FepA with Globular Domain Deletions Lacks Activity

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TonB-gated transporters have β-barrels containing an amino-terminal globular domain that occludes the interior of the barrel. Mutations in the globular domain prevent transport of ligands across the outer membrane. Surprisingly, FepA with deletions of the globular domain (amino acids 3 to 150 and 17 to 150) was previously reported to retain significant sensitivity to colicins B and D and to use ferric enterochelin, all in a TonB-dependent fashion. To further understand TonB interaction with the β-barrel, in the present study, proteins with deletions of amino acids 1 to 152, 7 to 152, 20 to 152, and 17 to 150 in fepA were constructed and expressed in a ΔfepA strain. In contrast to previous studies of fepA globular domain deletions, constructs in this study did not retain sensitivity to colicin B and conferred only marginal sensitivity to colicin D. Consistent with these observations, they failed to bind colicin B and detectably cross-link to TonB in vivo. To address this discrepancy, constructs were tested in other strains, one of which (RWB18-60) did support activity of the FepA globular domain deletion proteins constructed in this study. The characteristics of that strain, as well as the strain in which the ΔFhuA globular domain mutants were seen to be active, suggests the hypothesis that interprotein complementation by two individually nonfunctional proteins restores TonB-dependent activity.

TonB-gated transporters are located in the outer membranes of gram-negative bacteria, where they mediate the active transport of iron siderophores and vitamin B12 across the outer membrane. The energy for this process is transduced from the cytoplasmic membrane by a complex of cytoplasmic membrane proteins—TonB, ExbB, and ExbD (for reviews, see references 6 and 26). The crystal structures of TonB-gated transporters (also known as outer membrane receptors) reveal that they consist of a β-barrel that is occluded by an amino-terminal globular domain (also known as the “cork” or “plug” domain) (7, 10, 21). TonB protein physically interacts with the transporters (29), with at least one contact occurring between TonB and a region near the amino terminus of the globular domain, termed the TonB box (8, 23). Mutations within the TonB box prevent function of the transporter (3, 12, 22, 25). Recently, it has been reported that the amino-terminal globular domains of FhuA and FepA can be deleted without significant reduction in their activities and without alleviating their requirements for TonB (5, 28). In particular, deletion of the FepA globular domain (amino acids 17 to 150) resulted in a protein termed FepB, reported to support binding of ferric enterochelin (also known as ferric enterobactin), weak growth with ferric enterochelin as a sole source of iron, and significant sensitivity to colicins B and D (28). The latter three activities were also dependent upon TonB. The authors interpreted these data and data from hybrid transporters to suggest that the globular domain is not important for ligand recognition and that TonB does not function by interaction with the internal globular domain. Thus, TonB must interact within the β-barrel itself. To further examine TonB-barrel interactions, various deletions removing the globular domain of FepA were constructed for the present study.

The fepA gene was amplified as a BspHI fragment by PCR and cloned into the NcoI site of pBAD24 to create pKP515. Deletions in fepA were constructed in pKP515 by extra-long PCR as described previously (15) with primer sequences that are available upon request. DNA sequences of all plasmids were determined, and the absence of unintended base changes was confirmed. Deletion of amino acids 1 to 152 removed the entire globular domain up to an aromatic anchoring residue (trp) preceding the first β strand (7). Deletion of amino acids 7 to 152 was constructed to mimic the FhuA deletion (FhuAΔ5-160) characterized previously (5), with a less extensive deletion (residues 20 to 152) constructed to leave the TonB box (a region through which TonB demonstrably interacts [8]) intact. During these studies the work on FepB was reported, and so the identical protein FepAΔ17-150 was engineered as a control. Arabinose was added to the media for expression of wild-type FepA and three deletion proteins at chromosomal levels (final concentration, 0.25 μg/ml). FepAΔ1-152 required a final concentration of 10 μg of arabinose/ml to reach chromosomal levels (Fig. 1).

To determine if the constructs were correctly localized, strains expressing the FepA variants in KP1394 (fepA::kan rca::cat) were fractionated on sucrose density gradients as previously described (20). All of the mutant FepA derivatives localized as efficiently to the outer membrane as their wild-type parent (Fig. 2).

The ability of the deletion-containing FepA proteins to use ferric enterochelin as the sole iron source was evaluated. Ferric enterochelin was freshly obtained from culture supernatants of KP1344 (W3110, tonB::blaM) (20). While wild-type FepA could support ferric enterochelin-dependent growth (with zone sizes similar to those observed previously [28]), none of the FepA globular domain deletion proteins could support growth beyond that observed for KP1411 (W3110, fepA::kan
Since FepA and TonB are both required for sensitivity of Escherichia coli to colicins B and D, the sensitivity conferred by 17-150 was assessed in a fractionation of wild-type FepA: the mutants had similar profiles (not shown). Immunoblots of sodium dodecyl sulfate–11% polyacrylamide gels developed with α-FepA polyclonal antibody at 1:5,000 are shown. (A) KP1394/pKP515 (FepA wild type); (B) KP1394/pKP516 (FepAΔ1-152); (C) KP1394/pKP517 (FepAΔ7-152); (D) KP1394/pKP518 (FepAΔ20-152); and (E) KP1394/pKP519 (FepAΔ17-150).

The above results are in direct contrast with the previous results of Scott et al., who demonstrated TonB specificity for their Fepβ protein (28). The major difference between that study and this work is in the genetic backgrounds of the strains examined. Here the activity of FepAΔ17-150 was assessed in a W3110-derived background, whereas the identical Fepβ was assayed in strain KDF541 (F− thi entA proC trp rpsL recA fepA fluA cir). KDF541 is derived from RWB18-60 (27) and was a spontaneous mutant selected for its resistance to NADH oxidase (NADHox).

### Table 1. FepA globular domain deletion mutants do not support enterochelin-dependent growth

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Growth</th>
</tr>
</thead>
<tbody>
<tr>
<td>KP1411/pBAD24</td>
<td>aroB fepA</td>
<td>12 ± 0.5</td>
</tr>
<tr>
<td>KP1406</td>
<td>aroB tonB</td>
<td>No growth</td>
</tr>
<tr>
<td>KP1411/pKP515</td>
<td>aroB, fepAΔ1-152</td>
<td>21 ± 0</td>
</tr>
<tr>
<td>KP1411/pKP516</td>
<td>aroB, fepAΔ7-152</td>
<td>9 ± 0.9</td>
</tr>
<tr>
<td>KP1411/pKP517</td>
<td>aroB, fepAΔ20-152</td>
<td>15 ± 0.5</td>
</tr>
<tr>
<td>KP1411/pKP518</td>
<td>aroB, fepAΔ17-150</td>
<td>12 ± 0.3</td>
</tr>
</tbody>
</table>

* Size of growth zones due to ferric enterochelin supplied from KP1344-conditioned medium (in millimeters; disk size is 6 mm). Supernatants from KP1406 (W3110, tonB::blaM aroB) supported no growth for any of the strains (data not shown).

KP1411 was constructed by P1vir transduction of KP1410 with recA::cat from KP1286. KP1410 was constructed by P1vir transduction of KP1414 (W3110, aroB) (20) with fepA::kan from KP1072 (W3110, fepA::kan) (18).
isolated from it by a sequential selection for resistance to colicin B (ColB) and marginally sensitive to colicin D (ColD).

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Sensitivity to ColB</th>
<th>Sensitivity to ColD</th>
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<tbody>
<tr>
<td>KP1411/pBAD24</td>
<td>aroB fepA+</td>
<td>R&lt;sup&gt;a&lt;/sup&gt;</td>
<td>UD&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>KP1411/pKP515</td>
<td>aroB fepA+</td>
<td>3.9 x 10&lt;sup&gt;-5&lt;/sup&gt;</td>
<td>7.8 x 10&lt;sup&gt;-4&lt;/sup&gt;</td>
</tr>
<tr>
<td>KP1411/pKP516</td>
<td>aroB fepA/Δ1-152</td>
<td>R 0.2</td>
<td>R 0.2</td>
</tr>
<tr>
<td>KP1411/pKP517</td>
<td>aroB fepA/Δ7-152</td>
<td>R 0.2</td>
<td>R 0.2</td>
</tr>
<tr>
<td>KP1411/pKP518</td>
<td>aroB fepA/Δ20-152</td>
<td>R 0.2</td>
<td>R 0.2</td>
</tr>
<tr>
<td>KP1411/pKP519</td>
<td>aroB fepA/Δ17-150</td>
<td>R 0.2</td>
<td>R 0.2</td>
</tr>
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</table>

<sup>a</sup> Results are expressed as the highest dilution at which clearing of the bacterial lawn was observed.
<sup>b</sup> Undiluted colicin.
<sup>c</sup> Almost undetectable zones of killing.
<sup>d</sup> R, resistant.

The mutant FepA expressed by RWB193-MT912-59 does not contain the globular domain, whereas the mutant FepA encoded by RWB18-60 could potentially do so. If the FepA protein of RWB18-60 contains a globular domain, then one possibility for the difference between the two strains might be due to interprotein complementation. The globular domain from the inactive chromosomally encoded FepA might insert into the empty β-barrel of the plasmid-encoded globular domain of the FepA mutants (including RWB193-MT912-59) contain a fepA deletion of codons 55 to 359 replaced by a kan gene (1). In contrast, RWB18-60 contains an uncharacterized fepA mutation (16) and is also a lambda lysogen (M. A. McIntosh, personal communication).

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of the system, it is not unreasonable to expect that both the amino-terminal globular domains of TonB-gated transporters are indeed important for ligand transport, binding, and interaction with TonB (4, 8, 9, 12, 21, 24, 30). Given the complexity of the system, it is not unreasonable to expect that both the internal globular domain and the β-barrel are required for activity of the TonB-gated transporters and that each might still interact independently with TonB.

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REFERENCES