THE UNIVERSITY OF CALGARY

Identification of Regulators which act on the P2 Promoter of the *Pseudomonas aeruginosa regAB* Operon

by

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A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE

DEGREE OF MASTER OF SCIENCE

DEPARTMENT OF BIOLOGICAL SCIENCES

Calgary, Alberta

March, 2000

THE UNIVERSITY OF CALGARY

Abstract

Exotoxin A is one of the most toxic virulence factors produced by *Pseudomonas aeruginosa*. The primary level of regulation of ETA production is through the *regAB* operon. Two promoters, the P1 and the P2, are located directly upstream of this operon. The P1 promoter is active early in the growth cycle and its activity is independent of iron concentration. In contrast, the P2 promoter is only active later in the growth cycle and in low iron conditions. Surprisingly, very little is known about the regulation of the P2 promoter, therefore, identification and characterization of regulators that act on this promoter would enhance our understanding of the regulation of ETA. Previously, it was demonstrated that expression of *regA* is dependent on PvdS, an alternative sigma factor regulated by the global iron regulator Fur. We extensively analyzed the sequence upstream of *regAB* and identified an eight out of ten base pair match to the consensus sequence for PvdS binding. We expressed *pvdS* on a multicopy plasmid in *P. aeruginosa* strains IL-1, PA103 and PAO1. We observed that PvdS had a positive effect on the activity from the P2 promoter and ETA activity. Strain IL-1 contains a P2-*lacZ* fusion which is integrated onto the chromosome at the *regAB* locus. In *pvdS* knock-out derivatives of strains IL-1, PA103 and PAO1 we observed no expression from the P2 promoter, and little to no ETA activity. Complementation with *pvdS* restored P2 and ETA activity. These studies demonstrate that the alternative sigma factor PvdS acts as a regulator of ETA expression through the P2 promoter of the *regAB* operon. Strain IL-1 was also used for transposon mutagenesis and IL-1 mutants were screened by β-galactosidase assays for deregulation or upregulation of the P2 promoter. Two mutants which demonstrated irregular P2 activity were identified. Mutant H399 contained a transposon insertion in a gene coding for lactoglutathione lyase and the phenotype observed was demonstrated to be due to a polar effect of the transposon on the *regAB* operon. In mutant L522 the P2 promoter is down regulated in low iron conditions and the transposon is inserted into a putative gene coding for an activator of the P2 promoter. These studies suggest that at least two activators, PvdS and the mutated protein in mutant L522, regulate the P2 promoter of the *regAB* operon.

Acknowledgements

I would like to thank my supervisor, Dr. Doug Storey, for always providing me with tremendous support, encouragement, advice, and reassurance. Thank you also for allowing us all to conduct our research in such a fun and productive work environment and making coming to the lab each day something to look forward to.

All of my past and present colleagues, especially Michael Parkins, Claude Gallant, Amanda Kirkham, Andrea Rahn, Ryan Endersby, Isabelle Loubens, Sheri Lupul and David Erickson also deserve tremendous thanks for their support and advice throughout my degree. I would like to give extra-special thanks to my close friends Michael Parkins and Claude Gallant whom I am indebted to and who always gave of themselves to help me in any way with any size of problem and for their continuous encouragement, camaraderie and strength. I would also like to thank Alex Venter and Vandana Sood for their friendship, support, and advice. I am grateful for the support and friendship of Jessie Kaur and Nii Patterson. I would also like to thank the University of Calgary for financial support. In the Biological Sciences building, I would like to thank Dr. Ceri, Dr. Hynes, Dr. Sanderson, Dr. Voordouw, and Dr. Wong for allowing me to use equipment and lending advice.

Thank you to Linda McClelland and Peter Macklon for their encouragement early on. I am sure they do not realize what a difference they made.

Special thanks to my best friends, Cathy Landymore and Sandy Wong, for their encouragement and friendship throughout my degree.

Most of all, I would like to thank my wonderful parents, Daryl and Shalleen, and my brother Derek, as well as my Uncle Derek and Aunt Hilda for their love, patience, and encouragement.

Dedication

For those who have helped to shape me into who I am today and encouraged me to travel down this path. You know who you are.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

A	absorbance
α	alpha
ADP	adenosine diphosphate
ADPRT	ADP ribosyl transferase
AMP	adenosine monophosphate
Ap	ampicillin
β	beta
BIS	N,N'-methylene-bis-acrylamide
BLAST	basic local alignment search tool
bp	base pair
C-terminal	carboxy terminal
cAMP	cyclic AMP
Cb	carbenicillin
CF	cystic fibrosis
CIAP	calf intestine alkaline phosphatase
cm	centimetre
cpm	counts per minute
CTAB	cetyl trimethyl ammonium bromide
Da	dalton
dCTP	deoxycytidine triphosphate
ddH_2O	double distilled water
DMSO	dimethyl suphoxide
DNA	deoxynucleotide triphosphate
DNase	deoxyribonuclease
dNTP	deoxynucleotide triphosphate
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EF2	elongation factor 2
ELISA	enzyme-linked immunosorbent assay
ETA	exotoxin A

g	gram
Gm	gentamicin
GTE	glucose, Tris and EDTA buffer
IPTG	isopropyl-β-D-galactopyranoside
kb	kilobase
kDa	kilodalton
Km	kanamycin
L	litre
LB	Luria-Bertani broth
LPS	lipopolysaccharide
M	molar
MCS	multiple cloning site
mg	milligram
μg	microgram
mL	millilitre
μL	microlitre
mM	millimolar
μM	micromolar
mRNA	messenger RNA
MSG	mono sodium glutamate
MW	molecular weight
N	normal
N-terminal	amino terminal
NAD	nicotinamide adenine dinucleotide
Neo	neomycin
nt	nucleotide
OD	optical density
oNPG	O-nitrophenyl-β-D-galactopyranoside
ORF	open reading frame
P1	promoter 1 of the *regAB* operon
P2	promoter 2 of the *regAB* operon

PCR	polymerase chain reaction
PEG	polyethylene glycol
R	resistant
RNA	ribonucleic acid
RNAP	RNA polymerase
RNase	ribonuclease
rpm	rotations per minute
S	sensitive
σ	sigma
SDS	sodium dodecylsulphate
SF	stabilizing fragment
Sm	streptomycin
sp	species
Spec	spectinomycin
SSC	standard sodium citrate
T1	transcript 1 of *regAB*
T2	transcript 2 of *regA*
TAE	Tris-acetate-EDTA buffer
TBE	Tris-boric acid-EDTA buffer
TCA	tricholoacetic acid
TE	Tris EDTA
TEMED	N,N,N',N'-tetramethyl-1,2-diaminoethane
Tet	tetracycline
Tris	(hydroxymethyl) aminomethane
TSBDC	trypticase soy broth-dialyzed and chelexed
UV	ultraviolet
VBMM	Vogel Bonner minimal media
X-gal	5-chloro-4-bromo-3-indolyl-β-D-galactopyranoside
°C	degrees Celsius
Ω	omega

CHAPTER 1: INTRODUCTION

1.1 *Pseudomonas aeruginosa*

P. aeruginosa is a facultatively aerobic Gram negative bacillus which is ubiquitous in soil and aquatic environments. Pseudomonads have a great ability to adapt to diverse environments due to sparse nutritional requirements. The enormous metabolic diversity of this organism is highlighted by the ability to utilize over 80 organic compounds for growth and to survive at temperatures up to 42°C (Palleroni, 1984). *P. aeruginosa* is a common opportunistic pathogen that affects people who are immunocompromised or have underlying medical conditions (Bodey *et al.*, 1983).

1.1.1 Role in Infection

P. aeruginosa is an important human pathogen. Patients who have compromised host defenses appear to be the most susceptible to infections by this microorganism. *P. aeruginosa* is a common opportunistic pathogen in patients who are afflicted with diverse underlying medical conditions resulting in an impaired immune response. This pathogen can be commonly found in individuals who are victims of burns, cancers such as leukemia, diabetes, and cystic fibrosis (Bodey, 1983). In addition, *P. aeruginosa* infections are common among intravenous drug users and organ transplant recipients. *P. aeruginosa* has been demonstrated to be involved in 10 to 20% of nosocomial infections, including 10% of urinary tract infections, 9% of surgical wound infections, 11% of bacteriemias, and 17% of lower respiratory tract infections (Bennett, 1974). While it is uncommon to find *P. aeruginosa* among the normal flora of most healthy individuals, this bacterium is the most common pathogen cultured from patients who have been hospitalized for one week or more (Grogan, 1966). This pathogen has been demonstrated to have the ability to infect almost every part of the human body. *P. aeruginosa* infections have been associated with medical conditions such as endocarditis, pneumonia, dermatologic syndromes, external otitis, endophthalmitis, corneal infections, septicemia, and meningitis (Bodey, 1983). *P. aeruginosa* infections tend to be severe and often fatal. The importance of *P.*

aeruginosa in infection is evident by the demonstration of circulating antibodies to a number of virulence factors including exotoxin A and LPS (Cross *et al.*, 1980). Antibody titre levels have been demonstrated to correlate with survival of colonized cystic fibrosis patients. The intrinsic antibiotic resistance and the array of virulence factors produced by *P. aeruginosa* contribute largely to the diverse infections which can be attributed to this pathogen.

1.2 The Role of Iron in Bacterial Growth

Iron is the fourth most abundant element on earth and it is the second most abundant metal. A paradox exists in that even though iron is extremely abundant in the environment, it is often a limiting factor for bacterial growth due to its tendency to form insoluble ferric-hydroxide complexes in aerobic, neutral pH conditions. Iron is a reactive compound and, as such, has some intrinsic beneficial and detrimental properties. Iron exists on earth in two stable redox states, a soluble ferrous state (Fe^{2+}), and an oxidized, insoluble ferric state (Fe^{3+}). Although ferric iron is the most abundant in the environment, ferrous iron is the biologically active form of this element. Microorganisms have adapted to collect iron in the ferric state and reduce it to the ferrous state for use as cofactors for many essential enzymes. Ferrous iron is the active component of most oxygen carrier proteins, cytochromes, and several reductases. Because of its reactivity, iron is also a very hazardous material for cells to handle. Iron is the central catalyst of Fenton chemistry which ultimately results in the production of hydrogen peroxide and hydroxyl radicals that cause DNA damage. The Fenton reaction is depicted below:

$$2O_2^- + 2H^+ \rightarrow O_2 + H_2O_2$$

$$H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + OH^- + OH\bullet \quad \text{(causes DNA damage)}$$

$$Fe^{3+} + NADH \rightarrow Fe^{2+} + NADH^+ \quad \text{(to recycle the ferrous iron)}$$

Cells possess a system to combat hydroxyl radicals and the effects that they produce. This antioxidant system is composed of catalases, peroxidases and superoxide dismutases (Fridovich, 1978). This system uses iron as essential

cofactors and therefore, is only active in iron-rich conditions where hydroxyl radicals may be produced. It is essential to the cell that the level of intracellular iron be carefully monitored. Too little iron will cause the oxidative defence system to be depressed while too much iron will promote the Fenton reaction. Either situation can result in decreased growth or even death of the organism (Byers and Arceneaux, 1998).

When a microorganism invades a eukaryotic host, the host increases the iron stores in the form of ferritin, so the amount of free iron is depleted. Depriving the microorganism of iron benefits the host by increasing the vulnerability of microorganisms to oxidative killing by host phagocytes. Microorganisms have responded by developing sophisticated mechanisms for iron scavenging. As a facultative aerobe, *P. aeruginosa* has a particularly strong necessity for iron (Meyer *et al.*, 1996). *P. aeruginosa* produces two siderophores; pyoverdine and pyochelin which have high binding affinities for ferric iron. Other virulence factors include toxins which kill host cells to liberate host iron and a haem-binding protein which is able to collect iron from haem-containing compounds such as haemoglobin, haptoglobin and haemopexin (Letoffe *et al.*, 1998). These mechanisms enable *P. aeruginosa* to obtain iron and survive in the low iron environment of a eukaryotic host during an infection. Because of the importance of iron to all living things and the importance of its intracellular levels, it is not surprising that iron is critical to the regulation of many bacterial products. The level of extracellular iron is an important cue for the regulation of production of antioxidant enzymes and many virulence factors including siderophores of *P. aeruginosa* (Bjorn *et al.*, 1979).

1.3 Virulence Factors Produced by *P. aeruginosa*

The virulence of *P. aeruginosa* during an infection is multi-factorial. *P. aeruginosa* produces a wide array of virulence factors including both cell-associated and extracellular factors.

1.3.1 Cell-Associated Virulence Factors

P. aeruginosa has intrinsic antibiotic resistance. This can be attributed in part to the unique structure of the outer membrane. Pseudomonads contain a unique outer membrane which is highly impermeable to many antibiotics due to specialized porins in the outer membrane (Angus *et al.*, 1982; Bellido *et al.*, 1992). Like many Gram-negative bacteria, *P. aeruginosa* produces a chromosomally encoded β-lactamase for the breakdown of β-lactam antibiotics (Lodge *et al.*, 1993). Production of this enzyme is believed to contribute to the antibiotic resistance inherent to chronic pulmonary infections in cystic fibrosis patients. Furthermore, β-lactamases produced by *P. aeruginosa* have been extracted from CF sputum (Giwercman *et al.*, 1992). The production of numerous proton motive force-dependent multidrug efflux pumps are also believed to contribute to antibiotic resistance. To date, *P. aeruginosa* has been demonstrated to produce at least four efflux pump systems, MexAB-OprM, MexCD-OprJ, MexEF-OprN, and MexXY-OprM. The constitutively expressed MexAB-OprM efflux system (Poole *et al.*, 1993) confers resistance to β-lactams, β-lactam inhibitors, quinolones, chloramphenicol, tetracycline, trimethoprim, macrolides, sulfamethoxazole, and novobiocin. The MexCD-OprJ system (Poole *et al.*, 1996) provides resistance to chloramphenicol, tetracycline, quinolones, macrolides, and some fourth-generation cephalosporins. Chloramphenicol, trimethoprim, quinolones and carbapenems are effluxed by MexEF-OprN (Kohler *et al.*, 1997). Intrinsic resistance to aminoglycosides can be attributed to the MexXY-OprM efflux system (Aires *et al.*, 1999). Each of these mechanisms contribute to the intrinsic resistance of *P. aeruginosa* to antibiotics.

Pili are thought to play a major role in adhesion to epithelial cells (Doig *et al.*, 1988). As well, the type IV pili are important for twitching motility (Henrichsen, 1975). Non-pilus adhesins are believed to be involved in adherence to mucins which do not possess receptors for pili (Simpson *et al.*, 1992). However, the role of non-pilus adhesins has been questioned by Farinha *et al.* (1994), who

demonstrated that non-pilus adhesins do not contribute significantly to virulence in a mouse model.

P. aeruginosa produces a single polar flagellum which is important for motility and tissue invasion (Drake and Montie, 1988). The role of flagella in adhesion is controversial. While the flagellin protein itself is not an adhesin, there is an undetermined relationship between production of flagella and adhesion (Arora *et al.*, 1996). Flagellar motility is believed to be important during the initial colonization stages. However, because of their antigenicity, flagella-expression is lost once an infection has been established (Drake and Montie, 1988). Supporting this model is the finding that most CF isolates are non-motile (Mahenthiralingam *et al.*, 1994).

Alginate is a highly viscous linear exopolysaccharide composed of alternating mannuronic and guluronic acid residues. Overproduction of alginate is responsible for the mucoid phenotype characteristic of *P. aeruginosa* isolates from the CF lung (Doggett, 1969). Alginate production may be involved in resistance to dehydration, prevention of opsonization, impediment of phagocytosis, prevention of complement-mediated lysis, and may provide an ionic barrier to prevent the entrance of aminoglycosides (Govan and Fyfe, 1978). The production of alginate demands high levels of ATP and, as a result, production is highly regulated with multiple positive and negative regulators (May and Chakrabarty, 1994).

The lipopolysaccharide (LPS) of *P. aeruginosa* is typical of most Gram-negative bacteria. The O antigen of the LPS is often lost during a CF infection, causing the conversion from the LPS-smooth to LPS-rough phenotype (Goldberg and Pier, 1996). This LPS-type conversion is believed to occur because the O-antigen is extremely antigenic and loss of this portion of the LPS allows *P. aeruginosa* to evade the host immune response. During CF infections, the LPS of *P. aeruginosa* may up-regulate transcription of mucin genes in respiratory epithelial cells. Specifically, it is the lipid A portion of LPS which mediates this up-regulation.

The mechanism by which mucin expression is up-regulated is believed to be through modification of eukaryotic intracellular signal transduction pathways (Li *et al.*, 1997). Recently, it has been demonstrated that *P. aeruginosa* produces a unique lipid A portion of LPS, expressed exclusively in the CF lung. This unique lipid A is induced in the CF lung environment and may be involved in promoting bacterial resistance to cationic antimicrobial peptides and therefore, may enhance bacterial survival in the CF lung (Ernst *et al.*, 1999). In addition, lipid A of *P. aeruginosa* clinical isolates were demonstrated to have a stimulatory effect on the production of host IL-8 and resulted in an enhancement of the inflammatory response (Ernst *et al.*, 1999). These studies suggest that the LPS of *P. aeruginosa* may play an important role in the pathogenesis of CF.

1.3.2 Extracellular Virulence Factors

P. aeruginosa produces a number of extracellular virulence products which are secreted into the surrounding environment. Two phospholipase C proteins are produced, a hemolytic (PlcHR) and a nonhemolytic (PlcN) enzyme. While PlcHR has been demonstrated to be an important virulence factor, a role for PlcN has yet to be established (Ostroff *et al.*, 1990). PlcHR suppresses the respiratory burst of neutrophils and therefore, enhances bacterial survival during an infection (Terada *et al.*, 1999). Both phospholipase C enzymes act on eukaryotic phospholipids and may be involved in the breakdown of host cell membranes, as well as the major component of lung surfactant (Ostroff and Vasil, 1987). The breakdown of these host structures releases valuable nutrients and iron into the surrounding extracellular milieu for bacterial procurement.

Elastase and Las A protease are two enzymes believed to act synergistically in the break down of host tissues. Elastase is a zinc metalloprotease that can degrade a number of host tissue components, including elastin from lung tissues, complement proteins, immunoglobulins A and G, fibrin, serum α-1-proteinase inhibitor, γ-interferon, lysozyme and some types of collagen (Morihara, 1964; Schultz and Miller, 1974; Heck *et al.*, 1990; Doring *et al.*, 1981;

Morihara *et al.*, 1979; Horvat *et al.*, 1989; Jacquot *et al.*, 1985; Heck *et al.*, 1986). Las A protease has little elastolytic activity but acts to specifically enhance elastase activity. Las A protease is believed to enzymatically nick the elastin substrate to expose the elastin monomers and enable elastase to break down elastin at a higher rate (Peters and Galloway, 1990).

P. aeruginosa possesses a type III secretory system for the directed targeting of specific virulence factors directly into eukaryotic cells. The exoenzyme S regulon co-ordinately secretes effector proteins into the cytoplasm of eukaryotic cells by type III secretion (Yahr *et al.*, 1996b). At least eight proteins are believed to be secreted under the control of this operon and most have homologues to the Yersinia Yop type III secretory system (Yahr *et al.*, 1997). To date, only four of the eight putative effector proteins have been characterized: ExoS, ExoT, ExoU and ExoY. Exoenzyme S is an ADP-ribosyl transferase which requires cleavage by a eukaryotic cytoplasmic protein factor (FAS) for enzymatic activity (Iglewski *et al.*, 1978; Coburn *et al.*, 1991). *In vitro*, ExoS substrates include low molecular weight GTP-binding proteins such as Ras, the cytoskeletal protein vimentin, and soybean trypsin inhibitor (Coburn *et al.*, 1989a; Coburn *et al.*, 1989b; Kulich *et al.*, 1993). Recently, exoenzyme S has also been demonstrated to have a second activity independent of the enzymatic domain. ExoS has a cytotoxic ability to disrupt actin microfilaments and therefore, aid in resistance to phagocytosis (Frithz-Lindsten *et al.*, 1997). ExoT is also a FAS-dependent ADP-ribosyl transferase. While ExoS and ExoT share 75% amino acid identity and similar cytotoxicities, ExoT has only 0.2% of the ADP-ribosyl transferase activity of ExoS (Yahr *et al.*, 1996a). Because ExoS and ExoT share high amino acid identity, it has been speculated that they may be transcribed from duplicated genes which have evolved independently (Frank, 1997). ExoU is the most cytotoxic of the type III effector proteins, however, its enzymatic activity remains to be identified (Finck-Barbançon *et al.*, 1997). ExoY is an adenylate cyclase which requires an unidentified eukaryotic protein for activation (Yahr *et al.*, 1998). A fifth effector protein has been recently identified which causes apoptosis of macrophages and some epithelial cell lines (Hauser and

Engel, 1999). A sampling of *P. aeruginosa* strains has suggested that the exoenzyme S regulon is present in all strains tested and this implies that this regulon is a conserved mechanism of *P. aeruginosa* pathogenesis (Frank, 1997).

P. aeruginosa produces two siderophores; pyochelin and pyoverdine, for scavenging of iron. Siderophores are low molecular weight (500-1500 Da) compounds which are capable of solublizing ferric iron. Microorganisms that are able to grow at an enhanced rate will more efficiently express virulence factors and, as a result, be more virulent than those which grow at a reduced rate. Siderophores are therefore classified as virulence factors because they contribute to virulence by promoting growth of the microorganism. (Cox, 1982; Meyer *et al.*, 1996). Pyoverdine is the main siderophore produced by *P. aeruginosa* for iron scavenging. Pyoverdine is a fluorescent yellow-green protein which strongly chelates iron though catecholate and hydroxamate groups (Hohnadel and Meyer, 1988). The stability constant of ferripyoverdine is 10^{32}. The ability of this siderophore to chelate iron is extremely strong and it has even been shown have the capability to directly remove bound ferric iron from partially iron-saturated transferrin and lactoferrin *in vitro* (Meyer *et al.*, 1996; Xiao and Kisaalita, 1997). At least 78 kb of the *P. aeruginosa* genome is dedicated to the synthesis and function of pyoverdine (Tsuda *et al.*, 1995). Allocation of such a large portion of the chromosome alludes to the complexity of pyoverdine synthesis, and the importance of pyoverdine to the survival of this microorganism. The minor siderophore produced by *P. aeruginosa* is pyochelin. Pyochelin is a red, phenolic siderophore that can chelate many metal ions, including Fe^{3+}, Zn^{2+}, Cu^{2+}, Co^{2+}, Mo^{6+}, and Ni^{2+} (Cox and Graham, 1979; Visca *et al.*, 1992). The stability constant of ferripyochelin is only 10^5, so this siderophore's role in iron scavenging is thought to be limited and the main role may be in general mobilization of metal ions (Cox *et al.*, 1981).

1.3.2.1 Exotoxin A

Exotoxin A (ETA) is the most toxic virulence factor produced by *P. aeruginosa* (Liu, 1974). Molecular studies have predicted that approximately 95% of *P. aeruginosa* strains have a *toxA* gene, suggesting that this virulence factor is important to the pathogenicity of the microorganism (Vasil *et al.*, 1986). ETA is an ADP-ribosyl transferase which inhibits eukaryotic cell protein synthesis by inactivating elongation factor 2 (EF2) (Iglewski and Kabat, 1975). The reaction catalyzed by ETA is demonstrated below:

$$NAD^+ + EF2 \rightarrow ADP\text{-}ribose\text{-}EF2 + nicotinamide + H^+$$

ETA is translated from a monocistronic *toxA* transcript as a 638-amino acid precursor protein within the bacterial cell. During secretion of the protein, a 25-amino acid hydrophobic leader sequence is removed (Gray *et al.*, 1984). The inactive proenzyme form of the toxin then binds to the low-density lipoprotein receptor related protein on the surface of a eukaryotic cell (Kounnas *et al.*, 1992). The proenzyme enters the eukaryotic cell by receptor-mediated endocytosis. Within the endosome, ETA is cleaved by a eukaryotic membrane protease (furin) to free a 37 kDa fragment which includes the enzymatic domain (Kounnas *et al.*, 1992; Ogata *et al.*, 1990). At least two disulfide bonds of this catalytic form are reduced within the endosome and the fragment enters the endoplasmic reticulum (ER) by retrograde transport. This transport is facilitated by the last five C-terminal residues which resemble the ER retention sequence (Chaudhary *et al.*, 1990). Once in the ER, the catalytic ETA fragment is translocated into the cytoplasm where it can ADP-ribosylate EF2 and halt protein synthesis at the elongation step (Chaudhary *et al.*, 1990). Once protein synthesis is arrested, the eukaryotic cell begins to die by a cell death resembling apoptosis although the mechanism of cell death induced by ETA is unknown (Morimoto and Bonavida, 1992).

The crystal structure of the proenzyme form of ETA was solved to 3.0 Ångstrom resolution by Allured *et al.* (1986) and the catalytic domain complexed with an NAD analogue was solved to 2.3 Ångstrom resolution by Li *et al.* (1996).

These studies have revealed valuable information about the structural domains and enzymatic activity of ETA. The toxin is composed of three main domains. Domain Ia is the N-terminal domain and it is involved in eukaryotic cell recognition and binding to the receptor on cell surfaces. The second domain (domain II) is involved in the translocation process and is necessary for the catalytic domain to cross the membrane of the endoplasmic reticulum and enter the cytosol. This region of the protein contains many hydrophobic amino acids which are believed to insert into the lipid membrane to facilitate translocation (Hwang *et al.*, 1987). Domain III and to a lesser extent domain Ib, are required for catalytic activity. Domain III is involved in binding to both substrates NAD and EF2, and it is in this domain where disulfide bonds must be reduced within the endosome in order for the proenzyme form of ETA to become enzymatically active.

1.4 Regulation of Exotoxin A

The production of exotoxin A is not constitutive, it is regulated by many factors (Liu, 1973). *In vitro*, maximum ETA production is obtained when cultures are grown at a temperature of 32°C with aeration. The composition of the media was also shown to effect toxin production. The use of 1% glycerol as a carbon source and pure proteins or monosodium glutamate as a nitrogen source facilitates the production of maximal toxin yields, while the addition of nucleic acids inhibits toxin production (Liu, 1973). Exotoxin A expression is tightly regulated by extracellular iron concentrations. This regulation occurs at the level of transcription (Grant and Vasil, 1986). In high iron conditions (100 μM), *toxA* transcripts cannot be detected, while in low iron conditions *toxA* mRNA can be seen during late logarithmic and stationary phases of growth (Lory, 1986). Many regulators have been speculated to act on the *toxA* gene, but RegA is believed to be the most important. RegA is a positive regulatory protein which is required for the specific enhancement of ETA synthesis (Hedström *et al.*, 1986). The specific mechanism by which RegA enhances ETA expression remains unknown, however, it is speculated that RegA may interact with RNA polymerase to facilitate *toxA* transcription (Walker *et al.*, 1995). Because it has not been demonstrated that

RegA is able to bind to the *toxA* promoter, the specific mechanism by which RegA activates *toxA* transcription remains elusive (Hamood and Iglewski, 1990). Hedström *et al.* (1986) demonstrated that when *regA* was provided *in trans* to a *toxA*$^+$ but hypotoxigenic strain of *P. aeruginosa* (PA103-29), ETA production increased tenfold. ETA expression was completely abrogated in a *regAB*-knockout strain PA103Δ*regAB*::Gm, further confirming that the *regAB* locus is required for ETA production (Raivio *et al.*, 1996). Because RegA is required for ETA production, the environmental regulation of *toxA* expression is believed to mediated through the *regAB* operon.

1.5 The *regAB* Operon

The *regA* gene (originally termed *toxR*) has been cloned and sequenced (Wozniak *et al.*, 1987; Hindahl *et al.*, 1988). Transcript analysis of the 675 base pair *regA* gene revealed that RegA was composed of 249 amino acids (29 kDa) and that it contained a predicted hydrophobic transmembrane domain common to many membrane-bound proteins (Hindahl *et al.*, 1987). Using antibodies raised against a RegA protein overproduced in *E. coli*, Zimniak *et al.* (1989) confirmed that RegA localized to the membrane fraction of *P. aeruginosa* strain PA103.

The *regAB* operon of the hypertoxigenic strain PA103 is composed of the *regA* gene (777 bp) and the *regB* gene (228 bp) (Hindahl *et al.*, 1988; Wick *et al.*, 1990). The *regB* gene is located 6 nucleotides downstream of the termination codon of *regA* (Wick *et al.*, 1990). Northern blot analysis demonstrates that *regA* and *regB* are transcribed as an operon (Frank and Iglewski, 1988). The operon is preceded by two independent promoters which regulate the transcription of separate transcripts (Frank *et al.*, 1989; Storey *et al.*, 1990). The P1 promoter is located 164 base pairs upstream of the translational start site of *regAB* and is not tightly regulated by iron. The P2 promoter is only active in low iron conditions and it is located 75 nucleotides upstream of the *regAB* start codon. The activity and regulation of these two independent promoters will be discussed below.

1.5.1 The P1 Promoter

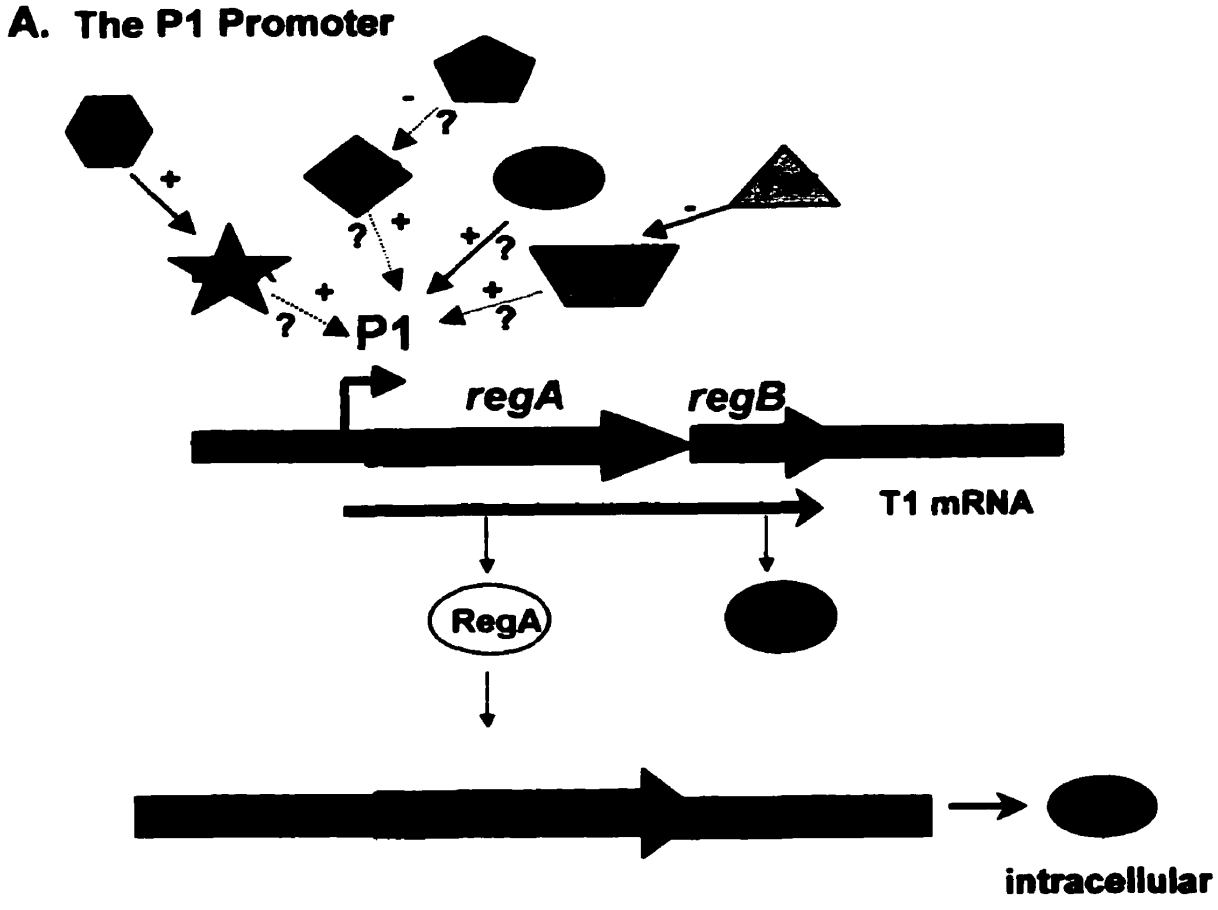
Figure 1A illustrates the regulation by the P1 promoter of the *regAB* operon on the *toxA* gene. Possible regulators that may act on this promoter are also shown. To date, at least seven regulators of the P1 promoter have been implicated as playing a role in the regulation of this promoter; *regB*, PtxR, PtxS, Vfr, LasR, Fur and PvdS. Transcription from the P1 promoter occurs during the early log phase of growth in both high and low iron conditions, albeit at reduced levels in high iron conditions (Frank and Iglewski, 1988). The T1 transcript is generated from the P1 promoter and this larger transcript contains both *regA* and *regB* (Frank and Iglewski, 1988). The RegA that is translated from the T1 transcript activates *toxA* transcription and the ETA protein that is produced remains primarily cell-associated. It has been postulated that this cell-associated ETA may act as a regulator by inhibiting P1 activity (Storey *et al.*, 1991) or it may localize to the membrane where it plays a role in facilitating its own secretion (Lory *et al.*, 1983).

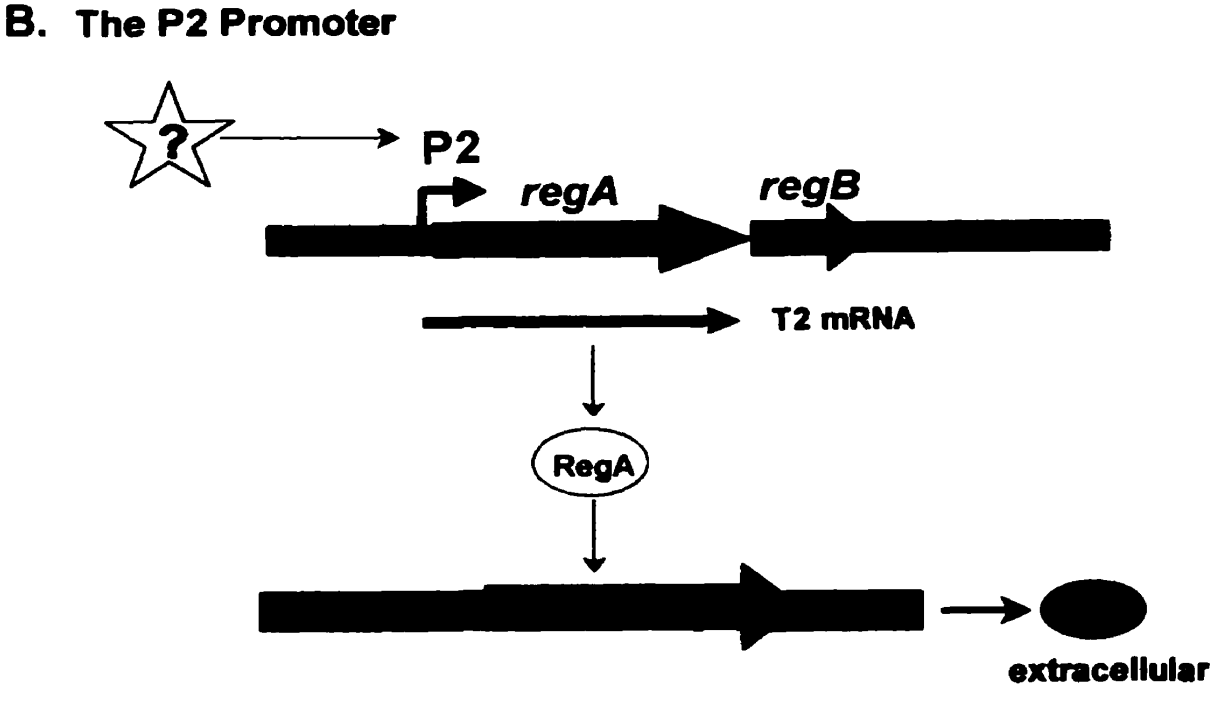
1.5.2 The P2 Promoter

The sequence of events following the activation of the P2 promoter are shown diagrammatically in Figure 1B. The P2 promoter is first expressed during late exponential growth and continues into stationary phase (Frank and Iglewski, 1988; Storey *et al.*, 1991). In contrast to the P1 promoter, this promoter is tightly regulated by the level of extracellular iron. In high iron conditions, transcription from the P2 promoter is undetectable (Frank and Iglewski, 1988). Transcription from the P2 promoter produces a smaller T2 transcript which contains *regA* but not *regB*. The RegA that is translated from the T2 transcript activates *toxA*. The ETA that is produced is secreted from the bacterial cell and can ADP-ribosylate the EF2 of host eukaryotic cells. The iron regulation of ETA production is believed to be mediated through this *regAB* promoter. Despite the tight iron regulation, a regulatory protein that acts on the P2 has not been identified.

Figure 1: Schematic representation of possible regulators that act on the P1 and P2 promoters of the *regAB* operon in *P. aeruginosa* hypertoxigenic strain PA103.

The *regAB* and *toxA* loci of PA103 are demonstrated diagrammatically. A (+) indicates activation, a (–) indicates repression. A broken arrow indicates an indirect regulatory effect and a (?) indicates that the role of the protein in regulation is only speculative. Refer to the text for a description of events. **A.** Model for regulation at the P1 promoter; **B.** Model for regulation at the P2 promoter. Adapted from Frank and Storey (1994).


A. The P1 Promoter
P1
regA
regB
T1 mRNA
RegA
intracellular


B. The P2 Promoter
P2
regA
regB
T2 mRNA
RegA
extracellular

1.5.3 Possible Regulators of the *regAB* Operon

1.5.3.1 *regB*

The *regB* gene is 228 nucleotides in length and it is the second gene of the *regAB* operon (Wick *et al.*, 1990). The *regB* gene is required for optimal ETA expression. If the *regB* locus is inactivated in strain PA103, ETA production is reduced fivefold (Wick *et al.*, 1990). However, RegA does not require *regB* in order to activate expression of *toxA*. It has been demonstrated that a functional *regB* gene is required in order for transcription from the P1 promoter to occur but activity from the P2 promoter is independent of the presence of a functional *regB* gene (Storey *et al.*, 1991). The *regAB* operon is, therefore, autogenously regulated through the P1 promoter and this regulation is facilitated by *regB*. The mechanism by which *regB* activates *regA* through the P1 promoter is unknown. *regB* codes for a predicted protein of 7527 Da, yet attempts to conclusively identify expression of the native RegB protein have been largely unsuccessful to date. The RegB protein has been expressed as a GST-fusion and a fusion protein of the GST protein mass plus approximately 7500 Da was detected, although at low levels (Raivio, 1995). The predicted RegB protein does not contain any known DNA-binding motifs but the requirement of a functional gene for activity from the P1 promoter demonstrates that *regB* does play an important regulatory role at this promoter. The prototrophic *P. aeruginosa* strain PAO1 lacks a *regB* start codon and the resulting lack of P1 activity is believed to account, in part, for the lower ETA production of this strain compared to the hypertoxigenic strain PA103 (Wick *et al.*, 1990).

1.5.3.2 PtxR and PtxS

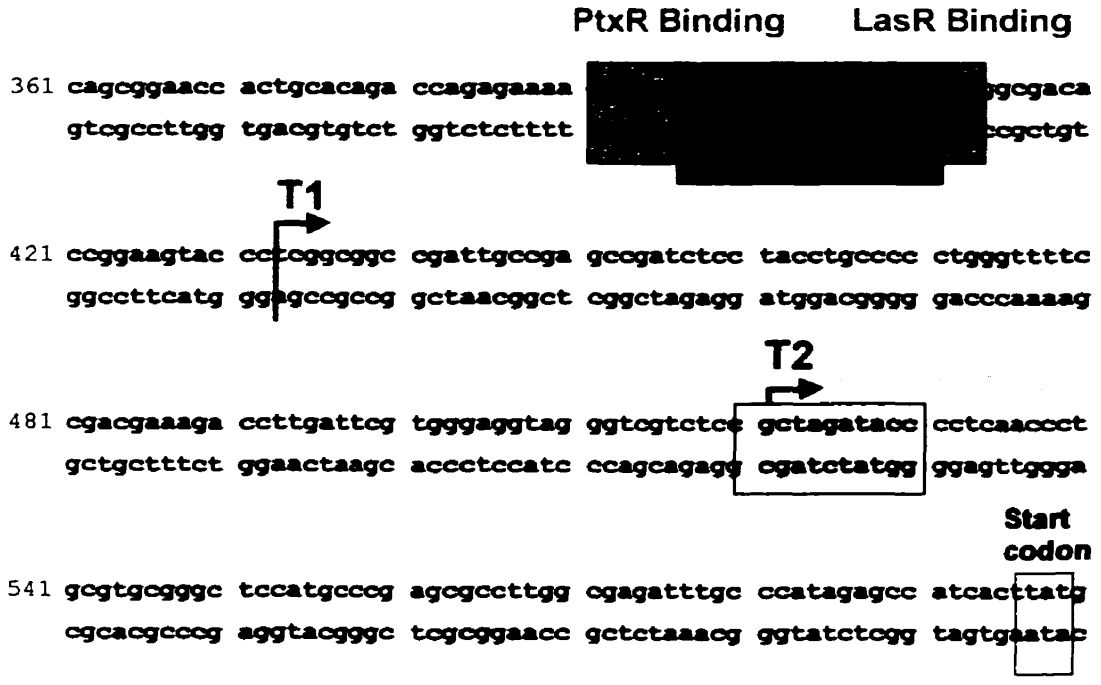
PtxR (Pseudomonas toxin regulator) has been identified as a positive regulator of ETA production in low iron conditions (Hamood *et al.*, 1996). The product of the *ptxR* gene codes for a 34 kDa protein that is a homologue of the LysR family of transcriptional activators (Hamood *et al.*, 1996). When PtxR is in multicopy, it increases transcription of both *regA* and *toxA*. Because of the

observed increase in *regA* transcription, it was hypothesized that the correlation between *ptxR* and an increase in ETA production is mediated through the *regAB* operon. The reporter plasmid pP11 was utilized to demonstrate that the effect of PtxR is mediated through the P1 promoter of the *regAB* operon. CAT expression by this plasmid was significantly less in a *ptxR*-deficient strain when compared to the parental strain PA103. The same *ptxR* knock-out strain was used to show that ETA is still produced, but at levels fivefold lower than in the wild-type strain. These data suggest that PtxR is not required for P1 activity but PtxR does act to increase transcription from this promoter (Hamood *et al.*, 1996). The predicted PtxR protein contains an N-terminal DNA binding motif which may bind to the P1 promoter region to enhance transcription. A putative consensus binding motif for PtxR is present near the P1 promoter transcriptional start site (see Figure 2). However, the exact mechanism of activation by PtxR remains to be elucidated.

Recently, a second gene was discovered to be involved in the regulation by PtxR. PtxS is a negative regulator of *ptxR* (Colmer and Hamood, 1998). PtxS belongs to the family of GalR-LacI repressors and contains an amino-terminal DNA-binding motif (Colmer and Hamood, 1998). This protein has been demonstrated to autoregulate its own synthesis by binding to its own promoter region and preventing transcription (Swanson *et al.*, 1999). However, the exact effect of PtxS on PtxR is unclear. It was demonstrated that in the presence of PtxS, the enhancement effect of PtxR on ETA production is reduced threefold. However, PtxS has not been demonstrated to bind to the promoter region of *ptxR*, so the negative effect on PtxR may be mediated through another regulator (Swanson *et al.*, 1999). To further complicate the regulation, the environmental signal to which PtxS responds is unknown at this time. PtxR, along with PvdS, has been demonstrated to positively regulate pyoverdine biosynthesis genes and the gene is essential for pyoverdine production. Because of the tight iron regulation of siderophore and ETA production, it is tempting to speculate that these two regulatory cascades may overlap in some way, however, evidence is currently unavailable. A further understanding of the regulation of *ptxR* should lead to a

Figure 2: Consensus sequences depicted in the region upstream of the *regAB* translational start codon.

The DNA sequence upstream of the *regAB* translational start codon is shown. The location of the T1 and T2 transcriptional start sites are shown by red arrows. Consensus sequences in this region are indicated by colored boxes: PtxR consensus binding sequence in purple, LasR consensus binding sequence in green, and PvdS consensus binding sequence in yellow. The translational start codon is indicated by a black outlined box.


PtxR Binding
LasR Binding
361 cagcggaacc actgcacaga ccagagaaaa
gtcgccttgg tgacgtgtct ggtctctttt
T1
421 ccggaagtac cctcggcggc cgattgccga gccgatctcc tacctgcccc ctgggttttc
ggccttcatg ggagccgccg gctaacggct cggctagagg atggacgggg gacccaaaag
T2
481 cgacgaaaga ccttgattcg tgggaggtag ggtcgtctcc gctagatacc cctcaaccct
gctgctttct ggaactaagc accctccatc ccagcagagg cgatctatgg ggagttggga
Start codon
541 gcgtgcgggc tccatgcccg agcgccttgg cgagatttgc ccatagagcc atcacttatg
cgcacgcccg aggtacgggc tcgcggaacc gctctaaacg ggtatctcgg tagtgaatac

better understanding of the regulation of the P1 promoter of the *regAB* operon.

1.5.3.3 Vfr

Vfr (virulence factor regulator) is a global virulence factor regulator that is required for ETA production (West *et al.*, 1994). West *et al.* (1994) demonstrated that *vfr* mutants do not produce a T1 transcript, suggesting that Vfr is a regulator required for P1 promoter activity. Vfr belongs to the cAMP-receptor (CAP/CRP) family of proteins and is a homologue of *Escherichia coli* CAP. The Vfr protein contains binding motifs for cAMP and RNA polymerase holoenzyme, as well as a helix-turn-helix DNA binding motif (West *et al.*, 1994). The role of Vfr in regulation of the P1 promoter of *regAB* was thought to be an indirect effect because CAP-like consensus binding sites are not present in the P1 or *toxA* promoter regions (West *et al.*, 1994). Furthermore, a consensus site was observed in the *lasR* and *algD* promoter sequences and there is evidence that Vfr directly regulates transcription of both of these genes. This has lead researchers to postulate that the regulatory effect of Vfr on the P1 promoter of *regAB* may be mediated through a second regulatory protein.

1.5.3.4 LasR

LasR is the transcriptional activator of the *las* quorum sensing system of *P. aeruginosa*. LasR activates the production of many virulence factors, including elastase, alkaline protease and exotoxin A (Gambello and Iglewski, 1991; Gambello *et al.*, 1993). Gambello *et al.* (1993) demonstrated that in a *lasR*-deficient derivative of PAO1, 30% less ETA was produced compared to the parental strain. They also showed that when *lasR* was added on a multicopy plasmid, ETA production increased two-fold in PAO1. This data demonstrates that LasR acts to enhance ETA production but it is not required for ETA expression. This group concluded that the effect of LasR on ETA production was mediated through the *toxA* promoter itself, and not mediated through either promoter of the *regAB* operon. However, it was later demonstrated that Vfr is required for *lasR* expression since Vfr binds to the *lasR* promoter in order to activate expression

(Albus *et al.*, 1997). In addition, a LasR putative binding consensus sequence is present upstream of the P1 promoter of the *regAB* operon (Figure 2). Given this evidence, it is enticing to hypothesize that the indirect regulatory effect of Vfr may be mediated through LasR. The enhancing effect of LasR on ETA production may be mediated through the P1 promoter of the *regAB* operon.

1.5.3.5 Fur

The ferric uptake regulator (Fur) is a global iron regulator also implicated in the regulation of the *regAB* operon. Fur is a DNA-binding repressor which requires Fe^{2+} as a cofactor to bind to the promoters of Fur-regulated genes and repress transcription in high iron conditions (Bagg and Neilands, 1987). In low iron conditions, Fur is not bound by ferrous iron and Fur-regulated genes are de-repressed. In order to investigate the role of Fur in regulation of ETA production, *E. coli* Fur protein was over-expressed in *P. aeruginosa* PA103 and PAO1 (Prince *et al.*, 1991). In the presence of multi-copy *E. coli fur*, transcription from the *toxA* and *regAB* loci was undetectable in both high and low iron conditions. This data suggested that Fur may play a role in the regulation of ETA production. Prince *et al.* (1991) performed transcript accumulation studies which suggested that Fur regulated ETA production through the P1 promoter of *regAB*. It was surprising to find that an iron regulated protein such as Fur would have a role in the regulation of the iron-independent P1 promoter, and not the tightly iron regulated P2 promoter. This data suggested that the iron regulation at the P2 promoter was not mediated through Fur. The *P. aeruginosa fur* gene has been cloned and found to be 53% identical to the *E. coli* protein (Prince *et al.*, 1993). Recently, the *P. aeruginosa* Fur protein was purified and demonstrated not to bind directly to the promoter regions of *toxA* or *regAB in vitro* (Ochsner *et al.*, 1995). It has therefore been postulated that the regulatory effect of Fur on the P1 promoter may be mediated through another factor.

1.5.3.6 PvdS

PvdS was originally identified as a regulatory gene involved in activating pyoverdine synthesis (Cunliffe *et al.*, 1995; Miyazaki *et al.*, 1995). The PvdS (pyoverdine sigma factor) protein shows homology to *E. coli* FecI, an alternative sigma factor. Based on this strong homology and the presence of a putative DNA-binding motif at the C-terminal end, PvdS has been postulated to act as an alternative sigma factor for RNA polymerase (Cunliffe *et al.*, 1995; Miyazaki *et al.*, 1995). PvdS expression is negatively regulated by iron. Furthermore, the promoter region of *pvdS* contains a Fur-box element. These data suggest that expression of *pvdS* is regulated by the Fur repressor. Purified *P. aeruginosa* Fur protein was used in DNA binding studies to confirm that Fur does bind to the promoter region of *pvdS* (Ochsner *et al.*, 1995). And it was confirmed that Fur regulated *pvdS* expression in response to iron concentrations. PvdS has been demonstrated to be involved in the regulation of both pyoverdine biosynthesis genes, and ETA production (Miyazaki *et al.*, 1995; Leoni *et al.*, 1996; Ochsner *et al.*, 1996).

A functional *pvdS* gene is required for ETA production and for transcription from the *regAB* operon (Ochsner *et al.*, 1996). The regulatory effect of PvdS on ETA production may therefore be mediated through the *regAB* operon. Recent evidence has suggested that the regulatory effect of PvdS on *regAB* may be mediated through PtxR. Vasil *et al.* (1998) have recently provided evidence that PvdS also regulates *ptxR*. The regulatory cascade postulated by this group suggests that Fur regulates the expression of *pvdS* in response to iron conditions. PvdS then activates the expression of *ptxR*, and PtxR regulates ETA production by activation of the P1 promoter of *regAB* and, possibly also the *toxA* promoter. This model suggests that the regulation of ETA production involves a complex regulatory cascade. Additional complexity is evident when variations in oxygen concentration are also considered. Recent evidence suggests that the mechanism of regulation at the *regAB* operon may be modified when the bacteria experience microaerophillic conditions (Ochsner *et al.*, 1996).

A consensus binding sequence for PvdS was identified by Rombel and co-workers in 1995, and this sequence is present at the promoter region of all genes known to be regulated by PvdS, including pyoverdine promoters C1 and J, *pvdA*, *pvdD*, and *pvdE* (Miyazaki *et al.*, 1995; Merriman *et al.*, 1995). A previously unrecognized eight out of ten base pair match to this consensus sequence at the P2 promoter of the *regAB* operon was identified during the present studies (Figure 2). These data suggest that PvdS may play a role in the iron regulation at the P2 promoter.

1.6 Project Objectives

Expression from the P2 promoter is tightly regulated, yet specific regulators of this promoter have not been identified. The main objective of this study was to identify a regulator which acts on the tightly iron-regulated P2 promoter of the *regAB* operon. In order to carry out this objective, we took two main approaches. First, knowing that the P2 promoter is tightly regulated by iron, we searched the region upstream of the *regAB* start codon for known consensus binding sequences of iron regulators. The alternative sigma factor PvdS is regulated directly by Fur and an eight out of ten base pair match to the PvdS binding sequence was identified at the start site of the P2 promoter. The goal of this approach was to investigate the role of PvdS in iron regulation from the *regAB* operon and the effects on exotoxin A expression. Our second approach was to search the *P. aeruginosa* chromosome for a previously unidentified regulator that acts on the P2 promoter. A regulator of the P2 promoter may be a repressor which can sense high iron conditions and prevent transcription from the P2. Alternatively, a regulator may activate transcription from the P2 promoter by an unknown mechanism solely in low iron conditions. We hypothesize that PvdS acts as an activator of the P2 promoter and that other regulators also act to regulate the P2 promoter of the *regAB* operon.

CHAPTER 2: MATERIALS AND METHODS

2.1 Strains and Plasmids

All strains used in this study are listed in Table 1 and plasmids in Table 2. All genetic manipulations were performed in *E. coli* JM109, unless otherwise indicated. Bacterial strains were stored in 15% glycerol at -80°C and plasmids were stored at -20°C.

2.2 Growth Media

2.2.1 Liquid Media

E. coli cultures were grown in Luria-Bertani (LB) broth (Sambrook *et al.*, 1989). One litre of LB broth contains 10 g tryptone (Difco Laboratories), 5 g yeast extract (Gibco-BRL), and 5 g of NaCl, pH 7.0.

P. aeruginosa cultures were grown in LB broth, trypticase soy broth dialysate chelexed (TSBDC), or Vogel Bonner minimal medium (VBMM). One litre of TSBDC (Liu, 1973) was prepared by mixing 30 g of trypticase soy broth (BDH Inc.) and 5 g of chelex 100 resin, 100-200 mesh sodium form (Bio-Rad Laboratories) in 90 ml of double-distilled water (ddH_2O) for 4-6 hours at room temperature. The dialysate was poured into 40 mm-width Spectr/Por dialysis tubing with a molecular weight cut-off of 6-8000 (Spectrum Laboratories). The tubing was dialyzed overnight in one litre of ddH_2O at 4°C and the liquid was then autoclaved. Prior to use, 20 ml of filter-sterilized 50% glycerol (BDH Inc.) and 20 ml of filter-sterilized 2.5M MSG (Sigma Chemical Co.) were added. VBMM (Vogel and Bonner, 1956) contains 3 g of trisodium citrate (EM Science Inc.), 2 g of citric acid (BDH Inc.), 10 g of K_2HPO_4 (BDH Inc.), 3.5 g of $Na(NH_4)HPO_4 \cdot H_2O$ (EM Science Inc.) and 0.2 g of $MgSO_4 \cdot 7H_2O$, pH 7.0 (Fisher Scientific) per litre.

Table 1: Bacterial Strains

Strain	Description	Reference
E. coli		
DH5α	φ80Δ *lacZ* ΔM15 Δ(*lacZYA-argF*) U169 *recA*1 *endA*1 *hsdR*17 ($r_K^-m_K^+$) *supE*44 *thi*$^-$ *d*$^-$	Liss, 1987
JM109	*endA*1 *recA*1 *gyrA*96 *thi*, *hsdR*17 (r_K^- m_K^+) *relA*1 *supE*44 Δ(*lac-proAB*) [F', *traD*36, *proAB*, *lacI*^{q}ZΔM15]	Yanisch-Perron *et al.*, 1985
S17-1	*pro thi recA hsdR* Tp^r Sm^r; chromosomally integrated RP4-2-Tc::Mu-Km::Tn7; mobilizer of plasmids carrying the R68-derived Mob region	Simon *et al.*, 1983
HB101	*supE*44 *ara*14 *galK*2 *lacY*1 Δ (*gpt-proA*)62 *rpsL*20 (Str^r) *xyl*-5 *mtl*-1 *recA*13 Δ (*mcrC-mrr*) *HsdS*$^-$(r^-m^-)	Sambrook *et al.*, 1989

Strain	Description	Reference
P. aeruginosa		
PAO1	*regA*$^+$ *regB*$^-$ prototrophic lab strain	Holloway *et al.*, 1979
PA103	*regA*$^+$ *regB*$^+$ hypertoxigenic lab strain	Liu, 1966
IL-1	PA103 *regA*::*lacZ* ;*P2-lacZ P1*$^-$ phenotype	Loubens, unpublished
PAO1Δ*pvdS*	PAO1 with a 460 bp deletion in the *pvdS* gene; Gm cassette at the *pvdS* locus; Gm^R	Ochsner, *et al.*, 1996
PA103 *pvdS* ::Gm	Gm cassette at the *Stu*I site of the *pvdS* gene in strain PA103; Gm^R	this study
IL-1 *pvdS*::Ω	Ω cassette at the *Stu*I site of the *pvdS* gene in strain IL-1; Sm^R/ Spec^R	this study

Table 2: Bacterial Plasmids

Plasmid	Description	Reference
pUC18/19	cloning and sequencing vector; Ap^R	Gibco/BRL
pUC181.8	pUC18 containing the 1.8 kb *PstI* stabilizing fragment (SF) for Pseudomonas replication; Cb^R	Frank and Iglewski, 1988
pUCP18	pUC18 containing the 1.8 kb stabilizing fragment (SF) for *Pseudomonas* replication; Cb^R	Schweizer, 1991
pBluescript II SK(+)	cloning and sequencing vector; Ap^R	Stratagene
pGEM-7Zf(+)	cloning and sequencing vector; Ap^R	Promega
pUCGm	plasmid containing the Tn*1696*-derived Gm^R cassette flanked by the pUC19 MCS; Gm^R	Schweizer,1993a
pLD201.1	1005 bp *pvdS* PCR product ligated in the *EcoR*I site of pBluescript; Ap^R	Donegan and Storey, unpublished
pTH*pvdS*	1.8 kb SF from pUC181.8 in the *Pst*I site of pLD201.1; Cb^R	this study
pHP45Ω	1.9 kb Sm^R/$Spec^R$ interposon cassette in plasmid pHP45; Ap^R; $Strep^R$/$Spec^R$	Prentki and Krisch, 1984
pJQ200SK	plasmid containing the *sacB* cassette; pBluescript II SK MCS; Gm^R	Quandt and Hynes, 1993
pBS*pvdS*::Ω	Ω ligated into the *Stu*I site of *pvdS*; *pvdS*::Ω ligated into the *EcoR*I site of pBluescript II SK; $Strep^R$/$Spec^R$; Ap^R	this study
pJQ*pvdS*::Ω	Ω ligated into the *Stu*I site of *pvdS*; *pvdS*::Ω ligated into the *Xho*I and *Xba*I sites of the MCS of pJQ200SK; $Strep^R$/$Spec^R$; Gm^R	this study

pJQ*pvdS*::Gm	Gm ligated into the *Stu*I site of *pvdS*; *pvdS*::Gm ligated into the *Xho*I and *Xba*I sites of the MCS of pJQ200SK; Gm^R	this study
pSUP102::Tn5-B30	pACYC184 suicide derivative; carries Tn5 derivative B30 transposon; Tet^R; Gm^R	Simon *et al.*, 1989
pZ1918	promoterless *lacZ* gene cassette flanked by pUC19 MCS; Ap^R	Schweizer, 1993b
pP21	P2-*cat* transcriptional fusion reporter vector; Ap^R	Storey *et al.*, 1990
pP11	P1-*cat* transcriptional fusion reporter vector; Ap^R	Storey *et al.*, 1990
pS_8E	2.4 kb *Sal*I-*Eco*RI fragment upstream of the *regAB* locus, contains the LysR-type regulator and lyase genes; cloned into pUC12; Ap^R	Loubens, unpublished
pLyLR	plasmid for H399 complementation studies; 2.4 kb S_8E fragment ligated into *Sal*I and *Eco*RI of pUCP18; Cb^R	this study
pH399	*Cla*I digest from H399; contains B30 transposon insertion site and flanking DNA; interrupted lyase gene but intact LysR-type regulator; cloned into pGEM7Zf(+); Ap^R, Tet^R	this study
pTnMod-OGm	plasposon (self-cloning minitransposon derivative) for transposon mutagenesis; Gm^R	Dennis and Zylstra, 1998

2.2.2 Solid Media

Solid media was prepared as for liquid media except agar (Gibco-BRL) was added to a final concentration of 1.5% before autoclaving. The solid media was poured into sterile petri plates before it solidified. TY solid media was used for conjugation experiments. TY media contains 8 g of tryptone (Difco Laboratories), 5 g of yeast extract (Gibco-BRL), 2.5 g of NaCl (BDH Inc.), and 15 g of agar (Gibco-BRL), pH 7.0, per litre.

2.2.3 Antibiotics

For maintenance of plasmids, antibiotics were added to liquid and solid media. All antibiotics were prepared as stock solutions in ddH_2O and stored at -20°C except tetracycline, which was dissolved in ethanol before storage at -20°C. For maintenance of plasmids in *E. coli*, antibiotics were added to a final concentration of 100 μg/ml ampicillin, 15 μg/ml gentamicin, 50 μg/ml streptomycin, 10 μg/ml tetracycline, or 50 μg/ml kanamycin. For growth of *P. aeruginosa* containing plasmids or chromosomal insertions, media contained antibiotics at final concentrations of 400 μg/ml carbenicillin, 50 μg/ml gentamicin, 500 μg/ml streptomycin, 500 μg/ml neomycin, or 100 μg/ml tetracycline. All antibiotics were purchased from Sigma Chemical Co.

2.2.4 Additives

To allow for blue/white selection on solid media, 800 μl of 50 mg/ml β-galactosidase chromogenic substrate X-gal (Gibco-BRL) in N,N-dimethyl formamide (Sigma Chemical Co.) and 500 μl of 1M isopropylthio-β-D-galactoside IPTG (Gibco-BRL) was added to 1 litre of solid media prior to pouring into sterile petri dishes.

In liquid TSBDC media, high iron conditions were achieved by adding 10 mg/ml $FeCl_3$ (Sigma Chemical Co.) in 1M HCl to a final concentration of 10 μg/ml. Low iron conditions were achieved by acid washing all glassware in 20% HCl for at least four hours, rinsing ten times with ddH_2O, and then autoclaving. Wherever possible, sterile iron-free plastic equipment was used in place of glassware.

2.3 DNA Methods

2.3.1 Plasmid Isolation

2.3.1.1 Rapid Mini-Prep Method

Small-scale preparations of plasmid DNA from *E. coli* were performed based on a modified procedure by Zhou *et al.* (1990). Briefly, bacteria were incubated overnight at 37°C in LB media containing the appropriate antibiotic and the cells were pelleted by centrifuging 1.5 ml of culture in a Biofuge microfuge at 13 000 rpm for 2 minutes. The supernatant was decanted, leaving 50-100 µl of liquid. The cells were suspended in this liquid by vortexing (American Scientific Products) and 300 µl of TENS buffer (10mM Tris-HCl, 1mM EDTA, 100mM NaOH and 5% SDS) was added to lyse the cells. Chromosomal DNA was precipitated by the addition of 150 µl of 3M sodium acetate (pH 5.2). The mixture was spun for 5 minutes to pellet the cellular debris and chromosomal DNA. The supernatant was transferred to a fresh tube, 4 µl of 1 mg/ml DNase-free pancreatic RNase A (Sigma Chemical Co.) was added and the tube was placed at 37°C for 20 minutes. Proteins were removed by extracting with an equal volume of 24:1 chloroform: iso amyl alcohol. Plasmid DNA was precipitated by adding twice the volume of ice cold 100% ethanol and placing the tube on ice for 10 minutes. The precipitate was centrifuged at 10 000 rpm in a Beckman microfuge at 4°C for 10 minutes. The DNA pellet was washed with 70% ethanol, dried, and resuspended in 40 µl of deionized water.

2.3.1.2 Alkaline Lysis Method

Plasmid DNA from *P. aeruginosa* cells was prepared in small-scale based on the alkaline lysis method developed by Birnboim and Doly (1979). Bacteria were grown overnight in LB media containing the appropriate antibiotic. The cells were pelleted by centrifuging 1.5 ml quantities in a Biofuge microfuge for 1 minute at 13 000 rpm and resuspended in 100 µl of ice-cold GTE buffer (50mM glucose, 10mM EDTA, 25mM Tris-HCl pH 8.0 and 4mg/ml lysozyme). The mixture was

stored at room temperature for 5 minutes to allow digestion by lysozyme. Cells were lysed by adding 200 µl of ice cold 0.1N NaOH / 1% SDS and incubated on ice for 5 minutes. Precipitation of chromosomal DNA was then performed by adding 150 µl of an ice cold solution of 3M with respect to potassium and 5M with respect to acetate (pH 4.8) and incubated on ice for 5 minutes, followed by centrifugation in a Beckman microfuge for 5 minutes at 4°C. Two serial extractions were performed with an equal volume of 25:24:1 phenol: chloroform: iso amyl alcohol for extraction of proteins. To precipitate the plasmid DNA, two volumes of ice cold 100% ethanol were added and the mixture was incubated on ice for 20 minutes, followed by centrifugation in a Beckman microfuge for 10 minutes at 4°C. The DNA pellet was washed once in 70% ethanol, dried, and resuspended in 50 µl TE (10mM Tris-Cl, 1mM EDTA) pH 8.0 and 1 µl of 1mg/ml DNase-free pancreatic RNase A was added.

2.3.1.3 CsCl Density Gradient Ultracentrifugation Method

Large-scale preparations of plasmid DNA from *E. coli* were performed using a modified CsCl density gradient ultracentrifugation method described by Garger *et al.* (1983). Bacteria were inoculated into 250 ml of LB media containing the appropriate antibiotic and grown overnight. The cells were pelleted at room temperature by centrifugation in a Beckman centrifuge for 10 minutes at 7000 rpm and then resuspended in 6 ml of GTE buffer (50mM glucose, 25 mM Tris-HCl pH 8.0, and 10mM EDTA). The cell walls were digested by the addition of 1 ml of 20 mg/ml lysozyme in GTE buffer and the solution was incubated at room temperature for 10 minutes. Next, 14 ml of 1% SDS / 0.2 N NaOH was added and the mixture was incubated on ice for 5 minutes to allow cell lysis to occur. Chromosomal DNA was precipitated by the addition of 7 ml sodium acetate (3M sodium and 5M acetate, pH 4.8) and the mixture was placed on ice for an additional 15 minutes before being centrifuged for 10 minutes at room temperature (10 000 rpm). The supernatant was collected and extracted twice with 12 ml of 25:24:1 phenol: chloroform: iso amyl alcohol. The aqueous phase was removed and the nucleic acids were precipitated by the addition of a 0.6 volume of isopropyl alcohol (BDH

Inc.) and incubated at room temperature for 10 minutes. The precipitate was collected by centrifugation at 5000 rpm for 15 minutes. The pellet was air dried at room temperature for one hour and then resuspended in 500 µl of deionized water. Next, one gram of cesium chloride was dissolved into the liquid and 80 µl of ethidium bromide (10 mg/ml in deionized water) was added. Finally, 10 µl of a 1:100 dilution (in water) of Triton X-100 was added and the solution was thoroughly mixed. The mixture was pipetted into Quick-Seal® tubes (Beckman) and carefully heat-sealed using a Beckman heat sealing device. The tubes were placed in a TLN-120 rotor, covered with spacers and plugs, and the plugs were torqued to 120 in.-lb (13.6 N·m). An Optima™ TLX Ultracentrifuge was used to spin the tubes at 120 000 rpm for 1 hour at 20°C, deceleration setting 5. The red plasmid band was visualized under a hand-held UV lamp (Fisher Scientific) and removed using an 18-gauge needle on a 1ml syringe. Ethidium bromide was removed from the plasmid DNA solution by extraction with an equal volume of n-butanol (BDH Inc.). The extraction was repeated until the bottom layer, which contains the DNA, was colorless (approximately 10 extractions). The CsCl salt was then removed from the DNA solution by overnight dialysis in 1l of TES dialysis buffer (1 litre contains 1.27 g of Tris-HCl, 0.236 g of Tris Base, and 1.68 g of EDTA) at 4°C.

2.3.1.4 Sequencing Mini-Prep Method

In order to purify high-quality plasmid DNA for sequencing, a modified mini alkaline-lysis/PEG precipitation procedure was used (Applied Biosystems Inc.). Bacteria were grown overnight in 15 ml of LB media containing the appropriate antibiotic. 1.5 ml aliquots were centrifuged at 13 000 rpm for 1 minute in a Biofuge microfuge. The cell pellets were resuspended in 200 µl of GTE buffer (50mM glucose, 25mM Tris-HCl pH 8.0, and 10mM EDTA pH 8.0), followed by the addition of 300 µl of 0.2 N NaOH / 1% SDS and incubated on ice for 5 minutes to allow the cells to lyse. Next, 300 µl of 3.0 M potassium acetate pH 4.8 was added and the mixture was precipitated by incubating a further 5 minutes on ice. Cellular debris was removed by centrifugation in a microfuge at 13 000 rpm for 10 minutes at room temperature. The supernatant was collected and 4 µl of 10 mg/ml DNase-

free RNase A was added. RNA was digested by incubating at 37°C for one hour and 30 minutes. The solution was extracted twice with 400 µl chloroform and the total DNA was precipitated with an equal volume of isopropanol. The DNA was pelleted by centrifugation at room temperature for 10 minutes (13 000 rpm) and washed once with 70% ethanol. The dried pellet was then dissolved in 32 µl of deionized water. Plasmid DNA was precipitated by adding 8 µl of 4 M NaCl and 40 µl of 13% polyethylene glycol 8000 (Sigma Chemical Co.). The sample was incubated on ice for 30 minutes and then centrifuged for 30 minutes at 4°C in a Beckman microfuge. The plasmid DNA pellet was washed in 70% ethanol, dried, and resuspended in 15 µl of deionized water.

2.3.2 Genomic DNA Isolation

Chromosomal DNA was isolated from *P. aeruginosa* using a modified version of the method described by Ausubel *et al.* (1991). A 5 ml culture of bacteria was grown overnight and the cells were collected by centrifugation at 5000 rpm in a Beckman centrifuge. Bacterial cells were resuspended in 1.5 ml of proteinase K solution (50mM NaCl, 2% SDS, and 400 mg of proteinase K) and incubated at 42°C for 30 minutes. Next, 250 µl of 5M NaCl and 250 µl of CTAB/NaCl solution (10% CTAB (Fisher Scientific) in 0.7 M NaCl) were added and the mixture was incubated at 65°C for 30 minutes. The mixture was then extracted four times with 25:24:1 phenol: chloroform: iso amyl alcohol and once with 24:1 chloroform: iso amyl alcohol. Next, 0.05 volumes of 5M NaCl and two volumes of 100% ethanol were added to precipitate the DNA. The mixture was placed on ice for one hour, or overnight at -20°C, and the precipitate was collected by centrifugation for 15 minutes at 4°C in a Beckman microfuge. The pellet was then washed in 70% ethanol, dried, and dissolved in 20-40 µl of deionized water. One µl of DNase-free RNase A was added and the mixture was incubated at 37°C for 15 minutes to allow the contaminating RNA to be digested before use. Chromosomal DNA was stored at -20°C.

2.3.3 Restriction Enzyme Digests

All restriction enzymes and buffers used were purchased from Gibco-BRL or Pharmacia BioTech Ltd. Plasmid digestions were performed in a total volume of 10 µl. Typically, 5 µg of plasmid preparation was added to 1 µl of the appropriate 10X React buffer (Gibco-BRL) or 1-2 µl of 10X All-Phor-One buffer (Pharmacia BioTech Ltd.), and 1 µl (1-20 units) of restriction enzyme in a total volume of 10 µl with deionized water. Digests were incubated at 37°C for one hour to overnight. Chromosomal digests were prepared as for plasmid digests except the volume of enzyme used was typically 3 µl (3 to 60 units) and the total volume was typically 50 µl.

2.3.4 Agarose Gel Electrophoresis

Agarose gel electrophoresis was used to separate digested DNA fragments. The gels were prepared using 0.8% agarose (Gibco-BRL) in 1XTAE buffer (4.84 g Tris base, 1.14 ml glacial acetic acid, 0.675 g EDTA pH 8.0 per litre). Prior to loading into the gels, samples were mixed with one fifth the volume of DNA tracking dye (0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol in water). Agarose gels were run in Mini Horizontal Agarose Submarine Units (Bio-Rad Laboratories) in 1XTAE buffer at voltages ranging from 25 to 100 volts. Gels were typically run with a 1 kb plus DNA ladder (Gibco-BRL) to allow for estimation of DNA size. Following electrophoresis, the gels were stained in 500 ml TAE buffer and 30 µl of a 10 mg/ml solution of ethidium bromide. DNA bands were visualized using Bio-Rad Gel Doc 2000.

2.3.4.1 Eckhardt Gels

In order to facilitate rapid screening of plasmids for the presence of DNA inserts, an Eckhardt gel method was used. A 50 ml- 0.7% agarose gel in 0.5XTBE buffer (0.5XTBE is 5.4 g Tris base, 2.75 g boric acid and 2 ml of 0.5M EDTA pH 8.0 in 1 litre of ddH_2O) was prepared. Once the gel was cooled to about 60°C, 500 µl of 20% SDS was added. Each sample was prepared for loading as follows: 10 µl of TE buffer was dropped onto a sterile petri plate (VWR). A toothpick was

used to resuspend a colony of cells in the buffer. The mixture was then transferred to a microfuge tube. Next, 10 µl of SRL buffer (25% sucrose in Terrific Broth; Terrific Broth contains 12 g bactotryptone, 24 g yeast extract and 4 ml glycerol in 900 ml of deionized water, autoclaved and cooled; add 100 ml of sterile 0.17M KH_2PO_4 / 0.72M K_2HPO_4) and 2 µl of 10 mg/ml RNase A was added and the samples were heated to 90°C for 10 minutes. The samples were then cooled to 4°C and 0.01 g of lysozyme was added to each sample. 10 µl of each sample was loaded into a well of the gel in 0.5% TBE loading buffer. The samples were allowed to lyse by sitting in the wells for 30 minutes before the gel was run at 20 volts for 10 minutes and then the voltage was adjusted to 100 volts for one hour. The gels was then stained and visualized as for a typical agarose gel.

2.3.5 Isolation of DNA from Agarose Gels

Once the DNA had been separated by agarose gel electrophoresis, the desired band of ethidium bromide stained-DNA was visualized by ultra-violet light and excised from the gel using a razor blade and placed in a microfuge tube for DNA isolation.

2.3.5.1 Gene Clean

DNA less than 10 kb in length was purified from the excised agarose using a Geneclean II kit as per manufacturer's instructions (Bio 101 Inc.).

2.3.5.2 Electroelution

DNA isolated from agarose gels which was greater than 10 kb in length was purified from the agarose slice using electroelution in order to minimize DNA shearing. The DNA-containing agarose slices were placed into 40 mm width Spectr/Por dialysis tubing (molecular weight cut-off of 6-8000 Da Spectrum Laboratories Inc.) with 1XTAE buffer. The tubing was placed into a Horizontal Agarose Submarine Unit (Hoefer Scientific Instruments) and covered with 1XTAE buffer. The gel slices in the dialysis tubing were orientated such that the DNA is placed nearest to the cathode. DNA was electroeluted overnight at 1 Volt per cm

of distance between the anode and the cathode. Following electroelution, the polarity of the electrodes was reversed and the voltage was increased to 100 volts for 30 seconds in order to free any DNA which had adhered to the tubing. The TAE containing the DNA was collected from the dialysis tubing and precipitated by adding one tenth the volume of 3M sodium acetate (pH 4.8) and twice the volume of 100% ethanol. The tubes were placed at -20°C for at least one hour and the precipitate was collected by centrifugation in a Beckman microfuge for 10 minutes at 4°C. The DNA pellet was washed twice in 70% ethanol, dried, and dissolved in 10 µl of deionized water.

2.3.7 Acrylamide Gel Electrophoresis

In order to increase the resolution of DNA bands on a gel, the PCR products of the arbitrary primed PCR technique were run on an acrylamide gel. First, 10 ml of a 1% agarose gel was prepared in 1XTAE buffer and poured into the bottom of the glass plates to serve as a plug to prevent acrylamide leakage. The acrylamide gel was prepared by first mixing 5 ml of 10XE buffer (49.6 g Tris base, 2.72 g Na_2EDTA, 2 g NaOH and 20.8 ml of glacial acetic acid in 1 litre of water, pH 7.8) with 39.05 ml of water in a 250 ml Erylenmyer flask. In a 15 ml tube (VWR), 2.5 g of acrylamide (Gibco-BRL) and 0.067 g of BIS (Bio-Rad Laboratories) was dissolved in 5.5 ml of water. The acrylamide / BIS solution was added to the flask containing the E buffer solution. Next, 350 µl of 10% ammonium persulfate (Bio-Rad Laboratories) and 45 µl of TEMED (Bio-Rad Laboratories) was added to the mixture. The mixture was swirled gently to mix and applied to the glass plates above the hardened agarose plug. Once the acrylamide gel had polymerized, the SE 600 Series Vertical Slab gel unit (Hoefer Scientific Instruments) was assembled and 1XE running buffer was added. The samples were prepared by adding DNA loading dye as for agarose gel electrophoresis. The gel was run at 80 volts for 5 minutes to allow the samples to enter the gel and then increased to 100 volts for 3-4 hours. Following electrophoresis, the DNA was stained with ethidium bromide and visualized as for agarose gel electrophoresis.

2.3.7 DNA Ligation

Prior to ligation, vector DNA was dephosphorylated with Calf Intestinal Alkaline Phosphatase (CIAP; Gibco-BRL). Vector DNA was digested with the appropriate restriction enzyme overnight in a final volume of 50 µl. Next, 30 µl of water, 10 µl of 10XCIAP buffer, and 5 µl of a 1:1000 dilution of CIAP enzyme (dilutions were performed on ice in CIAP dilution buffer) were added to the digestion mix. For restriction enzymes which produce 5'-protruding ends, the mixture was incubated for 30 minutes at 37°C, a further 5 µl of diluted CIAP was added and the mixture was again incubated for 30 minutes at 37°C. For restriction enzymes which produce 5'-recessed ends, the mixture was incubated for 15 minutes at 37°C then 15 minutes at 56°C, a further 5 µl of diluted CIAP was added and the mixture was again incubated for 15 minutes at 37°C then 15 minutes at 56°C. To stop the dephosphorylation reaction, the mixture was heated to 75°C for 15 minutes and extracted once with an equal volume of 25:24:1 of phenol: chloroform: iso amyl alcohol.

DNA fragments were typically ligated in a 10 µl final reaction volume using a 3:1 ratio of insert to dephosphorylated vector DNA. 2 µl of 5X ligation buffer was added to the insert and vector mixture and the volume was brought up to 9.5 µl with the addition of deionized water. 0.5 µl of T4 DNA ligase (Gibco-BRL) was added to the ligation mix and incubated at 16°C overnight or at room temperature for one hour to allow for ligation.

Blunt-end ligations were performed using the Takara DNA Blunting Kit, ligation reaction (Takara Shuzo Co., Ltd.) as per manufacturer's instructions.

2.3.8 DNA Transformation

Transformation of DNA into both *E. coli* and *P. aeruginosa* cells was performed. Plasmids and ligations were routinely transformed into *E. coli* by chemical means, unless efficiencies were poor, in which case electroporation was used. Electroporation was used to transform plasmids for transposon mutagenesis, and ligations into *P. aeruginosa*. Plasmid transformations into *P. aeruginosa* were routinely performed by chemical transformation.

2.3.8.1 Electroporation of *P. aeruginosa*

Electroporations were performed on *P. aeruginosa* using the method described by Smith and Iglewski (1989). For preparation of electro-competent cells, a 3 ml overnight culture was diluted into 50 ml of LB broth and incubated at 32°C with shaking until the OD_{600} reached 0.3 to 0.5. The cells were then harvested by centrifugation in a Beckman centrifuge at 5000 rpm for 10 minutes at 4°C. The cells were washed once with the same volume of sterile ice-cold 300mM sucrose, and once with half the volume of 300mM sucrose. The cells were then resuspended in 250 µl of ice-cold 300mM sucrose and chilled on ice for 30 minutes. Each electroporation mix consisted of 40 µl of electrocompetent *P. aeruginosa* cells and 1-5 µl of plasmid DNA or 5-10 µl of ligation DNA. Prior to electroporation, ligation mixtures were precipitated in 100% ethanol and washed twice with 70% ethanol to remove any salts which would interfere with the electroporation procedure. Each electroporation mixture was transferred into a chilled 0.2 cm gap cuvette (Bio-Rad Laboratories) and pulsed at 8kV/cm (1.6 kV for 0.2cm cuvettes) in a BioRad Gene Pulser II with a Pulse controller II, with 200Ω resistance and 25 µF capacitance. The cells were then added to 3 ml of LB broth and allowed to recover by incubation at 37°C for 1-2 hours. The cells were then plated onto solid media containing the appropriate concentration of antibiotics and incubated overnight at 37°C.

2.3.8.2 Electroporation of *E. coli*

Electrocompentent *E. coli* JM109 cells were prepared as described by Miller and Nickoloff (1995). An overnight culture of *E. coli* JM109 was diluted into 1 litre of LB broth and incubated with shaking at 37°C until the OD_{600} reached 0.4 to 0.5. The cells were centrifuged at 6000 rpm for 10 minutes and resuspended in 20 ml of sterile ice-cold ddH_2O. The cells were then washed two additional times in 10 ml of ice-cold ddH_2O. The cells were washed once in 10 ml of ice-cold 10% glycerol, then resuspended in 250 µl of ice-cold 10% glycerol. Aliquots of 40 µl were flash frozen in dry ice and stored at -80°C until required for use.

Electroporation of *E. coli* JM109 cells was performed essentially as for *P. aeruginosa* cells except the cuvettes were pulsed at 2.5kV in 0.2 cm cuvettes with 200Ω resistance and 25 μF capacitance.

2.3.8.3 Chemical Transformation of *P. aeruginosa*

Chemical transformation of plasmid DNA into *P. aeruginosa* cells was performed as described by Olsen *et al.* (1982). The *P. aeruginosa* strain was streaked onto a LB agar plate and incubated overnight at 37°C. The following morning, a single colony was used to inoculate 25 ml of LB and the culture was incubated with shaking for 2-3 hours until the OD_{540} reached 0.125. The cells were then centrifuged at 8000 rpm for 5 minutes in a Beckman centrifuge, resuspended in half the volume of sterile ice-cold 0.15M $MgCl_2$, and incubated on ice for 5 minutes. The cells were re-washed in half the volume of cold 0.15M $MgCl_2$ and incubated on ice for a further 20 minutes. The cells were centrifuged again and resuspended in 0.1 of the volume of ice-cold 0.15 M $MgCl_2$. For each transformation, 200 μl of competent cells were mixed with 1 μg of plasmid DNA and incubated on ice for 60 minutes. The transformation mixture was heat-shocked at 37°C for three minutes, incubated on ice for 5 minutes and re-incubated in 500 μl of LB broth for 1-2½ hours at 37°C. The cells were then plated onto VBMM agar plates containing the appropriate concentration of antibiotics and incubated overnight at 37°C.

2.3.8.4 Chemical Transformation of *E. coli*

E. coli JM109 or S17-1 competent cells were prepared by preparing an overnight culture in 2 ml of LB broth at 37°C. 500 μl of the overnight culture was added to 50 ml of LB broth and the culture was shaken at 37°C until the OD_{600} reached 0.45-0.55. The culture was chilled in ice water for 2 hours and the cells were collected by centrifugation at 5 000 rpm for 15 minutes in a Beckman centrifuge. The cells were resuspended in 1 ml of sterile ice-cold trituration buffer (100mM $CaCl_2$, 70mM $MgCl_2$, 40mM Na-Acetate, pH 5.5) and then diluted to 25 ml with the same solution. The cells were centrifuged at 5000 rpm for 10 minutes and

resuspended in 5 ml of cold trituration buffer. Next, 1.15 ml of sterile 80% glycerol was added drop-wise while the solution was agitated in ice water. The cells were then aliquotted into 1.5 ml microcentrifuge tubes in 200 µl quantities, flash frozen in dry-ice and stored at -80°C until ready for transformation.

One 200 µl aliquot of competent *E. coli* JM109 or S17-1 cells was used for each transformation. The tube was allowed to thaw on ice for 15 minutes before 10 µl of ligation mix or 1 µl of plasmid DNA was added. The mixture was placed on ice for 30 minutes and then heat shocked at 42°C for 2 minutes. The cells were allowed to recover in 1 ml of LB broth for 1-1½ hours at 37°C with shaking. The cells were then plated onto LB agar plates which contained the appropriate concentration of antibiotics and incubated overnight at 37°C.

2.3.8.5 Conjugation

For the transposon mutagenesis of *P. aeruginosa* strain IL-1, using the B30 transposon, the electroporation efficiencies were calculated to be quite low. As a result, conjugation procedures were also attempted.

2.3.8.5.1 Biparental Mating

The plasmid pSUP102::Tn5-B30 was transformed into competent S17-1 cells as described in section 2.3.8.4. *E. coli* strain S17-1 (pSUP102::Tn5-B30) was the donor strain for the conjugation while *P. aeruginosa* strain IL-1 was the recipient strain. Each strain was grown overnight in LB broth at 37°C and the following morning, the cultures were inoculated and grown in LB broth until the donor cells reached an OD_{600} of 0.5 and the OD_{600} of the recipient culture was 2. The two cultures were mixed together and then centrifuged in a Beckman centrifuge for 10 minutes at 8000 rpm to pellet the cells. The cells were resuspended in minimal LB broth and placed onto a sterile filter on a TY plate. The TY plate was incubated overnight at 37°C and conjugation occurred on the filter. The following morning, the filter was placed into 3 ml of sterile 8.5% saline solution and vortexed to resuspend the cells in the saline solution. The cells were plated onto VBMM plates containing 100 µg/ml of tetracyline. Transconjugants were then

picked onto LB agar plates containing 50 μg/ml of gentamicin to ensure that transposon integration was successful.

2.3.8.5.2 Triparental Mating

For the triparental mating technique, the procedure is identical to the biparental mating method with the following modifications. The donor strain used was *E. coli* DH5α (pSUP102::Tn5-B30) and an additional helper strain was required for plasmid mobilization. The helper strain used was *E. coli* HB101 (pRK2013). As for the biparental mating, the recipient strain was *P. aeruginosa* strain IL-1. Overnight cultures were inoculated into LB broth and incubated until the cell concentrations were $5X10^7$ for the donor and helper strains, and $2X10^8$ for the recipient strain. The three cultures were mixed and the procedure for the biparental mating was followed.

2.3.9 Southern Hybridization

Southern Blotting was performed as described by Ausubel *et al.* (1991) with some modifications.

2.3.9.1 Transfer to Nytran Membrane

Restriction enzyme digested DNA to be blotted was electrophoresed on an agarose gel to allow for separation of the fragments. The gel was then soaked in 0.25 M HCl for 8-10 minutes to allow the purines to be broken down. The gel was rinsed twice in deionized water and soaked in 1.0 M NaCl / 0.5 M NaOH for 15 minutes two times to allow the double stranded DNA to be denatured into single stranded DNA. The gel was then soaked twice for 15 minutes in 0.5 M Tris pH 7.4 / 1.5 M NaCl. The squash blot was then assembled by placing a piece of 20XSSC (175.3 g NaCl and 88.2 g Na-citrate per litre, pH 7) soaked Whatman 3M chromatography paper width-wise across a glass plate which was positioned across a shallow glass pan containing 20XSSC. The treated gel was placed wells down onto the soaked Whatman paper. A Nytran® plus membrane (Schleicher and Schuell) which was previously soaked in 10XSSC was placed on top of the gel

and the entire apparatus was covered with a large piece of saran wrap with a rectangular cut hole which is positioned exactly over the gel-membrane stack. Three to four pieces of Whatman 3M paper were placed over the membrane and a stack of paper towels was placed on top. Finally, one kilogram of weight was placed on top. The DNA was allowed to transfer to the Nytran membrane overnight at room temperature. The following morning, the blot was disassembled and the membrane was washed in 5XSSC for 5 minutes at room temperature. It was then wrapped in saran wrap and exposed to UV light for 5 minutes to cross-link the DNA to the membrane. The membrane was stored at 4°C. The squashed gel from the blotting procedure was stained in ethidium bromide and visualized to ensure complete transfer of nucleic acids as for the agarose gel electrophoresis procedure.

2.3.9.2 Probe Preparation

DNA fragments to be used as probes were digested, electrophoresed on an agarose gel, and isolated as described previously. DNA fragments were labelled with [γ^{32}P]dCTP (Dupont NEN Products) using an Oligolabelling Kit (Pharmacia) as per manufacturer's instructions. Unincorporated nucleotides were removed using a Nensorb 20 nucleic acid purification cartridge (NEN Research Products) or QIAquick ^{32}P nucleotide removal spin columns (Qiagen) as per manufacturer's instructions. After purification, 1 µl of labelled probe was tested in a Beckman Liquid Scintillation 6500 multi-purpose scintillation counter to ensure that the labelling reaction was successful before using the probe for hybridization.

2.3.9.3 Hybridization

The Nytran membrane was prehybridized for 1-4 hours at 42°C with shaking. Prehybridization solution was prepared in a 20 ml total volume by mixing together 6 ml of 20XSSC, 4 ml of 50X Denhardt's solution (10 g Ficoll 400, 10 g polyvinylpyrrolidone, and 10 g bovine serum albumin per litre), 2 ml of 10% SDS, and 7.9 ml of ddH_2O. The mixture was warmed to 42°C. 100 µl of 10 mg/ml DNA sodium salt from salmon testes (Sigma Chemical Co.) was boiled for 5 minutes

before being added to the prehybridization mixture. The prehybridization mixture was poured into a hybridization bag (Gibco-BRL) with the membrane. The bag was then closed with a heat sealer (Decosonic Inc.) and taped into a square plastic box for pre-hybridization.

Following prehybridization, the hybridization bag was opened and the prehybridization mixture was discarded and replaced with 20 ml of hybridization solution. Hybridization solution was prepared by mixing 6 ml of 20XSSC, 2 ml of 10% SDS, 10 ml of deionized formamide (Sigma Chemical Co.), and 2 ml of ddH_2O and prewarming to 42°C. The radiolabelled probe and 100 µl of salmon testes DNA (10mg/ml) were boiled for 5 minutes and added to the hybridization solution. The hybridization bag was again heat-sealed, taped to a plastic box, and placed in the 42°C water bath with shaking to allow the probe to hybridize to the membrane overnight.

2.3.9.4 Washing

Following overnight hybridization, the Nytran membrane was washed twice in 6XSSC / 0.1-0.5% SDS for 15 minutes at room temperature, and then twice in 1XSSC / 0.5-1% SDS for 15 minutes at 42°C to remove excess unhybridized probe. The membrane was blotted briefly on Whatman 3M paper and wrapped in saran wrap to prepare for radiography.

2.3.9.5 Visualization

The probed membrane was placed into an X-ray cassette with image intensifying screens. A Kodak Scientific Imaging film X-OMAT AR was placed into the cassette on top of the membrane. The film was allowed to be exposed to the membrane for 1 to 48 hours before development as per manufacturer's instructions.

2.3.9.6 Membrane Stripping

In some cases, membranes were probed with multiple probes. When this was the case, the first probe needed to be removed from the membrane before the

membrane could be probed with the subsequent probes. In order to remove a radiolabelled probe from a Nytran membrane, the membrane was heated in 100 ml formamide, 64 ml 20XSSC, and 36 ml of ddH_2O for 45 minutes at 65°C. The membrane was then rinsed in 2XSSC, wrapped in saran wrap, and stored at -80°C until ready for use. Autoradiography was used to monitor complete probe removal.

2.3.10 Colony Blotting

In order to facilitate screening of colonies for the correct chromosomal insert or the presence of a plasmid construct, a modification of the colony blotting protocol described by Schleicher and Schuell was used.

2.3.10.1 Transfer to Membrane

Single colonies were picked with sterile toothpicks onto replicate LB agar plates containing the appropriate concentration of antibiotics. The plates were incubated overnight at 37°C. One plate was stored at 4°C while the colonies on the duplicate plate were transferred to a Nylon membrane (Amersham Hybond-N^+) as follows. Briefly, the membrane was placed on top of the agar and the colonies were allowed to transfer for 2 minutes at room temperature. The membrane was then soaked on 6 ml of denaturing solution (1.5M NaCl, 0.5M NaOH) for 7 minutes with the colonies directed away from the solution. The membrane was then briefly blotted onto Whatman chromatography paper and placed onto 6 ml of neutralizing solution (1.5M NaCl, 0.5M Tris-HCl pH 7.2, 0.001M EDTA) for 3 minutes. The wash in neutralizing solution was repeated for a further 3 minutes before the membrane was washed onto 6 ml of 0.4M NaOH for 45 minutes in order to fix the membrane. The membrane was then rinsed in 5XSSC for a maximum of one minute before being wrapped in saran wrap and stored at 4°C until needed.

2.3.10.2 Probe Preparation

Probes to be used for the colony hybridization protocol were prepared in the same manner as for the Southern Blot (section 2.3.9.2).

2.3.10.3 Hybridization

The membrane was prehybridized for at least one hour at 65°C in 10 ml of prehybridization solution (10 ml of prehybridization solution contains 2.5 ml of 20XSSC, 1 ml of 50X Denhardt's solution, 0.5 ml of 10%SDS, 6 ml of ddH_2O, and 200 µl of salmon testes DNA which was previously boiled for 5 minutes). The prehybridization mixture and the membrane were added to a hybridization bag and the bag was heat-sealed, as for the Southern Blot. After the prehybridization period, the purified probe was boiled for 5 minutes and added to the prehybridization solution in the hybridization bag. The bag was then re-sealed and placed in the plastic container. The membrane was allowed to hybridize at 65°C overnight with shaking.

2.3.10.4 Washing

The following morning, the membrane was washed to remove all un-hybridized probe. For the first wash, the membrane was soaked in a solution of 2XSSC and 0.1% SDS for 10 minutes at room temperature with shaking. This first wash was repeated once. Next, the membrane was soaked in 1XSSC and 0.1% SDS for 15 minutes at 65°C. For the third wash, the membrane was soaked for 10 minutes in 0.1% SSC and 0.1% SDS at 65°C with shaking. Following these three washes, the membrane was blotted briefly on Whatman paper and wrapped in saran wrap to prepare for radiography.

2.3.10.5 Visualization

The probed membrane was placed into an X-ray cassette with image intensifying screens as for in the Southern Blot (section 2.3.9.5). A Kodak Scientific Imaging film X-OMAT AR was placed into the cassette on top of the membrane and the film was allowed to be exposed to the membrane for 1 to 48 hours before development as per manufacturer's instructions.

2.3.11 DNA Sequencing

Plasmid DNA for sequencing was prepared by performing the sequencing mini-prep described previously (section 2.3.1.4). PCR products for sequencing were purified using the Geneclean II procedure as described above (section 2.3.5.1). Automated sequencing was performed by the University Core DNA Services, University of Calgary, Calgary, Alberta. The automated sequencing reactions were performed with a "big dye terminal cycle sequencing ready reaction kit" using standard automated sequencing reactions in an ABI Prism™ DNA sequencer.

2.4 Biochemical Assays

2.4.1 β-Galactosidase Assays

β-galactosidase assays were performed on strain IL-1 to indicate P2 promoter activity and on all other constructs containing a *lacZ* reporter gene.

2.4.1.1 Microtitre Method

In order to facilitate screening of IL-1 transposon mutants, a modification of the microtitre β-galactosidase assay method by Kolmar *et al.* (1994) was used. Cultures were inoculated overnight in 0.5 ml of high or low iron TSBDC at 32°C with aeration. The following morning, 200 µl of each culture was transferred into a well of a Falcon microtitre plate (Becton Dickinson Labware). The microtitre plates were centrifuged in an IEC Centra GP8R centrifuge for 5 minutes. The supernatant was discarded and the cells were resuspended in 200 µl of Buffer Z (one litre of Buffer Z contains 8.55 g of Na_2HPO_4, 5.5 g of $NaH_2PO_4 \cdot H_2O$, 0.75 g of KCl and 0.246 g of $MgSO_4 \cdot 7H_2O$ in ddH_2O). Ten µl of the cell suspension was then transferred into a chloroform-resistant 96 well Microtest III ELISA plate (Becton Dickinson Labware) for assay. In a fume hood, 100 µl of Lysis Buffer (15 ml contains 3 ml of 10XBuffer Z, 5 µl of 10%SDS, 10.6 ml of ddH_2O, and 1.4 ml of β-mercaptoethanol) and 10 µl of chloroform was added to each well. The cells in the microtitre plate were allowed to lyse at room temperature for 5 minutes. Next,

100 µl of 4mg/ml o-nitrophenyl-β-D-galactopyranoside oNPG (Sigma Chemical Co.) in phosphate buffer (3 g K_2HPO_4 and 1 g of NaH_2PO_4 in 50 ml of ddH_2O) was added and the color was allowed to develop. To stop the reaction, 100 µl of 1 M Na_2CO_3 was added and the time before stopping the reaction was noted. The OD_{405} of the ELISA plate and the OD_{605} of the flexible microtitre plate was read using a Molecular Devices microplate reader.

2.4.1.2 Test Tube Method

In order to get a more precise measurement of the β-galactosidase activity of a strain, β-galactosidase assays were performed in test tubes in duplicate using a modified method of Miller (1972). One ml of an overnight culture to be assayed was centrifuged for 3 minutes in a Biofuge microfuge at 13 000 rpm to pellet the cells. The pellet was resuspended in 1 ml of 1XA Buffer (one litre contains 10.5 g of K_2HPO_4, 4.5 g of KH_2PO_4, 1 g of $(NH_4)_2SO_4$, and 0.5 g of sodium citrate·$2H_2O$ in ddH_2O). The cells could then be assayed immediately or stored at -80°C for later assay. Before performing the β-galactosidase assay, the OD_{600} of the cells was recorded for the calculation of β-galactosidase activity. To perform the assay, 100 µl of cells in 1XA buffer was added to a chloroform-resistant 6 ml Falcon tube (Becton Dickinson Labware). 900 µl of Buffer Z, 20 µl of 0.1% SDS and 40 µl of chloroform was added to each tube and mixed by vortexing for 1 minute. The tubes were incubated at room temperature for 5 minutes to allow the cells to lyse. Next, 200 µl of 4mg/ml oNPG in 1XA buffer was added to each tube and timing commenced. When the liquid in the tube turned a light straw yellow color (OD_{420} of approximately 0.5), the reaction was stopped by adding 500 µl of 1M Na_2CO_3 and the time was noted. Using a spectrophotometer (Beckman), the OD_{420} was recorded as an indication of the colorometric change in the reaction and the OD_{550} was recorded as an indication of the concentration of cellular debris. Units of β-galactosidase activity were calculated using the formula of Miller (1972):

$$\text{Miller Units} = 1000 \times \frac{OD_{420} - 1.75 \times OD_{550}}{t \times v \times OD_{600}}$$

where t is the time in minutes and v is the volume of the sample used in ml (0.1 ml).

2.4.2 *cat* Assay

Chloramphenicol acetyltransferase (CAT) reporter activity of plasmids pP21 and pP11 was assayed using a CAT ELISA kit (5 Prime→ 3 Prime Inc.) as per manufacturer's instructions. The absorbance at 405nm was measured using a Ceres uv9HDi microtitre plate reader (Mandel).

2.4.3 Protein Assay

In order to assay the concentration of proteins in each *cat* assay sample, a Bio-Rad colorimetric assay was used (Bio-Rad Laboratories). A microtitre plate method was selected in order to allow multiple samples to be assayed simultaneously. Bovine serum albumin standards were prepared and were assayed in five wells of each microtitre plate at the same time as samples were being assayed. Ten ml of each sample was applied to wells of a microtitre plate and 200 ml of diluted Bio-Rad dye reagent was added. The samples were mixed and allowed to incubate at room temperature for 15 minutes before the absorbance was measured at 595 nm in a Ceres uv900HDi microtitre plate reader (Mandel). Sample absorbances were compared to the standards for calculations of protein concentrations.

2.4.4 Exotoxin A Assay

Extracellular ADP-ribosyltransferase activity was assayed as described by Chung and Collier (1977). Cell-free bacterial supernatants to be assayed were collected by centrifugation in a Biofuge at 13 000 rpm. Ten μl of supernatant was mixed with 10 μl of urea-DTT solution (1 ml contains 0.02 g of DTT and 1 ml of 8M urea) and the mixture was incubated at room temperature for 15 minutes.

Following the incubation period, 25 µl of T-II-C (125 mM Tris pH7 and 100 mM DTT), 25 µl of EF2 from wheat germ extract (EF2 was prepared from wheat germ extract as described by Iglewski and Kabat (1975)), and 5 µl of ^{14}C labelled NAD (Amersham) were added. The mixture was incubated at room temperature for 40 minutes and the reaction was stopped by precipitating the proteins with 200 µl of 10% TCA. The precipitate was filtered over 0.45 µM nitrocellulose filters (Schleicher and Schuell). The filters were rinsed twice with 5% TCA and once with 75% ethanol. The filters were then placed into scintillation vials (Fisher Scientific) and air-dried for at least one hour. Five ml of liquid scintillation fluid was added to each vial and the vials were counted for ^{14}C in a Beckman Liquid Scintillation 6500 multi-purpose scintillation counter.

2.4.5 Protease and Elastase Assays

In order to qualitatively assess the production of virulence factors by the transposon mutants, two plate assays were performed. Elastase production was assessed by streaking the strains onto Reverse Elastin Plates. These plates contain Nutrient Agar (8 g/l Nutrient broth, 2% Noble agar, pH 7.5) with an elastin overlay (8 g/l Nutrient broth, 2% Noble Agar, 0.5% Elastin). Elastase production was indicated by a zone of clearance surrounding the bacterial colony after overnight incubation at 37°C, caused by the production of elastase which degrades the elastin in the overlay. The production of protease was monitored by streaking the strains onto Skim Milk plates and incubating overnight at 37°C. Skim Milk plates are composed of 850 ml Brain-Heart infusion agar (38 g BHI broth, 15 g Select agar and 850 ml ddH_2O) and 150 ml of Skim Milk (15 g skim milk powder and 150 ml ddH_2O). A zone of clearance around the bacterial colony indicated the production of proteases which are able to degrade components of the skim milk.

2.5 Growth Curve Methodology

ADP-ribosyltransferase, β-galactosidase and CAT ELISA assays were typically performed on samples collected over the course of a growth curve. Growth curves were performed over a 24-30 hour time period and samples were

collected at varying time intervals as indicated. All growth curves were repeated twice unless otherwise indicated.

2.5.1 Preparation of Materials

To remove any residual iron, all glassware used for growth curves was washed in 20% HCl for at least four hours, rinsed with ddH_2O ten times and then autoclaved. All volume measurements were performed with plastic tips or in plastic tubes in order to avoid introducing iron into low iron solutions. High and low iron materials were treated in the identical manner except that 10 μg/ml of $FeCl_3$ was added to high iron cultures.

2.5.2 Preparation of Cultures

Two days before a growth curve was to commence, cultures were streaked onto LB agar plates containing the appropriate antibiotic concentration. The following day, primary *P. aeruginosa* cultures were prepared by inoculating a single colony into 5 ml of TSBDC media in an acid-washed flask. The appropriate antibiotics were added and 5 μl of a 10 mg/ml stock of $FeCl_3$ was added to the cultures to allow the cells to grow under high iron conditions. Primary cultures were always incubated under high iron conditions so that activity from the P2 promoter of the *regAB* operon would be repressed. This ensures that the growth curve reflects the activation of the P2 promoter over time.

Secondary cultures in TSBDC were prepared on the morning of the growth curve. Each primary culture was used to inoculate both a high iron (10μg/ml) and a low iron culture. High iron secondary cultures were prepared by transferring a volume of cells from the primary culture such that the OD_{600} at time zero was 0.02. Low iron secondary cultures were prepared by pipetting a volume of cells from the primary culture to give an OD_{600} of 0.02 into an Eppendorf tube. The tube was then centrifuged in a Biofuge for 3 minutes at 13 000 rpm to pellet the cells. The supernatant, which contained iron from the primary culture, was discarded and the pellet was resuspended in fresh TSBDC and used to inoculate the secondary low iron culture.

Aliquots were removed at indicated time points. For all time points, the absorbance was recorded at 600nm as an indication of growth. Samples for β-galactosidase assays were centrifuged at 13 000 rpm in a Biofuge for 5 minutes and the supernatant was discarded. The cell pellets were resuspended in a volume of 1XA buffer such that the OD_{600} was approximately 1. Samples were stored at -80°C until β-galactosidase assays were performed on each sample in duplicate as described in section 2.4.1.2. Samples for exotoxin A assays were centrifuged for 5 minutes and 1 ml of supernatant was removed into a fresh Eppendorf tube. The tube was centrifuged for an additional 5 minutes and 800 μl of supernatant was transferred to a fresh Eppendorf tube. The supernatant was stored at -80°C until ADP-ribosyltransferase assays were performed. This method of cell-free sample collection ensures that only extra-cellular exotoxin A activity will be measured. Samples for *cat* assays were centrifuged for 5 minutes at 4°C in a Beckman microcentrifuge. The volume of sample removed was such that the final OD_{600} prior to centrifugation was approximately 5. The cell pellet was washed in 1 ml of cold 100mM Tris (pH 7.8) and recentrifuged for 2 minutes. The supernatant was discarded and the cell pellet was stored at -20°C.

2.6 PCR Methodology

All PCR reactions were prepared in a Biological Containment Hood which was cleared of contaminating DNA using an ultra-violet light. PCR reactions were performed in a Perkin Elmer GeneAmp PCR system 2400.

2.6.1 Primers

All primers used for PCR methods are listed in Table 3.

2.6.2 Arbitrary-Primed PCR

An arbitrary-primed PCR method was used to attempt to sequence the Tn5-B30 insertion site directly from the chromosomal DNA of mutant L522. The method used was a modification of Caetano-Anolles (1993) and it is depicted

Table 3: Primers Used for PCR

Oligo Name	Description	Sequence (5' to 3')
B30-neo	primer out from the Tn5-B30 transposon	GCCGGAGAACCTGCGTGCAATCCA
IS50R	primer out from the Tn5-B30 transposon	TAGGAGGTCACATGGAAGTCAGAT
arb1	Arbitrary primer	GGCCACGCGTCGACTAGTACNNNNNNNNNNGATAT
arb2	Arbitrary primer	GGCCACGCGTCGACTAGTAC
arb6	Arbitrary primer	GGCCACGCGTCGACTAGTACNNNNNNNNNNACGCC

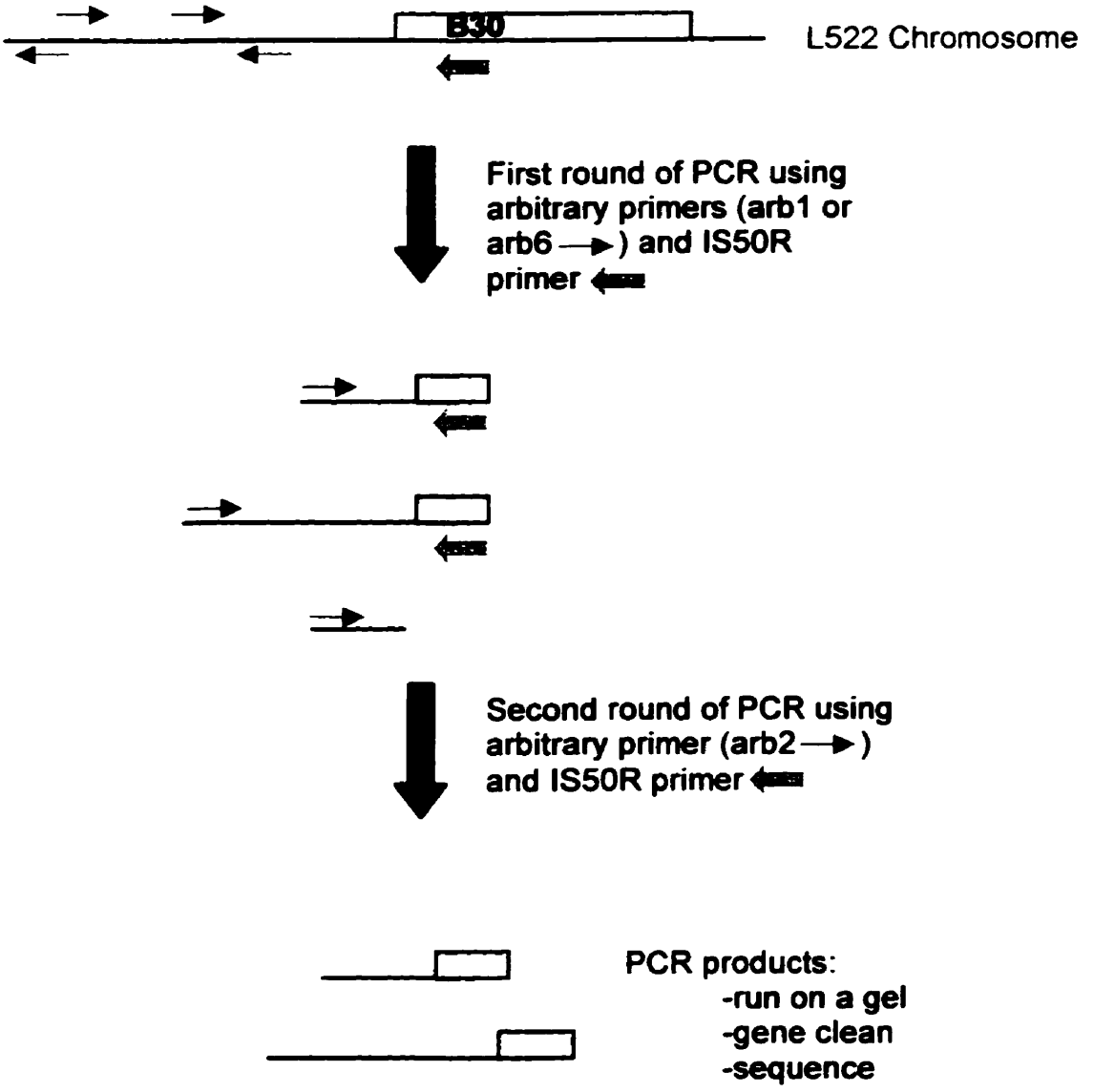
schematically in Figure 3. Two complete rounds of PCR were performed in this procedure to ensure specificity of resulting PCR products.

The reaction mix for the first round of PCR contained 5 l of 10X Taq PCR reaction buffer, 1 μl of arb1 or arb6 primer (1μM), 1 μl of transposon primer B30-neo or IS50R (1 μM), 2 μl of chromosomal DNA diluted 1:50, 4 μl of dNTP's (10 mM dATP, dTTP, dCTP, dGTP), 4 μl of 25 mM $MgCl_2$, 32.5 μl of ddH_2O, and 0.5 μl of Taq polymerase (Gibco-BRL). The chromosomal template used in the PCR reaction contained the B30 transposon inserted in the chromosome (mutant L522). A negative template control using IL-1 genomic DNA as a template was always run in parallel to aid in the identification and elimination of non-specific PCR products (products which were a result of amplification due to arbitrary primers only). The PCR cycle for the first round of PCR was 95°C for 5 minutes; five cycles of 94°C for 30 seconds, 30°C for 30 seconds, and 72°C for 1.5 minutes; followed by 30 cycles of 94°C for 30 seconds, 45°C for 30 seconds, and 72°C for 2 minutes; concluding with 5 minutes at 72°C and holding at 4°C.

The product of the first PCR reaction was used as a template for the second reaction. The arbitrary primer used for the second PCR reaction is more specific than the arbitrary primer used in the first reaction. The reaction mixture for the second PCR reaction consisted of 5 μl of 10X Taq PCR reaction buffer, 1 μl of arb2 primer (1μM), 1 μl of transposon primer B30-neo or IS50R (1 μM), 1 μl of the PCR product from the first round of PCR, 4 μl of dNTP's (10 mM dATP, dTTP, dCTP, dGTP), 4 μl of 25 mM $MgCl_2$, 33.5 μl of ddH_2O, and 0.5 μl of Taq polymerase. The PCR reaction for the second round of PCR was 29 cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1.5 minutes; a final extension of 72°C for 5 minutes; and 4°C to hold. The products of the second PCR reaction were run on an acrylamide gel (section 2.3.7) and bands which were unique to the PCR using the L522 chromosomal DNA as a template (compared to the products using the IL-1 chromosomal DNA as a template) were run on an agarose gel, isolated using the Geneclean II method and sent to be sequenced by the University of Calgary Core DNA Services.

Figure 3: Schematic representation of the arbitrary primed PCR technique.

Primer sequences are listed in Table 3. PCR conditions are described in section 2.6.2. The yellow box represents the B30 transposon and thin blue lines are the L522 chromosome. Thin arrows are arbitrary primers and thick arrows are transposon-specific primers.


B30
L522 Chromosome
First round of PCR using arbitrary primers (arb1 or arb6 ⟶) and IS50R primer ⟵
Second round of PCR using arbitrary primer (arb2 ⟶) and IS50R primer ⟵
PCR products:
-run on a gel
-gene clean
-sequence

2.6.3 Inverse PCR

A modified inverse PCR technique (Saiki *et al.*, 1988) was employed to attempt to sequence the chromosomal DNA flanking the transposon insertion in mutant L522. The protocol is summarized in Figure 4. The PCR primers utilized for this protocol were the B30-neo and IS50R primers. The PCR cycle was typically 95°C for 5 minutes, followed by 35 rounds of 95°C for 30 seconds, 66°C for 45 seconds, and 72°C for 3 minutes; followed by a final extension at 72°C for 10 minutes and then 4°C to hold. PCR was performed using Taq polymerase (Gibco-BRL) as per manufacturer's instructions.

2.7 Transposon Mutagenesis

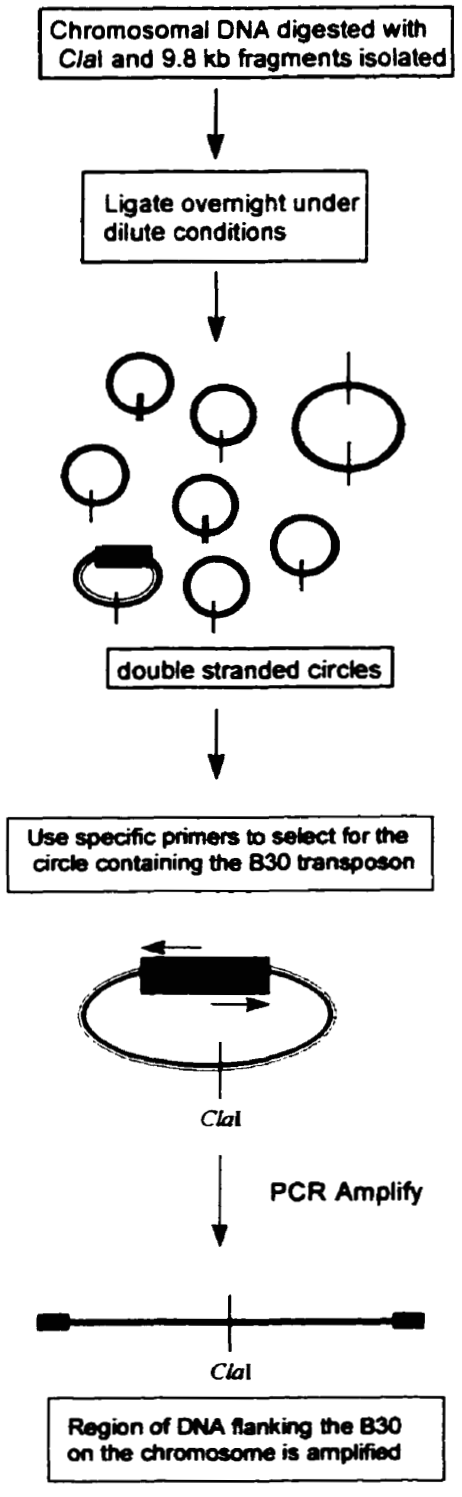
Transposon mutagenesis was performed on *P. aeruginosa* strain IL-1 using the transposon B30 from plasmid pSUP102::Tn5-B30. Both electroporation and conjugation procedures were employed for the generation of over 800 transposon mutants. All mutants were tested for tetracycline resistance by plating onto LB agar plates containing 100 μg/ml of tetracycline to confirm the presence of the transposon. Mutants were also tested for gentamicin sensitivity (transposition versus single-cross over plasmid integration) by picking mutants onto LB agar containing 50 μg/ml of gentamicin.

2.7.1 Screening of Mutants

Each transposon mutant was screened for anomalous P2 promoter activity. Figure 5 depicts the screening procedure. In a typical screening procedure, 90 mutants were screened. Each mutant, as well as the wild-type strain IL-1, was inoculated into 2 ml of TSBDC (low iron) and into 2 ml of TSBDC with 10 μg/ml of $FeCl_3$ (high iron). The 182 cultures were incubated overnight at 32°C with aeration. The following morning, β-galactosidase assays were performed in two microtitre plates. One microtitre plate contained the 90 mutants grown in high iron conditions and the other microtitre plate contained the 90 mutants grown in low iron conditions. In addition, the last six wells of each plate contained controls: two wells of IL-1 grown in high iron conditions, two wells of IL-1 grown in low iron conditions,

Figure 4: Schematic representation of the inverse PCR technique.

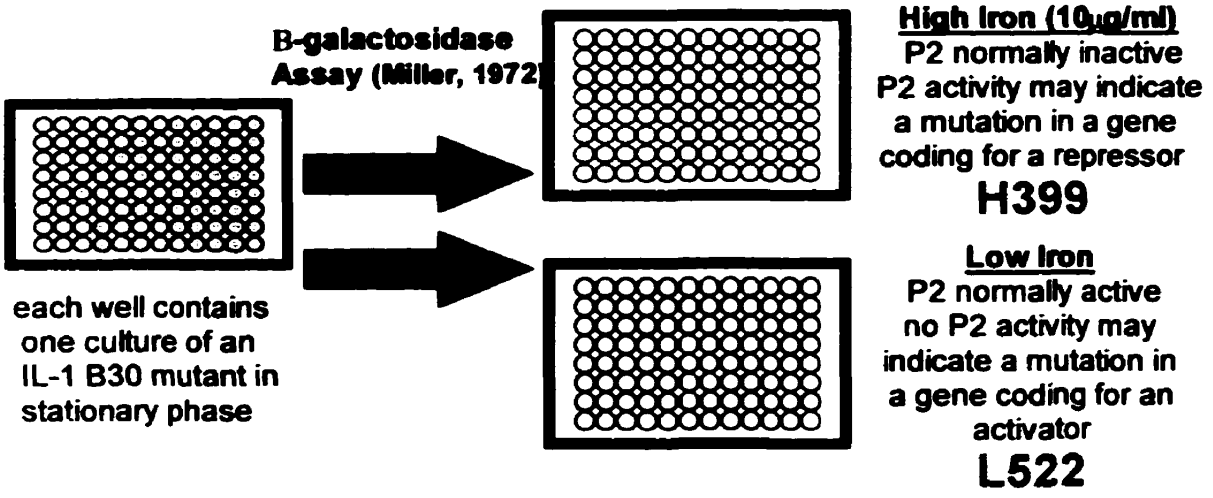
Black vertical lines indicate *Cla*I restriction sites in L522 chromosomal DNA. Red arrows indicate B30 transposon-specific primers B30-neo and IS50R. Primer sequences are listed in Table 3.



(modified from Saiki *et al*, 1988 and Gibco BRL, 1997)

and two wells of buffer Z. β-galactosidase assays were performed as described in section 2.4.1.1 and the OD_{405} was measured. For the assays from high iron cultures, any OD_{405} which was significantly higher than the OD_{405} of IL-1 grown in high iron was investigated further. This would indicate an activation of the P2 promoter in high iron conditions and could be due to a mutation in a repressor which acts on the promoter. For the assays of cultures grown in low iron conditions, any OD_{405} which was significantly lower than the OD_{405} of IL-1 grown in low iron was noted. The OD_{605} of these "putative mutants" was measured to investigate whether the low OD_{405} was actually due to low activity from the P2 promoter, or due to poor growth of the mutant. A transposon insertion which resulted in reduced P2 promoter activity in low iron conditions could be due to an insertion in an activator which acts on this promoter. All mutants which were identified by this screen were re-assayed using the test tube method for β-galactosidase detection (section 2.4.1.2).

Figure 5: Screening procedure used to identify mutants which may contain a mutation in a regulatory gene which acts on the P2 promoter of the *regAB* operon in IL-1. This procedure is outlined in detail in section 2.7.1.


B-galactosidase
Assay (Miller, 1972)
each well contains
one culture of an
IL-1 B30 mutant in
stationary phase
High Iron (10μg/ml)
P2 normally inactive
P2 activity may indicate
a mutation in a gene
coding for a repressor
H399
Low Iron
P2 normally active
no P2 activity may
indicate a mutation in
a gene coding for an
activator
L522

CHAPTER 3: RESULTS:
ROLE OF PVDS IN THE REGULATION OF EXOTOXIN A

In order to achieve our goal of identifying a regulator which acts on the iron-regulated P2 promoter of *regAB*, we explored the region upstream of the *regAB* start codon for consensus binding sequences. We concentrated our search on binding sequences of known iron regulators. One likely candidate was PvdS, an alternative sigma factor which is regulated by the Fur repressor.

3.1 Identification of PvdS Consensus Sequences

A PvdS consensus binding motif was identified by Rombel *et al.* (1995). Table 4 depicts the promoter regions of known or putative PvdS-regulated genes and shows the consensus binding motif for PvdS.

A nine out of ten base pair match to the PvdS binding consensus sequence had been identified previously in the promoter region of *toxA* (Rombel *et al.*, 1995) however, PvdS has not been demonstrated to directly regulate exotoxin A synthesis. We identified an eight out of ten base pair match to the PvdS binding consensus sequence within the *regAB* promoter region (Table 4). This suggests that the Fur-regulated PvdS alternative sigma factor may play a role in the regulation of the iron-regulated P2 promoter of the *regAB* operon. Our objective was to investigate whether PvdS regulates exotoxin A production, and if so, whether the regulation occurs directly on the *toxA* gene or through the *regAB* operon.

3.2 Construction of Plasmid pTH*pvdS*

The construction of plasmid pTH*pvdS* is outlined in Figure 6. The plasmid pTH*pvdS* and the vector control plasmid pUC181.8 were electroporated into *P. aeruginosa* strains IL-1, PA103, and PAO1. These three strains were chosen for our investigations because they each have unique properties which allow for specific aspects of regulation to be investigated. The *regAB* locus of each strain is depicted in Figure 7. *P. aeruginosa* strain IL-1 was utilized because it

Table 4: Promoter Region of PvdS-regulated Genes

Iron-regulated Promoter	Binding Sequence	Base pair match
P. aeruginosa pyoverdine promoter C1[a]	$^{-58}$GCTAAATCCC^{-49}	10/10
P. aeruginosa pyoverdine promoter J[a]	$^{-23}$GCTAAATACC^{-14}	9/10
P. aeruginosa pvdA promoter[b]	$^{-36}$CTTAAATTCA^{-27}	7/10
P. aeruginosa pvdD promoter[c]	$^{-37}$GCTAAATCCC^{-27}	10/10
P. aeruginosa pvdE promoter[c]	$^{-23}$GCTAAATACC $^{-14}$	9/10
P. aeruginosa exotoxin A promoter[a]	$^{-62}$CATAAATCCC^{-53}	9/10
P. putida siderophore promoter[a]	$^{-75}$CCTAAATCCT^{-66}	9/10
P. sp. strain M114 iron-regulated promoter[a]	$^{-92}$ACTAATTCCC^{-83}	8/10
P. aeruginosa regAB promoter P2[d]	$^{-77}$GCTAGATACC^{-68}	8/10
Consensus PvdS binding sequence	(G/C)CTAAATCCC	10/10

The consensus sequence similarity was identified by:

[a] Rombel *et al.*, 1995
[b] Miyazaki *et al.*, 1995
[c] Merriman *et al.*, 1995
[d] this work

Figure 6: Construction of plasmid pTH*pvdS*.

A 1005 bp fragment containing the *pvdS* gene from PA103 was digested with *Eco*RI from plasmid pLD201.1 and ligated into an *Eco*RI-digested pBluescript II SK(+) to produce plasmid pBS*pvdS*. This plasmid was digested with *Pst*I. The stabilizing fragment (SF) was excised from plasmid pUC181.8 by *Pst*I digestion and ligated into pBS*pvdS* to give plasmid pTH*pvdS*. This plasmid contains the *P. aeruginosa* origin of replication as well as the entire *pvdS* gene, including its own promoter region. This ensures that the plasmid is iron-regulated, as *pvdS* is on the chromosome. This drawing shows the construction of plasmid pTH*pvdS* but is not drawn to scale.

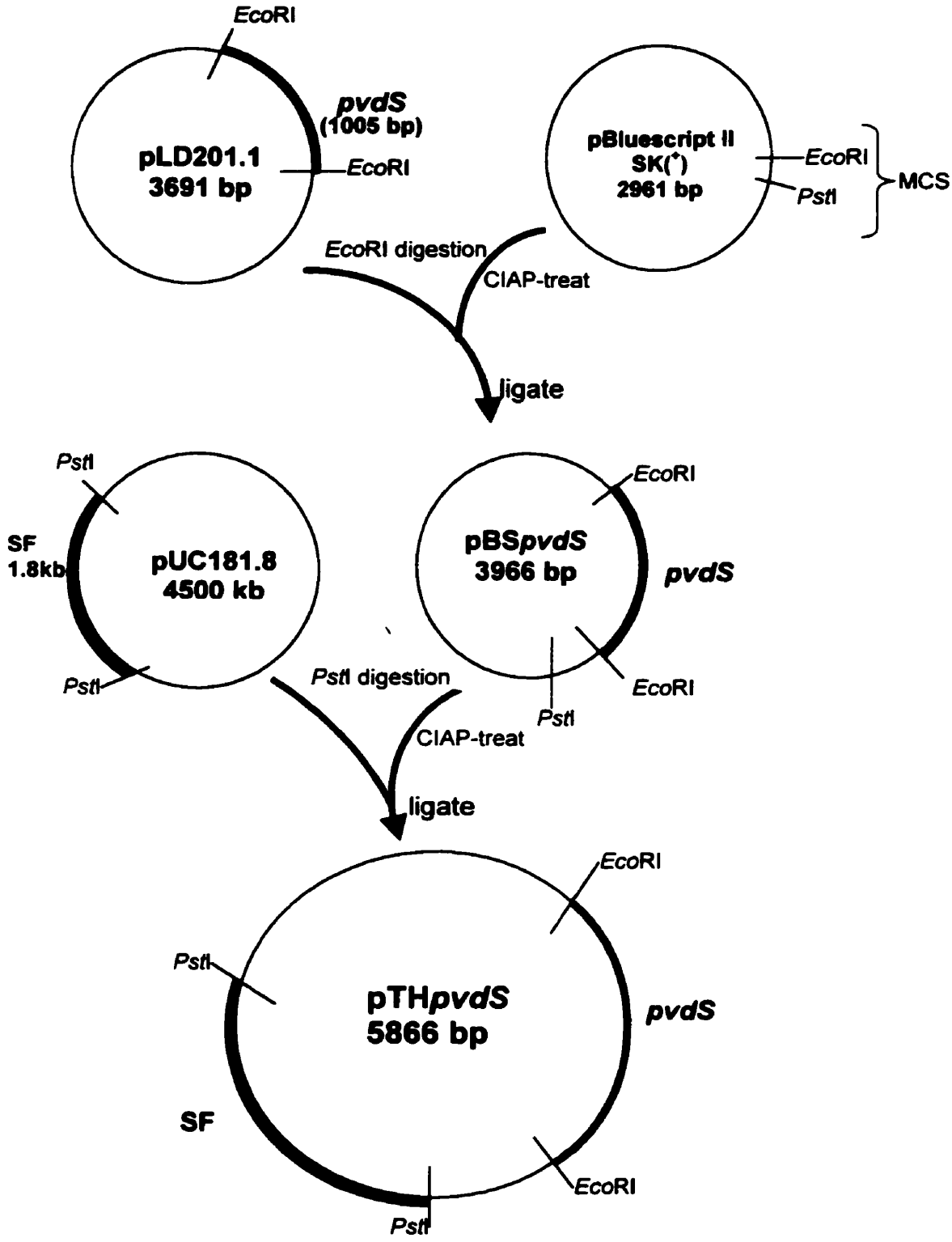
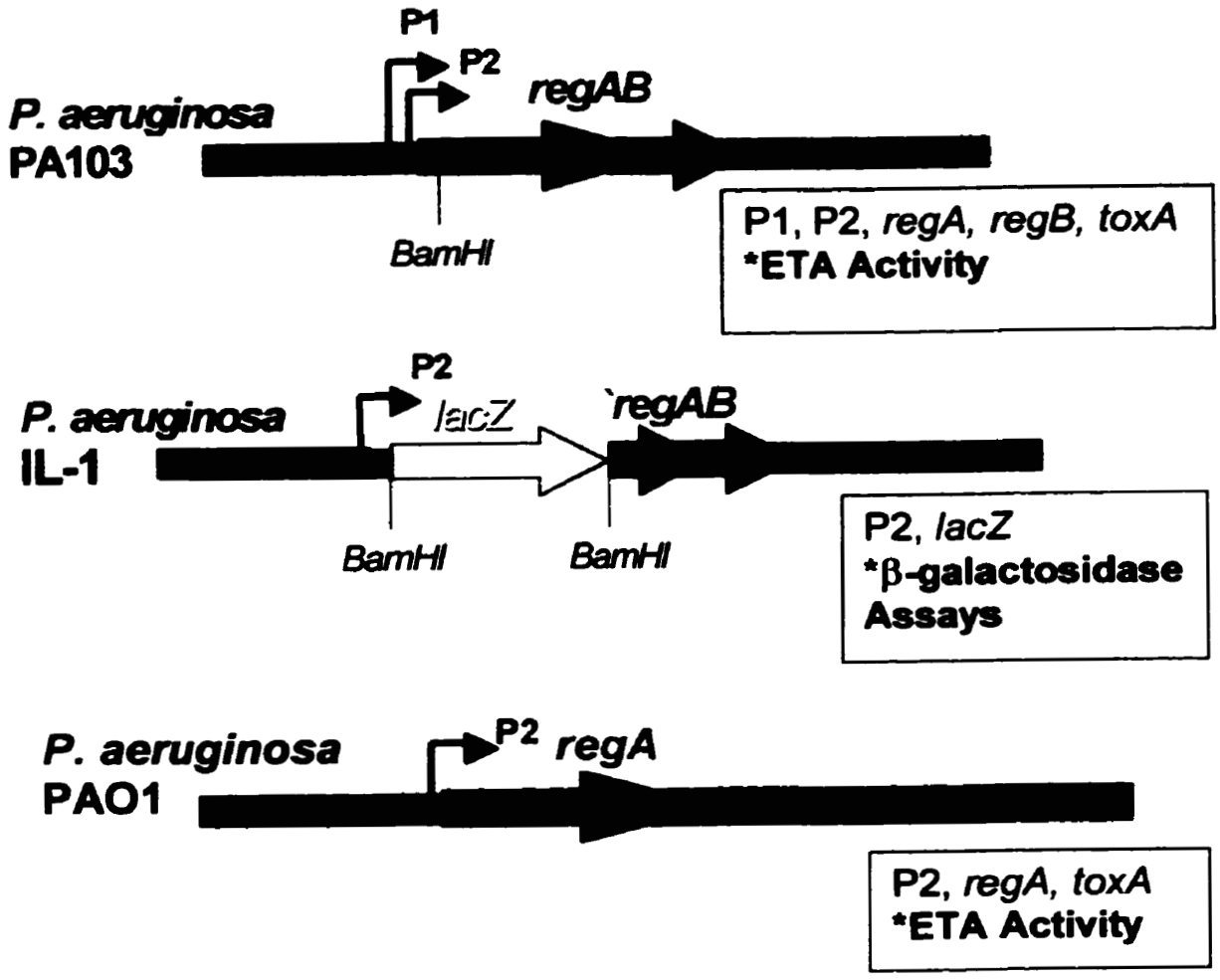

EcoRI
pvdS
(1005 bp)
pLD201.1
3691 bp
EcoRI
pBluescript II
SK(+)
2961 bp
EcoRI
PstI
MCS
EcoRI digestion
CIAP-treat
ligate
PstI
SF
1.8kb
pUC181.8
4500 kb
PstI
pBSpvdS
3966 bp
pvdS
EcoRI
EcoRI
PstI
PstI digestion
CIAP-treat
ligate
EcoRI
PstI
pTHpvdS
5866 bp
pvdS
SF
EcoRI
PstI

Figure 7: Schematic representation of the *regAB* locus in *P. aeruginosa* strains PA103, IL-1, and PAO1.

Chromosomal DNA is depicted by blue boxes and the *regAB* locus is shown by red arrows. The *lacZ* reporter gene is represented by a yellow arrow. All arrows indicate the direction of transcription of the open reading frame. Boxes to the right under each strain list relevant genes and promoters that are active in each strain and the type of assay performed on each strain to indicate activity.


P1
P2
regAB
P. aeruginosa
PA103
BamHI
P1, P2, regA, regB, toxA
*ETA Activity
P2
lacZ
'regAB
P. aeruginosa
IL-1
BamHI
BamHI
P2, lacZ
*β-galactosidase
Assays
P. aeruginosa
PAO1
P2 regA
P2, regA, toxA
*ETA Activity

contains a *lacZ* reporter gene insertion within the *regAB* open reading frame. The reporter construct prevents transcription from the P1 promoter in IL-1 because a functional *regB* is required for P1 activity and *regAB* is not transcribed in this strain. Transcription unique to the P2 promoter of the *regAB* operon can be monitored in this strain by performing β-galactosidase assays. *P. aeruginosa* strain PA103 is a hypertoxigenic laboratory strain, commonly used for exotoxin A studies. This strain is the parental strain of IL-1. The *regAB* locus of PA103 contains a functional *regA* and *regB* as well as both promoters. Exotoxin A assays can be performed on this strain as an indication of ETA production. This strain possesses the most complex regulation of exotoxin A production due to both promoters being functional. *P. aeruginosa* strain PAO1 was also used for these studies because it contains a *regAB* locus with less complex regulation of ETA production than PA103. This strain is similar to IL-1 at the *regAB* locus in that *regB* is not transcribed in this strain and, as a result, the P1 promoter is not active. All exotoxin A produced by PAO1 is due to the regulation by the P2 promoter at the *regAB* operon and the functional *toxA* gene. These three strains were used to investigate the effects of *pvdS* in multiple copies (pTH*pvdS*) on P2 promoter activity and on exotoxin A activity.

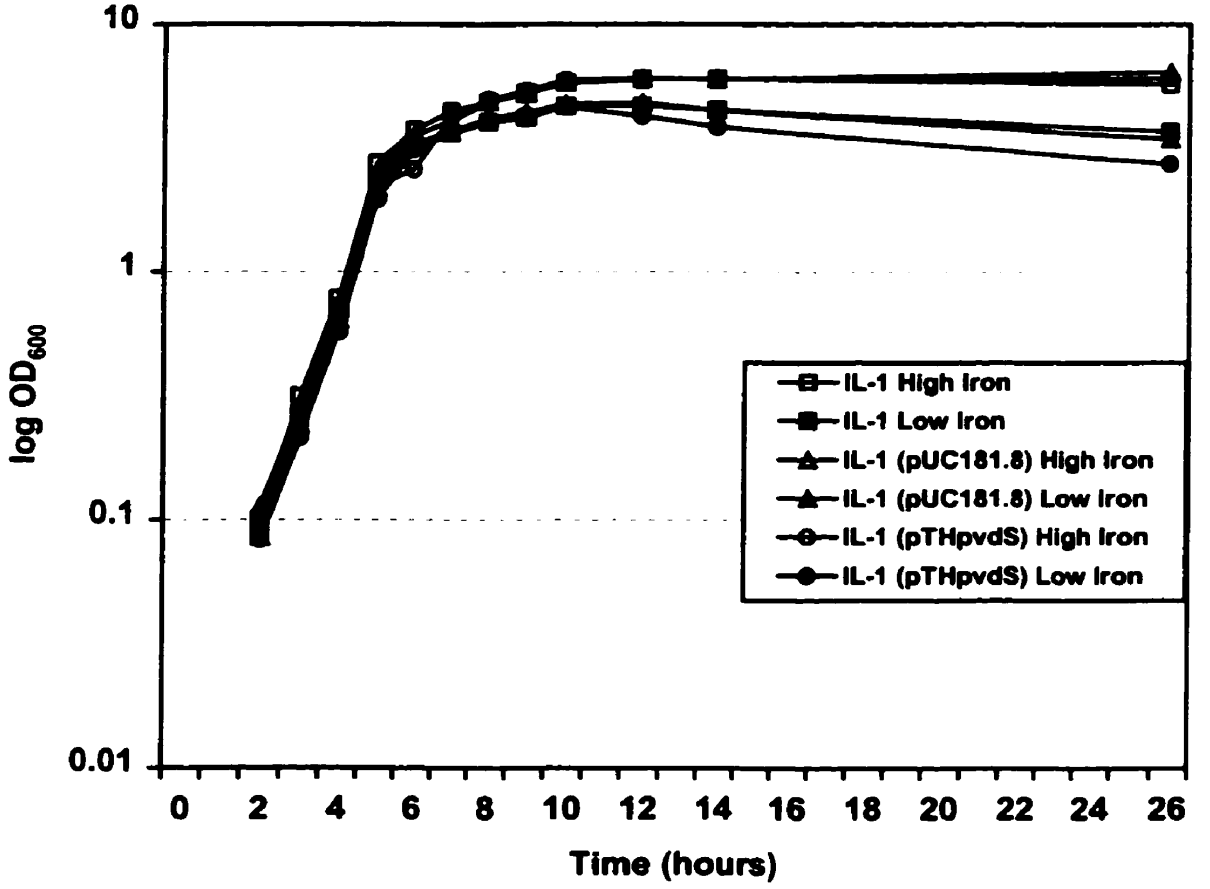
3.3 Effect of Multicopy *pvdS* on the P2 Promoter in IL-1

Plasmids pTH*pvdS* and the vector control pUC181.8 were electroporated into *P. aeruginosa* strain IL-1. A growth curve was performed on IL-1, IL-1 (pUC181.8) and IL-1 (pTH*pvdS*) in both high and low iron conditions. Growth was monitored by measuring the absorbance at 600nm at each time point and is depicted in Figure 8A. At each time point, samples were withdrawn from the culture and β-galactosidase assays were performed in duplicate to indicate activity from the P2 promoter in strain IL-1. The β-galactosidase activity of the strains over the course of a growth curve is shown in Figure 8B. The growth curve was repeated twice and the averages are shown.

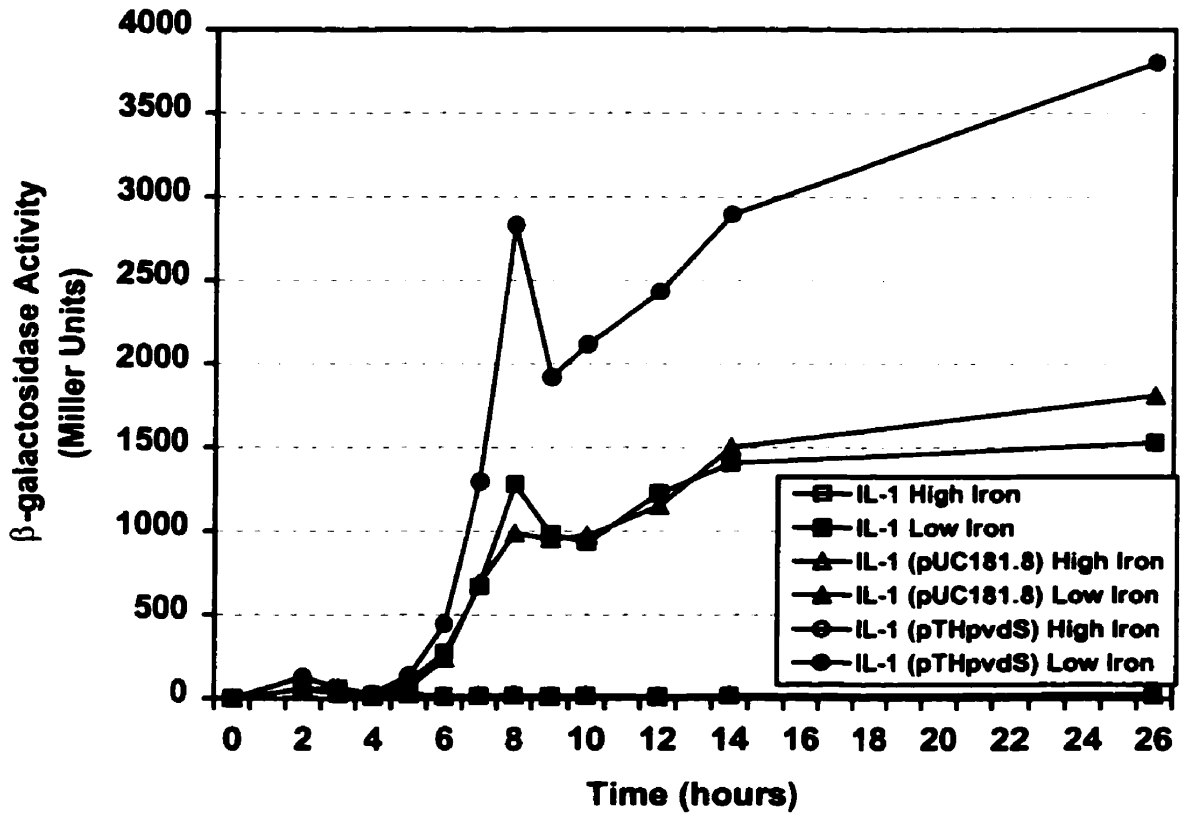
Figure 8: Growth curve and P2 activity of *P. aeruginosa* strains IL-1, IL-1 (pUC181.8), and IL-1 (pTH*pvdS*) in high and low iron conditions.

Primary cultures were grown overnight in TSBDC high iron conditions. Strain IL-1 (pUC181.8) and IL-1 (pTH*pvdS*) were grown in TSBDC plus 400 μg/ml of carbenicillin. Secondary cultures were inoculated to a starting OD_{600} of 0.02. Each strain was inoculated into both high iron and low iron TSBDC. The cultures of strain IL-1 (pUC181.8) and IL-1 (pTH*pvdS*) also contained 400 μg/ml of carbenicillin. Aliquots were removed at indicated time points, resuspended in 1XA buffer and frozen at -80°C. β-galactosidase assays were performed in duplicate. **A.** OD at 600nm and **B.** β-galactosidase assays of IL-1, IL-1 (pUC181.8), and IL-1 (pTH*pvdS*) in high and low iron conditions.

A.


10
1
0.1
0.01
log OD600
0
2
4
6
8
10
12
14
16
18
20
22
24
26
Time (hours)
IL-1 High Iron
IL-1 Low Iron
IL-1 (pUC181.8) High Iron
IL-1 (pUC181.8) Low Iron
IL-1 (pTHpvdS) High Iron
IL-1 (pTHpvdS) Low Iron

B.


4000
3500
3000
2500
2000
1500
1000
500
0
β-galactosidase Activity (Miller Units)
0
2
4
6
8
10
12
14
16
18
20
22
24
26
Time (hours)
IL-1 High Iron
IL-1 Low Iron
IL-1 (pUC181.8) High Iron
IL-1 (pUC181.8) Low Iron
IL-1 (pTHpvdS) High Iron
IL-1 (pTHpvdS) Low Iron

3.4 Effect of Multicopy *pvdS* on Exotoxin A Activity

3.4.1 *P. aeruginosa* Strain PA103

Plasmids pTH*pvdS* and pUC181.8 (the vector control) were electroporated into the *P. aeruginosa* hypertoxigenic strain PA103. A growth curve was performed on PA103, PA103 (pUC181.8) and PA103 (pTH*pvdS*) in both high and low iron conditions. Figure 9A shows the growth of the six cultures over time as indicated by the absorbance at 600nm. Cell-free supernatants were collected at each indicated time point and extracellular ADP-ribosyl transferase activity was measured by performing exotoxin A assays (Figure 9B). Each ETA assay was performed in triplicate and the entire growth curve was repeated twice and the averages are shown.

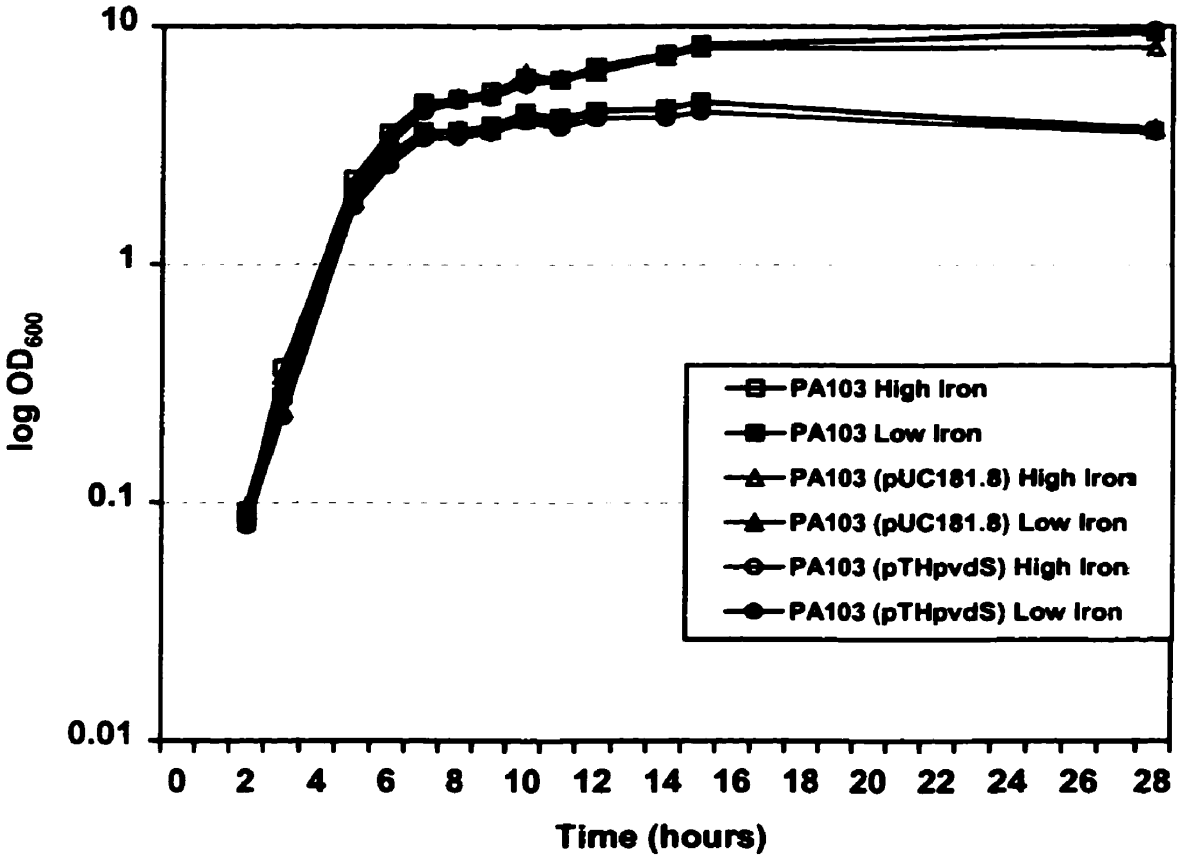
3.4.2 *P. aeruginosa* Strain PAO1

Plasmids pTH*pvdS* and pUC181.8 were electroporated into the phenotypically *regB*$^-$ strain PAO1. A growth curve was performed on PAO1, PAO1 (pUC181.8) and PAO1 (pTH*pvdS*) in both high and low iron conditions. The OD_{600} was measured at each time point and growth over the course of the growth curve is shown in Figure 10A. Exotoxin A assays were performed at each indicated time-point on the collected cell-free supernatants and the results are depicted in Figure 10B. Each ETA assay was performed in triplicate. Figure 10 shows the averages of two independent growth curves.

Figure 9: Growth curve and exotoxin A activities of *P. aeruginosa* strains PA103, PA103 (pUC181.8), and PA103 (pTH*pvdS*) in high and low iron conditions.

Primary cultures were grown overnight in TSBDC high iron conditions. Strain PA103 (pUC181.8) and PA103 (pTH*pvdS*) were grown in TSBDC plus 400 μg/ml of carbenicillin. Secondary cultures were inoculated to a starting OD_{600} of 0.02. Each strain was inoculated into both high and low iron TSBDC secondary cultures. The media of strains PA103 (pUC181.8) and PA103 (pTH*pvdS*) also contained 400 μg/ml of carbenicillin. Aliquots were removed at indicated time points, centrifuged three times to obtain cell-free supernatants and frozen at -80°C. Exotoxin A assays were performed in triplicate. **A.** Absorbance at 600nm and **B.** Exotoxin A assays of PA103, PA103 (pUC181.8), and PA103 (pTH*pvdS*) in high and low iron conditions.

A.


10
1
0.1
0.01
log OD600
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
Time (hours)
PA103 High Iron
PA103 Low Iron
PA103 (pUC181.8) High Iron
PA103 (pUC181.8) Low Iron
PA103 (pTHpvdS) High Iron
PA103 (pTHpvdS) Low Iron

B.

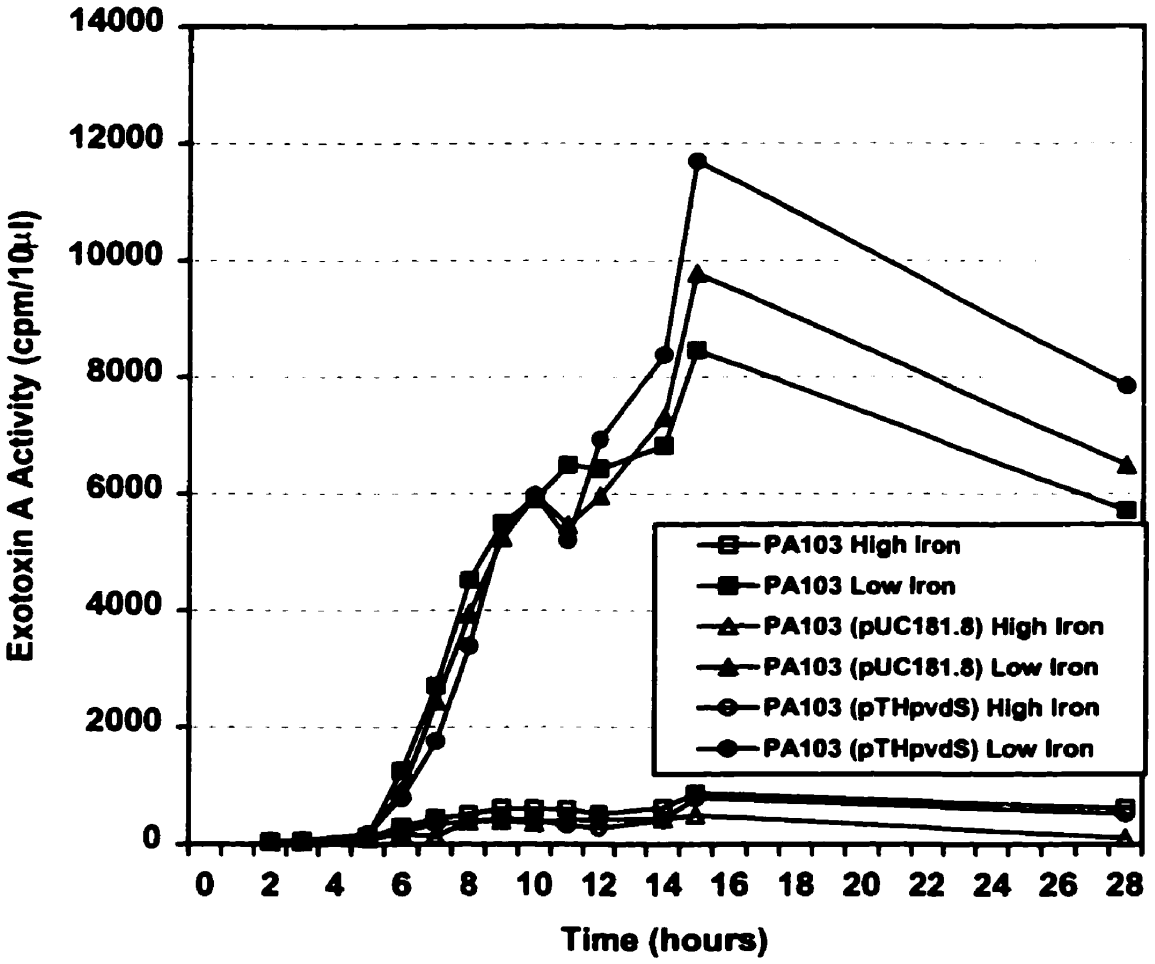
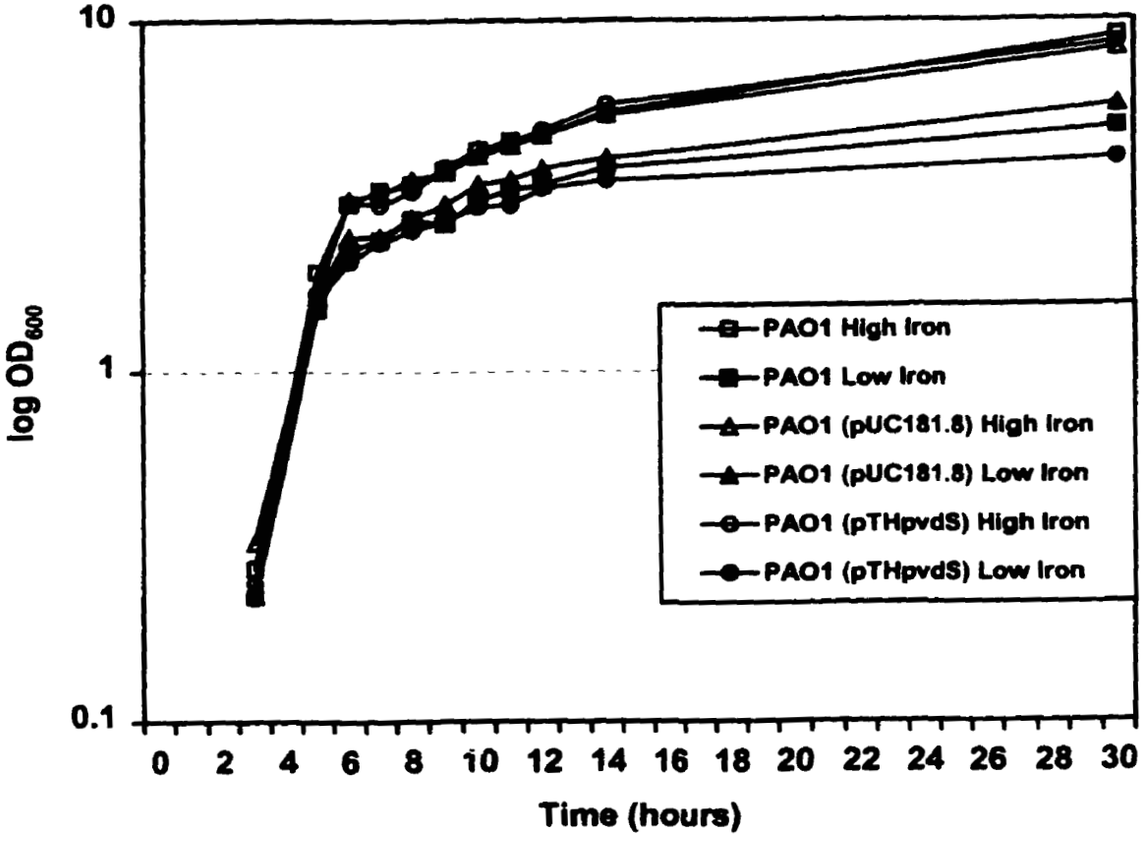

14000
12000
10000
8000
6000
4000
2000
0
Exotoxin A Activity (cpm/10µl)
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28
Time (hours)
PA103 High Iron
PA103 Low Iron
PA103 (pUC181.8) High Iron
PA103 (pUC181.8) Low Iron
PA103 (pTHpvdS) High Iron
PA103 (pTHpvdS) Low Iron

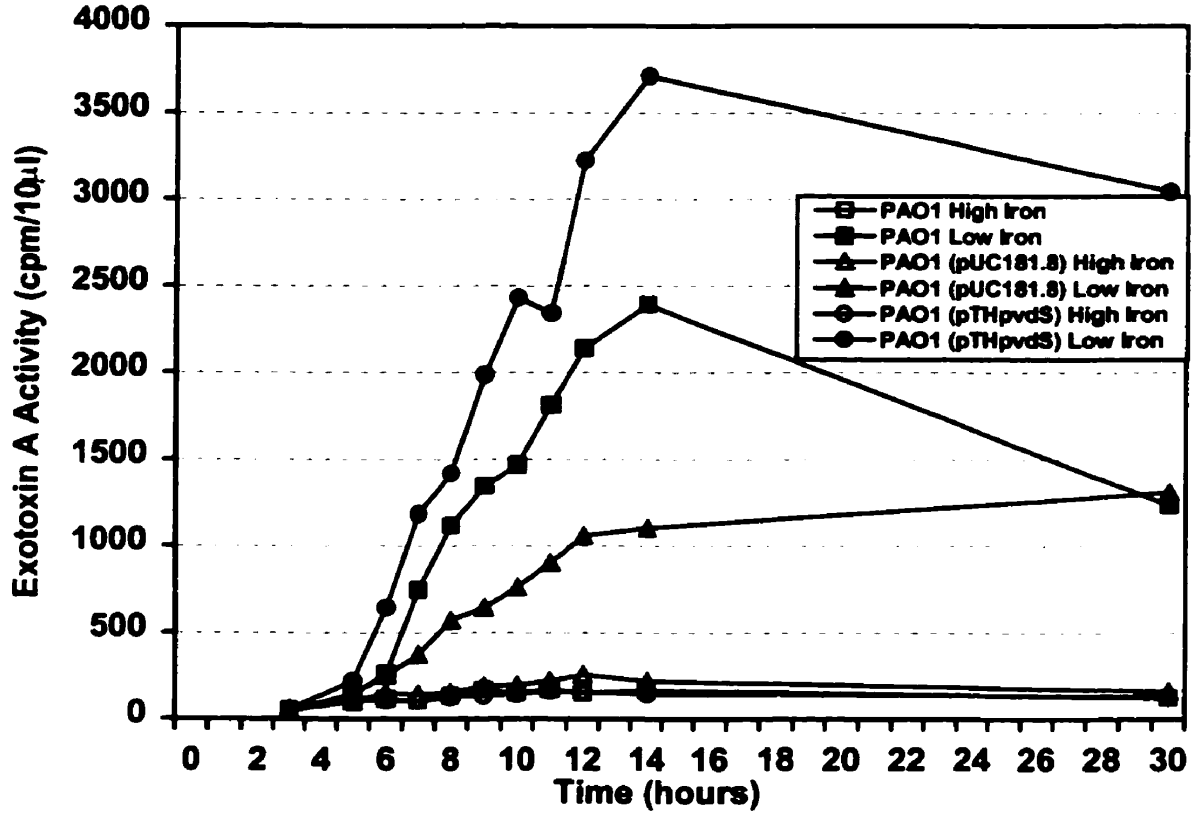
Figure 10: Growth curve and exotoxin A activities of *P. aeruginosa* strains PAO1, PAO1 (pUC181.8), and PAO1 (pTH*pvdS*) in high and low iron conditions.

Primary cultures were grown overnight in TSBDC high iron conditions. Strain PAO1 (pUC181.8) and PAO1 (pTH*pvdS*) were grown in TSBDC plus 400 μg/ml of carbenicillin. Secondary cultures were inoculated to a starting OD_{600} of 0.02. Each strain was inoculated into both a high and low iron flask containing TSBDC. The cultures of strains PAO1 (pUC181.8) and PAO1 (pTH*pvdS*) also contained 400 μg/ml of carbenicillin. Aliquots were removed at indicated time points, centrifuged three times to obtain cell-free supernatant and frozen at -80°C. Exotoxin A assays were performed in triplicate. **A.** OD at 600nm and **B.** Exotoxin A activities of PAO1, PAO1 (pUC181.8), and PAO1 (pTH*pvdS*) in high and low iron conditions.

A.


10
1
0.1
log OD600
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30
Time (hours)
PAO1 High Iron
PAO1 Low Iron
PAO1 (pUC181.8) High Iron
PAO1 (pUC181.8) Low Iron
PAO1 (pTHpvdS) High Iron
PAO1 (pTHpvdS) Low Iron

B.


Exotoxin A Activity (cpm/10µl)
4000
3500
3000
2500
2000
1500
1000
500
0
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30
Time (hours)
PAO1 High Iron
PAO1 Low Iron
PAO1 (pUC181.8) High Iron
PAO1 (pUC181.8) Low Iron
PAO1 (pTHpvdS) High Iron
PAO1 (pTHpvdS) Low Iron

3.4.3 *P. aeruginosa* Strain IL-1

Exotoxin A assays were also performed on strains IL-1, IL-1 (pUC181.8) and IL-1 (pTH*pvdS*) in order to determine whether the effect of *pvdS* in multiple copies acted on the *toxA* promoter directly in addition to its regulation of the P2 promoter of *regAB*. Although the *regAB* operon in strain IL-1 is not functional, the *toxA* region is identical to that of the hypertoxigenic wild-type derivative PA103. As a result, any exotoxin A produced by this strain is due to a RegA-independent regulator. To this end, exotoxin A assays were performed on strain IL-1. A growth curve was performed as described in Figure 8 and exotoxin A assays were performed at each time point on the collected cell-free supernatants. Each ETA assay was performed in duplicate and the results averaged (Figure 11). This growth curve was performed once. The results suggest that PvdS acts at the level of the *regAB* operon and not directly on the *toxA* promoter.

3.5 Construction of *pvdS* Knock-out Strains

The previous figures demonstrate that when *pvdS* is present in multiple copies, the activity from the P2 promoter is up-regulated. We next investigated the effects of interrupting the *pvdS* gene on the chromosome of strains IL-1, PA103 and PAO1. The activity from the P2 promoter in strain IL-1 and on exotoxin A activity in strains PA103 and PAO1 in these knock-out strains was investigated. In order to construct the knock-outs, the *pvdS* open reading frame was interrupted with an Ω cassette or a Gm cassette. The Ω cassette contains transcriptional and translational stops in all three open reading frames and confers streptomycin and spectinomycin resistance. The Gm cassette confers gentamycin resistance. The interrupted *pvdS* gene was constructed on a suicide plasmid which contained a *sacB* cassette (pJQ200SK) to facilitate the double-cross over onto the chromosome. The construction of IL-1 *pvdS*:: Ω is depicted in Figure 12. PA103 *pvdS*::Gm was constructed in the identical fashion except a *Sma*I-digested 850 bp Gm cassette was used instead of the 2 kb Ω fragment. The resulting plasmids, pJQ*pvdS*::Ω and pJQ*pvdS*::Gm, were electroporated into *P. aeruginosa* strains IL-1 and PA103 respectively. Colonies which demonstrated $Strep^R$ or Gm^R

Figure 11: Exotoxin A activities of *P. aeruginosa* strains IL-1, IL-1 (pUC181.8), and IL-1 (pTH*pvdS*) in both high and low iron conditions.

Cultures were prepared as described in Figure 8. Aliquots were removed at each indicated time point, centrifuged three times to obtain cell-free supernatants and frozen at -80°C. Exotoxin A assays were performed in duplicate on the supernatants.

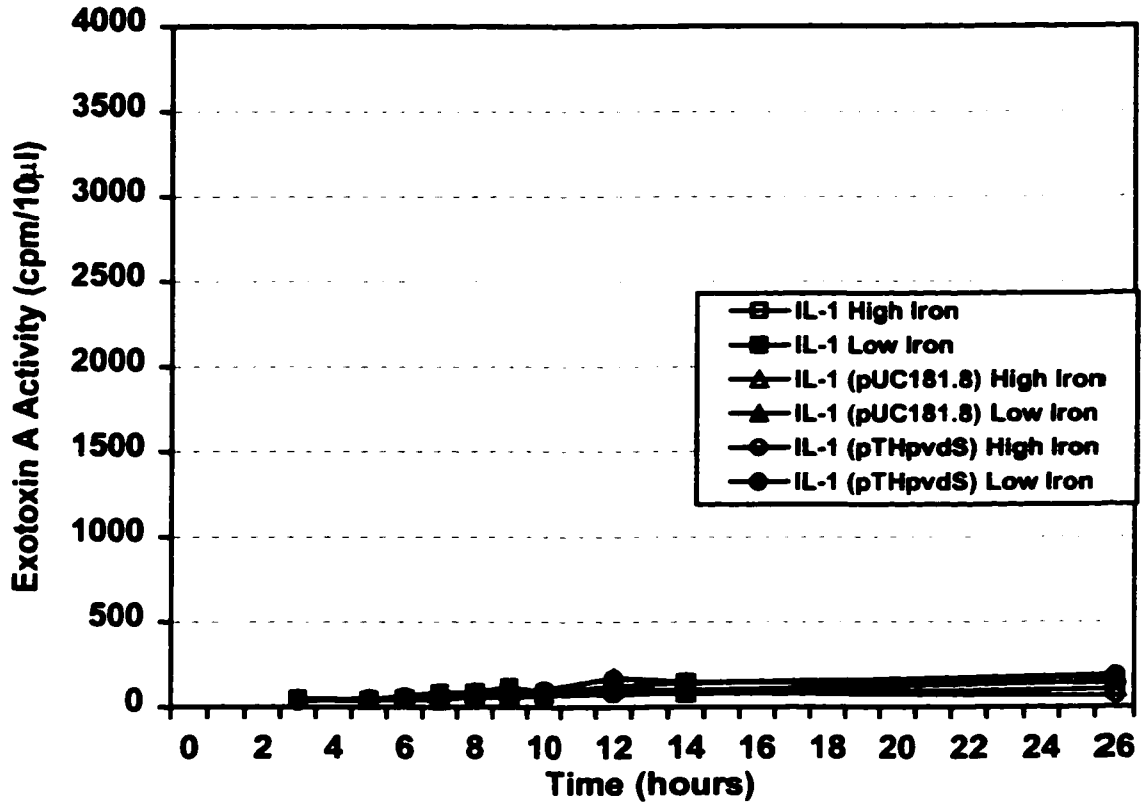
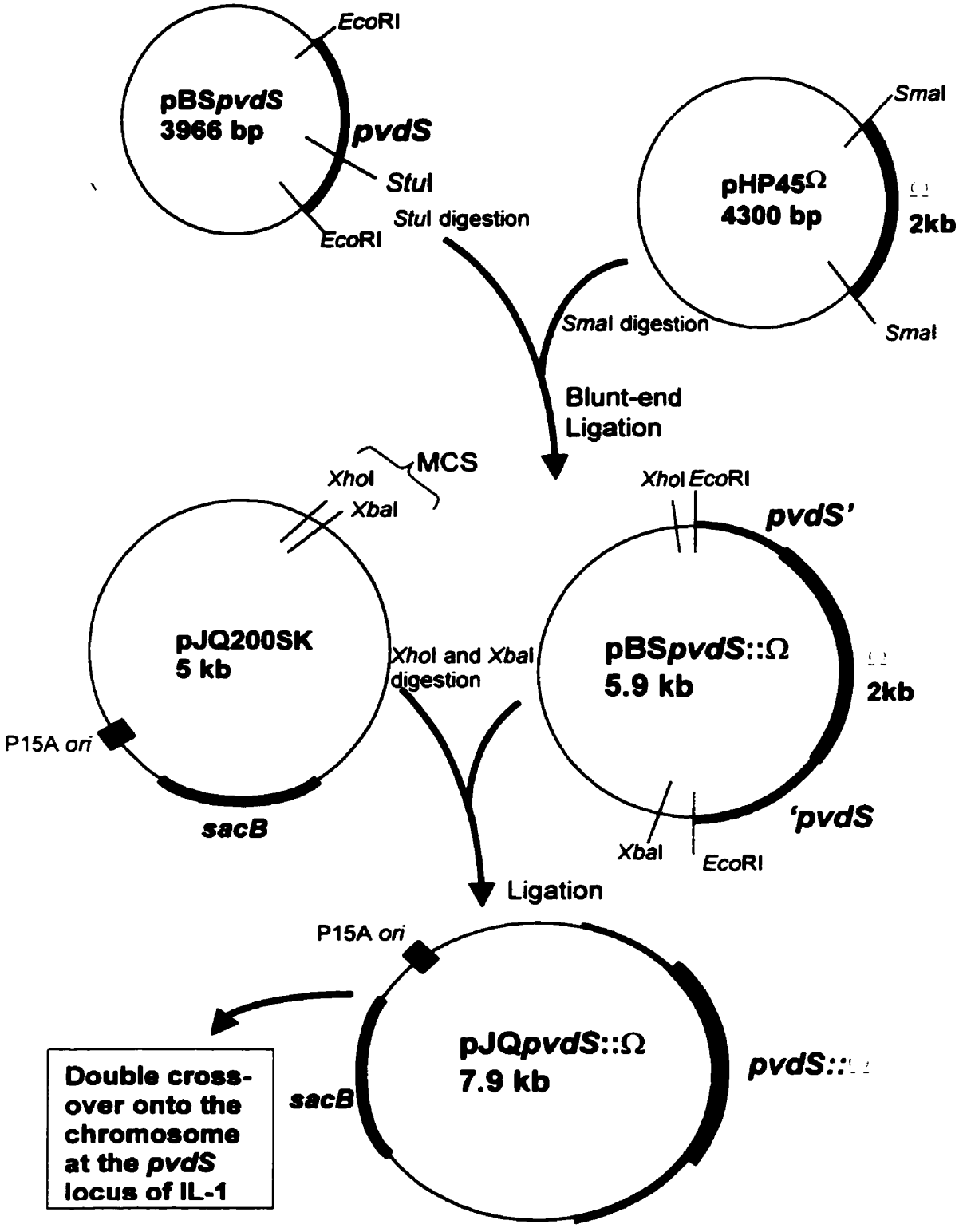

4000
3500
3000
2500
2000
1500
1000
500
0
Exotoxin A Activity (cpm/10µl)
0
2
4
6
8
10
12
14
16
18
20
22
24
26
Time (hours)
IL-1 High Iron
IL-1 Low Iron
IL-1 (pUC181.8) High Iron
IL-1 (pUC181.8) Low Iron
IL-1 (pTHpvdS) High Iron
IL-1 (pTHpvdS) Low Iron

Figure 12: Construction of strain IL-1 *pvdS*::Ω.

The 2 kb Ω cassette was isolated from plasmid pHP45 Ω by digestion with *Sma*I. pBS*pvdS* was digested with *Stu*I and the Ω fragment was ligated into the *pvdS* gene at the *Stu*I site by blunt-end ligation. The resulting plasmid pBS*pvdS*:: Ω was then digested with *Xho*I and *Xba*I, and ligated into the corresponding sites of pJQ200SK. pJQ200SK contains a *sacB* gene as well as a pBluescript SK(+) multiple cloning site which facilitated the construction. The resulting plasmid, pJQ*pvdS*:: Ω was then electroporated into *P. aeruginosa* strain IL-1 and the transformants were plated onto LB plates containing 500 μg/ml of streptomycin to select for the presence of the Ω cassette. Colonies were then picked onto LB plates containing 10% sucrose to select for double-cross overs. Putative mutants were picked onto duplicate LB plates and a colony hybridization was performed. Six colonies were selected and the chromosomal DNA was isolated and digested with *Eco*RI. A Southern blot was performed on the digested DNA (Figure 13). The sites in this drawing are not to scale.


EcoRI
pBSpvdS
3966 bp
pvdS
StuI
EcoRI
StuI digestion
SmaI
pHP45Ω
4300 bp
2kb
SmaI
SmaI digestion
Blunt-end
Ligation
MCS
XhoI
XbaI
pJQ200SK
5 kb
P15A ori
sacB
XhoI and XbaI
digestion
XhoI EcoRI
pvdS'
pBSpvdS::Ω
5.9 kb
2kb
'pvdS
XbaI
EcoRI
Ligation
P15A ori
pJQpvdS::Ω
7.9 kb
sacB
pvdS::
Double cross-over onto the chromosome at the pvdS locus of IL-1

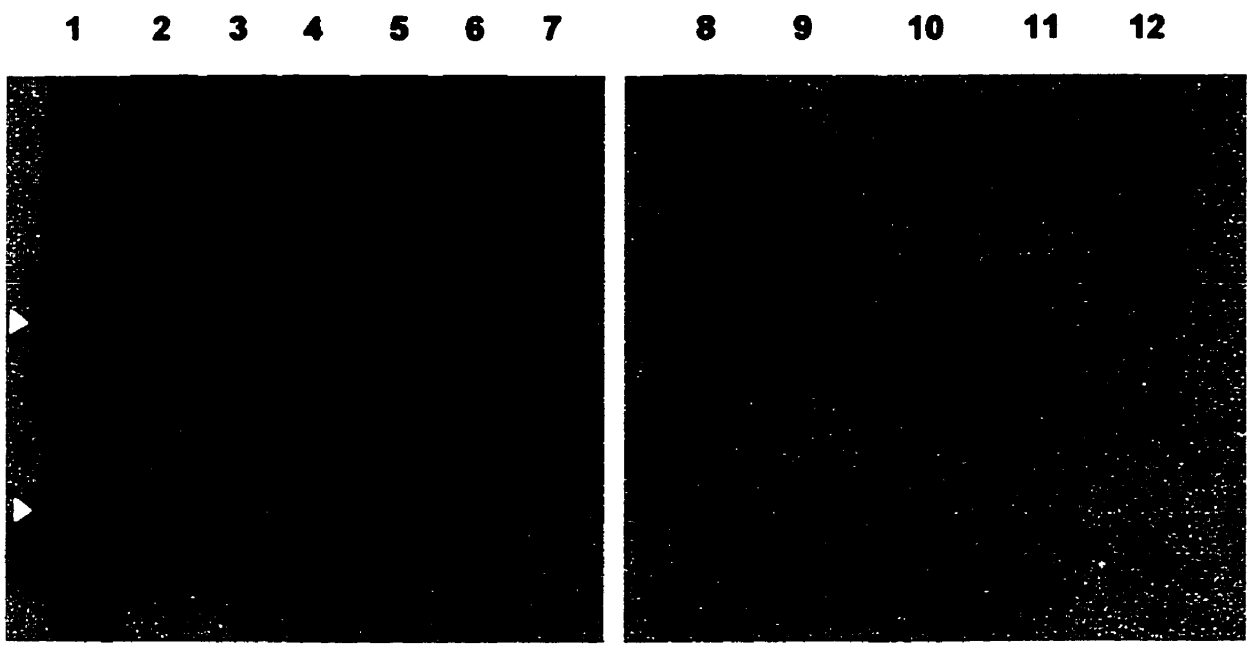
respectively were then picked onto LB agar plates containing 5% sucrose. This enabled selection for sucrose resistance, indicating those clones which contained the interrupted *pvdS* gene on the chromosome due to a double cross over event (loss of the *sacB* gene). A colony hybridization followed by a Southern blot was performed on putative knock-out strains. Figure 13 shows the results of the Southern blot probed with the 900 bp *pvdS* probe isolated from pLD201.1 and confirms the construction of strains IL-1 *pvdS*::Ω and PA103 *pvdS*::Gm. Strain PAO1Δ*pvdS* (a *pvdS* knock-out in PAO1) was obtained from Dr. M. Vasil (Ochsner *et al.*, 1996).

3.6 Effect of Multicopy *pvdS* on the P2 Promoter of IL-1 *pvdS*::Ω

Plasmids pTH*pvdS* and the control vector pUC181.8 were electroporated into strain IL-1 *pvdS*::Ω. A growth curve was performed on the three strains in both high and low iron conditions and growth was examined by recording the optical density at 600nm at each time point. The growth curve is shown in Figure 14A. At each indicated time point, samples were collected and β-galactosidase assays were performed in duplicate to indicate activity from the P2 promoter in strain IL-1 *pvdS*::Ω. The β-galactosidase activities of the strains at each indicated time point are demonstrated in Figure 14B. This growth curve was performed once. No activity was observed from the P2 promoter when the *pvdS* gene was knocked-out and these effects are complemented when *pvdS* is added *in trans*. We next wanted to investigate whether these effects at the *regAB* operon had an effect on exotoxin A production.

Figure 13: Southern Blot of *pvdS* knock-out strains IL-1 *pvdS*::Ω and PA103 *pvdS*::Gm probed with *pvdS*.

Putative knock-out strains were constructed as described in Figure 12. A Southern blot was performed on *Eco*RI digested chromosomal DNA with a 900 bp *pvdS* fragment isolated by *Eco*RI digestion from pLD201.1. The *pvdS* probe was labelled with ^{32}P-dCTP using an oligolabelling kit (Pharmacia). Lane 1 is *Eco*RI digested pLD201.1 (positive control); Lane 2 is PA103 chromosomal DNA digested with *Eco*RI; Lane 3-7 are chromosomal DNA isolated from putative knock-out strains (PA103 *pvdS*::Gm) and digested with *Eco*RI; Lane 8 is IL-1 chromosomal DNA digested with *Eco*RI; Lane 9-12 are chromosomal DNA isolated from putative knock-out strains (IL-1 *pvdS*::Ω) and digested with *Eco*RI. The clone in lane 7 was designated PA103 *pvdS*::Gm and lane 12 is IL-1 *pvdS*::Ω. White arrows indicate mobilization of 3 kb and 1 kb markers.


1
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9
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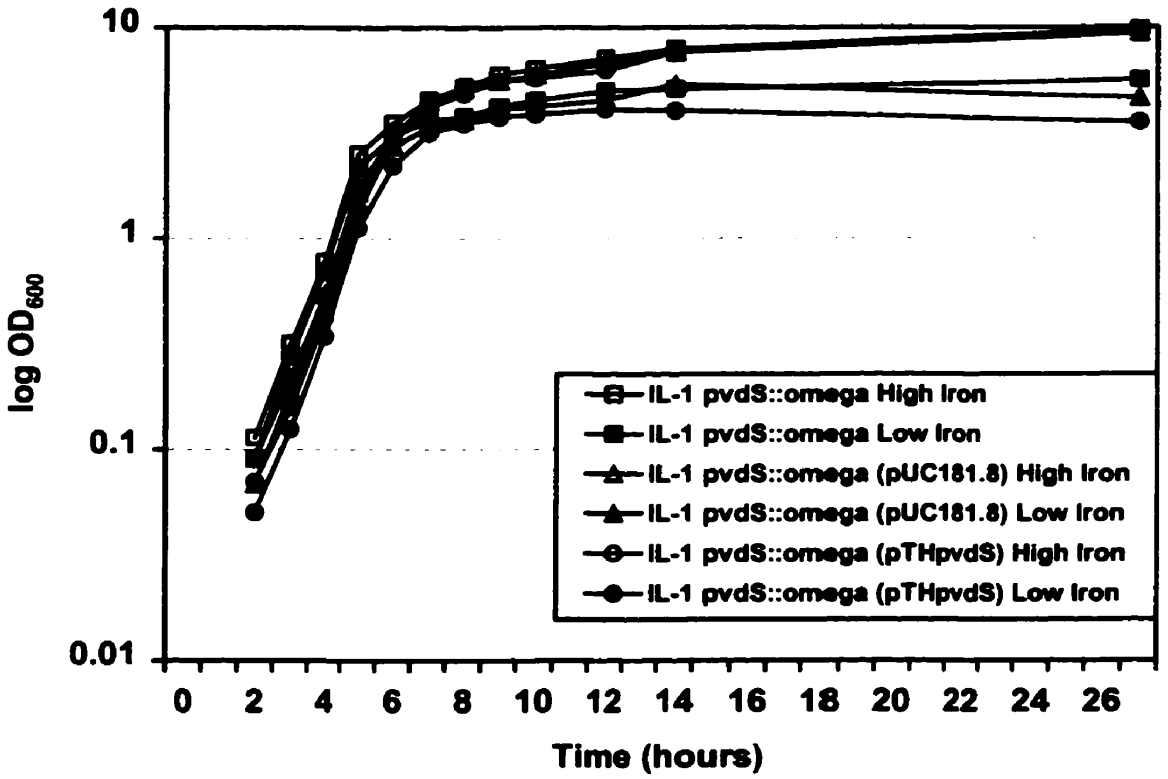
11
12

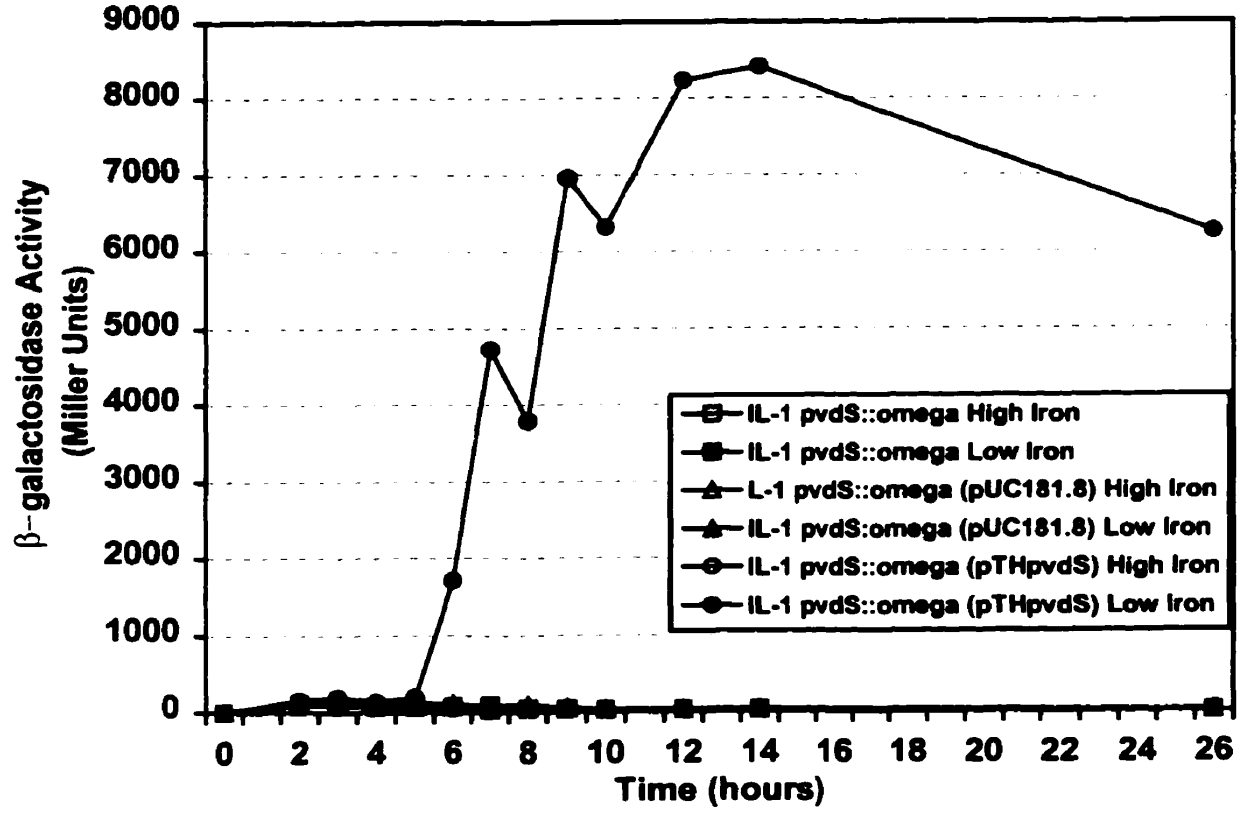
Figure 14: Growth curve and P2 activities of *P. aeruginosa* strains IL-1 *pvdS*::Ω, IL-1 *pvdS*::Ω (pUC181.8), and IL-1 *pvdS*::Ω (pTH*pvdS*) in high and low iron conditions.

Primary cultures were grown overnight in TSBDC high iron conditions. Strain IL-1 *pvdS*::Ω (pUC181.8) and IL-1 *pvdS*::Ω (pTH*pvdS*) were grown in TSBDC plus 400 μg/ml of carbenicillin. Secondary cultures were inoculated to a starting OD_{600} of 0.02. Each strain was inoculated in both high and low iron conditions in TSBDC and the cultures of strains IL-1 *pvdS*::Ω (pUC181.8) and IL-1 *pvdS*::Ω (pTH*pvdS*) also contained 400 μg/ml of carbenicillin. Aliquots were removed at indicated time points, resuspended in 1XA buffer and frozen at -80°C. β-galactosidase assays were performed in duplicate and the averages are shown. **A.** OD at 600nm and **B.** β-galactosidase assays of IL-1 *pvdS*::Ω, IL-1 *pvdS*::Ω (pUC181.8), and IL-1 *pvdS*::Ω (pTH*pvdS*) in high and low iron conditions.

A.


10
1
0.1
0.01
log OD600
0
2
4
6
8
10
12
14
16
18
20
22
24
26
Time (hours)
IL-1 pvdS::omega High Iron
IL-1 pvdS::omega Low Iron
IL-1 pvdS::omega (pUC181.8) High Iron
IL-1 pvdS::omega (pUC181.8) Low Iron
IL-1 pvdS::omega (pTHpvdS) High Iron
IL-1 pvdS::omega (pTHpvdS) Low Iron

B.


9000
8000
7000
6000
5000
4000
3000
2000
1000
0
β-galactosidase Activity (Miller Units)
0
2
4
6
8
10
12
14
16
18
20
22
24
26
Time (hours)
IL-1 pvdS::omega High Iron
IL-1 pvdS::omega Low Iron
L-1 pvdS::omega (pUC181.8) High Iron
IL-1 pvdS:omega (pUC181.8) Low Iron
IL-1 pvdS::omega (pTHpvdS) High Iron
IL-1 pvdS::omega (pTHpvdS) Low Iron

3.7 Effect of Multicopy *pvdS* on Exotoxin A Activity

3.7.1 *P. aeruginosa* Strain PA103 *pvdS*::Gm

Plasmids pTH*pvdS* and pUC181.8 were electroporated into *P. aeruginosa* strain PA103 *pvdS*::Gm. A growth curve was performed on the three strains in both high and low iron conditions. The OD_{600} was measured at each time point and growth over the course of the growth curve is demonstrated in Figure 15A. Exotoxin A assays were performed at each indicated time point in duplicate and the results are shown in Figure 15B. This growth curve was performed once.

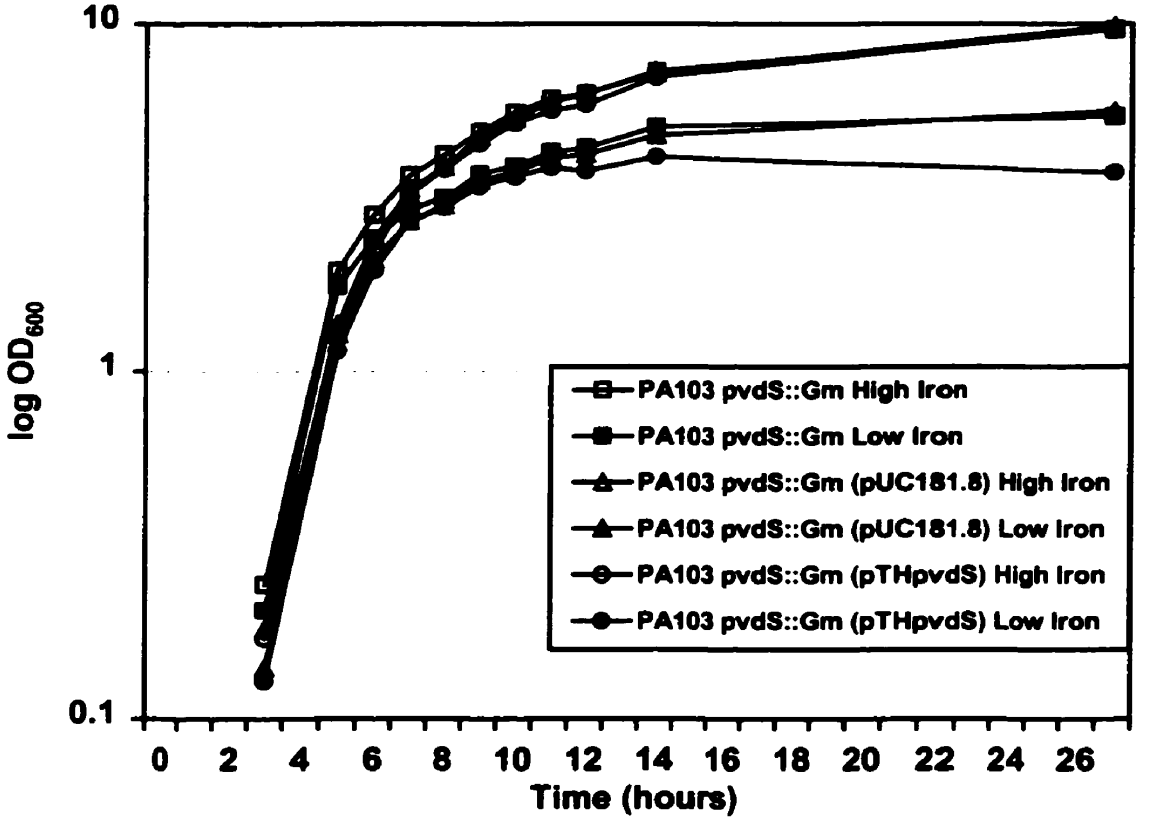
3.7.2 *P. aeruginosa* Strain PAO1Δ*pvdS*

The strain PAO1Δ*pvdS* was generously provided by Dr. M. Vasil (Ochsner *et al.*, 1996) and was used for these studies. This strain contains a deletion in a portion of the *pvdS* gene and an insertion of a gentamycin cassette within the gene on the chromosome of strain PAO1. Plasmids pTH*pvdS* and the vector control pUC181.8 were electroporated into strain PAO1Δ*pvdS* and a growth curve was performed on the three strains in both high and low iron conditions. Figure 16A shows the growth of the cultures over time as indicated by the absorbance at 600nm. Cell-free supernatants were collected at each time point and extracellular exotoxin A assays were performed (Figure 16B). Each ETA assay was performed in triplicate and the averages are shown. This growth curve was performed once. These experiments support the previous results and demonstrate that PvdS regulates exotoxin A production through the P2 promoter of the *regAB* operon.

Figure 15: Growth curve and exotoxin A activities of *P. aeruginosa* strains PA103 *pvdS*::Gm, PA103 *pvdS*::Gm (pUC181.8), and PA103 *pvdS*::Gm (pTH*pvdS*) in high and low iron conditions.

Primary cultures were grown overnight in TSBDC high iron conditions. Strains PA103 *pvdS*::Gm (pUC181.8) and PA103 *pvdS*::Gm (pTH*pvdS*) were grown in TSBDC plus 400 μg/ml of carbenicillin. Secondary cultures were prepared to a starting OD_{600} of 0.02 and each strain was inoculated into both a high iron and a low iron flask containing TSBDC. The cultures of strains PA103 *pvdS*::Gm (pUC181.8) and PA103 *pvdS*::Gm (pTH*pvdS*) also contained 400 μg/ml of carbenicillin. Aliquots were removed at indicated time points, centrifuged three times to obtain cell-free supernatant and frozen at -80°C. Exotoxin A assays were performed in duplicate. **A.** Absorbance at 600nm and **B.** Exotoxin A activities of PA103 *pvdS*::Gm, PA103 *pvdS*::Gm (pUC181.8), and PA103 *pvdS*::Gm (pTH*pvdS*) in high and low iron conditions.

A.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1
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log OD600
0
2
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6
8
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12
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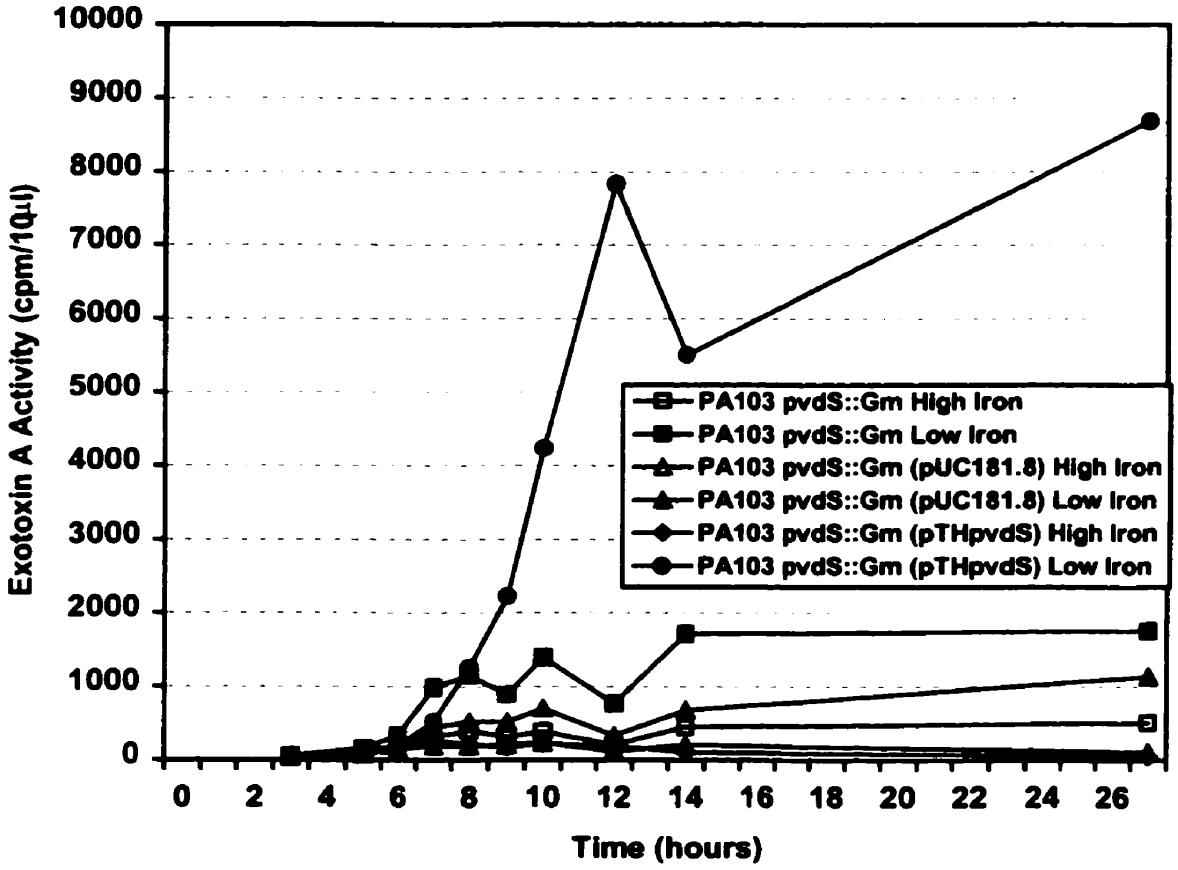
16
18
20
22
24
26
Time (hours)
PA103 pvdS::Gm High Iron
PA103 pvdS::Gm Low Iron
PA103 pvdS::Gm (pUC181.8) High Iron
PA103 pvdS::Gm (pUC181.8) Low Iron
PA103 pvdS::Gm (pTHpvdS) High Iron
PA103 pvdS::Gm (pTHpvdS) Low Iron

B.


10000
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7000
6000
5000
4000
3000
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Exotoxin A Activity (cpm/10µl)
0
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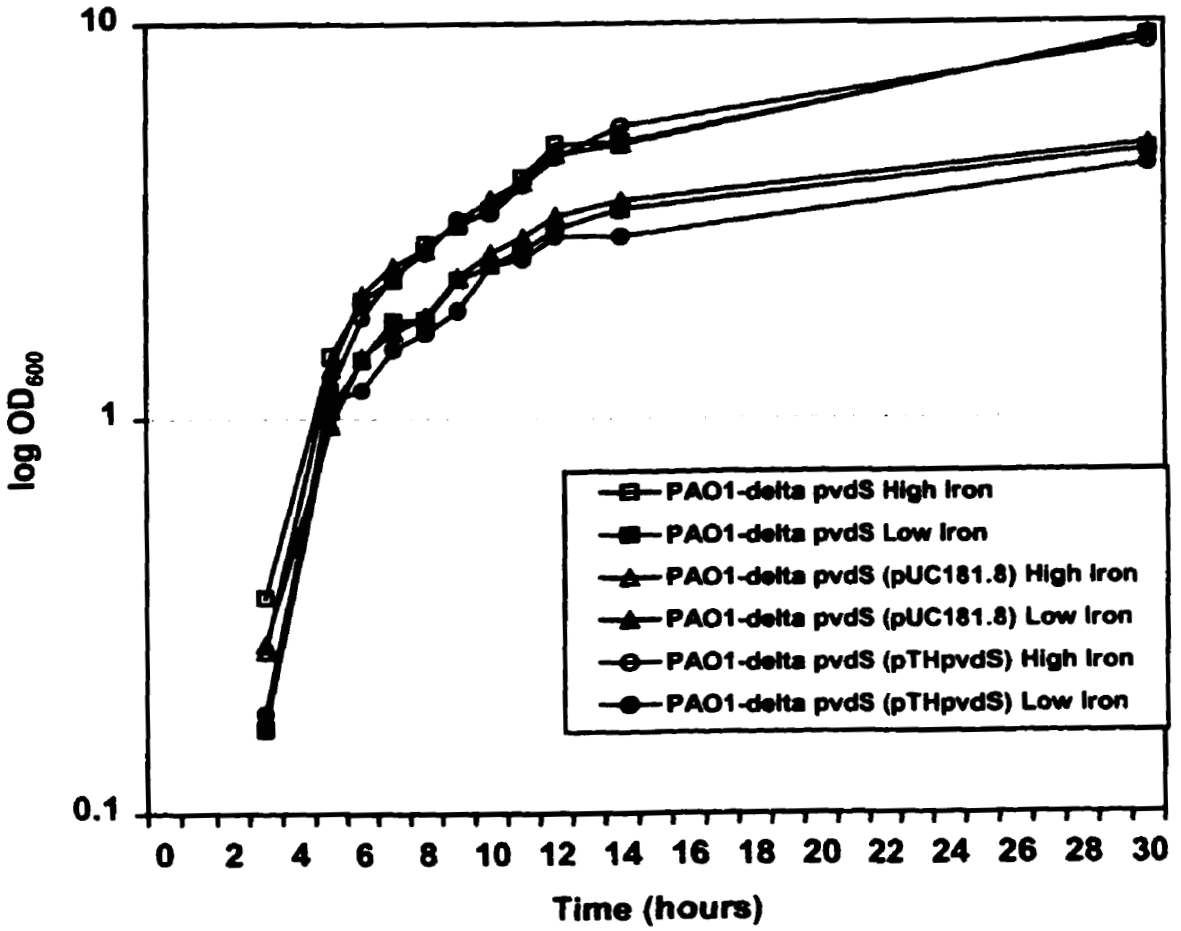
4
6
8
10
12
14
16
18
20
22
24
26
Time (hours)
PA103 pvdS::Gm High Iron
PA103 pvdS::Gm Low Iron
PA103 pvdS::Gm (pUC181.8) High Iron
PA103 pvdS::Gm (pUC181.8) Low Iron
PA103 pvdS::Gm (pTHpvdS) High Iron
PA103 pvdS::Gm (pTHpvdS) Low Iron

Figure 16: Growth curve and exotoxin A activities of *P. aeruginosa* strains PAO1Δ*pvdS*, PAO1Δ*pvdS* (pUC181.8), and PAO1Δ*pvdS* (pTH*pvdS*) in high and low iron conditions.

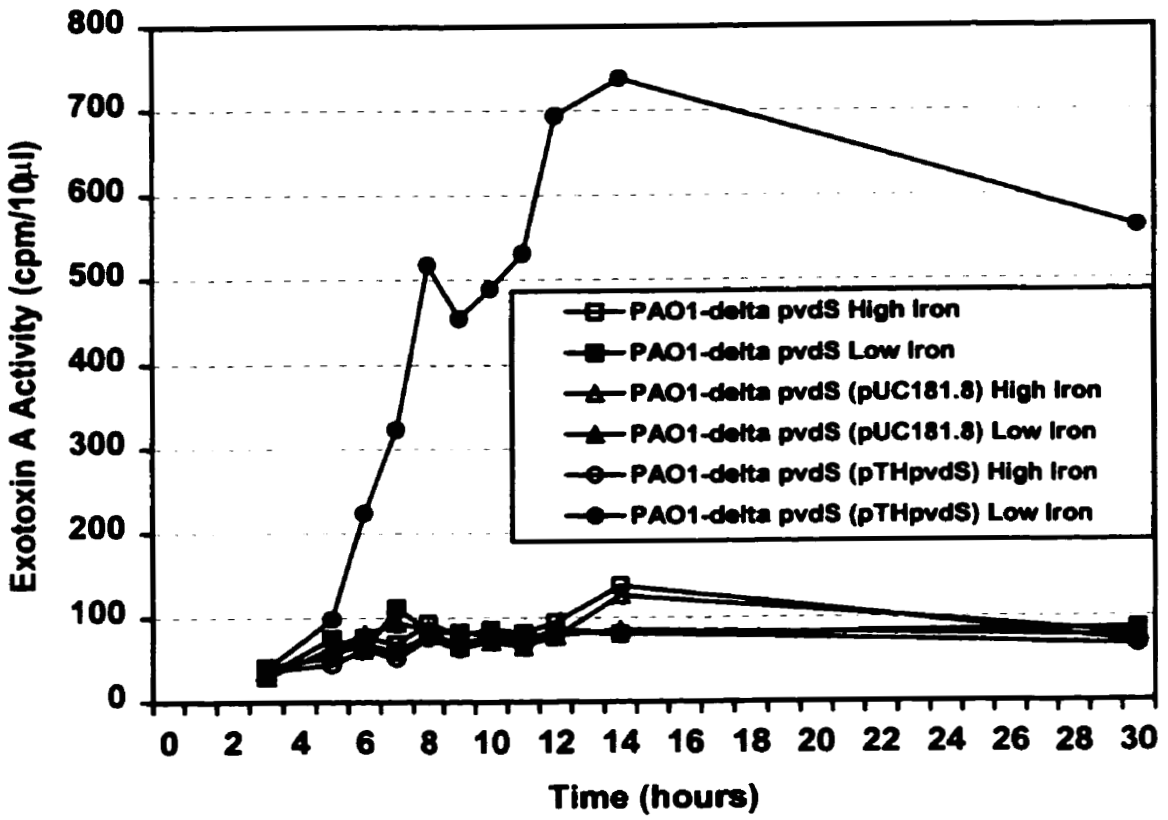
Primary cultures were grown overnight in TSBDC high iron conditions. Strain PAO1Δ*pvdS* (pUC181.8) and PAO1Δ*pvdS* (pTH*pvdS*) were grown in TSBDC plus 400 μg/ml of carbenicillin. Secondary cultures were prepared such that the starting OD_{600} was 0.02 and each strain was inoculated into high iron and low iron TSBDC. The secondary cultures of strains PAO1Δ*pvdS* (pUC181.8) and PAO1Δ*pvdS* (pTH*pvdS*) also contained 400 μg/ml of carbenicillin. Aliquots were removed at indicated time points, centrifuged three times and frozen at -80°C. Exotoxin A assays were performed in triplicate on each supernatant.

A. Absorbance at 600nm and **B.** Exotoxin A activities of PAO1Δ*pvdS*, PAO1Δ*pvdS* (pUC181.8), and PAO1Δ*pvdS* (pTH*pvdS*) in high and low iron conditions.

A.


10
1
0.1
log OD600
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
Time (hours)
PAO1-delta pvdS High Iron
PAO1-delta pvdS Low Iron
PAO1-delta pvdS (pUC181.8) High Iron
PAO1-delta pvdS (pUC181.8) Low Iron
PAO1-delta pvdS (pTHpvdS) High Iron
PAO1-delta pvdS (pTHpvdS) Low Iron

B.


Exotoxin A Activity (cpm/10µl)
800
700
600
500
400
300
200
100
0
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30
Time (hours)
PAO1-delta pvdS High Iron
PAO1-delta pvdS Low Iron
PAO1-delta pvdS (pUC181.8) High Iron
PAO1-delta pvdS (pUC181.8) Low Iron
PAO1-delta pvdS (pTHpvdS) High Iron
PAO1-delta pvdS (pTHpvdS) Low Iron

CHAPTER 4: RESULTS:
SEARCH FOR OTHER REGULATORS OF THE P2 PROMOTER

4.1 Transposon Mutagenesis of Strain IL-1

In order to attain our goal of identifying regulators which act on the P2 promoter of the *regAB* operon, transposon mutagenesis was performed on *P. aeruginosa* strain IL-1. This strain was chosen for these studies for many reasons. The parental strain is the hypertoxigenic lab strain PA103 which is typically used for studies of exotoxin A. Strain IL-1 was constructed by ligating a promoterless *lacZ* reporter gene from pZ1918 (Schweizer, 1993b) into a *Bam*HI site within the *regAB* open reading frame (see Figure 7 for a representation of the *regAB* locus of IL-1). This reporter gene insertion prevents transcription of *regAB*, resulting in a *regAB*$^-$ strain. The P1 promoter is not active in IL-1 because *regB* is not expressed, as a result, the P2 promoter controls transcription of the *lacZ* reporter gene. This strain facilitates our investigation of P2 promoter activity because activity is easily monitored through β-galactosidase reporter assays.

4.1.1 B30 Transposon

Transposon mutagenesis was performed on *P. aeruginosa* strain IL-1 using the B30 transposon from plasmid pSUP102::Tn5-B30 (Simon *et al.*, 1989). The transposon was electroporated into IL-1. The volume of plasmid DNA used in the reaction mix was typically 3 μl but this volume was modified to optimize the electroporation efficiency for each transposition. The efficiency of plasmid transformation into strain IL-1 using a control plasmid was typically 10^{-2} while the efficiency of transposon mutagenesis by transposon B30 was typically only $6X10^{-7}$. Because of this low efficiency of mutagenesis, conjugation was used as an alternative method of generating transposon mutants of IL-1. The triparental mating procedure did not result in improved efficiency of mutagenesis and was therefore discontinued. The biparental conjugation procedure was also attempted and this procedure resulted in the generation of many IL-1 transposon mutants. The efficiency of the biparental mating procedure was typically $7.5X10^{-5}$ and so

biparental mating was primarily utilized for the generation of B30-transposon mutants in *P. aeruginosa* strain IL-1.

4.1.2 pTnMod-OGm Plasposon

In order to facilitate the identification of the sequence surrounding the transposon insertion in mutants of IL-1, the plasposon pTnMod-OGm was also used for transposon mutagenesis (Dennis and Zylstra, 1998). This plasposon has many advantages over the B30 transposon. It is composed of Tn5 inverted repeats flanking an *E. coli* origin of replication and a Gm^R cassette. Rare restriction endonuclease multiple cloning sites are located in close proximity to the Tn5 inverted repeats in order to facilitate mapping of the transposon insertion on the chromosome of a transposon mutant. Following the identification of mutants H399 and L522 (see below), this plasposon was used in place of the B30 transposon because identification of the insertion site of the B30 in these two mutants was problematic. However, the putative mutants generated using this plasposon were not investigated further in these studies. Plasposon mutants were generated using the methods described for the B30 transposon mutagenesis procedure.

4.2 Identification of Mutants with Altered P2 Activity

The transposon mutagenesis procedures described above were performed on *P. aeruginosa* strain IL-1 to generate 806 mutants. These transposon mutants were then screened for irregular P2 promoter activity using the screening procedure described in section 2.7.1. Each mutant was screened in both high and low iron conditions to search for those which displayed P2 activity in high iron conditions, or low P2 activity in low iron conditions. Using this screening procedure, 32 putative mutants were identified. Nineteen of the mutants displayed the phenotype of low P2 activity in low iron conditions and 13 mutants showed high P2 activity in high iron conditions. β-galactosidase assays were then performed on these mutants using the more precise test tube method (section 2.4.1.2) after growth overnight in the appropriate iron conditions. This second round of

screening eliminated the false positives to leave five putative mutants. Mutant H399 displayed the phenotype of high P2 activity when grown in high iron conditions indicating the transposon in this mutant may be inserted into a gene which codes for a repressor that acts on the P2 promoter under high iron conditions. This mutant was used for further studies as it was the only putative mutant identified under high iron conditions. Mutants L522, L688, L742, and L799 were identified as displaying the phenotype of low P2 activity in low iron conditions. They each may contain an insertion of the B30 transposon in a gene which codes for an activator of the P2 promoter in low iron conditions. It was interesting that these four mutants displayed only reduced activity in low iron compared to IL-1, and not a total abrogation of activity. Mutant L522 displayed the lowest P2 activity of these four mutants and was chosen for further investigation.

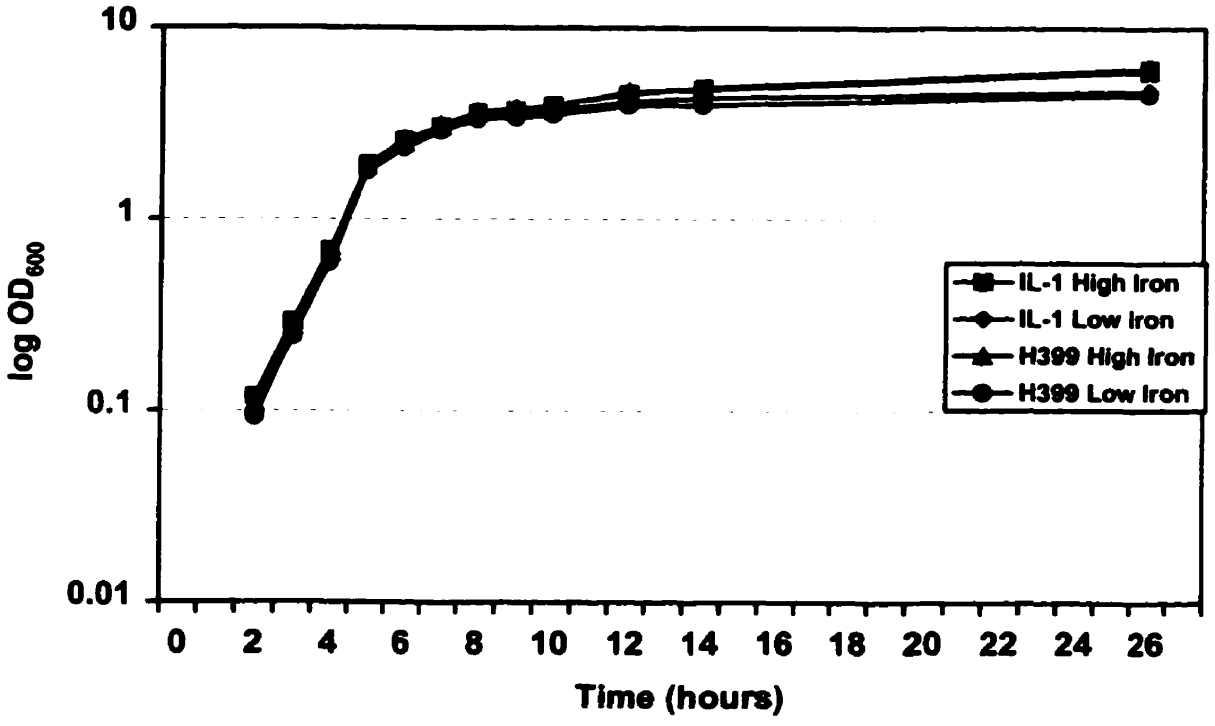
4.3 Mutant H399

A growth curve was performed on mutant H399 in order to investigate whether the transposon insertion had an effect on the normal housekeeping functions of this strain thereby causing a decrease in growth rate. The OD_{600} was recorded at each indicated time point in both high and low iron conditions. Growth was compared to the wild-type strain IL-1 and the average of three independent growth curves is shown in Figure 17A. At each time point, samples were collected and β-galactosidase assays were performed in duplicate to indicate activity from the P2 promoter. The β-galactosidase assay results are shown in Figure 17B and confirm that mutant H399 exhibits elevated *lacZ* expression in high iron conditions.

A phenotypic profile of mutant H399 was performed and the results are shown in Table 5. Resistance to tetracycline by H399 is due to the B30 transposon which contains a Tn10-derived Tet^R cassette. Sensitivity of mutant H399 to neomycin indicates that the B30 transposon is not inserted downstream of an active promoter on the chromosome. The B30 transposon contains a promoterless Neo^R gene upstream of the Tet^R gene. Qualitative protease and elastase assays indicate that the production of two other virulence factors does not seem to be effected by the transposon insertion.

Figure 17: Growth curve and β-galactosidase activities of *P. aeruginosa* strains IL-1 and H399 in high and low iron conditions.

Primary cultures were grown overnight in TSBDC high iron conditions. Secondary cultures were inoculated to a starting OD_{600} of 0.02 and each strain was inoculated into both a high iron and a low iron flask containing TSBDC. Aliquots were removed at indicated time points, resuspended in 1XA buffer and frozen at -80°C. β-galactosidase assays were performed in duplicate and averages of three growth curves are shown. **A.** Absorbance at 600nm and **B.** β-galactosidase activities of IL-1 and H399 in high and low iron conditions.


10
1
0.1
0.01
log OD600
0
2
4
6
8
10
12
14
16
18
20
22
24
26
Time (hours)
IL-1 High Iron
IL-1 Low Iron
H399 High Iron
H399 Low Iron

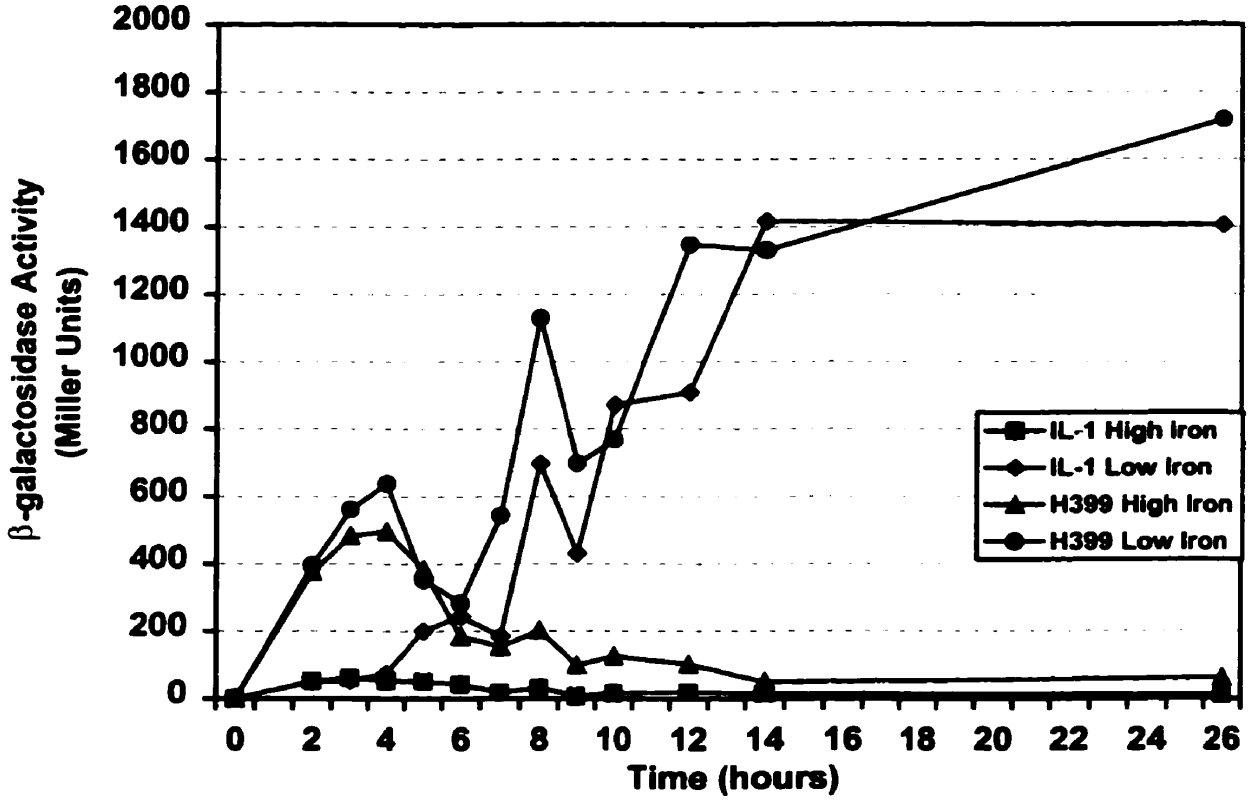

β-galactosidase Activity
(Miller Units)
2000
1800
1600
1400
1200
1000
800
600
400
200
0
0
2
4
6
8
10
12
14
16
18
20
22
24
26
Time (hours)
IL-1 High Iron
IL-1 Low Iron
H399 High Iron
H399 Low Iron

Table 5: Phenotype Profile of Mutant H399 vs. Wild-Type

Test Plate	IL-1	H399
Carbenicillin (400 μg/ml)	No growth	No growth
Neomycin (500 μg/ml)	No growth	No growth
Tetracycline (100 μg/ml)	No growth	Growth
Protease [a]	+ (0.5 mm clear zone)	+ (0.5 mm clear zone)
Elastase[b]	- (no zone of clearing)	- (no zone of clearing)

[a] PAO1 was a positive control and resulted in a 3 mm zone of clearing
[b] PAO1 was a positive control and resulted in a 2 mm zone of clearing

Genomic DNA was isolated from strains IL-1 and H399 and was digested with three different restriction enzymes that do not cut within the B30 transposon (*Eco*RI, *Cla*I and *Kpn*I). A Southern Blot was performed on the digested DNA using a 2.5 kb radiolabelled probe internal to the B30 transposon. The blot is shown in Figure 18 and confirms that the B30 transposon inserted into the chromosome of H399 only once and is absent from the chromosome of IL-1.

4.3.1 Effects of pP11 and pP21 in H399

As shown in Figure 17B, the P2 activity phenotype of mutant H399 was unusual. The P1 promoter is normally active in both high and low iron conditions and during the early log phase of growth. The activity of H399 in high iron could therefore be due to either an early P2 activity or to activity from the P1 promoter. In order to distinguish between these two types of activity, the expression from reporter plasmids pP11 and pP21 was investigated. The plasmid pP11 contains a P1-*cat* transcriptional fusion which allows the activity from a multicopy P1 promoter to be monitored by performing *cat*-ELISA assays (Storey *et al.*, 1990). Plasmid pP21 contains a P2-*cat* transcriptional fusion which, similarly expresses *cat* from a multicopy P2 promoter. Using these plasmids, we could differentiate between the two possible activities demonstrated by mutant H399 to determine whether or not this mutant contained a transposon insertion in a regulator of the P2 promoter.

Plasmids pP11 and pP21 were independently transformed into *P. aeruginosa* strains IL-1 and H399. Growth curves were performed in both high and low iron conditions and the absorbance at 600nm was measured over time. Figure 19A shows the pP11 growth curve and Figure 19B shows the pP21 growth curve. Each growth curve was performed a single time.

The complete genome sequence of *P. aeruginosa* strain PAO1 (Pseudomonas Genome Project) has revealed that this microorganism contains many *cat*-type genes. In order to test whether expression of these chromosomal genes would significantly affect our reporter gene expression, *cat*-ELISA assays were performed on non-transformed strains IL-1 and H399 in both high and low

Figure 18: Southern Blot of digested chromosomal DNA of mutant H399 and wild type IL-1 with a B30 internal probe.

A Southern Blot was performed on the chromosomal DNA of *P. aeruginosa* strains H399 and IL-1. A 2.5 kb internal B30 fragment was isolated by *Xho*I-*Eco*RV digestion from pSUP102::Tn5-B30 and used as a radiolabelled probe. Lane1 is *Eco*RI digested H399 chromosomal DNA; Lane 2 is *Cla*I digested H399 chromosomal DNA; Lane 3 is *Kpn*I digested H399 chromosomal DNA; Lane 4 is *Eco*RI digested IL-1 chromosomal DNA; Lane 5 is *Cla*I digested IL-1 chromosomal DNA; and Lane 6 is *Kpn*I digested IL-1 chromosomal DNA. Sizes from a λ-*Mlu*I ladder are indicated to the left of the blot.

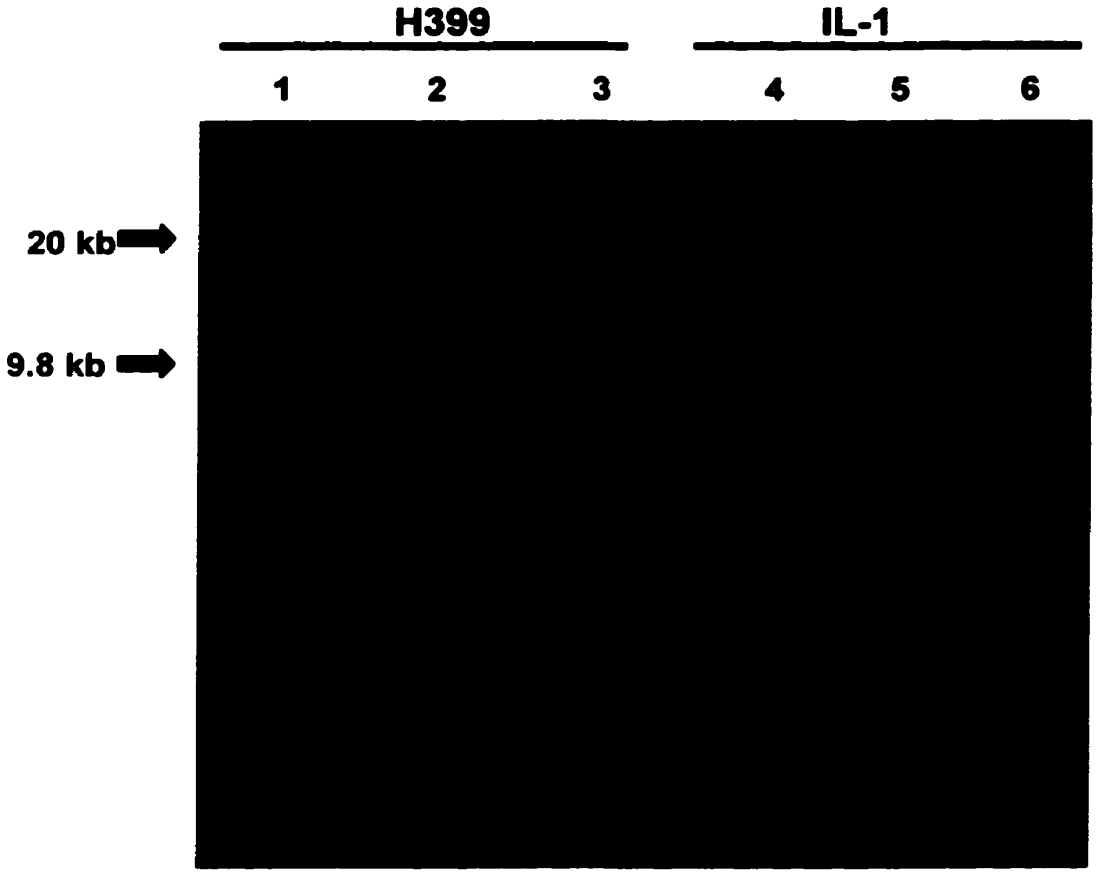

H399
IL-1
1
2
3
4
5
6
20 kb
9.8 kb

Figure 19

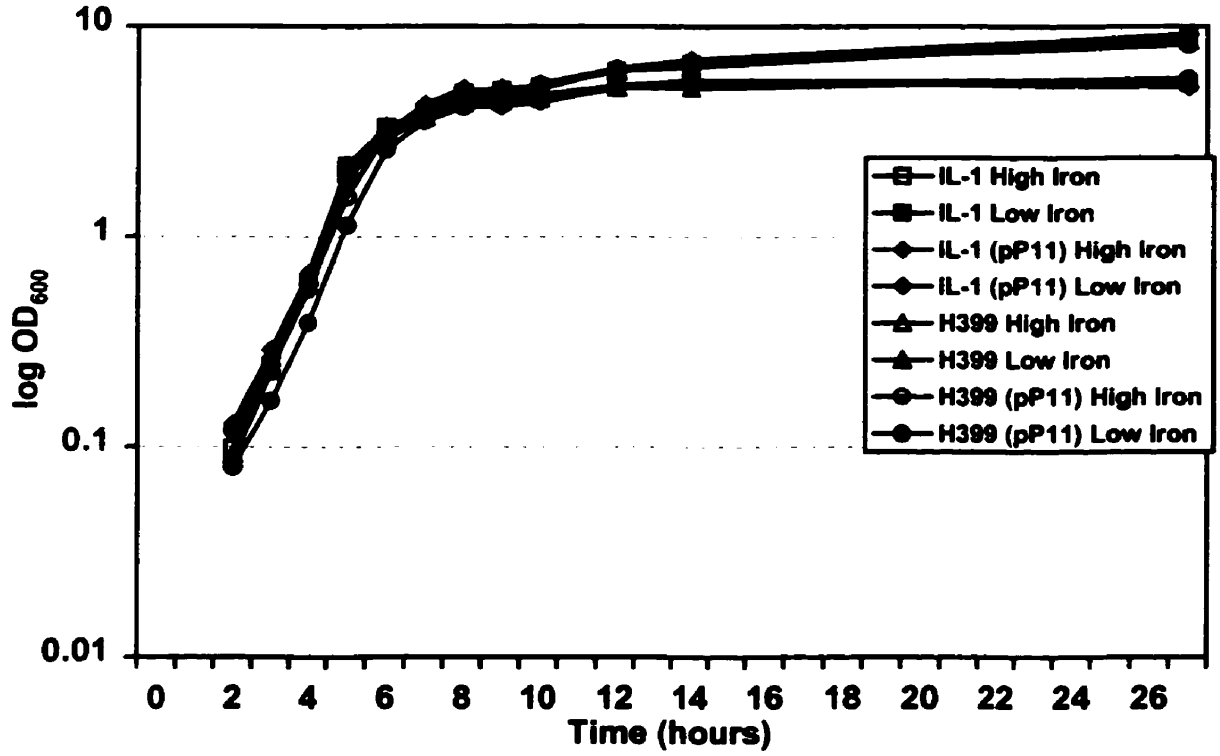
A: Growth curve of *P. aeruginosa* strains IL-1, IL-1 (pP11), H399 and H399 (pP11) in high and low iron conditions.

Primary cultures were grown overnight in TSBDC high iron conditions. Strains IL-1 (pP11) and H399 (pP11) were grown in TSBDC plus 400 μg/ml of carbenicillin. Secondary cultures were inoculated to a starting OD_{600} of 0.02 in both high and low iron TSBDC. High and low iron cultures of IL-1 (pP11) and H399 (pP11) also contained 400 μg/ml of carbenicillin. Aliquots were removed at each indicated time point and *cat*-ELISA assays were performed (Figure 21).

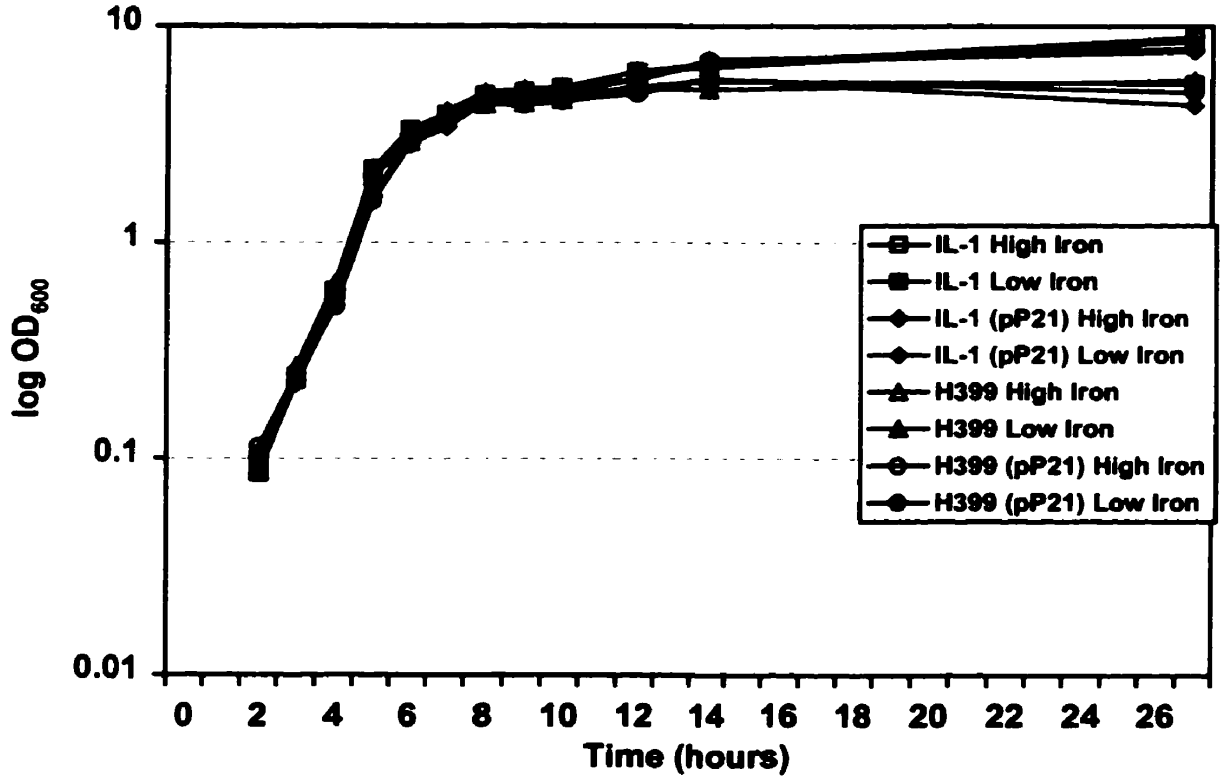
B: Growth curve of *P. aeruginosa* strains IL-1, IL-1 (pP21), H399 and H399 (pP21) in high and low iron conditions.

Primary cultures were grown overnight in TSBDC high iron conditions. Strains IL-1 (pP21) and H399 (pP21) were grown in TSBDC plus 400 μg/ml of carbenicillin. Secondary cultures were inoculated to a starting OD_{600} of 0.02. Each culture was started in both high and low iron conditions in TSBDC and cultures of IL-1 (pP21) and H399 (pP21) also contained 400 μg/ml of carbenicillin. Aliquots were removed at each indicated time point and *cat*-ELISA assays were performed (Figure 22).

A.


10
1
0.1
0.01
log OD600
0
2
4
6
8
10
12
14
16
18
20
22
24
26
Time (hours)
IL-1 High Iron
IL-1 Low Iron
IL-1 (pP11) High Iron
IL-1 (pP11) Low Iron
H399 High Iron
H399 Low Iron
H399 (pP11) High Iron
H399 (pP11) Low Iron

B.


10
1
0.1
0.01
log OD600
0
2
4
6
8
10
12
14
16
18
20
22
24
26
Time (hours)
IL-1 High Iron
IL-1 Low Iron
IL-1 (pP21) High Iron
IL-1 (pP21) Low Iron
H399 High Iron
H399 Low Iron
H399 (pP21) High Iron
H399 (pP21) Low Iron

iron conditions. Figure 20 shows the "background" CAT levels of these strains over the course of a growth curve. At each time point during the growth curves depicted in Figure 19A and 19B, samples were withdrawn from each culture and *cat*-ELISA assays were performed and compared to standards. The results of the pP11 growth curve are shown in Figure 21 and the results of the pP21 growth curve are in Figure 22.

4.3.2 Characterization of the Mutated Gene in H399

Genomic DNA from mutant H399 was digested with *Cla*I and separated on a 0.8% agarose gel. Fragments ranging in size from 7 kb to 12 kb were excised from the gel and electroeluted from the agarose. Simultaneously, pGEM-7Zf(+) vector DNA was digested with *Cla*I and CIAP treated. The H399 DNA fragments were ligated with the vector DNA and transformed into *E. coli* JM109 competent cells. The transformants were plated onto LB plates containing 10 μg/ml of tetracycline to select for the plasmid containing the B30 transposon. A Southern Blot and restriction enzyme digestion studies confirmed the presence of the B30 transposon in one plasmid. This plasmid, pH399, was prepared for sequencing and sent to the University Core DNA Services, University of Calgary. Primers used for sequencing included the IS50R primer which reads out from the B30 transposon, as well as the T7 and SP6 primers of the MCS of the vector pGEM-7Zf(+). Sequences obtained from the IS50R and SP6 primers overlapped to give 478 bp of common sequence located downstream of the B30 insertion on the H399 chromosome. BLAST analysis revealed that this region was homologous to a lactoylglutathione lyase enzyme, common to many bacteria. Figure 23 shows an amino acid alignment of this sequence downstream of the B30 insertion from strain H399, compared to other lactoylglutathione lyase bacterial protein sequences.

Figure 20: Background CAT levels of *P. aeruginosa* strains IL-1 and H399 in both high and low iron conditions.

Primary cultures were grown overnight in TSBDC high iron conditions. Secondary cultures were inoculated to a starting OD_{600} of 0.02 in both high and low iron TSBDC conditions. An aliquot volume of OD_{600} equal to 5 was removed at each indicated time point, washed in 100mM Tris (pH 7.8), and the cell pellet stored at -20°C. Cells were sonicated prior to performing *cat* ELISA assays.

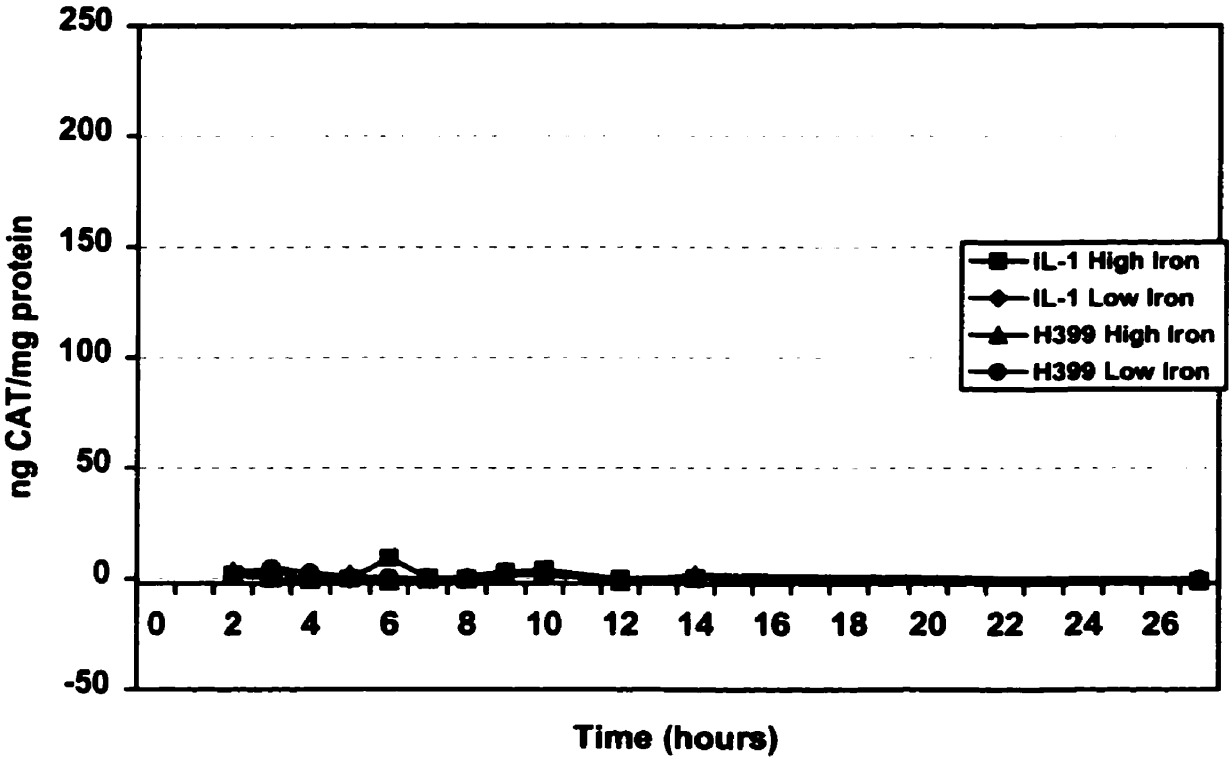

250
200
150
100
50
0
-50
ng CAT/mg protein
0
2
4
6
8
10
12
14
16
18
20
22
24
26
Time (hours)
IL-1 High Iron
IL-1 Low Iron
H399 High Iron
H399 Low Iron

Figure 21: CAT levels of *P. aeruginosa* strains IL-1 (pP11) and H399 (pP11) in both high and low iron conditions.

Cultures were prepared as described in Figure 19. A volume equal to $5X10^8$ cells was removed at each time point, washed in 100mM Tris (pH 7.8), and the cell pellet was stored at -20°C. Cells were sonicated prior to performing *cat* ELISA assays.

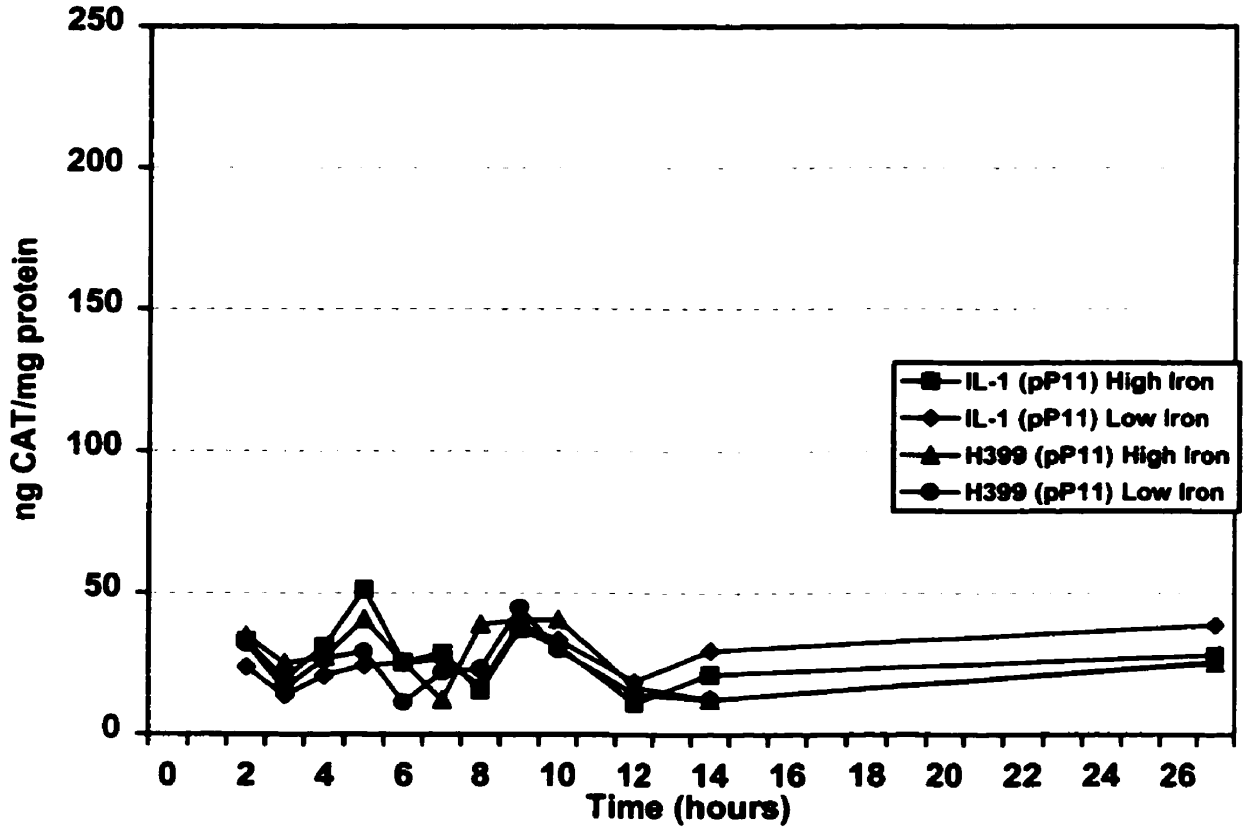

250
200
150
100
50
0
ng CAT/mg protein
0
2
4
6
8
10
12
14
16
18
20
22
24
26
Time (hours)
IL-1 (pP11) High Iron
IL-1 (pP11) Low Iron
H399 (pP11) High Iron
H399 (pP11) Low Iron

Figure 22: CAT activities of *P. aeruginosa* strains IL-1 (pP21) and H399 (pP21) in both high and low iron conditions.

Cultures were prepared as described in Figure 19. A volume equal to $5X10^8$ cells was removed at each indicated time point, washed in 100mM Tris (pH 7.8), and the cell pellet was stored at -20°C. Cells were sonicated prior to performing *cat* ELISA assays.

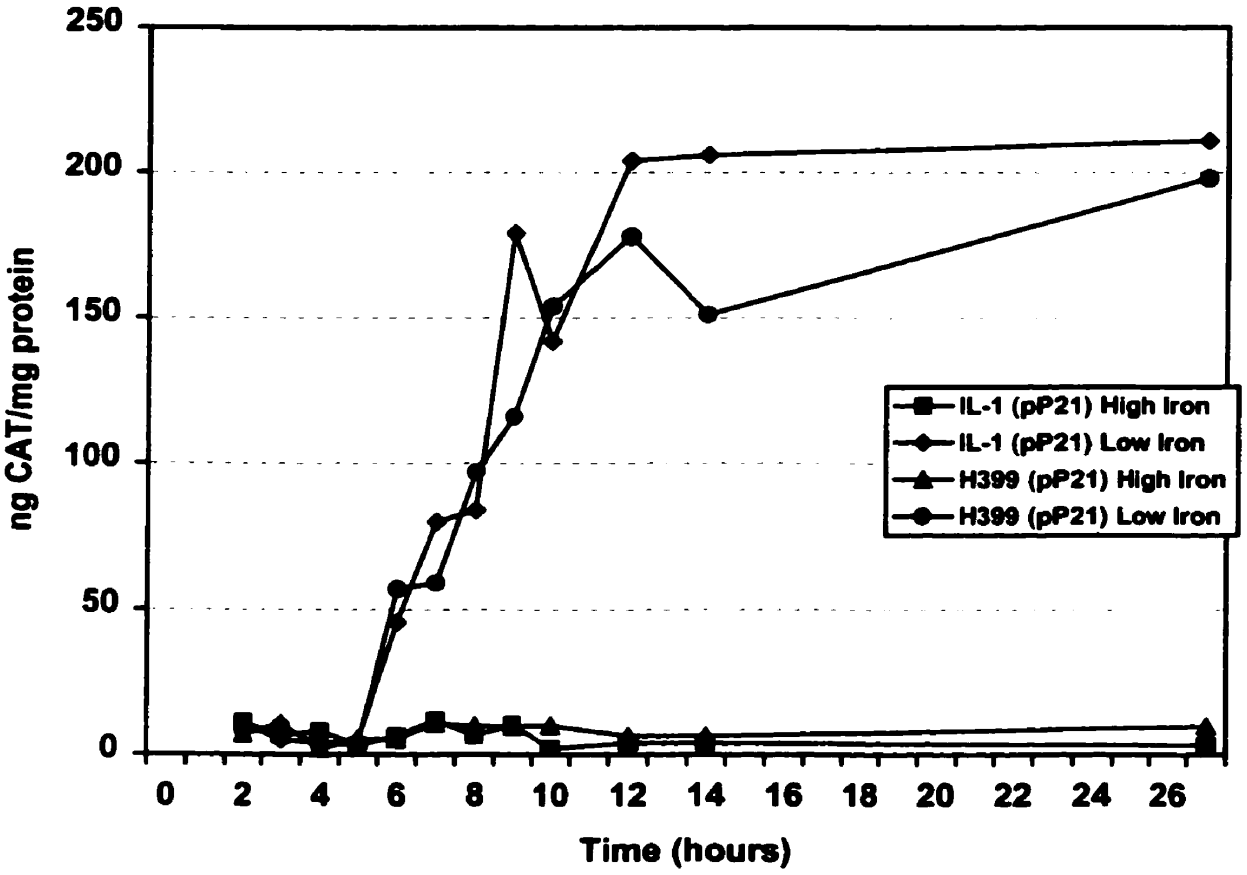

250
200
150
100
50
0
ng CAT/mg protein
0
2
4
6
8
10
12
14
16
18
20
22
24
26
Time (hours)
IL-1 (pP21) High Iron
IL-1 (pP21) Low Iron
H399 (pP21) High Iron
H399 (pP21) Low Iron

Figure 23: Amino acid alignment of a portion of the sequence from pH399 with the homologous region from other known lactoylglutathione lyase bacterial proteins.

The sequence from pH399 is shown in blue. Identical amino acids are shown in red and similar amino acids are pink. Non-homologous amino acids are black. All of the homologies shown are to the C-terminal end of the protein (Genbank accession No. Y14298, U32826; MacLean *et al.* (1998); Clugston *et al.* (1997); Genbank accession No. U06949).

H399	R	A	L	G	Y	R	V	T	R	E	A	G	P	M	Q	H	G	R	S	V	I	A	F	L	E	D	P	D	G	Y	K	V	E	L	I	Q
Neisseria meningitidis				G	G	N	V	V	R	E	A	G	P	M	K	H	G	T	T	V	I	A	F	V	E	D	P	D	G	Y	K	I	E	F	I	Q
Haemophilus influenzae	R	A	S	G	G	N	V	T	R	E	A	G	P	V	K	G	G	S	T	V	I	A	F	V	E	D	P	D	G	Y	K	I	E	F	I	E
Escherichia coli	R	Q	N	G	G	N	V	T	R	E	A	G	P	V	K	G	G	T	T	V	I	A	F	V	E	D	P	D	G	Y	K	I	E	L	I	E
Salmonella typhimurium	R	Q	N	G	G	N	V	T	R	E	A	G	P	V	K	G	G	S	T	I	I	A	F	V	E	D	P	D	G	Y	K	I	E	L	I	E
Vibrio parahaemolyticus	K	A	A	G	G	N	V	T	R	E	A	G	P	V	K	G	G	T	T	H	I	A	F	V	K	D	P	D	G	Y	M	I	E	L	I	Q

4.3.3 Complementation Studies

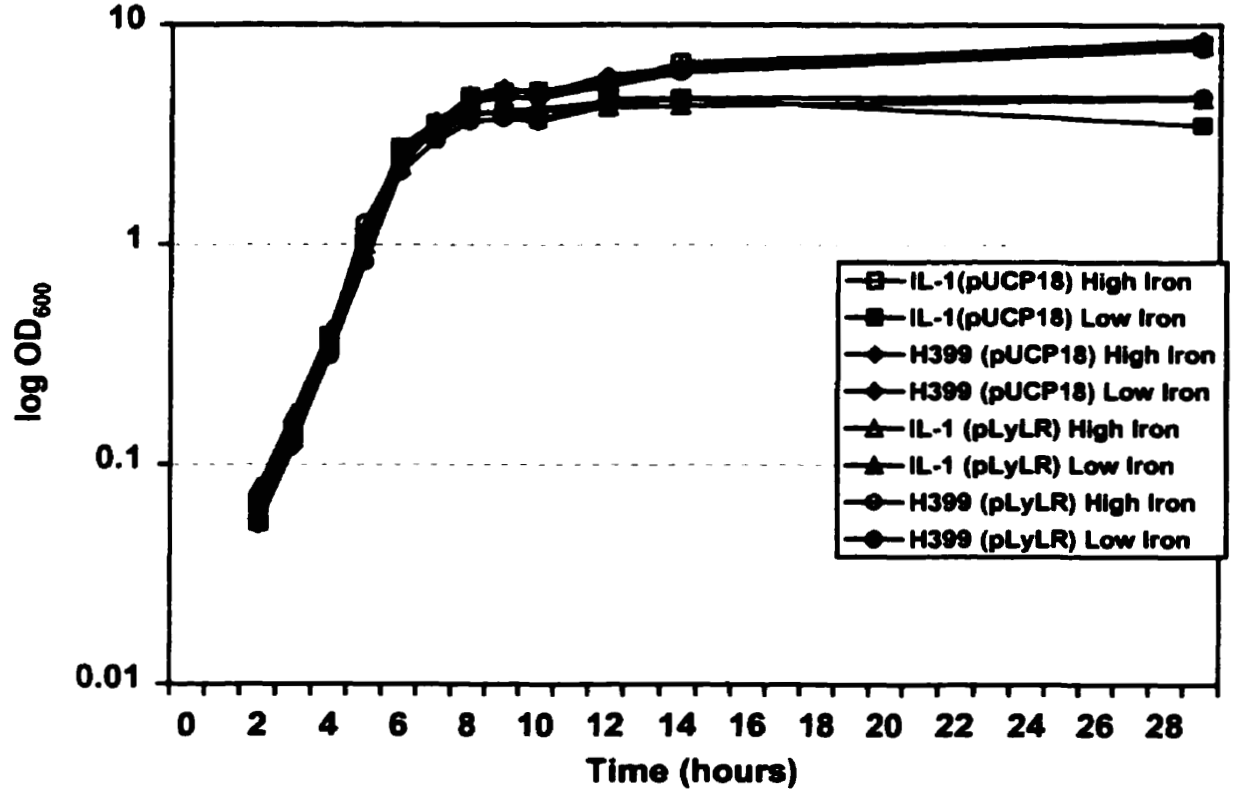
BLAST and genome analysis suggested that a lactoylglutathione lyase homologue is located on the *P. aeruginosa* chromosome approximately 1800 bp upstream of the *regAB* locus. This upstream region had previously been cloned into a pUC12 plasmid. The plasmid pS_8E (Loubens, unpublished) contains a 2.8 kb *SalI-Eco*RI fragment which includes the entire lactoylglutathione lyase gene as well as an upstream LysR-type regulator homologue. Plasmid pS_8E was digested with *SalI* and *Eco*RI and the resulting fragment was ligated into a pUCP18 vector which contains the *Pseudomonas* origin of replication. The resulting plasmid, pLyLR, was used for complementation studies. Plasmid pLyLR contains two functional genes, the lactoylglutathione lyase gene which is interrupted on the H399 chromosome and the uncharacterized LysR-type regulator homologue. The LysR-type regulator homologue gene was included on the complementation plasmid because it was a better candidate for possessing a role in the regulation of *regAB*. Also, the interruption of the proximal lactoylglutathione lyase gene on the H399 chromosome may affect transcription of the upstream LysR-type regulator and using pLyLR in the complementation studies would reveal if either gene was involved in causing the phenotype of mutant H399.

The complementation plasmid pLyLR and the vector control pUCP18 were transformed into strains IL-1 and H399. A growth curve was performed on these strains in both high and low iron conditions. Figure 24A shows the absorbance at 600nm at each time point. β-galactosidase assays were performed in duplicate to monitor the P2 activity of each strain at indicated time points. The β-galactosidase activities over the course of the growth curve are shown in Figure 24B. This growth curve was performed once and the results demonstrate that the plasmid did not complement mutant H399. Although the B30 transposon was chosen for these studies because it should not possess any genes which would read-through to the chromosome and cause polar mutations, our results suggest otherwise. The phenotype of H399 is, therefore, most likely due to a polar effect on the *regAB* operon which is in close proximity to the B30 insertion on the chromosome of

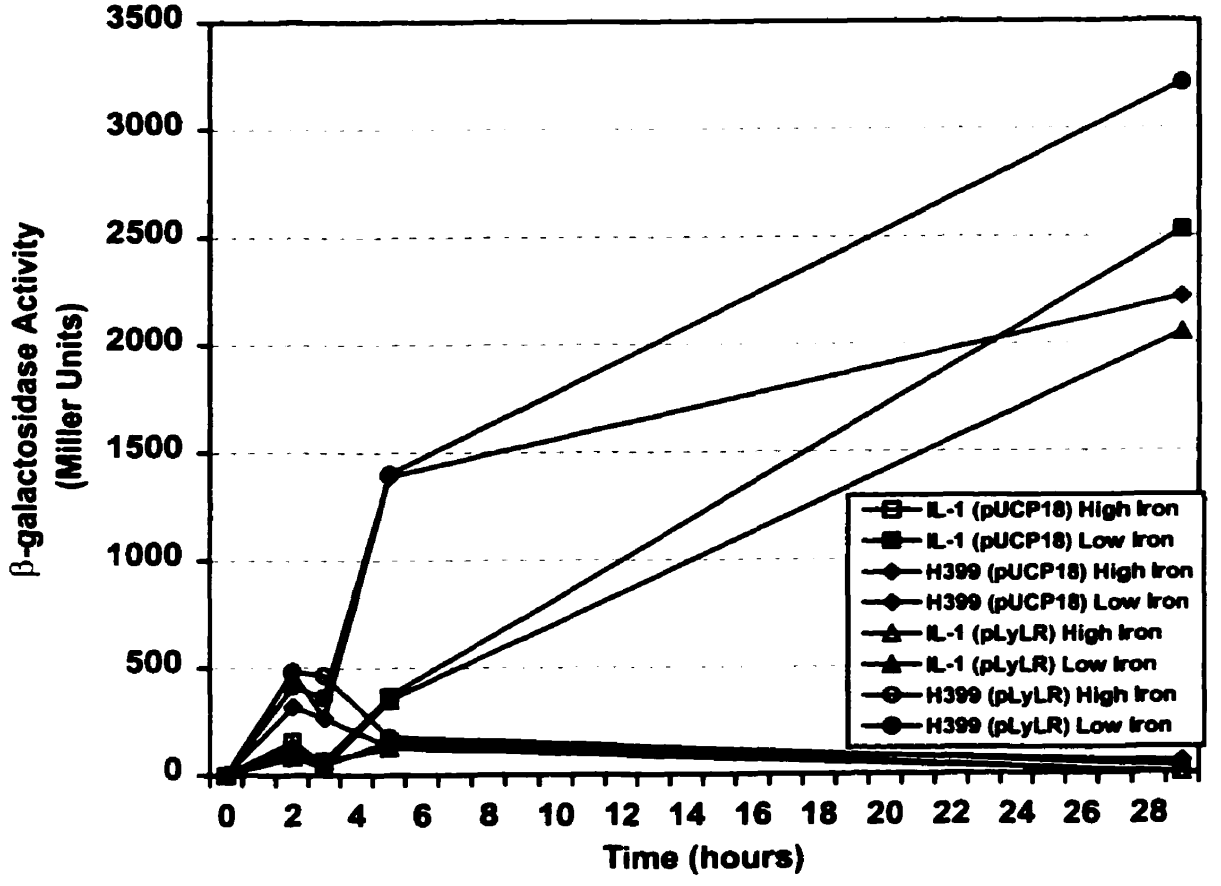
Figure 24: Growth curve of *P. aeruginosa* strains IL-1 (pUCP18), IL-1 (pLyLR), H399 (pUCP18), and H399 (pLyLR) in high and low iron conditions.

Primary cultures were grown overnight in TSBDC high iron conditions containing 400 μg/ml of carbenicillin. Secondary cultures were inoculated to a starting OD_{600} of 0.02. Each strain was inoculated into a high iron and a low iron flask of TSBDC containing 400 μg/ml of carbenicillin. Aliquots were removed at 2 hr, 3 hr, 5 hr, and 29 hr time points, resuspended in 1XA buffer, and stored at -80°C. β-galactosidase assays were performed in duplicate. **A.** Absorbance at 600nm and **B.** β-galactosidase assays of IL-1 (pUCP18), IL-1 (pLyLR), H399 (pUCP18), and H399 (pLyLR) in high and low iron conditions.

A.


10
1
0.1
0.01
log OD600
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
Time (hours)
IL-1(pUCP18) High Iron
IL-1(pUCP18) Low Iron
H399 (pUCP18) High Iron
H399 (pUCP18) Low Iron
IL-1 (pLyLR) High Iron
IL-1 (pLyLR) Low Iron
H399 (pLyLR) High Iron
H399 (pLyLR) Low Iron

B.


3500
3000
2500
2000
1500
1000
500
0
β-galactosidase Activity (Miller Units)
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28
Time (hours)
IL-1 (pUCP18) High Iron
IL-1 (pUCP18) Low Iron
H399 (pUCP18) High Iron
H399 (pUCP18) Low Iron
IL-1 (pLyLR) High Iron
IL-1 (pLyLR) Low Iron
H399 (pLyLR) High Iron
H399 (pLyLR) Low Iron

mutant H399 and not due to the interruption by the transposon.

4.3.4 Location of the Polar Mutation on the H399 Chromosome

Because of the proximity of the lactoylglutathione lyase gene to the *regAB* operon, and because the mutation was not complemented by pLyLR, we hypothesized that the phenotype displayed by mutant H399 may be due to a polar mutation. The B30 may be activating the P1 promoter of *regAB* by reading through the intergenic space, and not because it is interrupting the transcription of a regulator.

In order to confirm this hypothesis, the exact location of the B30 transposon with respect to the *regAB* operon was determined. The Southern Blot depicted in Figure 18 was stripped and re-probed with a 3.2 kb *Bam*HI-digested *lacZ* fragment from pZ1918. This probe hybridizes to the *lacZ* gene at the *regAB* locus of IL-1 and H399. The Southern Blot is shown in Figure 25. This probe hybridizes to the same chromosomal DNA fragment in mutant H399 as the B30 internal probe hybridizes, we can conclude therefore that the B30 transposon has inserted near the *regAB* locus in this mutant. The Southern Blot also shows that the band which the *lacZ* probe hybridizes to mutant H399 are larger than the comparable bands in IL-1. This suggests that the chromosomal fragment containing the *lacZ* gene in H399 is larger than the same fragment in IL-1, due to the 6.1 kb B30 transposon insertion on the chromosome of the mutant. Further sequencing analysis of plasmid pH399 confirmed the location of the B30 transposon on the H399 chromosome, and this is depicted schematically in Figure 26. This confirms the phenotype observed for mutant H399 was due to a polar mutation and is not a true mutation in a regulatory gene which acts on the P2 promoter of the *regAB* operon.

Figure 25: Southern Blot of IL-1 and H399 with a *lacZ* probe.

The Southern Blot of the chromosomal DNA from *P. aeruginosa* strains IL-1 and H399 shown in Figure 18 was stripped and re-probed with a 3.2 kb *Bam*HI-digested fragment from pZ1918. A portion of the Southern Blot probed with an internal B30 probe shown in Figure 18 is also shown for comparison. Lanes 1-6 are probed with the *lacZ* probe and lanes 7-9 are probed with the B30 internal probe. Lanes 1 and 7 are *Eco*RI digested H399 chromosomal DNA; Lanes 2 and 8 are *Cla*I digested H399 chromosomal DNA; Lanes 3 and 9 are *Kpn*I digested H399 chromosomal DNA; Lane 4 is *Eco*RI digested IL-1 chromosomal DNA; Lane 5 is *Cla*I digested IL-1 chromosomal DNA; and Lane 6 is *Kpn*I digested IL-1 chromosomal DNA. Note that *Cla*I cuts within the *lacZ* gene so two bands can be seen in the lanes containing *Cla*I digested DNA.

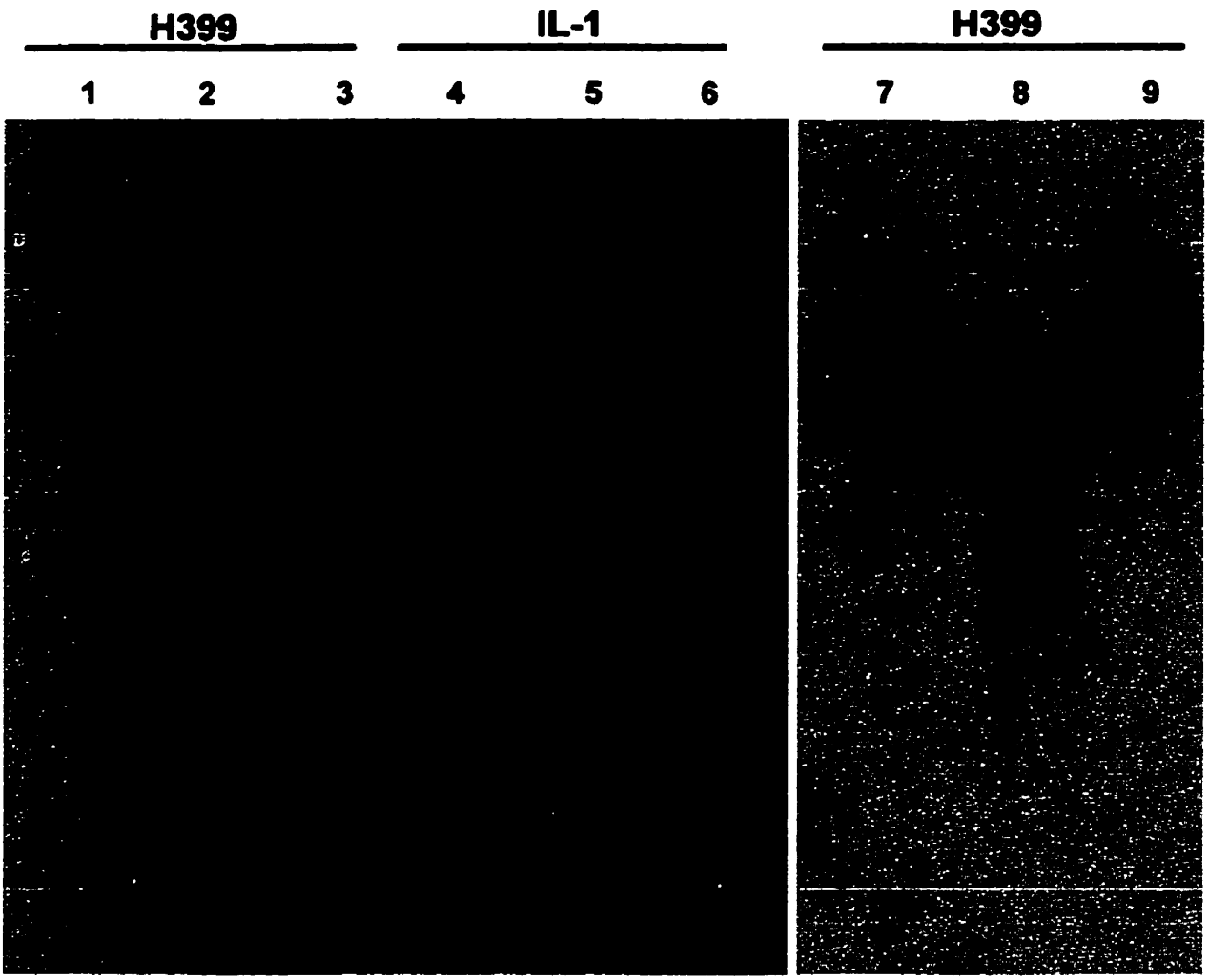
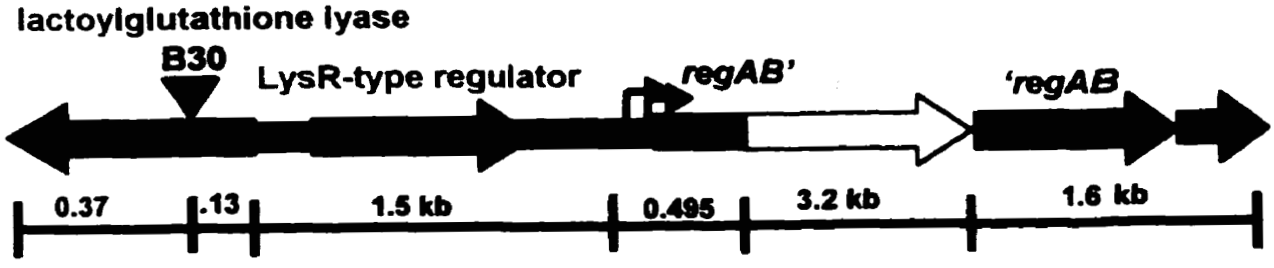

H399
IL-1
H399
1
2
3
4
5
6
7
8
9

Figure 26: Schematic representation of the position of the B30 transposon on the chromosome of mutant H399.

All known genes and gene homologues in this region are shown. Arrows represent the direction of transcription. Distances are listed below in kilobases and are not drawn to scale.


lactoylglutathione lyase
B30
LysR-type regulator
regAB'
'regAB
0.37
.13
1.5 kb
0.495
3.2 kb
1.6 kb

4.4 Mutant L522

A growth curve was performed on mutant L522 in both high and low iron conditions. The absorbance at 600nm was measured at each time point for the wild type IL-1 and the mutant L522. The growth curve was performed three times and the averages are shown in Figure 27A. At each indicated time point, samples were collected and β-galactosidase assays were performed in duplicate. Figure 27B shows the activity from the P2 promoter as indicated by the *lacZ* expression in each strain in both high and low iron conditions. The results indicate that the P2 activity of mutant L522 in low iron conditions is significantly lower than that of IL-1. The B30 transposon may have inserted into a gene which codes for an activator of the P2 promoter.

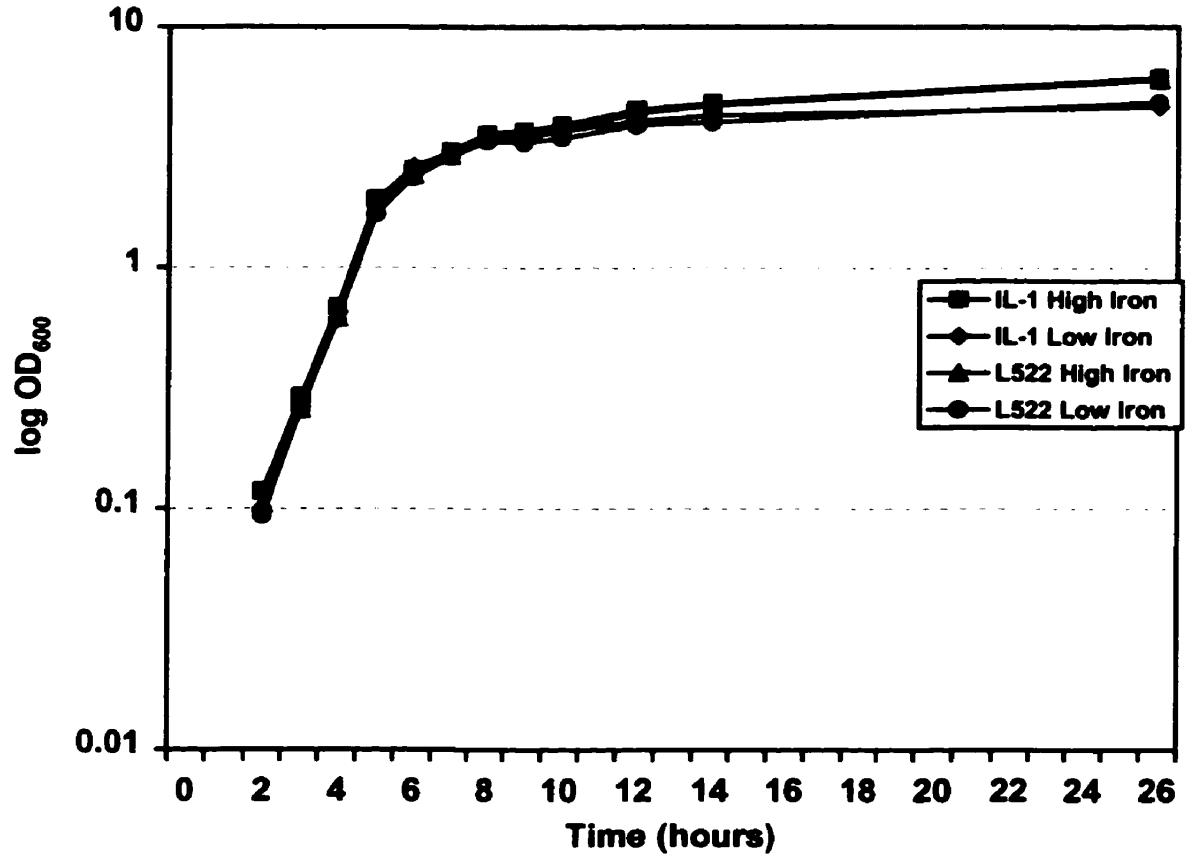
As with mutant H399, a qualitative phenotypic profile was performed on L522 and compared to the wild-type IL-1. The results are shown in Table 6. The resistance of L522 to tetracycline results from the B30 transposon, which possesses a Tet^R cassette. The B30 also contains a promoterless Neo^R cassette at the IS50L end. The neomycin resistance displayed by L522 implies that the transposon is inserted downstream of an active promoter on the chromosome of this mutant. The production of virulence factors (elastase and proteases) by L522 is not significantly different from wild type IL-1.

Chromosomal DNA was isolated from L522 and digested with three different restriction enzymes which do not cut within the B30 transposon (*Eco*RI, *Cla*I and *Kpn*I). The DNA was separated on a 0.8% agarose gel and blotted onto a Nytran membrane. A Southern Blot was performed using a probe internal to the B30 transposon and is shown in Figure 28. The blot demonstrates that the B30 transposon inserted only once on the chromosome of L522 and is absent from the chromosome of wild type IL-1.

Figure 27: Growth curve of *P. aeruginosa* strains IL-1 and L522 in high and low iron conditions.

Primary cultures were incubated overnight in TSBDC high iron conditions. Secondary cultures were inoculated to a starting OD_{600} of 0.02. Both strains were inoculated into high and low iron TSBDC and aliquots were removed at each indicated time point. Aliquots were removed at each indicated time point, resuspended in 1XA buffer, and frozen at -80°C. β-galactosidase assays were performed in duplicate and averages are shown. **A.** Absorbance at 600nm and **B.** β-galactosidase assays of IL-1 and L522 in high and low iron conditions.

A.


10
1
0.1
0.01
log OD600
0
2
4
6
8
10
12
14
16
18
20
22
24
26
Time (hours)
IL-1 High Iron
IL-1 Low Iron
L522 High Iron
L522 Low Iron

B.

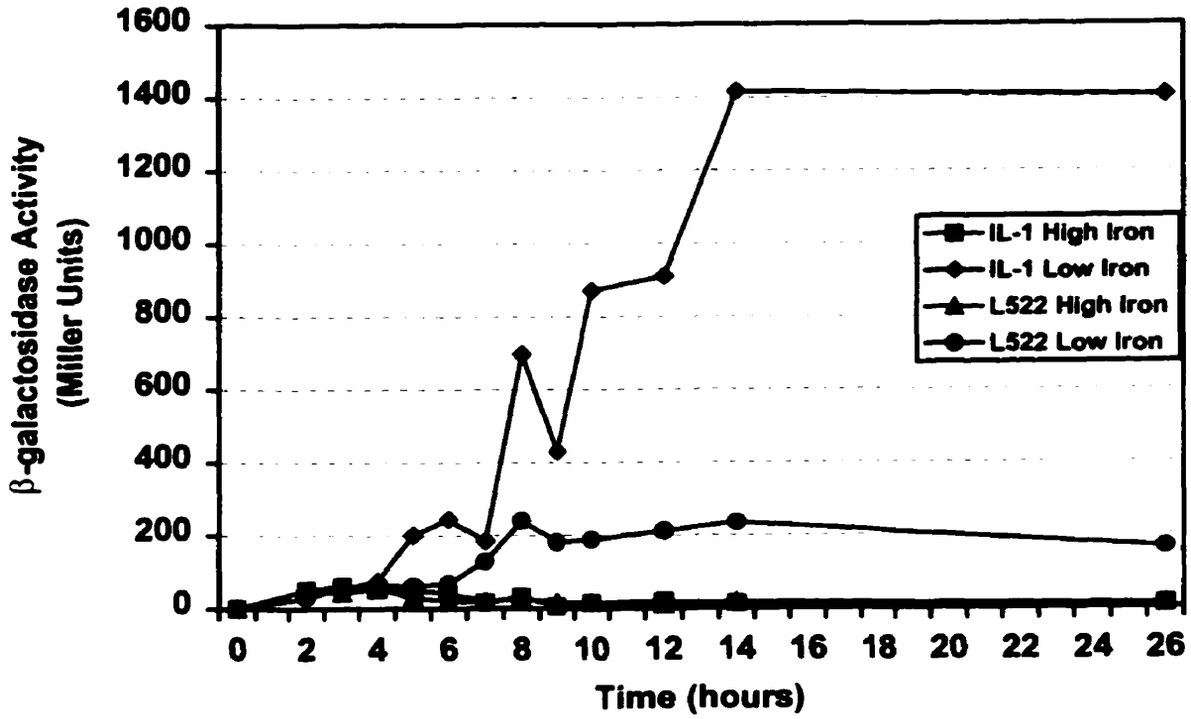

1600
1400
1200
1000
800
600
400
200
0
β-galactosidase Activity
(Miller Units)
0
2
4
6
8
10
12
14
16
18
20
22
24
26
Time (hours)
IL-1 High Iron
IL-1 Low Iron
L522 High Iron
L522 Low Iron

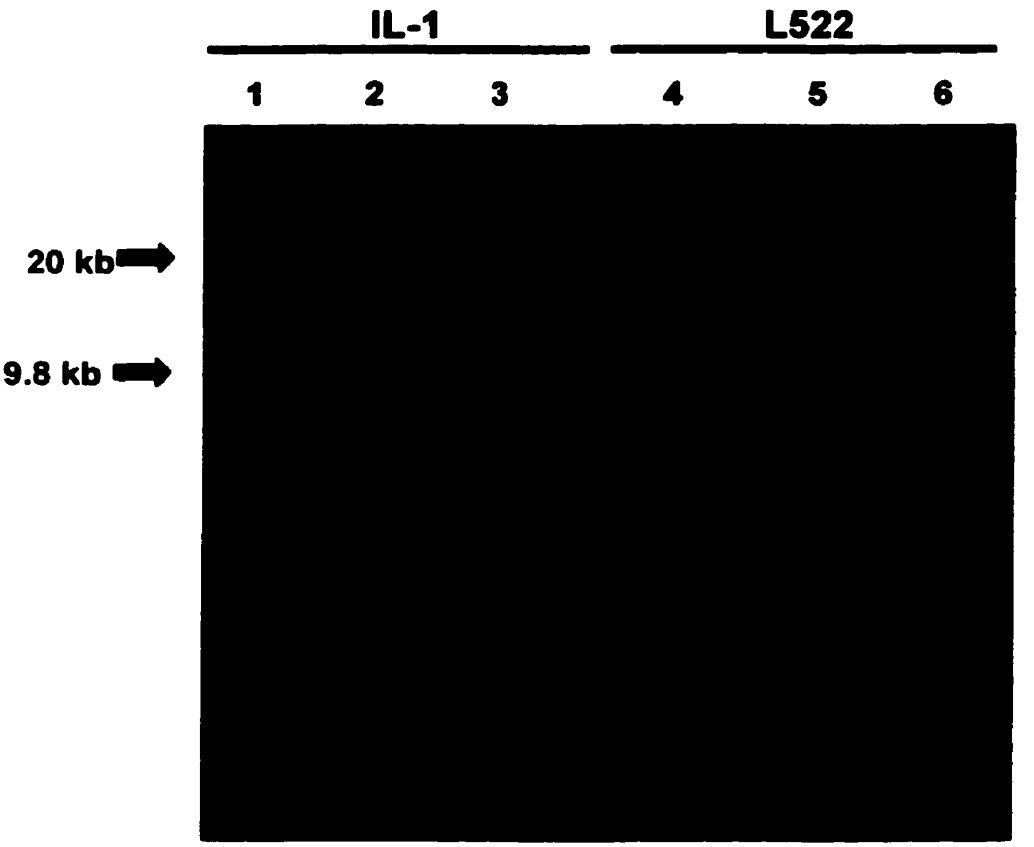
Table 6: Phenotypic Profile of Mutant L522 vs. Wild-Type

Test Plate	IL-1	L522
Carbenicillin (400 μg/ml)	No growth	No growth
Neomycin (500 μg/ml)	No growth	Growth
Tetracycline (100 μg/ml)	No growth	Growth
Protease [a]	+ (0.5 mm clear zone)	+ (0.5 mm clear zone)
Elastase[b]	- (no zone of clearing)	- (no zone of clearing)

[a] PAO1 was a positive control and resulted in a 3 mm zone of clearing
[b] PAO1 was a positive control and resulted in a 2 mm zone of clearing

Figure 28: Southern Blot of digested chromosomal DNA from IL-1 and L522 with an internal B30 probe.

A Southern Blot was performed on the chromosomal DNA of the wild-type IL-1 and mutant L522. A 2.5 kb internal B30 fragment was isolated by *Xho*I and *Eco*RV digestion from pSUP102::Tn5-B30 and used as a radiolabelled probe. Lane 1 is *Eco*RI digested IL-1 chromosomal DNA; Lane 2 is *Cla*I digested IL-1 chromosomal DNA; Lane 3 is *Kpn*I digested IL-1 chromosomal DNA; Lane 4 is *Eco*RI digested L522 chromosomal DNA; Lane 5 is *Cla*I digested L522 chromosomal DNA; and Lane 6 is *Kpn*I digested L522 chromosomal DNA. Sizes from a λ-*Mlu*I ladder are indicated to the left of the blot.


IL-1
L522
1
2
3
4
5
6
20 kb
9.8 kb

4.4.1 Effect of pP21 in L522

In order to verify that the phenotype of L522 is due to a true gene interruption and not a polar effect as with mutant H399, plasmid pP21 was used. The reporter plasmid pP21 contains a P2-*cat* fusion which expresses a *cat* reporter gene from a multicopy P2 promoter. If the phenotype displayed by L522 is due to a mutation in an activator of the P2 promoter then we would expect the CAT levels from pP21 to be similar to the *lacZ* activity from the chromosome. To this end, pP21 was electroporated into L522 and IL-1 and a growth curve was performed in high and low iron conditions (Figure 29). Background levels of CAT were measured (Figure 30) for both the mutant L522 and the wild-type strain IL-1 to verify that these levels would not skew the data obtained from the reporter plasmid pP21. At each time point during the growth curve depicted in Figure 29, samples were collected and *cat*-ELISA assays were performed. The results of the CAT levels from each strain transformed with pP21 confirm that the mutated gene in strain L522 is a positive regulator of the P2 promoter (Figure 31).

4.4.2 Evidence for a Novel Regulator of the P2 Promoter

To date, a regulator which acts on the P2 promoter has not been identified. However, data from Chapter 3 provides evidence that PvdS is a regulator of this promoter. Before further investigating the identity of the gene being interrupted on the chromosome of mutant L522, we wanted to examine whether the phenotype displayed by L522 was due to a mutation in the *pvdS* gene. Genomic DNA was isolated from IL-1 and L522 and the DNA was digested with various restriction enzymes. The digested DNA was separated on a 0.8% agarose gel and blotted onto a Nytran membrane. A Southern blot was performed with a probe to the *pvdS* gene and this blot is shown in Figure 32. The blot demonstrates that the *pvdS* gene in L522 is not interrupted by the B30 transposon. The hybridization bands of IL-1 and L522 are indistinguishable for each restriction enzyme digestion, so the *pvdS* locus of L522 is identical to that of IL-1. Also, when this blot was stripped and re-probed with an internal B30 probe, the pattern of the hybridization bands

Figure 29: Growth curve of *P. aeruginosa* strains IL-1, IL-1 (pP21), L522, and L522 (pP21) in high and low iron conditions.

Primary cultures were grown overnight in TSBDC high iron conditions. Strains IL-1 (pP21) and L522 (pP21) were grown in TSBDC plus 400 μg/ml of carbenicillin. Secondary cultures were inoculated to a starting OD at 600nm of 0.02. Each culture was inoculated into high and low iron TSBDC media. Cultures of IL-1 (pP21) and L522 (pP21) were supplemented with 400 μg/ml of carbenicillin. Aliquots were removed at indicated time points and *cat* assays were performed (Figure 30 and 31).

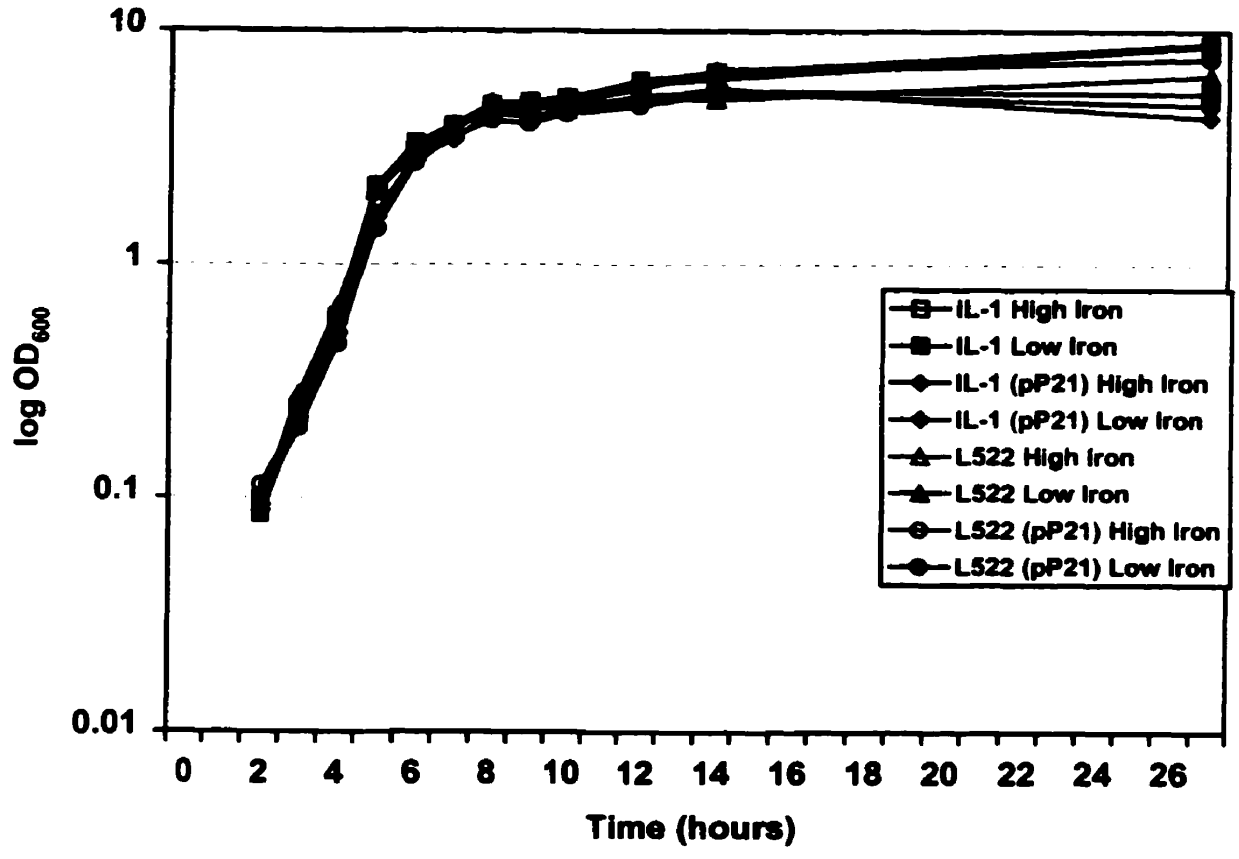

10
1
0.1
0.01
log OD600
0
2
4
6
8
10
12
14
16
18
20
22
24
26
Time (hours)
IL-1 High Iron
IL-1 Low Iron
IL-1 (pP21) High Iron
IL-1 (pP21) Low Iron
L522 High Iron
L522 Low Iron
L522 (pP21) High Iron
L522 (pP21) Low Iron

Figure 30: Background CAT levels of IL-1 and L522 in high and low iron conditions.

Primary cultures were grown overnight in TSBDC high iron conditions. Secondary cultures were inoculated to a starting OD_{600} of 0.02 in both high and low iron conditions. A volume equal to $5X10^8$ cells was removed at each time point, washed with 100mM Tris (pH 7.8), and the cell pellet was stored at -20°C. Cells were sonicated and *cat*-ELISA assays were performed.

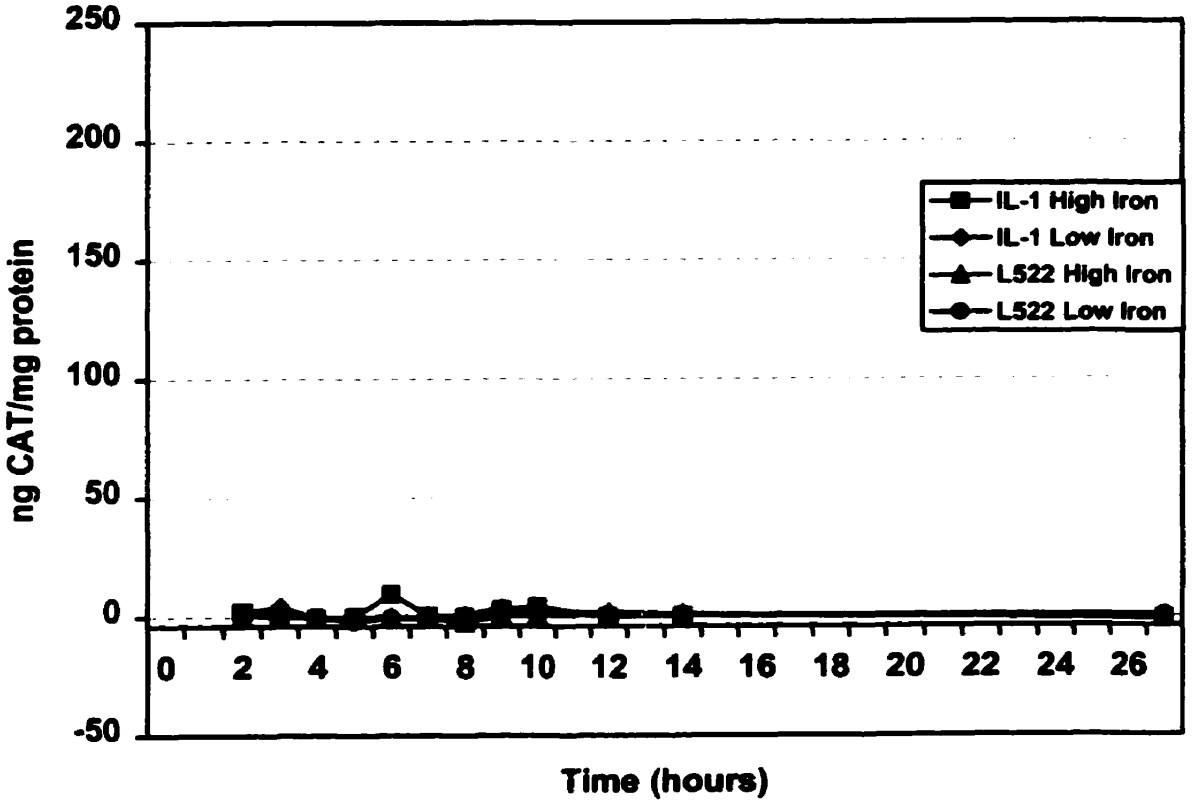

250
200
150
100
50
0
-50
ng CAT/mg protein
0
2
4
6
8
10
12
14
16
18
20
22
24
26
Time (hours)
IL-1 High Iron
IL-1 Low Iron
L522 High Iron
L522 Low Iron

Figure 31: CAT levels of *P. aeruginosa* strains IL-1 (pP21) and L522 (pP21) in high and low iron conditions.

Cultures were prepared as described in Figure 29. A volume equal to $5X10^8$ cells was removed at each indicated time point, washed in 100mM Tris (pH 7.8), and the cell pellet was stored at -20°C. Cells were sonicated prior to *cat*-ELISA assays being performed.

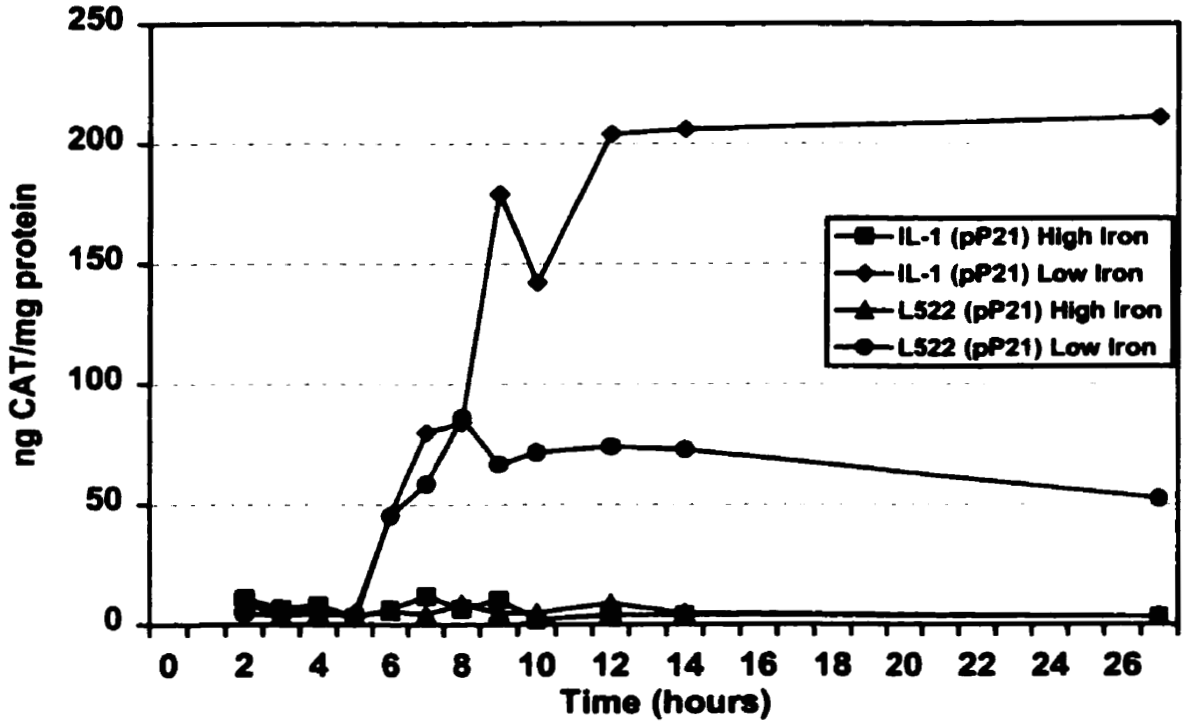
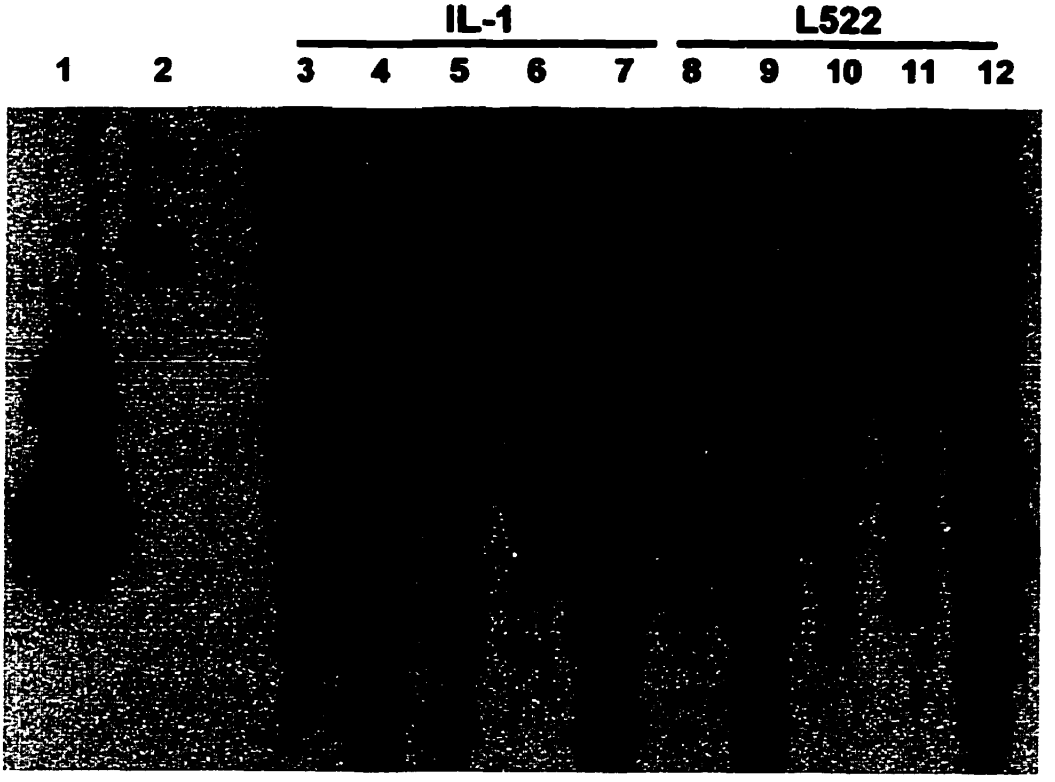

250
200
150
100
50
0
ng CAT/mg protein
0
2
4
6
8
10
12
14
16
18
20
22
24
26
Time (hours)
IL-1 (pP21) High Iron
IL-1 (pP21) Low Iron
L522 (pP21) High Iron
L522 (pP21) Low Iron

Figure 32: Southern Blot of digested IL-1 and L522 chromosomal DNA with a *pvdS* probe.

A Southern Blot was performed on the chromosomal DNA of IL-1 and L522. The chromosomal DNA was digested with five different restriction enzymes. A 900 bp *Eco*RI fragment from pLD201.1 was used as a *pvdS* probe. Lanes 3-7 contain IL-1 DNA digested with different restriction enzymes while Lane 8-12 contain L522 DNA digested with the same restriction enzymes. Lane 1 is undigested pLD201.1 (positive control); Lane 2 is undigested pSUP102::Tn5-B30 (negative control); Lane 3 is *Eco*RI digested IL-1 chromosomal DNA; Lane 4 is *Acc*I digested IL-1 chromosomal DNA; Lane 5 is *Kpn*I digested IL-1 chromosomal DNA; Lane 6 is *Bam*HI digested IL-1 chromosomal DNA; Lane 7 is *Sal*I digested IL-1 chromosomal DNA; Lane 8 is *Eco*RI digested L522 chromosomal DNA; Lane 9 is *Acc*I digested L522 chromosomal DNA; Lane 10 is *Kpn*I digested L522 chromosomal DNA; Lane 11 is *Bam*HI digested L522 chromosomal DNA; Lane 12 is *Sal*I digested L522 chromosomal DNA.


IL-1
L522
1
2
3
4
5
6
7
8
9
10
11
12

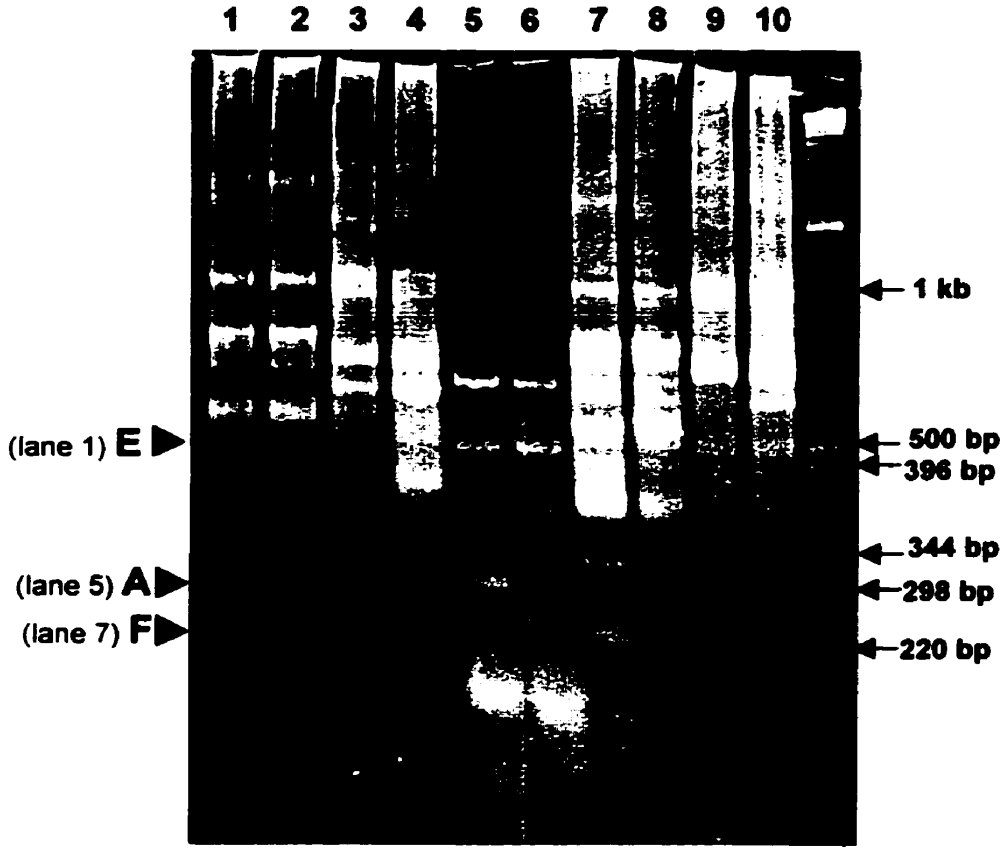
was different from those seen with the *pvdS* probe indicating that the B30 and the *pvdS* genes are at different locations on the chromosome of L522 (data not shown). The gene which is interrupted by the B30 transposon in L522 and codes for an activator of the P2 promoter is therefore a novel positive regulator of *regAB*.

4.4.3 Attempts to Clone the Gene Interrupted by the B30 in L522

Numerous conventional and novel methods have been employed in an attempt to identify the gene which the B30 transposon interrupts on the chromosome of mutant L522. Ligation of the transposon plus flanking DNA into vectors such as pBluescript II SK(+), pUC18, and pGEM-7Zf(+) have been unsuccessful, possibly due to the large size of the B30 transposon (6.1 kb). In addition, digestion within the transposon plus flanking DNA to yield smaller DNA fragments and ligation into these vectors has also been unsuccessful. Inverse PCR was also employed but PCR products were not generated using this method. Finally, arbitrary primed PCR was performed on the chromosomal DNA of IL-1 and L522. Using the arbitrary primed PCR technique, many PCR products are obtained because arbitrary primers are used. In order to distinguish between PCR products which are the result of only arbitrary primers and those which are the result of specific primers, the technique was performed on chromosomal DNA of IL-1 and L522 simultaneously. PCR products were run on an acrylamide gel and those bands which were unique to L522 were isolated and sequenced as potential clones. Figure 33 shows the results of an arbitrary primed PCR experiment and three putative unique PCR products were identified. One PCR product was sequenced and BLAST analysis revealed 95% homology to the *P. aeruginosa ampR* gene. A more detailed examination of the sequence uncovered that the B30 transposon may have inserted 101 base pairs downstream of the *ampR* stop codon, but not within a known gene. Unfortunately, these results could not be repeated and so the validity of this observation is questionable at this time.

Figure 33: Arbitrary Primed PCR of the chromosomal DNA of IL-1 and L522.

Arbitrary primed PCR was performed on chromosomal DNA of IL-1 and L522 using various primers. An acrylamide gel of the PCR products is shown. Products obtained using IL-1 chromosomal DNA as a template are run adjacent to products using L522 as a template for comparison. Bands unique to L522 are indicated with arrowheads to the right of the gel. Arrowhead A (lane 5) was identified as the sequence downstream of the *ampR* gene. Approximate sizes of DNA fragments from a 1kb-plus DNA ladder are shown to the right of the gel. Lane 1 and 2: primers arb1 and IS50R; Lanes 3 and 4: primers arb6 and IS50R; Lanes 5 and 6: primers arb1 and B30-neo; Lanes 7 and 8: primers arb1 and IS50R; Lanes 9 and 10: primers arb6 and IS50R. Lanes 1,3,5,7 and 9 are the results of the PCR using L522 chromosomal DNA as a template while lanes 2,4,6,8 and 10 used IL-1 DNA as a template.


1 2 3 4 5 6 7 8 9 10
1 kb
500 bp
396 bp
344 bp
298 bp
220 bp
(lane 1) E
(lane 5) A
(lane 7) F

CHAPTER 5: DISCUSSION

The *regAB* operon is regulated by two independent upstream promoters (Frank *et al.*, 1989). The P1 promoter is expressed early in growth under both high and low iron conditions (Frank and Iglewski, 1988). To date at least seven regulators have been proposed to act on this promoter. However, the exact mechanism of action by these various regulators on activity from the P1 promoter remains to be elucidated. Vfr, LasR, PtxR, *regB* and PvdS have all been demonstrated to positively regulate the P1 promoter while PtxS and Fur are believed to negatively regulate expression from this promoter. The P2 promoter of the *regAB* operon is only expressed late in growth, during the exponential and stationary phases (Frank and Iglewski, 1988). Expression from the P2 promoter is tightly regulated by the levels of environmental iron as indicated by transcription from this promoter occurring only in low iron conditions (Frank and Iglewski, 1988). The tight iron regulation of ETA expression is believed to be mediated through this promoter. Surprisingly, a regulatory protein which acts on the P2 promoter remains to be identified. We hypothesize that an iron-regulated activator mediates expression from the P2 promoter in low iron conditions. This regulator may act alone or in cooperation with other regulators to activate the P2 promoter. The primary objective of these studies was to identify such a regulator. Identification of this regulator would improve our knowledge of ETA production and aid in our understanding of the pathogenesis of *P. aeruginosa*.

5.1 Regulators of the P2 Promoter

As previously mentioned, a regulator of the tightly iron regulated P2 promoter of the *regAB* operon has not been identified to date. In order to attempt to identify regulators of the P2 promoter, transposon mutagenesis was performed on *P. aeruginosa* strain IL-1. All mutants were screened for abnormal P2 activity in both high and low iron conditions. Using this β-galactosidase screening procedure, two mutants were identified which contained a transposon insertion in a putative regulator of the P2 promoter of the *regAB* operon.

5.1.1 Transposon Mutant H399

Mutant H399 was identified from the initial screening procedure because it displayed elevated P2 activity in high iron conditions. The likely cause of this phenotype is an insertion in a putative repressor which acts on the P2 promoter in high iron conditions to prevent transcription. Growth curve analysis demonstrated that P2 promoter expression from this transposon mutant in high iron conditions only occurred during the first six hours of growth. In a typical *P. aeruginosa* strain, this is the phase of growth in which the P1 promoter, not the P2, would normally be active. In spite of the unexpected timing of the P2 promoter expression in mutant H399, we chose to investigate this mutant further. Multicopy reporter plasmids pP11 and pP21 were utilized to examine the activity from each of the two promoters independently. Growth curves performed with these reporter plasmids revealed that both the P1 promoter and the P2 promoter activities of mutant H399 were identical to those of the wild-type IL-1 (Figures 21 and 22). These unexpected results questioned our assumption that the phenotype of mutant H399 was the result of an insertion into a negative regulator of the P2 promoter. We began to explore the possibility that the H399 phenotype was the result of a polar effect by the transposon.

The region of the chromosome in which the transposon had inserted was cloned and sequenced. Sequence analysis revealed that the region shared strong homology with a lactoylglutathione lyase protein of many bacteria. Although this protein has not been characterized from *P. aeruginosa*, it is ubiquitous and has been studied in numerous other prokaryotic, as well as eukaryotic organisms. This gene has no homology to known regulatory proteins nor does it contain any putative DNA-binding motifs. Therefore, the role of this protein in the regulation of the P2 promoter was questioned. Using a probe to the *lacZ* gene, Southern Blot analysis suggested that the transposon had inserted in close proximity to the *regAB* locus in mutant H399 (Figure 25). When analyzed together with the sequence data, the location of the transposon in the chromosome of mutant H399 could be mapped to approximately 1.6 kb upstream of the *regAB* locus (Figure 26). Due to the upstream location of the insertion, we hypothesized that the phenotype

of H399 was due to a polar effect of the transposon on the P1 promoter. This observed polar effect was unexpected from this transposon. The B30 transposon was chosen for these studies due to its many positive characteristics such as having minimal target specificity and high stability once it has inserted into the genome. It also exhibits a low transposition frequency, enabling it to be an excellent transposon for transposon mutagenesis as it has a high probability of inserting into the genome of each mutant only once (Simon *et al.*, 1989). However, the Tn10-derived tetracycline resistance gene of the B30 transposon acts as a marker and does contain its own translational start site. This Tet^R cassette could be reading through the intergenic region on the chromosome of H399 to activate the P1 promoter, regardless of the iron conditions. This could account for the observed phenotype of H399. In order to conclusively confirm this hypothesis, complementation studies were performed. The lactoglutathione lyase gene was expressed on a multicopy plasmid in H399 and no complementation was observed (Figure 24). These studies verified that the observed phenotype of mutant H399 was due to a polar effect of the transposon insertion on the *regAB* locus and not due to a mutation in a gene which codes for a regulator of the P2 promoter of the *regAB* operon.

While mutant H399 is not useful for studies of the regulation of the *regAB* operon, it may be an interesting mutant for studies of the lactoylglutathione lyase gene of *P. aeruginosa*. The lactoylglutathione lyase enzyme is found in all living organisms, however, the function of this enzyme has not been clearly defined. Lactoylglutathione lyase catalyses the reaction of methylglyoxal and glutathione to D-lactic acid in the following two step reaction with the enzyme glyoxylase II (Racker, 1951).

$$\text{methylglyoxal} + \text{glutathione} \xrightarrow{\text{lactoylglutathione lyase}} \text{S-D-lactoylglutathione} \xrightarrow{\text{glyoxylase II}} \text{D-lactic acid}$$

The compound methylglyoxal is produced by intermediates of glycolysis and photosynthesis (glyceradehyde-3-phosphate and dihydroxyacetone phosphate) and is believed to be a cytotoxic metabolite (Richard, 1993). It has been

hypothesized that lactoylglutathione lyase may be involved in detoxification by removing excess methylglyoxal from the cell (Aronsson and Mannervik, 1977). The amino acid sequence alignment of the C-terminal portion of the lactoylglutathione lyase homologue from *P. aeruginosa* H399 with other bacterial lactoylglutathione lyase enzymes demonstrates extensive homology (Figure 23). It is interesting to note that the lactoylglutathione lyase gene of *Pseudomonas putida* shares very little homology with the lactoylglutathione lyase of *P. aeruginosa*, but strong homology with the corresponding enzyme of mammals and plants (Lu *et al.*, 1994). Lactoylglutathione lyase of *P. aeruginosa* shares strong homology with the analogous enzymes of other bacteria and yeast. These homology studies suggest that the enzyme has diverged through evolution to produce two distinct lactoylglutathione lyase enzymes, one most commonly found in mammals, plants and some bacteria such as *P. putida*, and the other more common in most prokaryotes and yeast. The ubiquitous nature of this enzyme suggests that it is important for cellular function yet these studies demonstrate that it is not critical as the knock-out mutant H399 did not demonstrate any significant growth defects in the conditions tested (Figure 17).

5.1.2 Transposon Mutant L522

Mutant L522 was identified from the screening of IL-1 transposon mutants as having reduced P2 activity in low iron conditions. The mutation in this strain may be in a regulatory gene which encodes an activator of the P2 promoter. Growth curve analysis confirmed that the P2 activity of L522 is approximately seven-fold lower after overnight growth in low iron conditions, when compared with the wild-type IL-1 (Figure 27). The P2 promoter is normally active only in low iron conditions and during the late log and stationary phases of growth. The P2 activity profile of L522 suggested that the low activity is from the P2 promoter since it was active after 6 hours of growth and only in low iron conditions. In order to confirm that the P2 activity phenotype of L522 was not due to a polar mutation as in mutant H399, plasmid pP21 was electroporated into IL-1 and L522. The P2 activity from the multicopy reporter plasmid in L522 remained over four-fold less than in strain

IL-1 (Figure 31). The difference in the P2 activity between the mutant L522 and the wild-type IL-1 was less in this multicopy experiment than when the activity from the chromosomal P2 promoter was observed. This was expected because the P2 promoter when present in multiple copies will not be regulated as efficiently as the single chromosomally encoded promoter due to the effects of regulators of the promoter being titrated out in the multicopy situation. However, the four-fold decrease in multicopy P2 activity observed from the mutant L522 indicated that the mutation in L522 was due to a transposon interruption of a gene coding for an activator of the P2 promoter. Furthermore, the phenotypic profile of L522 revealed that the transposon had inserted downstream of an active promoter (Table 6). The B30 transposon contains a promoterless neomycin resistance gene at the IS50R end. Because mutant L522 displayed a neomycin resistant phenotype, this suggested that the transposon had inserted downstream of an active promoter.

The results in Chapter 3 revealed that PvdS is a positive regulator of the P2 promoter of the *regAB* operon. In order to determine whether or not the phenotype observed for L522 was due to a transposon insertion within the *pvdS* gene, a Southern Blot was performed using the *pvdS* gene as a probe. The Southern Blot revealed that the mobility of the *pvdS* gene was identical in mutant L522 and the wild-type IL-1. This indicated that the transposon had not inserted into the *pvdS* gene of mutant L522 (Figure 32). This suggested that at least one other regulator besides PvdS acted on the P2 promoter of the *regAB* operon. Mutant L522 contains a transposon insertion in a second gene which codes for a positive regulator of the P2 promoter. Attempts to clone and sequence the chromosomal region surrounding the transposon insertion from mutant L522 have been unsuccessful to date. Conventional cloning techniques which were attempted included ligation of the B30 transposon and flanking chromosomal DNA into vectors such as pBluescript II SK(+), pUC18 and pGEM-7Zf(+) using a variety of different restriction enzymes. Inverse PCR techniques were also attempted and have proven ineffective. Arbitrary primed PCR was employed and one PCR product showed 95% homology to a region downstream of the *P. aeruginosa ampR* stop codon (Figure 33; Lodge *et al.*, 1993). Analyses of the putative B30

insertion site on the chromosome of mutant L522 using the annotated database of the *P. aeruginosa* PAO1 genome sequence have proven useful for identification of the location of the insertion on the chromosome. However, because L522 is a derivative of PA103, it is at times difficult to compare with the PAO1 genome database. Unfortunately, because the transposon was found to not actually interrupt a known gene of the PAO1 genome, the validity of these results are questionable.

5.2 The Role of PvdS in Exotoxin A Regulation

PvdS is an important Fur-regulated alternative sigma factor produced by *P. aeruginosa* in iron-limiting conditions believed to belong to the family of extracytoplasmic function (ECF) alternative sigma factors (Missiakas and Raina, 1998). The role of this transcriptional activator in the production of iron-regulated virulence factors was hypothesized due to its requirement for the production of the siderophore pyoverdine. We investigated the possibility that PvdS may also play a role in the activation of the P2 promoter of the *regAB* operon in low iron conditions.

The experiments in Chapter 3 indicated that the alternative sigma factor PvdS may play an important role in the regulation of exotoxin A expression. A previously unrecognized eight out of ten base pair match to the PvdS binding consensus sequence was identified in the region of the P2 promoter of the *regAB* operon (Figure 2; Table 4). This observation alluded to the possibility that PvdS may play a critical role in the regulation of the P2 promoter. When a plasmid containing the entire *pvdS* gene, including its own promoter region (pTH*pvdS*) was added *in trans* to *P. aeruginosa* strain IL-1, activity from the P2 promoter was enhanced more than two-fold relative to the wild-type (Figure 8). This enhancement was only observed when the cultures were grown in low iron conditions, indicating that the regulation remained dependent on environmental iron levels. The *pvdS* gene has previously been demonstrated to be regulated by the Fur repressor (Ochsner *et al.*, 1995). Figure 8 also demonstrates that the iron regulation by pTH*pvdS* on the P2 promoter was observed to be maintained, therefore, expression of *pvdS* from the multicopy plasmid continued to be regulated

by Fur, as it is on the chromosome. The levels of intracellular Fur were not titrated out during the course of the growth curve. The more than two-fold increase in P2 promoter expression was strong evidence that PvdS is an activator of the P2 promoter. Furthermore, when the *pvdS* gene of IL-1 was rendered non-functional in strain IL-1 *pvdS*::Ω, expression from the P2 promoter was not detected, irrespective of the iron conditions (Figure 14). P2 activity was only observed from this knock-out strain when *pvdS* was added *in trans* under iron limiting conditions. These data suggest that PvdS has an iron-regulated effect on expression from the P2 promoter of the *regAB* operon.

The primary role of RegA is in the transcriptional control of *toxA* (Hedström *et al.*, 1986). We next wanted to examine whether or not the positive regulatory effect of PvdS on RegA expression translated to elevated exotoxin A expression. In addition, we wanted to examine whether PvdS also regulated expression directly from the *toxA* promoter. For these studies, two *P. aeruginosa* strains were employed, PA103 and PAO1 (Figure 7). PA103 is a hypertoxigenic laboratory strain commonly used for studies of ETA expression. The *regAB* locus of PA103 contains both a functional P1 and P2 promoter. PAO1 is a well-characterized prototrophic laboratory strain which produces approximately 10-fold less ETA than PA103. Only the P2 promoter is functional at the *regAB* locus of this strain and, as such, the regulation of ETA expression in PAO1 is less complex than in PA103.

The effect of multicopy *pvdS* on ETA expression in strain PAO1 was more pronounced than in strain PA103. After overnight growth, ETA activity was enhanced greater than two-fold when *pvdS* was provided *in trans* (Figure 10). This enhancement was again only observed in low iron conditions due to the conservation of iron regulation. This dramatic increase in ETA expression was hypothesized to be due to the effects of PvdS on the P2 promoter of *regAB* operon because the P1 promoter is not functional in strain PAO1. Further evidence that the regulatory effect of PvdS is mediated through the P2 promoter of the *regAB* operon is presented in Figure 11. The *regAB* locus of strain IL-1 is interrupted by a *lacZ* cassette, so the RegA protein is not produced in this strain. In the absence of the transcriptional activator RegA, ETA is not produced from strain IL-1. The

extracellular ETA activity of IL-1 was monitored and activity was not detected when *pvdS* was present in multiple copies in either high or low iron conditions. These data indicate that in the absence of RegA, PvdS does not act on the *toxA* promoter directly. The regulatory effect of PvdS on ETA expression is mediated through the P2 promoter of the *regAB* operon.

Knock-out strains of *pvdS* in PA103 and PAO1 were studied in order to confirm that PvdS did not regulate the *toxA* gene directly. ETA activity was reduced but detectable in strain PA103 *pvdS*::Ω in low iron conditions and no activity was observable in high iron conditions. This low ETA activity in low iron conditions is believed to be due to the action of RegA produced by the activation of the P1 promoter in this highly regulated strain. However, when pTH*pvdS* was added, ETA activity increased almost five-fold in low iron conditions (Figure 15). These data confirm that PvdS increases the production of ETA in low iron conditions. Furthermore, it has been established that PvdS is not absolutely required for ETA activity in strain PA103, however, in its absence ETA activity is drastically reduced. In contrast, extracellular ETA activity from strain PAO1Δ*pvdS* was not detectable in either high or low iron conditions unless *pvdS* was added *in trans* under low iron conditions (Figure 16). These studies of *pvdS* knock-out strains further demonstrate that PvdS does not directly regulate the expression of ETA but the regulation is indirect. In low iron conditions PvdS up-regulates expression from the P2 promoter of the *regAB* operon, and the RegA produced by the activation of this promoter then acts to enhance transcription of *toxA*.

5.2.1 Regulation of the P2 Promoter of the *regAB* Operon by PvdS

It was previously suggested that PvdS regulated ETA activity by acting directly on the *toxA* promoter. Rombel *et al.* (1995) identified a 9 out of 10 base pair match to the PvdS consensus binding sequence 53 base-pairs upstream of the start codon for *toxA* and suggested that PvdS binds to that site. Studies presented here demonstrate that PvdS does not act directly on the promoter region of *toxA* in the absence of RegA, because no ETA production was observed from

IL-1 in high or low iron conditions even in the presence of multiple copies of PvdS (Figure 11).

Previous researchers had stated that a PvdS consensus binding site does not exist in the *regAB* promoter region (Rombel *et al.*, 1995; Ochsner *et al.*, 1996). However, we identified an 8 out of 10 base pair match to the PvdS binding consensus sequence 68 base pairs upstream of the *regAB* translational start, directly at the site of the P2 promoter (Table 4; Figure 2). The presence of this sequence is putative evidence that PvdS may act directly to regulate the *regAB* operon. Figure 8 demonstrates that when *pvdS* is present in multiple copies under the control of its own promoter in strain IL-1, iron regulation is maintained and expression from the P2 promoter increases two-fold. Furthermore, ETA activity increased markedly in *P. aeruginosa* strains PA103 and PAO1 when *pvdS* was added *in trans* (Figures 9 and 10). This *pvdS*-dependent enhancement of ETA activity is therefore mediated through the *regAB* operon. The ETA activity from strain IL-1 is negligible, regardless of iron conditions and *pvdS* copy-number indicating that the enhancement of ETA activity by PvdS requires a functional *regAB* operon (Figure 11). In *pvdS* knock-out derivatives of IL-1, PA103 and PAO1, activity from both *regAB* and *toxA* is only evident in low iron conditions in the presence of plasmid pTH*pvdS* (Figures 14, 15, and 16). The results of these experiments suggest that PvdS acts as a regulator of the P2 promoter of the *regAB* operon.

PvdS is believed to be an alternative sigma factor for RNA polymerase. This classification is based on protein homologies to known alternative sigma factors and the presence of a DNA-binding motif at the carboxyl-terminal end of PvdS (Cunliffe *et al.*, 1995; Miyazaki *et al.*, 1995). To date, however, PvdS has not been demonstrated to bind to either the RNA polymerase holoenzyme complex or to promoter DNA of any iron-regulated genes. In addition, it has been demonstrated that PvdS expression is regulated by iron through Fur, the global iron repressor (Ochsner *et al.*, 1996). PvdS is transcribed only in low iron conditions because in high iron conditions its transcription is repressed by Fur. Only when PvdS is transcribed is it able to bind to the region of the P2 promoter

and enable RNA polymerase to transcribe the *regAB* T2 transcript. Additional work with *pvdS* in which the native promoter was replaced with a p^{tac} promoter, suggested that the regulation by PvdS on ETA expression was more complex (Ochsner *et al.*, 1996). When this p^{tac}-*pvdS* plasmid was present in multiple copies, *regA* was expressed in both high and low iron conditions. Furthermore, in low iron conditions, both T1 and T2 transcripts could be seen. However, it was concluded from these studies that PvdS could not be demonstrated to directly regulate either of the promoters of the *regAB* operon. In addition, transcription of *toxA* could be observed only early in growth during aerobic conditions. However, during microaerophillic growth *toxA* was expressed at all time points suggesting that the regulation differed in response to varying oxygen conditions (Ochsner *et al.*, 1996). These studies suggest that the regulation of ETA expression involves at least one other regulator other than PvdS.

5.3 Regulation of the P2 Promoter of the *regAB* Operon

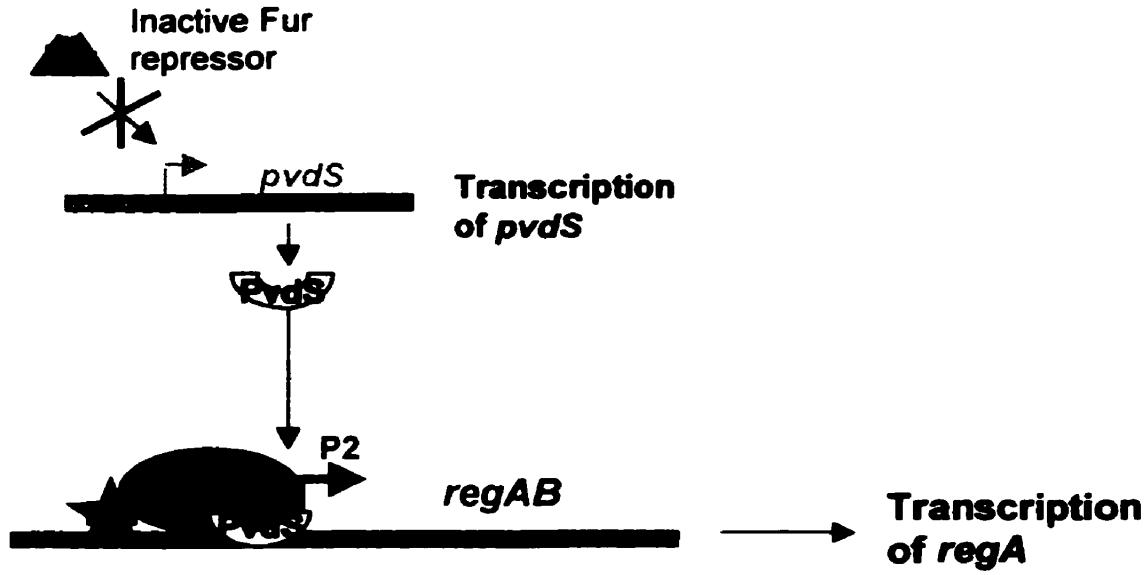
A model for the mechanism of regulation of the P2 promoter of the *regAB* operon is depicted in Figure 34. This model is based on the results herein, as well as from previous experiments and analogy to other bacterial regulatory systems. This model predicts that when PvdS is the only regulator of the P2 promoter, *regA* will be transcribed at low levels. A second positive regulator, "Ror" (Regulator of *reg*), is required for maximal transcription from the P2 promoter of the *regAB* operon. The gene which codes for this second activator is interrupted by the transposon in mutant L522 and Southern Blot analysis has revealed that the gene which encodes this regulator is not *pvdS* (Figure 32). When this second activator is mutated, activity from the P2 promoter is only 15% of wild-type P2 activity (Figure 27). This residual P2 activity in the absence of "Ror" is hypothesized to be due to basal levels of transcription by RNAP-PvdS. However, "Ror" is unable to initiate transcription from the P2 promoter in the absence of PvdS, as indicated by studies of an IL-1 *pvdS* knock-out strain (Figure14).

Figure 34: Model of regulation at the P2 promoter of the *regAB* operon.

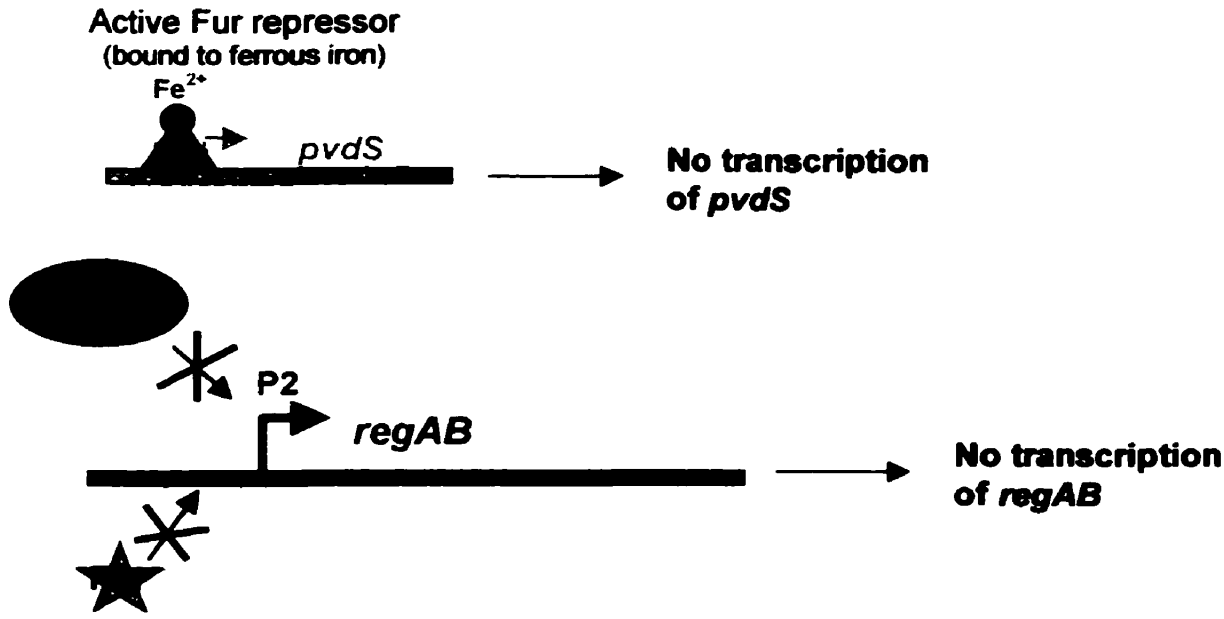
A. Regulation in low iron conditions. Transcription from the *regAB* operon is activated in the presence of the transcriptional activators PvdS and "Ror". The Fur repressor is inactive and, therefore, cannot bind to the promoter region of *pvdS* to prevent transcription. The PvdS produced then acts as an alternative sigma factor for RNA polymerase (RNAP) to activate transcription from the P2 promoter of the *regAB* operon. The uncharacterized activator of the P2 promoter ("Ror") also is predicted to interact with RNAP to enhance transcription.

B. Regulation in high iron conditions. Transcription from the *regAB* operon is prevented due to the absence of transcriptional activators PvdS and "Ror". The Fur repressor becomes active in high iron conditions and binds to ferrous iron (Fe^{2+}). The activated Fur-Fe^{2+} complex represses transcription from the *pvdS* promoter and, hence, PvdS is not transcribed. Without PvdS to act as an alternative sigma factor, RNAP is unable to bind to the promoter region and transcription from the *regAB* operon does not occur. The activator protein "Ror" is also unable to activate transcription from the P2 promoter of the *regAB* operon in high iron conditions due to either the inability to bind to the promoter region or the lack of transcription of "Ror" in high iron condition, possibly due to regulation by Fur.

A. Low Iron Conditions:



B. High Iron Conditions:



The model of regulation depicted in Figure 34 resembles the mechanism of regulation of the *degP* (*htrA*) gene of *Escherichia coli*. Much is known about the details of the regulation of this gene. DegP is a periplasmic protein which acts to proteolytically degrade misfolded proteins in the periplasm (Lipinska *et al.*, 1989). It has been demonstrated that the integrity of the bacterial cell envelope becomes compromised if misfolded protein are allowed to accumulate in the periplasm due to the non-specific insertion of these proteins into the outer membrane. Transcriptional activation of DegP is coordinated with the activation of other proteins which act in the periplasm to increase the number of correctly folded proteins and degrade any proteins which are not present in the correct conformation. Transcription of DegP is regulated by σ^E, an alternative sigma factor of the ECF-family (Lipinska *et al.*, 1988). In order for *degP* to be transcribed optimally, a second activator, CpxR, is also required. CpxR is the response regulator of a two-component regulatory system which activates the transcription of *degP* when it has been phosphorylated (Cosma *et al.*, 1995; Danese *et al.*, 1995; Missiakas and Raina, 1997). The alternative sigma factor σ^E, co-ordinately with CpxR, regulates the transcription of *degP*. In addition, σ^E independently regulates other genes, including *fkpA*, $rpoH_{P3}$, and $rpoE_{p2}$ (Danese and Silhavy, 1997; Rouvière *et al.*, 1995). In the regulatory mechanism of *degP* expression, CpxR also independently regulates other genes, including *dsbA*, *cpxP*, and *rotA* (Connolly *et al.*, 1997; Danese and Silhavy, 1997; Pogliano *et al.*, 1997).

Similarly, the alternative sigma factor PvdS regulates *regA* expression from the P2 promoter along with a second activator ("Ror"). Furthermore, like σ^E, PvdS independently regulates the expression of other genes, including those involved in the biosynthesis of the siderophore pyoverdine, *pvdA*, *pvdD*, and *pvdE* (Miyazaki *et al.*, 1995; Merriman *et al.*, 1995). It can be speculated that the second activator of the P2 promoter, "Ror", also may play a role in the regulation of other genes, independent of the regulation by PvdS. However, "Ror" is most likely not part of a two-component system like CpxR. Table 6 demonstrates that the production of the virulence factors elastase and proteases by the mutant L522 is not impaired by the

transposon insertion in this strain. This suggests that the mutation in the gene encoding "Ror" does not effect the production of other virulence factors. "Ror" is therefore not a general virulence factor regulator of *P. aeruginosa*. The *degP* system is involved in recognizing misfolded proteins whereas the P2 promoter is activated in response to low iron conditions. We expect the parallels between the two regulatory systems to diverge in terms of the mechanism of response to environmental conditions due to the lack of commonality in function between the two systems. However, it is interesting to recognize the parallels at the level of transcriptional regulation by an alternative sigma factor of the ECF family as well as a second activator protein.

5.4 Conclusions

In *P. aeruginosa*, expression of ETA is tightly regulated by the levels of extracellular iron. This iron regulation is believed to be directly mediated through the P2 promoter of the *regAB* operon. However, the regulatory cascade of ETA expression has remained elusive to date and regulators which act on the P2 promoter have not been previously identified. In these studies, we provided evidence that PvdS, a member of the ECF family of alternative sigma factors, regulates the expression of RegA from the P2 promoter. Investigations into the role of PvdS in the regulation of ETA were initiated following the identification of a previously unrecognized partial PvdS consensus binding sequence directly at the P2 promoter. We also provide evidence for the existence of a second positive regulator, "Ror", required for optimal P2 expression. This second regulator was discovered using transposon mutagenesis of an engineered *P. aeruginosa* reporter strain used to monitor P2 activity. The transposon mutant L522 contains a transposon insertion in a gene which codes for this second activator of P2 activity. When "Ror" is absent, expression from the P2 promoter is significantly reduced in low iron condition. These two positive regulators, PvdS and "Ror", act to co-ordinately regulate P2 activity. The expression of *pvdS* is regulated by the Fur repressor and, as such, is only expressed during low iron conditions. We believe that it is through PvdS that the expression of ETA is regulated by iron. The second

activator may also be regulated by iron at the level of transcription, however, further studies are required to characterize the expression and function of this regulator.

The results of the experiments presented herein provide exciting new evidence for the role of PvdS and a second activator in the regulation of ETA expression in *P. aeruginosa*. A further understanding of the regulation of the P2 promoter of the *regAB* operon will enhance the current model of ETA regulation and lead to a better understanding of the complex regulatory mechanism involved in the pathogenesis of *P. aeruginosa*.

5.5 Future Studies

Some questions remain to be answered regarding the regulation of the P2 promoter of the *regAB* operon of *P. aeruginosa*. Transcript accumulation studies should be performed to confirm the results of the *lacZ* reporter expression from strain IL-1. The transcription of *pvdS*, *regA* and *toxA* should be correlated for both high and low iron conditions after at least 6 hours of growth. These studies should be performed on *P. aeruginosa* strains IL-1, PA103, PAO1, and the *pvdS* knock-outs of each strain. These RNA studies should correlate with the β-galactosidase and ETA activities observed for each growth curve presented in Chapter 3. RNA studies would confirm that PvdS is an activator of the P2 promoter of the *regAB* operon by demonstrating elevated expression of the T2 transcript.

In addition, protein work needs to be performed to demonstrate that PvdS directly activates transcription from the P2 promoter and that PvdS is an alternative sigma factor that requires RNA polymerase. In order for these studies to be carried out, the PvdS protein requires over-expression and purification. The *pvdS* gene could be cloned into a protein expression vector with a His_6 tag, over-expressed in *E. coli*, and purified on a Ni^{2+} column. The purified recombinant PvdS protein could then be used in gel shift assays using a radiolabelled DNA fragment containing the putative PvdS-binding site upstream of the *regAB* locus. In addition, radiolabelled DNA fragments containing promoter sequences of genes known to be involved in pyoverdine synthesis and regulated by PvdS could also be used in

these studies to demonstrate that PvdS binds to these regions as binding studies have not been performed on these promoter regions to date. Gel shift studies could be performed using various combinations of PvdS and RNAP, as well as using different DNA fragments to confirm the PvdS binding site of PvdS-regulated genes. These studies would demonstrate that PvdS is a direct regulator of the P2 promoter and that PvdS is an alternative sigma factor for RNA polymerase.

Further studies are also required to characterize the unknown activator of the P2 promoter. Obviously, the chromosomal DNA surrounding the transposon insertion in mutant L522 requires identification. PCR techniques should be utilized to confirm or refute whether the transposon has indeed inserted just downstream of the *ampR* stop codon on the *P. aeruginosa* chromosome. If this is the case, the region should be further characterized to determine whether or not the transposon has inserted within an open reading frame and, if so, the protein encoded by the gene should be characterized. If these studies show that the transposon did not insert near the *ampR* gene, then another technique to identify the region in which the transposon had inserted would need to be employed. Because all other identification methods have been exhausted, direct sequencing from the L522 chromosome using the IS50R or B30-neo primers may be attempted. In any case, the gene in which the transposon has inserted in mutant L522 requires identification. The activator protein which is coded for by this gene requires characterization and should be demonstrated to be an activator of the P2 promoter. Once this activator is identified, more studies should be performed to characterize the role that this activator plays in the regulation of the P2 promoter and how this activator interacts with PvdS and/or RNA polymerase. In addition, the role that this activator plays in the regulation of other virulence factors produced by *P. aeruginosa* should be examined.

Lastly, more transposon mutants of IL-1 should be generated and screened in order to identify any other regulators which act on the P2 promoter of the *regAB* operon. In these studies, only 806 transposon mutants were generated and screened owing to the extensive and time-consuming nature of the β-galactosidase assay procedure. The screening procedure could be made more efficient if the

lacZ reporter gene on the chromosome of strain IL-1 was replaced with a *gfp* reporter gene at the *regAB* locus. Monitoring the expression of GFP could be a superior tool due to the increased speed of screening compared to screening the expression of β-galactosidase. Therefore, the screening procedure would be more efficient using this reporter strain. The PAO1 genome has been sequenced and revealed to contain an estimated 5500 genes which code for proteins. While many of these genes do not code for putative regulator proteins, obviously more screening needs to be performed in order to identify all regulators of the P2 promoter. We can conclude that the initial screen did not identify all regulators because PvdS was not identified. Furthermore, we would have expected to find all direct and indirect regulators of the P2 promoter using this screening method. Although Fur is an indirect regulator of the P2 promoter, we would not expect to find it using this screen because it has been demonstrated that in *P. aeruginosa*, *fur*⁻ mutants are lethal (Prince *et al.*, 1993). The screening method should also be optimized in order to allow for the screening of a larger number of transposon mutants.

The results of these proposed studies, along with the evidence presented herein, should allow for the construction of a precise model of the regulation of the P2 promoter of the *regAB* operon of *P. aeruginosa*.

CHAPTER 6: REFERENCES

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