FoxB of *Pseudomonas aeruginosa* Functions in the Utilization of the Xenosiderophores Ferrichrome, Ferrioxamine B, and Schizokinen: Evidence for Transport Redundancy at the Inner Membrane

Páraic Ó Cuív, Damien Keogh, Paul Clarke, and Michael O’Connell*

School of Biotechnology, Dublin City University, Glasnevin, Dublin 9, Ireland

Received 28 July 2006/Accepted 11 October 2006

Expression of the inner membrane protein FoxB (PA2465) of *Pseudomonas aeruginosa* in mutants of *Sinorhizobium meliloti* that are defective in the utilization of ferrichrome, ferrioxamine B, and schizokinen resulted in the restoration of siderophore utilization. Mutagenesis of foxB in *P. aeruginosa* did not abolish siderophore utilization, suggesting that the function is redundant.

Virtually all organisms display an absolute requirement for iron. Although iron is the fourth most abundant element on earth, it is rapidly oxidized and at neutral pH is virtually insoluble. Pathogens encounter conditions of iron limitation within infected tissues due to the action of iron binding proteins such as transferrin and lactoferrin that serve to prevent an infection from developing (9, 24, 25). To overcome these conditions of iron limitation, many bacteria synthesize high-affinity chelators termed siderophores that serve to deliver iron to the cell.

*Pseudomonas aeruginosa* occupies a diverse range of ecological niches, including soil and water habitats, and is a versatile opportunistic pathogen capable of infecting a range of eukaryotic species including animals, insects, and plants. Under iron-limiting conditions, *P. aeruginosa* produces two siderophores, pyoverdine and pyochelin, that have been shown to contribute to the virulence of the organism (8, 26). *P. aeruginosa* is predicted to encode up to 34 putative TonB-dependent receptors, many of whose ligands have not yet been characterized (7). Predictably, the organism has been shown to be capable of utilizing a wide range of xenosiderophores, including ferrichrome (17, 22), ferrioxamine B (3, 17, 19, 22), rhizobactin 1021, schizokinen, and aerobactin (16, 22). The number of siderophore receptors may explain the phenomenon of transport redundancy with multiple receptors capable of recognizing and transporting a particular siderophore. In contrast, there are few readily identifiable inner membrane siderophore transport systems relative to the predicted number of receptors (13, 21, 23). We report here the identification of the protein FoxB that functions in the utilization of ferrichrome, ferrioxamine B, and schizokinen across the inner membrane by a redundant mechanism.

Recently, the outer membrane receptors for ferrioxamine B and ferrichrome, FoxA (PA2466) and FiuA (PA4070), respectively, were identified (3, 17). In silico analysis, a putative transporter and a protein of unknown function, encoded by foxB (PA2465) and PA2464, respectively, which are located directly downstream of foxA, were identified. To investigate the possible role of FoxB and PA2464 in ferrioxamine B utilization, both genes were deleted and replaced with a kanamycin resistance cassette by allelic replacement in the pyoverdine and pyochelin biosynthesis mutant *P. aeruginosa* IA1 (2). Growth promotion assays of the resultant mutant *P. aeruginosa* PA2464-65km indicated that growth was indistinguishable from that of the parent strain when bacteria were grown under iron-limiting conditions in the presence of ferrioxamine B (Fig. 1). Analysis by the siderophore utilization bioassay confirmed that *P. aeruginosa* PA2464-65km was unaffected in the utilization of ferrioxamine B and that the utilization of ferrichrome, rhizobactin 1021, schizokinen, and aerobactin was similarly unaffected compared to that by *P. aeruginosa* IA1 (Table 1).

Previously we identified a novel protein, RhtX, functioning in the utilization of rhizobactin 1021 and schizokinen by *Sinorhizobium meliloti* 2011 (18, 21). To investigate the possible role of PA2464 and FoxB in rhizobactin 1021 and schizokinen utilization, a mutant was constructed by the insertion of an *Ω*-chloramphenicol resistance cassette in *P. aeruginosa*. Siderophore bioassay analysis indicated that *P. aeruginosa* strain SMc01511 is capable of utilizing both ferrichrome and ferrioxamine B to satisfy its iron requirements (20, 21). We have determined that the utilization of ferrichrome and ferrioxamine B across the inner membrane of *S. meliloti* 2011 is mediated by an ABC transport system comprising the periplasmic binding protein Sme01659, the inner membrane permease Sme01511 (*hmuU*), and the ATPase Sme01510 (*hmuV*) (P. Ó Cuív and M. O’Connell, unpublished data). Mutants with insertions in sme01659, sme01510, and sme01511, generated in...
an *S. meliloti* 2011*rhtX*-3 background, are defective in ferrichrome and ferrioxamine B utilization. The mutants were complemented, however, by pOC2465, further suggesting that FoxB functions in siderophore utilization (Table 1). It was noticeable that ferrichrome utilization was weaker than that of ferrioxamine B compared to utilization by *S. meliloti* 2011*rhtX*-3, suggesting that FoxB possibly has a greater specificity for the latter siderophore (data not shown).

In *Escherichia coli*, the utilization of hydroxamate siderophores across the inner membrane is dependent on the FhuCDB transport system. The ability of PA2464 and foxB to complement a *fluB* mutant, *E. coli* B1713, was examined. *E. coli* B1713 is a derivative of *E. coli* K-12 that is defective in enterobactin biosynthesis and that carries a λlacM insert in *fluB*. Neither PA2464 nor FoxB, individually or in combination, conferred upon *E. coli* B1713 the ability to utilize ferrichrome (Table 1). *E. coli* K-12 does not encode high-affinity outer membrane receptors for ferrioxamine B or rhizobactin 1021, schizokinen, and aerobactin, and there is no observable utilization of these siderophores as determined by siderophore bioassay analysis. Consequently, to further analyze the utilization of these siderophores the ferrioxamine B outer membrane receptor *foxA* from *Yersinia enterocolitica* (4) and the rhizobactin 1021, schizokinen, and aerobactin outer membrane receptor *iutA* from the *E. coli* virulence plasmid pColV-K311 (10, 21) were expressed in *trans*. The introduction of pOC2464, pOC2465, and pOC2464-65 did not confer upon *E. coli* B1713 expressing *foxA* the ability to utilize ferrioxamine B (data not shown). Similarly, the introduction of pOC2464, pOC2465, and pOC2464-65 did not confer upon *E. coli* B1713 expressing *iutA* the ability to utilize rhizobactin 1021, schizokinen, or aerobactin (data not shown).

FoxB is predicted to be 382 amino acids in length with a molecular mass of 43 kDa. The protein is predicted to be localized to the inner membrane, and the ability of FoxB to facilitate siderophore utilization in *S. meliloti* 2011 inner membrane siderophore transport mutants supports this prediction. FoxB is predicted to have eight transmembrane segments as determined by TMPred analysis (www.ch.embnet.org/software/TMPRED_form.html). A PepSY_TM transmembrane helix (PF03929) was identified in the FoxB amino acid sequence by using the Pfam database (http://www.sanger.ac.uk/Software/Pfam/). The PepSY_TM transmembrane helices are commonly found bounding a PepSY domain and serve to hold the domain to the exterior of the cell (27). The FoxB sequence was used to identify corresponding protein homologues in the GenBank database by using the BLASTP program (1). The highest percentages of identity to FoxB were those of PaerC_01001425 from *P. aeruginosa* C3719 (99%), Paer03000003 from *P. aeruginosa* UCBPP-PA14 (99%), PaerP_01005749 from *P. aeruginosa*

![FIG. 1. Growth promotion analysis of *P. aeruginosa* IA1 and PA2464-65km in response to iron limitation and ferrioxamine B. Cultures were supplemented with 2,2′-dipyridyl (500 μM) and ferrioxamine B (FB; 1 μM), as appropriate. OD600, optical density at 600 nm.](http://jb.asm.org/Downloaded from http://jb.asm.org/ on October 14, 2015 by University of Queensland Library)

**TABLE 1. Analysis of the role of FoxB in siderophore utilization**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Plasmid</th>
<th>Ferrichrome</th>
<th>Ferrioxamine B</th>
<th>Schizokinen</th>
<th>Rhizobactin 1021</th>
<th>Aerobactin</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. aeruginosa</em> IA1</td>
<td>pvd pch</td>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>P. aeruginosa</em> PA2464-65km</td>
<td>pvd pch PA2464 foxB</td>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>S. meliloti</em> 2011</td>
<td>Wild type</td>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>NT&lt;sup&gt;a&lt;/sup&gt;</td>
<td>+</td>
</tr>
<tr>
<td><em>S. meliloti</em> 2011<em>rhtX</em>-3</td>
<td>rhtX</td>
<td>pOC2465</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>NT&lt;sup&gt;a&lt;/sup&gt;</td>
<td>+</td>
</tr>
<tr>
<td><em>S. meliloti</em> 2011<em>rhtX</em>-3</td>
<td>rhtX</td>
<td>pOC2465</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>S. meliloti</em> 2011<em>rhtX</em>-3</td>
<td>rhtX</td>
<td>pOC2465</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>S. meliloti</em> 2011<em>rhtX</em>-3</td>
<td>rhtX</td>
<td>pOC2465</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>S. meliloti</em> 2011<em>rhtX</em>-3</td>
<td>rhtX</td>
<td>pOC2465</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>S. meliloti</em> 2011<em>rhtX</em>-3</td>
<td>rhtX</td>
<td>pOC2465</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>S. meliloti</em> 2011<em>rhtX</em>-3</td>
<td>rhtX</td>
<td>pOC2465</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

<sup>a</sup> NT, not tested.
PA7 (98%), Pfl_0120 from Pseudomonas fluorescens PToF-1 (67%), and PFL_0124 from P. fluorescens Pf-5 (66%). In all instances, the FoxB homologues are encoded in propinquity to putative siderophore outer membrane receptors. Recently, Benson et al. (5) reported the discovery of FegB, a novel transporter from Bradyrhizobium japonicum that functions in ferrichrome utilization. Although FegB displays limited identity to FoxB (19%), in silico analysis suggests that it also possesses eight transmembrane segments and a PepSY_TM helix (5; Ó Cuí and O’Connell, unpublished data).

Our results suggest that transport by P. aeruginosa of ferrichrome, ferrioxamine B, and schizokinen across the inner membrane is by a redundant mechanism. Inner membrane redundancy possibly explains the difficulty in identifying inner membrane transport systems in P. aeruginosa by traditional techniques such as transposon mutagenesis. In addition, despite overlapping ligand specificities, FoxB, FegB, and RhtX display limited identity to each other, suggesting that siderophore transporters may exhibit significant sequence diversity but possess common ligand specificities. Thus, it may be difficult to identify unusual siderophore transport proteins and to predict their ligand specificities by in silico analysis alone. A possible mechanism for the identification of single-unit inner membrane transport systems is by high-throughput complementation of a heterologous host(s). It would be relatively straightforward to construct a broad-host-range mobilizable expression vector that could be used to identify novel siderophore transporters by complementation of a heterologous host(s).

The virulence of P. aeruginosa has been found to correlate to its ability to acquire iron, and consequently siderophores have attracted attention as a means of delivering antimicrobials by a “Trojan horse” mechanism, thereby overcoming antibiotic efflux systems (6, 11, 12, 15). The discovery that inner and outer membrane transport of ferrioxamine B and ferrichrome is redundant is particularly interesting as it suggests that it would be difficult for a mutation to arise that would abolish utilization. This makes both siderophores attractive as scaffolds for the generation of siderophore-antibiotic conjugates. Mutations affecting siderophore uptake would likely be in a gene(s) encoding a component common to all siderophore transport systems such as the tonB gene and would likely result in a weakened strain because of pleiotropic effects.

Little is known about inner membrane siderophore transport in P. aeruginosa, and the identification of FoxB further augments our knowledge of this process. To date, the only characterized inner membrane siderophore transport protein identified in P. aeruginosa is FptX, which functions in pyochelin utilization (21). FoxB is the first characterized member of a novel family of proteins that functions in siderophore utilization and only the second inner membrane siderophore transporter to be identified in P. aeruginosa. The mechanism of function of the FoxB family remains to be determined. However, the inability of FoxB to mediate siderophore utilization in E. coli suggests a possible requirement for an additional transport protein(s) or a limited ability to respond to chemiosmotic transport gradients, and this remains to be elucidated. It also remains to be determined whether FoxB functions in the transport of or in the release of iron from the siderophores. From in silico analysis, several additional uncharacterized transporters can be readily identified that are encoded in the immediate vicinity of predicted outer membrane siderophore receptors in P. aeruginosa. It will be of interest to determine if these transporters function in siderophore utilization and if they represent the general mechanism for inner membrane siderophore transport by P. aeruginosa.

This publication has emanated from research conducted with the financial support of the Science Foundation Ireland and Enterprise Ireland.

We thank colleagues who sent strains and plasmids.

REFERENCES

22. Ó Cuí, P., P. Clarke, and M. O’Connell. 2006. Identification and charac-


