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# IRON ACQUISITION AND ITS CONTROL IN *PSEUDOMONAS AERUGINOSA*: MANY ROADS LEAD TO ROME

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## 1. ABSTRACT

Iron plays an important role in the pathogenesis and rhizosphere competence of the fluorescent group of pseudomonads and it is, thus, fitting that the characteristic fluorescence of these organisms is attributable to an iron-chelating molecule, pyoverdine. *Pseudomonas aeruginosa* is likely the best-studied member of this group, and while it synthesizes two siderophores, pyochelin and pyoverdine, it is also able to use a number of heterologous siderophores of fungal and bacterial origin and its genome is rich with homologues of iron-siderophore receptor genes, reflecting the enormous flexibility of the organism *vis-a-vis* iron carriers that it can use in nature. The ability to utilize a variety of heterologous siderophores is shared by other fluorescent pseudomonads and likely reflects both the importance of this vital nutrient for growth and survival and the need to compete with other microorganisms in the aquatic and terrestrial environments that they inhabit. Expression of the various receptors is, however, regulated, with receptor production responding positively to available siderophores only, and selection from multiple available siderophores based on their successful chelation of iron and subsequent transport. Thus, the superior siderophore in a given environment will upregulate the cognate receptor at the expense of other receptors. Such siderophore-dependent regulation of receptor gene expression is common in bacteria, particularly the fluorescent pseudomonads, and typically requires a signal transduction cascade that involves the receptor itself, whose binding to the siderophore initiates the cascade, as well as a regulatory protein pair that includes an environmentally-responsive so-called extracytoplasmic function (ECF) sigma factor, which activates receptor gene expression, and an anti-sigma factor that controls sigma factor activity. Despite the plethora of ferric siderophore receptors in *P. aeruginosa*, its genome sequence reveals a striking lack of obvious periplasmic and cytoplasmic membrane transport components capable of accommodating these molecules. Unlike e.g. *Escherichia coli*, then, where ferric siderophore permeases providing transport to the cytoplasm are clearly in evidence, iron-siderophore complexes in *P. aeruginosa* may be dissociated in the periplasm, with a common iron carrier then responsible for iron uptake into the cell interior.

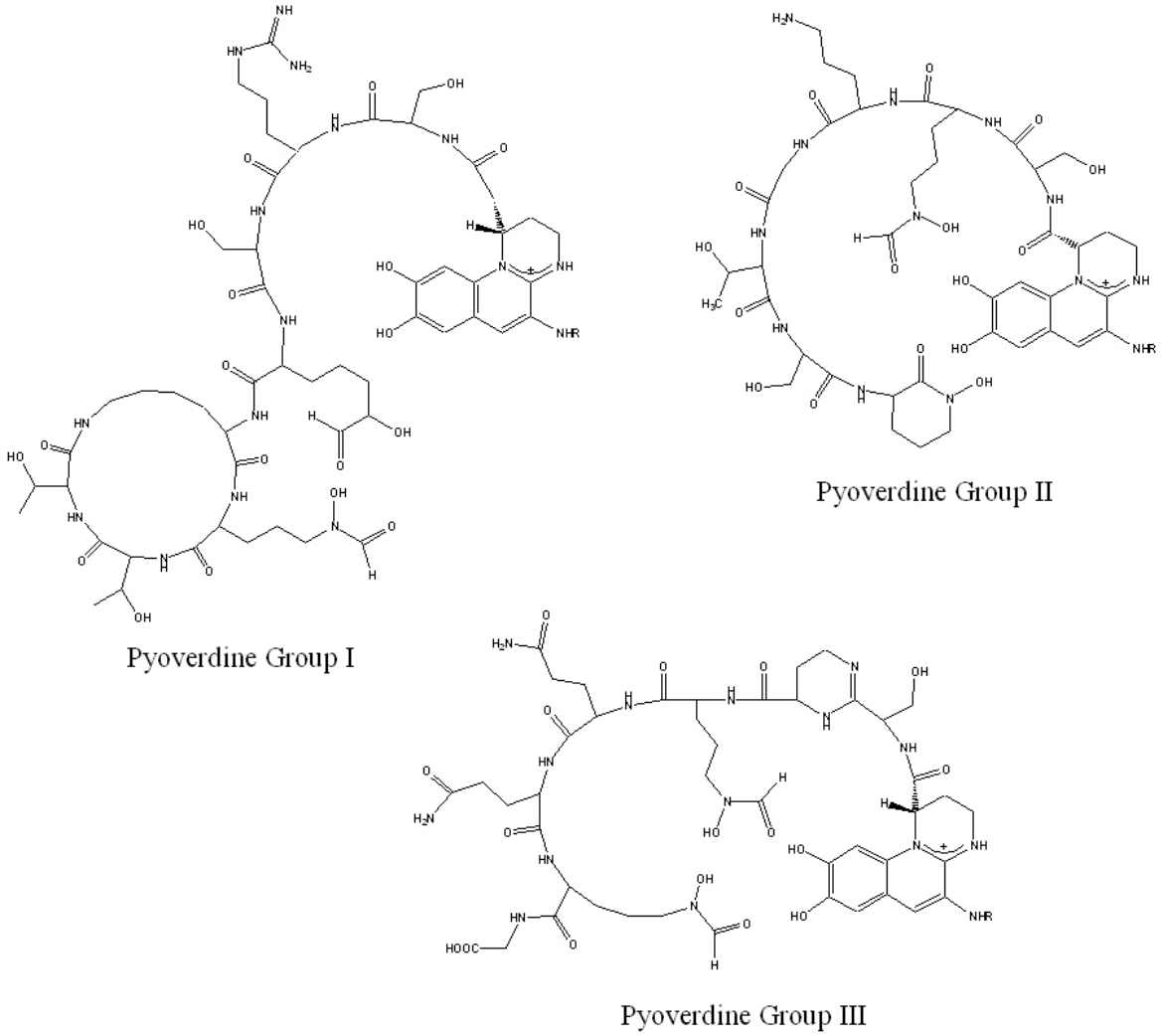


**Figure 1.** The pyoverdines of *P. aeruginosa*

## 2. INTRODUCTION

With few exceptions, iron is essential for microbial growth, being an indispensable cofactor of many important enzymes (1). Typically required by bacteria at micromolar concentrations, bioavailable (i.e. soluble) iron levels are characteristically low in aerobic environments [recent estimates indicate that Fe(III) is present at $10^{-9}$ M at pH 7.0 (2)]. As such, terrestrial and aquatic organisms, as well as mammalian pathogens [which encounter even lower freely available Fe(III) levels of ca. $10^{-18}$ M in the host due to its sequestration by transport and storage proteins (1)] have evolved a variety of mechanisms to acquire this essential nutrient from dilute environments. Predominant amongst these involves the synthesis of Fe(III)-chelating molecules (i.e. siderophores) and their cognate receptor proteins (3). As such, siderophore-mediated iron uptake plays an important role in bacterial growth and survival in the wild (4) and is crucial for the virulence of pathogenic organisms (5-7).

## 3. SIDEROPHORE-MEDIATED IRON TRANSPORT IN PSEUDOMONAS AND RELATED GENERA

Siderophore-mediated iron acquisition plays an important role in the virulence of pathogenic *Pseudomonas* sp. like *Pseudomonas aeruginosa* (8-12) and in the fitness (13-16) and biocontrol activity (17, 18) of fluorescent rhizosphere pseudomonads. Consistent with the importance of iron acquisition vis-à-vis *in vivo* growth and pathogenesis of *P. aeruginosa*, siderophores (19) and siderophore receptors (20-22) are produced by clinical isolates during infection and by *P. aeruginosa* in animal models of infection (23). The bulk of studies re. siderophore-mediated iron acquisition in *Pseudomonas* have focused on the fluorescent pseudomonads, in particular *P. aeruginosa*, *Pseudomonas putida* and *Pseudomonas fluorescens* (24, 25), although siderophore production and iron uptake has been described in other fluorescent pseudomonads (25-30) as well as non-

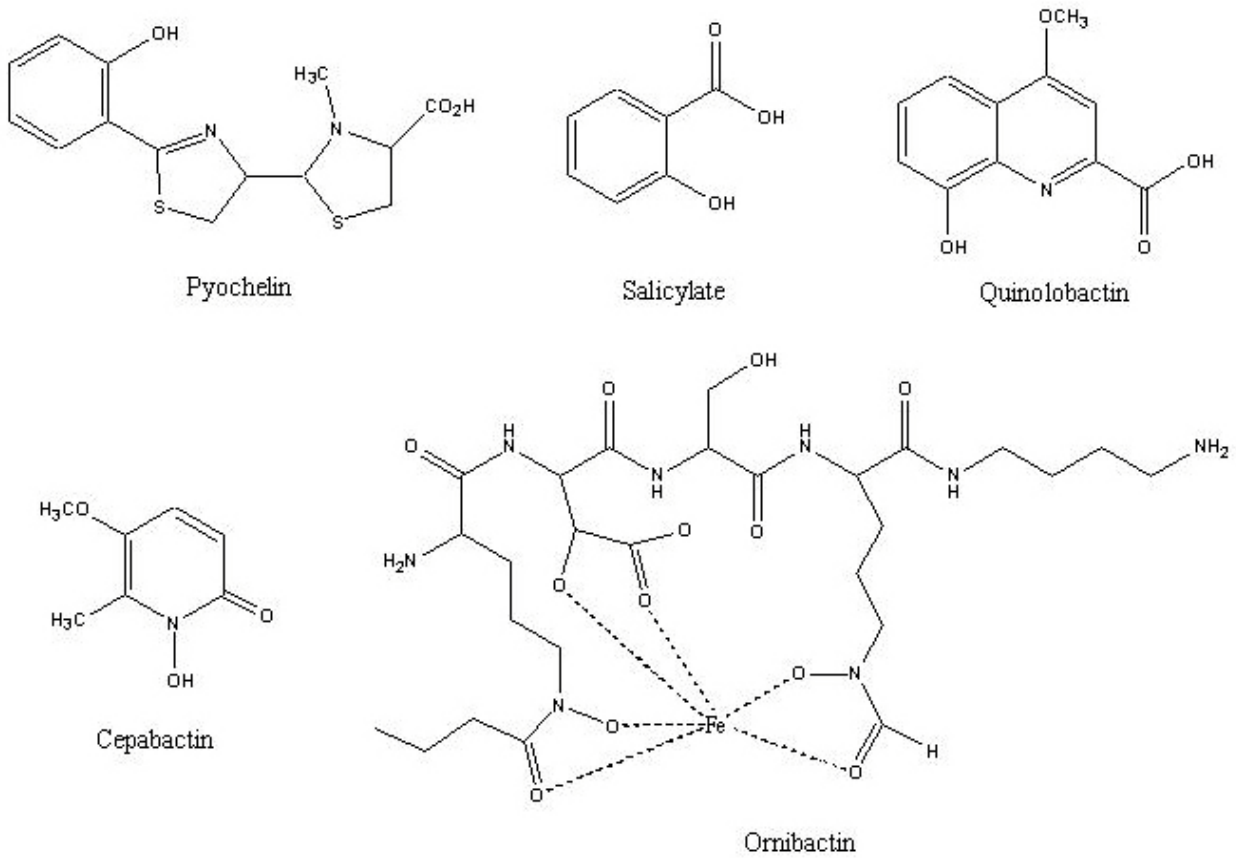


**Figure 2.** Endogenous siderophores of *Pseudomon*as and *Burkholderia* spp.

luorescent species (25, 30), including related organisms such as *Burkholderia* (formerly *Pseudomonas*) *cepacia* (31-36) and *Burkholderia* (formerly *Pseudomonas*) *pseudomallei* (37, 38). *P. aeruginosa*, which synthesizes two known siderophores, pyoverdine (39-41) (Figure 1) and pyochelin (42-44) (Figure 2), is quite striking in the range of heterologous siderophore molecules that it can use in its quest for iron (Figures 2-5), which include siderophores of other bacteria [aerobactin (45), enterobactin (45, 46), including its precursor 2,3-dihydroxybenzoic acid and breakdown product N-(2,3-dihydroxybenzoyl)-L-serine (47), pyoverdine/pseudobactin from other pseudomonads (48-50), and cepabactin (50)], fungal siderophores (deferrioxamines, deferrichrysin, deferrirubin, coprogen ) (50), synthetic chelators (e.g. nitrilotriacetic acid) (51) and naturally-occurring chelators such as citrate (42, 52) and myo-inositol hexakisphosphate (53). The organism and, indeed, many of the fluorescent pseudomonads and *B. cepacia* also synthesizes salicylic acid, which binds and promotes iron uptake (34, 36, 50, 54, 55), although its being a precursor of pyochelin suggests that it may not be intended as a siderophore as such (2), at least in *P. aeruginosa* and other pyochelin-producing species. A novel isoxazolidone siderophore, pseudomonine, has also been described in *P. fluorescens* (56, 57) and its biosynthesis appears to be related to salicylic acid (57). beta-lactam conjugates of synthetic (58-64) and natural siderophores such as pyoverdine (65-67) are also taken up by *P. aeruginosa* and prove to be potent antimicrobials. Some iron-siderophore receptors also function as pyocin receptors in *P. aeruginosa* (68,69).

### 3.1. Endogenous siderophores of fluorescent pseudomonads

#### 3.1.1 Pyoverdine

Also called pseudobactin in rhizosphere pseudomonads such as *P. putida* and *P. fluorescens*, this family of siderophore is produced by all fluorescent pseudomonads and is characterized by a conserved dihydroxyquinoline chromophore to which is attached a small dicarboxylic acid (or its monoamide) and a peptide chain of variable length and composition (24, 26) (Figure 1). The former is responsible for the fluorescence of the molecule and, indeed, the characteristic pigmentation of the fluorescent group of *Pseudomonas* sp. The peptide portion of individual pyoverdines varies in length from 6-12 amino acids and can contain a number of unsual amino acids (e.g. cyclo-delta N-hydroxyornithine, formyl-delta N-hydroxyornithine, diaminobutyric acid) as well as D and L-isomers of the more common amino acids (24). This variability in sequence and composition likely explains the noted specificity *vis-à-vis* pyoverdine utilization amongst the fluorescent pseudomonads, where e.g. a given strain of *Pseudomonas* will use its own pyoverdine but not that of other *Pseudomonas* strains (48, 70) and, indeed, suggests that the peptide moiety is involved in receptor recognition and binding. Amongst all *P. aeruginosa* strains examined

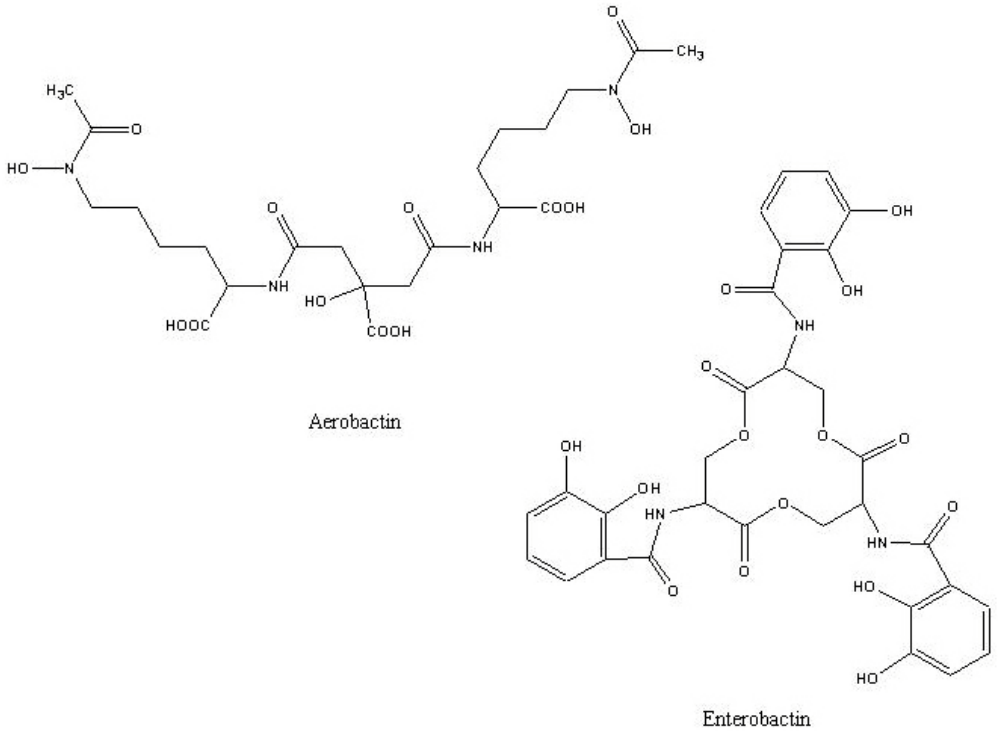


**Figure 3.** Heterologous bacterial siderophores used by *P. aeruginosa*

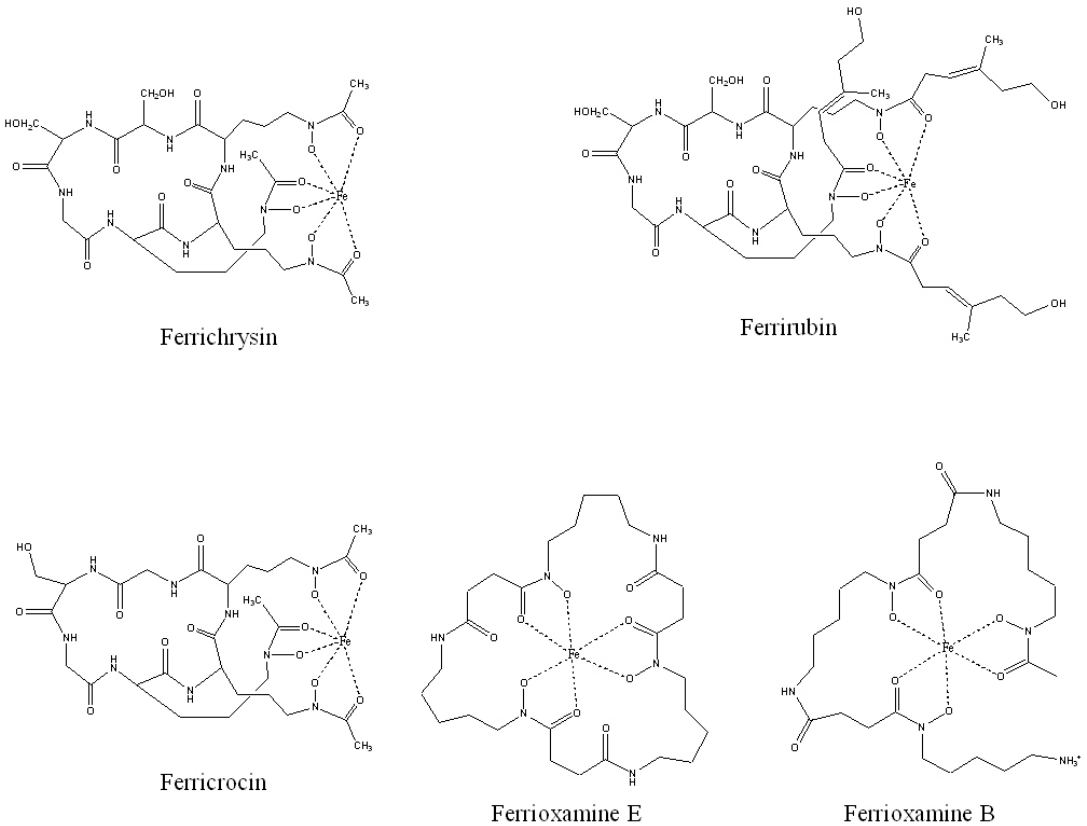


**Figure 4.** Fungal siderophores used by *P. aeruginosa*

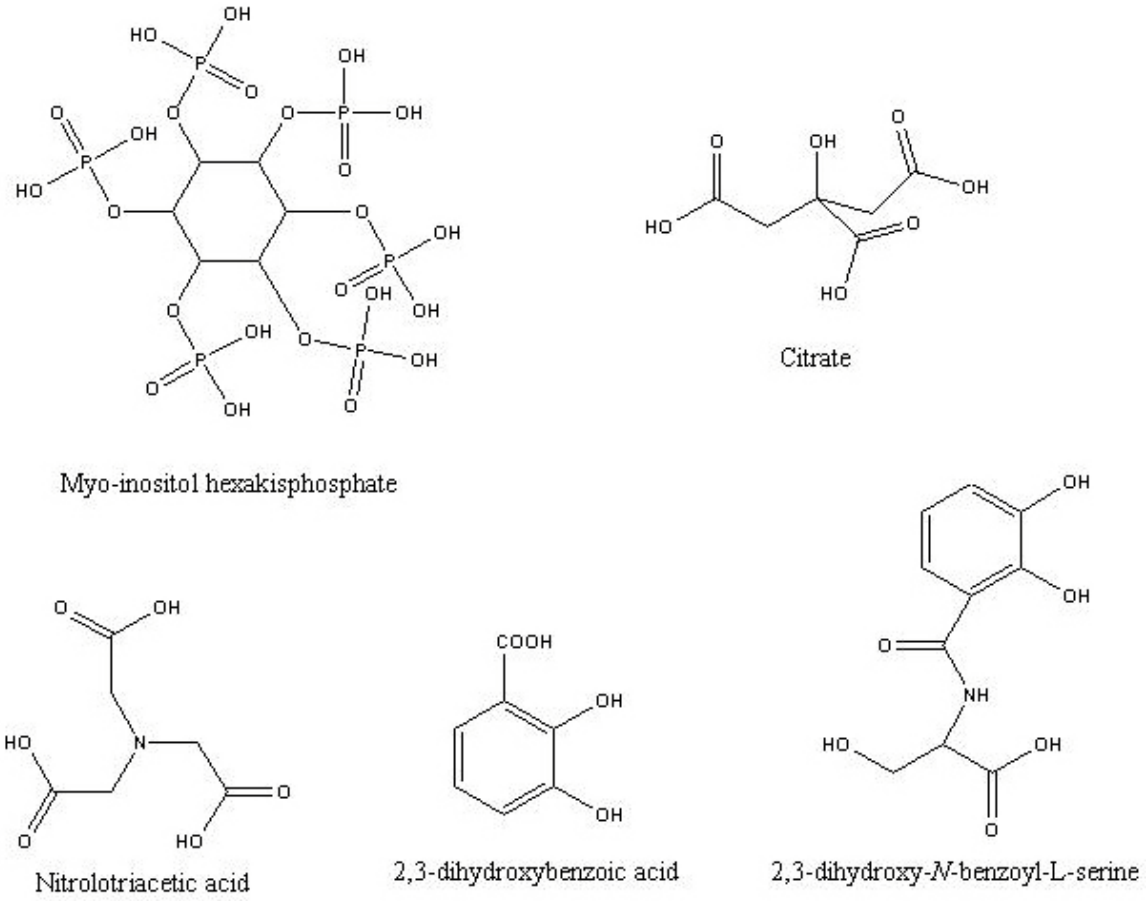


**Figure 5.** Assorted iron chelators used by *P. aeruginosa*

to date, only three structurally distinct pyoverdines have been described (dubbed types I, II and III; Figure 1), though much more variability has been noted amongst e.g. the pyoverdines/pseudobactins of *P. fluorescens* (24). Recently, a single example of a 4th pyoverdine has been reported, though it differs from type III only by the absence of L-Gln in the peptide moiety (71). Intriguingly, this diversity of pyoverdine structure forms the basis of a novel typing scheme dubbed siderotyping (30, 72).

Pyoverdine is effective at acquiring iron from transferrin (9, 73, 74) and lactoferrin (74) and this has obvious implications vis-à-vis *in vivo* growth and pathogenesis. Indeed, production of pyoverdine (75) and expression of pyoverdine biosynthetic genes (76) have been documented in *P. aeruginosa* growing *in vivo*. Moreover, mutants with deficiencies in pyoverdine biosynthesis (9-11) or transport (12) are effectively avirulent in animal models of infection. Pyoverdine itself appears also to be cytotoxic, as a consequence of its ability to stimulate the production of reactive oxygen species (77).

#### 3.1.1.1. Biosynthesis

A number of genes for the biosynthesis of pyoverdine have been identified to date (78-84) and cluster within a region of the *P. aeruginosa* chromosome referred to as the *pvd* locus (85), although an operon implicated in synthesis of the chromophore, *pvcABCD* (86, 87), occurs elsewhere. Mutations in cytochrome (88) and haem (89) biosynthetic genes also negatively impact pyoverdine biosynthesis by *P. fluorescens*, with the former proposed to interfere with pyoverdine maturation owing to lack of necessary oxidizing power (90). A gene, *repB*, encoding a homologue of the GacA response regulator of *P. fluorescens* (91) has also been implicated in pyoverdine biosynthesis in *Pseudomonas viridiflava* and is conserved in a number of fluorescent pseudomonads (27). Recently, too, a phosphopantetheinyl transferase, PcpS, involved in fatty acid biosynthesis has been implicated in the synthesis of pyoverdine (and pyochelin) (92). Mutants defective in the 'twin-arginine' translocation (TAT) system are also pyoverdine-deficient, indicating that at least some components of pyoverdine biosynthesis must be exported by this Sec-independent secretion system (93). The *pvd* locus appears to be involved in the non-ribosomal synthesis of the peptide moiety of pyoverdine although a recent paper has also implicated a *pvd* gene (*pvdL*) in chromophore synthesis (94), raising questions about the necessity of the *pvc* genes in pyoverdine biosynthesis. Genes for transport (and export?) of pyoverdine, including the ferric pyoverdine receptor, *fpvA* (95), also occur in the *pvd* locus (84). A limited number of pyoverdine biosynthetic genes have also been reported in other fluorescent pseudomonads (94, 96-98) where they invariably show homology to

known *P. aeruginosa pvd* genes (99), consistent with the presence of a conserved pathway for pyoverdine biosynthesis within the fluorescent pseudomonads.

#### 3.1.1.2. Regulation

Pyoverdine production in *P. aeruginosa* requires the product of a gene, *pvdS* (100, 101), which functions as a sigma factor (102, 103) and is essential for expression of several *pvd* genes (100, 101, 102, 84) as well as the *pvc* genes implicated in chromophore biosynthesis (87). The *pvd* pyoverdine biosynthetic genes carry a conserved iron starvation (IS) box (104-106) which is required for PvdS-mediated RNA polymerase binding to and transcription from *pvd* promoters (106). PvdS control of *pvc* expression is indirect and is mediated by PtxR, a positively regulator of the LysR family (107) that is required for *pvc* gene expression (87) and is itself controlled by PvdS (108). PvdS is a member of a family of sigma factors dubbed extracytoplasmic function (ECF) (109) which are typically regulated in response to environmental cues [see (110-112) for reviews]. These sigmas are widely distributed amongst bacteria with multiple examples in the same organism (113). ECF sigmas have also been implicated in siderophore biosynthesis in other pseudomonads, including *P. fluorescens* M114, where the PbrA sigma factor is required for pseudobactin$_{M114}$ production and expression of the pseudobactin$_{M114}$ biosynthetic genes (114), *P. putida* WCS358, where the PfrI ECF sigma is required for pseudobactin$_{358}$ production and expression of the corresponding biosynthetic genes (115), and *Pseudomonas* B10, where expression of a pseudobactin$_{B10}$ biosynthetic gene is promoted by the ECF sigma PsbS (116). In addition to pyoverdine biosynthetic genes, PvdS is required for production of the extracellular virulence factors exotoxin A (117) and endoprotease PrpL (118) as well as several other iron-regulated non-siderophore genes (84), including alkaline protease which requires PvdS for full expression (119). The rationale for co-regulation of these virulence factors with pyoverdine is for the most part unclear. The fact, however, that alkaline protease is able to release iron from transferrin (possibly making it available to pyoverdine), thereby stimulating growth of *P. aeruginosa* in cultures with transferrin as the sole iron source (119) may provide one possible connection. PrpL, too, is able to degrade lactoferrin (118) though its contribtution to iron acquisition and growth as a result remains to be studied.

The synthesis of pseudobactin358 also requires a second regularly gene, *pfrA*, whose product has been shown to activate siderophore biosynthetic gene expression in *P. putida* WCS358 (120). The protein is a homologue of the AlgR2 positive regulator of alginate biosynthesis in *P. aeruginosa* (121). Still, AlgR2 is not involved in pyoverdine biosynthesis in *P. aeruginosa*, although a recent study has confirmed that PfrA is required for heterologous expression of *P. aeruginosa pvd* genes in *P. putida* WCS358 (96), suggesting that an equivalent regulator may be involved in the control of pyoverdine biosynthesis in *P. aeruginosa.*

Pyoverdine production in *P. aeruginosa* increases in response to iron limitation as a result of increased expression of the corresponding biosynthetic genes. The iron control of gene expression is mediated by the product of a gene, *fur* (122), which like its well-characterized *E. coli* counterpart is a repressor that employs Fe(II) as a co-repressor and binds to a consensus operator sequence (Fur box) in the promoters of iron-regulated genes (123, 124) [see (125, 126) for reviews of the Fur repressor]. Thus, gene expression is repressed under high iron conditions and increases under low iron conditions (Figure 6A). Fur regulates a number of genes in *P. aeruginosa*, including a variety of regulatory genes, genes encoding metabolic or detoxifying enzymes and several genes/operons associated with iron acquisition, including those involved in the production and utilization of siderophores [reviewed in (83)]. As expected, *fur* mutants display constitutive pyoverdine production (122, 127, 128) and show reduced iron-responsiveness re. *pvd* gene expression (104). Paradoxically, however, *fur* mutants also exhibit a reduced ability to accumulate ferric pyoverdine (and ferric pyochelin) for reasons that have yet to be elucidated (128). Intriguingly, the *pvd* genes lack Fur boxes, although *pvdS* has a Fur box and iron-regulation of *pvdS* (100, 101) and Fur binding to its promoter (104) has been confirmed. Iron regulation of the *pvd* genes, thus, occurs indirectly, as a result of Fur control of *pvdS* expression (100, 101, 104) (Figure 6B) as appears to be the case for many of the iron-regulated genes of *P. aeruginosa* (84). Unlike *E. coli*, *fur* null mutants of *P. aeruginosa* are not obtainable, suggesting that *fur* is an essential gene in *P. aeruginosa* (122). Hybridization studies support the presence of *fur* homologues in *P. fluorescens*, *P. putida* and *Pseudomonas aureofaciens* (= *Pseudomonas chlororaphis*) (129) and a *fur* gene has been confirmed in *P. putida* WCS358 (115). The observation that *E. coli* Fur regulates expression of a pseudobactin biosynthetic gene from *P. fluorescens* M114 and binds to its promoter (130) also supports the presence of a Fur homologue in this organism and, indeed, a probable Fur repressor has been reportedly cloned from M114 (131). Finally, *fur* genes have been identified in *B. cepacia* and *B. pseudomallei* (132) and a search of the unfinished *P. fluorescens* genome sequence reveals a likely *fur* homologue whose product displays 98 % identity with *P. aeruginosa* Fur over 77 amino acids at the N-terminus, in agreement with reports of a *fur* homologue in this organism (133). Like *P. aeruginosa*, iron-regulation of the pseudobactin biosynthetic genes of *P. fluorescens* M114 and *P. putida* WCS358 is also mediated by Fur/iron regulation of the corresponding ECF sigma factors PbrA (134) and PfrI (115).

#### 3.1.1.3. Transport

The ferric pyoverdine receptor of *P. aeruginosa* is a ca.90 kDa outer membrane protein inducible under conditions of iron limitation (135, 136) and encoded by the *fpvA* gene (95). Hybridization studies suggest that homologues are present in a variety of *P. aeruginosa* strains as well as in *P. aureofaciens*, *P. putida* and *P. fluorescens* (129), and, indeed, receptors for ferric pseudobactin have been cloned and sequenced from *P. putida* WCS368 (*pupA*) (137) and *Pseudomonas fluorescens* M114 (*pbuA*) (138). A receptor for pyoverdine/pseudobactin has also been described in *P. syringae* (139) although the gene has not been cloned. As

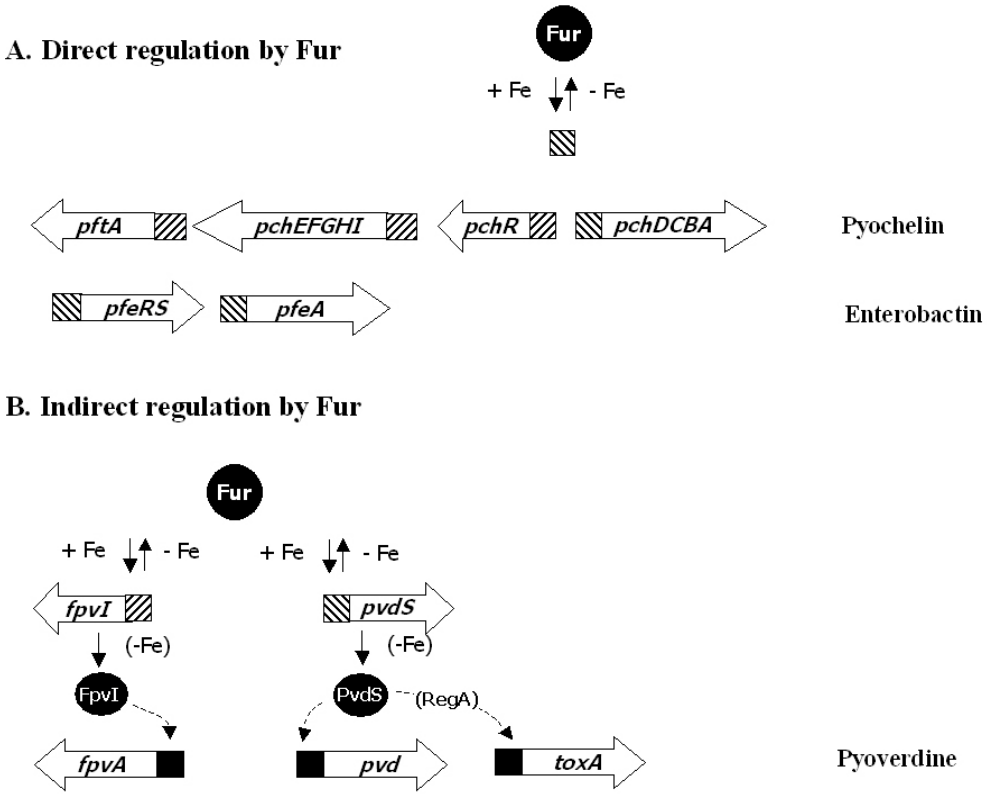


**Figure 6.** Schematic showing direct (A) and indirect (B) Fur-mediated iron regulation of pyochelin, enterobactin and pyoverdine biosynthetic and/or receptor genes in *P. aeruginosa*. Under iron replete conditions (+Fe) the Fur repressor binds to a so-called Fur box (shaded) in the promoter regions of siderophore (*pch*) and receptor (*pftA, pfeA*) genes (A) or ECF sigma factor genes (*pvdS, fpvI*) required for siderophore (*pvd*) and receptor (*fpvA*) gene expression (B) thereby blocking gene expression. Under iron-limiting conditions (-Fe), Fur is released from the promoters, permitting expression of the siderophore biosynthetic (*pch*) and receptor (*fptA, pfeA*) genes (A) and the ECF sigma factors (*pvdS, fpvI*) which subsequently activate expression of the biosynthetic (*pvd*) and receptor (*fpvA*) genes, as well as other genes influenced by the sigma factors e.g. exotoxin A (*toxA*) (B). The impact of PvdS on *toxA* expression is, however, indirect and likely mediated by the RegA transcriptional regulator (117). OM, outer membrane; PP, periplasm; CM, cytoplasmic membrane.

with pyoverdine biosynthesis, ferric pyoverdine uptake is compromised in TAT mutants, apparently because FpvA export is TAT-dependent (93). FpvA, PupA and PbrA share sequences typical of receptors whose activities are dependent upon the energy-coupling TonB protein [see (140, 141) for reviews of TonB involvement in siderophore-mediated iron uptake], and *tonB* genes have been reported in *P. aeruginosa* (142) (dubbed *tonB1*) and *P. putida* (143), and shown to be essential for siderophore-mediated iron uptake. A homologue of the *P. putida tonB* product is also present in the unfinished *P. fluorescens* genome sequence (http://www.ncbi.nlm.nih.gov/cgi-bin/Entrez/genom_table_cgi) although its role in siderophore-mediated iron uptake remains to be assessed. Intriguingly, two additional *tonB* genes [*tonB2* (144) and PA0695 (http://www.pseudomonas.com)] have been identified in *P. aeruginosa*, although neither have been shown to play a significant role in iron uptake. Both are, like the *P. putida tonB* gene (143), linked to homologues of *exbB* (dubbed *exbB* & PA0693) and *exbD* (dubbed *exbD* & PA0694) whose products are known to function as a complex with TonB in promoting energy-dependent, siderophore-mediated iron uptake across the outer membrane of e.g. *E. coli* [for a review on the subject see (140)]. The *tonB1* gene is not linked to *exbBD* homologues in *P. aeruginosa*, however, and mutant studies have confirmed that *exbB-exbD* are not required for iron (or haem) acquisition (144), raising the possibility that TonB1 works with the *exb* homologues (PA0693-PA0694) downstream of PA0695 or that it functions independently of such auxiliary proteins. In *E. coli*, ExbB-ExbD can be partially replaced by the products of the *tolQR* genes (145), and while homologues of these *tol* genes occur in *P. aeruginosa* and are, in fact, iron-regulated (146), they appear to be essential, precluding a ready assessment of their contribution to TonB1-dependent iron uptake. Suggestions that TonB1 might uniquely function independent of ExbBD homologues are, perhaps, supported by the fact that the protein possesses a novel N-terminal extension not reported in any TonB homologue described to date. While this extension might somehow replace the function provided by these auxiliary proteins, the observation that TonB1 function in *E. coli* is dependent

upon *E. coli* ExbBD (147) suggests that TonB1 does, in fact, function with comparable accessory proteins in its native host. The functional significance, then, of this extension is as yet unclear, though it does appear, at least, to be required for TonB1 stability (147), much like ExbBD contributes to TonB stability in *E. coli* (148-150). A number of C-terminal residues that are highly conserved amongst TonB proteins have been shown to be essential for TonB1 function in pyoverdine-mediated iron uptake (147) and may play a role in the interaction of the protein with the FpvA receptor. Interestingly, TonB1 also plays a role in multidrug efflux in *P. aeruginosa* (151, 152), as does a TonB homologue in *P. putida* (153). Given the importance of siderophore-mediated iron uptake to the virulence of *P. aeruginosa*, it is, perhaps, not surprising that *tonB1* mutants are also compromised for virulence in animal models of infection (12).

Although it has been generally accepted that siderophore receptors bind specifically to the ferrated siderophore only, purified FpvA has been shown to bind iron-free pyoverdine with an affinity very similar to that of ferric pyoverdine (154), and pyoverdine binding to FpvA in intact cells has been demonstrated (155). Indeed, FpvA on the cell surface appears to be saturated with iron-free siderophore under conditions of iron limitation although only iron-bound pyoverdine actually enters cells (155). One possible explanation is that iron-bound pyoverdine displaces iron-free pyoverdine on FpvA and, indeed, such displacement has been demonstrated *in vitro* (155) and *in vivo* (156). Remarkably, after ferric pyoverdine uptake and dissociation inside cells, pyoverdine is recycled back into the extracellular medium where it reloads onto FpvA (156,157). This explains earlier observations that much lower levels of pyoverdine than Fe(III) accumulate inside cells of *P. aeruginosa* during transport assays (158) and that ferrated pyoverdine is not accumulated by *P. aeruginosa* (159). Why ferric pyoverdine binding and uptake should require prior pyoverdine occupancy of the FpvA receptor remains to be determined, as does the site of dissociation inside the cell and mechanism of siderophore recycling to the cell surface. As well, it is not yet clear whether this displacement mechanism serves only to promote iron acquisition or whether it also plays a role in the receptor-mediated stimulation of FpvA, pyoverdine and virulence factor expression (see below). While this displacement mechanism of ferric siderophore uptake is so far unique to *P. aeruginosa*, receptor binding of iron-free siderophores has been reported in *Aeromonas hydrophila* (160). Recycling of siderophores, too, has been reported previously, with aerobactin apparently recycled by ColV plasmid-containing *E. coli* (161) and desferriferrichrome recycled by *P. putida* (162) and *Ustilago* spp. (163, 164).

#### 3.1.1.4. Receptor-mediated gene expression

FpvA, PupA and PbuA all possess additional sequences at the N-terminus not present in many ferric siderophore receptors but present in the well-characterized ferric dicitrate receptor, FecA, of *E. coli* (165, 166) where it plays an essential role, not in transport, but in FecA-dependent *fecA* gene expression. In *E. coli*, ferric dicitrate induces FecA expression following binding to its receptor, which 'transduces' the signal [via its N-terminus (167)] to a two component system that includes a cytoplasmic membrane spanning 'sensor', FecR, and a cytoplasmic ECF sigma factor, FecI, the latter of which promotes *fecA* gene expression [reviewed in (168)]. Similarly, FpvA has recently been shown to play a role in *fpvA* gene expression, mediated by its cognate siderophore, pyoverdine (169, 170). The positive influence of pyoverdine on FpvA production has been known for some time (171), although only recently has this been shown to occur at the level of *fpvA* gene expression and to involve the receptor itself (169, 170). Moreover, homologues of FecIR, dubbed FpvIR, are known to be involved, with FpvI as the ECF sigma factor and FpvR functioning as an apparent anti-sigma factor (169, 170) (Table 1), and FpvI stimulation of *fpvA* gene expression presumably dependent upon signals generated by FpvA upon ferric pyoverdine binding (Figure 7). This differs somewhat from FecR, which apparently modulates FecI activity and is, in fact, required for FecI activity (172). While there are no reports of PupA-dependent *pupA* expression, pseudobactin$_{M114}$ induction of its PbuA receptor gene in *P. fluorescens* M114 has been documented (173), although the involvement of PbuA in this has not been addressed. Still, an ECF sigma factor, PbrA, is required for expression of the receptor gene (134), which is inducible by pseudobactin$_{M114}$ (173), and it is tempting to speculate that PbuA mediates the positive pseudobactin influence on this gene via PbrA and a yet-to-be-identified sensor/anti-sigma factor component.

Interestingly, FpvA also plays a role in pyoverdine biosynthesis, with FpvA-deficient strains demonstrating a reduction in pyoverdine production as a result of reduced expression of *pvd* genes (174, 175). FpvA appears to mediate a positive influence of pyoverdine on *pvd* gene expression, in that the siderophore enhances *pvd* gene expression in wild type but not FpvA-deficient strains (174), in a process that involves the FpvR anti-sigma factor and PvdS (174). As with siderophore-dependent control of *fpvA* expression, then, siderophore interaction with FpvA initiates a signal transduction cascade involving the N-terminus of FpvA (175) that triggers the release of, in this case PvdS, by FpvR, with the sigma factor then free to activate *pvd* gene expression (Figure 7) (174). With FpvR functioning as an apparent anti-sigma factor for both PvdS and FpvI, what is presently unclear is whether the sigma factors occupy a common or different sites on FpvR. As expected given the role of PvdS in exotoxin A expression, the FpvA-FpvR-PvdS cascade also mediates pyoverdine stimulation of toxin production (Figure 7) (174). FpvA appears so far unique amongst ferric siderophore receptors in terms of its role in regulating siderophore biosynthesis, although the induction of pseudobactin$_{M114}$ biosynthetic gene expression by the cognate siderophore in *P. fluorescens* M114 (173) and the PbrA involvement in pseudobactin$_{M114}$ synthesis (see above) certainly implicates PbuA in a signal transduction cascade, with pseudobactin binding to PbuA serving to activate PbrA via as yet unknown sensor/anti-sigma factor. As well, pyoverdine$_{B10}$ induction of the *pbsA* pyoverdine biosynthetic gene in *Pseudomonas* strain B10 suggests a similar involvement of

**Table 1.** Known and putative iron receptors and their regulators in *Pseudomonas*[a]

| Receptor[b] | Regulator(s) | | | | | Reference(s) |
|---|---|---|---|---|---|---|
| | FecI/PupI-like ECF sigma factor | FecR/PupR-like sensor/anti-sigma factor | 2-component sensor kinase | 2-component response regulator | Other | |
| P. aeruginosa PAO | | | | | | |
| FpvA | FpvI (Pig32)[c] | FpvR[d] | --- | --- | --- | 95, 136, 169, 170, 174 |
| FptA | --- | --- | --- | --- | PchR[e] | 207, 208, 213, 214 |
| PfeA | --- | --- | PfeS | PfeR | --- | 46, 216, 221, 223 |
| FecA (PA3901)[f,g] | PA3899[g,h] | PA3900g,[h] | --- | --- | --- | 224 |
| PA0151 | --- | --- | --- | --- | --- | 224 |
| PA0192 | --- | --- | --- | --- | --- | 224 |
| FiuA | FiuI[h] | FiuR[h] (Pig17)[c] | --- | --- | --- | (83 |
| PirA | --- | --- | PirS (Pig19)[c] | PirR (pig19)[c] | --- | (83 |
| PA1302[i] | PA1300[h] | PA1301[h] | --- | --- | --- | 224 |
| PA1322 | --- | --- | --- | --- | --- | 224 |
| PA1365 | PA1363 | PA1364 | --- | --- | --- | 224 |
| UfrA (PA1910)[j] | PA1912 | PA1911 | --- | --- | --- | 224 |
| PA1922 | --- | --- | --- | --- | --- | 224 |
| PA2466 | PA2468[h] | PA2467[h] | --- | --- | --- | 224 |
| PA2911 | --- | --- | --- | --- | --- | 224 |
| PA3268 | --- | --- | --- | --- | --- | 224 |
| HasR | PA3410[h] (Pig25)[c] | PA3409[h] | --- | --- | --- | 224, 244 |
| PA4156 | --- | --- | --- | --- | --- | 224 |
| PA4168 | --- | --- | --- | --- | --- | 224 |
| PA4514[c] | --- | --- | --- | --- | --- | 224 |
| PA4675 | --- | --- | --- | --- | --- | 224 |
| PA4897[h] | PA4896[h] | PA4895[h] | --- | --- | --- | 224 |
| P. putida WCS358 | | | | | | |
| PupA | | | | | | |
| PupB | PupI | PupR | --- | --- | --- | 182, 193 |
| RF1 | --- | --- | --- | --- | --- | 184 |
| RF2[k] | --- | --- | --- | --- | --- | 184 |
| RF3[l] | --- | --- | --- | --- | --- | 184 |
| Pseudomonas sp. B10 | | | | | | |
| PupX | --- | --- | --- | --- | --- | 267 |
| Pseudomonas fluorecens M114 | | | | | | |
| PbuA | PbrA | ?[m] | --- | --- | --- | 114, 138 |
| B. cepacia | | | | | | |
| OrbA | --- | --- | --- | --- | --- | 237 |

[a] Where genes have not been shown to function as receptors/regulators, the designation of putative receptors and regulators is based on similarities to known siderophore/heme receptors and regulatory proteins as annotated by the *Pseudomonas aeruginosa* Community Annotation Project (see http://www.pseudomonas.com), and their proposed involvement in regulating the indicated putative receptors is based solely on their proximity (i.e. immediately adjacent) to the putative receptor genes. Regulatory genes fell into two groups, those showing homology to the iron starvation ECF sigma factors (e.g. FecI) and their corresponding sensor/anti-sigma factor (e.g. FecR) (see ref. 177) and those showing homology to the classical two-component regulatory system composed of a sensor histidine kinase and a response regulator (see ref. 222). [b]FpvA, ferric pyoverdine receptor; FptA, ferric pyochelin receptor; PfeA, ferric enterobactin receptor; FecA, ferric dicitrate receptor; FiuA, ferrioxamine B receptor; PirA, putative ferric enterobactin receptor; UfrA, unknown ferric siderophore receptor; HasR, heme uptake receptor; PupA, ferric pseudobactin$_{358}$ receptor; PupB, ferric pseudobactin$_{BN7/BN8}$ receptor; RF1, an incompletely characterized gene encoding a putative iron-siderophore receptor exhibiting 58.5 % identity with FpvA; RF2, probable receptor for pyoverdine$_{PAO}$ and pseudobactin$_{374}$; RF3, probable receptor for pseudobactin$_{7SR8}$ and pseudobactin$_{B10}$; PupX, ferric pseudobactin$_{B10}$ receptor; PbuA, ferric pseudobactin$_{M114}$ receptor; OrbA, ornibactin receptor. [c] First identified by Ochsner and Vasil (124) with their designation given in parentheses. [d] Also involved in control of pyoverdine biosynthesis through its action on PvdS, an ECF sigma factor required for expression of pyoverdine biosynthetic genes (174). [e] An AraC family regulator also involved in pyochelin production through its action on the *pch* genes of pyochelin biosynthesis (205, 213). [f] Role in ferric dicitrate uptake yet to be confirmed although *P. aeruginosa* has been shown to transport ferric citrate (42, 52). [g] Where receptor/regulator activity has yet to be confirmed experimentally the gene designation (i.e. PAxxxx) provided by the Pseudomonas Genome Project (http://www.pseudomonas.com) is reported. [h]Confirmed as iron-regulated (i.e. expression increases under iron limitation) (84). [i] Probable heme utilization protein/receptor. [j] First identified by Poole and colleagues (10). [k] Apparently inducible with pyoverdine$_{PAO}$ and pseudobactin$_{374}$. [l] Apparently inducible with pseudobactins B10 and 7SR8. [m]A sensor partner for the PbrA ECF sigma factor has yet to be identified

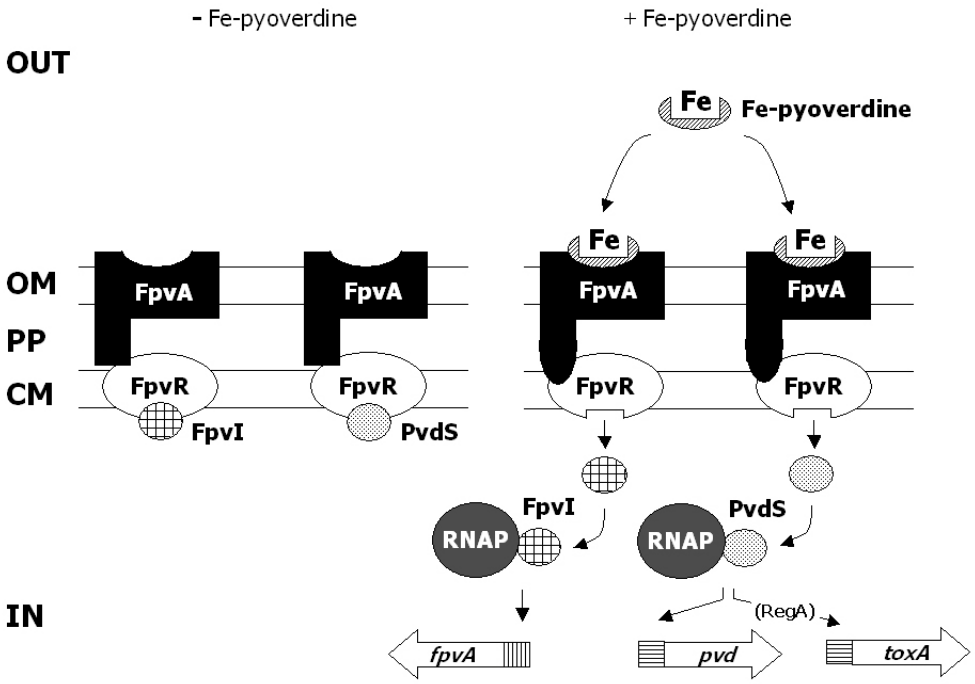


**Figure 7.** Schematic showing pyoverdine-dependent expression of its cognate biosynthetic (*pvd*) and receptor (*fpvA*) genes in *P. aeruginosa.* In the absence of pyoverdine (left), the ECF sigma factors necessary for *fpvA* (FpvI) and *pvd* (PvdS) expression are sequestered by the anti-sigma factor FpvR and unavailable to stimulate *pvd*/*fpvA* gene expression. In the presence of pyoverdine (right), the iron complex of this siderophore interacts with its outer membrane receptor, FpvA, causing a conformation change that effects release of the sigma factors by FpvR. These are then free to direct RNA polymerase (RNAP) to the *pvd*/*fpvA* genes, as well as additional pyoverdine-regulated genes such as *toxA* (exotoxin A). Again, the impact of PvdS on *toxA* expression is indirect and likely mediated by RegA.

this organism's ferric pyoverdine receptor in pyoverdine biosynthesis (176). Finally, the results of a previous study demonstrating pseudobactin$_{358}$-dependent expression of a pseudobactin$_{358}$ biosynthetic gene in *P. putida* WCS358 was suggestive of receptor (i.e. PupA) involvement, since utilization of the siderophore was probably needed for this upregulation (115). The involvement, if any, of PupA in pseudobactin$_{358}$ synthesis was not, however, examined in this study. Still, an earlier study revealed no difference in siderophore production by PupA-deficient mutants as compared to wildtype (137), suggesting that pseudobactin biosynthesis in *P. putida* WCS358, unlike pyoverdine biosynthesis in *P. aeruginosa*, has no receptor involvement.

Although *fpvA* expression is strictly iron-regulated, the gene lacks a binding site for the Fur repressor that typically mediates iron-regulated gene expression in *P. aeruginosa* (95). The *fpvI* gene [previously called *pig32* (124)] whose product promotes *fpvA* expression is, however, iron-regulated (170) and contains a Fur-binding site in its promoter (170, 124). Thus, as with the pyoverdine biosynthetic genes, whose iron regulation occurs via Fur control of expression of a required sigma factor, PvdS (see above), iron-regulation of *fpvA* occurs indirectly as a result of Fur control of FpvI (Figure 6B). Similarly, iron-regulation of the PbuA ferric pseudobactin receptor of *P. fluorescens* M114 is also mediated by Fur/iron regulation of the corresponding ECF sigma factor, PbrA (134).

PvdS, PbrA, PfrI (and FecI) belong to a subgroup of ECF sigmas that are iron-regulated (by Fur) and implicated in iron acquisition processes and dubbed iron-starvation sigmas (102, 177). These appear to be widespread amongst the fluorescent pseudomonads (177) and, indeed, several examples are readily identifiable in the *P. aeruginosa* genome sequence [see Table 1 and (177)]. Interestingly, these often occur in tandem with FecR/FpvR homologues and adjacent to putative siderophore receptor genes (Table 1). It is likely, therefore, that these regulatory gene pairs control expression of the receptor genes in response to hitherto unidentified, probably heterologous siderophores, whose binding (complexed with iron) to the cognate receptors ultimately triggers expression of the corresponding receptor genes.

#### 3.1.1.5. Uptake of heterologous pyoverdines by fluorescent pseudomonads

Despite the somewhat strain specificity of pyoverdine/pseudobactin recognition and uptake (30, 48, 70) many fluorescent pseudomonads are clearly able to utilize pyoverdines/pseudobactins produced by other fluorescent pseudomonads (24, 29, 48, 49, 50, 70, 178-182). In rhizosphere organisms this clearly enhances their rhizosphere 'competence' and ability to survive (15), while lack of heterologous siderophore utilization by plant deleterious pseudomonads and other plant pathogens likely forms the basis for pseudobactin-implicated biocontrol (70, 180). Utilization of heterologous pyoverdines/

pseudobactins occurs because different strains produce structurally identical pyoverdines or pyoverdines with conserved features likely important for receptor recognition [i.e. a single receptor can accommodate endogenous as well as identical or related heterologous pyoverdines (183-186)] or strains are able to express individual receptors specific for heterologous pyoverdines/pseudobactins (182, 184). Only three structurally unique pyoverdines have been described to date in *P. aeruginosa* (Figure 1) and any strain producing e.g. type I pyoverdine will obviously be able to utilize the pyoverdine produced by any other type I pyoverdine-producing strain (187). Similarly, *P. chlororaphis* ATCC 9446 and *P. fluorescens* ATCC 13525 produce the same pyoverdine (188) and, therefore, demonstrate cross-feeding with one another's siderophore. Several examples of *Pseudomonas* spp. utilizing structurally different heterologous pyoverdines are also known, though these pyoverdines invariably share some conserved feature within the peptide moiety [e.g. strains producing pyoverdines with a C-terminal cyclopeptide substructure tend to be able to utilize one another's pyoverdines despite differences in amino acid sequence of the peptide moiety (185, 189, 190)]. Presumably, this cyclopeptide structure is important for recognition by a given strain's ferric pyoverdine receptor and, as such, the receptor is able to recognize not only its own siderophore but also heterologous siderophores that share the same feature. In agreement with this, loss of the FpvA ferric pyoverdine receptor of *P. aeruginosa* PAO1 compromises iron uptake mediated by both the endogenous pyoverdine and the structurally different pyoverdine of *P. fluorescens* ATCC 13525, both of which demonstrate C-terminal cyclopeptidic moeities (185). Cross-utilization is also seen with *Pseudomonas* spp. producing structurally different pyoverdines with linear peptide chains, though this seems to be dependent upon a shared dipeptide (187) or tetrapeptide (191) sequence occurring at the same position within the peptide chains of the respective pyoverdines. Moreover, loss of a single receptor compromises utilization of the endogenous as well as heterologous linear pyoverdines (186), confirming once again that a single receptor is able to recognize both endogenous and heterologous pyoverdines, and highlighting the likely significance of the peptide tail, and features thereof, for receptor recognition.

Clearly, though, pseudomonads also utilize heterologous pyoverdines/pseudobactins with no shared sequences or substructure in the peptide moiety and in these instances there is evidence for expression of additional ferric pyoverdine/pseudobactin receptors specific for the heterologous siderophore (181, 183, 184). The best-characterized examples occur in *P. putida* WCS358 (Table 1), which readily utilizes the pseudobactins of a number of *Pseudomonas* strains as a result of expression of multiple ferric pseudobactin receptors (182, 184, 192). In most instances, the receptors are specific and induced by the heterologous siderophores (182, 184). This induction, in the case of *P. putida* WCS358 utilization of the pseudobactins of *P. putida* strains BN7 and BN8, is mediated by the pseudobactin$_{BN7/8}$ receptor, PupB (182), and sensor/anti-sigma factor (PupR) and ECF sigma factor (PupI) homologues of FecR/FpvR and FecI/FpvI (193) via a receptor-dependent signal transduction mechanism outlined earlier (Figure 7). It is likely that other fluorescent pseudomonads also express inducible receptors for heterologous pyoverdines/pseudobactins. Reported discrepancies between cross-feeding data (where cross-feeding was seen with heterologous siderophores) and binding/uptake data (where binding or uptake was not seen) (48) are, for example, explainable by an inducible ferric pyoverdine/pseudobactin receptor being present in the cross-feeding studies but, owing to lack of prior growth in the presence of the heterologous siderophore, being absent in the uptake/binding studies.

### 3.1.2. Pyochelin

A condensation product of salicylic acid and two cysteinyl residues [see (194) for detailed review of the enzymology of pyochelin biosynthesis] pyochelin (Figure 2) binds Fe(III) in a 2:1 stoichiometry and demonstrates a rather low affinity for iron in aqueous media ($5x10^5$ $M^{-1}$) (43). Still, the siderophore is effective at promoting iron uptake in *P. aeruginosa* (42) and acquiring iron from transferrin (195), and is implicated as a virulence determinant (8, 11), though the latter may relate to its ability to catalyze the formation of tissue-damaging free radicals (196-200). Interestingly, pyochelin also binds other transition metals [e.g. Mo(IV), Ni(II) and Co(II)] with appreciable affinity and is, in fact, implicated in the delivery of both Co(II) and Mo(IV) to *P. aeruginosa* cells (201).

#### 3.1.2.1. Biosynthesis and Transport

The genes for pyochelin biosynthesis have been identified and occur in two separate operons, *pchDCBA*, involved in the synthesis of the pyochelin precursor salicylic acid (202-204) and *pchEFGHI* (205, 206), though *pchHI* appear to have an export rather than synthetic function. The *fptA* gene (207) encoding the 75 kDa ferric pyochelin receptor (208) occurs immediately downstream of *pchI*. Hybridization studies have demonstrated a *fptA*-hybridizing signal in *P. aureofaciens*, *P. fluorescens*, *P. putida*, *B. cepacia* and several *P. aeruginosa* strains (209), although *fptA* genes were not identified and the ability of these organisms to utilize pyochelin was not assessed. Expression of *fptA* has been documented *in vivo*, using IVET (*in vivo* expression technology) (210), and the gene is inducible by human respiratory mucous *in vitro* (211), indicating that it likely pays a role in *in vivo* iron acquisition, particularly in lung infections. Pyochelin production has been documented in *B. cepacia* (31) where it appears to pay a role in pathogenesis (31, 212). A recent study also confirmed production of this siderophore by *P. fluorescens*, though not by *P. aureofaciens* or *P. putida* (209).

#### 3.1.2.2. Regulation

Production of pyochelin and the ferric pyochelin receptor is inducible under conditions of iron limitation (208), as a result of Fur regulation of the *fptA* receptor gene (124) and the *pchDCBA* (203) and *pchEFGHI* (205) biosynthetic operons. Pyochelin and FptA production also require the product of the *pchR* gene, encoding an AraC

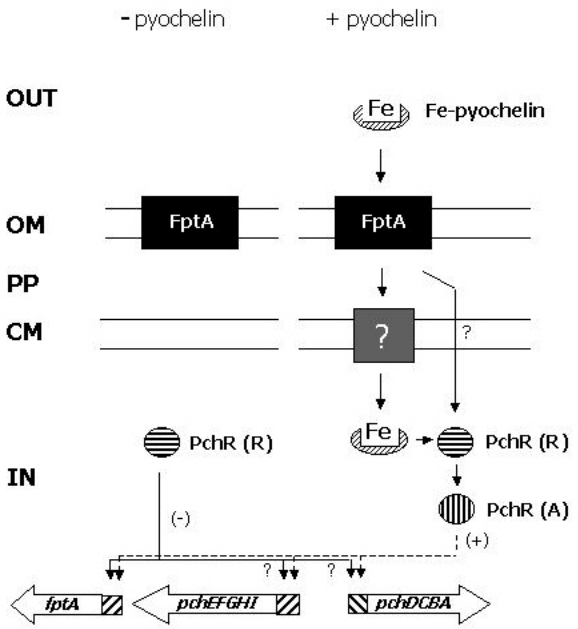


**Figure 8.** Schematic showing pyochelin-dependent expression of its cognate biosynthetic (*pch*) and receptor (*fptA*) genes in P. aeruginosa. In the absence of pyochelin (left) the regulatory protein, PchR exists in a repressor form (R) and blocks expression of *fptA* and, possibly, the pyochelin biosynthetic genes. In the presence of pyochelin (right), or more probably, ferric pyochelin, PchR is converted to a transcriptional activator (A) of the receptor and biosynthetic genes. Its is unclear, however, whether ferric pyochelin is a direct effector of PchR activator activity, following its transport across the cytoplasmic membrane via a hitherto unknown carrier, or whether it simply interacts with its receptor, FptA, on the cell surface and this is signalled to PchR via an as yet undiscovered signal transduction pathway. OM, outer membrane; PP, periplasm; CM, cytoplasmic membrane.

family regulator that is also regulated by Fur (213, 124) and required for expression of the *fptA* (214) and *pch* (205) genes. Intriguingly, pyochelin-deficient strains exhibit a marked deficiency in production of the ferric pyochelin receptor (171, 208) and its gene (214) that is reversed upon pyochelin supplementation (171), in a process mediated by PchR (214). In fact, PchR actually represses *fptA* expression in the absence of pyochelin and promotes its expression in the presence of this siderophore (214) (Figure 8). Similarly, expression of the *pchDCBA* and *pchEFGHI* operons is notably curtailed in pyochelin-deficient mutants but stimulated by exogenously added pyochelin, dependent, again, on the presence of *pchR* (205). Thus, this regulator mediates the positive effect of pyochelin (or more likely ferric pyochelin) on *fptA* and *pch* gene expression and, thus, production of pyochelin and its receptor (Figure 8). PchR activity may be modulated by the FptA receptor, inasmuch as mutants lacking FptA show reduced *fptA* gene expression (214), though the resultant defect in ferric pyochelin uptake would also negatively impact PchR stimulation of *fptA* expression should the iron-siderophore complex act directly on PchR. Still, in the absence of genes encoding an obvious cytoplasmic membrane permease for ferric pyochelin (see below) the likelihood that the complex actually reaches the cytoplasm to do so is questionable.

### 3.1.3. Quinolobactin

Like *P. aeruginosa*, *P. fluorescens* strain ATCC 17400 produces two siderophores, pyoverdine and a second siderophore dubbed quinolobactin (215) (Figure 2). Intriguingly, the receptor for ferric quinolobactin, reminiscent of FptA and pyochelin, is inducible by quinolobactin, which is, however, absent from the pyoverdine-producing wild type strain (215). Thus, a wild type strain lacks this receptor (unless purified siderophore is added to bacterial cultures) while a pyoverdine-deficient mutant produces substantial quantities of both quinolobactin and its receptor. Clearly, then, pseudomonads besides *P. aeruginosa* that synthesize multiple siderophores have mechanisms of prioritizing siderophore production and use (see below), perhaps utilizing control mechanisms similar to those described above.

### 3.1.4. Hierarchy of siderophore usage in *P. aeruginosa*

While *P. aeruginosa* is capable of producing two siderophores, pyoverdine and pyochelin, it clearly needs only a single delivery system for iron at a given time in a given environment and, indeed, pyoverdine-producing strains tend to produce very little pyochelin or ferric pyochelin receptor (208). The obvious explanation is that the superior iron chelator [typically pyoverdine, at least in aqueous medium (25)] binds available Fe(III) and interacts with its cognate receptor, upregulating receptor (and siderophore) biosynthesis at the expense of the siderophore and receptor genes for the inferior siderophore (i.e. pyochelin) (Figure 9). Such a hierarchy of siderophore usage also occurs in other pseudomonads that express multiple endogenous siderophores (see above) and likely extends to heterologous siderophores. Thus, enterobactin, an enterobacterial siderophore used by *P. aeruginosa* (see below) and exhibiting a markedly higher affinity for Fe(III) than either of the organism's endogenous siderophores (5), upregulates a transport system for ferric enterobactin at the expense of systems for the biosynthesis and uptake of pyoverdine or pyochelin (46, 216). In this way the cell invests energy in elaborating an uptake mechanism for the best available siderophore at a given time and place.

### 3.2. Heterologous siderophore uptake in *Pseudomonas*

*P. aeruginosa* and, indeed, many of the fluorescent pseudomonads are able to utilize a variety of heterologous siderophores, not only heterologous pyoverdines/pseudobactins (see above) but also additional siderophores of bacterial (45, 46, 47, 179) and fungal (50, 178) origin. Use of iron provided by phytosiderophores has also been reported in fluorescent rhizosphere pseudomonads, although this appears to involve ligand exchange between plant and bacterial siderophores (217). The use of multiple, heterologous siderophores by *P. aeruginosa* was predictable on the basis of early observations of multiple high-molecular weight iron-regulated outer membrane proteins (IROMPs) in this organism (218) and confirmed by

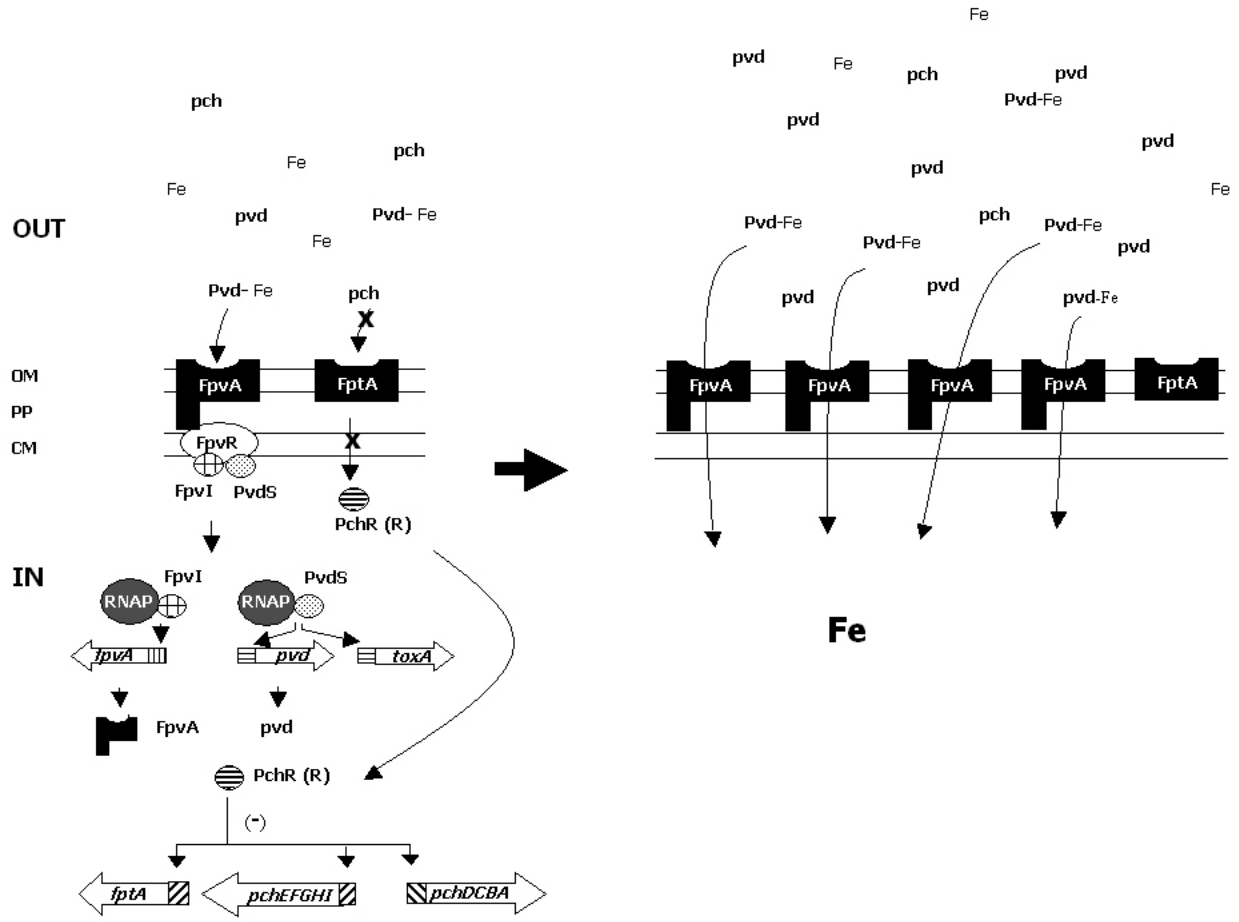


**Figure 9.** Schematic depicting the hierarchy of endogenous siderophore usage in *P. aeruginosa*. Although wild type *P. aeruginosa* produces both pyoverdine and pyochelin under iron-limiting conditions (left), only pyoverdine, the superior iron chelator binds Fe and subsequently interacts with its outer membrane receptor to initiate the signal transduction cascade that leads to pyoverdine biosynthetic (*pvd*) and receptor (f*pvA*) gene expression (see legend to Fig. 7 for a more detailed description). Unable to compete with pyoverdine for iron, only the iron-free form of pyochelin is present and is unable to interact with its receptor, FptA, for transport or initiation of a putative signal transduction pathway (see Fig. 8). PchR, thus, remains in its repressor (R) form and blocks expression of the pyochelin receptor and biosynthetic (?) genes. In this way the cell specifically amplifies production of the siderophore (and its transport system) that is better able to access available iron in a given environment (right). OM, outer membrane; PP, periplasm; CM, cytoplasmic membrane.

evidence for multiple receptor homologues in the genome sequence (Table 1). In many instances, use of heterologous siderophores requires induction of outer membrane receptors specific for the heterologous siderophores, a strategy used, in fact, by a number of bacteria [see (219, 177) for reviews of siderophore regulation of receptor gene expression]. Indeed, the non-fluorescent *Pseudomonas fragi*, which appears not to produce its own siderophore, is capable of utilizing a variety of heterologous bacterial and fungal siderophores and synthesizes a number of IROMPs as candidate receptors, at least one of which is siderophore inducible (220). Siderophore induction of receptor genes often involves regulatory protein pairs of the FecIR/FpvIR sort (see above and Figure 7) or the sensor kinase/response regulator sort (see below and Figure 10) and, strikingly, homologues of such regulator genes are often linked to putative siderophore receptor genes in the *P. aeruginosa* genome (Table 1). Its is tempting to speculate, then, that use of heterologous siderophores is common in *P. aeruginosa* because of the presence of an array of inducible receptors that respond specifically to these heterologous siderophores.

### 3.2.1. Enterobactin

A major catecholate siderophore of *E. coli* and other *Enterobacteriaceae*, enterobactin (Figure 3) promotes iron uptake into *P. aeruginosa* as a result of its induction of an outer membrane ferric enterobactin receptor PfeA (46, 216). Expression of *pfeA* requires the products of the linked *pfeRS* operon, which encodes response regulator (PfeR) and histidine kinase (PfeS) components of the superfamily of

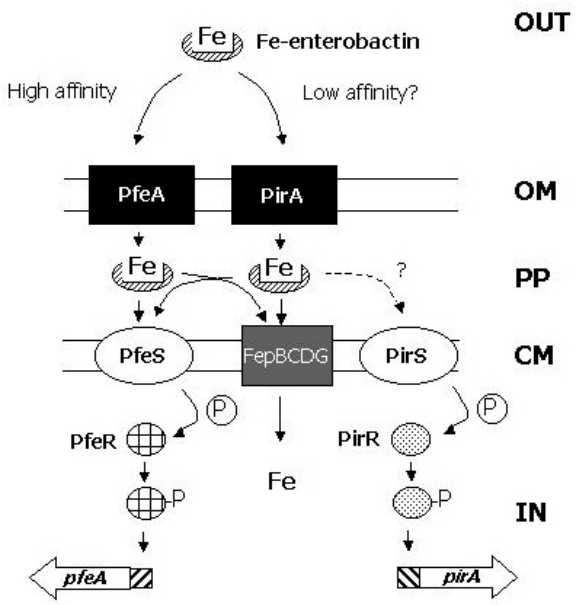


**Figure 10.** Schematic showing enterobactin-dependent expression of its cognate receptor gene *pfeA* in *P. aeruginosa*. Ferric enterobactin that is deposited in the periplasm by the PfeA receptor interacts with the PfeS sensor kinase, which ultimately phosphorylates the response regulator, PfeR, thereby activating it to drive expression of the *pfeA* gene. This process is independent of any transport of the ferric siderophore complex across the cytoplasmic membrane by permease components (FepBCDG). A second receptor able to accommodate ferric enterobactin, PirA, has been proposed to explain ferric enterobactin uptake and stimulation of PfeSR in the absence of PfeA. Apparently associated with its own phosho-relay two-component regulatory system, PirSR, it is likely that PirA also transports another, as yet unknown, siderophore as its primary substrate and that this provides for upregulation, via PirSR, of the *pirA* gene.

environmentally-responsive two-component regulatory protein pairs (221) [see (222) for a review of two-component regulatory systems]. Members of this superfamily utilize a phosphorelay mechanism to regulate gene expression, with an environmental stimulus triggering autophosphorylation of the histidine kinase and subsequent phosphoryl group transfer to the response regulator, which then activates target gene expression. In response to enterobactin, then, PfeRS function to activate *pfeA* expression via phosphotransfer form PfeS to PfeR (Figure 10) with enterobactin ultimately promoting PfeR binding to the *pfeA* promoter region (223). Expression of both *pfeA* and *pfeRS* is iron-regulated, mediated by the Fur repressor whose binding to the *pfeA* and *pfeR* promoters has been demonstrated (124). As such, enterobactin induction of *pfeA* occurs only under conditions of iron-limitation (46). Enterobactin inducibility of *pfeA* is independent of the PfeA receptor, distinguishing this system from the receptor-dependent ECF sigma factor-controlled systems described previously (223).

PfeA-deficient mutants still demonstrate enterobactin induction of *pfeA*, arguing for a second system for the transport of ferric enterobactin across the outer membrane in *P. aeruginosa*, since the siderophore must reach the cytoplasmic membrane PfeS component to initiate the phosphorelay mechanism (Figure 10). Consistent with this, PfeA-deficient mutants display growth, albeit reduced, in an enterobactin-supplemented iron-restricted minimal medium (223). A possible candidate for this second uptake system is the product of the *pirA* gene (a.k.a. PA0931; http://www.pseudomonas.com) (Table 1), which displays substantial homology to PfeA (83). Still, this gene is also linked to an operon encoding kinase/response regulator homologues of the superfamily of two-component regulators (PirRS; Table 1) and, thus, also likely siderophore-regulated. While it is unlikely that two systems would be present and specifically responsive to enterobactin in *P. aeruginosa*, it is possible that PirA promotes modest uptake of ferric enterobactin (while another, perhaps structurally-related siderophore is its intended ligand) sufficient to 'activate' PfeRS and, ultimately *pfeA* gene expression (Figure 10).

Genes encoding homologues of the periplasmic (*fepB*; a.k.a. PA4159) and cytoplasmic membrane (*fepC*, *fepD* and *fepG*; a.k.a. PA 4158, PA4169, PA4161) components of ferric enterobactin uptake in *E. coli* are readily identifiable in the *P. aeruginosa* genome sequence (http://www.pseudomonas.com) (224). Interestingly, *fepC* was identified as an iron-regulated gene in a recent DNA microarray study of iron-starvation-inducible genes in *P. aeruginosa* (84) though the other *fep* gene homologues were not. It is likely that these genes will, like the *pfeA* receptor gene, be responsive to enterobactin and, thus, not be inducible under iron limitation unless the siderophore is present. Why *fepC* was expressed under iron limitation in the absence of enterobactin is unclear, although it is noteworthy that the gene is transcribed divergently from the *fepBDG* homologues, perhaps because it plays an additional role in the cell unrelated to enterobactin uptake.

### 3.2.2. Citrate

The ability of citrate (Figure 5) to mediate iron uptake in *P. aeruginosa* has been known for some time (42). Like *E. coli*, citrate-mediated iron uptake in *P. aeruginosa* requires prior exposure to citrate, which induces expression of an outer membrane citrate-binding component (52). While a ferric citrate receptor has not yet been confirmed, a *fecA* homologue (PA3901) is present in the *P. aeruginosa* genome, adjacent to *fecIR/fpvIR* homologues (PA3899-3900) (Table 1) that probably mediate the citrate inducibility of *fecA*.

### 3.2.3. Ferrioxamine B

Ferrioxamine B (desferal) (Figure 4) is known to promote iron uptake in *P. aeruginosa* and to induce expression of a high-molecular weight IROMP that likely serves as its receptor (218). The ferrioxamine B receptor gene, *fiuA*, (a.k.a. PA0470) (Table 1) has recently been identified and is under the control of the FpvIR-like products of the adjacent *fiuIR* genes (83) (Table 1).

### 3.3. Uptake post-outer membrane

Siderophores and their cognate receptors function to deliver the ferric siderophore complex to the periplasm, where additional transport components are responsible for getting the iron to the cytoplasm. In *E. coli*, there are siderophore-specific periplasmic and cytoplasmic membrane components of the ABC permease family for the transport of ferric dicitrate (FecBCDE) and ferric enterobactin (FepBCDG), and a less specific transport system that accommodates hydroxamate siderophores (FhuBCD) that are internalized by a variety of different outer membrane receptors [reviewed in (3, 4)]. Transport post-outer membrane has received little if any attention in *Pseudomonas*, although *fepBCDG* homologues have been identified in the *P. aeruginosa* genome sequence (see above). Strikingly, however, no obvious periplasmic/cytoplasmic membrane transporter homologues are identifiable in the *pch-fptA* locus of ferric pyochelin biosynthesis and uptake, in the vicinity of *fecA-fecIR* genes of ferric dicitrate uptake, nor adjacent to any of the receptor homologues shown in Table 1. And while candidate ABC transporter homologues are present in the *pvd* locus (PA2408-2409; http://www.pseudomonas.com) and are iron- and PvdS-regulated (84), other genes in the putative operon that includes these ABC genes (e.g. PA02407 and PA02403) do not play a role in ferric pyoverdine uptake (84), suggesting that these ABC homologues do not as well.

The obvious similarities with pseudobactin biosynthesis and uptake in fluorescent rhizosphere organisms, and observations that cloned receptor genes for heterologous pseudobactins are alone sufficient to enable these organisms to utilize these heterologous siderophores (131, 182, 183, 225) suggests that periplasmic/cytoplasmic membrane permeases specific for any particular pseudobactin/pyoverdine may be absent from *Pseudomonas*. While one might envisage a broadly specific Fhu-like system, whereby any pyoverdine/pseudobactin delivered to the periplasm would be accommodated by a common transport system it is equally plausible, given the lack of obvious periplasmic/cytoplasmic membrane transporters for the other siderophores, that iron is generally removed from all siderophores (with the possible exception of enterobactin) in the periplasm and that an iron-specific transporter delivers the metal to the cytoplasm. Early studies of ferric pyoverdine uptake certainly suggested that iron and pyoverdine dissociated in the periplasm (158) though no mechanism was provided. Reports of reductase activity in *P. aeruginosa* capable of releasing iron from ferrated citrate (226), pyochelin (226) and pyoverdine (227, 228) suggest one possibility, although these activities were for the most part detected in the cytoplasm. Still, it is by no means certain that these activities play any role in iron uptake *in vivo*. Should iron be freed from siderophores in the periplasm, candidate iron-specific transporters include a homologue of the *E. coli* FeoAB Fe(II) transporter (229, 230) (PA4359-PA4358 in the *P. aeruginosa* genome; http://www.pseudomonas.com) and homologues of the *Serratia marcescens* SfuABC (231) (PA5216-5217 in the *P. aeruginosa* genome; http://www.pseudomonas.com) (224) and *Hemophilus influenzae* HitABC (232,233) (PA4687-4688 in the *P. aeruginosa* genome) (234) Fe(III) uptake systems. The FeoAB and SfuABC homologues have been shown to be iron-regulated in *P. aeruginosa* (84) though clearly any involvement of an Fe(II) transporter would require a reductive release mechanism in the periplasm. In light of recent evidence implicating a multicopper oxidase [responsible for the oxidation of Fe(II) to Fe(III)] in the use of Fe(II) as a sole iron source by *P. aeruginosa* under aerobic conditions, at least one of these Fe(III) transporters likely plays a role in the uptake of multicopper oxidase-generated Fe(III) as well (234).

### 3.4. Iron uptake in *B. cepacia*

Four siderophores are reportedly produced by *B. cepacia*, including pyochelin (31), cepabactin (a cyclic hydroxamate) (32), salicyclic acid (formerly azurechelin) (34, 36), and the ornibactins (33, 35), although a given strain might produce anywhere between 1 and all 4 (235). The organism is also able to use the *B. pseudomallei* siderophore, malleobactin (38), which has been shown to promote iron mobilization from transferrin to *B. cepacia* (37). Pyochelin and salicylic acid also mobilize iron from transferrin and lactoferrin for use by *B. cepacia* (37), and pyochelin production by *B. cepacia* has been correlated with severe pulmonary disease in cystic fibrosis (CF) patients (31). Still, salycylic acid and ornibactins predominate in isolates infecting the lungs of CF patients (235), suggesting that they are the important contributors to iron acquisition and, thus, growth, *in vivo*.

Ornibactins are linear hydroxamate-hydroxycarboxylate siderophores possessing a conserved tetrapeptide to which is attached one of three possible acyl groups (35). Two genes involved in ornibactin biosynthesis have been identified, encoding homologues of pyoverdine biosynthetic genes (236), likely reflecting the common presence of amino acids in both siderophore structures. The gene for the ferric ornibactin receptor, *orbA*, has also been cloned (237) and, consistent with its homology to the family of TonB-dependent receptors, *tonB-exbB-exbD* genes have been identified in *B. cepacia* and their involvement in iron acquisition confirmed (238). Receptor mutants (237) and mutants deficient in ornibactin biosynthesis (236) exhibit reduced virulence in animal models of infection, highlighting the importance of this iron uptake system for *in vivo* growth and pathogenesis. Interestingly, CepR, a quorum-sensing regulator that positively influences expression of an extracellular protease (and its cognate homoserine lactone) (239), negatively impacts ornibactin production (i.e. mutants show increased ornibactin levels) and expression of at least one ornibactin biosynthetic gene (240). This contrasts with *P. aeruginosa*, where the LasIR quorum-sensing system positively impacts pyoverdine production (241).

## 4. HAEM ACQUISITION

In addition to ferric iron provided by siderophores, many bacteria, including *P. aeruginosa*, can also acquire iron from haem and haem-containing proteins such as haemoglobin, often relying on cell surface receptors for haem that like siderophore receptors are TonB-dependent [for a review on the subject see (242,

243)]. Two distinct haem uptake systems have been described in *P. aeruginosa*, encoded by the *phu* and *has* loci, respectively (244). The *phu* genes include *phuR*, encoding an outer membrane haem receptor (Table 1) and *phuSTUVW*, encoding a transporter of the ATP-binding cassette (ABC) family. Both sets of genes are iron-regulated and possess Fur boxes that have been verified by footprinting studies (244). Deletion of *phuR* or *phuSTUV* greatly reduces growth of *P. aeruginosa* with haemin or haemoglobin as sole iron source, although residual growth is clearly apparent and likely attributable to the products of the *hasRA* operon (244). The *hasA* gene encodes an extracellular haem-binding protein (245) [a.k.a. haemophore (242)] that also facilitates utilization of haemoglobin iron while the *hasR* product is another haem receptor (Table 1) (244). Both genes are iron-regulated by Fur, and deletion of *hasR* severely compromises utilization of haemin or haemoglobin as iron sources (244).

The *P. aeruginosa* HasA protein is very similar to the well-characterized HasA haemophore of *S. marcescens* (246-248) and the recently described HasA protein of *P. fluorescens* (249, 250), both of which function in haem/haemoglobin utilization. Although the mechanistic details of haem capture and delivery by the HasA proteins are as yet unknown, the *S. marcescens* HasA protein (in the haem-bound and haem-free states) has been shown to bind to the HasR receptor with high affinity ($10^{-10}$ M) (251). The fact that *P. aeruginosa* HasA can functionally replace *S. marcescens* HasA (245) argues that whatever the uptake mechanism it is likely to be conserved between these systems. Moreover, whatever the mechanism or route of haem acquisition by *P. aeruginosa*, haem iron is ultimately released by a oxidative cleavage of the molecule by haem oxygenase, the product of the *pigA* gene [identified earlier as a Fur-/iron-regulated gene dubbed *pig4* (84, 124)] that is required for utilization of haem as an iron source (252).

Extracellular release of HasA in *S. marcescens* (253) and *P. fluorescens* (250) requires the products of a 3-gene operon, *hasDEF*, that encode a type I secretion apparatus of the ABC family [see (254, 255) for reviews of protein/peptide exporters of this family]. Homologues of *hasDEF* occur as a putative operon in *P. aeruginosa*, immediately downstream of *hasRA* (244), and although their role in HasA export has not been addressed, the observation that the *P. fluorescens* and *S. marcescens* exporters promote secretion of *P. aeruginosa* HasA (250) suggests that these HasDEF homologues do the same in *P. aeruginosa.*

While mutants lacking both the has- and *phu*-encoded haem acquisition systems are virtually unable to utilize haemin or haemoglobin (244), suggesting that only these two systems for haem acquisition exist in *P. aeruginosa*, a possible third haem receptor gene, PA1302, has been identified in the *P. aeruginosa* genome (Table 1). Intriguingly, this gene is linked to genes encoding FecR-/FpvR-like sensor (PA1301) and FecI/FpvI-like ECF sigma factor (PA1302) components (Table 1) that are strongly iron-regulated (46 to 200-fold increase in expression in iron-limited vs. iron-replete medium) (84) and, perhaps, involved in PA1302 expression. Similarly, the *hasR* haem receptor gene occurs adjacent to sensor (PA3409) and ECF sigma factor (PA3410) genes (Table 1), which are also iron-regulated (40-fold increase in expression in iron-limited vs. iron replete conditions) (84). Still, given the known direct Fur regulation of *hasR* expression (see above), clearly any iron-regulation of this receptor is not via iron/Fur control of PA3409-3410. What, if any, role these genes play in *hasR* expression remains to be elucidated. Consistent with the expected TonB-dependence of the haem receptors in *P. aeruginosa*, *tonB1* mutants (but not *tonB2* mutants) are compromised for utilization of haemin and haemoglobin as sole iron sources (144, 12).

The presence of multiple haem acquisiton systems in *P. aeruginosa* clearly emphasizes the importance of haem as an iron source, at least in certain environments. The need for multiple systems may reflect differences in affinity for haem of individual transporters and, as such, multiple systems may provide for efficient haem acquisiton over a range of environmental haem concentrations. The various haem acquisition systems may also operate optimally under different physiological conditions, ensuring haem iron acquisition in a variety of environments.

Haem-binding activity has been detected in the outer membrane of *B. cepacia* although its significance vis-à-vis haem iron acquisition is not known (256).

## 5. IRON STORAGE

To overcome problems of poor solubility, low availability and toxicity of iron, bacteria, including *P. aeruginosa*, not only express highly regulated systems for Fe(III) acquisition, but also appear to rely on intracellular iron storage proteins such as ferritins and bacterioferritins to both sequester the metal and to provide a reservoir of this essential nutrient [see (257) for a review on the subject]. Bacterioferritins have been identified in both *P. aeruginosa* (258, 259) and *P. putida* (260) where its expression is positively influenced by iron (260, 261).

## 6. CONCLUDING REMARKS

The versatility of the fluorescent pseudomonads in general and *P. aeruginosa* in particular as regards siderophore-mediated iron acquisition clearly provides an important competitive advantage in nature and likely necessitates a strategy based on multiple cell surface receptors and a common intracellular transporter for iron. This provides for needed flexibility in terms of siderophore utilization without devoting substantial resources (and genes) to the transport of what may be infrequently used ligands. The details, however, of intracellular iron transport, including the identity of cytoplasmic/periplasmic membrane transporter(s), as well as the site and mechanism of iron-siderophore dissociation remain to be elucidated. As expected, expression of individual receptors is highly regulated and under the ultimate control, in most instances, of a family of iron-starvation ECF sigma factors which appear to be particularly common in fluorescent

pseudomonads and other soil organisms (177), a reflection no doubt of a conserved strategy for iron acquisition based on siderophore-dependent expression of multiple ferric siderophore receptors in an environment where competition for iron is likely to be significant. Despite, however, this ability to utilize a variety of heterologous (and endogenous) siderophores to meet its iron needs, the organism clearly regulates expression of individual uptake systems and only the most effective (e.g. see Figure 9) at a given time and place is expressed. This provides for iron acquisiton in a variety of environments and in the presence of a variety of microbial competitors while at the same time preventing possible excess iron accumulation as a result of mutliple iron-siderophore transporters operating simultaneously. The fact, too, that systems for iron-siderophore acquisiton in *P. aeruginosa* are responsive to iron, mediated directly or indirectly by the Fur repressor, also guards against excess iron uptake and the attendant problems with toxicity.

The study of iron acquisition in *Pseudomonas* has, to date, focused exclusively on planktonic organisms as opposed to biofilms, though the latter may be the predominant mode of growth of organisms such as *P. aeruginosa* in nature (262). Unfortunately, details of siderophore biosynthesis and transports in biofilms, as well as their regulation and contribution to the growth of biofilm organisms are sorely lacking. IROMPS have been identified in *in vitro* grown biofilms (263) suggesting that ferric siderophore receptors are expressed, though their significance for biofilm growth and survival remains to be elucidated. A recent paper describing the negative impact of lactoferrin (and other iron chelators) on *P. aeruginosa* biofilm formation does, however, highlight the probable significance of iron availability for biofilm formation (264).

Similarly, the connection between cell density and siderophore biosynthesis, suggested by observations that defects in quorum-sensing negatively impact pyoverdine production (241) needs further investigation as do suggestions that pyoverdine itself may function as a quorum-sensing signal (via FpvA and the FpvR-PvdS regulatory proteins) (174) in controlling expression of iron-responsive genes. While pyoverdine as a general signaling molecule would provide for a connection between pyoverdine biosynthesis and uptake and expression of extracellular virulence factors such as exotoxin A, alkaline protease and the PrpL endoprotease, in that all of these are iron-regulated and possibly impacted by cell density, these apparently disparate activities may, in fact, have a common role in pyoverdine-mediated iron acquisition. The extracellular virulence factors could, for example, promote *in vivo* iron release through damage/destruction of host cells and proteins that sequester iron. Alternatively, their activities could free component parts (e.g. amino acids) of the siderophore to be used in its biosynthesis. Incorporation of growth medium-provided amino acids into pyoverdine has been demonstrated (158,265) and growth medium effects on pyoverdine production are known [several citations in (266)] and suggest that the nutritional status of the environment (as influenced e.g. by the action of these toxins/enzymes) can positively impact pyoverdine production by *P. aeruginosa.* Finally, the enzymology of pyoverdine/pseudobactin biosynthesis, in particular as it relates to chromophore biosynthesis needs to be resolved, and with it perhaps the function of the myriad iron- and PvdS-regulated genes of the *pvd* locus and the potential contribution of the PvdS-regulated virulence factors to this process.

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**Key Words:** Iron transport, *Pseudomonas*, *Pseudomonas aeruginosa*, Siderophore, Siderophore Biosynthesis, Siderophore Receptor, Pyoverdine, Pseudobactin, Pyochelin, Enterobactin, Heterologous Siderophore, Tonb, Fur Repressor, Regulation, ECF Sigma Factor, Haem, Review

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