Pyoverdine-Mediated Regulation of FpvA Synthesis in *Pseudomonas aeruginosa*: Involvement of a Probable Extracytoplasmic-Function Sigma Factor, FpvI

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A search of the *pvd* pyoverdine biosynthesis locus of *Pseudomonas aeruginosa* identified an open reading frame, PA2387, whose product exhibited a sequence similar to those of a number of so-called extracytoplasmic-function sigma factors responsible for siderophore-dependent expression of iron-siderophore receptors in *Escherichia coli* and *Pseudomonas putida*. Deletion of this gene, dubbed *fpvI*, compromised pyoverdine-dependent FpvA ferric pyoverdine receptor production and *fpvA* gene expression, while the cloned gene stimulated *fpvA* expression. A Fur-binding site was identified immediately upstream of *fpvI*, consistent with the observed iron-regulated expression of *fpvI* and *fpvA*.

With few exceptions, almost all bacteria require iron for growth and survival (27). Iron acquisition in nature is complicated, however, due to the low solubility of iron under aerobic conditions at neutral pH (27). Pathogenic organisms face similar restrictions in human hosts, since iron is generally sequestered intracellularly in heme-containing compounds, or in fluids, by iron-binding proteins such as lactoferrin and transferrin (39). Many bacteria overcome this problem by synthesizing high-affinity iron chelators called siderophores (29). Together with siderophore-specific outer membrane receptors, these facilitate the uptake of iron required to sustain growth and pathogenesis (28).

*Pseudomonas aeruginosa*, an opportunistic human pathogen (10), produces two known siderophores, pyoverdine (6) and pyochelin (5), in response to iron limitation. Pyoverdine is the superior chelator, at least at neutral pH (23), and is required for in vivo growth and virulence (24, 34, 48). Genes involved in the biosynthesis of pyoverdine localize in two gene clusters, the *pvc* operon (45, 46) and the *pvd* locus (22, 26, 36, 50), which are implicated in the synthesis of the chromophore and peptide moieties, respectively. The *fpvA* gene encoding the ferric pyoverdine receptor is also localized in the *pvd* cluster (22, 35, 36).

Although it is an essential nutrient for growth and pathogenesis, iron, in excess, is toxic to cells (12). Thus, uptake genes are tightly regulated by intracellular iron levels, mediated by the Fur repressor (8, 13, 38). Though a Fur homologue has been identified in *P. aeruginosa* (38), it does not directly regulate genes involved in pyoverdine biosynthesis. Rather, an alternative sigma factor, PvdS (7, 21, 26, 51), which positively regulates the expression of several pyoverdine biosynthetic genes (20, 52), is itself regulated by Fur (7, 20, 26). Pyoverdine control of FpvA expression has also been reported (11, 35), reminiscent of siderophore-dependent receptor gene expression in *Escherichia coli* (14) and *Pseudomonas putida* (17). In *E. coli*, ferric dicitrinate upregulates its receptor, FecA, and, via a two-component system, FecIR (49), which is responsive to FecA binding of its cognate siderophore (4, 49). Similarly, pseudobactin BN7/8 stimulates expression of its receptor, PupB, via FecIR homologues, dubbed PupIR (17). FecI is an extracytoplasmic-function (ECF) sigma factor (1) whose activity is controlled by FecR (30). Recently, a third example of this type of regulatory system was reported in *Bordetella bronchiseptica*, where *bupIR* gene products were shown to control expression of a putative siderophore receptor, BfrZ (37). In an effort to understand the basis of pyoverdine control of FpvA production in *P. aeruginosa*, then, FecIR homologues were sought in the *pvd* locus of the available PAO genome (http://www.pseudomonas.com) (47).

**MATERIALS AND METHODS**

**Bacterial strains and growth media.** Bacterial strains and plasmids used in this study are listed in Table 1. Routine growth for both *P. aeruginosa* and *E. coli* was performed in Luria-Bertani (LB) medium (Luria broth base; Difco). Growth under iron-limited conditions was performed by using iron-free BM2 succinate or glucose medium (35), which was made to be iron sufficient, as necessary, through the addition of 100 μM FeSO₄. Antibiotic selections used for *P. aeruginosa* included tetracycline (70 μg/ml in LB and 30 μg/ml in BM2 succinate), chloramphenicol (200 μg/ml in LB and 30 μg/ml in BM2 succinate) and kanamycin (for *ΔpvdA* strains only, 100 μg/ml in LB). For *E. coli*, tetracycline was used at 10 μg/ml (in LB) or 5 μg/ml (in BM2 glucose), chloramphenicol was used at 50 μg/ml (in LB) or 20 μg/ml (in BM2 glucose), and kanamycin was used at 50 μg/ml.

**DNA techniques.** Basic DNA procedures, including restriction endonuclease digestions, ligations, transformations, and agarose gel electrophoresis, were performed as described previously (24). Plasmid DNA isolation was performed by using the alkaline lysis method (24) or by using a plasmid Midi kit (Qiagen, Mississauga, Ontario, Canada). Genomic DNA was extracted from *P. aeruginosa* by using the method of Barcak et al. (3). DNA fragments for use in cloning were prepared as described previously (24).

**Cloning of *fpvI* and construction of a *ΔfpvI* mutant.** The *fpvI* gene was amplified by PCR by utilizing primers pf (5'-CATGGAATTCATGTTGGAAG GAATTCAGC-3'); the EcoRI site is underlined) and pfr (5'-AGCCTGAAATTC AATGCCTGAGAACG-3'; the EcoRI site is underlined). The PCR

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Strain or plasmid | Description* | Source or reference
--- | --- | ---
**P. aeruginosa**
K767 | PA01 prototroph | N. Gotoh, Kyoto Pharmaceutical University
K1120 | PA01 ΔpvdD | N. Gotoh, Kyoto Pharmaceutical University
K1203 | K1120 ΔpvdD | This study
K2100 | K1120 ΔfpvI | This study
K2102 | K1203 ΔfpvI | This study

**E. coli**
DHSa | supE44 ΔlacU169 (q80 lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1 | 2
SI7-1 | thi pro hsdR recA4 Tra+ | 43

Plasmids
pEX18tc | Broad-host-range gene replacement vector; Tc' | 15
pK1SmobucB | Broad-host-range gene replacement vector; Kan' | 40
pRK415 | Broad-host-range, low-copy-number cloning vector carrying MCS downstream of Plac; Tc' | 16
pMP190 | Broad-host-range, low-copy-number lacZ fusion vector; Cam' Sm' | 44
pEB3 | pMP190::fpvA-lacZ; Cam' | E. J. Blouin, unpublished
pAR001 | pRK415::fpvI | This study
pAR002 | pEX18tc::ΔfpvI | This study
pAR003 | pK1SmobucB::ΔfpvI | This study
pAR005 | pRK415 digested with Drai, digested with EcoRI, and cloned into EcoRI-digested pRK415, to yield pAR001. Nucleotide sequence confirmed that the fpvI gene was cloned in the same orientation as the lac promoter. | This study

mixture contained 50 ng of *P. aeruginosa* chromosomal DNA, 30 pmol of each primer, 0.2 mM (each) deoxyuridine triphosphate, 1 mM MgSO4, and 3% (vol/vol) dimethyl sulfoxide in 1× Thermopol buffer (New England Biolabs, Mississauga, Ontario, Canada), which was heated for 3 min at 95°C before the addition of 2 U of Vent DNA polymerase (New England Biolabs). The reaction was processed for 30 cycles of 1 min at 95°C, 20 s at 58°C, and 1 min at 72°C, followed by 10 min at 72°C. The resulting amplicon was purified by a Qiagen PCR purification kit (Qiagen), digested with EcoRI, and cloned into EcoRI-digested pRK415, to yield pAR001. Nucleotide sequence confirmed that the fpvI gene was cloned in the same orientation as the lac promoter.

To construct ΔfpvI mutants of *P. aeruginosa*, an internal deletion of the fpvI gene was first constructed in the gene replacement vector pK1SmobucB. This was accomplished by amplifying PCR products (by using the conditions and parameters described above) corresponding to sequence upstream and downstream of the deletion end points in fpvI by using primer pairs pfaeco (5'-GA TCGAATTCATGCTGCCTCTCGCGATGTC-3') and pfl (5'-GCTGTGCTCTTGCAGGTTGTG-3'), respectively. The ΔfpvI gene was excised from pAR002 by digestion with EcoRI and XhoI, and the resultant 1.8-kb fragment was cloned into EcoRI-XhoI-restricted pK1SmobucB to yield pAR003. This vector was then transformed into *E. coli* C600 (see Table 1) and mobilized into *P. aeruginosa* strains K1120 and K1203 by using a previously described protocol (45). By using chromosomal DNA (5 ng/μl) as a counterselective agent, kanamycin-resistant transconjugants were recovered and subsequently streaked onto LB agar containing 10% (vol/vol) sucrose. Sucrose-resistant colonies arising after 24 h of growth at 37°C were screened for the presence of a ΔfpvI chromosomal deletion by colony PCR (42) with primers pfp and pfp2 by using the conditions described above.

**Whole-cell extracts.** Whole-cell protein extracts were prepared from cultures of *P. aeruginosa* grown overnight in iron-sufficient BM2 succinate medium supplemented with the appropriate antibiotics for approximately 18 h at 37°C. To examine the impact of the cloned fpvI gene on fpvA-lacZ expression in a heterologous host, *E. coli* DH5α harboring pEB1 and either pAR001 (which carries fpvI) or pAR005 (vector control) was grown in iron-limited BM2 glucose minimal medium with the appropriate antibiotics to an OD600 of 0.5 before being assayed for β-galactosidase activity as described previously (25). To assess the impact of the cloned fpvI gene on fpvA-lacZ expression in a heterologous host, *E. coli* DH5α harboring pEB1 and either pAR001 (which carries fpvI) or pAR005 (vector control) was grown in iron-limited BM2 glucose minimal medium with the appropriate antibiotics to an OD600 of 0.5 before being assayed for β-galactosidase activity.

**RESULTS AND DISCUSSION.**

FIG. 1. Immunoblots of iron-limited P. aeruginosa whole-cell extracts developed with anti-FpvA antibodies. (A) Lane 2, K1120 (FpvI+); lane 3, K2100 (FpvI+); lane 4, K2100 carrying pAR001 (FpvI+). An immunoblot of K1120 grown in iron-supplemented minimal medium is shown in lane 1. (B) Lane 1, K1120 (FpvI+); lanes 2 and 3, K2100 (FpvI+ PvdD+); lanes 4 and 5, K2102 (FpvI+ PvdD+); lane 6, K2102 carrying pAR001 (FpvI+ PvdD-). Samples in lanes 3, 5, and 6 were prepared from cells supplemented with pyoverdine (100 μg/ml) during growth.

TABLE 2. Influence of FpvI on fpvA-lacZ expression

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant phenotype</th>
<th>β-Galactosidase activity (Miller units) with:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>No supplementation</td>
</tr>
<tr>
<td>K1120</td>
<td>FpvI+</td>
<td>1,400</td>
</tr>
<tr>
<td>K2100</td>
<td>FpvI+</td>
<td>256</td>
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<tr>
<td>K2100 (pAR001)</td>
<td>FpvI+</td>
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</tr>
<tr>
<td>DHH5a (pAR001)</td>
<td>FpvI+</td>
<td>106</td>
</tr>
<tr>
<td>DHH5a (pAR005)</td>
<td>FpvI-</td>
<td>3</td>
</tr>
<tr>
<td>K1203</td>
<td>FpvI+ PvdD-</td>
<td>616</td>
</tr>
<tr>
<td>K2102</td>
<td>FpvI+ PvdD-</td>
<td>370</td>
</tr>
</tbody>
</table>

a P. aeruginosa and E. coli strains harboring the fpvA-lacZ vector pEJB3 were cultured to log phase in antibiotic-supplemented iron-limited medium with or without the addition of 100 μg of pyoverdine/ml and assayed for β-galactosidase activity.  
b Data are a representation of experiments performed in triplicate.  
c —, not done.  
d PvdD-deficient owing to a deletion in the pvdD gene.

Iron regulation of fpvI. Since many iron uptake and regulatory genes, including that encoding the aforementioned PvdS ECF sigma factor, are themselves regulated by iron, with regulation mediated by the Fur repressor protein, it was of interest to assess regulation of fpvI by iron. By RT-PCR, expression of fpvI was clearly shown to increase under conditions of iron limitation (Fig. 2). Moreover, examination of the nucleotide sequence upstream of fpvI revealed the presence of a putative Fur box (Fig. 3, underlined), with 12 of 19 nucleotides matching the consensus Fur box sequence; indeed, Fur binding to this site has been previously confirmed (fpvI was previously identified as a Fur-regulated gene dubbed pgi32 [31]). Thus, the observation that expression of fpvA is governed by an iron-regulated probable sigma factor explains the known iron regulation of this receptor gene despite the absence of a Fur box upstream of the fpvA gene. This indirect iron regulation of gene expression is reminiscent of the iron-regulated expression of the pyoverdine biosynthetic (pvd) genes, which also lack Fur boxes and whose expression is governed by the iron-regulated ECF sigma factor PvdS (20, 21, 32, 52).

Involvement of pyoverdine in FpvI-mediated fpvA expression. In order to ascertain whether FpvI mediates the positive influence of pyoverdine on FpvA production, an fpvI deletion was created in a pyoverdine-deficient derivative of P. aeruginosa, K1203, and the ability of exogenously added pyoverdine...
to promote FpvA production in the resulting strain, K2102, was assessed. Immunoblotting with an anti-FpvA antibody confirmed previous findings that FpvA production was reduced in a pyoverdine-deficient (but FpvI+ strain (Fig. 1B, lane 2; compare with lane 1) and can be restored by the addition of exogenous pyoverdine (Fig. 1B, lane 3). In contrast, pyoverdine did not restore FpvA production in the FpvI strain K2102 (Fig. 1B, lane 5; compare with lane 4). Introduction of the cloned fpvI gene on plasmid pAR001 did, however, restore pyoverdine stimulation of FpvA production in this mutant (Fig. 1B, lane 6). Again, this effect occurred at the level of fpvA gene expression, with pyoverdine enhancing expression of the fpvA-lacZ fusion in the FpvI strain, K1203, but not in the FpvI strain, K2102 (Table 2). The observation that FpvI mediates the positive influence of pyoverdine on fpvA gene expression is reminiscent of PvdS and its mediation of the anti-sigma factor, FpvR (18, 41). Pyoverdine-dependent FpvI-mediated stimulation of fpvA expression appears also to be controlled by FpvA and FpvR (I. L. Lamont, personal communication).

ACKNOWLEDGMENTS

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