Crystal Structure of BamB Bound to a Periplasmic Domain Fragment of BamA, the Central Component of the β-Barrel Assembly Machine*

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The β-barrel assembly machinery (BAM) mediates folding and insertion of β-barrel outer membrane proteins (OMPs) into the outer membrane of Gram-negative bacteria. BAM is a five-protein complex consisting of the β-barrel OMP BamA and lipoproteins BamB, -C, -D, and -E. High resolution structures of all the individual BAM subunits and a BamD-BamC complex have been determined. However, the overall complex architecture remains elusive. BamA is the central component of BAM and consists of a membrane-embedded β-barrel and a periplasmic domain with five polypeptide translocation-associated (POTRA) motifs thought to interact with the accessory lipoproteins. Here we report the crystal structure of a fusion between BamB and a POTRA3–5 fragment of BamA. Extended loops 13 and 17 protruding from one end of the BamB β-propeller contact the face of the POTRA3 β-sheet in BamA. The interface is stabilized by several hydrophobic contacts, a network of hydrogen bonds, and a cation-π interaction between BamA Tyr-255 and BamB Arg-195. Disruption of BamA-BamB binding by BamA Y255A and probing of the interface by disulfide bond cross-linking validate the physiological relevance of the observed interface. Furthermore, the structure is consistent with previously published mutagenesis studies. The periplasmic five-POTRA domain of BamA is flexible in solution due to hinge motions in the POTRA2–3 linker. Modeling BamB in complex with full-length BamA shows BamB binding at the POTRA2–3 hinge, suggesting a role in modulation of BamA flexibility and the conformational changes associated with OMP folding and insertion.

The outer membrane of Gram-negative bacteria is essential for their viability and differs from the inner membrane in both lipid and protein compositions. Both leaflets of the inner membrane bilayer are made of phospholipids. However, the outer membrane has phospholipids in the inner leaflet and lipopolysaccharide (LPS) in the outer leaflet that bestow unique permeability properties to it. Whereas integral membrane proteins of the inner membrane have typical α-helical structures, their outer membrane counterparts are characterized by β-barrel transmembrane structures. Folding and membrane insertion of these β-barrel outer membrane proteins (OMPs) are essential processes carried out by a specialized multiprotein complex called the β-barrel assembly machine (BAM) as shown schematically in Fig. 1. The BAM complex in *Escherichia coli* is composed of five subunits, BamABCDE (1, 2). BamA is the central component of BAM. It is conserved in bacteria with two membranes as well as mitochondria and chloroplasts, and its deletion is lethal for the cells. In *E. coli*, null mutants of each of the lipoproteins BamB, -C, -D, or -E produce phenotypes ranging from lethality (bamD) to significant membrane defects (bamB) to barely detectable permeability increases (bamC and bamE) (2, 3). Nevertheless, the wide phylogenetic distribution of the lipoprotein subunits suggests that they play an important role in normal bacterial physiology. It has also been reported that bacteria missing a specific BAM lipoprotein may instead have an alternative subunit. Such is the case for *Caulobacter crescentus*, an α-proteobacterium in which a novel subunit (BamF) replaces the BamC lipoprotein found in γ-proteobacteria, highlighting the importance of the accessory BAM lipoproteins for bacteria in the wild (4). However, the specific role of the BAM lipoproteins in the mechanism of OMP folding and insertion remains ill defined.

High resolution structures of the individual BAM subunits have been reported (5–14). Genetic and biochemical studies have shown that BamA interacts directly with BamB and BamD, whereas BamC and BamE are in contact with BamD (1, 2, 15, 16). However, beyond this interaction map, structural information about the BAM complex architecture has remained elusive with the exception of the crystal structure of BamD in complex with the N-terminal domains of BamC (17).

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The abbreviations used are: OMP, outer membrane protein; BAM, β-barrel assembly machinery; POTRA, polypeptide translocation-associated; Ni-NTA, nickel-nitrilotriacetic acid.

**Background:** The β-barrel assembly machine (BAM) is essential for outer membrane protein (OMP) biogenesis and Gram-negative bacterial survival.

**Results:** BamB binds polypeptide translocation-associated domain 3 (POTRA3) of BamA.

**Conclusion:** BamB, binding at the hinge in the periplasmic domain of BamA, is poised to modulate BAM conformational changes.

**Significance:** The BAM complex architecture illuminates the mechanism of OMP assembly.
BamA is an essential protein consisting of a C-terminal β-barrel domain buried in the membrane and an N-terminal periplasmic domain that contains five polypeptide translocation-associated (POTRA) motifs (15, 18). These POTRA domains are thought to mediate binding to nascent OMPs by β-augmentation and serve as a scaffold for the other BAM subunits (15, 18). The null bamB mutation in E. coli produces the strongest phenotype among the non-essential BAM subunits (BamB, -C, and -E) (1, 3). To help understand the role of BamB in the BAM complex, we present the crystal structure of a fusion between BamB and the POTRA3–5 domains of BamA designed to stabilize the BamA-BamB interface. The structure defines an interaction interface between extended loops protruding from one side of the BamB β-propeller and the face of the β-sheet of BamA POTRA3. The physiological relevance of the observed interface is validated with mutagenesis and disulfide cross-linking experiments. Furthermore, previously described mutations that disrupt binding between BamA and BamB (15, 16) also map to the interface, providing additional functional validation of the structure presented in this report. Modeling of BamB binding to the entire periplasmic domain of BamA and the recently determined structure of full-length BamA (12) indicates that BamB is positioned to interact with both POTRA3 and POTRA2 and modulate the previously described hinge motion observed in the periplasmic domain in BamA (18, 19).

**EXPERIMENTAL PROCEDURES**

**Cloning, Expression, and Purification of the Chimeric Protein—**

The E. coli bamB gene lacking the signal sequence and the N-terminal Cys residue was amplified with AccuPrime Pfx DNA polymerase from E. coli K12 using PCR with primers U774 (5’-TTTAAAGGTACCGCCAGTCTGTTTAACACCGAAGATGTGG-3’) and L774 (5’-TTTAAAGAATTCTTAACGTGTAATAGAGTACACCGGTTCGTCTTTGCC-3’) that incorporate KpnI and EcoRI restriction sites at the 5’- and 3’-ends, respectively. POTRA3–5 was PCR-amplified from an existing plasmid (pMS488) encoding full-length E. coli bamA with primers U487 (5’-GTTCCAGGAAGCCATGGAAGCTGAAATCCAG-3’) and L466 (5’-CAAAGTTCCCGGGACCGGTGTTGCGCTC-3’) incorporating an NcoI restriction site at the 5’-end and an XmaI site at the 3’-end. This fragment was ligated into the pET28 vector adding a His6 tag and the tobacco etch virus protease cleavage site at the N terminus of POTRA3–5. The twelve-amino acid linker (linker 2) bridging BamA and BamB was incorporated via synthetic oligonucleotides U772 (5’-CACCAGAACCACCACCAGACCACGAA-AACCACCG-3’) and L772 (5’-CTAGCGGTGTTCTGTTGTTGTGTGCAC-3’) that generated Nhel and KpnI restriction sites, respectively. The two primers were phosphorylated with 10 units of T4 polynucleotide kinase in 1 mM ATP at 37 °C for 30 min, annealed, and ligated into the vector.

The resulting plasmid pMS988 was transformed into the E. coli BL21(DE3) cell line (Novagen). The transformed cells were plated on the kanamycin LB plates and grown overnight at 37 °C. A single colony from the plate was selected to start an overnight 100-ml LB/kanamycin culture, which was used to inoculate a 6-liter LB/kanamycin solution for protein expression the next day. The cells were grown until $A_{600}$ reached ~0.6 when the overexpression of the chimeric protein was induced
**BamA-BamB Structure**

with 1 mM isopropyl β-D-1-thiogalactopyranoside for 3 h. Cells were spun down, resuspended in buffer A (25 mM Tris, pH 8.0, 150 mM NaCl), and lysed using sonication. The cell debris was spun down at 31,000 × g for 30 min. The soluble fraction was loaded on a Ni-NTA (Qiagen) column equilibrated in buffer A. After extensive washing with buffer A and buffer B (25 mM Tris, pH 8.0, 150 mM NaCl, 25 mM imidazole), the protein was eluted with buffer C (25 mM Tris, pH 8.0, 150 mM NaCl, 150 mM imidazole). The His tag was cleaved with the tobacco etch virus protease overnight at 4 °C while dialyzing in buffer A. Finally, the protein was purified to 99% homogeneity by size exclusion chromatography (Superdex200, Amersham Biosciences) in buffer A. The eluted single protein peak was concentrated to 20 mg/ml and stored at ~70 °C.

**Protein Crystallization and Data Collection**—The BamA-BamB chimeric protein was crystallized at 16 °C using the sitting drop vapor diffusion method. Initial crystallization conditions were refined in a hanging drop in the following solution: 1.4 M ammonium sulfate, 9% 2-propanol, 0.1 M sodium acetate, pH 5.5. We used 1.5 μl of the 20 mg/ml protein mixed with 1.5 μl of mother liquor in a reservoir filled with 500 μl of precipitant solution. Crystals appeared within a few days. They were in the shape of a thin stick with the following dimensions: 100 × 10 × 5 μm. Harvested crystals were cryoprotected in 4 M malonate before they were flash frozen in liquid N2 at 100 K. The data collection was performed with a Rigaku R-Axis IV image plate system using CuKα radiation. The data were indexed and integrated using HKL2000 software (20), and the structure was determined by molecular replacement using PHENIX (21), which yielded an unambiguous solution. After resetting all atomic B-factors to the Wilson B-factor, positional and B-factor refinement of the model was carried out in PHENIX using the high resolution structures of BamB and the BamA POTRA domains as additional reference model restraints. Initially, one group B-factor was refined per residue, moving to two groups per residue in later rounds. After several rounds of refinement with these restraints interspersed with manual model rebuilding, the Rfree could not be further improved. At this point, removing the reference model restraints improved the Rfree by about 1% during refinement. Furthermore, refining individual B-factors also improved the Rfree by an additional 1%. Therefore, both strategies were adopted for the final rounds of refinement. Final refinement statistics are included in Table 1.

**Functional Assays: Cloning, Mutagenesis, Pulldowns, and Western Blotting—E. coli** strain JCM166 and the pZS21 plasmid encoding *E. coli* BamA were kind gifts from Dr. Thomas Silhavy (Princeton University). Mutations Y255A and T257A were introduced in *His* BamA-pZS21 using the QuikChange™ mutagenesis kit (Agilent Technologies) following the manufacturer’s recommendations. The pQLINK plasmid for dual expression of *His* BamA/BamB and its variants was constructed by first cloning *His* BamA and BamB individually into pQLINK using EcoRI and HindIII restriction sites. The expression cassettes were then combined into a single expression plasmid by restriction digestion with SwaI and Pael and ligation as described previously (22). BamA mutations V183C, S275C, and A375C as well as BamB mutation A129C were introduced into this plasmid using the QuikChange mutagenesis kit following the manufacturer’s recommendations. The correctness of all coding sequences was confirmed by DNA sequencing of all plasmids.

To deplete JCM166 of endogenous BamA, chemically competent JCM166 cells were transformed with the pZS21 plasmid encoding wild type or mutant forms of BamA; plated on 0.1% arabinose, 50 μg/μl kanamycin, 100 μg/μl ampicillin LB agar; and grown overnight at 37 °C. A single colony was used to inoculate 5 ml of LB containing 0.1% arabinose, 50 μg/μl kanamycin, and 100 μg/μl ampicillin. After overnight incubation at 37 °C, the cells were harvested by centrifugation, washed twice, and resuspended in fresh LB. Washed and resuspended cells were used to inoculate 5-ml LB cultures containing 50 μg/μl kanamycin and 0.1% fucose such that the initial A600 was ~0.05 unit. The cultures were grown in triplicates until they reached an A600 of ~0.6. The cells were diluted back to an A600 of 0.05 in LB containing 50 μg/μl kanamycin and 0.1% fucose and allowed to continue growing at 37 °C. When the cells reached an A600 of 0.6 again, they were diluted back to an A600 of 0.05 in LB containing 50 μg/μl kanamycin and 0.1% fucose for the third time. At this point, endogenously expressed BamA was depleted as cells transformed with a control pZS21 plasmid encoding GFP instead of BamA failed to support growth (data not shown). Cells containing pZS21 encoding BamA and its mutants continued to grow after the third dilution in 100 ml of LB supplemented with kanamycin and fucose. Cells were spun down when they reached an A600 of 0.6 and frozen at −20 °C overnight. The next day, the cells were thawed and lysed in 2 ml of the detergent mixture BugBuster™ supplemented with Halt™ protease and phosphatase inhibitor mixture (Thermo Scientific), 2 μl of Benzonase (Novagen), and 100 μg of lysozyme while rocking at room temperature for 1 h. The cell debris was spun down at 21,000 × g for 30 min. The clarified supernatant was loaded on 250 μl of a 50% slurry of Ni-NTA (Qiagen) pre-equilibrated with buffer D (10 mM Tris, pH 8.0, 150 mM NaCl, 0.5% Triton X-100). The column containing the beads and the cell supernatant was incubated for 30 min at room temperature while being gently shaken. The flow-through was collected, and the column was washed with 2 ml of buffer D supplemented with Halt protease and phosphatase inhibitor. The elution was performed with buffer E (buffer D supplemented with Halt protease and phosphatase inhibitor and 500 mM imidazole, pH 8.0) after a 5-min incubation on a shaker. The collected samples were mixed with a loading dye, boiled for 5 min, and loaded for SDS-PAGE. The gels were transferred on nitrocellulose membranes (GE Healthcare), which were then probed with BamA (1:20,000) or BamB (1:5,000) polyclonal antibodies raised against these proteins (Cocalico Biologicals, Inc.).

For the cysteine cross-linking experiments, a *bamB*-null strain of *E. coli* (8) was transformed with pQLINK plasmids for dual expression of *His* BamA and BamB cysteine mutants and plated on 100 μg/μl ampicillin LB agar. Single colonies were used to inoculate 50 ml of LB medium supplemented with 100 μg/μl ampicillin and allowed to grow until the A600 reached ~0.8. Expression was not induced, relying instead on leaky expression from the Ptac promoters of the plasmid. Cells were spun down, and the pellets were immediately resuspended in
the detergent mixture BugBuster solution supplemented with Halt protease and phosphatase inhibitor mixture, 2 µl of Benzonase nuclease (Novagen), 100 µg of lysozyme, and 10% N-ethylmaleimide to quench disulfide bond formation. Detergent-solubilized proteins were purified by Ni-NTA affinity chromatography as described above. The eluted fractions were split into two tubes and mixed with 2× loading dye containing N-ethylmaleimide with or without 200 mM DTT. Each of the samples under reducing and non-reducing conditions was loaded in duplicate for 7.5% SDS-PAGE and then blotted onto nitrocellulose. The membranes were cut in half with each half containing identical samples run on the same gel. One-half from each membrane was analyzed by Western blotting with BamA antibodies, whereas the matching half was analyzed with BamB antibodies as described above.

RESULTS

BamA-BamB Fusions for Crystallization and Structure Determination—BamB has been proposed to interact with the periplasmic POTRA domain of BamA (15, 16). However, attempts to copurify the soluble POTRA1–5 domain of BamA and a soluble, non-lipidated form of BamB failed to yield stable complexes. It was then reasoned that linking the two proteins together with a flexible linker would increase the local concentration of the binding partners and promote formation of a crystallizable complex. The C terminus of the periplasmic domain of BamA faces the outer membrane, whereas the N terminus of BamB is thought to be lipid-anchored to the outer membrane. Therefore, a series of fusion proteins were designed in which the C terminus of the E. coli BamA POTRA domain was linked to the N terminus of E. coli BamB.

The periplasmic domain of BamA is conformationally flexible due to a flexible hinge between POTRA domains 2 and 3 (18, 19). Therefore, constructs in which POTRA1–2 domains are removed would be expected to be more conformationally stable than the entire POTRA1–5 domain. To increase the chances of obtaining a crystallizable chimeric protein, three BamA fragments were thus targeted for fusion to BamB: (i) the complete, POTRA1–5 periplasmic domain, (ii) a POTRA3–5 fragment, and (iii) a POTRA4–5 fragment. In addition, three linkers with 16 (linker 1), 12 (linker 2), and 36 (linker 3) serine and glycine residues were incorporated in the fusions to minimize the chances that linker length restricted the formation of a productive complex. These design guidelines resulted in nine chimeric proteins (three BamA fragments and three linkers).

All nine chimeras were expressed with a cleavable N-terminal His tag for purification, and as expected, all yielded soluble proteins. They were purified to homogeneity, cleaved from the His tag, and subjected to crystallization trials. Two different chimeras produced crystals in the same crystallization condition. They consisted of POTRA3–5 connected to BamB with two different linkers (linker 1 and linker 2). The crystals grew as thin rods, and those with linker 2 (12 amino acids) diffracted slightly better and were thus selected for further characterization. Refinement of the crystallization conditions to 1.4 M ammonium sulfate, 9% 2-propanol, 0.1 M sodium acetate, pH 5.5 yielded single thin rod crystals. After cryoprotection, a native data set to a 3.1-Å resolution was collected from one of these crystals. Data collection statistics are shown in Table 1.

The structure of the chimera was solved by molecular replacement using data to a 3.3-Å resolution. Computation of rotation and translation functions using E. coli BamB as a search model led to an unambiguous solution. After fixing the BamB position in the crystal, similar calculations using a spliced model of E. coli BamA POTRA3–5 (19) also yielded a clear solution. However, inspection of the electron density map calculated with initial model phases did not show clear density for POTRA5 and several loops in both BamA and BamB. These segments of the structure were removed, and several iterations of refinement and model rebuilding resulted in a final model containing residues 32–392 of BamB and 176–346 in BamA (POTRA 3 and 4) with the following missing segments presumably due to conformational flexibility: in BamA, residues 203–214 and 287–293 (the flexible loops that connect the two α-helices in each POTRA domain (15, 18)); in BamB, residues 99–106 in loop 5 connecting the first and second β-propeller blades and 234–244 in loop 17 connecting the fourth and fifth blades. These loops are known to be conformationally labile, which has prevented their modeling or resulted in refinement with high temperature factors in previous crystal structures of isolated BamA and BamB (5, 7, 9, 11). As expected, no traceable density

| TABLE 1 |
| --- |
| **Data collection and refinement statistics** |
| **BamA-BamB** |
| Data collection |
| Wavelength (Å) | 1.5418 |
| Resolution (Å) | 1.90–3.10 (3.15–3.10) |
| Space group | P2₁ |
| Cell dimensions | a = 493 Å, b = 91.4 Å, c = 61.2 Å, β = 92.9° |
| Unique reflections | 9870 (484) |
| Completeness (%) | 99.9 (100) |
| Average redundancy | 3.7 (3.6) |
| Rmerge (%) | 11.0 (1.9) |
| Rwork (%) | 10.6 (57.1) |
| Rfree (%) | 8.0 (80.4) |
| Wilson B-factor | 0.090 (0.504) |
| CC1/2 (%) | 99.7 (0.819) |
| Wilson B-factor | 68.7 |

<sup>a</sup> Values in parentheses are for the highest resolution shell.

<sup>b</sup> Rmerge = 100 ∑ ||Ii(hkl) − 1/N ∑jIj(hkl)||/N ∑jIj(hkl) where I(hkl) is the ith measurement of each reflection hkl, 1/N ∑jIj(hkl) is the weighted mean of all measurements of hkl, and N is the number of unique reflections.

<sup>c</sup> The CC₁/₂ is the Pearson correlation coefficient between random halves of all the reflections of each measurement. CC = [2CC₁/₂/(1 + CC₁/₂)]^1/2 is defined by Karpus and Diederichs (32).

<sup>d</sup> CC_free is computed the same as CC₁/₂ but using the test set of reflections.

<sup>e</sup> CC_true is the Pearson correlation coefficient between observed and model calculated intensities (33). CC_true is computed the same as CC₁/₂ but using the test set of reflections.

<sup>f</sup> Root mean square.
was observed for the engineered Gly-Ser linker connecting BamA and BamB. Statistics for the final models refined to 3.1-Å resolution are shown in Table 1.

**BamA-BamB Structure and Interface**—As depicted in Fig. 2, the crystal structure shows that one end of the BamB β-propeller (green) interacts with the face of the β-sheet and the large loop connecting the β2- and β3-strands of the BamA POTRA3 domain (yellow). Approximately 911 Å² of surface area are buried at the interface, representing the largest protein-protein interface observed in the crystal as calculated by PDBePISA (23). No interactions are observed between BamB and the POTRA4 domain of BamA.

BamB is an eight-bladed β-propeller where three short loops connect the four β-strands within a blade and longer loops link the blades together. The BamB loops are numbered consecutively from blade 1 at the N terminus. Several loops in BamB adopt different conformations when bound to BamA compared with free BamB (Fig. 3A). Interblade loop 17, connecting the fourth and fifth blades, is known to be conformationally flexible (5, 7, 9, 11). The loop moves ~11 Å to make room for new conformations in loop 19, which harbors tyrosine 263, and loop 21 (linking the fifth and sixth blades) containing valine 286. In this new conformation, BamB Val-286 and Tyr-60 form a tight packing interaction with the side chain of BamA Pro-249 while its carbonyl oxygen hydrogen bonds with the side chain of BamB Tyr-263, contributing to the stability of the interface (Fig. 3B). The conformation of Tyr-263 is further stabilized by hydrogen bonding between the side chain oxygen of Gln-301 and the main chain amide nitrogen of Asn-264.

The BamA-BamB interface is stabilized by a network of hydrogen bonds (Fig. 4). The side chain of Gln-179 located on the β1-strand of BamA POTRA3 interacts with both Arg-77 and Glu-127 in BamB, whereas Thr-257 in the β3-strand of BamA POTRA3 is within hydrogen bonding distance of BamB Glu-150 and Arg-195. In addition, the phenolic hydroxyl of BamA Tyr-255 interacts with the main chain amide of BamB Tyr-60. The side chain of Arg-195 is at the heart of the BamA-BamB interface, and a cation-π interaction between its guanidinium group and the aromatic ring of BamA Tyr-255 (Fig. 4A, blue dotted line) further contributes to the BamA-BamB interaction. The electron density at the BamA-BamB interface is well defined as illustrated by an average kick map (24) displayed around interface residues (Fig. 4, B and C).

The extended BamB loop 13 links the third and fourth blades of the β-propeller. In the current structure, loop 13 is engaged in the interface with BamA, straddling the edge of the β2-strand in BamA POTRA3 (Fig. 5A). Although Leu-194 is packed against the BamB core, the side chain of Leu-192 makes a hydrophobic contact with BamA Leu-247. The straddling conformation of loop 13 is further stabilized by a hydrogen bond between the main chain nitrogen of BamA Leu-247 and the main chain carbonyl oxygen of BamB Ser-193. Therefore, although flexible in the structures of free BamB (5, 7, 9, 11), loop 13 is well defined in this structure and contributes to the stability of the BamB-BamA interface.

**Validation of the BamA-BamB Interface**—Two approaches were used to verify that the BamA-BamB interface observed in the crystallographic model is physiologically relevant. In the
first approach, BamA residues Tyr-255 and Thr-257 were individually mutated to alanine, and the mutants were tested for their ability to interact with BamB in vivo. As bamA is essential, a null mutant strain cannot be constructed. However, Silhavy and co-workers (1) developed a BamA depletion strain in which the normal bamA gene is deleted and another copy of wild-type bamA under the control of the inducible arabinose promoter is introduced. This strain (JCM166) grows normally in the presence of arabinose (inducer) but stops growing several generations after switching the culture to fucose (repressor) due to BamA depletion. This phenotype can be complemented by a plasmid encoding a functional copy of bamA under a constitutive promoter. It has been shown previously that the full-length bamA gene containing a His tag between the signal sequence and the first POTRA domain (HisBamA) cloned into the low copy number plasmid pZS21 can complement the JCM166
strains (15). Furthermore, \( \text{HisBamA} \) assembles into functional BAM complexes and can thus be used to examine BAM interac-
tions with other BAM subunits by pulldown assays (15).
Therefore, Y255A and T257A mutations were introduced into \( \text{HisBamA} \) and BamB.

The \( \text{HisBamA} \) mutants Y255A and T257A were able to support JCM166 growth in the presence of fucose, indicating that the mutants were expressed and functional (data not shown). JCM166 cells expressing \( \text{HisBamA} \), \( \text{HisBamA}(Y255A) \), or \( \text{HisBamA}(T257A) \) were thus grown in fucose-containing medium, disrupted with BugBuster, and subject to Ni-NTA purification followed by Western blotting to test the interactions with BamB. Both \( \text{HisBamA} \) and \( \text{HisBamA}(T257A) \) were able to pull down BamB (Fig. 6, right panel). Conversely, no BamB was detected in complex with \( \text{HisBamA}(Y255A) \) (Fig. 6, left panel), validating the role of Tyr-255 in stabilizing the BamA-BamB interface as defined in the crystal structure.

In a second approach to further validate the structure, disul-
fide engineering was used to probe the BamA-BamB interface. \textit{E. coli} BamA is devoid of reactive cysteines as its single N-ter-

crminal cysteine is blocked by lipidation. BamA contains two cyste-
ed residues in the \( \beta \)-barrel domain facing the outside of the cell (25). However, the periplasmic POTRA domains are devoid of cysteines. Therefore, we sought to introduce cysteine resi-

dues in BamA and BamB at positions that, according to the crystal structure, could allow formation of an interchain disul-

dide bond. Analysis of the interface revealed that the \( \beta \)-carbons of BamA Val-183 and BamB Ala-129 are 4.7 Å apart, which may allow disulfide bond formation across the interface if mutated to cysteines (Fig. 7A). A pQLINK plasmid (22) was thus con-
ducted for dual expression of \textit{E. coli} \( \text{HisBamA} \) and BamB (untagged) to test the ability of cysteine mutants to form disul-

dide bonds \textit{in vivo}.

Experiments were carried out in an \textit{E. coli bamB-null} strain (8) to prevent formation of complexes between \( \text{HisBamA} \) mutants and endogenous BamB. Cells transformed with plas-

mids encoding wild-type or cysteine mutants of \( \text{HisBamA} \) and BamB were grown to late log phase in rich medium and har-

evisted. Protein expression was not induced to minimize poten-
tial cross-linking artifacts due to overexpression. Instead, we relied on leaky expression from the pQLINK \( \text{P}_{\text{lac}} \) promoters (22). The cells were then lysed with BugBuster in the presence of an excess of \( N \)-ethylmaleimide to prevent disulfide bond for-

mation during sample processing. Detergent-solubilized pro-
teins were subject to Ni-NTA purification followed by SDS-

PAGE under both reducing and non-reducing conditions and

analyzed by Western blotting. As shown in Fig. 7, expression of \( \text{HisBamA}(V183C) \) together with BamB(A129C) leads to for-

mation of a high molecular weight band in non-reducing SDS-

PAGE that is reactive with both BamA and BamB antibodies.

This band disappears when the sample is treated with DTT, indicating that the species is \( \text{HisBamA}(V183C) \)-BamB(A129C) cross-linked by a disulfide. There is also a faint band that reacts with the BamB antibody and migrates at approximately the position of BamA (see Fig. 7C, upper right panel, V183C/A129C lane). This does not indicate that the BamB antibody cross-

reacts with BamA as the band is not observed in any other lane containing BamA. Instead, the band likely represents a degra-
dation product of the main cross-linked species as both disap-
pear upon treatment with DTT. Consistent with this result, control plasmids encoding \( \text{HisBamA}(V183C) \)-BamB(WT) or \( \text{HisBamA}(WT) \)-BamB(A129C) do not produce the cross-linked band that reacts with both antibodies. Furthermore, expression of BamB(A129C) together with \( \text{HisBamA} \) cysteine mutants in POTRA4 (\( \text{HisBamA}(S274C) \)) or POTRA5 (\( \text{HisBamA}(A375C) \)) also fails to produce high molecular weight bands that react with both BamA and BamB antibodies. This is not due to lack of expression of the mutant proteins as both BamA and BamB mutants are expressed at comparable levels (see Fig. 7C, bottom panel, for expression of BamB proteins). This strongly suggests that \( \text{HisBamA}(V183C) \)-BamB(A129C) form a disulfide bond \textit{in vivo} that is specifically facilitated by their close proximity in the

BamA-BamB interface observed in the crystal structure.

DISCUSSION

The physiological role of BamB is linked to its ability to bind BamA. This was revealed by mutations that disrupt BamB binding to BamA that are phenotypically similar to \( \text{bamB} \)-null mutations (1, 3, 8, 16). BamA mutations that abolished binding to BamB mapped to the POTRA3 motif in the periplasmic domain (15). This suggested that the soluble periplasmic domain of BamA and the lipid-free soluble form of BamB could be used to recapitulate the BamA-BamB complex. However, efforts to isolate a stable complex between these two proteins were not successful (data not shown). Therefore, we used the strategy of linking the two proteins together to generate a fusion protein for crystallization. As the C terminus of BamA POTRA domains and the N terminus of BamB both face the outer membrane, the C terminus of BamA was fused to the N terminus of BamB via flexible linkers. Several linker lengths were tested to minimize the chance that too short a linker could interfere with complex formation. To improve the chances of crystallization, not only the five-POTRA domain fragment of BamA was used in the fusions but also fragments encompassing POTRA3–5 and POTRA4–5. Whereas the five-POTRA frag-

ment is known to be conformationally flexible in solution due to a hinge between POTRA 2 and 3, the POTRA3–5 and POTRA4–5 fragments are relatively rigid (19). None of the POTRA4–5-containing fusions yielded crystals, consistent with the idea that POTRA3 is crucial for the BamA-BamB interaction. Fusions containing all five POTRA domains failed to yield crystals presumably due to their conformational flexibility. How-
ever, fusions consisting of POTRA3–5 linked to BamB with 16- or 12-amino acid linkers produced single crystals with those with the shorter linker diffracting slightly better and being selected for structure determination. The construct linking POTRA3 to BamB fusion with the longest (36-amino acid) linker did not stabilize the complex enough to yield crystals, or the long and flexible linker interfered with crystallization.

The lattice of the POTRA3–5-linker-BamB crystal displays several contact interfaces between BamA and BamB (Fig. 8). However, the interface shown in Fig. 2 buries the most surface area (911 Å²) and is the only one consistent with both previously reported mutagenesis studies as well as the mutagenesis and cysteine cross-linking experiments presented here. The extended loop 13 connecting the third and fourth blades of the BamB β-propeller is engaged in the interface with BamA POTRA3 and harbors conserved residues previously shown to be important for the interaction with BamA. Misra and co-workers (16) showed that the BamB loop 13 mutation R195A in combination with L192S or L194S BamB mutations causes a decrease or loss of interaction with BamA, respectively. Consistent with these results, the structure shows that residue Arg-195 is at the heart of the BamA-BamB interface (Fig. 3, in orange). In addition to a hydrogen bond interaction with BamA Thr-257, Arg-195 mediates cation-π interaction with BamA Tyr-255. Cation-π interactions are a common feature of protein-protein interfaces with approximately half of all protein complexes displaying at least one of these interactions (26). Arginine and tyrosine are the most frequent cation-π partners with planar stacking of the guanidinium and aromatic ring observed between BamB Arg-195 and BamA Tyr-255 being the
most common arrangement. The position of Arg-195 in the interface is stabilized by packing interactions of BamB loop 13 residues Leu-192 and Leu-194 (Fig. 5A, in orange) that straddle the β2-strand in BamA POTRA3 and contribute a main chain–main chain hydrogen bond to interface stability. It is therefore not surprising that two loop 13 mutations were necessary to disrupt the BamA–BamB interactions.

Mutation of the BamA side of the interface also disrupts complex formation. As shown in Fig. 6, BamA(Y255A) fails to interact with BamB through pulldown experiments. The mutation disrupts the cation–π interaction with BamB Arg-195 as well as a hydrogen bond to the BamB main chain, the amide nitrogen of BamB Tyr-60 (Fig. 4). The large difference in side chain volume between alanine and tyrosine likely contributes to the deleterious effect of the mutation. Conversely, BamA(T257A) was still able to support BamB pulldown. This mutation disrupts hydrogen bonding with BamB Arg-195 and/or Glu-150 (Fig. 4). However, Thr-257 does not mediate interactions with the BamB main chain, and its replacement with alanine does not create a large void at the interface. Therefore, the milder effect of the BamA T257A mutation compared with Y255A is consistent with their respective roles in stabilizing the BamA–BamB interface.

Two additional BamA mutations have previously been shown to modulate BamA–BamB binding. The β2-strand of BamA POTRA3 displays a β-bulge defined by amino acid Asp-241 (Fig. 5B, cyan). Kim et al. (15) investigated the importance of this structural feature in BamA by mutating Thr-243 or Val-245 to aspartate in an attempt to displace the β-bulge along the β2-strand (Fig. 5A, cyan). In their study, mutation T243D or V245D caused the decrease or loss, respectively, of the BamA–BamB interaction. Whereas the β-bulge and residue Asp-241 are not in direct contact with BamB in the structure, residues Thr-243 and Val-245 are in the section of the BamA β2-strand that interacts with BamB (Fig. 5B). Therefore, whether these mutations displaced the β-bulge toward the interface or introduced a negatively charged residue in the core-facing side of the BamA β2-strand, disrupting its conformation, the mutagenesis results are consistent with the interface observed in this structure.

Disulfide engineering is a powerful technique to probe structural models (27, 28). It produces a positive signal (disulfide formation) if the proteins are arranged as defined in the model. This provides an excellent complement to mutagenesis studies that produce a negative signal (loss of binding) when the interface is disrupted. We thus sought to introduce cysteines in BamA and BamB that would result in a disulfide bond if the proteins interact as defined in the crystal structure. Analysis of the BamA–BamB interface with the program Disulfide by Design (29) did not identify any pair of residues that would have a favorable geometry to form an interchain disulfide when mutated to cysteine. Nevertheless, manual inspection of the interface showed that the 4.7-Å distance between the β-carbons of BamA Val-183 and BamB Ala-129 may allow disulfide bond formation across the interface even though the geometry was not ideal. As shown in Fig. 7, expression of HisBamA(V183C) together with BamB(A129C) did lead to the formation of a disulfide cross-linked species that reacted with both BamA and BamB antibodies. The cross-linking efficiency is not very high, which is consistent with their proximity in the BamA–BamB interface but non-ideal geometry for disulfide bond formation. The disulfide cross-link is specific to this pair of residues. Expression of BamB(A129C) together with HisBamA(S274C) or HisBamA(A375C) with cysteines located in POTRA 4 and 5, respectively, did not produce cross-linked bands that react with both BamA and BamB antibodies. Instead, these BamA mutants produced faint cross-linked bands that only react with the BamA antibody and likely represent BamA dimers or BamA cross-links to other cysteine-containing E. coli proteins. Taken together, the mutagenesis and disulfide cross-linking experiments indicate that the BamA–BamB interface observed in the crystal structure is physiologically relevant.

The interactions between BamB and BamA POTRA3–5 are restricted to the interface with POTRA3 described above (Fig. 3). Although POTRA5 was present in the construct and could be accommodated in the crystal lattice without any clashes, the electron density for POTRA5 was very weak and broken. Therefore, POTRA5 is not included in the final model. This impacts the R factors of the refined model as the POTRA5 (and the linker) still contribute to the observed scattering. Nevertheless, the final model is of good quality and compares favorably with structures determined to similar resolution (see quality report for Protein Data Bank code 4PK1). The weak electron density for POTRA5 is likely due to its distal position from the point of interaction between BamA and BamB (Fig. 2) and the lack of stabilizing lattice contacts resulting in static disorder of its conformation. This was also observed in the crystal structure...
of POTRA4–5 where POTRA5 in one of the two molecules in
the asymmetric unit was ill defined due to static disorder (19).

Crystal structures of E. coli BamA POTRA1–4 as well as
small angle scattering data support a model in which the
periplasmic domain of BamA can adopt extended and bent con-
formations due to hinge motions around the link between
POTRA2 and POTRA3 (18, 19). Superimposing the current
POTRA3–4-BamB structure onto POTRA1–5 models indi-
cates that BamB is located at the hinge between POTRA2 and
POTRA3 and is positioned to interact with POTRA2 in the
extended conformation of BamA (Fig. 9A). Mutagenesis data
are consistent with this model. In addition to mutations in
BamB loop 13, Misra and co-workers (16) also reported that
mutation of either of two conserved aspartates in BamB loop 17
(Asp-246 or Asp-248; Fig. 5A, in orange) to alanine resulted in
weaker binding of BamB to BamA. However, the mutations
were not as destabilizing as those in loop 13 and did not result in
the membrane permeability phenotype associated with BamB
deletions. BamB Asp-246 in loop 17 and Leu-192 in loop 13 are
in a position to interact with the POTRA2 domain of BamA in
the extended conformation (Fig. 9A, orange space-filling mod-
els). Furthermore, loop 17 is not fully defined in the current
structure due to conformational flexibility (Fig. 9A, dotted line),
but given its position, it is likely to have additional contacts with
POTRA2 in full-length BamA.

Recently, two BamA structures that contain the β-barrel
domain and two (Haemophilus ducreyi) or all five POTRA
domains (Neisseria gonorrhoeae) were reported, providing the
first glimpse at the membrane component of BamA and its
relationship with its periplasmic domain (12). We superim-
posed the E. coli POTRA3–4-BamB structure presented here
with the POTRA3–4 domains in N. gonorrhoeae BamA to
generate a model of the BamA-BamB complex (Fig. 9B). The model
illustrates the position of BamB at the hinge between POTRA2
and POTRA3 as well as its location with respect to the mem-
brane. Most structures of BamB do not contain the first 10–12
amino acids of the mature protein, suggesting lability in its con-
formation. The only BamB structure containing the N-terminal
residues (Protein Data Bank 3Q7O) (11) displays an extended,
irregular structure followed by a short N-terminal helix for the
N-terminal segment. This is consistent with the model shown in
Fig. 9B where the β-propeller structure of BamB is not closely
associated with the membrane but tethered to the membrane
through an extended N-terminal segment terminated in the lipi-
dated cysteine characteristic of outer membrane lipoproteins.

Interestingly, BamA displays conformational flexibility both
within its periplasmic domain (18, 19) and in the relative positions
of the periplasmic and β-barrel domains (12). Furthermore, a con-
formational cycle has been proposed for the BamA-assisted OMP
folding and insertion that is modulated by mutation in some lipo-
protein subunits of BAM (30). As BamA by itself has recently been
reported to catalyze folding and insertion of substrate OMPs into
liposomes in vitro (31), one important role of the BAM lipopro-
teins may be to modulate BamA conformation and increase the
efficiency of the machinery (8). Therefore, understanding how the
BAM lipoproteins interact with BamA is crucial to define their
functional role. In accordance with the structure presented here,
we propose that binding of BamB at the hinge between POTRA2
and POTRA3 modulates the flexibility of the periplasmic domain
of BamA, increasing the efficiency of productive conformational
cycles responsible for the folding and insertion of outer membrane
proteins.

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