Understanding Tolerance to Biocides and its Consequences in Clinical Isolates of *Klebsiella pneumoniae* – How to Treat an ESKAPE Pathogen

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Contents

List of Abbreviations

HAI	Hospital-Acquired Infection
CAI	Community-Acquired Infection
AMR	Antimicrobial Resistance
ESKAPE	*Enterococcus faecalis, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, Enterobacter spp.*
ESBL	Extended-Spectrum β-Lactamase
KPC	*K. pneumoniae* Carbapenemase
OXA-48	Oxacillinase-48
NDM-1	New Dehli Metallo-β-lactamase 1
CRKP	Carbapenem Resistant *K. pneumoniae*
CRE	Carbapenem Resistant Enterobacteriaceae
MIC	Minimum Inhibitory Concentration
SMR	Small Multidrug Resistance
MATE	Multidrug And Toxic-compound Extrusion
MFS	Major Facilitator Superfamily
RND	Resistance Nodulation Division
ABC	ATP-Binding Cassette
ATP	Adenosine Triphosphate
UTI	Urinary Tract Infection
FDA	Food and Drug Administration
ENR	Enoyl Reductase
FASII	Fatty Acid Synthesis pathway II
MDR	Multidrug Resistant
LB	Luria-Bertani
BSAC	British Society of Antimicrobial Chemotherapy
MRSE	Methicillin-Resistant *Staphylococcus epidermidis*
MRSA	Methicillin-Resistant *S. aureus*
TRI	Triclosan
BAC	Benzalkonium Chloride
QAC	Quaternary Ammonium Compound
CHX	Chlorhexidine Digluconate
MHB	Mueller Hinton Broth
MHA	Mueller Hinton Agar
DMSO	Dimethyl Sulfoxide
ATCC	American Type Culture Collection
RCF	Relative Centrifugal Force
CSS	Combined Susceptibility Score
EtBr	Ethidium Bromide
CLSI	Clinical Lab Standards Institute
EUCAST	European Committee on Antimicrobial Susceptibility Testing
OD	Optical Density

1.0. Introduction to *Klebsiella pneumoniae*

1.1. *Klebsiella pneumoniae* and its Clinical Relevance

Klebsiella pneumoniae and other closely related species are Gram-negative bacteria found ubiquitously in nature; free in soil, water, and sewage, adhering to plant cell surfaces, and as commensal organisms in the gastrointestinal tracts of animals and humans (66, 126). *K. pneumoniae* was first isolated in 1882 from samples taken from the respiratory tracts of pneumonia patients however this bacterium can also cause opportunistic infections of the urinary tract and bloodstream (66). *K. pneumoniae* is often found as a commensal in the gut however colonisation rates vary widely based on geography; in the US and Australia there is approximately 4-6% colonisation in the community but in Asia carriage rates range from 18-87% of healthy individuals (59, 126).

Prolific biofilm formation enables host colonisation, adhesion to surfaces, and antimicrobial tolerance, aspects of the *K. pneumoniae* lifestyle which prime it for nosocomial transmission (119). As a result, *K. pneumoniae* is the 3rd leading cause of hospital-acquired infections (HAIs) and the 2nd leading cause of Gram-negative bloodstream infections in the US, and rates of colonisation in the US and Australia increase dramatically from 4-6% to 25% in those recently exposed to the hospital environment (66, 126). The primary sources of infection in hospitals are the hands of healthcare workers and fomites such as contaminated surfaces and instruments. While only transiently present on the skin, *K. pneumoniae*'s ability to colonise mucosal surfaces creates significant reservoirs for transmission and infection (66, 105). Bloodstream infections are often the result of dissemination of the bacteria from another site, commonly implicated sources include the urinary tract, gastrointestinal tract, intravenous or urinary catheters, and respiratory sites (82). Infections are normally nosocomial, generally occurring in the immunocompromised and other vulnerable groups such as neonates and the elderly, however some strains of *K. pneumoniae* can behave as a true non-opportunistic pathogen and cause community-acquired infections (CAIs) such as endophthalmitis, pneumonia, necrotising fasciitis, meningitis, and non-hepatic abscesses (55, 126).

In Europe, *K. pneumoniae* strains cause over 90,000 infections and 7,000 deaths annually, in part due to the rapid acquisition of antimicrobial resistance (AMR) mechanisms which

has placed this bacterial species in the ESKAPE pathogen groups alongside *Enterococcus faecium*, *Staphylococcus aureus*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter spp.* (54, 93, 125). Furthermore, owing to its ecology and genome plasticity, *K. pneumoniae* is implicated as a key amplifier and disseminator of AMR genes and has a close association with early reports of many AMR genes prior to their dispersal amongst other bacterial species. It has been shown that and multidrug resistant (MDR) phenotype can be passed from *K. pneumoniae* to *Escherichia coli* via conjugation (127). Examples of these genes include extended-spectrum beta-lactamases (ESBLs) such as *K. pneumoniae* Carbapenemase (KPC), Oxacillinase-48 (OXA-48), and New Delhi Metallo-β-lactamase-1 (NDM-1), which enable resistance to carbapenems (54, 125).

K. pneumoniae infections are commonly treated with β-lactams and other antibiotics that are effective against Enterobacteriaceae, however this species is intrinsically resistant to penicillins and many MDR strains have emerged due to exposure to antibiotics in clinical settings (66, 126). Carbapenem-resistant *K. pneumoniae* (CRKP), a subset of carbapenem-resistant Enterobacteriaceae (CRE), is among the most clinically threatening forms of this bacterial species. Despite their name, these strains are usually resistant to many other classes of antibiotic in addition to carbapenems as carbapenems are not the first line drugs for treatment. CRKP strains are becoming increasingly prevalent in hospitals in China, increasing from 1.8% of isolates in 2012 to 3.6% in 2015 (127). Several mechanisms of acquired resistance underpin high tolerance to carbapenems and other antimicrobials in these strains, including permeability alterations in the outer membrane, overexpression of efflux systems, and overproduction of a myriad of β-lactamase enzymes such as ESBLs and carbapenemases (45, 70, 85). Possession of a multitude of ESBLs increases tolerance of β-lactams to a greater extent than carriage of a single variety, minimum inhibitory concentrations (MICs) of ertapenem, imipenem, and meropenem have been shown to dramatically increase in cells with both bla_{NDM-1} and bla_{KPC-2} compared to those with only bla_{NDM-1} (127). One Chinese MDR CRKP isolate was found to co-harbour eight separate resistance genes: four ESBLs, bla_{NDM-1}, bla_{KPC-2}, bla_{CTX-M}, and bla_{SHV-1}, three fluoroquinolone resistance genes, *acc(6')-Ib, acc(6')-Ib-cr,* and *qnrB*, and a 16S rRNA methylase gene which provides aminoglycoside resistance, *rmtB* (127). Biofilms produced by *K. pneumoniae* can also reduce the effectiveness of some antimicrobials by interfering

with diffusion of the compounds (4, 108). It is for these reasons that investigating the effects of biocide tolerance on potential cross-resistance with antibiotics and influence on other phenotypic characteristics in clinical isolates of *K. pneumoniae* is of great importance.

2.0. Biocides and Antimicrobial Resistance

Antimicrobial compounds are a cornerstone of modern human society, but the principles behind their use are far more ancient. Many classes of antibiotics originate in fungal or bacterial species, having developed as a part of evolutionary arms races between different species of microorganisms vying for access to resources in the environment. Consequently, antimicrobial resistance mechanisms are a natural result of the selection pressure applied to microorganisms.

Antibiotic resistance is defined as an increase in the MIC of an antibiotic due to a permanent change in the cells, while antibiotic tolerance is defined as the ability of cells to survive an ordinarily lethal antibiotic exposure due to a reversible phenotypic state (13, 63). This definition applies to acquired resistance, while some species of microorganism are intrinsically resistant to specific antimicrobials due to the presence of wild-type resistance determinants in the core genome or the lack of a drug's specific target. The specific increase in an MIC caused by the development of resistance is not included in the definition, however in clinical research species-specific breakpoints that denote resistance, intermediate resistance, or susceptibility exist and are used to determine a strain's antibiotic susceptibility profile (52, 115). Resistance is essentially a heritable change in a lineage, while tolerance is essentially a transient form of resistance within a cell and is often related to the mechanism of a drug's action and the rate of bacterial growth (27, 114). Persistence is another related phenomenon, first discovered in 1944 by Professor Joseph Bigger, that occurs within subpopulations of "persister cells" during antibiotic exposure (8, 13). This is a growth-state dependent heterogenous response that occurs in a proportion of cells that survive antibiotic treatment by existing in a non-dividing and non-growing state. Cultures grown from persisters will have similar survival dynamics to the parental culture (52).

2.1. The Role of Efflux and Permeability in Antimicrobial Resistance

Efflux pump systems are intrinsic to bacterial species, having evolved as a means of removing toxic compounds from the cell, but can also be acquired *via* horizontal gene transfer (5, 67, 96). These systems are problematic in disinfection procedures and antibiotic therapy; they are often implicated as a first line of defence against antimicrobials by lowering the intracellular concentration of drugs enough to enable the development of other resistance mechanisms (22). Efflux pumps can be drug-specific or promiscuous in their substrate range, with the adaptive overexpression of the latter form being a cause of cross-resistance between diverse classes of antimicrobials (5, 21, 31, 60). There are five main drug efflux pump families, delineated on the basis of structure, homology, and energy source. Members of the Small Multidrug Resistance (SMR); Multidrug and Toxic compound Extrusion (MATE); and Major Facilitator Superfamily (MFS) families are located in the cytoplasmic membrane and extrude intracellular compounds across the cell membrane. The Resistance Nodulation Division (RND) and ATP-Binding Cassette (ABC) families are most prominent in Gram-negative bacteria and are typically tripartite in structure, composed of an efflux protein in the cytoplasmic membrane, a membrane fusion protein in the periplasm, and an outer membrane protein channel (46, 95, 96). Members of the ABC family rely on ATP as their source of energy, while the other families utilise the chemiosmotic proton gradient (96). Some members of the MATE family can use a sodium motive force instead of or in conjunction with the proton motive force (89). Efflux pumps can also contribute to biofilm formation, which can play a role in bacterial survival on surfaces and indwelling devices, AcrAB-TolC in *E. coli*, MexAB-OprM in *P. aeruginosa* and AdeFGH in *A. baumannii* are examples of drug efflux pumps that contribute to biofilm formation (2, 109). *P. aeruginosa* is exemplar of the effect decreased permeability and increased efflux can have on an antibiotic susceptibility profile, since many strains have an arsenal of efflux pumps that can be upregulated in response to antimicrobial exposure. Porins such as OprD are also commonly downregulated simultaneously with efflux pumps such as MexEF-OprN, MexAB-OprM, and MexXY in this bacterium (1, 93). Alterations to porins are also found in CRKP, contributing to their high levels of resistance (83).

Active efflux is a key contributor to the drug resistant phenotypes of MDR *K. pneumoniae* strains. AcrAB is a major drug efflux pump from the RND family present in a wide variety of bacteria, including *K. pneumoniae,* where its overexpression has been demonstrated to contribute to resistance to several antibiotics. Examples of the drugs extruded by AcrAB-TolC include oxacillin, cloxacillin, norfloxacin, erythromycin, tetracycline, and novobiocin, among others (5, 84, 86). Other RND pumps can also contribute to the efflux of antimicrobials, such as OqxAB, KexAB, KexD, KexEF, and EefABC, as well as KpnEF from the SMR family, which effluxes cefepime, ceftriaxone, colistin, rifampin, erythromycin, tetracycline, and streptomycin, KetM from the MATE family, which extrudes norfloxacin, ciprofloxacin, and cefotaxime, and KdeA from the MFS family, which acts on chloramphenicol and norfloxacin (84, 89, 94, 111). Due to the upregulation of efflux systems such as KpnEF in response to the stress caused by antimicrobials, it may be possible for biocide exposure to reduce susceptibility to antibiotics (111).

2.2. The Role of Biofilm in Antimicrobial Resistance

Biofilms are of great importance to the survival of bacteria in the environment, particularly during exposure to antimicrobial compounds. Environmental biofilms can facilitate the survival of an infectious dose of bacteria on abiotic surfaces, serving as a reservoir for transmission (103). The communities of microorganisms within these highly complex three-dimensional prokaryotic structures have been likened to multicellular organisms (28, 112). Extracellular matrix (ECM); the exopolysaccharides, eDNA, and proteins composing the biofilm, reduces the exposure of bacterial cells to many deleterious compounds, greatly increasing the MICs of these agents, as much as 10-1000-fold relative to the MICs for planktonic cells (6, 24, 28, 30, 108). The effects of antibiotic-degrading enzymes, such as β-lactamases, within the biofilm can enhance the reduction in antibiotic penetration further (4). Erythromycin and cetyltrimethyl ammonium bromide cannot efficiently penetrate biofilms (6). Ciprofloxacin and benzalkonium chloride penetration is unincumbered by biofilm, however the penetrative ability of an antibiotic or biocide is not correlated with its killing or removal efficiency. Other mechanisms related to bacterial growth in a biofilm may increase tolerance such as lowered metabolic activity due to specific microenvironments within the biofilm (4, 6, 124, 129). Older, more mature,

K. pneumoniae biofilms have greater tolerance of amikacin, this may be due to increased production of exopolysaccharide in older biofilms relative to younger biofilms (108). Biofilm-mediated antibiotic resistance can be difficult to define using the conventional definitions of resistance and tolerance because the resistance is caused by the differential expression of wild-type genes relative to planktonic cells and generally not due to mutations (63). In conjunction with their effect on antimicrobial efficacy biofilms can also facilitate the spread of resistance determinants between organisms, including different species of bacteria as biofilms can be densely inhabited by multiple species at once (28, 63, 110).

Bacterial growth during infection of human bodies is often found within biofilms, increasing the difficulty of successful treatment (24, 30). Biofilms on indwelling devices such as urinary and vascular catheters or endotracheal tubes cause 50-70% of all nosocomial infections, these infections, commonly caused by *S. aureus, S. epidermidis, E. faecalis, E. coli, K. pneumoniae*, and *P. aeruginosa*, are often recurrent and cause chronic inflammation (24, 30, 103). One study found that, among catheterised patients suffering from UTIs, 63% of *K. pneumoniae* isolates from urine were positive for *in vitro* biofilm production, slightly above the average of 60% for all strains involved (87). UTIs often recur within six months of recovery, biofilms likely contribute to this persistence (63). Host respiratory secretions can contribute to biofilm formation in the lungs, increasing the likelihood of ventilator-associated pneumonia (VAP) and worsening *K. pneumoniae* infections, among other pathogens, in patients with chronic obstructive pulmonary disease (COPD) (30, 103). For these reasons, biofilm formation is a particularly prolific virulence mechanism that has the additional benefit of increasing bacterial survival during exposure to antimicrobials.

2.3. Triclosan

2.3.1. Overview on Triclosan

Triclosan is a bisphenol biocide with a broad antibacterial spectrum and antifungal properties (73, 104). This biocide is classed as an aromatic ether, it is phenol substituted at C-2 with a 2,4-dichlorophenoxy group and at C-5 by a chloro group (43). The 2-D

chemical structure is visible in Fig. 1. Triclosan can be found in many formulations such as toothpastes, cosmetics, antiseptic soaps, detergents, lotions, and deodorants and materials such as plastics, food storage containers, garbage bags, kitty litter, paints, children's toys, and carpets (57, 128) however, its use has been restricted in the United States and Europe in recent years. In 2016, the FDA banned the sale of over-the-counter consumer antimicrobial soaps containing triclosan and 18 other chemicals on the grounds of resistance and endocrine dysfunction (65, 75). This is similar to the European Union, however triclosan can still be found as a preservative at specific concentrations in dermally applied cosmetic products, toothpastes, and mouthwash (100). Triclosan is now ubiquitous in the environment due to its use over several decades and the dearth of efficient removal processes, although enzymes capable of triclosan degradation do exist and may present an avenue for reducing its presence (75, 76, 113). This persistence in the environment is reason for continued research on the effects of triclosan and other antibacterial compounds on bacterial communities, particularly in the wake of the COVID-19 pandemic which saw a huge increase in the use of disinfectants (58, 65). Reformulations of triclosan may also appear on the market and contribute to the accumulation of this biocide. Similar to this study, most published research into the effects of triclosan on microorganisms focus on pure clinical samples and acute exposure to the chemical. Many of these studies associate triclosan with multidrug resistance (15). Further research should address the impacts of chronic triclosan exposure on microbial communities in a non-medical scenario. Existing literature indicates that communities can be biased towards clades containing commensal and pathogenic bacteria and that these communities have a higher relative abundance of efflux system components (17, 33).

Triclosan was once thought to be a non-specific antibacterial and antifungal agent but was found to act by inhibiting an enzyme responsible for lipid biosynthesis (73). The NAD(P)H-dependent enzyme, enoyl-acyl carrier protein reductase (ENR), is a component of fatty acid synthetase in the bacterial type II fatty acid biosynthesis (FASII) pathway and is encoded by the *fabI* gene (formerly *envM*) in bacteria such as *E. coli*, *S. aureus*, and *Francisella tularensis* (7, 57, 73, 91). In mycobacteria, such as *M. tuberculosis*, *inhA* is the *fabI* gene homolog (61, 91). ENR inhibition is bacteriostatic, however triclosan does appear to act upon multiple nonspecific targets and induce K^+ leakage at higher,

bactericidal concentrations (23, 99, 128). Structural analysis of triclosan in complex with ENR and NAD^+ revealed that triclosan is a site-directed potent inhibitor of the enzyme which acts by mimicking the natural substrate. The phenol ring of triclosan interacts with the nicotinamide ring with extensive pi-pi stacking and both rings of the triclosan molecule form Van der Waals contacts with residues within the active site of the enzyme's substrate binding pocket (57).

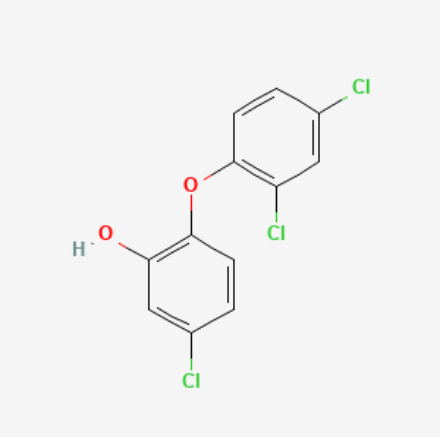

Fig. 1. Image depicting the 2-dimensional chemical structure of Triclosan. Triclosan is an aromatic ether, it is phenol substituted at C-2 with a 2,4-dichlorophenoxy group and at C-5 by a chloro group (43).

2.3.2. Triclosan Resistance and Cross-Resistance with Antibiotics

Increased triclosan tolerance can be readily acquired *via* serially passaging bacteria in increasing concentrations of the biocide and has been associated with changes in susceptibility to clinically relevant antibiotics (99, 128). Some bacteria are intrinsically resistant to triclosan, such as *P. aeruginosa*, which has reduced permeability, increased efflux, and a non-susceptible ENR isoenzyme, FabV. Similarly, *Streptococcus pneumoniae, E. faecalis* have FabK and *Bacillus subtilis* have FabL (15, 69, 128). Other intrinsically resistant bacteria produce triclosan-degrading enzymes, such as *Pseudomonas putida* strain TriRY and *Achromobacter xylosoxidans* spp. dentrificans strain TR1 (76, 128). These enzymes have the potential for horizontal gene transfer to susceptible species, and it has been shown that environmentally relevant triclosan concentrations can stimulate the conjugative transfer of MDR plasmids within and across genera (62). Acquired resistance mechanisms include mutations in the *fabI* gene encoding the target ENR protein, active

efflux due to overexpression of the global transcriptional activator *marA* and the *acrAB* efflux genes, and increased expression of the *fabI* gene (14, 74, 128). Of these resistance mechanisms, active efflux has the highest potential for causing cross-resistance with, or reducing susceptibility to, a broad spectrum of other antimicrobials. Mutations in *fabI* or its homologue *inhA* in *M. smegmatis* and *M. tuberculosis* selected for in the presence of triclosan have been shown to cause cross-resistance with diazaborines, a family of boron-containing antibacterial agents that target ENR, and isoniazid, one of the first line drugs for treating cases of tuberculosis (7, 91, 99, 128). Increased triclosan tolerance in *E. coli* K12 is often the result of single amino acid substitutions in ENR, the substituted residues, G93V, M159T, and F203L, have direct contact with triclosan during drug binding to the wild-type protein. Steric hindrance by valine due to the G93V mutation in the *fabI* gene produces a roughly 100-fold increase in the MIC at the cost of a decreased growth rate of approximately 40% in Luria–Bertani (LB) broth (57, 73). Similar mutations occur in the ENR protein under selection for diazaborine tolerance; G93S in the *E. coli fabI* gene and S94A in the *M. tuberculosis inhA* gene, with the latter also causing isoniazid resistance (7). Mutations in the target gene in response to triclosan present in the environment may derail current attempts to develop InhA inhibitors for the treatment of tuberculosis (91). *M. smegmatis* isolates selected for triclosan tolerance demonstrate co-resistance to isoniazid due to mutations in *inhA*, but mutants selected for isoniazid resistance retain triclosan susceptibility (128). This one-way relationship is likely due to mutations in the endogenous KatG enzyme, which activates isoniazid (91).

It is unclear whether adaptations to higher triclosan concentrations in the environment cause cross-resistance with clinically relevant antibiotics that do not target the ENR protein. A study by Cottell *et al* showed no association of antibiotic resistance and triclosan tolerance in strains of *S. aureus*, *E. coli*, and *Acinetobacter johnsonii*. While susceptibilities did appear to change in disc diffusion assays, the significant size differences in the zones of inhibition were not large enough to alter the classification of susceptible according to the British Society of Antimicrobial Chemotherapy guidelines (BSAC). Triclosan tolerant strains of *E. coli* were demonstrated to be significantly more susceptible to aminoglycosides (23). Studies have shown that clinical Methicillin Resistant *Staphylococcus epidermidis* (MRSE) isolates can have lower triclosan susceptibility (102).

Raised tolerances have also been verified in dermal, intestinal, and environmental microorganisms, some of which have clinical relevance (128). Conversely, several studies have demonstrated an increase in active efflux in isolates with higher triclosan tolerances. Upregulation of the AcrAB efflux system has been noted in *E. coli* and *Salmonella enterica* (74, 121), similarly upregulation of Mex efflux pumps in *P. aeruginosa* and *Rhodospirillum rubrum* (19, 79, 97). The OqxAB pump has also been shown to export triclosan in *E. coli* (37). The wide range of substrates transported by these pumps indicates that their upregulation in response to triclosan should affect bacterial susceptibility to other antibacterial agents, as is the case in MDR *P. aeruginosa* and other species (31, 60, 71, 104). TriABC, triclosan-specific pump from the RND family and composed of two fusion proteins, has also been discovered in a *P.* aeruginosa strain which had knockouts mutations in the MexAB-OprM, MexCD-OprJ, MexEF-OprN, MexJK, and MexXY efflux systems that normally contribute to increased triclosan tolerance (79). Some evidence does exist for the association of raised triclosan tolerance with ciprofloxacin (16, 99), chloramphenicol (10, 97), carbenicillin (97), and tetracycline (97). A study showed that *E. coli* acquired high levels of triclosan resistance after two sublethal exposures and subsequently demonstrated reduced susceptibility to chloramphenicol, erythromycin, imipenem, tetracycline, and trimethoprim as well as a number of biocides (11).

2.4. Benzalkonium Chloride

2.4.1. Overview on Benzalkonium Chloride

Benzalkonium chloride (BAC) is a cationic biocide with broad-spectrum activity, with the exception of bacterial endospores, that is commonly used in medical and food-processing environments (64, 80). Its 2-dimensional structure is shown in Fig. 2. It is also present in many consumer products such as eye drops, facial cleansers, sun protection creams, and hand sanitisers (38). It is one of many quaternary ammonium compounds (QACs), a group of antimicrobials that are thought to primarily act via non-specific disruption of cellular membranes (53). Membrane disruption is proposed to be achieved *via* firstly, adsorption of the QAC's positively charged head group, characterised by a positively charged quaternary nitrogen, to components of the bacterial cell envelope, and secondly,

solubilisation of the membrane by the long alkyl chains to form mixed micellar aggregates (34, 38, 122). BAC may also cause the denaturation of cellular proteins, as is hypothesised as its mechanism of action against non-enveloped viruses (37, 53). Superoxide stress is also a proposed effect of BAC on *E. coli* (9). Persistence of BAC in the environment is relatively minimal in comparison to triclosan, with 91-99% of BAC being removed in sewage plants (38).

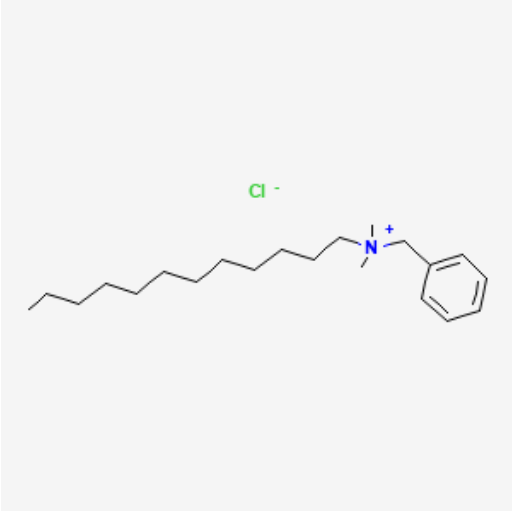

Fig. 2. Image depicting the 2-dimensional chemical structure of Benzalkonium Chloride. Benzalkonium is an aromatic amine and quaternary ammonium compound, commonly found as a biocide in its chloride salt form (44).

2.4.2. Benzalkonium Chloride Resistance and Cross-Resistance with Antibiotics

The consequences of exposure to BAC and other QACs on bacteria has been relatively well-studied from a variety of investigative avenues. Exposure to sub-inhibitory concentrations of BAC has been shown to enable the development of resistant clones of wild-type strains in a variety of bacterial species. 1-5% of cells can survive treatment with half the MIC of the biocide and maintain a morphology and growth rate similar to that of non-exposed cells, however the increased tolerance of BAC persists through serial transfers without the biocide (80). Due to the non-specific BAC mechanism of action, changes in the expression of genes, rather than point mutations, are often induced. Altered efflux and permeability is the most common form of resistance mechanism. The expression of the AcrAB-TolC is commonly upregulated in many bacteria, such as *K. pneumoniae*, as are QacE-I type exporters from the SMR family, MexAB-OprM in *P.*

aeruginosa, and OqxAB in *K. pneumoniae* and other Enterobacteriaceae (12, 25, 37, 38, 53, 80). Downregulation of porins such as OmpF has also been documented (9, 80). Osmotic and oxidative stress genes are often upregulated in response to the antimicrobial effects of BAC, and cell envelopes become more hydrophobic (9, 12, 53, 80). Only three non-stress factors have been found to be significantly upregulated in BAC-adapted *E. coli*, these are *ykfE*, b1171, and osmB. The b1171 gene has no known function, OsmB is a lipoprotein, and ykfE is an inhibitor of C-lysozyme with a potential protective function against damaging agents (80, 81). Changes in the expression of ribosomal subunits has also been noted and is thought to decrease protein aggregation under the stress caused by BAC (9, 53). Additionally, *P. aeruginosa* has been found to increase the content of phospholipids and fatty and neutral lipids in the periplasm as a means of sequestering BAC and decreasing the quantity that reaches the cell membrane (101).

The examination of antibiotic susceptibility profiles in BAC-adapted strains has primarily shown that resistance to higher BAC concentrations does not cause cross-resistance. Several studies have shown no increase in resistance, and others have shown an increase in amikacin, ceftazidime, ciprofloxacin, gentamycin, imipenem, ticarcillin, and tobramycin susceptibility in BAC-adapted *P. aeruginosa* (47, 88, 107). The previously mentioned upregulation of the *ykfE* gene may have the potential to cause cross-resistance with the cationic peptide polymyxin B, as the encoded protein has homology with VirK in *Salmonella*, a protein that confers resistance to this antimicrobial (26, 80). As for cross-resistance with other biocides, there is some evidence to suggest that BAC-adaptation raises the chlorhexidine tolerance in *S. enterica* Typhimurium, but not vice versa (11). Extensively drug resistant strains of *K. pneumoniae,* while capable of spreading in hospital environments, do not exhibit raised BAC tolerance (36, 40). While BAC exposure likely does not elicit cross-resistance, BAC has been shown to antagonise the activity of aminoglycosides if both agents are present at the same time (106).

2.5. Chlorhexidine Digluconate

2.5.1. Overview on Chlorhexidine

Chlorhexidine (CHX) is a divalent, cationic biguanide compound first discovered in the 1950's, but came to prominence as a common hospital disinfectant in the 1970's (48). The 2-dimensional structure of CHX is shown in Fig. 3. CHX is available in salt form, either diacetate, digluconate, or dihydrochloride, at concentrations ranging from 0.5-4% in consumer oral care solutions and in skin-antisepsis formulations which are used for disinfection prior to surgical procedures and catheter insertion (48, 78). The digluconate salt of CHX has much higher solubility (20% wt/vol) that the other forms and is used clinically as a 2% diluted solution combined with 70% iso-propyl alcohol (39, 130). Topical and oral usage of CHX has been shown to be beneficial in prevention of VAP (18, 48). This usage is related to its long residual activity and broad-spectrum activity, primarily against Gram-positive bacteria, including MRSA, due to the higher negative charge of their cell surfaces but CHX does have some activity against Gram-negative species, fungi, and some viruses (3, 39, 116). It is ineffective against bacterial spores and mycobacteria, likely due to spore coats and the prevention of biocide entry by the cell wall respectively (72). Mechanistically, CHX acts on the cell membrane, binding to anionic sites via its lipophilic groups, interfering with the osmolarity and integrity of the cell and causing leakage of protons and potassium ions at low concentrations and other cellular materials at higher concentrations (34, 39, 41). This mechanism is similar to that of QAC's however biguanides such as CHX bind more strongly to anionic regions of the cell membrane but hydrophobic regions of CHX do not become solubilised in the membrane, instead bridging adjacent phospholipid headgroups and displacing divalent cations that usually stabilise the membrane (34). Research into the presence of CHX in the environment is limited, however it has been confirmed to be ubiquitous in sludge samples from sewage treatment plants and has the potential to alter microbial populations (35).

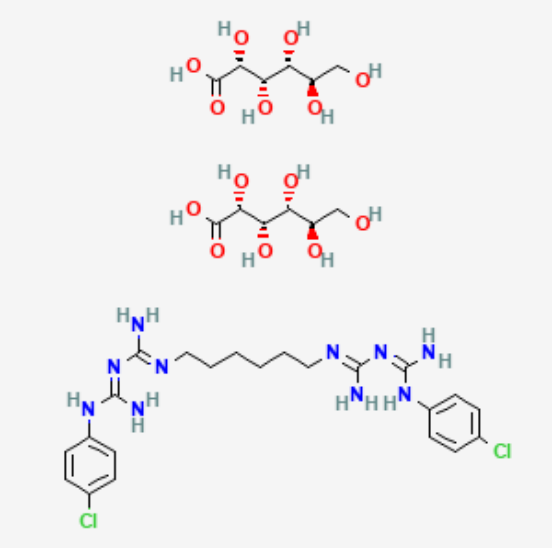

Fig. 3. Image depicting the 2-dimensional chemical structure of Chlorhexidine Digluconate. Chlorhexidine is a positively charged compound composed of two guanide units linked by a hexamethyl bridge. The gluconate/digluconate salt form is the most common, however it can also be found as chlorhexidine dihydrochloride and chlorhexidine diacetate (42).

2.5.2. Chlorhexidine Digluconate Tolerance and Cross-Resistance with Antibiotics

Both intrinsic and acquired tolerance to CHX has been well-documented in literature. Bacterial spores, mycobacteria, and the Gram-negative bacteria *Proteus* and *Providencia* are intrinsically tolerant by means of permeability barriers (98, 116). Acquired tolerance to CHX appears to be almost entirely dictated by increased levels of efflux. At least 11 efflux pumps are known to be implicated in staphylococcal tolerance to biocides, these are encoded by the *qacA/B/E/EΔ1/F/G/H/J/Z, smr,* and *norA* genes, some of which are known MDR genes (39, 92). Both overexpression of these pumps and mutation to alter their substrate specificities are involved in raising tolerance to biocides, either base-pair mutations in *smr* or overexpression of *norA* can raise the CHX MIC to 4 ug/mL in *S. aureus* (39, 56). Changes in the efflux capabilities of adapted strains often reflect wider changes in global regulatory systems (92). While the acquisition and expression of efflux determinants in response to biocides may not always directly raise antibiotic tolerance, the selective pressure imposed by CHX may contribute to multidrug resistance indirectly. CHX has been shown to induce HGT by conjugation in *E. coli,* and efflux pumps commonly selected for under biocide exposure are commonly present on MGE's that coharbour other resistance genes (51, 116). The *qacA* gene was first discovered on plasmid Tn4002,

which also encoded heavy metal and beta-lactam resistance determinants, and in staphylococci *qacA* has been found on many other plasmids and transposons that encode aminoglycoside, tetracycline, trimethoprim, and macrolide resistance genes (39, 116). Resistance to CHX in *K. pneumoniae* is associated with mutations in *phoPQ and smvR*, a two-component regulator and Tet repressor gene respectively, that upregulate the SmvA efflux pump from the MFS family. Five of six strains tested had cross-resistance with colistin, but adapting these strains to colistin did not cause CHX tolerance. (117). A correlation between decreased colistin susceptibility and CHX resistance has also been shown in *E. coli* and *A. baumannii* (116). CHX adaptation in *K. pneumoniae* also involves mutations in *ramR* gene, the repressor of potent MDR efflux pump AcrAB-TolC, which may be related to the cross-resistance with tetracycline, gentamicin, meropenem, and triclosan shown in some isolates of *Salmonella* spp. (51, 118). CHX has been shown to remain in saliva samples days after mouthwash application, this could pose a risk of resistance development as bacteria are constantly exposed to decreasing concentrations of CHX (38). A Taiwanese hospital where CHX was regularly used for hand hygiene saw the prevalence of MRSA isolates with a CHX MIC over 4 ug/mL increase from 1.7% to 46.7% in 15 years, although evidence shows that clinically relevant concentrations of CHX remain effective at killing less susceptible *S. aureus* under *in vivo* experimental conditions (116, 120). The rate of *K. pneumoniae* infections, including CRKP, has been shown to increase following CHX bathing of patients, indicating that it may enable the spread of dangerous pathogens despite evidence indicating that these strains do not have increased CHX MICs (40, 77). Mostly stable MIC increases of 500-fold and 32-fold can occur in *E. coli,* and *P.* aeruginosa respectively after low level exposure, leading to the recommendation that CHX should not be used in hand soaps to avoid the selection pressure on bacteria (48, 51).

2.6. Scope of the Study

This study aims to further the research into the consequences of biocide use. More experimentation is required to fully understand both the mechanisms underlying biocide-resistance and the ramifications of these mechanisms for antibiotic resistance. Given the ever-worsening AMR crisis, it is paramount that we maximise the stewardship of the antimicrobials we use.

Experimentation in this study focuses on the phenotypic changes associated with increased TRI, BAC, and CHX tolerance in clinical isolates of *K. pneumoniae*. Several assays were carried out to map the changes in biocide tolerance, antibiotic susceptibility, permeability, capsule formation, biofilm formation, and metabolic fitness in strains as they became adapted to higher biocide tolerances. Molecular work and further phenotypic testing will be carried out on these isolates in future as part of ongoing research in the lab.

3.0 Materials and Methods

3.1. General Methods

3.1.1. Media and Culture Conditions

Bacteria were grown in Mueller-Hinton Broth (MHB) (2.0 g/L beef infusion solids, 1.5 g/L starch, casein hydrolysate 17.5 g/L; Oxoid), LB broth (10 g/L tryptone, 5 g/L year extract, 5 g/L NaCl; Sigma), M9 minimal media (10x M9 salt solution (Na_2HPO_4, KH_2PO_4, NaCl, NH_4Cl) 20% (w/v) glucose, 1 M $MgSO_4$, 1 M $MgSO_4$, 1 M $CaCl_2$; Sigma) supplemented with casamino acids (1:500). Media were prepared using deionised water and sterilised by autoclaving.

Bacterial cultures were stored at -75°C as MHB cryostocks (50% Glycerol) and regularly used to streak 25 Mueller-Hinton Agar (MHA) plates. Plates were incubated aerobically at 37°C (REVCO ULTIMA Incubator Oven INB200) for 18 hours without shaking.

Overnight cultures were prepared by inoculating 2-3 colonies into 5mL MHB, LB broth, or M9 minimal media and incubated at 37°C (G24 Environmental Incubator Shaker, New Brunswick Scientific) for 18 hours with shaking at 220 rpm. In the case of biocide induction, overnight cultures were prepared in the same way, with 100 uL being used to inoculate 4.9 mL of MHB to a desired final concentration of either Triclosan, Benzalkonium Chloride, or Chlorhexidine Digluconate.

Overnight cultures were adjusted to 0.5 McFarland standard. Culture was added to 5 mL of Phosphate Buffered Saline (PBS) until the optical density at 600nm was between 0.08 and 0.10 on the spectrophotometer.

3.1.2. Antibiotics and Biocides

Filter sterilisation was carried out on stock antibiotics through 0.2 um Millex filters (Millipore) and stored at 4°C. Aztreonam powder (MP Biomedicals) was dissolved in dimethyl sulfoxide (DMSO) as a 1 mg/mL stock solution. Colistin sulfate powder (MP Biomedicals), meropenem trihydrate powder (United States Pharmacopeia), and tetracycline hydrochloride powder (Fisher Bioreagents) were dissolved in sterile distilled water as separate 1 mg/mL stock solutions. The tetracycline solution was protected from light. Ciprofloxacin powder (Sigma Aldrich) was dissolved in 0.1N hydrochloric acid as a 1

mg/mL solution. Chloramphenicol powder (Sigma Aldrich) was dissolved in ethanol as a 1 mg/mL stock solution. Each antibiotic stock solution was diluted with MHB to various desired working solution concentrations.

Triclosan powder (Sigma Aldrich) was dissolved in ethanol to produce a stock solution of 0.1 mg/mL and protected from light and stored at 4°C. Benzalkonium chloride powder (Sigma Aldrich) was dissolved in sterile distilled water at a stock concentration of 10 mg/mL and stored at room temperature. Chlorhexidine digluconate (20% aqueous solution (w/v) Thermo scientific) was diluted with sterile distilled water to produce a stock solution of 10 mg/mL which was stored at 4°C and protected from light. Chlorhexidine dihydrochloride powder (Sigma Aldrich) and chlorhexidine diacetate powder (Sigma Aldrich) were each dissolved in sterile distilled water and adjusted to pH 7 using a pH meter (Mettler-Toledo) at stock solution concentrations of 5 mg/mL and stored at 4°C.

3.1.3. Bacterial Strains

A full list of bacterial strains used in this study are listed in Fig. 4. Reference strains *K. quasipneumoniae* ATCC 700603 and *Escherichia coli* ATCC 25922 were used as controls throughout the study. Ten clinical isolates of *K. pneumoniae*, “318060”, “302216”, “318418”, “412967”, “412412”, “412594”, “412621”, “51596”, 51441”, and “50183” from St. James’ Hospital, Co. Dublin, were involved in experimentation. These strains were kindly provided by Dr. Brendan Crowley (St. James’ Hospital, Dublin, Ireland). Strain “51596” exhibited hetero-resistance to ertapenem in Kirby-Bauer assays, a colony growing adjacent to the antibiotic disk was isolated and dubbed “51596E”. Strains “412412”, “412621”, “302216”, “318418”, “51596E”, and “50183” were exposed to five different concentrations of TRI (0.3125 ug/mL, 0.625 ug/mL, 1.25 ug/mL, 2.5 ug/mL, and 5 ug/mL), BAC (3.9 ug/mL, 7.8 ug/mL, 15.625 ug/mL, 31.25 ug/mL, and 62.5 ug/mL), and CHX (15.625 ug/mL, 31.25 ug/mL, 62.5 ug/mL, 125 ug/mL, and 250 ug/mL) to produce 15 biocide-induced variants of each strain. Each of these six strains was also serially passaged in MHB five times without the presence of any biocide to control for the effects of serial passaging on the strains.

Bacterial Strain	Characteristics	Source/Reference
K. quasipneumoniae		
ATCC700603	Reference strain	Martins Lab Collection
E. coli		
ATCC25922	Reference strain	Martins Lab Collection
K. pneumoniae (Clinical Isolates)		
318060	Clinical Isolate	
412967	Clinical Isolate	
412594	Clinical Isolate	
51596	Clinical Isolate	
51441	Clinical Isolate	
412412	Clinical Isolate	St. James' Hospital
412621	Clinical Isolate	
302216	Clinical Isolate	
318418	Clinical Isolate	
51596	Clinical Isolate	
50183	Clinical Isolate	
K. pneumoniae (Induction Control)		
412412-C	Passaged without biocide 5 times	
412621-C	Passaged without biocide 5 times	
302216-C	Passaged without biocide 5 times	This Study
318418-C	Passaged without biocide 5 times	
51595E-C	Passaged without biocide 5 times	
50183-C	Passaged without biocide 5 times	
K. pneumoniae (Biocide-Induced)		
412412 TRI (P1-5)	Induced at 5 Concentrations	
412621 TRI (P1-5)	Induced at 5 Concentrations	
302216 TRI (P1-5)	Induced at 5 Concentrations	
318418 TRI (P1-5)	Induced at 5 Concentrations	
51596E TRI (P1-5)	Induced at 5 Concentrations	
50183 TRI (P1-5)	Induced at 5 Concentrations	
412412 BAC (P1-5)	Induced at 5 Concentrations	
412621 BAC (P1-5)	Induced at 5 Concentrations	
302216 BAC (P1-5)	Induced at 5 Concentrations	This Study
318418 BAC (P1-5)	Induced at 5 Concentrations	
51596E BAC (P1-5)	Induced at 5 Concentrations	
50183 BAC (P1-5)	Induced at 5 Concentrations	
412412 CHX (P1-5)	Induced at 5 Concentrations	
412621 CHX (P1-5)	Induced at 5 Concentrations	
302216 CHX (P1-5)	Induced at 5 Concentrations	
318418 CHX (P1-5)	Induced at 5 Concentrations	
51596E CHX (P1-5)	Induced at 5 Concentrations	
50183 CHX (P1-5)	Induced at 5 Concentrations	

Figure 4. List of Bacterial Strains used in the Study. ATCC strains 700603 (*K. quasipneumoniae*) and 25922 (*E. coli*) were used as controls. Clinical isolates were acquired from St. James' Hospital, Dublin. Clinical isolates were serially passaged in MHB and induced to various concentrations of TRI, BAC, and CHX, starting at half the MIC of the most susceptible strain (Passage (P) 1) and increasing in doubling concentrations (P2, P3, P4, P5) to eight times the lowest MIC (P5) for each biocide.

3.2. Assessment of Antimicrobial Tolerance

3.2.1. Assessment of Antibiotic Resistance Profiles – Kirby-Bauer Disk Diffusion Assay

Antibiotic resistance profiles were determined *via* the Kirby-Bauer method as per CLSI guidelines. Briefly, overnight bacterial cultures grown in MHB were diluted to 0.5 McFarland with PBS lawned on 25 mL MHA plates with sterile cotton swabs. Antibiotic disks (ThermoFisher Scientific) containing aztreonam (ATM30), cefpodoxime (CPD10), ertapenem (ETP10), ampicillin-clavulanic acid (AMC30), amikacin (AK30), ciprofloxacin (CIP5), tetracycline (TE30), and sulfamethoxazole-trimethoprim (SXT10) were stamped into the inoculated MHA plates with a disk dispenser. Piperacillin-tazobactam (TZP110) disks were used in the place of AMC30 disks for the testing of induced isolates. Plates were incubated aerobically at 37°C (REVCO ULTIMA Incubator Oven INB200) for 18 hours without shaking. Results were interpreted according to the EUCAST guidelines. CLSI guidelines were used in the case of tetracycline as EUCAST lacks figures.

3.2.2. Assessment of Biocide and Antibiotic Resistance – Microbroth Dilution Method

MICs were determined *via* the microbroth dilution method (29). Using a multichannel pipette, 100 uL of MHB was added to columns 1-11 in 96-well plates. For the initial biocide susceptibility testing, TRI, BAC, and CHX were diluted to concentrations of 40 ug/mL, 250 ug/mL, and 1000 ug/mL respectively. Using a multichannel pipette, 200 uL of each of these working solutions was added to separate wells of column 12 in 96-well plates. Dilution of each biocide was carried out by transferring 100 uL from one column to another, mixing, and repeating in descending order until column 3 was reached, at which the excess 100 uL was discarded. The wells of column 2 received no biocide to act as a growth control. Each row was inoculated using 15 uL of overnight bacterial culture grown in MHB and standardised to 0.5 McFarland with PBS, leaving the first column as a sterile control (media only). Inoculated 96-well plates were incubated aerobically at 37°C (REVCO ULTIMA Incubator Oven INB200) for 18 hours without shaking. The biocide-induced isolates were analysed after growing overnight without biocide present (denoted as "Pre") and with their respective induction concentrations, for example strain "50183-TRI-P5" was grown in MHB and MHB containing 5 ug/mL of TRI. Testing was carried out in duplicate over three technical replicates (on independent days) for each clinical isolate

and biocide combination. MIC values were taken as the first well in a row without turbid growth, this was determined by eye. Where there was discrepancy of 2-fold between technical replicates the most common value was taken as the MIC for the strain.

Biocide MIC testing was carried out in a similar fashion for the biocide-induced isolates, the sole differences being that the initial biocide concentrations were changed to 40 ug/mL TRI, and 250 ug/mL BAC and CHX, and the orientation of the biocide concentration gradient was switched. A starting concentration of 500 ug/mL CHX was used for the testing of the CHX-induced strains. The highest concentrations were in row H rather than column 12 and row A was divided into sterile control (wells 1-6) and growth control (wells 7-12). This enabled testing of a greater number of isolates per 96-well plate, reducing the number of plates required, with the trade-off of a slightly reduced testing range. Biocide-induced isolates were analysed after growing in both media only, and media containing the respective concentrations of biocide to which they were previously induced. Evaluation of the biocide-induced isolates was carried out in duplicate over three technical replicates on independent days, with the exception of CHX-induced strains which were evaluated in duplicate over one technical replicate. The MIC of chlorhexidine diacetate was also determined for parental, control, and TRI-induced strains in the same way as CHX.

Antibiotic MIC testing was carried out in the same fashion as the biocide MIC testing for the parental, control, and induced isolates. Working solutions of aztreonam, chloramphenicol, colistin, meropenem, and tetracycline were made by diluting the various stock solutions in MHB to final concentrations of 256 ug/mL, 128 ug/mL, 16 ug/mL, 16 ug/mL, 4 ug/mL, and 256 ug/mL respectively. The assay was carried out in duplicate for each strain-antibiotic combination over three replicates on independent days.

3.3. Induction of Biocide Tolerance in *K. pneumoniae* Clinical Isolates

Each parental isolate; “412412”, “412621”, “302216”, “318418”, “51596E”, and “50183”, was induced to a maximum of 5 ug/mL TRI, 62.5 ug/mL BAC, and 250 ug/mL CHX. These figures were chosen as eight times the lowest MIC across the parental strains, 0.625 ug/mL for TRI, 7.8125 ug/mL for BAC, and 15.625 ug/mL for CHX. Strains were induced to

tolerance through stepwise exposure to doubling concentrations of TRI, BAC, and CHX, beginning at half the MIC (denoted as P1) to a maximum of eight times the MIC (denoted as P5), producing five variants (P1-P5) of each parent strain per biocide. To begin the induction process, each parental isolate was grown in 5mL MHB with 220 rpm for 18 hours at 37°C (G24 Environmental Incubator Shaker, New Brunswick Scientific). 100 uL of each overnight culture was used to inoculate fresh MHB to a final volume of 5 mL at the desired biocide concentration in MHB and incubated under the same conditions for 24 hours. This step was repeated until the desired final concentration was reached for each biocide, with cryostocks and streaks on MHA being made for every strain. In some cases, the doubling biocide concentration killed the bacteria, when this occurred an overnight culture of the most tolerant strain was prepared and used to inoculate MHB at an intermediate biocide concentration. Each parental isolate was also passaged five times without biocide as a control.

3.4. Assessment of Efflux and Permeability – Ethidium Bromide Cartwheel Assay

This assay was carried out as described in Martins *et al*, 2011 (68). 25mL LB agar plates containing 2.0 ug/mL of Ethidium Bromide (EtBr) were prepared and wrapped in foil to protect them from light. Colonies from parental, control. and induced strains, and ATCC700603 were used to prepare overnight cultures in MHB. The inoculums were adjusted to 0.5 McFarland with PBS. Sterile cotton swabs were used to streak each culture onto LB-only and EtBr-containing LB plates in a cartwheel pattern with ATCC700603 as “north” followed in clockwise order by control strains, P1, P2, P3, P4, P5-induced strains, and parental strains. All isolates were analysed before and after exposure to their relevant concentrations of biocide. The plates were incubated for 18 hours (REVCO ULTIMA Incubator Oven INB200) and then viewed and imaged in an ImageQuant Las4000 using the EtBr UV (Trans-UV) setting with a 4 second exposure. Changes in the accumulation of EtBr were reflected in the level of fluorescence within a given bacterial streak on the plate, with less fluorescent strains having accumulated less EtBr due to either lower uptake or higher efflux.

3.5. Assessment of Capsule Production – Ludox Density Gradient Assay

A similar assay was carried out to that in Whiteway *et al*, 2022 (123), the sole difference being the standardisation of the overnight cultures to an optical density of 0.3 at OD_{600nm} rather than pelleting and resuspending overnight cultures in 1 mL of PBS. This change was made to reduce the variation in the thickness of bands as they appeared in the density gradient and to ascertain a more accurate measurement of migration. 250 uL of Ludox (LS colloidal silica, 30 wt. % suspension in H_2O) was added to a sterile 2.5 mL Eppendorf tube. 750 uL of overnight culture was then adjusted to an OD_{600nm} of 0.3 and added to the tube. Tubes were centrifuged at 4°C for 30 minutes at 9000 relative centrifugal force (RCF). The soft brake was used to maintain the gradient during deceleration. Using a ruler, the migration of bands of cells within the tubes was measured relative to the 1 mL mark. The assay was carried out for all parental, control and induced isolates, with the induced isolates being analysed before and after exposure to their respective concentrations of biocides.

3.6. Assessment of Biofilm Production

Overnight cultures were adjusted to 0.5 McFarland using M9 media. 100 uL of each dilution was added to 12 wells of two 96-well plate which were incubated at 37°C for 24 hours and 48 hours respectively to allow for biofilm maturation. The outside wells of both plates received 100 uL of M9 media without bacteria as a negative control. After incubation the plates were washed with 250 uL of PBS twice and dried. 125 uL of filter sterilised 0.1% crystal violet solution was added to each well and the plates were incubated for 15 minutes at 37°C. After incubation, the crystal violet was removed, and the plates were washed again with PBS. The plates were allowed to dry, then 150 uL of 30% glacial acetic acid was added. Following a 15-minute incubation the plates were read in a plate reader (LabNet Enduro GDA touch reader) at 575nm. Biofilms were quantified based on their optical densities relative to the sterile control and categorised as non-adherent ($OD < OD_{Control}$), weakly adherent ($OD_{Control} < OD < 2x\ OD_{Control}$), moderately adherent ($2x\ OD_{Control} < OD < 4x\ OD_{Control}$), or strongly adherent ($4x\ OD_{Control} < OD$). All strains were analysed in this way, in sextuplicate for 24 and 48 hours in one replicate each,

with the induced isolates being examined both before and after exposure to their relevant concentrations of biocide, with the exception of CHX post-exposure.

3.7. Assessment of Bacterial Metabolism – Growth Curves

Parental, control, ATCC700603, and all induced strains were grown overnight in MHB and adjusted to 0.5 McFarland with PBS. 100 uL of MHB was added to all wells in a 96 well plate and the adjusted overnights were used to inoculate three wells per strain, leaving three wells uninoculated as a negative control. Plates were then incubated overnight at 37°C with constant orbital shaking in a plate reader (Biotek; Epoch 2 Microplate Reader).

3.8. Statistical Tests

Data was expressed as mean ± standard deviation. Differences were tested by two-tailed t-test. P values < 0.05 were considered as statistically significant. Statistical testing, including determination of means, standard deviations, t-tests, and analysis were carried out in Microsoft Excel 365.

4.0. Results

4.1. Antimicrobial Tolerance of *K. pneumoniae* Clinical Isolates

Clinical isolates of *K. pneumoniae* (n=10) and ATCC700603 *K. quasipneumoniae* were tested *via* the Kirby-Bauer disk diffusion method to ascertain their antibiotic resistance profiles according to EUCAST guidelines. CLSI guidelines were used for tetracycline as EUCAST lacks figures. Ertapenem, cefpodoxime, aztreonam, sulfamethoxazole-trimethoprim, tetracycline, amikacin, ciprofloxacin, and amoxicillin-clavulanate disks were used, results are shown in Fig. 5. Clinical isolates were grouped based on the extent of their resistance profiles; isolates "50183", "51596", and "412967" were the most highly MDR, isolates "318060", "302216", "318418", "412594", and "51441" had an intermediate level of MDR relative to the other samples, and isolates "412412" and "412621" had a relatively low level of MDR. During the experiment, colonies from "51596" displayed hetero-resistance and grew within the zone of inhibition, adjacent to the ertapenem disk. This ertapenem resistant phenotype persisted when these colonies were re-streaked and tested in the same way, the resultant colonies were designated "51596E".

Antibiotic / Strain	ETP10		CPD10		ATM30		SXT10		TE30		AK30		CIP5		AMC30	
	1st Rep	2nd Rep	1st Rep	2nd Rep	1st Rep	2nd Rep	1st Rep	2nd Rep	1st Rep	2nd Rep	1st Rep	2nd Rep	1st Rep	2nd Rep	1st Rep	2nd Rep
ATCC700603	S	S	R	R	R	R	S	S	I	I	S	S	I	I	R	R
318060	S	S	R	R	R	R	S	S	S	S	S	S	S	S	R	R
302216	S	S	R	R	R	R	R	R	R	R	S	S	I	I	R	R
318418	S	S	R	R	R	R	R	R	R	R	S	S	I	I	R	R
412967	S	S	R	R	R	R	R	R	R	R	S	S	R	R	R	R
412412	S	S	S	S	S	S	S	S	S	S	S	S	I	I	R	R
412594	S	S	R	R	R	R	S	S	R	R	S	S	R	R	R	R
412621	S	S	S	S	S	S	S	S	S	S	S	S	S	S	R	R
51596	S	S	R	R	R	R	R	R	R	R	S	S	R	R	R	R
51596E	R	R	R	R	R	R	R	R	R	R	S	S	R	R	R	R
51441	S	S	S	S	S	S	R	R	R	R	S	S	I	I	R	R
50183	S	S	R	R	R	R	R	R	R	R	S	S	R	R	R	R

Figure 5; Antibiotic Susceptibility Profile of Clinical Isolates of *K. pneumoniae*. Profiles were determined *via* the Kirby-Bauer disk diffusion method. Zones of inhibition were interpreted based on EUCAST guidelines (CLSI guidelines for tetracycline) and categorised as Resistant ("R", red), Intermediate ("I", yellow), and Susceptible ("S", green). ATCC700603 was used as a control, having previously been characterised in the lab. Antibiotics: ETP10 (Ertapenem), CPD10 (Cefpodoxime), ATM30 (Aztreonam), SXT10 (Trimethoprim-Sulfamethoxazole), TE30 (Tetracycline), AK30 (Amikacin), CIP5 (Ciprofloxacin), AMC30 (Amoxicillin-Clavulanate). Strains selected for further testing are highlighted in blue.

4.1.1 Selection of Six *K. pneumoniae* Clinical Isolates

Six strains were selected from a group of ten based on their antibiotic resistance profiles ascertained from two technical replicates of Kirby-Bauer disk diffusion assay testing (Figure 5). Where antibiotic resistance profiles were identical, the sizes of zones of inhibition were considered. Isolates “412412” and “412621” were taken as low-MDR, isolates “302216” and “318418” were taken as intermediate-MDR, and isolates “51596E” and “50183” were taken as high-MDR.

4.2. Biocide Tolerance of Parental Strains and Control Strains

After selection of the clinical isolates, MIC testing *via* microbroth dilution was conducted in duplicate over three technical replicates for the biocides triclosan (TRI), benzalkonium chloride (BAC), and chlorhexidine digluconate (CHX) on strains “412412”, “412621”, “302216”, “318418”, “51596”, “51596E”, and “50183”. Results are shown in Fig. 6 below. Of the chosen clinical isolates “412412” had the highest tolerance to TRI at 2.5 ug/mL, with all other strains having an MIC of 0.625 ug/mL. Strains “302216” and “318418” had an MIC of 7.8125 ug/mL for BAC, half that of the other isolates. For CHX, strain “318418” had an MIC of 31.25 ug/mL, half that of all other tested strains. The strains chosen showed no clear association between high antibiotic tolerance and high biocide tolerance, with the intermediately MDR strains having the lowest biocide tolerance and low-MDR strain “412412” having the highest biocide tolerances of the tested strains.

Biocide / Strain	Triclosan MIC (ug/mL)	Benzalkonium MIC (ug/mL)	Chlorhexidine MIC (ug/mL)
ATCC700603	1.25	31.25	62.5
412412	2.5	31.25	62.5
412621	1.25	15.625	62.5
302216	0.625	7.8125	62.5
318418	0.625	7.8125	31.25
51596E	0.625	15.625	62.5
50183	0.625	15.625	62.5
412412-C	1.25	31.25	62.5
412621-C	0.625	31.25	62.5
302216-C	0.625	7.8125	62.5
318418-C	1.25	15.625	31.25
51596E-C	0.625	15.625	31.25
50183-C	0.625	15.625	31.25
Intra-Biocide Colour Key	Low Relative MIC	Medium Relative MIC	High Relative MIC

Figure 6; MIC of TRI, BAC, and CHX on selected clinical isolates. MICs were determined *via* the microbroth dilution method. Final MICs for Triclosan (TRI), Benzalkonium Chloride (BAC), and Chlorhexidine Digluconate (CHX) were taken as the most consistent result from the three technical replicates of microbroth dilution assays carried out in duplicate on ATCC700603 and the 6 clinical isolates selected for induction.

Similarly, MIC testing was carried out on the control derivatives of the clinical isolates. These isolates were passaged in liquid media without biocide to account for any potential adaptation to being passaged serially in liquid media and were designated with a "-C". There were some minor fluctuations in MIC's relative to the parental strains; strains "412412-C" and "412621-C" became 2-fold more susceptible to TRI while "318418-C" became 2-fold more tolerant. Strains "412621-C" and "318418-C" each became 2-fold more tolerant of BAC, and strains "51596E-C" and "50183-C" each became 2-fold more susceptible to CHX.

4.3. Biocide Tolerances of Triclosan-Induced Strains

All TRI-adapted strains were analysed by microbroth dilution to elucidate the MICs for TRI, BAC, and CHX were analysed to verify the induction of isolates and to reveal any cross-resistance or sensitisation. The experiment was carried out in duplicate over three technical replicates for each strain. Fig. 7 below shows the TRI MIC of each strain with respect to the level of induction. The MICs of TRI for every strain always outpaced the concentration of the biocide used in the induction process. Parental strains "318418", "51596E" and "50183" had the lowest TRI MICs (0.625 ug/mL) but exposure to half this value was sufficient to induce an MIC of 2.5 ug/mL in "51596E-TRI-P1" and "318418-TRI-

P1", and an MIC of 1.25 ug/ mL in "50183-TRI-P1". Exposure to this concentration of TRI also caused the MIC of parental strain "412621" to increase to 5 ug/mL in "412621-TRI-P1". The largest increase in TRI MIC occurred during the induction from P2 (0.625 ug/mL) to P3 (1.25 ug/mL) for isolate "318418", which increased 8-fold from 2.5 ug/mL to 20 ug/mL. Isolate "412621" was the only one to have a decrease in TRI MIC at any point, this occurred during induction from P4 (2.5 ug/mL) to P5 (5 ug/mL), where the MIC decreased by half from 20 ug/mL to 10 ug/mL.

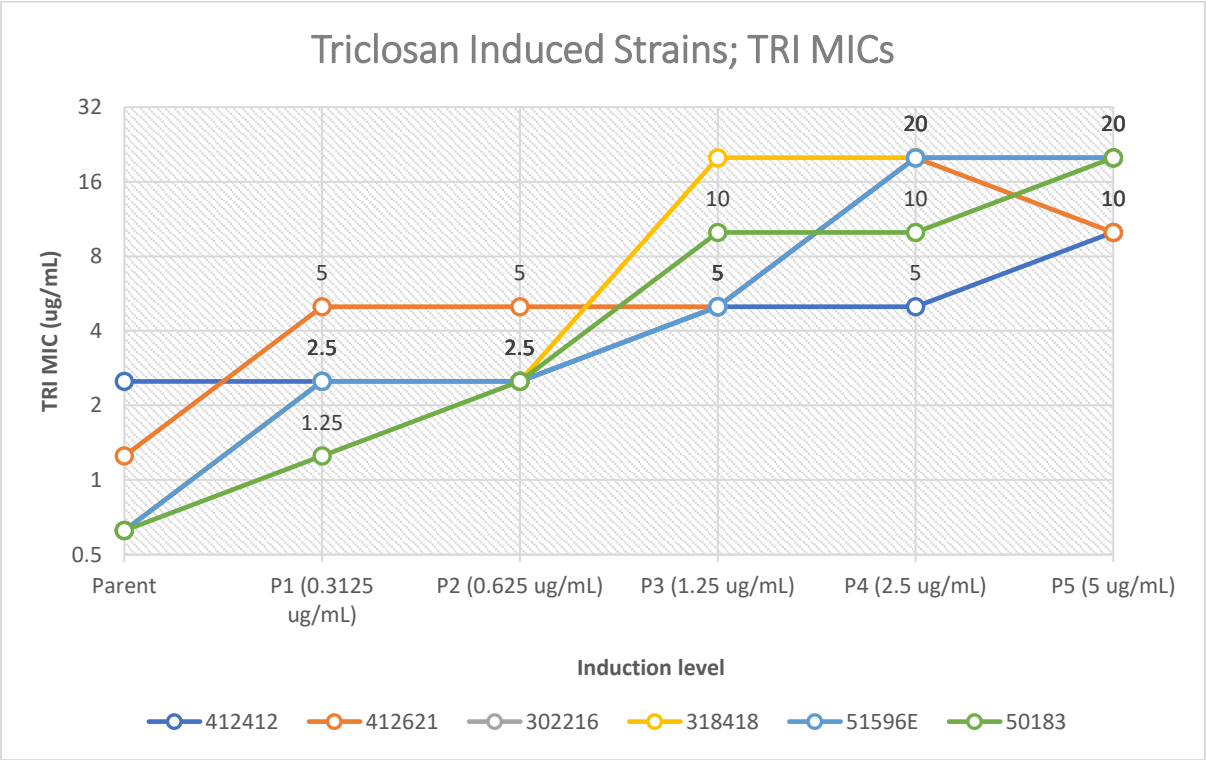

Figure 7. Triclosan MICs of Triclosan-adapted strains. The increasing triclosan MIC of each clinical isolate was graphed with respect to the level of induction. MIC testing *via* microbroth dilution was carried out in duplicate over three technical replicates for each strain. The most consistent value over all technical replicates was taken as the MIC for each strain.

Fig. 8 below shows the fold change in MIC for TRI, BAC, and CHX for each TRI-P5 strain and control strain relative to its parental isolate. Strain "50183-TRI-P5" demonstrated large increases in its tolerance of both BAC and CHX. The BAC MIC increased by 8-fold regardless of whether the induced strain had been grown overnight in 5 ug/mL of TRI, while the CHX MIC increased by 4-fold when the induced strain was grown with TRI, and 2-fold when grown in 5 ug/mL of TRI. The BAC MIC also increased for strains "302216-TRI-P5" and

"318418-TRI-P5", 8- and 4-fold respectively prior to TRI treatment and 4-fold for both after exposure. The CHX MIC for strain "318418-TRI-P5" also changed, in this case the isolate became 4-fold more susceptible regardless of TRI treatment prior to the assay. 2-fold decreases in CHX MICs were also seen in strains "412412-TRI-P5", "412621-TRI-P5", and "51596E-TRI-P5".

Biocide MIC; Fold Change Relative to Parent						
Strain	TRI Pre	TRI Post	BAC Pre	BAC Post	CHX Pre	CHX Post
412412-TRI-P5	4	4	0	0	-2	-2
412621-TRI-P5	8	8	0	0	0	0
302216-TRI-P5	32	32	8	4	0	0
318418-TRI-P5	32	32	4	4	-4	-4
51596E-TRI-P5	32	32	0	0	-2	-2
50183-TRI-P5	32	32	8	8	4	2

Figure 8. Fold Change in Biocide MICs for Control, and Triclosan-Adapted Strains at P5. MICs for TRI, BAC, and CHX were determined for the highest level of induction of TRI (P5: 5 ug/mL). MIC testing *via* microbroth dilution was carried out in duplicate over three technical replicates for each strain. The most consistent value over all technical replicates was taken as the MIC for each strain. "Pre" indicates an induced isolate grown in MHB and "post" indicates an induced isolate grown overnight in MHB with TRI. Colour indicates the magnitude of the fold change relative to the other strains examined. Darker greens indicate the larger increases in sensitivity, while darker reds indicate the larger increases in resistance.

The parental isolates and their TRI-induced counterparts were also tested against chlorhexidine diacetate, a different salt form of CHX. MIC data is shown in Fig. 9 below. Parental strain MIC values were equal to CHX for each isolate however chlorhexidine diacetate MICs begin to decrease for all isolates at the TRI-P3 induction (1.25 ug/mL). The magnitude of MIC difference between parental and induced isolates are shown in Fig. 10. The largest increase in chlorhexidine diacetate susceptibility occurred in "5159E-TRI-P5" and "412412-TRI-P5" which both had 32-fold decreases in their MIC relative to the parental strain. Strain "318418-TRI-P5" had the lowest initial resistance to chlorhexidine diacetate and had the lowest decrease in resistance at 4-fold.

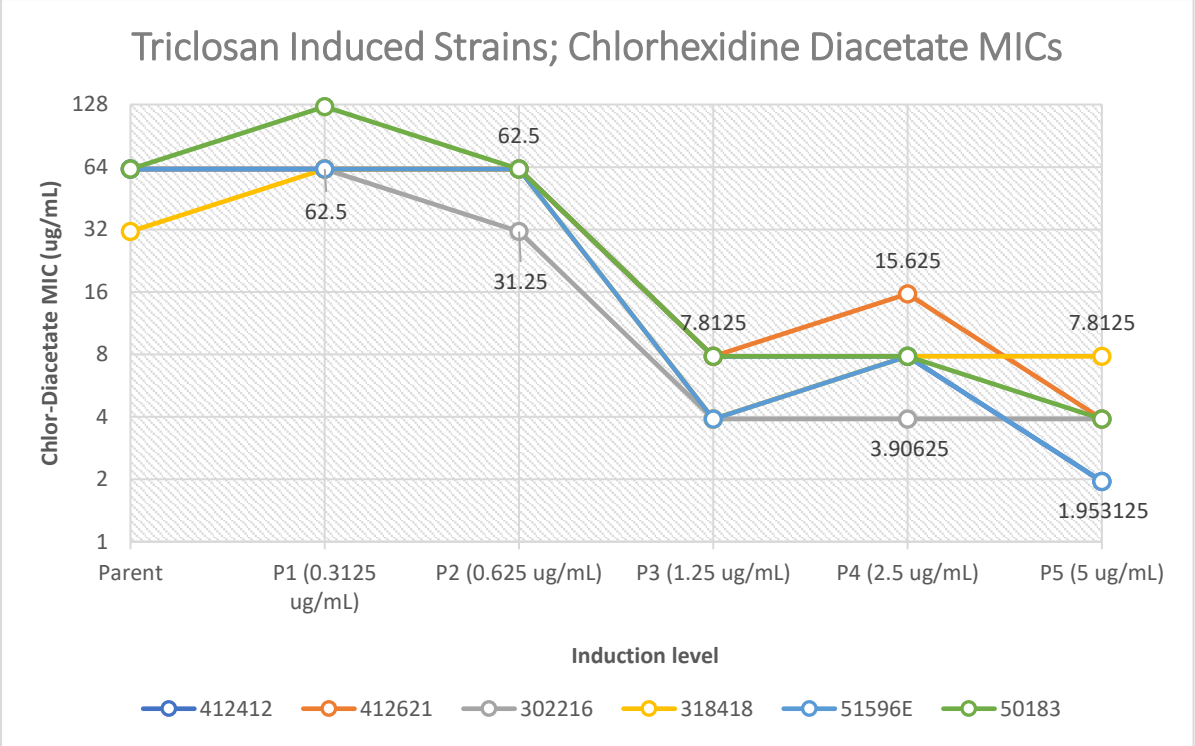

Figure 9. Chlorhexidine Diacetate MICs of Triclosan-Induced Strains. The decreasing chlorhexidine diacetate MIC of each clinical isolate was graphed with respect to the level of triclosan induction. MIC testing *via* microbroth dilution was carried out in duplicate over three technical replicates for each strain. The most consistent value over all technical replicates was taken as the MIC for each strain.

Strain	Parent Strain MIC (ug/mL)	Chlorhexidine Digluconate (CHX) MIC (ug/mL)	Chlorhexidine Diacetate MIC (ug/mL)
412412-TRI-P5	62.5	15.625	1.953125
412621-TRI-P5	62.5	31.25	3.90625
302216-TRI-P5	62.5	15.625	3.90625
318418-TRI-P5	31.25	31.25	7.8125
51596E-TRI-P5	62.5	15.625	1.953125
50183-TRI-P5	62.5	15.625	3.90625

Figure 10. Chlorhexidine Digluconate and Chlorhexidine Diacetate MICs. The CHX and chlorhexidine diacetate MICs of each TRI-P5 strain are shown. Only one value is shown for the parental MICs as they were the same for both forms of chlorhexidine. Red indicates higher MICs, and green indicates lower MICs. MIC testing *via* microbroth dilution was carried out in duplicate over three technical replicates for each strain. The most consistent value over all technical replicates was taken as the MIC for each strain.

4.4. Biocide Tolerances of Benzalkonium Chloride-Induced Strains

All BAC-induced strains were analysed via the microbroth dilution method to ascertain their MICs for TRI, BAC, and CHX. Increasing BAC MICs with respect to level of induction are shown in Fig. 11. Similar to the TRI-induced strains, the MIC of the induced strains always outpaced the level to which they were being induced. In parental strain "318418", exposure to half its BAC MIC (3.90625 ug/mL) induced a 4-fold increase in MIC to 31.25 ug/mL). After exposure to 62.5ug/mL of BAC, all BAC-P5 strains converged at an MIC of 125 ug/mL.

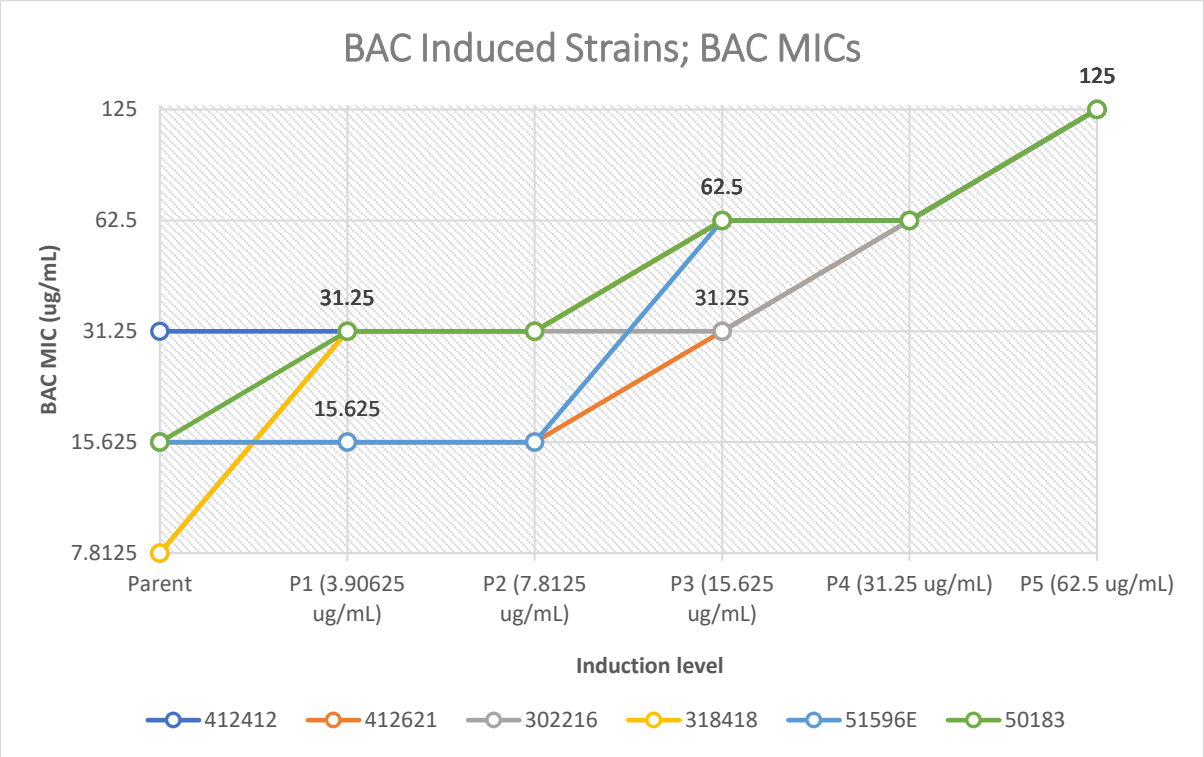

Figure 11. Benzalkonium Chloride MICs of Benzalkonium Chloride-adapted strains. The increasing BAC MIC of each clinical isolate was graphed with respect to the level of induction. MIC testing *via* microbroth dilution was carried out in duplicate over three technical replicates for each strain. The most consistent value over all technical replicates was taken as the MIC for each strain.

The fold change in TRI, BAC, and CHX MIC values for BAC-P5 strains are shown in Fig. 12 below. BAC MICs were higher in all BAC-P5 strains, with the exception of "302216-BAC-P5" and "50183-BAC-P5", after re-exposure to the concentration to which they were induced (62.5 ug/mL). Strains "302216-BAC-P5" and "50183-BAC-P5" maintained an 8-fold increase in BAC MIC relative to its parental strain regardless of whether or not they were grown in the presence of the biocide prior to testing. Strains "412412-BAC-P5",

"50183-BAC-P5", and "412621-BAC-P5" exhibited 2-fold increases in TRI MIC, post-, pre-, and both post- and pre-exposure to BAC respectively. Strains "412621-BAC-P5" and "50183-BAC-P5" both exhibited 2-fold increases in their CHX MICs both before and after re-exposure to BAC at 62.5 ug/mL. Conversely, strains "412412-BAC-P5" and "318418-BAC-P5" showed decreased CHX MICs, 4-fold and 2-fold respectively, after re-exposure to BAC.

Biocide MICs; Fold Change Relative to Parent						
Strain	**TRI Pre**	**TRI Post**	**BAC Pre**	**BAC Post**	**CHX Pre**	**CHX Post**
412412-BAC-P5	0	2	2	4	0	-4
412621-BAC-P5	2	2	4	8	2	2
302216-BAC-P5	0	0	8	8	0	0
318418-BAC-P5	0	0	4	8	0	-2
51596E-BAC-P5	0	0	4	8	0	0
50183-BAC-P5	2	0	8	8	2	2

Figure 12. Fold Change in Biocide MICs for Control, and Benzalkonium Chloride-induced Strains at P5. MICs for TRI, BAC, and CHX were determined for the highest level of induction of BAC (P5: 62.5 ug/mL). "Pre" indicates an induced isolate grown in MHB and "post" indicates an induced isolate grown overnight in MHB with BAC. Colour indicates the magnitude of the fold change relative to the other strains examined. Darker greens indicate the larger increases in sensitivity, while darker reds indicate the larger increases in resistance. MIC testing *via* microbroth dilution was carried out in duplicate over three technical replicates for each strain. The most consistent value over all technical replicates was taken as the MIC for each strain.

4.5. Biocide Tolerances of Chlorhexidine Digluconate-Induced Strains

CHX-induced strains at all levels were assayed *via* the microbroth dilution method to elucidate their respective TRI, BAC, and CHX MICs. The increasing CHX MICs with respect to level of induction are shown in Fig. 13. In the case of CHX-induction, the MIC did not always outpace the level to which the strain was induced. Strain "412621-CHX-P3" and "50183-CHX-P4" both had MICs equal to the level to which they were induced (62.5 ug/mL and 125 ug/mL CHX respectively). Similarly, strains "412412-CHX-P5", "318418-CHX-P5", "51596E-CHX-P5", and "50183-CHX-P5" were all shown to be inhibited by 250 ug/mL despite having been induced to and grown overnight at this concentration. Strains "412621-CHX-P5" and "302216-CHX-P5" had CHX MICs of 500 ug/mL, 2-fold higher than the final level of induction (250 ug/mL).

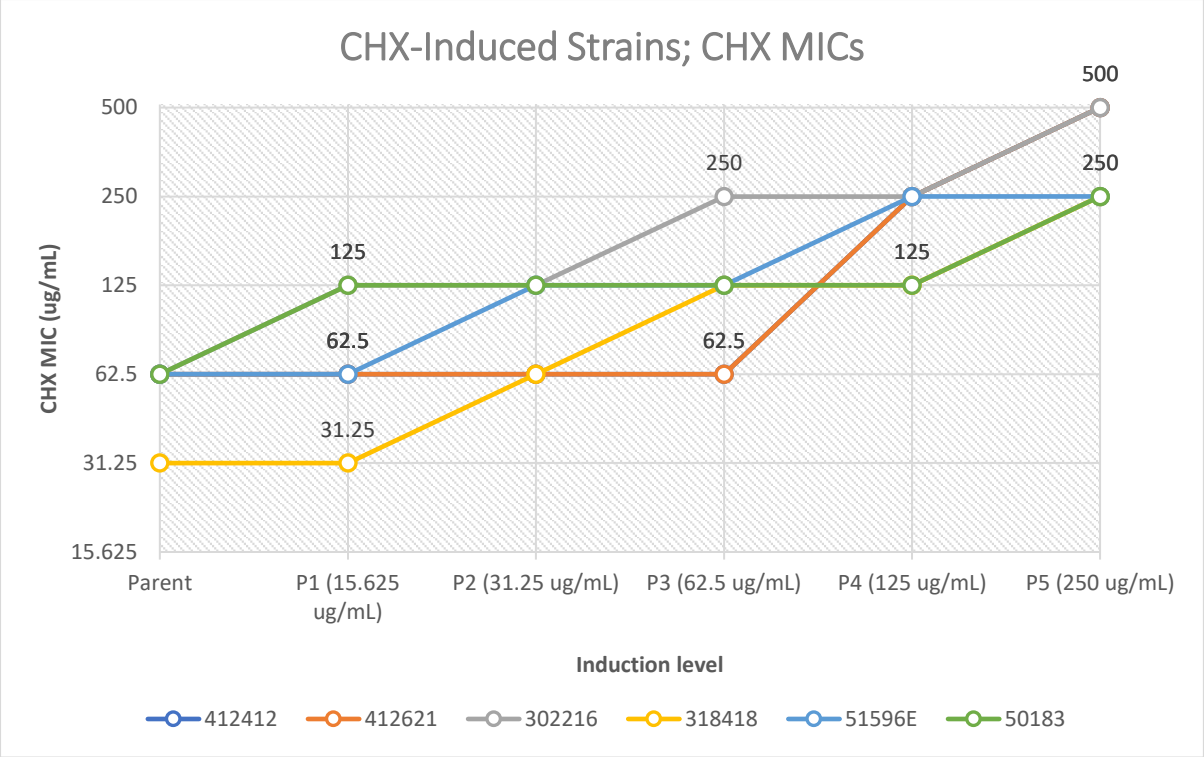

Figure 13. Chlorhexidine Digluconate MICs of Chlorhexidine Digluconate-adapted strains. The increasing CHX MIC of each clinical isolate was graphed with respect to the level of induction. MIC testing *via* microbroth dilution was carried out in duplicate over one technical replicate for each strain.

Fold changes in TRI, BAC, and CHX for the strains induced to the highest level of CHX tolerance are shown in Fig. 14. Across all six strains, 4-8-fold increases in CHX MICs occurred. Strains "302216-CHX-P5", "51596E-CHX-P5", and "50183-CHX-P5", each had 4-fold increases in their MICs relative to their parental strains prior to CHX re-exposure. This MIC persisted after re-exposure for the latter two strains but increased to 8-fold for "302216-CHX-P5", bringing it in line with "412412-CHX-5", "412621-CHX-P5", and "318418-CHX-P5" which all had 8-fold increases in their MICs both before and after re-exposure to CHX. Relative to their parental strains, TRI MICs decreased by 2-fold for strain "412621-CHX-P5" and 4-fold for "412412-CHX-P52, and "302216-CHX-P5", re-exposure to CHX, but not before. Strains "51596E-CHX-P5", and "50183-CHX-P5" were the only ones to have decreased TRI susceptibility without re-exposure to CHX, each had a 2-fold decrease in MIC which became a 4-fold decrease after CHX re-exposure. All CHX-P5 strains but "318148-CHX-P5" had increased BAC susceptibility following induction without re-exposure to CHX, but this strain was 2-fold more susceptible than its parent after re-

exposure was carried out. BAC MICs decreased 2-fold for “412412-CHX-P5”, “412621-CHX-P5”, and “50183-CHX-P5”, 4-fold for “302216-CHX-P5”, and 8-fold for “51596E-CHX-P5” prior to re-exposure and increased to 4-fold for “412412-CHX-P5” after.

Biocide MICs; Fold Change Relative to Parent						
Strain	TRI Pre	TRI Post	BAC Pre	BAC Post	CHX Pre	CHX Post
412412-CHX-P5	0	-4	-2	-4	8	8
412621-CHX-P5	0	-2	-2	-2	8	8
302216-CHX-P5	0	-4	-4	-4	4	8
318418-CHX-P5	2	0	0	-2	8	8
51596E-CHX-P5	-2	-4	-8	-8	4	4
50183-CHX-P5	-2	-4	-2	-2	4	4

Figure 14. Fold Change in Biocide MICs for Control, and Chlorhexidine Digluconate-induced Strains at P5. MICs for TRI, BAC, and CHX were determined for the highest level of induction of CHX (P5: 250 ug/mL). “Pre” indicates an induced isolate grown in MHB and “post” indicates an induced isolate grown overnight in MHB with CHX. Colour indicates the magnitude of the fold change relative to the other strains examined. Darker greens indicate the larger increases in sensitivity, while darker reds indicate the larger increases in resistance.

4.6. Antibiotic Susceptibility of Parental Strains and Control Strains

The results of antibiotic susceptibility testing of parental and control strains, carried out through Kirby Bauer disk diffusion, are shown in Fig. 14. Each antibiotic’s zone of inhibition was averaged over three replicates. and the average values of each antibiotic were used to tabulate a “combined susceptibility score” (CSS) for each strain. This score acts as a measure of multidrug resistance in a strain, it has a minimum of 8 (size of disks) and a maximum of 30 (cut-off point due to inaccuracies in measurements exceeding this value). The serial passaging of strains in MHB had no significant effect on the antibiotic zones of inhibition, with the exceptions of strains “412412-C” and “412621-C” for which piperacillin-tazobactam sensitivity increased slightly. These fluctuations had no bearing on the antibiotic susceptibility profiles of these strains.

Antibiotic / Strain	ETP10	CPD10	ATM30	SXT10	TE30	AK30	CIP5	TZP110	Combined Susceptibility Score
ATCC700603	27.1mm	13.2mm	14.1mm	19.5mm	18.7mm	23.5mm	24.3mm	23.8mm	20.5
412412	26.6mm	24.6mm	27.6mm	19.3mm	23.6mm	23mm	24.3mm	13.3mm	22.8
412621	27mm	27.6mm	28mm	29mm	28.3mm	23.6mm	30mm	10.3mm	25.5
302216	25.6mm	8mm	18.3mm	8mm	9mm	23.6mm	23.3mm	23.3mm	17.4
318418	29mm	9.6mm	19.5mm	8mm	9.3mm	21.3mm	24.6mm	25mm	18.3
51596E	18.3mm	8mm	8.3mm	8mm	8.3mm	19.6mm	16mm	19.3mm	13.25
50183	27.6mm	8mm	12.3mm	10mm	9mm	22.3mm	23mm	15.6mm	16
412412-C	30mm	29mm	30mm	19.6mm	22.3mm	22.3mm	23.6mm	18.6mm	24.5
412621-C	29.6mm	28.6mm	30mm	29.3mm	22mm	27mm	26mm	18.3mm	26.3
302216-C	28mm	8.3mm	20.6mm	8mm	8.6mm	21.6mm	24.6mm	23.3mm	17.9
318418-C	28.6mm	8.3mm	19.6mm	8mm	9mm	22mm	21.3mm	23.3mm	17.5
51596E-C	19.6mm	8mm	9mm	8mm	8mm	18.3mm	14mm	20.6mm	13.2
50183-C	26.3mm	8mm	12.3mm	8mm	8.3mm	21,3mm	22mm	13mm	14.9

Figure 14. Results of Kirby-Bauer Disk Diffusion Assay on ATCC700603, Parental, and Induction Control Strains. The disk diffusion assay was carried out over three technical replicates for each strain. Zones of inhibition were interpreted based on EUCAST guidelines (CLSI guidelines for tetracycline) and categorised as Resistant ("R", red), Intermediate ("I", yellow), and Susceptible ("S", green). Antibiotics: ETP10 (Ertapenem), CPD10 (Cefpodoxime), ATM30 (Aztreonam), SXT10 (Trimethoprim-Sulfamethoxazole), TE30 (Tetracycline), AK30 (Amikacin), CIP5 (Ciprofloxacin), TZP110 (Piperacillin-Tazobactam). Combined susceptibility score was calculated by averaging each strain's zone of inhibitions for the selected group of antibiotics, this was done to track changes in multidrug resistance as strains were passaged during induction of biocide resistance.

4.7. Antibiotic Susceptibility of Induced Isolates

The antibiotic susceptibilities of all parental, control, TRI-P5, BAC-P5, and CHX-P5 strains were tested *via* Kirby-Bauer disk diffusion and microbroth dilution method. Susceptibility to aztreonam, cefpodoxime, ertapenem, piperacillin-tazobactam, amikacin, ciprofloxacin, tetracycline, and sulfamethoxazole-trimethoprim were used in the Kirby-Bauer disk diffusion assay, and susceptibility to aztreonam, chloramphenicol, colistin, meropenem, and tetracycline were analysed with the microbroth dilution method. Zones of inhibition were used to calculate CSSs (Fig. 15) for every strain as a means to detect potential changes in multidrug resistance within an isolate as it underwent induction to TRI, BAC, and CHX tolerance. The CSSs of the different induction types pre- and post-exposure compared with that of the parent isolates via a two-tailed paired t-test. The only condition with a significant change in MDR was the BAC-post ($p = 0.038$). The two most MDR parental isolates, "51596E" and "50183", became slightly more susceptible to the panel

of antibiotics after induction to 5 ug/mL TRI, this increase in susceptibility occurred both before and after re-exposure of the adapted strains to this concentration of TRI. After induction to BAC, all BAC-P5 isolates, except “412621-BAC-P5”, and “50183-BAC-P5”, demonstrated increased susceptibility to the panel of antibiotics both before and after re-exposure to 62.5 ug/mL of BAC. The largest of these increases in susceptibility occurred in the induced versions of the two intermediately drug resistance parental isolates “302216-BAC-P5” and “318418-BAC-P5”, this was mostly due to the loss of sulfamethoxazole-trimethoprim, cefpodoxime, and aztreonam resistance. Overall, there were no significant changes in multidrug resistance in any of the strains after induction to 250 ug/mL of CHX (Pre-CHX p = 0.48, post-CHX p = 0.11).

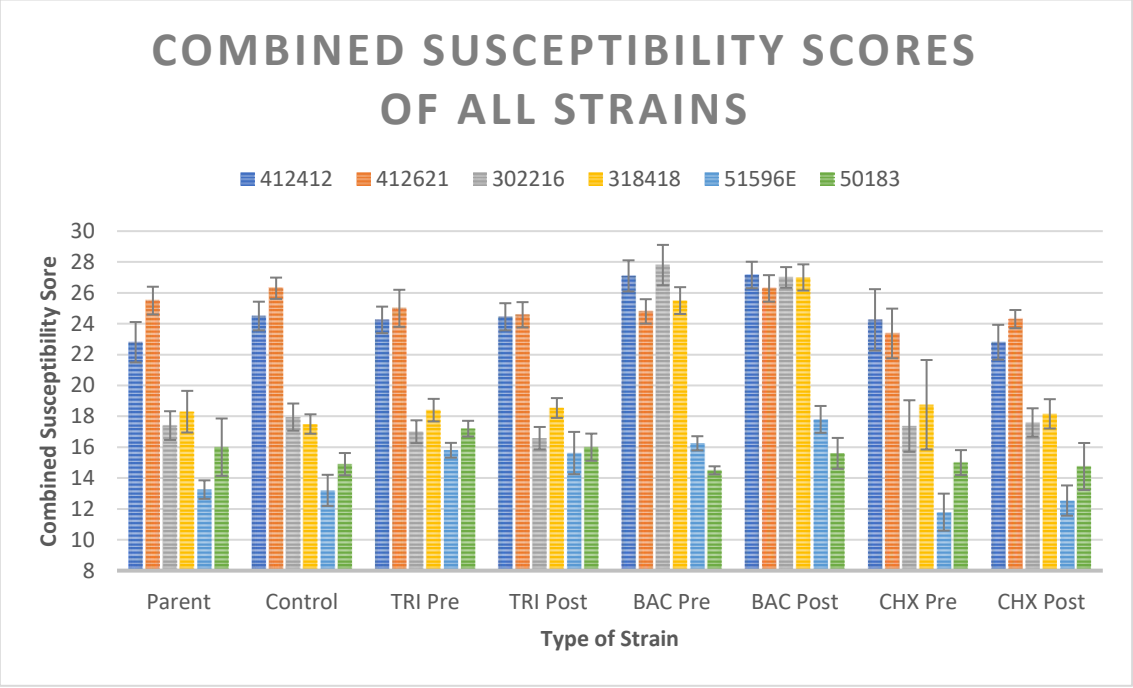

Figure 15. Combined Susceptibility Scores of Parental, Control, TRI-P5, BAC-P5, and CHX-P5 Strains. Zones of inhibition were measured around antibiotic disks from 8 different classes (aztreonam, cefpodoxime, ertapenem, piperacillin-tazobactam, amikacin, ciprofloxacin, sulfamethoxazole-trimethoprim, and tetracycline) and averaged over three technical replicates. These averages were then averaged within each strain type to calculate the average size of a zone of inhibition (combined susceptibility score). Larger scores indicate lower multidrug resistance. Error bars ±1 standard deviation, calculated from the three technical replicates carried out for independently for each strain.

4.7.1. Aztreonam Susceptibility

Susceptibility to aztreonam was analysed for all strains in both the disk diffusion and microbroth dilution assays, although aztreonam MIC results from the latter were discarded due to significant discrepancies with the expected results for the *E. coli* reference strain ATCC 25922 (an MIC of 64-128 ug/mL was reported). Results from the disk diffusion assay are shown in Fig. 16. Overall, significant changes in aztreonam susceptibility occurred across the six strains in the induction control (p=0.04), TRI-Pre (p=0.03), BAC-Pre (p=0.01), BAC-Post (p=0.01), and CHX-Pre (p=0.03) conditions. Isolates "302216", "318418", "51596E" all became more susceptible to aztreonam following induction to BAC-P5. Additionally, "51596E" also became more susceptible to the antibiotic following TRI-P5 and CHX-P5 induction. Strain "50183-TRI-P5" became more susceptible to aztreonam relative to its parental strain, however this susceptibility reverted upon re-exposure to the induction concentration. This change in zone of inhibition size was sufficient to change the EUCAST classification from resistant to susceptible, this was also the case for the aforementioned increase in susceptibility apparent in strains "302216-BAC-P5" and "318418-BAC-P5".

Aztreonam Zones of Inhibition (mm)								
Isolate	**Parent**	**Control**	**TRI-P5 Pre**	**TRI-P5 Post**	**BAC-P5 Pre**	**BAC-P5 Post**	**CHX-P5 Pre**	**CHX-P5 Post**
412412	27.6	30	30	30	29.6	30	28.3	29.6
412621	28	30	30	30	30	30	29.6	30
302216	18.3	20.6	20.3	17.6	29.6	30	18.3	18.3
318418	19.5	19.6	23.3	21	30	30	22	20.6
51596E	8.3	9	19.3	15.3	19	21.6	13.6	16
50183	12.3	12.3	25	13	15.6	18	17.3	19.3

Figure 16. Aztreonam Zones of Inhibition for All Parental, Control, and Induced Strains. Zones of inhibition were measured for all strains after Kirby-Bauer assays. The values above were determined from the average of the three technical replicates carried out for each strain. Biocide-induced strains were tested at the highest concentrations of induction for TRI, BAC, and CHX (P5) both before and after re-exposure to the concentration of the relevant biocide used in induction. Red indicates higher resistance and green indicates higher susceptibility to aztreonam. Two-tailed paired t-tests were carried out for each condition compared to the parental strain, the condition with a significant difference ($p<0.05$) were the control (p=0.04), TRI-Pre (p=0.03), BAC-Pre (p=0.01), BAC-Post (p=0.01), and CHX-Pre (p=0.03).

4.7.2. Cefpodoxime Susceptibility

Cefpodoxime zones of inhibition for each strain type analysed are shown in Fig. 17. Overall, no significant changes in cefpodoxime susceptibility were associated with any of the induction types. The high susceptibility of low-drug resistance parental isolates "412412" and "412621" remained unchanged throughout the induction process for all biocides. Similarly, the high tolerance of the high-MDR parental strains "51596E" and "50183" was also unaffected by the adaptation to high TRI, BAC, and CHX concentrations. Intermediately-drug resistant isolates "302216" and "318418" demonstrated an inversion of tolerance after being induced to tolerance of BAC however, changing in EUCAST classification from resistant to susceptible to cefpodoxime. This was also the case for strain "302216-CHX-P5", but only after being re-exposed to 250 ug/mL CHX prior to the assay. Strain "318418-CHX-P5" had a slight increase in susceptibility prior to re-exposure, but this was not sufficient to change its EUCAST classification.

Cefpodoxime Zones of Inhibition (mm)								
Isolate	**Parent**	**Control**	**TRI-P5 Pre**	**TRI-P5 Post**	**BAC-P5 Pre**	**BAC-P5 Post**	**CHX-P5 Pre**	**CHX-P5 Post**
412412	24.6	29	28.3	28.3	30	30	27.3	25
412621	27.6	28.6	26.6	26.6	25.3	26.6	23.3	23.6
302216	8	8.3	8.3	8	29.3	29.3	8.6	23.6
318418	9.6	8.3	10	9.6	28	30	14.6	8.3
51596E	8	8	8	10	8	8	8	8
50183	8	8	8	9	8	8	8	8

Figure 17. Cefpodoxime Zones of Inhibition for All Parental, Control, and Induced Strains. Zones of inhibition were measured for all strains after Kirby-Bauer assays. The values above were determined from the average of the three technical replicates carried out for each strain. Biocide-induced strains were tested at the highest concentrations of induction for TRI, BAC, and CHX (P5) both before and after re-exposure to the concentration of the relevant biocide used in induction. Red indicates higher resistance and green indicates higher susceptibility to cefpodoxime. Two-tailed paired t-tests were carried out for each condition compared to the parental strain, none of the conditions had a significant difference ($p<0.05$).

4.7.3. Ertapenem and Meropenem Susceptibility

Susceptibility to the carbapenem antibiotics ertapenem and meropenem were analysed *via* disk diffusion and microbroth dilution respectively. The results are shown in Fig. 18 and Fig. 19 respectively. Ertapenem data was analysed with two-tailed paired t-tests, comparing the various induction types to the parental strains. Ertapenem susceptibility in

BAC-Post exposure was the only condition with a significant change (p=0.01). All isolates induced to BAC-P5 demonstrated slight increases in susceptibility to ertapenem, however these increases are likely insignificant for strains “412412-BAC-P5” and “412621-BAC-P5” as the control strains for these isolates also had similarly sized zones of inhibition. Meropenem MIC data also showed slight increases in susceptibility for some of these strains, namely “412621-BAC-P5”, “302216-BAC-P5” post re-exposure, and “50183-BAC-P5”. The two most drug resistant parental isolates had increased ertapenem resistance when induced to CHX tolerance. While the magnitude of this increase was greatest in “51596E-CHX-P5”, the reduction of the ertapenem zone of inhibition for “50183-CHX-P5” was sufficient to cause reclassification as resistant under EUCAST guidelines. The MIC data for meropenem also indicates a 4-fold increase in meropenem resistance for “51596E-CHX-P5”, but not for “50183-CHX-P5”, which had a 2-fold decrease. MIC data also illustrated a 4-fold increase in meropenem resistance for “51596E-TRI-P5”, the strain with the highest original parental MIC for this antibiotic, however this was not corroborated by disk diffusion data for ertapenem, which had a slight increase in susceptibility for this strain.

Ertapenem Zones of Inhibition (mm)								
Isolate	**Parent**	**Control**	**TRI-P5 Pre**	**TRI-P5 Post**	**BAC-P5 Pre**	**BAC-P5 Post**	**CHX-P5 Pre**	**CHX-P5 Post**
412412	26.6	30	28.6	30	29.6	30	28	26.6
412621	27	29.6	28.3	28.3	30	29.6	26.6	30
302216	25.6	28	26.3	27.6	30	30	27.3	28.6
318418	29	28.6	26.3	28.6	29.6	30	25.6	28.6
51596E	18.3	19.6	20	19.6	23	25	11.6	13.3
50183	27.6	26.3	26.6	27.3	26	28.6	22.6	15

Figure 18. Ertapenem Zones of Inhibition for All Parental, Control, and Induced Strains. Zones of inhibition were measured for all strains after Kirby-Bauer assays. The values above were determined from the average of the three technical replicates carried out for each strain. Biocide-induced strains were tested at the highest concentrations of induction for TRI, BAC, and CHX (P5) both before and after re-exposure to the concentration of the relevant biocide used in induction. Red indicates higher resistance and green indicates higher susceptibility to ertapenem. Two-tailed paired t-tests were carried out for each condition compared to the parental strain, the only condition with a significant difference ($p<0.05$) was BAC-Post (p=0.01).

Meropenem MIC; Fold Change								
Strain	Parent MIC	Control	TRI-P5 Pre	TRI-P5 Post	BAC-P5 Pre	BAC-P5 Post	CHX-P5 Pre	CHX-P5 Post
412412	0.0625	0	0	0	0	0	2	2
412621	0.25	-2	-2	-2	-2	-4	-2	-2
302216	0.125	0	0	-2	0	-2	0	0
318418	0.0625	0	0	0	0	0	2	0
51596E	0.25	2	4	2	0	2	4	4
50183	0.5	0	-2	-4	-4	-4	-2	-2

Figure 19. Fold Change in Meropenem MIC relative to Parental Strains. Parental, control, and induced strains were assessed by microbroth dilution. The fold change in MIC relative to the parental strain was calculated using the most consistent MIC value from the three technical replicates carried out for each strain. Darker green indicates larger decreases in MIC while darker red indicates larger increases in MIC.

4.7.4. Piperacillin-Tazobactam Susceptibility

Susceptibility to piperacillin-tazobactam was tested by disk diffusion, results are shown in Fig. 20 below. Across the six strains, TRI-Pre (p=0.04), BAC-Post (p=0.04), and CHX-Post (p=0.002) showed significant changes in piperacillin-tazobactam susceptibility. Parental isolates “412412”, “412621”, and “50183” were initially classed as resistant to piperacillin-tazobactam but appeared to have differing responses to the induction process. Both “412412” and “412621” had increased susceptibility to the drug in their respective control strains while “50183-C” became slightly more resistant. The two former strains had increased susceptibility under all conditions, with “412412-TRI-P5” before re-exposure, “412412-BAC-P5”, “412412-CHX-P5” before re-exposure, and “412621-TRI-P5” being classed as sensitive to piperacillin-tazobactam under EUCAST guidelines. The “50183” isolate also became classed as sensitive after induction to CHX P5 after re-exposure. Isolates “302216” and “318418” only fluctuated slightly in their susceptibility to piperacillin-tazobactam throughout the induction process, while “51596E-TRI-P5” prior to re-exposure, “51596E-BAC-P5”, and “51596E-CHX-P5” after re-exposure became sensitive.

Piperacillin-Tazobactam Zones of Inhibition (mm)								
Isolate	Parent	Control	TRI-P5 Pre	TRI-P5 Post	BAC-P5 Pre	BAC-P5 Post	CHX-P5 Pre	CHX-P5 Post
412412	13.3	18.6	20	18.3	24.6	25	22.6	16.3
412621	10.3	18.3	21	16.3	15.3	18.6	12.6	17.6
302216	23.3	23.6	25	23.3	25.6	25.3	25.3	25.3
318418	25	23.3	25	24.6	22	24.6	27	28.6
51596E	19.3	20.6	26.6	19.6	27	29.6	19.6	23
50183	15.6	13	17	15.6	14	17	18.3	20.3

Figure 20. Piperacillin-Tazobactam Zones of Inhibition for All Parental, Control, and Induced Strains. Zones of inhibition were measured for all strains after Kirby-Bauer assays. The values above were determined from the average of the three technical replicates carried out for each strain. Biocide-induced strains were tested at the highest concentrations of induction for TRI, BAC, and CHX (P5) both before and after re-exposure to the concentration of the relevant biocide used in induction. Red indicates higher resistance and green indicates higher susceptibility to piperacillin-tazobactam. Two-tailed paired t-tests were carried out for each condition compared to the parental strain, the only conditions with a significant difference ($p<0.05$) were TRI-Pre ($p=0.04$), BAC-Post ($p=0.04$), and CHX-Post ($p=0.002$).

4.7.5. Amikacin Susceptibility

Susceptibility to amikacin was assessed by disk diffusion. The zones of inhibition recorded are displayed in Fig. 21. All parental isolates were classed according to EUCAST guidelines as susceptible. The only strain to become classed as resistant to amikacin was “51596E-CHX-P5”, all other strains either maintained their susceptibility levels or became slightly more susceptible to the drug. All BAC-P5 strains had significantly larger zones of inhibition relative to their parental strains after they were re-exposed to the biocide (BAC-pre: $p=0.01$, BAC-post: $p=0.0001$). In addition, strains “302216-BAC-P5”, “51596E-BAC-P5”, and “50183-BAC-P5” had increased susceptibility prior to re-exposure.

Amikacin Zones of Inhibition (mm)								
Isolate	**Parent**	**Control**	**TRI-P5 Pre**	**TRI-P5 Post**	**BAC-P5 Pre**	**BAC-P5 Post**	**CHX-P5 Pre**	**CHX-P5 Post**
412412	23	22.3	22	22.3	24.6	28.6	20.6	20.6
412621	23.6	22	23.6	22.3	23.3	27.6	21.6	23.3
302216	23.6	21.6	24.3	24.3	27.3	28.6	21.3	22.3
318418	21.3	22	23.3	23	23	27.6	23.6	21.3
51596E	19.6	18.3	18	23	22.3	25	16.3	15
50183	22.3	21.3	24	24.6	24.6	25.3	22	21.6

Figure 21. Amikacin Zones of Inhibition for All Parental, Control, and Induced Strains. Zones of inhibition were measured for all strains after Kirby-Bauer assays. The values above were determined from the average of the three technical replicates carried out for each strain. Biocide-induced strains were tested at the highest concentrations of induction for TRI, BAC, and CHX (P5) both before and after re-exposure to the concentration of the relevant biocide used in induction. Red indicates higher resistance and green indicates higher susceptibility to amikacin. Two-tailed paired t-tests were carried out for each condition compared to the parental strain, the only conditions with a significant difference ($p<0.05$) were BAC-pre ($p=0.01$) and BAC-post ($p=0.0001$).

4.7.6. Ciprofloxacin Susceptibility

Microbial sensitivity to ciprofloxacin was analysed through disk diffusion, results are shown in Fig. 22. Generally, changes in zones of inhibition size were consistent between the pre-and post-re-exposure replicates of the assay for induced strains, however only CHX-Pre and CHX-Post had a significant change in ciprofloxacin susceptibility ($p=0.03$ and 0.04, respectively). Strains adapted to 62.5 ug/mL of BAC (P5) tended to become more susceptible to ciprofloxacin, with strains “412412-BAC-P5”, “302216-BAC-P5”, and “318418-BAC-P5” changing in EUCAST classification from intermediately resistant to susceptible. Strains “412621-BAC-P5” and “51596E-BAC-P5” maintained their sensitivity and resistance respectively. Strain “50183-BAC-P5” was the only strain to become more resistant to ciprofloxacin under BAC-adaptation with an 11 cm reduction in its zone of inhibition relative to the parental isolate changing its classification from intermediate to resistant. The reductions in susceptibility seen in “50183-TRI-P5” prior to re-exposure and “50183-CHX-P5” were not as large but were still sufficient to also change the EUCAST classification. Strain “51596E-CHX-P5” also had a reduction in sensitivity following CHX adaptation but the parental isolate was already classed as resistant. Strain “302216-TRI-P5” had a reduction in ciprofloxacin susceptibility that was large enough to reclassify it as resistant under both pre- and post-re-exposure conditions, it was the only TRI-adapted strain to exhibit such a change relative to parental isolates.

Ciprofloxacin Zones of Inhibition (mm)								
Isolate	Parent	Control	TRI-P5 Pre	TRI-P5 Post	BAC-P5 Pre	BAC-P5 Post	CHX-P5 Pre	CHX-P5 Post
412412	24.3	23.6	23	24.3	28	28.6	23.6	23
412621	30	27	24.6	24.6	29	30	28	30
302216	23.3	24.6	15.6	15.6	29	30	21.6	22
318418	24.6	21.3	22.6	24.6	25.6	30	22.6	21.6
51596E	16	14	18.3	16	14.6	17.6	9	9
50183	23	22	21	23	12	12	16.3	17.6

Figure 22. Ciprofloxacin Zones of Inhibition for All Parental, Control, and Induced Strains. Zones of inhibition were measured for all strains after Kirby-Bauer assays. The values above were determined from the average of the three technical replicates carried out for each strain. Biocide-induced strains were tested at the highest concentrations of induction for TRI, BAC, and CHX (P5) both before and after re-exposure to the concentration of the relevant biocide used in induction. Red indicates higher resistance and green indicates higher susceptibility to ciprofloxacin. Two-tailed paired t-tests were carried out for each condition compared to the parental strain, the only conditions with a significant difference ($p<0.05$) were CHX-Pre ($p=0.03$) and CHX-Post ($p=0.04$).

4.7.7. Tetracycline Susceptibility

Tetracycline susceptibility was assessed with disk diffusion and microbroth dilution, these results are shown in Figs. 23 and 24 respectively. Increases in tetracycline resistance were consistently shown in both assays for the strain “412621-CHX-P5” under both conditions of CHX exposure during growth. This strain had 32-fold increase in MIC and a large increase in zone of inhibition size was sufficient to change the EUCAST classification from susceptible to resistant after re-exposure to 250 ug/mL CHX. Conversely, a 32-fold decrease in tetracycline MIC was seen in strain “318418-BAC-P5”, a 4-fold and 8-fold reduction in MIC also occurred in strain “302216-BAC-P5” under conditions of pre- and post-re-exposure respectively. These MIC decreases agreed with the disk diffusion results which illustrated a change of resistant to sensitive according to EUCAST guidelines. Changes in MIC and zone of inhibition size did not always agree, for instance “318418-TRI-P5” had a slight reduction in zone of inhibition but had a 16-fold increase in MIC, and strain “412412-CHX-P5” had a 32-fold increase in MIC relative to the parental isolate prior to re-exposure and no change following re-exposure but had a slight (2 cm) reduction in zone of inhibition. Strains “51596E-TRI-P5” and “50183-TRI-P5” also showed small 2-4-fold decreases in their tetracycline MICs but no change in susceptibility in the disk diffusion assay.

Tetracycline Zones of Inhibition (mm)								
Isolate	Parent	Control	TRI-P5 Pre	TRI-P5 Post	BAC-P5 Pre	BAC-P5 Post	CHX-P5 Pre	CHX-P5 Post
412412	23.6	22.6	21.6	22.6	26.3	28.3	21.6	21.6
412621	28.3	26	22	24	19.6	21	19.3	10.3
302216	9	8.6	8.3	8	28.6	25.6	8.3	8
318418	9.3	9	8.6	8.6	24.6	26	8	8
51596E	8.3	8	8	8	8	8	8	8
50183	9	8.3	8	8	8	8	8	8

Figure 23. Tetracycline Zones of Inhibition for All Parental, Control, and Induced Strains. Zones of inhibition were measured for all strains after Kirby-Bauer assays. The values above were determined from the average of the three technical replicates carried out for each strain. Biocide-induced strains were tested at the highest concentrations of induction for TRI, BAC, and CHX (P5) both before and after re-exposure to the concentration of the relevant biocide used in induction. Red indicates higher resistance and green indicates higher susceptibility to tetracycline. Two-tailed paired t-tests were carried out for each condition compared to the parental strain, however none of the conditions had a significant difference ($p<0.05$).

Tetracycline MIC; Fold Change								
Strain	Parent MIC	Control	TRI-P5 Pre	TRI-P5 Post	BAC-P5 Pre	BAC-P5 Post	CHX-P5 Pre	CHX-P5 Post
412412	4	0	0	0	0	4	32	0
412621	4	0	2	0	4	4	32	32
302216	128	0	-2	-4	-4	-8	0	-2
318418	256	-2	-16	-16	-32	-32	0	0
51596E	256	-2	-4	-2	-2	-2	0	0
50183	128	0	-4	-2	-2	0	0	0

Figure 24. Fold Change in Tetracycline MIC relative to Parental Strains. Parental, control, and induced strains were assessed by microbroth dilution. The fold change in MIC relative to the parental strain was calculated. Darker green indicates larger decreases in MIC while darker red indicates larger increases in MIC.

4.7.8. Sulfamethoxazole-Trimethoprim Susceptibility

Susceptibility to the combination of sulfamethoxazole and trimethoprim was assessed *via* disk diffusion. Results are shown in Fig. 25. The only cases of changes in susceptibility to this therapeutic occurred in the induced strains “302216-BAC-P5” and “318418-BAC-P5”, which went from resistant to susceptible regardless of whether they were grown in biocide prior to the assay. According to a two-tailed paired t-test comparing each induction group to the control, none of the induction conditions were associated with a significant change in drug susceptibility.

Sulfamethoxazole-Trimethoprim Zones of Inhibition (mm)								
Isolate	Parent	Control	TRI-P5 Pre	TRI-P5 Post	BAC-P5 Pre	BAC-P5 Post	CHX-P5 Pre	CHX-P5 Post
412412	19.3	19.6	20.3	19.6	24	16.6	21.6	19.6
412621	29	29.3	24	24.3	26.3	26.6	25.6	30
302216	8	8	8	8	23	17.3	8.3	8.3
318418	8	8	8	8	21.3	19.3	8	8
51596E	8	8	8.3	8	8	8	8	8
50183	10	8	8	8	8	8	8	8

Figure 25. Sulfamethoxazole-Trimethoprim Zones of Inhibition for All Parental, Control, and Induced Strains. Zones of inhibition were measured for all strains after Kirby-Bauer assays. Biocide-induced strains were tested at the highest concentrations of induction for TRI, BAC, and CHX (P5) both before and after re-exposure to the concentration of the relevant biocide used in induction. Red indicates higher resistance and green indicates higher susceptibility to sulfamethoxazole-trimethoprim. Two-tailed paired t-tests were carried out for each condition compared to the parental strain, however none of the conditions had a significant difference ($p<0.05$).

4.7.9. Chloramphenicol Susceptibility

Chloramphenicol susceptibility was assessed through microbroth dilution, results are shown in Fig. 26. The largest changes in susceptibility both occurred in TRI-P5 strains, namely an 8-fold increase in MIC for strain "302216-TRI-P5" and a 16-fold decrease in MIC for strain "318418-TRI-P5". The latter isolate appeared to also have increased tolerance of chloramphenicol in its control and BAC-P5 variants. CHX-induced isolates all had either no change or decreased chloramphenicol MICs.

Chloramphenicol MIC; Fold Change Relative to Parent								
Strain	Parent MIC	Control	TRI-P5 Pre	TRI-P5 Post	BAC-P5 Pre	BAC-P5 Post	CHX-P5 Pre	CHX-P5 Post
412412	64	2	0	0	0	2	-4	-2
412621	64	0	0	0	-2	0	-2	-2
302216	16	4	8	4	4	4	0	0
318418	64	-4	-16	0	0	0	-2	-2
51596E	32	-2	2	2	-4	-4	0	0
50183	64	-4	-4	-2	2	2	-4	-4

Figure 26. Fold Change in Chloramphenicol MIC relative to Parental Strains. Parental, control, and induced strains were assessed by microbroth dilution. The fold change in MIC relative to the parental strain was calculated. Darker green indicates larger decreases in MIC while darker red indicates larger increases in MIC.

4.7.10. Colistin Susceptibility

Colistin sensitivity was assessed *via* microbroth dilution, results are displayed in Fig. 27. All BAC-induced strains, with the exception of "412412-BAC-P5", had decreases in colistin MIC ranging from 2 to 4-fold in "412621-BAC-P5" to 32-fold in "51596E-BAC-P5". TRI-adapted strains also generally became more susceptible to colistin, with fold decreases in MIC ranging from 4 to 64-fold after re-exposure to TRI at 5 ug/mL prior to the assay. Some fluctuations in MIC also occurred in the control strains although there was no consistent trend. CHX-induced strains had higher colistin MICs in 5 of the 6 strains, three of which reached MICs of 32 ug/mL after re-exposure to CHX. These increases were 2 to 4-fold, with the exception of "412412-CHX-P5" which had a 16-fold increase in colistin resistance.

Colistin MIC; Fold Change								
Strain	**Parent MIC**	**Control**	**TRI-P5 Pre**	**TRI-P5 Post**	**BAC-P5 Pre**	**BAC-P5 Post**	**CHX-P5 Pre**	**CHX-P5 Post**
412412	2	4	0	-4	0	0	4	16
412621	8	0	0	-16	-2	-4	2	4
302216	16	-2	-8	-64	-4	-8	0	0
318418	4	2	-4	-16	-4	-8	2	2
51596E	16	-4	-2	-16	-32	-32	2	2
50183	4	0	-2	-8	-8	-8	2	2

Figure 27. Fold Change in Colistin MIC relative to Parental Strains. Parental, control, and induced strains were assessed by microbroth dilution. The fold change in MIC relative to the parental strain was calculated. Darker green indicates larger decreases in MIC while darker red indicates larger increases in MIC.

4.8. Efflux and Permeability of Parental, Control, and Induced Isolates

The relative levels of efflux and permeability in parental, control, and all biocide-induced isolates (P1-5) were analysed qualitatively in the EtBr cartwheel assay. Lowered accumulation of EtBr, which appeared in bacterial streaks as a decrease in fluorescence under UV-exposure, was indicative of either increased efflux, decreased permeability, or a combination of both factors. Fluorescence in the biocide-induced isolates relative to their respective parental strains was rated by eye from -2 to +2. Examples of EtBr cartwheel plates are shown in Fig. 28 and ratings of relative EtBr accumulation are shown in Fig. 28 below. Slight reductions in EtBr accumulation occurred in 2 of the control strains, "302216-C" and "318418-C". Elevated efflux or decreased permeability was common in TRI-induced strains with fluorescence decreasing in 5/6 TRI-P5 strains, 6/6 TRI-P4 strains,

6/6 TRI-P3 strains, 5/6 TRI-P2 strains, and 2/6 TRI-P1 strains prior to re-exposure. Re-exposure to TRI prior to the assay further maintained this level of fluorescence or decreased it further, with isolate “412412” having decreased fluorescence at all levels of TRI-induction post re-exposure. For BAC-induced strains, decreases in fluorescence were most predominant at the P2-level, with 5/6 strains being less fluorescent than their parent isolates, and most intense at the P3-level, with 3/6 strains very little fluorescence relative to their parent isolates. CHX-induced strains had their greatest reductions in fluorescence from the P3 level and up, with all strains having reduced fluorescence at this level and 5/6 having reduced fluorescence at both the P4 and P5 levels.

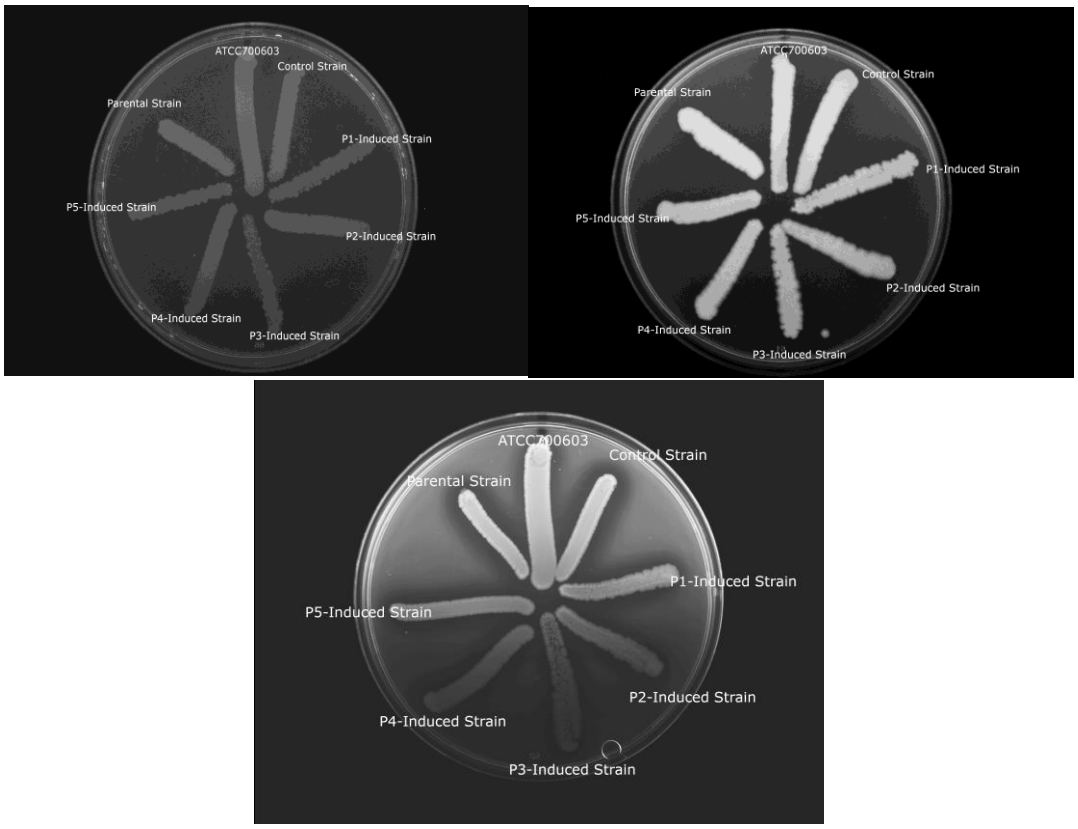

Figure 28. EtBr Cartwheel Assay Plates for Isolate 50183. Top left: Control plate without EtBr. Top right: CHX-P5 Prior to CHX re-exposure. Bottom: CHX-P5 after CHX re-exposure, in this picture P5 is representative of a -1 decrease in EtBr accumulation and P3 is representative of a -2 decrease in EtBr accumulation.

Strain	Control	TRI-P5 Pre	TRI-P5 Post	BAC-P5 Pre	BAC-P5 Post	CHX-P5 Pre	CHX-P5 Post
412412-P5	0	0	-2	0	0	-2	-2
412621-P5	0	-1	-1	-2	-2	-1	-1
302216-P5	-1	-1	-1	-1	0	-1	-1
318418-P5	-1	-1	-1	0	0	-2	-2
51596E-P5	0	-1	-1	-1	-1	-1	-1
50183-P5	0	-1	-1	-1	-1	0	-1
412412-P4	-	-1	-2	0	-1	-1	-1
412621-P4	-	-1	-1	-2	-2	-1	-1
302216-P4	-	-1	-1	0	-1	-2	-1
318418-P4	-	-1	-1	0	0	-2	-2
51596E-P4	-	-1	-1	0	0	-1	-1
50183-P4	-	-1	-1	-2	-2	0	-2
412412-P3	-	-1	-2	0	-1	-2	-2
412621-P3	-	-1	-1	-2	-2	-1	-1
302216-P3	-	-1	-1	-2	-1	-2	-1
318418-P3	-	-1	-1	0	0	-1	-1
51596E-P3	-	-1	-1	-1	-1	-1	-1
50183-P3	-	-1	-1	-2	-2	-1	-2
412412-P2	-	0	-2	-1	-2	-2	0
412621-P2	-	-1	-1	-1	-1	0	0
302216-P2	-	-1	-1	-2	-1	-2	-1
318418-P2	-	-1	-1	0	0	-1	-1
51596E-P2	-	-1	-1	-1	-1	0	0
50183-P2	-	-1	-2	-1	-1	0	-2
412412-P1	-	0	-2	0	0	0	0
412621-P1	-	0	0	-1	-1	0	0
302216-P1	-	0	-1	-1	-1	-1	-1
318418-P1	-	-1	-1	0	0	-1	-1
51596E-P1	-	-1	-1	-1	-1	0	0
50183-P1	-	0	0	-1	-2	-1	-1

Figure 28. Perceived Change in EtBr Accumulation. Strains were grown on plates containing EtBr and their fluorescence was assessed as a proxy for efflux and permeability. All control and biocide-induced strains are shown with scores indicating the relative change in fluorescence compared to their respective parental strains.

4.9. Capsule of Parental, Control, and Induced Isolates

Production of capsule was measured *via* the Ludox density gradient assay. Migration of the band of cells through the liquid is dependent on the amount of capsule present, greater penetration into the Ludox is indicative of lower capsule production. The average

distance travelled through the Ludox across three technical replicates is shown in Fig. 29 below. The changes observed in capsule expression are summarised in Fig. 30. Parental isolate "51596E" had significantly lower capsule production relative to the other parental isolates. This relatively low capsule production was maintained in this isolate throughout the induction process, appearing to decrease further in all three maximally induced strains after re-exposure to their respective biocides and prior to exposure in the "51596E-CHX-P5" strain. All TRI-P5 strains maintained their parental counterpart's level of capsule production prior to re-exposure to TRI. However, when grown overnight in 5 ug/mL of TRI, all strains had a significant decrease in capsule production. In the case of BAC-adaptation, strain "412621-BAC-P5" had decreased capsule production both before and after re-exposure while strains "412412-BAC-P5" and "318418-BAC-P5" had small but significant increases in their capsule production only following re-exposure. All CHX-P5 strains, with the exception of "50183-CHX-P5", had dramatically decreased capsule production after CHX induction prior to re-exposure. After re-exposure, "302216-CHX-P5" returned to the level of expression seen in the parental isolate, while "50183-CHX-P5" decreased its capsule expression.

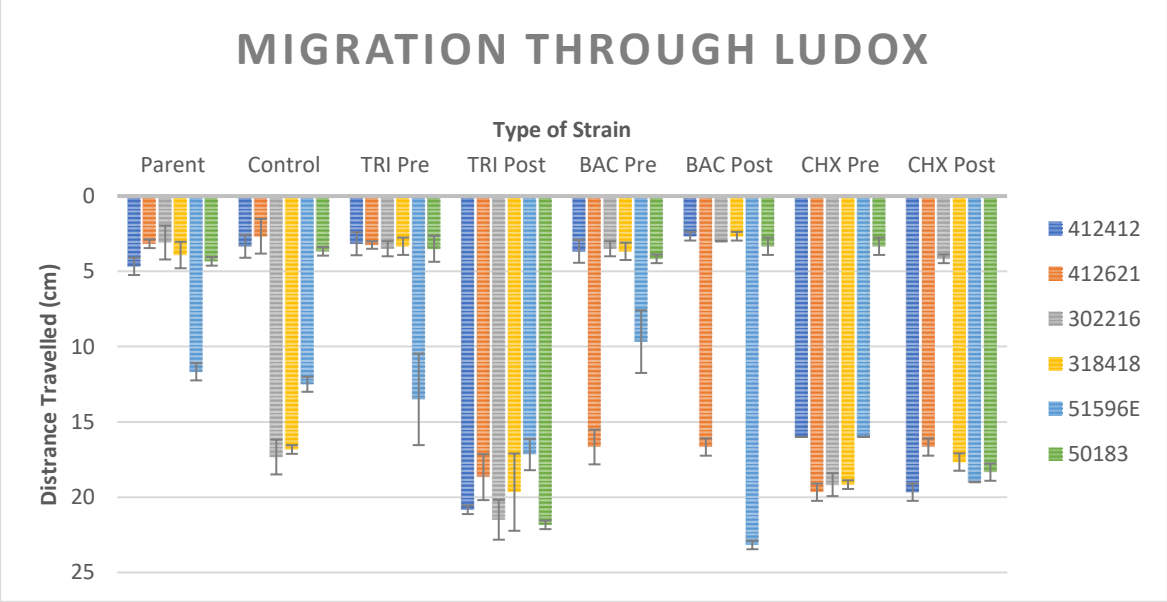

Figure 29. Migration of Cells through Ludox for Capsule Analysis. Parental, control, and induced isolates at the highest level of induction to TRI, BAC, and CHX respectively were analysed by centrifugation in ludox. Increased penetration through the ludox is caused by a reduction in capsule expression. Error bars ±1 standard deviation, calculated from the average values taken from the three technical replicates carried out for each strain.

Summary of Observed Changes in Capsule Expression							
Strain	**Control**	**TRI Pre**	**TRI Post**	**BAC Pre**	**BAC Post**	**CHX Pre**	**CHX Post**
412412	Increase	No change	Decrease	No change	No change	Decrease	Decrease
412621	No change	No change	Decrease	Decrease	Decrease	Decrease	Decrease
302216	Decrease	No change	Decrease	No change	No change	Decrease	No change
318418	Decrease	No change	Decrease	No change	No change	Decrease	Decrease
51596E	No change	No change	Decrease	No change	Decrease	Decrease	Decrease
50183	No change	No change	Decrease	No change	No change	No change	Decrease

Figure 30. Observed Changes in Capsule Expression Relative to Parental Isolates. All parental, control, and P5-induced strains were analysed. Green indicates a significant decrease in capsule production relative to the parental strain while red indicates a significant increase. Grey indicates no change in capsule expression following induction. A change was considered significant based on a p-value of less than 0.05, calculated from the three technical replicates carried out for each strain.

4.10. Biofilm of Parental, Control, and Induced Isolates

Biofilm growth was assessed after both 24 hours and 48 hours for parental, control, TRI-P5-induced, BAC-P5-induced, and CHX-P5-induced strains. The TRI and BAC isolates were analysed before and after re-exposure to their relevant biocides. CHX-induced isolates were only assessed prior to re-exposure. 24-hour and 48-hour biofilms are shown in Figs. 31 and 32 respectively. The biofilms were classified as non-adherent ($OD < OD_{Control}$), weakly adherent ($OD_{Control} < OD < 2x\ OD_{Control}$), moderately adherent ($2x\ OD_{Control} < OD < 4x\ OD_{Control}$), or strongly adherent ($4x\ OD_{Control} < OD$), these classifications are shown in Fig. 33. After 24 hours, the only significant changes in biofilm formation occurred in strains “412621-C”, “412412-BAC-P5” after re-exposure, and strains “318418-BAC-P5” and “51596E-BAC-P5” prior to re-exposure (p-values of 0.021, 0.0336, 0.0037, and 0.0069) respectively. After 48 hours of growth, significant changes in biofilm mass were only seen in strains “302216-C”, “318418-C”, “412412-BAC-P5” after re-exposure to BAC, and “51596E-CHX-P5” (p-values of 0.0211, 0.0009, 0.0207, and 0.0417 respectively). The only strain to undergo a significant change in biofilm production after both 24 and 48 hours was “412412-BAC-P5”, although this change was not sufficient to alter the classification of moderate biofilm formation found in the parental isolate. The only strain to have a significant change in biofilm formation and a change in classification was “51596E-CHX-P5” which went from moderate in the parental isolate to weak in the CHX-induced isolate.

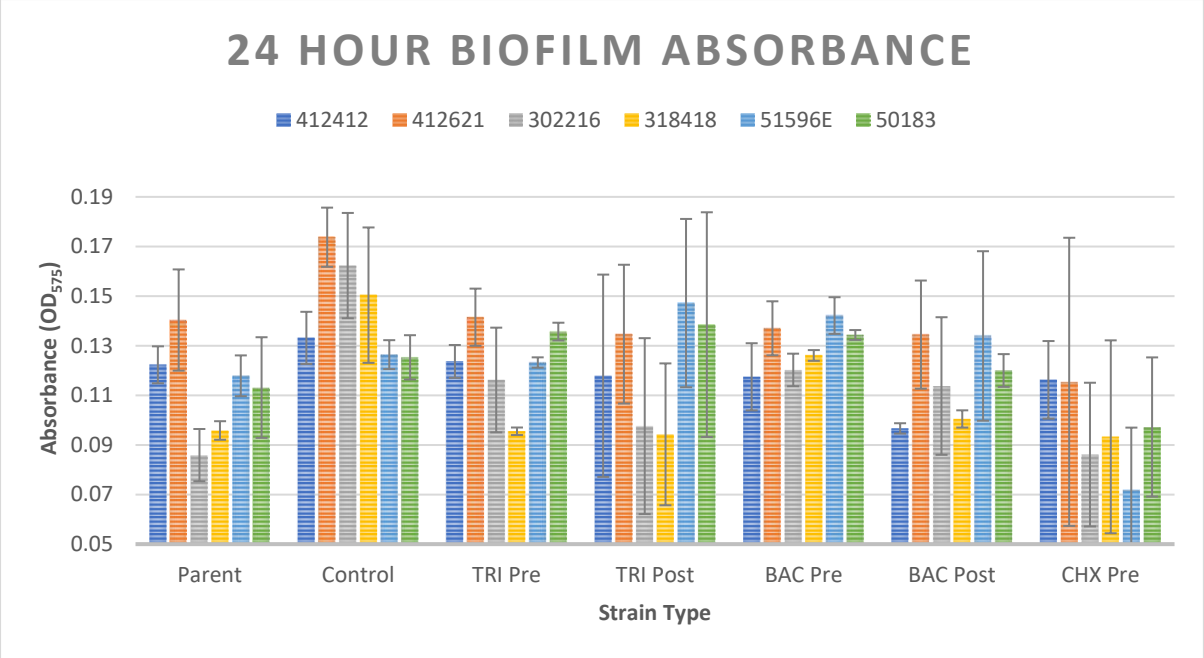

Figure 31. 24-Hour Biofilms for Parent, Control, Biocide-Induced Strains at the Highest Levels of Induction. TRI and BAC strains were assayed pre- and post-re-expose to the concentrations of TRI and BAC used in induction. CHX strains were only assayed prior to re-exposure. Results shown are the average of three technical replicates, error bars ±1 standard deviation. Two-tailed paired t-tests were carried out for each condition compared to the parental strains, however none of the conditions had a significant difference ($p<0.05$).

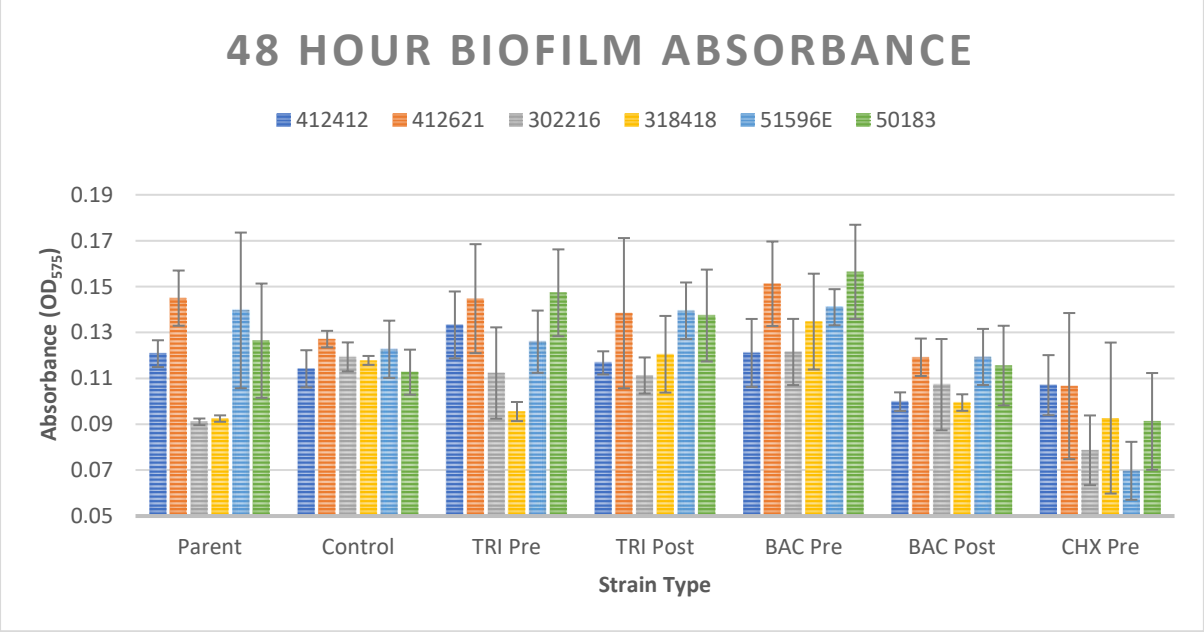

Figure 32. 48-Hour Biofilms for Parent, Control, Biocide-Induced Strains at the Highest Levels of Induction. TRI and BAC strains were assayed pre- and post-re-expose to the concentrations of TRI and BAC used in induction. CHX strains were only assayed prior to re-exposure. Results shown are the average of three technical replicates, error bars ±1 standard deviation. Two-tailed paired t-tests were carried out for each condition compared to the parental strains, however none of the conditions had a significant difference ($p<0.05$).

Strain (24 Hrs)	Parent	Control	TRI Pre	TRI Post	BAC Pre	BAC Post	CHX Pre
412412	Moderate	Moderate	Moderate	Moderate	Moderate	Moderate	Moderate
412621	Moderate	Moderate	Moderate	Moderate	Moderate	Moderate	Moderate
302216	Weak	Moderate	Moderate	Moderate	Moderate	Moderate	Weak
318418	Moderate	Moderate	Weak	Moderate	Moderate	Moderate	Moderate
51596E	Moderate	Moderate	Moderate	Moderate	Moderate	Moderate	Weak
50183	Moderate	Moderate	Moderate	Moderate	Moderate	Moderate	Moderate
Strains (48 Hrs)	**Parent**	**Control**	**TRI Pre**	**TRI Post**	**BAC Pre**	**BAC Post**	**CHX Pre**
412412	Moderate	Moderate	Moderate	Moderate	Moderate	Moderate	Moderate
412621	Moderate	Moderate	Moderate	Moderate	Moderate	Moderate	Moderate
302216	Moderate	Moderate	Moderate	Moderate	Moderate	Moderate	Weak
318418	Moderate	Moderate	Moderate	Moderate	Moderate	Moderate	Weak
51596E	Moderate	Moderate	Moderate	Moderate	Moderate	Moderate	Weak
50183	Moderate	Moderate	Moderate	Moderate	Moderate	Moderate	Weak

Figure 33. Classification of Biofilm Adherence. Biofilms produced by parent, control, and biocide-induced strains over 24 and 48 hours were classed based on their absorbance relative to the negative control. Weak adherence, shown in green, was defined as an optical density (OD) between 1x and 2x the OD of the control. Moderate adherence, shown in grey, was define as an OD between 2x and 4x the OD of the control. Each OD value was taken from the average of the six values produced for each strain in the single technical replicate carried out for each strain.

4.11. Metabolism of Parental, Control, and Induced Isolates

Growth curves were generated for all parental, control, and biocide-induced isolates at all levels of induction, and ATCC700603 as an internal control. The data from the three replicates of each strain was averaged, and then averaged by strain type to produce one curve for each isolate under the categories of parent, control, TRI-P5, TRI-P4, TRI-P3, TRI-P2, TRI-P1, BAC-P5, BAC-P4, BAC-P3, BAC-P2, BAC-P1, CHX-P5, CHX-4, CHX-3, CHX-P2, CHX-P1 strains. Graphs for TRI-induced (Fig. 35), BAC-induced (Fig. 36), and CHX-induced (Fig. 37), and a table with the rate of change in absorbance for each strain category over the first 5 hours of growth (Fig. 34) can be found below. TRI-P4 strains had a much lower final absorbance reading than the other TRI-induced strains. It also had a slightly lower growth rate than its parental isolate in the first 5 hours, but not as low as that found in the TRI-P3 and TRI-P2 strains. The lowest rate was seen in TRI-P3 strains however this average (0.10489) was skewed by the strain “318418-TRI-P3” which had a dramatically longer lag phase (in the first 5 hours the rate of change was 0.0072). TRI-P5 strains had an average rate of growth akin to that of the parental isolates. The BAC-induced strains had rates of growth in the first 5 hours that were similar to that of the parental strains, with

the exception of the BAC-P5 strains which had a lower rate and did not reach the same level of absorbance after 24 hours. The CHX-P5, CHX-P4, and CHX-P3 strains also displayed similar reductions in growth rate to that of the BAC-P5 strains.

Parent	TRI-P5	TRI-P4	TRI-P3	TRI-2	TRI-P1
0.132189	0.137455556	0.1286	0.104889	0.110578	0.125822
Control	**BAC-P5**	**BAC-P4**	**BAC-P3**	**BAC-2**	**BAC-P1**
0.137456	0.119577778	0.139133	0.134278	0.138211	0.134367
	CHX-P5	**CHX-P4**	**CHX-P3**	**CHX-2**	**CHX-P1**
	0.122688889	0.123278	0.110478	0.140744	0.13645

Figure 34. Average Growth Rates of Parent, Control, and Induced Strains. The rate of change in absorbance values over the first 5 hours of each growth curve was calculated to investigate the fitness costs related to biocide-induction. Red indicates strains with lower growth rates and green indicates strains with higher growth rates.

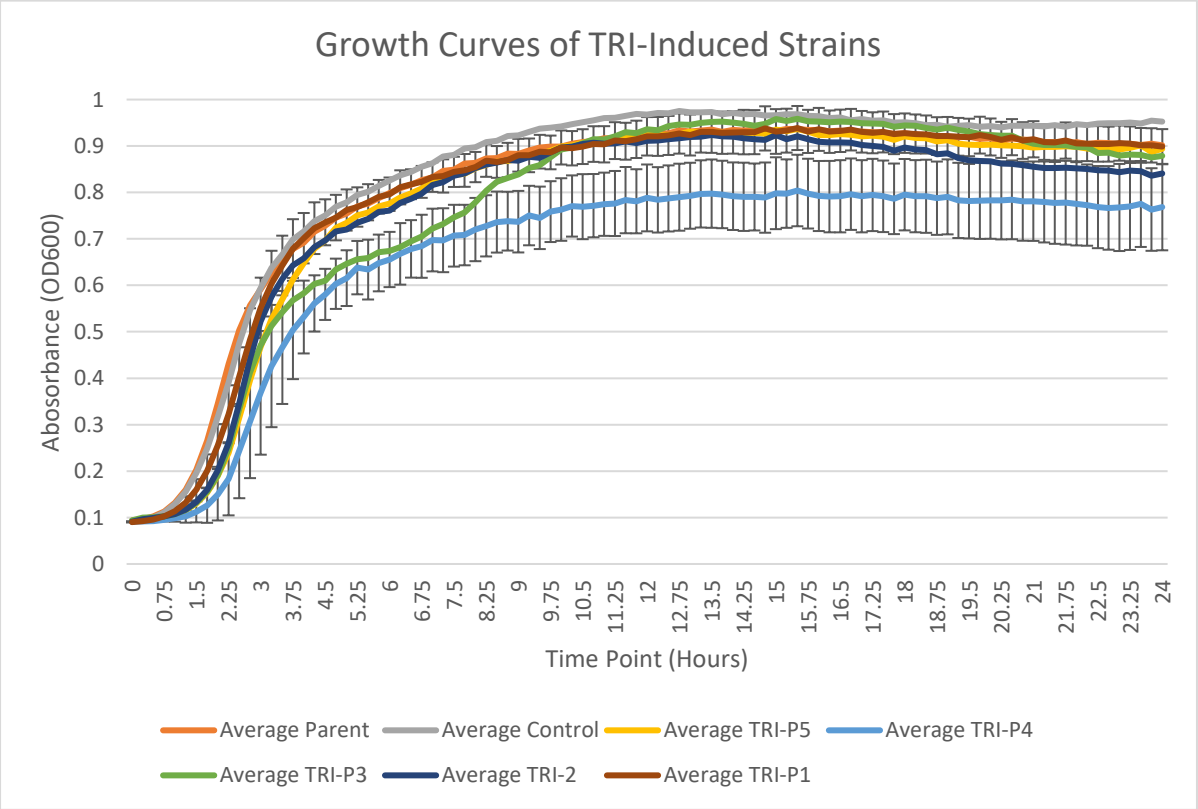

Figure 35. Growth Curves for Triclosan-Induced Strains. Growth curves for parental, control, and TRI-induced strains at all levels of induction are shown. Data shown is the average of three technical replicates for each of the six strains in each condition. Error bars (±1 standard deviation) shown are for the parental strains and TRI-P4.

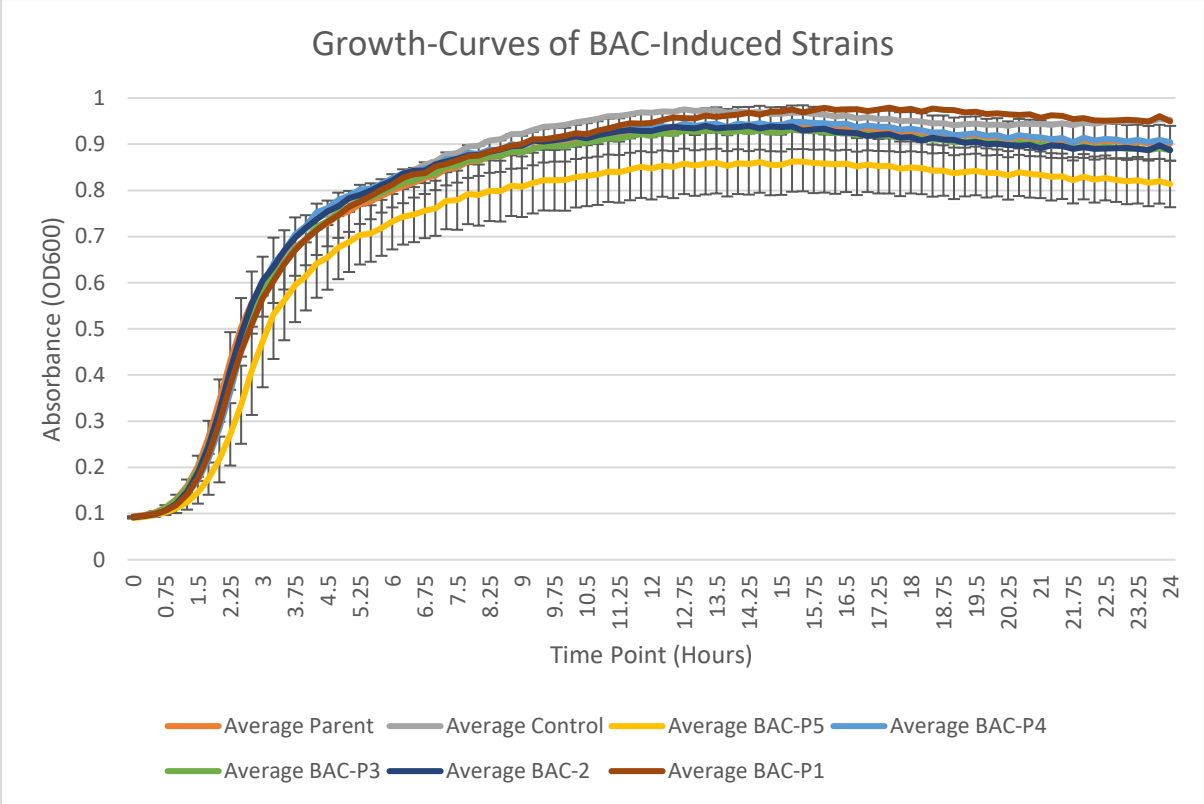

Figure 36. Growth Curves for Benzalkonium Chloride-Induced Strains. Growth curves for parental, control, and BAC-induced strains at all levels of induction are shown. Data shown is the average of three technical replicates for each of the six strains in each condition. Error bars (±1 standard deviation) shown are for the parental strains and BAC-P5.

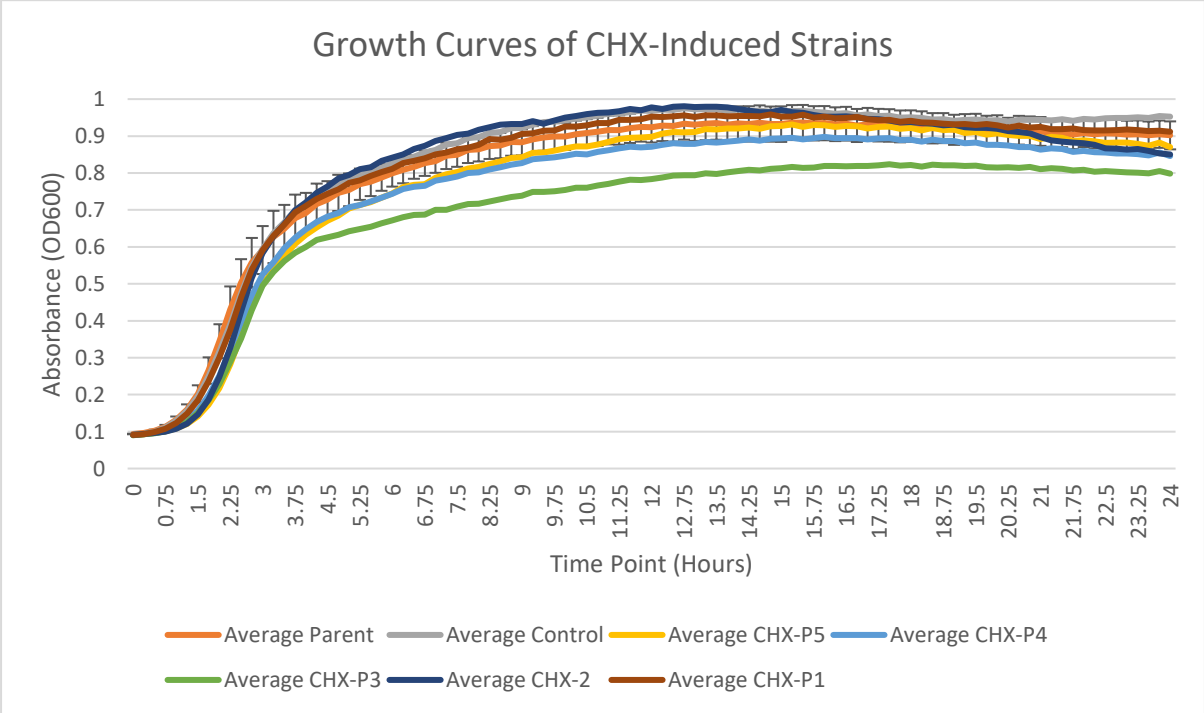

Figure 37. Growth Curves for Chlorhexidine Digluconate-Induced Strains. Growth curves for parental, control, and CHX-induced strains at all levels of induction are shown. Data shown is the average of three technical replicates for each of the six strains under each condition. Error bars (±1 standard deviation) shown are for the parental strains only, not shown for CHX-P3 due to a high standard deviation.

5.0. Discussion

5.1. The Consequences of Biocide Adaptation on Biocide Tolerance

5.1.1. Triclosan Adaptation and Biocide Tolerance

Biocide MIC testing results indicate that exposure to sub-inhibitory concentrations of TRI can induce increases in MIC that far exceed the concentration used. MICs in all TRI-induced strains were always at least double the concentration of the biocide to which they were exposed at every level of induction. This was repeatedly illustrated at multiple levels of induction but was most apparent in strain "318418-TRI-P3" for which the MIC increased from 2.5 ug/mL TRI in "318418-TRI-P2" to 20 ug/mL. When induced to the highest level of TRI used, 5 ug/mL, the fold increase in TRI MIC was constant regardless of whether the strain was re-exposed to 5 ug/mL of TRI prior to the microbroth dilution assay. This may indicate that the strains had become resistant to TRI rather than just tolerant. Given that TRI has a defined cellular target (the FabI protein) which, according to other research can mutate under the selective pressure of the concentrations used during induction in this study, this is to be expected. The existence of this cellular target is a point of concern for both the efficacy of this biocide and cross-resistance to first-line tuberculosis drugs.

The strains "302216-TRI-P5", "318418-TRI-P5", and "50183-TRI-P5" all demonstrated increases in their BAC MICs, but the experimentation carried out in this study does not provide any conclusive explanation for this. While, in the EtBr cartwheel assay, the permeability of these strains was shown to be slightly decreased relative to their parental strains, it may only be a factor in the "50183-TRI-P5" strain as it was the only one to also show an increase in CHX tolerance. In strain "318418-TRI-P5" there was a simultaneous 4-fold increase in CHX susceptibility while the CHX MIC for strain "302216-TRI-P5" remained unaffected.

The parental and TRI-induced strains were additionally tested to elucidate their MICs to chlorhexidine diacetate, an alternate salt form of CHX. While parental strain MICs to this antimicrobial agent were identical to their CHX MICs, TRI-induction had a sensitising effect in all isolates. The decreases in chlorhexidine diacetate increased with adaptation to higher concentrations of TRI, with strains "51596E-TRI-P5" and "412412-TRI-P5" having 32-fold reductions in their chlorhexidine diacetate MICs. It is unclear why this sensitising effect occurred for one form of chlorhexidine over the other, as the chlorhexidine

molecule is the active compound regardless of the accompanying salt molecules. There is no precedence for this in literature as different salts are taken to be synonymous. The differing solubility of these types of chlorhexidine salt may be the cause of this difference, however further research is required.

5.1.2. Benzalkonium Chloride Adaptation and Biocide Tolerance

Similar to the TRI-adapted strains, exposure to subinhibitory concentrations of BAC throughout the induction process always increased the MIC by 2-fold or more. All strains converged at an MIC of 125 ug/mL after exposure to 62.5 ug/mL of BAC. Unlike the TRI-induced strains however, there were some differences in BAC MICs depending on whether the BAC-P5 strains were exposed to 62.5 ug/mL prior to the assay. This was not the case in "302216-BAC-P5" and "50183-BAC-P5", but all other strains had double the BAC MIC if grown overnight in the presence of 62.5 ug/mL of BAC compared to when they were grown in MHB overnight without the biocide. This indicates that these strains can be induced to tolerate higher concentrations of BAC, but these mechanisms may not necessarily be constitutively active or heritable. Further research into mutations and changes in the transcriptome of these isolates may elucidate these mechanisms. The increases in MIC shown here are in agreement with those in literature, where increases of 2-fold to over 100-fold have been documented (20).

There were no clear trends in the susceptibilities of BAC-induced strains to other biocides, this indicates that cross-tolerance with other biocides with BAC is not a factor in these stains. This is despite changes in permeability and/or efflux in some strains, as shown in the EtBr cartwheel assay. While some 2-fold changes did occur in the TRI and CHX MICs of BAC-P5 strains, these are likely insignificant. The only case of a 4-fold change was sensitisation to CHX after strain "412412-BAC-P5" was re-exposed to BAC.

5.1.3. Chlorhexidine Digluconate Adaptation and Biocide Tolerance

CHX-induced strains, there were some cases (strains "412621-CHX-P3" and "50183-CHX-P4") in which the MIC was equal to the concentration to which the strains had been

induced, indicating that these strains were tolerant rather than resistant. Fold change in CHX MICs were consistent between CHX-P5 strains grown with and without 250 ug/mL CHX prior to the experiment, with strain “302216-CHX-P5” being the only one to have differing MICs before and after re-exposure (4-fold and 8-fold increases respectively). The increases in tolerance are in accordance with literature, where MIC increases of 2-fold to 200-fold have been reported (49, 50).

Strains induced to 250 ug/mL of CHX may have slightly increased susceptibility to TRI, but only after re-exposure to CHX prior to testing. 4 of the 6 CHX-P5 strains exhibited 2 or 4-fold increases in susceptibility to TRI after CHX re-exposure, but not before. This in conjunction with the chlorhexidine diacetate sensitisation of TRI-adapted strains may indicate a potential trade-off between the underlying mechanisms of tolerance in these strains. Further research in this area is required in order to establish a link between tolerance of these biocides. This being said however, the correlation between increased CHX tolerance and increasing BAC sensitivity was stronger than that seen in TRI susceptibility. Almost all CHX-P5 strains had increased BAC susceptibility which was not as dependent on re-exposure to CHX prior to testing as sensitivity was shown to increase in five of the six strains regardless. The increases in sensitivity to both TRI and BAC may be related a general decrease in the fitness of CHX-P5 strains, as shown by a slightly lower average rate of growth in the growth curve assay. CHX and BAC both attack the cell membrane, therefore it is unsurprising that the cellular changes induced in response to CHX could also diminish the activity of BAC.

5.2. The Effect of Biocide Adaptation on Antibiotic Resistance and its Association with Permeability and Efflux

No clear correlation between increased biocide tolerance and resistance to specific antibiotics was found. There was a slight increase in resistance to colistin in the strains induced to tolerate CHX-P5, although there were primary 2-4-fold in magnitude with the exception of “412412-CHX-P5” after re-exposure to CHX which had a 16-fold increase. The MIC of colistin did increase to 32 ug/mL in some of these strains with small fold increases being reflective of the initially high MICs in parental isolates. More research into this

relationship is paramount as colistin is a last line antimicrobial, the efficacy of which must be safeguarded. Aside from colistin, there were some sporadic increases in antibiotic resistance in some of the biocide-induced isolates, such as tetracycline resistance in strain "412621-CHX-P5". These changes were strain-specific rather than a trend associated with tolerance to any of the three biocides. This was also true for the loss of resistance in some strains, however more data on the mechanisms of resistance at play in the parental isolates is needed to fully understand these changes. The most common outcome for antibiotic susceptibility after induction of biocide-tolerance was an increase in sensitivity. This may be due to the fitness costs associated with biocide-adaptation, but the data indicates that the selective pressure imposed by biocides does not increase antibiotic resistance in most cases. As shown here, increases in tetracycline sensitivity occurring in conjunction with increased TRI tolerance have previously been reported, although the many studies report no correlation between TRI tolerance and cross-resistance to antibiotics (32, 90, 97). Similarly, there is no consensus in literature for strains induced to tolerate CHX and BAC, with papers indicating both increases and decreases in antibiotic sensitivity after biocide induction (49, 50). In strain-specific cases, such as the 32-fold increase in tetracycline sensitivity seen in strain "318418-BAC-P5", the sensitisation may be the result of the loss of a specific resistance gene or mobile genetic element. This could be investigated through genotypic analysis of these strains.

Increases in efflux and decreases in permeability of biocide-induced strains did not correlate with antibiotic resistance, however it may be a factor in increased resistance that occurred in some specific strains. This contrasts with much of the literature involving tolerance of biocides (19, 20, 51, 74, 128). The fitness costs imposed on the bacteria due to selection for biocide-tolerance may counteract the benefits to increased efflux and lowered permeability in strains that either did not become more antibiotic resistant or did not change in their sensitivities. Quantitative measures of changes in efflux and permeability would allow for more firm conclusions to be made. Furthermore, the permeability and efflux changes found in the biocide-adapted strains, while not directly increasing antibiotic resistance, may increase the occurrence of hetero-resistance in these strains. This should be pursued as an avenue of research in future as it may have indirect consequences on the therapeutic efficacy of antibiotics by contributing to the

development of resistance in biocide-adapted strains when they are exposed to antibiotics. Studies involving induction of biocide tolerance commonly involve non-clinical strains of *E. coli* and typically find some minor increases in antibiotic resistance, however the results discussed here do not support this conclusion. This disparity may be due to the different bacterial species used or the source of the strains used in this study, as all the strains used had pre-existing multidrug resistance. Furthermore, the directed evolution of biocide tolerance, as carried out in this study, may not be representative of the environmental conditions and selective pressures that environmentally-derived biocide-tolerant strains are exposed to, and may in turn select for alternative mutations (20).

5.3. The Effect of Biocide Adaptation on Capsule and Biofilm Production

Alterations in capsule expression associated with biocide-induction and biocide exposure were among the strongest phenotypic changes recorded. Significant changes in capsule expression occurred in the three control strains “412412-C”, “302216-C”, and “318418-C”, an increase in the former, and a decrease in the two latter strains. While significant, these changes were not as large as those that occurred in biocide-adapted strains, such as the CHX-P5 strains of isolates “412412”, 412621”, “302216”, “318418”, and “51596E”. The levels of capsule expression persisted after exposure to 250 ug/mL CHX in all of these strains with the exception of “302216” and “50183” which returned to the parental level and had decreased expression respectively.

While strains adapted to 5 ug/mL TRI did not have any change in their expression of capsule re-exposure to this concentration TRI after induction had a transient depressive effect on the production of capsule as all TRI-P5 strains had a significant reduction in capsule expression relative to their parental strain. This effect only occurred after strains were grown in the presence of 5 ug/mL TRI, indicating that this occurs in response to TRI exposure but is likely not a heritable characteristic within these strains. Further molecular research into the capsule production in these strains should be carried out to fully understand these findings.

Baseline level biofilm production after 24 hours and 48 hours appear to be unaffected by adaptation to biofilm production. After 24 hours only three of the maximally biocide-

induced strains and one control strain had significant changes in biofilm formation. The case was similar after 48 hours, indicating that adaptation to TRI, BAC, and CHX, have no effect on baseline biofilm formation. The growth of biofilms in the presence of these biocides and the resilience of biofilms to biocides was not tested but may present an interesting avenue of research in future.

5.4. The Effect of Biocide Adaptation on Bacterial Growth

Every strain involved in the study was analysed with growth curves in MHB to investigate if biocide-induction was associated with fitness costs. Growth curve data was averaged across all isolates within each level of TRI, BAC, and CHX, induction respectively. The rate of growth in the exponential phase was taken a measure of fitness. TRI-induction appeared to reduce the fitness of strains during P1, P2, P3, and P4, however the P5 level had a slightly raised rate of growth relative to parental strains. There is evidence in literature to corroborate the finding of reduced growth after adaptation to TRI (25). This raised rate of growth was equivalent to the increase seen in control strain data. BAC-induced strains only showed reduced growth at the highest level of induction. Of the levels of CHX induction, P3 strains had the largest fitness cost, which recovered slightly in the P4 and P5 levels of induction. The data also showed that, while strains did not always grow more slowly than parental isolates, they often entered stationary phase at a lower absorbance reading, indicating that they may reach lower total cell counts. Further experimentation is required to prove this, however.

6.0. Conclusion

The data collected in this study demonstrate the ability of *K. pneumoniae* isolates to adapt to high tolerances of TRI, BAC, and CHX when exposed to sub-inhibitory concentrations of these biocides. The adaptation to each biocide produced strains with varied phenotypic traits, with capsule production, efflux, permeability, and growth rates being altered relative to the parental isolates of these strains. Interestingly, there appears to be a link between TRI resistance and chlorhexidine diacetate sensitivity. This finding warrants further research. A potential link between colistin resistance and CHX was found, but

further experimentation is required to understand these findings. No other clear trends other than slight increases in susceptibility emerged in the antimicrobial susceptibility profiles of the biocide-induced isolates. Future research into the mutations and transcriptome changes in these TRI-, BAC-, and CHX-adapted strains should elucidate the mechanisms underlying these adaptations and their associated phenotypic changes. Observed differences between the antibiotic susceptibility profiles of lab-induced biocide-tolerant strains and those sourced from the environment may be due to the difference in selective pressures found when exposed to the single biocide in the laboratory and the litany of environmental pressures found in the real world. Furthermore, where tolerance of a biocide is induced, clinical isolates are rarely used. Their use in this study may have obfuscated the slight increases in antibiotic resistance seen in published literature involving reference strains as the clinical isolates had pre-existing multidrug resistance.

7.0. References

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