The capacity of numerous bacterial species to tolerate antibiotics and other toxic compounds arises in part from the activity of energy-dependent transporters. In Gram-negative bacteria, many of these transporters form multicomponent ‘pumps’ that span both inner and outer membranes and are driven energetically by a primary or secondary transporter component. A model system for such a pump is the acridine resistance complex of Escherichia coli. This pump assembly comprises the outer-membrane channel TolC, the secondary transporter AcrB located in the inner membrane, and the periplasmic AcrA, which bridges these two integral membrane proteins. The AcrAB–TolC efflux pump is able to transport vectorially a diverse array of compounds with little chemical similarity, thus conferring resistance to a broad spectrum of antibiotics. Homologous complexes are found in many Gram-negative species, including in animal and plant pathogens. Crystal structures are available for the individual components of the pump and have provided insights into substrate recognition, energy coupling and the transduction of conformational changes associated with the transport process. However, how the subunits are organized in the pump, their stoichiometry and the details of their interactions are not known. Here we present the pseudo-atomic structure of a complete multidrug efflux pump in complex with a modulatory protein partner from E. coli. The model defines the quaternary organization of the pump, identifies key domain interactions, and suggests a cooperative process for channel assembly and opening. These findings illuminate the basis for drug resistance in numerous pathogenic bacterial species.

A small, 49-residue protein, AcrZ, has recently been identified in genetic screens as a binding partner of AcrB that affects substrate preference. A mutant strain of E. coli lacking AcrZ is sensitive to some, but not all, antibiotics that are exported by the AcrAB–TolC pump. AcrZ is conserved in many enterobacterial lineages, suggesting that modulation by small proteins may be a recurring theme in the RND protein family to which AcrB belongs.

To investigate their interaction, AcrB was co-expressed with AcrZ carrying a carboxy-terminal histidine tag. The partner proteins co-purified through Ni²⁺ affinity and size-exclusion chromatography (Extended Data Fig. 1a), and co-crystals of the AcrBZ complex were obtained. Additionally, co-crystals were obtained of a ternary complex (Extended Data Fig. 1a, b and Extended Data Table 1). Electron density corresponding to AcrZ was apparent in the early, unbiased maps (Extended Data Fig. 2). AcrZ folds into a long, predominantly hydrophobic α-helix that fits into a wide groove in the transmembrane domain of AcrB (Fig. 1a, b and Extended Data Fig. 2). AcrBZ complex overlays well with the previously reported structure of AcrB bound to another potential partner, YajC (Extended Data Fig. 3a). The helical axis of AcrZ is inclined by nearly 45° with respect to the normal vector of the lipid bilayer, and although this is an unusual extent of inclination for a transmembrane α-helix, it optimizes the interfacial complementarity.

The interfacial residues are well conserved among the identified homologues of AcrZ, suggesting that analogous interactions are likely to occur for other RND family proteins (Extended Data Fig. 3b, c). We were able to model AcrZ residues 1–46, revealing that the amino terminus is in the periplasm and the C terminus is in the cytoplasm, where it interacts with the hydrophilic surface of AcrB, near the membrane (Fig. 1b). It seems possible that the reported effects of AcrZ on drug sensitivity might originate from the allosteric modulation of AcrB activity by AcrZ.

To stabilize the complete pump assembly, constructs were prepared in which the components were fused (Extended Data Fig. 4a, b). On the basis of the crystal structure of the AcrBZ complex, a construct was designed in which AcrA is fused through a flexible linker to the N-terminus of AcrZ, which was predicted to leave the individual structures unperturbed and to permit localization of AcrA to the periplasm while allowing AcrZ to cross the inner membrane with the correct topology and preserving its interactions with AcrB. This fusion harbours a histidine tag on the C terminus of AcrZ to facilitate Ni²⁺ affinity purification of the fusion protein. In a second engineered construct, we identified a loop in the periplasmic domain of AcrB where AcrA could be inserted with flexible linkers. Both the AcrA–AcrZ–His₅ and AcrA–AcrB fusions were stable during overexpression and purification.

At present, there is debate about the compositional stoichiometry of the AcrAB–TolC pump, with evidence favouring a 3:6:3 ratio for AcrB: AcrA:TolC:11–16. To enable the assembly of such a complex, we explored the co-expression of the fusion pairs AcrA–AcrB and AcrA–AcrZ–His₅. In the course of isolating the co-expressed recombinant proteins, we observed that the AcrA–AcrZ–His₅ fusion co-purified with both the AcrA–AcrB fusion and endogenous TolC. We subsequently designed co-expression vectors comprising fusion proteins of AcrA–AcrB, AcrA–AcrZ–His₅ and TolC, and found that the proteins remained associated during purification (Extended Data Fig. 4c). Assays of minimum inhibitory concentration (MIC) and drug efflux using the AcrAB and AcrAZ fusion proteins show that the engineered pump retains partial activity (Extended Data Fig. 5).

The assembly was further purified by the GraFix method, and analysis of the fractions by negative staining electron microscopy readily led to the identification of drumstick-shaped particles that matched the expected size and shape of the pump (Extended Data Fig. 6a). The crosslinked particles were subsequently imaged using cryo-electron microscopy (cryo-EM), which shows images of isolated and aggregated particles (Fig. 2a). Initial three-dimensional reconstruction was carried out using EMAN2 (ref. 18) without any imposed symmetry (Extended Data Fig. 6b), which suggested the presence of at least three-fold symmetry. As TolC and AcrB are known to be homotrimers, a subsequent three-fold symmetry enforced map was independently produced (Extended Data Fig. 6c). We then used RELION software to refine the particle classification and orientation determination, which improved the map greatly. The resolution of the final map was estimated to be ~16 Å (Extended Data Fig. 7a). This map was validated by a tilt-pair analysis (Extended Data Fig. 7b), which is
The hexameric assembly of AcrA resembles that of the homologous Pseudomonas aeruginosa TolC does not23. The organization of the AcrA–TolC subassembly is in and AcrA–TolC occur spontaneously, whereas that between AcrB and pull-down assays, which indicated that AcrA interacts with AcrB and TolC independently, whereas no interaction was observed between AcrB and TolC29. Our model also accounts for thermodynamic measurements, which suggest that the pairwise interactions of AcrA–AcrB and AcrA–TolC occur spontaneously, whereas that between AcrB and TolC does not29. The organization of the AcrA–TolC subassembly is in agreement with the dimensions of the homologous MexA–OprM complex from Pseudomonas aeruginosa24,25.

Our model shows that the assembly comprises an AcrB trimer, an AcrA hexamer and a TolC trimer. This is in accord with the 3:6 stoichiometry of the homologous CusA–CusB heavy-metal efflux complex15. The hexameric assembly of AcrA resembles that of the homologous MacA16 (Fig. 3a, b). The α-helical coiled coils (or ‘hairpins’) of AcrA pack into a cylinder that interacts with the periplasmic ends of the α-helical coiled coils portion of TolC. As TolC has an internal structural repeat, there are six quasi-equivalent contact surfaces that interact with the AcrA hairpins (Fig. 3a and Extended Data Fig. 8a).

The membrane-proximal domain and β-barrel domain of AcrA are involved in defined interactions with AcrB (Fig. 3a). One protomer of AcrA bridges the upper regions of subdomains PC1, PC2 and DC of AcrB (protomer 1 in Fig. 3a); this interaction is similar to that between CusA and CusB (Fig. 3c). However, the adjacent protomer of AcrA (protomer 2 in Fig. 3a) interacts with AcrB in a different manner: here, the membrane-proximal domain of AcrA shifts towards the PN2 subdomain of AcrB so that only the upper regions of PN2 and DC contact AcrA. The lipoyl domains of AcrA principally interact with each other to form a channel that is closed to the periplasm and make no predicted interactions with either AcrB or TolC.

The side-by-side packing of the β-barrel, lipoyl and α-helical hairpin domains of the six AcrA protomers generates a funnel-like structure with a sealed central channel along the three-fold axis. A chamber is formed by the β-barrel and lipoyl domains of AcrA, and the bottom of the chamber opens into the funnel of AcrB. The chamber is partially constricted near the middle of the lipoyl domain (Fig. 3c and Extended Data Fig. 8a). It then continues to a channel created by the helical hairpin domains, with a cogwheel shape at the end of these domains, like that of MacA (Fig. 3a, b). The modelled docking of the membrane-proximal domain of AcrA onto the surface of AcrB is consistent with the disruptive behaviour of mutants at that interface26,27.

A cross-section through the reconstruction reveals a conduit that is open to the cell exterior and runs from the transmembrane β-barrel of the detergent shell observed for aquaporin in the same detergent used in this study, namely n-dodecyl-β-d-maltopyranoside (DDM)21. The detergent also forms a layer around the transmembrane portion of AcrB (Fig. 2b, c).

One salient observation of our model is that there is no direct interaction between TolC and AcrB in the assembly, in contradiction to earlier models5,22. Instead, the two proteins are bridged in the periplasm entirely through AcrA. This is consistent with in vivo crosslinking and pull-down assays, which indicated that AcrA interacts with AcrB and TolC independently, whereas no interaction was observed between AcrB and TolC29. The modelled docking of the membrane-proximal domain of AcrA onto the surface of AcrB is consistent with the disruptive behaviour of mutants at that interface26,27.
Light Source synchrotron facility. The crystal structure was solved using molecular crystallized by vapour diffusion and diffraction data were collected at the Diamond and purified chromatographically. The AcrBZ and AcrBZ–DARpin complexes were Recombinant proteins were engineered with histidine tags, overexpressed in E. coli

METHODS SUMMARY

In isolation, TolC assumes a ‘closed’ resting state in which its periplasmic domain tapers to a near close, but to be accommodated in the continuous channel seen in the cryo-EM model, TolC must switch to an ‘open’ state\(^2\) (Fig. 2c and Extended Data Fig. 8b). The opening of TolC must result from its direct interactions with the \(\alpha\)-helical hairpins of AcrA. In this scenario, AcrB provides a platform for the proper assembly of hexameric AcrA suitable for presentation to TolC and opening of the channel. The AcrAB–TolC assembly forms in vivo without the requirement for substrates or proton-motive force\(^3\), suggesting that exogenous energy may not be required to open TolC in the context of the assembled pump. It seems likely that TolC remains open throughout the transport cycle, and we propose that the dilution of TolC from the closed to the open state is driven by both chelate cooperativity (due to the hexameric organization of AcrA) and allosteric cooperativity (associated with the breaking of interprotomer interactions in TolC).

The structure of the efflux pump presented here is likely to be similar to homologous assemblies in pathogenic species that infect humans such as Vibrio cholerae, P. aeruginosa, Neisseria meningitides and Salmonella enterica. The question arises as to how it might be feasible to counter the action of drug efflux in the treatment of bacterial infections. It seems paradoxical that a compound might exist that would achieve this, when the pump is well crafted to handle diverse compounds. Perhaps the protein–protein contacts that mediate the assembly might be favourable targets for such approaches.

METHODS SUMMARY

Recombinant proteins were engineered with histidine tags, overexpressed in E. coli and purified chromatographically. The AcrBZ and AcrBZ–DARpin complexes were crystallized by vapour diffusion and diffraction data were collected at the Diamond Light Source synchrotron facility. The crystal structure was solved using molecular replacement and refined. Freshly prepared specimens of the AcrABZ–TolC complex were applied to glycerol–glutaraldehyde gradients with ultracentrifugation\(^7\). The fractions were analysed by electron microscopy after either negative staining or rapid freezing under hydrated conditions. Individual particles were chosen from the cryo-EM images and were used for two-dimensional projection class averages and three-dimensional image reconstruction\(^14,15\). For in vivo activity tests of the engineered AcrABZ–TolC pump, expression vectors for the fusion proteins and controls were transformed into deletion strains. MICs were evaluated in the presence of antibiotics and efflux assays were conducted on fluorescent substrates.

Online Content Any additional Methods, Extended Data display items and Source Data are available in the online version of the paper; references unique to these sections appear only in the online paper.

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Author Contributions D.D., W.C. and B.F.L. designed the experiments; D.D. and N.R.J. purified and crystallized AcrBZ complexes. D.D. and B.F.L. solved the crystal structure of AcrBZ complexes. D.D. and B.F.L. solved the crystal structure of AcrBZ complexes. D.D. and N.R.J. purified AcrAB–ToIC complexes. Z.W., J.E.V. and W.C. obtained and analysed the single-particle cryo-EM data. D.D. and B.F.L. devised a model of AcrAB–ToIC based on the cryo-EM map. T.O.-A. and H.V. conducted MIC and efflux assays on the AcrAB–ToIC pump. D.D., J.E.V., W.C. and B.F.L. analysed results. All authors contributed to writing the manuscript.

Author Information Atomic coordinates and structure factors for the reported crystal structures have been deposited in the PDB under accessions 4C48 (AcrB/AcrZ/DARPIn) and 4CDI (AcrB/AcrZ). The cryo-EM map has been deposited in the Electron Microscopy Data Bank under accession EMD-5915. Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to B.F.L. (bjl20@cam.ac.uk) or D.D. (dd339@cam.ac.uk).
The DARPin gene was synthesized and protein was overexpressed and purified as described. Purified DARPin and AcrB complex were mixed at a molar ratio of 1:2 (AcrB monomer:DARPin monomer). The mixture was diluted with GF buffer 2 (20 mM Tris-HCl, pH 7.5, 150 mM sodium chloride, 0.03% DDM) to a concentration of 2–3 mg ml⁻¹, incubated at 4 °C overnight, then concentrated to 0.5 ml using a Vivaspin concentrator (MWCO = 100 kDa) and loaded onto a Superose 6 column equilibrated with GF buffer 2. Fractions containing purified AcrB–DARPin complex were pooled and concentrated to 15–20 mg ml⁻¹ using a Vivaspin concentrator (MWCO = 100 kDa) and dialyzed overnight against sample buffer using a 100 kDa MWCO dialysis membrane; the final concentration was 10–15 mg ml⁻¹.

**Cryocrystallization of AcrBZ and AcrBZ–DARPin.** The AcrBZ and AcrBZ–DARPin complexes were diluted to 10 mg ml⁻¹ using sample buffer. 9 mM n-octyl-β-D-thioglucoside (100 mM) was mixed with the AcrZ complex before the crystallization trials. The AcrBZ crystals were grown at 20 °C using the hanging-drop vapour diffusion method by mixing 4 μl of AcrB complex with 2 μl of reservoir solution (100 mM tricine, pH 7.4, 50 mM lithium sulphate, 5 mM cadmium chloride, 7% PEG 3000, 10% glycerol). The AcrBZ–DARPin complex was incubated with puromycin at 1 mM for 3 h at 4 °C before crystallization trials. Crystals were grown at 20 °C using the sitting-drop vapour diffusion method by mixing 400 ml of AcrB–DARPin complex with 200 ml of reservoir solution (100 mM HEPES, pH 7.5, 10 mM MgCl₂ and 12% (w/v) PEG 3350). Crystals appeared after 24 h and were set up using the crystallization trials and reached maximal size in 1 week. The crystals were transferred briefly into reservoir solution supplemented with 25% glycerol as cryoprotectant before flash freezing in nitrogen liquid.

**Crystallographic data collection and structure refinement.** Data sets were collected using beamlines I04-1 and I24 at the Diamond Light Source. The diffraction data were processed using X-ray Detector Software (XDS) and scaled using SCALA. Structures were solved by molecular replacement using Phaser with chain A of AcrB zymogen model and decoyed chain B (PDB accession 4DXS). The structures were included in the model, as well as additional acyl chains that were located mostly in the intra-protomer space in the transmembrane region of AcrB. For both AcrBZ and AcrBZ–DARPin complexes, the AcrB homotrimer is formed through crystallographic symmetry, so that any potential non-equivalence of the protomers is consequently lost.

**Expression and purification of the efflux pump assembly.** The constructs PET21a-acrBHis6, PET21a-acrZHis5 and pRSFDuet-1-acrA-polyGlySer-acrZHis5 were transformed into E. coli strain C43(DE3) supplemented with appropriate antibiotic(s) with shaking at 37 °C until an OD 600 of 0.5 was reached. The cells were harvested by centrifugation at 3,000 for 10 min at 4 °C, then washed three times by resuspension in 50 mM potassium phosphate buffer, pH 7.0, containing 5 mM MgSO₄ and sedimented by centrifugation at 3,000 for 10 min at 4 °C. The cells were harvested by centrifugation at 3,000 for 10 min at 4 °C, then washed three times by resuspension in 50 mM potassium phosphate buffer, pH 7.0, containing 5 mM MgSO₄ and sedimented by centrifugation at 3,000 for 10 min at 4 °C. Cell pellets were resuspended in lysis buffer (200 mM Tris- HCl, pH 8.0) with 100 μg ml⁻¹ lysozyme and sonicated at 4 °C for 1 h to digest the cell wall. The cells were lysed using a homogenizer (EmulsiFlex) at 15,000 psi for eight passages. Cell debris was pelleted by centrifugation at 125,755 g, 3 °C for 3 h. Debris was washed using primers AcrA_25GSF and AcrA_Infusion_Reverse and AcrZHis5-tolC1392 using the In-Fusion cloning method (Clontech), generating the construct pRSFDuet-1-acrA-polyGlySer-acrZHis5-tolC1392. A BamHI site was inserted into acrB by site-directed mutagenesis using PET21a-acrBHis6 as a template and primers AcrB_D328R and AcrB_D328M. The resulting construct was transformed into the NcoI site of pET21a-ccgccgccaccagagccaccacc, generating the construct pET21a-acrB_D328R. The NcoI-bounded acrBHis6, bounded by NcoI and XhoI sites, into the multiple cloning site (MCS) of expression vector pET21a. The resulting construct was transformed into the E. coli strain C43(DE3) lysogen.

**Overexpression and purification of the AcrBZ and the AcrBZ–DARPin complexes.** The constructs PET21a-acrBHis6, pRSFDuet-1-acrZHis5 were transformed into E. coli strain C43(DE3) LacI. The C-terminal histidines of AcrB were substituted to prevent non-specific association with the metal affinity matrix. Cells were grown in 20 °Y T medium with 100 μg ml⁻¹ carbenicillin and 50 μg ml⁻¹ kanamycin at 37 °C until the culture reached an absorbance, at 600 nm, of 0.5–0.6 and was then induced by the addition of 0.5 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) at 18 °C overnight. Cell pellets were resuspended in lysis buffer (400 mM sodium chloride, 200 mM Tris-HCl pH 8.0) with 100 μg ml⁻¹ lysozyme and sonicated at 4 °C for 1 h to digest the cell wall. The cells were lysed using a homogenizer (EmulsiFlex) at 15,000 psi for eight passages. Cell debris was pelleted by centrifugation at 9,000 g for 30 min. Culture supernatant was then concentrated by ultra centrifugation at 125,755 g, 3 °C for 3 h.

Membrane pellets were resuspended in buffer with protease inhibitors and were solubilized by adding 1.5% DDM and stirring at 4 °C for 3 h. Membrane pellets were solubilized by ultracentrifugation at 125,755 g, 3 °C.

Membrane pellets were resuspended in buffer with protease inhibitors and were solubilized by adding 1.5% DDM and stirring at 4 °C for 3 h. Membrane pellets were solubilized by ultracentrifugation at 125,755 g, 3 °C. The N-terminal histidines of AcrB were substituted to prevent non-specific association with the metal affinity matrix. Cells were grown in 20 °Y T medium with 100 μg ml⁻¹ carbenicillin and 50 μg ml⁻¹ kanamycin at 37 °C until the culture reached an absorbance, at 600 nm, of 0.5–0.6 and was then induced by the addition of 0.5 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) at 18 °C overnight. Cell pellets were resuspended in lysis buffer (400 mM sodium chloride, 200 mM Tris-HCl pH 8.0) with 100 μg ml⁻¹ lysozyme and sonicated at 4 °C for 1 h to digest the cell wall. The cells were lysed using a homogenizer (EmulsiFlex) at 15,000 psi for eight passages. Cell debris was pelleted by centrifugation at 9,000 g for 30 min. Culture supernatant was then concentrated by ultra centrifugation at 125,755 g, 3 °C.

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transport rates were determined between 85 to 185 s and 75 to 95 s for ethidium bromine and TMA-DPH, respectively. Assays were performed twice per day for three separate days, with different cell preparations used each day.

**GraFix preparation of the efflux pump assembly.** The GraFix procedure for the AcrABZ–TolC complex was carried out as described.4,7,42. Gradients for GraFix were generated by mixing GraFix buffer 1 (50 mM HEPES, pH 7.5, 400 mM NaCl, 0.03% w/v DDM, 10% v/v glycerol) and GraFix buffer 2 (50 mM HEPES, pH 7.5, 400 mM NaCl, 0.03% w/v DDM, 30% v/v glycerol, 0.15% v/v glutaraldehyde) using a gradient master (Gradient Master 107, BioComp Instruments), following the manufacturer’s recommendations for determining the parameters. 100 pmol of AcrABZ–TolC complex was loaded onto the top of the buffer cushion. Ultracentrifugation was carried out at 111,845 g, 4°C for 18 h (SW60 rotor, Beckmann). After centrifugation, the gradients were fractionated at 4°C by using a capillary to pump the gradient out from bottom to top, taking fractions of 200 μl. The same fractions from different centrifuge tubes were pooled, respectively. Glycine (1 M, pH 7.5) was added to the fractions to a final concentration of approximately 10 mg ml−1. Adsorption times were determined empirically. After blotting to dryness, the grid was washed by placing it onto a drop of deionized H2O for 5 s, blotted dry, and then repeated. The protein was stained by placing the grid carbon-side down on a 10 gm l−1 solution of uranyl acetate for 30 s, then dried and stored at room temperature. The sample was then imaged at ×20,000 magnification and 120 keV in a FEI Tecnai G2 microscope at the Multi Imaging Centre, University of Cambridge.

**Cryo-EM specimen preparation and data collection.** Samples were embedded in vitreous ice as per the following method. A 2 μl aliquot at approximately 2 mg ml−1 was applied onto hole carbon film supported by a 200-mesh R1.2/1.3 Quantifoil grid (Quantifoil) that had been previously washed and glow discharged. The grid was blotted and rapidly frozen in liquid ethane using a Vitrobot IV (FEI) with constant temperature and humidity during the process of blotting, and the grid was stored in liquid nitrogen before imaging. All grids were screened on a JEM3200FSC electron microscope (JEOL) operated at 300 keV, spot size 2, condenser aperture 70 μm, objective aperture 60 μm, and energy slit of the in-column filter of 20 eV. Images were recorded with a direct detection device (DDE) (DE-12 sk3k camera, Direct Electron, LP) operating in movie mode at a recording rate of 25 raw frames per second. A magnification of ×20,000 (corresponding to a calibrated sampling of 2.47 Å per pixel) and a dose rate of 25 electrons Å−2 s−1 were used to acquire a single movie, which spanned an area with a total exposure time of 2 s and a total of 1,281 frames. DDD images using movie frame mode were collected with a defocus range of −2.4 μm.

**Cryo-EM data processing and resolution evaluation.** Particle images were manually boxed with the EMAN2 program e2boxer.py with a box size of 256 × 256 pixels using the average sum of 48 raw frames per specimen area. The final frame average was computed from averages of every five consecutive frames to correct for the specimen movement during exposure similar to the procedure previously described. The particle intensity in each frame was weighted according to a radiation damage model (courtesy of B. Bannnes). Defocus of the particles in each frame average was determined by EMAN2 program e2ctf.py. The first reconstruction was done without any symmetry imposition or any initial model, using EMAN2 (v1.8). We started with 12,000 particle images and 8,400 particle images were selected manually according to the similarity in appearance to the class averages. The first map without any symmetry imposition appeared to have a six-fold symmetry. As previously observed, if the map is incorrect, the relative angle. Ideally all points would lie in exactly the same location; however, there is always some uncertainty in orientation determination, exacerbated in this case by radiation damage in the second image of the tilt pair and the strong pseudo hexagonal symmetry. As previously observed, if the map is incorrect, the relative angles will not correlate at all, and produce a nearly random distribution over the sphere. A clear cluster as observed in our case is an indication of successful validation, and our computed tilt angle of 10.4 degrees is a good match with the microscope setting of 10 degrees. The presence of some points at mirrored positions across the origin is an indication of weak handedness in the map, meaning that the map and its mirror image are difficult to distinguish at this resolution in some orientations.

**Model docking.** The merged map was low-pass filtered before rigid body docking of crystal structures using Coot and UCSF Chimera (http://www.cgl.ucsf.edu/chimera). The symmetrical partially opened model of TolC was used to prepare a fully opened model (PDB accession 2XMN)47. A homologous model of the inner sets of coated coils was devised based on the outer sets of coated coils, which was used to replace the inner sets of coated coils. The uncoiling movement of the inner sets of coated coils produced a fully opened TolC44.

The β-barrel domain, lipoyl domain and α-helical hairpin domain of each AcrA protein were modelled with chain C in the asymmetric unit of the crystal structure of AcrA (PDB accession 2FM1). A homology model of the membrane proximal domain of AcrA was devised based on the structure of MexA (PDB accession 2D4V). The individual domains of AcrA were fitted into the cryo-EM reconstruction using Coot, according to the features of the cryo-EM map and prior knowledge of the homohexameric Maca assembly (PDB accessions 3FFP and 4DKO). The closed-state TolC (PDB accession 1EK9), partially opened (PDB accession 2XMN) and fully opened model of TolC were separately fitted into the cryo-EM reconstruction, respectively. The AcrBZ trimer, the TolC trimer and the AcrA hexamer were treated as individual rigid bodies. These three rigid bodies were automatically fitted into the cryo-EM map by sequential fitting using Chimera. The AcrABZ–TolC model generated by Chimera was slightly adjusted manually to optimize the local fit into the density using Coot.45. The cross-correlation coefficient for fitting the AcrABZ–open TolC to the map is 0.924 and was calculated using the ‘fit in map’ function of Chimera.

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Extended Data Figure 1 | Co-purification of the AcrBZ and AcrBZ–DARPin complexes. a, Gel filtration profile of AcrBZ complex and SDS–polyacrylamide gel electrophoresis (SDS–PAGE) analysis of the peak fractions. b, Gel filtration profile of the AcrBZ–DARPin complex and SDS–PAGE analysis. The proteins were enriched by immobilized metal affinity chromatography (IMAC; results not shown) before the size-exclusion chromatography step. AcrZ has a heptahistidine tag on the C terminus as an IMAC affinity tag, and the C-terminal histidines of AcrB have been removed to prevent its binding to the matrix. Standard proteins thyroglobulin (669 kDa) and aldolase (158 kDa) (Bio-Rad) were eluted from the same column at volumes of 11.4 and 14.7 ml, respectively. See also Fig. 1.
Extended Data Figure 2 | Validation of the AcrBZ crystal structure. An AcrB protomer taken from the refined structure of the AcrBZ–DARPino complex at a resolution of 3.3 Å was used in molecular replacement of the 3.7 Å data of the AcrBZ complex (Extended Data Table 1). The model of AcrB without AcrZ was refined with REFMAC jelly-body and reference structure restraints, and a difference map calculated in which AcrZ does not contribute to the phases. Shown are the positive features of the difference map in the vicinity of AcrZ (green carbon atoms) taken by superposing the AcrB protomers from the AcrBZ–DARPino and AcrBZ (red) structures. The unbiased map shows the presence of AcrZ and indicates that the presence of DARPino does not disrupt the interactions with AcrB. The model of the AcrBZ–DARPino complex at a resolution of 3.3 Å was refined without AcrZ to generate a simulated annealing omit map, which confirmed the location of AcrZ (data not shown). See also Fig. 1.
Extended Data Figure 3 | The AcrBZ complex resembles AcrB–YajC, and AcrBZ interactions involve conserved residues. 

a, Superposition of AcrB–YajC (orange and yellow; PDB accession 2RDD) onto AcrBZ (red and green). See also Fig. 1. We were not able to identify an interaction between AcrB and C-terminal histidine-tagged YajC from E. coli, suggesting that the interaction is less avid than the AcrB–AcrZ pairing (data not shown). It is interesting to note that the contacting surface is also conserved in another RND protein, SecDF, which is involved in protein translocation and membrane insertion. Therefore, it seems likely that SecDF might be modulated by a similar helical peptide. Indeed, YajC forms a complex with SecDF and could act as an allosteric modulator in protein biogenesis. 

b, Sequence conservation of AcrZ. The secondary structure is annotated at the top and asterisks indicate residues that directly contact AcrB. 

c, Sequence variation of the surface of AcrB homologues, showing conservation of the surface that contacts AcrZ. Right-hand side includes the bound AcrZ in stick representation (green). The colour spectrum ranges from purple (most conserved) to blue (least conserved). This figure was made using ConSurf.
Extended Data Figure 4  | Schematic representations and purification of the fusion proteins used to assemble the efflux pump.  

**a**. The AcrA–AcrB fusion, with two flexible poly(Gly-Ser) linkers. The two C-terminal histidines of AcrB have been removed to prevent binding to the NTA matrix during co-purification.  

**b**. The AcrA–AcrZ–His₅ fusion and TolC co-expression construct. The numbers above the bars correspond to the residues of the protein, and, owing to the restriction site used for cloning, a single glycine residue was inserted after the start codon in both AcrA and AcrZ. The flexible poly(Gly-Ser) linker permits the protomers to manoeuvre.  

**c**. Co-purification of TolC with the AcrABZ complexes. SDS–PAGE of the eluate from gel filtration following nickel affinity chromatography purification. See also Fig. 2.
Extended Data Figure 5 | The fusion assemblies can drive efflux in vivo.
a, b, Drug-transport assays of the AcrAB and AcrAZ fusion proteins in the MCΔtolCΔacrAB strain show that the engineered pump retains partial activity for efflux of ethidium bromide (a) and trimethylammonium-diphenylhexatriene (b). The results show representative traces from six biological replicates. For ethidium bromide, initial influx rates of 10.3 ± 0.6, 6.4 ± 0.6 and 3.6 ± 0.4 arbitrary units (AU) per min were obtained for the non-expressing control cells, the cells expressing both fusion proteins plus TolC and the wild-type cells expressing AcrAB–TolC, respectively. For trimethylammonium-diphenylhexatriene, initial influx rates of 187 ± 6, 151 ± 3 and 54 ± 2 AU min⁻¹ were obtained for the non-expressing control cells, the cells expressing both fusion proteins plus TolC and the wild-type cells expressing AcrAB–TolC, respectively. For ethidium bromide, the drug-transport assay shows that the engineered pump retains partial activity for efflux, with influx rates of 10.3 ± 0.6, 6.4 ± 0.6, and 3.6 ± 0.4 AU min⁻¹ for the non-expressing control cells, the cells expressing both fusion proteins plus TolC, and the wild-type cells expressing AcrAB–TolC, respectively. For trimethylammonium-diphenylhexatriene, the drug-transport assay shows that the engineered pump retains partial activity for efflux, with influx rates of 187 ± 6, 151 ± 3, and 54 ± 2 AU min⁻¹ for the non-expressing control cells, the cells expressing both fusion proteins plus TolC, and the wild-type cells expressing AcrAB–TolC, respectively.

c, MIC data on antimicrobial susceptibility of E. coli MCΔtolCΔacrAB cells expressing wild-type AcrA–AcrB–TolC or the fusion proteins with TolC. These data indicate that the fusion of AcrA–AcrZ and the insertion of AcrA into AcrB both diminish the capacity for drug resistance. The plasmids pET21a-AcrB or pET21a-acrB328-polyGS-acrA-polyGlySer-acrB329 (AcrAB), together with plasmid pRSFDuet-1-acrA-polyGlySer-acrZHis5-tolC (AcrAZ-TolC), were transformed into MCΔtolCΔacrAB. Cells were tested for their ability to resist increasing concentrations of cytotoxic drugs. As a positive control, cells were transformed with plasmid pBAD_AcrA1-AcrB1-TolC, which encodes for the native AcrA–AcrB–TolC efflux pump.
Extended Data Figure 6 | Electron microscopy images and class averages of the AcrABZ–TolC complex. 

**a**, Negative-stain electron microscopy images of the purified AcrABZ–TolC complex after GraFix treatment. White circles indicate particles with long axis almost normal to the viewing plane; black circles show particles with the long axis parallel to the viewing plane.

**b, c**, Class averages from cryo-EM data and reconstructions with C1 symmetry and with C3 symmetry, respectively. The galleries in the side panels show representative two-dimensional class averages of the purified pump. The top shows views perpendicular to the long axis of the drumstick shape, the middle shows inclined views, and the bottom shows views along the long axis. The reconstructed images and cross-sections indicate the presence of a six-fold symmetry, which is consistent with six AcrA protomers. The pseudo-atomic model has three protomers each of AcrB, AcrZ and TolC, and as TolC and AcrB each have an internal structural repeat, they have pseudo six-fold symmetry at low resolution (cut view). The maps are consistent with each other. See also Fig. 2.
Extended Data Figure 7 | Resolution estimation and validation of the cryo-EM map. 

a, Fourier shell correlation (FSC) of two independently determined cryo-EM maps of the efflux pump assembly after their alignment by Foldhunter\textsuperscript{52}. b, Tilt-pair validation of the efflux pump assembly. Each point represents a pair of particles with experimentally known relative tilt. The radius indicates the computationally determined amount of tilt, and the azimuth indicates tilt direction. The red circle denotes particle pairs that cluster around the experimental tilt axis geometry, thus validating the map in Fig. 2.
Extended Data Figure 8 | Sections through the electron microscopy map.

(a) Slices 1–4: view of planes normal to the three-fold symmetry axis of the pump. (b) Slice 5 of the model assembly showing the opening of the TolC periplasmic domain. The same transverse plane through the cryo-EM map is shown with TolC in the closed state, as seen in the crystal structure of the isolated protein (left; PDB accession 1EK9), the partially open structure, made by mutations (middle; PDB accession 2XMN) and the modelled fully opened structure (right).
### Extended Data Table 1 | Crystallographic data and refinement statistics

| Crystal | AcrB/AcrZ/DARPin | AcrB/AcrZ |
|---------|------------------|-----------|
| Space group | R32 (H32) | R32 (H32) |
| Cell dimensions in hexagonal setting | | |
| \(a, b, c\) (Å) | 145.01, 145.01, 538.34 | 146.19, 146.19, 543.00 |
| \(\alpha, \beta, \gamma\) (°) | 90, 90, 120 | 90, 90, 120 |
| Resolution (Å) | 30.0-3.30 (3.46-3.30)* | 24.89-3.69 (3.99-3.69) |
| \(R_{\text{merge}}\), % | 13.4 (98.6) | 12.6 (117.0) |
| Total number of observations | 166,656 (22,416) | 244,483 (50138) |
| Unique reflections | 33,271 (4368) | 23,077 (4710) |
| Completeness, % | 99.8 (100.0) | 94.5 (94.8) |
| \(|I|/\sigma(I)\) (outer shell) | 11.0 (1.6) | 15.1 (2.1) |
| \(<|I|\>\) half-set correlation | 0.998 (0.585) | 0.999 (0.791) |
| B values Wilson plot/average in structure, Å² | 76.6/87.7 | 148/117.7 |
| Reflections used in refinement | 31,614 | 21,912 |
| Geometry: Ramachandran | | |
|favoured, allowed, outliers, % | 95.2; 4.6; 0.2 | 94.9; 4.9; 0.3 |
| Rms deviation from ideal values for bond lengths (Å) and bond angles (°) | 0.012, 1.456 | 0.011, 1.57 |
| Number of non-hydrogen atoms in refinement | 9452 | 7997 |
| Refinement \(R/R_{\text{free}}\) 5% reflections in test set, % | 28.3/32.4 | 33.9/36.1 |

*Values in parentheses are for highest-resolution shell
The PDB deposition codes for model and structure factors of AcrB/AcrZ/DARP in and AcrB/AcrZ are 4C48 and 4CDI respectively.