Cross-species chimeras reveal BamA POTRA and -barrel domains must be fine-tuned for efficient OMP insertion

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Cross-species chimeras reveal BamA POTRA and β-barrel domains must be fine-tuned for efficient OMP insertion

Introduction

The insertion of β-barrel containing integral outer membrane proteins (OMPs) into the outer membrane of Escherichia coli is achieved by the multi-protein β-barrel assembly machine, the BAM complex (Knowles et al., 2009; Hagan et al., 2011). In E. coli, this complex molecular machine consists of the OMP BamA (BamAEC; the subscript indicates the source of the BamA sequence used e.g. Ec refers to E. coli) and the four accessory lipoproteins (BamB–E). BamAEC is an essential protein in E. coli and belongs to the BamA/Omp85 family of proteins (Voulhoux et al., 2003; Anvari et al., 2012). The N-terminal periplasmic domain of BamAEC is composed of five polypeptide transport-associated (POTRA) motifs (POTRA1 to POTRA5) and a C-terminal β-barrel domain, which anchors the protein in the outer membrane (Knowles et al., 2009; Hagan et al., 2011). The POTRA domains have been shown to bind unfolded OMPs, being responsible for delivering them to the outer membrane, as well as scaffolding the BAM lipoproteins to the complex. BamD binds directly to POTRA5, while BamB associates through POTRA2 to POTRA5 (Kim et al., 2007; Knowles et al., 2008; Gatzeva-Topalova et al., 2010; Patel and Kleinschmidt, 2013). BamC and BamE do not bind BamAEC directly, but associate with the BAM complex through BamD (Kim et al., 2007). The recent crystal structures of BamAEC and its orthologues have demonstrated that in BamA proteins, the C-terminal domain folds into a 16-stranded β-barrel, over which the external loops (L1–L8) converge to form a covering dome (Noinaj et al., 2013; Albrecht et al., 2014; Ni et al., 2014). In E. coli, many of the loops are essential for BamAEC function, in particular L6, which is partially located within the barrel lumen because of an interaction between the barrel wall and the conserved VRGF amino acid motif at its tip (Delattre et al., 2010; Leonard-Rivera and Misra, 2012; Browning et al., 2013; Noinaj et al., 2013; Rigel et al., 2013; Albrecht et al., 2014; Ni et al., 2014).

BAM is an evolutionary conserved machine, components of which are found in eukaryotic mitochondria and chloroplasts, as well as Gram-negative bacteria (Voulhoux et al., 2003; Tommassen, 2010). BamA and BamD are essential in E. coli and found in all Gram-negative bacteria. However, the other components of the complex are less conserved, for example Neisseria species lack BamB.
while in some bacteria, additional accessory components are part of the BAM complex, such as Pal and BamF in *Caulobacter crescentus* and RmpM in *Neisseria meningitidis* (Volokhina *et al.*, 2009; Anwari *et al.*, 2010; 2012). During OMP insertion, BamA orthologues are thought to recognise the C-terminal residues of unfolded OMPs and it has been suggested that this is species-specific (Robert *et al.*, 2006; Paramasivam *et al.*, 2012). Biophysical analyses have suggested that bacterial BamA proteins function in a species-specific manner, being optimised for folding OMPs from that species (Robert *et al.*, 2006). Other studies are consistent with this as only BamA orthologues from closely related bacterial species can rescue BamA depletion in *E. coli* (Noi aj *et al.*, 2013; Ruhe *et al.*, 2013; Volokhina *et al.*, 2013). Thus, it is surprising that *E. coli* BamA can insert mitochondrial porins into its outer membrane (Wal ther *et al.*, 2010), while the analogous mitochondrial machinery can insert bacterial OMPs into the mitochondrial outer membrane (Walther *et al.*, 2009). Therefore, to investigate this species specificity in more detail, we generated a series of chimeric BamA fusion proteins, which carry either the POTRA or barrel domains of BamA orthologues from a wide range of proteobacteria. We demonstrate that the barrel domains, and to some degree, the POTRA domains of BamA orthologues are interchangeable and that for efficient complex function, the POTRA and barrel domains must be precisely tailored.

### Results

**Rescue of BamA depletion by β-barrel chimera fusion proteins**

As BamA orthologues were suggested to function in a species-specific manner (Robert *et al.*, 2006; Paramasivam *et al.*, 2012; Noi aj *et al.*, 2013; Ruhe *et al.*, 2013; Volokhina *et al.*, 2013), we reasoned that species specificity would reside in the POTRA domains, and not the β-barrels, as the POTRA domains are predicted to be the first point of contact between nascent OMPs, their periplasmic chaperones and the BAM complex. To test this hypothesis, we generated chimeric constructs in which the DNA encoding the *E. coli* BamA POTRA domains was fused in register to that encoding the barrel domains of BamA orthologues from a selection of phylogenetically diverse Gram-negative bacteria (Fig. 1A, Table S1 and Fig. S1). As BamAEC is essential in *E. coli*, we examined whether any of the barrel chimeras rescued loss of the native BamA. The DNA encoding each fusion was cloned into vector pET17b and transformed into the previously described *E. coli* K-12 depletion strain, JWD3 (Lehr *et al.*, 2010). In JWD3 chromosomally encoded BamAEc is only produced in the presence of arabinose, while in its absence, BamAEc expression is shut down and BamAEc levels are depleted by successive cell divisions resulting in cell death; depletion can be rescued by providing a functional plasmid-encoded copy of the *E. coli* bamA, such as pET17b/bamAEC (Browning *et al.*, 2013). Results in Fig. 1B show that, with the exception of the *Helicobacter pylori* fusion BamAEPH, all of the barrel chimeras supported growth in the absence of arabinose. While cells carrying the *Agrobacterium tumefaciens* BamAEA fusion grow particularly slowly, they can be repeatedly passaged in the absence of arabinose. Western blotting of total protein extracts and outer membrane preparations with anti-*E. coli* BamA POTRA antiserum demonstrated that all the chimeras were expressed and localised to the outer membrane (Fig. 1C and D), with the exception of BamAEPH. Interestingly, although the *Pseudomonas aeruginosa* BamAEpa, *A. tumefaciens* BamAEA and *N. meningitidis* BamAENm barrel chimeras support growth in liquid culture, Western blotting using anti-OmpF antiserum indicated that, in the absence of arabinose, the levels of OmpF, OmpC and OmpA porins were considerably lower than in cells producing BamAEc (Fig. 1C). Thus, although the barrels of most orthologues can be readily swapped to maintain viability, it is clear that some fusions function considerably better than others.

**Increased expression of β-barrel chimeras improves function**

During our study, we noted that the *B. japonicum* BAMAEPa, BamAEA and BamAENm barrel chimeras rescued depletion in liquid culture (Fig. 1B), but that JWD3 cells carrying these fusions did not form colonies on agar plates without arabinose (Fig. S2A). The reason for this is unclear, but may reflect increased stress associated with growth on solid surfaces. Therefore, to isolate chimeric constructs, which rescued depletion on solid medium, we passaged JWD3 cells, carrying pET17b/bamAEpa, pET17b/bamAEA or pET17b/bamAENm in liquid culture and plated cells onto agar plates without arabinose. Using this strategy, we isolated plasmids encoding BamAEPa, BamAEA and BamAENm constructs, which rescued BamA depletion on solid medium (Fig. 2A and S3). Surprisingly, sequencing of each plasmid construct failed to detect any differences in the DNA sequence encoding each chimera, but rather point mutations were identified in the sequence encoding the RNA I/II copy number control region of pET17b (Fig. S2B). To investigate whether these mutations had an impact on plasmid copy number, we isolated plasmid DNA from similar numbers of JWD3 cells transformed with either the initial plasmid constructs encoding BamAEPa, BamAEA and BamAENm or their cognate evolved plasmids. Agarose gel electrophoresis revealed more plasmid DNA was isolated from strains harbouring the evolved plasmids suggesting that the copy number of each plasmid had increased (Fig. 2B and S3). Western immunoblotting
of whole-cell fractions revealed that each of the strains harbouring the evolved plasmids produced higher levels of the chimeric BamA (Fig. 2 and S3) and in liquid culture growth rates under depletion conditions resembled those of strains harbouring native BamA Ec (Fig. S2C and D). Thus, the ability of these barrel chimeras to rescue depletion can be improved by increasing their cellular concentrations to that resembling native E. coli BamA.

TpsB β-barrels do not functionally substitute for BamA β-barrels

The BamA/Omp85 superfamily includes distantly related members such as the TpsB β-barrels of the two-partner secretion systems e.g. FhaC from Bordetella pertussis and EtpB from enterotoxigenic E. coli (Fleckenstein et al., 2006; Clantin et al., 2007; Meli et al., 2009). These TpsB β-barrels do not functionally substitute for BamA β-barrels.

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proteins recognise specific proteins rich in β-strands and secrete them across the outer membrane, whereas BamA orthologues fold β-stranded OMPs into the membrane. Thus, we wished to investigate whether a β-barrel domain derived from a TpsB protein would be able to functionally replace that of BamAEc. To test this, we fused the DNA encoding the BamAEc POTRA domains with that encoding the EtpB barrel, to generate an EtpB barrel chimera construct (BamAEtpB) (Table S1 and Fig. S1). Results in Fig. 3 demonstrate that while the BamAEtpB fusion was produced proteins recognise specific proteins rich in β-strands and secrete them across the outer membrane, whereas BamA orthologues fold β-stranded OMPs into the membrane. Thus, we wished to investigate whether a β-barrel domain derived from a TpsB protein would be able to functionally replace that of BamAEc. To test this, we fused the DNA encoding the BamAEc POTRA domains with that encoding the EtpB barrel, to generate an EtpB barrel chimera construct (BamAEtpB) (Table S1 and Fig. S1). Results in Fig. 3 demonstrate that while the BamAEtpB fusion was produced.
and folded into the outer membrane it was unable to rescue BamA depletion in JWD3, suggesting that only barrels from true BamA orthologues can functionally replace that of *Escherichia coli* BamA.

**Rescue of BamA depletion by POTRA chimera constructs**

To evaluate the role of the POTRA domains in BamA species-specificity, we generated a second series of chimeric constructs in which the POTRA domain of each orthologue was fused to the barrel of BamAEc (Table S1 and Fig. S1). Initially, gene fusions were subcloned into pET17b and their ability to rescue BamA depletion was examined in liquid medium in JWD3. Results in Fig. S4 show that only the *Salmonella enterica* serovar Typhimurium POTRA chimera (BamA*Sse*) supported growth of JWD3 cells in the absence of arabinose. As the cellular level of BamA is important (Aoki et al., 2008), we hypothesised increased expression of the fusion proteins would allow them to rescue depletion of BamA. Thus, the chimeric genes encoding the *Haemophilus influenzae*, *P. aeruginosa* and *N. meningitidis* POTRA domains (BamA*HIE*, BamA*PaE* and BamA*NmE*) were cloned into the high copy number (>100 copies) expression vector, pASK-IBA33plus (pASK). These new constructs contained an N-terminal His-tag to allow detection by immunoblotting; previous investigations revealed the His-Tag did not have an impact on function (Kim et al., 2007; Browning et al., 2013). Results in Fig. 4A show that leaky uninduced expression from pASK enabled the BamA*HIE* and BamA*NmE* POTRA chimeras to rescue BamA depletion, while the BamA*PaE* chimera did not. Interestingly, low-level induction of BamA*NmE* expression retarded growth, suggesting that elevated levels of BamA*NmE* are toxic. Western blotting of whole-cell lysates with anti-OmpF antiserum demonstrated that in cells expressing His-tagged versions of BamA*NmE* and BamA*PaE*, porin levels were comparable with those observed for BamA*Ec* (Fig. 4B). Furthermore, His versions of each POTRA chimera could be detected, but their levels were lower than that observed for BamA*Ec*, suggesting that these chimeric proteins may be unstable or poorly expressed (Fig. 4B). Thus, we conclude that the BamA*NmE* and BamA*PaE* POTRA chimeras can functionally
Mutational analysis of the His-BamA<sub>NmE</sub> and BamA<sub>Pa1–4</sub> POTRA constructs, in liquid medium was similar to theations required for this phenotype (Fig. S5). The background, and it was confirmed that only these muta-
E521G mutations were transferred into a clean plasmid.

amino acid conversion: R370C, R388G, E521G and
tor plates. Sequencing of the DNA encoding each
doing this, we isolated four candidates that rescued deple-
cells out onto nutrient agar plates lacking arabinose. By
cloned into pASK in the absence of arabinose and plated
E. coli

Neisseria gonorrhoeae

E. coli

E. coli

T4 of the

β-barrel has the capacity to clash with both R370 and R388 in BamA<sub>NmE</sub>. Correspondingly, the isolated

R370C and R388G mutations (Fig. 5D) alleviate this clash, restoring the charge balance observed in the Neisserial protein. Furthermore, extrapolating the side-chain orientations from the available <i>N. gonorrhoeae</i> BamA<sub>Ng</sub> crystal structure, E523 (in T3) is in close proximity to E363 (POTRA<sub>3</sub>), which is unfavourable from electrostatic point of view. This residue pair is non-conserved, and indeed such electrostatic repulsion is avoided in <i>E. coli</i> as position 363 in BamA<sub>Ec</sub> is alanine (Fig. 5E) (Noinaj <i>et al</i>, 2013). Presum-
ing a similar orientation of the Neisserial POTRA domains in our BamA<sub>NmE</sub> POTRA chimera we expect a potential for a similar clash between E363 and E521, as in BamA<sub>Ng</sub>, and this is relieved by the E521G/A substitutions making this new chimeric interface more BamA<sub>Ec</sub> like (Fig. 5E). Thus, we conclude that mutations in BamA<sub>NmE</sub> allow POTRA<sub>3</sub> to adopt different orientations with respect to the barrel domain.

Allosteric mutations improve the function of <i>P. aeruginosa</i> POTRA chimera constructs

The <i>P. aeruginosa</i> BamA<sub>Pae</sub> POTRA chimera failed to rescue BamA depletion (Fig. 4). Initial attempts to isolate improved versions of the BamA<sub>Pae</sub> chimera, by passaging plasmid constructs through the mutator strain XL-1 Red, also failed. Therefore, to examine which <i>P. aeruginosa</i> POTRA domains prevented the chimera from functioning in <i>E. coli</i>, we generated an additional set of chimeric constructs, cloned into pASK. Starting with BamA<sub>Pae</sub>, we pro-
progressively replaced the <i>P. aeruginosa</i> POTRA domains with those from <i>E. coli</i> to generate constructs which pos-
sessed <i>P. aeruginosa</i> POTRA<sub>1–4</sub> (BamA<sub>Pa1–4</sub>), POTRA<sub>1–3</sub> (BamA<sub>Pa1–3</sub>), POTRA<sub>1–2</sub> (BamA<sub>Pa1–2</sub>) and POTRA<sub>1</sub> only

<table>
<thead>
<tr>
<th>POTRA chimera.</th>
<th>Location of substitution.</th>
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<tbody>
<tr>
<td>His-BamA&lt;sub&gt;NmE&lt;/sub&gt; R370C</td>
<td>Neisseria meningitidis POTRA&lt;sub&gt;0&lt;/sub&gt;</td>
</tr>
<tr>
<td>His-BamA&lt;sub&gt;NmE&lt;/sub&gt; R388G</td>
<td>Neisseria meningitidis POTRA&lt;sub&gt;0&lt;/sub&gt;</td>
</tr>
<tr>
<td>His-BamA&lt;sub&gt;NmE&lt;/sub&gt; E521G</td>
<td>BamA&lt;sub&gt;Ec&lt;/sub&gt; barrel T3</td>
</tr>
<tr>
<td>His-BamA&lt;sub&gt;NmE&lt;/sub&gt; E521A</td>
<td>BamA&lt;sub&gt;Ec&lt;/sub&gt; barrel T3</td>
</tr>
<tr>
<td>BamA&lt;sub&gt;Pa1–4&lt;/sub&gt; T434I</td>
<td>BamA&lt;sub&gt;Ec&lt;/sub&gt; barrel L1</td>
</tr>
<tr>
<td>BamA&lt;sub&gt;Pa1–4&lt;/sub&gt; S436P</td>
<td>BamA&lt;sub&gt;Ec&lt;/sub&gt; barrel L1</td>
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<tr>
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<td>BamA&lt;sub&gt;Ec&lt;/sub&gt; barrel β2</td>
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<td>BamA&lt;sub&gt;Ec&lt;/sub&gt; barrel β2</td>
</tr>
<tr>
<td>BamA&lt;sub&gt;Pa1–4&lt;/sub&gt; E470G</td>
<td>BamA&lt;sub&gt;Ec&lt;/sub&gt; barrel β4</td>
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<td>BamA&lt;sub&gt;Ec&lt;/sub&gt; barrel β6</td>
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<td>BamA&lt;sub&gt;Ec&lt;/sub&gt; barrel β16</td>
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Table 1. Mutational analysis of the His-BamA<sub>NmE</sub> and BamA<sub>Pa1–4</sub> POTRA chimeras.

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Fig. 5. Mutational analysis of the His-BamA
NmE POTRA chimera.
A. The panel shows the composite structural model of the full-length BamA Ec, based on the full-length Neisseria gonorrhoeae BamA Ec structure (4K3B) (Noinaj et al., 2013) and Escherichia coli BamA Ec barrel structures (4C4V, 4N75) (Albrecht et al., 2014; Ni et al., 2014). The POTRA5 and POTRA6 structures (3OG5) have been taken from (Gatzeva-Topalova et al., 2010). The positions of residues R370, R388 in POTRA6 and E521 in turn T3 are shown.
B. The panel shows the potential POTRA5-barrel interactions for
BamA Ec1, BamA Ec3 and BamA Ec4, Fig. 6A). The ability of each construct to rescue BamA depletion was then examined in strain JWD3 in liquid culture and on agar plates. Results in Figs. 6B and S5 show that only constructs which carried both E. coli POTRA5 and POTRA6 could rescue BamA depletion. As these chimeric fusions carry E. coli POTRA domains, we examined whether fusions were detectable using Western blotting with anti-E. coli POTRA antiserum. Results in Fig. 5C reveal that the BamAPa1–4, BamAPa1–2 and BamAPa1 chimeras, which all rescue depletion, could be detected in whole-cell preparations of BamA-depleted cells (lanes 12–14). Western blotting using anti-P. aeruginosa BamA antiserum also demonstrated that the BamAPa1–4, BamAPa1–3, BamAPa1–2 and BamAPa1 chimeras were expressed in arabinose-grown cells; however, the level of each protein was considerably lower when BamA was depleted (Fig 6D). Thus, we conclude that differences in P. aeruginosa POTRA and POTRA6 prevent the BamAPa1 chimera from functioning in E. coli.

As the P. aeruginosa BamAPa1–4 chimera did not rescue BamA depletion (Fig. 6), we also attempted to mutate this plasmid construct by passaging it through the XL–1 Red mutator strain. Plasmid DNA, isolated from XL-1 Red cells, was transformed into JWD3 and cells were plated out onto an arabinose-free agar. Using this strategy, we isolated 14 mutant constructs that rescued BamA depletion. Unexpectedly, DNA sequencing of each chimera indicated that point mutations, which enabled BamAPa1–4 to function, were all located within the BamA Ec β-barrel domain and not in the POTRA domains (Table 1 and Fig. 7A). For five of these constructs (E470G, D614G, D614N, A654T and D746N), the DNA encoding each chimera was transferred into a clean plasmid background and each construct retained the ability to rescue depletion (Figs. 7B and S5). Western blotting with anti-OmpF antiserum indicated that these improved BamAPa1–4 chimeras could insert E. coli porins in the absence of E. coli BamA (Fig. 7C) and blotting with anti-P. aeruginosa BamA antiserum demonstrated that in the absence of arabinose, the cellular levels of these proteins were higher than that observed for the original BamAPa1–4 construct (Fig. 7D).

To understand the molecular basis for these gain-of-function mutations, we investigated the position of these amino acids in BamA. Many of these compensatory substitutions fall within β-strands (e.g. E470G and D614G) and would destabilise them, increasing barrel flexibility (Merkel and Regan, 1998) (Fig. 7E). Substitution D746N disrupts the network of salt bridges, which hold loops L4, L6 and L7 together, likely making the whole barrel domain less rigid (Fig. 7E). Other mutations, such as A654T and S657F, are predicted to alter the trajectory of L6 within the barrel (Fig. 7E). We have analysed the packing of the L6 loop in the available structures, which present six non-crystallographic copies of the loop from three different organisms. Structural superposition and B-factor analysis (Fig. S6A) reveal striking conservation of the trajectory of the loop within the barrel in all current structures, while on the outside of the barrel it displays a high degree of flexibility, as expected for a typical non-structured loop. The intra-barrel section of the L6 also appears to be extremely rigid as indicated by the very low B-factor values, and has a near identical match in all structures with a root-mean-square deviation from 0.22 to 0.67 Å, which is below the range of coordinate error for the structures. This extends beyond the C-alpha atoms to the side-chains, and is particularly evident for the VRGF motif, suggestive of the need for precise alignment of the structural elements of the loop for its functional activity (Fig. S6B). As L6 is precisely folded in all six available structures, and the L6 VRGF motif is tightly associated with the barrel (Fig. S6A), alterations of the loop will have a significant impact on the folded state of the β-barrel, particularly S657F, which would generate a direct steric clash with β16 (Noinaj et al., 2013; 2014; Albrecht et al., 2014; Ni et al., 2014). In addition, mutations in L1 (T434I and S436P) and β16 (N805S) are likely to
Fig. 6. Rescue of BamA depletion by the Pseudomonas aeruginosa POTRA chimeras.

A. The panel shows the P. aeruginosa POTRA chimeras used in this study. The Escherichia coli POTRA domains are shown in orange, while P. aeruginosa POTRAs are shown by blue hashing. The E. coli BamA barrel is depicted as a grey rectangle.

B. E. coli JWD3 cells, carrying the P. aeruginosa POTRA chimeras cloned into pASK (see panel A), were grown in Lennox broth supplemented with either arabinose (+Ara) or fructose (−Ara).

C. Detection of P. aeruginosa POTRA chimeras. The panel shows Western blots of normalised total cellular protein from JWD3 cells in panel B after 300 min of growth. Blots were probed with anti-E. coli BamA POTRA antiserum to detect BamAEc and P. aeruginosa POTRA chimeras, where possible. A non-specific band is used as a loading control (LC).

D. Detection of P. aeruginosa POTRA chimeras with anti-P. aeruginosa BamA antiserum. The panel shows Western blots of normalised total cellular protein from JWD3 cells in panel B after 300 min of growth. Blots were probed with anti-P. aeruginosa BamA antiserum to detect the various P. aeruginosa POTRA chimeras. A non-specific band is used as a loading control (LC).
affect the pairing of $\beta 1$ and $\beta 16$, lowering the kinetic barrier for barrel unfolding (Fig. 7E). Thus, we conclude that defects in the POTRA domain can be bypassed by substitutions in the barrel domain, which improve the alignment of POTRA relative to the barrel and/or facilitate the opening of the barrel.

**Discussion**

To understand the nature of the BAM species specificity, we generated a series of BamA $\beta$-barrel and POTRA chimeras. Bioinformatic analyses indicated that the barrel domains of BamA orthologues are more conserved than their corresponding POTRA domains (Arnold et al., 2010) and consistent with this, we found that most barrel chimeras were expressed, located in the outer membrane and rescued BamA Ec depletion (Fig. 1). Only the H. pylori barrel chimera failed to rescue BamA depletion. As we were unable to detect this protein product, this suggests that this fusion is likely unstable in E. coli. In contrast, Volokhina et al. (2013) investigated the species-specificity observed between BamA Ec and N. meningitidis BamA (BamANm), by expressing the full-length BamANm protein and similar barrel and POTRA chimeras in E. coli. Although protein products were detected and E. coli BamD was capable of binding to BamANm, all of these constructs failed to rescue depletion. In each case, gene expression was induced from plasmid pFP10 (Volokhina et al., 2013), while in our system, we relied on low-level leaky expression from either pET17b or pASK. Indeed, inducing expression of the BamANm POTRA chimera (Fig. 4A), even at low levels, retarded growth considerably, implying toxicity. Thus, we think it likely that the differences observed between the two studies are due to the different experimental systems employed.

It is of note that some barrel chimeras functioned poorly compared with the native protein. However, even slightly increasing the expression levels of these proteins greatly improved their ability to rescue depletion. As such BamA $\beta$-barrels can functionally replace that of E. coli, this would suggest that the mechanism of barrel-mediated OMP insertion has been conserved. Thus, it is quite surprising that the growth of strains expressing elevated levels of BamAAT or BamAENm were similar to the wild type on plates and in liquid medium, yet still poorly assembled porins into the outer membrane (Figs. 2 and S2).

While TpsB proteins, such as EtpB, are responsible for secreting TpsA proteins, e.g. EtpA, across the outer membrane (Fleckenstein et al., 2006; Meli et al., 2009) and BamA inserts many different OMPs into a lipid environment both recognise nascent $\beta$-strands and have a conserved VGRF motif in L6 (Delattre et al., 2010). However, our BamAENm barrel chimera failed to rescue BamA depletion in JWD3 (Fig. 3). Many of the recent crystal structures of BamA proteins demonstrate that the $\beta 1$ and $\beta 16$ $\beta$-strands of BamA $\beta$-barrels are unstably paired and it has been proposed that they separate to provide a template for OMP folding (Noinaj et al., 2013; 2014; Albrecht et al., 2014). Furthermore, it has been suggested that distortion and thinning of the lipid bilayer by the BamA $\beta$-barrel also facilitates OMP insertion (Noinaj et al., 2013; Gessmann et al., 2014). In contrast, TpsB barrels demonstrate a high degree of barrel stability at the $\beta 1$–$\beta 16$ interface, and thus, it is not surprising that the BamAENm chimera failed to rescue depletion (Clantin et al., 2007).

The POTRA domains act as the initial docking sites for chaperones and unfolded OMPs, as well as scaffolding the BAM lipoproteins and other associated proteins (Hagan et al., 2011; Webb et al., 2012). Therefore, we predicted that the majority of species-specificity would reside within the POTRA domains. While it is clear that the cellular levels of some POTRA chimeras were low (Fig. 4B), suggesting that the different POTRA domains affect the biogenesis or stability of the chimeric BamA proteins, several of the POTRA chimeras folded E. coli porins to levels approaching that of wild-type strains (Fig. 4B). We note that in the presence of arabinose, the levels of the POTRA chimeras are higher than in its absence (Fig. 4B). As chromosomally encoded BamAPa is expressed under these conditions in JWD3, this indicates that BamAPa is able to fold each chimeric protein more efficiently than when only each chimera is expressed. The gain-of-function chimeras also folded E. coli porins to levels approaching that of wild-type strains. The ability of these chimeras to assemble the porins suggests that in contrast to our initial hypothesis, no species specificity resides within the POTRA domains and the accessory lipoproteins, chaperones and nascent OMPs can interact with POTRA domains from diverse species to create a functional complex.

While the BamAPa POTRA chimera failed to rescue BamA depletion because of differences in POTRA and POTRAENm, the P. aeruginosa BamAPa1–4 POTRA chimera, which has P. aeruginosa POTRA1–4, could be made to function by introducing substitutions within the $\beta$-barrel (Figs. 7 and S5; Table 1). The locations of compensatory substitutions indicate that they will increase the flexibility of the BamAENm $\beta$-barrel, facilitating more efficient communication between the POTRA and barrel domains, while others will affect the pairing of $\beta 1$ and $\beta 16$, influencing barrel opening by lowering the activation energy of barrel unwrapping and facilitating strand invasion by the nascent OMP chain. Indeed, in one BamAENm structure, N805 stabilises the $\beta 16$ kink observed in the terminal $\beta$-strand (Noinaj et al., 2013; Albrecht et al., 2014). It is also of note that, L1 forms part of a pore by which the external loops of folding OMPs may exit BamA and so our substitutions could also affect this process (Noinaj et al., 2013; 2014; Albrecht et al., 2014; Ni et al., 2014).
Fig. 7. Mutational analysis of the BamA Pa1–4 POTRA chimera.

A. A topology model of the *Escherichia coli* BamA β-barrel (N422 to W810) derived from the *E. coli* BamA crystal structure (Albrecht et al., 2014). Amino acids within β-strand regions are shown as blue squares and those in external loops and periplasmic turns are shown as pink circles. Extracellular loops L1–L8 and periplasmic turns T1–T7 are indicated. The position of mutations, which enable the BamA Pa1–4 POTRA chimera to rescue BamA depletion in JWD3 cells on agar plates, is indicated (see Table 1). Images were prepared using PyMol (Schrodinger, 2010).

B. *E. coli* JWD3 cells, carrying BamA Pa1–4 POTRA chimeras cloned into pASK, were grown in Lennox broth supplemented with either arabinose (+ Ara) or fructose (−Ara). Blots were probed with anti-OmpF antiserum to detect OmpF, OmpC and OmpA, and a non-specific band is used as a loading control (LC).

D. Detection of *E. coli* porins after BamA depletion. The panel shows Western blots of normalised total cell protein from the JWD3 cells, carrying the various constructs in panel B, after 300 min of growth in Lennox broth supplemented with either arabinose (+ Ara) or fructose (−Ara). Blots were probed with anti-OmpF antiserum to detect OmpF, OmpC and OmpA, and a non-specific band is used as a loading control (LC).

E. The panels shows a model of the *E. coli* BamA β-barrel, based on the BamA Ec barrel structure (4C4V), where the missing L6 loop is replaced by L6 from the alternative BamA Ec structure (4N75) and the missing C-terminal residues are modelled in (Albrecht et al., 2014; Ni et al., 2014). A larger internal view of the barrel, focusing on the juxtaposition of L1, L6 and [J16 is shown. The location of mutations, which enable the BamA Pa1–4 POTRA chimera to rescue BamA depletion in JWD3 cells on agar plates, is indicated (see Table 1). Images were prepared using PyMol (Schrodinger, 2010).

In conclusion, our results demonstrate that the level of BamA expression is critical for the BAM to function, and that defects in the POTRA domains can be compensated for by substitutions within the barrel. The latter data suggest that the POTRA and barrel domains communicate during OMP biogenesis and that their interactions must be fine-tuned for efficient OMP folding. Importantly, our data reveal there is no strong amino acid template within BamA that confers species specificity for particular OMPs.

**Experimental procedures**

**Bacterial strains, growth conditions, plasmids and primers**

The bacterial strains, plasmids, DNA fragments and primers used in this study are detailed in Table S1. RLG221 was used as a standard *E. coli* K-12 strain throughout and all bacteria were cultured in Lennox broth [2% (w/v) peptone (Merck, Kenilworth, NJ, USA), 1% (w/v) yeast extract (Fisher Scientific, Loughborough, UK) and 170 mM NaCl] (Squire et al., 2010) and on nutrient agar (Oxoid, Basingstoke, Hampshire, UK). Ampicillin (100 μg ml⁻¹) was included in media where appropriate. Low-level protein expression was induced in cells carrying pASK plasmid derivatives by the addition of anhydrotetracycline to 2 ng ml⁻¹.

To determine the ability of plasmid constructs to rescue BamA depletion on solid media, the *E. coli* BamA depletion strain JWD3 was grown on agar plates in the presence or absence of 0.2% (w/v) arabinose (Lehr et al., 2010). To assess this in liquid media, JWD3 cells were grown in 50 ml of Lennox broth at 37 °C with shaking in the presence of 0.05% (w/v) arabinose or 0.05% (w/v) fructose, as a control, and optical density (OD₆₀₀) was monitored over time. After 300 min growth, cultures were sampled for analysis. All growth curves were done at least twice and representative curves are shown in figures. If constructs failed to rescue depletion in the presence of fructose, no further growth was detected after this point.

**Plasmid construction**

The DNA encoding each BamA orthologue was synthesised by Genscript (http://www.genscript.com) and cloned into pET17b using Ndel and XhoI. Each orthologue construct possessed the DNA encoding for the *E. coli* BamA signal sequence, ensuring efficient transit across the inner membrane, and were codon optimised for high-level expression in *E. coli* (Fig. S1 and Table S1). To aid gene manipulation, each ORF was purged of restriction sites and unique sites for Ndel, Nhel, BamHI and XhoI were introduced to facilitate easy swapping of the POTRA and barrel domains (Fig. S1). POTRA and barrel chimeras were, therefore, generated by subcloning the relevant Ndel-BamHI and BamHI-XhoI DNA fragments into pET17b/bamAEc. The POTRA and barrel chimeras are designated BamAEC and BamAXE, respectively, where X denotes the initials of the bacterial species from which the POTRA or barrel domains derives. pASK derivatives, carrying the DNA encoding BamA POTRA chimeras, were generated using PCR. DNA was amplified using the relevant pET17b construct as template, with primers BamABsaI and PetTerm. PCR products were cloned into pASK using BamHI and verified by DNA sequencing. All DNA constructs were verified by DNA sequencing.

The BamAEC barrel chimera construct was generated using PCR. The DNA encoding the EtpB barrel was amplified using primers EtpBUp and EtpBDown, with plasmid pJM1002 as template (Fleckenstein et al., 2006). Product was restricted with BamHI and XhoI, cloned into pET17b/bamAEc, and verified by DNA sequencing.

The swapping of individual POTRA motifs between BamAXE and BamAEC was achieved using mega-primer PCR (Sarkar and Sommer, 1990; Rossiter et al., 2011a). For POTRA chimeras BamAEC4, BamAXE3, BamAEC2 and BamAXE4 the first-round PCR product was generated using primer...
BamA1372Rev and primers Pa4Ec5, Pa3Ec4, Pa2Ec3 and Pa1Ec2 with pET17b/bamAEC as template. PCR products were used in a second round of PCR with primer PetPro and the POTRA chimera construct pET17b/bamAEC as template. For POTRA chimera BamAEC, the first-round PCR used primers Ec1Pa2 and BamA1372Rev with pET17b/bamAEC as template and primer PetPro and pET17b/bamAEC. In the second round PCR, Chimeras BamAEC and BamAEd were generated using primers BamA1372Rev and either Ec3Pa4 or Ec4Pa5 with pET17b/bamAEC as template. In the second round, PCR products were used with primer PetPro and pASK/bamA1–2 and pASK/bamA1–3, respectively, as template. All PCR products were cloned into pASK/bamAEC using NheI and BamH1 and verified by DNA sequencing.

Sample preparation and Western blotting

JWD3 cells, carrying various pET17b and pASK constructs were grown in 50 ml of Lennox broth at 37°C with shaking for 300 min in the presence of 0.05% (w/v) arabinose or fructose. The preparation of normalised total cellular protein samples, isolation of membrane fractions from cultures and the washing of membranes with urea were carried out as detailed Browning et al. (2013). Protein samples were resolved by SDS-PAGE and analysed using Western blotting as in (Rossiter et al., 2011b). E. coli BamA protein was detected using anti-E. coli POTRA BamA antiserum (Rossiter et al., 2011b), P. aeruginosa BamA using anti-BamA antiserum, OmpF, OmpC and OmpA proteins were detected using anti-OmpF antiserum and NarL using anti-NarL antibodies, all raised in rabbit. N-terminal His tags were detected using anti-His tag mouse monoclonal antibodies (Sigma-Aldrich, Gillingham, Dorset, UK). Blots were developed using the ECL Western Blotting Detection System (GE Healthcare, Little Chalfont, Buckinghamshire, UK).

Structural modelling of BamA

To generate the full-length composite model of the E. coli BamAEC protein (GenBank AAC73288), we used the X-ray barrel structure of BamAEC (4C4V) (Albrecht et al., 2014), splicing in the missing L6 loop from the alternative BamEC structure (4N75) (Ni et al., 2014) and manually building the missing C-terminal residues using Coot (Emsley et al., 2010). I-TASSER (Roy et al., 2010) was used with specific templates (3OG5) (Gatzeva-Topalova et al., 2010) to generate an additional model for POTRA1–5, arranged according to the full-length N. gonorrhoeae BamA90 POTRA orientation (4K3B) (Noinaj et al., 2014). The final models were manually optimised using Coot (Emsley et al., 2010). Structural superposition has been performed using Gesamt and SSM as implemented in CCP4 suite (Winn et al., 2011). Structural visualisations were done with PyMOL (Schrödinger, 2010).

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Conflict of interest

The authors declare no conflict of interest.

References


Supporting information

Additional supporting information may be found in the online version of this article at the publisher’s web-site.