

## PfeR, an Enterobactin-Responsive Activator of Ferric Enterobactin Receptor Gene Expression in *Pseudomonas aeruginosa*

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**PfeR (Regulator) and PfeS (Sensor), members of the superfamily of so-called two-component regulatory protein pairs, are required for the enterobactin-inducible production of the ferric enterobactin receptor (PfeA) in *Pseudomonas aeruginosa*. A *pfeR* knockout mutant failed to demonstrate enterobactin-inducible expression of a *pfeA-lacZ* fusion, indicating that PfeR acts at the level of *pfeA* gene expression. Consistent with this, PfeR overexpressed in *P. aeruginosa* bound, in bandshift assays, the promoter region of *pfeA*. Such binding was enhanced when PfeR-containing extracts were prepared from cells cultured in the presence of enterobactin, consistent with a model of PfeR as an enterobactin-responsive activator of *pfeA* expression. A region showing homology to the consensus binding sequence for the global iron repressor Fur was identified upstream of *pfeR*, suggesting that the *pfeRS* operon is iron regulated. As expected, expression of a *pfeR-lacZ* fusion in *P. aeruginosa* was increased under conditions of iron limitation. Enterobactin failed, however, to provide any enhancement of *pfeR-lacZ* expression under iron-limiting conditions, indicating that PfeR does not positively regulate *pfeRS* expression. A *pfeA* knockout mutant demonstrated enterobactin-inducible expression of a *pfeA-lacZ* fusion, indicating that the receptor is not required for the enterobactin inducibility of *pfeA* gene expression. Such mutants show growth, albeit reduced, in enterobactin-supplemented iron-limiting minimal medium, indicating that a second route of uptake across the outer membrane exists for ferric enterobactin in *P. aeruginosa* and may be important for the initial induction of *pfeA* in response to enterobactin.**

While nutrient acquisition is essential for bacterial growth and survival and bacteria have evolved a myriad of transport systems to avail themselves of a diversity of available nutrients, certain nutrients are absolutely required. In the case of iron, essential for all bacteria with the apparent exception of the lactobacilli, this acquisition is complicated by iron's low solubility under aerobic conditions at neutral pH (34). For animal pathogens which depend upon the host for nutrients, iron is similarly unavailable, being sequestered within cells or bound by high-affinity binding proteins such as transferrin and lactoferrin (44). Many bacteria have adapted to this iron limitation by synthesizing and secreting low-molecular-mass, high-affinity iron chelators, termed siderophores, which bind iron and deliver it to the bacterial cell via cognate cell surface receptors (31, 32). The effectiveness of siderophore-mediated iron uptake in overcoming host iron limitation contributes significantly to the virulence of many bacterial pathogens (6, 10, 16).

*Pseudomonas aeruginosa*, an important opportunistic pathogen of humans, synthesizes two known siderophores, pyoverdine (9) and pyochelin (8), and can utilize a variety of heterologous siderophores, including pyoverdines produced by other pseudomonads (22), ferrioxamine B (7), aerobactin (26), and enterobactin (26, 39). The ability to utilize a large number of siderophores presumably enhances the organism's ability to acquire iron under a range of environmental conditions and to compete with other microorganisms for iron. Outer membrane receptor proteins for ferric pyochelin (20), ferric enterobactin (39), and ferric pyoverdine (29, 37) have been identified in *P. aeruginosa*, and the genes encoding each of these, designated

*fptA*, *pfeA*, and *fpvA*, respectively, have been cloned and sequenced (2, 3, 11, 38).

Siderophore-mediated iron uptake systems in *P. aeruginosa* are globally regulated in response to environmental iron levels, mediated by the Fur repressor (40, 41). Expression of the receptors for ferric pyoverdine and ferric pyochelin is also positively regulated by the corresponding siderophore (14, 18, 19). Although the mechanisms by which receptor gene expression is enhanced by siderophores is unclear, the receptors themselves seem to play an important role. Indeed, mutants lacking FpvA fail to express the receptor gene (36), and FptA-deficient mutants show a marked decrease in *fptA* expression (19). Expression of the ferric pyochelin receptor gene also requires the PchR protein, a member of the AraC family of regulatory proteins (18). Apparently involved in mediating the pyochelin-dependent production of FptA, this protein activates *fptA* expression in the presence of pyochelin and represses receptor gene expression in the absence of this siderophore (19). Receptors involved in the uptake of heterologous siderophores are also regulated by their cognate ferric siderophores. A putative ferrioxamine B receptor in *P. aeruginosa* is inducible by ferrioxamine B (Desferal; Ciba-Geigy) (7), and the ferric enterobactin receptor is inducible by enterobactin (39). Upregulation of the PfeA receptor by enterobactin occurs at the expense of the ferric pyochelin and ferric pyoverdine receptors (11, 39), reflecting, perhaps, the need for only a single uptake system at any given time and the superior iron-chelating ability of enterobactin compared with pyochelin and pyoverdine.

Enterobactin-dependent production of PfeA is mediated by the products of the *pfeRS* operon (12). Members of the superfamily of two-component regulatory protein pairs, *pfeR* encodes a putative activator of *pfeA* gene expression while *pfeS* encodes a putative cytoplasmic membrane-associated histidine

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TABLE 1. Bacterial strains and plasmids used

Strain or plasmid	Relevant properties <sup>a</sup>	Source or reference
<b>Bacteria</b>		
<i>P. aeruginosa</i>		
PA01	Prototroph	A. Kropinski
PA06609	<i>met-9011 amiE200 rpsL pvd-9</i>	21
K407	Tn501 insertion mutant of PA06609 lacking PfeA	39
K800	PA06609 <i>pfeR::ΩTc</i> (previously called K609)	12
K802	Spontaneous streptomycin-resistant derivative of PA06609	Laboratory strain
K913	K802 <i>pfeA::Ω Hg</i>	This study
<i>E. coli</i>		
IR20	<i>aroB thi fepA</i>	17
S17-1	<i>thi pro hsdR recA Tra<sup>+</sup></i>	47
<b>Plasmids</b>		
pAK1900	Multicopy <i>E. coli-P. aeruginosa</i> shuttle vector; multicloning site within <i>lacZ</i> α fragment; Ap <sup>r</sup> , Cb <sup>r</sup>	R. Sharp
pCD3(A)	pAK1900 carrying <i>pfeRS</i> and <i>pfeA</i> on a 5.3-kb <i>SstI-BamHI</i> DNA fragment	11
pCD3(B)	pAK1900 carrying <i>pfeRS</i> and <i>pfeA</i> on a 5.3-kb <i>SphI-BamHI</i> fragment	11
pCD5	Deletion derivative of pCD3(A) carrying <i>pfeRS</i> only on a 2.5-kb insert	12
pUCP18	Multicopy <i>E. coli-P. aeruginosa</i> shuttle vector; Ap <sup>r</sup> , Cb <sup>r</sup>	46
pCD7	pUCP18 carrying <i>pfeRS</i> on a 2.5-kb <i>SstI-HindIII</i> fragment from pCD5 in the same orientation as P <sub>lac</sub> ; encompasses two potential <i>pfeR</i> start codons	This study
pCD8	pUCP18 carrying <i>pfeRS</i> on a 2.2-kb <i>BsrBI-HindIII</i> fragment from pCD5 in the same orientation as P <sub>lac</sub> ; only one potential <i>pfeR</i> start codon is retained	This study
pCD9	Derivative of pCD3(A) carrying <i>pfeA</i> , including 179 bp upstream of the start codon, on a 2.9-kb insert; directs synthesis of PfeA in K407	This study
pCD10	Deletion derivative of pCD3(A) carrying <i>pfeA</i> , including 131 bp upstream of the start codon, on a 2.9-kb insert; does not direct synthesis of PfeA in K407	This study
pCD11	Deletion derivative of pCD3(A) carrying <i>pfeR</i> on a 1.2-kb insert	This study
pCD12	pUCP18 carrying a 247-bp <i>EcoRI-RsaI</i> <i>pfeA</i> promoter fragment derived from pCD9	This study
pT7-7	pBR322 derivative carrying a MCS downstream of the strong gene 10 promoter of phage T7 and a Shine-Dalgarno site; Ap <sup>r</sup>	50
pVLT31	<i>E. coli-P. aeruginosa</i> shuttle expression vector; <i>lacI<sup>a</sup></i> , Tc <sup>r</sup>	13
pREX1	pVTL31::pfeR	This study
pSUP202	Gene replacement vector derived from pBR325; Mob <sup>+</sup> , Ap <sup>r</sup> , Tc <sup>r</sup> , Cam <sup>r</sup>	47
pCD13	pSUP202 <i>pfeA::ΩHg</i>	This study
pMP190	Low-copy-number, promoterless <i>lacZ</i> fusion vector; Cam <sup>r</sup> , Sm <sup>r</sup>	48
pCD14	<i>pfeA::lacZ</i> fusion derivative of pMP190; harbors a 782-bp <i>SalI</i> fragment derived from pCD3(B) encompassing the <i>pfeA</i> promoter region	This study
pCD15	<i>pfeR-lacZ</i> fusion derivative of pMP190; harbors a 750-bp <i>EcoRI-XmnI</i> fragment derived from pCD7 encompassing the <i>pfeR</i> upstream region	This study

<sup>a</sup> Ap<sup>r</sup>, ampicillin resistance; Cb<sup>r</sup>, carbenicillin resistance; Tc<sup>r</sup>, tetracycline resistance; Cam<sup>r</sup>, chloramphenicol resistance; Sm<sup>r</sup>, streptomycin resistance; MCS, multiple cloning site; Mob<sup>+</sup>, mobilizable.

kinase sensor which probably detects enterobactin and stimulates PfeR activity (12). According to current models for two-component regulators, such stimulation apparently involves phosphotransfer from a conserved C-terminal domain of the sensor to a conserved C-terminal domain of the activator which increases the affinity of the activator for its target DNA (35, 49). In the present report we examine the regulation of *pfeA* expression by PfeR, demonstrating that PfeR binds to the *pfeA* promoter and that such binding is enhanced by enterobactin.

#### MATERIALS AND METHODS

**Bacterial strains and plasmids.** The bacterial strains and plasmids used in this study are described in Table 1. Spontaneous streptomycin-resistant derivatives of *P. aeruginosa* were isolated by harvesting 5 ml of an overnight Luria (L) broth culture and plating the pellet (resuspended in 100 μl) on L agar containing 1,000 μg of streptomycin per ml. Distinct colonies appearing after 24 to 48 h of incubation at 37°C were recovered. Plasmid pCD8 was constructed by ligating a 2.1-kb *BsrBI-EcoRI* fragment of pCD5, encompassing *pfeRS*, into *SmaI-HindIII*-digested pUCP18. In constructing plasmid pCD9, plasmid pCD3(A), carrying a 5.3 kb-*SstI-BamHI* fragment encompassing both the *pfeRS* operon and *pfeA*, was linearized with *SstI* and partially digested with *PvuI*. A 7.68-kb *SstI-PvuI* fragment encompassing pAK1900 vector sequences and the *pfeA* gene was then isolated, blunt-ended with S1 nuclease, and recircularized. During S1 digestion, the *PvuI* site was destroyed and 16 bp of double-stranded DNA were

removed, leaving 179 bp upstream of the ATG start codon of *pfeA*. Plasmids pCD10 and pCD11 were constructed by exonuclease III digestion of plasmid pCD3(B) and pCD3(A), respectively, as described previously (11). Plasmid pCD12 was constructed by ligating a 247-bp *EcoRI-RsaI* fragment containing the *pfeA* promoter, derived from plasmid pCD9, into *EcoRI-SmaI*-restricted pUCP18. Plasmid pCD15 was constructed by isolating a 750-bp *EcoRI-XmnI* fragment of pCD7 carrying the *pfeR* upstream region, filling in the *EcoRI* overhang with Klenow fragment, and cloning the resultant fragment into *SalI*-restricted pMP190 made blunt ended with Klenow fragment.

**Media.** BM2 minimal medium (15), supplemented with 0.5 mM MgSO<sub>4</sub> and either 20 mM potassium succinate or 0.4% (wt/vol) glucose, was used as the iron-deficient medium throughout. Methionine (1 mM), shikimic acid (0.2 mM; Aldridge Chemical Co., Milwaukee, Wis.), and thiamine hydrochloride (10 μg/ml) were added to minimal media as required. L broth (1% [wt/vol] tryptone-0.5% [wt/vol] yeast extract-0.05% [wt/vol] NaCl) or brain heart infusion (BHI) (Difco) broth was employed as the rich medium as indicated. Antibiotics, including carbenicillin (100 μg/ml in minimal medium, 200 μg/ml in rich medium), tetracycline (10 μg/ml, *Escherichia coli*; 75 to 100 μg/ml, *P. aeruginosa*), ampicillin (100 μg/ml) and chloramphenicol (30 μg/ml, *E. coli*; 100 μg/ml [broth] and 200 μg/ml [agar], *P. aeruginosa*), were included in growth media as required. Solid media were prepared by adding 1.5% (wt/vol) Bacto-Agar (Difco).

**Preparation of enterobactin.** Enterobactin, as concentrated spent culture supernatant, was prepared from overnight 1-liter cultures of *E. coli* IR20 as described previously (39).

**Cell fractionation and SDS-PAGE.** Outer membrane fractions were isolated by differential Triton X-100 solubilization of cell envelopes as described previously (45). Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was conducted as previously described (27) with 9% (wt/vol) acrylamide

in the running gel and omitting  $\beta$ -mercaptoethanol from the sample loading buffer. Whole-cell protein extracts were prepared by harvesting 200  $\mu$ l of cell culture by centrifugation, resuspending the pellet in 100  $\mu$ l of gel loading buffer, and heating at 95°C for 5 min.

**DNA methods.** Standard methods were employed for isolation of plasmid DNA, digestion with S1 nuclease and restriction endonucleases, ligation, filling in of 5' overhangs with Klenow fragment, and separation of DNA fragments by agarose gel electrophoresis (43). Following electrophoretic separation, DNA restriction fragments were purified with the Prep-A-Gene DNA purification kit (BIO-RAD Laboratories Inc., Richmond, Calif.). Removal of unincorporated nucleotides following fill-in reactions with DNA fragments smaller than 500 bp was accomplished by using the Qiaquick PCR prep kit (Qiagen Inc., Chatsworth, Calif.). Plasmid DNA was transformed into *P. aeruginosa* and *E. coli* by using the protocols described by Berry and Kropinski (5) and Sambrook et al. (43), respectively.

**Site-directed mutagenesis of *pfeR* and construction of a PfeR overexpression vector.** Site-directed mutagenesis was carried out by using the Sculptor site-directed mutagenesis kit (Amersham) according to a protocol supplied by the manufacturer. The synthetic oligonucleotide 5'-GAG ATA TTC ACG AAC ATA TGA ATC ATT CTC ATA TTT C-3', carrying an imbedded *NdeI* site (underlined), was synthesized by the Core Facility for Protein and DNA Chemistry at Queen's University and used to introduce an *NdeI* site at the 5' end of *pfeR*, at the same time converting the GTG start codon to an ATG (see Fig. 3). The altered *pfeR* gene was subsequently excised on an *NdeI-HindIII* fragment and ligated into *NdeI-HindIII*-restricted plasmid pT7-7. This bacteriophage T7-based *E. coli* expression vector has a ribosomal binding site optimally located 8 bp upstream of the ATG start codon embedded in the *NdeI* restriction site (50). Thus, the *pfeR* gene and optimally spaced ribosome binding site could be recovered on a *XbaI-HindIII* fragment and cloned into the broad-host-range expression vector pVLT31 to generate pREX1 (see Fig. 3).

**Overexpression of PfeR and preparation of crude cell extracts.** To induce expression of *pfeR* on pREX1, *P. aeruginosa* PAO6609 harboring pREX1 (or pVLT31 in control studies) was grown overnight at 37°C in L broth containing tetracycline, diluted 1:10 into the same medium, and grown to an  $A_{600}$  of approximately 0.5. Isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG) was then added at a final concentration of 3 mM, and the culture allowed to grow at 37°C for an additional 5 h. To reduce the levels of PfeR produced following induction with IPTG, overnight cultures grown in L broth containing tetracycline (75  $\mu$ g/ml) were diluted 1:3 into the same medium and incubated at 18°C for 2 h. The culture was then induced with 1 mM IPTG and incubated a further 5 h at 18°C. Crude cell extracts for use in bandshift assays were prepared from cells harvested after induction in L broth at 18°C by resuspending the cells in 1 ml 50 mM Tris-hydroxymethylaminomethane (Tris)-HCl (pH 7.5)-10 mM MgCl<sub>2</sub>, subjecting them to one cycle of freeze-thaw, and sonicating them on ice at 50% power by using a Vibrosonic sonicator (Sonic and Materials, Danbury, Conn.). The disrupted cells were centrifuged at 50,000 rpm for 15 min in a Beckman TL100 ultracentrifuge, and the supernatants were recovered. If extracts were not used immediately in bandshift assays, glycerol was added to the supernatants (50% vol/vol final concentration), which were then stored at -80°C. In some experiments, crude cell extracts were prepared from cells induced in iron-deficient minimal medium. In these instances, *P. aeruginosa* PAO6609(pREX1) was grown overnight at 37°C in BM2 succinate minimal medium containing tetracycline (75  $\mu$ g/ml), diluted 1:1 into the same medium containing ethylenediamine di(*o*-hydroxyphenylacetic acid) (EDDHA) (25  $\mu$ g/ml) and incubated at 18°C for 2 h. IPTG (1 mM) was then added to cell cultures, which were incubated for an additional 3 h before the cells were harvested and suspended in 1 ml 25 mM Tris-HCl (pH 7.5)-10 mM MgCl<sub>2</sub>, and crude extracts were prepared as described above. Where indicated, enterobactin (25  $\mu$ l/ml concentrated culture supernatant) was added to cell cultures 1.5 h prior to harvesting.

**N-terminal amino acid sequence determination.** Overexpressed PfeR, present in outer membrane fractions prepared from PfeR-overproducing *P. aeruginosa* PAO6609(pREX1) induced at 37°C (see above), was separated by SDS-PAGE and electrophoretically transferred to ProBlott membrane (Applied Biosciences Inc., Mississauga, Ontario, Canada) in 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS) buffer according to a protocol provided by the manufacturer. Blotted proteins were stained with Coomassie brilliant blue, and the desired bands were excised with a razor blade and their N-terminal amino acid sequences were determined by the Core Facility for Protein and DNA Chemistry at Queen's University.

**Bandshift assays.** Bandshift assays were performed with the Pharmacia bandshift kit (Pharmacia LKB Biotechnology AB, Uppsala, Sweden) according to the low-salt, low-ionic-strength protocol supplied with the kit. The target DNA used was the 247-bp *pfeA* promoter fragment contained on plasmid pCD12, which was excised with *EcoRI* and *BamHI* and labelled with [ $\alpha$ -<sup>32</sup>P]dATP by using the Klenow fragment of DNA polymerase I (43).

**In vitro mutagenesis and gene replacement.** To construct a *pfeA* knockout mutant, the *pfeA* gene was recovered on a 3.2-kb *EcoRI* fragment from pCD9 and cloned into the unique *EcoRI* site in pSUP202. The recombinant vector was then restricted with *XhoI*, which cut once in *pfeA* and nowhere else on the plasmid, and the *XhoI* ends were filled in with Klenow fragment. A ca. 5-kb *SmaI* fragment of pHP45 $\Omega$ Hg carrying the HgCl<sub>2</sub> resistance-encoding  $\Omega$  Hg interposon was then ligated with this DNA and transformants carrying the pSUP202::

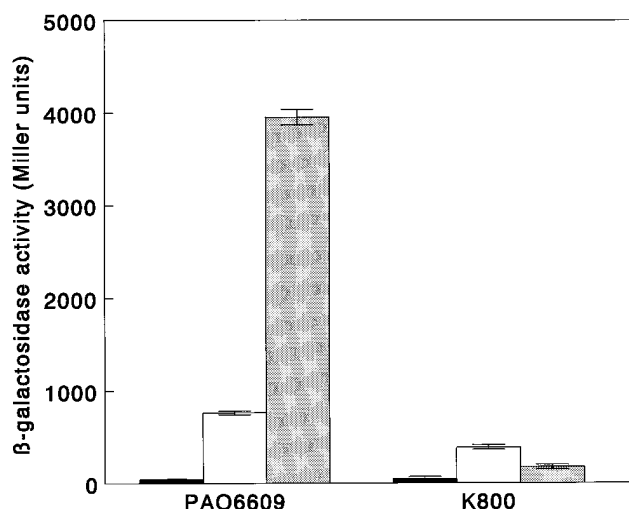


FIG. 1. Regulation of *pfeA* expression by PfeR and iron. PAO6609 and its PfeR<sup>-</sup> derivative K800 each carrying the *pfeA-lacZ* fusion vector pCD14 were cultured overnight in chloramphenicol-containing BHI broth alone (iron-rich medium) (filled bars) or with EDDHA (iron-limited medium) (open bars) or EDDHA and enterobactin (shaded bars). Following dilution (100-fold) into the same medium, cells were cultured to an  $A_{600}$  of 0.6 to 1.0 and assayed for  $\beta$ -galactosidase activity. Data are the means of three determinations  $\pm$  standard deviations (error bars).

*pfeA*:: $\Omega$ Hg construct selected on L agar containing tetracycline and chloramphenicol. Following confirmation that the  $\Omega$ Hg interposon had been inserted within the *pfeA* gene on pSUP202, the recombinant vector (pCD13) was introduced into *E. coli* S17-1 and mobilized into the streptomycin-resistant derivative of PAO6609 (K802) via conjugation as described previously (18). Transconjugants recovered on L agar plates containing streptomycin and HgCl<sub>2</sub> after 24 to 48 h of growth at 37°C were screened for sensitivity to carbenicillin. *P. aeruginosa* recipients carrying a  $\Omega$ Hg-inactivated *pfeA* gene on the chromosome were HgCl<sub>2</sub> resistant and carbenicillin sensitive. The PfeA deficiency of these recipients was confirmed by SDS-PAGE analysis of isolated outer membranes.

**Growth assays.** Overnight cultures of *P. aeruginosa* grown in BM2 succinate minimal medium were diluted into the same medium containing EDDHA (150  $\mu$ g/ml), with and without added enterobactin-containing *E. coli* culture supernatant (25  $\mu$ l/ml), to an  $A_{600}$  of 0.05. Cultures were shaken (200 rpm) in an orbital shaker at 37°C, and the increase in culture turbidity ( $A_{600}$ ) was monitored.

**$\beta$ -Galactosidase assays.** *P. aeruginosa* strains carrying the *lacZ* fusion vector pMP190 or its derivatives were grown overnight at 37°C in BHI broth containing chloramphenicol, EDDHA (30  $\mu$ g/ml), and enterobactin-containing concentrated *E. coli* culture supernatant (25  $\mu$ l/ml) as indicated. Following dilution (1/50 to 1/100) into the same media, cells were grown to mid- to late-log phase at 37°C, and aliquots (100  $\mu$ l) were assayed for  $\beta$ -galactosidase activity according to a previously described protocol (30).

## RESULTS

**PfeR-dependent expression of *pfeA*.** Insertional inactivation of *pfeR* effectively eliminates enterobactin-inducible production of the ferric enterobactin receptor PfeA by *P. aeruginosa* (12). To determine if this effect was mediated at the level of *pfeA* gene expression, a *pfeA-lacZ* fusion (pCD14) was constructed and the influence of a *pfeR* knockout mutation on *pfeA-lacZ* expression was assessed. As expected, *pfeA-lacZ* expression increased markedly in the PfeR<sup>+</sup> parent strain PAO6609 upon exposure to enterobactin, although a lower, but still significant, level of *pfeA* gene expression was evident under conditions of iron limitation in the absence of enterobactin (Fig. 1). The PfeR<sup>-</sup> strain, K800, also demonstrated low-level enterobactin-independent expression of *pfeA* under conditions of iron limitation, although the levels observed were about twofold less than that seen in PAO6609, suggesting that PfeR may be capable of limited enterobactin-independent ac-

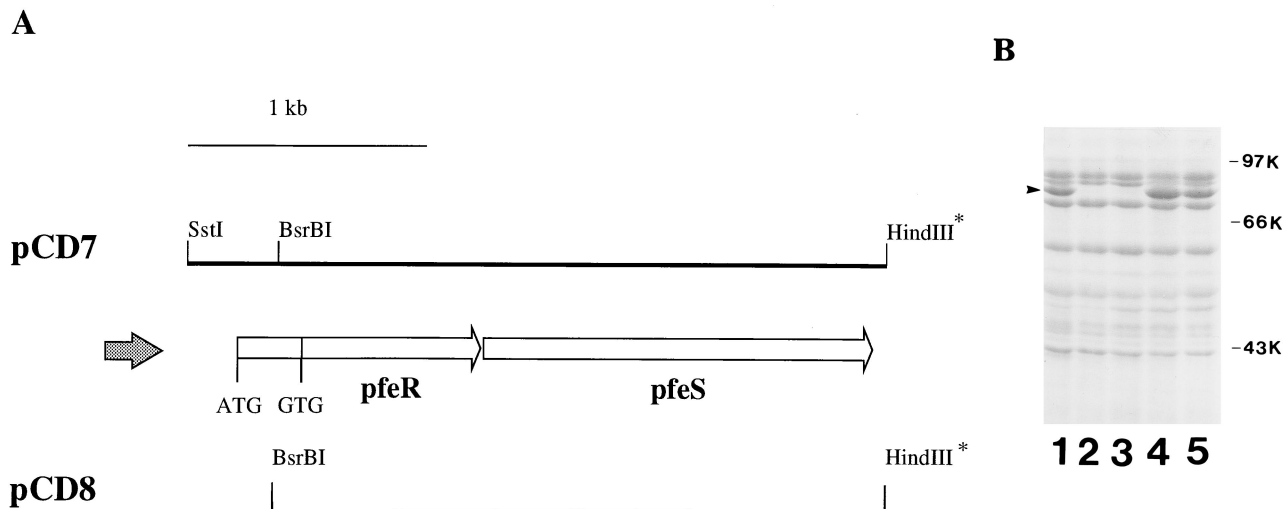


FIG. 2. (A) Physical map of the *pfeRS* operon detailing the positions of the GTG and ATG potential start codons in *pfeR* and the regions cloned into plasmid pUCP18 to yield pCD7 and pCD8. The position and orientation of the *lac* promoter of pUCP18 are indicated (shaded arrow). *HindIII*<sup>\*</sup>, restriction site derived from vector in which the genes were originally cloned. (B) Outer membrane proteins of *P. aeruginosa* PAO6609 (lane 1), K407 (lane 2), K800(pUCP18) (lane 3), K800(pCD7) (lane 4), and K800(pCD8) (lane 5), grown in BM2 succinate minimal medium with EDDHA (150  $\mu$ g/ml) and enterobactin. The ferric enterobactin receptor protein PfeA is indicated by the arrowhead. Samples were solubilized at 95°C for 5 min prior to electrophoresis. Molecular mass markers are shown on the right.

tivation of *pfeA*. In contrast to PAO6609, however, K800 failed to exhibit any increase in *pfeA-lacZ* expression in response to enterobactin (Fig. 1), indicating that PfeR is required for enterobactin-dependent activation of *pfeA* gene expression.

**Determination of the translational start site of *pfeR*.** A presumed activator of *pfeA* expression, PfeR is expected to bind to the *pfeA* promoter region. Overproduction of PfeR for the purpose of assessing this ability necessitated the cloning of *pfeR* into an expression vector. As two potential translation initiation sites, one a GTG codon and the other an ATG codon 201 bp upstream of the GTG codon, were previously identified for *pfeR* (12) (Fig. 2A), it was first necessary to determine the correct initiation site for *pfeR*. Thus, plasmids carrying *pfeS* and the *pfeR* open reading frame with both codons (pCD7) (Fig. 2A) or with the GTG codon but lacking the ATG codon (pCD8) (Fig. 2A) were introduced into the *pfeR* knockout strain K800, and production of PfeA was assessed. Both pCD7 and pCD8 complemented the *pfeR* mutation in strain K800, enabling PfeR-dependent production of PfeA in response to enterobactin (Fig. 2B), indicating that *pfeR* initiates at the GTG codon.

**Overexpression of PfeR.** Initially, the 5' end of the *pfeR* coding region was manipulated, by using site-directed mutagenesis, to enable cloning into the pT7-7 expression vector such that the *pfeR* initiation codon was spaced optimally with respect to the Shine-Dalgarno sequence resident on this vector (Fig. 3). Unfortunately, this construct failed to generate detectable levels of PfeR protein in *E. coli* (data not shown). Thus, the *pfeR* gene and upstream Shine-Dalgarno sequence were recovered from pT7-7 and cloned into the broad-host-range expression vector pVLT31 to yield pEX1 (Fig. 3). Introduction of this vector into *P. aeruginosa* PAO6609 and induction of *pfeR* with IPTG at 37°C yielded high levels of two proteins with molecular masses of 26 and 30 kDa, identifiable in whole-cell extracts (Fig. 4A). The mass of the larger protein was in agreement with that predicted for the translated *pfeR* product, although the N-terminal amino acid sequences of both proteins exactly matched the predicted N-terminal se-

quence of PfeR, indicating that both were PfeR. Treatment of these extracts with  $\beta$ -mercaptoethanol (200 mM) resulted in the loss of the lower-molecular-mass species (data not shown), indicating that it likely contained an intramolecular disulphide bond between the two cysteine residues present in this protein. Upon fractionation, these proteins were recovered with the outer membrane fraction along with a number of higher-molecular-mass proteins which were regularly spaced on the gel (Fig. 4B). Again, these all appeared to resolve into a single band upon treatment with mercaptoethanol (data not shown), suggesting that the higher-molecular-mass species were intermolecular disulphide-bonded versions of PfeR. Consistent with this, the N-terminal amino acid sequence of two of these matched the predicted PfeR sequence (data not shown). The cofractionation of overexpressed PfeR with outer membranes suggested that the protein was forming inclusion bodies, which precluded its immediate use in DNA-binding assays. In an effort to minimize inclusion body formation, therefore, induction of *pfeR* expression with IPTG was carried out at a reduced temperature (18°C). At this temperature, yields of PfeR were markedly reduced (Fig. 4C), although the protein was recoverable in the soluble fraction (data not shown).

**PfeR binds to the promoter region of *pfeA*.** Crude cell lysates prepared from the *pfeR* knockout mutant K800 or from *P. aeruginosa* PAO6609 carrying pVLT31 alone failed to alter the mobility of the 249-bp *pfeA* promoter-containing DNA fragment (Fig. 5, lanes 2 and 3) which encompasses the 179 bp upstream of *pfeA* shown to be required for PfeA production (see below). In contrast, extracts prepared from PfeR-overproducing PAO6609 carrying pREX1 altered the mobility of the promoter-containing fragment (Fig. 5, lane 4), consistent with PfeR interacting with this DNA. Competition with cold target DNA eliminated the mobility shift (Fig. 5, lane 5) while competition with cold unrelated DNA did not (Fig. 5, lane 6), confirming the specificity of the interaction.

According to accepted models of two-component systems, PfeR binding to the *pfeA* promoter region should be stimulated in response to enterobactin (presumably mediated by PfeS).

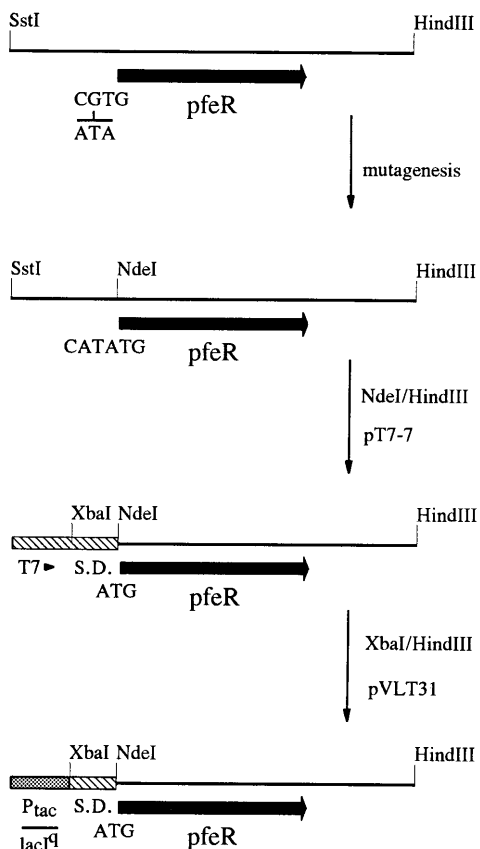


FIG. 3. Schematic showing the construction of the PfeR expression vector pREX1.

To assess the influence of enterobactin on PfeR binding, extracts were prepared from cells grown with and without enterobactin. Cells cultured in rich medium failed to demonstrate any difference in binding (as measured by amount of *pfeA*

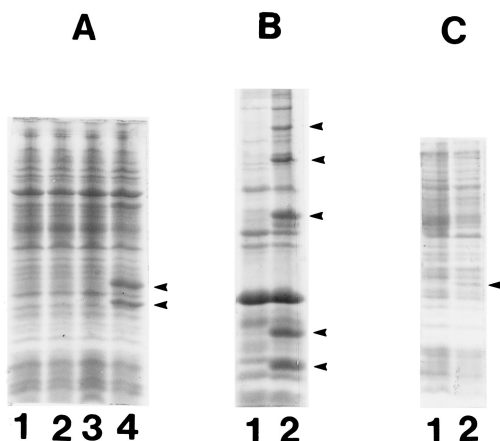


FIG. 4. Overexpression of PfeR in *P. aeruginosa* PAO6609. (A) SDS-polyacrylamide gel of whole-cell protein extracts prepared from PAO6609(pVLT31) (lanes 1 and 2) and PAO6609(pREX1) (lanes 3 and 4) grown at 37°C with (lanes 2 and 4) and without (lanes 1 and 3) IPTG. (B) Outer membrane proteins of PAO6609(pVLT31) (lane 1) and PAO6609(pREX1) (lane 2) grown at 37°C with IPTG. (C) Whole-cell protein extracts of PAO6609(pVLT31) (lane 1) and PAO6609(pREX1) (lane 2) grown at 18°C with IPTG. PfeR and PfeR aggregates are indicated by arrowheads. Samples were solubilized at 95°C for 5 min prior to electrophoresis.

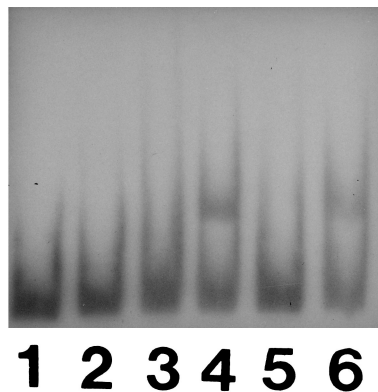


FIG. 5. Electrophoretic mobility of the <sup>32</sup>P-labelled *pfeA* promoter fragment (see Fig. 7A) incubated with no protein (lane 1) or crude cell extracts (50 µg protein) prepared from *P. aeruginosa* K609 (lane 2), PAO6609(pVLT31) (lane 3), or PAO6609(pREX1) (lane 4). Competition experiments were conducted for the pREX1 extracts by using excess cold target DNA (lane 5) or cold Epstein-Barr virus nuclear antigen (EBNA-1) DNA supplied with the Pharmacia band-shift kit (lane 6).

promoter DNA shifted), irrespective of the presence or absence of enterobactin (Fig. 6, lanes 2 and 3). Cells cultured in iron-deficient minimal medium, however, demonstrated a marked influence of enterobactin, with noticeably more promoter DNA shifting in the presence of extracts derived from enterobactin-grown cells (Fig. 6, lane 5) compared with extracts from cells cultured without enterobactin (Fig. 6, lane 4). Although the same concentration of total protein was employed in each case, it was evident that extracts prepared from cells cultured in rich medium (in the absence of enterobactin) shifted more of the *pfeA*-promoter DNA than did cells cultured without enterobactin in minimal medium (Fig. 6, compare lanes 2 and 4). Although it was not possible to quantitate PfeR levels in these extracts, this could be due to increased vector-mediated PfeR production in rich compared with minimal medium.

**Localization of the region upstream of *pfeA* necessary for PfeA production.** In order to localize the probable PfeR binding site in the region upstream of *pfeA*, the minimal region

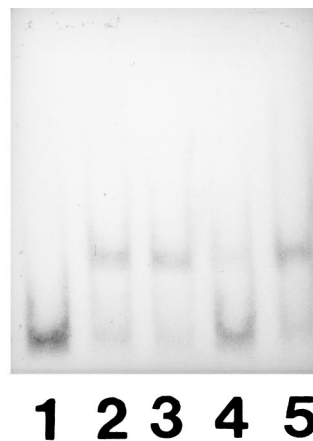


FIG. 6. Electrophoretic mobility of the <sup>32</sup>P-labelled *pfeA* promoter fragment (see Fig. 7A) incubated with no protein (lane 1) or with crude cell extracts (50 µg protein) prepared from *P. aeruginosa* PAO6609(pREX1) induced with IPTG in L broth (lanes 2 and 3) or in BM2 succinate minimal medium containing EDDHA (25 µg/ml) (lanes 4 and 5) with (lanes 3 and 5) and without (lanes 2 and 4) enterobactin.



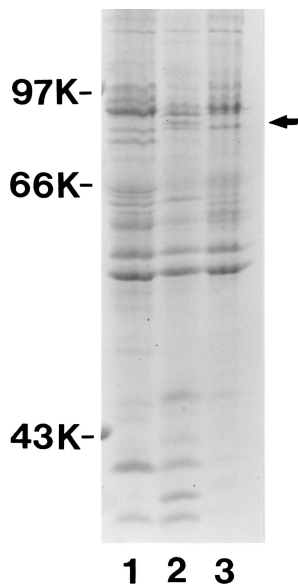


FIG. 10. Outer membrane protein profiles of *P. aeruginosa* PAO6609 (lanes 1 and 2) and its PfeA<sup>-</sup> derivative K913 (lane 3) grown in BHI broth without (lane 1) and with (lanes 2 and 3) enterobactin. The PfeA receptor protein is indicated by the arrow. Samples were solubilized at 95°C for 5 min prior to electrophoresis. Molecular mass markers are shown on the left.

transport of enterobactin across the outer membrane exists in *P. aeruginosa* and may be important for the enterobactin inducibility of the *pfeA* gene in K913.

## DISCUSSION

Control of the expression of individual genes in response to environmental stimuli affords bacteria the ability to quickly adapt to environmental change. Numerous bacterial adaptive

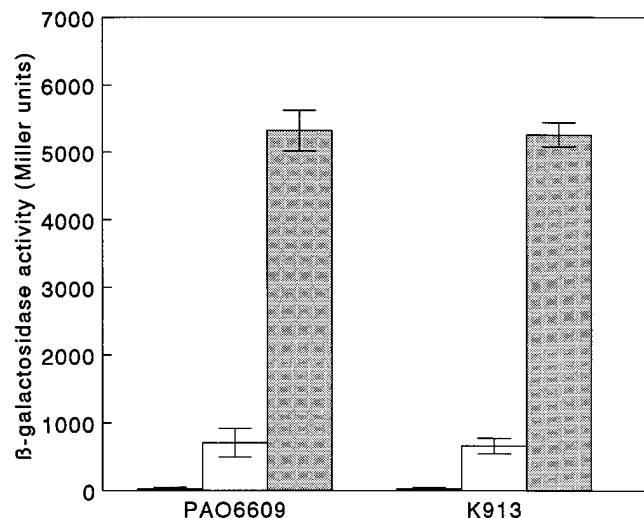


FIG. 11. Influence of PfeA on *pfeA* expression. *P. aeruginosa* strains PAO6609 and its PfeA<sup>-</sup> derivative K913, each carrying the *pfeA-lacZ* fusion vector pCD14, were cultured overnight in chloramphenicol-containing BHI broth alone (iron-rich medium) (filled bars) or with EDHHA (iron-limited medium) (open bars) or EDDHA and enterobactin (shaded bars). Following dilution (100-fold) into the same medium, cells were grown to an  $A_{600}$  of 0.6 to 1.0 and assayed for  $\beta$ -galactosidase activity. Data are the means of three determinations  $\pm$  standard deviations (error bars).

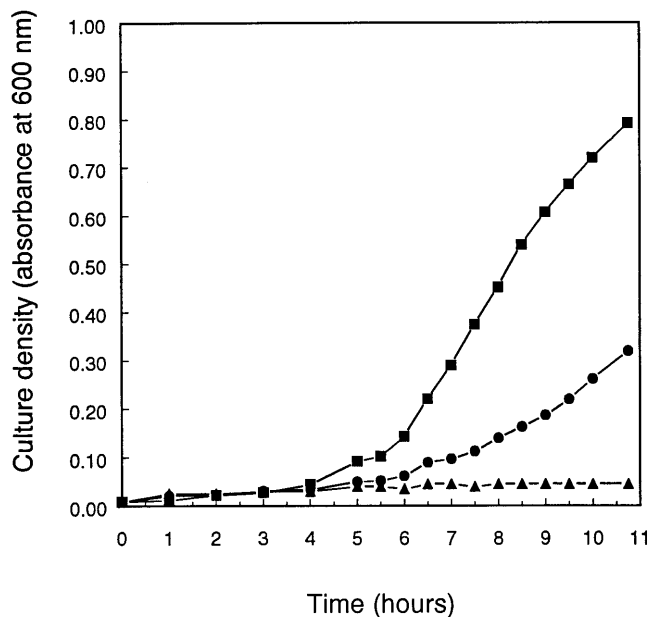


FIG. 12. Growth of *P. aeruginosa* strain PAO6609 (■) and its PfeA<sup>-</sup> derivative K913 (●) in BM2 succinate minimal medium containing EDDHA (150 g/ml) and enterobactin. Growth of both strains in the same medium in the absence of enterobactin (▲) is also shown.

responses are mediated by modular two-component protein pairs consisting of a histidine kinase (sensor), which detects an environmental signal, and a positive activator of target gene transcription (35). Enterobactin-dependent expression of the ferric enterobactin receptor protein (PfeA) in *P. aeruginosa* requires PfeS and PfeR, a sensor and regulator, respectively, belonging to this superfamily (12). As such, PfeR was expected to activate *pfeA* gene expression via an interaction with and upregulation of the *pfeA* promoter, as was demonstrated in this study. The failure of enterobactin to influence the binding of PfeR prepared from iron-rich cells to the *pfeA* promoter probably reflects the lack of PfeS in such cells. Although the levels of PfeR were elevated in these experiments because of overexpression from the multicopy expression vector, expression of *pfeS* off of the chromosome would likely be minimal given the demonstrated iron regulation of *pfeRS* expression. In the absence of PfeS, enterobactin-dependent activation of PfeR binding to the *pfeA* promoter is not possible. In contrast, cells cultured under iron-limiting conditions would express chromosomal *pfeS* and the sensor could activate PfeR in responses to enterobactin, thereby enhancing its binding to the *pfeA* promoter, as was observed.

The region upstream of *pfeA* necessary for *pfeA* expression and apparently involved in PfeR binding is characterized by the presence of two pairs of direct repeats. This is consistent with the presence of direct repeats in the binding sites of a number of other response regulators, particularly those of the OmpR subclass (1), to which PfeR belongs, and AlgR1 (23). The importance of one or both of the repeats upstream of *pfeA* in *pfeA* expression is highlighted by the striking reduction in PfeA production upon deletion of the first member of each repeat.

Although the binding of activators to target genes is typically stimulated by phosphorylation by the corresponding sensor component, regulators often exhibit constitutive target responses when overexpressed in the absence of their specific sensor partner (1). Potential explanations for this observation

include residual activity of the regulators in the absence of phosphorylation or low level phosphorylation as a result of cross talk with other active sensor proteins (1) or acquisition of phosphate from small phosphodonor molecules (35). Thus, PfeR still binds the *pfeA* promoter in the absence of enterobactin (and, presumably, activation by PfeS) when overexpressed in cells cultured in rich medium. The observed enterobactin-independent expression of *pfeA* under iron-limiting conditions which was decreased ca. twofold in the absence of PfeR (Fig. 1) suggests that PfeR may exhibit enterobactin (and PfeS)-independent binding to *pfeA* in vivo. Whether this contributes basal levels of PfeA under iron-limiting conditions to provide for the initial uptake of ferric enterobactin for interaction with PfeS and subsequent upregulation of *pfeA* expression is unknown. Nonetheless, the observation that mutants lacking PfeA still demonstrate enterobactin-inducible *pfeA* expression and are capable of acquiring ferric enterobactin indicates that a second route of transport across the outer membrane exists for ferric enterobactin which could also provide for the initial uptake of the iron-siderophore complex.

The nature of the secondary uptake pathway for ferric enterobactin across the outer membrane in *P. aeruginosa* is unknown. The siderophore-iron complex could be transported via a pathway for related molecules. In this vein, an iron-regulated catechol-specific outer membrane transport protein has recently been described in *P. aeruginosa* PAO1 that may be analogous to the Fiu and Cir proteins of *E. coli* (53). While the in vivo function of this protein is unclear, its apparent role in transporting catechol-substituted cephalosporins suggests that it could transport a range of catechol derivatives across the outer membrane, including ferric enterobactin.

Alternatively, ferric enterobactin could enter the periplasm via passive diffusion through porin channels. The minimum size limit of 500 Da required for siderophores to effectively bind iron may preclude their diffusion across the outer membrane of many gram-negative bacteria (33), but the relatively large porin channels present in the outer membrane of *P. aeruginosa* (4) apparently do allow passage of iron-siderophore complexes (28). Mutation resulting in the loss of the outer membrane protein OprF, which acts as a porin (51) with a molecular mass cutoff of 3,000 Da (4), eliminates uptake of desferriferrioxamine B and E, desferriferriochrysin, and desferriferrirosin in *P. aeruginosa* (28). While ferric enterobactin-mediated iron transport was not observed for cells uninduced for PfeA in the Meyer study (28), earlier experiments show that *P. aeruginosa* uninduced for PfeA, or Tn501 mutants lacking PfeA, showed readily measurable transport, if the ferric enterobactin levels used in the assay were increased 10-fold over the levels typically used in assays measuring high-affinity PfeA-mediated transport (39).

The ability of PfeA-deficient cells to activate *pfeA* gene expression contrasts with the observation that the receptors for the endogenous siderophores of *P. aeruginosa* (pyochelin and pyoverdine) appear to be directly involved in the upregulation of their own expression in response to the corresponding siderophore (36). Although as yet poorly defined in *P. aeruginosa*, receptor-dependent receptor gene expression is comparatively well characterized in *Pseudomonas putida*, where the PupB ferric pseudobactin BN7/8 receptor was shown to function both in transport and in signal transduction leading to receptor gene expression (24, 25). In this instance, PupB-dependent *pupB* expression is mediated by two proteins, PupR and PupI, the former apparently receiving a signal from PupB via an interaction with the receptor which it passes on to PupI, a sigma factor which stimulates *pupB* expression (25). A conformational change in PupB during transport apparently initiates

the signal transduction cascade, and the entry of the ferric pseudobactin into the cell is not required (25). The lack of a requirement for PfeA for *pfeA* expression and the observation that expression of *pfeA* correlates with an ability of cells to acquire ferric enterobactin (as demonstrated by enterobactin-dependent growth in iron-limited medium) argues that, for *pfeA* expression, the iron-siderophore complex must enter the cell, where it presumably interacts with PfeS.

The regulation of *pfeRS* by iron is consistent both with the presence of a Fur binding site upstream of *pfeR*, to which Fur binding has been confirmed (52), and with the specific need for these components under conditions of iron limitation. The observation that enterobactin fails to enhance *pfeRS* expression under iron-limiting conditions indicates that PfeR does not upregulate its own expression. That PfeR<sup>-</sup> mutants exhibit an increase in *pfeR* expression in iron-limited medium argues, however, that *pfeR* may be subject to negative autoregulation, a common feature of activator proteins (42). This effect of PfeR deficiency is not observed in cells grown with enterobactin, which appears to ameliorate the increased *pfeR* gene expression seen in PfeR<sup>-</sup> mutants. Although this is not understood at present, it is interesting to note that *pfeA* gene expression in a PfeR<sup>-</sup> mutant also decreases under iron-limiting conditions in the presence of enterobactin.

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