

Genomic fitness profiling of *Acinetobacter baumannii* reveals modes of action for common biocides and mechanisms of biocide-antibiotic antagonism

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Abstract

Biocides, such as antiseptics and disinfectants, are used ubiquitously for hygiene in households and for life-saving infection control in hospitals. An increasing concern is that the widespread use of biocides may contribute to the emergence and spread of multidrug-resistant bacteria. We performed transposon directed insertion site sequencing (TraDIS) to identify genes and key cellular pathways of the multidrug resistant nosocomial pathogen *Acinetobacter baumannii*, that affect host fitness during exposure to a panel of ten structurally-diverse and clinically-relevant biocides: silver nitrate, benzalkonium, cetyltrimethylammonium bromide (CTAB), chlorhexidine, triclosan, chloroxylenol, polyvidone iodine, bleach, glutaraldehyde and ethanol. Multiple genes encoding proteins localised either in the cell envelope or in the cytoplasm were shown to affect biocide susceptibility. These proteins are involved in multiple processes including fatty acid biogenesis, multidrug efflux, the tricarboxylic acid cycle, cell respiration and cell division, suggesting that these biocides may have intracellular targets in addition to their known effects on the cell envelope. Based on the importance of cell respiration genes for *A. baumannii* fitness on biocides, we proposed and confirmed that apart from triclosan, the other 9 biocides at sub-inhibitory concentration can dissipate the membrane potential and lead to *A. baumannii* tolerance to antibiotics that have intracellular targets. Our results support the concern that residual biocides in clinical or community environments can promote the development of antibiotic resistance in pathogenic bacteria.

Introduction

Multiple drug resistance (MDR) in bacterial pathogens is an alarming public health issue¹. Bacteria can resist drugs by expressing efflux pumps to export drugs out of the cell, altering outer membrane permeability to reduce drug accumulation, or expressing enzymes for drug inactivation by hydrolysis or modification². Alternatively, bacteria can gain intrinsic resistance mutations which prevent target recognition or cause target bypass. The Gram-negative opportunistic human pathogen *A. baumannii* is a significant worldwide threat for immunocompromised patients who are hospitalized in Intensive Care Unit (ICU) wards. This is due to the emergence of *A. baumannii* clonal lineages with high level resistance to antibiotics and tolerance to desiccation^{3,4}. The World Health Organisation (WHO) recently listed carbapenem resistant *A. baumannii* as a “top priority pathogen” for development of new therapies.

Biocides are widely used for disinfection and cleaning in both households and hospitals, but little is known about their impact on the emergence and spread of MDR infections. In contrast to antibiotics, the use of biocides is largely unregulated. The recommended in-use biocide concentrations are normally orders of magnitude higher than the bacterial minimum bactericidal concentration (MBC). Biocides are presumed to have multiple antibacterial targets or non-specific targets, reducing the likelihood of bacteria developing resistance to these compounds⁵. However, outbreaks of nosocomial MDR infections are not uncommon. There is evidence suggesting that biocide exposure at sublethal concentrations can select for antibiotic-resistant bacteria under laboratory conditions⁶, and clinical antibiotic resistant isolates typically have reduced biocide susceptibilities⁷. One of the most commonly known biocide and antibiotic

cross-resistance mechanisms is the activity of MDR efflux pumps, such as AcrAB-TolC from *Escherichia coli*⁸, AdeIJK and AdeABC from *Acinetobacter baumannii*⁹, and NorA from *Staphylococcus aureus*¹⁰.

Biocide modes of action are generally poorly characterized, except for cell lysis via interaction with the phospholipid membrane⁵. However, some biocides are known to have mode of action that start intracellularly. For example, silver ions can interact with the thiol group of exposed cysteine residues leading to enzyme inactivation and disruption of cellular iron homeostasis^{11,12}, and they also cause cell membrane proton leakage¹³. The known silver resistance determinants are primarily involved in reducing silver ion intracellular concentration, including membrane transporters for silver ion export¹⁴, and proteins for neutralization or reduction of silver ions to the inactive metallic form¹⁵. Triclosan targets an intracellular essential enzyme enoyl reductase (FabI) in fatty acid synthesis¹⁶. The two key determinants for high-level triclosan resistance are MDR efflux pumps and either mutations in the *fabI* gene or the acquisition of alternative Fab proteins not recognizable by triclosan¹⁷. Apart from the drug efflux pumps mentioned earlier, members from the proteobacterial antimicrobial compound efflux (PACE) family confer resistance to antibacterial surfactant compounds including chlorhexidine and benzalkonium¹⁸. These observations suggest the surfactant biocides are also likely to have intracellular targets, or these pumps can expel them from the membrane.

Knowledge of both biocide modes of action and tolerance mechanisms is important for improved biocide use for effective and sustainable infection control. Here we performed the first systematic genome-wide screen to identify genes that affect susceptibilities of an MDR *A. baumannii* strain to ten clinically important biocides (Table S1), using transposon-directed insertion-site sequencing (TraDIS)¹⁹.

Results

TraDIS experimental outcomes. TraDIS assays were performed on a global clone II multidrug resistant *A. baumannii* strain BAL062 transposon mutant library, containing >100,000 unique Tn5 insertions (1 insertion every 38 bp)²⁰, to assess the global genetic response to biocide treatments. Ten structurally distinct biocides were chosen for this study; these are all either listed as “essential medicines” by the WHO or commonly used in clinical settings and/or as household products (Table S1). The *A. baumannii* TraDIS library was exposed to a sub-inhibitory concentration of each biocide ($\frac{1}{4}$ × the minimum inhibitory concentration (MIC) of the wild-type strain), and genomic DNA was extracted for TraDIS sequencing and analysis as previously described²¹. Gene-wise mutant abundance data were compared to untreated control samples grown under identical conditions for the same length of time to identify mutations that affect fitness in the presence of biocides, while controlling for any general impacts on growth rate. Collectively, the biocide treatments revealed a range of genes harbouring a reduced mutant population (i.e. decreased insertion read counts; 3-120 genes), representing potential biocide tolerance determinants, or with an expanded mutant population (i.e. increased insertion read counts; 7-100 genes) whose inactivation is potentially beneficial under biocide-treatment (Table S1).

We examined genes previously associated with biocide resistance and found their functions reflected in the TraDIS results. For example, the MDR efflux pump-encoding operon *adeABC* and its activator genes (*adeRS*) showed significantly decreased insertion read counts (e.g. *adeB* had a 115.4-fold decrease) after treatment with benzalkonium, a known substrate of AdeABC; whereas no change was seen after treatment with glutaraldehyde, which is not known to be a substrate of AdeABC (Figure 1A).

Genes previously associated with antibiotic resistance were identified, for example as *lpsC* – a glycosyltransferase and LOS-synthesis gene involved in polymyxin resistance in *A. baumannii*²² but not previously implicated in biocide resistance – showed a 5.8-fold decrease in insertions following triclosan treatment (Figure 1A). We also revealed potential new roles in biocide resistance for previously characterised genes, e.g., the insertions in the pyrimidine synthesis gene *pyrC* showed a 35.9-fold increase in AgNO₃-treated cultures compared to the control (Figure 1A).

As proof-of-principle phenotypic validation of this TraDIS data, we measured the MICs of the four isogenic single-gene knockout Tn26 mutants from *A. baumannii* AB5075²³ for the genes outlined above: *adeB*, *lpsB*, *pyrC* and *adeR*, for benzalkonium, triclosan, AgNO₃ and glutaraldehyde respectively. In each case, the biocide MIC fold change for the mutant relative to wild-type was consistent with the TraDIS results (Figure 1B).

Outer membrane lipooligosaccharide. *A. baumannii* lacks a homolog of the O antigen ligase WaaL, which transfers O-antigen polysaccharide onto the outer-core of LPS, suggesting that *A. baumannii* LPS may comprise only lipid A and core oligosaccharide²⁴. We observed that insertions in a lauroyl acyltransferase encoding gene, *lpxL*, increased in frequency following treatment with the cationic surfactants chlorhexidine and cetyltrimethylammonium bromide (CTAB) (21- and 2.3-fold respectively), suggesting that this gene decreased fitness at sub-inhibitory concentrations of these two biocides (Figure 2A). The predominant glycolipid molecule in the outer leaflet of *A. baumannii* OM is hepta-acylated lipid A²⁵, whereas an *lpxL* mutant generates a hexa-acylated lipid A²⁶. This change in fatty acid profile may impact the effectiveness of these biocides against *A. baumannii*.

Cell surface polysaccharides. In *A. baumannii*, there are two gene clusters known to be involved in capsule polysaccharide biosynthesis (K-locus) and LOS outer core oligosaccharide biosynthesis (OC-locus) respectively²⁷. We identified 9 genes in the K-locus with significant changes in insertion counts after treatment with one or more biocides. For instance, insertions in the *gna* gene, encoding a UDP-glucose dehydrogenase, increased during treatment with chlorhexidine and bleach (207 and 3.2-fold, respectively), implicating capsule polysaccharide in biocide sensitivity.

Insertions in an initiating glycosyltransferase-encoding gene, *pglC*, had decreased read-coverage (9.9-fold) when treated with AgNO₃, while treatment with the other 9 compounds did not have an effect, suggesting this gene may mediate tolerance only for AgNO₃ (Figure 2A). *pglC* is located in the variable region of the K-locus, but its presence is conserved in this gene cluster across *A. baumannii* strains^{27,28}. It is required for the construction of capsular glycan repeat-units and glycosylation of glycoproteins²⁸, and

the deletion mutant affects capsule production not LOS²⁹. In addition to *pglC*, insertions in 7 other genes in the K-locus also displayed decreased frequency following AgNO₃ treatment (Figure 2A), implying that the capsular polysaccharide may play a specific role in AgNO₃ tolerance, whereas alteration of LOS glycoforms impacts susceptibility to biocides more broadly.

Insertions in seven genes in the OC-locus had altered frequency following biocide treatment (Figure 2A). For instance, insertions in the glycosyltransferase gene *lpsC* decreased 5.8-fold following exposure to benzalkonium, suggesting a specific role for *lpsC* in benzalkonium tolerance. Further, insertions in another glycosyltransferase gene, *gtrOC3*, dropped dramatically (78.8-fold) upon chlorhexidine treatment, suggesting *gtrOC3* mediates chlorhexidine tolerance (Figure 2A). Insertions in the glycosyltransferase gene *lpsB*, which is outside of the two polysaccharide biosynthesis loci and is involved in LOS core biosynthesis, also showed changes following exposure to 7 biocides (Figure 2A). Previously, it has been shown that disruption of these glycosyltransferases in *A. baumannii* produces truncated LOS^{30,31}. Thus, genes predicted to encode glycosyltransferases, that catalyze the linking of sugars of the LOS outer oligosaccharide core, may be important in biocide tolerance.

Chaperone-usher pilus. Another potential cell surface biocide tolerance determinant identified in this study is a chaperone-usher (CU) pilus assembly gene cluster *csuA/BABCDE* (Figure 2A)³², insertions in which decreased after treatment with eight biocides (Figure 2A). For example, insertions in all the *csu* genes decreased with most biocides: 2.2- to 3.2-fold decrease for AgNO₃, 2.8- to 3.8-fold decrease for benzalkonium, 7.2- to 8.4-fold decrease CTAB, and 5- to 7-fold decrease for triclosan (Figure 2A). Consistent with their known function as *csu* operon activators, insertions in the genes of the two-component system BfmRS were also shown to decrease following treatments with seven same biocides (Figure 2A). Interestingly, insertions in a transcriptional regulator (BAL062_01328) directly upstream of the *csu* operon also decreased in frequency following treatment with the same eight biocides and the fold change was similar to that of *csu* gene mutants (Figure 2A), indicating that BAL062_01328 encodes a potential local transcriptional activator of the *csu* operon. Together these results suggest that the mature pilus system is required for a biocide tolerance phenotype.

Membrane transport. Sixteen drug efflux systems have been characterized or identified in *A. baumannii*^{18,33}. One of the most clinically significant drug transporters in *A. baumannii* is a tripartite efflux system AdeABC³⁴, comprised of a membrane fusion protein (MFP) AdeA, a resistance-nodulation-division (RND) efflux pump AdeB, and an outer membrane factor (OMF) AdeC, respectively. This system is constitutively overexpressed in various clinical *A. baumannii* MDR strains, typically due to mutations in the two-component transcriptional activator genes *adeRS*³⁵.

Tn5 insertions in *adeB* significantly decreased in frequency 115.4-, 2.2-, 85.6-, 7.9- and 2.0-fold in the mutant pools that were treated by benzalkonium, cetrimonium, chlorhexidine, triclosan and chloroxylenol, respectively (Figure 2B), suggesting that *adeB* mediates resistance to these compounds. Benzalkonium and chlorhexidine have previously been characterized as AdeB substrates⁹. Surprisingly, changes in

frequency of Tn5 insertions in *adeA* and *adeC* were only seen in the mutant pools treated by benzalkonium and chlorhexidine (Figure 2B), where insertions in *adeA* had 3.3- and 78.8-fold decrease, and insertions in *adeC* had 2.4- and 11.2-fold decrease, respectively (Figure 2B). This suggests AdeABC functions as a tripartite system in pumping out these two compounds. These fold-changes were lower than the changes of *adeB* caused by the same compounds, indicating that there might be alternative OMF and MFP proteins that form a tripartite RND efflux system with AdeB.

Insertions in *adeR* decreased 7.6- and 26.7-fold, and those in *adeS* decreased 7.5- and 240-fold when treated by benzalkonium and chlorhexidine respectively, whereas no change was observed in the other three conditions that also selected against insertions in *adeB* (Figure 2B). These observations together suggest that benzalkonium and chlorhexidine could induce the overexpression of AdeABC through AdeRS³⁶, whereas the other three compounds cannot.

The other important RND system that has been characterized in clinical *A. baumannii* MDR strains is AdeIJK, which has a similar range of substrates as AdeABC⁹. The TraDIS data from this study for *adeIJK* is similar to that for *adeABC* (Figure 2B)^{9,37}. For instance, insertions in the OMF encoding gene *adeK* decreased 1.7-, 17-, 79.9-, 27.3-, and 10.0-fold after treatment with AgNO₃, benzalkonium, chlorhexidine, triclosan and chloroxylenol respectively (Figure 2B). The respective changes in frequency of insertions in the RND pump encoding gene *adeJ* and MFP encoding gene *adel* were lower than seen for *adeK*, suggesting AdeK might not only form a tripartite drug efflux system with Adel and AdeJ, but also with AdeAB (Figure 2C), consistent with previous reports^{38,39}. Also consistent with previous findings that the transcriptional regulator AdeN represses the transcription of *adeIJK*⁴⁰, the insertions in *adeN* increased 1.5-, 99.7-, 1.7- and 1.3-fold after treatment with benzalkonium, chlorhexidine, triclosan and chloroxylenol respectively (Figure 2B).

The frequency of Tn5 insertions in another RND membrane transporter gene BAL062_00031 and MFP encoding gene BAL062_00030 reduced 7.31- and 7.41-fold respectively when exposed to triclosan, suggesting these genes play a role in triclosan resistance (Figure 2B). There is no OMF gene located adjacent to BAL062_00030 and BAL062_00031. This system may use AdeK as its OMF partner, because *adeK* is the only OMF gene in which insertions reduced in frequency (27-fold) in the mutant pool exposed to triclosan (Figure 2B).

Four other drug efflux pumps were also implicated in mediating biocide resistance. The MFS transporter AmvA is a multidrug exporter with substrates including benzalkonium, and chlorhexidine^{41,42}. The frequency of insertions in *amvA* decreased 1.6- and 106.2-fold after exposure to benzalkonium and chlorhexidine respectively, and additionally showed a 19.4-fold decrease when treated by AgNO₃ (Figure 2B). Furthermore, the frequency of insertions in *amvR*, encoding a transcriptional repressor of *amvA*²⁰, increased 5.2- and 84.7-fold following exposure to AgNO₃ and chlorhexidine, respectively (Figure 2B). This suggests that AmvA may also recognize silver ions as a substrate or may transport other compounds related to silver detoxification.

The frequency of insertions in *tetR*, immediately upstream of a tetracycline efflux pump gene *tetA*, increased 21.1-fold following exposure to chlorhexidine (Figure 2B). TetR is a known transcriptional repressor of *tetA*⁴³, and this result suggested that *tetA* overexpression reduces susceptibility to chlorhexidine. In contrast, chlorhexidine did not affect the frequency of insertions in *tetA*, which is likely because TetR does not recognize chlorhexidine as a ligand and is not responsive to it. These are consistent with our previous finding that *E. coli* expressing *A. baumannii* TetA had a significant increase in chlorhexidine resistance level⁴⁴.

The other putative multidrug efflux pump highlighted in this study is an ABC transporter consisting of BAL062_01229, encoding a membrane permease, and BAL062_01230 encoding an ATPase. When exposed to AgNO₃ the frequency of insertions in BAL062_01229 and BAL062_01230 decreased 2.2- and 2.6-fold respectively (Figure 2B), suggesting this ABC efflux system plays a role in AgNO₃ tolerance.

Cell Division. Various genes that are involved in peptidoglycan (PG) synthesis, cell shape determination and cell division were shown to have a decreased frequency of Tn5 insertions following treatment with silver nitrate and several other biocides (Figure S1). Insertions in the bacterial rod shape determining genes *mreB*, *mreC*, *mreD*, *rodA* and *pbp2* decreased in frequency (4.8-, 6.3-, 4.7-, 4.8- and 4.0-fold respectively) when treated by AgNO₃, suggesting disruption of these genes resulted in greater sensitivity to AgNO₃. In line with the phenotype of these mutants, Ag⁺ solution has been shown to cause distortion in bacterial cell membranes and morphology⁴⁵. Hypochlorite and ethanol also showed similar effects on the frequency of these mutants, though with lower fold changes (Figure S1), suggesting they may also affect cell membranes and morphology.

The FtsZ-ring associated genes (*zipA*, *zapA* and *rlpA*) and six genes involved in peptidoglycan synthesis and hydrolysis were also found to have a role in tolerance to multiple biocides (Figure S1). Insertions in *zipA* decreased in frequency when treated with eight different biocides, for instance a 6.9-fold decrease in the AgNO₃ pool, a 3.0-fold decrease in the chloroxylenol pool, and a 2.5-fold decrease in the glutaraldehyde pool. The frequency of insertions in genes in the *pal-tolQ* operon, which is required for OM invagination during cell division, was strongly reduced (59.3 – 61.7-fold) upon chlorhexidine treatment.

The PMF was proposed to affect cell division because the cellular localization of MreB and FtsA (FtsZ-ring associated) proteins has been shown to be PMF-dependent⁴⁶. This may explain why TraDIS revealed that mutations in both electron transfer and cell division genes affect *A. baumannii* fitness in the presence of AgNO₃ and other biocides.

Central Metabolism and Respiration. Tn5 insertional inactivation of several TCA cycle genes had effects on *A. baumannii* susceptibility to silver nitrate. The frequency of insertions in the TCA cycle genes *sucC* and *sucD* encoding succinyl-CoA synthetase β and α subunits both showed a 12.3-fold increase upon silver nitrate treatment, suggesting that absence of succinyl-CoA synthetase increased resistance to silver nitrate (Figure S1). Similarly, previous work demonstrated that knockout strains of *E. coli* TCA cycle genes (Δ *sucB*, Δ *mdh*) were less sensitive to silver nitrate than the wild type¹². In contrast, insertions in two other

TCA cycle genes *acnA_1* encoding aconitate hydratase 1 and *icd_2* encoding isocitrate dehydrogenase showed 11.7- and 12.5-fold decreases after silver nitrate treatment, respectively (Figure S1), suggesting that these mutants had decreased fitness in silver nitrate. Silver nitrate may target SucC/SucD in *A. baumannii*, requiring alteration of fluxes through the TCA cycle, such that there is a heavier requirement for *icd_2* and *acnA*.

Another known antibacterial effect of AgNO₃ occurs via cytoplasmic membrane proton leakage and attenuated or ceased cell respiration¹³. We measured the membrane potential change upon exposure to AgNO₃ in *A. baumannii* AB5075 wild type strain using 3,3'-Diethyloxacarbocyanine iodide (DiOC₂(3)). AgNO₃ at as low as 1/32 MIC started to cause a drop in the membrane potential of cells at exponential phase in a dose-dependent manner (Figure 3B). The dissipation of membrane potential was reflected in decreased frequency of insertions in genes encoding proteins involved in electron/proton shuttling during respiration. For example, Tn5 insertions in *cydB* and *cydA_1*, encoding cytochrome D ubiquinol oxidase decreased by 5.5- and 7.0-fold respectively, and insertions in a ubiquinone biosynthesis gene BAL062_03562 decreased 4-fold (Figure S1). We hypothesize that these genes might be involved in maintaining the PMF to resist silver nitrate-induced cytoplasmic membrane proton leakage and the compromised electron suppliers from the TCA cycle.

An electron transport-related operon encoding cytochrome O (*cyo*) ubiquinol oxidase subunits had significantly reduced frequency of Tn5 insertions upon chloroxylenol, benzalkonium or ethanol treatment (Figure S1), suggesting that the electron transport chain is affected by these compounds, but in a different way to silver nitrate.

Biocide induced dissipation of membrane potential and impairment of membrane transport. The finding that cell division and cell respiration genes are required for fitness in the presence of multiple biocides indicated that, like AgNO₃, these molecules may cause dissipation of membrane potential as part of their activity. To explore this hypothesis, we first measured the membrane potential change of *A. baumannii* AB5075 on exposure to AgNO₃ using 3,3'-Diethyloxacarbocyanine iodide (DiOC₂(3)). As expected, given its known effect causing proton leakage, AgNO₃ caused a drop in membrane potential of exponential phase cells (Figure 3). This effect was apparent at as low as 1/32 MIC and was dose-dependent. We then measured the membrane potential of exponential-phase *A. baumannii* AB5075 following exposure to the remaining biocides at MIC. Based on the genetic fitness requirements revealed by TraDIS, we predicted that all biocides would impact membrane potential, with the exception of triclosan which was the only biocide that did not have respiratory among its resistance determinants. As shown in Figure 3A all compounds except for triclosan caused a drop in membrane potential. This suggests that dissipation of membrane potential might be a direct or downstream antibacterial effect of multiple biocides.

Cell membrane transport activities are energy dependent. If the biocides in this study do induce dissipation of membrane potential, they should also be able to compromise the solute efflux efficacy of the pumps that rely on membrane potential as energy source. Acriflavine is a fluorescent substrate for AdeB, AdeJ and AmvA (Figure 4A), which utilise PMF for substrate transport. Chlorhexidine and

benzalkonium can induce AdeABC expression but not AdelJK and AmvA; whereas triclosan does not affect the expression of any of these pumps. If benzalkonium and chlorhexidine induce the expression of AdeABC but have no impact on membrane potential, cells treated by either of these two biocides should have reduced acriflavine accumulation; however, if the biocides dissipate the membrane potential, despite efflux pump induction, higher acriflavine accumulation would be expected. To investigate the hypothesis above, we focused on how triclosan, ethanol, chlorhexidine and benzalkonium affect acriflavine accumulation in *A. baumannii* AB5075 wild type and its *amvA* transposon insertion mutant ($\Delta amvA$). As expected, at $\frac{1}{2}$ MIC, triclosan treatment did not lead to any change, whereas all the other three biocides increased acriflavine accumulation in the cells of both bacterial strains (Figure 4B). This suggests that, regardless of *adeB* induction, the dissipation of membrane potential induced by ethanol, chlorhexidine and benzalkonium significantly increased overall acriflavine accumulation.

Biocides' impact on antibiotic potency. The uptake of the poly-cationic aminoglycosides requires membrane potential (both the proton gradient (DH^+) and electrical potential (Dy) across the cytoplasmic membrane) and drug-sensitive ribosomal binding sites for irreversible drug uptake⁴⁷. Our next hypothesis is that the biocides that cause membrane potential dissipation can compromise aminoglycoside uptake and thus antagonise the killing effects of the antibiotic. As resistant bacterial strains accumulate less aminoglycoside antibiotic than sensitive strains⁴⁸, we used an aminoglycoside-sensitive strain *A. baumannii* ATCC17978 rather than the highly resistant AB5075 or BAL062 strain to test our hypotheses. We showed that apart from triclosan, the other biocides including benzalkonium, chlorhexidine, CTAB and ethanol at $\frac{1}{4}$ MIC increased *A. baumannii* ATCC17978 survival when treated with gentamicin (Figure 5A). Although benzalkonium, chlorhexidine and ethanol can induce AdeB expression, because AdeB does not confer resistance to gentamicin³⁹, the antagonism with these biocides is unlikely due to drug efflux. These observations raised the question of whether the biocides can also compromise the antibacterial effects of the other antibiotics that have different intracellular targets, such as fluoroquinolones and tetracyclines, and the ones targeting cell envelopes, such as colistin and β -lactams.

Uptake of tetracyclines into the cytoplasm is PMF dependent but likely involves passive diffusion of the uncharged form⁴⁹. Similarly, the zwitterionic form of the fluoroquinolones has been proposed to passively cross the cytoplasmic membrane, but due to their different protonation behaviour from tetracyclines, uptake of some fluoroquinolones has been shown to be negatively correlated with PMF^{49,50}. Comparing to the combination with triclosan or solo-antibiotic treatment, benzalkonium and chlorhexidine significantly increased *A. baumannii* survival rates with amikacin (another aminoglycoside), ciprofloxacin (fluoroquinolone) and tigecycline (glycylcycline) (Figure 5B). The observed biocide antagonism with amikacin and tigecycline is consistent with our hypothesis. In the case of ciprofloxacin, there are certain discrepancies in published data about the impact of PMF on the antibacterial effects of different fluoroquinolones⁵⁰. Although ciprofloxacin interaction patterns with triclosan, chlorhexidine and benzalkonium are similar to the other three antibiotics tested, we cannot conclusively state that dissipation of the membrane potential is the primary cause of the antagonism with biocides.

Dissipation of the membrane potential may not antagonise the antibacterial effects of imipenem and colistin, because the primary targets of these antibiotics are located on the cell surface. As expected, triclosan, benzalkonium and chlorhexidine at ¼ MIC have no impact on imipenem killing (Figure 5B). Synergism was observed between benzalkonium and colistin, but not for triclosan (Figure 5B). Chlorhexidine also seemed to marginally enhance colistin killing. The synergy between these compounds is likely because benzalkonium, chlorhexidine and colistin all targeting cell membranes. Collectively, our data suggest that the biocides that can dissipate membrane potential can promote *A. baumannii* tolerance to various classes of antibiotics which have intracellular targets.

Discussion

TraDIS enabled us to investigate the potential tolerance/resistance determinants and modes of action of 10 diverse biocides that are commonly used for disinfection in hospital and in personal hygiene products. These compounds have traditionally been thought to target the cell envelope, but our data suggests they have additional intracellular targets and need to penetrate through the cell envelope to exert their full antibacterial effects. Insertions in genes from multiple cellular pathways had statistically significant changes in frequency as a result of the biocide treatments, including the TCA cycle, electron transport chain, amino acid biosynthesis, nucleoside biosynthesis and biotin biosynthesis (SI 2). Various *A. baumannii* drug efflux pumps, such as AdeABC, AdeIJK and AmvA were shown to be tolerance/resistance determinants for multiple biocides, supporting the idea that these compounds may have intracellular target(s). The biocides also appeared to have diverse impacts on the cell surface. Transposon insertions in genes related to LOS and lipid A biosynthesis affected *A. baumannii* fitness in the presence of all of these 10 compounds. In contrast to LOS, capsular polysaccharide affected only AgNO₃ susceptibility out of the ten biocides tested.

While antibiotic stewardship has been a topic of increasing public health concern, the debate on whether biocide stewardship is necessary is still continuing⁵¹⁻⁵⁴. This can be largely explained by the fact that biocides' in-use concentrations are normally much higher than their MBCs. It should be emphasized that all of the biocides tested here are either WHO Essential Medicines or widely used hospital and household disinfectant products. In this study, we showed that the biocides at sub-MIC levels cause dissipation of the membrane potential which can significantly promote *A. baumannii* survival rate (tolerance) under treatment with various antibiotics that have intracellular targets, including aminoglycosides, ciprofloxacin and tigecycline. This is because membrane potential plays a critical role in drug uptake. However, they do not antagonise the killing effects of drugs targeting the cell envelope, such as colistin and imipenem.

Although drug efflux pumps only confer low level resistance to antibiotics, compared to their parental strains, bacteria that lack key MDR efflux systems are much less likely to develop MDR^{55,56}. Furthermore, intermittent antibiotic exposures have been shown to lead to more rapid evolution of tolerance and resistance⁵⁷⁻⁵⁹ and antibiotic tolerance facilitates the evolution of resistance⁶⁰. These works and our study together suggest that factors affecting antibiotic intracellular accumulation or drug target accessibility are important in the development of drug tolerance and AMR. Previous studies have typically

used direct measurement of bacterial susceptibilities to antibiotics and biocides to investigate co-selection of antibiotic resistance⁶¹. We propose that the principal concern with respect to biocide stewardship is not the emergence of biocide resistance or antibiotic cross-resistance, but rather the presence of residual biocides, especially chlorhexidine, benzalkonium and CTAB and others that are hard to remove from the environment, which could induce antibiotic tolerance and potentially AMR development upon the co-presence of antibiotics.

Declarations

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Figures

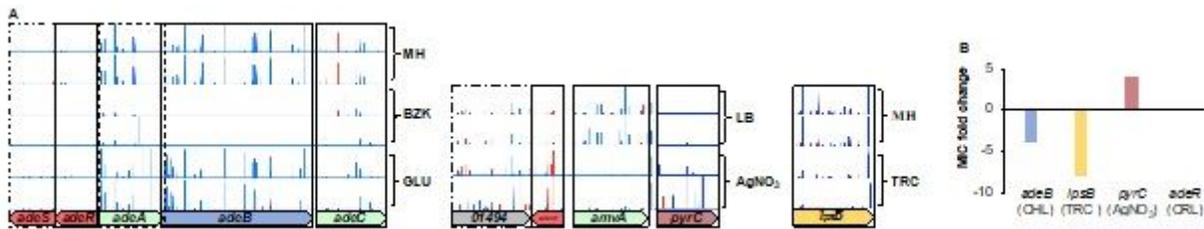


Figure 1

Biocide TraDIS data. (A) Representative genes and TraDIS insertion plots. Horizontal arrows represent gene length and direction, and gene names are within. Above each gene are lines representing reads mapped from each Tn5 insertion site, the height of the line represents the number of reads. The black box height represents a maximum of 100 reads in all conditions, except *lpsB* with 10 reads. (B) MIC fold change of four different mutants for relevant biocide compounds compared with the AB5075 wild-type strain. Biocides are listed in abbreviations (Table S1).

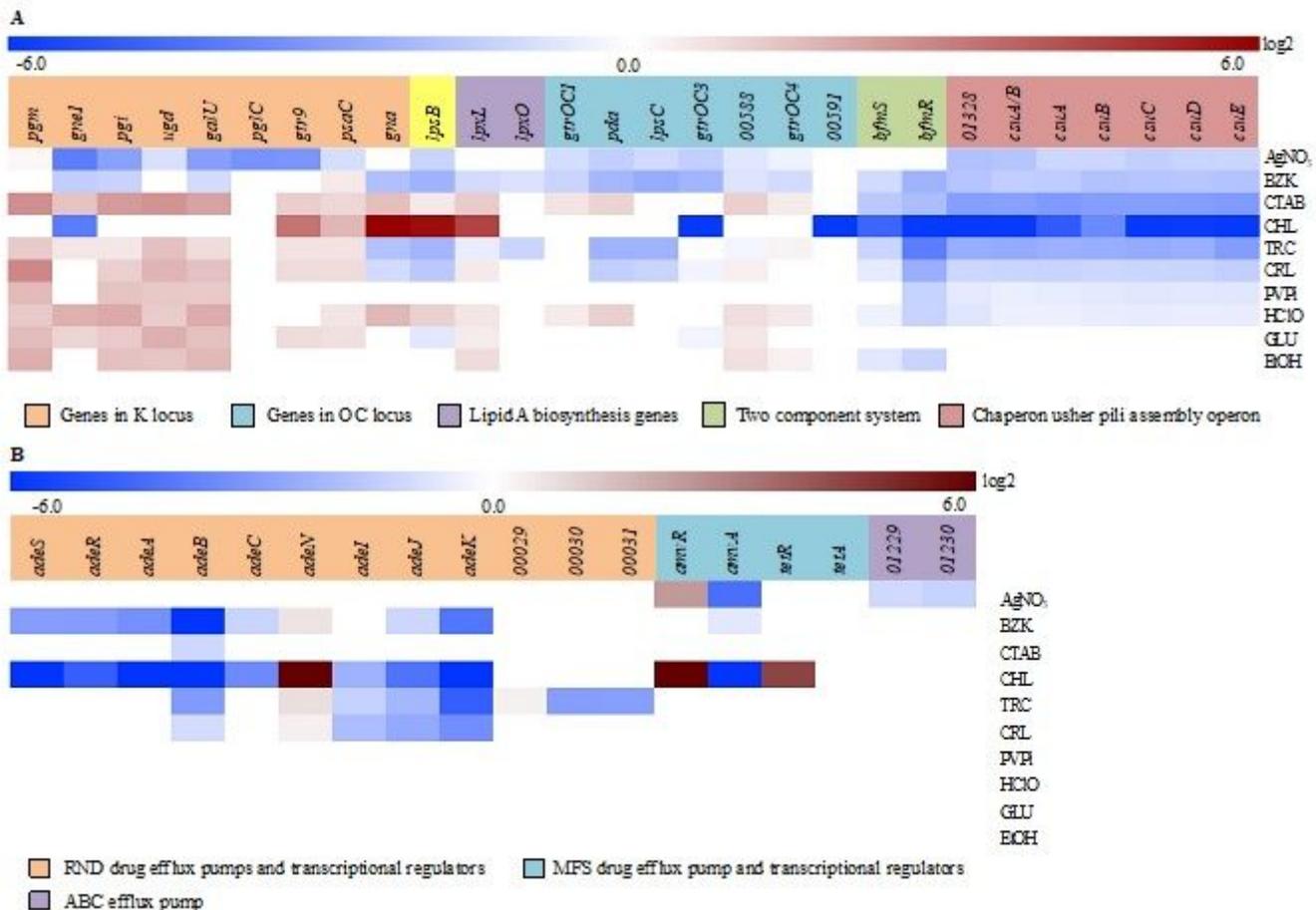


Figure 2

Genes with TraDIS insertion changes during biocide exposure. (A) Cell envelope-related genes. (B) Membrane transport-related genes. The colour-coded boxes represent the amount of Tn5 insertion read coverage fold change, for each representative gene (above) and for each biocide (righthand side; abbreviations as in Table S1), where blue indicates a larger decrease in Tn5 insertions and red shows an increase. Each gene's specific relationship to the cellular function is colour-coded, as stated below the heatmap.

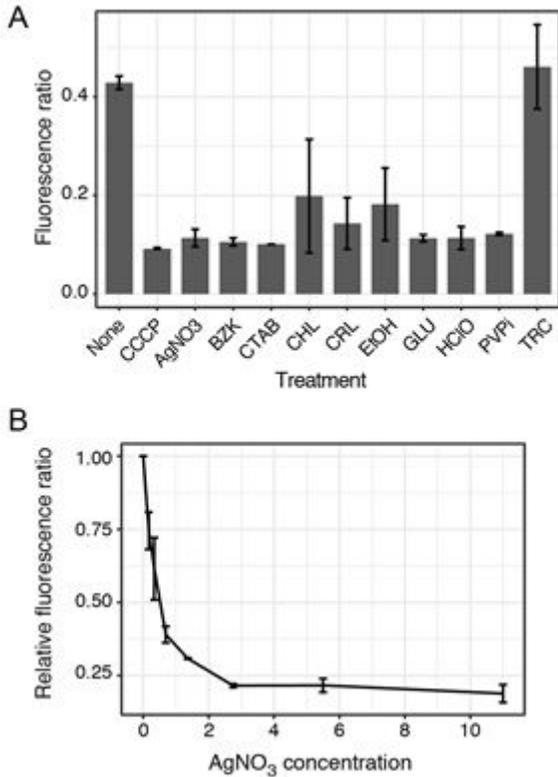


Figure 3

Effect of biocides on membrane potential. (A) Relative membrane potential reduction in *A. baumannii* after biocide exposure. Cells at OD₆₀₀ 0.4-0.6 were treated by the 10 biocides at 1xMIC, respectively. Two biological replicates were included. The membrane potential was measured with dye DiOC₂(3) which emits green fluorescence in the cytosol, but shifts towards red fluorescence as the dye molecules self-associate. The ratio of red versus green fluorescence of DiOC₂(3) shown here was calculated by population mean fluorescence intensities, which positively correlates with cytoplasmic membrane potential. (B) Dose-dependent relationship between AgNO₃ and membrane potential. Values on X-axis show AgNO₃ concentration (from 1/64 MIC to MIC), and 0 means no biocide treatment. The Y-axis shows the relative red/green fluorescence ratio change of each condition to no treatment. Two biological replicates were included.

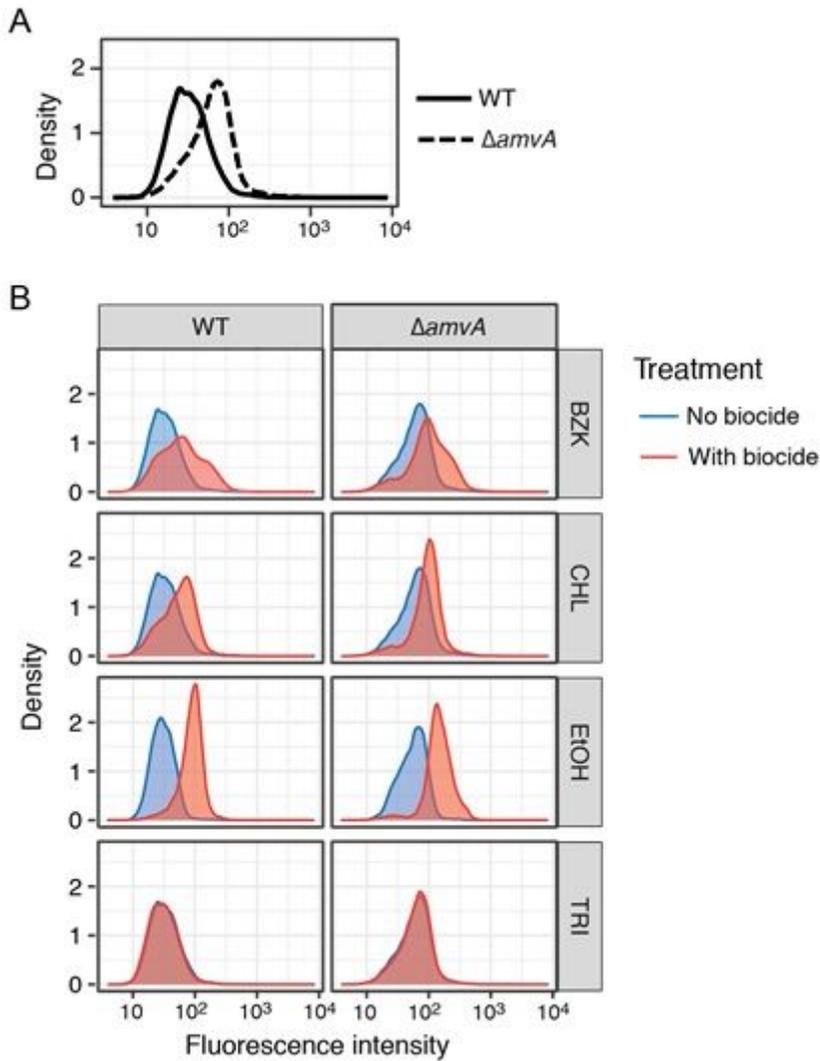


Figure 4

Biocides' impact on acriflavine intracellular accumulation. (A) Acriflavine accumulation difference between AB5075-UW wild type and the transposon mutant of acriflavine transporter *amvA*. (B) Acriflavine accumulation in both AB5075-UW wild type and $\Delta amvA$, upon the treatment of triclosan, ethanol, chlorhexidine or benzalkonium at $\frac{1}{2}$ MIC, respectively. Each curve shows the fluorescence intensity for 50,000 cells. The cell populations show distinct fluorescence profiles based on the concentration of acriflavine in the cell cytoplasm.

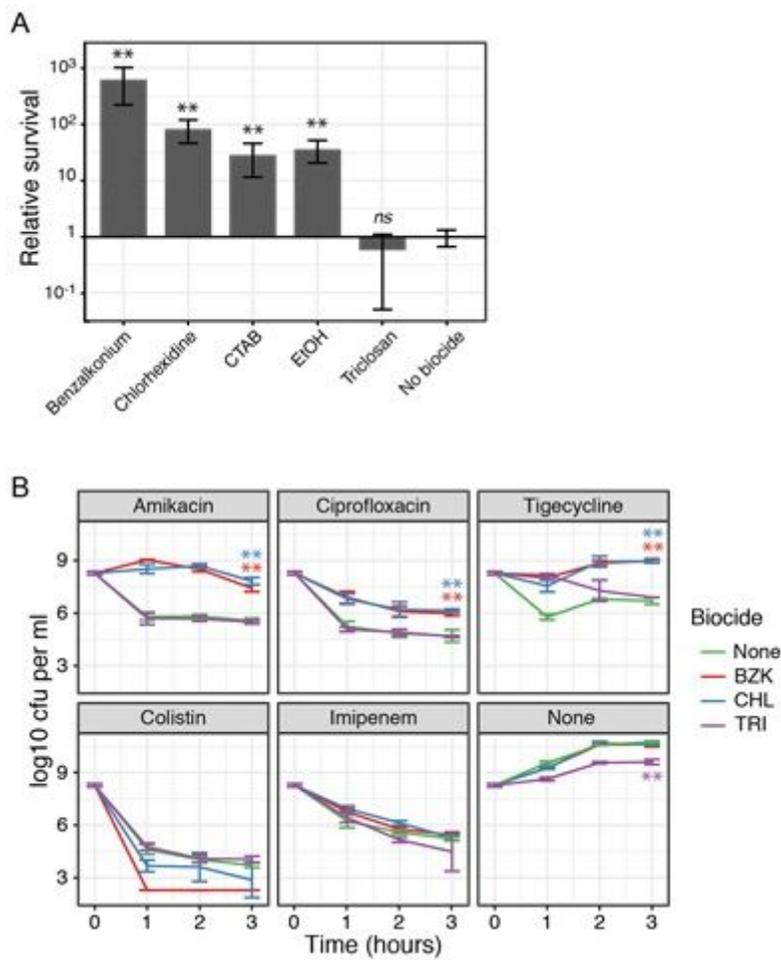


Figure 5

Biocides and antibiotics interactions in *A. baumannii*. (A) Biocides that dissipate membrane potential also reduce killing by gentamicin. Treatment of *A. baumannii* ATCC17978 with 4µg/ml Gentamicin (2x MIC) for 60 minutes killed 99.8% of cells. Addition of benzalkonium, ethanol, chlorhexidine or CTAB at ¼ MIC did not influence bacterial growth, but dramatically reduced killing by gentamicin, with survival in the presence of biocide 30-600 times higher than survival with gentamicin alone. Triclosan, which did not influence membrane potential at ¼ MIC, did not have this effect. Three biological replicates were included. Results shown are survival relative to gentamicin without biocide. Statistical significance was assessed by one-way ANOVA with Dunnett's post-hoc test. (B) Biocide antagonism with a broader range of antibiotics of intracellular targets. Treatment of *A. baumannii* ATCC17978 with 2xMIC of amikacin, ciprofloxacin or 3xMIC of tigecycline for 1 hour killed more than 99% of cells. After 1 hour, there was no significant killing effects observed for either of the three conditions, which was possibly due to persisters or drug tolerance of the minority of the cell population. Addition of benzalkonium or chlorhexidine at ¼ MIC did not affect bacterial growth, but significantly inhibit killing of these three antibiotics, with survival in the presence of biocide 100-1000 times higher than survival with antibiotic alone. Triclosan did not have antagonistic effect with any of the antibiotics. Colistin at 2xMIC killed more than 99% of cells within 1 hour. Addition of ¼ MIC of triclosan did not influence bacterial growth and colistin killing, but benzalkonium and chlorhexidine increased the killing rate for 10-100 times. None of the three biocides

affected killing by 2xMIC of imipenem. Three biological replicates were included. Statistical significance overall was assessed by mixed-repeated measures ANOVA, followed by a one-way ANOVA with Dunnett's post-hoc test at $t = 3$ if the first test showed a significant effect.

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