Regulation of Pyoverdine Biosynthesis in *Pseudomonas aeruginosa*

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ABSTRACT

Pseudomonas aeruginosa can acquire iron by internalizing ferric-pyoverdine via its outer membrane receptor, FpvA. Loss of FpvA in *P. aeruginosa* reduces the amount of pyoverdine produced relative to a wild-type strain, suggesting a positive role for FpvA in pyoverdine biosynthesis. Transcriptional analysis of genes found in the loci that encode the proteins required for biosynthesis of the peptide moiety of pyoverdine, *pvdD*, and the chromophore, *pvcAB*, was carried out in *P. aeruginosa* strains possessing and lacking FpvA. Decreased expression of a *pvdD-lacZ* fusion and the *pvcAB* transcript in the FpvA-deficient *P. aeruginosa* strain relative to wild-type was observed under iron limiting conditions. These observations, coupled with the homology of FpvA to the ferric-siderophore receptors PupB of *P. putida* and FecA of *E. coli*, led to a proposed model of receptor-mediated signalling of the pyoverdine biosynthetic genes via FpvA that is initiated by ferric-pyoverdine binding to FpvA. Unlike PupB and FecA, it appears that FpvA-deficiency has little affect on *fpvA* gene expression, since expression of *fpvA-lacZ* in an FpvA-deficient strain was almost equal to that seen in a wild-type ($FpvA^+$) strain. A pyoverdine-deficient strain of *P. aeruginosa* showed no difference in *pvdD-lacZ* expression as compared to wild-type under iron-limited conditions. In contrast, a decrease in expression of the *pvcAB* genes in a pyoverdine-deficient *P. aeruginosa* strain was observed under iron-limited conditions as compared to wild-type, suggesting that pyoverdine (or

ferric-pyoverdine) positively regulates its own expression via the *pvc* genes. Unexpectedly, the addition of exogenous pyoverdine to the growth medium further reduced *pvcAB* gene expression in both the pyoverdine-sufficient and pyoverdine-deficient *P. aeruginosa* strains. These data highlight the complexity of pyoverdine biosynthetic gene regulation, involving siderophore, receptor and iron. It is clear, however, that the exact nature of pyoverdine involvement remains to be elucidated. Nonetheless, FpvA plays a central role in expression of pyoverdine biosynthetic genes.

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LIST OF ABBREVIATIONS

Ap	ampicillin
A_{405}	absorbance reading at 405 nm
A_{420}	absorbance reading at 420 nm
A_{550}	absorbance reading at 550 nm
A_{600}	absorbance reading at 600 nm
bp	base pairs
BSA	bovine serum albumin
Cb	carbenicillin
Cm	chloramphenicol
CPM	counts per minute
Da	Dalton
DEPC	diethylpyrocarbonate
DMSO	dimethyl sulphoxide
dNTP	2'-deoxyribonucleoside-5'-triphosphates
D-TSB	deferrated tryptic soy broth
EDDHA	ethylene diamine-Di(*o*-hydroxyphenyl acetic acid)
EDTA	ethylene diamine tetra-acetic acid
g	gram
GET	glucose-EDTA-TrisCl solution (gentle lysis buffer)
IPTG	isopropylthio-b-D-galactoside
kb	kilobase pairs
kDa	kiloDalton
Km	kanamycin
L	litre
LB	Luria-Bertani Medium
mAmp	milliampere
μg	microgram
μL	microlitre

μM	micromolar
mg	milligram
mL	millilitre
MSG	monosodium glutamate or L-glutamic acid sodium salt
nm	nanometre
ONPG	*o*-nitrophenyl β-D-galactopyranoside
PBS	phosphate buffered saline
PIPES	piperazine-N, N'-bis-(2-ethanesulfonic acid)
psi	pounds per square inch
rpm	rotations per minute
SDS	sodium dodecyl sulphate
Sm	streptomycin
TAE	Tris-acetate/EDTA electrophoresis buffer
TBE	Tris-borate/EDTA electrophoresis buffer
Tc	tetracycline
Tris	2-amino-2-(hydroxymethyl)-1,3-propanediol
TSB	tryptic soy broth
UV	ultraviolet
v/v	volume per volume
w/v	weight per volume
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

Chapter 1: INTRODUCTION

1.1 Pseudomonads

The Pseudomonads have been divided into five distinct groups based on their 16S rRNA sequences (Palleroni, 1992). Characteristically, they are Gram-negative rods that employ aerobic chemoorganotrophic metabolism but never fermentative metabolism (Brock *et al.*, 1994). The members of the genus *Pseudomonas* have polar flagella and range in size from 0.5-1.0 μm by 1.5-4.0 μm. Though they cannot use a fermentative metabolism they can use compounds other than oxygen as electron acceptors (Brock *et al.*, 1994). A vast number of different compounds can be used by *Pseudomonas* spp. as both carbon sources and electron donors for energy production. Though most members of this genus are environmentally important in the degradation of plant and animal materials, certain species are pathogenic to plants and/or animals (Brock *et al.*, 1994). Some of these pathogenic pseudomonads are members of the RNA homology group I which include the so-called fluorescent pseudomonads *Pseudomonas aeruginosa, Pseudomonas fluorescens* and *Pseudomonas putida* (Palleroni, 1992). These bacteria produce a water-soluble yellow-green pigment, pyoverdine (also called pseudobactin), that fluoresces under ultraviolet (UV) light, hence the term fluorescent pseudomonads (Budzikiewicz, 1993).

1.1.1 *Pseudomonas aeruginosa*

P. aeruginosa has the ability to metabolize a wide variety of compounds which allows it to adapt to diverse nutritional conditions. Commonly, it is found in freshwater or soil environments (Botzenhart and Döring, 1993). This ubiquity coupled with increased life spans for many immunocompromised patients has allowed *P. aeruginosa* to become an important opportunistic pathogen, posing a danger in hospital settings (Mims *et al.*, 1995). Most infections caused by *P. aeruginosa* are seen in patients with burns, severe skin trauma, cystic fibrosis or patients who are immunocompromised in some way (Botzenhart and Döring, 1993). It must be noted, however, that eye and ear infections, as well as skin rashes caused by *P. aeruginosa* in immune competent individuals have also been documented (Mims *et al.*, 1995).

The pathogenicity of this organism is due to a number of virulence factors that it produces. Most of these, such as siderophores, exotoxin A and various extracellular proteases, are expressed under specific environmental conditions (e.g. that of low iron found in human hosts) allowing the bacterium to establish infection once inside a host (Litwin and Calderwood, 1993). In addition, *P. aeruginosa* possesses the ability to produce alginate, an exopolysaccharide, allowing for increased adherence capabilities, resistance from the immune system and creation of a favourable micro-environment inside the lung of cystic fibrosis patients (Botzenhart and Döring, 1993, Xie *et*

al., 1996). To further complicate matters, this bacterium is resistant to most common antibiotics due to outer membrane impermeability and the activity of multidrug efflux pumps (Brock *et al.*, 1994, Nikaido *et al.*, 1996). These characteristics likely contribute to *P. aeruginosa* being recognized as a frequent nosocomial pathogen (Thornsberry, 1996).

1.2 Iron as a requirement for life

All organisms, with the exception of lactobacilli, require iron to survive since the ease of oxidation and reduction of this molecule allows it to play a major role in enzymatic reactions within a cell as either a cofactor or a prosthetic group (Martinez *et al.*, 1990, Briat, 1992, Atlas and Bartha, 1993). For example, iron is required for the activity of cytochromes, hydroperoxidases and ribonucleotide reductases which all play a major role in cell growth and proliferation (Griffiths, 1987a, Briat, 1992). In contrast, too much iron in a cell can lead to toxicity through the generation of free radicals. Thus, the iron concentration in a cell must be moderated (Bagg and Neilands, 1987) and, as a result, iron transport systems are tightly regulated.

Bacteria require iron in the amount of 0.05-0.5 μM (Martinez *et al.*, 1990), though in the natural environment it is found at concentrations calculated to be 10^{-18} M at neutral pH under aerobic conditions (Litwin and Calderwood, 1993). These conditions permit the oxidation of ferrous ions (Fe^{2+}) to the ferric (Fe^{3+}) form which in turn precipitates as insoluble ferric

hydroxide ($Fe(OH)_3$) molecules (Griffiths, 1987a, Atlas and Bartha, 1993). This obstacle is surmounted through the direct use of host iron binding and transport molecules by bacteria or the secretion of high-affinity iron-binding compounds that allow the bacteria to acquire iron under very dilute conditions (see section 1.2.2).

1.2.1 Iron and pathogenesis

The human body contains between 3 and 5 grams of iron which is almost entirely sequestered by iron-binding proteins (Litwin and Calderwood, 1993). Extracellular iron-binding proteins like lactoferrin (in secretions), transferrin (in serum), hemoglobin (in blood) and their intracellular counterparts, heme, ferritin and haptoglobin, allow for the transport, storage and use of iron by the body while still maintaining a low iron concentration in the serum (Litwin and Calderwood, 1993, Mims *et al.*, 1995).

To establish an infection, bacteria must be able to get past a myriad of the hosts defenses, not the least of which is this low iron environment (Martinez *et al.*, 1990). The increase in susceptibility to infection by bacteria in hemochromatosis patients where there is excess iron in the body underlines this point (Litwin and Calderwood, 1993). Under normal conditions, the lack of iron acts in an antimicrobial fashion by maintaining the concentration of free iron in the body below that required by bacteria for growth (Martinez *et al.*, 1990). Moreover, the immune system responds to infection, toxin production or injury by the induction of hypoferremia in the

surrounding tissue so as to inhibit microbial proliferation (Litwin and Calderwood, 1993). Hence, pathogenic bacteria have developed methods to scavenge iron from the host to ensure their growth and survival in this environment (Griffiths, 1987b).

1.2.2 Iron scavenging

The acquisition of iron from the environment has evolved differently among the pathogenic bacteria. Some bacteria, such as *Neisseria* species (Mickelsen and Sparling, 1981), *Yersinia pestis* (Perry and Brubaker, 1979) and *Haemophilus influenzae* (Stull, 1987, Lee, 1992) have receptors that allow for the use of mammalian iron-binding (lactoferrin) or transport (hemoglobin, heme, transferrin) proteins directly. Others (e.g. *Escherichia coli, Vibrio cholerae*) use hemolysins to release iron from hemoglobin or heme (Waalwijk *et al.*, 1983, Stoebner and Payne, 1988). Still others, such as *Listeria* spp., produce soluble reductants that reduce ferric iron on carrier proteins, like transferrin, to the ferrous form so it can be transported into the cell (Deneer and Boychuk, 1992) Finally, many bacteria, including *P. aeruginosa*, release high affinity iron-binding compounds, called siderophores, which allow for meaningful iron acquisition even under low-iron conditions (Neilands, 1981, Martinez *et al.*, 1990). Successful acquisition of iron by bacteria in the host imparts an advantage with respect to establishment of infection. Thus, better iron scavengers appear to be more virulent.

1.3 Siderophores

These low molecular mass (between 500 and 1000 Daltons) compounds have the ability to chelate iron and deliver it to the cell via receptors at the cell surface (Neilands, 1981). High-affinity iron-binding molecules effective at mobilizing iron at low concentrations, siderophores, are produced under conditions of iron limitation (Atlas and Bartha, 1993). Siderophores must possess a functional group with a high affinity (an affinity constant of approximately 10^{30}) for ferric ions (Neilands, 1981). Most iron chelating molecules have hydroxamate or phenolate-catecholate groups where the Fe^{3+} molecule binds to the oxygen groups but other functional groups (i.e. citrate) or a mixture of these groups can also be incorporated into siderophore molecules used for iron binding (Neilands, 1981).

Iron-siderophore complexes are too big to be taken up through the porin channels of the outer membrane since these channels transport molecules of 600 Da or smaller (Braun, 1995). Therefore, specific outer membrane proteins, expressed only under low-iron conditions, act as receptors for these complexes (Neilands, 1982). With energy shuttled from the inner membrane, the outer membrane receptors transport the iron-siderophore complex into the periplasm where cytoplasmic membrane transporters bring it into the cytoplasm (Royt, 1990). At this point, or possibly in the periplasm, the iron is released from the siderophore by a

reductive mechanism and the siderophore is either broken down or re-released to the environment (Royt, 1990, Hallé and Meyer, 1992).

1.3.1 Siderophores in *P. aeruginosa*

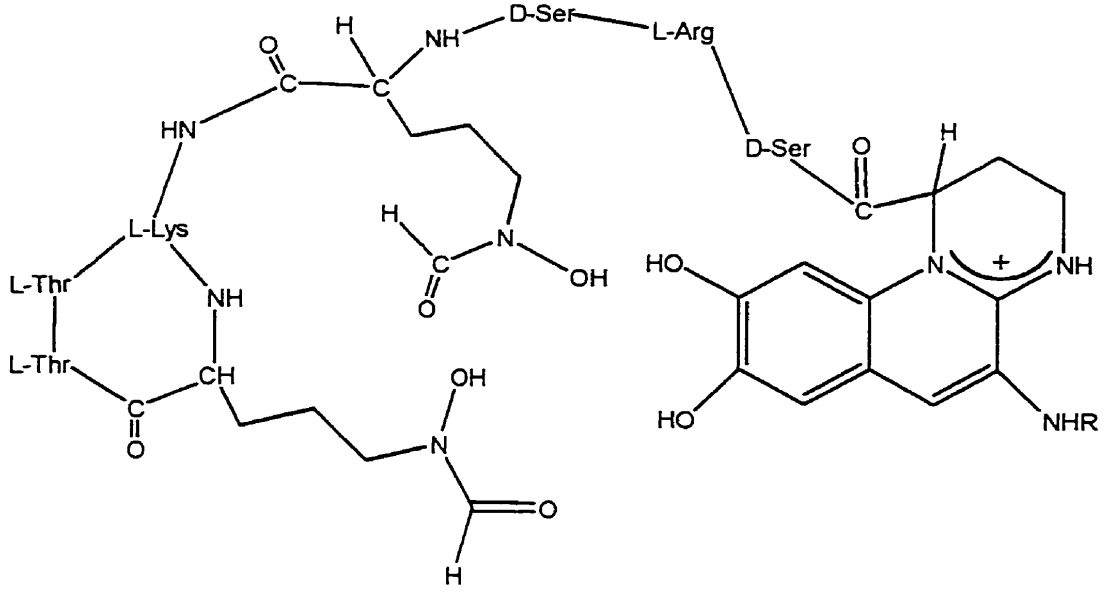
Two siderophores, pyoverdine and pyochelin (Fig. 1), are produced by *P. aeruginosa* as secondary metabolites (Budzikiewicz, 1993). Each has a different affinity for iron in aqueous media, suggesting that each is optimally useful under particular conditions. This led to the theory that under iron limiting conditions, both siderophores are produced at basal levels with the successful iron chelator being upregulated via a signalling mechanism involving the siderophore receptors (Gensberg *et al.*, 1992, Poole *et al.*, 1996a). Additionally, to assist its acquisition of iron in competition with other microorganisms, *P. aeruginosa* can use a variety of heterologous siderophores produced by other microorganisms, including fungi and other bacteria (see section 1.3.1.3).

1.3.1.1 Pyochelin

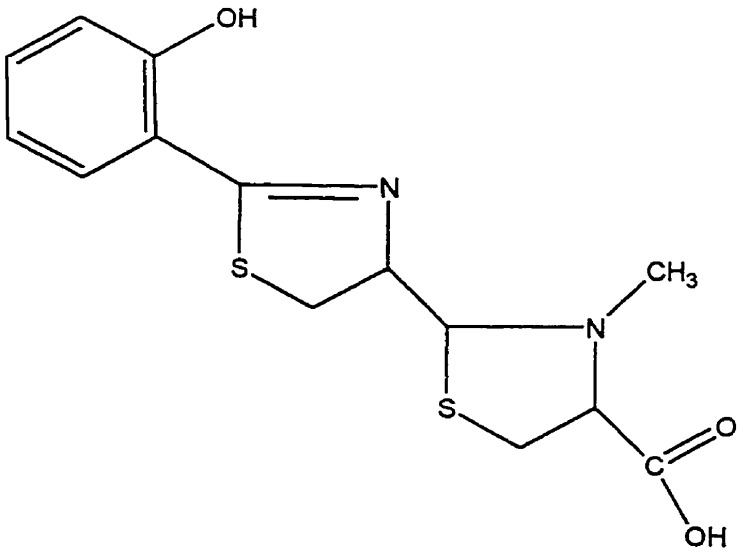
Pyochelin (2-[2-(*o*-hydroxyphenyl)-2-thiazolin-4-yl]-3-methyl-4-thiazolidinecarboxylic acid) is a hydrophobic, phenolate siderophore of low molecular mass (324 Da) that binds ferric iron with a 2:1 (pyochelin to Fe^{3+}) stoichiometric ratio (Fig. 1; Ankenbauer *et al.*, 1988). Even though it has a very low (5×10^5) affinity constant for iron, ferri-pyochelin contributes to

Figure 1: The siderophores produced by *P. aeruginosa* PAO1.
a) pyoverdine b) pyochelin

a) pyoverdine



b) pyochelin



virulence in *P. aeruginosa* (Cox, 1982, Ankenbauer *et al.*, 1988, Britigan *et al.*, 1997). Uptake of ferri-pyochelin is mediated by a major outer membrane receptor, FptA, (Ankenbauer, 1992). Exacerbation of infection by virulent *P. aeruginosa* strains producing this siderophore is thought to be as a result of the removal of iron from transferrin, stimulating growth of the bacteria (Cox, 1982, Ankenbauer *et al.*, 1988) as well as injury to tissue in the lungs brought about by oxygen radical formation catalyzed by ferri-pyochelin (Britigan *et al.*, 1997).

Pyochelin biosynthesis is encoded by the *pchDCBA* operon which is transcribed divergently from the *pchR* gene, and the *pchEF* operon transcribed in the same direction as the *pchR*, the latter encoding an AraC-like (Heinrichs and Poole, 1993) positive regulator of *pchDCBA* and *pchEF* expression (Serino *et al.*, 1997, Reimmann *et al.*, 1998). Pyochelin gene expression is negatively regulated by iron through repression by the Fur protein complexed to a ferrous iron corepressor (see section 1.5.1; Serino *et al.*, 1997). Interestingly, PchR plays a role in its own expression which is mediated negatively by iron, via the Fur protein and positively by PchR in response to pyochelin (Heinrichs and Poole, 1996, Serino *et al.*, 1997).

1.3.1.2 Pyoverdine

Pyoverdine is a water-soluble, yellow-green, fluorescent hydroxamate-catecholate pigment produced by *P. aeruginosa* that acts as a siderophore by binding iron with a 1:1 stoichiometry (Meyer and Abdallah, 1978, Barbhaiya

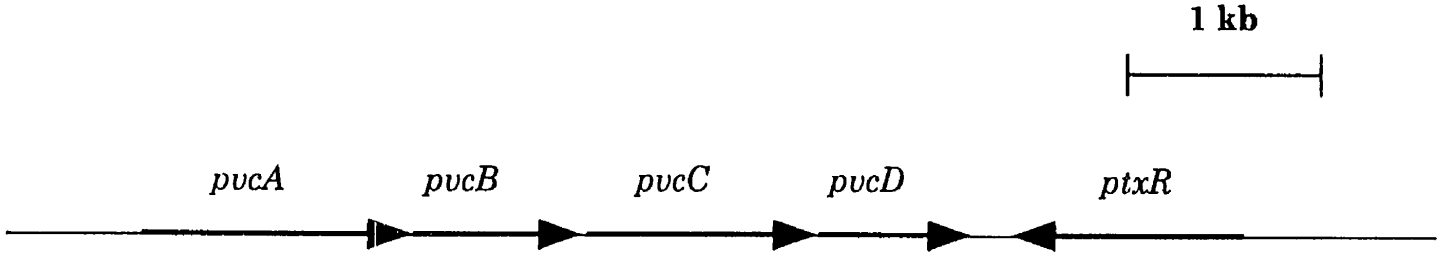
and Rao, 1985, Cornelis *et al.*, 1989). It is made up of a variable peptide moiety attached to a conserved 8-hydroxyquinoline-derived chromophore responsible for fluorescence of the molecule (Fig. 1; Cornelis *et al.*, 1989). Variability within the peptide chain occurs between strains of pseudomonads with three distinct types of pyoverdines occurring within *P. aeruginosa* strains (Meyer *et al.*, 1997). This variability can be used as a method of typing *P. aeruginosa* strains (Meyer *et al.*, 1997). For the PAO1 strain of *P. aeruginosa*, this peptide moiety is an octapeptide (D-Ser-L-Arg-D-Ser-L-N^{δ}-OH-Orn- L -Lys- L -Thr- L -Thr- L -N^{δ}-OH-Orn; Meyer *et al.*, 1997). In order for the organism to retrieve iron from ferric-pyoverdine, FpvA, an iron-regulated outer membrane protein (Poole *et al.*, 1993), acts a specific receptor for ferric-pyoverdine although the possibility of another, less specific, uptake system for ferric-pyoverdine also exists (Gensberg *et al.*, 1992, Meyer, 1992). Pyoverdine is important because it has a high affinity for iron, with an affinity constant of 10^{32} (Meyer *et al.*, 1990) and it has been shown to remove iron from transferrin in serum, probably assisting growth within, and ultimate colonization of, the human host by *P. aeruginosa* (Cox and Adams, 1985). Moreover, experiments studying the burned mouse and rat lung models of *P. aeruginosa* infection have shown that ferric-pyoverdine is required for infection and/or colonization, underlining the importance of ferric-pyoverdine to the virulence of *P. aeruginosa* (Meyer *et al.*, 1996, Poole *et al.*, 1996a).

Similar to other fluorescent pseudomonads (e.g. *P. putida*; Marugg *et al.*, 1985), the genes involved in the synthesis of the pyoverdine molecule are found in more than one area on the chromosome (Fig. 2; Ankenbauer *et al.*, 1986, Hohnadel *et al.*, 1986). The peptide moiety appears to be produced by enzymes encoded by genes found within the *pvd* locus at 47 minutes on the recalibrated PAO map (Tsuda *et al.*, 1995, Holloway, 1996). This locus includes genes coding for a peptide synthetase (*pvdD*; Merriman *et al.*, 1995), the ferric-pyoverdine outer membrane receptor (*fpvA;* Poole *et al.*, 1993), another synthetase (*pvdE*; McMorran *et al.*, 1996), an L-Ornithine N^5-Oxygenase (*pvdA;* Visca *et al.*, 1994) and an alternative sigma factor required for the expression of the *pvd* genes and controlled by iron concentration (*pvdS*; Cunliffe *et al.*, 1995, Miyazaki *et al.*, 1995). The synthesis of this peptidyl moiety is carried out by the products of these genes via a non-ribosomal mechanism (Merriman *et al.*, 1995).

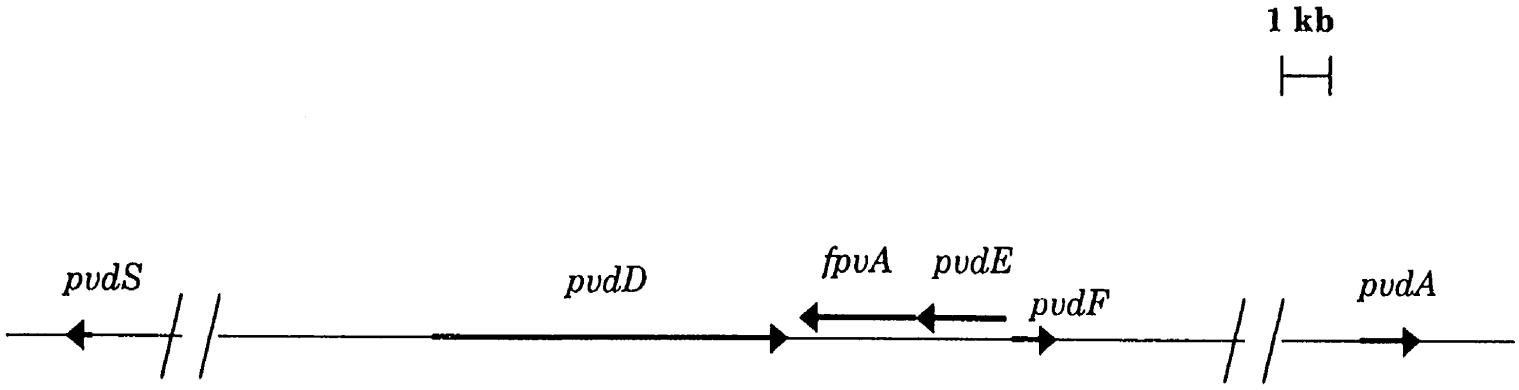
An operon, *pvc* (pyoverdine chromophore), that codes for the chromophore of the pyoverdine molecule can be found between 66-70 minutes on the chromosomal map of PAO (Stintzi *et al.*, 1996). This operon comprises four genes, *pvcABCD*, which when mutated cause a pyoverdine deficiency (Stintzi *et al.*, 1996 and submitted). Biosynthesis of the pyoverdine chromophore commences with the condensation of D-tyrosine with a L-2,4-diaminobutyric acid molecule possibly catalyzed by the product of *pvcA*, and is followed by further hydroxylation reactions apparently catalyzed by the

Figure 2: Genetic organization of the two major loci involved in pyoverdine biosynthesis.
a) The *pvc* operon with genes encoding the proteins required for biosynthesis of the chromophore.
b) The *pvd* locus containing genes encoding proteins required for biosynthesis of the peptide moiety, the outer membrane receptor and regulatory proteins.

a) *pvc* operon



b) *pvd* locus



pvcBCD gene products (Stintzi *et al.*, submitted). The *ptxR* gene, which is divergently transcribed downstream of this operon, appears to be a LysR-type transcriptional activator which plays a role in the activation of this operon (Stintzi *et al.*, submitted).

Finally, the 23 minute region on the recalibrated PAO chromosome has been shown to play a role in pyoverdine biosynthesis but no further information about this region is known at the present time (Hohnadel *et al.*, 1986, Stintzi *et al.*, 1996). The genes found in both the *pvd* region and the *pvc* operon are negatively regulated by iron (Rombel *et al.*, 1995, Tsuda *et al.*, 1995, Leoni *et al.*, 1996, Stintzi *et al.*, submitted). A binding sequence for the iron-regulatory protein, Fur (see section 1.5.1), was only found upstream of the *pvdS* gene and not the other genes in the *pvd* region or *pvc* operon (Poole *et al.*, 1993, Visca *et al.*, 1994, Rombel *et al.*, 1995, Stintzi *et al.*, submitted). Thus, PvdS expression responds to medium iron level (mediated by Fur) and, in turn, regulates *pvd* and *pvc* gene expression, explaining the iron-regulation of the latter genes (Leoni *et al.*, 1996, Crosa, 1997, Stintzi *et al.*, submitted). Surprisingly, neither Fur nor PvdS seem to be involved in the regulation of FpvA expression though it, too, is negatively iron-regulated (Poole *et al.*, 1993).

1.3.1.3 Heterologous siderophores

In addition to being able to use the two siderophores it produces, *P. aeruginosa* is able to use siderophores that are produced by other organisms

that may be living in the same environment, including fungi and other bacteria. These siderophores include ferric-enterobactin (Dean and Poole, 1993a) produced by members of the enterobacteriaceae, ferrioxamine B produced by actinomycetes (Cornelis *et al.*, 1987) as well as ferric-pyoverdines produced by other fluorescent pseudomonads (Hohnadel and Meyer, 1988, Meyer *et al.*, 1999). It has been shown that the ferric-enterobactin uptake system requires an iron regulated outer membrane receptor, PfeA, which has its expression induced by the presence of ferric-enterobactin in the environment via a two-component regulatory system (see section 1.5.5.1; Dean and Poole, 1993a). In fact, because of the better affinity constant of ferric-enterobactin (10^{52}; Griffiths, 1987b) as compared to ferric-pyoverdine or ferric-pyochelin, ferric-enterobactin is used preferentially by *P. aeruginosa* when it is in the environment. As such, pyoverdine and pyochelin production is reduced in the presence of ferric-enterobactin (Dean and Poole, 1993b). Desferal, a derivative of ferrioxamine B, prompts expression of a relatively high molecular weight iron-regulated outer membrane protein likely to be the ferrioxamineB receptor in *P. aeruginosa* (Cornelis *et al.*, 1987).

1.4 Iron-regulated outer membrane receptors

Under conditions of low iron in the environment, certain bacteria, including *P. aeruginosa*, produce high molecular weight (*ca.* 80 kDa) outer membrane proteins termed iron-regulated outer membrane proteins

(IROMPs) that are involved in the internalization of iron-siderophore complexes (Neilands, 1982). These receptors, which have a high affinity for their substrate, are required for iron uptake because the ferric siderophore complexes are too big to diffuse passively through the porins in the outer membrane (Neilands, 1982, Braun, 1995). In most cases, energy for this process of shuttling iron against its concentration gradient is supplied by the proton motive force of the cytoplasmic membrane which is apparently "coupled" to the outer membrane receptor by the cytoplasmic membrane-associated TonB protein (see section 1.4.3;Moeck and Coulton, 1998).

1.4.1 FptA

The major outer membrane receptor for ferric-pyochelin, FptA, is a 75 993 Da protein which is homologous to FpvA, PupA and PupB, all hydroxamate siderophore receptors (Ankenbauer and Quan, 1994). Thus, the type of siderophore that is transported by a given receptor is not apparent from homology studies. All of these receptors carry out TonB-dependent transport, as would be expected for FptA, yet a consensus TonB-binding sequence (TonB box) was absent from the N-terminus of the protein. Thus, it may interact in a different way with TonB than *E. coli* receptors do (see section 1.4.3; Ankenbauer and Quan, 1994, Poole *et al.*, 1996a). The interaction of FptA with TonB is expected to provide energy not normally available in the outer membrane (see section 1.4.3) for ferric-pyochelin uptake across the outer membrane. A putative Fur binding site (Fur box; see

section 1.5.1) was found in the *fptA* promoter and experimentally *fptA* was shown to be iron-regulated (Ankenbauer and Quan, 1994). Further, *fptA* expression was shown to be regulated by PchR, which activated *fptA* expression in the presence of pyochelin (dependent upon the presence of FptA) and repressed *fptA* expression in the absence of pyochelin (Heinrichs and Poole, 1996). It was, therefore, thought that FptA might directly signal pyochelin (or more probably ferric-pyochelin) abundance to PchR (Crosa, 1997).

1.4.2 FpvA

FpvA is an 86 kDa IROMP which acts as a receptor for ferric pyoverdine (Poole *et al.*, 1993). The *fpvA* gene (Poole *et al.*, 1993), found within the *pvd* locus at 47 minutes on the recalibrated PAO map, encodes this receptor (Tsuda *et al.*, 1995). FpvA is homologous to TonB-dependent receptors including the ferric-pseudobactin receptors, PupA and PupB, of *P. putida* WCS385 (Poole *et al.*, 1993). FpvA does not, however, appear to contain the consensus sequence termed the "TonB box" indicating that it, like FptA, may interact in a different way with TonB than *E. coli* receptors do (Poole *et al.*, 1996a). Again, the interaction with TonB is expected to provide energy for ferric-pyoverdine uptake that is not normally available in the outer membrane (see section 1.4.3).

An atypical N-terminal extension not present in most other ferric-siderophore receptors but present in PupB and FecA was identified in FpvA

and proposed to occur in the periplasm. This periplasmic extension is suspected to play a role in signal transduction, in a fashion resembling that of the *P. putida* WCS385 ferric-pseudobactin receptor, PupB (Koster *et al.*, 1994) and the ferric-dicitrate receptor, FecA, of *E. coli* (Kim *et al.*, 1997; see section 1.5.5).

1.4.3 The TonB-ExbB-ExbD complex

Transport of ferric siderophores across the outer membrane absolutely requires energy. Since there are no obvious modes of energy production in the outer membrane, the energy for these processes must be procured from somewhere else (Braun, 1995). This energy requirement is fufilled by the TonB-ExbB-ExbD complex and the proton motive force. TonB is a protein that is anchored by its N-terminal to the cytoplasmic membrane with a large fragment residing in the periplasmic space. ExbD is also a periplasmic protein that is anchored to the cytoplasmic membrane by its N-terminal end (Braun, 1995). ExbB, in contrast, is a cytoplasmic membrane-associated protein with its N terminus in the periplasm and possessing three membrane spanning regions with large loops present in the cytoplasm (Braun, 1995, Braun *et al.*, 1996). The role of TonB is to couple the energy of the proton motive force which is present across the cytoplasmic membrane to the outer membrane receptors. This appears to occur through physical interaction with the outer membrane receptors in response to the proton motive force and ligand occupying the receptor (Skare *et al.*, 1993, Moeck and Coulton, 1998).

Mutants lacking TonB or the TonB homologues that have been identified in *P. aeruginosa* (Poole *et al.*, 1996b) and *P. putida* (Bitter *et al.*, 1993) show no ferric siderophore uptake, consistent with the essential role TonB plays in this process (Braun, 1995).

Outer membrane receptors were thought to function as gated channels (Rutz *et al.*, 1992, Killmann *et al.*, 1993) but now, it seems that a "cork" domain acts to regulate transport through the channel (Ferguson *et al.*, 1998, Locher *et al.*, 1998, Buchanan *et al.*, 1999). The crystal structures of FhuA, the ferrichrome iron uptake protein (Ferguson *et al.*, 1998, Locher *et al.*, 1998), and FepA, the ferric-enterobactin receptor protein (Buchanan *et al.*, 1999), both from *E. coli*, have been elucidated with and without bound ferric-siderophore. Conformational changes in the N-terminal region of both these receptors, induced by the binding of ferric-siderophore to the receptor, are expected to allow TonB to bind to the receptor's TonB box that has been made accessible by the conformational change (Ferguson *et al.*, 1998). The energy transduced by the binding of TonB to the receptor induces a further conformational change in the cork domain of the receptor that opens a channel large enough for the iron-siderophore to pass through into the periplasm (Ferguson *et al*, 1998, Locher *et al.*, 1998). It has been postulated that TonB can physically interact with the outer membrane receptor either through a conformational change in the TonB protein that is still anchored to the cytoplasmic membrane (Fig. 3a; Braun, 1995) or by shuttling of the TonB

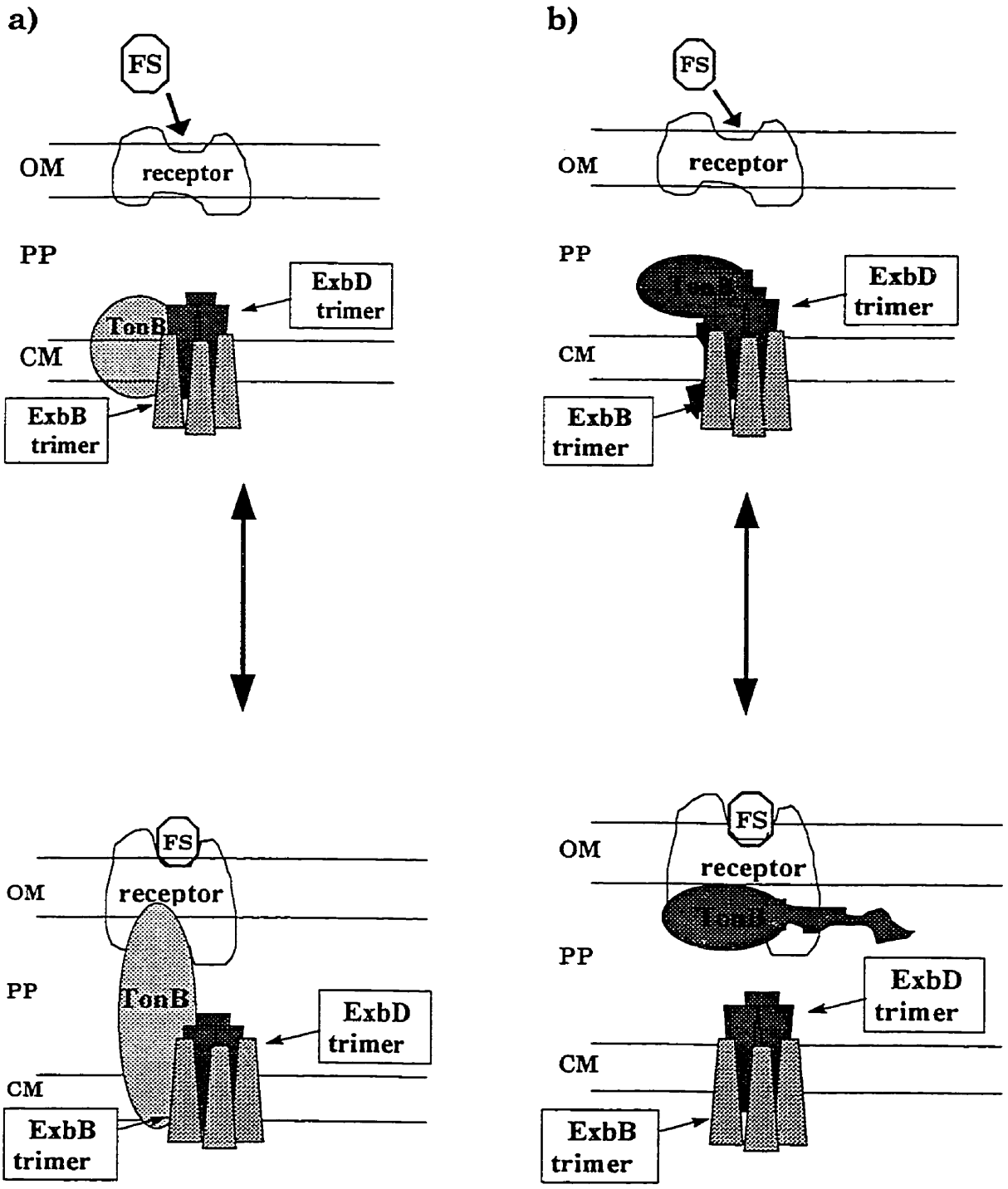
protein across the periplasm (Fig. 3b; Higgs *et al.*, 1998). The energized form of TonB transfers its energy to the receptor to promote iron-siderophore uptake and then needs to be re-energized. This probably occurs through interaction between TonB and ExbB-ExbD, the latter a heterohexameric complex forming an ion-translocating channel (Higgs *et al.*, 1998). Experiments using His-tagged ExbB bound to a nickel-nitriloacetate agarose column showed retention of both TonB and ExbD suggesting physical association between ExbB and TonB as well as ExbB and ExbD (Braun *et al.*, 1996). TonB has been shown to require both ExbB and ExbD to function properly, and mutants lacking these show defects in iron acquisition (Braun *et al.*, 1996). Partial function of TonB can be obtained via the interaction of TonB with TolQ and TolR, homologues of ExbB and ExbD, respectively, that are involved in uptake of the group A colicins via TolA (Braun, 1995, Braun *et al.*, 1996). A recent model of the arrangement of this complex has been put forward by Higgs *et al.* (1998; Fig. 3). Further, studies suggest that TonB is more likely to interact with a receptor to which a ligand has already bound allowing for a small amount of TonB to energize (as required) a large number of specific outer membrane receptors (Moeck and Coulton, 1998).

Figure 3: Models of TonB interaction with an outer membrane receptor. The ExbB and ExbD complexes provide energy to TonB from the proton motive force and stabilize TonB in the membrane.

a) Conformational changes in the outer membrane receptor and TonB upon ferric-siderophore binding allow for interaction between these two proteins.

b) A conformational change in the receptor induced by ferric-siderophore binding permits interaction between TonB and the outer membrane receptor as TonB shuttles between the two membranes.

FS, ferric-siderophore; OM, outer membrane; PP, periplasmic space; CM, cytoplasmic membrane


a)
FS
OM
receptor
PP
ExbD
trimer
TonB
CM
ExbB
trimer
b)
FS
OM
receptor
PP
ExbD
trimer
CM
ExbB
trimer
FS
OM
receptor
PP
TonB
ExbD
trimer
CM
ExbB
trimer
FS
OM
receptor
PP
ExbD
trimer
CM
ExbB
trimer

1.5 Iron uptake regulation

1.5.1 Fur

Ferric uptake regulation (Fur) proteins or homologues are found in several Gram-negative and Gram-positive bacteria including *E. coli* (Schäffer *et al.*, 1985), *Bacillus subtilis* (Bsat *et al.*, 1998), *Mycobacterium marinum* (Pagán-Ramos *et al.*, 1998), *Neisseria gonorrhoeae* (Berish *et al.*, 1993), *Synechococcus* sp. (Ghassemian and Straus, 1996), *Vibrio cholerae* (Crosa, 1997) and *P. aeruginosa* (Prince *et al.*, 1991). In Gram-negative bacteria, these proteins control the expression of iron-regulated genes such as those required for the synthesis of siderophores, their outer membrane receptors as well as certain virulence factors [e.g. exotoxin A (Prince *et al.*, 1993)]. Fur proteins, under conditions of high iron concentration, bind to these Fe^{2+} ions in the cytoplasm which then permits binding of the Fur-Fe^{2+} complex to the operator of these iron-regulated promoters, blocking binding of the RNA polymerase (Guerinot, 1994). The reducing environment of the cytoplasm likely maintains the iron as ferrous ions. The Fur binding region in *E. coli* has been identified as a consensus sequence of 19 bases (GATAATGATAATC ATTATC), dubbed the Fur box, which is often found in the promoters of iron-regulated genes (Bagg and Neilands, 1987b, Guerinot, 1994). When there is little iron in the environment, Fur occupancy of the operator is reduced, allowing the transcription of these genes. Other divalent cations of similar size (e.g. Co^{2+} and Mn^{2+}) can also associate with Fur, promoting Fur binding

to operator sequences, in some cases inducing iron starvation (Bagg and Neilands, 1987a).

In *E. coli*, the Fur protein has been identified as a 17 kDa polypeptide containing a high number of histidine residues, presumably present to bind the ferrous iron corepressor (Bagg and Neilands, 1987a). A Fur homologue identified in *P. aeruginosa* has been found to regulate genes involved in exotoxin A production as well as siderophore biosynthesis (Prince *et al.*, 1993). Comparison of the consensus Fur-binding sequence of *E. coli* with a putative Fur binding region in *P. aeruginosa* showed some homology, where any variation was most likely due to the tremendously different G+C content of these two organisms (Prince *et al.*, 1993). Also, the C-terminus of the *P. aeruginosa* Fur protein, which is predicted to be the site of DNA binding, is different from that of the *E. coli* Fur protein (Prince *et al.*, 1993), consistent with the *P. aeruginosa* Fur protein recognizing a slightly different operator sequence. Interestingly, while both siderophores produced by *P. aeruginosa* are known to be produced under low-iron conditions, pyochelin biosynthetic and transport genes exhibit putative Fur consensus sequences while genes involved in pyoverdine biosynthesis and transport do not (Ankenbauer and Quan, 1994, Serino *et al.*, 1997, Stintzi *et al.*, submitted). Instead, the pyoverdine biosynthetic genes appear to be regulated by PvdS, an alternative sigma factor, which is itself regulated by Fur (see section 1.5.2; Crosa, 1997, Stintzi *et al.*, submitted).

1.5.2 PvdS

The *pvdS* gene, found within the *pvd* locus, encodes an alternative sigma factor belonging to a σ^{70} subfamily called the extracytoplasmic function (ECF) sigma factors (Cunliffe *et al.*, 1995, Miyazaki *et al.*, 1995, Missiakas and Raina, 1998). Similarities between PvdS and FecI of *E. coli* and PupI of *P. putida* WCS358 were found in certain regions of the predicted PvdS protein. Unlike *fecI* and *pupI*, which form an operon with a second gene, *fecR* (Braun, 1997) and *pupR* (Koster *et al.*, 1994), respectively, the *pvdS* gene is not transcribed in an operon (Cunliffe *et al.*, 1995, Miyazaki *et al.*, 1995). The PupR and FecR proteins apparently function as signal transducers and it is possible that an unlinked homologue works with *pvdS* in *P. aeruginosa.*

A Fur box occurs upstream of the *pvdS* gene and Fur mediates iron-regulated expression of this gene (Cunliffe *et al.*, 1995). This permits iron-regulated expression of genes, such as *toxA* and the pyoverdine biosynthetic genes in *P. aeruginosa*, which depend on PvdS but lack their own Fur boxes (Leoni *et al.*, 1996, Ochsner *et al.*, 1996, Stintzi *et al.*, submitted).

1.5.3 PtxR

PtxR is a protein with similarity to the LysR family of transcriptional activators that was found, in *P. aeruginosa*, to play a role in exotoxin A expression as well as expression of the *pvc* operon (Hamood *et al.*, 1996, Stintzi *et al.*, submitted) and, thus pyoverdine biosynthesis. It is not a major activator of exotoxin A expression since PtxR mutants still show exotoxin A

production (Hamood *et al.*, 1996). In contrast, the loss of PtxR abolishes transcription of the *pvc* operon (Stintzi *et al.*, submitted). The *ptxR* gene is divergently transcribed downstream of the *pvc* operon which is located in the 66-70 minute region of the *P. aeruginosa* chromosome (recalibrated map; Holloway, 1996, Stintzi *et al.*, submitted). This genetic arrangement, where the activator is divergently transcribed from one of the operons it controls, is one of the characteristics of the LysR family of activator proteins (Henikoff *et al.*, 1988). Recent work has led to the hypothesis that PtxR is transcribed from two different promoters: an iron-regulated promoter active in late log and stationary phase under microaerobic conditions, and an iron-independent promoter that allows low-level constitutive expression of *ptxR* (Vasil *et al.*, 1998). The former is not directly regulated by Fur but does require PvdS, the Fur-regulated alternative sigma factor for expression (Vasil *et al.*, 1998).

1.5.4 LasR

Recently, LasR, a transcriptional activator involved with quorum sensing in *P. aeruginosa* (Gambello and Iglewski, 1991), was found to play a role in pyoverdine biosynthesis (Stintzi *et al.*, 1998). Through signalling from an effector molecule, a homoserine lactone or "autoinducer" called PAI-1, LasR mediates cell-density dependent upregulation of several extracellular virulence factors (Pesci and Iglewski, 1997). Studies relating to pyoverdine biosynthesis have shown that *lasR* mutants produce less siderophore than

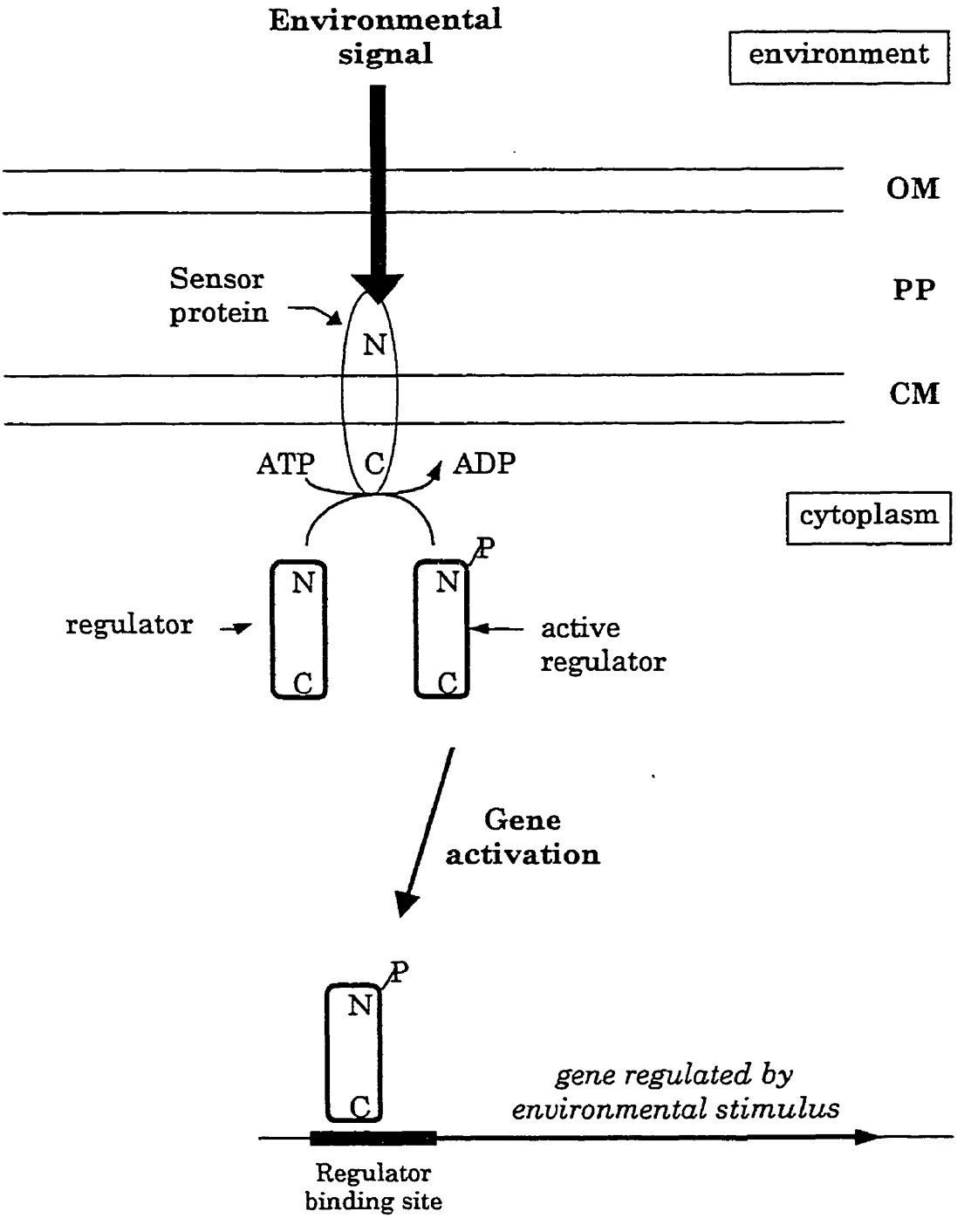
wild-type, apparently due to lowered expression of the *pvc* genes (Stintzi *et al.*, 1998).

1.5.5 Two-component regulatory systems

1.5.5.1 Classical

Classical signal transduction by a two-component regulatory system in bacteria involves the action of two proteins: a sensor and a regulator (Albright *et al.*, 1989). Examples include OmpR/EnvZ, NtrB/NtrC and PhoR/PhoB which are involved in expression of outer membrane porins, nitrogen assimilation and expression of the phosphate regulon, respectively (Albright *et al.*, 1989). Each of these pairs involves a component, the sensor, that senses an environmental signal and a component, the regulator, that initiates transcription of the required genes in response to a signal received from the sensor (Albright *et al.*, 1989). Among sensor proteins, there are five conserved regions in the C-terminus of these proteins that are thought to play a role in signal transduction (Albright *et al.*, 1989). The regulator proteins have homologies in the N-terminus with four main conserved regions (Albright *et al.*, 1989). Consequently, the model for signal transduction involves the N-terminus of the sensor protein receiving a signal which allows for autophosphorylation of the C-terminus of this protein and subsequent phosphorylation of the N-terminus of the regulator protein. The phosphorylated regulator then activates transcription of target genes by binding to appropriate promoter sequences (Fig. 4; Albright *et al.*, 1989). The

Figure 4: Classical two-component regulation. A sensor protein receives an environmental signal which is passed to a regulator protein by phosphorylation. The regulator then induces transcription of the target gene.
OM, outer membrane; PP, periplasmic space; CM, cytoplasmic membrane; P, phosphorylated; N, N-terminus of the protein; C, C-terminus of the protein


Environmental signal
environment
OM
PP
CM
Sensor protein
N
ATP
C
ADP
cytoplasm
P
N
C
N
C
regulator
active regulator
Gene activation
P
N
C
gene regulated by environmental stimulus
Regulator binding site

signal received by the sensor component may be a direct environmental signal or may be obtained indirectly through signalling from another receptor (Albright *et al.*, 1989). One example of a classical two-component regulatory system is associated with the regulation of iron uptake genes in *P. aeruginosa*, namely the ferric enterobactin receptor, PfeA (Dean and Poole, 1993b). Two genes, *pfeR* and *pfeS*, found in an operon upstream of the *pfeA* gene, encode two proteins that are homologous to previously identified sensor and regulator proteins (Dean and Poole, 1993b). In this case, the presence of ferric-enterobactin in the environment, under iron limiting conditions, is sensed by PfeS which activates PfeR which, in turn, is believed to stimulate *pfeA* transcription (Dean and Poole, 1993b).

1.5.5.2 Receptor-mediated

1.5.5.2.1 Ferric-pseudobactin transport in *Pseudomonas putida* WCS358

Pseudomonas putida WCS358 produces the pyoverdine-like siderophore, pseudobactin 358, under iron limiting conditions (Koster *et al.*, 1993). Ferric-pseudobactin 358 is transported into cells via PupA, an 85 kDa outer membrane receptor (Marugg *et al.*, 1989). Though most pseudomonads can only use their endogenous pseudobactin or pyoverdine, *P. putida* WCS358 can use several heterologous pseudobactins produced by other pseudomonads that grow in the plant root environment (Koster *et al.*, 1993). The presence of these heterologous pseudobactins in the environment induces the production of new high molecular weight outer membrane proteins in *P. putida* WCS358

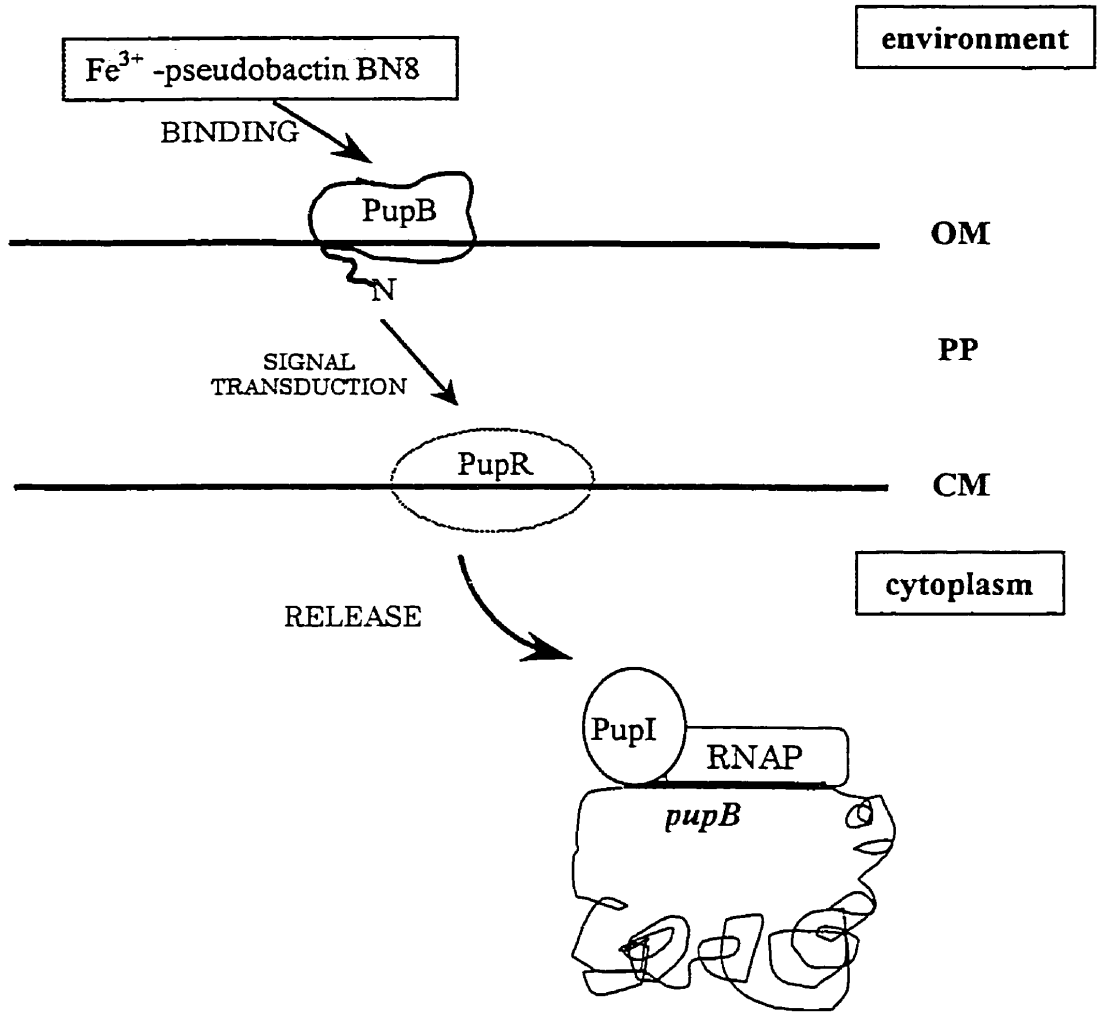
(Koster *et al.*, 1993). One of these outer membrane proteins has been identified as PupB, an 89 kDa receptor for the heterologous siderophores pseudobactin BN8 (produced by *Pseudomonas* BN8) and pseudobactin BN7 (produced by *Pseudomonas* BN7) (Koster *et al.*, 1993). PupB is homologous to TonB-dependent siderophore transporters in *E. coli* yet PupB does not have a "TonB box", indicating that it may, like FpvA in *P. aeruginosa*, interact with TonB in some novel way (Koster *et al.*, 1993). Like most TonB-dependent receptors, PupB is iron-regulated but its expression requires the presence of its substrate, ferric-pseudobactin BN8 or ferric-pseudobactin BN7 (Koster *et al.*, 1993). This inducibility in the presence of the substrate has been seen in *P. aeruginosa* in the case of the ferric-enterobactin receptor (Poole *et al.*, 1990) and in the *fec* system of *E. coli* (see section 1.5.5.2.2; Braun, 1997). PupB upregulation is mediated by an iron-regulated operon, found upstream of *pupB*, containing the genes *pupI* and *pupR* (Koster *et al.*, 1994). The predicted protein sequences indicate that PupI is homologous to members of the ECF sigma factors indicating that it is probably a cytoplasmic protein that acts as a positive regulator of *pupB* transcription (Koster *et al.*, 1994). PupR, on the other hand, is predicted to be a cytoplasmic membrane protein that appears to inhibit PupI activity in the absence of pseudobactin BN8 while stimulating PupI activity in the presence of this siderophore (Koster *et al.*, 1994). PupR stimulation of PupI requires a signal from PupB, apparently transduced via its periplasmic N-terminal domain during the conformational

change in the receptor resulting from its binding and transport of ferric-pseudobactin BN8 or BN7 (Fig. 5; Koster *et al.*, 1994). Involvement of the N-terminal extension of PupB in signalling to the *pupB* gene was demonstrated by experiments using a chimeric protein, where the N-terminal extension of PupB was used to replace the N-terminal domain of the PupA receptor. These experiments showed that binding and transport of ferric-pseudobactin 358, the siderophore transported by PupA, induced the upregulation of the *pupB* gene, indicating that the N-terminus of PupB specifically induces its own upregulation when siderophore binding to the receptor occurs (Koster *et al.*, 1994).

1.5.5.2.2 Ferric-dicitrate transport in *E. coli*

E. coli can use citrate as an iron chelator to deliver iron in the form of ferric-dicitrate to the cell. The *fec* locus encoding transport and regulatory genes for this system is found at 97.3 minutes on the *E. coli* K-12 chromosome (Veitinger and Braun, 1992). The genes found within this locus are arranged in two operons. The first contains genes encoding an outer membrane receptor, *fecA*, a periplasmic ferric-dicitrate binding protein, *fecB*, two cytoplasmic membrane proteins, *fecCD*, and a cytoplasmic protein that is associated with the inner membrane, *fecE*. The second operon, found upstream of *fecABCDE*, codes for two regulatory genes: *fecR*, a cytoplasmic membrane protein involved in signal transduction and *fecI*, a cytoplasmic

Figure 5: Receptor-mediated signal transduction in *P. putida* WCS358. The PupB receptor signals binding of ferric-pseudobactin to PupR through a conformational change in the receptor. PupR releases the ECF sigma factor PupI which induces upregulation of the *pupB* gene.
OM, outer membrane; PP, periplasmic space; CM, cytoplasmic membrane; N, N-terminus of the protein; RNAP, RNA polymerase


environment
Fe^{3+} -pseudobactin BN8
BINDING
PupB
OM
N
SIGNAL
TRANSDUCTION
PP
PupR
CM
cytoplasm
RELEASE
PupI
RNAP
pupB

protein (Braun, 1997, Welz and Braun, 1998). FecI belongs to the ECF subfamily of the σ^{70}-type factors that is involved in recognition of extra-cytoplasmic signals, in this case ferric-dicitrate, mediated by a cytoplasmic membrane protein (FecR) (Angerer *et al.*, 1995, Missiakas and Raina, 1998). It is responsible for the transcription of *fecABCDE* whose expression is, thus, FecIR-dependent (Angerer and Braun, 1998). Ferric-dicitrate is transported across the outer membrane in a TonB-, ExbB- and ExbD-dependent fashion via FecA (Zimmermann *et al.*, 1984). Both operons of the *fec* locus are controlled by the Fur protein but only *fecABCDE* is induced by ferric-dicitrate (Braun, 1997). Induction of the *fec* genes resembles that of the *P. putida pupB* gene since it involves signalling initiated by an outer membrane receptor and mediated by a cell membrane-associated protein (FecR or PupR) and an ECF sigma factor (FecI or PupI) (Koster *et al.*, 1994, Kim *et al.*, 1997, Braun, 1997). Not surprisingly, FecI/PupI and FecR/PupR are homologues and like PupB, FecA possesses an N-terminal, presumed periplasmic, extension critical for substrate- (ferric-dicitrate) mediated signal transduction via FecA, FecR and FecI (Kim *et al.*, 1997, Angerer and Braun, 1998). This signal cascade requires TonB and ExbBD to provide energy to FecA in order for signalling across the outer membrane to FecR to occur (Kim *et al.*, 1997). Interestingly, energy required for transport of ferric-dicitrate across the outer membrane is also derived from the TonB-ExbB-ExbD complex (Kim *et al.*, 1997).

1.6 Aim of this work

P. aeruginosa is quickly becoming an important nosocomial pathogen affecting immunocompetent and, more importantly, immunocompromised individuals. Since pyoverdine has been shown to be required, under certain circumstances, for the virulence of *P. aeruginosa*, it is important that the regulation of this molecule be elucidated. The aim of this work is to evaluate the involvement of FpvA in expression of the *fpvA* gene and/or the pyoverdine biosynthetic genes. Signalling via FpvA is expected to occur in a fashion similar to the receptor-dependent systems PupB and FecA in *P. putida* WCS385 and *E. coli*, respectively. In order to establish a link between FpvA and the pyoverdine biosynthetic genes at the level of transcription, *fpvA-lacZ*, *pvdD-lacZ* and *pvcA-lacZ* fusions were used to assess any influence of the receptor status on expression of the receptor and the pyoverdine biosynthetic genes. Owing to apparently very weak expression of the *pvcA-lacZ* fusion, ribonuclease (RNase) protection assays were used to assess FpvA involvement in the expression of the *pvc* operon. Additionally, exogenous pyoverdine was added to some trials to study its role in regulation of the *pvc* operon via the ferric-pyoverdine receptor.

Chapter 2: MATERIALS AND METHODS

2.1 Bacterial strains and plasmids

The bacterial strains and plasmids used for this work are highlighted in Tables 1 and 2, respectively.

2.2 Growth conditions

For general growth in rich medium, strains were grown in Luria-Bertani (LB) Broth (Difco, Detroit, MI) with 2 g/L additional NaCl (BDH Analar, Toronto, ON). Solid medium contained in addition 1.5% (w/v) agar (BDH, Toronto, ON). The iron-limited synthetic succinate medium contained 6 g $K_2HPO_4 \cdot 3H_2O$ (Sigma, St Louis, MO), 3 g KH_2PO_4 (BDH Assured, Toronto, ON), 1 g $(NH_4)_2SO_4$ (BDH Analar, Toronto, ON), 0.2 g $MgSO_4 \cdot 7H_2O$ (BDH Analar, Toronto, ON) and 4 g succinic acid $(CH_2COOH)_2$ (BDH Analar, Poole, England) per litre of double distilled water. This mixture was adjusted to pH 7.0 with the addition of NaOH pellets (BDH Analar, Toronto, ON). In order to use the iron-limited succinate medium in solid form, double strength liquid iron-limited succinate medium was added one to one to previously autoclaved and slightly cooled 3% (w/v) agar (Difco, Detroit, MI) in double distilled water. Auxotrophic strains were supplemented with 1 mM of the required amino acid (Sigma, St. Louis, MO and BDH, Toronto, ON) and 2 mM of adenosine (Sigma, St. Louis, MO), as required. For RNA isolation, deferrated tryptic soy broth (D-TSB) was used. The tryptic soy broth (TSB;

Table 1: Bacterial strains

Strain	Description*	Source or Reference
***E. coli* Strains**		
K340	*E. coli* S17-1 *thi, pro, hsdR* (rm^+), *recA* (derived from *E. coli* 294) carries plasmid RP4 derivative (amp; tet::Mu; km::Tn7) integrated into the chromosome Tc and Km sensitive, Ap and Tp resistant; tra^+ used in the mobilization of plasmids carrying a mob region	Simon *et al.*, 1983
K467	*E. coli* DH5α F^- ϕ80d*lacZ*, ΔM15 Δ(*lacZYA*$^-$, *argF*) U169 *deoR*, *recA1*, *endA1*, *hsdR17* (r_K^- m_K^+), *supE44* λ^-, *thi*-1, *gyrA96*, *relA1*	R. Sharp
***P. aeruginosa* Strains**		
K1120	*P. aeruginosa* PAO1 Δ*aphA* knockout obtained by gene replacement using partially deleted *aphA* from PAO1 stable mutation Km sensitive	N. Gotoh
K1183	*P. aeruginosa* K1120 Δ*fpvA* stable chromosomal *fpvA* deletion by gene replacement using pK18*mobsacB*	This study
K1203	*P. aeruginosa* K1120 Δ*pvdD* stable chromosomal *pvdD* deletion by gene replacement using pSUP202::Δ*pvdD*	This study

* Km, kanamycin; Tc, tetracycline

Table 2: Plasmids

Plasmid	Description*	Source or Reference
pAM1	pK18*mobsacB* containing *fpvA* deletion fragment from pTK608 cloned into *Sph*I site of this vector Km resistant	This study
pCR2.1	Plasmid containing T7 promoter and site for introduction of PCR fragments generated with *Taq* polymerase Unique *Hin*dIII and *Bam*HI sites for linearization Ap and Km resistant	Invitrogen
pCR2.1::*omlA*	pCR2.1 containing 443 bp fragment of *omlA* gene Used for riboprobe generation Km resistant	M. Vasil (Ochsner *et al.*, 1999)
pCR2.1::*pvcAB*	pCR2.1 containing 448 bp fragment encompassing a portion of the 3' end of the *pvcA* gene and a portion of the 5' end of the *pvcB* gene Km resistant	M. Vasil
pEJB3	*fpvA-lacZ* 2kb *Kpn*I-*Sal*I fragment encompassing promoter region of *fpvA* cloned into pMP190 Cm resistant	E. Blouin
pK18*mobsacB*	Mobilizable suicide vector used for gene replacement Blue/white screening Km resistant	Schäfer *et al.*, 1994

* Ap, ampicillin; Cm, chloramphenicol; Cb, carbenicillin; Km, kanamycin; Sm, streptomycin; Tc, tetracycline

Table 2 (continued)

Plasmid	Description*	Source or Reference
pMP190	Broad host range *lacZ* operon fusion vector (*uncQ*) Unique sites: *Sal*I, *Xba*, *Kpn*I, *Bgl*II, *Hin*dIII Cm and Sm resistant	Spaink *et al.*, 1987
pMP190::*PpvcA*	*pvcA-lacZ* 793 bp *Sal*I-*Kpn*I fragment of the *pvcA* promoter region cloned into pMP190 Cm resistant	S. Neshat
pPVR2	pAK1900::*fpvA* *fpvA* gene carried on a 4.8 kb *Sph*I fragment Ap or Cb resistant	Poole *et al.*, 1993
pSUP202::Δ*pvdD*	*pvdD* gene with 6.4 kb deletion cloned into the *Pst*I site of pSUP202 Tc and Cm resistant	I. Lamont
pTK608	pPVR2 containing a 4.6 kb *fpvA* gene fragment with a 198 bp deletion of an internal *Xho*I-*Xho*I fragment Ap or Cb resistant	T. Kon

* Ap, ampicillin; Cm, chloramphenicol; Cb, carbenicillin; Km, kanamycin; Sm, streptomycin; Tc, tetracycline

Difco, Detroit, MI) was deferrated by stirring 10 g sodium chelex-100 (100-200 mesh; BioRad Laboratories, Richmond, CA) in 10-fold concentrated TSB for 6 hours at room temperature. This mixture was then dialyzed in Spectra-Por number 2 dialysis tubing (Spectrum Medical Industries, Los Angeles, CA) against 850 mL of double distilled water for 16 hours at 4°C. The dialysate was brought to a volume of 910 mL, autoclaved and supplemented with 50 mM L-glutamic acid sodium salt (MSG, BDH, Toronto, ON) and 1% (v/v) glycerol (BDH, Toronto, ON) before use. Iron-replete media were prepared by the addition of 100-200 μM $FeCl_3$ (BDH Analar, Toronto, ON) prepared in 0.1N HCl (BDH Analar, Toronto, ON). For some studies, pyoverdine (provided by J-M. Meyer) was added to iron-limited media to a concentration of 20-50 μg/mL for RNA isolation or 100 μg/mL for β-galactosidase assays. All cultures were grown at 37°C, with the exception of cultures used for RNA isolation that were grown at 32°C. Broth cultures were placed on an orbital shaker at 190 rotations per minute (rpm) to allow for aeration. Antibiotics for the maintenance of plasmids were added at the concentrations indicated in Table 3.

2.3 DNA preparation and visualization

Small-scale plasmid DNA preparation was carried out using the alkaline lysis method as described by Sambrook *et al.* (1989). In brief, 1.5

Table 3: Antibiotic concentrations used to maintain plasmids in *E. coli* and *P. aeruginosa*

Antibiotic	*E. coli*	*P. aeruginosa*
Ampicillin	100 μg/mL	n/a*
Carbenicillin	n/a*	200 μg/mL
Chloramphenicol	30-50 μg/mL	200-400 μg/mL
Kanamycin	40 μg/mL	50 μg/mL
Tetracycline	10 μg/mL	50-100 μg/mL

* n/a, not applicable

mL of overnight culture was pelleted by centrifugation at 16 000 xg for 3 minutes. The pellet was resuspended in 100 μL of gentle lysis buffer [GET; 50 mM glucose (BDH, Toronto, ON), 10 mM ethylene diaminetetraacetic acid (EDTA; BDH, Toronto, ON) pH 8.0, 25 mM 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris-HCl; BDH, Toronto, ON) pH 8.0] then 200 μL of 0.2 N NaOH-1% (w/v) sodium dodecyl sulphate (SDS; ICN Biomedicals, Aurora, OH) was added and the mixture was incubated on ice for 5 minutes. To precipitate the proteins, 150 μL of a high salt solution [60 mL 5M potassium acetate (BDH, Toronto, ON), 11.5 mL glacial acetic acid (BDH, Toronto, ON), 28.5 mL H_2O] was added, and the mixture was incubated on ice for 10 minutes and then centrifuged at 16 000 xg for 10 minutes. The supernatant was transferred to a new tube where it underwent one phenol (100 mM Tris pH 8.0 equilibrated) extraction followed by two chloroform extractions. The DNA was precipitated from the aqueous phase with 0.7 volumes of isopropanol. Medium-scale plasmid DNA was prepared using the QIAGEN plasmid midi kit (QIAGEN, Mississauga, ON). Plasmid DNA was visualized on 0.8-1% (w/v) agarose gels (Gibco BRL Ultrapure, Gaithersburg, MD) prepared with and run in Tris-Acetate-EDTA (TAE) buffer (Sambrook *et al.*, 1989). Ethidium bromide was added to the gels at a concentration of 0.5 μg/mL in order to see the DNA bands under UV illumination. The size of DNA fragments was assessed by using λ DNA (Boehringer Mannheim,

Mannheim, Germany) digested with *Hind*III (Gibco BRL, Gaithersburg, MD) as a molecular size standard.

2.4 DNA digestion, isolation of fragments from agarose gels and ligations

Restriction enzyme digests were carried out following the directions and in the buffers provided by the manufacturer of the enzyme used (Gibco, Gaithersburg, MD, New England Biolabs, Beverly, MA or Promega Corporation, Madison, WI). For digests requiring two enzymes, digestion was carried out sequentially, using the Prep-a-gene binding matrix (BioRad Laboratories, Richmond, CA) to recover the DNA between digests. DNA fragments were isolated from agarose gels using the Prep-a-gene binding matrix (BioRad Laboratories, Richmond, CA) for fragments greater than 500 bp or the QIAquick gel purification kit (QIAGEN, Mississauga, ON) for fragments of 500 bp or less, again following the manufacturer's directions.

Ligations were performed using T4 DNA Ligase (Gibco, Gaithersburg, MD or New England Biolabs, Beverly, MA) using the buffer provided by the manufacturer. Insert and vector DNA in a 2:1 molar ratio was mixed with buffer and 1 unit of enzyme in 10μL final volume, and incubated overnight at 16°C as described by Sambrook *et al.* (1989). Some vectors provided for blue-white screening in bacteria harbouring them. This was exploited by the inclusion of 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) and

isopropylthio-β-D-galactoside (IPTG) in antibiotic plates being used to select for bacterial transformants, again as described by Sambrook *et al.* (1989). Typically, vectors with inserts yielded white colonies.

2.5 DNA transformation into competent cells

Competent *E. coli* cells were produced by harvesting 20 mL of cells (centrifuge at 2000 xg) at early log phase [absorbance at 600 nm (A_{600}) = 0.3-0.5]. These cells were then washed twice in 10 mL ice cold 100 mM $CaCl_2$ (BDH, Toronto, ON). Cells were resuspended in 700 μL of 100 mM $CaCl_2$ containing 15% (v/v) glycerol (BDH, Toronto, ON) so that they could be frozen at -75°C in 100 μL aliquots. To make competent *P. aeruginosa* cells, a 20 mL log phase culture (A_{600} = 0.4-0.6) was harvested at 2000 xg, washed twice in 10 mL ice cold 150 mM $MgCl_2$ (BDH, Toronto, ON) then resuspended in 150 mM $MgCl_2$ containing 15% (v/v) glycerol (BDH, Toronto, ON) so that the cells could be frozen in 100 μL aliquots at -75°C.

Before use, the competent cells were thawed for about 5 minutes on ice. Two to five microlitres of DNA was added to 100 μL competent cells which were then incubated on ice for 1 hour. The cells were then heat shocked for 5 minutes at 37°C (for *E. coli*) or 42°C (for *P. aeruginosa*) followed by 2 minutes on ice. Seven hundred microlitres of LB broth was then added and the cells were incubated for 1 hour at 37°C. This mixture was then plated on the appropriate antibiotic plate (see Table 3) in 10 and 100 μL

amounts. The remaining liquid was centrifuged at 16 000 xg for 1 minute, resuspended in 100 μL of LB broth and plated. Plates were then placed at 37°C for 16-36 hours to allow bacteria containing the plasmid DNA to grow. A control plate of competent cells with no added DNA was used to be sure the cells did not grow on the antibiotic plates without a plasmid present.

2.6 Biparental mating

Conjugal transfer of plasmid DNA between strains was carried out using *E. coli* K340 containing a mobilizable plasmid as the donor strain and a *Pseudomonas* strain as the recipient. The donor was grown overnight at 37°C with agitation in LB broth containing appropriate antibiotics to maintain the plasmid (Table 3). The recipient *Pseudomonas* strain was grown overnight at 42°C without agitation in order to minimize restriction endonuclease activity. Equal amounts (500 μL) of the two cultures were then mixed in an microfuge tube, centrifuged at 16 000 xg for 2 minutes then resuspended in 50 μL of LB broth. This mixture was spotted on an LB agar plate and allowed to grow overnight (about 16 hours) at 37°C. Controls of only donor cells and only recipient cells were treated in the same manner. The next day, 1 mL of LB broth was added to the plates such that the bacterial growth could be scraped off with sterile glass spreader and transferred to an microfuge tube. This mixture was then diluted 10-fold, 100-fold and/or 1000-fold and plated onto plates containing antibiotics that selected for plasmid-containing recipient

cells (for pK18*mobsacB* and its derivatives, 50 μg/mL kanamycin, and for pMP190 and its derivatives, 200 μg/mL chloramphenicol) and against the donor *E. coli* strain (either 10 μg/mL tetracycline or 0.5 μg/mL imipenem). These plates were left at 37°C for 24-48 hours. Colonies from these plates were then picked and screened for the plasmid in question.

2.6.1 Gene replacement to produce FpvA mutants

In order to produce an *fpvA* deletion mutant, biparental mating of K340 containing pAM1 (pK18*mobsacB*::Δ*fpvA*) with wild-type *P. aeruginosa* was carried out as described above. This plasmid provides for both kanamycin resistance and sucrose sensitivity and cannot replicate in *P. aeruginosa*. After conjugation, the strains were plated on to LB agar containing 50 μg/mL kanamycin, allowing only recipient containing the plasmid to grow, and 10 μg/mL tetracycline, to select against the donor strain. Since the plasmid cannot replicate in *P. aeruginosa*, kanamycin resistance (and sucrose sensitivity) of the cells demonstrate that the plasmid has entered into the chromosome at a region of homology, in this case the *fpvA* gene. Colonies picked off the conjugation plates were streaked onto LB agar plates containing either 10% (w/v) sucrose or 50 μg/mL kanamycin. In order to select for clones where the wild type *fpvA* gene was replaced by the deleted *fpvA* gene, single colonies from the sucrose plate were then spotted with a sterile toothpick in grids onto LB agar alone, LB with 10% (w/v) sucrose or LB with 50 μg/mL kanamycin. Any clones that grew on LB or

sucrose plates but were sensitive to kanamycin were screened for the absence of FpvA expression by phenotypic assays [lack of growth on iron-limited succinate agar + 150 μg/mL of the iron chelator ethylene diamne-di(*o*-hydroxyphenyl acetic acid) (EDDHA; Sigma, St. Louis, MO) indicates an FpvA$^-$ phenotype] and Western blots of outer membrane proteins using an anti-FpvA antiserum.

2.6.2 Gene replacement to produce a PvdD mutant

A biparental mating between K340 containing pSUP202::Δ*pvdD* and K1120 was performed with plating done on plates containing 50 μg/mL tetracycline, to select for plasmid-containing recipient cells, and 0.5 μg/mL imipenem, to counter-select against donor cells. Because pSUP202::Δ*pvdD* cannot replicate in *P. aeruginosa*, tetracycline resistant clones carried the plasmid in the chromosome Three colonies from the conjugation plate were inoculated independently into 5 mL LB broth and grown overnight at 37°C. These were then subcultured at a 1/1000 dilution into 5 mL fresh LB broth and again incubated overnight at 37°C. This was repeated daily over the course of 8 days to allow for replacement of the wild-type gene in the chromosome with the deleted gene from the plasmid, in some members of the population. Each culture was then diluted to 10^{-5}, 10^{-6} and 10^{-7}, and 100 μL of each dilution was spread onto LB agar. Just under eight hundred colonies from these plates were patched onto both LB agar and LB agar containing 100 μg/mL tetracycline. Colonies that showed tetracycline sensitivity were

then patched onto solid iron-limited succinate medium to look for a lack of fluorescence indicating abrogated pyoverdine expression due to loss of the wild-type *pvdD* gene.

2.7 Cell fractionation, polyacrylamide gel electrophoresis and Western immunoblotting

2.7.1 Outer membrane isolation

Bacteria were grown in 25 mL of iron-limited succinate medium overnight at 37°C with agitation. This entire amount was harvested by centrifugation at 11 100 xg for 10 minutes at 4°C. The pellet was resuspended in 15 mM Tris-HCl pH 8.0 (ICN, Aurora, OH) containing a pinch of DNase (Boehringer Mannheim, Mannheim, Germany) and a pinch of RNase (Boehringer Mannheim, Mannheim, Germany). This was put through a French pressure cell (American Instruments Corp., Silver Spring, MD) at 14 000 psi, then centrifuged at 1940 xg for 10 minutes at 4°C to remove whole cells. Two millilitres of the supernatant were transferred to ultra centrifuge tubes where 500μL of 10% (v/v) Triton X-100 (BDH, Toronto, ON) was added and the mixture was vortexed. This was centrifuged at 541 000 xg for 15 minutes in a TL 100.3 rotor in a TL-100 model ultracentrifuge (Beckman, Palo Alto, CA). The pellet was resuspended in 100 μL of ddH_2O and stored at -70°C. Between 5 and 10 μL of outer membrane was mixed with an equal volume of protein sample buffer [50 mM Tris-HCl (pH 6.8), 2% (w/v) SDS

(ICN Biomedicals, Aurora, OH), 0.1 % (w/v) bromophenol blue (BDH, Toronto, ON), 10% (v/v) glycerol (BDH, Toronto, ON)] and heated at 95°C for 5 minutes before being loaded onto an SDS-polyacrylamide gel (see section 2.7.3).

2.7.2 Whole cell extracts

In order to study FpvA expression in whole cell extracts, overnight cultures of *P. aeruginosa* grown in iron-limited succinate medium were concentrated 250 fold by harvesting 5 mL of culture and resuspending the cell pellet in 100 μL iron-limited succinate medium and 100 μL protein sample loading buffer. This mixture was heated at 95°C for 5 minutes then sonicated for 20 seconds at 40 % power using the Vibra Cell VC50T sonicator with a 3 mm probe (Sonics and Materials, Inc., Danbury, CT) in order to shear the nucleic acids and make the mixture less viscous. Before loading, the extract was centrifuged at 16 000 xg for 2 minutes, to remove cellular debris. Five to ten microlitres of whole cell extract (depending upon the size of the original bacterial pellet) were then loaded on a gel. Whole cell extracts from D-TSB grown cells were prepared by centrifuging 1 mL of overnight culture at 16 000xg for 3 minutes, resuspending the cell pellet in 50 μL of D-TSB and 50μL of protein sample loading buffer, and then heating the mixture at 95°C for 5 minutes. This mixture was then sonicated for 20 seconds (as above) and centrifuged for 2 minutes at 16 000 xg before loading 5 μL on an SDS-polyacrylamide gel.

2.7.3 Polyacrylamide gel electrophoresis (SDS-PAGE)

Eight or ten percent (w/v) polyacrylamide gels were poured as described by Sambrook *et al.* (1989). These gels were then run at 25-30 mAmps (constant current) per gel until the dye front has run to the end of the gel. Gels not being used for Western blotting were allowed to stain for 1 to 16 hours in Coomassie blue [47% (v/v) methanol (Commercial Alcohols, Brampton, ON), 6.5% (v/v) glacial acetic acid (BDH, Toronto, ON), 1.34 mM Coomassie brilliant blue R250 (United States Biochemical Corporation, Cleveland, OH)] then allowed to destain in a solution containing 30% (v/v) methanol-10% (v/v) glacial acetic acid in distilled water over a period of about 6 hours, during which the destain solution was replaced twice. These gels were then dried between sheets of cellophane on a model 543 gel dryer from BioRad Laboratories (Richmond, CA). For size comparisons, 1 μL of the low molecular weight marker (BioRad Laboratories, Richmond, CA) in 9μL of distilled water and 10 μL of protein sample buffer or 5 μL of rainbow marker (Amersham, Oakville, ON) mixed with 5 μL of protein sample buffer was heated at 95°C for 5 minutes then loaded on the gel with the other samples.

2.7.4 Western immunoblotting

Once the SDS-PAGE was complete, the proteins on the gel were transferred to a Protran nitrocellulose membrane (Schleicher and Schuell, Keene, NH) overnight at 25 mAmps (constant current) in a transfer apparatus (Hoefer Scientific Instruments, San Francisco, CA) containing

blotting buffer [20% (v/v) methanol, 14.5 g/L glycine (ICN, Aurora, OH), 3 g/L Tris (ICN, Aurora, OH), 1 g/L SDS (ICN, Aurora, OH)]. The next morning, the nitrocellulose was incubated with slight agitation for 1 to 1 ½ hours at room temperature in 10% (w/v) skim milk (Difco, Detroit, MI) prepared in phosphate buffered saline [PBS; 16.7 mM $NaH_2PO_4 \cdot 3H_2O$, 80.9 mM Na_2HPO_4, 1.5 M NaCl pH 7.4 (BDH, Toronto, ON)]. The membrane was then washed with PBS once for 15 minutes and twice for 5 minutes before the addition of the primary antibody, a polyclonal rabbit serum specific for FpvA. This antibody was added at a 1 in 10 000 dilution in 1% (w/v) BSA (Sigma, St. Louis, MO) in 10-20 mL PBS and incubated at room temperature for 1 hour. The nitrocellulose was washed in PBS once for 15 minutes then 4 times for 5 minutes, after which it was incubated for 1 hour in a 1 in 10 000 dilution of anti-rabbit IgG conjugated with horseradish peroxidase (Amersham, Oakville, ON) in 1% (w/v) BSA (Sigma, St. Louis, MO) in 10 mL PBS. The membrane was subsequently washed with PBS as above following the primary antibody step and the substrate for the horseradish peroxidase, Lumiglo, (Kirkegaard & Perry Laboratories, Gaithersburg, MD) was added as directed by the manufacturer. The blot was sealed in a plastic bag and exposed to X-OMAT AR film (Eastman Kodak, Rochester, NY) in an autoradiography cartridge for 1-5 minutes. The film was developed by an automatic developer, then aligned with the original blot to confirm the size of the developed band(s) on the autoradiography film. A rainbow marker

(Amersham, Oakville, ON) which had been run on the gel and transferred to the membrane during electrophoretic transfer, made this possible.

2.8 β-galactosidase assay

P. aeruginosa cultures carrying plasmid pMP190 and its derivatives were grown to late log phase (A_{600} = 0.7-1.5) in iron-limited succinate medium containing 100 μg/mL of chloramphenicol with or without added iron or pyoverdine. One-hundred microlitres of this culture was added to 900 μL Z buffer [60 mM $Na_2HPO_4 \cdot 7H_2O$, 40 mM $NaH_2PO_4 \cdot H_2O$, 10 mM KCl, 1 mM $MgSO_4 \cdot 7H_2O$ (BDH, Toronto, ON), 50 mM β-mercaptoethanol (BioRad Laboratories, Richmond, CA)] in 4 mL glass test tubes. In order to lyse the cells, 10 μL of 0.1% SDS (w/v) (ICN, Aurora, OH) and 20 μL of chloroform (BDH, Toronto, ON) was added to the tube, which was vortexed for 10 seconds and then incubated for 5 minutes at room temperature. For colorimetric assessment of the β-galactosidase activity, a substrate for β-galactosidase called *o*-nitrophenyl β-D-galactopyranoside (ONPG; Sigma, St. Louis, MO) was added at a concentration of 4 mg/mL in Z buffer. After addition of 200 μL of the ONPG solution, the tube was incubated at room temperature for 5-25 minutes, until a yellow colour developed. The reaction was stopped by the addition of 500 μL 1M Na_2CO_3 (BDH, Toronto, ON) when a light-yellow colour was visible. One millilitre of liquid from the tubes was transferred to disposable cuvettes and the absorbance was read at

wavelengths of 420 nm (A_{420}) and 550 nm (A_{550}). These two numbers along with the original culture density reading (A_{600}) were then used in the formula below to calculate transcriptional activity in Miller's units (Miller, 1972).

$$\text{Miller's units} = 1000 \times \left[\frac{A_{420} - (A_{550} \times 1.75)}{\upsilon \times t \times A_{600}} \right]$$

where υ = volume of culture added (in mL)
t = time of incubation with ONPG (in minutes)

2.9 RNA isolation

Any solutions used with RNA must be treated with an RNase inhibitor, such as diethylpyrocarbonate (DEPC) in order to stop any degradation of RNA caused by RNase contamination. In this case, the RNase inhibitor 0.1% (v/v) DEPC (ICN Biochemicals, Aurora, OH) was stirred into solutions for about 16 hours and these were then autoclaved for 30 minutes on the liquid cycle. Solutions containing Tris or EDTA were prepared in water treated with DEPC (DEPC-H_2O) as described above rather than being treated after being made. To prepare cell cultures for use in isolating RNA, cell suspensions were first prepared in D-TSB by scraping cells off LB agar plates yielding a final A_{600} of about 1. These were then diluted 1/500 in 15-20 mL of fresh D-TSB. The cultures were grown 10 or 12 hours, with addition of chloramphenicol (100 μg/mL) during the last 15 minutes of growth to interfere with the translation of mRNA into protein. Between 10 and 20 mL of the culture was cooled quickly on ice in pre-chilled, chloroform-treated Oak

Ridge tubes, then harvested by centrifugation at 5930 xg for 5 minutes at 4°C. Cells were resuspended in 3 mL of lysis buffer [0.15 M sucrose (BDH, Toronto, ON), 0.01 M sodium acetate (BDH, Toronto, ON), 1% (w/v) SDS (ICN Biochemicals, Aurora, OH)] preheated to 65°C and mixed at 65°C until the lysate was clear. The lysate was then mixed at 65°C for 5 minutes with 3 mL of preheated DEPC-H_2O-equilibrated phenol containing 1 mg/mL hydroxyquinoline (BDH, Toronto, ON) then centrifuged at 27 200 xg for 30 minutes at 4°C. The aqueous layer was transferred to new tubes such that the RNA could be precipitated overnight with 0.25 M sodium acetate pH 5.2 and 3 volumes of anhydrous ethanol (Commercial Alcohols, Brampton, ON) at -70°C. The following day, the RNA was centrifuged at 27 200 xg for 30 minutes at 4°C, then resuspended in 500 μL of DEPC-H_2O. This was split between two microfuge tubes and treated with 100 U of RNase-free DNaseI (Gibco BRL, Gaithersburg, MD) in a buffer of 0.6 M sodium acetate, 0.12 M $MgCl_2$, 0.02 M $CaCl_2$ (BDH, Toronto, ON) and 40 U of RNase inhibitor (RNasin; Promega Corporation, Madison, WI) at 37°C for 1 hour. The mixture was then extracted with DEPC-H_2O-equilibrated phenol/chloroform (1:1). The aqueous phase was precipitated using 0.5 volume of 7.5 M ammonium acetate (Fisher Scientific, Fairlawn, NJ) and 2.5 volumes of anhydrous ethanol at -70°C for 1 hour then centrifuged at 16 000 xg for 15 minutes at room temperature or 4°C. The RNA pellets were washed with 300 μL of 70% (v/v) ethanol and again centrifuged for 10 minutes at 16 000 xg.

Finally, the pellet was resuspended in 200-500 μL of 50% formamide in DEPC-H_2O and allowed to sit at 65°C for 5 minutes to hasten the dissolution of the RNA pellet. The RNA was quantified at 260 nm on a Beckman DU-600 series spectrophotometer (Beckman Instruments, Palo Alto, CA). One microgram of RNA was then visualized on a 0.8% (w/v) agarose gel made with and run in Tris-borate-EDTA buffer [TBE; 89 mM Tris-borate, 2mM EDTA (BDH, Toronto, ON)] to assess quality.

2.10 Ribonuclease (RNase) protection assay

RNA probes complementary to the transcript of interest (plasmids pCR2.1::*omlA* and pCR2.1::*pvcAB* for the *omlA* and *pvcAB* probes, respectively) as well as an RNA ladder (Ambion, Austin, TX) were labelled from a linearized DNA template with [α ^{32}P]-CTP (Amersham, Oakville, ON) using the Riboprobe T7 transcription system (Promega Corporation, Madison, WI), following the manufacturer's directions. The probe and ladder reactions were counted in a liquid scintillation counter (Beckman LS 6000LL, Palo Alto, CA) using the Cherenkov method, and 500 000 - 1 000 000 counts per minute (CPM) of the probe was then used in the hybridization mixture (see below).

Prior to hybridization of probe with RNA, 40 μg of previously isolated RNA in a volume of 50 μL was precipitated with 25μL of 7.5M ammonium acetate and 225 μL of anhydrous ethanol at -70°C for at least 1 hour. This

was centrifuged at 16 000 xg for 15 minutes and the pellet was washed with 200-300 μL of anhydrous ethanol. Finally, the pellet was resuspended in 25 μL of hybridization buffer [80% (v/v) formamide, 40 mM Piperazine-N, N'-bis-(2-ethanesulfonic acid) (PIPES; Sigma, St. Louis, MO), 1 mM EDTA, 0.4 M NaCl, 3.8% (v/v) DEPC-H_2O]. This RNA was then mixed with the probe of interest, heated at 95°C for 5 minutes and allowed to hybridize overnight (about 16-18 hours) at 65°C.

The following day, the mixture was incubated with a mixture of 2 μg/mL of RNase T1 (Gibco BRL, Gaithersburg, MD) and 40 μg/mL of RNase A (Sigma, St. Louis, MO) to digest any single stranded or unbound RNA. The bound probe plus RNA was DEPC-H_2O-equilibrated phenol-chloroform (1:1) extracted and precipitated using 20 μg of yeast tRNA (Gibco BRL, Gaithersburg, MD) as a carrier. The pellet was then resuspended in 4.5 μL of RNA gel loading buffer [80% (v/v) formamide (Sigma, St. Louis, MO, 10 mM EDTA (BDH, Toronto, ON), 0.1% (w/v) bromophenol blue (BDH, Toronto, ON), 0.1% (w/v) xylene cyanol (EM Science, Gibbstown, NJ)] before 4 μL was loaded onto an 8 M urea-5% polyacrylamide denaturing gel [5% of a 19:1 acrylamide/bisacrylamide mix (Sigma, St. Louis, MO)] prewarmed to 50°C by running it at 1450 V and/or 50W for 1 hour. As well, 7000 CPM of the ladder and 2000 CPM of the probe alone were loaded in separate lanes on the gel. This gel was allowed to run at 1450 V and/or 50 W on a model SA sequencing gel electrophoresis system (Gibco BRL Life Technologies, Gaithersburg, MD)

using TBE buffer until the xylene cyanol in the buffer had travelled about 30 cm, at which time the gel was affixed to 3MM chromatography paper (Whatman, Maidstone, England) and dried for 1 ½ to 2 hours under vacuum at 80°C. The gel with *pvcAB*-probed RNA was exposed at -70°C to BioMax MS film (Eastman Kodak, Rochester, NY) for 48-63 hours while the control gel (*omlA*-probed RNA) was exposed overnight (about 15 hours), both in the presence of intensifying screens (Fisher Scientific, Ottawa, ON). In addition, the gels were exposed to a phosphor screen (Molecular Dynamics, Sunnyvale, CA) so that intensities of the major protected fragments could be quantified using the ImageQuant software provided with the phosphorimager (Molecular Dynamics, Sunnyvale, CA). The *pvcAB* probe contains a fragment that is complementary to the end of the *pvcA* and the beginning of the *pvcB* mRNA which gives a major protected band of 448 bp. The *omlA* probe includes a 242 bp complementary sequence that is observed on the RNase protection assay gel. The quantity of *pvcAB* transcript obtained on the phosphor image was then divided by the amount of *omlA* transcript (an internal standard which did not vary with culture conditions; Ochsner *et al.*, 1999) to normalize the values to amount of RNA loaded per lane. In this way, relative amounts of *pvcAB* transcript from lane to lane in a given gel could be compared.

2.11 Growth determination and pyoverdine quantification

An overnight culture of *P. aeruginosa* grown in 5 mL of iron-limited succinate medium was centrifuged at 3500 xg and then resuspended in 2 mL of fresh iron-limited succinate medium. This resuspended cell culture was then diluted to an A_{600} value of 0.075 in 15-25 mL of fresh iron-limited succinate medium and grown at 37°C at 190 rpm in a 250 mL flask. One millilitre of culture was removed every hour from 4 to 9 hours and at 22.5 hours post inoculation. Values for A_{600} of the culture and A_{405} of the supernatant were measured using a Beckman series DU-650 spectrophotometer (Beckman, Palo Alto, CA) to measure cell density and pyoverdine production, respectively. The absorbance readings were then graphed to show amount of pyoverdine produced with respect to cell density over the course of growth of the strain.

CHAPTER 3: RESULTS

3.1 Construction of an FpvA knockout in *P. aeruginosa*

Given the homology of FpvA to the PupB receptor of *P. putida* and the fact that both receptors have N-terminal extensions predicted to be located in the periplasm, it is possible that FpvA mediates signals from the environment to genes within *P. aeruginosa*. In order to assess this, a *P. aeruginosa* strain was constructed that contained a stable chromosomal deletion within the *fpvA* gene yielding a lack of FpvA expression. To this end, a 4.6 kb fragment containing the *fpvA* gene sequence with an internal deletion of 198 bp was cloned from pTK608 into the *Sph*I site of pK18*mobsacB*. The construct, pAM1, was then transformed into *E. coli* K340 such that it could be conjugated into *P. aeruginosa* strains.

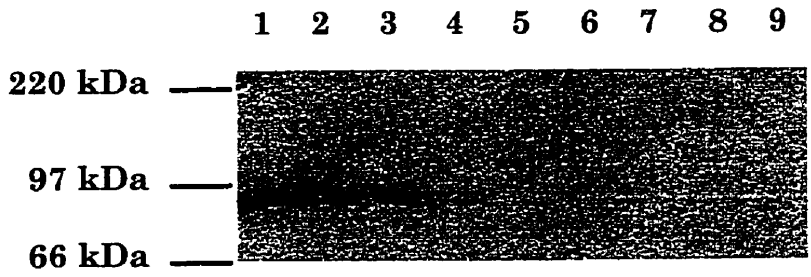
A conjugation between *E. coli* K340 (pAM1) and K1120, a kanamycin sensitive *P. aeruginosa* PAO1 strain, yielded approximately 1000 *P. aeruginosa* pAM1-containing (i.e. kanamycin resistant) colonies from the undiluted conjugation mixture and 20 colonies from a 1/100 diluted conjugation mixture. These colonies were expected to have the plasmid integrated in the chromosome since the kanamycin resistant phenotype was displayed and pAM1 could not otherwise replicate in *P. aeruginosa*. Two colonies were picked from the kanamycin plate and streaked for single colonies onto LB plates containing either 10% (w/v) sucrose or kanamycin. Twenty-four single colonies were subsequently picked from the sucrose plate

(to select for loss of pAM1) and patched onto LB agar containing kanamycin and LB agar alone. All 24 colonies showed growth on LB alone but not on kanamycin consistent with the loss of pAM1 in all cases. To check for the presence of the deletion in the *fpvA* gene, these were then tested by phenotypic assay on minimal media with or without 150 μg/mL EDDHA (FpvA-deficient strains cannot grow with EDDHA). Outer membranes from 8 clones that showed no growth in the presence of EDDHA were isolated for use in a Western immunoblot using anti-FpvA anti-serum (Fig. 6). One of the six clones showing a definite lack of FpvA expression (K1183; Fig. 6, lane 8) was then used in future work.

3.2 Pyoverdine production in FpvA$^+$ and ΔFpvA strains of *P. aeruginosa*

Preliminary examination of *P. aeruginosa* K1183 growing on iron-limited succinate agar plates revealed that the strain was less fluorescent (under UV light) than its parent, *P. aeruginosa* K1120. In order to assess whether pyoverdine production was reduced in an FpvA deficient strain such as *P. aeruginosa* K1183, the A_{405} of culture supernatants [and the cell density (A_{600})] was measured over the course of several hours of growth under iron-limited conditions and compare with *P. aeruginosa* K1120. *P. aeruginosa* K1120 (FpvA$^+$) showed an ever increasing production of pyoverdine with respect to cell density (A_{405}/A_{600}) (i.e. per cell) over the course of the assay

Figure 6: Confirmation of the FpvA-deficient phenotype. Western immunoblotting with anti-FpvA anti-serum was carried out on outer membrane preparations from *P. aeruginosa* in iron-limited succinate medium (transconjugants in lanes 4-9 showed the FpvA-deficient phenotype: a lack of growth on EDDHA-containing iron-deficient plates). Lane 1, *P. aeruginosa* K1120 (wild-type); lanes 2 and 3, transconjugants showing FpvA expression; lanes 4-9, transconjugants showing no FpvA expression

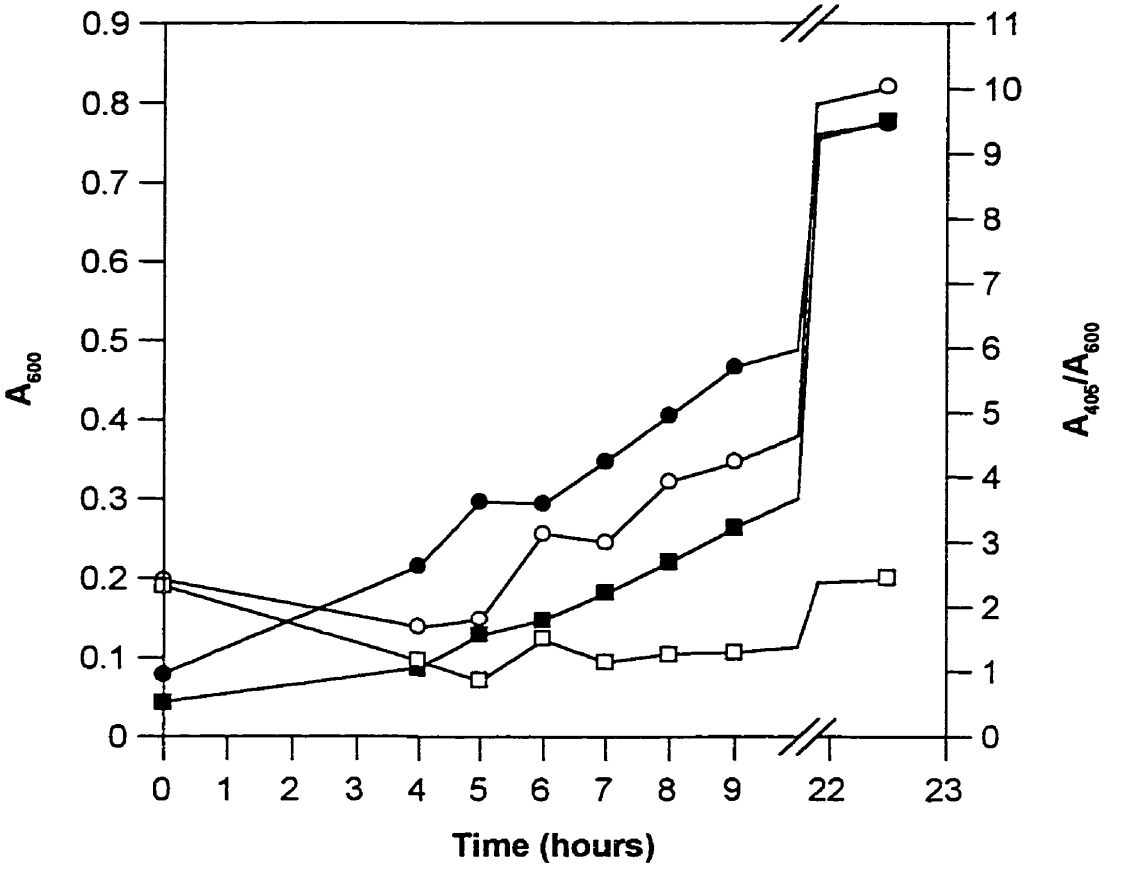

1 2 3 4 5 6 7 8 9
220 kDa
97 kDa
66 kDa

(Fig. 7). The highest amount of pyoverdine production was reached after 22.5 hours of growth with an A_{405}/A_{600} ratio of 10 (Fig. 7). Pyoverdine production when normalized for culture density (A_{405}/A_{600}) for *P. aeruginosa* K1183 never reached more than an A_{405}/A_{600} value of 2.4 (Fig. 7). This A_{405}/A_{600} value for *P. aeruginosa* K1183, reached at 22.5 hours post-inoculation, was 4-fold less than that of *P. aeruginosa* K1120 even though both strains have almost identical culture densities (Fig. 7). These observations indicate that the FpvA-deficient strain (*P. aeruginosa* K1183) exhibits a marked reduction in pyoverdine production, consistent with the hypothesis that FpvA plays a positive role in pyoverdine biosynthesis.

3.3 Transcriptional studies of *pvdD* in *P. aeruginosa* FpvA$^+$ and FpvA$^-$ strains using β-galactosidase assays

The homology of FpvA and the ferric-pseudobactin receptor, PupB, of *P. putida* as well as a correlation between lack of FpvA and pyoverdine deficiency (section 3.2) suggested that FpvA may be involved in a signal transduction pathway regulating pyoverdine biosynthetic gene expression. Since two main loci (*pvd* and *pvc*) are implicated in pyoverdine biosynthesis, the influence of FpvA status on pyoverdine production was initially assessed using *pvdD-lacZ* and *pvcA-lacZ* reporter fusions. The *pvdD* gene encodes a peptide synthetase involved in the synthesis of the peptide moiety of

Figure 7: Growth (A_{600}; filled symbols) and pyoverdine production with respect to culture density (A_{405}/A_{600}; open symbols) over time for *P. aeruginosa* strains K1120 ($FpvA^+$; circles) and K1183 ($FpvA^-$; squares). Cultures were grown for 9 hours in iron-limited succinate medium. Data are representative of 3 experiments.


0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
A_{600}
11
10
9
8
7
6
5
4
3
2
1
0
A_{405}/A_{600}
0 1 2 3 4 5 6 7 8 9 22 23
Time (hours)

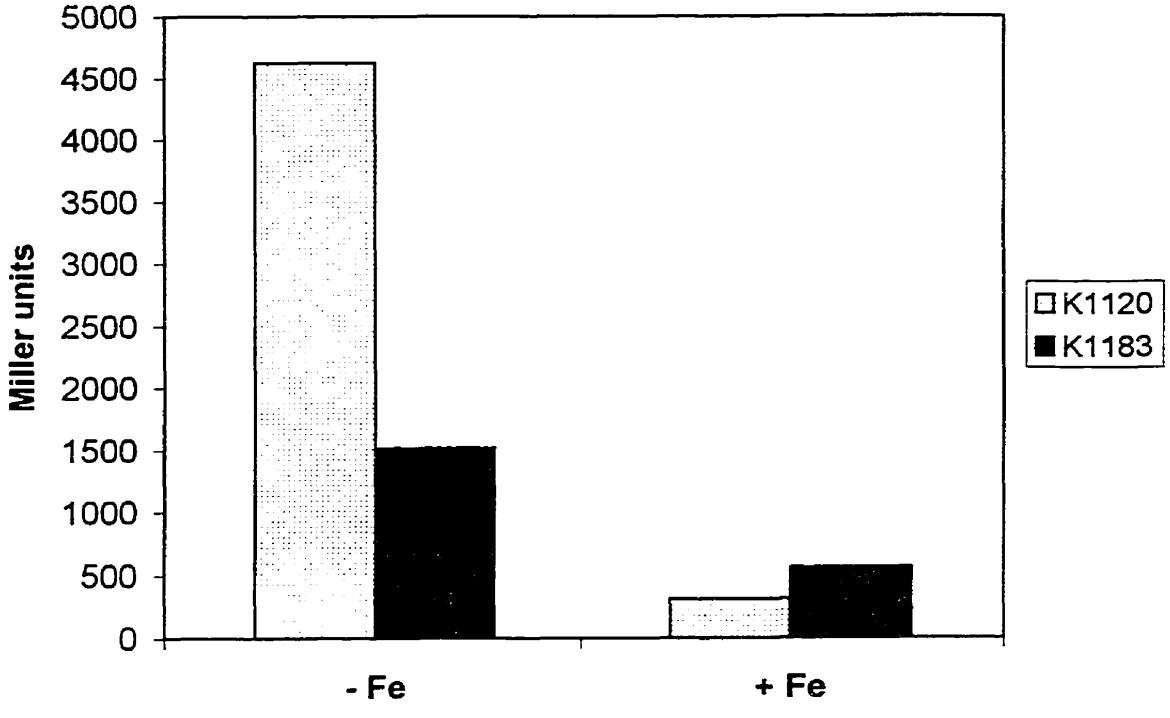
pyoverdine (Merriman *et al.*, 1995) and is expected to be representative of the genes in the *pvd* locus. The *pvcA* gene is the first in the operon that encodes the enzymes required for the biosynthesis of the chromophore of pyoverdine (Stintzi *et al.*, 1996 and submitted).

As expected, substantial enhancement (~15-fold) of *pvdD-lacZ* activity was seen under iron-limited conditions for *P. aeruginosa* K1120 as compared to iron-replete conditions (Fig. 8). In contrast, *P. aeruginosa* K1183 showed a markedly smaller increase (~3-fold) in *pvdD-lacZ* activity under iron-limited conditions versus iron-replete conditions (Fig. 8). As a result, a 3-fold decrease in *pvdD-lacZ* expression for *P. aeruginosa* K1183 was seen under iron-limited conditions relative to *P. aeruginosa* K1120 (Fig. 8). Thus, the FpvA deficiency of *P. aeruginosa* K1183 correlates with a reduction in *pvdD* gene expression, again consistent with a decrease in pyoverdine biosynthesis.

3.4 Transcriptional studies of *pvc* in *P. aeruginosa* FpvA$^+$ and FpvA$^-$ strains using RNase protection assays

The *pvcA-lacZ* fusion failed to show iron-regulated expression and consistently produced very low β-galactosidase activity (data not shown). To assess *pvc* expression, then, RNase protection assays were carried out in *P. aeruginosa* strains K1120 (FpvA$^+$) and K1183 (FpvA$^-$) using a *pvcAB* probe.

Figure 8: β-galactosidase activity of *P. aeruginosa* strains K1120 ($FpvA^+$) and K1183 ($FpvA^-$) containing the *pvdD-lacZ* fusion vector pMP190::P*pvdD*. Cultures were grown in iron-limited succinate medium containing 100 μg/mL chloramphenicol with (+Fe) or without (-Fe) 200 μM $FeCl_3$. Data are representative of 5 assays performed in triplicate. No more than 10% error was observed between replicates.


5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
Miller units
- Fe
+ Fe
K1120
K1183

To this end, RNA was isolated by hot-phenol extraction (Fig. 9) and was probed with a ^{32}P-labelled riboprobe, *pvcAB*. The expected 448 bp and 242 bp major protected fragments from probing with *pvcAB* and *omlA* (the internal control, see section 2.10), respectively, were observed in the RNase protection assays (see Fig. 10). Increased expression (by 10-fold) of *pvcAB* in *P. aeruginosa* K1120 (FpvA$^+$) was seen under conditions of iron-limitation versus iron-replete conditions (Fig. 10), indicating that the *pvc* operon is iron-regulated. In contrast, *P. aeruginosa* K1183 (FpvA$^-$) showed no increase in *pvcAB* expression under iron-limited conditions (Fig. 10). The higher level of *pvcAB* expression seen in *P. aeruginosa* K1183 relative to *P. aeruginosa* K1120 under iron-replete conditions may reflect some iron-limitation of the FpvA-deficient strain even in iron-replete media. In the end, a 3-fold increase was seen in *pvcAB* expression in *P. aeruginosa* K1120 as compared to *P. aeruginosa* K1183 under iron-limited conditions (Fig. 10). The decrease in expression of the *pvc* operon in an FpvA-deficient *P. aeruginosa* strain underlines the relationship between pyoverdine biosynthesis and the presence of the receptor in the outer membrane.

Figure 9: RNA isolated from *P. aeruginosa* by hot phenol extraction. A 0.8% agarose gel with 1 μg of RNA per lane. RNA was isolated from *P. aeruginosa* K1120 (lanes 1-3) and *P. aeruginosa* K1183 (lanes 4,5) from cultures that were iron-limited (lanes 1,4), iron-replete (lanes 2,5) and iron-limited with added pyoverdine (lane 3).

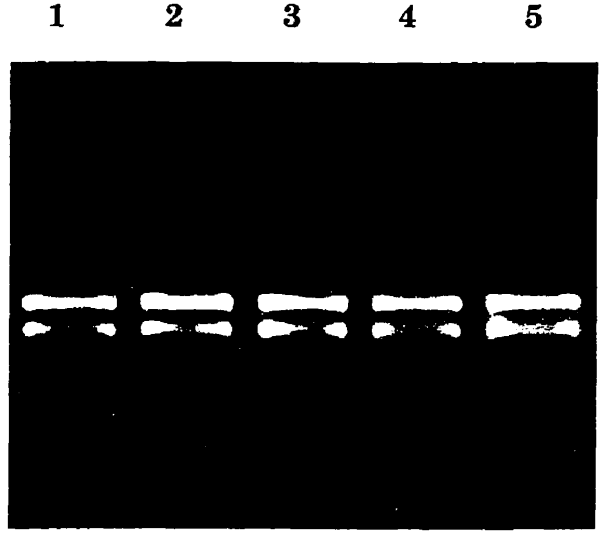

1
2
3
4
5

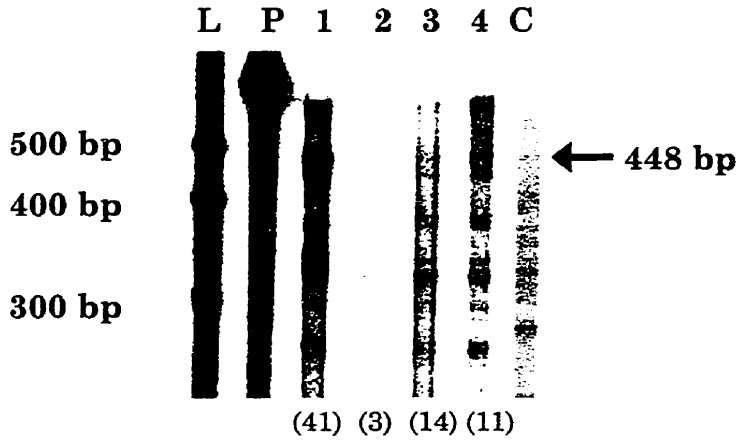
Figure 10: RNase protection assays of *P. aeruginosa* strains K1120 ($FpvA^+$) (lanes 1 and 2) and K1183 ($FpvA^-$) (lanes 3 and 4). RNA was extracted after 12 hours of growth from cultures grown in D-TSB (lanes 1,3) and D-TSB + 200 μM $FeCl_3$ (lanes 2,4).

a) RNA probed with *pvcAB* (the major protected fragment is at 448 bp). The numbers in parentheses under each lane represent the relative intensities of the major protected band normalized against *omlA* as described in section 2.10.

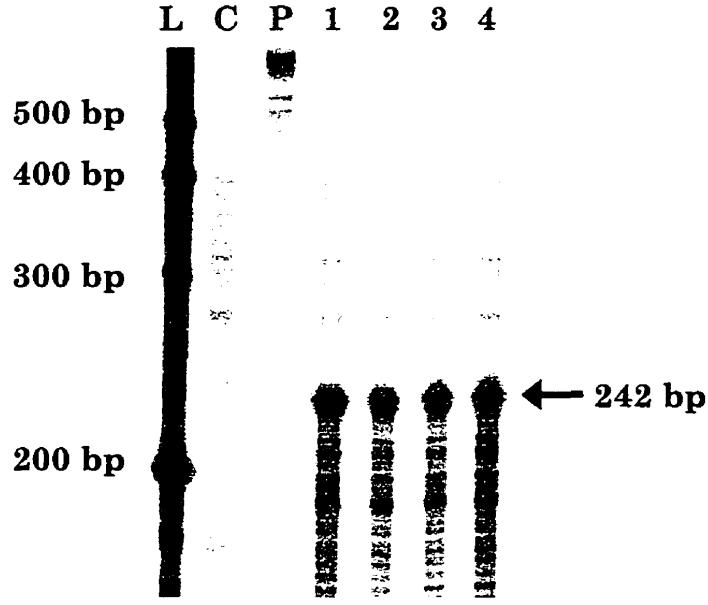
b) RNA probed with *omlA* (the major protected fragment is at 242 bp).

L, RNA size ladder; P, undigested probe; C, digested probe

a)


L P 1 2 3 4 C
500 bp
400 bp
300 bp
448 bp
(41) (3) (14) (11)

b)


L C P 1 2 3 4
500 bp
400 bp
300 bp
200 bp
242 bp

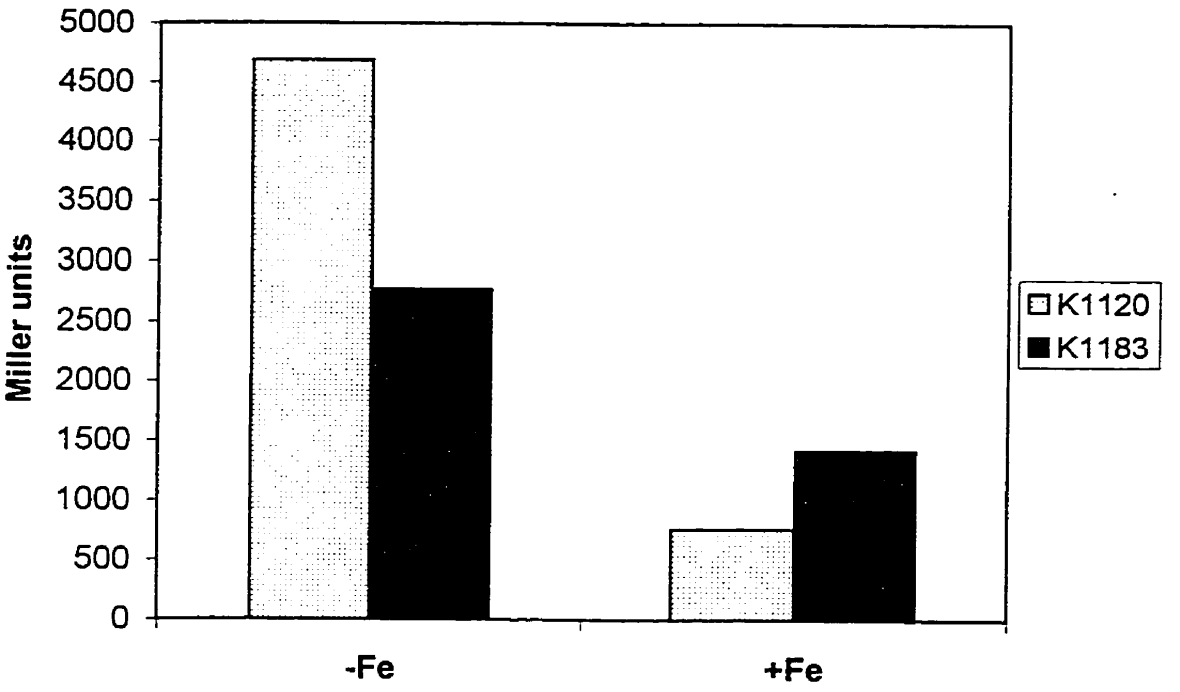
3.5 Transcriptional analysis of *fpvA* in *P. aeruginosa* FpvA$^+$ and FpvA$^-$ strains using β-galactosidase assays

Since PupB regulates its own expression, the possibility that FpvA similarly regulates *fpvA* expression was investigated. *P. aeruginosa* strains K1120 (FpvA$^+$) and K1183 (FpvA$^-$) harbouring pEJB3 (*fpvA-lacZ*) were grown in iron-limited and iron-replete conditions. As expected for a negatively iron-regulated gene, expression of the *fpvA-lacZ* fusion under iron-limited conditions was higher for both *P. aeruginosa* K1120 and K1183 (6-fold and 2-fold, respectively) than under iron-replete conditions (Fig. 11). The higher expression of *fpvA-lacZ* in *P. aeruginosa* K1183 compared to *P. aeruginosa* K1120 under iron-replete conditions may reflect some iron limitation of the former strain as a result of the absence of the ferric-pyoverdine receptor. Still, *P. aeruginosa* K1183 showed almost a 2-fold lower expression of *fpvA-lacZ* as compared to *P. aeruginosa* K1120 (Fig. 11) suggesting that FpvA might have a modest effect on *fpvA* gene expression.

3.6 Construction of a PvdD knockout in *P. aeruginosa*

In the FecA and PupB systems of *E. coli* and *P. putida*, respectively, ferric-siderophore binding to the receptor induces upregulation of the genes

Figure 11: β-galactosidase activity of *P. aeruginosa* strains K1120 ($FpvA^+$) and K1183 ($FpvA^-$) containing the *fpvA-lacZ* fusion vector pEJB3. Cultures were grown in iron-limited succinate medium containing 25 μg/mL chloramphenicol with (+Fe) or without (-Fe) 200 μM $FeCl_3$. Data are representative of 3 assays performed in triplicate. No more than 10% error was seen between replicates.


5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
Miller units
-Fe
+Fe
K1120
K1183

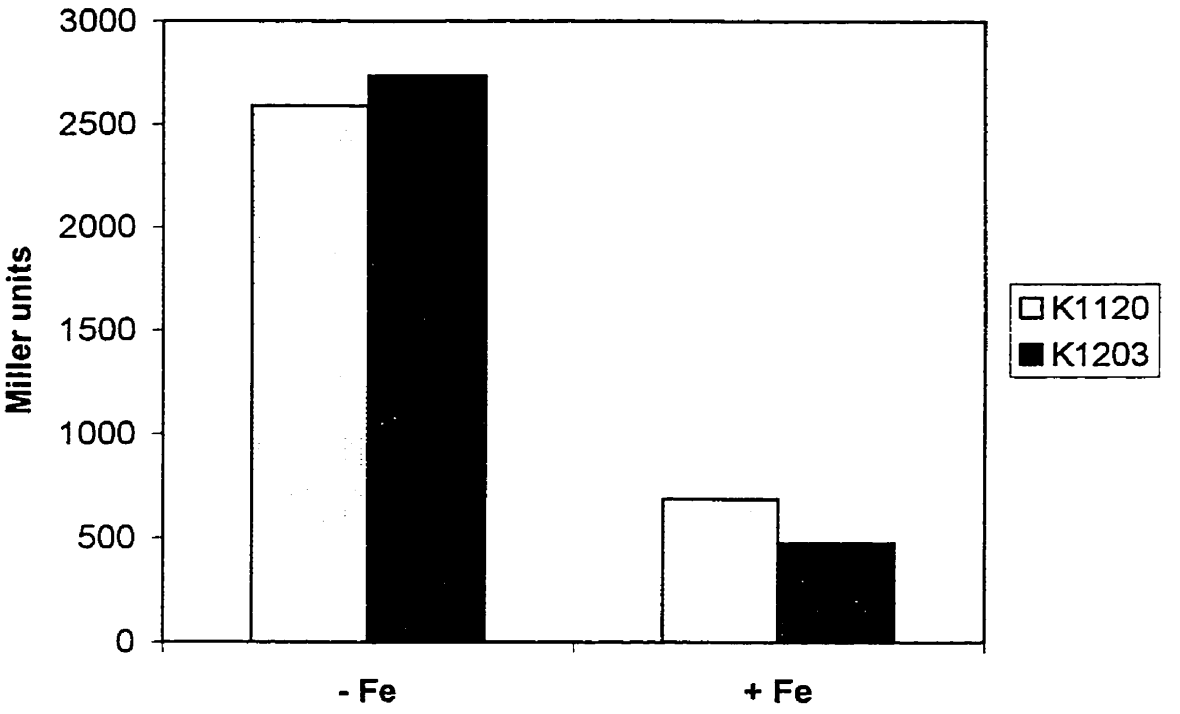
encoding the outer membrane receptor. Thus, the possibility exists that ferric-pyoverdine binding to its outer membrane receptor causes a signal to be mediated to the pyoverdine biosynthetic genes. A pyoverdine-deficient strain was, therefore, constructed, such that any effect ferric-pyoverdine had could be monitored by the addition of exogenous pyoverdine to the growth medium. The *pvdD* gene has been shown to be required for the production of pyoverdine and, so, an attempt was made to construct a *pvdD* deletion strain. The plasmid pSUP202 harbouring the *pvdD* gene containing a 6.4 kb deletion (pSUP202::Δ*pvdD*; from I. Lamont) was transformed into *E. coli* K340 such that it could be conjugated into *P. aeruginosa*. A conjugation between *E. coli* K340 (pSUP202::Δ*pvdD*) and *P. aeruginosa* K1120 yielded greater than 1000 pSUP202::Δ*pvdD*-containing (i.e. tetracycline resistant) colonies from the undiluted conjugation mixture and 137 colonies from a 1/100 diluted conjugation mixture. Three colonies were chosen and inoculated separately into 5 mL of LB broth and grown overnight. Each day over the course of 6 days, 5 μL of the overnight cultures were re-inoculated into 5 mL of fresh LB broth. This allowed for homologous recombination to occur between the deleted *pvdD* gene on the plasmid and the wild-type *pvdD* gene in the chromosome. Thirty-seven tetracycline sensitive clones, considered to no longer have the plasmid in their chromosome, were isolated and expected to contain either wild-type or deleted *pvdD*. The lack of the *pvdD* gene has been shown to correlate with lack of pyoverdine and, thus, lack of fluorescence

under UV light when grown on an iron-limited medium. So, *pvdD* deletion mutants were identified as non-fluorescent colonies on iron-depleted succinate medium. Eighteen of 37 tetracycline sensitive clones showed no fluorescence and one of these, dubbed *P. aeruginosa* K1203, was used in subsequent gene regulation studies.

3.7 Transcriptional analysis of *pvdD* in pyoverdine-deficient and pyoverdine-sufficient *P. aeruginosa* strains using β-galactosidase assays

If FpvA functions like PupB, it is expected that its signalling to the pyoverdine biosynthetic genes will involve ferric-pyoverdine binding to FpvA. Studies were undertaken, therefore, to assess if the presence or absence of pyoverdine had an impact on the expression of the *pvdD* and *pvcA* biosynthetic genes. Thus, the *pvdD-lacZ* fusion (pMP190::P*pvdD*) was introduced into *P. aeruginosa* K1120 (*pvdD*$^+$) and a pyoverdine deficient (*pvdD*$^-$) derivative, *P. aeruginosa* K1203, so that the influence of pyoverdine on *pvdD* expression could be assessed under iron-limited and iron-replete conditions. As expected, a significant increase in *pvdD-lacZ* expression was seen under iron-limited conditions as compared to iron-replete conditions for both *P. aeruginosa* K1120 (5- to 10-fold) and K1203 (15- to 20-fold; Fig. 12). Still, under iron limited conditions, *P. aeruginosa* K1120 (*pvdD*$^+$) never showed more than 1.5-fold more *pvdD-lacZ* activity as compared with *P.*

Figure 12: β-galactosidase activity of *P. aeruginosa* strains K1120 (*pvdD*$^+$) and K1203 (*pvdD*$^-$) containing the *pvdD-lacZ* fusion vector pMP190::P*pvdD*. Cultures were grown in iron-limited succinate medium containing 100 μg/mL chloramphenicol with (+Fe) or without (-Fe) 200 μM $FeCl_3$. Data are representative of 3 assays performed in triplicate. No more than 10% error was seen between replicates.


3000
2500
2000
1500
1000
500
0
Miller units
- Fe
+ Fe
K1120
K1203

aeruginosa K1203 (*pvdD*-) (Fig. 12) indicating that ferric-pyoverdine likely plays no role in the expression of *pvdD*. Moreover, the apparent involvement of FpvA in *pvdD* expression (Fig. 8) is independent of ferric-pyoverdine.

3.8 Transcriptional analysis of the *pvc* operon in pyoverdine-deficient and pyoverdine-sufficient *P. aeruginosa* strains using RNase protection assays

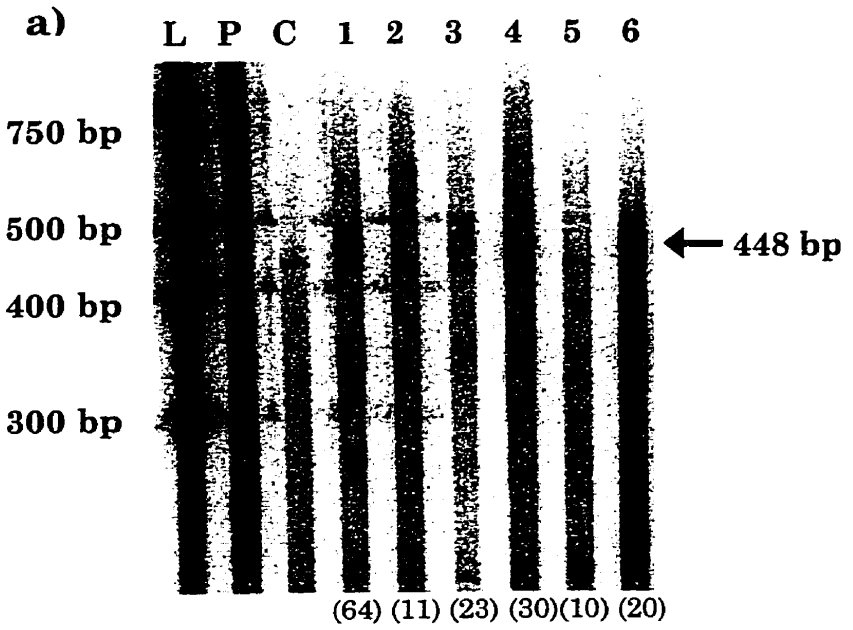
RNA extracted from *P. aeruginosa* K1120 (pyoverdine-sufficient) and *P. aeruginosa* K1203 (pyoverdine-deficient) strains grown under iron-limited and iron-replete conditions was used in RNase protection assays to assess the level of *pvcAB* expression in the presence and absence of pyoverdine. As expected, the level of *pvcAB* expression increased 6-fold under conditions of iron-limitation relative to iron-sufficiency for *P. aeruginosa* K1120 (Fig. 13). A significant increase in expression (3-fold) was also seen for *P. aeruginosa* K1203 under iron-limited as compared to iron-replete conditions (Fig. 13). *P. aeruginosa* K1120 showed approximately 2-fold higher expression of *pvcAB* than *P. aeruginosa* K1203 under iron-limited conditions (Fig. 13). The difference in *pvcAB* expression is postulated to be due to the lack of pyoverdine production by *P. aeruginosa* K1203. So, exogenous pyoverdine was added to see if the reduction in *pvcAB* expression could be reversed in *P. aeruginosa* K1203. Unexpectedly, a slight decrease (1.5-fold) in *pvcAB*

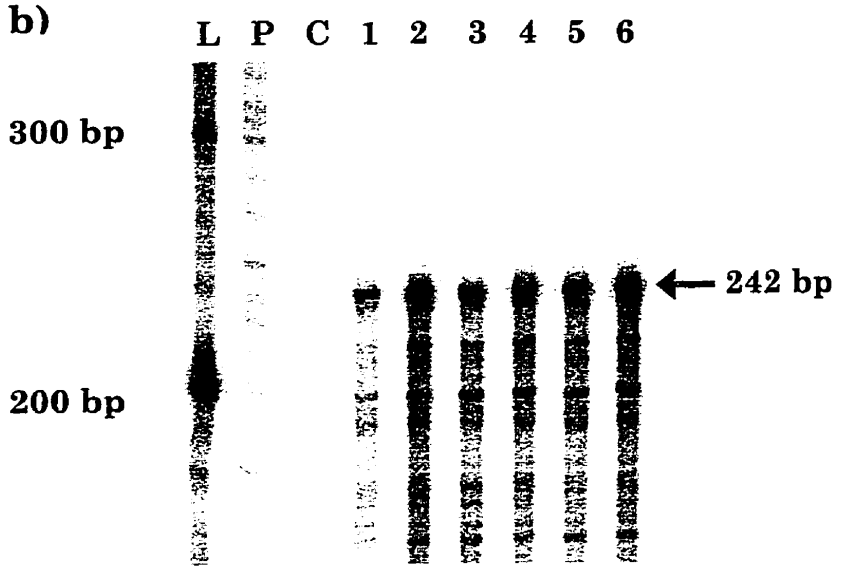
Figure 13: RNase protection assays of *P. aeruginosa* strains K1120 (*pvdD*$^+$) (lanes 1-3) and K1203 (*pvdD*$^-$) (lanes 4-6). RNA was extracted after 12 hours of growth from cultures grown in D-TSB (lanes 1 & 4), D-TSB + 200 μM $FeCl_3$ (lanes 2 & 5) and D-TSB + 50 μg/mL pyoverdine (lanes 3 & 6).

a) RNA probed with *pvcAB* (the major protected fragment is 448 bp). The numbers in parentheses under each lane represent the relative intensities of the major protected band normalized against *omlA* as described in section 2.10.

b) RNA probed with *omlA* (the major protected fragment is 242 bp).

L, RNA size ladder, P, undigested probe, C, digested probe


a)
L P C 1 2 3 4 5 6
750 bp
500 bp
400 bp
300 bp
448 bp
(64) (11) (23) (30)(10) (20)


b)
L P C 1 2 3 4 5 6
300 bp
200 bp
242 bp

expression was observed in *P. aeruginosa* K1203 upon the addition of pyoverdine under iron-limited conditions As well, a 3-fold decrease in *pvcAB* expression was seen with the addition of pyoverdine as compared to iron-limited conditions for *P. aeruginosa* K1120 (Fig. 13). Thus, addition of exogenous pyoverdine to the growth medium does not appear to restore wild-type *pvcAB* expression to the pyoverdine-deficient strain.

CHAPTER 4: DISCUSSION

Receptor-mediated signalling has been demonstrated in the ferric iron uptake systems PupB and FecA in *P. putida* and *E. coli*, respectively (Koster *et al.*, 1994, Kim *et al.*, 1997). In both cases, binding of a heterologous iron-uptake molecule (ferric-pseudobactin or ferric-dicitrate) to the receptor, under low iron conditions, transmits a signal via the N-terminus of the receptor to a cytoplasmic membrane associated protein. This is followed by release of an ECF sigma factor which in turn upregulates receptor, *pupB* or *fecA*, gene expression. Thus, the cells save energy by expressing the receptor only under conditions when it will be necessary. The homology of PupB to the ferric-pyoverdine receptor, FpvA, in *P. aeruginosa* and the fact that *fpvA* mutants show a deficiency in pyoverdine production led to the hypothesis that FpvA is involved in receptor-mediated signalling, most likely to a gene or possibly several genes involved in pyoverdine biosynthesis. Consequently, the goal of this work was to assess the role of the ferric-pyoverdine receptor, FpvA, in the expression of the pyoverdine biosynthetic genes at the level of *pvdD* and the *pvc* operon.

P. aeruginosa synthesizes two endogenous siderophores, pyoverdine and pyochelin. Each is expected to act as the ideal iron chelator under specific conditions. Thus, upregulation of the biosynthetic genes for only that siderophore which is successful in obtaining iron in a given environment, acts as an energy saving for the cells that would otherwise synthesize high levels

of both siderophores under iron-limiting conditions. Such regulation has already been demonstrated for pyochelin where high ferric-pyochelin levels in the environment induce the activator protein, PchR, to activate transcription of the pyochelin biosynthetic genes (Heinrichs and Poole, 1993, Serino *et al.*, 1997, Reimmann *et al.*, 1998). In the case of pyoverdine, successful pyoverdine chelation of ferric iron would generate ferric-pyoverdine molecules which would bind to the outer membrane receptor, FpvA, and be transported into the cell. A signal, induced by ferric-pyoverdine binding to the receptor, would be transduced via FpvA to the pyoverdine biosynthetic genes causing upregulation of these genes. It would follow, then, that the deletion of the *fpvA* gene causes reduced production of pyoverdine in *P. aeruginosa* strains. This has, in fact, been observed in a number of *fpvA* null mutants (Poole, unpublished observations). In a previous study, when a deletion was introduced into the *fpvA* gene, pyoverdine production appeared to be completely abolished with the inactivation of FpvA (e.g. *P. aeruginosa* K979; Table A, Appendix 1). The parent in this case, *P. aeruginosa* K337 (Table A, Appendix 1), is a low pyoverdine producer. Thus, the decrease in pyoverdine production seen in *P. aeruginosa* K979 was probably manifested as a complete loss of detectable fluorescence (i.e. pyoverdine) under UV light when the strain was grown on iron-limited media. In contrast, the FpvA-deficient strain used in this study, *P. aeruginosa* K1183, derived from *P. aeruginosa* K1120, a high pyoverdine producer, showed visible pyoverdine production as

fluorescence under UV light though it was at a lower level relative to *P. aeruginosa* K1120 (FpvA$^+$). Furthermore, it is expected that the genes in the *pvd* and *pvc* loci would be coordinately regulated since they are all required for the synthesis of pyoverdine. Thus, it is not surprising a lack of FpvA should have the same effect on both loci as observed by the comparable decrease in expression (3-fold) of both *pvdD* and *pvcAB* in *P. aeruginosa* K1183 relative to *P. aeruginosa* K1120.

A model of the FpvA signal transduction mechanism can be put forth that mimics that of PupB and FecA from *P. putida* and *E. coli*, respectively (sections 1.5.5.2.1 and 1.5.5.2.2). It is expected that ferric-pyoverdine binding to FpvA would induce a conformational change in the receptor that allows the N-terminal region to come into contact with a regulator protein, tentatively termed PvdR, which is expected to be a homologue of PupR and FecR. Physical interaction between PvdR and FpvA would, in turn, induce a conformational change in PvdR, which allows the release of an ECF sigma factor, provisionally termed PvdI, likely a homologue of PupI and FecI. PvdI would then encourage RNA polymerase binding to the promoters of the pyoverdine biosynthetic genes, inducing upregulation of pyoverdine production. Homologues of both PupR/FecR and PupI/FecI have been discovered within the *pvd* locus of the *P. aeruginosa* genome (Pseudomonas Genome Project, 1999). One of these PupI/FecI homologues, PvdS (Cunliffe *et al.*, 1995, Miyazaki *et al.*, 1995), is known to have a positive effect on genes in

the *pvd* locus (Leoni *et al.*, 1996, Ochsner *et al.*, 1996) and the *pvc* operon (Stintzi *et al.*, submitted) but investigations into its role in FpvA signal transduction did not show conclusively that PvdS expression is affected by FpvA (Vrionis, 1997). A second PupI/FecI homologue, SigR (Pseudomonas Genome Project, 1999), is presently being studied to assess its role in receptor-mediated signal transduction.

In this scenario, ferric-pyoverdine is expected to play a major role in the increase of pyoverdine biosynthesis. A *pvdD* null mutant, *P. aeruginosa* K1203, in contrast to the *fpvA* null mutant, showed only slight differences in expression of the pyoverdine biosynthetic genes. *P. aeruginosa* K1203 still expresses an intact and fully functional FpvA gene. So, perhaps, FpvA continues to produce a stimulatory effect on pyoverdine production independently of ferric-pyoverdine binding. On the other hand, since only one gene, *pvdD*, has been deleted in the pyoverdine biosynthetic pathway, perhaps a metabolite of pyoverdine is binding iron and inducing FpvA more weakly than the ferric-pyoverdine molecule.

The addition of pyoverdine to the growth medium in the RNase protection assays was expected to reverse the decline in *pvc* expression seen in *P. aeruginosa* K1203 (pyoverdine-deficient) as compared to *P. aeruginosa* K1120 (pyoverdine-producing) under iron limiting conditions. In fact, lower levels of expression of *pvc* were seen for both *P. aeruginosa* K1203 and *P. aeruginosa* K1120 with the addition of pyoverdine. It is possible that the

addition of siderophore (more correctly ferric-siderophore) early in the growth phase of *P. aeruginosa* would lower the iron-limitation sensed by these cells (by providing ferric iron so readily) so that any positive effect expected from ferric-pyoverdine-induced signalling by the receptor is counteracted by higher iron levels (i.e. less iron-limitation). This may, therefore, reflect the complexity of regulation of high-affinity iron uptake systems, where multiple signals play a role in pyoverdine biosynthetic gene expression.

In both PupB of *P. putida* and FecA of *E. coli*, it is the receptor gene itself which is regulated by receptor-mediated signalling (Koster *et al.*, 1994, Kim *et al.*, 1997). A previously constructed FpvA-deficient strain made by insertional mutagenesis (insertion of an ΩTc cartridge) showed no expression of the *fpvA-lacZ* fusion under iron-limited conditions (Poole *et al.*, 1996a) indicating similar receptor-dependent receptor gene expression for FpvA. In the current study, the FpvA-deficient strain, *P. aeruginosa* K1183, carries a small (198 bp) internal deletion, and expresses an *fpvA-lacZ* fusion at levels comparable to wild-type (*P. aeruginosa* K1120). This unexpected result might be due to the production of a C-terminally truncated FpvA receptor protein in *P. aeruginosa* K1183 that is non-functional with respect to iron uptake and unstable such that it degrades upon cell fractionation. This protein, though not detected by Western immunoblots in disrupted cells, could potentially bind ferric-pyoverdine *in vivo* and signal to the *fpvA* gene. At present, a null mutant lacking the full *fpvA* gene is being constructed to

unequivocally assess the role of FpvA in *fpvA* expression. If a partially functional FpvA was present in *P. aeruginosa* K1183, this may mean that the effect of the *fpvA* deletion was only partially felt by *pvdD* and *pvcAB*. Thus, larger differences in *pvdD* and *pvcAB* expression may be observed between *P. aeruginosa* wild-type and a completely deleted *fpvA* null mutant strain.

In summary, the ferric-pyoverdine outer membrane receptor, FpvA, in *P. aeruginosa* plays a positive role in the pyoverdine production by cells grown under iron-limiting conditions. Similarly, the genes encoding the enzymes involved in the biosynthesis of both the peptide moiety, the *pvd* locus, and the chromophore, the *pvc* operon, of pyoverdine are both affected by a lack of FpvA expression. These observations support the hypothesis that signalling mediated by FpvA affects the genes encoding the proteins required for pyoverdine production. However, the hypothesized ferric-pyoverdine requirement for this signalling could not be demonstrated by the methods employed in this study. The contrasting regulation of pyoverdine production (negatively by iron and positively by ferric-siderophores) likely renders it difficult to distinguish between the effects of iron versus ferric-siderophore availability on pyoverdine biosynthesis, at least in the strains examined here.

Suggested future work:

The growth rate of mutant strains [*P. aeruginosa* K1183 (FpvA$^-$) and *P. aeruginosa* K1203 (*pvdD*$^-$)] and the parental strain (*P. aeruginosa* K1120) was not always identical in the iron-limited medium. This would cause difficulties if any of the signalling that took place is cell density dependent. These assays should be performed again over the full growth cycle of the strains to see if growth phase does, in fact, affect the expression of the *fpvA*, *pvdD* and *pvc* genes.

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APPENDIX I: LIGAND BINDING IN THE FERRIC-PYOVERDINE RECEPTOR, FpvA, OF *Pseudomonas aeruginosa*

A. INTRODUCTION

The ability of an organism to scavenge iron *in vivo* appears to enhance the virulence of the organism. With the rise of *Pseudomonas aeruginosa* infections in nosocomial settings, coupled with its multiple antibiotic resistance, novel therapies would be helpful in preventing morbidity and mortality of *P. aeruginosa* infections. Such novel therapies could be aimed, for example, at iron transport mechanisms. By studying the ferric pyoverdine receptor, FpvA, and elucidating its functional mechanisms, one would hope to bring about the knowledge required to develop such new treatment options. Previous work has identified the region between tyrosine-350 and alanine-402 of FpvA as a putative binding region for ferric-pyoverdine (Kilburn, 1997). This study had as a goal to further define specific amino acid residues required for ferric pyoverdine binding to FpvA using random mutagenesis of the putative binding region. To facilitate this, unique restriction sites were engineered flanking the putative ferric-pyoverdine binding region in FpvA. This would permit excision, random mutagenesis and replacement of this region alone.

B. MATERIALS AND METHODS

B.1 Bacterial strains and plasmids

The bacterial strains and plasmids used in this study are highlighted in Tables A and B, respectively.

B.2 Growth conditions

See section 2.2.

B.3 DNA preparation and visualization

See section 2.3.

B.4 DNA digestion, isolation of fragments from agarose gels and ligations

See section 2.4.

B.4.1 T4 DNA polymerase treatment

Previously digested DNA containing a 3-prime overhang was treated with 1 unit of T4 DNA polymerase (New England Biolabs, Beverly, MA) per μg of DNA in the buffer provided by the enzyme distributor. The reaction mixture was supplemented with 50 μg/mL of BSA (New England Biolabs, Beverly, MA) and 100 μM of each deoxyribonucleoside triphosphate (dNTP). The reaction mixture was incubated at 12°C for 20 minutes then heat inactivated by incubation at 75°C for 10 minutes. The newly blunt-ended DNA was then gel purified for use in ligation reactions.

Table A: Bacterial strains†

Strain	Description*	Reference or Source
K337	*P. aeruginosa* ML5087 *ilv, thr, leu, met, pur, aphA* increased sensitivity to Km/Nm	Okii *et al.*, 1983
K979	*P. aeruginosa* K337 Δ*fpvA* stable *fpvA* deletion created by gene replacement using pK18*mobsacB*	This study
K1212	*E. coli* XL1-Blue MRF' Δ(*mcrA*)*183* Δ(*mcrCB-hsdSMR-mrr*)*173 endA1 supE44 thi-1 recA1 gyrA96 relA1* *lac*[F' *proAB lacI*^{q}*ZΔM15 Tn10*(Tc^{r})] Tc resistant	Stratagene

† Other strains used in this study were previously listed in Table 1 (p. 34).

* Km=kanamycin, Nm= neomycin, Tc=tetracycline

Table B: Plasmids†

Plasmid	Description*	Reference or Source
pAK1900	cloning vector MCS within *lacZ* gene can use blue/white screen Ap or Cb resistant	A. Kropinski
pAM4	PCR-amplified modified *fpvA*** gene cloned into *Hin*dIII site of pV1 in the opposite orientation to the *lac* promoter Ap or Cb resistant	This study
pAM5	PCR-amplified modified *fpvA*** gene cloned into *Hin*dIII site of pV1 in the same orientation as the *lac* promoter Ap or Cb resistant	This study
pRK415	low copy number vector *E. coli-P. aeruginosa* shuttle vector Tc resistant	R. E. W. Hancock
pV1	modified pAK1900 lacking MCS except for *Eco*RI and *Hin*dIII sites Ap or Cb resistant	This study
pVEC1	modified pRK415 lacking MCS except for *Eco*RI, *Sph*I and *Hin*dIII sites Tc resistant	This study

† Plasmids used in this study not listed in this table can be found in Table 2 (p. 35).

* Ap=ampicillin, Cb=carbenicillin, Tc=tetracycline, MCS=multiple cloning site

** the *fpvA* gene with unique *Bam*HI and *Sst*I sites on a 2.8 kb *Hin*dIII fragment

B.5 DNA transformation into competent cells

See section 2.5.

B.6 Biparental mating

See section 2.6.

B.7 Cell fractionation, polyacrylamide gel electrophoresis and Western immunoblotting

See section 2.7.

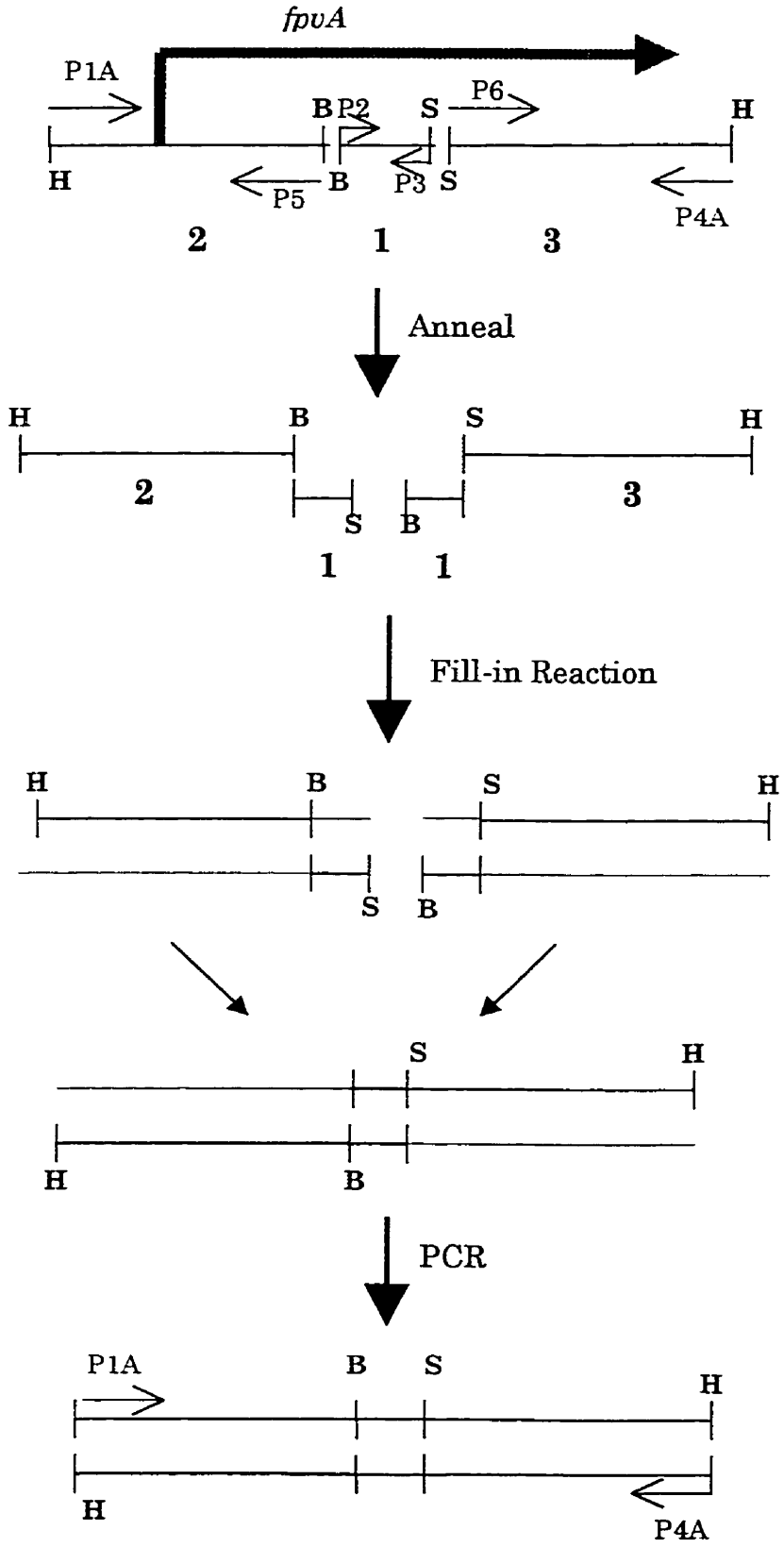
B.8 Polymerase chain reaction (PCR)

PCR was used to produce a full sized *fpvA* gene containing unique *Bam*HI and *Sst*I (*Sac*I) restriction sites flanking the putative ferric-pyoverdine binding region. The primers were made by Cortec DNA Laboratories (Queen's University, Kingston, ON) and used in the MiniCycler thermal controller (MJ Research, Watertown, MA) according to the programs below. All PCR was carried out using the enzyme Vent polymerase (New England Biolabs, Beverly, MA), in the thermopol buffer and $MgSO_4$ packaged with it. Each reaction mixture contained 10 % (v/v) dimethylsulphoxide (DMSO; BDH, Toronto, ON), 1x Thermopol Buffer (New England Biolabs, Beverly, MA), 60 pmol of each primer, 400 μM of each dNTP, 25-50 ng of template DNA, 0.5-1 unit of Vent DNA Polymerase (New England Biolabs, Beverly, MA) and enough sterile deionized water to make the reaction up to 100 μL. It was determined empirically that certain reactions required an

additional 2 mM $MgSO_4$. This requirement is stated with the PCR programs listed below. The reaction mixture was then overlaid with 100 μL mineral oil to prevent evaporation. All PCR products were visualized on 0.8% agarose gels to confirm quality and size of the amplified product. Before digestion with restriction enzymes or use in the overlap extension PCR, amplified fragments were purified using the QIAquick PCR purification kit (QIAGEN, Mississauga, ON).

The PCR fragment containing the unique sites flanking the putative binding region in FpvA was amplified with primers P2 (5'-CCAAGGGATC CGGCTGGTCG-3'), which introduced a *Bam*HI site, and P3 (5'-GGTGCCGA CCACGAGCTCGTGCT-3'), which introduced an *Sst*I site. The amplification was carried out with additional magnesium (2 mM $MgSO_4$) by undergoing heating at 94°C for 6 minutes, 30 cycles of 94°C for 1 minute, 56°C for 1 minute and 72°C for 1 minute followed by a final step of 72°C for 1 minute (Fig. A, fragment 1). The flanking regions of the *fpvA* gene were amplified without added magnesium using the primer pairs P1A (5'-AATTAAGCTTA AGCTTTTCAATGCCTGGCTCGAAGAGCGACCGGTCC-3') and P5 (5'-CGACCAGCCGGATCCCTTGG-3') as well as P6 (5'-AGCACGAGCTC GTGGTCGGCACC-3') and P4A (5'-AATTAAGCTTAAGCTTGGTAGCCGA GAAGCAGAACGCCCCGCATTG-3') in an amplification program of 6 minutes at 94°C, 30 cycles of 94°C for 1 minute, 56°C for 1 minute and 72°C for 2 minutes then a final step of 72°C for 1 minute (Fig. A, fragments 2 and

Figure A: Schematic of overlap extension PCR used to amplify a DNA fragment containing the *fpvA* gene with unique *Bam*HI and *Sst*I (*Sac*I) sites introduced by point mutations in primers P2, P3, P5 and P6. This strategy uses PCR products with complementary ends to act as templates after initial annealing and fill-in reactions.
B, *Bam*HI site; S, *Sst*I (*Sac*I); H, *Hin*dIII; 1, fragment 1 (P2/P3); 2, fragment 2 (P1A/P5); 3, fragment 3 (P6/P4A)


fpvA
P1A
B
P2
S
P6
H
H
B
P5
P3
S
P4A
2
1
3
Anneal
H
B
S
H
2
3
S
B
1
1
Fill-in Reaction
H
B
S
H
S
B
S
H
H
B
PCR
P1A
B
S
H
H
P4A

3, respectively). In later trials, longer flanking regions were used with primer P1A being replaced by primer "long P1" (5'-ACGTAAGCTTAAGCTT CATCCTGGCCAAGACCTTCG-3') and primer P4A being replaced by primer "Fpv-Hind2" (5'-AAGCTTAAGCTTGCAACCT GGCGATGGATGTC-3'). With these primers, the amplification was carried out in the presence of added magnesium (2 mM $MgSO_4$) following these steps: 94°C for 6 minutes, 30 cycles of 94°C for 1 minute, 59°C for 1 minute and 72°C for 2.5 minutes followed by a final step of 72°C for 1 minute.

To finally complete the PCR of the full length *fpvA* gene, the strategy employed was overlap extension PCR where the fragment of interest, *fpvA* containing two point mutations (producing unique *Bam*HI and *Sst*I sites), is amplified from three other DNA fragments (Fig. A, fragments 1, 2 and 3), initially created by PCR, that are allowed to anneal, undergo fill-in reactions and eventually act as a template for the final amplification steps (Ge and Rudolph, 1997; Fig. A). This was carried out by mixing equimolar amounts of fragments 1, 2 and 3 (P2/P3, P1A/P5 and P6/P4A, respectively) in a 100 μL reaction volume containing 10% (v/v) DMSO, 1X Thermopol buffer (New England Biolabs, Beverly, MA), 2 mM $MgSO_4$ (New England Biolabs, Beverly, MA), 400 μM of each dNTP and 1 unit of Vent DNA Polymerase (New England Biolabs, Beverly, MA) and overlaid with 100 μL of mineral oil. The reaction mixture was then allowed to sit at 94°C for 1 minute then cycle 7 times through 94°C for 1 minute, 56°C for 2 minutes and 72°C for 3

minutes. At this point, enough of the fragments had annealed and been filled-in to act as templates for the amplification of the full length *fpvA* gene so 60 pmol each of primers P1A and P4A were added through the mineral oil to the reaction mixture. The amplification program for the full length gene was resumed with 30 cycles of 94°C for 1 minute, 56°C for 2 minutes and 72°C for 3 minutes with a final step of 1 minute at 72°C. A longer amplified fragment containing the *fpvA* gene was amplified using the above procedure except P2/P3, Long P1/P5 and P6/Fpv-Hind2 (corresponding to fragments 1, 2 and 3, respectively in Fig. A) were mixed together as above except that the 72°C step was 4 minutes and 15 seconds long, and 60 pmol each of long P1 and Fpv-Hind2 primers were added once the annealing and fill-in cycles were complete (instead of P1A and P4A in Fig. A). To amplify this longer fragment, the elongation step at 72°C was increased to 4 minutes and 20 seconds long. The resulting fragment has *Hin*dIII sites on both ends for cloning purposes.

C. RESULTS

C.1 FpvA knockouts

Besides the *fpvA* null mutant described in section 3.1, an FpvA knockout of K337, a kanamycin sensitive auxotrophic *P. aeruginosa* strain, was obtained in the same manner for use in this study.

C.2 Site directed mutagenesis of areas flanking putative ferric-pyoverdine binding region in FpvA

To be able to carry out random mutagenesis solely on the putative ferric-pyoverdine binding region, unique restriction enzyme sites were introduced into the *fpvA* gene flanking this region (at serine-317 and leucine-431; Poole *et al.*, 1993). Primers used in PCR contained single (G to C) base changes which introduced unique *Bam*HI (at base pair 2179) and *Sst*I (at base pair 2521) sites upstream and downstream, respectively, of this region (Poole *et al.*, 1993). After purifications, an agarose gel of the three fragments used to amplify the complete *fpvA* gene (Fig. B) was used to confirm the quality and the amount of DNA that was to be used in the overlap extension PCR reaction. The full *fpvA* gene was then amplified using overlap extension PCR (Ge and Rudolph, 1997; Fig. A). The 2.8 kb fragment (Fig. C) was then gel purified to separate it from traces of the original three fragments so the entire *fpvA* gene could be used in cloning reactions. The larger fragment of approximately 4 kb containing the *fpvA* gene plus flanking sequence was amplified and gel purified in the same manner. Both fragments had *Hin*dIII sites on both ends that were used for cloning.

C.3 Modification of a vector for cloning of mutated *fpvA* gene

The unique sites (*Bam*HI and *Sst*I) engineered into the *fpvA* gene on the PCR fragment are usually found in the multi-cloning site of vectors. To

Figure B: PCR products used to form template in overlap extension PCR. A 0.8% agarose gel of purified PCR products amplified from 2 primers was used to assess the quality and size of the fragments. Lane1, primers P1A & P5 (fragment 2); lane 2, primers P6 & P4A (fragment 3); lane 3, primers P2 & P3 (fragment 1)

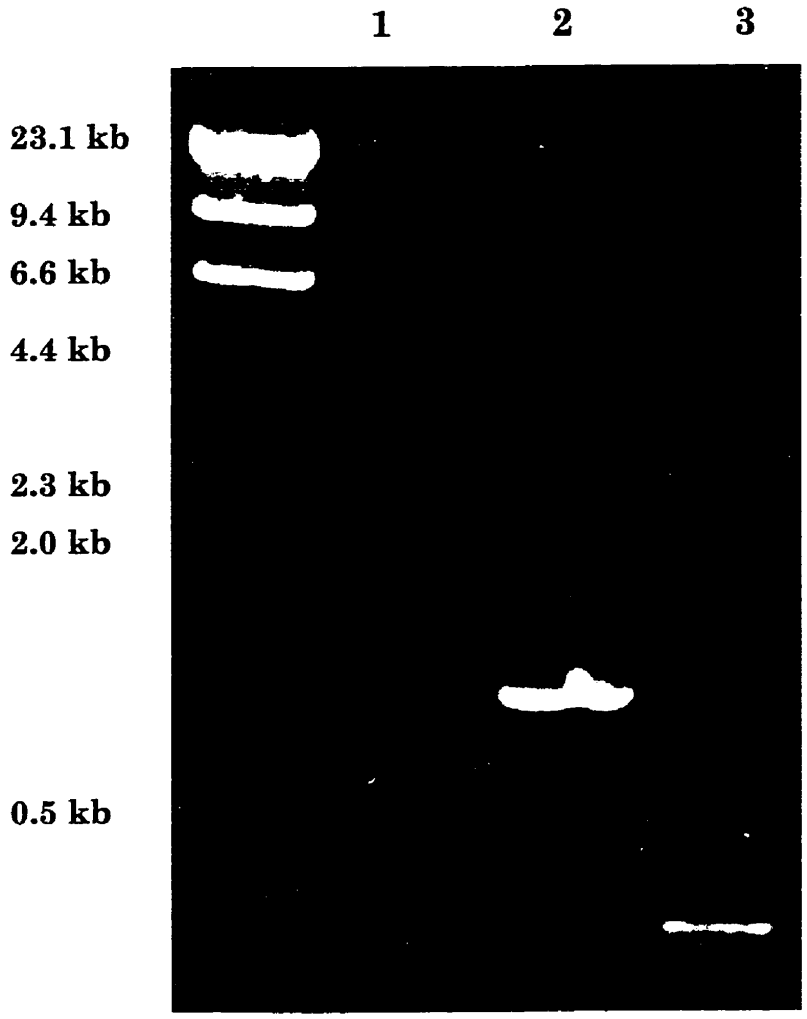
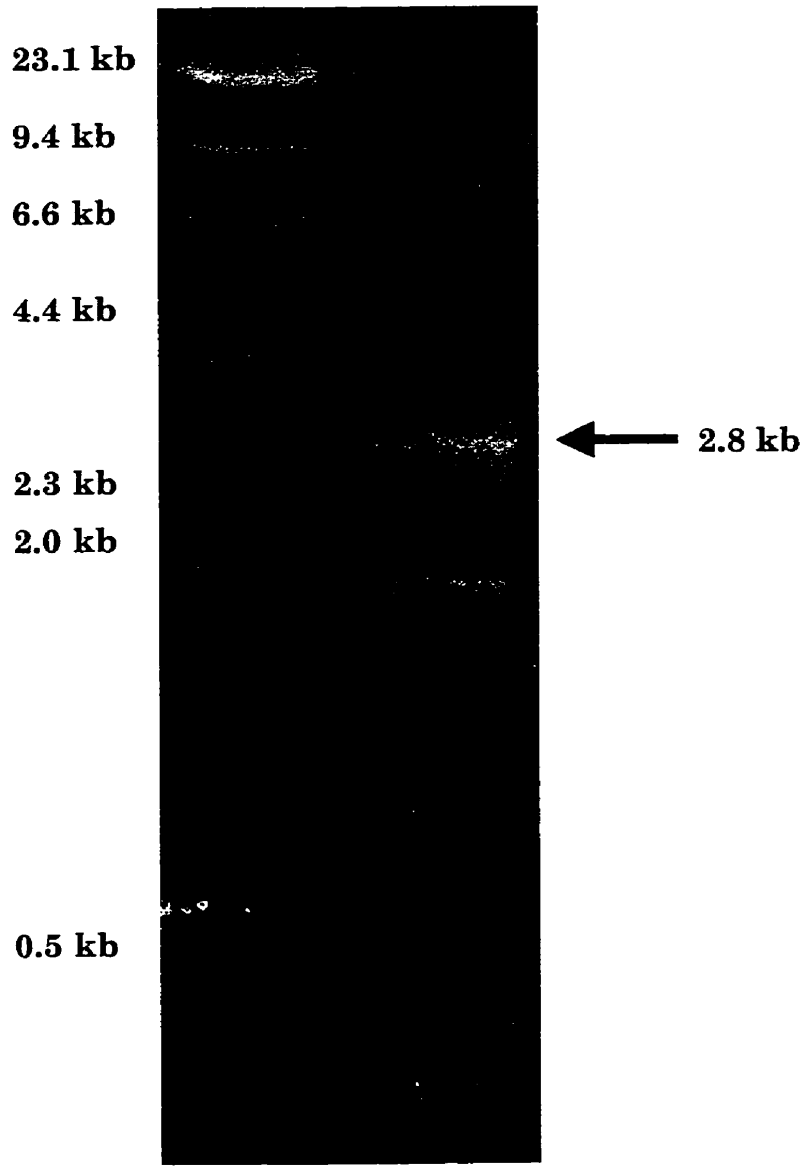

1
2
3
23.1 kb
9.4 kb
6.6 kb
4.4 kb
2.3 kb
2.0 kb
0.5 kb

Figure C: The product of overlap extension PCR. A 0.8% agarose gel showing the 2.8 kb fragment containing the *fpvA* gene as well as small amounts of the original PCR fragments used as template in the reaction.

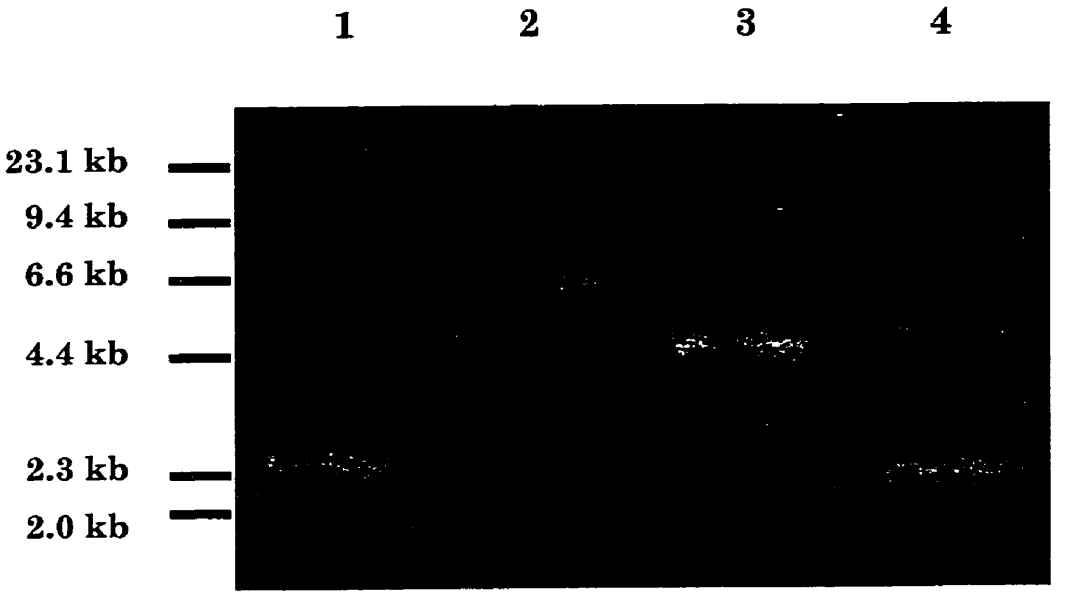

23.1 kb
9.4 kb
6.6 kb
4.4 kb
2.8 kb
2.3 kb
2.0 kb
0.5 kb

obtain a vector lacking these sites, the vectors pAK1900 and pRK415 were modified such that the *Bam*HI and *Sst*I sites were removed while retaining the *Hin*dIII site necessary for cloning of the *fpvA* PCR fragment. The vector pAK1900 was digested sequentially with *Sst*I and *Sph*I while the vector pRK415 was digested simultaneously with *Sst*I and *Pst*I, leaving 3' overhangs in each cases. Each vector was gel purified and treated with T4 DNA polymerase, in order to convert the sticky ends to blunt ends, before being recircularized in a ligation reaction. Half of the ligation reaction was transformed into competent *E. coli* K467 and several clones were picked for screening. Plasmid DNA from these clones were screened by separate digestions with *Bam*HI, *Sst*I and *Hin*dIII to show a loss of the *Bam*HI and *Sst*I sites and the retention of the *Hin*dIII site (Fig. D). The modified pAK1900 was named pV1 and the modified pRK415 was called pVEC1.

C.4 Cloning of the PCR-amplified *fpvA* fragment into the modified vector and screening for FpvA expression

Since the blue-white screening capability of the vectors was lost during their modification, the amplified *fpvA* gene carrying the unique *Bam*HI and *Sst*I sites bordering the ferric-pyoverdine binding site, was cloned, initially, into the *Hin*dIII site of the unmodified pAK1900. This allowed for the application of blue-white screening making it easier to find a clone

Figure D: Restriction endonuclease digests of pV1. A 0.8% agarose gel showing loss of the *Bam*HI (lane 2) and *Sst*I (lane 4) sites and retention of the *Hin*dIII (lane 3) site as compared to uncut pV1 DNA (lane 1).


1
2
3
4
23.1 kb
9.4 kb
6.6 kb
4.4 kb
2.3 kb
2.0 kb

containing the *fpvA* insert. The *fpvA* construct above and pV1 were digested separately with *Hin*dIII and each was separately gel purified and then ligated together. After transformation of half the ligation mixture into competent *E. coli* K467, about 60 colonies were screened by *Hin*dIII digestion of their plasmid DNA. Six of these clones contained the *fpvA* gene insert and in all cases the gene was in the opposite orientation to the *lac* promoter on the vector, as assessed by restriction mapping of the constructs. One of these insert-containing plasmids (called pAM4) was transformed into *P. aeruginosa* K979 (*P. aeruginosa* K337 ΔFpvA) to assess FpvA expression and activity from this construct. A phenotypic assay, where *P. aeruginosa* K979 (pAM4) was streaked onto iron-limited succinate medium plus 150 μg/mL of EDDHA, showed no growth, indicating no complementation of the *fpvA* deletion by this plasmid. To confirm this, a Western immunoblot of whole cell extracts from *P. aeruginosa* K979 (pAM4) was performed, and no expression of FpvA was detected using anti-FpvA anti-serum.

The orientation of the insert with respect to the *lac* promoter was originally not suspected to be a problem since the amplified *fpvA* gene carried its own promoter. Since no expression was being seen when the insert was in the opposite orientation to the *lac* promoter and no inserts in the same orientation as the *lac* promoter were isolated (perhaps due to overproduction lethality), it was decided that the ligation product should be transformed into an *E. coli* strain containing the $lacI^q$ gene to repress *lac* promoter activity.

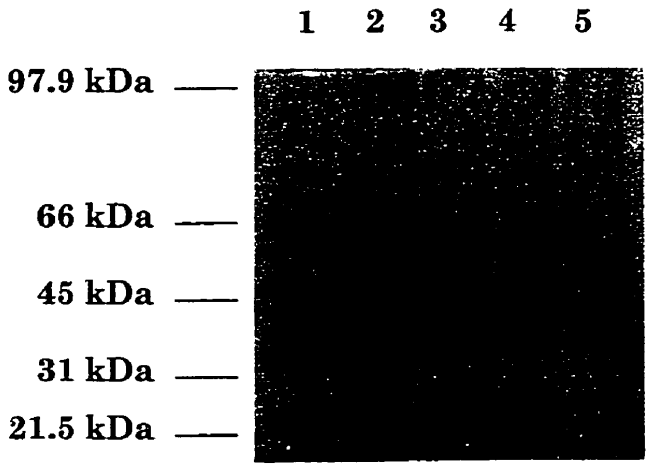
The *E. coli* K1212 was made competent and transformed with a new ligation mixture of *Hin*dIII-cut pV1 and the amplified *fpvA* gene. Sixteen colonies were screened by restriction mapping and five contained plasmid with the insert. One plasmid, pAM5, contained the insert in the same orientation as the *lac* promoter. The construct pAM5 was then transformed into *P. aeruginosa* K1183 (*P. aeruginosa* PAO1 ΔFpvA) to screen for FpvA expression. Phenotypic assays showed little growth on minimal medium containing 150 μg/mL EDDHA and Western immunoblotting of outer membrane preparations of three clones with the FpvA anti-serum showed FpvA expression in two of the three clones (Fig. E). The instability of this phenotype was further characterized by isolation of plasmid DNA from the *P. aeruginosa* K1183 (pAM5) clone that showed FpvA expression and transformation of this DNA into *P. aeruginosa* K1183. A Western immunoblot, using anti-FpvA anti-serum, of whole cell extracts prepared from 12 different *P. aeruginosa* K1183 (pAM5) clones showed FpvA expression in only one of these clones.

Since stable expression of FpvA has been seen from a 4.8 kb fragment containing the wild-type *fpvA* gene on pAK1900 in the same orientation as the *lac* promoter (pPVR2; Poole *et al.*, 1993), it was thought that the sequences flanking *fpvA* in pPVR2 may moderate FpvA expression. Thus, a longer (4.3 kb) fragment containing the *fpvA* gene with the engineered unique

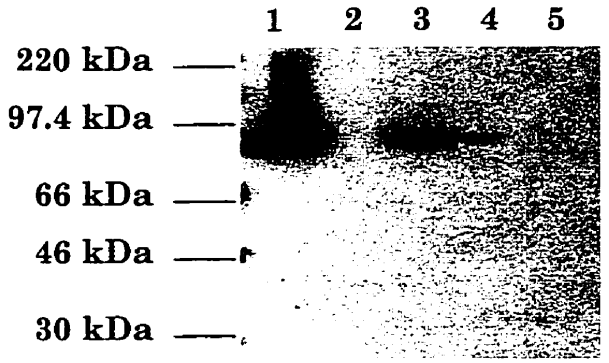
Figure E: Assessment of FpvA expression of *P. aeruginosa* K1183 harbouring pAM5 (pV1::*fpvA**). The outer membrane fraction of *P. aeruginosa* K1120 (wild-type; lane 1), *P. aeruginosa* K1183 (ΔFpvA; lane 2) and three clones of *P. aeruginosa* K1183 (pAM5) (lanes 3-5) were isolated from iron-limited cultures.
i) Coomassie stained 10% SDS-PAGE
ii) Western immunoblot using anti-FpvA antiserum

* the full length *fpvA* gene containing unique *Bam*HI and *Sst*I sites

i)


1 2 3 4 5
97.9 kDa
66 kDa
45 kDa
31 kDa
21.5 kDa

ii)


1 2 3 4 5
220 kDa
97.4 kDa
66 kDa
46 kDa
30 kDa

*Bam*HI and *Sst*I sites was cloned into pVEC1 at the *Hin*dIII site. This vector was chosen because other studies showed expression of FpvA even when the *fpvA* gene was inserted in the opposite orientation to the *lac* promoter (Q. Zhao, personal communication). Screening of clones obtained from ligation reactions that were carried out did not yield any vector containing insert. This was not pursued any further because a project involving the expression of the pyoverdine biosynthetic genes was considered a priority.

D. DISCUSSION

Lack of expression of FpvA from pAM4 and pAM5 did not allow for realization of the goal set forth in the introduction to the appendix. The project studying regulation of the pyoverdine biosynthetic genes took precedence over further cloning efforts which would most likely have produced a clone that expressed FpvA.

The 2.8 kb *fpvA*-containing fragment was cloned into pV1 and found to only insert in the orientation opposite to the *lac* promoter in *E. coli* DH5α, a strain that has little repression of the *lac* operon. On the other hand, in an *E. coli* strain carrying the $lacI^q$ gene, where tight repression of the *lac* operon is likely to occur, the fragment was found in the same orientation as the *lac* promoter but at a lower frequency than in the opposite orientation. This seems to indicate that expression of the *fpvA* gene from the *lac* promoter was lethal in *E. coli* such that only tight repression of this promoter (in $lacI^q$

strains) permitted cloning of *fpvA* in the same orientation as the *lac* promoter.

The 2.8 kb fragment containing the *fpvA* gene cloned in the same orientation as the *lac* promoter of pAM5 was unstable when the plasmid was introduced into *P. aeruginosa*, with FpvA expression being inconsistent and readily lost. Interestingly, a 4.8 kb *fpvA*-containing fragment originally cloned in pAK1900 in the same orientation as the *lac* promoter was stable and produced consistent yields of FpvA, possibly due to some moderating influence of the additional DNA sequence. It was expected, therefore, that the larger 4.3 kb fragment containing *fpvA* when cloned into pVEC1 would yield clones that stably expressed FpvA. Indeed, the parent of this vector (pRK415) containing an *fpvA* fragment of about this size showed FpvA expression even when cloned in the opposite orientation to the *lac* promoter (Q. Zhao, personal communication).

Since all the ligations were initially cloned into *E. coli* strains, one might expect to measure FpvA expression in *E. coli* to minimize the amount of transformations that are required. Unfortunately, FpvA is not, for reasons not yet understood, expressed in *E. coli* (i.e. undetectable even in Western immunoblots). Therefore, all constructs must be transformed into a *P. aeruginosa* strain before FpvA expression from a new clone can be determined.

Once FpvA expression was demonstrated, random mutagenesis using PCR with *Taq* DNA polymerase was going to be performed on the putative binding region, the ~300 bp between the newly engineered *Bam*HI and *Sst*I sites. ^{55}Fe-pyoverdine binding studies of FpvA-expressing clones were then going to be performed to see if any mutants demonstrating altered binding ability could be isolated. Sequence analysis of the DNA of the putative binding region (between the *Bam*HI and *Sst*I sites) would then yield clues as to which specific amino acid residues played a role in binding of ferric pyoverdine to its receptor, FpvA.