5) to which 5 μl of 0.5 M EDTA (pH 8.0) was added. Following incubation on ice for 10 min, the cells were centrifuged and the pellets were suspended in 0.5 ml of cold distilled H_2O and incubated on ice for 10 min. Cells were centrifuged, and the supernatants (periplasmic fractions) were kept. The pellets were suspended and the cells were lysed by two passages through a French pressure cell at 15,000 lb per in^2. The cell lysates were centrifuged at 100,000 x *g* for 1 h, and the supernatants (cytoplasmic fractions) were removed. The total membrane pellets were

suspended in distilled H_2O, and a sample was stored for further analysis. The remaining membranes were further fractionated into inner and outer membranes on sucrose gradients as described by Hancock and Nikaido (14) as modified by Hindahl and Iglewski (15) All fractions were stored at -20°C until analysis. Protein determination was by the method of Lowry with bovine serum albumin as a standard (28). Trichloroacetic acid precipitation of labeled fractions was done as described by Chung and Collier (3).

Elastase assay and elastase activation. Cultures were grown overnight at 37°C in Peptone Trypticase Soy broth (35). Elastase activity was measured by using Elastin Congo red (Sigma) as previously described (38). Activation studies were carried out by adding 100 μg of *E. coli* membrane fractions to 250 μl of culture supernatants from PAO1 and PAO-E64. Following incubation for 15 min at 37°C, these samples were assayed for elastolytic activity.

RESULTS

Subcloning of pRB1822 and analysis of lasA gene orientation. The plasmid pRB1822 (Fig. 1A) contains a 2.2 kilobase (kb) insert subcloned from the plasmid pHN3 (38). Both of these plasmids contain *P. aeruginosa* PAO1 chromosomal DNA which complements the *lasA1* lesion in the mutant PAO-E64 (35). Goldberg and Ohman (11) have reported that insertion of a gene block into the *Xho*I site inactivates the *lasA* gene. They noted that the *lasA* gene promoter was expressed in *E. coli* and the direction of *lasA* transcription was from *Xho*I toward the *Hin*dIII site (11). We confirmed and extended these observations by inserting the promoterless CAT gene block into the *Xho*I site of pRB1822 which contains 2.2 kb of *P. aeruginosa* DNA, compared with the 4 kb used by Goldberg and Ohman (11). Chloramphenicol resistance was achieved in only one direction (Fig. 1C). To further localize the *lasA* promoter, the *Sma*I-*Hin*dIII fragment of pRB1822 was inserted into pUC18 (pPS1816) and pUC19 (pPS1916) and the CAT gene block was inserted into the *Xho*I site of these plasmids. Chloramphenicol resistance occurred only when CAT was inserted in one direction such that transcription was from *Sma*I towards *Hin*dIII (Fig. 1B, D, E). These data indicate that a *P. aeruginosa* promoter was contained within approximately a 360 base pair (bp) region of DNA between the *Sma*I and *Xho*I sites (Fig. 1A).

Sequencing of the lasA gene. The 1.7 kb *Sma*I-*Hin*dIII fragment was put into the broad host range plasmid pLAFR (pPS1816-1). The plasmid pPS1816-1 complemented the mutation in PAO-E64, indicating that the 1.7 kb *Sma*I-*Hin*dIII fragment contained the *lasA* gene (data not shown). The 1.7 kb fragment of pPS1816 was sequenced and was found to contain 1712 bp of *Pseudomonas* DNA. Computer analysis of the sequence data showed several possible open reading frames

(ORF) (data not shown). When the codon usage algorithm of Gribskov et al. (13) was used, a large ORF with significant codon usage was detected starting at bp 105 and ending at bp 1290. Analysis of the ORF revealed that although the first methionine is encountered at bp 105, codon usage is not statistically significant until bp 160 (data not shown). No ribosome binding site (40) can be found upstream of bp 105; however, a ribosome binding site is seen starting at bp 148 with the sequence AGGAG. This binding site is 6 bp upstream from methionine (bp 159). The DNA sequence of the 1.7 kb fragment and the translated product of the ORF starting at bp 159 and ending at bp 1289 is shown in Fig. 2. This ORF theoretically codes for a 41,111 Da protein containing 377 amino acid residues.

Expression of the lasA gene product in an E. coli expression system. The pT7-7 vector contains the bacteriophage T7 promoter along with an inducible T7 RNA polymerase on a second plasmid (41). Rifampicin is used to inhibit the host RNA polymerase. Therefore, DNA inserted into the multicloning sites of the T7 promoter is expressed without concurrent expression of host proteins allowing one to radiolabel the protein of interest. The 1712 bp fragment of pPS1816 was cloned into the pT7-7 expression vector (pT7-17), and the expressed proteins were analyzed on SDS-PAGE (Fig. 3). A 40,000 Da protein (*lasA*) was seen when the T7 promoter was induced at 42°C with the addition of rifampicin (Fig. 3, lanes 3, and 4). The 40,000 Da *lasA* protein was not heat modifiable (Fig. 3, lanes 3,and 4).

To determine the cellular location of the 40,000 Da *lasA* protein *E. coli* containing the *lasA* protein was fractionated into periplasmic, cytoplasmic, and membrane fractions. Of the TCA precipitable radioactivity 93% was associated with the membrane fraction (Table 2). This total membrane fraction was further fractionated on a sucrose gradient to separate the inner and outer membranes. Of the TCA precipitable radioactivity 77% was associated with the outer membrane

fraction (Table 2). Analysis of these *E. coli* fractions on SDS-PAGE revealed that the only labeled protein was the 40,000 Da *lasA* protein found in the total membrane fraction and in the outer membrane fraction of induced pT7-17 *E. coli* (Fig. 5).

Activation of PAO-E64 culture supernatants with lasA protein. The elastolytic activity of culture supernatants of the parent strain PAO1 and the *lasA1* mutant strain, PAO-E64, were compared in the presence of outer membrane preparations from *E. coli* containing either the vector as a control or the *lasA* gene. PAO-E64 supernatants showed a 35% increase in activity when incubated with outer membranes containing the *lasA* protein (Table 3). Supernatants from the parent strain also showed an increase in elastolytic activity when incubated with membranes containing the *lasA* protein. Membranes from *E. coli* containing only the vector had no significant effect on the elastolytic activity of the culture supernatants. Treatment of either PAO1 or PAO-E64 elastase with *lasA* protein did not alter the apparent molecular weight of these proteins on SDS-PAGE (data not shown).

DISCUSSION

We have previously reported the cloning of two *P. aeruginosa* genes involved in elastase expression (38). One of these genes, *lasA*, complements the mutation in PAO-E64. This mutant produces an extracellular elastase which is the same size as the parent elastase and is proteolytic but is deficient in elastolytic activity at 37°C. We have sequenced and analyzed a 1712 bp *Sma*I-*Hin*dIII fragment which complements PAO-E64. Analysis showed that this DNA contained a large open reading frame which encoded a 377 amino acid protein with a predicted molecular weight of 41,111 (Fig. 2). When the *lasA* gene was expressed in *E. coli*, a protein, *lasA*, with an estimated molecular weight of 40,000 was found. Fractionation studies showed that this protein was associated with the outer membrane of *E. coli* (Table 2). Analysis of the deduced amino acid sequence suggested that the amino terminal region of the *lasA* protein (Fig. 2) is similar to other procaryotic signal sequences (20, 21). The location of the *lasA* protein in *P. aeruginosa* and the ability of its amino terminal region to function as a signal sequence are currently under investigation.

The 40,000 Da *lasA* protein predicted and observed in our study was considerably larger than the 31,000 Da value reported by Goldberg and Ohman (11). Goldberg and Ohman used an expression vector where the host proteins continue to be synthesized when the cloned gene is induced, making it difficult to identify unambiguously the correct product produced by the gene of interest. We used the pT7-7 system (41) to alleviate this problem.

Extracellular elastase from PAO-E64 was activated with *E. coli* membranes containing the *lasA* protein (Table 3). This activation raised the elastolytic activity of PAO-E64 supernatants to parental levels. This *lasA* protein also amplified the elastolytic activity of the parent PAO1. In both cases, treatment with the *lasA*

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TABLE 1. Bacterial strains, phages and plasmids

Strain, phage or plasmid	Genotype or phenotype	Source or Reference
E. coli		
HB101	general cloning host	(2)
JM107	general host for M13	(43)
K38	host for PT7 expression system HfrC (λ)	(36)
P. aeruginosa		
PAO1	Prototroph	(16)
PAO-E64	*lasA1* mutant of PAO1	(35)
Phage		
M13mp18/19	single stranded DNA sequencing vectors	(43)
Plasmids		
pT7-7	T7 RNA promoter vector	(41)
pGM1-2	inducible T7 RNA polymerase	(41)
pLAFR	IncP1, Tc^r, λ cos^+	(10)
pHN3	25 kb *Eco*RI insert in pLAFR, *lasA1*	(38)
pRB1822	pPS1875 2.2 kb *Sal*I-*Hin*dIII fragment ligated to pUC18, *lasA1*	(38)
pPS1816	*Sma*I deletion of pRB1822	This study
pPS1816-1	pPS1816 inserted into *Eco*RI site of pLAFR	This study
pPS1916	*Sma*I-*Hin*dIII fragment of pPS1816 in pUC19	This study
pT7-17	*Sma*I-*Hin*dIII fragment of pPS1816 in pT7-7	This study

TABLE 2. Localization of *lasA* protein in *E. coli*.

E. coli fractions containing *lasA*[1]	Without sucrose fractionation CPM[3]	Without sucrose fractionation % Total incorporation	With sucrose fractionation[2] CPM[3]	With sucrose fractionation[2] % Total incorporation
Periplasm	307	1.8%	307	1.6%
Cytoplasm	823	4.8%	823	4.3%
Total Membranes	16,023	93.4%		
Inner Membrane			3,353	17.4%
Outer Membrane			14,772	76.7%

[1] *E. coli* containing vector alone incorporated <500 cpm in any fraction.

[2] Membranes were fractionated on a sucrose gradient, inner and outer membranes were removed.

[3] Counts per minute (CPM) are based on 10 μg of total protein precipitated on trichloroacetic acid (TCA) filters after washing. See methods for details.

TABLE 3. Activation of *P. aeruginosa* extracellular elastase with *E. coli* membranes containing the *lasA* protein.[1]

E. coli membrane fractions	Elastolytic activity of culture supernatants[2]			
	PAO1 Specific Activity[3]	% increase in activity[4]	PAO-E64 Specific Activity[3]	% increase in activity[4]
None	6.6	---	4.0	---
Vector Membranes	7.2	9%	4.0	0%
lasA Membranes	8.4	22.6% ($p<.1$)	6.3	36% ($p<.001$)

[1] *E. coli* fractions demonstrated no elastolytic activity when 100 μg of total protein was used and when the fractions were incubated overnight at 37°C with Elastin Congo red as a substrate (see materials and methods).

[2] Supernatants were adjusted to 10 mg of total extracellular protein per ml.

[3] Elastase specific activity was determined from a standard curve with purified *P. aeruginosa* elastase. These experiments were repeated three times with equivalent results.

[4] Increase in elastolytic activity was determined as the increase in specific activity following preincubation with various membrane fractions as compared with culture supernatants preincubated with buffer alone.

FIG. 1 Restriction endonuclease map of of two *lasA* containing plasmids showing direction of lactose promoter (*lac i*) present in all recombinant plasmids (vector sequences not shown). **(A)** pRB1822, **(B)** pPS1816 *Sma*I deletion of pRB1822. **(C)** Insertion of chloramphenicol acetyltransferase (CAT) cartridge into pRB1822 showing direction of CAT insertion. **(D)** Insertion of CAT into pPS1816. **(E)** Insertion of CAT into pPS1916. Symbols: H, *Hin*dIII; M, *Sma*I; P, *Pst*I; S, *Sal*I; X, *Xho*I.

FIGURE 1.

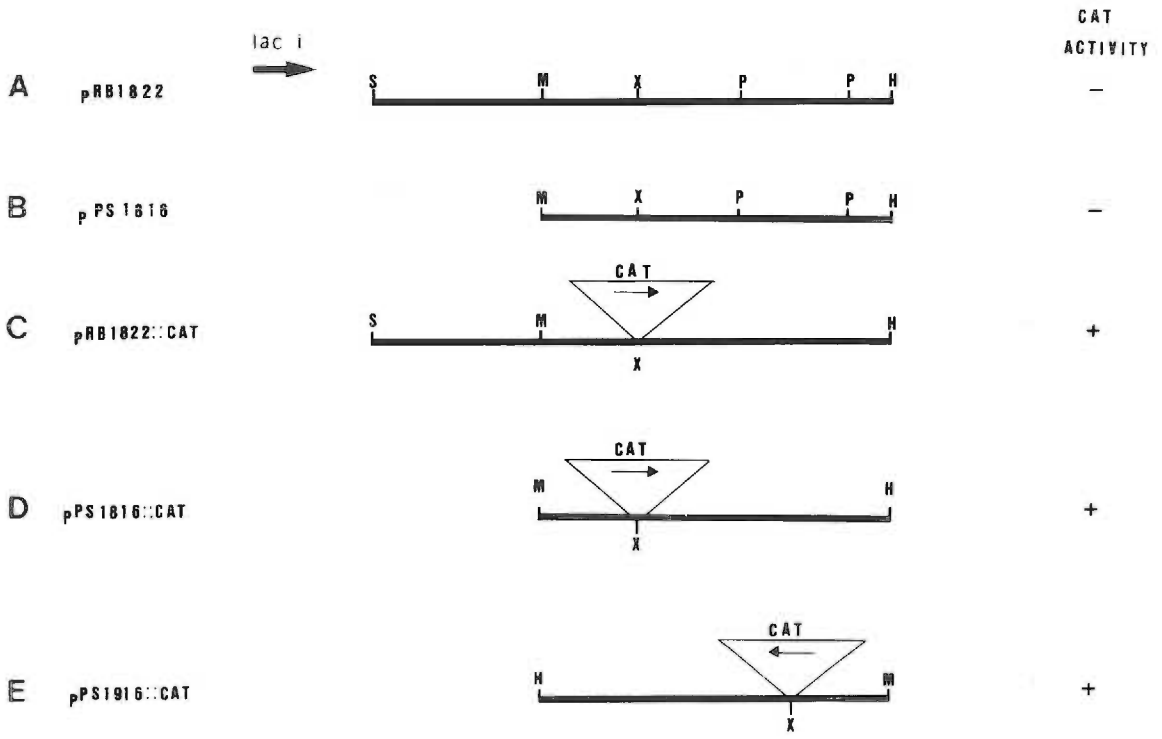

lac i
CAT ACTIVITY
A pRB1822
S M X P P H
–
B pPS1816
M X P P H
–
C pRB1822::CAT
CAT
S M H
X
+
D pPS1816::CAT
CAT
M H
X
+
E pPS1916::CAT
CAT
H M
X
+

FIG. 2. Graphic output of preferred codon usage according to Gribskov et al. (13). Graph shows large open reading frame in the third reading frame (*lasA*). Significant codon usage begins about bp 160 and ends about bp 1290, see arrows. Horizontal boxes indicate open reading frames, small vertical lines indicate rare codon usage.

FIGURE 2.

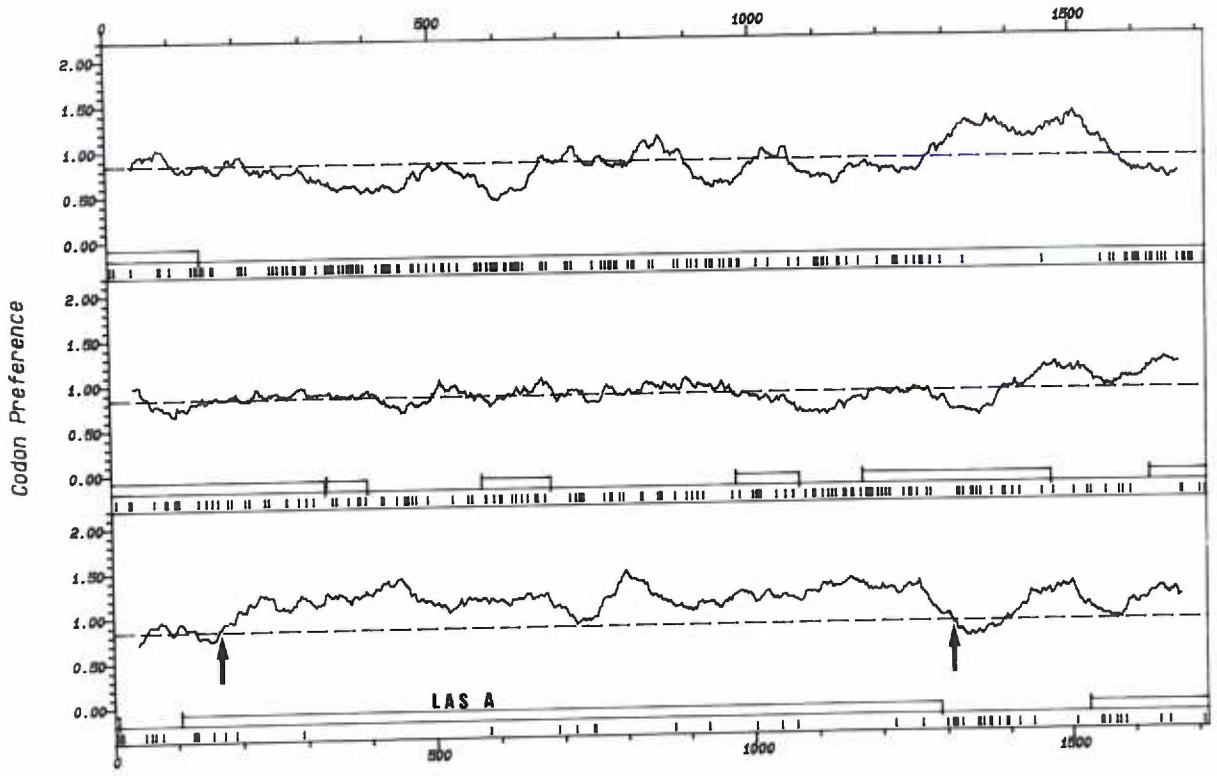
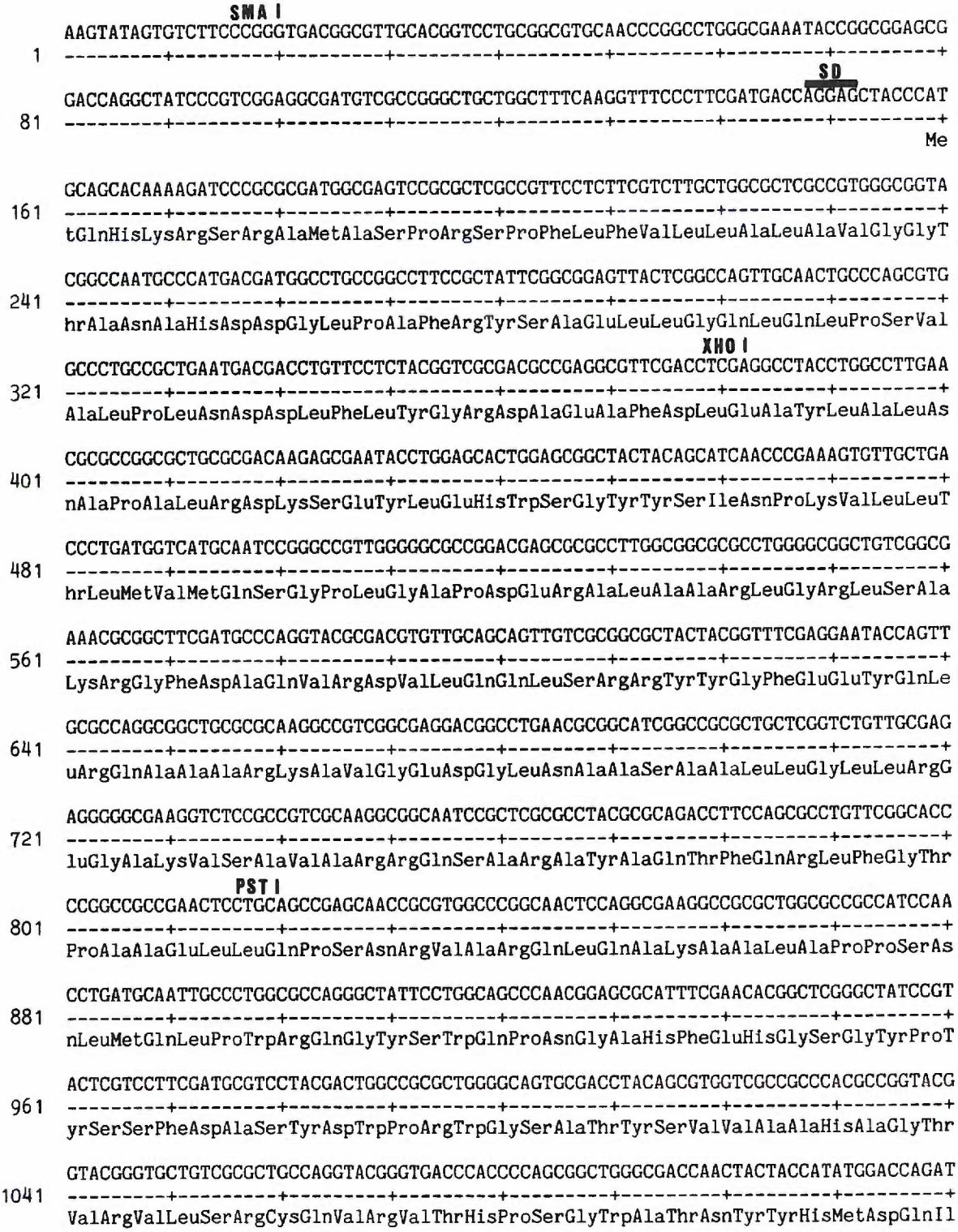


FIGURE 3.



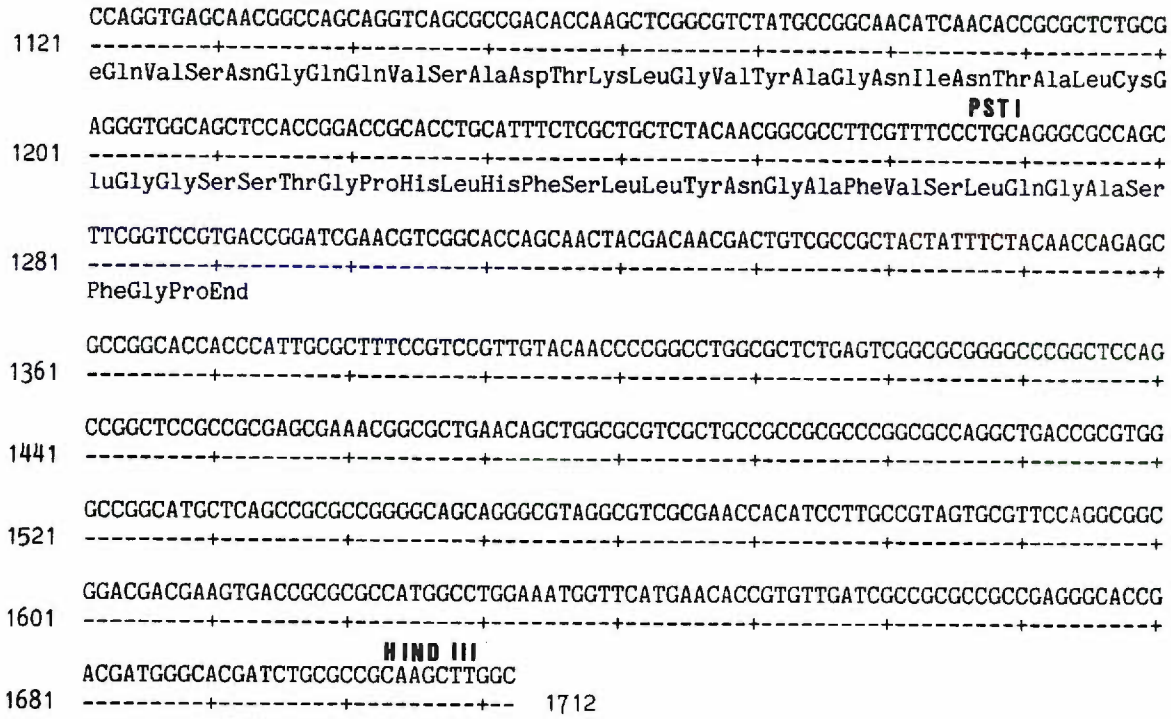


FIG. 3. Nucleotide sequence of the 1712 bp insert in pPS1816. The deduced amino acid sequence of the *lasA* protein starting at base pair 159 is shown below the DNA sequence. The Shine-Dalgarno box (SD) is shown as a broad line. Relevant restriction sites are shown.

FIG. 4. The *lasA* gene product in *E. coli* after induction of the T-7 promoter. **(A)** Autoradiograph of expressed *lasA* protein. **(B)** Coomassie Blue stained SDS-PAGE gel. Lanes in both panels: **1**, and **9**, [^{14}C]-methylated protein standards given in kilodaltons; **2**, Induction of T7 promoter and expression of *lasA* protein without rifampicin inhibition of host RNA polymerase; **3**, Expression of *lasA* protein with rifampicin (sample was heated at 100°C for 10 min); **4**, *lasA1* without heating; **5**, induced pT7-7 vector; **6**, *lasA1* expression without induction of T7 RNA polymerase and without inhibition of host RNA polymerase by rifampicin; **7**, pT7 vector alone (same conditions as 6); **8**, BioRad PAGE standards.

FIGURE 4.

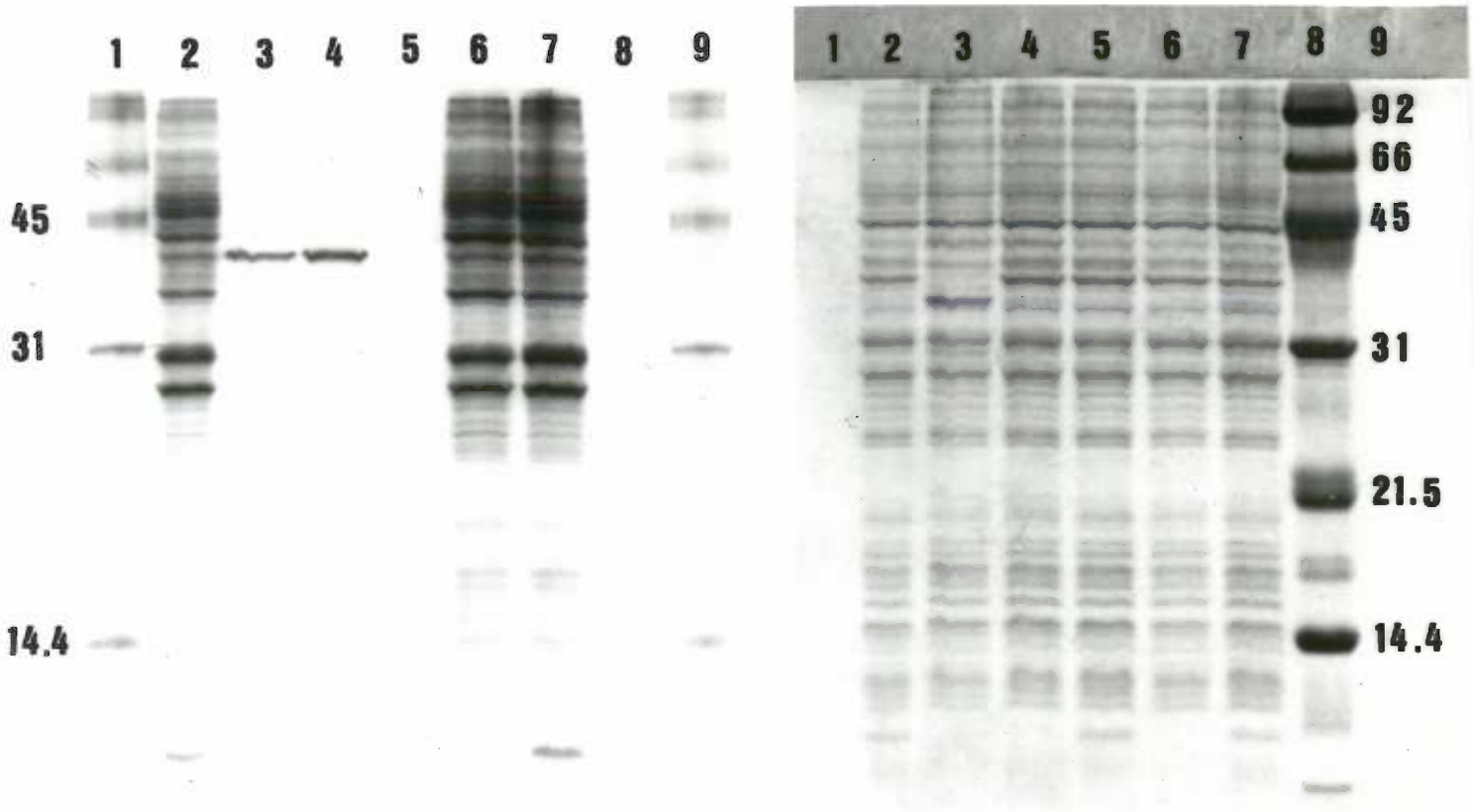
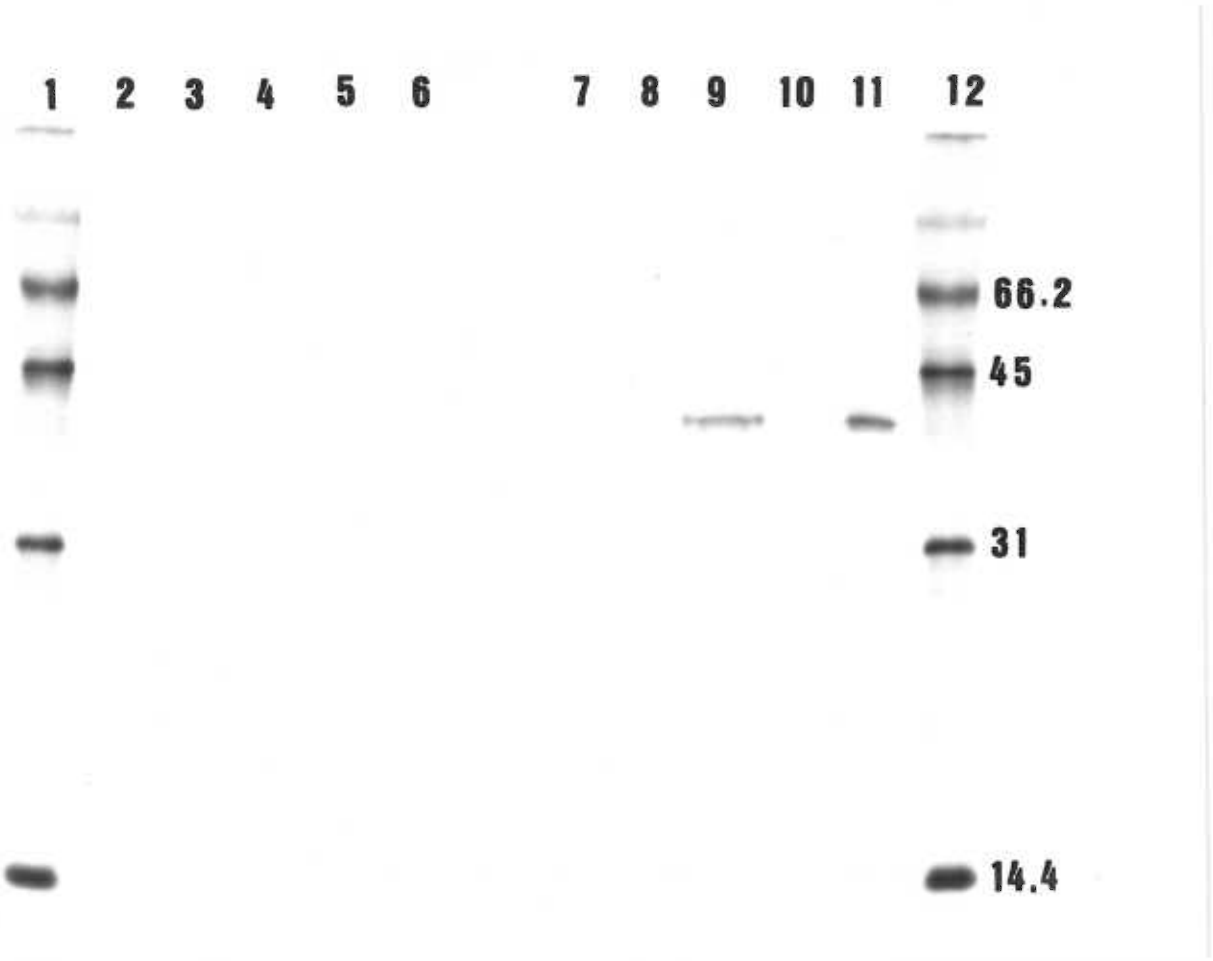


Fig. 5. Autoradiogram of cell fractions of *E. coli* containing the *lasA* protein. Lanes: 1,and 12, [^{14}C]-methylated standards given in kilodaltons; 2 to 6, *E. coli* containing the vector, pT7-7 (2. periplasmic; 3, cytoplasm; 4, total membranes; 5, inner membrane; 6, outer membrane); 7 to 11, *E. coli* containing the *lasA* clone, pT7-17 (7, periplasmic; 8, cytoplasm; 9, total membranes; 10, inner membranes; 11, outer membranes).

FIGURE 5.



IV. Summary and Discussion

Summary and Discussion

Pseudomonas aeruginosa is a ubiquitous organism found in nature and is therefore it is difficult to control exposure of at risk individuals. *P. aeruginosa* is noted for its ability to cause a chronic lung infection throughout the lives of the cystic fibrosis patients (4). This organism, is also the cause of localized infection of the skin, eye, wounds, urinary, intestinal, and reproductive system (3, 19, 20, 26, 28). *P. aeruginosa* is also able to cause systemic life threatening infections which occur in burn victims, immunocompromised persons, pneumonias, and surgical patients (6, 29, 30, 34). One problem in controlling *P. aeruginosa* infections is the high degree of antibiotic resistance found among *P. aeruginosa* isolates. This high degree of antibiotic resistance is thought to be due to the impermeability of the outer membrane to these substances (1, 35). The severity of *P. aeruginosa* infections coupled with its antibiotic resistance underscores the importance of gaining a better understanding of the virulence of this organism which may lead to a more effective control of these infections.

P. aeruginosa produces a variety of extracellular products which have been shown to be required for its virulence. Some of the factors implicated in the virulence of *P. aeruginosa* are exotoxin A, exoenzyme S, phospholipase C, alkaline protease, elastase, LPS, and alginate (15, 16, 25, 32). No one factor appears to be major virulence factor of this organism, rather virulence is multifactorial. Initially, investigators studied the virulence of *P. aeruginosa* using unrelated strains. This lead to conflicting evidence for the importance of any given virulence factors and failed to provide unambiguous data. Additionally, some of these studies have lead to conflicting information concerning the importance of different virulence factors (18, 23). A more direct approach has been to genetically alter a strain. This is frequently done by chemical or transposon mutagenesis. These genetic alterations allow the detection of a mutant strain deficient in the production of a single

virulence factor which may than be compared to the parent strain in an appropriate animal model. This approach has been used by several investigators (13, 25, 26, 27, 38). Ohman *et al.* (26, 27) isolated NTG mutants in PAO1 that were deficient in production or activity of elastase or exotoxin A. These mutant strains, when used in the appropriate animal model, demonstrated that exotoxin A was important in corneal infection (26), pulmonary infections (36), and in burn infections (27). Elastase was also found to be important in acute and chronic lung infections and in systemic infections of burned mice (2). Howe *et al.* used EMS to produce alkaline protease mutants in strains already deficient in elastase production. These mutants were avirulent in corneal infections. Furthermore, these studies showed that a protease, either elastase or alkaline protease, was necessary for initiating the corneal infection (13).

As stated above elastase is able to enhance infections caused by *P. aeruginosa.* Elastase seems to be important in localized infections and in systemic infections. Elastase is responsible for approximately 95% of the total extracellular proteolytic activity of most strains of *P. aeruginosa* and it is produced by 75% to 99% of the strains examined (37). Although elastase is generally referred to as a protease or metalloprotease it has very interesting substrate specificities. Elastase has been shown to degrade complement components (31), α-1 protease inhibitor (21, 22), immunoglobulin G and A (5, 11), and connective tissues such as collagen and elastin (10). One difficulty in studying elastase is that mutants previously used which are altered in elastase activity may have multiple mutations due to the use of chemical mutagenesis. Since little is known about the regulation, processing, and expression of elastase the most direct process to study elastase would be to clone genes responsible for elastase expression and using molecular methods, examined the function and gene product of those genes. Additionally, these genes could than be

mutated, by point mutagenesis or by deletion techniques, returned to the parent strain, by gene replacement, for analysis in animal models.

The possibility that several genes exist that control the levels of extracellular elastase can be inferred from the evidence that multiple genes are responsible for the excretion of various extracellular products of *P. aeruginosa.* It has been shown that some of the extracellular products of *P. aeruginosa* share common pathways for their excretion as indicated by the *xcp* (*e*xtra*c*ellular protein *h*yperproduction) phenotypes (38). These phenotypes have been grouped into two classes of mutants. Class 1 mutants are defective in the release of all extracellular products and in addition tend to be cell associated. Class 2 mutants seem to be defective in the formation of exoproducts on agar media but are able to excrete these products in broth cultures. Additionally, these class 2 mutants do not tend to accumulate cell associated exoproudcts as do class 1 mutants. Two mutants that allowed the production of extracellular proteins in the presence of high salts were examined in manuscript 1 and it was suggestive that these two mutants were part of what appears to be a regulatory operon for the excretion of extracellular products in *P. aeruginosa.* These mutants were also found to be defective in iron uptake underscoring the involvement of iron in the regulation of elastase yields which was first observed by Bjorn *et al.* (1a). The nature of this iron regulation remains unknown.

Since little is known about the regulation, processing, and/or secretion of elastase we set out to complement elastase negative strains in hopes of finding genes involved in elastase production. This was accomplished in several ways (manuscript 2). First a gene bank was made in a broad host range cosmid vector pLAFR (7). This vector was able to be transferred to chemical or transposon induced mutant strains of *P. aeruginosa* that were shown to be altered in their elastolytic activity (24, 27, manuscript 2). Second, by screening other elastase negative strains, environmental or clinical isolates, we hoped that additional genes would be

discovered that are involved in elastase production. One mutant that we were particularly interested in complementing was the strain PAO-E64. This mutant was previously shown to have little or no elastolytic activity at 37°C but it produced parental levels of extracellular elastase antigen (27). When elastase was purified from this strain it was found to have altered elastolytic activity at 37°C but it could be activated by lowering the temperature (J. Lile unpublished observations). This seemed to indicate that this elastase from PAO-E64 was somehow altered in its secondary structure inactivating its elastolytic activity at 37°C. Based on these data it has been concluded that the PAO-E64 had to be a mutation (*las*A) in the elastase structural gene (27). Several clones were found which complemented PAO-E64. Restriction analysis found these recombinant plasmids to contain a common 20 kb *Eco*RI fragment of DNA (manuscript 2). Using one of these clones, pHN13, subclones were generated, and a 2.2 kb *Sma*I-*Hin*dIII fragment was found which complemented the *lasA* mutation in PAO-E64. Since we believed this to be the structural gene for elastase, various attempts were made to produce fusion peptides in various plasmid vectors for expression of elastolytic activity or elastase antigen in *E. coli*. In addition, the amino terminus of elastase was sequenced and synthetic oligonucleotides were made to the region of the least codon permutations. Hybridization of these probes to the *lasA* clones would strongly suggest that the DNA which complemented PAO-E64 contained the elastase structural gene. All attempts at proving that the 2.2 kb *Sma*I-*Hin*dIII fragment contained the elastase structural gene were negative. It was at this time that a second clone, pRF1, was found which produced large amounts of proteolytic and elastolytic activity in an environmental isolate which had little proteolytic activity and no detectable elastolytic activity. This clone, pRF1, contained an 8 kb *Eco*RI fragment. When pRF1 was conjugated into PAO-E64 it did not complement of the *lasA* mutation. Thus we concluded that pRF1 and pHN13, encoded for different functions involved

in elastolytic activity in *P. aeruginosa.* Restriction analysis further indicated that these two clones contained unrelated fragments of *P. aeruginosa* DNA. Hybridization analysis further indicated that these two clones were unrelated by their inability to hybridize to each other in Southern blots. Interestingly, pRF1 hybridized with the synthetic oligonucleotides to the elastase protein. When *E. coli* containing pRF1 was examined for elastolytic activity small levels of activity was detected and elastase antigen was seen by Western blot analysis. Subcloning of this 8 kb fragment contained in pRF1 and its subsequent DNA sequence was done by Dr. R. Bever, (J. Bacteriology in press 1988). These data lead to two important conclusions, one was that the plasmid pRF1 contained the elastase structural gene as determined by antigen detection in *E. coli* and DNA hybridization to oligonucleotides made from the elastase amino acid sequence, and two, the mutation in PAO-E64 (*lasA*) was not a mutation in the structural gene, as originally reasoned, but in what appears to be a processing or activating gene. This assumption is based on the observations that PAO-E64 produces an elastase molecule that is altering in elastolytic activity at 37°C but normal in proteolytic activity, and that it produces parental levels of extracellular elastase antigen.

In order to better ascertain what the function of the *lasA* gene was we sequenced the 2.2 kb *Sma*I-*Hin*dIII fragment that complemented the *lasA* mutation in PAO-E64 (manuscript 3). The sequence of *lasA* revealed that one open reading frame (ORF) encoded for a protein of 41,000 daltons. This ORF was the only reading frame with significant codon usage as determined by codon usage and codon frequency algorithms (9). By cloning the *lasA* gene into the inducible T7 phage promoter vector (33) we were able to show that a protein of approximately 40,000 daltons was produced and that this protein was associated with the outer membrane of *E. coli.* Further analysis of this protein revealed that when outer membranes containing the *lasA* protein were incubated with PAO-E64 culture supernatants

activation of the elastase could be achieved. Control membranes from *E. coli* containing only the plasmid vector had little or no effect on elastase activity. The extracellular elastase produced by the strain PAO-E64 appears to have the same molecular weight as parental extracellular elastase (13a). Furthermore, its activation by the *las*A protein produced in *E. coli* failed to alter the apparent molecular weight (manuscript 3). Although we can not completely rule out proteolysis, our data suggests that the *las*A protein may stabilize or correctly fold the elastase molecule to confer its elastolytic activity. The exact nature of the modification of elastase by the *las*A protein will require additional experiments.

When the *lasA* sequence was compared to the elastase structural sequence no homology was seen either at a DNA or protein level, (R. Bever personal communication). However, we found significant protein homology with a β-lytic protease of *Myxobacter* and the *las*A protein; a score of 39%, using the NBRF protein data base. This protease of *Myxobacter* is also a zinc metalloprotease and has a preference for cleaving peptides which have a glycyl residue as the donor COOH group and/or a hydrophobic amino acid as a donor of the NH group. Several investigators have suggested that elastase may be synthesized as a larger precursor which is subsequently cleaved as the pre-elastase is excreted through the inner and outer membrane.

In conclusion, two genes involved in elastase production have been cloned and sequenced. The structural gene for elastase,*las*B, and a processing gene, *las*A. The processing gene, *las*A, appears to broaden the substrate specificity for elastase to include the substrate elastin. This was demonstrated by conversion of the elastase mutant strain PAO-E64 from protease$^+$/elastase$^-$ to protease$^+$/elastase$^+$. The exact nature of this modification is as yet unknown although it would appear not to be due to proteolytic processing of the elastase molecule. Further studies are needed to elucidate the exact process by which the *las*A protein activates/or processes elastase.

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V. Appendix

Computer analysis of the 41 kDa lasA protein

Computer analysis of the 41 kDa lasA protein

Computer analysis was done on the open reading frame of the 41 kDa *las*A protein (bp 159 to 1289), by using the Wisconsin genetics package version 5 (2).

Peptide sort (Table 1) displays peptides from a digest of a protein sequence (*las*A) using known enzymatic cleavage sites and by chemical cleavage as well (2). It sorts peptides by weight, position, and HPLC retention at pH 7.4, and shows the composition of each peptide. It also prints a summary of the whole protein showing the amount of each amino acid residue contained within the protein given in number, and mole percent for each amino acid, along with the total weight, pI, average weight for the amino acids, and total number of amino acids present. Table 1 is a summary of the *las*A protein showing the amino acid composition, mole % for each amino acid, molecular weight, and pI, but is excluding the enzymatic and chemical cleavage peptide data.

Wordsearch does a Wilbur and Lipman (8) search for similarity between a new sequence and any group of sequences. Wordsearch answers the following question: "What sequence(s) in the data base are similar to my sequence?" The output can be displayed with a companion program called segments. Table 2 shows the best scores, given as percent homology to the 41 kDa *las*A protein for sequences found in the NBRF protein data base, only the ten best scores are shown. Table 2 shows the name of the protein sequence file in the data base, the homology to the *las*A protein, the largest consecutive string of amino acids with absolute homology, and a brief description of the protein. The number of gaps required to achieve the best fit was taken from the segments program which makes an optimal alignment for the best segment of similarity between two sequences. Optimal alignments are found by inserting gaps to maximize the number of matches using the algorithm of Smith and Waterman (7) (data and printout not shown).

Peptidestructure makes a secondary structure prediction for a peptide sequence (*las*A) (1, 3-6). The predictions include, alpha helicies, beta sheets, and turns, in addition to, measures for antigenicity, flexibility, hydrophobicity, and surface probability. Plotstructure is the companion program which plots the peptidestructure file as parallel panels of a graph (Fig 1), or in a two dimentional squiggly representation (Fig 2). The data is useful in determining, at a theoretical level, regions that may be responsible for enzymatic activity, membrane anchoring, surface exposure used in receptor mediated binding, possible antigenic epitopes, how the protein of interest compares to similar proteins from other species, how a CRM is different from the wild type protein, and/or how a particular amino acid change may alter the protein structure of interest.

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Table 1. Amino acid composition of the open reading frame encoding the 41 kDa *las*A protein by using the Peptidesort program.

Summary for *las*A open reading frame

Residue	number	Mole %
Ala	54	14.324
Arg	27	7.162
Asn	13	3.448
Asp	15	3.979
Cys	2	0.531
Gln	24	6.366
Glu	13	3.448
Gly	32	8.488
His	10	2.653
Ile	3	0.796
Leu	47	12.467
Lys	8	2.122
Met	6	1.592
Phe	14	3.714
Pro	20	5.305
Ser	32	8.488
Thr	11	2.918
Trp	6	1.592
Tyr	19	5.040
Val	21	5.570

Molecular weight = 41,110.98

Amino acid residues = 377

Average amino acid residue weight 109.048

Charge (pI) = 7

Table 2. Protein homology for the *las*A protein as compared to the NBRF protein data base by using the Wordsearch and Segments programs.

Sequence[1]	Score[2]	With[3]	Gaps[4]	Documentation[5]
Lyyxb4	39	4	1	beta-lytic protease of Myxobacter
Wfbom	39	4	26	Mullerian inhibiting factor
Qqbe10	36	6	23	Hypothetical BOLF1 protein EB virus
Qqbe3	34	10	21	Hypothetical BHLF1 protein EB virus
Opnb7	33	5	17	Peroxidase Turnip
Wfhum	33	6	18	Mullerian inhibiting factor
Qqbe11	33	4	18	Probable glycoprotein of EB virus
Whrty	32	10	11	Tyrosine 3-hydroxylase
Qqbe3	32	8	16	Hypothetical BHLF1 protein EB virus
Wfbom	31	10	22	Mullerian inhibiting factor

[1] Name of protein file in database.

[2] Score is given as % homology to *las*A protein using exact amino acid matches only. Only the 10 highest scores are given.

[3] With is the largest consecutive string of amino acids with absolute homology.

[4] Gaps are the number of spaces needed to be inserted into the sequence to produce the best fit of homology. Data is from the segments program.

[5] Documentation is a brief description of the protein in the NBRF protein database.

Fig 1. Plot shows various data determined from the peptidestructure program as plotted by the Pepplot program for the 41 kDa *las*A protein.

FIGURE 1

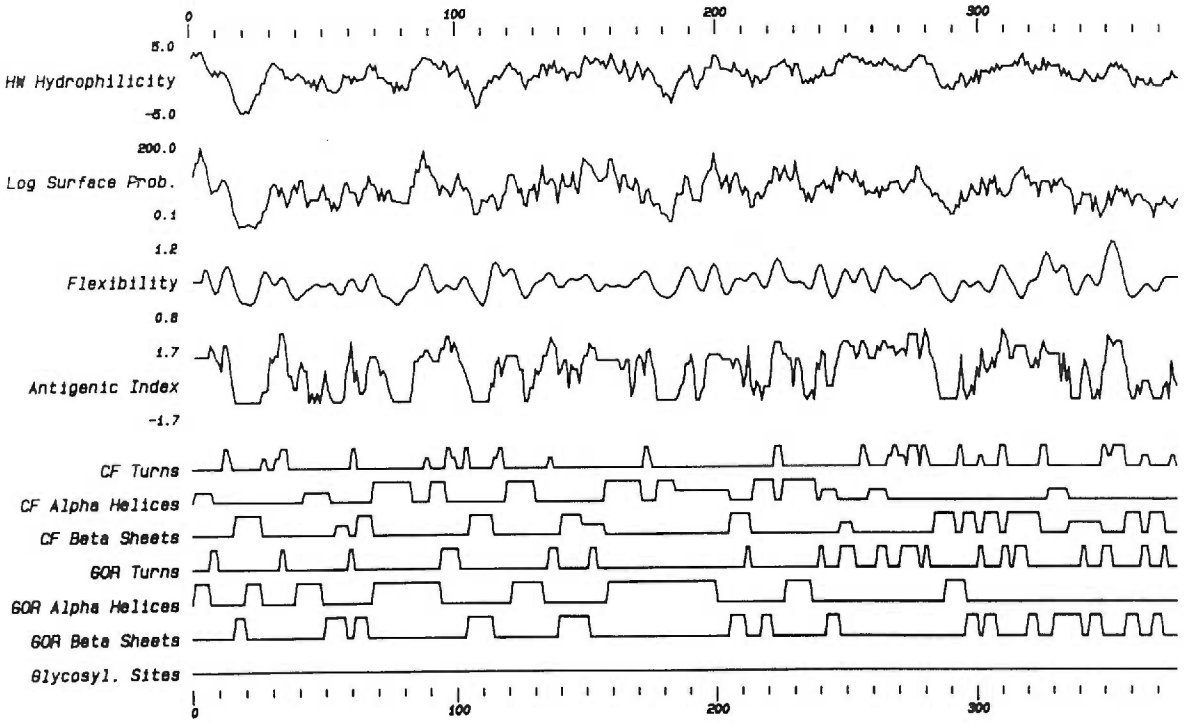
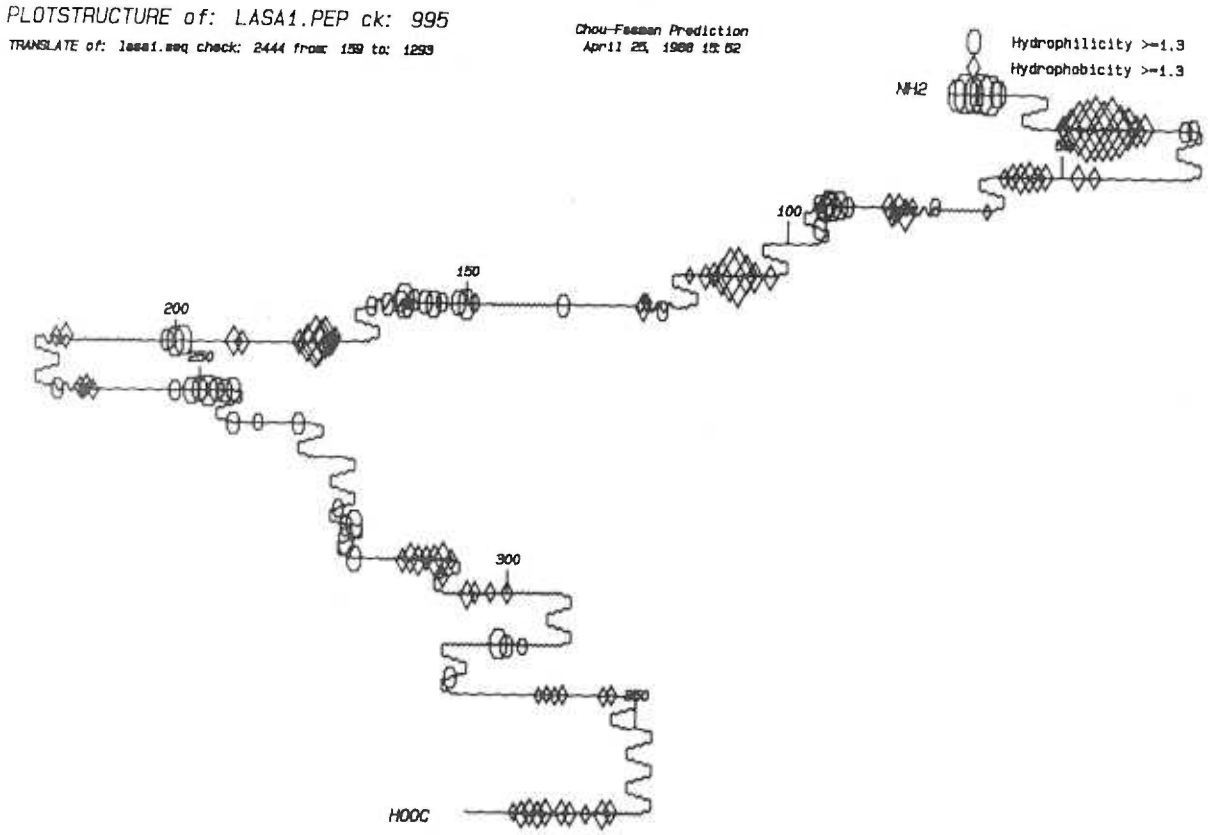

0
100
200
300
5.0
HW Hydrophilicity
-5.0
200.0
Log Surface Prob.
0.1
1.2
Flexibility
0.8
1.7
Antigenic Index
-1.7
CF Turns
CF Alpha Helices
CF Beta Sheets
GOR Turns
GOR Alpha Helices
GOR Beta Sheets
Glycosyl. Sites
0
100
200
300

Fig. 2. Two dimensional squiggly plot of the *las*A protein using Chou-Fasman predictions from the pepplot and peptidestructure programs. Hydrophobicity is plotted by using Kyte-Doolittle predictions. Hydrophobic amino acids are drawn as diamonds, hydrophilic regions are drawn as octagons. Refer to references for a complete description of symbols.

FIGURE 2.


PLOTSTRUCTURE of: LASA1.PEP ck: 995
TRANSLATE of: lasa1.seq check: 2444 from: 159 to: 1293
Chou-Fasman Prediction
April 25, 1988 15:52
Hydrophilicity >=1.3
Hydrophobicity >=1.3
NH2
100
150
200
250
300
HOOC