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Insertion Mutagenesis of the Ferric Pyoverdine Receptor FpvA of *Pseudomonas aeruginosa*: Identification of Permissive Sites and a Region Important for Ligand Binding

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Insertion of an 18-amino-acid-encoding sequence within the *fpvA* gene identified permissive sites at residues Y350, A402, R451, R521, and R558, consistent with these residues occurring in extramembranous loop regions of the protein. Insertions at R451, R521, and R558 did not adversely affect receptor function, although insertions at Y350 and A402 compromised ferric pyoverdine binding and uptake. The latter region likely contributes to or interacts with the ligand-binding site.

Iron is an essential nutrient whose acquisition by some bacteria is promoted by low-molecular-mass, high-affinity iron-chelating molecules termed siderophores (27). In conjunction with cell surface receptors specific for the Fe(III) complex of these siderophores (27, 28), they serve to facilitate iron acquisition under iron-limited conditions that predominate in animal and plant hosts (8, 19, 24). Indeed, siderophore production by human pathogens correlates with enhanced in vivo iron acquisition and growth and, thus, virulence (12, 14, 18).

Pseudomonas aeruginosa synthesizes two known siderophores, pyoverdine (13) and pyochelin (11), in response to iron limitation in vitro and in vivo (20). Pyoverdine displays higher affinity than does pyochelin for iron in buffered solutions (31) and is by far the superior siderophore in removing iron from transferrin (40) and in supporting growth in human serum (2). Moreover, the production of pyoverdine is correlated with enhanced in vivo growth and virulence (25, 31). The receptor for ferric pyoverdine is a ca. 80- to 90-kDa outer membrane protein (32) encoded by the *fpvA* gene (33). FpvA is a member of a family of receptors dependent on a cytoplasmic membrane-associated protein, TonB, which apparently couples the energized state of this membrane to the operation of these receptors (7, 35). A TonB homologue has been identified in *P. aeruginosa* (34).

Ferric siderophore receptors are described as gated channels, with a surface-exposed loop contributing to gate formation and acting as ligand-binding site (22, 23, 26, 36). In an effort to identify a potential gate/ligand-binding region of FpvA, we mutagenized putative external loop regions of the protein. We report here the identification of a region of FpvA necessary for ferric pyoverdine binding.

Strains and procedures. *P. aeruginosa* PAO1 is a wild-type strain and parent of the FpvA-deficient mutant K691, in which *fpvA* was disrupted by insertion of the tetracycline-resistant derivative of the Ω interposon (ΩTc) of plasmid pHP45ΩTc (16). The interposon was recovered on a 2-kb *Sma*I fragment and inserted into the *Sca*I site of *fpvA* on pPVR2 (a derivative of the cloning vector pAK1900) (33). This necessitated partial digestion of pPVR2 with *Sca*I and isolation of a 9-kb fragment representing full-length pPVR2. Transformants (*Escherichia coli* DH5α [3]) carrying pPVR2 with an ΩTc insert were selected on L agar containing ampicillin and tetracycline, and insertion of ΩTc within the *fpvA* gene was assessed by restriction analysis. The ΩTc-mutagenized *fpvA* gene was subsequently recovered on a 6.5-kb *Pst*I fragment and cloned into the unique *Pst*I site on plasmid pSUP202ΔTc (15). Following introduction into *E. coli* S17-1 (38), the vector was mobilized into *P. aeruginosa* PAO1 via conjugation (15). PAO1 derivatives carrying a chromosomal *fpvA*::ΩTc mutation were selected on L agar containing tetracycline and screened for the absence of plasmid-encoded carbenicillin resistance. The lack of FpvA in putative mutants was confirmed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis of isolated outer membranes. The iron-deficient succinate minimal medium has been described previously (32). Luria broth (Luria broth base; Difco) was employed as the rich medium throughout. Ampicillin (100 μg/ml), tetracycline (for *P. aeruginosa*, 200 μg/ml; for *E. coli*, 10 μg/ml), and carbenicillin (100 μg/ml) were included in growth media where appropriate. Due to the instability of the ΩTc insert in K691, K691 and plasmid-containing derivatives of this strain were always cultured in the presence of tetracycline. Bacteria were cultured at 37°C, with shaking (200 rpm) for broth cultures.

To generate a *tet*-linked DNA fragment encoding the cleavage site for the factor Xa protease, oligonucleotides T1 (5′-AGATCTTTATCGAGGGGCGGTTATCGAGGGGCGGTTATCGAGGGGCGGCCCGGGTGGCGCCCTGCA-3′), T2 (5′-GGGCGCCACCCGGGCCGCCCCTCGATAACCGCCCCTCGATAACCGCCCCTCGATAAAGATCT-3′), T3 (5′-AATTAGATCTCCCGGGTGGCGCCGAGCT-3′), and T4 (5′-CGGCGCCACCCGGGAGATCT-3′) were synthesized. T1 and T2 (50 pmol/μl each) were incubated at 70°C for 3 min followed by a 30-min incubation at room temperature to allow annealing of the oligonucleotides. The resultant duplex DNA carried internal restriction sites for *Bgl*II and *Sma*I, the coding sequence for the factor Xa cleavage site in all three reading frames, and a *Pst*I-compatible 3′ extension at one end; the other end was blunt. Oligonucleotides T3 and T4 were similarly annealed, yielding a duplex molecule possessing internal cleavage sites for *Bgl*II and *Sma*I and an *Sst*I-compatible 3′ extension at one end and an *Eco*RI-compatible 5′ extension

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at the other. The duplex molecules were ligated to a 2.1-kb *Eco*RI-*Pvu*II fragment of pBR322 (37) carrying the *tet* gene of this vector and cloned into *Sst*I-*Pst*I-restricted pAK1900 to yield pXa-1. The *tet*-factor Xa cartridge was used, ultimately, to insert 54 bp into various sites within the *fpvA* gene of plasmid pPVR2, thereby disrupting the FpvA receptor by the addition of 18 amino acids (including the factor Xa cleavage site). Although the intent was to use the inserted factor Xa cleavage sites to assess the topology of FpvA, the protease failed to cleave any of the modified FpvA. Still, the insertion of 54 bp/18 amino acids did afford the opportunity to examine the influence of the disruptions on FpvA receptor activity. Initially, then, the factor Xa sequence was recovered with the *tet* gene on a 2.1-kb *Sma*I fragment of pXa-1. This fragment was inserted into various sites within the *fpvA* gene of plasmid pPVR2 following partial digestion of pPVR2 with various enzymes whose digestion products were blunt ended (*Mst*I, *Nru*I, *Rsa*I, *Eco*RV, and *Hin*cII). Briefly, 1 μg of plasmid was restricted with 5 to 10 units of enzyme in the presence of 25 to 50 μg of ethidium bromide per ml for 15 to 30 min at 37°C. The conditions were optimized to produce a maximal yield of unit-length plasmids which were each cut once. Transformants (*E. coli* DH5α) carrying pPVR2 with an insert of the *tet*-factor Xa cartridge were selected on L agar supplemented with tetracycline and ampicillin. The site and orientation of the inserts were determined by restriction analysis and by sequencing with a primer (5′-GTGCCTGACTGCGTTAGC-3′) which anneals upstream of the pBR322 *tet* gene. The *tet* gene was subsequently excised by digestion with *Bgl*II followed by religation of the plasmids and selection of transformants which were ampicillin resistant but tetracycline sensitive. This resulted in insertion of 54 bp within *fpvA* encoding either the factor Xa cleavage site in all three reading frames (orientation 1) and insertion of 18 amino acids in FpvA or translational stop signals in all three reading frames (orientation 2) and no FpvA product.

Protocols for preparation of plasmid DNA, restriction digests, ligations, transformations and isolation of restriction fragments from agarose gels have been described previously (39, 42). DNA to be sequenced was purified with the Wizard Minipreps DNA purification system (Promega). DNA sequencing and oligonucleotide synthesis were performed by Cortec DNA Service Laboratories, Inc., Queen's University. Outer membranes were prepared as Triton X-100-insoluble cell envelopes isolated following disruption of cells with a French pressure cell (33) or a cell sonicator (Vibra Cell; Sonics & Materials, Inc.) (two bursts of 25 s at 50% power on ice) (39). Whole-cell protein extracts were prepared as described previously (30) with modifications. Briefly, 100 μl of overnight cell culture was harvested by centrifugation, resuspended in 30 μl of gel-loading buffer (2% [wt/vol] SDS–62.5 mM Tris-HCl [pH 8.0]–1% [vol/vol] glycerol), heated at 95°C for 5 min, and sonicated briefly. Following centrifugation (5 min at 15,000 rpm) to remove insoluble material, the whole-cell protein-containing supernatants were recovered. SDS-polyacrylamide gel electrophoresis was carried out as described previously (39) with 9% (wt/vol) acrylamide in the running gel. Western immunoblotting was carried out as described previously (39) with a rabbit anti-FpvA antiserum (33).

Uptake of ferric pyoverdine was assayed as described previously (32) with modifications. Cells were grown in iron-deficient succinate minimal medium supplemented with Casamino Acids (0.1% [wt/vol]; Difco) and antibiotics. Once cultures reached an A_{600} of 0.8 to 0.9, cells (5 ml) were harvested by centrifugation and resuspended in the same volume of nitrogen-free iron-deficient succinate minimal medium. Following incubation at 37°C with shaking for 30 min, aliquots (1 ml) were removed and added to 20 μl of a mixture of pyoverdine (3.5 mM) and $^{55}FeCl_3$ (0.29 μM), which had been previously incubated for 5 min at room temperature in a 10-ml disposable culture tube. Cells were vortexed gently, and aliquots (200 μl) were removed at intervals, harvested on membrane filters, washed with 10 ml of distilled water, dried, and counted in a scintillation counter (32).

Insertion mutagenesis of *fpvA*. Alignment of the predicted FpvA primary amino acid sequence with homologous receptor proteins identified several potential membrane-spanning and loop regions of FpvA (31, 33). Insertions of the 54-bp sequence encoding the factor Xa cleavage site within the *fpvA* gene of pPVR2 were achieved as outlined above, and insertion derivatives were expressed in the FpvA-deficient strain K691. Several hundred inserts within pPVR2 were screened, and many occurred outside the *fpvA* coding region. Those harboring the factor Xa coding sequences in the proper orientation within *fpvA* are described in Table 1. Although these derivatives were ultimately not susceptible to digestion with factor Xa, the insertion derivatives, but not FpvA itself, were cleaved by a nonspecific protease (subtilisin) in intact cells (data not shown), suggesting that the insertion sites were, nonetheless, surface accessible.

Most insertions yielded an FpvA product of slightly lower mobility than native FpvA in whole-cell extracts (Fig. 1A), consistent with the insertion of 18 additional amino acids, although an insertion at an *Rsa*I site at bp 1502 (pLK16-1 [Table 1]) failed to yield an FpvA product (Fig. 1A, lane 6). Regions of integral membrane proteins which tolerate amino acid insertions typically correspond to extramembranous loops (1, 4, 6, 10, 17) rather than membrane-spanning regions (1, 5). Indeed, the insertion site in most derivatives in Table 1 except $FpvA_{G473}$ was predicted to be a surface-exposed loop (31, 33). In each case, the proteins fractionated with the outer membrane (Fig. 1B), indicating that the insertions did not adversely affect proper export and localization of FpvA. The FpvA protein in each instance was also detectable in intact cells by using an FpvA-specific antiserum and indirect immunofluorescence (41) (data not shown). Some breakdown of the FpvA protein carrying an insertion at R451 (pLK161-3 [Table 1]) was evident in outer membrane extracts (Fig. 1B, lane 7), although this was not seen in whole-cell extracts (Fig. 1A, lane 7) and, therefore, must have occurred during the fractionation procedure.

Activity of FpvA derivatives. We then assessed the impact of the insertions on FpvA receptor activity. Growth of *P. aeruginosa* in minimal medium supplemented with the nonmetabolizable iron chelator EDDHA [ethylene diamine di (*o*-hydroxyphenyl acetic acid)] is dependent upon the production of pyoverdine and the presence of a functional ferric pyoverdine uptake system (32). Thus, the growth of K691 harboring the various *fpvA* insertion derivatives in medium containing EDDHA was measured. As expected, K691 itself grew very poorly, if at all, while the same strain harboring and expressing the wild-type *fpvA* gene (pPVR2) grew well (Table 1). Most of the FpvA insertion derivatives also provided for excellent growth of K691 in EDDHA-containing medium (Table 1), including those with insertions at R451 (pLK161-3), R521 (pLK32S-1), and R558 (pLK39S-1), indicating that insertions in these regions did not interfere with receptor function. In contrast, insertions at Y350 (pLK141-1) and A402 (pLK127-2) abolished the ability of these FpvA derivatives to support the growth of K691 in this medium (Table 1), consistent with these regions being important for FpvA activity. The apparent defect in activity of receptors $FpvA_{Y350}$ and $FpvA_{A402}$ was confirmed in pyoverdine-mediated iron uptake assays (Fig. 2). As ex-

TABLE 1. FpvA insertion derivatives[a]

Plasmid	Restriction endonuclease[b]	Insertion site		Growth in EDDHA-containing medium (A_{600} after 10 h)[e]	^{55}Fe-pyoverdine binding (pmol ± SD)[f]
		Nucleotide[c]	Amino acid[d]		
pLK141-1	*Rsa*I	1134	Y350	0.29	0.54 ± 0.21
pLK127-2	*Mst*I (*Fsp*I)	1288	A402	0.17	0.42 ± 0.26
pLK161-3	*Mst*I (*Fsp*I)	1434	R451	0.95	6.19 ± 1.72
pLK16-1	*Rsa*I	1502	G473	ND[g]	ND
pLK21S-1	*Nru*I	1645	R521	0.95	8.2 ± 0.59
pLK39S-1	*Nru*I	1757	R558	0.95	9.97 ± 1.66
pPVR2				0.99	11.80 ± 2.13
pAK1900				0.24	0.25 ± 0.21

[a] 54 bp of DNA was inserted into a variety of sites within the *fpvA* gene present on plasmid pPVR2, as described in the text. Insertion sites were determined by sequencing and restriction analysis.

[b] Indicates the enzyme which was used to cleave pPVR2 in the process of inserting 54 bp into *fpvA* and, thus, the restriction site at which the insert is found in the indicated plasmids.

[c] Indicates the nucleotide position of the 54-bp insert within the *fpvA* coding region, where the A of the ATG start codon is bp 1.

[d] Indicates the site of insertion of the 54-bp-encoded 18 amino acids within the mature FpvA protein, assuming that amino acids 1 to 28 of the precursor are cleaved by signal peptidase and that the mature protein begins with a Q residue. The indicated amino acid was actually lost as a result of the insertion.

[e] *P. aeruginosa* K691 harboring the indicated plasmids was inoculated into iron-deficient minimal medium supplemented with EDDHA (150 μg/ml) at an A_{600} of 0.05 to 0.10 and incubated at 37°C. Culture density (A_{600}) was monitored hourly for 10 h, and the final A_{600} value obtained is reported. The data are representative of three separate experiments carried out in duplicate.

[f] Outer membranes (100 μg) prepared from *P. aeruginosa* K691 carrying the indicated plasmids and cultured under iron-limiting conditions were incubated in the presence of $^{55}FeCl_3$-pyoverdine and harvested on membrane filters. Binding of ^{55}Fe was then assessed as described previously (21). Values reported are means of three determinations ± standard deviations and have been corrected for background levels of ^{55}Fe associating with membrane filters in the absence of added outer membranes.

[g] ND, not determined.

pected, K691 harboring wild-type *fpvA* (present on pPVR2) or an *fpvA* insertion derivative which did not adversely affect growth in EDDHA-containing medium (e.g., $FpvA_{R521}$ [pLK21S-1]) was proficient in ferric pyoverdine uptake (Fig. 2).

Given the functional importance of the region(s) of FpvA in the vicinity of residues Y350 and A402, it seemed likely that these regions were somehow involved in ligand (i.e., ferric pyoverdine) binding. To test this, outer membranes were prepared from K691 harboring native FpvA and various insertion derivatives and examined for binding of ferric pyoverdine, as described previously (21). As expected, K691 harboring the native receptor (pPVR2) demonstrated binding of ferric pyoverdine, as did K691 harboring insertion derivative $FpvA_{R521}$ (Table 1). $FpvA_{R521}$-expressing cells did demonstrate less binding of ferric pyoverdine than did cells expressing native FpvA, probably reflecting the decreased production of $FpvA_{R521}$ relative to the pPVR2-encoded native protein (Fig. 1, cf. lanes 3 and 5). In contrast, K691 harboring $FpvA_{A402}$ or $FpvA_{Y350}$ showed minimal ferric pyoverdine binding, comparable to levels observed for K691 carrying vector only (Table 1) or PAO1 cultured under iron-rich conditions (under which conditions FpvA is not induced) (0.64 ± 0.40 pmol of Fe). These data suggest that the region(s) of FpvA neighboring A402 and Y350 is important for ferric pyoverdine binding and may contribute to a binding site. Still, it cannot be ruled out that this region(s)

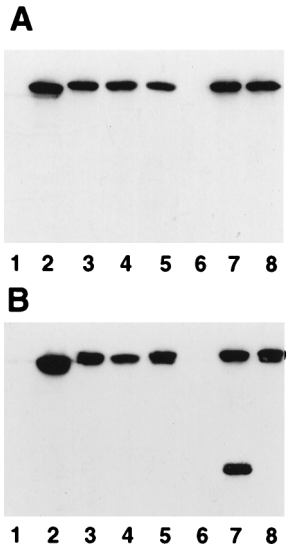

FIG. 1. Western immunoblot of whole-cell extracts (A) and outer membrane proteins (B) of *P. aeruginosa* K691 (lanes 1) and K691 harboring plasmids pPVR2 (lanes 2), pLK127-2 (lanes 3), pLK21S (lanes 4), pLK39S-1 (lanes 5), pLK16-1 (lanes 6), pLK161-3 (lanes 7), and pLK141-1 (lanes 8) probed with an FpvA-specific antiserum. Cells were cultured overnight in iron-deficient minimal medium and used to prepare whole-cell or outer membrane protein extracts which were subsequently electrophoresed on SDS-polyacrylamide gels and immunoblotted. In a typical experiment, 2.5 μl of outer membrane and 10 to 20 μl of whole-cell protein extracts were loaded onto SDS-polyacrylamide gels.

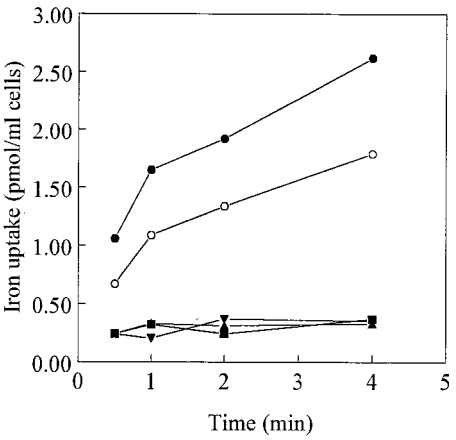

FIG. 2. Pyoverdine-mediated iron uptake by *P. aeruginosa* K691 harboring pAK1900 (■), pPVR2 (●), pLK127-2 (▲), pLK141-1 (▼), and pLK21S-1 (○). The data are representative of three separate experiments carried out in duplicate and are reported for cells at an A_{600} of 1.0.

interacts with the ligand-binding domain(s) of FpvA and its disruption indirectly impacts the ligand-binding site.

Ferric siderophore receptors appear to be gated porins, with the gate region functioning both to control access to the channel and as a ligand-binding site. Indeed, deletion of a single aspartic acid residue (D348) within the *E. coli* FhuA ferrichrome receptor obviates ligand binding (23), and this residue occurs in a region of the receptor whose deletion converts FhuA into a diffusion channel (22). Similarly, deletion of a region of the ferric enterobactin receptor FepA, implicated in ligand binding, also converts the protein into a nonspecific channel, indicating that the binding domain exists as part of a gate region in this receptor as well (26, 36). The identification here of a region of FpvA which is likely extramembranous and possibly involved in ligand binding suggests that the Y350-A402 region of FpvA may comprise part of a gate region for this receptor. Despite repeated attempts to delete this region of FpvA, however, we have failed to express the deletion derivative in *E. coli* or *P. aeruginosa*. Certain deletions of other ferric siderophore receptors appear also to be unobtainable (9). Intriguingly, the insertions at Y350 and A402 occur adjacent to basic amino acids, and arginine residues of the FepA gate region have recently been implicated in ferric siderophore binding (29). We are currently using site-directed mutagenesis to assess the significance of these and other residues near Y350 and A402 in ferric pyoverdine binding.

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