Monitoring the antibiotic darobactin modulating the β-barrel assembly factor BamA

Highlights

- Mechanical, kinetic, and energetic properties of BamA
- Properties change upon binding to an antibiotic
- Structural regions change mechanical stability and lifetime
- Structural regions change free-energy and mechanical rigidity

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In brief

Ritzmann et al. employ SMFS to investigate BamA, the central unit of the BAM complex of E. coli. They quantify how the antibiotic darobactin modulates the mechanical, kinetic, and energetic properties of the POTRA domains, the linker domain, and the β-hairpins of the transmembrane β-barrel of BamA.
Monitoring the antibiotic darobactin modulating the β-barrel assembly factor BamA

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SUMMARY

The β-barrel assembly machinery (BAM) complex is an essential component of Escherichia coli that inserts and folds outer membrane proteins (OMPs). The natural antibiotic compound darobactin inhibits BamA, the central unit of BAM. Here, we employ dynamic single-molecule force spectroscopy (SMFS) to better understand the structure-function relationship of BamA and its inhibition by darobactin. The five N-terminal polypeptide transport (POTRA) domains show low mechanical, kinetic, and energetic stabilities. In contrast, the structural region linking the POTRA domains to the transmembrane β-barrel conformation, which spans the outer membrane in Escherichia coli, the insertion and folding of β-barrel proteins into the outer membrane is facilitated by the β-barrel assembly machinery (BAM) complex. The BAM complex consists of five protein components. These are BamA, an integral OMP and a member of the Omp85 superfamily (Gentle et al., 2004; Voulhoux et al., 2003), and four lipoproteins, BamB, BamC, BamD, and BamE, which are anchored to the inner leaflet of the outer membrane (Wu et al., 2005). BamA, the central component of the BAM complex, consists of a C-terminal 16-stranded transmembrane β-barrel domain and five periplasmic N-terminal polypeptide transport (POTRA) domains (Gentle et al., 2005; Sánchez-Pulido et al., 2003). Although all components of the BAM complex are necessary to efficiently insert proteins into the outer membrane (Hagan et al., 2010), only BamA and BamD are essential (Malinverni et al., 2006; Voulhoux et al., 2003).

The exact mechanism by which β-barrel OMPs are rapidly folded and integrated into the outer membrane by the BAM complex in the absence of an energy source such as ATP presumably occurs via the “budding model” but remains debated (Höhr et al., 2018; Lee et al., 2019; Tomasek et al., 2020). The first step of the insertion process is initiated by hydrogen bonding of a C-terminal signal sequence of the incoming substrate to the N-terminal β-strand of BamA (Lee et al., 2019; Robert et al., 2006; Xiao et al., 2021). β-sheets of the substrate are then thought to fold, starting from the C-terminus into the interior of the β-barrel. Upon reaching sufficient folding, the substrate is released into the outer membrane through a lateral gate formed by the first N-terminal and the last C-terminal β-strand of the transmembrane BamA β-barrel (Tomasek and Kahne, 2021). Compared to most other β-barrel-forming OMPs, the first and the last β-strand of the BamA β-barrel establish loose interactions. This results in a structurally dynamic region, referred to as the gate region, that takes a central role in inserting, folding, and releasing OMPs into the outer membrane (Noinaj et al., 2013). However, which mechanical and kinetic properties of the BamA β-barrel retain the unique dynamic state of the gate region remain to be characterized.

Because the BAM complex is essential for Gram-negative bacteria, it renders a promising antibiotic target. Compared to other essential components of Gram-negatives, the BAM complex is located at the cellular periphery and thus directly exposed to the external environment. Recently, the natural compound darobactin was identified to inhibit BamA. Darobactin is a bicyclic heptapeptide produced by Photorhabdus, found in the microbiome of...
entomopathogenic nematodes (Imai et al., 2019). The recent structure of the BAM complex bound to darobactin revealed darobactin binding to the functionally relevant gate region of the BamA β-barrel (Kaur et al., 2021). In order to inhibit BamA, darobactin mimics the C-terminal β-signal of native BamA substrates and binds to the first and last β-strand of the BamA β-barrel, which seals the lateral gate. Although the structure of the BamA-darobactin complex describes in great detail how darobactin inhibits BamA, it does not describe how darobactin affects the mechanical, kinetic, and energetic properties of BamA. Thus, quantifying how darobactin modulates these properties at a resolution of individual secondary structural elements would complement the structure of the BAM complex and allow us to draw a more complete picture of the darobactin-mediated BamA inhibition. Additionally, such insight is relevant for the targeted engineering of novel antibiotics, which could inhibit BamA similarly to darobactin.

Here, we characterize quantitatively and structurally how darobactin modulates the mechanical, kinetic, and energetic properties of BamA in the native outer membrane. Thereto, we apply atomic force microscopy (AFM)-based single-molecule force spectroscopy (SMFS), which allows us to quantify the mechanical properties of individual membrane proteins and to map them to their structural regions such as α-helices, β-hairpins, or polypeptide loops (Engel and Gaub, 2008; Oesterhelt et al., 2000; Thoma et al., 2017). The results provide an important and unique insight into the mechanisms by which darobactin inhibits BamA, guiding toward the mechanistic understanding of how to functionally inhibit BamA.

RESULTS

BamA stabilizes distinct structural segments

To characterize BamA in the native membrane, we prepared outer membrane vesicles (OMVs) enriched with BamA (Figure 1A). For SMFS, the OMVs were adsorbed to mica where they opened up and formed planar membrane patches with diameters ranging from 100 nm to 1 μm (Figure 1B). Membrane patches of densely packed BamA showed corrugated topographs, elevated 10–15 nm in height from the mica (Figure 1C). We then pushed the tip of the AFM cantilever onto BamA-enriched regions with a force of 1 nN for 0.5 s to promote the non-specific attachment of the tip to the N-terminal region (Thoma et al., 2018a, 2018b). Such FD curves showed the mechanical extension and stretching of the N-terminal region, which was followed by the stepwise unfolding of structural segments, until a single BamA was fully unfolded and extracted from the outer membrane. We repeated this procedure thousands of times to identify the reoccurring mechanical unfolding pattern specific to BamA in the absence of the antibiotic darobactin.

To compare the FD curves recorded upon mechanically unfolding single BamA, we aligned them in the force contour length space, where the prominent force peak at a contour length of 406 aa was used as a reference (Figures 2C and S1A). The alignment revealed 15 force peak classes (Figures 2D and S1B). Each of the force peak classes detected the unfolding of a structural segment of BamA in response to mechanical stress (Thoma et al., 2018a, 2018b). Counting from the N-terminal region, which was picked up by the AFM tip, we used the contour length of each force peak class to assign the structural segment BamA, unfolded in response to mechanical stress (Figure 2E). The first five force peak classes describe the unfolding of the five N-terminal POTRA domains of BamA (P1–P5), followed by the force peak class describing the short linker region (LR) between the
POTRA domain P5 and the transmembrane β-barrel. The next force peak classes are located in the β-barrel region of BamA and describe the unfolding of one β-hairpin after the other until the eight β-hairpins (H1–H8) are completely unfolded. One additional force peak class located between β-hairpins H6 and H7 corresponds to the unfolding of extracellular loop 6, which is locked in a lid-like (LL) structure to seal the β-barrel lumen from the extracellular space (Maier et al., 2015).

In summary, the FD curves recorded upon mechanically unfolding single BamA from the native outer membrane reveal a sequence of force peaks, each of which assigns the unfolding of a structural segment of the membrane protein. The forces at which each segment unfold are a direct measure of their mechanical stability.

**Darobactin increases mechanical stability of BamA**

Next, we unfolded BamA in the presence of darobactin in order to determine how darobactin binding changes the mechanical stability of BamA. At first sight, the force peak classes and thus the structural segments stabilizing BamA in the absence and presence of darobactin were very similar (Figures S1 and S2). Thus, darobactin did not change the structural segments BamA stabilizes against mechanical unfolding. Additionally, the mechanical forces required to unfold the individual POTRA domains were mostly unaffected by darobactin, except for POTRA domain P5, which increased from 136 pN to 149 pN (to ≈110%); Figure 3). Similarly, the mechanical stability of the LR increased weakly in the presence of darobactin. In large contrast, however, the forces required to mechanically unfold structural segments of the β-barrel region of BamA increased considerably in the presence of darobactin. In principle, every structural segment of the β-barrel, most of which representing a single β-hairpin, required higher forces to unfold than in the absence of darobactin. Upon adding up these forces, we found that 1.45 ± 0.15 nN (mean ± sd) was required to unfold all structural segments of the BamA β-barrel in the absence and that 1.67 ± 0.08 nN was required to completely unfold the β-barrel in the presence of darobactin.
Darobactin modulates free-energy landscape

The mechanical forces at which proteins unfold depend on the pulling speed (i.e., the loading rate describing the force applied over time) (Bippes and Muller, 2011; Evans, 2001; Evans and Ritchie, 1997). In response to mechanically applied force membrane proteins, stepwise unfold structural segments (Bippes and Muller, 2011), such as that observed here for BamA. The mechanical properties and the kinetic or energetic stability of the structural segments at equilibrium (i.e., no force applied) can be approached by measuring their unfolding forces over a broad range of pulling speeds. We hence applied SMFS in the dynamic force spectroscopy (DFS) mode and mechanically unfolded BamA over a range of retraction speeds (Method Details) in the absence and presence of darobactin (Figures S1 and S2).

The DFS data show that the mean forces required to unfold individual structural segments of BamA increase linearly with the logarithm of the loading rate (Figures 4 and S3). Fitting the Bell-Evans model (Bell, 1978; Evans, 2001; Evans and Ritchie, 1997) to the DFS plots allows us to extrapolate the mechanical, kinetic, and energetic properties of each structural segment at equilibrium (Bippes and Muller, 2011). The fits approximate that the distance $x_u$ that every structural segment has to be stretched along the pulling trajectory to reach the transition state toward unfolding and the unfolding rate $k_0$. The $x_u$ values can be used to describe the width of the free-energy valley hosting the folded state of a structural segment (Serdiuk et al., 2014). Accordingly, a larger $x_u$ value describes a wider free-energy valley that can host a larger number of conformational substates of the structural segment. The reciprocal of $k_0$ represents the lifetime of a structural segment. Using $x_u$ and $k_0$, the height of the free-energy barrier $\Delta G^*$ stabilizing a structural segment against unfolding and the mechanical stiffness $\kappa$ (i.e., spring constant) of the segment can be calculated. These free-energy landscape parameters, which were approximated for each structural segment of BamA in the presence and in the absence of darobactin (Table 1), are described in the following.
Darobactin widens free-energy wells

The $x_u$ values of the five POTRA domains increased from 0.32 nm to 0.39 nm (to $\approx 122\%$) in the presence of darobactin (Table 1). The only POTRA domain, which slightly decreased $x_u$ upon darobactin binding was P5. Among all structural segments of BamA, the LR showed the smallest $x_u$ values in the absence (0.17 nm) and presence (0.20 nm) of darobactin. In contrast, the structural segments (e.g., $\beta$-hairpins) of the transmembrane $\beta$-barrel region widened their free-energy wells from 0.33 nm to 0.76 nm (to $\approx 235\%$) in the presence of darobactin. The strongest increase in $x_u$ was observed for $\beta$-hairpin H6, which moved its distance to the transition state from the initial 0.33 nm to 1.05 nm and thus increased the width of the free-energy well to 315%.

The results show that darobactin binding to BamA only slightly widens the $x_u$ values of the POTRA domains and the LR, but considerably increases the $x_u$ values of the $\beta$-barrel domain. Thus, darobactin binding increases the $x_u$ value of almost every structural segment of BamA. Consequently, the free-energy valleys accommodating the structural segments widen so that they could host more and/or different conformational states. Amongst all structural segments of BamA, the region linking the POTRA domains to the transmembrane $\beta$-barrel show the smallest $x_u$ values, thus indicating to display the smallest conformational variability amongst all segments in the absence and in the presence of darobactin.

Darobactin increases kinetic stability

On average, the POTRA domains decreased their $k_0$ values to 24% in the presence of darobactin and thus considerably increased their lifetimes (Table 1). Amongst all structural segments of BamA, the LR showed the largest $k_0$ value of 1.05 s$^{-1}$ in the absence of darobactin, which upon darobactin binding, reduced to 0.53 s$^{-1}$. In stark contrast, the structural segments (e.g., $\beta$-hairpins) of the $\beta$-barrel of BamA decreased on average their $k_0$ values to 6% in the presence of darobactin.

In summary, every structural segment of BamA lowers the unfolding rate $k_0$ and thus increases lifetime upon darobactin binding. Naturally, by their insertion into the outer membrane, the $\beta$-hairpins of the transmembrane $\beta$-barrel expose high lifetimes, which in the presence of darobactin, is further increased by orders of magnitude. In contrast, the shortest lifetime is observed for the polypeptide stretch linking the POTRA domains to the transmembrane $\beta$-barrel.

Darobactin increases energetic stability

Darobactin binding of BamA increased the free-energy barriers stabilizing the structural segments against mechanical unfolding (Table 1). On average, the $\beta$-hairpins of the BamA $\beta$-barrel increased their $\Delta G^0_{u}$ values drastically from 25.8 kBT in the absence to 45.0 kBT in the presence of darobactin. Whereas the weakest free-energy barrier increase in individual $\beta$-hairpins was to 103% (H8), the strongest increase was to 229% (H5), and the average increase in all $\beta$-hairpins forming the $\beta$-barrel was to 171%. At the same time, the average $\Delta G^0_{u}$ values of the POTRA domains and the LR increased slightly from 21.7 kBT to 23.7 kBT.

Taken together, whereas the free-energy barriers of all $\beta$-hairpins and the LL structure of the $\beta$-barrel increase on average to 171% upon darobactin binding, the POTRA domains and the gate region increase the free-energy barriers on average to only 109%. We thus conclude that the binding of darobactin to BamA considerably increases the energetic stability of the $\beta$-barrel but only slightly that of the soluble POTRA domains and LR.
In the absence of darobactin, the spring constants $k_0$ of the five POTRA domains and the transmembrane $\beta$-barrel reduced considerably (to $\approx 77\%$) upon darobactin binding. One exception was POTRA domain P5, which slightly increased stiffness from 2.67 to 2.95 N m$^{-1}$ (to $\approx 111\%$). Among all structural segments of BamA, the LR showed the highest stiffness both in the unbound (5.69 N m$^{-1}$) and in the darobactin-bound (4.40 N m$^{-1}$) state. In the absence of darobactin, the structural segments representing individual $\beta$-hairpins of the transmembrane $\beta$-barrel showed spring constants ranging from 1.02 N m$^{-1}$ (H5) to 4.09 N m$^{-1}$ (H1). Upon darobactin binding, the $\beta$-hairpins considerably decreased spring constants now ranging from 0.25 N m$^{-1}$ (H5) to 2.68 N m$^{-1}$ (H8). The largest relative decrease in mechanical stiffness was measured for $\beta$-hairpin H6, which lowered from 1.85 N m$^{-1}$ (unbound state) to 0.40 N m$^{-1}$ (to $\approx 22\%$). In the presence of darobactin, structural segments of the $\beta$-barrel on average reduced their spring constants to $\approx 42\%$ of their initial values.

In summary, upon darobactin binding, BamA reduces the structural stiffness of almost every structural segment. In the unbound state, the mechanically stiffest segments are the LR and the N-terminal $\beta$-hairpin H1. Additionally, the C-terminal $\beta$-hairpin H8, which together with H1, forms the gate region of BamA, shows a relatively high spring constant (mechanical stiffness) in the unbound state. Upon darobactin binding, we find that these and the other $\beta$-hairpins of the transmembrane $\beta$-barrel region considerably reduce mechanical stiffness.

**DISCUSSION**

SMFS experiments on integral $\beta$-barrel OMPs have thus far observed common mechanical unfolding pathways. By applying a mechanical pulling force to one terminal end, individual $\beta$-hairpins forming the transmembrane $\beta$-barrel unfold and extract sequentially from the membrane. This sequential unfolding of $\beta$-hairpins was observed for several OMPs, including OmpA (Bosshart et al., 2012b), OmpG (Sapra et al., 2009), FhuA (Thoma et al., 2012), LamB (Thoma et al., 2017), and BamA (Thoma et al., 2018a, 2018b). The findings presented here confirm the previous studies.

The extensive data recorded in our study allow us to identify an additional force peak class of BamA, which locates between the POTRA domains and the $\beta$-barrel and is referred to as LR. Thus, upon mechanically pulling its N-terminal end, BamA displays 15 distinct force peak classes, each originating from the mechanical unfolding of a structural segment. The soluble POTRA domains and the LR, which are located at the N-terminus of BamA, unfold first at comparably low forces. Afterwards, the region linking POTRA domains and transmembrane $\beta$-barrel unfolds. Eight of the nine following force peak classes correspond to the mechanical unfolding of individual $\beta$-hairpins and display relatively high mechanical stability, such as that previously measured for other OMPs (Thoma et al., 2012, 2017).
Upon mechanically unfolding BamA in the absence and in the presence of darobactin, we observe the same 15 force peak classes, describing the same sequential unfolding pathways (Figure S4). This observation indicates that a characteristic network of interactions stabilizes the same structural segments of BamA in the absence and presence of darobactin. The finding is in full agreement with the structural model of BamA that shows no large structural rearrangement upon darobactin binding (Kaur et al., 2021). However, the force peak classes recorded in the presence of darobactin display increased unfolding forces (to $\approx 116\%$) as compared to those recorded in the absence of darobactin. Thus, darobactin considerably increases the mechanical stability of BamA. This stabilizing effect is most prominent for the $\beta$-barrel region of BamA (Figure 5). Thereby, the first four N-terminal $\beta$-hairpins H1–H4 were the most stable $\beta$-hairpins with the first N-terminal $\beta$-hairpin H1 showing the highest mechanical stability. This observation indicates that the first $\beta$-hairpin H1 forms a mechanically stable gate together with the last C-terminal $\beta$-hairpin H8 of the BamA $\beta$-barrel. Upon binding to this gate region, darobactin further stabilizes $\beta$-hairpins H1 and H8 as well as the other $\beta$-hairpins of the $\beta$-barrel. However, darobactin also increases the stability of the only essential POTRA domain P5 (Bos et al., 2007). Although the structural model does not provide conclusive evidences for a specific interaction of POTRA domain P5 and darobactin, our data show that the properties of POTRA domain P5 are modulated via the darobactin-mediated inhibition of BamA. Interestingly, it has been previously observed that darobactin-resistant strains show mutations of residue F394 in the POTRA domain P5 (Imai et al., 2019). The darobactin-dependent modulation of the mechanical stability of P5 thus adds insight into the manifold interactions of darobactin with BamA.

To further investigate the complex effects of darobactin on the structural properties of BamA, we employed SMFS in the dynamic mode. For every structural segment of BamA, we find a linear relationship between the mean unfolding force and the logarithm of the mean loading rate. This suggests that the folded state of every segment is separated from the unfolded state by a single free-energy barrier (Evans and Ritchie, 1997). We observe the general trend that darobactin increases the distance...
to the transition state ($x_t$ values), increases the lifetime, enhances the free-energy barrier, but lowers the mechanical stiffness (or rigidity) of almost every structural segment of BamA (Figure 5). As these changes apply at different magnitudes in the structural segments, we will discuss them as follows in detail.

One prominent effect is observed for the structural region linking the POTRA domains to the transmembrane $\beta$-barrel. This LR shows the lowest kinetic stability amongst all structural segments of BamA in the absence and presence of darobactin. Interestingly, the LR also shows the smallest transition state distances in the absence and presence of darobactin, thus indicating to adopt the smallest conformational variability amongst all structural segments of BamA. Moreover, the LR exposes the highest spring constant amongst all structural segments of BamA, thus showing the highest mechanical stiffness. Together, the low kinetic stability and conformational variability and the high mechanical stiffness of the LR provide important functional insight into how the POTRA domains connect to the $\beta$-barrel domain.

Another remarkable insight is provided for the transmembrane $\beta$-barrel. Although darobactin increases the height of every free-energy barrier stabilizing a structural segment of BamA (Figure 5), which means that all segments stabilize energetically, the highest increase (to $171\%$) was observed for the structural segments (e.g., $\beta$-hairpins) of the transmembrane $\beta$-barrel. In the unbound state, the mechanically stiffest segments of BamA are the LR and the N-terminal $\beta$-hairpin H1. Additionally, the N-terminal $\beta$-hairpins H2, H3, and H4 and the C-terminal $\beta$-hairpin H8 show a relatively high mechanical stiffness in the unbound state. Comparatively, $\beta$-hairpins H5–H7 are among the mechanically most flexible regions of BamA. This suggests that if a substrate is funneled into the membrane by the two relatively stiff and stable $\beta$-hairpins H1 and H8, which form the lateral gate of BamA, the $\beta$-hairpins H5–H7 provide a mechanically flexible region such as that needed to enlarge the transmembrane $\beta$-barrel and to insert and fold the substrate. As darobactin considerably changes the mechanical stiffnesses of almost every structural region of BamA as well as their mechanical, kinetic, and energetic stabilities, these fine-tuned properties come out of balance. Particularly, the stiffnesses of all structural segments of the $\beta$-barrel decrease. In summary, the data thus show that in the presence of darobactin, the $\beta$-barrel domain of BamA increases its relatively high energetic stability and decreases its mechanical stiffness to structurally soften. Most importantly, however, darobactin binds between $\beta$-hairpins H1 and H8, thus sealing the lateral gate to prevent substrate inclusion (Kaur et al., 2021).

Taken together, we observe darobactin to mechanically stabilize BamA considerably. The stabilizing effects are most prominent for the entire $\beta$-barrel domain of BamA, although darobactin only binds to the lateral gate region (Kaur et al., 2021). The free-energy landscape parameters characterizing the properties of every structural segment of BamA describe how darobactin modulates the mechanical, kinetic, and energetic properties of BamA in great detail (Figure 5). Together with the structural models of BamA in the unbound state and in the darobactin-bound state, the parameters provide mechanistic insight into how the rather complex BamA machinery is inhibited. Further SMFS studies may be conducted in the presence of $\beta$-signals of varying OMPs to investigate how transient interactions modulate the mechanical, kinetic, and energetic properties of BamA in contrast to darobactin. Similarly, the effects of different antibiotic compounds targeting the BAM complex could be studied by SMFS to elaborate on current findings. Gaining a deeper understanding of different factors shaping BamA-mediated OMP insertion and folding will be beneficial for the rational design of novel antibiotic compounds against the BAM complex and will hopefully advance our efforts to resolve the current antimicrobial resistance crisis.

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Daniel J. Müller (daniel.mueller@bsse.ethz.ch).

**STAR METHODS**

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## STAR METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Bacterial and virus strains** | | |
| BL21(DE3)omp8 | (Philipov et al., 1998) | N/A |
| **Chemicals, peptides, and recombinant proteins** | | |
| LB Agar, powder (Lennox L agar) | Thermo Fisher Scientific | Cat# 22700025 |
| Ampicillin sodium salt | Sigma-Aldrich | Cat# A9518 |
| LB medium (Difco) | Becton Dickinson | Cat# BD 244610 |
| Isopropyl-β-D-thiogalactoside | Sigma-Aldrich | Cat# I6758 |
| Dulbecco’s phosphate buffered saline with added magnesium and calcium | Sigma-Aldrich | Cat# D8662 |
| **Deposited data** | | |
| Structure of BamA complex | (Gu et al., 2016) | PDB: 5D0O |
| Structure of BamA bound to darobactin | (Kaur et al., 2021) | PDB: 7NRI |
| **Oligonucleotides** | | |
| Step1_fwd: 5’-GGTGGCGGCTCTGGTTCCGGTTCTGCTGAAGGGTTCTGAGTGATA-3’ | This Work | N/A |
| Step1_rev: 5’-CCAGAGCCGCCACGCGCAGAAAAGAACCCTTAAGGTGGGCCGGC-3’ | This Work | N/A |
| Step2_fwd: 5’-GGTACGAAGAGCTCTTTCTTCGCGTGGCCGGTGG-3’ | This Work | N/A |
| Step2_rev: 5’-GAAGCCTTCTTCCTACAAGGACCTTTCTACGCTGAC-3’ | This Work | N/A |
| **Recombinant DNA** | | |
| pY161 | (Thoma et al., 2018a, 2018b) | N/A |
| **Software and algorithms** | | |
| Pymol (Version 2.4.2) | Schrodinger | http://www.pymol.org |
| Prism (Version 8.4.3) | Graphpad Software | http://www.graphpad.com |
| SMFS Data Analysis Procedure | (Thoma et al., 2017) and this work | N/A |
| Nanoscope Analysis software (version 1.8) | Bruker | http://nanoscaleworld.bruker-axs.com/nanoscaleworld/ |
| **Other** | | |
| OMCL-RC800PSA cantilevers | Olympus | Cat# OMCL-RC800PSA |
| 450 nm filter unit | Merck Millipore | Cat# S2HVU02RE |
| Python codes for FD curve analysis of the data published in this work | This Work | https://doi.org/10.5281/zenodo.5647099 |

### RESOURCE AVAILABILITY

#### Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Daniel J. Müller (daniel.muller@bsse.ethz.ch)

#### Materials availability
This study did not generate new unique reagents. Plasmids and bacterial strains produced in this study will be available upon request.

#### Data and code availability
All data reported in this paper will be shared by the lead contact upon request.
All original code has been deposited at Zenodo and is publicly available as of the date of publication (MuellerLabETHZ, 2021). DOIs are listed in the key resources table.

Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

E. coli BL21(DE3)omp8 (Prilipov et al., 1998) was used to prepare outer membrane vesicles under culture conditions as described in Method Details.

METHOD DETAILS

Cloning

Plasmid pNR48, which was used to overexpress BamA into the E. coli outer membrane was cloned in two steps, in which an N-terminal flexible linker was attached to facilitate unfolding experiments. First the sequence coding for FFSARGGSGSGS was inserted in front of the newly inserted sequence with primers 5’-GGTAACGAAGAAGGCTTCTTTTCTGCGCGTGG-3’ and 5’-CCAGAGCCGCCACGCGCAGAAAAGAAACCGTATACGGTGGCGGCGC-3’ with the QuikChange PCR method (Cormack and Caserta, 2002). In a second step the sequence coding for NEEG was inserted in front of the newly inserted sequence with primers 5’-GTTAACGGAAGGCTTCTTCTTCTGCCTGCGTG-3’ and 5’-GAAGCCTTCTTCTACGGTATACGCGTGCGGCG-3’ again using the QuikChange PCR method.

Preparation of outer membrane vesicles (OMVs) enriched with BamA

The plasmid pNR48 was transformed into E. coli BL21(DE3)omp8 (Prilipov et al., 1998). OMVs were prepared as described. Briefly, the E. coli were grown on a Luria-Bertani (LB) Agar plate (Lennox L Agar, Invitrogen) supplemented with 100 µg ml−1 ampicillin (Ampicillin sodium salt, Sigma) at 37°C. A single colony was picked and grown over night in LB medium (Difco, Becton Dickinson) supplemented with 100 µg ml−1 ampicillin at 37°C under constant shaking at 220 rpm. 300 ml LB medium in a baffled Erlenmeyer flask containing 100 µg ml−1 ampicillin were inoculated 1:100 with the overnight culture and grown at 37°C, 220 rpm while the optical density at 600 nm (OD600) was closely monitored. Overexpression of BamA was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG, Sigma) when OD600was 0.4 was reached. After induction the cells were incubated until the onset of saturation in growth was reached. The cells were removed by centrifugation at 10’000 xg for 10 min. The supernatant was sterile-filtered using a 450 nm filter unit (Merk Millipore) and stored overnight at 4°C. After the adsorption, the sample was gently washed with DPBSS to remove non-adsorbed membranes. For SFMS in presence of darobactin, the sample was centrifuged for 15 min at 16’100 xg at 4°C and the pellet was resuspended in 40 ml DPBSS. The OMVs were washed in an additional centrifugation step at 108’000 xg for 1 h and resuspended in 1 ml of DPBSS. The resulting solution was stored in aliquots at −80°C.

Single-molecule force spectroscopy (SMFS) and dynamic force spectroscopy (DFS)

For each SMFS experiment, an aliquot containing 5 µl OMVs in DPBSS was thawed at 4°C and filled up to 50 µl with DPBSS. The sample was centrifuged for 15 min at 16’100 xg at 4°C and the pellet was resuspended in 40 µl DPBSS. 5 µl of the resulting sample were diluted in 45 µl DPBSS and adsorbed to freshly cleaved mica. After 15 min the sample was rinsed several times with DPBSS to remove non-adsorbed material. The sample was filled up to 1.5 ml and covered with a silicon skirt to prevent evaporation. SMFS was performed at ≈25°C using a commercial AFM (Nanowizard II Ultra, JPK Instruments) and OMCL-RC800PSA cantilevers (Olympus), which were calibrated with the thermal noise method (Butt and Jaschke, 1995). Membrane patches were located by contact mode AFM imaging. The tip of the AFM cantilever was pushed onto densely packed regions of the membrane for 500 ms with a force of 1 nN to non-specifically attach BamA. DFS was conducted at six different retraction speeds (500, 700, 1’000, 3’000, 4’500 and 6’000 nm s−1). At least five different cantilevers were used for each experimental condition to minimize errors due to uncertainties in cantilever spring constant calibration. SMFS experiments conducted at different retraction speeds were recorded in mixed and random order to reduce the influence of external factors on the experimental outcome. For SFMS in presence of darobactin, the sample preparation, AFM imaging and SMFS were conducted in DPBSS containing 20 µM darobactin. An external 16-bit data acquisition card (NI PCI-6221, National Instruments) was used to record SMFS data at high-frequencies.

Force–distance curve-based AFM (FD-Based AFM) imaging of OMVs

OMVs enriched with BamA were diluted (1:50) in DPBSS and adsorbed onto freshly cleaved mica for 15 min at room temperature. After the adsorption, the sample was gently washed with DPBSS to remove non-adsorbed membranes. FD-based AFM imaging of OMVs was performed with a Nanoscope Multimode 8 (Bruker, USA) operated in PeakForce Tapping mode in DPBSS at room temperature as described (Pfreundschuh et al., 2014; Thoma et al., 2018a, 2018b). In FD-based AFM, the AFM cantilever approaches and retracts from a biological sample while raster-scanning in a pixel-by-pixel manner to record FD curves. Thereby the distance of the cantilever tip and the sample distance is measured for each pixel to estimate the height profile of the sample topography. The AFM was placed in a temperature-controlled acoustic isolation box and equipped with a 120-µm piezoelectric J scanner and fluid cell. AFM topographs were recorded using PEAKFORCE-HiRs-F-A (Bruker Nano Inc., USA) cantilevers having a nominal spring
constant of 0.4 N m\(^{-1}\), a resonance frequency of \(165\) kHz in liquid, and a sharpened silicon tip with a nominal radius of \(1\) nm. Before imaging, cantilevers were calibrated by ramping on the mica surface and the thermal noise method (Butt and Jaschke, 1995). AFM topographs were recorded by applying an imaging force of 100–120 pN at 2 kHz oscillation frequency, with a vertical oscillation amplitude of 30 nm and a resolution of 512\(\times\)512 pixels. Image post-processing and analysis were performed using the Nanoscope Analysis software v.1.8.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

**SMFS data analysis**

For each experimental condition, the FD curves were transformed to force versus contour length space using the worm-like chain (WLC) model (Bustamante et al., 1994),

\[
F(x) = \frac{k_BT}{P} \left[ \frac{1}{4} \left( 1 - \frac{x}{L} \right)^2 - \frac{1}{4} + \frac{x}{L} \right] \tag{Equation 1}
\]

where \(k_B\) is the Boltzmann constant, \(T\) the absolute temperature in Kelvin, \(P\) the average polypeptide persistence length (assuming a length of 0.36 nm per amino acid (aa)), \(x\) the extension in nm and \(L\) the polypeptide contour length in aa. A fixed persistence length of 0.4 nm was used (Bosshart et al., 2012a). The BamA unfolding curves were aligned to each other in force versus contour length space using a self-written software. In the next step the position of every force peak in every FD curve was detected. To assign individual force peaks to force peak classes, we used the OPTICS clustering from scikit learn (Ankerst et al., 1999; Pedregosa et al., 2011) to automatically cluster the force peaks of one SMFS data set recorded at 1'000 nm s\(^{-1}\) retraction speed in presence of darobactin. After the force peak positions were clustered and the mean contour lengths determined, we used the resulting force peak clusters as a template to classify the FD curves recorded under other experimental conditions.

The aligned and classified FD curves were analyzed as described (Spoerri et al., 2019; Thoma et al., 2017). Briefly, the loading rate of every force peak was determined by the slope of a linear fit to the last data points before of the rupture event described by the respective force peak in the force-time curve (Alsteens et al., 2015). For each experimental condition the rupture forces and loading rates of all force peaks belonging to a force peak class were binned and the resulting histograms were fitted with Gaussians to determine the most probable rupture force and loading rate (Figure S3). The resulting means and standard errors of each force peak class were fitted with the Bell-Evans model (Bell, 1978; Evans, 2001; Evans and Ritchie, 1997),

\[
F^* = \frac{k_BT}{k_B} \left( \frac{x_{uf}}{k_B T_0} \right) \tag{Equation 2}
\]

where \(F^*\) is the most probable rupture force in pN and \(r\) is the loading rate in pN s\(^{-1}\) (Figure 4). Both values allowed to approximate the distance \(x_u\), separating the free-energy valley of the folded structural segment from the unfolded state, and the unfolding rate \(k_0\) of the structural segment. The parameter \(\Delta G_u\) describing the height of the free-energy barrier stabilizing a structural segment against unfolding was calculated using

\[
\Delta G_u = -k_BT_0 \ln(\tau_0 k_0) \tag{Equation 3}
\]

where \(\tau_0\) is the Arrhenius frequency (Gräter et al., 2005), for which we chose \(10^{-8}\) s\(^{-1}\) (Zocher et al., 2012). The parameter \(\kappa\), which describes the stiffness of a structural segment, was calculated by:

\[
\kappa = \frac{2\Delta G_u}{x_u^2} \tag{Equation 4}
\]

Errors were propagated using the python uncertainties package (Lebigot, 2018).