Requirement for YaeT in the Outer Membrane Assembly of Autotransporter Proteins

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Autotransporters constitute the largest group of secreted proteins in gram-negative bacteria. Autotransporter secretion involves the insertion of a carboxy-terminal beta barrel into and the translocation across the outer membrane of an amino-terminal domain across the outer membrane. Here, we demonstrate that secretion of autotransporters from several organisms requires the outer membrane assembly factor YaeT.

YaeT (Omp85), a highly conserved protein (7, 8), is required for gram-negative outer membrane protein assembly (5, 29, 30). In cells that lack YaeT, membrane insertion, folding, and/or oligomerization of many outer membrane proteins, including LamB, OmpA, TolC, OmpF/C, phospholipase A, and PhoE in Escherichia coli and PorA, PorB, PilQ, FrpB, and OMPLA in Neisseria meningitidis, are defective (5, 23, 28–30). In addition, processing of the neisserial autotransporter IgA1 protease at the cell surface is impaired (28), consistent with a defect in its assembly in the outer membrane. Given these findings, we explored whether YaeT is required for the outer membrane assembly of autotransporters more generally.

YaeT is required for secretion of Shigella flexneri autotransporters IcsA and SepA. Since YaeT is essential in E. coli (29, 30) and N. meningitidis (28), we postulated that it would be essential in S. flexneri. Therefore, we generated a derivative of S. flexneri wild-type strain 2457T (14) in which YaeT could be depleted (Table 1). On solid medium, the depletion strain did not grow in the absence of arabinose because in this strain expression of yaeT is under the control of the arabinose promoter. In rich liquid medium, growth was measured under replete (0.2% arabinose) or depleting (0.1% fucose) conditions. Under depletion conditions, the depletion strain’s growth rate rapidly dropped off, whereas under replete conditions, it grew as well as the wild-type strain under either condition (Fig. 1A). Thus, under these conditions, yaeT is essential for growth in S. flexneri. At 3 h of depletion, YaeT expression was less than 5% that of the wild-type or of the deletion strain grown under replete conditions (Fig. 1B). Of note, the disruption in yaeT did not have significant polar effects on hlpA (skp), the gene that lies immediately downstream of yaeT and that encodes the periplasmic chaperone Skp, since levels of Skp were unaltered (Fig. 1B).

Like many autotransporters, after assembly in the outer membrane, the Shigella autotransporters IcsA and SepA are proteolytically processed, releasing the mature amino-terminal domains into the extracellular milieu. In wild-type cells, proteolytic processing of IcsA at the cell surface is inefficient, such that a significant amount of full-length mature IcsA is detectable at the cell surface and in cell pellets (Fig. 1C) (6, 10, 24). In contrast, SepA is efficiently processed and is undetectable on the surface and in cell pellets (Fig. 1D) (2, 12). Upon YaeT depletion, although SepA remained undetectable in cell pellets, IcsA was reproducibly reduced or absent in that fraction (Fig. 1C and D).

To examine the role of YaeT in the secretion of IcsA and SepA, we examined culture supernatants for proteolytically processed amino-terminal domains of protein that had been newly secreted after depletion of YaeT. Cells were grown for 3 h under conditions of depletion or repletion, washed to remove spent supernatant, resuspended in fresh medium, and grown again for 2 h. Proteins that then accumulated in the culture supernatants of cells grown under depletion conditions represent proteins secreted after depletion of YaeT. The processed amino-terminal domains of IcsA and SepA were both dramatically reduced in the culture supernatants of YaeT-depleted cells compared to those of replete or wild-type cells (Fig. 1C and D), indicating that YaeT is required for proper expression and/or secretion of IcsA and SepA in S. flexneri.

YaeT depletion leads to DegP-dependent instability of unassembled IcsA. Given the described role for the periplasmic protease/chaperone DegP in the efficient secretion of IcsA (22) and in proteolytic degradation of misassembled outer membrane proteins and given the observation that depletion of YaeT leads to accumulation of some outer membrane proteins...
in the periplasm (29), we tested whether the observed reduction in levels of IcsA might be due to DegP-dependent degradation. We generated derivatives of strain 2457T in which either DegP alone or both DegP and YaeT could be depleted (Table 1). Under depletion conditions, the DegP depletion strain and DegP YaeT double depletion strain were defective for growth at 42°C (data not shown), as previously reported for E. coli degP mutants (15, 26). Under depletion conditions at 30°C, the growth rate of the DegP depletion strain was similar to that of the wild-type, but that of the double depletion strain dropped off more rapidly than that of the YaeT depletion strain (Fig. 2A), as has been noted previously in E. coli (29).

The decrease in viability of cells lacking both YaeT and DegP compared with cells lacking YaeT alone demonstrates that in the absence of YaeT, DegP activity increases the time to cell death, indicating that DegP provides an advantage to these cells.

TABLE 1. Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant genotype</th>
<th>Construction, source, or reference</th>
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<tbody>
<tr>
<td>Wild type 2457T</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BS109</td>
<td>pWR100 icsA::lacI</td>
<td></td>
</tr>
<tr>
<td>MBG283</td>
<td>pWR100 sepA::pSJ581 Ap'</td>
<td>Integrated pSJ581 suicide vector using conjugation</td>
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<tr>
<td>SJ588</td>
<td>pWR100 sepA::pSJ581 Ap'</td>
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</tr>
<tr>
<td>SJ603</td>
<td>pBAD-yaeT Ap'</td>
<td>P1 (SJ588) × 2457T</td>
</tr>
<tr>
<td>SJ518</td>
<td>pBAD-yaeT Ap'</td>
<td>P1 (JCMI66) × SJ518</td>
</tr>
<tr>
<td>SJ447</td>
<td>pBAD-degP Sp'</td>
<td>Transformed with pBAD-degP</td>
</tr>
<tr>
<td>SJ455</td>
<td>pBAD-degP Ap'</td>
<td>P1 (BW25113 degP::kan) × SJ447</td>
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<td>DegP depletion strain</td>
<td>pBAD-degP ΔdegP Sp'</td>
<td>Excision of kan using FLP recombinase</td>
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<td>Transformed with pBAD-yaeT</td>
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<td>YaeT depletion strain</td>
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<td>Sp' Ap'</td>
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<td>Sp' Ap'</td>
<td>Transformed with p-brkA</td>
</tr>
<tr>
<td>JCM166 p-NG162</td>
<td>Sp' Ap'</td>
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<td>Sp' Ap'</td>
<td>Transformed with p-yaeT</td>
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<td>JCM158 p-BrkA</td>
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<td>Sp' Ap'</td>
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<tr>
<td>Plasmids</td>
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<td>Sp'</td>
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<td>pBAD-yaeT</td>
<td>Ap'</td>
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<td>pNG162 brkA ΔbrkA Sp'</td>
<td>NruI-HindIII fragment containing brkA from pDO6935 inserted into the EcoRI-HindIII sites of pNG162</td>
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<tr>
<td>pDO6935</td>
<td>pBluescript II SK(-) PbrkA brkA Ap'</td>
<td></td>
</tr>
<tr>
<td>pGP704</td>
<td>Suicide vector Ap'</td>
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<td>pIB264</td>
<td>pBAD-aidA Ap'</td>
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<td>pNG162</td>
<td>Expression vector, pSC101 ori lacI Ap'</td>
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<tr>
<td>pSJ265</td>
<td>pBAD-araC Ap'</td>
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</tr>
<tr>
<td>pSJ581</td>
<td>pBAD-araC Ap'</td>
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CiaI-SphI fragment containing aidA from pIB264 inserted into the same sites of pNG162

XbaI-SphI fragment containing degP inserted into same sites of pSJ262

XbaI-SphI fragment containing 2457T yaeT inserted into same sites of pBAD18

NruI-HindIII fragment containing brkA from pDO6935 inserted into the EcoRI-HindIII sites of pNG162

BsrRI-EcoRI fragment from pBAD24 inserted into same sites of pAM238

pGP704 containing ∼2 kb internal EcoRI-EcoRV fragment of segA

in the periplasm (29), we tested whether the observed reduction in levels of IcsA might be due to DegP-dependent degradation. We generated derivatives of strain 2457T in which either DegP alone or both DegP and YaeT could be depleted (Table 1). Under depletion conditions, the DegP depletion strain and DegP YaeT double depletion strain were defective for growth at 42°C (data not shown), as previously reported for E. coli degP mutants (15, 26). Under depletion conditions at 30°C, the growth rate of the DegP depletion strain was similar to that of the wild-type, but that of the double depletion strain dropped off more rapidly than that of the YaeT depletion strain (Fig. 2A), as has been noted previously in E. coli (29). The decrease in viability of cells lacking both YaeT and DegP compared with cells lacking YaeT alone demonstrates that in the absence of YaeT, DegP activity increases the time to cell death, indicating that DegP provides an advantage to these cells.

In cells depleted of DegP and YaeT, the amount of IcsA present in cell pellets was reproducibly increased compared to
that in cells depleted of YaeT alone (Fig. 2B), indicating that the decrease in IcsA levels upon YaeT depletion is at least partially dependent on DegP and is not solely an effect on icsA expression. Moreover, this indicates that, in the absence of YaeT, IcsA is accessible to DegP in the periplasm. There was no effect on SepA levels (data not shown).

For these experiments, since cell lysis occurred after prolonged growth under depletion conditions, the time of depletion and of growth in fresh medium were reduced to 3.0 h total (1.5 h each); the reduction of YaeT levels at 1.5 h was comparable to that at 3 h (data not shown). For the double depletion strain, IcsA was not detected in the culture supernatant even under replete growth conditions (data not shown); this was surprising, since growth under this condition was not impaired (Fig. 2A), but it might reflect altered stoichiometry of DegP and YaeT, since in this strain, the two proteins are expressed from plasmids of different copy numbers (Table 1).

In the absence of YaeT, IcsA is not exposed at the cell surface. The decrease in processed amino-terminal domains of IcsA and SepA in culture supernatants could represent either a decrease in outer membrane assembly of the protein or a decrease in proteolytic processing per se. To distinguish between these possibilities, we tested whether the mature amino-terminal domain of IcsA was exposed on the surface of YaeT- and DegP-depleted cells, as it normally is on wild-type cells (10, 12). IcsA was detectable on significantly fewer YaeT- and DegP-depleted cells and was qualitatively less bright on these cells than on wild-type or DegP-depleted cells, indicating that YaeT is required for the translocation of IcsA to the cell surface (Fig. 2C and Table 2). It was not possible to distinguish whether the IcsA on the surface of some YaeT-depleted cells reflected residual full-length IcsA or proteolytically processed IcsA associated with these cell pellets (Fig. 2B). As discussed above, the reason for the relatively low frequency of IcsA secreted by the YaeT DegP depletion strain grown in arabinose is unclear.

In the absence of YaeT, IcsA is not assembled in the outer membrane. Once folded and assembled in the outer membrane, beta-barrel proteins retain a stable compact conformation during sodium dodecyl sulfate-polyacrylamide gel electro-
phoresis analysis unless heated above their melting point (27).
Therefore, whether a protein is assembled in the outer membrane
can be assessed by its migration on sodium dodecyl
sulfate-polyacrylamide gel electrophoresis gels. We took ad-
vantage of these observations to test whether, in the absence of
YaeT, IcsA is assembled in the outer membrane.
As has been observed for other beta-barrel proteins (27),
when cell pellets of wild-type S. flexneri
were heated to only 30°C, the major IcsA band migrated faster than when pellets
were heated to 100°C (Fig. 2D, left panel, wild type). The
faster-migrating band in the 30°C sample likely represents
folded and outer membrane-assembled protein, and the slower-
migrating band in the 100°C sample likely represents unfolded
denatured protein. In contrast to the wild-type strain, upon
depletion of both YaeT and DegP, the major band in the 30°C
sample migrated at the same rate as unfolded denatured pro-
tein (Fig. 2D, right panel, YaeT DegP depletion), indicating
that YaeT is required for folding of IcsA. This result is con-
sistent with a requirement for YaeT for the assembly of IcsA in
the outer membrane.
Taken together, the accessibility of IcsA to DegP in the
periplasm in the absence of YaeT and the loss of the fast-
migrating form in the absence of YaeT and DegP indicate that
YaeT is required for the assembly of the IcsA beta barrel in the
outer membrane. The requirement for YaeT for insertion of
two other beta-barrel-containing outer membrane proteins,
TolC and OmpA, has been observed (29); like IcsA, each of these accumulates in the periplasm in the absence of YaeT and DegP.

YaeT is required for the secretion of autotransporters from other gram-negative bacilli. To test whether autotransporters from other organisms also require YaeT for secretion, we assayed secretion of the diffusely adherent *E. coli* autotransporter AIDA-I and the *Bordetella* sp. autotransporter BrkA in an *E. coli* YaeT depletion background. Normally, upon its secretion, the proteolytically processed amino-terminal domains of AIDA-I and BrkA remain noncovalently associated with the bacterial surface and, hence, are present in cell pellets (3, 20). Following growth under YaeT depletion conditions, the levels of the proteolytically processed amino-terminal domains of AIDA-I or BrkA in cell pellets were reduced compared to those of wild-type cells or cells grown under replete conditions (Fig. 3A and B), indicating that, as for IcsA and SepA, YaeT is required for outer membrane assembly of AIDA-I and BrkA. Although some species specificity has been observed for YaeT recognition of outer membrane proteins (23), our findings indicate that *Bordetella* BrkA is sufficiently well recognized by *E. coli* YaeT for efficient assembly. The levels of full-length mature AIDA-I or BrkA did not increase (Fig. 3B and data not shown), suggesting that, as was observed for IcsA, in the absence of proper outer membrane assembly, these improperly processed autotransporters may be unstable. To determine whether the residual amount of processed BrkA associated with the pellet under fucose conditions represented protein that had been translocated to the cell surface prior to YaeT depletion, at 3 h of depletion we removed surface-associated proteins with proteinase K, as described previously (4), and then examined whether BrkA accumulated upon continued growth under depletion conditions. Proteinase K treatment removed the cell-associated proteolytically processed BrkA (Fig. 3C, left three lanes), without detectable degradation of periplasmic proteins (Fig. 3C, bottom panel). Upon subsequent growth under depletion conditions, no BrkA was associated with the cell pellets (Fig. 3C, right three lanes), indicating that in the absence of YaeT, no BrkA is translocated to the cell surface.

In conclusion, we show that autotransporters from *S. flexneri*, *E. coli*, and *Bordetella* sp. are dependent on YaeT for proper outer membrane assembly, suggesting that all members of the autotransporter family may depend on this general outer membrane assembly apparatus. Moreover, in the case of *S. flexneri* IcsA, our results are consistent with a model in which YaeT is specifically required for early steps in outer membrane assembly, including insertion of the beta barrel into the outer membrane and the translocation of the mature amino terminus across the outer membrane.

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