Understanding benzalkonium chloride tolerance and its implications in antibiotic resistance and other phenotypes in clinical isolates of *Klebsiella pneumoniae*

A dissertation carried out for the degree of M. Sc. in the Faculty of Science, Technology, Engineering, and Mathematics, Trinity College Dublin

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Declaration

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Summary

In this study, clinical isolates of *Klebsiella pneumoniae*, a Gram-negative pathogen associated with hospital acquired infections, were tested for both phenotypic and genotypic changes following adaptation to the commonly used disinfecting agent benzalkonium chloride. Changes in antibiotic susceptibility and susceptibility to other disinfectants were assessed using broth microdilution, and changes in colony morphology and capsule density were also determined. Assays using ethidium bromide and Hoechst H33342 fluorescent dye were also performed to observe changes in efflux activity and accumulation following adaptation to benzalkonium chloride.

It was found that bacterial cells which had been repeatedly exposed to sub-inhibitory concentrations of benzalkonium chloride showed greater tolerance to the biocide, but this did not correspond to a change in antibiotic resistance profile or confer cross-resistance with other biocides. Capsule density and efflux/accumulation were found to be the most impacted phenotypes in the adapted strains, with strains showing decreased capsule densities and increased efflux following adaptation.

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Table of contents

List of figures

List of tables

List of abbreviations

Abbreviations	Full name
BAC	Benzalkonium Chloride
BAC^t	Benzalkonium chloride tolerant
bp	Base pair
BSC	Biological safety cabinet
BSI	Blood Stream Infection
CCCP	Carbonyl cyanide 3-chlorophenylhydrazine
c*Kp*	Classical *K. pneumoniae*
CLSI	Clinical Laboratory Standards Institute
CPZ	Chlorpromazine
CR-*Kp*	Carbapenem resistant *K. pneumoniae*
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ECDC	European Centre for Disease Prevention and Control
EEA	European Economic Area
EPI	Efflux Pump Inhibitor
EtBr	Ethidium Bromide
EU	European Union
EUCAST	European Committee on Antimicrobial Susceptibility Testing
GDPR	General Data Protection Regulation
GNB	Gram-negative bacilli
HIA	Heat-inactivated
HMV	Hypermucoviscous
hv*Kp*	Hypervirulent *K. pneumoniae*
LB	Luria-Bertani broth
LPS	Lipopolysaccharide
MBC	Minimum bactericidal concentration
MHA	Mueller-Hinton agar
MHB	Mueller-Hinton broth

MIC	Minimum inhibitory concentration
NCBI	National Centre for Biotechnology Information
OMP	Outer membrane proteins
PBP	Penicillin-binding protein
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
PMZ	Promethazine
QAC	Quaternary ammonium compound
RNA	Ribonucleic acid
RND	Resistance nodulation division
RT-qPCR	Reverse transcription quantitative polymerase chain reaction
SEM	Scanning electron microscopy
TEM	Transmission electron microscopy
TLR4	Toll-Like Receptor 4
T_m	Melting temperature
TZ	Thioridazine
UTI	Urinary tract infections
WHO	World Health Organisation
WT	Wild-type

Chapter 1: Introduction

1.1 *Klebsiella pneumoniae* general introduction

*Klebsiella pneumonia*e is part of the lower classifications of the *Enterobacteriaceae* family of Gram-negative organisms. *K. pneumoniae* is commonly associated with nosocomial infection of the urinary tract, as well as blood stream infections (BSI), among others. It is also part of the ESKAPE group of pathogens, as designated by the World Health Organisation (WHO) in 2017, which also comprises *Enterococcus faecium*, *Staphylococcus aureus*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter spp*. The ESKAPE pathogens are designated priority pathogens, for which new and novel antibiotics are urgently needed. They are considered to be of great concern for human health globally due to their high rates of antimicrobial resistance leading to reduced options for treatment of serious infections. This leads to an increased burden on the healthcare sector and poorer patient outcomes, and in severe cases increased death rates (1).

K. pneumoniae is commonly associated with high levels of antibiotic resistance. In 2021, antibiotic resistant *K. pneumoniae* was the third most commonly reported isolate to the WHO/European Centre for Disease Prevention and Control (ECDC), after *Escherichia coli* and *Staphylococcus aureus* (2). In addition, co-resistance is common, with more than 20% of reported isolates in 2021 showing resistance to two or more of the antibiotics under surveillance by the EDCD and WHO (3). Multi-drug resistance in *K. pneumoniae* will be discussed further in this chapter.

1.2 *Klebsiella pneumoniae* hospital acquired infections

K. pneumoniae is often implicated in catheter associated urinary tract infections and systemic infections of hospitalised patients. These infections are often difficult to treat, as many clinical *K. pneumoniae* isolates are resistant to several antibiotics. Recent data from the ECDC found that 35,000 people die from antibiotics-resistant infections every year in the European Union (EU)/European Economic Area (EEA). Of particular concern is the observed increase in resistance to last line antibiotics such as carbapenems and third generation

cephalosporins. The number of reported carbapenem-resistant *K. pneumoniae* isolates increased by 31% in the year 2019-2020, and a further 20% in the year 2020-2021 (4).

1.3 Virulence and virulence factors

K. pneumoniae strains which are of particular concern to human health can generally be divided into two classes; classical *K. pneumoniae* (*cKp*) and hyper-virulent *K. pneumoniae* (*hvKp*). Strains classed as *cKp* are typically hospital acquired, while *hvKp* strains tend to be community acquired and cause more severe infections in otherwise healthy individuals, including metastatic infections not commonly associated with Gram-negative bacilli (GNB) (5,6).

Several virulence factors have been identified in *K. pneumoniae*. These include, but are not limited to, the presence and composition of the bacterial capsule and the presence of lipopolysaccharides, adhesion factors such as type 1 and type 3 pili, and siderophores which facilitate iron carriage (7).

1.3.1 Capsule

K. pneumoniae produces a viscous, complex polysaccharide capsule. Analyses performed by Dutton and Paulin (1980) found that the capsule is made up of complex polysaccharides consisting of repeating subunits (8,9). The polysaccharide composition varies between different strains of *K. pneumoniae*. These variations give rise to different strain specific serotypes, known as K serotypes, which can be used to distinguish between strains in clinical settings. At least 79 known K serotypes have been identified, though serotypes K1 and K2 are the ones primarily associated with bacteraemia (10). While most clinical infections are caused by a relatively small number of serotypes, more diversity is seen among strains implicated in UTIs (11,12).

The *K. pneumoniae* capsule and its associated mucoviscosity plays a vital role in virulence and the establishment of infection through protecting against phagocytosis and serum killing (13–16).

The plasmid-mediated *rmpA* virulence gene regulates capsular polysaccharide biosynthesis and carriage of this gene is associated with the *hvKp* HMV phenotype (17).

1.3.2 Lipopolysaccharide

Lipopolysaccharide (LPS) is composed of the O antigen, made up of repeating oligosaccharide units, the core oligosaccharide, and lipid A (18,19). LPS and associated LPS modifications are a known method of virulence and contributor to hypervirulence in *hvKp,* by both protecting *K. pneumoniae* against host immune defences, and by stimulating the production of cytokines and chemokines through interactions between lipid A and Toll-Like Receptor 4 (TLR4) . In addition, *K. pneumoniae* can mask itself from certain host immune responses through LPS modifications (20).

1.3.3 Fimbriae

Bacterial fimbriae, also known as pili, aid in bacterial adhesion to surfaces. Two types of fimbriae, Type 1 and Type 3 fimbriae, have been identified in *K. pneumoniae.* Fimbriae promote adhesion of bacteria to host epithelial cells have been shown to be important in colonisation of the human urinary tract, including the formation of biofilms on urinary catheters, contributing to the development of UTIs (21,22).

1.3.4 Outer membrane proteins

Several outer membrane proteins (OMPs) have been shown to be implicated in *K. pneumoniae* virulence, examples including OmpA, peptidoglycan-associated lipoprotein (Pal), and murein lipoprotein (LppA) (23).

1.3.5 Siderophores

Siderophores are small iron-carrying molecules which are secreted into the extracellular space and aid in the acquisition of environmental Fe^{3+}. The siderophores found in *K. pneumoniae* are: enterobactin, yersinabactin, aerobactin, and salmochelin. Salmochelin and aerobactin in particular are common in *hvKp* strains and are associated with invasive disease (24,25).

The expression of siderophores has been shown to be increased 6- to 10-fold in hv*Kp* strains compared to c*Kp* strains. A possible link has also been established between the production of aerobactin and mucoviscosity in *K. pneumoniae* strains (26,27)

1.3.6 Biofilm

Biofilms consist of a community of cells embedded in an extracellular matrix of proteins, polysaccharides and DNA. *K. pneumoniae* biofilms may colonise the respiratory tract, gastrointestinal tract, or the urinary tract, especially in patients with tracheal tubes or indwelling catheters (28) (24).

In *K. pneumoniae*, biofilm formation depends mainly on two surface structures; capsular polysaccharides and type 3 fimbriae. The capsular polysaccharides form the structure of the biofilm matrix and facilitate communication within the biofilm, while adhesion of the biofilm is mediated by the type 3 fimbriae (7,29).

Bacterial communities that exist in biofilms have greater tolerance to antimicrobial peptides and phagocytosis than planktonic cells (30).

1.4 Treatment of *Klebsiella pneumoniae* infections

The classes of antibiotics under surveillance by the WHO/EDCD are: third-generation cephalosporins (cefotaxime, ceftriaxone, ceftazidime), carbapenems (imipenem, meropenem), fluoroquinolones (ciprofloxacin, levofloxacin, ofloxacin), and aminoglycosides (gentamicin, tobramycin). Additionally, colistin or tigecycline may be used as last-line antibiotics for the treatment of carbapenem resistant *K. pneumoniae,* (*CR-Kp*) (3) (31)

1.4.1 β-lactam antibiotics

β-lactams include penicillins, cephalosporins, and carbapenems among others. β-lactams target penicillin-binding proteins (PBPs) 2 and 3. PBPs are essential for peptidoglycan

biosynthesis, and targeting of these by β-lactam antibiotics inhibits this process, thus destabilising the bacterial outer membrane (32,33).

K. pneumoniae is often more susceptible to third-generation cephalosporins such as cefotaxime and ceftazidime than E. coli, likely due to the presence of the large outer membrane porin OmpK35. The loss of OmpK35 may contribute to resistance to these antibiotics (34).

Carbapenems have the broadest spectrum of all β-lactam antibiotics for both Gram-negative and Gram-positive pathogens, and are therefore often used as treatment options for multi-drug resistant infections (35). High levels of resistance to third-generation cephalosporins and carbapenems have been reported in *K. pneumoniae.* In 2021, 42% of countries in the WHO European Region reported resistance percentages of 50% or above for third-generation cephalosporins, while 33% reported carbapenem resistance in 25% or more *K. pneumoniae* isolates (2).

Unlike other β-lactams, carbapenems are not sensitive to β-lactamases (33,36). The current treatment options for carbapenem-resistant *Enterobacterales* are the last-line antibiotics colistin and tigecycline (37).

1.4.2 Fluoroquinolones

Fluoroquinolones, such as ciprofloxacin, levofloxacin and ofloxacin, are typically used to treat invasive infections such as respiratory and gastrointestinal infections (38). They target DNA synthesis by inhibiting DNA gyrase and topoisomerase IV, (39).

Due to their efficacy in treating severe invasive infections, fluoroquinolones are widely used to treat severe *K. pneumoniae* infections, though the overuse of fluoroquinolones have led to increased resistance in clinical isolates (38). Resistance to fluoroquinolones has been found to be associated with amino acid substitutions in the *gyrA* and *parC* genes, as well as acquisition of the *qnrA* and *qnrB* genes (40).

1.4.3 Aminoglycosides

Aminoglycosides, examples of which include tobramycin, gentamicin and amikacin, are a group of antibiotics which inhibit protein synthesis by targeting the ribosome (41,42). The use of aminoglycosides for the treatment of *K. pneumoniae* infections has largely been replaced by other drugs such as carbapenems and fluoroquinolones (24).

Widespread resistance to aminoglycosides may occur as a result of enzymes such as the 16S rRNA methylase, or chromosomally through alterations in permeability (43–45).

1.4.4 Tigecycline

Tigecycline is often used in combination with colistin to treat carbapenem-resistant *K. pneumoniae* infections (46). Resistance to tigecycline in *K. pneumoniae* has been attributed to the plasmid encoded resistance-nodulation-division (RND) superfamily efflux gene cluster, TMexCD1-TOprJ1 (47), as well as the AcrAB-TolC efflux pump, also of the RND superfamily (46).

1.4.5 Colistin

Colistin, part of the polymyxin class of antibiotics, is one of the last line antibiotics used to treat carbapenem-resistant *K. pneumoniae* infections (31). The exact mechanism of action of colistin is not fully understood, though it is known to target the LPS and the Gram-negative cell wall (48,49). Susceptibility testing for colistin is generally not performed as part of routine AST in hospitals, making surveillance of developing resistance difficult.

1.4.6 Combinations therapies

Many *K. pneumoniae* infections are multi-drug resistant, making single antibiotic treatment (monotherapy) challenging. One way of combating MDR-*Kp* infections is through the use of combinations therapies. Ideally, the use of two or more antibiotics may slow down the evolution of resistance (50). Studies on the use of combination therapies to treat Gram-negative infections have shown improved patient outcomes, including decreased mortality

rates. These studies typically included the use of a β-lactam in combination with a fluroquinolone or aminoglycoside (51,52).

Combination therapy may be beneficial where monotreatment with a β-lactam antibiotic is likely to fail, such as with carbapenem-resistant infections. Combination of a carbapenem with colistin or tigecycline has proven beneficial for patient outcomes relative to monotherapies with the same antibiotics (53,54).

1.5 Multi-drug resistance

In the context of antimicrobial susceptibility, the term "resistance" refers to an increase in the MIC of a strain to a given antibiotic or compound as a result of a permanent change to the cell, while the term "tolerance" refers to the ability of a strain to survive concentrations of a given substance at levels which may ordinarily be lethal to the cell, as a result of a reversible phenotypic change (55). Multi-drug resistance, defined as resistance to three or more different classes of antibiotics (56), is an increasing concern in Irish and other EU hospitals, with *K. pneumoniae* being a known reservoir of antibiotic resistance genes (57).

High levels of antibiotic resistance have been observed in *K. pneumoniae*. Resistance may develop as a result of the overreliance of antibiotics, mutations in genes which alter antibiotic targets, or through the acquisition of virulence genes through horizontal gene transfer (HGT) (36).

An increase in antimicrobial resistance was seen following the SARS-CoV-2 pandemic, possibly accelerated by the increased use of antimicrobial surface disinfectants (58).

1.6 Permeability and efflux

Diffusion across the bacterial outer membrane is necessary in order for a drug or compound to enter Gram-negative bacteria. This process is typically mediated by porins, which form channels in the outer membrane, allowing for entry into the cell. β-lactams especially seem to

be dependent on this mode of transport (34,59). Highly drug resistant *K. pneumoniae* strains often have a reduced amount of porins (60).

1.6.1 Porins

Porin channels allow for the diffusion of drugs or other compounds into the cell through the outer membrane. The major porins in *K. pneumoniae* are OmpK35 and OmpK36, which are homologues of *E. coli* OmpF and OmpC respectively (59,61). OmpK35 and OmpK36 have been shown to form larger, more permeable channels than their *E. coli* homologues, making them better at diffusing lipophilic and large compounds (34).

Antimicrobial resistance in *K. pneumoniae* is often associated with the loss of porins, possibly, particularly OmpK35 (60).

1.6.2 Efflux pumps

Efflux pumps are found intrinsically in the membrane of Gram-negative bacteria, which gives rise to intrinsic resistance to some antibiotics through the reduction of the intracellular concentration of these, as they are being exported out of the cell (62). Certain efflux pumps can transport multiple classes of antibiotics, giving rise to an MDR phenotype (63).
Efflux pumps can be divided into primary and secondary transporters. Primary transporters utilise the energy from ATP hydrolysis to facilitate efflux, while secondary transporters use the energy from the electrochemical membrane potential (64). Secondary transporters, particularly those in the RND family, are the most clinically significant efflux pumps (63). These will be discussed in further detail below.

1.6.3 RND-type efflux pumps

Multidrug efflux pumps are a known mechanism for removing harmful chemicals in *K. pneumoniae.* The most prominent type of efflux pumps in Gram-negative bacteria is the RND family, comprising the AcrAB, OqxAB, EefAB, and KexD efflux pumps (65). RND efflux pumps are comprised of an inner membrane protein, a periplasmic protein and an outer

membrane protein, with the inner membrane protein having the ability to recognise and bind various substrates for extrusion (66).

RND-type efflux pumps also play a crucial role in colonisation of the human gastrointestinal tract, and in virulence (67).

1.6.3.1 AcrAB-TolC

The AcrAB-TolC efflux pump is the most well characterised efflux pump of the RND family. It has been demonstrated to be essential for virulence in Gram-negative pathogens (68,69) and strains which overexpress this pump have been found to have higher levels of antibiotic resistance (70). Additionally, AcrAB-TolC has been shown to be important in contributing to biocide tolerance in *K. pneumoniae*, with an increase in *acrA* expression observed in strains with increased tolerance (71).

1.6.3.2 RamR-RamA

RamA is a global regulator in *K. pneumoniae* which has been shown to be implicated in resistance to multiple drugs and compounds (72). RamA is also the regulator of the *acrAB* genes, and mutations in *ramR*, the transcriptional regulator of *ramA*, causing overexpression of *ramA* has been shown to upregulate the AcrAB-TolC efflux system.

Exposure of K. pneumoniae to benzalkonium chloride (BAC) has previously been shown to select for *ramA* overexpressing mutants (73). Additionally, Park and Yoon (2023) found that *ramA* increases its transcription upon treatment with BAC in *Salmonella enterica serovar typhimurium*, and that the absence of *ramA* made the strain susceptible. Complementation of the *ramA* mutant with *acrA* in trans restored the resistance, suggesting a relationship between the *ramA* regulated AcrAB-TolC efflux pump and BAC susceptibility (74)

RamA mediated alterations have been shown to decrease susceptibility of *K. pneumoniae* to antibiotics Colistin E and Polymyxin B, both of which target Lipid A of the lipopolysaccharide. (72)

1.6.4 Efflux pump inhibitors

Efflux pump inhibitors (EPIs), promote the intracellular accumulation of antimicrobial agents or other substances, by inhibiting efflux activity. The use of EPIs has been shown to decrease antibiotic resistance, or delay the development of resistance (75). The EPIs used in this study are explained further below.

1.6.4.1 Phenothiazines

Phenothiazines are a group of drugs primarily used in the treatment of psychiatric disorders. Phenothiazines have been shown to inhibit the AcrAB-TolC efflux pump system, though the exact mechanism on inhibition remains unknown (76,77).
Chlorpromazine (CPZ), the most widely studied of the phenothiazines, has been shown to be a substrate for the AcrB subunit of the AcrAB-TolC efflux pump in *S. typhimurium* and *E. coli* (78). Other phenothiazines which have been shown to inhibit efflux activity include thioridazine (TZ) and promethazine (PMZ) (77,79).

1.6.4.2 carbonyl cyanide m-chlorophenylhydrazine

carbonyl cyanide m-chlorophenylhydrazine (CCCP) acts by reducing the transmembrane potential required to maintain efflux activity (80,81). Combining CCCP with other antimicrobial agents has been shown to reduce antimicrobial resistance in certain MDR bacterial strains (81,82).

1.7 Benzalkonium chloride general introduction

Benzalkonium chloride is a cationic surfactant with broad-spectrum bactericidal activity against bacteria, fungi, parasites and lipophilic viruses (83,84)
BAC, and other quaternary ammonium compounds (QACs), are commonly used disinfectants in domestic, industrial, and healthcare settings, and are also used as preservatives in cosmetics and pharmaceutical products (85,86).

The primary mode of action of BAC is to perturb the lipid bilayers of the bacterial inner and outer membranes through interactions of the positively charged alkyl groups of BAC with the

negatively charged head groups of the membrane phospholipids. This results in cell lysis through the release of lytic enzymes, and subsequent and leakage of cytoplasmic contents into the extracellular space (87–89).

The antimicrobial efficacy of QACs corresponds to the lipophilicity of the compound, which is determined by the alkyl-chain length, with a length of C12 to C16 eliciting the highest biocidal activity (87,90,91).

1.8 Benzalkonium chloride tolerance

Bacteria may be exposed to sub-inhibitory concentrations of BAC through improper usage, or through environmental contamination. This can create a selective pressure, driving the emergence of strains with decreased susceptibility to BAC, with potential cross resistance to clinically relevant antibiotics (85,92).

Changes in BAC MIC have been found to be associated with a change in the membrane phospholipid composition, or with the overexpression of multidrug efflux pumps, typically through the acquisition of *qac*-genes through horizontal gene transfer (93,94). Conflicting evidence exists regarding the effects of BAC on biofilm formation, though some studies have demonstrated increased biofilm formation in response to BAC in Gram-negative bacteria (95–97)

1.8.1 Changes in membrane composition

Nordholt et al (2021) described that periodic disinfection of *E. coli* with BAC selected for a tolerant subpopulation with mutations in the *lpxM* locus, which is associated with Lipid A biosynthesis, leading to a possible change in cell surface charge which may explain the reduction in BAC susceptibility (98). Other studies have shown that exposure to benzalkonium chloride induces the expression of genes involved in fatty acid metabolism in *Bacillus cereus*, causing changes in membrane composition (99).

1.8.2 Upregulation of efflux pumps

The expression of efflux pumps is a known mechanism of tolerance to BAC, with the acquisition of *qac*-genes accounting for many reported cases of tolerance (97).

Exposure of bacteria to common household biocides has been shows to increase efflux pump expression in Gram-negative organisms (100). Additionally, Romanova et al. (2006) were able to restore susceptibility (to pre-adaptation levels) of an adapted L. monocytogenes strain when efflux pump inhibitors were added to the growth medium, suggesting at least a partial role of efflux pumps in benzalkonium chloride susceptibility in this organism (101).

Additionally, as discussed in 1.6.2.1 above, the AcrAB-TolC efflux pump has been shown to be implicated in BAC tolerance (71).

1.9 Cross-resistance with antibiotics

Some studies have suggested a link between benzalkonium chloride resistance and the emergence of increased antibiotic resistance (86,92,97,102).

It has been shown that exposure of bacteria to biocides may cause decreased susceptibility to other antimicrobials agents, likely through an overlap in resistance mechanisms between the biocide and antimicrobial compound (85,103,104).

Dissemination of BAC from household, clinical and agricultural use has been shown to alter the composition of antimicrobial resistance genes present in activated sludge environments (92). *K. pneumoniae* is already a known trafficker of antimicrobial resistance genes, primarily due to horizontal gene transfer (57). Additionally, acquisition of multi-drug efflux pumps which remove BAC from the cell may also remove clinically relevant antibiotics, leading to cross resistance (105).

1.10 Scope of this study

This study aims to investigate the effects of benzalkonium chloride tolerance on multi-drug resistant clinical isolates of *Klebsiella pneumoniae*, as well as the possible impacts on antibiotic resistance and cross-resistance to other biocides. Experiments were carried out for the assessment of metabolism, general phenotypic changes, and changes in accumulation and efflux profile. A combination of phenotypic and genotypic experiments were used.

The BAC tolerant (BAC^t) strains used in this study were generated during a previous study through continuous passage in increasing concentrations of BAC over several days. A schematic outlining the adaptation is shown in Figure 1 below:

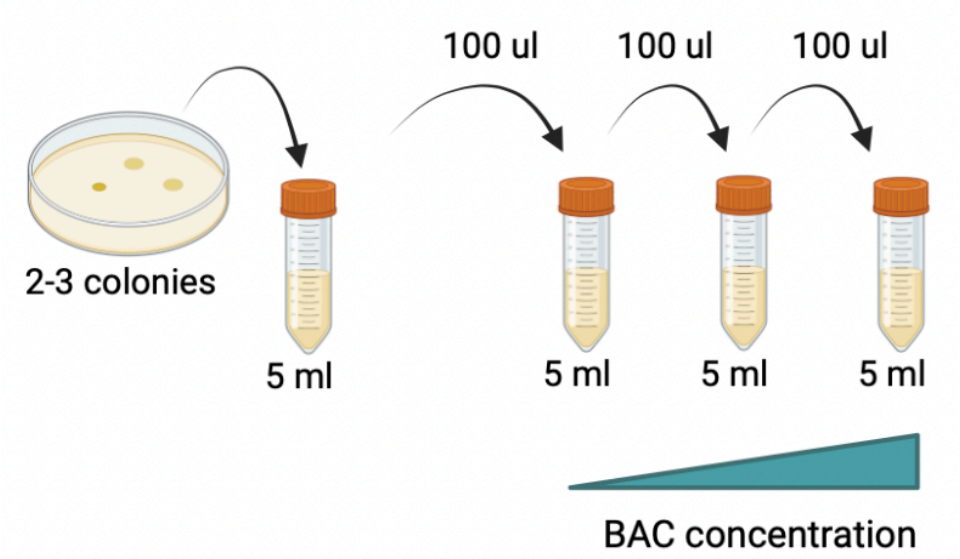


Figure 1 Schematic showing the serial passage method used for the adaptation of the BAC^t strains. Image created using BioRender (www.biorender.com)

Chapter 2: Methods and Materials

2.1 Strains used in study

The strains used for this study have been outlined in Table 1 below.

Bacterial strain	Strain type	Source
K. pneumoniae		
ATCC 700603	Reference strain	Martins lab collection
K. pneumoniae (clinical isolates)		
412412 WT	Clinical isolate	St. James's Hospital
412621 WT	Clinical isolate	St. James's Hospital
302216 WT	Clinical isolate	St. James's Hospital
318418 WT	Clinical isolate	St. James's Hospital
51596E WT	Clinical isolate	St. James's Hospital
50183 WT	Clinical isolate	St. James's Hospital
K. pneumoniae (adapted strains)		
412412 BAC^t	Biocide induced	Martins lab collection
412621 BAC^t	Biocide induced	Martins lab collection
302216 BAC^t	Biocide induced	Martins lab collection
318418 BAC^t	Biocide induced	Martins lab collection
51596E BAC^t	Biocide induced	Martins lab collection
50183 BAC^t	Biocide induced	Martins lab collection
E. coli		
ATCC 25922	Reference strain	Martins lab collection

Table 1 Strains used in this study. Clinical isolates were kindly donated from St. James's Hospital. Benzalkonium chloride tolerance was induced in the adapted strains by passage through increasing concentrations of the biocide on five separate occasions. This adaptation was performed as part of a previous study carried out in the laboratory. K. pneumoniae ATCC 700603 was used as a reference strain for all experiments involving the clinical isolates. E. coli ATCC 25922 was used as a control for antibiotic susceptibility testing when comparing MICs to CLSI guidelines.

The *K. pneumoniae* clinical isolates used for this study were obtained from St. James's Hospital in Dublin. The strains are known to be respiratory isolates, though specific information regarding patient treatment history has not been disclosed due to GDPR. A *K. pneumoniae* reference strain, ATCC 700603, was used as a reference standard for all experiments, and an *E. coli* reference strain, ATCC 25922, was used as a reference for antibiotic susceptibility testing.

2.2 Storage and growth conditions

2.2.1 Culture and storage of bacterial strains

The two types of media used for bacterial cultures in this project were Mueller-Hinton broth (MHB) (2.0 g/L beef infusion solids, 1.5 g/L starch, casein hydrolysate 17.5 g/L; oxoid) and Luria-Bertani broth (LB) (10 g/L tryptone, 5 g/L year extract, 5 g/L NaCl; Sigma). Mueller-Hinton agar (MHA) was also used. Media was sterilised by autoclaving before use.

Freezer stocks were prepared from MHB overnight cultures in 50% (v/v) glycerol and stored at -75° C. These were used to prepare fresh streaks on MHA plates, which were incubated overnight (approx. 18 hours) at 37° C (REVCO ULTIMA Incubator Oven INB200) and subsequently stored at 4° C.

Overnight cultures were prepared by inoculating 5 ml liquid media (MHB or LB) with 2-3 colonies. Media used for overnight cultures was supplemented with benzalkonium chloride unless otherwise specified. Overnight cultures were incubated overnight in a shaking incubator (Labwit Scientific) at 37° C with 200 rpm shaking.

2.2.2 Preparation and storage of antibiotic and biocides

Biocides and antibiotics were prepared as master stocks and diluted to working concentrations from these as needed. The preparation, concentrations and storage conditions of the biocides and antibiotics used are outlined in Table 2 on the next page.

Preparation and storage of biocide and antibiotic stocks

Compound	Source	Solvent	concentration	Storage (°C)
Benzalkonium chloride	Sigma Aldrich	Sterile H_2O	10 mg/ml	25° C
Triclosan	Sigma Aldrich	Ethanol	0.1 mg/ml	4° C
Virkon	Antec International	Sterile H_2O	4% w/v	4° C
Meropenem trihydrate	United States Pharmacopedia	Sterile H_2O	1 mg/ml	-20° C
Tetracycline hydrochloride	Fisher bioreagents	Sterile H_2O	1 mg/ml	-20° C
Amikacin	Thermo-Fisher Scientific	Sterile H_2O	1 mg/ml	-20° C
Colistin sulphate	MP Pharmaceuticals	Sterile H_2O	1 mg/ml	-20° C
Tobramycin	Thermo-fisher Scientific	Sterile H_2O	1 mg/ml	-20° C
Gentamicin	Sigma Aldrich	Sterile H_2O	1 mg/ml	-20° C
Aztreonam	MP pharmaceuticals	DMSO	1 mg/ml	-20° C
Kanamycin	Sigma Aldrich	Sterile H_2O	1 mg/ml	-20° C
Cefpodoxime	Sigma Aldrich	Sterile H_2O	1 mg/ml	-20° C
Chloramphenicol	Sigma Aldrich	Ethanol	1 mg/ml	-20° C

Table 2 Preparation and storage conditions of biocides and antibiotics used in this study. DMSO = dimethyl sulfoxide. All master stocks were diluted to a working concentration of 128 μg/ml in sterile H_2O before being used in experiments, regardless of the solvent used to make up the master stock.

The storage temperatures for the antibiotics listed in the table above indicate the storage temperatures of the master stocks. When diluted to working concentration (128 μg/ml),

working stocks were stored at 4° C prior to use. All working stocks were diluted in sterile H_2O. This was done to ensure that only the antibiotic concentration changed between dilutions.

2.3 Metabolic and phenotypic changes

Experiments were carried out to assess changes inf phenotype and metabolism following adaptations. This included looking at colony morphology of wild-type and BAC^t strains and noting differences in these as a result of adaptation, measuring the growth rate in the presence and absence of BAC, and assessing changes in capsule density and mucoviscosity.

2.3.1 Growth curve

A growth curve was performed to measure the growth rate of the strains over 18 hours in the presence and absence of BAC.

100 µl MHB was added to the wells of a 96-well plate (SARSTEDT). When strains were grown in the presence of biocide, the biocide was diluted in MHB to a concentration corresponding to half the MIC of the strain to be tested and 100 µl added to the appropriate wells.

Overnight cultures grown in MHB were adjusted to 0.5 McFarland standard (0.08-0.1 OD_{600}) in sterile phosphate buffered saline (PBS). 5 µl of the adjusted overnight culture was added to each well of the 96-well plate. Triplicates were performed for each strain. One row of wells was left un-inoculated to act as a sterility control and to be used for blank subtraction.

The plate was left in a microplate reader (Bio-Tek) for 18 hours at 37° C with 200 rpm shaking. An OD_{600} absorbance reading was taken every 15 minutes.

Results were graphed using GraphPad Prism 9.

2.3.2 String test for hypermucoviscosity

Bacteria were streaked on MHA and incubated overnight at 37° C. A sterile loop was applied to a single colony and gently lifted. Strains were defined as having a hypermucoviscous (HMV) phenotype if a string of 5 mm or longer formed.

2.3.3 Ludox® capsule densities

An experiment measuring the capsule densities of the strains pre- and post-adaptation was performed using Ludox® colloidal silica (Thermo-Fisher Scientific), based on a modified version of the protocol developed by Dorman et al. (106).

10 ml overnight cultures grown in LB broth supplemented with BAC were centrifuged at 4000g for 20 minutes in a benchtop centrifuge (Eppendorf 5810 R) at 4° C. The pellet was resuspended in PBS and the suspension adjusted to OD_{600} 4.0.

Ludox® colloidal silica was diluted to a final volume of 500 µl in sterile water to concentrations varying in density by 0.01 g/cm^3 to form micro-gradients. The following formula was used to calculate the amount of Ludox® per dilution: $V = \frac{\rho - 0.99}{0.22}$, where V = the volume of Ludox needed for the dilution, ρ = final density in g/cm^3, 0.22 = density of Ludox®, 0.99 = density of water.

100 µl of the bacterial suspension was carefully applied to the top of each micro-gradient in.a 1.5 ml microfuge tube. The gradients were centrifuged at max speed (13,000g) for 10 minutes in a benchtop microfuge (Eppendorf 5424 R).

The centrifuged gradients were read by eye and the density was defined as the lowest density value (g/cm^3) at which the suspension remained above the gradient line.

2.4 Antimicrobial susceptibility testing by broth microdilution

100 µl MHB was added to columns 1-11 of a 96-well plate. 200 µl of the compound to be tested was added to column 12. Using a p200 multi-channel pipette, the compound of interest

was serially diluted by pipetting 100 µl from column 12 to column 11, then from column 11 to column 10, repeating this until reaching column 3 and discarding the remaining liquid.

Columns 3 to 12 contain the compound of interest at increasing concentrations, while columns 1 and 2 contain no compound. Column 1 was left un-inoculated to act as a sterility control, and column 2 was inoculated to act as a growth control.

5 µl of an overnight culture grown in MHB was adjusted to 0.5 McFarland standard (OD_{600} 0.08-0.1) in sterile PBS and each well from column 2 through to column 12 were inoculated with the suspension. The plate was incubated at 37° C overnight (16-18 hours) without shaking and read by eye the following day. The MIC was defined as the lowest concentration at which no growth could be detected by the naked eye.

In some cases, the protocol was adjusted to fit the conditions to be tested.

2.4.1 Benzalkonium chloride susceptibility testing

The MIC was performed as described above using a starting concentration of 800 µg/ml BAC.

2.4.2 Benzalkonium chloride minimum bactericidal concentration

In order to measure the minimum bactericidal concentration (MBC), an MIC plate was prepared the day before, as described above, and incubated overnight.

The following day, 100µl MHB was added to each well of a new 96 well plate. A plate replicator containing 96 prongs, corresponding to the wells of a 96-well plate, was sterilised by submerging in ethanol and flaming using a Bunsen burner. The sterile plate replicator was then placed onto the MIC plate so that the prongs of the replicator reached the bottom of each well. The plate replicator was then moved to the new MBC plate, transferring cells from the wells of the MIC plate to the corresponding wells of the MBC plate. This method of transferring cells from the MIC plate to fresh media in an MBC plate allows any cells that may still be alive, but have inhibited growth in the presence of the tested compound, to grow

once the stress of the compound is removed. By replicating the whole MIC plate from the previous day, the MIC and MBC can easily be compared by simply comparing the wells of the two plates.

The plate was incubated at 37° C overnight and read the following day. Similarly to an MIC, the MBC was defined as the lowest concentration at which no growth could be detected by the naked eye.

2.4.3 Antibiotic susceptibility testing

The MIC was performed as above with starting concentrations of 128 μg/ml, corresponding to CLSI (Clinical Laboratory Standards Institute) standard concentrations. Antibiotic stocks were diluted as per the preparation method stated in Table 2.

Plates were incubated at 37° C for 16-18 hours and results were compared to CLSI breakpoints to determine the sensitivity profile. In addition to the *K. pneumoniae* strains used in this study, and *E. coli* reference strain was used as a control when comparing with CLSI breakpoints. Where no CLSI breakpoints exist for K. pneumoniae for a given antibiotic against *K. pneumoniae*, European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines were used instead.

2.4.4 Susceptibility tests for other disinfectants

Disinfectant start concentrations were as follows: 16 μg/ml triclosan, 4% (w/v) virkon, 100% (v/v) ethanol

As both triclosan and virkon are light sensitive compounds, the broth microdilution was performed with the lights of the biological safety cabinet (BSC) turned off, and the 96-well plates were wrapped in tinfoil before transferring to the incubator.

2.4.5 MICs of efflux pump inhibitors

In order to determine the effects of efflux pump inhibitors on BAC tolerance, broth microdilutions were performed for three phenothiazine EPIs in order to determine the concentrations needed for future assays. The phenothiazines used were PMZ, CPZ, and TZ. The starting concentrations for all phenothiazines were chosen to be 800 μg/ml.

As phenothiazines are light sensitive, the assay was carried out in low-light conditions and the plates wrapped in tinfoil before incubating at 37° C overnight.

2.4.6 Effect of efflux pump inhibitors on BAC tolerance

MHB was added to columns 1-11 of a 96-well plate and BAC added to the wells of column 12. Serial dilutions were performed on wells 12 through to well 3, as described above. The EPI to be tested was then added to columns 3-12 to a final concentration corresponding to 0.5 the MIC of the EPI, based on the results obtained in 2.4.5 above.

2.5 Changes in accumulation and efflux following adaption

Assays were carried out to assess the permeability of the *K. pneumoniae* strains. An accumulation assay using Hoechst fluorescent dye was performed using a protocol developed by Paixão et al. (107).

2.5.1 EtBr cartwheel assays

A cartwheel assay using ethidium bromide (EtBr) was performed to asses efflux and permeability, following the protocol developed by Martins et al. (108)

20 ml LB-agar plates were prepared supplemented with EtBr to concentrations of 0 μg/ml, 0.5 μg/ml, 1 μg/ml, 1.5 μg/ml, 2 μg/ml and 2.5 μg/ml. Preparation was performed in low-light conditions as EtBr is light sensitive.

Overnight cultures were adjusted to 0.5 McFarland standard in sterile PBS. With a sterile swab, a single stroke of each adjusted culture was placed in a predetermined position on an agar plate of each EtBr concentration.

Plates were covered in tinfoil to protect from light and incubated for 16-18 hours in at 37° C (REVCO ULTIMA Incubator Oven INB200).

Following incubation, the plates were read and photographed in a transilluminator to determine the minimum concentration of EtBr at which fluorescence was observed.

2.5.2 Hoechst concentration optimisation

The concentration of Hoechst dye used for the fluorescence assays was determined by performing a serial dilution in a black clear bottom 96 well plate (Corning), starting at 8 μM dye and measuring fluorescence in the reference strain ATCC 700603 over 30 minutes in a fluorescent plate reader (BioTek Synergy H1 Hybrid Reader). The concentration that produced the best fluorescent signal without impacting readability was chosen for subsequent assays. To ensure that viability was not impacted, a drop from each well was spotted on an agar plate and incubated over night at 37° C (REVCO ULTIMA Incubator Oven INB200).

2.5.3 Accumulation using Hoechst fluorescent dye

The following assay was carried out following a protocol developed by Paixão et al. (107). CCCP was used as an efflux pump inhibitor on the basis that CCCP acts as an uncoupler of oxidative phosphorylation, thus removing energy from RND-type efflux pumps , which are the main types of efflux pumps in *Klebsiella spp.* (65,82).

5 ml overnight cultures grown in LB broth were inoculated into 25 ml fresh LB broth in 250 ml conical flasks, adjusted to an OD_{600} of approximately 0.1. The cultures were incubated at 37° C with 200 rpm shaking and reading taken regularly until a final OD_{600} of 0.6 was reached.

Overnight cultures were centrifuged at 4000g and 4° C for 20 minutes in an Eppendorf 5810 R centrifuge (Eppendorf) and adjusted to OD_{600} 0.3 in sterile PBS. 160 µl PBS was added to the first column of a clear bottom black 96-well plate (Corning) to act as a negative control, and a heat-inactivated (HIA) positive control strain was added to the second column. 160 µl of the adjusted cultures were then added to the remaining wells, with four replicates performed per strain. Right before the start of the assay, 40 µl of a 10 µM Hoechst dye stock was added to each well, for a final concentration of 2 µM.

The plate was read in a BioTek Synergy H1 fluorescent microplate reader for 30 minutes with the following parameters: 37° C, read every 60 seconds, 355nm excitation, 460nm emission. After 30 minutes the plate was removed from the plate reader and CCCP immediately added to a final concentration of 10 µM, before reading again for an additional 30 minutes with the same parameters as previously.

Results were downloaded onto an Excel document and graphed in GraphPad Prism.

2.5.4 RT-qPCR primer design

Primers were designed using the National Centre for Biotechnology Information (NCBI) GenBank primer design tool using the following parameters:

PCR product size: 60 to 200 bp
Primer melting temperature (T_m): 58.0° C to 62.0°C, Max T_m difference = 2° C
PCR product T_m: 70° C to 90° C
Primer size: 18 to 26 bp, optimal 22 bp
Primer GC content (%): min 30.0%, max 70.0%

Sequences used for primer design were chosen from the GenBank gene database. Primers were ordered from Integrated DNA Technologies (www.eu.idtdna.com).

Primers used for this study are shown in Table 3 below on page 24.

2.5.5 Primers used for this study

RT-qPCR primers			
Gene	**primer sequence (5'-3')**	**Tm (°C)**	**GC content (%)**
ramA fw	CGATTCGCAACAGACCTTTACC	56.2°C	50.0 %
ramA rv	ACTGTGGTTCTCTTTGCGGTAG	57.1° C	50.0 %
acrA fw	GACTTGGTTTGTTCTGATGGCG	56.7° C	50.0 %
acrA rv	CAGGTAAAAGCGCAGGAAGTAG	55.8° C	50.0 %
gyrA fw	GTGTCGTTGGTGACGTAATCG	56.0° C	52.4 %
gyrA rv	GTCGATGGAACCAAAGTTACCC	55.8° C	50.0 %

Table 3 Primers used for RT-qPCR in this study. Primers were designed using the GenBank primer design tool

The primers used in this study are displayed in Table 3 above. Primers were ordered from Integrated DNA Technologies (www.eu.idtdna.com)

2.5.6 RNA extraction

Overnight cultures used for RNA extraction were prepared in 10ml of LB broth supplemented with BAC. 2ml of the overnight culture was spun at max speed (13,000g) in a benchtop microfuge (Eppendorf) and supernatant decanted, leaving the pellet to be used for RNA extraction.

RNA extractions were performed using the New England Biolabs Monarch total RNA extraction kit as per manufacturer's instructions.

RNA concentrations were measured using a DeNovix DS-11 spectrophotometer. RNA samples were stored at -20° C.

2.5.7 RT-qPCR of efflux genes

RT-qPCRs were performed using the Promega GoTaq® 1-Step RT-qPCR kit according to manufacturer's instructions. Reactions were prepared in triplicates in the wells of an Applied Biosystems MicroAmp™ Optical 96-well Reaction Plate (Thermo-Fisher Scientific).

The 1-step RT-qPCR reaction was performed using an Applied Biosystems OneStepPlus Real-Time PCR system

Thermo cycling was set to the following conditions:

Step	Cycles	Temperature	Time
Reverse transcription	1	37° C	15 minutes
Reverse transcriptase inactivation and DNA polymerase activation	1	95° C	10 minutes
Denaturation	40	95 ° C	10 seconds
Annealing		60° C	30 seconds
Extension		72° C	30 seconds

Table 4 thermocycling conditions used for 1-Step RT-qPCR. The initial reverse transcription step converts RNA to cDNA. Data was collected during each annealing step.

Chapter 3: Antimicrobial susceptibility

3.1 Introduction

Exposure of bacteria to biocides such as BAC has been shown to cause decreased susceptibility to antimicrobial agents, likely due to an overlap in resistance mechanisms between the biocide and other antimicrobial compounds (85,103,104). A decrease in susceptibility may be caused by changes in membrane permeability, expression of efflux pump, or the acquisition of efflux pump genes among others. Concerns have been raised that exposure to benzalkonium chloride may lead to cross resistance with antibiotics (85,92).

In this chapter, *K. pneumoniae* strains were assessed for changes in susceptibility to BAC, as well as other disinfectants and different classes of antibiotics. The broth microdilution method was used to determine the MICs and MBCs and results were analysed to determine changes in susceptibility following adaptation of the strains to BAC. As mentioned in Chapter 1.9, there have been speculations around the emergence of increased antibiotic resistance following repeated BAC exposure. Therefore, a variety of antibiotics from different classes were tested to asses changes in antibiotic susceptibility following adaptation. Additionally, the strains were assessed for changes in susceptibility to other biocides.

3.2 Results

3.2.1 Assessing the susceptibility of *Klebsiella pneumoniae* to benzalkonium chloride

Susceptibility testing for benzalkonium chloride was performed for all strains, including the *K. pneumoniae* reference strain ATCC 700603. Both the MIC and MBC was determined for all strains.

3.2.1.1 Assessing the changes in benzalkonium chloride minimum inhibitory concentrations following adaptation

In order to determine the BAC MICs of all *K. pneumoniae* strains, broth microdilutions were performed for each strain. The results displayed in Table 5 below shows the MIC values obtained from three replicates performed on independent days. A four-fold change in MIC or greater was defined as being a true change in MIC, as a two-fold deviation is within the normal technical variability for antimicrobial susceptibility testing (109).

Benzalkonium chloride MICs (μg/ml)

Bacterial strains	Wild-type	BAC^t
412412	25	100
416621	12.5	100
302216	12.5	100
318418	12.5	100
51596E	12.5	100
50183	12.5	100
ATCC 700603	25	NA

Table 5 Minimum inhibitory concentrations for benzalkonium chloride (μg/ml). MIC values were obtained from three biological replicates, which were performed on three separate days. NA = Not Applicable.

All clinical isolates had an MIC of 12.5 μg/ml before adaptation, except for isolate 412412, which had an MIC of 25 μg/ml. All clinical isolates had the same MIC (100 μg/ml) post adaptation. Interestingly, the reference strain had a higher MIC than all but one of the WT clinical isolates.

3.2.1.2 Assessing the changes in benzalkonium chloride minimum bactericidal concentrations following adaptation

An assay was performed to determine the MBC of all strains used for the study. The results of this are shown in Table 6 on page 28.

The MBC of the wild-type strains were generally greater than the MIC and comparable to that of the BAC^t strains. In the BAC^t strains, the MBC was the same as the MIC (100 μg/ml for all strains).

Benzalkonium chloride MBCs (μg/ml)

Bacterial strains	WT	BAC^t
412412	50	100
416621	100	100
302216	100	100
318418	50	100
51596E	100	100
50183	50	100
ATCC 700603	50	NA

Table 6 Minimum bactericidal concentrations for benzalkonium chloride (μg/ml). Values represent the most consistent results based on three separate experiments performed on different days. NA = Not Applicable.

3.2.2 Investigating the effects of increased benzalkonium chloride tolerance on antibiotic susceptibility

MICs of selected antibiotics were performed using the broth microdilution method in order to determine the effects of BAC adaptation on antibiotic susceptibility. The results of these microdilutions are displayed in Table 7 on page 29. values shown are the values obtained from the independent replicates. Susceptibilities were determined by comparing the results obtained to CLSI breakpoints, and EUCAST breakpoints in the case of kanamycin. A four-fold change in MIC or greater was defined as being a true change in MIC.

Of the six WT clinical isolates, four were MDR based on the results obtained, and of the six BAC^t strains, only two were MDR. Strains 318418 WT and 50183 WT, which were MDR prior to adaptation, lost this phenotype following adaptation due to their loss of resistance/intermediate resistance to colistin.

Antibiotic MICs (µg/ml)

β-lactams								
	412412	**412621**	**302216**	**318418**	**51596E**	**50183**	**ATCC**	**E.coli**
MEM	1 (1)	1 (0.5)	0.5 (0.5)	1 (0.5)	2 (1)	0.5 (1)	0.5	0.5
AZ	2 (0.5)	2 (2)	2 (1)	**128** (0.5)	**128 (128)**	**128 (128)**	**128**	0.5
CFPD	4 (0.5)	4 (**8**)	**16** (4)	**128** (1)	**128 (128)**	**128 (64)**	**32**	0.5
Aminoglycosides								
	412412	**412621**	**302216**	**318418**	**51596E**	**50183**	**ATCC**	**E.coli**
AK	2 (1)	2 (1)	2 (2)	4 (1)	**16 (16)**	2 (2)	1	4
CN	0.5 (NG)	1 (0.5)	1 (1)	0.5 (0.5)	**64 (64)**	0.5 (0.5)	**16**	2
K	2 (1)	1 (1)	2 (1)	1 (0.5)	**32 (32)**	2 (1)	**32**	8
TOB	0.5 (NG)	0.5 (0.5)	0.5 (1)	0.5 (0.5)	**32 (32)**	1 (0.5)	**16**	1
Polymyxins								
	412412	**412621**	**302216**	**318418**	**51596E**	**50183**	**ATCC**	**E.coli**
COL	0.5 (**8**)	1 (0.5)	**16** (4)	2 (0.5)	1 (0.5)	**8** (0.5)	**16**	0.5
Chloramphenicol								
	412412	**412621**	**302216**	**318418**	**51596E**	**50183**	**ATCC**	**E.coli**
C	**32 (64)**	**64 (64)**	**64(128)**	**64 (64)**	**128 (32)**	**32 (64)**	**32**	4
Tetracyclines								
	412412	**412621**	**302216**	**318418**	**51596E**	**50183**	**ATCC**	**E.coli**
TET	**4 (8)**	**16 (16)**	**32** (8)	**16 (8)**	**32 (128)**	**32 (128)**	**16**	**2**

Table 7 Minimum inhibitory concentrations for selected antibiotics measured by broth microdilution. Values in brackets represent the MIC post-adaptation (BAC^t). Values in bold indicate resistance, while underlined values indicate intermediate resistance. Values marked in red indicate a 4-fold change or greater.
MEM = Meropenem, AZ = Aztreonam, CFPD = cefpodoxime, AK = Amikacin, CN = Gentamicin, K = Kanamycin, TOB = tobramycin, COL = Colistin, C = Chloramphenicol, TET = Tetracycline, NG = no growth

All WT strains showed resistance or intermediate resistance to at least one β-lactam antibiotic, with all being resistant or intermediately resistant to cefpodoxime. Only three of the six BAC^t clinical isolates were resistant to at least one β-lactam antibiotic, and one, 302216 BAC^t showed intermediate resistance to cefpodoxime. Two strains (412412 and 318418) became susceptible to cefpodoxime following adaptation, and 318418 became susceptible to aztreonam. All strains were susceptible to meropenem, except for the wildtype strain 51596E, which showed intermediate resistance at 2 μg/ml.

Strain 51596E was the only strain resistant to all aminoglycosides (amikacin, gentamicin, tobramycin and kanamycin). An MIC for gentamicin and tobramycin could not be determined for strain 412412 BAC^t as no growth was present in any of the wells containing the antibiotic, suggesting that the MIC is likely lower than the 0.5-128 μg/ml range tested.

Little change in susceptibility was observed for most antibiotic, with the exception of Colistin, where a change was observed for four different strains, three of which involved a change in susceptibility profile. Strain 412412 showed a change from susceptible (0.5 μg/ml) to resistant (8 μg/ml) post adaptation. Strains 318418 and 50183 showed a change from intermediate to susceptible (2 μg/ml to 0.5 μg/ml) and resistant to susceptible (8 μg/ml to 0.5 μg/ml) respectively. Strain 302216 showed a 4-fold decrease in MIC, from 16 μg/ml to 4 μg/ml, though while this does indicate an increase in susceptibility, it does not confer a clinically relevant change in susceptibility, as the MIC is still above the threshold for resistance.

All strains were resistant to the tetracycline and chloramphenicol, both before and after adaptation.

3.2.3 Investigating the effects of increased benzalkonium chloride tolerance on susceptibility to other disinfectants

Broth microdilutions were performed for each strain for the disinfectants triclosan, Virkon, and ethanol to determine their MICs. Table 8 on page 30 shows the data obtained from three independent replicates.

Very little variation in MIC was observed between the strains. Triclosan MICs ranged from 1 μg/ml to 2 μg/ml, Virkon MICs ranged from 0.25% w/v to 0.5% w/v, and ethanol MICs ranged from 6.25% v/v to 12.5% v/v. A slight reduction in ethanol MIC was observed in all clinical isolates following adaptation, except for strain 302216 for which no change was observed.

Disinfectant MICs (μg/ml)

	Triclosan (μg/ml)		**Virkon** (% w/v)		**Ethanol** (% v/v)	
Strains	**WT**	**BAC^t**	**WT**	**BAC^t**	**WT**	**BAC^t**
412412	2	1	0.5	0.25	12.5	6.25
412621	1	2	0.5	0.25	12.5	6.25
302216	1	1	0.5	0.25	6.25	6.25
318418	2	2	0.5	0.25	12.5	6.25
51596E	2	1	0.5	0.5	12.5	6.25
50183	1	1	0.25	0.25	12.5	6.25
700603	1	NA	0.5	NA	6.25	NA

Table 8 Minimum inhibitory concentrations of triclosan, Virkon, and ethanol, measured by the broth microdilution method. Triclosan concentrations are measured in μg/ml, Virkon as % (w/v) and ethanol as % (v/v). NA = Not Applicable

3.3 Discussion

The results obtained in 3.2.1 show that repeated exposure to BAC can increase tolerance in clinical isolates of *K. pneumoniae.* While an increase in MIC was observed for the BAC^t strains relative to their corresponding WT strain, less difference was seen in MBC, with all WT strains having exhibiting an MBC similar to that of the corresponding BAC^t strains. The BAC^t MBCs corresponded to the MICs for all BAC^t strains. Interestingly, strains 412412 WT and ATCC 700603, which had the greatest MICs of the non-adapted strains, only showed a 2-fold change between the MIC and MBC. Overall, it appears that adaptation to BAC through continuous exposure increases the concentration of BAC required to inhibit growth of *K. pneumoniae*, as demonstrated by the increase in MIC, but not for killing.

It does not appear that there is a correlation between increased tolerance to BAC and antibiotic resistance, as most strains showed similar BAC MICs, but all had varying levels of antibiotic resistance, and a greater BAC MIC after adaptation did not correspond to greater resistance to antibiotics. However, it cannot be excluded that tolerance may have an impact on susceptibility to antibiotics not tested in this study. For example, it has been demonstrated that exposure of *E. coli* and *Chronobacter sakazakii* to sub-inhibitory concentrations of BAC may cause reduced susceptibility to ciprofloxacin (110,111), which was not included in this study. Some changes were observed in susceptibility to colistin, but both changes from susceptible to resistance, and resistant to susceptible were observed, so it is unlikely that a single change as a result of BAC exposure was the cause of this.

The reference strain ATCC 700603 showed a greater level of antibiotic resistance when compared to the clinical isolates used in this study. This was expected, as ATCC 700603 is an MDR strain. An MDR reference strain was chosen in favour of a more susceptible reference strain, as a high level of antibiotic resistance is more in line with the clinical picture when discussing *Enterobacteriaceae*, which tend to have a high level of antibiotic resistance.

Chapter 4: Phenotypic and metabolic changes

4.1 Introduction

Experiments were carried out to assess what, if any, phenotypic and metabolic changes occur in cells following adaptation to BAC.

It has been demonstrated that induced tolerance to BAC may change bacterial morphology and ultrastructure, with cells appearing thinner and longer, with a more rough looking cell surface under scanning electron microscopy (SEM) and transmission electron microscopy (TEM) (86). In this chapter, changes in the cell surface were assessed by performing a string test for hypermucoviscosity and a capsule density assay for changes in capsule production following adaptation.

A bacterial growth curve was also performed to determine changes in growth rate and survival of wild-type vs. BAC^t strains.

4.2 Results

4.2.1 Observing changes in colony morphology following adaptation to benzalkonium chloride

Changes in colony morphology and mucoidy was observed by eye on plates grown overnight on MH agar. It was observed that the BAC^t strains were generally less mucoid and appeared drier when compared to their corresponding WT strains. Colonies of WT strains were also larger than those of the corresponding BAC^t strains. The exception to this was strain 50183, where no change in colony morphology was obvious between the WT and adapted strain.

Though most changes were minimal, the most obvious changes in colony morphology were observed in strains 302216, 318418, and 51596E. An example of visible changes in colony morphology is provided in Figure 2 on page 34.

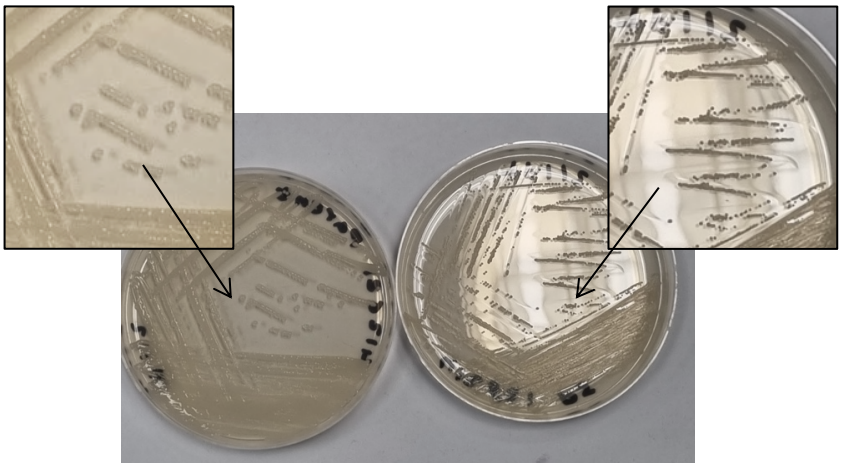
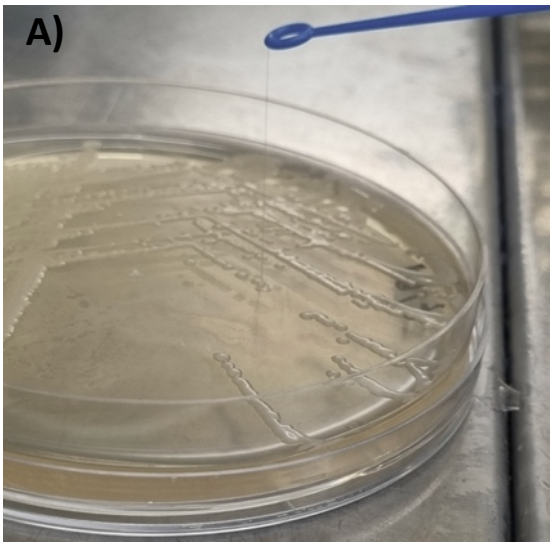


Figure 2 412621 WT (left) and 412621 BACt (right). The colonies formed by the WT strain grow noticeably larger colonies compared to the BACt strain. The colonies formed by 412621 BACt are also drier than the WT colonies, which were slightly mucoid.

4.2.2 Assessing strains for hypermucoviscosity using string testing



B)

	WT	BAC^t
412412	3 mm	1 mm
412621	1 mm	0 mm
302216	>20 mm	1 mm
318418	>10 mm	0 mm
51596E	3 mm	0 mm
50183	1 mm	1 mm
ATCC	1 mm	NA

Figure 3 a) positive string test for strain 302216 WT, showing formation of a stringer >5 mm when touched with a sterile loop. b) table showing string test results for all strains, comparing results pre- and post-adaptation. Four of the WT strains (412621, 302216, 318418, and 51596E) were mucoid, with two strains (302216 and 318418) being HMV. None of the BAC^t strains were mucoid or HMV.

A string test was performed to assess the strains for hypermucoviscosity (see Figure 3.a on page 34). Strains which formed a string >5mm in length when a colony was touched with a sterile loop were considered to exhibit a HMV phenotype. Four WT strains were mucoid, with two strains (302216 and 318418) being HMV. None of the BAC^t strains had a mucoid phenotype. The results of the string test are displayed in Figure 3.b on page 34 above.

4.2.3 Observing the effects of benzalkonium chloride on growth rates over 18 hours

Strains were grown in the presence and absence of BAC at 37° C for 18 hours. The strains grown in the presence of BAC were grown with half the MIC, based on the results obtained in Chapter 3.2.1.1.

Results of the 18 hour growth curves are shown in Figure 4 on page 36, and in Figure 5 on page 37. Results are based on the mean values of three technical replicates from one representative experiment. It was not possible to perform multiple independent experiments due to time constrains.

When comparing the growth rates of BAC^t strains relative to their wild-type in the absence of BAC, most strains showed an elongated lag phase, this being most apparent in strain 412621 BAC^t. All BAC^t strains reached a similar OD_{600} to the corresponding wild-type strain during stationary phase, except for 412621 which performed considerably poorer, only reaching a maximum OD_{600} of 0.5, compared to 0.9 for the wild-type. However, strain 302216 BAC^t performed slightly better than 302216 WT, reaching an OD_{600} just above 1.0, where the wild-type only reached a maximum OD_{600} of slightly above 0.8.

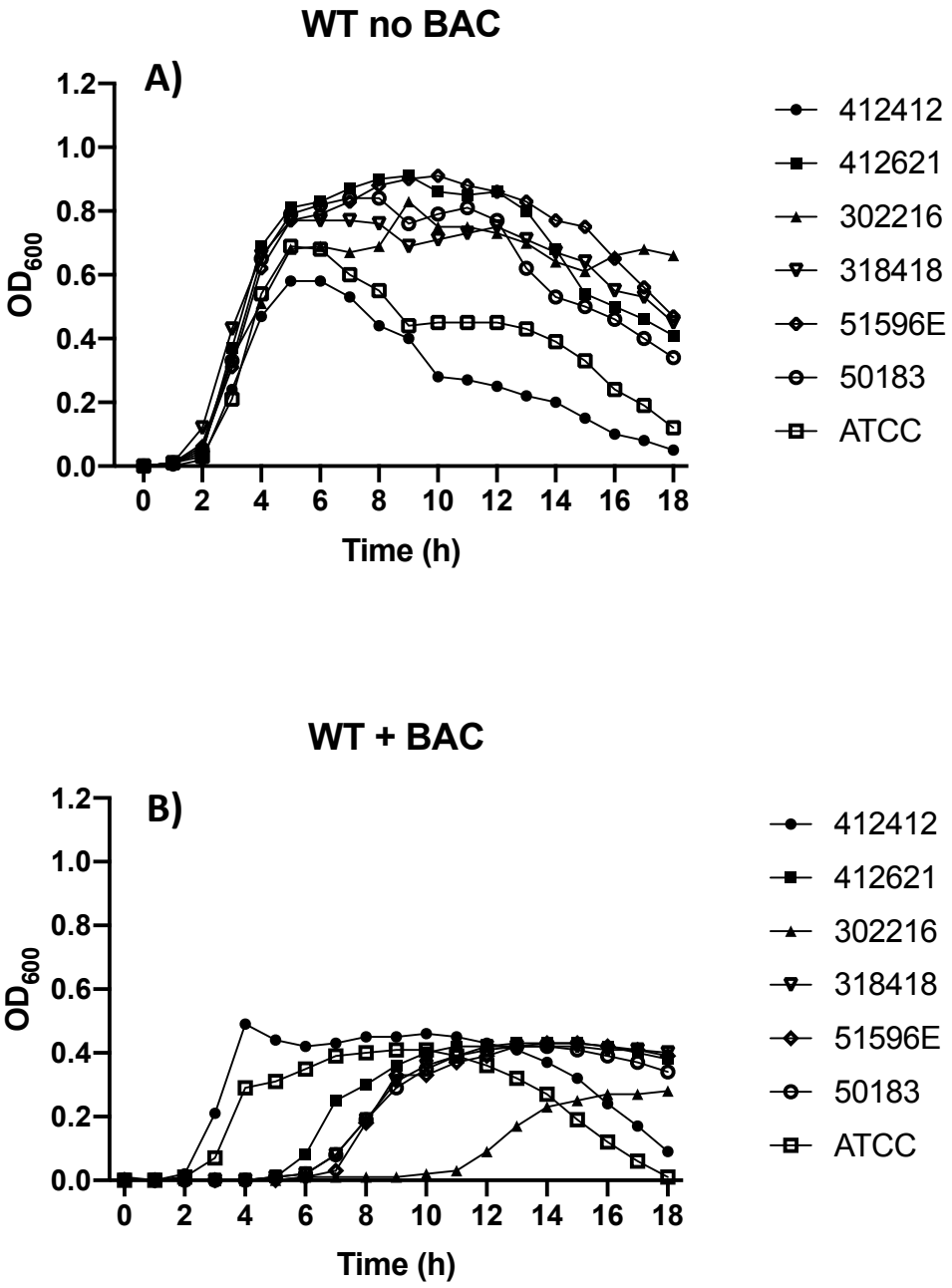


Figure 4 Graphs showing growth rates of wild-type strains in the absence (a) and presence (b) of BAC at 0.5 MIC. The data shown represents the mean values of three technical replicates from one experiment.

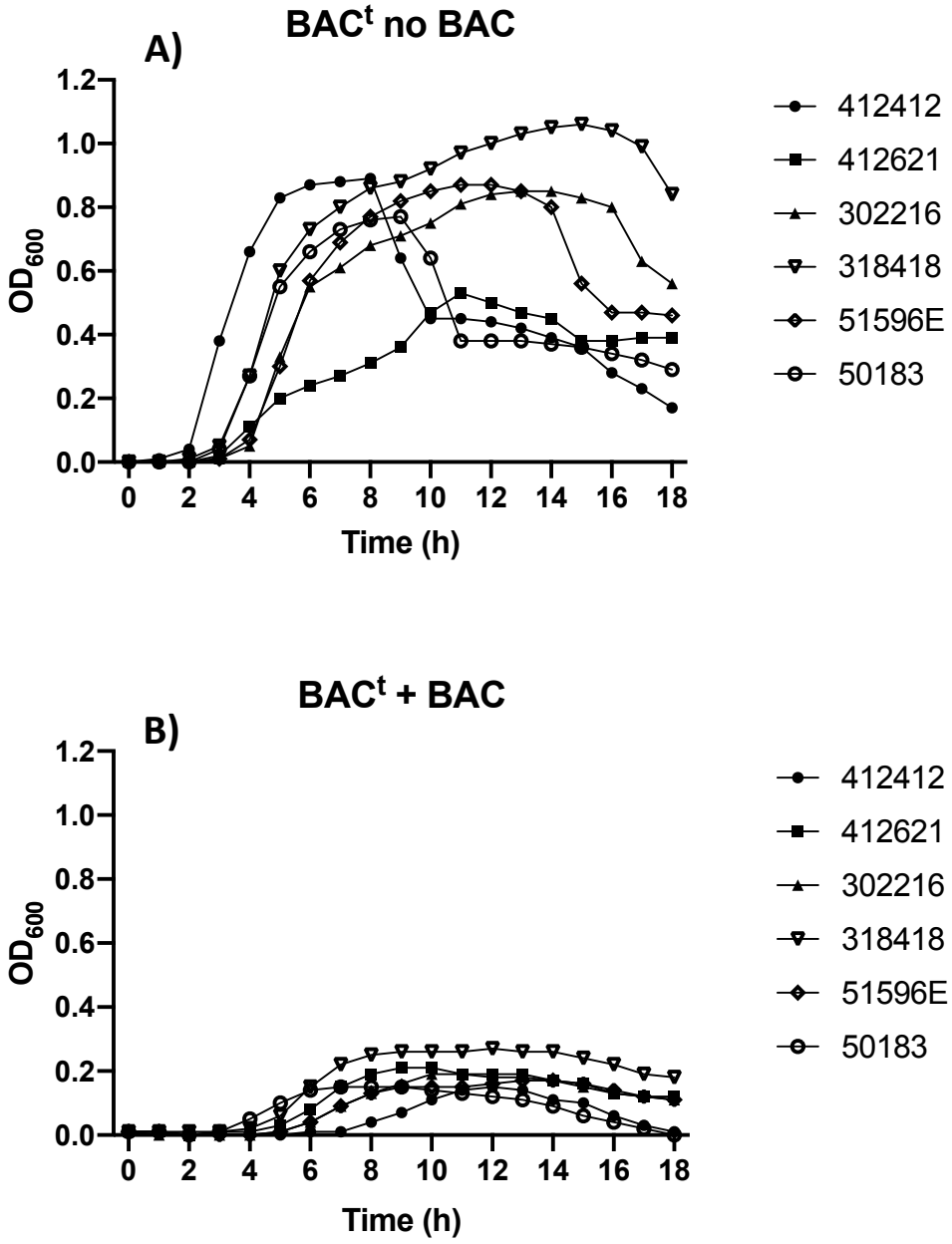


Figure 5 Graphs showing growth rates of BAC^t strains in the absence (a) and presence (b) of BAC at 0.5 MIC. The data shown represents the mean values of three technical replicates from one experiment.

All wild-type strains except for 412412 performed better than the reference strain ATCC 700603 in the absence of BAC. However these two strains performed the best in the presence of BAC, reaching a similar maximum OD_{600} to the other strains while having a shorter lag

phase. All BAC^t strains grew at a similar rate in the presence of BAC, though they all had a longer lag phase and reached lower a lower optical density when compared to the wild-type strains. Interestingly, the strain 412412 BAC^t performed the worst of all the tolerant strains, but performed the best of the wild-type strains.

4.2.4 Measuring changes in capsule density following adaptation

A Ludox® capsule density assay was performed to measure the changes in capsule densities following adaptation. The results of this assay are shown in Figure 6 below.

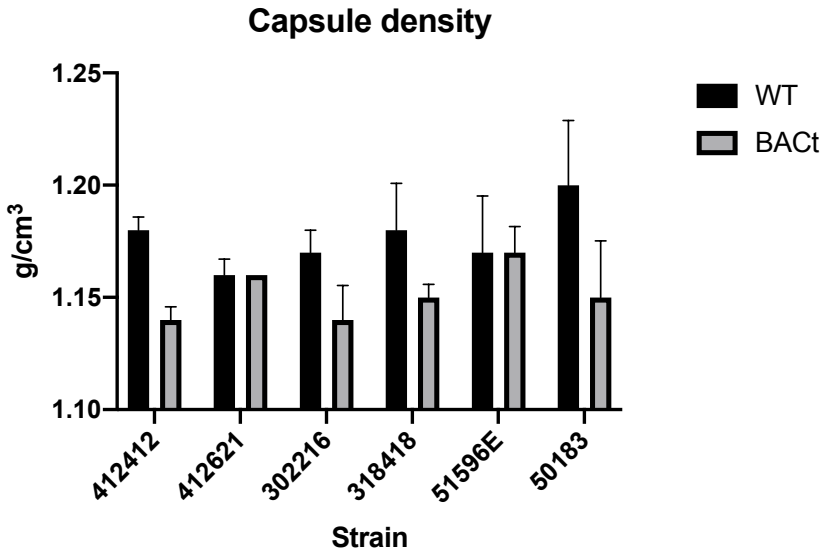


Figure 6 Graph showing changes in capsule densities of clinical isolates before and after adaptation to BAC. Wild-type strains generally had a higher capsule density when compared to the adapted strains, with the exception of strains 412621 and 51596E. Data is based on the mean values of three separate experiments. Error bars represent the standard error of the mean.

Capsule densities were lower in all adapted strains relative to the wild-type, except for strains 412621 and 51596E, for which the densities post adaptation remained the same as for the wild-type. The greatest changes in density were observed for strains 412412 with a change from 1.18 g/cm^3 (WT) to 1.14 g/cm^3 (BAC^t) and 50183 with a change from 1.20 g/cm^3 (WT) to 1.15 g/cm^3 (BAC^t).

4.3 Discussion

It is apparent that a fitness cost is associated with BAC tolerance, as observed by the poorer growth rates of BAC^t strains relative to the WT. None of the wild-type strains reached a maximum OD_{600} greater than 0.5 in the presence of BAC, indicating that the cells struggle to grow in the presence of the biocide. This was even more apparent in the BAC^t strains, where all strains performed poorer in the presence of BAC than the wild-type strains. While the growth rate of the BAC^t strains when exposed to BAC were lower, they are still growing at much higher concentrations of BAC (50 μg/ml for all strains) than the wild-type strains (between 6.25 μg/ml and 12.5 μg/ml), which is further evidence that a fitness cost is associated with tolerance to higher BAC concentrations.

Altered colony morphology as a result of adaptation was noted by the slightly smaller colonies produced by the BAC^t strains, in particular the very noticeably smaller colonies produced by 412412 BAC^t. This appears to be consistent with the findings in 3.2.4 showing decreased capsule production in BAC^t strains. Additionally, the change in mucoviscosity and hypermucoviscosity could also be explained by a change in capsule production. Changes in capsule densities also seem to be consistent with previous studies showing changes in cell morphology under scanning electron microscopy (SEM) following BAC adaptation in *K. pneumoniae* (86).

As not all mucoid strains are HMV, a string test was performed to indicate which strains possessed this phenotype. A string test served only to determine whether or not a strain is HMV, and cannot be used to quantitatively determine the extent of mucoviscosity (112). Since the HMV phenotype is associated with virulence, it is possible that the wild-type strains 302216 and 318418, which lost this phenotype following adaptation are less virulent . It has also been found that *Salmonella* strains treated with commercial biocides showed less invasiveness in Caco-2 cells (113). An infection assay could be performed to confirm this.

Chapter 5: Permeability and efflux

5.1 Introduction

Efflux is known to be implicated in BAC tolerance. MICs of different efflux pump inhibitors (EPIs) were determined by broth microdilution and 0.5 x EPI MICs was used to assess the effects of EPIs on BAC MIC. An EtBr cartwheel assay was performed to assess differences in permeability between the WT and BAC^t strains, and later an accumulation assay using Hoechst fluorescent dye was performed to investigate changes in accumulation pre- and post-adaptation.

Finally, since BAC exposure has been shown to select for RamA over expressing mutants (73), RT-qPCR was performed on all clinical isolates to measure the expression of *ramA* relative to the reference strain ATCC 700603. Additionally, RT-qPCR was also performed for the *acrA* gene, which encodes part of the AcrAB-TolC efflux pump. This was done based on the knowledge that *acrA* expression is linked with RamA activity and the link between acrA expression and BAC susceptibility demonstrated by Park and Yoon (2023) (74).

5.2 Results

5.2.1 Establishing phenothiazine minimum inhibitory concentrations

The broth microdilution method was used to observe the effects of adaptation to BAC on the MICs of the EPIs (phenothiazines) against the studied strains. The results of these are displayed in Table 9 on page 40.

Both the WT and BAC^t strains all showed low susceptibility to EPIs, with adaptation having no impact on susceptibility, as most strains had the same MIC for each EPI before and after adaptation. MICs also varied very little between the EPIs tested, with most having MICs between 200 μg/ml and 400 μg/ml, the only exception being the MIC of PMZ for strain 302216 WT, which was 100 μg/ml.

	Phenothiazine MICs (μg/ml)		
	CPZ	**PMZ**	**TZ**
412412			
WT	400	200	400
BAC^t	400	200	400
412621			
WT	400	400	400
BAC^t	400	400	800
302216			
WT	200	100	200
BAC^t	400	200	400
318418			
WT	400	400	400
BAC^t	400	200	400
51596E			
WT	200	200	200
BAC^t	200	200	200
50183			
WT	400	200	400
BAC^t	400	400	400
ATCC 700603			
Reference strain	400	400	400

Table 9 Table showing the minimum inhibitory concentrations of phenothiazines in μg/ml. The results are based on three different replicates performed on separate days. CPZ = chlorpromazine, PMZ = promethazine, TZ.= thioridazine

5.2.2 Analysing the effects of efflux pump inhibitors on benzalkonium chloride susceptibility

MICs were performed by broth microdilution using varying concentrations of BAC with half the MIC of the EPI to be tested. EPI MICs were based on the results from 5.2.1 above. The results are listed in Table 10 on page 41.

Little to no change was observed in BAC susceptibility for the WT strains, except for 50183 WT, where no growth was observed in the wells when grown in the presence of CPZ and TZ. The BAC^T strains showed a reduction in susceptibility in the presence of CPZ and TZ, with the exception of strains 50183 BAC^T, where no change was observed in the presence of TZ, and only a two-fold reduction was observed in the presence of CPZ. PMZ had the least effect

on susceptibility in the BAC^T strains, with all MIC values being identical to, or within a 2-fold range of the MIC in the absence of PMZ.

All MICs for the reference strain ATCC 700603 where within a two-fold range of the MIC in the absence of EPIs.

Effects of EPIs on BAC MICs (μg/ml)

	No EPI	CPZ	PMZ	TZ
412412				
WT	25	12.5	25	12.5
BAC^t	100	25	100	25
412621				
WT	12.5	12.5	12.5	25
BAC^t	100	25	100	50
302216				
WT	12.5	12.5	6.25	12.5
BAC^t	100	25	100	25
318418				
WT	12.5	12.5	12.5	12.5
BAC^t	100	12.5	50	25
51596E				
WT	12.5	12.5	12.5	12.5
BAC^t	100	25	100	25
50183				
WT	12.5	NG	12.5	NG
BAC^t	100	50	50	100
ATCC 700603				
Reference strain	25	50	25	12.5

Table 10 MICs of BAC + 0.5 MIC EPIs, compared to the MIC in the absence of EPIs. Almost all BAC^T strains showed a reduction in MIC in the presence of CPZ and TZ, with PMZ having the least effect on the MIC. Little to no change in MIC is seen for the wild-type strains. Results are based on three replicates performed on separate days. EPI = efflux pump inhibitor, CPZ = chlorpromazine, PMZ = promethazine, TZ.= thioridazine

5.2.3 Assessing changes in permeability using EtBr cartwheels

An EtBr cartwheel assay was performed to asses efflux and permeability. A low level of fluorescence indicates increased efflux or decreased permeability, as less EtBr is being taken in to or extruded from the cells. An example of the EtBr plates for the WT and BAC^t strains at 1 μg/ml EtBr is shown in Figure 7 on page 43.

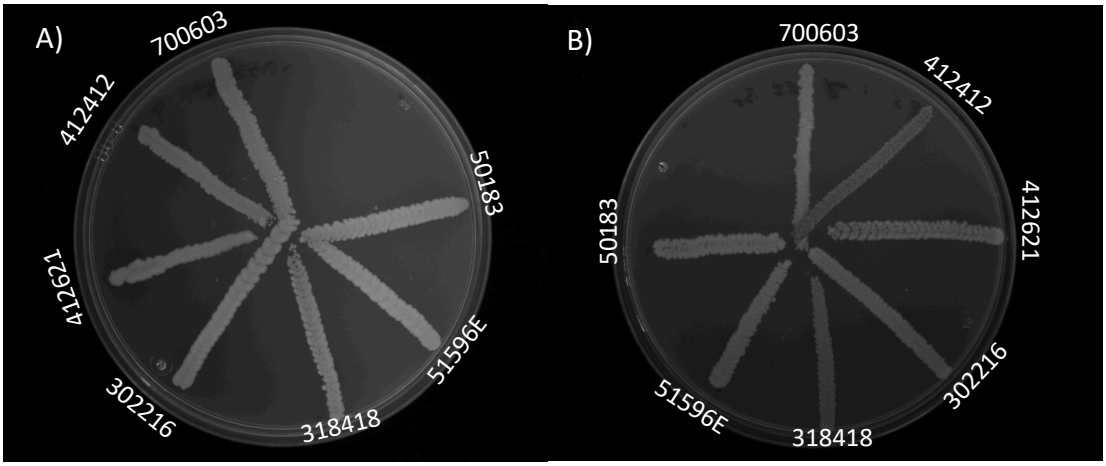


Figure 7 EtBr cartwheels showing permeability in strains pre-and post-adaptation at 1 μg/ml EtBr. The reference strain ATCC 700603 was used as a control on for both wild-type and BAC^t. A) Wild-type strains B) BAC tolerant strains.

Based on the results shown in Figure 7 above, it appears that strains 412412 BAC^t and 318418 BAC^t show increased efflux activity or lower permeability than the other BAC^t strains, and their corresponding WT strains, as these exhibited the least amount of fluorescence. Overall, permeability or efflux appears to be greater in the WT strains, as greater fluorescence is observed for these relative to the BAC^t, which would suggest a greater uptake of EtBr.

5.2.4 Determining the optimal Hoechst concentration for use in future assays

A Hoechst accumulation assay was performed using only the reference strain ATCC 700603 and no EPI. This was done to determine the optimal concentration to be used for future accumulation assays. In a previous experiment (results not shown), a heat-inactivated (HIA) control strains was found to exhibit a fluorescence of 15,000. The purpose of this control strain is to compare the results from cells with active efflux activity to that of a strain exhibiting no efflux activity. The HIA strain was also used a s a control in subsequent experiments. A decision was made to not include this strain on the graphed results as the large difference in fluorescence would make the results more difficult to read.

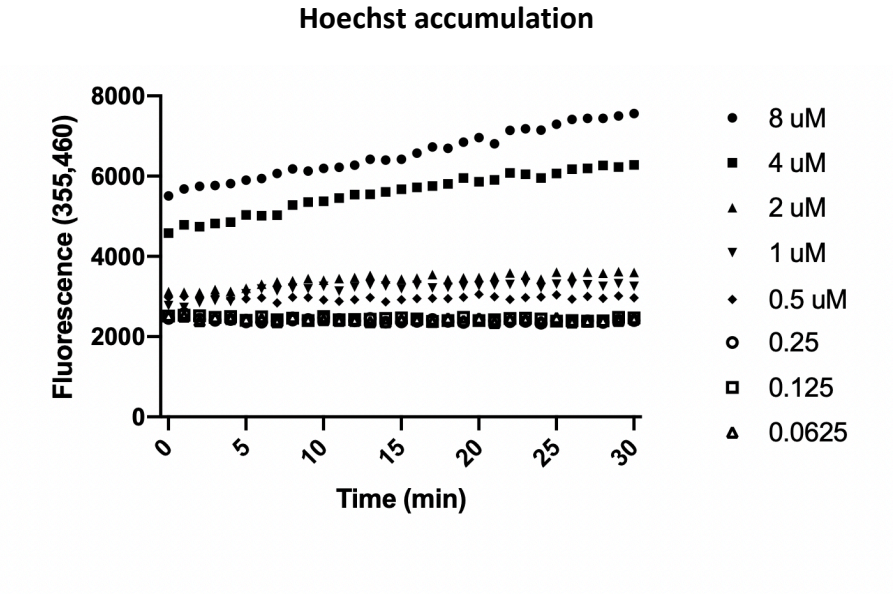


Figure 8 accumulations of Hoechst dye at different concentrations in reference strain ATCC 700603. 2 μM was chosen as the optimal concentration for future assays.

Figure 8 above shows the accumulation of Hoechst dye in the reference strain ATCC 700603 over a period of 30 minutes. 2 μM was picked as the optimum concentration for use in future assays as this was the concentration at which the greatest fluorescence signal was produced, which was not so high that it would interfere with the ability to observe differences in accumulation between strains.

5.2.5 Determining the effects of BAC adaptation on susceptibility to CCCP

A broth microdilution was performed to ensure that the chosen concentration of 10 μM did not affect BAC susceptibility. The results are shown in the table 11 on page 45.

Impact of CCCP on BAC susceptibility

	MIC (μg/ml)		MBC (μg/ml)	
	WT	**BACt**	**WT**	**BACt**
412412	25	100	50	100
412621	15.5	100	25	100
302216	12.5	100	12.5	100
318418	12.5	100	25	100
51596E	12.5	100	25	100
50183	12.5	100	25	100
ATCC 700603	25	NA	50	NA

Table 11 Effects of 10 μM CCCP on the MIC and MBC of all strains used for the accumulation assay. No change was seen in the MIC for either the WT or BAC^t strains, and only a small change in MIC was observed for some of the WT MBCs. No change in MBC was observed for the BAC^t strains. CCCP = carbonyl cyanide 3-chlorophenylhydrazone

5.2.6 Measuring Hoechst dye accumulation to assess efflux and permeability

An accumulation assay using Hoechst fluorescent dye was performed to assess permeability of the strains. The results of this assay are shown in Figure 9 on page 46. A heat-inactivated strain was used as a control (results not shown).

For strains 412621, 302216, and 318418, fluorescence was lower in the BAC^t strains compared to the WT. For 51596E, little difference was seen in fluorescence between the WT and BAC^t strain. The assay was not performed for 412412 BAC^t or 50183 BAC^t, so no comparison can be made to the WT strains.

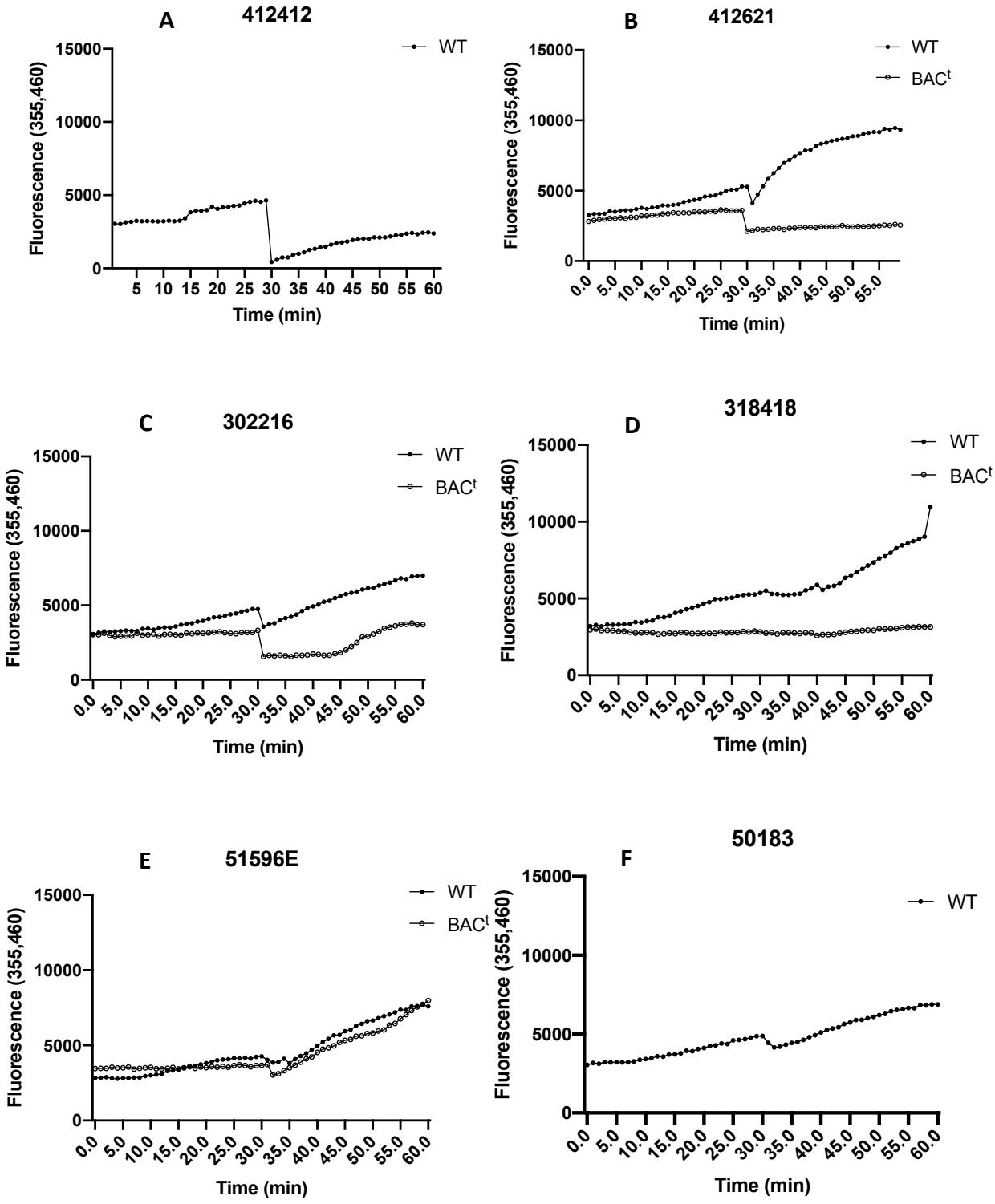


Figure 9 Results of accumulation assay using Hoechst fluorescent dye, measured at 355nm excitation, 460nm emission for 30 minutes, followed by the addition of 10 μM CCCP before measuring for an additional 30 minutes. Results were not obtained for strains 412412 BAC^t and 50183 BAC^t. Values represent the mean of three biological replicates from a single experiment.

5.2.7 Measuring changes in *ramA* and *acrA* expression using RT-qPCR

RT-qPCRs were performed to observe changes in expression of the RND efflux pump associated genes *ramA* and *acrA*. The results of these are displayed in Figures 10 and 11 respectively.

Based on the results shown in Figure 10 below, *ramA* expression is increased, to varying levels, in four out of the six BAC^t strains relative to the corresponding WT strain. For strain 302216, this was only a very slight increase, with the greatest increase seen for strains 412412 and 51596E. For strains 412621 and 318418, a slight decrease in *ramA* expression was observed following adaptation. In the case of *acrA* (see Figure 11 on page 48), there was an increase in expression in four of the adapted strains relative to the WT, with the greatest increase seen for strain 412412.

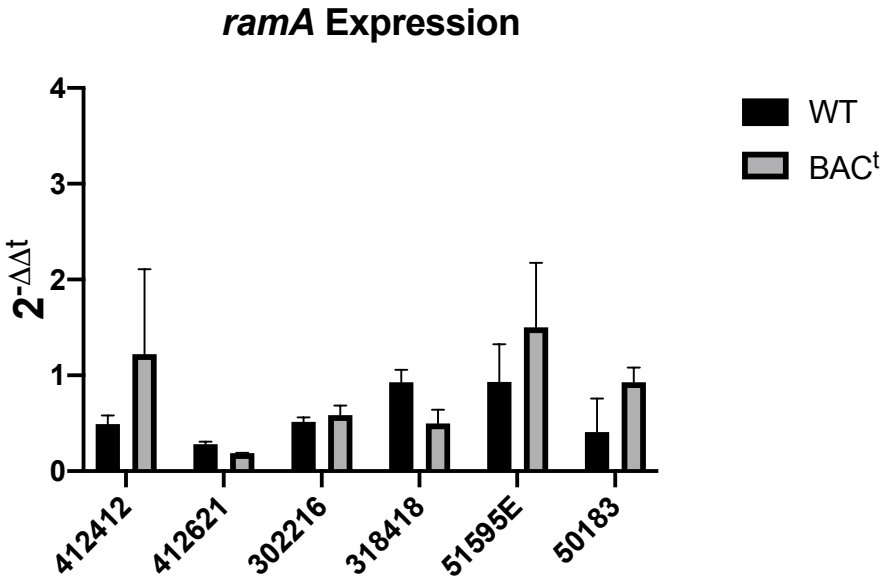


Figure 10 ramA expression measured by RT-qPCR showing altered expression following adaptation. Greater *ramA* expression was seen post adaptation for strains four out of the six strains tested, with strains 412412 BAC^t and 51595E BAC^t showing the greatest change in expression. Results are quantified relative to the reference strains ATCC 700603 (results not shown). Error bars represent the standard error of the mean based on three technical replicates.

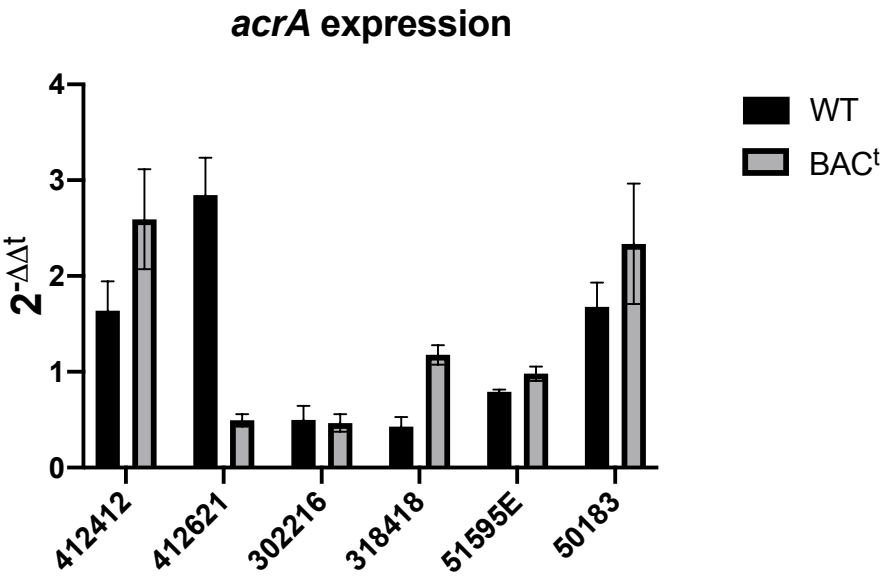


Figure 11 *acrA* gene expression measured by RT-qPCR showing altered expression following adaptation. An increase in expression post adaptation is observed in four of the six strains tested, with the greatest increase observed in strain 412412 BAC^t. Results are quantified relative to the reference strains ATCC 700603 (results not shown). Error bars represent the standard error of the mean based on three technical replicates.

5.3 Discussion

Based on the accumulation assay, it appears that neither the wild-type or BAC^t strain 318418 is sensitive to CCCP, as no drop in fluorescence is observed upon addition of CCCP. The difference in accumulation between 302216 WT and 302216 BAC^t is consistent with the results seen in the cartwheel assay in 5.2.3, as less fluorescence is seen for 302216 BAC^t, suggesting lower permeability compared to the WT.

The results of the Hoechst accumulation assay appear to be consistent with that of the EtBr cartwheel assay, with the exception being strain 412412 WT for which fluorescence was lower than for most other WT strains, and more comparable to that of some of the BAC^t strains, while this is not reflected in the cartwheel assay. The cartwheel assay is, however, a

crude method for observing changes in permeability, and not a quantitative method, so the results of the Hoechst accumulation assay may be more accurate. It was not possible to perform the assay for strain 412412 BAC^t due to the poor growth rate in the presence of BAC, and the assay did not work for 50183 BAC^t and a repeat was not possible due to time constraints.

The altered expression levels of *ramA* and *acrA* were mostly consistent, with an increase in expression being observed for both genes in strains 412412, 51596E, and 50183. Verry little change in expression was observed for strain 302216, and for strain 412621 a decrease in expression was observed for both strains following adaptation. The only strain which showed an inconsistent change in expression was strain 318418, for which *ramA* expression was decreased, while *acrA* expression was increased. The increased expression of both *ramA* and *acrA* in 412412, 51596E and 50183 BAC^t strains, and of *acrA* in 318419 BAC^t is consistent with the existing literature (71). The results of the RT-qPCR are preliminary only and based on a single replicate. It is therefore not possible to make any conclusions based on the results, and these can only be compared to the other results obtained in this study.

Chapter 6: General discussion and future perspectives

6.1 Benzalkonium chloride susceptibility

The results obtained in this study show that repeated exposure to sub-lethal concentrations of benzalkonium chloride can drive BAC tolerance in clinical isolates of *K. pneumoniae*. When these strains were assessed for phenotypic changes post-adaptation, changes in capsule density and increased efflux, or decreased permeability, were found to be the most consistent changes observed.

Based on the results observed in Chapter 3, repeated exposure of *K. pneumoniae* to BAC can increase the MIC up to 8-fold. While the maximum MIC reached for any of the clinical isolates (100 μg/ml) is still below the average concentration used in hospital surface disinfectants (1200-2400 μg/ml) and BAC-based soaps (1000-10,000 μg/ml), it is close to the in-use concentrations for pharmaceutical products (100-200 μg/ml) and BAC-based domestic antiseptic products (200-400 μg/ml) (114). These concentrations, however, assume that the product is being used correctly and do not account for improper or off-label uses which may dilute the concentration to sub-inhibitory levels.

While the MIC increased as a result of BAC exposure, the MBC remained similar to that of the WT strains, suggesting that the changes which made the cells more tolerant are not enough to protect from killing at high enough concentrations. The MBC is based on the cells which remained alive after incubation with BAC for 16-18 hours and does not give an indication of any potential increased survival over time. A way to determine if the BAC^t strains have a better or poorer survival rate over time relative to the WT would be to perform a time-kill assay with readings taken every hour and count the CFU/ml to determine the surviving fraction at each time point.

The experiments conducted as part of this study used benzalkonium chloride in isolation, while most disinfectants and preservatives contain BAC in combination with other compounds (97). It is therefore not possible to conclude whether the effects observed when using BAC in isolation would be the same as when used alongside other compounds. Studies

using commercially available BAC-based disinfectants would need to be done to determine if similar changes would be observed upon exposure to such products.

6.2 Antibiotic susceptibility

Very little change in susceptibility was observed when comparing the WT and BAC^t strains, and the most common changes were a decrease in MIC, with four WT strains being MDR compared to only two BAC^t strains. As discussed in Chapter 3.3, it is possible that these strains may be resistant to other antibiotics, or classes of antibiotics, not tested as part of this study, such as ciprofloxacin (110). No fluoroquinolone antibiotics were tested either, despite some of these being under surveillance by the WHO/ECDC. Determinations of MDR phenotype could also only be based on the four groups on antibiotics tested, it cannot be excluded that all strains may possess a greater level of resistance to other antibiotics not included in this study. Future experiments may be done to include a wider range of antibiotics and antibiotic classes in order to better determine the full extent of BAC exposure and tolerance on antibiotic susceptibility. It is also possible that a greater change in susceptibility would have been observed if the adaptation had been done on strains with a lower level of antibiotic resistance. Since the strains used in this study were originally chosen for adaptation due to their high levels of antibiotic resistance in order to better mimic real-life clinical cases, it is possible that these already possess many of the mutations that would confer higher levels of antibiotic resistance. In order to better asses the effects of BAC exposure on antibiotic susceptibility, strains with lower levels of susceptibility could be included in future studies.

Since no experiments were performed using non-adapted control strains passaged in media containing no biocide, it is not possible to conclude whether any of the changes reported in this study are as a direct result of the adaptation, or if these would have occurred naturally. This is especially true for the very large increase in susceptibility to cefpodoxime and aztreonam observed in strain 318418 post adaptation, which could be due to the loss of a plasmid. In order to determine this, similar experiments should be carried out using clinical isolates which were repeatedly passaged through media containing no biocide.

6.3 Changes in capsule density and colony morphology

The loss of capsule density associated with BAC adaptation is consistent with other studies which have shown alterations in the cell surface following exposure to BAC (86). This is also consistent with the loss of mucoviscosity and smaller colonies observed when comparing the WT and BAC^t strains. While a string test is a good indicator of HMV phenotype, it is not a quantitative test, so the extend of hypermucoviscosity in strains 302216 WT and 318418 WT cannot be determined. In order to quantify this, cells may be centrifuged at low speed and the supernatant measured, on the bases that highly mucoviscous strains sediment to a lesser extent at lower speed (112).

It is possible that a reduction in virulence may be associated with adaptation to BAC, based on the loss of mucoviscosity and HMV phenotype demonstrated in Chapter 4, since there is a known link between virulence and mucoviscosity (16). In order to determine this, infection assays in macrophages would need to be carried out to allow for comparison of infectivity pre- and post-adaptation.

It has been demonstrated that repeated exposure of *E. coli* to BAC may select for a tolerant genotype with mutations in the Lipid A associated *lpxM* locus, resulting in altered cell surface charge (98). It is possible that a similar mutation may be present in the clinical isolates following adaptation. Amplification and sequencing of the *lpxM* locus would have to be done to confirm this.

6.4 Changes in efflux and permeability

Based on the results obtained in Chapter 5, it appears that efflux plays an important role in BAC tolerance. This is consistent with other studies which have observed similar trends to those seen in these results (101,104,113). The preliminary results of the Hoechst accumulation assay show reduced accumulation in most of the BAC^t strains tested, with an increased rate of accumulation upon the addition of CCCP, suggesting that efflux was occurring prior to the addition.

The increase in expression of *ramA* and *acrA* observed following adaptation in strains 412412, 51596E and 50183, and the increase in *acrA* expression in 318418, are consistent

with what has been reported in the literature in regards to biocide tolerance (71). The overexpression of *ramA* in some of the BAC^t strains may be due to mutations in *ramR*, which regulates the transcription of *ramA*. To determine if this is the case, the *ramR* gene would need to be isolated and sequenced to screen for mutations.

6.5 General conclusion

In conclusion, this work expands upon the existing body of knowledge surrounding the effects of repeated BAC exposure in *K. pneumoniae*. It was found, using the broth microdilution method, that BAC tolerance had little impact on the susceptibility of the antimicrobials tested, though further testing of a wider range of antimicrobials is needed.

Capsule densities were found to be reduced upon adaptation, which is in line with the existing literature, additionally, adapted strains tended to be less mucoid than their corresponding wild-type strain, which may suggest that they are also less virulent. Future experiments to determine the virulence of adapted vs. non-adapted strains should be done to confirm this.

Finally, it was demonstrated that efflux and permeability may be altered as a result of BAC adaptation. In line with these results, the *ramA* and *acrA* efflux pump genes were also upregulated in some adapted strains, and that adaptation affects efflux and permeability, which is in line with what has previously been reported in the literature. However, further replicates of these experiments should be performed in order to determine their reproducibility.

Overall, the work carried out in this study yielded several results which could benefit from further exploration in the future, particularly within the context of permeability and efflux.

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