

University of Dundee

Structure of the MacAB-ToIC ABC-type tripartite multidrug efflux pump

Fitzpatrick, Anthony W. P.; Llabres, Salome; Neuberger, Arthur; Blaza, James N.; Bai, Xiao-Chen; Okada, Ui

Published in:
Nature Microbiology

DOI:
[10.1038/nmicrobiol.2017.70](https://doi.org/10.1038/nmicrobiol.2017.70)

Publication date:
2017

Document Version
Peer reviewed version

[Link to publication in Discovery Research Portal](#)

Citation for published version (APA):

Fitzpatrick, A. W. P., Llabres, S., Neuberger, A., Blaza, J. N., Bai, X-C., Okada, U., Murakami, S., van Veen, H. W., Zachariae, U., Scheres, S. H. W., Luisi, B. F., & Du, D. (2017). Structure of the MacAB-ToIC ABC-type tripartite multidrug efflux pump. *Nature Microbiology*, 2, 1-8. [17070]. <https://doi.org/10.1038/nmicrobiol.2017.70>

General rights

Copyright and moral rights for the publications made accessible in Discovery Research Portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from Discovery Research Portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain.
- You may freely distribute the URL identifying the publication in the public portal.

Take down policy

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Structure of the MacAB-TolC ABC-type tripartite multidrug efflux pump

Anthony W.P. Fitzpatrick^{1*}, Salomé Llabrés², Arthur Neuberger³, James N. Blaza⁴, Xiao-chen Bai¹, Ui Okada⁵, Satoshi Murakami⁵, Hendrik W. van Veen³, Ulrich Zachariae^{2,6}, Sjors H.W. Scheres¹⁺, Ben F. Luisi⁷⁺, Dijun Du^{7+*}

¹ Medical Research Council Laboratory of Molecular Biology, Cambridge Biomedical Campus, Francis Crick Avenue, Cambridge CB2 0QH, U.K.

² Computational Biology, School of Life Sciences, University of Dundee, Dow Street, Dundee DD1 5EH, U.K.

³ Department of Pharmacology, University of Cambridge, Tennis Court Road, Cambridge CB2 1PD, U.K.

⁴ MRC Mitochondrial Biology Unit, Cambridge Biomedical Campus, Hills Road, Cambridge, CB2 0XY

⁵ Department of Life Science, Tokyo Institute of Technology, 4259-J2-17, Nagatsuta, Midori-ku, Yokohama, 226-8501, Japan

⁶ Physics, School of Science and Engineering, University of Dundee, Nethergate, Dundee DD1 4NH, U.K.

⁷ Department of Biochemistry, University of Cambridge, Tennis Court Road, Cambridge CB2 1GA, U.K.

*These authors contributed equally to this work.

+Corresponding authors: dd339@cam.ac.uk, bfl20@cam.ac.uk, scheres@mrc-lmb.cam.ac.uk

Abstract

The MacA-MacB-TolC assembly of *Escherichia coli* is a transmembrane machine that spans the cell envelope and actively extrudes substrates, including macrolide antibiotics and polypeptide virulence factors. These transport processes are energized by the ATPase MacB, a member of the ATP-binding cassette (ABC) superfamily. We present an electron cryo-microscopy structure of the ABC-type tripartite assembly at near-atomic resolution. A hexamer of the periplasmic protein MacA bridges between a TolC trimer in the outer membrane and a MacB dimer in the inner membrane, generating a quaternary structure with a central channel for substrate translocation. A gating ring found in MacA is proposed to act as a one-way valve in substrate transport. The MacB structure features an atypical transmembrane domain (TMD) with a closely packed dimer interface and a periplasmic opening that is the likely portal for substrate entry from the periplasm, with subsequent displacement through an allosteric transport mechanism.

Key words: ABC transporter, drug efflux pump, multi-drug resistance, macrolide

43 transporter, toxin transporter

44

45

46 The MacA-MacB-TolC assembly (hereafter, MacAB-TolC) contributes to drug
47 resistance and virulence phenotypes in *E. coli* and other Gram-negative bacteria that
48 include pathogenic species. These pumps drive not only the efflux of macrolide
49 antibiotics ¹, but also the transport of outer membrane glycolipids ², lipopeptides ³,
50 protoporphyrin ⁴, and polypeptide virulence factors including the heat-stable
51 enterotoxin II ⁵. It has been proposed that the pumps are involved in outer membrane
52 maintenance through transport of lipopolysaccharides ⁶. The transport processes are
53 coupled to input of metabolic energy by the ABC transporter MacB ^{7,8}.

54

55 Insight into how ABC transporters energise translocation of compounds comes from
56 structural and functional data for transporters that export substances or import
57 nutrients ⁹⁻¹⁴. These ABC transporters have TMDs that contain substrate-binding
58 pockets and nucleotide-binding domains (NBDs) that enable binding and hydrolysis
59 of ATP. Some ABC family membranes function as sensors that modulate ion channels
60 ¹⁵. The available data for ABC transporters support an ‘alternating access’ mechanism
61 in which the transporter alternates between inward open, occluded, and outward open
62 states to transport substrates across the membrane bilayer. Also, an ‘outward-only’
63 mechanism has been proposed that can account for transport of substrates by certain
64 ABC exporters ^{16,17}. These exporters, which are capable of transporting large
65 substrates such as lipids and peptides, may intercept their substrates from within the
66 membrane in an outward-facing binding pocket, after which a conformational change
67 decreases affinity for the ligand, causing it to be displaced into the exterior
68 compartment. For both importers and exporters, the conformational changes are
69 governed by ATP binding-associated NBD dimerization, and ATP hydrolysis-
70 associated NBD dissociation ¹⁸⁻²⁰.

71

72 MacB is an atypical ABC family transporter, consisting of an N-terminal NBD and
73 four transmembrane (TM) helices. The MacB periplasmic domain is positioned
74 between the first and second TM helices (Supplementary Fig. 1) and is expected to
75 mediate interactions with MacA. Another distinguishing aspect of MacB is that it
76 might accept some substrates such as heat stable enterotoxin II from the periplasm,
77 suggesting that MacB’s transport mechanism is different from conventional models
78 for ABC transporters ⁵. To gain insight into the details of the interactions between the
79 subunits and how the transporter works in the context of the pump assembly, we
80 undertook structure determination of the complete assembly. Our results show how an

81 ABC transporter is engaged in a tripartite pump complex and reveals that a gating ring
82 in MacA acts as a one-way valve in substrate transport. The MacB structure features
83 an atypical transmembrane domain (TMD). A portal is identified in the periplasm that
84 may serve as the entrance for substrate into the pump.

85

86 **Results**

87 **Engineering stable MacAB-TolC assemblies with functional activity.** Preparation
88 of the complete MacAB-TolC assembly for structural studies is challenging, as the
89 complex is liable to dissociate during purification. An engineering approach to
90 stabilise the pump was pursued by fusing components through flexible linkers that
91 were anticipated to maintain the native structure while co-localising the components
92 and so favouring their interactions. We noted that the C-terminus of MacB and N-
93 terminus of MacA are expected to be in proximity on the cytoplasmic side and that
94 MacA has a single N-terminal transmembrane helix. Therefore, the fusion of MacA to
95 the C-terminus of MacB with a flexible poly glycine-serine peptide was anticipated to
96 preserve the proper membrane topology of the components (Supplementary Fig. 1).
97 Another consideration is that the compositional stoichiometry of the MacAB-TolC
98 pump is unclear, with proposed models having a 3:6:2 or 3:6:6 ratio for
99 TolC:MacA:MacB²¹⁻²³. The co-expression of the MacB-MacA fusion protein and
100 TolC enable the assembly of a complex with either possible stoichiometries. In the
101 assembly with a 3:6:2 ratio, the four excess, fused subunits of MacB would remain
102 disordered and in close proximity of the complex, but without being recruited into the
103 structured assembly. Such a fusion, constructed with a hexa-histidine tag at the C-
104 terminus of MacA (Supplementary Fig. 1), could be expressed and co-purified with
105 TolC. Complex formation required full length TolC, and was greatly diminished if the
106 C-terminus was truncated, consistent with results from functional analyses²⁴. Assays
107 show that the fusion complex is likely to be active *in vivo* to confer macrolide
108 resistance in a drug-hypersensitive *E. coli* strain lacking expression of the tripartite
109 efflux pump AcrAB-TolC (Supplementary Fig. 2).

110 We also prepared constructs to stabilise the full pump using disulphide-linkages
111 that were chosen from the proximity of residues MacA Asp271 and MacB Gly465
112 seen in the cryo-EM structure of the fusion-stabilised pump (described below). Co-
113 expression of these single cysteine mutants without fusion of MacA to MacB showed
114 that MacA D271C could be co-purified with hexa-histidine-tagged MacB G465C, and
115 the interaction was disrupted by reducing agent, indicating disulphide bond formation
116 between the two components. Moreover, the disulphide bond-stabilised MacAB can
117 capture TolC to form a full pump assembly and is fully active *in vivo* (Supplementary
118 Fig. 2; Supplementary Fig. 3). We used the same procedure to prepare the native

119 MacAB-TolC pump without fusion or disulphide-linkages, but unable to obtain the
120 full pump assembly. Future work will be required to develop a procedure to capture
121 the full assembly with native, wild type components to corroborate the models
122 presented here.

123

124 **Quaternary structure of the MacAB-TolC pump.** Cryo-EM structure determination
125 by single-particle analysis produced three maps of the constituent parts of the
126 tripartite pump for the fusion-stabilised pump with different overall resolutions
127 (Supplementary Fig. 4). The disulphide-bond stabilised pump yielded a better map for
128 the TolC and MacA portions (Supplementary Fig. 5), whereas the MacB part was
129 better defined in the fusion-stabilised pump. We therefore prepared a hybrid map by
130 combining the MacA-TolC portion (from the disulphide-stabilised pump), and the MP
131 domain of MacA and MacB (from the fusion-stabilised pump). Local resolution
132 analysis of this hybrid map showed a range from 3.0 Å to 8.0 Å (Supplementary Fig.
133 4; Supplementary Fig. 5). The density readily accommodates the individual crystal
134 structures of TolC, MacA and MacB with some adjustments (see methods section)
135 (Fig. 1; Supplementary Fig. 6). The pump has an elongated shape and contains three
136 protomers of TolC and six of MacA. In the fusion-stabilised pump, a hexamer of
137 MacA can be visualised and each is fused to MacB. It is therefore expected that six
138 MacB protomers are present, but only density for an ordered MacB dimer is observed.
139 The remaining four MacB subunits do not have a structured interaction with the core
140 of the assembly and are disordered and distributed over the periphery of the assembly
141 outside of the central core, which is visible as areas with diffuse density in the images
142 of 2D classification (Supplementary Fig. 4b). In the density map of the disulphide-
143 stabilised pump, only a MacB dimer is observed (Supplementary Fig. 7). Six subunits
144 of MacA are recruited into the assembly, but only two are situated to form a
145 disulphide link to MacB. The other four MacA molecules assemble into the MacA
146 hexamer, but do not form disulphide links with MacB. Thus, the disulphide-stabilised
147 MacAB-TolC pump shows a stoichiometry of 3:6:2 for TolC:MacA:MacB, which is
148 in agreement with the structure obtained for the fusion-stabilised pump.

149 The locations of TolC and MacB demark the boundaries of the inner and outer
150 membranes, respectively, and the long axis of the pump assembly through those
151 membranes is roughly 320 Å. This is similar to the dimension seen for another class
152 of envelope-spanning transport machine, namely the AcrAB-TolC multi-drug efflux
153 pump powered by the electrochemical proton gradient²⁵⁻²⁷. The NDB domain of
154 MacB extends roughly 44 Å into the cytoplasm.

155

156 **A MacB dimer in the pump assembly.** Consistent with results from biophysical

157 experiments ²⁸ and the crystal structure of a homologue ²⁹, MacB forms a homodimer
158 in the MacAB-TolC assembly. The crystallographic model of the dimeric MacB fits
159 well into the cryoEM map with rigid-body adjustments to the quaternary structure
160 (Fig. 2, Supplementary Fig. 8). As the cryoEM density and crystallographic
161 coordinates were obtained by independent experiments, the good agreement validates
162 the structure models. The region of the map around the MacB protomers clearly
163 resolves three domains and their secondary structural elements: NBD, TMD and
164 periplasmic domain (PLD)(Supplementary Fig. 8). The TMD comprises four TM
165 helices, two of which (TM1 and TM2) have elongated extensions into the periplasm
166 that form the stalk of the globular PLD. TM1 is preceded by an N-terminal helix of
167 roughly 20 residues that skirts along the inner leaflet of the cytoplasmic membrane
168 before making an abrupt turn at nearly a right angle into the interior of the lipid
169 bilayer. This N-terminal helix corresponds to the ‘connecting helix’ found in other
170 ABC transporters ¹⁴. The dimer interface in the membrane is packed tightly without
171 space to accommodate a transport substrate. There is no shared TM helix that crosses
172 over between the protomers of the MacB dimer like that seen in the ABC family drug
173 transporter Sav1866 ⁹. Like MacB, the recently solved structure of the heterodimeric
174 ABC transporter ABCG5/8 also reveals a N-terminal NBD and absence of shared TM
175 helices that cross over between the protomers of the dimer; however, its TMD
176 contains 6 TMH and distinct from 4 seen for MacB, and its packing differs as well ¹⁴.

177 The PLD of MacB bears N-terminal and C-terminal subdomains (PSN and PSC,
178 respectively). It is interesting to note that PSN is a structural homologue of the PN/PC
179 subdomains of the RND transporter AcrB, while PSC presents no similarity to AcrB
180 ³⁰. The two NBDs of the MacB dimer are physically separated, and in this state, they
181 cannot be engaged with the nucleotide.

182 Unexpectedly, the MacB dimer has an opening between the periplasmic
183 extensions of TM1 and TM2. Density was observed inside this opening, which could
184 not be assigned to any of the three protein components making up the pump and does
185 not fit the detergent molecules (DDM or DMNG) used in the sample preparation
186 (Supplementary Fig. 9). The identity of this molecule could not be established by
187 mass spectrometry and is unclear due to the limited local resolution of the map (Fig.
188 2). This molecule, which occludes the opening, may be a natural transport substrate.
189 Some of the substrates of MacB, such as matured heat-stable enterotoxin II and
190 lipopolysaccharide precursors, are likely captured by the transporter from the
191 periplasm ^{5,6}. It seems likely that the opening that we observe is the access point for
192 these and other periplasmic substrates of the MacAB-TolC assembly.

193

194 **MacA forms a nanotube channel with a gating ring.** The cryoEM map clearly

195 reveals the four structural modules of MacA: the α -helical hairpin, lipoyl, β -barrel and
196 membrane proximal (MP) domains (Supplementary Fig. 6a)³¹. The density for the N-
197 terminal transmembrane helix is not clear for either the engineered fusion or the
198 cysteine disulphide stabilised assembly, indicating that the helix may not make a
199 defined interaction with the core of the complex. The modular domain organization of
200 MacA is common to the large class of periplasmic proteins that mediate tripartite
201 pump assemblies in Gram-negative bacteria²³. In isolation, MacA forms a hexameric
202 assembly, much like that observed for AcrA in the AcrAB-TolC efflux pump^{25,31}.
203 The cryoEM map of MacAB-TolC reveals that the same hexameric arrangement
204 occurs in the full pump assembly, with some requirement for structural adjustment for
205 the linker regions between the β -barrel and MP domains (Supplementary Fig. 6a). The
206 helical hairpin regions pack into a cylindrical assembly to form an α -helical barrel
207 quaternary structure³². The helical hairpin domains engage the open-state TolC
208 through helix-turn-helix motifs, the lipoyl and β -barrel domains form stacked annular
209 rings, and the membrane proximal domain skirts over the surface of the periplasmic
210 domain of MacB, as we will describe further below.

211 Loops in the MacA lipoyl domains present six glutamine residues (Q209) that
212 form an inter-protomer hydrogen-bonding network (Supplementary Fig. 10a). These
213 residues are not in the same plane, as seen in the crystal structure of *E.coli* MacA³¹.
214 The aperture observed in the cryoEM model is too narrow for substrates to pass
215 readily (Fig. 1b). This glutamine residue is conserved in MacA homologues from
216 divergent species³¹. We substituted Q209 to A but found that the mutant was still able
217 to efflux the transport substrate erythromycin *in vivo* with similar level to the wild
218 type protein, suggesting that substrates can somehow readily pass through the aperture
219 in the native protein (Supplementary Fig. 11). To explore this further, molecular
220 dynamics (MD) simulations were undertaken and show that erythromycin makes
221 favorable interactions with the Q209 hydrogen-bonding network, which partially
222 disrupt the network and enable the substrate to pass through the opening
223 (Supplementary Fig. 12). Steered MD simulations show that inward transfer induces a
224 larger conformational change of the loops than outward transfer. As a consequence,
225 opening the gate in the inward direction requires more non-equilibrium work than in
226 the direction of efflux, similar to a one-way valve (Supplementary Fig. 12). The
227 aperture is specific to the MacA proteins in the MacAB-TolC pump, as there is no
228 such feature in the AcrA of AcrAB-TolC assembly²⁵.

229

230 **Interactions between MacA and MacB.** The β -barrel and MP domains of MacA
231 mediate the interaction with the periplasmic domain of MacB, in agreement with the
232 reported functional data^{1,33} and the observation that the MP domain is required for

233 MacA and MacB to associate in solution (with nanomolar dissociation constant)³³.
234 Three MacA MP domains contact one MacB protomer, with one MP contacting the
235 PSN and two contacting the PSC. These interactions distort the hexameric ring
236 formed by the MacA MP domains to generate a more oval shape that accommodates
237 the two-fold symmetry of the MacB dimer (Fig. 3a; Supplementary Fig. 7b). The β -
238 barrel domains of MacA dock to the periplasmic domain of MacB (Fig. 3a). Three
239 MacA β -barrel domains contact MacB in non-equivalent ways, with one interacting
240 with the PSN, another contacting the PSC and the third bridging between the PSC and
241 the PSN of the adjacent MacB (Fig. 3a-c). The lipoyl domains form a hexameric ring
242 but do not contact MacB. Comparison of the crystal structure and cryoEM model of
243 MacB indicates movement in the periplasmic domain of MacB to accommodate
244 MacA (Supplementary Fig. 6c).

245

246 **Interfacial contacts between TolC and MacA.** Using focussed refinement³⁴ of the
247 disulphide-bond stabilised pump assembly, the MacA-TolC section could be resolved
248 at 3.3 angstroms, offering unprecedented view of the details of the molecular
249 interfaces (Supplementary Fig. 13). A short helix-turn-helix motif in the α -helical
250 hairpin domain of MacA meshes with the helix-turn-helix motif of TolC in the
251 periplasmic end (Fig. 1; Supplementary Fig. 13), similar to the observations seen for
252 the homologous AcrA interaction with TolC^{25,35}. The MacA tip regions make slightly
253 different interactions with the intra-protomer and inter-protomer grooves of TolC
254 (Supplementary Fig. 13b,c). Further evidence for this interaction comes from *in vitro*
255 affinity binding studies of site-directed mutants, *in vivo* drug resistance measurements
256³⁶, and the behaviour of chimeric proteins containing the tip regions of the α -helical
257 barrel of TolC that were found to bind to MacA³⁷. Lee *et al.* (2013) explored the
258 functional and physical interaction of two TolC homologues from *Vibrio vulnificus*
259 with MacA in *E. coli* using site-directed mutational analyses and chemical cross-
260 linking, and they found that conserved residues at the aperture tip region of the α -
261 hairpin of the TolC proteins play an essential role in the formation of the functional
262 MacAB-TolC pump³⁸. Taken together, these findings suggest that TolC has
263 conserved tip-to-tip interactions with MacA in Gram-negative bacteria. The
264 interaction between MacA and TolC directly opens the periplasmic end of TolC to
265 form a long, continuous channel that is roughly 25 to 30 Å wide with small variation
266 (Fig. 1b; Supplementary Fig. 6b). Thus, TolC is held in an open state in the pump, a
267 situation which is similar to that observed in the RND-based AcrAB-TolC pump²⁵.

268

269 **Discussion**

270 While the MacAB-TolC assembly studied here shares some organizational similarities

271 with the AcrAB-TolC multi-drug efflux pump, there are also some important
272 differences that impact on functionality. Although the periplasmic domains of the
273 inner membrane transporters MacB and AcrB are involved in contact with the
274 periplasmic partner, they interact in completely different ways and have different
275 stoichiometries. In the AcrAB-TolC pump, a trimer of TolC is engaged with a
276 hexamer of AcrA and a trimer of AcrB, yielding a 3:6:3 protomer stoichiometry for
277 TolC:AcrA:AcrB, while the protomer stoichiometry for the MacAB-TolC pump is
278 3:6:2. Another notable difference between the two pumps is a constriction point in the
279 MacA formed by a conserved glutamine ring in the lipoyl domain. This ring may act
280 as a gate through which substrates may passively move and could help to prevent
281 backflow in situations where the efflux pump operates against a concentration
282 gradient. Thus, this loop may act as a gating ring that favours substrate translocation
283 in the outward direction. A broadly similar gating mechanism may be involved in the
284 capsular polysaccharide transporter Wza, where a hydrogen-bonding ring (formed by
285 residues Y110) may selectively permit phosphorylated polysaccharide to pass through
286 a constrictive barrier (Supplementary Fig. 10b)³⁹. The absence of a gating ring in the
287 AcrAB-TolC pump might be due to the absence of any continuous opening between
288 the pump and the periplasmic compartment or cytoplasm during the transport process
289 through which substrates might leak.

290

291 The MacAB-TolC structure provides some clues as to the transport mechanism.
292 Like the AcrAB-TolC pump, the MacAB-TolC assembly may also accept some
293 transport substrates from the periplasm. One of its substrates, the heat-stable
294 enterotoxin II, is transported as a precursor across the inner membrane to the
295 periplasm by the Sec machinery, where it undergoes maturation⁵. This matured form
296 likely enters the MacAB-TolC pump from the periplasmic side⁵. The opening we
297 observe in MacB could be the entry point for such substrates, and accordingly we
298 refer to this as a portal. Density that might be attributed to a bound ligand was found
299 at this putative portal of MacB (Fig. 2; Supplementary Fig. 9). For the substrates that
300 gain access from the periplasmic side, MacB possibly uses a transport mechanism that
301 shares certain features of the ‘outward-only’ model advanced for some ABC
302 transporters, whereby the substrate-binding pocket remains in an outward facing
303 conformational state^{16,17}. The substrates enter the pump through the portal.
304 Conformational changes of this outward facing state are coupled with ATP
305 hydrolysis, resulting in a switch that decreases binding affinity for the substrate and
306 cause it to be jettisoned into the channel of the pump and then transported across the
307 cell envelope to the exterior through the tripartite assembly. In contrast, the ABC
308 transporters adopting an ‘outward-facing’ mechanism capture substrates from the lipid

309 bilayer and move them across the membrane. For MacB, transport through the
310 periplasm would involve allosteric coupling of conformational change propagated
311 from the NBD through the TMD to PLD, which is supported by the finding that
312 interaction of MacA and MacB is stimulated by the presence of ATP and contributes
313 to recruitment of TolC into the pump assembly⁷.

314

315 Similar to the structure of the human ABCG5/ABCG8 sterol transporter¹⁴, MacB has
316 a tightly packed dimer interface in the TMD, and this interface does not appear to
317 have space required to accommodate a transport substrate. For small substrates like
318 erythromycin and protoporphyrin, MacB may drive the transport through the
319 membrane, but this would require re-organization of the dimer interface during the
320 process. This mode of operation is likely to be similar to the alternating access model
321 for ABC-transporters^{14,16}. The details of the mechanism of ATP binding and
322 hydrolysis by MacB in the full pump are unclear presently and require further
323 investigation. However, based on the available data, we envisage that the MacAB-
324 TolC ABC-type tripartite assembly is a highly versatile machinery that can
325 accommodate both periplasmic-entry and transmembrane crossing substrates by using
326 different transport mechanisms, both driven by the energy of ATP binding and
327 hydrolysis.

328

329 **Figure legends**

330

331 **Figure 1 | A pseudo-atomic model for the MacAB-TolC pump. a**, The density map
332 for the pump. Five protomers are colour-coded: TolC (blue), MacA (red, orange and
333 yellow), and MacB (purple). **b**, A sliced view of pump. **c**, Pseudo-atomic model based
334 on docked crystal structures. A trimer of TolC crosses the outer membrane and its
335 helical end protrudes into the periplasm, where it engages a hexamer of MacA. The β -
336 barrel and membrane proximal (MP) domains of MacA mediate the interaction with
337 the periplasmic domain of MacB, which is a dimer. The map shown in **a** was prepared
338 by combining maps for the MacB portion (Supplementary Fig. 4d) and the higher
339 resolution map for the MacA/TolC portion (Supplementary Fig. 5b).

340

341 **Figure 2 | Structure of MacB. a**, Linear representation of MacB. The domains and
342 subdomains in **b** and **c** are color-coded as in panel **a**. **b**, View into the plane of the
343 membrane. **c**, View from the periplasm in the perpendicular direction of the
344 membrane. Unidentified, elongated density shown in grey occludes the region
345 between the periplasmic extensions of TM1 and TM2, which is referred to as the
346 MacB portal. The dash lines delimit the boundary of inner membrane. The NBD

347 locates in the cytoplasmic side and the PLD (PSN and PSC) in the periplasmic side.
348 PSN: periplasmic subdomain N; PSC: periplasmic subdomain C; NBD: nucleotide-
349 binding domain; TM: transmembrane helix; IM: inner membrane.
350

351 **Figure 3 | Interactions between MacA and MacB.** **a**, Top view of the MacA-MacB
352 interface, viewing from the periplasm in the perpendicular direction of the membrane.
353 Each MacB periplasmic domain interacts with three MacA protomers. These
354 interactions distort the hexameric ring formed by the MacA MP domains to generate a
355 more oval shape that accommodates the two-fold symmetry of the MacB dimer. **b** and
356 **c**, Side view of the interactions between three MacA and one MacB. A rotation of 180
357 degrees relates the view in **c** relative to **b**. For clarity, only the β -barrel and MP
358 domains of MacA, and the periplasmic domain of MacB are shown. PLD: periplasmic
359 domain; PSN: periplasmic subdomain N; PSC: periplasmic subdomain C; MP:
360 membrane proximal domain.
361

362 Methods

363 **Construction of vectors for overexpression of MacAB-TolC complex.** The *macA*, *macB* and *tolC*
364 genes were amplified from genomic DNA of *E. coli* strain W3110. The *macA* gene was first amplified
365 using primer pairs MacAGS_F: 5'-
366 GGTGGGAGCGGCGGTGGTGGCTCTGGTGGCGGCGGTAGTATGAAAAAGCGGAAAACCGT
367 GAAGAAGC -3' and MacAinf_R: 5'-
368 GGTGGTGGTCTCGAGTTGTGCAGCTCCTGGTTTGGCCTCA -3'; and it was amplified again
369 using primer pairs MacAGSinf_F: 5'-
370 GGCACGAGAGGGATCCGGTGGGAGCGGCGGTGGTGGCTCT -3' and MacAinf_R. The *macB*
371 gene was amplified using primer pairs MacBinf_F: 5'-
372 AAGGAGATATACATATGACGCCTTTGCTCGAATTAAGGATATTCGTC -3' and MacBinf_R:
373 5'- GCTCGAATTCGGATCCCTCTCGTGCCAGAGCATCTACTGGGTCCAGTCG -3'.

374 The DNA fragment of *macB* was inserted into the *Nde I* and *BamH I* digested pET20b vector using
375 In-Fusion cloning method, generating the construct pET20b-*macB*; The DNA fragment of *polyGlySer*-
376 tagged *macA* was then inserted into the *BamH I* and *Xho I* digested pET20b-*macB* using In-Fusion
377 cloning method, generating the construct pET20b-*macB-polyGS-macA-6His*.

378 The *tolC* gene was amplified using primers TolCinf_F: 5'-
379 AAGGAGATATACATATGAAGAAATTGCTCCCCATTCTTATCGGCC-3' and
380 TolCFLAGXhoI_R: 5'-
381 GAGCTCGAGTCACTTATCGTCGTCATCCTTGTAATCGTTACGAAAGGGTTATGACCGTTAC
382 TGGT -3'; and it was amplified again using TolCinf_F and TolCFLAG_inf_R: 5'-
383 TTGAGATCTGCCATATGTCACTTATCGTCGTCATCCTTGTAATCGTTACG-3'. The resulting
384 DNA fragment of *tolC-FLAG* was inserted into the *Nde I* digested pRSFDuet-1 using In-Fusion cloning
385 method, generating the construct pRSFDuet-*tolC-FLAG*.

386 The *macAB* genes were amplified from genomic DNA of *E. coli* K12 strain W3110 using primers
387 MacAB_F 5'- AAGGAGATATACATATGAAAAAGCGGAAAACCGTGAAGAAGCGT -3' and
388 MacAB_R 5'- GGTGGTGGTGGCTCGAGCTCTCGTGCCAGAGCATCTACTGGATCCAG -3', and
389 were inserted into the *Nde I* and *Xho I* digested pET20b plasmid using In-fusion ligation kits, generating

390 construct pET20b-*macAB*_{6His}.

391 The MacA_D271C and MacB_G465C mutants were produced by site-directed mutagenesis using
392 pET20b-*macAB*_{6His} as a template and primer pairs MacAD271C_F: 5'-
393 CGACGCCGAAAAGGTTAACTGCGCTATTTTCTATTACGC -3'/MacAD271C_R: 5'-
394 GCGTAATAGAAAATAGCGCAGTTAACCTTTTCCGGCGTTCG -3' and
395 MacBG465C_F: 5'- ATGTCCGGGCGAGTTATGTGCCAGTCGTGGCTTAAC -3'/MacBG465C_R:
396 5'- GTTAAGCCACGACTGGCACATAACTCGCCCGGACAT -3', generating construct pET20b-
397 *MacA*_{D271C}*MacB*_{G465C_6His}
398

399 **Overexpression and purification of *E. coli* MacAB fusion with TolC.** Genes encoding AcrA and
400 AcrB were deleted from the chromosome of *Escherichia coli* strain C43 (DE3). The resulting C43
401 (DE3) Δ *acrAB* strain was transformed with plasmids pET20b-*macB-polyGS-macA-His*₆ expressing the
402 MacB-MacA fusion with a C-terminal his-tag and pRSFduet-*tolC-FLAG* expressing full-length TolC
403 with a C-terminal FLAG-tag. A single colony taken from an agar plate with selective antibiotics was
404 used to inoculate 20 ml of LB medium containing carbenicillin at 100 μ g ml⁻¹ and kanamycin at 50 μ g
405 ml⁻¹ in a 50 ml centrifuge tube. The culture was grown in an orbital shaker at 37 °C, 220 rpm for 4 h. 10
406 ml of the culture was used to inoculate 1 litre of 2xYT medium with antibiotics in a 2 L baffled flasks.
407 The culture was grown in an orbital shaker at 37 °C, 220 rpm and was induced at absorbance of
408 A₆₀₀=0.5-0.6 using 0.25 mM IPTG. The temperature was then dropped to 20 °C and the culture
409 incubated overnight. Cells were harvested by centrifugation, and pellets from 10 L culture were re-
410 suspended in 200 ml of lysis buffer composed of 20 mM Tris (pH 8.0), 400 mM NaCl.

411 To the cell suspension was added 1 tablet of EDTA-free protease inhibitor mixture tablet per 50 ml,
412 lysozyme to a final concentration of 5 mg ml⁻¹, and DNase I to a final concentration of 5 U ml⁻¹. The
413 mixture was incubated at 4 °C for 1 h and then passaged 8 times through a high-pressure homogenizer
414 (Emulsiflex) at 15,000 psi at 4 °C. The lysate was centrifuged at 9,000xg for 30 mins at 4 °C to remove
415 the cell debris, and the supernatant was ultra-centrifuged at 125,775xg for 4 h at 4 °C to pellet the
416 cellular membrane. The cellular membrane pellet from 10 L culture was re-suspended in 200 ml of
417 lysis buffer. EDTA-free protease inhibitor mixture tablet was added to membrane suspension at 1 tablet
418 per 50 ml, and n-dodecyl- β -D-maltoside (DDM) was added to a final concentration of 1.5 % w/v. The
419 mixture was gently stirred at 4 °C for 3 h. The membrane solution was then clarified by ultra-
420 centrifugation at 125,775xg for 30 min at 4°C. Imidazole (5M pH: 7.5) was added to the supernatant to
421 a final concentration of 10 mM, and 100 ml of the mixture was applied onto two collected HiTrap
422 Chelating 1 ml column charged with Ni²⁺ and equilibrated with lysis buffer containing 20 mM
423 imidazole and 0.05 % DDM. Two columns were washed with 50 ml of lysis buffer supplemented with
424 0.03 % decyl maltose neopentyl glycol (DMNG) and 50 mM imidazole. The his-tagged MacAB-TolC
425 complex protein was eluted using lysis buffer supplemented with 0.03 % DMNG and 500 mM
426 imidazole. The eluate was buffer exchanged to sample Buffer-I containing 20 mM Tris pH 8.0, 400
427 mM NaCl, 0.03% DMNG using HiTrap Desalting column.

428 The MacAB-TolC complex was further purified by ANTI-FLAG M2 affinity resin. 0.5 ml of
429 ANTI-FLAG M2 affinity resin (Sigma, A2220) in a column was washed by three sequential
430 application of 1 ml of 0.1 M glycine HCl, pH 3.5, then the resin was washed with 5 ml buffer
431 containing 20 mM Tris (pH 7.5) and 150 mM NaCl followed by 5 ml of Buffer-I, allowing a small
432 amount of buffer to remain on the top of the column. The ANTI-FLAG M2 affinity resin in the
433 chromatography column was re-suspended in the above buffer-exchanged protein solution. The
434 mixtures were rotated gently at 4 °C for 1 h and loaded on the chromatography column, allowing the
435 residue protein solution to drain. The resin was washed by loading 10 ml of sample buffer-II containing

436 20 mM NaPi (pH 7.6), 300 mM NaCl and 0.03 % DMNG and was suspended in 0.5 ml of 1 mg ml⁻¹
437 FLAG-peptide in Buffer-II. The mixture was rotated gently at 4 °C for 30 min, and then was
438 centrifuged at 10,000 rpm in an eppendorf centrifuge for 1 min at 4 °C to spin down the resin. The
439 supernatant was removed with a narrow-end pipette tip and loaded on a mini chromatography column
440 to remove the trace amount of resin, collecting the flow through. The resin was re-suspended in 1 ml of
441 sample Buffer-II and was centrifuged at 10,000 rpm in an eppendorf centrifuge for 1 min at 4 °C to spin
442 down the resin. The supernatant was removed with a narrow-end pipette tip and loaded on a mini
443 chromatography column to remove the trace amount of resin, collecting the flow through. This step
444 was repeated 3 times. Fractions containing purified MacAB–TolC complex were pooled and
445 concentrated to 0.5 mg ml⁻¹ using a Vivaspin column (MWCO: 100 kDa). Amphipol A8-35 (100 mg
446 ml⁻¹) was mixed with the protein solution with a mass ratio of amphipol A8-35 to protein of 4:1. The
447 mixture was incubated at 4 °C for 3 h. Polystyrene beads (Bio-Beads SM-2) were then added to the
448 protein/DMNG/amphipol A8-35 mixture with a mass ratio of Bio-Beads SM2 to detergent of 10:1. The
449 mixture was gently rotated at 4 °C overnight to remove DMNG. The detergent-exchanged MacAB–
450 TolC complex was 5-fold diluted using 20 mM NaPi (pH 7.6), 300 mM NaCl and glutaraldehyde (25
451 % w/v) was added to a final concentration of 0.1 % w/v. The cross-linking reaction was incubated on
452 ice overnight and then glycine (1 M, pH 7.5) was added to the sample to a final concentration of 80
453 mM to quench further crosslinking. The mixtures were concentrated to 100 µl using a Vivaspin
454 concentrator (MWCO=100 kDa) and was loaded onto a Superose 6 3.2/300 column equilibrated with
455 Buffer-III containing 20 mM NaPi (pH 7.6), 300 mM NaCl and 0.025 % amphipol A8-35. Fractions
456 containing purified MacAB–TolC complex were pooled and concentrated to 2 mg ml⁻¹ using a
457 Vivaspin column (MWCO: 100 kDa) and embedded in vitreous ice.

458
459 **Overexpression and purification of disulfide-engineered *E. coli* MacAB-TolC.** The C43 (DE3)
460 *ΔacrAB* strain was transformed with plasmids pET20b-*MacA*_{D271C}*MacB*_{G465C-6His} expressing the
461 *MacA*_{D271C} and *MacB*_{G465C} mutants with a C-terminal 6xHis-tag on *MacB*, and pRSFduet-*tolC*-
462 *FLAG* expressing full-length TolC with a C-terminal FLAG-tag. The *MacA*_{D271C}*MacB*_{G465C}-TolC
463 complex was overexpressed and purified as described above for MacAB fusion-TolC complex without
464 cross-linker treatment. Co-expression of the *MacB*-*MacA* fusion and truncated TolC at residue 464 was
465 also tried but did not yield a stable complex.

466
467 **Cell growth for drug sensitivity assay.** Overnight cultures from glycerol-stocks of *E. coli* *ΔacrAB*,
468 harbouring pET20b-derived plasmid encoding fused *MacA*-*MacB*_{E170Q} (control) or fused wildtype
469 *MacAB* in one set of experiments or cysteine mutants *MacA*_{D271C} *MacB*_{G465C}, *MacA*_{D271C}
470 *MacB*_{G465C}_{E170Q}, or wild-type *MacAB* in another set of experiments, were grown in LB
471 containing 25 mM glucose and 100 µg ml⁻¹ carbenicillin. Cultures were grown for about 16 h at 37°C,
472 and then used to inoculate fresh medium. Once *E. coli* cells had reached OD₆₀₀ of 0.8, the cells were
473 diluted to OD₆₀₀ of 0.06 in fresh medium containing 0.07 mM IPTG in the wells of a 96-well plate to
474 which antibiotics were added as indicated in Supplementary Fig. 2. Growth was followed over time at
475 OD₆₀₀ at 37 °C in a Versamax microplate reader (Molecular Devices).

476 The same experimental procedure was followed for the cell growth-based sensitivity assay with *E.*
477 *coli* *ΔacrAB*, harbouring pET20b-derived plasmid encoding wild-type *MacAB*, *MacA*_{Q209A} *MacB*,
478 *MacA*_{Q210A} *MacB*, or no *MacAB* (control) in the presence of 0 – 88.9 µg ml⁻¹ erythromycin
479 (Supplementary Fig. 11).

480
481 **Electron microscopy data collection.** For cryo-EM of the fusion-stabilised pump, aliquots of 3 µl of

482 purified MacAB-TolC in amphipols at a concentration of 2 mg ml⁻¹ were applied to glow-discharged
483 holey carbon grids (Quantifoil Au R1.2/1.3, 300 mesh), blotted with filter paper to remove excess
484 sample, and plunge-frozen in liquid ethane slush using an FEI Vitrobot Mark IV. Zero-energy-loss
485 images of the frozen, hydrated grids were acquired automatically using an FEI Titan Krios electron
486 microscope operated at 300 kV with Legikon on a Gatan K2-Summit detector in super-resolution
487 counting mode⁴⁰. A GIF-Quantum energy filter (Gatan) was used with a slit width of 20 eV to remove
488 inelastically scattered electrons. Twenty movie frames were recorded each with an exposure time of
489 800 ms using a dose rate of ~2.5 electrons Å⁻² s⁻¹ (approximately 5 electrons pixel⁻¹ s⁻¹) at a calibrated
490 magnification of 36,764× (yielding a pixel size of 1.36 Å at the sample level). The final dataset is
491 composed of 2,136 micrographs with defocus values ranging from -1.6 to -2.6 μm.

492 For cryo-EM of the disulphide-stabilised pump, a strategy based on PEGylated gold grids was
493 chosen to improve grid stability and the distribution and number of particles^{41,42}. The UltrAufoil grids
494 (UltrAuFoil[®] Holey Gold grid R1.2/1.3, 300 mesh, Quantifoil Micro Tools GmbH) were glow-
495 discharged at 20 mA for 90 s, and immersed in anaerobic ethanol containing 5 mM SPT-0011P6
496 (SensoPath Technologies). The grids were incubated with the reagent for 2 days at room temperature in
497 an anaerobic glovebox, and were then washed 3 times in absolute ethanol to remove excess reagent and
498 allowed to air-dry. A Vitrobot IV (FEI) was used to prepare grids in a controlled atmosphere (4 °C, 100
499 % relative humidity). A 2.5 μl aliquot of purified MacAB-TolC at a concentration of 2 mg ml⁻¹ in
500 amphipols was applied, and the grids were blotted at a force of '-10' for 8-16 s before plunging into
501 liquid ethane. The grids were stored in liquid nitrogen before imaging. Zero-energy-loss images of
502 frozen-hydrated MacAB-TolC particles were recorded automatically on an FEI Titan Krios electron
503 microscope at 300 kV with a GIF Quantum energy filter set with a slit width of 20 eV. A Gatan K2-
504 Summit direct electron detector was used in super-resolution counting mode at a nominal magnification
505 of 36,765, corresponding to a calibrated physical pixel size of 1.36 Å, and a dose rate of 2.7 electrons
506 per square angstrom per second (approximately 5 electrons per pixel per second). Exposures of 16 s
507 were dose fractionated into 20 movie frames, leading to an accumulative dose of about 45 electrons/Å²
508 on the specimen. A total of 869 movie stacks were collected with a defocus range of -1.2 to -2.5 μm.
509

510 **Image Processing.** For the fusion-stabilised pump, all super-resolution frames were corrected for gain
511 reference, binned by a factor of 2 from 0.68 Å pixel⁻¹ to 1.36 Å pixel⁻¹, and motion-corrected using
512 MOTIONCORR⁴³. Aligned frames were then summed into average micrographs, which were then used
513 to estimate the contrast transfer function (CTF) using Gctf⁴⁴. All subsequent image-processing steps
514 were performed using RELION⁴⁵. Templates for reference-based particle picking were obtained from
515 (reference-free) 2D classification of a subset of manually picked particles. The templates were low-pass
516 filtered to 20 Å to limit reference bias, and used to automatically pick⁴⁶ 180,602 particles from all
517 micrographs. Selection of suitable 2D classes from a 2D classification with all auto-picked particles
518 yielded 111,656 particles, which were further, classified using 3D classification. An initial model
519 composed of the crystal structures of TolC and MacA (PDB accession numbers 3FPP and 1EK9,
520 respectively) and a homology model of MacB was converted into a density map using e2pdb2mrc.py
521 from EMAN2⁴⁷ and low-pass filtered to 60 Å to limit reference bias during classification. The two
522 largest classes, comprising 92,913 particles, were selected for subsequent 3D auto-refinement (without
523 symmetry) to an overall resolution of 6.2 Å. Beam-induced motion correction and radiation-damage
524 weighting (particle polishing) was used to increase the signal-to-noise ratio of the individual particles
525⁴⁸. A further round of 2D classification resulted in a final dataset of 91,162 polished particles.

526 Initial 3D refinement of the polished particles resulted in a map with an improved overall
527 resolution of 5.2 Å. Masked 3D refinement and classification with partial signal subtraction³⁴ was used

528 to improve the density for each of the three sub-complexes. In the overall map, the density of the inner
529 membrane protein MacB was relatively weak owing to structural flexibility in this region (as is also
530 visible in the 2D class averages, see Supplementary Fig. 4b). Masked 3D classification with partial
531 signal subtraction and without alignment on this region yielded a more homogeneous subset of 17,154
532 particles. Masked refinement of this subset lead to a reconstruction of MacB with improved density to a
533 resolution of 5.3 Å (Supplementary Fig. 4c,d). Masked refinements with partial signal subtraction of
534 the MacA and TolC regions were performed imposing C6 and C3 symmetry, respectively, yielding
535 improved maps to 4.0 and 4.6 Å resolution (Supplementary Fig. 4c,d).

536 For the disulphide-stabilised pump, the software MotionCor2 was used for whole-frame motion
537 correction and dose weighting, CTFFIND4 for estimation of the contrast transfer function parameters,
538 RELION-1.4 and RELION-2.0/beta packages for all other image processing steps. A particle subset
539 was manually picked up to calculate reference-free 2D class averages, which was then used as
540 templates for automated particle picking of the entire data set. The templates were lowpass filtered to
541 20 Å to limit model bias. 71,462 particles were picked automatically from a total of 792 micrographs
542 with good ice thickness. Then initial runs of 2D and 3D classifications were used to remove the
543 heterogeneous particles, as well as the false positive particles from the auto-picking. 27,614 particles
544 were selected for a first 3D auto-refinement with C3 symmetry. This generated a reconstruction with a
545 resolution of 4.4 Å that clearly showed density for TolC, the α -helical hairpin, lipoyl and β -barrel
546 domains of MacA; however, the density for the other portions of the pump was poor. This particle
547 subset was then used for a focused 3D auto-refinement⁴⁹ by applying a soft mask around TolC, the α -
548 helical hairpin, lipoyl and β -barrel domains of MacA, and subtracting the residual signal for the rest of
549 the pump. This yielded a reconstruction with a resolution of 3.3 Å (Supplementary Fig. 5b,c).

550 This particle subset was also used for a focused 3D classification³⁴ by applying a soft mask around
551 the MP domains of MacA and the PLD domains of MacB, subtracting the residual signal for the rest
552 part of the pump, and using the orientation information of the particles from the first 3D auto-
553 refinement without perform any alignments. This yielded a homogenous subset of 11,158 particles.
554 This particle subset with partial signal subtraction was submitted to 3D auto-refinement imposing C2
555 symmetry and using small angular sampling and local search, generating a reconstruction for the MP
556 domains of MacA and the PLD domains of MacB with a resolution of 8.0 Å (Supplementary Fig. 5b,c;
557 Supplementary Fig. 7b).

558 All resolution estimates were calculated using separately refined half-reconstructions in RELION,
559 using the Fourier shell correlation at 0.143 criterion⁵⁰ (Supplementary Fig. 4c; Supplementary Fig. 5c).
560 Local resolution variations were calculated using the RELION wrapper to the ResMap⁵¹ program
561 (Supplementary Fig. 4d; Supplementary Fig. 5b).

562
563 **Model docking and refinement.** The crystal structure of trimeric TolC (PDB code: 1EK9) was docked
564 into the cryoEM map using Chimera. The model was adjusted manually to optimize the local fit to
565 density using Coot. Chain B from the crystal structure of MacA (PDB code: 3FPP) including β -barrel
566 domain, lipoyl domain and α -helical hairpin domain were fitted to the density map by using Chimera.
567 A homology model of the MP domain of MacA was built based on the crystal structure of MexA (PDB
568 code: 2V4D) and was fitted to the density. A homology model of *E.coli* MacB was built based on the
569 crystal structure of *Acinetobacter baumannii* MacB²⁹. The model was docked into the cryoEM map,
570 and the periplasmic and NBD domains of MacB were rigid body fitted to the density by using Chimera.
571 To optimize the local fit to density, the periplasmic domain was further adjusted manually using Coot
572 based on the crystal structure of the periplasmic domain of MacB from *Actinobacillus*
573 *actinomycetemcomitans* (PDB code: 3FTJ). The model for the complete pump was refined using

574 REFMAC “jelly-body” refinement using secondary-structure restraints to maintain proper
575 stereochemistry. Ramachandran outliers were corrected semi-automatically in Coot and MolProbity
576 statistics were computed to ensure proper stereochemistry. The model of the whole pump complex was
577 validated by computing a FSC with the density map (Supplementary Fig. 14).
578

579 **Molecular Dynamics Simulations.** All simulations were performed with the software package
580 GROMACS5⁵². The amber99sb force field⁵³ was used for the protein. Erythromycin was
581 parameterized by using amber99sb atom types in conjunction with RESP2 (HF/6-31G(d)) charges⁵⁴, as
582 implemented in the Antechamber module of the AMBER12 software package⁵⁵. The TIP3P explicit
583 water model⁵⁶ was used for solvation of the protein together with Joung and Cheatham ion parameters
584⁵⁷, at a NaCl concentration of 0.15M. Unbiased molecular dynamics simulations of 200 ns length were
585 carried out on the MacA structure obtained by cryo-EM to equilibrate the system. Subsequently, a set
586 of 2×100 individual force-probe (steered) molecular dynamics simulations was performed⁵⁸, in which
587 erythromycin was propagated along the pore axis in inward and outward direction by a moving
588 harmonic potential with a force constant of 50 kJ mol⁻¹ Å⁻² and at a constant velocity of 2.5 Å ns⁻¹.
589

590 **Data availability.** The cryoEM map and the atomic model have been deposited in the Protein Data
591 Bank and EMDB under accession numbers [xxxx](#) and [xxxx](#), respectively. The data that support the
592 findings of this study are available from the corresponding authors upon request.
593

594 References

- 595 1. Kobayashi, N., Nishino, K. & Yamaguchi, A. Novel macrolide-specific ABC-type efflux
596 transporter in *Escherichia coli*. *J. Bacteriol.* **183**, 5639–44 (2001).
- 597 2. Vallet-Gely, I. *et al.* Association of hemolytic activity of *Pseudomonas entomophila*, a versatile
598 soil bacterium, with cyclic lipopeptide production. *Appl. Environ. Microbiol.* **76**, 910–21
599 (2010).
- 600 3. Cho, H. & Kang, H. The PseEF efflux system is a virulence factor of *Pseudomonas syringae*
601 *pv. syringae*. *J. Microbiol.* **50**, 79–90 (2012).
- 602 4. Turlin, E. *et al.* Protoporphyrin (PPIX) efflux by the MacAB-TolC pump in *Escherichia coli*.
603 *Microbiologyopen* **3**, 849–59 (2014).
- 604 5. Yamanaka, H., Kobayashi, H., Takahashi, E. & Okamoto, K. MacAB is involved in the
605 secretion of *Escherichia coli* heat-stable enterotoxin II. *J. Bacteriol.* **190**, 7693–8 (2008).
- 606 6. Lu, S. & Zgurskaya, H. I. MacA, a periplasmic membrane fusion protein of the macrolide
607 transporter MacAB-TolC, binds lipopolysaccharide core specifically and with high affinity. *J.*
608 *Bacteriol.* **195**, 4865–72 (2013).
- 609 7. Lu, S. & Zgurskaya, H. I. Role of ATP binding and hydrolysis in assembly of MacAB-TolC
610 macrolide transporter. *Mol. Microbiol.* **86**, 1132–43 (2012).
- 611 8. Tikhonova, E. B., Devroy, V. K., Lau, S. Y. & Zgurskaya, H. I. Reconstitution of the
612 *Escherichia coli* macrolide transporter: the periplasmic membrane fusion protein MacA
613 stimulates the ATPase activity of MacB. *Mol. Microbiol.* **63**, 895–910 (2007).
- 614 9. Dawson, R. J. P. & Locher, K. P. Structure of a bacterial multidrug ABC transporter. *Nature*
615 **443**, 180–5 (2006).
- 616 10. Ward, A., Reyes, C. L., Yu, J., Roth, C. B. & Chang, G. Flexibility in the ABC transporter
617 MsbA: Alternating access with a twist. *Proc. Natl. Acad. Sci. U. S. A.* **104**, 19005–10 (2007).
- 618 11. Gutmann, D. A. P., Ward, A., Urbatsch, I. L., Chang, G. & van Veen, H. W. Understanding
619 polyspecificity of multidrug ABC transporters: closing in on the gaps in ABCB1. *Trends*

- 620 *Biochem. Sci.* **35**, 36–42 (2010).
- 621 12. Doshi, R., Woebking, B. & van Veen, H. W. Dissection of the conformational cycle of the
622 multidrug/lipidA ABC exporter MsbA. *Proteins* **78**, 2867–72 (2010).
- 623 13. Choudhury, H. G. *et al.* Structure of an antibacterial peptide ATP-binding cassette transporter
624 in a novel outward occluded state. *Proc. Natl. Acad. Sci. U. S. A.* **111**, 9145–50 (2014).
- 625 14. Lee, J.-Y. *et al.* Crystal structure of the human sterol transporter ABCG5/ABCG8. *Nature* **533**,
626 561–4 (2016).
- 627 15. Aittoniemi, J. *et al.* SUR1: a unique ATP-binding cassette protein that functions as an ion
628 channel regulator. *Philos. Trans. R. Soc. B Biol. Sci.* **364**, 257–267 (2009).
- 629 16. Locher, K. P. Mechanistic diversity in ATP-binding cassette (ABC) transporters. *Nat. Struct.*
630 *Mol. Biol.* **23**, 487–93 (2016).
- 631 17. Perez, C. *et al.* Structure and mechanism of an active lipid-linked oligosaccharide flippase.
632 *Nature* **524**, 433–8 (2015).
- 633 18. Khare, D., Oldham, M. L., Orelle, C., Davidson, A. L. & Chen, J. Alternating access in maltose
634 transporter mediated by rigid-body rotations. *Mol. Cell* **33**, 528–36 (2009).
- 635 19. Woo, J.-S., Zeltina, A., Goetz, B. A. & Locher, K. P. X-ray structure of the Yersinia pestis
636 heme transporter HmuUV. *Nat. Struct. Mol. Biol.* **19**, 1310–5 (2012).
- 637 20. Korkhov, V. M., Mireku, S. A. & Locher, K. P. Structure of AMP-PNP-bound vitamin B12
638 transporter BtuCD-F. *Nature* **490**, 367–72 (2012).
- 639 21. Song, S., Kim, J.-S., Lee, K. & Ha, N.-C. Molecular architecture of the bacterial tripartite
640 multidrug efflux pump focusing on the adaptor bridging model. *J. Microbiol.* **53**, 355–64
641 (2015).
- 642 22. Zgurskaya, H. I., Weeks, J. W., Ntrel, A. T., Nickels, L. M. & Wolloscheck, D. Mechanism of
643 coupling drug transport reactions located in two different membranes. *Front. Microbiol.* **6**, 100
644 (2015).
- 645 23. Du, D., van Veen, H. W. & Luisi, B. F. Assembly and operation of bacterial tripartite multidrug
646 efflux pumps. *Trends Microbiol.* **23**, 311–9 (2015).
- 647 24. Yamanaka, H., Izawa, H. & Okamoto, K. Carboxy-terminal region involved in activity of
648 Escherichia coli TolC. *J. Bacteriol.* **183**, 6961–4 (2001).
- 649 25. Du, D. *et al.* Structure of the AcrAB-TolC multidrug efflux pump. *Nature* **509**, 512–5 (2014).
- 650 26. Daury, L. *et al.* Tripartite assembly of RND multidrug efflux pumps. *Nat. Commun.* **7**, 10731
651 (2016).
- 652 27. Kim, J.-S. *et al.* Structure of the tripartite multidrug efflux pump AcrAB-TolC suggests an
653 alternative assembly mode. *Mol. Cells* **38**, 180–6 (2015).
- 654 28. Lin, H. T. *et al.* MacB ABC transporter is a dimer whose ATPase activity and macrolide-
655 binding capacity are regulated by the membrane fusion protein MacA. *J. Biol. Chem.* **284**,
656 1145–54 (2009).
- 657 29. Okada, Ui; Yamashita, Eiki; Neuberger, Arthur; W. van Veen, Hendrik; Murakami, S. Crystal
658 structure of tripartite-type ABC transporter, MacB from Acinetobacter baumannii. *Submitt.*
659 *Artic.* (2016).
- 660 30. Xu, Y. *et al.* Crystal structure of the periplasmic region of MacB, a noncanonic ABC
661 transporter. *Biochemistry* **48**, 5218–25 (2009).
- 662 31. Yum, S. *et al.* Crystal structure of the periplasmic component of a tripartite macrolide-specific
663 efflux pump. *J. Mol. Biol.* **387**, 1286–97 (2009).
- 664 32. Calladine, C. R., Sharff, A. & Luisi, B. How to untwist an alpha-helix: structural principles of
665 an alpha-helical barrel. *J. Mol. Biol.* **305**, 603–18 (2001).

- 666 33. Modali, S. D. & Zgurskaya, H. I. The periplasmic membrane proximal domain of MacA acts as
667 a switch in stimulation of ATP hydrolysis by MacB transporter. *Mol. Microbiol.* **81**, 937–51
668 (2011).
- 669 34. Bai, X., Rajendra, E., Yang, G., Shi, Y. & Scheres, S. H. W. Sampling the conformational
670 space of the catalytic subunit of human γ -secretase. *Elife* **4**, (2015).
- 671 35. Jeong, H. *et al.* Pseudoatomic Structure of the Tripartite Multidrug Efflux Pump AcrAB-TolC
672 Reveals the Intermeshing Cogwheel-like Interaction between AcrA and TolC. *Structure* **24**,
673 272–6 (2016).
- 674 36. Xu, Y. *et al.* The tip region of the MacA alpha-hairpin is important for the binding to TolC to
675 the Escherichia coli MacAB-TolC pump. *Biochem. Biophys. Res. Commun.* **394**, 962–5 (2010).
- 676 37. Xu, Y. *et al.* Functional implications of an intermeshing cogwheel-like interaction between
677 TolC and MacA in the action of macrolide-specific efflux pump MacAB-TolC. *J. Biol. Chem.*
678 **286**, 13541–9 (2011).
- 679 38. Lee, M. *et al.* The α -barrel tip region of Escherichia coli TolC homologs of Vibrio vulnificus
680 interacts with the MacA protein to form the functional macrolide-specific efflux pump MacAB-
681 TolC. *J. Microbiol.* **51**, 154–9 (2013).
- 682 39. Dong, C. *et al.* Wza the translocon for E. coli capsular polysaccharides defines a new class of
683 membrane protein. *Nature* **444**, 226–9 (2006).
- 684 40. Suloway, C. *et al.* Automated molecular microscopy: the new Legimon system. *J. Struct. Biol.*
685 **151**, 41–60 (2005).
- 686 41. Meyerson, J. R. *et al.* Self-assembled monolayers improve protein distribution on holey carbon
687 cryo-EM supports. *Sci. Rep.* **4**, 7084 (2014).
- 688 42. Russo, C. J. & Passmore, L. A. Electron microscopy: Ultrastable gold substrates for electron
689 cryomicroscopy. *Science* **346**, 1377–80 (2014).
- 690 43. Li, X. *et al.* Electron counting and beam-induced motion correction enable near-atomic-
691 resolution single-particle cryo-EM. *Nat. Methods* **10**, 584–90 (2013).
- 692 44. Zhang, K. Gctf: Real-time CTF determination and correction. *J. Struct. Biol.* **193**, 1–12 (2016).
- 693 45. Scheres, S. H. W. RELION: implementation of a Bayesian approach to cryo-EM structure
694 determination. *J. Struct. Biol.* **180**, 519–30 (2012).
- 695 46. Scheres, S. H. W. Semi-automated selection of cryo-EM particles in RELION-1.3. *J. Struct.*
696 *Biol.* **189**, 114–22 (2015).
- 697 47. Tang, G. *et al.* EMAN2: an extensible image processing suite for electron microscopy. *J.*
698 *Struct. Biol.* **157**, 38–46 (2007).
- 699 48. Scheres, S. H. Beam-induced motion correction for sub-megadalton cryo-EM particles. *Elife* **3**,
700 e03665 (2014).
- 701 49. Scheres, S. H. W. Processing of Structurally Heterogeneous Cryo-EM Data in RELION.
702 *Methods Enzymol.* **579**, 125–57 (2016).
- 703 50. Scheres, S. H. W. & Chen, S. Prevention of overfitting in cryo-EM structure determination.
704 *Nat. Methods* **9**, 853–4 (2012).
- 705 51. Kucukelbir, A., Sigworth, F. J. & Tagare, H. D. Quantifying the local resolution of cryo-EM
706 density maps. *Nat. Methods* **11**, 63–5 (2014).
- 707 52. Abraham, M. J. *et al.* GROMACS: High performance molecular simulations through multi-
708 level parallelism from laptops to supercomputers. *SoftwareX* **1**, 19–25 (2015).
- 709 53. Hornak, V. *et al.* Comparison of multiple Amber force fields and development of improved
710 protein backbone parameters. *Proteins* **65**, 712–25 (2006).
- 711 54. Wang, J., Cieplak, P. & Kollman, P. A. How well does a restrained electrostatic potential

712 (RESP) model perform in calculating conformational energies of organic and biological
713 molecules? *J. Comput. Chem.* **21**, 1049–1074 (2000).
714 55. Case. *AMBER 12*. (University of California, 2012).
715 56. Jorgensen, W. L., Chandrasekhar, J., Madura, J. D., Impey, R. W. & Klein, M. L. Comparison
716 of simple potential functions for simulating liquid water. *J. Chem. Phys.* **79**, 926 (1983).
717 57. Joung, I. S. & Cheatham, T. E. Determination of alkali and halide monovalent ion parameters
718 for use in explicitly solvated biomolecular simulations. *J. Phys. Chem. B* **112**, 9020–41 (2008).
719 58. Grubmüller, H., Heymann, B. & Tavan, P. Ligand binding: molecular mechanics calculation of
720 the streptavidin-biotin rupture force. *Science* **271**, 997–9 (1996).
721
722

723 **Acknowledgements**

724 This work was supported by the Wellcome Trust (B.F.L.), HFSP (B.F.L., H.W.v.V., S. M.), Marie
725 Curie International Outgoing Fellowship (A.W.P.F.), the UK Medical Research Council
726 (MC_UP_A025_1013, to SHWS), Wellcome Trust ISSF award (grant number: WT097818MF), the
727 Scottish Universities' Physics Alliance (U.Z. and S.L.) and MRC Mitochondrial Biology Unit (Grant
728 number: U105663141). A.N. is the recipient of a Herchel-Smith Scholarship. We thank Jeremy
729 Skepper for help using the EM microscope and imaging facility at Cambridge University, Hong Zhou
730 for providing access to the electron microscope at the University of California at Los Angeles, Jake
731 Grimmitt and Toby Darling for support with high-performance computing at the MRC Laboratory of
732 Molecular Biology, Stuart Rankin for help with computing on the High-Performance Computing
733 System at the University of Cambridge and Len Packman for mass spectrometry analyses. We thank
734 the staff of Diamond Light Source for access to the eBIC facility. The EM maps have been deposited in
735 the Electron Microscopy Databank.

736

737 **Author contributions**

738 D.D., B.F.L. and S.H.W.S. designed the project. D.D. purified the fusion and disulphide-linkage
739 stabilized MacAB–TolC complexes. D.D., A.W.P.F., X.B. and J.N.B. obtained and analysed the single-
740 particle cryo-EM data. U.O. and S.M. built the homology model of MacB. D.D. and B.F.L. devised a
741 model of MacAB–TolC based on the cryo-EM map. A.N. and H.W.v.V. conducted MIC assays on the
742 MacAB–TolC pump. S.L. and U.Z. carried out molecular dynamics simulations of MacA. D.D., B.F.L.
743 and S.H.W.S. wrote the paper. All authors contributed to editing the manuscript.

744

745 **Additional information**

746 **Supplementary information** is available for this paper.

747 **Correspondence and requests for materials** should be addressed to D.D., B.F.L. or S.H.W.S.

748 ORCID ID of Dijun Du: 0000-0002-1546-8939

749 ORCID ID of Ben Luisi: 0000-0003-1144-9877

750 ORCID ID of Sjors Scheres: 0000-0002-0462-6540

751

752 **Competing interests**

753 The authors declare no competing financial interests.