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The response of *Pseudomonas aeruginosa* to iron: genetics, biochemistry and virulence

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Summary

During the past decade significant progress has been made towards identifying some of the schemes that *Pseudomonas aeruginosa* uses to obtain iron and towards cataloguing and characterizing many of the genes and gene products that are likely to play a role in these processes. This review will largely recount what we have learned in the past few years about how *P. aeruginosa* regulates its acquisition, intake and, to some extent, trafficking of iron, and the role of iron acquisition systems in the virulence of this remarkable opportunistic pathogen. More specifically, the genetics, biochemistry and biology of an essential regulator (Ferric uptake regulator – Fur) and a Fur-regulated alternative sigma factor (PvdS), which are central to these processes, will be discussed. These regulatory proteins directly or indirectly regulate a substantial number of other genes encoding proteins with remarkably diverse functions. These genes include: (i) other regulatory genes, (ii) genes involved in basic metabolic processes (e.g. Krebs cycle), (iii) genes required to survive oxidative stress (e.g. superoxide dismutase), (iv) genes necessary for scavenging iron (e.g. siderophores and their cognate receptors) or genes that contribute to the virulence (e.g. exotoxin A) of this opportunistic pathogen. Despite this recent expansion of knowledge about the response of *P. aeruginosa* to iron, many significant biological issues surrounding iron acquisition still need to be addressed. Virtually nothing is known about which of the distinct iron acquisition mechanisms *P. aeruginosa* brings to bear on these questions outside the laboratory, whether it be in soil, in a pipeline, on plants or in the lungs of cystic fibrosis patients.

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Introduction

Iron limitation has profound consequences for all but a few microbial organisms so far identified on our planet. The paucity of soluble, biologically useful iron in aerobic environments, where the bulk of life resides on earth, is as much a dilemma to planktonic microrganisms trying to sustain themselves in vast areas of the South Pacific (Behrenfeld and Kolber, 1999) as it is to microbial pathogens attempting to initiate and sustain commensal or pathological relationships with other organisms (Calderwood and Mekalanos, 1987; Litwin and Calderwood, 1993; 1994; Crosa, 1997). Typical bacterial organisms (e.g. *Escherichia coli*) require $\approx$0.3–1.8 μM of iron for optimal growth, whereas the concentration in soil is $<$0.1 μM and only 10^{-9} μM in a mammalian host. Moreover, iron in an aerobic environment (Fe^{3+}) is extremely insoluble (10^{-18} μM) at pH 7 (Braun and Killmann, 1999). The concentration used in most laboratories for iron-replete conditions is 10–100 μM. Accordingly, the diverse array of microbes requiring iron have evolved remarkably sophisticated mechanisms to scavenge iron from the usually plentiful, but biologically unusable, sources in the environment in which they reside. Microbes can obtain iron from human-made structures (e.g. biocorrosion of steel; Dzierzewicz *et al*., 1997), sea water (Butler, 1998) and by the outright robbery, battery and murder of other prokaryotic and eukaryotic organisms (Cornelissen and Sparling, 1994). Some of the strategies used include: (i) production of powerful iron-binding compounds (siderophores), (ii) direct utilization and uptake of host iron-binding proteins (Cornelissen and Sparling, 1994), (iii) reduction of the insoluble form of iron (Fe^{3+}) to the soluble usable form (Fe^{2+}) (Coulanges *et al*., 1997), (iv) enzymatic degradation of iron-binding compounds (e.g. transferrin) (Wolz *et al*., 1994a) and (v) production of lethal compounds (exotoxins) (Bjorn *et al*., 1978; Calderwood and Mekalanos, 1987) that may eliminate competitors for usable iron resources. Yet the actual procurement of iron is only one ramification of the problem that microbes face with respect to their use of this nutriment. Aerobic metabolism, in contrast to microaerobic or anaerobic processes, requires the highest concentrations of usable iron. However, by definition, aerobic organisms are the ones most likely to incur

oxidative damage from reactive oxygen intermediates generated by Fenton-type reactions catalysed by this metal (Miller and Britigan, 1995; 1997). In response to this predicament, respiring microbes need ways to (i) sense the level of iron they have ingested, (ii) terminate its further intake and (iii) sequester potentially toxic levels (Andrews, 1998). Along with clever mechanisms to acquire iron, microbes have developed sophisticated genetically and biochemically controlled mechanisms to avoid the consequences of overindulgence.

Pseudomonas aeruginosa is a magnificent paradigm of a microbe in which to analyse the regulatory processes relating to the acquisition and metabolism of iron. Except in unusual circumstances, *P. aeruginosa* is obliged to respire oxygen or nitrogenous compounds, and these processes require significant amounts of iron. It is also found in a remarkable variety of environments, which furnish a variety of exigencies, both in terms of how to obtain iron from its surroundings and how to deal with potentially toxic levels once it starts to increase its intake of iron. *P. aeruginosa* can be isolated from moist soils (e.g. riparian soil) throughout the world, it can be pathogenic for plants (Rahme *et al*., 1995; 1997) or can even provide them with protection (De Meyer *et al*., 1999); it can be found tenaciously associated with state-of-the-art medical devices (e.g. endoscopes; Struelens *et al*., 1993); and it can survive for decades in the lungs of humans with very specific genetic maladies (e.g. cystic fibrosis). Consequently, in comparison with many frank pathogens that need to acquire iron only from the limited ecological niches to which they are restricted, i.e. a human host, *P. aeruginosa* has to use an assortment of strategies for accessing iron and for regulating its intake.

The effort to dissect the molecular biology and genetics of iron-regulated gene expression in *P. aeruginosa* only began in earnest several years after the observation that the production of an extracellular toxin, exotoxin A (ETA), which is produced by nearly all strains of *P. aeruginosa* (Vasil *et al*., 1986), is negatively influenced by iron. As ETA had previously been found to inhibit protein synthesis in eukaryotic cells by the same enzymatic mechanism as diphtheria toxin (DT) (Iglewski and Kabat, 1975; Iglewski *et al*., 1977), it was reasonable to question whether production of ETA was controlled by iron-like DT production (Qiu *et al*., 1995; White *et al*., 1998). Bjorn *et al*. (1978) reported that relatively low levels of iron, which had little effect on the growth rate of *P. aeruginosa*, caused a significant increase in the amount of ETA detected in culture supernatant compared with cells growing in more iron-replete conditions. Subsequently, they extended their initial observation by demonstrating that production of an extracellular haemagglutinin and an extracellular proteolytic activity are co-ordinately regulated by iron (Bjorn *et al*., 1979). At about the same time, the two siderophores of *P. aeruginosa*, known as pyoverdine and pyochelin, were extensively characterized at the biochemical level and, predictably, biosynthesis of these iron-chelating compounds by *P. aeruginosa* was likewise found to be negatively regulated by iron (Cox, 1980; Cox *et al*., 1981; Cox and Adams, 1985). Several studies illustrated the potential importance of pyoverdine, pyochelin and iron acquisition during infection (Sokol and Woods, 1984; Ankenbauer *et al*., 1985; Cox, 1985; Sokol, 1987), but it was not until 1993 that a genetic element was identified that co-ordinately regulates production of both ETA and siderophores (Prince *et al*., 1993). This regulatory gene encodes a homologue of the ferric uptake regulator (Fur) that had previously been described and characterized in the greatest detail in *E. coli* (Hantke, 1984; Neilands, 1990). Until then, the molecular and genetic analysis of iron-regulated responses in *P. aeruginosa* largely proceeded along two parallel paths, one relating ETA expression and the other concerned with the biosynthesis of siderophores. However, since Fur was identified in *P. aeruginosa*, it has become increasingly apparent that the regulatory processes affecting the production of both virulence determinants are highly integrated, and that Fur is central to understanding the role that ETA and siderophores play in the biology of iron metabolism in *P. aeruginosa*.

Genetics and biochemistry of *Pseudomonas aeruginosa* Fur

In the most basic model of the iron regulatory mechanism, Fur is a classical prokaryotic aporepressor that requires iron (corepressor) in order to bind to a target sequence ('Fur box') in the promoter region of iron-regulated genes and block their transcription when the level of intracellular iron (Fe^{2+}) reaches a certain threshold (Neilands, 1990). Although it is likely that many aspects of this model are true, this model omits many other important truths about the role of Fur in prokaryotic biology and about genus- or species-specific properties and functions of Fur.

The presence of Fur in pseudomonads was first identified by Prince *et al*. (1991) in a search for novel genes that might regulate expression of ETA. Multiple copies of a plasmid carrying the *E. coli fur* gene repressed *toxA* transcription even under iron-limiting conditions, and *P. aeruginosa* produced a protein that specifically cross-reacts with antibody made against *E. coli* Fur (Prince *et al*., 1991). In a subsequent report, Prince *et al*. (1993) complemented an *E. coli fur* null mutant with a *P. aeruginosa* DNA fragment. Sequencing of the complementing gene revealed that *P. aeruginosa* Fur is 53% identical and >70% similar to *E. coli* Fur. Perhaps the most surprising finding was that *fur* is an essential gene in *P. aeruginosa*, in contrast to its dispensable nature in other organisms including *E. coli*, several *Vibrio* spp. and *Yersinia pestis*

(Litwin and Calderwood, 1993; 1994; Staggs *et al*., 1994; Touati *et al*., 1995). Subsequent to the report by Prince *et al*. (1993), Fur was also found to be essential for *P. putida* and *Neisseria gonorrhoeae* (Berish *et al*., 1993; Venturi *et al*., 1995). Perhaps it is not coincidental that both of these organisms, like *P. aeruginosa*, are obligate respirers. Interestingly, with regard to species where *fur* is a non-essential gene, Touati *et al*. (1995) discovered that Fur is conditionally essential in *E. coli*. A double *fur* and *recA* mutant of *E. coli* could not be grown aerobically. These investigators suggested that a cell lacking the RecA protein could not repair DNA damage, resulting from the generation of reactive oxygen intermediates caused by the inability to control its intake of iron. A third mutation in *tonB* that ostensibly blocked the uncontrolled uptake of iron and oxidative damage under aerobic conditions in the *fur*$^{-}$ *rec*$^{-}$ mutant restored the ability of the triple mutant to grow aerobically. An additional layer of complexity was added to this question when investigators recently reported that the level of iron in a Fur mutant of *E. coli* was actually 2.5× lower than in a wild-type cell (Abdul-Tehrani *et al*., 1999). We also reported a seemingly paradoxical decrease in the uptake of iron charged siderophores in a *P. aeruginosa* mutant carrying a missense mutation in the *fur* gene (Hassett *et al*., 1996). These, and an ever-increasing number of reports, suggest that Fur plays a more global role in bacterial physiology than simply turning off genes involved in the acquisition of iron, and that this role may vary from species to species.

Another highly noteworthy difference between *P. aeruginosa* Fur (PA-Fur) and more than 20 other Fur proteins so far identified, is that PA-Fur lacks a highly conserved Gly-X-Cys-(2–5)X-Cys motif that is present in the C-termini of almost all the other Fur proteins, except for Fur from other *Pseudomonas* spp. or the closely related species *Rhizobium leguminosarum* (Fig. 1). There is also another motif with two Cys residues [His-(2X hydrophobic)-Cys-(2X)-Cys] that is present in the Fur proteins from the organisms that contain the Gly-X-Cys-(2–5)X-Cys motif, but which is absent in those lacking this motif. At the present time, it is not clear why *P. aeruginosa* and these other species lack these motifs. The Cys residues in these motifs had initially been suggested to play a role in the metal-binding properties of Fur (Saito *et al*., 1991). In this regard, we reported that there are differences in the DNA footprint patterns of PA-Fur and EC-Fur on identical promoters in the presence of zinc versus manganese (Ochsner *et al*., 1995). X-ray absorption spectroscopy analysis of EC-Fur recently revealed that it contains a separate binding site for zinc, in addition to its iron-binding site in the C-terminal domain (Jacquamet *et al*., 1998). That is, the aporepressor form of EC-Fur is already bound to zinc. Whether the difference between the binding of these proteins to their DNA target in the presence of zinc is related to the presence or absence of this C-terminal Cys motif is not clear at the present time. It is tempting to speculate that such a C-terminal Cys motif could also affect the redox status of those Fur proteins in which it is present. The presence or absence of this motif might account for some of the differences we have observed in the behaviour of PA-Fur and EC-Fur in the presence or absence of zinc, and it could ultimately impact on the response of *P. aeruginosa* and *E.*

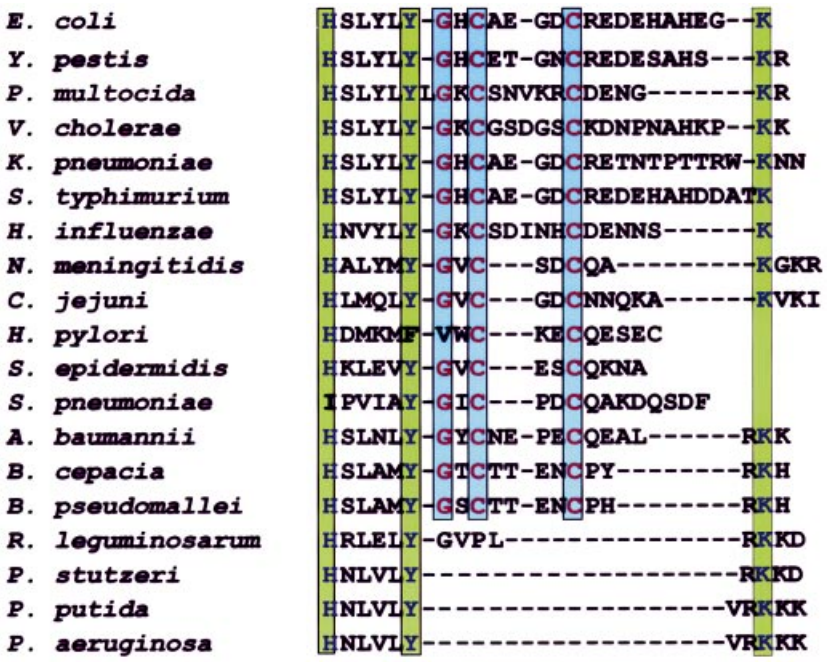

Fig. 1. The C terminus of Fur illustrating the Gly-X-Cys-(2–5)X-Cys motif absent in *P. aeruginosa* and related species.

coli to environmental levels of either zinc, iron or both metals.

The gene structure of *P. aeruginosa fur* is intricate and differs in various aspects from *E. coli fur*, which has been reported to be autoregulated and influenced by the catabolite repressor protein (De Lorenzo *et al*., 1988). How these regulatory effects on *fur* expression affect the biology of iron acquisition in *E. coli* is not understood. In *P. aeruginosa*, autoregulation has not been observed. The *fur* gene promoter does not contain a sequence similar to a consensus iron box, nor does the purified Fur bind to the *fur* promoter. An effect of other regulatory proteins *in trans* on *fur* expression has not been reported, but the *fur* gene in *P. aeruginosa* does have tandem activator elements in its promoter.

Two independent promoters influence *P. aeruginosa fur* expression, and the regulatory region of *fur* extensively overlaps with a divergently expressed gene (*omlA*) encoding an outer membrane lipoprotein (Ochsner *et al*., 1999a). In fact, the distal (T1) *fur* promoter is located within the coding sequence of *omlA*, and the sole *omlA* transcript and the *fur* T1 transcript overlap by over 150 bp. Despite this intimate association, a functional link between OmlA and Fur could not be established. However, environmental conditions that influence the expression of *fur* in *P. aeruginosa* have not been identified. The proximal *fur* promoter (P2) has a consensus σ 70-type sequence, whereas the mapped distal promoter (P1) does not match any known promoter consensus sequences and these may require auxiliary factors for optimal activity. Supporting this hypothesis is the recent identification of two tandem activator elements roughly 100 bp and 200 bp upstream of *fur*-P1, the presence of which increased P1 activity twofold and fourfold respectively (Ochsner *et al*., 1999a). An insertion between the distal promoter and the proximal *fur* promoter causes a twofold to fourfold decrease in the level of Fur in *P. aeruginosa* and leads to a mutant Fur phenotype. That is, iron-regulated genes are derepressed (e.g. *toxA*), and the mutant becomes sensitive to reactive oxygen intermediates. It is estimated that there are at least between 10 000 and 40 000 copies of Fur in *P. aeruginosa*. Although it is possible that a decrease in Fur to 5000 copies would severely alter the ability of Fur to regulate its estimated 50 direct targets, it seems more likely that there are additional functions besides direct gene repression that Fur fulfills in *P. aeruginosa* as well in other organisms. Alternatively, it is possible that Fur is compartmentalized in *P. aeruginosa* or other organisms, however, this issue has not been examined.

Even though Fur is essential to *P. aeruginosa*, it has been possible to isolate and characterize Fur mutants of *P. aeruginosa*. Hantke (1984) originally reported that manganese-resistant mutants of *E. coli* frequently carry a mutation that could be complemented by the *fur* gene. This method was applied to *P. aeruginosa* to isolate several distinct classes of *fur* mutants that were more extensively characterized (Barton *et al*., 1996; Ochsner *et al*., 1999a). One class of mutants (H86R or H86Y) with changes in the putative highly conserved iron-binding domain of Fur, constitutively expressed siderophores, but the pattern of iron-regulated expression of *toxA* was unaltered. Another mutant class (A10G), with a change in or near the proposed DNA-binding domain of Fur, was hyperconstitutive for siderophore production, but was conditionally constitutive for iron-regulated expression of *toxA*. Purified A10G mutant Fur also does not bind to a Fur-box *in vitro*, even at high concentrations. Expression of *toxA* in this mutant was iron regulated under aerobic conditions (20% O_2), but was constitutive under microaerobic conditions (8–10% O_2). The A10G mutation could have altered the conformation of Fur under aerobic versus microaerobic growth and thereby caused this unusual phenotype. This scenario is not likely because an identical phenotype was found in another mutant that has a nucleotide change in the noncoding region of *fur*. This mutation causes a decrease in the level of Fur by four to fivefold, but of course it would not alter the structure of Fur. These data strongly suggest that the DNA-binding function of Fur cannot be solely responsible for the fact that *fur* is an essential gene in *P. aeruginosa*. Additional effects of these mutations have been described. Hassett *et al*. (1996) unexpectedly found that the A10G mutant, in particular, was defective in the ferripyoverdine- and ferripyochelin-mediated uptake of iron. Furthermore, the Fur mutants exhibited a significant lag in growth under aerobic conditions, but not under microaerobic conditions. These data emphasize the elaborate scope of the biology of Fur in *P. aeruginosa* and raise additional interesting questions about its molecular architecture and function.

Regulation of ETA production as a paradigm of a Fur-controlled system

Molecular and genetic studies on the iron-regulated expression of ETA began with the cloning of the gene (*toxA*) encoding this toxin and with the identification of a positive regulatory gene (*regA* formerly *toxR*) required for the optimal expression of ETA (Hedstrom *et al*., 1986; Frank *et al*., 1989; Storey *et al*., 1990; 1991; Wick *et al*., 1990). Transcription of *toxA* was clearly demonstrated to be negatively influenced by iron, which did not affect the stability of *toxA* transcripts *per se*, rather it appeared to control transcription initiation of *toxA* (Lory, 1986). A single major transcriptional start site in the *toxA* gene was detected in cells grown under iron-limiting conditions, but transcripts were absent from cells grown under iron-replete conditions. Examination of the promoter region of the *toxA* gene revealed the absence of any consensus -10 or -35 RNA

polymerase binding sites. These data suggested that expression of *toxA* requires one or more accessory factors for optimal expression. Further supporting this hypothesis was an earlier finding that *toxA* could not be expressed from its own promoter in *E. coli*. Hedstrom *et al*. (1986) reported the cloning of a gene, *toxR*, later named *regA*, that complemented a chemically mutagenized, toxin-deficient strain of *P. aeruginosa*. This gene had a positive influence on the expression of ETA production, but multiple copies of this gene did not alter the pattern of iron-regulated ETA expression. Transcription of the *regA* is also negatively regulated by iron, and involves two promoters and growth phase-regulated expression of both promoters (Storey *et al*., 1990). Early on, it was thought that *regA* was part of a two-gene operon that also encoded a gene called *regB* (Wick *et al*., 1990; Storey *et al*., 1991). Subsequently, the existence of *regB* as a gene was questioned because: (i) although the mRNA initiated from the P1 promoter does continue through the region assigned to *regB*, this putative gene has extremely poor codon usage for *P. aeruginosa*; *(*ii) a constitutively expressed transcript encoding a protein highly homologous to chloramphenicol acetyl transferase starts at the proposed translational initiation site for *regB*; and (iii) *P. aeruginosa* strain PA01 lacks a translational initiation codon for *regB*. Perhaps the region called *regB* does not actually encode a protein, but instead influences the stability of the *regA* message.

RegA also appears to have a very limited role in the regulation of other *P. aeruginosa* genes besides *toxA*. Using two-dimensional gel electrophoresis, Wolz *et al*. (1994b) found only a single 45 kDa protein that was present in wild-type culture supernatants and not present in supernatants of a *regA* insertion mutant. Using this method, no differences were detected between the wild-type and the mutant whole cells, despite the fact that there were at least 69 proteins in these gels whose expression was dependent on iron limitation.

As both *toxA* and *regA* are negatively influenced by iron at the transcriptional level, the search for a new regulatory gene that could control both genes or mediate the iron-regulated expression of *toxA* through regulating the expression of *regA* was initiated. A candidate regulatory element was identified when Prince *et al*. found that multiple copies of *fur* from *E. coli* repressed transcription of *toxA* and *regA* under iron-limiting conditions, and that *P. aeruginosa* produces a protein highly homologous to Fur from *E. coli* (Prince *et al*., 1991). These data suggested that a Fur-like protein in *P. aeruginosa* might repress *toxA* and *regA* and provided a testable model. That is, ETA expression should be constitutive in a Fur mutant and a purified Fur would be expected to bind to the promoter of *toxA* or *regA*, if it directly regulated these genes. As mentioned above, constitutive expression of ETA in Fur mutants was conditional. It was dependent on microaerobic conditions and on the kind of Fur mutant examined. Moreover, purified active PA-Fur failed to bind to either the *toxA* or *regA* promoter even at high Fur concentrations (Ochsner *et al*., 1995; Barton *et al*., 1996). Data available at that time clearly indicated that one or more additional regulatory genes were situated between *fur*, *regA* and *toxA* and that Fur did not directly regulate these genes.

Because Fur co-ordinately regulates the production of ETA and the biosynthesis of siderophores (Prince *et al*., 1993; Barton *et al*., 1996), the newly identified siderophore regulatory genes were strong candidates for a *toxA* regulatory gene. The *pchR* gene product belongs to the AraC class of regulators and controls the production of the siderophore pyochelin (Heinrichs and Poole, 1993; 1996). Another candidate, *pvdS*, encodes a protein similar to alternative sigma factors belonging to the ECF class (extracytoplasmic factor) of regulatory proteins(Venturi *et al*., 1995). It regulates synthesis of the yellow fluorescent siderophore, pyoverdine (Cunliffe *et al*., 1995; Miyazaki *et al*., 1995; Leoni *et al*., 1996; Stintzi *et al*., 1999). The *pvdS* gene fulfilled all the necessary criteria for a Fur-dependent regulatory factor that could mediate iron control of both *regA* and *toxA* expression. On the other hand, although Fur binds to the *pchR* promoter with high affinity, deletion of this gene in *P. aeruginosa* had no effect on ETA production, thereby eliminating this regulator as a candidate for mediating the co-ordinate control of siderophore and ETA production.

In the model shown in Fig. 2, we propose that *pvdS* regulates the expression of *regA* and another regulatory gene designated *ptxR*. Both *regA* and *ptxR* are required for the optimal expression of *toxA*. The *ptxR* gene product belongs to the LysR class of prokaryotic regulatory proteins (Hamood *et al*., 1996; Vasil *et al*., 1998) and it positively influences the expression of *toxA*, although it is not essential for *toxA* expression. We recently reported that Fur does not directly regulate the expression of *ptxR*, but it does so through *pvdS*. Preliminary data from our laboratory suggest that PtxR activates transcription of *regA* as well as influencing the production of siderophores by activating transcription of an operon involved in the synthesis of the chromophore of pyoverdine (Stintzi *et al*., 1999). Adjacent to the *pvdS* gene are two genes we have designated *pvdY* and *pvdX*, which may also play a role in the increasingly complex regulatory circuit controlling expression of *toxA* and the synthesis of pyoverdine. Transcription of the *pvdY* gene is dependent on iron limitation, and this iron control is mediated through PvdS (M. L. Vasil, Z. Johnson and U. A. Ochsner, unpublished observations). On the other hand, *pvdX* expression is positively regulated by iron and is repressed by PvdS. PA-Fur does not bind to the promoter regions of either *pvdY* or *pvdX*. PvdY and PvdX lack significant homology to any proteins of known function. It is possible that PvdY is an accessory transcription factor that post-translationally enhances the activity of

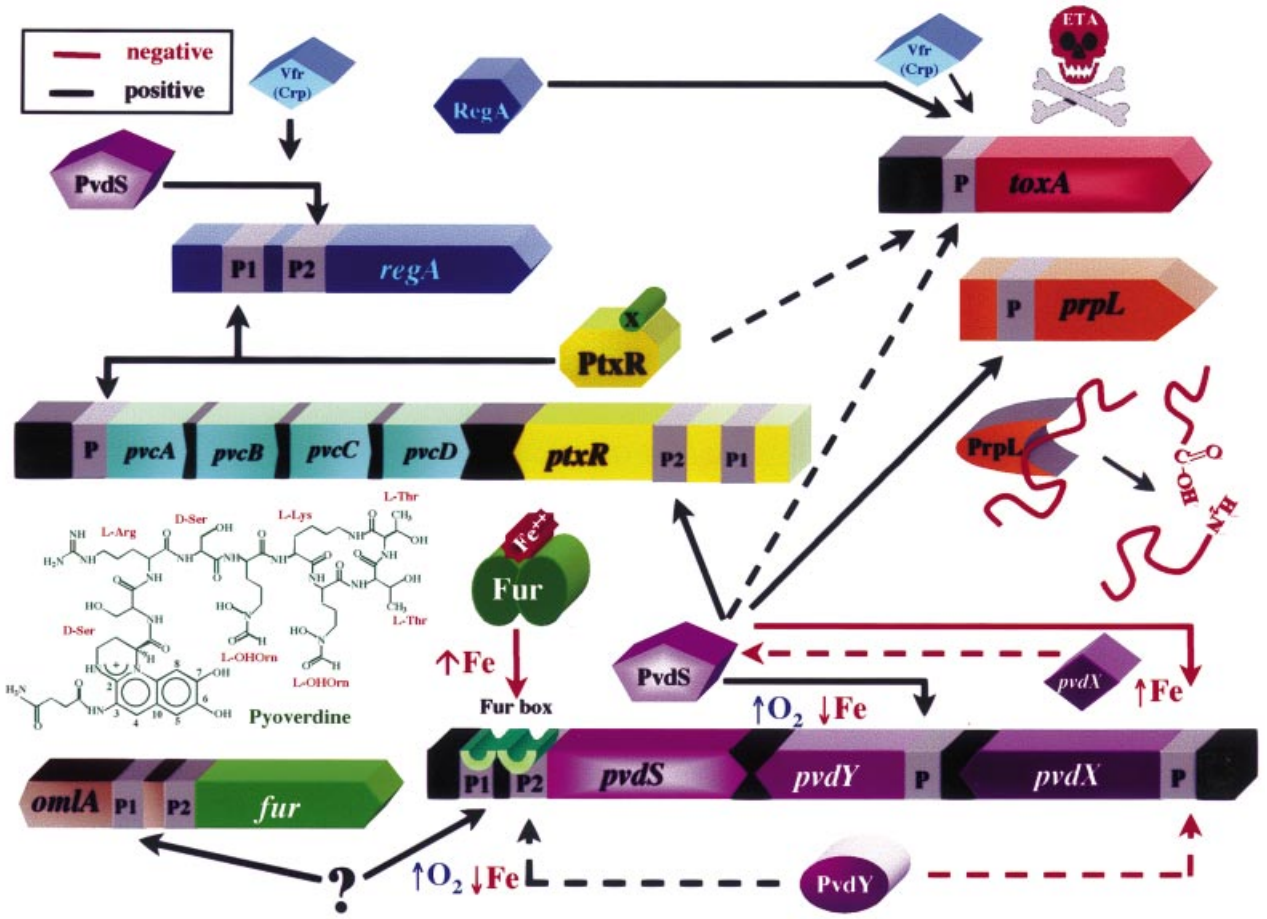

Fig. 2. Working model of a Fur-dependent iron regulatory circuit in *Pseudomonas aeruginosa*. Under iron-replete conditions, Fe^{++} binds to apoFur. Fe-activated Fur then specifically, and with high affinity, recognizes a sequence (Fur box) in the promoter of the *pvdS* gene, which encodes an alternative σ factor. The response of *pvdS* to environmental iron and oxygen is supported by experimental data. The different responses of *pvdY* and *pvdX* to iron have been experimentally determined. However, the proposed functions for these genes is entirely speculative in nature at the present time. The 'X' figure associated with PtxR is a hypothetical small activator molecule necessary for the DNA-binding specificity of LysR-type regulators. Solid lines with arrows shown in this model indicate that there are experimental data supporting the proposed positive or negative effect, whereas broken lines illustrate hypothetical effects, which have no supporting data at the present time. ETA, exotoxin A.

PvdS, but this is entirely speculative at this time. In contrast, as PvdX is produced only under iron-replete conditions, it could be involved in inactivating any residual PvdS that might still be present when Fur has repressed further transcription of *pvdS*. Such a scenario could prevent further *pvdS*-induced uptake of iron once a potentially toxic threshold level of iron has been reached. Alternatively, a motif search has revealed that PvdX has some similarity to NusG, a transcriptional terminator. Perhaps PvdX is involved in terminating transcription of the *pvdS* gene.

Interestingly, optimal expression of *pvdS* and *pvdY* occurs under aerobic conditions and both are highly repressed under microaerobic conditions (M. L. Vasil, U. A. Ochsner and Z. Johnson, unpublished). However, the impact of this oxygen control of these genes on the expression of *toxA* and *regA* has not been studied.

It is worthwhile to note that with respect to *Pseudomonas* sp., *toxA*, *regA, ptxR* and *pvdY* (Fig. 2) are only found in *P. aeruginosa*, and homologous sequences are not detected in the species most closely related to *P. aeruginosa*, such as *P. fluorescens* and *P. putida* (Vasil *et al*., 1998). On the other hand, *pvdS* and *fur* homologues are present in other pseudomonads. Perhaps, *toxA, regA, ptxR and pvdY* represent a relatively recent acquisition by the more virulent *P. aeruginosa* species, or they provide a specific function for *P. aeruginosa* in mammalian infections that are significantly less frequently associated with other *Pseudomonas* spp.

One additional level of regulation that is imposed on the expression of *toxA* and *regA* relates to the requirement for a Crp homologue for the optimal expression of these genes. West *et al*. (1994) identified a gene, designated *vfr*, that

encodes a protein highly homologous to the catabolite repressor protein of *E. coli*. Vfr binds to the *toxA* and *regA* promoters, but it does not appear that it strictly plays a role in the iron-influenced regulation of ETA expression. The function of this protein in the physiology of *P. aeruginosa* is obscure as the levels of cAMP vary little during the growth of this organism (Siegel *et al*., 1977; Collier *et al*., 1996). There have been reports attempting to identify the influence of carbon and nitrogen sources on the production of ETA, but none have been connected to the Vfr protein or its reported effect on the expression of *regA* or *toxA* (Somerville *et al*., 1999).

Finally, a single report provided data suggesting that the *las* quorum-sensing system of *P. aeruginosa* affects the expression of ETA (Gambello *et al*., 1993). A LasR quorum-sensing mutant was reported to have only a 1.5-fold-decreased yield of ETA based on the measurement of its ADP-ribosyl transferase activity. Despite these limited data, many investigators frequently state that the LasR-regulated quorum-sensing system regulates the expression of ETA, without considering the possible complex pleiotrophic effects of the quorum-sensing system on *P. aeruginosa*. Despite careful quantitative measurements of ETA protein and *toxA* transcripts in defined *lasR* and *rhlR* deletion mutants and in *lasR,rhlR* double mutants, we have been unable to demonstrate any effect of the quorum-sensing system on the expression of ETA. It is possible that quorum sensing in some very limited, indirect way affects the expression of ETA. A recent report indicated that the quorum-sensing systems of *P. aeruginosa* affect the expression of the TypeII (Xcp) secretion system that would be used by ETA (Chapon-Hervé *et al*., 1997).

Other Fur-regulated genes of *P. aeruginosa*

Several *P. aeruginosa* genes involved in iron acquisition have been isolated by studying mutants that exhibited growth defects under iron-limited conditions. Potential 'Fur-boxes' in the promoter regions of these genes were reported, however, the direct binding of Fur was demonstrated only after purified Fur protein from *P. aeruginosa* was available (Ochsner *et al*., 1995). Roughly 30 additional *Pseudomonas* iron-regulated genes ('*pigs*') were isolated in a cycle selection procedure based on the affinity of purified Fur protein for DNA sequences containing a Fur binding site *in vitro* (Ochsner and Vasil, 1996). The corresponding genes were subsequently isolated, the direct binding of Fur in their promoter regions was demonstrated by DNase I footprinting, and their expression was shown to be derepressed under iron-deplete conditions by RNase protection (Ochsner and Vasil, 1996). In this report, we also provided a compilation of Fur-box elements for most of the known *P. aeruginosa* Fur-regulated genes. The data suggested that the consensus Fur-box for *P. aeruginosa* is basically identical to one compiled for *E. coli*. More recently, however, Escolar *et al*. (1998) experimentally addressed the composition of a consensus Fur-box for *E. coli* Fur. Using natural or synthetic DNA targets, Dnase I footprinting and missing-T assays, they found that the Fur-box for Fur from *E. coli* is composed of a minimum of three repeats of the hexameric motif GATAAT, rather than a palindromic 19 bp target sequence. Further experimental evidence is required to know whether this is true for the Fur-box motif of *P. aeruginosa*.

All *P. aeruginosa* genes for which experimental evidence of direct Fur control has been obtained are listed in Table 1. With a few exceptions, the Fur-regulated genes have been mapped using pulse-field gel electrophoresis followed by Southern blotting, and it appears that they are localized randomly rather than clustered over the chromosome (Ochsner et al., 1995).

A large group of Fur-regulated genes are involved in siderophore-mediated iron acquisition and encode ferrisiderophore receptors, siderophore biosynthetic enzymes and regulatory proteins. Although *P. aeruginosa* PAO1 may produce as many as eight different outer membrane receptors for ferrisiderophores, some of these may be used by different siderophores that have very similar but non-identical structures. They include *fpvA* (Poole *et al*., 1993) and *fptA* (Ankenbauer and Quan, 1994) encoding the receptors for the two endogenous siderophores, ferripyoverdine and ferripyochelin. The other receptors appear to be specific for ferrisiderophores from heterologous sources, which have been found to promote growth of *P. aeruginosa* under iron-restricted conditions (Meyer, 1992). Ferrienterobactin utilization requires the PfeA receptor, which is induced in the presence of enterobactin by the action of the PfeR–PfeS two-component regulatory system encoded immediately upstream of *pfeA* (Dean and Poole, 1993a, b; Dean et al., 1996). A second, lower-affinity ferrienterobactin uptake system has been postulated to exist in *P. aeruginosa*, and may involve the PirA receptor together with the PirR–PirS regulatory system, which are highly identical to PfeA and PfeR–PfeS. Ferrioxamine B uptake depends on the FiuA receptor and on the regulators FiuR and FiuS, which are responsible for the induction of FiuA in the presence of this compound (U. A. Ochsner, unpublished). The specificities of the additional receptors PiuA, PfuA and UfrA have not yet been determined.

Siderophore production is strictly iron regulated, however, only the pyochelin biosynthetic genes are under direct control of Fur, while pyoverdine synthesis is indirectly iron regulated through the Fur-controlled alternative sigma factor PvdS (see below). The phenolate-type siderophore, pyochelin, is synthesized from one molecule of salicylate and two molecules of cysteine. The *pchDCAB* operon provides enzymes for the synthesis (PchBA) and activation (PchD) of salicylate as well as a putative thioesterase

Table 1. Fur-regulated genes in *P. aeruginosa*.

Class	Gene or operon	Function	Source or reference (accession number)
Ferri-siderophore receptors	*fpvA*	Ferri-pyoverdine receptor	Poole *et al*. (1993) (U07359)
	fptA	Ferri-pyochelin receptor	Ankenbauer and Quan (1994) (U03161)
	pfeA	Ferri-enterobactin receptor	Dean and Poole (1993) (M98033)
	piuA (pig12–ORF1)	Unknown	Ochsner and Vasil (1996) (AF051690)
	fiuA (pig17A)	Ferrioxamine receptor	Ochsner and Vasil (1996) (AF51691)
	pirA (pig19A)	Unknown	Ochsner and Vasil (1996) (AF051692)
	pfuA (pig31)	Unknown	Ochsner and Vasil (1996) (AF051693)
	ufrA	Unknown	K. E. Poole (unpublished) (U33150)
Siderophore biosynthesis	*pchABCD*	Pyochelin biosynthesis	Serino *et al*. (1997) (X82644)
	pchEF	Pyochelin biosynthesis	Reimmann *et al*. (1998) (AF074705)
Haem uptake	*phuR (pig20)*	Haem/haemoglobin receptor	Ochsner *et al*. (1999a) (AF055999)
	phuSTUVW	ABC transporter for haem uptake	Ochsner *et al*. (1999a) (AF055999)
	hasRADEF	Haem receptor, haemophore, ABC transporter for HasA export	Ochsner *et al*. (1999a) (AF127223)
Iron acquisition (other systems)	*feoAB*	Fe(II) uptake	This work
	iutAB-1	Iron utilization proteins	This work
	iutAB-2	Iron utilization proteins	This work
Alternative sigma factors (ECF)	*pvdS*	Regulator of pyoverdine and exotoxin A synthesis	Cunliffe *et al*. (1995); Ochsner *et al*. (1995)
	pigDE (pig4DE)	Unknown	Ochsner and Vasil (1996)
	fiuIR (pig17IR)	Regulation of ferrioxamine receptor gene *fiuA*	Ochsner and Vasil (1996) (AF051691)
	pig25	Unknown	Ochsner and Vasil (1996)
	pig32	Pseudogene upstream of *pvdA*	Ochsner and Vasil (1996)
Two-component regulatory system	*pfeRS*	Regulation of ferri-enterochelin receptor gene *pfeA*	Dean *et al*. (1996); Dean and Poole (1993) (L07739)
	pirRS (pig19RS)	Regulation of receptor gene *pirA*	Ochsner and Vasil (1996) (AF056192)
Other regulators	*pchR*	Regulation of *pchABCD* and *pchEF* pyochelin biosynthetic genes	Heinrichs and Poole (1993) (L11657)
	pigF (pig4F)	Negative regulator of cell division (putative)	Ochsner *et al*. (1996)
Metabolic and detoxifying enzymes	*fumC–sodA*	Alternative fumarase, Mn-cofactored superoxide dismutase	Hassett *et al*. (1997) (U59458)
	nuoA	Oxidoreductase (putative)	Ochsner and Vasil (1996)
Miscellaneous	*tonB (pig13)*	Energy-dependent transporter	Ochsner and Vasil (1996); Poole *et al*. (1996) (U23764)
	tolQRA (pig6)	Pyocin killing, colicin tolerance	Dennis *et al*. (1996); Ochsner and Vasil (1996) (U39558)
	pigAC (pig4AC)	Unknown	Ochsner and Vasil (1996)
	piuC (pig7)	Unknown	Ochsner and Vasil (1996) (AF051690)
	piuB (pig12-ORF2)	Unknown	Ochsner and Vasil (1996)
	pig23	Unknown	Ochsner and Vasil (1996) (AF051690)

(PchC) (Serino *et al*., 1997). The *pchEF* operon encodes dihydroaeruginoic acid synthetase and pyochelin synthetase, which catalyse the conversion of salicylate to dihydroaeruginoate and the subsequent formation of pyochelin from that intermediate (Reimmann *et al*., 1998). Although both the *pchDCAB* and *pchEF* operons are Fur regulated, they are also positively affected by the AraC-like regulator PchR, which is under the control of Fur itself (Heinrichs and Poole, 1993). PchR has been demonstrated to function as both an activator and a repressor, depending on the presence or absence of pyochelin, in controlling the expression of *fptA* and *pchR* (Heinrichs and Poole, 1996).

Living in variable environmental niches, *P. aeruginosa* has evolved alternative iron uptake systems besides those involving siderophores. Two distinct haem acquisition systems have recently been described. The first system, *phu*, is closely related to the haem uptake systems of yersiniae. The *phu* locus contains the *phuR* haem receptor gene and the *phuSTUVW* operon encoding a typical periplasmic binding-protein-dependent ABC transport system

(Ochsner *et al*., 1999b). The *phuR* gene and the *phuS-TUVW* operon are transcribed from divergent promoters that are co-regulated by Fur binding to the intergenic region. The second system, *has*, contains five genes in a Fur-regulated operon and is structurally related to the haem uptake system of *Serratia marcescens*. The *hasR* gene encodes an outer membrane receptor and *hasA* encodes an extracellular haem-binding protein, a so-called haemophore, which appears to be exported by a type I secretion apparatus encoded by the *hasDEF* gene products (Letoffe *et al*., 1998; Ochsner *et al*., 1999b). Mutants affected in either the *phu* or *has* locus exhibit severely reduced growth with haem or haemoglobin as the sole iron source (Ochsner *et al*., 1999b).

Two additional iron utilization loci, *iutAB-1 and iutAB-2*, encode factors highly homologous to the HitA and HitB proteins of *Haemophilus influenzae*, which are required for iron acquisition from various iron chelates (Sanders *et al*., 1994). The *iutAB-1 and iutAB-2* operons are similar to each other and are directly regulated by Fur. However, the precise role of these systems has not yet been elucidated.

Most uptake systems, including those for ferrisiderophores and haem, depend on the TonB protein, which functions as an energy transducer in coupling the energized state of the cytoplasmic membrane to outer membrane receptor function. The *tonB* gene was isolated by complementation of a *P. putida tonB* mutant that was unable to grow on iron-deficient minimal medium (Poole *et al*., 1996) and is strongly regulated by Fur (Ochsner and Vasil, 1996). Interestingly, the *tonB* gene could be deleted in the pyoverdine-deficient strain PAO6609, but several attempts to create a *tonB* mutant of PAO1 failed (Poole *et al*., 1996; U. A. Ochsner and M. L. Vasil, unpublished).

The solubility of iron salts at neutral pH is extremely low under aerobic conditons, but increases under microaerobic or anaerobic conditions, and iron is more soluble in the ferrous state. It is thus not surprising that *P. aeruginosa* contains a ferrous iron uptake system, *feoAB*, which is under the control of Fur and Anr (U. A. Ochsner, unpublished). The well-studied *feoAB* operon in *E. coli* encodes the small protein FeoA and the cytoplasmic membrane protein FeoB, which harbours an ATPase motif, suggesting that ferrous iron uptake may be ATP driven (Kammler *et al*., 1993).

A number of Fur targets appear to link iron starvation directly to even more basic physiological processes than the process of iron acquisition. The *fumC–sodA* operon encodes alternative enzymes for the optimal maintenance of crucial cellular processes under iron-limiting growth conditions (Hassett *et al*., 1997). In analogy to the studies in *E. coli*, the FumC gene, fumarase, does not require iron for activity, in contrast to the *fumA* and *fumB* gene products (Park and Gunsalus, 1995). Similarly, the SodA superoxide dismutase functions with Mn(II) as cofactor and replaces the Fe(II)-cofactored SodB for oxidative stress defence if iron becomes limited. The gene products in the *pig14* operon exhibit a high degree of identity to the components of the *E. coli* proton-translocating NADH:ubiquinone oxidoreductase (Weidner *et al*., 1993). The *pig4* region contains the *pig4F* gene and the divergently transcribed *pig4ACDE* operon, which are co-regulated by Fur owing to two closely spaced tandem Fur-boxes in the intergenic region. The Pig4F gene product is highly identical to the SulA protein of *S. marcescens*, which is an inhibitor of cell division that belongs to the SOS system (Freudl *et al*., 1987), suggesting that *P. aeruginosa* may be capable of actively halting cell division upon encountering severe iron restriction. The *pig6* region has been characterized as the *orf1-tolQRA* operon, which is involved in pyocin AR41 killing and appears to be essential for *P. aeruginosa* (Dennis *et al*., 1996). The Tol proteins of *E. coli* are involved in transport of colicins and phages across the cell envelope (Sun and Webster, 1987). Interestingly, *P. aeruginosa* Tol proteins were functionally unable to complement *E. coli tol* mutants, although *P. aeruginosa* TolQ was able to complement the iron-limited growth of an *E. coli exbB* mutant (Dennis *et al*., 1996).

Besides the Fur-regulated genes listed in Table 1, many additional genes are regulated by iron indirectly through Fur-controlled regulators. Iron regulation of the *toxA* and *regA* genes, required for exotoxin A production, is mediated by the Fur-controlled alternative sigma factor PvdS, as described above. PvdS also controls the expression of several genes involved in pyoverdine production (Cunliffe *et al*., 1995; Miyazaki *et al*., 1995; Venturi *et al*., 1995; Leoni *et al*., 1996) and a gene encoding an extracellular protease, *prpL* (Fig. 2). The hydroxamate-type siderophore pyoverdine is a water-soluble, yellow-green fluorescent compound that consists of a highly conserved dihydroxyquinoline chromophore linked to the amino terminus of a peptide arm of varying structures in different *Pseudomonas* strains (Briskot *et al*., 1986; Cornelis *et al*., 1989). The peptidic moiety of different pyoverdines is crucial in the recognition process between siderophores and their receptors. The partly cyclic octapeptide of pyoverdine from *P. aeruginosa* PAO1 contains two molecules of L-N^5-hydroxy ornithine, which is formed from ornithine by the *pvdA* gene product, L-ornithine N^5-oxygenase (Visca *et al*., 1994). An updated corrected structure (shown in Fig. 2) was derived from mass spectrometry and two-dimensional nuclear magnetic resonance (Briskot *et al*., 1989; Demange *et al*., 1990). The pyoverdine peptide is synthesized by a non-ribosomal mechanism that involves the homodimeric 273 kDa PvdD peptide synthetase encoded by the *pvdD* gene (Merriman *et al*., 1995). Also required for pyoverdine synthesis are *pvdE* encoding an ABC transporter component and *pvdF* (Rombel *et al*., 1995; McMorran *et al*., 1996). The formation of the pyoverdine chromophore involves several enzymes

encoded in the *pvcABCD* gene cluster, and *pvcABCD* expression requires the *ptxR* gene encoding a LysR-type transcriptional activator (Stintzi *et al*., 1999).

The *lipA* gene encoding an extracellular lipase is also indirectly iron regulated, but the specific *lipA* regulator is not known (Wohlfarth *et al*., 1992). Additional candidate regulators encoded by Fur-controlled genes have been identified and include alternative sigma factors homologous to PvdS as well as two-component regulatory systems (Table 1).

Genes expressed under iron-replete conditions

Potential virulence determinants produced under iron-limiting conditions, such as in the milieu of a host, have clearly been a focus of research in microbial pathogenesis. However, the expression of a growing number of *P. aeruginosa* genes has been found to be upregulated under high-iron conditions. The molecular genetic basis for this response to high-iron concentrations is largely unknown, and may involve iron-responsive regulators and/or iron-responsive RNA secondary structure elements that impose transcriptional or translational effects. The proteins that are produced at higher levels during the growth of *P. aeruginosa* under iron-replete conditions are listed in Table 2. Typically, these factors contain iron and play roles in iron storage and oxidative stress defence. The response to high-iron conditions appears appropriate as a mechanism to avoid the uncontrolled generation of deleterious reactive hydroxyl radicals in the presence of high concentrations of transition metals, such as iron, owing to increased levels of Fenton-type reactions (Miller and Britigan, 1997). *P. aeruginosa* possesses two bacterioferritin genes, *bfrA* and *bfrB*, which encode the a and b subunits that are present in variable proportions in the bacterioferritin 24mer (Moore *et al*., 1994; Ma *et al*., 1999). Expression of *bfrA* is constitutive during the exponential growth phase and becomes iron regulated upon transition into the stationary phase (Ma *et al*., 1999), whereas *bfrB* is strongly upregulated under high-iron conditions during all growth phases (U. A. Ochsner, Z. Johnson and M. L. Vasil, unpublished). At least three catalases are present in *P. aeruginosa*, among which KatA appears to contribute the bulk of the total cytoplasmic catalase activity. The KatA enzyme requires haem iron as a cofactor, and depends on *bfrA* for optimal activity. Interestingly, the *katA* gene is located immediately upstream of *bfrA*, suggesting that bacterioferritin may play a role in feeding iron or haem into catalase (Ma *et al*., 1999). Similarly, the second bacterioferritin gene *bfrB*, is located adjacent to *ahpA*, which encodes a haem-containing alkylhydrogen peroxidase (U. A. Ochsner and M. L. Vasil, unpublished). The iron-cofactored SodB superoxide dismutase is produced exclusively during growth in high-iron media, whereas the expression of the manganese-cofactored SodA occurs only under low-iron conditions (Hassett *et al*., 1993; Hassett *et al*., 1995). The *pvdX* gene, which is located in a cluster together with *pvdS*/*pvdY*, and a highly similar gene, *pvdX-2*, which is located downstream of the *ahpCF* alkylhydrogen peroxidase operon, are also expressed in response to high-iron conditions. However, the roles of the *pvdX* and *pvdX-2* gene products in iron metabolism are unknown. The only other proteins that show significant homology to PvdX are present in *Mycobacterium tuberculosis* and in *Synechocystis* sp., however their function is also unknown.

Contribution of iron-regulated genes to virulence

Perhaps it is somewhat surprising that only a limited number of genes involved in the iron acquisition system of *P. aeruginosa* have yet been examined for their role in experimental infections. Nevertheless, it is clear that iron modulates the outcome of a model *P. aeruginosa* infection (Sokol and Woods, 1984; Meyer *et al*., 1996). Well-defined mutants deficient in the production of exotoxin A have been examined in a variety of animal models. However, in some cases, *toxA* was shown to contribute to virulence, but in other instances no difference between the wild type and the *toxA* mutant was observed (Hirakata *et al*., 1993). In the latter situation, it is not clear whether there was no effect of a *toxA* mutation on virulence, or whether the different methods used for assessing virulence in these

Table 2. *P. aeruginosa* genes activated under high-iron conditions.

Class	Gene or operon	Function	Source or reference (accession number)
Iron storage	*bfrA*	Bacterioferritin A	Ma *et al*. (1999) (AF047025)
	bfrB	Bacterioferritin B	Ma *et al*. (1999)
Oxidative stress defence	*katA*	Catalase	Ma *et al*. (1999) (AF047025)
	ahpA	Alkylhydrogen peroxidase	This work
	sodB	Fe(II)-cofactored superoxide dismutase	Hassett *et al*. (1993; 1995)
Others	*pvdX*	Unknown	This work
	pvdX2	Unknown	This work

models were not sensitive enough to detect a difference between the virulence of the wild type and that of the mutant. For instance, we recently reported that no differences were detected between the ability of a well-defined *toxA* deletion mutant and the ability of the wild-type parental strain to colonize heart valves or to disseminate to other organs in an experimental endocarditis model in rabbits. In this model, endocarditis is initiated on the right side or left side of the heart by inducing a thrombotic lesion on the heart valves with a sterile catheter and then introducing *P. aeruginosa* intravenously (Xiong *et al*., submitted). Once the infection is initiated, colonization of the valves is assessed and seeding of the infecting strain from the valves to the kidneys or spleen is measured. This model infection mimics human endocarditis in a number of ways, including histopathology of the valves and the different outcomes of right-sided and left-sided infections (Jackson, 1994). The predominant environmental difference between the left side and right side of the heart is the level of oxygen. The left-sided oxygen tension is $\approx$40 mmHg greater than on the right side of the heart. This difference is virtually identical to the difference between aerobic and microaerobic growth conditions mentioned above, relating to the expression of *toxA* in a Fur mutant. Although no impact of a *toxA* deletion was seen in this model, we did find that a *pvdS* deletion mutant was significantly affected in its ability to colonize the left-sided valves compared with the wild-type parental strain. No differences were observed between the mutant and the wild-type strain in their ability to colonize the right-sided valves in this model. The mutant was also significantly reduced in its ability to seed the spleen and kidneys from the heart in the left-sided model than the parental strain. Although there was a clear difference between the *pvdS* mutant and the wild-type strain in the left-sided model, as mentioned above, there was no observable difference between a *toxA* deletion mutant and the parental strain to induce endocarditis in this model. Perhaps other *pvdS* controlled genes besides *toxA* are required for full virulence in this model. Such a candidate (see Fig. 2) could be a *pvdS*-regulated gene, *prpL* (PvdS regulated endoprotease Lysyl-class), which we recently identified (P. J. Wilderman, U. A. Ochsner and M. L. Vasil, unpublished).

Concluding remarks

In the past decade, a significant amount of progress has been made relating to the mechanisms of the genetic regulation of the iron acquisition systems of *P. aeruginosa*. Much of this work was done prior to the availability of the sequence of the genome of a single *P. aeruginosa* strain, PAO1. However, the sequence of the genome of this strain has not diminished the importance of that information, rather it has enhanced it. It has also raised more interesting and worthwhile questions about the iron acquisition systems and about the biology and pathogenesis of *P. aeruginosa* in general than it has answered. Just some of the high-priority issues relating to the influence of iron on the biology of *P. aeruginosa* that need to be addressed in the coming years include: (i) a more complete understanding of how oxygen tension and the production of reactive oxygen intermediates influence the physiology of *P. aeruginosa* in iron-deficient or iron-replete environments, (ii) a better understanding of iron-trafficking mechanisms in this organism and (iii) further identification and characterization of genes that are positively influenced by iron. In addition, at the present time there is virtually no information about the iron-regulatory systems *P. aeruginosa* uses in any of its natural environments outside the laboratory. Although there is a strong sense that iron has a major influence on the outcome of infections caused by this organism, there is essentially no information about the genetic or biochemical mechanisms that impact on iron in an infected mammalian host. As many important players have now been identified and the technologies (e.g. microchip arrays and immunofluorescence microscopy) for examining these questions have improved considerably in the past few years, it should be possible to make significant progress in the near future towards understanding the mechanisms by which iron influences the outcome of a host–*P. aeruginosa* confrontation. Finally, although it has become clear that *P. aeruginosa* has an extraordinarily redundant assortment of iron acquisition and regulatory genes, it is astonishing that the Achilles' heel of this organism may actually be the essential nature of the single most important gene that regulates these processes, *fur*. Perhaps this apparently fatal flaw can be exploited to generate novel therapeutic agents against this formidable and opportunistic pathogen.

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