

1 The membrane-proximal domain of the periplasmic adapter
2 protein plays a role in vetting substrates utilising channels 1
3 and 2 of RND efflux transporters

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15 **Running title:** PAP controls access to RND substrate channels

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25 **Abstract**

26 Active efflux by resistance-nodulation-division (RND) efflux pumps is a major
27 contributor to antibiotic resistance in clinically relevant Gram-negative bacteria.
28 Tripartite RND pumps, such as AcrAB-TolC of *Salmonella enterica* serovar
29 Typhimurium, comprise of an inner membrane RND transporter, a periplasmic adaptor
30 protein (PAP) and an outer membrane factor. Previously, we elucidated binding sites
31 within the PAP AcrA (termed binding boxes) that were important for AcrB-transporter
32 recognition. Here, we have refined the binding box model by identifying the most
33 critical residues involved in PAP-RND binding and show that the corresponding RND-
34 binding residues in the closely related PAP AcrE are also important for AcrB
35 interactions. In addition, our analysis identified a membrane-proximal domain (MPD)-
36 residue in AcrA (K366), that when mutated, differentially affects transport of substrates
37 utilising different AcrB efflux-channels, namely channels 1 and 2, supporting a
38 potential role for the PAP in sensing the substrate-occupied state of the proximal
39 binding pocket (PBP) of the transporter and substrate vetting. Our model predicts that
40 there is a close interplay between the MPD of the PAP and the RND transporter in the
41 productive export of substrates utilising the PBP.

42

43 **Importance**

44 Antibiotic resistance greatly threatens our ability to treat infectious diseases. In Gram-
45 negative bacteria, overexpression of tripartite efflux pumps, such as AcrAB-TolC,
46 contributes to multidrug resistance because they export many different classes of
47 antibiotics. The AcrAB-TolC pump is made up of three components: the periplasmic
48 adaptor protein (PAP) AcrA, the RND-transporter AcrB, and the outer-membrane
49 factor TolC. Here, we identified critical residues of AcrA that are important for its

50 function with AcrB in *Salmonella enterica* serovar Typhimurium. Also, we show that
51 AcrA shares these critical residues with AcrE, a closely related PAP, explaining their
52 interoperability with AcrB. Importantly, we identified a residue in the membrane-
53 proximal domain of AcrA that when mutated affected how different substrates access
54 AcrB and impacted downstream efflux *via* TolC channel. Understanding the role that
55 PAPs play in the assembly and function of tripartite RND pumps can guide novel ways
56 to inhibit their function to combat antibiotic resistance.

57

58 **Introduction**

59 Antibiotic resistance is one of the greatest global public health challenges and
60 threatens our ability to effectively treat and prevent infectious diseases (1). In clinical
61 isolates of Gram-negative bacteria, the Resistance-Nodulation-Division (RND) family
62 of efflux pumps are frequently upregulated and associated with multidrug resistant
63 phenotypes (2-6). Tripartite RND pumps span the double membrane of Gram-negative
64 bacteria and consist of an inner membrane RND transporter, a periplasmic adaptor
65 protein (PAP), and an outer membrane factor (OMF) (7, 8). The AcrAB-TolC pump is
66 the principal RND efflux system in Enterobacteriaceae, including *Salmonella enterica*.
67 It can export a wide range of structurally different compounds, including clinically
68 relevant antibiotics such as β -lactams and fluoroquinolones (9, 10).

69

70 Gram-negative bacteria encode a wide repertoire of RND transporters, which typically
71 pair with a single cognate PAP and an OMF to form tripartite pumps that have varied
72 substrate specificities and physiological roles (10-16). The *S. enterica* genome
73 encodes five RND pumps: AcrAB-TolC, AcrEF-TolC, AcrAD-TolC, MdtABC, and
74 MdsABC (10). The AcrEF-TolC pump possesses a similar substrate profile to AcrAB-

75 TolC, but its expression is silenced by H-NS under laboratory conditions (17). The
76 PAPs comprise four domains: α -helical domain, lipoyl domain, β -barrel domain and
77 the membrane-proximal domain (MPD) (18). The α -helical domain has a coiled-coil
78 arrangement and appears to interact with the α -barrel domain of the OMF (18, 19).
79 The lipoyl domain is involved in stabilising the self-assembly of the PAPs within the
80 tripartite efflux pump. The β -barrel domain is flexibly linked to the MPD, and both
81 domains appear to interact with the porter domain of the RND-transporter (20). The
82 MPD of RND-associated PAPs is critical for the assembly and function of AcrAB-TolC
83 in *E. coli* and *S. enterica* (21, 22). In the PAPs ZneB and CusB of the tripartite ZneCAB
84 and CusABC RND efflux pumps, respectively, the MPD appears to play an important
85 role in substrate acquisition and presentation to the metal pumping RND transporters
86 (23, 24). Additionally, in the related ABC-transporter-associated PAP MacA, the MPD
87 has been demonstrated to be involved in direct binding of possible pump substrates
88 (25) and has been suggested to be involved in substrate vetting (18).

89

90 Previous studies have shown that AcrA and AcrE are interchangeable in *S. enterica*
91 (24-26), whereas MdtA and MdsA can only function with their cognate RND-
92 transporters (22). Previously, we showed that the regions of PAP-transporter contact
93 are relatively compact and discrete. Based on homology models of the PAPs
94 in *Salmonella*, we found these regions to be highly conserved between AcrA and AcrE,
95 while differing significantly between divergent PAPs, such as MdtA and MdsA,
96 providing a possible explanation of the observed interoperability of AcrA and AcrE.
97 The 3D RND-interaction sites can be delineated into discrete linear sequences, which
98 we have dubbed “binding boxes”, that map to the β -barrel domain (boxes 1–5) and the
99 MPD (6–9). Disruption of a few key residues within the binding boxes 1 and 4-6

100 mapping to the exposed β -barrel loops and the MPD, abrogated transport, suggesting
101 an important role for this region in AcrB-binding (22).

102

103 Here, we set out to further validate the “binding boxes” model of PAP-RND interaction
104 by phenotypic profiling of site-directed mutants targeting the β -barrel and
105 membrane-proximal domains. We specifically sought to describe the efflux profiles of
106 substrates that have been suggested to utilise different AcrB-efflux channels (26-28).

107

108 **Results**

109 **Refining the binding box model of PAP-RND interactions**

110 Previously, using disruptive site-directed mutagenesis (SDM), we demonstrated that
111 discrete stretches of residues, which we dubbed “binding boxes” based on their spacial
112 proximity (19, 20, 29, 30), control PAP-RND complex formation and recognition of
113 cognate PAP-RND pairs (22). Here, we set out to refine our binding box model by
114 generating and testing the effects of more subtle and conservative mutations to identify
115 the PAP-residues most critical for RND-binding (Fig. 1). Specifically, residues which
116 were previously shown to be important for RND-binding (22) were mutated to residues
117 with similar properties to produce conservative mutations, while residues, the previous
118 mutation of which led to limited functional impact were subjected to more disruptive
119 mutagenesis. Mutated versions of AcrA were expressed in the *Salmonella* SL1344
120 Δ 4PAP strain, which lacks all four known RND-associated PAPs (AcrA, AcrE, MdtA
121 and MdsA) (22). The effects of the mutations on efflux function were assessed using
122 ethidium bromide efflux assays and antimicrobial susceptibility testing.

123

124 The PAPs reside in the periplasm and are embedded in the plasma membrane by a
125 lipid anchor and/or a transmembrane helix and are composed of several well-defined
126 domains (18, 31). From the plasma membrane outward, these are the
127 membrane-proximal domain (containing boxes 6-9), the β -barrel domain (containing
128 boxes 1-5), the lipoyl domain, and the α -hairpin domain (18). The G58F mutant
129 mapping to box 1 was previously shown to impair efflux function (22). Here, the R59A
130 mutant was produced to investigate whether other residues in proximity to box 1 had
131 a role in RND-binding. The R59A mutant caused an intermediate impairment of efflux
132 function, between that of the Δ 4PAP and the WT complement strain, confirming the
133 important role of box 1 (Fig. 2 and Table S2). Residues which were mutated as pairs
134 in our previous study were separated to identify the most critical residue. The T270F-
135 T271F mutant mapping to box 4 was separated into T270D and T271D for disruptive
136 mutagenesis, and T270A and T271A mutants as more subtle mutations. The T271D
137 mutant significantly impaired efflux, whilst the T270D mutant had no effect.
138 Furthermore, the T271A mutation still caused a mild impairment of efflux function (Fig.
139 2 and Table S2), suggesting that T271 is a critical residue in efflux function. The
140 G272P-S273P mutant mapping to box 4 previously impaired efflux function, therefore,
141 a more conservative mutation was produced to determine the role of G272-S273. The
142 G272A-S273A mutant had no effect on efflux activity, indicating that these residues
143 can tolerate neutral mutations (Fig. 2 and Table S2). The F292G mutant mapping to
144 box 5 was previously shown to affect both AcrB- and AcrD-binding (22, 32). Hence,
145 the F292V mutant was produced to determine whether a more subtle change would
146 still affect efflux function. Like the F292G phenotype, the F292V mutant also resulted
147 in significantly abrogated efflux (Fig. 2 and Table S2), indicating that F292 is critical
148 for efflux function. The Q310F mutant mapping to box 6 was previously shown to have

149 no impact on efflux function, and the Q311F reported here, similarly did not influence
150 efflux function detectably (Fig. 2 and Table S2). Therefore, both residues were
151 simultaneously mutated into Q310P-Q311P, which resulted in significant impairment
152 of efflux function, indicating that double glutamine residues may provide some
153 functional redundancy and that the presence of a glutamine residue may be critical in
154 this position (Fig. 2 and Table S2).

155

156 Mutations mapping to box 6, including R315F and R318A, were previously shown to
157 have no impact on efflux function. Therefore, several novel mutations mapping to box
158 6 were produced (P317G, P317F and R318F), which did not have any impact on efflux
159 function (Fig. 2 and Table S2). Novel mutations mapping to box 8 (I344F-G344F and
160 K346A) also had no observable influence on efflux function (Fig. 2 and Table S2).
161 G363 in box 9 was shown by us and other studies to be critical for efflux function (22,
162 32, 33). Further mutations in box 9 were produced to investigate whether other
163 residues also play a role in RND-binding. However, Q365F, K366D, R368F and R368A
164 had no effect on efflux function, suggesting that only G363 is critical for efflux function
165 (Fig. 2 and Table S2). Western blotting verified that the observed effects of the mutants
166 with impaired efflux were not due to changes in protein expression levels or stability
167 (Fig. S1A).

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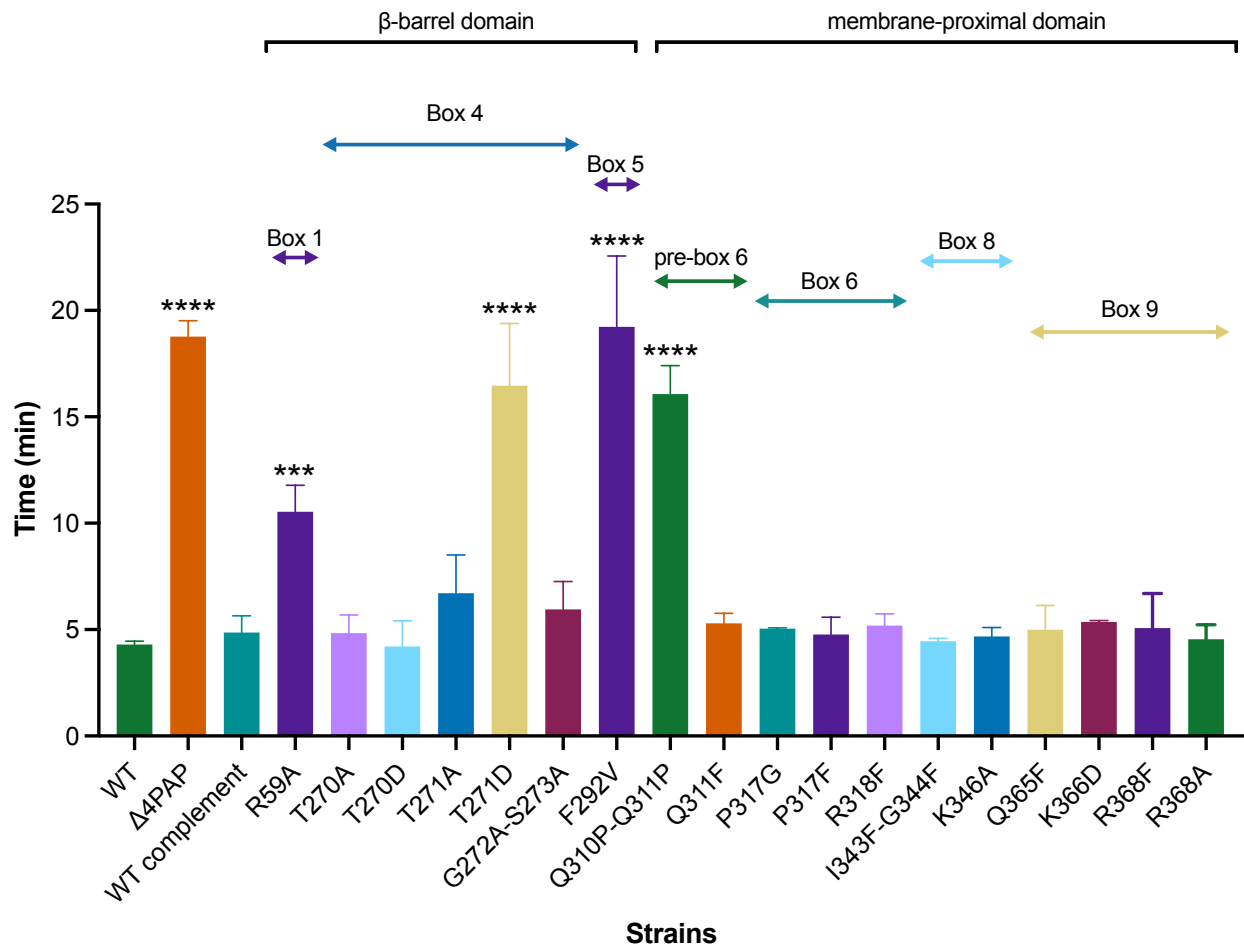
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172 **Figure 1.** Multiple sequence alignment of *Salmonella* AcrA and AcrE combined with
 173 the mapping of the secondary structure derived from the experimentally defined
 174 structure of *E. coli* AcrA (PDB 5V5S, chain G) (20). Identical residues are coloured red
 175 and similar residues are coloured yellow. The PAP-binding boxes implicated in RND-
 176 binding (22) are numbered 1 to 9 and depicted using blue rectangles. Figure created
 177 using Esript 3.0 (34).



178

179 **Figure 2. Efflux of ethidium bromide by the $\Delta 4PAP$ strain complemented with**
 180 **mutated versions of AcrA.** Data presented are the mean of three biological replicates
 181 and are shown as the time taken for the fluorescence to decrease by 25% +/- SD.
 182 Bacteria were treated with ethidium bromide and the proton-motive force dissipator
 183 CCCP for 1 hour and then re-energised with glucose. Annotation above indicates the
 184 mapping of each mutation to its binding box, as well as the domain mapping of
 185 respective boxes. Data were analysed by one-way ANOVA and compared to the WT
 186 complement using Dunnett's test. Significantly different strains are denoted with *** (P
 187 ≤ 0.001) or **** ($P \leq 0.0001$).

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190 **AcrE and AcrA share conserved binding boxes that are responsible for their**
191 **interoperability relative to AcrB**

192 The binding boxes between AcrA and AcrE were previously shown to be highly
193 conserved in *Salmonella* Typhimurium (Fig. 1), potentially explaining the observed
194 interchangeability between the two PAPs (22). To validate their functional role in AcrE,
195 SDM was used to mutate the residues, corresponding to the most critical binding box
196 residues previously identified in AcrA (22) - namely G57, R58, T270, F291, R293 and
197 G362 (Fig. 1). The effect of the mutations was assessed by ethidium bromide
198 accumulation assays and antimicrobial susceptibility testing in the Δ 4PAP Δ acrF strain
199 (22). This strain lacks all four RND-associated PAPs and the cognate RND-transporter
200 AcrF, thereby allowing the impact of AcrE mutations on AcrB-binding to be determined.
201 All mutations corresponding to the critical residues of AcrA (AcrE G57F, R58A, T270D,
202 F291G, R293F and G362F) also had a significant effect on efflux function and
203 antimicrobial susceptibility (Fig. 3 and Table S3). Consistent with this, the mutation of
204 phenotypically neutral residues in AcrA, corresponding to the AcrE T216F, K365D and
205 R367D respectively, also had no impact on efflux function (Fig. 3 and Table S3). The
206 observed effects of the mutations tested stemmed from their impact on the function of
207 the protein and were not due to changes in expression levels or stability of the variant
208 alleles, as validated by Western blotting, with a possible exception of G57F (Fig. S1B).

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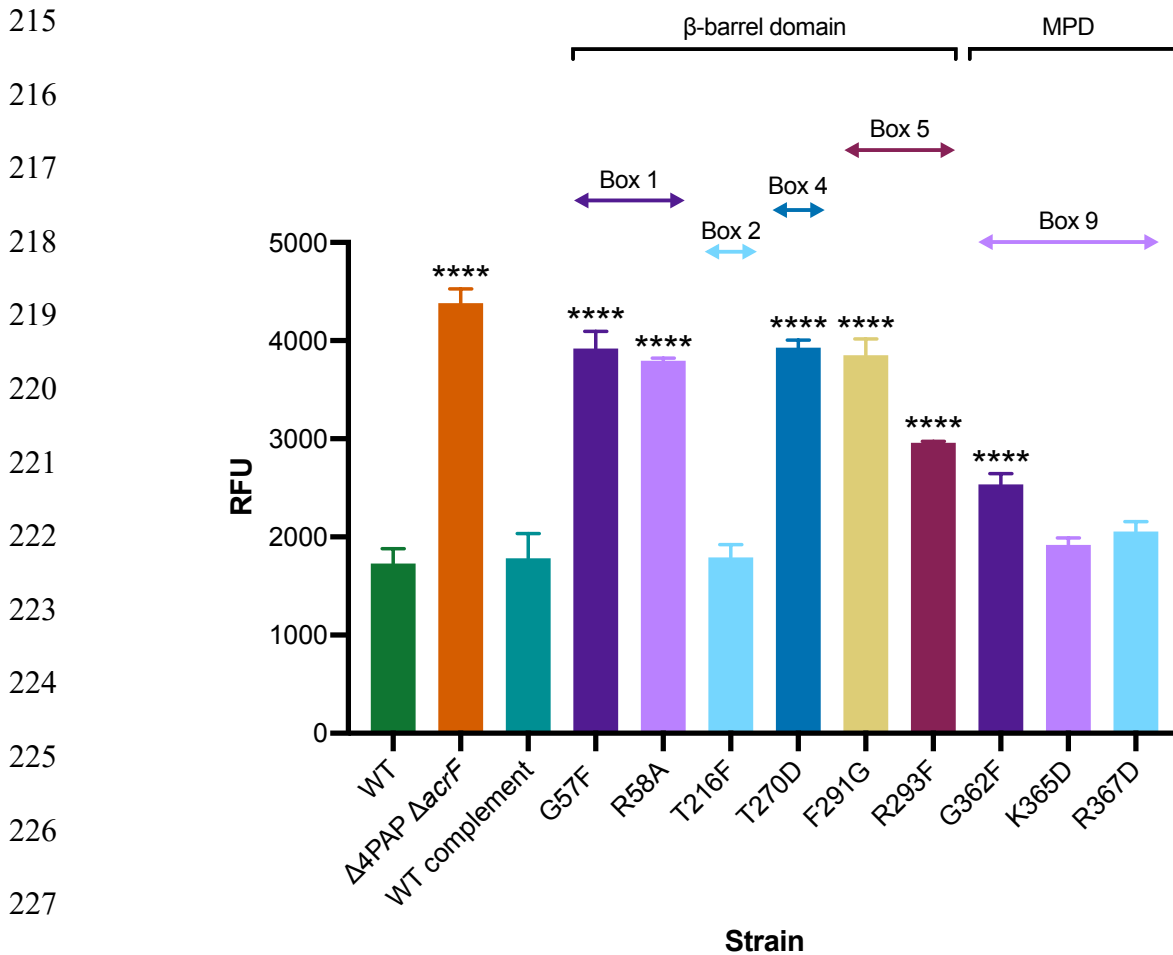
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229 **Figure 3. Accumulation of ethidium bromide in $\Delta 4\text{PAP } \Delta \text{acrF}$ strain**
230 **complemented with mutated versions of AcrE.** Data represented are the mean of
231 three biological replicates showing maximum RFU values after 30 min of ethidium
232 bromide exposure +/- SD. Annotation above indicates the mapping of each mutation
233 to its binding box, as well as the domain mapping of respective boxes. Data were
234 analysed by one-way ANOVA and compared to the WT complement strain using
235 Dunnett's test. Significantly different strains are indicated with **** ($P \leq 0.0001$). MPD,
236 membrane-proximal domain.

237

238 The above results confirm the conservation of function of the binding boxes between
239 AcrA and AcrE, which explains their interoperability in conjunction with AcrB.

240 **Potential role for the membrane-proximal domain of AcrA in vetting substrate**
241 **access to channel 1 and channel 2**

242 The refinement of the binding box model of PAP-RND interaction led to the discovery
243 of an AcrA mutant with a peculiar phenotype. The K366D AcrA mutant mapping to box
244 9 (Fig. 1), did not alter ethidium bromide efflux or susceptibility (Fig. 2), but showed a
245 distinct antimicrobial susceptibility profile to other antimicrobials tested (Table S2).
246 Notably, the K366D mutation in AcrA conferred differential effects depending on the
247 physicochemical properties of the compounds tested. The K366D mutant displayed
248 greater than two-fold reduction in MIC values to high-molecular-mass drugs (HMMDs),
249 such as doxorubicin, erythromycin, fusidic acid and novobiocin ($M > 500$ g/mol) and
250 low-molecular-mass drugs (LMMDs), such as chloramphenicol, clindamycin, linezolid,
251 and minocycline ($M < 500$ g/mol), compared to WT AcrA complement. However, the
252 K366D mutant had no impact on the MIC values for planar aromatic cations (PACs),
253 including acriflavine, berberine, benzalkonium chloride, crystal violet, ethidium
254 bromide, methylene blue and rhodamine 6G (Table 1).

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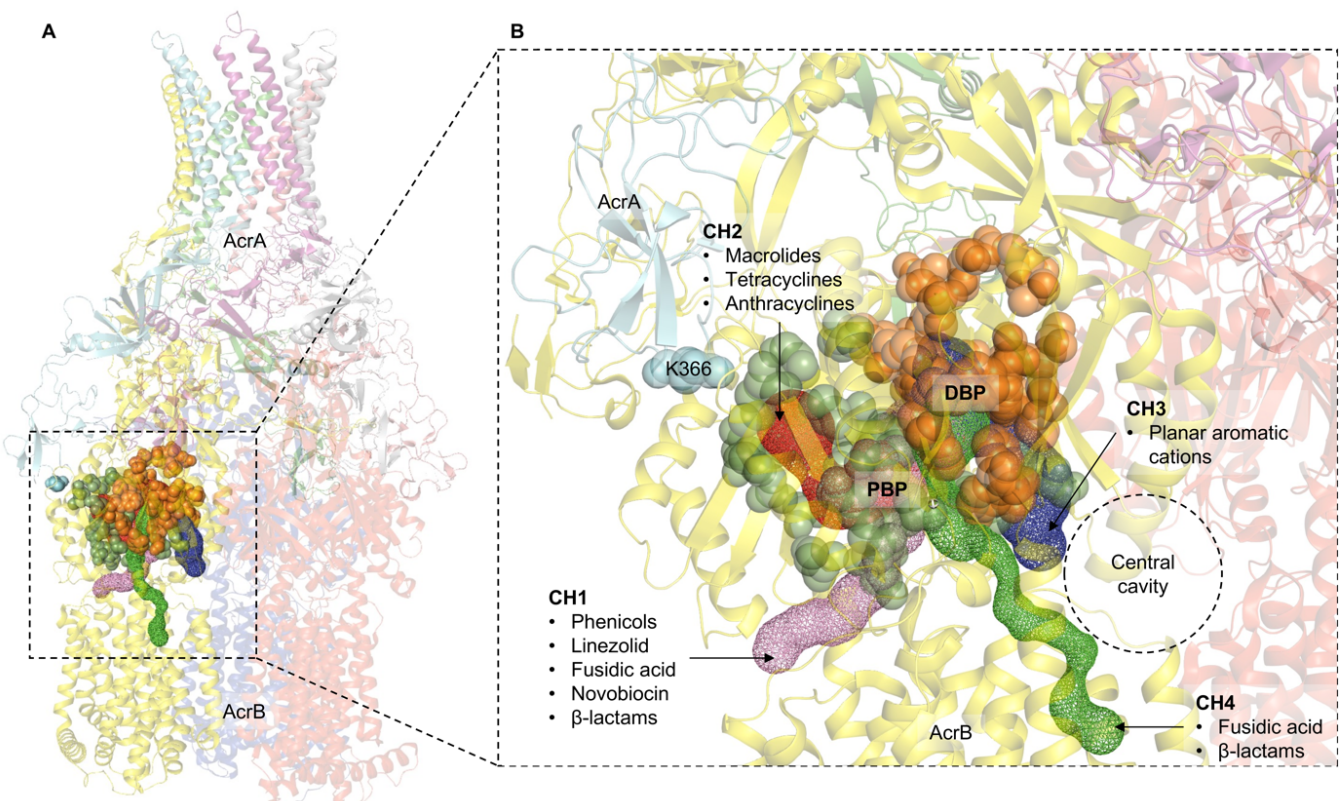
265 **Table 1.** Antimicrobial susceptibility of Δ 4PAP strain complemented with F292V,
266 Q311F or K366D AcrA to drugs with different physicochemical characteristics.

Strain	MIC (μ g/mL)														
	HMMDs				LMMDs				PACs						
	ERY	DOX	FA	NOV	CHL	CLI	MIN	LZD	ACR	BZK	BER	CV	EB	MB	R6G
WT	128	1024	1024	512	4	512	2	512	256	64	>1024	64	1024	1024	1024
Δ 4PAP	4	2	8	2	0.5	4	0.25	16	16	4	128	2	16	8	4
WT complement	<u>64</u>	<u>64</u>	<u>256</u>	<u>64</u>	<u>4</u>	<u>128</u>	<u>1</u>	<u>128</u>	<u>64</u>	<u>32</u>	<u>>1024</u>	<u>16</u>	<u>128</u>	<u>128</u>	<u>128</u>
F292V AcrA	4	2	8	2	0.5	4	0.25	16	16	4	128	2	16	8	4
Q311F AcrA	64	64	256	64	4	128	1	128	64	32	>1024	16	64	128	128
K366D AcrA	16	16	32	16	1	32	0.25	16	64	32	>1024	16	64	128	64

267 Underlined values highlight values for the Δ PAP strain complemented with wild-type
268 AcrA. Bold values are at least >2-fold different than the parent strain. HMMDs, high-
269 molecular-mass drugs; ERY, erythromycin; DOX, doxorubicin; FA, fusidic acid; NOV,
270 novobiocin; LMMDs, low-molecular-mass drugs; CHL, chloramphenicol; CLI,
271 clindamycin; MIN, minocycline; LZD, linezolid; PACs, planar aromatic cations; ACR,
272 acriflavine; BZK, benzalkonium chloride; BER, berberine; CV, crystal violet; EB,
273 ethidium bromide; MB, methylene blue; R6G, rhodamine 6G.

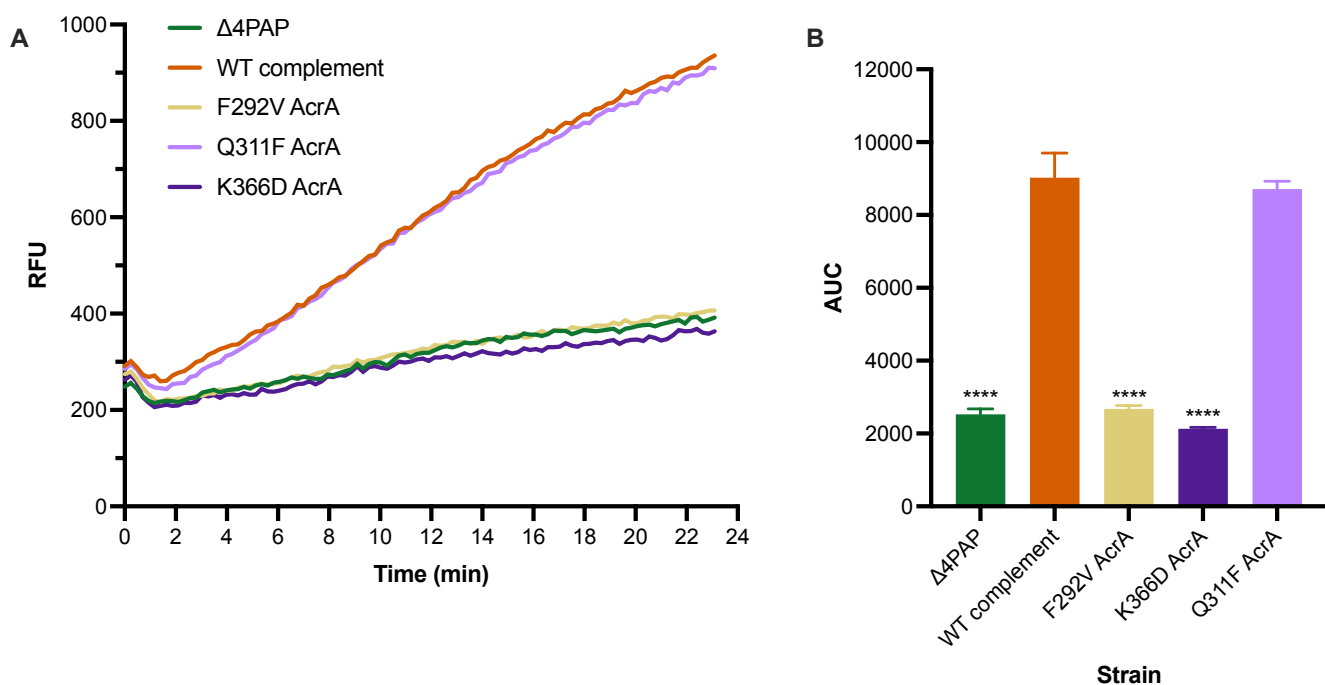
274
275 Previous studies have associated molecular weight of the substrate-drugs with the
276 preferred channel access and binding pocket validation by the RND-transporter (26-
277 28, 35). The RND-transporter AcrB has multiple substrate entry channels identified,
278 which are used by drugs depending on their physicochemical properties (26, 27, 36)
279 (Fig 4). LMMDs have been proposed to preferentially enter through channel 1 (CH1),
280 whilst HMMDs are thought to enter AcrB through channel 2 (CH2) (36-38). The cleft
281 entrance of CH2 has been previously suggested to be impacted by the membrane-
282 proximal domain (MPD) of AcrA (26). This is seen in cryo-EM structures of the AcrAB-

283 TolC complex, which show that AcrA interacts with the PC1 and PC2 subdomains of
284 AcrB (19, 39). PACs on the other hand, are preferentially taken up through channel 3
285 (CH3), which starts from the vestibule formed by the central cavity of the three AcrB
286 protomers and leads directly to the deep binding pocket (DBP) (26). The entrance of
287 the recently proposed channel 4 (CH4) is in the groove formed by TM1 and TM2 and
288 leads to the DBP (40). The location of CH3 and CH4 within AcrB suggests that the
289 MPD of AcrA should not have a direct steric impact on the drug access (Fig. 4).



290 **Figure 4. A.** The crystal structure of the trimeric AcrB transporter and the hexameric
291 AcrA assembly (TolC not shown for clarity). The different substrate entry pathways are
292 shown as coloured channels, and the binding pockets are indicated by coloured
293 spheres. **B.** Zoomed-in view of the substrate channels and the binding pockets relative
294 to K366 of AcrA. The green and orange spheres correspond to the space-fill
295 representation of the residues lining the proximal binding pocket (PBP) and the deep
296 binding pocket (DBP), respectively. K366 is in the membrane-proximal domain of AcrA
297 and impacts the residues lining the PBP and the entrance of channel 2 (CH2). Channel

298 1 (CH1) also feeds into the PBP, so is likely to be impacted by changes in K366.
299 Channel 3 (CH3) starts from the central cavity and leads to the DBP. Similarly, channel
300 4 (CH4) starts from the groove formed by TM1/TM2 and leads to the DBP. Therefore,
301 CH3 and CH4 are unlikely to be directly impacted by K366 substitutions.
302
303 Therefore, to understand how the K366D AcrA mutation affects the susceptibility of
304 the Δ 4PAP strain to HMMDs and LMMDs, but not to PACs, we designed more specific
305 antimicrobial sensitivity screens to be able to better differentiate the usage of the
306 access channels by respective substrates and the impact of the K366D mutation on
307 specific channels.



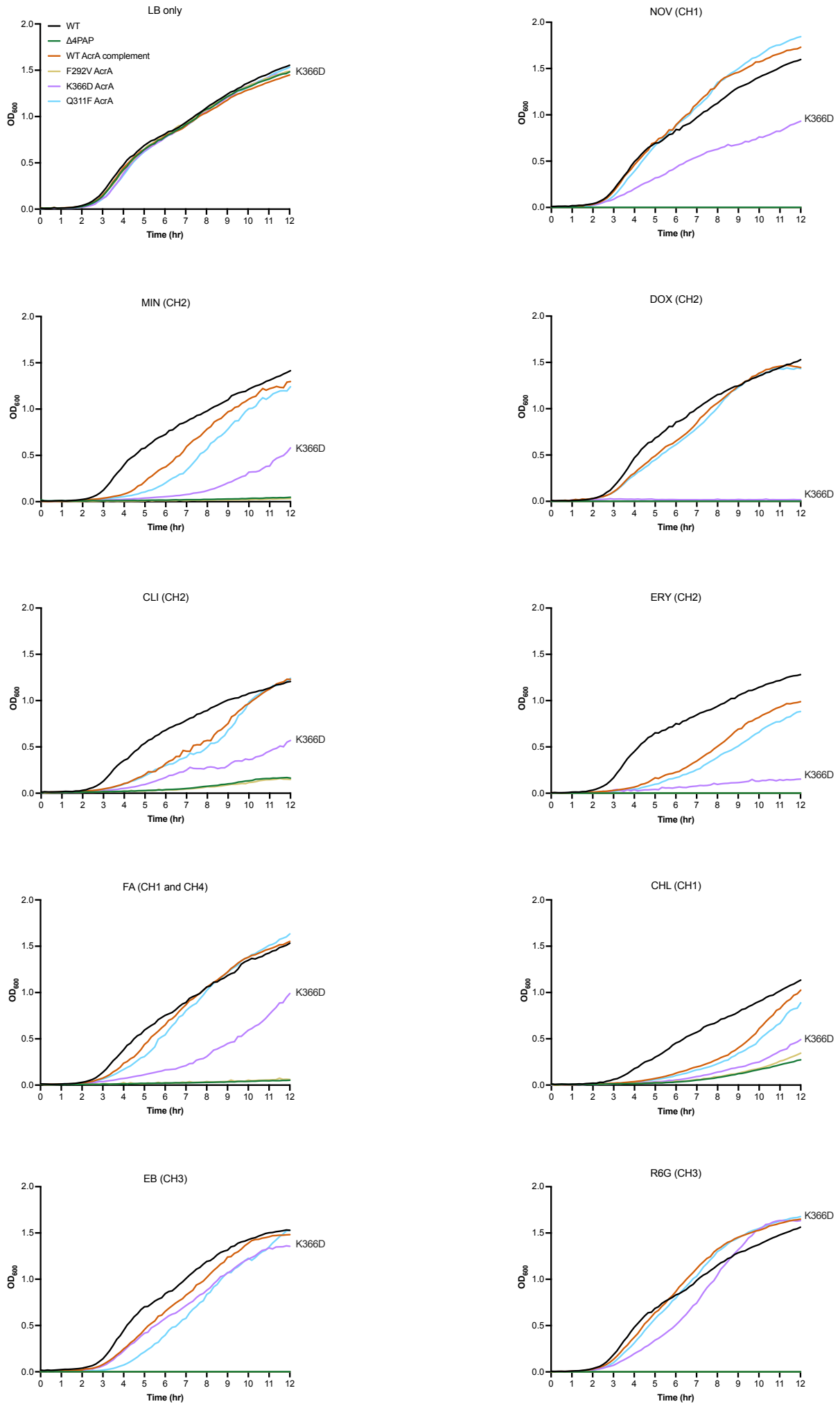
308 **Figure 5. A) Efflux of doxorubicin over time in Δ 4PAP strain complemented with**
309 **mutated versions of AcrA.** Bacteria were treated with doxorubicin and the proton-
310 motive force dissipater CCCP for one hour and then re-energised with glucose. Efflux
311 was monitored by increasing RFU due to extracellular doxorubicin. Data presented
312 are the mean of three biological replicates. **B) Area under curve (AUC) analysis for**
313 **doxorubicin efflux over time.** Data shown are the mean AUC of the three biological

314 replicates shown in panel A. Data were analysed by one-way ANOVA and compared
315 to the WT complement using Dunnett's test. Strains with a significantly different AUC
316 are indicated with **** ($P \leq 0.0001$).

317

318 To further clarify the impact of the K366D mutation on specific channels, we monitored
319 growth in the presence of efflux-substrates at $0.25 \times \text{MIC}$ for K366D AcrA, using the
320 efflux-impaired F292V and phenotypically neutral Q311F as controls. The growth
321 kinetics data showed that, compared to the WT complement, the K366D AcrA mutant
322 grew poorly or not at all in the presence of CH2 substrates doxorubicin, erythromycin,
323 minocycline, and clindamycin (Fig. 6). The K366D AcrA mutant also displayed growth
324 defects when grown in CH1 substrates, including novobiocin, fusidic acid and
325 chloramphenicol (Fig. 6). Notably, the K366D AcrA mutant did not have any
326 observable growth defects when grown in CH3 substrates, such as ethidium bromide
327 and rhodamine 6G (Fig. 6). This data shows disproportionate impact of the K366D on
328 substrates utilising CH1 and CH2 (27, 28), while substrates documented to utilise
329 CH3, such as ethidium bromide and rhodamine 6G (26) appear relatively unaffected.

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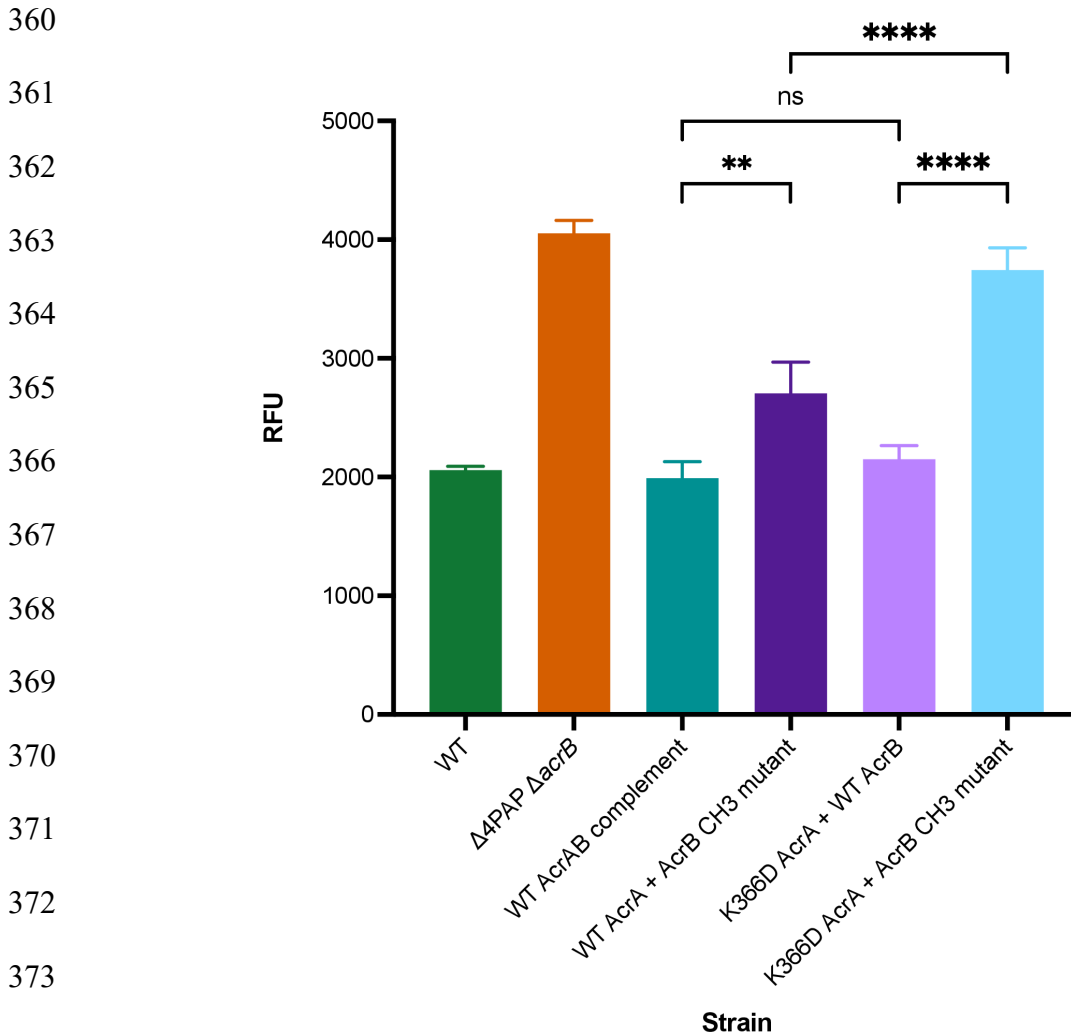
332 **Figure 6. Growth kinetics of Δ 4PAP strain complemented with mutated versions**
333 **of AcrA.** Abbreviations and concentrations of drugs used: CHL, 0.5 μ g/mL
334 chloramphenicol; CLI, 8 μ g/mL clindamycin; DOX, 4 μ g/mL doxorubicin; EB, 16 μ g/mL
335 ethidium bromide; ERY, 4 μ g/mL erythromycin; FA, 8 μ g/mL fusidic acid; MIN,
336 0.25 μ g/mL minocycline; NOV, 4 μ g/mL novobiocin; R6G, 16 μ g/mL rhodamine 6G.
337 Brackets indicate the preferred channel utilised by the substrate: CH1, channel 1;
338 CH2, channel 2; CH3, channel 3; CH4, channel 4. Data shown are the mean OD₆₀₀
339 values of three biological replicates. Concentrations of drugs are 0.25 \times MIC of K366D
340 AcrA.

341

342 **AcrB mutant with impacted channel 3 function supports the role of K366 in**
343 **ensuring productive efflux of channel 1 and channel 2 substrates**

344 The above data, combined with the location of the K366D mutation, strongly suggests
345 that it may affect substrates entering through CH1 and CH2, but not CH3. To further
346 increase assay sensitivity and avoid interference from substrates that use CH1-3
347 promiscuously, such as ethidium bromide, we used an AcrB mutant (A33W, T37W,
348 N298W AcrB) with impacted CH3 function (26), which allows for better separation of
349 efflux signal arising from CH1 and CH2. An AcrB CH3 mutant (A33W T37W N298W
350 AcrB), which was previously shown to impact the export of PACs (26). The AcrB CH3
351 mutant displayed an intermediate level of efflux impairment between that of Δ 4PAP
352 Δ acrB and the WT *acrAB* complement strain (Fig 7). Furthermore, the AcrB CH3
353 mutant displayed increased susceptibility to PACs (Table S4) and impaired growth in
354 the presence of 32 μ g/mL ethidium bromide (Fig. S2). Importantly, when present in
355 combination with the AcrB CH3 mutant, the K366D mutation abrogated ethidium
356 bromide efflux even further, to a level comparable to that of the Δ 4PAP Δ acrB strain

357 (Fig. 7). Antimicrobial susceptibility testing also showed that the K366D AcrA with the
358 AcrB CH3 mutation displayed increased susceptibility to substrates compared to the
359 K366D AcrA or AcrB CH3 mutants alone (Table S4).



375 **Figure 7. Accumulation of ethidium bromide in Δ4PAP ΔacrB strain**
376 **complemented with K366D AcrA and the AcrB CH3 mutant (A33W T37W N298W**
377 **AcrB).** Data shown are the mean of three biological replicates showing maximum RFU
378 values after 30 minutes of ethidium bromide exposure. Data were analysed by one-
379 way ANOVA and corrected for multiple comparisons using Tukey's test. Significantly
380 different strains are indicated with ** ($P \leq 0.01$) or **** ($P \leq 0.0001$). ns, not significant.

381

382 Next, the ability of the double mutant (K366D AcrA + AcrB CH3 mutant) to export
383 doxorubicin was measured. The WT AcrA combined with the AcrB CH3 mutations
384 showed a similar level of doxorubicin efflux as the WT AcrAB complement, whilst the
385 K366D AcrA mutation with WT AcrB strain displayed impaired doxorubicin efflux. The
386 double mutant showed complete impairment of doxorubicin efflux, like that of the
387 $\Delta 4\text{PAP } \Delta \text{acrB}$ strain (Fig. 8). The doxorubicin efflux assay results were further
388 validated by growing the K366D AcrA and the AcrB CH3 mutant strains in the presence
389 of doxorubicin. The double mutant failed to grow in the presence of 2 $\mu\text{g/mL}$
390 doxorubicin like the $\Delta 4\text{PAP } \Delta \text{acrB}$ strain. The K366D AcrA mutant with WT AcrB
391 displayed impaired growth in the presence of 8 $\mu\text{g/mL}$ doxorubicin, whilst the AcrB
392 CH3 mutant with WT AcrA had no observable growth defect (Fig. S3). The
393 concentration dependent effect of doxorubicin growth inhibition is consistent with the
394 blockage of CH2, and gradual saturation of CH1, the function of which is partially
395 impacted by K366D mutation. Western blotting verified that the phenotypic effects of
396 the AcrB CH3 mutation were not due to changes in protein expression or stability (Fig.
397 S1C). In summary, the AcrB CH3 disruption has a clearly pronounced additive effect
398 compared to K366D acting on its own, consistent with the role of the MPD of the PAP
399 in the control of CH1 and CH2 substrates. These data further support the essential
400 role that K366, and the MPD in general play in the transport of CH1 and CH2
401 substrates.

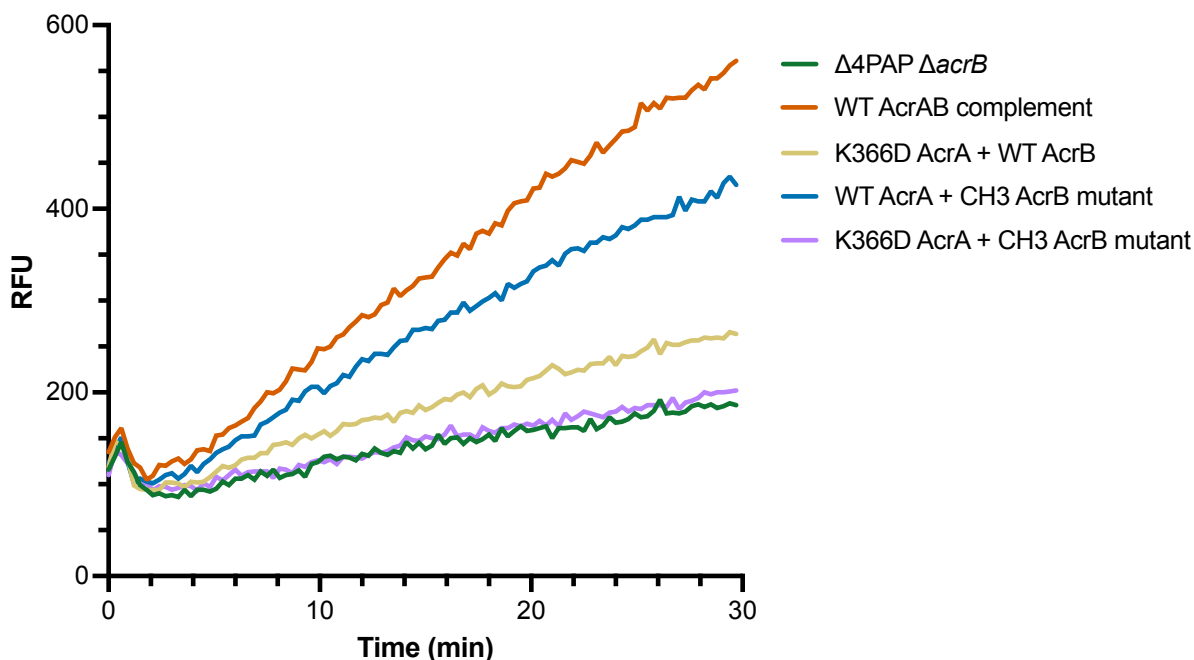
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408 **Figure 8. The effect of the K366D AcrA and the AcrB channel 3 (CH3) mutation**

409 **on doxorubicin export** Efflux of doxorubicin over time in $\Delta 4\text{PAP } \Delta\text{acrB}$ strain

410 complemented with K366D AcrA and the AcrB channel 3 (CH3) mutant. Bacteria were

411 treated with doxorubicin and the proton-motive force dissipater CCCP for 1 hour and

412 then re-energised with glucose. Efflux was monitored by increasing RFU due to

413 extracellular doxorubicin. Data presented are the mean of three biological replicates.

414 AcrB CH3 mutant refers to A33W T37W N298W AcrB.

415

416 **K366D also impacts channel 1 substrate transport**

417 Like chloramphenicol, linezolid also uses CH1 (28) and consistent with the

418 interpretation that K366D impacts efflux through this channel, we observed a similar

419 result for linezolid, with clearly pronounced concentration-dependent effect, which is

420 most pronounced at 8 $\mu\text{g/mL}$ and above (Fig. S4). This data suggests that K366 is

421 also somehow involved in either active substrate vetting or surveillance of the

422 substrate-bound state of the transporter, as discussed in detail in the section below.

423 Discussion

424 In this study, we set out to refine the previously reported “binding box” model of PAP-
425 RND interaction (22) by generating and characterising additional subtle and more
426 conservative PAP mutations. Consistent with the model’s prediction, we report that the
427 AcrE residues that correspond to the previously identified critical residues in AcrA
428 conserve their functional significance, as evidenced by targeted mutagenesis.
429 Furthermore, we have been able to refine the effect of the previous, rather blunt
430 mutations by separating previously reported double mutant AcrA T270F-T271F into
431 AcrA T270A, T270D, T271A and T271D, which enabled us to narrow down the T271
432 as a crucial residue for efflux function. Likewise, the function of F292 also seems to
433 be critical for efflux function, with even subtle mutations (AcrA F292V) resulting in
434 complete destabilisation of the AcrAB-ToIC assembly. The R59A mutant, which is in
435 proximity to G58, resulted in intermediate impairment of efflux function, validating the
436 previously reported role for box 1, and the PAP β -barrel domain in functional tripartite
437 pump assembly.

438

439 During the refinement of the binding box 9, which belongs to the membrane-proximal
440 domain of the PAP, we identified an AcrA mutant (K366D) with a peculiar phenotype.
441 The K366D mutant had no impact on ethidium bromide efflux or susceptibility to PACs.
442 However, it had significantly increased susceptibility to HMMDs and LMMDs
443 compared to WT complement. Structural analysis of the available RND tripartite
444 assemblies (20, 29, 30) indicated that K366 is in proximity to the proposed entry of the
445 CH2 (Fig. 4). We hypothesised that if this were indeed the case, as substrates and
446 drugs exhibit clear channel access preferences, K366D will disproportionately affect
447 substrates using CH1 and CH2, but not CH3 or CH4. Consistent with this prediction,

448 we observed that the K366D mutant exported doxorubicin, a CH2-substrate, very
449 poorly. Furthermore, the K366D mutant displayed growth defects in the presence of
450 several additional CH1-substrates (chloramphenicol, fusidic acid, and linezolid), as
451 well as CH2-substrates (doxorubicin, erythromycin, and minocycline), but not CH3-
452 substrates (ethidium bromide and rhodamine 6G).

453

454 Notably, the disruption of CH3 in AcrB had a clearly pronounced additive effect on the
455 AcrA K366D mutant acting in a WT AcrB background, consistent with the role of the
456 PAP MPD in the control of CH1 and CH2 access to the respective substrates.
457 Intriguingly, in addition to the straightforward effect of K366D on the CH2 entry, the
458 linezolid data presented here suggests that there is also a measurable impact on the
459 CH1-substrates. This necessarily requires some level of allosteric communication,
460 because K366 is too far from the proposed entrance of CH1 (26, 27, 41).

461

462 At this stage, the available data doesn't provide a definitive answer as to how the MPD
463 of the PAP may impact on the apparent substrate preference and selection. However,
464 the analysis of the available PAP-RND complex structures (20, 29, 30) (Fig. 4),
465 provides hints to the possible mechanism of the K366 action. One straightforward
466 possibility, based on the location of K366 near the suggested entry of CH2 (26), and
467 the flexibility of its side-chain is that it may affect CH2 substrate access and kinetics
468 by playing the role of a "cap" on the tunnel entrance, possibly sensing, and even
469 partially coordinating the incoming substrate. However, the effect of K366D also
470 extends to CH1-substrates, such as linezolid and chloramphenicol, while K366 is
471 located too far away from the suggested CH1 entry points to be directly involved in
472 any active substrate vetting. Thus, it is tempting to suggest that the K366, and the

473 MPD as a whole may be involved in a more generalised sensing of the substrate
474 occupied state of the PBP (which is the convergence of CH1 and CH2) (27, 41), and/or
475 the potential propagating of the “substrate-occupied” signal upwards *via*
476 conformational change in the PAP leading to TolC engagement and channel opening
477 as previously suggested (8). Strikingly, this interpretation is directly supported by the
478 very recent *in situ* cryo-electron tomography structure of the assembled AcrAB-TolC
479 pump (42), which displays strong and differential association of the MPDs of PAP
480 protomers I and II with the PC1 and PN1/PC2 domains of AcrB respectively, with the
481 latter in particular suggested to be associated with sensing the MBX3132 drug-
482 occupied state of the transporter, and providing a conformational signal to TolC,
483 affecting its channel gating. Additionally, for the first time, the *in-situ* structure also
484 unambiguously identifies the location of the C-terminal helices and the membrane-
485 associated N-terminal tails of AcrA (42), that appear to occupy a crevasse on the AcrB-
486 surface that may also plausibly account for CH1 effects reported here, possibly
487 providing additional sensory/allosteric input. Such sensory input may be allosterically
488 conveyed to engagement of the OMF partner protein during the initial assembly of the
489 tripartite complex, or possibly provide directionality of the L-T-O transition during the
490 efflux cycle, which is compatible with the mechanistic model of RND pump cycling
491 suggested recently (8).

492

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497

498 **Materials and methods**

499 **Bacterial strains and growth conditions**

500 All strains were derived from *Salmonella enterica* serovar Typhimurium strain SL1344
501 (henceforth referred to as *S. Typhimurium*), a pathogenic strain first isolated from an
502 experimentally infected calf (43). All strains were grown in LB broth at 37°C with
503 aeration.

504

505 **Growth kinetic assays**

506 Overnight cultures (~10⁹ cfu/mL) of test strains were diluted to a starting inoculum of
507 10⁶ cfu/mL in a 96-well plate. Where appropriate, the test strains were diluted in LB
508 broth supplemented with antibiotics. Growth was monitored over 12 hr in a FLUOstar
509 OMEGA plate reader (BMG Labtech, Germany).

510

511 **Site directed mutagenesis**

512 Mutations in *AcrA* was generated using the plasmid *pacrA* (pET-20b (+) carrying the
513 *acrA* gene from *S. Typhimurium* SL1334 with C-terminal 6xHis-tag). Mutations in both
514 *AcrA* and *AcrB* was generated using the plasmid *pacrAB* (pET-20b (+) carrying the
515 *acrAB* operon from *S. Typhimurium* SL1334 with a C-terminal 6xHis-tag). Mutations in
516 *AcrE* were generated using the plasmid *pacrE* (pTrcHis2-TOPO carrying the *acrE*
517 gene from *S. Typhimurium* SL1334 with a C-terminal 6xHis-tag). All site-directed
518 mutagenesis (SDM) reactions were carried out using the QuikChange Lightning SDM
519 Kit (Agilent, USA). The mutations were verified by sequencing (Eurofins Genomics,
520 UK). Primers used for all the SDM reactions are listed in Table S1.

521

522 **Ethidium bromide accumulation and efflux assay**

523 The efflux activity of strains was assessed by measuring ethidium bromide
524 accumulation and efflux as previously described (44).

525

526 **Doxorubicin efflux assay**

527 Doxorubicin efflux was measured in a similar manner to ethidium bromide efflux, with
528 some changes. Cells were grown to an OD₆₀₀ of 0.6 and washed with efflux buffer (20
529 mM potassium phosphate buffer with 5 mM magnesium chloride) three times. Carbonyl
530 cyanide *m*-chlorophenylhydrazone (CCCP) and doxorubicin were added at a final
531 concentration of 100 µM and 20 µM, respectively. Cells were incubated at 37°C with
532 aeration for 1 hour. Following incubation, cells were washed with efflux buffer three
533 times. Cells were energised with 25 mM glucose and doxorubicin efflux was measured
534 over 30 min at excitation and emission wavelengths of 485 and 620-10 nm,
535 respectively.

536

537 **Antimicrobial susceptibility**

538 The agar doubling dilution method was used to determine the minimum inhibitory
539 concentrations (MICs) of various antimicrobials and dyes according to Clinical and
540 Laboratory Standards Institute guidance (45). All MICs were repeated at least three
541 times.

542

543 **Western blotting**

544 Wild-type and mutant AcrA were expressed in SL1344 Δ4PAP from *pacrA* plasmids.
545 Wild-type and mutant AcrE were expressed in SL1344 Δ4PAP Δ*acrF* from *pacrE*
546 plasmids. Wild-type and mutant AcrB were expressed in SL1344 Δ4PAP Δ*acrB* from
547 *pacrAB* plasmids. Cultures were grown to an OD₆₀₀ of 0.4 without induction. Cells were

548 harvested and lysed in 10 mM Tris-HCl, 1 mM disodium EDTA, pH 8.0, supplemented
549 with complete EDTA-Free Protease Inhibitor tablets (Roche, Switzerland) and
550 100 µg/mL lysozyme using sonication. Membrane fractions were harvested, separated
551 using a 12% SDS-PAGE gel for AcrA and AcrE and 8% SDS-PAGE gel for AcrB, and
552 transferred to a PVDF membrane. The His-tagged proteins were blotted using anti-6x
553 His tag HRP-conjugated monoclonal antibody (Invitrogen, USA) and detected using
554 Clarity Western ECL Substrate (Bio-Rad, USA) on an Amersham 680 Imager (Cytiva,
555 USA).

556

557 **Molecular visualisation of substrate channels**

558 The location of the substrate channels 1-3 within the RND-transporter AcrB were
559 calculated using CAVER software v3.0 (46) as described previously (26). For
560 visualisation of the recently reported channel 4, we used the CAVER-output kindly
561 provided by K. M. Pos (personal communication), as reported in Tam et al. (28).
562 PyMOL (Molecular Graphics System, Version 2.0 Schrödinger, LLC.) was used for 3D
563 rendering of molecular structures and the substrate channels discussed.

564

565 **Statistical analysis**

566 Experiments were carried out at least three times on separate occasions. Data shown
567 are the mean of at least three biological replicates, and where shown, error bars
568 indicate standard deviations. All statistical comparisons were performed using one-
569 way ANOVA with multiple comparisons in GraphPad Prism 9.2 software (GraphPad
570 Software LLC).

571

572

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578

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