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FpvA Receptor Involvement in Pyoverdine Biosynthesis in *Pseudomonas aeruginosa*

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Alignment of the *Pseudomonas aeruginosa* ferric pyoverdine receptor, FpvA, with similar ferric-siderophore receptors revealed that the mature protein carries an extension of ca. 70 amino acids at its N terminus, an extension shared by the ferric pseudobactin receptors of *P. putida*. Deletion of *fpvA* from the chromosome of *P. aeruginosa* reduced pyoverdine production in this organism, as a result of a decline in expression of genes (e.g., *pvdD*) associated with the biosynthesis of the pyoverdine peptide moiety. Wild-type *fpvA* restored *pvd* expression in the mutant, thereby complementing its pyoverdine deficiency, although a deletion derivative of *fpvA* encoding a receptor lacking the N terminus of the mature protein did not. The truncated receptor was, however, functional in pyoverdine-mediated iron uptake, as evidenced by its ability to promote pyoverdine-dependent growth in an iron-restricted medium. These data are consistent with the idea that the N-terminal extension plays a role in FpvA-mediated pyoverdine biosynthesis in *P. aeruginosa*.

Iron is an essential cofactor for many microbial enzymes and, as such, is required for growth of most bacteria (20). This need for iron is, however, complicated by the low solubility and, thus, bioavailability of this element in nature (20, 45). Many bacteria overcome this problem by synthesizing high-affinity iron-chelating molecules termed siderophores that, together with cell surface receptors specific for the iron-siderophore complexes, serve to provide the organism with iron under the most nutritionally dilute conditions (7). Significantly, pathogenic organisms also encounter an iron-limited environment in the host, where siderophore-mediated iron uptake plays an equally important role in growth and, thus, pathogenesis of many disease-causing bacteria (21, 55).

Pseudomonas aeruginosa is an opportunistic human pathogen associated with infections of compromised individuals (15). The organism produces two known siderophores, pyoverdine (11) and pyochelin (10), and can utilize a number of siderophores produced by other microorganisms (9, 38, 52), which likely explains the large number of ferric-siderophore receptor homologues identified in the recently completed genome sequence (66). Production of pyoverdine has been documented in vivo (22), consistent both with the in vivo induction of pyoverdine biosynthetic genes (24) and a demonstrated role for this siderophore in promoting in vivo growth and pathogenesis (41, 48, 67). Pyoverdine is, by far, the superior iron chelator in aqueous medium (62), exhibiting a stability constant of 10^{24} at pH 7 (40). A mixed hydroxymate-catecholate siderophore, pyoverdine is characterized by a conserved hydroxquinoline chromophore bound to an amino acid tail of variable length and composition (8). The latter appears to be responsible for the strain specificity of pyoverdine utilization in *P. aeruginosa* (42). Two gene clusters involved in pyoverdine biosynthesis have been identified, which are responsible for synthesis of the chromophore (*pvc*) (64, 65) and peptide (*pvd*) (13, 25, 35, 56, 69) moieties of the siderophore, respectively. The *fpvA* gene (51) encoding the ferric pyoverdine receptor (50) also maps to the *pvd* gene cluster (37).

Pyoverdine production occurs in response to iron limitation, and its production decreases with increasing iron availability (39). This iron-regulated expression is mediated by a homologue of the Fur repressor protein shown to regulate iron-siderophore systems in *Escherichia coli* (4, 54). Fur only indirectly regulates pyoverdine biosynthesis in *P. aeruginosa*, via the alternate sigma factor PvdS (74), which positively regulates several pyoverdine biosynthetic genes (12, 36, 44) but is itself regulated by Fur (12, 36, 44, 47). More recently, a LysR-type transcriptional activator of the *pvc* genes, PtxR, has been described in *P. aeruginosa* (23, 65) and is itself regulated by PvdS (71).

An interesting feature of FpvA is its N-terminal extension that may be unique to certain receptors involved in signal transduction. Both the FecA ferric dicitrate receptor of *E. coli* (53, 73) and the PupB ferric pseudobactin BN7/8 receptor of *Pseudomonas putida* WCS358 (33) share this extension and are involved in receptor-dependent expression of the corresponding receptor genes (26, 34). Such receptor-dependent signaling of gene expression involves an atypical two-component regulatory system, including a cytoplasmic membrane-associated component (FecR and PupR) and a cytoplasmic alternate sigma factor of the extracytoplasmic factor family (FecI and PupI) (2, 34, 70). Signaling involves the novel N termini of these receptors (32, 34), which has, in the case of FecA, been shown to physically associate with the cytoplasmic membrane component FecR (14), which itself interacts with FecI (14, 63). Ligand binding to the outer membrane receptor ultimately activates or releases the alternate sigma factors, which then stimulate receptor gene expression (6). In the present study, we assessed the significance of FpvA vis-à-vis expression of genes associated with pyoverdine-mediated iron uptake and noted that pyoverdine production and expression of pyoverdine biosynthetic genes are reduced in *fpvA* knockouts. As

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
P. aeruginosa		
K767	Wild-type PAO1	
K1660	K767 Δ*fpvA*	This study
K1661	K1660 *attB*::*fpvA*[a]	This study
K1662	K1660 *attB*::*fpvA**[a,b]	This study
E. coli		
DH5α	*endA hsdR17 supE44 thi-1 recA1 gyrA relA1* Δ*(lacZYA-argF)U169 deoR* [Φ*80dlac*Δ*(lacZ)M15*]	(3)
MM294	*supE448 rfbD1 spoT1 thi-1 endA1 hsdR17 pro*	(59)
Plasmids		
pAK1900	*E. coli-P. aeruginosa* shuttle cloning vector; Ap^r/Cb^r	A. Kropinski, Queen's University
pPVR2	pAK1900::*fpvA*	(51)
pEX18Tc	Broad-host-range gene replacement vector; Tc^r	(27)
pJSS2	pEX18Tc::Δ*fpvA*	This study
pRK2013	Broad-host-range helper/mobilization vector; Km^r	(16)
pRK415	Broad-host-range cloning vector; Tc^r	(30)
pJSS4	pRK415::*fpvA*	This study
pJSS5	pRK415::*fpvA**[b]	This study
mini-CTX1	*P. aeruginosa* chromosome integration vector; Tc^r	(28)
pJSS6	Mini-CTX1::*fpvA*	This study
pJSS8	Mini-CTX1::*fpvA**[b]	This study
pMP190	Broad-host-range, low-copy-number *lacZ* fusion vector; Cm^r Sm^r	(60)
pMP190::*pvdD*	pMP190::*pvdD-lacZ*; Cm^r	(57)

[a] Carries the *fpvA* genes in the chromosome at the *attB* site.
[b] 5′ deletion of the *fpvA* gene, corresponding to 64 of the first 66 amino acids of the mature protein.

expected, FpvA-mediated pyoverdine production requires the N-terminal extension of the protein.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1. Plasmid pJSS4, a pRK415 derivative carrying the wild-type *fpvA* gene in the same orientation as the resident *lac* promoter of this vector, was constructed by releasing the gene on a ca. 4.7-kb *Sph*I fragment from pPVR2 and, following polishing of the ends with Klenow fragment, cloning it into *Pst*I-restricted and Klenow-treated pRK415. Plasmid JSS5 is a pRK415 derivative carrying a truncated *fpvA* (dubbed *fpvA**) gene that encodes a receptor that retains the signal sequence but lacks 64 of the first 66 amino acids (V3 to Q66) of the mature protein. Initially, the 3′ end of the gene was recovered on a 2.4-kb *Eco*RI-*Pst*I fragment from pPVR2 and cloned into *Eco*RI-*Pst*I-restricted pRK415 to yield pJSS3. To eliminate the sequence encoding V3 to Q66 of the mature FpvA protein, the 5′ end of *fpvA* was then amplified by PCR in two parts, one upstream of the region to be deleted and one downstream. The upstream region (450 bp), which encompassed the promoter, ribosome-binding site, start codon, and signal sequence, was amplified with Vent polymerase (New England Biolabs, Inc., Mississauga, Ontario, Canada) and primers JS12 (5′-GTCACTG CAG**AATTC**TCAATGC-CTGGCTCGAAGCGCGAC-3′; *Pst*I site underlined and *Eco*RI site in bold) and JS11 (5′-CACGAAGCTTTTCCTGCGCCTGGA CATATC-3′; *Hin*dIII site underlined). The downstream region (700 bp) was amplified with Vent polymerase and primers JS13 (5′-CTGGAAGCTTGGCA ATGCGATAACCATCAG-3′; *Hin*dIII site underlined) and Shen2 (5′-GGCCC TTGAATTCATGGGTAGGT-3′; *Eco*RI site underlined). Both reaction mixtures contained 50 ng of pPVR2 template DNA, 40 pmol of each primer, 0.2 mM each deoxynucleoside triphosphate, 2 mM $MgSO_4$, 10% (vol/vol) dimethyl sulfoxide, and 2 U of Vent DNA polymerase in 1× thermal reaction buffer (New England Biolabs). The mixtures were heated for 2 min at 94°C and then subjected to 30 cycles of 1 min at 94°C, 30 s at 57°C, and 2 min at 72°C. Following purification of the PCR products (with a QIAquick PCR purification kit [Qiagen, Inc., Mississauga, Ontario, Canada]) and restriction digestion with *Pst*I and *Hin*dIII (upstream fragment) or *Hin*dIII and *Eco*RI (downstream fragment), the appropriately deleted 5′ end of *fpvA* was reconstructed following a three-piece ligation with *Pst*I-*Eco*RI-restricted pRK415. The 5′ end of *fpvA* containing the deletion was then liberated on a 1.1-kb *Eco*RI fragment, which was cloned into *Eco*RI-restricted pJSS3. One plasmid in which the 5′ and 3′ ends of *fpvA** were in the proper orientation, pJSS5, expressed a FpvA protein of the expected size, as confirmed by Western immunoblotting. Nucleotide sequencing of *fpvA** confirmed its proper construction.

The Δ*fpvA* derivative of PAO1 strain K767, dubbed K1660, was constructed with gene replacement vector pEX18Tc, in which sequences upstream and downstream of *fpvA* were cloned. Sequences upstream of the *fpvA* coding region were amplified by PCR with primers JS7 (5′-CAGCGAACCGCTCCATCTG-3′; anneals ca. 750 bp upstream of *fpvA* start codon) and JS8 (5′-CGGATCCAGGA CTGAGACCGTG-3′; *Bam*HI site underlined; anneals ca. 20 bp upstream of *fpvA* start codon). Sequences downstream of *fpvA* were amplified with primers JS9 (5′-GCGTTCTGCTTCTCGGCTAC-3′; anneals ca. 35 bp downstream of the *fpvA* stop codon) and JS10 (5′-GCAACCTGGCGATGGATG-3′; anneals ca. 750 bp downstream of the *fpvA* stop codon). Reaction mixture formulations and amplification parameters were as described above. The upstream fragment was then purified with the QIAquick-spin PCR Purification Kit (Qiagen, Inc.) and cloned into pCR-Blunt II-TOPO (Invitrogen Corp., Carlsbad, Calif.) with the Zero Blunt PCR Cloning Kit and a protocol supplied by the manufacturer. Following sequencing of the insert to ensure that no errors had been introduced by PCR, the 700-bp upstream fragment was released from the TOPO vector by digestion with *Eco*RI and *Bam*HI and cloned into *Eco*RI-*Bam*HI-restricted pEX18Tc to yield pJSS1. The downstream PCR product was similarly purified, cloned into pCR-Blunt II-TOPO, and sequenced. This fragment was then released by digestion with *Bam*HI and *Pst*I and cloned into *Bam*HI-*Pst*I-restricted pJSS1 to yield pJSS2. Δ*fpvA*-carrying pJSS2 was then introduced into *E. coli* strain S17-1 and mobilized into wild-type *P. aeruginosa* PAO1 strain K767 via conjugation as previously described (49). Cells of K767 carrying pJSS2 in the chromosome were selected on tetracycline and chloramphenicol (25 μg/ml; to counterselect the donor *E. coli*). Following selection on Luria-Bertani (LB) agar containing 10% (wt/vol) sucrose, sucrose-resistant colonies were screened for the presence of the *fpvA* deletion by PCR with primers JS7 and JS10. Loss of FpvA in K1660 was ultimately confirmed by Western immunoblotting (see below).

Introduction of wild-type or truncated *fpvA* into the chromosome of strain K1660 was achieved with the mini-CTX1 vector and a protocol developed by Hoang and coworkers (28). The wild-type gene was first recovered on a 4.3-kb *Pst*I fragment from plasmid pPVR2 and cloned into *Pst*I-restricted mini-CTX1. The resultant vector, pJSS6, was introduced into *E. coli* S17-1 and mobilized into K1660 via conjugation (49). Following selection on tetracycline and chloramphenicol (25 μg/ml; counterselection), *fpvA* was recovered in the chromosome (at the ϕCTX *attB* site) in the absence of plasmid backbone sequences with

recombinase-encoding plasmid pFLP2. This plasmid was mobilized from *E. coli* S17-1 as described above with selection on carbenicillin. Tetracycline-sensitive, carbenicillin-resistant colonies that had lost pJSS6 plasmid backbone sequences from the chromosome were then streaked onto LB agar containing 10% (wt/vol) sucrose to recover those that had subsequently lost pFLP2. Several putative *fpvA*-carrying derivatives of K1600 were screened for restoration of FpvA production by Western immunoblotting.

Similarly, *fpvA** was cloned into mini-CTX1, although by a two-step procedure. Initially, the 5′ end of *fpvA*, including the start codon, signal sequence, ribosome-binding site, and promoter regions, were recovered on a 450-bp *Eco*RI-*Hin*dIII fragment from pJSS5 and cloned into *Eco*RI-*Hin*dIII-restricted mini-CTX1 to yield pJSS7. A 3.1-kb *Hin*dIII fragment of pJSS4 carrying an *fpvA* gene encoding sequences beyond the first 66 amino acids of mature FpvA was then cloned into *Hin*dIII-restricted, alkaline phosphatase-treated pJSS7. Restriction analysis identified those plasmids that carried the 3′ end of *fpvA* in the proper orientation with respect to the 5′ end of the truncated gene, one of which was saved and designated pJSS8. This plasmid was then mobilized from *E. coli* S17-1, and a *fpvA**-carrying K1660 derivative (dubbed K1662) was selected exactly as described above for K1661. Expression of the truncated FpvA* protein in K1662 was confirmed by Western immunoblotting.

Growth conditions. LB broth (1% [wt/vol] Difco Tryptone, 0.5% [wt/vol] Difco yeast extract, 0.5% [wt/vol] NaCl) was used as the rich medium throughout. The iron-deficient succinate minimal medium used has been described previously (39).

Bacteria were cultured at 37°C with shaking (200 rpm). Strains were maintained on LB agar, although succinate minimal cultures were always inoculated with bacteria previously grown on succinate minimal agar. The following antibiotics were included in growth media as required: tetracycline (*P. aeruginosa*, 100 μg/ml; *E. coli*, 10 μg/ml), carbenicillin (400 μg/ml), ampicillin (100 μg/ml), kanamycin (50 μg/ml), and chloramphenicol (*P. aeruginosa*, 150 μg/ml; *E. coli*, 30 μg/ml).

DNA methods. Standard protocols were used for restriction endonuclease digestions, ligations, treatment of DNA with Klenow fragment or calf intestinal alkaline phosphatase, and agarose gel electrophoresis (58). The alkaline lysis method (58) or a plasmid midi kit (Qiagen Inc.) was used to isolate plasmids from *E. coli* DH5α. DNA fragments used for cloning were extracted from agarose gels with Prep-A-Gene (Bio-Rad Laboratories, Richmond, Calif.) as recommended by the manufacturer. *E. coli* cells were made competent by the $CaCl_2$ method (58) or, when supercompetent cells of *E. coli* were required, the method of Inoue et al. (29). Oligonucleotides for use in PCRs were chemically synthesized by Cortec DNA Services Inc., Kingston, Ontario, Canada. Nucleotide sequencing with universal or custom primers was also carried out by Cortec DNA Services Inc.

Growth assay. *P. aeruginosa* grown overnight in iron-deficient succinate minimal medium was subcultured into the same medium supplemented with ethylene diamine di(o-hydroxyphenyl)acetic acid (EDDHA; 1.5 to 3 mg/ml) to a final A_{600} of 0.1. In some experiments, pyoverdine (100 μg/ml) (38) was also included in the growth medium.

Pyoverdine assays. Overnight cultures of *P. aeruginosa* grown in iron-deficient succinate minimal medium were harvested by centrifugation, washed twice with the same medium, and subcultured into the same medium to a final A_{600} of 0.1. Pyoverdine production was then assessed by measuring the A_{405} of 1-ml aliquots of cell-free culture supernatants (40) taken hourly during growth at 37°C. Alternatively, 1-ml cell-free samples were recovered and hydroxamate nitrogen of pyoverdine was measured by a modification of a previously described protocol (18). Values for both assays were normalized to A_{600} to provide a measure of per-cell pyoverdine production.

β-Galactosidase assay. *P. aeruginosa* bacteria carrying pMP190 and its derivatives were grown overnight in iron-deficient succinate minimal medium supplemented with chloramphenicol. Following subculture into the same medium lacking chloramphenicol with or without $FeCl_3$ (100 μM), cells were grown to late log phase before being harvested and assayed for β-galactosidase activity as described previously (43).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western immunoblotting. Whole-cell extracts (31) were subjected to sodium dodecyl sulfate–(10% [wt/vol]) polyacrylamide gel electrophoresis (61) and Western immunoblotting (75) with an FpvA-specific rabbit polyclonal antiserum (51).

RT-PCR. RNA was prepared from strains of *P. aeruginosa* subcultured in succinate minimal medium with or without iron (200 μM $FeCl_3$) supplementation to an A_{600} of ca. 0.7 with a Qiagen RNeasy mini kit (Qiagen) in accordance with the manufacturer's instructions. Reverse transcription (RT)-PCR was performed on 1 to 100 ng of RNA with a Qiagen OneStep RT-PCR kit (Qiagen) and primer pairs specific for *pvcA* (5′-CATCGAACAGGTCCAGTTGCC-3′ and 5′-ATCCTCACCAGGTCGCCGAACA-3′), *pvdD* (5′-GAAAGCGCGGCCTACCATA-3′ and 5′-ATCGTCGTCTCGACCTGCCGAG-3′), and *rpsL* (5′-GCAACTATCAACCAG-3′ and 5′-GCTGTGCTCTTGCAG-3′). Reaction mixtures were heated for 30 min at 50°C, followed by 15 min at 95°C and then 30 cycles of 45 s at 94°C, 45 s at 58°C, and 60 s at 72°C before finishing with 10 min at 72°C. RT-PCR products were stored at 4°C until visualized by agarose gel electrophoresis. RT-free PCRs were carried out on all RNA templates to control against genomic DNA contamination.

RESULTS AND DISCUSSION

Role of FpvA in pyoverdine production. Alignment of FpvA with homologous ferric-siderophore receptors (Fig. 1) demonstrated that the mature FpvA protein, like the ferric pseudobactin receptors of *P. putida*, possesses an extension at the N terminus of ca. 70 amino acids that is not shared by other ferric-siderophore receptors. In the case of the PupB ferric pseudobactin BN7/BN8 receptor, this extension plays a role in PupB-mediated signal transduction associated with upregulation of *pupB* gene expression (34). To assess the possible involvement, therefore, of FpvA in receptor-dependent expression of *fpvA*, the entirety of the *fpvA* coding region was deleted from the chromosome of wild-type *P. aeruginosa* PAO1 strain K767. Intriguingly, the resultant strain, K1660, was, unlike its parent, nonfluorescent on iron-deficient minimal succinate plates, suggesting that it was deficient in pyoverdine biosynthesis. Examination of pyoverdine production as a function of growth in an iron-limited medium confirmed the pyoverdine deficiency of the FpvA-deficient K1660 strain (Fig. 2). As expected, then, K1660 also failed to grow in iron-limited minimal medium supplemented with the nonmetabolizable iron chelator EDDHA (Fig. 3), a phenotype typical of *P. aeruginosa* with defects in pyoverdine production or utilization. Two loci, *pvd* and *pvc*, are known to be associated with pyoverdine biosynthesis in *P. aeruginosa* (65, 69). Using an available *pvdD-lacZ* fusion vector, loss of *fpvA* in K1660 was correlated with an inconsistent decline in *pvdD* expression of twofold or less (as a measure of expression of genes of the *pvd* locus, which are expected to be coregulated [12, 36, 44]) (data not shown). Beare and colleagues reported a similar ca. 2.5-fold decline in expression in another *pvd* gene, *pvdF*, in an FpvA knockout, also with a *lacZ* reporter (P. A. Beare et al., Pseudomonas 2001, Brussels, Belgium, abstr. PS146, 2001). With RT-PCR, however, it was clear that the FpvA-deficient strain indeed exhibited reduced *pvdD* expression during growth under iron limitation relative to its $FpvA^+$ parent (Fig. 4B, c.f. lanes 2 and 4). The FpvA-producing wild-type strain produced surprisingly modest levels of *pvdD* under iron-limiting conditions (Fig. 4, compare lane 2 with lane 1), likely reflecting the fact that RNA for RT-PCR was harvested from cells early during growth in an iron-limiting medium (at an optical density at 600 nm of 0.7), when they would be only moderately iron limited. The identification of *pvdD* transcripts in wild-type cells (K767) growing under iron-replete conditions (Fig. 4, lane 1), although perhaps unexpected, was consistent with the earlier demonstration that L-broth-grown (i.e., iron-replete) cells of *P. aeruginosa* produce some FpvA receptor protein (and other iron-regulated receptors) (46). Thus, *P. aeruginosa* apparently expresses these iron-regulated genes at low levels under iron-replete conditions and methods such as RT-PCR are sensitive enough to detect this.

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FptA    ------------------------------------------------------------
PbuA    ------QEWTLNIPSQPLAQALQTLGQQTSLQIIYSPESLQGLRSTALNGRYQDDESLKA 54
FhuE    ------------------------------------------------------------
FauA    ------------------------------------------------------------
PupA    ------QEWTLDIPAQSMNSALQALAKQTDTQLLYSPEDIGGLRSSALKGRHDLQSSLRI 54
FpvA    ------QEVEFDIPPQALGSALQEFGRQADIQVLYRPEEVRNKRSSAIKGKLEPNQAITE 54
PupB    ---AAQAQADFDIPAGPLAPALAHFGQSAHILLSYPTALTEGRSTSGLAGRFDIDQGLAI 57
pa2466  AEAAAEQARPYAIPAGQLGDVLNRFAREAGITLSATPAQTGGYSSQGLRGSFTVQQGLAR 60

FptA    ---------DARKDG------------ETELPDMVISGESTSATQ------PPGVTTLG- 32
PbuA    MLN--GTGIRYQRDGNTVTVLGPATGSAMELAPTNVNASRLGATTEGSNSYTTGGVTIG- 111
FhuE    ------------------------APATEETVIVEG-SATAPDDGENDYSVTSTSAGT 33
FauA    -------------------QEARTGNDIAQLPAISVTGREISDLTEGTNAYTTEAMSTAT 41
PupA    LLQGTGLRYQIDGNTVTVTASAAAKDGQIELSATNVNSAGLGETTEGTGSYTTRVTSTAT 114
FpvA    LLRGTGASVDFQGN-AITISVAEAADSSVDLGATMITSNQLGTITEDSGSYTPGTIATAT 113
PupB    LLAGTGLEAS-RGANASYSLQASASTGALELSAVSISGKAPGSTTEGTGLYTTYSSSSST 116
pa2466  LLADTPLEAEDQGDGSFVLREAPAKDGDVLN-MQAVEVFALGNNLGSTDGYLATHSQIAT 119
                                        :                    .

FptA    KVPLKPRELPQSASVIDHERLEQQNLFSLDEAMQQATGVTVQPF--QLLTTAYYVRGFKV 90
PbuA    KGVHSLKETPQSVTVMTRKMLDDQNLNTIEQVMEKTPGITVYDS--PMGGKYFYSRGFRM 169
FhuE    KMQMTQRDIPQSVTIVSQQRMEDQQLQTLGEVMENTLGISKSQA--DSDRALYYSRGFQI 91
FauA    GLTLSPRETPQSVSVVTRQQIEDQGLTDTGAILATAPGISVTRS--DSNRYSFSARGFTI 99
PupA    KMNLSIRETPQTITVVTRQRMDDQHLGSMNEVLTQTPGITMSQD--GGERFNIYSRGSAI 172
FpvA    RLVLTPRETPQSITVVTRQNMDDFGLNNIDDVMRHTPGITVSAY--DTDRNNYYARGFSI 171
PupB    RLNLTPRETPQSLTVMTRQRLDDQRLTNLTDALEATPGITVVRDGLGSESDSYWSRGFAI 176
pa2466  KTSKPLLETSQTVSVITREQIDDTASKTVQQAMRYTPGIFTGQVGASNRYDYVVMRGFAD 179
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FIG. 1. Multiple-sequence alignment of FpvA and its homologues. Proteins related to FpvA were identified with BLAST (1) (http://www.ncbi.nlm.nih.gov/BLAST/), and the mature sequences (putative signal peptidase cleavage sites were identified by the method of Nielsen et al. [46] [http://www.cbs.dtu.dk/services/SignalP/]) were aligned with CLUSTALW (68) (http://www.ebi.ac.uk/clustalw/). Only the N termini are shown. FptA, *P. aeruginosa* Fe(III)-pyochelin receptor (accession number P42512); PbuA, *P. putida* M114 Fe(III)-pseudobactin M114 receptor (accession number Q08017); FhuE, *E. coli* receptor for Fe(III)-coprogen, -ferrioxamine B, and -rhodotorulic acid (accession number P16869); FauA, *Bordetella pertussis* Fe(III)-alcaligin receptor (accession number AAD26430); PupA, *P. putida* WCS358 Fe(III)-pseudobactin 358 receptor (accession number P25184); FpvA, *P. aeruginosa* Fe(III)-pyoverdine receptor (accession number P48632); PupB, *P. putida* WCS358 Fe(III)-pseudobactin BN7/BN8 receptor (accession number P38047); PA2466, probable *P. aeruginosa* TonB-dependent receptor (accession number D83337).

In contrast to that of *pvdD*, expression of the *pvc* genes did not appear to be altered in K1660 relative to its wild-type parent strain under conditions of iron limitation (data not shown), although the very weak expression of *pvcA* detected likely precluded detection of modest changes in *pvc* gene expression. In any case, FpvA control of pyoverdine biosynthesis occurs via the peptide biosynthetic genes at least.

Interestingly, introduction of the cloned wild-type *fpvA* gene (on plasmid pJSS4) into K1660 failed to restore pyoverdine production (Fig. 2) or growth on EDDHA-supplemented (i.e., iron-restricted) minimal medium (Fig. 3) despite the restored production of FpvA in the mutant (Fig. 5, lane 4). Still, introduction of this gene into the chromosome of K1660 (at the ϕCTX *attB* site with the mini-CTX1 vector of Hoang et al. [28], yielding strain K1661), which similarly restored FpvA production (Fig. 5, lane 5), did restore pyoverdine production (Fig. 2) and growth in EDDHA-supplemented medium (Fig. 3). Not unexpectedly, the restored pyoverdine production paralleled an increase in *pvdD* gene expression (Fig. 4B, lane 6). One explanation for the failure of the plasmid-borne *fpvA* gene to complement the pyoverdine and growth deficiencies of K1660 is that the presence of multiple copies of this gene, in particular its upstream promoter region, might have titrated out a positive regulator associated with expression of both *fpvA* and genes of pyoverdine biosynthesis. Still, a previous report showed that plasmid-borne *fpvA* was able to complement the growth defect of an FpvA-deficient mutant (31), although the *fpvA* mutant in this instance was constructed by insertional inactivation and no gene sequences were deleted. It was possible, therefore, that any growth restoration seen resulted from recombination between chromosomal *fpvA* sequences and the plasmid-borne gene. Screening of a *P. aeruginosa* gene library for sequences able to restore pyoverdine biosynthesis and growth (on EDDHA) of pJSS4-harboring K1660, in an attempt to identify such a regulatory gene, have so far failed.

Intriguingly, the only ferric-siderophore receptors to be previously characterized with respect to their involvement in signal transduction are FecA and PupB. Unlike, FpvA, however, these receptors transport, and thus respond to, siderophores not produced by the organism itself. Presumably, this provides a way for the cell to sense the presence of these siderophores, upregulating the transport genes only when they are likely to be of use. The need to couple pyoverdine biosynthesis to receptor utilization, presumably of the ferrated siderophore, is less clear. *P. aeruginosa* is capable of producing two siderophores, although only one is likely needed at any given time. Perhaps the coupling of siderophore uptake via FpvA to pyoverdine biosynthesis is a way of ensuring that pyoverdine is effective at chelating and, thus, providing iron to the organism in a particular environment before investing energy in produc-

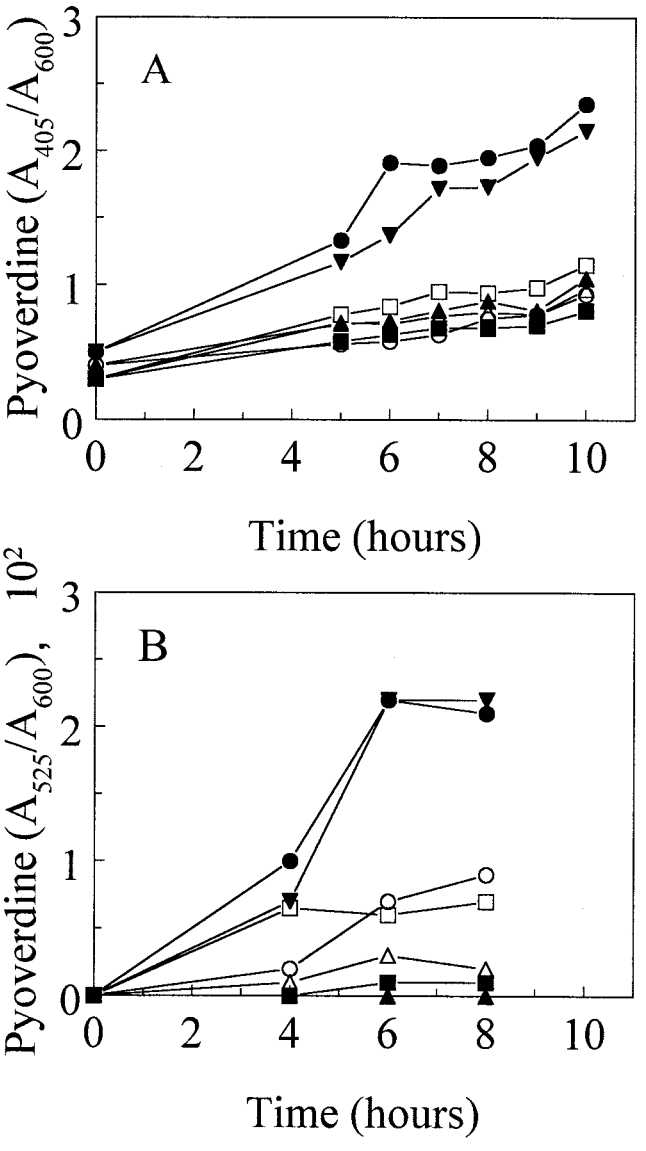

FIG. 2. Pyoverdine production by *P. aeruginosa* strains K767 (●), K1660 (▲), K1660(pRK415) (□), K1660(pJSS4) (○), K1660(pJSS5) (■), K1661 (▼), and K1662 (△) as a function of growth in iron-deficient succinate minimal medium. Pyoverdine levels were estimated by measuring the A_{405} of the cell-free culture supernatant (A) and hydroxamate nitrogen at A_{520} (B). Values are normalized with respect to culture density, so as to provide a measure of per-cell pyoverdine production. All strains grew equally well in the minimal medium, plateauing at an A_{600} of ca. 1.4 after 7 h of growth. The results are representative of three independent experiments run on different days. Note that data points for K1662 in panel A are masked by data points of other strains in the lower part of the graph.

ing this siderophore. With two endogenous and potentially multiple heterologous siderophores to choose from, this would ensure that the cell invests in pyoverdine only when it is prudent to do so. In the presence, for example, of enterobactin (which is typically a better chelator than pyoverdine [19]) or under conditions in which pyochelin is the better chelator, the resultant failure of pyoverdine to acquire iron would, through its failure to interact productively with FpvA, ensure that energy is not wasted synthesizing a siderophore that is ineffective at acquiring iron. This mirrors what is seen with respect to FpvA expression, inasmuch as strains deficient in pyoverdine production exhibit reduced synthesis of this protein (17). The observation here that the levels of the N-terminally truncated FpvA* protein were noticeably reduced relative to those of wild-type FpvA (Fig. 5, compare lanes 6 and 7 with lanes 4 and 5) is therefore consistent with the reduction in pyoverdine seen in FpvA*-expressing *P. aeruginosa*. That pyoverdine-deficient strain K1660(pJSS4) produced wild-type levels of FpvA (Fig. 5, lane 4) likely reflects the multicopy nature of the plasmid-borne *fpvA* gene in this instance and promotion of *fpvA* gene

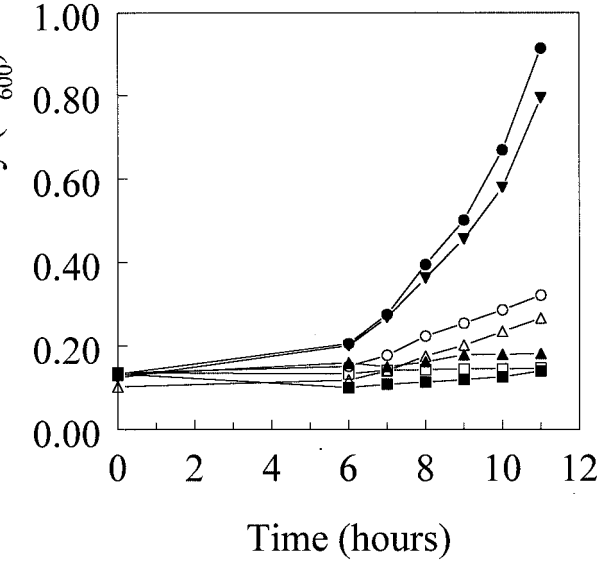

FIG. 3. Growth of *P. aeruginosa* strains K767 (●), K1660 (▲), K1660(pRK415) (□), K1660(pJSS4) (○), K1660(pJSS5) (■), K1661 (▼), and K1662 (△) in EDDHA-supplemented (3 mg/ml) succinate minimal medium. The results are representative of three independent experiments run on different days.

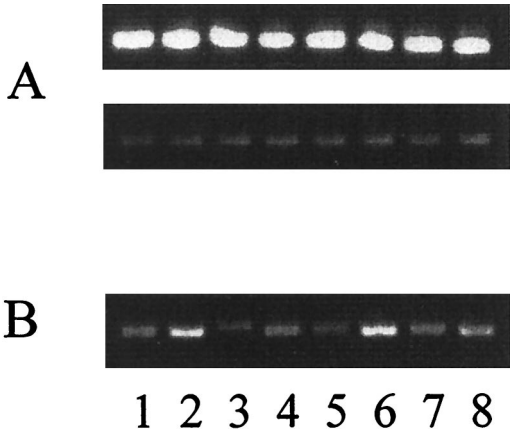

FIG. 4. *rpsL* (A) and *pvdD* (B) expression in *P. aeruginosa* cultured under iron-sufficient (odd-numbered lanes) and iron-deficient (even-numbered lanes) conditions measured by RT-PCR of total RNA (24 ng, top of panel A; 4 ng, bottom of panel A; 4 ng, panel B) isolated from strains K767 (lanes 1 and 2), K1660 (lanes 3 and 4), K1661 (lanes 5 and 6), and K1662 (lanes 7 and 8).

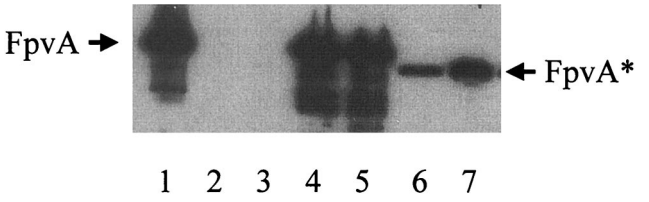

FIG. 5. Western immunoblot showing FpvA production in *P. aeruginosa* strains K767 (lane 1), K1660 (lane 2), K1660(pRK415) (lane 3), K1660(pJSS4) (lane 4), K1661 (lane 5), K1660(pJSS5) (lane 6), and K1662 (lane 7) grown in iron-limited succinate minimal medium. Whole-cell extracts were prepared from stationary-phase cells and probed with antibodies to FpvA following electrophoresis and electroblotting as described in Materials and Methods.

expression from the resident *lac* promoter of pJSS4 parent plasmid pRK415, both of which would provide some escape from the usual pyoverdine controls.

Involvement of the N terminus in FpvA-mediated pyoverdine production. The involvement of FecA and PupB in signal transduction is intimately tied to the unique N-terminal extensions of these receptors (32, 34). To asses, then, the importance of the N terminus of FpvA vis-à-vis the receptor's involvement in pyoverdine biosynthesis, a gene encoding a truncated version of FpvA lacking 64 of the first 66 amino acids (V3 to Q66) of the mature protein (dubbed FpvA*) was constructed. Like wild-type FpvA-encoding pJSS4, FpvA*-encoding pJSS5 did not promote enhanced pyoverdine production by K1660 (Fig. 2) or growth of K1660 in the presence of EDDHA (Fig. 3), although the truncated protein was expressed (Fig. 5, lane 6). Unlike K1661, however, where introduction of the wild-type *fpvA* gene at the ϕCTX *attB* site of K1660 complemented the growth and pyoverdine production defects, the *fpvA** gene of K1662 did not promote enhanced pyoverdine production (Fig. 2) or growth in the presence of EDDHA (Fig. 3). As expected, FpvA* was also unable to restore wild-type *pvdD* expression levels in K1662 (Fig. 4B, compare lane 8 with lanes 2 and 6). Nonetheless, the truncated protein was produced in K1662 (Fig. 5, lane 7) and this strain did demonstrate pyoverdine-enhanced growth in EDDHA-supplemented medium, something also seen in wild-type PAO1 strain K767 but not in FpvA-deficient stain K1660 (Fig. 6). Growth of *P. aeruginosa* in the presence of EDDHA is typically dependent upon the presence of a functional ferric-pyoverdine uptake system and is thus a good measure of pyoverdine-mediated iron acquisition. Thus, FpvA* is presumably competent for transport but defective with regard to its role in pyoverdine biosynthesis. An N-terminally truncated FecA receptor was similarly competent for ferric dicitrate transport but unable to induce *fecA* gene expression in this case (32). It is likely, therefore, that FpvA, specifically its N terminus, plays a role in a signal transduction process leading to activation of the *pvd* genes of pyoverdine biosynthesis. PvdS is known to activate *pvdD* gene expression (12), suggesting that this extracytoplasmic sigma factor mediates the influence of FpvA on pyoverdine biosynthesis. Interestingly, homologues of *fecIR*/*pupIR* have been identified in the *pvd* locus (PA2387 and PA2388), as revealed by the recently completed *P. aeruginosa* genome sequence (66). Deletion of these genes does not, however, impact pyoverdine production by *P. aeruginosa* (A. Redly, unpublished data).

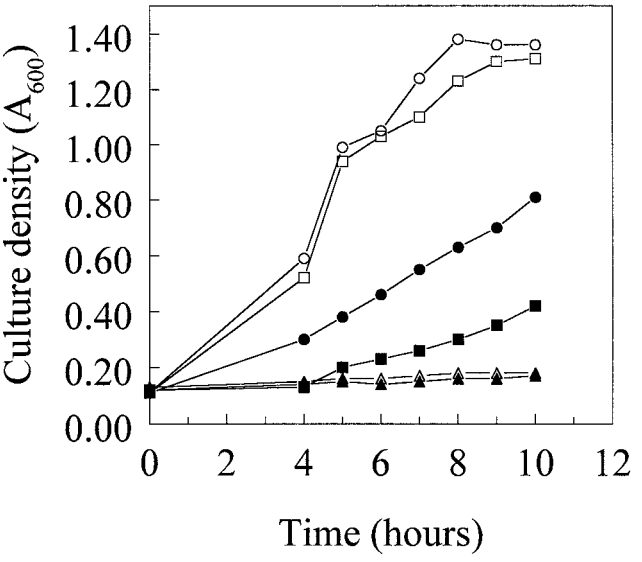

FIG. 6. Influence of exogenously added pyoverdine on growth of *P. aeruginosa* strains K767 (circles), K1660 (triangles), and K1662 (squares) in EDDHA-supplemented (1.5 mg/ml) succinate minimal medium. Filled symbols, no pyoverdine added; open symbols, 100 μg of pyoverdine added per ml.

While FpvA appears to be unique among ferric-siderophore receptors in terms of its role in regulating siderophore biosynthesis, PupA, the receptor for the endogenous pseudobactin of *P. putida* WCS358, also possesses an N-terminal extension (Fig. 1) and is thus anticipated to play some role in the regulation of gene expression. While this remains to be elucidated, the results of a previous study demonstrating pseudobactin 358-dependent expression of a pseudobactin 358 biosynthetic gene was suggestive of receptor (i.e., PupA) involvement, since utilization of the siderophore was probably needed for this upregulation (72). The involvement, if any, of PupA in pseudobactin 358 synthesis was not, however, examined in this study. Still, an earlier study revealed no difference in siderophore production by PupA-deficient mutants compared to the wild type (5), suggesting that pseudobactin biosynthesis in *P. putida* WCS358, unlike pyoverdine biosynthesis in *P. aeruginosa*, has no receptor involvement.

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