FvbA is required for vibriobactin utilization in *Pseudomonas aeruginosa*

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Bacteria acquire iron through a highly specific mechanism involving iron-chelating molecules termed siderophores. The Gram-negative bacterium *Pseudomonas aeruginosa* can utilize siderophores produced by other micro-organisms to facilitate iron uptake. Here we show that a *P. aeruginosa* strain deficient in siderophore production can use the *Vibrio cholerae* siderophore vibriobactin as an iron source. In addition, we identified a *P. aeruginosa* gene, PA4156 (*fvbA*), encoding a protein highly homologous to the *V. cholerae* vibriobactin receptor (ViuA). A *P. aeruginosa* mutant in the two endogenous siderophores (pyoverdine and pyochelin) and in *fvbA* was unable to utilize vibriobactin as an iron source. Additionally, preliminary analyses revealed the involvement of vibriobactin, Fur protein and an IclR-type regulator, FvbR (PA4157), in *fvbA* regulation.

INTRODUCTION

Iron is an essential element for nearly all known organisms, including most bacteria, which require iron for survival and growth. For pathogenic bacteria, iron acquisition is a major challenge, since this metal exists in insoluble mineral complexes or bound to mammalian high-affinity iron-binding proteins (Skaar, 2010). Therefore, bacteria have developed several mechanisms to obtain iron. One such system consists of iron-specific chelators termed siderophores (for a recent review, see Chu *et al.*, 2010). Under iron-limiting conditions, bacteria synthesize and secrete siderophores into the environment. The ferric siderophore is transported, in an energy-dependent manner, into the cell through a highly specific receptor protein located in the outer membrane (Braun & Hantke, 2011; Cornelis *et al.*, 2008; Noinaj *et al.*, 2010). In Gram-negative bacteria, the transport of the ferric siderophore is driven by the TonB-ExbB-ExbD protein complex that couples cytoplasmic membrane electrochemical potential to the outer membrane receptor (Noinaj *et al.*, 2010). The expression of the TonB-dependent siderophore receptor is tightly regulated by the level of iron (Noinaj *et al.*, 2010). Furthermore, in several cases, the siderophore itself acts as a signal and induces the expression of its cognate receptor (Beare *et al.*, 2003; Llamas *et al.*, 2006; Neilands, 1995). The binding of the siderophore to its receptor induces the expression of

various genes, including genes required for siderophore uptake, and can be regulated through several regulatory pathways. One of the mechanisms involves an extracytoplasmic function (ECF) sigma/anti-sigma factor system (Visca *et al.*, 2002). For example, in *Escherichia coli*, ferric citrate binds to an outer membrane receptor, FecA, that transmits the signal across the membrane to a transmembrane anti-sigma factor, FecR. This induces a conformational change, which allows the release of the FecI sigma factor protein from FecR, leading to activation of transcription of the *fecABCDE* operon (Braun *et al.*, 2005). Another regulatory pathway involves two-component systems, for example Pfs/R, which are required for the enterobactin-dependent production of the ferrienterobactin receptor PfsA in *Pseudomonas aeruginosa* (Dean & Poole, 1993). Finally, Arc-type transcription regulators have been shown to regulate the synthesis of siderophores and siderophore receptors. In *P. aeruginosa*, the AraC-type regulator PchR activates the expression of both the pyochelin and the ferripyochelin receptor (Heinrichs & Poole, 1996). These various mechanisms enable bacteria to directly respond to the binding of a specific siderophore and to tightly regulate the expression of relevant genes.

The opportunistic pathogen *P. aeruginosa* is known to produce and utilize two chemically distinct siderophores, pyochelin (Cox, 1980) through the FptA receptor (Ankenbauer & Quan, 1994; Hoegy *et al.*, 2009; Michel *et al.*, 2007), and pyoverdine (Cox & Adams, 1985) through two receptors, FpvA and FpvB (Poole *et al.*, 1993; Schalk, 2008). In addition to its endogenous siderophores, *P. aeruginosa* can utilize siderophores produced by other micro-organisms. It has been shown that *P. aeruginosa* has

Abbreviations: ECF sigma factor, extracytoplasmic function sigma factor; EDDA, ethylenediamine di-ortho-hydroxyphenylacetic acid; FURTA, Fur titration assay.

Two supplementary figures are available with the online version of this paper.

two receptors that mediate the uptake of the *E. coli* siderophore enterobactin, PfeA and PirA (Dean & Poole, 1993; Ghysels *et al.*, 2005). In addition, *P. aeruginosa* is capable of utilizing the *Mycobacterium paratuberculosis* siderophore mycobactin through FemA (Llamas *et al.*, 2008), ferrichrome through FiuA, ferrioxamine through FoxA (Llamas *et al.*, 2006), citrate through FecA (Cox, 1980), aerobactin through ChtA (Cuív *et al.*, 2006), and the siderophores cepabactin (Mislin *et al.*, 2006), desferrichrysin, desferricrocine and coprogen (Meyer, 1992) through unidentified receptors. It is interesting to note that the above-mentioned receptors probably represent only a part of the total iron-acquisition potential of *P. aeruginosa*. An *in silico* analysis of the *P. aeruginosa* genome has revealed an impressive total of 34 genes encoding putative TonB-dependent outer membrane receptors (Cornelis & Matthijs, 2002). However, only a fraction of these genes have been experimentally validated thus far. Combined with the endogenous siderophores, the utilization of heterogeneous siderophores may provide *P. aeruginosa* with the ability to 'steal' iron from other micro-organisms, providing a competitive advantage in the environment.

The facultative anaerobic bacterium *Vibrio cholerae* produces only one known catechol siderophore, called vibriobactin (Griffiths *et al.*, 1984). Since both *V. cholerae* and *P. aeruginosa* are common in aquatic environments, we investigated whether *P. aeruginosa* can utilize vibriobactin for iron uptake. In the current study we show that *P. aeruginosa* can utilize vibriobactin, a siderophore produced by *V. cholerae*, as an iron source when grown in iron-deficient medium, through a newly identified receptor, FvbA (PA4156). Additionally, we show that *fvbA* expression is tightly regulated by Fur, by vibriobactin itself as well as by an IclR-type regulator, FvbR (PA4157).

METHODS

Bacterial strains and growth conditions. Bacterial strains and plasmids used in this study are listed in Table 1. M9 minimal medium was used as a low-iron medium (Becton Dickinson; containing 0.4 % glucose, 2 mM MgSO₄, 0.1 mM CaCl₂). The iron chelator ethylenediamine di-ortho-hydroxyphenylacetic acid (EDDA; Fluka) was added to the growth medium to induce iron starvation. Purified siderophores (EMC Microcollections GmbH) were dissolved in double-distilled water (deferoxamine and ferrichrome), methanol (vibriobactin; Bio-Lab) or DMSO (enterobactin and aerobactin; Sigma). For induction of *fvbR* overexpression, arabinose was added to a final concentration of 1 mM.

Construction of *P. aeruginosa* strains. The triple mutant *PAO1ΔpvdDΔpchEF fvbA::pSE-PA4156IS* was constructed as follows: a 420 bp DNA fragment corresponding to a region at the middle of *fvbA* gene was amplified and cloned into the TOPO cloning vector (Invitrogen) using 5'-AAGCTTTTCTCCACAAACGACAACAA-3' and 5'-TCTAGACTAGAAGGTGCCCTTGATGC-3' as primers. The resulting plasmid, pSE-PA4156IS, was constructed in *E. coli* DH5 α , purified, and transferred into *P. aeruginosa* *PAO1ΔpvdDΔpchEF* by electroporation (Choi & Schweizer, 2006). Transconjugates were selected on LB agar (Becton Dickinson) containing carbenicillin

(200 μ g ml⁻¹), resulting in a marked *PAO1ΔpvdDΔpchEF fvbA::pSE-PA4156IS* (PASE2) strain.

For *fvbA* complementation, the *fvbA* gene (coordinates 4653 103–4650 208 on the *P. aeruginosa* chromosome) was amplified by PCR using 5'-AGAGAAAGCTTGAAATAGGCGCTGCGGTA-3' and 5'-AGAGGGATCCGATGCCTTCGATGTTGCTGT-3' as primers. The PCR product was digested with *Hind*III and *Bam*HI and ligated with *Hind*III/*Bam*HI-digested pUCP18. The resulting plasmid, pSE-PA4156COM, was purified and transferred into *PAO1ΔpvdDΔpchEF fvbA::pSE-PA4156IS* (PASE2) strain by electroporation (Choi & Schweizer, 2006) to create strain PASE3.

The *fvbA*–*gfp* promoter fusion strain was constructed as follows: a fragment containing the *fvbA* promoter (500 bp upstream from the *fvbA* start codon; coordinates 4650 374–4650 874 on the *P. aeruginosa* chromosome) was amplified by PCR using 5'-AAAAGCTTAGGTCGTAGAGACCGCTGAG-3' and 5'-AAGAAATTCGCGTTCAAGGATCTTCTCCA-3' as primers. The PCR product was digested with *Hind*III and *Eco*RI and ligated with a *Hind*III/*Eco*RI-digested plasmid with a *gfp* transcription fusion in mini-CTX to create pSE-PR4156GFP. This plasmid was constructed in *E. coli* DH5 α , purified and transferred into *E. coli* SM10 (by chemical transformation). The plasmid was mobilized from *E. coli* SM10 into *P. aeruginosa* PAO1 by conjugation. Transconjugates were selected on *Pseudomonas* Isolation Agar (Becton Dickinson) containing tetracycline (100 μ g ml⁻¹). The insertion was confirmed by PCR analysis. One transconjugate, PASE4, was further characterized for its GFP expression.

The triple mutant *PAO1ΔpvdDΔpchEF PA4157::pSE-PA4157IS* was constructed as follows: a 395 bp DNA fragment corresponding to a region at the middle of PA4157 gene was amplified by PCR using 5'-ATATGAATTCCTCAGCGTCTCTACGACCT-3' and 5'-ATATAAGCTTAGCAGGTGGCTGGTCAGTT-3' as primers. The PCR product was digested with *Hind*III and *Eco*RI and ligated with *Hind*III/*Eco*RI-digested pEX18Amp. The resulting plasmid, pSE-PA4157IS, was constructed in *E. coli* DH5 α , purified, and transferred into *P. aeruginosa* *PAO1ΔpvdDΔpchEF* by electroporation (Choi & Schweizer, 2006). Transconjugates were selected on LB agar (Becton Dickinson) containing carbenicillin (200 μ g ml⁻¹), resulting in a marked *PAO1ΔpvdDΔpchEF fvbR::pSE-PA4157IS* strain (PASE7).

Strain PASE6, which overexpresses PA4157, was constructed as follows: a 805 bp DNA fragment containing the PA4157 gene (coordinates 4652 714–4653 518 on the *P. aeruginosa* chromosome) was amplified by PCR using 5'-TATAGAATTCATGCCGACGCCCGG-3' and 5'-ATATGGATCCGCCGGGCTGAGGTGGCTC-3' as primers. The PCR product was digested with *Eco*RI and *Bam*HI and ligated with *Eco*RI/*Bam*HI-digested pJN105 plasmid to create pED-PA4157OE. This places the *fvbA* gene under the arabinose-inducible promoter P_{BAD}. The resulting plasmid was constructed in *E. coli* DH5 α , purified, and transferred into *P. aeruginosa* *PAO1ΔpvdDΔpchEF* by electroporation (Choi & Schweizer, 2006), and transconjugates were selected on LB agar (Becton Dickinson) containing carbenicillin (200 μ g ml⁻¹).

RNA isolation, RT-PCR, and real-time PCR analysis. PASE1, PASE5 and PAO1 overnight cultures were diluted into fresh M9 minimal medium to OD₅₉₅ 0.01 and grown to mid-exponential phase (OD₅₉₅ 0.5) twice. The culture was then distributed into fresh tubes and EDDA (25 μ g ml⁻¹), FeCl₃ (100 μ M) or vibriobactin (2.5 μ M) was added and cultures were grown for an additional 3 h. RNeasy Protect Bacteria Reagent (Qiagen) was added to stabilize the RNA. Total RNA was isolated using an RNeasy spin column (including an on-column DNase I step) according to the manufacturer's instructions (Qiagen). cDNA was synthesized from 2 μ g RNA using the SuperScript II Synthesis System and random hexamers (Invitrogen). Real-time PCR was performed using a StepOnePlus

Table 1. Strains and plasmids used in this studyAbbreviations: Amp^r, ampicillin resistance; Carb^r, carbenicillin resistance; Gm^r, gentamicin resistance; Km^r, kanamycin resistance.

Strain or plasmid	Description	Reference or source
<i>P. aeruginosa</i> strains		
PAO1	Wild-type	Holloway <i>et al.</i> (1979)
PASE1	PAO1 Δ pvdD Δ pchEF	Banin <i>et al.</i> (2005)
PASE2	PAO1 Δ pvdD Δ pchEF <i>fvbA</i> ::pSE-PA4156IS	This study
PASE3	PAO1 Δ pvdD Δ pchEF <i>fvbA</i> ::pSE-PA4156IS carrying pSE-PA4156COM	This study
PASE4	PAO1 Δ pvdD Δ pchEF carrying pSE-PR4156GFP	This study
PASE5	PAO1, <i>ISphoA/hah PA4157</i>	Jacobs <i>et al.</i> (2003)
PASE6	PAO1 Δ pvdD Δ pchEF carrying pED-PA4157OE	This study
PASE7	PAO1 Δ pvdD Δ pchEF <i>fvbR</i> ::pSE-PA4157IS	This study
<i>E. coli</i> strains		
DH5 α	F' <i>endA1 hsdR17 supE44 thi-1 recA1 gyrA relA1 Δ(lacZYA-argF)U169 deoR</i>	Woodcock <i>et al.</i> (1989)
SM10 λ pir	<i>thi-1 thr leu tonA lacY supE recA</i> ::RP4-2-Tc::Mu Km ^r λ pir	de Lorenzo & Timmis (1994)
H1717	<i>aroB shuF</i> :: λ placMu	Leoni <i>et al.</i> (1996)
ECSE3	H1717 carrying pSE-pvdSFURTA	This study
ECSE1	H1717 carrying pSE-4156FURTA1	This study
ECSE2	H1717 carrying pSE-4156FURTA2	This study
Plasmids		
pUCP18	Broad-host-range cloning vector; Amp ^r	Schweizer (1991)
pSE-PA4156COM	pUCP18 carrying <i>fvbA</i> gene for complementation; Carb ^r	This study
TOPO-TA	PCR product cloning vector; Amp ^r	Invitrogen
pSE-PA4156IS	TOPO carrying 420 bp PCR product corresponding to a region in <i>P. aeruginosa fvbA</i> fragment; Carb ^r	This study
mini-CTX1	Self-proficient integration vector with <i>tet</i> , V-FRT-attPMCS, <i>ori</i> , <i>int</i> and <i>oriT</i> ; Tc ^r	Hoang <i>et al.</i> (2000)
pSE-PR4156GFP	<i>fvbA</i> promoter- <i>gfp</i> transcription fusion in Mini-CTX- <i>lacZ</i> ; Tc ^r	This study
pJN105	<i>araC</i> -pBAD (broad-host-range vector); Gm ^r	Newman & Fuqua (1999)
pED-PA4157OE	pJN105 carrying PA4157 gene for overexpression; Carb ^r	This study
pBR322	Broad-host-range cloning vector; Amp ^r	Covarrubias & Bolivar (1982)
pSE-4156FURTA1	pBR322 carrying PA4156 full-length promoter; Amp ^r	This study
pSE-4156FURTA2	pBR322 carrying PA4156 truncated promoter; Amp ^r	This study
pSE-pvdsFURTA	pBR322 carrying <i>pvdS</i> promoter; Amp ^r	This study
pEX18Amp	Broad-host-range gene replacement vector; <i>sacB</i> ; Amp ^r	Hoang <i>et al.</i> (1998)
pSE-PA4157IS	pEX18 Amp carrying 395 bp PCR product corresponding to a region in <i>P. aeruginosa</i> PA4157 fragment; Carb ^r	This study

Real-Time PCR system (Applied Biosystems). The 20 μ l PCR included 15 ng cDNA, 1 $\times$ Fast SYBR Green Master Mix (Applied Biosystems) and 0.4 μ M each primer. The primer pairs used were: *pvdS*-RT-PCR-58F (5'-GATAACCGTACGATCCTGGTGAAT-3') and *pvdS*-RT-PCR-138R (5'-CGCATCCTGGACCACATCTT-3'), PA4156-RT-PCR-1920F (5'-CGCCAGCTACCGCTTCAA-3') and PA4156-RT-PCR-2013R (5'-CCGGTTTTCCTGCATGTAGGT-3'), PA1684-2F (5'-TGAGCAGCCTTACCGTCTATCA-3') and PA1684-88R (5'-CCAGG-GTCAAGCGATGT-3'), PA4157-RT-PCR370F (5'-TGGTACCGCA-GCGCCTAT-3') and PA4157-RT-PCR437R (5'-GTATTGTGCATGG-GCAGGATATC-3'), and *pfeA*-RT-PCR-F (5'-CTACGGGCAAAGCA-CCAGTT-3') and *pfeA*-RT-PCR-R (5'-GCTGTACTCGATGCCCA-GTTC-3'). The reactions were performed in Optical 8-Tube Strips (Applied Biosystems) at 95 °C for 20 s, followed by 40 cycles of 95 °C for 30 s and 60 °C for 30 s. Data were collected after each cycle. Following the PCR, a melting curve was performed from 60 to 95 °C. A standard curve was performed for each primer pair to check the efficiency of the reactions.

Construction of Fur consensus logo. Fur binding sequences of 36 iron-regulated genes in *P. aeruginosa*, identified by two microarray studies (Ochsner *et al.*, 2002; Palma *et al.*, 2003), were extracted from

a cooperative Fur binding model designed by van Oeffelen *et al.* (2008). The sequence logo was generated using Weblogo at <http://weblogo.berkeley.edu>.

Fur titration assay (FURTA). FURTA was performed as described by Stojiljkovic *et al.* (1994). The putative promoter region *P_{fvbA}* was amplified by PCR using 5'-GGCTGCGAAGTATTCAACG-3' and 5'-GCAGGGTGGACAGCGTGAC-3' as primers to obtain a 305 bp fragment containing the full-length promoter including the predicted Fur box, and 5'-GGCTGCGAAGTATTCAACG-3' and 5'-GGAT-CTTCTTTTCCATTTCAGGCTATCACGCCACC-3' as primers to obtain a 214 bp fragment containing the truncated promoter without the Fur box. PCR products were cloned into *EcoRV*-digested pBR322 (NEB) to create pSE-4156FURTA1 and pSE-4156FURTA2. The resulting plasmids were transformed into *E. coli* H1717 and sequenced. *E. coli* H1717 strains carrying plasmids were grown on MacConkey lactose agar (Difco) supplemented with 100 μ M ampicillin and different FeCl₃ concentrations (0, 20, 40, 50, 60, 70, 120 or 150 μ M), and colony phenotype was examined after 24 h incubation at 37 °C. Red colonies (Lac⁺) indicate Fur protein binding to the Fur box in the promoter region. The promoter of *pvdS* (from base pairs 2 721 925 to 2 722 175) cloned into pBR322 (pSE-*pvdS*FURTA) and

transformed into *E. coli* H1717 served as a positive control for the FURTA experiment.

RESULTS AND DISCUSSION

Vibriobactin supports the growth of a *P. aeruginosa* siderophores mutant under iron-deficient conditions

It is known that strains of *P. aeruginosa* deficient in pyoverdine and pyochelin production fail to grow in iron-limited minimal medium containing the synthetic iron chelator EDDA, probably because they are incapable of utilizing EDDA-bound iron for growth (Ankenbauer *et al.*, 1986). Based on this phenotype, we examined the ability of a *P. aeruginosa* mutant in siderophores production to grow in the presence of the *V. cholerae* siderophore vibriobactin and EDDA. The *P. aeruginosa* double mutant in the *pvdD* and *pchEF* genes (PASE1) was grown in iron-deficient minimal medium with and without EDDA, or EDDA and vibriobactin. As mentioned above, EDDA inhibited the growth of the *P. aeruginosa* PASE1 mutant compared with the wild-type PAO1 (Fig. 1, black bars). However, the addition of vibriobactin restored the growth of this mutant in EDDA-containing medium (Fig. 1, grey bars). This result suggests that *P. aeruginosa* can utilize vibriobactin as an iron source, perhaps through a vibriobactin-specific transport system.

FvbA is a ferric vibriobactin receptor in *P. aeruginosa*

In order to identify the ferrivibriobactin transport system in *P. aeruginosa*, we examined the *P. aeruginosa* genome and searched for genes with high homology to the *V. cholerae* vibriobactin uptake system. We identified one ORF, PA4156, annotated as a probable TonB-dependent receptor (*Pseudomonas* Genome Project database) with 48 % similarity to ViuA (Protein ID YP_001217728). ViuA

is a 74 kDa outer membrane protein that serves as a receptor for ferric vibriobactin in *V. cholerae* (Stoebner *et al.*, 1992). Bioinformatic analysis revealed that the *P. aeruginosa* PA4156-encoded protein contained TonB-dependent receptor characteristics, including an N-terminal 'plug' domain (residues 55–171) and a predicted β -barrel structure. Therefore, we named the PA4156 gene *fvbA* for ferric vibriobactin receptor. To test whether FvbA was responsible for vibriobactin uptake in *P. aeruginosa*, we constructed a triple mutant, PASE2 (*PAO1* Δ *pvdD* Δ *pchEF* *fvbA*::pSE4156IS), containing an insertion in the *fvbA* gene in the background of the PASE1 siderophores-deficient mutant and examined the effect of vibriobactin on its growth in iron-deficient medium. Similar to the parent strain PASE1, PASE2 grew in M9 medium and failed to grow in M9 containing EDDA. However, unlike the parental strain, vibriobactin was unable to restore the growth of PASE2 (Fig. 1, white bars), suggesting that FvbA is indeed required for vibriobactin utilization in *P. aeruginosa*. In support, complementation of the *fvbA* mutation *in trans* restored the ability of the PASE3 mutant strain to utilize vibriobactin (Fig. 1, striped bars).

In addition to the siderophore-specific outer membrane receptor, there are periplasmic and inner membrane proteins responsible for the transport of the ferric siderophores across the periplasm and the inner membrane. Interestingly, downstream of the *fvbA* gene there is a gene cluster, the *fepBDG* operon (PA4159–PA4161) and the *fepC* gene (PA4158), which are annotated as genes that may be involved in ferric enterobactin utilization. The FepB and FepC proteins in *E. coli* have been shown to be located in the periplasm and in the cytoplasmic membrane, respectively, and transport catecholate ferric siderophores (Pierce & Earhart, 1986). Since both enterobactin and vibriobactin belong to the catecholate siderophore family, we examined the involvement of these genes in vibriobactin uptake. For this we used the PASE1 strain to construct a mutant carrying an insertion in the *fepBDG* operon. Growth in the presence of EDDA and vibriobactin

Fig. 1. Effect of vibriobactin on growth of *P. aeruginosa* strains in iron-deficient conditions. *P. aeruginosa* strains PAO1 (black bars), PASE1 (grey bars), PASE2 (white bars) and *fvbA* complemented mutant PASE3 (hatched bars) were grown overnight on M9 minimal medium with or without 25 μ g EDDA ml⁻¹ or 25 μ g EDDA ml⁻¹ and 2.5 μ g vibriobactin ml⁻¹. All cultures were incubated at 37 °C with shaking (250 r.p.m.). Bacterial growth (OD₅₉₅) was measured after 12 h. Error bars, SD.

was not impaired in this mutant (data not shown), suggesting either that this gene cluster is not involved in vibriobactin uptake or that there is a redundancy in these uptake systems.

The *fvbA* promoter contains a Fur binding sequence

Our next goal was to understand how *fvbA* expression is regulated in the bacterium. Many of the iron-regulated genes are controlled by the ferric uptake regulator (Fur), a repressor that responds to the intracellular iron concentration. Under iron-replete conditions, Fur binds Fe(II), undergoes changes in configuration and binds to specific promoter sequences, 19 bp long, known as Fur boxes (Escobar *et al.*, 1999). Sideropore receptors are among the Fur-regulated genes in *P. aeruginosa*; moreover, the promoter region of PA4156 has been demonstrated to contain a Fur binding consensus sequence (van Oeffelen *et al.*, 2008). In order to determine whether this putative Fur box (Fig. 2a) is functional we performed the FURTA (Stojiljkovic *et al.*, 1994). In this assay an enterobactin-deficient *E. coli* strain carrying a *fhuF::lacZ* chromosomal fusion with low affinity for Fur-Fe(II) was used as a surrogate; the presence of a high-copy plasmid with a Fur binding site titrates out the Fur pool, resulting in de-repression of *fhuF::lacZ* and in a Lac⁺ phenotype (Stojiljkovic *et al.*, 1994). We examined two constructs, one with the full-length *fvbA* promoter containing the

putative Fur box (ECSE1) and one with a truncated promoter, missing this motif (ECSE2). The ECSE1 strain carrying *fvbA* full-length promoter demonstrated a red colony phenotype (Lac⁺) at iron concentrations up to 120 µM, indicating that the Fur protein can bind the *fvbA* promoter (Fig. 2b). The ECSE2 strain carrying a truncated *fvbA* promoter without the Fur predicted sequence displayed a Lac⁺ phenotype at iron concentrations lower than 40 µM (similar to the negative control strain), indicating that the Fur protein was unable to bind the truncated promoter (Fig. 2b). These results demonstrate that the predicted Fur box sequence indeed serves as the Fur binding site in the *fvbA* promoter. As a positive control we used the *pvdS* promoter region. PvdS is an ECF sigma factor that is required for pyoverdine synthesis and is known to be repressed under iron-replete conditions due to the presence of a Fur binding sequence in its promoter region (Cunliffe *et al.*, 1995). This construct showed a strong Lac⁺ phenotype even at iron concentrations above 150 µM. Therefore, we conclude that the Fur box of *fvbA* is most likely not a strong Fur binding sequence.

Vibriobactin specifically induces *fvbA* expression

The presence of a Fur box in the regulatory region of the *fvbA* gene suggests that iron availability affects its expression. Using real-time PCR we examined the effect of iron on *fvbA* expression. As a control, we also examined the expression of *pvdS*. Consistent with previous data, both

Fig. 2. Characterization of a Fur box in the *fvbA* promoter region. (a) Fur consensus sequence predicted in the *fvbA* promoter region. The Fur consensus sequence logo was derived from Fur binding sites of 36 iron-controlled genes in *P. aeruginosa* identified by microarray studies. Letter heights indicate the frequency with which a given base is represented at each position. The *fvbA* putative Fur box is presented below the schematic. Upper-case type represents residues that match the base in the corresponding position within the Fur consensus sequence. (b) FURTA. H1717 *E. coli* strains containing the *fvbA* putative full-length promoter region or a truncated promoter without the Fur box were grown on MacConkey lactose agar plates supplemented with 100 µM ampicillin and 20, 40, 50, 60, 70, 120 or 150 µM FeCl₃. *E. coli* strain H1717 containing the *pvdS* promoter was used as a positive control, and H1717 alone as a negative control. Plates were incubated overnight at 37 °C and LacZ activity was determined by colony colour (red for Lac⁺ and white for Lac⁻).

(b)

Strain/ construct	Lac ⁺ phenotype (FeCl ₃)
P _{pvdS} positive control	≥150 µM
H1717 negative control	≤40 µM
<i>fvbA</i> full-length promoter	≤120 µM
<i>fvbA</i> truncated promoter	≤40 µM

pvdS and *fvbA* were repressed, 17- and 1.5-fold, respectively, when iron was added to the medium (100 μ M; Fig. 3). The fairly low repression by iron of *fvbA* expression supports the results obtained with the FURTA assay, suggesting a weak Fur box. However, in some cases, the siderophore itself can act as a signal for the expression of its cognate receptor. For example, the expression of pyoverdine uptake genes is enhanced in the presence of pyoverdine (Beare *et al.*, 2003), and *pfeA* expression is activated by enterobactin (Dean & Poole, 1993). To examine whether *fvbA* expression can also be induced by the siderophore itself, we measured the transcript level of *fvbA* in the presence of vibriobactin (*pvdS* expression was also examined as a control). The expression of *fvbA* was not altered by the addition of EDDA, but was induced 12-fold when vibriobactin was added (Fig. 3). These results show that *fvbA* is specifically induced in the presence of vibriobactin. Supporting these findings, similar results were observed with an *fvbA* promoter fusion to GFP (see Supplementary Fig. S1). As expected, *pvdS* expression was induced 2.6-fold in the presence of EDDA. However, the addition of vibriobactin resulted in an approximately fivefold reduction in *pvdS* transcript levels, further indicating that in the presence of vibriobactin, *P. aeruginosa* is able to efficiently obtain iron from the environment (Fig. 3). To further characterize the *fvbA*-specific response to vibriobactin, we used a *fvbA* promoter fusion to GFP to test the ability of different siderophores to induce *fvbA*

expression. Generally, siderophores can be divided into three major groups according to their chemical structure: hydroxamate siderophores (including aerobactin, ferrichrome and deferoxamine), catecholate siderophores (including vibriobactin and enterobactin) and carboxylate siderophores (Miethke & Marahiel, 2007). As can be seen in Fig. 4, none of the tested siderophores, except for vibriobactin, was able to induce the expression of *fvbA*. This also included enterobactin, which, similarly to vibriobactin, belongs to the catecholate siderophore family. Furthermore, while enterobactin was able to induce the expression of its cognate receptor, *pfeA* (Supplementary Fig. S2), no change in *pfeA* transcript levels was observed in the presence of vibriobactin (data not shown). Our results support the hypothesis that despite the fact that both enterobactin and vibriobactin are catecholate siderophores, they seem to have distinct uptake systems in *P. aeruginosa*. Ghysels and colleagues have shown that a *P. aeruginosa* double mutant in the two enterobactin receptors, *pfeA* (PA2688) and *pirA* (PA0931), fails to utilize enterobactin as an iron source (Ghysels *et al.*, 2005), indicating that there is no additional enterobactin uptake system in the genome. Likewise, here we showed that *fvbA* expression was not induced by enterobactin (Fig. 4), and that the two enterobactin-uptake systems (i.e. PfeA and PirA, which are intact in our triple mutant PASE2) cannot support vibriobactin uptake.

Fig. 3. Vibriobactin induces *fvbA* expression in *P. aeruginosa*. *P. aeruginosa* mutant strain PASE1 was grown in M9 to the mid-exponential phase (OD_{595} 0.5) and then exposed for 3 h to the following conditions: M9, M9+EDDA (25 μ g ml^{-1}), M9+FeCl₃ (100 μ M) and M9+vibriobactin (2.5 μ g ml^{-1}). Total RNA was isolated and the relative quantities of *fvbA* (black bars) and *pvdS* (white bars) transcripts were measured using real-time PCR. PA1684 was used as a housekeeping reference gene and M9 alone was used as the reference condition. Error bars, SD.

Fig. 4. Siderophore-mediated FvbA expression in *P. aeruginosa*. *P. aeruginosa* mutant strain PASE4 carrying a chromosomal *fvbA* promoter-*gfp* fusion was grown in M9 to the mid-exponential phase (OD_{595} 0.5) and then exposed for 4 h to different siderophores: vibriobactin, enterobactin, aerobactin, ferrichrome or deferoxamine (DFO). *fvbA* expression was assessed by measuring fluorescence. The specific activity, i.e. relative fluorescence units (RFU) versus growth (OD_{595}), was determined, and the fold change in specific activity in response to siderophore addition is presented. Error bars, SD.

FvbR is involved in *fvbA* regulation

The expression of *fvbA* is induced in the presence of vibriobactin; however, a multiple sequence alignment with similar ferric siderophore receptors revealed that the FvbA receptor lacks the N-terminal extension (data not shown), a typical feature of TonB receptors involved in signal transduction. Since this region has been shown to take part in receptor-dependent gene expression (Noinaj *et al.*, 2010), the autoregulation of FvbA by the canonical sigma/anti-sigma factor is highly unlikely. Interestingly, the adjacent gene downstream to *fvbA*, PA4157, is annotated as a probable transcriptional regulator of the IclR family and contains a helix–turn–helix DNA binding motif in the N-terminal domain. Members of the IclR family are known to regulate diverse metabolic processes, including glycerol metabolism in *Streptomyces avermitilis* (Bolotin & Biro, 1990), degradation of acyl-homoserine lactones in *Agrobacterium tumefaciens* (Zhang *et al.*, 2004), catabolism of aromatic acids in *Pseudomonas* (Arias-Barrau *et al.*, 2004) and catechol catabolism in *Rhodococcus opacus* (Eulberg & Schlömann, 1998). The IclR regulators are very diverse and can act as activators or repressors, or can have a dual transcriptional activity (for a review, see Molina-Henares *et al.*, 2006).

In order to examine the possible involvement of PA4157 in the regulation of *fvbA*, we measured transcript levels of *fvbA* in the wild-type strain and the PA4157 mutant strain (PASE5) in the presence or absence of vibriobactin. Previously, we observed a 12-fold induction in *fvbA* expression in the presence of vibriobactin in the PASE1 mutant (Fig. 3). Real-time PCR analysis showed that vibriobactin was also able to induce *fvbA* expression in the wild-type strain, although to a lesser extent (Fig. 5a). Interestingly, the expression of *fvbA* in the PA4157 mutant remained high, independently of the presence of vibriobactin in the medium, suggesting that PA4157 plays a role in the regulation of *fvbA* and may act as a repressor (Fig. 5a). Based on these results, we termed PA4157 FvbR for ferric vibriobactin regulator. To further understand the involvement of FvbR in *fvbA* regulation, we used the PASE1 mutant to construct strains that either over-expressed or were defective in *fvbR* and examined their ability to utilize vibriobactin for growth. The ability of the PASE7 triple mutant to utilize vibriobactin was intact (Fig. 5b); however, overexpression of *fvbR* resulted in complete growth inhibition of this strain, despite the presence of vibriobactin in the medium (Fig. 5b), a phenotype similar to that of the PASE2 mutant strain (Fig. 1). These results further support the real-time PCR analysis that suggested that FvbR acts as a repressor of *fvbA* transcription. To further understand the mechanism of *fvbA* regulation we examined the expression of *fvbR* in the presence of vibriobactin by real-time PCR. Surprisingly, the expression of *fvbR* was induced more than threefold in the presence of vibriobactin (data not shown). We hypothesize that perhaps vibriobactin acts as an effector and titrates out FvbR, allowing the expression of *fvbA* and utilization of

Fig. 5. Involvement of FvbR in *fvbA* regulation. (a) Effect of *fvbR* on *fvbA* expression. *P. aeruginosa* PAO1 and *fvbR* mutant strain PASE5 were grown in M9 to the mid-exponential phase (OD₅₉₅ 0.5) and then were exposed for 3 h to the following conditions: M9 or M9 containing vibriobactin (2.5 µg ml⁻¹). Total RNA was isolated and the relative quantity of *fvbA* transcripts was measured using real-time PCR. PA1684 was used as a housekeeping reference gene and PAO1 grown in M9 alone was used as the reference condition. Error bars, SD. (b) Effect of *fvbR* mutation and overexpression on the utilization of vibriobactin in iron-deficient conditions. *P. aeruginosa* triple mutant strain PASE7 (grey bars) and *fvbR* overexpression strain PASE6 (white bars) were grown overnight on M9 minimal medium with or without 25 µg EDDA ml⁻¹ or 25 µg EDDA ml⁻¹ and 2.5 µg vibriobactin ml⁻¹. All cultures were incubated at 37 °C with shaking (250 r.p.m.). Bacterial growth (OD₅₉₅) was measured after 12 h. Error bars, SD.

iron for growth. When vibriobactin levels fall below a certain threshold, FvbR is no longer titrated and can then act as a repressor of *fvbA*. This mechanism can allow fine-tuning and increased sensitivity to changes in vibriobactin concentrations. The regulation of IclR transcriptional activity by small molecules has been reported in several

bacterial species and in different metabolic pathways (Molina-Henares *et al.*, 2006). In fact, many IclR-type regulators contain a small molecule effector binding domain at their C terminus; however, only a few effectors have been identified. For example, the IclR transcriptional regulator in *E. coli* recognizes two antagonist ligands and can act as a corepressor (upon pyruvate binding) or activator (upon glyoxylate binding) of the glyoxylate bypass operon (Lorca *et al.*, 2007). To the best of our knowledge this is the first report of an IclR-like regulator involved in iron uptake, and future work will be required to establish its mode of regulation.

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