Contribution of efflux to colistin heteroresistance in a multidrug resistant Acinetobacter baumannii clinical isolate

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Abstract

Purpose. The mechanisms underlying colistin heteroresistance in Acinetobacter baumannii are not fully understood. Here, we investigated the role of efflux in colistin-heteroresistant populations of a multidrug-resistant (MDR) A. baumannii clinical isolate.

Methodology. Three colistin-resistant A. baumannii strain variants isolated from the same clinical sample were studied for the presence of heteroresistance to colistin by drug susceptibility testing, genotyping and drug resistance target mutation analysis. The existence of active efflux was studied by synergism assays with efflux inhibitors, real-time efflux activity measurements and analysis of the mRNA transcriptional levels of selected efflux pump genes in response to colistin.

Results. All of the strain variants belong to the ST218, international clonal lineage II. Different colistin susceptibility levels were observed among the three strain variants, indicating that colistin-heteroresistant subpopulations were being selected upon exposure to colistin. No mutations were found in the genes lpxACD and pmrAB, which are associated with colistin resistance. The results showed the existence of synergistic interactions between efflux inhibitors and colistin and ethidium bromide. Real-time efflux assays demonstrated that the three strain variants had increased efflux activity that could be inhibited in the presence of the inhibitors. The efflux pump genes adeB, adeJ, adeG, craA, amvA, abeS and abeM were found to be overexpressed in the strain variants in response to colistin exposure.

Conclusion. This study shows that efflux activity contributes to colistin heteroresistance in an MDR A. baumannii clinical isolate. The use of efflux inhibitors as adjuvants of the therapy can resensitize A. baumannii to colistin and prevent the emergence of drug resistance.

INTRODUCTION

Acinetobacter baumannii is a major pathogen that is responsible for hospital- and community-acquired infections. Its increased capacity to develop antibiotic resistance, combined with the ability to persist in hospital environments, makes A. baumannii a public health concern [1]. Carbapenems are the first choice to treat multidrug-resistant (MDR) A. baumannii infections and, as such, resistance rates to carbapenems are increasing worldwide [2]. Tigecycline and minocycline have been used to treat carbapenem-resistant A. baumannii strains [3], but resistance to both has already been reported [4, 5]. As a consequence, colistin is one of the last resources to treat these infections [6]. Although the incidence of colistin-resistant strains remains low, it is increasing due to intensified use in clinical settings [7–10].
Resistance to polymyxins differs among Gram-negative bacterial species and includes lipopolysaccharide (LPS) modification, complete loss of LPS, capsule formation, MgrB truncation, plasmid-mediated mcr genes and efflux mechanisms [11, 12]. Colistin acts through the disruption of the outer membrane. Colistin resistance in A. baumannii may occur due to the presence of mutations in the genes involved in lipid A biosynthesis (lpxA, lpxC and lpxD), leading to the complete loss of LPS [13] or, due to mutations in the genes that encode the two component system PmrAB, which are responsible for PmrC activation and phosphoethanolamine addition to lipid A [14, 15]. Although a controversial topic, colistin resistance has also been associated with efflux mechanisms [16–19]. Several efflux pumps encoded by A. baumannii have been implicated in the development of MDR phenotypes. Nevertheless, these are rarely and indirectly associated with colistin resistance or heteroresistance [20].

It is known that, besides the well-described heteroresistance of bacteria from clinical isolates of patients under therapy, some strains of A. baumannii harbour a small persistent proportion of colistin-resistant cells in their populations [6]. Under selective pressure, these persistent colistin-resistant variants rapidly overtake the susceptible population and give rise to isolates with high-level resistance to colistin [6, 13]. In this study, we evaluated the contribution of efflux mechanisms to the maintenance of a colistin-resistance profile without the presence of known drug resistance-associated mutations in an A. baumannii clinical isolate. Our aim was to provide a better understanding of the mechanisms underlying the efflux-mediated response and the development of heteroresistance to colistin by identifying the mechanisms involved, and to avert the development of heteroresistance.

**METHODS**

**Isolation, characterization and drug susceptibility testing**

The three A. baumannii strain variants were isolated from the same bronchial secretion specimen collected from a patient hospitalized in an intensive care unit presenting with pneumonia and a urinary tract infection after bladder reconstruction due to a carcinoma. The sample was collected before initiation of the therapy for A. baumannii infection. The specimen was cultured on blood agar plates at 37°C, 5% CO2 for 48–72 h. Single colonies were subcultured for identification and drug susceptibility testing (DST) using the Vitek GNI+cards (bioMérieux, Marcy l’Etoile, France). Due to discrepancies in the colistin susceptibility testing results obtained for different colonies, three of these were selected and sent to our laboratory for further analysis. These were named AB1, AB2 and AB10, and are referred to as strain variants throughout this study.

Confirmatory DST was performed at our laboratory by disk diffusion (Oxoid, UK) (Table S1, available in the online version of this article), Etest for colistin (bioMérieux) and using a selective culture medium to detect Gram-negatives presenting reduced susceptibility to polymyxins (SuperPolymyxin medium) [21, 22]. The determination of the minimum inhibitory concentrations (MICs) of colistin sulphate (Sigma-Aldrich, MO, USA) was performed by the two-fold broth microdilution method (reference method) using non-coated microtitre polystyrene plates (Greiner, Frickenhausen, Germany) in cation-adjusted Mueller–Hinton broth (CAMHB, Oxoid) [23]. The MICs were also assessed using the twofold broth macrodilution method in CAMHB using glass test tubes. The results were interpreted according to the EUCAST clinical breakpoints [21].

The morphology of the colonies was evaluated using MacConkey agar (Difco, Becton and Dickinson, Sparks, MD, USA). The growth rates were assessed on CAMHB by measuring the OD600 hourly from 0 to 8 h and after 24 h of incubation at 37°C.

*A. baumannii* ATCC 19606 (hereafter referred to as ATCC) was used as a control. For the testing using the SuperPolymyxin medium, A. baumannii ATCC and Klebsiella pneumoniae FF61198 were included as polymyxin-susceptible and polymyxin-resistant control strains, respectively.

**Genotyping by ERIC-PCR and MLST**

Strain similarity was analysed using the enterobacterial repetitive intergeneric consensus sequence-based PCR (ERIC-PCR) with the primers ERIC1 and ERIC2 [24] as described in the Supplementary Material. Multilocus sequence typing (MLST) was carried out using seven housekeeping genes (gltA, gyrB, recA, cnp60, gdhB, gpi and rpoD) according to Bartual et al. [25].

**PCR amplification and DNA sequencing of genes associated with colistin resistance**

The presence of mutations in the genes lpxA, lpxC, lpxD, pmrA, pmrB and pmrC was investigated by PCR using the primers and conditions described in Table S2. The presence of the mcr-1 gene was investigated by PCR as described previously [26].

**Membrane permeability evaluation**

The strains were grown at 37°C, until they reached an OD600 of 0.6. The samples were incubated with the efflux inhibitors at concentrations ranging from 90 to 22.5 µM for 1 h at room temperature. Afterward, the cells were collected by centrifugation at 16 060 g for 10 min, the supernatant was discarded, and the pellet was resuspended in the same volume of a saline solution and assayed for membrane damage using the Live/Dead BacLight Bacterial Viability kit (Molecular Probes, Life Technologies, Oregon, USA) according to the manufacturer’s instructions. Fluorescence was measured using a Synergy HT multi-mode microplate reader (BioTek Instruments, Vermont, USA) with the following filters: 485/20 (excitation) and 528/20 (emission) for green and 590/35 (emission) for red. The green to red ratio was determined, the values were normalized and the results are presented as the percentage of intact membranes (±SD) compared with the control (no treatment).
Synergism assays with efflux inhibitors

The MICs of the efflux inhibitors, antibiotics and EtBr were determined as described above. The efflux inhibitors tested were verapamil, thioridazine, chlorpromazine, 1-[1-naphthylmethyl]-piperazine (NMP), phe-arg-β-naphthylamide (PAβN) and carbonyl cyanide-m-chlorophenylhydrazone (CCCP). The MICs of the antibiotics and EtBr were determined in the presence of the efflux inhibitors at the same molar concentration of 22.5 µM (≤1/8 MIC; Table 1), a concentration that showed no effect on the membrane permeability of these strains (Fig. S1). The modulation factor (MF) [27] was used to quantify the effect of each efflux inhibitor on the MIC values.

Evaluation of efflux activity by real-time fluorometry

The accumulation and efflux of EtBr was evaluated by a semi-automated fluorometric method using a Rotor-Gene 3000 thermocycler (Corbett Research, Sydney, Australia) [28, 29]. The EtBr accumulation and efflux assays were performed as previously described [30], except that the efflux inhibitors were tested at 22.5 µM. The effect of the inhibitors was quantified through the determination of the relative final fluorescence (RFF) index as described previously [31]. For the efflux assays, we selected thioridazine, since it was the most efficient efflux inhibitor in promoting intracellular accumulation of EtBr at the concentration tested. Each assay was performed in triplicate and the results are presented as the average of three independent assays (±SD).

PCR amplification of efflux pump-encoding genes

Selected genes encoding efflux pumps in A. baumannii were screened by PCR with the primers and conditions described in Table S3.

### Table 1. MICs of colistin, EtBr and efflux inhibitors against the A. baumannii isolates, as determined by broth microdilution

<table>
<thead>
<tr>
<th>Compound</th>
<th>ATCC</th>
<th>AB1</th>
<th>AB2</th>
<th>AB10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antibiotic</td>
<td>µg ml⁻¹</td>
<td>µM</td>
<td>µg ml⁻¹</td>
<td>µM</td>
</tr>
<tr>
<td>COL</td>
<td>2</td>
<td>–</td>
<td>8</td>
<td>–</td>
</tr>
<tr>
<td>EtBr</td>
<td>512</td>
<td>–</td>
<td>1024</td>
<td>–</td>
</tr>
<tr>
<td>Efflux inhibitors</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TZ</td>
<td>64</td>
<td>157.3</td>
<td>64</td>
<td>157.2</td>
</tr>
<tr>
<td>CPZ</td>
<td>64</td>
<td>180.1</td>
<td>64</td>
<td>180.1</td>
</tr>
<tr>
<td>VP</td>
<td>2048</td>
<td>4170.6</td>
<td>1024</td>
<td>2085.3</td>
</tr>
<tr>
<td>PAβN</td>
<td>1024</td>
<td>1971.2</td>
<td>1024</td>
<td>1971.2</td>
</tr>
<tr>
<td>NMP</td>
<td>512</td>
<td>2262.3</td>
<td>256</td>
<td>1131.2</td>
</tr>
<tr>
<td>CCCP</td>
<td>64</td>
<td>312.8</td>
<td>32</td>
<td>156.4</td>
</tr>
<tr>
<td>EtBr</td>
<td>512</td>
<td>–</td>
<td>1024</td>
<td>–</td>
</tr>
</tbody>
</table>

CCCP, carbonyl cyanide-m-chlorophenylhydrazone; COL, colistin; CPZ, chlorpromazine; EtBr, ethidium bromide; MIC, minimum inhibitory concentration; NMP, [1-(1-naphthylmethyl)-piperazine]; PAβN, phe-arg-β-naphthylamide; TZ, thioridazine; VP, verapamil. –, not applicable.

Efflux pump gene expression analysis

RNA extraction

The strains were grown overnight in CAMHB with and without colistin at half MIC. Total RNA was isolated using the Direct-zol RNA kit (Zymo Research, California, USA) according to the manufacturer’s instructions. RNA was treated with RNase-free DNase I (QIAGEN, Hilden, Germany) for 30 min at room temperature.

cDNA synthesis and quantitative real-time PCR

cDNA was prepared with 0.5 µg of each RNA sample using random priming with the NZY First-Strand cDNA Synthesis kit (Nzytech, Lisbon, Portugal) as per the manufacturer’s protocol. cDNA was mixed with PowerUp SYBR Green Master Mix (Applied BioSystems, Life Technologies) and 10 pmol of each primer (Table S3). The efflux pump genes evaluated were adeB, adef, adeG, craA, amvA, abeS and abeM. The relative expression of the efflux pump genes was determined by comparing the relative quantity of mRNA in the presence of colistin to that in the non-exposed condition using the comparative quantification 2⁻ΔΔCt method [32]. A level of relative expression equal to 1 indicated that the expression level was identical to that for the non-exposed strain. Genes showing expression levels above 1, when compared with the non-exposed strain, were considered to be overexpressed. Relative expression levels above 2 were considered to be significantly overexpressed. The data were normalized to the A. baumannii 16S rDNA reference gene.

Time–kill assays

Time–kill assays were performed as previously described [30]. Colistin was tested at ¼ MIC (Table 2; macrodilution MIC) with and without 22.5 µM thioridazine (the most efficient efflux inhibitor). The cultures were sampled for the determination of colony-forming units (c.f.u.) ml⁻¹ hourly from 0 to 6 and after 24 h of incubation at 37°C.
colonies were counted after 24 h of incubation at 37 °C. The limit of detection of the assay was 20 c.f.u. ml⁻¹. Each assay was repeated twice.

Statistical analysis
Statistical analysis was carried out using Student’s t-test. A P value of <0.05 was considered to be statistically significant, and P values of <0.01 and <0.001 were considered to be highly significant (two-tailed tested).

RESULTS

Growth features and genetic relationship
The three A. baumannii strain variants exhibited different colony morphologies and growth rates (Fig. 1). AB2 displayed round and shiny colonies that were similar to those produced by the ATCC strain (Fig. 1a). AB10 was characterized by larger, opaque and flattened colonies, whereas AB1 combined two types of colonies: the first type was small and shiny and the second resembled those presented by AB2 and the ATCC strain. When grown in CAMHB, all produced a homogenous growth, although they varied in their growth rates (Fig. 1b). AB1 and ATCC presented similar growth rates, whereas AB10 grew more quickly. By contrast, AB2 showed impaired growth, grew slowly, and at the end of 24 h it had not been able to reach the same OD₆₀₀. The heteroresistance phenotype was stable after growth in colistin-free medium and the colony morphology remained unchanged.

ERIC-PCR analysis confirmed the isogenic nature of the three strain variants (Fig. 1c). MLST analysis revealed that all of the colistin-resistant variants belonged to the ST218, clonal complex (CC) 92, international clonal lineage II.

Antimicrobial resistance
The colistin susceptibility results are presented in Table 2. According to the Vitek method, the strain variants only differed from each other in their susceptibility to colistin. AB1 and AB2 were colistin-susceptible and AB10 was colistin-resistant. Colistin susceptibility was further assayed by Etest, and using this method only AB1 was found to be susceptible to colistin. However, discrete colonies were observed within the zone of inhibition, indicating the presence of heteroresistant subpopulations among the strain variants.

Detection of colistin resistance-associated mutations
Analysis of the complete lpxACD and pmrCAB operons showed no mutations resulting in amino acid changes among the colistin-resistant strain variants. The mcr-1 gene was not detected in these isolates.

Synergistic activity between colistin and efflux inhibitors
The MICs of colistin and EtBr were determined in the presence of the efflux inhibitors (Table 3). Thioridazine, NMP and CCCP were the most efficient compounds in decreasing the MICs of colistin for all isolates, except for AB10, yielding 8- to 256-fold MIC decreases. With respect to EtBr, NMP and CCCP reduced the MICs by 8- to 1024-fold for the ATCC and AB1, while thioridazine only reduced the MICs by 2-fold. To give a better overview of the impact of efflux pump activity on the resistance phenotype of the strain variants, we also performed

<table>
<thead>
<tr>
<th>Table 2. Colistin susceptibility determined using different methods</th>
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<tbody>
<tr>
<td>A. baumannii</td>
</tr>
<tr>
<td>----------------</td>
</tr>
<tr>
<td>ATCC</td>
</tr>
<tr>
<td>AB1</td>
</tr>
<tr>
<td>AB2</td>
</tr>
<tr>
<td>AB10</td>
</tr>
</tbody>
</table>

*Colistin breakpoints: susceptible, ≤2 µg ml⁻¹; resistant, >2 µg ml⁻¹ [21].
†Colistin resistance, growth at 3.5 µg ml⁻¹; colistin susceptibility, no growth at 3.5 µg ml⁻¹ [22].

MIC, minimum inhibitory concentration.
synergism assays for other antibiotics (Table S4). It was noted that there was a general decrease of the MICs for the majority of the antibiotics in the presence of CCCP, suggesting the involvement of efflux pumps that are mainly dependent on the proton motive force in their resistance phenotype.

![Fig. 1. Growth features and genetic relationship of the A. baumannii strain variants. (a) Morphology of the colonies presented by the A. baumannii strain variants and the ATCC strain grown on MacConkey agar after 24 h of incubation at 37 °C. (b) Growth curves of the A. baumannii strain variants and the ATCC strain monitored for 24 h. (c) ERIC-PCR patterns for the A. baumannii strain variants and the ATCC strain.](image)

**K. pneumoniae FF61198**
MIC colistin: 128 µg ml⁻¹

**A. baumannii AB1**
MIC colistin: 8 µg ml⁻¹

**A. baumannii AB2**
MIC colistin: 32 µg ml⁻¹

**A. baumannii AB10**
MIC colistin: 128 µg ml⁻¹

![Fig. 2. Evaluation of the colistin resistance of the strain variants growing on SuperPolymyxin medium. The three A. baumannii strain variants were able to grow on the SuperPolymyxin medium that contained colistin at 3.5 µg ml⁻¹. The A. baumannii strain variants produced light lavender (characteristic of non-fermenting Gram-negative bacteria) and dark-centred mucoid colonies. The dark centres are more evident in AB1 and AB10 than in AB2. No growth was obtained for the ATCC strain, since it is susceptible to colistin. K. pneumoniae, used as colistin-resistant positive control, produced dark blue/brown mucoid colonies, which are characteristic of lactose-fermenting Gram-negative bacteria.](image)
**Table 3. Synergistic effect of efflux inhibitors on the microdilution MIC values of colistin and EtBr against the *A. baumannii* isolates**

<table>
<thead>
<tr>
<th>Strain</th>
<th>No EI</th>
<th>+TZ</th>
<th>+CPZ</th>
<th>+VP</th>
<th>+PAβN</th>
<th>+NMP</th>
<th>+CCCP</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATCC (COL&lt;sup&gt;0&lt;/sup&gt;)</td>
<td>6</td>
<td>0.025</td>
<td>0.025</td>
<td>32</td>
<td>32</td>
<td>32</td>
<td>32</td>
</tr>
<tr>
<td>AB1 (COL&lt;sup&gt;0&lt;/sup&gt;)</td>
<td>128</td>
<td>128</td>
<td>128</td>
<td>128</td>
<td>128</td>
<td>128</td>
<td>128</td>
</tr>
<tr>
<td>AB2 (COL&lt;sup&gt;0&lt;/sup&gt;)</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>AB10 (COL&lt;sup&gt;0&lt;/sup&gt;)</td>
<td>1024</td>
<td>1024</td>
<td>1024</td>
<td>1024</td>
<td>1024</td>
<td>1024</td>
<td>1024</td>
</tr>
<tr>
<td>AB10 (COL&lt;sup&gt;0&lt;/sup&gt;)</td>
<td>1024</td>
<td>1024</td>
<td>1024</td>
<td>1024</td>
<td>1024</td>
<td>1024</td>
<td>1024</td>
</tr>
</tbody>
</table>

Changes ≥fourfold are indicated in boldface. Efflux inhibitors (EIs) were used at 22.5 µM. A fourfold or greater reduction in the MIC values of COL/EtBr in combination with the EIs was considered significant. CCCP, carbonyl cyanide-m-chlorophenylhydrazone; COL, colistin; CPZ, chlorpromazine; EI, efflux inhibitor; EtBr, ethidium bromide; MF, modulation factor; MIC, minimum inhibitory concentration; NMP, 1-[1-naphthylmethyl]piperazine; PAβN, phe-arg-β-naphthylamide; R, resistant; S, susceptible; TZ, thioridazine; VP, verapamil.

**Assessment of real-time efflux activity**

Afterward, the efflux inhibitors were evaluated for their ability to inhibit the efflux activity of the strain variants on a real-time basis, using EtBr as fluorescent efflux substrate. The EtBr accumulation/efflux equilibrium concentration was determined as follows: 0.25 µg ml<sup>-1</sup> for the ATCC and AB1; 0.5 µg ml<sup>-1</sup> for AB2, and 1 µg ml<sup>-1</sup> for AB10. These results showed that AB2 can handle higher concentrations of EtBr than their counterparts and the ATCC strain, which is suggestive of the presence of more active efflux systems in this strain variant. The inhibitor that presented the highest EtBr accumulation index was thioridazine, followed by chlorpromazine, verapamil and NMP (Fig. 3a; Table 4).

To further confirm the efflux activity of the strain variants, we performed EtBr efflux assays in the presence of thioridazine. As shown in Fig. 3(b), efflux takes place in the presence of glucose, which can be inhibited in the presence of thioridazine. The results also confirmed that AB2 possesses higher efflux activity, followed by AB1 and the ATCC strain, in comparison with AB10, which revealed reduced EtBr efflux activity (Fig. 3b).

**Efflux pump gene expression analysis in response to colistin**

To connect the physiological evidence found above, which is suggestive of the presence of active efflux systems, with the molecular genetic basis expected to support it, we analysed the effect of exposure to subinhibitory concentrations of colistin on the expression levels of seven efflux pumps of *A. baumannii* that are known to have EtBr as a substrate [20, 35] (Fig. 3c). The results showed a different pattern of expression between the ATCC and the strain variants. The ATCC strain predominantly expressed the *amvA*, followed by *craA*, although the latter was expressed at low levels. Among the strain variants, AB1 did not overexpress any of the pumps tested, whereas AB2, with the sole exception of *adeG*, presented increased expression of all of the genes, in particular *abeM*, *craA* and *abeS*. AB10 also overexpressed all of the genes, with *adeM* being predominant.

**Synergy studies with time-kill assays**

Time-kill studies were performed to evaluate the time-response profiles of colistin alone and in combination with thioridazine (Fig. 4). After 24 h of exposure to colistin alone (at ¼ MIC) and to thioridazine alone (at 22.5 µM, 1/8 MIC), bacterial regrowth was observed for both drugs, confirming that they do not affect bacterial viability at the concentrations used. Thioridazine in combination with colistin at subinhibitory concentrations displayed rapid bacterial killing after just 1 to 2 h of exposure. No regrowth was observed at the end of the 24 h period of exposure for any of the cultures. These results showed that the combination between colistin and thioridazine, at subinhibitory concentrations, is bactericidal against colistin-resistant *A. baumannii*.

**DISCUSSION**

Heteroresistance defines the presence of different subpopulations within an isolate that exhibit varying susceptibilities towards an antimicrobial agent [36, 37]. Bacterial heteroresistance can be intrinsic or acquired and the mechanisms involved in its development resemble those involved in general antimicrobial resistance and rely on genetic and non-genetic mechanisms [37]. The genetic mechanisms include the presence of mutations or the gene duplication of
drug-target genes and regulatory systems, while the non-
genetic mechanisms involve the modulation of the bacterial
response to the presence of noxious compounds in the envi-
ronment [38, 39]. Clinically, heteroresistance causes great
concern, as the heteroresistant proportion of the bacterial
population may be selected and become predominant dur-
ing therapy, leading to treatment failure [40]. The purpose
of the present study was to unveil the impact of efflux
mechanisms in the emergence of colistin resistance in
A. baumannii.

The sequence type found by MLST was the ST218 (CC92), a
lineage that is associated with MDR and extensively drug-
resistant A. baumannii and has spread worldwide [41].
Despite their isogenicity, the strain variants presented a
clear heterogeneity in the colonies produced, which is one
of the characteristics of heteroresistant subpopulations [42].

It is recognized that the resistance phenotype and morpho-
types may be unstable, mainly in the case of intrinsic hetero-
resistance, and may reverse after some passages in drug-free
medium [43]. The heteroresistance phenotype of these
strain variants was maintained even when they were grown
without antibiotic pressure. Since the phenotype was stable,
we searched for mutations in the genes that have been
described as being associated with colistin resistance in
A. baumannii [11, 12] in all of the strain variants, but none
were detected. Nevertheless, we cannot exclude the involve-
ment of other genes that have not yet been described as
being associated with colistin resistance in these strain var-
iants, which will be the subject of further investigation. The
maintenance of a heterogeneous phenotypic expression of
resistance has been proposed to occur as an adaptive strat-
egy to ensure bacterial survival under environmental
changes [44]. Taken together, these data indicated that

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**Fig. 3.** Evaluation of the presence of efflux activity in the A. baumannii strain variants. (a) Effect of efflux inhibitors on the accumulation of EtBr. Only the most efficient efflux inhibitors are shown (see Table 4); (b) Effect of thioridazine on the efflux of EtBr. (c) Quantification of the relative mRNA expression levels of a panel of efflux pump genes. The results were considered significant when *P*<0.05, and highly significant when **P**<0.01 and ***P***<0.001.
heteroresistance may be induced by antibiotic treatment in these strain variants and prompt us to explore other mechanisms that could be involved in colistin resistance, namely the contribution of efflux mechanisms to the emergence of resistance to colistin.

The synergism assays showed the existence of a synergistic interaction between the efflux inhibitors and colistin, as well as EtBr, a clear indication that efflux was involved. These results demonstrated that colistin resistance can be reversed by the use of efflux inhibitors such as those tested, pointing towards a role of efflux inhibitors as adjuvants in the treatment of colistin-resistant *A. baumannii* clinical isolates. Our synergism data are in accordance with a previous study [18] in which it was shown that colistin resistance can be reduced in combination with CCCP in *A. baumannii* clinical isolates. Our study demonstrated the same effect for compounds that are used in clinical practice (e.g. thioridazine or chlorpromazine). These inhibitors have a wide range of activity, acting on several efflux pumps rather than being specific for a single efflux system [45]. While the mode by which these compounds inhibit drug efflux is not yet fully understood, it is known that these inhibit drug efflux due to their interference with the energy required for the activity of the pumps rather than inhibiting the pump [30]. Is plausible to assume that these compounds behave similarly with respect to *A. baumannii* efflux systems. Nevertheless, more studies will be necessary to confirm this hypothesis.

We did not observe a straight relationship between the effect of the inhibitors on the MIC values of colistin and the efflux pump gene expression assays. Our analysis revealed a non-specific and increased response to the exposure to colistin elicits a global stress response in these strain variants. The ATCC strain demonstrated a more restricted response, only showing overexpression of the *amvA* and *craA* genes. In contrast, AB1 did not express any of the genes evaluated, revealing a resistance phenotype related to an early stage of exposure to colistin, with a slow awakening

### Table 4. Relative final fluorescence values (RFF) based on the accumulation of EtBr for the *A. baumannii* strain variants and the ATCC strain in the presence of efflux inhibitors, and correlation with colistin phenotype and genotype

<table>
<thead>
<tr>
<th></th>
<th>ATCC</th>
<th>AB1</th>
<th>AB2</th>
<th>AB10</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>COL phenotype</strong></td>
<td>COL^R-</td>
<td>COL^R-</td>
<td>COL^R-</td>
<td>COL^R-</td>
</tr>
<tr>
<td><strong>COL genotype</strong></td>
<td>Wild-type</td>
<td>Wild-type</td>
<td>Wild-type</td>
<td>Wild-type</td>
</tr>
<tr>
<td><strong>RFF of the efflux inhibitors</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VP</td>
<td>0.24±0.04</td>
<td>1.10±0.04*</td>
<td>0.43±0.00*</td>
<td>0.81±0.03*</td>
</tr>
<tr>
<td>TZ</td>
<td>1.03±0.06</td>
<td>2.02±0.05*</td>
<td>8.19±0.83*</td>
<td>4.82±0.34***</td>
</tr>
<tr>
<td>CPZ</td>
<td>0.20±0.02</td>
<td>0.58±0.21</td>
<td>3.13±0.05***</td>
<td>1.30±0.37*</td>
</tr>
<tr>
<td>PAβN</td>
<td>-0.35±0.10</td>
<td>-0.03±0.01</td>
<td>-0.02±0.08</td>
<td>0.16±0.005</td>
</tr>
<tr>
<td>NMP</td>
<td>-0.20±0.09</td>
<td>0.08±0.02</td>
<td>1.78±0.12</td>
<td>-0.03±0.02</td>
</tr>
<tr>
<td>CCCP</td>
<td>-0.54±0.02*</td>
<td>0.02±0.01</td>
<td>-0.19±0.05**</td>
<td>1.22±0.11**</td>
</tr>
</tbody>
</table>

Values in boldface (RFF≥1) indicate enhanced accumulation of EtBr in the presence of an efflux inhibitor. Negative values indicate that isolates accumulate less EtBr in the presence of an efflux inhibitor than those in the drug-free condition. The results were considered significant when *P*<0.05, and highly significant when **P**<0.01 and ***P**<0.001. CCCP, carbonyl cyanide-m-chlorophenylhydrazone; COL, colistin; CPZ, chlorpromazine; EtBr, ethidium bromide; MIC, minimum inhibitory concentration; NMP, 1-[1-naphthylmethyl)piperazine; PAβN, phe-arg-β-naphthylamide; R, resistant; S, susceptible; TZ, thioridazine; VP, verapamil.

**Fig. 4.** Time–kill curves for colistin in the presence and absence of thioridazine. Colistin was tested at ¼ MIC (see Table 2 for MIC values) with and without 22.5 µM of thioridazine. A drug-free control was included in each assay. Samples were collected hourly from 0 to 6 h and after 24 h to determine bacterial viability.
of its efflux pumps. The normal levels of expression of ABI’s efflux pumps and its activity is sufficient to acquire a low-level resistance to colistin by efflux, as confirmed by the reduction of colistin resistance by the efflux inhibitors (Table 3) and the increased efflux activity detected by the real-time fluorometry assays (Table 4). Overall, these results showed the different efflux-mediated responses that the colistin-heteroresistant subpopulations can display during treatment with colistin. The outcome will depend on the variant selected.

The role of efflux pumps in colistin-heteroresistant populations and their contribution to the establishment of drug-resistant phenotypes in MDR A. baumannii clinical isolates has not, to the best of our knowledge, been described before. Only recently, the Emr transporter was found to be associated with adaptation to osmotic stress and resistance to colistin in A. baumannii [46]. Our study provided evidence that efflux is an important player in the emergence of phenotype resistance to colistin in A. baumannii. Treatment of A. baumannii infections is problematic due to its widespread MDR, the existence of heteroresistance and the lack of novel drugs for the treatment of these serious infections. The use of an efflux inhibitor as an adjuvant to the therapy can resensitize A. baumannii to colistin, as demonstrated in this study, and may be considered as a last-resource therapeutic option for the control of A. baumannii MDR infections.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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