Benzalkonium chloride antagonises aminoglycoside antibiotics and promotes evolution of resistance

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ABSTRACT

Background: Biocide disinfectants are essential tools in infection control, but their use can inadvertently contribute to emergence of antibiotic-resistant bacteria. In this study we systematically examine the effect of the biocide benzalkonium chloride, which is primarily used for surface disinfection but is also present as a preservative in many consumer products, on the activity of aminoglycoside antibiotics in \textit{Acinetobacter baumannii}.

Methods: The effect of subinhibitory BAC on aminoglycoside treatment of \textit{A. baumannii} ATCC17978 was investigated using time-to-kill assays, MIC determination, directed evolution experiments, fluctuation tests and labelled gentamicin accumulation assays. Further MIC determinations and directed evolution experiments were performed with additional Gram-negative ESKAPE pathogens.

Findings: In \textit{A. baumannii} ATCC17978, BAC prevents gentamicin killing and drastically increases the frequency at which resistant mutants emerge, through reducing intracellular antibiotic accumulation. BAC also increases the MIC of multiple aminoglycoside antibiotics (kanamycin, tobramycin, streptomycin, gentamicin and amikacin). BAC promotes the emergence of mutants with reduced gentamicin susceptibility in other Gram-negative ESKAPE pathogens but does not always alter the MIC. These effects occur at BAC concentrations which are similar to residual levels in high-use environments, and just below the concentration range for BAC when used as a preservative in eye drops and ear drops.

Interpretation: Our results suggest that subinhibitory BAC has the potential to antagonise aminoglycoside activity and promote the emergence of bacterial mutants with reduced susceptibility. We suggest that the extremely widespread use of BAC in clinical and home settings and its long half-life mean there is potential for these interactions to occur in the environment, or in patients who use BAC-containing products while taking aminoglycosides to treat skin, eye or ear infections, although such co-exposure is likely to be rare. We suggest that biocide stewardship is needed to prevent the types of exposure that can contribute to antibiotic resistance.

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1. Introduction

Antimicrobial resistance (AMR) is one of the major global public health challenges of the 21\textsuperscript{st} century [1]. Bacteria become resistant to antibiotics through chromosomal mutations or the acquisition of resistance genes; this genetic resistance is detected by routine surveillance methods and has been the target of the majority of research to date [2,3]. However, bacteria also employ strategies for transient tolerance or resistance to antibiotics, which
Collectively have a major impact on the efficacy of antibiotic treatment and also drive the evolution of genetic resistance [4–6]. In addition to essential development of new drugs, there is a need for better understanding of the factors contributing to bacterial survival of antibiotic treatment even in the absence of heritable resistance determinants, with a view to altering these factors where possible to improve treatment outcomes.

Benzalkonium chloride (BAC) is a broad-spectrum biocide of the quaternary ammonium compound family [7]. The name refers to a group of compounds comprising a dimethyl benzyl ammonium chloride structure with a variable length (C8–18) alkyl chain, which are typically found as a mixture in commercial formulations [7]. Some favourable properties of BAC are its broad antimicrobial activity (against fungi, algae, bacteria and viruses), its long half-life, and low toxicity. BAC is used at high concentrations (≥0.05%) for surface disinfection in healthcare and home settings, and is used at lower concentrations (0.002–0.02%) as a preservative in consumer health products such as eye drops and intranasal sprays. In both contexts BAC is extremely widespread, for example BAC is the most common preservative used in ophthalmologic formulations [8], and is one of the most common active ingredients in household disinfectants [7,9]. Like other biocides, the use of BAC is largely unregulated, and its widespread use in hospitals and home environments has led to concern over its possible impact on antibiotic resistance [7,10,11]. Genetic adaptation to BAC, as a result of repeat low-level exposure, can cause cross-resistance to other antibiotics, with ampicillin, cefotaxime and sulfamethoxazole the most commonly reported [11]. Although numerous studies have explored how prior BAC adaptation can lead to reduced antibiotic susceptibility through selection for specific mutations (such as those promoting antibiotic efflux), other interactions between BAC and antibiotics, for example effects on tolerance or effects of co-exposure, have not been explored in depth.

Acinetobacter baumannii is a notorious pathogen of the “ESKAPE” group of bacterial species, which collectively are responsible for the majority of antibiotic-resistant infections [12,13]. It causes a range of severe infections (e.g. wound, respiratory) in vulnerable patients, and multidrug-resistant clones are now widespread [14]. For this reason A. baumannii is ranked by the World Health Organisation as a “critical” priority pathogen for which new antibiotics are urgently needed [15]. A. baumannii has a particular ability to survive for extended periods of time on surfaces, and this property contributes to its ability to seed outbreaks [16,17]. The efficacy and downstream consequences of different disinfectants is therefore of particular importance for the management of this pathogen. BAC kills A. baumannii by disrupting cell membranes at high concentrations, and by causing protein aggregation at low concentrations [18]. Recently, we found that sub-inhibitory concentrations of BAC dissipate the membrane potential of A. baumannii and reduce killing by the aminoglycosides gentamicin and amikacin, both of which depend on active transport for uptake into cells [19]. Here, we have investigated the effects of sub-inhibitory BAC on aminoglycoside tolerance and resistance in A. baumannii, with the aim of determining whether residual BAC in healthcare and home environments has the potential to reduce aminoglycoside efficacy.

We show that BAC antagonises both the killing and growth-preventing activity of gentamicin and other aminoglycosides, and dramatically increases the frequency at which A. baumannii colonies arise in the presence of gentamicin. Sub-MIC levels of BAC prevent intracellular accumulation of gentamicin in A. baumannii, but do not increase base mutation rates. Finally, we present evidence that sub-MIC BAC can also promote the emergence of clones with reduced gentamicin susceptibility in other Gram-negative ESKAPE pathogens. We suggest that potential effects of BAC on antibiotic efficacy should be considered when designing disinfection strategies for different environments, and that BAC-containing personal care products should be avoided by patients who are taking aminoglycosides to treat skin, wound or ear infections.

2. Methods

2.1. Bacterial strains and growth conditions

The bacterial strains used in this study were A. baumannii ATCC17978 (American Type Culture Collection), A. baumannii ABS075 [19], E. coli BW25113 [20], Enterobacter cloacae NCTC 9394 [Public Health England], K. pneumoniae ATCC43816 (American Type Culture Collection) and K. pneumoniae R201207 [36]. Bacteria were grown in Luria-Bertani medium (Benton-Dickinson) for routine maintenance or in Mueller-Hinton II medium (Benton-Dickinson) for antibiotic and biocide experiments, with agar added at 1.5% (w/v) when needed. Cultures were incubated at 37 °C, and liquid cultures were aerated by shaking at 200 RPM. The growth curve was performed in a 96-well format with 100 μl media +/- BAC per well, inoculated with an overnight culture of A. baumannii ATCC17978 (approx.
10^{10} \text{ cfu ml}^{-1} \) at 1/200 dilution. The plate was sealed with an Aeroseal™ gas-permeable membrane and incubated in a Pherestar plate reader with shaking, and optical density was measured every six minutes. Antibiotics (gentamicin, amikacin, streptomycin, kanamycin and tobramycin) and BAC (CAS 63449-41-2) were all purchased from Sigma. All antibiotics used were sulfate salt forms, with the exception of kanamycin.

### 2.2. Time to kill assay

Cultures of A. baumannii ATCC17978 (10 ml volume) were inoculated with overnight cultures of bacteria at a 1/20 dilution and grown in 50 ml cell culture flasks at 37 °C, 200 RPM to exponential phase (OD = 0.6). BAC (0.4 \text{ µg ml}^{-1}) and gentamicin (2 \text{ µg ml}^{-1}) were added and the cultures returned to incubate at 37 °C, 200 RPM. At set time points, cultures were sampled, serially diluted in phosphate-buffered saline (PBS), spot-plated on LB agar plates (5 µl volume, entire dilution series) and the plates incubated overnight to enumerate viable bacteria.

### 2.3. Evolution of gentamicin-resistant mutants

10 ml bacterial cultures were started by inoculating with overnight cultures at a 1/100 dilution. Cultures were grown to late exponential phase (OD = 1.0) at 37 °C, 200 RPM. 100 µl samples of each culture were serially diluted in PBS and the entire series spot-plated onto Mueller-Hinton agar supplemented with gentamicin (0.4 \text{ µg ml}^{-1}) with or without added BAC (0.6 \text{ µg ml}^{-1}). BAC pre-treatment was performed by adding 4 \text{ µg ml}^{-1} BAC to late exponential-phase cultures (or leaving as untreated controls) and growing for a further four hours. The stability of gentamicin resistance phenotypes was determined by plating 100 µl of serially diluted late exponential phase cultures on Mueller-Hinton II agar plates supplemented with 2 \text{ µg ml}^{-1} gentamicin with or without added sub-MIC BAC, and incubating overnight. Single colonies were then patch-plated onto nonselective media, and these patched colonies were then transferred onto Mueller-Hinton II agar with 2 \text{ µg ml}^{-1} gentamicin and grown overnight. Colonies were scored as resistant to gentamicin (at the concentration used in the plate) if they showed continuous growth along the length of the patch.

### 2.4. Fluctuation test

A. baumannii ATCC17978 was grown to late exponential phase (OD = 1.0) and used to seed 20 individual 10 ml cultures with a calculated starting inoculum of 1000 cells. Of these, 10 cultures were supplemented with BAC at 2 \text{ µg ml}^{-1} (the highest amount determined not to inhibit growth) and 10 cultures were not. Cultures were incubated in 50 ml Falcon tubes at 37 °C, 200 RPM for 24 hours, and plated on LB agar supplemented with 25 \text{ µg ml}^{-1} rifampicin. The numbers of rifampicin-resistant colonies arising from the replicate cultures were used to calculate the base mutation rate with and without added BAC according to the method of Hamon and Ycart [37], implemented at http://www.lcbp.upmc.fr/bzrates.

### 2.5. Measurement of minimum inhibitory concentration

The minimum inhibitory concentrations of aminoglycosides and BAC were determined by broth microdilution in cation-adjusted Mueller-Hinton II medium as described [21]. Clinical and Laboratory Standards Institute methods were followed for inoculum and antibiotic preparation, broth microdilution, and for interpretation of results [22]. Experiments were performed in at least biological duplicate.

### 2.6. Gentamicin-Texas Red conjugation

The Texas Red-gentamicin conjugate was prepared by combining Texas Red-X succinimidyl esters (Life Technologies, T6134), dissolved in DMF, with an excess of gentamicin in potassium carbonate buffer (pH 10, 100 mM) and incubating at 4 °C on a rotating wheel for 8 days as previously described [23,24]. The conjugate was stored at -20 °C, protected from light, and was diluted in water to generate working stocks for experiments.

Purity of the conjugate was investigated as follows: a 3 cc CiSe Seppak column was used for sample clean up prior to LC/MS-MS analysis. The column was equilibrated by flushing twice with 500 µl acetonitrile, followed by flushing 3 times with 0.2% formic acid in water, and a 20 µl aliquot of the GT-TR sample was diluted in 500 µl 0.2% formic acid in water was loaded to the column. The column was then washed 3 times with 500 µl 0.2% formic acid, and sample eluted with 2 volumes of 500 µl 98% acetonitrile and 0.2% formic acid in water. Eluent was dried to completion at 55 °C in a vacuum centrifuge, and resuspended in 200 µl 0.2% formic acid. LC/MS-MS analysis was performed using a Q-Exactive + (Thermo Fisher Scientific) mass spectrometer equipped with a Vanquish UPLC (Thermo Fisher Scientific) and an Accucore Vanquish C18+ (2.1 x 100 mm) column (Thermo Fisher Scientific) at a column temperature of 45 °C. The mobile phase A (MPA) contained 99.9% water and 0.1% formic acid and the mobile phase B (MPB) 99.9% acetonitrile and 0.1% formic acid. Gentamicin-Texas Red was injected (2 µl) onto the column and separated by gradient elution over 15 min with a flow rate of 0.3 µl min⁻¹. An initial concentration of MPB of 5% was held for 1 min, before increasing to 30% over 4 min then to 95% over 3 min and held at 95% for 3 min. MPB was then reduced to 5% over 1 min and held for 3 min. The column eluent was directed into the ionization source of the mass spectrometer operating in positive ion mode. The spray voltage was set to 3.5 kV, with a capillary temperature of 320 °C, sheath gas flow of 10 and 5-lens RF level of 60. Precursors from 200 to 2000 m/z were scanned at 140k resolution, with an AGC target of 3 x 10⁶ and a maximum injection time of 100 ms. The 5 most intense ions in the survey scan were fragmented using a normalized collision energy of 30 with a precursor isolation width of 1.4 m/z. The MS/MS method had a minimum signal requirement value of 1.6 x 10⁷ for MS² triggering, an AGC target value of 8 x 10³, maximum ion injection time of 50 ms. MS² scan resolution was set at 35 k, and dynamic exclusion was set to 10 seconds.

### 2.7. Gentamicin-Texas Red uptake assays

Gentamicin uptake assays were performed as follows: A. baumannii ATCC17978 was grown to OD = 0.6 in Mueller-Hinton II medium. 500 µl culture aliquots were transferred to 2 ml sterile Eppendorf tubes, with or without BAC added at 4 µg.ml⁻¹, and gentamicin-TR was added to each reaction at a final gentamicin concentration of 1 µg.ml⁻¹. Reactions were protected from light and incubated for 30 minutes at 37 °C 200 RPM. Cells were then pelleted by centrifugation at 8000 x g for 1 minute, washed with 400 µl PBS, and the pellet resuspended in 1 ml DMSO and stored at -20 °C prior to measurement. Gentamicin uptake assays were performed in the same way with A. baumannii AB5075, except that cells were incubated with 4 µg.ml⁻¹ BAC and 500 µg.ml⁻¹ gentamicin-TR.

Photophysical measurements were performed with a FLS980 photoluminescence spectrometer (Edinburgh Instruments) equipped with a Xe1 Xenon Arc Lamp (450 W ozone free, excitation range 230 nm – 1000 nm) for steady-state measurements. Excitation (λex) was performed at 550 nm and emission spectra were recorded in DMSO at 28 °C with 1 nm step-size, 0.1s integration time, and slit-width of Δλex = Δλem = 1.5 nm for AB5075 or Δλex = Δλem = 5 nm for ATCC17978.
2.8. Statistics and reproducibility

All microbiological experiments reported in the manuscript are from three biological replicates, defined as samples originating from separate overnight cultures each started from a single, separate colony. Technical replicates were used for the 96-well plate format experiments – MIC measurements and growth curves – and were defined as wells seeded with the same culture. Statistical significance for microbiological growth and survival experiments was determined by ANOVA on log-transformed data. Correction for multiple testing was done using either the Bonferroni correction, or Dunnett's post-hoc test. Parametric statistics were used because bacterial growth and viable count data are generally considered to be log-normally distributed [25,26], though the Shapiro-Wilk test was also used and the assumption of normality was not rejected. Experiments were not blinded, and no data were excluded. Specific numbers of replicates for each experiment (biological and technical), and details of the specific statistical test(s) used, are provided in the results section and the Fig. legends.

2.9. Role of funding source

The funders had no role in study design, data collection, data analyses or interpretation, or writing of the manuscript.

2.10. Ethics

No human or animal subjects were used in this study. All work was carried out in accordance with the Macquarie University Institutional Biosafety Committee guidelines (application ID 5201401141).

3. Results

3.1. Benzalkonium chloride protects A. baumannii from killing by gentamicin

The minimum inhibitory concentrations of gentamicin and BAC for A. baumannii ATCC17978, a common laboratory strain, were determined by broth microdilution to be 0.5 μg ml⁻¹ and 16 μg ml⁻¹, respectively. Time-to-kill assays were performed to determine the effect of different sub-MIC BAC levels on gentamicin activity. Cultures of A. baumannii were grown to exponential phase in rich medium then exposed to 2 μg ml⁻¹ gentamicin, with or without added sub-MIC BAC, and the number of viable cells measured over time (Fig. 1A). With no BAC, the A. baumannii population decreased exponentially to <0.2% of the original population after 3 hours (from 4.5*10⁶ cfu ml⁻¹ to 8*10⁵ cfu ml⁻¹). Addition of BAC at concentrations of 1-4 μg ml⁻¹ significantly improved survival (p < 0.001, mixed repeated measures ANOVA). At 1 μg ml⁻¹ BAC viable counts stayed constant over 3 hours. The presence of BAC at 2 μg ml⁻¹ or 4 μg ml⁻¹ prevented killing by gentamicin to the extent that A. baumannii proliferated in these conditions.

Tolerance by slow growth is a phenomenon seen for many antibiotic/pathogen combinations [5]. To determine whether BAC prevents gentamicin killing of A. baumannii ATCC17978 by reducing the growth rate, growth curves were performed. Growth was delayed by 4 μg ml⁻¹ BAC (p < 0.001, mixed repeated measures ANOVA), but not by BAC at 1-2 μg ml⁻¹ (Fig 1B). The concentration of BAC needed to reduce growth was higher than that required to dramatically reduce gentamicin killing (Fig 1A), suggesting that the mechanism of reduced gentamicin killing was not tolerance by slow growth.

BAC increases the rate of emergence of A. baumannii colonies in the presence of above-MIC gentamicin

A. baumannii ATCC17978 showed reduced killing by gentamicin in the presence of sub-MIC BAC. As tolerance to antibiotics can increase the window for evolution of resistance, we then tested how sub-MIC BAC influenced the frequency at which colonies arose on gentamicin-containing media. A. baumannii ATCC17978 cultures were grown to late exponential phase (approx. 10¹⁰ cfu ml⁻¹), then viable counts measured on gentamicin-supplemented media at 1, 2 or 4 μg ml⁻¹ with increasing levels of sub-MIC BAC (the breakpoint for gentamicin resistance is 4 μg ml⁻¹ in A. baumannii [27]). Supplementation with sub-MIC BAC dramatically increased cfu per ml of A. baumannii ATCC17978 at all gentamicin concentrations (Fig. 2A, p < 0.01, one-way ANOVA with Dunnett’s post-hoc test). Increased gentamicin selection reduced the number of colonies arising (from ~10⁶ with 1 μg ml⁻¹ gentamicin to 10² with 4 μg ml⁻¹ gentamicin), and also appeared to increase the amount of BAC supplementation needed to give rise to higher colony counts. We next sought to determine if the changes in cfu per ml required the continued presence of BAC. A. baumannii ATCC17978 cultures were grown to early exponential phase then either treated with 4 μg ml⁻¹ BAC, or left untreated, for four hours prior to washing cells and plating on gentamicin-containing media (Fig. 2B). Note that no neutraliser was used in these experiments, so it is possible that some positively-charged BAC remained bound to cells. Pre-treatment with BAC had no effect on colony formation at 1 μg ml⁻¹ gentamicin, and a very small effect on colony formation on 2 μg ml⁻¹ gentamicin (p < 0.05). Treated and untreated cells all showed the expected dramatic increase in cfu per ml with BAC supplementation of the agar (p < 0.01, one-factor ANOVA with Dunnett’s post-hoc test). These results show that BAC promotes the growth of A. baumannii ATCC17978 in the presence of gentamicin primarily through a mechanism that requires co-occurrence of both biocide and antibiotic. Finally, we sought to investigate the heritability of the reduced gentamicin susceptibility observed when BAC was present, by isolating individual colonies from gentamicin-only or gentamicin + BAC agar plates on non-selective media, then regrowing these on gentamicin-containing agar. Colonies from agar plates containing 2 μg ml⁻¹ gentamicin with or without BAC were patch-plated onto agar plates without antibiotic, grown overnight at 37 °C, and then patched again onto Mueller-Hinton ll agar supplemented with 2 μg ml⁻¹ gentamicin. Colonies showing full growth along the length of the patch on 2 μg ml⁻¹ gentamicin were scored as having an acquired reduced-susceptibility phenotype. Results are shown in Fig. 2C. Addition of BAC increased the numbers of reduced-susceptibility mutants that grew on gentamicin-containing agar following passage without selection, by 8-fold at 2 μg ml⁻¹ BAC (p < 0.05) and >2000-fold at 4 μg ml⁻¹ BAC (p < 0.01). This effect was evident even though the majority of colonies that emerged when BAC was present were

Fig. 1. Sub-MIC BAC prevents gentamicin killing of A. baumannii ATCC17978. (a) Survival of late exponential-phase cultures following addition of gentamicin at 4x MIC (2 μg ml⁻¹). Results shown are mean and standard deviation of three biological replicates. Statistically significant differences in survival relative to gentamicin-only were determined by two-factor repeated measures ANOVA with Bonferroni correction for multiple testing. ***p < 0.001 (alpha = 0.016), n = 3 (b) Effect of sub-MIC BAC on growth of A. baumannii ATCC17978. ***p < 0.001 relative to growth without BAC added, two-factor repeated measures ANOVA with Bonferroni correction for multiple testing (alpha = 0.016). Results shown are the mean and 95% confidence interval from three biological and two technical replicates.
showed only a transient reduction in susceptibility to gentamicin; 70% of colonies grown on 2 \( \mu \)g ml\(^{-1}\) BAC, and 94% of colonies grown on 4 \( \mu \)g ml\(^{-1}\) BAC, reverted to the susceptibility of the parent strain following re-isolation. Overall, these results demonstrate that BAC dramatically increases growth of \textit{A. baumannii} ATCC17978 in the presence of above-MIC levels of gentamicin, that this effect requires the continued presence of BAC, and that BAC promotes both transient and acquired (in this case meaning maintained through one passage of growth on non-selective media, or roughly 20 generations) reductions in susceptibility to gentamicin.

### Table 1

Minimum inhibitory concentrations of aminoglycoside antibiotics against \textit{A. baumannii} ATCC17978 in the presence of BAC. Antibiotics used are amikacin (AMK), kanamycin (KAN), gentamicin (GEN), streptomycin (STR) and tobramycin (TOB). MICs were measured in biological duplicate.

| [BAC] \( \mu \)g ml\(^{-1}\) (fold change) | AMK | GEN | KAN | STR | TOB |
|----------------------------------------|-----|-----|-----|-----|-----|
| 0                                     | 0.25| 0.25| 0.25| 2   | 0.0625 |
| 0.25                                  | 0.25| 0.25| 0.25| 4 (2)| 0.125 (2) |
| 0.5                                   | 0.5 (2)| 1 (2)| 0.5 (2)| 8 (4)| 0.125 (2) |
| 1                                     | 0.5 (2)| 1 (2)| 0.5 (2)| 16 (8)| 0.25 (4) |
| 2                                     | 0.5 (2)| 2 (4)| 0.5 (2)| 16 (8)| 0.25 (4) |
| 3                                     | 1 (4)| 2 (4)| 1 (4)| 32 (16)| 0.25 (4) |
| 4                                     | 1 (4)| 4 (8)| 1 (4)| 32 (16)| 0.5 (4) |

3.2. BAC increases the minimum inhibitory concentration of aminoglycoside antibiotics

We next determined how BAC affects resistance to gentamicin and other aminoglycoside antibiotics (i.e. the concentration required to prevent growth, rather than killing), by measuring their MICs in the presence of increasing amounts of BAC (Table 1). Note that although a low inoculum was used in order to minimise the chance of reduced-susceptibility mutants arising, this possibility cannot be excluded. Gentamicin was antagonised by BAC at concentrations of 0.5 \( \mu \)g ml\(^{-1}\) and above, with an 8-fold increase in MIC observed at 4 \( \mu \)g ml\(^{-1}\) BAC. All other aminoglycosides tested (streptomycin, tobramycin, kanamycin and amikacin) were also antagonised by BAC. Maximum MIC fold-changes ranged from 4–16, and gentamicin and streptomycin MICs were both increased to at or above their respective clinical breakpoints (4 \( \mu \)g ml\(^{-1}\) and 16 \( \mu \)g ml\(^{-1}\)) defined by EUCAST or the FDA \cite{27,28}. The minimum concentration of BAC required to increase the MIC of an antibiotic was 0.25 \( \mu \)g ml\(^{-1}\) (the lowest tested), and all antibiotics were antagonised by 0.5 \( \mu \)g ml\(^{-1}\) BAC. These results demonstrate that BAC antagonises the growth-preventing activity of aminoglycoside antibiotics in \textit{A. baumannii} ATCC17978, and that this effect occurs over a concentration range of 0.5–4 \( \mu \)g ml\(^{-1}\).
3.3. BAC reduces intracellular accumulation of gentamicin, but does not affect mutation rates

We then investigated the potential mechanism by which BAC may influence tolerance and resistance to gentamicin and other aminoglycoside antibiotics. Bacteria can increase their mutation rates in response to stress; these stress-induced mutagenesis programs can contribute to evolution of antibiotic resistant mutants. To test the possibility that sub-MIC BAC may promote evolution of mutants showing reduced gentamicin susceptibility (Fig 2) by increasing the base mutation rate, we performed Luria-Delbrück fluctuation tests in the absence of antibiotic selection (see Methods). *A. baumannii* ATCC17978 mutation rates, bounded by their 95% confidence intervals, were $6.26 \times 10^{-10}$ to $1.68 \times 10^{-9}$ in the absence of BAC, and $6.14 \times 10^{-10}$ to $1.57 \times 10^{-9}$ when supplemented with BAC at $2 \mu \text{g ml}^{-1}$ (the highest concentration at which the growth rate was not affected, Fig 1b). Therefore, the mechanism by which BAC promotes growth and colony formation in the presence of gentamicin does not depend on general elevation of the mutation rate.

Next, we investigated intracellular accumulation of gentamicin in the presence of BAC, using a gentamicin-Texas Red (TR) conjugate. The effectiveness of the labelling reaction to produce the fluorophore-conjugated gentamicin was confirmed by mass spectrometry (see Methods), which showed that roughly 96.5% of the TR present was antibiotic-conjugated. *A. baumannii* ATCC17978 was grown to late exponential phase, then incubated with labelled gentamicin at $1 \mu \text{g ml}^{-1}$ (2x MIC) for 30 minutes, with or without $4 \mu \text{g ml}^{-1}$ BAC. The final viable count did not differ between treatments. Cells were then collected, resuspended in DMSO, and the fluorescence of the gentamicin-TR measured (Fig. 3a). As the measured fluorescence of

![Fig. 3. BAC reduces intracellular levels of gentamicin in *A. baumannii*. (a) Accumulation of a gentamicin-Texas Red conjugate after 30 minutes in *A. baumannii* ATCC17978. (b) Accumulation of gentamicin-Texas Red in *A. baumannii* AB5075 after 30 minutes. Results shown are the mean and 95% confidence interval of three biological replicates. Note that different detection settings were used for the two strains, so fluorescence values are not comparable. An instrumental artefact at 660nm is visible in ATCC17978 spectra (denoted by *).

![Fig. 4. BAC affects gentamicin activity in other ESKAPE pathogens. Number of colonies arising on gentamicin-containing agar plates in the presence of sub-MIC BAC. Gentamicin was added at 0.5 $\mu \text{g ml}^{-1}$ for *E. coli* K12, *E. cloacae* ATCC9394 and *K. pneumoniae* ATCC43816, and at 2 $\mu \text{g ml}^{-1}$ for *K. pneumoniae* RH201207, corresponding to 2x MIC. **p<0.01 relative to no BAC supplementation, one-way ANOVA with Dunnett’s post-hoc test. Overall significance was <0.001 all data series except for *K. pneumoniae* RH201207, for which BAC supplementation had no significant effect.](image_url)
intracellular gentamicin–TR in ATCC17978 was close to the detection limit of the instrument, the experiment was also performed using a gentamicin-resistant strain, A.baumannii AB5075, with gentamicin–TR added at 500 μg.ml⁻¹ (Fig. 3b). The presence of 4 μg ml⁻¹ BAC reduced the fluorescence detected from intracellular gentamicin–TR by ~2.25-fold in both strains, with no overlap in 95% confidence intervals. These results demonstrate that sub-MIC BAC reduces cellular accumulation of gentamicin.

3.4. BAC also antagonises gentamicin in other ESKAPE pathogens

Finally, we tested the effect of BAC on gentamicin in a selection of Gram-negative bacteria; Escherichia coli K12, Klebsiella pneumoniae ATCC43816, Enterobacter cloacae ATCC9394 and K. pneumoniae RH201207. The gentamicin MIC of all strains was 0.25 μg ml⁻¹ except for K. pneumoniae RH201207, which possesses an aminoglycoside acetyltransferase gene and has an MIC of 1 μg ml⁻¹. Note that the EUCAST breakpoint for Enterobacteriales, which includes these three species, is 2 μg ml⁻¹ [27]. BAC MICs were 16 μg ml⁻¹ for E. coli K12 and both K. pneumoniae strains, and 32 μg ml⁻¹ for E. cloacae ATCC9394. As shown in Fig. 4, the presence of sub-MIC BAC significantly increased the number of colonies recovered on 2 × MIC gentamicin (p < 0.01 compared to no BAC, one-way ANOVA with Dunnett’s post-hoc test) for E. coli K12 (max 10000-fold increase), E. cloacae ATCC9394 (2500-fold increase) and K. pneumoniae ATCC43816 (31-fold increase), but not for K. pneumoniae RH201207. Effects were seen at slightly elevated concentrations of BAC: 2 μg ml⁻¹ for E. coli, and 4 μg ml⁻¹ for E. cloacae and K. pneumoniae ATCC43816. We also tested whether the gentamicin MIC was affected in each of these bacterial strains, and found that only E. coli K12 had an increased gentamicin MIC in the presence of BAC (increased to 1 μg ml⁻¹ with 4 μg ml⁻¹ BAC). Overall these results show that BAC can also antagonise the effects of gentamicin in other Gram-negative pathogens, and that this effect is strain-specific.

4. Discussion

Biocides are crucial for infection control, but the potential for these agents to inadvertently drive AMR cannot be ignored. Here, we have shown that the widely-used biocide BAC can interfere with aminoglycoside antibiotics in A. baumannii. This antagonism manifests as abrogation of killing activity, increased MICs, and a marked increase in the frequency of emergence of reduced-susceptibility mutants. Unlike the majority of previously documented biocide-related antibiotic resistance, the process we identify here does not require prior biocide exposure and adaptation.

BAC reduced the amount of gentamicin accumulated in exposed cells, which presumably underpins its effects on antibiotic activity. A reduction in intracellular antibiotic levels could be a consequence of reduced import, or of increased export due to induction of aminoglycoside efflux pumps. We recently showed that BAC dissipates membrane potential in A. baumannii when present at low concentrations [29], and speculate that this activity underpins BAC-amino-glycoside antagonism, as import of aminoglycosides is known to be membrane potential-dependent [30]. Though we did not investigate efflux pump expression, the transcriptional response of A. baumannii ATCC17978 to sub-MIC (5 μg ml⁻¹) BAC has been explored by other researchers [18]. Although several efflux pump genes (aceI, adeAB, macA and ermA) are induced in response to BAC, these pumps are not known to transport aminoglycosides [31,32], and do not contribute to fitness during sub-MIC aminoglycoside selection [33], with the exception of AdeAB which has a small (2-fold) effect on aminoglycoside resistance when overexpressed in an efflux-deficient background [33,34]. As the transporters induced by BAC have only a very subtle effect on aminoglycoside resistance, we consider that the antagonistic effects of BAC are more likely to be a consequence of reduced uptake due to disruption of membrane potential rather than increased efflux. Note that BAC antagonism of antibiotics through efflux pump induction has been reported in P. aeruginosa, in which increased expression of mexCD-oprJ following BAC exposure resulted in increased resistance to tetracycline and ciprofloxacin [10]. The precise mechanism of BAC-aminoglycoside antagonism, and the range of bacteria affected, merits further study.

Sub-lethal BAC can severely compromise aminoglycoside efficacy at certain concentrations, resulting in MIC increases to at or above the relevant clinical breakpoints for some drugs (Fig 3), and an exponential increase in the number of colonies with reduced gentamicin susceptibility (Fig 2). Key to understanding the impact this phenomenon may have in the clinic is the question of how frequently these concentrations of BAC are encountered. BAC is not used in internal medicines, so the biocide and antibiotic interactions we identify here are not likely to occur within a patient undergoing aminoglycoside treatment for internal infections. However, BAC is used as a preservative in eye drops, ear drops (including aminoglycoside ear drops) and decongestant nasal sprays, and as an antimicrobial in some wound disinfectants and soaps, meaning co-exposure could occur in patients who use such products while taking aminoglycoside antibiotics for external infections e.g. wound, sinus, ear or eye. In formulations where BAC is used as a preservative, concentrations are usually in the 20–100 μg ml⁻¹ range; this is higher than the concentrations used in our study, but is sub-MIC for many clinical pathogen isolates and BAC-adapted strains (MICs 7–1024 μg ml⁻¹, reviewed in [7]). Biofilm formation during infection may increase the window for antagonistic interactions between BAC and aminoglycosides due to poor penetration of biofilms leading to sub-MIC antimicrobial levels, and BAC-adapted strains of multiple bacterial species (such as P. aeruginosa, Listeria monocytogenes and Salmonella spp.) show increased biofilm formation [35–37]. There is also scope for co-occurrence of antibiotic and biocide at near-MIC concentrations outside of the patient, as a consequence of heavy use of BAC for cleaning. Although BAC levels in cleaning products and disinfectants are much higher than those used in our study (>0.05%), residual BAC is a cause for concern. While typical residual levels on surfaces following the use of BAC products are unknown, we note that BAC levels in wastewater from high-use settings (hospitals, laundries) are 2 mg.L⁻¹ – 6 mg.L⁻¹ [38–40]; concentrations which antagonise aminoglycosides in our lab experiments. Many biocides are now banned as additives in over-the-counter personal care products due to their off-target effects on antibiotic resistance [41,42]. Here, we have identified a distinct and broad-acting mechanism by which BAC, which is not currently restricted, can compromise one of the most important classes of antibiotics. This finding is in addition to a wealth of literature suggesting that adaptation of bacteria to BAC can also lead to reduced antibiotic susceptibility [7,11]. Although co-occurrence of the precise concentrations of biocide and antibiotic which produce antagonistic effects is likely to be rare, we suggest that the extensive use of BAC-containing products, its long half-life, and the long-term consequences of co-exposure (by driving evolution of reduced-susceptibility mutants) mean that the potential for these interactions should not be ignored. We suggest that biocide stewardship [43] is essential in order to use these agents effectively without driving resistance to vital antibiotics.

**Contributors**

Francesca L Short jointly conceived and designed the study, supervised preliminary experiments, performed experiments, analysed and interpreted data and wrote the manuscript. Victor Lee and Rafa Mamun performed experiments and analysed data. Robert Malmberg and Koushik Venkatesan designed and supervised the gentamicin–Texas Red conjugation and transport experiments and interpreted results. Liping Li and Monica I Espinosa contributed to study and experiment design and interpretation of results. The MQ UBAR
conducted preliminary experiments and analysed results, with supervision from Francesca L. Short and Monica I Espinosa. Ian T Paulsen jointly conceived and designed the study, and contributed to interpretation of results. All authors revised the manuscript and read and approved the final version. Underlying data has been verified by Francesca L. Short, Victor Lee and Rafa Mamun.

**Declaration of Competing Interest**

The authors declare that they have no financial interests to disclose.

**Data sharing**

No large-scale datasets are reported.

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**Supplementary materials**

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