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Classifying β-Barrel Assembly Substrates by Manipulating Essential Bam Complex Members

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ABSTRACT

The biogenesis of the outer membrane (OM) of *Escherichia coli* is a conserved and vital process. The assembly of integral β-barrel outer membrane proteins (OMPs), which represent a major component of the OM, depends on periplasmic chaperones and the heteropentameric β-barrel assembly machine (Bam complex) in the OM. However, not all OMPs are affected by null mutations in the same chaperones or nonessential Bam complex members, suggesting there are categories of substrates with divergent requirements for efficient assembly. We have previously demonstrated two classes of substrates, one comprising large, low-abundance, and difficult-to-assemble substrates that are heavily dependent on SurA and also Skp and FkpA, and the other comprising relatively simple and abundant substrates that are not as dependent on SurA but are strongly dependent on BamB for assembly. Here, we describe novel mutations in *bamD* that lower levels of BamD 10-fold and >25-fold without altering the sequence of the mature protein. We utilized these mutations, as well as a previously characterized mutation that lowers wild-type BamA levels, to reveal a third class of substrates. These mutations preferentially cause a marked decrease in the levels of multimeric proteins. This susceptibility of multimers to lowered quantities of Bam machines in the cell may indicate that multiple Bam complexes are needed to efficiently assemble multimeric proteins into the OM.

IMPORTANCE

The outer membrane (OM) of Gram-negative bacteria, such as *Escherichia coli*, serves as a selective permeability barrier that prevents the uptake of toxic molecules and antibiotics. Integral β-barrel proteins (OMPs) are assembled by the β-barrel assembly machine (Bam), components of which are conserved in mitochondria, chloroplasts, and all Gram-negative bacteria, including many clinically relevant pathogenic species. Bam is essential for OM biogenesis and accommodates a diverse array of client proteins; however, a mechanistic model that accounts for the selectivity and broad substrate range of Bam is lacking. Here, we show that the assembly of multimeric OMPs is more strongly affected than that of monomeric OMPs when essential Bam complex components are limiting, suggesting that multiple Bam complexes are needed to assemble multimeric proteins.

The assembly of integral β-barrel outer membrane proteins (OMPs) into the outer membrane (OM) of Gram-negative bacteria is essential for cell growth and viability. OMP targeting and OM integration depend on an assembly pathway comprising semiredundant periplasmic chaperones and the heteropentameric OM-associated β-barrel assembly machine (Bam). The primary constituents of the periplasmic chaperone network in *Escherichia coli* are the proline isomerases SurA and FkpA, the bifunctional protease/chaperone DegP, and the prefoldin-like chaperone Skp. The Bam complex is composed of BamA (itself a β-barrel) and four associated lipoproteins, BamB, BamC, BamD, and BamE (1–3).

Although OMPs require the Bam complex for folding into the OM, the individual or combinatorial deletion of OMP chaperones or dispensable Bam complex components differentially influences the assembly of various OMP families, suggesting that there are categories of substrates with divergent dependencies for efficient assembly (3, 4). We previously demonstrated two classes of substrates (3): one class contains large, low-abundance, and complex substrates (e.g., LptD and FhuA) that are heavily dependent on the chaperone SurA and uniquely require Skp and FkpA. The other class contains simple abundant substrates (e.g., the general porins and maltoporin LamB) that exhibit lower dependence on SurA than the former class but are strongly dependent on BamB for proper assembly.

The simple plentiful substrates described in the second class are orders of magnitude more abundant than the complex substrates with intricate folding pathways. Both monomeric and multimeric proteins are found within this substrate category. Given this, we wondered if the assembly requirements for multimeric proteins would differ from those for monomeric proteins in this class.

We reasoned that differential assembly requirements of high-abundance multimers relative to monomers might be revealed in strains of *E. coli* in which the OMP assembly machinery is limiting. Previous work has shown that when BamA or BamD is depleted, OMP assembly stalls, and eventually the cells will become nonviable (1, 2, 5). Here, we utilized mutations that reduce endogenous

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TABLE 1 Strains[a] used in this study

Strain	Relevant characteristics	Source or reference
JCM158	MC4100 Ara^r	1
JCM290	JCM158 Δ*bamD* Δ(λ*att-lom*)::*bla* P_{BAD} *bamD araC*	2
PTM767	JCM158 *bamA101* Tn*5*	This study
PTM825	JCM158 Δ*bamD nadB*::Tn*10* pZS21::*bamD*	This study
PTM826	JCM158 Δ*bamD nadB*::Tn*10* pZS21::*bamD* L13P	This study
PTM829	JCM158 Δ*bamD nadB*::Tn*10* pZS21::*bamD*-4C→T	This study
PTM928	JCM158 Δ*malK-bla*$_{(fwd)}$-*nudF-tolC*$_{PandSS}$-*lamB*$_{ΔSS}$	This study
PTM929	JCM158 Δ*malK-bla*$_{(fwd)}$-*nudF-tolC*$_{PandSS}$-*lamB*$_{ΔSS}$	This study
PTM940	PTM928 *bamA101* Tn*5*	This study
PTM941	PTM929 *bamA101* Tn*5*	This study
PTM942	PTM929/pZS21::*bamD* Δ*bamD nadB*::Tn*10*	This study
PTM943	PTM928/pZS21::*bamD*-4C→T Δ*bamD nadB*::Tn*10*	This study
PTM945	PTM929/pZS21::*bamD*-4C→T Δ*bamD nadB*::Tn*10*	This study
PTM946	JCM158 *bla*$_{(fwd)}$-*nudF-tolC*$_{PandSS}$-*ompA*$_{ΔSS}$	This study
PTM948	JCM158 *bla*$_{(fwd)}$-*nudF-tolC*$_{PandSS}$-*ompA*$_{ΔSS}$	This study
PTM949	PTM946 *bamA101* Tn*5*	This study
PTM950	PTM946/pZS21::*bamD* Δ*bamD nadB*::Tn*10*	This study
PTM952	PTM946/pZS21::*bamD* L13P Δ*bamD nadB*::Tn*10*	This study
PTM953	PTM948 *bamA101* Tn*5*	This study
PTM954	PTM948/pZS21::*bamD*-4C→T Δ*bamD nadB*::Tn*10*	This study
PTM955	PTM928 Δ*malT*	This study
PTM956	PTM955 *bamA101* Tn*5*	This study
PTM957	PTM955/pZS21::*bamD*-4C→T Δ*bamD nadB*::Tn*10*	This study
PTM958	PTM955/pZS21::*bamD* L13P Δ*bamD nadB*::Tn*10*	This study
PTM959	PTM955/pZS21::*bamD* Δ*bamD nadB*::Tn*10*	This study

[a] JCM158 is a spontaneous arabinose-resistant (Ara^r) mutant. *bla*$_{(fwd)}$, *bla* gene oriented in the same direction as *tolC*$_{PandSS}$-*lamB*$_{ΔSS}$ or *tolC*$_{PandSS}$-*ompA*$_{ΔSS}$.

levels of the essential members of the Bam complex (BamA and BamD) in order to create an assembly bottleneck at the site of OM insertion and determine the subsequent effect on OM folding of an array of OMPs with divergent properties. In this work, we identify mutations in the signal sequence and the ribosomal binding site of the essential lipoprotein BamD that lower its levels >10-fold and >25-fold, respectively, and we show that *E. coli* can tolerate a dramatic reduction in BamD levels with minimal effects on growth and viability. These novel *bamD* mutations allow us to interrogate the functionality of the Bam complex by limiting the number of complete functional Bam complexes in the cell. We show that the impact of BamA/D restriction on OMP assembly in Bam-limited strains affects some OMPs far more dramatically than others, raising the intriguing possibility that certain OMPs (or OMP classes) have higher affinity for Bam.

MATERIALS AND METHODS

Bacterial strains, media, and culture conditions. All strains used in this study are isogenic derivatives of *E. coli* MC4100 constructed by generalized transduction with bacteriophage P1 (6) and are listed in Table 1. All plasmids used in this study are listed in Table 2. To construct the fusions of *tolC*$_{PandSS}$-*lamB*$_{ΔSS}$ (the *tolC* promoter and signal sequence [*tolC*$_{PandSS}$] fused to *lamB* lacking its native signal sequence and promoter [*lamB*$_{ΔSS}$]) and *tolC*$_{PandSS}$-*ompA*$_{ΔSS}$ (*tolC*$_{PandSS}$ fused to *ompA* lacking its native signal sequence and promoter [*ompA*$_{ΔSS}$]), the fusion fragments were created via two sequential PCRs. For the *tolC*$_{PandSS}$-*lamB*$_{ΔSS}$ fragment, the first PCR used genomic DNA as a template to amplify the intragenic region between *tolC* and the upstream gene *nudF*, the promoter and signal sequence of TolC attached to the beginning of *lamB* without a signal sequence using primer *tolC-lamB* reaction1 FWD (5′-AGATCGCTGAGATAGGTGCCTCACTGATTAAGCATTGGTAA TGTTAATGTCCTGGCACTAATAGTGA-3′) and primer *tolC-lamB* reaction1 REV (5′-CACCGCCGCTACCTGTCCAACCAATACCGGA ACGTGCATAGCCGTGGAAATCAACGGCCTGGCTCAACGAACT GAA-3′). The forward primer is composed of the last 41 bp of *bla* and the first 26 bp of the intragenic region upstream of *tolC*. The reverse primer is composed of the complement of the last 21 bp of the signal sequence of *tolC* and the first 55 bp of *lamB* lacking the signal sequence. The second PCR amplified *bla* from plasmid pTB263, using forward primer *tolC-lamB* reaction2 FWD (5′-GTGGAGGATTTAAGCCATCTCCTGATGACGCATAGTCA GCCCATCATGAATGTTGCTGTCGATGACAGGTTGTTACAAAGGG AGAAGGGCTATCCGCTCATGAGACAATAACCCTGATAAATGCTT CAAT-3′) and the PCR product from reaction 1 as the reverse primer. The forward primer for reaction 2 is composed of the 90 bp immediately upstream of *malK* and the first 40 bp of *bla*. The resultant PCR fragment carries the gene for ampicillin resistance and the promoter and signal sequence of *tolC* fused to *lamB* lacking a signal sequence. The PCR fragment carrying the fusion was then introduced into the strain by homologous recombination using previously described methods (7). The *tolC*$_{PandSS}$-*ompA*$_{ΔSS}$ fusion was generated using the same 2-step PCR approach, with the primer *tolC-ompA* reaction1 REV (5′-GGTACTGGG ACCAGCCCAGTTTAGCACCAGTGTACCAGGTGTTATCTTTCGGA GCGGCCTGGCTCAACGAACTGAA-3′) and the primer *tolC-lamB* reaction 1 FWD primer described above. The reverse primer in this reaction is composed of the last 21 bp of *tolC* and the first 55 bp of *ompA* without a signal sequence. The second PCR used the primer *tolC-ompA* reaction 2 FWD (5′-AAGATTAAACATACCTTATACAAGACTTTTTTTTCATAT GCCTGACGGAGTTATCCGCTCATGAGACAATAACCCTGATAAAT GCTTCAAT-3′) and the PCR product from reaction 1 as the reverse

TABLE 2 Plasmids used in this study

Plasmid	Relevant characteristics	Source or reference
pZS21::*bamD*	*bamD* cloned into pZS21::*luc*	32
pZS21::*bamD*$_{RBS}$	*bamD*-4C→T	This study
pZS21::*bamD*$_{SS}$	*bamD* L13P	This study

primer. The forward primer in reaction 2 is composed of 51 bp located 174 bp upstream of *ompA* and the first 40 bp of *bla*. The resultant PCR fragment carries the gene for ampicillin resistance and the promoter and signal sequence of *tolC* fused to *ompA* lacking a signal sequence. The PCR fragment carrying the fusion was then introduced into the strain by homologous recombination using previously described methods (7). *malT* is also deleted in strains carrying the *lamB* fusion construct, as the promoter of *malK* was partially responsible for driving expression of the construct; with the introduction of Δ*malT*, the expression of *lamB* relies solely on the *tolC* promoter.

Random mutagenesis and screening of *bamD*. Random PCR mutagenesis of *bamD* was performed using the GeneMorph II EZClone method (Stratagene), according to the manufacturer's instructions. Mutagenized pools of pZS21::*bamD* were transformed into the *bamD* depletion strain (JCM290), and transformants were screened for sensitivity on SDS-EDTA at 37°C in the absence of arabinose.

Media and chemicals. All strains were grown in Luria-Bertani (LB) broth or agar at 37°C, unless otherwise indicated. When appropriate, strains were grown in LB supplemented with 100 μg/ml ampicillin, 25 μg/ml kanamycin, 25 μg/ml tetracycline, or 20 μg/ml chloramphenicol.

Sensitivity assays. Sensitivity of the strains was also determined by efficiency of plating (EOP) assay. Strains were grown overnight and were serially diluted and replica plated onto LB plates supplemented with either 0.8 mM EDTA–0.5% SDS or MacConkey agar plates supplemented with 0.2% maltose. Spots were allowed to dry, and the plates were incubated overnight at 37°C, 30°C, or 24°C.

Western blot analysis. For whole-cell lysates, 1-ml samples of strains were pelleted (16,000 × *g*, 1 min) and resuspended at a volume equal to the optical density at 600 nm (OD_{600})/14 (for LptD and FhuA blots) or OD_{600}/6 (for all other blots) for strains harvested in the exponential phase of growth. For strains harvested in stationary phase, 250-μl samples of strains were pelleted (16,000 × *g*, 1 min) and resuspended at a volume equal to the optical density at 600 nm (OD_{600})/48 (for LptD and FhuA blots) or OD_{600}/24 (for all other blots). Reducing and nonreducing LptD blots were performed as previously described (8). FhuA blots were performed using a polyvinylidene difluoride (PVDF) membrane (Bio-Rad, Hercules, CA), and all others were performed with a nitrocellulose membrane (Whatman GmbH, Dassel, Germany). Immunoblotting was performed using the following antibodies at the indicated dilutions: anti-LamB antibody (which cross-reacts with OmpA), 1:30,000; anti-BamA antibody, 1:20,000; anti-LptD antibody, 1:7,000; anti-FhuA antibody, 1:2,500; anti-TolC antibody, 1:30,000; anti-LptE antibody, 1:20,000; anti-BamD antibody, 1:5,000; anti-BamC antibody, 1:20,000; anti-BamE antibody, 1:20,000; and anti-DegP, 1:20,000. For FhuA blots, goat anti-mouse secondary antibody conjugated to horseradish peroxidase (HRP) was used at a dilution of 1:5,000 (Bio-Rad). For all other blots, donkey anti-rabbit secondary antibody conjugated to horseradish peroxidase was used at a dilution of 1:8,000 (GE Healthcare). Immunoblots were visualized using Luminata Classico Western HRP substrate (EMD Millipore Corporation, MA). The anti-TolC antibody was a gift from R. Misra; the anti-BamB, anti-BamD, and anti-LptE antibodies were a gift from D. Kahne; and the anti-FhuA antibody was a gift from J. Coulton.

Growth assays. Overnight cultures grown at 37°C were diluted 1:100 in 2 ml of LB in 24-well plates. The optical density at 600 nm (OD_{600}) was measured every 10 min for 16 h, with continuous orbital shaking at 37°C, 30°C, or 24°C.

qRT-PCR analysis. RNA was prepared from 1×10^8 cells from exponentially growing cells using the Qiagen RNAprotect and RNeasy system, including the optional on-column DNase I treatment described in the manual. The amount of RNA present in each strain was quantitated, and the same amount of RNA was used in the cDNA for each strain being analyzed. cDNA was made using the RNA Applied Biosystems high-capacity cDNA reverse transcription kit, according to the protocol described in the manual. The cDNA was normalized to 5 ng/μl before experimentation. Reverse transcription-quantitative PCR (qRT-PCR) was performed using an Applied Biosystems Step One Plus real-time PCR machine in 10-μl reaction mixtures using PerfeCTa SYBR green FastMix ROX by Quanta Biosciences, Inc. For comparative analysis, transcript levels were normalized to the level of the housekeeping gene *hfq*. All assays were performed in biological triplicates.

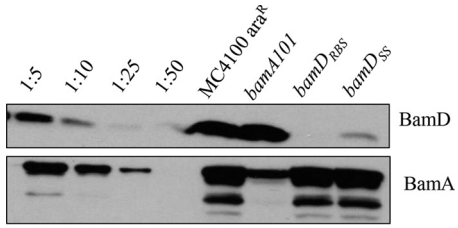

FIG 1 Effect of *bamD* down mutations and *bamA101* on levels of BamA and BamD. Strains were grown at 37°C in LB until reaching an OD_{600} between 0.7 and 0.8; next, whole-cell lysates were analyzed via SDS-PAGE for levels of BamA and BamD. Dilutions of the wild type (MC4100 Ara^r) of 1:5, 1:10, 1:25, and 1:50 are included to quantify the fold change of BamA and BamD levels in *bamA101* and the *bamD* down mutants, respectively.

RESULTS

Genetic screen for BamD-limited mutants. A transposon insertion in the *bamA* promoter that lowers the expression of BamA by approximately 90% (*bamA101*) was previously isolated in a screen for mutants of *E. coli* that are resistant to contact-dependent growth inhibition. BamA levels in the *bamA101* mutant are reduced by ~10-fold (Fig. 1), with surprisingly little impact on viability (9), making *bamA101* a convenient background for the characterization of Bam-limited OMP assembly *in vivo*. However, no analogous mutants that would aid in determining the consequences of reducing levels of the essential lipoprotein BamD have been described.

Mutant strains of *E. coli* in which the concentration of BamD at the OM is sufficiently reduced to compromise Bam function without affecting the sequence of the mature OM-localized protein were obtained in a screen for recessive *bamD* mutations. This was achieved through PCR-based random mutagenesis of a low-copy-number plasmid bearing the *bamD* open reading frame (ORF) and 5′ untranslated region (UTR) (pZS21::*bamD*) (Table 1). A pool of mutated plasmid (pZS21::*bamD**) was introduced into JCM290, a strain in which the expression of chromosomal *bamD* is regulated by the arabinose-inducible P_{araBAD} promoter; in this background, only the plasmid-borne *bamD* allele is expressed when arabinose is excluded from the growth medium. We then screened the resulting transformants for growth on LB plates seeded with SDS-EDTA in the absence or presence of arabinose, expecting that a significant reduction in the expression of *bamD* from pZS21::*bamD* would, like previously described loss-of-function *bamD* mutations (2), cause increased OM permeability (as indicated by the failure to grow in the presence of SDS-EDTA). pZS21::*bamD** plasmid was purified from recessive SDS-EDTA^s mutants (i.e., transformants that exhibit sensitivity only in the absence of arabinose) and sequenced to determine the specific genetic lesion causing the OM permeability phenotype in each case.

Of the mutant plasmids that were sequenced, two carried mutations that do not lie within the region encoding the mature protein but would be expected to influence BamD levels: one muta-

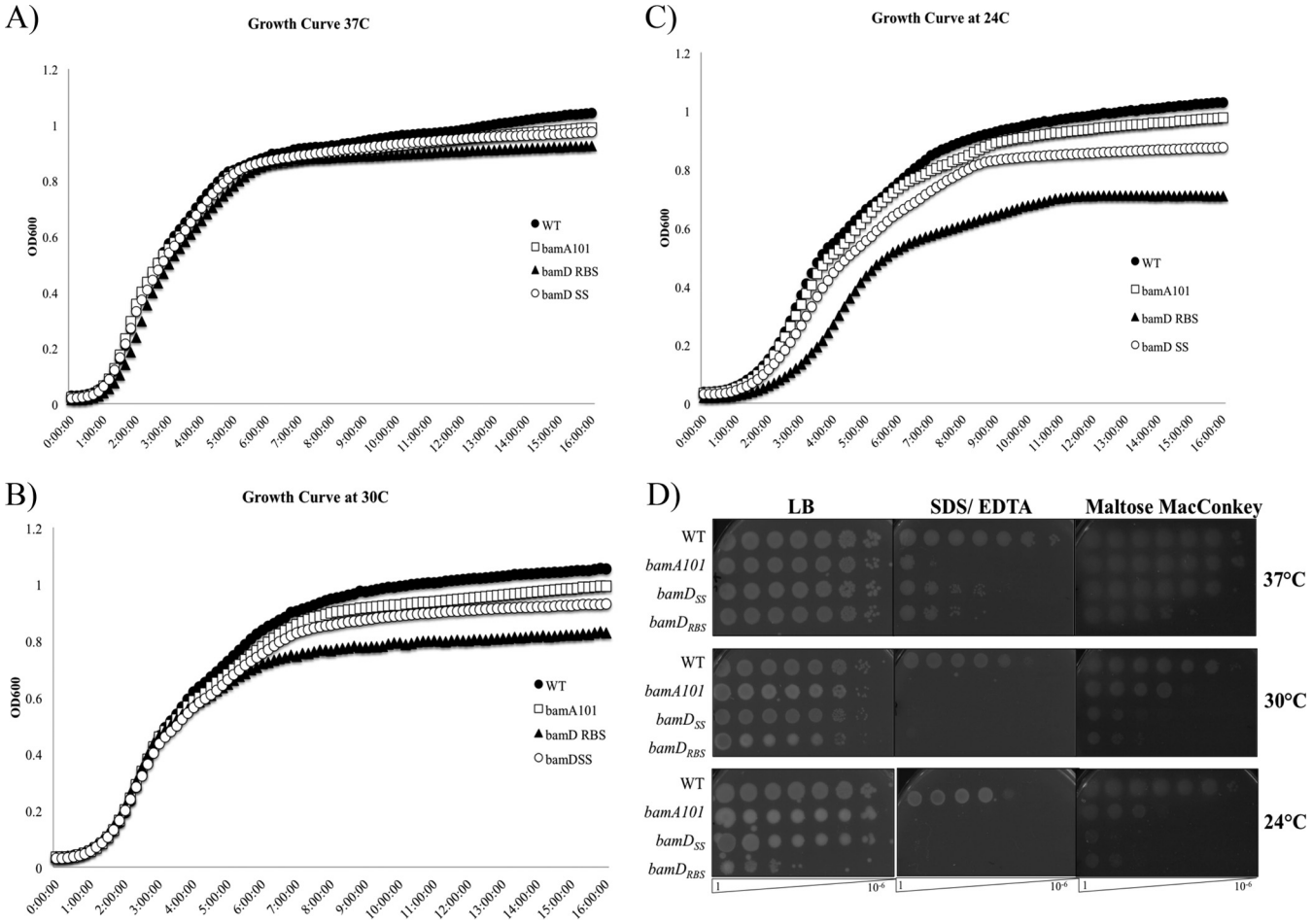

FIG 2 Effects of *bamA101*, $bamD_{RBS}$, and $bamD_{SS}$ on growth and OM permeability. (A to C) Growth of strains in LB over a 16-h period with OD_{600} measurements taken every 10 min. Growth curves were determined in triplicate, and a representative curve is shown here. (D) EOP assays with 10-fold serial dilutions were performed on LB, LB supplemented with 0.5% SDS–0.8 mM EDTA, or maltose-MacConkey agar with wild-type (WT; MC4100 Ara^r), *bamA101*, $bamD_{SS}$, and $bamD_{RBS}$ strains at 37°C, 30°C, and 24°C.

tion affects the sequence of the predicted Shine-Dalgarno sequence upstream of the translation start site (*bamD*-4C→T, herein referred to as $bamD_{RBS}$ [RBS, ribosome binding site]), and the other leads to alteration of the hydrophobic N-terminal signal sequence (SS) that is required for the targeting of BamD to the periplasm (*bamD* L13P, herein referred to as $bamD_{SS}$). These mutations presumably impact the translational efficiency or stability of the *bamD* transcript and the secretory protein (Sec)-dependent secretion of full-length BamD precursor, respectively.

Because neither mutation maps to the sequence encoding the mature processed BamD lipoprotein, we expected that the OM permeability phenotype caused by these *bamD* mutations is a consequence of reduced BamD levels. To quantify the reduction in BamD levels resulting from the $bamD_{RBS}$ and $bamD_{SS}$ mutations, we performed Western blotting using whole-cell extracts of each mutant strain. We found that these mutations indeed cause decreases in BamD levels; specifically, the BamD down mutations lower levels of BamD approximately 10-fold ($bamD_{SS}$) or >25-fold ($bamD_{RBS}$) relative to the wild type (Fig. 1). Importantly, reducing BamD synthesis or secretion does not impact BamA levels (Fig. 1), nor do levels of other accessory lipoproteins in the Bam complex change in the presence of any of the aforementioned mutations (see Fig. S1 in the supplemental material), which is expected, as OM lipoprotein targeting is a Bam-independent process (10).

Characterizing mutations that lower the essential components of the Bam complex. Given that these mutations significantly lower the levels of functional Bam complexes, we wanted to determine if these mutations affected cell viability or growth rate. Growth at the physiological temperature of 37°C was unimpaired for all three mutants, with the $bamD_{RBS}$ mutant showing only a very minor reduction in final population density relative to a wild-type strain (Fig. 2A). When the temperature was lowered to 30°C, there was a minor but detectable impact on the growth rates of all three strains in late-exponential phase, with the growth defect in the $bamD_{RBS}$ mutant being the most severe (Fig. 2B). When growth was monitored at 24°C, we observed a marked decrease in the growth rate and final density of the $bamD_{RBS}$ strain (Fig. 2C). These results indicate that the essential OMP assembly factors BamA and BamD are limiting factors for growth when levels are reduced ≥10-fold, particularly at reduced temperatures.

To more thoroughly assess the extent to which *bamA101* and the *bamD* down mutations lead to increased OM permeability, we performed assays of efficiencies of plating (EOPs) in the presence

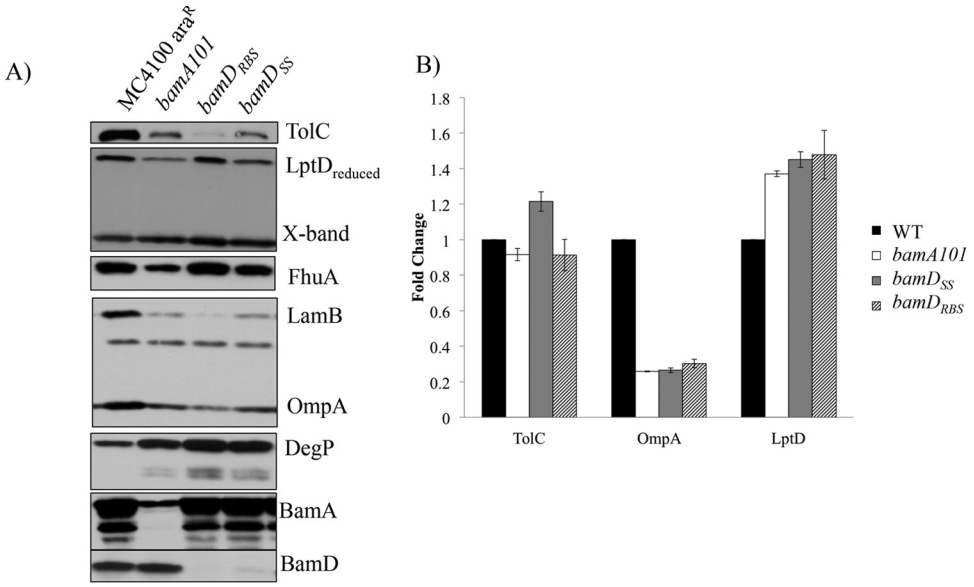

FIG 3 Effects of *bamA101*, $bamD_{RBS}$, and $bamD_{SS}$ on the assembly of model OMPs. (A) Strains were grown in LB at 37°C to an OD_{600} of ~0.4 to 0.55, and whole-cell lysates were harvested and subjected to SDS-PAGE analysis. The levels of TolC, LptD, FhuA, LamB, OmpA, and DegP, as well as BamA and BamD, were assayed via Western blotting. X-band is a cross-reacting band of approximately 55 kDa, shown here as a loading control. (B) Strains were grown in LB at 37°C to an OD_{600} of ~0.4 to 0.55 and harvested for RNA extraction and subsequent synthesis of a cDNA library. The fold changes in transcription for *tolC*, *ompA*, and *lptD* were determined for the *bamA101* strain and the *bamD* down mutants compared to the wild type. Values are the means ± the standard deviations.

of bile salts (a selective component of MacConkey agar) and an anionic detergent. At 37°C, the *bamA101*, $bamD_{SS}$, and $bamD_{RBS}$ mutants grow well on LB, but on LB supplemented with the detergent SDS, all three strains exhibit a viability defect, with EOPs reduced by at least four to five orders of magnitude (Fig. 2D). Consistent with the slight cold sensitivity observed during growth in rich liquid media, sensitivity to detergent is exacerbated at lower temperatures, with increased sensitivity to SDS observed at 30°C and 24°C (Fig. 2D). The same trend holds for growth on bile salts: the RBS mutant $bamD_{RBS}$ strain displays a slight sensitivity to bile salts at 37°C, and this defect is greatly increased at 30°C and 24°C (Fig. 2D). *bamA101* and $bamD_{SS}$ strains are also sensitive to bile salts at the lower temperatures of 30°C and 24°C (Fig. 2D). This increase in OM permeability suggests that the barrier function and OMP assembly may be impaired in *bamA101*, $bamD_{RBS}$, and $bamD_{SS}$ mutants.

Substrates affected by lowering the number of Bam complexes in the cell. The OM permeability defects observed for mutants expressing *bamA101* and both *bamD* down mutations suggest that OMP assembly may be impaired in these mutants. To address this hypothesis, and to determine what classes of substrates might be affected, we examined the impact of *bamA101* and the *bamD* down mutants on various Bam substrates. Since unassembled OMPs are rapidly degraded, we determined the effect of these mutations on protein levels, expecting that a reduction in whole-cell OMP levels is an indicator of failed assembly at the OM.

Comparing the effects of *bamA101*, $bamD_{RBS}$, and $bamD_{SS}$ on an array of OMP substrates revealed that the three mutations similarly impact the steady-state levels of model OMPs (Fig. 3A). In particular, mutants expressing *bamA101* and $bamD_{SS}$, which both lower the level of an essential Bam complex component ~10-fold, appear to exhibit very similar defects, while the OMP assembly defects in the mutant expressing $bamD_{RBS}$, which lowers BamD levels >25-fold, are more pronounced. An examination of the protein levels in *bamA101*, $bamD_{RBS}$, and $bamD_{SS}$ mutants during mid-log phase (OD_{600}, ~0.4 to 0.55) revealed a significant decrease in the levels of LamB and OmpA, two abundant OMPs whose assemblies are known to be compromised following mutation of Bam components or periplasmic chaperones (Fig. 3A). Levels of the export channel TolC are also reduced in all three strains, which is noteworthy in light of the fact that these are the first reported mutations that have been shown to compromise TolC assembly *in vivo*. TolC does not require any of the known OMP chaperones for assembly, and null mutations in *bamB*, which are known to compromise the assembly of porins, instead lead to a measurable increase in the levels of TolC in the OM (4, 11, 12). Levels of LptD are also impacted to various degrees in all three mutants, and FhuA assembly is moderately affected in the *bamA101* mutant (Fig. 3A). Besides OmpA, FhuA, and LptD (which forms a heterodimer with LptE), OMPs that are strongly impacted by *bamA101* are oligomeric proteins. This appears to be true not just for abundant multimers, like LamB, but also for lower-abundance multimers, like TolC. OMPs that are highly dependent on SurA for assembly, LptD and FhuA, also appear to be moderately affected by reductions in the number of functional Bam complexes in the cell, although perhaps to different degrees (Fig. 3A). The levels of substrates were also assayed in late-log phase for mutants expressing *bamA101* and the *bamD* down mutations, with similar results. Levels of LamB, TolC, and LptD were strongly affected in all three mutants; however, levels of OmpA were no longer impacted (see Fig. S2 in the supplemental material).

The σ^E stress response is activated in *bamA101* and *bamD* down mutants. Activation of the σ^E envelope stress response is known to upregulate small RNAs (sRNAs) that in turn downregulate the expression of some substrates to alleviate the load on the

biogenesis complex (13). To determine which of these decreases in protein levels were due to defective assembly and subsequent proteolysis and which were caused by repression of synthesis, we performed quantitative PCR (qPCR). The levels of *tolC* and *lptD* mRNA were unaffected in *bamA101*, *bamD*$_{RBS}$, and *bamD*$_{SS}$ mutants; similarly, *fhuA* has been shown to behave as *lptD*, and transcription is unaffected by activation of the σ^E stress response (14). However, levels of *ompA* mRNA were severely reduced in all three strains, measuring less than a third of those of the wild type (Fig. 3B). This suggests that TolC and LptD are truly defective for assembly in *bamA101*, *bamD*$_{RBS}$, and *bamD*$_{SS}$ mutants and that OmpA protein levels are low in the mutant backgrounds due to decreased expression rather than an assembly defect.

The decrease in *ompA* mRNA levels suggests that the σ^E stress response is induced in these strains likely because unfolded OMPs are accumulating in the periplasm. To assess if the σ^E stress response was activated in our strains with reduced BamA or BamD, we examined levels of the chaperone/protease DegP, which is strongly induced following σ^E activation. Western blot analysis of DegP levels in the *bamA101*, *bamD*$_{RBS}$, and *bamD*$_{SS}$ strains shows much higher levels than those in the wild type for all three mutants and reveals the products of DegP autoproteolysis (indicated by the doublet below the primary DegP band in immunoblots), indicating that DegP is present and acting as a protease (Fig. 3A). qRT-PCR analysis of *rpoE*, the gene that encodes the σ^E alternate sigma factor and is itself positively regulated by this product, also reveals that *rpoE* transcript abundance is increased by approximately 3-fold in the *bamA101*, *bamD*$_{RBS}$, and *bamD*$_{SS}$ strain backgrounds (see Fig. S3 in the supplemental material). Taken together, these results indicate that the σ^E response is strongly induced in all three mutants.

***ompA* and *lamB* constructs that are unregulated by the σ^E stress response.** We wished to assess whether the assembly of LamB or OmpA was affected by the mutations that limit the essential Bam components without the potential confounding factor of σ^E-dependent downregulation of gene expression. To address this question, we created chromosomal fusions of either *lamB* or *ompA* to the promoter and signal sequence of *tolC*, a gene whose expression is not affected by *bamA101* or either *bamD* down mutant (see Fig. 3B). Among the strains with the *tolC* promoter and signal sequence fused to *lamB* lacking its native signal sequence and promoter, LamB still exhibited assembly defects in the *bamA101*, *bamD*$_{RBS}$, and *bamD*$_{SS}$ strains, suggesting that the lowered levels of LamB in these mutants are due to a bona fide assembly defect rather than a decrease in transcription (Fig. 4A). In contrast, the *bamA101*, *bamD*$_{RBS}$, and *bamD*$_{SS}$ strains with the *tolC* promoter and signal sequence fused to *ompA* did not have decreased levels of OmpA compared to the wild type (Fig. 4B). This suggests that any decrease observed in OmpA levels in strains with lowered Bam components was due to a concomitant decrease in gene expression caused by activation of the σ^E stress response.

Combinatorial effects of *bamA101* and *bamD* down mutations. BamA and BamD are both essential for OMP assembly and are present in a 1:1 ratio within the complex (12), suggesting that incomplete Bam complexes lacking either BamA or BamD are nonfunctional and prompting the prediction that a simultaneous reduction in levels of BamA and BamD would not cause additional defects beyond those observed when only one of these two factors becomes limiting. Indeed, the defects in the *bamA101* *bamD*$_{SS}$ double mutant, in which both mutations lower levels of BamA or BamD 10-fold, appear to be no worse than those in either of the single mutants (Fig. 5). The OMP assembly defect of the *bamA101* *bamD*$_{SS}$ double mutant appears to be consistent with that of the single mutant with the stronger assembly defect (Fig. 5A). In the *bamA101* *bamD*$_{SS}$ double mutant, levels of TolC, LamB, and LptD are decreased to about the same extent as those in the more severe single mutant. Similarly, the OM permeability defects of the *bamA101* *bamD*$_{SS}$ double mutant on maltose-MacConkey agar are no worse than those of the single mutant with the more severe mutation (Fig. 5B). Taken together, the results suggest that the phenotypes of the mutants expressing *bamA101* and the *bamD* down mutations individually are due to the lowered number of functional Bam complexes present in the cell, since the double mutations do not exacerbate any of the single-mutant OMP phenotypes.

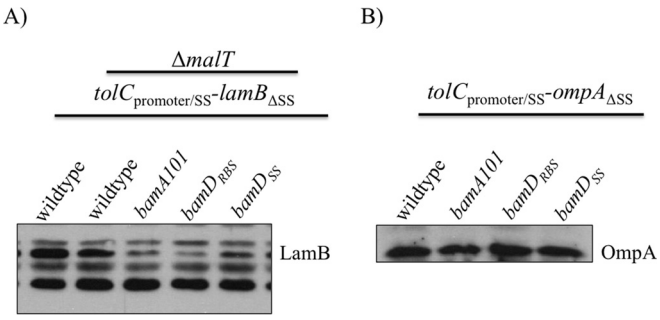

FIG 4 Assembly of fusion proteins specified by *tolC*$_{PandSS}$-*lamB*$_{\Delta SS}$ and *tolC*$_{PandSS}$-*ompA*$_{\Delta SS}$, which are not regulated by σ^E. Strains were grown in LB at 37°C to an OD_{600} of ~0.6 to 0.85, and whole-cell lysates were harvested and subjected to SDS-PAGE analysis. Strains have *bamA101*, *bamD*$_{SS}$, or *bamD*$_{RBS}$, combined with a fusion of the promoter and upstream intragenic region and signal sequence of *tolC* with either *lamB* or *ompA* without the signal sequence. The resulting construct encodes a wild-type mature LamB or OmpA protein that cannot be regulated by σ^E, as the *tolC* promoter and signal sequence are not impacted by σ^E. (A) Levels of LamB in the wild-type, *bamA101* mutant, *bamD*$_{SS}$ mutant, and *bamD*$_{RBS}$ mutant strains were assessed via Western blotting. (B) Levels of OmpA in the wild-type, *bamA101*, *bamD*$_{SS}$, and *bamD*$_{RBS}$ strains were assessed via Western blotting.

The *bamA101* *bamD*$_{RBS}$ double mutant can be constructed, but it is extremely sick and exhibits frequent suppressor outgrowth. Accordingly, we have not done additional experiments with this double mutant. This result suggests that the levels of functional Bam complex are lower in the double mutant than in a strain carrying the *bamD*$_{RBS}$ mutation alone. For reasons described in the Discussion, we feel this combinatorial effect reflects the physical properties of the OM.

DISCUSSION

The Bam complex is a heteropentameric complex that exists in a 1:1:1:1:1 ratio (12); thus, we expected that reducing the intracellular concentration of one or the other essential component would have a similar effect on biogenesis, as mutations in either would lower the number of functional Bam complexes in the cell by the same degree. Indeed, upon depletion of either BamA or BamD, OMP assembly eventually stalls completely, and the strains become nonviable when levels of BamA or BamD are sufficiently reduced (1, 2, 5). The well-characterized transposon mutant *bamA101*, which lowers the expression of BamA by 10-fold (9), has surprisingly little effect on the efficiency of OMP assembly in particular and cell viability in general, despite the extent to which

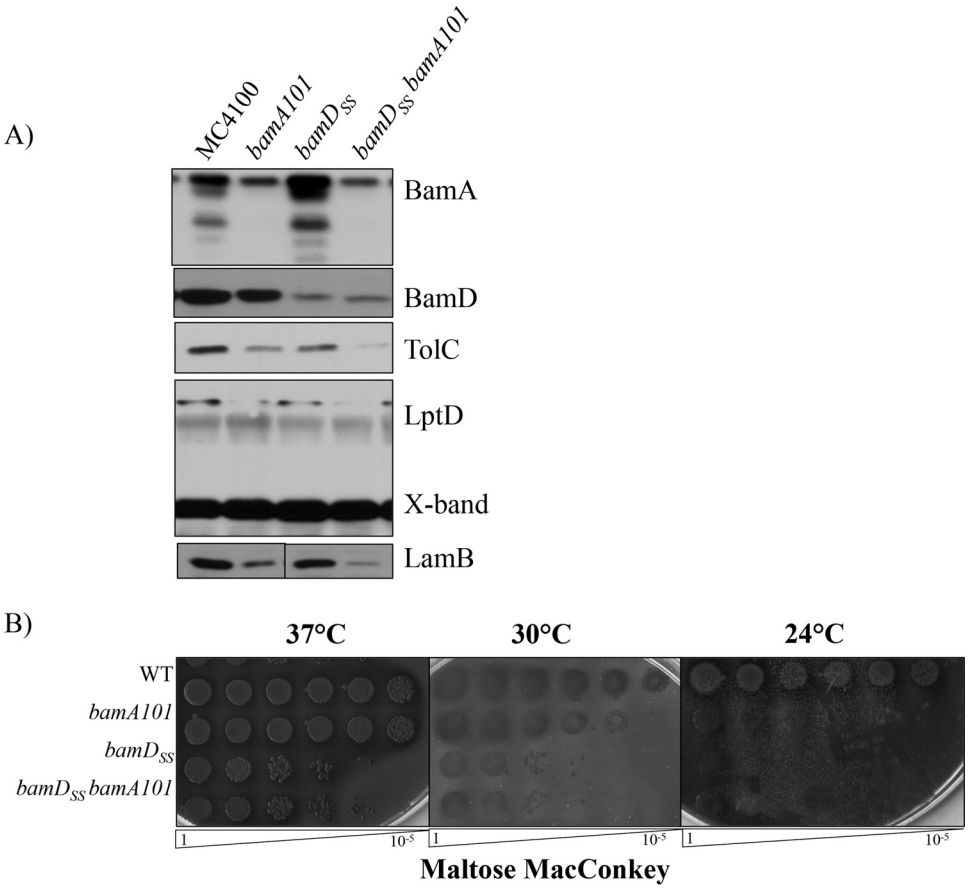

FIG 5 Effects of $bamD_{SS}$ and *bamA101* double mutation are not combinatorial. (A) Strains were grown in LB at 37°C to an OD_{600} of 0.700 to 0.850, and whole-cell lysates were analyzed by SDS-PAGE for the levels of model OMPs. X-band is a cross-reacting band of approximately 55 kDa, shown here as a loading control. (B) EOP assays with 10-fold serial dilutions were performed with *bamA101*, $bamD_{SS}$, and $bamD_{SS}$ *bamA101* strains on maltose-MacConkey agar to assess the OM permeability defect of the double mutant compared to the single mutants at 37°C, 30°C, and 24°C.

BamA levels are decreased. In this work, we identified $bamD_{SS}$, a mutation in the signal sequence of *bamD* that lowers BamD protein levels approximately 10-fold relative to the wild type. In keeping with our prediction, this *bamD* mutant displays very similar OMP assembly and permeability defects to those observed in the *bamA101* mutant. This indicates that lowering the level of Bam holocomplexes in the cell has the same effect on biogenesis, regardless of which essential component of the Bam complex is limiting. Strikingly, levels of BamD can be lowered >25-fold below wild-type levels without significantly compromising growth or viability. Although the $bamD_{RBS}$ mutant has stronger biogenesis defects than the *bamA101* or $bamD_{SS}$ mutant, decreased viability is observed only when mutant strains are grown at subphysiological temperatures, suggesting that the levels of functional Bam complex can be severely reduced before the cell becomes nonviable, at least under laboratory conditions.

As hydrophobic interactions are weakened at low temperatures, the growth defect at low temperatures might indicate that hydrophobic interactions may be important for interaction of the Bam complex with substrate or for the interaction of the Bam complex members with one another. Reductions in temperature will also further decrease the fluidity of the characteristically rigid OM (15, 16), which could exacerbate assembly defects of certain classes of proteins. The kinetics of OMP folding into a membrane is strongly influenced by the physical properties of the membrane, and temperature can strongly affect the biophysical properties of the OM, leading to changes in the rate of OMP folding with temperature (17–20). Alternatively, or in addition, it seems likely that there is an increased requirement for periplasmic proteolysis in the BamA/BamD down mutant strains. Indeed, we have been unable to introduce null mutations of the important periplasmic quality control factor *degP* into the strains carrying the *bamA* and *bamD* down mutations. Since DegP exhibits significantly lower protease activity at low temperatures (21), this might lead to a toxic buildup of misfolded proteins and contribute significantly to the cold sensitivity of the *bamA* and *bamD* down mutant strains.

Lowering both BamA and BamD 10-fold by combining *bamA101* and $bamD_{SS}$ does not lead to an additive increase in OMP assembly or OM permeability defects. This is consistent with the presence of BamA and BamD in a 1:1 ratio in the Bam complex and with the finding that a full complex is required for assembly of substrates. However, when BamA levels are lowered 10-fold and the level of BamD is lowered >25-fold via $bamD_{RBS}$, synergistic defects are observed. This combinatorial effect may be due to the distinct assembly pathways for BamA and BamD. These two essential factors are targeted to the OM by independent transport pathways and only form functional BamAD complexes after OM integration. Because the rate of BamAD complex formation is

ultimately a function of BamA and BamD concentrations at the OM, we suggest that the combinatorial reduction in BamA and BamD levels further reduces the effective concentration of functional BamAD-containing complexes and restricts the growth of the cell. This effect is likely compounded by the limited diffusion of OM-integral BamA: although the BamD lipoprotein can freely diffuse in two dimensions within the phospholipid-containing inner leaflet of the OM, the β-barrel domain of BamA likely exhibits restricted mobility within the OM due to the relatively low fluidity of the lipopolysaccharide (LPS)-containing outer leaflet (15) and cannot effectively engage in a diffusional search for binding partners. We suggest, therefore, that the synergism observed when both BamA and BamD levels are reduced reflects insufficient formation of functional Bam complexes.

Previous work has revealed two classes of Bam substrates. Proteins, such as LptD and FhuA, which are large OMPs with complex assembly pathways that are heavily dependent on the chaperone SurA and exhibit an unusual requirement for Skp and FkpA (3), comprise one class. Perhaps unsurprisingly, LptD and FhuA are both somewhat affected by reductions in the essential components of the Bam complex. The fact that the effect on these complicated low-abundance substrates is comparatively minor might suggest that this class of proteins is given preference by the Bam machine for assembly.

A second class contains smaller and much-more-abundant substrates, such as the porins, which exhibit lower dependence on SurA but are strongly dependent on BamB (4, 22, 23). This work elucidates differences in the assembly requirements for multimeric and monomeric high-abundance substrates that cannot be attributed to changes in gene expression, thus revealing a third class, multimeric substrates, such as LamB, that are affected by decreasing one of the two essential components of the Bam complex.

Abundant multimeric OMPs are not the only family of proteins that exhibit assembly defects when the number of Bam complexes in the cell is reduced: the assembly of the multimer TolC is also compromised in Bam-limited backgrounds. TolC is not a high-abundance substrate (11), nor does it require BamB or known periplasmic chaperones for assembly (as do the porins), and although it is an atypical substrate comprising a single β-barrel formed from three identical subunits, it does not exhibit the complex assembly requirements of LptD and FhuA. The TolC assembly defect observed in Bam-limited strains is particularly striking, as the assembly of this efflux protein is unperturbed by null mutations in periplasmic chaperones or nonessential Bam complex members (4, 11). Thus, the effects of these reductions in BamA or BamD do not correlate with simple or complex substrates; rather, we found that *bamA101*, $bamD_{SS}$, and $bamD_{RBS}$ led to a marked decrease in the levels of multimeric proteins of various types.

The assembly defect of oligomeric proteins in *bamA101* and the BamD down mutants is intriguing when considered together with the growth defects of the BamD down mutant strains at low temperatures. Because the relatively rigid OM becomes even less fluid at lower temperatures, the kinetic barrier to both OMP integration and multimer assembly is likely raised as the temperature decreases. Proposed models for Bam function imply that a single Bam complex assembles only one monomer at a time (24, 25), raising questions about how multimers are assembled in the nonfluid OM. The OMP assembly pathway as it pertains to multimerization of oligomers, and how the Bam complex participates, is still not fully elucidated (5, 26, 27). It has been suggested that some multimeric OMPs, such as PhoE and TolC, do not proceed through a folded monomeric intermediate form, but rather, trimerization occurs concomitantly with folding and assembly and may help precipitate folding in the first place (28, 29), while other OMPs, like LamB, may proceed though folded monomeric intermediates that then trimerize upon insertion in the OM (30). If multimeric OMPs (e.g., LamB) indeed multimerize before or during OM integration, this would necessitate a mechanism by which individual neighboring Bam complexes could act coordinately to simultaneously integrate multiple monomers of compatible OMP species. The specific susceptibility of multimers to lowered quantities of Bam machines in the cell may indicate that multiple Bam complexes are needed to efficiently assemble multimeric proteins into the OM. The fact that OMPs appear not to exist in a sea of LPS, but rather in OMP islands, may provide a key to understanding the complex problem of multimer assembly in a nonfluid environment (31).

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REFERENCES

1. **Wu T, Malinverni J, Ruiz N, Kim S, Silhavy TJ, Kahne D.** 2005. Identification of a multicomponent complex required for outer membrane biogenesis in *Escherichia coli*. Cell **121:**235–245. http://dx.doi.org/10.1016/j.cell.2005.02.015.
2. **Malinverni JC, Werner J, Kim S, Sklar JG, Kahne D, Misra R, Silhavy TJ.** 2006. YfiO stabilizes the YaeT complex and is essential for outer membrane protein assembly in *Escherichia coli*. Mol Microbiol **61:**151–164. http://dx.doi.org/10.1111/j.1365-2958.2006.05211.x.
3. **Schwalm J, Mahoney TF, Soltes GR, Silhavy TJ.** 2013. Role for Skp in LptD assembly in *Escherichia coli*. J Bacteriol **195:**3734–3742. http://dx.doi.org/10.1128/JB.00431-13.
4. **Charlson ES, Werner JN, Misra R.** 2006. Differential effects of *yfgL* mutation on *Escherichia coli* outer membrane proteins and lipopolysaccharide. J Bacteriol **188:**7186–7194. http://dx.doi.org/10.1128/JB.00571-06.
5. **Werner J, Misra R.** 2005. YaeT (Omp85) affects the assembly of lipid-dependent and lipid-independent outer membrane proteins of *Escherichia coli*. Mol Microbiol **57:**1450–1459. http://dx.doi.org/10.1111/j.1365-2958.2005.04775.x.
6. **Silhavy TJ, Berman ML, Enquist LW.** 1984. Experiments with gene fusions. Cold Spring Harbor Laboratory Press, Plainview, NY.
7. **Datsenko KA, Wanner BL.** 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc Natl Acad Sci U S A **97:**6640–6645. http://dx.doi.org/10.1073/pnas.120163297.
8. **Ruiz N, Chng S-S, Hiniker A, Kahne D, Silhavy TJ.** 2010. Nonconsecutive disulfide bond formation in an essential integral outer membrane protein. Proc Natl Acad Sci U S A **107:**12245–12250. http://dx.doi.org/10.1073/pnas.1007319107.
9. **Aoki SK, Malinverni JC, Jacoby K, Thomas B, Pamma R, Trinh BN, Remers S, Webb J, Braaten BA, Silhavy TJ, Low DA.** 2008. Contact-dependent growth inhibition requires the essential outer membrane protein BamA (YaeT) as the receptor and the inner membrane transport

protein AcrB. Mol Microbiol **70:**323–340. http://dx.doi.org/10.1111/j.1365-2958.2008.06404.x.
10. **Okuda S, Tokuda H.** 2011. Lipoprotein sorting in bacteria. Annu Rev Microbiol **65:**239–259. http://dx.doi.org/10.1146/annurev-micro-090110-102859.
11. **Werner J, Augustus AM, Misra R.** 2003. Assembly of TolC, a structurally unique and multifunctional outer membrane protein of *Escherichia coli* K-12. J Bacteriol **185:**6540–6547. http://dx.doi.org/10.1128/JB.185.22.6540-6547.2003.
12. **Hagan CL, Kim S, Kahne D.** 2010. Reconstitution of outer membrane protein assembly from purified components. Science **328:**890–892. http://dx.doi.org/10.1126/science.1188919.
13. **Rhodius VA, Suh WC, Nonaka G, West J, Gross CA.** 2006. Conserved and variable functions of the sigma(E) stress response in related genomes. PLoS Biol **4:**e2.
14. **Vertommen D, Ruiz N, Leverrier P, Silhavy TJ, Collet J-F.** 2009. Characterization of the role of the *Escherichia coli* periplasmic chaperone SurA using differential proteomics. Proteomics **9:**2432–2443. http://dx.doi.org/10.1002/pmic.200800794.
15. **Nikaido H.** 2003. Molecular basis of bacterial outer membrane permeability revisited. Microbiol Mol Biol Rev **67:**593–656. http://dx.doi.org/10.1128/MMBR.67.4.593-656.2003.
16. **Ricci DP, Silhavy TJ.** 2012. The Bam machine: a molecular cooper. Biochim Biophys Acta **1818:**1067–1084. http://dx.doi.org/10.1016/j.bbamem.2011.08.020.
17. **Danoff EJ, Fleming KG.** 2015. Membrane defects accelerate outer membrane β-barrel protein folding. Biochemistry **54:**97–99. http://dx.doi.org/10.1021/bi501443p.
18. **Kleinschmidt JH.** 2006. Folding kinetics of the outer membrane proteins OmpA and FomA into phospholipid bilayers. Chem Phys Lipids **141:**30–47. http://dx.doi.org/10.1016/j.chemphyslip.2006.02.004.
19. **Marsh D, Shanmugavadivu B, Kleinschmidt JH.** 2006. Membrane elastic fluctuations and the insertion and tilt of beta-barrel proteins. Biophys J **91:**227–232. http://dx.doi.org/10.1529/biophysj.105.079004.
20. **Burgess NK, Dao TP, Stanley AM, Fleming KG.** 2008. Beta-barrel proteins that reside in the *Escherichia coli* outer membrane *in vivo* demonstrate varied folding behavior *in vitro*. J Biol Chem **283:**26748–26758. http://dx.doi.org/10.1074/jbc.M802754200.
21. **Spiess C, Beil A, Ehrmann M.** 1999. A temperature-dependent switch from chaperone to protease in a widely conserved heat shock protein. Cell **97:**339–347. http://dx.doi.org/10.1016/S0092-8674(00)80743-6.
22. **Sklar JG, Wu T, Gronenberg LS, Malinverni JC, Kahne D, Silhavy TJ.** 2007. Lipoprotein SmpA is a component of the YaeT complex that assembles outer membrane proteins in *Escherichia coli*. Proc Natl Acad Sci U S A **104:**6400–6405. http://dx.doi.org/10.1073/pnas.0701579104.
23. **Vuong P, Bennion D, Mantei J, Frost D, Misra R.** 2008. Analysis of YfgL and YaeT interactions through bioinformatics, mutagenesis, and biochemistry. J Bacteriol **190:**1507–1517. http://dx.doi.org/10.1128/JB.01477-07.
24. **Stegmeier JF, Andersen C.** 2006. Characterization of pores formed by YaeT (Omp85) from *Escherichia coli*. J Biochem **140:**275–283. http://dx.doi.org/10.1093/jb/mvj147.
25. **Noinaj N, Kuszak AJ, Balusek C, Gumbart JC, Buchanan SK.** 2014. Lateral opening and exit pore formation are required for BamA function. Structure **22:**1055–1062. http://dx.doi.org/10.1016/j.str.2014.05.008.
26. **Hagan CL, Silhavy TJ, Kahne D.** 2011. β-Barrel membrane protein assembly by the Bam complex. Annu Rev Biochem **80:**189–210. http://dx.doi.org/10.1146/annurev-biochem-061408-144611.
27. **Knowles TJ, Scott-Tucker A, Overduin M, Henderson IR.** 2009. Membrane protein architects: the role of the BAM complex in outer membrane protein assembly. Nat Rev Microbiol **7:**206–214. http://dx.doi.org/10.1038/nrmicro2069.
28. **Masi M, Duret G, Delcour AH, Misra R.** 2009. Folding and trimerization of signal sequence-less mature TolC in the cytoplasm of *Escherichia coli*. Microbiology **155:**1847–1857. http://dx.doi.org/10.1099/mic.0.027219-0.
29. **Jansen C, Heutink M, Tommassen J, de Cock H.** 2000. The assembly pathway of outer membrane protein PhoE of *Escherichia coli*. Eur J Biochem **267:**3792–3800. http://dx.doi.org/10.1046/j.1432-1327.2000.01417.x.
30. **Ureta AR, Endres RG, Wingreen NS, Silhavy TJ.** 2007. Kinetic analysis of the assembly of the outer membrane protein LamB in *Escherichia coli* mutants each lacking a secretion or targeting factor in a different cellular compartment. J Bacteriol **189:**446–454. http://dx.doi.org/10.1128/JB.01103-06.
31. **Rassam P, Copeland NA, Birkholz O, Toth C, Chavent M, Duncan AL, Cross SJ, Housden NG, Kaminska R, Seger U, Quinn DM, Garrod TJ, Sansom MSP, Piehler J, Baumann CG, Kleanthous C.** 2015. Supramolecular assemblies underpin turnover of outer membrane proteins in bacteria. Nature **523:**333–336. http://dx.doi.org/10.1038/nature14461.
32. **Rigel NW, Schwalm J, Ricci DP, Silhavy TJ.** 2012. BamE modulates the *Escherichia coli* beta-barrel assembly machine component BamA. J Bacteriol **194:**1002–1008. http://dx.doi.org/10.1128/JB.06426-11.