

## Molecular and functional characterization of a novel efflux pump, AmvA, mediating antimicrobial and disinfectant resistance in *Acinetobacter baumannii*

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**Objectives:** *Acinetobacter baumannii* has emerged as an important nosocomial pathogen in hospitalized patients, and causes a multitude of infections with significant morbidity and mortality. The aim of this study was to elucidate the role of a novel efflux pump in *A. baumannii*.

**Methods:** The open reading frame ABAYE1518, annotated as a putative Methyl Viologen resistance protein in the genome of strain *A. baumannii* AYE, exhibits >50% similarity with members of the major facilitator superfamily (MFS) multidrug efflux pumps. The antimicrobial susceptibility profiles of *Escherichia coli* KAM32 cells carrying the putative efflux pump were monitored by broth dilution method. Different efflux pump inhibitors were used for fluorimetric efflux assays. The functions of the putative efflux pump were confirmed in *A. baumannii* by insertional inactivation and complementation. Its expression in clinical isolates was analysed by reverse transcriptase-PCR.

**Results:** *E. coli* cells carrying the pump had decreased susceptibility to some antibiotics, disinfectants, dyes and detergents, with enhanced efflux activity. The pump was inactivated in a clinical isolate of *A. baumannii* AC0037 and further characterization confirmed its role in antimicrobial resistance by active efflux. We found increased expression of the pump in clinical isolates that also exhibited elevated tolerance to antibacterial agents.

**Conclusions:** This report describes the functions of a novel resistance determinant, a member of the MFS efflux pumps, for the first time in *A. baumannii*.

**Keywords:** MFS efflux pumps, drug:H<sup>+</sup> antiporter-2, disinfectant tolerance, nosocomial pathogens

### Introduction

The management of infections due to *Acinetobacter baumannii* is greatly hindered by its intrinsic and acquired resistance to a wide variety of antimicrobial agents.<sup>1</sup> In addition to enzyme-mediated resistance, overexpression of efflux pumps is thought to be an important factor in the multiple resistance phenotype of *A. baumannii*.<sup>2</sup> In Gram-negative bacteria, a subset of inner membrane proteins in the major facilitator superfamily (MFS) act as efflux pumps to decrease the intracellular concentrations of multiple toxic substrates and confer multidrug resistance.<sup>3</sup>

The MFS drug transporters are classified as: (i) drug:H<sup>+</sup> antiporter-1 (DHA1) family, with 12 transmembrane segments; or (ii) drug:H<sup>+</sup> antiporter-2 (DHA2) family, with 14 transmembrane segments.<sup>4</sup> Well-studied examples such as QacA and NorA of *Staphylococcus aureus*, KmraA of *Klebsiella pneumoniae*, and SmvA of *Salmonella enterica* serovar Typhimurium belong

to the latter family.<sup>5–8</sup> Though CraA (DHA1 family) has been recently characterized in *A. baumannii*,<sup>9</sup> the functions of other chromosomally borne DHA2 family efflux pumps are still unknown and this report discusses the unprecedented functions of one such efflux pump.

In our preliminary report, we demonstrated that β-lactamases, aminoglycoside-modifying enzymes, target gene mutations and efflux pumps were responsible for the multidrug-resistant (MDR) behaviour of the clinical strains isolated during 2005–07 from central Ohio tertiary care hospitals.<sup>10</sup> *A. baumannii* AC0037 is one such MDR isolate from our collection (isolated from the respiratory tract of a patient in Ohio State University Medical Center in central Ohio, in 2007), which displayed decreased susceptibility to fluoroquinolones, β-lactams, aminoglycosides, as well as commercially available disinfectants, dyes and detergents.<sup>10</sup>

Therefore, studies were performed in *A. baumannii* AC0037 to investigate the contributions of the putative efflux pump belonging to the DHA2 family in conferring antibiotic and disinfectant resistance.

## Materials and methods

### Bacterial strains, growth media and chemicals

*Escherichia coli* KAM32 ( $\Delta$ acrAB  $\Delta$ ydhE) and *A. baumannii* AC0037 were used for genetic studies.<sup>10,11</sup> Bacteria were grown at 37°C in Luria–Bertani (LB) and agar (Difco Laboratories, Sparks, USA). Different antibiotics, detergents, dyes and inhibitors used in this study were purchased from Sigma–Aldrich.

### Cloning of the putative efflux pump

Genomic DNA from *A. baumannii* AC0037 was extracted using the DNeasy Tissue kit (Qiagen, Valencia, CA, USA). The open reading frame (ORF) from *A. baumannii* AC0037 was amplified using AmvA-F/R primers by PCR as described previously,<sup>10</sup> with minor modifications (Table 1). The PCR products were purified by using the QiaQuick PCR purification kit (Qiagen), digested with PstI and BamHI, and then cloned into similarly digested pUC18 vector. At least two independently generated recombinant plasmids were sequenced bidirectionally using a CEQ 8000 (Beckman Coulter Instruments Inc., Palo Alto, CA, USA) capillary electrophoresis system to rule out mutations introduced during PCR and analysed by BLAST ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)). The recombinant plasmid denoted pUC18-AmvA was transformed into *E. coli* KAM32 to generate *E. coli* KAM32/pUC18-AmvA.

### MICs

MICs were determined according to the guidelines of the CLSI by the broth dilution method in Mueller–Hinton broth (Difco, Sparks, USA).<sup>12</sup> Susceptibility was tested in duplicate and the tests were repeated three times. The MIC values are presented as means and variation about the mean was always <10%. The different classes of disinfectant formulations used were: Virkon-S (an oxidizing agent: peroxygen blend and organic acid) (Antec International, UK); Wex-cide-128 (a phenolic agent) (Wexford Laboratories, USA); Synergize [a glutaraldehyde disinfectant containing quaternary ammonium compound (QAC)] (Preserve International, USA); chlorhexidine gluconate (MP Biomedicals, LLC); benzalkonium chloride (a QAC) (MP Biomedicals, LLC); and Parvosol II RTU (a synergized QAC with inert ingredients) (Hess & Clark Inc., USA).

**Table 1.** Primers used in this study

| Primer     | Primer sequence (5' to 3')       | Source     |
|------------|----------------------------------|------------|
| AmvA-F     | GACACTGCAGGGCAGCTTGAGAACATTTCTCT | this study |
| AmvA-R     | CCAGGGATCCATTACTTCTTCGGAAA       | this study |
| AmvA-RT1   | TGTATTCCCTCATTAATATCCC           | this study |
| AmvA-RT2   | TTACTTCTCGGAAA                   | this study |
| 16S rRNA-F | TCGATGCAACGCGAAGAAC              | this study |
| 16S rRNA-R | CGTAAGGCCATGATGACTT              | this study |
| aadA1-F    | ACAGATATCATGAGGGAAAGCGGTGATC     | 15         |
| aadA1-R    | TACGGTCGACTTATTGCTACTACCTTGGTGA  | 15         |

### Analysis of growth kinetics

The growth inhibition assay was done as described previously, with minor modifications.<sup>10</sup> The efflux pump inhibitors used in this study were carbonyl cyanide 3-chlorophenylhydrazone (CCCP), reserpine, verapamil and phenyl-arginine- $\beta$ -naphthylamide (PA $\beta$ N) (Sigma, St Louis, MO, USA).<sup>13,14</sup> Efflux inhibitors had no intrinsic antibacterial activity against clinical isolates at the concentration used in the experiments.<sup>10</sup>

### Accumulation studies

Accumulation of ethidium bromide was monitored as described previously.<sup>15</sup> Briefly, *A. baumannii* cells were grown to mid-log phase, harvested and suspended in 1x PBS (0.136 M NaCl/0.0026 M KCl/0.01 M Na<sub>2</sub>HPO<sub>4</sub>/0.00176 M KH<sub>2</sub>PO<sub>4</sub>, pH 7.0) to an optical density at 600 nm (OD<sub>600</sub>) of 0.2. Ethidium bromide was added at a concentration of 10 mg/L. Aliquots were taken at different time intervals, harvested, suspended in 1 mL of 0.1 M glycine/HCl buffer (pH 2.3) and incubated for 6 h at 37°C. Then, the fluorescence of the supernatant was measured at excitation 530 nm and emission 600 nm. Where indicated, the proton-motive force uncoupler CCCP was added to the assay mixture at a final concentration of 25 mg/L. This assay was also performed using acriflavine as substrate, and the fluorescence of the supernatant was measured at excitation 468 nm and emission 499 nm.

### Construction of deletion mutant in MDR strain *A. baumannii* AC0037

To evaluate the role of amvA in *A. baumannii* AC0037, attempts were made to generate an amvA deletion mutant. Kanamycin resistance plasmid pCR2.1-TOPO (Invitrogen, Carlsbad, CA, USA), unable to replicate in *A. baumannii*, was used as a suicide vector.<sup>16</sup> The recombinant plasmid pInt-amvA was introduced into the kanamycin-susceptible, ticarcillin-resistant *A. baumannii* AC0037 by electroporation (Eppendorf, Hamburg, Germany). Selection of the amvA null mutant was made on plates containing 50 mg/L kanamycin and 80 mg/L ticarcillin. Inactivation of the amvA gene by insertion of pInt-amvA was confirmed by PCR amplification and DNA sequencing, and the null mutant was designated as AC0037 $\Delta$ amvA.

### Complementation studies with AmvA deletion mutant

The aadA1 gene was PCR amplified using AC0019 (accession number EU977568.1) as a template DNA with primers aadA1-F/R (Table 1). The amplicon was ligated into the EcoRV site of the *A. baumannii*–*E. coli* shuttle vector pWH1266,<sup>17</sup> transformed into *E. coli* Top10 cells and selected on LB plates containing 200 mg/L streptomycin. Plasmid DNA from the streptomycin-resistant transformant was denoted as pWH-Spc. The obtained plasmid, pWH-Spc, was modified by cloning a PCR-amplified wild-type amvA gene from *A. baumannii* strain AC0037 into the PstI site of pWH-Spc, to yield a recombinant plasmid pWH-amvA. Electroporation of the recombinant plasmid pWH-amvA into the *A. baumannii* AC0037 $\Delta$ amvA mutant resulted in the AC0037 $\Delta$ amvA $\Omega$ amvA mutant. Selection of the complemented mutant was made on plates containing 200 mg/L streptomycin and 50 mg/L kanamycin.

### RNA preparation and reverse transcriptase–PCR (RT–PCR) analysis

To detect amvA gene expression, RT–PCR was performed.<sup>18,19</sup> Total RNA from different clinical isolates grown in LB broth until the exponential growth phase was extracted by using the RNA miniprep kit (Stratagene, La Jolla, CA, USA). The quality of samples was checked electrophoretically and quantification was done spectrophotometrically. Quantification of amvA RNA transcripts was carried out by reverse transcription using

500 ng of RNase-free DNase-treated total RNA with the Titan One Tube RT-PCR System (Roche Inc., USA). The primers used for *amvA* (AmvA-RT1/2) and 16S rRNA (16SrRNA-F/R) are shown in Table 1. The RT-PCR was carried out for 25 PCR cycles and cycling parameters were followed according to the manufacturer's instructions (Roche). The RT-PCR products (10 µL) were analysed by agarose gel electrophoresis (1.5%, w/v) and visualized by ethidium bromide staining. The bands of the *amvA* gene were subjected to densitometric analysis using the image scanning software Quantity one 4.1.1 (Bio-Rad). The expression levels were standardized relative to the transcription levels of 16S rRNA (a housekeeping gene) for each isolate. Two independent cultures were measured and the results were found to be consistent. As a control for DNA contamination of RNA samples, non-RT reactions (i.e. standard PCRs) were carried out. In no instance was a product obtained in the absence of the RT reaction mixture. *P* values of <0.05 were considered statistically significant.

### Data analysis

The assays were performed in triplicate on independent occasions to confirm reproducibility. All data are presented as means±SEM.

## Results

### Cloning of the putative ORF from *A. baumannii* clinical isolate AC0037

The ORF ABAYE1518, annotated as a putative Methyl Viologen resistance protein in strain *A. baumannii* AYE (accession number: CU459141.1),<sup>20</sup> exhibits 55% identity with *Salmonella* Typhimurium SmvA (accession number: NP\_460533.1). Using the ABAYE1518 gene sequence as template, primers were designed to amplify the ORF from the clinical isolate *A. baumannii* AC0037. The amplified ORF was designated AmvA (*A. baumannii* Methyl Viologen and antimicrobial resistance protein).

AmvA was cloned into the pUC18 vector to yield the recombinant plasmid pUC18-AmvA (Table 1). Sequencing revealed one complete ORF (100% identity with ABAYE1518) in the construct. Two independently generated clones were sequenced to ensure authenticity of the sequence. Further analyses revealed that *amvA* was a 1479 bp gene with a start and stop codon, specifying a 492 amino acid protein with a calculated mass of 52.8 kDa.

As per the Basic Local Alignment Search Tool (BLAST) at www.ncbi.nlm.nih.gov, AmvA exhibited different degrees of identity with other MFS transporters, such as SmvA from *Salmonella* Typhimurium (with 55.2% identity and 76.4% similarity),<sup>8</sup> KmRA from *K. pneumoniae* (with 52.1% identity and 74.5% similarity)<sup>7</sup> and QacA from *S. aureus* (with 28.7% identity and 53.5% similarity).<sup>5</sup>

Multiple sequence alignment using CLUSTAL W<sup>21</sup> indicated that AmvA is composed of 14 α-helical transmembrane segments [Figure S1, available as Supplementary data at JAC Online (<http://jac.oxfordjournals.org/>)] and is a member of the DHA2 transporter family.<sup>4</sup>

### Contributions of the efflux pump AmvA in mediating antimicrobial resistance

To evaluate the role of *amvA* in antimicrobial resistance, the MICs of various compounds were determined. AmvA-carrying *E. coli* cells had decreased susceptibility to different classes of antibiotics, disinfectants, detergents and dyes, as shown in Table 2.

Similar increases in the MICs of various antimicrobial compounds were observed on expressing AmvA under the control of an arabinose-inducible (pBAD/Myc-His; Invitrogen, Carlsbad, CA) promoter in *E. coli* (data not shown). AmvA expression in *E. coli* was linked to a subtle decrease in antibiotic susceptibility, but to a significant increase in resistance to disinfectants, detergents and dyes.

### Growth inactivation profiles of *E. coli* carrying AmvA

To decipher whether *amvA* confers resistance via active efflux, screening for a potential efflux phenotype was accomplished by determining the MICs of different substrates (erythromycin, Methyl Viologen and ethidium bromide) in the presence and absence of efflux pump inhibitors (25 mg/L), as described previously.<sup>15</sup> The results displayed the following MICs for *E. coli* KAM32/pUC18-AmvA towards different substrates in the absence of CCCP: 5 mg/L erythromycin; 200 mg/L Methyl Viologen; and 8 mg/L ethidium bromide.

The addition of CCCP decreased the MICs of AmvA-carrying cells for the same substrates (2 mg/L erythromycin, 64 mg/L Methyl Viologen and 2 mg/L ethidium bromide) (Figure 1). Similar reductions in the MICs were found upon using PAβN (data not shown). In independent experiments, neither reserpine nor verapamil decreased the MICs (data not shown). Overall, the findings indicated that the *amvA* gene product conferred decreased susceptibility to various antimicrobial compounds through an active efflux mechanism.

### Phenotypic characterization of efflux pump in *A. baumannii* AC0037

To evaluate the role of *amvA* in *A. baumannii* AC0037, attempts were made to generate an *amvA* deletion mutant. A 792 bp internal fragment was obtained (restriction site at position of 590 and 1376 bp) upon digesting the full-length *amvA* gene using HindIII (NEB, Beverly, MA, USA). This was subsequently inserted into the pCR2.1-TOPO vector using T4 DNA ligase (NEB) to yield the recombinant plasmid pInt-amvA, which was then introduced into strain AC0037 by electroporation to yield AC0037Δ*amvA*.

Susceptibility testing indicated that the deletion of *amvA* resulted in increased susceptibility to several of the antibiotics and most of the disinfectants or dyes tested (Table 2). The growth rate of AC0037Δ*amvA* in the presence of Methyl Viologen, erythromycin, ethidium bromide and TPPCI was abrogated as compared with wild-type AC0037 (data not shown).

### Fluorimetric efflux assay using *A. baumannii* amvA null mutant

To elucidate whether resistance caused by AmvA was due to active efflux, a fluorimetric experiment (using CCCP) to determine the rate of ethidium bromide/acriflavine accumulation (loss of fluorescence indicated efflux activity) was performed. The substrate accumulation data indicated that the efflux was most efficient in the wild-type AC0037 strain, whereas it was less efficient (increased accumulation of 2.5-fold for ethidium bromide and 3.5-fold for acriflavine) in the AC0037Δ*amvA* mutant (Figure 2). The addition of CCCP increased the ethidium

**Table 2.** MICs of various antimicrobial agents for *E. coli* (KAM32/pUC18 and KAM32/pUC18-AmvA) and *A. baumannii* (AC0037, AC0037ΔamvA and AC0037ΔamvAΩamvA) strains used in this study

| Compound              | MIC (mg/L) for strains |                  |                          |                     |              |                          |                  |  |
|-----------------------|------------------------|------------------|--------------------------|---------------------|--------------|--------------------------|------------------|--|
|                       | <i>E. coli</i>         |                  |                          | <i>A. baumannii</i> |              |                          |                  |  |
|                       | KAM32/pUC18            | KAM32/pUC18-AmvA | fold change <sup>a</sup> | AC0037              | AC0037 ΔamvA | fold change <sup>a</sup> | AC0037ΔamvAΩamvA |  |
| Amikacin              | 1                      | 2                | 2                        | 256                 | 256          | 1                        | 256              |  |
| Ciprofloxacin         | 0.002                  | 0.004            | 2                        | 120                 | 60           | 2                        | 120              |  |
| Erythromycin          | 4                      | 20               | 5                        | 64                  | 16           | 4                        | 64               |  |
| Minocycline           | 0.5                    | 1                | 2                        | 2                   | 2            | 1                        | 2                |  |
| Nalidixic acid        | 1                      | 1                | 1                        | 256                 | 256          | 1                        | 256              |  |
| Norfloxacin           | 0.06                   | 0.24             | 4                        | 512                 | 256          | 2                        | 512              |  |
| Novobiocin            | 4                      | 12               | 3                        | 120                 | 60           | 2                        | 120              |  |
| Rifampicin            | 2                      | 2                | 1                        | 2                   | 2            | 1                        | 2                |  |
| Tetracycline          | 0.5                    | 1                | 2                        | 64                  | 64           | 1                        | 64               |  |
| Trimethoprim          | 0.125                  | 0.125            | 1                        | 64                  | 64           | 1                        | 64               |  |
| Acridine Orange       | 16                     | 128              | 8                        | 200                 | 50           | 4                        | >128             |  |
| Acriflavine           | 2                      | 32               | 16                       | 128                 | 16           | 8                        | 128              |  |
| Benzalkonium chloride | 1                      | 6                | 6                        | 48                  | 12           | 4                        | 32               |  |
| Chlorhexidine         | 2                      | 8                | 4                        | 32                  | 16           | 2                        | 32               |  |
| DAPI                  | 0.5                    | 32               | 64                       | 2                   | 0.25         | 8                        | 2                |  |
| Deoxycholate          | 125                    | 2000             | 16                       | >2058               | 512          | 4                        | >1024            |  |
| Ethidium bromide      | 4                      | 96               | 24                       | 1024                | 128          | 8                        | >512             |  |
| Methyl Viologen       | 64                     | 1024             | 16                       | 800                 | 50           | 16                       | >512             |  |
| Pyronin Y             | 4                      | 8                | 2                        | 4                   | 2            | 2                        | 4                |  |
| Rhodamine 123         | 8                      | 8                | 1                        | 128                 | 128          | 1                        | 128              |  |
| SDS                   | 50                     | 450              | 9                        | >256                | 128          | 2                        | 256              |  |
| TPPCL                 | 8                      | 128              | 16                       | 1500                | 186          | 8                        | 1024             |  |

DAPI, 4',6-diamidine-2-phenylindole; TPPCL, tetraphenylphosphonium chloride.

<sup>a</sup>Fold change is the ratio of MICs for pUC18-AmvA to pUC18 and MICs for AC0037 to AC0037ΔamvA.

bromide/acriflavine levels, which eventually reached a plateau in both AC0037 and AC0037ΔamvA strains (Figure 2). However, upon inactivating a single gene we did not observe a complete loss in the resistance profile. Thus, it is important to state here that other efflux pumps in the genome of the bacteria may also have a role in conferring decreased susceptibility to these agents.

We also tested the effect of monovalent cations, such as Na<sup>+</sup> and Li<sup>+</sup>, in the efflux process, because the activities of several multidrug efflux pumps are coupled with monovalent cations.<sup>22</sup> The addition of either NaCl or LiCl showed no difference in the efflux activity compared with that of the control strain (data not shown). These results pointed to the actual role of the AmvA as a proton-dependent efflux pump that conferred resistance to numerous antimicrobial agents in *A. baumannii*.

### Complementation studies

The role of *A. baumannii* amvA was confirmed by performing complementation experiments, as described in the Materials and methods section. The expression of amvA in the complemented strain was confirmed at the RNA level (data not shown). Susceptibility testing showed that complementation with the wild-type gene restored the ability of the deletion

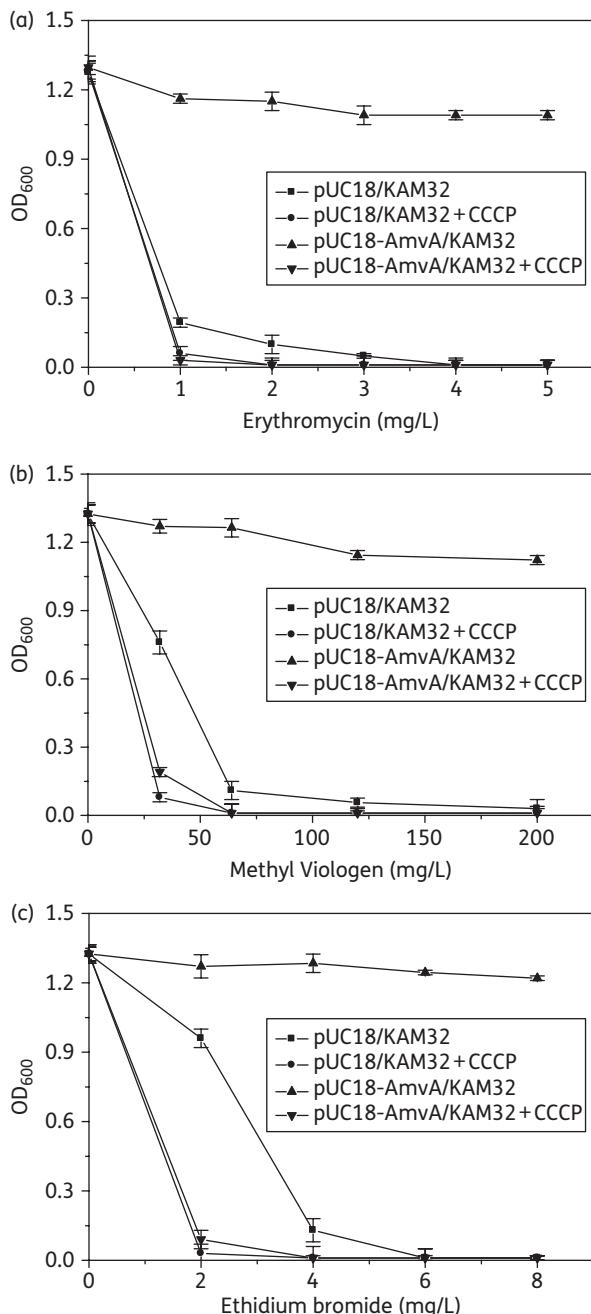
mutant to confer resistance to several of the antibiotics and most of the disinfectants, dyes and detergents (Table 2). These observations evidently confirmed the contributions of AmvA in mediating antimicrobial resistance in *A. baumannii*.

### Analysis of amvA expression in MDR clinical isolates

PCR amplification of AmvA using genomic DNA clearly indicated the presence of AmvA in all the clinical isolates in our collection (data not shown).

For the current study, a few MDR strains (AC0019, AC0037, AC0045, AC0029, AC0030 and AC0050) were chosen and the expression of AmvA was monitored by RT-PCR. As shown in Figure 3, the expression levels of the amvA gene were higher in drug-resistant strains than in a susceptible strain (AC0030).

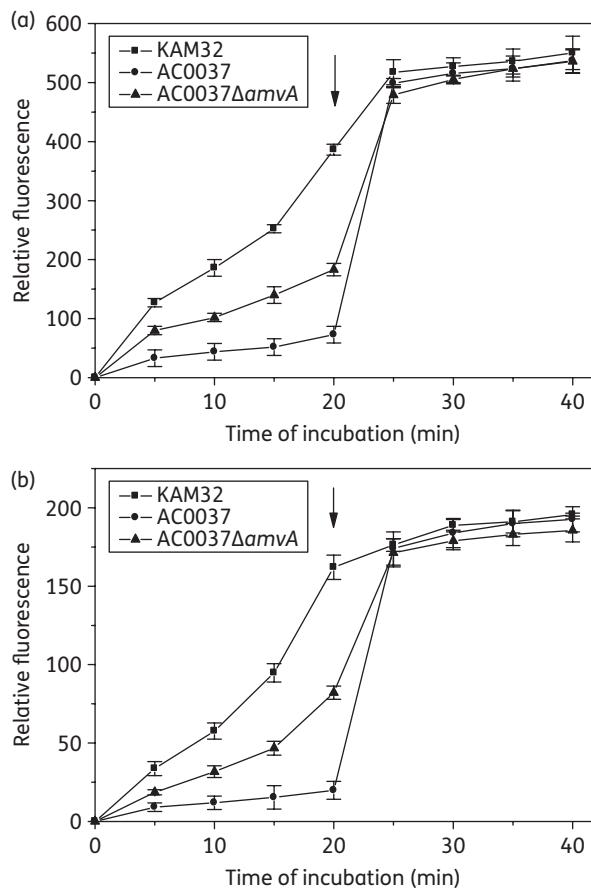
The results of two independent experiments showed that the relative amvA gene expression was ~2-fold higher in clinical isolate AC0029 [with moderate MICs of ciprofloxacin (<32 mg/L), erythromycin (12 mg/L), Methyl Viologen (256 mg/L) and ethidium bromide (64 mg/L)] (Figure 3) and ~4-fold higher in clinical isolates AC0019, AC0037, AC0045 and AC0050 that exhibited high MICs of ciprofloxacin (>72 mg/L), erythromycin (>32 mg/L), Methyl Viologen (800 mg/L) and ethidium bromide (>512 mg/L) (Figure 3).



**Figure 1.** *In vitro* inactivation profiles of *E. coli* KAM32 cells carrying AmvA. The cells of KAM32/pUC18-AmvA and KAM32/pUC18 were grown to mid-log phase ( $OD_{600}=0.2$ ). At this point, the cultures received different concentrations of erythromycin (a), Methyl Viologen (b) and ethidium bromide (c), either alone or in the presence of 25 mg/L CCCP. Ranges of results obtained for the duplicate experiments are shown by error bars.

## Discussion

Numerous outbreaks caused by MDR *A. baumannii* from different parts of the USA are appearing very rapidly.<sup>23</sup> Active extrusion by efflux pumps (including members of the MFS family) is thought to be one of the important factors in the multiple resistance

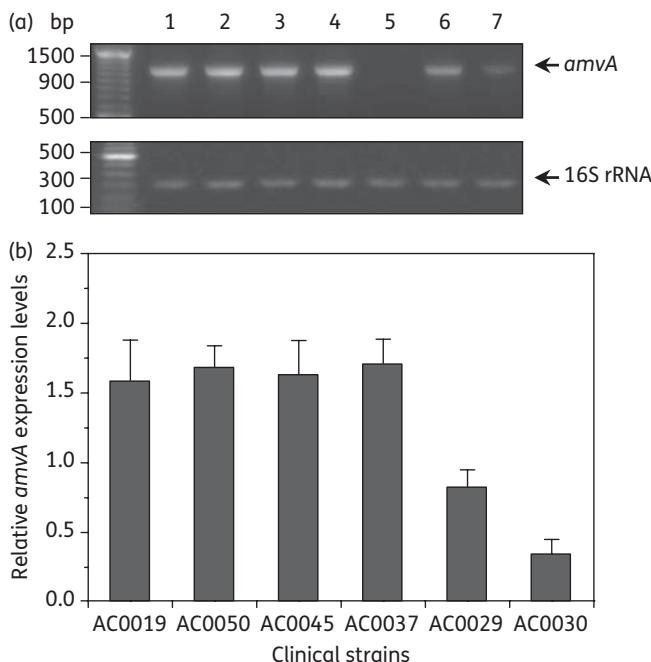


**Figure 2.** Effect of the addition of CCCP on the accumulation of ethidium bromide (a) or acriflavine (b) by *A. baumannii* AC0037, *A. baumannii* amvA null mutant (AC0037 $\Delta$ amvA) and *E. coli* KAM32. The accumulation study using 10 mg/L substrate was performed as described previously.<sup>15</sup> The arrow indicates the timepoint when the inhibitor was added to the assay mixture. The relative fluorescence intensity (y-axis) represents the level of accumulated ethidium bromide or acriflavine. The graph shows the fluorescence value of the supernatant collected in the absence (5–20 min duration) and presence of inhibitor CCCP (25–40 min duration). Each datapoint represents the mean $\pm$ SD of three independent experiments.

phenotype of *A. baumannii*.<sup>24,25</sup> In this study, we characterized the functions of a putative efflux pump designated as AmvA from a clinical strain, *A. baumannii* AC0037.

Expression of amvA in the heterologous host *E. coli* KAM32 caused decreased susceptibility to several agents, such as Acriflavin, acriflavine, benzalkonium chloride, DAPI, deoxycholate, ethidium bromide, Methyl Viologen, SDS and TPPCl. Members of the DHA2 family, such as QacA of *S. aureus*, are known to confer resistance to a wide range of structurally dissimilar monovalent and bivalent cationic antimicrobial compounds.<sup>5</sup> KmrA of *K. pneumoniae* is involved in mediating resistance against acriflavine, DAPI, Hoechst 33342, TPPCl, Methyl Viologen and ethidium bromide.<sup>7</sup> LfrA of *Mycobacterium smegmatis* is known to confer resistance to acriflavine, benzalkonium chloride, ethidium bromide and fluoroquinolones.<sup>26</sup>

The efflux gene was inactivated in the MDR clinical isolate *A. baumannii* AC0037 and its role in conferring resistance to



**Figure 3.** Expression of the *amvA* gene from different clinical strains of *A. baumannii*. (a) RT-PCR analysis of the *amvA* gene. Lane 1, AC0019; lane 2, AC0050; lane 3, AC0045; lane 4, AC0037; lane 5, AC0037Δ*amvA*; lane 6, AC0029; and lane 7, AC0030. (b) Relative expression (*n*-fold) determined from data in (a) by densitometric analysis against the expression level of pansusceptible strain AC0030. The expression of the 16S rRNA gene was used as the internal control. *amvA* expression was not found in AC0037Δ*amvA*. Each bar represents the average value of two independent experiments and the error bars represent the standard deviations.

broad-spectrum compounds was confirmed. The expression of AmvA was found to be higher in a few representative clinical isolates in our study that exhibited very high MICs of carbapenems, cephalosporins, aminoglycosides and fluoroquinolones. Studies are warranted to decipher the influence of AmvA on the antimicrobial resistance phenotype of *A. baumannii* clinical isolates in different geographical areas.

## Conclusions

In conclusion, using genetic tools, we have demonstrated the importance of the AmvA multidrug efflux pump, belonging to the MFS family, in mediating antimicrobial resistance for the first time in an MDR clinical isolate, *A. baumannii* AC0037.

## Sequence accession number

The nucleotide sequence data reported in this paper have been deposited in the GenBank nucleotide sequence database under accession number FJ911554.

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## Transparency declarations

None to declare.

## Supplementary data

Figure S1 is available as Supplementary data at JAC Online (<http://jac.oxfordjournals.org/>).

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