The Effect of Iron Limitation on the Transcriptome and Proteome of *Pseudomonas fluorescens* Pf-5

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Abstract

One of the most important micronutrients for bacterial growth is iron, whose bioavailability in soil is limited. Consequently, rhizospheric bacteria such as *Pseudomonas fluorescens* employ a range of mechanisms to acquire or compete for iron. We investigated the transcriptomic and proteomic effects of iron limitation on *P. fluorescens* Pf-5 by employing microarray and iTRAQ techniques, respectively. Analysis of this data revealed that genes encoding functions related to iron homeostasis, including pyoverdine and enantiopyochelin biosynthesis, a number of TonB-dependent receptor systems, as well as some inner-membrane transporters, were significantly up-regulated in response to iron limitation. Transcription of a ribosomal protein L36-encoding gene was also highly up-regulated during iron limitation. Certain genes or proteins involved in biosynthesis of secondary metabolites such as 2,4-diacetylphloroglucinol (DAPG), orfamide A and pyrrolnitrin, as well as a chitinase, were over-expressed under iron-limited conditions. In contrast, we observed that expression of genes involved in hydrogen cyanide production and flagellar biosynthesis were down-regulated in an iron-depleted culture medium. Phenotypic tests revealed that Pf-5 had reduced swarming motility on semi-solid agar in response to iron limitation. Comparison of the transcriptomic data with the proteomic data suggested that iron acquisition is regulated at both the transcriptional and post-transcriptional levels.

Introduction

*Pseudomonas fluorescens* Pf-5 is a soil bacterium that was first described for its capacity to protect cotton seedlings from infection by *Rhizoctonia solani* and *Pythium ultimum* [1], [2]. Subsequently, the potential of Pf-5 to control other plant pathogenic fungi and bacteria was demonstrated [3], [4]. The biocontrol activities of Pf-5 can be attributed largely to its secretion of a broad spectrum of bioactive secondary metabolites [5], and approximately 6% of the 7.1 Mbp genome of Pf-5 is dedicated to secondary metabolite production [6]. The secondary metabolites produced by Pf-5 that have been demonstrated to be antagonistic to plant pathogens are pyrrolnitrin, pyoluteorin, hydrogen cyanide, rhizoxin analogs, and 2,4-diacetylphloroglucinol (DAPG) [1], [2], [7], [8], [9]. In addition, Pf-5 produces orfamide A, a recently identified biosurfactant compound which was shown to have anti-microbial activity [10].

One of the most important micronutrients for bacterial growth is iron, which is an essential cofactor in a number of important enzymes involved in energy metabolism. Iron is abundant in soil but, under aerobic conditions, it exists primarily in the insoluble ferric oxide form [11], which is not available for microbial growth. Due to the scarcity of available iron in many microbial habitats as well as the toxicity of free iron at elevated concentrations, bacteria employ a variety of mechanisms to regulate intracellular iron concentrations [12]. A mechanism of particular importance is the production and secretion of siderophores, which are small molecules that are secreted from the cell and sequester iron from the environment through high-affinity interactions [12], [13]. Iron-complexed siderophores can then be taken up by the bacteria via siderophore specific cell-surface receptors and transport systems [14]. The production and secretion of siderophores is thought to confer a competitive advantage on the producing organism, since siderophore-bound iron is not available to surrounding organisms lacking specific uptake systems for the ferric-siderophore complexes. The production of siderophores by biological control bacteria in the rhizosphere may also suppress plant disease by sequestering iron in a form that is not available to pathogens, resulting in their iron starvation [15].

*P. fluorescens* Pf-5 produces and secretes two siderophores, a pyoverdine and enantiopyochelin [16]. The capacity to produce these compounds is encoded in distinct gene clusters in the genome of Pf-5 that contain genes for siderophore biosynthesis, regulation, and membrane transport, including exporting the unloaded siderophores and importing iron-siderophore complexes [6], [16], [17]. In addition, Pf-5 encodes a number of TonB-dependent receptors that take up siderophores produced by other microorganisms [17], putative haem uptake membrane transporters, mechanisms for direct uptake of ferrous iron, and iron storage proteins such as bacterioferritins. Pf-5 can also utilize iron complexes of plant-produced compounds such as iron-phytosiderophore and iron-citrate [18]. As in other gamma-proteobacteria,
Iron acquisition by *Pseudomonas* spp. is under the control of the ferric uptake regulator (Fur) [19], [20]. Fur forms a homodimer complex with iron(II) and acts as a transcriptional repressor by binding to specific DNA sequences (Fur boxes) in the operator regions of target genes [21]. Transcriptional repression is relieved under low iron conditions, when iron(II) dissociates from the Fur complex and affinity for the Fur box is reduced. Fur is a global regulator of iron uptake, thus transcriptional control occurs both directly and also indirectly via the expression of regulatory RNAs, sigma factors, and other transcriptional regulators [19], [22], [23], [24], [25], [26].

Studies investigating the effect of iron limitation on global gene expression in the opportunistic human pathogen *Pseudomonas aeruginosa* PAO1 and the plant pathogen *Pseudomonas syringae* DC3000 have been performed [27], [28], [29]. In both organisms, the transcription of more than 300 genes was significantly influenced by iron [27], [28], [29]. Despite the importance of rhizospheric bacterial biocontrol agents, no studies have investigated the global effects of iron limitation in these organisms. Therefore, this study was undertaken to observe the global transcriptomic and proteomic changes in Pf-5 when faced with iron limitation. Here, we observed a wide ranging physiological effect on *P. fluorescens* Pf-5 by iron limitation, including changes to the expression of important biocontrol factors.

**Results and Discussion**

**Growth of Pf-5 in Iron-limited and Iron-amended Media**

Iron commonly occurs in the forms of two oxidation states, i.e., the +2 and +3 oxidative states. In the presence of oxygen and when pH is above 5, iron(II) will oxidize rapidly to iron(III) in a solution [30]. Nevertheless, different physiological consequences of FeCl₂ and FeCl₃ supplementation on cell cultures grown under oxygenic conditions have been observed, such as in a study on eukaryotic Vero monkey kidney cells [31]. Additionally, a study with *Pseudomonas putida* conducted by Molina et al. [32], showed that overnight cultures supplemented with either FeCl₂ or FeCl₃ had different growth biomass, which might suggest different levels of iron bioavailability or chemistry of the two iron sources used. Therefore, we employed both FeCl₂ and FeCl₃ in our study to observe whether usage of these two iron sources has any differential effects on Pf-5 at the molecular level, besides looking at the effect of iron limitation of *P. fluorescens* Pf-5.

The growth rate of *P. fluorescens* Pf-5 was higher in M9 minimal medium supplemented with either 100 μM FeCl₂ or 100 μM FeCl₃ than in non-amended M9 medium. Additionally, the total cell biomass was higher during stationary phase in both FeCl₂- and FeCl₃-amended cultures than in non-amended medium (Figure S1). Unlike observations reported for *P. putida* [32], we observed no notable difference between the growth rates and final cell densities of Pf-5 cultures supplemented with equal amounts of FeCl₂ versus FeCl₃ under our conditions (Figure S1). In the non-amended medium, Pf-5 produced a yellow-green fluorescent pigment characteristic of pyoverdine production.

**General Assessment of Iron Limitation Microarray Datasets**

The transcription of 180 and 121 genes increased or decreased by at least 2-fold, respectively, in iron-starved cells as compared to cells receiving FeCl₂ supplementation (Figure 1A; Table S1). Transcription of only 38 and 86 genes increased or decreased by at least 2-fold, respectively, in iron-starved cells versus cells grown in medium supplemented with FeCl₃ (Figure 1B; Table S1). Many of the genes undergoing transcriptional changes were known or predicted to be involved in iron homeostasis, allowing the delineation of the genes encoding iron uptake machinery in Pf-5. There was significant overlap between genes transcriptionally regulated in the two experiments although a number of genes involved in iron homeostasis (i.e., PFL_0909, PFL_3496, PFL_4008 and PFL_5555) had significantly higher transcriptional response upon FeCl₂ supplementation versus FeCl₃ supplementation, as discussed below (Table S1).

The disparity in transcriptional levels observed between the experiments might be due to differences in iron bioavailability or chemical properties of the iron sources used.

The genes found in the Pf-5 genome have been divided into 18 functional categories ([http://cmr.jcvi.org/cgi-bin/CMR/shared/RoleList.cgi](http://cmr.jcvi.org/cgi-bin/CMR/shared/RoleList.cgi)). Under iron-limited conditions, genes in the role categories of “transport and binding proteins” and “regulatory function” were notably up-regulated (Figure S2). In the category for “regulatory function”, many of the genes consisted of extra-cytoplasmic function (ECF) sigma factors that are adjacent to TonB-dependent receptor genes that may function in iron uptake (see below) [17].

qRT-PCR validation was performed on the microarray data using a subset of 22 differentially regulated genes (Table S2). In experiments utilizing FeCl₂ as the iron source, the microarray data generally corresponded well with qRT-PCR data with a Pearson correlation coefficient of 0.86 (Figure S3). Minor discrepancies were observed between the qRT-PCR and microarray results for three genes (PFL_1649, PFL_4410 and PFL_5963). Such occurrences are not unique and several other studies observed similar discrepancies [33], [34]. In the case of FeCl₃, a good correlation was observed, with a Pearson correlation coefficient of 0.93 (Figure S3).

**Iron Limitation Increased Transcription of Iron Acquisition Systems**

Genes for the biosynthesis and uptake of the siderophores pyoverdine and enanto-pyochelin were more highly expressed in iron-limited cultures of Pf-5 than in iron-amended cultures (Figure 1). Within the up-regulated pyoverdine biosynthesis gene clusters, the most highly expressed gene was *pvdS* (PFL_4190), which encodes the extra-cytoplasmic function (ECF) sigma factor PvdS, a transcriptional regulator of pyoverdine biosynthesis genes (Table 1). Recently, Hassan et al. [35] identified putative PvdS-controlled promoter regions upstream of 16 genes in the pyoverdine biosynthesis gene clusters of Pf-5 based on their similarity to the PvdS-controlled promoters of *P. syringae* [25] and *P. aeruginosa* [27]. In this study, all genes directly downstream of a putative PvdS-controlled promoter region were transcriptionally up-regulated, although not always by more than 2-fold (data not shown). Within the enanto-pyochelin gene cluster (PFL_3488-3497), the gene most highly regulated by iron was *pehR* (PFL_3497), an AraC family regulator that controls transcription of genes for the biosynthesis and transport of enanto-pyochelin [36] (Table 1). Genes (PFL_3498 to PFL_3503) adjacent to the currently defined enanto-pyochelin cluster were also up-regulated in iron-limited medium (Table 1). These genes encode proteins with predicted functions in membrane transport and may have roles in the uptake of iron-bound enanto-pyochelin [16], [36].

In addition to siderophores, Pf-5 has haem acquisition systems encoded by three distinct clusters within the genome (PFL_4625-29, PFL_5263-66 and PFL_5374-80) [35], [37]. The haxA gene (PFL_5377), which encodes a protein homologous to a haemophore produced by *P. aeruginosa* [37], and haxB (PFL_5380), which encodes an ECF sigma factor, were highly up-regulated in iron-limited medium (Table 2). Pf-5 also has genes for an alternative haem uptake system that is homologous to the *phu* operon of *P. aeruginosa* [58]. In the *phu* operon of Pf-5, the genes encoding a
sigma factor (PFL_4625) and haem oxygenase (PFL_4628, hemO) as well as a putative haem ABC transporter (PFL_5266, phuT) were up-regulated by more than 2-fold under iron-limited conditions (Table 2). The haem oxygenase is involved in haem degradation to release the bound iron [39]. Its orthologues in P. aeruginosa and P. syringae were also highly up-regulated when iron was limited [27], [29] (Table S1).

Motifs characteristic of Fur binding sites, which strongly resemble the Fur binding site of P. syringae [20], were identified between 14 and 209 nt 5’ of predicted translational start sites for 38 genes in the Pf-5 genome [35], including three genes in the pyoverdine biosynthesis region (PFL_4080, fpaC; PFL_4189, pvdL; and PFL_4190, pvdS) and two genes involved in haem uptake (PFL_5378, hasR; and PFL_5380, hasF). Of these 38 genes, transcripts of 27 were significantly up-regulated in iron-limited culture medium (Table S3). Similar to iron limitation studies in P. aeruginosa [27], [28] and P. syringae [20], [29], we observed that some genes encoded downstream of putative Fur binding motifs (e.g. PFL_2491 encoding a TetR regulator) were not iron-regulated, possibly because other factors controlling their transcription may counteract and obscure the effect of iron under the conditions of this study [40].

Transcriptional Effects on Extra-cytoplasmic Sigma Factors and TonB-dependent Receptors

The genome of Pf-5 includes 28 genes predicted to encode ECF sigma factors, many of which are likely to control iron homeostasis...
18 of the genes encoding these ECF sigma factors are situated adjacent to or near genes encoding anti-sigma factors and TonB-dependent receptors that contain N-terminal signaling domains. Upon receiving an external signal, the N-terminal signaling domains of these receptors will interact with anti-sigma factors, which then release the bound sigma factors thereby allowing the sigma factors to participate in the transcription of target genes [41]. These regulatory systems are involved in cell-surface signaling, which bacteria use to detect signals from the environment and transmit them into the cytoplasm [41]. Of the 18 sigma factor genes described above, 13 have consensus Fur binding sites upstream from their translational start sites that are likely to control their transcription [35] (Table S4). A Fur binding site is also upstream of the gene encoding the FpvI sigma factor (PFL_4080), which is unusual as its cognate anti-sigma factor (PFL_2903, fprR) and TonB-dependent receptor for pyoverdine (PFL_4092) are located distally within the genome [17].

### Table 1. Transcriptional regulation by iron of genes for siderophore biosynthesis and transport.

| Genes                          | Annotated functions                                          | Fold change (log2) |
|-------------------------------|--------------------------------------------------------------|--------------------|
| PFL_2901                      | hypothetical protein                                          | 1.71 NS            |
| PFL_4079                      | L-ornithine 5-monoxygenase PvdA                              | 1.81 NS            |
| PFL_4080                      | RNA polymerase sigma-70 factor, ECF subfamily, PvdD         | 2.84* 1.02*        |
| PFL_4082                      | efflux ABC transporter, ATP-binding/permease protein         | 1.09 NS            |
| PFL_4083                      | efflux transporter, outer membrane factor lipoprotein, NodT family | 1.09 NS            |
| PFL_4086                      | chromophore maturation protein PvdP                         | 1.15 NS            |
| PFL_4093                      | non-ribosomal peptide synthetase PvdD                        | 1.74 NS            |
| PFL_4095                      | non-ribosomal peptide synthetase PvdD                        | 1.19 NS            |
| PFL_4096                      | siderophore-interacting protein                              | 1.17 1.17          |
| PFL_4169                      | PepSY-associated TM helix domain protein                     | 2.39 1.48          |
| PFL_4171                      | conserved hypothetical protein                               | 1.44 NS            |
| PFL_4178                      | MbtH-like protein                                            | 3.99 4.73          |
| PFL_4189                      | non-ribosomal peptide synthetase PvdL                        | 2.59 NS            |
| PFL_4190                      | polymerase sigma-70 factor, ECF subfamily, PvdS             | 5.07 3.94          |

| Enantio-pyochelin biosynthesis gene cluster                                      |
|---------------------------------------------------------------------------------|
| Genes                          | Annotated functions                                          | Fold change (log2) |
| PFL_3483                      | RNA polymerase sigma-70 factor, ECF subfamily               | 1.87 NS            |
| PFL_3484                      | sigma factor regulatory protein, putative                   | 1.14 NS            |
| PFL_3490                      | enantio-pyochelin biosynthetic protein PchC                 | 1.21 NS            |
| PFL_3491                      | saccharopine dehydrogenase PchK                             | 1.30 NS            |
| PFL_3492                      | enantio-pyochelin synthetase PchF                           | 1.11 NS            |
| PFL_3495                      | ABC transporter, permease/ATP-binding protein, putative, PchH | 1.60 NS            |
| PFL_3496                      | salicyl-AMP ligase PchD                                    | 1.11* 0.45*        |
| PFL_3497                      | regulatory protein PchR                                     | 3.41 1.53          |
| PFL_3498                      | TonB-dependent outermembrane enantio-pyochelin receptor FetA | 2.01 NS            |
| PFL_3499                      | PepSY-associated membrane protein                           | 1.70 NS            |
| PFL_3500                      | iron-chelate uptake ABC transporter, FeCT family, periplasmic iron-chelate-binding protein, putative | 1.87 NS            |
| PFL_3501                      | iron-chelate uptake ABC transporter, FeCT family            | 1.23 NS            |
| PFL_3502                      | iron-chelate uptake ABC transporter, FeCT family, ATP-binding protein | 1.23 NS            |
| PFL_3503                      | transporter, major facilitator family                       | 1.06 NS            |

* Pf-5 genes that contain Fur binding motifs upstream [35].

Values are statistically different as determined using MeV software with P-value ≤0.01. NS means not significant when analyzed with SAM at FDR <1% in this study. Numbers in bold denote fold changes that surpass the significant differential expression threshold defined in this study, i.e. equal to or exceeding 2-fold change for transcript levels.

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down-regulated (PFL_4912) in the iron-limited media (Tables 1 and 2). No Fur boxes were found upstream of these genes [35].

TonB-dependent receptors rely on the accessory proteins ExbB, ExbD and TonB for energy transduction [43]. Two of the five annotated tonB-exbB-exbD clusters in the Pf-5 genome were regulated by iron; PFL_0225-27 was significantly down-regulated in iron-limited cultures (when compared to cells grown in culture supplemented with FeCl3) and PFL_6067-69 was significantly up-regulated. P. aeruginosa PAO1 encodes a tonB2-exbB-exbD locus PA0197-99, which is orthologous to PFL_0225-27 of Pf-5 (Table 2). This cluster has not been reported to be iron-regulated but is strongly up-regulated under sulfate starvation conditions [33]. Cornelis et al. [19] suggest that there is an overlap between the iron and sulfur regulons in P. aeruginosa. Because PFL_6067-69, which has no orthologous cluster in PA01, was the only exbB-exbD-tonB cluster significantly up-regulated in cells of Pf-5 grown in iron-limited culture medium, it is likely to function in iron acquisition. The orthologous cluster in P. syringae was also transcriptionally up-regulated under iron-limited conditions [29] (Table S1).

### Table 2. Transcriptional regulation by iron of genes for iron acquisition, oxidative stress response and iron storage.

| Genes          | Annotated functions                                      | Fold change (log2) | Iron-deprived (-FeCl2) | Iron-deprived (-FeCl3) |
|----------------|----------------------------------------------------------|--------------------|------------------------|------------------------|
| PFL_4625*      | RNA polymerase sigma-70 factor, ECF subfamily            | 1.70               | 0.85                   |
| PFL_4628       | heme oxygenase HemO                                      | 2.28               | NS                     |
| PFL_5266*      | hemin ABC transporter, periplasmic heme-binding protein PhuT | 1.01               | 0.37                   |
| PFL_5377       | heme acquisition protein HasAp                           | 3.70               | NS                     |
| PFL_5379       | sigma factor regulatory protein HasS                     | 2.74               | NS                     |
| PFL_5380*      | RNA polymerase sigma-70 factor, ECF subfamily, Hasl     | 1.72               | NS                     |
| PFL_3498       | **fetA**                                                 |                    |                        |
| PFL_0932       | TonB-dependent receptor                                   | 1.49               | NS                     |
| PFL_3177       | TonB-dependent outer membrane receptor                    | 1.14               | NS                     |
| PFL_4912       | TonB-dependent outer membrane receptor                    | NS                 | −1.21                  |
| PFL_5377       | RNA polymerase sigma-70 factor, ECF subfamily            | 1.05               | NS                     |
| PFL_0932       | TonB-dependent receptor                                   | 1.49               | NS                     |
| PFL_3177       | TonB-dependent outer membrane receptor                    | 1.14               | NS                     |
| PFL_4912       | TonB-dependent outer membrane receptor                    | NS                 | −1.21                  |
| PFL_0225       | TonB2 protein                                            | NS                 | −2.83                  |
| PFL_0226       | TonB system transport protein ExbB2                      | −0.99              | −2.35                  |
| PFL_6067       | TonB system transport protein ExbB1                      | 3.40               | NS                     |
| PFL_6068       | TonB system transport protein ExbD1                      | 2.85               | NS                     |
| PFL_6069       | periplasmic energy transduction protein TonB1             | 3.55               | 1.52                   |
| PFL_0573       | iron ABC transporter permease FbpB                       | 1.35               | NS                     |
| PFL_0574       | iron ABC transporter substrate-binding protein FbpA       | 2.08               | 1.06                   |
| PFL_0910       | zinc(II)-iron(III) family metal cation transporter permease | 2.44             | NS                     |
| PFL_3255       | ferrous iron permease EfeU                               | 2.76               | 1.25                   |
| PFL_5964*      | ferric iron ABC transporter, FeT family, periplasmic ferric iron-binding protein, putative | 1.78               | NS                     |
| PFL_0909       | superoxide dismutase, Mn, SodA1                          |                    |                        |
| PFL_4826       | superoxide dismutase (Fe) SodB                           | −1.53              | −1.13                  |
| PFL_4858*      | bacterioferritin-associated ferredoxin, putative         | NS                 | 4.83                   |
| PFL_5555       | bacterioferritin A                                       | −2.48*             | −0.72*                 |
| PFL_5556       | catalase KatA                                            | −1.37              | −0.70                  |

* Pf-5 genes that contain Fur binding motifs upstream [35].
* Values are statistically different as determined using MeV software with P-value <0.01. NS means not significant when analyzed with SAM at FDR <1% in this study. Numbers in bold denote fold changes that surpass the significant differential expression threshold defined in this study, i.e. equal to or exceeding 2-fold change for transcript levels.

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Transcription of Genes Involved in Iron Transport Across Inner Membrane

In addition to ferric-siderophore uptake systems, other mechanisms are employed by bacteria to take up iron(II) and iron(III) in
a free form. In anaerobic or reducing conditions, iron(II) is stable and soluble and can freely enter bacterial cells through the outer membrane porins of gram-negative bacteria [37]. Once in the periplasm, iron(II) is taken across the inner membrane by divalent metal uptake transporters [44]. Haritha et al. [45] performed a bioinformatic analysis, utilizing the TransportDB database [46], to identify the metal transportome of Pf-5. One of the predicted metal transporters is a gene encoding a zinc(II)-iron(II) permease family metal cation transporter (PFL_0910), which was significantly over-transcribed under iron limitation condition in our study, suggesting a role in the uptake of free iron (Table 2).

Many bacteria have the capacity to transport ferrous iron into the cytoplasm, and homologues of EfuUOB of *Escherichia coli*, which facilitates uptake of iron(II) in acidic conditions [47], are present in many bacterial genera. The PFL_3255 membrane transporter, which was described by Haritha et al. [45] as an iron transporter, has 58% identity to the Efu protein of *E. coli* K12. In our study, this gene was up-regulated strongly under iron limiting conditions (Table 2). A similar effect was observed for its orthologue in *P. syringae* [29] (Table S1). In *E. coli*, this operon is regulated by Fur and induced by acidic conditions [47]. A previous analysis of the Pf-5 genome did not detect a Fur box upstream of the gene [35] but this binding motif was found when a HMM search was performed with HMMER2 [48] at a lower E-value parameter of 1e-1 (data not shown).

Several genes encoding components of ABC transport systems predicted to be involved in iron(III) uptake across the cytoplasmic membrane were highly transcribed under the iron limiting condition used in our study. These include the genes located in PFL_5963-64 and PFL_0573-74 operons, which are homologous to the well-characterized iron(III) transporters HitABC of *Hemophilus influenzae* [49] and SiaABC of *Serratia marcescens* [50], respectively (Table 2). In concordance, transcription of the orthologous genes in *P. aeruginosa* is enhanced under iron-limited conditions [27], [28] (Table S1).

Oxidative Stress Response and Iron Storage

Bacterial oxidative stress responses are associated with iron homeostasis due to the role of iron in production of reactive oxygen species such as superoxide (O$_2^-$) and hydroxyl radicals (HO$_2^-$) [51]. The presence of iron(II) inside the cell can result in the production of HO$_2^-$ through the Fenton reaction (iron(II) + $H_2O_2$ $\rightarrow$ iron(III) + HO$_2^-$). To counter this, excess cellular iron(II) is converted to iron(III) through ferroxidase activity imparted by bacterioferritins [52]. In the iron-limited medium, PFL_5555, a gene encoding a non-haem binding bacterial ferritin (PA4235) [53], was down-regulated in Pf-5 (Table 2). Similar results were also observed in *P. syringae*, possibly because the need for iron storage was reduced under low-iron conditions [29] (Table S1). In addition, a bacterioferritin-associated ferredoxin gene (PFL_4858) was up-regulated by Pf-5 in iron-limited medium, as observed previously in *P. aeruginosa* [27], [28] and *P. syringae* [29] (Table 2; Table S1). The bacterioferritin-associated ferredoxin mobilizes iron stored in bacterioferritin B, and PFL_4858 gene is adjacent to a bacterioferritin B-encoding gene (PFL_4859), implying their cooperative nature [54].

Transcriptional changes were also observed for genes encoding oxidative stress response proteins such as superoxide dismutases, which counter reactive oxygen species by converting O$_2^-$ to H$_2$O$_2$. In Pf-5, a gene (PFL_4826) coding for a superoxide dismutase that utilizes iron as cofactor (SodB) was down-regulated in iron-limited conditions, presumably due to the reduced availability of iron in the cells (Table 2). Conversely, a manganese-based superoxide dismutase (PFL_0909, sodD) was up-regulated. This gene is part of the _fagE-fumC-orfX-sodD_ operon (PFL_0906-09) that is involved in oxidative stress responses in *P. aeruginosa* [55], [56], [57]. This operon may be regulated by Fur, since it is encoded downstream of a putative Fur binding motif [35]. PFL_3556, a homologue of PA1236, which encodes the KatA catalase of *P. aeruginosa*, was down-regulated by iron limitation (Table 2). This gene is adjacent to the bacterioferritin A gene (PFL_5555) in the Pf-5 genome, which was also down-regulated under iron-limited conditions, as described above. The genetic organization of the two genes is similar in Pf-5 and *P. aeruginosa* PAO1, where the genes are known to be expressed from different transcripts [52]. In *P. aeruginosa*, a functional bacterioferritin A is critical to the catalase activity of KatA [52]. Similarly, the regulation of _katA_ and bacterioferritin A could be linked in Pf-5.

Transcription of Respiratory Chain Genes

*Pseudomonas* spp. have extensively branched respiratory chain systems with multiple terminal cytochrome oxidases, thereby possessing the flexibility to use the electron transfer chain that is most suitable for the specific environmental conditions encountered [58], [59]. In aerobic metabolism, the respiratory chain typically uses proteins that require iron cofactors [27]. When Pf-5 was grown in the iron-limited medium, genes encoding cytochrome c-type biogenesis proteins (PFL_1684-88) and subunits of _ebk3_ cytochrome c oxidases (PFL_1922-25, PFL_2834) were down-regulated (Table 3; Table S1), whereas genes encoding subunits of a second _ebk3_ cytochrome c oxidase (PFL_1918-1921) were not regulated significantly by iron. Similarly, in *P. aeruginosa*, transcription of the _ebk3_ cytochrome c oxidase subunit II gene (PA1556), but not its paralogous gene (PA1553), is lower in an iron-limited versus an iron-replete medium [28]. In contrast, transcription of genes encoding subunits of a cytochrome _o_ ubiquinol oxidase (PA1317-21, _cyoABCDE_) increased under iron limitation in *P. aeruginosa* [27], [59]. In Pf-5, only one gene (PFL_5037) in the _cyoABCDE_ cluster (PFL_5033-37) was up-regulated in the iron-limited medium, and that effect was observed only in the experiment evaluating the influence of FeCl$_3$ (Table 3). Transcription of a second _cyoABCD_ cluster (PFL_4732-35) was not influenced by iron. Several other genes that encode participants in the electron transport chain, such as those coding for ubiquinol-cytochrome _c_ reductase (PAFL_5078-80), cytochrome _c4_ (PFL_0084) and a cytochrome _c_ family protein (PFL_5004), were down-regulated in cells grown under iron limitation (Table 3). As described for other bacteria [60], [61], iron availability appears to alter the preferred branch of the electron chain utilized by Pf-5 growing in an aerobic environment, providing a mechanism for the bacterium to conserve iron demands on the cell.

The Effect of Iron Limitation on Swarming Motility

Iron limitation generally resulted in reduced transcription of some genes in the flagellar biosynthesis clusters (PFL_1629-73) and (PFL_4477-85) (Table 3). For example, transcription of _fliA_ (PFL_1667) was reduced in the iron-limited culture compared to the FeCl$_3$-supplemented culture. The _fliA_ gene encodes a sigma factor controlling flagellar biosynthesis and motility in *P. aeruginosa* [62] and its reduced expression in Pf-5 may account for the decreased expression of flagellar biosynthesis genes. We evaluated swarming motility of Pf-5 on 0.6% agar plates containing minimal media supplemented with various concentrations of iron, and observed that the diameters of the swarming colonies were proportional to iron concentration of the medium (Figure 2). Our observation is in contrast to previous reports that motility of *P. aeruginosa*...
**Iron Limitation Affects Transcription of Ribosomal Protein L36 Gene**

A ribosomal protein L36 gene (PFL_3806) was highly up-regulated under iron-limited conditions in Pf-5 (Table 4). Interestingly, a putative Fur binding site was observed upstream of the gene [35]. A second non-annotated ribosomal protein L36 gene was identified at coordinates 6351985-6351869 in Pf-5 (RefSeq number: NC_004129.6). In *P. aeruginosa*, two paralogues of ribosomal protein L36 (C⁺ and C⁻ forms) have been found [68]. The L36 C⁺ form has a ‘CXXC.CXXXH’ motif that forms a metal-binding zinc-ribbon motif [69], which is absent in the C⁻ form. PFL_3806 corresponds to the C⁻ protein. The transcription of ribosomal protein L36-encoding genes was not significantly affected by iron limitation in *P. aeruginosa* or *P. syringae* [27], [28], [29]. The reason for the high-level transcription of this gene by Pf-5 under iron-limited conditions remains to be answered. The gene product could be involved in iron homeostasis in Pf-5, reminiscent of the functions of some ribosomal proteins in zinc homeostasis in other bacterial species [28].

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**Table 3. Transcriptional regulation by iron of genes involved in respiratory chain components and flagellar biosynthesis.**

| Genes | Annotated functions | Fold change (log₂) |
|-------|---------------------|--------------------|
|       |                     | Iron-deprived (-FeCl₂) | Iron-deprived (-FeCl₃) |
| **Respiratory chain gene clusters** | | | |
| PFL_0084 | cytochrome c₄ | −1.18* | −0.46* |
| PFL_1684 | cytochrome c₅-biogenesis protein CcmE | −1.24 | NS |
| PFL_1685 | cytochrome c₅-biogenesis protein CcmF | −1.04 | NS |
| PFL_1687 | cytochrome c₅-biogenesis protein Cycl | −1.10 | NS |
| PFL_1917 | cytochrome c oxidase accessory protein CcoG | −1.58 | −0.72 |
| PFL_1922 | cytochrome c oxidase, cbb3-type, subunit III, CcoP₂ | NS | −1.29 |
| PFL_1923 | cytochrome c oxidase, cbb3-type, CcoQ subunit | −1.00 | −1.36 |
| PFL_1924 | cytochrome c oxidase, cbb3-type, subunit II, CcoO₂ | −1.57 | −1.47 |
| PFL_1925 | cytochrome c oxidase, cbb3-type, subunit I, CcoN₂ | NS | −1.57 |
| PFL_2834 | cytochrome c oxidase, cbb3-type, subunit I, CcoN₃ | −1.57 | −0.74 |
| PFL_5004 | cytochrome c family protein | −1.18 | −1.17 |
| PFL_5037 | cytochrome o ubiquinol oxidase, subunit II, CyoA₂ | NS | 1.55 |
| PFL_5078 | ubiquinol-cytochrome c reductase, cytochrome c₁, putative | −1.20 | NS |
| PFL_5080 | ubiquinol-cytochrome c reductase, iron-sulfur subunit PetA | −1.41 | NS |
| **Flagellar biosynthesis gene clusters** | | | |
| PFL_1636 | sigma-54 dependent DNA-binding response regulator FleR | −1.41 | −1.16 |
| PFL_1637 | flagellar hook-basal body complex protein FlIE | −1.66 | NS |
| PFL_1638 | flagellar M-ring protein FliF | −1.74* | −0.87* |
| PFL_1639 | flagellar motor switch protein FliG | −1.75* | −0.70* |
| PFL_1640 | Flagellar assembly protein FliH | −1.18 | NS |
| PFL_1641 | flagellum-specific ATP synthase Fli | −1.12 | NS |
| PFL_1646 | flagellar hook-length control protein FliK | −1.23 | NS |
| PFL_1647 | flagellar protein FiiL | −1.38 | NS |
| PFL_1648 | flagellar motor switch protein FiiM | −1.47 | NS |
| PFL_1649 | flagellar motor switch protein FiiN | −1.96 | NS |
| PFL_1652 | flagellar biosynthetic protein FiiQ | −1.04 | NS |
| PFL_1656 | conserved hypothetical protein | −1.87 | NS |
| PFL_1657 | 3-oxoacyl-(acyl-carrier-protein) synthase III, putative | −1.32 | −0.68 |
| PFL_1664 | flagellar biosynthesis protein FliA | −1.48 | NS |
| PFL_1666 | flagellar synthesis regulator FleN | NS | −1.55 |
| PFL_1667 | motility sigma factor FliA | NS | −1.39 |
| PFL_4480 | flagellar basal-body rod protein FlgB | −1.08 | NS |

*Values are statistically different as determined using MeV software with P-value <0.01. NS means not significant when analyzed with SAM at FDR <1% in this study. Numbers in bold denote fold changes that surpass the significant differential expression threshold defined in this study, i.e. equal to or exceeding 2-fold change for transcript levels.

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reb-homologous Genes were Highly Transcribed under Iron Limitation

Two genes (PFL_0183 and PFL_0184) that were highly transcribed by Pf-5 grown in the iron-limited medium (Table 4) are similar to reb genes of Caedibacter taeniospiralis [70], which putatively encode refractile inclusion bodies (R-bodies). R-bodies are cylindrical structures in the bacterial cell, which are composed of insoluble proteinaceous ribbons and are associated with toxicity [71], [72]. For example, the R-bodies produced by C. taeniospiralis are thought to kill a sensitive paramecium [73]. R-bodies have been observed in P. aeruginosa [74] and other pseudomonads, such as Pseudomonas taeniospiralis [75] and Pseudomonas avenae [76], but have not been observed in P. fluorescens. Of the four published P. fluorescens genomes (Pf-5, SBW25, Pf0-1 and WH6), only Pf-5 has genes predicted to encode R-bodies (PFL_0180, PFL_0183 and PFL_0184) [6], [77], [78]. The synthesis of R-bodies has been associated with mobile elements such as plasmids and prophages [71], [72], but there is no evidence of recent horizontal acquisition of the reb genes by Pf-5.

Effect of Iron Limitation on Transcription of Genes Involved in Secondary Metabolite Biosynthesis

Iron is known to influence the production of secondary metabolites by Pseudomonas spp., such as the phytoxins syringomycin and syringotoxin produced by P. syringae [79], [80]. Accordingly, transcription of many secondary metabolite biosynthesis genes by Pf-5 was influenced by iron amendment of the medium. As shown in a previous study by Blumer and Haas [81], hydrogen cyanide biosynthesis genes (PFL_2577-79) were down-regulated in iron-limited medium (Figure 1; Table 4; Table S1). Blumer and Haas [81] suggested that the ANR regulator, which controls hydrogen cyanide production by binding to the promoter of the hcnABC cluster, is sensitive to iron concentration as it contains Fe-S clusters. A lack of iron restricts the assembly of the Fe-S clusters, potentially disabling its function. Within the DAPG biosynthesis cluster, genes encoding the transcriptional repressor PhlF (PFL_5953) and the biosynthetic enzyme PhlA (PFL_5954) were up-regulated in Pf-5 grown in the iron-limited medium (Table 4). Within the orfamide A biosynthesis cluster, genes encoding a transcriptional regulator of the LysR family (PFL_2143) and the non-ribosomal peptide synthetase OfaC (PFL_2147) were up-regulated in the iron-limited medium (Table 4). We did not observe significant, 2-fold or greater, transcriptional effects of iron on other biosynthetic genes within the other known secondary metabolic gene clusters in Pf-5 (i.e., pyoluteorin, pyrrolnitrin, or rhizoxin analogs).

Comparison with Pf-5 gacA Mutant Strain Transcriptional Profile

Hassan et al. [35] investigated the effect of a gacA mutation on the transcriptome of P. fluorescens Pf-5 and found that many genes
involved in iron homeostasis were negatively regulated by the GacS/GacA two-component regulatory system. According, there is a significant degree of overlap between the transcriptomic responses of Pf-5 to gacA deletion and to iron limitation (Figure 3). For example, approximately half (43 of 84) of the genes that were up-regulated by more than 2-fold in response to gacA deletion, were also significantly up-regulated by 2-fold or more under iron-limited conditions (Table S1). These loci include 14 genes encoding ECF sigma factors, the fgaA-funcG-ofx-xodA operon (PFL_0906-09), pchR (PFL_3497), the pyoverdine biosynthesis cluster, the cbbB-cbbD-tobB cluster (PFL_6067-69), and genes encoding a bacterioferritin-associated ferredoxin (PFL_4858) and haem oxygenase HemO (PFL_4628) (Table S1). Conversely, approximately one third (43 of 122) of the genes positively regulated by the Fur regulator were up-regulated by more than 2-fold and were significantly down-regulated by at least 2-fold under iron-limited conditions (Table S1). These loci include the hydrogen cyanide synthase genes (PFL_2577-79), cytochrome c oxidase cbOb-type gene cluster (PFL_1922-25), iron-based superoxide dismutase (PFL_4826) and flagellar-related regulators (PFL_1666-67) (Table S1). The overlapping gacA and iron regulons signifies that some iron genes involved in iron homeostasis are controlled by both iron and the GacS/GacA signal transduction cascade. It is possible that the Fur regulator interacts with a component of the GacS/GacA signal transduction cascade, although this needs to be experimentally demonstrated. A recent study [82] showed that both Fur and iron concentration affect gacA gene expression in P. syringae, but we did not observe a significant influence of iron on transcription of gacA in this study.

While the mechanism remains uncharacterized, a regulatory architecture linking iron and the GacA/GasS cascade might provide fine-tuning of iron homeostasis in conjunction with other environmental cues.

### Overview of Proteomic Analyses and Comparison with Transcriptional Profile

A shotgun proteomic analysis was conducted to complement our transcriptomic study and extend the results to an analysis of protein expression. The culture conditions used were identical to those used in the microarray studies. A total of 547 proteins were detected in the experiment comparing iron deprivation with either FeCl2 or FeCl3 supplementation. Of these, 168 were significantly up-regulated and 132 proteins were down-regulated in the iron-limited compared to the cultures supplemented with FeCl3 (Table S5). Likewise, in the experiment comparing iron deprivation to FeCl3 supplementation, 184 proteins were significantly up-regulated and 129 were significantly down-regulated under iron limitation (Table S5). In both the FeCl2 and FeCl3-based experiments, a greater proportion of proteins defined as being in the “protein synthesis” role category were down-regulated (Figure S4). The correlation of differentially expressed proteins detected in the FeCl2 and FeCl3 experiments was strong (Pearson correlation coefficient = 0.91) (Figure 4). Overall, an under-representation of membrane-associated proteins (estimated 3.8%), as determined from the GRAVY scores, probable transmembrane domains and predicted localizations of the detected proteins, was observed in our proteomic experiment (compared to estimated 28% of the whole predicted proteome of Pf-5). Other proteomic studies utilizing iTRAQ have also observed an apparent under-representation of membrane-associated proteins [83], [84], which could be the result of inherent difficulties in membrane protein solubilization and digestion by protease [85].

A moderate correlation between the transcriptomic and proteomic data was observed (Figure 5). Of the 385 proteins differentially expressed by Pf-5 in iron-limited versus iron-replete media (either the FeCl2 or FeCl3 form), 70 were also significantly regulated at the transcriptional level. Of these 70 proteins, 16 showed opposite trends in regulation at the transcriptional and translational levels (Figure 3; Table S5). These patterns are likely to be related to the complexity of regulation and are commonly observed in studies comparing transcript and protein abundance of bacteria under a range of experimental conditions [86], [87], [88], [89], [90]. For example, in Leptospira interrogans, only 25% of the outer membrane proteins that were regulated significantly by temperature were correspondingly regulated at the transcriptional level [90]. Post-transcriptional regulation involving translational

### Table 4. Transcriptional regulation by iron of selected genes.

| Genes       | Annotated functions                  | Fold change (log2) | Iron-deprived (-FeCl₂) | Iron-deprived (-FeCl₃) |
|-------------|--------------------------------------|--------------------|------------------------|------------------------|
| PFL_3806   | ribosomal protein L36 RpmJ            | 3.20               | 2.67                   |                        |
| PFL_0183   | RebB protein                          | 0.91               | 1.25                   |                        |
| PFL_0184   | RebB protein                          | NS                 | 1.31                   |                        |
| PFL_2143   | transcriptional regulator, LuxR family| NS                 | 1.48                   |                        |
| PFL_2147   | Nonribosomal peptide synthetase OfaC  | 1.41               | NS                     |                        |
| PFL_2577   | hydrogen cyanide synthase HcnA        | NS                 | −1.49                  |                        |
| PFL_2578   | hydrogen cyanide synthase HcnB        | −1.26              | −1.49                  |                        |
| PFL_2579   | hydrogen cyanide synthase HcnC        | NS                 | −1.36                  |                        |
| PFL_5953   | transcriptional repressor PhlF         | 2.42               | NS                     |                        |
| PFL_5954   | 2,4-diacetylphloroglucinol biosynthesis protein PHIA | 2.44               | NS                     |                        |

* PF-5 genes that contain Fur binding motifs upstream [35]. NS means not significant when analyzed with SAM at FDR <1% in this study. Numbers in bold denote fold changes that surpass the significant differential expression threshold defined in this study, i.e. equal to or exceeding 2-fold change for transcript levels.

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Iron Limitation Effect on Pseudomonas fluorescens

PL1050 bacterioferritin-associated ferredoxin
PL1050 fagA protein
PL1050 fumate hydratase class II
PL1050 conserved hypothetical protein
PL1450 iron regulated transcription activator
PL1470 MnTH-like protein-related protein
PL1870 conserved hypothetical protein
PL1870 acetate-CoA ligase
PL1870 pyochelin synthetase
PL1870 non-ribosomal peptide synthetase
PL1870 conserved hypothetical protein
PL1870 tryptophan synthase alpha subunit
PL1870 tryptophan synthase beta subunit
PL1870 malto-oligosyltrehalose synthase
PL1870 conserved hypothetical protein
PL1870 conserved hypothetical protein
PL1870 conserved hypothetical protein
PL1870 non-ribosomal peptide synthetase
PL1870 exodeoxyribonuclease VII large subunit
PL1870 acetyltransferase GHAT family
PL1870 response regulator/HD domain protein
PL1870 metallo-beta-lactamase family protein
PL1870 60-kDa SS-Fe(II) ribonucleoprotein
PL1870 ribosomal protein L3E
PL1870 conserved hypothetical protein
PL1870 conserved hypothetical protein
PL1870 non-ribosomal peptide synthetase
PL1870 sigma factor regulatory protein PchR putative
PL1870 L-ornithine 5-monooxygenase
PL1870 conserved hypothetical protein
PL1870 siderophore utilisation protein
PL1870 lipoprotein putative
PL1870 membrane protein putative
PL1870 iron(III) ABC transporter periplasmic
PL1870 PepST-associated membrane protein
PL1870 oxidoreductase 20G-Fe(II) oxygenase
PL1870 DNA-binding response regulator
PL1870 regulatory protein PchR putative
PL1870 RNA polymerase sigma factor FecI
PL1870 sigma factor regulatory protein FecR
PL1870 TonB system transport protein ExkB
PL1870 RNA polymerase sigma-70 factor ECT
PL1870 TonB system transport protein ExkD
PL1870 superoxide dismutase Mn
PL1870 ferric siderophore transporter periplasmic
PL1870 conserved hypothetical protein
PL1870 cytochrome c oxidase chb3-type subunit
PL1870 cytochrome c oxidase chb3-type CoQ
PL1870 hydrogen cyanide synthase MccB
PL1870 arginine/ornithine antiporter
PL1870 oxidoreductase short chain dehydrogenase
PL1870 arginine deiminase
PL1870 alcohol dehydrogenase II
PL1870 oxygen-independent coproporphyrinogen
PL1870 cytochrome c oxidase chb2-type subunit
PL1870 cytochrome c family protein
PL1870 oxidoreductase FAD-binding
PL1870 motility sigma factor III
PL1870 universal stress protein family
PL1870 membrane fusion protein putative
PL1870 major facilitator superfamily protein
PL1870 cytochrome c-type biogenesis protein
PL1870 ornithine carbamoyltransferase
PL1870 superoxide dismutase (Fe)
PL1870 sulfate ABC transporter periplasmic
PL1870 carboxylate kinase
PL1870 sigma-54 dependent DNA-binding response regulator
PL1870 probable antioxidant protein P455
PL1870 2-octaprenyl-2-methyl-6-methoxy-14-b-cyclopentadiene
PL1870 glucose-6-phosphate 1-dehydrogenase
PL1870 sigma-54-binding protein
PL1870 arginine/ornithine antiporter
PL1870 conserved hypothetical protein
PL1870 aspartate ammonia-lyase
efficiency as well as mRNA and protein stability is particularly important in the regulation of iron homeostasis. In \textit{P. aeruginosa}, the Fur-controlled regulatory RNAs \textit{prrF1} and \textit{prrF2}, which have homologues in Pf-5 \cite{91}, mediate the translation of mRNAs encoding iron-containing proteins \cite{92}. Additionally, regulatory RNA sequence of iron-responsive-like elements, which might confer post-transcriptional regulation by iron, have been detected in bacterial mRNA \cite{93}, \cite{94}. Many of the 70 genes regulated only at the translational level in this study have annotated functions in primary metabolism, such as amino acid and nucleotide synthesis. For example, the proteomic analysis identified 42 ribosomal proteins to be less abundant in the iron-limited versus iron-supplemented medium, which correlates with the lower growth rate of Pf-5 observed under iron-limited conditions.

The differences in expression levels of detected proteins between FeCl$_2$ and FeCl$_3$-supplemented cultures were not as extensive as those observed at transcriptional level. In both experiments (FeCl$_2$ and FeCl$_3$), proteins involved in iron homeostasis, including pyoverdine and enantio-pyochelin biosynthesis and transport, were up-regulated in the iron-limited medium. A number of highly-expressed proteins (\textit{i.e.} PFL\_4008, PFL\_4089, PFL\_4092 and PFL\_4179) involved in pyoverdine biosynthesis were not over-expressed significantly at the transcriptional level, suggesting that they are subject to post-transcriptional control (Table 5; Table S5). Two proteins encoded by genes (PFL\_3496 and PFL\_3500) in the enantio-pyochelin cluster were over-expressed in the iron-limited medium (Table 5). In addition to the siderophore biosynthesis proteins, components of the alternative haem uptake (Phu) system (PFL\_4628 and PFL\_5266) and a homologue (PFL\_5964) of the HitABC iron[III] uptake system were also over-expressed under iron limitation (Table 5). Notably, the Fur protein (PFL\_0824) was over-expressed by Pf-5 grown under iron-limited conditions, a phenomenon that was observed previously in \textit{Neisseria meningitidis} \cite{95}. It is intriguing that Fur was over-expressed in a condition where it is unlikely to perform its role as an iron-dependent repressor. Over-expression of Fur under iron-limited conditions may serve to maintain Fur abundance above a minimum threshold level in order to facilitate a more effective regulatory control \cite{96}. Besides that, it was reported that Fur in its apo form can still function actively in regulation \cite{97}.

The abundance of several proteins involved in the oxidative stress response of Pf-5 was also altered by iron levels. The iron-independent fumarate hydratase (PFL\_0907) \cite{98} was over-expressed under iron-limited conditions while catalase KatA (PFL\_5556) and iron-dependent superoxide dismutase (PFL\_4826) were under-expressed at both the transcriptional and protein levels (Table 5). Another probable iron-independent fumarate hydratase (PFL\_4328), which was not regulated by iron at the transcriptional level, was over-expressed at the protein level under iron-limited conditions (Table 5). Interestingly, a predicted bacterioferritin (PFL\_0613, a homologue of PA4880 of \textit{P. aeruginosa}) was under-expressed in the iron-limited medium but was not significantly regulated by iron at the transcript level (Table 5). This observation is consistent with the known post-transcriptional regulation of PA4880 by PrrF1 and PrrF2 in \textit{P. aeruginosa} \cite{92}.

We also observed repression of the hydrogen cyanide biosynthetic protein PFL\_2579, supporting similar findings from a
previous study [81]. Interestingly, other proteins contributing to fungal antagonism by Pf-5, including a pyrrolnitrin biosynthetic protein (PFL_3604), a chitinase (PFL_2091), and a chitin binding protein (PFL_2090) were up-regulated under iron-limiting conditions. In line with the phenotypic observation of reduced motility of Pf-5 under low iron condition, our proteomic experiments detected decreased abundance of a flagellin protein (PFL_1629) from cultures grown in iron-limited media versus iron-replete media (Table 5).

Effects of Iron Limitation on Expression of Proteins Containing Iron-sulfur Cluster Binding Motifs and the Machinery Involved in Iron-sulfur Cluster Biogenesis

Iron-sulfur clusters play a very important role in the activity of many enzymes in bacteria [99]. Under iron limitation, a number of Pf-5 genes encoding proteins with iron-sulfur clusters displayed decreased transcription (Table 6). In addition, many genes encoding proteins containing iron-sulfur cluster binding motifs were not affected at a transcriptional level, but their corresponding protein abundance was lower in cells grown under iron-limitation (Table 6), suggesting post-transcriptional control. A similar pattern of iron-regulated expression of iron-sulfur proteins was seen in *V. cholerae* [100], and also in *E. coli* where the regulatory RNA RyhB controls the expression of proteins containing iron-sulfur clusters [101]. Another possible explanation for the lower abundance of proteins containing iron-sulfur clusters, but not their corresponding transcripts, under iron-limited conditions is that the iron-sulfur cluster itself might protect the proteins against intracellular protease degradation [102].

Besides the effects on proteins containing iron-sulfur clusters, we also observed the consequence of iron limitation on certain genes (PFL_4964, *iscU*; and PFL_4966, *iscR*) in the well-conserved iron-sulfur cluster assembly operon *iscRUA* (PFL_4959-66) [103]. In *E. coli*, IscR functions as a repressor for the *iscRUA* operon when bound to an iron-sulfur cluster, which can be disrupted when the iron level is low, resulting in derepression of the operon [103]. In our study, iron limitation resulted in transcriptional up-regulation of *iscR* but down-regulation of *IscU* production in Pf-5 (Table 6). The apparent conflict in the influence of iron on expression of two genes in a single polycistronic transcript could be due to post-transcriptional regulation of IscU. In *E. coli*, the post-transcriptional regulation of the *iscRUA* polycistronic transcript is mediated by RyhB [101] through partial degradation of the transcript, leaving only the transcript segment encoding IscR intact and capable of protein expression [104]. It is yet to be ascertained if *P. fluorescens* Pf-5 employs similar post-transcriptional regulatory control of its *iscRUA* operon.

Conclusions

In this study, we examined the transcriptomic and proteomic impact of iron limitation on the biocontrol bacterium *P. fluorescens* Pf-5 by evaluating iron-limited cultures in comparison to both FeCl₂- and FeCl₃-amended cultures. The effects of both forms of iron were very similar despite some variation in levels of transcriptional regulation for some genes. Under iron limitation, we observed changes in transcription of genes encoding iron homeostasis functions such as siderophore biosynthesis and other iron uptake systems. Based on the transcriptional data, it seems likely that, of the five *exbB-exbD-tonB* transport clusters found in Pf-5, the PFL_6067-69 gene cluster encodes proteins facilitating energy transduction of various TonB-dependent receptors expressed under iron limitation. Iron limitation resulted in a number of unexpected responses, particularly the increased transcription of the ribosomal protein L36 gene as well as reduced flagellar biosynthesis gene expression and motility. Interestingly, certain genes or proteins involved in biosynthesis of secondary metabolites such as 2,4-diacetylphloroglucinol (DAPG), orfamide A and

![Figure 5. Scatterplot of fold changes of transcript versus protein expression.](image-url)
Iron regulation of selected genes and proteins.

| Genes       | Annotated functions                                      | Fold change (log2) | Iron-deprived (-FeCl₂) | Iron-deprived (-FeCl₃) |
|-------------|----------------------------------------------------------|--------------------|------------------------|------------------------|
|             |                                                          | Transcript         | Protein                | Transcript             | Protein                |
| PFL_0613    | bacterioferritin family protein                          | NS                 | −0.76                  | NS                     | −1.33                  |
| PFL_0824    | ferric uptake regulation protein Fur                     | NS                 | 0.89                   | NS                     | 1.27                   |
| PFL_0907    | fumarate hydratase, FumC₁                                 | 3.93               | 3.89                   | 2.16                   | 4.22                   |
| PFL_1629    | flagellin FliC                                           | NS                 | −0.26                  | NS                     | −0.69                  |
| PFL_2090    | chitin-binding protein                                   | NS                 | 1.52                   | NS                     | 2.09                   |
| PFL_2091    | chitinase ChIC                                           | NS                 | 1.97                   | NS                     | 2.11                   |
| PFL_2579    | hydrogen cyanide synthase HcnC                           | NS                 | −1.63                  | −1.36                  | −1.75                  |
| PFL_3496    | salicyl-AMP ligase PchD                                   | 1.11               | 2.12                   | NS                     | 2.02                   |
| PFL_3500    | FeCT family iron-chelate ABC transporter periplasmic iron-chelate-binding protein | 1.87               | 2.91                   | NS                     | 2.88                   |
| PFL_3604    | tryptophan halogenase PmA                                | NS                 | 1.84                   | NS                     | 1.79                   |
| PFL_4088    | chromophore maturation protein PvdN                      | NS                 | 2.88                   | NS                     | 3.57                   |
| PFL_4089    | chromophore maturation protein PvdO                      | NS                 | 1.36                   | NS                     | 1.82                   |
| PFL_4092    | TonB-dependent outermembrane ferripoverdine receptor FpvA | NS                 | 1.88                   | NS                     | 1.18                   |
| PFL_4179    | diaminobutyrate−2-oxoglutarate aminotransferase PvdH     | NS                 | 0.90                   | NS                     | 1.38                   |
| PFL_4328    | fumarate hydratase FumC₃                                  | NS                 | 1.47                   | NS                     | 1.00                   |
| PFL_4628    | heme oxygenase HemO                                      | 2.28               | 2.49                   | NS                     | 3.41                   |
| PFL_4826    | superoxide dismutase (Fe) SodB                           | −1.53              | −1.01                  | −1.13                  | 0.32                   |
| PFL_5266    | hemin ABC transporter periplasmic hemin-binding protein PhuT | 1.01               | 1.02                   | NS                     | 1.22                   |
| PFL_5556    | catalase KatA                                            | −1.37              | −3.19                  | NS                     | −2.75                  |
| PFL_5964    | FeT family ferric iron ABC transporter periplasmic ferric iron-binding protein | 1.78               | 1.65                   | NS                     | 1.94                   |

NS means not significant when analyzed with SAM at FDR <1% in this study. Numbers in bold denote fold changes that surpass the significant differential expression threshold defined in this study, i.e. equal to or exceeding 2-fold and 1.5-fold changes for transcript and protein levels respectively.

1Transcript level of these gene differed significantly between cells grown in iron-limited versus iron-amended media when analyzed with SAM at FDR <5%.

Table 5.

Materials and Methods

Bacterial Strains and Growth Conditions

For microarray and proteomic analyses, Pseudomonas fluorescens Pf-5 was grown in M9 minimal media supplemented with 100 μM calcium chloride (CaCl₂), 2 mM magnesium sulphate (MgSO₄) and 0.4% glucose [107]. The iron-replete cultures were supplemented with either 100 μM iron(II) chloride (FeCl₂) or 100 μM iron(III) chloride (FeCl₃), while iron-limited cultures were not supplemented with iron in any form. Both iron stock solutions were prepared by dissolving the iron chloride crystals in deionized water. The cultures were grown aerobically with shaking at room temperature to late exponential phase; optical densities (at 600 nm wavelength) of harvested cultures were approximately 0.7 for iron-limited cultures and 1.1 for iron-amended cultures (Figure S1). The pH of iron-limited and iron-supplemented culture media was tested and found to be close to neutral.

RNA Extraction

Extraction of RNA was performed using the PureLink Microto-Midi Total RNA Purification System (Invitrogen) with an initial extraction using Trizol reagent (Invitrogen) as per the manufacturer’s instructions. The concentration and purity of mRNA extracted was determined spectrophotometrically using a NanoDrop ND1000 spectrophotometer (NanoDrop Technologies).

cDNA Synthesis and Labeling

Reverse transcription of RNA samples was performed using the SuperScript Plus Indirect cDNA Labeling System (Invitrogen) with random hexamer primers. Approximately 7 μg of total RNA was used for cDNA synthesis in each reaction. The cDNA samples were labeled with either Alexa Fluor 555 or Alexa Fluor 647 (Invitrogen).

Microarray Experiments

The quality of labeled cDNA was confirmed spectrophotometrically using a Nanodrop ND1000 spectrophotometer (NanoDrop Technologies), prior to hybridization. Microarray slides spotted with 70-mer DNA oligonucleotides representing almost all of the...
Table 6. Iron regulation of selected genes encoding proteins with iron-sulfur cluster binding motifs or involved in iron-sulfur cluster assembly.

| Genes     | Annotated functions       | Fold change (log2) | Iron-deprived (-FeCl2) | Iron-deprived (-FeCl3) |
|-----------|---------------------------|-------------------|------------------------|------------------------|
| PFL_0330  | formate dehydrogenase subunit beta FdsB | N.S. | -1.85 | -0.62 |
| PFL_1208  | ferredoxin FdsA           | N.S. | -1.97 | N.S. |
| PFL_1241  | ferredoxin-NADP reductase Fpr_1 | -0.65 | -1.44 | N.S. |
| PFL_1666  | flagellar synthesis regulator FleN | N.S. | ND | -1.55 |
| PFL_1912  | oxygen-independent coproporphyrinogen III oxidase HemN | -1.23 | ND | -1.52 |
| PFL_1917  | cytochrome c oxidase accessory protein CooG | -1.58 | ND | -0.72 |
| PFL_1929  | aconitate hydratase 1 AcnA | -1.44 | ND | -0.95 |
| PFL_2063  | isopropylmalate isomerase large subunit LeuC | N.S. | -0.68 | N.S. |
| PFL_2578  | hydrogen cyanide synthase HcnB | -1.26 | -0.29 | -1.49 |
| PFL_2633  | bifunctional aconitate hydratase 2/2-methylisocitrate dehydratase AcnB | 0.29 | -1.57 | N.S. |
| PFL_2835  | sulfite reductase (NADPH) hemoprotein subunit beta CysL_1 | -1.45 | -2.51 | -0.51 |
| PFL_2869  | cytochrome c oxidase accessory protein CooG | -1.04 | ND | N.S. |
| PFL_2917  | oxidoreductase membrane protein, FAD-binding | -1.93 | ND | -2.31 |
| PFL_3902  | NADH dehydrogenase subunit G NuoG | -0.75 | -1.38 | N.S. |
| PFL_3904  | NADH-quinone oxidoreductase, I subunit Nuol | -1.03 | ND | N.S. |
| PFL_4801  | fumarate hydratase, class I | -0.76 | -4.92 | N.S. |
| PFL_4858  | bacterioferritin-associated ferredoxin, putative | N.S. | NA | 4.83 |
| PFL_4964  | scaffold protein IscU | 0.87 | -0.76 | N.S. |
| PFL_4966  | iron-sulfur cluster assembly transcription factor IscR | N.S. | ND | 1.29 |
| PFL_5080  | ubiquinol-cytochrome c reductase, iron-sulfur subunit PetA | -1.41 | ND | N.S. |
| PFL_5693  | biotin synthase BioB | 1.69 | -1.57 | N.S. |
| PFL_5877  | dihydroxy-acid dehydratase IlvD | N.S. | -3.14 | N.S. |

* Pf-5 genes that contain Fur binding motifs upstream [35]. NS means not significant when analyzed with SAM at FDR <1% in this study. ND means protein is not detected from database search of the proteomic mass spectrometry data. Numbers in bold denote fold changes that surpass the significant differential expression threshold defined in this study, i.e. equal to or exceeding 2-fold and 1.5-fold changes for transcript and protein levels respectively.

open reading frames annotated in the genome of Pf-5 [35]. Each microarray experiment consisted of at least three biological replicates and three technical replicates. Flip-dye experiments were also performed to ensure a lack of dye bias in the results. The hybridization procedure was performed as described previously [108]. Hybridized slides were scanned with an Axon 4000B scanner with GenePix 4.0 software. The microarray data was analyzed with Spotfinder (TIGR) and normalized using the LOWESS algorithm in the TIGR-MIDAS package with block mode and a smoothing parameter value of 0.33. Genes significantly regulated by iron were identified using the Statistical Analysis of Microarrays (SAM) algorithm with a false discovery rate (FDR) of less than 1% [109]. Comparisons between transcriptional profiles were performed with MultiExperiment Viewer (MeV) 4.1 software [110]. A fold change of at least 2 was considered to be significantly differentially expressed. The microarray datasets have been deposited in the Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo/) with the accession number GSE29322. The details provided for the microarray datasets are in compliance with the MIAME guideline [111].

qRT-PCR Validation of Microarray

Quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) analyses were conducted on a selection of differentially expressed genes to validate the microarray results. For each sample, 2.5 μg of RNA was reverse-transcribed to cDNA with the SuperScript VILO cDNA Synthesis Kit (Invitrogen) according to the manufacturer’s instructions. The resulting cDNA samples were used as template for 10 μl real-time PCR reactions containing EXPRESS SYBR GreenER qPCR SuperMix with Premixed ROX (Invitrogen) and gene-specific primers. The primers were designed using Primer3 software [112] and tested for their amplification efficiencies. Genes encoding ribosomal protein S7 and S12 (PFL_5586 and PFL_5587, respectively) were utilized as internal controls versus experimental samples [113].

Motility Assay

Motility tests were performed to determine the effect of iron-limited conditions on swarming phenotypes. Tests were performed on 0.6% agar-containing M9 minimal media plate supplemented with 100 μM CaCl2, 2 mM MgSO4 and 0.4% glucose. Varying concentrations of FeCl2, from 0 to 100 μM, were added into the agar plates. Overnight cultures of Pf-5 were
diluted to OD<sub>600</sub> = 0.2, 2 μl was placed on the center of the agar plate, and plates were incubated at 25°C for two days. A galA mutant [JL4577] [35] served as a swarming motility negative control.

**iTRAQ Labeling of Protein Samples**

Cultures of *P. fluorescens* Pf-5 cultures were grown in M9 broth medium as described above. The bacterial pellets from cultures were washed with phosphate-buffered saline solution (pH 7.4) before being lysed in lysis buffer [20 mM HEPES, 150 mM sodium chloride (NaCl), DNase I (Sigma-Aldrich), and protease inhibitor cocktail (Sigma-Aldrich)] by mechanical lysis in a FastPrep FP120 bead beater (BIO101/Savant, Q-Biogene) at 5.5 Throw for 30 seconds. The concentrations of the harvested protein extracts were determined using Bradford assays (Bio-Rad) and the quality examined with SDS-PAGE. An 8-plex iTRAQ (isobaric tag for relative and absolute quantitation) system (Applied Biosystems) was used. The protein samples were reduced and alkylated with 100 mM tris-2-carboxyethylphosphine (TCEP) and proteolysed with trypsin for 16 hours at 37°C. The samples were labeled with iTRAQ tags (Applied Biosystems) according to the manufacturer’s protocol. Three biological replicates of iron-limited cells (labeled with iTRAQ 113, 116 and 119) were used. The samples were grown in three batches where two batches consisted of iron-limited, FeCl<sub>2</sub>-supplemented cultures or iron-limited versus FeCl<sub>3</sub>-supplemented cultures (constrained due to limited number of iTRAQ labels available).

**Strong Cation Exchange Fractionation**

Strong cation exchange chromatography was performed in an Agilent 1100 quaternary HPLC pump (Agilent Technologies) with a PolyC PolySulfoethyl A 200 mm × 2.1 mm 1.5 μm 200A column. The buffers used were buffer A [5 mM phosphate, 25% acetonitrile (CH<sub>3</sub>CN), pH 2.7] and buffer B [5 mM phosphate, 350 mM potassium chloride (KCl), 25% CH<sub>3</sub>CN, pH 2.7]. Labeled samples were suspended in buffer A and loaded into the column. The peptides were separated as the buffer B concentration was increased from 10% to 45% over a period of 70 minutes. Subsequently, the buffer B concentration was quickly increased to 100% for 10 minutes at a flow rate of 300 μl/min. The fractions collected were dried in a SpeedVac prior to analysis with nanoLC electrospray (MS/MS).

**Mass Spectrometry**

NanoLC electrospray (MS/MS) was performed using the Agilent 1100 nanoLC system (Agilent Technologies) and QStar Elite MS/MS system (Applied Biosystems). The samples were resuspended in 100 μl of loading/desalting solution [0.1% trifluoroacetic acid (TFA), 2% CH<sub>3</sub>CN and 97.9% H<sub>2</sub>O]. 40 μl of each sample was loaded into a reverse phase peptide Captrap (Michrom BioResources) and then desalted with the desalting solution at 10 μl per minute for 15 minutes. After this procedure, the trap was switched on line with a ProteoCol column (150 μm × 10 cm C18 3 μm 300A) (SGE GmbH). In Channel 1, the buffer consisted of 97.9% H<sub>2</sub>O, 0.1% formic acid (HCOOH), 2% CH<sub>3</sub>CN, Channels 2A and 2B contained 99.9% H<sub>2</sub>O, 0.1% HCOOH and 90% CH<sub>3</sub>CN, 9.9% H<sub>2</sub>O, 0.1% HCOOH, respectively. To elute the peptides from the column, the concentration of Channel 2B was changed from 5% to 100% at 500 nL per minute for a period of 93 minutes in three linear gradient steps. After peptide elution, the column was flushed with Channel 2B buffer and equilibrated with 95% Channel 2A buffer for 8 minutes before the next sample was injected. Peptides eluted from the reverse phase nanoLC were analyzed in an information-dependant acquisition mode (IDA) with positive ion nanoflow electrospray. A TOFMS survey scan was performed (m/z 300–1600, 0.5 second) in the IDA mode. The three most intense multiply charged ions (counts >70) were put into MS/MS analysis sequentially. A modified Enhanced All Q2 transition setting was utilized, which favours low mass ion detection so that iTRAQ label intensity signals were enhanced for quantification. The MS/MS spectra (mass range m/z 100–1600) were collected for 2 seconds.

**Proteomic Data Analysis**

ProteinPilot 4.0 software (Applied Biosystems) was used to analyze the MS/MS data generated. The amino acid sequence database for *P. fluorescens* Pf-5 was obtained from the NCBI nr database. A thorough ID search was performed using the Paragon method with Biological modifications selected in ID Focus, False Discovery Rate analysis enabled and Background Correction setting used. The iTRAQ reagent correction factors were entered into the iTRAQ Isotope Correction Factors table. For quantification purpose, the software will select peptides that meet its default set of criteria, such as having peptide ID confidence of at least 15.0%, iTRAQ modification and a good signal-to-noise ratio. The results of this analysis were filtered to include only proteins with at least 2 peptides detected in the MS/MS spectra with 95% confidence and having Unused ProtScore ≥2, which is the measure of the protein identification confidence where a score of 2 corresponds to a 99% confidence limit. Protein abundance ratios from comparison of iron-limited culture against iron-supplemented cultures from each sampling batch were calculated. The ratios for each category (i.e. either iron-limited versus FeCl<sub>2</sub>-supplemented cultures or iron-limited versus FeCl<sub>3</sub>-supplemented cultures) from all the batches were subsequently averaged and reported. A fold change of at least 1.5 was considered significant [89].

**Bioinformatics Analysis**

Useful information pertaining to iron-sulfur cluster binding motifs and predicted operons were garnered from Pseudomonas Genome Database [114] and EcoCyc [115]. Membrane association characteristics of the detected proteins from the proteomic experiment were evaluated as follows: hydrophobicity properties were calculated as grand average of hydropathy (GRAVY) scores [116] with Sequence Manipulation Suite [117], transmembrane spanning domains were examined using TMHMM v2.0 [118], and predicted localization of proteins were inferred using PSORTb v3.0.2 [119]. Conversions of identifiers between databases were facilitated by db2db software in bioDBnet [120].

**Supporting Information**

Figure S1 Growth curves of *P. fluorescens* Pf-5 in iron-limited minimal medium and in minimal medium amended with either FeCl<sub>2</sub> or FeCl<sub>3</sub>.

Figure S2 Role categories of genes (as defined by Hassan et al. [35]) of microarray data.
Figure S3 Validation of microarray data. (DOC)

Figure S4 Role categories of genes (as defined by Hassan et al. [35]) analysis of iTRAQ proteomic data. (DOC)

Table S1 Iron-regulated genes identified by SAM analysis using a false discovery rate (FDR) of less than 1% and at least a 2-fold change in transcript level. (DOC)

Table S2 Primer sequences for qRT-PCR validation of microarray data. (DOC)

Table S3 Regulation of genes with predicted upstream Fur binding sites. (DOC)

Table S4 Regulation of clusters containing genes encoding sigma factors, TonB-dependent receptors, and anti-sigma factor regulators as determined by SAM analysis. (DOC)

Table S5 Comparison of proteomic data from iTRAQ experiments versus transcriptomic data from microarray experiments. (XLS)

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Author Contributions

Conceived and designed the experiments: SGT ITP. Performed the experiments: CKL KA SG. Analyzed the data: CKL KA SG JEL. Contributed reagents/materials/analysis tools: ITP. Wrote the paper: CKL.

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