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

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15

16 **Abstract**

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

23

24 Introduction

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

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

44 AedG homologs are distributed widely across the *Acinetobacter* genus

45 Genes encoding AedG were previously identified on plasmids carried by *A. baumannii, A.*
46 *bereziniae, A. defluvii, A. johnsonii, A. nosocomialis, A. seifertii, A. ursingii* and *Acinetobacter*

47 strains with no species designation (8). These genes were nearly identical; only one nucleotide
48 difference was present between sub-sets of genes, producing proteins with either a tyrosine
49 or histidine at the predicted cytoplasmic boundary of TM helix 13 in the proteins expressed.
50 The genes were each located downstream of a gene cluster comprised of *adeABC* and *adeRS*
51 (Figure 1A), which encode a tripartite RND efflux system, AdeABC, and its two-component
52 transcriptional regulator, AdeRS (refs). The 3' ends of *adeC* in the *aedG* linked clusters were
53 formed by an inverted repeat sequence that was also found adjacent to a nearby IS*18* (Figure
54 1A) (8). The locations of these repeat sequences suggested that the intervening sequence,
55 comprised of the IS*18* transposase gene, *adeRS* and *adeABC*, may be mobilised by the IS*18*
56 transposase. These loci also carried a partial *adeS* gene (3' end) downstream of the *aedG*
57 homologs, that could be related to an alternative organisation of these genes in an ancestral
58 locus (Figure 1A).

59 We sought to find additional homologs of *aedG* and determine whether they are also
60 associated with IS*18*/*adeRS*/*adeABC*. We initially queried the NCBI nr/nt database using
61 tblastn with the AedG sequence encoded in *A. nosocomialis* AC1530 plasmid pAC1530 and
62 found close to 300 high scoring hits from strains designated in 23 *Acinetobacter* species and
63 *Acinetobacter* strains with no species designation. Surveys of the local genetic environments
64 of the high scoring hits showed one of two general arrangements. Either *aedG* was located
65 downstream of the IS*18*/*adeRS*/*adeABC* cluster, as in pAC1530, or *aedG* was located between
66 homologs of *adeAB* and *adeRS* gene pairs and *adeC* and the IS*18* transposase gene were
67 absent, as in *A. baumannii* ABF9692 plasmid pABF9692 (Figure 1B). It is not unusual for *adeC*
68 to be absent from *adeAB* loci in *Acinetobacter* genomes, and AdeAB can function with AdeK
69 as an alternative outer membrane protein (8, 10). Insertion elements were also seen in some

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

72 To identify additional homologs of AedG, we queried the NCBI experimental clustered protein
73 database using the amino acid sequence of AedG encoded by pAC1530. This search identified
74 69 clusters whose representative members had above 65 % amino acid sequence identity
75 with the query AedG sequence across at least 95 % of its length. Proteins in the top scoring
76 cluster were encoded by *Acinetobacter* species. The local genetic environments of these
77 homologs resembled those of *aedG* in pAC1530 or pABF9692. Other high scoring clusters
78 included *Acinetobacter* proteins encoded by *aedG* homologs in an alternative genetic
79 environment. For example, the genetic environment of *aedG* homologs in the *Candidatus*
80 Acinetobacter avistercoris isolate 5402 metagenome assembled genome (MAG) and an *A.*
81 *bereziniae* GD03393 plasmid resembled that in pABF9692 but included *adeC* (Figures 1B and
82 S1). Of note, the *A. bereziniae* GD03393 plasmid harboured a second *aedG* homolog similar
83 to pAC1530 (Figure S1).

84 The proteins in lower scoring clusters were encoded by species outside the *Acinetobacter*
85 genus. The genes encoding these proteins were frequently adjacent to divergently
86 transcribed *tetR* family regulator genes that may control expression of the *aedG* homologs.
87 Some non-*Acinetobacter* aedG homologs were adjacent to RND efflux pump genes. For
88 example, the *aedG* homolog in the MAG of Proteobacteria bacterium isolate CTSoil_107
89 (likely *Pleomorphomonas* based on GyrB and RpoD sequence similarity) was adjacent to
90 distant homologs of *adeRS*/*adeAB* (Figure S2), and the *aedG* homolog from *Pelagibacterium*
91 sp. SCN 63-126 ABS74_C0016 was encoded adjacently to a novel RND efflux system (Figure
92 1B). These results demonstrate a tendency for genes encoding AedG to be co-localised with

93 those encoding RND efflux systems, specifically AdeAB(C) in *Acinetobacter* species. AedG and
94 AdeAB(C) may cooperate in the efflux of substrates from the cytoplasm to outside the cell.

95 A phylogenetic analysis was performed to examine the relationships of AedG homologs with
96 other members of the DHA2 family. This analysis showed that AedG homologs are most
97 closely related to the AmvA (also known as AedF) (9, 11) and SmvA (12) efflux pumps from
98 *Acinetobacter* and *Salmonella*, respectively (Figure 1C). Pairwise amino acid sequence
99 similarity scores confirmed the relatedness of AedG homologs and AmvA/SmvA (Figure S3).

100 <u>AedG confers resistance to multiple antibiotics, biocides and dyes</u>

101 Based on the proximity of *aedG* genes to homologs of *adeAB(C)* in *Acinetobacter* species and
102 the similarity of AedG variants to drug transporters in the DHA2 family, it seemed likely that
103 AedG pumps would mediate resistance to one or more antimicrobials. We profiled the
104 antimicrobial resistance potential of the AdeG pump encoded in pAC1530 to eight
105 antimicrobials and compared resistance levels to those conferred by the multidrug efflux
106 pump AmvA in the same expression system (9, 11). The *aedG* homolog from pAC1530 was
107 synthesised and cloned into the pTTQ18 expression plasmid (Amp^r) behind the *tac* promoter
108 (13) with a C-terminal RGSH6 tag and sequence verified (Twist Bioscience). The *amvA* gene
109 from *A. baumannii* ATCC 17978 (also known as AedF) was cloned previously (9). AedG
110 conferred increased resistance to all eight antimicrobials tested when expressed in *E. coli*
111 BL21 cells and promoted higher levels of resistance to tetracycline, DAPI, ethanol and
112 ethidium bromide than AmvA (Figure 2A). These results suggest that AedG can recognise a
113 broad spectrum of antimicrobials similar to AmvA and is a new multidrug resistance protein
114 in *Acinetobacter*.

115 <u>AedG functions via an active efflux mechanism</u>

116 The ability of AedG to confer resistance to the nucleic acid intercalating dye ethidium bromide
117 allowed us to test efflux using real-time fluorometric transport assays. *E. coli* BL21 cells
118 expressing AedG, AmvA or no additional protein (negative control) were loaded with 10 µM
119 ethidium bromide in the absence of energy. The cells were washed, and re-energised and
120 ethidium efflux monitored by fluorescence changes. Both AmvA and AedG promoted the
121 active export of efflux ethidium bromide, as seen in fluorescence decrease over time relative
122 to control cells (Figure 2B). This result confirms efflux as the mechanism of resistance
123 mediated by AedG and that AedG is a new multidrug efflux pump.

124 Conclusions

125 The genes encoding AedG are part of the *Acinetobacter* accessory genome. This is in contrast
126 to AmvA, its closest relative, which is encoded in the core genome of *A. baumannii* and many
127 other *Acinetobacter* species. AmvA expression is not readily responsive to antimicrobial
128 treatment, and recent work showed that it may have a physiological function in polyamine
129 efflux (14). The organisation of *aedG* genes in *Acinetobacter*, downstream of *adeAB(C)* and in
130 proximity to *adeRS* homologs (Figures 1A and 1B), suggests that these genes may be
131 controlled by AdeRS and co-expressed with *adeAB(C)*. As such AedG homologs may play a
132 more significant role in antimicrobial resistance than AmvA in isolates that carry them.
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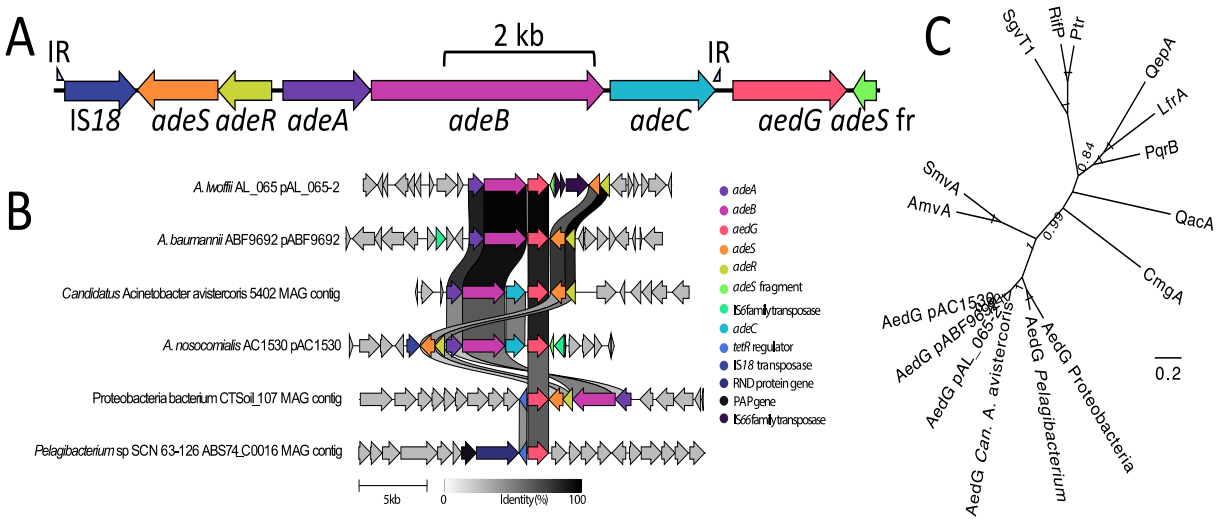


Figure 1. (A) Organisation of the local genetic environment of the *aedG* homolog in *A. nosocomialis* AC1530 plasmid pAC1530 and related plasmids, including the locations of surrounding genes and inverted repeat (IR) sequences that flank the IS*18* transposase gene and *adeC*. (B) Alignment of loci carrying an *aedG* 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 (14). (C) The amino acid sequences of AedG homologs from the lineages represented in (B) were aligned to the sequences of other representative DHA2 family proteins using MUSCLE (15), and phylogeny inferred using MrBayes v3.2.6 (16). The node labels show posterior probability values.

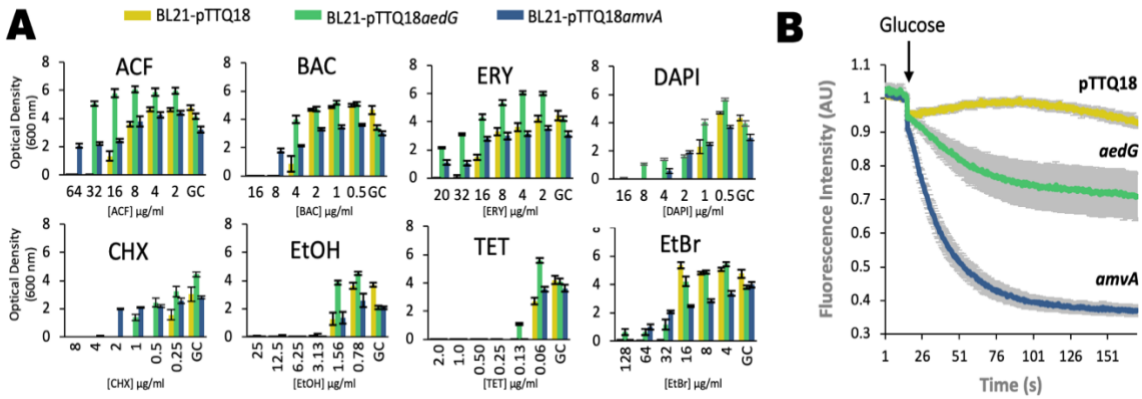
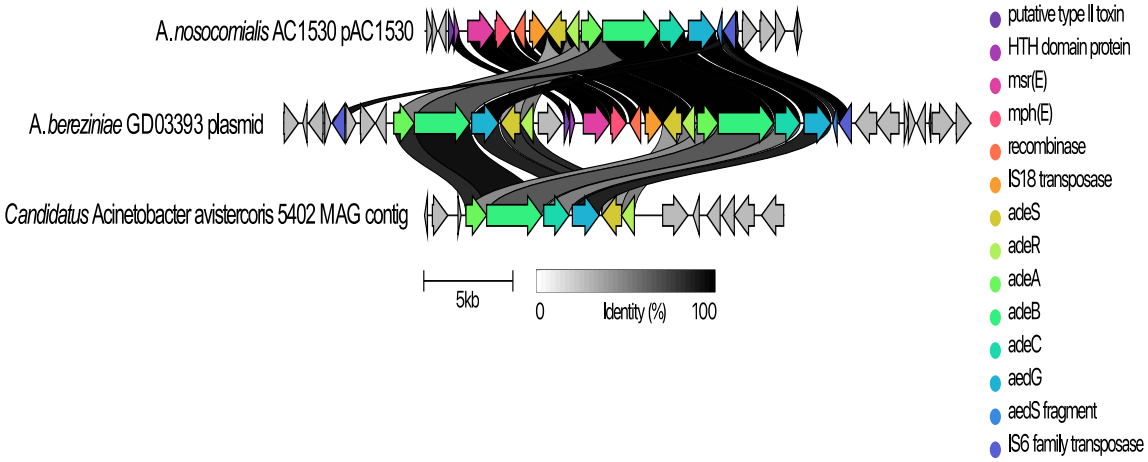


Figure 2. (A) *E. coli* BL21 cells carrying empty pTTQ18-*aedG* or pTTQ18-*amvA* were examined in minimum inhibitory concentration (MIC) assays for resistance to a broad range of antimicrobials, including the antibiotics tetracycline (TET) and erythromycin (ERY); the biocides benzalkonium chloride (BAC),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



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

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

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

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

169 clinker v0.0.25 (14).

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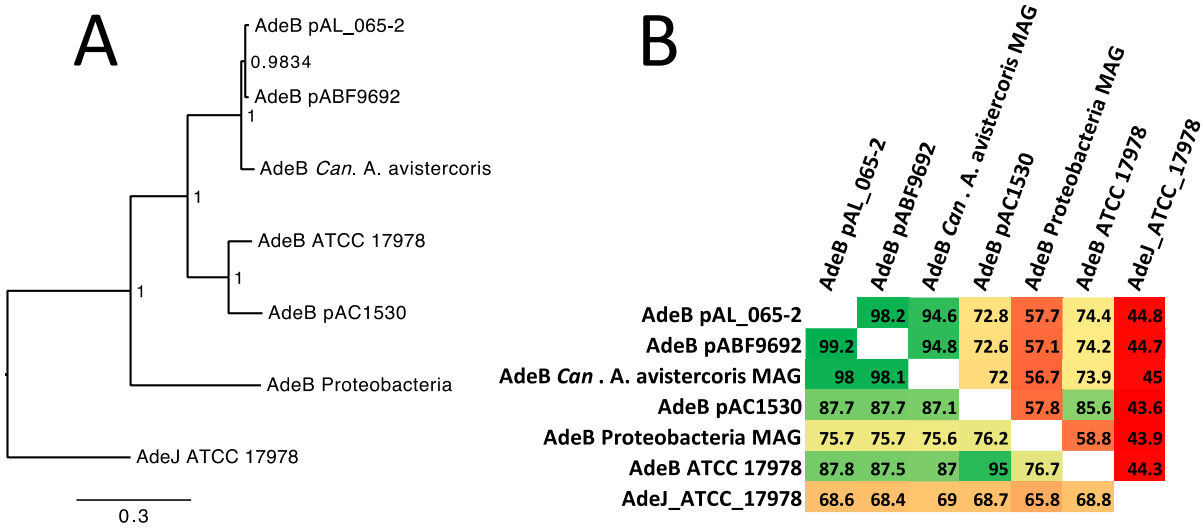

Figure S2. (A) The phylogenetic tree of AdeB homologs from representative *Acinetobacter* lineages (Figure 1B). The MAG of Proteobacteria bacterium isolate CTSoil_107, the *A. baumannii* ATCC 17978 chromosome, and the AdeJ sequence from the *A. baumannii* ATCC 17978 chromosome were aligned using MUSCLE (15). The phylogeny of these sequences was inferred using MrBayes v3.2.6 (16). 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 (17) with a BOLSUM 50 scoring matrix.

	AedG pAL_065_2	AedG pABF9692	AedG *Can.* A. avistercoris MAG	AedG pAC1530	AedG *Pelagibacterium* MAG	AedG Proteobacterium 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 (17) with a BOLSUM 50 scoring matrix.

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