

Cloning and Nucleotide Sequence Analysis of the Ferripyoverdine Receptor Gene *fpvA* of *Pseudomonas aeruginosa*

KEITH POOLE,* SHADI NESHAT, KATHLEEN KREBES, AND DAVID E. HEINRICHS

*Department of Microbiology and Immunology, Queen's University,
Kingston, Ontario, Canada K7L 3N6*

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Pseudomonas aeruginosa K437 lacks the ferripyoverdine receptor and, as a result, grows poorly on an iron-deficient minimal medium supplemented with ethylenediamine-di(*o*-hydroxyphenylacetic acid) (EDDHA) and pyoverdine. By using a phagemid-based *in vivo* cloning system, attempts were made to clone the receptor gene by complementing this growth defect. Several recombinant phagemids carrying *P. aeruginosa* chromosomal DNA which provided for good growth on EDDHA-pyoverdine-containing medium and which concomitantly restored production of the ferripyoverdine receptor in strain K437 were isolated. These phagemids contained a common 4.6-kb *Sph*I fragment which similarly restored production of the receptor in K437. Nucleotide sequencing of the *Sph*I fragment revealed a single large open reading frame, designated *fpvA* (ferripyoverdine uptake), of 2439 bp. The predicted translation product of *fpvA* has a molecular mass of 89,395 Da. N-terminal amino acid sequence analysis of the purified ferripyoverdine receptor confirmed *fpvA* as the receptor gene. Moreover, it indicated that the receptor is initially synthesized as a precursor with a signal sequence of 27 amino acids which is cleaved to yield the mature protein. The deduced FpvA polypeptide exhibited homology to regions shown to be conserved in TonB-dependent receptor proteins. FpvA also shared strong homology (41.3% identity) with the PupA protein of *Pseudomonas putida* WCS358. This protein is the receptor for the iron-bound form of pseudobactin, a compound structurally very similar to pyoverdine.

With the possible exception of some lactobacilli, all bacteria require iron for growth (43). The acquisition of this nutrient is, however, complicated by its low solubility in nature (43). To overcome this problem, many bacteria synthesize and release low-molecular-mass iron-binding compounds called siderophores which chelate iron with generally high affinity and deliver it back to the cell via cell surface receptors specific for the iron-siderophore complex (41, 42). Interestingly, this strategy for acquiring iron also appears to be important for the growth of pathogenic bacteria in the host (11) which is iron limiting for bacterial growth (8, 22, 32, 52). Not surprisingly, then, siderophore-mediated iron transport systems contribute to the virulence of bacterial pathogens (11, 13, 21, 29, 31, 40, 48, 54, 56, 58, 63).

Pseudomonas aeruginosa synthesizes two known siderophores, pyoverdine (12) and pyochelin (10), in response to conditions of iron limitation. The organism is also capable of utilizing a number of so-called heterologous siderophores (produced by other microorganisms) to transport iron into the cell (9, 34, 37, 46). The production of pyochelin is associated with improved *in vivo* growth and increased virulence (11) perhaps partly attributable to a demonstrated ability of the siderophore to remove transferrin-bound iron (56, 59). Pyoverdine, by far the superior siderophore in removing iron from transferrin *in vitro* (59), facilitates the growth of producing strains in human serum and in the presence of human transferrin (1). This siderophore is known to be produced *in vivo* during colonization of the lungs of patients with cystic fibrosis (23), where it may play an important role in enabling this colonization to occur. Indeed, animal infection model studies indicate that lung

colonization by *P. aeruginosa* is dependent upon the production of pyoverdine (45a).

Two receptors for ferripyochelin, of 14 kDa (57) and 75 kDa (24) molecular mass, respectively, in *P. aeruginosa* have been reported; the gene for the latter has recently been cloned (2). The receptor for ferripyoverdine has been identified as an 80-kDa (38) or 90-kDa (44) protein in strain PAO1. It is likely, however, that the proteins are identical and that the 80-kDa molecular mass reported by Meyer et al. (38) is an underestimate (37a). An 85-kDa iron-regulated outer membrane protein which functions as the pyocin Sa receptor in a clinical isolate of *P. aeruginosa* also appears to be a receptor for ferripyoverdine (20).

We report here the cloning and nucleotide sequencing of the gene encoding the ferripyoverdine receptor of *P. aeruginosa* PAO. Interestingly, the receptor is highly homologous to the ferric pseudobactin receptor PupA of *Pseudomonas putida* (6).

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth media. *P. aeruginosa* CD10(pADD214) was used in the *in vivo* cloning of the ferripyoverdine receptor (see below) and has been described previously (14). *P. aeruginosa* K437 (44) is a ferripyoverdine receptor-deficient derivative of strain K372 (24), itself a pyochelin receptor-deficient derivative of PAO6609 (*met-9011 amE200 rpsL pvd9*) (27). *Escherichia coli* 5K (45) was employed in the subcloning of DNA prior to its introduction into *P. aeruginosa* strains. pPVR1 is a derivative of phagemid pADD214 (14) carrying *P. aeruginosa* chromosomal DNA which restores synthesis of the ferripyoverdine receptor in K437. pPVR2 is a pAK1900 (55) derivative carrying the *fpvA* gene on a 4.6-kb *Sph*I fragment. The iron-deficient

* Corresponding author.

succinate minimal medium used in these studies has been described (44) and was supplemented with methionine (1 mM) as required. L broth (16) was employed as the rich medium throughout. Solid media were obtained via the addition of Bacto Agar (Difco; 1.5% [wt/vol]), Tetracycline (100 µg/ml), carbenicillin (200 µg/ml), and ampicillin (100 µg/ml) were included in growth media when appropriate.

Outer membranes and SDS-PAGE. Outer membranes were prepared by differential Triton X-100 solubilization of cell envelopes as previously described (53). Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was performed as previously described (35), with 9% (wt/vol) acrylamide in the running gel.

Pyoverdine. Pyoverdine was prepared as described previously (44) and quantitated by using the molar extinction coefficient for this compound at 380 nm (17).

In vivo cloning of the ferripyoverdine receptor. The ferripyoverdine receptor gene of *P. aeruginosa* was cloned by the in vivo cloning system described by Darzins and Casadaban (14). Briefly, a phage lysate was prepared from *P. aeruginosa* CD10 cells harboring the mini-D replicon pADD214 (14) by thermal induction as previously described (15). The lysate (0.15 ml) was subsequently used to infect *P. aeruginosa* K437 (0.15 ml of an overnight culture) on L-broth plates at 30°C. After 3 h, the cells were recovered from the plates by suspension in iron-deficient succinate minimal medium and, following several washes in this medium, plated on iron-deficient succinate minimal plates supplemented with tetracycline, ethylenediamine-di(*o*-hydroxyphenylacetic acid) (EDDHA; 200 µM), and pyoverdine (25 µM). Transductants appearing after 48 h at 37°C were recovered, and outer membranes were prepared from these following growth under iron-limiting conditions for examination on SDS-polyacrylamide gels. Those expressing the 90-kDa ferripyoverdine receptor were retained for further study.

DNA methodology. Plasmid DNA was routinely prepared by the alkaline lysis procedure (50). For sequencing purposes, the DNA was purified on CsCl₂ gradients (50). Restriction endonucleases and T4 ligase were obtained from GIBCO-BRL or Pharmacia-LKB and used according to manufacturer's instructions or as described by Sambrook et al. (50). Transformation of *E. coli* (50) and *P. aeruginosa* (4) with plasmid DNA has been described. Restriction fragments were isolated, as required, from agarose gels (0.8 to 1.5% [wt/vol] with GeneClean (BIO 101 Inc., La Jolla, Calif.) or Prep-a-gene (Bio-Rad Laboratories, Mississauga, Ontario, Canada) glass matrices as detailed by the manufacturers. For subcloning of *P. aeruginosa* DNA and introduction of cloned DNA into *P. aeruginosa*, the *E. coli*-*P. aeruginosa* shuttle cloning vector pAK1900 (55) was used. This vector carries the *bla* gene and pBR322/pRO1600 origins of plasmid pQF10 (19) plus the multicloning site, *lacZ* gene, and fl origin of plasmid pGEM3Zf⁺ (Promega Biotec, Madison, Wis.).

Nucleotide sequence determination and analysis. DNA for sequencing was obtained as CsCl₂-purified double-stranded template and sequenced by the Centres of Excellence Core Facility for Protein/DNA Chemistry at Queen's University. Overlapping sequence from both strands was obtained by using a series of custom-synthesized primers. Nucleotide and deduced amino acid sequences were analyzed by using the PC Gene software package (Intelligenetics, Inc., Mountain View, Calif.).

Growth assays. Bacterial growth was assayed by monitoring the change in A_{600} of cultures inoculated at an A_{600} of

0.05 to 0.10 with stationary-phase cells and shaken (200 rpm) at 37°C.

Purification of the ferripyoverdine receptor. Enhanced production of the ferripyoverdine receptor was achieved by culturing *P. aeruginosa* K372 in iron-deficient minimal medium in the presence of ZnSO₄ (0.1 mM). Eight liters of an overnight cell culture were harvested by centrifugation (10,000 × *g*, 10 min), resuspended in 80 ml of 20 mM Tris-HCl (pH 8.0), and disrupted by three passages in a French pressure cell (15,000 lb/in²). Outer membranes were isolated (53), resuspended in 100 ml of 2% (vol/vol) Triton X-100–20 mM Tris-HCl (pH 8.0) and centrifuged (180,000 × *g*, 45 min). The pellets obtained were extracted twice with 80 ml of 2% (vol/vol) Triton X-100–20 mM Tris-HCl (pH 8.0)–1 M NaCl before being resuspended in 40 ml of 1% (wt/vol) Zwittergent 3-14 (Calbiochem, San Diego, Calif.). Following centrifugation (180,000 × *g*, 45 min), the resultant Zwittergent-soluble material was recovered and loaded onto a 5-ml DEAE-Sepharose 4B-Cl (Pharmacia-LKB) column (1.5 by 3.0 cm) equilibrated with 1% (wt/vol) Zwittergent 3-14–20 mM Tris-HCl (pH 8.0) (column buffer). The column was then washed with 10 ml of column buffer and then with 5 ml of column buffer containing 0.1 M NaCl. Bound protein was eluted from the column with a 40-ml linear gradient of NaCl (0.1 to 0.4 M) in column buffer and collected in 1-ml fractions. Fractions enriched for the 90-kDa ferripyoverdine receptor were pooled and electrophoresed on preparative (3-mm-thick) SDS-polyacrylamide gels (85 V through the stack and 120 V through the running gel). Following electrophoresis, gels were immersed in 4 M sodium acetate to visualize protein bands (26). The band corresponding to the ferripyoverdine receptor was excised with a razor blade, cut into smaller cubes, and soaked in distilled water for 10 min. The protein-containing gel cubes were then loaded into the sample trap of a Little Blue Tank electroeluter and concentrator (ISCO, Lincoln, Nebr.) containing 0.1× SDS-PAGE buffer supplemented with 0.1% (wt/vol) SDS. The trap was placed in the electrode chamber containing 1× SDS-PAGE buffer, and the protein was eluted for 4 h at 25 mA (constant current).

Preparation of antibodies to the ferripyoverdine receptor. Electroeluted ferripyoverdine receptor (50 µg in a 1:1 mixture of saline and Freund's incomplete adjuvant) was used to immunize a New Zealand White rabbit on days 0, 4, 7, 11, 27, 41, and 52. Three days after the final immunization, the animal was bled, and serum was recovered from clotted blood.

N-terminal amino acid sequence determination. Ferripyoverdine receptor used for N-terminal amino acid sequence determination was recovered as an enriched preparation from a DEAE-Sepharose 4B-Cl column (see above). Following electrophoresis on SDS-polyacrylamide gels, the receptor-containing preparation was electrophoretically transferred to ProBlott membrane (Applied Biosciences Inc., Mississauga, Ontario, Canada) in 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS) buffer as detailed by the manufacturer. Proteins were visualized with Coomassie blue by a procedure provided by the manufacturer, and the band corresponding to the ferripyoverdine receptor was excised with a razor blade. N-terminal amino acid sequence determination was carried out on the electroblotted protein by the Centres of Excellence Core Facility for Protein and DNA Chemistry at Queen's University.

Nucleotide sequence accession number. The nucleotide sequence of *fpvA* is registered in the GenBank data base under accession number L10210.

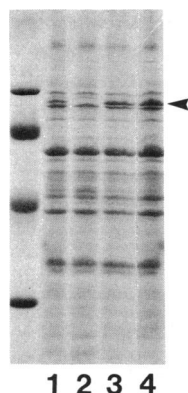


FIG. 1. Outer membrane proteins of iron-limited *P. aeruginosa* strains K372 (lane 1), K437 (lane 2), K437/pPVR1 (lane 3), and K437/pPVR2 (lane 4). The ferripyoverdine receptor FpvA is indicated by an arrowhead. Molecular mass standards (at left) are as follows: phosphorylase B, 97.4 kDa; bovine serum albumin, 66.2 kDa; ovalbumin, 42.7 kDa; carbonic anhydrase, 31 kDa. Samples were solubilized at 95°C for 5 min prior to electrophoresis.

RESULTS

In vivo cloning of the ferripyoverdine receptor gene (*fpvA*). The siderophore-deficient strain K372 is incapable of growth on iron-deficient minimal medium containing EDDHA unless supplemented with pyoverdine (27, 44). A mutant of K372 lacking the ferripyoverdine receptor (K437) shows markedly reduced growth on EDDHA-containing minimal medium supplemented with pyoverdine compared with that of K372 (44). As a result, K437 forms smaller colonies than K372 following overnight growth on iron-deficient EDDHA-pyoverdine minimal plates. Initially, then, attempts were made to clone the ferripyoverdine receptor gene by complementing this growth defect. By using the *in vivo* cloning system of Darzins and Casadaban (14), several clones which showed enhanced growth (formed larger colonies) on EDDHA-pyoverdine-supplemented minimal plates were obtained. Several of these expressed an outer membrane protein with the same molecular mass (ca. 90 kDa) as the ferripyoverdine receptor following growth under iron-limiting conditions (e.g., Fig. 1, lane 3). Phagemid DNA was isolated from all clones expressing the 90-kDa protein and subjected to restriction analysis. A common 4.6-kb *SphI* fragment which was sufficient to direct the expression of the 90-kDa outer membrane protein was identified in these phagemids (Fig. 1, lane 4). The *SphI* fragment-encoded protein facilitated enhanced growth of K437 in minimal medium containing EDDHA and pyoverdine and reacted with a polyclonal antiserum raised against the ferripyoverdine receptor (data not shown), confirming that it was, indeed, the receptor.

Nucleotide sequence of the ferripyoverdine receptor gene. The 4.6-kb *SphI* fragment directing the synthesis of the ferripyoverdine receptor in strain K437 was sequenced on both strands in its entirety (Fig. 2). A single, large open reading frame, designated *fpvA* (ferripyoverdine uptake), was identified on the *SphI* fragment. Of three potential start codons (at nucleotides 1100, 1145, and 1190), only the first two were preceded by sequences resembling ribosome-binding sites. Initiation seems likely at the second methionine residue, however, since the resultant product would possess a typical N-terminal signal sequence, consistent with the outer membrane location of the ferripyoverdine

receptor. The predicted FpvA product initiating at the second methionine residue possesses 797 residues (excluding the methionine residue), for a molecular mass of 89,395 Da. A sequence identical to the 21 N-terminal amino acids of the purified ferripyoverdine receptor (QEVEFDIPXQALG SALQEFGR) was identified in this predicted FpvA protein beginning at amino acid 27 (excluding the initiation methionine), confirming that *fpvA* was, indeed, the receptor gene. Moreover, it indicated that FpvA is initially synthesized with a signal sequence of 27 amino acids which is removed to yield a mature protein of 770 amino acids and a 86,245 Da molecular mass. This is in reasonable agreement with the 90-kDa molecular mass estimated for the protein on SDS-polyacrylamide gels (Fig. 1) (44). Typical of many bacterial outer membrane proteins, the predicted FpvA product exhibited a high β -sheet content, a net negative charge (-21), and a C-terminal phenylalanine residue. Interestingly, the GC content of *fpvA* (60.7%) is somewhat lower than the average GC content of *P. aeruginosa* (67%) (18).

A smaller open reading frame lacking a start codon was identified upstream of the *fpvA* gene on the 4.6-kb *SphI* fragment, terminating with a TGA stop codon at bp 995. The codon usage of the translated product was typical of *P. aeruginosa* genes (data not shown), indicating that this open reading frame undoubtedly forms part of a gene. The region between this stop codon and the first methionine of the *fpvA* open reading frame is markedly AT rich (60%) in contrast to the *fpvA* coding region (39.3% AT) and the *P. aeruginosa* genome as a whole (33% AT [18]). Sequences with strong homology to known promoters were absent from this upstream region, although two regions with limited homology to the canonical -35 (cTaaAgA, at nucleotide 1036) and -10 (TtTAtT, at nucleotide 1061) regions of *E. coli* σ^{70} -type promoters were identified in this region.

Homology to TonB-dependent receptors. In *E. coli*, nutrient transport dependent upon outer membrane proteins requires the product of the *tonB* gene (7, 47). These so-called TonB-dependent receptors show very little overall homology, although specific domains within the mature sequences appear to be conserved amongst this group of proteins (36) and in the PupA ferric pseudobactin receptor of *P. putida* (6) and in the FoxA ferrioxamine B receptor of *Yersinia enterocolitica* (3). Although a TonB protein and a TonB-dependent mechanism have yet to be demonstrated for *P. aeruginosa*, the conservation of these domains in FpvA would be consistent with ferripyoverdine uptake utilizing such a mechanism. Of the three most conserved domains found in TonB-dependent receptors (3, 6), regions corresponding to domains II and III were readily identifiable in FpvA and at the same relative positions as in the other receptors (Fig. 3). Furthermore, the invariant asparagine and aspartate residues of region II were conserved in FpvA and the invariant glycine residues of region III were similarly conserved in this protein (Fig. 3). Interestingly, however, the previously invariant threonine and valine residues of region I, the so-called TonB box, were not conserved in FpvA (Fig. 3).

Homology to the *P. putida* PupA protein and other ferrisiderophore receptors. A scan of the GenBank and Swiss Protein data bases identified the PupA and PupB proteins of *P. putida* WCS358 and the FhuE protein of *E. coli* as the most homologous to FpvA (Fig. 4). PupA (6) and PupB (5) are receptors for the iron-bound form of pseudobactin, the pyoverdine-like siderophore produced by *P. putida*, while FhuE is the receptor for the fungal siderophores coprogen and rhodotorulic acid (51). Alignment of the deduced amino acid sequences revealed that the homology is highest be-

1 ACACCGGCCGATCAGCGAAACCGCAACAACATCCGCGACCTCCACATCAGCTCCATCAATATATTCATCCTGGCCAGACCTTCGGCTCGATGCTGT
101 CTTCTGTGGATCGGCTTACGCTCCAGGCTACTGGCTTCGTCCTCAATCCGCGCCCATCACTGGCTTCGTGATGGCTCCTGTACATGAAG
201 GGACCCTCGAAACAGGTGATCCTTCCATCGTCAGTCGCGCCAGGTCGCTTCCAGCGGGTCGCGAACTTCGAGCGTTTTCTCTCCCGA
301 ACCCATCTTCTGCTCAGCGAACAGGAGGCCCCGGCAAGACCGTCCAGAGCTTGGAACTGCGCGACGTTTCGCTACAGCCCCCGCGTCGAAGGCAGC
401 GAACCGCTCCTATCGGCGCTATCAATTCGCTATCGCCCAAGGTGACATCGTCTTATCGCGCGGAGAACGGCTGCGCGAAGACACCTGATCAAGC
501 TGCTGCTCGGCTCTATCGCCGAGTCCGGCGAGATCCTTCTCAACGGAGAACCGGTGAGCGCGAGACCCGCGACGATTACCGCCAGCTCTCAGCAC
601 CATCTTCGCGGACTACTACTATTCGACGACCTGGTCCAGGCAAGCAGGCTTCGTTGCAGAACGCGCAATATCTGCAACGCGTGGAAATCGCCAC
701 AAGTCAGCGTTCGACGGAATGGCGCGACCCAGGCTTCCAGCCAGGCTTCCAGCGCGAGCGCAAGCGCTTGCCTGATCAATGCTTGGCTGAAAGCGCGG
801 TCCTGGCTTCGACGAATGGCGCGACCCAGGCTTCCAGGCTTCTACACCGAACTGTTCCGCGACCTGAAACGCGAGCGGCAAGACCAT
901 CATCGTATCAGCCAGGATGATCGCTATTGAAATGGCGGACAGTTGATAAGGCTCTCGCGAGCAAGGTCGTCAGGAAACCGAGCGCGTGAAC
1001 TTTTTCATCGGTTTTCCGTTGGTAAACGTCATATAAGAGAATTGAGAAGCATCTCATTATTTCGCAACTGCCAACCTAAGAGCAATACCCCA
S.D.
1101 M P A P H G L S P L S K A F L M R R A F Q R R I L P H S L A M A L S
T G C C A G C A C C A C G G T C A G T C C T C G A G T A G G C G T T T G A T G C G C G T G C C T C C A A C G C A G G A T T C T C C C A C T C G C T C G C C A T G C C C T G A G
S.D.
1201 L P L A G Y V Q A I Q E V E F D I P P Q A L G S A L Q E F G R Q A D
C C T G C C G C T G C G G G A T A T G C C A G G C G A G G A A G T C G A G T T C G A C A T C C C T C C C A G G C G T G G G C A G C C A C T C C A G G A G T T C G C C G C C A G G C G A T
1301 I Q V L Y R P E E V R N K R S S A I K G K L E P H Q A I T E L L R
A T C C A G G T G C T G T A T C C C C G A A G A G G T C C G A A C A A G C G T A G C A G T G C G A T C A A G G C A A G C T G G A G C G A A C C A G G C A T T A C C G A G C T G C T C G T G
1401 G T G A S V D F Q G N A I T I S V A E A A D S S V D L G A T M I T S
G C A C C G C G C A T C G T G G A C T T C A G G G C A A T G C G A T A A C C A T C A G C G T C G C G A G G C G G C A G C A G C G T C G A T C T C G G C C C A C C A T G A T C A C C T C
1501 N Q L G T I T E D S G S Y T P G T I A T A T R L V L L T P R E T P Q
C A A C A G T T G G G C A C C A C C C G A G G A C A G C G G C T C C T A T A C G C C A G G C A C T A T C G C C A C G G C A C C C G C C T G G T G C T G A C T C C G C G A G A G C C C G A G
1601 S I T V V T R Q N M D D F G L N M I D D V M R H T P G I T V S A Y
T C G A T C A C C G T G C C A C C G C A G A A C A T G A C G A C T T C G C C C T C A A C A A C T C G A C G A C T C A T G C C C A T A C C C G G G C A T C A C C G T C G G C C T A C G
1701 D T D R N N Y Y A R G F S I N M F Q Y D G I P S T A R N V G Y S A G
A C A C T G C C G C A A C A C T A T G C C C G C G C T T C T C G A T C A A C A A C T C C A G T A C G A C G G A T T C C C T C G A C G G C C G C A A C G T C G C C T A C T C C G C G G G
1801 N T L S D M A I Y D R V E V L K G A T G L L T G A G S L G A T I N
C A A T C A G C T C A G T G A C A T G G C C A T C A G C C G G T G A A G T A C T C A A G G G G C C A G C C G C C T G C T C A C C G T G C G G G T C C C T C G G C C A C A T C A A C
1901 L I R K K P T H E F K G H V E L G A G S W D N Y R S E L D V S G P
C T G A T C C G A A G A A C C T A C C A T G A A T T C A A G G C C A T G T C G A A C T G G G C G G G C A G T T G G A C A A T T A C C G T C C G A A C T G G A C G T C A G C G C C A C
EcoRI
2001 L T E S G N V R G R A V A A Y Q D K H S F M D H Y E R K T S V Y Y G
T G C C G A G T C G G C A A C G T C C G C G C G G C G G T A G C C C T A C C A G A T A A G C A C T C G T T C A T G G A C A C T A C G A G C G A A A A A C C A G C G C T T A T T A C G G
2101 I L E F D L N P D T M L T V G A D Y Q D N D P K G S G W S G S F P
C A T C C T C G A G T C G A T C G A A T C C G A C A C C A T G C T T A C C G T C G G C G C G A C T A T C A G G A C A C G A T C C C A A G G A T C G G G T G T G C G G C A G C T T C C C G
2201 L T F D S Q G N R N D V S R S F N N G A K W S S W E Q Y T R T V F A
C T C T C G C A C C A G G G C A C C G C A A C G A C G T C C C C G T C C T T C A A C A C G G A G C C A A G T G G A G C A G T T G G A G A G T A C A C C C G C C C C T T C G C C A
2301 M L E H N F A N G W V G K V Q L D H K I N G Y H A P L G A I M G D W
A C C T C G A G C A A A T T T C G C C A C C G C T G G T C G G C A A G G T G C A A C T C G A T C A C A A G A T C A A C G G T A C C A C G C C C C C T C G G C G C A T C A T G G C G A C T G
2401 P A P D N S A K I V A Q K Y T G E T K S N S L D I Y L T G P F Q
G C C G A C C G G A C A C A C G C C A A G T C G T T G C G C A A G T A C A C C G C G A A C C A A G A G C A A C T C G T G G A T A T C T A T C T C A C C G C C C C T T C C A G T T C
2501 L G R E H E L V V G T S A S F S H W E G K S Y M N L R N Y D N T T
C T C G T C G C G A C A G C A G C T G G T G T C G C C A C T C G G C T C C T T C C C A C T G G G A G G C A A G A G C T A C T G G A A C T C G C C A C T A C G A C A C C A C C C G
2601 D D F I N W D G D I G K P D W G T P S Q Y I D D K T R Q L G S Y M T
A G C A C T C A T C A A C T G G G A C G G C A T A G G C A A G C C C A C T G G G T A C C C C T C C C A G T A C A T C G A T G A C A A G C C C G C A G T T G G C A G C A T A C A T G A C
KpnI
2701 A R F N V T D D L N L F L G G R V D Y R V T G L N P T I R E S G
C G C C G C T T C A A T G T G A C C G A T G A C C T G A A C C T T C C T A G G T G G A C G C T G G T C G A C T A C C G G T A A C C G C C T G A A T C G A C C A T T C G C A G T C C G G A
Sall
2801 R F I P Y V G A V Y D L N D T Y S V Y A S Y T D I F M P Q D S W Y
C G C T T C A T C C C T A C G T C G G C G G G T C A G C A C T G A A C A T A C C T A C C G T C T A C G C C A G T A T A C C G A C A T T C A T C A T C G C C G A G C A G T T G G T A T C
2901 R D S S N K L L E P D E G Q N Y E I G I K G E Y L D G R L N T S L A
G C G A C A G C A G C A A C A C T G C T C G A A C C C A G C A A G G C C A G A C T A C G A G A T C G G C A T C A A G G T G A A T A T C T C G A C G G A C G A C T C A A T A C C A G T C C G C
3001 Y F E I H E E N R A E E D A L Y N S K P T M P A I T Y A Y K G I K
C T A C T T C G A G A T T C A T G A G G A A A C C G C G G A A G A C G C C C T C T A C A A C A G A A A C C G A C C A A C C C G C C A T C A C T A C G C C T A C A G G G C A T C A A G
3101 A K T K G Y E A E I S G E L A P G W Q V Q A G Y T H K I I R D D S
G C C A A G C A A G G G C T A C G A G G C G A A A T C T C G G G T G A C T G G C C A G G T T C A G G T T C A G G T G G C T A T A C C C A A G A T C A T T C G C G A C A C A G T
3201 G K K V S T W E P Q D Q L S L F T S Y K F K G A L D K L T V G G G A
G A A A G A G G T T C A C C T G G A G C C T C A G G A C A G T T G A G C C T T C A C C A G T A C A A G T T C A A G G G C C C C T G G A C A A G C T C A C G G T C G G C G G C G G C G C
3301 R W Q G K S W Q M V N P R R R W E K F S Q E D Y W L V D L M A R Y
C C G C T G C A G G C A A G A G C T G G C A G A T G G T C A A T C C C G C A G A C C T G G G A A A G T T C C C A G G A A C T A C T G C T G T G G C A T T C G C T G C G A G T T C G C T G C G A G T T C G C T G C G A G T T C G C T G C G A G T T G C C A G C A G T G C C G A A C T C G G E
3401 Q I T D K L S A S V N V M N V F D K T Y Y T N I G F Y T S A S Y G
C A G A T C A C C G A C A A G C T G T C A G C C A G C C T C A A C G T C A A C A C G T C T C G A C A A G A C T A C A C C A C A C A C T C G G T T T C A C C T C G G A C T C T A T G C G C
3501 D P R N L M F S T R W D F -
A T C C A C G A A C C T G A T G T T C A G C A C T C G C T G G A C T T C T G A G C C C A G C T G C G A A A A A G A A C C C C C C A A T G C G G G G C G T T C T G C T C T C G G C T A C C C A
3601 C G C G C C A T C G C C G A A G A C G C C T G T C G G C A G C A C C A A A T G A T T C C T G T A C A G A A A A A A G A G C C C G C C T G A A G G C G G G C T T T G A A C G G A A C G G A C C T
3701 G A G A T C A G C G C C G A A G C T G C A C A A A C T C A G G G T G T C G A T G A T T G T C G T G C C G G G A T C A G C A C C C A C C G G T T C G A C A C G C T C A G C C C G A
3801 T C G C T C G G C C A T C G A G C C T C C A C C G C C T C C A C T G G A T G G C A T T G T G C T G C G C G C G C C A C C A G G C G T G A C C T T C G C C T C A G A C C G G G T A A T C
3901 C A T C G C T T C A G G C C T C G T C G A G A T C T G T T C A A C T C C C A G G A C A C T T C A G T T C G T G C T G C T G C G A T T C G C T G C G A G T T C G C T G C G A G T T G C G A A C T C G G E
4001 C C G A T C T C C G C T T C C G C C A G T C G C T C A T C C T C C A G C G C T G C T C A T C G T C G T G G C G G A C A G A T C G A T G A C C A G G C C G T T C G A T A G C T G
4101 G C C C G C T C G G A A A C A T G A C A C C A G C A G T T C C A C C C G C A T C G C C C A C G G A T A G G T T C G G A C T G C C T C G G C G T C G G C C G A T C T G T C C A G A A C G
4201 C T T C G A C C T G A C C G G T G C C G T G C A T C G A T C C A G C G A C G A A A G C A C C T G C C G C C A C G C T G C T C A G C G G G C G C G A C A T C C A T C G C A G G T G C C
4301 G C C A G G C A C C A T C C C G C A G G T T G A A A A C C C C T T C G G G G T G C T C T C A G C A A T T G C T C G G C G A T T C G C A A C C A T A T G T C C C A C T C G G C A C C T C G
PstI
4401 C G A C C A G C C C A G A G G C A C G G C A G A C A C C C T T G A C C G A T G A C G A T C C C G A C G C C A T T G C G A G G G T T T G T A A C A G T G C A C A G A C C G A A A
4501 C T C G G A T G A A C A A C A G C G G C A A C C T T C G C G C T G C C G T T G A G C G C A C C A G C A A C G A C T C A C G C C T G C C C C T C G A T

I		II	
BtuB	4	SPDTLVVTAN	BtuB 565 IANLFDKRY
Cir	4	DGETMVVTAS	Cir 608 VLNLFDKDL
FecA	21	SGFTLSVDAS	FecA 705 VKNIFDQDY
FepA	10	HDDTIVVTAA	FepA 674 VDNLFDKRL
FhuA	5	DEDTIVVTAA	FhuA 680 VNNLFDREY
IutA	4	DDETFVVSAN	IutA 660 IENLFDRDY
FoxA	1	-DDTIEVTAK	FoxA 650 VNNIADKKY
FhuE	4	TEETVIVEGS	FhuE 661 VNNLFDKTY
PupA	66	DGNTVTVTAS	PupA 739 VNNIADKTY
FpvA	66	QGNAITISVA	FpvA 736 VNNVFDKTY
			* *
III			
BtuB	105	QRVEYIRGPRSAVYGSDAIGGVNIIITTR	
Cir	106	ERIEVVRGPMSSLYGSDALGGVNNIITTK	
FecA	190	DAIDVVRGGGAVRYGPQSVGGVNVFVTRA	
FepA	120	ERIEVLRGPARARYGNGA-GGVNIIITTK	
FhuA	127	ERAEIMRGPVSVLYGKSSGPGLLNMVSKR	
IutA	103	HHIEVIFGAT-SLYGGGSGTGLINIVTKK	
FoxA	121	ERIDVIKGPSSALYQGSIPGGVMMTSKR	
FhuE	120	ERVEVVRGATGLMTGTGNPSAAINMVRKH	
PupA	202	DRIEIVRGATGLMTGAGDPSAVVNVIRKR	
FpvA	201	DRVEVLKGTGLLTGAGSLGATINLIRKK	
		* *	

FIG. 3. Conserved sequences in TonB-dependent receptors and FpvA. The numbers at the left represent the positions of the first amino acid in each homology region in the mature sequence of the protein. Identical amino acids are indicated by asterisks. Sequences were taken from reference 3, in which the original sequence papers are referenced.

tween FpvA and PupA (41.3% identical residues and 14.8% conserved changes) and FpvA and PupB (40.6% identical residues and 13.3% conserved changes) though still substantial between FpvA and FhuE (35% identical residues and 12.1% conserved changes). Multiple alignment of the mature sequences of all four receptor proteins (Fig. 4) revealed that fully 15% of the residues were identical in all proteins with an additional 33.5% of the residues judged as being similar. Moreover, the regions of homology and similarity shared by all proteins were spaced regularly along the length of the sequences, interrupted by regions of marked variability. This situation has been noted previously for the porin family of outer membrane proteins (28, 60), for which it was determined that conserved regions represented transmembranous domains and the variable regions formed extramembranous loops.

DISCUSSION

The FpvA protein is predicted to consist of 797 amino acids (89,395 Da) processed to a mature protein of 770 amino acids (86,245 Da). This is based on the assumption, however, that translation of the protein initiates at the second methionine identified in the *fpvA* open reading frame. While this seems reasonable given the presence of a putative Shine-Dalgarno sequence immediately upstream and a typical signal sequence downstream, the observed homology of FpvA to the ferric pseudobactin receptors PupA and PupB raises some doubts. The Pup proteins possess unusual signal sequences which are larger than normal (44 to 47 residues) and with the basic amino acids positioned more centrally

within the sequence rather than at the N terminus (5, 6). As with typical signal sequences, however, the basic residues are followed by a long stretch of hydrophobic residues. Interestingly, if FpvA actually initiates at the first methionine residue of the *fpvA* open reading frame, the resultant FpvA precursor would possess a Pup protein-like signal of 42 amino acids with the basic residues occurring upstream of a hydrophobic sequence but between residues 11 and 22 of the precursor and not at the N terminus. In any case, the functional significance of such an unusual signal sequence has yet to be determined.

Although a *tonB* gene has yet to be identified for *P. aeruginosa*, the presence in FpvA of sequences apparently conserved in TonB-dependent receptors certainly suggests that ferripyoverdine uptake utilizes a TonB-like mechanism. Indeed, we have recently expressed the *P. aeruginosa* ferric enterobactin receptor PfeA in *E. coli*, in which it was capable of mediating ferric enterobactin uptake (16). Not only does this indicate that the PfeA receptor must be able to interact with the *E. coli* TonB protein, but it also indicates that a TonB-like mechanism must operate in *P. aeruginosa*. Furthermore, a *P. aeruginosa* TonB homolog would be expected to resemble the *E. coli* TonB protein, since both appear to be capable of recognizing the same receptor. Whether the recognition site on the receptor proteins (the TonB box) is the same for both TonB proteins is questionable, however, given that FpvA shows very little homology to the *E. coli* TonB box (homology region I of TonB-dependent receptors). It is possible, however, that the TonB proteins tolerate some variation (e.g., conservative substitutions) in the so-called TonB box of receptor proteins, accounting for the variation in this region of FpvA. Alternatively, the lack of conservation of the TonB box sequence in FpvA could indicate that this region is not the critical site of interaction between the TonB proteins and the receptors.

Although the region upstream of the *fpvA* gene did not exhibit strong homology to known promoter sequences, some homology to the *E. coli* σ^{70} class of promoter was evident. This lack of strong homology to known promoters is not surprising, in that *Pseudomonas* promoters are, in general, poorly characterized and often exhibit little or no homology to known enteric promoters (49). The sequence upstream of *fpvA* is unusual, nonetheless, in that it is relatively AT rich, in contrast to the overall GC richness of the *P. aeruginosa* genome and the *fpvA* coding region. Whether this relates to promoter function or activity or to regulation of *fpvA* expression is unclear. It is interesting to note, however, that part of the AT richness of this upstream region is attributable to 2 oligo(dT) tracts (at nucleotides 1001 to 1005 and 1013 to 1016). Such tracts have been identified in the region upstream of the promoter of the *ompF* gene which encodes the osmoregulated OmpF porin protein in *E. coli* (30). These oligo(dT) tracts are involved in the binding of a transcriptional activator, OmpR, which operates as part of a two-component regulatory system responsive to medium osmolarity (30). Interestingly, the ferripyoverdine receptor of *P. aeruginosa* has recently been shown to be positively regulated by pyoverdine (20). Moreover, the ferric pseudobactin receptors of *P. putida* (33) and the ferric enterobactin receptor PfeA of *P. aeruginosa* (16)

FIG. 2. Nucleotide sequence of the 4.6-kb *SphI* fragment carrying the ferripyoverdine receptor gene *fpvA*. The deduced amino acid sequence of a large open reading frame corresponding to *fpvA* is indicated. Potential translation start sites (M) and Shine-Dalgarno sequences (S.D.) for FpvA are underlined. The start of the mature protein is indicated by an arrow. Selected restriction sites are highlighted.

FPVA	Q---EVEFDIPQALGSAQEFGRQADIQVLRPEEVRNKRSSAIGKLE	47
PUPA	Q---EWTLDIPQASNSALQALAKQTDQLLSPEDIIGLRSSALGRHD	47
PUPB	AAQQAQDFDIPAGLAPALAHFGQSAHILLSYPTALTEGRSTSLGRFDD	50
FHUE	-----	7
1		
FPVA	PNQAITELLRGTSASVDFQGMNITISVAEAA-DSSVDLGTAMITSNQLGT	96
PUPA	LQSSLRILLQCTGLRYQIDGNTVTVTASAAAKDQIELSATNVNSAGLGE	97
PUPB	IDQGLAILLAGTGLEAS-RGANASYSLQASASTGALELSAVSISGKAPGS	99
FHUE	-----AAPATEETVIVEGSATA	17
2 3		
FPVA	ITEDSGSYTPGTIATATLVLVTPRETPQSIITVTVTRQNMDDDFLNNIDDVM	146
PUPA	TTETGTSYTRVSTATKNNLSIRETPQTITVTVTRQNMDDQHLGSMNEVL	147
PUPB	TTETGLTYTSSSSSRNLNTPRETPQSLTVTRQRLDDQRLNLTLDAL	149
FHUE	FDGGENDYSVTSTAGTKMQGTQRDIPQSVTVTVSQQMEDDQLQTLGEMV	67
4		
FPVA	RHTPGITVSAYD--DTRNNYVARGFSLNMFQVDCIPSTARNVGVSAGNTL	194
PUPA	TQTPGITMSQDG--GERFNLYSRGSAINIYQFDGVTVYQDNQTRNMFSTL	195
PUPB	EATPGITVVRDLGSESDSWSRGAFAIQNYEVDGVPPTSTRLDNVSQS---	196
FHUE	ENTLIGISKQAD--SDRALYYSRFGQIDNYMVDGIPITYPESR-WNLGDAL	114
5 6 7		
FPVA	SDMAIYDRVEVLKQATGLLGTAGSLGATINLIRKPT-HEFKGHVELGAG	243
PUPA	MDVGLYDRIEIVRGATLTDGTGAGDPSAVNVVIRKPT-REFKSHIQAGVG	244
PUPB	--MAMFORVEIVRGATGLISGMGNFSATINLIRKPT-AEAQASITGAEAG	243
FHUE	SDMALFRVEIVRGATGLMTGTGNFSAANMVRKHATSREFKGDVSAEYG	164
8 9		
FPVA	SWDNYRSELVDSGPLTESGMVGRRAVAAYQDKHSFMDHYERKTSVYVYGL	293
PUPA	SNWYRAEADVSRGLTDDGRVRFPAKQDNHTFMDWYTDQRDLVQAGVG	294
PUPB	NWDRYGTGFDVSGPLTETGNIIRGRFVADYKTEKAWIDRYNQSQLMYGIT	293
FHUE	SNWKERVADVLSGPLTETDGGKTRAIRVGGYQNNDSWLDRYNSEKTFPSIV	214
10 11		
FPVA	EPDLNFDITMLTVGADYQNDPKGSGWSSGFFLFDSCQNRDVSRSFNNGA	343
PUPA	EADVTDITVARPGLDRGTLYKNGVAP---GVPIIYTNQPTNFRSHISDA	341
PUPB	EPDLSSEDTLLTGVFSYLRSDIDSLRSG-LPFRFSTGERTNLRKRSINLNAAP	342
FHUE	DADLGDITLTVAGFYQRIDVMSPTW--GGLPRWMTDGSNSYDRAARSTAP	263
12		
FPVA	KWSSWEQYTRTVFA-NLEHNFGWGVCKVQ---LDHK-----INCHVA	382
PUPA	RWG-VDDYTTNYTFCLEQLLADHWQFKLAAAYNDVD-----RDSFSS	383
PUPB	DWS-VNDHEQTSFPTSI EQQLGNWGSQKIE--LTHA-----ENKFE	381
FHUE	DWA-VNDKEINKVMTLKKQFADTQATLNATHSEVDFDSKMYVDAYVN	312
13		
FPVA	PLGAIMG-DWPAPDNSAKIVAQK---VTGETKSNLSDIYLTGPFQFLGREH	429
PUPA	YYSTTTNRSYVLELDGSTEISAGI--VTAKHQKGVDAITLQGFQLLQGT	431
PUPB	LFNFAGELNPGDGLSOL-FVR--PSGTPRDNLDLVATYQPSLFGREH	428
FHUE	KADGMLVGPYSNYGCPDFVGGTGWNSGKRKVDALDLDADGSEYELFGHQ	362
14 15		
FPVA	ELVVGTSAS-FSH---WEGKSYNLRNVDNTDDFINWDDGIGKPDWGT	474
PUPA	ELIVGYN---YLE---YENKH---RGDSGPDVNFYDWDNQTFPKPGDDE	472
PUPB	ELITGTMFLSQRENTPSWGWRYDYAGSPAGADINLFWNDGKSAKP---A	475
FHUE	NLMFGGYSK---QNNRYFSSW---ANIFPDEIGSFYFNNGFPQDWSWSP	406
16 17		
FPVA	--PSQYIDDKRTQLGSLYNTARFVNTDLDLFLQGRVVDYRVV-----GL	516
PUPA	IIPGLIQNYSNRQSGYFVASRFLNLTDDLHLILGARASNYRFDYALWRIGN	522
PUPB	FVESGKSSIDEDQYAAVLTSRFSVTDLHLILGSRILNWRKRTSDRPYGG	525
FHUE	--QSLAQDDTTHMKSALYATRVTLADPLHLILGARVYTNWRVDTLTYSMK	454
18 19		
FPVA	NPT---IRESGRFIPYVAGVLDLMDTYSVYASVYTDIFMPQDSWYRDSNK	563
PUPA	EPAPYKMERGVVTPYAGIVYDLTNEQSVYASVYTDIFKPKQNN---VDITGK	570
PUPB	EETEVRNEENGVTPIYAGVYDLDLWLSYASYTKIFNPQAGAWVDDESNK	575
FHUE	NHT-----TPYAGLVFDINDNWSYASYTSIFQPQNDR---DSSGK	492
20 21		
FPVA	LLEPDEGQNYEIGKEVYDGLRSLTSLAYFEIHEENRA---EDALYNSK	610
PUPA	PLDPEVGNELGKWEFLEGLRANALYVNRKDLA---ESTN---EVV	615
PUPB	PLDPMEGVCHYELGKTHLNGKRLNSLAVFLEQDHLAILATQRLYSAE	625
FHUE	YLAPITGNNGELGKSDWMSRLTTLAIFRIEQDNVAQSTG-----TPI	537
22 23		
FPVA	PTNPAITAYVKGIKAKTKYAEVLESSELAPGWVQAGYTHKIRIRDSGKK	660
PUPA	PDSSGLLASRAVDAETKGVVDVVELSGEVLPGNVVFTGYSHRTEADAGKR	665
PUPB	QDQTS-----KGI E L E R E L A E G W Q A S A G Y S V T D A D D O R	663
FHUE	PGSNGETAYKAVDGTVSKGVEFELNGAITDNWQLTFGATRYIAEDNEGA	587
24 25		
FPVA	VSTWEPQDQLSFTSYKFGALDKLTVGGGARWQKSWQMVNRRRWEKF	710
PUPA	LTPQLPMDTFRFVNTYRPLPGEWEKLTLLGGVNVNSKS--TLNFARYNSHV	713
PUPB	INTNLPRNSFKTFSYRHLPLDKITIGGGVNVQSKVGDALH-----TF	707
FHUE	VNPNLPRNTVVKMFTSYRPLPV-MPELTVGGGVNQRVYTDVTVPYGTFR	636
26		
FPVA	SQEDYVLDLMLARYQITDKLSASVNMVNFVKTYYLNI-GFYTSASYGDP	759
PUPA	TQDDYFVTSIMARYINESLAATLNVNNIFDKKYAGMAGSY--GHYGAP	761
PUPB	SQGSYAVTNLMARYDINQHLGSSVNLNVDREY-----NGVYGTPT	748
FHUE	EQGSYALVDLFRYQVTKNPSLQGNVNNLFDKTYDYNVEG---SIVYGTPT	683
27		
FPVA	RNLKFSRWDF 770	
PUPA	RNATVTLRYDF 772	
PUPB	RNVMTSFKYSF 759	
FHUE	RNFSITGVQF 694	

FIG. 4. Multiple alignment of the amino acid sequences of the FpvA, PupA, PupB, and FhuE siderophore receptor proteins. Residues identical in all sequences (*) and similar residues (.) are indicated. The alignment was carried out by using the CLUSTAL algorithm developed by Higgins and Sharpe (25) and included in the PC Gene software package. Predicted membrane-spanning regions of FpvA are indicated in boldface letters and numbered.

are similarly regulated by the corresponding siderophore via a two-component regulatory system. It is tempting, therefore, to speculate that a pyoverdine-responsive two-component system regulates expression of FpvA and that the oligo(dT) tracts are necessary for the binding of a transcriptional activator.

The observed homology between FpvA and the ferric pseudobactin receptors PupA and PupB is not surprising given that pseudobactin and pyoverdine are synonyms for a group of *Pseudomonas* siderophores which are characterized by an N-terminal dihydroxyquinoline moiety and a C-terminal hydroxyornithine residue joined by a peptide chain of varied composition. Indeed, it is only the differing composition of the peptide chain which distinguishes the various pyoverdines and pseudobactins. The high degree of homology to FhuE, the receptor for ferric coprogen, ferric rhodotorulic acid, and ferrioxamine B, by contrast, is unexpected. These latter siderophores are structurally very different from the pseudobactins and pyoverdines, although coprogen and rhodotorulic acid, like the pyoverdines and pseudobactins, do contain hydroxyornithine (62). Whether this would explain some similarity in these receptors is unknown. The homology between FpvA, PupA, PupB, and FhuE is even more puzzling in light of the poor homology between FhuE and FoxA, the ferrioxamine B receptor of *Y. enterocolitica* (3). Indeed, FoxA shows more homology to FhuA, the ferrichrome receptor of *E. coli*, than to FhuE (3) despite the fact that FhuE and FoxA share a common substrate. In the absence of substrate-related conservation of sequence in FpvA, PupA, PupB, and FhuE, the likeliest explanation is that the observed homologies relate to evolutionary relatedness of these receptors. It is interesting to note, for example, that the GC contents of FpvA (this study) and PupA (6) are lower than the average GC content of these organisms. One explanation is that the genes originated outside of *P. aeruginosa* and *P. putida*, where they evolved for some time before being acquired by their present "hosts".

One advantage of the homology between FpvA and a number of different receptors is the opportunity to develop a topology map of FpvA by using strategies based on secondary structure prediction models and the assumption that conserved regions represent transmembranous domains and that variable regions represent loops (3, 5, 28, 61). Using this approach, we have developed a model which is characteristic of the models developed for other ferrisiderophore receptors (3, 5, 39). We are currently assessing the accuracy of this model experimentally.

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