The Heterologous Siderophores Ferrioxamine B and Ferrichrome Activate Signaling Pathways in *Pseudomonas aeruginosa*

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Pseudomonas aeruginosa **secretes two siderophores, pyoverdine and pyochelin, under iron-limiting conditions. These siderophores are recognized at the cell surface by specific outer membrane receptors, also known as TonB-dependent receptors. In addition, this bacterium is also able to incorporate many heterologous siderophores of bacterial or fungal origin, which is reflected by the presence of 32 additional genes encoding putative TonB-dependent receptors. In this work, we have used a proteomic approach to identify the inducing conditions for** *P. aeruginosa* **TonB-dependent receptors. In total, 11 of these receptors could be discerned under various conditions. Two of them are only produced in the presence of the hydroxamate siderophores ferrioxamine B and ferrichrome. Regulation of their synthesis is affected by both iron and the presence of a cognate siderophore. Analysis of the** *P. aeruginosa* **genome showed that both receptor genes are located next to a regulatory locus encoding an extracytoplasmic function sigma factor and a transmembrane sensor. The involvement of this putative regulatory locus in the specific induction of the ferrioxamine B and ferrichrome receptors has been demonstrated. These results show that** *P. aeruginosa* **has evolved multiple specific regulatory systems to allow the regulation of TonB-dependent receptors.**

In most environments, the amount of free iron (approximately 10^{-9} M) is below the concentration required by most microorganisms for growth (49). To fulfill their requirements for iron, bacteria have developed several strategies, including (i) the reduction of ferric to ferrous ions, (ii) the direct acquisition of iron from host proteins, (iii) the secretion of high-affinity iron-chelating compounds, called siderophores, and (iv) the uptake of heterologous siderophores (23). The transport of (heterologous) siderophores and heme/hemophores through the outer membranes of gram-negative bacteria is an energy-dependent process that needs the function of the ExbB-ExbD-TonB system. This system transduces the proton-motive force of the cytoplasmic membrane to the high-affinity outer membrane receptors, which are therefore also known as TonB-dependent receptors (48). The synthesis of siderophores and their transport systems is negatively regulated by the Fur repressor protein (18). In addition, some siderophore transport systems are also positively regulated by the presence of their cognate siderophores (12). This level of regulation often involves extracytoplasmic function (ECF) sigma factors (56). The ECF family of sigma factors constitutes a group of environmentally responsive transcriptional factors of the RpoD (σ^{70}) family that control a wide range of bacterial functions (39). The ECF factors involved in the regulation of iron uptake systems have been classified as the iron starvation class of sigma factors (35). One well-studied example is ferric dicitrate uptake by *Escherichia coli* (10). Binding of ferric dicitrate to the outer membrane receptor FecA generates a signal that is transmitted by the N terminus of FecA, located in the

periplasm, to the C terminus of the cytoplasmic membrane FecR. This protein functions as an anti-sigma factor inhibiting the function of the ECF sigma factor FecI in the absence of ferric dicitrate. After induction, FecI binds to the RNA polymerase core complex and directs it to the promoter region of the genes required for ferric dicitrate transport (*fecABCDE*). Similar regulatory systems, also called cell surface signaling, have been identified in various bacteria, including *Pseudomonas putida* (31, 32) and *Pseudomonas aeruginosa* (6).

P. aeruginosa is a remarkably versatile pathogen. This bacterium, which grows in soil, freshwater, and saltwater habitats, is not only an important opportunistic pathogen for humans but is also able to infect a large range of other species, including plants, insects, and nematodes (*Caenorhabditis elegans*) (57). Under iron-limiting conditions, *P. aeruginosa* secretes two siderophores, pyoverdine and pyochelin. Ferri-pyoverdine binding to the outer membrane receptor FpvA induces, via a cell surface signaling pathway consisting of two ECF sigma factors, transcription of the *fpvA* gene and also that of the genes required for the production of pyoverdine as well as the genes encoding exotoxin A and the protease PrpL (6, 34). Moreover, the presence of a second pyoverdine receptor, FpvB, in *P. aeruginosa* has recently been reported (21). Besides these two pyoverdine receptors and the characterized receptor for pyochelin (FptA) (2, 27), this bacterium also produces receptors for heterologous iron sources: there are two receptors, PhuR and HasR, involved in the utilization of heme (44), and two, PfeA and PirA, that mediate the uptake of enterobactin (14, 22). However, these receptors are probably just a small sample of the real iron acquisition potential of *P. aeruginosa*, because in silico analysis of its genome revealed an impressive total of 34 genes encoding putative TonB-dependent outer membrane receptors (11). Here we report that two of these receptors, PA2466 (FoxA) and PA0470 (FiuA), are indeed

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TABLE 1. Bacterial strains and plasmids used for this study

Strain or plasmid	Relevant characteristics[a]	Reference
E. coli strains		
DH5α	*supE44* Δ*lacU169* (φ80 *lacZ*ΔM15) *hsdR1 recA1 endA1 gyrA96 thi-1 relA1*	25
HB101	*supE44 hsdS20 recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1*	9
P. aeruginosa strains		
PAO25	PAO1 *leu arg*	24
PAO1	Wild type	28
PA2466 (*foxA*)	PAO1 with insertion of IS*lacZ*/hah transposon in nucleotide 266 of the PA2466 (*foxA*) gene (ID 19564)	28
PA2467 (*foxR*)	PAO1 with insertion of IS*lacZ*/hah transposon in nucleotide 231 of the PA2467 (*foxR*) gene (ID 9095)	28
PA0470 (*fiuA*)	PAO1 with insertion of IS*phoA*/hah transposon in nucleotide 378 of the PA0470 (*fiuA*) gene (ID 34018)	28
PA0471 (*fiuR*)	PAO1 with insertion of IS*phoA*/hah transposon in nucleotide 822 of the PA0471 (*fiuR*) gene (ID 53020)	28
PA0472 (*fiuI*)	PAO1 with insertion of IS*phoA*/hah transposon in nucleotide 39 of the PA0472 (*fiuI*) gene (ID 52421)	28
pvdD pchEF	PAO1 with unmarked allelic *pvdD pchEF* mutations	21
pvdD pchEF PA0470	Allelic mutant of PA40470 (*fiuA*) in *pvdD* and *pchEF* mutant background	21, 22
Plasmids		
pBSL99	Source of the Km cassette; Ap^r Km^r	1
pMMB67EH	IncQ broad-host-range plasmid; *lacI*^q Ap^r	20
pMP220	IncP broad-host-range *lacZ* fusion vector; Tc^r	53
pRK600	Helper plasmid with *oriColE1*, *mobRK2*, and *traRK2*; Cm^r	16
pUC18	Cloning vector with *oriColE1*, *rop* mutant, α-*lacZ* mutant	42
pUCMA8	pUC18 carrying at the SmaI site the 2.5-kb PCR fragment containing the *P. aeruginosa* PAO1 *foxI-foxR* (PA2468-PA2467) genes; Ap^r	This study
pMUM8	pMMB67EH carrying the 824-bp SacI-SalI insert from pUCMA8 containing the *foxI* (PA2468) gene; Ap^r	This study
pMPR8b	*foxA* (PA2466) promoter fragment cloned upstream of *lacZ* gene in pMP220; Tc^r	This study
pMPR8bKm	pMPR8b carrying at the BgIII site, in the opposite direction from the *lacZ* gene, a 1.2-kb BamHI fragment of pBSL99 containing a Km cassette; Km^r Tc^r	This study
pMMBFiuI	pMMB67EH carrying the 742-bp XbaI-HindIII PCR fragment containing the *fiuI* (PA0472) gene; Ap^r	This study
pMPFiuA	*fiuA* (PA0470) promoter fragment cloned upstream of *lacZ* gene in pMP220; Tc^r	This study
pMPFiuAKm	pMPFiuA carrying at the BgIII site, in the opposite direction from the *lacZ* gene, a 1.2-kb BamHI fragment of pBSL99 containing a Km cassette; Km^r Tc^r	This study
pMPR2911	PA2911 promoter fragment cloned upstream of *lacZ* gene in pMP220; Tc^r	This study
pMPR2911Km	pMPR2911 carrying at the BgIII site, in the opposite direction from the *lacZ* gene, a 1.2-kb BamHI fragment of pBSL99 containing a Km cassette; Km^r Tc^r	This study
pMPR3268	PA3268 promoter fragment cloned upstream of *lacZ* gene in pMP220; Tc^r	This study
pMPR3268Km	pMPR8 carrying at the BgIII site, in the opposite direction from the *lacZ* gene, a 1.2-kb BamHI fragment of pBSL99 containing a Km cassette; Km^r Tc^r	This study

[a] Ap^r, Cm^r, Km^r, and Tc^r, resistance to ampicillin, chloramphenicol, kanamycin, and tetracycline, respectively.

involved in the transport of the heterologous siderophores ferrioxamine B and ferrichrome, respectively. In addition, we also show the involvement of both receptors in a signaling pathway that regulates their own synthesis in response to the presence of their cognate ligands.

MATERIALS AND METHODS

Bacterial strains, plasmids, culture media, and growth conditions. The bacterial strains and plasmids used for this study are listed in Table 1. The *P. aeruginosa* PAO1 wild-type strain and all the *P. aeruginosa* transposon insertion mutants used were from the comprehensive *P. aeruginosa* transposon mutant library at the University of Washington Genome Center (28). The locations of the mutations were confirmed by PCR with primers flanking the insertion sites. The strains' unique identifiers are given in the table, and further information on these mutants can be found at http://www.genome.washington.edu/UWGC/Pseudomonas/index.cfm.

Bacterial strains were routinely grown in liquid Luria-Bertani medium (50) at 37°C on a rotary shaker operated at 200 revolutions per min. For experiments on the effect of the iron concentration on gene expression, *P. aeruginosa* cells were grown in liquid CAS medium [3.18 mM Ca(NO$_3$)$_2$, 1 mM MgSO$_4$, 50 mM PIPES, pH 6.8, containing 1 mM K$_2$HPO$_4$, 1% (wt/vol) Casamino Acids, and 1% (wt/vol) glycerol] supplemented with either 50 μM FeCl$_3$ (iron-rich conditions) or 400 μM 2,2′-bipyridyl (iron-restricted conditions). The ferrioxamine B and ferrichrome stock solutions (20 mM) were prepared by dissolving desferrioxamine B mesylate (Desferal) and ferrichrome (Sigma) in a Tris-HCl, pH 8.8, solution without (iron-free form) or with 20 mM FeCl$_3$ (iron-loaded form). Pseudobactin PS358 (8) was used at a 20 μM final concentration. When required, antibiotics were used at the following final concentrations (μg ml^{-1}): ampicillin, 100; chloramphenicol, 30; kanamycin, 25 (for *E. coli*) and 200 (for *P. aeruginosa*); piperacillin, 25; and tetracycline, 10 (for *E. coli*) and 20 (for *P. aeruginosa*).

General molecular biology methods. Standard molecular biology techniques were used for DNA manipulations (50). DNAs from plasmids pUCMA8 and pMMBFiuI were sequenced by the dideoxy sequence termination method (51) with Ampli*Taq* DNA polymerase (Big Dye Terminator v3.1 cycle sequencing kit; Applied Biosystems). PCR amplifications were carried out using the Expand high-fidelity system (Roche) containing 0.2 ng μl^{-1} *P. aeruginosa* PAO1 chromosomal DNA, a 100 μM concentration of each deoxynucleoside triphosphate, a 0.5 μM concentration of each primer, and 5% (vol/vol; final concentration) dimethyl sulfoxide. The primers used for amplification of the wild-type PA2468 and PA2467 genes were Puma8F (5′-CTGGCCAACATCGAATTGT-3′) and
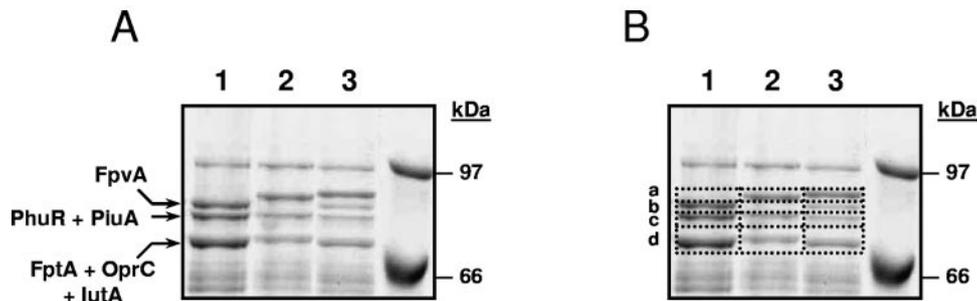
FIG. 1. SDS-PAGE analysis of outer membrane proteins from *P. aeruginosa* PAO25. *P. aeruginosa* PAO25 was grown under iron-limiting conditions without siderophores (lane 1), with 20 μM ferrioxamine B (iron-loaded) (lane 2), or with 40 μM ferrichrome (iron-loaded) (lane 3) until late log phase. Outer membrane-enriched preparations were prepared as described in Materials and Methods and separated in a 7.5% acrylamide gel. (A) The positions of the TonB-dependent receptor proteins detected by MALDI-MS in cells grown under iron-limiting conditions are indicated on the left (arrows), and those of the molecular size markers are indicated on the right. (B) The different samples used for MALDI-MS analyses are shown.

Puma8R (5′-GAGACGGTCTGGCTGGTTT-3′), and those for the amplification of PA0472 were FiuI5X (5′-TATTCTAGATGATCAGCAAAGGTTCGGC-3′) and FiuI3H (5′-AAAAAGCTTCGATCGTCGGCGCTCACC-3′).

Construction of *lacZ* transcriptional fusions and β-galactosidase assay. Transcriptional fusions were constructed by cloning DNA segments with the promoter regions of the PA0470, PA2911, PA2466, and PA3268 genes of *P. aeruginosa* PAO1, amplified by PCR as EcoRI-XbaI fragments with appropriate primers, into the EcoRI-XbaI sites of pMP220. The fusion constructs were confirmed by DNA sequencing and transferred from *E. coli* DH5α to *P. aeruginosa* by triparental mating using the helper plasmid pRK600 as described before (16). β-Galactosidase activity from the fusion plasmid was measured as described previously (38). Each assay was run in duplicate at least three times, and the data given are averages.

Cell envelope preparations. *P. aeruginosa* cells were grown until late log phase, and fractions enriched for outer membrane proteins were isolated by centrifugation of ultrasonically disrupted cells (30 min at 10,000 × *g*) followed by extraction with 1% (wt/vol) Sarkosyl (17). Protein profiles were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in gels containing 7.5% acrylamide as described by Laemmli (33). Samples were incubated for 10 min at 95°C prior to electrophoresis, and proteins were stained with Coomassie blue.

In-gel digestion of proteins and sample preparation for MALDI-mass spectrometry (MALDI-MS) fingerprint mapping. Protein bands of interest were excised from Coomassie-stained SDS-PAGE gels. The excised gel bands were washed twice with 400 μl of 50 mM ammonium bicarbonate in 50% (vol/vol) acetonitrile to destain the proteins and dehydrate the gel pieces and then dried completely in a vacuum centrifuge for 30 to 45 min. The dried gel pieces were rehydrated in 50 mM ammonium bicarbonate, pH 8, containing 6.25 ng/μl trypsin (sequencing grade; Promega), and the proteins were digested for 12 to 16 h at 37°C. The peptides were then recovered from the gel particles by performing two extractions. For the first extraction, 50 μl of 1% (vol/vol) trifluoroacetic acid was added, and the samples were vortexed for 15 min. The second extraction was performed with 100 μl of 0.1% (vol/vol) trifluoroacetic acid in 50% (vol/vol) acetonitrile. Peptide extracts were combined and concentrated by reducing the final volumes of the extracts to 20 to 25 μl in a vacuum centrifuge. The tryptic peptides were then desalted and concentrated in reversed-phase C$_{18}$ Zip Tip pipette tips (Millipore) according to the manufacturer's recommendations. Briefly, Zip Tips were washed with 50% (vol/vol) acetonitrile and equilibrated with 0.1% (vol/vol) trifluoroacetic acid. Peptide extracts were then applied to the Zip Tips, washed with 0.1% (vol/vol) trifluoroacetic acid, and eluted in 3 μl of a saturated matrix solution of 5 μg/μl α-cyano-4-hydroxycinnamic acid in 0.1% (vol/vol) trifluoroacetic acid and 50% (vol/vol) acetonitrile. A 0.5-μl sample of the mixture was immediately spotted onto the matrix-assisted laser desorption ionization (MALDI) target and allowed to dry and crystallize.

MALDI-MS and database searching. The molecular masses of the tryptic peptides (peptide mass fingerprinting) were determined in a MALDI-time of flight mass spectrometer (4700 Proteomics analyzer; Applied Biosystems), using an acceleration voltage of 20 kV. MS spectra were searched against the NCBI database, using Protein Prospector MS-Fit v4.0.5 software, available at http://prospector.ucsf.edu, to identify the proteins. The protonated trypsin autodigestion products at *m/z* 842.510 and 2,211.104 were used for internal calibration of

the MALDI-MS spectra. The MALDI-MS resolution for the peptides was ~10,000, and the mass accuracy was within 0.01 to 0.02 Da. For database searching of peptide mass fingerprint data, the mass tolerance was set at 20 ppm.

Computer-assisted analysis. In silico analysis of the PAO1 genome was performed at http://www.pseudomonas.com. Signal peptides were predicted using the SignalP 3.0 server available at http://www.cbs.dtu.dk/services/SignalP (7).

RESULTS

TonB-dependent receptors under iron-limiting conditions. *P. aeruginosa* PAO1 genome analysis showed that this bacterium has the potential to produce 34 different TonB-dependent receptors (11). We first wanted to establish how many of these receptors are produced under iron-limiting conditions. Therefore, cell envelope preparations, enriched for outer membrane proteins by Sarkosyl extraction, of cells grown in liquid CAS medium supplemented with 400 μM of 2,2′-bipyridyl (iron-limiting conditions) were subjected to SDS-PAGE analysis. At least three high-molecular-weight major outer membrane proteins could be observed in these samples (Fig. 1A, lane 1). In order to unequivocally determine the nature of these proteins, the gel bands shown in Fig. 1B were excised and subjected to tryptic digestion, followed by MALDI-mass spectrometry for peptide mass mapping. The first visible band (sample 1b) contained the pyoverdine receptor FpvA, the second band (sample 1c) contained mainly the heme/hemophore receptor PhuR, and the third band (sample 1d) contained the pyochelin receptor FptA (Table 2). Besides PhuR, sample 1c also contained the putative TonB-dependent receptor PA4514. The amount of this protein was considerably lower than that of PhuR, as judged by the smaller number of tryptic peptides that matched PA4514 (Table 2). Sample 1d not only contained FptA but also had the TonB-dependent receptor PA4675 and the putative TonB-dependent outer membrane protein OprC (PA3790) (Table 2). In addition, in sample 1a, in which no protein band was visible (Fig. 1), another putative TonB-dependent receptor encoded by PA4168 was detected. This protein, which is the second pyoverdine receptor, named FpvB (21), has been visualized previously (26). Although the presence of the FpvA, PhuR, and FptA receptors in the outer membrane of *P. aeruginosa* has been reported previously, this analysis showed the presence of four other TonB-dependent receptors under iron-limiting conditions, which brings the total

TABLE 2. TonB-dependent receptors of *P. aeruginosa* identified by MALDI-MS analysis[a]

Sample	Rank	Protein name	PA number[b]	No. of peptides matching protein	% Protein coverage	Protein score[c]
1a	1	FpvA	PA2398	12	20	1.6e+08
	2	FpvB	PA4168	11	17	0.61e+04
2a	1	Probable TonB-dependent receptor	PA2466	29	44	1.2e+10
3a	1	Probable hydroxamate-type ferrisiderophore receptor	PA0470	42	55	4.1e+17
1b	1	FpvA	PA2398	49	58	1.4e+25
2b	1	FpvA	PA2398	23	30	3.2e+08
	2	Probable TonB-dependent receptor	PA3268	16	22	1.6e+05
3b	1	FpvA	PA2398	23	25	1.8e+08
	2	Probable TonB-dependent receptor	PA3268	17	25	7e+05
1c	1	PhuR	PA4710	47	68	5.8e+22
	2	Hydroxamate-type ferrisiderophore receptor (PiuA)	PA4514	16	28	2.3e+06
2c	1	Hydroxamate-type ferrisiderophore receptor	PA4514	36	53	1.9e+14
	2	PhuR	PA4710	32	42	3.4e+13
	3	Probable TonB-dependent receptor	PA2911	19	28	1.3e+09
3c	1	Probable TonB-dependent receptor	PA2911	18	30	7.9e+08
	2	Hydroxamate-type ferrisiderophore receptor	PA4514	12	18	1.1e+05
1d	1	FptA	PA4221	48	70	9e+21
	2	OprC	PA3790	28	41	6.6e+10
	3	Probable TonB-dependent receptor (IutA)	PA4675	20	29	1.3e+07
2d	1	Probable TonB-dependent receptor	PA4675	33	43	2.1e+11
	2	OprC	PA3790	22	34	9.6e+07
3d	1	OprC	PA3790	52	67	6.1e+20
	2	Probable TonB-dependent receptor	PA4675	26	33	1e+10

[a] For the mass peptide fingerprint search, only mass deviations of <20 ppm were allowed (high-stringency search).
[b] PA number in the *P. aeruginosa* genome annotation project (http://www.pseudomonas.com).
[c] Scores are $-10 \log(P)$ values, where P is the probability that the observed match is a random event (http://prospector.ucsf.edu). Protein scores given are significant ($P < 0.05$).

to seven. This means that the 27 other putative *P. aeruginosa* TonB-dependent receptors require additional or other inducing conditions. To substantiate this hypothesis, *P. aeruginosa* was grown under iron-limiting conditions in the presence of various heterologous siderophores.

Induction of *P. aeruginosa* outer membrane proteins by ferrioxamine B and ferrichrome. In order to determine the inducing conditions for other TonB-dependent receptors, *P. aeruginosa* was grown in the presence of different iron sources, such as the iron-loaded form of the heterologous siderophores ferrioxamine B, ferrichrome, and pseudobactin PS358. In the presence of pseudobactin PS358, *P. aeruginosa* did not express any other readily detectable outer membrane proteins compared with those induced under normal iron-restricted conditions (data not shown). However, new outer membrane proteins with different apparent molecular weights were expressed when *P. aeruginosa* was cultured in the presence of ferrioxamine B or ferrichrome (Fig. 1, lanes 2 and 3, respectively). The expression of these inducible proteins was completely suppressed upon growth under iron-rich conditions, even if ferrioxamine B or ferrichrome was present (not shown). Apparently, both the absence of iron and the presence of the heterologous siderophores are needed for induction.

To determine the nature of the induced proteins, the gel bands shown in Fig. 1B (lanes 2 and 3) were excised and analyzed by MALDI-MS. A protein database search revealed that the ferrioxamine B-induced protein (Fig. 1B, lane 2, sample 2a) was the putative TonB-dependent receptor PA2466 (Table 2), whereas the ferrichrome-induced protein (Fig. 1B, lane 3, sample 3a) showed high identity with the putative TonB-dependent receptor PA0470 (FiuA) (Table 2). These two identified proteins (PA2466 and PA0470) could not be detected in the corresponding gel fragment in lane 1 (sample 1a). To determine whether any other receptors were induced by ferrioxamine B or ferrichrome, the gel fragments corresponding to regions b, c, and d were also analyzed by MALDI-MS analysis. This analysis showed that the proteins in region b (lanes 2 and 3) contained, in addition to FpvA, the TonB-dependent receptor PA3268, whereas in region c (lanes 2 and 3), the previously identified PA4514 protein (PiuA) and the novel putative TonB-dependent receptor PA2911 were detected (Table 2). Proteins in sector d (lanes 2 and 3) were shown to be the previously identified OprC and PA4675 proteins (Table 2). These results indicate that the addition of ferrioxamine B and ferrichrome not only resulted in the induction of a specific receptor but that both siderophores possibly also induced PA2911 and PA3268 synthesis, since these pro-

A

B
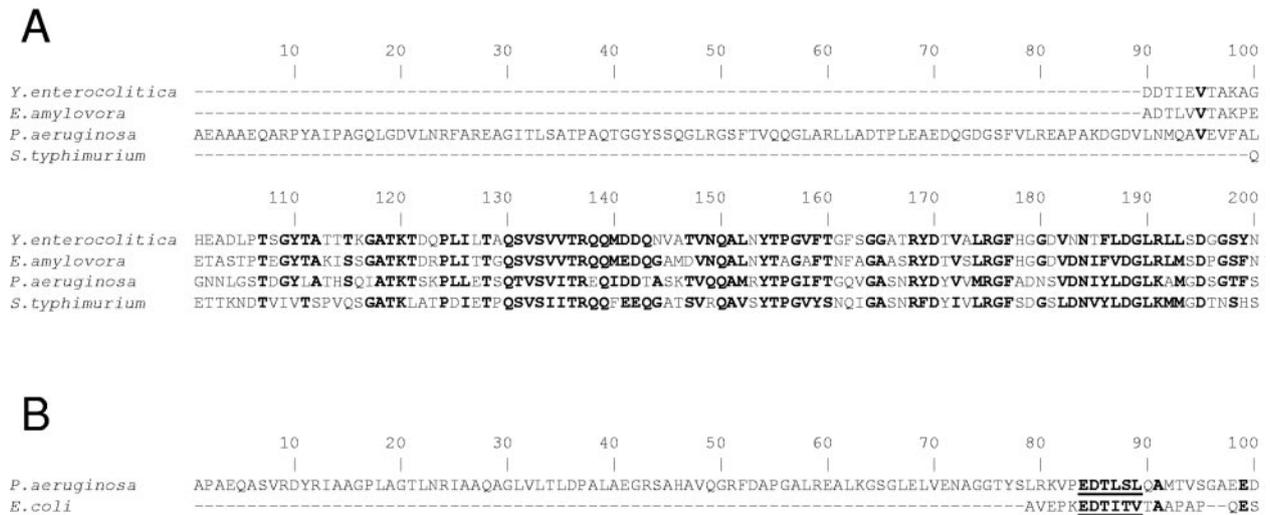
FIG. 2. N-terminal extension of mature *P. aeruginosa* FoxA and FiuA proteins. (A) N-terminal extension of mature *P. aeruginosa* FoxA compared to mature *Y. enterocolitica*, *E. amylovora*, and *S. enterica* serovar Typhimurium FoxA proteins. (B) N-terminal extension of mature *P. aeruginosa* FiuA compared to mature *E. coli* FhuA. The proposed TonB box sequences are underlined. Positions at which identical or similar residues are present in at least three of the four sequences are shown in bold. The numbering of amino acid residues is indicated.

teins were not detected in the absence of these siderophores (samples 1b and 1c, respectively).

Characteristics of ferrioxamine B- and ferrichrome-induced proteins. The ferrioxamine B-induced protein PA2466 is an 820-amino-acid protein that contains a predicted signal peptide of 47 amino acids and has a predicted molecular mass of 85,273 Da (after cleavage of the signal peptide). The PA2466 gene is annotated in the Pseudomonas Genome Project database (www.pseudomonas.com) as a probable TonB-dependent receptor gene named *optS* (*o*uter *p*rotein *S*). A BLAST search of the *P. aeruginosa* PA2466 protein sequence showed 43 to 47% identities with the characterized TonB-dependent ferrioxamine B FoxA receptors of *Salmonella enterica* serovar Typhimurium (29), *Yersinia enterocolitica* (5), and *Erwinia amylovora* (15). By analogy with these proteins, we propose renaming the *P. aeruginosa* PA2466 gene *foxA*. The *foxA* gene contains a strong putative Fur box in its promoter region, suggesting its regulation by the Fur repressor protein. This is consistent with the observation that the synthesis of FoxA is repressed under iron-rich conditions, even in the presence of ferrioxamine B (not shown). The mature FoxA protein contains an N-terminal periplasmic extension (Fig. 2A), which is a typical feature of TonB-dependent receptors involved in cell surface signaling pathways (30). Moreover, the two open reading frames adjacent to the *foxA* gene are annotated as a probable transmembrane sensor gene (PA2467) and a probable σ^{70} gene of the ECF subfamily (PA2468), respectively. The PA2467 (designated *foxR* in this work) and PA2468 (designated *foxI*) genes are clustered in an operon that also contains a putative Fur box in its promoter region. In fact, Ochsner and coworkers (46) have shown that expression of the *foxI-foxR* operon considerably increases under iron-limiting conditions.

The ferrichrome-induced protein encoded by the PA0470 gene is a protein of 802 amino acids that contains a predicted signal peptide of 26 amino acids and has a predicted molecular mass of 85,489 Da (after cleavage of the signal peptide). This gene is annotated in the *P. aeruginosa* genome as a probable hydroxamate-type ferri-siderophore receptor gene named *fiuA*. The FiuA protein shows 31.6% identity with the ferrichrome receptor FhuA of *E. coli* (19), but in contrast to FhuA, the mature FiuA protein contains an N-terminal extension (Fig. 2B). The *fiuA* gene contains a putative Fur box in its promoter region and is preceded by two genes, named *fiuR* (PA0471) and *fiuI* (also called *fiuS*) (PA0472), encoding a probable transmembrane sensor and an ECF sigma factor, respectively. The expression of the *fiuI-fiuR* gene cluster has been shown to be under the control of the iron-responsive Fur repressor protein (45).

Involvement of the Fox system and ferrioxamine B in the production of FoxA. To analyze the function of the putative ECF sigma factor FoxI (PA2468), the *foxI* gene was amplified by PCR, cloned into the pMMB67EH plasmid (yielding pMUM8) (Table 1), and introduced in *P. aeruginosa*. In pMUM8, *foxI* is constitutively expressed from the *Ptac* promoter, and this plasmid also contains the *lacI*q gene, which allows regulated expression of the sigma factor. The introduction of pMUM8 into *P. aeruginosa* PAO1 resulted in the specific induction of an outer membrane protein in both noninduced cultures (Fig. 3) and cultures induced with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) (not shown). The induction by FoxI was independent of the presence or absence of ferrioxamine B in the culture medium, and the apparent molecular weight of the FoxI-induced protein was similar to that of the ferrioxamine B-induced receptor FoxA (Fig. 3A). Subsequently, the pMUM8 plasmid was transferred to the PA2466 mutant strain, which contains a transposon insertion in the *foxA* gene (Table 1). Both the FoxI- and ferrioxamine B-induced outer membrane proteins were absent in the *foxA* mutant (Fig. 3A), indicating that the protein is in fact FoxA.

The involvement of ferrioxamine B and FoxI in regulating the production of FoxA was tested further using the plasmid pMPR8bKm, which contains the putative promoter region of
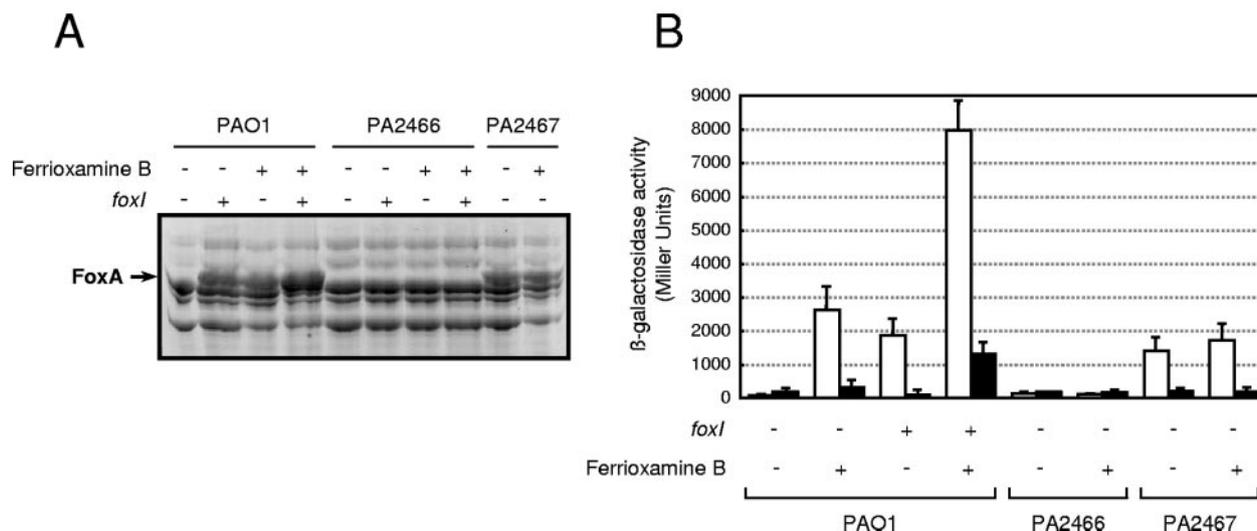
FIG. 3. Analysis of Fox signaling system. *P. aeruginosa* PAO1 (wild type), PA2466 (*foxA* mutant), and PA2467 (*foxR* mutant) bearing the plasmid pMMB67EH (*foxI* negative) or pMUM8 (*foxI* positive) were grown under iron-restricted conditions without (−) or with (+) 20 μM ferrioxamine B (iron-free form) until late exponential growth phase. (A) Cell envelope preparations enriched for outer membrane proteins by Sarkosyl extraction were separated in a 7.5% acrylamide gel. The position of the ferrioxamine B receptor FoxA (PA2466) is indicated on the left. (B) *P. aeruginosa* strains containing the plasmid pMPR8bKm (P*foxA*::*lacZ* transcriptional fusion) and the plasmid pMMB67EH (*foxI* negative) or pMUM8 (*foxI* positive) were grown under iron-restricted conditions (white bars) or iron-rich conditions (black bars) in either the presence (+) or absence (−) of 20 μM ferrioxamine B (iron-free form).

the *foxA* gene fused to a promoterless *lacZ* reporter gene. As shown in Fig. 3B, the addition of ferrioxamine B to the iron-restricted culture medium of the wild-type PAO1 strain led to a 33-fold increase in the activity of the *foxA* promoter. Overexpression of *foxI* has a similar effect and resulted in a 25-fold increase in *foxA* expression in the absence of ferrioxamine B and a 100-fold increase in the presence of the siderophore, both under iron-restricted conditions. This effect of ferrioxamine B and FoxI on *foxA* expression was severely reduced under iron-rich conditions (Fig. 3B). The requirement of FoxI for *foxA* expression is consistent with FoxI being a sigma factor that directs the expression of *foxA* in the presence of ferrioxamine B.

By analogy with other cell surface signaling systems, the activity of FoxI would be regulated by the transmembrane sensor FoxR (PA2467), which would act as an anti-sigma factor, and by the FoxA protein itself. To test this, first the production of FoxA in the PA2467 *foxR* mutant was examined. As expected, the FoxR mutant showed induction of FoxA independent of ferrioxamine B (Fig. 3A). However, the IS*lacZ*/hah transposon used to generate the PA2467 gene mutation could have polar effects on the expression of the downstream gene PA2466 encoding FoxA (28). Therefore, the involvement of FoxR in *foxA* expression was also examined using the pMPR8bKm plasmid. Also, in this situation, the expression of *foxA* in the *foxR* mutant occurred in the absence of ferrioxamine B (Fig. 3B) and was higher under iron-restricted than iron-rich conditions. These data are consistent with FoxR being an anti-sigma factor for FoxI, which affects FoxA production in the absence of ferrioxamine B through its inhibitory effect on FoxI activity. In fact, the specific binding of FoxR to FoxI has been previously reported (36). In addition, the level of *foxA* promoter activity in the PA2466 *foxA* mutant strain was also examined. *foxA* promoter activity in the presence of ferrioxamine B was completely abolished in this mu-

tant (Fig. 3B), which indicates that the ferrioxamine B signal was not properly transmitted.

All of these data are consistent with a model in which binding of ferrioxamine B to FoxA results in signal transmission to FoxR and, consequently, increased activity of FoxI, enabling *foxA* expression.

Involvement of the Fiu system and ferrichrome in the production of FiuA. The role of the Fiu system in the ferrichrome-mediated production of FiuA was analyzed in a similar way. The *fiuI* gene was constitutively expressed in *P. aeruginosa* from the plasmid pMMBFiuI. Introduction of this plasmid into the *P. aeruginosa* wild-type PAO1 strain resulted in the specific induction of an outer membrane protein (Fig. 4A) with an apparent molecular weight similar to that of the ferrichrome-induced receptor FiuA (Fig. 4A). Both the FiuI- and ferrichrome-induced proteins were absent in the PA0470 (*fiuA*) mutant (Fig. 4A), confirming that in both cases the induced protein was the FiuA receptor. The role of FiuI was analyzed using a *fiuI* (PA0472) mutant strain. As shown in Fig. 4A, this strain did not produce FiuA after growth in ferrichrome. Complementation of the *fiuI* mutation with the pMMBFiuI plasmid resulted in normal amounts of FiuA in both the absence and presence of ferrichrome (Fig. 4A), indicating that FiuI is required for FiuA production. Subsequently, the reporter construct pMPFiuAKm, in which the *fiuA* promoter region was cloned in front of a *lacZ* gene (Table 1), was used to show that FiuI is a sigma factor that causes expression from the *fiuA* promoter. The presence of ferrichrome under iron-restricted conditions led to a sevenfold increase in the activity of the *fiuA* promoter (Fig. 4B). This induction, however, was lacking in the PA0472 *fiuI* mutant strain (Fig. 4B). Constitutive expression of *fiuI* from the pMMBFiuI plasmid in both the wild-type PAO1 and *fiuI* mutant strains resulted in 13.5- and
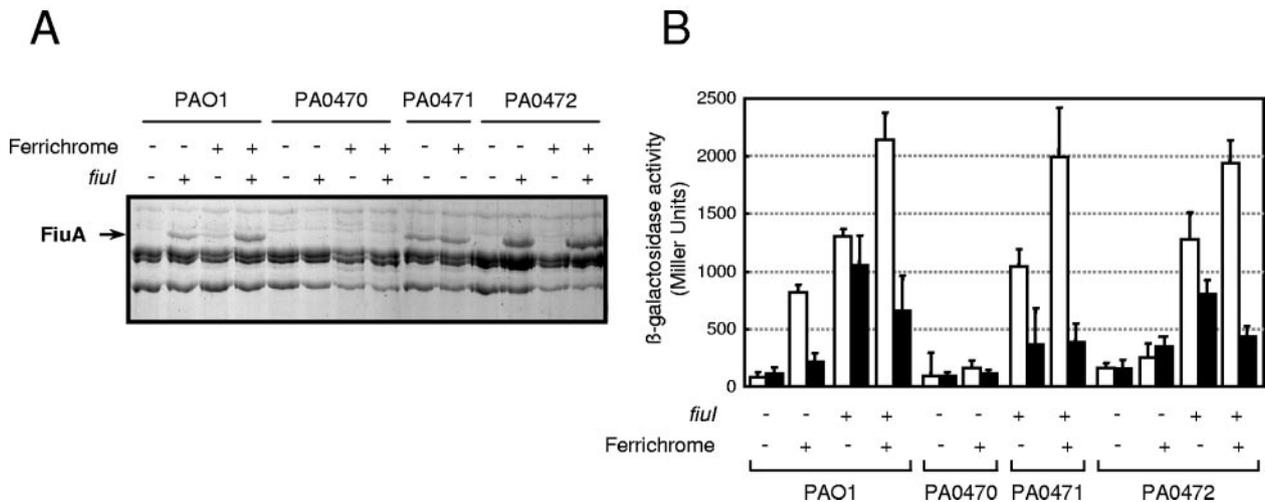
FIG. 4. Analysis of Fiu signaling system. *P. aeruginosa* PAO1 (wild type), PA0470 (*fiuA* mutant), PA0471 (*fiuR* mutant), and PA0472 (*fiuI* mutant) bearing the plasmid pMMB67EH (*fiuI* negative) or pMMBFiuI (*fiuI* positive) were grown under iron-restricted conditions without (−) or with (+) 40 μM ferrichrome (iron-free form) until late exponential growth phase. (A) Cell envelope preparations enriched for outer membrane proteins by Sarkosyl extraction were separated in a 7.5% acrylamide gel. The position of the ferrichrome receptor FiuA (PA0470) is indicated on the left. (B) *P. aeruginosa* strains containing the plasmid pMPFiuAKm (P*fiuA*::*lacZ* transcriptional fusion) and the plasmid pMMB67EH (*fiuI* negative) or pMMBFiuI (*fiuI* positive) were grown under iron-restricted conditions (white bars) or iron-rich conditions (black bars) in either the presence (+) or absence (−) of 20 μM ferrichrome (iron-free form).

20-fold increases in *fiuA* promoter activity in the absence and presence of ferrichrome, respectively (Fig. 4B).

The role of FiuR in the ferrichrome-mediated signaling pathway was analyzed using a *fiuR* mutant (PA0471 strain) (Table 1). As expected, the production of FiuA in the *fiuR* mutant was independent of the presence or absence of ferrichrome (Fig. 4A) due to the constitutive expression of *fiuA* in this mutant (Fig. 4B). This is consistent with a role for FiuR as an anti-sigma factor for FiuI. The constitutive expression of *fiuA* in PA0471 was higher under iron-restricted than iron-rich conditions (Fig. 4B). In contrast, *fiuA* promoter activity was completely abolished in the PA0470 *fiuA* mutant, even in the presence of ferrichrome (Fig. 4B). This confirmed the role of the FiuA receptor in sensing and transmitting the signal that induces its own synthesis.

These data are in agreement with a model in which the FiuA receptor transmits a signal to FiuR after sensing the presence of ferrichrome, which increases FiuI activity and, in turn, *fiuA* expression.

Role of FoxA and FiuA in the uptake of ferrioxamine B and ferrichrome. FoxA and FiuA are part of a signaling pathway, induced by ferrioxamine B and ferrichrome, respectively, that regulates their own synthesis. To determine whether these receptors are, in addition to their role in signal transduction, also involved in the uptake of their corresponding ligands, the growth of the PA2466 (*foxA*) and PA0470 (*fiuA*) mutants under iron-restricted conditions supplemented with either ferrioxamine B or ferrichrome was assayed. *P. aeruginosa* cells were grown in CAS medium containing 800 μM 2,2′-bipyridyl (iron-restricted conditions) supplemented with different concentrations of the iron-free forms of the siderophores. At higher siderophore concentrations (10 and 100 μM), no growth differences between the mutants and the wild-type strain were observed (data not shown). But at lower siderophore concentrations (0.5 and 1 μM), a very slight defect in growth rate was detected in the PA2466 mutant growing in fer-

rioxamine B and in the PA0470 mutant growing in ferrichrome (not shown). In order to rule out any effect caused by the endogenous siderophores pyoverdine and pyochelin, a *P. aeruginosa* PA0470 mutant in a siderophore-free background (*pvdD pchEF* mutant) was used. As reported before (21, 22), the *pvdD pchEF* mutant could not grow in the presence of 100 μM of the iron chelator 2,2′-bipyridyl unless an exogenous source of iron, i.e., ferrioxamine B or ferrichrome, was present in the medium (Fig. 5A). The mutation of PA0470 (*fiuA*) in this genetic background significantly delayed growth in the presence of ferrichrome, whereas this mutation had no effect on growth in the presence of ferrioxamine B (Fig. 5A). In agreement with that observation, the ferrichrome receptor FiuA was not detectable in this mutant, whereas the ferrioxamine B receptor FoxA could be detected (Fig. 5B). These results show that FiuA is directly involved in the uptake of ferrichrome but that *P. aeruginosa* is also able to take up this siderophore via another receptor(s).

Analysis of PA2911 and PA3268 gene expression. Proteomic analysis showed that ferrioxamine B and ferrichrome not only induce the synthesis of the specific receptors FoxA and FiuA, respectively, but that they both also seem to induce the production of two other TonB-dependent receptors, i.e., PA2911 and PA3268 (Table 2). In order to confirm this result, the reporter constructs pMPR2911Km and pMPR3268Km were generated, in which the promoter regions of the PA2911 and PA3268 genes, respectively, were fused to a promoterless *lacZ* gene. Subsequently, these constructs were introduced into the *P. aeruginosa* wild-type PAO1 strain. In contrast to what was expected, the addition of ferrioxamine B or ferrichrome to the iron-restricted medium did not result in the induction of PA2911 and PA3268 expression (not shown). Also, overexpression of the sigma factors *foxI* and *fiuI* from pMUM8 and pMMBFiuI, respectively, in the strains containing the reporter constructs did not result in increased expression of the PA2911 and PA3268 genes (not shown). These results show that nei-

A

B
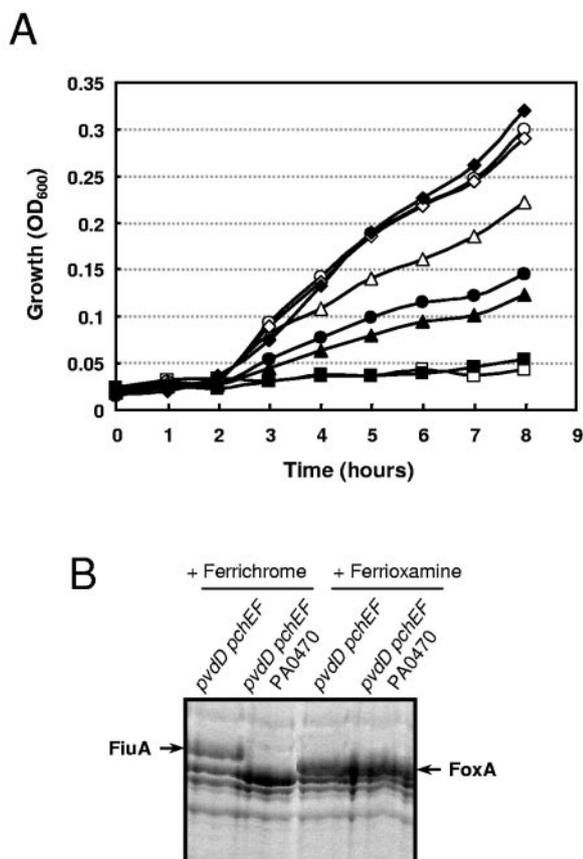
FIG. 5. Growth phenotype of *P. aeruginosa* PA0470 (FiuA) mutant. (A) *P. aeruginosa* PAO1 *pvdD pchEF* (white symbols) and *pvdD pchEF* PA0470 (black symbols) mutant strains were grown in CAS medium containing 100 μM 2,2′-bipyridyl and no siderophores (squares), 0.5 μM ferrichrome (triangles), 2 μM ferrichrome (circles), or 0.5 μM ferrioxamine B (diamonds) (iron-free forms of the siderophores were used). Growth is expressed as an increase in the optical density measured at 600 nm. A representative growth curve from three separate experiments is shown. (B) SDS-PAGE analysis of outer membrane proteins from *P. aeruginosa* PAO1 *pvdD pchEF* mutant and its isogenic *fiuA* mutant (*pvdD pchEF* PA0470) after overnight growth in CAS medium containing 100 μM 2,2′-bipyridyl and 50 μM ferrichrome or ferrioxamine B, respectively. The position of the ferrichrome receptor FiuA (PA0470) is indicated on the left, and that of the ferrioxamine B receptor FoxA (PA2466) is indicated on the right.

ther these siderophores nor the signaling pathway activated by them is directly involved in the induction of PA2911 and PA3268 synthesis and that the presence of these proteins in siderophore-containing cultures must have another reason.

DISCUSSION

P. aeruginosa contains an impressive number of genes (34) coding for TonB-dependent receptors. Using a proteomic approach, we showed that at least seven of these TonB-dependent outer membrane receptors are produced in significant amounts under iron-limiting conditions (Fig. 1 and Table 2). The expression of some of these receptors, but not all of them (i.e., IutA and OprC), has been shown previously by microarray analysis to be induced under similar conditions (46, 47). This could be due to the fact that growth conditions (different

carbon sources or iron chelators) can influence the expression of TonB receptor genes (21), which means that the receptors expressed under one condition could not be expressed under another one. In addition, we have identified four TonB-dependent receptors that are induced by the presence of heterologous siderophores. The receptors PA2911 and PA3268 were detected in cells cultured in the presence of either ferrioxamine B or ferrichrome (Table 2), whereas two other receptor genes, PA0470 (*fiuA*) and PA2466 (called *foxA* in this work), were specifically induced under iron-restricted conditions in the presence of the heterologous siderophores ferrichrome and ferrioxamine B, respectively.

Most siderophores fall into two chemical groups, namely, catecholates and hydroxamates. Ferrichrome and ferrioxamines both belong to the latter group. Ferrichrome is produced by a number of fungi, whereas ferrioxamines are produced by different bacterial species. In addition to these ferrioxamine-producing species, many other bacteria have also been shown to utilize ferrioxamines. In *E. coli*, a low level of ferrioxamine B transport is mediated by the outer membrane FhuE receptor (52), and the existence of a second ferrioxamine B receptor has been proposed (40, 41). *Y. enterocolitica*, *S. enterica* serovar Typhimurium, and *Serratia marcescens* take up ferrioxamine B efficiently through an outer membrane protein encoded by a Fur-repressible gene (5), although it has not been determined whether the synthesis of these receptors is induced by ferrioxamine. In *Vibrio vulnificus*, a ferrioxamine B receptor has been recently identified whose synthesis is induced by the presence of the siderophore under iron-limiting conditions via an AraC-like regulator (3, 54). This work shows that in *P. aeruginosa*, ferrioxamine B and ferrichrome regulate the production of their own outer membrane receptors (FoxA and FiuA, respectively) through a cell surface signaling pathway composed of the receptor itself, an ECF sigma factor, and an anti-sigma factor. These signaling systems show mechanistically high similarities to the *E. coli* FecIRA and *P. putida* PupIRB systems (10, 32). However, whereas the anti-sigma factors FecR and PupR are required for maximal activities of the corresponding sigma factors (32, 43), our data show that FoxR and FiuR are not required for maximal activities of FoxI and FiuI, respectively.

It has been stated previously that, based on unpublished data, the FiuA receptor is involved in the uptake of ferrioxamine B, and that the FiuR and FiuI proteins are responsible for the induction of FiuA in the presence of this compound (55). However, our analysis shows that the FiuA (PA0470) receptor is induced by ferrichrome, whereas the FoxA protein (PA2466) is induced by ferrioxamine B. This result was also confirmed by analyzing a *P. aeruginosa fiuA* mutant strain. In this mutant, the ferrioxamine-induced protein could be still detected, whereas the ferrichrome-induced protein was not detectable (Fig. 4A and 5B). In addition, the *fiuA* promoter activity was not increased in the presence of ferrioxamine B (not shown), and the growth of the *fiuA* mutant was compromised when ferrichrome was used as a source of iron, but not when ferrioxamine B was used (Fig. 5A). However, very recent data suggest that not only FoxA but also FiuA facilitates ferrioxamine B uptake (4). These two proteins share high sequence identity (40%). This probably means that FiuA can functionally replace FoxA for the uptake of ferrioxamine B in

the absence of this main ferrioxamine B receptor and vice versa, although its synthesis is not induced by the siderophore. This is consistent with the fact that growth of the *foxA* mutant in ferrioxamine B and of the *fiuA* mutant in ferrichrome was not completely abolished (not shown and Fig. 5A), suggesting the involvement of other receptors in the uptake of the siderophores. In silico analysis of the *P. aeruginosa* PAO1 genome has already suggested a certain degree of redundancy among the putative receptor genes in such a way that different receptors would facilitate the uptake of a single siderophore (11), as is the case for the siderophores pyoverdine and enterobactin (21, 22).

The described FoxIRA and FiuIRA surface signaling systems are not the first examples of siderophore-mediated regulation in *P. aeruginosa*. The ferri-enterobactin receptor gene (*pfeA*) is not only repressed by Fur in the presence of iron but is also subject to positive control by the enterobactin-responsive PfeR-PfeS two-component regulatory system (13). The involvement of alternative sigma factors in the regulation of pyoverdine uptake has also been reported (6, 10, 32). In fact, analysis of the *P. aeruginosa* genome sequence revealed the presence of 10 other ECF sigma factors associated with an anti-sigma-like factor (56). The involvement of these regulatory systems in the expression of siderophore receptors provides an additional level of regulation, enabling *P. aeruginosa* to respond to the presence of siderophores in the environment as well as to levels of intracellular iron. This seems to be an important issue for environmental bacteria, as other species of *Pseudomonas* also contain large numbers of ECF sigma factors (37, 56). In agreement with that, a genome-wide analysis of TonB-dependent receptors involved in regulatory pathways has indicated that these signaling systems are commonly found in several environmental bacteria but are only seldom present in dedicated human and animal pathogens (30).

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