BonA from *Acinetobacter baumannii* Forms a Divisome-Localized Decamer That Supports Outer Envelope Function

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**ABSTRACT** *Acinetobacter baumannii* is a high-risk pathogen due to the rapid global spread of multidrug-resistant lineages. Its phylogenetic divergence from other ESKAPE pathogens means that determinants of its antimicrobial resistance can be difficult to extrapolate from other widely studied bacteria. A recent study showed that *A. baumannii* upregulates production of an outer membrane lipoprotein, which we designate BonA, in response to challenge with polymyxins. Here, we show that BonA has limited sequence similarity and distinct structural features compared to lipoproteins from other bacterial species. Analyses through X-ray crystallography, small-angle X-ray scattering, electron microscopy, and multiangle light scattering demonstrate that BonA has a dual BON (Bacterial OsmY and Nodulation) domain architecture and forms a decamer via an unusual oligomerization mechanism. This analysis also indicates this decamer is transient, suggesting dynamic oligomerization plays a role in BonA function. Antisera recognizing BonA shows it is an outer membrane protein localized to the divisome. Loss of BonA modulates the density of the outer membrane, consistent with a change in its structure or link to the peptidoglycan, and prevents motility in a clinical strain (ATCC 17978). Consistent with these findings, the dimensions of the BonA decamer are sufficient to permeate the peptidoglycan layer, with the potential to form a membrane-spanning complex during cell division.

**IMPORTANCE** The pathogen *Acinetobacter baumannii* is considered an urgent threat to human health. *A. baumannii* is highly resistant to treatment with antibiotics, in part due to its protective cell envelope. This bacterium is only distantly related to other bacterial pathogens, so its cell envelope has distinct properties and contains components distinct from those of other bacteria that support its function. Here, we report the discovery of BonA, a protein that supports *A. baumannii* outer envelope function and is required for cell motility. We determine the atomic structure of BonA and show that it forms part of the cell division machinery and functions by forming a complex, features that mirror those of distantly related homologs from other bacteria. By improving our understanding of the *A. baumannii* cell envelope this work will assist in treating this pathogen.

**KEYWORDS** *Acinetobacter baumannii*, cell division, cell envelope, outer membrane proteins
Acinetobacter baumannii is a notorious “red alert” pathogen, considered an urgent threat to human health by international infectious disease control agencies (1–4). As a member of the gammaproteobacterial family Moraxellaceae, A. baumannii is genetically and physiologically divergent from well-studied model Gram-negative Enterobacteriaceae such as Escherichia coli. A. baumannii has a unique cell envelope that protects it from disinfectants and desiccation that readily kill other bacterial species, allowing it to persist for long periods on artificial surfaces in hospitals (5, 6). In addition, A. baumannii is notorious for its innate and acquired antibiotic resistance (2). It is currently estimated that as many as 50% of all A. baumannii infections in the United States are caused by strains resistant to carbapenems and many strains acquire polymyxin resistance during treatment (7, 8).

Like other Gram-negative bacteria, A. baumannii has a cell envelope consisting of an inner and outer membrane. This dual membrane encloses the periplasm, a crowded compartment that contains a thin layer of peptidoglycan (9). The outer membrane of A. baumannii is an intricate structure, consisting of an asymmetric lipid bilayer with an inner leaflet composed of phospholipids and an outer leaflet composed of lipooligosaccharide (LOS) (10). The LOS-derived surface of the outer membrane acts as a barrier to hydrophobic molecules (11). In addition to LOS and phospholipids, the outer membrane contains numerous proteins that are either integrated into or anchored in the membrane (12).

To maintain the integrity of the outer membrane, Gram-negative bacteria actively maintain its lipid asymmetry and coordinate its biogenesis rate with the overall rate of cell growth. In addition, the outer membrane must be constricted in conjunction with the peptidoglycan cell wall during division (13). To achieve this, Gram-negative bacteria have evolved a network of interlinked pathways for the construction and maintenance of the outer membrane (12, 14–22). Despite considerable progress in understanding how these pathways function in E. coli, in many cases, the proteins that constitute them are not well characterized, and additional pathways likely remain to be identified (12, 20, 23).

In species divergent from E. coli, such as A. baumannii, these knowledge gaps are much more substantial.

Among these knowledge gaps is the role of dual-BON domain proteins, a widespread family of outer envelope proteins in Gram-negative bacteria. Dual-BON family proteins contain a pair of Bacterial OsmY and Nodulation (BON) domains, which fold into a conserved αβ sandwich (24). They possess a signal peptide targeting them to the periplasm, and some family members possess a lipobox with an N-terminal acylated cysteine, mediating peripheral outer membrane association (25, 26). They lack conserved residues indicative of an enzyme active site, though some family members bind phospholipids (27). Archetypical members of this dual-BON domain family are the outer membrane-associated lipid-binding protein DolP (formerly YraP) and the soluble periplasmic protein OsmY, both of which play a role in the construction and maintenance of the bacterial outer envelope (25–29). OsmY is an abundant periplasmic protein in E. coli induced in response to stressors such as osmotic shock, heat shock, acidic pH, and bile salts (25, 30). Recently, it was shown that OsmY functions as a chaperone, enhancing the stability of periplasmic proteins and the assembly of a subset of outer membrane proteins (31).

DolP is a lipoprotein widely present in Gram-negative bacteria. In E. coli and Neisseria meningitidis, it localizes to the inner leaflet of the outer membrane via an N-terminal lipid anchor (32–34). DolP was initially identified in E. coli as a lipoprotein whose expression is induced under cell envelope stress and it forms part of the σE regulon (35). Mutants of E. coli, N. meningitidis, and Salmonella enterica lacking DolP are compromised in outer membrane integrity, rendering the cells more sensitive to agents like the detergent sodium dodecyl sulfate (SDS) or the antibiotic vancomycin (26, 27, 29, 33, 35, 36). Possibly as a result of impaired outer membrane integrity, loss of DolP leads to attenuation of virulence in rodent models of infection (26).

There has been significant recent progress in determining the function of DolP in E. coli, suggesting multifaceted roles in regulating cell division and aiding outer membrane protein
BonA from *Acinetobacter baumannii* is a member of a diverse family of dual-BON domain outer membrane lipoproteins. Analysis of *A. baumannii* genomes showed that they encode only one BON domain family protein, and sequence analysis of this lipoprotein suggests dual-BON domains, a terminal lipobox with an acyl-anchoring cysteine residue, and N- and C-terminal extensions (Fig. 1A). BonA shows substantial sequence divergence from DoIP from *E. coli* (23% amino acid identity) and *Neisseria* spp. (24% identity) and is even more distantly diverged from OsmY from *E. coli* (20% identity) (see Table S1 in the supplemental material). A phylogenetic tree confirmed the distant evolutionary relationship between BonA and other dual-BON domain lipoproteins identified in a HMMER search of the reference proteome database (Fig. 1B; see also Table S1) (41). BonA belongs to a distinct clade clustering with proteins from other species of the family *Moraxellaceae*. A C-terminal proline-rich extension is present in BonA and other related sequences from *Acinetobacter* and *Moraxella* species but is absent from DoIP and OsmY (Fig. 1A; see also Fig. S1).

**BonA** is localized to the divisome and its deletion prevents motility. The distant evolutionary relationship between BonA and other dual-BON family proteins poses the question of whether these proteins share a conserved function. To address this, we sought to determine the subcellular localization and physiological role of BonA. Mutants of the well-characterized *A. baumannii* type strain ATCC 19606 and clinical
isolate ATCC 17978 were constructed (ΔbonA). Antibodies raised to BonA detected the protein in wild-type A. baumannii ATCC 19606, but not in the ΔbonA strain, when membrane extracts were analyzed by SDS-PAGE and immunoblotting (Fig. 2A). BonA was not detected in soluble cell extracts after ultracentrifugation, indicating it is
BonA from *Acinetobacter baumannii* entirely membrane-associated (see Fig. S2A). Upon Western blotting of BonA from membrane extracts, we noted multiple bands corresponding to the protein (Fig. 2A). This pattern is reminiscent of proteins modified by O-linked glycosylation mediated by the enzyme PglL, which glycosylates serine residues in motifs similar to those in the BonA C-terminal extension (see Fig. S1) (42, 43). To test whether O-linked glycosylation was present, we analyzed cell extracts from wild-type *A. baumannii* ATCC 17978 and a *pggL* deletion mutant (*ΔpggL*). In both strains the patterns of BonA on SDS-PAGE were analogous, suggesting that PglL does not glycosylate BonA (see Fig. S2B). The pattern of electrophoresis observed for BonA may be due to an unknown posttranslational modification or to proteolytic processing of the protein. To monitor the subcellular localization of BonA, cell membrane extracts were fractionated via a sucrose gradient, followed by immunoblotting, revealing that BonA colocalizes with the outer membrane porin Omp38 and not the inner membrane NADH-quinone oxidoreductase subunit NuoG (Fig. 2D; see also Fig. S2C).

While the relative abundance of proteins present in *A. baumannii* ATCC 19606 *ΔbonA* membranes was similar to the wild type, the outer membrane fraction from the *ΔbonA* strain progressed further into the sucrose gradient. This suggests that its structure or composition is altered, leading to an increase in density (Fig. 2D). No significant increase in sensitivity to SDS, vancomycin, or tetracycline was observed in the *ΔbonA* strain (see Table S2), suggesting that loss of BonA does not impair the integrity of the outer membrane in *A. baumannii*. A common phenotype for surface defects in *A. baumannii* is loss of motility on a swarm plate: while *A. baumannii* lacks flagella, twitching motility is observed in some strains of this species, thought to be mediated by the type IV pilus (44). Type IV pili are dynamic protein filaments that are assembled and secreted from the cell via a large protein complex that spans the bacterial cell envelope (45). ATCC 19606 is nonmotile, so this phenotype could not be tested in this strain (see Fig. S2D); however, loss of motility was observed in the *A. baumannii* ATCC 17978
Delta bonA strain, which could be complemented by the addition of bonA in trans (Fig. 2E). The loss of twitching motility observed in the ATCC 17978 Delta bonA mutant suggests that BonA plays either a direct or indirect role in the assembly or maintenance of the motility machinery.

Like BonA, DolP is a lipoprotein anchored to the outer membrane. DolP localizes to the divisome where it plays a role in regulating peptidoglycan remodeling during cell division (32). To determine whether BonA shares a common localization, we used the antibodies to monitor BonA in single cells via immunofluorescence microscopy. Consistent with localization to the divisome, fluorescence corresponding to BonA was observed as a central band in what appeared to be elongated, early-stage dividing cells. This band constricted in concert with the cell division septum (Fig. 2C; see also Fig. S3). No fluorescence beyond background was observed in Delta bonA cells (Fig. 2C; see also Fig. S3). To investigate the native structure of BonA, membrane extracts were solubilized in detergent and analyzed by blue-native PAGE. The vast majority of BonA was detected at a molecular weight of ~60 kDa (Fig. 2B). Longer exposure of the immunoblots revealed a smaller proportion of BonA was detected as a larger oligomeric species (250 to 300 kDa) (Fig. 2B).

Crystal structure of BonA. To gain insight into the structural organization of BonA, as well as its architecture at the outer membrane, we solved its crystal structure. Crystal trials were performed with full-length BonA, as well as several truncation constructs. High-quality crystals were only obtained for N-terminally truncated BonA, missing the 27 amino acids (aa) after its lipid-anchoring cystine. The structure of this protein, designated BonA-27N, was solved at 1.65 Å by single-wavelength anomalous dispersion (SAD) phasing, using selenomethionine labeled protein. The structure of BonA-27N was built and refined from the resulting electron density maps (Fig. 3A; see also Table S3). The crystal structure of BonA-27N consists of two alpha/beta-sandwich BON domains that interact extensively via the external face of their three-strand beta-sheets (Fig. 3A). In contrast to the structure of DolP in which both domains adopt a canonical BON domain fold (27), in the BonA structure, alpha-helix 1 (alpha H1) of BON domain 1 (BON1) does not adopt the expected BON domain conformation of running parallel to the BON domain beta-sheet. Rather, it is displaced from the rest of the domain (Fig. 3A). The 39 aa of the C-terminal extension of BonA-27N (aa 196 to 235) are disordered in the crystal structure. This region of BonA is not present in DolP or OsmY and is predicted to be largely unstructured (Fig. 1A; see also Fig. S1).

Analysis of BonA-27N crystallographic symmetry reveals that it exists as a dimer, aligned with the crystallographic 2-fold axis (Fig. 3B). Analysis with the molecular interface prediction tool PISA (46) predicts that this interface is bona fide. The symmetrical BonA-27N dimer interacts via an extensive interface encompassing both BON domains (Fig. 3B). The interface is stabilized by alpha H1 of BON domain 2 (BON2), which substitutes for the displaced alpha H1 of BON1, thus completing the alpha/beta-sandwich fold of BON1 (Fig. 3C). This interaction of alpha H1 of BON2 with BON1 is largely mediated by hydrophobic interactions (Fig. 3D), with Tyr118 and Met122 of alpha H1 of BON2 extending deeply into a hydrophobic pocket created by the displacement of alpha H1 of BON1 (Fig. 3E). While the interactions between alpha H1 of BON2 and BON1 are largely hydrophobic, the dimer interface of BonA-27N is mediated by a mixture of interaction types, including 14 hydrogen bonds and four salt bridges (Fig. 3F). The interface also contains two symmetrical, highly solvated pockets, which trap a total of 34 water molecules, as well as two Zn^{2+} ions which were present at a high concentration in the crystallization solution (Fig. 3G). In DolP from E. coli, alpha H1 of BON2 is responsible for binding to anionic phospholipids present in the outer membrane, with lipid binding mediating the divisome localization of DolP (27). In the BonA-27N structure, alpha H1 of BON2 is largely buried at the dimer interface and would be unable to access lipids. Further, BonA lacks conserved residues present in this helix of DolP required for lipid binding (27). This suggests that BonA localizes to the divisome by some other means.

A recent study by Wu et al. supports the physiological relevance of the BonA dimer, demonstrating via a global proteomic approach that interaction occurs between BonA
The crystal structure of BonA-27N reveals a dual-BON domain architecture that dimerizes via an α-helix swap mechanism. (A) The crystal structure of BonA-27N shown as a rainbow cartoon N terminus (blue) to C terminus (red) displays a dual-BON domain architecture with displaced α-helix 1 (αH1) of BON domain 1. (B) Dimer of BonA-27N observed in crystallo. (C) A key interface of the BonA-27N dimer involves the displacement of αH1 of BON1 by α-helix 1 (αH1) of BON2 of the opposing BonA molecule. αH1 of BON2 is amphipathic and interacts with the opposing molecule largely through hydrophobic interactions shown in panels D and E. As shown in panels F and G, the BonA dimer interface consists of both hydrophobic and polar interactions and is highly solvated.
molecules in A. baumannii cells (47). This study identified intermolecular cross-links between lysines 50, 59, and 65 of neighboring BonA molecules in A. baumannii cells. In the BonA-27N structure, lysines 59 and 65 are located in H1 of BON1 and are within proximity to their dimer equivalent in our BonA-27N structure (see Fig. S4). Lysine 50 is unresolved in the crystal structure, but given this region of BonA is crucial for oligomerization, it is also a plausible candidate for cross-linking based on our data.

**BonA decamers under physiological conditions through interactions mediated by its N-terminal extension.** Our structural and biochemical analysis indicated that BonA oligomerizes in A. baumannii cells and as a recombinant protein. To investigate the oligomeric state of BonA, the mature recombinant protein (lacking its signal sequence) was analyzed by size exclusion chromatography (SEC). In the absence of detergent, BonA migrated predominately as a high-molecular-weight species, with some disassociation to a lower-molecular-weight species observed. To gain a more precise understanding of the molecular weight of this oligomer, purified BonA was analyzed by analytical ultracentrifugation, revealing the presence of a single species with a molecular mass of ~240-kDa (see Fig. S5A). The molecular mass of the BonA oligomer was confirmed by size exclusion chromatography coupled multiangle laser-light scattering (SEC-MALS), which indicated this species has a molecular mass of 233 kDa, which is consistent with a decamer, while the smaller species has a mass of 23 kDa, corresponding to a BonA monomer (Fig. 4B). To determine whether the N- or C-terminal extensions flanking the core BonA BON domains were responsible for oligomerization, truncation constructs lacking the N-terminal 27 aa succeeding the lipobox and/or the C-terminal 45 aa were analyzed via SEC-MALS (Fig. 1A and 4A). Removal of the C-terminal extension increased the tendency of BonA to aggregate but did not affect the oligomeric state of the protein, with a decamer of 205 kDa observed for the truncated protein (Fig. 4C). Conversely, loss of the 27 N-terminal amino acids abrogated oligomerization, with only a monomeric species of ~22 kDa observed (Fig. 4D). Loss of both the N- and C-terminal regions also resulted in a monomeric protein, further confirming the role of the N terminus of BonA in oligomerization (Fig. 4E). In conclusion, BonA forms a decamer that requires its N-terminal extension and undergoes spontaneous disassociation into a monomeric species in solution. The monomeric nature of BonA-27N in solution contrasts with the dimer observed in its crystal structure, suggesting that weak interactions between monomers of this truncated protein are selected for during crystallization.

To understand the basis of oligomerization of BonA, both full-length and BonA-27N were analyzed via size exclusion coupled small-angle X-ray scattering (SEC-SAXS) (see Fig. S5; see also Table S3). Despite the C-terminal extension, which largely lacks predicted secondary structure and was disordered in the BonA-27N crystal structure, SAXS scattering indicates that decameric BonA forms a compact particle in solution with maximum dimensions of ~164 Å (see Fig. S5F and G). In contrast, SAXS scattering indicates that BonA-27N is highly flexible in solution with maximum dimensions of 107 Å, which is indicative of an unstructured and fully extended C terminus (see Fig. S5J and K). These differences between decameric and monomeric BonA suggest that intermolecular interactions stabilize the C terminus of the oligomeric form of the protein.

Molecular envelopes of full-length and BonA-27N were modeled based on SAXS scattering data. For full-length BonA, C5 symmetry was imposed, based on the decameric organization of the oligomer and the dimer observed in the crystal structure. The resulting molecular envelope was prlate, with dimensions of ~172 by 102 Å. Five dimers of the BonA-27N crystal structure could be modeled with C5 symmetry into a bulge at the center of the envelope. The N and C termini of all molecules are oriented in the same direction, which is required by the lipid anchored N terminus of BonA. Regions truncated or disordered in the BonA-27N crystal structure could be accommodated by the remainder of the molecular envelope (Fig. 4F). The molecular envelope of BonA-27N was indicative of a monomer, with dimensions of ~133 by 40 Å. The crystal structure of BonA-27N could be modeled into a bulge at one end of the envelope, with additional space accounting for the unstructured C-terminal extension.
FIG 4  BonA forms a decamer with pentameric symmetry mediated by its N terminus. (A) A cartoon schematic of BonA showing its two central BON domains, with N- and C-terminal extensions with
(Continued on next page)
(Fig. 4G). The simulated scattering curves for both envelopes were an excellent fit for the experimental data (Fig. 4H).

To validate the SAXS-based modeling of the BonA decamer, we further investigated BonA via negative-stain electron microscopy (NS-EM). Initial analysis of EM grids prepared with native BonA did not contain discrete particles. To stabilize the decamer, on-column glutaraldehyde cross-linking was performed, stabilizing BonA as first a dimeric and then a decameric species with increasing glutaraldehyde concentration (see Fig. S5B). NS-EM of the cross-linked sample revealed largely uniform monodisperse particles (see Fig. S5C). Two-dimensional (2D)-class averages derived from these images are suggestive of a particle with dimensions compatible with the BonA SAXS envelope and C5 symmetry, as predicted by other analyses (Fig. 4I).

**Disruption of the BonA dimer interface destabilizes the decamer and affects protein function.** To gain insight into the role of the BonA dimer in stabilizing the decamer, we expressed and purified a series of BonA variants with amino acid substitutions in hydrophobic residues of αH1 of BON2 that form part of the dimer interface (Fig. 5A). Two double substitutions (Y118S/A2125D and M122D/A129D) and one quintuple substitution (Y118S/I121D/M122D/A125D/A129D) were generated to maximize disruption of the BonA dimer interface. All mutant variants expressed and purified as the wild type, predominately decameric species were observed, with an increase in the amount of monomer present in all mutants. Wild-type and mutant BonA variants were purified concurrently and the decameric species were isolated and incubated for 0, 24, or 72 h at room temperature before the rate of decamer disassociation was monitored by SEC. All mutants displayed a higher rate of disassociation than the wild type, indicating that disruption of the BonA dimerization interface also partially destabilizes the BonA decamer (Fig. 5B; see also Table S4). The lack of total disruption of the decamer in the BonA mutants is consistent with the role of the BonA N-terminal extension in decamerization. The stability of the wild-type and BonA decamers was also tested using differential scanning fluorimetry and light scattering. Wild-type BonA fluorescence exhibited two inflection points at 54.3 and 60.5°C, the latter corresponding to an increase in light scattering indicating protein denaturation, with the former likely corresponding to decamer disassociation (Fig. 5C; see also Table S4). Inflection point 1 was shifted compared to wild type by −9 and −7°C for the Y118S/A2125D and M122D/A129D mutants, respectively, further indicating destabilization of the decamer in these mutants. Inflection point 1 was not observed for the quintuple mutant, and inflection point 2 was shifted by −10.4°C, indicating this mutant was further destabilized (Fig. 5C; see also Table S4).

To assess the effect of mutations on the function of BonA in *A. baumannii*, wild-type and mutant BonA constructs were introduced into the *A. baumannii* ATCC 17978 ΔbonA strain. The multiple copies of the complementation plasmid meant all complemented strains produced more BonA than wild-type *A. baumannii* ATCC 17978 (Fig. 5D and E). The migration pattern of the mutant BonA variants on blue-native PAGE was distinct from the wild-type complement strain, with a compact BonA species present at ~66 kDa and less-diffuse species present at ~240 kDa. However, the profile of the BonA mutants was more similar to that of the uncomplemented wild-type strain in this experiment, making the significance of these differences difficult to interpret. Interestingly,
in all complemented strains a diffuse higher-order species at ~1,000 kDa was observed, suggesting BonA is present in a larger oligomer or complex (Fig. 5D). As a readout for BonA functionality, the swarming motilities of wild-type, ΔbonA, and complemented strains were assessed (Fig. 5F). While motility was restored in A. baumannii ATCC 17978 ΔbonA complemented with wild-type BonA, none of the mutant BonA variants complemented the motility defect, indicating these variants have compromised functionality and that BonA oligomerization is important for function.

DISCUSSION

In this work, we identify BonA, a member of the bacterial dual-BON domain family of proteins, produced by A. baumannii and encoded by other members of the family Moraxellaceae. We demonstrate that BonA is anchored to the outer membrane where it plays a role in maintaining membrane structure and is required for twitching motility. Through structural analysis, we show that BonA possesses unique structural features and forms a divisome localized decamer that is important for the protein’s function (Fig. 6). We show that while BonA shares a common outer membrane and divisome localization to DolP from E. coli and Neisseria spp. (26, 28, 35), its loss does not lead to
The gross defects in outer membrane permeability observed in DolP deletion mutants of these species. Further, purified DolP is monomeric and its divisome localization is mediated by phospholipid binding, while BonA is a decamer that lacks the conserved lipid-binding residues found in DolP (27). Analysis of DolP from *E. coli* membranes indicates it forms diffuse oligomers that echo those observed in membrane-derived BonA (29). However, whether the oligomerization mechanism we demonstrate for BonA extends to DolP and whether the conformation of monomeric BonA is analogous to DolP remain to be determined. Furthermore, whether, like DolP, BonA interacts transiently with the BAM complex to mitigate protein folding stress is an interesting question for future study (29).

The change in outer membrane density associated with the loss of BonA suggests a significant alteration in the structure or composition of this membrane or the physical membrane-peptidoglycan links. Consistent with this, the loss of twitching motility observed in the *A. baumannii* ATCC 17978 ΔbonA mutant, likely mediated by an outer-envelope spanning type IV pilus (44), is suggestive of a perturbed outer envelope. These data are also consistent with our previous finding that BonA is upregulated in response to outer membrane destabilizing polymyxins (38, 39) and broadly indicate a role for BonA in supporting optimal outer membrane function.

While further work is required to determine the precise role of BonA in the outer membrane, our structural analysis provides important insights into BonA function. We show that BonA forms a decamer that is ~172 Å in length. In the context of the periplasmic space, where the nominal distance between the outer and inner membranes is ~200 Å (48), outer membrane-anchored BonA would span the majority of the periplasm if extending perpendicular from the membrane (Fig. 6). In this configuration, BonA would penetrate the peptidoglycan layer and would be capable of interacting with proteins embedded in the inner membrane, thus bridging the inner and outer membranes. When localized to the site of cell division, BonA could tether the outer membrane to the peptidoglycan or the membrane-spanning divisome complex (Fig. 6). In support of this hypothesis, in-cell cross-linking data show interactions occur between BonA and OmpA in *A. baumannii* (47), with OmpA playing a role in tethering the outer membrane to the peptidoglycan (49). The transient nature of BonA oligomerization is also consistent with a role in coordinating a dynamic process during cell division. If BonA is indeed important for coordinating the outer envelope during cell division, its role in maintaining membrane integrity and providing a scaffold for divisome proteins will be critical for the organism’s survival.
division, its loss would lead to improper remodeling of this structure, which is consistent with the ΔbonA phenotypes we observe.

The cell envelope provides a key defense for *A. baumannii* against antimicrobial compounds and environmental stress. To effectively combat *A. baumannii* infection and its persistence in the hospital environment, we must develop strategies to overcome the outer envelope’s defenses. To do so, a robust understanding of the key factors required for outer membrane construction and maintenance is required. Our work on BonA informs this understanding and provides insights into the role of this protein in supporting outer membrane function in *A. baumannii*.

**MATERIALS AND METHODS**

**Protein sequence analysis.** To determine the relationship between distantly related dual-BON domain family members, we constructed a tree of BonA homologs, identified with a HMMER search of the reference proteomes database using BonA as a query sequence (41). BonA homologs identified in the HMMER search were curated to only include proteins with a dual-BON domain architecture and a lipobox sequence determined using SignalP 5.0 (50). This yielded 896 sequences, which were further reduced for tree construction using CD-Hit to filter sequence with a pairwise similarity of <75%, yielding 565 sequences (see Table S1) (51). These 565 protein sequences, plus OsmY from *E. coli* as a sequence to define the root branch, were aligned using MUSCLE (52) implemented in the phylogenetic analysis program MEGAX v.10.1.7 (53), which was subsequently used as the input for constructing a maximum-likelihood (ML) phylogenetic tree to infer evolutionary relationships for this protein family. The best amino acid substitution model was inferred using MEGAX which compared 56 different models; the Jones-Taylor-Thornton model with a gamma distribution of 1 was selected. To infer tree topology, the default ML heuristic method ML nearest-neighbor-interchange was applied, and initial trees were made with Neighbor-Joining and BioNJ algorithms. The final tree was built by including all residues and bootstrapping with 100 replicates.

**Strain propagation, maintenance, and antimicrobial susceptibility testing.** *E. coli* and *A. baumannii* strains were propagated in lysogeny broth (LB) and LB agar at 37°C, with antibiotic selection to maintain plasmids where appropriate. Antimicrobial susceptibility was conducted with CLSI guidelines using the broth microdilution method and cation adjusted Muller Hinton broth. MICs were defined as ≥80% reduction in growth, and significance considered as ≥2 concentration increase or decrease in MIC relative to the wild-type control.

**Construction of ΔbonA strains in *A. baumannii*.** Plasmid DNA, genomic DNA, and PCR products were purified using relevant kits from Bioneer, Qiagen, and Promega, respectively, according to the manufacturers’ instructions. The *A. baumannii* ΔbonA mutants were constructed as described previously (54), with minor modifications. Briefly, the kanamycin resistance cassette was PCR amplified from pKD4 using disruption primers containing ≥80 bases of homology to the bonA flanking sequence (as described in Table S5 in the supplemental material). The resultant fragments were gel purified and introduced into *A. baumannii* strains ATCC 17978 and ATCC 19606 by electroporation as previously described (55), with selection on LB agar, supplemented with 50 μg/ml kanamycin. The mutations were confirmed by PCR amplification using primers flanking the insertion, followed by Southern hybridization of genomic DNA digested with EcoRV, probed with kanamycin- and bonA-specific digoxigenin-labeled probes, as described previously (56).

For complementation, the full-length bonA sequence plus 500 nucleotides upstream of the translational start site (deemed to include the native promoter) were PCR amplified from ATCC 19798 with forward and reverse complementation primers encoding 5’ AatII and EcoRI restriction sites, respectively. The resultant fragments were digested and ligated into the *E. coli-Acinetobacter* shuttle vector, pWH1266 (57). To create Y118S/A125D, M122D/A129D, and Y118S/I121D/M122D/A125D/A129D mutant BonA complementation constructs, gene blocks identical to the PCR-amplified sequence, aside from the stated mutations, were synthesized and cloned into pWH1266. The pbonA wild-type and mutant constructs were confirmed by sequencing before electroporation into the respective mutant strains as described previously, with the pWH1266 vector-only used as a control.

**Twitching motility assays.** Twitching motility was assessed as described previously (44). Briefly, a 1-μl drop of stationary-phase culture was placed onto the center of a 0.25% modified LB agarose and incubated at 37°C for up to 48 h. Three independent experiments were performed for each.

**BonA antiserum generation.** Polyclonal rabbit antiserum for the detection of BonA was generated at the Monash Animal Research Platform from recombinant proteins purified in-house. Rabbids were serially injected with purified protein (10 mg/ml) in combination with complete (first injection) or incomplete (subsequent injections) Freund adjuvant, over 1 to 3 months, with clarified rabbit sera periodically tested for reactivity to the target protein. Once acceptable levels of reactivity were achieved, rabbits were euthanized, and clarified sera were collected and stored at −80°C.

**Isolation and fractionation of membranes from *A. baumannii*.** *A. baumannii* cells were cultured in LB media and grown to an optical density at 600 nm (OD600) of 0.6 before harvesting. Membranes were purified and subsequently fractionated by sucrose density fractionation (60:55:45:40:35% [wt/wt]) as described previously (58).

**Detection and localization of BonA in *A. baumannii* cell extracts by Western blotting.** For the detection of BonA in cell extracts, 50-μg portions of isolated total membranes were analyzed by 10% SDS-PAGE or 5 to 16% blue-native (BN)-PAGE (59) and was subsequently analyzed by Western blotting.
against BonA (antibody dilution, 1:20,000). To determine the cellular localization of BonA, 30-μl aliquots of each fraction from the sucrose gradient were separated by 10% SDS-PAGE for Coomassie blue staining and subsequent Western blotting as described above.

**Localization of BonA in A. baumannii cells by immunofluorescence microscopy.** Bacterial cultures were grown to mid-log phase in LB media at 37°C with shaking (200 rpm). Then, 500 μl of culture medium was centrifuged (4,000 × g, 5 min, 4°C), washed twice in phosphate-buffered saline (PBS), and resuspended in 500 μl of PBS. Eight-well, coverglass-bottom chambers (Sarstedt) were coated with 0.01% (vol/vol) poly-lysine (Sigma-Aldrich, P8920) for 10 min at room temperature before excess poly-lysine was removed. Afterward, 200 μl of bacterial cell suspension was immobilized onto each well. To ensure a monolayer of bacteria was formed at the bottom of each well, chamber slides were subjected to centrifugation (4,000 × g, 3 min, 4°C), followed by several washing steps to remove nonadhered cells. The monolayer of bacteria was then fixed with a mixture of paraformaldehyde (2% [wt/vol]) and glutaraldehyde (0.2% [vol/vol]) for 5 min at 4°C, which was then washed with PBS to remove excess fixatives. To reduce auto-fluorescence caused by the background, samples were treated with the fluorescence quencher NaBH₄ at a concentration of 0.1% (wt/vol) in PBS, followed by several washing steps with PBS. Samples were then permeabilized with Triton X-100 (0.001% [vol/vol] in PBS), followed by three washing steps with PBS.

Before antibody staining, samples were blocked with 5% (wt/vol) bovine serum albumin (BSA) in PBS for 1 h at room temperature, followed by incubation with anti-BonA antisera (1:1,000 in 5% [wt/vol] BSA in PBS) for 1-h mixing by rotary inversion at room temperature. Samples were washed thoroughly with PBS to remove excess antisera. Secondary staining was carried out for 45 min at room temperature using anti-rabbit immunoglobulin G (lgG)-Alexa Fluor 488 (Thermo Fisher, A-11008) diluted to 1:3,000 (in 5% BSA in PBS), followed by several washing steps to remove excess antibody. Olympus IX-81 inverted fluorescence microscope equipped with Olympus Cell-M software was used to visualize bacterial samples in a 20× objective with a fluorescein isothiocyanate (FITC) filter.

**Protein expression and purification.** DNA encoding full-length BonA and BonA-C45 were amplified by PCR, with C-terminal NcoI and Xhol restriction sites and cloned into a PET20b derived vector which added a 10× N-terminal His tag, followed by a TEV cleavage site, via restriction digest and ligation. Gene blocks encoding Y118S/A125D, M122D/A129D, and Y118S/I121D/M122D/A125D/A129D mutant full-length BonA constructs were synthesized and cloned into the modified PET20b derived vector as for the wild-type protein. The resulting vector was transformed into E. coli BL21(DE3) C41 cells. DNA encoding BonA-27N and BonA-27N-45C were amplified by PCR, minus stop codon, with C-terminal Ndel and Xhol restriction sites and cloned into petT22b vector which added a 6× C-terminal His tag. The resulting vectors were transformed into E. coli BL21(DE3) C41 cells. Protein expression was performed in terrific broth (12 g NaHPO₄, 11.55 g KH₂PO₄, 10 g glycerol) with 100 mg/ml ampicillin for selection. Cells were grown at 37°C until they reached an OD₆₀₀ of 1.0 induced using 0.3 mM IPTG (isopropyl-β-D-thiogalactopyranoside), followed by further growth 14 h at 25°C. For selenomethionine-labeled BonA-27N, the BonA-27N construct was transformed into the methionine auxotrophic E. coli strain Crystal Express (DE3). Cells were grown in M9 minimal medium containing 100 mg/liter of each amino acids (minus methionine) and 50 mg/liter selenomethionine. Cells were harvested by centrifugation, lysed using a cell disruptor (Emulsiflex) in Ni-binding buffer (50 mM Tris, 50 mM NaCl, 20 mM imidazole [pH 7.9]) plus 0.1 mg/ml lysozyme, 0.05 mg/ml DNase I, and Complete protease cocktail inhibitor tablets (Roche). The resulting lysate was clarified by centrifugation and applied to Ni-agarose resin, followed by washing with 10× column volumes of Ni-binding buffer, and elution of bound proteins with a step gradient of Ni-agarose buffer (50 mM Tris, 500 mM NaCl, 500 mM imidazole [pH 7.9]) of 5, 10, 25, and 50%. Eluted fractions containing recombinant protein were pooled and applied to a 26/600 Superdex S200 size exclusion column equilibrated in SEC buffer (50 mM Tris, 200 mM NaCl [pH 7.9]). The recombinant protein was then pooled concentrated to 10 mg/ml, snap-frozen, and stored at −80°C.

**Size-exclusion chromatography multangle light scattering.** The absolute molecular masses of BonA-FL and truncated variants were determined by SEC-MALS. First, 100-μl protein samples (1 to 5 mg/ml) were injected onto a Superdex 200 10/300 GL size exclusion chromatography column in 20 mM Tris–200 mM NaCl (pH 7.9) at 0.6 ml/min with a Shimadzu LC-20A. The column eluent was fed into a DAWN Helos II MALS detector (Wyatt Technology), followed by an Optilab T-Rex differential refractometer (Wyatt Technology). Light scattering and differential refractive index data were collected and analyzed using ASTRA 6 software (Wyatt Technology). Molecular masses and estimated errors were calculated across individual eluted peaks by extrapolating to Zimm plots with a refractive index increment (dn/dc) value of 0.1850 ml/g. SEC-MALS data are presented with the absorbance (280 nm) plotted alongside fitted the molecular masses (M).

**Analytical size exclusion chromatography of wild-type mutant BonA.** Wild-type and Y118S/ A125D, M122D/A129D, and Y118S/I121D/M122D/A125D/A129D mutant BonA was purified concurrently as described above. Protein concentration was normalized to 3 mg/ml, and 200 μl was loaded onto a Superdex 200 Increase 10/300 column equilibrated in 50 mM Tris–200 mM NaCl (pH 7.9) after 0, 24, and 72 h of incubation at 22°C. Chromatograms were analyzed based on known decamer and monomer peaks, and the peaks were calculated.

**Differential scanning fluorimetry of wild-type mutant BonA.** Fluorescence at 330 and 350 nm and light scattering of wild-type and mutant BonA were recorded as a function of temperature using a Prometheus NanoDSF instrument, with a BonA concentration of 5 mg/ml in 50 mM Tris–200 mM NaCl (pH 7.9). Due to the lack of tryptophan residues in BonA, the fluorescence recorded resulted from tyrosine residues.

**Protein crystalization, data collection, and structure solution.** Purified BonA proteins were screened for crystallization conditions using commercially available screens (~800 conditions). Crystals grew from drops containing BonA-27N in medium composed of 0.2 M zinc acetate, 0.1 M sodium acetate, and 20% PEG 3350 (pH 4.5), and the crystals were optimized from this starting condition. The crystals were
cryoprotected by increasing PEG 3350 concentration to 30% and flash cooled in liquid N₂. Diffraction data were collected at 100 K at the Australian Synchrotron on selenomethionine-labeled crystals and processed in the space group P3₂12₁ at 1.65 Å. Heavy atom sites were located, phases were obtained using single-wavelength anomalous dispersion (SAD), and the initial model was built using Autosol from the Phenix package (60). Eight heavy atom sites were located; four of these sites were selenium, and four of these sites were zinc. The BonA-N27 model was improved manually in Coot and refined using Phenix refine and Refmac (60–62). Analysis of the BonA-27N crystal structure was performed using the Phenix and CCP4 packages, noncrystallographic interfaces were predicted using PISA (46, 60, 63).

**Small-angle X-ray scattering.** Small-angle X-ray scattering (SAXS) was performed using Coflow SEC-SAXS at the Australian Synchrotron (64). Purified BonA and BonA-27N were analyzed at a preinjection concentration of 10 mg/ml. Scattering was collected over a q range of 0.0 to 0.3 Å⁻¹. A buffer blank for each SEC-SAXS run was prepared by averaging 10 to 20 frames before or after protein elution. Scattering data from peaks corresponding to BonA and BonA-27N were then buffer subtracted and scaled across the elution peak and compared for interparticle effects. Identical curves (S–10) from elution were then averaged to provide curves for analysis. Data were analyzed using the PRIMUS package, SCAtter, and DAMMIF modeler (65).

**Analytical ultracentrifugation.** Sedimentation velocity (SV) determination was carried out in a Beckman Coulter Optima analytical ultracentrifuge using an An-50 Ti 8-hole rotor. BonA-FL (370 μl) at concentrations ranging from 0.25 to 2 mg/ml was loaded into a 12-mm path-length centerpiece and centrifuged at 40,000 rpm for ~6 h at 20°C. Scans were collected every 20 s using absorbance optics (at 230, 240, and 280 nm, with a radial range of 5.8 to 7.2 cm and a radial step size of 0.005 cm). We used 50 mM Tris–200 mM NaCl (pH 7.9) as the buffer. Data were analyzed with SEDFIT using the continuous c(s) distribution model (66). SEDNTERP was used to calculate the partial specific volume, the buffer density, and the viscosity at 15 and 20°C.

**On-column cross-linking and negative-stain electron microscopy.** To stabilize the BonA decamer an "on-column" cross-linking method was used. Initially, 200 μl of glutaraldehyde solution (0.05 to 0.5% in dH₂O) was injected to a preequilibrated Superdex 200 10/300 column in buffer (20 mM HEPES, 150 mM NaCl [pH 7.4]). The column was run at 0.25 ml/min for 20 min (5 ml buffer). Subsequently, the column flow was paused, and the injection loop was flushed using buffer, followed by injection of purified BonA (200 μl at 10 mg/ml). Subsequently, the column was run at 0.25 ml/min, and 0.5-ml fractions were collected. Collected fractions were immediately quenched by the addition of 50 μl of 50 mM Tris (pH 7.5). The crosslinking efficiency was visualized by running the individual fractions on a 10% SDS gel, and cross-linked fractions were flash-frozen for NS-EM analysis.

Native and cross-linked BonA were serially diluted in buffer (20 mM HEPES, 150 mM NaCl [pH 7.4]), and 5 μl was spotted onto freshly glow-discharged carbon-coated 200-mesh copper grids (Pelco), followed by blotting to remove all but a thin film of protein solution. Blotted grids were fixed with the tungsten-based Nano-W stain (Nanoprobes) by adding the stain to each grid, followed by 60 s of incubation and blotting; this was repeated three times prior to air drying. The grids were imaged on a 120-keV Tecnai Spirit G2 microscope (FEI) equipped with a 4 K FEI Eagle camera. Images were processed, particles were picked, and 2D classes were generated using the RELION package (v2.1) (67).

**Data availability.** The crystallographic coordinates and associated structure factors for BonA are available in the Protein Data Bank (PDB) under accession code 6V4V. Small-angle X-ray scattering data for BonA full-length and BonA-27N are available in the SASBDB with the accession codes SASDJW3 and SASDJX3. The accession numbers of protein sequences used to construct the phylogenetic tree are provided in Table S1 in the supplemental material.

**SUPPLEMENTAL MATERIAL**

Supplemental material is available online only.

**FIG S1,** JPG file, 0.4 MB.

**FIG S2,** JPG file, 0.8 MB.

**FIG S3,** JPG file, 2.2 MB.

**FIG S4,** JPG file, 0.2 MB.

**FIG S5,** JPG file, 0.8 MB.

**TABLE S1,** XLSX file, 0.1 MB.

**TABLE S2,** XLSX file, 0.01 MB.

**TABLE S3,** XLSX file, 0.01 MB.

**TABLE S4,** XLSX file, 0.01 MB.

**TABLE S5,** XLSX file, 0.01 MB.

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R.G., F.C.M., R.A.D., E.H., and T.L. conceived and designed the experiments. R.G., F.C.M., R.A.D., M.B., A.K., S.D.G., and N.E.S. performed the experiments. R.G., F.C.M., R.A.D., M.B., S.D.G., P.M.L., E.H., and T.L. analyzed the data. R.G., S.B., A.Y.P., C.G., J.L., E.H., T.L., and N.E.S. contributed reagents/materials/analysis tools. R.G. and T.L. wrote the paper. All authors edited and approved the manuscript.

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