Activation of the *Escherichia coli* β-barrel assembly machine (Bam) is required for essential components to interact properly with substrate

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Contributed by Thomas J. Silhavy, January 24, 2012 (sent for review January 2, 2012)

The outer membrane (OM) of Gram-negative bacteria such as *Escherichia coli* contains lipoproteins and integral β-barrel proteins (outer-membrane proteins, OMPs) assembled into an asymmetrical lipid bilayer. Insertion of β-barrel proteins into the OM is mediated by a protein complex that contains the OMP BamA and four associated lipoproteins (BamBCDE). The mechanism by which the Bam complex catalyzes the assembly of OMPs is not known. We report here the isolation and characterization of a temperature-sensitive lethal mutation, *bamAE373K*, which alters the fifth polypeptide transport-associated domain and disrupts the interaction between the BamAB and BamCDE subcomplexes. Suppressor mutations that map to codon 197 in *bamD* restore Bam complex function to wild-type levels. However, these suppressors do not restore the interaction between BamA and BamD; rather, they bypass the requirement for stable holocomplex formation by activating BamD. These results imply that BamA and BamD interact directly with OMP substrates.

Omp85 | membrane protein folding | conditional lethal | allele-specific

The outer membrane (OM) of *Escherichia coli* functions primarily as a robust permeability barrier that insulates the cell against a variety of potentially cytotoxic agents in the extracellular milieu. The cell is able to adapt to and mediate exchange with its environment through the regulated synthesis and assembly of integral OM proteins, which participate in a diverse array of cellular processes. Whereas integral proteins found in the inner membrane exclusively contain hydrophobic α-helical transmembrane domains, integral OM proteins (OMPs) instead adopt an amphipathic β-barrel conformation within the membrane (1).

Following synthesis, the aggregation-prone, unfolded precursor OMP is translocated across the inner membrane, is processed to its mature form, and is stabilized during transit through the crowded, aqueous periplasm by periplasmic chaperones, which deliver the nascent OMPs to the OM-associated β-barrel assembly machine (Bam). There, OMPs are folded and integrated into a rigid asymmetric lipid bilayer in the absence of an obvious energy source while OM integrity is maintained faithfully (1–3).

Bam is a heteropentamer (4) composed of a very highly conserved OM β-barrel (BamA) and four OM lipoproteins (BamBCDE) that dock with BamA (4–6). The hub of the physical complex is the BamA N-terminal extension, a periplasmic beaded chain comprising five structurally homologous polypeptide transport-associated (POTRA) domains that are numbered sequentially from the N terminus (7, 8). A stable association between BamA and the nonessential factor BamB requires most of the periplasmic domain of BamA. Conversely, only the POTRA 5 (P5) domain is necessary for the interaction of BamA with the essential factor BamD, which in turn mediates the apparently indirect association of BamA with BamC and BamE (4, 5, 8).

BamA and BamD are indispensable in *E. coli*, and depletion of either leads to rapid accumulation of unassembled OMPs in the periplasm followed by cell death (5, 6, 9, 10). Because structure–function analysis of BamA–D has the potential to yield significant insight into the general mechanism of OMP assembly [a process conserved across bacteria, chloroplasts, and mitochondria (11)], we sought to analyze the BamA–D interaction genetically and biochemically. We report here the isolation and characterization of mutations that disrupt the physical interaction between the BamAB and BamCDE subcomplexes. Suppressor analysis suggests that the P5 domain controls the activity of BamD and that the BamAB and BamCDE subcomplexes can function efficiently in the absence of a tight association provided that BamD is activated genetically.

Results

Screen for Conditional Lethal Alleles of *bamA*. We sought to identify BamA residues that influence function by screening mutations in *bamA* that cause impaired cell growth at 37 °C. A low-copy plasmid containing *bamA* (p*bamA*$^+$) was mutagenized (*Materials and Methods*) and was introduced into JCM320, a strain in which expression of only the chromosomal *bamA* gene can be induced or repressed by the addition of arabinose or fucose, respectively, to the growth medium (6, 8). Only the plasmid-borne, mutagenized (*bamA**) allele is expressed in the JCM320/p*bamA** background when arabinose is excluded.

To obtain conditional lethal alleles of *bamA*, p*bamA** transformants of JCM320 were screened for arabinose-dependent growth at 37 °C. Uninteresting (e.g., null) mutations were avoided by simultaneously screening for arabinose-independent growth at low temperature (24 °C). We describe here one mutation identified in the screen, *bamA5*, which confers a temperature-sensitive (ts$^-$) growth phenotype. Haploid *bamA5* strains, such as DPR881, do not grow above 24 °C.

Mutation in the P5 Domain of BamA Is Lethal Under Conditions That Require Efficient OMP Assembly. DNA sequence analysis reveals that the *bamA5* mutation is a transition at codon 373 (GAA to AAA) causing a Glu-to-Lys substitution; accordingly, the *bamA5* mutant gene henceforth is referred to as "*bamAE373K*," and the mutant protein as "BamA^{E373K}." This residue maps to the P5 domain, the POTRA domain most proximal to the C-terminal β-barrel domain of BamA. The recently solved structure of BamA P5 shows that the Glu^{373} side chain is solvent-exposed (7), and phylogenetic analysis reveals a high degree of conservation at this position across Gram-negative bacteria (Fig. S1).

The conditional inducer-dependence of JCM320/p*bamAE373K* suggests that the BamA^{E373K} protein is not functional at high temperature or that the functionality it retains is not sufficient to

Author contributions: D.P.R., C.L.H., D.K., and T.J.S. designed research; D.P.R. and C.L.H. performed research; D.P.R., C.L.H., D.K., and T.J.S. analyzed data; and D.P.R., C.L.H., D.K., and T.J.S. wrote the paper.

The authors declare no conflict of interest.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1201362109/-/DCSupplemental.

MICROBIOLOGY

sustain rapid growth. To assess the functionality of BamA^{E373K} in the nonpermissive condition, growth was monitored before and after depletion of inducer during incubation at 37 °C. In the presence of arabinose, the growth rate of JCM320/p*bamAE373K* was comparable to the growth rate of JCM320 containing p*bamA*$^{+}$. Upon depletion of wild-type BamA, growth of the strain expressing *bamAE373K* slowed and stalled at a rate comparable to that observed for the empty vector control (Fig. 1*A*).

Depletion of *bamA* causes a significant decrease in the steady-state levels of all OMPs because of a combination of misassembly, misfolding, down-regulation, and degradation of Bam substrates (5, 6, 12). To determine the impact of *bamAE373K* on OMP assembly, the levels of several OMPs were evaluated after growth in the presence of either arabinose or fucose. Although levels of various OMPs (including LamB and BamA itself) are normal in JCM320/p*bamAE373K* when arabinose is present, the depletion of wild-type BamA in this background leads to a significant decrease in the steady-state levels of all OMPs tested (including BamA^{E373K}), indicating a global defect in β-barrel assembly (Fig. 1*B*). This defect is comparable to that observed upon depletion of *bamA*$^{+}$ in the presence of empty vector, suggesting that *bamAE373K* exhibits a null-like phenotype in the nonpermissive growth condition. Additionally, we observe a sharp reduction in OMP levels at steady state even when a haploid *bamAE373K* strain is grown at the permissive temperature (Fig. S2). Thus, even under permissive conditions, function of the BamA^{E373K} mutant protein is compromised.

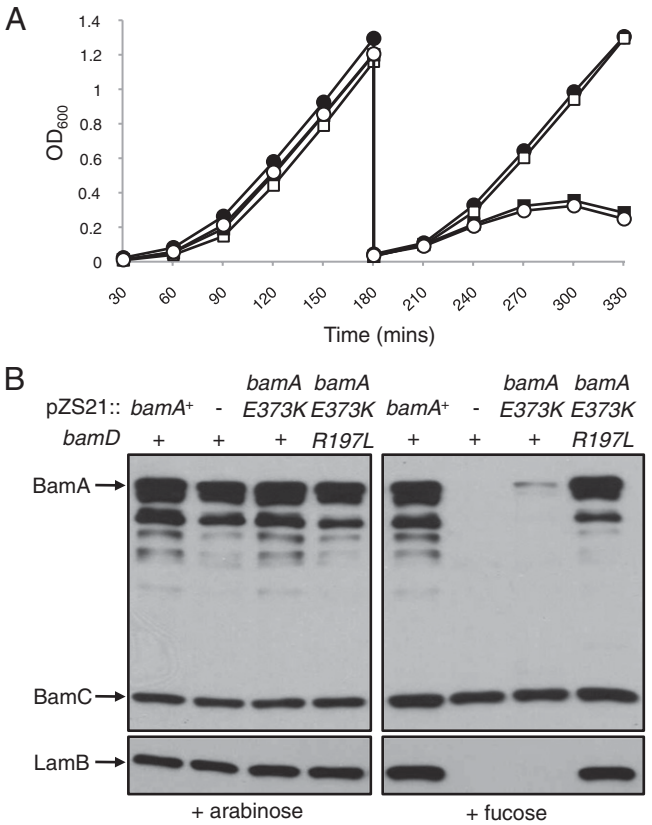

Fig. 1. *bamAE373K* is a lethal mutation at 37 °C. (*A*) Overnight cultures of JCM320 containing pZS21 (open circles), p*bamA*$^{+}$ (closed circles), or p*bamAE373K* (*bamD*$^{+}$, closed squares; *bamDR197L*, open squares) were subcultured into LB medium containing D-fucose to repress expression of chromosomal *bamA*. Cultures were grown at 37 °C and back-diluted at 3 h. (*B*) Cultures of the strains shown in *A* were grown in the presence of arabinose (*Left*) or fucose (*Right*) to OD_{600} = ~1 and subjected to SDS/PAGE followed by immunoblotting for the proteins indicated.

BamA^{E373K} Is Properly Assembled. A simple explanation for the observed phenotypes of *bamAE373K* is that perturbation of Glu^{373} causes BamA instability or misfolding. Because BamA is itself an OMP, it is dependent on Bam for assembly. Consequently, the significant reduction in BamA^{E373K} levels upon depletion of *bamA*$^{+}$ does not allow us to distinguish between a BamA instability/misfolding defect and a functional defect, because levels of folded BamA^{E373K} would decrease in either case.

To determine whether the *bamAE373K* mutation affects BamA biogenesis, we sought to assess the assembly state of BamA^{E373K} in a *bamA*$^{+}$ background. If BamA^{E373K} is simply folding defective or unstable, then *bamA*$^{+}$ should complement the ts^{-} phenotype of *bamAE373K* but should not cause an increase in BamA^{E373K} levels. However, if BamA^{E373K} is assembly competent and stable but nonfunctional, then the BamA^{E373K} concentration should be comparable to that of BamA^{WT} when *bamA*$^{+}$ is supplied *in trans*. We find that comparable amounts of BamA^{WT} and BamA^{E373K} are present in *bamA*$^{+}$ strains, suggesting that the E373K mutation does not significantly affect the biogenesis or stability of BamA. However, we consistently observe some proteolysis of BamA^{E373K} upon purification (Fig. 2*A*, *Upper Right*, lanes 2 and 4), likely reflecting increased proteolysis of BamA^{E373K} in cell extracts (*SI Results*).

***bamAE373K* Disrupts the Interaction Between BamA and BamD.** The wild-type BamABCDE complex is stable and can be purified as an intact unit (4, 6). To determine if the *bamAE373K* mutation affects the interactions of BamA with BamB and BamCDE, we His_6-tagged and expressed the BamA^{WT} and BamA^{E373K} proteins. Upon purification of the tagged BamA variants, we observed no difference between the wild-type and mutant protein in the amount of coprecipitated BamB (Fig. 2*A*). However, we found that the amounts of coeluted BamC, BamD, and BamE are all reduced significantly by the E373K substitution (Fig. 2*A*). Furthermore, although purified wild-type BamAB and BamCDE subcomplexes can be combined in vitro to form five-member holocomplexes, the E373K mutation prevents holocomplex formation (Fig. 2*B*). These results clearly show that this P5 mutation specifically affects the physical interaction between the BamAB and BamCDE subcomplexes.

In light of the data presented in this section, we conclude that the observed destabilization of BamA–CDE in a *bamAE373K* background occurs because Glu^{373} is critically important for the physical interaction between the two Bam subcomplexes. Because the association between BamAB and BamCDE likely is stabilized by direct binding of BamA to BamD, we suggest that replacing Glu^{373} with Lys cripples Bam by preventing a productive association between these two essential complex components.

Suppressor Mutations in *bamD* Restore Bam Complex Function. The biochemical and genetic evidence described thus far implicates Glu^{373} in some vital aspect of the BamA–BamD relationship. We reasoned that, if so, we potentially could isolate suppressors of the thermosensitivity of *bamAE373K* that map to *bamD*. We selected ts^{+} revertants of *bamAE373K* and mapped the affected loci. Four independent revertants were obtained. One of these four mutations was found to be a true revertant that restores Glu at position 373. The remaining three are extragenic suppressors that map to a single codon in *bamD*, resulting in the mutation of a highly conserved residue (Arg^{197}) in the C-terminal domain of BamD to Leu, Ser, or His. The facts that suppressors are obtained at frequencies comparable to true reversion and that all three of them map to the same codon suggest an allele-specific relationship between the original mutation and the suppressors. The allele

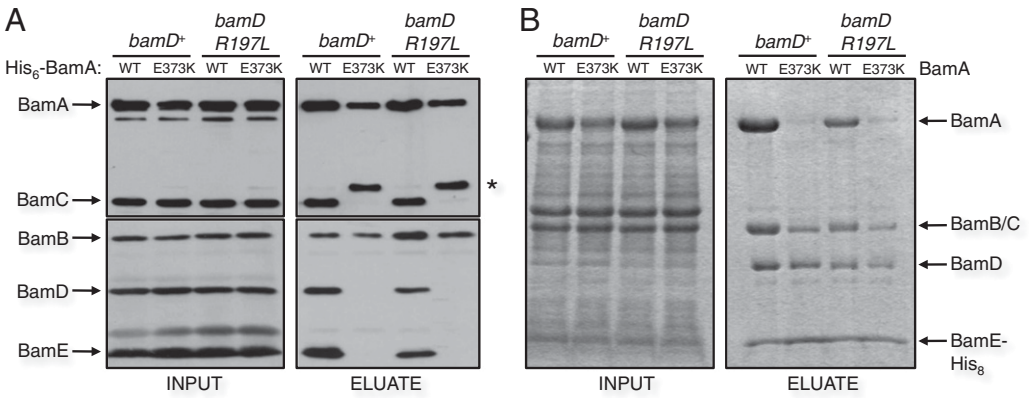

Fig. 2. *bamAE373K* disrupts the BamAB–BamCDE interaction. (*A*) Affinity purification was performed in *bamD*$^+$ and *bamDR197L* strains containing either pHis-BamA$^+$ or pHis-BamA^{E373K}. Samples were subjected to SDS/PAGE and immunoblotting for BamA–E. Protein levels are shown before (*Left*) and after (*Right*) purification. The band indicated by the asterisk corresponds to an N-terminal fragment of BamA^{E373K}. (*B*) Extracts derived from solubilized *E. coli* membranes containing either wild-type or mutant BamAB and BamCDE$_{His}$ complexes were mixed in vitro and then purified by Ni-NTA chromatography using BamE-His$_8$. Eluates were subjected to SDS/PAGE and Coomassie blue staining.

encoding the Leu substitution, the first to be isolated, is referred to here as "*bamDR197L*," and its protein product is referred to as "BamD^{R197L}." Although this suppressor is discussed specifically below, it is important to note that both the Ser and His substitutions phenocopy *bamDR197L* by every assay we have conducted.

The *bamDR197L* mutation is a strong suppressor that reverses the deleterious phenotypes associated with *bamAE373K*: OMP levels return to normal (Fig. 1*B*), and the growth rate of the suppressed strain is indistinguishable from that of wild type (Fig. 1*A*). Additionally, the robustness of the permeability barrier in *bamAE373K bamDR197L* at 37 °C (see Fig. 3) is especially remarkable, given that the *bamAE373K* mutant does not grow at this temperature even in the absence of antibiotics. The complete suppression of *bamAE373K* by compensatory mutations in *bamD* further supports the hypothesis that *bamAE373K* defects are a consequence of a nonproductive interaction between BamA and BamD. This allele of *bamD* is a gain-of-function mutation as evidenced by the fact that it is dominant to *bamD*$^+$ in a *bamAE373K* background; that is, it suppresses the conditional lethality of *bamAE373K* even in the presence of wild-type BamD.

The *bamD* mutants described here could represent classical interactive suppressors that restore a direct physical interaction that was disrupted by the *bamAE373K* mutation. Additional evidence supporting such an allele-specific interaction between BamA and BamD could be obtained were it shown that the *bamDR197L* mutation weakens the interaction between BamD and wild-type BamA. Upon purification of His$_6$-tagged Bam components from either whole cells or in vitro experiments using overexpressed proteins, we found that the amount of BamA^{WT} that associates with BamD^{R197L} compared with BamD^{WT} is somewhat reduced (Fig. 2). Thus, the interaction between BamA^{WT} and BamD^{R197L} is compromised, suggesting that *bamDR197L* causes partial destabilization of an otherwise wild-type complex.

We suspected that *bamDR197L* might inhibit Bam function in a *bamA*$^+$ background, perhaps causing defects similar to those observed for *bamAE373K* in a *bamD*$^+$ context. However, when we replaced wild-type *bamD* of MC4100 with *bamDR197L* and evaluated the phenotype of the resulting *bamDR197L bamA*$^+$ strain, we found this mutant to be indistinguishable from wild type with respect to growth rate, steady-state OMP levels, and OM permeability (Fig. 3 and Fig. S3). Thus, BamD^{R197L} is functionally compatible with both BamA^{WT} and BamA^{E373K}. Said differently, the identity of residue 373 of BamA seems to be inconsequential in the presence of *bamDR197L*. In support of this conclusion, the lethality associated with a *bamA* mutation in which Glu^{373} is replaced by Arg (*bamAE373R*) also is suppressed completely by *bamDR197L*. Although *bamDR197L* can suppress these lethal *bamA* mutations, it does not bypass the requirement for *bamA* altogether; *bamA* cannot be deleted in a *bamDR197L* background.

***bamDR197L* Does Not Restore Stability of a Complex Containing BamA^{E373K}.** If *bamDR197L* were indeed an allele-specific suppressor of *bamAE373K*, it would follow that the dissociated complex observed in *bamAE373K* should be restored by *bamDR197L*. Therefore we attempted to copurify BamCDE with BamA^{E373K}B in either a *bamD*$^+$ or *bamDR197L* background to determine the stability of the BamA–BamD interaction in each context. Surprisingly, the amount of BamD^{R197L} that copurifies with BamA^{E373K} is not appreciably different from the amount of BamD^{WT} that copurifies with BamA^{E373K} (Fig. 2*A*). Moreover, the Bam holocomplex

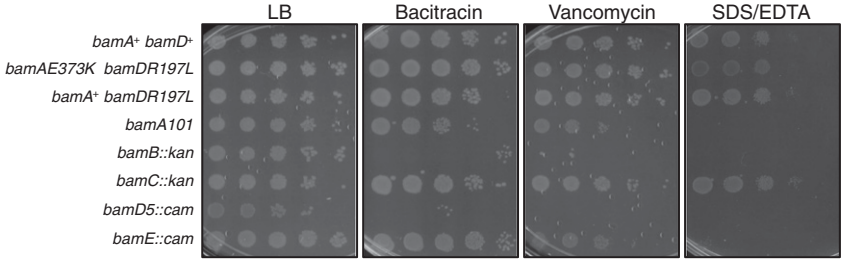

Fig. 3. *bamDR197L* restores the OM permeability barrier in a *bamAE373K* mutant. Serial 10-fold dilutions of stationary-phase cultures of the strains indicated were prepared in 96-well plates and then were spotted onto LB medium alone or onto LB medium supplemented with 625 μg/mL bacitracin, 60 μg/mL vancomycin, or 0.5% SDS + 1.0 mM EDTA and were incubated overnight at 37 °C.

cannot be detected after chemical crosslinking in either a *bamAE373K* or *bamAE373K bamDR197L* mutant background, suggesting that the BamA–BamD interaction is indeed dissociated and not simply weakened (*SI Results* and Fig. S4). These paradoxical results suggest that *bamDR197L* reverses all phenotypes associated with *bamAE373K* without stabilizing the disrupted Bam complex.

As shown in Fig. 2*B*, the complete Bam holocomplex can be reconstructed in vitro from overexpressed BamAB and BamCDE subcomplexes (13, 14). Consistent with the results shown in Fig. 2*A*, the R197L mutation in BamD does not significantly increase the amount of $BamA^{E373K}B$–$CD^{R197L}E$ holocomplex reconstructed from mutant subcomplexes (Fig. 2*B*). These results show that, despite complete suppression of the *bamAE373K* phenotype, $BamD^{R197L}$ does not restore the interaction with $BamA^{E373K}$. Taken together, our data suggest that restoration of Bam complex function in *bamAE373K* strains can be accomplished without restoring complex stability.

$BamD^{R197L}$ Has Increased Activity Compared with Wild-Type BamD. We considered the formal possibility that a slight increase in complex stability, although not detectable biochemically, is sufficient to restore function to Bam. This idea is conceivable, because a ∼10-fold reduction in *bamA* expression causes only minor defects in OMP maturation (15), suggesting that OM biogenesis can be sustained even when the concentration of Bam holocomplex is greatly diminished. The experiments presented below argue against this possibility and suggest instead that *bamDR197L* is a gain-of-function mutation.

It seems likely that $BamCD^{R197L}E$ interacts less well with $BamA^{WT}$ than does BamCDE (Fig. 2). If holocomplex formation is important for function in a *bamDR197L* $bamA^+$ background, then it is reasonable to expect that reducing the holocomplex levels by limiting the expression of *bamA* would negatively affect growth of this strain. To test the prediction that holocomplex levels are important in a *bamDR197L* strain, we reduced the levels of $BamA^{WT}$ protein by decreasing the expression level of *bamA*. We introduced into this strain a transposon located upstream of *bamA* that reduces expression of this gene by an order of magnitude; this allele is referred to as "*bamA101*" (15). Unexpectedly, not only is the *bamDR197L bamA101* double mutant viable, the *bamDR197L* allele in fact suppresses the growth defect observed in a *bamA101* strain (Fig. 4).

It is important to note that the OM permeability barrier is robust in both *bamDR197L* single and *bamAE373K bamDR197L* double mutants; indeed, both strains are equivalent to wild type in this respect. Although mutations known to cause modest defects in Bam function lead to increased OM permeability (5, 15, 16), the *bamDR197L* and *bamAE373K bamDR197L* mutants are as resistant to these compounds as wild type (Fig. 3). Taken together, results presented in this section argue that the mutant protein can do something the wild-type protein cannot, suggesting that suppression of *bamAE373K* and *bamA101* by *bamDR197L* is the result of an increase in BamD activity.

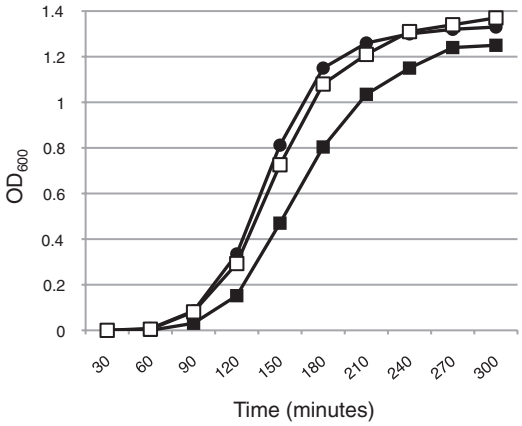

Fig. 4. *bamDR197L* suppresses the growth defect caused by a reduction in *bamA* expression. Overnight cultures of DPR909 (circles), DPR959 (closed squares), and DPR960 (open squares) were subcultured into LB medium and grown at 37 °C. OD was measured every 30 min.

Discussion

We describe amino acid substitutions at a single residue within the periplasmic domain of BamA that profoundly impair Bam and prevent efficient OMP assembly. This residue (Glu^{373}) is situated on a solvent-exposed surface of P5, which is the only POTRA domain required for binding of BamA to BamD, the only essential POTRA in the BamA homolog of *Neisseria meningitidis* (17), and apparently the most evolutionarily ancient of the five POTRA domains (17, 18). Replacement of Glu^{373} with Lys (E373K) prevents growth above room temperature on rich medium and greatly destabilizes the interaction between BamA and BamD. Replacement of Glu^{373} with Arg (E373R) is lethal. These findings seem to indicate a critical role for Glu^{373} in initiating or maintaining the physical BamA–BamD association.

Compensatory mutations in BamD that suppress all defects conferred by *bamAE373K* in an apparently allele-specific manner do not restore the stable physical interaction between BamA and BamD. These findings suggest that the stability of BamAD is not a reliable proxy for the fidelity of the reaction catalyzed by the pair and that the defects observed in a *bamAE373K* background cannot be attributed solely to the physical dissociation of the two essential Bam components.

Both $BamA^+$ and $BamA^{E373K}$ are fully functional in a *bamDR197L* background, suggesting that Glu^{373} is not crucial for BamA activity per se. Said differently, it appears that $BamA^+$ and $BamA^{E373K}$ are equally competent to promote OMP assembly, but $BamA^{E373K}$ is incompatible with wild-type BamD. Because *bamDR197L* restores complete functionality to a machine containing $BamA^{E373K}$ in a manner independent of complex stability, and because the phenotype of *bamDR197L* is the same in the presence of either $bamA^+$ or *bamAE373K*, we suggest that the *bamAE373K* phenotype in fact reflects the absence of BamD function rather than a lack of BamA function. In other words, the *bamAE373K* mutation dissociates the BamAB and BamCDE subcomplexes; this dissociation does not inactivate BamA, but it inactivates BamD. We propose that *bamDR197L*, a gain-of-function mutation, is a bypass suppressor that activates BamD without the need for a stable BamA–BamD interaction.

It has been established previously that the P5 domain interacts with BamCDE; however, the results presented here suggest that a stable association between the P5 domain and BamCDE is not required for OMP assembly if BamD is otherwise activated. We propose that, in the wild-type complex, BamD is "activated" via an interaction with the P5 domain of BamA. Glu^{373} is required for the interaction between BamD and the P5 domain and might be involved directly in BamD activation. We do not know how the $BamD^{R197L}$ mutation overcomes the deleterious effects of the E373K substitution, but it likely stabilizes an active form of BamD. The fact that a mutation in BamD improves OMP assembly without significantly changing how it interacts with $BamA^{E373K}$ implies that BamD must affect the assembly process through a direct interaction with substrates.

The fact that various unrelated substitutions at this position (R197L, R197S, R197H) suppress *bamAE373K* implies that the specific identity of this residue is not important for BamD activity per se. Arg^{197} is situated within a highly conserved patch on the surface of the BamD C-terminal domain (Fig. S5) that previously has been suggested to mediate an interaction with BamA (4, 5, 19,

20). Our findings suggest that this surface also may play a role in the activity of BamD.

Early characterization of BamD demonstrated the importance of the C-terminal domain for complex stability and Bam function. C-terminal truncations that pare the final tetratricopeptide repeat of BamD have been shown to disrupt the physical interactions between BamD and all other complex members. In addition, these truncations cause marked defects in OMP assembly at elevated temperatures, suggesting that the efficiency of β-barrel assembly is reduced in strains expressing these BamD variants (4, 5). The fact that Arg^{197} is found within in the C-terminal domain of BamD, which is implicated in the interaction(s) between BamA and BamD, is consistent with our suggestion that this residue is intimately involved in some aspect of the interaction and communication between these two essential factors.

In conclusion, we have identified a mutation, *bamAE373K*, that prevents association of the two essential Bam subcomplexes, BamAB and BamCDE, indicating that the P5 domain of BamA controls the activity of BamD in the wild-type complex. Suppressor analysis shows that the need for stable subcomplex association can be bypassed without affecting the activity of the machine. Therefore, we suggest that the functions performed by the essential members of these subcomplexes, BamA and BamD, affect the OMP assembly reaction directly. We predict that the mutant proteins described here will provide useful tools for analyzing the general β-barrel assembly mechanism.

Materials and Methods

Bacterial Strains, Plasmids, and Growth Conditions. Strains used in this study are listed in Table S1. Media were prepared as previously described (21). XL-1 Red (Stratagene) mutagenesis and site-directed mutagenesis (QuikChange; Stratagene) were conducted according to manufacturer's specifications. Relevant oligonucleotides are listed in Table S2. In strains producing TetR, expression of *bamA* from pZS21 was induced with 25 μg/mL tetracycline (22).

Affinity Purification. Bam copurification from strains DPR821, DPR822, DPR989, and DPR990 was performed essentially as described (23), except that no crosslinking agents were used, and cells were lysed in BugBuster solution (Novagen) containing lysozyme (5 μg/mL), DNase I (50 μg/mL), RNase I (50 μg/mL), and 1 mM PMSF. Western blots were prepared as previously described (23).

In Vitro Reconstruction of Mutant Bam Complexes. Wild-type and mutant BamAB and BamCDE complexes were overexpressed as described previously using plasmids pSK38, pSK46, pCH121, pCH123, and pBamE-His (14). Cells were lysed by French press in 20 mM Tris (pH 8), 150 mM NaCl, and 1 mM PMSF. The lysate was centrifuged at 5,000 × *g* for 10 min at 4 °C; then the supernatant was collected and ultracentrifuged at 100,000 × *g* for 30 min at 4 °C. The membrane pellet was solubilized in 20 mM Tris (pH 8), 150 mM NaCl, 2% (vol/vol) TX-100, and 2 μg/mL lysozyme by incubation on a rocker at 24 °C for 1 h. The solutions then were ultracentrifuged again at 100,000 × *g* for 30 min at 4 °C. The clarified solutions containing mutant and wild-type BamCDE complexes were mixed with the solutions containing mutant and wild-type BamAB complexes such that the BamAB proteins were in excess. The resulting Bam complexes then were isolated by Ni-nitrilotriacetic acid (NI-NTA) affinity chromatography using the His_8 tag on BamE. The Ni column was washed with 20 mM Tris (pH 8), 0.05% N-dodecyl-β-D-maltoside (DDM), and 40 mM imidazole, and the complexes were eluted in 20 mM Tris (pH 8), 0.05% DDM, and 200 mM imidazole. These eluates were run on SDS/PAGE (4–20% gradient gel) and stained with Coomassie blue.

ACKNOWLEDGMENTS. This work was supported by National Institute of General Medical Sciences Grant GM34821 (to T.J.S.) and National Institute of Allergy and Infectious Disease Grant AI081059 (to D.K.). C.L.H. is supported by a National Science Foundation graduate research fellowship.

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