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The Pyocin Sa Receptor of *Pseudomonas aeruginosa* Is Associated with Ferripyoverdin Uptake

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We have used Tn*5* mutagenesis to obtain a mutant resistant to pyocin Sa. When grown in iron-deficient succinate medium this mutant lacked an 85-kDa iron-regulated outer membrane protein (IROMP), and expression of a 75-kDa IROMP was increased compared with that in the parent strain. The mutant was deficient in pyoverdin biosynthesis and showed a 95% decrease in transport of ferripyoverdin purified from the parent strain, suggesting that the 85-kDa IROMP is the specific receptor for ferripyoverdin and pyocin Sa. The mutant compensated for the deficiency in pyoverdin biosynthesis and transport by exhibiting a fourfold increase in ferripyochelin transport. The low-level transport of ferripyoverdin in the Sa-resistant mutant, which extended to heterologous pyoverdins from other strains, suggests that *Pseudomonas aeruginosa* has a second ferripyoverdin uptake system of lower affinity and broader specificity.

The expression of a high-affinity iron uptake system is an important component in the adaptation of cells to growth conditions encountered in vivo (11). The iron-binding glycoproteins lactoferrin and transferrin ensure that the level of extracellular iron in the body available to invading organisms is far below their growth requirements (2). To overcome this nutrient deprivation, some bacteria, including *Pseudomonas aeruginosa*, derepress an iron uptake system based on low-molecular-weight iron-chelating compounds (siderophores), which compete with transferrin and lactoferrin for host iron and deliver it to the cell via interaction with an iron-regulated outer membrane protein (IROMP) receptor (11). *P. aeruginosa* produces two siderophores, pyochelin and pyoverdin. Pyochelin is produced by all strains of *P. aeruginosa* (5), and two uptake systems, involving a 14-kDa ferripyochelin binding protein in the outer membrane (OM) (22) and a 75-kDa IROMP (12), have been identified.

Studies of ferripyoverdin uptake have been complicated by the marked heterogeneity of this system. Strains of *P. aeruginosa* produce several different pyoverdins, each having the characteristic yellow-green fluorescent 2,3-diamino-6,7-dihydroxyquinoline chromophore attached to a peptide backbone varying from 6 to 10 amino acid residues (6). Three pyoverdin-mediated iron-uptake groups have been identified from amongst a series of type strains and clinical isolates, on the bases of growth promotion studies, labelled uptake studies, and amino acid composition of purified pyoverdins (3). Antibodies raised against an 80-kDa IROMP from two of the groups reacted only against strains producing the same pyoverdin group (3), and several reports have indicated heterogeneity in molecular weight and immunological cross-reactivity of ferripyoverdin receptors from different strains (16, 18, 20). These data point to significant variation in ferripyoverdin IROMP structure which is sufficient to discriminate between pyoverdins having only subtle structural differences.

In an attempt to gain further insight into the regulation and structure of the ferripyoverdin uptake system, we have reinvestigated the receptors for the colicinlike S pyocins. Three classes of pyocin, R, F, and S, have been identified on the bases of structural and chemical properties (9, 13, 19). Phage-tail-like R and F pyocins are produced by more than 90% of *P. aeruginosa* strains, and colicinlike, trypsin-sensitive S pyocins are produced by approximately 70% of isolates (8). Susceptibility to one or more classes of pyocin occurs in 100% of isolates.

The S-type pyocins are of particular interest because their lethal activity appears to be mediated via high-molecular-weight IROMPs. Iron-rich conditions decreased the absorption and lethality of pyocin S2, whereas under iron-deficient conditions, susceptibility was significantly enhanced and accompanied by the appearance of an OM protein which appeared to act as the S2 receptor (17). Pyocin S2-resistant mutants lacking a minor IROMP have been described, but competition data suggested that this IROMP did not have a role in iron uptake (17). Govan (10) identified a new pyocin Sa, produced by *P. aeruginosa* J1003, by screening for activity against a strain resistant to pyocins S1 and S2. A further survey of 1,000 strains with a mutant resistant to S1, S2, and Sa failed to demonstrate additional S pyocin receptors in this strain.

In order to characterize the Sa receptor, we screened our laboratory culture collection for other susceptible strains. Only one clinical isolate, 0:9, from more than 20 laboratory and clinical strains tested was sensitive, and Tn*5* mutagenesis (21) was used to obtain a resistant mutant. Pooled neomycin-resistant exconjugants were plated in soft agar over a surface culture of J1003 grown for 6 h at 30°C and inactivated by exposure to chloroform vapor for 15 min. After overnight incubation at 37°C, approximately 100 exconjugants were picked from the center of the zone of inhibition, but on restreaking against the producer strain, only one mutant, designated PH1, showed no growth inhibition. When discs saturated with ferripyoverdin were placed on the soft agar overlay containing the sensitive strain 0:9, distinct zones of growth were detected, indicating that ferripyoverdin could inhibit killing by pyocin Sa. Outer

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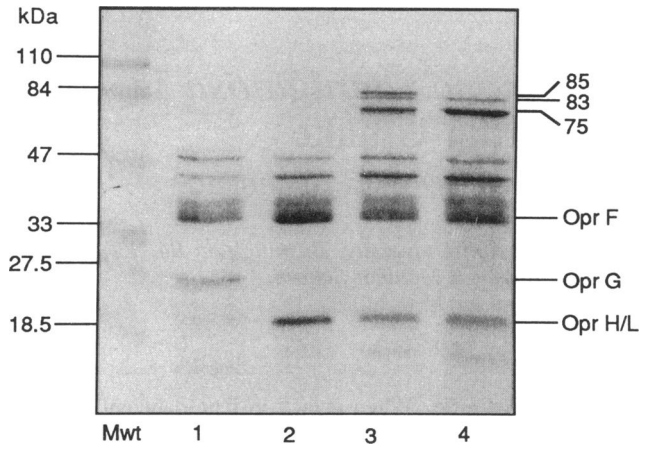

FIG. 1. SDS-polyacrylamide gel electrophoretogram of outer membranes prepared from strains of *P. aeruginosa* grown in iron-deficient succinate medium with (lanes 1 and 2) or without (lanes 3 and 4) 50 μM $FeSO_4$. Lanes 1 and 3, 0:9; lanes 2 and 4, pyocin Sa-resistant mutant PH1.

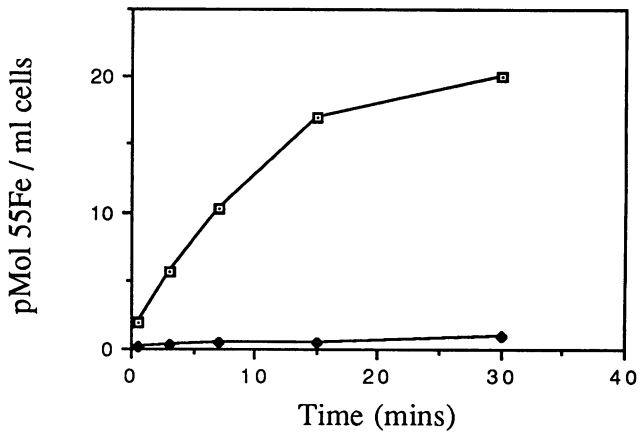

FIG. 2. Pyoverdin-mediated iron ($^{55}Fe^{3+}$) transport by *P. aeruginosa* 0:9 (⊡) and PH1 (◆) grown in iron-deficient succinate medium. The uptake mixture contained pyoverdin (80 μg/ml), $^{55}FeCl_3$ (115 nM), and 1 ml of cells at an optical density at 470 nm of 1.0. Data are representative from four experiments.

membranes were prepared by the Sarkosyl solubilization method (7) and analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (14). OM protein profiles from 15-h cultures of PH1 and 0:9, grown in succinate minimal medium (15) at 37°C are shown in Fig. 1. After growth without added iron, three major IROMPs of 75, 83, and 85 kDa were induced in the OM. The Sa-resistant mutant PH1 specifically lacked an 85-kDa IROMP (lane 4), and expression of the 75-kDa IROMP was increased compared with that in 0:9. Growth in succinate medium with added iron confirmed that these proteins are iron repressible (lanes 1 and 2). The 25-kDa OM protein G (OprG) expressed by 0:9 under iron-rich growth conditions (lane 1) was partially repressed in PH1 (lane 2) by approximately 50%, as determined by densitometry. A 10-fold decrease in the pyoverdin fluorescence peak for PH1 culture supernatants was noted (not shown), indicating that a component of a pyoverdin siderophore biosynthesis-uptake operon was inactivated. In addition, PH1 was unable to grow in succinate medium supplemented with the nonutilizable chelator ethylene diamine di(*o*-hydroxyphenyl) acetic acid (400 μM; EDDHA), indicating perturbed pyoverdin production or transport.

Pyoverdin was purified from 0:9 culture supernatants, and uptake studies were performed as described by Poole et al. (18). Uptake profiles for 15-h stationary-phase cells of 0:9 and PH1 grown in iron-deficient succinate medium are shown in Fig. 2. By using a 1:50 molar ratio of ^{55}Fe to pyoverdin, the uptake rate of PH1 was 0.03 pmol of Fe per min per ml of cells compared with 0.57 pmol of Fe per min per ml of cells in 0:9, a reduction of 95%. Increasing the molar ratio of iron to pyoverdin did not affect uptake.

Ferripyochelin transport assays were undertaken to determine if the increased expression of the 75-kDa IROMP was associated with elevated transport incurred by using this siderophore, since pyochelin binding and transport have been associated with a 75-kDa IROMP in late-logarithmic or early-stationary phase cells (12). Pyochelin was purified from PH1 culture supernatants as described by Heinrichs et al. (12), and uptake assays were performed with 15-h stationary-phase cells grown in succinate medium as described by Cox (4). The initial rate of ferripyochelin uptake in PH1 was 0.4 pmol of Fe per min per ml of cells compared with 0.1 pmol of Fe per min per ml for 0:9 (Fig. 3).

In this work we have isolated a mutant resistant to *P. aeruginosa* pyocin Sa which lacks an 85-kDa IROMP and shows greatly reduced ferripyoverdin uptake. The inhibition of pyocin Sa killing by competition with ferripyoverdin strongly suggests that the 85-kDa IROMP is both the pyocin Sa receptor and a ferripyoverdin transporter. Indeed, this may be the same transporter as the 90-kDa IROMP identified by Poole et al. (18), although it remains to be determined whether this strain produces a pyoverdin molecule from the same uptake group (3) and is sensitive to pyocin Sa. Using a mutant deficient in the 90-kDa IROMP, these workers also provided evidence for a second uptake system (18). The residual uptake noted in this study also suggests a second transporter, although it was not sufficient to permit growth in the presence of EDDHA. Evidence for a second uptake system has also been found with the related fluorescent pseudomonad *P. putida* (1). The *pupA* gene coding for the

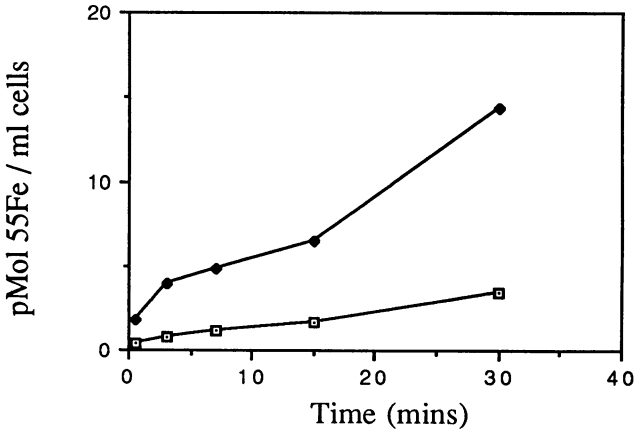

FIG. 3. Pyochelin-mediated iron ($^{55}Fe^{3+}$) transport by *P. aeruginosa* 0:9 (⊡) and PH1 (◆) grown in iron-deficient succinate medium. The uptake mixture contained pyochelin (60 μM), $^{55}FeCl_3$ (115 nM), and 1 ml of cells at an optical density at 470 nm of 1.0. Data are representative from three experiments.

85-kDa pseudobactin 358 receptor was identified, and *pupA* mutants were obtained by marker exchange. These mutants still showed 30% uptake, whilst uptake of other pseudobactins was not affected. In this present study the Sa mutant showed only 5% uptake of parental siderophore. Uptake experiments with chromatographically distinct pyoverdins from other strains all showed low uptake of approximately 5% both in PH1 and in the parent strain 0:9 (data not shown). It therefore seems likely that the 85-kDa IROMP is a specific high-affinity ferripyoverdin receptor, whereas a second receptor, which has low affinity for a wider range of pyoverdins, exists.

The ferripyochelin transport assays described in this work support the finding that the 75-kDa IROMP is a second pyochelin transporter (12) and suggest that PH1 compensates for the deficiency in pyoverdin biosynthesis and transport by derepressing further the pyochelin-based system. This effect seems to be restricted to an increased ability to transport the siderophore complex, rather than to produce siderophore, since we did not detect any differences in pyochelin production between PH1 and 0:9. Similarly, with the growth conditions used in this study, the increase in ferripyochelin transport correlated with the 75-kDa IROMP transporter rather than the well-characterized 14-kDa ferripyochelin binding protein (22).

The perturbations in expression of OM proteins in PH1 were not seen only after growth in iron-depleted media. When grown in iron-rich conditions, which represses synthesis of IROMPs, expression of OprG in PH1 was reduced by approximately 50%. Whilst the role of OprG remains unclear, its expression has been shown to increase when the level of available iron is similarly increased (24), and we are now reviewing its role in iron uptake.

In summary, our data indicate that the site of interaction of pyocin Sa is a ferripyoverdin receptor and provides evidence for a second low-affinity pyoverdin uptake system. Whilst the deficiency in pyoverdin transport and synthesis could be attributed to downstream effects exerted by Tn*5* insertion into an operon (23) and the increased ability to transport ferripyochelin could be a response to the increased iron deficiency imposed by the inability to transport ferripyoverdin, the altered expression of OprG under iron-rich conditions, where pyoverdin-mediated iron transport is not thought to operate, was surprising. Moreover, the observation that the receptor for this pyocin is a critical component of the iron uptake system and yet few strains are susceptible to it lends further credence to the notion that pyoverdin biosynthesis and uptake are highly heterogeneous amongst *P. aeruginosa* strains. Sequence analysis will be required to determine the extent of the similarities of the systems. We are now cloning the site of the transposon insertion to determine whether a pyoverdin synthesis-uptake operon has been inactivated or if a larger iron-regulated operon has been identified.

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