AedG, a new weapon in *Acinetobacter's* fight against antibiotics

Varsha Naidu[a,b], Bhumika Shah[b,c], Claire Maher[a,b], Ian T. Paulsen[b,c], Karl A. Hassan[a,b*]

Affiliations

[a]School of Environmental and Life Sciences, University of Newcastle, Callaghan, NSW 2308, Australia

[b]ARC Centre of Excellence in Synthetic Biology, Macquarie University, North Ryde, NSW, Australia

[c]School of Natural Sciences, Faculty of Science and Engineering, Macquarie University, North Ryde, NSW, Australia

***Corresponding Author**

School of Environmental and Life Sciences, University of Newcastle, Callaghan, NSW, Australia. Phone: +6124921 7236. Email: karl.hassan@newcastle.edu.au

18 **Abstract**

19 A novel multidrug efflux pump, AdeG from the Drug:H^+ antiporter 2 family was discovered in
20 *Acinetobacter* multiresistance plasmids. Here, we profiled the antimicrobial resistance
21 potential and examined the distribution of this gene. Putative homologs of this novel efflux
22 pump were found in many *Acinetobacter* species and other Gram-negative species. The
23 pump conferred resistance to at least eight diverse antimicrobials. These results show that
24 the novel pump is a new determinant in the *Acinetobacter* resistance arsenal.

25

26 **Introduction**

27 The success of pathogenic *Acinetobacter* species in clinical settings is at least partly due to
28 their drug resistance capabilities. Multidrug efflux pumps are significant contributors of
29 resistance in *Acinetobacter*, as a single pump will frequently confer resistance to a broad
30 range of antimicrobials. All *Acinetobacter* species encode efflux pumps that may confer
31 resistance to antibiotics (1). Many of these pumps are found in the core genomes of the
32 species, if not the genus, suggesting that they have been vertically inherited in these
33 lineages for a considerable time (1, 2, 3). Others appear to be associated with mobile
34 genetic elements, such as plasmids and transposons and have a scattered distribution in
35 sub-sets of phylogenetically distant strains, suggesting horizontal transfer between strains
36 (4, 5). For example, some *Acinetobacter* strains encode the tetracycline efflux pump, TetB,
37 that was originally characterised in *Escherichia coli* on the transposon Tn*10* and is found in
38 other Gram-negative species (6, 7).

39 We recently identified genes encoding a putative multidrug efflux pump from the Drug:H^+
40 antiporter 2 (DHA2) family of the Major Facilitator Superfamily (MFS) of transport proteins
41 in several *Acinetobacter* plasmids (8). Here we demonstrate that the novel DHA2 family
42 efflux pump can mediate resistance to a range of antimicrobials, and genes encoding
43 homologs of the pump are widespread, but always associated with an *adeAB(C)* locus in
44 *Acinetobacter* species. We call the novel efflux pump AedG, as *Acinetobacter* efflux pump
45 from the DHA2 family member G (9).

46

47

48

49 **Methods**

50 **Bacterial growth conditions, Cloning, PCR and Complementation.**

51 Bacterial strains were grown at 37 °C with shaking (200 rpm) in Mueller Hinton (MH) broth
52 or LB broth or on LB plates supplemented with 100 mg/L ampicillin, and IPTG where
53 necessary (see below). The coding region of *aedG* together with an RGSH6 epitope tag at its
54 3 end was synthesised and cloned into the pTTQ18 plasmid (10), followed by next-
55 generation sequence verification by Twist Bioscience. The recombinant plasmid carring the
56 *amvA* gene (pTTQ18$_{amvA}$) was obtained from our previous work (9). The empty plasmid
57 (pTTQ18) and recombinant plasmids (pTTQ18$_{aedG}$ and pTTQ18$_{amvA}$) were transformed into
58 chemically competent *E. coli* BL21 cells. Transformants were selected on LB plates
59 containing 100 mg/L ampicillin and colony PCR was used to confirm positive insertion of
60 plasmids.

61

62 **Antimicrobial susceptibility testing**

63 Antimicrobial susceptibility assays were conducted in MH broth supplemented with 0.05
64 mM IPTG using the broth microdilution method as described (11). Assays for each
65 antimicrobial was performed with three biological replicates and four technical replicates,
66 resulting in twelve replicates in total. Cell growth was measured spectrophotometrically
67 using the PHERAstar FSX (BMG LABTECH, Germany).

68

69 **Whole Cell transport assay**

70 *E. coli* BL21 cells carrying either pTTQ18 (empty vector), pTTQ18$_{aedG}$, or pTTQ18$_{amvA}$ were
71 grown in replicates of three in LB broth supplemented with 100 mg/L ampicillin to OD_{600} =
72 0.6, followed by induction of gene expression with IPTG (0.2 mM) for 1h. The cells were

73 washed three times in transport assay buffer (20 mM HEPES-NaOH (pH 7), 145 mM NaCl, 5
74 mM KCl) and resuspended in the same buffer to an OD_{600} = 1. Acriflavine (2 μM) or
75 ethidium (10 μM) and CCCP (10 μM) were added, and after 30 minutes incubation at 37 °C,
76 the cells were washed three times in transport assay buffer and resuspended in the same
77 buffer. Transport reaction was initiated at 37 °C by adding 1% glucose and a change in
78 fluorescence was measured using the PTI QuantaMaster 8000 (HORIBA Scientific) with an
79 excitation and emission wavelengths of 530 nm and 610 nm, respectively.

80

81 **Distribution of AdeG and its phylogenetic relationship to other DHA2 family proteins**

82 The *adeG* loci from *Acinetobacter* plasmids and their surrounding genes were aligned and
83 visualised using clicker v0.0.25 (12). The amino acid sequences of AedG homologs from
84 different lineages were aligned to the sequences of other representative DHA2 family
85 proteins using MUSCLE (13), and phylogeny inferred using MrBayes v3.2.6 (14).

86

87 **Results and discussion**

88 <u>AedG homologs are distributed widely across the *Acinetobacter* genus</u>

89 Genes encoding AedG were previously identified on plasmids carried by *A. baumannii, A.*
90 *bereziniae, A. defluvii, A. johnsonii, A. nosocomialis, A. seifertii, A. ursingii* and *Acinetobacter*
91 strains with no species designation (8). These genes were nearly identical, as only one
92 nucleotide difference was present between sub-sets of genes, producing proteins with
93 either a tyrosine or histidine at the predicted cytoplasmic boundary of TM helix 13 in the
94 proteins expressed. The genes were each located downstream of a gene cluster comprised

95 of *adeABC* and *adeRS* (Figure 1A), which encode a tripartite RND efflux system, AdeABC, and
96 its two-component transcriptional regulator, AdeRS (refs). The 3' ends of *adeC* in the *aedG*
97 linked clusters were formed by an inverted repeat sequence that was also found adjacent to
98 a nearby IS*18* (Figure 1A) (8). The locations of these repeat sequences suggested that the
99 intervening sequence, comprised of the IS*18* transposase gene, *adeRS* and *adeABC*, may be
100 mobilised by the IS*18* transposase. These loci also carried a partial *adeS* gene (3' end)
101 downstream of the *aedG* homologs, that could be related to an alternative organisation of
102 these genes in an ancestral locus (Figure 1A).

103 We sought to find additional homologs of *aedG* and determine whether they are also
104 associated with IS*18*/*adeRS*/*adeABC*. We initially queried the NCBI nr/nt database using
105 tblastn with the AedG sequence encoded in *A. nosocomialis* AC1530 plasmid pAC1530 and
106 found close to 300 high scoring hits from strains designated in 23 *Acinetobacter* species and
107 *Acinetobacter* strains with no species designation. Surveys of the local genetic environments
108 of the high scoring hits showed one of two general arrangements. Either *aedG* was located
109 downstream of the IS*18*/*adeRS*/*adeABC* cluster, as in pAC1530, or *aedG* was located
110 between homologs of *adeAB* and *adeRS* gene pairs and *adeC* and the IS*18* transposase gene
111 were absent, as in *A. baumannii* ABF9692 plasmid pABF9692 (Figure 1B). It is not unusual
112 for *adeC* to be absent from *adeAB* loci in *Acinetobacter* genomes, and AdeAB can function
113 with AdeK as an alternative outer membrane protein (8, 15). Insertion elements were also
114 seen in some of the loci, such as that in *A. lwoffii* strain AL_065 pAL_065-2, where insertion
115 sequence elements appear to have disrupted *adeS* (Figure 1B).

116 To identify additional homologs of AedG, we queried the NCBI experimental clustered
117 protein database using the amino acid sequence of AedG encoded by pAC1530. This search

118 identified 69 clusters whose representative members had above 65 % amino acid sequence
119 identity with the query AedG sequence across at least 95 % of its length. Proteins in the top
120 scoring cluster were encoded by *Acinetobacter* species. The local genetic environments of
121 these homologs resembled those of *aedG* in pAC1530 or pABF9692. Other high scoring
122 clusters included *Acinetobacter* proteins encoded by *aedG* homologs in an alternative
123 genetic environment. For example, the genetic environment of *aedG* homologs in the
124 *Candidatus* Acinetobacter avistercoris isolate 5402 metagenome assembled genome (MAG)
125 and an *A. bereziniae* GD03393 plasmid resembled that in pABF9692 but included *adeC*
126 (Figures 1B and S1). Of note, the *A. bereziniae* GD03393 plasmid harboured a second *aedG*
127 homolog similar to pAC1530 (Figure S1).

128 The proteins in lower scoring clusters were encoded by species outside the *Acinetobacter*
129 genus. The genes encoding these proteins were frequently adjacent to divergently
130 transcribed *tetR* family regulator genes that may control expression of the *aedG* homologs.
131 Some non-*Acinetobacter* aedG homologs were adjacent to RND efflux pump genes. For
132 example, the *aedG* homolog in the MAG of Proteobacteria bacterium isolate CTSoil_107
133 (likely *Pleomorphomonas* based on GyrB and RpoD sequence similarity) was adjacent to
134 distant homologs of *adeRS*/*adeAB* (Figure S2), and the *aedG* homolog from *Pelagibacterium*
135 sp. SCN 63-126 ABS74_C0016 was encoded adjacently to a novel RND efflux system (Figure
136 1B). These results demonstrate a tendency for genes encoding AedG to be co-localised with
137 those encoding RND efflux systems, specifically AdeAB(C) in *Acinetobacter* species. AedG
138 and AdeAB(C) may cooperate in the efflux of substrates from the cytoplasm to outside the
139 cell.

140 A phylogenetic analysis was performed to examine the relationships of AedG homologs with
141 other members of the DHA2 family. This analysis showed that AedG homologs are most
142 closely related to the AmvA (also known as AedF) (9, 16) and SmvA (17) efflux pumps from
143 *Acinetobacter* and *Salmonella*, respectively (Figure 1C). Pairwise amino acid sequence
144 similarity scores confirmed the relatedness of AedG homologs and AmvA/SmvA (Figure S3).

145 AedG confers resistance to multiple antibiotics, biocides and dyes

146 Based on the proximity of *aedG* genes to homologs of *adeAB(C)* in *Acinetobacter* species
147 and the similarity of AedG variants to drug transporters in the DHA2 family, it seemed likely
148 that AedG pumps would mediate resistance to one or more antimicrobials. We profiled the
149 antimicrobial resistance potential of the AdeG pump encoded in pAC1530 to eight
150 antimicrobials and compared resistance levels to those conferred by the multidrug efflux
151 pump AmvA in the same expression system (9, 16). The *aedG* homolog from pAC1530 was
152 synthesised and cloned into the pTTQ18 expression plasmid (Amp^r) behind the *tac* promoter
153 (10) with a C-terminal RGSH6 tag and sequence verified (Twist Bioscience). The *amvA* gene
154 from *A. baumannii* ATCC 17978 (also known as AedF) was cloned previously (9). AedG
155 conferred increased resistance to all eight antimicrobials tested when expressed in *E. coli*
156 BL21 cells and promoted higher levels of resistance to tetracycline, DAPI, ethanol and
157 ethidium bromide than AmvA (Figure 2A). These results suggest that AedG can recognise a
158 broad spectrum of antimicrobials similar to AmvA and is a new multidrug resistance protein
159 in *Acinetobacter*.

160 AedG functions via an active efflux mechanism

161 The ability of AedG to confer resistance to the nucleic acid intercalating dye ethidium
162 bromide allowed us to test efflux using real-time fluorometric transport assays. *E. coli* BL21

163 cells expressing AedG, AmvA or no additional protein (negative control) were loaded with 10
164 µM ethidium bromide in the absence of energy. The cells were washed, and re-energised
165 and ethidium efflux monitored by fluorescence changes. Both AmvA and AedG promoted
166 the active export of efflux ethidium bromide, as seen in fluorescence decrease over time
167 relative to control cells (Figure 2B). This result confirms efflux as the mechanism of
168 resistance mediated by AedG and that AedG is a new multidrug efflux pump.

169 **Conclusions**

170 The genes encoding AedG are part of the *Acinetobacter* accessory genome. This is in
171 contrast to AmvA, its closest relative, which is encoded in the core genome of *A. baumannii*
172 and many other *Acinetobacter* species. AmvA expression is not readily responsive to
173 antimicrobial treatment, and recent work showed that it may have a physiological function
174 in polyamine efflux (18). The organisation of *aedG* genes in *Acinetobacter*, downstream of
175 *adeAB(C)* and in proximity to *adeRS* homologs (Figures 1A and 1B), suggests that these
176 genes may be controlled by AdeRS and co-expressed with *adeAB(C)*. As such AedG
177 homologs may play a more significant role in antimicrobial resistance than AmvA in isolates
178 that carry them.

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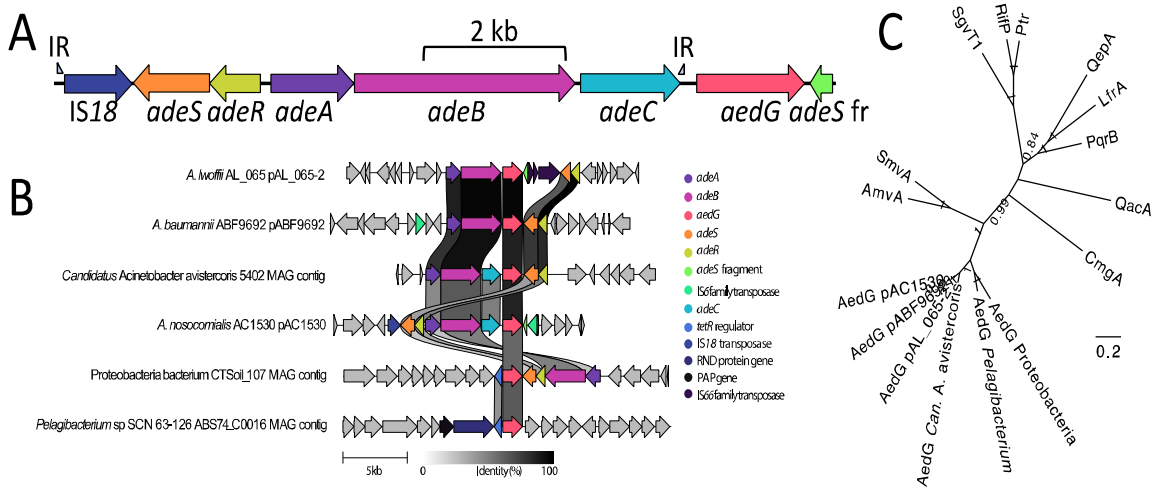

180

181 Figure 1. (A) Organisation of the local genetic environment of the *aedG* homolog in *A.*
182 *nosocomialis* AC1530 plasmid pAC1530 and related plasmids, including the locations of
183 surrounding genes and inverted repeat (IR) sequences that flank the IS*18* transposase gene
184 and *adeC*. (B) Alignment of loci carrying an *aedG* homolog in selected bacterial isolates.
185 Genes highlighted by colour and labels are found in more than one of the loci shown, or are
186 features noted in the text. This figure was made using clinker v0.0.25 (12). (C) The amino
187 acid sequences of AedG homologs from the lineages represented in (B) were aligned to the
188 sequences of other representative DHA2 family proteins using MUSCLE (13), and phylogeny
189 inferred using MrBayes v3.2.6 (14). The node labels show posterior probability values.

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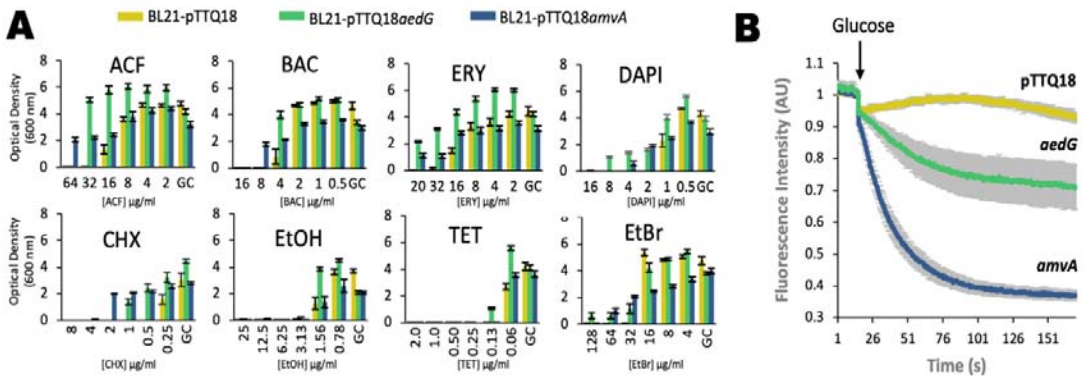

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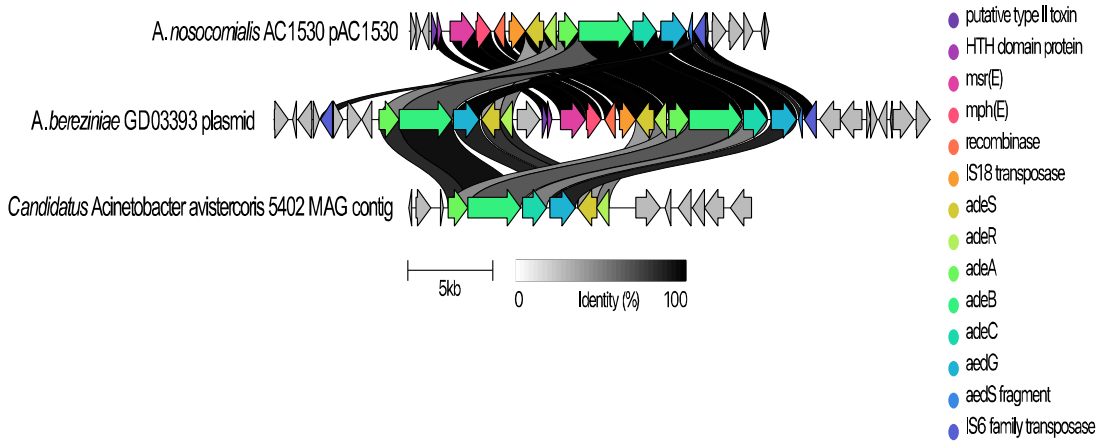
193 Figure 2. (A) *E. coli* BL21 cells carrying empty pTTQ18-*aedG* or pTTQ18-*amvA* were examined
194 in minimum inhibitory concentration (MIC) assays for resistance to a broad range of
195 antimicrobials, including the antibiotics tetracycline (TET) and erythromycin (ERY); the
196 biocides benzalkonium chloride (BAC), chlorhexidine (CHX), and ethanol (EtOH); and the
197 antimicrobial dyes 4′,6-diamidino-2-phenylindole (DAPI), acriflavine (ACR) and ethidium
198 bromide (EtBr). Resistance assays were performed as previously described (9). (B) Ethidium
199 transport from *E. coli* BL21 cells carrying either pTTQ18, pTTQ18-*amvA*, or pTTQ18-*aedG*.
200 Cells were loaded with 10 µM ethidium, transport was initiated by adding glucose and efflux
201 monitored fluorescently (Ex:530nm; Em:610nm) in a PTI QuantaMaster 8000 (HORIBA
202 Scientific) as described previously (9).

203



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205 Figure S1. Local genetic organisation of the *adeG* loci in *A. bereziniae* GD03393 plasmid. One

206 gene cluster shows high sequence similarity to the *aedG* locus in *A. nosocomialis* AC1530

207 pAC1530 and an adjacent cluster shows high similarity to the *aedG* locus in the *Candidatus*

208 Acinetobacter avistercoris 5402 metagenome assembled genome. This figure was made

209 using clinker v0.0.25 (12).

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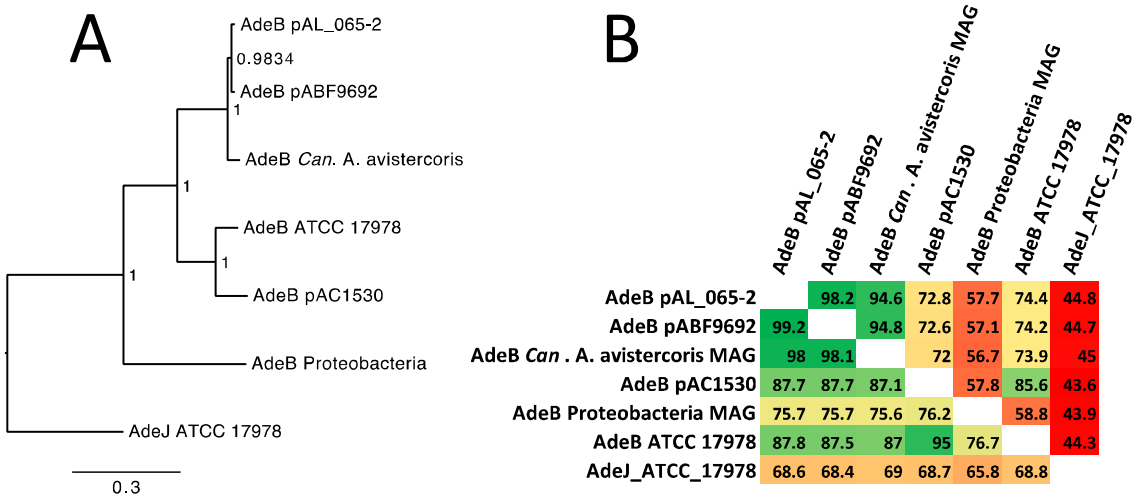

Figure S2. (A) The phylogenetic tree of AdeB homologs from representative *Acinetobacter* lineages (Figure 1B). The MAG of Proteobacteria bacterium isolate CTSoil_107, the *A. baumannii* ATCC 17978 chromosome, and the AdeJ sequence from the *A. baumannii* ATCC 17978 chromosome were aligned using MUSCLE (13). The phylogeny of these sequences was inferred using MrBayes v3.2.6 (14). The node labels show posterior probability values. (B) Pairwise amino acid sequence similarity (bottom left) and identity (top right) scores for the proteins shown in (A). These values were determined using MatGat version 2.0 (19) with a BOLSUM 50 scoring matrix.

	AedG pAL_065_2	AedG pABF9692	AedG *Can.* A. avistercoris MAG	AedG pAC1530	AedG *Pelagibacterium* MAG	AedG Proteobacteria MAG	AmvA	SmvA	PqrB	CmgA	QacA	QepA	LfrA	SgvT1	RifP	Ptr
AedG pAL_065-2		98	88.1	97	72.6	66.6	42.7	43.5	36.2	33.8	34.2	35.5	32.9	30.9	31.2	30.9
AedG pABF9692	99.2		89.1	99	72.4	67.2	42.9	43.5	36.4	33.5	34.4	36.2	32.8	30.6	31.6	31
AedG *Can.* A. avistercoris MAG	93.5	94.2		88.9	70.1	66.6	44.9	43.7	35.8	34.4	33	35.7	33.5	32.5	32.2	30.9
AedG pAC1530	98.6	99.4	94.2		72.4	67	42.7	43.5	35.8	33.6	33.8	36	32.2	30.4	31.2	31
AedG *Pelagibacterium* MAG	83.9	83.5	83.3	83.7		71.7	43	45.3	38	32.5	32.1	38.3	33.9	32	34.7	34.6
AedG Proteobacteria MAG	82.6	82.4	81.2	82.6	84.4		41.5	43.1	37.2	34.7	31.8	36.7	35.7	32	32.2	32
AmvA	66.5	66.1	67.5	66.3	65.3	62.6		55.2	33	32	28.7	30.7	33.6	27.9	31.2	31.6
SmvA	67.1	67.5	67.7	67.7	67.1	63.4	76.4		37.4	34.2	32.3	35.5	32.4	31.7	34.9	31.5
PqrB	57.1	57.1	54.9	56.9	56.5	57.1	59.4	59.2		39.7	36	45.8	47.8	40	40.4	38.1
CmgA	58.5	58.3	58.3	58.1	56.2	57.5	59.3	56.6	59.8		33	35.8	33.3	32.5	32.5	34.7
QacA	57.2	57.2	56.8	56.8	54.5	56	53.5	54.1	60.3	55.3		33.1	33.5	29.4	30.3	31.5
QepA	55.6	55.8	57.3	55.8	55.2	55.4	54.4	56.4	64.8	55.6	59.9		42.4	31.3	35.7	35.2
LfrA	54.4	54.2	53.2	53.8	53.4	55.7	58.5	54.8	69.2	54.6	57.8	63.4		34	33	31.4
SgvT1	52.6	52.5	55.3	53.4	53.2	52.6	52.1	51.3	59.4	53.8	52.8	53.6	53.2		50.3	48.2
RifP	52.9	53.1	53.3	52.9	54.2	53.3	54.4	53.6	59	51.5	55	57.1	53.8	68.5		62.5
Ptr	53.6	54.4	56.7	54.2	57.2	55.9	55.7	53.7	56.3	56.3	54.3	56.4	55.4	66.8	77.8	

Figure S3. Pairwise amino acid sequence similarity (bottom left) and identity (top right) scores for members of the DHA2 family of efflux proteins. These values were determined using MatGat version 2.0 (19) with a BOLSUM 50 scoring matrix.

Conflicts of Interest

None

Funding information

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Ethical Approval

N/A

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