

How we learnt about iron acquisition in *Pseudomonas aeruginosa*: a series of very fortunate events

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Abstract The ferric uptake repressor (Fur) of *Pseudomonas aeruginosa*, and a wide assortment of other prokaryotic organisms, has been mostly regarded as a negative regulator (repressor) of genes involved in iron acquisition (e.g., expression and utilization of siderophores) or of iron-regulated genes involved in virulence (e.g., toxins). However, there is an emerging picture of an even broader role for this protein in basic bacterial biology. Evidence has now accumulated indicating that Fur acts in a positive manner as well, and that it has a considerably wider impact on gene expression than originally perceived. We discovered that in *P. aeruginosa* Fur directly (i.e., negatively) regulates the expression of two, nearly identical tandem small (<200nt) RNA transcripts (sRNA). Our initial experiments showed that these Fur-regulated sRNAs (PrrF) affected expression of certain genes we initially thought might be directly, but positively, regulated by Fur. However, with discovery of the Fur-regulated sRNAs, first in *Escherichia coli* and then in *P. aeruginosa*, it became clear that Fur, in at least some cases, exerts its positive regulatory

effect on gene expression by repressing the expression a negative regulatory factor (i.e., PrrF), which acts at the posttranscriptional level. While a clear picture was already available regarding the function of genes (see above) that are directly repressed by Fur (negative regulation), the functional classes of genes that are influenced by Fur-repressed sRNAs (positive regulation) had not been identified for *P. aeruginosa*. Accordingly we established a set of rigorous criteria, based on microarray experimental data, to identify the cohort of genes that are likely to be directly influenced by Fur-regulated PrrFs. More than 60 genes that fulfilled these strict criteria were identified. These include genes encoding proteins required for the sequestration of iron (e.g., bacterioferritins) and genes encoding enzymes (superoxide dismutase) vital to defense against iron catalyzed oxidative stress. More notably however, we identified more than 30 genes encoding proteins involved in carbon catabolism and aerobic or anaerobic respiration that are regulated by PrrFs. A significant number of genes encoding enzymes (e.g., aconitase, citrate synthase) involved in the TCA cycle are controlled by the PrrFs however, in quite a few instances there are genes encoding proteins with redundant functions (i.e., aconitase, citrate synthase) that do not appear to be influenced in any way by PrrFs. Based on our microarray experiments, as well as on phenotypic data, we propose

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that the Fur regulated sRNAs (i.e., PrrFs) exert a powerful regulatory influence that permits the sparing of vital metabolic compounds (e.g., citrate) during periods of iron limitation. These and other data to be presented indicate that Fur controlled gene expression in bacteria like *P. aeruginosa* is considerably more imperative and intricate than previously appreciated.

Keywords *Pseudomonas aeruginosa* · Iron acquisition · Ferric uptake regulator · Pyoverdine signaling · Regulatory sRNAs · Intermediary metabolism · Biofilms

The dynamic control of intracellular iron concentrations is paramount to virtually all biological systems. One aspect of this issue is that, especially in an aerobic environment, biologically useful iron (i.e., Fe^{2+}) is extremely limiting or it is highly insoluble (i.e., Fe^{3+}). Accordingly, biological entities have evolved efficient mechanisms to acquire this nutrient from the insoluble form, which is generally in plentiful quantities. On the other hand, further acquisition of iron above biologically useful concentrations can have dire consequences for cells. Excess free iron will catalyze the generation of highly reactive oxygen and nitrogen intermediates that will damage all known biological macromolecules. This conflict, in a major way is dealt with in a diverse array of pathogenic and commensal prokaryotic microbes, by repressor proteins, which play the key role in controlling iron homeostasis at the level of transcription. The ferric uptake regulator (Fur) serves this function in many bacteria. In fact, in the opportunistic pathogen *Pseudomonas aeruginosa* Fur (PA-Fur) is an essential protein that negatively controls (represses) the expression of genes involved in the acquisition of environmental iron, including those that contribute to its virulence (Vasil and Ochsner 1999). For example, PA-Fur controls the production of: (i) extracellular proteinases, which degrade host iron binding proteins (Wilderman et al. 2001) (ii) low molecular weight, high affinity, iron binding compounds (i.e., siderophores) (Ochsner et al. 1996) (iii) two heme uptake systems and (Ochsner et al. 2000) (iv) a potent extracellular toxin (exotoxin A) (Prince et al. 1991; Ochsner et al. 1995).

Perhaps unexpectedly, Fur in *P. aeruginosa*, and in an increasing number of other organisms, also behaves as a positive regulatory factor (Wilderman et al. 2004), by contrast to its more familiar nature as a repressor. The mechanism by which this positive regulation occurs was obscure until the recent discovery of the Fur-regulated small RNA (sRNA) transcript called RyhB by Masse and Gottesman in 2002 (Masse and Gottesman 2002). In *P. aeruginosa* the transcription of two small RNAs (PrrF1 and PrrF2) is directly repressed by PA-Fur (Wilderman et al. 2004). These small RNAs in turn negatively control the expression of an array of genes at the post-transcriptional level under iron limiting conditions. Thus, the positive regulatory nature of Fur is mediated through its negative regulation of other negative regulatory entities (i.e., PrrF1 & PrrF2). PrrF-regulated genes are induced under iron-replete conditions and are involved in iron storage, defense against oxidative stress, as well as basic intermediary metabolism and very likely, other key cellular processes (Vasil, Oglesby, Farrow and Pesci unpublished observations). The ability of Fur to regulate both iron starvation induced genes, as well as those expressed under iron replete conditions (i.e., through Fur-regulated expression of sRNAs) considerably expands the scope of its influence on gene expression *P. aeruginosa* and in other bacteria that have similar regulatory sRNAs.

In this review we will provide greater detail regarding some of the remarkable intricacies of the Fur-dependent gene regulation in *P. aeruginosa*, as well as the rather astonishing influence that it has on the basic biology and virulence of this premier opportunistic pathogen.

Regulation of expression, molecular architecture and function of PA-Fur

The *fur* gene of *P. aeruginosa* expresses a single 15.2 kDa polypeptide that most likely assembles immediately into a dimer, which appears to be its basic functional unit in vivo. Of note, relatively little is known regarding the expression of the *fur* gene in *P. aeruginosa* except that it has two promoters and that neither seems to be iron

regulated or auto-regulated (Ochsner et al. 1999). Both promoters are required for the high levels of PA-Fur that are found in *P. aeruginosa*. There are on the order of 5,000–10,000 molecules of PA-Fur per cell, which is consistent with the levels that were reported for *Vibrio cholerae* (>7,000/cell) (Watnick et al. 1997). This level is at least as high as the most plentiful prokaryotic regulatory protein known, the leucine-response protein. While as discussed below, expression of many genes are influenced by Fur, far less of these are actually directly regulated by Fur. Based on some published and unpublished observation with a Fur mutant that is unable to bind DNA (Barton et al. 1996), we propose that PA-Fur may provide additional vital cellular functions, besides its ability to repress gene expression through its DNA binding properties. It is plausible that the metal binding capacity (i.e., iron) of PA-Fur also plays a vital role in defense against oxidative stress under iron-replete conditions. In support of this view are the observations of Touati et al. with *fur* mutants in *E. coli* (Touati et al. 1995). They reported that *fur*[−], *recA*[−] double mutants of *E. coli* are unable to grow aerobically, but they can grow anaerobically by fermentation. Introduction of a third mutation (i.e., *tonB*), which inhibits iron transport or, by the decrease of intracellular iron levels through other means, restores the growth of the double mutant under aerobic conditions. Perhaps, Fur is essential in *P. aeruginosa* as a consequence of its requirement to respire, which generates considerable potential for oxidative stress, and its inability to generate ATPs through anaerobic fermentation.

The molecular structure of Fur from any organism was largely unclear until 2002 when Pohl et al. reported the crystal structure of the PA-Fur dimer (Pohl 2003). Although, earlier data clearly identified that the DNA binding function of *E. coli* Fur was associated with an N-terminal domain and that the mature protein bound zinc (Coy and Neilands 1991; Coy et al. 1994; Jacquamet et al. 1998). Additionally, based on alignments of Fur homologs from a wide assortment of gram negative organisms and a handful of homologous proteins from gram-positive bacteria (e.g., *Bacillus* and *Staphylococcus*), although there is a considerable level of similarity between

these proteins, there are also some notable differences. For example, Fur from *E. coli* and other closely related organisms (e.g., *V. cholerae* & *Shigella* spp.) have four cysteine residues, two of which had, early on, been shown to play a role in binding to zinc (Coy et al. 1994). By contrast, Fur from *P. aeruginosa* only has a single Cys residue in its entire sequence. Moreover, the Fur proteins most similar to *E. coli* Fur have a Cys-4X-Cys motif at their C-termini, which was completely lacking in *P. aeruginosa* Fur (Pohl et al. 2003). The contribution of some of these dissimilarities to the structure, and possibility to the function of Fur from in the organisms which produce them, has begun to be addressed based on the crystal structure of PA-Fur and more recently based on additional biophysical and crystallographic data from *E. coli* Fur (Pecqueur et al. 2006). For example, Pohl et al. found that the single Cys residue of PA-Fur is associated with its dimerization domain and that it does not participate in metal binding (e.g., of the structural zinc or the regulatory iron) (Pohl et al. 2003). However in *E. coli* Fur, two Cys residues coordinate zinc binding, which is crucial to its dimerization. Moreover, these two zinc binding Cys residues are located far from the corresponding zinc binding region of PA-Fur, which lacks any Cys residues. Thus, while the overall dimeric structure of PA-Fur and *E. coli* Fur are still quite similar, the involvement of zinc in the formation of these structures is contrasting (Pohl et al. 2003; Pecqueur et al. 2006).

The function of the additional two Cys residues found in the Cys-4 X-Cys motif of *E. coli* Fur is still not clear, but it has been proposed that they may be involved in redox sensing because a similar motif (Cys-2X-Cys) is found in Fur-like proteins that function as redox stress sensors (e.g., FurA of *M. tuberculosis*), rather than in iron-mediated gene regulation (Pecqueur et al. 2006). In any case, it is curious that PA-Fur, and Fur from very closely related organisms (e.g., *Pseudomonas putida*) lack this distinct C-terminal motif.

While as mentioned above, PA-Fur rapidly forms as a dimer after its synthesis, in vivo a single dimer may not be sufficient to effect the repression of an iron starvation inducible gene.

Others and we have performed extensive DNase I footprinting assays on a wide assortment of Fur-regulated genes from *P. aeruginosa* and *E. coli*. Fur from either of these organisms never protects <27–30 bp in footprint assays (Ochsner and Vasil 1996; Escolar et al. 2000). Based on these data and modeling of the docking of PA-Fur dimers with DNA (Pohl et al. 2003) it is likely that it takes at least two dimers to cause the repression of a Fur-regulated gene in *P. aeruginosa*. It is also worth noting that there are genes in *P. aeruginosa* that exhibit an extended footprint with PA-Fur, which would likely require even more than just two dimers (Ochsner and Vasil 1996; Escolar et al. 2000). Taken together these and other observations discussed above could account for the need to maintain high levels (~5,000–10,000/cell) of Fur in comparison to other regulatory proteins (see above).

The scope of Fur repression

With the advent of molecular genomics it became possible to more comprehensively address questions relating to the breadth of the impact that a particular regulatory factor might have on gene expression. Approximately, a decade ago two powerful methods were independently developed to identify genes that are directly regulated by Fur. The first was the Fur Titration Assay (FURTA), which employed a genetic approach to identify Fur-regulated genes originally in *E. coli* and subsequently in a variety of other organisms (Stojiljkovic et al. 1994; Tsolis et al. 1995; Fassbinder et al. 2000; Rau et al. 2005). We developed an alternative molecular approach that enabled us to identify relatively small DNA genomic fragments (200–500 bp) that bound purified PA-Fur with high affinity (Ochsner and Vasil 1996). This method called SELEX-like cycle selection (systematic evolution of ligands by exponential enrichment) consisted of in vitro DNA-Fur interaction, binding of this DNA-Fur complex to anti-Fur antibody, purification of the antibody:PA-Fur:DNA complex on protein G, and PCR amplification and then cloning of the DNA fragment that bound PA-Fur. *P. aeruginosa* DNA fragments were isolated and cloned after

several rounds of selection were then characterized in mobility shift assays with PA-Fur and in DNase I footprinting assays. Finally, the DNA fragments were transcribed in vitro, the transcripts labeled and then used in RNase protection assays to demonstrate that the fragment encoded an iron-regulated gene. Using this method we identified twenty-five separate DNA fragments that very likely represented actual Fur-regulated genes. Based on the sequence of four of these fragments, they were immediately recognized as known genes of *P. aeruginosa*, while 16 others had not been previously identified in *P. aeruginosa*. Even though the sequence of the *P. aeruginosa* genome was not available at that time (1996) translation of the DNA sequence we obtained from these Fur-binding fragments provided some clues about their function due to their partial similarities with known proteins in other organisms, mainly *E. coli*. Overall, based on this approach we identified the following classes of genes as directly Fur-regulated (i) 2 two-component regulators (ii) five alternative sigma factors (iii) five possible siderophore receptors (iv) two distinct heme uptake systems and (v) an assortment of other genes (e.g., *tonB*) that could be reasonably associated with iron acquisition. There were two other groups of genes that answered this molecular selection that are worthy of further note. The first group included four genes that did not encode proteins with similarity to any other protein of known function, and the second group was comprised of two sRNAs that are adjacent to a cluster of genes involved in heme uptake (*phuSTUVW*) we had identified with this method (Ochsner and Vasil 1996; Ochsner et al. 2000). However, at that time we were unable to ascribe any function to these sRNAs since they did not have any effect on the expression of any of these genes (Ochsner et al. 2000). Ultimately, the sRNAs turned out to be PrrF1 and PrrF2 (see above and below) (Wilderman et al. 2004). Based on these and other observations, it became clear that Fur imparts a great deal of its influence on iron-regulated gene expression through its direct control on other regulatory factors (sigma factors, two component regulators, sRNAs) that are required for gene expression linked to iron homeostasis. Accordingly, perhaps a reason why

we did not identify any iron-regulated genes encoding known virulence factors (e.g., exotoxin A) in our SELEX-like experiments, is that Fur mostly directly regulates genes (e.g., sigma factors) which are, in turn, essential for the expression such genes (e.g., *toxA*).

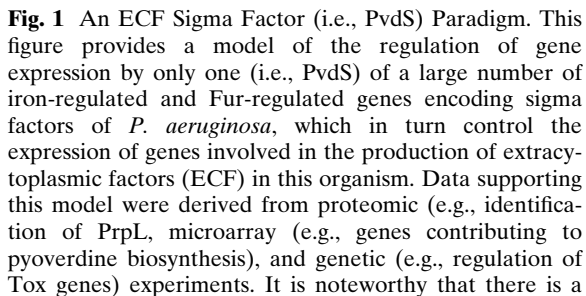
The recognition of this *modus operandi* of Fur in terms of its impact on the expression of virulence related loci led us to focus our further efforts on one or more of the Fur-regulated genes encoding ECF sigma factors or other types of regulators that we identified in our SELEX-like experiments. For various reasons we ultimately concentrated our efforts on PvdS that had been independently identified about a year earlier by Lamont and others as a gene that is required for the expression of the high affinity siderophore, pyoverdine (Cunliffe et al. 1995; Miyazaki et al. 1995). Based on proteomics, microarray data, and genetic data (e.g., reporter genes) we ultimately determined that PvdS, not only controls an assortment of genes involved in the biosynthesis and the secretion or release of pyoverdine in *P. aeruginosa*, we and our colleagues also found that PvdS is required for the optimal expression of the outer membrane pyoverdine receptor (*fpvA*), as well as two well characterized virulence determinants (Lamont et al. 2002; Shen et al. 2002; Redly and Poole 2003).

One is a powerful extracellular protease (PrpL) that is able to degrade lactoferrin and other iron binding proteins (Wilderman et al. 2001). PrpL expression clearly contributes to the virulence of *P. aeruginosa* in relevant animal models. PrpL mutants were significantly altered in their ability to compete with the wild type parent in a chronic pulmonary infection in rats. We also demonstrated that PvdS is required for the optimal expression of the well-established virulence determinant, exotoxin A (ETA) (Ochsner et al. 1996). These data and additional data from other experimental animal models (see below) indicated that the scope of PvdS regulation was evidently broader than its ability to regulate the biosynthesis of pyoverdine. Figure 1 illustrates this paradigm of Fur and PvdS regulation of gene expression. Based on the fact that we identified 4 additional ECF type sigma factors and an assortment of other types of regulators

in addition to PvdS during our SELEX-like experiments, it became increasingly obvious that Fur has, by far, a broader regulatory grasp than had been previously appreciated. Moreover, data discussed below will illustrate the intricacies associated with its regulation of gene expression mediated through its influence on the expression of a significant number other regulatory proteins (e.g., PvdS).

The role of pyoverdine expression in signaling and pathogenesis

Additional complexities associated with the Fur-regulated expression of PvdS became even more evident during our investigations on the influence of PvdS on virulence factor expression and on the pathogenesis of *P. aeruginosa* in experimental animal models. We initially observed that not only was PvdS expression controlled directly by PA-Fur, but that it is also influenced by oxygen tension (Ochsner et al. 1996). That is, PvdS is optimally expressed under iron-limiting conditions in an aerobic (20% O₂) environment and is >10-fold repressed in an iron-limited, microaerobic (8–10% O₂) environment. Since we had also determined that PvdS is required for the expression of virulence determinants such as exotoxin A (ETA), we investigated whether different environmental levels of oxygen might have distinct influences on the role of PvdS in a model infection (Xiong et al. 2000). While, endocarditis caused by *P. aeruginosa* is a relatively rare infection in humans there is a model of this infection in rabbits that enabled us to examine the effect of different oxygen tensions on the virulence of a PvdS mutant. In rabbits, as well as in humans, the prevailing O₂ tensions in the right-side and left-side heart chambers differ by ~50 mm Hg (40 vs. 90 mm Hg, respectively), which is roughly the difference between the levels of O₂ that we had used in our experiments described above to examine the effect of O₂ tension on the in vitro expression of PvdS. In the rabbit model, as in human endocarditis, *P. aeruginosa* forms a biofilm-like nidus of infection on damaged heart valves where it can also seed other tissues (e.g., kidneys & spleen). It is



possible in the rabbit model to initiate infection by inducing *P. aeruginosa* (wild type or PvdS mutant) to establish infection either on the right (tricuspid valve) or left side (aortic valve) of the heart, where the only difference between these infections would be the levels of oxygen as described above. Remarkably, in all parameters that were measured (e.g., number of bacteria colonizing the valves; the ability to seed to other organs) there were no differences between the wild type parent and the PvdS mutant when the infection was initiated on the right side of the heart (40 mm Hg). However, when the infection was first induced on the left side of the heart (90 mm Hg), the PvdS mutant was significantly reduced in its ability to survive as well as the wild type parent on the aortic valves and in its ability to seed to other organs (i.e., spleen & kidneys) which are highly oxygenated. These data graphically illustrate the distinctive role that Fur

The remarkable association between the PvdS mediated control of pyoverdine biosynthesis, expression of *tox*A and *prp*L, provided a rationale for examining whether pyoverdine itself had any influence on gene expression in *P. aeruginosa*, such as occurs with other small molecular weight compounds (e.g., homoserine lactones) involved in quorum sensing processes (Lamont et al. 2002).

The first question examined in terms of this hypothesis was: whether pyoverdine perhaps regulates the expression of genes involved in its own biosynthesis. Based on our transcriptional analysis of a *pvdS* mutant we had identified a large cluster of genes that are required for the biosynthesis of pyoverdine (Ochsner et al. 2002). This cluster included genes encoding multiple non-ribosomal peptide synthases and an assortment of other proteins, including ones with unknown functions.

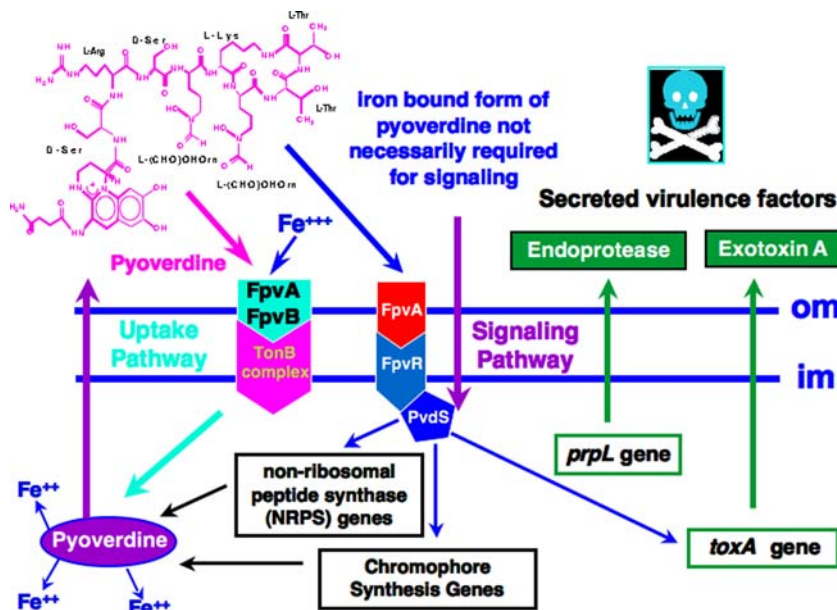
Our initial experiments addressed whether purified pyoverdine when exogenously added to a mutant (*pvdF*[−]), unable to synthesize pyoverdine itself, could induce the expression of other genes that are involved in its synthesis (e.g., *pvdE*). These experiments were done with a *pvdF* mutant carrying a *pvdE::lacZ* reporter. The data clearly indicated that pyoverdine does have a positive influence on the genes involved in its biosynthesis. We then examined the expression of *tox*A in a mutant (*pvdF*[−]) unable to synthesize pyoverdine and found that the levels of *tox*A transcripts (*tox*A::*lacZ* reporter), as well as the level of ToxA protein (western blot), were severely decreased in a mutant (*pvdF*[−]) that was unable to produce pyoverdine due to a mutation in a biosynthetic gene. This was also true for the *prp*L expression. However, when exogenous purified pyoverdine was provided to the *pvdF* mutant, expression of both *tox*A and *prp*L were completely restored to wild type levels. Finally, a key experiment was performed that addressed whether the uptake of pyoverdine was required for the signaling pathway (Lamont et al. 2002). When, *tox*A expression was examined in a mutant that was defective in the production of the pyoverdine receptor (FpvA[−]) this strain produced levels of *tox*A similar to those produced by the biosynthetic mutant (*pvdA*), but unlike the PvdA mutant, *tox*A expression in the receptor mutant did not return to wild type levels when exogenous pyoverdine was provided to the FpvA receptor mutant. These data and other from Lamont et al. decidedly indicate that the interaction of pyoverdine with its receptor, FpvA, is *sine qua non* for its signaling properties (Lamont et al. 2002; Beare et al. 2003; James et al. 2005).

While it might seem that this apparent signaling effect of pyoverdine on the expression of genes that are not directly related to its biosynthesis is merely due to the ability of pyoverdine to acquire and supply iron to *P. aeruginosa*, there are two unassailable facts that considerably diminish this possibility. First, it is known that iron free pyoverdine can bind to its receptor (FpvA) with only a moderately lower affinity (<20X) than the iron-loaded pyoverdine (Schalk et al. 2001). Therefore, it is quite probable that pyoverdine can initiate this signaling pathway in its iron-free form that would be considerably more plentiful under the iron limiting conditions when *tox*A and *prp*L are optimally expressed (Ochsner et al. 1996; Wilderman et al. 2001). An even more compelling argument is the fact that expression of the genes (i.e., *tox*A and *prp*L), which are the target of pyoverdine signaling, are only induced under conditions of iron starvation and repressed in an iron-replete environment. If pyoverdine was simply gathering iron for the bacterial cell then it would be expected that the expression of these genes would be repressed by the increased levels of iron, **NOT** induced as they clearly are by pyoverdine. This pyoverdine-mediated signaling processes is illustrated in the model presented in Fig. 2.

It is also noteworthy that we identified yet another pyoverdine receptor (FpvB) in *P. aeruginosa* however, its expression is not affected by pyoverdine and it does not appear to be involved in the pyoverdine signaling pathway shown in Fig. 2 (Ghysels et al. 2004). It should also be noted that its expression is governed by the utilization of different carbon sources than those that control expression of FpvA (e.g., glycerol vs. succinate) (Ghysels et al. 2004).

While at the present time pyoverdine-mediated signaling seems to be limited to the biosynthesis of pyoverdine and the expression of *tox*A and *prp*L, it is possible that signaling extends to the expression of other genes that may be involved in virulence or otherwise contribute to its survival in the plethora of environments in which *P. aeruginosa* resides. The role of PvdS and PvdS-controlled genes in the endocarditis models as described above may be such an example, since we did not detect any diminution in the virulence

Fig. 2 Model of pyoverdine uptake and signaling pathways. While the FpvA pyoverdine receptor plays a role in both the uptake pathway and the virulence factor signaling pathway the alternative pyoverdine receptor FpvB only plays a role in the uptake pathway



of a ToxA deficient mutant on either side of the heart in this model, as we did with the PvdS mutant on the left side. Another example where pyoverdine mediated signal may have an impact on gene expression, other than with those described herein, is in relation to the development of biofilms as described in the following section.

PA-Fur and PvdS mediated iron regulated gene expression in biofilm development

Singh et al. first described the most compelling association between iron and biofilm development in *P. aeruginosa* (Singh et al. 2002). They showed that human lactoferrin blocks biofilm development by this organism. This inhibitory activity occurred at concentrations below those that are lethal or prevent growth. These investigators proposed that by chelating iron and thereby lowering intracellular levels, lactoferrin actually stimulates twitching motility, a specialized form of movement across a solid surface mediated by pili. Consequently, iron limitation (i.e., through the iron binding capacity of lactoferrin) actually inhibits biofilm formation due to the constant movement (i.e., twitching) of the bacteria under these conditions, which in turn,

restricts the ability of *P. aeruginosa* to establish static foci that could ultimately develop into mature biofilms (Singh et al. 2002).

Based on these data and others, they proposed that a significant level of intracellular iron is required for biofilm development, which is well above the concentrations that are ordinarily required for planktonic growth. Once the iron level reaches a theoretically acceptable level, *P. aeruginosa* can then cease movement (twitching) and organize into complex biofilms.

In terms of the known role of PA-Fur and PvdS in iron homeostasis in *P. aeruginosa*, there were assortment pertinent questions emanating from the observations recounted above about the role of iron in biofilm development. Based on the availability of an assortment of well-defined mutants (e.g., those affecting the biosynthesis pyoverdine or another siderophore, pyochelin) we took a genetic approach to examine several aspects of this process (Banin et al. 2005). We first examined the ability of mutants defective in the biosynthesis of pyoverdine or pyochelin to establish the complex mushroom-like structures that are produced by the wild type parental strain. While the mutant that is deficient in the production of the lower affinity siderophore, pyochelin (*pchA*⁻), produced complex biofilms like the parental wild type, the strain (*pvdA*⁻) that was

unable to express the high affinity siderophore (pyoverdine) was only able to form flat uniform layer of bacteria on a glass surface that was notably similar to that produced by the wild type in the presence of lactoferrin. A PvdS deficient strain formed flat biofilms like the PvdA mutant, and complementation of both mutants with the corresponding genes resulted in strains that were able to form the complex mushroom-like structures seen with the parental wild type biofilms. Moreover, an FpvA (pyoverdine receptor) mutant also formed poorly developed biofilms like the PvdA and PvdS mutants, thereby indicating that interaction of the pyoverdine with the organism and probably the resultant iron uptake are critical to mature biofilm development.

By contrast with the mutants that are associated with the regulation of pyoverdine synthesis (PvdS), its biosynthesis (PvdA), or its uptake (FpvA), none of 12 other ECF sigma factor mutants nor 10 siderophore receptor mutants exhibited any deficiency in their ability to produce complex wild type like biofilms. The single exception to these results was associated with a mutant that is deficient in a putative ferric dicitrate uptake system. This exception was uncovered when it was found that when a PvdA mutant was provided ferric citrate, but not ferric chloride, it was then able to form the complex mushroom-like structures characteristic of wild type biofilms even though it could not produce pyoverdine. An inspection of the annotation of the *P. aeruginosa* genome revealed that there is a set of genes in this organism (PA3899–PA3901), which encode proteins with extensive similarities to the ferric dicitrate uptake (Fec) system in *E. coli* (Staudenmaier et al. 1989). Construction of a double mutant (i.e., *pvdA* and an insertion in PA3901) led to a strain that was unable to form mature biofilms even in the presence of ferric dicitrate. These and other data indicated the uptake of environmental iron through some, but not all, transport systems available to *P. aeruginosa* is crucial to the development of the complex biofilms examined in this study. Supportive of this view were additional data we obtained with a Fur mutant (i.e., a strain that expresses significantly lower levels of Fur or a missense Fur mutant) (Barton et al. 1996; Ochsner et al. 1999). Since, a

Fur mutant should be derepressed for the expression of iron uptake systems, such as those associated with pyoverdine and pyochelin, it would be predicted that such a mutant would be able to form complex biofilms even in the presence of powerful iron chelators such as lactoferrin, in contrast to the parental wild type that cannot establish biofilms under these conditions. This is precisely what was observed with the PA-Fur mutant. It forms highly structured biofilms even in the presence of lactoferrin levels that normally inhibit their formation in the parental wild type.

As a reflection of the variety of environments in which *P. aeruginosa* can exist, perhaps it is not surprising that more than a single iron uptake system can contribute to the development and maturation of biofilms. It is also tempting to wrongly assume that one kind of biofilm, like those seen in the study described above, will be completely subject to the same rules that govern biofilms, which form in a mammalian host or on plants. Nonetheless, it is probable that there are some basic truths that can be garnered from studies like these. After all, we have now drawn a connection between the regulation of pyoverdine biosynthesis, through PvdS, and the ability of *P. aeruginosa* to establish a biofilm like nidus on the heart valves of rabbits. However, in this case another very important variable, (i.e., oxygen tension) in addition to iron had a significant influence on the outcome of a biofilm related process. Clearly, a more complete understanding of the pathways, iron regulatory or otherwise, leading to formation of these fascinating structures will indeed further provide further valuable contributions about key biological and physiological processes involved in the formation of biofilms associated with human disease.

The positive regulation of iron dependent gene expression by PA-Fur—the role of regulatory RNAs

While Fur was originally identified as a negative regulatory factor, as early as 1989 its positive effect on the iron inducible expression of bacterioferritin and superoxide dismutase was also

described. In one of these early studies, Andrews et al. prophetically suggested that this type of Fur gene regulation might occur through a regulatory RNA (antisense), while others maintained that positive regulation might still occur through the direct binding of Fur to the promoters of the positively regulated genes (e.g., *sodB*) (Niederhoffer et al. 1990; Dubrac and Touati 2000).

We initiated our analysis of positive Fur regulation once our microarray analysis of iron dependent gene expression in *P. aeruginosa* revealed that not only are there >300 repressible genes (i.e., iron starvation inducible) in this microbe, but there are also >400 genes that demonstrate iron inducible expression (Ochsner et al. 2002). We further examined the expression of one of these (i.e., *bfrB*) and showed that its iron-induced expression is clearly Fur dependent, as it is in *E. coli* (Wilderman et al. 2004). As others had (see above) with these positively Fur-regulated genes, we initially proposed that PA-Fur might regulate the expression of *bfrB* by binding to its upstream promoter region, but that PA-Fur binding to this region might be more complex than when it regulates the expression of iron repressible genes.

However, through a series of extremely fortunate events, Shelley Payne informed us that Masse and Gottesman had identified a Fur-repressed sRNA transcript (RyhB) that in turn negatively controls the expression of iron inducible genes (e.g., *bfr*, *sodB* and the *sdh* operon) at the posttranscriptional level. Upon contacting Masse and Gottesman they generously shared their data with us, before its publication, about the relationships between this sRNA (RyhB) and Fur-regulated control of iron inducible genes. They mentioned at that time that they had examined the *P. aeruginosa* genome for sequences similar to RyhB, but had found none despite the fact that other gram-negative organisms (e.g., *Shigella flexneri* and *V. cholerae*) have sequences homologous to RyhB (Davis et al. 2005; Mey et al. 2005; Oglesby et al. 2005; Payne et al. 2006). Nevertheless, in a continuing series of fortunate events, in collaboration with Masse, Gottesman and Fitzgerald (Wilderman et al. 2004) we were able to identify sequences in the intergenic region (IG) of the genome of

P. aeruginosa, which remarkably fulfilled all the criteria we had established for a similar regulatory RNA in *P. aeruginosa*. We queried the IG using a program called PATSCAN for a sequence that included a consensus Fur binding site (14/19 match) followed by a spacer of <200 bp, and a stem-loop structure, then followed by at least three T nucleotides. Based on this search we found that an IG region between the 3' ends of two genes actually contains two, nearly identical sequences that matched all our criteria extremely well. Then in yet another set of fortunate events, we discovered that we had already extensively characterized the expression of these tandem sRNAs. They are situated at the end of the Phu operon (see above) and we had identified them as being directly Fur regulated because they answered our SELEX-like protocol for Fur-regulated genes nearly a decade earlier (Ochsner 1996; Ochsner et al. 2000). Moreover, we had demonstrated that while the transcription of both sRNAs are influenced by iron, one is also repressed by heme, but neither is involved in the regulation of the Phu operon (Ochsner et al. 2000). In any case, it was not until we identified them as possible RyhB analogs that we began to realize their potential role in iron homeostasis and Fur-regulated gene expression in *P. aeruginosa*.

It appears that RyhB-type sRNAs control gene expression at the post-transcriptional level through pairing with the mRNA of their target genes and subsequent degradation of the sRNA:mRNA duplex by RNaseE (Masse et al. 2003). Target genes that had been identified include ones that are involved in iron storage, resistance to oxidative stress, and intermediary metabolism and are the same genes that had been found to be iron inducible, mainly in *E. coli*, over the preceding years. In our initial characterization of PrrF-regulated genes we also provided data indicating that the same types of genes are also targets for PrrF regulation. However, since there are noteworthy differences between the physiology of *P. aeruginosa* and *E. coli* (mainly aerobic vs. facultative) we thought that a more complete analysis of PrrF regulated genes might reveal some interesting insights into the role of these sRNAs in the basic biology of *P. aeruginosa*. To address similar questions in *E. coli* and *Vibrio*

cholerae, Masse and others took an approach where they over-expressed these Fur-regulated sRNAs and then asked which genes were repressed during ectopic expression of RyhB (Masse et al. 2005). We decided to take an alternative approach where we identified potential targets by setting up a series of criteria that would have to be met for a gene to be called PrrF regulated.

Below are the rational, experimental approach and the outcomes of a set of experiments we performed to identify *P. aeruginosa* genes that have a substantial probability of being highly influenced by the PrrF sRNAs.

Experimental protocol

1. Comparison of transcriptional profiles of wild type grown under iron replete and iron limiting conditions.
2. Comparison of transcriptional profiles of wild type and PAO1 Δ PrrF1,2 mutant under iron limiting conditions.
3. Comparison of transcriptional profiles of a Δ PrrF1,2 mutant with a Δ PrrF1,2 complemented mutant.

Conditions

Cells were grown to stationary phase in media with glycerol as the main carbon source and glutamate as a main nitrogen source. These experiments were performed using microarrays to evaluate the transcriptional profiles of the strains listed above.

Criteria required for a gene to be designated as a PrrF1,2-regulated gene

1. Gene must show increased expression under Fe^{2+} limitation in the Δ PrrF1,2 mutant as compared to wild type parent.
2. The expression of the genes identified above must have returned to no change (NC) when

the Δ PrrF1,2-complemented mutant was compared to the wild type parent under Fe^{2+} limiting conditions.

3. Genes meeting conditions 1 and 2 above must also be increased in when the wild type parent is grown under Fe^{2+} replete conditions as compared to when it is grown under Fe^{2+} limiting conditions (i.e., the expression of the gene must be induced by Fe^{2+}).

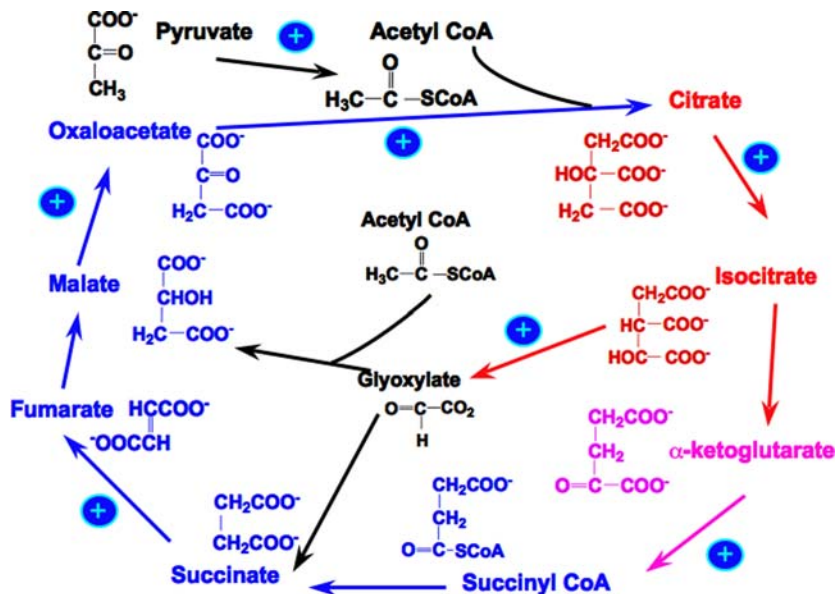
Outcome

Based on these criteria we identified the following classes of genes that we consider to be influenced by one or both of the sRNAs in *P. aeruginosa* under the conditions of the experiment.

- 2 genes encoding bacterioferritin
- 3 genes encoding transcriptional regulators (AraC, LysR, CpxR)
- 8 genes encoding proteins involved in environmental or oxidative stress (RecA, SodB, LexA, heat shock proteins)
- 4 genes encoding proteins of unknown function
- 30 genes encoding enzymes involved carbon metabolism (e.g., TCA cycle) & aerobic respiration
- 6 genes encoding proteins involved in anaerobic respiration

It is clear to see that most of the genes fulfilling our criteria are involved in carbon metabolism or aerobic respiration. While this may not be particularly surprising since it was already known that RyhB has a strong influence on the expression of the succinate dehydrogenase operon in *E. coli*, as well as on other genes encoding enzymes of the TCA cycle (Masse and Gottesman 2002; Masse et al. 2005), a more detailed analysis of this group of PrrF regulated genes of *P. aeruginosa* revealed some interesting insights about the possible role of these regulatory sRNAs in its physiology. Because some, but not all, genes encoding TCA cycle enzymes appear to be controlled by PrrF we thought that it might be informative to see the enzymes encoded by the PrrF regulated genes in perspective to the

Fig. 3 The effect of PrrF sRNAs on the expression of genes encoding TCA cycle and Glyoxylate Shunt enzymes. The steps in these pathways where a one or more genes encoding enzymes are involved in that reaction are indicated by ⊕



entire TCA cycle. Accordingly, Fig. 3 illustrates the individual steps in the TCA cycle and shows where some, but not all, of the genes encoding TCA cycle enzymes are regulated by one or both of the PrrF sRNAs. Moreover, it is interesting to note that *P. aeruginosa* exhibits a remarkable redundancy with regard to the genes encoding TCA cycle enzymes (Fig. 4). For example, while other organisms may only have one, or at the most two, genes encoding an aconitase (Gruer and Guest 1994; Somerville et al. 2002), *P. aeruginosa* actually has three genes encoding homologous proteins with this activity, two annotated as AcnA type enzymes, and one annotated as AcnB (see annotation of *P. aeruginosa* genome www.Pseudomonas.com). In this regard it is interesting to note (see Fig. 4) that the two genes encoding AcnA type enzymes are regulated by the PrrF sRNAs, but the third gene encoding AcnB is not (Vasil unpublished observations). These and other observations regarding PrrF-regulated genes encoding TCA cycle enzymes provide a glimpse into an additional layer of depth with regard to the impact of these sRNAs, as well as Fur, on the complex physiology of *P. aeruginosa*. For example, by analogy with the two classes of aconitase of *E. coli* it is worthwhile to mention that the aconitase enzymes themselves (e.g., AcnB) have a significant impact on gene

regulation and influence on susceptibility of the organism to redox stress (Tang and Guest 1999; Tang et al. 2004). What is more, it has been shown that the iron-free form (apo-AcnB) of AcnB influences, albeit indirectly, the transcription of flagellar (i.e., *flhC*) genes in *Salmonella enterica* (Tang et al. 2004).

On the other hand, while there are some notable similarities between the impact that RyhB has on gene expression and the impact that the PrrF sRNAs have on *P. aeruginosa* gene expression, there are some curious differences between certain reported phenotypes associated with RyhB in *E. coli* and ones we have observed that are associated with a PrrF mutant. Masse and Gottesman reported that when RyhB was over produced in *E. coli* this organism showed poor growth on media when succinate was provided as the sole carbon source (Masse and Gottesman 2002; Masse et al. 2005). In contrast, we found that while a PrrF mutant grew as well as the wild type parent on media where glycerol was the primary carbon source, this *P. aeruginosa* mutant exhibited growth arrest after ~3 h on media where the major carbon source was succinate. The wild type parent grew well on either carbon source (Vasil, Wilson and Oglesby unpublished data). It is worth noting at this point that in our biofilm studies (see above) we did not observe

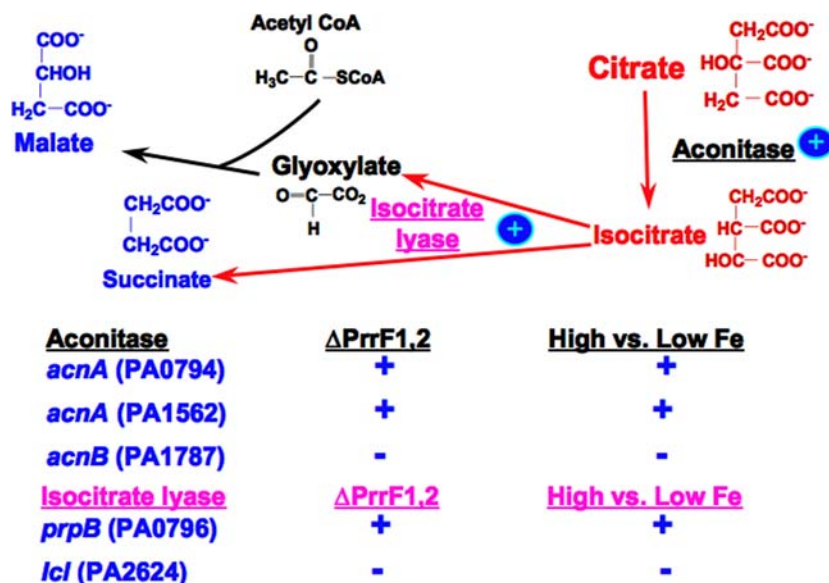


Fig. 4 The redundancy of *P. aeruginosa* genes encoding TCA and Glyoxylate enzymes. While the expression of genes encoding aconitase are controlled by PrrF sRNAs *P. aeruginosa* has three such genes and only two are affected by these regulatory sRNAs. In the table below the top of the figure a + indicates that that particular gene was either

highly expressed under iron limiting conditions in a Δ PrrF1,2 mutant in comparison the expression of that gene under the same conditions in the wild type parent, or that it showed significantly increased expression in the wild parental strain as compared to that strain under iron limiting conditions

any defects in the ability of a PrrF mutant to produce the complex mature biofilms like the parental wild type (Banin et al. 2005). However, based on the differences we observed in terms of the planktonic growth of the PrrF mutant when succinate was the carbon source it would be worthwhile to re-examine the ability of the PrrF mutant to form biofilms on different carbon sources than those we used in the biofilm studies described above. In any case, the observations regarding the differences described above pertaining to the growth of the PrrF mutant, in contrast to an *E. coli* strain that over expresses RyhB, clearly indicate attempts to always draw analogies, no matter how tempting, between the impact of Fur-regulated gene expression on the basic physiology of these two gram negative pathogens can have significant drawbacks.

Finally, Masse et al. in their examination of RyhB-regulated genes proposed that this regulatory process is a mechanism by which an organism can spare iron in an iron limiting environment, because many of the proteins (e.g., bacterioferritin) encoded by RyhB-regulated genes have high

requirements for iron (Masse et al. 2005). Consequently, their expression is shutoff by RyhB during iron limiting conditions. While this is a compelling rationale for RyhB, and even PrrF mediated regulation, it may be too limited in scope. We proposed based on the data presented above, and on other data from our laboratory (in preparation) that this pattern of gene regulation also provides for the sparing of key metabolic intermediates (e.g., succinate) under iron limiting conditions, that would otherwise be degraded through intermediary metabolism (i.e., TCA cycle) when iron would be more plentiful. We also believe that further dissection of the intricacies regarding the impact of Fur, PrrF sRNAs, and other regulators (e.g., AcnB) they control, may reveal additional unexpected tiers of control on iron homeostasis in *P. aeruginosa*.

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