Examination of cationic antimicrobial tolerance in *Escherichia coli* to identify phenotypic and genotypic adaptations

by

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Abstract

Cationic antimicrobial (CA) agents describe a variety of positively charged antimicrobials that are widely used in many clinical, agricultural and industrial facilities to disinfect and prevent microbial growth. Increased tolerance to CAs by Gram-negative bacteria is a growing problem because CA tolerant bacteria frequently confer therapeutic antimicrobial cross-resistance. Previous studies have shown that CA tolerant bacteria frequently exhibit alterations in lipid modification pathways, up-regulation of efflux pumps and porins as a mechanism of tolerance but these changes have yet to be consistently identified in experiments containing the same species or strain exposed to different CAs. In this thesis, *E. coli* K12 was adapted to increasing concentrations of CAs, specifically, benzalkonium chloride, cetrimide bromide, chlorhexidine hydrochloride and colistin sulphate that belong to different antimicrobial classes to determine phenotypic and genotypic changes over 20-40 sub-cultures. It was revealed that CAs belonging to similar classes had similar growth phenotypes, antimicrobial cross-resistance and genotypic alterations. Experiments exploring the stability of CA-tolerant phenotypes when CA selection is removed over a 10-day period among revealed a dependence on previous CA exposure. Genotypic analysis involved identification of repeatedly identified single nucleotide variants (SNVs) in lipopolysaccharide biosynthesis pathways, antimicrobial transcriptional regulators, transposable elements, and to a lesser extent in efflux pump genes. This study suggests that CA adaptation may be dependent upon how each CA specifically disrupts the cell membrane, since each CA disrupts the membrane at potentially different outer membrane targets. It also reveals new insights and genetic markers associated with CA tolerance.
Acknowledgements

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List of Abbreviations (in alphabetical order)

ALX, alexidine dihydrochloride
AMK, amikacillin
AMP, ampicillin
AMOX, amoxicillin
AMR, antimicrobial resistance
AST, antimicrobial susceptibility testing
ATP, adenosine triphosphate
BG, bisbiguanide
Br, bromide
BZE, benzethonium
BZK, benzalkonium chloride
BZK-R, benzalkonium-resistant
Ca, calcium
CA, cationic antimicrobial
CAZ, ceftazidime
CDF, cationic diffuser facilitator
CDAB, cetyl dimethylammonium bromide
CET, cetrimide bromide
CET-A, cetrimide-adapted
CHG, chlorhexidine digluconate
CHL, chloramphenicol
CHX, chlorhexidine hydrochloride
CHX-A, chlorhexidine-adapted
CHX-R, chlorhexidine-resistant
CIP, ciprofloxacin
Cl. chlorine
CLSI, Clinical Laboratory Standards Institute
CPC, cetylpyridinium chloride
CPC-A, cetylpyridinium-adapted
COL, colistin sulphate
COL-A, colistin-adapted
CTAB, cetyltrimethylammonium bromide
CTX, cefotaxime
Da, Dalton
DDAB, didodecyldimethyl ammonium bromide
DDAC, didecyldimethyl ammonium chloride
DMAB, didecyldimethyl ammonium bromide
DG, davis glucose
DMSO, dimethyl sulphoxide
DNA, deoxyribonse nucleic acid
DOM, domiphen bromide
ECA, enterobacterial common antigen
EPS, extrapolymeric substance
ERY, erythromycin
ETC, electron transport chain
EUCAST, European Committee on Antimicrobial Susceptibility Testing
GENT, gentamycin
H$_2$SO$_4$, hydrogen sulphate
IMP, imipenem
K, potassium
KAN, kanamycin
LB, lysogeny broth
LPS, lipopolysaccharide
MATE, multidrug and toxin extruder
MDR, multidrug resistant
MFS, major facilitator superfamily
Mg, magnesium
Mg/L, milligram per litre
µg/ml, microgram per millilitre
MHB, mueller hinton broth
MIC, minimum inhibitory concentration
MV, methyl viologen
NA, nalidixic acid
Ng, nanogram
NOR, norfloxacin
OD, optical density
OMP, outer membrane protein
OMV, outer membrane vesicles
PACE, proteobacterial antimicrobial compound efflux
PEN, penicillin
PG, peptidoglycan
PHMB, polyhexamethylene biguanide
PIP, piperacillin
PMXB, polymyxin B
QAC, quaternary ammonium compound
rDNA, ribosomal deoxyribose nucleic acid
RND, resistance nodular division
SDS, sodium dodecyl sulphate
SFX, Sulfamethoxazole
SMR, small multidrug resistance
SNV, single nucleotide variant
Spp., species
STR, streptomycin
TAZ, tazobactam
TET, tetracycline
TLN, triclosan
TMP, trimethoprim
TOB, tobramycin
TSB, tryptic soy broth
WGS, whole genome sequencing
WHO, World Health Organisation
WT, wild type
CHAPTER 1. INTRODUCTION

1.1. Antimicrobials and their classifications

Antimicrobial is a general term used to describe substances including medicines that kill or slowly inhibit the growth of microorganisms when treating human or environmental surfaces. This group can be classified into 2 main groups: biocides and antibiotics. Because biocides and antibiotics range in antimicrobial activity, other specific terms can be included to indicating how they inhibit growth or kill may be used: “-static,” refers to agents which inhibit growth (e.g., bacteriostatic, fungistatic, and sporistatic) and “-cidal,” refers to agents which kill the target organism (e.g., sporicidal, virucidal, and bactericidal). "Biocide" is an overarching term that describes a broad-spectrum chemical agent such as detergents, preservatives, antiseptics, and disinfectants, and possess either bacteriostatic (inhibit) or bacteriocidal (kill) properties towards the growth of microorganisms. Antiseptics are biocides that kill or inhibit the growth of microorganisms and are typically used on living tissues, whereas disinfectants are similar to antiseptics, but are used on inanimate objects or surfaces. An antibiotic is a naturally occurring or synthetic medicine designed to kill or slow the growth of bacteria and some fungi, generally at low concentrations in or on living tissue and are therapeutically administered.

Biocides are now known to encompass a number of different types of compounds including detergents, quaternary ammonium compounds (QACs), bisbiguanides (BG), chlorine compounds, phenolics, iodine, alcohols, hydrogen peroxide, silver compounds and dyes. Historically, biocides and other antimicrobial agents have been employed in a variety of forms for a number of centuries. Historical empirical approaches included using vinegar and honey for cleansing wounds and copper and silver vessels for storing portable water in the 17th century. Later, reports stated the use
of iodine as a wound disinfectant, the use of chlorine water in obstetrics and phenol (carbolic acid) as a wound dressing in the antiseptic cleansing prior to surgery\(^4\). Over the course of the 20\(^{th}\) century, other chlorine releasing agents and some QACs were introduced into use as reviewed by Russell (2002)\(^3\). By the mid 1940’s, common biocides in use included phenolics, iodine, alcohols, hydrogen peroxide, silver compounds and dyes, with a lot of the agents remaining in use today\(^4\). The most important agents introduced within the last 60 years include amphoteric surfactants, bisphenols including triclosan (TLN) and biguanides including chlorhexidine (CHX) and alexidine (ALX)\(^3\).

1.1.1. Cationic antimicrobials

Cationic antimicrobial agents (CAs) comprise a chemically diverse range of antimicrobial compounds that possess a positive charge at neutral pH\(^5\) (Figure 1.1). CAs are commonly used to disinfect and sterilize in clinics\(^6\), food preparation facilities\(^7\), households\(^8\), agriculture/aquaculture\(^9\), and industrial facilities\(^2,10\). In clinics, antiseptics and disinfectants are commonly used as part of infection control practices to prevent the spread of nosocomial infections by disinfecting surfaces\(^2\). The mechanism of action of CAs is their adsorption and penetration into the bacterial cell envelope; thereon follows a reaction with negatively charged outer membrane (lipids and proteins) followed by membrane disruption; this then causes cell leakage of cytoplasmic contents (including Magnesium (Mg\(^{2+}\)) and Calcium (Ca\(^{2+}\)) ions, degradation of nucleic acids and proteins and eventual cell death\(^5\). There are numerous biocide CAs in clinical use as reviewed by Maillard (2005)\(^6\), however, due to the focus of this thesis, the following subsections will discuss three of the most frequently used CAs classes in antiseptic products and therapeutic medications: QACs, BGs, and polymyxins (PMX).
**Figure 1.1** Examples of cationic antimicrobial chemical structures. Panels show QACs benzalkonium chloride; cetrimide bromide: domiphen bromide and cetyltrimethylammonium bromide: bisbiguanides chlorhexidine hydrochloride and alexidine dihydrochloride: biguanide polyhexamethylene biguanide and polymyxins polymyxin B and E.
1.1.2. Quaternary ammonium compounds (QACs)

QACs possess two distinct regions in their molecular structure, 3-4 hydrophobic acyl or aryl compound groups attached to one or more permanently charged nitrogen cations resulting in a quaternary bound ammonium atom\(^2,11\) (Figure 1.1). Commonly used QACs such as benzalkonium chloride and cetrimide bromide are found in a number of products including disinfectants, surfactants, antiseptic creams and cosmetic products including shampoos, fabric softeners and detergents. QACs are relied upon for a variety of clinical disinfection purposes, including preoperative disinfection of skin, oral rinses, eye drops, and disinfection solutions and wipes\(^2\).

QACs are membrane active agents, and therefore have a mechanism of action that targets the outer and cytoplasmic membranes of bacteria\(^2\). Specifically, QACs mechanism of action includes the progressive adsorption of the cationic nitrogen headgroup to acidic phospholipid headgroups situated on the outer or cytoplasmic membrane of the bacterium\(^11\). This leads to decreased fluidity of the cell membrane, creating voids within the membrane due to lipid vesicilization\(^11\). Reactive oxygen species (chemically reactive species containing oxygen) and reactive nitrogen species (chemically reactive species containing nitrogen) are generated which cause oxidative stress, thus denaturing proteins and DNA\(^12,13\). Eventual lysis of the bacterial cell is induced, and subsequent solubilization of phospholipids and proteins into QAC/phospholipid micelles occurs\(^11\) (Figure 1.2B).

1.1.3. Bisbiguanides

Bisbiguanides (BG) are a class of chemically related compounds known for their bactericidal properties\(^2\). BGs, such as CHX (Figure 1.1), are widely used as antiseptics and can be found in items such as oral hygiene products, surgical handwashes, as well as topical
Figure 1.2. A cartoon diagram of known CA mechanisms of action at a Gram-negative bacterial outer membrane surface. The proposed mechanisms of action are compared between bisbiguanide (BG) (A), QACs (B) and polymyxin (PMX) (C).\textsuperscript{11,14,15}
ointments/washes for wound infections\textsuperscript{11}. BGs have similar chemical features to QACs in the sense that they are positively charged at nitrogen groups but at neutral pH and their main target site of bacterial entry is the membrane\textsuperscript{11}. BGs have a similar mechanism of action to the QACs since BG cationic charges associate strongly with the anionic phospholipids and acidic protein sites in the cell membrane\textsuperscript{16}. Like other CAs, BGs can subsequently induce displacement of membrane associated divalent cations (Mg\textsuperscript{2+}, Ca\textsuperscript{2+})\textsuperscript{17}. Unlike QACs, the hydrophobic regions of membrane associated BGs do not become solubilized within the hydrophobic membrane core. The carbon chain length of BGs are shorter (6-8 C atoms) when compared to QAC aryl groups (12-18 C atoms). Since the shorter BG hydrophobic region is less flexible, it cannot penetrate deep enough into the bilayer to displace lipids like many QACs. Hence, BG specific mechanism of action creates gaps or bridges in between paired phospholipid headgroups and acidic proteins, displacing the associated divalent cations, causing eventual ion leakage that results in cell lysis and death\textsuperscript{17} (Figure 1.2A).

Polyhexamethylene biguanide (PHMB) is a polymeric biguanide that is commonly used as a disinfectant in the food industry; it can also be found in cosmetics, leather preservatives, contact lens disinfectants, in treatment of hatching eggs, fibers and textiles and technical fluids like cutting oils and glues as well as a disinfectant in swimming pools\textsuperscript{2}. PHMB, like CHX, is a membrane-acting agent that impairs the integrity of the outer membrane of Gram-negative bacteria\textsuperscript{2}. PHMB is comprised of repeating basic biguanide units connected by hexamethylene hydrocarbon chains, which provides the compound with a cationic and amphipathic structure\textsuperscript{18}. PHMB is different to other biguanides such as CHX and ALX in the sense that PHMB causes domain formation of the acidic phospholipids of the cytoplasmic membrane\textsuperscript{18}. The sequence of events includes i) rapid attraction of PHMB toward the negatively charged bacterial outer membrane, with specific
adsorption to phosphate-containing compounds; ii) the integrity of the outer bacterial membrane is impaired, and subsequent attraction of PHMB to the cytoplasmic membrane ensues; iii) binding of PHMB to phospholipids occurs, with an increase in inner membrane permeability (K⁺ loss) accompanied by bacteriostasis; and iv) complete loss of membrane function follows with precipitation of intracellular constituents and a bactericidal effect².

1.1.4. Polymyxins

Polymyxins (PMX) are therapeutic antimicrobial peptides that possess a polycationic charge at neutral pH¹⁴. PMXs were discovered over 6 decades ago and were derived from Bacillus polymyxa¹⁹. PMXs are amphiphilic, where they have net cationic charges and possess hydrophilic and hydrophobic/lipophilic regions; typically, a cyclic heptapeptide ring, attached to a tripeptide which in turn is attached to a hydrophobic fatty acid chain (Figure 1.1)²⁰. Only PMXs B and E (colistin) are used clinically, frequently as last resort therapeutic antibiotics in the treatment of multidrug resistant Gram-negative bacterial infections²¹. PMXB is normally applied topically to treat eye, ear and skin infections, while colistin is used to treat diarrhea in children²¹.

PMXs, like colistin, target the bacterial cell membrane, specifically, due to the polycationic peptide ring which interacts with the lipid A portion of negatively charged lipopolysaccharides (LPS), enabling colistin to penetrate through the outer membrane, potentially forming a pore and subsequently displacing Mg²⁺ and Ca²⁺²². Insertion between the phospholipids of the cytoplasmic membrane leads to loss of membrane integrity and eventual bacterial cell death²³ (Figure 1.2C).

1.2. The problem of antimicrobial resistance (AMR)

Antimicrobial resistance (AMR) as determined by the World Health Organisation (WHO), is defined as:
“the ability of a microorganism (like bacteria, viruses, and some parasites) to stop an antimicrobial (such as antibiotics, antivirals and antimalarials) from working against it. As a result, standard treatments become ineffective, infections persist and may spread to others\textsuperscript{24}.”

AMR is a biological process that develops in bacteria over a period of time, due to selective pressure exerted by the antimicrobial\textsuperscript{25}. As new AMR mechanisms are being discovered, it is becoming clear to healthcare professionals that AMR poses a global threat\textsuperscript{24}. AMR mechanisms threaten our ability to treat infections, resulting in prolonged illness and even fatality\textsuperscript{24}. According to the report by Jim O’Neill, it is predicted that by 2050, 10 million lives/annum and a 100 trillion USD worth of economic output are at risk due to the rise of AMR infections\textsuperscript{26}. Although new antimicrobials are being developed, not nearly enough are in development and are expected to be completely ineffective against extremely multidrug resistant (MDR) bacteria. Therefore, it is critical to study AMR mechanisms to identify key new targets for therapeutic intervention and treatment. The report by Jim O’Neill in 2016 proposes that seven main interventions need to be implemented in order to attempt to tackle the growing crisis of AMR: i) introduce a global public awareness campaign; ii) improve sanitation and reduce the spread of infection; iii) reduce unnecessary use of antimicrobials; iv) improve global surveillance of drug resistant antimicrobials and their use; v) promote new and rapid diagnostics to reduce the unnecessary use of antimicrobials; vi) promote development and use of vaccines and other alternatives and vii) improve the number, pay and recognition of people working in infectious disease\textsuperscript{26}. Implementation of these interventions in a timely manner will ideally tackle the global burden of AMR\textsuperscript{26}. 
1.2.1. Critical priority AMR Gram-negative bacterial infections

According to the WHO, carbapenem resistant Gram-negative bacteria, in particular Enterobacteriaceae, are an emerging AMR problem and effectual cause of nosocomial acquired infections that pose a significant threat to public health\textsuperscript{27}. In November 2017, the WHO published its first ever list of antibiotic-resistant “priority pathogens” and this was undertaken to guide and promote research and development of new antibiotics to try and address the growing issue of global resistance. The WHO list is divided into three categories according to the urgency of need for new antibiotics: critical, high and medium priority and consists of \textit{Acinetobacter} spp., \textit{Pseudomonas} spp. and various Enterobacteriaceae (including \textit{Klebsiella} spp. and \textit{Escherichia coli}) (Table 1.1). These opportunistic pathogens can cause severe and often fatal infections and have become resistant to many relevant antibiotics, including carbapenems and third generation cephalosporins, which are the first line treatment for treating MDR bacterial infections\textsuperscript{1}. According to the WHO: “the second and third tiers in the list – the high and medium priority categories – contain other increasingly drug-resistant bacteria that cause more common diseases including gonorrhoea and food poisoning caused by \textit{Salmonella} spp.” (Table 1.1)\textsuperscript{1}. 
Table 1.1. A summary of WHO priority AMR pathogens

<table>
<thead>
<tr>
<th>CRITICAL PRIORITIES</th>
<th>HIGH PRIORITIES</th>
<th>MEDIUM</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Acinetobacter baumannii</em></td>
<td><em>Enterococcus faecium</em></td>
<td><em>Streptococcus pneumoniae</em></td>
</tr>
<tr>
<td>carbapenem-resistant</td>
<td>vancomycin-resistant</td>
<td>penicillin-non-susceptible</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td><em>Staphylococcus aureus</em></td>
<td><em>Haemophilus influenzae</em></td>
</tr>
<tr>
<td>carbapenem-resistant</td>
<td>methicillin-resistant</td>
<td>ampicillin-resistant</td>
</tr>
<tr>
<td><em>Enterobacteriaceae</em></td>
<td><em>Vancomycin-intermediate &amp; resistant</em></td>
<td><em>Shigella spp.</em></td>
</tr>
<tr>
<td>carbapenem-resistant</td>
<td><em>Helicobacter pylori</em></td>
<td>fluoroquinolone-resistant</td>
</tr>
<tr>
<td>extended spectrum</td>
<td>clairthomycin-resistant</td>
<td></td>
</tr>
<tr>
<td>beta-lactamase producing</td>
<td>Campylobacter spp. fluoroquinolone-resistant</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Salmonellae</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td>fluoroquinolone-resistant</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Neisseria gonorrhoeae</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td>cephalosporin-resistant</td>
<td></td>
</tr>
<tr>
<td></td>
<td>fluoroquinolone-resistant</td>
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</tbody>
</table>
1.2.2. The relevance of *E. coli* as a model organism to study AMR

*Escherichia coli* is a Gram-negative facultative anaerobic, rod shaped bacterium that is commonly found in the gut and lower intestines of most animals including humans\(^\text{28}\). The majority of *E. coli* strains are non-pathogenic and are part of the normal microbiota of the gut, however, some pathogenic serotypes can cause illness in humans, including diarrhea, vomiting, abdominal pain and fever\(^\text{29}\). Fecal–oral transmission is the major route of pathogenic infection that cause disease, and most cases of *E. coli* infection and illness are caused by improper food handling, cross-contamination of food utensils, consuming dairy products that have been left out too long or stored at the incorrect temperature, consuming foods that are not cooked to the correct temperature or duration of time, especially meats and poultry, consuming raw seafood products, drinking unpasteurized milk and consuming raw produce that has not been properly washed\(^\text{30}\).

*E. coli* can be grown and cultured easily and inexpensively in a laboratory setting and has been intensively investigated for over 60 years\(^\text{29}\). *E. coli* is the most widely studied prokaryotic model organism, and an important species in the fields of biotechnology and microbiology, where it has served as the host organism for the majority of work with recombinant DNA\(^\text{31}\). The increased availability of genome sequences has provided the basis for comprehensive understanding of organisms at the molecular level. Besides sequence data, a large number of experimental and computational resources are required for genome-scale analyses. *E. coli* K-12 has been one of the best characterized organisms in molecular biology\(^\text{29}\). *E. coli* K-12 BW25113 is a common laboratory strain that was created in the laboratory of Barry L. Wanner and was utilized in a method using the bacteriophage lambda red recombination system to perform gene disruptions with double-stranded PCR products\(^\text{32}\). *E. coli* K-12 BW25113 later became the parental strain for the ‘Keio collection’, a major resource consisting of approximately 4,000 non-essential single-gene deletion mutants\(^\text{33,34}\). The ‘Keio collection’ provides a valuable resource not only for systematic
analyses of unknown gene functions and gene regulatory networks but also permits genome-wide testing of mutational effects in *E. coli* K-12 BW25113. Additionally, a complete set of cloned genes corresponding to each essential and non-essential gene were cloned into the pCA24N expression vector with and without a green fluorescent protein C-terminal fusion tag and are known as the ‘ASKA collection’. The ASKA collection allows systematic gene complementation and functional analyses of the Keio mutants to examine phenotype. Whole genome sequences are available for BW25113 and its two closely related K-12 strains, MG1655 and W3110. Hence, *E. coli* K12 BW25113 and its derivatives are being used in countless laboratories for a variety of studies, including systematic phenotypic surveys and synthetic biology efforts.

Despite the genetic characterization of *E. coli*, nearly 1/3 of the genes in the *E. coli* genome (designed as genes beginning with a ‘y’) have unknown function and/or are hypothetical functions, many of these are predicted to target the cell membrane, emphasizing the need to continue genotypic and phenotypic studies of *E. coli*.

### 1.3. CA resistance in Gram-negative species

CAs used as antiseptics and disinfectants can describe a wide variety of chemicals, each with their own working concentrations, properties, chemical modifications and counterion formulations (gluconate; acetate; Cl⁻, Br⁻, H₂SO₄⁻). In addition to chemical property variations, there is considerable confusion and controversy in determining what defines bacterial CA susceptibility, CA concentration thresholds or break points since there are no reference strains or agreed upon CA compounds for biocide testing thus far. Standard biocide susceptibility testing methods are not available from the Clinical Laboratory Standards Institute (CLSI) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST). There is also
considerable ambiguity in what defines minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) since most biocides are bactericidal. The problem is biocide ‘resistance’ and ‘tolerance’ are terms often used interchangeably in published articles leading to confusion when compared to antibiotics. The review article published by Cerf et al. (2010)\textsuperscript{49} attempts to define biocide ‘resistance’ and ‘tolerance’ as follows: ‘resistance’ should be used to describe bacterial killing (i.e. MBC values), while ‘tolerance’ should be used to describe bacterial adaptation to inhibitory biocide concentrations and used when describing MIC values of more than 2-fold change as compared to the WT strain\textsuperscript{45,49}.

Although most biocides are effective at killing Gram-negative pathogens at the manufacturer’s recommended high concentrations (0.001-10% w/v)\textsuperscript{6,8}, many Pseudomonas and Acinetobacter spp. are intrinsically more tolerant to high concentrations of cationic biocides as compared to Enterobacteriaceae (see Table 1.2 for E. coli examples). Enterobacteriaceae species can also rapidly acquire tolerance to high concentrations of CA biocides when exposed to prolonged sub-inhibitory concentrations. CA resistance has been shown in E. coli, with QAC resistance demonstrating up to 4-fold increases in MIC\textsuperscript{50,51} (Table 1.2). Reduced biocide susceptibility by opportunistic pathogens is associated with higher rates of hospital outbreaks (as reviewed by Weber et al. (2007)\textsuperscript{52}, making biocide tolerance an important AMR aspect to consider with respect to environmental and clinical AMR stewardship initiatives.
Table 1.2. A summary of published MICs (mg/L) for biocide susceptible and biocide tolerant or biocide adapted *E. coli* strains.

<table>
<thead>
<tr>
<th>Species</th>
<th>n</th>
<th>CHX</th>
<th>BZK</th>
<th>BZE</th>
<th>CET</th>
<th>DMAB</th>
<th>CPC</th>
<th>TLN</th>
<th>CAZ</th>
<th>CTX</th>
<th>IMP</th>
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<td></td>
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<tr>
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<td>25</td>
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<tr>
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<tr>
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<td>ND</td>
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<td>8</td>
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<td>Bore et al. 200750</td>
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<td>ND</td>
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<td>ND</td>
<td>800</td>
<td>250</td>
<td>ND</td>
<td></td>
<td>Ishikawa et al. 200251</td>
</tr>
</tbody>
</table>

ND; not determined, BZK-A; benzalkonium-adapted species, CTAB-A; cetyltrimethylammonium bromide adapted species.

**Biocide abbreviations:** BZK; benzalkonium, CHX; chlorhexidine, BZE; benzethonium, CET; cetrimide, CTAB; cetyltrimethylammonium bromide, CPC; cetylpyridinium chloride, DMAB; didecyldimethylammonium bromide, TLN; triclosan

**Antibiotic abbreviations:** AMK; amikacin, AMP; ampicillin, AMOX; amoxicillin, CAZ; ceftazidime, CHL; chloramphenicol, CIP; ciprofloxacin, COL; colistin, CTX; cefotaxime, ERY; erythromycin, GEN; gentamicin, IMP; imipenem, KAN; kanamycin, NA; nalidixic acid NOR; norfloxacin, PEN; penicillin, PMXB; polymyxin B, PIP; piperacillin, SFX; sulfamethoxazole, STR; streptomycin, TAZ; tazobactam, TET; tetracycline, TOB; tobramycin, TMP; trimethoprim.
1.3.1. CA tolerance and adaptation in Enterobacteriacea

There have been many \textit{in vitro} laboratory experiments conducted to artificially ‘adapt’ various lab cultured Gram-negative species to study biocide CA tolerance. These biocide ‘adaptation’ (also known as ‘exposure’) experiments involve gradually exposing a pure or mixed bacterial culture to increasing concentrations of a CA (most frequently BZK and CHX) over multiple subcultures (5-40 sub-cultures) ultimately producing a culture that can tolerate higher biocide MICs (ranging from 2-100 fold) as compared to the initial ‘un-adapted’ strain (Table 1.2). In most studies, the ‘stability’ of the newly acquired biocide tolerant culture is verified by repeated sub-culturing in media lacking biocide followed by sub-culturing in media at the adapted biocide MIC value\textsuperscript{55,56}. Many adaptation studies have also examined the fitness of the CA-adapted species, where the majority consensus suggests biocide adaptation came at significant cost (5-50% reduction) in their overall growth as compared to initial un-adapted strains\textsuperscript{51,57,58}. However, some cationic biocide adapted strains showed no difference in fitness and even gained fitness as well as virulence as was the case for \textit{Salmonella}\textsuperscript{58,59}. It remains unclear if specific CAs have a greater fitness cost in certain species/ genera due to lack of studies examining more than 3 different classes of biocide CAs in a single study.

With respect to MIC values achieved by CA-adapted species, mostly modest (2-6 fold changes) and occasionally high (20-50 fold changes) MIC values were obtained for various Enterobacteriacea as compared to their initial starting strains\textsuperscript{50,60–62}. Factors influencing final CA-adapted MIC values may be related to the genetic background of starting strain/serotype used for adaptation\textsuperscript{61,63} and variations in the total number of sub-cultures generated during the adaptation experiment\textsuperscript{64}. Unfortunately, experimental variations in the number of sub-cultures (or lack of reported values) required to generate the final biocide adapted species making it difficult to determine if background species or final sub-culture numbers are more influential on final CA
adaptation thresholds. For example a study examining CHX adaptation of various *Klebsiella* spp. used a method that involved a fixed total of 10 sub-cultures for all experiments and reported differences in the final CA concentrations tolerated by each strain\(^6^4\). This suggests that different strains/serotypes were more adept than others to acquire higher CA tolerance supporting the importance of species/serotype background. The importance of CA adaptation order was also demonstrated in *Enterobacter* spp. experiments\(^6^1\). Adaptation of *Enterobacter* species to benzalkonium (BZK) demonstrated increased susceptibility to cetrimide (CET) and cetylpyridinium chloride (CPC), yet the same strains adapted first to CPC showed reduced susceptibility to BZK\(^6^1,^6^3\). As a result, growing consensus from previous adaptation studies, in Enterobacteriaceae, suggests that different CA tolerance mechanisms may be responsible for adaptation to different antimicrobials (BZK versus CHX for example), rather than a single generic CA tolerant pathway or mechanism\(^5^0,^5^1,^6^4\). However, further evidence in support of this theory has not been shown to date. Hence, biocide adaptation to one CA does not necessarily confer cross-resistance to other CAs or biocides and highlights the importance of understanding the mechanisms of biocide action, cellular targets, and biocide synergies/antagonism, especially when many antiseptics/disinfectants are formulated in commercial biocide solutions.

1.3.2. CA contributions to antimicrobial cross-resistance in *E. coli*

The consequences of increased CA tolerance by Enterobacteriaceae extend beyond increased tolerance to other biocides, as numerous studies surveying antibiotic cross-resistance of biocide adapted strains have demonstrated increased tolerance to particular antimicrobials (Table 1.2). CA-adapted strains have frequently demonstrated low level increases (≥2 fold) in antibiotic MIC values to one or more antibiotics tested as compared to the un-adapted strain or MIC value
ranges reported for collections representing each species (Table 1.2). For example, CA-adapted *E. coli* strains have shown antibiotic cross-resistance to β-lactams: ampicillin, amoxicillin, penicillin and macrolide erythromycin51,54, third generation cephalosporins ceftazidime, cefotaxime50,65,66, as well as naladixic acid and aminoglycoside tobramycin67. Additionally, CA-adapted strains also demonstrated reduced susceptibility to similar classes of CAs as well as antibiotic cross-resistance testing suggesting tolerance to one CA class may enhance tolerance to others. For example, the type of CA used for *Salmonella* spp. adaptation to BZK, CPC, chlorhexidine digluconate (CHG), and CET increases MIC value, CA tolerance and cross-resistance to various antimicrobials such as ampicillin, trimethoprim-sulfamethoxazole, nalidixic acid and tetracycline57,61,63. Furthermore, differences in MIC values to these four antimicrobials were also noted for CHX-adapted *S. enterica* serovars in two separate studies57,63; increased resistance was only demonstrated to tetracycline in both studies. This may suggest that species/serovar/serotype selection for biocide adaptation is an important factor for antimicrobial cross-resistance determination.

Regarding the impact of the final MIC values attained for a CA-adapted strains and its influence on antibiotic cross-resistance, CHX-adapted *K. pneumoniae* (4-8-fold increase in MIC from the un-adapted strain) demonstrated significant increases in MIC ranges towards many clinically relevant antibiotics tested64. Similar cross-resistance values were noted in *E. coli* strains adapted to DDAC that exhibited ≥3 fold MIC from the un-adapted starting strain68. These adaptation studies suggest that Enterobacteriaceae strains with higher final CA-adapted MIC values may concomitantly increase antibiotic resistance. It should be noted that other studies examining CA-tolerant strains tested for their antibiotic cross-resistance have also demonstrated
no significant differences in antibiotic cross-resistance\textsuperscript{8,69,70}. These outcomes may be explained by many of the same factors discussed above as well as reduced culture fitness, and intrinsic differences in background CA tolerance due to variations between species/strain/serovar. Hence, bacterial CA tolerance, CA adaptation and cross-resistance experiments would benefit from further studies using known organisms, to address experimental irreproducibility, reference strain continuity, and more in-depth CA tolerance mechanism exploration.

Finally, CA adaptation has been reported to increase tolerance to other CAs, specifically aminoglycosides\textsuperscript{51,54} and CA peptides such as PMXs\textsuperscript{64,71} in various Enterobacteriaceae, including \textit{E. coli} (Table 1.2). Based on the solubility, size, and net charge differences of these CA compounds at neutral pH, it would seem logical that tolerance to any CA would increase its tolerance of other CAs. However, antimicrobial cross-tolerance/resistance by Enterobacterial CA-adapted strains to both CA biocides or other antimicrobials has not been convincingly demonstrated in any species to date\textsuperscript{50,51,54}. Some explanations for this may be due to the inherent differences between antimicrobial chemical properties such as size, hydrophobicity, aromaticity, net charge as well as mechanism(s) of drug action, and molecular mechanisms associated with its tolerance (Table 1.2). How the CA/drug disrupts the membrane, the concentrations at which it can do so, the target surface and/or membrane proteins/lipids it targets, whether or not the compound can pass through outer membrane porins or bypass porins in favour of self-promoted uptake/direct membrane penetration are all variables that may predict CA-antibiotic cross-resistance patterns but at the present time there are insufficient experiments to support this.
1.3.3. Mechanisms of CA tolerance

There are three main mechanisms of AMR and tolerance in bacteria; efflux pumps\textsuperscript{72,73}, lipid biosynthesis and transport\textsuperscript{74} and outer membrane porin down regulation\textsuperscript{75} (Figure 1.3). In addition, other AMR systems including transcriptional regulators such as marR\textsuperscript{76}, ramR\textsuperscript{77}, soxS\textsuperscript{78} and PhoPQ\textsuperscript{79} and lipid biosynthesis enzymes such as lpxL\textsuperscript{80} and lpxM\textsuperscript{81} have also been associated with CA tolerance mechanisms in bacteria\textsuperscript{82,83}. Similar to some antibiotics (aminoglycosides), the mechanism of tolerance to CAs involves alterations of the bacterial membrane that reduce the entry of the antimicrobial into the bacterial cell. Previous studies have shown an association between mechanisms of CA tolerance and up-regulation of efflux pumps\textsuperscript{50,84}, as well as porin down-regulation\textsuperscript{50,51,84,85} and altered lipid biosynthesis enzyme expression\textsuperscript{86,87}. More importantly it should be noted that different genes/proteins are often altered depending on the type of CA the cell was exposed to (ie. QAC versus BG) suggesting that although CAs disrupt the membranes, they may not share specific CA tolerance biomarkers\textsuperscript{64}. CAs such as QACs are also known to denature proteins as well as disrupt the membrane, therefore, QAC-mediate disruption may influence electron transport chain activity as well and increase the level of reactive oxygen species radicals in the cell\textsuperscript{50}. Therapeutic CAs such as COL are known to target the LPS on the surface of bacterial cells, therefore, the most commonly identified mechanism of tolerance can be observed via changes in LPS in a number of studies\textsuperscript{51,88}. Since CA tolerance impacts many membrane-associated systems, including reducing drug permeability and membrane lipid composition alteration\textsuperscript{11,51}, each mechanism is discussed in further detail in the following sections below (as summary is provided in Figure 1.3).
Figure 1.3. A cartoon summary of membrane proteins alterations caused by CA adaptation in various Enterobacteriaceae.
1.3.3.1. Outer membrane proteins (OMPs)

In Gram-negative bacteria, β-barrel forming proteins known as OMPs play numerous roles for the cell. Many OMPs (also known as porins) permit passive nutrient diffusion/osmoregulation (OmpC, OmpF) and participation in dedicated efflux and transport complexes (TolC). OMPs also help stabilize and maintain membrane integrity (OmpA) or possess enzymatic activity such as proteolysis (OmpT). Since porins regulate outer membrane permeability by limiting the entry of most compounds into the cell, porins are known to influence resistance to antimicrobials, such as β-lactams, tetracycline, chloramphenicol, and fluoroquinolones\(^7\) when their expression and accumulation are altered; as reviewed by Fernandez and Hancock, 2012\(^7\). After surveying transcriptomic and proteomic experiments involving CA-adapted strains of Enterobacteriaceae, many OMPs previously associated with MDR are also linked to biocide adaptation.

OMP TolC is an archetypical member of the outer membrane efflux protein family and a component of numerous Resistance Nodular Division (RND) (AcrAB) and Major Facilitator Superfamily (MFS) (EmrAB) efflux systems in Enterobacteriaceae such as *E. coli*\(^8\). In transcriptomic studies of QAC-adapted *E. coli*, the outer membrane component of *acrAB* efflux system, *tolC* was shown to be significantly up-regulated in addition to *acrAB* as well as other *tolC*-dependent efflux systems\(^5\). TolC participates in a variety of multipartite membrane complexes responsible for iron siderophore release, toxin/ metabolite expulsion, and efflux pumps; hence, inactivation of TolC impairs cellular repair mechanisms and promotes metabolic shutdown as reviewed by Zgurskaya et al., 2011\(^8\). Hence, *tolC* up-regulation likely has beneficial pleiotropic effects in QAC adapted strains beyond efflux-mediated tolerance mechanisms.
*E. coli* CA adaptation experiments have identified similar patterns of porin expression and accumulation by general diffusion porins, OmpC and OmpF, as observed for MDR Enterobacteriaceae. In QAC tolerant and adapted *E. coli* strains, OmpC is up-regulated, while OmpF is down-regulated. OmpF is slightly larger in pore size than OmpC and is down-regulated under conditions of high osmolarity and high oxidative stress (as reviewed by Falagas 2005), similar to conditions that would be induced by QAC exposure. Under similar nutrient, oxidative, and stress culturing conditions, *E. coli* *ompC* expression increases, suggesting that biocide adaptation triggers similar stress responses regulating porin expression. Due to the membrane disruptive actions of biocides, *in vitro* porin channel conductance experiments in artificial membranes cannot determine if biocides enter these pores, however, both pores can passively diffuse molecules \(< 600\) Da in size. It is clear that OmpC and OmpF regulation is similar between cationic biocide adapted and antimicrobial resistant *E. coli* but its contributions in other CA tolerant genera remains hypothetical.

OMPs associated with membrane stability were identified among CA-adapted species. OmpA is one of the most abundant proteins in Gram-negative membranes. It has poor channel conductance as compared to OmpC and OmpF, but it confers an important pathogenic role with respect to host cell adhesion and invasion as reviewed by Confer and Alayew (2013). OmpA plays a stabilizing role for Gram-negative outer membranes due to its interactions with peptidoglycan (PG) and LPS. Biocide adaptation is known to alter LPS and PG through direct contact, therefore, the loss of OmpA may either facilitate membrane alterations or is a consequence of altered LPS content in CA-tolerant bacterial membranes. Up-regulation of OMPs
responsible for membrane stability are an expected tolerance adaptation, especially under conditions where membranes must ward off the disruptive effects of CAs.

BZK, cetyltrimethylammonium bromide (CTAB), and CHX-tolerant species also demonstrated \textit{ompW} up-regulation in \textit{E. coli}\textsuperscript{50,85}. Although it is unclear if BZK or CTAB can pass through OmpW pore as this has not been determined to date, OmpW has been shown to participate in QAC herbicide methyl viologen (MV) efflux as mediated by SMR efflux pump EmrE in \textit{E. coli}\textsuperscript{98}. QAC adapted \textit{E. coli} showed down-regulation of \textit{ompT}, a protease with specificity for cleaving paired basic residues\textsuperscript{99}, which is highly induced in response to heat shock and protein over-expression\textsuperscript{100}. In \textit{E. coli}, OmpT confers resistance to urinary CA peptides \textsuperscript{101,102}, regulates outer membrane vesicle biogenesis\textsuperscript{103}, and requires LPS for protease activity in \textit{in vitro} experiments\textsuperscript{104}. It remains unclear as to why \textit{ompT} is down-regulated as a consequence of QAC adaptation. Based on OmpT’s varied roles and regulation, it may be due to stress response signaling changes or perhaps a reduction in specifically modified LPS caused by CA adaptation.

\textbf{1.3.3.2. Efflux pumps}

In comparison to all other CA-resistant mechanisms, efflux pumps are the most well characterized and play an important part in reducing biocide susceptibility. Efflux pump activity expels drugs that enter cells and is fueled by primary active adenosine triphosphate (ATP) or secondary active proton motive force driven pumps. Efflux activity prevents drug action or targeting by expelling compounds that enter the cell. Gram-negative bacteria such as \textit{E. coli} intrinsically possess a variety of distinct efflux pump systems, however, clinically relevant resistance to most antimicrobials including biocide CAs is conferred by an assortment of chromosomally encoded single or multi-component (OMP-dependent) efflux pump systems.
OMP-dependent efflux systems form a multipartite complex spanning both the plasma membrane (AcrB), the periplasm (AcrA) and outer membrane (TolC) of E. coli to completely remove the target compound, as reviewed by Li et al 2015\textsuperscript{105}. In CA-adapted Enterobacterial studies reported so far, OMP-dependent efflux systems belonging to the resistance-nodulation division (RND) family play a large role in reducing CA susceptibility and are significantly upregulated in most CA-adapted species, including E. coli\textsuperscript{50}. Biocide-selective acrAB orthologues are present in at least one or more copies within the genomes of Enterobacteriaceae and are often significantly upregulated in biocide-resistant and biocide-adapted species\textsuperscript{50}.

OMP-dependent efflux systems are augmented by the activities of single component/OMP-independent efflux pumps that reside exclusively within the plasma membrane. As their name implies, these efflux pumps are not reliant on TolC or OMP in the outer membrane and frequently confer biocide tolerance when expressed as a single gene (as reviewed by Slipski et al 2017\textsuperscript{106}). OMP-independent biocide selective efflux pumps include many transporter families: small multidrug resistance (SMR;\textsuperscript{107}), proteobacterial antimicrobial compound efflux (PACE;\textsuperscript{108}), cation diffusion facilitator (CDF;\textsuperscript{109}), multidrug and toxin extruder (MATE) and MFS\textsuperscript{110}. OMP-independent efflux pump systems enhance CA tolerance mechanisms in a variety of important ways. These pumps are conditionally expressed\textsuperscript{111} and/or induced by oxidative and stress responses\textsuperscript{83}, such as MATE members NorM\textsuperscript{112} and SMR qac genes\textsuperscript{113}. Many OMP-independent efflux pumps are laterally inherited via integrons and multidrug-resistance plasmids\textsuperscript{106} which expand a cells’ ability to recognize and efflux a broader range of CAs not recognized by dominant OMP-dependent efflux systems\textsuperscript{111,114}. Elevated tolerance to QACs and/or CHX have also been demonstrated in over-expression studies of MFS members mdtM\textsuperscript{115}, mdfA and emrD\textsuperscript{83,114}, but none
of the efflux pump has been directly identified in transcriptomic/proteomic analyses of QAC or CHX adapted \textit{E. coli} strains. MATE member \textit{mdtK} (\textit{ydhE}) which has previously shown BZK (2-fold) tolerance when over expressed in \textit{E. coli}\textsuperscript{16}, suggests that particular CAs may induce specific efflux pumps. By comparison to AcrAB pumps, most OMP-independent efflux pumps only modestly enhance CA tolerance (> 2-6 fold MIC values) based on overexpression studies as reviewed by Slipski \textit{et al} 2017\textsuperscript{106}, emphasizing their supporting role in efflux-mediated AMR.

1.3.3.3. Transcriptional regulators, lipid modifiers, and other membrane proteins

When considering the multiple mechanisms of action induced by biocide CAs, the development of CA tolerance/adaptation may force cells to overcome multiple stressors at the membrane rather than a single target. Therefore, it is not surprising that many of the transcriptional regulators target a variety of membrane proteins. Analyses of CA tolerant and adapted Enterobacterial species have identified altered up-regulation of numerous transcriptional regulators (\textit{marR}, \textit{ramR}, \textit{soxS}, \textit{phoPQ}) in studies involving antimicrobial exposure, oxidative damage, and stress. Many of these systems control the expression of various efflux pump systems and antimicrobial-resistance genes\textsuperscript{82,83}. QACs and CHX have been shown to up-regulate similar efflux pump regulators, \textit{marRA} and \textit{soxS}, in \textit{E. coli}; both transcriptional regulators are known to positively regulate dominant pump \textit{acrAB-tolC}\textsuperscript{84,117}.

In addition to efflux system regulation, transcriptional regulators that control LPS modifications have been implicated in CA-adapted Enterobacterial studies. In \textit{E. coli} and \textit{Salmonella enterica} serovar Typhimurium, \textit{pmrD} regulates the expression of the \textit{pmrHFIJKLM} (also known as \textit{arnBCADTEF} operon), which enhances resistance to CA peptides including PMXB by 4-amino-4deoxy-\(\alpha\)-L-arabinose modifications to lipid A\textsuperscript{118,119}. In \textit{E. coli}, PmrD acts as
a connector between the PmrAB and PhoPQ two component systems as reviewed by Dalebroux et al (2014). LPS alterations have demonstrated increased resistance to PMXs as well as other CA peptides as reviewed by Olaitan et al 2014. Therefore, it makes sense that they may play an additional role in biocide CA tolerance. Other PMX resistance associated genes rfaL, yefI, rfc, rfbX have been identified from PHMB-adapted E. coli. LPS O-antigen polysaccharide modifying enzymes rfaL(waaL), yefI (wbbK), rfc (wbbH), rfbX (wzx) were up-regulated in PHMB adapted E. coli strains, suggesting that modifications of LPS O-antigen sugars are increased in cationic biocide-adapted E. coli. QAC exposure in E. coli is known to alter LPS properties, suggesting an potential role for PMX-like LPS modifications in other CA tolerance mechanisms.

Outer membrane lipoproteins have been up-regulated in CA-adapted E. coli. The lipoprotein, vacJ/ mlaA, is an outer membrane component of the Mla pathway involved in phospholipid transport system. MlaA directly interacts with OmpC and OmpF porins to maintain the phospholipid-LPS asymmetry of the outer membrane. MlaA enhances outer membrane vesicle (OMV) formation and polymyxin resistance. Up-regulation of osmB, a hyperosmotic stress inducible lipoprotein in CA-adapted E. coli membranes may suggest that osmotic stress inducible proteins as well as OMV regulatory proteins are required to counteract the membrane disruptive actions of CAs. SoxRS-regulated electron transport chain (ETC) components were also up-regulated in BZK adapted E. coli and include flavoprotein FldA and cytosolic fumarate reductase FumC. The increased oxidative stress inducible components as well as superoxide dismutase SodA up-regulation may help offset the chronic oxidative damage caused by CA exposure in these adapted cells. Additionally, CA-adapted E. coli and S. Typhimurium both demonstrated a variety of energy production, nucleotide metabolism, and carbohydrate
metabolism pathway components that were variably up and down-regulated; many which were biased towards MarAR and SoxSR inducible systems\textsuperscript{50,126}. It is clear that many proteins responsible for membrane stability, maintenance, and lipid biosynthesis / modifications contribute towards CA tolerance mechanisms in addition to oxidative stress induced responses, however, due to the lack of experiments comparing alterations caused by different CA classes in the same experiment it is hard to pinpoint shared and unique mechanisms of CA tolerance and resistance attributed to CA adaptation. Future experiments will ideally clarify the cause and effect relationships linked to CA adaptation/tolerance to determine what phenotypic and genotypic alterations are essential for CA tolerance to develop.

1.4. Thesis objectives and hypotheses

The main aim of the study is to understand CA tolerance mechanisms by generating and characterizing \textit{E. coli} strains adapted to various CAs. The main hypothesis for the project is as follows:

\textit{“E. coli K-12 strain BW25113 adapted to different CA classes (QACs, BGs, and PMXs) will result in similar phenotypic and genotypic alterations”}.

To address the main hypothesis, the following sub-hypotheses for the project were examined:

1. Are CA tolerant phenotypes easily lost when CAs are removed from growth medium? The phenotypic stability of CA tolerance after the removal of CA selection over 10 days will gradually diminish CA tolerance phenotypes among CA-adapted \textit{E. coli} strains.
2. Are CA-adapted *E. coli* more or less fit than un-adapted *E. coli* under similar growth conditions? The growth fitness of CA-adapted strains will be compromised as compared to un-adapted *E. coli* strains.

3. Does CA tolerance enhance cross-resistance to therapeutic antibiotics and other biocides? CA-adapted strains will enhance antimicrobial cross-resistance profiles as compared to the un-adapted *E. coli* strain.

4. Does CA adaptation result in genotypic alterations ie. single nucleotide variants (SNV)? If yes, are SNVs similar or different among similar CA-adapted strains? *E. coli* adapted to different CAs will confer similar genetic alterations (SNVs) in gene and pathways involving the outer membrane, lipid biosynthesis and trafficking, OMP/porins, and efflux pump systems.

CA-adapted *E. coli* K12 BW25113 strains were generated employing gradual exposure to four different CAs, BZK, CET, CHX, and COL involving the broth culture CA adaptation procedure described by Bore *et al.* 2007 in triplicate; resulting in a total of 3 independently adapted strains per drug tested. The antimicrobial susceptibility of these 12 strains was compared to un-adapted *E. coli* and experiments involving phenotypic stability and growth fitness was conducted by growing CA-adapted strains and un-adapted strains in various broth media. The genetic alterations present within each CA-adapted strain was determined via Illumina MiSeq whole genome sequencing (WGS) and bioinformatic analysis to map and characterize SNVs associated with each CA tolerance phenotype.
CHAPTER 2. MATERIALS AND METHODS

2.1. E. coli species, strains and chemicals used in study

CAs and chemicals used in this study were obtained from Tokyo Chemical Industry (TCI) America (OR, USA), Millipore Sigma (MA, USA), Fisher Scientific and VWR (Appendix i). *E.coli* K-12 BW25113$^{33}$ was obtained from the Coli Genetic Stock Centre (CGSC; [http://cgsc2.biology.yale.edu](http://cgsc2.biology.yale.edu)).

2.2. E. coli cryopreservation, stab culture methods, and growth conditions

CA-adapted *E. coli* sub-cultures were cryopreserved in Lysogeny Broth (LB) media (1% tryptone, 0.5% yeast extract, 0.5% sodium chloride), containing a final concentration of 16% (v/v) glycerol and stored at -80°C. LB+glycerol as a cryopreservant was selected based on prior CA adaptation experiments$^{50}$. Samples were cryopreserved when ODs reached stationary phase from overnight incubation (LB media containing 16% v/v glycerol). Stab cultures of 1% LB agar were also prepared in line with standard protocol$^{127}$, incubated overnight at 37°C in a non-shaking incubator and then stored at 4°C. The viability of the cryopreserved stocks was tested by inoculating 5 ml of LB per sample and incubating overnight at 37°C until turbidity was observed. All *E. coli* cultures were grown in a shaking incubator (New Brunswick Excella E25) at 37°C in this study unless otherwise noted.

2.3. E. coli adaptation to CAs

*E. coli* BW25113 CA adaptation experiments were performed in LB broth medium using a repeated sub-culturing method described by Bore *et al.* 2007$^{50}$ with modifications listed below
Briefly, dimethyl sulfoxide (DMSO) cryopreserved strains of *E. coli* K12 BW25113 were grown overnight (18 hours) in LB medium and subsequently diluted $10^{-2}$ into sterile LB containing one of four different CAs, BZK, CET, CHX and COL (Appendix i) at final concentrations ranging from 0.2-6µg/ml; these concentrations were equivalent to 20% of the respective MIC value for the CA based on the un-adapted WT strain. Triplicate cultures were inoculated for each CA to be tested and all test tubes incubated for 20–24 hours with shaking (150-170 rpm). The next day, cultures with the highest CA concentration and turbidity (growth) were selected and re-inoculated ($10^{-2}$ dilution) into three new tubes of LB media, one at the same CA concentration and the remaining two at 2 and 4 µg/mL more CA than tube one, respectively. This sub-culturing cycle was repeated for each CA-adapted culture (three replicates total) until its gradual CA tolerance was unchanged after five consecutive subcultures and it reached a $> 2$-fold higher MIC value than the original un-adapted *E. coli* BW25113 strain. COL adapted strains reached 32 subcultures both QAC adapted strains reached 40 subcultures (BZK and CET), and CHX adapted *E. coli* reach 20 subcultures; CHX adaptation experiments were extended to 30 subcultures to verify phenotypic enhancements were unaltered after 20 subcultures. *E. coli* BW25113 was also sub-cultured 40 times in LB only for use as un-adapted WT controls in all experiments. All sub-cultures were cryopreserved throughout the experiment as described above. *E. coli* BW25113 adapted to each of the four CAs were generated in biological triplicate resulting in a final total of 12 CA-adapted strains for analysis.
Figure 2.1. Diagram flow charts showing A) the CA adaptation process and B) the phenotypic stability testing experiment.
2.4 Antimicrobial Susceptibility Testing (AST) methods

A broth microdilution AST method was used to determine MIC values as described by Balouiri et al. 2016. Briefly, final sub-culture cryopreserved stocks of each CA-adapted or un-adapted E. coli (three biological replicates per CA in technical triplicate) were inoculated into sterile LB medium and grown for 18 hours with shaking. The overnight cultures were sub-cultured into fresh tubes containing 50% of the final CA concentration they were adapted to (40 µg/mL BZK; 50 µg/mL CET; 1 µg/mL CHX; 55 µg/mL COL) and grown for 22-24 hours with shaking. Prior to inoculation for AST, overnight culture turbidity was adjusted spectrophotometrically to obtain an optical density at 600 nm (OD\textsubscript{600nm}) of 1.0 units with LB. The standardized OD\textsubscript{600nm} 1.0 adjusted cultures were diluted 10\textsuperscript{-2} into 96 well microtitre plates containing 2-fold serial dilutions of an antimicrobial stock solution (1-50 mg/mL) in LB broth medium to a final volume of 200 µL/well. For antimicrobials stocks that required solubilization in ethanol or DMSO, control wells containing these adjuvants with and without inoculated culture were used as optical baselines. A total of 17 antimicrobial compounds were included for AST and are highlighted in bold in Appendix i. Triplicate CA-adapted/un-adapted E. coli strains were also repeated in technical triplicate (n=6) by AST and microplates were incubated for 18 hours with shaking (150 rpm) before OD\textsubscript{600nm} measurement by a Multiskan Spectrum UV-Vis microplate reader (ThermoFisher, MA). MIC values were defined as the lowest concentration of CA tested per adapted strain that resulted in an OD\textsubscript{600nm} value that was indistinguishable from the uninoculated control well (Student’s t-test p-values ≥ 0.001). Significant changes in MIC were determined as a +/- 4-fold change as compared to the un-adapted WT control strain. Since AST involved a 2-fold serial
dilution of CAs the +/- 4-fold was deemed to be the minimum threshold and therefore, accounts for log 2 error.

2.5. Growth curve experiments for fitness determination

CA-adapted strain fitness was determined by comparing the growth of each strain in 96 well microplate rich and defined broth cultures to the un-adapted E. coli WT strain. 96 well microplate growth curves were set up as described for AST with the exception that only one specified CA concentration was selected for each CA to be tested. Final sub-inhibitory CA concentrations at 20% of the E. coli WT strain MIC was tested for each strain and compared to the growth of the un-adapted strain in media only. Growth curves were measured spectrophotometrically (OD$_{600 \text{ nm}}$) every 30 minutes over 24 hrs in Biotek Synergy Neo2 Hybrid Multimode reader. CA-adapted and un-adapted strains were grown in a variety of rich media: LB, LB + 0.2% (w/v) Glucose, Mueller Hinton broth (MHB), Tryptic Soy broth (TSB). Defined media was also tested: minimal nine salts (M9)$^{127}$ and Davis Glucose (DG)$^{127}$ minimal media to measure bacterial fitness in various growth conditions.

2.6. Phenotypic stability testing of CA-adapted strains when CA is removed

The stability of CA tolerance by CA-adapted strains grown in the absence of CA was determined using broth sub-culturing each CA-adapted E. coli strains in LB without CA for 10 days (summarized in Figure 2B). Each day of the 10-day sub-culturing stability experiment, the culture (-CA) was re-inoculated at $10^2$ dilution into LB media containing CA at 50% of the drug concentration they were cryopreserved at (40µg/ml; CET 50µg/ml; CHX 1µg/ml; COL 55µg/ml).
All stability broth testing was grown for 22-24 hours with shaking and OD$_{600\text{nm}}$ were measured for a minimum of 3 replicates/CA-adapted strains (n=3). To determine the stability of the MICs obtained following adaptation, CA-adapted strains were grown in the presence of CA and then sub-cultured in LB without CA for 10 days (Figure 2.1B). Each day of the 10-day sub-culturing stability experiment, AST was performed.

### 2.7. Whole genome sequencing analyses of CA-adapted strains

Genomic DNA was isolated from the final CA-adapted broth culture for each of the three biological replicates as well as the un-adapted *E. coli* WT strain using Invitrogen Purelink Microbiome DNA isolation kits in order to capture SNV changes within the population (ThermoFisher Scientific, MA). Genomic DNA (30-100 µl of 10-30 ng/µL) was extracted from 22-24 hour cultures grown in LB containing 50% its respective final adapted CA (BZK (40µg/ml; CET (50µg/ml); CHX (1µg/ml); COL (0.5µg/ml). Genome sequencing was performed by MicrobesNG (https://microbesng.uk/), which was supported by the BBRSC (grant number BB/L024209/1). An Illumina-MiSeq system (Illumina, Inc., CA) was used to sequence genome at a minimum of 30x coverage (Appendix ii), and all strains were verified to be *E. coli* BW25113 K-12 based on their 16S rDNA sequences and alignment to the BW25113 reference map (Genbank sequence CP009273.1). Genomic sequences for each CA-adapted strain replicate are available to download at the MicrobesNG project link (https://microbesng.uk/portal/projects/2626E4CE-05C7-45FC-A54C-EF0DE0B8500/) and will be uploaded to NCBI in June 2019. Trimmed paired reads were generated and assembled using the MicrobesNG in-house pipeline where the *E. coli* BW25113 (CP009273.1) was used as the mapping reference.
SNV analysis was performed using Geneious® next generation sequencing bioinformatics software (v 11.1.5) to identify SNVs for each CA-adapted strain to the *E. coli* BW25133 reference genome. Briefly, the trimmed paired reads were assembled to provide contigs of the reads mapped to the reference genome. Contigs were reviewed to determine how the reads map against the reference genome and to ensure that the reads mapped to the expected distance apart, based upon the insert size specified when the reads were set up. A consensus sequence was generated to enable sequence base visualization (consensus of the reads only). Annotation and prediction of low and high coverage contig alignments was calculated so that low coverage regions could be excluded when SNVs were called. Gene annotation using Geneious® was performed to create an annotation track of SNVs added to the reference sequence. SNV annotations in coding and non coding regions was generated in separate tables. SNV annotations were then compared to the BW25113 reference genome to enable the filtering out of SNVs that were of low coverage. SNVs identified in the un-adapted WT strain grown for 40 subcultures (to account for genetic drift in LB media) were also identified and excluded from SNV analysis. SNV information was then extracted and exported into Microsoft Excel for further analyses.

### 2.8. Statistical analyses used

Data analysis was performed using Microsoft Excel 2016. Statistically significant differences in growth between CA-adapted and un-adapted strains were determined using a two-tailed Student’s t-test using 2 -tailed paired heteroscedastic where \( p \)-values \( \leq 0.001 \) were deemed significantly different.
CHAPTER 3. RESULTS

Adaptation, cross resistance to antimicrobials, stability testing, and growth curve experiments were conducted and led by N. Cartwright with experimental assistance from Kari Green. Whole genome sequencing was performed by Microbes NG and analysis was conducted by N. Cartwright using Geneious® software v 11.1.5.

3.1 *E. coli* adaptation to CA differs depending on the antimicrobial class it was adapted to

To remain consistent with previous experiments that ‘adapted’ lab cultured *E. coli* strains to biocides in broth media, *E. coli* BW25113 was adapted in LB at gradually increasing concentrations of one of four different CAs (BZK, CET, CHX, COL) over 20-40 sub-cultures (Figure 3.1) to produce a CA-adapted *E.coli* strain with an MIC ≥ 2 fold as compared to the initial ‘un-adapted’ BW25113 strain (Table 3.1). CA-adapted BW25113 strains were sub-cultured in triplicate and the adaptation experiment stopped when visible growth was not observed in the highest concentration after 5 sub-cultures; hence, a total of 12 CA-adapted bio-replicates (3 per each of the 4 CAs tested) were generated and we refer to them as strains based on their subtle difference in antimicrobial tolerance and fitness phenotypes as well as genotypes as discussed in further results sections in this chapter. Broth microdilution AST methods were selected as the primary method to determine MIC values of each CA-adapted strain. As compared to the un-adapted WT strain (WTG0), each CA-adapted strain showed a significant increase in MIC value (≥4 fold) as compared to WTG0 towards its respective CA with the exception of CHX adapted strains (Table 3.1) For CHX, only one CHX adapted strain (CHXG20R1) showed an increase in MIC value (4-fold) compared to the WTG0, suggesting that the culture adapts to CHX at variable
rates. Surprisingly, COL adapted BW25113 demonstrated a 300-450-fold increase in MIC values (Table 3.1). When assessing the CA adaptation concentrations attained by each strain over the 20-40 days, as shown in Figure 3.1, the plotted outcome for QAC and COL adapted strain shows a linear increase in drug versus time; the only exception were the CHX adapted strains, which showed a stepwise increase due to the low concentrations of CHX and shorter time frame we examined. This suggests that BW25113 gradually and slowly adapted to increasing amounts of CAs for each subculture. This is important considering, parabolic and/or sigmoidal curves are often observed in antibiotic adaptative evolution experiments which imply that antimicrobial threshold must be reached prior to any gain in antimicrobial resistance\textsuperscript{129,130}. Taken altogether the results of CA gradual adaptation demonstrate that BW25113 is capable of tolerating all four CAs but at significantly different concentrations as reflected by MIC values.
Figure 3.1. A summary of the gradually adapted maximum CA concentration achieved by each *E. coli* BW25113 subculture grown in LB broth in triplicate. CA concentrations (y-axis) are reported for each sub-cultured strain (x-axis) capable of turbid growth in LB media containing the specified concentration of CA after 22 hrs at 37°C. A summary of gradual adapted *E. coli* BW25113 strains (R1-R3 shown in panel legends) to increasing concentrations of **A)** BZK, **B)** CET, **C)** CHX and **D)** COL are shown.
Table 3.1 Breakdown of each adapted strain replicate and their final MIC as compared to un-adapted WT.

<table>
<thead>
<tr>
<th>Experiment identifier</th>
<th>CA used for adaptation identifier</th>
<th>Adapted MIC (µg/ml)</th>
<th>WT MIC (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BZKG40R1</td>
<td>BZK</td>
<td>144</td>
<td>18</td>
</tr>
<tr>
<td>BZKG40R2</td>
<td>BZK</td>
<td>72</td>
<td>18</td>
</tr>
<tr>
<td>BZKG40R3</td>
<td>BZK</td>
<td>72</td>
<td>18</td>
</tr>
<tr>
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<td>30</td>
</tr>
<tr>
<td>CETG40R2</td>
<td>CET</td>
<td>240</td>
<td>30</td>
</tr>
<tr>
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<td>CET</td>
<td>240</td>
<td>30</td>
</tr>
<tr>
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</tr>
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<td>2.4</td>
</tr>
<tr>
<td>CHXG20R3</td>
<td>CHX</td>
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<td>2.4</td>
</tr>
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<td>1</td>
</tr>
<tr>
<td>COLG32R3</td>
<td>COL</td>
<td>&gt;450</td>
<td>1</td>
</tr>
</tbody>
</table>

Experimental strains generated: R1; replicate strain 1, R2; replicate strain 2, R3; replicate strain 3 per CA used for adaptation of the WT G0 strain. G, refers to subculture generation.
3.2. CA-adapted *E. coli* show limited cross-tolerance to antimicrobials and antibiotics

Previous studies of CA-adapted strains have shown increased tolerance to various antimicrobials beyond their own CAs\textsuperscript{51,131}. To determine if CA-adapted BW25113 strains generated herein have increased or decreased cross-tolerance as compared to WTG0, broth microdilution AST was repeated using a more extensive antimicrobial library. CAs including BZK and CET as well as additional QACs and BGs were included as well as therapeutic antibiotics representing PMXs, β-lactams, aminoglycosides, glycopeptides, quinolones, oxazolidinones, sulfonamides, tetracyclines, macrolides and ansamycins (Table 3.2). Overall, QAC adapted strains (BZK and CET) showed significantly higher MIC values (≥4 fold increase) to CPC and insignificant but higher (2-fold increases) to CDAB, CTAB, DOM as compared to WTG0. QAC adapted strain AST results for anionic disinfectant triclosan (TLN) demonstrated insignificant but higher MIC increases (2-fold) as compared to WTG0 suggesting that BZK and CET adaptation only marginally improved TLN tolerance. Previous studies have shown CA exposure (BZK) can increase cross-tolerance to TLN (≥5–100 fold increase in MIC as compared to the WT strain)\textsuperscript{61}. Our AST results show low level increases in tolerance suggesting TLN mechanisms of action may have some overlap with CA mechanisms of tolerance. BZK and CET adapted strains exhibited greater susceptibility (2–16 fold) to a Gram-positive specific antibiotic vancomycin (VAN) as compared to the WT (Table 3.2). This finding suggests that QAC adaptations, particularly CET adapted strains, may alter the outer membrane and/or PG content to allow VAN easier access to the cell wall that is not possible in WT Gram-negative *E. coli*. 
Table 3.2: Summary of MIC values of each adapted replicate strain when tested against other antimicrobial compounds. Values in bold indicate >2 fold change from the WTG0 value

<table>
<thead>
<tr>
<th>Strain tested</th>
<th>BZK</th>
<th>CET</th>
<th>CHX</th>
<th>COL</th>
<th>ERY</th>
<th>ALX</th>
<th>CEF</th>
<th>CDAB</th>
<th>CPC</th>
<th>CTAB</th>
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<tr>
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<td>2</td>
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<td>1</td>
<td>&gt;32</td>
<td>&gt;64</td>
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<td>120</td>
<td>4</td>
<td>0.5</td>
<td>256</td>
<td>4</td>
<td>1</td>
<td>&gt;32</td>
<td>&gt;64</td>
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<td>0.5</td>
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<td>4.8</td>
<td>1</td>
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<td>2</td>
<td>1</td>
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<td>8</td>
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<td>1</td>
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<td>2</td>
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<tr>
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<td>30</td>
<td>0.5</td>
<td>2</td>
<td>&gt;450</td>
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<td>4</td>
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<table>
<thead>
<tr>
<th>Strain tested</th>
<th>DOM</th>
<th>DOXY</th>
<th>KAN</th>
<th>LIN</th>
<th>MER</th>
<th>PMXB</th>
<th>Rif</th>
<th>SMX-TMP</th>
<th>TLN</th>
<th>VAN</th>
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<tbody>
<tr>
<td>WTG0</td>
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<td>0.25</td>
<td>256</td>
</tr>
<tr>
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<td>32</td>
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<td>8</td>
<td>2048</td>
<td>0.015</td>
<td>NA</td>
<td>256</td>
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<td>128</td>
</tr>
<tr>
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<td>16</td>
<td>8</td>
<td>2048</td>
<td>0.015</td>
<td>NA</td>
<td>256</td>
<td>16</td>
<td>0.5</td>
<td>128</td>
</tr>
<tr>
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<td>8</td>
<td>8</td>
<td>2048</td>
<td>0.03</td>
<td>NA</td>
<td>256</td>
<td>16</td>
<td>0.5</td>
<td>128</td>
</tr>
<tr>
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<td>8</td>
<td>2048</td>
<td>0.015</td>
<td>NA</td>
<td>256</td>
<td>16</td>
<td>0.5</td>
<td>128</td>
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<tr>
<td>CETR2G40</td>
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<td>NA</td>
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<td>0.25</td>
<td>256</td>
</tr>
<tr>
<td>COLR1G32</td>
<td>4</td>
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<td>16</td>
<td>32</td>
<td>0.03</td>
<td>128</td>
<td>0.125</td>
<td>32</td>
<td>0.0078</td>
<td>64</td>
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<tr>
<td>COLR2G32</td>
<td>4</td>
<td>0.5</td>
<td>8</td>
<td>32</td>
<td>0.03</td>
<td>256</td>
<td>0.125</td>
<td>32</td>
<td>0.0078</td>
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</tr>
<tr>
<td>COLR3G32</td>
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<td>256</td>
<td>0.125</td>
<td>32</td>
<td>0.0078</td>
<td>32</td>
</tr>
</tbody>
</table>

Abbreviation of antimicrobials: CET, BZK, CHX, COL, erythromycin (ERY), alexidine dihydrochloride (ALX), ceftazidime (CEF), cetyldiethyl ammonium bromide (CDAB), cetylpyridinium chloride (CPC), cetyl trimethyl ammonium bromide (CTAB), dimethyldidodecylammonium bromide (DDAB), domiphen bromide (DOM), doxycycline (DOXY) kanamycin (KAN), linezolid (LIN), meropenem (MER), polymyxin B (PMXB), rifamycin (Rif), trimethoprim-sulfamethoxazole (SMX-TMP), triclosan (TLN) and vancomycin hydrochloride (VAN).
CHX adapted strains showed no significant cross-tolerance to any of the antimicrobials we tested; all MIC values were either similar or insignificantly susceptible (2-fold reduction in MIC) as compared to WTG0 for the majority of antimicrobials tested, including alexidine (ALX) (Table 3.2). The results for ALX are striking when considering ALX chemically differs from CHX by the presence of two ethylhexyl moieties on each end of the molecule in contrast to the $p$-chlorophenyl groups of CHX. The ethylhexyl-end groups of ALX, are suggested to influence the ability of a biguanide to perturb LPS and lipid domains in the cytoplasmic membrane and this might, in turn, affect resistance patterns and cellular targets observed\textsuperscript{132}. Previous studies using colourimetric biochemical \textit{E. coli} membrane assays comparing ALX and CHX mechanisms of action show that both agents must saturate a number of envelope targets before penetration into the cytosol is possible and that ALX possessed a higher affinity towards these target sites than CHX\textsuperscript{16}. Differences in the BG mechanism of action and target specificity between CHX and ALX may account for the lack of cross-tolerance in the CHX adapted strains expected. All three COL-adapted strains exhibited increased tolerance to PMXB as compared to WTG0 (Table 3.2), indicating that tolerance to one PMX confers cross-tolerance to other PMXs, similar to findings for QAC adapted strains. COL adapted strains demonstrated significant increases in susceptibility ($\leq$4-fold reduction in MIC as compared to WTG0) to the majority of antimicrobials tested, including QACs (CPC, CTAB, CDAB, DDAB), anionic disinfectant TLN, and therapeutic antibiotics ERY, DOX, DOM, LZD, RIF and VAN (Table 3.2). To explain the high susceptibility to most antimicrobials we observed by COL adapted \textit{E. coli}, it is likely that adaptation has resulted in significant changes to the outer membrane, particularly targeting lipid A as observed in previous
studies (as reviewed by Biswas et al. 2012). This would explain why COL adapted E. coli are more susceptible to most antimicrobials we tested, each with variable mechanisms of action.

In conclusion, these AST findings indicate that E. coli K-12 adaptation to QACs and PMXs only increases tolerance to its respective antimicrobial classes (i.e. QAC to QAC and PMX to PMX) and does not enhance cross-tolerance/resistance to other CAs or antimicrobials. E. coli CHX adaptation appears to be the exception to this trend, since it failed to enhance tolerance to ALX. This may highlight differences in BG mechanisms of action or highlight differences in cellular targets of each CHX and ALX during adaptation.

### 3.3 CA-adapted E. coli have similar growth phenotypes in rich media but reduced growth in minimal defined media

Since antimicrobial adaptation is known to come at significant growth fitness costs to some microorganisms, growth time-course (growth curve) experiments were performed for each CA-adapted strain in rich (LB +/- glucose, TSB, MHB) and minimal media (DG, M9). 24 hr growth curve experiments infer growth from OD$_{600}$ nm values over 30 minute time points in 96 well microtitre plates. These experiments were used to compare how CA adaptation altered the growth rate and cell titre (OD$_{600}$nm) with respect to WTG0. Growth curves of each CA adapted strain were compared to WTG0 in the presence and absence of sub-inhibitory CA concentrations as listed in Figures 3.2 – 3.4. Overall, CA-adapted strains demonstrated robust growth in all rich media tested with or without sub-inhibitory concentrations of CAs added as compared to WTG0. Some minor exceptions in rich media growth were noted; QAC adapted strains demonstrated a 2-4 hr extension of the lag-phase (Figures 3.2–3.3) and CHX adapted strains demonstrated slightly higher stationary
phase OD\textsubscript{600nm} values (OD\textsubscript{600nm} = 0.2 unit gain; Figure 3.2-3.3) as compared to WTG0. Although these findings were statistically non-significant (p>0.001), the findings were reproducible. This suggests that both QAC adapted strains have delayed growth fitness in rich medium, in contrast to CHX which appears to reach higher cell titres than WTG0 at stationary phase.

In minimal media, the growth curves of nearly all CA-adapted strains with and without sub-inhibitory CA concentrations added to the growth media demonstrated non-significant (\(p\)-value = \(\geq0.001\)) delays in lag phase growth at 2-6 hrs as well as reduced OD\textsubscript{600 nm} values at stationary phase for all strains. Again, despite these findings being statistically non-significant (p>0.001), the findings were reproducible. The only exception was CHX adapted strains which maintained the same growth rate when compared to WTG0 (Figure 3.4). This suggests that CA addition in a more defined and lower osmolarity minimal growth medium has a significant impact on CA adapted strain fitness in contrast to rich media. An explanation for why minimal media is more detrimental to culture fitness is that CAs may adsorb to the diverse organic components in rich media reducing the antimicrobial concentration exposed to cells. In minimal medium, there are fewer components for CAs to adsorb onto upon increasing the exposure of cell and drug. Therefore, as observed in previous studies, \textit{E. coli} CA adaptation comes at fitness cost when cultures are exposed to CAs in defined minimal medium.

3.4. QAC adapted \textit{E. coli} have unstable tolerance phenotypes as compared to other CAs used for adaptation

Previous CA adaptation studies have examined the phenotypic ‘stability' of each CA-adapted strain when the selective pressure exerted by an antimicrobial compound is removed.
Typically, antimicrobial adaptation stability is determined from sub-culturing experiments, conducted over days to weeks, in growth medium lacking the antimicrobial compound (for examples refer to Méchin et al., 1999\textsuperscript{134} and Gradel et al., 2005\textsuperscript{135}). In this study, all 12 CA-adapted strains and the WTG0 were grown overnight without selection and repeatedly sub-cultured in medium lacking CA over a period of 10 days in LB broth. After each sub-culture day, the strains were re-exposed to media containing CAs at 50% of the final drug concentration they were cryopreserved at (refer to Table 3.1) and the results are summarized in Figure 3.5. \textit{E. coli} strains adapted to COL and CHX maintained a stable CA tolerant phenotype to their respective CAs over the entire 10-day period. In contrast, BZK and CET-adapted strains lost tolerance to their respective CAs, quickly after the second day of sub-culturing in LB medium (Figure 3.5). These results indicate that antimicrobial tolerance phenotypes of CA-adapted \textit{E. coli} BW25113 are stable for CHX and COL CAs, but not for QACs when selection is completely removed.
Figure 3.2. A summary of growth curves reached by each *E. coli* BW25113 subculture grown in LB broth (A-D) and MHB broth (E-H). Optical densities (y-axis) are reported as an average of each sub-cultured strain over the course of 24 hours (x-axis). A summary of gradual adapted *E. coli* BW25113 strains to sub-inhibitory concentrations of **A and E)** BZK, **B and F)** CET, **C and G)** CHX and **D and H)** COL are shown.
Figure 3.3. A summary of growth curves reached by each *E. coli* BW25113 subculture grown in TSB broth (A-D) and LB plus glucose broth (E-H). Optical densities (y-axis) are reported for each sub-cultured strain over the course of 24 hours (x-axis). A summary of gradual adapted *E. coli* BW25113 strains to sub-inhibitory concentrations of *A* and *E*) BZK, *B* and *F*) CET, *C* and *G*) CHX and *D* and *H*) COL are shown.
Figure 3.4. A summary of growth curves reached by each *E. coli* BW25113 subculture grown in DG broth (A-D) and M9 broth (E-H). Optical densities (y-axis) are reported as an average for each sub-cultured strain over the course of 24 hours (x-axis). A summary of gradual adapted *E. coli* BW25113 strains to sub-inhibitory concentrations of A and E) BZK, B and F) CET, C and G) CHX and D and H) COL are shown.
Figure 3.5 A summary of the initial stability experiment for each *E. coli* strain adapted to A) BZK, B) CET, C) CHX and D) COL are shown as compared to WTG0. The stability of each individual CA-adapted *E. coli* strain sub-cultured without selection in LB medium was measured over 10 days. On the x-axis, the growth outcome for each CA-adapted strain after each day when re-exposed to LB containing CA concentrations equal or greater than WTG0 MIC are plotted. All strains were compared to the WTG0 strain. In each panel, LB broth containing CAs at the following concentrations were measured: (BZK = 40µg/ml; CET = 50µg/ml; CHX = 1µg/ml and COL = 55µg/ml).
To follow up on these findings, the stability experiment was repeated, with one modification. At the very start of this experiment all CA-adapted strains were grown with CA selection to begin with (i.e. in media containing CA concentrations at 50% of the final concentration they were cryopreserved at) and then sub-cultured without selection in LB medium over a period of 10 days. At each daily time point, a complete broth microdilution AST panel was measured to precisely determine each strains respective CA tolerance (Figure 3.6). Interestingly, CA tolerant stability experiment AST results revealed that nearly all CA-adapted strains maintained a stable CA phenotype (>2-fold increase in MIC from WTG0) until day 10 of the experiment (Figure 3.6). On day 10, all CA-adapted strains began to show moderate reductions in MIC values that were still above the MIC of WTG0 with the exception of CHX adapted strains; CHX adapted strains demonstrated MIC value fluctuations of ≥ 8 µg/ml over the 10-day period that never decreased (Figure 3.6). This suggests that QAC and COL-adapted E. coli strains may be more prone to losing their CA-adapted phenotypes over time without CA selection in the medium as compared to CHX. This observation is consistent with other stability experiment findings (formaldehyde, glutaraldehyde/BZK compound, oxidizing compound, tar oil phenol, iodophor tested) that show changes in MIC values were only identified after a long period of drug removal and growth in media only\textsuperscript{135}. Unfortunately, most CA tolerant stability studies have been performed with QACs; in QAC-adapted strains (BZK) resistance was maintained after several passages in broth without QACs\textsuperscript{135}. Therefore, the CA tolerant stability experiments performed herein indicate that CA tolerance is quickly lost for QACs if CA tolerance is not added after cryopreservation. However, phenotypic CA tolerance is maintained over longer periods when CA tolerance of the strain is verified and then removed.
Figure 3.6 A summary of the stability experiment outcomes to determine MIC values for *E. coli* BW25113 strain adapted to A) BZK, B) CET, C) CHX and D) COL. Each CA-adapted *E. coli* subculture was grown in LB without CA selection for the indicated number of days plotted on the x-axis followed by AST to determine the MIC value of the culture to its respective CA (y-axis). The strains were cryopreserved at each daily time point in triplicate.
These stability experiments also indicate that QAC adapted strains have the least stable CA tolerance as compared to CHX and COL adapted strains suggesting that QAC adapted strains may have cellular alteration that are less permanent or less specific than those caused by CHX and COL due to their broad mechanism of action.

3.5. Whole genome sequencing (WGS) identifies different amounts of SNV to gene ratios within genes and non-coding regions of each CA-adapted *E. coli*

Previous studies of CA-adapted *E. coli* have demonstrated an association between down-regulated porin expression and increased efflux pump activity, specifically by QAC-adapted *E. coli*51,52,88,136. Lipid alterations have also been observed in other proteobacteria and summarized in a review by Tezel and Pavlostathis137. To examine what, if any, genetic alterations occurred in the genomes of CA-adapted *E. coli*, next generation WGS was performed on extracted genomic DNA isolated from each strain at 30X minimum sequencing coverage. 30X WGS coverage was deemed to be sufficient for SNV calling based on the size of the *E. coli* BW25113 genome (4631469 bp; 4.63 Mbp), N50 values (>100,000 bp) and >95% coverage as discussed by Chen et al 2015138. The N50, N75, and L75 values can also be found in Appendix ii. These values are an important measure of the quality of the assembled genome and based on fragmented contigs of different lengths. N50 values in this project exceeded 100MB ranging from 142,000-204,000 bps for all CA adapted strains, therefore, based on N50 and % coverage values the quality of our sequencing was acceptable for SNV calling139. SNVs were identified in each CA-adapted strain genome in both coding (gene) and non-coding (intergenic) regions after sequence alignment to the reference genome and comparison un-adapted BW25113 grown only in LB only for 40 subcultures
Based on the assembled genomes and using BW25113 sequence as reference template, the complete list of detailed SNVs found in each CA-adapted genome are provided in Appendix Table iii.

To address the final hypothesis of this study, which states:

“E. coli adapted to different CAs will confer similar genetic alterations (SNVs) in gene and pathways involving the outer membrane, lipid biosynthesis and trafficking, OMP/porins, and efflux pump systems”,

SNV analysis of each CA adapted strain (n=12) was performed using Geneious® software. SNV analysis was used to identify genes frequently targeted by SNVs in each CA adapted genome to identify if efflux, porin and lipid biosynthesis genes were specifically altered by CA exposure. This analysis focused on whether the same coding and non-coding SNVs occurred in E. coli strains adapted to the same CA and/or between different CAs used for adaptation. Tables 3.3 and 3.4 summarize total SNVs identified within more than one CA-adapted strains’ genome, in either coding or non-coding regions. Since genetic alterations may be random, repeatedly identified SNVs that occurred between the same CA adapted strain genomes or amongst the genomes of different CAs were focused on for this study. This approach was taken in an effort to identify genetic regions/genes that may be linked to each CAs specific mechanism of action and tolerance.

Prior to gene analysis, SNV frequency of occurrence trends were compared between all 12 CA-adapted strains. Based on crude SNV totals per genome, the greatest number of SNVs occurred within BZK (53-101 SNVs) and CET (97-110 SNVs) adapted strain genomes (Tables 3.3 and 3.4). CHX and COL-adapted strains had slightly lower total SNVs; CHX-adapted had 59-74 SNVs and
Table 3.3. A summary of the number of coding SNVs repeatedly identified for CA-adapted *E. coli* BW25113 strains.

<table>
<thead>
<tr>
<th>CA used</th>
<th>Strain name</th>
<th>Total number SNVs</th>
<th>Total number coding SNVs</th>
<th>Gene identified with SNV (total number of SNVs)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BZKG40R2</td>
<td>53</td>
<td>33</td>
<td>avtA (1): rpoC (1): rob (1): No gene in location (1)</td>
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<tr>
<td>CHX</td>
<td>CHXG20R1</td>
<td>60</td>
<td>18</td>
<td>cdaR (1)<em>: mlaA (2): eutE (1): yghQ (14)</em></td>
</tr>
<tr>
<td></td>
<td>CHXG20R2</td>
<td>59</td>
<td>30</td>
<td>marR (18)<em>: mlaA (7): yhiS (+insH) (5)</em></td>
</tr>
</tbody>
</table>

Bolded genes highlight genes with one or more SNVs identified in more than one CA adapted strain genome. Genes with asterisk highlight frameshift mutations.
Table 3.4. A summary of the number of SNVs identified in non-coding regions for adapted *E. coli* BW25113 replicate strains

<table>
<thead>
<tr>
<th>CA used</th>
<th>Strain name</th>
<th>Total number SNVs</th>
<th>Total number non-coding SNVs</th>
<th>Upstream genes (total number of non-coding SNVs per region)</th>
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</thead>
<tbody>
<tr>
<td>CET</td>
<td>CETG40R1</td>
<td>110</td>
<td>18</td>
<td><em>lacZ</em> (10): <em>IS1</em> (1); gfcA; <em>insA</em> (6); <em>mlaA</em>; <em>ydfC</em> (1)</td>
</tr>
<tr>
<td></td>
<td>CETG40R2</td>
<td>97</td>
<td>25</td>
<td><em>insA</em> (3); <em>ydiJ</em>; <em>pfkB</em> (13); <em>IS5</em> (9)</td>
</tr>
<tr>
<td></td>
<td>CETG40R3</td>
<td>96</td>
<td>30</td>
<td><em>insA</em> (3); <em>lacZ</em> (3); <em>ydiJ</em>; <em>pfkB</em> (12); <em>IS5</em> (9); <em>trmL</em> (3)</td>
</tr>
<tr>
<td>BZK</td>
<td>BZKG40R1</td>
<td>101</td>
<td>24</td>
<td><em>lacZ</em> (1): <em>lon</em> (11); <em>IS4</em> (6); <em>murB</em> (2); <em>fimA</em> (3); <em>rob</em>; <em>creA</em> (1)</td>
</tr>
<tr>
<td></td>
<td>BZKG40R2</td>
<td>53</td>
<td>20</td>
<td><em>lacZ</em> (2): tRNA (1); <em>murB</em> (2); <em>fimE</em> (1); <em>mdtM</em> (14)</td>
</tr>
<tr>
<td></td>
<td>BZKG40R3</td>
<td>58</td>
<td>3</td>
<td><em>ompX</em>; <em>rhaA</em> (3)</td>
</tr>
<tr>
<td>CHX</td>
<td>CHXG20R1</td>
<td>60</td>
<td>42</td>
<td><em>lon</em> (12): <em>mdfA</em> (9); <em>mlaA</em>; <em>yfdC</em> (12); <em>trmL</em> (1); <em>fimE</em> (8)</td>
</tr>
<tr>
<td></td>
<td>CHXG20R2</td>
<td>59</td>
<td>29</td>
<td><em>lacZ</em> (4): <em>IS5</em> (6); <em>trmL</em> (1); <em>fimE</em> (11); <em>fimA</em> (11); <em>yfdC</em> (1)</td>
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<tr>
<td></td>
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<td>29</td>
<td><em>lacZ</em> (4); <em>IS5</em> (9); <em>ynaJ</em> (2); <em>fimE</em> (16)</td>
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<tr>
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<tr>
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<td>COLG32R2</td>
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<td>6</td>
<td><em>mlaA</em>; <em>yfdC</em> (6)</td>
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<td>COLG32R3</td>
<td>40</td>
<td>1</td>
<td><em>acrE</em> (1)</td>
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</tbody>
</table>

N/A: no genes identified or not applicable. Bolded non-coding regions highlight non-coding regions with one or more SNVs identified in more than one CA adapted strain genome. More details on SNV locations and the intergenic non-coding region are summarized in Appendix iii.
COL-adapted 18-56 SNVs in examined genome sequences. This outcome may suggest that QAC exposure exerts greater genetic alteration as compared to intermediate CHX and COL SNV totals.

It is noteworthy that the ratio of coding SNVs per total SNVs (SNV coding/ SNV total) was highest for COL-adapted strains (0.89 – 1). SNV coding/total ratios for QAC-adapted strains were intermediate (0.67 – 0.84 for CET, 0.63 – 0.94 for BZK) in value and SNV coding/total ratios for CHX-adapted (0.29 –0.61) were lowest among comparison of all CA adapted strain genomes. These SNV coding ratio values suggest that CHX-adapted strain genomes have fewer SNVs in genes as compared to SNVs in QAC or COL-adapted strains, which may indirectly relate to how each drug/compounds’ mechanism of action exerts selective pressure to alter each genome. To elaborate, COL is believed to have the most focused mechanism of action, which primarily targets and alters lipid A biosynthesis and trafficking as reviewed by Olaitan et al. 2014. In contrast, QACs (BZK and CET) and CHX have multiple mechanisms of action focused not only on outer membrane disruption but also include protein denaturation (CHX and QACs) as well as oxidative damage (QACs). Therefore, the observation that more non-specific and non-coding SNVs occur in antiseptic adapted strains may not be as surprising given the many multiple mechanisms of action by these biocides.

3.5.1 Repetitive SNVs were most frequently identified in various lipid biosynthesis, trafficking, and modifying genes or non-coding regulatory regions of each CA-adapted E. coli strain

To further examine SNV differences between each CA adapted genome, Figures 3.7 and 3.8 were generated and showing network diagrams of all coding and non-coding SNVs that may affect promoter/ enhancer regions of the genome; identified genes and potential regulatory non-coding regions in both figures are grouped based on their function and operon locations.
Figure 3.7 Network diagrams illustrating coding and non-coding SNVs identified from WGS of CET (purple) and BZK (green) adapted strains. Legends in the panels indicate the meaning of symbols and arrows shown in the diagrams.
Figure 3.8 Network diagrams illustrating coding and non-coding SNVs identified from WGS of CHX (blue) and COL (red) adapted strains. Legends in the panels indicate the meaning of symbols and arrows shown in the diagram.
In Figures 3.7-3.8, SNVs repeatedly located in coding (filled circles) or non-coding (empty circles) regions within putative upstream regulatory/promoter regions of nearby genes are coloured and form ‘bullseyes’ that can easily be visualized as genetic regions with repetitive SNV targets for each genome. The more ‘bullseyes’ found on a network map, identify genes/ regulatory non-coding regions with SNVs which may be interpreted as less random SNV occurrence among independently CA adapted strains. Based on the hypothesis we expected that most repetitive SNV to gene/non-coding (or ‘bullseyes’) in Figures 3.7-3.8 would preferentially target efflux systems, porins, and lipid biosynthesis and/or lipid trafficking systems which were seen but were different for each CA used to adapt E. coli. More specifically, SNV ‘bullseyes’ were mainly observed in LPS biosynthesis or LPS trafficking pathways amongst all 4 CA-adapted strains and targeted genes located in the lpx genes (lpxCLM) msbA, mlaA or the waa operon (Figures 3.7-3.8).

Repetitive identification of deleterious SNVs (frame shifts, substitutions, indels) in mlaA in 2/3 CHX-adapted strains as well as BZK adapted strain (BKZG40R2) are important to note. MlaA is an outer membrane lipoprotein\textsuperscript{140} implicated in a retrograde phospholipid trafficking pathway; MlaA is involved in maintaining outer membrane lipid asymmetry by removing mis-localized outer leaflet phospholipids and transporting them back to the inner membrane. Since BZK and CHX perturbs LPS molecules as part of their mechanisms of action\textsuperscript{11}, deleterious mutations to mlaA suggest inhibition of phospholipid recycling and may be a mechanism of tolerance toward CHX and BZK agents. SNVs were also noted in the upstream non-coding region of mlaA single CA adapted strains CETG40R1, CHXG20R1, and COLG40R2 highlighting the potential value of mlaA and its upstream non-coding regions as a biomarker for multiple CA adapted strains.
Repetitive deleterious SNVs found in *waaY (rfaY)* in all 3 COL-adapted strains (Figure 3.9) suggest core lipid A glycosylation is significantly affected by prolonged *E. coli* COL adaptation. WaaY is a heptose specific LPS core kinase which catalyses phosphorylation of the second heptose residue in the inner core of LPS\(^{141}\). Deletions mutants of *waaY* in *E. coli* have previously shown enhanced resistance towards human cathelicidin antimicrobial peptide (LL-37) and suggest COL adaptation also targets this gene.

Repetitive SNVs were identified in essential genes *lpxC* (COL-adapted), *lpxM* (CET-adapted), *lpxL* (CET and BZK adapted) which are all located in unrelated (*trans*) operons of the *E. coli* genome but all that function as lipid A biosynthesis enzymes to maintain outer membrane integrity\(^{142}\). Most SNVs identified in *lpx* genes were non-deleterious (in frame codon substitutions) and altered a specific codon. For example, SNVs in LpxM altered codons D211A in 2/3 BZK adapted strains and LpxC had I186N alterations in 3/3/ COL adapted strains (Figure 3.9). Hence, SNV mutations in *lpx* genes may serve as useful predictive genetic markers of CA adaptation and enhanced CA tolerance in *E. coli*. It is important to note, that repetitive non-deleterious (codon substitutions) SNVs were identified within *msbA*, an ATP-dependent lipid A-core flippase\(^{143}\), in 2/3 BZK and 1/3 CET-adapted strain (CETG40R1) (Figures 3.7, 3.9). SNVs in BZK adapted genomes altered a single codon, V178G and in the single CETG40R1 altered A207V both were located in loop regions of the membrane transmembrane strand domain of the MsbA protein (Figure 3.9). *msbA* mutants have been previously reported to coincide with and rescue *lpxL* null mutations\(^{144}\) (Figures 3.7, 3.9). *msbA* over-expression has been shown to rescue *lpxL* null mutants by increasing LPS translocation of tetra-acylated lipid A\(^{145}\). SNVs in both *msbA* and *lpxL* were noted in genomes of single CA adapted strains CETG40R1 and BZKG40R1, verifying the
Figure 3.9 Repetitive SNVs identified within the coding regions of CA adapted strains shown as protein maps. Black and grey sections highlighted on each protein map indicate transmembrane strands and functional domains respectively. Coloured highlighting behind protein maps (shown as rectangles) indicate LPS biosynthesis and transport genes (yellow) and transcriptional regulation (blue) genes with SNVs shown as triangles (refer to in legend in the figure panel).
importance of these compensatory mutations in QAC adapted strains. The presence of nsbA-lpxL SNVs may suggest that acylated lipid A modifications may one option to contribute to QAC adaptation and reaffirm the co-association between these lipid biosynthesis genes.

3.5.2 WGS of CA-adapted strains identifies SNVs few CA adapted strain efflux pump genes and outer membrane assembly proteins

SNVs were identified within efflux pump gene acrB and the upstream non-coding region of mdfA in BZK, CHX, and COL-adapted strain genomes (Figures 3.7 and 3.8). However, only COL-adapted strains identified repetitive SNVs in acrB (Figure 3.8). MdfA is a major facilitator superfamily transporter that recognizes a broad range of cation-selective efflux pump, particularly chloramphenicol. AcrB is part of a dominant multipartite efflux pump system (AcrAB-ToIC) in resistance nodulation and cell division (RND) superfamily involved in the efflux of a wide variety of cationic and lipophilic molecules, including some QACs. The lack of repetitive SNVs identification within efflux pump genes and regulatory regions of more than one CA-adapted strain is not surprising, since efflux pump regulation commonly occurs at the RNA transcript and protein translation level in response to stressors, as noted in previous QAC adapted E. coli studies. Therefore, it is surprising that repetitive SNVs were identified within acrB in COL-adapted strains that appear to alter many codons (Figure 3.9) but the functional outcome is unclear as these codon alterations have not been experimentally examined to date; further experimental validation and testing will ideally reveal the functional outcome of these codon variants with respect to enhanced COL tolerance.

SNVs identified in fimbrial associated OMPs, ompX (BZKG40R3) and sfmD (CETG40R1) genes and OMP lipoproteins bamA (COLG40R3) and bamD (CETG40R1) of the β-
barrel assembly machinery (BAM) complex\textsuperscript{153} responsible for proper OMP folding and insertion were identified in only one CA-adapted strain genome but are worth mentioning (Figures 3.7-3.8). Previous mutational studies of \textit{ompX}, a porin that associates with type 1 fimbriae, showed that \textit{E. coli} strains had impaired motility, increased extrapolymeric substance (EPS) production (typically observed during biofilm formation), and increased tolerance to sodium dodecyl sulfate (SDS) and hydrophobic antibiotics\textsuperscript{154} when \textit{ompX} was mutated. The \textit{sfdD} gene is a chaperone-usher protein that is typically cryptically expressed in \textit{E. coli} but when expressed increases type fibrial formation and surface attachment to epithelial cells. Interestingly, in other the remaining 2 BZK adapted strains SNVs were noted in \textit{yghO} (BZKG40R1) and \textit{yghQ} (BZKG40R2) suggesting each BZK adapted strain may target alter different biofilm and/or surface attachment genes besides \textit{ompX} (Figure 3.7). The identification of SNVs in only one CA adapted strain may still be worthwhile to explore in future studies, as these may include SNVs that act as compensatory mutations to maintain fimbriae formation and OMP secretion. Further experimental analyses of these genes should be undertaken to determine how these gene mutations affect CA tolerance and how they may compensate for other SNVs within specific CA adapted strains.

\textbf{3.5.3 WGS of CA-adapted strains identify SNVs in previously identified antimicrobial resistance genes}

Many repetitive SNVs were also identified in coding/non-coding regions in CA-adapted \textit{E. coli} to different drugs that are known for their influence on antibiotic resistance genes. Repetitive SNVs in the antimicrobial resistance DNA-binding transcriptional regulator \textit{rob}, part of the \textit{marA/soxS/rob} regulon\textsuperscript{155}, was identified in 2/3 BZK and CET adapted strain genomes. Rob is a transcriptional activator known to bind the \textit{mar-sox-rob} regulons upstream of \textasciitilde 50 genes\textsuperscript{156}
involved in enhanced oxidative stress\textsuperscript{157}, solvent tolerance\textsuperscript{158}, antibiotic resistance\textsuperscript{159} and heavy metal tolerance\textsuperscript{160}. Interestingly, all rob SNVs identified from BZK and CET-adapted strain genomes altered the same codon W109G which is located in a protein region in between the helix-turn-helix (HTH\textsubscript{18}) and GRYL-like domain of the transcription factor (Fig. 3.9). Rob is known to regulate antibiotic resistance, as well as superoxide resistance\textsuperscript{157} and organic solvent tolerance\textsuperscript{158}, indicating that W109G may be an important variant and genetic marker for antimicrobials like QACs that have multiple mechanisms of action.

Repetitive SNVs were identified in 2/3 CET adapted strain \textit{yhdP} genes at different codons A401P and L1230Q (Figure 3.9); YhdP is a regulatory polypeptide necessary for maintaining the outer membrane permeability barrier in \textit{E. coli} by altering enterobacterial common antigen (ECA) levels and increases \textit{E. coli} susceptibility to VAN resistance when deleted\textsuperscript{161}. The association between ECA and QAC adaptation may be a useful direction to explore in future experiments.

An exciting and novel finding in 2 of 3 COL adapted strain genomes was the identification of deleterious (frame-shift causing) SNVs in \textit{sbmA}, an antimicrobial peptide transport gene (Figure 3.8). Bacterial mutants and knockouts of \textit{sbmA} have demonstrated resistance to antimicrobial peptides including microcin\textsuperscript{162,163}; therefore, \textit{sbmA} may be an intrinsically occurring \textit{E. coli} biomarker to predict COL resistance.

Finally, as we expected from previous COL resistance studies\textsuperscript{14}, repetitive SNVs in \textit{pmrA} (\textit{basS}) in 3/3 COL adapted genomes both specifically altered codon R39P of this two-component sensor kinase protein (Figure 3.9). Activation of the PmrAB two component system leads to the upregulation of the \textit{pmrCAB} and \textit{arnBCADTEF-pmrE} (also called \textit{pmrHFIJKLM-ugd}) operons that mediate the synthesis and transfer of phosphatidylethanolamine (PEtN) and 4-amino-4-deoxy-
L-arabinose (L-Ara4N), respectively, to lipid A. PEtN and L-Ara4N modifications of lipid A in COL-resistant strains are well documented and considered to be a primary mechanism of COL resistance. Their identification in 2/3 COL adapted strains suggest multiple pathways of COL adaptation and lipid A modification have occurred in this study, emphasizing its importance. SNVs were identified in DNA-binding transcriptional regulator yghO in 2/3 BZK (and BZKG40R1) adapted strain genomes (Figure 3.9). YghO influences antimicrobial resistance in bacteria grown as a biofilm, by an as yet unidentified functional mechanism (Figure 3.7). Biofilm formation is known to be a multicellular mechanism of antimicrobial resistance employed by E. coli as well as many other Proteobacteria to resist CAs as well as antibiotics.

3.5.4 WGS of CA-adapted E. coli strains identified most SNVs within transposon and prophage regions

SNV network diagrams also highlighted a number of transposases/prophages in coding and non-coding regions by all CA-adapted strain genomes examined herein (Figures 3.7-3.8). Monitoring SNVs in transposable elements is important since, SNVs may alter the movement and regulation of transposons and prophages, ultimately changing the rate of recombination and multidrug resistance gene transfer in bacteria. Repetitive SNVs were identified in genes and non-coding regions of insertion sequence (IS) transposase ins genes (e.g. insA, insH) and IS5 transposase interrupted LPS rhamnosyltransferase wbbL gene in CET, BZK, and CHX-adapted strains (Fig. 3.7-3.8). Transposases such as InsH1 often facilitate the mobilization and transmission of antimicrobial resistance genes and factors. Mutations and disruption of the wbbL gene interrupted by IS5 element have been shown to affect the synthesis of O-antigen of LPS, termed the rfb-50 mutation, which may contribute to antiseptic CA tolerance. Repetitive SNVs identified
in e14 prophage gene, stfP (ycfK) were also noted in CET and BZK-adapted strains (Figure 3.7). StfP has been shown to be upregulated in E. coli during antimicrobial drug exposure and associated with oxidative stress\textsuperscript{169}, which is known to be induced upon QAC exposure\textsuperscript{12}. An increase in SNVs within transposons and prophage genes after prolonged exposure to stress(ors)\textsuperscript{170–172} and antimicrobials\textsuperscript{173} in E. coli and other bacteria. In experiments conducted herein, prolonged QAC and CHX exposure also appears to increase SNV occurrences in transposable elements, likely due to the variable mechanisms of action by QACs and CHX. SNVs were also identified in non-coding regions adjacent to insertion elements, insA, insH1 in 2/3 CET-adapted strain genomes (Figure 3.7) and SNVs in cryptic prophage CP4-6 insH region of CHX-adapted strains (Figure 3.8). It is important to note that COL-adapted strain genomes had the lowest amount of repetitive SNVs in transposable element genes, which may suggest COL exerts less of generalized stress response as compared to antiseptics or exerts more focused selective pressure on lipid A as compared to cationic biocides.

3.5.5 WGS of CA-adapted strains identified SNVs within other essential genes and regulatory upstream non-coding regions

As discussed above, essential lipid biosynthesis genes, including lpxL and lpxM were also SNV targets, together with other outer membrane essential genes including bamA and bamD. However, essential genes associated with RNA/DNA replication, rpoC, rpoB and gyrB had repetitive SNVs in CET and BZK-adapted strains and essential 30S ribosomal subunit 1 gene rpsA required for protein translation had repetitive SNVs in CET-adapted strains. Mutations occurring in many of these essential genes we identified with SNVs after QAC/CHX adaptation, have also been noted in rpoC\textsuperscript{174,175}, rpoB\textsuperscript{176}, gyrB\textsuperscript{177}, and rpsA\textsuperscript{178} genes from various antibiotic resistant
bacteria. Although we did not observe increased cross-resistance to antibiotics among our CA adapted strains in this study, our findings suggest that antiseptic exposure conditions could eventually select SNVs in essential genes that enhance antimicrobial cross-resistance to therapeutic drugs.

Non-coding regions were included in the gene network analysis if they occurred within upstream regions of a gene. Without performing a transcriptomic or proteomic analysis of each CA-adapted strain (a future project direction), we cannot confidently determine how non-coding SNVs alter gene expression. In light of this, only repetitive SNVs occurring in upstream genetic regions will be discussed here. BZK adapted strain genomes, identified repetitive SNVs within non-coding regions that potentially regulate murB as well as coding gene mipA (Figure 3.7), and both participate in peptidoglycan (PG) assembly and outer membrane integrity. Over expression of mipA can result in altered PBP1 binding and compromised membrane integrity\textsuperscript{179}, while murB is an essential gene\textsuperscript{33} that catalyzes the second committed step of PG synthesis\textsuperscript{180}. To date, PG assembly genes have been speculated to participate in antiseptic tolerance, but have yet to be directly identified\textsuperscript{181} and highlighting the potential importance of these SNV findings.

As discussed above repetitive SNVs were also found upstream of ydiJ and pfkB in CET-adapted strains, both of which are associated energy generation as they encode for putative flavin adenine dinucleotide (FAD)-linked oxidoreductase\textsuperscript{182} and 6-phosphofructokinase 2 enzyme, respectively. It has been noted that deletion of either ydiJ or pfkB have no apparent effect on cell growth but their individual deletions may impact growth on particular sugars\textsuperscript{182,183}. It is worth noting here, that repetitive SNVs were located with in other energy generation pathway related genes, specifically in dmlA of CET adapted strain genomes (Figures 3.7, 3.9). DmlA is a D-malate
dehydrogenase that is essential for aerobic growth on D-malate\textsuperscript{184}; SNVs identified in *dmlA* alter a single specific codon N270T, located quite close to the conserved isocitrate dehydrogenase region (244-263 aa) of the protein, which may alter the function of this enzyme. It is unclear how coding and non-coding SNVs may alter the regulation these energy generation proteins and further experimental analysis is needed. Since many energy generating proteins reside within the inner membrane, it is not surprising that CA adapted strains would impact these systems, and some SNVs may be compensate for membrane changes during CA adaptation.

As noted above, greater emphasis was placed on repetitively occurring SNVs in genes or non-coding regions, but most SNVs we identified only occurred in a single CA adapted strain genome (1/3 genomes). Despite their single occurrence in one CA-adapted strain, some of these SNVs also occurred in strains adapted to different CAs; therefore, some are worthy of noting. The Venn diagram shown in Figure 3.10, highlights all of the repetitive SNVs identified in coding and upstream non-coding gene regions. Of particular note within the centre of the Venn diagram, is multiple antibiotic resistance (MAR) transcriptional regulator, MarR and outer membrane lipoprotein MlaA (discussed in section 3.5.1 above; Figure 3.10) and were identified in at least one adapted strain per drug (MarR) or within ¾ of the CA adapted strain genomes (MlaA). *MarR* is part of the *marRAB* operon that that negatively regulates the expression of its own operon and represses expression of genes with a *mar-sox-rob* regulon as reviewed by Wales and Davis. 2015\textsuperscript{7}. *MarR* regulates expression for a network of genes involved in antimicrobial resistance and includes outer membrane porin gene *ompF*, and efflux pump genes *acrAB-tolC*. Previous QAC and CHX-adapted studies have reported up-regulated efflux pump regulators linked to MarRA and
Figure 3.10. Venn diagram illustrating highly repetitive SNVs identified within one CA adapted strain genome and/or within multiple CAs. Bolded genes has SNVs in 2/3 or 3/3 strain genomes adapted to the same CA. Overlapping coloured circles denote genes that are shared between specific CAs. The diagram shows genes with identified SNVs for each CA adapted strain encircled by colour; BZK (Green), CET (purple), CHX (blue) and COL (red).
SoxS, which positively regulate dominant pump AcrAB-ToIC in *E. coli*\textsuperscript{84,117}. The marRAB operon is induced by antibiotics\textsuperscript{7} (such as salicylate, tetracycline, and chloramphenicol) and was previously shown to be upregulated in BZK-adapted strain transcriptomic studies\textsuperscript{50}. Most but not all marR SNVs were deleterious which suggest that the loss of this repressor should enhance mar-sox-rob regulon but future studies are needed to validate how marR mutants may act as a mechanism of CA tolerance. As previously noted at the start of this section, the majority of repetitive SNVs were found within the QAC-adapted strains as compared to CHX and COL-adapted strains. A number of SNVs were also identified as being shared across multiple CAs (CET, BZK and CHX) including *lon* (ATP-dependent protease responsible for degradation of misfolded proteins), *trmL* (methyltransferase) and a number of prophages/transposons (*cp4-6* and *insH*). The number of different types of genes identified between CET, BZK and CHX again supports the observation that multiple mechanisms of tolerance are developing as compared to COL-adapted strains. This would suggest that many random and compensatory genetic alterations are occurring specific for each CA we tested. Efflux pump acrB was also identified to SNVs within a single CA-adapted strain which also suggest a common tolerance mechanism triggered by BZK and COL.

In relation to the thesis hypothesis, this study showed that each CA used for *E. coli* K12 BW25113 adaptation resulted phenotypic as well as genotypic alterations that validated the hypothesis. The most highly repetitive SNVs were observed in transcriptional regulators associated with stress and antimicrobial tolerance, lipid A biosynthesis and LPS transport genes, a variety of outer membrane proteins, efflux pump *acrB*, and transposable elements with all three mechanisms together observed in some drug classes alone eg; BZK (*ompX; acrB* and *lpxL*). However, as stated earlier very few repetitive SNVs occurred at identical positions in the same
genes between strain genomes adapted to different CAs, where QACs BZK and CET has the closest relationship. This suggests that the types and number of highly repetitive SNVs may not be directly used to predict tolerance to a particular CA but with more experimental analyses may have use as a genetic antiseptic tolerance prediction tool.
CHAPTER 4. DISCUSSION

4.1. Summary of main findings

In summary, this project has identified that *E. coli* K12 BW25113, when exposed to increasing drug concentrations can produce CA tolerant strains that differ in antimicrobial tolerance, CA phenotypic stability and genotype depending on the type of CA used for initial drug adaptation. In terms of CA tolerant stability of each adapted strain, the loss of CA tolerance significantly differed depending upon the CA it was adapted to and if selection was initially verified in the starting culture at day 0 of the experiment. QAC adapted *E. coli* had the least stable CA-tolerant phenotypes after prolonged removal of drug and the addition of QAC at day 0 also had a big impact on the stability of the CA tolerant phenotype. Growth curve studies demonstrated that CA-adapted *E. coli* had similar growth phenotypes in rich media but reduced and delayed lag phase growth in minimal media. Furthermore, CA-adapted strains showed limited cross-resistance to antimicrobials and antibiotics unless they were closely chemically related, with the exception of CHX-adapted strains. Lastly, WGS identified few common SNVs within genes and non-coding regions of various CA-adapted *E. coli* but frequently identified genes with SNVs in operons and pathways related to outer membrane integrity and biosynthesis. These are important findings as the differences between each CA may be linked to its specific mechanism(s) of action. Hence, the findings of this study have provided new insights and genetic targets to explore related to CA adaptation and tolerance mechanisms.
4.2 Benefits and limitations of study

There are certain benefits to the approaches undertaken within this project. Laboratory based experiments for CA adaptation in a single antimicrobial susceptible reference *E. coli* K-12 strain allowed us to generate CA-tolerant reference strains and genomes free from other confounding antimicrobial cross-resistance mechanisms. This enabled the specific measurements of how exposing the same reference *E. coli* strain to various CAs could be compared. Undertaking this research in a laboratory based controlled environment also removed any external factors that may be found in the natural environment such a mobile genetic element acquisition that carry many antimicrobial, heavy metal and biocide resistance genes with them\textsuperscript{7,185}.

There were also limitations to conducting this type of project. The use of only one *E. coli* strain adapted to four drugs may make it difficult to compare to other *E. coli* strains as well as other bacterial species used in adaptation studies. However, our development of stable *E. coli* strains with a collection of single gene knockouts as part of the Keio\textsuperscript{33} and ASKA\textsuperscript{35} clone collections in Japan make this experiment extremely valuable for genetically validating other CAs and their identified SNV targets in future molecular experiments. Furthermore, only 4 CA drugs were included in the study and it would have been useful to include more CAs to expand CA classes and include other classes to further validate the CA patterns we observed. With regards to specific experiments, as lack of cross-resistance was observed with the CAs tested it would have been useful, time permitting to have tested more antibiotics in each respective class to ensure that the patterns being observed were consistent among other closely related drug types. Growth curve experiments could have also been improved by growing cells as biofilms, a more relevant *E. coli* growth physiology associated with antiseptic tolerance\textsuperscript{186} and explored different media
formulations to explore other growth conditions. Lastly, SNV data provided useful information regarding alterations in coding and non-coding regions, however, this data cannot determine if RNA transcripts, translated proteins or synthesized lipids are affected, which is where the future work of this project will begin and is ongoing by other members of the Bay lab. SNVs that cause frame-shifts of significant DNA sequence insertions are the only alterations that can be easily interpreted directly from sequenced genomes without corresponding transcriptomic, proteomic and/or lipidomic analysis.

4.3 Findings in relation to hypotheses

4.3.1 Discussion of the first thesis sub-hypothesis

The first sub-hypothesis of this study that has been addressed is as follows:

“The phenotypic stability of CA tolerance after the removal of CA selection over 10 days will gradually diminish CA tolerance phenotypes among CA-adapted E. coli strains”.

The results of CA tolerance stability testing confirm that QACs but not CHX or COL adapted strains lose their tolerance to CAs faster over a 10 day period when grown in media lacking their respective CA (Figure 3.5). When this experiment was repeated, MICs for CA-adapted strains remained stable however stability diminished by day 10 (except CHX). Previous studies have demonstrated a loss of CA tolerant stability over time by various proteobacteria including E. coli, suggesting that CA stability is temporary and prone to rapid loss\textsuperscript{134,135}. Considering the number of essential gene SNVs identified in QAC adapted strain genomes we examined this is likely due to the higher fitness costs exerted on E. coli to genetically adapt to phenotypically tolerate higher
QAC concentration exposures as compared to the un-adapted WT strain. Therefore, this hypothesis is only partially valid and is dependent on the type of antimicrobial used to initially adapt *E. coli*.

**4.3.2 Discussion of the second thesis sub-hypothesis**

The second thesis sub hypothesis stated:

“The growth fitness of CA-adapted strains will be compromised as compared to un-adapted *E. coli* strains”.

In order to characterize the dynamics of CA adaptation, it was important to quantify how the adapted strains compared to the original un-adapted WT strain by comparing planktonic cell growth. Previous adaptation studies have shown that CA adaptation came at a significant cost when the fitness of CA-adapted species (BZK\textsuperscript{58}, CHX\textsuperscript{58} and CTAB\textsuperscript{51}) was measured. However, some CA-adapted strains showed no difference in fitness and even gained fitness\textsuperscript{58,59}. Despite these conflicting studies, this project indicated CA adapted *E. coli* growth/fitness was only compromised in minimal media, suggesting that more defined and lower osmotic minimal media may be an important factor for CA adaptation. If we were to repeat this CA adaptation experiment again, only this time in minimal media it is likely that the CA adaptation time and final CA tolerance would be much lower as compared to rich medium. Therefore, based on this study we can conclude that this sub-hypothesis is valid for CA-adapted *E. coli* K-12.

**4.3.3 Discussion of the third thesis sub hypothesis.**

The third thesis sub-hypothesis states that:

“CA-adapted strains will enhance antimicrobial cross-resistance profiles as compared to the un-adapted *E. coli* strain”.

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Previous CA biocide cross-tolerance studies have reported low to moderate (2-6 fold) to high (20-50) increases in MIC values (2-6 fold) for CA adapted Enterobacteriaceae and *Pseudomonas* spp. strains when compared to their respective un-adapted strains\(^{50,60,62}\). In contrast, other studies have reported enhanced susceptibility to other biocides by CA-adapted Enterobacteriaceae strains\(^{61,63,87}\). Due to differences in experimental design and species/strain differences it is difficult to determine any unifying theme or trend for any particular CA in the studies cited above, including *E. coli* studies. There are three main variables that make comparison between these studies difficult: 1) strain/species differences, 2) drug concentration differences and exposure lengths, and 3) the chemical formulation of the CA used for strain adaptation. Although studies specifically exploring variables 1) to 3) have yet to be properly and independently conducted, the outcome of this study provides a framework to now test these experimental parameters. Regarding variable 1, my study shows that QACs adapted and COL-adapted strains conferred cross-tolerance to closely related chemicals expect CHX (Table 3.2). In this study, chlorhexidine hydrochloride (CHX) was used for CHX adaptation studies instead of chlorhexidine digluconate (CHG) which is more commonly used as a disinfectant and antiseptic in commercial formulations; in addition, CHX formulation is less soluble than CHG but releases very different counterions, e.g. HCl vs digluconate in solution. The counterion itself may also exert some selective pressure during adaptation as increased anionic charges may exert an adaptive osmotic regulatory pressure on the cells. Further experimental studies are needed to determine the validity of CA formulations. Hence, it can be suggested that biocide adaptation to one CA biocide does not necessarily confer cross-resistance to other CA biocides and highlights the importance of
understanding the mechanisms of biocide action, cellular targets, and biocide synergies/antagonism.

A number of studies analysing antibiotic cross-resistance of biocide adapted strains have demonstrated increased resistance to particular antimicrobials\(^{50,51,54}\). In this project, cross-resistance was observed in both the QAC strains for a few of the antibiotics tested, however what was interesting to note was that increased susceptibility of all 3 COL-adapted strains to nearly all of the antibiotics tested was observed suggesting a strain with a very permeant cell membrane, particularly due to COL adapted *E. coli* strain susceptibility to Gram-positive selective vancomycin. In previous studies, QAC and CHX biocide adapted *E. coli* strains have shown antibiotic cross-resistance to a number of antibiotics, including \(\beta\)-lactams and macrolides\(^{51,54}\) and third generation cephalosporins\(^{50,65,66}\) but my findings did not show increased resistance to these compounds (Table 3.2). It is possible that in some of these *E. coli* strains/ isolates, there may have already been some pre-existing antimicrobial resistance genes present to confer these phenotypes or perhaps the strains had prior antibiotic exposure before CA adaptation in these studies; the lack of any genotypic information for strains used in these experiments prevents any further speculation.

Altogether, the third sub-hypothesis appears to be valid for only QAC and COL-adapted strains but not for CHX-adapted strains based on our studies findings comparing antimicrobial cross-resistance/tolerance values collected under the same conditions. Again, understanding the mechanism of action is important here, particularly as variations in CA cross-tolerance profiles were observed in this study.
4.3.4. Discussion of the fourth sub-hypothesis of the thesis.

The fourth sub hypothesis of this thesis states:

“*E. coli* adapted to different CAs will confer similar genetic alterations (SNVs) in gene and pathways involving the outer membrane, lipid biosynthesis and trafficking, OMP/porins, and efflux pump systems”.

WGS performed on each CA-adapted strain in this study revealed that a number of SNVs were repetitively identified for each CA-adapted strain and between strains. The total amount of SNVs in coding and non-coding regions differed between each CA-adapted strain suggesting that CA mechanism of action influences different genetic alterations of *E. coli* K12. With respect to the fourth sub-hypothesis itself, repetitive SNVs were found in greatest frequency in transposable insertion element genes and non-coding regions as well as LPS modification/trafficking systems (*waaY, lpxCLM, mlaA, msbA*) of the CA-adapted strains. SNVs identified in genes and upstream non-coding regions in other pathways involving the membrane such as efflux pump systems (*acrB, mdfA*), outer membrane proteins (*ompX, bamAD, sfmD*), and peptidoglycan synthesis (*mipA, murB*) were also observed in some but not all CA-adapted partially supporting the fourth sub-hypothesis. CA adaptation also revealed repetitive SNV occurrence rates in genes/ non-coding regions associated with many transcriptional regulators (*marR, rob, pmrB, yghQ*) known for antimicrobial resistance gene regulation, biofilm formation (*yghQ*), DNA/RNA replication (*rpoBC, gyrA*) and protein translation/ folding (*rpsA, lon, trmL*) between various CA adapted strain genomes (Figures 3.7-3.8). However, these SNVs (including lipid biosynthesis/trafficking genes and transposases/prophages) were not consistently or repetitively identified in the same genes/ non-coding regions between all four sets of CA adapted strains which may interpreted in two ways.
The first interpretation is that therapeutic CA, COL exerts various amounts of selective pressure on *E. coli* K12 as compared to biocide CAs, CHX and QACs. Although all these compounds act to disrupt the cell membrane(s) and only COL is known to target lipid A specifically, where as QACs and CHX may target multiple anionic lipids and proteins forming a number of cellular targets for alteration. Greater amounts of SNVs located within transposable elements of QAC and CHX-adapted *E. coli* as compared to COL adapted strains may provide some evidence to support this. Transposable elements have been shown not only to regulate host gene expression but are often co-opted by the host to serve new cellular functions as reviewed by Navarro, 2017\(^{187}\) and ‘hop’ within the genome potentially causing deleterious mutations and altered gene expression\(^{187}\). In addition, prophages can also contribute in rapid genome editing of the bacterial DNA chromosome or extrachromosomal plasmids\(^{188}\), and also influencing rapid evolution of bacteria under stress\(^{188}\). A number of repetitive SNVs in similar regions of transposable elements were observed in CET, BZK and CHX-adapted strains, but very few were observed in COL-adapted strains. It is therefore entirely possible that transposable elements have been targeted in the biocide CA-adapted strains as a mechanism of stress response and creating a way to attempt to spread the possibility of antimicrobial resistance genes.

The second interpretation of the SNV data is that the mechanism of CA action and the stress it exerts on the cell plays a much bigger role in *E. coli* adaptation to CAs than assumed by the sub-hypothesis. As discussed in the results 3.5 section, QAC and CHX mechanisms of action\(^{11}\) may place greater pressure on the cell to overcome a number of stressors beyond the sub-hypothesis pathways: lipid alterations, efflux pump enhancements and porin downregulation. In contrast, COL resistance is know to be associated with lipid A modifications specifically\(^{14}\). QACs
and CHX both disrupt membranes, denature proteins but so far, only QACs have experimental evidence showing they increase reactive oxygen/ nitrogen species and inducing DNA damage\textsuperscript{189}. The identification of SNVs in genes associated with various transcriptional regulators, such as rob, marR, and pmrB (COL-adapted E.coli only) suggest that different stress pathways may be triggered and more transcriptional analysis such as RNA-seq or qPCR may shed more light on how these are regulated and perhaps give more insight into how mechanism of action or cellular targets influence CA genotypic adaptation.

Therefore, CA adapted E. coli strains do not share identical lipid, efflux and porin targets but do alter similar components related to these pathways that may be influenced by CA mechanism of action and/or the number of cellular targets they interact with to drive selective pressure. In relation to the overall hypothesis, E. coli K-12 strain BW25113 adapted to different CA classes (QACs, BGs, and PMX) has resulted in similar phenotypic and genotypic alterations (to a degree) and so therefore the project hypothesis can be concluded as being proven.

4.4 Future directions

Future work on this project and strains should focus on transcriptomic, proteomic and lipidomic approaches to determine whether specific genes, proteins, or lipids respectively have increased or decreased as a result of each CA adaptation. Furthermore, repeating CA adaptation experiments with different CA biocides eg: ALX, CPC, DDAB, and different bacterial strains eg; Pseudomonas spp., Acinetobacter baumannii could be performed to determine if phenotypic and genotypic alterations are consistently observed based on the findings for E. coli and the 4 CAs tested. It is also important that follow up work be undertaken further to the genetic targets
identified via WGS. One approach could be through site directed mutagenesis (SDM), where genes with specific SNV codon substitutions could be generated in clones and transformed into *E. coli* Keio collection library mutants of BW25113\(^3\) (single gene deletion mutants) to determine if phenotypic alterations are caused by particular genes of interest identified from the WGS findings. Constructs from the ASKA collection\(^4\), a plasmid library built in *E. coli* K-12 where each gene has been cloned in pCA24N (-) could be used for SDM experiments, to complement Keio mutants (assuming it is not an essential gene). Comparing the WT gene to the mutant gene (with SNVs we observed) by AST can determine if CA tolerance is altered and estimate an MIC value. This future work could provide further insight into the process of how CA adaptation occurs and identify phenotypic and genotypic targets in CA tolerance which are still uncertain.
References


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121. Ekiert, D. C., Bhabh, G., Isom, G. L et al. Architectures of lipid transport systems for the bacterial


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Appendix i A summary of chemicals and antimicrobials used in this study.

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## Appendix ii N50 and N75 values for WGS of CA-adapted strains

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**Appendix iii: Complete list of SNVs for all adapted *E. coli* BW25113 adapted strains.**
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No genes in location.
| CET1 | 275515 | 275515 | 1 | 1 | A -> G | ACT -> ACC | 246 | SNP (transversion) | Novel | 43.90% | 1.00E-14 | 270018 | mph = in+ | Undetermined | BW/RI/11_0256 |
| CET1 | 275642 | 275642 | 1 | 1 | K-Q | G-Q-C | 235 | SNP (transversion) | Substitution | 41.80% | 1.90E-13 | 270014 | mph = | BW/RI/11_0256 |
| CET1 | 275651 | 275651 | 1 | 1 | K-Q | G-Q-C | 235 | SNP (transversion) | Substitution | 41.80% | 1.90E-13 | 270014 | mph = | BW/RI/11_0256 |
| CET1 | 275649 | 275649 | 2 | 1 | T-M | C-A | G-Q-A | 235 | SNP (transversion) | Substitution | 37.45% | 2.70E-10 | 270011 | mph = in+ | BW/RI/11_0256 |
| CET1 | 275652 | 275652 | 1 | 1 | K-Q | G-Q-C | 246 | SNP (transversion) | Substitution | 41.80% | 1.90E-13 | 270014 | mph = | BW/RI/11_0256 |
| CET1 | 275647 | 275647 | 4 | 1 | L1 + AV | CAA + GCG | CTAAG + GCATG | 257 | SNP (transversion) | Substitution | 36.00% | 2.70E-10 | 270011 | mph = in+ | BW/RI/11_0256 |
| CET1 | 275643 | 275643 | 1 | 1 | MP-MQ | CGG-GAC | 246 | SNP (transversion) | Substitution | 37.45% | 2.70E-10 | 270011 | mph = in+ | BW/RI/11_0256 |
| CET1 | 275649 | 275649 | 2 | 1 | T-M | C-A | G-Q-A | 235 | SNP (transversion) | Substitution | 37.45% | 2.70E-10 | 270011 | mph = in+ | BW/RI/11_0256 |
| CET1 | 275642 | 275642 | 1 | 1 | K-Q | G-Q-C | 235 | SNP (transversion) | Substitution | 41.80% | 1.90E-13 | 270014 | mph = in+ | BW/RI/11_0256 |
| CET1 | 275647 | 275647 | 4 | 1 | L1 + AV | CAA + GCG | CTAAG + GCATG | 257 | SNP (transversion) | Substitution | 36.00% | 2.70E-10 | 270011 | mph = in+ | BW/RI/11_0256 |
| CET1 | 275643 | 275643 | 1 | 1 | MP-MQ | CGG-GAC | 246 | SNP (transversion) | Substitution | 37.45% | 2.70E-10 | 270011 | mph = in+ | BW/RI/11_0256 |
| CET1 | 275642 | 275642 | 1 | 1 | K-Q | G-Q-C | 235 | SNP (transversion) | Substitution | 41.80% | 1.90E-13 | 270014 | mph = in+ | BW/RI/11_0256 |
| CET1 | 275647 | 275647 | 4 | 1 | L1 + AV | CAA + GCG | CTAAG + GCATG | 257 | SNP (transversion) | Substitution | 36.00% | 2.70E-10 | 270011 | mph = in+ | BW/RI/11_0256 |
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| CET1 | 275642 | 275642 | 1 | 1 | K-Q | G-Q-C | 235 | SNP (transversion) | Substitution | 41.80% | 1.90E-13 | 270014 | mph = in+ | BW/RI/11_0256 |
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| CET1 | 275652 | 275652 | 1 | 1 | K-Q | G-Q-C | 235 | SNP (transversion) | Substitution | 41.80% | 1.90E-13 | 270014 | mph = in+ | BW/RI/11_0256 |
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| CET1 | 275647 | 275647 | 4 | 1 | L1 + AV | CAA + GCG | CTAAG + GCATG | 257 | SNP (transversion) | Substitution | 36.00% | 2.70E-10 | 270011 | mph = in+ | BW/RI/11_0256 |

**Note:** The table above represents a portion of the SNP (single nucleotide polymorphism) data extracted from the document, detailing the changes in nucleotide sequences and their implications. Each row provides the position, type of change, and significance of the change in the context of bacterial genomes. The columns include the accession numbers and other relevant identifiers to track the specific changes within the bacterial genomes. The data help in understanding the genetic variations and their potential impacts on bacterial traits and functions.
insH-1 (U)
Substitution
31.8% -> 38.2%
A -> G
BW25113_1687; BW25113_1723
244 -> 247
Substitution
T -> G
GW01103.7
GW01103.9
1
Substitution
218
GGAACGCACT -> AACTTATTGA
BW25113_1687; BW25113_1723
30.7% -> 31.3%
165
SNP (transition)
Substitution
0.518
1800547
1
57.70% -> 54.5%
BW25113_1687; BW25113_1723
2095224
1800572
2287018
Substitution
BW25113_1855
1.4E-319
1848366
2287008
9.70E-100
Substitution
insH-1 (U)
1933819
C -> A
27.20%
dmlA
1877011
AAT -> ACT
98.10%
115 -> 124
BW25113_1800
BW25113_0259
2
BW25113_1687; BW25113_1723
1800559
2
BW25113_1687; BW25113_1723
marR
151 -> 162
2286365
1800562
1933819
1.9E-148
TGTAG -> GCATC
2283593
AT -> TC
1876977
1800557
5
25.8% -> 29.0%
SNP (transversion)
BW25113_0259
305
A -> C
0.518
2.00E-75
0.361
2283618
1
A -> G
2283608
1615340
1
8.4E-132
43.8% -> 50.6%
ydiJ (U): pfkB (U)
6E-225
BW25113_1800
GCTCC -> CACTA
1
Substitution
1800536
Substitution
1802972
214
4.20E-163
marR
C -> TG
2
1
39.7% -> 41.2%
Frame Shift
31.70%
dmlA
1800529
Substitution
1876968
1
1
insH-1 (U)
wbbL +insH1
TTACCACA -> AACACTAT
Substitution
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1802991
insH-1 (U)
2286996
1
3
Frame Shift
2283626
1
A -> C
Insertion
ydjF
1802552
2286362
2286991
Truncation
N -> T
Substitution
insH-1 (U)
CC -> GT
1933819
insH-1 (U)
TTTTA -> ACACC
2283608
1615357
0
54.8% -> 55.6%
Substitution
1
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BW25113_1687; BW25113_1723
73
1800575
5
1
BW25113_1800
1
187 -> 188
2017013
43.6% -> 46.2%
3
3.60E-213
BW25113_0259
1879874
214
insH-1 (U)
2095224
2283617
1
173 -> 189
4.20E-97
0.515
0.304
1
34.7% -> 34.9%
TCG,CTC -> TTT,ATG
2286357
SNP (transversion)
TCG,CTC -> TTT,ATG
1
1800575
32.40%
55.20%
0
A -> T
1
Substitution
1
1879331
1613650
2
I -> F
5
1877006
1877006
Substitution
GAT -> GCT
1800555
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7
25.0% -> 25.1%
wbbL +insH1
123 -> 124
1802549
214 -> 218
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**Table 1: Comparison of Mutations**

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**Note:** Mutations are represented in bold, and the control gene is noted in parentheses. P-values are provided for each mutation comparison.
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3124728

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COL1 3793706 3793706 3 1 R -> S TCT -> GAG AGA -> TTC 83 -> 87 Substitutions Substitution 27.7% -> 31.4% 2.40E-63 3798682 wild type (O) 3798682
COL1 3793728 3793728 11 2 HIS1 -> MDL TCTAAAATTTA -> GAATTCGAGATTAAATGAGAT -> AGG,GGAGAATCTTC 58 -> 80 Substitutions Substitution 36.9% -> 42.6% 2.56E-58 3798570 wild type (O) 3798570
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COL1 3793739 3793739 1 1 K -> G T -> G GAG -> GGG 105 SNP (transversion) Substitution 0.629 1.54E-19 3798690 wild type (O) 3798690
COL1 3793745 3793745 1 1 D -> Y TC -> GA GAC -> TTC 100 -> 107 Substitutions Substitution 34.9% -> 31.0% 1.46E-78 3798690 wild type (O) 3798690
COL1 3793749 3793749 1 1 A -> G GCC -> CCT 105 Insertion Frame Shift 0.276 1.84E-70 3798681 wild type (O) 3798681
COL1 3793750 3793750 1 1 A -> G EGT -> GCC 105 SNP (transversion) None 0.259 7.16E-64 3798685 wild type (O) 3798685
COL1 3793756 3793756 1 1 K -> S TT -> AAG AAG -> CTG 190 Substitutions Substitution 32.6% -> 26.5% 7.59E-40 3798612 wild type (O) 3798612
COL1 3793768 3793768 1 1 T -> Y GC -> TAT GTA -> CAT 30 Substitutions Substitution 31.1% -> 29.8% 1.53E-32 3798645 wild type (O) 3798645
COL1 3794004 3794004 2 1 KEFY -> NRLK TTACACCT -> AGGAGCTT 37 Substitutions Substitution 26.5% -> 25.9% 2.56E-28 3797556 wild type (O) 3797556
COL1 3794095 3794095 9 1 RLE -> RIGA TACACAG -> CACCCCTT TCTTCATT -> GAAGCGGT 15 -> 45 Substitutions Substitution 31.1% -> 46.9% 2.15E-33 3797225 wild type (O) 3797225
COL1 3794109 3794109 1 1 K -> L A -> G TTT -> CTT 42 SNP (transversion) Substitution 0.548 7.76E-23 3797225 wild type (O) 3797225
COL1 3794131 3794131 2 1 A -> G 94 -> 95 Deletion Frame Shift 0.256 1.06E-17 3797220 wild type (O) 3797220
COL1 3794141 3794141 1 1 E -> A TG -> GCA 12 Substitutions Substitution 0.518 1.80E-07 3797315 wild type (O) 3797315
COL1 3794174 3794174 1 1 T -> A 56 Substitutions Substitution 0.417 7.76E-17 3797225 wild type (O) 3797225
COL1 3794230 3794230 1 1 KNY -> VRP TCAACCT -> CCGAAAAGAAGT -> AAGGTTTTCCCGCC 34 -> 46 Substitutions Substitution 27.8% -> 31.6% 1.76E-13 3797225 wild type (O) 3797225
COL1 3794147 3794147 14 1 KEFY -> SQFR CCGAAAAGAAGT -> AAGGTTTTCCCGCC 35 -> 39 Substitutions Substitution 25.6% -> 28.0% 1.84E-20 3797225 wild type (O) 3797225
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COL1 3794154 3794154 5 2 VM -> LQ GISAGC -> TGSGAG GCTATG -> CTCGAA 88 -> 92 Substitutions Substitution 25.9% -> 25.8% 1.38E-44 3797595 wild type (O) 3797595
COL1 3794186 3794186 2 1 T -> G SFATC -> TGATC 68 -> 73 Substitutions Substitution 30.8% -> 31.9% 1.10E-103 3797625 wild type (O) 3797625
COL1 3794201 3794201 6 1 WP -> QH ARAAE -> TGCTT GCTTT -> CAGCAT 63 -> 65 Substitutions Substitution 46.0% -> 47.7% 3.04E-90 3798752 wild type (O) 3798752
COL1 3794217 3794217 6 1 HC -> VR TEAA -> CAGT AAT,CAA -> AGT,AAA 59 -> 64 Substitutions Substitution 48.7% -> 49.9% 3.88E-52 3798735 wild type (O) 3798735
COL1 3794226 3794226 5 2 NS -> SV GAATT -> TACCT AAT,ACT -> AGT,AAA 58 -> 61 Substitutions Substitution 31.9% -> 37.9% 3.84E-57 3798746 wild type (O) 3798746
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COL1 3794233 3794233 1 1 A -> G 68 SNP (transversion) Substitution 0.280 4.00E-44 3797974 wild type (O) 3797974
COL1 3794243 3794243 1 1 A -> Y TCT -> YCA 59 SNP (transversion) Substitution 27.105 1.00E-42 3797975 wild type (O) 3797975
COL1 3794246 3794246 10 1 K -> R GC -> AAG 77 SNP (transversion) Substitution 1.20E-92 3.92E-65 3798696 wild type (O) 3798696
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COL1 4052785 4052785 1 1 T -> A 89 SNP (transversion) Substitution 1.28E-12 4.32E-09 wild type (O) 4052785

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