BamE directly interacts with BamA and BamD coordinating their functions

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Abstract

The β-barrel assembly machinery (Bam) complex facilitates the assembly of outer membrane proteins (OMPs) in gram-negative bacteria. The Bam complex is conserved and essential for bacterial viability and consists of five subunits, BamA-E. BamA is the transmembrane component, and its β-barrel domain opens laterally to allow folding and insertion of incoming OMPs. The remaining components are regulatory, among which only BamD is essential. Previous studies suggested that BamB regulates BamA directly, while BamE and BamC serve as BamD regulators. However, specific molecular details of their functions remain unknown.

Our previous research demonstrated that BamE plays a specialized role in assembling the complex between the lipoprotein RcsF and its OMP partners, required for the Regulator of Capsule Synthesis (Rcs) stress response. Here, we used RcsF/OmpA as a model substrate to investigate BamE function. Our results challenge the current view that BamE only serves as a BamD regulator. We show that BamE also directly interacts with BamA. BamE interaction with both BamA and BamD are important for function. Our genetic and biochemical analysis show that BamE stabilizes the Bam complex and promotes bidirectional signaling interaction between BamA and BamD. This BamE function becomes essential when direct BamA/BamD communication is impeded.

Graphical Abstract
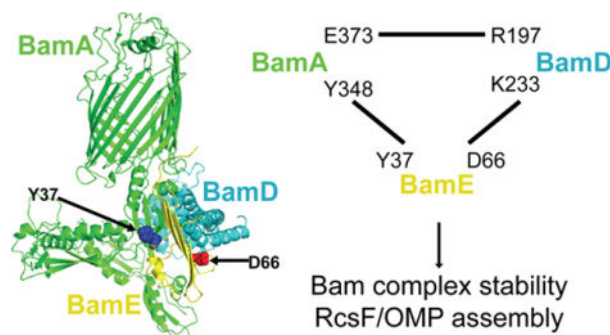
The Bam complex is essential for folding and inserting outer membrane proteins in gram-negative bacteria and is a key target for antibiotic development. However, the specific functions of its

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components, especially BamE, are poorly understood. Our study demonstrates that BamE directly interacts with essential components BamA and BamD, and plays a critical role in coordinating their activities, promoting the assembly of challenging substrates, such as the stress-sensing RcsF/OMP complex.

Keywords

outer membrane biogenesis; bacterial cell envelope; membrane proteins

INTRODUCTION

Cells of gram-negative bacteria are surrounded by two membranes, a cytoplasmic and an outer membrane (OM) (Saha *et al.*, 2021). The outer membrane has a distinct structure, being an asymmetrical bilayer and containing a large number of β-barrel OM proteins (OMPs) (Sun *et al.*, 2022). The assembly of OMPs is facilitated by the β-barrel assembly machinery (Bam) complex (Tomasek & Kahne, 2021). Bam complex is highly conserved and essential for bacterial viability, making it an attractive target for antibiotic development (Overly Cottom *et al.*, 2023).

In the best-studied *Escherichia coli* model system, the Bam complex is a stable heteropentamer formed by BamA-E subunits (Wu *et al.*, 2005, Sklar *et al.*, 2007). Most of the Bam complex research thus far has been focused on BamA. BamA is the only transmembrane component of the Bam complex as it contains a β-barrel domain. Its β-barrel domain is quite unusual because it can open up laterally, providing a site for folding and insertion of the incoming OMP substrates (Shen *et al.*, 2023, Doyle *et al.*, 2022, Lee *et al.*, 2019, Tomasek *et al.*, 2020, Wu *et al.*, 2021). Additionally, BamA is the most conserved component of the complex and is found in all gram-negative bacteria, and also two-membrane organelles of bacterial origin, such as mitochondria and chloroplasts (Misra, 2012, Diederichs *et al.*, 2021, Webb *et al.*, 2012). BamA also contains a periplasmic region made up by five POTRA (polypeptide transport–associated) domains. The POTRA domains scaffold four lipoprotein components BamB, C, D, and E, tethered to OM periphery by their N-terminal lipid modifications. In *E. coli*, BamD is the only essential component (Malinverni *et al.*, 2006); BamB,C,E are individually dispensable but collectively essential for viability (Tellez & Misra, 2012, Sklar *et al.*, 2007, Rigel *et al.*, 2012, Hart & Silhavy, 2020). Mutant variants of BamA have been reported that allow it to function in the absence of the Bam lipoproteins (Hart & Silhavy, 2020, Tellez & Misra, 2012, Hart *et al.*, 2020), indicating that BamA alone is sufficient for the OMP assembly, while Bam lipoproteins likely play a regulatory role, although their specific mechanistic functions remain unknown. As the lipoprotein components are also less conserved in other species (Webb *et al.*, 2012), they likely have specialized functions.

Previous studies have suggested that OMP assembly involves a cycle of coordinated conformational changes in BamA and BamD (Lee *et al.*, 2018, Hagan *et al.*, 2015, Hagan *et al.*, 2013, Pavlova *et al.*, 2013, McCabe *et al.*, 2017). During this cycle, the transition between active conformation states of both proteins is facilitated by a signaling interaction

that relies on a dynamic electrostatic network between BamA's POTRA5 domain and BamD (McCabe *et al.*, 2017, Sinnige *et al.*, 2015). Introducing mutations at the BamA/BamD interface deregulates the two essential components, preventing them from adopting functionally compatible conformations (Ricci *et al.*, 2012, McCabe *et al.*, 2017).

Mutations at the BamA/BamD interface also separate the Bam complex into two subcomplexes, BamAB and BamDCE (Malinverni *et al.*, 2006, Ricci *et al.*, 2012, McCabe *et al.*, 2017). While the stability of the heteropentameric Bam complex is not required for its function (Ricci *et al.*, 2012, McCabe *et al.*, 2017), this observation laid a foundation for the widely accepted model, in which BamB regulates BamA directly, while BamE and BamC serve as BamD regulators (Hart & Silhavy, 2020). BamB increases the efficiency of the OMP assembly *in vivo* and *in vitro* (Hagan *et al.*, 2010, Hagan & Kahne, 2011, Roman-Hernandez *et al.*, 2014, Mahoney *et al.*, 2016). BamE and BamC remain the most mysterious components of the Bam complex, as their deletion does not affect OMP assembly.

Our previous research provided the first direct evidence of the specialized functions of Bam lipoproteins by demonstrating that BamE is uniquely required for the assembly of the OM complex between the lipoprotein RcsF and its OMP partners (Tata & Konovalova, 2019, Konovalova *et al.*, 2016). The RcsF/OMP complexes function in the Regulator of Capsule Synthesis (Rcs) stress response, allowing RcsF to monitor integrity of the OM (Lach *et al.*, 2023, Tata *et al.*, 2021). Identification of the RcsF/OMP complexes as the first substrate of the Bam complex that requires BamE activity, presented an opportunity to study a molecular function of BamE. Our work suggested that BamE coordinates BamA and BamD to prevent a formation of an aberrant off-pathway dead-end complex between RcsF and BamA, that can sequester BamA from its function in the OMP assembly (Tata *et al.*, 2021, Tata & Konovalova, 2019). This BamA sequestration by RcsF is a molecular reason for synthetic lethal interactions of *bamE* with mutations that lower Bam complex levels or efficiency, for example, those caused by *bamB* null mutations(Hart *et al.*, 2019, Tata & Konovalova, 2019).

The purpose of this study is to investigate how BamE coordinates the activities of BamA and BamD. Our results challenge the current view that BamE only serves as a BamD regulator. We demonstrate that BamE can directly interact with BamA, independently of other Bam components. Using genetic and biochemical analysis, we show that BamE stabilizes BamA/BamD complex and facilitates bidirectional signaling interaction among them. BamE function becomes essential when mutations at the BamA/BamD interface disrupt their direct communication, and this essentiality is dependent on RcsF. As BamE and its BamA and BamD interaction interface are more broadly conserved than RcsF, we propose that BamE is particularly important for assembling challenging substrates that impede the ability of BamA and BamD to communicate directly during their assembly pathway.

RESULTS

BamE stabilizes BamA-BamD interaction *in vivo* and *in vitro*.

BamA/D interaction was reported to be destabilized *in vivo* in the *bamE* mutant strain (Sklar *et al.*, 2007). We confirmed this finding using pull-down experiments with Strep-

BamA as the bait; BamD co-purified with BamA in a BamE-dependent manner (Fig. 1A). BamE is required for BamA/BamD coordination only in the presence of RcsF, suggesting that RcsF binding to BamA may prevent direct BamA/BamD communication. Therefore, we sought to clarify the underlying relationship between BamE, RcsF, and the stability of the BamAD complex. For this, we tested whether BamA/D destabilization results from the absence of BamE or stalling of RcsF on BamA by comparing the stability of the BamA/D complex with or without RcsF. We observed that BamE was required for BamA/BamD co-purification regardless of the presence of RcsF (Fig. 1A).

Previous studies suggested that BamE only interacts with BamD based on *in vivo* pull-down experiments (Malinverni *et al.*, 2006, Ricci *et al.*, 2012). However, when the first Bam complex structures became available, they revealed direct BamA/BamE contact sites (Bakelar *et al.*, 2016, Iadanza *et al.*, 2016). Nonetheless, whether BamE can interact with BamA directly in the absence of the other Bam components, namely BamD, has not been tested. Therefore, we decided to examine the interactions between the BamA/BamE/BamD subunits *in vitro* (Fig. 1B). We expressed and purified soluble (without lipid modification) BamE-Strep and BamD-Strep proteins and the detergent-refolded His-BamA, mixed the components at the equimolar ratio, and subjected them to the Ni-NTA purification. To our surprise, we observed that BamE co-purified with BamA (Fig. 1B), providing the first experimental evidence for direct BamE/BamA interaction. In contrast, BamD did not efficiently co-purified with BamA (Fig. 1B) despite a large interaction interface between the two proteins observed in the various Bam complex structures. However, the efficiency of BamD co-purification with BamA improved significantly in the presence of BamE (Fig. 1B), demonstrating that BamE stabilizes BamA/BamD interaction *in vitro*.

Examination of BamE/BamA and BamE/BamD interface.

To facilitate further functional studies, we investigated BamA/BamE and BamD/BamE interaction interface more closely to identify mutations that disrupt corresponding interactions. In various *E. coli* Bam complex structures, these interfaces are nearly identical: BamE interaction with the Potra 5 domain of BamA is facilitated by several hydrogen bonds (Fig 2A, and S2, S3 Table S1), while BamE interaction with BamD involves both hydrogen bonds and a salt bridge interaction (Fig. 2A and S2, S3, and Table S2). Although the interaction residues are conserved in many but not all species (Fig. S3), Collab Fold Multimer modeling (Mirdita *et al.*, 2022) revealed a high degree of conservation of interaction interfaces between the corresponding protein pairs, suggesting that the proteins likely co-evolved (Fig. S2, Tables S1,S2).

We then selected several possible residues on BamE that facilitate BamA or BamD interaction for further analysis using the following criteria: the residues established polar contacts in several independent Bam complex structures; structure-based *in silico* mutagenesis of the residues disfavored the corresponding interaction; the residues were well separated from each other on the BamE structure and away from the BamA/BamD interaction interface to facilitate interpretation of the downstream genetic analysis. Here, we report on two of such BamE residues, Y37 and D66 (Fig. 2A).

BamE Y37 residue falls on the BamA interface and interacts with several hydrogen bonds with Y348 residue of BamA (Fig. 2A, Fig. S4). Y37 residue is within an unstructured loop of BamE, and its mutation is not expected to disrupt BamE folding (Fig. S4). We generated *bamE(Y37P)* mutation because, based on the *in silico* mutagenesis, proline was the only substitution that resulted in a decrease in hydrogen bonding at the interface and therefore is most likely a disruptor of BamE/BamA interaction (Fig. S4). Indeed, when we purified the BamE(Y37P) variant, it was unable to bind to His-BamA *in vitro* (Fig. 2B).

BamE D66 falls within the BamD interface (Fig. 2A, Fig. S5)) and forms the salt bridge with BamD K233 residue. D66 is also located in an unstructured loop on the opposite surface of the BamE molecule, and its mutation was also not expected to disrupt BamE folding but weakened BamE/BamD interface (Fig. S5). Introducing charge-substitution mutation, D66R, resulted in a loss of the BamE/BamD interaction *in vitro* (Fig. 2C).

We concluded that the BamE interactions with both BamA and BamD we observed *in vitro* are facilitated by the same interface that has been observed in the Bam complex structures. Furthermore, these interfaces are conserved in many other bacterial species, which suggests that they are functionally significant.

Both BamA/BamE and BamE/BamD interactions is required for function.

Our initial observation revealed that BamE directly interacts with BamA and BamD, promoting the stability and function of the Bam complex. To further investigate the mechanism by which BamE achieves this, we aimed to distinguish between BamE's role as a structural bridge between these essential components and its ability to regulate their conformations. If BamE functions solely as a structural bridge, disrupting either the BamE/A or BamE/D interfaces should result in a loss-of-function phenotype, mimicking the phenotype observed in the *bamE* mutant. However, if BamE plays a regulatory role for BamA or BamD, mutations in the BamE/A or BamE/D interfaces may produce distinct phenotypes, suggesting that one interface is more crucial for complex stability and/or RcsF/OMP assembly than the other.

To address the BamE function, we conducted *in vivo* phenotypic characterization of *bamE(Y37P)*, *bamE(D66R)*, and the double mutants. For this, we cloned *bamE* with or without corresponding mutations into a pTrc99a vector (Amann *et al.*, 1988). We employed a leaky expression from the trc promoter (without an inducer) to ensure physiological protein levels (Fig. S6), and tested phenotypes in the complementation experiments in the *bamE* background.

First, we examined the impact of the mutations on the Bam complex stability *in vivo* using pull-down experiments (Fig. 3). Surprisingly, we found that not only the BamE(D66R) variant failed to co-purify with BamD or BamA (Fig. 3A), but also disrupted the interaction between BamA and BamD evident from the reciprocal Strep-BamA pull-down (Fig. 3B). On the other hand, *bamE(Y37P)* mutation had no impact the complex stability, and the *bamE(D66R Y37P)* mutant phenocopied *bamE(D66R)* (Fig. 3A,B). Because the Y37P and D66R mutations resulted in different outcomes, we concluded that rather than serving as

a bridge BamE, BamE acts as an allosteric regulator, stimulating BamD to interact with BamA, thereby stabilizing the BamADE complex.

Because the Bam complex stability does not always correlate with function (McCabe *et al.*, 2017), we used RcsF/OMP assembly as a direct functional readout. In the absence of BamE cells fail to assemble RcsF complex with partner OMPs (Lach *et al.*, 2023), but we use OmpA as a convenient readout in the formaldehyde crosslinking experiments (Tata & Konovalova, 2019, Tata *et al.*, 2021). RcsF/OmpA complex is significantly reduced in the absence of BamE, and instead, RcsF accumulated on BamA (Fig. 3C). Even though *bamE(D66R)* mutation led to the destabilization of BamA/D interaction, it had no impact on RcsF crosslinking (Fig. 3C). In contrast, *bamE(Y37P)* displayed increased RcsF/BamA crosslinking, but still assembled RcsF/OmpA complexes (Fig. 3C). We interpret this intermediate phenotype as an indication that in the *bamE(Y37P)* strain, a significant fraction of BamA exists in the conformation prone to RcsF binding in the off-pathway conformation, while the remaining fraction of still capable of assembling RcsF/OmpA complex. Importantly, in the *bamE(D66R Y37P)* mutant RcsF/OmpA assembly was significantly reduced, and RcsF/BamA crosslinking was increased, phenocopying *bamE* strain (Fig. 3C).

The formation of off-pathway RcsF/BamA complex leads to the BamA sequestration from its function in the OMP assembly, underlying the RcsF-dependent synthetic lethal interaction of *bamE* with *bamB* null and several other *bam* mutant alleles (Tata & Konovalova, 2019, Hart *et al.*, 2019, Tata *et al.*, 2021). Therefore, we also tested the *bamE* mutants for their ability to complement *bamE bamB* synthetic lethality (Fig. 3D). We introduced the plasmids into the AK-1255 [*bamE bamB8* P$_{BAD}$ *bamE*] background that contains a loss-of-function allele of *bamB* and *bamE* under control of arabinose inducible promoter (Tata *et al.*, 2021). Growth of this strain in the absence of arabinose requires the presence of a functional *bamE* allele on a plasmid (Fig. 3D). Consistent with our crosslinking experiments, both *bamE(Y37P)* and *bamE(D66R)* mutants were able to grow, while the double mutant behaved like the EV control (Fig. 3D).

We also tested *bamE(Y37G)* and *bamE(D66G)* mutants to establish whether the observed phenotypes were due to specific amino acid substitution. *bamE(Y37G)* phenocopied *bamE(Y37P)* in every assay (Fig. S8). *bamE(D66G)* mutant had a somewhat different phenotype, as, unlike *bamE(D66R)*, it did not destabilize the BamADE complex (Fig. S9). However, when combined with Y37P mutation, it resulted in the same outcome in complex stability, RcsF assembly, and growth assays (Fig. S9). It suggests that D66G is a milder version of D66R mutation and uncovers that BamA co-purification with BamE(Y37P) was likely facilitated by BamD.

Together, our results demonstrated that both BamE/BamA and BamE/BamD interactions are functionally important but not equivalent. BamE/BamD interaction is more important for promoting BamA/BamD interaction and overall BamADE complex stability, as D66R mutation alone destabilized BamADE complex. However, even without the stable association, BamE can directly promote BamA function, as only when Y37P was introduced cells lost the ability to assemble RcsF.

BamE becomes essential when direct communication between BamA and BamD is compromised.

To ensure proper OMP assembly, BamA and BamD interact to undergo coordinated conformational changes (McCabe *et al.*, 2017, Ricci *et al.*, 2012). The stable BamA/BamD association is not required for OMP assembly, however, the signaling interaction between BamA and BamD that regulates the necessary conformational changes in both components is essential. As our results suggest that BamE plays a similar function, we hypothesized that BamE may become more important when BamA/BamD interaction is altered by the mutations at the BamA/BamD interface.

The interaction between BamA and BamD is facilitated by an electrostatic network involving the E373 and R197 residues (Hagan *et al.*, 2015, Hart & Silhavy, 2020). The *bamA(E373K)* mutation is lethal because it prevents the activation of BamD, and BamD remains in the functionally incompatible conformation. The *bamA(E373A)* mutation is less severe, allowing for some interaction between BamA and BamD (Ricci *et al.*, 2012).

We observed that *bamA(E373A)* exhibits synthetic lethal interaction with *bamE* null (Fig. 4A, Fig. S10, S11A), which is consistent with previous studies (Rigel *et al.*, 2012). *bamE* null derivative strains could not grow under standard laboratory conditions (LB media at 37°C), but could grow under permissive conditions (glucose minimal medium at 30°C) (Fig. S10, S11) because slower growth lowers the demand for the Bam complexes. We found that *bamA(E373A) bamE* synthetic lethal phenotype was also *rcsF*-dependent (Fig. S10), suggesting that the underlying reason is the RcsF-sequestration of BamA. To further investigate the role of BamE, we introduced *bamE* point mutants and tested them in complementation experiments (Fig. 4A, Fig. 5, Fig. S11A). *bamA(E373A) bamE(D66R)* mutant was not viable under standard conditions (Fig. 4a, Fig. S1A). This result suggests that BamE and BamA play partially overlapping functions in regulating BamD conformations.

bamD(R197L) is a gain-of-function mutation that enables BamD to adopt a conformation that is compatible with BamA(E373K), circumventing BamA regulation (Ricci *et al.*, 2012). *bamD(R197L)* restores the viablity of *bamA(E373K)* mutant without restoring Bam complex stability (Hagan *et al.*, 2015). *bamD(R197L)* is also compatible with *bamA(WT)* allele and does not appear to cause OMP assembly defects (McCabe *et al.*, 2017, Ricci *et al.*, 2012). However, *bamD(R197L)* strain cannot assemble RcsF, similar to the *bamE* strain (Tata & Konovalova, 2019), suggesting that it is at least somewhat defective in coordinating with BamA.

Despite its gain of function nature, *bamD(R197L)* mutation is also synthetic lethal with *bamE* (Rigel et al., 2012), and this phenotype is also *rcsF*-dependent (Tata & Konovalova, 2019)(Fig. 4B, Fig. S11B). We then introduced *bamE* point mutants in this background, we observed that unlike *bamA(E373A) bamE(D66R)*, the *bamD(R197L) bamE(D66R)* mutant was viable (Fig. 4B, S11B), indicating that BamD(R197L) bypasses not only regulation by BamA but also by BamE. However, *bamD(R197L) bamE(D66R Y37P)* strain remained not viable (Fig. 4B, S11B), suggesting that the regulation of BamA by BamE was still required.

This result suggests that BamE and BamD play a partially overlapping function in regulating BamA conformations.

When we assayed the Bam complex stability in both backgrounds under the permissive conditions, we observed BamADE complex destabilization in all mutants, including *bamE(Y37P)* (Fig. 4C–F), demonstrating that BamE/BamA interaction becomes more important for complex stability when the BamA/BamD interface is weakened by either *bamA(E373A)* or *bamD(R197L)* mutations. Additionally, these result explains the discrepancy between the *in vitro* and *in vivo* pull-down assays with BamE(Y37P) (Fig. 2 and Fig. 3), as the co-elution of BamA with BamE(Y37P) *in vivo* is mediated by BamD (Fig. 4C–F).

Taken together, our analysis summarized in Table 1 and Fig. 5 supports the model that BamE does not simply bridge BamA and BamD for complex stability but rather acts as a bifunctional regulator. BamE enables bidirectional signaling between BamA and BamD, and this function becomes essential when the direct communication between BamA and BamD is compromised.

DISCUSSION

BamE was the last component of the Bam complex to be identified because of its small size, which made it difficult to detect during the initial Bam complex purification (Wu *et al.*, 2005, Sklar *et al.*, 2007). However, the lack of significant phenotypes in the *bamE* strain hindered its functional studies. Nonetheless, BamE quickly emerged as a BamD regulator because of reported mutations in *bamD* or *bamA* that separated the stable Bam complex into two separate BamAB and BamCDE subcomplexes (Malinverni *et al.*, 2006, Ricci *et al.*, 2012). This suggested that BamE associated with the Bam complex exclusively through BamD. All subsequent studies interpreted the role of BamE in regulating BamA conformations indirectly through BamD, despite the fact that pairwise subunit interactions were never tested. When the structures of the Bam complex emerged, they uncovered a direct BamA/BamE interaction interface. However, it remained unclear whether this interaction was simply driven by adjacent BamD or had any functional significance. Here, we provide the first direct evidence that BamE interacts directly with BamA and BamD, and both interactions are important for function. We show that despite a large interface predicted by the Bam complex structures, BamA and BamD cannot stably associate with each other in the absence of BamE. BamE promotes the stability of the Bam complex, but our study presents compelling evidence, discussed below, that BamE achieves this by regulating the conformations of BamA and BamD, rather than simply bridging the two components.

The first line of evidence comes from the analysis of Bam complex stability. The mutations that disrupt pairwise BamE/BamA and BamE/BamD interactions *in vitro* do not have the same phenotypic outcomes *in vivo*. BamE/BamD interaction is clearly more important for overall complex stability, suggesting that BamE allosterically regulates BamD's ability to associate with BamA. BamE(Y37P) does not interact with BamA *in vitro*, but *in vivo* BamA still co-purifies with BamE(Y37P) because of the indirect interaction facilitated by BamD. Only when BamA/BamD or BamE/BamD interfaces are weakened by corresponding

bamA(E373A), *bamD(R197L)*, or *bamE(D66G)* mutations, BamA no longer co-purified with BamE(Y37P).

The second line of evidence comes from the functional assays using RcsF/OMP assembly as a readout. Our previous work suggested that the underlying defect in the *bamE* strain is the inability of BamD and BamA to functionally coordinate with each other, as depletion of *bamD* phenocopied *bamE* null (Tata & Konovalova, 2019). Here we show that destabilization of BamA/BamD interaction is not sufficient to cause a phenotype as the *bamE(D66R)* mutation does not affect RcsF/OMP assembly. Only when BamE/BamA regulation is additionally perturbed cells lose the ability to assemble RcsF/OMP.

The third line of evidence comes from the genetic interaction of *bamE* mutations with mutations affecting the BamA/BamD interface (Fig. 5). *bamA(E373A) bamE(D66R)* synthetic lethality suggests that BamE and BamA play a partially overlapping function in regulating BamD. On the other hand, *bamD(R197L)* bypasses *bamE(D66R)* but not *bamE(D66R Y37P)*, suggesting that BamE and BamD play a partially overlapping function in regulating BamA. The phenotypic similarity between *bamD(R197L)* and the *bamE* strain, including the accumulation of RcsF on BamA (Tata & Konovalova, 2019), indicates that BamA adopts a similar defective conformation in both strains, and these findings are consistent with *in vivo* biochemical evidence (Rigel *et al.*, 2012, Rigel *et al.*, 2013). Together these results provide evidence that BamE can promote bidirectional signaling interaction from BamA to BamD and from BamD to BamA, thereby providing an alternative route for BamA/BamD coordination. This is why BamE becomes essential when direct BamA/BamD coordination is impaired.

Several independent studies have isolated suppressors of *bamE* synthetic lethal interactions (Hart *et al.*, 2020, Hart & Silhavy, 2020, Tata & Konovalova, 2019, Tellez & Misra, 2012, Tata *et al.*, 2021). Despite the prevalent model of BamE being a BamD regulator, these suppressor mutations were always found in *bamA*, never in *bamD*. The bifunctional regulatory model of BamE proposed in this study provides an explanation for this paradox. Our model suggests that BamE regulates BamA both directly and indirectly via BamD. Therefore, *bamE* suppressors need to bypass both arms of regulation. Indeed, several *bamA* suppressors that we found to alleviate RcsF-sequestration of BamA were independently reported to be partial bypass suppressors of *bamD* (Tellez & Misra, 2012, Misra *et al.*, 2015, Hart & Silhavy, 2020).

It is important to emphasize that BamE is only essential for BamA/BamD coordination in the presence of RcsF and is otherwise completely dispensable for viability or general OMP assembly in a *rcsF* background (Hart *et al.*, 2019, Tata & Konovalova, 2019). So, why is BamE specifically required for RcsF/OMP assembly? The RcsF/OMP complex is clearly a challenging substrate for the Bam complex, as it requires coordination of OMP folding and RcsF surface exposure. At some point during RcsF/OMP complex assembly, BamA and BamD may lose the ability to communicate directly, relying on BamE as an alternative route for functional coordination. While RcsF sequestration of BamA can explain the phenotypes of the *bamE* mutant in *E. coli* (Hart *et al.*, 2019, Tata & Konovalova, 2019), it is interesting

that BamE, including its BamA and BamD interfacing residues, is more widely conserved than RcsF, suggesting the presence of additional substrates.

The lack of viability observed in *bam* mutants is often attributed to severely impaired OMP assembly consistent with the loss of function of the Bam complex. However, the example of RcsF illustrates how a defect in the assembly of a single substrate can affect overall OMP assembly indirectly by sequestering BamA. To better understand the function(s) of the Bam complex, it is crucial to identify additional model substrates rather than relying solely on growth phenotypes. The Bam complex is responsible for assembling a structurally diverse set of OMPs. Some OMPs exist as homo- or hetero-oligomers and may have periplasmic, plug, or large extracellular domains that are translocated during the folding of a β-barrel (Konovalova *et al.*, 2017). Assembly of these challenging substrates may require specialized activities of the Bam complex. Identifying such substrates will be crucial for studying the functional roles of individual Bam components.

MATERIALS AND METHODS.

All the bacterial strains used in this study are listed in Table S3. For media and growth conditions, refer to the Supplemental Materials and Methods.

In vitro pull-down experiments.

Equimolar amount of each purified protein (5μM) was incubated in 200 μl total reaction volume in buffer C (25mM Tris-HCl pH8.0, 150mM NaCl, 0.05% n-Dodecyl-beta-Maltoside (DDM) for 1 hour at room temperature, and then mixed with 100 μL of Ni-NTA His Bind® Resin (EMD Millipore), pre-equilibrated with buffer C. Beads were loaded into Pierce Centrifuge Columns (Thermo Fisher Scientific), washed four times with 500 μL of buffer C, and eluted three times with 50 μL buffer C with 250 mM imidazole. Each fraction (20 μL each) was analyzed on a 15% SDS-PAGE (SDS–polyacrylamide gel electrophoresis) and visualized with Imperial Protein Stain (Thermo Fisher Scientific). Bio-Rad Image Lab was used to quantify gels from independent replicates. For quantification, the intensity of corresponding bands was normalized to the level of the bait protein His-BamA. Graphs represent mean +/− SD. Statistical analysis was performed by using one-way ANOVA using GraphPad Prism.

In vivo pull-down experiments.

Cells from mid-log cultures grown in LB at 37°C or under permissive conditions as indicated were harvested by centrifugation, washed twice with phosphate buffer saline (PBS) and resuspended in 400μL of membrane solubilizing buffer (25 mM Tris–HCl pH 8.0, 150 mM NaCl, 20mM MgCl2, 1% DDM, 0.1mM PMSF, and 1X Protease Inhibitor Cocktail, 2mg/mL lysozyme, and 8U/mL benzonase). Samples were incubated at room temperature for 60 min with mild rotation and cell debris were removed by high-speed centrifugation 15000g for 15min.

For BamE-His pull-down, the clarified lysate (200 μl) was mixed with 100μl of 50% suspension of Ni-NTA His Bind® Resin (EMD Millipore), pre-equilibrated with buffer C and incubated for 1 h at 4°C with mild rotation. Beads were loaded into Pierce Centrifuge

Columns (Thermo Fisher Scientific), columns were washed with 10 times of 400 μL of buffer C and eluted with buffer C containing 250mM of imidazole.

For Strep-BamA pull-down, the clarified lysate (200 μl) was mixed with 100μl of 50% suspension of Strep-Tactin beads (IBA Life sciences), pre-equilibrated with buffer C (25 mM Tris–HCl pH 8.0, 150 mM NaCl and 0.05% DDM), and treated as described above. The proteins were eluted with buffer C containing 5mM deshthiobiotin.

All the input and elution fractions were analyzed by immunoblotting.

In vivo crosslinking and immunoblot analysis.

In vivo formaldehyde crosslinking on cells from mid-log cultures grown in LB at 37 °C, and the subsequent immunoblot analysis were performed as described (31). Immunoblots were visualized using the ChemiDoc MP Imaging System (Bio-Rad) and quantified using Image Lab (Bio-Rad). All figures are representative images from at least three independent biological replicates, graphs represent quantification of independendent replicates mean +/− SD. Statistical analysis was performed by using one-way ANOVA using GraphPad Prism.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

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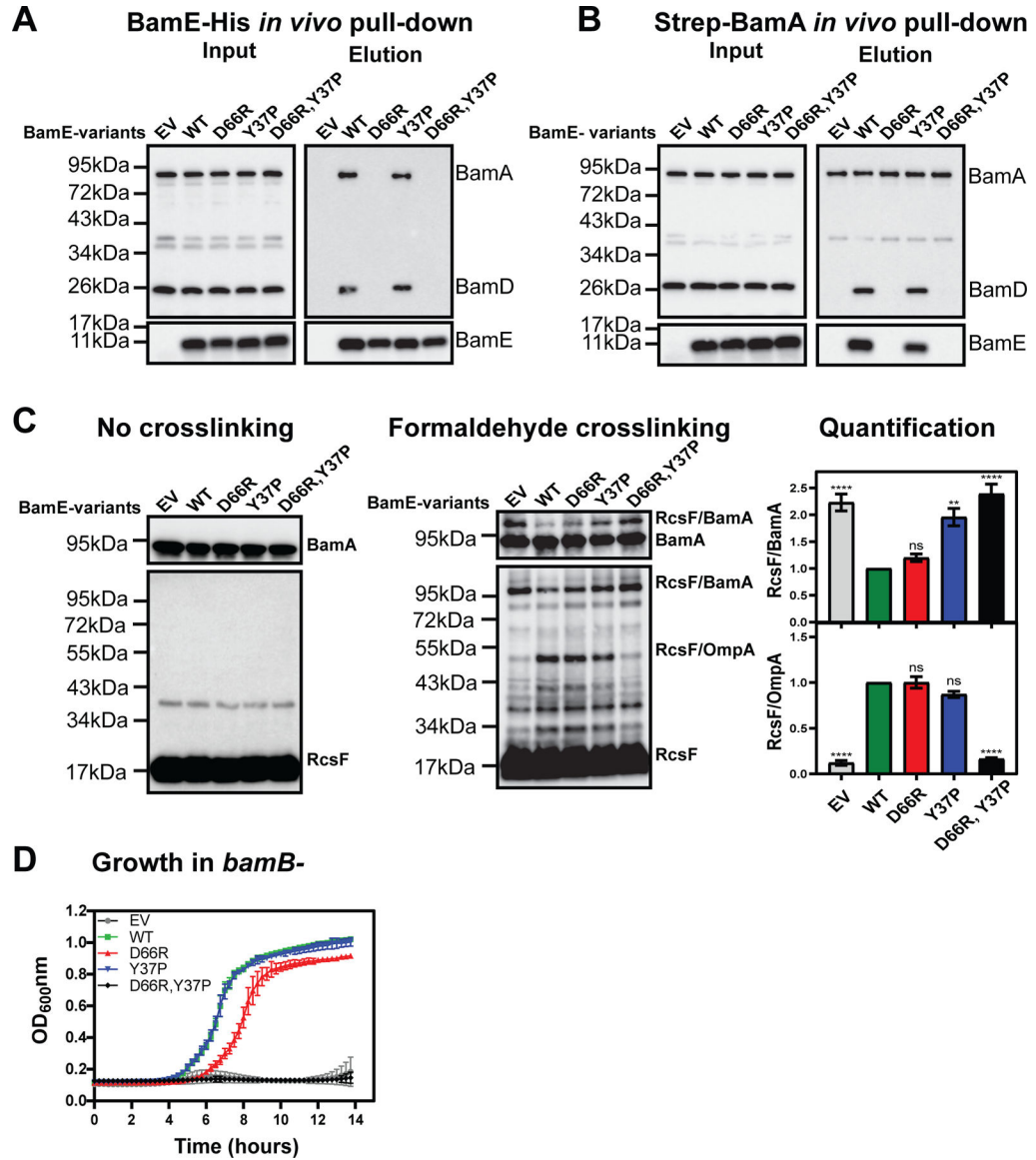
A — Strep-BamA *in vivo* pull-down

B — His-BamA *in vitro* pull-down
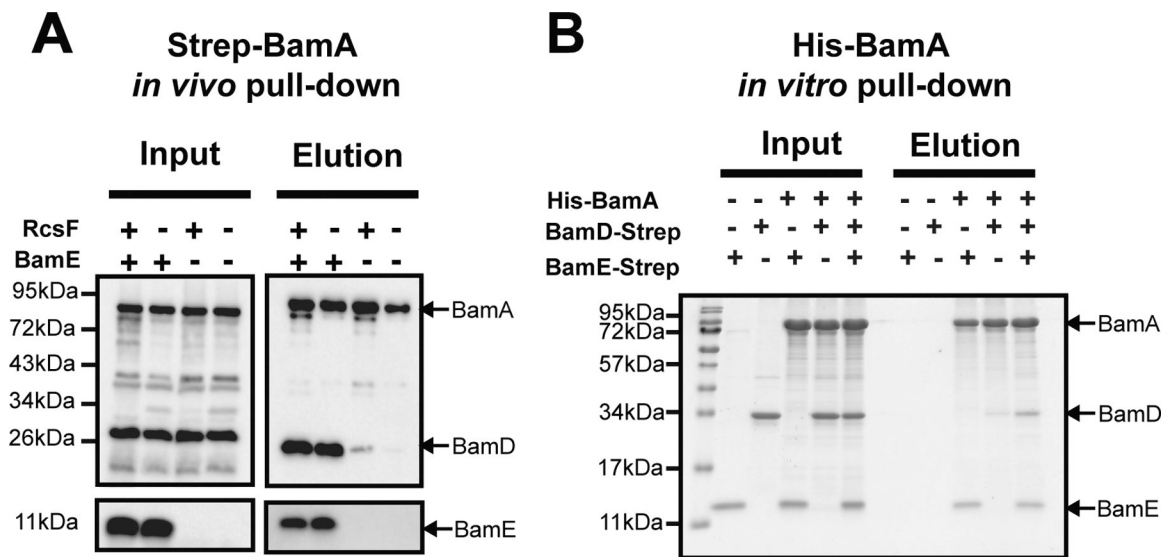
Figure1. BamE stabilizes BamA-BamD interaction *in vivo* and *in vitro*.
(**A**) Effect of BamE and RcsF on the stability of BamA and BamD interaction using *in vivo* pull-down assay with Strep-BamA expressed from the low copy pZS21 plasmid (Tata *et al.*, 2021). Total cell lysates containing solubilized membranes were subjected to Streptacin purification. The figure represents the immunoblot analysis of input and elution fractions with α-BamA, BamD, and BamE antibodies. For immunoblot quantifications of independent biological replicates, see Fig. S1A. (**B**) *In vitro* interaction assay using purified components. Individual proteins were purified separately, mixed at the equimolar ratio, and subjected to a Ni-NTA purification. The figure represents the SDS-PAGE analysis of input and elution fractions visualized by the Imperial Protein Stain. For immunoblot quantifications of independent biological replicates, see Fig S1B.
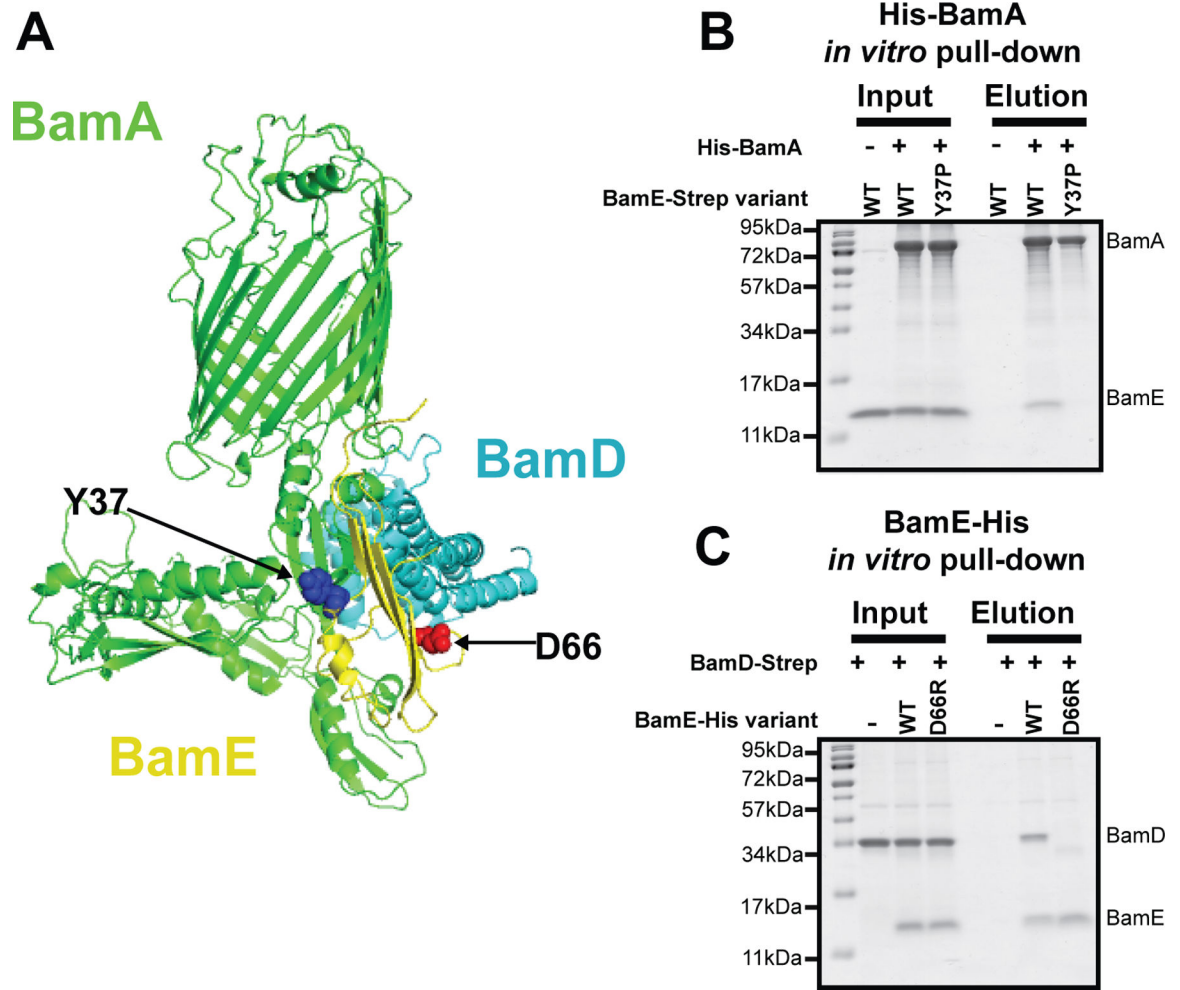
Figure 2. BamE/BamA and BamE/BamD interaction interface.
(**A**) The structure of Bam Complex (PDB: 5ekq (Bakelar *et al.*, 2016)), BamA (green), BamE (yellow), BamD (blue) are shown with the remaining components omitted for clarity. Y37 and D66 residues of BamE chosen for this study are visualized by blue and red spheres (**B, C**). *In vitro* interaction assay using purified components. Individual proteins and mutant derivatives were purified separately, mixed at the equimolar ratio, and subjected to Ni-NTA purification. The figure represents the SDS-PAGE analysis of input and elution fractions visualized by the Imperial Protein Stain. (**B**) *In vitro* interaction between His-BamA and BamE-Strep variants. (**C**) *In vitro* interaction between BamD-Strep and BamE-His variants.

Figure 3. Characterization of *bamE* mutants.

(**A, B**) Effect of *bamE* mutations on the stability of BamA/BamD/BamE interaction *in vivo* using Ni-NTA pull-down of BamE-His variants (**A**) or Streptactin pull-down of Strep-BamA in the presence of BamE-His variants (**B**). Empty vector (EV) pTrc99a, and its derivatives with indicated *bamE-His* alleles were transformed in the MT-567 [*bamE bamA//pZS21::Strep-bamA*] background, and resulting strains were subjected to the *in vivo* pull-down followed by immunoblot analysis as described in Fig. 1. For immunoblot quantifications of independent biological replicates, see Fig S7. (**C**) Immunoblot analysis of *in vivo* formaldehyde crosslinked samples probed with α-BamA (Top) and α-RcsF antibodies (bottom). For band validation, see (Tata & Konovalova, 2019) Quantification of RcsF/BamA and RcsF/OmpA crosslinking bands based on three independent biological replicates relative to the WT control, mean+/− standard error of the mean (SEM). Statistical analysis was performed by using one-way ANOVA in comparison with the WT control.

n.s. = P ≥ 0.05, **P <0.01, ****P < 0.0001. (**D)** Growth curve analysis. EV and plasmids with indicated *bamE*-His8 alleles were transformed in the AK-1255 [*bamE bamB8 P_{BAD}-bamE]* background and propagated in the presence of arabinose to allow BamE expression from the chromosomal locus. Overnight cultures were used to inoculate media without arabinose at the approximate cell density of 10^5 cell/ml to allow depletion of BamE expressed from the chromosomal locus. Growth was monitored continuously at 37°C in the Bioteck plate reader.
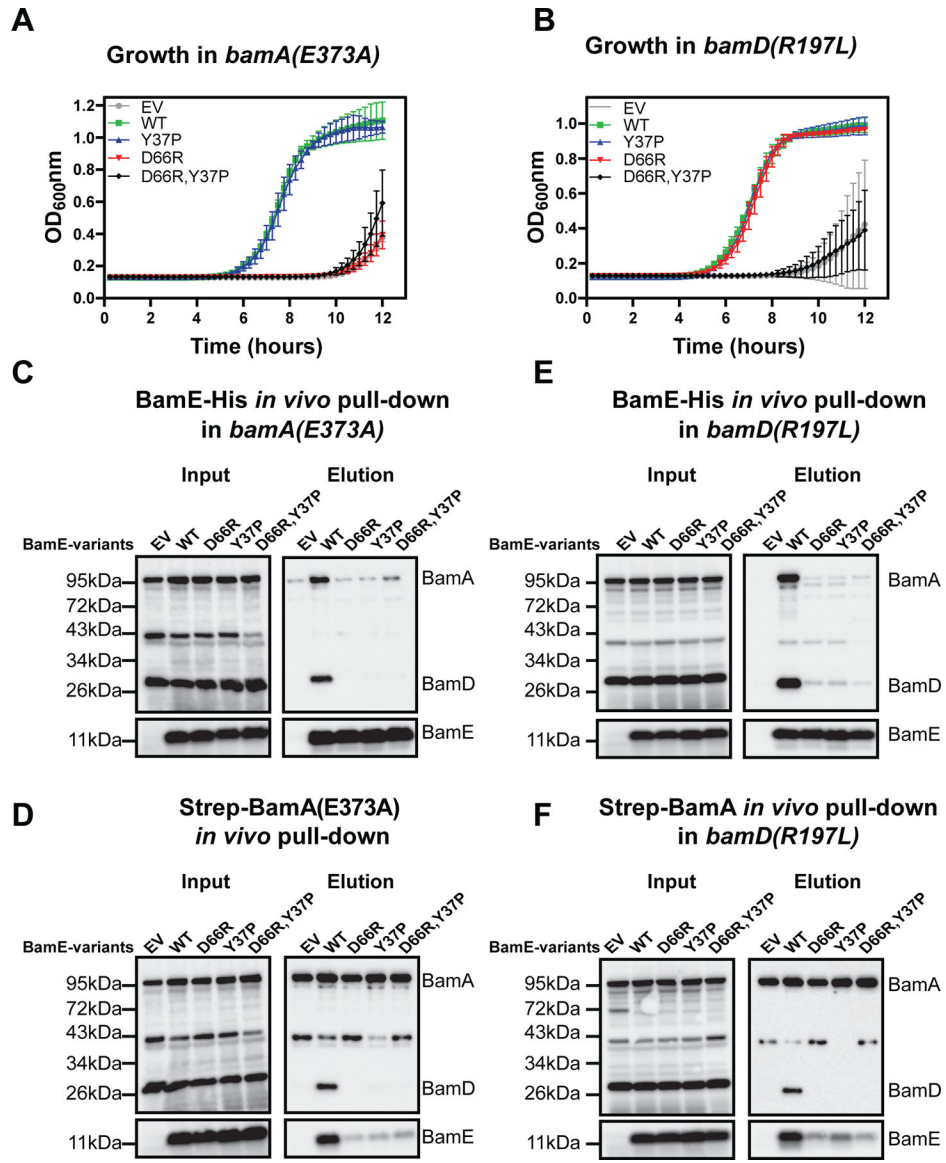
Figure 4. Characterization of *bamE* mutants in the *bamA(E373A)* and *bamD(R197L)* backgrounds.

EV and vectors with indicated *bamE* alleles were transformed in the SK-130 [*bamE bamA* P$_{BAD}$-*bamA* // pZS21:Strep-*bamA(373A)*] and MT-171 [*bamE::cm bamD(R197L) nadB::Tn10*] and propagated under permissive conditions (M9 glucose minimal media at 30°C). **(A,B)** Growth curves of *bamA(E373A)* (A) and *bamD(R197L)*(B) derivate strains in LB at 37°C. **(C-F)** *in vivo* BamA/BamD/BamE complex stability assayed using Ni-NTA pull-down of BamE-His variants **(C, E)** or Streptactin pull-down of Strep-BamA in the presence of BamE-His variants **(D, F)**. Experiments were performed using culture grown under permissive conditions and processed as described in Fig. 3. For immunoblot quantifications of independent biological replicates see Fig S12.
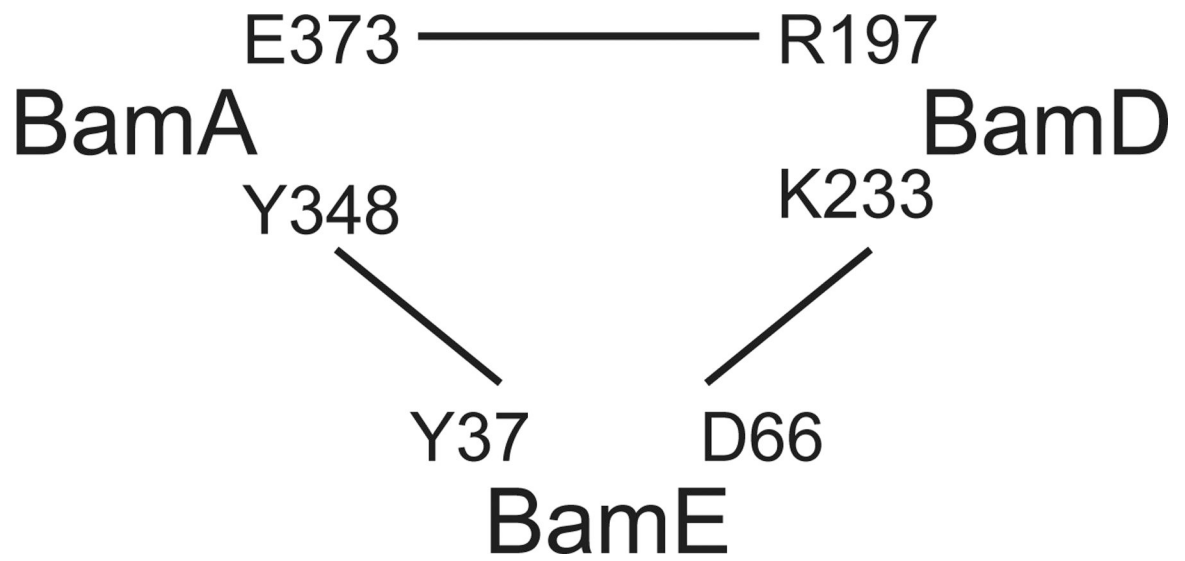
Figure 5. BamE interacts with both BamA and BamD to promote their stable association and functional coordination.

The residues utilized for genetic analysis to explore the functionality of the protein interfaces are indicated.

Table 1.

Summary phenotypes of strains used in this study.

bamE/rcsF allele	RcsF crosslinking		Growth in LB at 37°C				BamAD complex stability		
	OmpA	BamA	*bam+*[1]	*bamB-*	*bamA E373A*	*bamD R197L*	*bam+*[1]	*bamA E373A*	*bamD R197L*
bamE(WT)	+++	+	+	+	+	+	+	+	+
bamE	+	+++	+	–	–	–	–	–	–
bamE rcsF	NA	NA	+	+	+	+	–	ND[3]	ND[3]
bamE(D66R)	+++	+	+	+	–	+	–	–	–
bamE(Y37P)	+++	++	+	+	+	+	+	–	–
bamE(D66R Y37P)	+	+++	+	–	–	–	–	–	–

[1] *bam+* indicated WT alleles for all BamA-D components.

[2] NA, not applicable.

[3] ND, not determined.