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# Crystal Structure of BamD. An Essential Component of the β-Barrel Assembly Machinery of Gram Negative Bacteria

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## Abstract

Folding and insertion of integral β-barrel proteins in the outer membrane is an essential process for Gram-negative bacteria that requires the β-barrel Assembly Machinery (BAM). Efficient OMP folding and insertion appears to require a consensus C-terminal signal in OMPs characterized by terminal F or W residues. The BAM complex is embedded in the outer membrane and, in *E. coli*, consists of the β-barrel BamA and four lipoproteins BamBCDE. BamA and BamD are broadly distributed across all species of Gram-negative bacteria whereas the other components are present in only a subset of species. BamA and BamD are also essential for viability suggesting that these two proteins constitute the functional core of the bacterial BAM complex. Here we present the crystal structure of BamD from the thermophilic bacteria *Rhodothermus marinus* refined to 2.15 Å resolution. The protein contains 5 tetratricopeptide repeats (TPRs) organized into two offset tandems, each capped by a terminal helix. The N-terminal domain contains three TPRs and displays remarkable structural similarity with proteins that recognize targeting signals in extended conformations. The C-terminal domain harbors the remaining 2 TPRs and previously described mutations that impair binding to other BAM components map to this domain. Therefore, the structure suggests a model where the C-terminal domain provides a scaffold for interaction with BAM components, while the N-terminal domain participates in interaction with the substrates, either recognizing the C-terminal consensus sequence or binding unfolded OMP intermediates.

## Introduction

The outer membrane of Gram-negative bacteria is unique and essential for its survival. This semi-permeable membrane is typically asymmetric composed of phospholipids on the inner leaflet and primarily lipopolysaccharide (LPS) on the outer leaflet [1]. Proteins associated with the outer membrane can be divided into three groups based on their architecture: (i) lipoproteins, which do not contain transmembrane domains and are attached to the OM by lipid modification of their N-terminal cysteine residue [2]; (ii) Outer Membrane Proteins (OMPs), characterized by a transmembrane β-barrel architecture [3]; and (iii) periplasm-



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spanning export channels, whose trans-outer-membrane components can have β-barrel structures as in TolC [4], or a novel α-barrel architecture typified by Wza [5; 6].

Transmembrane β-barrel OMPs carry out a variety of functions in bacteria including nutrient and waste product exchange with the environment, cell adhesion and fulfillment of structural and enzymatic roles [3]. Folding and insertion of OMPs in the outer membrane is an essential process that requires a multiprotein complex in the outer membrane known as β-barrel Assembly Machinery (BAM) [7; 8]. In *E. coli*, this complex is composed of BamA, a β-barrel OMP also known as Omp85/YaeT, and four lipoproteins, BamBCDE (previously known as YfgL, NlpB, YfiO and SmpA respectively) [8; 9]. Periplasmic chaperones such as SurA assist in shuttling nascent OMPs to the BAM complex [10]. The process was recently reconstituted *in vitro* indicating that SurA and BAM can mediate OMP folding and insertion into membranes [11]. *In vivo*, the efficiency of the process appears to depend on a consensus sequence at the C-terminus of β-barrel OMPs [12; 13]. The last residue in OMPs is almost always F or W and, starting from the C-terminus, positions 3, 5, 7 and 9 are hydrophobic residues with Y frequently occupying position 3 [13]. Thus, it is likely that this signal is important for one or more mechanistic steps in OMP folding and insertion.

BamA is essential for viability [8; 14; 15] and homologs are retained in mitochondria and chloroplasts where they also mediate insertion of β-barrel proteins [16]. BamD is the only BAM lipoprotein required for viability [8; 9; 17; 18]. Both BamA and BamD are broadly distributed in Gram-negative bacteria [19] which, together with their requirement for viability, suggest that they constitute the core of the BAM complex. The phylogenetic distribution of BamB, C and E is more restricted [19], and individual deletion of their genes in *E. coli* result in membrane permeability defects but not loss of viability [8; 9].

The structural information necessary to understand the molecular mechanisms of OMP folding and insertion mediated by BAM is beginning to emerge. BamA contains a large periplasmic domain with five POlypeptide-TRanslocation-Associated (POTRA) repeats in addition to its transmembrane β-barrel. Crystallographic and solution scattering data revealed a superhelical arrangement of the POTRA domains [20; 21; 22] thought to interact with nascent OMPs and perhaps nucleate folding of their β-strands [20; 21; 23]. Structures of the non-essential lipoproteins BamB and BamE have recently been reported [24; 25; 26; 27]. However, structural data on the essential lipoprotein BamD has remained elusive, although sequence analysis suggested that the protein contains tetratricopeptide repeats [19]. Here we report the crystal structure of BamD from *Rhodothermus marinus* refined to 2.15 Å resolution and discuss the implications for the role of BamD in the BAM-mediated mechanism of OMP folding and insertion.

## Results and Discussion

### Crystal Structure of BamD

A lipid-free, soluble form of BamD from the thermophilic bacteria *Rhodothermus marinus* (rmBamD) was expressed in *E. coli* from a plasmid encoding the mature protein (amino acids 23–280) with the N-terminal cysteine mutated to alanine. The structure was determined using single anomalous dispersion methods and seleno-methionine substituted protein. Data collected from native crystals was then used to refine the structure to 2.15Å resolution (Table 1). The final model contains residues 30–280 and is thus only missing seven amino acids from the N-terminus due to conformational flexibility.

The structure of rmBamD consists of five tetratricopeptide repeats (TPR) arranged in two offset tandem arrays (Figure 1). TPRs are 34 amino acid degenerate sequences that fold into a helix-turn-helix motif [28; 29]. TPRs are typically arranged in tandem arrays such that the

two antiparallel helices (A and B) from one repeat pack against the A helix of the next motif. The tandem is frequently terminated by a capping helix. Packing several TPRs in this manner results in a right-handed superhelical structure [30]. In rmBamD however, three N-terminal TPRs (TPR1–3, red, green and blue in Figure 1) are capped by a helix that is continuous with the A helix of TPR 4 (light blue and yellow). As a consequence, the two C-terminal TPRs (TPR4–5 yellow and orange) are offset from the N-terminal three, breaking the superhelical arrangement. rmBamD is therefore a very elongated molecule measuring more than 95 Å between the N-terminus and the capping helix of TPR5 (light orange). In general, the turns connecting the A and B helices are short and fairly typical. TPR3 however is notable by the extended loop that connects the two helices (magenta in Figure 1).

Mapping the electrostatic surface potential on the structure of rmBamD reveals a striking distribution of charge (Figure 2). One side of the molecule, harboring the turns linking the TPRs helices, is positively charged whereas the opposite side displays a strong electronegative character. Interestingly, the offset between the N and C-terminal domains creates a negatively charged semicircular groove at the bottom of rmBamD, approximately 24 Å in diameter that may be important for interaction with other BAM components.

## BamD across Gram-negative bacteria

BamD is essential in *E. coli* [8; 17; 18] as well as *Neisseria* meningitidis [31], and together with BamA is thought to constitute the core of the BAM machinery. Consistent with its essential role, BamD homologs are ubiquitous in Gram-negative bacteria [17; 19]. We used hidden Markov models to iteratively search the KEGG database for BamD homologs using jackhmmer (part of the HMMER package [32]). The alignment was manually edited in Jalview [33] to eliminate a few sequences that did not contain the invariant cysteine at the mature N-terminus that becomes lipidated in BamD [34]. This resulted in 607 sequences with broad representation of Gram-negative bacteria.

The length of BamD is approximately 240–300 amino acids (from the translational start) in most bacteria, with rmBamD being 280 amino acids long. In *E. coli* BamD is 245 amino acids and the alignments suggest that the protein is missing the last helix capping TPR5 in rmBamD (Figure 3A). Conversely, BamD homologs in Pseudomonadales including *Moraxella*, *Acinetobacter* and *Pseudomonas*, have an extended C-terminus that makes them 340–380 amino acids long. This extension does not have clear sequence similarity to any other protein and may represent additional TPR repeats.

To evaluate conserved features in BamD we subjected the aligned sequences to analysis with the Consurf server, which uses Bayesian methods to score the rate of evolution at each aligned position [35]. Figure 3A shows the alignment of rmBamD with *E. coli* and *Neisseria* sequences for reference. The results mapped on the structure of rmBamD (Figure 3B), show that the N-terminal domain is generally more conserved than the C-terminal domain. The extended loop connecting the A and B helices of TPR3 together with TPR4 form a deep groov. e lined with conserved residues suggestive of a protein:protein interaction surface. Consistent with this hypothesis, C-terminal truncations of *E. coli* BamD resulted in impaired binding to BamA as well as the accessory lipoproteins BamC and BamE [9; 17]. These truncations map to the beginning of TPR5 in rmBamD and would not only eliminate the entire TPR5 but likely result in destabilization of TPR4 as well. Therefore, we suggest that the extended loop of TPR3 together with the C-terminal domain of BamD harbor the determinants for interaction with other BAM components.

## Potential roles for BamD in OMP biogenesis

The structure of rmBamD N-terminal domain is strikingly similar to a number of proteins involved in recognition of C-terminal targeting signal sequences (Figure 4). The right-handed twist in the TPR tandem creates a concave face lined by the TPR's the A helices. TPR domains in proteins such as the Hsp-Organizing-Protein (Hop), Tom70 (a component of the mitochondrial Translocase of the Outer Membrane) and FKBP52 use this concave surface to recruit molecular chaperones Hsp70 or Hsp90 by binding their C-terminal tails in an extended conformation [36; 37; 38] (Figure 4A, B and C). Similarly, the C-terminal Peroxisomal Targeting Signal-1, present at the C-terminus of proteins destined to the peroxisome, is bound by PEX5 in the concave face of its TPR domain (Figure 4D), although a second cluster of TPRs also contributes to the binding [39]. Notably, the targeting signal peptides are all bound in a similar extended conformation in these proteins. Since the structure of the N-terminus of rmBamD is very similar to TPR domains in these proteins (Figure 4), BamD may have an analogous role, recognizing the C-terminal consensus sequence observed in the vast majority of OMPs: starting at the C-terminus, positions 1, 3, 5, 7 and 9 are hydrophobic amino acids with 1 being almost always F or W and 3 preferentially Y [13]. Given the conservation of this C-terminal signature in OMPs across species, it is likely that they are important for one or more mechanistic steps of OMP folding and insertion. BamD is ideally suited to recognize the OMP signal.

The N-terminal domain of BamD is also structurally similar to chaperones of the Type Three Secretion System (TTSS) such as IpgC, SycD and PcrH [40; 41; 42] (Figure 4E and F). Crystal structures of IpgC and PcrH in complex with their targets show the concave face of the chaperone 3-TPR tandem binding 8–13 amino acids long peptides in extended conformations, spanning the entire face of the TPR tandem (Figure 4E and F) [41; 42]. By analogy to these proteins it is possible that BamD binds unfolded OMPs in an extended conformation prior to folding and insertion into the Outer Membrane. Interestingly, the receptor for β-barrel protein import in mitochondria (Tom70) also has TPR domains that interact with precursor β-barrels prior to translocation through the TOM pore, in addition to the TPRs binding Hsp70 and Hsp90 mentioned above [43; 44].

BamD is associated with the membrane by its lipidated amino terminal cysteine [34]. All but 7 amino acids at the N-terminus are defined in the structure of rmBamD. Therefore the N-terminal domain must be proximal to the membrane. Conversely, BamC and BamE, the other two BAM lipoproteins known to interact directly with BamD, have disordered N-terminal extensions [25; 27; 45; 46]. Both BamE and BamC are thought to bind the C-terminus of BamD [9; 17]. Therefore, currently available data on the BAM complex components can be consolidated into a working model described in Figure 5. BamA, with its periplasmic five-POTRA domain recognizes nascent OMPs [14] and may nucleate formation or β-strands or β-hairpins in its substrates [20; 21; 22; 23.] BamA interacts directly with BamD, whose C-terminal domain provides a scaffold for the binding of BamC and BamE, which in turn stabilize the complex with BamA. C-terminal truncations of BamD are thus tolerated in *E. coli*, but result in loss of BamC and weakened binding of BamE to the complex [9; 17]. The N-terminal domain of BamD plays an essential role in BAM function. We suggest that it may be required to recognize the C-terminal consensus signal in OMPs or perhaps interact with unfolded OMP precursors. BamB interacts directly with BamA independently of BamCDE [9; 11; 17]. As a non-essential component of BAM, BamB may increase the efficiency of the complex or assist in the folding and insertion of a specific subset of substrates either recognizing their C-terminal OMP signal or nucleating β-strand formation [24.]

## Materials and Methods

### Protein Cloning, Expression and Purification

The gene fragment coding for mature BamD (amino acids 23–280) was PCR amplified from *Rhodothermus marinus* genomic DNA. The primers incorporated NdeI and XhoI restriction sites at the 5' and 3' ends of the fragment respectively and also mutated the N-terminal cysteine (C23) to alanine. The amplified fragment was ligated into a modified pET28 vector that incorporates an N-terminal His-tag followed by the recognitions site for the TEV protease. The resulting plasmid (pMS716) thus drives the expression of a His-tagged mature rmBamD C23A. Upon cleavage with TEV protease, amino acid GHM from the tag remain fused to the N-terminus of the protein.

*E.coli* Rosetta (DE3) cells (Novagen) were transformed with plasmid pMS716 and selected in LB agar plates containing 50 μg/ml kanamycin. A single colony was used to inoculate 100 ml of LB supplemented with 50 μg/ml kanamycin and the culture grown overnight. A 10 ml aliquot was then diluted into 1L of LB with kanamycin and allowed to grow at 37º C to an $OD_{600}$ of 0.6. Expression was induced by addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG, Gold Bio Inc.) and incubation for 3 hours at 37º C. Cells were harvested by centrifugation, resuspended in a buffer A (25 mM TrisHCl pH 8.0, 150 mM NaCl) supplemented with a protease inhibitor cocktail (Complete EDTA-free, Roche) and frozen until used. After thawing, cells were lysed on ice by sonication. Cell debris were removed by centrifugation at 16,000 rpm for 30 min at 4º C and the supernatant applied to a 10 ml Ni-NTA column (Qiagen) pre-equilibrated with buffer A. The protein was allowed to bind the Ni-NTA beads in batch for 2 hours at 4º C. The resin was collected in a column and washed with 5 column volumes of buffer A, followed by a wash with 5 column volumes of buffer A containing 25 mM imidazole. The protein was then eluted with buffer A supplemented with 200 mM imidazole. TEV protease was added to the elution pool and dialyzed overnight at 4º C in Buffer A supplemented with 10 mM DL-Dithiothreitol (Gold Bio Inc.) to clave the His tag. The cleaved protein was concentrated and loaded on a size exclusion column (HiLoad 26/60 Superdex 200, Amersham Pharmacia Biotech) pre-equilibrated with buffer A and eluted in the same buffer. Purified rmBamD protein was concentrated to 15 mg/ml and stored at -80º C until needed.

Seleno-methionine substituted rmBamD was prepared as described above with the following modifications to the bacterial cultures. Overnight cultures of *E. coli* Rosetta (DE3) transformed with pMS716 were spun down and resuspended in 3 L M9 minimal medium supplemented with 50 μg/ml Kan, and 4.0 g/l glucose. Cultures were grown at 37º C to $OD_{600}$ of 0.4. Methionine synthesis was then inhibited by addition of 100 mg/l D-lysine, D-phenylalanine, and D-threonine; 50 mg/l D-isoleucine and D-valine. The culture was also supplemented with 120 mg/l of seleno-methionine (Acros Organics) and incubation continued at 37º C until $OD_{600}$ was 0.6. Cultures were cooled on an ice bath for 10 minutes, expression induced by addition of 1 mM IPTG followed by incubation overnight at 20º C. Protein purification was performed as describe above. Final protein concentration was 7.5 mg/ml.

### Protein Crystallization

Crystallization screening with purified rmBamD was carried out using numerous commercial and homemade screens at various protein concentrations. Several conditions yielded crystals, but best conditions appeared in Wizard screens (Emerald Biosystems). Further optimization was performed using the hanging drop vapor diffusion method (2.0 μl protein: 2.0 μl precipitant) at 16º C. The best native crystals were obtained form a precipitant solution containing 3.25 M NaCl and 0.1 M Tris pH 8.0 and a protein solution at 7.5 mg/ml

concentration. Crystals were harvested, transferred directly into 4.0 M malonate pH 7.0 and flash frozen in a stream of $N_2$ at 100 K. Crystals of seleno-methionine substituted rmBamD were obtained in 2.7 M NaCl, 0.1 M Tris pH 7.5, and 0.2 M MgCl2. Cryo-protection was achieved in 4 M malonate as well.

### Structure Determination

A dataset to 2.7Å resolution collected at the peak wavelength for selenium from seleno-methionine substituted rmBamD crystals was used to solve the structure by Single Anomalous Dispersion using the AutoSol module of Phenix [47]. Three selenium sites were readily identified (*Rhodothermus marinus* BamD has three non-terminal methionines) and used to calculate SAD phases followed by density modification. The resulting electron density map was readily interpretable and a partial model was automatically built into this map with the AutoBuild module of Phenix. Manual rebuilding using Coot [48] interspersed with model refinement in Phenix were carried out until no further improvement of the R-factor could be achieved. Amino acids 30 to 280 could be unambiguously modeled and refined. The final model statistics are shown in Table 1.

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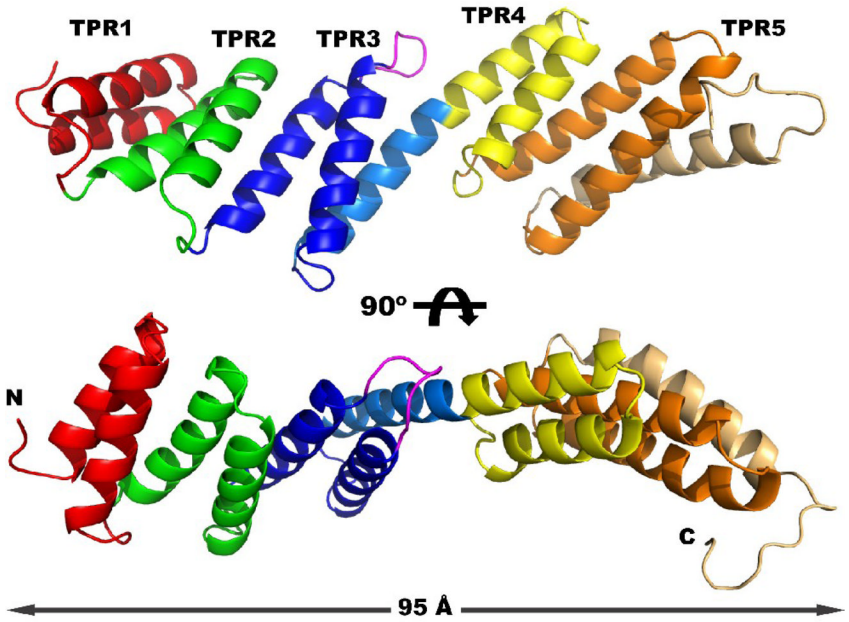


**Figure 1. Crystal structure of rmBamD**

Two views of rmBamD related by a 90º rotation. The N-terminal domain contains TPRs 1–3 (red, green and blue respectively) and is capped by a helix (light blue). The C-terminal domain contains TPRs 4 and 5 (yellow and orange) and the final capping helix (light orange). An extended loop in TPR3 is labeled magenta.

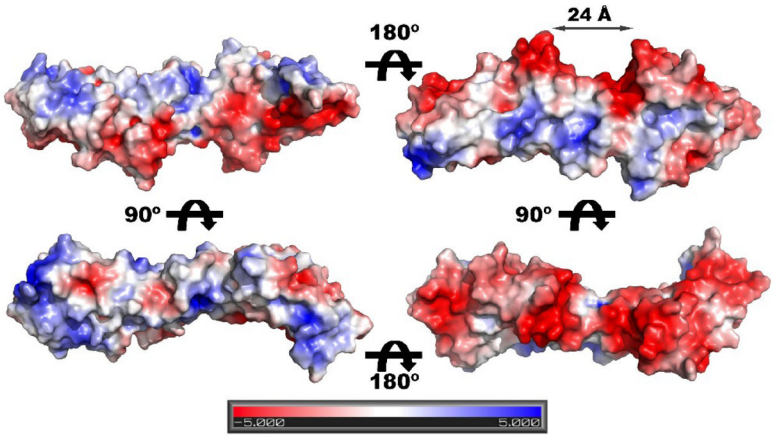


**Figure 2. Electrostatic surface potential of rmBamD**

The distribution of positive (blue) and negative (red) surface potential is mapped to a surface representation of rmBamD. For reference, the orientation of the molecule in the left panels is the same as in Figure 1. A negatively charged, semicircular groove between the N and C-terminal domains 24Å in diameter is labeled.

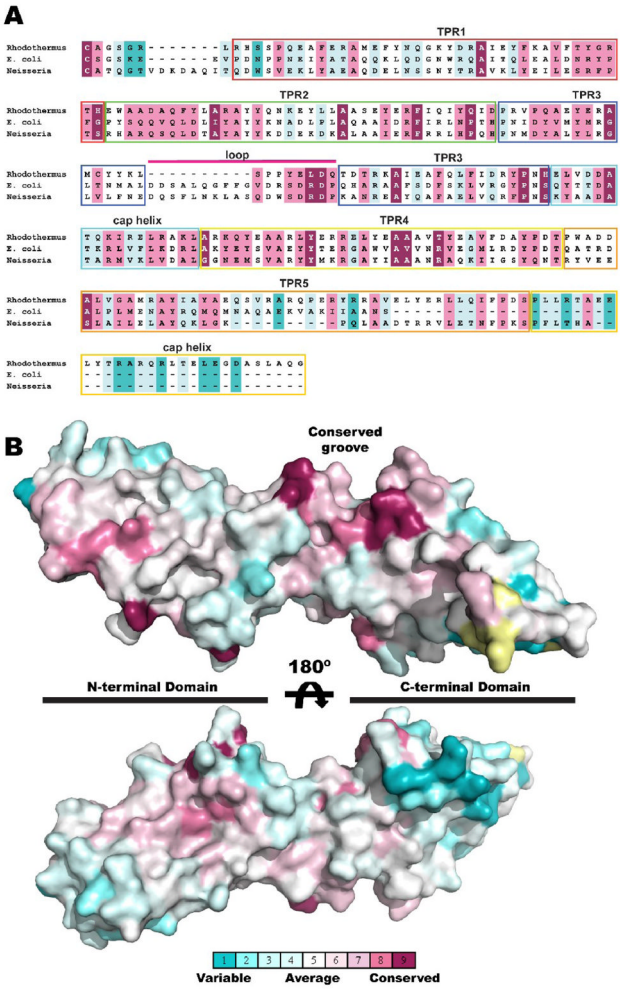


**Figure 3. Sequence conservation in BamD**

A sequence alignment of BamD homologs (see text) was analyzed with the Consurf sever. Conservation scores are normalized so that the average score is zero and the standard deviation is 1. Thus rapidly evolving positions are negative (colored cyan) and slowly evolving positions are positive (colored purple) while positions with an average rate of evolution are colored white. (A) Aligned sequences of BamD from Rhodothermus marinus (Rhodothermus), Escherichia coli (E. coli) and Neisseria meningitidis (Neisseria) colored by the Consurf scores. Structural elements are labeled and highlighted in boxes colored as in Figure 1. (B) Conservation scores mapped on a surface representation of the rmBamD structure showing a groove between the extended loop in TPR3 and the C-terminal domain lined with slowly evolving residues.

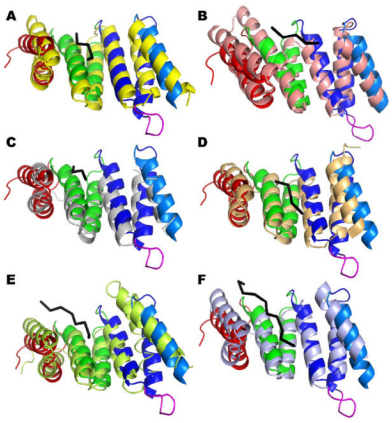


**Figure 4. The N-terminal domain of rmBamD is similar to proteins binding extended polypeptides**

The N-terminal domain of rmBamD (colored as in Figure 1) is superimposed to TPR domains of Hop (A, yellow), FKBP52 (B, salmon), Tom70(C, gray) and PEX5 (D, light orange); as well as the chaperones PcrH (E, light green) and IpgC (light blue). All these proteins bind extended polypeptides (shown as black C-alpha traces) in the concave face of the TPR tandem.

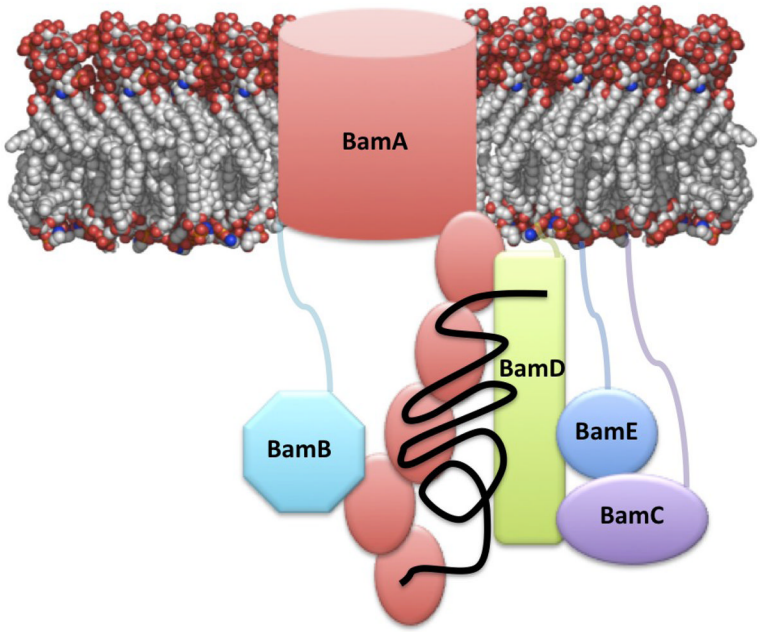


**Figure 5. Working model of the BAM complex**

BamA and BamD constitute the core of the complex. BamA is proposed to recognize and help fold nascent OMPs with its five POTRA repeats (show as orange ovals). The N-terminal domain of BamD may assist in this folding role, or may recognize the consensus sequence at the C-terminus of OMPs. BamC and E interact with the C-terminal domain of BamD and help stabilize the complex. BamB interacts with BamA independently of BamC, D and E.

**Table 1**

Data Collection and Refinement Statistics.

| | Native BamD | SeMet BamD |
|---|---|---|
| **Data Collection** | | |
| Space Group | R3[a] | R3[a] |
| Unit Cell: *a*=*b* (Å) | 114.37 | 114.22 |
| *c* (Å) | 77.63 | 77.62 |
| Wavelength (Å) | 1.0000 | 0.9792 |
| Resolution (Å)[b] | 70.0–2.15 (2.23–2.15) | 50.0–2.7 (2.80–2.70) |
| $R_{sym}$[c] (%) | 7.3 (33.7) | 8.5 (22.8) |
| *I* / s | 20.3 (2.7) | 15.2 (3.7) |
| Data Completeness (%) | 99.7 (99.0) | 97.7 (87.7) |
| Redundancy | 2.8 (2.6) | 3.6 (2.7) |
| **Phasing** | | |
| FOM before DM[d] | | 0.36 |
| FOM after DM | | 0.69 |
| **Refinement Statistics** | | |
| Resolution (Å) | 57.2 - 2.15 (2.26 – 2.15) | |
| Reflections in work set | 19492 | |
| Reflections in test set | 1032 | |
| Protein atoms | 2117 | |
| Water molecules | 152 | |
| $R_{work}$ (%) | 16.9 (20.7) | |
| $R_{free}$[e] (%) | 21.0 (27.9) | |
| Mean *B*-value protein (Å$^2$) | 37.1 | |
| Mean *B*-value water (Å$^2$) | 41.5 | |
| RMS dev. bonds (Å) | 0.007 | |
| RMS dev. angles (°) | 1.007 | |

[a] The hexagonal axis setting was used.

[b] Values in parentheses are for the highest-resolution shell.

[c] $R_{sym}=\Sigma_h\Sigma_i |(I_i(h)-< I(h)>|/ \Sigma_h\Sigma_I I_i(h)$, where $I_i(h)$ is the I-th measurement of reflection h, and <I(h)> is the weighted mean of all measurements of h.

[d] Figure Of Merit before Density Modification

[e] $R_{work} = \Sigma|F_{obs}-F_{calc}|/\Sigma F_{obs}$ where $F_{obs}$ = observed structure factor amplitude and $F_{calc}$ = structure factor calculated from model. $R_{free}$ is computed in the same as $R_{work}$, but using the test set of reflections.