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ANALYSIS OF CIS- AND TRANS-ACTING FACTORS INVOLVED IN REGULATION OF LASA PRODUCTION, A SECOND ELASTASE PRODUCED BY PSEUDOMONAS AERUGINOSA

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By

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The Ohio State University
1995

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Department of Microbiology
DEDICATION

To my husband Tim
ACKNOWLEDGEMENTS

I would like to express gratitude to my adviser, Dr. Aldis Darzins, for his guidance and support during the last several years. I also wish to thank Dr. Darrell Galloway for allowing me to work on this project, and for his support in the last year. Encouragement and advice from the other members of my committee, Dr. Neil Baker and Dr. William Strohl, has been greatly appreciated. I would like to acknowledge Dave Brown for his help in the production of antiserum to LasR. Thanks also go to Patty Truax and Mary Russell for their friendship and support. I would like to thank my parents for their lifelong encouragement. I am most indebted to my husband Tim, for his enormous faith in me, his understanding, and his ability to make me laugh.
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<tr>
<td>AAI</td>
<td><em>Agrobacterium</em> autoinducer</td>
</tr>
<tr>
<td>ADP</td>
<td>adenosine diphosphate</td>
</tr>
<tr>
<td>Ap</td>
<td>ampicillin</td>
</tr>
<tr>
<td>AP</td>
<td>alkaline phosphatase</td>
</tr>
<tr>
<td>bp</td>
<td>base pairs</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>Cb</td>
<td>carbenicillin</td>
</tr>
<tr>
<td>CF</td>
<td>cystic fibrosis</td>
</tr>
<tr>
<td>Cm</td>
<td>chloramphenicol</td>
</tr>
<tr>
<td>dATP</td>
<td>deoxyadenosine triphosphate</td>
</tr>
<tr>
<td>dCTP</td>
<td>deoxycytidine triphosphate</td>
</tr>
<tr>
<td>dGTP</td>
<td>deoxyguanosine triphosphate</td>
</tr>
<tr>
<td>dNTP</td>
<td>deoxynucleoside triphosphate</td>
</tr>
<tr>
<td>dTTP</td>
<td>deoxythymidine triphosphate</td>
</tr>
<tr>
<td>ECR</td>
<td>elastin congo-red</td>
</tr>
<tr>
<td>EF-2</td>
<td>elongation factor 2</td>
</tr>
<tr>
<td>EMSA</td>
<td>electrophoretic mobility shift assay</td>
</tr>
<tr>
<td>ETA</td>
<td>exotoxin A</td>
</tr>
<tr>
<td>GCG</td>
<td>Genetics Computer Group</td>
</tr>
<tr>
<td>h</td>
<td>hours</td>
</tr>
<tr>
<td>HSL</td>
<td>homoserine lactone</td>
</tr>
<tr>
<td>HTH</td>
<td>helix-turn-helix</td>
</tr>
<tr>
<td>IL-2</td>
<td>interleukin 2</td>
</tr>
<tr>
<td>kb</td>
<td>kilobases</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodaltons</td>
</tr>
<tr>
<td>Km</td>
<td>kanamycin</td>
</tr>
<tr>
<td>LD</td>
<td>lethal dose</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>mA</td>
<td>milliamperes</td>
</tr>
<tr>
<td>min</td>
<td>minutes</td>
</tr>
<tr>
<td>MW</td>
<td>molecular weight</td>
</tr>
<tr>
<td>NAD</td>
<td>nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NK</td>
<td>natural killer</td>
</tr>
<tr>
<td>nt</td>
<td>nucleotides</td>
</tr>
<tr>
<td>PAI</td>
<td><em>Pseudomonas</em> autoinducer</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>pI</td>
<td>isoelectric point</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
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<tr>
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<tr>
<td>PIA</td>
<td><em>Pseudomonas</em> isolation agar</td>
</tr>
<tr>
<td>PLC</td>
<td>phospholipase C</td>
</tr>
<tr>
<td>PMN</td>
<td>polymorphonuclear</td>
</tr>
<tr>
<td>PVDF</td>
<td>polyvinylidene difluoride</td>
</tr>
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<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>Tc</td>
<td>tetracycline</td>
</tr>
<tr>
<td>VAI</td>
<td><em>Vibrio</em> autoinducer</td>
</tr>
<tr>
<td>WT</td>
<td>wild type</td>
</tr>
<tr>
<td>X-gal</td>
<td>5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside</td>
</tr>
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CHAPTER I
INTRODUCTION

_Pseudomonas aeruginosa_ as an Opportunistic Pathogen.

**Occurrence.** _Pseudomonas aeruginosa_ is a member of the eubacterial family _Pseudomonadaceae_, a diverse group of bacteria well-adapted to living in a wide variety of ecological niches, including soil, water, plants, insects, and sewage. _P. aeruginosa_ is described as a ubiquitous microorganism, but requires a moist environment for survival. _P. aeruginosa_ can be routinely cultured from soil, especially the rhizosphere, and from plants and vegetables, but it is rare to find the bacteria in pristine aquatic environments (Botzenhart and Döring, 1993). It is present in low amounts in treated water supplies, but can be readily isolated from polluted water reservoirs, sink and shower drains, toilets, pools and whirlpools (Rhame, 1980; Botzenhart and Rüden, 1987). The ability of _P. aeruginosa_ to utilize numerous compounds as carbon and energy source allows it to multiply in nutrient-poor environments, including purified water (Rhame, 1980).

_P. aeruginosa_ can be recovered from feces of only 4% to 12% of healthy humans and is rarely cultured from home environments, yet causes 10% to
20% of hospital-acquired infections (Bodey et al., 1983). The fact that *P. aeruginosa* causes infections only in patients who are compromised, either physically or immunologically, makes it understandable that the bacteria is found more often in the hospital environment and that most infections occur while patients are hospitalized (Botzenhart and Döring, 1993). As an opportunistic pathogen *P. aeruginosa* is quite successful. Table 1 shows conditions that predispose patients to infection with *P. aeruginosa*, and the types of infection most often associated with each condition.

**Infections caused by *P. aeruginosa***. *P. aeruginosa* can cause infections at many different locations (Bodey et al., 1983). Infections of the skin are either associated with moist skin, humid climates, or existing sores, or are secondary infections from bacteremic spread of the bacteria. Infections of the spinal cord, heart, gastrointestinal tract, and bones and joints are rare but may be severe or fatal. *P. aeruginosa* causes 10% of all cases of meningitis in cancer patients. Typhlitis, a necrotizing infection of the colon in neutropenic patients, requires surgery to remove the infection, and if infection is not localized, mortality rates are very high. Urinary tract infections are usually nosocomial, most often in patients who have been catheterized, and can progress to bacteremic infections. *P. aeruginosa* causes approximately 15% of nosocomial pneumonias, especially in patients with heart disease, tracheal intubations, tracheostomies, and hematologic malignancies, and is associated with high mortality rates (50%-80%) in these patients. Approximately 7% to 18% of Gram negative bacillary septicemia is caused by *P. aeruginosa*. The
**Table 1.** Conditions predisposing to infection with *P. aeruginosa.*

<table>
<thead>
<tr>
<th>Condition</th>
<th>Prevalent Infection(s)</th>
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<tbody>
<tr>
<td>leukemia, neutropenia</td>
<td>typhlitis, septicemia</td>
</tr>
<tr>
<td>cancer</td>
<td>meningitis, pneumonia, septicemia</td>
</tr>
<tr>
<td>drug addiction</td>
<td>endocarditis, osteomyelitis</td>
</tr>
<tr>
<td>neonatal period</td>
<td>meningitis, diarrhea</td>
</tr>
<tr>
<td>urinary catheterization</td>
<td>urinary tract infection</td>
</tr>
<tr>
<td>vascular catheterization</td>
<td>suppurative thrombophlebitis</td>
</tr>
<tr>
<td>tracheostomy</td>
<td>pneumonia</td>
</tr>
<tr>
<td>diabetes, elderly</td>
<td>malignant otitis externa</td>
</tr>
<tr>
<td>corneal ulcer</td>
<td>keratitis, panophthalmitis</td>
</tr>
<tr>
<td>burn wound</td>
<td>cellulitis, septicemia</td>
</tr>
<tr>
<td>Cystic Fibrosis</td>
<td>pneumonia</td>
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</tbody>
</table>
infections are acquired almost exclusively in the hospital and are especially prevalent in neutropenic patients. Prognosis of the patient’s underlying disease determines the chance for recovery from bacteremic infection, but *P. aeruginosa* bacteremia is associated with higher mortality rates than those for all other Gram negative bacillary septicemias.

Important community-acquired infections of the eye and ear are also caused by *P. aeruginosa* (Bodey et al., 1983). Nearly 70% of all cases of swimmer’s ear (otitis externa) are caused by *P. aeruginosa*, and in elderly and diabetic patients this infection can progress to malignant otitis externa, where the infection may spread and cause paralysis of facial and cranial nerves. Mortality associated with malignant otitis externa is approximately 25%, and this increases if nerve paralysis has already occurred when patients present for treatment. *P. aeruginosa* is one of the major pathogens isolated from middle ear infections in patients with chronic suppurative otitis media (Proctor, 1973). *P. aeruginosa* is the most common Gram negative bacteria causing infection of corneal ulcers, which are caused by trauma or even use of extended wear contact lenses. If left untreated, infection can progress within hours to days from keratitis to panophthalmitis resulting in possible loss of the eye.

Two groups, burn patients and cystic fibrosis (CF) patients, represent extremely important hosts for *P. aeruginosa* infection. Burn deaths due to bacterial infection prior to the introduction of the first generation of antimicrobial agents were usually due to bacteremic infection caused by the Gram positive bacteria *Staphylococcus aureus* and *Streptococcus pneumoniae* (Holder, 1993). Effective control of these pathogens resulted in a shift to Gram
negative sepsis as the most common bacteremic infection (Holder, 1993). *P. aeruginosa* is the most common organism causing Gram negative septicemia in burn patients, and mortality associated with *P. aeruginosa* infection has been reported to be as high as 77% (Mason, 1986). In a recent report, *P. aeruginosa* was associated with 25% of septic deaths, but the overall rates of infection and mortality associated with infection are decreasing (Holder, 1990).

Cystic fibrosis (CF), the most common lethal genetic disease among caucasians, is caused by mutations in a membrane protein involved in ion transport, CFTR, Cystic Fibrosis Transmembrane Conductance Receptor (Riordan *et al.*, 1991). Mutations in CFTR have been found to interfere with chloride and sodium ion transport through the epithelial cell membrane (Li *et al.*, 1988; Boucher *et al.*, 1986) resulting in production of dehydrated secretions by exocrine glands in the liver, pancreas, gall bladder, intestines, and airways. Those viscous secretions are responsible for the two fundamental pathophysiological symptoms of CF, pancreatic and respiratory insufficiency, resulting in malnutrition and chronic pulmonary infections, respectively. Pulmonary dysfunction, mainly due to chronic infections, is believed to be responsible for approximately 90% of CF deaths (Gilligan, 1991). *S. aureus* is typically the first organism to infect the CF lung, and colonization occurs soon after birth (Gilligan, 1991). As the disease progresses, colonization with *P. aeruginosa* becomes more common, and one study reported the incidence of *P. aeruginosa* in respiratory tract cultures of all CF patients to be approximately 60% (Keramidas, 1987). Nearly all CF patients will eventually become chronically infected with *P. aeruginosa*, which is now recognized as the most
important bacterial pathogen in CF lung disease. *P. aeruginosa* is very difficult to completely eradicate with antibiotic therapy, and recurrent episodes of decreased respiratory function frequently require rehospitalization for intravenous antibiotic therapy (Matthews and Drotar, 1984). While the life expectancy of CF patients has increased within recent years, with many patients surviving into their thirties, *P. aeruginosa* usually remains in the airways of patients, causing destruction of the lung tissue, until their premature death.

**Virulence Factors Produced by *P. aeruginosa***

**Cell-associated factors.** Many cell surface-associated structures have been identified as virulence factors in *P. aeruginosa*. The lipopolysaccharide (LPS) is similar in structure to enterobacterial LPS, with a variable O-specific side chain attached via a core polysaccharide to Lipid A (Wilkinson, 1983). Like endotoxin of other Gram negative bacteria, LPS of *P. aeruginosa* activates macrophages and the complement pathway (Kropinski et al., 1985) while also conferring resistance to opsonization, phagocytosis, and the bacteriocidal action of complement (Engels et al., 1985; Young, 1972). Pili are polar, filamentous appendages constructed from identical protein monomers (i.e., pilin) which have the capability of retraction (Bradley, 1974). The importance of pili has been demonstrated for adherence of *P. aeruginosa* to proteolytically modified buccal epithelial cells and pili are now well established as an adhesin of *P. aeruginosa* (Woods et al., 1980). Protease treatment
reportedly destroys cell surface fibronectin, exposing new receptor sites for pili (Woods et al., 1981b). The single polar flagella of *P. aeruginosa* was shown to be involved in virulence by examination of flagellin mutants in the burned mouse model, and is believed to contribute to virulence at an early stage of infection (McManus *et al.*, 1980; Montie *et al.*, 1982). The mucoid phenotype of *P. aeruginosa* is due to production of an extracellular polysaccharide, alginate, which is a linear copolymer of L-guluronic acid and D-mannuronic acid (Linker and Jones, 1964). This mucoid phenotype is most often associated with lung infections of CF patients, and approximately 80% of CF isolates are mucoid (Pedersen *et al.*, 1991). Alginate contributes to virulence by inhibiting phagocytosis (Speert, 1985) and serving as an adhesin (Baker and Svanborg-Edén, 1989).

**Extracellular factors.** Many extracellular products produced by *P. aeruginosa* are also considered virulence factors. Leucocidin, or cytotoxin, exists as a cell-associated precursor that is released upon lysis of the *P. aeruginosa* cell (Scharmann, 1976), and causes lysis of target cells by the formation of transmembrane pores (Lutz *et al.*, 1987). Phospholipase C (PLC) was first identified by Liu (1974) as a heat-labile hemolysin. PLC activity is actually due to the presence of two distinct enzymes, one of which is not hemolytic (Ostroff and Vasil, 1987). These enzymes may contribute to virulence of *P. aeruginosa* infections by degradation of phosphatidylcholine in lung surfactant, production of immunomodulators from phosphatidylcholine (Vasil *et al.*, 1991), or by cytotoxic activity (Berk *et al.*, 1987). Iron limitation
is an effective mechanism of host defense which inhibits growth of invading microorganisms by limiting the availability of iron, and has been termed nutritional immunity (Weinberg, 1984). This is accomplished by binding of extracellular iron to transferrin and lactoferrin, which have extremely high affinities for iron. Compounds that are involved in iron scavenging by *P. aeruginosa*, such as the siderophores pyochelin (Cox and Graham, 1979) and pyoverdin (Cox and Adams, 1985), have indeed been implicated as important virulence factors (Cox, 1982).

Exotoxin A (ETA) is an extracellular enzyme that transfers the adenosine 5'-diphosphate-ribose (ADP-ribose) moiety from \( \text{NAD}^+ \) to elongation factor 2 (EF-2) to inhibit protein synthesis (Iglewski and Kabat, 1975; Iglewski *et al.*, 1977). ETA is the most toxic factor produced by *P. aeruginosa*, with an \( \text{LD}_{50} \) of 0.2 \( \mu \text{g} \) for intra-peritoneal injection in mice (Iglewski *et al.*, 1977). After thirty years of investigation, current research on ETA is focused on the molecular analysis of ETA structure-function and regulation of ETA production. Exoenzyme S is a second ADP-ribosylating enzyme produced by *P. aeruginosa*, which shares more biochemical similarities with cholera toxin, since it can ADP-ribosylate multiple protein targets (Coburn *et al.*, 1989a; 1989b) and requires a eukaryotic accessory factor for full enzymatic activity (Coburn *et al.*, 1991). While exoenzyme S has not been definitively shown to be a toxin, it does appear to be involved in pathogenesis (Nicas *et al.*, 1985; Nicas and Iglewski, 1985). Early studies on exoenzyme S are confusing, since it appears that at least two different, unrelated proteins
were being examined (N. Baker, personal communication), and the role of exoenzyme S in adherence is now in dispute (Baker et al., 1991). Cloning of the gene for the 49 kDa active form of exoenzyme S (Kulich et al., 1994) should allow more definitive studies on the role of exoenzyme S in virulence of *P. aeruginosa*.

**Extracellular proteases produced by *P. aeruginosa***

Numerous proteases or proteolytic activities have been reported to be produced and secreted by *P. aeruginosa*, and are considered to be important virulence factors. Only those proteases that have been purified and well characterized are discussed here.

**Alkaline protease.** Alkaline protease (AP) is a 48 kDa zinc metalloprotease that was originally purified and characterized by Morihara (1964). While it is not the major protease produced by *P. aeruginosa*, it has been analyzed and implicated in numerous virulence studies. Sequence analysis of the cloned alkaline protease gene (*aprA*) from two strains of *P. aeruginosa* revealed extensive homology to the PrtB and PrtC proteases of *Erwinia chrysanthemi* (52%) and a metalloprotease of *Serratia marcescens*, PrtSM (53%) (Okuda et al., 1990; Duong et al., 1992). AP is secreted by a pathway distinct from that used for secretion of other extracellular enzymes and is homologous to the *E. coli* α-hemolysin secretion pathway (Duong et al.,
Thus, AP does not contain a signal sequence and does not appear to be produced in a precursor form, as are the other *P. aeruginosa* proteases.

**Elastase.** Elastase, a 33 kDa zinc metalloprotease, is the most abundant and active protease produced by *P. aeruginosa*. Morihara originally purified this protease and demonstrated its ability to degrade elastin fibers (Morihara, 1964; Morihara et al., 1965). Sequence analysis of the cloned gene (*lasB*) revealed significant homology to thermolysin of *Bacillus thermoproteolyticus*, neutral protease of *Bacillus subtilis*, and Smp, a metalloprotease of *S. marcescens* (Bever and Iglewski, 1988). Other metalloprotease genes sequenced since then have also been found to possess homology to elastase throughout the entire sequence: HA/protease of *Vibrio cholera* (Häse and Finkelstein, 1991), vibriolysin of *Vibrio proteolyticus* (David et al., 1992), and hemolytic metalloproteases of *Legionella pneumophila* (Black et al., 1990) and *Vibrio anguillarum* (Milton et al., 1992). In fact, Häse and Finkelstein (1993) have classified many bacterial metalloproteases into the homology groups elastase-like, thermolysin-like, and serratia-like. The thermolysin-like and elastase-like groups are themselves closely related, suggesting a common ancestor. The crystal structure of elastase has been solved (Thayer et al., 1991) and found to display remarkable similarity to the three-dimensional structure of thermolysin (Matthews et al., 1972). Alignment of the three-dimensional structure and primary sequence of elastase with those of the extensively studied thermolysin allowed prediction of putative zinc binding and active site residues (Bever and Iglewski, 1988; Thayer et al., 1991).
Elastase is produced as a 53.6 kDa preproelastase that accumulates in the inner membrane in the presence of signal peptidase inhibitors (Kessler and Saffrin, 1988a). Removal of the signal peptide during translocation into the periplasm results in the formation of a 51 kDa proelastase I (Kessler and Saffrin, 1988a). This form was shown to accumulate in *E. coli* and *P. aeruginosa* cells expressing lasB containing a mutation in the active site of elastase (His-223), indicating that cleavage of this form occurs through an autoproteolytic event (McIver et al., 1991; 1993). Autoproteolytic processing of proelastase I yields a 33.5 kDa inactive proelastase II associated with an 18 kDa propeptide (Kessler and Saffrin, 1988b). The propeptide appears to serve as an inhibitor of elastase, remaining non-covalently associated with proelastase II, perhaps to protect the cell from proteolytic damage prior to secretion (Kessler and Saffrin, 1994). Translocation across the outer membrane and activation of proelastase II to mature active 33 kDa elastase has not been extensively studied and appears to be a complex event, requiring dissociation of propeptide from proelastase II, a potential proteolytic event involved in activation of proelastase II, and secretion across the outer membrane (Kessler and Saffrin, 1988a). Since the N-terminal sequence of proelastase II (Kessler et al., 1992) matches that of mature elastase (Bever and Iglewski, 1988), any proteolytic processing that may occur must take place at the C-terminus of proelastase II.

**LasA.** LasA is a 22 kDa protease produced by *P. aeruginosa*, and while it has only been recently purified (Peters and Galloway, 1990), the structural
gene for LasA, \textit{lasA}, was actually the first protease gene to be cloned from \textit{P. aeruginosa} (Goldberg and Ohman, 1987a). Confusion over the identity of LasA arose from the initial characterization of a nitrosoguanidine mutant of PAO1, PAO-E64, that produced reduced levels of elastolytic activity, yet parental levels of elastase antigen (Ohman \textit{et al.}, 1980a). The mutation in PAO-E64 was designated \textit{lasA} \textit{I} and presumed to be a missense mutation in the elastase structural gene. The \textit{lasA} gene was cloned nearly simultaneously by Goldberg and Ohman (1987a) and Schad \textit{et al.} (1987) by complementation of the \textit{lasA} \textit{I} lesion in PAO-E64. Goldberg and Ohman (1987a) hypothesized that the \textit{lasA} gene product was either the elastase structural gene or was involved in elastase processing. In their report on cloning of \textit{lasA}, Schad \textit{et al.} (1987) also reported cloning of the true elastase structural gene, \textit{lasB}, by complementation of two other mutants deficient in elastolytic activity, PA-E15 and PAO-E105. The identification of \textit{lasB}, not \textit{lasA}, as the elastase structural gene was confirmed by hybridization to degenerate oligonucleotide probes designed from the N-terminal sequence of mature elastase. In another report, Goldberg and Ohman (1987b) suggested that the \textit{lasA} gene product has a role in secretion of elastase. These authors also demonstrated that a cell extract of \textit{E. coli} expressing \textit{lasA} was able to enhance the elastolytic activity of culture supernatants from a \textit{lasA} deletion mutant, FRD2128, and concluded that the \textit{lasA} gene product also has a function in activating proteolytic elastase somehow to broaden the substrate specificity to include elastin.

Sequence analysis of the \textit{lasA} gene (Schad and Iglewski, 1988) revealed the presence an open reading frame encoding a 41 kDa protein with 39%
homology to the amino acid sequence of the beta-lytic protease of *Myxobacter* 495, now called *Lysobacter enzymogenes*. This homology indicated that LasA may possess proteolytic activity. Since then, only two other proteases have been identified as having significant homology to LasA, the beta-lytic protease of *Achromobacter lyticus*, which is 95% homologous to the beta-lytic protease of *Lysobacter enzymogenes* (Li et al., 1990), and the N-terminal 40 amino acids of a novel zinc metalloprotease of *Aeromonas hydrophila*, AhP (Loewy et al., 1993). AhP, which showed 69% homology to the N-terminus of the active fragment of LasA, is able to hydrolyze peptide cross-links in fibrin dimers. Thus, LasA appears to belong to a small family of metalloproteases that hydrolyze peptide cross-links in complex macromolecules, such as peptidoglycan (β-lytic proteases), fibrin (AhP), and elastin (LasA).

While no one had been able to demonstrate proteolytic cleavage of mature elastase by LasA-containing extracts, the possibility still existed that processing of proelastase II by LasA broadened the substrate specificity of elastase to include elastin. However, Peters and Galloway (1990) presented data indicating that LasA does not function by acting on elastase. They purified a 22 kDa active fragment of LasA that was able to enhance the elastolytic, but not proteolytic, activity of purified elastase 10-fold. Moreover, LasA also enhanced the elastolytic activity of three enzymes belonging to different protease classes: thermolysin, human neutrophil elastase, and proteinase K. This result indicates that LasA, which possesses poor proteolytic activity, may act on the elastin substrate to allow general
proteases, with poor or no elastolytic capabilities, to degrade elastin. Several inconsistencies between the size and pI of the active LasA fragment and the published sequence of lasA led to the resequencing of lasA (Darzins et al., 1990). A sequencing mistake by Schad and Iglewski (1988) in the stop codon of lasA was corrected, and the lasA open reading frame was extended to yield an active fragment of the correct size and pI.

Further studies have led to the classification of LasA as a second elastase of P. aeruginosa. Purified LasA was found to have some elastolytic activity in two different assay systems, a conductimetric assay (Saulnier et al., 1992; Peters et al., 1992) and the elastin-congo red dye release assay (Peters et al., 1992). It has also been demonstrated that LasA is able to enhance elastase-mediated degradation of elastin by pre-exposure of elastin to LasA, followed by removal of LasA and exposure to elastase (Peters et al., 1992). In addition, elastase purified from an isogenic lasA mutant of PAO1, AD1825, possesses the same specific activity as elastase from PAO1 (Peters et al., 1992). LasA has also been shown to enhance the elastolytic activity of P. aeruginosa AP (Wolz et al., 1991). Thus, degradation of elastin by P. aeruginosa appears to involve separate enzymatic activities of LasA and elastase, and perhaps other proteases, on the elastin substrate.

Information from the analysis of genetically defined mutants in lasA and lasB have confirmed the biochemical evidence for the importance of LasA in elastolysis. Residual elastolytic activity in culture supernatants of lasA mutants has ranged from 8% (Goldberg and Ohman, 1987b) to 60% (Toder et al., 1994) parental levels, however these mutants do not appear to be deficient
in proteolytic activity. Residual elastolytic activity in a lasB mutant, PAO-B1, grown on agar plates is abolished in a lasA lasB double mutant, PAO-A1B1 (Toder et al., 1994). Interestingly, residual elastolytic activities of PAO-B1 and PAO-A1B1 in culture supernatants are equivalent (Toder et al., 1994). These results are consistent with findings in this laboratory, where it has been found that the lasB mutant PAO1E produces larger zones of elastin hydrolysis on plates than the lasA mutant AD1825, and PAO-LAB, a double lasA lasB mutant, produces no elastolytic zone (Appendix; Table 12). No difference has been observed in elastolytic activity in culture supernatants of PAO1E and PAO-LAB. These findings indicate that the in vivo effect of LasA on elastolysis may be more pronounced on solid surfaces than in liquid media.

Another interesting finding concerning LasA occurred when Kessler et al. (1993) noticed similarities of LasA to the previously described staphylolytic enzyme of P. aeruginosa (Zyskind et al., 1965). Staphylolytic enzyme was recently repurified, found to digest pentaglycine efficiently but casein inefficiently, and shown by SDS-PAGE to be a protein of approximately 19 kDa (Brito et al., 1989). LasA shares biochemical similarities to a larger group of lytic endopeptidases (Strominger and Ghysen, 1967), and the deduced amino acid sequence of LasA shares homology to two described lytic proteases. Kessler et al. (1993) examined the lasA mutant FRD2128 and found no staphylolytic activity. Staphylolytic enzyme purified from PAO1 is identical to LasA in the 15 N-terminal amino acids. Purified LasA is able to cleave pentaglycine, and anti-LasA antibodies react with staphylolytic enzyme. Thus, LasA can be considered a staphylolytic protease. This has intriguing
implications for virulence of *P. aeruginosa*, since *P. aeruginosa* infection in the CF lung is usually secondary to infection by *S. aureus* (Gilligan, 1991).

**LasD.** Recently, a novel protease was purified from *P. aeruginosa* culture supernatants. LasD is a 23 kDa protease that also exhibits staphylolytic activity (Park and Galloway, 1995). These investigators demonstrated that both LasA and LasD are able to cleave synthetic peptide substrates at internal Gly-Gly bonds and to hydrolyze β-casein at the sequence NKK->IGKFQ. Pentaglycine hydrolysis by both proteases can be inhibited by metalloprotease inhibitors, while β-casein hydrolysis is inhibited by serine protease inhibitors, indicating that LasA and LasD may cleave different substrates by different proteolytic mechanisms.

**The Role of Extracellular Proteases in Virulence of *P. aeruginosa*.**

Avirulent strains of *P. aeruginosa* have not been described due to the large arsenal of virulence factors produced by the bacterium. The wide array of infections caused by this opportunistic pathogen has led to the development of numerous animal models. For these reasons, results from virulence studies of *P. aeruginosa* are typically not as clear as for many other bacterial pathogens. However, after approximately thirty years of study, there is quite extensive evidence, both direct and indirect, for the role of *P. aeruginosa* proteases in virulence.
Evidence for protease production in infections. *P. aeruginosa* proteases are produced by stains isolated from corneal infections (Kreger and Gray, 1978), acute lung infections (Woods et al., 1986), middle ear infections (Jin et al., 1991), and systemic infections, with systemic isolates often producing higher levels of protease than isolates from localized infections (Janda et al., 1980). In one study of isolates from the lungs of CF patients, 65% of the isolates, both mucoid and nonmucoid, produced elastase, and those from recently infected patients produced higher levels than isolates from chronically infected patients (Jagger et al., 1983).

Recent studies measuring transcript levels in the sputa of CF patients have indicated that the genes for both elastase and LasA are actively transcribed during chronic infection (Storey et al., 1992a; 1992b). However, Döring et al. (1985) were unable to detect bacterial proteases in CF sputa using a sensitive RIA for detection. These investigators noted that neutralizing antibodies to elastase and alkaline protease were present in serum IgG preparations at 1 to 5 x 10³-fold excess over the level of proteases in the sputum. They suggest that, due to the high serum antibody titers, the proteases produced by *P. aeruginosa* are, for the most part, complexed with antibodies in chronic infection. Thus the contribution of bacterial proteases to lung damage may be more significant in the early stages of infection, before high levels of neutralizing antibodies can be raised. Another group, however, has been able to demonstrate uninhibited elastase activity due to a metalloprotease (*P. aeruginosa* elastase) in CF sputum samples (Bruce et al.,
1985). In addition, production of the exopolysaccharide alginate by *P. aeruginosa* results in the formation of microcolonies (Lam *et al.*, 1980). The extracellular matrix, which has been shown to contain metalloprotease activity, appears to trap the secreted proteases (Anastassiou *et al.*, 1989). This may lead to a more protected localized environment where the action of bacterial proteases is not neutralized by antibodies.

**Evidence for protease involvement in tissue destruction.**

Progressive loss of pulmonary function in CF patients chronically infected with *P. aeruginosa*, coupled with the observation by Bruce *et al.* (1985) of fragmented and exfoliated elastin fibers in the lungs of autopsied CF patients, has made the secreted proteases of *P. aeruginosa* obvious candidates as mediators of tissue destruction in the CF lung. This may be especially true in light of the increasing number of proteases produced by the organism.

Hamster tracheal explants were used to study tissue destruction by protease-producing and protease-deficient strains of *P. aeruginosa* (Baker, 1982). Protease-producing strains or purified elastase, but not protease-deficient strains, cause exfoliation and disorganization of the tracheal epithelium after 12 h exposure. Purified elastase and AP are able to impair motility and disrupt the structure of respiratory ciliary axonemes (Hingley *et al.*, 1986), which may lead to impaired clearance of *P. aeruginosa* from the CF lung. Elastase is able to solubilize human lung elastin 10 times more effectively than human neutrophil elastase, and is approximately 50 times more active on human lung elastin than on bovine nuchal ligament elastin, which is the elastin substrate
commonly used (Hamdaoui, 1987). Removal of fibronectin was shown to be important for adherence of \textit{P. aeruginosa} to buccal epithelial cells (Woods \textit{et al.}, 1981). Elastase treatment of human lung fibroblast monolayers was found to decrease levels of fibronectin on the cell surface and increase adherence of \textit{P. aeruginosa} to the cells (Azghani \textit{et al.}, 1992). Interstitial lung fibroblasts and fibronectin in the extracellular matrix and are important in repair of tissue damage (Proctor, 1987), thus degradation of fibronectin could also be important in the extent and course of \textit{P. aeruginosa} infection in the lung (Azghani \textit{et al.}, 1992).

Destruction of connective tissue is of obvious importance in the invasiveness of acute \textit{P. aeruginosa} infections. Collagen is a major structural component of all connective tissues, and degradation of interstitial collagen types I and III, which are found in the skin, lungs, and blood vessel walls, are degraded by elastase (Kessler \textit{et al.}, 1977; Heck \textit{et al.}, 1986b). Basement membranes are located at the junctions of epithelial cells and connective tissue, and are functionally important in ultrafiltration of the blood, serving as a barrier to bacterial invasion and metastasis of malignant cells, and providing physical support to blood vessels and the lens capsule. Collagen IV and laminin are the major components of basement membranes, and both are degraded by elastase and alkaline protease (Heck \textit{et al.}, 1986a; 1986b). Bejarno \textit{et al.} (1989) demonstrated fragmentation of intact basement membranes from bovine anterior lens capsules and lungs, with degradation of collagen IV, laminin, and other non-collagenous components. Elastase is able to alter the structure and destroy the barrier function of tight junctions in an
epithelial cell line that is able to form tight junctions in vitro, which may also allow penetration and dissemination of *P. aeruginosa* in acute infections (Azghani *et al.*, 1993).

**Evidence for involvement in interference with immune function.** Nonspecific immune mechanisms are the first line of defense against any invading pathogen. One such defense is the sequestering of available iron by binding to transferrin and lactoferrin. Initial studies on the acquisition of iron by the siderophore pyoverdin from transferrin at equimolar concentrations demonstrated a requirement for elastase for the release of iron from transferrin (Döring *et al.*, 1988). However, when pyoverdin is present at the higher concentrations found in culture supernatants, the siderophore is able to acquire iron from transferrin without elastase, but the rate of acquisition is enhanced by elastase (Wolz *et al.*, 1994). Another interesting aspect of the interaction of elastase and transferrin is that cleavage of Fe-transferrin by elastase results in the formation of iron chelates that are effective catalysts of hydroxyl radical formation via the Haber-Weiss reaction (Britigan and Edeker, 1991). This may contribute to oxidant-mediated tissue damage at the site of infection. Cleavage of transferrin and lactoferrin was found to occur in the lungs of CF patients infected with *P. aeruginosa* (Britigan *et al.*, 1993).

Interrelated protease-activated and protease-generating cascades present in the circulation, such as the Hageman factor/kallikrein and kininogen/kinin systems, enhance the inflammatory response through the regulated production of biological mediators. Both Hageman factor and
prekallikrein are activated by a proteolytic mechanism, and elastase has been shown to activate both of these proteases in vitro (Holder and Neely, 1989; Shibuya et al., 1991). Figure 1, taken from Neely et al. (1994), shows a simplified scheme of the consequences of uncontrolled activation of the Hageman factor/kallikrein system by trauma or infection with *P. aeruginosa*. *P. aeruginosa* proteases can also interfere with the infiltration of phagocytic cells to an area of infection by inhibiting monocyte and polymorphonuclear (PMN) leukocyte chemotaxis and chemiluminescence, an indicator of the oxidative burst response (Kharazmi et al., 1984a; 1984b; Kharazmi and Nielsen, 1991). *P. aeruginosa* proteases have also been shown to inhibit natural killer (NK) cell cytotoxicity perhaps by degradation of surface receptors on the NK cell (Pedersen and Kharazmi, 1987).

Human neutrophil elastase, normally contained within the phagolysosomes of PMN cells, is released into the extracellular environment during frustrated phagocytosis and following cell death, and appears to be a major contributor to tissue damage during chronic inflammation, such as is caused by *P. aeruginosa* infection of the CF lung (Döring, 1994). *P. aeruginosa* elastase is able to inactivate two of the major inhibitors of neutrophil elastase, α1-proteinase inhibitor and bronchial mucosal proteinase inhibitor (Morihara et al., 1979; Johnson et al., 1982) and thus may contribute to neutrophil elastase-mediated tissue damage.

The complement cascade is important in chemotaxis of immune cells to a site of infection and in promotion of opsonic and non-opsonic phagocytosis.
Figure 1. Simplified scheme of the human Hageman factor/kallikrein proteolytic cascade in the circulation. Solid thin arrows represent proteolytic or enzymatic conversions. Dashed arrows represent mediators of conversions. Thick arrows represent the pathological consequences of uncontrolled activation of these cascades. Taken from Neely et al. (1994).
Elastase and alkaline protease are able to inactivate complement-derived chemotactic and phagocytic factors (Schultz and Miller, 1974), and to degrade complement components, including C1q and C3, the recognition units for the classical and alternative pathways of complement activation, respectively (Schultz and Miller, 1974; Hong and Ghebrehiwet, 1992).

Antibody-mediated opsonic phagocytosis is commonly recognized as the major mechanism of acquired resistance to P. aeruginosa. The original observation that P. aeruginosa elastase is able to degrade human immunoglobulins IgG, IgA, and secretory IgA in vitro (Döring et al., 1981) has been followed by studies investigating the effects of this degradation. Holder and Wheeler (1984) demonstrated that normal human IgG that has been cleaved by elastase loses its protective effect when used to treat P. aeruginosa-infected burned mice. They also found that plasma IgG levels continue to drop until death in burned mice infected with protease-producing strains of P. aeruginosa, but level off post-burn in mice infected with protease-deficient strains. Additional studies have shown that the IgG cleavage products, either generated in vitro with elastase or isolated from CF sputa, inhibit opsonophagocytosis of P. aeruginosa cells by PMN cells (Fick et al., 1984; Bainbridge and Fick, 1989).

T cell cytotoxicity appears to play a role in acquired resistance to P. aeruginosa by a poorly understood mechanism (Campa et al., 1993), but may be due to antibody-dependent activation of bactericidal effector T cells (Markham et al., 1991). Infection with P. aeruginosa leads to impaired T cell-mediated immunity (Campa et al., 1993). The role of P. aeruginosa proteases
in immunosuppression is supported by recent findings that elastase and AP selectively cleave CD4 molecules on human T-lymphocytes (Pedersen et al., 1987). *P. aeruginosa* proteases inhibit interleukin-2 (IL-2)-dependent T cell proliferation by degradation of IL-2 (Theander et al., 1988). In addition, AP and elastase act synergistically to inactivate gamma interferon by limited proteolysis, resulting in loss of antiviral and macrophage-activating properties of the cytokine (Horvat et al., 1989; Horvat and Parmely, 1988).

**Evidence for protease involvement in animal models of infection.**

The burned mouse model of Stieritz and Holder (1975) involves subjecting shaved mice to a controlled non-lethal burn, followed by subcutaneous inoculation with *P. aeruginosa* to initiate infection. When compared to protease-producing strains, protease-deficient mutants of *P. aeruginosa* were found to be less virulent and less invasive in this model (Pavlovskis and Wretlind, 1979). More recent studies have tried to take into account host factors as well as bacterial factors. Neely et al. (1994) have proposed that the amount of total circulating protease activity is very low in normal individuals, due to a balance of circulating proteases and protease inhibitors. Trauma, such as a burn or bacterial infection, results in a shift towards proteases, raising the total circulating protease activity. Burn and infection together result in heightened levels of protease activity. Once above a certain threshold, which varies depending on the infecting organism, the chance of death due to sepsis increases with increasing protease activity. These investigators demonstrated that circulating protease activity is increased by
challenge of burned mice with *P. aeruginosa*. Since elastase is able to inactivate serum protease inhibitors, its effect on circulating protease activity may be significant.

A characteristic feature of the early phases of septic shock, which distinguishes septic shock from other types of shock, is hypotension with low vascular resistance. Low vascular resistant shock in guinea pigs does not seem to be due to endotoxin, but is induced by intravenous injection of *P. aeruginosa* elastase (Khan *et al.*, 1993). These authors suggest that the role of elastase may be in the activation of endogenous mediator systems which are known to induce a hypotensive response, such as the Hageman factor/kallikrein-kinin system. In another sepsis model involving CaCl₂-treated mice injected intramuscularly with *P. aeruginosa*, the protease deficient strain, PA103, was able to grow in the muscle, but unable to disseminate to the liver unless co-injected with elastase (Tamura *et al.*, 1992). Immunization with elastase toxoid inhibited dissemination of a protease-producing strain, but did not affect proliferation at the site of injection, suggesting that elastase may be involved in invasiveness of the organism.

Two animal models of lung infection have been used to examine the effect of proteases on virulence. The LasA-deficient strain, PAO-E64, was found to be less virulent in a rat chronic lung infection model involving transtracheal implantation of agar beads seeded with bacteria (Woods *et al.*, 1982). The same *lasA* mutant was also less virulent in an acute lung infection model in which bacteria are directly inoculated intratracheally (Blackwood *et al.*, 1983). It is important to point out that most of the studies involving
protease-deficient strains of *P. aeruginosa* were not done using isogenic or genetically defined mutants. Indeed many of the classic strains used for these studies, such as PA103 and PAO-E64, are believed to contain more than one mutation involved in expression of proteases and other virulence factors.

**Regulation of Protease Production in *P. aeruginosa***.

**Environmental factors affecting protease production.** Early studies indicated that protease production was inhibited by the presence of high levels of iron in the growth medium (Bjorn et al., 1979). This result has been difficult to duplicate, and, in fact, other investigators have shown that iron is required, along with zinc, for optimal production of proteases in minimal media (Jensen et al., 1980). The issue of iron regulation may be easier to resolve now that the *P. aeruginosa fur* gene, encoding an iron repressor protein, has been cloned (Prince et al., 1993). Olson and Ohman (1992) reported that calcium and zinc ions are required for efficient production and processing of elastase. Zinc alone stimulates production of LasA, but calcium may be required for efficient processing of LasA precursor. In addition, zinc has been found to have an inhibitory effect on transcription of a *lasB::lacZ* translational fusion, as measured by mRNA levels, but still stimulate production of β-galactosidase activity (Brumlik and Storey, 1992). This result indicates that zinc regulates elastase expression at the level of translation.
Identification of trans-regulatory factors involved in protease production. A gene involved in regulation of protease production has been cloned by its ability to restore zones of proteolysis to a protease-null strain, PA103-AP2, which contains a mutation in the gene for alkaline protease (Gambello and Iglewski, 1991). This gene, designated lasR, was found to encode a 27 kDa protein with 27% amino acid identity to LuxR, a transcriptional regulator of luminescence in the symbiotic marine bacterium V. fischeri. LuxR responds to a freely diffusible factor, termed autoinducer, to activate luminescence (Engebrecht and Silverman, 1984; 1987; Devine et al., 1988). Analysis of a genetically defined lasR deletion mutant, PAO-R1, for the presence of specific transcripts revealed that the lasR gene is required for transcription of lasB, the elastase structural gene (Gambello and Iglewski, 1991), lasA, the LasA structural gene (Toder et al., 1991), and aprA, the AP structural gene (Gambello et al., 1993). In addition, PAO-R1 exhibited reduced levels of β-galactosidase activity associated with a toxA::lacZ translational fusion, and it has been suggested that LasR is a global regulator of virulence factors in P. aeruginosa (Gambello et al., 1993).

Homology of LasR to LuxR led Passador et al. (1993) to examine regions flanking lasR for the presence of a gene involved in autoinducer production, similar to the V. fischeri luxI gene. They identified an open reading frame downstream of lasR, transcribed in the same orientation, that exhibits 35% amino acid homology to LuxI of V. fischeri. The gene was designated lasI since E. coli cells expressing lasI produce a diffusible factor that is able to induce
β-galactosidase expression in the presence of lasR in E. coli containing a lasB::lacZ translational fusion. The diffusible factor was purified from culture supernatants of both P. aeruginosa PAO1 and E. coli cells expressing lasI by the ability to activate lasB::lacZ expression in the presence of lasR in E. coli, and identified as N-(3-oxododecanoyl) homoserine lactone (Pearson et al., 1994). This compound is related to the autoinducer molecule of V. fischeri, N-(3-oxohexanoyl) homoserine lactone, also known as VAI (Eberhard et al., 1981), and the P. aeruginosa compound has been termed PAI, for Pseudomonas autoinducer (Pearson et al., 1994). See Figure 2 for the structures of autoinducer molecules.

Purified PAI, however, is only able to induce lasB::lacZ expression in PAO-R1 when lasR is expressed on a multicopy plasmid from the lac promoter, not when lasR is under control of its own promoter (Pearson et al., 1995). However, ethyl acetate extracts of PAO1 culture supernatants are able to induce lasB::lacZ expression in PAO-R1 when lasR is expressed from its own promoter, indicating that another factor is present in PAO1 culture supernatants that may affect expression of lasR. This compound, named factor 2, has been purified, identified as N-butyrylhomoserine lactone (Figure 2), and shown to be produced by a gene other than lasI (Pearson et al., 1995). V. fischeri has also recently been shown to produce two autoinducer molecules in addition to VAI, N-hexanoyl-homoserine lactone and N-octanoyl-homoserine lactone (Figure 2), with the former being produced by luxI, and the latter being produced by a novel gene, ain (Kuo et al., 1994).
**Figure 2. Autoinducer molecules.** Shown are the chemical structures of known autoinducer molecules along with their common designation, for example VAI. Homoserine lactone is abbreviated HSL. The structure of the *V. harveyi* autoinducer, not described in the text, is taken from Cao and Meighen (1989).
Recently, a second trans-acting gene involved in regulation of protease production has been cloned from *P. aeruginosa* PAO1 and designated *vfr*, for virulence factor regulator, by complementation of previously characterized nitrosoguanidine mutants of PA103 that are deficient in production of both exotoxin A and proteases (Ohman *et al.*, 1980b; West *et al.*, 1994a; 1994b). Sequence analysis of the *vfr* gene revealed an open reading frame coding for a 28.5 kDa protein that exhibits 67% identity and 91% similarity to the *E. coli* cyclic AMP receptor protein, also known as CAP or Crp. The *vfr* gene can restore catabolite repression to an *E. coli crp cya* mutant in the presence of cAMP, but the *E. coli crp* gene is unable to complement either the exotoxin A-deficient or protease-deficient phenotype of the *vfr* mutants. Thus, it seems that Vfr can bind cAMP and recognize the *E. coli* CAP-binding site. Since an *E. coli* consensus CAP-binding site has been detected upstream of *lasR* (Gambello *et al.*, 1993), the effect of a *vfr* mutation on protease production may be indirect and mediated by LasR.

**Quorum Sensing Regulatory Systems of Bacteria**

**Bacterial communication.** Most of the research on regulation of bacterial gene expression has focussed on intra-cellular signalling mechanisms for controlling the response of individual bacterial cells to internal and environmental stimuli. The classical two-component signalling systems, which involve transfer of an environmental signal from the sensor component to the response regulator component via phosphorylation events, are widely
distributed, well-studied systems for regulation of gene expression in bacteria. *Inter*-cellular signalling has been less extensively studied and is commonly believed to be limited to bacteria that undergo morphological changes.

Recently, Kaiser and Losick (1993) reviewed the best studied bacterial systems in which cell-to-cell communication is important, summarizing the specific functions of this form of communication. *Enterococcus faecalis* and *Bacillus subtilis* produce pheromones, termed mating factor and competence factor, respectively, to facilitate genetic exchange between cells. Formation of aerial mycelia in some *Streptomyces spp.* requires multicellular cooperation for the production of high levels of a morphogenic protein known as sporulation associated protein, while in other species the production of small molecules stimulates sporulation. *Anabaena spp.* produce an intercellular gradient of a morphogen to establish a one-dimensional pattern of heterocyst development. *Vibrio fischeri*, *Myxococcus xanthus*, and *B. subtilis* monitor the concentration of small molecules, which are either freely diffusible or secreted from the cell, to measure cell density for the production of light, entry into the early stages of fruiting body formation (Downard and Toal, 1995), and entry into sporulation, respectively. The authors conclude that cooperative behavior among bacteria is actually quite common in the prokaryotic world, and that molecular biological approaches to old questions will likely result in many more examples of intercellular communication among bacteria.

**The LuxR family of transcriptional activators.** The modular nature of the individual components of classical two-component regulatory systems
has led to the classification of signal-transducing proteins into superfamilies and families based on homologies within various domains. Each individual component of the classical two-component systems is made up of two domains. Sensor proteins are made up of an N-terminal sensor domain that receives the environmental signal, and a C-terminal transmitter domain, that autophosphorylates following a conformational change upon receipt of the stimulus, then transmits the signal to the response regulator. Response regulator proteins contain an N-terminal receiver domain that receives the signal, via phosphorylation, from the transmitter module of the sensor protein. The C-terminal regulator domain is then activated, via a conformational change following phosphorylation of the receiver domain, and can regulate expression of the target gene(s), typically by activating transcription.

The number of proteins exhibiting homology to one or more of these functional domains is increasing so rapidly that reviews on the families of signal-transducing proteins are usually outdated before their release. However, based upon homologies in the C-terminal regulator domain, three main superfamilies of response-regulatory proteins have been identified (Gross et al., 1989). Members of the OmpR superfamily all contain homology in the C-terminal DNA-binding domain and activate transcription from $\sigma^{70}$-dependent promoters. Members of the FixJ superfamily contain a short variable region in between the N-terminal and C-terminal domains, and a C-terminal domain with a helix-turn-helix (HTH) DNA-binding motif (Pabo and Sauer, 1984). Members of the NtrC superfamily contain a large central domain with a
consensus nucleotide-binding motif and a C-terminal DNA-binding domain. Members of this superfamily only activate transcription from promoters that require the alternative sigma factor RpoN.

Shortly after the luxR gene was sequenced (Engebrecht and Silverman, 1987; Devine et al., 1989), Henikoff et al. (1990) published a report on the use of nucleotide sequence databases for the identification of protein similarities by translation of the DNA database in all six reading frames. Using the predicted amino acid sequence of LuxR, these authors detected homology in the C-terminus of LuxR to the C-terminal domain of FixJ and other proteins, some of which were known to be activator proteins. In addition, LuxR was found to exhibit homology over its entire length to a 28 kDa open reading frame upstream of the uvrC gene of E. coli, the significance of which has only recently become clear. In all, Henikoff and co-workers identified 10 proteins that had extensive homology in the C-terminus, some of which were further grouped into families based on homology in the N-terminus of the protein. Since that time, numerous proteins with homology to the C-terminus of FixJ have been identified, most of which also possess the classical two-component system N-terminal receiver domain and can thus be classified as members of the FixJ superfamily of response regulators. Two interesting exceptions are two sigma factors, the SigH protein of B. subtilis and the Sig70 protein of E. coli, which were identified as having homology in the region including, but not limited to, the HTH DNA-binding motif (Stout et al., 1991).

Homology in the N-terminal domain had classified LuxR and UvrC-28K, an E. coli protein of unknown function, as a family within the FixJ superfamily.
LasR of *P. aeruginosa* was identified as the third member of the LuxR family (Gambello and Iglewski, 1991). Since proteases were known to be produced in the later stages of growth, it was suggested that LasR was sensing a starvation-induced metabolite in response to high cell densities. The UvrC-28K protein was originally identified as an open reading frame located upstream of the *E. coli* excision repair gene *uvrC* (Sharma et al., 1986). The gene for the UvrC-28K protein was essentially reisolated by investigators searching for genes able to suppress a block in cell division, and was renamed *sdiA*, for suppressor of division inhibition (Wang et al., 1991). The *sdiA* gene product, SdiA, activates transcription from *ftsQAZ*, a gene cluster required for cell division.

Still, the implications of the homology between these transcriptional activators did not attain real significance until the report of another LuxR homolog in *Agrobacterium tumefaciens*, TraR, which is involved in conjugal transfer of the nopaline-type Ti plasmid (Piper et al., 1993). Identification of the *traR* gene was published along with a report that identified the diffusible conjugation factor required for pTi transfer as *N*-oxooctanoyl homoserine lactone (Figure 2), a compound closely related to the *V. fischeri* autoinducer of LuxR (Zhang et al., 1993). Thus, both of the signalling components regulating plasmid transfer in *A. tumefaciens* were similar to the *lux* regulatory system. When the *lasI* gene was sequenced shortly after and reported to be homologous the *V. fischeri* autoinducer synthetase gene *luxI* (Passador et al., 1993), it became apparent that autoinducer-mediated activation of gene expression in
response to high cell densities may be a common mechanism for sensing population size in bacterial cultures.

Additional transcriptional activator genes have been cloned and sequenced with gene products that exhibit homology to LuxR in both the N-terminal and C-terminal domains, and the LuxR family of transcriptional activators has expanded (Table 2). The LuxR family now also includes _A. tumefaciens_ TraR for regulation of octopine-type Ti plasmid transfer (Fuqua and Winans, 1994), RhiR of _Rhizobium leguminosarum_ b.v. _viciae_ for expression of rhizosphere genes (Cubo _et al._, 1992), PhzR of _Pseudomonas aureofaciens_ involved in regulation of phenazine antibiotic production (Pierson _et al._, 1994), and _Erwinia carotovora_ CarR for regulation of production of the antibiotic carbapenem (McGowan _et al._, 1995). Of particular interest was the finding that _P. aeruginosa_ produces a second LuxR homolog, known as RhlR, which is involved in production of the biosurfactant rhamnolipid (Ochsner _et al._, 1994). LasR and RhlR are only 23% identical, and RhlR actually displays the highest homology to SdiA of _E. coli_, indicating that _lasR_ and _rhlR_ are separate genes. This was the first report of two LuxR homologs in the same species that regulate expression of unrelated genes. While _A. tumefaciens_ also produces two LuxR homologs, the _traR_ genes are plasmid-borne, regulate the expression of related genes, and would not be present in the same cell. Multiple LuxR homologs are also reported to exist in _Er. carotovora_, regulating the production of carbapenem production (CarR) and exoenzyme production (ExpR), but the sequence for ExpR has not yet been published (see Table 2).
Table 2. Components of the autoinducer-mediated family of signal transduction.

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Activator</th>
<th>AI Synthetase</th>
<th>Autoinducer *</th>
<th>Phenotype Regulated</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>V. fischeri</em></td>
<td>LuxR</td>
<td>LuxI</td>
<td>VAI, N-(3-oxohexanoyl) HSL</td>
<td>light production</td>
</tr>
<tr>
<td></td>
<td>*</td>
<td>Ain</td>
<td>N-octanoyl HSL</td>
<td>*</td>
</tr>
<tr>
<td><em>A. tumefaciens</em></td>
<td>TraR&lt;sub&gt;rop&lt;/sub&gt;</td>
<td>TraI&lt;sub&gt;rop&lt;/sub&gt;</td>
<td>AAI</td>
<td>nopaline pTi transfer</td>
</tr>
<tr>
<td></td>
<td>TraR&lt;sub&gt;oct&lt;/sub&gt;</td>
<td>TraI&lt;sub&gt;oct&lt;/sub&gt;</td>
<td>AAI</td>
<td>octopine pTi transfer</td>
</tr>
<tr>
<td><em>P. aeruginosa</em></td>
<td>LasR</td>
<td>LasI</td>
<td>PAI</td>
<td>protease production</td>
</tr>
<tr>
<td></td>
<td>RhlR</td>
<td>(RhlII) &lt;sup&gt;b&lt;/sup&gt;</td>
<td>factor 2, VAI, AAI</td>
<td>rhamnolipid production</td>
</tr>
<tr>
<td></td>
<td>LasR&lt;sup&gt;?&lt;/sup&gt;</td>
<td>?</td>
<td>AAI</td>
<td>protease, unknown</td>
</tr>
<tr>
<td><em>E. carotovora</em></td>
<td>CarR</td>
<td>CarI</td>
<td>VAI</td>
<td>carbapenem production</td>
</tr>
<tr>
<td></td>
<td>(ExpR)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>ExpI</td>
<td>VAI</td>
<td>exoenzyme production</td>
</tr>
<tr>
<td><em>R. leguminosarum</em></td>
<td>RhiR</td>
<td>?</td>
<td>AAI</td>
<td>rhizosphere genes</td>
</tr>
<tr>
<td><em>P. aureofaciens</em></td>
<td>PhzR</td>
<td>?</td>
<td>VAI</td>
<td>phenazine production</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>SdiA</td>
<td>?</td>
<td>AAI</td>
<td>cell division</td>
</tr>
<tr>
<td><em>En. agglomerans</em></td>
<td>EagI</td>
<td>?</td>
<td>VAI</td>
<td>?</td>
</tr>
<tr>
<td><em>S. marcescens</em></td>
<td>?</td>
<td>?</td>
<td>VAI</td>
<td>?</td>
</tr>
<tr>
<td><em>Er. herbicola</em></td>
<td>?</td>
<td>?</td>
<td>VAI</td>
<td>?</td>
</tr>
<tr>
<td><em>C. freundii</em></td>
<td>?</td>
<td>?</td>
<td>AI</td>
<td>?</td>
</tr>
<tr>
<td><em>Pr. mirabilis</em></td>
<td>?</td>
<td>?</td>
<td>AI</td>
<td>?</td>
</tr>
<tr>
<td><em>H. alveii</em></td>
<td>?</td>
<td>?</td>
<td>AI</td>
<td>?</td>
</tr>
<tr>
<td><em>Rh. aquatilis</em></td>
<td>?</td>
<td>?</td>
<td>AI</td>
<td>?</td>
</tr>
</tbody>
</table>

*Abbreviations: HSL, homoserine lactone; VAI, *Vibrio* autoinducer, N-(3-oxohexanoyl) HSL; AAI, *Agrobacterium* autoinducer, N-(3-oxooctanoyl) HSL; PAI, *Pseudomonas* autoinducer, N-(3-oxododecanoyl) HSL.

<sup>b</sup> DNA sequence not yet published.
**Autoinducers and LuxI homologs.** While the LuxR family of transcriptional activators cannot be considered members of the classical two-component regulatory systems, since signal transfer does not occur by phosphorylation, there are indeed two components involved in response to an environmental signal. While *in vitro* binding of *V. fischeri* autoinducer (VAI) to LuxR has not been demonstrated, extensive molecular genetic analysis of LuxR has resulted in the proposed mechanism of autoinducer-mediated activation of transcription (Fuqua *et al.*, 1994). At low cell densities, basal level transcription of the autoinducer synthetase gene, luxI, results in production of autoinducer, which diffuses from the cell. Autoinducer accumulates in the surrounding medium until it reaches a sufficient concentration inside the cells to interact with LuxR. This interaction results in a conformational change that activates LuxR to allow it to function as a transcriptional activator. Thus, the autoinducer molecule serves the same function phosphorylation does in the classical response regulator molecule, and can be considered the second component of autoinducer-mediated signal transduction systems.

In addition to the autoinducer molecules produced by *P. aeruginosa* (PAI) and *A. tumefaciens* (AAI), the presence of autoinducer activity in culture supernatants (Bainton *et al.*, 1992) or the presence of genes encoding for production of autoinducer activity (Swift *et al.*, 1993) have been detected in many bacteria using a sensitive lux bioassay as a screening technique. The following bacteria were found to produce small molecules with autoinducer activity: *Serratia marcescens, Erwinia herbicola, Erwinia carotovora, P.*
*aeruginosa, Citrobacter freundii, Enterobacter agglomerans, Proteus mirabilis, Hafnia alvei, and Rhanella aquatilis* (Table 2). The autoinducer activity was purified from *P. aeruginosa, S. marcescens, Er. herbicola,* and *Er. carotovora,* and identified as VAI (Bainton et al., 1992). Thus, *P. aeruginosa* produces at least three, and perhaps four, different N-acyl-homoserine lactone molecules, since AAI has also been detected in *P. aeruginosa* culture supernatants (Pearson et al., 1994). The structures for all autoinducer molecules known are shown in Figure 2.

Cloning and sequencing has led to the identification of many of the autoinducer synthetase genes based on homology to the *luxI* gene of *V. fischeri* (Table 2). In *Er. carotovora,* the *carI* gene, involved in production of the antibiotic carbapenem (Swift et al., 1993), and the *expl* gene involved in production of cell wall-degrading exoenzymes (Pirhonen et al., 1993) are different genes exhibiting 70% identity at the amino acid level. The *luxI* homolog encoding production of autoinducer activity in *En. agglomerans, eagI,* has also been sequenced (Swift et al., 1993). LuxI homologs have also been identified for both the octopine- and nopaline-type Ti plasmids (Fuqua and Winans, 1994; Hwang et al., 1994). Finally, a gene encoding a second LuxI homolog in *P. aeruginosa, rhlI,* is reported to exist downstream of the *rhlR* gene (J. Reiser, personal communication).
Regulation of the *V. fischeri* lux Operon as a Model System.

**General features.** Early analysis of regulation of luminescence in *V. fischeri* resulted in the knowledge of the structure and freely-diffusible nature of the autoinducer molecule, VAI (Eberhard *et al.*, 1981; Kaplan and Greenberg, 1985), and of the genes required for response to and synthesis of VAI, *luxR* and *luxI*, respectively (Engebrecht and Silverman, 1984). The arrangement of the genes in the *lux* operon is shown in Figure 3. The intergenic region between *luxR* and *luxI* contains an *E. coli* consensus CAP-binding site (Engebrecht and Silverman, 1987; Devine *et al.*, 1988) and a 20 bp inverted repeat that has been identified as the *lux* operator by site-directed mutagenesis (Devine *et al.*, 1989). This operator is both required and sufficient for autoinducer-dependent activation of *luxICDABE* by LuxR.

Catabolite repression of luminescence is mediated by CRP-cAMP in the presence of low levels of LuxR, but CRP-cAMP is not required for activation of *luxICDABE* transcription when LuxR is present at high levels (Dunlap and Greenberg, 1985; 1988). In fact, in the absence of LuxR, CRP-cAMP represses transcription of *luxICDABE* (Dunlap and Greenberg, 1985). CRP-cAMP also enhances transcription of the *luxR* gene in the presence of LuxR (Shadel and Baldwin, 1992b). LuxR is also capable of both positive and negative autoregulation (Figure 3). Negative autoregulation seems to occur when VAI is present at high concentrations (Dunlap and Greenberg, 1988; Dunlap and Ray, 1989; Shadel and Baldwin, 1991). Positive autoregulation occurs when VAI is present at lower levels and is enhanced by CRP-cAMP (Shadel and
**Figure 3.** Schematic arrangement of the *lux* operon and interaction of regulatory gene products with promoters. The *luxR* and *luxI* genes are represented by thick arrows. The *luxCDABE* genes are shown as a thin arrow. The promoters for *luxR* and *luxICDABE* are labelled $P_L$ and $P_R$, respectively. The shaded box represents the *lux* operator. Thick arrows from the active LuxR-VAI complex and large circles labelled - and + represent the action of LuxR when high levels of VAI are present. The thin arrow from the active LuxR-VAI complex and small circle labelled + represent the action of LuxR on $P_L$ when low levels of VAI are present.
Figure 3.
Baldwin, 1991; 1992a). Recently, it was found that *E. coli* mutants deficient in production of the GroESL molecular chaperones are weakly luminescent when transformed with *luxR luxICDABE* (Dolan and Greenberg, 1992). This effect appears to be due to facilitation of binding of VAI to LuxR by the GroESL proteins (Adar and Ulitzer, 1993).

**Functional analysis of the LuxR protein.** LuxR has been overproduced in *E. coli*, where it is functional as determined by induction of *luxICDABE*, and purified as an inclusion body (Kaplan and Greenberg, 1987). The purified protein was found to bind DNA by filter-binding assays, but binding was not *lux*-specific. Also, the purified LuxR was unable to bind $^3$H-VAI. It took seven years for any *in vitro* activity to be demonstrated with LuxR (Stevens *et al.*, 1994). In the meantime, investigators have identified the regions of LuxR required for *in vivo* activity, by examination of *luxR* mutants, usually in *E. coli*. Initial studies on hydroxylamine-generated mutants defined two domains (Slock *et al.*, 1990; Shadel *et al.*, 1990). An N-terminal sensing domain, in which mutations cause a requirement for higher levels of VAI to induce luminescence, is considered to be involved in autoinducer binding. A C-terminal domain was proposed to be involved in DNA binding due to the loss of activator function caused by mutations in this region and homology to the FixJ superfamily of transcriptional activators.

The functional domains of LuxR are shown in Figure 4. A series of N-terminal deletions of LuxR were expressed in *E. coli* containing *luxICDABE*
and analyzed for the ability of various deletions to activate *lux* gene expression (Choi and Greenberg, 1991). This analysis determined that residues 10 to 20 are needed for autoregulation of *luxR*, while residues 162 to the C-terminus are capable of high level autoinducer-independent transcriptional activation. Deletions up to residue 138 are inactive, suggesting that the region from residue 20-162 is able to mask the activator function of the C-terminal domain. Deletions from the C-terminus were used to identify a region from residues 211-250 that is required for transcriptional activation of *luxICDABE*, but not for negative autoregulation of *luxR* (Choi and Greenberg, 1992a). Deletion beyond residue 211 resulted in loss of negative autoregulation, supposedly due to loss of the ability to bind DNA. The HTH of LuxR spans residues 196-210. Dominance tests with mutant and wild-type forms of *luxR* show evidence for multimerization of LuxR, since *luxR* genes encoding mutations in the C-terminal DNA-binding and activator domains are dominant over wild-type genes (Choi and Greenberg, 1992b). A region mapping to residues 116-161 appears to be involved in multimerization of LuxR. Analysis of a number of deletions for $^3$H-VAI binding *in vivo* has defined nearly the entire N-terminal domain, starting somewhere between residues 10 and 58 and ending between residues 162 and 193, as being required for VAI binding (Hanzelka and Greenberg, 1995).

The only report of *in vitro* activity associated with LuxR is DNA-binding activity associated with the purified C-terminal transcriptional activator domain, residues 155-250 (Stevens *et al.*, 1994). The 10 kDa LuxR fragment,
**Figure 4. Functional domains of LuxR.** Numbers denote the amino acid residues. The C-terminal transcriptional activator domain is shown separated from the N-terminal domain by a thin line to represent the functional nature of the C-terminal domain in the absence of any N-terminal residues. Limits of the region involved in autoinducer binding are shown by the dashed bracket. Residues involved in negative autoregulation of *luxR* are solid (a). The region of the N-terminal domain involved in masking the activator function of the C-terminal domain are stippled, with the heavily-stippled region (b) also required for multimerization of LuxR. Residues involved in DNA binding are denoted with diagonal stripes, with the thick stripes (c) representing the HTH of LuxR. The C-terminal region required for transcriptional activation is shown by vertical stripes (d).
purified from *E. coli*, was shown by electrophoretic mobility shift assays to bind to a region just upstream of the *lux* box, but not to the *lux* box itself. Binding of the C-terminal LuxR fragment is enhanced by addition of RNA polymerase, as seen by an improved footprint in DNaseI-protection studies, indicating synergistic binding to the *lux* promoter region. The investigators reported some of the obstacles that have hindered *in vitro* analysis of LuxR. Formation of inclusion bodies when LuxR is overexpressed in *E. coli*, the requirement for GroESL for proper formation of active LuxR, and the fact that LuxR is membrane-associated in *V. fischeri* (Kolibachuk and Greenberg, 1993) have all hindered purification of active full-length LuxR. In addition, a number of proteins reportedly recognize sequences in the *lux* regulatory region, making analysis of LuxR-containing extracts difficult. While the results reported by these investigators are significant, there exists the possibility that full-length LuxR may behave differently *in vitro* from the C-terminal activator domain.

**Areas of current research.** Clearly the recent findings on LuxR function will pave the way for structure-function studies on LuxR homologs. Likewise, the expanding list of homologous proteins will make identification of key residues easier. However, it is also clear that active, full-length activator proteins will eventually need to be purified for use in *in vitro* studies, with perhaps the ultimate goal being demonstration of DNA binding or activation of transcription *in vitro* in the presence of autoinducer, activator, and target DNA. One aspect of the existence of this family of autoinducer-mediated signal transduction systems that is currently being investigated is the
interchangeability of system components. Gray et al. (1994) have demonstrated that LasR and LuxR are able to induce expression from target promoters of the homologous system in the presence of their own cognate autoinducer, although induction of lasB expression by LuxR-VAI was less efficient than by LasR-PAI. In addition, since P. aeruginosa and Er. carotovora are known to produce more than one autoinducer-mediated signal transduction system, an interesting area of research in the future will be analysis of cross-talk between the systems in vivo. In fact, it has already been shown that rhlR mutants of P. aeruginosa are deficient in elastase production (Ochsner et al., 1994).

LuxR has only been shown to function at two divergently transcribed promoters, the luxR promoter and the luxICDABE promoter. The fact that some of the autoinducer signal transduction systems appear to be regulons, with multiple target genes located at different places on the chromosome, requires analysis of relative promoter activity and possible hierarchical expression. Recently, Seed et al. (1995) examined the regulation of the lasI gene by transcript mapping and determined there to be different transcription initiation sites depending on whether LasR is present or absent. Comparison of autoinducer-mediated induction of single-copy lacZ fusions of lasI and lasB indicated that ten-fold higher levels of PAI are required for induction of the lasB promoter than are required for lasI induction, thus suggesting an autoinduction hierarchy. The fact that lasI possesses no lux box may indicate that LasR recognizes a unique high affinity binding site present in the lasI promoter region.
A recent study by Huisman and Kolter (1994) has interesting implications in the area of autoinducer synthesis, a subject that has not received much attention. These authors discovered that homoserine lactone (HSL), without an acyl side chain, acts as an intracellular starvation signal in *E. coli*, inducing expression of the stationary phase sigma factor, $\sigma^a$. They propose that homoserine lactone is synthesized when intermediates of the threonine biosynthetic pathway accumulate under starvation conditions, regardless of the cell density. Induction of $\sigma^a$ synthesis by HSL results in increased stress resistance of individual starved cells. At high cell densities, acylation of homoserine lactone allows the signal molecule to diffuse across membranes, thus converting it to an intercellular signal molecule. It remains to be determined whether HSL serves as a starvation signal in other bacteria with autoinducer-responsive regulatory systems.

**Goals of this Project**

This project was initiated to examine the regulation of LasA production by *P. aeruginosa*. Initial studies focused on the effects of environmental factors on LasA production and on the analysis of the protease-hyperproducing strain, PA220 (Pavlovskis *et al.*, 1977). While these studies yielded some interesting patterns, the complexity of the systems made interpretation of the results difficult. Cloning of *lasR* and the report that the presence of a functional *lasR* gene is required for transcription of *lasA* and *lasB* (Gambello and Iglewski, 1991; Toder *et al.*, 1991) warranted a closer examination of the
effects of this \textit{trans}-acting factor on \textit{lasA} expression. At the same time, studies were initiated in an attempt to identify which regions upstream of the \textit{lasA} open reading frame are required for transcription initiation and promoter function. The original hypothesis was that \textit{LasR} interacts with a binding site(s) upstream of the \textit{lasA} promoter to activate transcription. Construction and analysis of an isogenic \textit{lasR} mutant, purification of \textit{LasR}, and functional definition of the \textit{lasA} promoter region were all achieved in an attempt to substantiate this hypothesis. The ultimate goal was to demonstrate an \textit{in vitro} interaction of \textit{LasR} with the \textit{lasA} promoter region, such as transcription activation or DNA-binding activity. While this goal was not achieved, many unexpected results were obtained, and an increased understanding of the complexity of \textit{lasA} regulation has been achieved.
CHAPTER II
Materials and Methods

Bacterial Strains, Plasmids, and Culture Conditions

Tables 3 and 4 list the bacterial strains and plasmid vectors, respectively, used in this study, along with genotypes, relevant descriptions, and the source or reference for each strain or plasmid. Plasmids constructed in this study are described in Table 5, with pertinent details on construction given in the results sections of Chapter III and Chapter IV. Oligonucleotides synthesized for this study were purchased from Ransom Hill Bioscience (Ramona, CA) and are described in Table 6.

*E. coli* and *P. aeruginosa* strains were maintained at 37°C on LB agar: Miller's LB broth base (Gibco BRL, Gaithersberg, MD)[10 g/L Select peptone 140, 5 g/L Select yeast extract, 10 g/L NaCl] plus 1.5% Select agar (Gibco BRL). Liquid cultures were routinely grown in LB broth with shaking at 37°C. Nutrient agar and nutrient broth (Difco Laboratories, Detroit, MI) were used during analysis of the PAO-LR phenotype. Pseudomonas Isolation Agar (PIA, Difco Laboratories) was used for selection of *P. aeruginosa* strains following triparental matings with *E. coli*. For production and purification of PAI, *P. aeruginosa* PAO1 was grown in A medium [8 mM (NH₄)₂SO₄, 33 mM KH₂PO₄, 60 mM K₂HPO₄, 5 mM Na-Citrate (Ausubel *et al.*, 1990)] supplemented with
Table 3. Bacterial strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
<th>Source or Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Escherichia coli strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DH5α</td>
<td><em>endA hsdR17(r_{B}^m_{B}^-) supE44 thi-1 recA1 gyrA(Nal^r) relA1</em> Δ(lacZYA-argF) φ80lacZΔM15</td>
<td>Hanahan, 1983</td>
</tr>
<tr>
<td>BL21 (DE3)</td>
<td>F^-ompT r_{B}^-m_{B}^-; contains a λ prophage carrying an inducible T7 RNA polymerase gene</td>
<td>Studier and Moffatt, 1986</td>
</tr>
<tr>
<td>HB101</td>
<td>Δ(gpt-proA) leuB6 thi-1 lacY1 hsdS20 recA rpsL20(Str^r) ara-14 galK2 xyl-5 mtl-1 supE44 mcrB_{B}</td>
<td>Boyer and Rolland-Dussoix, 1969</td>
</tr>
<tr>
<td><strong>Pseudomonas aeruginosa strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAO1</td>
<td>prototroph; WT protease production</td>
<td>Holloway et al., 1979</td>
</tr>
<tr>
<td>PA103</td>
<td>protease negative clinical isolate</td>
<td>Liu, 1966</td>
</tr>
<tr>
<td>PAO-LR</td>
<td>Tc^- ΔlasR::tet</td>
<td>This study</td>
</tr>
<tr>
<td>PAO-LI</td>
<td>Tc^- ΔlasI::tet</td>
<td>This study</td>
</tr>
<tr>
<td>PAO-LRI</td>
<td>Tc^- ΔlasRlasI::tet</td>
<td>This study</td>
</tr>
<tr>
<td>PAO-LAB</td>
<td>Cb^+ Tc^- lasB::bla lasA::tet</td>
<td>Appendix</td>
</tr>
<tr>
<td>Plasmid</td>
<td>Description</td>
<td>Source or Reference</td>
</tr>
<tr>
<td>-------------</td>
<td>------------------------------------</td>
<td>-----------------------------------</td>
</tr>
<tr>
<td><strong>Escherichia coli vectors</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pUC18</td>
<td>Ap&lt;sup&gt;r&lt;/sup&gt;; ColE1 ori; general cloning vector</td>
<td>Yanisch-Perron et al., 1985</td>
</tr>
<tr>
<td>pK18</td>
<td>Km&lt;sup&gt;r&lt;/sup&gt;; pUC18 derivative; general cloning vector</td>
<td>Pridmore, 1987</td>
</tr>
<tr>
<td>pBR322</td>
<td>Ap&lt;sup&gt;r&lt;/sup&gt;, Tc&lt;sup&gt;r&lt;/sup&gt;; ColE1 ori; general cloning vector</td>
<td>Sutcliffe, 1979</td>
</tr>
<tr>
<td>pNOT19</td>
<td>Ap&lt;sup&gt;r&lt;/sup&gt;; pUC18 derivative; gene replacement vector</td>
<td>Schweizer, 1992</td>
</tr>
<tr>
<td>pRK2013</td>
<td>Km&lt;sup&gt;r&lt;/sup&gt;; ColE1 ori, RK2 Tra; triparental mating helper plasmid</td>
<td>Figurski and Helinski, 1979</td>
</tr>
<tr>
<td>pACYC184</td>
<td>Tc&lt;sup&gt;r&lt;/sup&gt; Cm&lt;sup&gt;r&lt;/sup&gt;; P15A ori; general cloning vector</td>
<td>Chang and Cohen, 1978</td>
</tr>
<tr>
<td>pET-3a</td>
<td>Ap&lt;sup&gt;r&lt;/sup&gt;; ColE1 ori; contains T7 φ10 gene promoter, terminator and translation initiation signals</td>
<td>Rosenberg et al., 1987</td>
</tr>
<tr>
<td><strong>Pseudomonas aeruginosa vectors</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pMOB3</td>
<td>Km&lt;sup&gt;r&lt;/sup&gt; Cm&lt;sup&gt;r&lt;/sup&gt;; source of oriT and sacB cassette for gene replacement</td>
<td>Schweizer, 1992</td>
</tr>
<tr>
<td>pCP13</td>
<td>Tc&lt;sup&gt;r&lt;/sup&gt; Km&lt;sup&gt;r&lt;/sup&gt;; IncP, λcos&lt;sup&gt;+&lt;/sup&gt;; broad host range cosmid vector</td>
<td>Darzins and Chakrabarty, 1984</td>
</tr>
<tr>
<td>pUCP18</td>
<td>Ap&lt;sup&gt;r&lt;/sup&gt;; pUC18 derivative; contains 1.8 kb stabilizing fragment of pRO1614</td>
<td>Schweizer, 1991</td>
</tr>
<tr>
<td>pQF50</td>
<td>Ap&lt;sup&gt;r&lt;/sup&gt;; broad host range lacZ transcriptional fusion vector</td>
<td>Farinha and Kropinski, 1990</td>
</tr>
</tbody>
</table>

*Abbreviations: Ap<sup>r</sup>, ampicillin resistant; Tc<sup>r</sup>, tetracycline resistant; Km<sup>r</sup>, kanamycin resistant; Cm<sup>r</sup>, chloramphenicol resistant.*
Table 5. Plasmids constructed for this study.

<table>
<thead>
<tr>
<th>Plasmid No.</th>
<th>Size (kb)</th>
<th>Vector</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>pLO1763</td>
<td>5.0</td>
<td>pK19</td>
<td>2.3 kb <em>lasA</em> <em>SalI-HindIII</em> clone in <em>SalI-HindIII</em> sites of pK19; Km</td>
</tr>
<tr>
<td>pLO1831</td>
<td>3.1</td>
<td>pUC18</td>
<td>364 bp <em>SmaI-XhoI</em> subclone of <em>lasA</em> in <em>SmaI-SalI</em> sites of pUC18; Ap</td>
</tr>
<tr>
<td>pLO2841</td>
<td>4.3</td>
<td>pK19</td>
<td>1.6 kb <em>lasR</em> <em>SmaI-EcoRI</em> clone in <em>SmaI-EcoRI</em> sites of pK19; Km</td>
</tr>
<tr>
<td>pLO2842</td>
<td>10.7</td>
<td>pK19</td>
<td>8.0 kb <em>lasB</em> <em>EcoRI</em> clone in <em>EcoRI</em> site of pK19; Km</td>
</tr>
<tr>
<td>pLO2845</td>
<td>4.3</td>
<td>pNOT19</td>
<td>1.6 kb <em>SmaI-EcoRI</em> fragment containing <em>lasR</em> (as <em>EcoRI-HindIII</em> fragment of pLO2841) in <em>EcoRI-HindIII</em> sites of pNOT19; Ap</td>
</tr>
<tr>
<td>pLO2856</td>
<td>7.8</td>
<td>pQF50</td>
<td>1.0 kb <em>SalI-XhoI</em> (BL) subclone of <em>lasA</em> in <em>SmaI</em> site of pQF50;[+]; Ap</td>
</tr>
<tr>
<td>pLO2901</td>
<td>7.2</td>
<td>pQF50</td>
<td>365 bp <em>SmaI-XhoI</em> (BL) subclone of <em>lasA</em> in <em>SmaI</em> site of pQF50;[+]; Ap</td>
</tr>
<tr>
<td>pLO2906</td>
<td>7.3</td>
<td>pQF50</td>
<td>480 bp <em>XmnI-XhoI</em> (BL) subclone of <em>lasA</em> in <em>SmaI</em> site of pQF50;[+]; Ap</td>
</tr>
<tr>
<td>pLO2923</td>
<td>5.1</td>
<td>pNOT19</td>
<td>1.4 kb <em>EcoRI-AvaI</em> (BL) Tc cassette of pBR322 in <em>EcoRV</em> deletion of <em>lasR</em> in pLO2845; Ap, Tc</td>
</tr>
<tr>
<td>pLO2927</td>
<td>10.9</td>
<td>pNOT19</td>
<td>5.8 kb <em>NotI mob</em> cassette of pMOB3 in <em>NotI</em> site of pLO2923; Ap, Tc, Cm</td>
</tr>
<tr>
<td>pLO3086</td>
<td>4.5</td>
<td>pK19</td>
<td>m34 exonuclease III-generated deletion of <em>lasA</em> (from <em>Clal</em> site of pLO1763; <em>KpnI</em> as protected site); Km</td>
</tr>
<tr>
<td>pLO3090</td>
<td>4.8</td>
<td>pK19</td>
<td>q5 exonuclease III-generated deletion of <em>lasA</em> (from <em>Clal</em> site of pLO1763; <em>KpnI</em> as protected site); Km</td>
</tr>
<tr>
<td>pLO3152</td>
<td>7.2</td>
<td>pQF50</td>
<td>431 bp <em>EcoRI-XhoI</em> (BL) fragment of pLO3086 (m34 <em>lasA</em> deletion) in <em>SmaI</em> site of pQF50;[+]; Ap</td>
</tr>
<tr>
<td>pLO3166</td>
<td>7.2</td>
<td>pQF50</td>
<td>375 bp <em>EcoRI-XhoI</em> (BL) fragment of pLO3179 (m75 <em>lasA</em> deletion) in <em>SmaI</em> site of pQF50;[+]; Ap</td>
</tr>
<tr>
<td>Plasmid No.</td>
<td>Size</td>
<td>Vector</td>
<td>Description*</td>
</tr>
<tr>
<td>------------</td>
<td>-------</td>
<td>--------</td>
<td>--------------</td>
</tr>
<tr>
<td>pLO3175</td>
<td>4.5 kb</td>
<td>pK19</td>
<td>m41 exonuclease III-generated deletion of lasA (from ClaI site of pLO1763; KpnI as protected site); Km</td>
</tr>
<tr>
<td>pLO3176</td>
<td>4.6 kb</td>
<td>pK19</td>
<td>m42 exonuclease III-generated deletion of lasA (from ClaI site of pLO1763; KpnI as protected site); Km</td>
</tr>
<tr>
<td>pLO3179</td>
<td>4.4 kb</td>
<td>pK19</td>
<td>m75 exonuclease III-generated deletion of lasA (from ClaI site of pLO1763; KpnI as protected site); Km</td>
</tr>
<tr>
<td>pLO3181</td>
<td>7.5 kb</td>
<td>pQF50</td>
<td>727 bp EcoRI-XhoI [BL] fragment of pLO3090 (q5 lasA deletion) in SmaI site of pQF50; [+]; Ap</td>
</tr>
<tr>
<td>pLO3185</td>
<td>7.3 kb</td>
<td>pQF50</td>
<td>512 bp Sall-Xmnl subclone of lasA (as SmaI-Xmnl fragment of pLO1763) in SmaI site of pQF50; [+]; Ap</td>
</tr>
<tr>
<td>pLO3193</td>
<td>8.1 kb</td>
<td>pK18</td>
<td>5.4 kb EcoRV lasI clone in SmaI site of pK18; Km</td>
</tr>
<tr>
<td>pLO3196</td>
<td>4.1 kb</td>
<td>pK18</td>
<td>1.4 kb EcoRV-Sall subclone of lasI (as Sall fragment of pLO3193) in Sall site of pK18; Km</td>
</tr>
<tr>
<td>pLO3289</td>
<td>3.1 kb</td>
<td>pUC18</td>
<td>393 bp Sau3AI-XhoI subclone of lasA in SmaI-Sall sites of pUC18; Ap</td>
</tr>
<tr>
<td>pLO3291</td>
<td>7.2 kb</td>
<td>pQF50</td>
<td>393 bp Sau3AI-XhoI [BL] subclone of lasA in SmaI site of pQF50; [+]; Ap</td>
</tr>
<tr>
<td>pLO3296</td>
<td>5.0 kb</td>
<td>pK19</td>
<td>700 bp EcoRI-Sall subclone of lasI (as EcoRI fragment of pLO3196) in EcoRI site of pLO2841; joining lasR and lasI at EcoRI site; Km</td>
</tr>
<tr>
<td>pLO3298</td>
<td>5.0 kb</td>
<td>pNOT19</td>
<td>2.3 kb SmaI-Sall fragment containing lasR lasI (as SmaI fragment of pLO3296) in SmaI-HindIII [BL] sites of pNOT19; Ap</td>
</tr>
<tr>
<td>pLO3299</td>
<td>5.0 kb</td>
<td>pNOT19</td>
<td>1.4 kb EcoRI-AvaI [BL] Tc cassette of pBR322 in PstI [BL] deletion of lasR lasI in pLO3298; Ap, Tc</td>
</tr>
<tr>
<td>Plasmid No.</td>
<td>Size</td>
<td>Vector</td>
<td>Descriptiona</td>
</tr>
<tr>
<td>------------</td>
<td>-------</td>
<td>--------</td>
<td>--------------</td>
</tr>
<tr>
<td>pLO3301</td>
<td>10.8 kb</td>
<td>pNOT19</td>
<td>5.8 kb NotI mob cassette of pMOB3 in NotI site of pLO3299; Ap, Tc, Cm</td>
</tr>
<tr>
<td>pLO3308</td>
<td>4.1 kb</td>
<td>pNOT19</td>
<td>1.4 kb EcoRV-SalI subclone of lasI (as BamHI [BL] fragment of pLO3196) in SmaI-HindIII [BL] sites of pNOT19; Ap</td>
</tr>
<tr>
<td>pLO3310</td>
<td>5.2 kb</td>
<td>pNOT19</td>
<td>1.4 kb EcoRI-AvaI [BL] Tc cassette of pBR322 in ClaI-PstI [BL] deletion of lasI in pLO3308; Ap, Tc</td>
</tr>
<tr>
<td>pLO3394</td>
<td>11.0 kb</td>
<td>pNOT19</td>
<td>5.8 kb NotI mob cassette of pMOB3 in NotI site of pLO3310; Ap, Tc, Cm</td>
</tr>
<tr>
<td>pLO3409</td>
<td>4.1 kb</td>
<td>pK19</td>
<td>1.4 kb SalI-PstI subclone of lasA (with 512 bp SalI-XmnI [BL] fragment in opposite orientation) in SalI-PstI sites of pK19; Km</td>
</tr>
<tr>
<td>pLO3413</td>
<td>7.8 kb</td>
<td>pQF50</td>
<td>1.0 kb SalI-XhoI subclone of lasA (as BamHI-XhoI [BL] fragment of pLO3409) in SmaI site of pQF50; [+] Ap</td>
</tr>
<tr>
<td>pLO3425</td>
<td>6.8 kb</td>
<td>pUCP19</td>
<td>2.3 kb SmaI-SalI fragment containing lasR lasI (as BamHI fragment of pLO3296) in BamHI site of pUCP18; [-]; Ap</td>
</tr>
<tr>
<td>pLO3427</td>
<td>6.1 kb</td>
<td>pUCP19</td>
<td>1.6 kb SmaI-EcoRI fragment containing lasR (as BamHI-EcoRI fragment of pLO2841) in BamHI-EcoRI sites of pUCP19; [-]; Ap</td>
</tr>
<tr>
<td>pLO3573</td>
<td>3.4 kb</td>
<td>pUC18</td>
<td>719 bp PCR product of lasR (generated using oligonucleotides LR429 and LR1148) as SmaI-XbaI fragment in SmaI-XbaI sites of pUC18; Ap</td>
</tr>
<tr>
<td>pLO3575</td>
<td>5.3 kb</td>
<td>pET-3a</td>
<td>719 bp PCR product of lasR (as Asel-BamHI fragment of pLO3573) in Ndel-BamHI sites of pET-3a; Ap</td>
</tr>
<tr>
<td>pLO3600</td>
<td>5.2 kb</td>
<td>pUCP19</td>
<td>719 bp PCR product of lasR (as EcoRI-HindIII fragment of pLO3573) in EcoRI-HindIII sites of pUCP19; [+] Ap</td>
</tr>
<tr>
<td>Plasmid No.</td>
<td>Size</td>
<td>Vector</td>
<td>Description*</td>
</tr>
<tr>
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<td>--------------</td>
</tr>
<tr>
<td>pLO3604</td>
<td>7.3 kb</td>
<td>pQF50</td>
<td>497 bp EcoRI-XhoI [BL] fragment of pLO3175 (m41 lasA deletion) in SmaI site of pQF50; [+]; Ap</td>
</tr>
<tr>
<td>pLO3611</td>
<td>7.4 kb</td>
<td>pQF50</td>
<td>552 bp EcoRI-XhoI [BL] fragment of pLO3176 (m42 lasA deletion) in SmaI site of pQF50; [+]; Ap</td>
</tr>
<tr>
<td>pLO3622</td>
<td>5.8 kb</td>
<td>pACYC184</td>
<td>1.6 kb SmaI-EcoRI fragment containing lasR (as BamHI-EcoRI [BL] fragment of pLO2841) in BamHI [BL] site of pACYC184; [+]; Cm</td>
</tr>
<tr>
<td>pLO3626</td>
<td>5.6 kb</td>
<td>pACYC184</td>
<td>1.4 kb EcoRV-SalI fragment of lasI (as BamHI fragment of pLO3196) in BamHI site of pACYC184; [+]; Cm</td>
</tr>
<tr>
<td>pLO3629</td>
<td>6.5 kb</td>
<td>pACYC184</td>
<td>2.3 kb SmaI-SalI fragment containing lasR lasI (as BamHI fragment of pLO3296) in BamHI site of pACYC184; [+]; Cm</td>
</tr>
<tr>
<td>pLO3710</td>
<td>4.5 kb</td>
<td>pK19</td>
<td>1.8 kb EcoRI-Smal subclone of lasB (as Smal fragment of pLO2842) in SmaI site of pK19; Km</td>
</tr>
<tr>
<td>pLO3711</td>
<td>7.8 kb</td>
<td>pQF50</td>
<td>1.0 kb EcoRI-StuI subclone of lasB (as Smal-StuI fragment of pLO3710) in SmaI site of pQF50; [+]; Ap</td>
</tr>
<tr>
<td>pLO3719</td>
<td>7.6 kb</td>
<td>pQF50</td>
<td>818 bp AluI-StuI subclone of lasB in SmaI site of pQF50; [+]; Ap</td>
</tr>
<tr>
<td>pLO3766</td>
<td>3.5 kb</td>
<td>pK19</td>
<td>778 bp PCR product of lasB (generated using oligonucleotides LB135 and LB913) as KpnI-XbaI fragment in KpnI-XbaI sites of pK19; Km</td>
</tr>
<tr>
<td>pLO3768</td>
<td>7.6 kb</td>
<td>pQF50</td>
<td>778 bp PCR product of lasB (as HincII-Smal fragment of pLO3766) in SmaI site of pQF50; [+]; Ap</td>
</tr>
</tbody>
</table>

* Abbreviations: [BL], sites end-filled with Klenow enzyme; [+], insert in same orientation as P<sub>lac</sub>, P<sub>Tc</sub>, or promoterless lacZ gene. Ap, Cm, Km, Tc: resistance to ampicillin, chloramphenicol, kanamycin, and tetracycline, respectively.
Table 6. Oligonucleotides constructed for this study.

<table>
<thead>
<tr>
<th>Oligonucleotide Name</th>
<th>Sequence</th>
<th>Position in gene</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>LA724 5'-CGACATCGCCTCCGACG</td>
<td>lasA nt 706 to 724 (Figure 12)</td>
<td>Primer extension mapping of lasA</td>
<td></td>
</tr>
<tr>
<td>LA755 5'-CGAAGGGAAACCTTGAAAGC</td>
<td>lasA nt 736 to 755 (Figure 12)</td>
<td>S1 nuclease mapping of lasA</td>
<td></td>
</tr>
<tr>
<td>LR433 5'-GCCATAGCGGTACGTTC</td>
<td>lasR nt 417 to 433 (Gambello and Iglewski, 1991)</td>
<td>Cloning of lasR</td>
<td></td>
</tr>
<tr>
<td>LR429 5'-GGGAATTCTAGATTAATGGCCTTGGTTGACG</td>
<td>lasR nt 429 to 444 (Gambello and Iglewski, 1991)</td>
<td>PCR primer for lasR protein fusion in pET-3a</td>
<td></td>
</tr>
<tr>
<td>LR1148 5'-AGGGTACCAGTCATGAG</td>
<td>lasR nt 1133 to 1148 (Gambello and Iglewski, 1991)</td>
<td>PCR primer for lasR protein fusion in pET-3a</td>
<td></td>
</tr>
<tr>
<td>LB135 5'-GGGAATTCTAGATCGACG</td>
<td>lasB nt 135 to 150 (Bever and Iglewski, 1988)</td>
<td>PCR primer for lasB promoter analysis</td>
<td></td>
</tr>
<tr>
<td>LB913 5'-AGGGTACCCGGGATCCCTCCACTGATCGA</td>
<td>lasB nt 898 to 913 (Bever and Iglewski, 1988)</td>
<td>PCR primer for lasB promoter analysis</td>
<td></td>
</tr>
</tbody>
</table>

a Oligonucleotide names consist of a gene designation (LA for lasA, LR for lasR, and LB for lasB), followed by a number designation which refers to the 5' nt of the oligonucleotide.

b Underlined nt represent generated restriction sites.

c Positions are based on published sequences cited or Figure 12.
0.4% glucose, 0.05% yeast extract, and 1mM MgSO\(_4\) after autoclaving, as described by Pearson et al. (1994). Antibiotic concentrations were as follows: ampicillin (Ap), 100 µg/ml in agar media, 25 µg/ml in liquid media; kanamycin (Km), 20 µg/ml; chloramphenicol (Cm), 25 µg/ml; tetracycline (Tc), 25 µg/ml in LB agar and broth, 300 µg/ml in PIA; carbenicillin (Cb), used in place of Ap for \(P.\ aeruginosa\) cultures, 300 µg/ml in agar media, 100 µg/ml in liquid media.

**Standard DNA Isolation and Manipulation Techniques**

Restriction enzyme digestion reactions, ligation reactions, transformation of \(E. coli\), and routine plasmid DNA isolation were all done according to established protocols (Sambrook et al., 1989), with plasmid DNA prepared from \(E. coli\) by the boiling miniprep method of Holmes and Quigley (1981), and from \(P. aeruginosa\) by the alkaline lysis miniprep procedure (Birnboim and Doly, 1979). For DNA sequencing, plasmid DNA was prepared by a modification of the boiling miniprep: 160 µl 5% CTAB (cetyltrimethylammonium bromide; Sigma Chemical Co., St Louis, MO) was added to the DNA solution following removal of the chromosomal DNA pellet, and the plasmid DNA was precipitated by spinning for 15 min, followed by resuspension of the DNA in 1.2 M NaCl and re-precipitation with ethanol. For agarose gel electrophoresis, the buffer system of Loening (1967) was used and contained 36 mM Tris-HCl (pH 7.7), 30 mM NaH\(_2\)PO\(_4\), and 1 mM EDTA. DNA restriction or PCR fragments were isolated from ethidium bromide-stained agarose gels using the GeneClean II kit (Bio101, La Jolla, CA), which is based on the method of Vogelstein and Gillespie (1979).
Genomic DNA was isolated from overnight cultures of *P. aeruginosa* by modification of the method of Woo *et al.* (1992). Bacterial cells (1.5 ml) were pelleted in Eppendorf tubes by microcentrifugation for 2 min and washed once in 1 ml of TN buffer [10 mM Tris-HCl (pH 8.0), 10 mM NaCl]. Washed cells were resuspended in 135 µl TN, followed by addition of an equal volume of TN-2% Triton X-100 and 30 µl of a 5 mg/ml solution of lysozyme. Following incubation for 30 min at 37°C, 15 µl proteinase K (20 mg/ml) was added, the tubes were mixed by inversion and incubated for 2 h at 65°C. The DNA was then ready for use or could be stored at -20°C. Complete restriction enzyme digestion was obtained by using the DNA at 1/5 to 1/10 the total volume of the digestion reaction.

DNA fragments were labelled by three different methods: end-labelling with T4 polynucleotide kinase, end-filling with Klenow enzyme, and random primed labelling with Klenow enzyme. For all three labelling protocols, unless otherwise stated, unincorporated nucleotides were removed by centrifugation through a bed of Sephadex G-50 (Sigma Chemical Co.) as described in Sambrook *et al.* (1989). The number of cpm/µl was determined by scintillation counting using ScintiVerse liquid scintillation fluid (Fisher Scientific, Pittsburgh, PA). DNA fragments (50 ng) were continuously-labelled with [α-32P]-deoxycytidine triphosphate (25 µCi; Amersham, Arlington Heights, IL) using the Random Primed DNA Labeling Kit of US Biochemical (Cleveland, OH) as instructed by the manufacturer.
Oligonucleotides and DNA fragments were end-labelled with T4 polynucleotide kinase (Gibco BRL). The amount of dephosphorylated DNA fragment or oligonucleotide required to yield 5 to 10 pmol of 5' ends was calculated using equation 1:

\[
\frac{\text{pmol 5' ends}}{\mu\text{g DNA}} = \frac{N}{\text{MW}} \times \frac{10^8 \text{ pmol}}{\mu\text{mol}}
\]

where \( N \) is the number of strands in the DNA to be labelled. \( \text{MW} \) is the molecular weight of the DNA and is calculated as (number of bp) \( \times \) (660 \( \mu\)g/\( \mu \)mol) for double stranded DNA fragments or as (number of bases) \( \times \) (330 \( \mu\)g/\( \mu \)mol) for oligonucleotides. DNA fragments were dephosphorylated with calf intestinal alkaline phosphatase (Boehringer Mannheim, Indianapolis, IN) for 30 min at 37°C in 50 mM Tris-HCl, 0.1 mM EDTA, (pH 8.5). Labelling reactions were carried out as recommended by the enzyme supplier and contained 5 to 10 pmol of 5' ends of the DNA to be labelled, 25 \( \mu \)Ci [\( \gamma \)-\( ^{32} \)P] adenosine triphosphate (Amersham), and 10 U T4 polynucleotide kinase in reaction buffer [70 mM Tris-HCl (pH 7.6), 10 mM MgCl\(_2\), 100 mM KCl, 1 mM 2-mercaptoethanol].

DNA fragments were also end-labelled by filling in a recessed 3' end of a linear DNA fragment with the Klenow fragment of \( E. \) coli DNA Polymerase I (Gibco BRL). An appropriate labelled [\( \alpha \)-\( ^{32} \)P]-dNTP (Amersham) was chosen for the restriction enzyme used to generate each linear DNA fragment. The amount of DNA in the labelling reaction varied from 0.1 to 100 ng and did not seem to dramatically affect the labelling efficiency. Labelling reactions
contained 25 μCi of the labelled dNTP, 200 μM of the other three dNTPs, and 2 U Klenow enzyme in 1X Klenow reaction buffer [50 mM Tris-HCl (pH 8.0), 10 mM MgCl₂, 50 mM NaCl] and were incubated for 15 min at room temperature. A 5 min chase reaction with 200 μM of unlabelled dNTP (same one as the labelled dNTP) was done to ensure complete end-filling. Reactions were stopped by incubation at 65°C for 10 min.

DNA sequences were determined by the dideoxy chain termination method of Sanger et al. (1977). Double stranded plasmid DNA was denatured by the addition of 2 μl of 2 M NaOH to 18 μl DNA and incubation at 65°C for 5 min. Tubes were chilled on ice, and 6 μl water, 7 μl 3 M Na-acetate, and 75 μl 95% ethanol added. After 5 min, the DNA was collected by centrifugation for 20 min. Denatured DNA was annealed to 1 to 3 pmol universal or reverse sequencing primer (New England Biolabs, Beverly, MA) by incubation at 42°C for 30 min. Sequencing reactions were carried out using Sequenase Version 2.0 (US Biochemical) according to the instructions of the manufacturer, and included 0.5μCi deoxycytidine [5'-α-³⁵S]-thiotriphosphate (Amersham) as the labelled dNTP and 7-deaza dGTP to resolve compressions. Reactions were resolved by electrophoresis through 6% denaturing polyacrylamide gels [5.7% acrylamide, 0.3% bis-acrylamide, 50% urea] in modified TBE buffer [130 mM Tris-HCl, 45 mM boric acid, 2.5 mM Na₂-EDTA] at a constant power of 70 Watts. Urea was removed from the gel by soaking in a solution of 10% acetic acid, 12% methanol for 30 min. The gel was then transferred onto Whatman 3M filter paper (Whatman, Hillsboro, OR), dried under vacuum for 1 h at 80°C,
and exposed to autoradiography film at room temperature. DNA sequence analysis was performed using MacVector version 4.1.4 (International Biotechnologies, New Haven, CT) or Wisconsin Genetics Computer Group (GCG) software (Devereux et al., 1984).

The polymerase chain reaction (PCR) was used to generate DNA fragments when appropriate restriction enzyme sites were not available. PCR reactions (100 μl total volume) contained 400 ng plasmid template, 40 pmol each oligonucleotide primer, 100 μM each dNTP, 1.5 mM MgCl₂, and 2 U Taq polymerase (Promega, Madison, WI) in PCR buffer [50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% Triton X-100]. No oil overlay was necessary since the thermal cycler used employs a heated lid unit to prevent evaporation of samples (Hot Bonnet; MJ Research, Watertown, MA). The DNA template was initially denatured by heating at 95°C for 5 min, and the reaction was started by the addition of Taq polymerase followed by two cycles of low stringency amplification: denaturation at 95°C for 5 min, annealing at 45°C for 5 min, and extension at 72°C for 5 min. This was immediately followed by 30 cycles of high stringency amplification: denaturation at 95°C for 1 min, annealing at 65°C for 1 min, and extension at 72°C for 1 min. The extension reaction during the final cycle was increased to 10 min at 72°C to completely fill in the ends. Cloning of PCR products was facilitated by purification of the correct-sized fragment from agarose gels prior to digestion with restriction enzymes at primer-derived sites.
**DNA Hybridization Techniques**

Southern hybridization (Southern, 1975) of restriction endonuclease digested DNA was accomplished by vacuum transfer of DNA from 0.7% agarose gels to Nytran nylon membrane (Schleicher and Schuell, Keene, NH) using the VacuGene vacuum blotting apparatus of Pharmacia (Uppsala, Sweden), according to manufacturer's instructions. Depurination with 0.25 N HCl, denaturation with 1.5 M NaCl, 0.5 M NaOH, and neutralization with 1 M Tris, 2 M NaCl (pH 5.0) were all accomplished by exposing the gel to the solutions for 5 min at a vacuum pressure of 40 cm H$_2$O. DNA was transferred to the membrane for 1 h with 20X SSC [3 M NaCl, 0.3 M Na-Citrate, pH 7.0]. DNA was immobilized on the nylon membrane by baking for 1 h at 80°C. SSPE was used in prehybridization and hybridization solutions [a 1X SSPE solution is 0.18 M NaCl, 10 mM sodium phosphate (pH 7.7), 1 mM EDTA]. Membranes were prehybridized for 2 h at 65°C in 6X SSPE, 10X Denhardt's solution, 1% SDS, 0.2 mg/ml sheared, denatured salmon sperm DNA. Membranes were hybridized to continuously-labelled probes overnight at 65°C in 6X SSPE, 5X Denhardt's, 1% SDS, 0.1 mg/ml sheared, denatured salmon sperm DNA. Following hybridization, the membranes were washed under three conditions in order of increasing stringency. Wash solutions and temperatures were as follows: (1) 5X SSPE, 0.2% SDS at room temperature, twice for 10 min each; (2) 1X SSPE, 0.5% SDS at 37°C, twice for 10 min each; (3) 0.1X SSPE, 0.5% SDS at 65°C, once for 1 h. Membranes were wrapped in plastic wrap and exposed at -70°C to autoradiography film with intensifying screens (Lightning Plus; DuPont NEN, Wilmington, DE).
Recombinant *E. coli* containing *lasR* or *lasI* in a subgenomic library were detected by colony hybridization (Grunstein and Hogness, 1975) to either a labelled oligonucleotide probe (*lasR*) or a labelled DNA fragment generated with restriction enzymes (*lasI*). Recombinant colonies were patched onto master LB-Km plates containing no filter disks and onto Nytran nylon filter disks (Schleicher and Schuell) overlaid on LB-Km, and incubated overnight at 37°C. The protocol for colony hybridization was as recommended by Schleicher and Schuell. Cells were lysed by placing the filters, colony side-up, on Whatman 3M paper saturated with 0.5 N NaOH, in a pan suspended over boiling water for 5 min. The membranes were neutralized by placing on Whatman 3M paper saturated with 1 M Tris-HCl, pH 8.0 for 5 min, then on Whatman 3M paper saturated with 1 M Tris-HCl, pH 8.0, 1.5 M NaCl for 5 min. To reduce background hybridization to cellular material, membranes were placed on Whatman 3M paper saturated with 10% SDS for 3 min, followed by a final wash in 2X SSC. DNA was immobilized on the membrane by baking for 1 h at 80°C. Membranes were prehybridized for 2 h at 65°C in 5X SSPE, 3X Denhardt's solution, 0.3% SDS, 0.1 mg/ml sheared, denatured salmon sperm DNA. Hybridization and wash conditions varied for the type of probe used. The *lasR* oligonucleotide LR433 (8 x 10⁵ cpm/ml) was hybridized to the membranes for 24 h at 49°C in 5X SSPE, 2X Denhardt's, 0.3% SDS, 50 µg/ml sheared, denatured salmon sperm DNA. Membranes were washed twice in 1X SSPE, 0.1% SDS for 30 min each at room temperature, and once for 20 min at 49°C, followed by autoradiography. For the cloning of *lasI*, a *lasR* restriction fragment (8 x 10⁴ cpm/ml) was hybridized overnight at 65°C in 4X
SSPE, 5X Denhardt's, 1% SDS, 0.2 mg/ml sheared, denatured salmon sperm DNA. Membranes were washed twice at room temperature for 15 min each in 2X SSPE, 0.2% SDS, then twice at room temperature for 15 min each in 1X SSPE, 0.1% SDS.

Transfer of DNA into *P. aeruginosa*

Plasmids containing a broad host range replicon and an origin of transfer \((oriT)\) could be mobilized from *E. coli* DH5α into *P. aeruginosa* by triparental matings with *E. coli* HB101/pRK2013 providing the transfer functions \(\textit{in trans}\) (Figurski and Helinski, 1979). For patch matings, a loopful each of the DH5α donor, HB101 helper, and *P. aeruginosa* recipient strains were mixed in a small patch on an LB plate and incubated overnight at 37°C to allow plasmid transfer. The cells were transferred to a 16 x 125 mm culture tube, resuspended in 2 ml PBS (145 mM NaCl, 2.2 mM KH₂PO₄, 4.2 mM Na₂HPO₄) by vortexing, and plated on PIA containing the appropriate antibiotic. For filter matings, each strain was grown overnight in liquid LB containing appropriate antibiotics, and 0.5 ml of each culture filtered through a Swinex-25 disc filter holder (Millipore, Marlborough, MA) attached to a 5 ml syringe to transfer the cells onto a 0.45 μm filter (25 mm, MF-Millipore type HA). The filter was then aseptically transferred to an LB plate and incubated overnight. The filter was transferred to a 16 x 125 mm culture tube, and the cells resuspended and plated as for patch matings.
Plasmids containing a broad host range replicon but no oriT had to be introduced into \textit{P. aeruginosa} by a modification of the transformation method of Olsen \textit{et al.} (1982; S. Wahl, personal communication). Cells from a late-logarithmic phase culture (O.D.\textsubscript{600} of 1.0 to 1.5) were pelleted and washed once by resuspending in 0.5 ml 0.1 M CaCl\textsubscript{2} per ml of culture, followed by incubation on ice for 5 min. Following centrifugation, the pelleted cells were resuspended in 0.1 ml 0.1 M CaCl\textsubscript{2} per ml of culture, followed by incubation on ice for 20 min. Cells were then diluted with an equal volume of either 0.1 M CaCl\textsubscript{2}, for immediate use, or 50\% glycerol, for freezing of competent cells in dry ice-ethanol and storage at -70\(^\circ\)C. Competent cells were transformed by addition of 10 \(\mu\)l plasmid DNA to 0.3 ml cells, followed by incubation on ice for 1 h. Cells were heat-shocked at 48 to 49°C for 3 min, and immediately placed on ice for 5 min. To allow expression of plasmid-borne genes, cells were grown in LB for 1.5 h at 37°C prior to plating on PIA containing the appropriate antibiotic.

\textbf{Enzyme Assays}

The elastolytic activity of \textit{P. aeruginosa} strains was measured both on solid media and in liquid cultures. On solid media containing an elastin overlay, a zone of clearing of the elastin fibers is produced where elastolytic enzymes are secreted and diffuse into the agar media. LB overlay plates were made by the addition of 2.5 mg/ml bovine nuchal elastin (ICN Pharmaceuticals, Costa Mesa, CA) or elastin-congo red (Sigma Chemical Co.) to sterile, molten LB agar, once it had cooled to approximately 65°C. Five ml of the elastin-agar
suspension was then overlaid onto LB plates and allowed to solidify. Nutrient agar overlay plates were made in the same manner. Strains patched onto the overlay plates were incubated at 37°C until zones of clearing were evident. Zones sizes were measured from the edge of the bacterial growth, and the relative zone sizes of different strains were compared as an indication of the amount of elastolytic activity produced.

Elastolytic activity in the supernatants of liquid cultures was determined using elastin-congo red as substrate and measuring the release of congo red from the insoluble elastin molecules. Microcentrifuge tubes containing 10 mg elastin-congo red in 0.95 ml 0.1 M Tris-HCl (pH 7.0) were equilibrated to 37°C, and 0.05 ml of the culture supernatant to be assayed was added to start the reaction. The tubes were capped tightly and shaken vigorously in a horizontal position at 37°C for 4 h. Following incubation, the tubes were centrifuged to remove insoluble elastin, and the absorbance values (A\textsubscript{495}) of the resulting supernatants read against a reagent blank. Units of elastolytic activity are reported as A\textsubscript{495} per ml supernatant.

β-Galactosidase activity of \textit{E. coli} and \textit{P. aeruginosa} cultures containing \textit{lacZ} transcriptional fusions was determined by the method of Miller (1972). Some of the optional protocols given by Miller were used as follows. Cell membranes were disrupted with 40 µl chloroform and 20 µl 0.1% SDS instead of toluene. Instead of correcting for cell debris interference by a measurement of light scattering at 550 nm, the assay tubes were spun in a microcentrifuge for 15 min to remove the cellular debris. The molar extinction coefficient for \textit{o-}
nitrophenol under the assay conditions was reported to be 4500 M$^{-1}$. Standard Miller units (A$_{420}$ min$^{-1}$ ml$^{-1}$) were then converted to specific activity (nmol o-nitrophenol produced min$^{-1}$ ml$^{-1}$) by division by the extinction coefficient. Actual calculation of values was done using equation 2:

$$U = \frac{A_{420}}{\text{min} \times A_{600}} \times \frac{1 \text{ ml rxn}}{\text{ml sample}} \times \frac{\text{mmol}}{4500 \text{ ml}} \times \frac{10^6 \text{ nmol}}{\text{mmol}}$$

**Standard Protein Analysis Techniques**

Protein was quantitated by the method of Bradford (1976) using bovine gamma globulin as the standard. Proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using the buffer system of Laemmli (1970). For immunoblot analysis, proteins were transferred from SDS-PAGE gels to nitrocellulose membranes (Schleicher and Schuell) by electrophoretic transfer (Towbin et al., 1979) using a semi-dry transfer apparatus (Trans-Blot SD; Bio-Rad Laboratories, Richmond, CA) as recommended by the manufacturer. Transfer buffer contained 25 mM Tris, 192 mM glycine, 20% methanol (pH 8.3). Transfers for 140 mm × 120 mm × 1.5 mm gels were done at 540 mA for 30 min. All solutions were made up in TBS (10 mM Tris, 0.5 mM NaCl, pH 7.5). Membranes were blocked for 1 h at room temperature in 2% skim milk (Difco Laboratories) with constant rotation on a variable speed rotator (Scientific Products, McGaw Park, IL). Polyclonal anti-LasR antiserum in 1% skim milk was incubated with the membranes overnight at 4°C. Blots were washed three times for 10 min each at room temperature in 0.1% Tween-20, followed by incubation with 15 μCi $^{125}$I-Protein
A (Sigma Chemical Co.) in 1% skim milk for 3 h at room temperature. Blots were washed as above and exposed to x-ray film with intensifying screens (DuPont NEN) at -70°C. For N-terminal sequence determination, proteins were transferred onto Westran PVDF membrane (Schleicher and Schuell) using CAPS buffer as the transfer buffer [10 mM 3-(cyclohexylamino)-1-propanesulfonic acid, 10% methanol, pH 11.0] as described by Matsudaira (1987). The PVDF membrane was stained with 0.1% Coomassie Blue R-250 (Bio-Rad Laboratories) in 50% methanol for 5 min, then destained in 50% methanol, 10% acetic acid for 5 to 10 min, and rinsed in deionized water. The desired band was excised with a scalpel and submitted for N-terminal sequence analysis to the Biochemical Instrument Center (The Ohio State University, Columbus, OH).

**Overproduction and Purification of LasR Inclusion Bodies**

Overexpression of lasR in *E. coli* was accomplished using the pET-3a vector of Rosenberg *et al.* (1987) to generate a translational fusion of LasR to T7 gene 10 in pL03575 as described in Chapter III. Following transformation of the expression strain BL21 (DE3) (Studier and Moffatt, 1986) with pL03575, overexpression of lasR was carried out within a few days due to instability of the plasmid. Strain BL21 (DE3) exhibits leaky expression of T7 RNA polymerase, so induction with IPTG was not necessary for overproduction of LasR. BL21 (DE3)/pLO3575 and BL21 (DE3)/pET-3a were inoculated into 250 ml LB-Ap at a starting O.D.₆₀₀ of 0.05, grown to an O.D.₆₀₀ of 0.7 (approximately 2.5 h), then harvested 3 h later by centrifugation at
15,000 x g, 4°C, for 15 min. Cells were washed once in 10 ml PBS, then resuspended in 30 ml lysis buffer [50 mM Tris-HCl (pH 8.0), 1 mM EDTA, 100 mM NaCl, 0.1 mM PMSF] for BL21 (DE3)/pLO3575 or 10 ml lysis buffer for BL21 (DE3)/pET-3a. Cells were disrupted by treatment in a French pressure cell three times at 15,000 psi.

The recent review by Fischer et al. (1993) was used as a guide for optimization of conditions for isolation, solubilization, and renaturation of LasR inclusion bodies. The insoluble fraction, including inclusion bodies, was sedimented by low-speed centrifugation at 5000 x g, 4°C for 15 min. The supernatant resulting from this spin was saved as the cell-free extract. Contaminating proteins, which include membrane proteins, were solubilized by washing the pellet in 30 ml lysis buffer containing 5 M urea, five times for 15 min each, with shaking at room temperature. Following each wash, the inclusion bodies were collected by centrifugation for 15 min at 15,000 x g. The supernatant of the first wash was saved for analysis. Inclusion bodies were then washed twice in 30 ml lysis buffer, resuspended in 5 ml lysis buffer, aliquotted into 1.5 ml screw-cap tubes, and stored at -70°C.

Treatment of LasR inclusion bodies with 4 M, 6 M, and 8 M guanidine-HCl resulted in degradation of the protein. However, LasR could be solubilized in 8 M urea. Pelleted inclusion bodies were resuspended in lysis buffer containing 8 M urea (2 ml per ml of inclusion body suspension) and incubated at room temperature for 3 h. Soluble, denatured LasR was used directly in various assay systems by 100-fold dilution into the assay buffer to dilute the denaturant and allow partial renaturation. Alternatively, the denaturant was
removed by sequential dialysis against lysis buffer containing 2 M urea and lysis buffer containing 1 M urea. Centrifugation of dialyzed samples removed any LasR that had precipitated during renaturation. The amount of soluble LasR was quantitated by the method of Bradford (1976).

Preparation of Antiserum to LasR

Denatured LasR inclusion bodies were used to generate polyclonal antiserum in a New Zealand white rabbit (Mohican Valley Rabbits, Loudenville, OH). Approximately 150 µg of denatured LasR was separated from contaminating proteins in the inclusion body preparation by SDS-PAGE. The 12.5% gel was stained with Coomassie Blue R-250 (Bio-Rad Laboratories) and destained in 10% methanol, 10% acetic acid. The 27 kDa band corresponding to overexpressed recombinant LasR was excised from the gel with a scalpel, and the polyacrylamide fragments were cut into small pieces and dried in a SpeedVac (Savant Instruments, Farmingdale, NY). The resulting pellet was resuspended in 2 ml PBS using a syringe with a 19-G needle, then emulsified in Complete Freunds adjuvant (Sigma Chemical Co.). Pre-immune serum (5 ml) was collected from the rabbit prior to subcutaneous injection at ten different sites. Protein for booster immunizations was prepared as above except Incomplete Freunds adjuvant (Sigma Chemical Co.) was used instead of Complete Freunds. Booster immunizations were administered at 12 and 43 days post-primary immunization. Test bleeds (5 ml) were taken from the medial artery after 34 and 58 days, and the final bleed (70 ml) was done by cardiac puncture after nine weeks. The antibody titer of
the serum was determined by immunoblotting against LasR inclusion bodies.
Serum was aliquotted and stored at -70°C.

Partial Purification of Pseudomonas Autoinducer (PAI)

$N$-Acyl-homoserine lactones were extracted from culture supernatants
with ethyl acetate (Eberhard et al., 1981; Pearson et al., 1994). A crude ethyl
acetate extract containing PAI was prepared from culture supernatants of
PAO1 grown to early stationary phase in 700 ml supplemented A medium.
Cells were pelleted by centrifugation at 22,000 x g for 15 min. The culture
supernatant was filtered through a non-sterile 0.45 μm PVDF filter (Acrodisc
LC; Gelman Sciences, Ann Arbor, MI), and the volume of supernatant
measured. Supernatant fluid was extracted with an equal volume of ethyl
acetate containing 0.1 ml/L acetic acid, and the top organic phase saved. The
extraction was repeated, and the two organic phases were pooled. Residual
water was removed by slowly adding approximately 5 g of MgSO$_4$ until
vigorous shaking and stirring had no effect on the amount or appearance of
MgSO$_4$ in the extract. Filtration through Whatman 3M paper removed the
MgSO$_4$ from the extract. The extract was evaporated in a rotary evaporator
(Brinkman Instruments, Westbury, NY) at 48°C. The green residue was
extracted with 15 ml ethyl acetate-acetic acid and the extract saved. This was
followed by dissolving the residue in 10 ml 95% ethanol and evaporation at
48°C. The ethyl acetate-acetic acid and ethanol extraction steps were
repeated twice, and the three ethyl acetate extracts pooled. The volume of the
extract was reduced to 9 ml by evaporation and resuspension in ethyl acetate-acetic acid. The extract was aliquotted into 1.5 ml screw-cap tubes and stored at -70°C.

To determine the amount of PAI activity in the extract, a bioassay was developed using a genetically defined lasI mutant of PAO1 designated PAO-LI. This strain is deficient only in the PAI synthase gene lasI, so LasR-dependent expression of target genes should be restored by exogenous addition of the diffusible PAI molecule. A lasA::lacZ transcriptional fusion, pLO2856, was transformed into PAO-LI, and β-galactosidase activity determined as an indication of lasA expression. Carbenicillin was present at all times to select for maintenance pLO2856. An overnight culture of PAO-LI/pLO2856 grown in supplemented A medium was diluted into fresh supplemented A medium at an O.D.₆₆₀ of 0.1 and stored on ice until the assay was started. Prior to use in the bioassay, ethyl acetate was removed from the extract by evaporation in a SpeedVac (Savant Instruments), and the pellet was redissolved in sterile water. Each bioassay contained 2 ml of the diluted culture in 16 x 125 mm culture tubes, and was started by the addition of varying volumes of the extract. Assay cultures were grown for 5 h at 37°C with shaking, after which 0.15 ml of each culture was assayed for β-galactosidase activity.

RNA Isolation and Transcript Mapping

Solutions for RNA isolation and manipulation were treated overnight with 0.2% DEPC (Sigma Chemical Co.) to inactivate RNases, followed by
autoclaving to inactivate the DEPC. Cellular RNA was isolated from 50 ml cultures of PAO1, grown in LB for 12 h, by the guanidinium isothiocyanate-hot phenol method of Rothmel et al. (1991). Harvested cells were washed once in PBS, then resuspended in 2 ml of 4 M guanidinium isothiocyanate solution (prepared as described in Maniatis et al., 1982) to lyse the cells, then transferred to a sterile, RNase-free, disposable 17 x 100 mm culture tube. The tube was placed at 60°C, and an 18-G needle-syringe unit used to shear the chromosomal DNA until the mixture was no longer viscous. An equal volume (2 ml) of hot (60°C) nucleic acid grade phenol (Gibco BRL), equilibrated with RNase-free TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0), was added, and the lysate again forced through the needle, keeping the tube at 60°C. One ml of solution A [0.1 M Na-acetate (pH 5.2), 10 mM Tris-HCl (pH 7.4), 1 mM EDTA] was added, followed by 2 ml chloroform:isoamyl alcohol (24:1). The tube was spun for 20 min in a clinical centrifuge (International Equipment Co., Needham Heights, MA) at the highest setting. The aqueous layer was removed and extracted twice with an equal volume (3 ml) of phenol:chloroform (1:1). Nucleic acids were precipitated from the aqueous phase by addition of 6 ml cold 95% ethanol, incubation at -70°C for 20 min, and spinning for 20 min in the clinical centrifuge. The pellet was air dried and resuspended in 1 ml solution B [0.1 M Tris-HCl (pH 7.4), 50 mM NaCl, 10 mM MgCl₂]. DNA was digested for 1 h at 37°C by the addition of 10 units human placental RNase inhibitor (RNasin; Ambion, Austin, TX) and 10 units RNase-free DNase (Ambion). SDS and EDTA were then added to final concentrations of 0.2% and 10 mM, respectively, to provide the conditions necessary for proteinase K treatment (1
μg added) for 30 min at 37°C. The RNA preparations were then extracted twice with an equal volume of phenol:chloroform, precipitated with 2 volumes cold 95% ethanol, and stored at -70°C until needed. Samples of the RNA preparations were collected by centrifugation in a microcentrifuge, dried in a SpeedVac (Savant Instruments), and resuspended in RNase-free water. RNA concentrations were quantitated spectrophotometrically by reading the absorbance at 260 nm.

Dephosphorylated restriction fragments for use in low resolution S1 nuclease mapping were purified from agarose gels, ethanol-precipitated from 0.3 M Na-acetate, washed twice in 70% ethanol to remove salts, and end-labelled with T4 polynucleotide kinase. Unincorporated label was removed by precipitating with 95% ethanol, with salmon sperm DNA (75 μg/ml) as a carrier. The probe was prepared for S1 nuclease analysis by extraction with phenol:chloroform, reprecipitation with 95% ethanol, and two washes with 70% ethanol. Probe pellets were dried in a SpeedVac (Savant Instruments) and resuspended in 100 μl RNase-free water.

A strand-specific DNA probe for use in high resolution S1 nuclease mapping was prepared by modification of the method of Dixon (1984). Instead of single stranded phage DNA, double stranded DNA (pLO1831) was used as a template for primed synthesis of a probe from oligonucleotide LA755. This would be expected to reduce the intensity of the signal due to competition by the antisense plasmid DNA strand, but should not otherwise affect the results. For synthesis of the labelled antisense DNA probe, 4 μg pLO1831 was denatured as for sequencing reactions and hybridized with 40 ng LA755 for 30
min at 42°C. Extension from the annealed primer was achieved using Sequenase Version 2.0 (US Biochemical) in the reaction buffer supplied by the manufacturer. The initial labelling reaction (10 min, 37°C) contained 10 μCi [α-32P] deoxycytidine triphosphate (Amersham), 6 mM DTT, and 0.3 μM dATP, dGTP, and dTTP. To allow for extension and synthesis of high molecular weight probes, all four dNTPs were added at a final concentration of 100 μM, and the reaction continued an additional 10 min. Probe was prepared for S1 nuclease analysis by extraction and precipitation as described above.

For S1 nuclease mapping studies, a modification of the method of Sambrook et al. (1989) was used. Probe (1-5 x 10^5 cpm) was mixed with 50 to 100 μg PA01 RNA and lyophilized in a SpeedVac (Savant Instruments). Yeast tRNA (Boehringer Mannheim) was added so that all reactions contained the same amount of total RNA. Samples were resuspended in 30 μl S1 hybridization buffer [40 mM PIPES (pH 6.4), 1 mM EDTA (pH 8.0), 0.4 M NaCl, 80% formamide] by vigorous pipetting. Nucleic acids were fully denatured by incubation of the reaction tubes at 85°C for 10 min. Tubes were then rapidly transferred to a water bath set at 65°C and allowed to hybridize overnight. The following components were added to each reaction tube while still submerged at 65°C: 294 μl ice-cold S1 buffer [50 mM NaCl, 30 mM Na-acetate (pH 4.6), 1 mM Zn-acetate, 5% glycerol], 10 U RNasin, 3 μg salmon sperm DNA, and 140 U S1 nuclease (Gibco BRL). Tubes were rapidly mixed and transferred to a 37°C water bath for 30 min. Reactions were stopped by extraction with 300 μl phenol:chloroform. The aqueous phase was then
precipitated by addition of 33 μl Na-acetate and 600 μl cold 95% ethanol for 15 min at -70°C. Nucleic acids were pelleted by centrifugation and dried briefly in a SpeedVac (Savant Instruments). Pellets were resuspended in 6 μl water and 5 μl denaturing loading dye [95% formamide, 20 mM EDTA, 0.05% Bromophenol Blue, 0.05% Xylene Cyanol FF] and analyzed by electrophoresis in 6% denaturing polyacrylamide gels, as for sequencing reactions. Low resolution S1 reactions were analyzed next to end-labelled size standards, while high resolution S1 reactions were analyzed alongside a sequencing ladder generated from pLO1831 and LA755.

The primer extension system of Promega was used as directed by the manufacturer. PAO1 RNA (50 μg) was hybridized to 200 fmol end-labelled oligonucleotide LA724 by heating to 90°C for 3 min to denature the RNA, then transferring reaction tubes to 58°C for 20 min and cooling at room temperature for 10 min. Following reaction with AMV reverse transcriptase for 30 min at 42°C, reactions were precipitated at -20°C for 20 min by addition of 7 μl water, 6 μl 3 M Na-acetate, and 75 μl cold 95% ethanol. Nucleic acids were pelleted by centrifugation and resuspended in 6 μl water and 4 μl denaturing loading dye. Reaction products were analyzed by electrophoresis in 6% denaturing polyacrylamide gels, alongside a sequencing ladder generated from pLO1763 and LA724.

**Generation of 5′ deletions of the lasA promoter region**

Exonuclease III (Gibco BRL) was used to generate deletions from the 5′ end of lasA in a unidirectional manner (Henikoff, 1987). Exonuclease III
cannot attack a 3' overhang, so digestion of pLO1763 with *Kpn*I effectively blocked digestion of vector sequences by exonuclease III, while digestion with a restriction enzyme leaving a 5' overhang or blunt end allowed deletion of *lasA* sequences. Initial experiments in which pLO1763 was digested with *Kpn*I and *Sal*I resulted in poor deletion, perhaps due to incomplete digestion with *Sal*I. *Cla*I was chosen as an alternative 5' overhang since it is located only 100 bp from the *Sal*I site. While there is another *Cla*I site in pLO1763 located within the promoter region of *lasA*, this site is blocked by methylation in strain DH5α. Thus, 10 μg pLO1763 was digested with *Kpn*I and *Cla*I and subjected to exonuclease III digestion as described by Henikoff (1987), except that all volumes were doubled so that 10 μl of each time point could be analyzed by agarose gel electrophoresis. Prior to transformation of DH5α, religated samples were digested with *Cla*I, so any plasmids that had not been deleted by exonuclease III would be linearized and thus would not be transformable. Kanamycin resistant colonies were screened for deletion by restriction analysis of plasmid DNA, and the end-points of the deletions were determined by DNA sequencing.

**Electrophoretic Mobility Shift Assay**

DNA-binding proteins present in *P. aeruginosa* extracts were analyzed by the electrophoretic mobility shift assay (EMSA). Buffer systems in the EMSA are most simply derived from the buffer of the protein to be assayed, so the lysis buffer for *P. aeruginosa* cell-free extract preparation [20 mM HEPES
(pH 7.9), 2 mM EDTA, 2 mM DTT, 1 mM PMSF, 10% glycerol] was chosen to be essentially 2X the desired EMSA reaction buffer [10 mM HEPES (pH 7.9), 1 mM EDTA, 1 mM DTT, 0.5 mM PMSF, 10% glycerol, 60 mM KCl]. Monovalent salt (KCl) was omitted from the lysis buffer, because this parameter is often varied to maximize binding activity. Cell-free extracts were prepared from *P. aeruginosa* LB cultures grown for 14 to 16 h at 37°C. Cells were harvested by centrifugation at 22,000 x g, 4°C, for 15 min, and resuspended in 25 ml lysis buffer per L culture. Cells were disrupted by treatment in a French pressure cell three times at 15,000 psi. The extracts were cleared of unbroken cells and membrane fragments by centrifugation at 40,000 x g, 4°C, for 1 h. The supernatant was carefully removed from the pellet, aliquotted, and stored at -70°C. DNA-binding activity was stable for up to 2 months.

DNA probes for EMSA were end-labelled by filling in a recessed 3' end with Klenow enzyme. The general procedure for EMSA was as described by Ausubel *et al.* (1990), with use of the high ionic strength buffer system (50 mM Tris, 380 mM glycine, 2 mM EDTA, pH 8.5) making buffer recirculation unnecessary. Generally the binding reactions were initiated by combining two components in equal volumes: a probe mixture containing the labelled probe fragment, nonspecific competitor DNA (Poly [dI-dC] • Poly [dI-dC]; Sigma Chemical Co.), acetylated BSA (New England Biolabs), KCl, and glycerol; and a protein mixture in lysis buffer. Typical reaction conditions after combination of the two components were: 10 mM HEPES (pH 7.9), 1 mM EDTA, 1 mM DTT, 0.5 mM PMSF, 10% glycerol, 60-100 mM KCl, 10 ng/μl Poly dI-dC, 0.25 μg/μl
BSA, 1-5 x 10^4 cpm probe, and 10-300 µg *P. aeruginosa* extract. Binding reactions were allowed to reach equilibrium for 20 min at 30°C. Loading dye containing 0.5% Bromophenol Blue (BioRad Laboratories), and 0.5% Xylene Cyanol FF (BioRad Laboratories) and all of the reaction components, except probe and extract, was added to each binding reaction at 5% the final volume, prior to loading on a pre-run, nondenaturing polyacrylamide gel [4% acrylamide, 0.05% bis-acrylamide, 2.5% glycerol] in Tris-glycine-EDTA buffer. Electrophoresis was carried out in a cold room for approximately 2.5 h at 200 mA. The gel was transferred to Whatman 3M filter paper, dried in a gel dryer under vacuum at 80°C for 1 h, and exposed to autoradiography film with intensifying screens (DuPont NEN) at -70°C.
CHAPTER III

The Importance of LasR in Expression of the lasA Gene

Introduction

The regulatory locus lasR was originally cloned by its ability to complement the non-elastolytic phenotype of a clinical isolate of P. aeruginosa, strain PA103 (Gambello and Iglewski, 1991). Cloning of the linked locus responsible for autoinducer production, lasI, has also been reported (Passador et al., 1993). Comparison of a genetically defined mutant with its isogenic parent is generally preferred to analysis of clinical strains that could contain multiple defects leading to a given phenotype. While Gambello and Iglewski (1991) have created a genetically defined mutant in lasR, PAO-R1, they have been unable to create an isogenic mutant in lasI (Pearson et al., 1995). While the effect of lasR on transcription of the lasA gene has been investigated (Toder et al., 1991), a quantitative analysis has not been reported.

Most of the studies on the mechanism of autoinducer-mediated transcription activation by LasR have involved the use of a lasB::lacZ translational fusion, pTS400, or its derivatives, in either E. coli or PAO-R1. The level of β-galactosidase activity in PAO-R1 containing pTS400 is 0.3% the level seen in PAO1/pTS400 (Pearson et al., 1993). When E. coli/pTS400-1.7,
which also contains lasR, is grown in the presence of exogenous autoinducer, β-galactosidase activity was 40-fold higher than when lasR was absent and levels were nearly half those seen in PAO1 (Passador et al., 1993). Thus, it has been established that LasR and autoinducer are both required and sufficient for transcription of lasB.

This project extends the initial characterization of the role of LasR in expression of lasB to examine the role of LasR in lasA expression. The three main goals of this study were to:

1. perform a quantitative analysis of the effect of an isogenic lasR mutation on expression of lasA in P. aeruginosa strain PAO1,
2. determine the ability of LasR and PAI to activate lasA transcription, and
3. purify LasR and PAI for use in various in vitro analyses of lasA promoter function.

**Results**

**Cloning of lasR and lasI from P. aeruginosa PAO1.**

The lasR gene was originally cloned on an 11 kb EcoRI fragment and shown to reside on a 1.6 kb SmaI-EcoRI fragment (Gambello and Iglewski, 1991). A subgenomic library of P. aeruginosa strain PAO1 was generated by digestion of chromosomal DNA with SmaI and EcoRI followed by
electrophoresis on 0.7% agarose. A window of DNA fragments ranging from 1.4 kb to 2.5 kb was excised from the gel and the DNA isolated. The chromosomal DNA fragments were then ligated to the pUC-based vector pK19 that had been digested with Smal and EcoRI. \textit{E. coli} strain DH5α was transformed with the ligation mixture. Approximately 450 Km\textsuperscript{T}, lac\textsuperscript{-} colonies were analyzed by colony hybridization using an end-labelled oligonucleotide probe, LR433, which matches the published sequence of lasR from nt 417 to 433 (Gambello and Iglewski, 1991). Hybridization of the lasR probe, followed by stringent washes and autoradiography revealed one colony that hybridized strongly. Plasmid DNA isolated from this colony exhibited the expected restriction fragments and DNA sequence indicating that the PAO1 lasR gene had been cloned.

The report of Passador \textit{et al}. (1993) on the cloning and sequencing of lasI indicated that the autoinducer-encoding gene was linked to the lasR gene and contained on a 5.4 kb EcoRV fragment spanning the EcoRI site used to clone lasR. An SspI-EcoRI restriction fragment of the lasR clone was used to screen a second subgenomic library of PAO1, created as described above by isolation of a 4 kb to 6 kb window of PAO1 chromosomal DNA digested with EcoRV. Three colonies that hybridized strongly all contained plasmid DNA with the expected restriction fragments and DNA sequence, indicating that lasI had been cloned.
Construction and analysis of lasR and lasI gene replacement mutants in PA01.

The gene replacement system of Schweizer (1992) was used to generate genetically defined, isogenic mutants of PA01 in lasR and lasI. This system relies on the conditionally lethal nature of the sacB gene contained on the gene replacement plasmid, which allows for selection of recombinants that have lost vector sequences in a second recombination event by simply plating recombinants on media containing 5% sucrose. Refer to Figure 5 for a restriction map of the lasR lasI locus in PA01. The original lasR SmaI-EcoRI clone, pLO2841, was digested with EcoRI and HindIII, and the lasR insert transferred into pNOT19, that had been digested with EcoRI and HindIII, to generate pLO2845. This plasmid was digested with EcoRV to delete most of the lasR coding region, and the 1.4 kb Tc\textsuperscript{r} gene of pBR322, which had been digested with EcoRI and AvaI and end-filled with Klenow enzyme, was inserted in place of the lasR EcoRV fragment. The resulting plasmid, pLO2923, was rendered mobilizable by insertion of the 5.8 kb NotI fragment of pMOB3, which contains oriT and sacB, into the unique NotI site. The resulting plasmid, designated pLO2927, is shown in Figure 6.

An EcoRV-SalI subclone (pLO3196) of the original lasI EcoRV clone (pLO3193) was digested with BamHI and end-filled with Klenow enzyme. The 1.4 kb lasI fragment was inserted into pNOT19 that had been digested with HindIII and SmaI and the ends blunted with Klenow to remove the PstI site in the polylinker. The resulting plasmid, pLO3308, was digested with ClaI and
Figure 5. Restriction map of the lasR lasI locus in PAO1. Plain arrows represent the lasR and lasI coding regions. Abbreviations: Sm, SmaI; RV, EcoRV; P, PstI; S, SalI; C, ClaI; E, EcoRI.
**Figure 6.** Gene replacement plasmids for generation of isogenic *lasR* and *lasI* mutants of PAO1. pLO2927, *lasR* gene replacement plasmid; pLO3394, *lasI* gene replacement plasmid; pLO3301, *lasR lasI* gene replacement plasmid. Thin lines represent pNOT19 sequences. The origin of replication (oriV) and β-lactamase gene (Ap) of pNOT19 and the origin of transfer (oriT), Cm<sup>r</sup> gene (Cm), and sacB gene (medium arrow) of the mob cassette are all labelled. The thick arrow represents the Tc<sup>r</sup> gene of pBR322. The *lasR*, *lasI*, and *lasR lasI* sequences are represented by the unfilled, lightly stippled, and heavily stippled boxes, respectively, with arrows showing the direction of the deleted open reading frames. Underlined restriction sites were used to subclone *lasR* or *lasI*. The large X next to the *EcoRV* site of pLO3394 indicates that that site was destroyed during plasmid construction.
Figure 6.
PstI and end-filled to delete the lasI coding region. The 1.4 kb Tc\(^r\) gene of pBR322, prepared as above, was inserted in place of the lasI deletion, to create pLO3310. In order to delete both lasR and lasI, the two genes first had to be rejoined in their native conformation at the EcoRI site. The lasR clone, pLO2841, was linearized with EcoRI, and the 0.8 kb EcoRI fragment of the lasI subclone, pLO3196, was inserted in the correct orientation, to generate pLO3296. This plasmid was digested with SmaI, and the 2.3 kb insert transferred into pNOT19 that had been digested with SmaI and HindIII and end-filled with Klenow to remove the PstI site from the polylinker. The resulting plasmid, pLO3298, was digested with PstI and end-filled with Klenow to delete most of both lasR and lasI. Again, the Tc\(^r\) gene of pBR322, prepared as above, was inserted in place of the deletion to create pLO3299. The mob cassette of pMOB3 was inserted into the unique NotI sites of pLO3310 and pLO3299 as described above.

The three gene replacement plasmids for recombination of the deleted forms of lasR, lasI, and lasR lasI into the PAO1 chromosome are designated pLO2927, pLO3394, and pLO3301, respectively (Figure 6). These constructs were introduced into PAO1 by triparental mating and recombinants were selected on PIA-Tc plates. Individual tetracycline-resistant recombinants were then streaked onto LB-Tc-5% sucrose to select loss of plasmid sequences by double recombination. All of the Tc\(^r\) suc\(^r\) colonies examined displayed decreased zones of elastolysis on LB agar plates containing elastin-congo red (LB-ECR).
Figure 7. Southern analysis of isogenic \textit{lasR} and \textit{lasI} mutants of PAO1.

Chromosomal DNA was digested with \textit{SmaI} (A) or \textit{SmaI} and \textit{BamHI} (B and C). Blots were probed with the 1.6 kb \textit{SmaI-EcoRI} fragment of pLO2841.

Lanes are labelled according to the chromosomal DNA sample loaded: O1, PAO1; LR, PAO-LR; LI, PAO-LI; LRI, PAO-LRI. The arrow marks the location of the 4.0 kb \textit{SmaI} fragment of PAO1.
Figure 8. Restriction map of the *lasR lasI* locus in PAO1 and isogenic mutants PAO-LR, PAO-LI, and PAO-LRI. Plain arrows represent the *lasR* and *lasI* coding regions. The dashed arrows represent the Tc\(^r\) gene of pBR322. Abbreviations: Sm, *Sma*I; RV, *EcoRV*; P, *Pst*I; S, *Sal*I; C, *Cla*I; E, *EcoRI*. The sites used for Southern analysis are boxed.
Figure 8.
One Tc\textsuperscript{r} suc\textsuperscript{r} recombinant from each mating with pLO2927, pLO3394 and pLO3301 was chosen for further analysis and designated PAO-LR, PAO-LI, and PAO-LRI, respectively. Chromosomal DNA was isolated from each mutant and compared to PAO1 by Southern analysis using a 1.6 kb Smal-EcoRI lasR probe (Figure 7). The lasR and lasI genes in PAO1 are contained on a 4.0 kb Smal fragment. This fragment was converted to a 4.8 kb Smal fragment in PAO-LR, a 1.9 kb Smal-BamHI fragment in PAO-LI and a 0.8 kb Smal-BamHI in PAO-LRI. These results are consistent with the predicted chromosomal arrangement of the mutants as depicted in Figure 8.

The mutants were initially characterized by comparing the sizes of the zones of clearing on LB-ECR plates. All three mutants produced very small zones compared to PAO1 after 24 h incubation (Table 7). However, after 48 h incubation, the mutants produced significant zones of elastin clearing. These values are significantly higher than those seen for a lasA lasB double mutant, PAO-LAB (see Appendix 1 for description), and the protease negative clinical isolate, PA103, which both which both fail to produce zones of elastolysis on LB-ECR plates (Table 7). However, when culture supernatants of overnight cultures grown in liquid LB medium were analyzed for elastolytic activity, PAO-LR produced significantly lower levels of elastolytic activity, and more closely resembled PA103 and PAO-LAB in the amount of activity produced (Table 7).

PAO-LR was then analyzed for elastase activity in the culture supernatant at various stages of growth in LB (Figure 9). After 20 h of growth,
Table 7. Elastolytic activity of isogenic lasR and lasI mutants.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Elastolytic Activity</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>LB-ECR a</td>
<td>24 h</td>
<td>48 h</td>
</tr>
<tr>
<td>PAO1</td>
<td></td>
<td>2.5</td>
<td>5.5</td>
<td></td>
</tr>
<tr>
<td>PA103</td>
<td></td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>PAO-LR</td>
<td></td>
<td>0.5</td>
<td>3.5</td>
<td></td>
</tr>
<tr>
<td>PAO-LI</td>
<td></td>
<td>0.5</td>
<td>3.5</td>
<td></td>
</tr>
<tr>
<td>PAO-LRI</td>
<td></td>
<td>0.5</td>
<td>3.5</td>
<td></td>
</tr>
<tr>
<td>PAO-LAB</td>
<td></td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

a Zones of digestion were measured from the edge of bacterial growth after 24 or 48 h incubation at 37°C.

b Strains were grown in LB broth for 18 h at 37°C. Culture supernatants were collected and assayed for 4 h at 37°C.

c ND - not determined.
Figure 9. Elastolytic activity in culture supernatants of an isogenic lasR mutant of PAO1. Culture supernatants were collected and assayed for elastolytic activity with elastin-congo red as substrate. Assays were done in triplicate. Differences in activity for PA103 and PAO-LR were found to be statistically significant at all time points when compared to PAO1, yet were not significant until 20 h when compared to each other (p ≤ 0.005). Open symbols represent growth (O.D.600). Filled symbols represent elastolytic activity (A495 ml⁻¹). Circles, PAO1; squares, PAO-LR; triangles, PA103.
PAO-LR levels of elastase activity increased sharply while the elastolytic activity of strain PA103 never increased. Activity in PAO-LR reached maximum levels after 28 h of approximately 16% WT. To determine if the residual elastolytic activity seen during stationary phase coincided with increased transcription of \textit{lasA}, PAO-LR was transformed with a \textit{lasA::lacZ} transcriptional fusion, pLO2856, which contains the entire regulatory region of \textit{lasA}, and with the fusion vector, pQF50. As seen in Figure 10, PAO-LR showed increased levels of \(\beta\)-galactosidase activity beginning at 8 h, with activity ranging from approximately 10 to 20% WT and 4- to 7-fold vector levels. PA103 \(\beta\)-galactosidase levels never increased above those seen for vector alone.

**Analysis of \textit{lasA} promoter activity in \textit{E. coli}.*

To determine if LasR and autoinducer are sufficient for \textit{lasA} promoter activity in \textit{E. coli}, as they are for the \textit{lasB} promoter, the ability of \textit{E. coli} cells containing \textit{lasR} and \textit{lasI}, both separately and together, to express a \textit{lasA::lacZ} transcriptional fusion, was analyzed. Many \textit{P. aeruginosa} promoters are not active in \textit{E. coli} due to the requirement for transcriptional activators not present in \textit{E. coli}. To ensure expression of \textit{lasR} and \textit{lasI}, the genes were subcloned, both separately and in their native configuration, into pACYC184 under control of the constitutive promoter of the Tc gene to create pLO3622 (\textit{lasR}), pLO3626 (\textit{lasI}), and pLO3629 (\textit{lasR lasI}). See Table 5 for detailed descriptions of these plasmids. There appears to be a transcription terminator
Figure 10. β-Galactosidase activity due to a plasmid-borne lasA::lacZ transcriptional fusion in an isogenic lasR mutant of PA01. Assays were done in triplicate. Differences in activity for PA103 and PAO-LR were found to be statistically significant at all time points when compared to PAO1, yet were not significant until 9 h when compared to each other (p ≤ 0.005). Open symbols represent growth (O.D.₆₀₀). Filled symbols represent β-galactosidase activity (nmol ONPG hydrolyzed min⁻¹ ml⁻¹). Circles, PAO1; squares, PAO-LR; triangles, PA103. The symbols x and + represent activity and growth, respectively, of one vector control PAO1/pQF50. Vector controls in all other strains showed similar results.
after lasR, and the lasR gene product is required for transcription from the lasI promoter (Passador et al., 1993). By analogy to the lux system and preliminary analysis of lasI expression (Seed et al., 1995), the expectation for pLO3629 is that transcription of lasR driven by the Tc promoter would generate a monocistronic message coding for production of LasR. The inactive LasR molecule can then combine with autoinducer produced by basal level transcription of lasI to activate LasR, which can then induce transcription from P_{lasI}, resulting in additional production of autoinducer to levels required for LasR-mediated activation of other target genes such as lasA and lasB.

The p15A replicon of pACYC184 is compatible with the pMB1 replicon of pLO2856 (lasA::lacZ). Thus, *E. coli* DH5α/pLO2856 was transformed with pLO3622, pLO3626, or pLO3629, and Ap\(^{r}\) Cm\(^{r}\) colonies were analyzed for the presence of the expected plasmids by restriction analysis of plasmid DNA. The ability of the lasR and lasI gene products to activate transcription of the lasA promoter was determined by measuring β-galactosidase activity in early stationary phase (Table 8). The presence of lasR alone resulted in essentially no increase in activity, while lasI alone or both lasR and lasI were responsible for a 1.8-fold or 2.9-fold increase, respectively. The β-galactosidase levels in *E. coli*, however, are still approximately 700-fold lower than those obtained in *P. aeruginosa* PAO1. To determine whether lasR and lasI were being expressed in *E. coli*, the same analysis was done using a lasB::lacZ transcriptional fusion, pLO3711. In this case, lasR and lasI were sufficient for an 80-fold increase in
Table 8. Activity of lasA and lasB promoters in *E. coli*.

<table>
<thead>
<tr>
<th>Strain</th>
<th>lacZ Fusion</th>
<th>lasR</th>
<th>lasI</th>
<th>β-Gal Activity</th>
<th>Relative β-Gal Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>DH5α/pLO2856</td>
<td>lasA</td>
<td>-</td>
<td>-</td>
<td>0.08 ± 0.005</td>
<td>1.0</td>
</tr>
<tr>
<td>DH5α/pLO2856/pACYC184</td>
<td>lasA</td>
<td>-</td>
<td>-</td>
<td>0.08 ± 0.005</td>
<td>1.0</td>
</tr>
<tr>
<td>DH5α/pLO2856/pLO3622</td>
<td>lasA</td>
<td>+</td>
<td>-</td>
<td>0.09 ± 0.004</td>
<td>1.1</td>
</tr>
<tr>
<td>DH5α/pLO2856/pLO3626</td>
<td>lasA</td>
<td>-</td>
<td>+</td>
<td>0.14 ± 0.04</td>
<td>1.8</td>
</tr>
<tr>
<td>DH5α/pLO2856/pLO3629</td>
<td>lasA</td>
<td>+</td>
<td>+</td>
<td>0.23 ± 0.02</td>
<td>2.9</td>
</tr>
<tr>
<td>PA01/pLO2856</td>
<td>lasA</td>
<td>+</td>
<td>+</td>
<td>160.00 ± 2.72</td>
<td>2000.0</td>
</tr>
<tr>
<td>DH5α/pLO3711</td>
<td>lasB</td>
<td>-</td>
<td>-</td>
<td>0.09 ± 0.004</td>
<td>1.0</td>
</tr>
<tr>
<td>DH5α/pLO3711/pACYC184</td>
<td>lasB</td>
<td>-</td>
<td>-</td>
<td>0.09 ± 0.004</td>
<td>1.0</td>
</tr>
<tr>
<td>DH5α/pLO3711/pLO3629</td>
<td>lasB</td>
<td>+</td>
<td>+</td>
<td>7.50 ± 0.06</td>
<td>83.3</td>
</tr>
<tr>
<td>PA01/pLO3711</td>
<td>lasB</td>
<td>+</td>
<td>+</td>
<td>195.00 ± 9.47</td>
<td>2166.7</td>
</tr>
</tbody>
</table>

*Refers to the transcriptional fusion contained in pQF50-based plasmids pLO2856 and pLO3711.*

*Refers to the presence of the lasR and lasI genes in compatible pACYC184-based plasmids pLO3622, pLO3626, and pLO3629.*

*Cultures were grown for 10 h at 37°C and assayed in triplicate for β-galactosidase activity, reported as nmol ONPG hydrolyzed min⁻¹ ml⁻¹. Differences in activity compared to the pACYC184 control were found to be statistically significant, with worst case values of p ≤ 0.04.*
β-galactosidase levels. This result is consistent with those reported by Passador et al. (1993) who observed a 40-fold increase in expression of a lasB::lacZ translational fusion in E. coli in the presence of lasR by addition of exogenous autoinducer.

The failure to activate the lasA promoter in E. coli indicates that another P. aeruginosa factor may be required for LasR-mediated induction. Attempts were made to clone the additional factor from a P. aeruginosa PAO1 genomic library using the indicator strain DH5α/pLO2856/pLO3629. A positive clone would be expected to impart a lac+ phenotype on DH5α/pLO2856/pLO3629 by the ability of the cloned gene product to interact with LasR or E. coli RNA polymerase and activate transcription from the lasA promoter. However, no lac+ colonies were obtained following triparental mating of two different PAO1 libraries (EcoRI in cosmid pCP13 and HindIII in cosmid pCP13) with DH5α/pLO2856/pLO3629 and subsequent screening of approximately 30,000 Ap r Cm r Tc r colonies on antibiotic plates containing X-gal. The expected probability of identifying any given gene in the PAO1 libraries used in this study is >99.9% (A. Darzins, personal communication). It is therefore very likely that the gene for the putative activator was adequately represented.
Purification of LasR and PAI.

Overexpression of lasR was accomplished using the T7 expression vector, pET-3a. The lasR clone, pLO2841, was used as a template for PCR with oligonucleotides LR429 and LR1148 to amplify the region from the start codon at bp 429 to the stop codon at bp 1148 of the published sequence of lasR (Gambello and Iglewski, 1991). The PCR product was digested with SmaI and XbaI and cloned into pUC18 that had been digested with the same restriction enzymes. The resulting plasmid, pLO3573, was confirmed to contain the correct structure by DNA sequencing. Since a PCR-generated construct would be used for overexpression of lasR, it is important to confirm that no mutations were introduced during PCR that would cause the purified recombinant LasR to be inherently non-functional. The lasR PCR product was taken from pLO3573 as an EcoRI-HindIII fragment and cloned into the broad host range vector pUCP19 to create pLO3600. For comparison, lasR and lasR lasI cloned fragments were also placed into pUCP18 to create pLO3427 and pLO3425, respectively. All three plasmids were transformed into PAO-LR. Transformants were analyzed on LB-Cb-elastin plates for zones of elastin hydrolysis (Table 9). Since zone sizes on elastin plates are significantly smaller than on elastin-congo red plates, significant differences are more difficult to determine. The vector control, PAO-LR/pUCP18 was unable to digest elastin fibers after 48 h incubation. However, the lasR PCR product in pLO3600 was clearly able to complement the elastolytic phenotype of PAO-LR just as well as the lasR-containing fragments derived from the lasR
Table 9. Complementation of PAO-LR with cloned and PCR-generated \( \textit{lasR} \) fragments.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Multicopy gene (^a)</th>
<th>( P_{lac} ) Orientation (^b)</th>
<th>Zone Size (^c) (mm from edge)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAO1/pUCP18</td>
<td>none</td>
<td>NA</td>
<td>2.0</td>
</tr>
<tr>
<td>PAO-LR/pUCP18</td>
<td>none</td>
<td>NA</td>
<td>0.0</td>
</tr>
<tr>
<td>PAO-LR/pLO3427</td>
<td>( \textit{lasR} )</td>
<td>[-]</td>
<td>0.5</td>
</tr>
<tr>
<td>PAO-LR/pLO3425</td>
<td>( \textit{lasR lasI} )</td>
<td>[-]</td>
<td>1.5</td>
</tr>
<tr>
<td>PAO-LR/pLO3600</td>
<td>( \textit{lasR PCR} )</td>
<td>[+]</td>
<td>1.0</td>
</tr>
</tbody>
</table>

\(^a\) Gene contained in the polylinker of pUCP18.

\(^b\) Orientation of the \( \textit{lasR} \) promoter to the \( \textit{lacZ} \) promoter, where [+] designates the same orientation, and [-] designates the opposite orientation.

\(^c\) Measured after 48 h at 37\(^\circ\)C.
genomic clone. Thus, in theory, overexpression of the PCR-amplified lasR gene should lead to the production of active LasR protein.

The lasR insert of pLO3600 was liberated by digestion with AseI and BamHI and transferred into pET-3a that had been digested with NdeI and BamHI. The resulting plasmid, pLO3575, contains an in-frame fusion at the first codon (Met) of lasR to the first codon of the T7 φ10 protein of pET-3a (Figure 11). E. coli strain BL21 (DE3) was transformed with pLO3575, and whole cell lysates were examined for overproduction of a 27 kDa polypeptide by SDS-PAGE. Since BL21 (DE3) exhibits leaky expression of T7 RNA polymerase, induction with IPTG was not required. When various cellular fractions were analyzed by SDS-PAGE, an abundant polypeptide with an Mr of approximately 27 kDa was observed in the insoluble fraction of BL21 (DE3)/pLO3575 but not BL21 (DE3)/pET3a (Figure 12, lanes 3-4). Microscopic examination of the two strains demonstrated the presence of refractile inclusion bodies in BL21 (DE3)/pLO3575 that were absent in BL21 (DE3)/pET3a. Purified inclusion bodies were electrophoresed on an SDS-polyacrylamide gel and transferred to PVDF membrane. The membrane was stained with Coomassie Blue R-250 and the 27 kDa band excised from the membrane. N-terminal protein sequencing of the 27 kDa product revealed the following amino acid residues: A-L-V-D-G. This sequence matches the deduced N-terminal sequence of LasR (MALVDG) except for the missing methionine as the first amino acid (Gambello and Iglewski, 1991). Taken together, the results
**Figure 11.** Plasmid construct for T7 RNA polymerase-mediated **overexpression of lasR.** The DNA sequence at the pET-3a::lasR junction is shown beneath the plasmid map. The NdeI/AseI junction is underlined and labelled. The ribosome binding site (RBS) of the \(\phi 10\) gene is shown in underlined italics. The ATG of lasR is labelled. In the map of plasmid pLO3575, the \(\beta\)-lactamase gene (Ap, thin arrow) and origin of replication (ori) are labelled. The thick arrow represents the lasR open reading frame. The stippled box represents the T7 promoter and \(\phi 10\) RBS. The pET-3a::lasR junction is marked by *. The thin filled box represents T7 transcription terminators that may help stabilize messages.
Figure 12. Overproduction of LasR in BL21 (DE3) and localization in inclusion bodies. Cellular fractions of BL21 (DE3) containing either pET-3a or pLO3575 were analyzed by SDS-PAGE on a 15% gel stained with Coomassie Blue R-250. Sizes of the molecular weight standards are shown to the right. The arrow marks the location of the overexpressed 27 kD polypeptide. Lanes: 1, pET-3a whole cell lysate; 2, pLO3575 whole cell lysate; 3, pET-3a insoluble fraction; 4, pLO3575 insoluble fraction; 5, MW standard; 6, pET-3a cell-free extract; 7, pLO3575 cell-free extract; 8, pET-3a membrane wash; 9, pLO3575 membrane wash.
indicated that LasR partitions into insoluble inclusion bodies when overexpressed in *E. coli* BL21 (DE3).

Conditions for overproduction of LasR and for the purification, solubilization, and renaturation of inclusion bodies were optimized for both yield and purity. However, significant amounts of LasR were lost during solubilization in 8 M urea and in the renaturation process. During removal of the denaturant, a large fraction of the protein presumably assumes incorrect secondary structures that precipitate from solution. The final yield of soluble LasR in 1 M urea was approximately 10.6 μg per ml of cell culture. This protein was to be used in *in vitro* transcription reactions and electrophoretic mobility shift assays to study LasR function.

Since LasR requires autoinducer for activation, PAI was partially purified from a PAO1 culture supernatant. An ethyl acetate extract should contain the various *N*-acyl derivatives of homoserine lactone which have been found in *P. aeruginosa* culture supernatants, such as PAI [N-(3-oxododecanoyl) homoserine lactone], AAI [N-(3-oxooctanoyl) homoserine lactone (Pearson et al., 1994), VAI [N-(3-oxohexanoyl) homoserine lactone (Bainton et al., 1992), and factor 2 (N-butyrylhomoserine lactone (Pearson et al., 1995). The extract was tested for autoinducer activity in a bioassay using PAO-LI/pLO2856 (*lasA::lacZ*). Since PAO-LI harbors a defective *lasI* gene, supplying autoinducer exogenously should activate transcription of the *lasA::lacZ* reporter fusion. Addition of increasing amounts of the ethyl acetate extract to the bioassay resulted in activation of *lasA* expression in a dose-dependent and saturable manner (Figure 13). Maximal induction of the bioassay strain by
Figure 13. Autoinducer activity of an ethylacetate extract of PAO1 culture supernatant. The extract was diluted 100-fold for use in the bioassay with PAO-LI/pLO2856. Various volumes of the diluted extract were added to 2 ml of the bioassay strain, which was then grown for 5 h at 37°C and assayed for β-galactosidase activity. Assays were done in triplicate.
exogenously added extract resulted in 7.5-fold higher levels of β-galactosidase activity compared to the bioassay strain in the absence of exogenous extract.

The LasR inclusion body preparation was used for the production of polyclonal antiserum in rabbits. In immunoblot analysis, the antiserum recognized LasR inclusion bodies at a 1:5000 dilution, while the pre-immune serum from the same rabbit did not react at any dilution tested (data not shown). When antiserum was used to detect LasR in cell-free extracts of *P. aeruginosa*, only two protein bands were recognized by anti-LasR antiserum that were not recognized by the pre-immune serum (data not shown). One highly cross-reactive protein was too small to be full-length LasR (Figure 14), and the identity of this protein has not been determined. Only after prolonged exposure was a faint band observed comigrating with LasR inclusion body. This indicates that LasR is probably present at very low levels in cell-free extracts of PAO1 (Figure 14, lane 3). A 27 kDa protein was also detected in PAO-LR cell-free extracts (Figure 14, lane 4). This may be due to cross-reactivity of anti-LasR antibodies with RhlR, a second LuxR homolog of *P. aeruginosa* (Ochsner *et al.*, 1994). An *rhlR* mutant, strain 65E12 (kind gift of J. Reiser), also produced an anti-LasR reactive 27 kDa protein, as did the parent strain, PG201 (Figure 14, lanes 5-6). Thus, in the *lasR* and *rhlR* mutant strains the antiserum may be recognizing the homologous protein, RhlR and LasR, respectively, while the parent strains produce both of the homologs. Since RhlR has not been purified, and numerous attempts to
Figure 14. Immunoblot analysis of *P. aeruginosa* cell extracts with anti-LasR antiserum. Antiserum was used at a 1:1000 dilution, and bound antibodies were detected with $^{125}$I-Protein A. The blot was exposed for 6 days at -70°C. The autoradiogram was digitized and the background of the image was subtracted using NIH Image software (National Institutes of Health, Bethesda, MD). Lines in lane 2 represent the locations of the prestained molecular weight standards when the autoradiogram was lined up with the immunoblot. The arrows represent the location of the 2 proteins specifically recognized by anti-LasR antiserum, with * marking the 27 kD protein. Lanes: 1, LasR inclusion bodies; 2, MW standard; 3, 50 µg PAO1 cell-free extract; 4, 50 µg PAO-LR cell-free extract; 5, PG201 whole cell lysate; 6, 65E12 (*rhlR*) whole cell lysate.
Figure 14.
generate a double lasR rhlR mutant were unsuccessful, this issue could not be resolved.

A large degree of variability in the levels of the 27 kDa protein in different cell-free extracts was observed and seemed to depend on the amount of membrane contamination. It is interesting to note that whole cell lysates (lanes 5-6, Figure 14) contain higher levels of the 27 kDa protein than cell-free extracts. While cell fractionation studies have not been attempted, this observation indicates that LasR may be membrane associated, as has been demonstrated for LuxR (Kolibachuk and Greenberg, 1993).

**Discussion**

The goals of this project were to examine the regulation of the lasA gene of *P. aeruginosa*. Since lasA has been reported to be regulated by an autoinducer-activated regulatory protein, LasR, in response to cell density, an initial aim was to more closely examine the effects of LasR on lasA expression. The lasR deletion mutant of PAO1, designated PAO-LR, was found to produce low levels of elastolytic activity, especially during later stages of growth. It has been previously reported that a lasR mutant of *P. aeruginosa* is able to express a lasB::lacZ reporter fusion very inefficiently, producing only 0.3% parental levels of β-galactosidase (Passador et al., 1993). Expression of lasB, therefore, appears to be very dependent on the presence of a functional lasR gene. The finding that PAO-LR produced nearly 20% parental levels of β-galactosidase
from a lasA::lacZ reporter fusion suggested that while LasR is important for maximal expression of lasA, the lasA gene is expressed in the absence of functional LasR. This result also raises important questions concerning the cause of the residual elastolytic activity of PAO-LR and potential differences in the regulation of lasA and lasB expression.

Induction of lasA expression in PAO-LR preceded the production of low level elastolytic activity by several hours (Figures 5 and 6). Since, by itself, LasA is not an efficient elastase and requires the presence of other proteases for efficient elastin degradation, this gap between lasA expression and elastolysis may be due to the absence of proteases for LasA-enhanced elastolysis in the culture supernatant until later in stationary phase. Expression of a lasA::lacZ promoter fusion in PAO-LR was still induced at the log to stationary phase transition, while the vector control produced the same low level of β-galactosidase activity throughout the growth curve (Figure 10). This result may indicate the interaction of another cell density-responsive regulator at the lasA promoter. The clinical isolate PA103 consistently exhibited lower levels than PAO-LR of elastolytic activity and lasA expression, yet also exhibited cell density dependent induction of lasA expression. Thus, PA103 may contain a mutation in another gene, in addition to lasR, that has a small effect on lasA expression. Since PA103 is also considered an exotoxin A overproducer, the clinical strain probably contains multiple regulatory defects (Liu, 1973).
The significant amount of residual elastolytic activity produced by PAO-LR on solid media is in disagreement with the reported activity of a previously characterized mutant, PAO-R1 (Gambello and Iglewski, 1991). This mutant contains the same chromosomal deletion as PAO-LR yet has been previously characterized as non-elastolytic, and reported to produce zones of elastolysis comparable to a lasA lasB double mutant (Gambello and Iglewski, 1991; Toder et al., 1991). These investigators used Nutrient Agar based-elastin congo red (NA-ECR) plates to determine elastolytic activity. Even when analyzed on NA-ECR, PAO-LR retained near WT levels after 48 h of incubation (data not shown). Thus, PAO-LR appears to retain higher levels of elastolytic activity on plates than in liquid culture. In addition, the elastolytic activity of lasB mutants on plates is abolished by an additional mutation in lasA, which has no effect on elastolytic activity in culture supernatants (Appendix, Table 12; Toder et al., 1994). Taken together, these results indicate that the in vivo effect of LasA on elastin degradation is more pronounced on solid surfaces, and may be due to enhanced expression of lasA by a LasR-independent mechanism. Differential gene expression on solid surfaces has been shown to occur for pilT of P. aeruginosa, a gene involved in pili assembly and twitching motility, by an unknown mechanism (P. Truax, personal communication).

Two other mutants constructed in this study, PAO-LI and PAO-LRI, both produced comparable results to PAO-LR in elastolytic activity and lasA expression. A defined mutant in lasI has not been previously described. If LasR were able to respond to an autoinducer molecule produced by a lasI-independent reaction for activation of protease expression, a lasI mutant would
be expected to produce higher levels of elastolytic activity than a lasR mutant. However, activation of LasR in PAO-LI by other autoinducer molecules, such as factor 2 (Pearson et al., 1995), did not result in significant activation of target gene expression in the assay systems examined. Likewise, PAI produced by lasI in PAO-LR was not able to significantly enhance activation of protease expression by other regulatory proteins, such as RhlR (Ochsner et al., 1994). However, the presence of lasI in E. coli did result in a small (2X) induction of lasA expression in the absence of lasR (Table 8). In this system, lasI in pLO3626 is present in multiple copies and overexpressed from the constitutive Tc promoter of pACYC184. Thus, the induction observed may be due to a high level of LasI which results in the production of high levels of PAI. At higher levels, PAI may be able to induce other autoinducer-responsive regulators, such as RhlR, that actually possess a higher affinity for their cognate autoinducer molecules, to activate expression of lasA.

In E. coli, the presence of both lasR and lasI resulted in inefficient induction of the lasA promoter, with three-fold higher levels of β-galactosidase activity than when neither lasR or lasI were present, but very efficient induction of the lasB promoter, with 80-fold higher β-galactosidase levels. Since LasR and LasI appear to be sufficient for induction of the lasB promoter, but not for the lasA promoter, additional factors are clearly involved in lasA expression in P. aeruginosa. In addition, while LasR-mediated induction of lasB in the system is significant, the overall level of β-galactosidase activity, and
thus lasB promoter strength, is still approximately 26-fold lower than that obtained in P. aeruginosa PAO1.

This result is in contrast to the result of Passador et al. (1993) who observed only two-fold lower β-galactosidase levels in E. coli. The lasB::lacZ fusion constructed by these investigators is a translational fusion, thus the difference observed could reflect translational effects seen in their fusion that are not seen with pLO3711. In addition, in pLO3629 lasI is under control of its own promoter, so the endogenously produced autoinducer may be present at lower concentrations than the exogenous autoinducer used by Passador et al. (1993). These investigators used culture supernatants from E. coli containing lasI under control of the lac promoter as their source of autoinducer. Thus, the conditions of their system may result in higher levels of PAI leading to artificially high levels of β-galactosidase activity in E. coli. The implication of the relatively poor expression of lasB in E. coli described here is that, even though LasR and PAI are sufficient for expression of lasB, maximal expression of lasB may require the presence of additional P. aeruginosa factors that are missing in E. coli. However, differences between E. coli and P. aeruginosa RNA polymerases, especially in the sigma subunits, may lead to inefficient activation of E. coli RNA polymerase by LasR, resulting in lower level expression.

All attempts at cloning additional trans-acting factors required for induction of lasA expression in E. coli failed. This experiment may have been unsuccessful for a number of reasons. First, the gene encoding an additional
factor may contain both EcoRI and HindIII sites. It may, therefore, be
necessary to use a PAO1 chromosomal library created with different
restriction enzymes. Second, it is conceivable that the cosmid clone harboring
the activating gene may encode a gene product that is toxic for \textit{E. coli}. Third,
the promoter of the cloned gene may itself be inactive in \textit{E. coli}, requiring a \textit{P. aeruginosa} factor(s) not found in \textit{E. coli}. If this were true, expression of the
cloned gene would only occur when the DNA fragment containing the gene is
positioned close enough to, and in the same orientation as, a promoter on the
cosmid vector. Lastly, more than one locus may be required for activation of
LasR-mediated induction of \textit{lasA} transcription. With either of the last two
possibilities, the chances for getting the correct configuration of cloned genes in
a single cosmid are very low, making this approach to cloning the additional
factor(s) required for \textit{lasA} promoter function extremely difficult.

Typically, the identification of factors involved in protease production
have been based on the complementation of clinical isolates that are deficient
in proteolytic or elastolytic activity. To date, only three regulators able to
affect protease production in \textit{P. aeruginosa} have been identified: LasR, RhlR,
and Vfr. The large and increasing number of proteases produced by \textit{P. aeruginosa}, all of which may be independently regulated, makes an approach
based on restoration of proteolytic activity difficult for the identification of loci
involved in regulation of a specific protease, such as \textit{LasA}. Transposon
mutagenesis of strain PAO1 containing either a plasmid-borne or chromosomal
\textit{lasA::lacZ} transcriptional fusion should result in the identification of mutants
exhibiting decreased \textit{lasA} expression by screening on media containing X-gal.
Interrupted genes can be cloned by marker rescue and screened with the cloned genes for known regulators: lasR, lasI, rhlR, rhlI, and ufr. This approach should identify novel loci involved in regulation of lasA, one of which may allow transcription of lasA in E. coli.

The ethyl-acetate extract of PAO1 culture supernatants was able to restore expression of the lasA::lacZ fusion to PAO-LI in a dose-dependent manner (Figure 13). Saturation of this response resulted in levels of β-galactosidase activity that were typically lower than the levels produced by PAO1. Different growth conditions in the PAI bioassay may account for this difference. A maximal induction of 7.5-fold occurred at saturation. This is significantly lower than the 57-fold induction by PAI reported for lasB in E. coli (Pearson et al., 1994). These investigators reported that ethyl-acetate extracts induce lasB expression to the same level as purified PAI, so the use of an extract is probably not causing inhibition of lasA expression, but this possibility cannot be excluded. The difference in induction levels also may be caused by differences in response at the lasA and lasB promoters. However, the most likely explanation is that in E. coli lasR is present on a multicopy plasmid, so saturation with exogenous autoinducer results in higher level LasR-mediated expression of target genes. A bioassay in the native environment of PAO-LI, where lasR is present as a single chromosomal copy, more accurately represents the effect of autoinducer on gene expression.

Numerous difficulties were encountered with the polyclonal anti-LasR antiserum. The most perplexing was the inability to demonstrate loss in
PAO-LR of a 27 kDa protein recognized by the antiserum (Figure 14). This problem may be caused by the presence of RhlR in PAO-LR cells. While the overall amino acid sequences of LasR and RhlR are only 31% identical, certain domains are more highly conserved. Studies on RhlR have been performed in strain PG201, which does contain a functional lasR gene (U. Ochsner, personal communication). However, attempts to generate a double lasR rhlR mutant in PG201 were complicated by the small zone of elastolytic activity produced by this strain on solid media, making phenotypic screening of potential mutants difficult (data not shown). In addition, numerous attempts to delete rhlR in PAO1 by gene replacement were unsuccessful. Thus, a double lasR rhlR mutant could not be generated to resolve problems encountered with single mutants and determine the quality of the antiserum.

LasR was overproduced in E. coli, where it formed insoluble inclusion bodies, and purified away from contaminating proteins. The original motive behind purification of LasR was to use the soluble, renatured LasR in in vitro systems, as has been accomplished with ToxR of P. aeruginosa in transcription assays (Walker et al., 1994) and EMSA (Walker et al., in preparation). However, no in vitro activity could be demonstrated with purified LasR in either in vitro transcription reactions with the lasB promoter (data not shown), or in EMSA with either the lasA or lasB promoters (see Chapter IV). By analogy to the LuxR system, several factors may be responsible for this lack of activity. The molecular chaperones GroESL, which are present in vivo in E. coli, may be required for proper interaction of LasR and PAI. Activation of LuxR appears to involve non-covalent interaction with autoinducer in the N-
terminal domain to unmask the C-terminal transcriptional activator domain and allow function of the regulatory protein (Choi and Greenberg, 1991). Thus, an in vitro system may require the proper stoichiometric ratios of LasR, PAI, and GroESL to achieve a non-covalent association resulting in activation of LasR. Transcriptional activators that are activated by covalent modification, such as the classical two-component response regulators, or that do not require activation, such as ToxR, may be easier to work with in vitro.
CHAPTER IV
The Importance of cis-Acting Factors in Expression of the lasA Gene

Introduction

Gene expression involves several fundamental elements that are common to all genes, such as a promoter, which is bound by RNA Polymerase to initiate transcription, and a ribosome binding site for translation initiation. In addition, expression of many genes is regulated by increasing or decreasing the efficiency of transcription or translation, and often, many regulatory processes are involved in expression of a single gene. Which regulatory events occur at which genes is controlled solely by the DNA sequence of the genes themselves, based on the presence or absence of sequence or structural motifs that are recognized by DNA-binding proteins. Once bound, the DNA-binding proteins, either directly or through interactions with other proteins, effect the positive or negative regulatory events. Thus, while it is common to think of the main function of DNA as coding for the production of proteins, without the correct motifs in the DNA or RNA, the regulated production of proteins is lost.

Therefore, in addition to examining the effects of accessory proteins, such as LasR, which regulate expression of lasA and investigating the mechanism of action of these regulatory proteins, another very important
aspect in understanding lasA regulation is the definition of the lasA DNA sequences involved in various regulatory processes. The goals of this study were:

1. to determine the transcription initiation site of lasA and the DNA sequence of the upstream region in order to define the lasA promoter and other potential sequence motifs that may be involved in lasA regulation,
2. to delineate the lasA regulatory region by examining the transcriptional activity of various lasA upstream gene fragments fused to a lacZ reporter gene, and
3. to determine whether any regions of lasA are bound by sequence-specific DNA-binding proteins in P. aeruginosa extracts.

Results

Mapping the transcription initiation site of lasA.

Determining the transcription start point of a particular gene is often simplified by knowing the N-terminal amino acid of the gene product. The amino terminus of the precursor form of LasA, however, has not been determined experimentally. Two different start codons separated by approximately 100 bp have been proposed based on independent determinations of lasA gene sequence, size and pI of purified, mature LasA, and size of the unprocessed form expressed in E. coli (Schad and Iglewski, 1988; Darzins et al., 1990). This made it difficult to initially predict an
approximate location for the transcription start site. For this reason the transcription initiation point was first localized by low resolution S1 analysis using end-labelled restriction fragments and comparison to known size standards (data not shown). A single S1-protected fragment was observed for all probes, and the size of this fragment was the same for both a SalI-XhoI and SmaI-XhoI probe, indicating that the start site was located downstream of the unique SmaI site located at bp 631 (Figure 15). Low resolution S1 analysis localized the lasA transcription initiation site to a region approximately 25 to 60 bp downstream of the SmaI site. This region is approximately 110 nt upstream of the proposed ATG start of Schad and Iglewski (1988) and 250 nt upstream of the proposed TTG start of Darzins et al. (1990). To avoid ambiguity, start points and other reaction products have been localized with respect to this site rather than to potential start codons.

The lasA sequences previously reported begin at the SmaI site (Schad and Iglewski, 1988; Darzins et al., 1990). Since the transcription start point was localized to a region only 25 to 60 bp downstream of the SmaI site, a σ^{70} promoter, if present, could be split by the SmaI site between the -10 and -35 elements. In addition, transcriptional activators, such as LasR, are expected to bind upstream of the promoter between -200 and -20 (Collado-Vides et al., 1991). Therefore, the nucleotide sequence upstream of the SmaI site had to be determined. To generate the sequence shown in Figure 15, overlapping subclones were constructed, spanning the region from the SalI site used to
**Figure 15.** Sequence of the regulatory region of *lasA*. Overlapping subclones spanning the region from *SalI* (bp 1) to *Sau3AI* (bp 785) were sequenced on both strands. The sequence of the sense strand of *lasA* is shown. The two potential initiation codons of the precursor form of LasA are underlined and labelled Met (Schad and Iglewski, 1988) and Leu (Darzins *et al.*, 1990). Oligonucleotides used for S1 nuclease (LA755) or primer extension (LA724) mapping studies are complementary to the sense strand in the regions underlined and labelled LA755 and LA724. The transcription initiation site is underlined and labelled +1. A putative σ^70^ promoter is boxed and labelled -10 and -35. The 5' ends of smaller S1-protected fragments are double-underlined. The location of the potential stem-loop and pseudoknot of the *lasA* mRNA are shown by the shaded boxes.
Figure 15.
clone \textit{lasA} (Darzins \textit{et al.}, 1990) to the \textit{Sau3AI} site at bp 785, and sequenced on both strands.

From the low resolution S1 results, an oligonucleotide primer (LA755, 5'-CGAAGGGAAACCTTGAAAGC, Figure 15), complementary to the sense strand approximately 75 bp from the mapped start point, was synthesized for use in high resolution S1 analysis. A \textit{SmaI}-\textit{XhoI} subclone of \textit{lasA}, pLO1831, served as template for extension from LA755 to produce a continuously-labelled probe. The results from high resolution S1 analysis of PAO1 RNA with the synthesized probe are shown in Figure 16. The two largest S1-protected fragments are indicated by arrowheads (lanes 1 and 2) and correspond to two 5' ends of the RNA located at bp 657 and bp 659 and labelled +1 in Figure 15. The bracket in Figure 16 denotes a ladder of smaller S1-protected fragments with 5' ends that correspond to bp 672 to 675, which are double-underlined in Figure 15.

Multiple attempts at primer extension analysis using LA755 were unsuccessful, and reaction products always included a ladder of products ending within the first 20 nt from the 3' end of LA755 (data not shown). One possible explanation for this is that secondary or tertiary structures in the RNA were preventing reverse transcriptase from extending to the 5' end, resulting in inefficient polymerization and premature termination. To address this possibility, the sequence from bp 657 to bp 757 of Figure 15 was analyzed for secondary structure using the method of Jaeger \textit{et al.} (1989). A potential stem-loop structure with a favorable free energy of -18 kcal was identified (Figure 17A). This alone, however, may not be a strong enough structure to
Figure 16. High resolution S1 nuclease mapping of the *lasA* transcription initiation site. The sequence of the sense strand is shown to the left, with the 5′ ends of reaction products underlined. Arrowheads mark the position of the largest S1-protected fragments, and the bracket marks the position of a ladder of smaller protected fragments. Lanes: A, C, G, T are the Sanger sequencing reactions of the antisense strand generated from pLO1831 and LA755; 1-3, S1 protection reactions with 50 μg, 100 μg, and 0 μg PAO1 RNA, respectively.
Figure 16.
prevent reverse transcriptase from extending from LA755 through the region. Analysis of single stranded regions of the RNA for sequences complementary to nucleotides within the loop revealed the sequence 5' - CUGG (+16 to +19), able to base pair with the sequence 5' - CCAG (+41 to +44) found in the loop. These bases are double-underlined in Figure 17A. Tertiary structures in RNA molecules due to interactions between loops and single stranded regions have been described (Studnicka et al., 1978). These structures, known as pseudoknots, are hairpin loops with a single strand folded back to pair with the bases in the loop, and generally involve less than 5 nt so that the single strand does not pass completely through the loop. Figure 17B is a graphic representation of the potential pseudoknot structure of the lasA mRNA. These structures are stabilized by stacking of the two double helical segments formed by paired nucleotides in the stem and the pseudoknot (Pleij et al., 1985). Notably, the 5' ends of the ladder of smaller S1-protected fragments observed in Figure 16 are located in the same region as the bases in the pseudoknot as seen in Figure 15. It is possible, therefore, that pseudoknot formation allowed the probe to be susceptible in that region to digestion by S1 nuclease. The presence of this pseudoknot has not, however, been confirmed experimentally.

A second oligonucleotide primer (LA724, 5' - CGACATCGCCTCCGAGGG, Figure 15) was synthesized which should destabilize these structures on the RNA, as shown in Figure 17A. Primer extension analysis of PAO1 RNA using LA724 confirmed the predicted transcription initiation site (Figure 18). The two major extended products indicated by arrowheads (lane 1) corresponded exactly to the 5' ends of the two larger protected fragments
Figure 17. Potential secondary and tertiary structures of the *lasA* leader RNA. (A.) Sequence of the *lasA* mRNA within 80 nt of the 5' end showing the potential stem-loop structure. Watson-Crick base pairs are shown by :, while -- denotes a non-Watson-Crick interaction. Underlined nt in the loop can base hybridize with the double-underlined nucleotides to form the indicated pseudoknot interactions. The free energy of the stem-loop structure is also shown. The double and thick overlines represent the regions of the mRNA shown that would hybridize to oligonucleotides LA755 and LA724, respectively. (B.) Graphic representation of the potential *lasA* pseudoknot. The bottom structure is stabilized by stacking of the two double helices, as indicated by the direction of the equilibrium.
A.

\[
\begin{array}{c}
A \quad C \quad C \\
G \\
G \\
C: :G \\
G :G \\
A :U \\
| \\
G :C \\
G :C \\
C :G \\
C :G \\
C :G \\
\end{array}
\]

\[\Delta G = -18 \text{ kcal}\]

\[\text{pseudoknot interactions:}\]

5' CCAG 3'

3' GGUC 5'

B.

\[\text{Figure 17.}\]
**Figure 18.** Primer extension mapping of the *lasA* transcription initiation site. The sequence of the sense strand is shown to the left, with the 5' ends of reaction products underlined. Arrowheads mark the position of the prominent extension products. Lanes: A, C, G, T are the Sanger sequencing reactions of the antisense strand generated from pL01831 and LA724; 1-2, primer extension reactions with 50 µg and 0 µg PAO1 RNA, respectively.
Figure 18.
determined by S1 mapping (Figure 16). No additional products were seen that mapped to the same region as the smaller S1-protected fragments. Therefore, transcription of the lasA gene appears to be initiated at two different nt located at bp 657 and 659 (Figure 15).

**Analysis of the lasA promoter region.**

The region upstream of the transcription start point of lasA was examined for potential $\sigma^{70}$ promoter sequences by comparison to other Pseudomonas promoters. Deretic et al. (1989) classified twelve Pseudomonas promoters as $\sigma^{70}$ (rpoD) promoters. From these promoters a consensus sequence was derived which is as follows: TTGACR (-35 region) and TATRRT (-10 region), where R represents C or T and the most invariant bases are underlined. The region upstream of the transcription start site of lasA was found to contain an appropriately positioned sequence homologous to the consensus promoter labelled -10 and -35 in Figure 15. The lasA sequence was also compared to a recent compilation of 29 $\sigma^{70}$ promoters from streptomycetes (Strohl, 1992), which, like pseudomonads, contain DNA with a high G+C content. The results of this analysis also identified the -10 and -35 regions shown in Figure 15.

Since lasA and lasB are co-regulated in some manner by LasR, a comparison of the two promoter regions may identify regions of homology to which LasR or other regulatory proteins may bind. The lasB promoter has been classified as a *P. aeruginosa* virulence promoter by Deretic et al. (1989).
This designation was based on the lack of homology to other known promoters and the role of this gene in pathogenesis. These same investigators also noted limited homology of the lasB promoter region to some members of the σ\(^{54}\) class of promoters. However, a σ\(^{54}\) (rpoN) mutant of P. aeruginosa strain PAK produces parental levels of elastase (Totten et al., 1990). Thus, transcription of lasB is probably not initiated by an RpoN-containing RNA polymerase. Taking into account, however, that lasA and lasB are co-regulated by LasR, and that a putative σ\(^{70}\) promoter was identified upstream of the lasA transcriptional start site, it was logical to examine the lasB sequences for a potential σ\(^{70}\) promoter. The nucleotide sequence and transcription initiation point of lasB have been previously reported (Bever and Iglewski, 1988; Mohr et al., 1990). Inspection of the sequence upstream of the lasB transcription initiation site for homology to the P. aeruginosa σ\(^{70}\) consensus promoter identified an appropriately positioned sequence which is labelled -10 and -35 in Figure 19A.

The promoter region of lasA was then aligned with the sequence upstream of the lasB transcription start site to produce the best homology within 150 bp of +1. Two regions of considerable homology between the aligned sequences were found and are referred to as Box I and Box II (Figure 19A). Box I displays 75% homology and is centered around -86 of lasA and -111 of lasB. Box II is 69% homologous and is centered around -51 of lasA and -71 of lasB. Fuqua et al. (1994) have generated a lux box consensus sequence based on
**Figure 19. Homologies of the lasA regulatory region.** Homologous bases are shown by I. Gaps introduced to generate the best fit are denoted by --.

(A.) Homology to the lasB regulatory region. Regions of high homology are boxed and labelled Box I (thick dashed box) and Box II (shaded box). Putative $\sigma^{70}$ promoters are double-underlined and labelled -10 and -35. Transcription start sites are underlined and labelled +1.

(B.) Alignment of the lux boxes of lasB and lasA to the functionally defined lux operator of luxI. Sequence characteristics are marked as in (A). The lux boxes are unshaded, except where the lasA lux box overlaps with Box II. The overlapping region is outlined with a dashed box.
Figure 19.
homology between the functionally defined \textit{lux} operator of \textit{V. fischeri} and the target promoters of LuxR homologs. These investigators have identified a \textit{lux} box adjacent to the -35 of \textit{lasB}, as seen in Figure 19B. When examined for homology to the improved consensus sequence, the \textit{lasA} regulatory region also contained an appropriately positioned region with homology to the \textit{lux} box consensus (Figure 19B). This \textit{lux} box overlaps Box II identified by homology to \textit{lasB}. However, many sequences located within the regulatory region displayed better homology to the \textit{lux} box consensus, thereby making it difficult to speculate on the functional significance of any of the \textit{lux} elements without experimental data.

\textbf{Identification of regions required for \textit{lasA} promoter activity.}

Quantitation of \textit{lasA} promoter activity was accomplished using transcriptional fusions in pQF50, a promoter probe vector containing a promoterless \textit{lacZ} gene. The \textit{lacZ} gene contains its own translation initiation sequences, so β-galactosidase activity serves as a reporter of \textit{lasA} transcription initiation at sites fused to the promoterless \textit{lacZ}, independent of any translational regulation of \textit{lasA}. The \textit{SalI}-\textit{XhoI} fragment of \textit{lasA} (Figure 15) in pLO2856, used as the full-length promoter fusion, contains all of the regulatory region available, since the \textit{SalI} site was used to clone \textit{lasA} (Darzins \textit{et al.}, 1990), and a minimal amount of coding region.

The level of β-galactosidase activity produced by PAO1/pLO2856 and PAO1/pQF50 was determined at various stages of growth in LB broth. The
β-galactosidase activity of the control culture remained essentially unchanged over the 12 h growth period (Figure 20). The β-galactosidase levels of PAO1/pLO2856 did not begin to accumulate until the culture reached a certain cell density, at the transition from exponential to stationary phase, as would be expected for a gene controlled by a quorum-sensing system. During the exponential phase, β-galactosidase activity was low and, in fact, decreased. The best explanation for this decrease is that β-galactosidase carried over from the inoculum of an overnight starter culture was responsible for activity seen at low cell densities. Since transcription of lasA was not induced until an O.D.₆₀₀ of approximately 2, the amount of β-galactosidase would remain constant during logarithmic growth, while the cell density increases. This would cause an artificial decrease in β-galactosidase activity, which is calculated by dividing the absorbance of the assay product, ONP, by the optical density of the culture.

In order to identify regions of the lasA gene required for promoter function, a series of 5'-deletions of lasA was generated and cloned into pQF50. The relative amount of β-galactosidase activity for various deletions in strain PAO1 was determined approximately 5 h after entry into stationary phase and compared to pLO2856 as an indication of promoter strength (Figure 21). Deletion up to the XmnI site in pLO2906 resulted in a 50% loss of promoter activity. This was not due to the loss of a promoter upstream of the XmnI site, since the SalI-XmnI fragment alone in pLO3185 contained no promoter
Figure 20. β-Galactosidase activity in PAO1 due to a plasmid-borne lasA::lacZ transcriptional fusion at various stages of growth. Assays were done in triplicate. Similar results were obtained in more than five separate experiments. Open symbols represent growth (O.D.₆₀₀). Filled symbols represent β-galactosidase activity (nmol ONPG hydrolyzed min⁻¹ ml⁻¹). Circles, PAO1/pQF50; squares, PAO1/pLO2856.
activity. To determine if the region upstream of the XmnI site could function like an enhancer in either orientation, as has been reported for the AlgR1 binding site of the *P. aeruginosa* *algC* gene (Fujiwara et al., 1993), the orientation of the *SalI-XmnI* fragment was reversed in pLO3413. Inversion of the *SalI-XmnI* fragment resulted in the loss of 80% of promoter activity, indicating that there was no enhancer-like element contained within the *SalI-XmnI* fragment. The fact that only 50% of *lasA* promoter activity was lost when the *SalI-XmnI* fragment was deleted, but 80% lost when the fragment was inverted may instead indicate the presence of a negative regulatory element on the *SalI-XmnI* fragment that functions better when moved closer to the promoter.

The m41 deletion in pLO3604 contains only 17 bp upstream of the *XmnI* site, yet retained 80% of the promoter activity, compared to 50% retained by pLO2906 (Figure 21). This indicates that perhaps the *XmnI* site interrupts a site involved in promoter function, and that the m41 deletion includes an intact, or nearly intact, site. Figure 22 shows the sequence of the *lasA* promoter region and marks the deletions analyzed, the *lux* box, and Box I and Box II. Based on the above information, it was necessary to determine if any related boxes span the *XmnI* site. Homology searches for sequences related to the *lasA* Box I, Box II, or *lux* box revealed the presence of a site with 70% homology to Box II spanning the *XmnI* site on the opposite strand. This site was named Box II'.

Removal of the region between the m34 deletion in pLO3152 and the *Sau3AI* site of pLO3291 resulted in essentially no change in promoter
Figure 21. Relative β-Galactosidase activity associated with 5′ deletions of the lasA regulatory region in plasmid-borne lacZ transcriptional fusions in PAO1. Plasmid designations are given at the left. Shown are pertinent restriction sites (Sal, SalI; Xmn, XmnI; Sau, Sau3AI; Sma, SmaI; Xho, XhoI) and deletion endpoints of exonuclease III-generated deletions (q5, m42, m41, m34, m75). The arrow labelled P represents the potential lasA promoter. β-Galactosidase assays were done in triplicate after 10 h growth in LB. Plasmid pLO2856 was assayed with every construct, and relative β-galactosidase levels are averages from at least three different experiments. Differences in activity were compared for each subsequent deletion, except pLO3185 and pLO3413, by the students T test. Deletions which resulted in a significant decrease in promoter activity (p ≤ 0.02) are indicated by (+), while (-) indicates that the deletion resulted in no significant difference in promoter activity from the previous construct. The pole markers represent various DNA motifs: shaded square, Box II′ and Box II; open square, Box I; open circle, lux box.
Figure 21.
Figure 22. Locations of DNA motifs and deletions analyzed in the lasA regulatory sequence. Endpoints of subclones and exonuclease III deletions are marked with arrows and the restriction site or deletion name. Identified DNA motifs are boxed and labelled: Box II and Box II', shaded box; Box I, thick dashed box; lux box, unshaded box, or shaded and dashed box where it overlaps with Box II. The potential stem-loop and pseudoknot are double-underlined and labelled.
strength, even though the deleted region contained Box I (Figure 21). This result indicates that the homology between lasA and lasB in Box I is not important for lasA promoter strength in this assay system. Finally, a second major decrease in promoter strength was caused by deletion of only 18 bp between -58 (pLO3291) and -40 (pLO3166). The m75 deletion in pLO3166 contains an intact promoter, but essentially no upstream sequences (Figure 22). The fact that pLO3166 exhibited no promoter activity indicates that at least some sequences other than a -10 and -35 site are required for the lasA promoter to function. The presence of either the lux box or Box II in pLO3291 was sufficient for nearly half the promoter activity. However, since the lux box and Box II overlap in lasA, the two sites could not be separated.

The original assumption that regions identified by high homology between the lasA and lasB promoter regions could serve as binding sites for regulatory proteins also implies that such sites would serve a similar function in regulation of the two genes. Thus, the effect of deletion of Box II in lasB may indicate the importance of this site in lasA promoter activity. Box II was separated from the lux box of lasB by PCR, using oligonucleotides LB135 and LB913 as primers and pLO3710 as template. The PCR product contained nt 135 to 913 of the published lasB sequence (Bever and Iglewski, 1988), where the 3' end at nt 913 is one-half of the unique StuI site of lasB. Using primer-derived restriction sites, the PCR product was cloned into pQF50 to create pLO3768. This construct was compared to two subclones of lasB in pQF50, both of which also contain one-half of the StuI site at the 3' end. An EcoRI-StuI fragment containing the full-length promoter and an AluI-StuI fragment
were cloned into pQF50 to create pLO3711 and pLO3719, respectively (Figure 23 and Table 10). The results in Table 10 indicate that the amount of β-galactosidase activity produced from the fusions was essentially the same regardless of whether both Box II and the \textit{lux} box (pLO3719) or only the \textit{lux} box (pLO3768) were present. Thus Box II does not appear to be important for \textit{lasB} promoter function in this assay system. It is interesting that deletion of the region between \textit{Alul} and \textit{EcoRI} resulted in a 50% loss of promoter activity, as was seen for the \textit{lasA} promoter when the region upstream of the \textit{XmnI} site at -144 was removed. The region was analyzed for sequences related to Box II of \textit{lasA} and \textit{lasB}, and several sites with limited homology (55%) were found. However, since the \textit{lasB} sequence does not extend to the \textit{EcoRI} site, and there is no further evidence for the location of upstream elements, this finding was not pursued.

\textbf{Examination of \textit{P. aeruginosa} extracts for proteins able to bind \textit{lasA} promoter fragments.}

Regions that were identified as having a role in \textit{lasA} promoter function could contain binding sites for potential DNA-binding proteins involved in \textit{lasA} regulation. The electrophoretic mobility shift assay (EMSA) was used to determine whether various regions of the \textit{lasA} promoter could be bound by proteins in \textit{P. aeruginosa} extracts. The most obvious candidate protein for binding to the \textit{lasA} promoter region was \textit{LasR}, so cell-free extracts were prepared from \textit{P. aeruginosa} strains PAO1 and PAO-LR. Since either Box II or
**Figure 23.** Locations of DNA motifs and deletions analyzed in the *lasB* regulatory sequence. Endpoints of subclones and the PCR135 product are marked with arrows and the restriction site or PCR135. Identified DNA motifs are boxed and labelled: Box II, shaded box; Box I, thick dashed box; *lux* box, unshaded box.
Table 10. Promoter activity of lasB::lacZ transcriptional fusions in *P. aeruginosa* PAO1.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Site at 5' End</th>
<th>Distance from +1</th>
<th>β-Gal Activity&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>pLO3711</td>
<td>EcoRI</td>
<td>ca. - 290</td>
<td>198.08 ± 9.47</td>
</tr>
<tr>
<td>pLO3719</td>
<td>AluI</td>
<td>- 96</td>
<td>100.91 ± 3.01</td>
</tr>
<tr>
<td>pLO3768</td>
<td>PCR135</td>
<td>- 57</td>
<td>110.69 ± 4.00</td>
</tr>
<tr>
<td>pQF50</td>
<td>NA</td>
<td>NA</td>
<td>7.93 ± 0.18</td>
</tr>
</tbody>
</table>

<sup>a</sup> Strains were grown for 10 h at 37°C and assayed in triplicate for β-galactosidase activity, reported as nmol ONPG hydrolyzed min<sup>-1</sup> ml<sup>-1</sup>. Differences in the activity of pLO3719 and pLO3768 were compared to the full-length *lasB* promoter fusion, pLO3711, and found to be statistically significant (p ≤ 0.003).
the lux box was responsible for nearly 50% of lasA promoter activity, the Sau3AI-TaqI fragment of lasA was chosen for initial analysis by EMSA. For all EMSA figures, refer to Figure 24 for a restriction map of the lasA gene. Figure 25 shows that both PAO1 and PAO-LR extracts contain a protein, or proteins, that bind to the Sau3AI-TaqI fragment and retard its mobility in the gel producing a distinct band following autoradiography. Both PAO1 and PAO-LR extracts also produced one major shifted complex for two other DNA fragments containing the lasA promoter examined by EMSA, m34-TaqI and m42-RsaI (data not shown). Fragments that were bound by a protein or proteins in the extracts usually produced only one major shifted complex whose mobility was efficiently retarded by the extracts. I will refer to the major shifted complex of each probe as C1, for complex 1, even though different proteins may be causing the shift for different DNA fragments.

To determine whether RNA polymerase was able to bind to these fragments, purified P. aeruginosa RNA polymerase (a gift from S. Walker) was analyzed by EMSA with the m42-RsaI probe (Figure 26). RNA polymerase was able to bind to the m42-RsaI fragment (lanes 3-5), but the Polymerase-probe complex, C2, had a much lower mobility than the major complex, C1, observed with PAO1 extract (lane 2). The addition of both RNA Polymerase and PAO1 extract to the binding reaction inhibited binding of RNA Polymerase (lanes 6-7).

Competition experiments are commonly performed during EMSA analysis of crude protein preparations to determine if the shift complex
Figure 24. Restriction map of the lasA gene. The lasA open reading frame and promoter are shown as the open and solid arrows, respectively. Abbreviations: Sa, SalI; X, XmnI; S, Sau3AI; Sm, SmaI; T, TaqI; R, RsaI; Xh, XhoI; P, PstI; H, HindIII.
Figure 25. Detection of DNA-binding activity to the lasA promoter region in PAO1 and PAO-LR cell-free extracts. The 185 bp Sau3AI-TaqI fragment containing the lasA promoter was purified as a TaqI fragment of pLO3289, end-labelled, and analyzed by EMSA. The arrow labelled P marks the location of the probe fragment, while the arrow labelled C1 marks the major shifted complex. Lanes: 1, no extract; 2, 200 μg PAO1 extract; 3, 200 μg PAO-LR extract.
Figure 26. DNA-binding activity of *P. aeruginosa* RNA polymerase to the *lasA* regulatory region. The 420 bp m42-*RsaI* fragment containing the *lasA* regulatory region sufficient for full promoter activity was purified as an *EcoRI-RsaI* fragment from pLO3176, end-labelled, and analyzed by EMSA with purified *P. aeruginosa* RNA polymerase in the absence or presence of 50 µg PAO1 extract. The arrow labelled P marks the location of the probe fragment, while the arrow labelled C1 marks the major shifted complex, and the arrow labelled C2 marks the RNA polymerase-DNA complex. Lanes: 1, no protein; 2, 50 µg PAO1 extract; 3, 2 µl RNA polymerase; 4, 10 µl RNA polymerase; 5, 20 µl RNA polymerase; 6, 2 µl RNA polymerase + 50 µg PAO1 extract; 7, 10 µl RNA polymerase + 50 µg PAO1 extract.
detected is caused by a sequence-specific interaction between protein and DNA or by a non-specific DNA binding protein. Non-specific competitor DNA, in the form of poly dI-dC, is already present in each binding reaction. Unlabelled restriction fragments of the lasA gene were added, in concentrations up to 200-fold higher than the concentration of the labelled m42-RsaI probe, prior to addition of the PAO1 extract and incubation of the reaction mixture. If the detected DNA-protein complex were due to binding of a transcriptional activator to a binding site upstream of the lasA promoter, then the m42-SmaI fragment, which contains the regulatory region of lasA responsible for full promoter activity but not the intact promoter, should compete effectively, while lasA fragments outside of the regulatory region would not compete. However, Figure 27 clearly shows that while the m42-SmaI fragment did inhibit formation of C1, other lasA fragments, which were not expected to be effective competitors, also efficiently inhibited C1 formation.

The C1 bands in Figure 27 were subjected to densitometry analysis using NIH Image software to determine the percent inhibition of each competitor DNA fragment (Table 11). All of the lasA fragments tested inhibited very efficiently (90% inhibition or better) at a concentration 200X higher than the probe concentration. The m42-XhoI fragment, which is the same fragment found to give full promoter activity in pQF50, also inhibited efficiently at a 20X relative concentration. However, the m42-SmaI fragment, which contains the regulatory region upstream of the lasA promoter, was less efficient as a competitor. In fact, the SmaI-XhoI
Figure 27. Competition analysis of the ability of lasA DNA fragments to inhibit lasA regulatory region DNA-protein complex formation. The 420 bp m42-RsaI fragment was purified as an EcoRI-RsaI fragment from pLO3176, end-labelled, and analyzed by EMSA in the presence of increasing concentrations of competitor DNA fragments. 50 µg of PAO1 extract was present except where indicated. 2, 20, and 200 represent the fold-excess of competitor DNA over probe DNA. (A.) Lanes: 1, no extract, no competitor; 2, no competitor; 3-5, m42-SmaI fragment of lasA; 6-8, PstI fragment of lasA; 9-10, Km\(^r\) gene fragment. (B.) Lanes: 1, no extract, no competitor; 2, no competitor; 3-4, m42-XhoI fragment of lasA; 5-7, SmaI-XhoI fragment of lasA.
Figure 27.
Figure 27 (Continued)

B.

[Diagram showing lanes labeled 1 to 7 with markers C1 and P, and fold excess competitor on the x-axis (0, 20, 200, 2, 20, 200).]
Table 11. Inhibition of lasA promoter DNA-protein complex formation in PAO1 extracts by competitor DNA fragments.

<table>
<thead>
<tr>
<th>Competitor DNA</th>
<th>% Inhibition a</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2:1 b</td>
</tr>
<tr>
<td>m42-XhoI</td>
<td>ND c</td>
</tr>
<tr>
<td>m42-SmaI</td>
<td>0%</td>
</tr>
<tr>
<td>SmaI-XhoI</td>
<td>52%</td>
</tr>
<tr>
<td>PstI-PstI</td>
<td>79%</td>
</tr>
<tr>
<td>Km gene fragment</td>
<td>ND</td>
</tr>
</tbody>
</table>

a Densitometry analysis of autoradiograms in Figure 27 was performed using NIH Image. The areas under the peaks corresponding to the shift complexes were calculated and % inhibition determined versus the control lanes containing no competitor DNA.

b These values refer to the stoichiometry of competitor DNA:probe DNA present in the binding reactions.

c ND - not determined.
fragment, which contains only the -10 of the promoter and the leader RNA of lasA, competed more efficiently than the m42-SmaI fragment, giving 52% inhibition when present at only twice the concentration of the probe. The most efficient lasA fragment, however, was the PstI fragment, located nearly 800 bp downstream of the promoter, which gave nearly 80% inhibition at a 2X relative concentration. Two unrelated DNAs were found to be relatively inefficient competitors. A 430 bp NarI-StyI fragment of the Km\textsuperscript{r} gene of pK19, which contains only coding region, did not inhibit C1 formation at all at a 20X concentration, and only inhibited 62% at 200X. Also, poly dI-dC present in 670X and 6700X excess over probe still only inhibited 31% and 48%, respectively.

The finding that regions of lasA, such as the SmaI-XhoI and PstI fragments, located outside of the promoter/regulatory region efficiently inhibited formation of the C1 complex of the m42-RsaI probe indicates that DNA sequences on these fragments may themselves be recognized and bound by the protein(s) responsible for m42-RsaI binding. Several different DNA fragments were isolated for use as probes in EMSA analysis with PAO1 extract. The m42-SmaI fragment, which was an inefficient competitor, did have its mobility retarded by the presence of PAO1 proteins (Figure 28A), but the efficiency of DNA-protein complex formation was relatively much lower than observed for the m42-RsaI fragment (data not shown). The SmaI-XhoI fragment, which was an efficient competitor, was bound efficiently by a protein or proteins in PAO1 extract to generate one major shifted complex (Figure
**Figure 28.** Detection of DNA-binding activity in extracts of PAO1 to various *lasA* DNA fragments. The major shift complex of each fragment is marked by an arrow labelled C1. Probe bands are marked with an arrow labelled P and a number representing the size of the probe fragment in bp. (A.) m42-SmaI probe. Lanes: 1, no extract; 2-4, 50 μg, 100 μg, and 200 μg PAO1 extract, respectively. (B.) SalI-XmnI (lanes 1-4) and SmaI-XhoI (lanes 6-9) probes. Lanes: 1 and 6, no extract; 2 and 7, 20 μg PAO1 extract; 3 and 8, 50 μg PAO1 extract; 4 and 9, 150 μg PAO1 extract; 5, m42-RsaI probe + 50 μg PAO1 extract.
Figure 28.
Figure 28 (Continued)

B.
The SalI-XmnI fragment of lasA was also efficiently bound, with the formation of one major complex (Figure 28B). Surprisingly, numerous attempts to demonstrate the ability of the PstI fragment, which was an extremely efficient competitor, to be bound by protein in a PAO1 extract were unsuccessful, with only very inefficient complex formation observed (data not shown). In addition, two other DNA fragments from the lasA coding region, the XhoI-PstI and PstI-HindIII fragments, were not bound at all by extract proteins (data not shown).

Discussion

Another major goal of this study was to analyze DNA sequences upstream of lasA and determine their role in regulating gene expression. Transcript mapping of the lasA message and sequence analysis of the region upstream of the transcription start site resulted in identification of a potential σ70-type promoter. Two rules have been established by McClure (1985), based on homology to the E. coli σ70 consensus (Figure 29), regarding the assignment of potential promoters. First, promoters should possess at least two of the three most highly conserved bases in the -10 region; and second, they should contain at least one of the most highly conserved TTG bases in the -35 region. As seen in Figure 29, the putative promoter assignment for lasA fits this rule for both the E. coli and P. aeruginosa consensus sequences. An exonuclease III-generated deletion (m75) of lasA was shown to contain the putative
**Figure 29.** Comparison of the putative lasA promoter to *E. coli* and *P. aeruginosa* consensus σ70 promoters. Highly conserved bases are underlined, with the most invariant nucleotides shown in a larger font size. R represents either A or G.

<table>
<thead>
<tr>
<th></th>
<th>-35</th>
<th>-10</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em> Consensus</td>
<td>TTGACA</td>
<td>TATAAT</td>
</tr>
<tr>
<td><em>P. aeruginosa</em> Consensus</td>
<td>TTGACR</td>
<td>TATRRT</td>
</tr>
<tr>
<td><em>lasA</em></td>
<td>TTGTCT</td>
<td>CACGGT</td>
</tr>
</tbody>
</table>

-35 and -10 refer to the positions relative to the transcription start site.
promoter with essentially no upstream sequences. Fusion of this deletion to a promoterless \textit{lacZ} gene resulted in no promoter activity (Figures 18 and 19). The inclusion of only 18 additional bases upstream of the m75 deletion to a \textit{Sau}3AI site, resulted in 40\% the promoter activity of the full-length \textit{lasA} fusion. These 18 bp encompass two sites that were identified by sequence homology: Box II, with homology to the region upstream of the \textit{lasB} promoter, and a \textit{lux} box, with homology to the \textit{lux} operator of \textit{V. fischeri}. Thus, it seems that one or both of these sites is necessary for \textit{lasA} promoter function in \textit{P. aeruginosa}.

Deletion of Box II from \textit{lasB} did not affect \textit{lasB} promoter activity (Figure 23; Table 10). While this may imply that Box II is also not necessary for \textit{lasA} promoter activity, several arguments can be made that support the idea that Box II does function in the \textit{lasA} promoter. First, a second site with 69\% homology to Box II, designated Box II', overlaps a 16 bp region further upstream that is responsible for another 30\% of \textit{lasA} promoter activity. Second, the lack of function for Box II in the \textit{lasB} promoter has only been determined for one assay of promoter function under one set of environmental conditions. Box II may represent a binding site for a regulatory protein that responds to environmental conditions differently at the two promoters. Third, since LasR is sufficient for activation of the \textit{lasB} promoter but not the \textit{lasA} promoter, the \textit{lux} boxes of \textit{lasA} and \textit{lasB} were compared to the \textit{lux} box consensus sequence (Figure 30). The \textit{lasB} \textit{lux} box exhibits 85\% homology to the consensus, while the \textit{lasA} \textit{lux} box displays 70\% homology. Even more significant is the observation that there are six bases in the consensus that
Figure 30. Alignment of the *lasA* and *lasB lux* boxes to the *lux box* consensus sequence. Invariant bases of the consensus sequence are boxed. Bases matching the consensus are marked with I. Box II of *lasA* is underlined.
are absolutely conserved among the eight promoters used to generate the consensus, including lasB. The lasA lux box is missing two of these conserved bases. Thus, the presence of a weaker lux box for lasA may enhance the importance of a regulatory protein binding at Box II, while a stronger lux box for lasB reduces the importance of any interaction at Box II. Small differences in promoter strength, such as may be occurring by deletion of the lasB Box II, may be undetectable with a multicopy reporter fusion. Finally, just as the lasA lux box may have evolved to become less optimal for LasR recognition, Box II of lasB may have evolved to become less functional for recognition of the potential DNA-binding protein. Differences in the relative strengths of protein binding sites in the lasA and lasB promoters may serve a purpose in regulation by establishing hierarchies of DNA-protein interactions.

To confirm this lasA promoter assignment, it may not be helpful to use site-directed mutagenesis to generate promoter-down mutations, since it has been shown that a lasA::lacZ fusion containing only the -10 and -35 region of lasA is not active. However, it may be possible to confirm the promoter designation by the generation of promoter-up mutations fused to lacZ. Such mutations may allow LasR-independent transcription of the lasA promoter, resulting in high level expression of the lasA promoter in PAO-LR or E. coli. Additional mutations can be made to analyze the importance of sites upstream of the lasA promoter in activation of gene expression. The promoter deletion and sequence analysis of this study have resulted in the identification of sites that may function in cis in regulation events occurring at the lasA promoter. The importance of Box II' can be confirmed by an internal deletion,
with the removal of only Box II' by PCR, fused to lacZ. If a similar decrease in
promoter activity is detected, site-directed mutagenesis can be used to identify
important bases in Box II'. Likewise, the importance of Box II can be
assessed by deletion of the portion not overlapping the lasA lux box (Figure 30).
To examine the importance of the lux boxes, sites that should be altered first
are the six bases that are conserved in the lux box consensus sequence. Only
two of the four bases conserved in lasA can be changed without affecting Box
II (Figure 30). Alteration of these sites may result in the loss of promoter
activity in P. aeruginosa. An optimal lux box can also be created for lasA to
see if LasR is then sufficient for expression of lasA in E. coli.

Based on the results obtained in this study, it was assumed that sites
within the lasA promoter region could serve as binding sites for regulatory
proteins. Examination of various regions of the lasA gene for the ability to be
bound by proteins in a P. aeruginosa extract has led to the identification of at
least one protein with sequence-specific DNA binding activity. EMSA analysis
of P. aeruginosa cell extracts for the ability to bind lasA promoter fragments
revealed that a protein or proteins in both PAO1 and PAO-LR extracts were
able to bind different DNA fragments containing the intact lasA promoter, the
smallest being a 154 bp Sau3AI-TaqI fragment (Figure 25). No difference
between PAO1 and PAO-LR in probe migration patterns was observed when
PAI extract was added, and high levels of PAI actually inhibited DNA binding in
the assay (data not shown). In addition, the presence of multiple copies of lasR
in PAO1 did not result in the identification of any additional shifted complexes.
Thus, no DNA-protein complex in EMSA analysis of cell extracts could be
attributed to LasR, perhaps due to recognition of LasR binding sites by the second LuxR homolog of *P. aeruginosa*, RhlR. In addition, purified LasR did not possess any DNA-binding activity. As discussed in Chapter III, difficulties associated with a covalently modified transcriptional activator protein may be causing the inability to observe *in vitro* function attributable to LasR. Removal of the N-terminal domain of LasR may be necessary to prevent masking of the C-terminal functional domain, as was necessary for demonstration of DNA binding activity of LuxR (Choi and Greenberg, 1991; Stevens *et al.*, 1994). Other transcription factors that are self-regulated through the inhibitory effects of the N-terminus include FixJ of *Rhizobium meliloti* (Kahn and Ditta, 1991), which belongs to the same superfamily of transcriptional activators as LuxR and LasR, and σ$^{70}$ and σ$^{32}$ of *E. coli* (Dombroski *et al.*, 1993).

*P. aeruginosa* RNA polymerase was also not responsible for formation of the major shift complex of the lasA promoter region, but purified RNA polymerase was able to bind to lasA promoter fragments to produce a complex of extremely low mobility in EMSA. The finding that binding of RNA polymerase to the lasA promoter was inhibited by the addition of PAO1 extract indicates that the protein(s) involved in formation of the C1 complex may compete with RNA polymerase for shared or overlapping binding sites (Figure 26). However, numerous attempts to map the binding site of the protein(s) involved in C1 formation by footprint analysis with 1,10-phenanthroline-copper complex (Sigman *et al.*, 1991) were unsuccessful. The reasons for
failure to map the binding site of this protein(s) in the lasA promoter region are not clear, but may be purely technical in nature. It is possible that the protein(s) does not cover the minor groove of the DNA at the binding site, in which case, a footprint with 1,10-phenanthroline-copper would not be detected, since the nuclease activity of this reagent is restricted to the minor groove (Sigman et al., 1991). Further attempts to map the binding site may require the use of alternative footprinting techniques, such as DNaseI protection or methylation interference.

To determine whether the observed DNA-protein complex was due to binding of a non-specific DNA-binding protein, the ability of various DNA fragments to competitively inhibit formation of the DNA-protein complex was determined (Figure 27; Table 11). The lasA promoter-protein complex appears to be due to binding of a sequence-specific DNA-binding protein since the KmR gene was significantly less efficient as a competitor than the other DNA fragments tested. Both the m42-SmaI fragment, which contains the -35 region of the lasA promoter and sequences upstream, and the SmaI-XhoI fragment, which contains the -10 of the lasA promoter and sequences downstream, were able to inhibit protein binding to the m42-RsaI fragment. This indicated that two binding sites are present on the m42-XhoI fragment of lasA. In addition, the PstI-PstI fragment located well within the coding region of lasA competed binding to the promoter region very efficiently, indicating the presence of another binding site nearly 800 bp downstream of the promoter. Distantly located regulatory elements involved in positive and negative
regulation events have been identified within the *P. aeruginosa* *alg*$_D$ gene and the *V. fischeri* *lux*$_D$ gene, respectively (Wozniak, 1994; Shadel and Baldwin, 1992a).

The ability of competing DNA fragments to be recognized and bound by protein(s) in a PAO1 extract was determined (Figure 28). Both the m42-*Sma*I and *Sma*I-*Xho*I fragments exhibited retarded motility in EMSA, with the *Sma*I-*Xho*I fragment more efficiently bound. The finding that the *Pst*I-*Pst*I fragment of *lasA*, which was a very efficient competitor, was poorly bound by extract proteins may indicate cooperativity between binding sites on the *Pst*I-*Pst*I fragment and in the promoter region. In addition, the *Sal*I-*Xmn*I fragment of *lasA* was also bound by PAO1 extracts, while two other fragments were not retarded at all in EMSA. The inability of two *lasA* DNA fragments to be recognized and bound by any proteins in the PAO1 extracts also indicates that the interactions of extracts with other DNA fragments are sequence-specific. While it cannot be stated conclusively that the same protein(s), is binding to the *lasA* DNA fragments, this hypothesis is supported by the observation that one complex was formed for each probe, and other complexes typically were not visible except upon prolonged exposure of the EMSA gels. In addition, the shift patterns for different probes are similar, with regard to both the relative mobilities of the probe and shift complexes and the amount of probe shifted at a given concentration of extract (see Figure 28B).

Thus, evidence presented here suggests that a protein(s) in *P. aeruginosa* extracts binds in a specific manner to the *lasA* promoter region (m42-*Sma*I fragment), and to both a 5' distal site (*Sal*I-*Xmn*I fragment) and
proximal and distal sites located 3' to the lasA promoter (SmaI-XhoI and PstI-PstI fragments, respectively). The downstream binding site on the PstI-PstI fragment did not appear to be important for transcription activation since lasA::lacZ transcriptional fusions containing the SalI-PstI and SalI-PstI-PstI regions of lasA produced similar levels of β-galactosidase activity (data not shown). However, the use of a multi-copy assay system may have caused the absence of an observed difference, especially if the protein involved in interaction with the DNA is present at extremely low levels and the affinity of the protein for different binding sites varies.

Attempts to purify the protein(s) by DNA-affinity chromatography using the lasA promoter region were also unsuccessful (data not shown). However, the easily detectable shift product and the relative ease of the EMSA make it a very good assay system to follow purification of the protein(s) involved in formation of the DNA-protein complex by classical chromatography techniques. This approach was used successfully to purify the V. harveyi LuxR protein, a transcriptional activator required for luminescence in this relative of V. fischeri that is neither activated by autoinducer nor homologous to LuxR of V. fischeri (Swartzman and Meighen, 1993). While the specificity of the DNA-protein interactions for only certain lasA fragments indicates that these interactions do serve some functional significance, it is impossible to speculate on the process(es) in which they are involved without purification of the protein(s).
Summary

This study has examined two different aspects of regulation of the *P. aeruginosa* lasA gene. First, the effects of one known regulator of lasA expression, LasR, were examined in detail. While LasR appears to be important for maximal expression of lasA, other factors are required in addition to LasR for expression of lasA and are able to activate lasA expression in the absence of LasR. Second, the lasA gene was analyzed for cis-acting sites involved in expression. The lasA transcription initiation site and promoter were identified, and several potential protein-binding sites have been identified based on homology to lasB and the lux operator of *V. fischeri*. Two regions containing these potential binding sites, spanning only 16 and 18 bp, were determined to account for 70% of lasA promoter strength. Finally, the presence of a DNA-binding protein able to specifically bind to the lasA promoter, and to sites upstream and downstream of the promoter, was detected in extracts of *P. aeruginosa*. 
LIST OF REFERENCES


Figure 31. Restriction map of the lasA locus in isogenic mutants of P. aeruginosa strain PAO1. (A.) lasA mutant AD1825. The lasA gene was insertionally disrupted in the PAO1 chromosome by homologous recombination with pBR322 containing the lasA internal PstI fragment. The shaded region represents the inserted plasmid and is not shown to scale. The interrupted lasA open reading frame is shown by the arrow labelled lasA '. (B.) lasA lasB mutant PAO-LAB. The lasA gene was insertionally disrupted in the chromosome of a PAO1 lasB mutant, PAO1E (Wolz et. al., 1991), by homologous recombination with pACYC184 containing the lasA internal PstI fragment. The Tc' gene of pACYC184 was first replaced with the Tc' locus of RP4 (tetA tetR). The striped region represents the inserted plasmid and is not shown to scale. Abbreviations: S, SalI; X, XhoI; P, PstI; E, EcoRI; H, HindIII.
Table 12. Elastolytic activity of isogenic lasA and lasB mutants.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>LB-ECR $^a$</th>
<th>Specific Activity $^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAO1</td>
<td>WT</td>
<td>8.0</td>
<td>77.0</td>
</tr>
<tr>
<td>PAO1E</td>
<td>lasB</td>
<td>3.0</td>
<td>3.0</td>
</tr>
<tr>
<td>AD1825</td>
<td>lasA</td>
<td>1.0</td>
<td>51.0</td>
</tr>
<tr>
<td>PAO-LAB</td>
<td>lasA lasB</td>
<td>0</td>
<td>4.0</td>
</tr>
</tbody>
</table>

$^a$Zones of elastin digestion were measured in mm from the edge of bacterial growth after 48 h at 37°C.

$^b$Strains were grown in LB medium for 16 h, and culture supernatants collected and assayed for 4 h at 37°C. Specific activity is reported as $A_{495}$ per mg protein. Assays were done in duplicate.