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Tn5 Insertion Mutants of *Pseudomonas aeruginosa* Deficient in Surface Expression of Ferripyochelin-Binding Protein

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Transposon (Tn5) insertion mutants were isolated in *Pseudomonas aeruginosa* PAO. These mutants were screened for expression of the ferripyochelin-binding protein with monoclonal antibody in a whole-cell immunoblot assay. Fourteen mutants were identified which did not express ferripyochelin-binding protein on the cell surface. These mutants did not take up ^{59}Fe-labeled pyochelin and grew slowly in the presence of iron chelators.

Bacteria require iron for the establishment and maintenance of infections (13). Most iron in mammalian hosts is complexed to iron-binding glycoproteins such as transferrin or lactoferrin (21). To successfully compete for iron, bacteria have evolved specific high-affinity mechanisms to acquire iron (11). These iron acquisition systems are composed of specific iron chelators termed siderophores, which are secreted by most microorganisms (11), and outer membrane proteins which act as specific receptors for the iron-siderophore complexes in gram-negative bacteria (12).

Previously, we have identified an outer membrane protein which binds iron in complex with the siderophore pyochelin (17). This ferripyochelin-binding protein (FBP) has a molecular weight of approximately 14,000 and is regulated by the iron concentration of the culture medium (17). This protein has been purified (18), and both rabbit polyclonal sera (19) and mouse monoclonal antibodies (20) which react specifically with FBP have been prepared. Both monoclonal and polyclonal antibodies block the binding of ^{59}Fe-labeled pyochelin to isolated outer membranes of *Pseudomonas aeruginosa* (19, 20). Antibodies to FBP also inhibit ferripyochelin uptake by whole cells of *P. aeruginosa* (19). By using indirect immunofluorescence techniques with monoclonal antibodies, FBP was shown to be exposed on the surface of *P. aeruginosa* grown in low-iron medium (20).

In the present study, transposon (Tn5) mutagenesis was used to isolate mutants of strain PAO which do not express FBP on their surface. The effect of this mutation on ferripyochelin transport was examined. This study provides genetic evidence that FBP is involved in ferripyochelin uptake in *P. aeruginosa*.

P. aeruginosa PAO, originally described by Holloway et al. (8), was used for isolation of mutants. *Escherichia coli* HB101(pUW964) (22), kindly provided by Alison Weiss, University of Virginia School of Medicine, Charlottesville, was used to introduce Tn5 insertions into the chromosome of strain PAO. Bacteriophage E79tv-2 (10) was kindly provided by Allan Godfrey, University of Calgary, Calgary, Alberta, Canada. *E. coli* 1830(pJB4J1) (2) was used to construct a Tn5 probe for hybridization experiments.

Bacteria were grown in Luria broth (10 g of tryptone, 10 g of NaCl, 5 g of yeast extract per liter) for conjugation experiments. Tn5 insertion mutants were selected on M9 agar supplemented with 0.5% glucose and 750 μg of neomycin per ml (Sigma Chemical Co., St. Louis, Mo.). *P. aeruginosa* strains were grown in M9 medium extracted with 8-hydroxyquinoline (15) to assay for expression of FBP and for ferripyochelin transport assays. Assays to measure growth of the mutants were done in the same medium supplemented with either 200 μg of transferrin per ml (Sigma) or 200 μM ethylenediamine-*N*,*N*′-diacetic acid (EDDA). For the detection of pyochelin, cultures were grown in 0.5% Casamino Acids (Difco Laboratories, Detroit, Mich.), deferrated as previously described (16).

The vector pUW964 constructed by Weiss et al. (22) was used to introduce Tn5 insertions into *P. aeruginosa* PAO. This plasmid is able to replicate in *E. coli* but not in *P. aeruginosa*. Therefore, it can be used as a suicide plasmid to deliver Tn5 into *P. aeruginosa*. The conjugal transfer of pUW964 was done by filter matings of PAO and HB101(pUW964). Mid-log-phase cultures were mixed in a 5:1 ratio of donor to recipient and filtered through a 0.45-μm-pore-size filter (Nalgene Labware Div., Nalge/Sybron Corp., Rochester, N.Y.). The filter was transferred to L agar and incubated at 37°C for 3 to 4 h. Bacteria were removed from the filter by vortexing into phosphate-buffered saline, pH 7.2. The bacteria were washed once with phosphate-buffered saline, suspended in phosphate-buffered saline, and refiltered. This filter was placed on M9 agar with 750 μg of neomycin per ml to select for recombinants containing Tn5 insertions. The Nm^r colonies were also scored for trimethoprim resistance to confirm that the plasmid was not maintained. Controls of PAO and HB101(pUW964) alone were processed identically and plated as described above to determine the frequency of spontaneous mutation.

If the bacteria were plated on neomycin agar immediately after incubation of the filter on L agar, 67% of the recombinants were also resistant to trimethoprim, indicating maintenance of the plasmid. Presence of the plasmid was confirmed in Nm^r Tp^r exconjugants by agarose gel electrophoresis of plasmid extracts of these strains. No plasmid was detectable in Nm^r Tp^s isolates (data not shown). When recombinants were selected on neomycin agar overnight and passed in L broth for 18 to 24 h, less than 5% were Tp^r, indicating that stable maintenance or integration of the plasmid rarely occurred.

Mutants not expressing FBP were identified by a lack of reaction with monoclonal antibodies to FBP (MCA-47) in a whole-cell immunoblot assay (20). Colonies which appeared negative in this assay were retested in triplicate for confirmation. Approximately 4,900 neomycin-resistant exconjugants were screened by this method from five independent

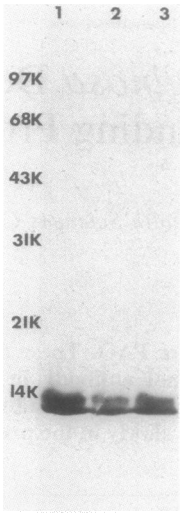

FIG. 1. Immunoblot analysis of outer membrane proteins of FBP mutants. Outer membranes were electrophoresed by 12.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The proteins were electrophoretically transferred to nitrocellulose and reacted with MCA-47. The blots were reacted with protein A-peroxidase and developed with HRP color reagent (Bio-Rad Laboratories, Richmond, Calif.). Molecular weight markers are indicated on the left. Lanes: 1, PAO; 2, FBP-2; 3, FBP-28. K, Kilodaltons.

matings. Fourteen mutants which did not react with monoclonal antibody to FBP were identified.

These mutants were also examined for expression of FBP on the surface by indirect immunofluorescence techniques by using MCA-47 antibodies to FBP (20). Whereas MCA-47 reacts on the surface of PAO cells (20), no fluorescence was observed with any of the mutants (data not shown), indicating that no FBP was present on the surface of these mutants.

Since the whole-cell immunoblot and immunofluorescence assays demonstrated that FBP was not surface exposed in these mutants, outer membranes were isolated from these strains as previously described (17) to determine whether FBP was present in the membrane fraction. Outer membrane preparations were electrophoresed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (9) and stained with Coomassie blue. In all mutants examined, a protein with the same molecular weight as FBP was seen. There were no significant differences in the outer membrane protein profile between any of the mutants and the parent strain (data not shown).

Outer membrane preparations were electrophoresed and transferred to nitrocellulose with a Transblot apparatus (Hoeffer Scientific Instruments, San Francisco, Calif.) for 30 min at 1 A. The nitrocellulose blots were blocked with 3% bovine serum albumin in 10 mM Tris (pH 7.4)–0.9% NaCl (Tris-saline) incubated with MCA-47 (19) ascites fluid, followed by incubation with horseradish peroxidase-conjugated protein A (Sigma). All the mutants had FBP present in the outer membrane which reacted with MCA-47. An immunoblot of FBP-2 and FBP-28 reacted with MCA-47 is shown in Fig. 1. The other mutants gave an identical reaction. These results indicate that FBP is present in the outer membrane of these mutants, although it is not exposed on the surface.

To determine whether the FBP present in these mutants was functional in iron acquisition, ferripyochelin uptake assays were done. Cultures were grown to a density of 10^8 CFU/ml. ^{59}Fe-labeled pyochelin was added to the cultures, and the amount of ^{59}Fe accumulated was determined at 10-min intervals. PAO continued to accumulate ^{59}Fe throughout the 30-min assay (Fig. 2). The mutants FBP-2, FBP-19, and FBP-28 did not accumulate ^{59}Fe-pyochelin during this time (Fig. 2). Also, their initial binding of ^{59}Fe-pyochelin was much lower than that of PAO and not significantly different from that of controls. Similar results were obtained with the other mutants. Therefore, surface exposure of FBP is required for ferripyochelin uptake.

To determine whether the FBP in the membranes of the mutant strains was capable of binding ferripyochelin, ^{59}Fe-labeled pyochelin binding to outer membranes isolated from these strains was examined. When 100 μg of outer membrane protein was reacted with ^{59}Fe-labeled pyochelin (17), there was no difference between the amount of ^{59}Fe bound by PAO and the amount bound by the mutant strains tested. When outer membrane preparations isolated from these mutants were incubated with ^{59}Fe-labeled pyochelin, electrophoresed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and subjected to autoradiography (16), the ^{59}Fe bound specifically to FBP in mutants FBP-2, FBP-4, and FBP-28. The reaction was identical to that of the PAO control (data not shown). These data indicate that the Tn*5* insertions in these mutants have no apparent effect on the function of this protein in binding ferripyochelin.

Since Tn*5* insertions may cause polar mutations which would affect the synthesis of other gene products (1), the amount of pyochelin produced by the mutants was compared with that produced by the parent strain. Pyochelin was purified from cultures grown in 50 ml of Casamino Acids medium as previously described (4, 16). Fluorescence was measured at 440 nm while the sample was being excited at 350 nm in a Spectro-glo fluorometer (Gilson, Middleton, Wis.). The pyochelin concentration was determined from fluorescence values obtained from a standard curve constructed by using known amounts of pyochelin. PAO produced 5.12 μg of pyochelin per ml in Casamino Acids medium. All of the mutants produced parental levels of pyochelin, which ranged from 4.8 to 5.16 μg/ml, with a mean of 5.05 μg/ml. Therefore, the mutation affecting expression of FBP does not affect production of pyochelin.

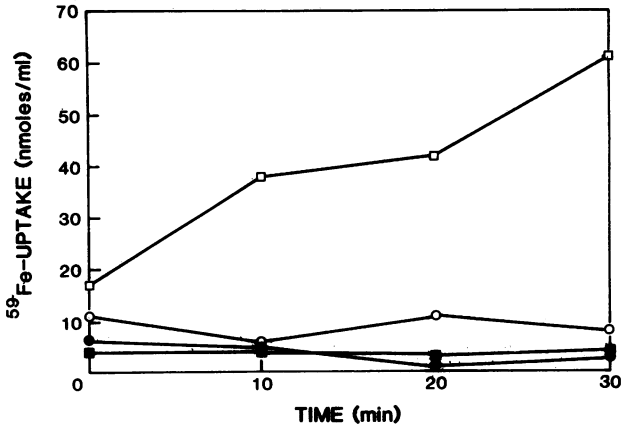

FIG. 2. Ferripyochelin uptake by FBP mutants. Uptake was initiated by the addition of ^{59}Fe-labeled pyochelin. Samples (1 ml) were removed at indicated intervals and filtered. The amount of iron accumulated was calculated from a standard curve. Symbols: □, PAO; ○, FBP-2; ●, FBP-19; ■, FBP-28. These experiments were done three times with comparable results.

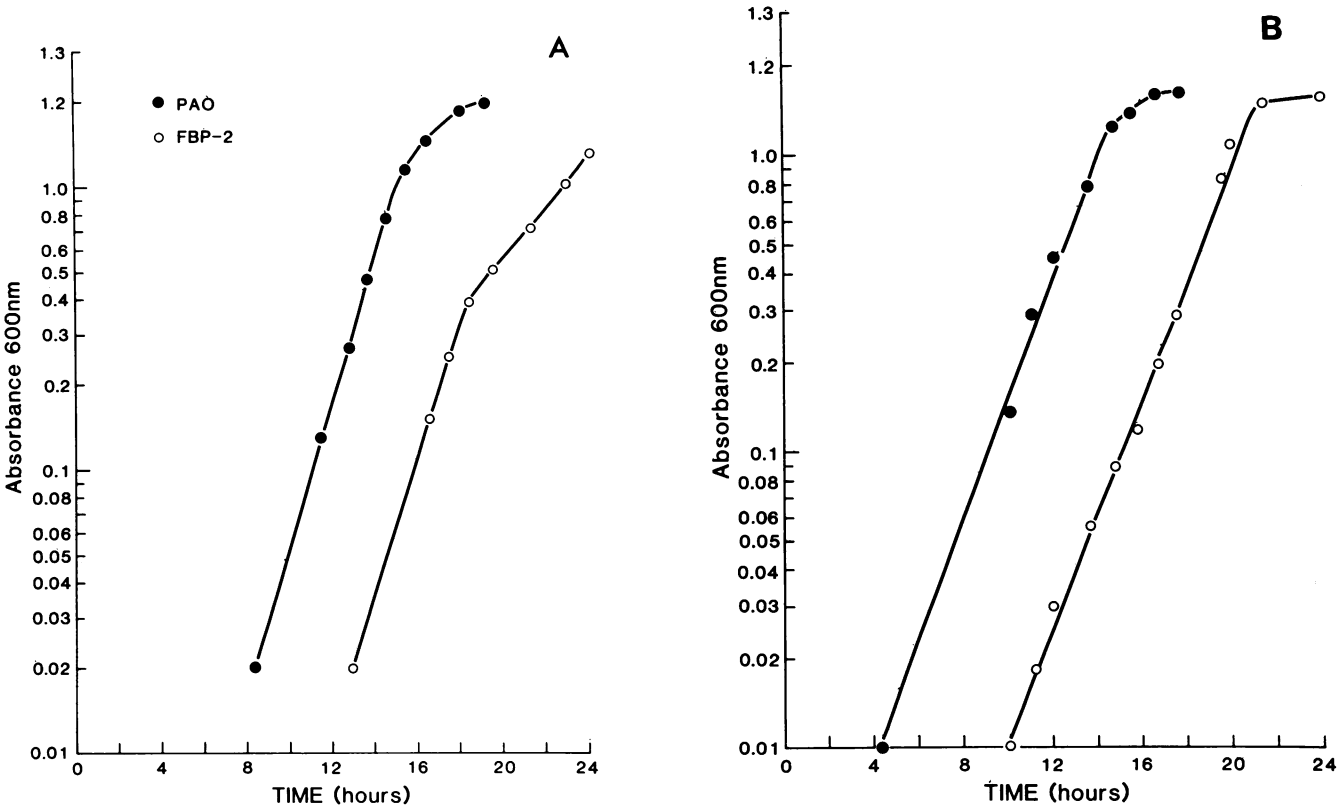

FIG. 3. Comparison of growth of PAO and FBP-2 in the presence of iron chelators. Cultures were started at a density of 10^5 CFU/ml in M9 medium with (A) 100 μg of transferrin per ml or (B) 200 μM EDDA. Growth was determined by measuring the A_{600} at selected intervals. These values represent the means of two experiments.

To determine the specificity of these mutations for iron uptake, the ability of the mutants FBP-2, FBP-8, FBP-15, and FBP-16 to transport [^{14}C]lysine and [^{14}C]glucose was also examined. Cultures were grown to log phase in M9 glucose medium. For glucose uptake experiments, the glucose concentration in this medium was reduced to 0.05%. [^{14}C]lysine (2 μCi; 324 mCi/mmol) or [^{14}C]glucose (10 μCi; 358 mCi/mmol) was added, and 1-ml samples were removed at selected intervals, filtered through 0.45-μm-pore-size filters (Millipore Corp., Bedford, Mass.), and washed extensively. The filters were dissolved in Scintiverse (Fisher Scientific Co., Pittsburgh, Pa.), and the amount of radioactivity was determined in a Beckman LS 6800 scintillation counter (Beckman Instruments, Inc., Fullerton, Calif.). There was no significant difference in the amount of either [^{14}C]glucose or [^{14}C]lysine accumulated in the mutants compared with the parental strain. Therefore, this mutation appears to be specific for iron transport and not a general transport defect.

The effect of the FBP mutation on growth in the presence of iron chelators was examined. PAO and FBP-2 were grown overnight in M9 medium with 200 μM EDDA. The cultures were adjusted to the same optical density (A_{600} = 0.2) and diluted to a concentration of 10^5 CFU/ml in the same medium. Growth was determined by measuring the A_{600} (Fig. 3A). FBP-2 grew in the presence of EDDA, but the lag phase was approximately 6 h longer than that of PAO. Once FBP-2 entered log phase, the growth rate was approximately the same as that of PAO in medium supplemented with EDDA (Fig. 3A). Similar results were obtained with mutants FBP-4, FBP-21, and FBP-28.

When FBP-2 and PAO were grown in medium supplemented with transferrin, similar results were observed (Fig. 3B). The cultures were grown overnight in M9 with 20 mM $NaHCO_3$, 0.5% glucose, and 100 μg of transferrin per ml and diluted to an initial concentration of 10^5 CFU/ml in the same medium. FBP-2 grew in the presence of transferrin, but the lag phase was approximately 5 h longer than that of the parent strain. But, as observed with EDDA in the medium, once FBP entered log phase, the rate of growth was the same as that of PAO (Fig. 3B).

There are several possible explanations for the difference in lag phase between the mutants and the parent strain. Ferripyochelin uptake may be more important in the initial stages of growth, or the longer lag may simply reflect time needed to switch to an alternate mechanism for iron acquisition. A third explanation is that since FBP is not exposed on the surface of these mutants, internalization of ^{59}Fe-labeled pyochelin may occur more slowly.

To determine whether the FBP mutation was cotransducible with neomycin resistance, the transducing phage E79tv-2 (10) was used to transfer neomycin resistance from this mutant to the parental strain. An E79tv-2 lysate of FBP-2 was prepared by the soft agar method. A 1:10 dilution (10^9 CFU/ml) of an overnight culture of PAO was centrifuged and suspended in 0.01 M Tris (pH 7.4)–0.15 M NaCl–0.01 M $MgSO_4$ (TMN buffer). The culture was mixed with E79tv-2 at a multiplicity of infection of 1. The mixture was incubated for 15 min at 37°C and centrifuged to remove unadsorbed phage. The culture was suspended in TMN buffer and plated on L agar plus 750 mg of neomycin per ml. Transductants were restreaked on selective media and as-

sayed for FBP expression in the whole-cell immunoblot assay. The FBP mutation was cotransducible with neomycin resistance at a frequency of 50%. The ability of six FBP-2 transductants to take up ^{59}Fe-labeled pyochelin was examined. All were unable to accumulate ^{59}Fe in a complex with pyochelin (data not shown). Therefore, the mutation resulting in loss of FBP expression on the cell surface was cotransducible with the inability to take up ferripyochelin.

The location of Tn*5* insertions in seven mutants was determined by using a ^{32}P-labeled DNA probe specific for Tn*5* to hybridize to chromosomal DNA restriction fragments. Chromosomal DNA from PAO, FBP-2, FBP-4, FBP-6, FBP-8, FBP-15, FBP-16, and FBP-28 was isolated by the method of Clarke and Carbon (3). Plasmids pJB4J1 and pUW964 were isolated by the procedure of Hansen and Olson (7). Restriction enzymes were used according to the recommendations of the manufacturers (GIBCO Laboratories, Grand Island, N.Y.; Bethesda Research Laboratories, Inc., Gaithersburg, Md.). Chromosomal DNA was digested with *Eco*RI and electrophoresed on a 0.7% agarose gel in 0.89 M Tris-borate–0.02 M EDTA. *Eco*RI was chosen because no restriction sites for *Eco*RI are contained in Tn*5*. The DNA fragments were electrophoretically transferred to Gene Screen Plus (New England Nuclear Corp., Boston, Mass.) by using a Hoeffer Transblot apparatus.

The Tn*5*-specific DNA probe was prepared from the internal *Hin*dIII fragment of Tn*5*. The 3.3-kilobase (kb) *Hin*dIII fragment of pJB4J1 was electroeluted from an agarose gel as described by Perbal (14). This fragment was nick translated by using a Bethesda Research Laboratories Nick Translation Kit according to the recommendations of the manufacturer. Hybridization was done at 65°C with Gene Screen Plus according to the recommendations of the manufacturer. Digests of pJB4J1 and pUW964 were run as controls.

The Tn*5* probe hybridized to a single 6.6-kb *Eco*RI fragment in FBP-2 and FBP-4. This probe hybridized to a 6.1-kb fragment in FBP-15, FBP-16, and FBP-28 and to an 8.8-kb fragment in FBP-8. Two Tn*5* insertions were found in the FBP-6 mutant: one in a 6.1-kb fragment identical to FBP-2 and FBP-4 and one in an 11.5-kb fragment. These data suggest that there are at least three sites where Tn*5* may insert, resulting in mutations with the same phenotype. The remaining seven mutants were not characterized because they were additional siblings from the same matings as these mutants. Presumably, the insertion in the 11.5-kb fragment of FBP-6 has nothing to do with FBP expression since this mutant has the same phenotypic properties as FBP-2 and FBP-4. The Tn*5* insertions in these mutants presumably affect processing or secretion of FBP to the cell surface since the protein present in isolated membrane fractions is functional in binding ferripyochelin.

It is interesting that of approximately 5,000 recombinants screened from five independent matings, no mutants were identified which were completely negative in FBP expression. All had the same phenotype, i.e., expression of FBP in the membrane but no surface exposure. There are at least two possible explanations for the inability to isolate FBP-negative mutants. It is possible that Tn*5* does not insert in the FBP structural gene or that a complete loss of FBP results in a lethal mutation. It seems unlikely that the loss of FBP would be lethal, since *P. aeruginosa* may also acquire iron with pyoverdin (6). Further, pyochelin-negative mutants are able to grow and acquire iron (5).

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