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

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Abstract

A novel multidrug efflux pump, AadT from the Drug:H^+ antiporter 2 family, was discovered in *Acinetobacter* multidrug resistance plasmids. Here, we profiled the antimicrobial resistance potential and examined the distribution of this gene. Putative homologs of this efflux pump were encoded in many *Acinetobacter* species and other Gram-negative species, and were genetically associated with novel variants of *adeAB(C)*, which encodes a major tripartite efflux pump in *Acinetobacter*. The AadT pump conferred decreased susceptibility to at least eight diverse antimicrobials, including antibiotics erythromycin, tetracycline; biocides chlorhexidine; and dyes ethidium bromide and DAPI. These results show that AadT is a new determinant in the *Acinetobacter* resistance arsenal and may cooperate with variants of AdeAB(C).

30 **Introduction**

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

43 We recently identified genes encoding a putative multidrug efflux pump from the Drug:H^+
44 antiporter 2 (DHA2) family of the Major Facilitator Superfamily (MFS) of transport proteins in
45 several *Acinetobacter* plasmids (8). Here we demonstrate that the novel DHA2 family efflux
46 pump can decrease susceptibility to a range of antimicrobials, and that genes encoding
47 homologs of the pump are widespread, but always associated with a variant of the *adeAB(C)*
48 locus in *Acinetobacter* species. We call the novel efflux pump AadT, as the *Acinetobacter*
49 antimicrobial drug transporter (9).

50

51

52

53 **Methods**

54 Bacterial growth conditions, Cloning, PCR and Complementation

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

64

65 Antimicrobial susceptibility testing

66 Antimicrobial susceptibility assays were conducted in cation adjusted MH broth
67 supplemented with 0.05 mM IPTG using the broth microdilution method as described (11).
68 Assays for each antimicrobial were performed with three biological replicates and four
69 technical replicates, resulting in twelve replicates in total. Cell growth was measured
70 spectrophotometrically as OD_{600} endpoint readings using the PHERAstar FSX (BMG LABTECH,
71 Germany).

72

73 Whole Cell transport assay

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

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

84

85 Distribution of AadT and its phylogenetic relationship to other DHA2 family proteins

86 Variants of *aadT* were identified in NCBI databases using NCBI blast searches (see detailed
87 descriptions in the text). Representative *aadT* loci and their surrounding genes were aligned
88 and visualised using clicker v0.0.25 (12). The amino acid sequences of representative AadT
89 homologs from different lineages were aligned to the sequences of other representative
90 DHA2 family proteins using MUSCLE (13), and phylogeny inferred using MrBayes v3.2.6 (14).
91 The pairwise sequence identity and similarity of the representative proteins was determined
92 using MatGat (15). The same approaches were used to infer the phylogeny and sequence
93 similarities of representative homologs of adeB.

94

95 **Results and discussion**

96 AadT homologs are distributed widely across the *Acinetobacter* genus

97 Genes encoding AadT were previously identified on plasmids carried by *A. baumannii, A.*
98 *bereziniae, A. defluvii, A. johnsonii, A. nosocomialis, A. seifertii, A. ursingii* and *Acinetobacter*

99 strains with no species designation (8). These genes were nearly identical, as only one
100 nucleotide difference was present between sub-sets of genes, producing proteins with either
101 a tyrosine or histidine at the predicted cytoplasmic boundary of TM helix 13. The genes were
102 each located downstream of a gene cluster comprised of variants of *adeABC* and *adeRS*
103 (Figure 1A), which encode a tripartite RND efflux system, AdeABC, and its two-component
104 transcriptional regulator, AdeRS (16). The 3' ends of *adeC* in the *aadT* linked clusters were
105 formed by an inverted repeat sequence that was also found adjacent to a nearby IS*18* (Figure
106 1A) (8). The locations of the repeat sequences suggested that the intervening sequence,
107 comprising the IS*18* transposase gene, *adeRS* and *adeABC*, may be mobilised by the IS*18*
108 transposase. These loci also carried a partial *adeS* gene (3' end) downstream of the *aadT*
109 homologs, that could be related to an alternative organisation of these genes in an ancestral
110 locus (Figure 1A).

111 We sought to find additional homologs of *aadT* and determine whether they are also
112 associated with IS*18*/*adeRS*/*adeABC*. We initially queried the NCBI nr/nt database using
113 tblastn with the AadT amino acid sequence encoded in the *A. nosocomialis* AC1530 plasmid
114 pAC1530 and found close to 300 high scoring hits from strains designated in 23 *Acinetobacter*
115 species and *Acinetobacter* strains with no species designation. Surveys of the local genetic
116 environments of the high scoring hits showed one of two general arrangements. Either *aadT*
117 was located downstream of the IS*18*/*adeRS*/*adeABC* cluster, as in pAC1530, or *aadT* was
118 located between homologs of *adeAB* and *adeRS* gene pairs and *adeC* and the IS*18* transposase
119 gene were absent, as in *A. baumannii* ABF9692 plasmid pABF9692 (Figure 1B). It is not unusual
120 for *adeC* to be absent from *adeAB* loci in *Acinetobacter* genomes, and AdeAB can function
121 with AdeK as an alternative outer membrane protein (8, 17). Insertion elements were also

122 seen in some of the loci, such as that in *A. lwoffii* strain AL_065 pAL_065-2, where insertion
123 sequence elements appear to have disrupted *adeS* (Figure 1B).

124 To identify additional homologs of AadT, we queried the NCBI experimental clustered protein
125 database using the amino acid sequence of AadT encoded by pAC1530. This search identified
126 69 clusters whose representative members had above 65 % amino acid sequence identity
127 with the query AadT sequence across at least 95 % of its length. Proteins in the top scoring
128 cluster were encoded by *Acinetobacter* species. The local genetic environments of these
129 homologs resembled those of *aadT* in pAC1530 or pABF9692. Other high scoring clusters
130 included *Acinetobacter* proteins encoded by *aadT* homologs in an alternative genetic
131 environment. For example, the genetic environment of *aadT* homologs in the *Candidatus*
132 Acinetobacter avistercoris isolate 5402 metagenome assembled genome (MAG) and an *A.*
133 *bereziniae* GD03393 plasmid resembled that in pABF9692 but included *adeC* (Figures 1B and
134 S1). Of note, the *A. bereziniae* GD03393 plasmid harboured a second *aadT* homolog similar
135 to pAC1530 (Figure S1).

136 The proteins in lower scoring clusters were encoded by species outside the *Acinetobacter*
137 genus. The genes encoding these proteins were frequently adjacent to divergently
138 transcribed *tetR* family regulator genes that may control expression of the *aadT* homologs.
139 Some non-*Acinetobacter aadT* homologs were adjacent to RND efflux pump genes. For
140 example, the *aadT* homolog in the MAG of Proteobacteria bacterium isolate CTSoil_107 (likely
141 *Pleomorphomonas* based on GyrB and RpoD sequence similarity) was adjacent to putative
142 distant homologs of *adeRS*/*adeAB* (Figure 1), and the *aadT* homolog from *Pelagibacterium* sp.
143 SCN 63-126 ABS74_C0016 was encoded adjacently to a novel RND efflux system (Figure 1B).
144 These results demonstrate a tendency for genes encoding AadT to be co-localised with those

145 encoding RND efflux systems, specifically variants of AdeAB(C) in *Acinetobacter* species. AadT
146 and AdeAB(C) may cooperate in the efflux of substrates from the cytoplasm to outside the
147 cell, with AadT moving substrates from the cytoplasm to the periplasm and AdeAB(C) moving
148 substrates from the periplasm out of the cell (18).

149 A phylogenetic analysis was performed to examine the relationships of AadT homologs with
150 other members of the DHA2 family. This analysis showed that AadT homologs are most
151 closely related to the AmvA (also known as AedF) (9, 19) and SmvA (20) efflux pumps from
152 *Acinetobacter* and *Salmonella*, respectively (Figure 1C). Pairwise amino acid sequence
153 similarity scores confirmed the relatedness of AadT homologs and AmvA/SmvA (Figure S2).

154 Phylogenetic and pairwise amino acid sequence similarity studies were conducted to examine
155 the relationships of AdeB homologs encoded adjacently to *aadT* variants (Figure 1B). The
156 results (Figure 1D and 1E) showed that the AdeB pump encoded in pAC1530 is most similar
157 to the chromosomally encoded AdeB pump from *A. baumannii* ATCC 17978 (95 % amino acid
158 sequence similarity; Figure 1E). The AdeB pumps encoded in pAL_065-1, pABF9692 and the
159 MAG of *Candidatus Acinetobacter avistercoris* 5402 showed greater than 98 % amino acid
160 sequence similarity to each other, but were less than 88 % similar to the chromosomal AdeB
161 or AdeB encoded on pAC1530 (Figures 1D and 1E). The putative AdeB homolog encoded by
162 the Proteobacteria CTSoli_107 isolate showed low similarity to all other AdeB homologs
163 (Figure 1E). These results indicate that at least two distict putative *adeAB(C)* loci have been
164 acquired on different *Acinetobacter* plasmids and formed an association with *aadT*. As above,
165 the *A. bereziniae* GD03393 plasmid carries both of these loci (Figure S1).

166

167 <u>AadT confers resistance to multiple antibiotics, biocides and dyes</u>

168 Based on the proximity of *aadT* genes to homologs of *adeAB(C)* in *Acinetobacter* species and
169 the similarity of AadT variants to drug transporters in the DHA2 family, it seemed likely that
170 AadT pumps would mediate resistance to one or more antimicrobials. We profiled the
171 antimicrobial resistance potential of the AadT pump encoded in pAC1530 to eight
172 antimicrobials and compared resistance levels to those conferred by the multidrug efflux
173 pump AmvA in the same expression system (9, 19). The *aadT* homolog from pAC1530 was
174 synthesised and cloned into the pTTQ18 expression plasmid (Amp^r) behind the *tac* promoter
175 (10) with a C-terminal RGSH6 tag and sequence verified (Twist Bioscience). The *amvA* gene
176 from *A. baumannii* ATCC 17978 (also known as AedF) was cloned previously (9). AadT reduced
177 susceptibility to all eight antimicrobials tested when expressed in *E. coli* BL21 cells and
178 promoted higher levels of tolerance to tetracycline, DAPI, ethanol and ethidium bromide than
179 AmvA (Figure 2A). These results suggest that AadT can recognise a broad spectrum of
180 antimicrobials similar to AmvA and is a new multidrug transport protein in *Acinetobacter*.

181

182 <u>AadT functions via an active efflux mechanism</u>

183 The ability of AadT to confer tolerance to the nucleic acid intercalating dye ethidium bromide
184 allowed us to test efflux using real-time fluorometric transport assays. *E. coli* BL21 cells
185 expressing AadT, AmvA or no additional protein (negative control) were loaded with 10 µM
186 ethidium bromide in the absence of energy. The cells were washed, and re-energised and
187 ethidium efflux monitored by fluorescence changes. Both AmvA and AadT promoted the
188 active export of efflux ethidium bromide, as seen in fluorescence decrease over time relative
189 to control cells (Figure 2B). This result confirms efflux as the mechanism of tolerance
190 mediated by AadT and that AadT is a new multidrug efflux pump.

191

192 **Conclusions**

193 The genes encoding AadT are part of the accessory genomes of more than 20 *Acinetobacter*
194 species. This is in contrast to AmvA, its closest known relative in *Acinetobacter*, which is
195 encoded in the core genome of *A. baumannii* and other *Acinetobacter* species. AmvA
196 expression in *Acinetobacter* is not readily responsive to antimicrobial treatment, and recent
197 results from our groups showed that it may have a physiological function in polyamine efflux
198 (21). The organisation of *aadT* genes in *Acinetobacter*, downstream of *adeAB(C)* variants and
199 in proximity to *adeRS* homologs (Figures 1A and 1B), suggests that *aadT* genes may be
200 controlled by AdeRS and co-expressed with *adeAB(C)*. As such AadT homologs may play a
201 more significant role in antimicrobial resistance than AmvA in the isolates that carry them.
202 That *aadT* has formed a genetic assocication with several novel RND efflux pump genes,
203 mostly homologs of *adeAB(C)*, suggests a selective advantage to this arrangement, which as
204 mentioned above, may relate to cooperative transport activities of these pumps (18, 22).
205 The discovery of genes encoding novel variants of AdeAB(C) and AdeRS in *Acinetobacter*
206 plasmids is significant. These genes were found in hundreds of *Acinetobacter* sequences. The
207 variant AdeAB(C) pumps may have different substrate preferences to the chromosomally
208 encoded pumps. Details of these substrate preferences will be of significant interest in
209 determining their importance to antibiotic resistance in pathogenic *Acinetobacter.* The role
210 of the plasmid encoded AdeRS system is also of significant interest. This system seems likely
211 to control expression of the plasmid encoded *adeAB(C)* genes and as mentioned above may
212 control *aadT* gene expression. It is also possible that the plasmid encoded AdeRS systems
213 could promote expression of chromosomal *adeAB(C)* homologs, which are tightly regulated
214 in many strains.

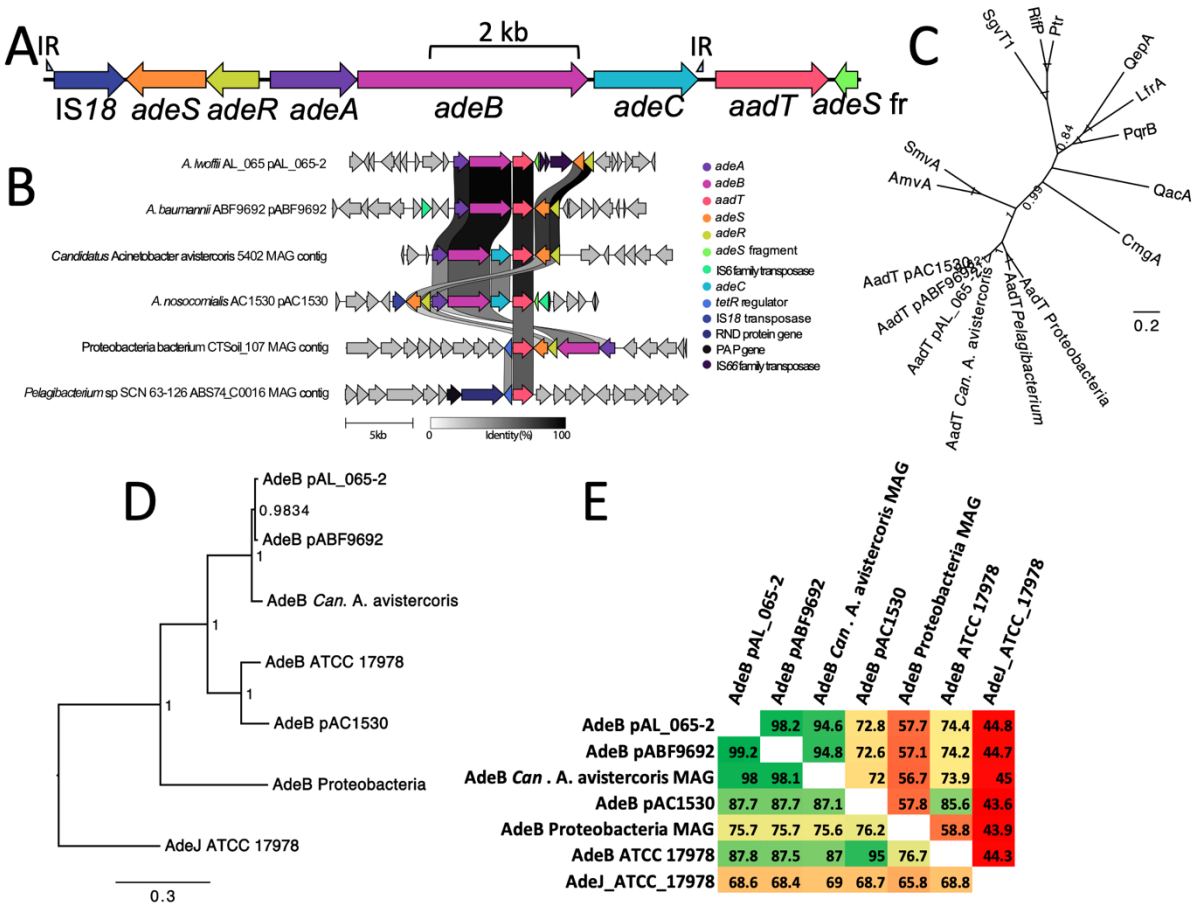


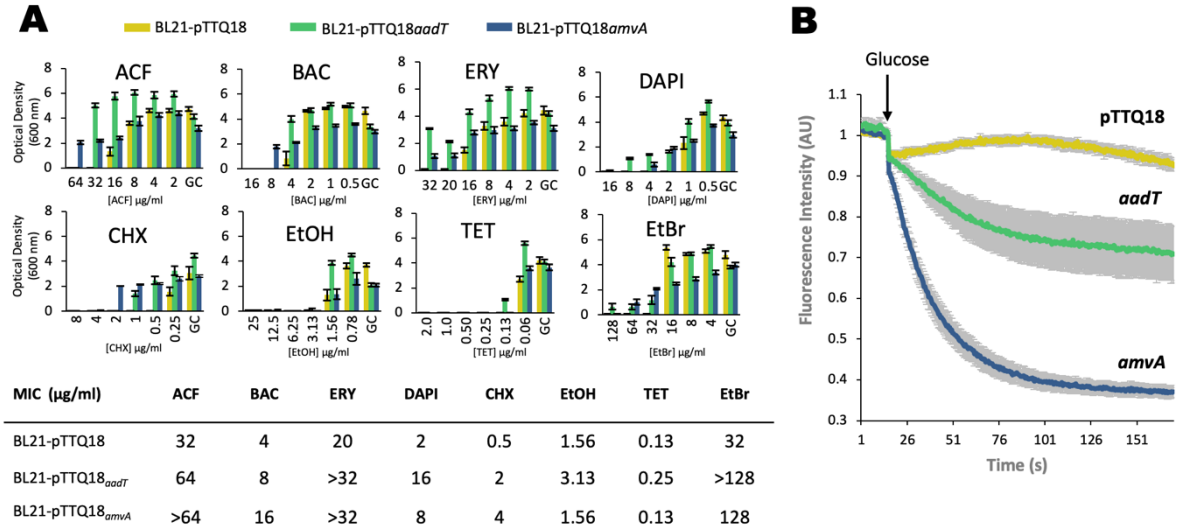
Figure 1. (A) Organisation of the local genetic environment of the *aadT* homolog in *A. nosocomialis* AC1530 plasmid pAC1530 and related plasmids, including the locations of surrounding genes and inverted repeat (IR) sequences that flank the IS*18* transposase gene and *adeC*. (B) Alignment of loci carrying an *aadT* homolog in selected bacterial isolates. Genes highlighted by colour and labels are found in more than one of the loci shown, or are features noted in the text. This figure was made using clinker v0.0.25 (12). (C) The amino acid sequences of AadT homologs from the lineages represented in (B) were aligned to the sequences of other representative DHA2 family proteins using MUSCLE (13), and phylogeny inferred using MrBayes v3.2.6 (14). The node labels show posterior probability values. (D) The putative AdeB homologs depicted in panel (B), and the AdeJ sequence from the *A. baumannii* ATCC 17978 chromosome were aligned using MUSCLE (13) and the phylogeny of these sequences was inferred using MrBayes v3.2.6 (14) and is shown in the tree. The node labels

228 show posterior probability values. (E) Pairwise amino acid sequence similarity (bottom left)

229 and identity (top right) scores for the proteins used in the phylogenetic analysis (D). These

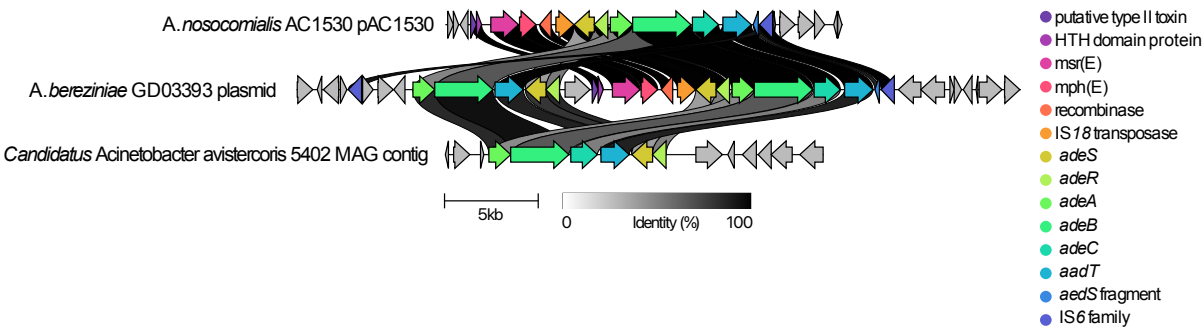
230 values were determined using MatGat version 2.0 (15) with a BOLSUM 50 scoring matrix.

231



MIC (µg/ml)	ACF	BAC	ERY	DAPI	CHX	EtOH	TET	EtBr
BL21-pTTQ18	32	4	20	2	0.5	1.56	0.13	32
BL21-pTTQ18$_{aadT}$	64	8	>32	16	2	3.13	0.25	>128
BL21-pTTQ18$_{amvA}$	>64	16	>32	8	4	1.56	0.13	128

Figure 2. (A) *E. coli* BL21 cells carrying empty pTTQ18-*aadT* or pTTQ18-*amvA* were examined in minimum inhibitory concentration (MIC) assays for resistance to a broad range of antimicrobials, including the antibiotics tetracycline (TET) and erythromycin (ERY); the biocides benzalkonium chloride (BAC), chlorhexidine (CHX), and ethanol (EtOH); and the antimicrobial dyes 4',6-diamidino-2-phenylindole (DAPI), acriflavine (ACR) and ethidium bromide (EtBr). The OD_{600} measurements were taken as endpoint readings after 24h growth. Resistance assays were performed as previously described (9). (B) Ethidium bromide transport assays in *E. coli* BL21 cells carrying either pTTQ18, pTTQ18-*amvA*, or pTTQ18-*aadT*. Cells were loaded with 10 µM ethidium, transport was initiated by adding glucose and efflux monitored fluorescently (Ex:450nm; Em:509nm) in a PTI QuantaMaster 8000 (HORIBA Scientific) as described previously (9). The total area under the curve (AUC) was calculcated after transport initiation by glucose for each strain BL21 pTTQ18 (150); pTTQ18-*amvA* (72); and pTTQ18-*aadT* (120). The grey shaded area represents the standard error of three biological replicates per strain.



248

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

250 gene cluster shows high sequence similarity to the *aadT* locus in *A. nosocomialis* AC1530

251 pAC1530 and an adjacent cluster shows high similarity to the *aadT* locus in the *Candidatus*

252 *Acinetobacter avistercoris* 5402 metagenome assembled genome. This figure was made using

253 clinker v0.0.25 (12).

254

	AadT pAL_065_2	AadT pABF9692	AadT *Can.* A. avistercoris MAG	AadT pAC1530	AadT *Pelagibacterium* MAG	AadT Proteobacterium MAG	AmvA	SmvA	PqrB	CmgA	QacA	QepA	LfrA	SgvT1	RifP	Ptr
AadT 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
AadT 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
AadT *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
AadT 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
AadT *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
AadT 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 S2. 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 (15) with a BOLSUM 50 scoring matrix.

260 **Conflicts of Interest**

261 None

262

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267

268 **Ethical Approval**

269 N/A

270

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