The structure of *Acinetobacter*-secreted protease CpaA complexed with its chaperone CpaB reveals a novel mode of a T2SS chaperone–substrate interaction

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Members of the *Acinetobacter baumannii*-calcoaceticus complex are nosocomial pathogens frequently causing multidrug-resistant infections that are increasing at alarming rates. *A. baumannii* has become the Gram-negative bacterium with the highest rate of multidrug resistance. As such, it is categorized by the World Health Organization as a critical priority for the research and development of new antimicrobial therapies. The zinc-dependent metalloendopeptidase CpaA is a predominant substrate of the type II secretion system (T2SS). CpaA is also a virulence factor of medically relevant *Acinetobacter* strains that specifically degrade the human glycoprotein coagulation factor XII and not its deglycosylated form, but the mechanism for this specificity is unknown. CpaB is a membrane-anchored T2SS chaperone that interacts with CpaA and is required for its stability and secretion. Here, we report the crystal structure of the CpaAB complex at 2.6-Å resolution, revealing four glycan-binding domains in CpaA that were not predicted from its primary sequence and may explain CpaA’s glycoprotein-targeting activity. The structure of the complex identified a novel mode for chaperone–protease interactions in which the protease surrounds the chaperone. The CpaAB organization was akin to zymogen inactivation, with CpaB serving as a prodomain that inhibits catalytically active CpaA. CpaB contains a C-terminal tail that appears to block access to the CpaA catalytic site, and functional experiments with truncated variants indicated that this tail is dispensable for CpaA expression and secretion. Our results provide new insight into the mechanism of CpaA secretion and may inform the future development of therapeutic strategies for managing *Acinetobacter* infections.

The *Acinetobacter baumannii*-calcoaceticus complex is comprised primarily of *A. baumannii*, *Acinetobacter nosocomialis*, *Acinetobacter calcoaceticus*, and *Acinetobacter pittii*, nosocomial pathogens that are a frequent cause of serious multidrug-resistant infections associated with high mortality rates (1) worldwide in immunocompromised individuals (2). *Acinetobacter* species possess several protein secretion systems, including type I, type VI, and type II secretion systems (T2SS), which contribute to virulence (3–7). A zinc-dependent metalloendopeptidase CpaA is the most abundant T2SS substrate (4) and CpaA expression and secretion is conserved across several medically-relevant *Acinetobacter* species (4, 8). Deletion of CpaA results in attenuation of *A. nosocomialis* M2, establishing the importance of this T2SS substrate in virulence (8). CpaA had been previously shown to cleave factor V and deregulate blood coagulation (9), and is required for virulence against *Galleria mellonella* larvae and is critical for dissemination of *A. nosocomialis* to the spleen in a murine pulmonary model of infection (8). The human coagulation factor XII (fXII) was recently reported as a substrate of CpaA (10). It was shown that CpaA cleaves factor XII at two sites Pro^{279}–Thr^{280} and Pro^{308}–Thr^{309} located in the proline-rich domain of fXII between proline and O-linked glycosylated threonine. Deglycosylation of fXII prevents cleavage by CpaA (10). However, why CpaA specifically targets glycosylated proteins is not understood.

Membrane-bound T2SS chaperone CpaB is required for the stability and secretion of CpaA (4). This is reminiscent of the *Burkholderia glumae* type II substrate, LipA, which requires a membrane bound, lipase-specific foldase (Lif), to be folded into its active form and secreted (11). Therefore, CpaB and Lif belong to a unique class of T2SS, membrane-bound chaperones, which possess evident functional analogies. Their role in T2SS is reminiscent of the type III secretion system (T3SS), where the chaperones are soluble cytoplasmic proteins that aid in the specific targeting of substrates to the secretion apparatus.

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The abbreviations used are: T2SS, type II secretion system; Lif, lipase-specific foldase; SAXS, small angle X-ray scattering; SAD, single-wavelength anomalous diffraction; βME, β-mercaptoethanol; CD, catalytic domain; PD, prodomain.

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The atomic coordinates and structure factors (code 6O38) have been deposited in the Protein Data Bank ([http://wwwpdb.org/](http://wwwpdb.org/)).

This article contains Tables S1–S4 and Figs. S1–S6.

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The structure of CpaA complexed with its chaperone CpaB

in folding, stabilization, and/or regulating secretion of T3SS substrates (12, 13). However, CpaB and Lif chaperones do not present any sequence similarity and are different in size and pl. CpaB is the first reported membrane-bound, periplasmic chaperone required for secretion of a protease via a T2SS.

CpaA and CpaB interact strongly and the C-terminal periplasmic domain of CpaA is sufficient for its chaperone function and interaction with CpaA, whereas the transmembrane domain of CpaB keeps it tethered to the inner membrane preventing its secretion with CpaA (8). Although the necessity of CpaB for secretion of CpaA has been established, whether its role in secretion of CpaA goes beyond stabilizing CpaA has not been determined. One possibility is that this interaction inactivates CpaA preventing self-intoxication of the cell by the proteolytic activity of CpaA. However, CpaB binding appears not to block the proteolytic activity of CpaA against human factor V (8).

The lack of structural information for the CpaAB complex, as well as for each of the proteins alone, hinders the development of strategies to treat antibiotic-resistant infections through the inhibition of CpaA. The basis behind the specificity of CpaA for glycosylated FXII is unknown (14). Here, we present the crystal structure of the complex of CpaA in complex with the C-terminal periplasmic domain of its chaperone CpaB at 2.6-Å resolution. These studies reveal novel insight into chaperone–substrate interaction, proteolytic regulation, and substrate-specificity of an important T2SS substrate and chaperone. The results of this study constitute a significant advance in the development of novel antivirulence treatments for Acinetobacter infections designed based on the structure of CpaA.

Results

Overall structure of the CpaAB complex

We determined the crystal structure of metallopeptidase CpaA in complex with the C-terminal periplasmic domain of its chaperone CpaB by the single-wavelength anomalous diffraction (SAD) method using crystals of selenomethionine-incorporated protein to 2.6-Å resolution (Fig. 1, Fig. S1, and Table 1). The protein constructs and residue numbering used in this study compared with reference sequences of the proteins in Acinetobacter are shown on Fig. S2. The electron density maps are clearly interpretable for the majority of the complex and the density explicitly defined the main and side chain atoms (Fig. S3). There are six copies of the complex in the asymmetric unit. Each copy is composed of one CpaA bound to one CpaB establishing the stoichiometry of the complex as 1:1. Due to specific arrangement of chaperone–protease assembly where the protease surrounds the chaperone (Fig. 1), interactions between CpaA and CpaB are prevalently intradimer contacts. This leads to an average buried surface area of 2099.7 Å² between CpaA and CpaB. The next largest buried surface area is 560 Å² that occurs between two CpaA molecules. The large buried surface area of ∼2099.7 Å² suggests the dimer of CpaA and CpaB observed in the crystal structure is likely to occur in solution. The structure reveals a modular architecture of CpaA that contains four very similar predominantly β-sheet repeats (domains 1 to 4), and a fifth domain with peptidase activity (catalytic domain) (Fig. 1).

Small angle X-ray scattering (SAXS) of CpaAB demonstrates the solution state is a 1:1 complex

To unambiguously establish the solution state and solution structure of CpaA we performed a SAXS analysis. SAXS profiles for CpaAB matched the crystal structure of the 1:1 complex very well with a χ^2 value of 1.02 (Fig. 2). The ab initio envelope reconstruction from the SAXS profile resulted in an envelope that closely resembles the 1:1 CpaAB crystal structure. These results demonstrate the crystal structure of the CpaAB complex is representative of the solution state of CpaAB, and that CpaA wraps around CpaB upon interaction in solution.

Comparison of CpaAB to a known T2SS chaperone:substrate complex

Each molecule of CpaB is embedded into the core of a CpaA molecule through multiple interactions (Fig. 1). The only other type II secretion chaperone:substrate complex studied of Lif/LipA suggested a model in which the chaperone wraps around the substrate and protects the cargo. In contrast, the CpaAB complex reveals entirely different arrangement of chaperone–protease interaction. Each CpaB chaperone is surrounded by the CpaA protease (Fig. 3). This novel finding extends the understanding of chaperone–substrate interplay.

CpaA contains a canonical zinc metalloprotease domain

The metalloprotease catalytic module immediately follows repeat 4 and spans residues 372–574 (Figs. 1 and 4A, and Fig. S2). This domain of CpaA resembles several features of the canonical metzincin superfamily of metallopeptides including a characteristic α+β-fold, extended zinc-binding motif HEXXXHXXGXXH, and a conserved Met-turn (15) (Fig. 4B). The three histidine residues (His⁴⁹⁸, His⁵⁰², and His⁵⁰⁸) are responsible for binding the catalytic zinc ion, the glutamate residue (Glu⁴⁹⁹) is the general base/acid residue involved in catalysis, whereas the Met-turn (Met⁵²⁷) assists in the folding and provides stability to the catalytic domain (Fig. 4A and Fig. S2) (17). To confirm these canonical metalloprotease features, we generated a Glu⁴⁹⁹ to Ala point mutation and tested CpaA activity in vitro. Mutation of CpaA Glu⁴⁹⁹ to Ala produced a catalytically inactive protease incapable of degrading Factor V (Fig. 5).

Another important structural feature observed is a stretch of glycines (CpaA Gly⁴⁶⁶, Gly⁴⁶⁸, Gly⁴⁶⁹, and Gly⁴⁷¹) (Fig. 4B). In other metzincin superfamily members, this conserved glycine stretch likely confers plasticity to the active site cleft and facilitates recognition of the O-glycan–induced conformation (16). Finally, the peptidase domain also shows high structural similarity to adamalysins/ADAMs.

CpaA possesses four glycan-binding–like domains

The four tandem repeats of CpaA span residues 2–94 (repeat 1), 95–190 (repeat 2), 191–284 (repeat 3), and 285–371 (repeat 4). Pairwise superposition of repeats confirms they all share a similar fold (Fig. 4C and Table S1). Structural similarity search
using the DALI server (17) revealed that these four repeats resemble glycan-binding domains. Enterohemorrhagic Escherichia coli, zinc metalloprotease StcE (PDB ID 4DNY) (Fig. 4D) specifically targets densely O-glycosylated substrates (16) and shows structural similarity with all four repeats (Fig. 4, E–H): Dali Z-score: 10.6, r.m.s. deviation 2.2 Å for repeat 1; Dali Z-score: 8.4, r.m.s. deviation 2.7 Å for repeat 2; Dali Z-score: 10.4, r.m.s. deviation 1.9 Å for repeat 3; Dali Z-score: 8.3, r.m.s. deviation 1.9 Å for repeat 4). The four repeats form a large cavity with a buried surface area of 2,119 Å² taken up by CpaB in the complex. This large cavity may accommodate substrates during proteolysis. Each CpaB molecule contacts all four repeats potentially blocking access for binding of glycoprotein substrates.

**CpaA putative substrate-binding cleft**

The structure showed a large cavity in the center of CpaA formed by the contribution from all subdomains of CpaA including the four tandem repeats and a peptidase domain. This cavity comprises the active site located in the peptidase domain and a large concave surface toward the catalytic site that may serve for substrate recognition. However, the identity, recognition, and mode of interaction of CpaA proteolytic substrates remain unknown.
Pairwise comparison of the six molecules of CpaA in the asymmetric unit showed negligible r.m.s. deviations ranging from 0.155 to 0.371 Å. A majority of the differences are in loops of the protease domain, whereas the glycan-binding repeats remain unchanged.

The structure and organization of CpaA provide insight into potential substrate specificity. Structural superposition of CpaA with metzincin superfamily members revealed several unique structural elements that are typical for cleavage of densely O-glycosylated substrates. StcE is a 100 kDa metallo-protease that specifically interacts with O-glycosylated substrates (18, 19) and its INS domain has the highest DALI score for all CpaA repeats.

The StcE INS glycan-binding domain contains a cluster of conserved solvent-exposed residues, Trp189, Arg191, and Tyr214 (Fig. 4B), suitable for binding glycoproteins (16). Pairwise superposition of the INS domain with each of the four CpaA tandem repeats shows that all four repeats retain the solvent-exposed loop containing a conserved tryptophan residue at the same position, whereas the other two residues of the triad are not conserved (Fig. 4, D–G). Curiously, only residue Lys34 of the triad analogs in repeat 1 is involved in chaperone binding (Fig. 4D) and the corresponding loop is tilted toward the substrate-binding cleft, whereas the analogous loops in all other repeats are exposed to the solvent (Fig. 4A).

**CpaB shares similarity to a protease prodomain**

Metallopeptidase chaperone CpaB has no significant sequence similarity to any known protein. However, it shows high structural similarity with the prodomain of *Bacteroides fragilis* toxin (PDB ID 3p24, Dali score 5.8, r.m.s. deviation 2.8 Å), a zinc-dependent metallopeptidase (20). CpaB consists of a large twisted antiparallel β-sheet (strands β1 and β3–β8), which vertically traverses the whole domain (Fig. 6A, Fig. S4). Strand β3 is twisted and split between the vertical antiparallel β-sheet and a small two-stranded antiparallel β-sheet (strands β2 and β3).
The structure of CpaA complexed with its chaperone CpaB

A. Peptidase domain and substrate binding cleft.

B. Key residues: His 502, Gly-rich loop, His 498, His 508, Met 527.

C. Repeats 1 to 4.

D. Tyr214, Trp189, Arg191, Arg213.

E. Tyr56, Trp32, Lys34, Lys193.

F. Repeat 2/INS: Trp126, Val150, Gln128.

G. Repeat 3/INS: Trp220, Trp244, Ser222.

H. Repeat 4/INS: Trp315, Ala339, Pro317.
The structure of CpaA complexed with its chaperone CpaB

The interaction between protease CpaA and chaperone CpaB in CpaAB complex resembles interactions between the catalytic domain (CD) and the prodomain (PD) of fragilysin-3 (PDB ID 3p24) (Fig. 6, D and E) (20). The catalytic fragilysin-3 moiety is unique within the metzincin metallopeptidases as it shows high structural similarity despite negligible sequence identity to adamalysins/ADAMs (20). The PD in fragilysin-3 is thought to confer latency maintenance for the protein and serves as a chaperone that assists in the folding and stabilization of the fragilysin-3 catalytic domain. In the fragilysin-3 structure, the PD does not cap the CD but is attached to a lateral surface (Fig. 6E). The PD prevents access to the active-site cleft through the C-terminal segment, which runs in an extended conformation across the entire CD active site in the opposite orientation that substrates occupy. As in other prodomains, the PD of fragilysin-3 prevents access of substrates to active-site clefts of zymogens.

In addition to structural similarity between CpaB and the PD of fragilysin-3, superposition of CpaA peptidase domain/CpaB with fragilysin-3 reveals a similar organization of the domains and functional elements (Fig. 6, D and E). The CD of fragilysin-3 like CpaA also contains an extended zinc-binding motif and a conserved methionine within a tight 1,4-β-turn, the Met-turn (15, 21–25). The extended flexible fragment of the PD in fragilysin-3 traverses the catalytic domain of fragilysin similarly to the tail of the chaperone CpaB in CpaAB complex and provides the coordination partner for catalytic zinc (Fig. 6, D and E).

The fragilysin-3 PD plays a role in latency maintenance and also functions as a chaperone that assists in the folding and stabilization of the CD (20). The inhibitory mechanism follows an “aspartate switch” that has been described for proastacin and ADAMs/adamalysins (26). According to this mechanism, an aspartate of the PD coordinates and inactivates the catalytic zinc ion by replacing the solvent molecule usually found in mature CDs primed for catalysis (27). The solvent is further linked to a general base/acid, which is generally a glutamate. In CpaA this general base/acid glutamate appears as Glu499. The structural conservation suggests a similar mechanism of inhibition occurs for CpaA by CpaB.

The structure and organization of CpaA:CpaB complex provide insight into chaperone–substrate interaction and multiple roles for this interaction can be proposed. Thus, in the following sections we attempted to evaluate some of them.

CpaB does not protect CpaA from self-proteolysis

Previously, we have observed that CpaA is unstable in the absence of CpaB (8). To investigate whether CpaB prevents the self-proteolysis of CpaA inside Acinetobacter cells, we looked at protein levels of CpaAE499A (catalytically inactive CpaA, Fig. 5) in the absence of CpaB by Western blot analy-

The CpaB tail blocks access to the CpaA catalytic site

In the CpaAB complex, the chaperone CpaB makes an extended network of polar interactions with the protease domain of CpaA (Table S2). The chaperone CpaB β-sheet strands β4–β7 (residues Asp110, Arg113, Thr131, Asp136, Gln142, Asp149, Gln153, and Lys155) interacts with CpaA helix 2 (residues Ser392, Leu393, Arg397, Glu400, and Asp531). In addition, the CpaB tail embeds in the catalytic active site of CpaA creating contacts between Gly167, Thr169, Asp170, and Glu172 of the CpaB tail and Cys670, Ser492, and catalytic zinc coordination partners His498, His502, and His508 of CpaA (Fig. 6B).

The structure of CpaA complexed with its chaperone CpaB

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The structure of CpaA complexed with its chaperone CpaB

A

B

C

D

E

CpaAB complex

Fragilysin-3
The structure of CpaA complexed with its chaperone CpaB

CpaB is required for stability and secretion of CpaA (8). Furthermore, CpaB interacts with all four glycan-binding domains of CpaA as well as the protease domain (Fig. 1). To investigate what interactions are required for stability and secretion of CpaA we generated CpaA glycan-binding domain deletions and tested expression and secretion of CpaA (Fig. S6). Deletion of one or more glycan-binding domains was sufficient to destabilize CpaA (Fig. S6). All glycan-binding domains of CpaA are required for its stability. Additionally, CpaA and CpaB interact through CpaB’s tail and the CpaA CD (Fig. 6B). The CpaB tail embeds in the catalytic active site of CpaA creating contacts between Gly167, Thr169, Asp170, and Glu172 of the CpaB tail and Cys470, Ser192, and catalytic zinc coordination partners His498, His502, and His506 of CpaA (Fig. 6B). To test the role of the CpaB tail domain in stability and secretion of CpaA we created point mutations of CpaB Asp170 and deleted the tail domain (CpaBΔ167–178) (Fig. 7). The mutations were selected to interfere with the zinc coordination; either by changing the net charge or the length of the interaction. All CpaB mutant variants were expressed and stable (Fig. 7A). We did not observe any effect of CpaB tail on CpaA stability (Fig. 7B) and secretion (Fig. 7C), not even when the entire C-tail was removed. Taken together these results indicate that CpaB C-tail does not assist on CpaA stability and secretion.

Discussion

The zinc-dependent metalloendopeptidase CpaA is secreted by the T2SS and plays an important role in Acinetobacter virulence (4, 8, 14). It requires a chaperone CpaB for correct folding, stability, and secretion (8). The protease and chaperone are encoded adjacent to each other in the genome and interact directly in the periplasm (8). However, the mechanism of the interaction as well as a role of the chaperone is unclear. We identified the molecular interactions involved in CpaA secretion through binding to its membrane-bound chaperone by solving the crystal structure of the CpaA in the presence of the C-terminal periplasmic domain of CpaB.

The structure showed that CpaA is reminiscent of metzincins (15) and possesses their superfamily features such as characteristic α+β-fold subdomains (1 to 4) with a glycan-binding domain—like fold, extended zinc-binding motif HEXX-HXXGXXH, a conserved Met-turn and a glycine-rich stretch in the catalytic domain. Mutagenesis of Glu499 to alanine abolishes CpaA-mediated cleavage of Factor V, confirming the structurally predicted catalytic Glu499 is required for the proteolytic activity of CpaA. CpaA bears structural and functional similarities to the E. coli–secreted glycoprotease StcE. However, both proteases share negligible sequence identity and exhibit different domain organization (16). There is a large sub-

Figure 6. Structural similarity with fragilysin-3 suggests an inhibitory mechanism for CpaAB. A, structural overlay of CpaB with the prodomain (PD) of fragilysin-3 (PDB ID 3p24, Dali Z-score: 5.8, r.m.s. deviation 2.8). Upper and lower panels are related by 90° rotation and shows CpaB in orange and PD in blue. B, CpaA/CpaB interface. Interacting residues are shown as sticks, zinc as a red sphere, and CpaA and CpaB are colored gray and orange, respectively. C, an electrostatic surface representation of CpaAB. Inset shows binding of the chaperone tail to a shallow solvent-exposed pocket in the catalytic domain of CpaA. Residues making hydrogen bonds are shown as sticks and colored white or orange, respectively. Zinc ion is shown in magenta. Hydrogen bonds are presented by dotted black lines. Zinc coordination environment is shown by a solid lines. D and E, structural similarity between CpaA and fragilysin-3 suggests an analogous inhibitory mechanism. Catalytic zinc coordination ligands and canonical Met in Met-turn are shown as sticks. CpaA and CpaB are colored gray and orange, respectively (D). CD and PD of fragilysin-3 are shown in magenta and blue, respectively (E).
The structure of CpaA complexed with its chaperone CpaB

strate-binding cleft in the center of CpaA formed by the residues from four structurally similar tandem repeats and the peptidase domain. A glycine-rich stretch on the peptidase domain region, contributing to the cleft, confers high conformational freedom to the polyglycine backbone and facilitates recognition of the O-glycan induced conformation. The presence of glycan-like binding domains explains why glycosylation of proteins is required for CpaA activity (14). Our data suggests a potential mechanism by which CpaA specifically targets its substrates through the interactions of its glycan-binding domains with the glycan epitopes on target proteins (10).

CpaB and Lif belong to a novel class of T2SS chaperones, which possess evident functional analogies. In contrast to the functionally related Lif chaperone required for folding and secretion of LipA (11), chaperone CpaB does not engulf the cognate effector protein but instead is surrounded by the protease. This is an unusual arrangement and provides new understanding of chaperone binding and possible function. The results presented here suggest a putative regulatory mechanism more akin tozymogen regulation. The CpaAB complex is stabilized by an extended network of hydrogen bonds and salt bridges between CpaA and CpaB (Table S2). These interactions ensure a strong interaction between the two proteins. CpaB tail residues Gly167–His175 form several interactions with CpaA. Despite the requirement of CpaB for secretion and stability of CpaA, the tail region of CpaB is not responsible for those functions.

In the absence of CpaB, CpaA is cleaved into a 30-kDa fragment. We determined that this fragment is not generated by an autocatalytic cleavage event in the absence of CpaB. In addition to the stabilizing protein–protein interactions, the CpaB tail (residues Gly167–His175) forms hydrogen bonds with the amino backbone groups of Cys370, Ser492, and His508, and the imidazole nitrogen atoms of His498 and His508 in the CpaA catalytic domain (Fig. 6, B and C). A zinc ion required for CpaA peptidase activity is found at the catalytic site, also located at the interface between CpaA and CpaB. The catalytic triad of His498, His502, and His508 of CpaA make a zinc-coordination sphere that adopts tetrahedral coordination geometry (Fig. 6C). In addition, Asp170 of the CpaB tail is the fourth coordination ligand for the zinc ion. One possibility is that CpaB limits accessibility to the catalytic triad of CpaA and is a possible mechanism of inactivation of the CpaA to prevent self-intoxication of the cell prior to secretion. Therefore, the CpaB chaperone may serve as an immunity protein that shields Acinetobacter from CpaA peptidase activity.

We have previously shown that CpaB lacking its membrane domain is secreted along with CpaB (8). Displacement of CpaB through a putative interaction with the T2SS machinery would be required to dissociate the chaperone from the protease. It is tempting to speculate that CpaB can additionally contribute to target CpaA to the secretion machinery.

The elucidation of the structure of CpaA is one important step toward the design of novel antivirulence treatments for Acinetobacter infections through the structure-based development of CpaA-specific inhibitors. This study opens a new page in the understanding of chaperone–secreted protein interactions and extends our knowledge of type II substrate secretion and regulation providing a novel targeted strategy to the elimination of multidrug-resistant nosocomial infections.

Experimental procedures

Strains, plasmids, and growth conditions

Bacterial strains and plasmid used in this study can be located in Table S3. E. coli Stellar and A. nosocomialis M2 cells were grown in Lennox broth (LB) at 37 °C. pWH1266-based plasmids were selected with tetracycline (5 µg/ml).

Protein expression, purification, and crystallization

Selenomethionine-substituted CpaAB (SeMet CpaAB) was generated using the same expression construct (pETDUET-CpaAHis-CpaB) and E. coli BL21 Rosetta 2 (Novagen) strain as described (8). The constructs employed express the mature form of CpaA and a soluble form of CpaB lacking its transmembrane domain. The constructs are represented in Fig. S2. Cells were grown in SelenoMet Medium (M9 minimal media supplemented with glucose, vitamins, and amino acids, Molecular Dimensions Ltd., MD12-501), ampicillin (100 mg/ml), and chloramphenicol (12.5 mg/ml) at 37 °C until the A600 reached ~0.6. The following amino acids were added for 15 min before isopropyl 1-thio-β-D-galactopyranoside induction at 30 °C for 12 h: lysine, phenylalanine, and threonine (100 mg/liter each) and isoleucine, leucine, valine, and dl-selenomethionine (50 mg/liter each). Purification was performed under the same conditions as described (8). Briefly, cells were pelleted at 8,000 × g for 10 min, washed with 30 mM Tris, pH 8.0, and resuspended in 20 ml of 50 mM NaH2PO4, 300 mM NaCl, and 10 mM imidazole, pH 8.0. Cells were lysed with two rounds of a cell disruptor using 35 k.p.s.i. (Constant System Ltd., Kennesaw, GA). Cell lysates were centrifuged at 11,000 rpm for 30 min, 5 mM β-mercaptoethanol (βME) was added and the lysate was passed over a nickel-nitrioltriacetic acid–agarose column (Gold Bio, St. Louis, MO). After washing the column with 50 mM NaH2PO4, 5 mM βME, 300 mM NaCl, and 25 mM imidazole, pH 8.0, and 50 mM NaH2PO4, 300 mM NaCl, and 50 mM imidazole, pH 8.0, protein was eluted with 50 mM NaH2PO4, 5 mM βME, 300 mM NaCl, and 250 mM imidazole, pH 8.0. Further purification was done on a Superdex 200 gel filtration column in 100 mM NaCl, 10 mM HEPES, pH 7.2. SeMet derivative crystals were obtained by sitting drop vapor diffusion at 18 °C by mixing 1.0 µl of protein (15 mg/ml) and 1.0 µl of seed stock. To prepare seed stock, the 1-µl drop containing the original clustered crystals (grown from 1.3 M magnesium sulfate, 0.1 M MES buffer, pH 6.5) was mixed with 9 µl of well solution (1.28 M magnesium sulfate, 0.1 M MES buffer, pH 6.5) and pipetted up and down. Crystals were cryoprotected by transfer to a well solution including 25% glycerol for 30 s, plunged into liquid nitrogen, and kept at 100 K during data collection.

Data collection and structure determination

Because both CpaA and CpaB have no sequence homologs the structure of the complex was determined by selenium SAD. The data collection was performed at the selenium peak wavelength to 2.6-Å resolution at the Advanced Photon Source 23-IDD beamline. Data were processed with HKL-3000 (28).
The structure of CpaA complexed with its chaperone CpaB

The anomalous completeness of the data were 98.3% (87.2% in outer shell), with an anomalous multiplicity of 12.0 overall (7.0 in outer shell). Anomalous correlation between half-sets was 0.760 in the inner shell and 0.280 overall. SHELEXC, SHELDX, and SHELEXE (29) were used in pipeline for substructure determination and phase approximation. SHELEXC found 72 selenium atoms of 72 possible selenium sites. Phasing was done with MLPHARE (30) followed by density modification carried out by DM (30). The initial model was built by Buccaneer (30) and refined in Refmac5 (31) coupled with Parrot (30) for further density modification and manual model correction in COOT (32). The structure model was refined with Refmac5 to $R_{\text{work}}/R_{\text{free}}$ factors as 23.6/28.5%. Then the refinement was carried on with Phenix (33) to the final $R_{\text{work}}/R_{\text{free}}$ factors as 21.5/23.3%. The Ramachandran plot showed that 100% of residues were allowed with 96.84% in the preferred region. The electron density maps are clearly interpretable for the majority of the complex with a few exceptions for the N terminus of CpaA chains C (residue range 2–35, 40–48, 53–58, and 84–90) and F (residues 2–5, 29–32, and 53–56) and for the N-terminal residues of CpaB chain G (residues 1–31, 50–65), chain H (residues 1–41, 50–65), chain K (residues 1–39, 49–66), chain L (residues 1–28, 50–65), chain M (residues 1–31, 50–65), and chain N (residues 1–36, 50–64), which are disordered. X-ray data and refinement statistics are shown in Table 1.

Small angle X-ray scattering

Data for the CpaAB complex were collected at the SIBYLS beamline 12.3.1 at ALS using standard procedures (34), and analyzed using the ATSAS package, version 2.8.3 (35). A series of 0.3-s exposures were collected at 0.5, 1, and 2 mg/ml of CpaAB in 10 mM HEPES, pH 7.4, 150 mM NaCl in triplicate. The first 12 exposures of each collection were analyzed and merged using SAXS FrameSlice (sibyls.als.lbl.gov/ran). The triplicate concentration runs were assessed and merged using PRIMUS. Experimental profiles were compared with the structure using CRYSOL. Ab initio model generation was performed in DAMMIF, and the filtered average envelope of 10 models was obtained by DAMAVER. SUPCOMB was used to align structures and SAXS reconstructions.

Generation of CpaA and CpaB point mutations and deletions

The polymerase chain reaction (PCR) primers used for site-directed mutagenesis are listed in Table S4. To generate a catalytically inactive, and the truncated variants of CpaA, pWH-cpaA-his-cpaB (4) was used as the template. To generate single and double amino acid substitutions, and the truncations in CpaB, pWH-cpaA-cpaB-his (8) was used as template. PCR was carried out using Phusion DNA Polymerase (Thermo Scientific). Site-directed mutagenesis was performed according to the method described by Fisher and Pei (36). Truncation variants were generated using the In-fusion HD EcoDry cloning kit. The ligation products were transformed into E. coli Stellar cells and transformants were selected in LB-agar supplement with tetracycline. Transformants were subcultured and the plasmids were purified and verified by sequencing. The pWH-based constructs were then electroporated into electrocompetent A. nosocomialis M2 ΔcpaAB::frt strains.

Immunoblotting

Bacterial secretion whole-cell and supernatant samples were prepared as published previously (4, 8). Briefly, cultures were grown to 0.5 AU/ml, and 0.5 AU was pelleted by centrifugation and resuspended in 50 μl of 1 × Laemmli buffer for the whole-cell samples. Supernatant samples were obtained by TCA-precipitating cell-free supernatants as published previously (7). Proteins were resolved by SDS-PAGE analysis and transferred to a nitrocellulose membrane by semidry transfer and probed with either polyclonal anti-histidine (1:2000, Pierce), polyclonal anti-CpaA (1:1000 (8)), and/or monoclonal anti-RNA polymerase (1:2000, Biologend). Western blots were probed with IRDye-conjugated secondary antibodies and visualized with an Odyssey CLx imaging system (LI-COR Biosciences, Lincoln, NE).

Factor V cleavage assay

CpaA-His or CpaAE499A-His were purified out of the supernatant of A. nosocomialis M2. Briefly, A. nosocomialis ΔcpaAB::frt carrying pWH-cpaA-his-cpaB or pMFH32 was grown in LB supplemented with tetracycline to mid-log phase. His-tagged proteins were purified from cell-free supernatants through nickel affinity chromatography as above. The purified proteins were concentrated, and the buffer was changed to 20 mM HEPES, 150 mM NaCl, and 50% glycerol, pH 7.4, using Amicon Ultra centrifugal filter units.

The Factor V cleavage assay was carried out according to Kinsella et al. (8). Briefly, 1.6 μg of purified CpaA or CpaAE499A (in 20 mM HEPES, 150 mM NaCl, and 50% glycerol, pH 7.4) was mixed with 11 ng of human Factor V (Abcam, Cambridge, MA) in a total volume of 20 μl (20 mM HEPES and 150 mM NaCl, pH 7.4). All samples were incubated at 37 °C for 30 min. Factor V cleavage was monitored by Western blotting by resolving 8.2 ng of Factor V per lane on an 8% polyacrylamide gel. Factor V was detected using polyclonal sheep anti-human Factor V (Thermo Fisher, 1:1,000), polyclonal rabbit anti-sheep Ig (Fc-specific, Sigma, 1:12,000), and IRDYE® 800CW goat anti-rabbit IgG (LI-COR, 1:15,000). CpaA-His was detected with monoclonal mouse anti-His6 (Thermo Fisher, 1:1000) and IRDYE® 680RD goat anti-mouse IgG (LI-COR, 1:15,000).

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