Resistance–nodulation–division efflux pumps play a key role in inherent and evolved multidrug resistance in bacteria. AcrB, a prototypical member of this protein family, extrudes a wide range of antimicrobial agents out of bacteria. Although high-resolution structures exist for AcrB, its conformational fluctuations and their putative role in function are largely unknown. Here, we determine these structural dynamics in the presence of substrates using hydrogen/deuterium exchange mass spectrometry, complemented by molecular dynamics simulations, and bacterial susceptibility studies. We show that an efflux pump inhibitor potentiates antibiotic activity by restraining drug-binding pocket dynamics, rather than preventing antibiotic binding. We also reveal that a drug-binding pocket substitution discovered within a multidrug resistant clinical isolate modifies the plasticity of the transport pathway, which could explain its altered substrate efflux. Our results provide insight into the molecular mechanism of drug export and inhibition of a major multidrug efflux pump and the directive role of its dynamics.
AcrB is a homotrimeric integral membrane protein that forms part of the tripartite AcrAB-TolC efflux pump (Fig. 1a, b and Supplementary Fig. 1). Energized by the proton-motive force, AcrB transports a broad variety of toxic substances, including antibiotics, outside of the cell through a channel formed by the periplasmic adaptor protein, AcrA, and outer membrane channel, TolC. It is constitutively expressed channel formed by the periplasmic adaptor protein, AcrA, and substances, including antibiotics, outside of the cell through a proton-motive force, AcrB transports a broad variety of toxic homologues forming the most clinically relevant pumps, has in many pathogenic Gram-negative bacteria and, with its pump inhibition and their synergy with antibiotics. One fundamental aspect of its structure that remains unresolved concerns the role of its structural dynamics, which are often crucial for protein function.

Previous structural and biochemical work has enabled drug-binding pockets and efflux pathways for AcrB to be proposed. Drug transport is purposed to occur via cooperative rotation between three distinct monomer conformations: loose (L), tight (T), and open (O) (Fig. 1c). Where, in the L-state, drugs gain access to the proximal binding pocket (PBP) through entrance channels. Upon a conformational change to the T-state, the drug is then moved towards the distal binding pocket (DBP) before being transported through the exit channel of the state, the drug is then moved towards the distal binding pocket and then through the exit channel. The distal binding pocket (DBP) is also postulated to be involved in drug transport.

In this work, we reveal that the β-naphthylamide (PAβN) antibiotic and the well-studied phenylalanine-arginine-β-naphthylamide (PAβN) efflux pump inhibitor (EPI) 5,7,15,29, 30, we were able to further understand the structural and functional consequences of substrate and inhibitor binding and clinically relevant mutation.

In this work, we reveal that the PAβN EPI restricts the intrinsic motions of the drug-binding pockets as part of its mechanism of action and is effective against both AcrBWT and AcrBG288D. We also demonstrate that an EPI can dually bind to the two binding pockets of AcrB.
to AcrB alongside an antibiotic, without affecting its inhibitory action. We discover that an MDR mutation in acrB impacts upon the structural dynamics of the efflux translocation pathway, likely contributing to its modified substrate efflux. Structural dynamics therefore have a critical role in the inhibition and substrate efflux of AcrB. Understanding the effect of these dynamics on structure and function is critical for successful assessment of resistance–nodulation–division (RND) efflux pump mechanism and inhibition, especially in relation to MDR-conferring mutations.

Results

HDX-MS of AcrB. HDX-MS is a solution-based method which can provide molecular-level information on local protein structure, stability, and dynamics.31–33 HDX occurs when backbone amide hydrogens are made accessible to D2O solvent through structure unfolding and H-bond breakage; HDX is fast within unfolded regions and slow within stably folded regions (i.e. α-helices, β-sheet interiors), where local structural fluctuations which expose an otherwise protected amide hydrogen to solvent transiently are required for HDX to occur. In order to decipher the effect of drug binding and mutation on AcrB structural dynamics, we performed differential HDX (ΔHDX) analysis between two conditions (e.g. drug-bound and drug-free), which is a sensitive approach for detecting associated structural perturbations between two different protein states (Fig. 1d).33–35

We optimized HDX-MS conditions on Escherichia coli (E. coli) AcrB solubilized within n-Dodecyl-β-D-Maltopyranoside (DDM) detergent micelles achieving 72% peptide coverage (Supplementary Fig. 2). A relative fractional deuterium uptake analysis of AcrB revealed that many of its residues form part of stable structures, inferred from the longest labelling time points (0.5–1 h) required for substantial deuteration incorporation (Supplementary Figs. 3, 4). Its most structurally dynamic regions being discovered within the subdomains of the Porter Domain (PC1, PC2, PN1, and PN2) which, notably, host the main drug-binding pockets (Fig. 1a, b and Supplementary Table 1). Lack of extensive HDX was observed within the TM domains, likely afforded by their protection within the hydrophobic environment of the detergent micelle.

EPI restricts drug-binding pocket dynamics. First, we examined how CIP and PAβN binding affects AcrBWT structural dynamics. CIP is a licensed antibiotic in clinical use and has been demonstrated to bind to the DBP, PC1/PC2 cleft and central cavity by MD simulations and X-ray crystallography.15,29 While PAβN, an EPI and substrate of AcrB, has been shown to bind to similar areas of AcrB as many antibiotics, this binding may be at distinct sites.5,15,29

In the presence of CIP only a few regions within the PN2 subdomain, central cavity, R2 domain (TM 7–12) and Iα-helix demonstrated significant differential HDX (Fig. 2a, b), suggesting that CIP binding only subtly alters the structural motions of AcrBWT. The presence of PAβN gave comparably increased HDX within the PN2 subdomain. However, in stark contrast to CIP, inhibitor binding led to HDX reduction throughout extensive parts of the PC1/PC2 cleft of the drug-binding pockets and within the connecting-loop (Fig. 2a, b and Supplementary Fig. 5). This could signify inhibitor-induced structural stabilization of the drug-binding pocket entrances. HDX of the Iα-helix did not significantly change upon addition of PAβN, whereas HDX was substantially increased upon CIP binding (Fig. 2a), possibly because the inhibitor weakens the coupling between the R1 and R2 TM domains. Notably, switch-loop spanning peptides with reduced HDX were detected when PAβN was present, which may reflect a binding interaction and/or structural stabilization (Fig. 2a, b and Supplementary Fig. 5).

Together, our HDX data support an inhibitory mode of action by which an EPI primarily acts to impart concerted restraint on AcrB structural dynamics, notably restricting the drug-binding pockets, connecting- and switch-loops.

Multi-copy 1-µs long MD simulations of AcrBWT bound to PAβN confirmed that—in agreement with the previous literature—the inhibitor could stably bind to the DBP, straddling the switch-loop and establishing strong interactions with the hydrophobic trap (HT); a peculiar region of the DBP rich in phenylalanes and involved in EPI binding (Supplementary Fig. 6).

To further understand the dynamics of AcrB in the presence of substrates, Root Mean Square Fluctuation (RMSF) and first hydration shell profiles were compared to the HDX-MS data. The average number of water molecules in the first amide NH solvation shell computed by MD simulations has been found to correlate well with HDX.37 A reduced hydration shell should therefore imply reduced HDX, due to the decrease in specific interactions between amide N–H bonds and the solvent. However, protein HDX is complex, with neighbouring residues having significant differences in their solvent interactions, this combined with the stark contrast between MD simulation and HDX-MS experimental time scales (µs to ms versus seconds to hours) means that a simple quantitative comparison can often be incomplete. Nevertheless, comparisons to MD calculated hydration profiles can provide informative qualitative interpretation of protein HDX.

MD analysis of AcrBWT-PAβN (T monomer) and apo AcrBWT (L monomer) revealed that binding of PAβN is accompanied by an overall rigidification of the protein (Supplementary Fig. 7; see also Supplementary Discussion and Supplementary Tables 2, 3), which involves large patches of the DBP, PBP, switch-loop, as well as the exit channel gate (EG), CH1, and CH2 channels.8 In particular: (i) regions containing residues belonging/adjacent to the switch-loop that were found to directly interact with PAβN become more rigid in its presence, the extent of HDX protection upon PAβN binding (as revealed by the HDX-MS data) correlating with the formation of hydrogen bonds between the EPI and residues of (and nearby) the DBP; ii) the switch-loop itself (residues 615–620) features moderately enhanced hydration, whereas the nearby segments (residues 612–614 and 621–624) are overall dehydrated with respect to apo AcrBWT. This supports an interaction between PAβN and the switch-loop region, which we anticipate being a key factor in mediating the mode of action of this EPI. More generally, the structural stabilization that occurs upon PAβN binding might prevent local, as well as distal, functional movements that are key to substrate efflux along the transport pathway.

Overall, these data agree with a model for inhibitor action, which has been proposed to work by trapping AcrB in a conformation, possibly a T-like state, which prevents adequate functional rotation and substrate transport.16

EPI and antibiotic can dually bind to AcrB. To fully understand EPI action, it is essential to consider its activity in the presence of antibiotic substrates, especially considering the emerging importance of drug combination therapies for treating bacterial infection.38 Antibiotic susceptibility assays against E. coli confirmed the ability of the PAβN EPI to potentiate antibiotic activity (Fig. 2c). PAβN increased antibiotic susceptibility for a range of antimicrobial AcrB substrates (ciprofloxacin (CIP), tetracycline (TET), and chloramphenicol (CHL)) in a substrate-dependent
manner, with better effectiveness observed at lower PAβN concentrations for CIP than for TET and CHL.

To explore the effect of an antibiotic on efflux inhibition we performed HDX-MS in the presence of both CIP and PAβN (AcrBWT-CIP-PAβN). We anticipated that the presence of equimolar CIP may interfere with PAβN binding, thereby affecting its ability to prevent functionality of the transporter through dynamic restraint. This was not the case. The presence of CIP did not alter the action of the PAβN inhibitor, as revealed by the strikingly similar differential HDX profiles for both AcrBWT-PAβN and AcrBWT-CIP-PAβN (Fig. 2a, b and Supplementary Fig. 5). Consequently, we investigated the possibility that PAβN acts by outcompeting CIP binding to AcrB. To test this, we exploited the innate fluorescence of CIP to perform fluorescence polarization binding and competition assays. Interestingly, we found that CIP binds with comparable affinity to both AcrBWT ($K_D$ of 74.1 ± 2.6 µM from Su et al.39) and a preformed AcrBWT-PAβN complex ($K_D$ of 67.3 ± 13.2 µM) (Fig. 3a), and that titration of PAβN EPI could not effectively outcompete CIP binding from a AcrBWT-CIP complex (Fig. 3b). These data suggest that antibiotic and inhibitor may be able to simultaneously bind at different sub-sites within the voluminous DBP.

To further support this hypothesis, we performed blind docking calculations and MD simulations on AcrBWT-PAβN-CIP (Supplementary Figs. 8, 9). Importantly, both drugs stably bind to the DBP within the T-state monomer, with PAβN partly occupying the HT and CIP lying in proximity of the PBP/DBP interface (Fig. 3c). The simultaneous binding of CIP and PAβN has similar effects on the flexibility and hydration of the DBP as the binding of PAβN only (Fig. 2a, b and Supplementary Fig. 10; see Supplementary Discussion). Several interactions contribute to stabilize this configuration, including the formation of hydrogen bonds between the substrates and several residues of the DBP (Supplementary Table 4) and stable intermolecular hydrogen bonds between the two ligands (Supplementary Fig. 9).

Overall, our data support the hypothesis that PAβN does not compete or prevent antibiotic binding (competitive inhibition). Instead, we propose that it inhibits AcrB function by enforcing a more restrained state, thus, reducing the frequency and
Molecular docking and multi-copy long MD simulations reveal stable interactions of CIP (orange) and PAβN (cyan) to AcrBWT T-state monomer and show their likely binding locations. EG = exit channel gate (blue spheres), SL = switch-loop (yellow), and HT = hydrophobic trap (purple). All computational data can be found in Supplementary Table 2 and Supplementary Figs. 8, 9.

MDR-conferring G288D mutation affects drug-binding pocket dynamics. We next turned our attention to the substitution mutation, G288D (AcrBG288D), which was found to cause resistance to some drugs (e.g. CIP) in AcrB WT having increased HDX reduction within PC1 and R2 (TM 7-12) (Supplementary Table 3). Similar conclusions emerged from the comparison between AcrBG288D-CIP-PAβN and AcrBWT-CIP-PAβN. Indeed, MD simulations of the former complex revealed that, even upon G288D substitution, CIP and PAβN can stably occupy the DBP at the same time (Fig. 5a). As in AcrBWT-CIP-PAβN, stabilizing interactions include several substrate contacts with the AcrBG288D protein, also involving D288 (Supplementary Figs. 14, 15 and Supplementary Table 7), as well as intermolecular hydrogen bonds between the two ligands (Supplementary Table 8).

Fluorescence polarization binding and competition assays support that a ternary AcrBG288D-CIP-PAβN is also possible (Fig. 5b, c): (i) CIP binds to a preformed AcrB G288D complex (K_D of 22.7 ± 2.9 μM) with similar, albeit slightly higher, affinity compared to CIP binding to AcrBWT-PAβN (K_D of 67.3 ± 13.2 μM); (ii) titration of the PAβN inhibitor could not effectively outcompete CIP binding from AcrBG288D-CIP, as was found for AcrBWT-CIP (Fig. 3b). Taken together, the fluorescence polarization binding assays and MD simulation data advocate that AcrBG288D is inhibited by PAβN in a similar manner as AcrBWT.

These findings were supported by bacterial susceptibility assays on E. coli containing overexpressed AcrBG288D. AcrBG288D was previously discovered within Salmonella clinical isolates and found to have increased and decreased susceptibility to MIN and CIP antibiotics, respectively. We chose, therefore, to study these AcrB substrates in the presence of the inhibitor PAβN to observe
what, if any, effect would be seen with the different AcrB genotypes. PAβN incubation led to increased MIN and CIP antibiotic susceptibility for both AcrBWT and AcrBG288D (Fig. 5d). AcrBG288D being more susceptible to PAβN than AcrBWT. The decreased susceptibility of AcrBG288D to CIP, found in Salmonella30, was not recapitulated in our assays using the laboratory E. coli strain MG1655. This may be due to CIP efflux via another transporter found in E. coli but not in Salmonella. However, the associated increased susceptibility to MIN was observed (Fig. 5d), supporting that G288D has a profound impact on AcrB substrate efflux within both E. coli and Salmonella.

**Discussion**

In summary, we found that binding of an EPI, PAβN, restricts AcrB dynamics and could not be outcompeted by an antibiotic, CIP, whose activity it potentiates. Fluorescence binding, MD simulations, and docking studies supporting the existence of a ternary protein–EPI–antibiotic complex. Endorsing the theory that RND-pump inhibitors act through an “altered-dynamics” mechanism, obstructing the translocation of substrates rather than preventing their binding and recognition.

Furthermore, we reveal that an MDR-conferring AcrB drug-pocket substitution, G288D30, modifies the structural dynamics of the translocation pathway in a substrate-independent manner. Our previous MD simulations30 show that the G288D substitution increases the gyration radius and hydration at and around the DBP. This disruption could subsequently lead to the observed allosteric action on farther AcrB regions. In turn, these changes may alter the energetic barrier for substrate binding and transport during functional rotation and, consequently, be the ultimate cause for the altered substrate efflux caused by this mutation.
conformation remaining the same even when the surrounding membranes display different curvatures\(^5\). This coupled with the agreement found between our HDX-MS data and MD simulations support that the structural dynamic behaviour uncovered here informs on the native protein state, although further studies investigating the effect of lipids on its dynamics are necessary to understand the system in its entirety.

We anticipate that the findings reported here will be important not only for establishing the general role of structural dynamics in modulating AcrB multidrug binding and efflux, which are hard to elucidate from biochemical and high-resolution structural data alone, but also for defining how naturally occurring mutations and EPI interactions affect its structure function.

**Methods**

**Plasmid construction.** An overexpression plasmid containing AcrB with a C-terminal 6xHis tag (AcrB-6xHis) was constructed from a pET15b-AcrB-sGFP-6xHis plasmid from Reading et al.\(^6\). Briefly, the sGFP sequence was deleted and a 6xHis-tag placed at the C-terminus of AcrB followed by a stop codon using the Q5 site-directed mutagenesis kit (New England Biolabs)—AcrB contains two Histidine residues at its C-terminus, therefore, this construct resulted in AcrB having an 8xHis histidine tag. The G288D mutation was then generated from this pET15b-AcrB-6xHis plasmid using the Q5 site-directed mutagenesis kit (New England Biolabs). All constructs were verified by DNA sequencing (Eurofins MWG). Primers used are reported in Supplementary Table 9.

pBR322-AcrB plasmids were generated for bacterial susceptibility assays. Briefly, pBR322 was linearized with HindIII and EcoRI restriction enzymes (New England Biolabs), acrB genes with its natural promoter, including the "marbox" sequence, was then amplified from K-12 *Escherichia coli* chromosomal DNA (Zyagen Labs) and cloned into the pBR322 vector using In-Fusion\(^\text{®}\) HD cloning (Takara Bio). A 6xHistidine tag sequence was included in the reverse primer to provide a 6xHis tag at the C-terminus of AcrB (pBR-AcrB\(^\text{WT}\)). The G288D mutation was generated from this pBR-AcrB\(^\text{WT}\) plasmid using the Q5 site-directed mutagenesis kit (New England Biolabs). All constructs were verified by primer walking DNA sequencing (Eurofins MWG). Primers used are reported in Supplementary Table 9.

**AcrB overexpression and purification.** pET15b-AcrB-6xHis plasmid containing AcrB wildtype (AcrB\(^\text{WT}\)) or G288D mutant (AcrB\(^\text{G288D}\)) was transformed into C43 (DE3)ΔacrAΔkanR E. coli cells. 7 ml of an overnight LB culture was added to 1 L of pre-warmed LB culture containing 100 μg/ml ampicillin and 30 μg/ml kanamycin and grown at 37 °C until an OD of 0.6–0.8 was reached. The culture was induced with 1 mM IPTG and grown for 16–18 h at 18 °C. At which point the cells were harvested by centrifugation at 4200 × g for 30 min and washed with ice-cold phosphate buffer saline (PBS).

Cell pellets were immediately resuspended in buffer A (50 mM sodium phosphate, pH 7.4, 300 mM sodium chloride) and supplemented with a protease inhibitor tablet (Roche), 100 μM PMSF, 1 μM Benzonase, and 5 mM β-mercaptoethanol (β-ME). The cell suspension was then passed twice through a microfluidizer process (Microfluidics) at 25,000 psi and 4 °C. Insoluble material was removed by centrifugation at 20,000 × g for 30 min at 4 °C. Membranes were then pelleted from the supernatant by centrifugation at 200,000 × g for 1 h at 4 °C. Membrane pellets were resuspended to 40 mg ml\(^{-1}\) in ice-cold buffer A supplemented with a protease inhibitor tablet (Roche) and 100 μM PMSF, and homogenized using a Potter-Evahjem Teflon pestle and glass tube. AcrB was extracted from homogenized membranes by overnight incubation with 1% (w/v) n-dodecyl-β-D-maltoside (DDM) detergent (Anatrace) at 4 °C with gentle agitation. Insoluble material was then removed by centrifugation at 100,000 × g for 1 h at 4 °C. The sample was then filtered through a 22 μm filter (Fisher Scientific) and loaded onto a 1 ml HiTrap column (GE Healthcare) equilibrated in buffer B (50 mM sodium phosphate, pH 7.4, 300 mM sodium chloride, 20 mM imidazole, 10% (w/v) glycerol, 0.03% (w/v) DDM). The column was washed with 5 CVs of buffer B and then with 10 CVs of buffer C (50 mM sodium phosphate, pH 7.4, 300 mM sodium chloride, 20 mM imidazole, 10% (w/v) glycerol, 0.03% (w/v) DDM). A flow rate of 1 ml/min was used during HiTrap and SEC purification. Peak fractions eluted from the SEC column containing pure AcrB were pooled, spin concentrated using a 100 K MWCO concentrator (Amicon\(^\text{®}\)), and spin filtered before being flash frozen and stored at −80 °C. SDS-PAGE electrophoresis was used to assess AcrB purification and protein concentration was

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**Here, to examine the structural dynamics of AcrB we performed HDX-MS within DDM detergent micelles and supported our findings with MD simulations completed within POPG/POPG (2:1 ratio) lipid bilayers. Detergent and amphipol membrane mimetics have been used extensively to obtain structural information for the determination of drug binding interactions and efflux mechanisms of AcrB. However, these systems do not provide a lipid environment, which can modulate membrane protein structure and function.**\(^8\) Recent studies have used SMA lipid particle (SMALP) technology and liposome reconstitution to capture AcrB within a lipid environment and solved its structure at high-resolution using cryo-EM\(^9\). \(^11\)–\(^13\). The resulting structures were largely consistent with high-resolution crystal structures solved in DDM detergent micelles, with homotrimeric AcrB
calculated using a Cary 300 Bio UV–Vis spectrophotometer (Varian) with a calculated extinction coefficient of ε280 = 89,730 M⁻¹ cm⁻¹.

**Circular dichroism spectroscopy.** Circular dichroism spectroscopy (CD) spectra were recorded with an Aviv Circular Dichroism spectrophotometer, Model 410 (Biomedical Inc., Lakewood, NJ, USA), with specially adapted sample detection to eliminate scattering artefacts. Multiple CD scans were averaged, the buffer background subtracted, and zeroed and minimally smoothed using CDTool. A final protein concentration of 0.3–1.5 mg ml⁻¹ was used in a quartz rectangular or circular cell (Bioanalytical systems). For thermal protein unfolding the mean residue ellipticity at 222 nm was monitored with increasing temperature.

**Native mass spectrometry.** Purified AcrB was buffer exchanged into MS buffer (200 mM ammonium acetate, pH 7.4, 0.03% (w/v) DDM or Triton X-100) using a centrifugal buffer exchange device (Micro Bio-Spin 6, Bio-Rad) according to the manufacturer’s instructions. Native mass spectrometry experiments were performed either on a Synapt G2-Si mass spectrometer (Waters) or a Thermo Scientific Q Exactive UHMR hybrid Quadrupole-orbitrap mass spectrometer. For experiments on the Synapt G2-Si mass spectrometer the instrument settings were used: 1.5 kV capillary voltage, source temperature of 25 °C, argon trap collision gas, 180 V trap collision voltage, 120 V cone voltage, and 50 V source offset. Data were processed and analyzed using MassLynx v.4.1 (Waters). Native mass spectrometry data on a Thermo Scientific Q Exactive UHMR hybrid Quadrupole-orbitrap mass spectrometer was acquired at resolving power 8750 at m/z 400 in the m/z range 2000–30,000. The instrument was optimized for transmission and desolvation of integral membrane proteins. Critical parameters throughout were relative pressure of 6, capillary temperature 250 °C, S-Lens RF level 0, in-source trapping 200 and the HCD energy was 300%. Data were analyzed by the use of Xcalibur software 4.3 and Biopharma Finder 3.1 (both Thermo Fisher Scientific). Deconvoluted spectra were acquired using Biopharma Finder 3.1 in sliding window mode. The following settings were used: Output mass range 10,000–100,000 Da, deconvolution mass tolerance 10 ppm, sliding window merge tolerance 30 ppm and minimal number of detected intervals.

**Preparation of ligands for hydrogen/deuterium mass spectrometry.** Ciprofloxacin (CIP) antibiotic and Phe-Arg-β-naphthylamide dihydrochloride (PAβN) inhibitor were both purchased from Sigma Aldrich. Stock concentrations of CIP (10 mg/ml) and PAβN (10 mg/ml) were prepared in 0.1 N HCl and water, respectively. As demonstrated previously, a primary consideration before carrying out HDX-MS is to ensure close to full binding to the target protein under off. Data were processed and analyzed using MassLynx v.4.1 (Waters). Native mass spectrometry experiments on a Thermo Scientific Q Exactive UHMR hybrid Quadrupole-orbitrap mass spectrometer was acquired at resolving power 8750 at m/z 400 in the m/z range 2000–30,000. The instrument was optimized for transmission and desolvation of integral membrane proteins. Critical parameters throughout were relative pressure of 6, capillary temperature 250 °C, S-Lens RF level 0, in-source trapping 200 and the HCD energy was 300%. Data were analyzed by the use of Xcalibur software 4.3 and Biopharma Finder 3.1 (both Thermo Fisher Scientific). Deconvoluted spectra were acquired using Biopharma Finder 3.1 in sliding window mode. The following settings were used: Output mass range 10,000–100,000 Da, deconvolution mass tolerance 10 ppm, sliding window merge tolerance 30 ppm and minimal number of detected intervals.

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**Hydrogen/deuterium mass spectrometry.** Hydrogen/deuterium mass spectrometry (HDX-MS) was performed on an HDX nanoAcquity ultra-performance liquid chromatography (UPLC) Synapt G2-Si mass spectrometer system (Waters Corporation). Optimized peptide identification and peptide coverage for AcrB was performed from undeuterated controls. The optimal sample workflow for HDX-MS of AcrB was as follows: 5 μl of AcrB (15 μM) was diluted into 95% of either buffer D or deuterated buffer D at 20 °C. After fixed times of deuterium incubation samples were mixed with 100 μl of formic acid-DDM quench solution to provide a quenched sample at pH 2.5 and final 0.075% (w/v) DDM concentration. 80 μl of the quenched sample was then loaded onto a 50 μl sample loop before being injected into an Acquity UPLC (Waters) at a flow rate of 200 μl/min) maintained at 20 °C. The peptide fragments were trapped onto an Acquity BEH C18 1.7 μm VANGUARD pre-column (Waters) for 3 min. The peptic fragments were then eluted using an 8–40% gradient of 0.1% formic acid in acetonitrile at 40 μl/min into a chilled Acquity UPLC BEH C18 1.7 μm, 110 × 100 mm column. The columns were both maintained at 0 °C. The eluted peptides were ionized by electrospray into the Synapt G2-Si mass spectrometer. MS² data was acquired with a 20–30 V trap collision energy ramp for high-energy acquisition of product ions. Argon was used as the trap collision gas at a flow rate of 2 ml/min. Leucine-enkephalin was used for lock mass accuracy control and the mass spectrometer was calibrated with sodium iodide. The online Enzyme“ pepscolumn was washed three times with pepscolumn (1.5 M Gu-HCl, 4 % MeOH, 0.8% formic acid), as recommended by the manufacturer, and a blank run was performed between each sample to prevent significant pepscolumn carry-over between runs. All samples and controls were manually injecting using the FASTA file of the corresponding amino acid sequence of E. coli AcrB retrieved from the Uniprot database (Uniprot Id: P31224). Next, 100 homology models were generated for
Supplementary Table 10). Namely, we calculated the C

calculated upon alignment of the T monomer of the protein to the reference frame.

and reorientations of the substrates during the simulations, their RMSDs were

the MD trajectory. In particular. To evaluate the magnitude of the displacements

respect to the initial (docking) structure after alignment of the whole trimer. The

runs were subjected to all-atom molecular dynamics (MD) simulations (each of 1

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AcrB. The topology and the initial coordinate

complex, to consider the largest number of putative binding modes, we purposely

selected docking poses with an orientation different than that reported previously

(Supplementary Fig. 16).

Molecular dynamics simulations. All of the 52 complexes selected from docking

runs were subjected to all-atom molecular dynamics (MD) simulations (each of 1 µs in length) performed with the AMBER18 package.

Protein-ligand interaction network. All the RMSD calculations of switches described in the previous paragraph. As a

result, we calculated the Cα RMSD of the substrate (setting the cut-off to 3 Å), which returned respectively 11, 9, 15, and 17 different poses for

the AcrBWNT–PAβN, AcrBWNT–CIP–PAβN, AcrB2G88D–PAβN, and AcrB2G88D–CIP–PAβN, respectively. The following analyses were performed to evaluate their agreement with HDX-MS data.

Comparison with HDX-MS data. RMSFs and hydration properties of each system were compared with a proper reference state according to the current knowledge about the most likely conformations assumed by AcrB in the absence of ligands or complexed with substrates and inhibitors106. For instance, to account for conformational changes of AcrB induced by inhibitor binding, PAβN-bound and apo AcrB structures were considered in their T- and L-state, respectively. The T-state was also considered for systems containing both PAβN and CIP (AcrBWNT–CIP–PAβN and AcrB2G88D–CIP–PAβN), hyposthesizing their stability in this conformation, as evidenced by the RMSD analyses conducted on our trajectories (Supplementary Figs. 6, 9, 12, 14). The list of reference states used for each analysis are reported in Supplementary Table 11.

Post-processing of MD trajectories. MD trajectories were analyzed using cpptraj (http://ambermd.

org/GetAmber.php). Clustering of the ligand trajectory was carried out using the average-linkage hierarchical agglomerative clustering method implemented in cpptraj. During the MD simulations, the lengths of all the R–H bonds were constrained with the SHAKE algorithm. Coordinates were saved every 100 ps. The Particle mesh Ewald algorithm was used to evaluate long-range electrostatic forces with a non-bonded cut-off of 9 Å.

Interaction network. Interactions stabilizing the complexes were analyzed by considering residues within 3.5 Å of each substrate in the last 300 ns of the MD trajectories. Hydrogen bonds were identified through geometrical criteria, were calculated using cpptraj after structural alignment of each trajectory as described in the previous paragraph.

Hydration properties. Residue-wise average numbers of waters within the first (second) hydration layer were calculated with cpptraj using a distance cut-off of 3.4 (5.0) Å between the nitrogen of the protein and the water oxygens.

Metasatellite data files including processed DynaX files have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD019047. HDX-MS meta-data have been provided in Supplementary Data files 1–4 with this paper. Source data are provided with this paper.

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Nature Research | Reporting Summary

Further information on research design is available in the Nature

Research Reporting Summary linked to this article.

Data availability

Data supporting the findings of this manuscript is available from the corresponding authors upon reasonable request. The molecular dynamics trajectories will be available anyone at any time by sending an e-mail to Attilio Vittorio Vargiu (vargiu@dsf.unica.it).

Table 11. List of reference states used for each analysis are reported in Supplementary Table 11.

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Author contributions
E.R., Z.A., A.V.V, L.J.V.P., and A.P. designed the research. E.R., Z.A., A.M.L., and H.F. performed all experiments and analyses, except for molecular modelling and bacterial susceptibility assays. C.F., G.M., and A.V.V. carried out docking, molecular dynamics and performed post-molecular dynamics analyses. X.W.K., V.R., J.S., and E.M.G. performed bacterial susceptibility assays and analysis. A.K. and Z.A. performed UHMR Native MS measurements and analysis. E.R., P.J.B., P.R., A.V.V, L.J.V.P., and A.P. supervised the project. E.R., Z.A., and A.P. wrote the manuscript with input from the other authors.

Competing interests
The authors declare no competing interests.

Additional information
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