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Dissection of β -barrel Outer Membrane Protein Assembly Pathways through Characterizing BamA POTRA 1 Mutants of *Escherichia coli*

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Summary

BamA of *Escherichia coli* is an essential component of the hetero-oligomeric machinery that mediates β -barrel outer membrane protein (OMP) assembly. The C- and N-termini of BamA fold into trans-membrane β -barrel and five soluble POTRA domains, respectively. Detailed characterization of BamA POTRA 1 missense and deletion mutants revealed two competing OMP assembly pathways, one of which is followed by the archetypal trimeric β -barrel OMPs, OmpF and LamB, and is dependent on POTRA 1. Interestingly, our data suggest that BamA also requires its POTRA 1 domain for proper assembly. The second pathway is independent of POTRA 1 and is exemplified by TolC. Site-specific cross-linking analysis revealed that the POTRA 1 domain of BamA interacts with SurA, a periplasmic chaperone required for the assembly of OmpF and LamB, but not that of TolC and BamA. The data suggest that SurA and BamA POTRA 1 domain function in concert to assist folding and assembly of most β -barrel OMPs except for TolC, which folds into a unique soluble α -helical barrel and an OM-anchored β -barrel. The two assembly pathways finally merge at some step beyond POTRA 1 but presumably before membrane insertion, which is thought to be catalyzed by the trans-membrane β -barrel domain of Bam A.

Introduction

Assembly of β -barrel outer membrane proteins (OMPs) in *Escherichia coli* is facilitated by the Bam complex, comprised of five proteins: BamABCDE, of which BamA and BamD are essential (Wu *et al.*, 2005; Doerrler and Raetz, 2005; Werner and Misra, 2005; Malinverni *et al.*, 2006; Sklar *et al.*, 2007a). BamA is a member of the Omp85 family of proteins that are essential for OMP biogenesis in bacteria, mitochondria, and chloroplast (Gentle *et al.*, 2004; Knowles *et al.*, 2009). BamA folds into two distinct domains: an N-terminal soluble domain assembled from five POTRA (polypeptide translocation associated) domains and a C-terminal trans-membrane domain assumed to fold as a β -barrel. High-resolution structures of the BamA POTRA domains have been determined (Kim *et al.*, 2007; Knowles *et al.*, 2008; Gatzeva-Topalova *et al.*, 2008). Experimental data have indicated the involvement of POTRA domains in assisting β -barrel OMP assembly (Kim *et al.*, 2007; Knowles *et al.*, 2008). It has been proposed that POTRA-OMP interactions may be mediated through β augmentation, in which a β -strand of the POTRA domain is augmented by the addition of a β -strand from the substrate (Kim *et al.*, 2007; Knowles *et al.*, 2008). These initial interactions could then lead to the assembly of the entire OMP β -barrel, followed by its

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insertion into the outer membrane presumably with the help of the β -barrel domain of BamA.

Prior to interacting with the Bam complex, the nascent mature OMP polypeptides recently released into the periplasm by the Sec machinery must stay assembly competent. For this, most β -barrel OMPs rely on a major periplasmic chaperone SurA (Lazar and Kolter, 1996; Rouvière and Gross, 1996; Vertommen *et al.*, 2009; this study). However, SurA is not essential despite a severe effect on OMP biogenesis in a *surA* null mutant, indicating the existence of alternative but less efficient pathways for keeping OMPs assembly competent (for reviews, see Mogensen and Otzen, 2005; Ruiz *et al.*, 2006a; Knowles *et al.*, 2009). It has been shown that SurA interacts with BamA (Sklar *et al.*, 2007b; Vuong *et al.*, 2008), and this interaction presumably results in the handing over of SurA-bound OMPs to BamA for assembly and insertion into the outer membrane. Genetic analysis revealed that deletion of the POTRA 1 domain does not disrupt the interactions of BamA with the BamBCDE lipoproteins, but drastically reduces the level of β -barrel OMPs (Kim *et al.*, 2007). Therefore, it is conceivable that the BamA Δ POTRA 1 mutant is defective in its ability to receive SurA bound to OMPs.

Aberrant OMP assembly induces envelope stress response, which activates sigma E and Cpx regulons (Gerken *et al.*, 2010). The members of these regulons include genes that encode for OMP assembly factors, such as the Bam complex proteins and SurA, a major periplasmic protease DegP, and small regulator RNAs that inhibit translation of OMP mRNA (for reviews, see Raivio and Silhavy, 1999; Johansen *et al.*, 2006). The collective actions of these factors help reduce envelope stress by enhancing folding/degradation of OMPs and inhibiting their synthesis.

Suppressor analysis has been a valuable tool for identifying genes involved in the biogenesis of outer membrane components. One of the genes identified through this analysis was *lptD* (*imp*) (Sampson *et al.*, 1989). It is now known that LptD (Imp) is involved in LPS transport (Bos *et al.*, 2003). Suppressors of an *imp* allele, *imp4213*, led to the isolation of null mutations in *bamB* (Eggert *et al.*, 2001) and a mutation affecting the POTRA 3 domain of BamA (Ruiz *et al.*, 2006). In this study we sought suppressors of a different *imp* allele than previously used, in an attempt to isolate novel mutations in genes involved in outer membrane biogenesis. One such suppressor mutation identified a novel allele of *bamA*, *bamA66*, which resulted in the deletion of a single residue from the POTRA 1 domain.

Cells expressing BamA Δ R64 from a low-copy-number plasmid displayed a dramatic defect in the biogenesis of most β -barrel OMPs except that of TolC. Data showed that BamA Δ R64 is severely defective in its ability to interact with SurA. Consistent with this, the POTRA 1 domain of BamA was shown to interact with SurA in close proximity to R64, the POTRA 1 residue that was deleted in BamA Δ R64. Analysis of BamA Δ R64 and a POTRA 1 deletion mutant, BamA Δ R36-K89, allowed the dissection of two distinct β -barrel OMP assembly pathways, one of which is dependent on SurA-POTRA 1 interactions and is followed by most β -barrel OMPs, while the other, which is independent of SurA-POTRA 1 interactions, is followed by TolC. BamA's independence from SurA suggests that it too belongs to the TolC pathway. Both pathways ultimately merge, and in the case of TolC past POTRA 1, for BamA-dependent insertion into the outer membrane.

Results

Isolation and initial characterization of BamA Δ R64

The *imp208* allele of *lptD* (*imp*) confers hypersensitivity towards a variety of noxious compounds (Sampson *et al.*, 1989). This hypersensitivity phenotype was used to isolate

suppressor mutations by growing cultures on medium containing bacitracin. Though the majority of suppressor mutations conferring bacitracin resistance identified null alleles of either *yfgM* or *bamB* or missense mutations within *imp208* (E. Charlson, E. Coon, and R. Misra, unpublished data), in revertant number 66 the suppressor mutation was found to be genetically linked to *bamA*. DNA sequence analysis of the entire *bamA* gene from this revertant revealed a deletion of the R64 codon within the $\alpha 2$ helix of the POTRA 1 domain of BamA. This mutant *bamA* allele is named *bamA66* and the protein is referred to as BamA Δ R64. In an *lptD*⁺ (*imp*⁺) background, *bamA66* displays a slight sensitivity to vancomycin and rifampin and causes a moderate reduction (20–30%) in OMP levels, but is synthetically lethal with the depletion of BamB, conditionally lethal in the absence of DegP or SurA; i.e., no growth at or above 37°C and 40°C, respectively. All of these observations point to an OMP biogenesis defect of BamA Δ R64 that is exacerbated in the absence of other OMP assembly factors.

Low expression of BamA Δ R64 confers acute phenotypes

To further assist in the investigation of BamA Δ R64's defects, we introduced the chromosomal *bamA66* allele into a low-copy number plasmid pZS21-*bamA*_{6His} (Kim *et al.*, 2007). The presence of a 6×His tag at the N-terminal end of BamA is useful for affinity purification to study protein-protein interactions and the detection of BamA. Additionally, the low level constitutive expression of *bamA* from pZS21 is driven from the *tet* promoter, thus making BamA synthesis from this construct impervious to σ^E -mediated regulation. This uncoupling of σ^E -mediated up-regulation of BamA synthesis during envelope stress proved to be important in dissecting BamA Δ R64's functional defect (see below), which otherwise remains masked to some degree when BamA Δ R64 is expressed from its native, σ^E -regulated, chromosomal locus. Construction of strains harboring pZS21-*bamA* and pZS21-*bamA66* plasmids required transformation into a Δ *bamA* strain expressing BamA from an arabinose-inducible P_{BAD} promoter of a pBAD33-*bamA* plasmid. Subsequent curing of pBAD33-*bamA* was readily achieved from the Δ *bamA* (pZS21-*bamA*) strain at 37°C; however, curing of pBAD33-*bamA* from the Δ *bamA* (pZS21-*bamA66*) strain was only possible at 30°C. Due to the *ts* phenotype of the Δ *bamA* (pZS21-*bamA66*) strain, all subsequent experiments were carried out at 30°C unless otherwise stated.

Effects of BamA Δ R64 on β -barrel OMP biogenesis

The acute conditional lethal phenotype of *bamA66* when expressed from pZS21 indicated a severe OMP biogenesis defect associated with the reduced synthesis of BamA Δ R64. To assess the extent of the OMP biogenesis defects we analyzed envelope proteins enriched for integral membrane proteins, including OMPs, by two-dimensional isoelectric focusing/SDS-PAGE. Images of individual gels from *bamA*⁺ and *bamA66* protein samples in Figs. 1A and B, respectively, were overlaid in Fig. 1C to aid in direct relative comparison. A total of thirty protein spots (including isoforms labeled as a, b or c), numbered in Figs. 1A and B, and listed in Fig. 1E, were identified by MS/MS analysis, of which twenty belong to fifteen different OMP species (Fig. 1C). The level of fourteen OMPs, including BamA Δ R64, were down significantly in the *bamA66* (pZS21) mutant (Figs. 1C and D). Strikingly, intensities of the three TolC spots increased 6.0, 6.9 and 4.9 fold (p values of 0.0009, 0.009, and 0.000; n=2) in the *bamA66* (pZS21) strain compared to the *bamA*⁺ (pZS21) strain. For better visualization of BamA and LptD (Imp) spots, this region of the gel was enlarged in Fig. 1D. Note that the LptD (Imp) spot is readily detectable by Coomassie blue staining in wild type strain samples where BamA is expressed from its normal chromosomal copy but only weakly when it is expressed from pZS21 (Fig. 1D). We believe that this difference is due to reduced BamA expression from pZS21 (Fig. 1D), which in turn lowers OMP levels in general. The migration of certain OMPs, including BamA, OmpA, LamB, and TolC, as multiple spots during two-dimensional gel electrophoresis is a commonly observed

phenomenon, and such heterogeneity is attributed to the existent of differently charged isoforms (Molloy *et al.*, 2000) or insufficiently denatured species (Berven *et al.*, 2003).

We also fractionated envelopes by sucrose density gradients and determined OMP levels by Coomassie blue staining and Western blots (Fig. S1 and Fig. S2). In the parental strains (pZS21-*bamA*⁺) the major β -barrel OMPs—OmpC, OmpF, and OmpA—were present in fractions with buoyant density of 1.226 g/Cm³, which are typical values for the wild type outer membrane (Fig. S1 and Fig. S2A). However, in the mutant strain (pZS21-*bamA66*) these proteins peaked in fractions of a much lower buoyant density (1.181 g/Cm³) owing to a profound defect in the biogenesis of β -barrel OMPs (Fig. S1 and Fig. S2A). TolC and LptD (Imp) were analyzed by Western blots from peak outer membrane fractions. The level of TolC in the outer membrane fractions of the *bamA66* (pZS21) mutant was substantially greater than that present in the outer membrane fractions of the wild type *bamA* (pZS21) strain (Fig. S2B). Considerably lower levels of LptD (Imp) were detected in the outer membrane fractions of *bamA66* (pZS21) envelopes (Fig. S2C). To evaluate relative effects of *bamA66* (pZS21) and Δ *surA* on OMPs, we also analyzed OMPs from wild type and Δ *surA* strains grown at 30°C by 2D gels and sucrose density gradients and found the effects of Δ *surA* on OMPs to be similar to that exerted by *bamA66* (pZS21) (Fig. S1 and Fig. S3).

The migration of several proteins as multiple spots during isoelectric focusing (Fig. 1) and their presence in multiple fractions during sucrose gradient analysis (Fig. S1 and Fig. S2) made it somewhat difficult to assess the extent of *bamA66* effect on various OMPs. To circumvent this, we performed a side-by-side comparison of TolC and BamA with four other β -barrel OMPs using equal amounts of proteins from purified cell envelopes (Fig. 2). Consistent with the data presented in Fig. 1 and Fig. S2, TolC levels were higher in the *bamA* mutant (pZS21-*bamA66*) than in the parental strain (pZS21-*bamA*⁺) (Fig. 2A; lanes 1 and 2). In contrast to elevated TolC levels, the levels of LamB, OmpA, OmpC, and OmpF in the *bamA* mutant decreased significantly while no changes in the level of AcrA, a control inner membrane protein, were observed (Fig. 2A and B; lanes 1 and 2). Interestingly, an increase in TolC level was no longer apparent when the mutant BamA Δ R64 protein was expressed from the arabinose-inducible promoter of pBAD33 or synthesized from its native chromosome promoter (Fig. 2A and B; lanes 3–4 and 5–6). Thus the severity of the BamA Δ R64's effect on OMPs was connected to its expression levels, which were lowest from pZS21 and highest from pBAD33 (Fig. 2C). Together these analyses showed that low expression of a mutant BamA protein negatively affected the biogenesis of several β -barrel OMPs, including BamA, LamB, LptD (Imp), OmpA, OmpC, and OmpF, but not that of TolC. OMP data from *surA*⁺ and Δ *surA* strains are shown in Fig. 2, Fig. S1 and Fig. S3. Although the overall effects of Δ *surA* on OMPs were similar to that of *bamA66* (pZS21), the level of BamA, expressed from its native chromosomal location, went up in the Δ *surA* strain compared to the isogenic *surA*⁺ strain (Fig. 2C; lanes 7–8).

Reverse transcription quantitative PCR analyses to assess gene expression levels

We quantified the levels of *tolC* mRNA, through RT-qPCR, to determine whether transcription or a post transcription process in TolC biogenesis is enhanced when the biogenesis of other β -barrel OMPs is severely impaired in the *bamA66* (pZS21) mutant (Fig. 1, Fig. 2, Fig. S1, and Fig. S2). The expression of *tolC* remained unchanged in either mutant (Fig. 3), indicating that increased TolC levels in the mutant backgrounds reflect benefit TolC incurs at a post-transcriptional step of its biogenesis. In contrast to *tolC*, the expression of *ompF* and *lamB*, which are indirectly regulated by the activated σ^E system, was significantly impaired in the mutant strain (Fig. 3). Expression of the two σ^E -regulated genes, *lptD* (*imp*) and *degP*, went up considerably in the *bamA66* (pZS21) mutant, with no significant changes in the expression of *bamA* from the pZS21 plasmid (Fig. 3). Since the transcription of *bamA66* is similar to wild type *bamA* when expressed from pZS21, the low BamA Δ R64

levels seen in Fig. 1 and Fig. 2 are likely due to a defect in some post-transcriptional step, such as protein folding or assembly. It is important to note that despite increased expression of *lptD* (*imp*) in the *bamA66* (pZS21) mutant, the levels of LptD (Imp) were significantly down (Fig. S2C), indicating that the assembly of LptD (Imp) depends on BamA POTRA 1. A parallel analysis in a *surA* mutant background showed that when gene expression was affected, the effects were somewhat milder than that seen in a *bamA66* (pZS21) background (Fig. 3).

Interestingly, the expression of *cpxP*, a gene regulated by the CpxAR two-component signal transduction system, went up in the mutant BamA background (Fig. 3). This is consistent with our recent observation that the CpxAR two-component system is not only activated in response to aberrant β -barrel OMP assembly, but is necessary to reduce envelope stress under these conditions (Gerken *et al.*, 2010). Thus, an increase in *degP* expression is likely the result of the combined effect of activated σ^E and CpxAR regulons.

Distinguishing the effects of *bamA66* (pZS21) on OMP assembly from synthesis

The above analysis indicated that the assembly, but not the synthesis of BamA Δ R64 and LptD (Imp) was compromised in a *bamA66* (pZS21) background. However, since the synthesis of OmpF and LamB was drastically inhibited in the mutant BamA background (Fig. 3), reduced OmpF and LamB levels in Fig. 1 and Fig. 2 (lanes 1 and 2) reflect a sum total of defects in synthesis and assembly. In order to distinguish the effects of *bamA66* (pZS21) on OmpF and LamB assembly from their synthesis, we synthesized these proteins free from any native regulation by expressing them under the transcription/translation control of the pBAD24 plasmid. To verify the σ^E -independent expression of OmpF and LamB from pBAD24-*ompF* and pBAD24-*lamB*, these plasmids were transformed into wild type and Δ *rseA* backgrounds. The synthesis of most β -barrel OMPs produced under their native transcription/translation control is expected to be severely inhibited in the absence of RseA, the negative regulator of σ^E . Indeed, the levels of chromosomally expressed LamB, OmpA, OmpC, and OmpF were dramatically reduced in a Δ *rseA* background (Fig. 4A). In contrast, no significant change in OmpF and LamB levels were observed when they were expressed from pBAD24 plasmids (Fig. 4A). Note that pBAD24-*ompF* and pBAD24-*lamB* also lack OmpR and CpxR binding sites, and hence the activated forms of these regulatory proteins during envelope stress cannot influence plasmid-borne OmpF and LamB synthesis.

Plasmids expressing OmpF and LamB free of σ^E -mediated down-regulation were introduced in the *bamA66* (pZS21) background deleted of the chromosomal copy of *ompF* or *lamB* so that plasmid-borne OmpF and LamB levels could be monitored independent from their chromosomally expressed levels. OmpF and LamB expression from plasmids was optimized so as to closely match the levels produced from the chromosomal genes. In the *bamA66* (pZS21) background, the plasmid-borne OmpF and LamB levels were reduced by over 3 fold (Figs. 4B and C). Expectedly, the effects of *bamA66* (pZS21) on these proteins were less severe than when they were expressed under their native transcription/translation control elements, because in addition to assembly their synthesis was inhibited by the activated σ^E regulon (Fig. 3). Lanes 1 and 2 of Figs. 4B and C show a combined assembly and synthesis defect in a *bamA66* (pZS21) background, resulting in 25 and 12 fold reduction in OmpF and LamB levels, respectively.

As a control, we examined the expression of a periplasmic protein, PhoA, produced under the identical transcription/translation control of pBAD24 as that of pBAD24-*ompF* and pBAD24-*lamB*. No appreciable effect of *bamA66* (pZS21) on plasmid-borne PhoA was observed in either a *bamA66* (pZS21) Δ *ompF* or *bamA66* (pZS21) Δ *lamB* background (Figs. 4B and C), indicating that the negative effect of *bamA66* (pZS21) was specific to the β -barrel OMPs, OmpF and LamB, and was not caused by their reduced expression from the

plasmid replicon. These results showed that although the bulk of the effect of *bamA66* (pZS21) on the chromosomally-expressed OmpF and LamB proteins is inflicted at their synthesis, the rendering of OMP synthesis immune to such regulatory inhibition exposes the residual effect of BamA Δ R64 on OmpF and LamB assembly. From this analysis we conclude that the assembly of OmpF and LamB, like that of LptD (Imp), depends on the wild type POTRA 1 domain of BamA.

Biogenesis of TolC depends on BamA even in a *bamA66* (pBAD33) background

It was intriguing that while the biogenesis of fourteen different β -barrel OMPs was severely impaired in a *bamA66* (pZS21) background, TolC levels went up significantly (Fig. 1, Fig. 2, and Fig. S2). This at first appears at odds with our earlier observations showing that TolC assembles in a BamA/BamD-dependent manner (Werner and Misra, 2005; Malinverni *et al.*, 2006). Why then was TolC biogenesis not impaired in the mutant BamA Δ R64 background? We considered the possibility that TolC, which assembles independent of SurA, may also bypass the POTRA 1 domain of BamA but rely on other POTRA domains and/or the β -barrel domain of BamA for insertion into the outer membrane. If so, TolC's biogenesis will meet the same fate as other β -barrel OMPs when BamA Δ R64 is depleted from the cell. Since BamA Δ R64 from pZS21-*bamA66* is constitutively expressed at low levels, we opted to use a Δ *bamA* strain whose survival is dependent on *bamA66* expression from the arabinose-inducible promoter of pBAD33.

An overnight culture of Δ *bamA* (pBAD33-*bamA66*) grown at 30°C in LB (0.1% arabinose) was diluted 1:100 in LB supplemented with or without arabinose and growth was resumed at 30°C. When the cultures reached early stationary growth phase (OD₆₀₀ of 1.0) they were diluted 1:50 with the same respective fresh medium and growth was resumed (Fig. 5A). Throughout the growth period, aliquots of bacterial samples were withdrawn and OMPs were analyzed by Western blot from cell amounts equalized based on OD₆₀₀. TolC and LamB were readily detected from cells grown in the presence of arabinose (Fig. 5B). However, LamB levels from BamA Δ R64-depleted cells (i.e., grown in the absence of arabinose) collected at 3, 4 and 5 hours after the first dilution dropped dramatically (Fig. 5B). In contrast to LamB, no significant reduction in TolC levels was noted during the same time points of BamA Δ R64 depletion. It should be noted that because LamB synthesis, unlike that of TolC, is subject to inhibition by the activated σ^E regulon (Fig. 3), a drop in LamB levels prior to that of TolC was to be expected. When BamA Δ R64 was further depleted after the second dilution, LamB became undetectable and TolC levels also dropped dramatically (Fig. 5B; hours 9 to 11 without arabinose). Since at these later time points cells ceased to grow without arabinose, a drop in TolC level is unlikely due to dilution of TolC in newly born cells. Biogenesis of OmpC, OmpF, and OmpA followed the same trend as that of LamB (data not shown). The levels of AcrA, an inner membrane-anchored periplasmic protein, did not decrease during the entire growth period.

These results reiterated our previous conclusions that the biogenesis of TolC, like other β -barrel OMPs studied here, is ultimately dependent on BamA though the dependence of the two groups of OMPs on BamA appears to be different: OmpF, LamB, and LptD (Imp) require wild type POTRA 1 for their assembly while TolC does not. However, subsequent to the POTRA 1 step, the two assembly pathways appear to converge perhaps at the point of BamA's β -barrel domain, which may aid in the insertion of all β -barrel OMPs.

A BamA POTRA 1 deletion mutant produces similar albeit a more severe phenotype than BamA Δ R64

Though the expression of BamA Δ R64 from a low-copy-number plasmid (pZS21) had a severe negative effect on most β -OMPs, TolC biogenesis was not inhibited. It is possible

that despite the low levels of BamA Δ R64, it retains some residual POTRA 1 activity that is sufficient for TolC assembly but not that of other β -barrel OMPs. We therefore constructed a POTRA 1 deletion mutant (BamA Δ R36-K89) lacking R36 to K89 residues of the POTRA 1 domain of BamA (A21 to R91). Though the mutant lost most of the POTRA 1 domain, it retained the first 15 residues of mature BamA so as to facilitate its detection by antibodies raised against the A₂₁-Q₃₅ BamA peptide.

The expression of BamA Δ R36-K89 without concurrent expression of wild type BamA from the chromosome or a compatible plasmid replicon did not support bacterial growth on LBA at any growth temperature. However, on glycerol minimal medium at 30°C we were able to delete the chromosomal wild type *bamA* gene as long as the expression of BamA Δ R36-K89 was fully induced from pBAD33 by the addition of arabinose. This conditional phenotype allowed us to examine the effects BamA Δ R36-K89 on rich medium under wild type BamA depletion conditions and on minimal medium under steady state growth in the complete absence of wild type BamA. We first carried out wild type BamA depletion experiments with strains expressing wild type *bamA* from the pBAD33 replicon and a second copy of the wild type or mutant *bamA* allele from a compatible plasmid replicon of pZS21. As expected, in the presence of pZS21*bamA*, depletion of wild type BamA from pBAD33 by the addition of fucose had no negative effect on LamB levels (Fig. 6A), since constitutive expression of wild type BamA from pZS21 provided adequate activity for LamB assembly (also see Fig. 2). In contrast, depletion of wild type BamA from cells expressing BamA Δ R64 or BamA Δ R36-K89 from pZS21 led to an immediate reduction in LamB levels after the second dilution in medium supplemented with fucose, with the effect on LamB during these growth periods being more severe in cells expressing BamA Δ R36-K89 than BamA Δ R64 (Figs. 6B and C). However, despite the severe negative effect of BamA Δ R36-K89 on LamB, no significant alterations in TolC levels were observed (Figs. 6B and C). We also examined the effects of BamA Δ R36-K89 on OMPs under steady-state growth conditions and in the complete absence of wild type BamA (Fig. S4). Cultures for this experiment had to be grown on glycerol minimal medium at 30°C, since strains expressing BamA Δ R36-K89 as the sole BamA species cannot grow on rich medium. The data showed normal TolC biogenesis in cell expressing BamA Δ R36-K89, while the biogenesis of LamB, LptD, OmpA, OmpC, and OmpF was adversely affected (Fig. S4). These results showed that TolC assembles independent of the POTRA 1 domain of BamA.

Assembly of BamA depends on its POTRA 1 domain

We consistently observed that the steady-state levels of the two BamA POTRA 1 mutant proteins were significantly lower than that of wild type BamA (Fig. 1, Fig. 2, and Fig. S4). This prompted us to consider a possibility that the two BamA mutants not only interfere with the assembly of several other β -barrel OMPs, but also their own. To test this possibility, we took advantage of the experiment in which wild type BamA was depleted from strains concomitantly expressing either BamA Δ R64 or BamA Δ R36-K89 from an arabinose- and σ^E -independent promoter of pZS21 (Fig. 6B and C). We reasoned that if the mutant BamA proteins are defective in their own assembly, then as the wild type BamA protein is being depleted by the addition of fucose, the levels of mutant BamA proteins may also decrease together with the levels of other BamA POTRA 1-dependent β -barrel OMPs.

We chose to analyze protein samples from strains expressing wild type BamA from pBAD33 and BamA Δ R36-K89 from pZS21 because BamA Δ R36-K89 confers a more severe OMP biogenesis defect than BamA Δ R64 and migrates faster than wild type BamA on a polyacrylamide gel, thus allowing for better detection and quantification. Moreover, BamA Δ R36-K89 can be readily detected by antibodies raised against the first 15 mature BamA residues that are still present in the mutant protein. In samples obtained at 3, 4, and 5 hours of growth after first dilution, the levels of BamA Δ R36-K89 in the presence of

arabinose, which allowed wild type BamA synthesis, or fucose, which inhibited wild type BamA synthesis, were very similar (Fig. 6D). Thus, during these growth periods neither the level of mutant BamA (Fig. 6D) nor that of LamB (Fig. 6C) was affected, indicating the persistence of low levels of wild type BamA sufficient for OMP assembly. However, after diluting the culture a second time the level of BamA Δ R36-K89 in the presence of fucose dropped significantly compared to its level in the presence of arabinose (Fig. 6D). This decrease in BamA Δ R36-K89 levels coincided with a significant drop in LamB levels (Fig. 6C), reflecting an OMP assembly defective environment. We surmise from these results that the assembly of BamA, like that of several other β -barrel OMPs, is dependent on the POTRA 1 domain. Note that the BamA A₂₁-Q₃₅ peptide antibodies used in Fig. 6D recognize BamA Δ R36-K89 better than wild type BamA. Therefore, it is not possible to make a direct quantitative comparison between the mutant and wild type BamA proteins.

Region encompassing R64 of POTRA 1 α 2 interacts with SurA

Although two independent studies have shown that SurA interacts with BamA in a cross-linker-dependent manner (Sklar *et al.*, 2007b; Voung *et al.*, 2008), it has not been determined where in BamA these interactions occur. A previous mutagenesis analysis revealed that deletion of the POTRA 1 domain causes an acute OMP biogenesis defect without affecting mutant BamA's interactions with the Bam lipoproteins (Kim *et al.*, 2007). Thus the defect observed in the POTRA 1 deletion mutant might be due to impaired interactions with SurA. Consistent with this, BamA Δ R64, in which R64 the α 2 helix of POTRA 1 has been deleted, confers OMP biogenesis defects similar to that seen in a Δ surA mutant (Fig. 2, Fig. S1 and Fig. S3). Therefore, it is possible that BamA Δ R64 is defective in interacting with SurA.

To test this possibility, we first set out to determine whether the POTRA 1 domain of BamA interacts with SurA. We generated eight variants of BamA in which D28, R49, R64 or D74 was substituted with alanine and cysteine to assess the importance of these charged residues in BamA's function (alanine substitutions) and to conduct SPDP-mediated site-specific cross-linking. SPDP, a membrane permeable cross-linker with a 6.8 Å spacer arm length, mediates amine-to-sulfhydryl cross links. The charged side chains of the four POTRA 1 residues, D28, R49, R64, and D74, which are relatively well conserved among Omp85 orthologs (Voulhoux *et al.*, 2003), project outward and in different directions from each other (Fig. 7A). None of the alanine/cysteine substitutions produced a phenotype, i.e., vancomycin sensitivity or synthetic/conditional phenotype in a Δ bamB background, indicating that the mutant BamA proteins maintained normal structure-function.

In parallel with SPDP, we also carried out DSP-mediated cross-linking analysis because we anticipated that DSP-mediated BamA-SurA cross-linking should be identical in all mutants since no amine-reactive group was altered in the four BamA derivatives (DSP mediates amine-to-amine cross links). We pulled down BamA-SurA complexes via BamB_{6His}, which forms stable complexes with BamA in a cross-linker-independent manner (Vuong *et al.*, 2008). Freshly grown bacterial cultures were divided into three aliquots: two of which were treated with DSP or SPDP, while the third one received no cross-linker. After cross-linking, cells were lysed and BamB_{6His} was immuno-precipitated using 6 $\times$ His-specific antibodies. Eluates, containing BamB_{6His} and proteins cross-linked with it, were subjected to Western blot analysis and BamA and SurA were detected using TolC/BamA and SurA antibodies, respectively, and BamB by HisProbe-HRP (Fig. 7B).

In all cases, regardless of the absence or presence of a cross-linker, BamB_{6His} was able to pull down similar amounts of BamA (Fig. 7B, top panel). When DSP was used for cross-linking, wild type BamA and its four cysteine-derivatives pulled down SurA with similar efficiencies (Fig. 7B, bottom panel). However, when SPDP was used even the wild type

BamA protein, which contains two natural cysteine residues in its β -barrel domain, pulled down SurA, albeit not as strongly as when DSP was used: the ratio of SPDP- to DSP-mediated SurA from three independent reactions was 0.4 ± 0.1 . The same was true for BamA_{D28C} and BamA_{R49C}, showing SPDP- to DSP-mediated SurA ratios of 0.5 ± 0.2 and 0.4 ± 0.2 , respectively. This ratio slightly increased in the case of BamA_{D74C} (0.7 ± 0.2) but remained below 1, showing no significant SPDP-specific cross-linking mediated by D74C. In contrast, the SPDP-mediated cross-linking of SurA by BamA_{R64C} was consistently greater than that mediated by DSP; at 2.1 ± 0.8 this ratio was three to five times higher compared to wild type BamA or the three other cysteine derivatives. We also calculated SPDP/DSP ratios of SurA as a function of BamA, which was directly pulled down by BamB_{6His}, and found that only the R64C variant had a greater ratio (>2.6 fold) than the wild type protein. These results suggested that SurA not only interacts with the POTRA 1 domain of BamA, but it interacts at close vicinity to the R64 residue of the $\alpha 2$ helix of POTRA 1.

BamA Δ R64 is defective in its interaction with SurA

Since POTRA 1 of BamA interacts with SurA, it is likely that the OMP biogenesis defect of BamA Δ R64, in which R64 has been deleted, stems from its inability to properly interact with SurA. This is a particularly strong possibility given that our data indicated that SurA interacts with BamA in the vicinity to R64 (Fig. 7B). To test this, we carried out BamB_{6His}-mediated pull down experiments similar to that described in Fig. 7, except that we only used DSP for cross-linking. As expected, in the absence of DSP, BamA, but not SurA, could be efficiently pulled down by His-tagged BamB; however, the amounts of BamA Δ R64 present were approximately 60% of wild type BamA (Fig. 8A; lanes 2 and 5). The lower amount of BamA Δ R64 detected by silver staining is likely due to lower starting BamA Δ R64 levels present in the cell when expressed from pBAD33 but could also be due to an aberrant BamB_{6His}-BamA Δ R64 interaction. Because SurA is pulled down as a function of BamA, the amount SurA present with BamA Δ R64 was expected to be proportionally less ($\approx 60\%$) than wild type BamA if there was no defect in BamA Δ R64-SurA interactions. However, even after factoring in this reduction, when DSP was used for cross-linking, an eightfold ($n=4$) lower level of SurA was pulled down by BamA Δ R64 compared to wild type BamA (Fig. 8B; lanes 1 and 4). Therefore, BamA Δ R64 is defective in its ability to properly interact with SurA.

Discussion

In this study we isolated and characterized two BamA POTRA 1 mutants of *E. coli*. One of the mutants, BamA Δ R64, carried an in-frame deletion of codon R64 and was isolated as a suppressor of *imp208* (Sampson *et al.*, 1989), a mutant allele of another essential gene, *lptD* (*imp*), whose product facilitates the transport of LPS to the outer membrane (Bos *et al.*, 2004). The second BamA mutant, BamA Δ R36-K89, was constructed by site-directed mutagenesis that deleted a majority of the POTRA 1 domain. Both mutants behaved similarly, except that BamA Δ R36-K89 displayed a significantly more severe phenotype with respect to β -barrel OMP biogenesis defects than BamA Δ R64. Curiously, however, neither mutant produced a negative effect on TolC. The effects of BamA Δ R64 on OMPs were most noticeable when the mutant BamA protein was expressed at low levels from pZS21, but masked to a large degree in a wild type background, but not in a Δ *bamB*, Δ *degP*, or Δ *surA* background, when expressed at higher levels from the chromosome or pBAD33.

We found that BamA Δ R64 is severely defective in its ability to interact with SurA. Moreover, our data strongly indicated that SurA interacts with the $\alpha 2$ helix of POTRA 1 and in close proximity to R64. Therefore, the inability of BamA Δ R64 to properly interact with SurA is likely to interfere with the efficient 'handover' of SurA-bound OMP cargo to the Bam complex to complete the final stages of assembly and insertion into the outer

membrane. However, given that TolC bypasses the need for SurA, it is expected to be immune to a defective SurA-POTRA 1 interaction in the BamA Δ R64 mutant. Depletion of BamA Δ R64 inhibited TolC biogenesis but well after the biogenesis of SurA-dependent OMPs was inhibited. Since BamA Δ R64 is already defective in SurA-POTRA 1 interactions, the lowering of BamA Δ R64 levels during depletion assays is expected to first affect those OMPs that assemble through the SurA pathway compared to those that bypass this step. This scenario is similar to when BamA Δ R64 is expressed constitutively at a very low level from pZS21. Ultimately, when BamA Δ R64 levels are severely exhausted from the outer membrane, the steps leading to the insertion of OMPs into the outer membrane will be inhibited. At this stage, assembly of all β -barrel OMPs, including that of TolC, which depend on BamA for insertion into the outer membrane (Werner and Misra, 2005), will be inhibited.

It is worth noting that the biogenesis of LamB and OmpF, and other β -barrel OMPs such as OmpA and OmpC, in BamA POTRA 1 mutants is expected to be inhibited at two additional steps apart from the step involving SurA-POTRA 1 interactions: one of these entails translational inhibition via σ^E -regulated small RNAs (Johansen *et al.*, 2006; Papenfort *et al.*, 2006), while the other concerns the inhibition of OMP assembly at stages involving LPS (Laird *et al.*, 2004). By expressing *lamB* and *ompF* from a σ^E -independent promoter, we were able to distinguish the effects of the BamA mutant on β -barrel OMP assembly from synthesis. Unlike LamB and OmpF, LptD synthesis is up-regulated in a BamA Δ R64 mutant background (Fig. 3). Therefore, a reduction in LptD levels in the BamA Δ R64 mutant grown on rich medium indicates a defect in LptD assembly. This would in turn interfere with LPS biogenesis, leading to a defect in LamB and OmpF assembly. Although it may not be possible to render LamB and OmpF assembly independent of LPS, we noted that LptD levels were unaffected in cells expressing BamA Δ R64 from pBAD33 and grown on glycerol minimal medium (Fig. S4). Lower LamB and OmpF levels under these growth conditions must then be due to reduced synthesis, as a result of σ^E activation, and impaired assembly at the step of BamA POTRA 1. Thus, in BamA POTRA 1 mutants lower levels of β -barrel OMPs, expressed from their native chromosomal locations, must reflect a sum of effects resulting from the inhibition of OMP assembly and synthesis.

We have previously reported that TolC follows a unique assembly pathway that is independent of the periplasmic chaperones SurA and Skp (Werner *et al.*, 2003), *de novo* lipid synthesis (Werner and Misra, 2005), and BamB (Charlson *et al.*, 2006). In fact TolC appears to benefit when the assembly of most β -barrel OMPs is compromised either in the absence of BamB (Charlson *et al.*, 2006), SurA (Vertommen *et al.*, 2009; Fig. 2, Fig. S1, and Fig. S3), or in a BamA Δ R64 (pZS21) background (Fig. 1, Fig. 2, Fig. S1, and Fig. S2). We have shown here that the increase in TolC levels in *bamA66* (pZS21) and Δ *surA* backgrounds is not due to an increase in *tolC* transcription (Fig. 3). Therefore, elevated TolC levels could be due to its enhanced assembly/trimerization or, as we have previously suggested, reduced competition for insertion into the outer membrane (Charlson *et al.*, 2006). However, given that the transcript levels of many OMPs are reduced, presumably due to σ^E /small RNA-mediated inhibition, TolC may also benefit from reduced competition at the level of Sec-mediated translocation across the inner membrane. The same may also apply for wild type BamA, whose levels, like that of TolC, go up in the absence of SurA (Vertommen *et al.*, 2009; Fig. 2 and Fig. S3). Like *tolC*, transcription of *bamA* in a Δ *surA* strain grown at 30°C does not change significantly (Fig. 3). Therefore, an increase in BamA levels in a Δ *surA* background may also be due to reduced competition for translocation across the inner membrane, assembly in the periplasm or insertion into the outer membrane.

Little is known about the assembly of BamA. Like TolC, BamA appears to assemble independent of SurA (this study; Vertommen *et al.*, 2009). Both proteins contain large

periplasmic soluble domains comprised mainly of α -helices (TolC) or a combination of α -helices and β -strands (BamA). It is possible that the presence of the large soluble domains and their intrinsic folding properties render the folding of these two proteins independent of known periplasmic folding factors. In support of this notion, we recently showed that a small quantity of signal sequence-less mature TolC can fold and trimerize in the cytoplasm, but was unable to properly insert into the inner membrane (Masi *et al.*, 2009).

A previous mutagenesis analysis showed that deletion of POTRA 1 did not affect integrity of the BamA complex, while deletions of POTRA 2 and 5 interrupted BamA's interactions with BamB and BamBCDE, respectively (Kim *et al.*, 2007). Of these lipoproteins, only BamD is essential and is thought to stabilize the BamA complex through its interactions with BamCE (Malinverni *et al.*, 2006). The two BamA mutants analyzed here have intact POTRA 2–5 domains; therefore, their interactions with BamBCDE are not expected to be affected. On the other hand, low BamA Δ R64 and BamA Δ R36-K89 levels indicate that their biogenesis is severely impaired. This defect is not at the level of transcription, and it is unlikely that the translation and translocation steps of the mutant proteins are defective. Moreover, since BamA assembles independent of SurA, low mutant BamA levels cannot be attributed to their reduced capacity to interact with SurA. The fact that the presence of wild type BamA elevates the level of the mutant BamA protein deleted for its POTRA domain suggests that POTRA 1 is involved in BamA assembly (Fig. 6D). Improper assembly of the BamA POTRA 1 mutant proteins would lead to their misfolding and degradation. This same misfolding and degradation probably occur when LamB and OmpF are unable to assemble properly due to a defective POTRA 1-SurA interaction, resulting in their low levels even when expressed from a σ^E -independent promoter (Fig. 4). Consistent with the view that BamA promotes self-reception/assembly, we discovered that the depletion of wild type BamA in a mutant BamA Δ R36-K89 background significantly reduced the mutant BamA levels, just as the levels of other β -barrel OMPs were being reduced. Therefore, a positive correlation between wild type BamA and mutant BamA levels, when expressed in the same background, reflects that wild type BamA aids in the assembly of the POTRA 1 mutant BamA proteins. The SurA-independent assembly of BamA implies that incoming nascent BamA polypeptides either bind directly or through an unidentified factor, to the POTRA 1 domain of already assembled OM-localized BamA. It is also possible that elevated BamA Δ R36-K89 levels in the presence of the wild type BamA protein may reflect stabilization of the mutant BamA protein by mixed BamA oligomer formation.

The molecular mechanism of how OMPs interact with BamA is unclear. *In vitro* peptide binding assays (Robert *et al.*, 2006; Knowles *et al.*, 2008) have highlighted the importance of the POTRA domains of BamA in substrate binding. The OMP-POTRA interactions are thought to occur via β -augmentation (Kim *et al.*, 2007) and *in vitro* data exist supporting this notion (Knowles *et al.*, 2008). Despite these binding data, it is not clear as to how the Bam complex mediates insertion of β -barrel OMPs. Robert *et al.* (2006) showed that BamA can form channels *in vitro*, presumably through their OM-anchored β -barrel domain, and suggested that the diameter of these channels, formed by BamA tetramers, are large enough to accommodate passage of partially folded OMPs. These authors envisioned the insertion of OMPs in between the subunits of the BamA complex. However, the oligomeric states of BamA and the BamA complex have been disputed (Kim *et al.*, 2007) and the *in vivo* existence and physiological relevance of BamA channels are unproven at this point. In a recent exciting work, Hagan *et al.* (2010) reported reconstitution of an OMP assembly system. This opens the door for a deeper *in vitro* investigation of OMP assembly and insertion.

Several models have been proposed to explain how BamA may facilitate assembly and insertion of the β -barrel OMPs (Knowles *et al.*, 2009). The assembly pathways appear to

diversify immediately after mature monomers of β -barrel OMPs are released from the Sec machinery (Fig. 9). It is likely that the intrinsic folding properties of various OMP monomers dictate their dependence on periplasmic chaperones. For example, large, intrinsically stable periplasmic domains of certain OMPs, such as TolC and BamA, may aid in their rapid folding and thus avoid aggregation in the periplasm. On the other hand, OMPs such as LamB and OmpF, which fold exclusively as trans-membrane β -barrels, presumably interact with periplasmic chaperones to remain assembly competent. Assembly of LptD is also dependent on SurA (Vertommen *et al.*, 2009; Fig. S3 of this study), thus putting it in the same pathway as LamB and OmpF (Fig. 9). These proteins are then delivered to BamA via SurA-POTRA 1 interactions. Our results indicate BamA also requires POTRA 1 for its own assembly, a step bypassed by TolC. The SurA-dependent and independent assembly pathways then appear to merge past POTRA 1, possibly at a step requiring BamA-mediated membrane insertion (Fig. 9). As mentioned above, in the absence of BamB, which makes direct contact with BamA (Sklar *et al.*, 2007b; Vuong *et al.*, 2008), the biogenesis of SurA/POTRA 1-dependent OMPs is impaired but that of TolC is enhanced (Charlson *et al.*, 2006). Thus the merger of the two assembly pathways appears to occur past the steps involving SurA/POTRA 1 and BamB/POTRA 2. The conditional viability of the BamA POTRA 1 deletion strain suggests the existence of a secondary, albeit inefficient, assembly pathway for β -barrel OMPs. Whether this involves delivery of OMPs to the remaining POTRA domains or another protein is unclear at this point.

A variance from TolC/BamA and other typical β -barrel OMPs has been the assembly of PulD, a secretin from *Klebsiella oxytoca* required for pullulanase secretion (d'Enfert *et al.*, 1989). PulD assembles into a large dodecameric structure and inserts into the outer membrane independent of BamA (Collin *et al.*, 2007). Guilvout *et al.* (2006) have suggested that the interaction of PulD with PulS, a pilotin lipoprotein, in the periplasm may facilitate PulD's targeting to the outer membrane via the Lol-based lipoprotein sorting pathway (Tokuda and Matsuyama, 2004). An integral outer membrane lipoprotein, Wza, which is involved in capsule secretion, folds into a novel trans-membrane α -helical barrel and a large periplasmic domain assembled from α -helices and β -strands (Dong *et al.*, 2006). Given that it lacks a trans-membrane β -barrel, its large soluble periplasmic domain may drive folding independently of SurA, while targeting and insertion into the outer membrane may be facilitated by the Lol system, a combination of the Lol/Bam systems, or through another mechanism yet to be determined. Clearly more work is needed to fully comprehend the insertion mechanism of OMPs containing a trans-membrane β -barrel or α -barrel.

Experimental Procedures

Bacterial strains, plasmids and media

All bacterial strains and plasmids used in this study are listed in Table 1. Luria broth (LB), Luria broth agar (LBA), and M63 minimal media supplemented with glycerol and casamino acids (0.4% and 0.1%, respectively [w/v, final]) were prepared as described previously (Silhavy *et al.*, 1984). When necessary, growth media was supplemented with L-arabinose (0.1% w/v), D-fucose (0.05% w/v), ampicillin (50 $\mu\text{g ml}^{-1}$), chloramphenicol (Cm, 12.5 $\mu\text{g ml}^{-1}$), Kanamycin (Kan, 25 $\mu\text{g ml}^{-1}$), and tetracyclin (10 $\mu\text{g ml}^{-1}$).

DNA methods

Plasmid clones used for this study were created using PCR amplified fragments, containing introduced restriction endonuclease sites, of MC4100 chromosomal genes: *bamA* (*XbaI-SphI*), *ompF* (*BspHI-HindIII*), *lamB* (*EcoRI-HindIII*) and *phoA* (*EcoRI-XbaI* and *XbaI-SalI*). Primers used for PCR amplification are listed in Table S1. Digested PCR fragments were ligated into appropriately digested pBAD33 and pBAD24 plasmids (Guzman *et al.*, 1995).

and the resulting clones were confirmed by DNA sequencing. The *ompF* PCR fragment (*Bsp*HI-*Hind*III) was inserted into pBAD24 digested with *Nco*I and *Hind*III restriction enzymes. Cloning of the *phoA* gene was performed in two steps; first, the *phoA* PCR fragment (*Eco*RI-*Xba*I) containing the region encoding for the signal sequence of PhoA was introduced into pBAD24. This plasmid was then used to clone the mature portion of PhoA (PCR fragment containing *Xba*I-*Sal*I). The BamA variants used in this study were created using the QuikChange Lightning site-directed mutagenesis (SDM) kit (Stratagene) as per manufacturer's instructions. Mutations were created on the pZS21-*bamA*_{6his} (Kim *et al.*, 2007) and pBAD33-*bamA* (*Xba*I-*Sph*I clone) plasmid templates and transformed into JM109 competent cells. Mutations were confirmed by DNA sequencing. Primers used for SDM are available upon request.

RNA isolation and real-time quantitative PCR

Total RNA was extracted from 10 ml cells grown to log phase (OD₆₀₀ ~0.8) at 30°C using TRIzol Max Bacterial RNA Isolation Kit (Invitrogen). Following extraction, total RNA was purified with the RNeasy kit (Qiagen). cDNA was synthesized from 10 µg of RNA using 100 pM random hexamer primer (Integrated DNA Technologies) and M-MuLV Reverse Transcriptase (New England Biolabs). After reverse transcription, samples were treated with 5 units of RNaseH (New England Biolabs) for 20 min at 37°C and cDNA was purified with QIAquick PCR purification kit (Qiagen). To measure target RNA transcripts, 300 nM of each primer and 10 ng of cDNA was added to SYBR Green PCR Master Mix (Applied Biosystems) per each 20 µl reaction. Primers, shown in Table S1, were designed as per SYBR Green PCR Master Mix and RT-PCR Reagents manufacturer's protocol. Critical threshold (Ct) values were measured using ABI Prism 7900HT Sequence Detection System (Applied Biosystems). Relative quantification of target transcripts was calculated according to the 2^{-ΔΔCt} method (Livak and Schmittgen, 2001) using two endogenous control genes, *ndh* and *ftsL*, whose expression did not change by mutations used in this study. Each PCR reaction was performed in triplicate. Relative fold changes in transcript levels and standard deviation were calculated from two independent experiments (n=2).

Cross-linking and immunoprecipitation

Bacterial subcultures containing 100 ml LB, appropriate antibiotics and 0.1% (w/v) arabinose were inoculated 1:100 with overnight cultures and grown at 30°C to an OD₆₀₀ of approximately 0.8. For cross-linking, each 100 ml culture (equalized according to OD) was then separated into 25 ml aliquots, centrifuged and washed with 20 mM phosphate buffer (pH 7.4). Cells were resuspended in 2.5 ml of 20 mM phosphate buffer and cross-linked using either DSP [dithiobis(succinimidylpropionate)] (0.5 mM; Pierce) or SPDP [*N*-succinimidyl 3-(2-pyridyldithio) propionate] (0.2 mM; Pierce) for 30 min at 37°C on a rocker. Dimethyl sulfoxide (DMSO; 25 µl) was added to cells not receiving cross-linker. Reactions were quenched with 40 mM Tris-HCl (pH 7.5) and 2.5 mM L-cysteine. For experiments where only DSP was used, L-cysteine was not included as a quencher. After cross-linking, cells were pelleted and washed with 10 mM Tris-HCl (pH 8.0) and lysed by freeze-thawing in 0.1 ml lysis buffer (50 mM Tris-HCl [pH 8.0], 100 mM NaCl, 0.1 mM EDTA, 0.1% Triton, and 5.0 µg ml⁻¹ lysozyme). After incubation on ice for 30 min, proteins in the cell lysates were solubilized under gentle conditions using 1 ml BugBuster protein extraction reagent (Novagen), 0.5 µl Benzonase nuclease (Novagen), and 0.1 mM (final) phenylmethylsulfonyl fluoride (PMSF) for 20 min at room temperature on a rotating mixer. Insoluble debris was removed by centrifugation for 25 min at 20,000 × *g* and supernatants containing the extracted proteins were precipitated using the IP50 protein G immunoprecipitation kit (Sigma-Aldrich). Incubation of 300 µl extract with 0.4 µg Penta-His antibody (QIAGEN) was done overnight at 4°C on a rocker followed by incubation with protein G beads for two hours. 0.5 M NaCl was used in the first two washes to increase

stringency. Samples were prepared for SDS-PAGE by eluting immunoprecipitates from columns with 45 μ l sodium dodecyl sulfate (SDS) sample buffer containing β -mercaptoethanol at 95°C for 5 min. Proteins from 10 μ l of eluates were separated on an 11% SDS-polyacrylamide gel and visualized using the SilverQuest silver staining kit (Invitrogen) or Western blot analysis.

Preparation of cell envelopes

Bacterial subcultures, inoculated with 1:100 of overnight grown cultures, were incubated at 30°C in LB containing appropriate supplements to an OD₆₀₀ of approximately 1.0. Equalized cells based on OD₆₀₀ were centrifuged, washed with 10 mM Tris-HCl (pH 7.5) and resuspended in lysis buffer (2 mM PMSF, 100 mM Tris-HCl [pH 7.5], 10 mM MgCl₂, and 0.25 μ g ml⁻¹ DNase I [Sigma]). Samples were passed through a French Pressure Cell and centrifuged at low speed to remove unlysed cells. Membranes were collected from the supernatant by ultracentrifugation at 105,000 $\times$ g for an hour at 4°C. The resulting pellet was washed and resuspended in 10 mM Tris-HCl (pH 7.5) at a volume 1:1000 of the starting subculture to yield approximately 25,000 μ g ml⁻¹ total protein. For SDS-PAGE, proteins from resuspended envelopes were first quantified using Coomassie Protein Assay Reagent (Pierce). Based on protein concentration, samples were equalized by diluting with 1X sample buffer containing SDS and β -mercaptoethanol to ~800 μ g ml⁻¹ total protein (~8 μ g total protein loaded per lane) or ~200 μ g ml⁻¹ total protein (~2 μ g total protein loaded per lane) for staining with Coomassie blue or Western blot analysis, respectively.

Two-dimensional gel electrophoresis

Wild type and mutant (*bamA66* or Δ *surA*) strains were subcultured in 100 ml LB at 30°C to an OD₆₀₀ of 1.0. Envelopes were obtained from equalized amounts of cells as described above using the French Press lysis method. Following ultracentrifugation, membrane fractions containing outer and inner membrane proteins were resuspended in 100 μ l 10mM Tris-HCl (pH 7.5). Enrichment of outer membrane proteins was performed by incubating 90 μ l of resuspended membrane fraction in 810 μ l 0.1 M sodium carbonate (pH 11.00) at 4°C for an hour (Molloy *et al.*, 2000). Treated envelopes were pelleted using a Beckman Coulter Optima TLX ultracentrifuge and TLA 120.2 rotor. Samples were centrifuged at 89,000 $\times$ g for one hour at 4°C, washed with 10 mM Tris-HCl (pH 7.5), and centrifuged again for 30 min. The remaining pellet was resuspended in 50 μ l 10 mM Tris-HCl (pH 7.5) and quantified using Coomassie Protein Assay Reagent (Pierce). Two-dimensional electrophoresis was performed using the carrier ampholine method of isoelectric focusing (O'Farrell, 1975) by Kendrick Labs, Inc (Madison, WI). Isoelectric focusing of 200 μ g total protein per sample was carried out in glass tubes of inner diameter 2.0 mm, using 1% pH 4–6 (GE Healthcare, Piscataway, NJ) and 1% 5–8 (GE Healthcare, Piscataway, NJ) for 9600 volt-hrs. 1 μ g of an IEF internal standard, tropomyosin, was added to each sample prior to loading. After equilibrium in SDS sample buffer (10% glycerol, 50 mM dithiothreitol, 2.3% SDS and 0.0625 M Tris-HCl, pH 6.8), each tube gel was sealed to the top of a stacking gel that overlays a 10% acrylamide slab gel (0.75 mm thick). SDS slab gel electrophoresis was carried out for about 4 hours at 15 mA/gel. The following proteins (Sigma Chemical Co., St. Louis, MO) were added as molecular weight markers: myosin (220,000), phosphorylase A (94,000), catalase (60,000), actin (43,000), carbonic anhydrase (29,000), and lysozyme (14,000). The gels were stained with Coomassie blue and dried between cellophane sheets. Selected spots of unknown identity were excised from the dried gels, rehydrated in water, and identified by LC/MS/MS analysis at the University of Arizona Proteomics Consortium. Computerized comparisons of 2D gels from wild-type and mutant samples were performed by scanning gels with a laser densitometer (Model PDSI, Molecular Dynamics Inc, Sunnyvale, CA). The images were analyzed using Progenesis PG240 software with TT900 (version 2006, Nonlinear Dynamics). The general method of computerized analysis for these

pairs included image warping with TT900 software followed by automatic spot finding, background subtraction (average on border), matching, and quantification in conjunction with detailed manual checking.

Western blot analysis

Whole cell analysis of total protein was performed with normalized volumes (equal to 100 μ l at OD₆₀₀ of 1.0) taken from log-phase grown cultures. Cells were pelleted and resuspended in sample buffer containing SDS and β -mercaptoethanol. After heating for 5 min at 100°C, samples were analyzed by SDS-PAGE containing 4 M urea if necessary to resolve OmpC and OmpF. Proteins were transferred onto PVDF Immobilon-P membrane (Milipore) using a Mini Trans-Blot Cell (Bio-Rad) and incubated in primary antibody for 1.5 hours. Primary rabbit antibodies and dilutions used were: AcrA (1:16,000), GroEL (1:50,000), LamB (1:10,000), LptD (1:5,000), OmpF/C/A (1:16,000), PhoA (1:10,000 [5 Prime–3 Prime Inc.]), SurA (1:5,000), TolC (1:5,000), TolC/BamA (1:25,000), and the N-terminal mature peptide BamA_{A21-Q35} (1:2,000 [Kim *et al.*, 2007]). Proteins tagged with 6 $\times$ His were detected using HisProbe-HRP (1:12,500 for immunoprecipitated BamB_{6his}) as described previously (Vuong *et al.*, 2008). Following incubation with primary antibody, membranes were incubated with goat anti-rabbit HRP-conjugated immunoglobulin G secondary antibodies for an hour. Conjugate detection was carried out by 5 min incubation in Immuno-Star HRP chemiluminescent and the Molecular Imager ChemiDoc XRS system (Bio-Rad). Protein bands were quantified using Bio-Rad Quantity One software.

Separation of outer and inner membrane by sucrose density gradient analysis

Cell fractionation and envelope isolation was performed by French Press lysis and ultracentrifugation as described above. Envelopes from wild-type and mutant strains were separated into inner and outer membrane fractions using a six step sucrose density gradients (30–55%, w/v) as described previously (Misra *et al.*, 2000). In total, 19 fractions of 250 μ l each were collected from top of the centrifugation tube, diluted in sample buffer, and subject to SDS-PAGE containing 4 M urea. Proteins were visualized using Coomassie blue staining and Western blot analysis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig 1.

Two-dimensional (2D) gel electrophoresis analyses of envelope fractions enriched for integral membrane proteins. **(A and B)** Coomassie blue stained gels containing protein samples from strains expressing *bamA*⁺ **(A)** and *bamA66* **(B)** from pZS21. **(C)**. Individual 2D gel images from **A** and **B** are overlaid to show relative protein abundance. Gel images containing protein samples from wild type and *bamA66* strains are colored green and magenta, respectively.. Overlapping areas within a spot common in both wild type and mutant appear black; predominance of green indicates that the corresponding protein spot is more abundant in wild type than mutant, and predominance of magenta indicates the opposite. **(D)** A montage of individual Coomassie blue stained 2D gels containing envelope samples from wild type and mutant strains analyzed in **(A and B)**. Areas outlined by green show a close-up view of gel regions encompassing BamA and LptD (Imp) spots. All numbered and labeled protein spots were identified using LC/MS/MS and are listed in **(E)**. Isolation of envelopes and enrichment of integral membrane proteins were carried out as described in the Experimental procedures' section.

Fig 2.

Comparative analysis of selected envelope proteins from wild type, *bamA*66, and Δ *surA* strains grown at 30°C. Wild type BamA and BamA Δ R64 were expressed from pZS21 (low level constitutive expression), pBAD33 (induced with 0.1% arabinose), or from the native chromosomal location. Lanes 7 and 8 contain envelope samples prepared from *surA*⁺ and Δ *surA* strains, respectively. Proteins were detected either by Western blot analysis using antibodies shown in the figure (A and C) or by SDS(urea)-PAGE analysis followed by Coomassie blue staining (B). AcrA serves as both an envelope preparation and gel loading control.

Fig 3.

An examination of relative gene expression in the *bamA66* (pZS21) and Δ *surA* mutants using quantitative real-time PCR analysis. RNA was isolated from mid-log phase grown cultures at 30°C. Relative quantification of gene transcripts was performed in triplicate using the $2^{-\Delta\Delta C_T}$ method and both *ndh* and *ftsL* as reference genes. Relative fold-changes in gene expression and error bars representing standard deviation are shown (n=2).

Fig 4.

The effects of $\Delta rseA$ and *bamA66* (pZS21) on OmpF and LamB protein levels when expressed from the arabinose P_{BAD} promoter contained on the pBAD24 vector. (A) To determine *ompF* and *lamB* sensitivity to σ^E -mediated down-regulation, the levels of OmpF and LamB, expressed from either their native chromosomal location (left panel) or from the pBAD24 plasmid (middle and right panels, respectively), were evaluated in wild type (WT) and $\Delta rseA$ (Δ) backgrounds. The chromosomal status of *ompF* and *lamB* is shown below each panel. For Western blot analysis, 5 ml of LB containing ampicillin was inoculated with overnight grown cultures and grown at 37°C. Upon reaching an OD₆₀₀ of approximately 0.4, 0.001% arabinose (w/v, final) was added to all cultures for 30 min. After induction, cultures were chilled on ice and whole cell lysates were analyzed by SDS-PAGE. Proteins were detected by Western blots using appropriate polyclonal antibodies. (B and C) Expression of *ompF* (B) and *lamB* (C) from the chromosome or pBAD24 in backgrounds expressing wild type (WT) or mutant BamA Δ R64 (Δ R64) from pZS21. Methods were followed as described in (A) with the exceptions that whole cell lysates were obtained from cultures grown at 30°C and inoculated with 0.001% or 0.005% arabinose for 45 or 60 minutes (B and C, respectively). The pBAD24-*phoA* construct was used as an induction control and GroEL as a sample loading control.

Fig 5.

The effects of BamA Δ R64 depletion on cell growth and TolC/LamB levels. **(A)** Cells deleted of chromosomal *bamA* and harboring pBAD33-*bamA*66 were grown in LB at 30°C with (circles) and without (squares) arabinose (0.1%, w/v). After the first dilution from overnight cultures, OD₆₀₀ was measured from samples taken every hour. **(B)** Western blot analysis to detect TolC, LamB, and AcrA from equal number of cells (based on OD₆₀₀) withdrawn at time points indicated by downward arrows in **(A)**. An open downward arrow at the 3 h time point indicates that only the sample from cells grown without arabinose was analyzed. AcrA serves as a sample loading control.

Fig. 6.

The effects of a BamA POTRA 1 deletion ($\Delta R36-K89$) on LamB, TolC, and BamA $\Delta R36-K89$ levels in strains depleted of wild type BamA. (**A to D**) Strains used were deleted of chromosomal *bamA* and simultaneously expressed wild type BamA from pBAD33 (arabinose inducible promoter is inhibited by fucose) and wild type (**A**) or mutant BamA (**B** and **C**) from pZS21 (low level constitutive expression). Cultures were grown overnight at 30°C with appropriate antibiotics and 0.05% arabinose (final). Overnight cultures were subsequently diluted (first dilution) to an OD₆₀₀ of 0.025 in 5 ml of fresh medium containing appropriate antibiotics and 0.05% arabinose (Ara) or 0.05% D-fucose (Fuc). After five hours (average OD₆₀₀ 0.8), cultures were again diluted (second dilution) in fresh medium as before and growth was resumed. Samples were taken at hours 3, 4, and 5 (1st dilution) and 8, 9 and 10 (2nd dilution) every hour and prepared for Western blot analysis as described in Fig 6B. (**D**) Samples used in Fig 6C were further analyzed on a 7.5% polyacrylamide gel to separate the two BamA species; wild type BamA and the faster migrating BamA $\Delta P1$. Antibodies specific to the N-terminal of BamA were used to visualize protein levels. The ratios of BamA $\Delta P1$ levels when expression of wild type BamA was inhibited (fucose; Fuc) to the levels of BamA $\Delta P1$ when wild type was induced (arabinose; Ara) are shown along with standard deviation (n=2). Note that BamA N-terminal antibodies recognize BamA $\Delta P1$ better than wild type BamA. BamA $\Delta P1$ is short for BamA $\Delta R36-K89$.

Fig 7.

BamA POTRA 1-SurA cross-linking using four BamA POTRA 1 cysteine variants. **(A)** A view of the BamA POTRA 1 structure (pdb 2qdf) with its N-terminus rotated in front. Residues R64 and R49 (red) with D28 and D74 (blue) represent the locations of the four BamA cysteine substitutions used for SPDP-mediated cross-linking with SurA. **(B)** For cross-linking, $\Delta bamA \Delta bamB$ (pBAD24-BamB_{6His}) strains expressing wild type BamA or BamA cysteine variants from pBAD33 were grown at 30°C in LB plus arabinose (0.1%, w/v) and three equal culture aliquots were treated with DSP, SPDP or solvent. Immunoprecipitation was carried out as described in the Experimental procedures' section. 6×His-tagged BamB was used as bait and the levels of BamB_{6His} and co-immunoprecipitated BamA and SurA from eluents were determined by Western blot analysis using Hisprobe-HRP, TolC/BamA antibodies, and SurA antibodies. SurA levels relative to BamB were quantified for each lane containing either DSP or SPDP cross-linker.

The amounts of SurA for each cross-linker are shown as SPDP:DSP ratios $\pm$ standard deviation (n=3).

Fig 8.

DSP-mediated cross-linking of BamAΔR64 and SurA. $\Delta bamA \Delta bamB$ (pBAD24-BamB_{6His}) strains containing either pBAD33bamA (lanes 1–3) and pBAD33bamA66 (lanes 4–6) were grown in LB at 30°C and cross-linked using DSP as described in the Experimental procedures' section. Eluents from DSP cross-linked cells (lanes 1 and 4) and uncross-linked cells (lanes 2 and 5) were analyzed by SDS-PAGE, followed by detection of protein by silver staining (**A**) or Western blots (**B**). BamB 6×His and SurA were visualized using Hisprobe-HRP and SurA antibodies, respectively. Lanes 3 and 6 contain control samples from cells receiving no cross-linker and His antibody.

Fig 9.

A cartoon showing distinct pathways for assembly and insertion of integral outer membrane proteins (OMPs) in *Escherichia coli*. (A) Newly synthesized precursor OMPs traverse the inner membrane (IM) via the SecYEG complex and, upon cleavage of the signal sequence, enter the periplasmic compartment. Here, they are kept soluble/assembly-competent through interactions with chaperones, like SurA, or interact with factors that target them to the outer membrane (OM). The first two assembly pathways split at this step based on their SurA-dependence (LptD [Imp], LamB, and OmpF) or SurA-independence (BamA and TolC). TolC, being SurA-independent, also bypasses a requirement for the BamA POTRA 1 domain, which interacts with SurA. BamA on the other hand appears to require its own POTRA 1 domain for efficient assembly. The remaining two pathways rely on a specific OM-targeting factor, PulS (PulD) or Lol (Wza). Insertion of PulD into the OM has been shown to be independent on BamA, but may become dependent on the Lol system after PulD's interactions with PulS, which is a lipoprotein. It is not known whether Wza gains entry into the OM exclusively through the Lol system or in addition requires the Bam complex. (B) Disruption of POTRA 1 negatively impacts the assembly of BamA and the SurA dependant OMPs, LptD, OmpF and LamB, leading to increased proteolysis and activation of envelope stress responses (σ^E and Cpx). The activated σ^E and Cpx systems attempt to reduce envelope stress by inhibiting synthesis of several OMPs, including LamB and OmpF, and elevating synthesis of factors that either degrade misfolded OMPs (DegP) or enhance OMP folding (SurA and Bam complex members).

Table 1

Bacterial strains and plasmids used in this study.

Strains or plasmids	Relevant characteristics	Source or reference
Strains		
JM109	e14 ⁻ (McrA ⁻) <i>recA1 endA1 gyrA96 thi-1 hsdR17</i> (rK-mK+) <i>supE44 relA1 Δ(lac proAB)</i> [F' <i>traD36 proAB lacIqZΔM15</i>]	Promega
MC4100	F- <i>araD139 Δ(argF lac)U139 rspL150 relA1 fibB5301 ptsF25 deoC1 thi-1 rbsR</i>	Casadaban (1976)
RAM1292	MC4100 <i>Δara714</i>	Werner and Misra (2005)
RAM1446	RAM1292 <i>ΔsurA::Kan^r</i>	Gerken <i>et al.</i> (2010)
RAM127	MC4100 <i>ΔlamB106 ΔompF80 imp208</i>	Sampson <i>et al.</i> (1989)
RAM1413	RAM127 <i>bamA66</i>	This study
RAM913	MC4100 <i>ΦdegP::lacZ Tn10::zad</i>	Werner <i>et al.</i> (2003)
RAM1414	RAM1413 <i>Tn10::zad</i>	This study
RAM1420	RAM1292 <i>ΔbamA::Kan^r pBAD33-bamA</i>	This study
RAM1421	RAM1292 <i>ΔbamA::Kan^r pBAD33-bamA66</i> (BamAΔR64)	This study
RAM1422	RAM1292 <i>ΔbamA::Kan^r pBAD33-bamAPI</i> (BamAΔR36-K89)	This study
RAM1423	RAM1421 (<i>bamA66</i>) <i>Tn10::zad</i> (Kan ^s , cured of pBAD33- <i>bamA66</i>)	Gerken <i>et al.</i> (2010)
RAM1424	RAM1421 (<i>bamA⁺</i>) <i>zad::Tn10</i> (Kan ^s , Cm ^s , cured of pBAD33- <i>bamA66</i>)	This study
RAM1425	RAM1424 (<i>bamA⁺</i>) <i>ΔdegP::Kan^r</i>	This study
RAM1426	RAM1423 (<i>bamA66</i>) <i>ΔdegP::Kan^r</i>	This study
RAM1427	RAM1424 <i>ΔsurA::Kan^r</i>	This study
RAM1428	RAM1423 <i>ΔsurA::Kan^r</i>	This study
RAM1429	RAM1424 <i>ΔbamB::Kan^r pBAD24-bamB_{6His}</i>	This study
RAM1430	RAM1423 <i>ΔbamB::Kan^r pBAD24-bamB_{6His}</i>	This study
RAM1431	RAM1292 <i>ΔbamA::scar pBAD33-bamA</i>	John Werner
RAM1432	RAM1431 pZS21- <i>bamA_{6His}</i> (Cm ^s , cured of pBAD33- <i>bamA</i>)	This study
RAM1433	RAM1431 pZS21- <i>bamA_{6His}</i> (BamAΔR64) (Cm ^s , cured of pBAD33- <i>bamA</i>)	This study
RAM1434	RAM1431 pZS21- <i>bamA_{6His}</i> (BamAD28A) (Cm ^s , cured of pBAD33- <i>bamA</i>)	This study
RAM1437	RAM1431 pZS21- <i>bamA_{6His}</i> (BamAR49A) (Cm ^s , cured of pBAD33- <i>bamA</i>)	This study
RAM1438	RAM1431 pZS21- <i>bamA_{6His}</i> (BamAR64A) (Cm ^s , cured of pBAD33- <i>bamA</i>)	This study
RAM1439	RAM1431 pZS21- <i>bamA_{6His}</i> (BamAD74A) (Cm ^s , cured of pBAD33- <i>bamA</i>)	This study
RAM1440	RAM1431 pZS21- <i>bamA_{6His}</i> (Cm ^r)	This study
RAM1441	RAM1431 pZS21- <i>bamA_{6His}</i> (BamAΔR64) (Cm ^r)	This study
RAM1442	RAM1431 pZS21- <i>bamA_{6His}</i> (BamAΔR36-K89) (Cm ^r)	This study
RAM1443	RAM1292 <i>ΔbamB::scar pBAD24-bamB_{6His}</i>	Vuong <i>et al.</i> (2008)
RAM1444	RAM1443 <i>ΔbamA::Kan^r pBAD33-bamA</i>	This study

Strains or plasmids	Relevant characteristics	Source or reference
RAM1445	RAM1443 $\Delta bamA::Kan^r$ pBAD33- <i>bamA</i> (BamAD28C)	This study
RAM1447	RAM1443 $\Delta bamA::Kan^r$ pBAD33- <i>bamA</i> (BamAR49C)	This study
RAM1448	RAM1443 $\Delta bamA::Kan^r$ pBAD33- <i>bamA</i> (BamAR64C)	This study
RAM1449	RAM1443 $\Delta bamA::Kan^r$ pBAD33- <i>bamA</i> (BamAD74C)	This study
RAM1456	RAM1292 pBAD24	This study
RAM1457	RAM1292 $\Delta rseA::Kan^r$ pBAD24	This study
RAM1458	RAM1292 $\Delta ompF::scar$ pBAD24- <i>ompF</i>	This study
RAM1459	RAM1458 $\Delta rseA::Kan^r$	This study
RAM1460	RAM1292 $\Delta lamB::scar$ pBAD24- <i>lamB</i>	This study
RAM1461	RAM1460 $\Delta rseA::Kan^r$	This study
RAM1462	RAM1432 pBAD24	This study
RAM1463	RAM1433 pBAD24	This study
RAM1464	RAM1432 $\Delta ompF::Cm^r$ pBAD24- <i>ompF</i>	This study
RAM1465	RAM1433 $\Delta ompF::Cm^r$ pBAD24- <i>ompF</i>	This study
RAM1466	RAM1432 $\Delta ompF::Cm^r$ pBAD24- <i>phoA</i>	This study
RAM1467	RAM1433 $\Delta ompF::Cm^r$ pBAD24- <i>phoA</i>	This study
RAM1468	RAM1432 $\Delta lamB::Cm^r$ pBAD24- <i>lamB</i>	This study
RAM1469	RAM1433 $\Delta lamB::Cm^r$ pBAD24- <i>lamB</i>	This study
RAM1470	RAM1432 $\Delta lamB::Cm^r$ pBAD24- <i>phoA</i>	This study
RAM1471	RAM1433 $\Delta lamB::Cm^r$ pBAD24- <i>phoA</i>	This study
Plasmids		
pBAD24	Amp ^r ; Expression vector; arabinose inducible promoter	Guzman <i>et al.</i> (1995)
PBAD24-ompF	<i>ompF</i> (<i>Bsp</i> HI/ <i>Nco</i> I- <i>Hind</i> III) clone	Henri Gerken, this study
PBAD24-lamB	<i>lamB</i> (<i>Eco</i> RI- <i>Hind</i> III) clone	Henri Gerken, this study
PBAD24-phoA	<i>phoA</i> (<i>Eco</i> RI- <i>Xba</i> I- <i>Sal</i> I) clone	Henri Gerken, this study
pBAD24-bamB6His	<i>bamB</i> clone (BamB containing C-terminal 6-His tag)	Vuong <i>et al.</i> (2008)
pBAD33	Cm ^r ; Expression vector; arabinose inducible	Guzman <i>et al.</i> (1995)
pBAD33-bamA	<i>bamA</i> (<i>Xba</i> I- <i>Sph</i> I) clone	Phu Vuong
pZS21	Kan ^r ; Expression vector	Lutz <i>et al.</i> (1997)
pZS21-bamA6His	<i>bamA</i> clone (BamA containing N-terminal 6-His tag)	Kim <i>et al.</i> (2007)