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NOTES

The Essential β-Barrel Assembly Machinery Complex Components BamD and BamA Are Required for Autotransporter Biogenesis▽

Amanda E. Rossiter,[1]§ Denisse L. Leyton,[1]§ Karina Tveen-Jensen,[1] Douglas F. Browning,[1] Yanina Sevastsyanovich,[1] Timothy J. Knowles,[2] Katie B. Nichols,[3] Adam F. Cunningham,[1] Michael Overduin,[2] Mark A. Schembri,[3] and Ian R. Henderson[1]*

School of Immunity and Infection[1] and School of Cancer Sciences,[2] University of Birmingham, Birmingham, United Kingdom, and School of Chemistry and Molecular Biosciences, University of Queensland, Brisbane, Australia[3]

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Autotransporter biogenesis is dependent upon BamA, a central component of the β-barrel assembly machinery (BAM) complex. In this report, we detail the role of the other BAM components (BamB-E). We identify the importance of BamD in autotransporter biogenesis and show that BamB, BamC, and BamE are not required.

Gram-negative bacteria possess a double membrane barrier that is instrumental in protecting the bacteria against the external environment and maintaining viability (4, 24). However, this unique feature poses a formidable obstacle to both the insertion of proteins into the outer membrane and the secretion of proteins across it. Most integral outer membrane proteins (OMPs) form a β-barrel structure (38), and the recently described β-barrel assembly machinery (BAM) complex (40) has been implicated in the insertion and/or folding of nearly all known OMPs (7, 20, 39). BAM has now been well characterized as a hetero-oligomeric complex composed of five proteins, one integral β-barrel membrane protein termed BamA and four outer membrane-associated lipoproteins, BamB-E (22). BamA is the central component and forms a pore with a diameter of approximately 2.5 nm in the outer membrane (30). Although other components are crucial to the functional integrity of the complex (2, 19, 34, 40), only BamA and BamD are universally essential for both bacterial cell viability and OMP biogenesis (27). Why this is, however, remains unclear.

Autotransporters (ATs) represent a large family of secreted proteins that are widespread among Gram-negative bacteria (5, 14, 16). Their name is derived from the supposition that translocation across both membranes occurs independently of accessory factors and is attributable to their conserved architecture (11). Superficially, all ATs have a three-domain architecture with an N-terminal signal sequence that mediates inner membrane translocation, a central passenger domain representing the secreted effector protein, and a C-terminal β-barrel domain that inserts into the outer membrane and facilitates the translocation of the passenger domain into the external milieu (1, 6, 28). There is now a significant body of evidence demonstrating that the biogenesis of ATs requires BamA (18, 25, 31, 33), although how this occurs remains speculative. Furthermore, the precise role of the other BAM components is not known. In order to define their functions, we used Pet, an archetypal AT that is a functionally well-characterized cytotoxin from the human pathogen enteroaggregative *Escherichia coli* (EAEC) strain 042 (8), the Sat cytotoxin from uropathogenic *E. coli* (UPEC) strain CFT073 (9), and the phase-variable OMP antigen 43 (Ag43) from laboratory *E. coli* strain MG1655 (17, 36). This is the first report detailing the role of each component of the BAM complex in AT biogenesis.

BamA and BamD are required for AT biogenesis. We first examined the role of the essential BamA and BamD proteins in Pet biogenesis. Full-length Pet protein was expressed under the control of its native promoter in BamA (25) and BamD (27) depletion strains by transforming them with the *pet*-containing recombinant plasmids pCEFN1 (8) and pACYC184/*pet*, respectively. In the respective depletion strains, expression of *bamA* or *bamD* is under the control of the P_{BAD} promoter, such that expression is induced in the presence of arabinose but repressed in its absence. To construct pACYC184/*pet*, the *pet* gene, together with its native promoter, was PCR amplified from pCEFN1 using primers GGGATCCGGAAGACGGTTG TTGCG and CGGGATCCGGTTAGCTCTGAATTAAG and the product was cloned into the BamHI site of pACYC184. After growth under both replete (with arabinose) and depletion (without arabinose) conditions, the culture supernatant fractions were harvested from the BamA and BamD depletion strains. Secreted proteins were precipitated using trichloroacetic acid (26, 31), resolved by sodium dodecyl sulfate-polyacrylamide electrophoresis (SDS-PAGE), and detected by Coomassie staining (32). Analysis of proteins in whole-cell extracts followed established protocols (19). Results in Fig. 1 illustrate that when both BamA and BamD are depleted from the cells, secretion of Pet into the culture medium is severely diminished. These results confirm previous

* Corresponding author. Mailing address: School of Immunity and Infection, University of Birmingham, Birmingham B15 2TT, United Kingdom. Phone: 44 121 4144368. Fax: 44 121 4143599. E-mail: i.r.henderson@bham.ac.uk.
§ These authors contributed equally.
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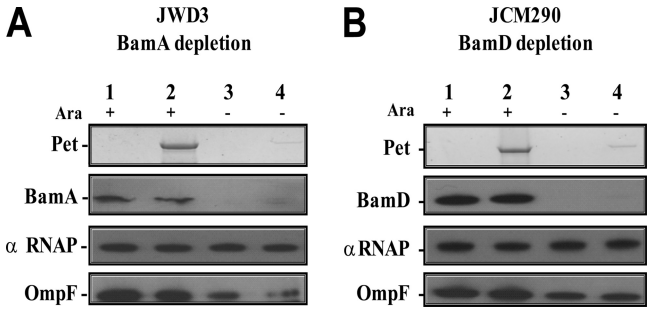

FIG. 1. BamA and BamD are required for Pet secretion. Shown are Coomassie blue-stained SDS-PAGE of trichloroacetic acid-precipitated supernatant proteins and Western blot analysis of whole-cell extracts from cultures of BamA depletion strain *E. coli* JWD3 (A) and BamD depletion strain *E. coli* JCM290 (B). In panel A, *E. coli* JWD3, carrying either the empty pSPORT1 vector (lanes 1 and 3) or pCEFN1 (lanes 2 and 4), was grown in LB medium containing 100 μg/ml ampicillin supplemented with either 0.2% L-arabinose (Ara) (+) or 0.2% D-fructose (−). In panel B, *E. coli* JCM290, carrying either pACYC184/*pet* (lanes 2 and 4) or the empty vector, was grown in LB medium containing 30 μg/ml chloramphenicol supplemented with either 0.05% L-arabinose (+) or 0.05% D-fructose (−). For both panels A and B, overnight cultures were diluted into a volume of 50 ml (OD_{600} = 0.025) and grown at 37°C with shaking for 3 h. Cultures were harvested, and supernatant proteins were precipitated with 10% trichloroacetic acid. BamA, BamD, and OmpF were detected using antiserum raised in a rabbit, and the α subunit of RNA polymerase (αRNAP) was detected using mouse monoclonal antibodies (Neoclone). Blots were developed using the ECL Plus Western blotting detection system (GE Healthcare). Like OmpF levels, under BamA and BamD depletion conditions, Pet levels are severely diminished whereas the levels of RNAP remain constant.

observations that BamA is required for AT biogenesis and demonstrate for the first time an essential role for BamD in AT biogenesis.

BamB, BamC, and BamE are not required for AT biogenesis. While BamA has been implicated in the biogenesis of all examined ATs (18, 33), the roles of the nonessential components of the BAM complex (BamB, BamC, and BamE) have not yet been fully investigated. In order to determine the role of these accessory factors in AT biogenesis, *bamB*, *bamC*, and *bamE* deletion mutants were constructed in laboratory strain *E. coli* HB101 and wild-type UPEC strain CFT073 using the λ Red system (3). In each mutant, the absence of the relevant endogenous protein in whole-cell lysates was confirmed by Western immunoblotting (Fig. 2). Anti-BamE antibodies were previously described (21, 23); to raise antibodies to BamB and BamC, constructs encoding these proteins but lacking their acylation site were synthesized *de novo* and cloned into the vector pET22b$^+$ (Genscript). Proteins were overexpressed and purified from the periplasm of *E. coli* strain BL21(DE3) (Novagen) and purified by nickel affinity chromatography. These proteins were used to immunize rabbits using a standard 3-month immunization program (Eurogentec), and the antibodies were purified by affinity purification. For analysis of Pet biogenesis, *E. coli* HB101 and its mutant derivatives were transformed with pCEFN1 and the empty vector pSPORT1 (8). Overnight cultures were diluted 1:100 into fresh medium and grown to an optical density at 600 nm (OD_{600}) of 1.0. The cells were pelleted, and the supernatant and outer membrane

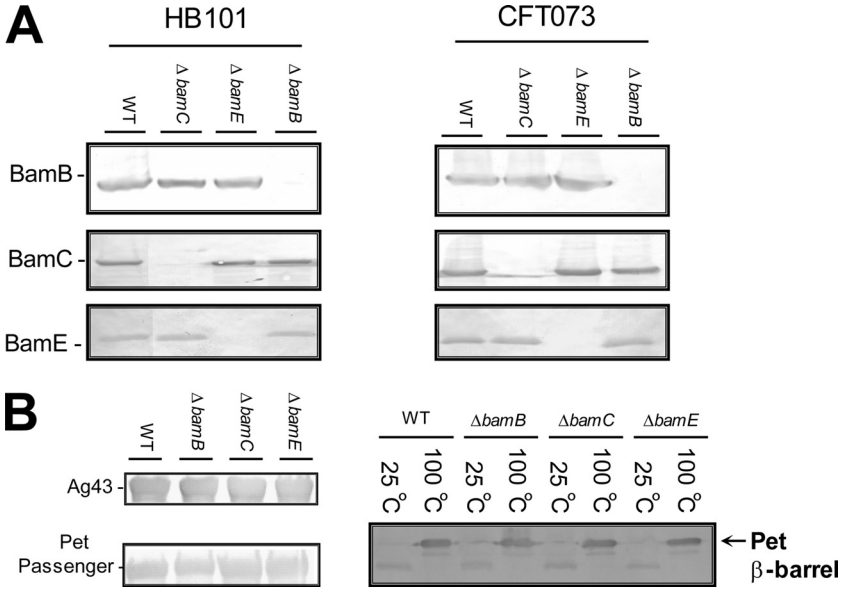

FIG. 2. BamB, BamC, and BamE are not required for AT secretion. (A) Construction of *bamB*, *bamC*, and *bamE* null mutations in *E. coli* HB101 and *E. coli* CFT073. Whole-cell lysate of each mutant was prepared by spinning down 1 ml of an overnight culture and resuspending it in Laemmli buffer. Samples were separated by SDS-PAGE and Western immunoblotted with antibodies raised to BamB, BamC, and BamE, confirming the absence of the relevant protein from each mutant. (B) Western immunoblotting was performed on the BamB, BamC, and BamE null mutant strains expressing Pet and Ag43. The Pet passenger domain was prepared from supernatant fractions after filter sterilization and precipitation with a final volume of 10% trichloroacetic acid. No difference in the accumulation of Pet in culture supernatant was noted. The Pet β-barrel was detected by Western immunoblotting of outer membrane fractions with anti-Pet β-domain antibodies after heating to either 100°C or 25°C; no differences in β-domain levels or heat modifiability were detected. For analysis of Ag43 biogenesis, the Ag43 passenger domain was released from the cell surface using the standard heat release assay (60°C for 2 min) and detected by Western immunoblotting with Ag43-specific antiserum. No differences in the levels of Ag43 were detected. These data indicate that AT secretion is not significantly influenced by BamB, -C, or -E. WT, wild type.

fractions were prepared as described previously (17, 26). Supernatant proteins were separated by SDS-PAGE, and Pet was detected by Western immunoblotting using a polyclonal rabbit antiserum generated toward the Pet passenger domain (8), secondary goat anti-rabbit antibodies conjugated with alkaline phosphatase (AP), and the AP substrate 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (32). Accumulation of Pet in the culture medium was unaffected by the absence of BamB, BamC, or BamE (Fig. 2). To gain a more complete picture of Pet biogenesis, we also examined the production of the C-terminal β-barrel of Pet. OMPs were purified and resolved by SDS-PAGE; Western immunoblotting was performed as described above but with a polyclonal rabbit antiserum recognizing the Pet β-barrel. To raise antibodies, the Pet β domain was cloned into the expression vector pMAL-c2X (New England BioLabs, Herts, United Kingdom) and the resulting maltose-binding protein–Pet–β-barrel fusion was purified in accordance with the manufacturer's instructions before immunization of rabbits (Eurogentec) and subsequent harvesting of serum. In each case, the levels of the Pet β-barrel were unaffected by the absence of the BAM components, and the β-barrel remained heat modifiable (Fig. 2), indicating that it was inserted in the outer membrane in its native conformation. To ensure that these effects were not specific to Pet, we examined the influence of these mutations on another AT, namely, biofilm-promoting Ag43. *E. coli* HB101 and its knockout derivatives were transformed with *agn43*-containing plasmid pCO2 (35). Expression levels were monitored using a previously described heat release assay which detects Ag43 release from the bacterial cell surface (17, 35). The resulting samples were analyzed by SDS-PAGE and Western immunoblotting with anti-Ag43 antibodies (Fig. 2). The levels of Ag43 were unaffected, indicating that BamB, BamC, and BamE are not required for the translocation of Ag43 to the exterior of the cell. These observations were confirmed for production of the Sat AT cytotoxin in the wild-type strain and mutant versions of UPEC CFT073 (data not shown).

While secretion is unaffected, it remains possible that folding of the passenger domain is abnormal. To test this hypothesis, we quantified the functional activity of Pet and Ag43 as a direct indicator of protein folding. To calculate the enzymatic activity of Pet, supernatant fractions from *E. coli* HB101 and isogenic *bamB*, *bamC*, and *bamE* deletion strains expressing Pet were harvested at an OD_{600} of 1.0 and concentrated 100-fold using Vivaspin 20 columns (Sartorius Stedim Biotech) with a molecular weight cutoff of 50,000 as previously described (13). Pet isolated from culture supernatants was buffer exchanged into 50 mM Tris-HCl (pH 7.5), and 30 μg of protein was assayed for protease activity using azocasein as the substrate as described previously (29). One (arbitrary) unit of protease activity was defined as the protease activity resulting in an increase in A_{440} of 0.001 in 16 h at 37°C. The Ag43 cell-cell aggregation assay was performed as previously described (15, 17). In all of the mutants, the enzymatic activity of Pet and the ability of Ag43 to mediate cell-cell aggregation were indistinguishable from those of the wild-type organism (Fig. 3), indicating that BamB, BamC, and BamE are not required for folding of the passenger domain.

Concluding remarks. In agreement with other studies, our results confirm that BamA is required for AT biogenesis (18, 33). However, this study has highlighted the additional requirement of BamD for AT biogenesis. This observation is consistent with all previous studies which have demonstrated the essential nature of BamD in OMP assembly and reinforce the concept that BamA and BamD work in concert (10, 27). Previous investigations have demonstrated that different OMPs are assembled into the outer membrane via different routes. Thus, porins such as OmpF and LamB, as well as the omptin family member OmpT, have severe biogenic defects in the absence of BamB, while, in contrast, levels of TolC increase in a BamB mutant (2, 10). Similarly, TolC levels are unaffected by loss of BamE whereas biogenesis of porins is marginally affected (2, 34, 37). We conclude from our data that, like TolC, the AT biogenesis pathway does not require the nonessential lipoproteins BamB, BamC, and BamE. However, in contrast to TolC, AT levels do not increase in the absence of BamB. These studies suggest that there are at least two distinct pathways for OMP assembly in *E. coli*, one which is dependent on BamB, -C, and -E (the porins) and one which is independent of these other factors (the ATs and TolC), both of which converge on the core of the BAM complex formed by the BamA and BamD subunits.

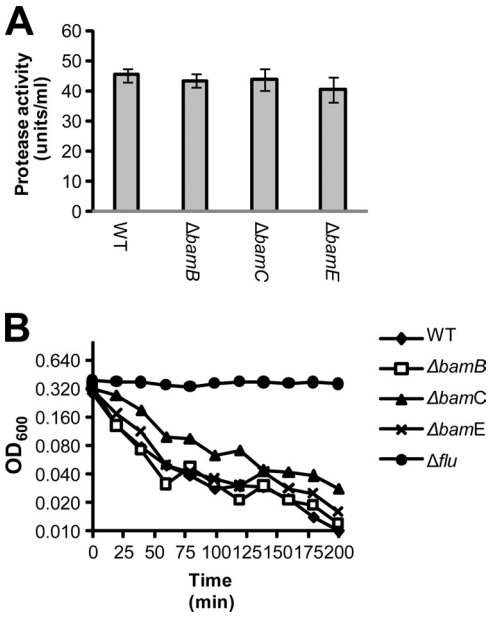

FIG. 3. BamB, BamC, and BamE are not required for folding of the passenger domains. If the AT passenger domains do not fold correctly, they will not be able to perform their extracellular functions. To determine if BamB, BamC, or BamE influences the folding of the passenger domain, the functional activity of the Pet and Ag43 ATs was characterized in wild-type (WT) *E. coli* HB101 and isogenic derivatives. (A) The enzymatic activity of Pet was calculated using an azocasein assay. Protease activity was expressed in arbitrary units. There was no difference in the functional activity of Pet harvested from the mutant strains and that of Pet harvested from the wild-type organism, indicating that Pet produced from the mutants is not aberrantly folded. (B) The ability of Ag43 to mediate cell-cell aggregation was quantified in wild-type *E. coli* HB101 and isogenic derivatives. No difference in the rate of sedimentation between the mutants and the parent strain was observed. In contrast, the *E. coli* HB101 strain lacking the gene encoding Ag43 (Δ*flu*) did not aggregate at all. These data indicate that Ag43 is not aberrantly folded.

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