Structural and functional analysis of the promiscuous AcrB and AdeB efflux pumps suggests different drug binding mechanisms

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Upon antibiotic stress Gram-negative pathogens deploy resistance-nodulation-cell division-type tripartite efflux pumps. These include a H+/drug antiporter module that recognizes structurally diverse substances, including antibiotics. Here, we show the 3.5 Å structure of subunit AdeB from the Acinetobacter baumannii AdeABC efflux pump solved by single-particle cryo-electron microscopy. The AdeB trimer adopts mainly a resting state with all protomers in a conformation devoid of transport channels or antibiotic binding sites. However, 10% of the protomers adopt a state where three transport channels lead to the closed substrate (deep) binding pocket. A comparison between drug binding of AdeB and Escherichia coli AcrB is made via activity analysis of 20 AdeB variants, selected on basis of side chain interactions with antibiotics observed in the AcrB periplasmic domain X-ray co-structures with fusidic acid (2.3 Å), doxycycline (2.1 Å) and levofloxacin (2.7 Å). AdeABC, compared to AcrAB-TolC, confers higher resistance to E. coli towards polyaromatic compounds and lower resistance towards antibiotic compounds.
The Gram-negative opportunistic pathogen *Acinetobacter baumannii* exhibits a high level of multidrug resistance (MDR) to drugs including carbapenems and the last-resort antibiotics tigecycline and colistin. This feature, paired with its persistence in hospital settings, contributes to frequent nosocomial outbreaks of *A. baumannii* infection. Following the increasing emergence of strains that are non-susceptible to all clinically used antibiotics, the WHO has ranked carbapenem-resistant *A. baumannii* first place in its global priority pathogen list.

The superfamily of resistance-nodulation-cell division (RND) efflux pumps plays a key role in intrinsic MDR in Gram-negative bacteria. These tripartite complexes comprise an RND transporter in the inner membrane that acts as a secondary active H+/drug antiporter extruding a vast spectrum of structurally unrelated drugs through a periplasmic membrane fusion protein channel connected to an outer membrane channel factor across the outer membrane. RND transporters of the Hydrophobe/Amphiphile Efflux 1 (HAE-1) family are composed of 12 transmembrane (TM) helices, which form the TM domain (TMD). Two helices emerging between TM1 and TM2 and between TM7 and TM8 form a large periplasmic region that is divided into the inner membrane-proximal porter domain and the distal funnel domain. The porter domain is built up by the four subdomains PN1, PN2, PC1 and PC2, while the funnel domain consists of the DN and DC subdomains.

The best characterized RND transporter is the HAE1 family member AcrB from *Escherichia coli*. The first solved structure of AcrB was a symmetric homotrimer comprising three protomers in the so-called loose (L) conformation (thus denoted as LLL). Later efforts yielded high-resolution crystallographic structures of asymmetric AcrB trimers adopting loose (L), tight (T) and open (O) conformations (LTO) in the apo state and with bound substrate molecules. Substrate binding was observed only for the L and T protomers, at the access pocket (AP) and deep-binding pocket (DBP), respectively. These binding pockets are connected to the periplasm via four channels (CH1–CH4) and with bound substrate molecules. In the O conformation, the AP, DBP and all channels are closed, although there is an exit channel from the (closed) DBP site leading to the funnel domain. Structures of the tripartite RND complexes AcrA-AB-ToIC from *E. coli* and MexAB-OprM from *Pseudomonas aeruginosa* have also been determined using single-particle cryo-electron microscopy (cryo-EM).

The current hypothesis is that any of the protomers within the AcrB trimer can adopt any of the three conformations L, T, and O. During drug efflux, the protomers cycle through these states in a concerted and consecutive manner, so that drugs are sequestered from the periplasm (and the outer leaflet of the inner membrane), followed by binding at the AP and/or DBP. In the T conformation, protons can enter the TMD via water channels and protonate the charged residues D407 and/or D408, thus changing the electrostatics inside the TMD. This leads to a conformational change of the TMD and energy transduction towards the porter domain, which in turn leads to the closure of the AP, DBP, and CH1–CH4 channels and the opening of the exit channel. The efflux activity of AcrB is readily inhibited by molecules binding in an area of the DBP known as the hydrophobic trap. Recently, we constructed and crystallized a soluble fusion of the two periplasmic AcrB loops (AcrBper) together with pyranopyridine-based inhibitors (e.g., MBX3132) and rhodamine 6G, which helped us expand our understanding of substrate and inhibitor recognition.

The three *A. baumannii* RND transporter complexes AdeABC, AdeFGH and AdeIJK were identified and characterized mainly in their natural host but also heterologously in *E. coli*. The constitutively expressed transporter complex AdeIJK is responsible primarily for intrinsic drug resistance in *A. baumannii,* with overexpression showing cytotoxic effects. AdeABC and AdeFGH are strongly regulated and are involved in acquired drug resistance. Single point mutations in the regulators of both pump complex genes are enough to induce their strong overexpression in strains exposed to low concentrations of drugs. AdeABC was found to be upregulated in most clinical MDR strains, whereas AdeFGH overexpression is less common. Therefore, we focused our efforts on obtaining structural information for AdeB of *A. baumannii*. The broad substrate spectrum of AdeB, like that of *E. coli* AcrB, comprises β-lactams such as carbapenems and cephalosporins, fluoroquinolones, tetracyclines (including tigecycline), chloramphenicol, macrolides, trimethoprim, ethidium, rifampicin and novobiocin. Unlike AcrB, however, AdeB was reported to confer resistance to aminoglycoside antibiotics in *A. baumannii* BM445145 and BM468924. Others reported in both *A. baumannii* ATTCC17978 and by heterologous expression in *E. coli* that the resistance against aminoglycosides is not clearly attributable to the expression of the AdeB efflux pump alone, and it was suggested that AdeABC overexpression observed in clinical strains appears essential, but not the sole factor for the increased resistance against aminoglycosides. In this study, we aim to elucidate the previously uncharacterized determinants of substrate polyspecificity in AdeB.

For structural elucidation of this RND transporter, we reconstituted AdeB in Salipero Nanodiscs sustaining a native-like lipid environment. Here, we show the single-particle cryo-EM structure of trimeric AdeB in the OO0 conformation and in the L’OO conformation. Molecular docking studies followed by free-energy calculations indicate binding of ethidium and rhodamine 6G to the L’ protomer, suggesting a role of this conformational state in initial drug uptake in the overall catalytic drug transport mechanism. In addition, we analyze the role of key residues in the binding of nine different substrates, based in part on three crystal structures of AcrBper in complex with the known AcrB and AdeB substrates levofloxacin, doxycycline and fusidic acid. These three high-resolution structures show previously unknown binding modes and add to our understanding of the substrate promiscuity of RND transporters.

**Results**

**Structural investigation of *A. baumannii* AdeB shows two distinct conformations.** AdeB from *A. baumannii* was produced in *E. coli* C43(DE3) ΔacrAB and purified by immobilized metal affinity chromatography (IMAC) followed by size exclusion chromatography (SEC). To determine the structure of *A. baumannii* AdeB in a native-like lipid environment, the protein was reconstituted into Salipero Nanodiscs (Supplementary Fig. 1). After confirming the integrity and trimeric state of the protein by native polyacrylamide gel-electrophoresis and negative stain electron microscopy (EM), samples were vitrified on holey carbon grids and analyzed by cryo-EM (Supplementary Fig. 2, statistics in Supplementary Table 1). A C3-symmetric density map could be solved up to a resolution of 3.5 Å (Fig. 1 and Supplementary Figs. 3 and 4). Our resulting structural model was compared to published structures of the RND transporters AdeB29,30, AcrB10, MexB31, MtrD32 and CusA33 (Supplementary Table 2) and found to adopt the previously reported OOO conformation (Figs. 1A, C and 2A, D). However, not all particles adopted this trimeric arrangement. After classification of all protomers, we found that approximately 10% of the protomers adopt an intermediate state, i.e. 30% of particles adopt a trimeric arrangement of two O protomers together with a previously uncharacterized conformation. Based on the comparison with the AcrB and AdeB structures (Supplementary Table 2), this protomer structure corresponds to neither L nor T conformations, hereafter referred
to as L*, thus constituting the AdeB trimer L*OO (Figs. 1B and 2B, C and Supplementary Figs. 5 and 6). We solved the structure of AdeB in this newly described conformation with an overall resolution of 3.95 Å for the L* protomer and 3.84 Å for the L*OO trimer (Figs. 1 and 2, statistics in Supplementary Table 3 and Supplementary Figs. 5 and 6). The overall architecture of the L* protomer most closely resembles the T conformation of AcrB (overall RMSD (Cα’s): 0.68 Å with the T conformation of AcrB, compared with 0.89 Å with the L conformation of AcrB)(Fig. 2G, H). Interestingly, the comparison of the AdeB L* protomer with the AdeB T protomer structures in the access/binding/extrusion (LTO, PDB: 7KGI) and the binding/extrusion/extrusion (TOO, PDB: 7KGG) conformations30, indicated the lowest overall RMSD’s between AdeB L* and other AdeB protomers of 1.81 Å and 1.55 Å, respectively (Supplementary Table 2 and Supplementary Fig. 6).

However, a unique arrangement of the PN2 and PC1 subdomains could be observed in the L* conformation. Whereas the structure of the PC1 subdomain, including the switch loop, is more similar to the L conformation of AcrB (local PC1 RMSD (Cα’s): 1.32 Å with the T conformation of AcrB, 1.18 Å with the L conformation of AcrB), the structure of PN2 subdomain largely differs from both the L and T conformations (local PN2 RMSD (Cα’s): 1.83 Å with the T conformation of AcrB, 2.1 Å with the L conformation of AcrB) (Supplementary Table 2, Fig. 2G, H, and Supplementary Fig. 6A, B). Analogous to the L conformation of AcrB and AdeB, the DBP (comprising the PN2 and PC1 subdomains) is closed in the L* conformation (Fig. 2E, F and Supplementary Figs. 5A and 6B).

Using the CAVER Analyst software34, we identified three entry tunnels proceeding towards the (closed) DBP of the L* protomer (Fig. 2B, C). These tunnels resemble CH1–CH3 in AcrB6,7,11. As observed in structures of AcrB, the closure of these entry tunnels appears concomitant with the opening of an exit tunnel in the O conformation of AdeB (Fig. 2B, C). Since the DBP in the AdeB L* conformation is in a closed state (Fig. 2E, F), we conducted molecular docking calculations of ethidium and rhodamine 6G to the AdeB L*, L, and T protomers30 and found binding of these substrates proximal to the switch loop in the AP of the L* and L protomer (Supplementary Fig. 7 and Supplementary Table 4). Top-docking poses were energy minimized to refine protein-ligand interactions, and approximate free energies of binding (ΔG) were estimated (see Methods). The calculated ΔG values appear the highest in the DBP of the T protomer for both substrates and substantially smaller in the AP of the L and L* protomers. The L* protomer has a wider cleft between the PC1 and PC2 subdomains and the recently solved LTO AdeB trimer30 indicated ethidium bound to the AP. Possibly, the L* drug binding might elicit clamping of the substrate (due to a PC2 subdomain movement, Supplementary Fig. 6A, B). Alternatively, the L* drug binding might induce opening of the DBP and conversion to the T state, yielding the TOO protomer with bound ethidium (AdeB-Et-I)30.

Fig. 1 Cryo-EM density maps of AdeB. A Side view of AdeB density in the OOO conformation at an overall resolution of 3.54 Å. B Side view of AdeB density in the L*OO conformation at 3.84 Å resolution. C Top view of AdeB density in the OOO conformation. D Top view of AdeB density in the L*OO conformation. The densities for the protomers are shown from light to dark red for the O conformation and in green for the L* conformation. The approximate boundary of the inner membrane embedded part of AdeB is indicated by dashed lines. The dimensions of the densities corresponding to the transmembrane, porter and funnel domains are indicated in (A) and (B). The densities are displayed at cutoff levels 0.0356 (OOO) and 0.0343 (L*OO) in ChimeraX (https://www.rbvi.ucsf.edu/chimerax).
As the ethidium-bound structures of AdeB were published after submission of this manuscript and our efforts to obtain co-structures with novobiocin were unsuccessful, we set out to determine binding of various drugs to the open-state DBP of AcrB, for which we had previously established a successful methodology for the determination of the AcrB periplasmic part (AcrBper) drug co-structures. Based on these AcrB per co-structures, we analyzed the activity of 20 single-substitution DBP variants of AdeB with known substrates for both AdeB and AcrB and compared these activities with DBP variants of AcrB.

Structural investigation of substrate binding to AcrBper. We obtained crystal co-structures of trimeric AcrBper in the LLT conformation with doxycycline (2.1 Å), fusidic acid (2.3 Å) and levofoxacin (2.7 Å) by soaking pre-grown AcrBper/DARPin crystals in the presence of doxycycline (6.25 mM), fusidic acid (5 mM) or levofoxacin (5 mM), respectively (Fig. 3 and Supplementary Figs. 8 and 9). Doxycycline appears to bind in a congruent manner to the DBP groove as reported for minocycline and tetracycline antibiotics within the DBP, where DXT-2 interacts with the side chains of S135, F136, V139, F178, Y327, M573, F610, F615, F617 and F628. This second binding site is expected to have a lower affinity, as reflected by its higher B-factor (Supplementary Table 5). The levofoxacin binding site within the DBP overlaps substantially with the doxycycline (2.1 Å), fusidic acid (2.3 Å) and levofoxacin (2.7 Å) binding sites.

Fig. 2 Structural models of AdeB in the OOO and L’OO conformations. A Side view of the C3-symmetric structure of AdeB in the OOO conformation solved at 3.54 Å. The approximate boundary of the inner membrane embedded part of AdeB is indicated by dashed lines. The dimensions of the transmembrane, porter and funnel domains are indicated. B From the same sample, we obtained a structure of AdeB in the L’OO conformation at 3.84 Å resolution shown in side view. C Top view of AdeB in the L’OO conformation. The L’ conformation (Chain A of the PDB entry: 7BQQ) is depicted in green, and the O conformation in light and dark red. Tunnels were calculated with CAVER Analyst. Channels 1, 2 and 3, leading to the closed DBP, are marked in the L’ conformation. The PC1 and PC2 subdomains constitute an open cleft in L’ conformation (channel 2 entrance). All entry sites are closed in the O conformation, and an exit channel is opened. In the O conformation, the PC1 and PC2 subdomains are in close contact constituting a closed cleft. D Top view of the C3-symmetric structure of AdeB in the OOO conformation. E The DBP cave (wheat surface and side chains in sticks) and groove (light blue) regions are closed in the L’ conformation F Rhodamine 6G (R6G) derived from the co-structure of AcrBper (PDB: 5ENS), superimposed on the AdeB L’ conformation (green cartoon). Binding of R6G (pink sticks) to the DBP in this conformation is not possible. G A superposition on the PN1 subdomain of the L’ conformation (green cartoon) with the AcrB L (blue cartoon) and H the AcrB T conformation (yellow cartoon) (PDB: 4DX5) shows clear deviations in the PN2 and PC1 subdomains. Latter subdomains in L’ are arranged in a different state compared to the L and T conformations, while the PN1 and PC2 subdomains are congruent. The switch loop (L’, green) is shifted to the inside of the DBP compared to the switch loop in the AcrB T conformation (yellow, H), congruent to the switch loop conformation in the L conformation of AcrB (blue, G). AP, access pocket; DBP, deep-binding pocket.
Fig. 3 Crystal structures of doxycycline (DXT; 2.1 Å), fusidic acid (FUA; 2.3 Å) and levofoxacin (LFX; 2.7 Å) bound to the DBP of the AcrBper protomer in the T conformation. A, B The DXT-soaked AcrBper crystals yielded two non-proteinaceous densities within the DBP, which we assigned to the molecules DXT-1 (A) and DXT-2 (B). While the DXT-1 binding position at the DBP groove is similar to the one previously reported for the tetracycline antibiotic minocycline8, 10, 18, DXT-2 is located at the DBP cave region, where it interacts with the side chains of S135, F136, V139, Y327, M573, F610, F615, F617 and F628. C Relative orientation of both DXT molecules within the DBP. D The LFX binding site within the DBP overlaps substantially with the binding site previously reported for rhodamine 6G (R6G)18. Like R6G, LFX interacts mainly with the hydrophobic side chains of F178, F610 and F628. E The FUA binding site resides at a more proximal part of the DBP. In addition to extended hydrophobic interactions within the binding pocket, the 3-hydroxyl group and carboxylate moiety on either side of the FUA molecule are involved in hydrogen bonding with the G616 and F136 main chains, respectively. Ligands are represented as sticks (carbon = blue-green (DXT), gray (FUA), salmon (LFX); nitrogen = blue; oxygen = red; fluoride = pale blue). The 2Fo–Fc electron density maps (blue-colored mesh) are contoured at 0.8σ. Residues involved in ligand binding are shown as sticks (carbon = yellow; nitrogen = blue; oxygen = red; sulfur = gold). Water molecules are represented as cyan-colored spheres. Hydrogen bonds are shown as red dashed lines, with the numbers representing the H-bond distances in Å.
with the binding site previously reported for rhodamine 6G\textsuperscript{22} (Figs. 3D, 4E, F, 5 and Supplementary Figs. 8B and 9B). Like rhodamine 6G, levofoxacin interacts mainly with the hydrophobic side chains of F178, F610 and F628. We found fusidic acid bound to a more proximal part of the DBP, where it interacts with the side chains of S135, F136, V139, Y327, Q569, V571, M573, F615, F617, G619, F628 and L668 (Figs. 3E and 4G and Supplementary Figs. 8C and 9C). In addition, the fusidic acid 3-hydroxyl group forms a hydrogen bond with the G616 mainchain carbonyl oxygen, while the fusidic acid carboxyl group is

**Fig. 4 Substrate binding to the AcrB T conformation.** Binding sites within the AcrB DBP for **A** minocycline (MIN; carbon = green; PDB: 4DX5), **B** doxycycline (DXT-1) and **C** doxycycline (DXT-2); carbon = blue-green; this study), **D** doxorubicin (DOX; carbon = violet; PDB: 4DX7), **E** rhodamine 6G (R6G; carbon = pink; PDB: 5ENS), **F** levofoxacin (LFX; carbon = salmon; this study), **G** fusidic acid (FUA; carbon = gray; this study) and **H** puromycin (PUY; carbon = orange; PDB: 5NC5). AcrB residues involved in ligand binding are shown as sticks (AcrB; carbon = yellow; nitrogen = blue; oxygen = red; sulfur = gold) and by number, with colored numbers representing the corresponding AdeB residues.
Molecular determinants of substrate binding in the DBP of AdeB. From the AcrB/AcrBper co-structures determined in this study, we identified the interaction sites of levofloxacin, doxycycline and fusidic acid, as well as the residues involved in binding rhodamine 6G (PDB: 5ENS\textsuperscript{18}), minocycline (PDB: 4DX5\textsuperscript{10}) and doxorubicin (PDB: 4DX7\textsuperscript{10}) (Figs. 3 and 4). Based on these structural insights, we constructed 20 single-substitution DBP variants of AdeB. In the first set of variants, 10 residues involved in substrate binding to AcrB/AcrBper were exchanged with Ala at homologous positions in AdeB. Six of these residues are conserved between AcrB and AdeB. A second set was generated based on non-conserved amino acids, i.e. ten residues in the DBP of AdeB were substituted with their counterparts in AcrB. As a control, we used site-directed mutagenesis on AcrB for those homolog positions, which showed in AcrB the largest drug susceptibility effects upon substitution (Supplementary Table 6 and Supplementary Fig. 10). We then tested the drug efflux capacity of the AdeB variants in drug-agar-plate dilution assays and compared it to that of wildtype AcrB (Supplementary Figs. 10, 11, 12, 13). Drug concentrations were adjusted to yield an intermediate growth of cells producing wildtype AdeB, so that both less active and hyperactive variants could be identified within one experimental setup. Results (from three independent biological repeats) were analyzed by counting the dilution steps showing cell growth. The inactive proton transport relay variant AdeB D407N was used as a negative control in this study, and the number of dilution steps was subtracted from all other dilution step numbers. Furthermore, to compare the activity with that of wildtype AdeB, we subtracted the number of dilution steps showing cell growth for \textit{E. coli} cells harboring wildtype AdeB (Fig. 6).

Overall, we show that all tested AcrB substrates (fusidic acid, doxorubicin, tetracyclerinophosphonium, ethidium, rhodamine 6G, chloramphenicol, doxycycline, minocycline, and levofloxacin) are transported by wildtype AdeB (Fig. 6 and Supplementary Figs. 11, 12, 13). AcrB conferred in general less susceptibility than AdeB, except for tetracyclerinophosphonium. Furthermore, we observed a strong tendency towards much higher AcrB-conferred resistance to all the non-polyaromatic compounds, and doxorubicin, but this effect was more moderate on minocycline and doxycycline. The overall discrepancy in resistance between AcrAB-TolC and AdeABC might be explained by the slightly lower levels of the heterologously expressed \textit{ade}B and perhaps \textit{ade}AC genes (Supplementary Figs. 11 and 12). However, AdeABC seems to confer higher resistance than AcrAB-TolC towards those compounds having three or more aromatic rings (ethidium, tetracyclerinophosphonium, rhodamine 6G), despite the lower expression levels.

Below we compare the AdeB variants to wildtype AdeB, all of which display similar expression levels (Supplementary Figs. 11 and 12).

**Rhodamine 6G, tetracyclerinophosphonium and ethidium share a binding site in AcrB.** Co-structures with ethidium and rhodamine 6G bound to the DBP of AdeB and AcrB, respectively, have been reported recently\textsuperscript{18,30}. These molecules share characteristics of polyaromaticity (three rings or more) and an overall positive charge with tetracyclerinophosphonium, for which there is no co-structure with any of the known RND transporters. In AcrB, rhodamine 6G interacts mainly with the aromatic side chains of F136, F178, Y327, and F628 (F623 in AdeB) inside the hydrophobic trap by π–π stacking, as well as with Q176 (Fig. 4E)\textsuperscript{18}. Y327 and Q176 are conserved residues amongst RND transporters\textsuperscript{35}. In AcrB and AdeB, Ala substitution of these residues resulted in a marked increase in rhodamine 6G susceptibility (Fig. 6 and Supplementary Table 6), which suggests a similar binding mode for this drug in the AcrB and AdeB DBP (Fig. 6, Supplementary Table 6, and Supplementary Figs. 10 and 11). The AdeB variants F136A, F178A, Y327A, Q292A and F623A appear to have a similar, but not identical, resistance phenotype to rhodamine 6G, ethidium, and tetracyclerinophosphonium, which suggests a shared binding site localized around these residues within the AdeB DBP. Indeed, superimposition of the AdeB T protomer-ethidium co-structure with the AcrBper-rhodamine 6G co-structure shows a common binding plane for these molecules (Supplementary Fig. 14)\textsuperscript{18,30}. The effects of Ala substitutions are less pronounced for ethidium (except for F178A), but other substitutions appear to influence resistance to rhodamine 6G as well (E89A, Q176A, F277A).

The AdeB variant G135S has a strong lowering effect on resistance to rhodamine 6G, whereas its resistance to ethidium and tetracyclerinophosphonium is hardly affected. The E151Q and W568V variants also negatively affect the resistance against rhodamine 6G, whereas there is no change in their resistance to ethidium, but these variants confer a better than wildtype resistance towards tetracyclerinophosphonium.

The position of the F610 side chain in AcrB, part of the hydrophobic trap, is occupied by T605 in AdeB. Despite the important role of F610 in substrate binding/transport in AcrB\textsuperscript{26,36,37} including binding of rhodamine 6G\textsuperscript{18} (Supplementary Table 6 and Supplementary Fig. 14), the transport of rhodamine 6G, tetracyclerinophosphonium and (to a lower extent) ethidium is disturbed in the AdeB variant T605F (Fig. 6 and Supplementary Fig. 15). By contrast, transport of levofloxacin, chloramphenicol, minocycline, doxycycline, chloramphenicol, doxycycline, minocycline, and levofloxacin are transported by wildtype AdeB (Fig. 6 and Supplementary Figs. 11, 12, 13). AcrB conferred in general less susceptibility than AdeB, except for tetracyclerinophosphonium. Furthermore, we observed a strong tendency towards much higher AcrB-conferred resistance to all the non-polyaromatic compounds, and doxorubicin, but this effect was more moderate on minocycline and doxycycline. The overall discrepancy in resistance between AcrAB-TolC and AdeABC might be explained by the slightly lower levels of the heterologously expressed \textit{ade}B and perhaps \textit{ade}AC genes (Supplementary Figs. 11 and 12). However, AdeABC seems to confer higher resistance than AcrAB-TolC towards those compounds having three or more aromatic rings (ethidium, tetracyclerinophosphonium, rhodamine 6G), despite the lower expression levels.

Below we compare the AdeB variants to wildtype AdeB, all of which display similar expression levels (Supplementary Figs. 11 and 12).
and fusidic acid is better than AdeB wildtype in this variant. We suggest that in AdeB, rhodamine 6G is shifted slightly in the DBP compared to its location in AcrB (and to the position of ethidium (Supplementary Figs. 7 and 14)), using the extended space near T605. This might explain the severe negative effect on rhodamine 6G resistance, but less so on ethidium resistance, of cells harboring the AcrB Y327A variant, whereas the contrasting effects are observed in AdeB Y327A variant, susceptibilities are strongly decreased in the AdeB Y327A variant, whereas in AcrB, this substitution does not alter the susceptibility compared to wildtype AcrB. For Y327A, F136A causes an increase in resistance towards levo-oxacin and chloramphenicol, whereas in AcrB, this substitution does not cause a strong susceptibility effect (Fig. 6 and Supplementary Table 6).

### Improved transport of levofloxacin and correlation to chloramphenicol

Concomitant with larger susceptibilities to rhodamine 6G, ethidium and tetraphenylphosphonium caused by the F136A (except with ethidium), F277A (rhodamine 6G only), and F178A (except with ethidium) substitutions, susceptibilities to levofloxacin and chloramphenicol are, in contrast, substantially reduced (i.e., higher resistance is observed), particularly for the F136A and Y327A AdeB variants (Fig. 6 and Supplementary Figs. 11 and 12). Compared to wildtype AcrB, AcrB variants F136A and F178A do not confer any difference in susceptibility to doxycycline (DXT) and 0.01 µg/ml levofoxacin (LFX). All experiments were performed in biological triplicates. The last dilution steps showing cell growth were documented and averaged (Supplementary Figs. 11 and 12). The numbers indicate the calculated difference to AdeB wildtype after subtraction of the negative control (D407N). Positive results (green shadings) indicate increased resistance to the drug compared to AdeB WT, negative results (red shadings) indicate decreased resistance. E. coli AcrB, wildtype A. baumannii AdeB, inactive AcrB variant (D407N) and AdeB deep-binding pocket (DBP) variants (E89A, F136A, Q176A, F178A, Q292A, Y327A, M570A, T605A, F623A, E89Q, G135S, Q292K, W568V, E151Q, A180S, T605F, W610F, N276D, F277I, A180S, T605F, W610F, N276D, F277I) and AcrB, wildtype A. baumannii AdeB, inactive AcrB variant (D407N) were measured twice, once in biological triplicates with the single Ala-substitutions (E89A, F136A, Q176A, F178A, F277A, Q176A, F178A, Q292A, Y327A, M570A, T605A, F623A, E89Q, G135S, Q292K, W568V, E151Q, A180S, T605F, W610F, N276D, F277I). Plate dilution assays were performed with E. coli BW25113 ΔacrB ΔacrD ΔmdtBC prRSFdueFX_MS_adeAC harboring pET24_acrB or p7XC3H_adeB WT and mutants. Dilution series of overnight cultures with an OD_{600nm} of 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6} and 10^{-7} (6 dilution steps) were spotted on Mueller-Hinton Agar plates containing 50 µg/ml kanamycin, 50 µg/ml carbencillin and 20 µM IPTG, with or without (control plate) the tested drug. Plates were supplemented with the following compounds: 2 µg/ml fusidic acid (FUA), 8 µg/ml doxycycline (DOX), 60 µg/ml rhodamine 6G (R6G), 60 µg/ml ethidium (ETH), 250 µg/ml tetrathorphosphonium (TPP), 1 µg/ml chloramphenicol (CAM), 1 µg/ml minocycline (MIN), 1 µg/ml doxycycline (DXT), and 0.01 µg/ml levofoxacin (LFX). All experiments were performed in biological triplicates. The last dilution steps showing cell growth were documented and averaged (Supplementary Figs. 11 and 12). The numbers indicate the calculated difference to AdeB wildtype after subtraction of the negative control (D407N). Positive results (green shadings) indicate increased resistance to the drug compared to AdeB WT, negative results (red shadings) indicate decreased resistance. E. coli AcrB, wildtype A. baumannii AdeB, inactive AcrB variant (D407N) were measured twice, once in biological triplicates with the single Ala-substitutions (E89A, F136A, Q176A, F178A, Q292A, Y327A, M570A, T605A, F623A, E89Q, G135S, Q292K, W568V, E151Q, A180S, T605F, W610F, N276D, F277I).

### Table 6

| Drug | MIC (µg/ml) |
|------|-------------|
| FUA  | 2           |
| DOX  | 8           |
| R6G  | 60          |
| ETH  | 60          |
| TPP  | 250         |
| CAM  | 1           |
| MIN  | 1           |
| DXT  | 1           |
| LFX  | 0.01        |

**Fig. 6 Drug susceptibility profiles of E. coli harboring AdeB deep-binding pocket variants.** Analysis of plate dilution assays with E. coli AcrB, wildtype A. baumannii AdeB, inactive AcrB variant (D407N) and AcrB deep-binding pocket (DBP) variants (E89A, F136A, Q176A, F178A, Q292A, Y327A, M570A, T605A, F623A; E89Q, G135S, Q292K, W568V, E151Q, A180S, T605F, W610F, N276D, F277I). Plate dilution assays were performed with E. coli BW25113 ΔacrB ΔacrD ΔmdtBC prRSFdueFX_MS_adeAC harboring pET24_acrB or p7XC3H_adeB WT and mutants. Dilution series of overnight cultures with an OD_{600nm} of 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6} and 10^{-7} (6 dilution steps) were spotted on Mueller-Hinton Agar plates containing 50 µg/ml kanamycin, 50 µg/ml carbencillin and 20 µM IPTG, with or without (control plate) the tested drug. Plates were supplemented with the following compounds: 2 µg/ml fusidic acid (FUA), 8 µg/ml doxycycline (DOX), 60 µg/ml rhodamine 6G (R6G), 60 µg/ml ethidium (ETH), 250 µg/ml tetrathorphosphonium (TPP), 1 µg/ml chloramphenicol (CAM), 1 µg/ml minocycline (MIN), 1 µg/ml doxycycline (DXT), and 0.01 µg/ml levofoxacin (LFX). All experiments were performed in biological triplicates. The last dilution steps showing cell growth were documented and averaged (Supplementary Figs. 11 and 12). The numbers indicate the calculated difference to AdeB wildtype after subtraction of the negative control (D407N). Positive results (green shadings) indicate increased resistance to the drug compared to AdeB WT, negative results (red shadings) indicate decreased resistance. E. coli AcrB, wildtype A. baumannii AdeB, inactive AcrB variant (D407N) were measured twice, once in biological triplicate with the single Ala-substitutions (E89A, F136A, Q176A, F178A, Q292A, Y327A, M570A, T605A, F623A) and once in biological triplicate with E89Q, G135S, Q292K, W568V, E151Q, A180S, T605F, W610F, N276D, F277I.
on susceptibilities toward levofloxacin/chloramphenicol compared to rhodamine 6G/ethidium/tetraphenylphosphonium might indicate a shared binding site for levofloxacin and chloramphenicol, as well as mutually exclusive binding of the three polyaromatic compounds rhodamine 6G, ethidium and tetraphenylphosphonium. Compared to the latter cationic polyaromatic compounds (with delocalized charge), levofloxacin and chloramphenicol comprise only one aromatic ring, and levofloxacin contains a localized negative charge (and an additional positive charge at lower pH), whereas chloramphenicol contains a local zwitterion. Despite these obvious physicochemical differences, the AcrBper/levofloxacin co-structure shows the binding of levofloxacin at the same planar level as the binding of rhodamine 6G, albeit slightly shifted towards Y327 (Fig. 4E, F). This slight deviation in orientation is also apparent compared to ethidium binding in the T protomer of AdeB (Supplementary Fig. 14B).

The main interaction partners of levofloxacin seem to be the conserved AdeB residues F178 and F628 (F623 in AdeB), as well as F615 (W610 in AdeB). Removal of hydrophobic bulky residues (F136A and Y327A) in the lower part (entrance) of the DBP cave region markedly increased the levofloxacin and chloramphenicol pump activity in AdeB. Ile or Ala substitution of F277, a residue located further up in the DBP groove, also causes higher levofloxacin and chloramphenicol pump activity. Moreover, introduction of Phe in the T605F variant, also increases levofloxacin and chloramphenicol pump activity leading to higher resistance against these compounds, whereas removal of the phenyl ring of F610 in AcrB (homologous to the T605 position), causes a strong reduction in resistance against levofloxacin and chloramphenicol (Supplementary Table 6). We suggest that levofloxacin and chloramphenicol share a similar binding mode in AdeB. The binding region in AdeB appears to be different for levofloxacin and chloramphenicol compared to rhodamine 6G, ethidium and tetraphenylphosphonium. The shared binding site for levofloxacin with rhodamine 6G is that seen in the AcrB co-structures and supported by the mutational analysis data (Supplementary Table 6, except for F136A), does not seem to hold true for AdeB.

It appears that AdeB binding and transport of the tested substrates changes strongly upon removal of Phe and Tyr side chains in the DBP, and these exchanges result in different phenotypes in AcrB. For AdeB, substitutions like Q176A (which leads to a hyperactivity resistance phenotype for chloramphenicol and a severe reduction in resistance to rhodamine 6G), most likely affect the orientation(s) of the nearby F178 and F136 side chains, leading to a change in the binding properties. Similarly, G135S is expected to influence the conformational freedom of F136, leading to suboptimal binding of rhodamine 6G and slightly more favorable conditions for binding and/or transport of chloramphenicol, minocycline, doxycycline, and levofloxacin (Fig. 6 and Supplementary Figs. 12 and 14).

Minocycline, doxycycline, fusidic acid and doxorubicin, but also chloramphenicol and levofloxacin, appear to be less preferred substrates of AdeB, considering the lower susceptibility (higher resistance) effects of many of the side chain substitutions near the DBP. Ala-substitutions in general hardly affect the activities against these drugs (except for chloramphenicol and levofloxacin with higher resistances upon Ala-substitution). Almost all substitutions leading to homolog AcrB residues increase resistance to these compounds.

Exceptions are the resistance observed for these drugs by G135S (no effect on resistance to fusidic acid and doxorubicin), and A180S and N276D, which either have no effect or decrease resistance to most compounds. The AdeB T605F variant clearly confers higher resistance to cells, implying a more favorable interaction site especially for chloramphenicol and levofloxacin. Substitution of F277 with Ile or Ala resulted in a variant of AdeB that conferred higher resistance compared to wildtype AdeB for minocycline, doxycycline, fusidic acid, doxorubicin, chloramphenicol, and levofloxacin.

The DBP of AdeB is highly lipophilic (13 non-polar residues, compared with 11 in AcrB and 4 in AcrD) with a moderate propensity for hydrogen bonding (13 side chain hydrogen bond donors/acceptors, compared with 16 in AcrB and 20 in aminoglycoside-transporting E. coli homolog AcrD). Although AdeB contains three negatively charged side chains (compared with two in AcrB and two in AcrD), it has no positively charged side chains (compared with two in AcrB and four in AcrD).

We assume that AdeB variants E89A, E89Q, E151Q, N277D and Q292K change the resistance phenotype by altering the balance of charges in the DBP. Removal of a negative charge in variants E89A, E89Q, and E151Q increases resistance to all compounds that we tested except rhodamine 6G. The additional negative charge in N277D decreases resistance to all compounds except the tetracyclines minocycline and doxycycline. Insertion of a positive charge in Q292K improves transport of all tested compounds.

In sum, it appears that AdeB displays much higher preference for polyaromatic compounds (with three or more aromatic rings), and that single substitutions changing the polyaromatic interaction sites within the DBP greatly decrease pump activity. These variants, however, transport more hydrophilic compounds, especially levofloxacin and chloramphenicol, much more efficiently. Furthermore, the lower susceptibilities conferred by many of the AdeB variants compared to wildtype AdeB suggest that levofloxacin, chloramphenicol, and other hydrophilic compounds are less efficiently transported out of the E. coli cell by the wildtype AdeB pump.

Discussion

AdeABC is a major factor in MDR of A. baumannii, but it is tightly regulated in wildtype strains. So-called “slow” porins, which have low pore-forming activity, contribute to the tight outer membrane of A. baumannii. Its low permeability, in synergy with multidrug efflux pumps, is a strong defense mechanism against toxic compounds. By contrast, the well-characterized E. coli AcrAB-ToIC complex acts in synergy with a rather porous outer membrane, yet porination appears to only affect permeation of a distinct set of toxins. For some hydrophobic compounds, outer membrane permeation was shown to be higher in A. baumannii compared to E. coli. RND transporter complexes spanning the outer and inner membranes need to overcome different challenges in these organisms. Previous reports and this study illustrate the extraordinarily broad substrate spectrum of AdeB, bestowing it with a promiscuity that is anticipated to be even higher than that observed for AcrB. Owing to the relatively fast permeation of some substrates across the more porous outer membrane, E. coli needs to react immediately. The presumed resting state of constitutively expressed AcrB is the LLL conformation, which has multiple channels and binding sites per trimeric complex. A current hypothesis, based on cryo-EM and in situ cryo-electron tomography studies, suggests that AcrAB diffuses along the inner membrane, transiently forming complexes with ToIC. Substrate binding leads to the instant activation of the transport cycle by closing a channel formed by AcrA and opening the periplasmic α-barrel of ToIC. In A. baumannii, the AdeIJK efflux pump is constitutively expressed and might be comparable in its role to AcrAB-ToIC in E. coli. The recent apo and eravacycline-bound structures revealed an asymmetric trimeric setup, with the protomers in three distinct states. Our single-particle cryo-EM results in this work show that 90% of imaged AdeB protomers
remain inaccessible to substrates from the periplasm in the O conformation. Just 10% of analyzed protomers adopt the L* conformation. We assume that the high prevalence of particles in the OOO conformation is not an artifact, as similar trimeric conformations have been observed in another cryo-EM structure of AdeB and a crystal structure of Campylobacter jejuni CmeB. A recent study of the structure of symmetric AdeB found particles only in the O conformation. Despite the O conformation containing no substrate binding sites, Su et al. presented molecular docking of AdeB substrates bound to AdeB. This apparent contradiction might be explained using homology modeling of AdeB with one of the published homologous RND (co-)structures (likely in a T conformation). However, since the homology modeling procedure was neither indicated nor described as such, the impression was conveyed that the solved AdeB OOO structure included substrate binding sites. The recent report from the same lab on the symmetric and asymmetric trimeric structures obtained in presence of ethidium, on the other hand, gives valuable insights. The asymmetric structures reveal multiple trimeric protomer states i.e., (i) binding/extrusion (TOO), (ii) binding/extrusion/resting (TO-Resting), and (iii) access/binding/extrusion (LTO). The O and resting states are the only states without drug bound, and the L and T states are only present in an ethidium bound state. Whereas the L state binds one ethidium molecule in the AP, the T protomer in the TOO state binds two ethidium molecules in the DBP and one in the AP, the T protomer in the TO-Resting and LTO states, bind one ethidium molecule in the DBP and AP each.

In this report, we describe a previously undiscovered protomer conformation by single-particle cryo-EM. The L* conformation, assumed to be present in only 30% of AdeB trimers in the cell, revealed three substrate entry channels per protomer, corresponding to AcrB CH1–CH3, which lead to the (still closed) DBP. We propose that L* is the first conformation in the transport cycle of AdeB. Substrate binding via L* might lead to an induced fit conformational change (possibly to a drug-bound T conformation), priming the inner membrane pump for assembly into an active AdeABC tripartite complex. Molecular docking experiments indicate the binding of rhodamine 6G and ethidium to the L* protomer near the experimental ethidium molecule in the L state (Supplementary Fig. 7 and Supplementary Table 4). Whereas it is not possible to discern whether the L* conformation is recurring in the postulated AdeB LTO cycle, that state appears to be the only one bearing entrances from the periplasm in the absence of substrates. This would allow initial substrate entry in case drugs enter the periplasm, since the OOO state lacks substrate entry and binding sites. As the OOO and L*OOO conformations and no other conformations are observed in the absence of drugs, we suggest the drug extrusion cycle starts with drug binding at the L* protomer. Binding of drugs might trigger the L* to T transition, possibly favored by the counterclockwise neighboring O protomer with a tilted PN1 subdomain. This would result in the TOO structure, as observed by Morgan et al. (AdeB-Et-I, PDB: 7KGG). The other, clockwise neighboring protomer, might subsequently convert to the L state and bind substrate in the AP, leading to the LTO structure AdeB-Et-III (PDB: 7KGI). Further drug uptake and release is proposed to occur in analogy to the E. coli AcrB LTO transport cycle.

The highly reduced number of accessible binding sites together with wildtype gene repression might contribute to a tight regulation of AdeB, when the adeABC operon is expressed. In comparison, to date, no structures of trimeric AcrB in the OOO conformation have been reported. A trimeric arrangement with more than one O conformation was calculated to be energetically unfavorable and to hypothetically induce release of AcrA, leading to a disassembly of the complex. The observed OOO conformation of AdeB in the absence of its interaction partners AdeA and AdeC is consistent with this hypothesis. In the asymmetric AdeB structure AdeB-II_Et, an additional, possibly intermediate state was observed, which was designated as resting state. This resting state shows structural resemblance to the previous observed C state in the MexAB-OprM tripartite structure. It was suggested that this C-state precedes the O-state. It therefore appears that the single-particle Cryo-EM analysis reveals additional energy states of the RND protomers, hence conformations, which represent intermediate states between the three main L, T and O states derived from crystal structures.

For RND-type antibiotic exporters, both the existence of multiple substrate entry sites and the variable nature of the AP and DBP contribute to the broad substrate polyspecificity observed. For AcrB, four distinct substrate entry sites have been described. However, all entry channels are thought to converge in the DBP of the T conformation, from which the substrates are expelled through the exit channel upon transition from the T to the O conformation. From superposition of the known substrate coordinates (8–10,15,18 (Figs. 4 and 5), we conclude that substrates share partially overlapping binding sites within the groove and cave regions of the entire DBP. Largely planar, heterocyclic compounds including doxycycline and levofloxacin fit into the DBP groove and upper cave region, interacting efficiently with polar side chains and hydrophobic residues of the Phe-rich area. Non-planar molecules like fusidic acid and puromycin are found at a more proximal part of the DBP. These molecules appear to bind to the AP-DBP interface, below the switch loop at a similar position as reported for erythromycin in the AP of the L conformation. The co-structures with erythromycin, fusidic acid and puromycin might represent states close to the L–T transition. In general, each co-structure can be interpreted as a snapshot of a single compound along its transport pathway. These snapshots are likely to represent local energy minima, where the substrate is bound to its preferred interaction site within the DBP. Nevertheless, upon extrusion, each drug is thought to interact with several transient binding sites. In fact, each binding mode observed in the co-structures (Fig. 4) described above might reflect a possible position that any of the drugs can take, albeit with different affinities and hence probabilities. We hypothesize that the overlapping binding sites illustrated by superposition of the different co-structures (Fig. 5) represent the transport pathway of any of the drugs during catalysis. In this way, drug substrates slide from underneath the switch loop towards the DBP, from where these drugs are actively guided upon the T–O transition through the exit tunnel.

The higher resistances conferred to E. coli by AdeB towards polyaromatic compounds as compared to more hydrophilic drugs and the decrease of resistance for the polyaromatic compounds upon Ala substitutions, may imply that the L* conformation, which includes three substrate transport channels (Fig. 2B), is closer to a drug binding state than might be anticipated at first sight. The reported molecular docking poses are one indication that rhodamine 6G and ethidium might initially bind in the AP of the L* protomer (Supplementary Fig. 7). Our mutagenesis analysis with AdeB indicates furthermore that a slight change in binding properties elicits differential effects, resulting in worse or much better than wildtype transport efficiencies. The effect of substitutions in AcrB, however, are either increasing the susceptibility of all drugs tested (e.g., rhodamine 6G, tetraphenylphosphonium, levofloxacin, apart from F136A for tetraphenylphosphonium and levofloxacin) or have no effect. For chloramphenicol, e.g., only Y327A and F610A increase susceptibility and the three other substitutions are without effect. In contrast, substitutions like F136A and Y327A in AdeB greatly increase the resistance towards levofloxacin and chloramphenicol.
Of interest, all T protomers shown by Morgan et al.30, contain next to the binding of one or two ethidium molecules in the DBP, always an ethidium molecule in the AP. This contrasts with AcrB, where the DBP has been found open in the T state even in the absence of substrates40 and thus far has not shown to be binding drugs to both AP and DBP in the same protomer. We speculate that the binding of drugs to the AcrB DBP might occur via conformational selection. For AdeB, the mechanism might be rather an induced fit. The AP of the L* protomer is occupied by the drug, which might induce the opening of the DBP. Polyaromatic substrates might be acting subsequently more effective with the aromatic side chains of the AdeB DBP and allow for tight donor and acceptors. The L* state might be a state only present in the absence of drugs in the L3O0 conformation, next to the O0O conformation. Once drugs are present, the other protomers like L and T with drug bound might influence the conformation of their neighboring partners within the trimer. The TOO state (AdeB-I-Et) observed by Morgan et al.30 might represent likewise an initial state prior to the LTO state (AdeB-III-Et).

This report indicates that substrate binding to RND transporters cannot be understood only by substitution-based functional analysis, nor exclusively using co-structures. Indeed, much more structural information is needed to understand AdeB (and AcrB) substrate binding, including information on other conformational states, which could possibly be inferred from analyses of inhibitor binding or substitution variants, complemented by in silico studies. Moreover, the insights from one specific RND transporter cannot easily be extrapolated to homologous transporters. To understand the efflux phenotype of pumps in their cognate environments, these must be studied in their native hosts, as the synergy between OM permeability and efflux pumps has been evolutionary developed.

**Methods**

**Cloning of adeB, adeA and adeC, site-directed mutagenesis of adeB.** The genes encoding AdeA (ABAYE1821), AdeB (ABAYE1822) and AdeC (ABAYE1823) were PCR-amplified from the genomic DNA of A. baumannii AYE. The adeB gene was cloned into pNiTA cat via FX-cloning43. All adeB mutants were constructed by site-directed mutagenesis using inverse PCR with the template pNiTA adeB. For further experiments, wildtype adeC (WT) and the resulting mutants were sub-cloned by FX-cloning into p7XC3H145. The genes adeA and adeC were inserted into the plasmid pRSFDuetFX-Ms, a modified version of pRSFDuet-1 (Novagen, Merck, Germany) (Supplementary Fig. 16). First, adeA was cloned into pNiTA cat by FX-cloning then subcloned into pRSFDuetFX-Ms with the same method. The adeC gene was cloned into pRSFDuetFX-Ms with a restriction site KpnI and Pacl. The identities of adeB-His10 (WT and mutants), adeA-Myc and adeC-Strep were confirmed by Sanger sequencing. All primers are listed in Supplementary Table 7.

**Expression and purification of AdeB.** *E. coli* C43(D3E) ∆acrAB42 harboring p7XC3H_adeB was grown in TB-medium (12 g/l tryptone, 24 g/l yeast extract, 5 g/l glycerol, 2.31 g/l KH2PO4, 12.5 g/l K2HPO4) with 50 µg/ml kanamycin to an OD600 of 1.2. Protein expression was induced with 0.5 mM IPTG and proceeded overnight at 20 °C. Cells were harvested and resuspended at 2 ml/g wet weight cells in 20 mM Tris-HCl pH 8.5, 500 mM NaCl, 2 mM MgCl2 supplemented with 10 µg/ml lysozyme, 10 µg/ml DNase and 200 µM PMSF. The cell suspension was stirred for 1 h and then lysed by two runs through Stansted SPCH-EP 10 pressure cell homogenizer (Homogenizing Systems Ltd, UK) at 14–28 kPsi. The lysate was then centrifuged in two steps, first for 45 min at 16,264 x g (Sorvall GSA rotor) to remove cell debris, then for 1 h at 186,010 x g (Beckmann 45 Ti rotor) to isolate the cell membranes. The membrane pellet was resuspended at 4 ml/g wet weight membranes in 20 mM Tris-HCl pH 8.5, 500 mM NaCl, then diluted in 8 ml 20 mM Tris-HCl pH 8.5, 150 mM NaCl, 1% glycerol. The mix was supplemented with 10 mM imidazole and 1 % N-Dodecyl-b-2-maltopyranoside (DDM). Membrane proteins were solubilized for 1.5 h. After centrifugation of the mixture for 1 h at 180,000 x g (Beckmann 45 Ti rotor), the supernatant was incubated with Ni-NTA (Qiagen, 0.2 ml of 50% slurry per ml supernatant) for 1 h. The loaded resin was washed consecutively with 20 mM Tris-HCl pH 8.5, 150 mM NaCl, 1% glycerol supplemented with 0.03% DDM and (1) 10 mM, (2) 30 mM, (3) 50 mM and (4) 100 mM imidazole, respectively. Washing steps 1 and 2 were performed with 15 bed volumes of buffer, steps 3 and 4 with 5 bed volumes of buffer. AdeB was eluted with the same buffer containing 100 mM imidazole. Purified AdeB was concentrated with Amicon 100 Ultra-15, 100 kDa cutoff to 1 ml and applied to SEC using a Superose 6 10/300 increase column equilibrated with 20 mM Tris-HCl pH 8.5, 150 mM NaCl, 0.03% DDM. All purification steps were performed at 4 °C.

**Reconstitution of AdeB into salipro nanodiscs.** The scaffold protein SapA was expressed and purified as previously described46 with some modifications. All growth media were supplemented with 0.4 µg/ml kanamycin, but no tetracycline was added. Cultures were incubated overnight at 20 °C after induction and cells were lysed by two passages through Stansted SPCH-EP-10 pressure cell homogenizer (Homogenizing Systems Ltd, UK) at 21 kpsi. In addition, imidazole concentrations during IMAC were reduced to 20 mM in the first washing steps and 100 mM in the buffer for elution flaps. The remaining exposed side and main-chain hydrogen bonding donors and acceptors. The L* state might be a state only present in the absence of drugs in the L3O0 conformation, next to the O0O conformation. Once drugs are present, the other protomers like L and T with drug bound might influence the conformation of their neighboring partners within the trimer. The TOO state (AdeB-I-Et) observed by Morgan et al.30 might represent likewise an initial state prior to the LTO state (AdeB-III-Et).

This report indicates that substrate binding to RND transporters cannot be understood only by substitution-based functional analysis, nor exclusively using co-structures. Indeed, much more structural information is needed to understand AdeB (and AcrB) substrate binding, including information on other conformational states, which could possibly be inferred from analyses of inhibitor binding or substitution variants, complemented by in silico studies. Moreover, the insights from one specific RND transporter cannot easily be extrapolated to homologous transporters. To understand the efflux phenotype of pumps in their cognate environments, these must be studied in their native hosts, as the synergy between OM permeability and efflux pumps has been evolutionary developed.

**Cryo-EM sample preparation and data collection.** For the structural analysis of AdeB by cryo-EM, two datasets from consecutive purifications were recorded. For the first data set, purified AdeB-Salipro particles were concentrated to 0.68 mg/ml in 20 mM Tris-HCl pH 8.5, 150 mM NaCl. After exchanging into fresh buffer, the sample was dialyzed for an additional 3 h. The reconstitution mix was concentrated and purified by SEC using a Superose 6 10/300 increase column equilibrated with the same buffer. After the addition of AdeB to the reconstitution mix, all steps were performed at 4 °C.

**Cryo-EM data processing.** A total of 1997 micrograph stacks from both datasets were aligned with UCSF MotionCor2. The contrast transfer function (CTF) parameters were estimated using GCTF3. Further processing steps were performed with Relion 3.05. A small set of approximately 1000 particles was picked manually, 2D classes were generated and models for automated picking were selected. We extracted 381,635 particles after autopicking. Poor-quality particles were removed after each of several iterative 2D classification rounds. We used 192,349 particles as an input for an unsupervised 3D classification with a low-pass filtered reference map of trimeric *E. coli* AcrB pdb (2GHP). Correction of per-particle defocus and beam-induced motion was performed with 132,326 selected particles. After a high-resolution auto-refinement with applied C3 symmetry, a density map of AdeB in O0O conformation was obtained at 3.5 Å global resolution. The same set of particles was expanded based on their C3-symmetry, which resulted in triple the number of particles rotated along their symmetry axis. Two monomers were subtracted from each particle, resulting in 397,038 monomer particles. An unsupervised 3D classification was performed using a soft monomer mask and C3 symmetric AdeB as a reference map. The classification was performed without image alignment and the regularization parameter T was set to 15. In a total of 10 classes, one homogenous class comprising 35,170 particles showed an altered conformation. The class was selected for a high-resolution auto-refinement without applied symmetry, which resulted in the density map of the AdeB L* conformation.
at 3.95 Å global resolution. The same particles were trimmed again by re-verting the subtraction. Then, 280 duplicated particles were removed and a high-resolution auto-rotation averaging using the XLAB package was performed. Local resolution estimations were performed for all maps using the Local resolution implementation of Relion 3.0. An initial structure of AcrB was generated with Phyre2, using the T (for AcrB T) and O (for AcrB O) conformations from PDB 4DX1 as a starting model. In a Phenix real space refinement, the model was adapted to the calculated densities. The resulting structure was optimized using Coot and validated with MolProbity.

AcrBper expression, purification, and crystallization. Overexpression, purification and crystallization of AcrBper and DARPin clone 1108_19 were performed as previously described, with minor changes. In brief, purified AcrBper and DARPin were mixed in a molar ratio of 1:1. AcrBper/DARPIn crystals were grown at 18 °C in hanging drops containing 1.5 µl AcrBper/DARPIn solution (total protein concentration of 15 mg/ml) and 1.5 µl reservoir solution over 800 µl precipitant solution (0.1 M MES pH 6.5, 0.21 M NaCl, 11.5% PEG 4000) in the reservoir well.

Soaking of AcrBper/DARPIn crystals with doxycycline (DXT), fusidic acid (FUA), and levofloxacin (LFX). Solutions of doxycycline (50 mM), fusidic acid (19 mM) and levofloxacin (19 mM) were prepared in AcrBper purification buffer (10 mM HEPES pH 7.2, 0.2 M NaCl, 1 mM DTT, 10% glycerol). These stock solutions were mixed in a molar ratio of 1:1 and 1:5. Protein crystals were transferred into 1 µl soaking solutions and incubated over 800 µl reservoir solution for one week at 18 °C. For cryo-protection, the crystals were briefly soaked in a solution containing 15–25% PEG 200/300 (the respective substrate) in reservoir solution.

X-ray data collection and structure determination. X-ray data were collected at the beamlines P13 and P14 (Deutsches Elektronen Synchrotron, Hamburg, Germany) and PX1 (Synchronroton SOLEIL, Paris, France). Datasets were processed using XDS and AIMLESS from the CCP4 suite 27. The same set of R-free reflections (5% of the AcrB per apo dataset) was used for all datasets. Phases of the ligand-bound AcrBper and DARPin structures were derived from Phaser phenix-restrained refinement (REFMACS or phenix.refine from the PHENIX package) using the AcrBper/DARPIn apo apo-protein coordinates (PDB ID code 5ENS) as an input model. The first model was built using Coot, followed by restrained refinement using REFMACS or phenix.refine. For the assigned ligands (doxycycline, fusidic acid and levofloxacin), were taken from the Coot model dictionary. Ligands (doxycycline and levofloxacin) occupancies were refined using the respective option in phenix.refine. Structure validation was accompanied by data quality analysis using Molprobity. Packer maps were generated using Polder Maps from the PHENIX package. Statistics from data processing and refinement are listed in Supplementary Table 5. Ligasite files were generated using LiggPig 41. Figures were prepared using PYMOL (www.pymol.org).

Drug-agar-plate docking assays in E. coli. To test the drug efflux capability of different AcrB and AcrB variants, agar-plate docking assays were performed as described previously, with some modifications. E. coli BW25113(DE3) AcrB per AcrB ΔmdtBC harboring pRSFDSEX_MS adeA adeC together with pET24 AcrB per, pET24 AcrB ΔmdtBC, pET24 AcrB ΔmdtBC ΔadeA WT, inactive mutants D407N or D407A were transformed into the E. coli strain. After selection on LB plates containing 50 µg/ml kanamycin, 50 µg/ml carbenicillin and 20 µg/ml IPTG. After harvesting of cells, lysis experiment was performed on plates without kanamycin and carbenicillin. Expression levels were determined from overnight cultures grown in medium containing 50 µg/ml kanamycin, 50 µg/ml carbenicillin, and 20 µM IPTG. After harvesting of cells, lysis was performed by shearing force with FastPrep-24 (MP) and samples were solubilized in 1% DDM for 1 h. After centrifugation for 10 min at 191,531 × g in a TLA 100 rotor, AcrB ΔmdtBC (or variants) AcrB ΔmdtBC ΔadeA WT, inactive mutant D407N, T605F or N276D were grown to an OD600 of 0.5–0.6 at 37 °C, then incubated with 20 µM IPTG. After 1.5 h, cells were harvested, washed with 20 ml potassium phosphate pH 7.0, 1 mM MgSO4, 0.2% glucose and resuspended in the same buffer. The cell suspension was adjusted to an OD600 of 2. After the addition of ethidium bromide in a final concentration of 20 µM, the accumulation was observed with Tescan Microprobe Reader (Tescan, Switzerland) at excitation and emission wavelengths of 535 and 610 nm, respectively. Expression levels in the same cell suspensions were analyzed as described in the previous section.

Molecular docking. Molecular docking calculations targeting AdeB were performed using two different packages, namely AutoDock VINA, and the recently developed immersive synergistic docking function based on convolution neural networks. Re-docking of ethidium onto the structure of AdeB with PDB ID: 7KGI was successful using both codes (Supplementary Table 4). For consistency, the same input files, structures, and settings (in particular, the same box center and dimensions) were employed with both the docking programs. Default settings were used in all cases, except for the exhaustiveness parameter (giving a measure of the exhaustiveness of the local search), which was set to 512 (default 8). Protein and ligand input files in PDBQT format were prepared with AutoDock Tools. Protein flexibility was considered indirectly by employing five different conformations of AdeB. Namely, the R protonator in 7KGI, the T protonator in 7KGI and 7KGG, and the L and T protonators in 7KGI were aligned to the L* monomer in 7B8Q, also included in the pool of receptor structures. Ligand input configurations were obtained from quantum/mechanical optimizations at the density functional theory level (B3LYP/6–31G** in implicit solvent, as detailed in Mallorci et al. The two compounds were considered flexible during docking (the number of rotatable bonds being 4 and 8 for ethidium and rhodamine 6G, respectively). Two sets of guided docking runs were performed for all ligands using two rectangular boxes of dimensions 30 × 30 × 30 Å³ partly overlapping and centered at two different sites: the first one at the center of mass of ethidium bound in the AP of the L monomer in 7KGI and the other one at the center of mass of the two molecules of ethidium bound in the DBP of the T monomer in PDB ID 7KGG (Supplementary Fig. 17). For each program employed, each receptor structure, and each docking site, the top 10 docking poses were retained, totaling to 200 modes of binding per compound (10 poses × 5 AdeB structures × 2 programs × 2 sites). Top-scoring poses were energy-optimized to refine protein-ligand interactions using the molecular mechanics general potential AMBER99SB-ILDN force field detailed in Basciu et al. For each optimized binding mode of both ethidium and rhodamine 6G, the (pseudo) free-energy of binding ΔG was evaluated as detailed in previous work, using the Molecular Mechanics–Generalized Born Surface Area (MM-GBSA) method.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability. Atomic coordinates and structure factors reported in this paper have been deposited in the Protein Data Bank under accession numbers 7B8F (AdeB-OOO), 7B8Q (AdeB-L*OO), 7B8R (AcrBper/DARPIn in complex with Doxycycline), 7B8S (AcrBper/DARPIn in complex with fusidic acid), 7B8T (AcrBper/DARPIn in complex with Levofloxacin). Atomic coordinates that were used and support the findings of this study are available in the Protein Data Bank under accession numbers 5ENS, 7KGI, 7KGG, 7KGD, 7B8P, 7B8Q, 7B8R, 7B8S, 7B8T, 7KGD, 7KGG, 7KGI, 7KSI, 7KSI. Source data for Supplementary Figs. 1, 3, 4, 10–13 and 15 are provided with this paper. Source data are provided with this paper.

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Author contributions

A.O.C., J.W., H.S., A.S.F. and K.M.P. designed experiments. A.O.C. and J.W. performed AdeB production, membrane preparation, and protein purification. A.O.C. performed reconstitution and cryo-EM sample preparation. A.O.C. and A.S. collected cryo-EM data. A.O.C. and J.R. processed cryo-EM data and solved the structure of AdeB. J.W. and H.S. performed AcrBper and DARPin production, protein purification and crystallization, collected synchrotron data and determined crystal structures. A.O.C. (AdeB/AcrB substitutions), J.W. (AdeB/Ala substitutions), and J.K. (AcrB/Ala substitutions) performed biochemical/microbiological assays and western blot experiments. A.V.V. and G.M. conducted docking, molecular mechanics, and free-energy calculations. A.O.C., J.W., J.K., A.S.F. and K.M.P. contributed to manuscript preparation.

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Competing interests

The authors declare no competing interests.

Additional information

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