JOURNAL OF BACTERIOLOGY, Feb. 2003, p. 1261–1265 Vol. 185, No. 4
0021-9193/03/$08.00+0 DOI: 10.1128/JB.185.4.1261–1265.2003

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

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Received 1 October 2002/Accepted 21 November 2002

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 dicitrate 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 $FeSO_4$. 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 Δ*aphA* 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 extracted from agarose gels by using Prep-A-Gene (Bio-Rad Labs, Richmond, Calif.) in accordance with the manufacturer's instructions. Nucleotide sequencing of plasmid-borne DNA was carried out by Cortec DNA Services, Inc. (Kingston, Ontario, Canada). Nucleotide sequence alignments were performed using the GENESTREAM website (http://xylian.igh.cnrs.fr/bin/align-guess.cgi) (33).

Cloning of *fpvI* and construction of a Δ*fpvI* mutant. The *fpvI* gene was amplified by PCR by utilizing primers pff (5′-CATGGAATTCTGGTAGTTGGAAG GAATCCAGC-3′; the *Eco*RI site is underlined) and pfr (5′-AGCTGAATTCC AGTTGCCTGAGTCAATTCCAG-3′; the *Eco*RI site is underlined). The PCR

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description[a]	Source or reference
P. aeruginosa		
K767	PAO1 prototroph	N. Gotoh, Kyoto Pharmaceutical University
K1120	PAO1 Δ*aphA*	N. Gotoh, Kyoto Pharmaceutical University
K1203	K1120 Δ*pvdD*	A. Meldrum, unpublished
K2100	K1120 Δ*fpvI*	This study
K2102	K1203 Δ*fpvI*	This study
E. coli		
DH5α	*supE44* Δ*lacU169* (ϕ80 *lacZ*Δ*M15*) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	2
S17-1	*thi pro hsdR recA* Tra^+	43
Plasmids		
pEX18tc	Broad-host-range gene replacement vector; Tc^r	15
pK18*mobsacB*	Broad-host-range gene replacement vector; Kan^r	40
pRK415	Broad-host-range, low-copy-number cloning vector carrying MCS downstream of P*lac;* Tc^r	16
pMP190	Broad-host-range, low-copy-number *lacZ* fusion vector; Cam^r Sm^r	44
pEJB3	pMP190::*fpvA-lacZ*; Cam^r	E. J. Blouin, unpublished
pAR001	pRK415::*fpvI*	This study
pAR002	pEX18tc::Δ*fpvI*	This study
pAR003	pK18*mobsacB*::Δ*fpvI*	This study
pAR005	pRK415 digested with *DraI*, religated (Δ*DraI*)	This study

[a] Cam^r, chloramphenicol resistance; Kan^r, kanamycin resistance; Tc^r, tetracycline resistance; Sm^r, streptomycin resistance; MCS, multiple cloning site; P*lac*, *lac* promoter.

mixture contained 50 ng of *P. aeruginosa* chromosomal DNA, 30 pmol of each primer, 0.2 mM (each) deoxynucleoside triphosphate, 1 mM $MgSO_4$, 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 VentR 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 Qiaquick PCR purification kit (Qiagen), digested with *Eco*RI, and cloned into *Eco*RI-digested pRK415, to yield pAR001. Nucleotide sequencing confirmed that the *fpvI* gene was cloned in the same orientation as the *lac* promoter.

To construct Δ*fpvI* mutants of *P. aeruginosa*, an internal deletion of the *fpvI* gene was first constructed in the gene replacement vector pK18*mobsacB*. 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 pflaeco (5′-GATCGAATTCATGCTGCCTCTCGCGATGTC-3′; the *Eco*RI site is underlined) and pflakpn (5′-CGTAGGTACCGGCACTGAGGAATCGCAG-CA-3′; the *Kpn*I site is underlined) and pfrakpn (5′-CGTAGGTACCAACGCGATGAAGCACTGTC-3′; the *Kpn*I site is underlined) and pfrahind (5′-GACTAAGCTTGTGTTCCAGGTACTGGCTCTG-3′; the *Hin*dIII site is underlined), respectively. The downstream fragment was purified by using a Qiaquick PCR purification kit (Qiagen), digested with *Kpn*I and *Hin*dIII, and cloned into *Kpn*I-*Hin*dIII-restricted pEX18Tc. The resulting vector was then digested with *Eco*RI and *Kpn*I, and the *Eco*RI-*Kpn*I-digested upstream fragment was cloned into this vector, yielding the Δ*fpvI* plasmid pAR002. The Δ*fpvI* gene was excised from pAR002 by digestion with *Eco*RI and *Xmn*I, and the resultant 1.8-kb fragment was cloned into *Eco*RI-*Xmn*I-restricted pK18*mobsacB* to yield pAR003. This vector was then transformed into *E. coli* S17-1 and mobilized into *P. aeruginosa* strains K1120 and K1203 by using a previously described protocol (45). By using chloramphenicol (5 μg/ml) as a counterselective agent, kanamycin-resistant transconjugants were recovered and subsequently streaked onto LB agar containing 10% (wt/vol) sucrose. Sucrose-resistant colonies arising after 24 h of growth at 37°C were screened for the presence of a Δ*fpvI* chromosomal deletion by colony PCR (42) with primers pff and pfr 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 or iron-deficient BM2 succinate medium following subculture (1:99 dilution) into the same medium and growth to an optical density at 600 nm (OD_{600}) of approximately 0.7. Two 1.5-ml aliquots were centrifuged sequentially in the same microcentrifuge tube at 13,000 rpm (Biofuge *pico*; Heraeus Instruments) and resuspended in 400 μl of phosphate-buffered saline (1.7 mM NaH_2PO_4, 8.1 mM Na_2HPO_4, 145 mM NaCl). An equal volume of loading buffer (4% [wt/vol] sodium dodecyl sulfate [SDS], 20% [vol/vol] glycerol, 250 mM Tris-HCl [pH 6.8]) was then added, after which the samples were boiled for 5 min and sonicated on ice for 10 s (by using setting 40 on a Vibra Cell sonicator [Sonic and Materials Inc., Danbury, Conn.]). Total cell protein concentrations were determined using a BCA protein assay kit (Pierce, Rockford, Ill.) according to the manufacturer's instructions.

SDS-polyacrylamide gel electrophoresis and immunoblotting. Whole-cell extract samples were electrophoresed on 10% (wt/vol) SDS-polyacrylamide gels (53) and electrotransferred onto Immobilon-P polyvinylidene difluoride membranes (Millipore) as described previously (54). Equal loading of protein in all wells was confirmed by rapid Coomassie staining of duplicate gels (9). Membranes were probed with a polyclonal anti-FpvA antibody as described previously (36).

β-Galactosidase assay. *P. aeruginosa* strains containing promoter-*lacZ* fusions were grown in iron-limited BM2 succinate medium supplemented with the appropriate antibiotics for approximately 18 h at 37°C. In some experiments, pyoverdine was included at 100 μg/ml. Cultures were then diluted 1:49 into the same medium without antibiotics and grown at 37°C to an OD_{600} of ~0.7 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 pEJB3 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 OD_{600} of ~0.5 before being assayed for β-galactosidase activity.

RNA isolation and RT-PCR. RNA was isolated by using the RNeasy RNA isolation kit (Qiagen) in accordance with the manufacturer's instructions. Samples were then treated with RQ1 DNase (Promega, Madison, Wis.) in accordance with the manufacturer's instructions, and RNA was quantitated by spectrophotometry. Reverse transcription (RT)-PCR was carried out by using intragenic primers specific for *fpvI* (namely, piff [5′-ACTGGAATTCCAGCGAGCAGGAGTCGTCTT-3′] and pifr [5′-ACTGGAATTCTTGCGCAACAGGAAGGAAC-3′]) and *rpsL* (namely, rpsL1 [5′-GCAACTATCAACCAGCTG-3′] and rpsL2 [5′-GCTGTGCTCTTGCAGGTTGTG-3′]) and the One Step RT-PCR kit (Qiagen) by following the manufacturer's instructions. PCR conditions were as described above, except that the annealing temperature was 59°C. Non-RT controls were run to ensure that no DNA contaminated the RNA samples.

RESULTS AND DISCUSSION

Role of FpvI in *fpvA* expression. Examination of the *Pseudomonas* genome (*Pseudomonas* genome project website, http://www.pseudomonas.com) by using BLASTX (National Center for Biotechnology Information webpage, http://www.ncbi.nlm

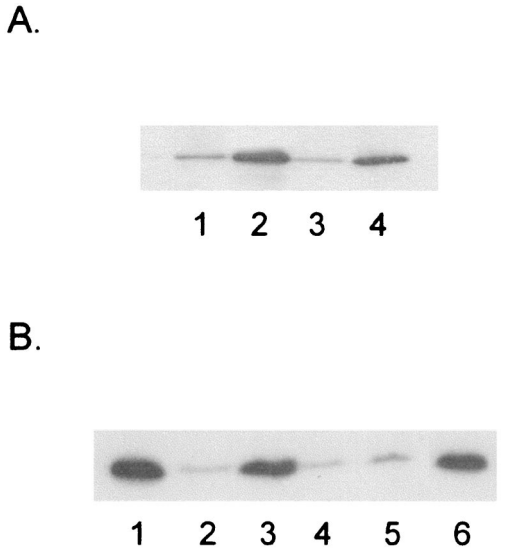

FIG. 1. Immunoblots of iron-limited *P. aeruginosa* whole-cell extracts developed with anti-FpvA antibodies. (A) Lane 2, K1120 ($FpvI^+$); lane 3, K2100 ($FpvI^-$); lane 4, K2100 carrying pAR001 ($FpvI^+$). An immunoblot of K1120 grown in iron-supplemented minimal medium is shown in lane 1. (B) Lane 1, K1120 ($FpvI^+$); lanes 2 and 3, K1203 ($FpvI^+$ $PvdD^-$); 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.

.nih.gov/BLAST) revealed two open reading frames, PA2387 and PA2388, whose deduced products exhibited homology to FecIR and PupIR, and they were subsequently designated *fpvI* and *fpvR* (in accordance with the nomenclature suggested by I. Lamont, University of Otago). The *fpvI* and *fpvR* genes were transcribed divergently from one another and were localized within the so-called *pvd* locus between the pyoverdine biosynthetic genes *pvdK* (19) and *pvdA* (50). FpvI showed almost 30.6% identity (59% overall similarity) to FecI (accession no. AAA23766), 31.6% identity (63.3% overall similarity) to BupI (accession no. CAB71123), and 29.5% identity (60.1% overall similarity) to PupI (accession no. CAA54870). To assess its role in FpvA production, then, an in-frame *fpvI* deletion was constructed in *P. aeruginosa* strain K1120, yielding K2100. Although FpvA production increased substantially in $FpvI^+$ strains under iron restriction (compare lanes 1 and 2 in Fig. 1A), loss of *fpvI* in K2100 abolished this iron-limited increase in FpvA (Fig. 1A, lane 3). FpvA production in iron-limited K2100 was, however, restored by the vector-borne *fpvI* gene present on plasmid pAR001 (Fig. 1A, lane 4), confirming a role for this probable ECF sigma factor in FpvA expression under iron-limiting conditions. To assess whether FpvI was influencing FpvA at the level of gene expression, an *fpvA-lacZ* fusion vector, pEJB3, was introduced into $FpvI^+$ and $FpvI^-$ *P. aeruginosa*, and β-galactosidase activity was determined for cells cultured under iron-limiting conditions. As seen in Table 2, loss of *fpvI* in K2100 reduces *fpvA* expression approximately fivefold relative to that of the $FpvI^+$ parental strain, K1120. Moreover, the cloned *fpvI* gene restored *fpvA* expression to wild-type levels in K2100 and stimulated the expression of the *fpvA-lacZ* fusion in *E. coli* (Table 2). In contrast, *pvdD-lacZ* expression, previously demonstrated to be dependent on the PvdS ECF sigma factor (21), was not stimulated by FpvI in *E. coli* (data not shown). These data are consistent with FpvI directly and positively promoting *fpvA* gene expression.

TABLE 2. Influence of FpvI on *fpvA-lacZ* expression[a]

Strain	Relevant phenotype	β-Galactosidase activity (Miller units) with[b]:	
		No supplementation	Pyoverdine supplementation
K1120	$FpvI^+$	1,400	—[c]
K2100	$FpvI^-$	256	—
K2100 (pAR001)	$FpvI^+$	1,455	—
DH5α (pAR001)	$FpvI^+$	106	—
DH5α (pAR005)	$FpvI^-$	3	—
K1203	$FpvI^+$ Pvd^-[d]	616	1,749
K2102	$FpvI^-$ Pvd^-	370	298

[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] Pyoverdine 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 *pig32* [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

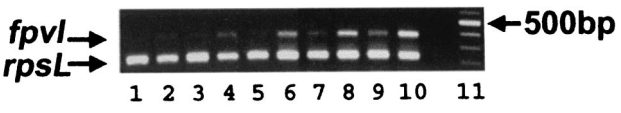

FIG. 2. *fpvI* expression in *P. aeruginosa* strain PAO1 K767, measured by RT-PCR of RNA (4 ng) isolated from cells cultivated under iron-sufficient (odd-numbered lanes) and iron-deficient (even-numbered lanes) conditions. The reactions were carried out for 26 (lanes 1 and 2), 27 (lanes 3 and 4), 28 (lanes 5 and 6), 29 (lanes 7 and 8), and 30 (lanes 9 and 10) cycles. *fpvI* and the *rpsL* (control) products are highlighted. Lane 11, 100-bp ladder with a 500-bp marker highlighted.

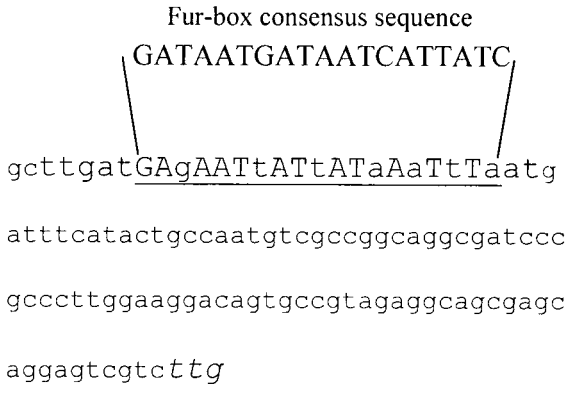

FIG. 3. Nucleotide sequence of the 5′ upstream region of *fpvI*, highlighting a putative Fur box. The Fur box (underlined) and FpvI translational initiation codon (italicized) are highlighted. Matches to the consensus Fur box sequence are capitalized.

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 positive influence of this siderophore on *pvd* gene expression, the latter in a process involving the receptor itself and an 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

The Δ*pvd* strain K1203 was engineered by using a Δ*pvdD* plasmid provided by I. L. Lamont (University of Otago).

This work was supported by an operating grant from the Canadian Institutes of Health Research (formerly the Medical Research Council of Canada) to K.P. K.P. is a Canadian Cystic Fibrosis Foundation (CCFF) Scholar. G.A.R. is the recipient of a CCFF graduate student scholarship.

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