



Functions of the BamBCDE Lipoproteins Revealed by Bypass Mutations in BamA

Elizabeth M. Hart,^a Thomas J. Silhavy^a

^aDepartment of Molecular Biology, Princeton University, Princeton, New Jersey, USA

ABSTRACT The heteropentameric β -barrel assembly machine (BAM complex) is responsible for folding and inserting a diverse array of β -barrel outer membrane proteins (OMPs) into the outer membrane (OM) of Gram-negative bacteria. The BAM complex contains two essential proteins, the β -barrel OMP BamA and a lipoprotein BamD, whereas the auxiliary lipoproteins BamBCE are individually nonessential. Here, we identify and characterize three *bamA* mutations, the E-to-K change at position 470 (*bamA*_{E470K}), the A-to-P change at position 496 (*bamA*_{A496P}), and the A-to-S change at position 499 (*bamA*_{A499S}), that suppress the otherwise lethal Δ *bamD*, Δ *bamB* Δ *bamC* Δ *bamE*, and Δ *bamC* Δ *bamD* Δ *bamE* mutations. The viability of cells lacking different combinations of BAM complex lipoproteins provides the opportunity to examine the role of the individual proteins in OMP assembly. Results show that, in wild-type cells, BamBCE share a redundant function; at least one of these lipoproteins must be present to allow BamD to coordinate productively with BamA. Besides BamA regulation, BamD shares an additional essential function that is redundant with a second function of BamB. Remarkably, *bamA*_{E470K} suppresses both, allowing the construction of a BAM complex composed solely of BamA^{E470K} that is able to assemble OMPs in the absence of BamBCDE. This work demonstrates that the BAM complex lipoproteins do not participate in the catalytic folding of OMP substrates but rather function to increase the efficiency of the assembly process by coordinating and regulating the assembly of diverse OMP substrates.

IMPORTANCE The folding and insertion of β -barrel outer membrane proteins (OMPs) are conserved processes in mitochondria, chloroplasts, and Gram-negative bacteria. In Gram-negative bacteria, OMPs are assembled into the outer membrane (OM) by the heteropentameric β -barrel assembly machine (BAM complex). In this study, we probe the function of the individual BAM proteins and how they coordinate assembly of a diverse family of OMPs. Furthermore, we identify a gain-of-function *bamA* mutant capable of assembling OMPs independently of all four other BAM proteins. This work advances our understanding of OMP assembly and sheds light on how this process is distinct in Gram-negative bacteria.

KEYWORDS BAM complex, *Escherichia coli*, Gram-negative bacteria, outer membrane, outer membrane biogenesis

The distinctive asymmetric structure of the outer membrane (OM) contributes to its essential function and its distinctive permeability properties. Large and hydrophobic molecules, including many antibiotics, are excluded from entering the cell due to the strong lateral interactions between lipopolysaccharide (LPS) molecules that make up the outer leaflet of the OM. Conversely, small and hydrophilic nutrients and signaling molecules are able to cross through β -barrel outer membrane proteins (OMPs) that function as generalized or specialized porins (1, 2). OMPs further contribute to the selective permeability of the OM by maintaining envelope integrity and participating in toxic molecule efflux (2, 3).

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Address correspondence to Thomas J. Silhavy, tsilhavy@princeton.edu.

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While all β -barrel OMPs share the common feature of a β -barrel domain, which is an antiparallel β -sheet folded into a cylinder, the size and complexity of OMPs differ significantly (3, 4). The dimensions of the β -barrel domain can vary from 8 strands to 26 strands (5, 6) in *Escherichia coli*, and recently, a 36-strand β -barrel was described in the Gram-negative microbe *Flavobacterium johnsoniae* (7). OMPs can have additional functional domains (3), including passenger domains (8, 9), protein-protein interaction motifs (6, 10, 11), and extracellular modules (12). Furthermore, OMPs are found as monomers (5), dimers (13), trimers (14–16), and in higher oligomeric states (17, 18), and several are folded around lipoprotein plugs (3, 19, 20).

The β -barrel assembly machine (BAM complex) is a heteropentameric complex responsible for the assembly of OMPs into the OM. BAM is composed of four lipoproteins, BamBCDE, and one OMP, BamA. Only BamA and BamD are essential for viability in wild-type cells, while BamBCE are individually nonessential (21–23). The BAM complex lipoproteins scaffold around the periplasmic domain of BamA to form a top hat in the OM (24–29). The vestibule formed by the BAM complex lipoproteins and the periplasmic domain of BamA protects OMP substrates during the initial stages of folding that occur at the periplasmic face of the OM (30–33).

The current model of OMP assembly suggests that BamD regulates OMP engagement with the complex, and the bulk of the folding process relies on BamA. Substrate recognition by BamD represents an assembly checkpoint; abnormal binding results in substrates being rejected from the BAM machinery while proper binding allows for assembly to proceed (31, 34–36). Once the quality of the OMP substrate has been confirmed, BamD communicates to BamA to engage with the OMP substrate (34, 35, 37, 38). BamA then catalyzes OMP folding and membrane insertion, though the mechanism through which this occurs is under current investigation (21, 30, 32, 33, 39, 40).

Despite the diversity of OMP substrates, all are assembled by the BAM complex through utilization of distinct assembly pathways. For example, the assembly of the LPS insertase LptD around its lipoprotein plug LptE (19, 41, 42) is orders of magnitude slower than the assembly of simple, smaller multimers like LamB (14, 43), and assembly of these two substrates relies on different BAM lipoproteins (44). The mechanism by which the BAM complex prioritizes substrate selection and regulates the assembly of the entire OMP profile of the cell remains unknown but likely requires one or more of the BAM lipoproteins.

Mutants that lack one of the nonessential lipoproteins display only minor defects in OMP assembly and OM integrity (21, 23). Double mutants of the accessory lipoproteins exhibit varying phenotypes ranging from the modest temperature sensitivity of a $\Delta bamB \Delta bamC$ mutant to the more pronounced conditional lethality of the $\Delta bamB \Delta bamE$ mutant (23, 45, 46). A $\Delta bamB \Delta bamC \Delta bamE$ triple mutant is lethal. Thus, BAM complex function correlates with the number of accessory lipoproteins present, indicating that BamBCE likely share a redundant function(s) that becomes increasingly essential only when more than one has been removed. The functional redundancy of the BAM lipoproteins complicates studies of their individual importance.

Here, we identify suppressor mutations that allow viability of otherwise lethal lipoprotein deletions ($\Delta bamB \Delta bamC \Delta bamE$, $\Delta bamD$, and $\Delta bamC \Delta bamD \Delta bamE$). In these suppressed strains, the function(s) of the remaining BAM lipoprotein(s) becomes increasingly important. We utilize genetic analysis to probe these functions by comparing and contrasting how suppressors identified in one deletion background behave in another. Strikingly, one of the *bamA* suppressor mutations allows cells to survive without any of the BAM lipoproteins. Thus, none of the redundant roles of the lipoproteins are essential for BAM complex catalytic activity, and all must function to increase the efficiency of OMP assembly via regulatory mechanisms. The suppressor mutations described here identify key residues in BamA that help define the active site of this remarkable protein-folding factor.

TABLE 1 Summary of *bamA* suppressor phenotypes

<i>bamA</i> allele	Phenotype				
	OM barrier	Growth	β -barrel stability	MRL-494 resistance	Basal Rcs activation
<i>bamA</i> _{E470K}	+	+	–	+	+
<i>bamA</i> _{A496P}	+	+	+	–	+
<i>bamA</i> _{A499S}	+	+	+	–	–

RESULTS

Suppression of $\Delta bamB \Delta bamC \Delta bamE$. Previous studies have shown that the stress-sensing lipoprotein RcsF is a substrate of the BAM complex (20, 47). During assembly, RcsF is threaded through the lumen of an abundant OMP to anchor the lipidated amino terminus of the protein in the outer leaflet of the OM (20). This complex, interlocked structure represents a challenging substrate for the BAM complex to assemble. Indeed, several BAM complex mutations, notably the $\Delta bamB \Delta bamE$ double mutant, can be suppressed by simply removing RcsF (48, 49).

The discrepancy in viability between $\Delta bamB \Delta bamE$ mutant and $\Delta bamB \Delta bamC \Delta bamE$ mutant cells led us to test for suppression of the $\Delta bamB \Delta bamC \Delta bamE$ mutant by removal of RcsF. We used linkage disruption to quantitate the ability to inherit *bamB::kan* in a $\Delta bamC \Delta bamE$ mutant background either in the presence or absence of RcsF. As expected, kanamycin-resistant colonies were not recovered in $\Delta bamC \Delta bamE$ mutant transductants, and deletion of *rscF* did not increase the frequency of *bamB::kan* integration (see Table S1 in the supplemental material). Thus, the lethality of the $\Delta bamB \Delta bamC \Delta bamE$ triple mutant is not due to stalled assembly of RcsF.

We conclude that in a strain lacking BamBE (with or without RcsF), BamC function becomes critical. This is noteworthy because, in a wild-type strain, taking away BamC has no obvious consequence (21). In this triple mutant, we can now use suppressor analysis to probe the function of BamC.

We isolated spontaneous suppressor mutations that allowed for survival of $\Delta bamC \Delta bamE$ mutant cells under conditions of BamB depletion in the absence of RcsF. Targeted sequencing of *bamA* identified these suppressor mutations as the A-to-P change at position 496 (*bamA*_{A496P}) and the A-to-S change at position 499 (*bamA*_{A499S}), both of which alter residues located on extracellular loop 3 of the BamA β -barrel domain (24).

The E-to-K change at position 470 (*bamA*_{E470K}) confers resistance to the BamA inhibitor MRL-494 (50), and other changes at codon E470 have also been identified as suppressors of additional assembly-defective mutations (30, 51). Previously, we showed that *bamD* can be deleted in a *bamA*_{E470K} mutant background, demonstrating that BamA^{E470K} bypasses the requirement for BamD in OMP assembly (52). The potent suppression demonstrated by *bamA*_{E470K} led us to investigate if this mutation was able to suppress other toxic BAM complex deletions.

We monitored both the growth and OM permeability barrier in the *bamA*_{E470K}, *bamA*_{A496P} and *bamA*_{A499S} strains both in the presence and absence of RcsF (Table 1; Fig. 1A and B). Cells expressing these suppressor alleles showed similar barrier function, as measured by ability to grow on medium containing antibiotics and growth, as cells expressing wild-type *bamA*. In contrast to the *bamA*_{E470K} strain, the *bamA*_{A496P} and *bamA*_{A499S} strains are not resistant to MRL-494 and exhibit a stable β -barrel domain (Fig. 1C and D).

To test for suppression of the $\Delta bamB \Delta bamC \Delta bamE$ strain by *bamA*_{E470K}, *bamA*_{A496P} and *bamA*_{A499S}, we again used linkage disruption. Kanamycin-resistant *bamB::kan* transductants were isolated from $\Delta bamC \Delta bamE$ *bamA*_{E470K} mutant cells in the presence or absence of RcsF (Table S1). *bamB::kan* was inherited by the $\Delta bamC \Delta bamE$ *bamA*_{A496P} and $\Delta bamC \Delta bamE$ *bamA*_{A499S} mutant cell only in the absence of RcsF. The inability of BamA^{A496P} and BamA^{A499S} to promote viability of the $\Delta bamB \Delta bamC \Delta bamE$ mutant cells in the presence of RcsF suggests that these mutants struggle to assemble

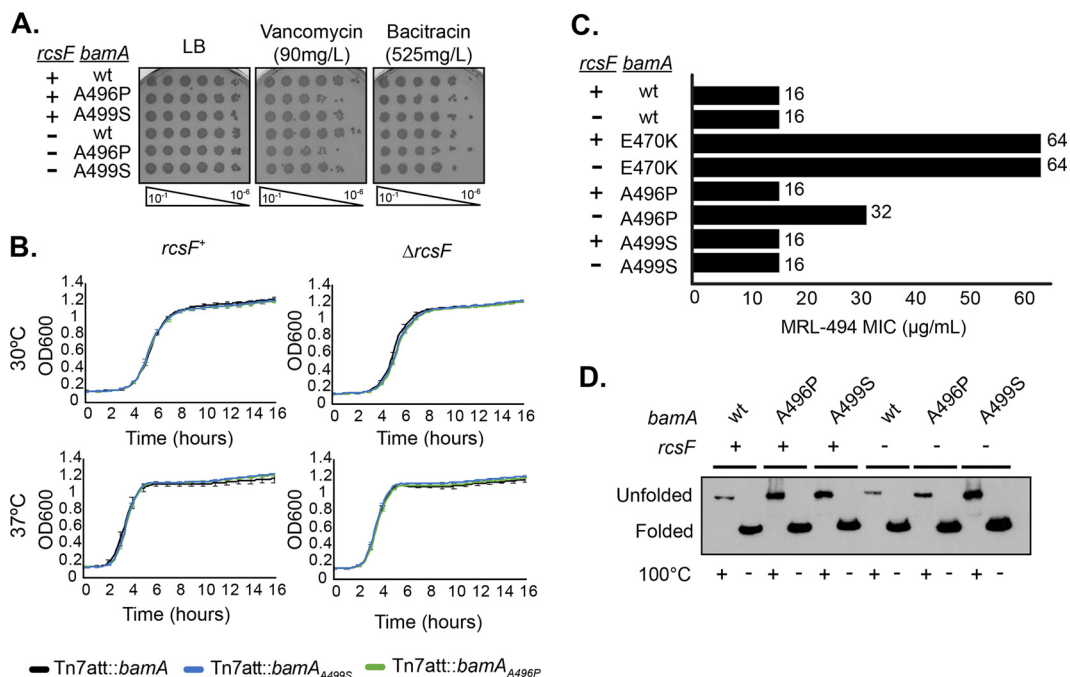


FIG 1 Phenotypic characterization of *bamA*_{A496P} and *bamA*_{A499S} mutations. (A) The indicated strains were normalized by OD₆₀₀ serially diluted, and spotted onto medium containing vancomycin, bacitracin, or erythromycin. Plates were grown overnight at 37°C. (B) The indicated strains were normalized by OD₆₀₀ inoculated into LB, and grown overnight at 30°C or 37°C. Error bars represent standard error of the mean (SEM). (C) MRL-494 resistance of the *bamA* suppressor alleles was evaluated using the MIC protocol. Data represents the average of two biological replicates. (D) Heat modifiability of BamA mutants. Exponentially growing cultures were normalized by OD₆₀₀, lysed, and either boiled (denatured) or incubated at room temperature (nondenatured) for 10 min. Samples were electrophoresed at 4°C and probed for BamA.

RcsF/OMP complexes when the nonessential lipoproteins are absent. Thus, *bamB* can be deleted in cells expressing all three of the BamA suppressors in the absence of BamC and BamE. However, for two of the suppressors, *bamA*_{A496P} and *bamA*_{A499S}, *rscF* must be deleted (Table 2).

To conclusively demonstrate that the BamA suppressors support viability in the absence of all three accessory lipoproteins, we built clean deletion strains in which *bamB*, *bamC*, and *bamE* are directly deleted or disrupted. PCR and immunoblot analysis (Fig. 2A and B) confirm that the gene and protein products are absent from these strains.

Phenotypic characterization of the $\Delta bamB \Delta bamC \Delta bamE$ null strains revealed that all strains exhibit growth defects (Table 3), permeability (Fig. 2C), and OMP assembly defects (Fig. 2D), suggesting that the OM barrier is impaired. Notably, the levels of all OMPs tested are reduced in this background rather than defective assembly of only a subset of OMP substrates (Fig. 2D).

We conclude that the critical function of BamC that becomes apparent in strains lacking BamB and RcsF can be suppressed by *bamA*_{E470K}, *bamA*_{A496P} and *bamA*_{A499S}.

TABLE 2 Summary of suppression phenotypes

<i>bamA</i> allele	BAM mutant			
	$\Delta bamD$	$\Delta bamC \Delta bamE$	$\Delta bamB \Delta bamC \Delta bamE$	$\Delta bamB \Delta bamC \Delta bamE$ BamD depletion
<i>bamA</i> _{E470K}	+	+	+	+
$\Delta rcsF \text{ } bamA$ _{E470K}	+	+	+	-
<i>bamA</i> _{A496P}	+	+	-	-
$\Delta rcsF \text{ } bamA$ _{A496P}	+	+	+	-
<i>bamA</i> _{A499S}	-	-	-	-
$\Delta rcsF \text{ } bamA$ _{A499S}	+	+	+	-

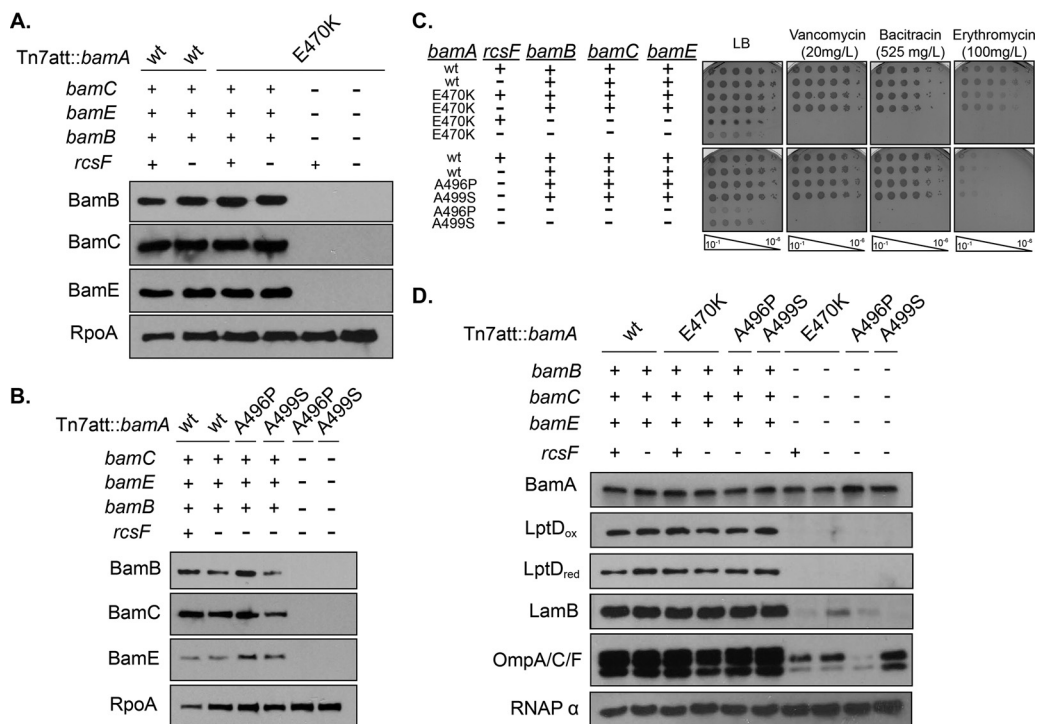


FIG 2 *bamA*_{E470K}, *bamA*_{A496P}, and *bamA*_{A499S} mutations promote viability of $\Delta bamB \Delta bamC \Delta bamE$ mutants. (A and B) Immunoblot analysis of stationary phase suppressed $\Delta bamB \Delta bamC \Delta bamE$ mutants expressing *bamA*_{E470K} (A) or *bamA*_{A496P} or *bamA*_{A499S} (B) probing for BamB, BamC, and BamE. RpoA served as a loading control. (C) The indicated strains were normalized by OD₆₀₀, serially diluted, and spotted onto medium containing vancomycin, bacitracin, or erythromycin. Plates were incubated overnight at 30°C. (D) Immunoblot analysis of stationary-phase cultures. Samples were electrophoresed and probed for a variety of β -barrel OMPs. RpoA served as a loading control.

Suppression of $\Delta bamD$. As noted above, *bamA*_{E470K} bypasses the requirement for BamD (52). Since *bamA*_{E470K}, *bamA*_{A496P}, and *bamA*_{A499S} can all suppress the $\Delta bamB \Delta bamC \Delta bamE$ mutant in strains lacking RcsF, we tested whether the new suppressor mutations would also be able to bypass the function of BamD.

TABLE 3 Viability assessment of $\Delta bamB \Delta bamC \Delta bamE$, $\Delta bamD$, and $\Delta bamC \Delta bamD \Delta bamE$ mutants in stationary-phase overnight cultures

Genotype	CFU/ml \pm SD ($\times 10^8$) (n = 3)
Tn7att:: <i>bamA</i>	11.5 \pm 1.82
Tn7att:: <i>bamA</i> _{E470K}	11.7 \pm 3.09
Tn7att:: <i>bamA</i> _{A496P}	12.4 \pm 0.55
Tn7att:: <i>bamA</i> _{A499S}	13.9 \pm 1.10
$\Delta rcsF$ Tn7att:: <i>bamA</i>	11.0 \pm 1.31
$\Delta rcsF$ Tn7att:: <i>bamA</i> _{E470K}	11.6 \pm 2.32
$\Delta rcsF$ Tn7att:: <i>bamA</i> _{A496P}	12.7 \pm 0.84
$\Delta rcsF$ Tn7att:: <i>bamA</i> _{A499S}	12.5 \pm 3.98
$\Delta bamB \Delta bamC \Delta bamE$ Tn7att:: <i>bamA</i> _{E470K}	1.08 \pm 0.19
$\Delta bamB \Delta bamC \Delta bamE \Delta rcsF$ Tn7att:: <i>bamA</i> _{E470K}	0.32 \pm 0.33
$\Delta bamB \Delta bamC \Delta bamE \Delta rcsF$ Tn7att:: <i>bamA</i> _{A496P}	0.32 \pm 0.18
$\Delta bamB \Delta bamC \Delta bamE \Delta rcsF$ Tn7att:: <i>bamA</i> _{A499S}	0.463 \pm 0.68
<i>bamD::kan</i> Tn7att:: <i>bamA</i> _{A496P}	0.241 \pm 0.08
<i>bamD::kan \Delta rcsF</i> Tn7att:: <i>bamA</i> _{A496P}	0.587 \pm 0.23
<i>bamD::kan \Delta rcsF</i> Tn7att:: <i>bamA</i> _{A499S}	0.611 \pm 0.13
$\Delta bamC \Delta bamD \Delta bamE$ Tn7att:: <i>bamA</i> _{E470K}	1.16 \pm 0.49
$\Delta bamC \Delta bamD \Delta bamE \Delta rcsF$ Tn7att:: <i>bamA</i> _{E470K}	1.35 \pm 0.33
$\Delta bamC \Delta bamD \Delta bamE$ Tn7att:: <i>bamA</i> _{A496P}	0.385 \pm 0.06
$\Delta bamC \Delta bamD \Delta bamE \Delta rcsF$ Tn7att:: <i>bamA</i> _{A496P}	0.732 \pm 0.02
$\Delta bamC \Delta bamD \Delta bamE \Delta rcsF$ Tn7att:: <i>bamA</i> _{A499S}	0.298 \pm 0.06

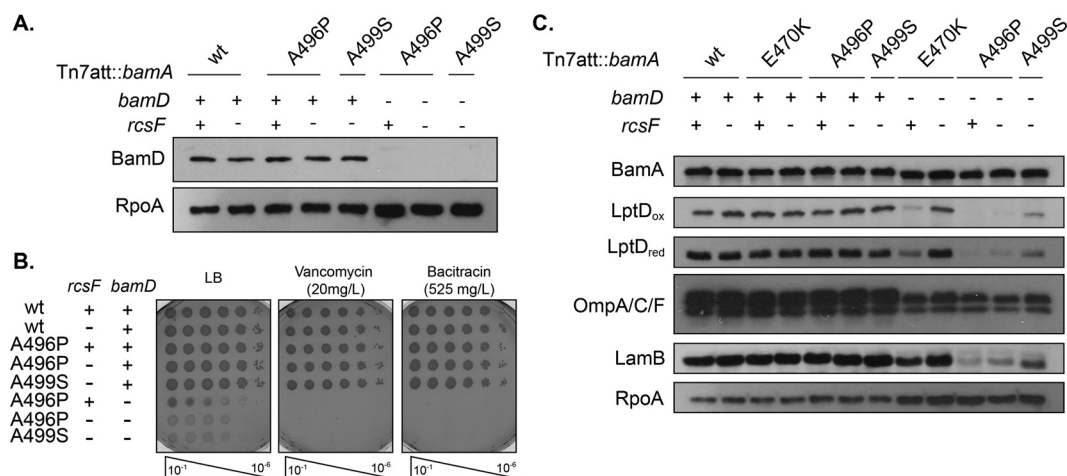


FIG 3 *bamA*_{A496P} and *bamA*_{A499S} mutations bypass the essentiality of BamD. (A) Immunoblot analysis of stationary-phase *bamD* null strains expressing *bamA*_{A496P} or *bamA*_{A499S}. RpoA was used as a loading control. (B) The indicated strains were normalized by OD₆₀₀, serially diluted, and spotted onto medium containing vancomycin or bacitracin. Plates were grown overnight at 30°C. (C) Stationary-phase cultures were normalized by OD₆₀₀, electrophoresed, and probed for the indicated proteins. RpoA served as a loading control.

The ability to delete *bamD* in cells expressing *bamA*_{A496P} and *bamA*_{A499S} using linkage disruption with a P1 lysate carrying a *bamD::kan* disruption allele linked to a nearby *nadB::Tn10* marker. Cells expressing *bamD* in diploid were able to inherit the *bamD::kan* allele along with *nadB::Tn10*. Conversely, *bamA*⁺ strains (in the presence or absence of *rscF* [$\pm rscF$]) were unable to inherit *bamD::kan*, demonstrating the essentiality of BamD. Cells expressing *bamA*_{A496P} were able to inherit *bamD::kan* in both the presence and absence of *rscF*, whereas cells expressing *bamA*_{A499S} were able to receive *bamD::kan* only in a $\Delta rcsF$ background (see Table S2 in the supplemental material). The requirement of *rscF* to be deleted in order for BamA^{A499S} to allow viability of cells lacking BamD highlights the difficult nature of RcsF/OMP complex assembly. We show that this effect is specific to RcsF and not Rcs signaling, as deletion of the response regulator of the Rcs stress response, *rscB*, which disrupts the signal transduction pathway downstream of RcsF, does not allow *bamD* to be disrupted in a *bamA*_{A499S} mutant background (Table S2).

We have observed that the levels of the Rcs stress response are noticeably higher in the *bamA*_{E470K} and *bamA*_{A496P} strains than in *bamA*_{A499S} strains (see Fig. S1 in the supplemental material). Since unassembled RcsF could accumulate in a cellular location where it might activate the stress response, this may reflect the fact that assembly of RcsF/OMP complexes is more efficient in *bamA*_{A499S} strains and is instead rejected from the BAM machine by BamA^{E470K} and BamA^{A496P}. If so, then this would explain why the presence of RcsF is more of a problem in the *bamA*_{A499S} strain than in the other two suppressors.

Based on these results, we constructed clean deletion strains in which *bamD* is directly disrupted in *bamA*_{A496P} ($\pm rcsF$) and $\Delta rcsF$ *bamA*_{A499S} mutant backgrounds (Table 2). PCR and immunoblot analysis (Fig. 3) confirmed that BamD protein was absent in these strains.

We characterized the suppressed *bamD* mutants expressing *bamA*_{A496P} and *bamA*_{A499S} and observed similar defects to those in *bamD::kan* *bamA*_{E470K} strains (52). Cells exhibited growth (Table 3), OM permeability (Fig. 3B), and OMP assembly defects (Fig. 3C).

We conclude that *bamA*_{E470K}, *bamA*_{A496P}, and *bamA*_{A499S} suppressors can all bypass the essential requirement for BamD.

Suppression of $\Delta bamC$ $\Delta bamD$ $\Delta bamE$. Previous studies have demonstrated that the BAM complex can be functionally dissected into two subcomplexes of BamAB and BamCDE. Structural studies have identified contacts between BamAD, BamAC, and

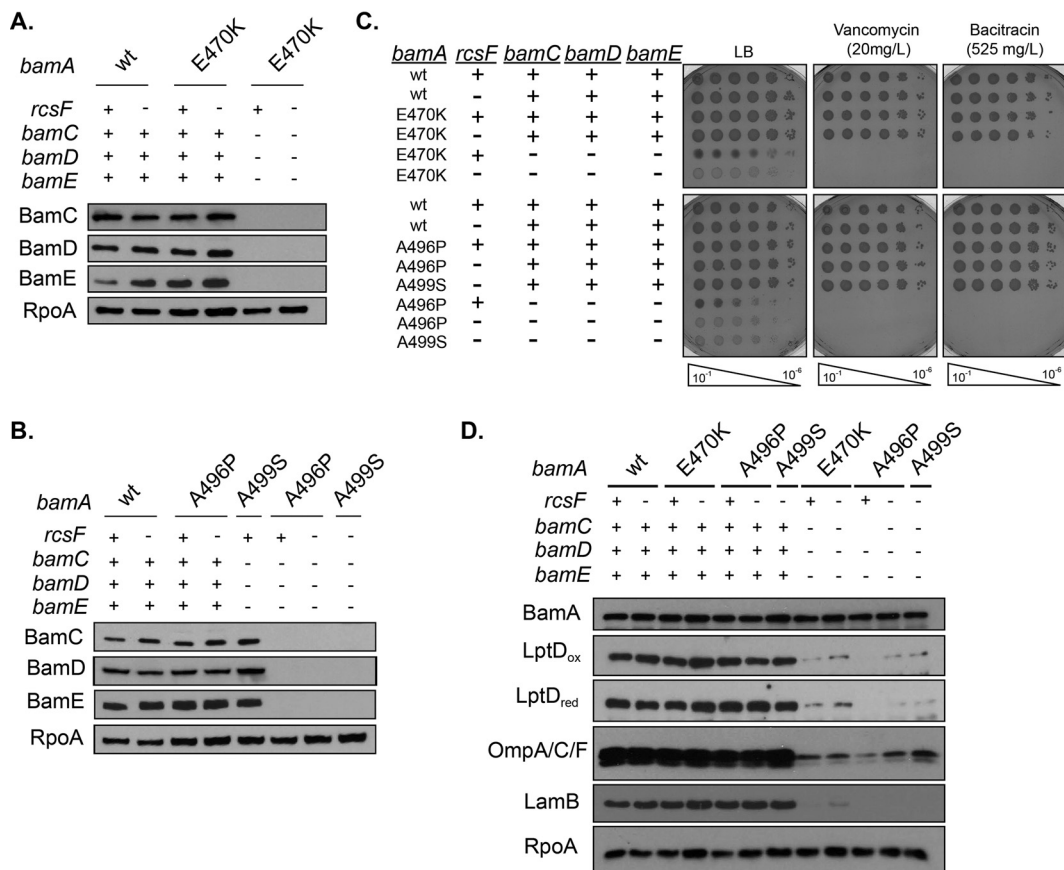


FIG 4 *bamA* alleles promote viability of $\Delta bamC \Delta bamD \Delta bamE$ mutants. (A and B) Immunoblot analysis probing for BamC, BamD, and BamE in stationary-phase $\Delta bamC \Delta bamD \Delta bamE$ mutants expressing *bamA*_{E470K} (A) or *bamA*_{A496P} or *bamA*_{A499S} (B). RpoA served as a loading control. (C) Cells were normalized by OD₆₀₀, serially diluted, and spotted onto medium containing the indicated antibiotics. Plates were incubated overnight at 30°C. (D) Stationary-phase cultures were analyzed by immunoblot analysis probing for OMPs. RpoA was used as a loading control.

BamAE (24); however, only the interaction between the BamAD proteins is essential (37, 38), leading to the hypothesis that the interaction of BamC and BamE to BamA is largely mediated through BamD. Since the essential function of BamD is bypassed by *bamA*_{E470K} ($\pm rcsF$) (52), *bamA*_{A496P} ($\pm rcsF$), and *bamA*_{A499S} $\Delta rcsF$, we predicted that *bamC* and *bamE* could be deleted in these suppressor backgrounds as well.

We assayed our ability to delete *bamD* using linkage disruption experiments with the P1 phage carrying a *bamD::kan* allele linked to a *nadB::Tn10* marker described earlier. The *bamD::kan* deletion allele could not be inherited by haploid *bamD* cells lacking BamC and BamE ($\pm rcsF$), reflecting the essentiality of BamD in cells expressing *bamA*⁺. Coinheritance of *bamD::kan* with the *nadB::Tn10* marker was also observed in $\Delta bamC \Delta bamE$ mutant cells expressing *bamA*_{E470K} and *bamA*_{A496P}, regardless of the presence of RcsF. The inheritance of the marker was observed only in $\Delta bamC \Delta bamE$ *bamA*_{A499S} mutant cells that lacked RcsF (Table S2). Based on these results, clean disruption strains were constructed, and PCR and immunoblot analyses (Fig. 4A and B) confirmed that these strains lacked BamC, BamD, and BamE.

We characterized the phenotypes of the suppressed $\Delta bamC \Delta bamD \Delta bamE$ mutant strains. Similar to suppressed $\Delta bamD$ mutants, cells lacking BamC, BamD, and BamE have growth defects (Table 3), OM barrier (Fig. 4C), and OMP assembly defects (Fig. 4D).

We conclude that in *bamB*⁺ $\Delta bamD$ strains carrying the *bamA* suppressors, BamC and BamE have little, if any, effect on OMP assembly.

A minimal BAM complex. The identification of suppressor alleles that promote viability of $\Delta bamB \Delta bamC \Delta bamE$ and $\Delta bamC \Delta bamD \Delta bamE$ mutant cells led us to

wonder if we could construct a viable strain with a minimal BAM complex lacking all of the lipoproteins, BamBCDE. Our strategy was to deplete BamD from the background that had the most stringent requirement for suppression, the $\Delta bamB \Delta bamC \Delta bamE$ mutant strain (Table 2). Again, we utilized an arabinose-inducible ectopic copy of *bamD* in a genetic background expressing the *bamA* suppressor alleles in which the native loci of *bamD*, *bamC*, *bamE*, and *bamB* were deleted. In cells expressing *bamA*_{A496P} or *bamA*_{A499S}, *rscF* was also deleted, as these alleles cannot suppress the $\Delta bamB \Delta bamC \Delta bamE$ mutant when RcsF is present. Depletion of *bamD* by growing the strains on the anti-inducer fucose showed that the *bamA*_{E470K} ($\pm rcsF$) mutant supported growth, albeit weakly, whereas $\Delta rcsF$ *bamA*_{A496P} and $\Delta rcsF$ *bamA*_{A499S} mutants did not support growth in the absence of the BAM complex lipoproteins (see Fig. S2 in the supplemental material).

To monitor cell viability over the course of BamD depletion, we passaged exponentially growing cells expressing the above-mentioned depletion constructs in arabinose or fucose. In control strains expressing wild-type *bamA*, cells inoculated into fucose survived two subcultures, or about 12 doublings, before the cells died (Fig. 5A). The depletion of the BamD protein can also be monitored by immunoblot analysis. In the $\Delta bamB \Delta bamC \Delta bamE \Delta rcsF$ *bamA*_{A496P} and $\Delta bamB \Delta bamC \Delta bamE \Delta rcsF$ *bamA*_{A499S} mutant backgrounds, the cells grown in arabinose survived due to the viability of the $\Delta bamB \Delta bamC \Delta bamE$ mutant backgrounds (Fig. 5B and C, red; Table 2). Cells grown in fucose lysed before reaching the mid-log-phase back dilution benchmark, suggesting that this genetic background is hypersensitive to BamD depletion (Fig. 5B and C, black; Table 2).

In contrast, $\Delta bamB \Delta bamC \Delta bamE$ *bamA*_{E470K} mutant cells ($\pm rcsF$) were able to survive depletion of BamD and grow past the point at which wild-type cells die due to a lack of BamD (Fig. 5D and E). Removal of RcsF accelerated the growth of these strains (Fig. 5E). The BamD-depleted $\Delta bamB \Delta bamC \Delta bamE \Delta rcsF$ *bamA*_{E470K} mutant cells, however, could not be successfully isolated on agar plates (Fig. 5E). BamD-depleted $\Delta bamB \Delta bamC \Delta bamE$ *bamA*_{E470K} mutant cells that express RcsF could be successfully isolated on agar plates, but viability of these cells is severely compromised (Fig. 5D; see also Fig. S2). The absence of BamBCDE in the depleted strain was confirmed by immunoblot analysis (see Fig. S3 in the supplemental material).

The seemingly paradoxical behavior of RcsF in the *bamA*_{E470K} suppressor strain merits further comment. As noted above, we suggest that the assembly of the RcsF/OMP complexes is defective in *bamA*_{E470K} strains (Fig. S1). Accordingly, the presence of RcsF does not further compromise BAM function significantly in this mutant, but the mislocalized protein does activate the Rcs stress response. We suspect that removal of RcsF in the $\Delta bamB \Delta bamC \Delta bamE$ *bamA*_{E470K} mutant background accelerates growth because the mutant cells do not waste energy producing capsule. However, if RcsF is present, the capsule produced strengthens the cell envelope enough to allow individual colonies to be isolated on agar plates (53, 54).

We monitored OMP assembly in the depleted $\Delta bamB \Delta bamC \Delta bamD \Delta bamE$ *bamA*_{E470K} mutant cells (Fig. S3). OMP levels were strongly reduced in this strain and were decreased in comparison to the isogenic $\Delta bamB \Delta bamC \Delta bamE$ *bamA*_{E470K} strain that was grown in the presence of arabinose to induce expression of BamD. *BamA*^{E470K}, though, was able to successfully assemble detectable levels of OMPs in the absence of BamBCDE.

We conclude that in the absence of all four of the BAM lipoproteins, *BamA*^{E470K} can assemble OMPs well enough to support cell viability.

BamB and BamD have a redundant function in OMP assembly. It is clear from the analysis of the phenotypes of the $\Delta bamD$, $\Delta bamC \Delta bamD \Delta bamE$, $\Delta bamB \Delta bamC \Delta bamE$, and $\Delta bamB \Delta bamC \Delta bamD \Delta bamE$ strains carrying the various *bamA* suppressors that *bamA*_{A496P} and *bamA*_{A499S} cannot tolerate the simultaneous deletion of *bamB* and *bamD* (Table 2). These results suggest that BamB and BamD share a redundant function in OMP assembly.

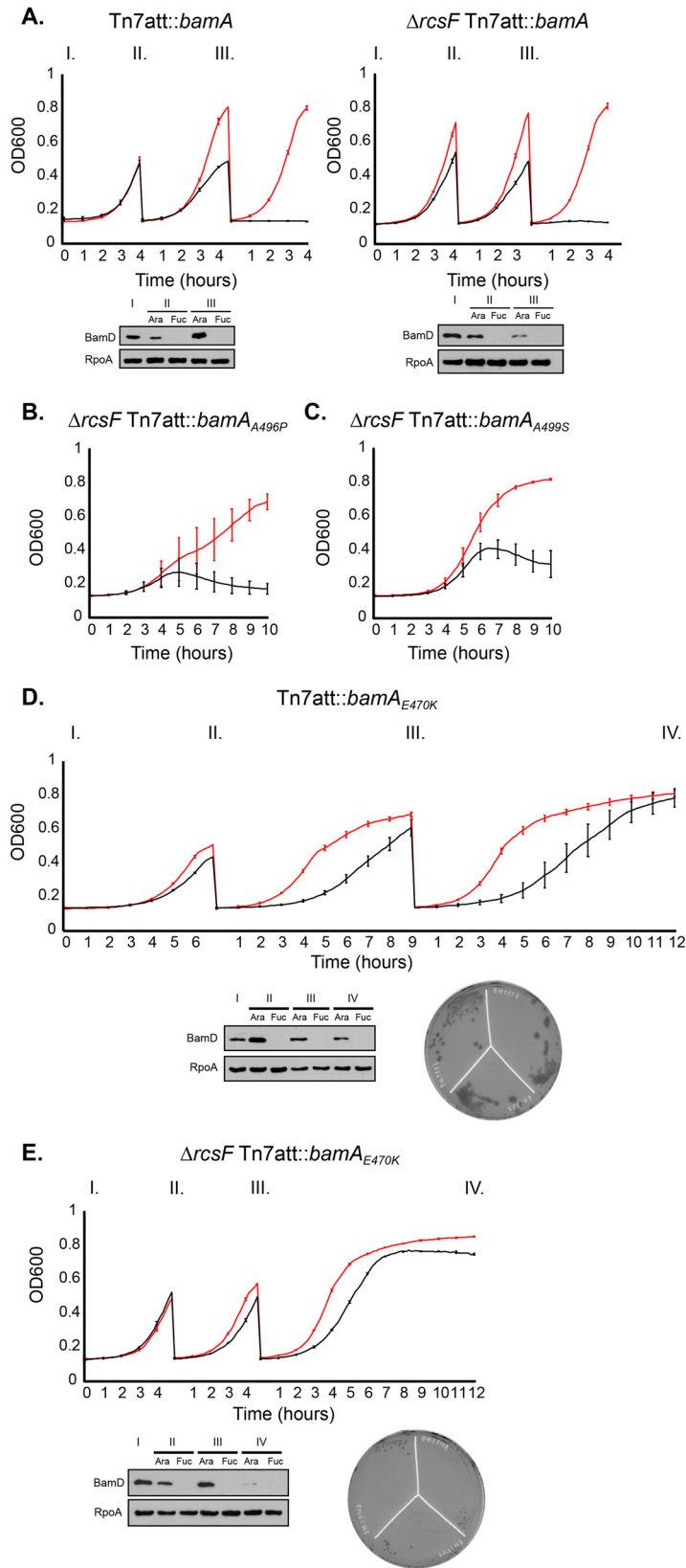


FIG 5 BamA^{E470K} allows for cell viability in the absence of BamB, BamC, BamD, and BamE. Overnight cultures of wild-type and $\Delta rcsF$ strain controls (A) or $\Delta bamB \Delta bamC \Delta bamE$ mutants expressing an arabinose-inducible copy of *bamD* and the labeled *bamA* alleles ($\pm rcsF$) (B to E) were grown overnight at 30°C in medium supplemented with arabinose. Cells were normalized by OD₆₀₀ and

(Continued on next page)

We depleted BamD from *bamB* mutants in strains expressing the *bamA* suppressor alleles ($\pm rcsF$) (Fig. 6). Cells carrying an ectopic copy of *bamD* expressed from an arabinose-inducible promoter were inoculated into medium containing arabinose or fucose and cycled in exponential phase to deplete BamD. Cells expressing *bamA*⁺ were unable to survive BamD depletion in either *bamB*⁺ (see Fig. S4 in the supplemental material) or *bamB* null backgrounds (Fig. 6A) due to the essentiality of BamD. The *bamA*_{A496P} ($\pm rcsF$) and *bamA*_{A499S} $\Delta rcsF$ strains were able to survive BamD depletion, as previously demonstrated (Fig. S4), but were unable to survive BamD depletion in *bamB* null backgrounds (Fig. 6C and D). Thus, in *bamA*_{A496P} and *bamA*_{A499S} mutant backgrounds, BamB and BamD have a redundant function.

In contrast, *bamA*_{E470K} promoted survival in the absence of BamB and BamD (Fig. 6B). Intriguingly, *bamB::kan* $\Delta rcsF$ *bamA*_{E470K} mutant cells survived longer than the wild-type control during BamD depletion; however, these cells eventually lysed, suggesting that BamA^{E470K} only promotes survival in the absence of BamB and BamD when RcsF was present. This phenotype mimics the ability of $\Delta bamB$ $\Delta bamC$ $\Delta bamE$ $\Delta rcsF$ *bamA*_{E470K} mutant cells to grow during BamD depletion; however, individual cells were not able to survive on agar plates. As described in more detail in Discussion, this demonstrates that BamD has two essential functions, one that is redundant with BamB and another that is unique.

DISCUSSION

In this study, we have used three *bamA* suppressor mutations, *bamA*_{E470K}, *bamA*_{A496P}, and *bamA*_{A499S}, to provide insight into the functions performed by the BAM complex lipoproteins during OMP assembly. These studies were facilitated by the knowledge that the interlocked RcsF/OMP complex poses a significant assembly challenge for the BAM complex. The lipidated amino terminus of RcsF is exposed on the cell surface by being threaded through the lumen of an OMP (20, 47). Efficient formation of the complex requires BamE (47), and the conditional lethal phenotype of a $\Delta bamB$ $\Delta bamE$ double mutant can be suppressed by simply removing RcsF (48, 49). Phenotypes conferred by one of these *bamA* suppressor alleles, *bamA*_{A499S}, are apparent only in strains in which *rcsF* is deleted and suppression by the *bamA*_{A496P} suppressor allele sometimes requires the absence of RcsF as well.

In a $\Delta rcsF$ mutant background, all three *bamA* mutations bypass the normally essential requirement for BamD. This reinforces the conclusion that BamD does not perform a critical role in OMP folding and membrane insertion but rather functions as an assembly checkpoint to prevent BamA from engaging defective substrates (35, 36, 52). Indeed, *bamA*_{E470K} and *bamA*_{A496P}, but not *bamA*_{A499S}, can suppress the $\Delta bamD$ mutant even in cells that produce RcsF (Table 2). Therefore, *bamA*_{E470K}, *bamA*_{A496P}, and *bamA*_{A499S} suppress the $\Delta bamD$ mutant by bypassing the assembly checkpoint imposed by BamD on BamA.

We have shown that the suppression phenotypes conferred by the *bamA* suppressor alleles to $\Delta bamC$ $\Delta bamD$ $\Delta bamE$ strains are identical to those conferred to the $\Delta bamD$ strain (Table 2). This correlation confirms that the deletion of $\Delta bamD$ likely dissociates most of the BamC and BamE proteins from the BamAB subcomplex, explaining the ability of the same *bamA* suppressor mutations to suppress both conditions. In wild-type cells, we propose that BamD mediates interaction of BamC and BamE with the BAM holocomplex. In the absence of BamD, BamC and BamE are not required for OMP assembly, as they can be removed with little consequence to cell viability in the suppressor strains.

FIG 5 Legend (Continued)

inoculated into medium containing arabinose or fucose. Cultures were grown to an OD₆₀₀ of ~0.5 and back-diluted to cycle cells during logarithmic growth. Growth was monitored by OD₆₀₀ and BamD depletion was confirmed by immunoblot analysis probing for BamD and RpoA as a loading control. The red line indicates growth in medium supplemented with arabinose, and the black line indicates growth in medium supplemented with fucose. Each depletion curve represents the average of three biological replicates and error bars indicate SEM.

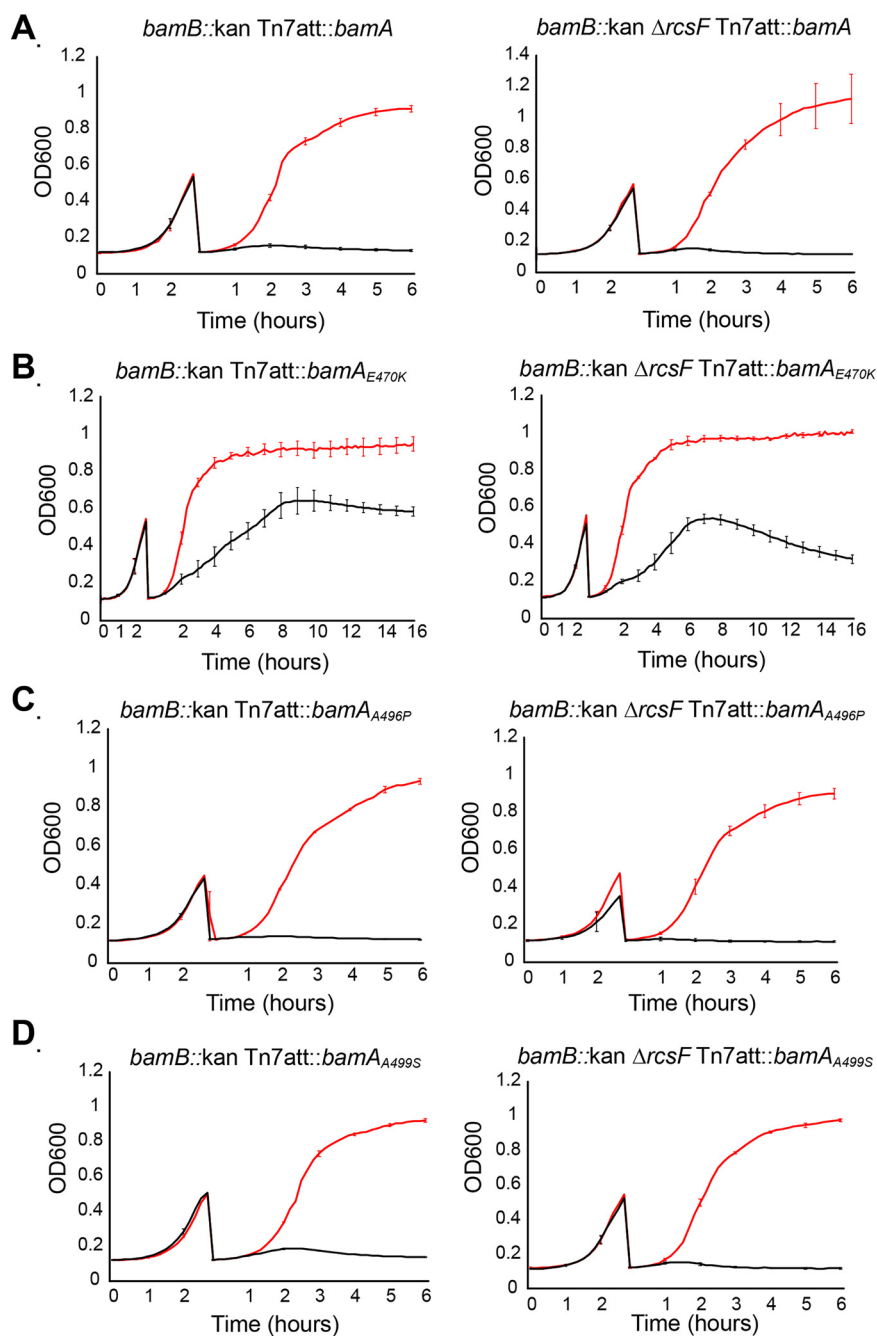


FIG 6 BamB and BamD have redundant functions. Depletion of BamD from an arabinose-inducible P_{BAD} promoter in *bamB* null backgrounds expressing the wild-type *bamA* or the *bamA* suppressor alleles in the presence or absence of RcsF. The red line indicates growth in medium supplemented with arabinose, and the black line indicates growth in medium supplemented with fucose. *bamA*⁺ (A), *bamA*_{E470K} (B), *bamA*_{A496P} (C), or *bamA*_{A499S} (D) strains in the presence or absence of *rcsF*. Cells were inoculated into medium supplemented with arabinose (inducer) or fucose (repressor). Cultures were back-diluted at an OD₆₀₀ of ~0.5. Depletions were performed at 37°C.

Our results demonstrate that the nonessential BAM lipoproteins, BamBCE, all share a redundant function. Indeed, in an otherwise wild-type strain lacking RcsF, this function can be performed by any one of the three, even the enigmatic BamC. It is also clear that this function can be bypassed by suppressor mutations in *bamA* that bypass the essential function of BamD. Thus, the redundant function shared by these three lipoproteins is to facilitate proper cooperation between the essential proteins BamA

and BamD. In the absence of all three lipoproteins, BamAD coordination is lethally disrupted, resulting in cell death.

In addition to this shared redundant function, the nonessential lipoproteins also have unique functions. BamE is required to assemble the surface-exposed RcsF/OMP interlocked complex (47). Also, in the absence of BamE, BamB prevents the lethal jamming of the BAM complex by RcsF (48, 49). The absence of BamB also reduces the assembly of the abundant porins but does not affect the assembly of lower volume substrates, such as the LptD/E complex (44). Indeed, for reasons discussed below, we think it is likely that BamB has an additional function(s).

The synthetic lethality of *bamB* and *bamD* deletion in the *bamA*_{A496P} and *bamA*_{A499S} suppressor backgrounds led us to identify a functional redundancy between BamB and BamD (Table 2). Thus, despite the fact that BamA^{A496P} and BamA^{A499S} do not require BamD for function, in its absence, BamB becomes essential. Alternatively stated, BamD has two essential functions as follows: (i) regulation of BamA activity (35, 37, 38, 52, 55) and (ii) a function shared specifically with BamB. In the absence of *bamB*, the second function of BamD becomes essential even in genetic backgrounds that have bypassed the first function. This demonstrates that BamB has two functions, one that is redundant with BamC and BamE and one that is redundant with BamD.

Previous *in vitro* studies have demonstrated that BamB and BamD are individually sufficient to assemble substrate BamA (55). Additionally, BamA folding is impaired in *bamB* mutants under conditions of BamD depletion (56). If the redundant function of BamB and BamD affects only the assembly of substrate BamA, then it is possible that this function is catalytic. If this is so, then the *bamA*_{E470K} suppressor mutation must affect not only the catalytic function of BamA but also its properties as an assembly substrate. We would predict that *bamA*_{E470K} mutant cells are able to survive in the absence of both BamB and BamD because BamA^{E470K} self-assembles without the aid of any of the BAM lipoproteins. Alternatively, if the redundant function of BamB and BamD extends to other OMP substrates beyond BamA, then this function may be regulatory as well, rather than catalytic.

The potency of the *bamA* suppressor alleles prompted us to test if they would support cell viability in the absence of all four BAM lipoproteins. Cells expressing BamA^{A496P} and BamA^{A499S} could not survive in the absence of BamB, BamC, BamD, and BamE. Incredibly, we observed that strains expressing *bamA*_{E470K} were able to survive in the absence of BamBCDE; however, cells require RcsF to be present in order to grow on agar medium. We propose that the capsule produced in the Δ *bamB* Δ *bamC* Δ *bamD* Δ *bamE* *bamA*_{E470K} mutant cells offers mechanical support to the weakened OM, whereas deletion of *rscF* prevents production of the protective capsule (53, 54).

The synthetic phenotype of *bamB* and *bamD* deletion in *bamA*_{A496P} and *bamA*_{A499S} mutants was predictive of the inability of these cells to survive in the absence of all four BAM lipoproteins.

Thus, we have identified a genetic background that permits cell survival despite removal of all of the BAM complex lipoproteins, suggesting that BamA alone is able to catalyze the folding and insertion of OMPs into the OM. The BAM complex lipoproteins, then, may have evolved to improve the accuracy and efficiency of OMP assembly and aid with assembly of different OMP substrates, such as RcsF/OMP complexes. This result is consistent with *in vitro* work demonstrating that BamA can catalyze OMP insertion on its own (55, 57, 58). However, the *in vitro* work is done under optimized conditions with a single OMP substrate. Cell survival is a much more demanding test that requires a potent suppressor mutation.

We have shown that the *bamA*_{E470K} mutant bypasses the requirement for BamD, the redundant function that BamD shares with BamB, and the redundant function shared by the three nonessential lipoproteins BamBCE. However, in striking contrast to the observation that the phenotypes conferred by this *bamA* suppressor to Δ *bamC* Δ *bamD* Δ *bamE* strains are identical to that conferred to the Δ *bamD* strain (Table 2), the *bamA*_{E470K} suppressed Δ *bamC* Δ *bamD* Δ *bamE* mutant grows far better than the sup-

pressed $\Delta bamB \Delta bamC \Delta bamD \Delta bamE$ mutant. Thus, there must be an additional function for BamB that we have not yet identified.

All of the BAM lipoproteins are located in the periplasm. Residue E470 is located on the fourth β -strand with its side chain pointing into the lumen, and residues A496P and A499S are located on extracellular loop 3 (24). None of these locations are near the periplasm, but they clearly alter BamA activity in a striking manner. Recent work shows that considerable barrel folding occurs within the BamA barrel (33), and residue E470 has been demonstrated to lie close to a stalled substrate (30). Extracellular loop 3 is near the seam of BamA as a part of the exit pore, which must open to allow substrates into the OM (40). We suggest that these residues are an important part of the active site of this remarkable protein-folding catalyst.

Accepted evolutionary hypotheses posit that mitochondria and chloroplasts are descended from diderm bacteria (59, 60). Both organelles have an OM containing β -barrel proteins that are inserted by an Omp85 family protein, Sam50 in mitochondria and OEP80 in chloroplasts (61–64). However, additional accessory lipoproteins involved in organelle OMP assembly do not share sequence similarity to the BAM complex lipoproteins (65). Mitochondria have a number of accessory proteins that aid OMP assembly by Sam50, but they are located on the cytoplasmic side of the mitochondrial OM, not the intramembrane space, which would be the equivalent of the bacterial periplasm (66–68). Accessory proteins of the chloroplast β -barrel assembly complex have not been characterized (65). Both mitochondria and chloroplasts are located in a constant, never-changing environment and are responsible for assembling only a small set of substrates (69–71). The bacterial lipoproteins evolved to broaden the substrate specificity and fortify BAM to allow function in many different, often hostile environments. Indeed, BamA and BamD are proposed to be the ancestral components of the BAM complex in proteobacteria, and many of the nonessential BAM lipoproteins are highly conserved as well (72).

MATERIALS AND METHODS

Bacterial strains and growth conditions. All strains, plasmids, and oligonucleotides are listed in Table S3 in the supplemental material of this study. Strains were constructed using standard microbiological techniques as previously described (73). Strains were grown in LB medium supplemented with 0.2% arabinose, 0.1% fucose, 50 mg/liter kanamycin, 25 mg/liter tetracycline (high), 10 mg/liter tetracycline (low), 20 mg/liter chloramphenicol (high), 10 mg/liter chloramphenicol (low), 525 mg/liter bacitracin, and 20 mg/liter vancomycin when appropriate. Unless otherwise noted, all strains were grown at 30°C. *bamD* and *bamA* deletion alleles originated from previous studies (21, 22). Construction of the λ_{att} *bamD* and *bamA* depletion alleles is described elsewhere (21–23). Other deletion alleles originated from the Keio collection, Carol Gross Tn10 collection, or Blattner collection (74–76). FLP recombination target (FRT)-flanked resistance cassettes were removed by use of the FLP recombinase system as previously described (77).

Efficiency of plating assay. Stationary-phase cultures were normalized by optical density at 600 nm (OD_{600}), serially diluted, and spotted onto the indicated medium. Plates were incubated at 30°C overnight. In the case of depletions, cells were grown overnight in medium containing 0.2% arabinose and diluted in LB medium.

Immunoblot analysis. Stationary-phase cultures were normalized by OD_{600} and lysed in a mixture of BugBuster (Millipore), Benzonase (1:100; Sigma-Aldrich), 1 M $MgCl_2$ (1:100), and protease inhibitor cocktail (1:100; Sigma-Aldrich) for 10 min at room temperature. Samples were diluted 1:2 in 4 \times Laemmli sample buffer (Bio-Rad) supplemented with β -mercaptoethanol for reduced samples. Samples were boiled for 10 min and electrophoresed on a 10% (BamA, OmpA/C/F, LamB, RNA polymerase [RNAP] α , BamD, and BamC), 8% (LptD), or 15% (BamE) SDS-PAGE gel. Proteins were transferred to a nitrocellulose membrane and immunoblotted with rabbit polyclonal primary antibody probing for anti-BamA (1:25,000), anti-LamB (1:11,500), anti-LptD (1:10,000), anti-BamC (1:60,000), anti-BamE (1:5,000), anti-BamD (1:10,000), anti-OmpA/C/F (1:25,000), or anti-RpoA (1:50,000). Donkey anti-rabbit IgG-horseradish peroxidase secondary antibody (GE Healthcare) was used at a dilution of 1:10,000.

Heat modifiability assay. Samples were normalized by OD_{600} and lysed in BugBuster (Millipore), Benzonase (1:100; Sigma-Aldrich), 1 M $MgCl_2$ (1:100), and protease inhibitor cocktail (1:100; Sigma-Aldrich) at room temperature for 10 min. Samples were diluted 1:2 in 4 \times Laemmli sample buffer (Bio-Rad) supplemented with β -mercaptoethanol and divided in two. One aliquot was boiled for 10 min (denatured/unfolded protein), and the other was incubated at room temperature for 10 min (nondenatured/folded protein). Samples were electrophoresed on a 10% SDS-PAGE gel at 4°C, and immunoblotting was carried out as described above.

MIC assay. Evaluation of MIC was performed as previously described (50, 78). Briefly, 2-fold serial dilutions of MRL-494 were made in dimethyl sulfoxide (DMSO) to a concentration 50-fold higher than the

target concentration to account for dilution upon treatment. The tested MRL-494 concentrations ranged from 64 $\mu\text{g/ml}$ to 0.03125 $\mu\text{g/ml}$. Overnight cultures of the indicated strains were first diluted 1:50 and then diluted 1:1,000 in fresh medium. A total of 98 μl of the cell dilution was inoculated into a 96-well plate, and 2 μl of the appropriate MRL-494 concentration was added. The plate was incubated overnight at 37°C, and the OD₆₀₀ was measured at 16- and 24-h time points in a BioTek Synergy H1 plate reader.

Cell viability measurements. The number of viable cells in an overnight culture reflects the growth rate and viability of the strain. Viability was assessed by measuring CFU (CFU per milliliter). Stationary-phase cultures were diluted, plated onto LB medium, and grown overnight at 30°C. The number of colonies was used to determine CFU per milliliter. Measurements were performed in biological triplicate.

Linkage disruption assay. P1 phage carrying *bamD::kan nadB::Tn10* or *bamB::kan xyz::cam* was used to transduce the indicated strains at 30°C. Transductants were selected for integration of the marker allele (i.e., tetracycline for *nadB::Tn10* and chloramphenicol for *xyz::cam*), and colonies were patched to kanamycin to check for integration of the allele of interest. The number of kanamycin colonies over total number of transductants was used to calculate linkage frequency. Calculations represent the average of at least three transductions. In the case of linkage disruption to ascertain viability of ΔbamC ΔbamD ΔbamE strains, cells carried a *bamE::cam* allele and were cross-patched to chloramphenicol to ensure that integration of *bamD::kan nadB::Tn10* did not transduce wild-type *bamE*. Similarly, in linkage disruption experiments testing viability of ΔbamB ΔbamC ΔbamE mutant cells, *bamC* was amplified by colony PCR from kanamycin-resistant transductants to ensure that *bamB::kan xyz::cam* integration did not transduce wild-type *bamC*.

BamD liquid depletion. Cells were grown overnight at 30°C in LB medium supplemented with 0.2% arabinose. Cultures were normalized to an OD₆₀₀ of 0.01 and inoculated into LB medium containing either arabinose or fucose in 2-ml volumes in a 24-well plate (Sarstedt). Cultures were grown shaking at the indicated temperature until cells reached an OD₆₀₀ of 0.5 in a BioTek Synergy H1 plate reader. Cells were then diluted 1:50 into fresh medium, and growth was repeated. Back dilutions were performed until cell survival of wild-type control ceased. At each back dilution, samples were collected for immunoblot analysis as described above. Growth measurements were performed in biological triplicate, and immunoblot analysis with anti-BamD to ensure depletion of BamD was performed on one biological replicate.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.6 MB.

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E.M.H. and T.J.S. designed the research. E.M.H. performed the research. E.M.H. and T.J.S. wrote the manuscript.

We declare no competing interests.

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