MOLECULAR REGULATION OF IRON UPTAKE IN

PSEUDOMONAS AERUGINOSA.

by

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SUMMARY

The microbial demand for iron is often met by the elaboration of siderophores into the surrounding medium and expression of cognate outer membrane receptors for the ferric siderophore complexes. Conditions of iron limitation, such as those encountered *in vivo*, cause *Pseudomonas aeruginosa* to express two high-affinity iron-uptake systems based on pyoverdin and pyochelin. These systems will operate both in the organism's natural habitat, soil and water, where the solubility of iron at neutral pH is extremely low, and in the human host where the availability of free iron is too low to sustain bacterial growth due to the iron-binding glycoproteins transferrin and lactoferrin. Cross-feeding and radiolabelled iron uptake experiments demonstrated that pyoverdin biosynthesis and uptake were highly heterogeneous amongst *P. aeruginosa* strains, that growth either in the presence of pyoverdin or pyochelin resulted in induction of specific IROMPs, and that induction of iron uptake is siderophore-specific.

The *P. aeruginosa* Tn5 mutant PH1 is deficient in ferripyoverdin uptake and resistant to pyocin Sa, suggesting that the site of interaction of pyocin Sa is a ferripyoverdin receptor. Additional Tn5 mutants appeared to exploit different strategies to achieve pyocin Sa-resistance, involving modifications in expression of pyoverdin-mediated iron uptake, indicating that complex regulatory systems exist to enable these organisms to compete effectively for iron.

Modulation of expression of IROMPs prompted a study of the mechanism of uptake of a semi-synthetic C(7) α-formamido substituted cephalosporin BRL 41897A. Sensitivity to this agent correlated with expression of the 75 kDa ferri-pyochelin receptor and demonstrated the potential of high-affinity iron uptake systems for targeting of novel antibiotics.

Studies with ferri-pyoverdin uptake-deficient mutant PH1 indicated that expression of outer membrane protein G (OprG), which is usually expressed under iron-rich conditions and repressed under iron-deficient conditions, was perturbed. Attempts were made to clone the *oprG* gene using a degenerate probe based on the N-terminal amino acid sequence. A strongly hybridising HindIII restriction fragment was cloned and sequenced, but failed to reveal an open reading frame corresponding to OprG. However, there appears to be good evidence that a part of the gene coding for the hydrophilic membrane-associated ATP-binding component of a hitherto uncharacterised periplasmic-binding-protein-dependent transport system has been isolated. The full organisation and sequence of the operon, and substrate for this putative transport system, are yet to be elucidated.

ACKNOWLEDGEMENTS

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ABBREVIATIONS

A	ampere(s)
ATP	adenosine triphosphate
°C	degrees centigrade
CAA	casamino acids medium
CAA agar	CAA + 1.5% bacto agar
CAS agar	chrome azurol S agar
CDM-Fe	chemically defined medium with no added iron
CF	cystic fibrosis
CIP	calf intestinal alkaline phosphatase
cm	centimetre(s)
ddH$_2$O	double-distilled water
DMF	N,N-dimethyl formamide
DMSO	dimethyl sulphoxide
DNA	deoxy-ribonucleic acid
EDDHA	ethylene diamine di(o-hydroxyphenyl) acetic acid
EDTA	ethylene diamine tetra-acetic acid
F	Farad
-Fe	no added iron
g	acceleration due to gravity
g	gramme(s)
h	hour(s)
IATS	International Antigenic Typing Scheme
IPTG	isopropylthio-ß-D-galactoside
IROMPs	iron-regulated outer membrane proteins
kV	kilovolt(s)
l	litre(s)
L-agar	LB + 1.5% bacto agar
LB	Luria broth
M	mole(s) per litre
µg	microgramme(s)

μl	microlitre(s)
mg	milligramme(s)
ml	millilitre(s)
mm	millimetre(s)
MIC	minimum inhibitory concentration
min	minute(s)
MOPS	3-[N -Morpholino]propane-sulphonic acid
nm	nanometre(s)
O.D.	optical density
OM	outer membrane
OMP	outer membrane protein
RNA	ribonucleic acid
rpm	revolutions per minute
s	second(s)
s/n	supernatant
Sarkosyl	N-Lauryl Sarcosine
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SSC	standard saline citrate (0.15M NaCl/0.015 trisodium citrate)
ssDNA	single-stranded DNA
STE	0.1M NaCl/ 10mM Tris.Cl pH 8.0/1mM EDTA pH 8.0
TAE	Tris-acetate/EDTA buffer
TBE	Tris-borate/EDTA buffer
TE	10mM Tris.Cl pH 8.0/1mM EDTA pH 8.0
Tris	tris (hydroxymethyl) amino ethane
V	volt(s)
v/v	volume by volume
w/v	weight by volume
X-gal	5-bromo-4-chloro-3-indolyl-ß-D-galactoside
Ω	ohm(s)

1. INTRODUCTION.

1.1. Preamble.

Many bacteria, when encountering conditions of low iron availability, have the capacity to elaborate into the surrounding environment low-molecular-weight iron-chelating molecules (siderophores) which, when complexed with iron, are delivered into the cell via interaction with their cognate receptors on the bacterial cell surface (Bullen & Griffiths, 1987b). *Pseudomonas aeruginosa* utilises two siderophore-mediated iron uptake systems based on pyochelin and pyoverdin, which operate both in the organism's natural habitat (soil and water), where the solubility of iron at neutral pH is extremely low, and in the human host. Here the anti-infective, anti-neoplastic defence system of "iron withholding" is in operation (Weinberg, 1992), in which the availability of free iron is too low to sustain bacterial growth due to the iron-binding glycoproteins transferrin and lactoferrin (Griffiths, 1990; 1991; 1993). The ability of *P. aeruginosa* to compete with transferrin and lactoferrin for iron, coupled with production of a number of virulence determinants (Smith, 1990) and an innate resistance to antibiotics, especially when growing as a biofilm (Anwar *et al.*, 1991), has established this organism as a leading cause of morbidity and mortality in the immunocompromised host, such as in AIDS patients (Franzetti *et al.*, 1992; Lozano *et al.*, 1992), and is the primary pathogen in the respiratory tracts of cystic fibrosis (CF) patients (Woods *et al.*, 1991). Surgery, catheterisation, tracheostomy, corneal abrasions or severe burns also allow this opportunistic pathogen to create infections in the hospital environment, possibly leading to life-threatening sepsis or severe tissue damage (Visca *et al.*, 1992a; Wick *et al.*, 1990). The ubiquitous nature of *P. aeruginosa* is highlighted in the case of a multiply-injured intensive care patient who became infected by coming into contact with contaminated holy water (Greaves and Porter, 1992).

1.2. The Gram-negative bacterial membrane.

Gram-negative bacteria are surrounded by a double membrane system, consisting of an inner (cytoplasmic) and an outer membrane, which are separated by the periplasm (Figure 1.1). The cytoplasmic membrane contains proteins required for generating and maintaining an electrochemical potential, and also proteins which utilise this potential for the active transport of biologically important molecules (Ames and Joshi, 1990). The periplasm (or periplasmic space) contains components of the active transport systems and detoxifying enzymes, while the outer membrane serves as a permeability barrier to allow passage of small hydrophilic molecules into the periplasm (Postle, 1990).
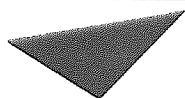
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Figure 1.1. Schematic representation of the outer membrane of a Gram-negative bacterium. LPS = lipopolysaccharide; PL = phospholipid; P = protein; LP = lipoprotein. (From Sleytr *et al.*, 1988).

It has been shown that the properties of the outer membrane are highly variable, according to the conditions encountered (Brown *et al.*, 1990; Gilbert *et al.*, 1990; Brown *et al.*, 1991); altered expression of porins in the outer membrane have been shown in response to growth conditions (Bell *et al.*, 1991; Yamano *et al.*, 1993; Siehnel *et al.*, 1992), with decreased expression of porins to correlate with increased resistance to antimicrobial agents (Yamano *et al.*, 1990; Chamberland *et al.*, 1989; Woodruff and Hancock, 1988). Quantitative and qualitative alterations in lipopolysaccharide profiles have also been implicated in antibiotic resistance (Leying *et al.*, 1992). The known outer membrane proteins of *P. aeruginosa* and their suspected functions are summarised in Table 1.1.

Protein	Mol. wt.	Function
IROMP	75-90 kDa	Ferrisiderophore binding/transport (Chapters 4, 5 and 6)
OprC	70 kDa	Porin (Yoshihara and Nakae, 1989)
Esterase	55 kDa	Growth on acyl-esters (Ohkawa et al., 1979)
OprP	48 kDa	Phosphate transport (Hancock et al., 1982; Worobec et al., 1988; Poole and Hancock, 1986; Siehnel et al., 1988)
OprB	46 kDa	Glucose porin (Hancock and Carey, 1980; Trias et al., 1988)
OprD	45.5 kDa	Imipenem/basic amino acid selective porin (Yoshihara and Nakae, 1989; Quinn et al., 1986; Trias et al., 1989)
OprE	44 kDa	Porin (Yoshihara and Nakae, 1989)
OprF	38 kDa	Porin/structural (Woodruff et al., 1986; Gotoh et al., 1989; Woodruff and Hancock, 1989)
OprG	25 kDa	Unknown (Yates et al., 1989)
OprH	21 kDa	Stabilising Mg^{2+}-deprived cells (Nicas and Hancock, 1983; Bell and Hancock, 1991)
OprL	20.5 kda	Structural/lipoprotein (Mizuno, 1979)
FBP	9-14 kDa	Ferripyochelin binding and transport (Sokol and Woods, 1983)
OprI	9 kDa	Structural/lipoprotein (Mizuno and Kageyama, 1979; Cornelis et al., 1989; Duchêne et al., 1989)

Table 1.1. Outer membrane proteins of *P. aeruginosa* (adapted from Hancock, 1987; Hancock et al., 1990).

The role of OprF has been show to be both as a structural protein required for the maintenance of the integrity of the outer membrane, analogous to the OmpA protein of *E. coli* (Gotoh et al., 1989), and as a major porin, forming water-filled channels through the outer membrane (Finnen et al., 1992). The importance of this protein in the outer membrane of *P. aeruginosa* has prompted studies involving synthetic peptides, analogous to OprF epitopes, as immunogens in a synthetic vaccine against *P. aeruginosa* infection (Hughes et al., 1992). The pore size of OprF is still a subject of debate; Yoshihara and Nakae (1989) found no pore-forming activity due to OprF in reconstituted liposome membranes, whereas Bellido et al. (1992) suggest that the pore formed by OprF is sufficiently large for the passage of a tetrasaccharide, and that OprF is the major porin protein for the passage of large compounds across the outer membrane, with other porins such as OprB, OprC, OprD and OprE, being involved in the passage of smaller compounds. OprF has been demonstrated to be involved in ferric siderophore uptake (Meyer, 1992), and is discussed in Chapter 5.

There are a number of outer membrane proteins whose expression is regulated by the concentration of available iron; when little iron is available, such as *in vivo* or in iron-depleted media, a group of OM proteins in the range 75-90 kDa are expressed in the outer membrane which have been shown to be immunogenic in the CF lung (Cochrane et al., 1988; Cochrane et al., 1987; Anwar et al., 1984), whereas, in conditions of high available iron, a 25 kDa protein, OprG, is expressed in the outer membrane. Little is known about this protein; it has been implied that it may play a part in uptake of

quinolones into *P. aeruginosa* cells, in that resistant strains were isolated which showed considerably reduced expression of OprG, or that its role may be to facilitate iron uptake under conditions of abundant iron (Yates *et al.*, 1989; Yates, 1992). The role of OprG is reviewed in Chapter 7.

1.3. Bacterial iron acquisition.

Reduced intermediates of molecular oxygen, such as superoxide and hydrogen peroxide, are ubiquitous products of normal aerobic metabolism; most cells require protection from the potentially damaging effects of excessive amounts of these radicals although they are poorly reactive in aqueous solution and unable to modify DNA, lipid and protein molecules (Gutteridge and Quinlan, 1992; Minotti, 1993). The presence of iron greatly enhances the toxicity of these radicals by catalysing the formation of hydroxyl radicals. Therefore, free iron levels are maintained at a low level (approximately 10^{-18} M) *in vivo* in part by the presence of the extracellular glycoproteins transferrin and lactoferrin (Griffiths, 1987a). This low concentration of free iron cannot support the growth of bacteria, while the presence of complement in plasma confers a bactericidal activity against invading bacteria (Bullen *et al.*, 1992). During infection, the host reduces the total amount of iron in serum (bound to transferrin), possibly by releasing lactoferrin from polymorphonuclear leucocytes, which has a higher affinity for iron than transferrin. The Fe^{3+}-lactoferrin complexes are then thought to be internalised by macrophages and eliminated by the reticuloendothelial system (Ward, 1987). Alternatively, the iron in transferrin may be diverted to intracellular storage in ferritin molecules (Munro, 1990; Crichton and Ward, 1992).

The low concentration of free iron in host tissues restricts the growth of infecting bacteria, thus acting as a non-specific defence mechanism, and so, in order for the bacterium to obtain iron, it produces iron-chelating molecules, termed siderophores, which are also able to convert polymeric ferric oxyhydroxides, present in aerobic environments, into soluble chelates which are substrates for high affinity transport mechanisms (Sriyosachati and Cox, 1986). Binding of the ferric-siderophore complex to its cognate receptor on the outer membrane of the bacterial cell is followed by release of iron via a reductive mechanism located on the cytoplasmic membrane (Meyer *et al.*, 1987; Hallé and Meyer, 1992a; Hallé and Meyer, 1992b) which may be internalised and stored in a bacterioferritin (Mielczarek *et al.*, 1992).

Alternatively, pathogenic bacteria may utilise heme-compounds, released from tissues damaged by extracellular proteases of bacterial origin, or utilise host iron-binding molecules directly (Otto *et al.*, 1992).
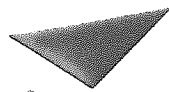
Figure 1.2. Bacterial siderophore-mediated iron uptake. sid = siderophore (Griffiths, 1987b).

Such siderophore-mediated iron uptake mechanisms have been described in a wide variety of organisms, including *Escherichia coli*, *Aeromonas* species (Byers *et al.*, 1991), *Klebsiella* species (Tarkkanen *et al.*, 1992), *Pseudomonas pseudomallei* (Yang *et al.*, 1993), *Pseudomonas roseus fluorescens* (Vande Woestyne *et al.*, 1992, *Azotobacter vinelandii* (Serdal Sevinc and Page, 1992; Menhart and Viswanatha, 1990), *Rhizobium meliloti* (Reigh and O'Connell, 1993), *Bacillus subtilis* (Peters and Warren, 1968), *Erwinia* species(Persmark *et al.*, 1992; Ishimaru and Loper, 1992; Expert *et al.*, 1992), *Acinetobacter baumannii* (Echenique *et al.*, 1992), *Pasteurella multocida* (Choi-Kim *et al.*, 1991), *Bordetella pertussis* (Agiato and Dyer, 1992), *Mycobacterium* species (Sritharan and Ratledge, 1989; 1990), *Vibrio cholerae* (Goldberg *et al.*, 1991), and *Candida albicans* (Sweet and Douglas, 1991),

Interesting exceptions are found in *Neisseria meningitidis* and *Haemophilus influenzae* (Harkness *et al.*, 1992), which express human-specific ferritransferrin receptors on their outer membranes, and thus acquire iron by a more direct route from the host (Bhatnagar and Frasch, 1990; Schryvers and Gray-Owen, 1992; Williams and Griffiths, 1992; Griffiths *et al.*, 1993); this phenomenon may indicate the closer relationship of these organisms to their hosts than the opportunistic pathogen *P. aeruginosa*.

1.4. Transport of iron through the bacterial envelope.

Internalisation of ferric siderophore complexes, with the ultimate aim of meeting the intracellular iron requirement, is complicated by the double membrane surrounding

Gram-negative bacteria; on one hand, the formation of an outer membrane confers a number of survival advantages, such as increased resistance to toxic substances (e.g. antibiotics and host defence proteins), with the additional benefit of lipopolysaccharide on the outer surface which serves to mask the outer membrane proteins from the action of the host's defence mechanisms. On the other hand, transport of biologically important molecules across the outer membrane is more complex, and requires that there be some method of energising the outer membrane to facilitate active transport. This system has been extensively studied in *E. coli*, and a cytoplasmic membrane protein, TonB, identified, which provides an essential energy link between the cytoplasmic and outer membranes, enabling Gram-negative bacteria to actively take up molecules which would be excluded by the size limit of their porin channels (Bagg and Neilands, 1987).Interestingly, ferrioxamine B-mediated iron uptake is TonB-independent (Nelson and Szaniszlo, 1992).

Compound	Outer membrane receptor
Fe^{3+} -aerobactin	IutA
Fe^{3+} -ferrichrome	FhuA
Albomycin	FhuA
Fe^{3+} -coprogen	FhuE
Fe^{3+} -rhodotorulic acid	FhuE
Fe^{3+} -dicitrate	FecA
Fe^{3+} -enterobactin/dihydroxybenzoyl-serine	FepA
Fe^{3+} -catecholic cephalosporins/dihydroxybenzoyl-serine	Fiu
Fe^{3+} -catecholic cephalosporins/dihydroxybenzoyl-serine	Cir
Vitamin B_{12}	BtuB
Colicin B	FepA
Colicin D	FepA
Colicin M	FhuA
Colicin Ia, Ib	Cir
Phage T1	FhuA
PhageØ80	FhuA

Table 1.2. TonB-dependent transport across the outer membrane of *Escherichia coli*.

In addition to ferric siderophores and vitamin B12, some colicins and phages also utilise these outer membrane proteins for attachment and internalisation into the bacterial cell, with the additional requirement of ATP to supply energy for the release of substrates into the periplasmic space. *E. coli* mutants lacking a functional TonB protein were unable to transport these substrates, but receptor binding was unaffected. In addition, OM receptors with altered "TonB boxes" (i.e., regions of interaction of the receptor with the TonB protein) were incapable of transporting their substrates across the outer membrane, indicating that a physical interaction between TonB and the outer membrane receptors is occurring. TonB boxes are composed of short amino acid sequences close to the N-terminus, which display close homology (Sauer *et al.*, 1987):

FhuA TonB box	Asp-Thr-Ile-Thr-Val
FhuE TonB box	Glu-Thr-Val-Ile-Val
Cir TonB box	Glu-Thr-Met-Val-Val
BtuB TonB box	Asp-Thr-Leu-Val-Val
Iut TonB box	Glu-Thr-Phe-Val-Val
FepA TonB box	Asp-Thr-Ile-Val-Val

The TonB protein is functionally and physically unstable, having a half-life of only 10 min at 42°C. A number of gene products have been found which stabilise TonB, of which the best characterised is the ExbB protein. Mutations in *exbB* cause reduced levels of vitamin B_{12} and ferric siderophore transport (Wooldridge *et al.*, 1992), which induces overproduction of enterobactin and also confers increased resistance to B-group colicins (Eick-Helmerich *et al.*, 1987). Reductions in the levels of ExbB protein have also been shown to reduce the half-life of TonB, to as little as two minutes. ExbB is a 26 kDa cytoplasmic membrane protein which shares 26.3 % amino acid identity with the TolQ protein, which is involved in TonB-independent sensitivity to A-group colicins. ExbB, the TolQR proteins and FhuA have been shown to stabilise TonB, probably by physical interactions, while TonB activity also depends on the ExbBD proteins, whose function can be partially replaced by the TolQR proteins (Postle, 1990).
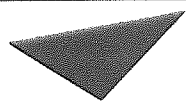
Figure 1.3. Model showing current view of iron uptake into *E. coli* (Braun *et al.*, 1991).

Iron transport across the outer membrane probably occurs in the following manner: the ferric siderophore (in this case, ferrichrome) binds to its receptor, FhuA. TonB binds to FhuA and induces FhuA to release the ferrichrome complex into the periplasmic space, where it binds to the FhuD protein. FhuD passes ferrichrome on to the FhuB protein which, in an energy-requiring process, translocates ferrichrome across the cytoplasmic membrane (Köster and Böhm, 1992). The energy for this step is donated by the FhuC protein, which possesses a typical ATP-binding region (Becker *et al.*, 1990; Schultz-Hauser *et al.*, 1992b). Once inside the cytoplasm, iron(III) is reduced to iron(II) by a reductive mechanism, deferri-ferrichrome is acetylated, and the modified product excreted into the culture medium (Braun *et al.*, 1991; Hartmann and Braun, 1980; Schneider *et al.*, 1981).

In *Pseudomonas* sp., the proteins required for the transport of ferric siderophores across the outer membrane are less well understood; however, there has already been found some homology between both the *pupA* and *pupB* structural genes (which encode the ferric-pseudobactin receptors PupA and PupB in *Pseudomonas putida* strain WCS358) and TonB-dependent receptor proteins of *E. coli* (Bitter *et al*, 1991; Koster *et al*, 1993). The presence of TonB, together with ExbB and ExbD proteins, has been confirmed by Bitter *et al.* (1993), in complementation experiments using mutants resistant to catecholic cephalosporins. The ferripyoverdin receptor of *P. aeruginosa* has also been cloned and sequenced, and it too demonstrates conserved regions characteristic of TonB-

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dependent receptor proteins, as well as 41.3% identity with the PupA receptor of *P. putida* WCS358 (Poole *et al.*, 1993).

1.5.1. Siderophores from *Pseudomonas* sp.: pyoverdin.

The fluorescent pseudomonads, i.e. *P. aeruginosa*, *P. putida*, *P. fluorescens*, *P. ovalis*, *P. mildenbergii*, *P. reptilivora*, *P. geniculata* and *P. calciprecipitans* are characterised by the biosynthesis in iron-deficient conditions of yellow-green, water-soluble compounds (Demange *et al.*, 1987). This pigment was first named "bacterial fluorescein" or "fluorescin", although this name was changed in 1958 to pyoverdine, and more recently to pyoverdin, in order to avoid any confusion with the chemically-defined fluorescein and fluorescin (Meyer *et al.*, 1987). Speculation about the importance of pyoverdins in bacterial cell growth was fuelled initially by the observation that in certain media, such as succinate or fumarate media, *P. mildenbergii* produces almost 300mg of pyoverdin per litre of growth medium, with a dry cell weight of only 500mg. This observation led Newkirk and Hulcher to state: "It seems unlikely that so much of the metabolic effort of *P. mildenbergii* would be directed toward the accumulation of these substances (the pyoverdins) if there was no biological function for them" (Newkirk and Hulcher, 1969).

The pyoverdins of *P. aeruginosa* are structurally related to the pseudobactins and pyoverdins from other fluorescent pseudomonads (Meyer *et al.*, 1987; Linget *et al.* 1992; Demange *et al.*, 1990b), azotobactin from *Azotobacter vinelandii* (Demange *et al.*, 1987) and azoverdin from *Azomonas macrocytogenes* (Linget *et al.*, 1992). These are highly water-soluble yellow-green compounds which all share a 2,3-diamino-6,7 dihydroxyquinoline chromophore (Michels and Taraz, 1991; Taraz *et al.*, 1991; Demange *et al.*, 1990a) and two N-hydroxyornithine residues attached to a varying amino acid backbone of 6 - 10 residues which allows the optimum arrangement of the three bidentate complexing groups (Demange *et al.*, 1987; Morel *et al.*, 1992; Saalfrank *et al.*, 1991). These are the hydroxamate group in the middle of the peptide chain, the catechol group of the chromophore, and the hydroxamate group of the terminal N - hydroxy-piperidone ring, thus forming a very stable 1:1 octahedric Fe(III)-pyoverdin complex with an association constant $K = 10^{32}$ (Wendenbaum et al, 1983). The ability of pyoverdins to form such stable complexes has led to the suggestion that they be used to treat patients with secondary iron overload, which is ultimately associated with hepatocellular damage (Jégo *et al.*, 1993; Jégo *et al.*, 1992; Morel *et al.*, 1992), as an alternative to current deferration therapies, which involve the use of the siderophore desferrioxamine B to eliminate aluminium and iron overload; however, desferrioxamine B is utilisable by a number of bacterial species as well as the fungus *Rhizopus*, thereby enhancing their pathogenicity (Boelaert *et al.*, 1993). Interestingly, Lytton *et al.*(1992) describe the use

of a fluorescent derivative of desferrioxamine B, NBD-DFO, as an indicator of chelatable iron in tissues under conditions of iron-mediated cell damage, iron overload, and diseases of iron imbalance; it seems likely that pyoverdins may be put to the same use. However, pyoverdin may not be used for the elimination of aluminium, since, in experiments using pseudobactin and iron(III) analogs, no change in absorbance characteristic of pseudobactin complex formation was observed upon addition of aluminium to a pseudobactin-rich supernatant (Fekete and Barton, 1991). It was also found that scandium(III)- and yttrium(III)-pseudobactin complexes were formed, but that they enhanced pseudobactin production; since these metals do not have a reduced divalent state, the reductase enzymes would be unable to release the metal ions from these complexes, and even if these metals were released into the cytoplasm, they would not function as indicators of iron status, since the bacterium utilises the reduced divalent form of iron as a co-repressor in the negative regulation of siderophore synthesis (Neilands, 1990).

Isolation and purification of pyoverdins from *Pseudomonas aeruginosa* PAO1 has been described (Meyer and Abdallah, 1978). For analytical purposes, further purification by HPLC (Andriollo *et al.*, 1992) or ion-exchange chromatography (Fuhrmann, 1991) can be performed, which may then yield other minor compounds from the original *Pa* -Fe(III) complex, designated *Pa A* -Fe(III) (Figure 1.4.) , *Pa B*-Fe(III) and *Pa C*-Fe(III), with the major compound desigated *Pa*-Fe(III). However, it is thought that *Pa C* is an artifact and not a true pyoverdin, since it cannot be detected in the HPLC direct analysis of the culture medium during siderophore production (Meyer and Abdallah, 1978).

The structures of *Pa A*, *Pa B* and *Pa C* differ only in the chromophore, with all three having the same peptide chain, and with the amino acids possessing the same stereochemistry (Demange *et al*, 1987).
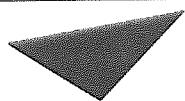
Figure 1.4. The structure of pyoverdin *Pa* (Morel *et al.*, 1992; Jégo *et al.*, 1993).

$$Pa \quad \ldots\ldots\ldots R = CH_2 - CH_2 - CONH_2$$
$$Pa\,A \ldots\ldots\ldots R = CH_2 - CH_2 - COOH$$
$$Pa\,C \ldots\ldots\ldots R = CH_2 - CH_2 - COO\,CH_3$$
$$Pa\,B \ldots\ldots\ldots R = CO - CH_2 - CH_2 - COOH$$
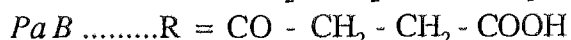
Pyoverdin *Pa A* is the acid form of *Pa,* in which the succinamide group is rapidly hydrolysed to the acid form. This reaction occurs at higher pH, and so may explain why, in young cultures where the pH is still fairly close to 7.0, the amount of *Pa A* is relatively low in comparison to older cultures where the pH has risen to around 9.0. At higher pH, pyoverdin *Pa A* is the major compound which is isolated.

Pyoverdin *Pa B* is probably formed due to replacement of the succinic acid moiety observed in pyoverdin *Pa A* with α-ketoglutarate.

The structures of pyoverdins from other fluorescent pseudomonads have been elucidated, with three major pyoverdins being isolated from each strain, differing only in their chromophore (as for *Pseudomonas aeruginosa* PAO1). Each of the three major compounds isolated for each species contains the same amino acid sequence, with this sequence differing between species (Table 1.2.):
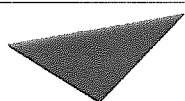
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Table 1.3. Sequence of several pyoverdins and of azotobactin D as determined by FAB Mass Spectrometry. Chr represents the chromophore, OHAsp is β-threo-hydroxyaspartic acid, OHOrn and cOHOrn being N^δ-hydroxyornithine and its cyclised form, respectively (Demange et al., 1987; Morel et al., .1992).

Taraz et al. (1991) found that, in P. aeruginosa culture supernatants containing pyoverdins, a related siderophore, desferriferribactin, could be isolated which was identical to pyoverdin in all ways except the chromophore. It was therefore suggested that these compounds are precursors in the biosynthesis of pyoverdins (Taraz et al., 1991; Linget et al., 1992). However, the role of these compounds in iron acquisition remains unclear.

Poole et al. (1991) demonstrated that a 90 kDa OM protein was responsible for ferripyoverdin binding and uptake in a P. aeruginosa mutant deficient in pyoverdin biosynthesis and uptake. Other workers identified ferripyoverdin receptors in different P. aeruginosa strains of different weights (Meyer et al., 1990; Hohnadel and Meyer, 1988; Aznar and Alcaide, 1992). The high degree of variability of the peptide backbone of pyoverdins has been shown to correlate with the specificity of pyoverdins for their cognate outer membrane receptors, in cross-feeding studies of pyoverdins from different P. aeruginosa type strains, which demonstrated variable rates of ferripyoverdin uptake

into bacterial cells dependent on the pyoverdin type (Cornelis *et al.*, 1987; Chapter 4). The heterogeneity of cognate receptors for these various pyoverdins was also demonstrated by SDS-PAGE of OM proteins from cells grown under conditions of iron deprivation (Cornelis *et al.*, 1987; Chapter 4), together with immunological studies of the 75-90 kDa IROMPs (Smith *et al.*, 1991). Further cross-feeding studies (Cornelis *et al.* 1989) suggested that pyoverdins could be placed in three groups in respect of their growth-promoting activity and their ability to facilitate ^{59}Fe uptake in each producer strain. Finally, a mutant of *P. aeruginosa* which was only capable of secreting a precursor of the pyoverdin chromophore was found to be incapable of iron transport, despite the fact that the secreted chromophore precursor chelated iron tightly, demonstrating that the peptide backbone of the pyoverdin molecule is necessary for receptor binding (Longerich *et al.*, 1993; Shanzer *et al.*, 1993). Experiments to determine siderophore cross-feeding occurring naturally in the rhizosphere revealed that siderophores with invariant structures, such as ferrioxamine B and ferrichrome, were utilisable by considerably more of the organisms tested than were pyoverdin-type siderophores; additionally, organisms producing pyoverdin-type siderophores were able to utilise at least one other siderophore (Jurkevitch *et al.*, 1992). Conversely, in the rhizosphere, pyoverdin produced by *P. fluorescens* was found to have growth-stimulating effects on *Bacillus polymyxa*, which is a siderophore deficient, nitrogen-fixing, potential growth-promoting rhizobacterium for forage plant species (Chiarini *et al.*, 1993).

The pyoverdin synthesis genes from *P. aeruginosa*, *P. syringae*, *P. putida* and *P.seudomonas* sp. B10 have been compared, and considerable DNA homology was found in certain regions of DNA, suggesting that some regions are more highly conserved than others (such as the genes involved in synthesis of the chromophore), and that they share a common ancestry (Rombel and Lamont, 1992). These data suggest that the ability to elaborate complex, highly variable siderophores which require specific receptors is a mechanism by which iron may be delivered to the pyoverdin-producing organism while rendering it unavailable to competing species (Buyer and Leong, 1986). Inhibition of the growth of competing species is also achieved by the elaboration into the environment of pyocins, the bacteriocins of *P. aeruginosa*, i.e. antibiotic substances which have the characteristic property of only being lethal to other *P. aeruginosa* or closely related organisms (Govan, 1978; 1986; Sano *et al.*, 1990; Sano, 1993; Sano and Kageyama, 1993). These features of fluorescent pseudomonads may explain their ability to compete successfully with and suppress plant root pathogens in the rhizosphere (DeWeger *et al.*, 1988; O'Sullivan and O'Gara, 1992; Buyer *et al.*, 1993).

The steps involved in pyoverdin biosynthesis have been studied in various *P. aeruginosa* mutants deficient in synthesis of this siderophore, and it was found that synthesis of *N*-hydroxyornithine was followed by peptide synthesis and finally, chromophore synthesis (Visca *et al.*, 1992). *N*-hydroxyornithine residues, which are incorporated into the peptide backbone of pyoverdin and act as iron binding groups, are

synthesised by the action of an enzyme unique to bacteria and fungi. This enzyme may be utilised as a target site for new antimicrobial agents, thereby interfering in the ability of the pathogen to obtain iron from the host (Visca *et al.*, 1992).

Extensive studies have been undertaken concerning the pyoverdin-like siderophore pseudobactin (Magazin *et al.*, 1986), produced by *P. putida*. The outer membrane protein receptor for pseudobactin in *P. putida* WCS 358, designated PupA, has been cloned (Marugg *et al.*, 1989) and sequenced (Bitter *et al.*, 1991), and elements of the biosynthetic pathway have been identified (Marugg *et al.*, 1988). At least 15 genes distributed over five gene clusters have been found to be required for the synthesis of pseudobactin 358, with high-affinity uptake of ferric-pseudobactin complexes involving an 86 kDa IROMP, designated PupA (Leong *et al.*, 1990). However, a mutant of *P. putida* WCS358 lacking the PupA receptor for pseudobactin 358 retained 30% residual uptake of this siderophore (Bitter *et al.*, 1991), which suggested that a second pseudobactin uptake system was present. A second receptor gene for pseudobactins BN7 or BN8, designated *pupB*, has been identified and sequenced (Koster *et al.*, 1993). This outer membrane protein, like PupA, was shown to be expressed under conditions of low iron concentration, but the presence of pseudobactin BN7 and BN8 was also required for maximal expression of PupB (Leong *et al.*, 1991), as was the presence of a functional *pupR* locus upstream of the *pupB* gene. The role of the *pupR* locus has been compared to the role of *fecR*, which is involved in iron dicitrate uptake in *E. coli* K-12. In this system, five transport genes for ferric dicitrate, *fecABCDE*, form an operon; transcription of *fecABCDE* is under the control of two genes, termed *fecI* and *ferR* upstream of *fecA* (the gene for the ferric dicitrate outer membrane receptor protein FecA), where the periplasmic FecR protein acts as a sensor for ferric citrate, and interacts with the cytoplasmic membrane-associated FecI. The FecI protein contains a region close to the carboxy-terminal end which is typical for DNA-binding regulatory proteins. The interaction between FecR and FecI causes induction of the FecBCDE periplasmic-binding-protein-dependent transport mechanism for ferric dicitrate (Schultz-Hauser *et al.*, 1992a).The presence of citrate and iron in the periplasmic space is required for induction (Van Hove *et al.*, 1990; Staudenmaier *et al.*, 1989).

The presence of a second pseudobactin uptake system has been demonstrated in *Pseudomonas* sp. strain M114. However, in contrast to the second uptake system of *P. putida* WCS358, no residual pseudobactin M114 uptake was observed in a mutant lacking the 89 kDa pseudobactin M114 receptor, the mutant utilising a different siderophore, pseudobactin MT3A, in iron uptake via an 81 kDa IROMP (Morris *et al.*, 1992). An iron regulated promoter has been isolated from *Pseudomonas* sp. strain M114, and found to show some DNA homology to four other iron-regulated pseudomonad promoters, as well as to the 19 basepair Fur-binding consensus sequence in *E. coli*. (O'Sullivan and O'Gara, 1991), as has a gene involved in ferric-siderophore dissociation (O'Sullivan *et al.*, 1990).

1.5.2. Siderophores from *Pseudomonas* sp.: pyochelin.

P. aeruginosa PAO1 (ATCC 15692) can also produce a second siderophore called pyochelin (Cox and Graham, 1979) which is structurally different to the pyoverdins (Demange *et al.*, 1987). Pyochelin comprises a salicyl ring bonded to a thiazoline ring which is itself bonded to a *N*-methylthiazolidine ring and is presumed to be biosynthesized from salicylic acid and two cysteinyl residues (Cox *et al.*, 1981). Pyochelin is produced in smaller amounts than pyoverdin, and seems less efficient at binding iron, having an association constant for iron $K = 2.4 \times 10^5$ (compared to $K = 10^{32}$ for pyoverdin) at neutral pH.

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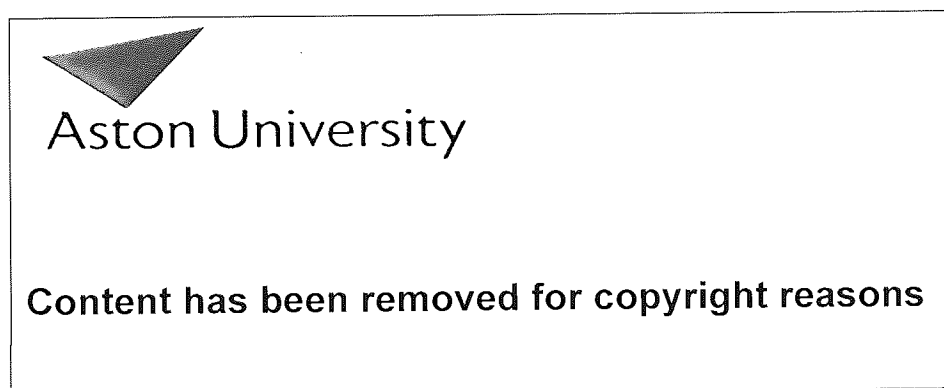
Figure 1.5. The structure of pyochelin from *Pseudomonas aeruginosa* PAO1 (Cox *et al*, 1981)

Visca *et al.* (1992) noted that, in contrast to pyoverdin, pyochelin could form complexes with various transition metals, that pyochelin secretion was repressible by metal concentrations in excess of 100 μM, and that these metal-pyochelin complexes were growth-promoting. These observations led to the notion that pyochelin may be involved in uptake of transition metals other than iron into *Pseudomonas* sp. cells. There may also be a requirement for the uptake of zinc, as Höfte *et al.* (1992) noted that both pyoverdin and pyochelin production were enhanced in the presence of this metal.

It would appear from the binding constants of pyoverdin and pyochelin that pyochelin is of little importance in bacterial iron acquisition in growth media containing little or no free iron. This supposition was supported by experiments with siderophore-deficient mutants of *Pseudomonas aeruginosa* PAO1, using a pyoverdin- and pyochelin-deficient mutant, IA1, a pyochelin-deficient mutant, IA5, a pyoverdin-deficient mutant, 211-5, and the wild type, PAO1. Strain IA5 can grow just as well as its parent, PAO1, in serum and in the presence of transferrin. Conversely, both pyoverdin mutants (IA1 and 211-5) had severely retarded growth, with the only apparent advantage of 211-5 over IA1 being that it exhibited an accelerated log phase at the later stages of the growth curve, in minimal medium. Strain 211-5 had no observable advantage over IA1 when grown in

human serum. Pyochelin synthesis in PAO1 can be inhibited when grown in glucose-minimal media, and so this may explain the identical growth chracteristics of 211-5 and IA1 in this medium. If these mutants are grown in media which allow pyochelin synthesis, mutant 211-5 still does not grow as well as IA5, although it does reach almost the same cell density. If pyochelin is added to the growth media, almost identical growth characteristics are observed, indicating that 211-5 may be unable to produce pyochelin at a sufficient rate (Ankenbauer *et al*, 1985). This inability to produce sufficient pyochelin under certain growth conditions, coupled with the possibility that pyochelin binds to serum albumin (as do other phenolate siderophores, thus rendering them unavailable) may explain the need for PAO1 to produce an alternative siderophore (pyoverdin) when iron is limited. However, pyochelin utilisation (but not necessarily production) is ubiquitous in both *P. aeruginosa* clinical isolates (Cox and Graham, 1979) and *P. cepacia* clinical isolates (Sokol, 1986), suggesting that this siderophore may be more important in pathogenicity than recent studies indicate.

Two iron-regulated outer membrane protein (IROMP) uptake systems have been identified for this siderophore. Firstly, a 14 kDa ferri-pyochelin binding protein was detected (Sokol and Woods, 1983) and mutants deficient in surface expression of this protein showed reduced ferripyochelin uptake (Sokol, 1987). Secondly, a 75 kDa IROMP has been shown to be associated with ferripyochelin uptake using protease protection, in which the 75 kDa protein was protected from trypsin digestion by binding of ferripyochelin and labelled iron transport studies (Heinrichs *et al.*, 1991; Ankenbauer, 1992; Ankenbauer and Quan, 1994). Mutants deficient in expression of the 14 kDa protein were still able to transport ferripyochelin, whereas mutants deficient in expression of the 75 kDa protein were unable to transport ferripyochelin; these observations led Ankenbauer (1992) to suggest that the 14 kDa OM protein may act to stabilise ferripyochelin binding to the 75 kDa receptor, while Heinrichs *et al.* (1991) suggested that the 14 kDa system operated in logarithmic phase cells, whereas the 75 kDa IROMP transporter was of greater importance in late logarithmic and early stationary phase, where iron deficiency becomes more acute. An additional 68 kDa OMP was noted by Visca *et al.* (1992) in response to pyochelin-mediated transition metal (other than iron) uptake, although it is not known if there is any role of this protein in ferripyochelin uptake.

Heinrichs and Poole (1993) have cloned and sequenced a transcriptional activator of pyochelin and ferripyochelin receptor synthesis (PchR), and identified a region upstream of *pchR* with homology to the consensus binding region for the Fur repressor. Pyochelin receptor synthesis has been shown to be inducible in the presence of pyochelin (Gensberg *et al.*, 1992); therefore, PchR may be regulated posttranscriptionally by pyochelin in activating pyochelin receptor synthesis, and may possibly also enhance production of pyochelin (Heinrichs and Poole, 1993).

1.5.3. Other siderophores utilised by *Pseudomonas* sp.

In addition to the siderophores pyoverdin and pyochelin, *P. aeruginosa* can utilise a number of exogenous siderophores which it does not synthesise, such as heterologous pyoverdins from other pseudomonads (Cornelis *et al.*, 1989; Chapter 4) and enterobactin (Poole *et al.*, 1990).
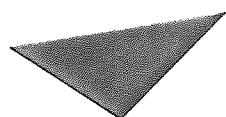
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Figure 1.6. The structure of enterobactin (Tor *et al.*, 1992).

Enterobactin is secreted under conditions of iron restriction by *E. coli*, *K. pneumoniae*, *S. typhimurium* and some species of *Shigella*. This compound, which is the cyclic triester of 2,3-dihydroxybenzoyl-serine, removes iron from iron-binding proteins and promotes bacterial growth (Brock *et al.*, 1991); enterobactin has the highest binding constant for any ferric chelator, at around 10^{52} at neutral pH (Griffiths, 1987b). It is proposed that hydrolytic cleavage of the bonds between the 2,3-dihydroxybenzoyl-serine residues by the action of ferric enterobactin esterase is required to release iron from the very stable ferric complex (Brickman and McIntosh, 1992). Enterobactin may serve an additional purpose in infection, since it has been shown that enterobactin is cytotoxic to mouse T-cells, thereby causing immunosuppression of the host (Autenreith *et al.*, 1991).

Growth of a *P. aeruginosa* mutant deficient in pyoverdin production in the presence of enterobactin and EDDHA resulted in the expression of a novel 80 kDa IROMP, which is comparable to the 81 kDa ferric enterobactin receptor FepA in *E. coli*, and enhanced growth on minimal media (Poole *et al.*, 1990). The ferric enterobactin

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receptor in *P. aeruginosa*, PfeA, has been cloned and sequenced (Dean and Poole, 1993a), and was found to have 60% homology to the *E. coli* FepA protein, as well as immunological cross-reactivity (Rutz *et al.*, 1991). The role of iron in the regulation of PfeA was confirmed with the finding of a Fur box upstream of *pfeA*, overlapping a putative promoter sequence. Induction of this receptor due to presence of enterobactin, was found to be regulated by two proteins, PfeR in the cytoplasm and PfeS located on the cytoplasmic membrane, whose amino acid sequences showed considerable homology to the regulator and sensor components, respectively, of the superfamily of regulatory protein pairs (Dean and Poole, 1993b).

Salicylic acid, a precursor in the synthesis of pyochelin (Ankenbauer *et al.* 1991), also appears to act as a siderophore in *P. fluorescens* CHAO (Meyer *et al.*, 1993), *P. aeruginosa* (Visca *et al.*, 1993) and *P. cepacia* (Sokol *et al.*, 1992; Visca *et al.*, 1993). Its role in iron uptake is discussed in greater depth in Chapter 4.

Pseudomonas sp. strain X40, under iron limiting conditions, has been shown to produce and utilise the siderophore aerobactin, a dihydroxamate siderophore previously found only in the *Enterobacteriaceae*, together with two IROMPs, of which at least one is believed to act as the cognate receptor for this siderophore. These IROMPs were found to be dissimilar to the aerobactin receptor of *E. coli* (Buyer *et al.*, 1991).

A variety of other siderophores have been found to operate in *Pseudomonas* sp., such as citrate in *P. aeruginosa* (Cox, 1980; Harding and Royt, 1990), ferrichrome (Jurkevitch *et al.*, 1992) and mugineic acid (Jurkevitch *et al.*, 1993) in *P. putida*, and cepabactin (Meyer *et al.*, 1989) and the ornibactins, which are linear hydroxamate tetrapeptide siderophores (Stephan *et al.*, 1993), in *P. cepacia*.

The considerable variation in siderophore-mediated iron uptake systems in pseudomonads clearly demonstrates the vital role of this element in metabolism.

1.6. Regulation of siderophore expression by the *fur* gene product.

All bacteria, with the notable exception of the lactobacilli, require iron for the synthesis of a variety of both heme- and non heme-containing enzymes. It is generally assumed that aerobic growth will create a greater demand for iron, for the synthesis of cytochromes. In iron-limited environments, incoming iron into the cell is diverted away from cytochrome synthesis, thus directing the cell into a more fermentative mode, with organic acids accumulating inside the cell rather then being oxidised through the TCA cycle. The intracellular level of iron is controlled by regulation of the uptake mechanisms, iron being stored intracellularly inside ferritin molecules, so that the toxic effects of unbound iron, such as the catalytic generation of free radicals, can be avoided. Ferritin synthesis in eukaryotic systems is controlled by binding of a repressor protein to the mRNA, when the intracellular iron concentration is low; upon increased intracellular iron

concentration, the repressor dissociates from the mRNA, allowing it to be translated into ferritin protein molecules (Munro, 1990). Less is known about regulation of bacterioferritin synthesis in prokaryotes; no consensus Fur-binding site has been identified has been identified upstream of the *E. coli* K-12 bacterioferritin gene (*bfr*), although it is suggested that a Fur-regulated antisense mRNA may base-pair with the *bfr* transcript in the absence of Fe^{2+}, leading to repression of the *bfr* gene. In the presence of iron, antisense mRNA production is repressed by the Fe^{2+}-Fur complex, leading to derepression of the *bfr* gene (Andrews *et al.*, 1989)

The bacterial response to low intracellular iron concentrations is generally the elaboration of siderophores into the environment, coupled with production of their cognate receptors, for the capture and delivery of iron(III) to the cell. A constitutively derepressed mutant of *E. coli* was isolated which expressed siderophore biosynthesis and ferric siderophore uptake systems irrespective of iron availabilty, and designated a *fur* (ferric uptake regulation) mutant. It was considered that this mutant could have been deficient in a repressor. This repressor was cloned and sequenced (Schäffer *et al.*, 1985), and shown to regulate β-galactosidase synthesis in an *in vitro* transcription-translation system from *lac Z* fused in the aerobactin operon. Activity was found to be totally dependent on the presence of a divalent heavy-metal ion as corepressor, and it was reasoned that the biologically active metal species is iron(II). Divalent ions chemically close to Fe^{2+}, such as Co^{2+} or Mn^{2+}, can mimic the effect of Fe when complexed with Fur; however, these elements are present in such small amounts in the environment in relation to iron that they could not interfere appreciably with the complexation of iron with Fur, and thus iron regulation of genes (Hennecke, 1990). The Fur repressor may become deactivated by decreased Fe(II), resulting in dissociation from the operator and allowing synthesis of systems for siderophore synthesis and transport.

The Fur protein is unusually rich in histidine residues, and has a molecular mass of 17 kDa; it lacks significant homology with any of the known DNA-binding proteins (Briat, 1992). In *E. coli* iron-regulated promoter regions studied, the metalloregulatory Fur-Fe^{2+} complex binds to a highly A + T rich palindromic 19 base pair consensus DNA recognition sequence (the "iron box"), of the nucleotide sequence 5'-G A T A A T G A T A A T C A T T A T C - 3' (Figure 1.7.).
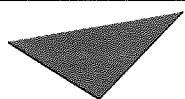
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Figure 1.7. Proposed mechanism of action of the Fur repressor (Litwin and Calderwood, 1993).

In the aerobactin operon, the occupation of two contiguous binding sites is required for complete repression, with the primary binding site at -35 bases from the transcription start site, and the secondary binding site covering -10 bases and the transcription start site. The Fur repressor may cover the binding sites as if the Fur-Fe^{2+} complex wraps around the DNA in a screw-like fashion, probably binding as a dimer (Neilands, 1990).

The *fur*-repressor gene itself is negatively regulated by its own gene product (which requires heavy metal activation), with the repressor having an affinity for its own operator 40 times less than that for the operator site in the *iuc* promoter of the aerobactin operon, where *iucABCD* are the biosynthetic genes for aerobactin (Bagg and Neilands, 1987; Neilands, 1990). Although there is much less information regarding regulation of iron uptake in *Pseudomonas aeruginosa*, it is becoming increasingly apparent that there is

a *fur*-like gene in this species also, which would appear to span 7kb of DNA and have similar functions to the *E. coli fur* gene (Prince *et al.*, 1993). It should be emphasised that siderophore receptor expression may be induced in the presence of the specific siderophore (Van Hove *et al.*, 1990; Staudenmaier *et al.*, 1989; Leong *et al.*, 1991; Chapter 4), indicating that a means of fine adjustment of the expression of siderophore-mediated iron-uptake systems exists.

The Fur repressor protein is not involved only in regulation of genes whose products participate in iron uptake systems; Privalle and Fridovich (1993) noted that Fur represses biosynthesis of superoxide dismutase, which was also repressible by the binding of the global repressor ArcA (aerobic respiration control) at a similar site (Tardat and Touati, 1993), while Hantke (1987) noted a reduced ability to uptake succinate in *fur* mutants, suggesting that Fur may act as a positive regulator of this system. This supposition is supported by the findings of Foster and Hall (1992), who noted both negative and positive regulatory effects due to Fur in *Salmonella typhimurium*. Additionally, a number of virulence factors, such as Shiga toxin from *S. dysenteriae*, diphtheria toxin from *C. diphtheriae*, and exotoxin A from *P. aeruginosa* have been found to be Fur-regulated (Litwin and Calderwood, 1993; Mekalanos, 1992; Prince *et al.*, 1991).

No Fur repressor protein has yet been isolated in *Vibrio anguillarum*; however, iron concentration in the medium has been found to correspond with repression of mRNA for iron transport genes in addition to the increased expression of antisense mRNAs; thus, antisense mRNAs may constitute a novel mechanism of regulation of iron uptake systems (Salinas *et al.*, 1993).

Undermodification of tRNAs may also occur under conditions of iron stress in *E. coli* and *S. typhimurium* when grown in the presence of transferrin, lactoferrin or ovotransferrin, in which the altered tRNAs are those which recognise codons with a 5'-uridine, and include the tRNAs for phenylalanine, tyrosine, tryptophan and serine (Griffiths, 1987b). These modifications may act to increase the rate of spontaneous mutations when cells are subjected to environmental stress. Interestingly, this process does not appear to be influenced by Fur (Griffiths, 1991).

1.7. Iron and anaerobiosis.

In *E. coli*, the expression of a variety of anaerobic functions is mediated by the FNR (to denote the defects in fumarate and nitrate reduction) protein, which activates transcription of specific genes, such as those involved in the anaerobic utilisation of fumarate and nitrate as terminal electron acceptors, in response to lowered oxygen levels (Sawers, 1991). It is predicted that the FNR protein contains a DNA-binding domain and a nucleotide-binding domain, and it has been shown that FNR contains an N-terminal

cluster of four cysteine residues resembling a metal, probably iron, binding site, which may perform a redox-sensing function in converting FNR to its active DNA-binding conformation in conditions of oxygen depletion. Ferrous iron may signal the onset of oxygen depletion by inducing the transcriptionally active DNA-binding form of FNR (Green and Guest, 1993). Once more aerobic conditions are encountered, ferrous iron may be oxidised to the ferric form and be released from FNR, rendering it inactive. In this way, *E. coli* may detect the extent of availabilty of oxygen by the intracellular concentration of Fe^{2+} (Green *et al.*, 1991). Mutations in the *fnr* gene which affect the putative iron binding site give rise to non-functional FNR proteins which activate anaerobic metabolism under aerobic conditions (Guest, 1992). In contrast to this hypothesis, Niehaus *et al.* (1991) concluded that the switch of FNR from aerobic to anaerobic conformation is not regulated by the intracellular iron level and binding of ferrous iron under physiological conditions.

An FNR-like protein, ANR, has been identified in *P. aeruginosa* (Sawers, 1991), and the *anr* gene sequenced; it showed 51% identity with the *fnr* gene at the amino acid level, and retained the putative iron-binding site seen in FNR. However, the role of iron in activation of ANR is unknown (Zimmermann *et al.*, 1991), although it has been shown to activate transcription by upstream binding of the *arcDABC* operon, involved in the generation of ATP from arginine, under anaerobic conditions (Gamper *et al.* 1991; Verhoogt *et al.*, 1992; Galimand *et al.*, 1991). The role of iron, if any, in the activation of ANR is yet to be established.

1.8. Pseudomonas aeruginosa - pathogenesis of infection in cystic fibrosis (CF) patients.

CF is the most prevalent of the fatal inherited diseases in white populations, resulting in an average survival time for sufferers of 25-30 years (Høiby and Koch, 1990). The genetic defect responsible for CF occurs in the CFTR (cystic fibrosis transmembrane conductance regulator protein), characterised by cellular impermeability to chloride ions in sweat duct epithelial cells, CF pancreatic duct cells, and CF airway epithelium, contributing to the increased viscosity and osmotic potential of mucous secretions which are less easily cleared from the lung by ciliary action (Pedersen, 1992). Once a *P. aeruginosa* infection has become established in the CF lung, it generally persists, with cross-infection between CF patients at treatment centres also occurring (Fegan *et al.*, 1991; Høiby and Koch, 1990; Richardson *et al.*, 1991) since these patients use various equipment for diagnosis and therapy, any of which may be contaminated (Høiby, 1988). *P. aeruginosa* isolates from CF patients generally are auxotrophic (Taylor *et al.*, 1992), siderophore-producers (Haas *et al.*, 1991), and producers of alginate (Pedersen *et al.*, 1992a) in combination with other virulence factors.

The major stages of *P. aeruginosa* lung infection are acquisition of the bacteria; adhesion of the bacteria to the surface of the host tissue (Prince, 1992; Saiman *et al.*, 1990); bacterial proliferation and invasion, leading to tissue damage and clinical symptoms; recruitment of non-specific and specific immunological defence mechanisms (Brett *et al.*, 1992; Sørensen *et al.*, 1990); and, if the patient survives, elimination of the bacteria or establishment of chronic infection (Table 1.4.).
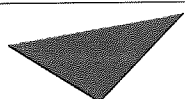
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Table 1.4. Pathogenesis of chronic *P. aeruginosa* infection in CF (Høiby and Koch, 1990).

Traditional therapy consists of administration of antibiotics either singly, or in combinations (Bosso *et al.*, 1990; Geddes, 1988), generally resulting in clinical improvement despite the fact that the organism is rarely eradicated (Ferguson *et al.*, 1991). Such management of chronic infections has led to 90% of sufferers surviving more than 10 years of chronic infection, whereas prior to this only 54% survived more than 5 years (Hoiby and Koch, 1990). A more radical treatment for end-stage lung disease in CF lies in the form of transplantation, either of heart and lungs, or of lungs alone (Lewiston *et al.*, 1990). Saiman *et al.* (1989) have suggested that pilin proteins may be used as a vaccine, since pili are instrumental in initial binding of *P. aeruginosa* to

respiratory epithelium. Semi-synthetic antibiotics which utilise bacterial iron uptake systems for delivery of antibiotics into the cell are showing considerable potential, and may become instrumental in eradication of chronic *P. aeruginosa* infections (see chapter 6).

P. aeruginosa infection is notoriously difficult to eradicate due to its innately high resistance to antibiotics (Yoshihara and Nakae, 1992), its ability to evade host immune response (Pedersen, 1992), and its ability to acquire essential nutrients, notably iron (Griffiths, 1990), from the host. The production of a number of virulence factors, which enhance the pathogenicity of this organism, are briefly reviewed.

1.9. Virulence factors of Pseudomonas aeruginosa.

A number of virulence factors have been described in *P. aeruginosa* , which enable it to invade, colonise, exploit, and ultimately cause the death of its host. However, despite the apparently high pathogenicity of this organism, *P. aeruginosa* is unable to cause disease in a host whose immune system is not impaired, and if the host is in otherwise good health (Bergan, 1981). Pseudomonal proteases (elastase and alkaline protease) inhibit the function of phagocytes and natural killer and T lymphocytes by cleaving the CD4 molecule on T-helper cells, inhibiting IL-1 and IL-2 activity, cleaving immunoglobulins, and inactivating complement components. The proteases of *P. aeruginosa*, and possibly toxins such as exotoxin A and S, may be important in the establishment of the initial colonisation by preventing the host from mounting a local immune response, and by inhibiting non-specific defence mechanisms (Wick *et al*, 1990). These virulence factors are summarised below:
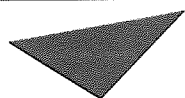
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1.9.1. Elastase.

Pseudomonal elastase, a zinc metalloprotease with a broad substrate specificity, cleaves a number of biologically important host molecules such as elastin, collagen, immunoglobulins, some complement components, and human transferrin (but not lactoferrin), releasing iron to the bacteria and inactivating the host immune response (reviewed by Gambello and Iglewski, 1991). Elastase is similar to several other bacterial proteases that have been well characterised, such as the neutral proteases of the *Bacillus* species, an extracellular zinc metalloprotease from *Legionella pneumophila* and the HA/protease from *Vibrio cholera*, and has been demonstrated to cause tissue destruction by its proteolytic enzyme activity. Elastase is distinguished from alkaline protease, another proteolytic enzyme produced by *P. aeruginosa*, by its ability to degrade elastin, which is present in lung tissue (28%) to allow expansion and contraction. However, the elastolytic activity of elastase is far exceeded by its proteolytic activity, the elastolytic activity being approximately a quarter that of *Bacillus* thermolysin, and a seventh of that of purified human neutrophil elastase; conversely, the proteolytic activity of elastase toward casein is four times that of trypsin, and about ten times that of pseudomonal alkaline protease. Therefore, elastase may be regarded as a proteolytic enzyme with some elastolytic activity.

Elastin degradation during a *P. aeruginosa* infection is usually achieved in concert with at least one other protein, LasA, which "nicks" the elastin structure, thereby rendering it more susceptible to degradation by elastase (the *lasB* gene product). The activity of LasA also enhances the elastolytic activity of other (non-pseudomonal) proteases, such as thermolysin, human neutrophil elastase, and proteinase K (Peters *et al.*, 1992). Any other proteases that may be present, particularly the pseudomonal alkaline protease, enhance elastin degradation (Freck-O'Donnell and Darzins, 1993). It has been shown that elastolysis may occur in the absence of elastin, since a *P. aeruginosa* LasB mutant still showed some elastolytic activity due to the combined activities of LasA and alkaline protease (Wolz *et al.*, 1991), and to LasA alone (Toder *et al.*, 1991).

Elastase is recovered from the supernatant of appropriate *P. aeruginosa* cultures, and appears to have the molecular size of 33 kDa. Analysis of the elastase structural gene (*lasB*) suggests that a preproelastase of 498 amino acid residues is processed and secreted as a mature protein of 301 amino acids. Preproelastase, weighing 53.6 kDa, appears to be transported across the cytoplasmic membrane where the signal peptide sequence is removed to yield another precursor in the periplasm, proelastase I, of approximately 50 kDa. Proelastase I is rapidly cleaved to yield the smaller (33.5 kDa) proelastase II and a 17 kDa component; this 17 kDa component is thought to remain associated with proelastase II, thereby inhibiting its proteolytic activity. Proelastase II passes out through

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the outer membrane, possibly aided by the 17 kDa protein, where it is found as the 33 kDa mature protein:
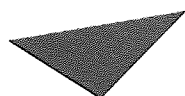
Figure 1.8. Schematic diagram of the postulated mechanism of elastase secretion. IM = inner membrane; PG = peptidoglycan; OM = outer membrane (Galloway, 1991)

McIver *et al.* (1993) produced a *lasB* mutant of *P. aeruginosa* by gene replacement resulting in an elastase with an amino acid replacement at the active site. The resulting defective elastase was found consequently to have reduced activity, with a concomitant decrease in secretion, indicating that autoproteolytic processing of proelastase is necessary for efficient secretion of elastase, or that the active site is important for binding of cleaving enzymes. Reduced secretion of the defective elastase was attributed to processing by other proteolytic enzymes at the same site.

Wolz *et al.* (1991) have shown that processing of LasA is independent of LasB, since LasA secretion was unaffected in a LasB mutant, and suggest that alkaline protease, an autoproteolytic process, or another unknown protease is responsible for LasA processing.

Expression of elastase has been shown to be regulated by the presence of zinc and iron (Jensen *et al.*, 1980), where zinc is required for optimal elastase secretion, and the absence of iron has also been shown to have a slight stimulatory effect on elastase secretion (Brumlik and Storey, 1992). Since elastase is a zinc metalloprotease, this finding is not surprising. The increase in secretion of elastase under low-iron conditions is interesting, since elastase secretion is beneficial to *P. aeruginosa* when it causes disease. However, the effect of iron is small when compared to the regulation of expression by the transcriptional activator, LasR (Gambello and Iglewski, 1991). LasA production is also stimulated by the presence of zinc, where zinc is postulated to be involved in the processing of the inactive 41 kDa LasA protein to its active 21 kDa form. Calcium was also shown to enhance processing of both elastase and LasA, especially

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under conditions of low zinc availability, where it is thought to be involved in alternative processing mechanisms (Olson and Ohman, 1992).

The *lasR* gene has been cloned and sequenced (Gambello and Iglewski, 1991), and it has been shown that the LasR protein is required for transcription of the elastase structural gene (*lasB*) and the *lasA* gene to occur (Toder *et al.* 1991). Passador *et al.* (1993) identified a second elastase regulatory gene, *lasI*, which is involved the synthesis of a small effector molecule which is readily diffusible, termed PAI (*P. aeruginosa* autoinducer). Both *lasI* and *lasR* genes are required for maximum *lasB* expression, while experiments performed in *E. coli* indicate that the LasR protein and PAI are sufficient for expression of *lasB*; therefore, the LasR virulence regulator protein requires the diffusible inducer molecule PAI, produced by *P. aeruginosa*. The *lasR/I* autoinducer system shows homology to the *luxR/I* system for bioluminescent *Vibrio* sp. and antibiotic production by *Erwinia carotovora* (Jones *et al.* 1993).

1.9.2. Alkaline protease.

As previously stated, alkaline protease may participate in elastin degradation in the presence of LasA, and it potentiates the elastolytic activity of elastase with LasA. Alkaline protease has been shown to be independent of the general secretion mechanisms, as it does not appear to possess a signal sequence which is cleaved during transport across the inner membrane (Guzzo *et al.*, 1991; Duong *et al.*, 1992). In common with elastase and LasA, alkaline protease secretion appears to be regulated by LasR at the transcriptional level (Gambello *et al.*, 1993).

1.9.3. Exotoxin A and exoenzyme S.

P. aeruginosa produces two distinct ADP-ribosyl transferase toxins, exotoxin A and exoenzyme S. Exoenzyme S appears to cause tissue damage in lung and burn infections by acting on vimentin, a cellular structural component, and GTP-binding proteins (Wick *et al.*, 1990). Exoenzyme S production, which has been shown to be unrelated to extracellular iron concentration, is involved in the dissemination of infection from the site of colonisation into the bloodstream, often resulting in fatal sepsis (Frank and Iglewski, 1991). Its role as an adhesin, facilitating cell binding to host respiratory tract epithelium, has also been demonstrated (Baker *et al.*, 1991). Exotoxin A (the *toxA* gene product) is the most toxic *P. aeruginosa* product, which acts to halt eukaryotic protein synthesis at the level of chain elongation by causing ADP-ribosylation of elongation factor 2, in an identical manner to that of diphtheria toxin, thereby causing cell death (Hamood *et al.*, 1992). It appears that exotoxin A may form a transmembrane pore,

which is an essential feature of its toxicity (Gambale *et al.*, 1992).

Regulation of exotoxin A activity involves a number of different elements; transcription of the *toxA* gene is repressed when the iron concentration in the medium is high, and derepressed when the iron concentration is low, (Frank and Iglewski, 1988) and this regulation by iron is thought to occur through the *regAB* operon. The *regAB* operon, a positive activator of *toxA* transcription, is controlled by two promoters, where transcription from the P1 promoter occurs early in the growth cycle and is not tightly iron-regulated, while transcription from the P2 promoter occurs late in the growth cycle and is tightly iron-regulated (Storey *et al.*, 1991). Prince *et al.* (1991) showed that the *E. coli fur* gene, when present in multiple copies in *P. aeruginosa*, repressed exotoxin A production and transcription of *regA.*. A *fur* gene has been found in *P. aeruginosa*, which was shown to coordinately regulate siderophore and exotoxin A production (Prince *et al.*, 1993)

1.9.4. Alginate and lipopolysaccharide.

Alginate is a secreted exopolysaccharide which is rarely expressed by *P. aeruginosa* in its natural environment (soil and water); however, alginate production results in development of a mucoid, capsule-like coating found in 94% of the cases of *P. aeruginosa* lung infections in CF patients. CF patients colonised with non-mucoid *P. aeruginosa* showed similar lung capacity and body weight profiles as uncolonised CF patients (Pedersen *et al.*, 1992a). Mucoidy of *P. aeruginosa* lung infections is associated with unusually high resistance to natural host defense and clearance mechanisms, eliciting hyperimmune response from the host, and may be triggered by the osmolarity of lung secretions in CF patients (Mohr *et al.*, 1991). Conversion to a mucoid phenotype may be associated with rearrangement of *P. aeruginosa* chromosomal DNA (Shortridge *et al.*, 1991), which is itself associated with qualitative or quantitative changes in extracellular virulence factors and LPS serotype (Woods *et al.*, 1991). *P. aeruginosa* lipopolysaccharide (LPS) has been shown to be immunogenic, as evidenced by very high IgA anti-lipid A titres in CF sputum samples (Kronberg *et al.*, 1992), and LPS composition may vary depending on environment (Kelly *et al.*, 1990). It is suggested that LPS production may, like alginate, act as a barrier to the host immune system, as well as being highly immunogenic, thereby causing the tissue damage associated with hyperimmune complex formation in the CF lung.

1.9.5. Pyocyanin.

Pyocyanin (*N*-methyl-1-hydroxyphenazine) is a blue secondary metabolite of *P.*

product, leukopyocyanin, which itself can reduce both oxygen and Fe(III). The ability of leukopyocyanin to reduce Fe(III) has been demonstrated to be sufficient for the removal of iron from transferrin (Cox, 1986).
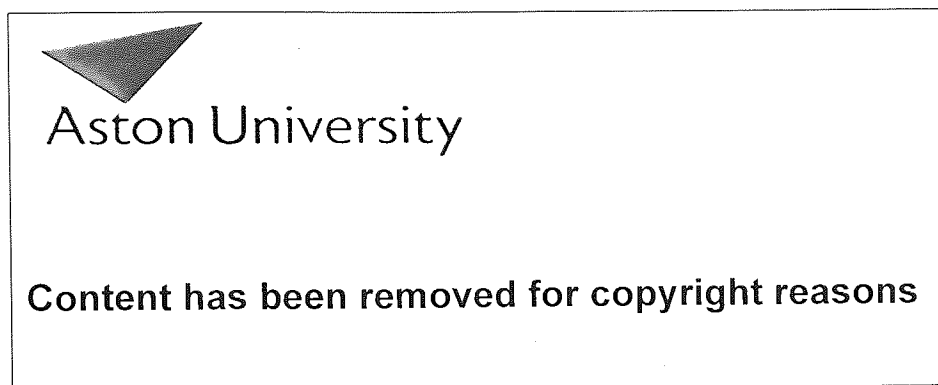
Figure 1.9. The structure of pyocyanin (Budzikiewicz, 1993).

In addition, pyocyanin may undergo redox cycling under aerobic conditions, resulting in the generation of superoxide and hydrogen peroxide. These free radicals may account for much of the bactericidal activity of pyocyanin (Britigan et al., 1992). Ferripyochelin, a complex of iron and the P. aeruginosa siderophore pyochelin, is also capable of catalysing the generation of free radicals, in this case the more potent hydroxyl radicals (Coffman et al., 1990). Interaction of pyocyanin and ferripyochelin has been shown to result in the generation of hydroxyl radicals, and cause synergistic free radical damage to host tissues at the site of P. aeruginosa infection (Britigan et al., 1992). P. aeruginosa appears to protect itself from the bactericidal properties of pyocyanin by increased production of antioxidant enzymes, such as superoxide dismutase, catalase and peroxidase, to eliminate the potentially toxic effects of oxygen reduction products (Hassett et al., 1992).

1.10. Aims of the study.

Understanding the role of iron in the pathogenicity of Pseudomonas aeruginosa may prove to be fundamental to the development of treatments to eradicate Pseudomonas aeruginosa infections in the human host. The aim of this research was to investigate some of the mechanisms involved in iron uptake in Pseudomonas aeruginosa. In response to iron limitation, Pseudomonas aeruginosa expresses a number of IROMPs; Chapters 4 and 5 describe the studies made on these proteins, which led to an investigation into the utilisation of these proteins in the delivery of antimicrobial agents against this notoriously resistant and durable pathogen, described in Chapter 6. The role of a positively iron-regulated outer membrane protein, OprG, was examined in Chapter 7.

2. MATERIALS

2.1. Bacterial strains.

P. aeruginosa IA1, a Pvd-, Pch- mutant of PAO1 (ATCC 15692) was obtained from Dr C. D. Cox (Ankenbauer *et al.*, 1985).

P. aeruginosa PAO1 (ATCC 15692) was obtained from the Aston Collection.

The *P. aeruginosa* clinical isolates 0:1, 0:3, 0:4, 0:5, 0:6, 0:7, 0:8, 0:9, 0:10, 0:11, 0:12, 0:13, 0:14, 0:15 and 0:16 were from cystic fibrosis patients, donated by Dr. Ty Pitt (Division of Hospital Infection, Public Health Laboratory Service, Colindale Avenue, London).

P. aeruginosa PH1, a mutant of 0:9 deficient in both pyoverdin production and the ferri-pyoverdin receptor, was obtained from the Aston Collection (Smith *et al.*, 1992).

J1003, a pyocin Sa-producing *P. aeruginosa* strain, was donated by Dr. J.R. Govan (Department of Bacteriology, University of Edinburgh Medical School, Edinburgh).

E. coli XL-1 (*supE44 hsdR17 recA1 endA1 gyrA46 thi relA1 lac⁻* ; F'[*proAB⁺ lacI^q lacZ*ΔM15 Tn10(*tet^r*)])was supplied by Stratagene Ltd., Cambridge, England.

E. coli HB101 (*supE44 hsdS20*(r_B⁻m_B⁻) *recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1*) was obtained from the Aston Collection.

The pBluescript phagemid vector, which contains a multicloning site inside a functional *lacZ* gene to allow blue/white colour selection of recombinant phagemids on suitable selection medium, a ColE1 origin of replication used in the absence of R408 helper phage, and an f1 origin of replication for use with R408 helper phage,was supplied by Stratagene Ltd., Cambridge, England.

R408 helper phage, which provides all of the gene products necessary for nicking, displacing, re-circularising and packaging single-stranded phage particles from the pBluescript vector, was supplied by Stratagene Ltd., Cambridge, England.

The monoclonal antibody mAb C108 was prepared using a purified preparation of an 85

kDa IROMP from *P. aeruginosa* AK1282, an LPS-defective mutant of *P. aeruginosa* PAO1 (Smith *et al.*, 1991).

2.2. Chemicals.

All chemicals and reagents not specified in the text were supplied by BDH Chemicals Ltd., (Poole, Dorset), Sigma Chemical Co. (Poole, Dorset), and Fisons (Loughborough, Leics.) and were of Analar grade or equivalent.

The antibiotic BRL 41897A, a C(7)α - formamido substituted cephalosporin as described by Critchley *et al*, (1991), was kindly donated by SmithKline Beecham Pharmaceuticals.

2.3. Radiochemicals.

Iron (^{55}Fe-III) chloride in aqueous solution in 0.5M HCl; 1 ml at 20 mCi/ml, E.I. DuPont de Nemours & Co. Ltd. (Wilmington, DE, USA).

Deoxyadenosine [γ-^{32}P] triphosphate in aqueous solution; 25 μl at 10 mCi/ml, Icn Radiochemicals (Irvine, CA, USA).

Deoxyadenosine 5'-[α-^{35}S] triphosphate, triethylammonium salt in 20 mM dithiothreitol aqueous solution; 25 μl at 10 mCi/ml, Amersham International plc, (Amersham, Bucks).

2.4. Complex media.

Luria broth = 1% tryptone/ 0.5% yeast extract/ 0.5% NaCl pH 7.5

SOC recovery medium   =   2% Bacto tryptone
                          0.5% Bacto yeast extract
                          10mM NaCl
                          2.5mM KCl
                          10mM MgCl$_2$
                          10mM MgSO$_4$
                          20mM glucose

CAA = casamino acids medium = 0.5% casamino acids/ 0.4% MgSO$_4$.7H$_2$O

SuperBroth = 35g Bacto-tryptone/ 20g yeast extract/ 5g NaCl/ 1 ml 5M NaOH in 1 l.

2.5. Chemically defined media.

CDM-Fe (chemically defined medium) was made

CDM-Fe was made from 10x stock solutions to the same specification as CDM10, 10 being the theoretical optical density at 470nm which can be obtained by growth of cells in this medium (Noy, 1982) but without the addition of iron:

10x glucose solution = 72g/l
10x CDM (1 litre)   =   0.462g KCl
                        0.292g NaCl
                        5.57g K_2HPO_4
                        52.8g $(NH_4)_2SO_4$

100x $MgSO_4.7H_2O$ (100 ml) = 0.986g autoclaved separately.
10x MOPS adjusted to pH 7.4 (1 litre) = 104.6g

1x CDM-Fe solution (1 litre) was prepared by mixing the above stock solutions as follows: 100ml CDM/ 100ml MOPS/ 690ml ddH_2O/ 10ml $MgSO_4.7H_2O$ and autoclaving the resulting solution. CDM-Fe agar was prepared in a similar manner, but with the addition of 1.5% Bacto agar before autoclaving. After the solution had cooled to 60°C, 100ml of the sterile 10x glucose solution was added aseptically.

Succinate medium = (per litre), 6.0g K_2HPO_4, 3.0g KH_2PO_4, 1.0g $(NH_4)_2SO_4$, 0.2g $MgSO_4.7H_2O$, 4.0g sodium succinate, and the pH adjusted to 7.0 by addition of NaOH prior to sterilisation (Meyer and Abdallah, 1978). Iron-replete succinate medium was supplemented with 100µM $FeCl_3$. For induction studies, purified pyochelin or pyoverdin were added at 10 µg/ml and 50 µg/ml, respectively.

2.6. Equipment.

Automatic pipettes: Gilson pipetman, P-20, P-200, P-1000 and P-5000 (Anachem Ltd., Luton, Beds.).
Balances: Sartorius 1702 and Sartorius U4800P Universal (Sartorius Instruments Ltd., Belmont, Surrey).
Centrifuges: Beckman J2-21, Beckman TL100 Ultracentrifuge (Beckman Instruments

Ltd., High Wycombe, Bucks.) and MSE Micro Centaur microfuge

DNA drier: Savant SpeedVac SVC100 (Savant Instruments Inc., Farmingdale, NY, USA).

Electroporation equipment: Bio-Rad Gene Pulser, Bio-Rad Pulse Controller, Bio-Rad electroporation cuvettes (Bio-Rad Laboratories Ltd., Watford, Herts.).

Freeze drier: Edwards Modylo freeze dryer (Edwards High Vacuum Ltd., Crawley, Sussex).

French press: Amicon Corp., High Wycombe, Bucks.

Gel electrophoresis equipment: Bio-Rad Mini-Protean system and Bio-Rad 21 x 40 cm Sequi-Gen sequencing cell (Bio-Rad Laboratories Ltd., Watford, Herts.).

Gel drier: Bio-Rad Model 583 gel drier (Bio-Rad Laboratories Ltd., Watford, Herts.).

Heated water bath: Grant JB1 (Grant Instruments (Cambridge) Ltd., Barrington, Cambridge).

Heating block: Techne Dri-Block DB-2A (Techne (Cambridge) Ltd., Duxford, Cambridge).

Hybridisation oven: Hybaid mini-hybridisation oven (Hybaid Ltd., Teddington, Middlesex).

Immunoblotting apparatus: Trans Blot Cell (Bio-Rad Laboratories Ltd., Watford, Herts.).

Incubators: Gallenkamp orbital shaking incubator (Gallenkamp, London) and Heraeus B6060 (Heraeus Equipment Ltd., Brentwood, Essex).

pH meter: Corning pH meter 220 (Ciba Corning Diagnostics Ltd., Sudbury, Suffolk).

Photographic equipment: Polaroid DS 34 Direct Screen Instant Camera (Polaroid Corporation, Cambridge, MA, USA) and Ricoh Mirai 35mm SLR camera (Ricoh Co. Ltd., Tokyo, Japan).

Power supplies: Bio-Rad computer-controlled electrophoresis power supply and Bio-Rad 200/2.0 power supply (Bio-Rad Laboratories Ltd., Watford, Herts.)

Scintillation counter: Packard 1600TR liquid scintillation analyzer (Canberra Packard, Pangbourne, Berks.).

Shaker: Hybaid HB-SHK 1 shaker (Hybaid Ltd., Teddington, Middlesex).

Spectrophotometers: LKB Ultrospec 4050 (LKB Biochrom, Cambridge) and Unicam SP8000 scanning UV spectrophotometer (Pye Unicam Instruments Ltd., Cambridge).

Spectrophotometer cuvettes: plastic macro (Gallenkamp, Loughborough, Leics.), quartz (Hellma, Westcliffe-on-Sea, Essex).

Syringes: Hamilton precision syringes 25µl and 50 µl (Hamilton Bonaduz AG, Switzerland).

Transilluminator: UVP TM 20 Transilluminator (UVP, San Gabriel, CA, USA).

Whirlimixers: Kinematica Vibrofix VF1 S3 Electronic Whirlimixer (Kinematica, Switzerland).

3. EXPERIMENTAL METHODS

3.1. Measurement of bacterial cell growth in liquid medium.

Changes in cell concentration during bacterial growth were followed using spectrophotometric measurement. At low cell concentrations the light scattered by a bacterial cell suspension is directly proportional to the cell concentration in the suspension, as expressed by the Beer-Lambert Law:

$$O.D. = Log (Io / I)$$

where Io = intensity of incident light and
I = intensity of the emergent light provided that the light path is constant

This relationship is obeyed between an optical density (O.D.) of 0.03 and 0.3 (Kenward, 1975). Measurements of absorbance were made at a wavelength of 470nm ($O.D._{470}$) to minimise absorption by media constituents and bacterial metabolic products such as pyocyanin. An O.D. of 1.0 at 470nm indicates a concentration of approximately 1×10^9 cfu.ml^{-1} (Anwar, 1981)

To determine bacterial growth characteristics in liquid medium, batch culture was carried out in Erlenmeyer flasks containing not more than 20% of their volume of appropriate media. Cells were grown at 37°C, agitated at 180 rpm on an orbital shaker, and growth of bacteria was measured by determining changes in $O.D._{470}$ of the culture with time.

3.2. Bacterial viable counts.

These were determined by using the spread plate method (Crone, 1984). Viable counts were performed on cell suspensions by preparing suitable serial dilutions in growth medium to yield between 30 and 300 colonies per plate. Volumes of 100 μl were plated in triplicate for each dilution on predried Luria agar plates. Plates were incubated at 37°C overnight and colonies counted. The viable count for the original suspension was calculated by multiplying the mean number of colony-forming units from a triplicate set of plates by the dilution factor.

3.3. Freezing bacterial samples.

Cells from an overnight culture on L-agar supplemented with antibiotics as appropriate were suspended in 1 ml LB + 20% glycerol and stored at -70°C.

3.4. Preparation of electrocompetent *E. coli* XL-1 Blue cells.

The method of electroporation used was one outlined by Dower *et al* (1988). This method, in addition to being the most efficient in terms of transformants generated, is also useful in cases when other methods of mobilising plasmid vectors into bacteria are ineffective. *E. coli* XL-1 Blue (Stratagene) was used throughout the cloning experiments, and was prepared as follows: XL-1 Blue cells were grown to an $O.D._{600}$ of 0.5-1.0 in 500 ml LB medium. The cells were chilled in the flask briefly on ice and centrifuged at 4000 x g for 15 min at 4°C. The ionic strength of the suspension was reduced by washing the cells as follows: the cells from a 500 ml culture were resuspended in 500 ml of cold 1 mM HEPES (pH 7.0), centrifuged as before, resuspended in 250 ml of cold 1 mM HEPES (pH 7.0), centrifuged as before, resuspended in 10 ml of 10% glycerol, centrifuged, and finally resuspended in 1 ml 10% glycerol. The ice-cold cell suspension was stored in aliquots of 40 µl in sterile microfuge tubes, and snap-frozen in liquid nitrogen. The electrocompetent cells were stored at -70°C until required, for up to 6 months.

3.5. Transformation of *E. coli* XL-1 Blue cells by electroporation.

The concentrated cells were thawed slowly on ice. Chilled recombinant plasmid DNA was added, and the cell mix was decanted into a pre-chilled 0.2 cm gap electroporation cuvette (Bio-Rad Laboratories, Richmond, CA). The cuvette was placed in the pre-chilled cuvette carrier of a Gene Pulser and Pulse Controller (Bio-Rad) and subjected to a single 2.5 kV pulse, capacitance and resistance of the power supply having been set to 25 µFarads and 200 Ω, respectively, to produce a nominal time constant for the capacitor discharge of 5 ms. The time constant was recorded at each application of the electric field, to determine the actual time constant. The cuvette was removed from the apparatus, and 1 ml sterile SOC medium added. The cuvette contents were gently shaken, and carefully poured into a sterile bijou bottle. The bottles were incubated at 37°C in a shaker for 45 min, to allow expression of plasmid-borne antibiotic resistance. Transformed cells were then selected by plating out 100µl aliquots onto L-agar plates containing ampicillin (100 µg/ml) and tetracycline (12.5 µg/ml), to whose surfaces had

been applied 40 μl X-gal (20 mg/ml in DMF) and 4 μl 0.84M IPTG using a sterile glass spreader. The plates were incubated overnight at 37°C, and then stored at 4°C for a further 24 h to allow blue-white colour selection to develop; a blue colony indicated that the *lacZ* gene of the inserted plasmid had not been interrupted by an insertion of "foreign" DNA, whereas a white colony indicated that the bacterial cells harboured a recombinant plasmid.

3.6. Insertion mutagenesis of *Pseudomonas aeruginosa* using transposon Tn5.

The method used was a modification of one devised by Sokol (1987). The Tn5 transposon on plasmid pUW 964 was in the host *E. coli* HB 101. The conjugal transfer of pUW 964/Tn5 from *E. coli* to *P. aeruginosa* was performed by a filter mating. The filter was placed on L-agar for 4 h at 37°C, then removed from the plate and placed in 10 ml sterile PBS (phosphate buffered saline) and vortexed to disaggregate the cells; 100μl of this suspension was plated out onto PIA (*Pseudomonas* isolation agar) containing 750μg/ml neomycin to determine the efficiency of transfer. The remainder was passed through a second membrane filter, which was placed on M9/Nm 750 agar overnight. The filter was placed in 10 ml LB and shaken at 37°C for 18 h. One hundred μl was added to 10 ml M9/Nm 750 and shaken at 37°C overnight, for use in pyocin selection experiments.

3.7. Selection of pyocin Sa-resistant Tn5 mutants.

A loopful of an overnight culture of the pyocin Sa-producing strain *P. aeruginosa* J1003, suspended in 1 ml saline, was spread onto succinate agar in a glass plate, leaving approx. 1 cm clear from the rim of the plate. The J1003 cells were allowed to grow for 5 h at 30°C, and then killed by exposure to chloroform vapour for 20 min. The plate was air-dried at 37°C. Five hundred μl of Tn5-mutagenised *P. aeruginosa* cells were mixed with 5 ml molten soft succinate agar solidified with 0.75% agar and supplemented with neomycin (750 μg/ml) at 37°C, and poured onto the surface of the succinate agar. The plate was incubated overnight at 37°C.

Any colonies growing within the zone of pyocin deposition were picked onto fresh succinate agar plates. These cells were subjected to a second round of pyocin selection, as follows; an inoculum of J1003 cells was prepared as before and applied as a streak across succinate agar. The J1003 cells were incubated and killed as before. Tn5-mutagenised *P. aeruginosa* cells suspected of being pyocin-resistant were streaked, using a toothpick, across the line of killed J1003 cells, and the plate incubated overnight at

37°C. Uninterrupted growth of mutant cells across the zone of production of pyocin was interpreted as pyocin resistant.

3.8. Isolation and purification of pyoverdin.

The method of Poole *et al* . (1991) was used. Briefly, a 500ml overnight culture of *P. aeruginosa* grown in iron-deficient succinate medium, was harvested by centrifugation at 15,000 x g at 4°C for 10 minutes, and the supernatants decanted and freeze-dried. The lyophilised material was resuspended in 12 ml double-distilled water and, following centrifugation at 27,000 x g for 10 minutes at 4°C to remove insolubles, was extracted once with an equal volume of ethyl acetate. The organic phase was discarded and solid NaCl added to the aqueous phase to saturation. The aqueous phase was extracted twice with 0.5 volume phenol:chloroform (1g/ml) and the organic phases pooled. Following addition of 2 volumes diethyl ether to the organic phase, the precipitated pyoverdin was pelleted by centrifugation at 12,000 x g for 10 minutes at 4°C, and subsequently washed three times with 3 ml diethyl ether. The pellet was air-dried, suspended in double distilled water to 20 mg/ml, and stored at -20°C.

3.9. Isolation and purification of pyochelin.

The procedure of Meyer *et al.* (1989) was adapted as follows. Cells were grown to early stationary phase in casamino acids medium (Ankenbauer *et al.*, 1985), removed by centrifugation and the supernatant acidified to pH 3.0 with glacial acetic acid. After extraction with 0.5 vol of ethylacetate, the organic phase was evaporated to dryness under reduced pressure. The crudely purified pyochelin was dissolved in 1 ml methanol. This preparation was purified further by chromatography through a 45 x 1.5 cm column of Sephadex LH-20 (Pharmacia) in methanol. Fractions were spotted onto Whatman 3MM paper and sprayed with 0.1M $FeCl_3$ in 0.1M HCl in order to detect iron chelating activity. Positive fractions were stored dry at 4°C.

3.10. Cross feeding of pyoverdin from different *Pseudomonas aeruginosa* strains with the siderophore deficient mutant IA1.

Succinate agar supplemented with EDDHA (400µg/ml) were seeded with a lawn of log-phase IA1 cells. After allowing the cell suspension to permeate the agar surface for 30 minutes, sterile filter paper discs (12.7mm) were placed in the centre of each plate. To

each disc was added 50μl filter-sterilised supernatant from each of 16 *P. aeruginosa* isolates from the laboratory collection. The chelating power of each supernatant had been standardised using the siderophore assay (Schwyn and Neilands, 1987). After incubation at 37°C overnight, the plates were categorised into : no growth, poor growth and good growth. Supernatants which resulted in growth of IA1 were noted.

3.11. ^{55}Fe-Pyoverdin uptake.

The general procedure of Poole *et al.*, (1991) was used. Cells were grown in succinate medium to late logarithmic phase and harvested by centrifugation at 4°C. The cells were washed twice with succinate medium, resuspended to an O.D.$_{470}$ of 1.0, and equilibrated at 37°C for 15 min prior to transport assays. During equilibration, no increase in O.D.$_{470}$ was observed. To 1 ml of cell suspension was added 80 μg pyoverdin and 115 nM ^{55}FeCl$_3$ (32.18 mCi/mg, Amersham) at 37°C. Ferri-pyoverdin uptake was assayed by withdrawing 200 μl samples and filtration through 0.2 μm cellulose acetate membranes (Whatman). The membranes were washed twice with 10 ml volumes of saline and allowed to air dry. The activity retained on the membranes was determined by scintillation counting on the ^{3}H channel of a Packard TR1600 betacounter using Optiphase Hisafe II scintillant (LKB-Bromma).

3.12. ^{55}Fe-Pyochelin uptake.

The cells were harvested and washed as described above for the ^{55}Fe-pyoverdin uptake studies. Pyochelin (10 mM in methanol) and ^{55}FeCl$_3$, were added to final concentrations of 20 μg/ml and 115 nM, respectively, in succinate medium 15 min prior to the start of an uptake experiment. Uptake was initiated by addition of cells at 37°C to a final OD$_{470}$ of 1.0. Cell filtration and scintillation counting were as described above, except that cells were washed with 0.5M HCl.

3.13. Siderophore assay.

The method devised by Schwyn & Neilands (1987) was used.

3.14. Preparation of bacterial outer membranes - sarkosyl method.

Outer membranes (OMs) were prepared by the method of Filip *et al.*, (1973). Cells were grown under various conditions at 37°C in an orbital shaking incubator and harvested by centrifugation at 5000 x *g* for 10min at 4°C. The resulting pellet was washed twice in 0.85% saline and then suspended in 10ml sterile distilled water. Cells were broken by 6 x 30 second pulses of sonication in an ice bath with 30 second intervals for cooling. N-lauryl sarcosinate (sarkosyl) was added to a final concentration of 2% v/v. After incubation at room temperature for 30 min, any remaining unbroken cells were removed by centrifugation at 5000 x *g* for 10 min. The supernatant was centrifuged at 100,000 x *g* for 3 hours at 4°C. The OM pellet was washed and suspended in distilled water and stored at -20°C.

3.15. Lowry protein assay.

The protein content of OM preparations was determined by the method of Lowry *et al.*, (1951) modified by Peterson (1977), using bovine serum albumin (BSA) as the standard.

3.16. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE).

Separation of OM proteins was carried out by gel electrophoresis using a Mini-Protean system, according to the methods described by Lugtenberg *et al.*, (1975), as modified by Anwar *et al.*, (1983c). The running and stacking gel were prepared as described in table 3.1., and polymerisation was initiated by addition of N,N,N,'N'-tetramethylethylene diamine (TEMED). The running gel was poured between glass plates separated by 1.5mm plastic spacers and allowed to set for 10min. A spray of electrode buffer on top of the gel ensured complete polymerisation. This buffer solution was removed and the stacking gel was cast in a similar manner. A teflon comb was inserted between the plates to create wells for sample application.

Samples were denatured at 100°C for 10min with an equal volume of sample buffer before loading onto the gel. The electrode buffer contained 0.025M Tris, 0.19M glycine and 0.1% SDS. In the Mini-Protean system a constant voltage of 200V was applied across the gel and electrophoresis continued until the tracking dye had migrated to within 0.5cm of the bottom of the gel. Gels were stained for protein with 0.1% Coomassie brilliant blue R-250 in 50% methanol / 10% acetic acid for 60min. Gels were

subsequently destained in 10% methanol/ 20% acetic acid and photographed using diffuse transmitted light.

Pre-stained molecular weight markers (Sigma) consisted of:

Protein	molecular weight
Bovine albumin	66 000
Egg albumin	45 000
Rabbit muscle glyceraldehyde-3-phosphate dehydrogenase subunit	36 000
Bovine erythrocyte carbonic anhydrase	29 000
Bovine pancreas trypsinogen (PMSF treated)	24 000
Soybean trypsin inhibitor	20 100
Bovine milk α-lactoglobulin	14 200

CONSTITUENT	RUNNING GEL 12%	STACKING GEL	SAMPLE BUFFER
stock I	3.0ml		
stock II		2.0ml	
10% w/v SDS	0.3ml	0.12ml	5.0ml
1.5M Tris pH8.8	3.7ml		
0.5M Tris pH6.8		3.0ml	2.5ml
distilled water	4.7ml	6.4ml	5.0ml
TEMED	0.03ml	0.032ml	
10% w/v AMPS	0.04ml	0.04ml	
glycerol			2.5ml
2-mercaptoethanol			0.25ml
5% bromophenol blue			0.2ml

stock I = 44% w/v acrylamide and 0.8% w/v N,N'-methylene-bis-acrylamide (Bis)

stock II = 30% w/v acrylamide and 0.8% w/v N,N'-methylene-bis-acrylamide (Bis)

AMPS = Ammonium persulphate (freshly prepared)

TEMED = N,N,N,'N'-Tetramethylethylene diamine

Table 3.1. Compositions of running gel, stacking gel and sample buffer for SDS-PAGE.

3.17. Western blotting (immunoblotting).

Following separation by SDS-PAGE, proteins were transferred onto nitrocellulose membranes (0.45 μm pore size, Bio-Rad) according to the western blotting method modified from Towbin *et al* (1979). Transfer was carried out in a Mini Trans-Blot cell (Bio-Rad). The polyacrylamide gel and nitrocellulose membrane were rinsed in transblot buffer (25mM Tris, 192mM glycine and 20% v/v methanol, pH 8.3), placed together, sandwiched between filter paper and scotch brite pads (soaked in buffer) and placed within a perforated plastic cassette. This assembly was fitted into the transblot apparatus filled with ice cold buffer. Blotting was performed at 100V for one hour. Transfer efficiency could be determined by staining the gels after transfer.

Transferred proteins were immunodetected using antisera. Blots were first blocked for one hour with gentle agitation in TBS-Tween and probed for at least 3 h at 4°C with appropriate antisera diluted in TBS-Tween. Blots were then rinsed thoroughly in TBS three times, and soaked with gentle agitation in TBS-Tween containing 0.25μg/ml protein A-horse radish peroxidase conjugate (Sigma) for 3 hours at 4°C. This solution was removed, and the blots rinsed again in TBS. Blots were visualised with freshly prepared developing solution (H_2O_2 0.01% v/v and 4-chloro-1-naphthol 25μg/ml in 10mM Tris. Cl pH 7.4). Colour development was stopped by washing with distilled water.

3.18. Determination of MICs.

MICs were determined by serial dilution in succinate medium (pH 7.0), with an inoculum of 10^6 cfu/mL. The tube cultures were incubated at 37°C for 72 h, with the MIC defined as the lowest concentration of antibiotic that inhibited visible bacterial growth.

3.19. ^{55}Fe-BRL 41897A uptake.

P. aeruginosa IA1 cells were grown in succinate medium to late logarithmic phase and harvested by centrifugation at 4°C. The cells were washed twice with succinate medium, resuspended to an OD at 470 nm (OD_{470}) of 1.0, corresponding to 10^9 cells, and equilibrated at 37°C for 15 min prior to transport assays. During equilibration, no observable increase in OD at 470 nm was observed. To 1 mL of cell suspension was added BRL 41897A to 0.0617 mM and ^{55}FeCl$_3$ (32.18 mCi/mg, Amersham) to 115 nM at 37°C. ^{55}Fe-BRL 41897A uptake was assayed by withdrawing 0.2 mL samples and

filtration through 0.2 μm cellulose acetate membranes (Whatman). The membranes were washed twice with 10 mL volumes of 0.5 M HCl and allowed to air dry. The activity retained on the membranes was determined by scintillation counting on the ^{3}H channel of a Packard TR1600 betacounter using Optiphase Hisafe II scintillant (LKB-Bromma).

3.20. Electroblotting proteins to PVDF membranes for subsequent microsequencing.

PVDF (Bio-Rad) membrane was cut to size and pre wetted in 100% methanol (for 2-3 secs) and distilled water (for 2-3 secs), then equilibrated in CAPS buffer (10mM 3 [cyclohexylamino]-propane sulphonic acid, 10% methanol pH 11) for at least 15 minutes. SDS–PAGE gels were allowed to equilibrate in a large volume of CAPS buffer for 15 minutes to remove contaminating tris and glycine. The blotting apparatus was as for western blotting, except that transfer was at 0.5 A for 20 minutes, in CAPS buffer. Following transfer, the PVDF membrane was rinsed in distilled water (3 washes, 5 minutes each), stained for 5 minutes in Coomassie brilliant blue 0.1% in 50% methanol, and destained for 15 minutes with 50% methanol, 10% acetic acid. The protein band of interest was cut from the membrane, air-dried, and stored at -20°C prior to microsequencing.

3.21. Preparation of plasmid DNA.

The method of Birnboim and Doly (1979) was used. Larger preparations were scaled up as appropriate, according to the methods of Birnboim and Doly (1979) and Ish-Horowicz and Burke(1981), as described by Sambrook *et al* (1989). Where necessary, plasmid DNA was purified by caesium chloride equilibrium density centrifugation (Sambrook *et al* ,1989).

3.22. Preparation of bacterial chromosomal DNA.

The method used was one reported by Chesney *et al* (1979). A 20ml overnight culture of *P. aeruginosa* in LB medium was centrifuged at 8,000 x *g* for 10 minutes at 4°C. The bacterial pellet was suspended in 3ml Tris/ sucrose (10% sucrose, 0.05M Tris.Cl pH 8.0) supplemented with 0.5ml freshly-prepared lysozyme solution (10 mg/ml in 0.25M Tris.Cl pH 8.0), and placed on ice for 10 minutes. A 0.6ml aliquot of 5% SDS was added to the tube, which was then immediately vortexed vigorously for 10 seconds

and incubated at 37°C for 30 minutes.

A 0.1ml aliquot of proteinase K (1mg/ml in 15mM NaCl and 10mM Tris.Cl at pH 7.5) was added, and incubated at 37°C for 60 min. The chromosomal DNA was purified by caesium chloride equilibrium density centrifugation.

3.23. Spectrophotometric determination of the amount of RNA or DNA.

For quantitating the amount of DNA present in the sample, O.D. readings were taken at 260nm and 280nm. The reading at 260nm allowed calculation of the concentration of nucleic acid in the sample; an O.D. of 1 corresponds to approximately 50 μg/ml for double-stranded DNA. The ratio between the readings at 260nm and 280nm (O.D.$_{.260}$/O.D.$_{.280}$) provides an estimate of the purity of the nucleic acid. Pure preparations of DNA have O.D.$_{.260}$/ O.D.$_{.280}$ values of 1.8. Contamination of the sample with protein or phenol will cause a significantly lower O.D.$_{.260}$ / O.D.$_{.280}$ value, and accurate quantitation of the amount of DNA will not be possible.

3.24. Preparation of dialysis tubing.

The tubing was cut into convenient lengths (10-20 cm) and boiled for 10 min in a large volume of 2% (w/v) sodium hydrogen carbonate and 1 mM EDTA (pH 8.0). The tubing was rinsed thoroughly in water, and autoclaved in water at 20 lb/sq. in. for 10 min in a loosely capped jar.

3.25. Agarose gel electrophoresis.

Agarose solutions (0.8-1.5%) were prepared by dissolving electrophoresis-grade agarose in 0.5 x TBE or 1.0 x TAE at elevated temperature in a microwave oven. Upon cooling to approx. 45°C, gels were cast in UV-transparent trays according to the manufacturers instructions and electrophoresed at 2V/cm. Ethidium bromide could be incorporated into the gel to approx. 10μg/ml. The DNA bands, complexed with ethidium bromide, were visualised on a UV transilluminator. Photographs were taken where necessary.

3.26. Southern blotting.

The procedure of Southern *et al* (1975) was modified as follows: after staining and photographing, gels were immersed in 5-10 volumes of denaturing solution (0.6M NaCl/ 0.2M NaOH) and incubated at room temperature with gentle shaking for 30 min. The denaturing solution was replaced with the same volume of neutralising solution (1.5M NaCl/ 0.5M Tris.HCl, pH 7.0) and shaken gently at room temperature for a further 30 min. The blotting apparatus was set up as described by Hames and Higgins(1988). The DNA fragments were allowed to transfer from the gel to the filter overnight. After transfer, the paper towels and 3MM paper were carefully removed. The location of the wells in the gel were visible as indentations in the filter, and these were marked on the filter with a soft pencil; the orientation of the filter was also marked by cutting off one corner. The filter was carefully peeled away from the gel and soaked in 4x SSC for 5 min. The gel was stained with ethidium bromide to verify that the DNA fragments had transferred to the filter. The filter was blotted dry, left to air-dry at room temperature for 20 min, and then sandwiched between sheets of 3MM paper and baked at 80°C for 2 h.

3.27. Labelling of synthetic oligonucleotides by phosphorylation with bacteriophage T4 polynucleotide kinase.

Synthetic oligonucleotides lacking a 5' phosphate were synthesised by Dr. J. Fox (Alta BioSciences, Birmingham University). They were end-labelled with ^{32}P using the method of Sambrook *et al* (1989) as follows:

oligonucleotide (10 pmoles/µl)	1.0 µl
*10x bacteriophage T4 polynucleotide kinase buffer	2.0 µl
[γ—^{32}P]ATP (specific activity 5000 Ci/mmole; 10 mCi/ml in aqueous solution)(10 pmoles)	5.0 µl
ddH$_2$O	11.4 µl

*10x bacteriophage T4 polynucleotide kinase buffer =

  0.5 M Tris.Cl (pH 7.6)
  0.1 M MgCl$_2$
  50 mM dithiothreitol
  1 mM spermidine.HCl
  1 mM EDTA (pH 8.0)

One μl (≈ 8 units) of T4 polynucleotide kinase was added to the reaction mixture and incubated at 37°C for 45 min. The oligonucleotide DNA was purified from unincorporated ^{32}P-ATP through a NucTrap push column (Stratagene) in accordance with the manufacturer's instructions.

3.28. Recovery of DNA from agarose gels.

This was performed using GeneClean (Bio 101) in accordance with the manufacturer's instructions.

3.29. Dephosphorylation of linearised plasmid DNA.

The procedure of Simsek *et al* (1973) was used. Ten-20 μg closed circular plasmid DNA was digested with a 2-3 fold excess of the desired restriction enzyme for 1 h; total digestion was verified by agarose gel electrophoresis, using undigested plasmid DNA as a marker. When digestion was complete, the sample was extracted with phenol:chloroform and the DNA recovered by ethanol precipitation. The DNA pellet was resuspended in 90 μl of Tris.Cl (pH 8.3), and stored at -20°C. A small aliquot (approx. 0.5 μg) was stored separately, to be used later to determine the efficiency of dephosphorylation. To the remainder of the DNA was added 10 μl of 10x CIP dephosphorylation buffer (10 mM $ZnCl_2$/ 10 mM $MgCl_2$/ 100 mM Tris.Cl pH 8.3) with the appropriate amount of CIP, and incubated under the appropriate conditions:

	Protruding 5' termini	Blunt or recessed termini
Amount of CIP required	1 unit/ 100 pmoles	1 unit/ 2 pmoles
Incubation conditions	30 min at 37°C	15 min at 37°C. Then add another aliquot of CIP and continue incubation for a further 45 min at 55°C

At the end of the incubation period, SDS and EDTA (pH 8.0) were added to 0.5% and 5mM, respectively. Proteinase K was added to 100μg/ml. The reaction mixture was incubated at 56°C for 30 minutes and extracted once with phenol and once with phenol:chloroform. The linearised dephosphorylated plasmid DNA was recovered by ethanol precipitation, resuspended in 10μl TE and stored at -20°C.

3.30. Ligation of plasmid DNA using T4 DNA ligase.

Ligation reactions were prepared as follows:

1 μg vector DNA

2-10 μg insert DNA

5 μl 10x ligation buffer (Stratagene)

0.5 μl 10 mM ATP, pH 7.5

0.5 μl T4 DNA ligase at 4 units/μl (Stratagene)

ddH$_2$O up to a volume of 50 μl

The ligation reaction was allowed to proceed overnight at 4°C.

3.31. Pre-hybridisation of nitrocellulose filters.

A hybridisation oven (Hybaid) was used in accordance with the manufacturer's instructions. The filter was wetted in 6x SSC, placed on top of the nylon mesh, rolled up and placed inside a hybridisation bottle. The bottle was placed in the oven at the appropriate temperature for 10 min to wet the filter thoroughly. The SSC was decanted from the bottle, and replaced with 10 ml pre-hybridisation buffer (10 x Denhardt's solution/ denatured salmon sperm DNA @ 0.05 mg/ml/ 6x SSC; 100x Denhardt's solution = 2% bovine serum albumin/ 2% Ficoll/ 2% polyvinylpyrollidone). The filter was incubated for 1 hr at the hybridisation temperature.

3.32. Hybridisation of nitrocellulose filters.

After pre-hybridisation, the labelled oligonucleotide was carefully pipetted directly into the buffer, and the bottle shaken to thoroughly disperse the label. The cap was replaced and the bottle incubated overnight at the hybridisation temperature.

The buffer containing the probe was discarded, and replaced with 50 ml 6x SSC/ 0.1% SDS which was pre-warmed to 5°C lower than the hybridisation temperature (T_{dmin}). The filter was washed for 5 min in the oven, and a further two washes were performed, as before. A fourth 2 min wash was performed at the T_{dmin}. A final room temperature wash was performed. The filter was wrapped flat in cling film and subjected to autoradiography at -70°C.

3.33. Immobilisation of recombinant DNA libraries.

Bacteria containing recombinant plasmids were spread onto L-agar plates containing ampicillin (100 μg/ml) and tetracycline (12.5 μg/ml), and incubated at 37°C until the bacterial colonies were just visible. Nitrocellulose filters were laid onto the surface of the plates, and the orientation marked by puncturing the filter and agar with a sterile pipette tip. The filter was removed and laid, colony side up, onto a fresh L-agar plate containing ampicillin (100 μg/ml) and tetracycline (12.5 μg/ml). A replica filter was prepared in the same way. The filters were incubated at 37°C until the colonies were 1-2 mm in diameter. Each filter was then peeled off the agar plate and laid, colony side up, onto a stack of three sheets of Whatman 3MM filter paper saturated with denaturation solution (1.5M NaCl/ 0.5M NaOH) in a plastic tray, for 5 min. The filter was transferred, keeping it horizontal, onto a second stack of filter paper soaked in neutralisation buffer (1.5M NaCl/ 1M Tris-HCl, pH 7.5). After 5 min, the filter was carefully transferred to a third stack of filter paper soaked in 4 x SET buffer (20 x stock SET buffer has the composition; 3M NaCl/ 20mM EDTA/ 0.4M Tris-HCl, pH 7.8) for a further 5 min. The filter was then placed onto dry 3MM paper and left at room temperature until it was dry (15-30 min). The filter was sandwiched between sheets of 3MM paper, kept flat between a pair of glass plates, and baked in an oven at 80°C for 2 h.

3.34. Pre-hybridisation and hybridisation of filter-bound colony DNA.

This was performed as before, with the important initial step of removing bacterial debris from the surface of the baked nitrocellulose filter. This was achieved while the filter was soaking in 6 x SSC, prior to insertion into a hybridisation bottle, by gently rubbing the surface of the filter with a piece of filter paper or paper towel soaked in 6 x SSC.

3.35. Obtaining and purifying single-stranded (ss) DNA.

E coli XL-1 Blue cells harbouring the recombinant plasmid of interest were grown overnight in 10 ml LB containing ampicillin (100 μg/ml) and tetracycline (12.5μg/ml). Three ml of Superbroth was inoculated with sufficient cells to yield an O.D.$_{600}$ of 0.1, and shaken at 37°C in a 100 ml conical flask. When the O.D.$_{600}$ reached 0.3 ($\approx$2.5 x 10^8 bacteria/ml), R408 helper phage were added at a multiplicity of infection of 20:1 (phage to cells). The culture was incubated at 37°C with shaking for a further 8 hours. The culture was transferred to microfuge tubes and centrifuged at 13,000 rpm for

2 min at room temperature. The supernatant was transferred to fresh microfuge tubes in 1.2 ml aliquots. To each tube was added 300 µl of a 3.5M ammonium acetate, pH 7.5/ 20% PEG solution. The tube contents were mixed by inversion and allowed to stand at room temperature for 20 min, followed by centrifugation in a microfuge at 13,000 rpm for 20 min. The supernatant was thoroughly drained, taking care not to dislodge the phage pellet. The pellet was resuspended in 300 µl TE, followed by addition of an equal volume phenol:chloroform. The tube was vortexed for 1 min, and spun for 1 min in a microfuge. The upper aqueous phase was transferred to a fresh microfuge tube, ensuring that none of the white interface was transferred. This extraction process was repeated until no white interface was formed. An equal volume of chloroform was added to each tube, the tubes vortexed as before, and spun for 2 min in a microfuge. The aqueous phase was transferred to a fresh tube. The ssDNA was precipitated by addition of an equal volume of 7.5M ammonium acetate, pH 7.5 and 2 volumes cold 100% ethanol, and allowed to stand at -70°C for 30 min. The tubes were spun in a microfuge at 13,000 rpm for 20 min to sediment the precipitated ssDNA, and the supernatants discarded. The ssDNA pellets were rinsed with 70% ethanol, and centrifuged as before. The supernatant was discarded, and the pellets were dried and resuspended in 20µl water. The ssDNA was analysed by applying aliquots onto a 1% agarose gel; R408 runs at approx. 4 kb when compared to a double-stranded DNA marker, while pKS+ ssDNA with no insert runs at approx. 1.6 kb when compared to a double-stranded DNA marker.

3.36. DNA sequencing of DNA by dideoxy-mediated chain-termination.

DNA sequencing was performed using the dideoxy-mediated chain-termination method (Sanger *et al*,1977) and Sequenase Version 2.0 (united States Biochemical) in accordance with the manufacturer's instructions.

3.37. Gel electrophoresis of sequencing reactions.

Polyacrylamide gel electrophoresis was performed using a Bio-Rad Sequi-Gen nucleic acid sequencing system in accordance with the manufacturer's instructions. Samples were denatured at 75°C for 2 min, then resolved on 7M urea, 4% or 6% 0.4 mm polyacrylamide gels. After pouring, but before the gel had set, a sharktooth comb was inserted, flat side first, 5mm into the gel. Once polymerisation was complete, the comb was removed, the top of the gel was washed with 1x TBE, and the comb was re-inserted so that the teeth just made contact with the top of the gel so as to separate samples without distorting the gel.

Gels were pre-run at 45W for at least one hour, then 1µl-4µl of each denatured sample loaded into four adjacent wells in the order GATC or TCGA. Electrophoresis was at constant power (40 - 45W) to maintain gel temperature at 45-50°C throughout electrophoresis. Upon completion of electrophoresis, the apparatus was disassembled and the plates carefully prised apart so as not to dislodge the gel. The gel was immersed in 10% acetic acid/ 10% methanol for 15 minutes, then transferred to 3mm Whatman paper, covered in cling film and dried under vacuum at 80°C for 1 hour.

After drying, the gel was exposed to Kodak X-omat AR film for 24-48 hours at room temperature in a light proof cassette. After exposure, the film was developed (under Kodak 1A Safelight) by immersion in developer (Kodak) for 3 min, washing in water for 1 min, then immersing it in fixer (Kodak) for 3 minutes and finally washing with water.

4. CROSS-FEEDING EXPERIMENTS

4.1. Introduction.

Pseudomonas aeruginosa has at least two siderophore-mediated iron uptake systems based on pyochelin and pyoverdin. These systems will operate both in the organism's natural habitat, soil and water, where the solubility of iron at neutral pH is extremely low, and in the human host where the availability of free iron is too low to sustain bacterial growth due to the iron-binding glycoproteins transferrin and lactoferrin (Griffiths, 1991). Evidence is now emerging that transport of different ferri-pyoverdin and ferri-pseudobactin complexes is mediated via various IROMPs, some of which have broad specificity for several complexes and some of which are specific for a single complex. It seems likely that these interactions are controlled possibly by subtle differences in the amino acid backbone of the siderophore. Amongst P. aeruginosa strains Cornelis et al., (1989) noted three pyoverdin uptake groups and polyclonal antisera raised against an 80 kDa IROMP only reacted against strains producing the same siderophore. This antiserum was subsequently shown to inhibit ferri-pyoverdin transport in this strain, confirming that the 80 kDa IROMP was a transporter (Meyer et al., 1990). Poole et al., (1991) have identified a 90 kDa IROMP ferri-pyoverdin transporter in the pyoverdin-deficient strain 6609. A mutant deficient in expression of this protein still showed low uptake of ferri-pyoverdin, providing evidence for a second transport system. Similar evidence has been obtained in a strain made resistant to P. aeruginosa pyocin Sa. This mutant lacked an 85 kDa IROMP and was deficient in ferri-pyoverdin transport, but it did still show 5% residual uptake (Smith et al., 1992).

Although it is now becoming clear that P. aeruginosa has multiple iron transport systems, the contribution of these systems to growth in vivo is unclear. Ankenbauer et al., (1985) obtained mutants deficient in one or both siderophores and concluded that pyoverdin was more important for growth in the presence of serum or purified transferrin. However, the inability of the pyoverdin-deficient (pvd-) strain to grow in serum was attributed to a failure to synthesise pyochelin rather than to the siderophore being unable to acquire iron from transferrin.

In an attempt to understand the regulation of expression of iron uptake systems in P. aeruginosa, iron uptake in a mutant deficient in both pyoverdin and pyochelin production was investigated. In this chapter, induction of specific IROMPs in response to growth either in the presence of pyoverdin or pyochelin is demonstrated and that induction of iron uptake is siderophore-specific.

4.2. Results.

4.2.1. Agar plate cross-feeding experiments.

Preliminary experiments involved applying crude filter-sterilised culture supernatants from various *P. aeruginosa* clinical isolates to a sterile filter paper in the centre of a CDM-Fe agar plate containing 400μM EDDHA which had been seeded with a lawn of IA1 cells. Cross-feeding was indicated by the presence of a halo of bacterial growth around the filter paper after incubation at 37°C for 24 h

.

Supernatants from *P. aeruginosa* isolates which produced:

a) Good halos	b) Poor halos	c) No halos
S.T. 0:17	S.T. 0:12	S.T. 0:14
S.T. 0:16	S.T. 0:7	S.T. 0:13
S.T. 0:15	S.T. 0:6	S.T. 0:11
S.T. 0:9		S.T. 0:10
S.T. 0:5d		S.T. 0:8
		S.T. 0:3
		S.T. 0:1

where   no halos = no visible bacterial growth around the filter paper disc

poor halos = bacterial growth around the filter less than 2 mm larger than the diameter of the filter paper disc

good halos = bacterial growth around the filter greater than 2 mm larger than the diameter of the filter paper disc

4.2.2. Growth curves of IA1 in the presence of exogenous siderophores.

Growth curves of IA1 in CDM-Fe medium in the presence of supernatants from clinical isolates (10% v/v) which produced halos were determined, cell density being measured spectrophotometrically at 470nm at 1 hour intervals. The chelating power of the added supernatants was standardised by first measuring the chelating power of the supernatants using the CAS siderophore assay (Schwyn and Neilands, 1987), and then diluting the supernatants as appropriate with CDM-Fe medium. In addition, the supernatant from S.T.0:3, a clinical isolate which did not produce cross-feeding (as indicated by the agar plate experiment) was used.
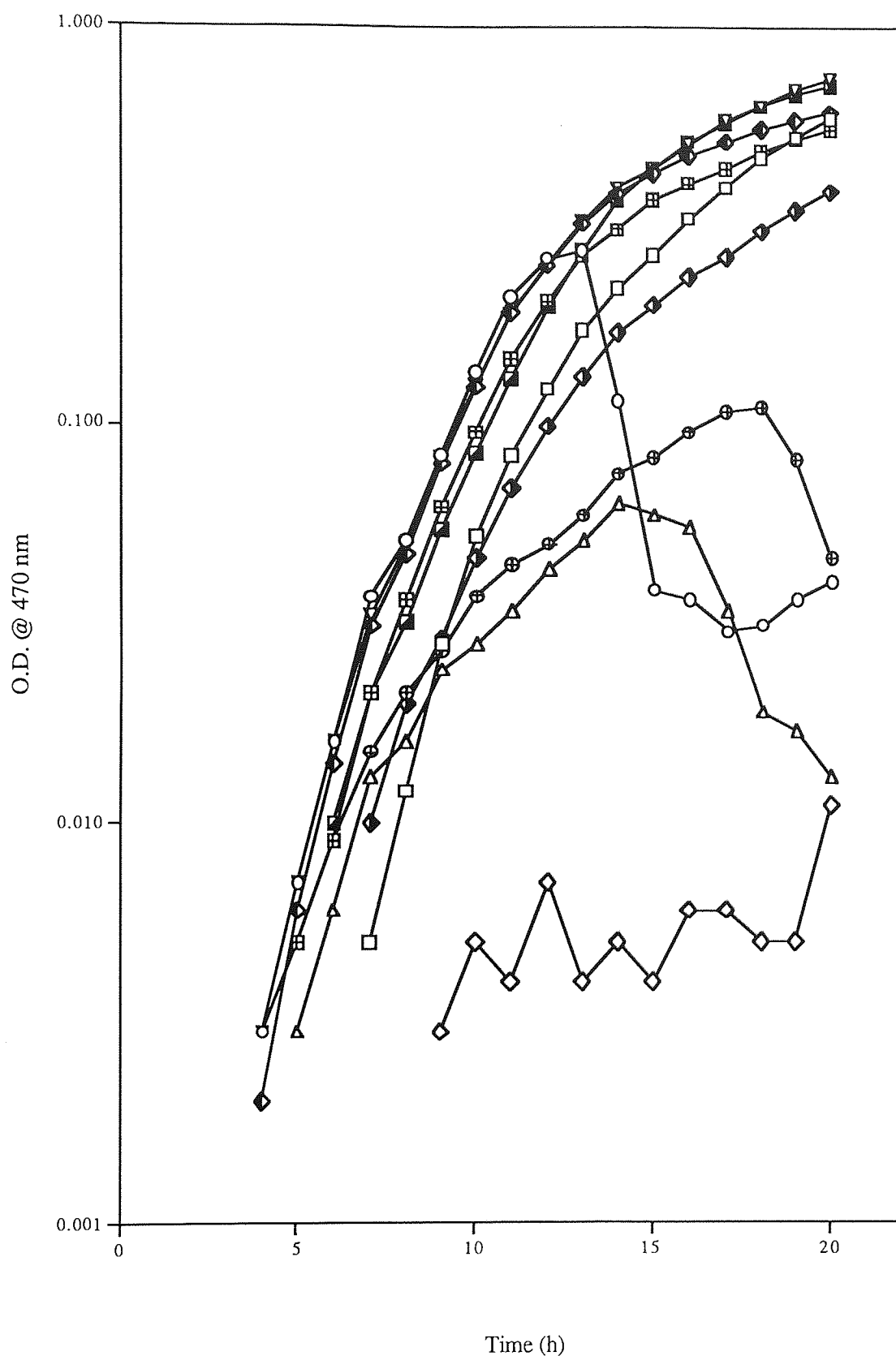
Figure 4.1. Growth curves for IA1 grown in CDM-Fe supplemented with supernatant (10% v/v) from *P. aeruginosa* IA1 (□), and the clinical isolates S.T. 0:3 (◇), S.T. 0:5d (○), S.T. 0:6 (△), S.T. 0:7 (⊞), S.T. 0:9 (◆), S.T. 0:12 (⊕), S.T. 0:15 (▽), S.T. 0:16 (▨) and S.T. 0:17 (◆).

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The growth curve results were in general agreement with the agar plate cross-feeding results, in that supernatants from both S.T. 0:12 and S.T. 0:6 allowed comparatively poor growth, while supernatant from S.T. 0:3 totally inhibited growth. However, supernatant from S.T. 0:7, in contrast to the agar plate result, enhanced the growth of IA1 even more than did supernatant from S.T. 0:17 which was classed as a good cross-feeder. The growth curve for IA1 supplemented with S.T. 0:5d supernatant was similar to growth curves produced by other good cross-feeders (i.e. S.T. 0:7, S.T. 0:9, S.T. 0:16 and S.T. 0:15) up to 12 h, after which the cells began to clump, giving rise to the low readings $O.D._{470}$.

4.2.3. SDS-PAGE of outer membrane proteins of P. aeruginosa clinical isolates.

Outer membranes of *P. aeruginosa* clinical isolates representing 17 IATS serotypes, as well as IA1, were prepared from cells grown in CDM-Fe to stationary phase, and susbjected to SDS-PAGE (Figure 4.2).
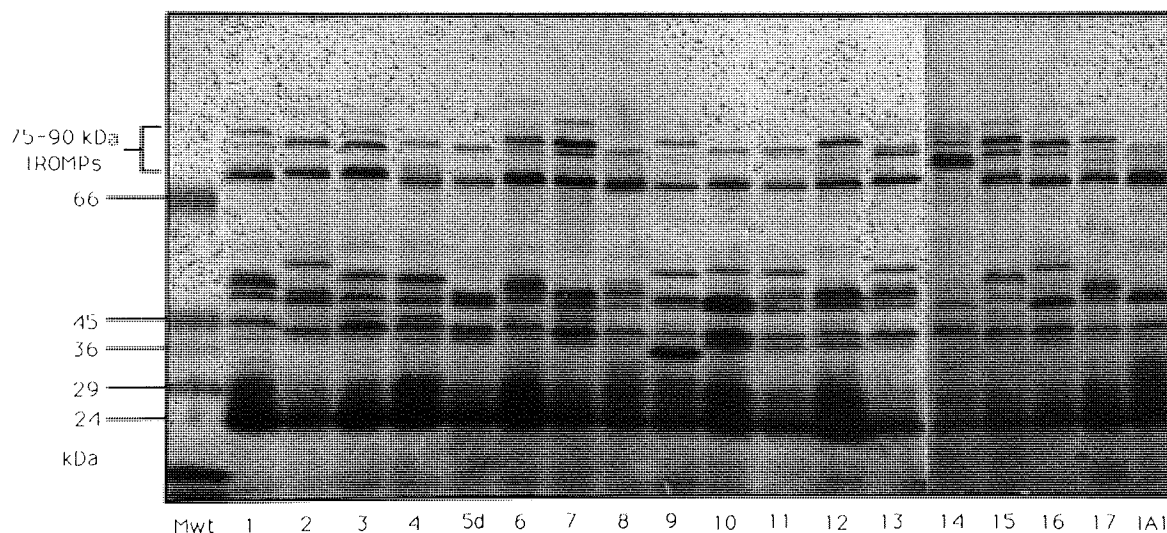
Figure 4.2. SDS-PAGE of outer membranes from *P. aeruginosa* IA1 and 17 clinical isolates grown in iron-deficient CDM-Fe medium. Lanes are labelled by isolate type. Mwt=molecular weight markers.

The heterogeneity of IROMPs between isolates can clearly be seen. Additionally, the IROMPs of S.T. 0:9 and S.T. 0:12 appear similar, yet supernatants from these isolates were good and poor cross-feeders to IA1, respectively. Under iron-replete conditions, the IROMPs were repressed (Figure 4.9, lane 5).

4.2.4. Thin layer chromatography of purified pyoverdins.

Pyoverdins from isolates which demonstrated cross-feeding with IA1, namely S.T.0:5d, S.T.0:6, S.T.0:7, S.T.0:9, S.T.0:12, S.T.0:15 and S.T.0:17, were purified for further experiments, as well as pyoverdins from PAO1 (the parent strain of IA1) and S.T.0:3, a clinical isolate where the supernatant did not promote growth of IA1 in the agar plate experiment. These purified pyoverdins were analysed by thin layer chromatography in methanol:water (70:30 v/v) as described by Bitter *et al.* (1991).

Pyoverdin type:	R_F
PAO1	0.38
S.T.0:3	0.41
S.T.0:5d	0.21
S.T.0:6	0.25
S.T.0:7	0.47
S.T.0:9	0.39
S.T.0:12	0.22
S.T.0:15	0.30
S.T.0:16	0.32
S.T.0:17	0.32

4.2.5. Effect of growth in the presence of purified pyoverdins on the OMPs of IA1.

Preliminary experiments adding crude supernatants from cultures of other strains of *P. aeruginosa* to IA1 indicated that growth could be either increased or inhibited depending on the strain, suggesting that IA1 could be cross-fed from some siderophores, but not from others. In order to assess whether these effects were reflected in changes in OM protein profiles, siderophores were purified and added to cultures of IA1 in succinate medium. Succinate medium was selected since this has been reported to give good yields of pyoverdin (Meyer and Abdallah, 1978). The IROMP profiles are shown in Figure 4.3.

kDa

IROMPs

85
83
81
75

1   2   3   4   5   6

kDa

IROMPs

85
83
81
75

7   8   9   10   11   12
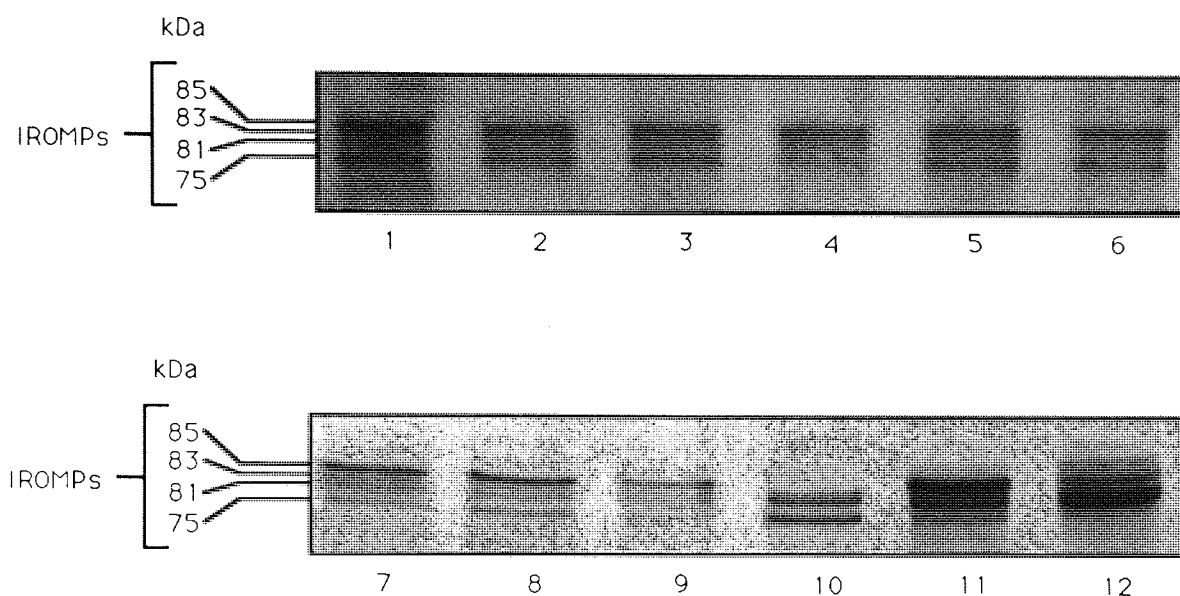
Figure 4.3. Outer membranes from IA1 cells grown in succinate medium supplemented with pyoverdin from PAO1 (lane 1), S.T. 0:5 (lane 2), S.T. 0:6 (lane 3), S.T. 0:7 (lane 4), S.T. 0:9 (lane 5), S.T. 0:12 (lane 6), S.T. 0:15 (lane 7), S.T. 0:16 (lane 8), S.T. 0:17 (lane 9); succinate medium supplemented with pyochelin (lane 10), supernatant from an overnight culture of PAO1 (10% v/v)(lane 11) and succinate medium alone (lane 12).

The distribution and expression of IROMPs of IA1 grown in the presence of different purified pyoverdins (lanes 1-9) all appeared to be the same. However, when IA1 was grown in the presence of pyochelin (lane 10), the 85 kDa IROMP was almost absent while the 83, 81 and 75 kDa IROMPs were expressed more strongly. When grown in the presence of PAO1 supernatant (lane 11), expression of IROMPs was biased towards the 81 and 85 kDa proteins. IA1 grown in succinate medium alone (lane 12) exhibited especially increased expression of the 81 kDa IROMP, while the 75 kDa IROMP was poorly expressed.

4.2.6. Radiolabelled iron uptake studies.

Since it appeared that there was a relationship between growth conditions and expression of IROMPs in IA1, radiolabelled iron uptake experiments were performed on IA1 cells which had been preconditioned by growth in the presence of purified pyoverdins; additionally, pyoverdin from S.T. 0:3 as well as the non-metabolisable chelator EDDHA were used on non-preconditioned IA1 cells, since preconditioning was

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not possible due to the growth inhibition of IA1 by these siderophores.

Figure 4.4. S.T. 0:5d pyoverdin-mediated iron ($^{55}Fe^{3+}$) uptake by *P. aeruginosa* IA1 grown in iron-deficient succinate medium (■), supplemented with 50 μg/ml S.T. 0:5d pyoverdin (□); S.T. 0:16 pyoverdin-mediated iron ($^{55}Fe^{3+}$) uptake by *P. aeruginosa* IA1 grown in iron-deficient succinate medium (▲), supplemented with 50 μg/ml S.T. 0:16 pyoverdin (△). The uptake mixture contained pyoverdin (80 μg/ml), $^{55}FeCl_3$ (115 nM) and 1 ml of cells at O.D.$_{470}$ 1.0.

Figure 4.5. S.T. 0:9 pyoverdin-mediated iron ($^{55}Fe^{3+}$) uptake by *P. aeruginosa* IA1 grown in iron-deficient succinate medium (■), supplemented with 50 µg/ml S.T. 0:9 pyoverdin (□); S.T. 0:15 pyoverdin-mediated iron ($^{55}Fe^{3+}$) uptake by *P. aeruginosa* IA1 grown in iron-deficient succinate medium (▲), supplemented with 50 µg/ml S.T. 0:15 pyoverdin (△). The uptake mixture contained pyoverdin (80 µg/ml), $^{55}FeCl_3$ (115 nM) and 1 ml of cells at O.D.$_{470}$ 1.0.

Figure 4.6. S.T. 0:6 pyoverdin-mediated iron ($^{55}Fe^{3+}$) uptake by *P. aeruginosa* IA1 grown in iron-deficient succinate medium (■), supplemented with 50 µg/ml S.T. 0:6 pyoverdin (☐); S.T. 0:12 pyoverdin-mediated iron ($^{55}Fe^{3+}$) uptake by *P. aeruginosa* IA1 grown in iron-deficient succinate medium (▲), supplemented with 50 µg/ml S.T. 0:12 pyoverdin (△). The uptake mixture contained pyoverdin (80 µg/ml), $^{55}FeCl_3$ (115 nM) and 1 ml of cells at O.D.$_{470}$ 1.0.

Figure 4.7. S.T. 0:7 pyoverdin-mediated iron ($^{55}Fe^{3+}$) uptake by *P. aeruginosa* IA1 grown in iron-deficient succinate medium (■), supplemented with 50 μg/ml S.T. 0:7 pyoverdin (□); S.T. 0:17 pyoverdin-mediated iron ($^{55}Fe^{3+}$) uptake by *P. aeruginosa* IA1 grown in iron-deficient succinate medium (▲), supplemented with 50 μg/ml S.T. 0:17 pyoverdin (△). The uptake mixture contained pyoverdin (80 μg/ml), $^{55}FeCl_3$ (115 nM) and 1 ml of cells at O.D.$_{470}$ 1.0.
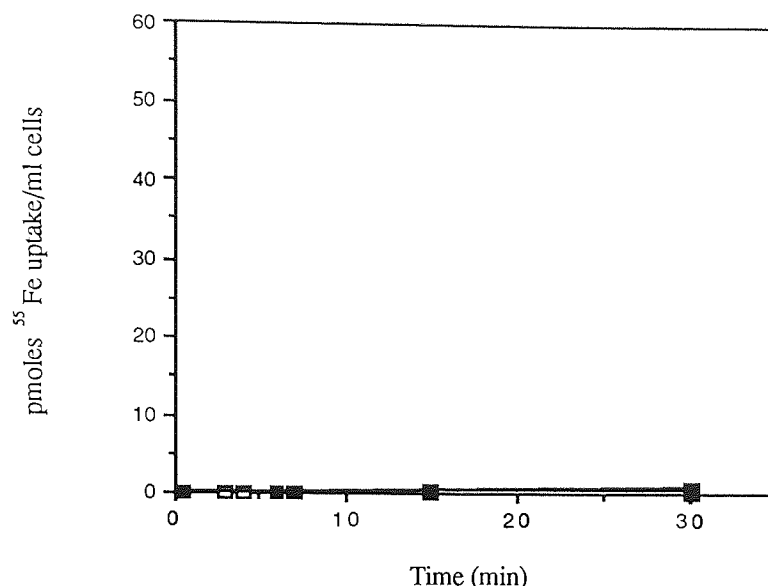
Figure 4.8. S.T. 0:3 pyoverdin-mediated iron ($^{55}Fe^{3+}$) uptake by *P. aeruginosa* IA1 grown in iron-deficient succinate medium (□); EDDHA-mediated iron ($^{55}Fe^{3+}$) uptake by *P. aeruginosa* IA1 grown in iron-deficient succinate medium (▇). The uptake mixture contained pyoverdin (80 μg/ml) or EDDHA (43.2 μg/ml), $^{55}FeCl_3$ (115 nM) and 1 ml of cells at O.D.$_{470}$ 1.0.

All uptake graphs are shown with the same axes, to emphasise the differences in uptakes of different ^{55}Fe-siderophores. The pyoverdin-mediated uptake results for S.T. 0:5d, S.T. 0:16, S.T. 0:9 and S.T. 0:15 pyoverdins (figures 4.4 and 4.5) were similar, in that growth of IA1 in the presence of these pyoverdins prior to assay (preconditioning) increased the accumulation of radiolabelled iron from 20.14, 14.92, 23.29 and 27.78 pmoles ^{55}Fe/ml cells to 42.62, 41.33, 42.63 and 48.63 pmoles ^{55}Fe/ml cells, respectively, approximately double that of non-preconditioned cells, after 30 min. A similar phenomenon was observed for pyoverdins from S.T. 0:6 and S.T. 0:12 (figure 4.6), except the accumulation of ^{55}Fe was much reduced, at 0.44 and 1.43 pmoles ^{55}Fe/ml cells for non-preconditioned cells rising to 2.98 and 3.76 pmoles ^{55}Fe/ml cells respectively for preconditioned cells.

The ^{55}Fe-pyoverdin uptake profile for S.T. 0:17 pyoverdin was different to those for S.T. 0:5d, S.T. 0:16, S.T. 0:9 and S.T. 0:15 pyoverdins in that uptake for preconditioned cells was 53.93 pmoles ^{55}Fe/ml cells, but comparatively high at 39.76 pmoles ^{55}Fe/ml cells for non-preconditioned cells. Conversely, the ^{55}Fe-pyoverdin uptake profile for S.T. 0:7 pyoverdin by preconditioned cells was 56.905 pmoles ^{55}Fe/ml cells, but comparatively low at 9.81 pmoles ^{55}Fe/ml cells for non-preconditioned cells (figure 4.7).

Uptake of ^{55}Fe via S.T. 0:3 pyoverdin and EDDHA (figure 4.8) by non-

preconditioned cells was extremely low at 0.41 and 0.47 9.81 pmoles ^{55}Fe/ml cells, respectively.

Since uptake experiment results were highly variable without obvious alterations in IROMP expression, more detailed studies were performed using pyoverdins from PAO1 and the chromatographically-distinct pyoverdin produced by the isolate S.T. O:12, and pyochelin.

4.2.7. Effect of exogenous pyoverdins and pyochelin on IROMP expression.

Figure 4.9. SDS-PAGE of outer membranes prepared from *P. aeruginosa* IA1 grown in iron-deficient succinate medium (lane 1), supplemented with 50 µg/ml PAO1 pyoverdin (lane 2), 50 µg/ml O:12 pyoverdin (lane 3), 10 µg/ml pyochelin (lane 4) and 100µM FeCl$_3$ (lane5).

In succinate medium alone IA1 strongly expresses 3 IROMPS of 81, 83 and 96 kDa (lane 1), whereas addition of pyoverdin from the parental strain PAO1 (lane 2) causes induction of an 85 kDa IROMP and partially represses the 96, 83 and 81 kDa IROMPs. Addition of chromatographically-distinct pyoverdin from strain O:12 (lane 3) induces the 85 kDa IROMP, and partially represses the 81 kDa IROMP. Addition of purified pyochelin increased expression of a 75 kDa IROMP only (lane 4). Growth in iron-replete succinate medium (lane 5) represses all high molecular weight IROMPs.

4.2.8. Immunoblotting.

Figure 4.10. shows a Western blot of the IROMPs reacted with mAb C108 which was raised against an 85 kDa IROMP (Smith *et al.,* 1991a).
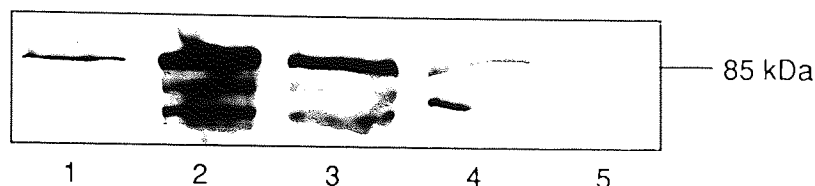
Figure 4.10. Immunoblot probed with mAb C108. Lanes are as described in fig.4.9.

No reaction was seen against IROMPs prepared from iron-replete cells (lane 5). Weak activity was seen against IROMPs of IA1 alone (lane 1) or supplemented with pyochelin (lane 4), whereas a strong reaction with some cross-reactivity with lower molecular weight proteins was seen against IROMPs from cells grown in the presence of added pyoverdins (lanes 2 and 3).

4.2.9. Ferri-pyoverdin uptake after growth with pyochelin or pyoverdin.

Since cross-feeding with purified pyoverdins caused changes in IROMP profiles, labelled iron transport studies were performed to determine whether ferri-siderophore uptake was affected. Figure 4.11. shows iron uptake from ferri-pyoverdin after growth in succinate medium with and without addition of pyoverdin or pyochelin.
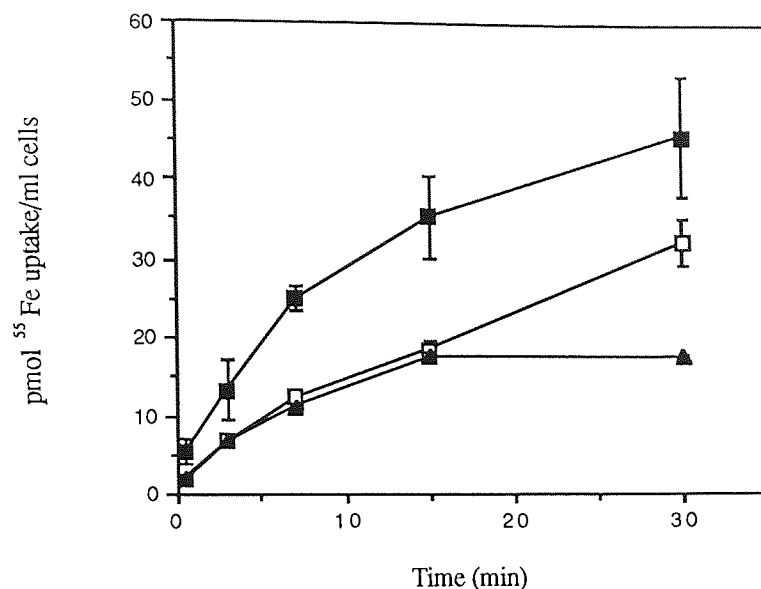
Figure 4.11. PAO1 pyoverdin-mediated iron ($^{55}Fe^{3+}$) uptake by *P. aeruginosa* IA1 grown in iron-deficient succinate medium (□), supplemented with 50 μg/ml PAO1 pyoverdin (■) or supplemented with 10 μg/ml pyochelin (▲). The uptake mixture contained pyoverdin (80 μg/ml), $^{55}FeCl_3$ (115 nM) and 1 ml of cells at O.D.$_{470}$ 1.0.

Growth in the presence of pyoverdin caused a 2.8-fold increase in the inital rate of iron uptake from ferri-pyoverdin. The rate increased from 1.27 pmol Fe/min/10^9 cells without induction to 3.57 pmol Fe/min/10^9 cells with growth in the presence of pyoverdin. Growth in medium supplemented with pyochelin had no effect on ferri-pyoverdin uptake. Incubation of cells with mAb C108 did not affect uptake (data not shown).

4.2.10. Ferri-pyochelin uptake after growth with pyochelin or pyoverdin.

Figure 4.12. shows the results of ferri-pyochelin uptake studies.
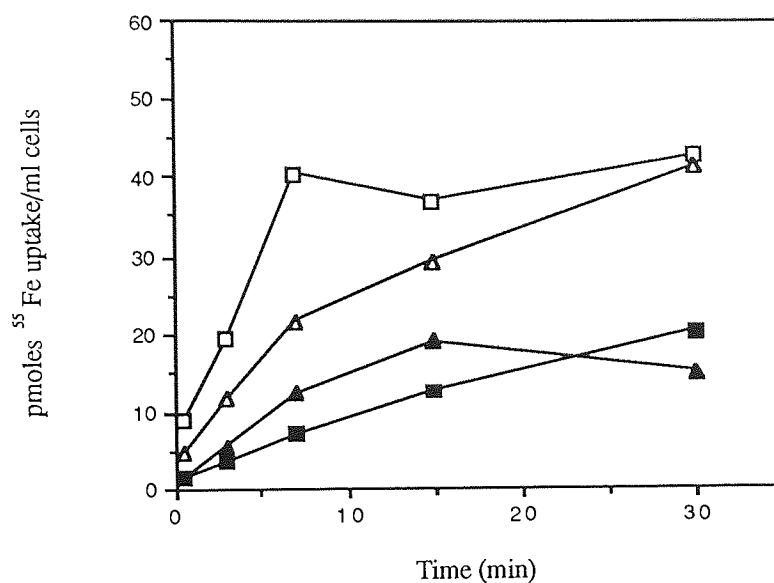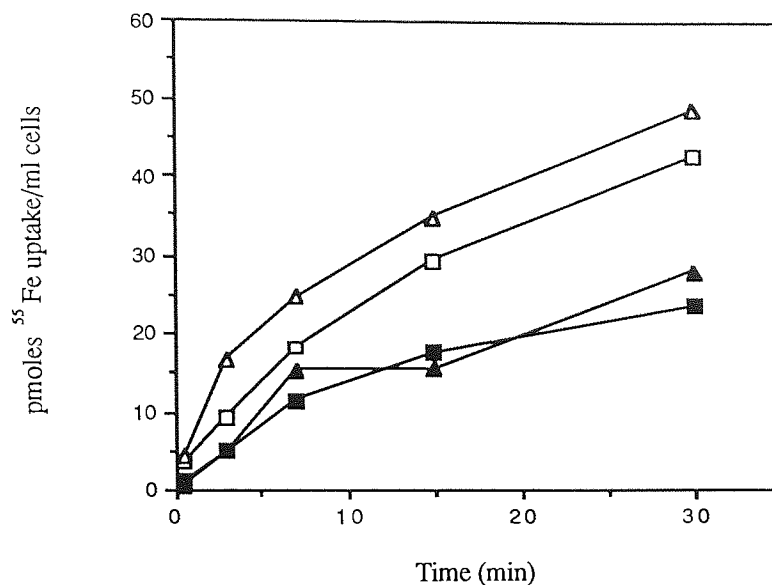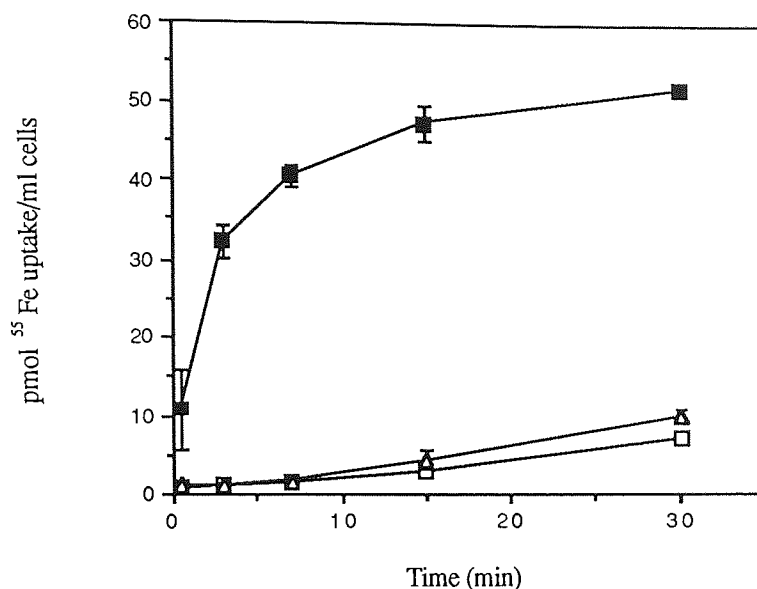

Figure 4.12. Pyochelin-mediated iron ($^{55}Fe^{3+}$) uptake by *P. aeruginosa* IA1 grown in iron-deficient succinate medium (□), supplemented with 50 μg/ml PAO1 pyoverdin (△) or supplemented with 10 μg/ml pyochelin (■). The uptake mixture contained pyochelin (60 μM), $^{55}FeCl_3$ (115 nM) and 1 ml of cells at O.D.$_{470}$ 1.0.

Similar to the observations made for ferri-pyoverdin uptake, ferri-pyochelin uptake was increased only after growth with pyochelin. The increase in initial rate of uptake was much greater, rising from 0.3 pmol Fe/min/10^9 cells to 10.6 pmol Fe/min/10^9 cells. Pyoverdin pre-treatment had no effect; the rate of iron uptake being 0.3 pmol Fe/min/10^9 cells.

4.2.11. Pre-treatment with chromatographically distinct pyoverdins.

The R$_f$ values for PAO1 and 0:12 pyoverdins were determined as 0.38 and 0.22, respectively. Ferri-pyoverdin uptake profiles for *P. aeruginosa* IA1 using pyoverdin from strain 0:12, with and without growth in the presence of this siderophore are shown in Figure 4.13.
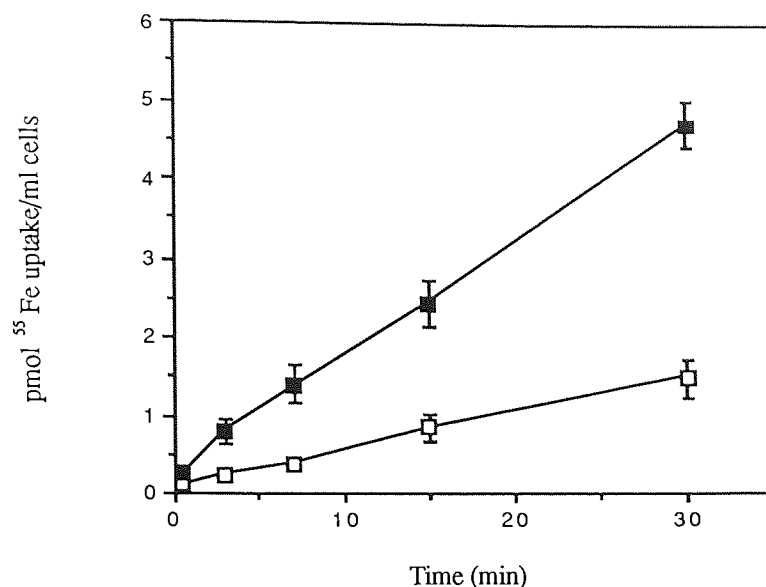
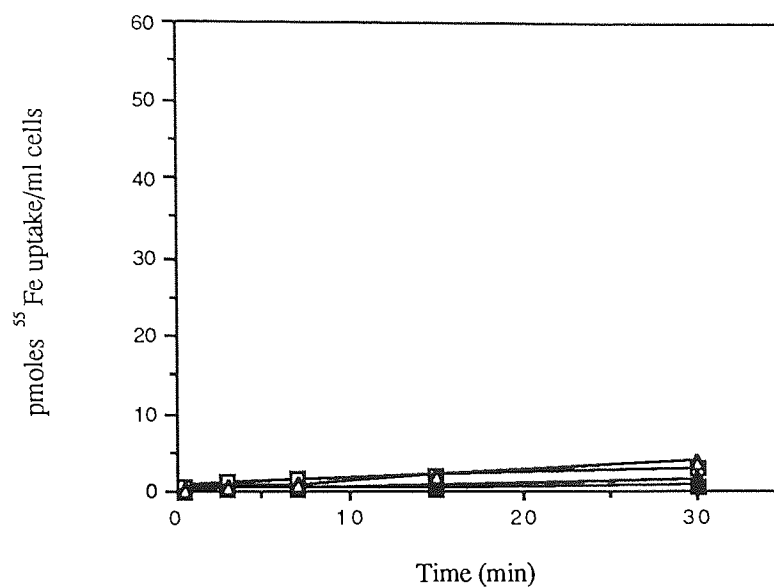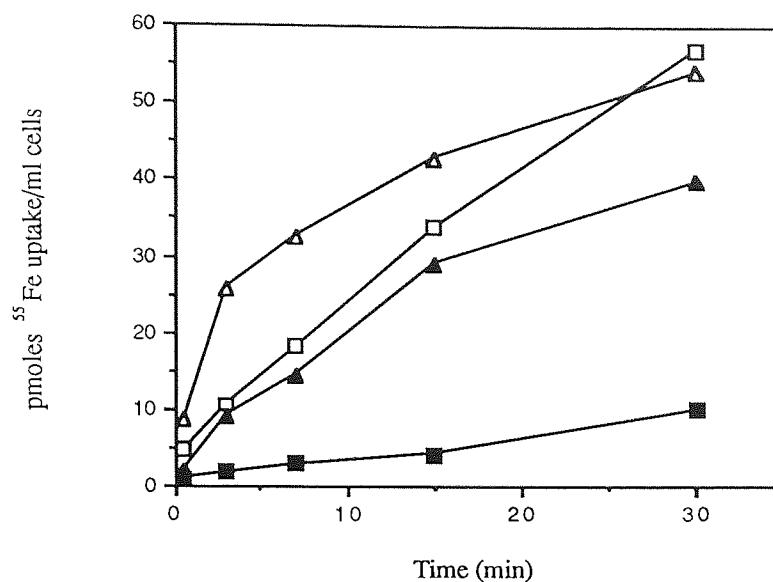Figure 4.13. 0:12 pyoverdin-mediated iron ($^{55}Fe^{3+}$) uptake by *P. aeruginosa* IA1 grown in iron-deficient succinate medium alone ($\square$) or supplemented with 50 µg/ml O:12 pyoverdin ($\blacksquare$). The uptake mixture contained pyoverdin (80 µg/ml), $^{55}FeCl_3$ (115 nM) and 1 ml of cells at O.D.$_{470}$ 1.0.

Increased uptake was noted with cells grown in the presence of siderophore, but the rate and extent of uptake, even after pre-treatment, were significantly less than that seen with PAO1 pyoverdin (Fig. 4.11.). The pre-treatment experiments were repeated using either PAO1 or 0:12 pyoverdin and uptake experiments performed with 0:12 and PAO1 pyoverdins, respectively. Figure 4.14. shows the uptake profiles.
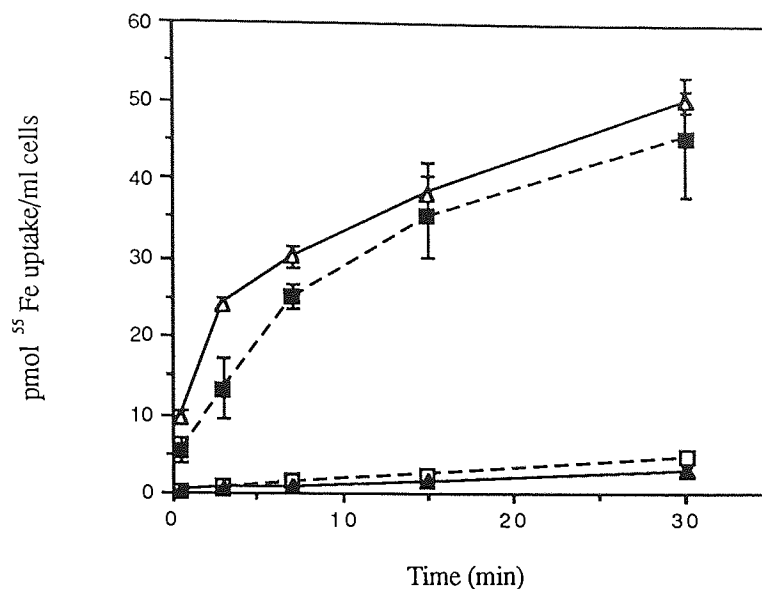
Figure 4.14. Pyoverdin cross-induction and uptake studies. Iron ($^{55}Fe^{3+}$) uptake by *P. aeruginosa* IA1 via PAO1 pyoverdin grown in iron-deficient succinate medium supplemented with 50 μg/ml PAO1 pyoverdin (■) , or O:12 pyoverdin (Δ); iron ($^{55}Fe^{3+}$) uptake by *P. aeruginosa* IA1 via S.T. 0:12 pyoverdin grown in iron-deficient succinate medium supplemented with 50 μg/ml PAO1 pyoverdin (▲) or O:12 pyoverdin (□). The uptake mixture contained pyoverdin (80 μg/ml), $^{55}FeCl_3$ (115 nM) and 1 ml of cells at $O.D._{470}$ 1.0. The dashed lines are duplicated from figures 4.11. and 4.13. for comparison.

The increased uptake was the same, irrespective of which pyoverdin had been added to the growth medium.

4.2.12. Effect of growth in the presence of salicylic acid on OMPs of IA1.

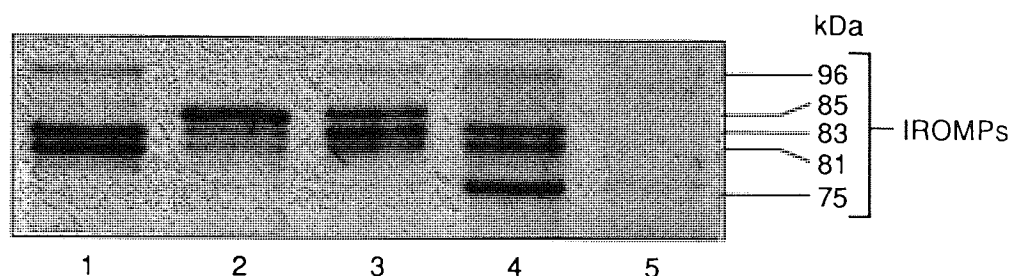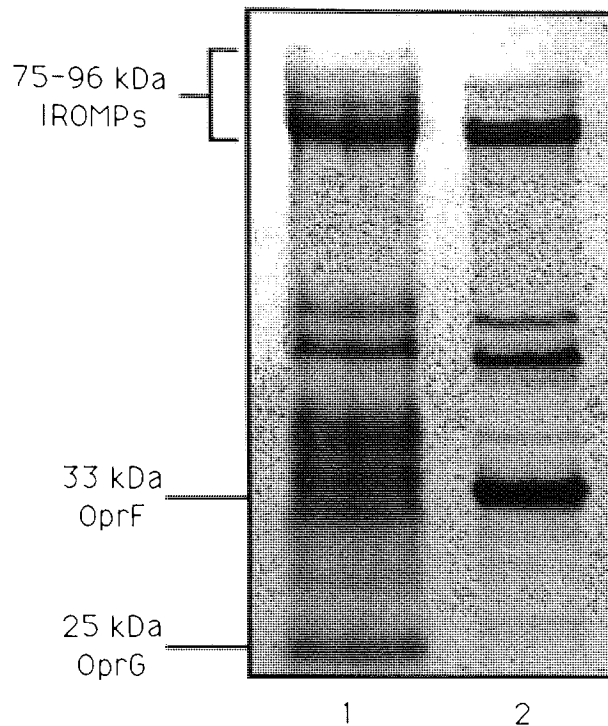
Figure 4.15. SDS-PAGE of outer membranes prepared from *P. aeruginosa* IA1 grown in iron-deficient succinate medium (lane 1) and supplemented with 20 µg/ml salicylic acid (lane 2).

When IA1 cells were grown in succinate medium in the presence of salicylic acid (lane 2), there was a considerable increase in expression of OprF when compared to IA1 cells grown in succinate medium alone (lane 1).

4.3. Discussion

In this work it was shown that *P. aeruginosa* can respond specifically to the presence of siderophore in the growth medium and not simply to the iron-deficiency evoked by that siderophore, based on the finding that growth in the presence of pyoverdin induces only ferri-pyoverdin uptake and growth in the presence of pyochelin only increases ferri-pyochelin uptake. This may go some way to explaining why *P. aeruginosa* retains at least two siderophore-mediated iron uptake systems, despite the observation that the binding constant of iron for pyoverdin (10^{32}) (Wendenbaum *et al.*, 1983) is significantly greater than that of pyochelin (10^5 at acid pH), which in addition requires two molecules to bind one molecule of iron (Cox & Graham, 1979). It is speculated that *P. aeruginosa* produces low amounts of each siderophore in iron-deficient growth environments and monitors which siderophore is most effective at complexing iron and delivering it to the cell before becoming committed to, and inducing, iron uptake via that route. Although the observations reported in this work were made with the siderophore-deficient strain IA1, inducible uptake with the siderophore-competent parent strain PAO1 was also noted (data not shown).

The finding that the increased uptake of ferri-pyochelin after growth in pyochelin-supplemented medium correlated with increased expression of a 75 kDa IROMP, supports the findings of Heinrichs *et al.*, (1991) that this IROMP is a ferri-pyochelin binding protein. No changes in expression of the 14 kDa ferri-pyochelin binding protein (Sokol & Woods, 1983) were observed, although these studies were carried out using early stationary phase cells when this uptake system is thought to be less important (Heinrichs *et al.*, 1991).

Several groups have reported IROMPS in the range 80-90 kDa being associated with ferri-pyoverdin uptake in different strains (Meyer *et al.*, 1990; Poole *et al.*, 1991). The finding that exogenous pyoverdins could be classed as good, poor or non-cross-feeders was in agreement with the observations of Cornelis *et al.*, (1989); however, this tripartite classification system could not be extended to the IROMPs of different clinical isolates, which displayed a large heterogeneity of IROMPs in response to endogenous pyoverdins (figure 4.2). In this work, ferri-pyoverdin uptake correlated with expression of an 85 kDa IROMP, although it was not possible to inhibit uptake with the anti-85 kDa IROMP mAb. However, other workers have shown only poor interaction with whole cells, possibly as a result of being shielded by lipopolysaccharide or because the mAb was raised against an epitope which is not surface-exposed (Smith *et al.*,1991). It was interesting to note that uptake by IA1 of ferri-pyoverdins from other strains occurred at different rates, suggesting that either the receptor for its own pyoverdin had a broader substrate specificity, with not all ferri-pyoverdin complexes being taken up at the same rate, or that a second ferri-pyoverdin receptor exists. The induction studies with different pyoverdins including those from PAO1 and strain 0:12, suggest that the same receptor is

induced (figure 4.3., lanes 1-9; figure 4.9., lanes 1, 2 and 3), and this was supported further by the increased reactivity with the anti-85 kDa IROMP mAb (Fig. 4.10., lanes 1, 2 and 3). Pre-treatment with either pyoverdin resulted in the same rate of uptake; pre-treatment with 0:12 resulted in the same induction of PAO1 ferri-pyoverdin uptake as pretreatment with PAO1 pyoverdin itself. The reverse was also true, with PAO1 pyoverdin pretreatment resulting in the same induction of 0:12 ferri-pyoverdin uptake as with 0:12 pyoverdin itself. Competition studies with ^{55}Fe-0:12 pyoverdin and non-radioactive Fe-PAO1 complex proved inconclusive (data not shown). It was expected that the rate of uptake of ^{55}Fe-0:12 complex would be reduced in the presence of Fe-PAO1 pyoverdin complex due to competition at the receptor. However, the opposite was observed. This was attributed to transport of ^{55}Fe as the ^{55}Fe-PAO1 complex which resulted from equilibration between the two pyoverdins. These data suggest therefore that with the pyoverdins and strains used in this study, the uptake and induction phenomena require just one receptor. However, Poole *et al.*, (1991) have noted residual uptake of ferri-pyoverdin complex in a mutant deficient in a 90 kDa IROMP and suggested that this is due to a second transport system. Interestingly, these workers were unable to demonstrate an induction phenomenon in this strain. Similarly, it was not possible to demonstrate induction in a strain rendered unable to transport ferri-pyoverdin through resistance to *P. aeruginosa* pyocin Sa (Smith *et al.*, 1992). Both these strains lacked a major IROMP associated with ferri-pyoverdin uptake, which suggests that the IROMP must be present for the induction phenomenon reported in this work to be seen. Radiolabelled iron uptake studies revealed that uptake of ferripyoverdin from S.T. 0:3 and ferric-EDDHA was 3.8% and 4.4% respectively, compared to uptake of PAO1 pyoverdin in non-preconditioned IA1 cells after 30 min, indicating that a non-specific route of entry for these structurally unrelated chelators exists; if a second pyoverdin-specific uptake system does indeed exist in *P.aeruginosa*, it appears to be non-inducible. However, it cannot be ruled out that there is a second uptake system, and the functions of other high molecular weight IROMPs, notably the 81 and 83 kDa proteins, remain to be determined.

Visca *et al.* (1993) conclusively demonstrated that azurechelin, a siderophore produced by *Pseudomonas cepacia* (Sokol *et al.*, 1992) is salicylic acid, which is also a siderophore of *P. fluorescens* CHAO (Meyer *et al.*, 1992) and *P. aeruginosa* PAO1 (Meyer, 1992). When IA1 was grown in the presence of salicylic acid (figure 4.15.). increased expression of OprF occurred. This finding was unexpected, since salicylic acid is a precursor of pyochelin and, if acting as a siderophore, might be expected to utilise the same uptake pathway as pyochelin. However, Burns and Clark, (1992) have shown that porin synthesis in *P. cepacia* can be reduced by growth in the presence of salicylates, while Rosner *et al.*, (1991) also showed a reduction in expression of OmpF porin in *E. coli* when grown in the presence of salicylates, but their observations were made with cells grown in iron-replete media, in which there may be no requirement for salicylic acid

as a siderophore.
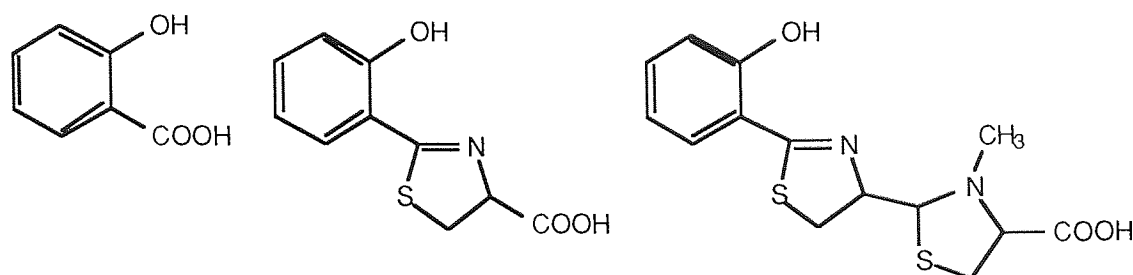
Figure 4.16. The structures of salicylic acid, aeruginoic acid and pyochelin, respectively.

Meyer (1992) showed that salicylic acid uptake by an OprF mutant (H636) of *P. aeruginosa* PAO1 was the same as the parent strain, demonstrating that OprF was not involved in salicylic acid uptake, but that the siderophores desferriferrioxamine B, desferriferrioxamine E, desferriferrichrycin and desferriferricrocin may be acquired by *P. aeruginosa* PAO1 via OprF; it is further suggested that OprF may act as the suspected second uptake mechanism for other ferrisiderophores.

It was interesting to note that the growth of IA1 in succinate medium alone was only marginally increased by addition of exogenous siderophores from other clinical isolates (figure 4.1.), suggesting that, although IA1 is deficient in production of pyoverdin and pyochelin, it may still employ a hitherto uncharacterised siderophore-mediated iron uptake system. IA1 grown in succinate alone expreses IROMPs of 81 and 83 kDa, (figure 4.9., lane 1) to which no function has yet been ascribed. Yamada *et al.*, (1970) described the isolation of a precursor to pyochelin, which was named aeruginoic acid. Since pyochelin and its precursor salicylic acid are known to be siderophores of *P. aeruginosa*, it seems likely that aeruginoic acid may also function in this way, possibly in concert with either the 81 or 83 kDa IROMPs. Additionally, one or more of these IROMPs may be involved in uptake of ferric-ornibactin (Stephan *et al.*, 1993), a peptidic siderophore resembling pyoverdin but lacking a chromophore, isolated from *P. cepacia*.

Poole *et al.*, (1990) have noted an induction phenomenon in *P. aeruginosa*, but with the enterobacterial siderophore enterobactin. Growth inhibition of a pyoverdin-deficient strain imposed by the non-metabolisable chelator ethylene diamine-di(o-hydroxyphenol acetic acid) could be reversed by addition of enterobactin. A novel 80 kDa IROMP was detected and enterobactin-grown cells transported ferri-enterobactin in an energy-dependent manner at low iron concentrations. Leong *et al.*, (1991) have noted an induction response to exogenous siderophore in the related fluorescent pseudomonad *P. putida*. In their study, the gene coding for a second pseudobactin uptake system (*pupB*), located on plasmid pMK15, was identified by its ability to render transformed strains no

longer susceptible to starvation by pseudobactin BN7, the native siderophore of strain BN7. However, an additional OM protein was expressed in strain A124/pMK15 only after growth in the presence of pseudobactin BN7. Moreover, these workers noted new OM proteins when strains were grown in the presence of pseudobactin B10 and pyoverdin, but not deferriferrichrome A. This work extends these observations to *P. aeruginosa* and to siderophores which are not structurally related, i.e. pyoverdin and pyochelin. At the present stage it is unclear how the signal of siderophore present in the growth medium is detected by the cell, but evidence is now emerging for positive and negative iron regulators in *P. aeruginosa*. Prince *et al.*, (1991; 1993) have identified a Fur homologue in *P. aeruginosa* and have also noted that the *E. coli fur* gene will regulate exotoxin A expression. Several workers have identified positive regulators of iron-regulated gene expression, notably the *regA* gene in exotoxin A expression (Wick *et al.*, 1990) and LasI/LasR regulation of both elastase and exotoxin A expression (Gambello and Iglewski, 1991; Gambello *et al.*, 1993; Passador *et al.*, 1993) in *P. aeruginosa* and a transcriptional activator associated with a gene from the siderophore biosythesis/uptake cluster of a fluorescent plant pseudomonad (O'Sullivan and O'Gara, 1991).

In summary it has been shown that *P. aeruginosa* can respond specifically to the presence either of pyoverdin or pyochelin in the growth medium by induction of siderophore receptors in the outer membrane.

5. MECHANISMS OF RESISTANCE TO PYOCIN Sa IN PSEUDOMONAS AERUGINOSA.

5.1. Introduction.

Bacteriocins are protein or protein-complex antibiotics produced by a wide variety of bacterial species, whose bactericidal activity is generally directed towards strains of the same or closely related species, pyocins being the bacteriocins of *P. aeruginosa* (Govan, 1986). Three classes of pyocin, R, F, and S, have been identified on the bases of structural and chemical properties (Govan, 1978; Kuroda and Kageyama, 1983; Sano and Kageyama, 1981). Phage-tail-like R and F pyocins are produced by more than 90% of *P. aeruginosa* strains, and colicinlike (i.e. similar to the bacteriocins of *E. coli*), trypsin-sensitive S pyocins are produced by approximately 70% of isolates (Fyfe *et al.*, 1984). Susceptibility to one or more classes of pyocin occurs in 100% of isolates.

The S-type pyocins are of particular interest because their lethal activity appears to be mediated via high-molecular-weight IROMPs. Although pyocin production appears to be unaffected by iron concentration (Govan, 1986), but rather by regulatory genes whose activities are governed by the bacterial response to DNA damage (Matsui *et al.*, 1993), iron-rich conditions decreased the absorption and lethality of pyocin S2, whereas under iron-deficient conditions, susceptibility was significantly enhanced and accompanied by the appearance of an OM protein which appeared to act as the S2 receptor (Ohkawa *et al.*, 1980).

Pyocin S2-resistant mutants lacking a minor IROMP have been described, but competition data suggest that this IROMP (designated Fe-b) did not have role in iron uptake (Ohkawa *et al.*, 1980). Additionally, the IROMP profiles of the parent strain appeared to be identical to that of *P. aeruginosa* PAO1 (Ohkawa *et al.*, 1980); thus, it may be deduced that Fe-b correlates with the 83 kDa IROMP, which Gensberg *et al.* (1992) showed to be non-inducible by the presence of pyoverdin or pyochelin. Govan (1986) identified a new pyocin, Sa, produced by *P. aeruginosa* J1003, which exhibited similarly iron-dependent bactericidal activity.

In an attempt to clarify the method of uptake of pyocin Sa, work was undertaken to determine the mechanisms of resistance in transposon Tn5-mutagenised *P. aeruginosa*.

5.2. Results.

5.2.1. Isolation of a pyocin Sa-resistant Tn5 mutant from susceptible P. aeruginosa.

The laboratory culture collection was screened, using pyocin Sa produced by *P. aeruginosa* strain J1003, to determine *P. aeruginosa* strains susceptible to the pyocin. Only one clinical isolate, S.T. 0:9, from more than 20 laboratory and clinical strains tested was sensitive, and Tn5 mutagenesis (Sokol, 1987) followed by selection of pyocin Sa-resistant Tn5 mutants, was performed. Only one mutant, designated PH1, showed no growth inhibition due to pyocin Sa on agar.

5.2.2. Pyoverdin competition.

When discs saturated with ferripyoverdin were placed on the soft agar overlay containing the sensitive strain S.T. 0:9, distinct zones of growth were detected, indicating that ferripyoverdin could inhibit killing by pyocin Sa.

5.2.3. SDS-PAGE analysis of outer membrane proteins.

Outer membranes were prepared by the Sarkosyl solubilisation method (Filip *et al.*, 1973) and analysed by SDS-PAGE (Laemmli, 1970). OM protein profiles from 15 h cultures of PH1 and S.T. 0:9, grown in succinate minimal medium (Meyer and Abdallah, 1978) at 37°C are shown in Figure 5.1.
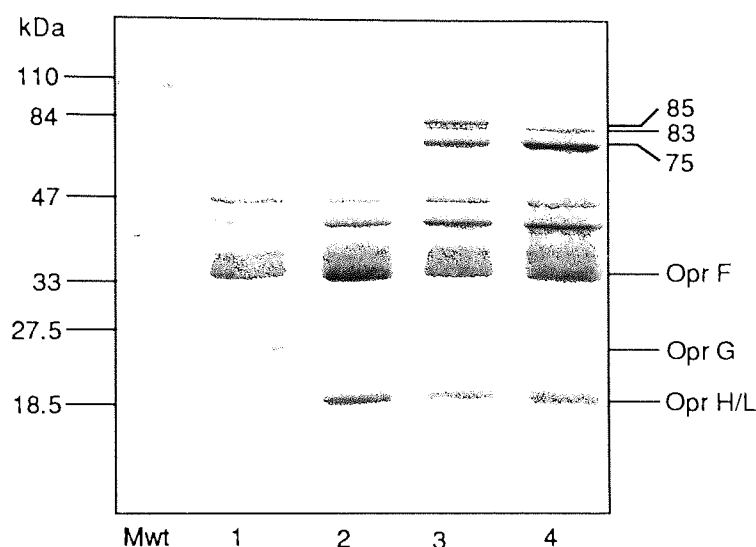
Figure 5.1. SDS-polyacrylamide gel electrophoretogram of outer membranes prepared from strains of *P. aeruginosa* grown in iron-deficient succinate medium with (lanes 1 and 2) or without (lanes 3 and 4) 50μM FeSO$_4$. Lanes 1 and 3, S.T. 0:9; lanes 2 and 4, pyocin Sa-resistant mutant PH1.

After growth without added iron, three major IROMPs of 75, 83, and 85 kDa were induced in the OM. The Sa-resistant mutant PH1 specifically lacked an 85 kDa IROMP (lane 4), and expression of the 75 kDa IROMP was increased compared with that in S.T. 0:9. Growth in succinate medium with added iron confirmed that these proteins were iron repressible (lanes 1 and 2). The 25 kDa OM protein G (OprG) expressed by S.T. 0:9 under iron-rich growth conditions (lane 1) was partially repressed in PH1 (lane 2) by approximately 50%, as determined by densitometry.

Total siderophore production was estimated by measuring the relative fluorescence of the culture supernatants, using an excitation filter at 360 nm and by reading the emission at 445 nm in a spectrofluorimeter (Cornelis *et al.*, 1987). A 10-fold decrease in the fluorescence peak for PH1 culture supernatants was noted, indicating that a component of a siderophore biosynthesis-uptake operon was inactivated. In addition, PH1 was unable to grow in succinate medium supplemented with the non-utilisable chelator EDDHA (400μM), indicating perturbed pyoverdin production or transport.

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5.2.4. Radiolabelled iron uptake assays.

Pyoverdin was purified from S.T. 0:9 culture supernatants, and uptake studies were performed as described by Poole *et al.*(1991). Uptake profiles for 15 h stationary-phase cells of S.T. 0:9 and PH1 grown in iron deficient succinate medium are shown in Figure 5.2.
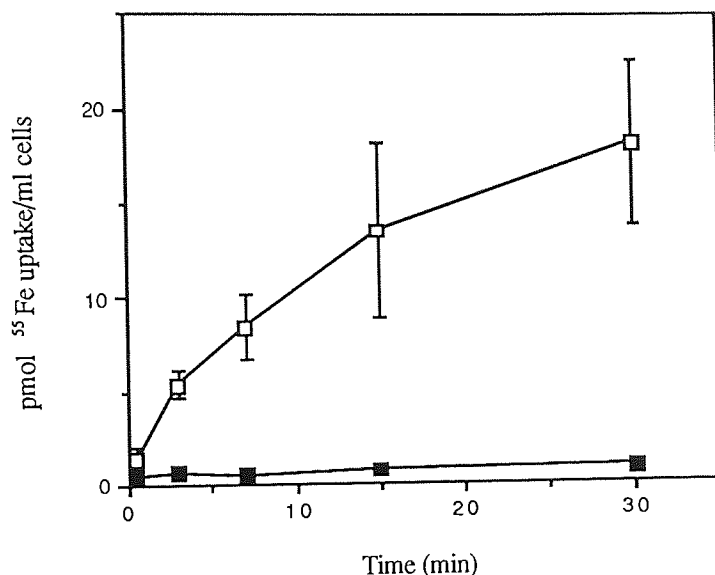
Figure 5.2. Pyoverdin-mediated iron (^{55}Fe^{3+}) transport by *P. aeruginosa* S.T. 0:9 (□) and PH1 (■) grown in iron-deficient succinate medium. PH1 error bars are not visible, since they are smaller than the plot symbol. The uptake mixture contained pyoverdin (80µg/ml), ^{55}FeCl$_3$ (115nM), and 1 ml of cells at an optical density at 470 nm of 1.0.

By using a 1:50 molar ratio of ^{55}Fe to pyoverdin, the uptake rate of PH1 was 0.03 pmol ^{55}Fe/min/ml cells compared with 0.61 pmol ^{55}Fe/min/ml cells in S.T. 0:9, a reduction of 95%. Increasing the molar ratio of iron to pyoverdin did not affect uptake.

Ferripyochelin transport assays were undertaken to determine if the increased expression of the 75 kDa IROMP was associated with elevated transport incurred by using this siderophore, since pyochelin binding and transport have been associated with a 75 kDa IROMP in late logarithmic or early stationary phase cells (Heinrichs *et al.*, 1991). Pyochelin was purified from PH1 culture supernatants as described by Heinrichs *et al.* (1991), and uptake assays were performed with 15 h stationary phase cells grown in succinate medium as described by Cox (1980) (Figure 5.3.).
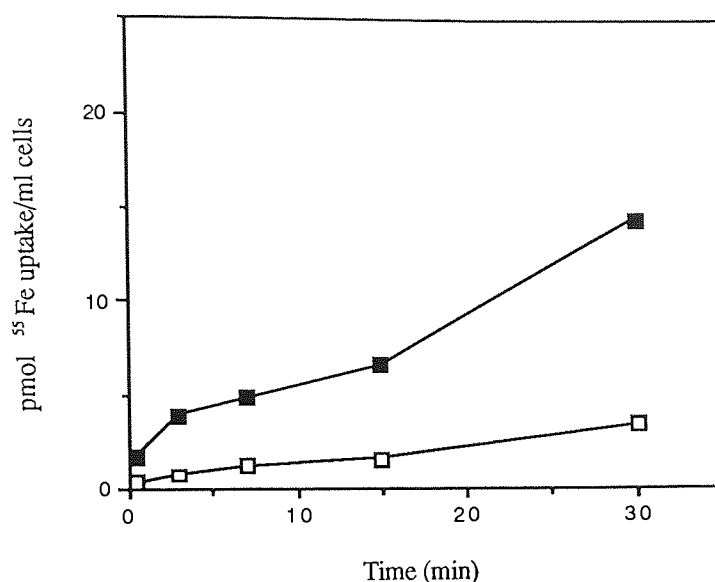
Figure 5.3. Pyochelin-mediated iron ($^{55}Fe^{3+}$) transport by *P. aeruginosa* S.T. 0:9 (□) and PH1 (■) grown in iron-deficient succinate medium. The uptake mixture contained pyochelin (60μM), $^{55}FeCl_3$ (115nM), and 1 ml of cells at an O.D.$_{-470}$ 1.0.

The initial rate of ferripyochelin uptake in PH1 was 0.4 pmol ^{55}Fe/min/ml cells compared with 0.1 pmol ^{55}Fe/min/ml cells for S.T. 0:9

5.2.5. Pyoverdin induction and cross-feeding studies on PH1.

PH1 cells were grown in succinate medium, and in succinate medium supplemented with S.T. 0:9 pyoverdin (80 μg/ml), and used in ^{55}Fe-pyoverdin uptake experiments. Very little growth occurred in the pyoverdin-supplemented culture, indicating that iron was rendered virtually unavailable to PH1 cells.
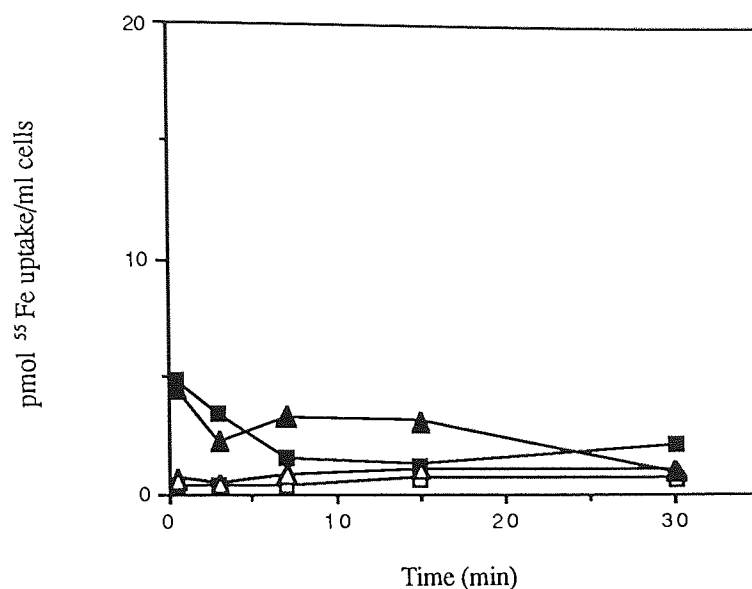
Figure 5.4. S.T. 0:9 pyoverdin- ($\square$) or EDDHA- ($\blacksquare$) mediated iron ($^{55}Fe^{3+}$) transport by *P. aeruginosa* PH1 grown in iron-deficient succinate medium, and S.T. 0:9 pyoverdin- ($\triangle$) or EDDHA- ($\blacktriangle$) mediated iron ($^{55}Fe^{3+}$) transport by *P. aeruginosa* PH1 grown in iron-deficient succinate medium supplemented with 80 μg/ml S.T. 0:9 pyoverdin. The uptake mixture contained pyoverdin (80 μg/ml), $^{55}FeCl_3$ (115 nM), EDDHA (43.2 μg/ml) and 1 ml of cells at $O.D._{470}$ 1.0.

The graph shows that the highest uptake was observed with EDDHA-solubilised $^{55}Fe^{3+}$, as opposed to S.T. 0:9 pyoverdin-solubilised $^{55}Fe^{3+}$; irrespective of preconditioning in the presence of S.T. 0:9 pyoverdin. Similarly, S.T. 0:9 pyoverdin preconditioning appeared to have little effect on rate of S.T. 0:9 pyoverdin-mediated $^{55}Fe^{3+}$ uptake.
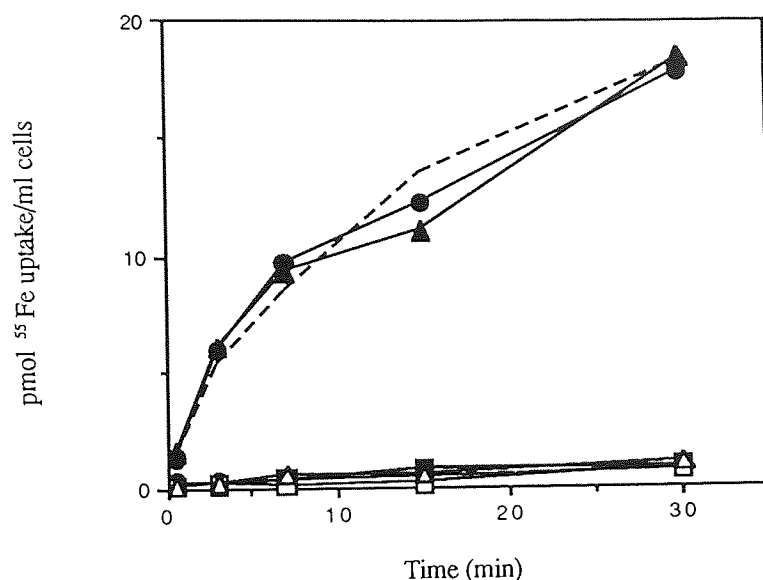
Figure 5.5. S.T. 0:7 pyoverdin- (●), S.T. 0:12 pyoverdin- (■) or S.T. 0:16 pyoverdin- (▲) mediated iron ($^{55}Fe^{3+}$) transport by *P. aeruginosa* S.T. 0:9 grown in iron-deficient succinate medium; S.T. 0:7 pyoverdin- (O), S.T. 0:12 pyoverdin- (□) or S.T. 0:16 pyoverdin- (Δ) mediated iron ($^{55}Fe^{3+}$) transport in *P. aeruginosa* PH1 grown in iron-deficient succinate medium. The uptake of S.T. 0:9 ferripyoverdin by S.T. 0:9 is repeated (dashed line) for comparison. The uptake mixture contained pyoverdin (80 μg/ml), $^{55}FeCl_3$ (115 nM) and 1 ml of cells at $O.D._{470}$ 1.0.
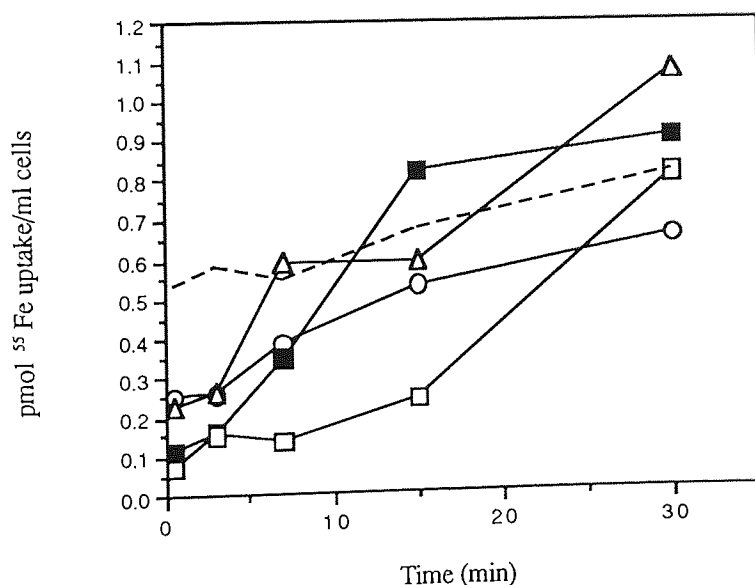
Figure 5.6. S.T. 0:12 pyoverdin-mediated iron ($^{55}Fe^{3+}$) transport in *P. aeruginosa* S.T. 0:9 (■) grown in iron-deficient succinate medium; S.T. 0:7 pyoverdin (O), S.T. 0:12 pyoverdin- (□) or S.T. 0:16 pyoverdin- (Δ) mediated iron ($^{55}Fe^{3+}$) transport in *P. aeruginosa* PH1 grown in iron-deficient succinate medium. The uptake of S.T. 0:9 ferripyoverdin by PH1 is repeated (dashed line) for comparison. The uptake mixture contained pyoverdin (80 μg/ml), $^{55}FeCl_3$ (115 nM) and 1 ml of cells at $O.D._{470}$ 1.0. Note reduced scale of y-axis for clarity.

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The uptakes of S.T. 0:7 and S.T. 0:16 ferripyoverdins by S.T. 0:9 (Figure 5.5.) were similarly high, and comparable to the uptake of S.T. 0:9 pyoverdin by this strain. The uptake of S.T. 0:12 ferripyoverdin by S.T. 0:9, along with the uptakes of S.T. 0:7, S.T. 0:12 and S.T. 0:16 ferripyoverdins by PH1 (Figure 5.6.) were all similarly low, with the rate of uptake by the mutant PH1 being similar to that of the parent for S.T. 0:12 ferripyoverdin.

5.2.6. SDS-PAGE analysis of the IROMPs of additional pyocin Sa-resistant mutants.

Ten more Tn5 mutagenesis experiments were performed, and pyocin Sa-resistant mutants selected as before. SDS-PAGE was performed on the outer membranes of these mutants when grown in iron-deficient succinate medium (Figure 5.7.). As can be seen from the figure, the levels of expression of IROMPs differ, and are summarised in Table 5.1.
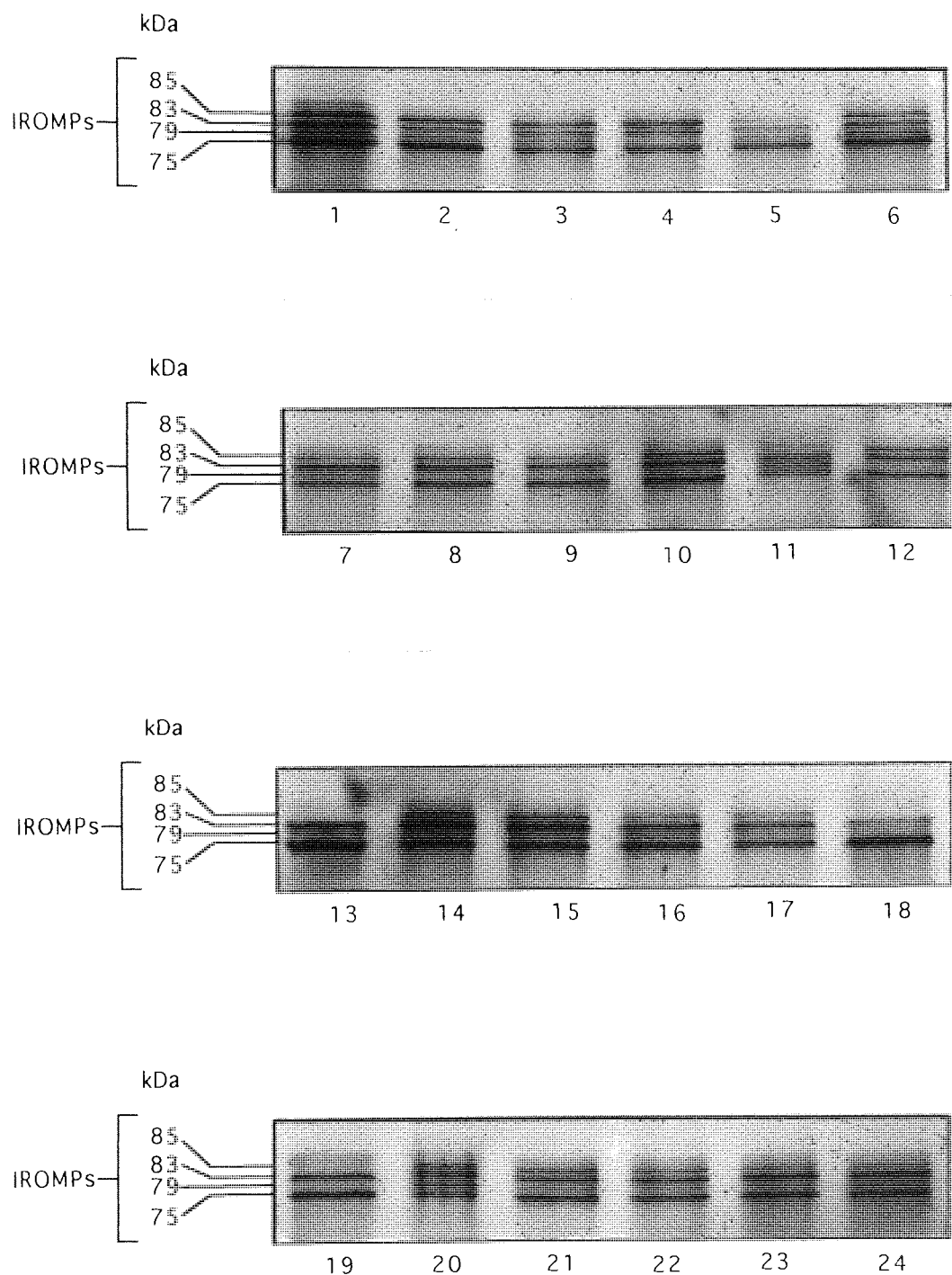
Figure 5.7. SDS-PAGE of outer membranes prepared from pyocin Sa-resistant Tn5 mutants grown in iron-deficient succinate medium. Lane 1 = A1(1); lane 2 = A7(1); lane 3 = G11(2); lane 4 = A1(3); lane 5 = B8(3); lane 6 = E1(3); lane 7 = G2(3); lane 8 = F1(4); lane 9 = F10(4); lane 10 = A9(5); lane 11 = D11(5); lane 12 = A7(6); lane 13 = D10(7); lane 14 = A11(8); lane 15 = B5(8); lane 16 = C1(8); lane 17 = H5(8); lane 18 = H7(8); lane 19 = C3(9); lane 20 = E4(9); lane 21 = E7(9); lane 22 = C6(10); lane 23 = F6(10); lane 24 = 0:9.

5.2.7. Ferripyoverdin-uptake studies on additional pyocin Sa-resistant mutants.

The rate of uptake of ^{55}Fe-pyoverdin after 30 min was determined for each mutant, as was the relative fluorescence of each culture supernatant. Both sets of data are shown on the same graph for ease of comparison (Figure 5.8.).
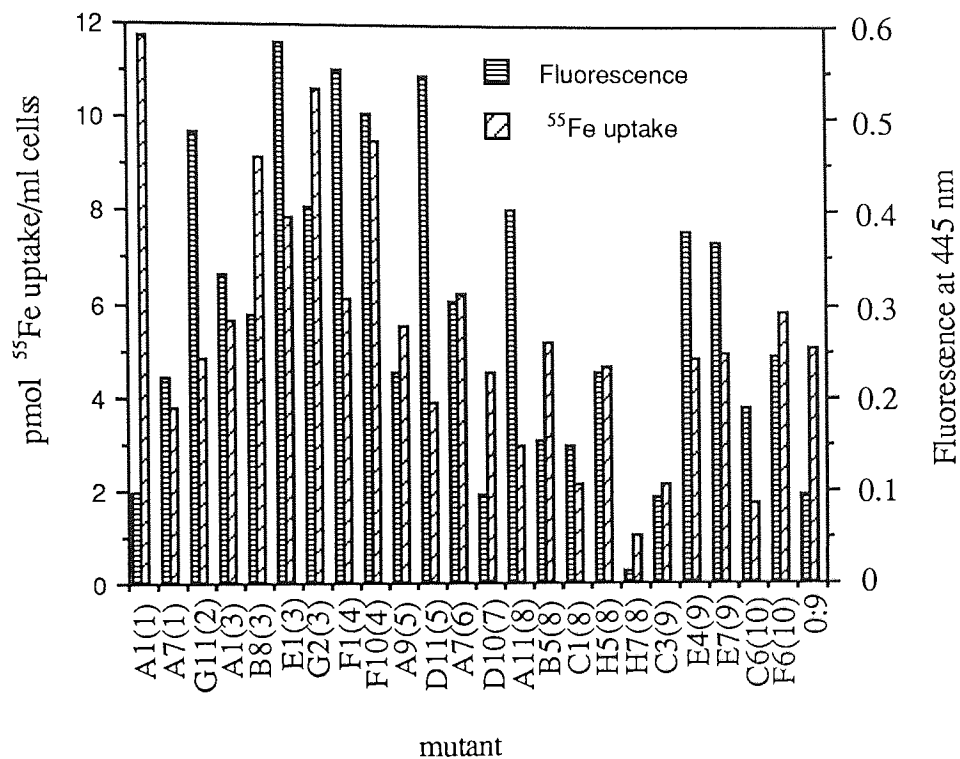
Figure 5.8. Uptake of ^{55}Fe-pyoverdin after 30 min, and fluorescence of culture supernatant for pyocin Sa-resistant mutants.

The data presented in the SDS-PAGE of pyocin Sa-resistant mutant OMs, and in mutant ferripyoverdin uptake and pyoverdin production, are summarised in Table 5.1. Ferripyoverdin uptake and pyoverdin production is expressed as a percentage of that of the parent, S.T. 0:9.

	% ^{55}Fe uptake:	% pvd production:	IROMP alterations:
A1(1)	232	102	none.
A7(1)	74	236	none.
G11(2)	95	516	none.
A1(3)	111	355	none.
B8(3)	181	307	enhanced 75 kDa IROMP.
E1(3)	155	621	enhanced 75 kDa IROMP.
G2(3)	210	434	none.
F1(4)	120	591	none.
F10(4)	188	539	none.
A9(5)	109	240	none.
D11(5)	76	583	all IROMPs poorly expressed.
A7(6)	122	323	none.
D10(7)	89	102	85 kDa IROMP missing, enhanced 75 kDa IROMP.
A11(8)	58	430	none.
B5(8)	102	163	none.
C1(8)	42	156	none.
H5(8)	92	241	none.
H7(8)	20	14	85 kDa IROMP missing, enhanced 75 kDa IROMP.
C3(9)	42	98	85 kDa IROMP missing, enhanced 75 kDa IROMP.
E4(9)	95	405	none.
E7(9)	97	392	none.
C6(10)	33	202	none.
F6(10)	114	261	none.
0:9	100	100	none.

Table 5.1. Uptakes of ^{55}Fe-S.T. 0:9 pyoverdin and pyoverdin production expressed as percentages of the values for S.T. 0:9, and alterations in IROMP profiles for additional pyocin Sa-resistant Tn5 mutants.

5.3. Discussion.

In this work, a mutant resistant to *P. aeruginosa* pyocin Sa which lacks an 85 kDa IROMP and shows greatly reduced ferripyoverdin uptake has been isolated. The inhibition of pyocin Sa killing by competition with ferripyoverdin strongly suggests that the 85 kDa IROMP is both the pyocin Sa receptor and a ferripyoverdin transporter. A similar phenomenon was reported by Liu and Shokrani (1978), in which some *P. aeruginosa* strains were rendered resistant to pyocins in the presence of pyoverdin and pyochelin (which these workers termed 'pyochelins'). This 85 kDa IROMP appears to perform the same function as the 90 kDa IROMP identified by Poole *et al.*(1991), although it remains to be determined whether this strain produces a pyoverdin molecule from the same uptake group (Cornelis *et al.*, 1989). Using a mutant deficient in the 90 kDa IROMP, these workers also provided evidence for a second uptake system (Poole *et al.*, 1991). A second uptake system has also been found in the related fluorescent pseudomonad *P. putida* (Bitter *et al.*, 1991; Leong *et al.*, 1991; Bitter *et al.*, 1993). The *pupA* gene coding for the 85 kDa pseudobactin 358 receptor was identified, and *pupA* mutants were obtained by marker exchange. These mutants still showed 30% uptake, whilst uptake of other pseudobactins was not affected. Growth of this mutant in the presence of pseudobactins BN7 or BN8 (which are not produced by the parent) induced a second 87 kDa IROMP, designated pupB. The residual uptake noted in this study also suggests a second transporter, although it was not sufficient to permit growth in the presence of EDDHA. In agreement with Poole *et al.*, (1991), this putative second ferripyoverdin uptake mechanism was not inducible in PH1 by growth in the presence of pyoverdin (figure 5.4.). Indeed, it would appear that iron uptake in this ferripyoverdin receptor-deficient mutant was poorer if the parent pyoverdin were used as a siderophore rather than the chelator EDDHA. Additionally, cross-feeding both S.T. 0:9 and PH1 with exogenous pyoverdins (figures 5.5. and 5.6.) indicate that S.T. 0:9 possesses only one ferripyoverdin receptor, since ferripyoverdin derived from strain S.T. 0:12 is transported equally poorly in both S.T. 0:9 and PH1, and transport of S.T. 0:7 and S.T. 0:16 ferripyoverdins in PH1 was reduced to that of the parent pyoverdin, indicating that the 85 kDa IROMP was also the route of uptake for these siderophores. In this present study, the pyocin Sa mutant showed only 5% uptake of the parental pyoverdin. It therefore seems likely that the 85 kDa IROMP is a specific high-affinity ferripyoverdin receptor, whereas a second receptor, which has lower affinity for a wider range of pyoverdins, also exists, although it would appear to be different to the secondary system in *P. putida*, since the rate of uptake is very much lower, and the corresponding IROMP appears to be non-inducible. Meyer (1992) has shown that OprF may take part in uptake of a wide range of exogenous siderophores, including ferripyoverdin; a porin route of entry would explain the higher uptake of ferric-EDDHA compared to that of ferripyoverdin in PH1 (figure 5.4.), since ferric-EDDHA complexes are smaller than ferripyoverdins.

The ferripyochelin transport assays described in this work support the finding that the 75 kDa IROMP is a second ferripyochelin transporter (Heinrichs *et al.*, 1991), and suggest that PH1 compensates for the deficiency in pyoverdin biosynthesis and transport by derepressing further the pyochelin-based system. This effect seems to be restricted to an increased ability to transport the siderophore complex, rather than to produce siderophore, since no differences in pyochelin production between PH1 and S.T. 0:9. were detected. Similarly, with the growth conditions used in this study, the increase in ferripyochelin transport correlated with the 75 kDa IROMP transporter rather than the well-characterised 14 kDa ferripyochelin binding protein (Sokol and Woods, 1983).

The perturbations in expression in OM proteins in PH1 were not seen only after growth in iron-depleted media. When grown in iron-rich conditions, which repress synthesis of IROMPs, expression of OprG in PH1 was reduced by approximately 50%. Whilst the role of OprG remains unclear, its expression has been shown to increase when the level of available iron is similarly increased, and it is suggested that OprG may participate in low-affinity iron uptake (Yates *et al.*, 1989).

Analysis of the IROMPs and ferripyoverdin uptake/pyoverdin biosynthesis data from the additional pyocin Sa-resistant mutants suggested that resistance to pyocin Sa may be accomplished by different mechanisms. The most obvious means by which resistance to pyocin might be achieved is by elimination of the outer membrane binding site, previously demonstrated to be the 85 kDa ferripyoverdin receptor. However, only mutants D10(7), H7(8) and C3(9) appear to utilise this strategy, with a concomitant increase in expression of the 75 kDa IROMP. This result would indicate that the reduction in ferripyoverdin uptake, and therefore iron, is compensated for by emphasising ferripyochelin uptake, as already seen for PH1. A similar effect was observed by Meyer *et al.*, (1992), in a mutant of *P. fluorescens* CHAO unable to produce pyoverdin, which showed considerably enhanced ferric-salicylic acid uptake. Mutant H7(8) appears to behave similarly to PH1, in that they both lack an 85 kDa IROMP, show increased expression of the 75 kDa IROMP, and have greatly reduced levels of ferripyoverdin uptake and pyoverdin production. C3(9) is similar, except that pyoverdin production appears normal. Although D10(7) appears to lack the 85 kDa IROMP, it is still capable of ferripyoverdin uptake at 89% that of the parent. This result contradicts studies made on the mutant PH1, and may be due to a reversion to the parent phenotype during the interval between outer membrane preparations and ferripyoverdin uptake experiments. Workers at The Department of Infection, Medical School, The University of Birmingham, had encountered similar problems with mutants when stored at -70°C in glycerol-supplemented medium, which led them to employ a different method of storage (personal communication).

The majority of the mutants are considerable over-producers of pyoverdin, with only six of the mutants producing pyoverdin at less than double the parent rate, suggesting that resistance to pyocin Sa may be achieved, at least in part, by

overproducing pyoverdin to promote competition for the ferripyoverdin receptor.

Five of the mutants, A7(1), A11(8), C1(8), C6(10) and D11(5), appear to utilise both mechanisms in combination, such that low ferripyoverdin uptake is accompanied by overproduction of pyoverdin. D11(5) is different to the others in this group in that all of the IROMPs are partially repressed.

Two of the mutants, A1(1) and B5(8), exhibited normal levels of pyoverdin production, with normal (B5(8)) or increased (A1(1)) ferripyoverdin uptake. These mutants may be revertants (Cornelis *et al.*, 1992), or may indicate that, although the outer membrane receptor for ferripyoverdin and pyocin Sa is shared, transport across the inner membrane may require different systems, and a defect in the system utilised by pyocin Sa may lead to resistance. Thomas and Valvano (1993) have reported that cloacin DF13 (a bacteriocin secreted by *Enterobacter cloacae*) and ferric aerobactin share the same IutA receptor in *E. coli* K-12, but that different internalisation systems are utilised by these compounds.

The mechanisms of resistance to pyocin Sa indicate the complexity of regulation of pyoverdin secretion and uptake; Koster *et al.*, (1993) have shown that a second pyoverdin uptake system (*pupB*) may be induced in *P. putida* by growth in the presence of one of the two cognate pseudobactins, possibly regulated by *pupR*. Regulation of a siderophore uptake system has also been demonstrated in the ferric dicitrate transport system of *E. coli* K-12, where the periplasmic FecR protein acts as a sensor for ferric citrate, and interacts with the cytoplasmic membrane-associated FecI which, in turn, induces the FecBCDE periplasmic-binding-protein-dependent transport mechanism for ferric dicitrate (Van Hove *et al.*, 1990; Staudenmaier *et al.*, 1989).

In summary, these data suggest that the site of interaction of pyocin Sa is a ferripyoverdin receptor. While the deficiency in pyoverdin transport and synthesis in PH1 could be attributed to downstream effects exerted by Tn5 insertion into an operon (Weiss *et al.*, 1983) and the increased ability to transport ferripyochelin could be a response to the increased iron deficiency imposed by the inability to transport ferripyoverdin, the altered expression of OprG under iron-rich conditions, where pyoverdin-mediated iron transport is not thought to operate, was surprising. Moreover, the observation that the receptor for this pyocin is a critical component of the iron uptake system and yet few strains are susceptible to it lends further credence to the notion that pyoverdin biosynthesis and uptake are highly heterogeneous amongst *P. aeruginosa* strains. The additional Tn5 mutants appeared to exploit different strategies to achieve pyocin Sa-resistance, indicating that complex regulatory systems exist to enable these organisms to compete effectively for iron.

6. UPTAKE OF BRL 41897A, a C(7) α-FORMAMIDO SUBSTITUTED CEPHALOSPORIN, BY *PSEUDOMONAS AERUGINOSA.*

6.1. Introduction.

Many bacteria, when encountering conditions of low iron availability, have the capacity to elaborate into the surrounding environment low-molecular-weight iron-chelating molecules (siderophores) which, when complexed with iron, are delivered into the cell via interaction with their cognate receptors on the bacterial cell surface (Bullen & Griffiths, 1987b; Neilands, 1993). Exploitation of these iron-transport mechanisms may provide alternative routes by which antibiotics could enter pathogenic bacteria, thus avoiding the more usual porin route of entry (Minnick *et al.*, 1992). Dolence *et al.*, (1991) synthesised various siderophore-antibiotic conjugates, in which N^5-acetyl-N^5-hydroxy-L-ornithyl-N^5-acetyl-N^5-hydroxy-L-ornithyl-N^5-acetyl-N^5-hydroxy-L-ornithine, the functionally instrumental component of the fungal siderophore ferrichrome, was incorporated into both carbacephalosporin and oxamazin-type ß-lactam antibiotics. These antibiotics were designed with the additional purpose of being used in deferration therapy without the associated administration of antibiotics to eliminate the risk of septicaemias, since conventional treatment for iron overload uses naturally occurring siderophores which may be utilised by several types of microbes to promote their own growth *in vivo*. As expected, these compounds were transported into susceptible cells via their ferrichrome-hydroxamate iron transport systems. Since fluorescent *Pseudomonas* spp. possess a Fe^{3+}-ferrichrome uptake system, (Jurkevitch *et al.*, 1992), it may be assumed that semi-synthetic compounds of this type may enter *Pseudomonas* spp. via this route.

Watanabe *et al.*, (1987) investigated the mode of action of E-0702, a novel cephalosporin, on various bacteria, including *E. coli* and *P. aeruginosa*, by selecting resistant mutants. It was found that the mechanism of resistance did not fall into one of the three basic categories of resistance, i.e. inactivation of the antibiotics by ß - lactamase, alteration of the targets (penicillin-binding proteins, or PBPs), or reduced penetration of the outer membrane as a result of alteration of porin proteins, but by a defect in the *tonB* gene product, thus deactivating TonB-dependent iron transport systems.

Curtis *et al.*, (1988) went on to show that catecholic ß-lactam antibiotics are transported actively into *E. coli* K-12 cells via the iron-regulated outer membrane proteins Fiu and Cir, controlled by TonB, such that both *tonB* and *fiu cir* mutants showed a comparably reduced susceptibility to the test compounds. This finding indicates that both Fiu and Cir are both jointly and specifically involved in TonB-mediated uptake of

catecholic ß-lactam antibiotics. Nikaido and Rosenberg (1990) have also confirmed this finding, and suggest that the proteins Fiu and Cir, whose functions were hitherto unknown, may be involved in the recapture of 2,3-dihydroxybenzoic acid and 2,3-dihydroxybenzoyl serine (the hydrolytic products of enterobactin), while Hantke (1990) showed that these breakdown products may act as siderophores.

Interestingly, Brochu *et al.*, (1992) have shown that two new siderophore-ß-lactam conjugates, one a spermidine-based catechol siderophore-carbacephalosporin (JAM-2-263) and the other an N^5-acetyl-N^5-hydroxy-L-ornithine tripeptide hydroxamate siderophore-carbacephalosporin (EKD-3-88), strongly inhibited the growth of *Escherichia coli* X580, with resistance in this organism to JAM-2-263 arising from loss of expression of the 74 kDa Cir protein, while EKD-3-88 resistance was accompanied by loss of the 78 kDa Fhu protein, demonstrating the requirement of Cir and Fhu in uptake of these compounds into the cell.

Basker *et al.*, (1984) showed that the 6α-substituted penicillin BRL 36650 is active against both Enterobacteriaceae and *Pseudomonas aeruginosa*, while Basker *et al.*, (1986) showed that, while naturally-occurring 7α-formamidocephalosporins have only poor antibacterial activity, some semi-synthetic derivatives displayed outstanding broad-spectrum activity. Critchley *et al.*, (1991) went on to show that BRL 41897A, a C(7) α-formamido substituted cephalosporin (figure 6.1.), was once again able to utilise the Fiu and Cir proteins of *E. coli* to gain entry into the cell, and also that this compound exhibited considerable activity against *P. aeruginosa* under conditions of iron-limitation.
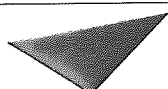
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Figure 6.1. Structure of C(7)α-formamido cephalosporins. BRL 41897A, X = OH; BRL 42948A, X = H (Critchley *et al.*, 1991).

In this chapter, the role of the iron uptake systems of *P. aeruginosa* in the uptake of the C(7) α-formamido substituted cephalosporin BRL 41897A is examined.

6.2. Results.

6.2.1. Effect of iron on the sensitivity to BRL 41897A.

Table 6.1. shows the MIC of BRL 41897A in *P. aeruginosa* under different growth conditions.

Strain	MIC (mg/l)			
	+Fe	-Fe	-Fe+pch	1/8 MIC
IA1	------	16.0	4.0	------
PH1	>96	1.8	------	------
PAO1	------	4.0	------	7.5
0:9	>384	3.6	------	------

Table 6.1. MICs of BRL 41897A for the four *P. aeruginosa* strains used in this study when grown in succinate medium (-Fe), succinate medium supplemented with 0.1 mM $FeCl_3$ (+Fe), and the MIC of BRL 41897A for *P. aeruginosa* IA1 in succinate medium supplemented with 50 mg/l pyochelin (+pch). To examine the effect of growth in a sub-inhibitory concentration of antibiotic, PAO1 cells were grown in succinate medium supplemented with 0.375 mg/l (1/8 MIC) BRL 41897A prior to determination of MIC.

Under conditions of plentiful iron (0.1 mM), no MIC could be determined for *P. aeruginosa* 0:9 or the pyoverdin biosynthesis/transport deficient derivative PH1; however, under conditions of low iron, the MICs of PH1, 0:9 and PAO1 were within the range 1.8 - 4.0 mg/l. When grown under iron-limited conditions, the MIC for IA1, a pyoverdin/pyochelin biosynthesis mutant of PAO1, was four times greater than the highest MIC obtained for the other strains used in the same conditions. When IA1 inoculum was grown under low iron conditions and supplemented with pyochelin (50 mg/l), the MIC was found to be 4.0 mg/l, within the range of MICs found for PH1, 0:9 and PAO1. When PAO1 was grown in the presence of a sub-inhibitory concentration of BRL 41897A (an eighth, or 0.375 mg/l), and these cells used as the basis of determining the MIC, it was found that the MIC rose slightly, to 7.5 mg/l

6.2.2. Pyochelin competition.

Competition experiment. 0.05 mL of pyochelin (10mM in methanol) was applied to a sterile 9mm fibre filter disc, and allowed to dry at 37°C for 30 min. 0.1 mL of an overnight culture of *P. aeruginosa* IA1 grown in succinate medium was spread onto the

surface of a succinate agar plate containing 20 mg/l BRL 41897A, the seeded plate was surface-dried for 15 min at 37°C, and the pyochelin-pretreated disc was placed onto the surface of the agar.

The plate was incubated at 37°C for 24 h, after which time a distinct zone of bacterial growth was observed around the pyochelin-loaded filter paper disc.

6.2.3. ^{55}Fe-BRL 41897A transport assays.

Strain IA1 was chosen for these studies because it has been shown that its iron uptake systems and sensitivity to BRL 41897A could be modulated by siderophore pretreatment (Gensberg *et al.*, 1992; this work). Radiolabelled iron transport experiments were performed to determine the kinetics of accumulation when complexed with BRL 41897A. The method of Critchley *et al*, (1991) to determine uptake of radiolabelled ferric-antibiotic complexes, in which nitrilotriacetic acid (NTA) was utilised as a source of solubilised ferric iron, was not used, since NTA was readily utilisable as a source of ferric iron for IA1 in the experiments (data not shown), as demonstrated by Meyer and Hohnadel (1992). Figure 6.2. shows uptake profiles for cells grown in succinate medium supplemented with 50 mg/l pyochelin, and for control cells.
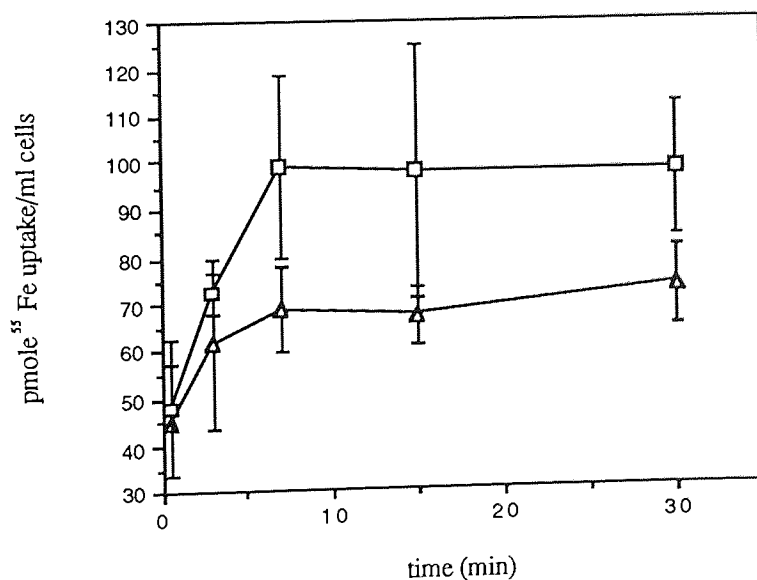
Figure 6.2. BRL 41897A-mediated ^{55}Fe uptake (pmoles ^{55}Fe/ml cells at O.D.$_{470}$ 1.0) in *P. aeruginosa* IA1 grown in succinate medium (△), and in succinate medium supplemented with pyochelin (□).

When IA1 cells had been pre-conditioned with pyochelin prior to assay, an increased accumulation of ^{55}Fe-BRL 41897A uptake was observed (97.82 pmoles ^{55}Fe/mL cells, compared to 72.72 pmoles ^{55}Fe/mL cells, after 30 min). Pretreatment with pyoverdin did not increase uptake compared to non-pretreated control cells (data not shown). Although there was some inter-experimental variability, uptake was essentially complete after 10 min. and the pyochelin pretreated cells acquired more iron.

6.2.4. SDS-PAGE analysis of outer membrane proteins.

Figure 6.3. shows the effect of growth in the presence of sub-inhibitory concentrations of BRL 41897A on outer membrane proteins.
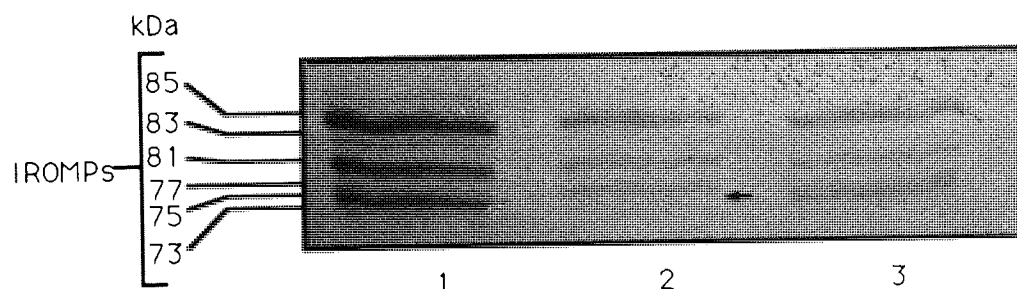
Figure 6.3. SDS-PAGE of *P. aeruginosa* PAO1 outer membranes from cells grown in succinate medium alone (lane 1), supplemented with 0.375 mg/l BRL 41897A (lane 2), and supplemented with 0.094 mg/l BRL 41897A (lane 3).

Growth in 1/8 MIC (lane 2) appears to have a similar distribution of IROMPs to control cells, with the notable exception of the 75 kDa protein, which is absent (arrowed). Reducing to 1/32 MIC did not result in any significant changes compared to control.

6.3. Discussion.

It can be seen that the susceptibility of *P. aeruginosa* to BRL 41897A is modulated by the presence of iron in the growth medium, implicating the high affinity iron uptake systems as possible mediators of transport. In contrast to Critchley *et al.*, (1991), it was not possible to measure an MIC against strains grown in iron-supplemented succinate medium, nor could an MIC for the *p*-hydroxy derivative BRL 42948A be measured under either iron-replete or iron-depleted conditions (data not shown). The mutant IA1, which lacks the ability to produce siderophores but is not deficient in expression of their cognate receptors, displays an MIC four times higher than its parent, PAO1. It has been shown that IA1 can respond specifically to the presence of siderophore in the growth medium and not simply to the iron-deficiency imposed by that siderophore (Gensberg *et al.*, 1992); it therefore seems likely that the IA1 cells, not having been exposed either to pyoverdin or pyochelin, have comparatively fewer numbers of these siderophore receptors in the outer membrane compared to PAO1, which is capable of self-induction due to endogenous siderophore synthesis. When IA1 was grown in the presence of pyochelin, which we have shown to increase ferripyochelin transport by increased synthesis of the 75 kDa IROMP receptor, the MIC fell to a similar level to the parent strain PAO1. This result suggested that the iron-antibiotic complex was exploiting the ferripyochelin transport system for entry into the cells.

The MIC for PH1, a mutant of the *P. aeruginosa* clinical isolate 0:9, was slightly lower than that for its parent (0.73-3.0 mg/l compared to 2.25-5.0 mg/l) in iron-deficient medium. This strain lacks the 85 kDa ferripyoverdin receptor and overexpresses the 75 kDa ferripyochelin receptor, possibly to counteract the lack of iron uptake via ferripyoverdin (Smith *et al.*, 1992). These data indicate that the antibiotic is not entering via the ferripyoverdin system. However, overexpression of the ferripyochelin receptor correlates with its increased susceptibility, again suggesting a ferripyochelin receptor route of entry for the antibiotic. A simple disk competition assay also supported the hypothesis that ferripyochelin and ferric-BRL 41897A are competing for the same receptor

Iron transport studies (Table 6.2. and Figure 6.2.) also confirmed that more iron, and presumably more antibiotic, accumulated in cells preconditioned with pyochelin. Preconditioning cells with pyoverdin prior to transport assays had no effect on the rate of uptake of ^{55}Fe-BRL 41897A.

When PAO1 cells were grown in the presence of a sub-inhibitory concentration of BRL 41897A prior to use in MIC determinations, a slightly higher MIC was observed, indicating that the iron-antibiotic complex was unable to signal the cells to produce ferri-pyochelin receptors. These data led to investigation into the effect of growth in the presence of sub-inhibitory concentrations of BRL 41897A on expression of outer

membrane proteins. Figure 6.3. shows the SDS-PAGE outer membrane protein profiles of PAO1 after growth in 0.094 mg/l (1/32 MIC) and 0.375 mg/l (1/8 MIC) of BRL 41897A. Growth of PAO1 in 1/32 MIC showed minor alterations in expression of the 83 and 73 kDa proteins when compared to the outer membrane protein distribution in PAO1 grown in succinate alone, but this was not reflected in a change in MIC (data not shown). However, growth in 1/8 MIC showed considerable reduction in expression of the 75 kDa ferripyochelin receptor, which may explain the increased MIC. Competition between a sub-inhibitory concentration of antibiotic and pyochelin for the pyochelin receptor may reduce effective iron transport via this route, leading to decreased derepression of this system and dependence on other iron-uptake systems to compensate for the deficit.

Bitter et al., (1993) studied three different catecholic cephalosporins (E-0702, M-14659 and M205113) against Pseudomonas putida, and noted only small differences in MIC in mutants deficient in pseudobactin transport. These data support the hypothesis that the pyoverdin/pseudobactin transport system is not the primary uptake route for these catechol-substituted compounds. Interestingly, Hazumi et al. (1992) demonstrated that resistance of P. aeruginosa PAO2146 to the catecholic cephem antibiotic BO-1341, was accompanied by elevated expression of both an 84 kDa outer membrane protein and a 55 kDa periplasmic protein when cells were grown in iron-replete media. Since BO-1341 contains a 6,7-dihydroxyisoquinolinium moiety, and given that an 84 kDa IROMP is involved in resistance to BO-1341, it is possible that BO-1341 enters susceptible PAO 2146 cells via the high-affinity ferripyoverdin uptake system, and that resistance to this antibiotic is achieved, in part, by derepression of this system in iron-replete conditions which may allow binding but not transport of the compound; the role of the 55 kDa periplasmic protein in resistance to BO-1341 is unknown. In iron-depleted succinate medium, in which the ferripyoverdin-transport systems of both parent and mutant are derepressed, the MICs for both mutant and parent were the same.

In all of the experiments conducted, the cells were grown to early stationary phase. It therefore seems likely that the reported 14 kDa ferripyochelin receptor (Sokol and Woods, 1983) was not involved in antibiotic uptake, since this receptor is present early in log phase, to be replaced by the 75 kDa ferripyochelin receptor in late log phase. This hypothesis may be verified by experiments using a P. aeruginosa mutant deficient in the 14 kDa ferripyochelin receptor (Heinrichs et al., 1991). It seems unlikely that the ferric-antibiotic complex may enter in significant amounts through the porin-OprF channels (Meyer, 1992) in light of the difference in MIC for cells grown with and without iron, and the apparent ferripyochelin-inducible uptake of ^{55}Fe-BRL 41897A.

Critchley et al., (1991) showed that in E. coli BRL 41897A exploited the Fiu and Cir outer membrane proteins for entry into the cell, and not the FepA protein (responsible for ferrienterobactin uptake). Further, Nikaido and Rosenberg (1990) suggest that the E. coli proteins Fiu and Cir may be involved in the recapture of the hydrolytic products of

enterobactin, which occur spontaneously in growth media, such as 2,3-dihydroxybenzoic acid and 2,3-dihydroxybenzoyl serine.
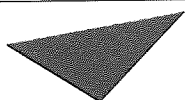
Figure 6.4. Structures of 2,3-dihydroxybenzoic acid, and 2,3-dihydroxybenzoylserine, respectively (Griffiths, 1987b).

It is unlikely that the function of this mechanism is to facilitate reconstruction of enterobactin molecules, since, although *P. aeruginosa* demonstrates inducible ferrienterobactin uptake and utilisation (Poole *et al.*, 1990), it does not produce enterobactin. Hantke (1990) showed that in *E. coli*, ferric-dihydroxybenzoylserine acts as a siderophore, delivering iron to the cell via FepA, Fiu and, to a lesser extent Cir, while ferric-dihydroxybenzoate was taken up by Fiu, Cir and, to a lesser extent, by FepA. In view of the findings, it would appear that the ferripyochelin receptor behaves in the same way as *E. coli* Fiu or Cir, in respect of BRL 41897A uptake.

An interesting alternative to the use of semi-synthetic iron-chelating antibiotics may be to utilise naturally-occurring, iron-containing, cyclic decapeptides called ferrocins which also contain the iron-binding component of the fungal siderophore ferrichrome (Katayama *et al.*, 1992).
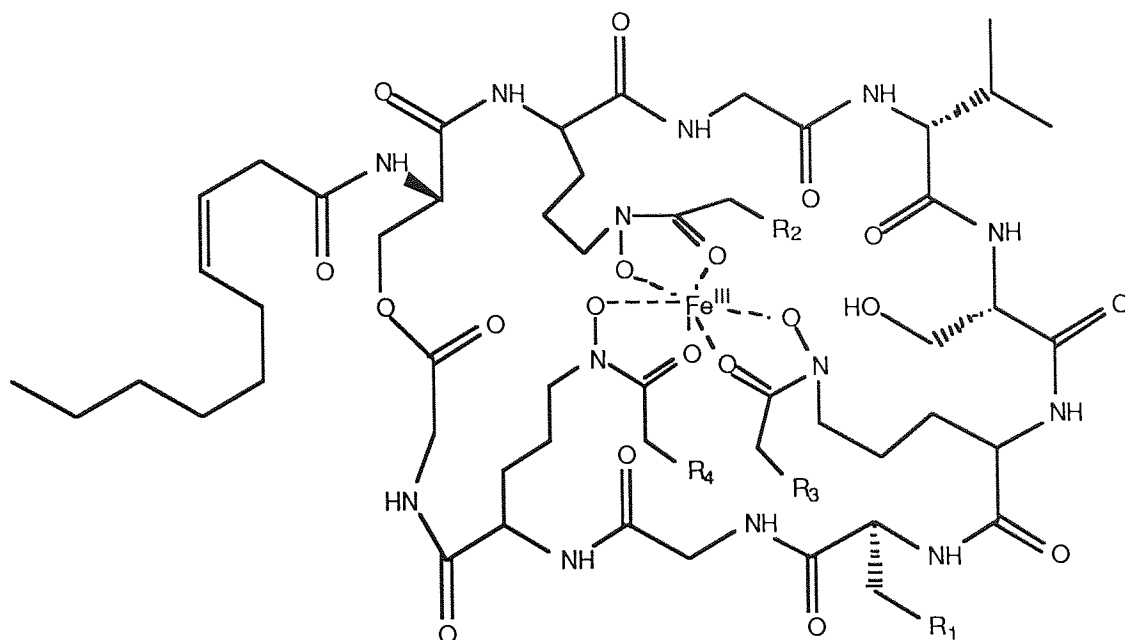
Figure 6.5. The structure of ferrocins A, B, C and D. Ferrocin A: R_1 = H, R_2, R_3 and R_4 = H. Ferrocin B:R_1 = OH, R_2, R_3 and R_4 = H. Ferrocins C and D: R_1 = H, R_2, R_3 = H, and R_4 =CH_3. (Katayama *et al.*, 1992).

These compounds were shown to be produced by *P. fluorescens* in both iron-depleted and even more so in iron-replete media, and were active against *P. aeruginosa*.in iron-deficient conditions, although the mode and site of action is unknown. Despite the apparent siderophore-like structure of ferrocins, it is more likely that they function to suppress growth of competing *P. aeruginosa*, and possibly other organisms, in the rhizosphere.

More than 90% of cystic fibrosis (CF) patients suffer from progressive deterioration in respiratory function due to colonisation by *P. aeruginosa* (Woods *et al.*, 1991) and, more recently, *P. cepacia* (Lewin *et al.*, 1990). Since pyochelin utilisation is ubiquitous in both *P. aeruginosa* clinical isolates (Cox and Graham, 1979) and *P. cepacia* clinical isolates (Sokol, 1986), the therapeutic potential of BRL 41897A and related compounds is considerable.

7. CLONING AND SEQUENCING OF IRON-REGULATED OUTER MEMBRANE PROTEIN G.

7.1. Introduction.

In anaerobic environments, ferrous ion concentrations may reach 100mM at pH 7.0, whereas in aerobic conditions, oxidation of ferrous to ferric ion occurs, with a concomitant decrease in solubility (Neilands, 1993). Precipitation of ferric ion to form polymeric ferric oxyhydroxides created a bacterial requirement to elaborate iron-solubilising molecules (siderophores). Much attention has been paid to the role of high affinity iron uptake systems, which allow pathogenic bacteria to compete with host iron-binding proteins such as transferrin and lactoferrin, and their role in pathogenicity (Griffiths, 1990). However, in the absence of these proteins and in an iron-replete environment in which high-affinity uptake systems are repressed, a requirement for solubilisation of ferric ion remains. Yates et al., (1989) observed a direct relationship between media iron concentration and expression of a 25 kDa outer membrane protein in *Pseudomonas aeruginosa*, termed outer membrane protein G (OprG). Little is known of this protein; Anwar et al., (1984) demonstrated an antibody response to OprG using serum from a CF patient, indicating that OprG expression occurs in the iron-limited conditions encountered in the CF lung, while Cochrane et al., (1988) showed repression of OprG in a rat lung model. Both Chamberland et al., (1989) and Yates et al., (1989) suggest quinolone resistance may be related to reduced expression of OprG, implying that it acts as a porin, while Yoshihara and Nakae (1989), using reconstituted liposome swelling assays, found that OprG had little or no pore-forming activity. Evidence that OprG is involved in iron uptake was provided in studies on a pyoverdin biosynthesis and uptake-deficient Tn5 mutant (Smith et al., 1992), in which OprG expression was reduced by 50% in iron-replete conditions. Conversely, Angerer et al. (1992) described a ferric iron uptake system in *Serratia marcescens* which is not dependent on either an outer membrane receptor or TonB under high-iron conditions, in which sufficient ferric iron for bacterial growth, solubilised with oxaloacetate, citrate and sodium pyrophosphate, is delivered across the cytoplasmic membrane via the SfuABC proteins.

In an attempt to clarify the structure and function of OprG, work was undertaken to isolate and sequence the *OprG* gene.

7.2. Results.

7.2.1. Protein purification.

Outer membranes were prepared from an overnight 500 ml liquid culture of IA1 cells in L-broth, and subjected to SDS-PAGE. The OMPs were electroblotted onto PVDF membrane, and the membrane was stained and dried. The OprG band was excised from the blot and subjected to amino-acid microsequencing (courtesy of Dr. P. Williams, Department of Pharmaceutical Sciences, The University of Nottingham). The procedure was repeated, so that two entirely separate OprG preparations could be microsequenced. The N-terminal amino acid sequences of both OprG preparations were compared and found to be identical, as follows:

$$NH_2\text{-Asp-Ile-Gln-Gly-His-Lys-Ala-Gly-Asp-Ile-}$$
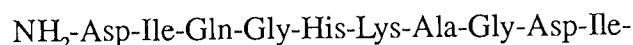
or, using one-letter code:

$$NH_2\text{-D-I-Q-G-H-K-A-G-D-I-}$$

A degenerate oligonucleotide probe was constructed based on the first 6 amino acids revealed by microsequencing, by referring to both the amino-acid sequence and the codon usage chart for *Pseudomonas aeruginosa* (Wada *et al.*, 1992), of the following sequence:

$$5'\text{-GAW ATW CAR GGQ CAW AA-3'}$$
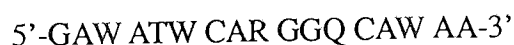
where G = guanine, A = adenine, T = thymine, C = cytosine, and
W = C or T, R = A or G, and Q = inosine.

7.2.2. DNA manipulations.

P. aeruginosa IA1 chromosomal DNA was digested (in 1 μg aliquots) with the restriction enzymes Kpn I, EcoRI, BamH1 and Hind III, run on a 1% agarose gel at 70 V for 3 h 15 min, and the DNA fragments were Southern blotted onto nitrocellulose membrane. The oligonucleotide probe was end-labelled using ^{32}P-ATP, and the radiolabelled probe was hybridised with the fixed DNA on the Southern blot. The hybridisation temperature is the temperature at which perfect hybrids of the oligonucleotide probe with target DNA are stable, but mismatched hybrids are unstable; this is achieved by hybridising at about 5°C below the temperature (T_d) at which a

perfectly-matched hybrid will be half-dissociated. The T_d can be estimated from the equation:

$$T_d = 4°C \text{ per GC base pair} + 2°C \text{ per AT base pair}$$

For the mixed oligonucleotide, a hybridisation temperature 5°C lower than the lowest T_d ($T_{d_{min}}$) was used. The blot was washed three times with 50 ml 6xSSC at 39°C for 5 min each, followed by a wash at 44°C for 2 min, and finally rinsed in 6xSSC at room temperature.

The probed Southern blot was subjected to autoradiograpy.
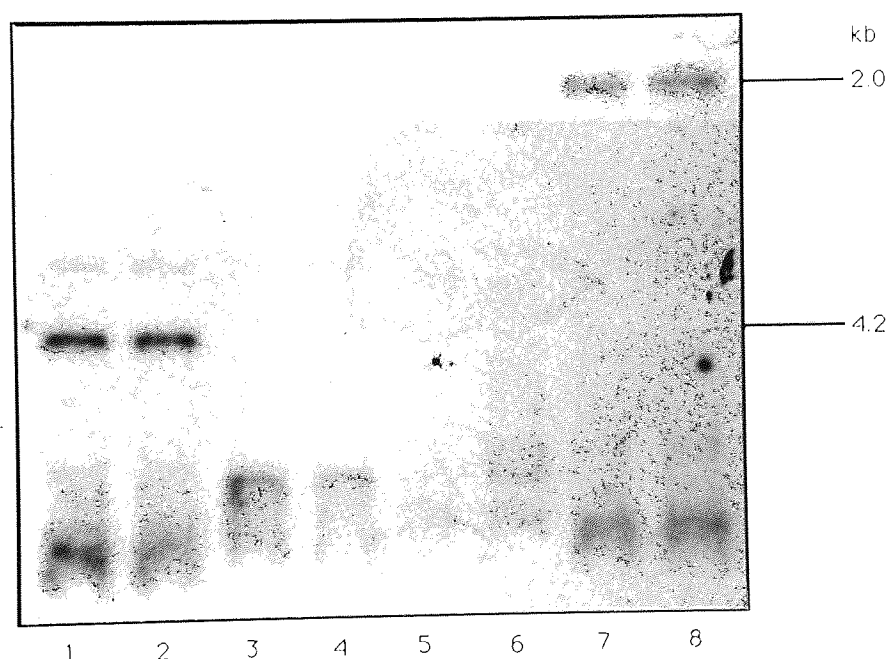
Figure 7.1. Autoradiograph of oligonucleotide-probed Southern blotted *P. aeruginosa* IA1 genomic DNA. Lanes 1 and 2 = HindIII; lanes 3 and 4 = BamHI; lanes 5 and 6 = EcoRI; lanes 7 and 8 = KpnI.

It was found that a 4.2 kb fragment of genomic DNA generated by Hind III digestion hybridised with the oigonucleotide probe, as well as a 1.9 kb fragment as a result of Kpn I digestion. It was decided to use the larger Hind III fragment for subsequent cloning and sequencing, because of the higher probability that the entire gene coding for OprG would be present. DNA fragments produced by Hind III digestion with lengths between 3.4 and 5 kb were recovered from the agarose gel using the GeneClean procedure.

The recovered HindIII-cut fragments were ligated into similarly-cut pKS+ plasmid, and the recombinant plasmids were used to transform competent XL-1 *E. coli* by electroporation. Aliquots of the transformed cells were spread onto L-agar plates containing ampicillin and tetracycline, which had been surface-seeded with 40 µl X-gal (20 mg/ml in DMF) and 4 µl 0.84M IPTG. The plates were incubated overnight at 37°C and incubated for a further 36 h at 4°C to allow the blue-white selection to develop. Approximately 1500 white colonies (therefore containing pKS+ plasmids with insert) were produced by this method, which were individually picked onto duplicate L-agar plates containing ampicillin and tetracycline. Cells from each colony were transferred to nitrocellulose filters (making careful note of their orientation using pencil and punch marks). The DNA from the colonies was fixed to the filters, the filters were probed using the same conditions as for the Southern blot, and then subjected to autoradiography.

There was a total of 28 possible "positives", i.e. colonies containing DNA which hybridised with the oligonucleotide probe. These positives were picked onto fresh duplicate L-agar plates containing ampicillin and tetracycline, and again subjected to blotting, hybridisation and autoradiography.
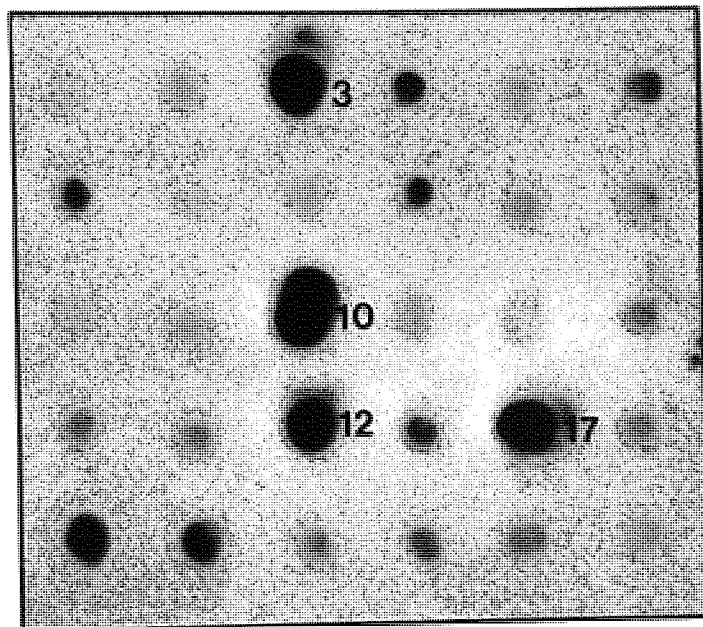
Figure 7.2. Autoradiograph of oligonucleotide-probed colony-blotted genomic DNA. The colonies harbouring DNA complementary to the probe are numbered.

It was found that four of the colonies unequivocally contained DNA to which the radiolabelled oligonucleotide probe bound strongly; these "positive" XL-1 strains were designated pKS+/3, pKS+/10, pKS+/12 and pKS+/17 (according to their numbered location during the previous selection procedures). Cells from these colonies were harvested and stored at -70°C.

Plasmid DNA was extracted from 1 ml cultures of each the four "positives" and cut with HindIII to verify that they all contained identical-length inserts in their plasmids; this was found to be the case, with all four plasmid types harbouring a 4.2 kb insert, presumably of *P. aeruginosa* IA1 genomic DNA.

XL-1/pKS+/3, 10, 12 and 17 DNA preparations were subjected to multiple digestions and agarose gel electrophoresis, and their individual restriction fragment patterns compared to each other, to establish that they were identical. Restriction fragment distributions were very similar, only subtle differences being observed in one or two bands on the agarose gel, while all other bands appeared to be of identical weight. The most striking difference in fragment distribution between the four cell types was produced when subjected to PstI digestion.
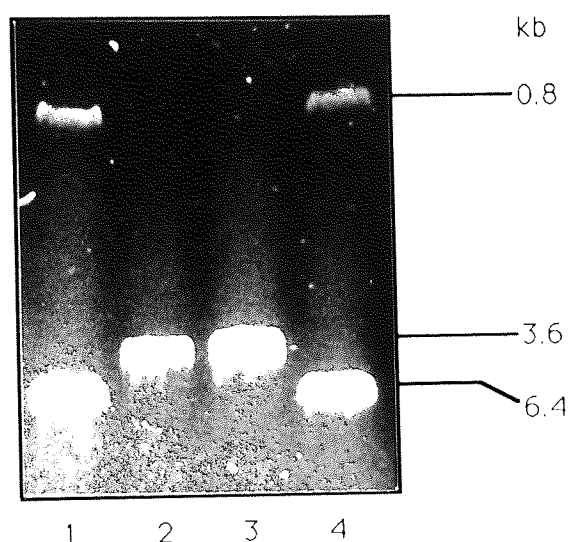
Figure 7.3. Agarose gel electrophoresis of pKS+/3, 10, 12 and 17 plasmid DNA digested with PstI. Lane 1 = pKS+/3; lane 2 = pKS+/10; 3 = pKS+/12; lane 4 = pKS+/17.

Plasmid DNA from cell types 3 and 17 appeared to be identical, with release of an 800 bp upon PstI digestion, while that from cell types 10 and 12 was also identical, producing a large band at approximately 3.6 kb; this 3.6 kb band was subsequently found to be a doublet.

Further digestions of type 12 and 17 plasmid DNA revealed that these plasmids differed only in the orientation of their 4.2 kb inserts:
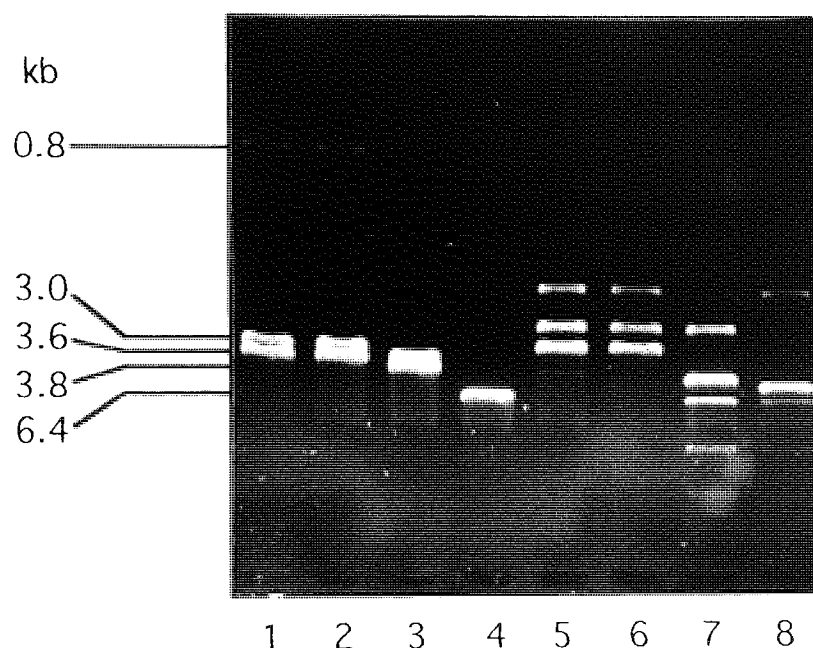
Figure 7.4. Agarose gel electrophoresis of pKS+/12 (lanes 1, 3, 5 and 7) and pKS+/17 (lanes 2, 4, 6 and 8) plasmid DNA. Lanes 1 and 2 = HindIII/PstI double digest; lanes 3 and 4 = PstI; lanes 5 and 6 = HindIII/BamHI double digest; lanes 7 and 8 = BamHI.

Since all four "positives" harboured identical-length genomic inserts, cell type 17 was arbitrarily selected for further DNA manipulations. Plasmid DNA was extracted from a 500 ml culture of type 17 cells in LB containing ampicillin (100 µg/ml) and tetracycline (12.5 µg/ml), and the plasmid DNA, composed of pKS+ plasmid with its 4.2 kb genomic DNA insert, was exposed to a range of restriction enzymes. The digests were run on a 0.8% agarose gel at 70 V.
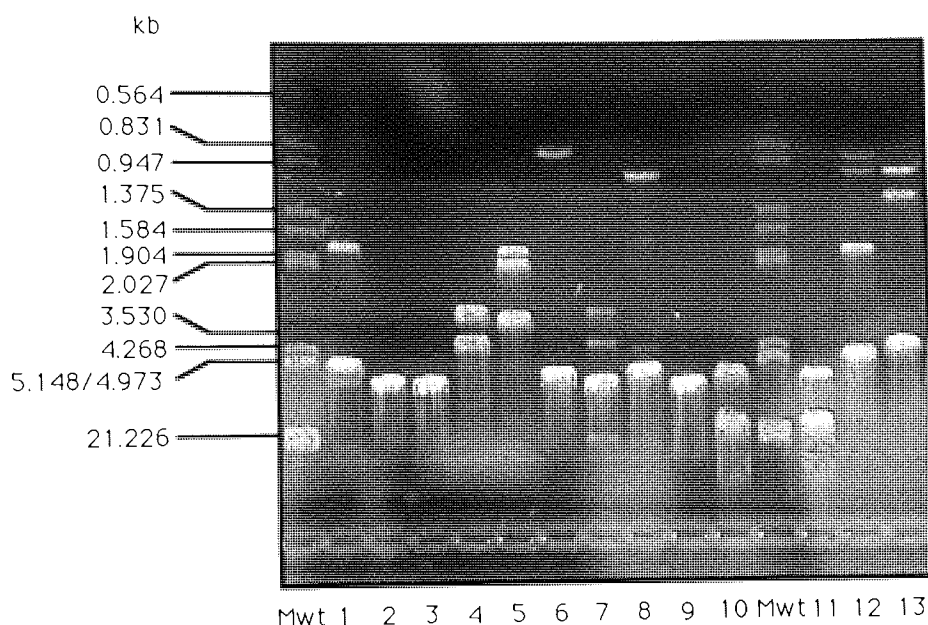
Figure 7.5. Multiple restriction digests of recombinant pKS+ plasmid 17. Mwt =molecular weight markers; lane 1 = BamHI; lane 2 = EcoRI; lane 3 = EcoRV; lane 4 = HindIII; lane 5 = KpnI; lane 6 = PstI; lane 7 = SalI; lane 8 = SmaI; lane 9 = XbaI; lane 10 = XhoI; lane 11 = HpaI; lane 12 = SphI; lane 13 = StuI

The DNA fragments in the gel were transferred to nitrocellulose by Southern blotting and subjected to hybridisation and autoradiography, in order to identify fragments of the 4.2 kb insert which contained the DNA sequence complementary to the radiolabelled oligonucleotide probe.
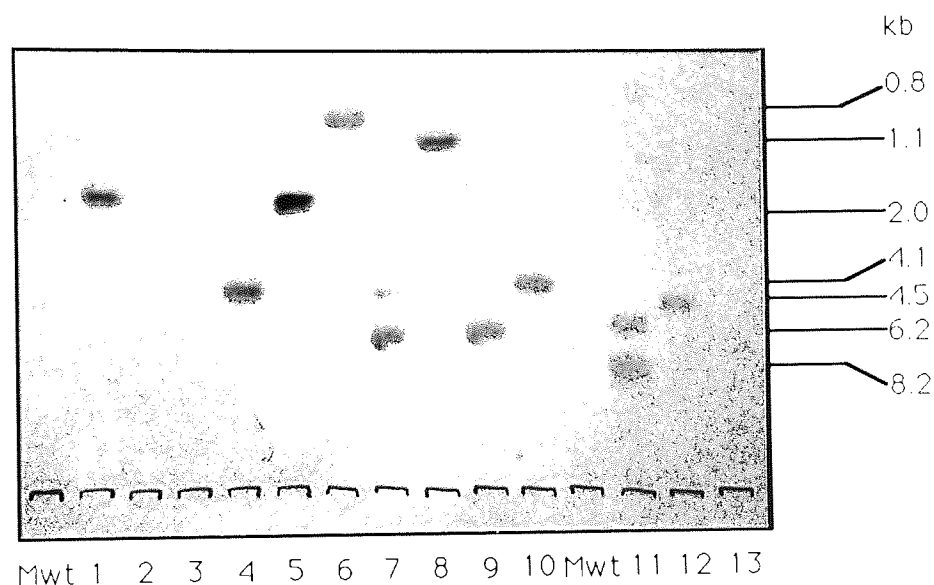
Figure 7.6. Autoradiograph of probed Southern blot of pKS+/17 restriction fragments. Lanes are as described previously in figure 7.5.

The smallest fragment of DNA which reacted with the probe was generated by Pst I digestion. This 800 basepair fragment was recovered and ligated into pKS+ plasmid. Single-stranded DNA was prepared from these cells, and used in dideoxy chain termination sequencing reactions.

7.2.3. Sequencing.

Due to the very high G-C content of *P. aeruginosa* genomic DNA (63.3% in this case), a large number of gel artifacts (compressions) occurred due to secondary folding of the DNA during electrophoresis. To overcome this problem, formamide was incorporated into the sequencing gel at up to 40% v/v in an attempt to increase the denaturing conditions prevailing during electrophoresis. However, formamide gels were found to be far less manageable, would liquify after extended run times, and produced extremely blurred bands following autoradiography and so their use was discontinued.

The nucleotide analog dITP was substituted for dG in sequencing reactions in an attempt to relieve compression artifacts; use of this analog did considerably reduce (but

not abolish) compressions, but produced BAFLs (bands across four lanes), another type of gel artifact, in their place. Additionally, bands were far less visible, and blurred, resulting in considerably less readable sequence obtainable from autoradiographs. The analog 7-deaza-dGTP was found to produce the best results, almost eliminating BAFLs and compressions, and also yielding clear bands following autoradiography. Both normal and extended sequencing reactions were run on 4% and 6% sequencing gels and the complete nucleotide sequence of the insert was deduced from the resulting autoradiographs. Initial sequence data revealed that the 800 basepair Pst I fragment contained a section of the multicloning site (MCS) from the "parent" pKS+/4.2 kb insert construct. This additional section of MCS allowed inversion of the insert in the same plasmid, by double digestion with Hind III and Pst I followed by recovery of fragments and ligation:
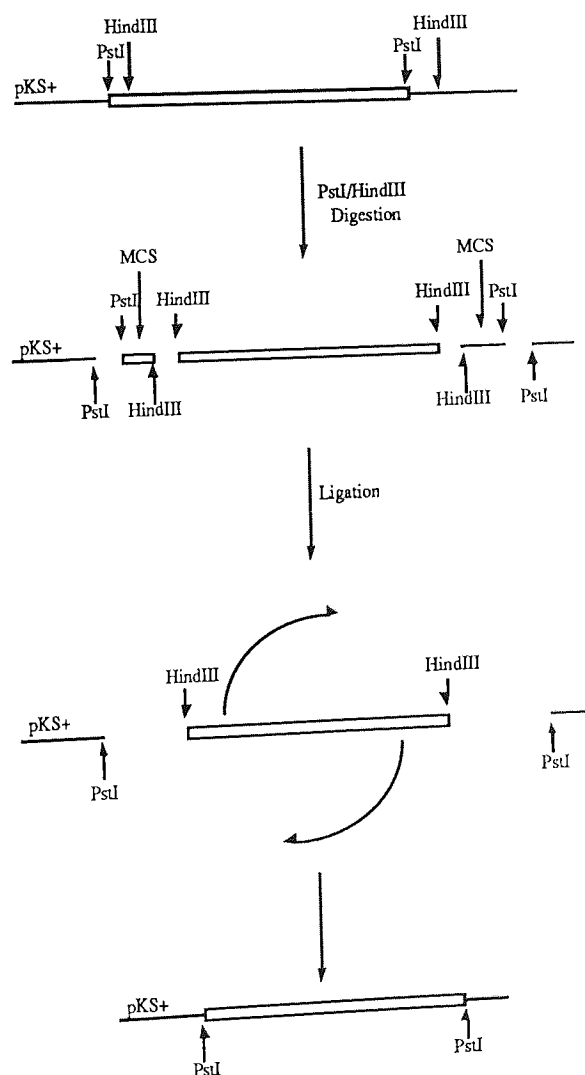
Figure 7.7. Restriction digestion, inversion and ligation of pKS+/17 insert DNA.

XL-1 *E. coli* cells were transformed with the ligation products, and cells containing the pKS+ plasmid with its insert in the opposite orientation were selected. Both normal and extended sequencing reactions were performed on both cell types, so that ssDNA of the insert could be produced from both ends of the insert. An EcoRV site was found near the centre of the insert, and so it was cut into two pieces which were ligated into similarly-prepared pKS+ and pSK+, so that the entire insert, including a 30-50 bp overlap region, could be clearly sequenced. Each of the two pieces of the insert was sequenced in both directions using the 7-deaza-dGTP analog, as well as with the dI analog and dG, ensuring that the definitive sequence of the fragment was obtained.

```
           10          20          30          40          50
  1234567890 1234567890 1234567890 1234567890 1234567890
  CTGCAGGCGC TTCAGCTCGA TCTGCATTTC CTTGCGCACG TTCAGGTCCA      50
  L  Q  A  L    Q  L  D    L  H  F    L  A  H  U    Q  U  Q

  GCGCCGACAG CGACTCGTCG AGCAGCAGCA CCTTGGGCCG GCTGGCCAGC     100
   R  R  Q    R  L  U  E    Q  Q  H    L  G  P    A  G  Q  R

  GCGCGGGCCA GGGCGATGCG TTGCTGCTGG CCACCGGACA ACTGGTCGGC     150
   A  G  Q    G  D  A    L  L  L  A    T  G  Q    L  U  G

  GCGGCGCTTG CCGACCTCCG GCAGCTTCAC CAGGTCGAGC ATGCGGCTCA     200
   A  A  L  A    D  L  R    Q  L  H    Q  U  E  H    A  A  H

  CGGTGGCGTC GATCTCGCTG CGCGGGCGGC CCTGCATCTC CAGGCCGAAG     250
   G  G  U    D  L  A  A    R  A  A    L  H  L    Q  A  E  A

  CCGATGTTGT CGGCGACGCT CATGTGCGGG AACAGCGCAT AACTCTGGAA     300
   D  U  U    G  D  A    H  U  R  E    Q  R  I    T  L  E

  CACCGTGTTG ACCGGGCGGC GGAACGGCGG CAGGCCTTCC ATGTGTTCGC     350
   H  R  U  D    R  A  A    E  R  R    Q  A  F  H    U  F  A

  CATAGAGGCG GATGCTGCCG GAACTGGGCT GTTCGAAGCC GGCGATGAGG     400
   I  E  A    D  A  A  G    T  G  L    F  E  A    G  D  E  A

  CGGAGGAGGG TGGTCTTGCC GCAGCCGGAA GGGCCGAGGA GGGTGAAGAA     450
   E  E  G    G  L  A    A  A  G  R    A  E  E    G  E  E
                  ──────────────────▶
  TTCGTTGGCG TGGATATCCA GGGACACATC GTCAAGGGCC TTCAGGCCTT     500
   F  U  G  U    D  I  Q    G  H  I    U  K  G  L    Q  A  L

  GGCCGGGGGT ACCGAATTCC ATGCAGATCC GCTCGATGGC TATCGCGCTG     550
   A  G  G    T  E  F  H    A  D  P    L  D  G    Y  R  A  G

  GTCATGCTGA ATTCCATGCA GTCAAGCAGT TGTTATTGTT GAGGTTTTGC     600
   H  A  E    F  H  A    U  K  Q  L    L  L  L    R  F  C

  GGTTTCTGGG GCCCGCTTCA CCTGCTGCGG TTCGATAATG ACCGGGGCGC     650
   G  F  W  G    P  L  H    L  L  R    F  D  N  D    R  G  A

  CGCGGGCGCA GAACGATAGA TGCGGCCAAT AAGGGATCGA ACAGGCCAAT     700
   A  G  A    E  R  .  M    R  P  I    R  D  R    T  G  Q  S

  CTGGTGGCGC TGGAAGAAGA CTGCGGATGC TCTCTTGTCG TCTCTTTCGG     750
   G  G  A    G  R  R    L  R  M  L    S  C  R    L  F  R

  ACAGGAAACT CGGACCTGTC CGAGTCTCCC GGGCGGCTGG CTGACTTTAA     800
   T  G  N  S    D  L  S    E  S  P    G  R  L  A    D  F  K

                                                            804
  GCTT
   L
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Figure 7.8. DNA sequence of the HindIII-PstI IA1 genomic fragment which bound to the probe. The probe-binding site and direction is indicated by the arrow.

7.3. Discussion.

The sequence data were very disappointing (figure 7.8), in that only 16 of the 17 nucleotides of the probe bound to the genomic DNA fragment, indicating that the sequenced genomic fragment was not part of the gene coding for OprG. Additional evidence for this finding was to be found when comparing the amino acid sequence obtained by microsequencing the protein itself to the implied amino acid sequence translated from the DNA:

Amino acid sequence of protein: $NH_2-D-I-Q-G-H-K-A-G-D-I-$
Translated sequence from DNA: $NH_2-D-I-Q-G-H-I-V-K-G-L-$

although the absence of a start (ATG) codon upstream of the oligonucleotide binding site eliminates the possibility of translation of this sequence.

Finally, the presence of a stop codon (TAG) at position 667 on the fragment was too close to the start of the region of probe binding, at position 463, leaving only 204 nucleotides to code for OprG; this could clearly not be the case, since this protein is approximately 25 kDa in weight, corresponding to approximately 750 nucleotides in length.

The reason for the failure in isolating the DNA coding for OprG may lie in the preparation and microsequencing of the protein itself. The Southern blot of IA1 genomic DNA fragments (figure 7.1.) clearly shows the probe binding to a 4.2 kb HindIII fragment and to a 1.9 kb KpnI fragment. Probe binding to immobilised colony DNA (figure 7.2.) also clearly revealed the presence of regions of hybridisation. Similarly, probe binding to fragments of pKS+/17 plasmid DNA (figures 7.5. and 7.6.) again clearly demonstrated the location of hybridising DNA sequence. The possibility that there was more than one 4.2 kb HindIII fragment which could bind the radiolabelled probe, with one fragment being "false" and the other "true" was also unlikely, as indicated in figure 7.3. The difference in the restriction fragment patterns in lanes 1 and 4 when compared to those in lanes 2 and 3 reflected the orientation of the 4.2 kb HindIII fragment in plasmid pKS+. The sequenced fragment was an 804 bp HindIII-PstI fragment of the initial 4.2 kb HindIII fragment indicating that the location of the PstI site in the 4.2 kb fragment was approximately 800 bp from one of the HindIII ends. Depending on orientation of this 4.2 kb fragment in pKS+, which also possesses a unique PstI site close to its HindIII site (clustered together in the plasmid's multiple cloning site), PstI digestion of the 7.2 kb recombinant plasmid (approximately 3 kb plasmid + 4.2 kb insert) would yield either fragments of 0.8 and 6.4 kb, or 3.8 and 3.4 kb (as shown in figure 7.3.) Double digestions, using a combination of HindIII and PstI, created identical restriction fragment patterns, as did HindIII/BamHI double digestions

(figure 7.4.). Therefore, none of the other three positives, namely pKS+/3, pKS+/10 or pKS+/12, could have been harbouring the "true" genomic DNA fragment, if one exists. The fact that all four (out of approximately 1500) transformed XL-1 *E. coli* colonies contained IA1 genomic DNA which could bind the radiolabelled probe proved to be "false" suggests that there is no "true" fragment, there being only a one-in-sixteen chance of this occurring (assuming only one "false" and one "true" fragment).

It is possible that the oligonucleotide probe was incorrectly synthesised, having the sequence 5'-GAW ATW CAR GGQ CAW AT-3' instead of the specified 5'-GAW ATW CAR GGQ CAW AA-3'. However, if this were the case, some probe-binding to genomic DNA fragments would still be evident, which was not observed. Therefore, inaccuracies in the preparation and amino acid microsequencing of the protein itself may be the cause of failure to isolate and sequence the gene coding for OprG.

Since a section of *P. aeruginosa* genomic DNA had been sequenced, the sequence data were analysed using GeneWorks and PC Gene, and the GenBank-European Molecular Biology Laboratory sequence database was searched by means of the GCG DNA-Protein analysis software package.
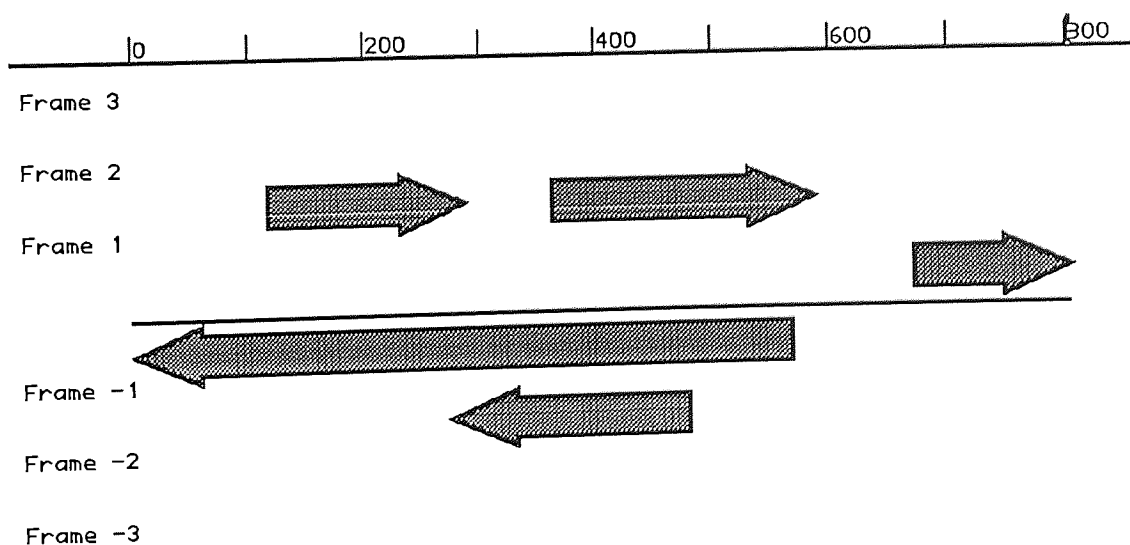
Figure 7.9. Open reading frames of 804 basepair *P. aeruginosa* IA1 genomic fragment. The putative OprG DNA is represented in frame 1.

```
          10          20          30          40          50
 1234567890  1234567890  1234567890  1234567890  1234567890
 AAGCTTAAAG  TCAGCCAGCC  GCCCGGGAGA  CTCGGACAGG  TCCGAGTTTC   50
 CTGTCCGAAA  GAGACGACAA  GAGAGCATCC  GCAGTCTTCT  TCCAGCGCCA  100
 CCAGATTGGC  CTGTTCGATC  CCTTATTGGC  CGCATCTATC  GTTCTGCGCC  150
 CGCGGCGCCC  CGGTCATTAT  CGAACCGCAG  CAGGTGAAGC  GGGCCCCAGA  200
 AACCGCAAAA  CCTCAACAAT  AACAACTGCT  TGACTGCATG  GAATTCAGCA  250
 TGACCAGCGC  GATAGCCATC  GAGCGGATCT  GCATGGAATT  CGGTACCCCC  300
 GGCCAAGGCC  TGAAGGCCCT  TGACGATGTG  TCCCTGGATA  TCCACGCCAA  350
 CGAATTCTTC  ACCCTCCTCG  GCCCTTCCGG  CTGCGGCAAG  ACCACCCTCC  400
 TCCGCCTCAT  CGCCGGCTTC  GAACAGCCCA  GTTCCGGCAG  CATCCGCCTC  450
 TATGGCGAAC  ACATGGAAGG  CCTGCCGCCG  TTCCGCCGCC  CGGTCAACAC  500
 GGTGTTCCAG  AGTTATGCGC  TGTTCCCGCA  CATGAGCGTC  GCCGACAACA  550
 TCGGCTTCGG  CCTGGAGATG  CAGGGCCGCC  CGCGCAGCGA  GATCGACGCC  600
 ACCGTGAGCC  GCATGCTCGA  CCTGGTGAAG  CTGCCGGAGG  TCGGCAAGCG  650
 CCGCGCCGAC  CAGTTGTCCG  GTGGCCAGCA  GCAACGCATC  GCCCTGGCCC  700
 GCGCGCTGGC  CAGCCGGCCC  AAGGTGCTGC  TGCTCGACGA  GTCGCTGTCG  750
 GCGCTGGACC  TGAACGTGCG  CAAGGAAATG  CAGATCGAGC  TGAAGCGCCT  800
 GCAG                                                        804
```

Figure 7.10. Complementary nucleotide sequence of the HindIII-PstI IA1 genomic fragment. The probe-binding site and direction is indicated by the arrow.

Comparison of the *P. aeruginosa* IA1 genomic fragment sequence with others in the GCG database showed that, out of the 40 library nucleotide sequences that showed closest homology, all but four displayed homology to the complementary DNA strand (Figure 7.9., frames -1, -2 and -3). The closest DNA match was with the *E. coli potG* gene, showing 62.1% identity in a 494 basepair overlap (at positions 300 to 794 in frame -3). The *potG* gene is one of a cluster of four genes (*potF, potG, potH* and *potI*) which are involved in putrescine transport, where potF is a periplasmic substrate-binding protein, potG a membrane-associated protein with a nucleotide-binding domain, and potH and potI are transmembrane proteins probably forming the channel for putrescine (Pistocchi *et al.*, 1993). Similar homology was found with the related *E. coli* spermidine-putrescine transport system, encoded by *potA, potB, potC* and *potD*.

64.5% identity in a 443 basepair overlap (in frame -3 at positions 360 to 803) was found with open reading frame (ORF) 4 in the *Azotobacter vinelandii* molybdate transport operon, whose products, molA, molB, molC and molD (in four open reading frames) show strong homologies to proteins of periplasmic transport systems. The protein encoded in ORF 4 showed extensive homology with other membrane-associated proteins of the ATP-binding cassette (ABC) superfamily of transport systems (Luque *et al.*, 1993).

62.7% identity in a 495 basepair overlap (in frame -3 at positions 277 to 772) was found with the *S. marcescens sfuC* gene, a component of the periplasmic-binding-protein-dependent iron transport system (sfuABC) in which the periplasmic sfuA protein is probably the site of primary iron-binding while the sfuB and sfuC proteins are associated with the cytoplasmic membrane. The sfuC protein shows strong sequence homology to the nucleotide-binding proteins of periplasmic-binding-protein-dependent transport systems (Angerer *et al.*, 1990; Angerer *et al.*, 1992).

58.8% identity in a 364 basepair overlap (in frame -3 at positions 307 to 670) was found with the *P. aeruginosa BraF* gene, a component of the high-affinity branched-chain amino acid transport system (BraCDEFG), in which BraC is a periplasmic binding protein for branched-chain amino acids, BraD and BraE are integral membrane proteins, and BraF and BraG are putative nucleotide-binding proteins (Hoshino and Kose, 1990; Hoshino *et al.*, 1992).

Although acting on widely different substrates (e.g. sugars, amino acids, peptides, ions and vitamins), bacterial periplasmic-binding-protein-dependent transport systems are generally composed of a membrane-bound complex, usually comprising between two and four membrane-bound proteins, and a soluble periplasmic protein, typically transporting their respective substrates with high affinity and achieving large concentration gradients. The action of these systems is composed of binding of the substrate to the periplasmic protein, followed by interaction with the membrane associated complex (usually composed of two highly hydrophobic membrane-spanning proteins), possibly aided by another, hydrophilic membrane-associated protein which bears an ATP-binding site (Ames and Joshi, 1990). Presumably, this hydrophilic protein utilises ATP as the energy source to cycle the delivery of substrate through the cytoplasmic membrane.

Although the results of the database search are not conclusive, there appears to be good evidence that the sequenced *P. aeruginosa* DNA codes for the hydrophilic membrane-associated ATP-binding component of a hitherto uncharacterised periplasmic-binding-protein-dependent transport system. The full organisation and sequence of the operon, and substrate for this putative transport system, are yet to be elucidated.

8. CONCLUDING REMARKS

The ability of *P. aeruginosa* to acquire iron, coupled with production of a number of virulence determinants has established this organism as a leading cause of morbidity and mortality in the immunocompromised host.

Throughout this thesis, emphasis has been placed on the role of iron in bacterial growth, and the lengths to which *P. aeruginosa* will go in order to acquire iron from its environment. While the high-affinity iron uptake systems provide the means by which *P. aeruginosa* can sustain an infection, they may also constitute a target for novel antimicrobial agents.

The results of this study have provided evidence that *P. aeruginosa* is able to regulate finely its iron uptake mechanisms in response to its environment. This is hardly surprising, in view of the complex interactions of organisms in environments where iron is a limiting factor and the ability to respond to and utilise available siderophores may confer a considerable survival advantage. Such inducible uptake has been noted for a variety of exogenous siderophores in pseudomonads, and appears to be the norm. The lack of inducible expression of a second ferripyoverdin receptor in *P. aeruginosa* suggests that there is only one receptor in this organism, unlike *P. putida* in which a second inducible ferripyoverdin uptake system has been identified. *P. aeruginosa* utilises pyochelin as its second siderophore, which may play an important part during infection, based on the observation that all clinical isolates of *P. aeruginosa* recovered from the CF lung appear to be able to utilise pyochelin even if they do not produce this siderophore. The role of ferripyochelin, in conjunction with pyocyanin, in free radical-mediated tissue damage may also be important in sustaining an infection *in vivo*.

Uptake of the catecholic cephalosporin BRL 41897A via the ferripyochelin receptor suggests that this receptor may also participate in uptake of the hydrolytic products of enterobactin, which occur normally in the environment and demonstrate chelating activity. Induction and uptake experiments using dihydroxybenzoyl-serine and dihydroxybenzoate as siderophores may add credence to this hypothesis.

Salicylic acid acts as a siderophore in some *Pseudomonas* species, and may be utilised in the biosynthesis of pyochelin. An intermediate in the biosynthesis of pyochelin, aeruginoic acid, has been found in culture supernatants. Although no function of this compound has been described, it seems likely that it may act as a means of solubilising ferric iron. Once again, induction and uptake experiments are required to confirm this putative role.

Ferrocins, a new type of iron-containing peptide antibiotic, have been described, although their mode and site of action have not yet been elucidated; their structures seem somewhat similar to ferric-siderophore complexes, and it is suggested that they may enter susceptible cells via IROMP receptors. There is the additional possibility that they are, in fact, S-type pyocins.

The sequence data generated from the putative *OprG* gene were initially disappointing, in that an unrelated section of the *P. aeruginosa* genome was finally sequenced. However, the probability that the sequenced region encoded part of the hydrophilic membrane-associated ATP-binding component of a hitherto uncharacterised periplasmic-binding-protein-dependent transport system was some consolation. It is hoped that further sequencing may ultimately reveal the substrate for this system.

In summary, the data presented in this thesis indicate that expression of siderophore-mediated iron uptake systems in *P. aeruginosa* is exquisitely regulated in response to the environment, and that novel antibiotics may capitalise on the microbial demand for iron. It is likely that genes involved in a novel periplasmic-binding-protein-dependent transport system have been isolated.

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CAS agar = chrome azurol S agar comprised (in 1 litre distilled water) 100ml 10x CDM/ 100ml 10x MOPS/ 10ml 100x MgSO$_4$.7H$_2$O/ 590ml ddH$_2$O/100ml 10x glucose/15g agar (prepared as for CDM-Fe agar above) +[60.5mg chrome azurol S in 50 ml ddH$_2$O + 10ml 1mM FeCl$_3$ in 10mM HCl + 72.9mg cetrimide in 40ml ddH$_2$O - autoclaved separately and added later].

5x gel loading buffer = 0.5% SDS/ 25% glycerol/ 0.25% bromophenol blue/ 0.05M EDTA.

phenol:chloroform = equal amounts of phenol and chloroform (w/v) equilibrated by extraction several times with 0.1M Tris.Cl (pH 7.6). The equilibrated mixture was stored under 0.01M Tris.Cl (pH 7.6) at 4°C in a dark glass bottle to prevent the entry of light.

Siderophore reagent
The siderophore assay reagent is composed of the following stock solutions:
10mM cetrimide = 91mg in 25ml ddH$_2$O
1mM FeCl$_3$.6H$_2$O in 10mM HCl = 22μl concentrated HCl + 6.8mg FeCl$_3$.6H$_2$0 in 25ml ddH$_2$O
2mM CAS (chrome azurol S) = 30.3mg in 25ml ddH$_2$O
  The siderophore reagent was prepared in a 100ml glass bottle and wrapped in foil to prevent light from degrading the reagent. Six ml cetrimide was poured into the bottle, followed by 30ml ddH$_2$O. A 1.5ml aliquot of 1mM FeCl$_3$.6H$_2$O in 10mM HCl solution was added, followed by 7.5ml CAS solution. 4.307g anhydrous piperazine was weighed out into a plastic universal bottle, which was then filled to approximately two-thirds with ddH$_2$O. The universal was filled with 6.25ml concentrated HCl. The contents of the universal were poured into the 100ml bottle, which was then topped up to 100ml with ddH$_2$O. Finally, 101.6mg of 5-sulphosalicylic acid was added (this compound acts as a "shuttle" to accelerate the reaction). The bottle was shaken and stored at 37°C for a few hours to allow the shuttle to dissolve.

0.5x TBE = 0.045M Tris-borate/ 0.001M EDTA

Codon Usage in *Pseudomonas aeruginosa* per thousand genes (West and Iglewski, 1988).