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Efflux in *Acinetobacter baumannii* can be determined by measuring accumulation of H33342 (bis-benzamide)

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Objectives: Overexpression of efflux pumps in *Acinetobacter baumannii* is a common mechanism of multidrug resistance in this nosocomial pathogen. Increased efflux pump expression is often assumed from MICs of antibiotics and dyes, without measurement of efflux levels. This study describes a safe, rapid and simple 96-well plate assay that measures the accumulation of a fluorescent dye, Hoechst (H) 33342.

Methods: The growth kinetics of three resistant and three susceptible Singaporean clinical isolates of *A. baumannii* in the presence of carbonyl cyanide-*m*-chlorophenylhydrazone (CCCP) and phenylalanine-arginine-β-naphthylamide (PAβN) were studied to determine non-inhibitory concentrations for use in the assay. Accumulation of H33342 was measured in these clinical isolates with and without efflux inhibitors. Accumulation was also measured in an *adeB* efflux pump deletion mutant and its parental strain to assess the ability of the assay to identify altered efflux in strains lacking efflux pumps. Results were compared with data from accumulation assays with ethidium bromide and norfloxacin.

Results: Increased accumulation, indicative of reduced efflux, was observed in AB211Δ*adeB* compared with parental strain AB211. Clinical isolates demonstrated different levels of accumulation of H33342. The addition of both CCCP and PAβN significantly increased the accumulation of H33342. The pattern of norfloxacin accumulation broadly reflected H33342 accumulation. Ethidium bromide showed a different pattern of accumulation in clinical isolates.

Conclusions: The measurement of the intracellular accumulation of H33342 in real time allowed a comparison of efflux activity between strains of *A. baumannii*.

Keywords: MDR, resistance–nodulation–division, antibiotics

Introduction

Acinetobacter baumannii is a Gram-negative nosocomial pathogen, capable of causing opportunistic infections such as pneumonia, skin and soft tissue infections, urinary tract infections and bacteraemia in immunocompromised patients, particularly those in the intensive care unit. Isolates are often multidrug[1] or even pan-drug[2] resistant and are able to resist desiccation; therefore, they persist for long periods of time in the hospital environment.[3,4] As a consequence, this pathogen poses a major threat to human health worldwide.

A. baumannii exhibits intrinsic multiple drug resistance (MDR) to a range of antibiotics due to chromosomally encoded enzymes, an innate expression of efflux pumps and low membrane permeability. Various chromosomally encoded efflux systems and outer membrane porins (OMPs) have been identified as contributing to MDR in *A. baumannii*.[5] Best characterized is the AdeABC resistance–nodulation–division (RND) family system, first identified in *A. baumannii* BM4454, an MDR clinical isolate. *adeABC* is overexpressed in some clinical isolates and is associated with resistance to aminoglycosides, β-lactams, fluoroquinolones, tetracyclines, tigecycline, macrolides, chloramphenicol and trimethoprim.[6,7] In BM4454, another RND system, AdeIJK, gives innate MDR and has a different substrate range from AdeABC.[8] When *adeIJK* is inactivated, the MICs of β-lactams, fluoroquinolones, tetracyclines, tigecycline, lincosamides, rifampicin, chloramphenicol, co-trimoxazole, novobiocin and fusidic acid are decreased, while susceptibility to aminoglycosides is

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unaffected.[8,9] The AdeFGH RND system, also identified in BM4454 by the Courvalin team, confers MDR when overexpressed, but did not contribute to innate resistance to the antibiotics tested.[9,10] Inactivation of AdeFGH decreased the MICs of fluoroquinolones, chloramphenicol, trimethoprim, clindamycin, tetracyclines, tigecycline and sulfamethoxazole, but not of aminoglycosides or β-lactams.[10] There are also several other chromosomally encoded non-RND efflux pumps in *A. baumannii*, such as CraA in strain ATCC 19606,[11] AmvA in clinical isolate AC0037,[12] AbeM in ATCC 19606[13] and AbeS in AC0037.[14]

Compared with other Gram-negative bacteria, *Acinetobacter* species possess a relatively small number of OMPs with porin-like activity. The expression of OMPs can be regulated in response to antibiotic stress and confer MDR. For example, membrane expression of OmpA38, OmpA32, CarO and OmpW in an MDR strain highly resistant to tetracycline, DU202, has been shown to decrease in the presence of sub-MIC levels of tetracycline.[15] Reduced expression of OMPs has been observed in MDR clinical isolates worldwide and may contribute towards resistance to carbapenems. A reduction in the expression of OMPs has been seen in imipenem-resistant clinical isolates with no identified efflux mechanisms.[16–18]

Levels of efflux and membrane permeability in *A. baumannii* are often inferred from the MICs of antibiotics, and changes in pump expression are assumed without direct measurement. Cellular accumulation of antibiotics in Gram-negative bacteria has previously been assessed using radiochemical and fluorescent techniques.[19,20] Accumulation of ethidium bromide has been measured in *A. baumannii* using a fluorometric assay to assess the contribution of efflux pumps to antibiotic accumulation and drug susceptibility.[7] Ethidium bromide is a DNA intercalating dye; its fluorescence increases when intercalating with DNA, and this feature can be used to differentiate between intracellularly and extracellularly located compound. However, problems have been identified with the use of ethidium bromide as an indicator of cellular accumulation. The compound gives a low quantum yield and is self-quenching at high intracellular levels, so cellular accumulation can appear lower than it actually is.[21] In addition, ethidium bromide is toxic and its use in laboratories is being phased out. Therefore, a safe, rapid and easy method is needed to measure accumulation in *A. baumannii* and provide reliable information on levels of efflux and membrane permeability in this organism.

The bis-benzamide dye Hoechst (H) 33342 is a fluorescent dye that is readily taken up by living cells and fluoresces with a high quantum yield upon binding to DNA and when in the hydrophobic environment of the lipid membrane. It displays low toxicity and low mutagenicity when used at a concentration of 10 μM,[22] making it an ideal fluorescent probe for the measurement of cellular accumulation. H33342 has been used to characterize membrane transport activity in mammalian cells[23] and *Lactococcus lactis*,[24] to investigate the contribution of the ABC efflux pump VcaM to MDR in *Vibrio cholerae*[25] and to assess efflux activity in *Escherichia coli* and *Salmonella enterica*.[25] Previously in this laboratory, an assay of H33342 accumulation was developed as a single, rapid test for MDR Gram-negative Enterobacteriaceae.[26] In the present study, the H33342 accumulation assay was adapted for use with *A. baumannii* and accumulation was studied in several MDR *A. baumannii* clinical isolates and efflux pump gene-deletion mutants.

Methods

Strains, media and chemicals

Nine strains of *A. baumannii* were used throughout. AB211 is a tigecycline-resistant, post-therapy clinical isolate and AB211Δ*adeB* is a mutant created by Hornsey *et al.*[27] by insertional inactivation of *adeB* in AB211. S1, S2, S3, R1, R2 and R3 were provided by the Network for Antimicrobial Resistance Surveillance (Singapore) as clinical isolates that showed an antibiotic resistance profile similar to highly resistant (R) and more susceptible (S) isolates found causing infection in Singaporean hospitals (see Table 1). AYE was used as a reference MDR strain; bioinformatic analyses revealed that AYE was representative of *A. baumannii* as it contained all previously described efflux pump genes and the majority of predicted efflux pumps (AciBASE: http://acinetobacter.bham.ac.uk; L. Evans and L. J. V. Piddock, unpublished data). All clinical isolates and AYE were confirmed as *A. baumannii* using a *gyrB* multiplex PCR.[28,29] All strains were routinely cultured on Luria-Bertani (LB) agar and in LB broth. All chemicals were from Sigma–Aldrich (Poole, Dorset, UK).

Determination of MICs and H33342 toxicity

The MICs of antibiotics and efflux inhibitors (EIs) were determined by the agar doubling dilution method according to BSAC standard methodology.[30] The MICs of imipenem and meropenem were determined by Etest (bioMérieux, Hampshire, UK). Resistance was determined using BSAC-recommended breakpoints for *A. baumannii*. Where MIC breakpoints were unavailable, EUCAST non-species-specific breakpoints were used. MDR was defined as resistance to three or more antimicrobial classes. Toxicity of H33342 was determined by overnight incubation of 1 μL of S1–S3 and R1–R3 at mid-logarithmic growth phase [optical density (OD) at 600 nm of 0.6] at 37°C on agar containing doubling concentrations of H33342.

Growth kinetics

Bacterial strains were grown with aeration in LB broth at 37°C overnight. A 4% inoculum (120 μL in 3 mL) of bacterial culture was added to fresh LB broth. This suspension was incubated with aeration at 37°C until the cells reached an OD at 600 nm of 0.6 (=10^8 cfu/mL). Cells were harvested by centrifugation at 2200 **g** for 10 min at room temperature and resuspended in 3 mL of sterile LB broth. The OD at 600 nm of the suspension was measured and adjusted to 0.5 to standardize the number of cells in each culture and to simulate the conditions used in the H33342 accumulation assay. An aliquot of 196 μL of this suspension was added to each well of a clear 96-well microtitre tray, along with 4 μL of EI at the required concentration (see the Results section). The OD at a wavelength of 600 nm was measured over 16 h in a BMG FLUOstar Optima (BMG, Aylesbury, UK) at 37°C. Each experiment included three biological replicates and three technical replicates for each bacterial strain. The FLUOstar is sensitive to an OD at 600 nm of between 0.0 and 4.0, and reproducibility is ±0.010 for the OD range 0.0–2.0 (www.bmglabtech.com). The difference in final OD at 16 h was calculated, and values returning a P value of ≤0.05 from a Student's t-test were taken as significant.

Accumulation assays of H33342, ethidium bromide and norfloxacin

The H33342 bis-benzamide assay was carried out as described by Coldham *et al.*,[26] with the following modifications; strains were grown to an OD at 600 nm of 0.6 and, once resuspended in PBS at room temperature, were adjusted to an OD at 600 nm of 0.1, 0.2, 0.3 or 0.5. Centrifugation steps were carried out at 2200 **g**. The wells of a black

microtitre tray (Corning, Amsterdam, The Netherlands) were inoculated with either 180 μL of cell suspension or 176 μL of cell suspension with 4 μL of the EI carbonyl cyanide-*m*-chlorophenyl hydrazone (CCCP) or phenylalanine-arginine-β-naphthylamide (PAβN) at the required concentration (see the Results section). Fluorescence was measured and data were analysed as previously described.[26] The level at which maximum fluorescence was reached and remained unchanged within the time period of the assay was taken as the steady-state accumulation level. In order to quantitatively compare the efflux rate of the strains, the time needed for a 4-fold increase in dye fluorescence after H33342 injection was calculated. Each assay was repeated three times with three biological replicates. Differences in accumulation between clinical isolates and AYE were analysed for statistical significance using Student's *t*-test; a P value ≤ 0.05 was considered significant.

Ethidium bromide assays were carried out essentially as the H33342 accumulation assays described above, except that cultures were resuspended in 1 M sodium phosphate buffer with 5% glucose. A 1 mM ethidium bromide stock solution was prepared and 20 μL was injected to give a final concentration of 0.1 mM in the assay. Fluorescence was measured over 117 min at excitation and emission wavelengths of 530 nm and 600 nm, respectively, in a FLUOstar Optima. Norfloxacin assays were carried out as previously by Mortimer and Piddock.[31]

Results

Phenotypic characterization of strains

AYE, R1, R2 and R3 were MDR clinical isolates, with resistance to more than three classes of antibiotics including β-lactams (including carbapenems), fluoroquinolones and aminoglycosides. Isolate R2 was also resistant to colistin. Strain AYE and isolates R2 and R3 were resistant to tigecycline. Isolates S1, S2 and S3 were resistant to fewer antibiotics; S1 and S3 were susceptible to ciprofloxacin, S2 and S3 were susceptible to colistin, S1 was susceptible to tigecycline and S1, S2 and S3 were susceptible to carbapenems (Table 1).

Growth in the presence of EIs and toxicity of H33342

To determine non-inhibitory concentrations of EIs for use in the H33342 accumulation assay, the MICs of CCCP and PAβN for strain AYE and isolates S1–S3 and R1–R3 were determined. For all isolates other than S3, the MICs of CCCP and PAβN were 256 and 1024 mg/L. The MICs of CCCP and PAβN were one dilution lower for isolate S3. Growth kinetics in the presence of sub-MIC concentrations of EIs were then assessed to simulate conditions in the H33342 accumulation assay and ensure no inhibition of growth. Concentrations of EIs that did not restrict growth in the 2 h time period of the H33342 accumulation assay were used in all experiments. Concentrations of 50 μM CCCP and 100 μM PAβN had no effect on growth under conditions used in the H33342 assay (data not shown). The MICs of H33342 and ethidium bromide were >256 mg/L for all isolates.

H33342 can be used to determine efflux in A. baumannii

Previous work in this laboratory has shown that different levels of accumulation of H33342 can be distinguished for different strains of Enterobacteriaceae with an H33342 concentration of 2.5 μM.[26] With the *A. baumannii* isolates studied, accumulation of 2.5 μM H33342 with cultures at an OD at 600 nm of 0.5 was able to detect different levels of accumulation. Heat-killed cells, which rapidly accumulate H33342, were included as a maximum fluorescence level control.

The ability of the assay to identify altered efflux in strains lacking efflux pumps was assessed with AB211 and AB211Δ*adeB*. *AB211ΔadeB* contains an inactivated *adeB* gene, which encodes the inner membrane component of the AdeABC RND efflux pump. Previous studies have shown that this efflux pump deletion mutant is inhibited by a lower concentration of tigecycline than the parental strain.[27] AB211Δ*adeB* accumulated H33342 at an increased rate, with a 4-fold fluorescence increase reached at 331 s after injection compared with 1656 s in the parental strain. The steady-state level of H33342 was also significantly higher in the mutant when compared with the parental strain, suggesting a reduced level of efflux (Figure 1).

When data for the clinical isolates were compared with those for AYE, differing levels of accumulation were seen for isolates S1–S3, R1 and R2 (Figure 2a). S1, S3, R1 and R2 showed significantly higher levels of accumulation of H33342 than AYE, whereas isolate S2 accumulated significantly lower levels of H33342 when compared with AYE. Levels of norfloxacin accumulated in the clinical isolates followed the same pattern as H33342 accumulation.

Table 1. MIC values of clinically relevant antibiotics for *A. baumannii* (mg/L)

Strain	NAL	CIP	KAN	GEN	CAZ	IPM[a]	MEM[a]	PIP	AMP	AMP+SUL	CHL	TGC	TET	ERY	CST	TMP
AYE	>1024	128	>1024	1024	>1024	0.38	>32	512	512	32	512	2	>1024	512	2	>1024
S1	8	0.5	1	<2	>1024	0.094	0.38	<2	32	8	128	0.12	4	8	8	16
S2	32	16	512	2	>1024	0.094	0.5	64	512	64	128	2	16	16	1	8
S3	32	0.5	1	64	>1024	0.094	0.25	32	512	64	128	2	8	8	2	32
R1	1024	8	>1024	>1024	>1024	>32	>32	512	512	32	256	0.5	>1024	512	2	32
R2	>1024	256	>1024	>1024	>1024	>32	>32	256	1024	64	128	1	1024	512	4	128
R3	1024	128	>1024	256	>1024	>32	>32	1024	1024	64	256	2	>1024	1024	2	128

NAL, nalidixic acid; CIP, ciprofloxacin; KAN, kanamycin; GEN, gentamicin; CAZ, ceftazidime; IPM, imipenem; MEM, meropenem; PIP, piperacillin, AMP, ampicillin; AMP+SUL, ampicillin+sulbactam; CHL, chloramphenicol; TGC, tigecycline; TET, tetracycline; ERY, erythromycin; CST, colistin; TMP, trimethoprim.

[a]MIC values determined by Etest.

H33342 accumulation in the presence of EIs

The contribution of different classes of efflux pump to the accumulation of H33342 in strain AYE and clinical isolates S1–S3 and R1–R3 was further investigated with the addition of EIs. The fold change in fluorescence in the steady-state phase ± the addition of EIs was calculated for each isolate.

Addition of CCCP caused a significant increase ($P \leq 0.05$) in the level of H33342 accumulated at steady-state for all isolates with the largest fold changes of 2.15 and 2.00 seen in isolates S2 and R3, respectively (Figure 2b). Isolates S1, S3, R1 and R2 and strain AYE showed fold increases in H33342 accumulation of 1.42–1.80.

Addition of PAβN caused the largest increase in accumulation of H33342 in isolate S3, with a fold change of 1.89 (Figure 2b). There was also a significant increase in accumulation in all other isolates, but the increase was generally significantly greater in the presence of CCCP.

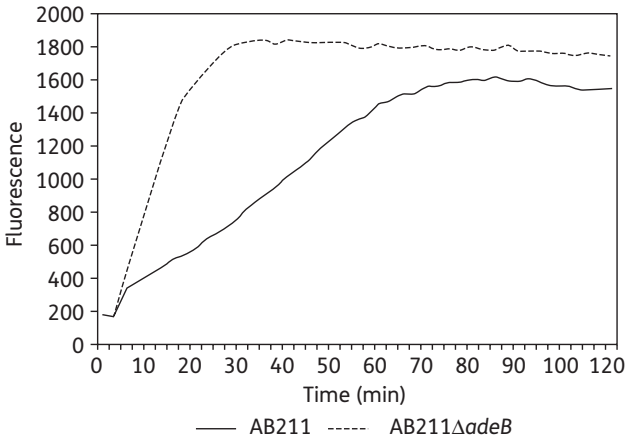

Figure 1. Accumulation of H33342 by AB211 and AB211Δ*adeB*. Data displayed are a representative example of at least three separate experiments.

Ethidium bromide accumulation

To make a comparison with data obtained with H33342, accumulation of ethidium bromide was measured as it is possible that ethidium bromide may be a substrate for different efflux pumps. Isolates S1–S3 and R1–R3 all showed a significant difference in accumulation of ethidium bromide compared with reference strain AYE (Figure 3a). R1 and R2 showed less accumulation than S1–S3. Isolate R3 and strain AYE accumulated more ethidium bromide than R1 and R2, similar to that seen for isolates S1–S3.

Addition of CCCP caused a significant increase ($P \leq 0.05$) in the level of ethidium bromide accumulated at steady-state for strain AYE and isolates R1 and R2, with a 1.54-, 1.60- and 2.59-fold increase, respectively (Figure 3b). Addition of CCCP had a smaller effect on the level of ethidium bromide accumulated in S1, with a 1.10-fold increase observed. CCCP had no effect on the level of ethidium bromide accumulated in S2, S3 and R3.

Addition of PAβN also significantly increased ($P \leq 0.05$) the level of ethidium bromide accumulated in AYE, R1 and R2, with a 1.2-fold increase in strain AYE and a 1.4-fold increase in both R1 and R2 (Figure 3b). PAβN had no effect on the accumulation of ethidium bromide by isolates S1, S2, S3 and R3.

Discussion

Increased efflux as a result of overexpression of efflux pumps is a common mechanism of MDR in *A. baumannii*, and resistance to a wide range of antibiotics can arise from a single mutation in a gene.[6] An increase in the MIC of a number of different classes of antibiotics is often assumed to be the result of an overexpression of efflux pumps, but levels of efflux are not always directly

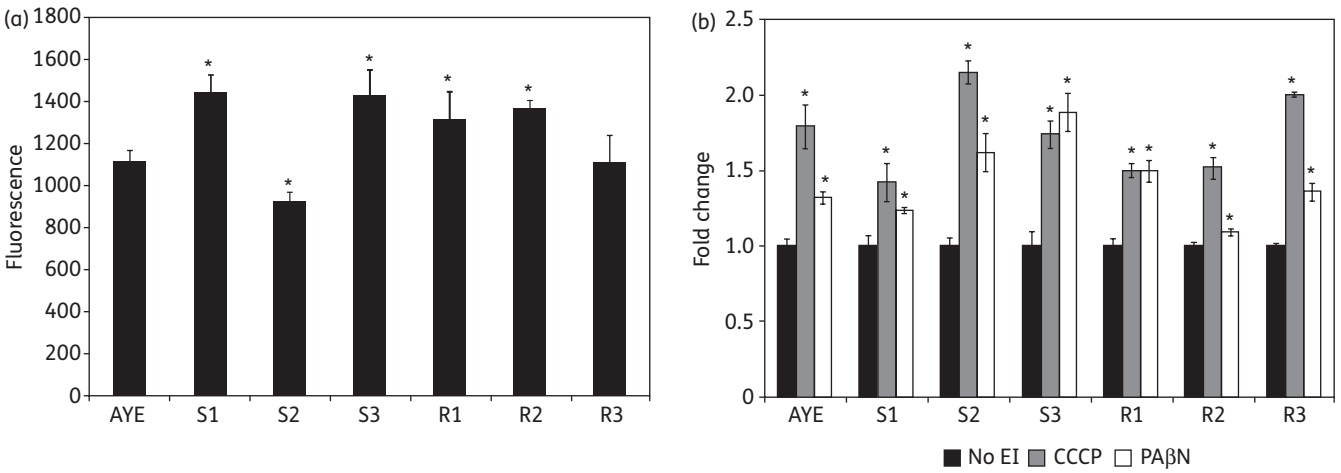

Figure 2. (a) Steady-state levels of accumulation of H33342 by strain AYE and clinical isolates S1, S2, S3, R1, R2 and R3 at 117 min of exposure. *Significant differences in value for the clinical isolates compared with AYE, indicating values returning a *P* value of ≤0.05 from a Student's *t*-test. (b) Fold difference in levels of H33342 accumulated by AYE and clinical isolates S1, S2, S3, R1, R2 and R3 at steady-state ± EI. *Significant differences in value in the presence of EI compared with the absence of EI, indicating values returning a *P* value of ≤0.05 from a Student's *t*-test. Data are displayed as a representative example of at least three separate experiments. The standard deviations represent variations between three biological replicates.

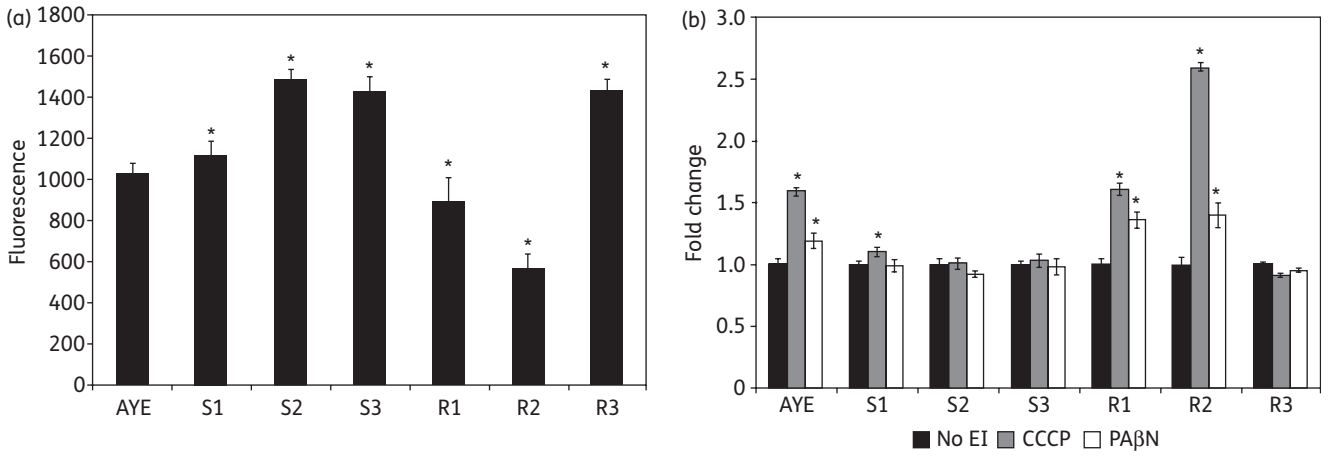

Figure 3. (a) Steady-state levels of accumulation of ethidium bromide by strain AYE and clinical isolates S1, S2, S3, R1, R2 and R3 at 117 min of exposure. *Significant differences in value for the clinical isolates compared with AYE, indicating values returning a P value of $\leq$0.05 from a Student's t-test. (b) Fold difference in levels of ethidium bromide accumulated by AYE and clinical isolates S1, S2, S3, R1, R2 and R3 at steady-state ± EI. *Significant differences in value in the presence of EI compared with the absence of EI, indicating values returning a P value of $\leq$0.05 from a Student's t-test. Data are displayed as a representative example of at least three separate experiments. The standard deviations represent variations between three biological replicates.

measured. A sensitive and specific method is needed so that changes in efflux can be measured safely, quickly and simply.

Measurement of the accumulation of H33342 provides a means of characterizing MDR strains of *A. baumannii* and assessing the role of efflux in antibiotic resistance. The assay is carried out in a 96-well plate and provides a rapid and easy way of inferring levels of efflux by measuring the accumulation of a fluorescent dye. Further assessment of the role of efflux in MDR can be investigated with the addition of EIs to inhibit different classes of efflux pump. This method can also be used to determine whether alterations in outer membrane permeability contribute to the phenotype as changes in dye accumulation due to altered outer membrane protein levels can be assessed.[26]

This study demonstrates the suitability of the H33342 accumulation assay, previously developed in this laboratory to measure H33342 accumulation in Enterobacteriaceae,[26] for use with *A. baumannii*. The H33342 assay revealed differences in levels of accumulation between AYE and five of the six clinical isolates tested.

The addition of EIs highlighted the contribution of different classes of efflux pump to efflux levels in isolates. In AYE, a previously characterized MDR isolate, the addition of CCCP, which dissipates the proton motive force required by several efflux pumps, caused a significant increase in H33342 accumulation, suggesting reduced efflux due to the inhibition of active efflux. The addition of PAβN, which inhibits RND transporters such as *Pseudomonas aeruginosa* MexAB[32] and *E. coli* AcrB,[33] also resulted in higher levels of H33342 accumulation, i.e. reduced levels of efflux. However, this reduction was not as great as that seen with CCCP, suggesting that RND pumps contribute to efflux in AYE or that CCCP perturbs other cellular activities, as found by Henderson.[34]

Accumulation of H33342 also detected a significant difference in dye accumulation in an efflux pump mutant (Figure 1) compared with the parental strain. AB211Δ*adeB*, which lacks the RND pump AdeABC due to deletion of *adeB*, accumulated a much higher level of dye than the parental strain AB211. This effect has been previously shown in the *adeB* mutant, BM4454-1, and its parental strain, BM4454, using an ethidium bromide accumulation assay.[7]

The level of H33342 accumulated at steady-state did not always correlate with the MICs of antibiotics for the clinical isolates. It was hypothesized that strains exhibiting higher MICs of antibiotics would display increased levels of efflux, thus accounting, in part, for their MDR phenotype. *A. baumannii* has been shown to possess various different antibiotic resistance mechanisms.[5] However, the clinical isolates also possessed numerous antibiotic resistance genes, including *tem*, *oxa*, *ampC* and *aph*, and these presumably mask the effect of efflux on the phenotype when measured by MIC.

Comparison of the accumulation of H33342 and ethidium bromide in the clinical isolates of *A. baumannii* revealed different patterns of accumulation of the dyes, suggesting that these two compounds might be substrates for different pumps. Low levels of accumulation of ethidium bromide in isolates R1 and R2 suggest higher levels of efflux and may provide an explanation for the MDR phenotype displayed by these isolates. It may be that ethidium bromide and antibiotics tested in this study are substrates for some of the same efflux pumps. In the presence of CCCP, the efflux of ethidium bromide was most affected in strain AYE and isolates R1 and R2. This was not surprising as these clinical isolates showed the highest level of efflux of this dye. The presence of CCCP gave no significant decrease in efflux in isolates R3 and S2, the isolates that showed the least ethidium bromide efflux. Interestingly, these two isolates were those that showed the highest levels of efflux of H33342 and displayed the largest decrease in efflux of this dye with the addition of CCCP. This suggests that clinical isolates of *A. baumannii* produce efflux pumps with different substrate specificities and these may be at different levels. The addition of PAβN affected the efflux of ethidium bromide in a similar way to CCCP, although the increase in accumulation was not as high as that seen with CCCP.

In summary, the H33342 accumulation assay previously developed for assessment of cellular permeability in Enterobacteriaceae has been adapted to provide a safe, cheap, rapid and sensitive test for the measurement of accumulation in *A. baumannii*. This assay can be used to compare clinical isolates of *A. baumannii* and assess the role of individual efflux pumps or other proteins by comparing deletion mutants with their parental strain. Using EIs, the accumulation of H33342 can be used to evaluate the contribution of efflux pumps to the MDR phenotype commonly seen in clinical isolates. However, the disparity between data acquired with H33342 and ethidium bromide emphasizes the need to use more than one substrate to measure efflux when researching the role of efflux in MDR.

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Transparency declarations

None to declare.

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