Understanding the genetics and evolution of antimicrobial resistance in *Escherichia coli*

by

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A thesis submitted to the Faculty of Graduate and Postdoctoral
Affairs in partial fulfillment of the requirements
for the degree of

Doctor of Philosophy

in

Biology

Carleton University
Ottawa, Ontario

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ABSTRACT

Antimicrobial resistance (AMR) has emerged as a global public health challenge. The central goals of my PhD research are to better understand AMR evolution, with the long-term goal of developing novel strategies to mitigate the problem of AMR. This involves gaining comprehensive insights into the evolutionary trajectories of resistance evolution, investigation of the availability of resistance mutations and their pleiotropic consequences, and identification of genetic targets for the prevention/slowing of resistance evolution.

In a systematic study, I have investigated the effects of different chromosomal quinolone resistance mutations, and the pleiotropic effects of such mutations in *Escherichia coli*. I identified 50 spontaneous resistance mutants on nalidixic acid, ciprofloxacin, and levofloxacin, with mutations in regions of *gyrA*, *gyrB*, and *marR*, which are known contributors of resistance in quinolones. Resistant isolates had increased resistance levels, widespread cross-resistance to other quinolones, and overall significant costs of resistance in the absence of antibiotic. To investigate novel strategies to combat AMR, I carried out a small RNA screen on a quinolone resistant *gyrA* background, to identify alternative drug targets. This screen led to the identification of 30 genes whose knockdown reduced the level of gyrase-mediated quinolone resistance in *E. coli*. Finally, to gain understanding of the network of genes contributing to intrinsic and phenotypic resistance, I have studied *E. coli*'s two-component signal transduction systems (TCS). TCSs are involved in bacterial responses to many stresses, including

antibiotics. I examined interactions between antibiotic stress and other environmental stressors in a set of eight TCS mutants. Mutants showed different types of responses to antibiotics and stressor+antibiotic combinations.

In this research, I have used novel approaches to understand bacterial resistance evolution and to combat AMR. On an applied level, these studies have implications for public health strategies. This research can help lead to better selection of appropriate antibiotics, alternative drug targets involved in resistance, and has prospects for development of new therapeutic approaches for combating AMR.

ACKNOWLEDGEMENTS

First and foremost, I would like to express my sincere gratitude to my supervisor, **Dr. Alex Wong**, for introducing an inexperienced PhD student to the fascinating world of bacterial evolution and genetics. I am always amazed and constantly inspired to see your critical thinking, scientific abilities, wisdom, and compassion. Thank you, for providing me this opportunity to pursue my research. Through your kind, patient and supportive supervision, today I am a better researcher with broader perspectives and overall a new evolved version of myself. It was my great honour to pursue a PhD degree under your supervision, and an incredible journey that I will proudly remember.

I would like to thank my committee members, Dr. Ashkan Golshani and Dr. Thien-Fah Mah, who have been extremely helpful and guided me with valuable comments and feedback that broadened my research avenues.

I would like to acknowledge all the people that made my whole PhD journey memorable, and I am grateful to every one of them. I could not have enjoyed my experience at Carleton without the camaraderie of the Wong lab. I am privileged to have worked with and learned from such a fantastic group of scientists. Special thanks to my lab members – Ahlam, Prabh, Melissa, Trevor, Janelle, Tom, Gaby, Jess, Andrew, Nicole, Amanda, Bryn, Leah, Katie, and Aaron. I am appreciative of all the conferences, dinners, celebrations, parties, and other outside the lab fun activities. The lab 220 and my

grad room 232 has been my home during all these years, and I will miss it dearly when I am gone.

Thank you, to my other Nesbitt friends – Chanchal, Gigi, Bhaswati, Brenda, Houman for being great support and for all the wonderful memories. I would also like to extend my appreciation to Nesbitt Biology staff at Carleton for all their support. Thank you, Laura, Lisa, Darlene, SarahAnne, Courtney, Ed, and Taye for making Nesbitt a happier place to work.

Thanks to my family, without whom I wouldn't be anywhere near where I am at now. To my husband, Mayank for all the support and for providing me with the unlimited supply of coffee just the way I like it. My little brother, Avichal for always supporting me. You are one person to whom I can speak my mind. Manda, thank you for being part of our family. You are such a beautiful soul. Last but not the least, *Papa* and *Mummy*. I cannot pen down anything to express my feelings of gratitude and appreciation for you both. I am here today, that is the fruit of your hard work, sacrifices, and blessings. Thank you for shaping me into the person I have become. Thank you for always supporting me. Thank you for everything! *Always papa's little girl* ☺

DEDICATION

This is for you – Manya

~ The apple of my eye ~

सर्वं ज्ञानं मयि विद्यते (ऋग्वेद)

Sarv gyanam mayi vidyate (RigVed)

(All that I have to learn is within me)

STATEMENT OF CONTRIBUTION

The thesis, “**Understanding the genetics and evolution of antimicrobial resistance in *Escherichia coli***” is composed of three studies.

Chapter 2, “The mutational landscape of quinolone resistance in *Escherichia coli*”, is the result of experiments primarily designed and carried out by myself with contributions from Dr. Alex Wong. Dr. Alex Wong contributed to the computational analysis of the whole-genome sequencing data. A version of this chapter is currently under review at PLOS One.

Chapter 3, “An sRNA screen for reversal of quinolone resistance in *Escherichia coli*”, is the result of experiments primarily designed and carried out by myself with guidance from Dr. Alex Wong. Dr. Vandana Sharma and Dr. Yohei Yokobayashi (Okinawa Institute of Science and Technology) provided the small RNA expression vectors. Dr. Aaron Hinz made the allelic constructs for λ -Red recombinase technique. A version of this chapter is currently under review at G3.

Chapter 4, “Two-component regulatory networks as determinants of stress responses and antimicrobial resistance in *Escherichia coli*”, is the result of experiments primarily designed and carried out by myself with contributions from Dr. Alex Wong.

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LIST OF ABBREVIATIONS

μg	Microgram
μl	Microlitre
μM	Micromolar
ATP	Adenosine triphosphate
bp	Base pair
C	Celsius
DNA	Deoxyribonucleic acid
g	Gram
l	Litre
M	Molar
mM	Millimolar
mm	Millimeter
ml	Millilitre
mRNA	Messenger RNA
ng	Nanogram
OD	Optical density
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
rpm	Revolutions per minute
WT	Wild-type
w/v	Weight by volume

Chapter 1

General Introduction

1.1 Antimicrobials and the evolution of antimicrobial resistance (AMR)

With the breakthrough discovery of sulphonamides in the 1930's, followed by penicillin in the 1940's, antibiotics revolutionized medicine. During 1950's and 1960's, the “golden age” of antibiotic discovery saw the development of many new drug classes. The golden age was followed by the development of synthetic or semisynthetic derivatives of natural substances with improved spectrum and pharmacokinetic properties (Debabov 2013; Ventola 2015). Multiple classes of antibiotics were launched during this era, including the β -lactam antibiotics, aminoglycosides, tetracyclines, macrolides, glycopeptides, polymyxins, and fluoroquinolones. Since 1990, pleuromutilins, lipoglycopeptides, oxazolidinones, and derivatives of older classes were launched. Antibiotic therapy made treatment of infections, surgery, transplantation, chemotherapy, neonatal care and prosthetic surgery safer and more efficient.

However, soon the inevitability of antimicrobial resistance (AMR) evolved. AMR evolution is part of an ancient process of natural selection wherein organisms producing, and organisms targeted by antibiotics, have been in an arms race against each other (D’Costa *et al.* 2006; Wright and Poinar 2012; Andersson and Hughes 2017). As most antibiotics are natural products (Allen *et al.* 2010), resistance genes are already present in the producers, and thus are easily shared with other bacteria, including potential human pathogens (Davies and Davies 2010). Clinically relevant resistance appeared soon after the introduction of mass-produced antibiotics (Barber and Rozwadowska-Dowzenko 1948), with the first multi-drug resistant (MDR) enteric bacteria found within a few years (Watanabe 1963). Thereafter, there has been increasing use, overuse, and misuse of antibiotics in healthcare, agriculture, and livestock.

Major health organizations like the Centers for Disease Control (CDC), the World Health Organization (WHO) and the European Center for Disease Prevention and Control (ECDC) have recognized MDR as an emergent global crisis (Blair *et al.* 2014; Roca *et al.* 2015). In 2017, the WHO issued a list of “Priority Pathogens” categorized into critical, high, and medium priority for which new antibiotics are urgently required. 9/12 pathogens listed are Gram-negative bacteria, including all three critical priority pathogens (carbapenem-resistant Enterobacteriaceae (CRE), *Acinetobacter baumannii*, and *Pseudomonas aeruginosa*). The Canadian Antimicrobial Resistance Alliance (CARA 2015) reported the prevalence of AMR in a range of infectious pathogens among Canadian patients from 2007-2016 and presented the most common pathogens as *Escherichia coli* (23%; resistant), *Staphylococcus aureus* (MRSA; 73%), and *P. aeruginosa* (15%). In addition to impacting public health, AMR is also burdening the

economy. The World Bank has predicted that AMR will increase global healthcare costs by \$300 billion to \$1 trillion per year by 2050 (World Bank 2018).

1.1.1 *Challenges of antibiotic discovery*

Drug discovery has slowed down dramatically, leading to an innovation gap with a dry antibiotic development pipeline (Palumbi 2001; Levy and Marshall 2004; Spellberg *et al.* 2008; Fischbach and Walsh 2009; Andersson and Hughes 2010; Davies and Davies 2010; Silver 2011; Fair and Tor 2014; Boeckel *et al.* 2014; Ventola 2015). The reasons for the inadequate supply of new antibacterials are complex, but are in part due to the failure of industry and academia to identify new drug leads in the past 20 years (Payne *et al.* 2007; Gwynn *et al.* 2010; Livermore 2011).

All antibiotics developed during the golden age were discovered by empirical screening via inhibition of growth assays, and their mechanisms of action were determined much later (Silver 2011). In the 1990s, an alternative approach was pursued, focused on synthetic small molecules that bind to different essential targets. This knowledge led to a huge expansion of industrial efforts in which companies ran high-throughput screens (HTSs) on dozens of different targets. For example, GlaxoSmithKline (GSK) (1995-2001) ran 67 *in vitro* target-directed screens against over half a million compounds in search of potential antimicrobials. 16 of these screens resulted in hits, where a “hit” is a chemically tractable, low-micromolar potency inhibitor of the target with 10 times selectivity for bacterial over mammalian enzymes. Of these initial hits, only five were potent candidate leads (a “lead” has the qualities of “hit” as well as

antibacterial activity due to inhibition of the *in vitro* targeted enzyme) with Gram-positive antibacterial activity. However, none of their sustained efforts generated successful new drug leads due to narrow spectrum of activity (Payne *et al.* 2007; Tommasi *et al.* 2015). At AstraZeneca (AZ), 65 HTS yielded 19 leads for Gram-positive infections, but no leads for Gram-negatives were identified (Tommasi *et al.* 2015). These screening approaches were profoundly unsuccessful for various reasons, including a lack of chemical diversity, the compounds were unable to cross Gram-negative bacterial cell walls, or a lack of correlation between activity in a biochemical assay and whole-cell activity. Such failures ultimately halted interest in novel antibacterials on the part of many major pharmaceutical companies.

Given the failure of HTS for antibiotic discovery, research strategies have turned to other novel methods to help bridge the innovation gap of antibiotic discovery. The use of whole-cell phenotypic screening approaches, for example, overcomes the limitation of a lack of translation of activity from biochemical assays, but this kind of screening requires follow-up mechanism of action studies. The screening of natural products can provide access to chemical diversity and it has unraveled known and new scaffolds (Cox *et al.* 2017). Thus, the search for novel stand-alone antibacterials with novel mechanisms of action continues to be an imperative measure to continue the fight against antibiotic-resistant bacterial infections. Challenges in drug discovery, along with accelerating drug resistance, mean that additional approaches should be explored.

Recent efforts have identified novel antibacterials using innovative technologies and conceptual approaches. With the help of novel culture methods, for example, the new

compound teixobactam was discovered that inhibits cell wall synthesis in pathogenic bacteria, such as *S. aureus* and *Mycobacterium tuberculosis* (Piddock 2015; Ling *et al.* 2015). Other strategies include antibiotic adjuvants or antibiotic potentiators (as discussed in chapter 3 of this thesis) that can either block bacterial resistance mechanisms or enhance the antimicrobial action of a drug. The pursuit of antibiotic adjuvants helps to avoid the re-discovery of known drugs, and has the advantage that it lessens the continuous effort in the challenging and expensive identification of new targets that are essential for bacterial survival (Kalan and Wright 2011; Farha and Brown 2013; Gill *et al.* 2015; Brown 2015; Wright 2016; Melander and Melander 2017). Some examples of antibiotic adjuvants include clinically-approved β -lactamase inhibitors that restore the activity of β -lactam antibiotics (Drawz *et al.* 2014). Inhibitors of aminoglycoside-modifying enzymes (AME) have also been developed, but none of them have been clinically approved (Ramirez and Tolmasky 2010; Labby and Garneau-Tsodikova 2013). Another set of potential adjuvant targets are the signaling and regulatory pathways used by bacteria to detect antibiotics and in turn activate resistance mechanisms. Some examples include inhibition of BlaR1 or MecR1 mediated identification of β -lactam antibiotics in *S. aureus* (Boudreau *et al.* 2015), interference with the SOS DNA repair pathway (Alam *et al.* 2016), and interference with the two-component signaling system (TCS) pathways that have a role in antimicrobial intrinsic resistance or tolerance (as discussed in chapter 4 of this thesis) (Worthington and Melander 2013).

1.2 Classification of Antibiotics

Antibiotics, of natural or synthetic origin, can be classified by various criteria, including their activity spectrum, origin, or mode of action. On the basis of spectrum, antibiotics can be active against a wide range of Gram-negative and Gram-positive bacteria (broad-spectrum antibiotics), for example tetracyclines and chloramphenicol. Others have activity against limited range of bacterial species (narrow-spectrum antibiotics), for example macrolides and vancomycin (Campbell *et al.* 2001; Brandis *et al.* 2014).

Antibiotics attack infections either by inhibiting growth of (bacteriostatic), or killing (bactericidal), the infecting bacteria. Antibiotics usually target essential bacterial pathways that are sufficiently different from or not present in human cells to avoid toxicity. The common targets of antibiotics are cell wall synthesis, protein synthesis, folate synthesis, DNA replication, and RNA transcription (Figure 1.1, Table 1.1).

1.2.1 Cell wall synthesis inhibitors

β -lactams are the largest group of antimicrobials used worldwide, and include penicillins, cephalosporins, carbapenems, and monobactams. These antibiotics are cell wall synthesis inhibitors and share a common chemical structure, the β -lactam ring. They target bacterial cell wall biosynthesis by binding and inactivating penicillin-binding proteins (PBPs) involved in cross-linking peptidoglycan (Walsh 2000; Poole 2004). They are commonly used to treat various bacterial infections caused by Gram-positive (penicillins) and -negative bacteria (cephalosporins, carbapenems, monobactams).

Figure 1.1 Mode of action of some commonly used antibiotics and their mechanisms of resistance (Crofts *et al.* 2017).

1.2.2 Ribosomal inhibitors

Some antibiotics target the ribosome and inhibit different stages of protein synthesis. Some examples are aminoglycosides (such as kanamycin, amikacin, gentamicin, tobramycin, and streptomycin) that disrupt protein synthesis by binding to the 30S subunit of the ribosome, blocking translation (Jana and Deb 2006). Tetracyclines are broad-spectrum, bacteriostatic antibiotics that bind to a different site on the 30S ribosomal subunit and block elongation during translation. Macrolides are bacteriostatic antibiotics, mainly effective against Gram-positive bacteria. They bind to the polypeptide

exit tunnel in the 50S subunit of the ribosome, thus blocking peptidyl transfer and protein elongation. Chloramphenicol is a broad-spectrum bacteriostatic antibiotic that binds to the 50S ribosomal subunit, inhibiting the peptidyl transferase of the ribosome (Walsh 2000).

1.2.3 Folate synthesis inhibitors

Bacteria are dependent on folate synthesis for production of nucleic acids, making the enzymes of this pathway targets for antibiotics. Sulfonamides and trimethoprim are important folate synthesis inhibitors, which inhibit the dihydropteroate synthetase (DHPS) and dihydrofolate reductase (DHFR) respectively. These antibiotics possess broad spectrum activity and are helpful in the treatment of urinary and respiratory tract infections (Hawser *et al.* 2006).

1.2.4 DNA gyrase inhibitors

Fluoroquinolones (FQs) are broad-spectrum antibiotics that bind to the type II topoisomerase enzymes DNA gyrase and topoisomerase IV (Topo IV). They are involved in the control of DNA supercoiling, transcription, and chromosome decatenation (Hooper 2001a; b). FQs stabilize the DNA-gyrase complex after cleavage of the DNA and thereby arrest DNA replication, transcription or decatenation, eventually leading to cell death (Ince and Hooper 2003).

1.2.5 *RNA polymerase inhibitors*

Rifamycin antibiotics are a group of antibiotics that bind to the β -subunit of the bacterial RNA polymerase (RpoB), and sterically inhibit the elongation of the newly synthesized mRNA transcript (Conde and Lapa E Silva 2011). Rifampicin is the most popular broad-spectrum bactericidal antibiotic of the rifamycin class and is used in the treatment of tuberculosis (Walsh 2003).

1.2.6 *Membrane inhibitors*

Antimicrobial peptides are considered as potential therapeutic agents as they are not commonly administered. The peptides often interact with the bacterial cytoplasmic cell membrane and disrupt the membrane (Hancock and Sahl 2006). Polymyxins are a class of cyclic peptides (such as colistin) that interact and disrupt the outer membrane (lipopolysaccharide) of Gram-negative bacteria, leading to increased permeability and cell death.

Table 1.1 Different antibacterial mechanisms of action and their targets.

Antibiotic class	Molecular target	Function inhibited	Examples
β -lactams	PBPs	Peptidoglycan synthesis	Penicillin, Ampicillin, Cephalosporins, Carbapenems
Aminoglycosides	rRNA of 30S ribosome subunits	Protein synthesis	Gentamicin, Tobramycin, Amikacin, Streptomycin, Kanamycin
Trimethoprim, Sulphonamides*	DHFR, DHPS	Folic acid synthesis	Trimethoprim, Sulfamethoxazole
Quinolones	Topoisomeraes (DNA gyrase, topoisomerase IV)	DNA replication	Nalidixic acid, Fluoroquinolones: Ciprofloxacin, Levofloxacin, Moxifloxacin
Macrolides	rRNA of 50S ribosome subunit	Protein synthesis	Erythromycin, Clarithromycin, Azithromycin
Tetracyclines	rRNA of 30S ribosome subunit	Protein synthesis	Tetracycline
Glycopeptides	D-Ala-D-Ala of lipid II	Peptidoglycan synthesis	Vancomycin, Teicoplanin
Chloramphenicol	rRNA of 50S ribosome subunit	Protein synthesis	Chloramphenicol
Rifamycins	RNA polymerase	RNA synthesis	Rifampicin
Polymyxins	Cell membrane	Disrupt cell membrane	Polymyxin B, Colistin

*Because these antibiotics target two different steps in the same synthesis pathway, they are often used synergistically.

1.3 Resistance mechanisms

There are primarily two types of AMR, namely, intrinsic and acquired. Intrinsic resistance or innate resistance involves all of the inherent characteristics of a particular microorganism, such as physiological and genetic features, that contribute to its baseline resistance to antibiotics. Classically, intrinsic resistance is defined by the inherent capabilities of a bacterium to be resistant to certain antibiotics, due to impermeability of their cell wall, lack of target, or upregulated MDR efflux pumps (Nikaido 1989; 1994; Alekshun and Levy 2007; Fernández and Hancock 2012). Gram-negative bacteria, for example, are insensitive to many clinically effective antibiotics because many molecules cannot penetrate their double membrane cell wall structure (Cox and Wright 2013).

Studies of the intrinsic resistome have indicated a number of additional, non-conventional genes that can contribute to relative intrinsic resistance level and complement intrinsic resistance (Wright 2010; Cox *et al.* 2014). These genes fall under two categories of genes. The first category includes those genes whose inactivation can make bacteria more resistant to antibiotics, such as a mutation in a transcriptional repressor of an MDR efflux pump (Martinez and Baquero 2000; Martinez *et al.* 2007; 2011a; Fajardo *et al.* 2008).

The other set of elements are those whose inactivation make bacteria more susceptible to antibiotics. For example, in *P. aeruginosa* the inducibility of chromosomal β -lactamase limits the efficacy of several β -lactam antibiotics that are effective in other bacterial species (Martinez 2012). More recently, apart from the aforementioned classical resistance genes, several other elements from a variety of functional categories have been recognized to contribute to intrinsic resistance, including elements of bacterial general

metabolism that respond to the presence of molecular signals, such as an antibiotics or another environmental stimuli (Fajardo and Martinez 2008; Fajardo *et al.* 2008; Girgis *et al.* 2009; Davies and Davies 2010; Sengupta *et al.* 2013).

One such metabolic situation where the bacterial intrinsic resistome is unveiled is when bacteria gain phenotypic resistance under specific growth conditions. Bacterial phenotypic resistance is a relatively unexplored area, and is a non-inherited, temporary survival mechanism for a bacterium to respond to surrounding environmental cues, e.g., stress, temperature, nutrient conditions, and antibiotics (Levin and Rozen 2006; Fernández and Hancock 2012; Olivares *et al.* 2013; Hughes and Andersson 2017). Bacteria display rapid metabolic downregulation or upregulation by alterations in their transcriptome, depending on the extracellular environmental conditions. Certain environmental stress responses in bacteria provide them with temporary tools to survive and increase the chances to resist antibiotic exposure, for example, tolerance to polymyxins or antimicrobial peptides can be mediated by low concentrations of magnesium or calcium (McPhee *et al.* 2003; Mulcahy *et al.* 2008). Therefore, phenotypic tolerance under metabolic control allow a certain proportion of the bacterial population to survive and become resistant despite the presence of antibiotics (Martinez and Rojo 2011).

Two-component signal transduction systems (TCSs) (chapter 4 of this thesis) constitute the most prevalent form of signal transduction used by bacteria to respond to changes in their surroundings. TCSs manage the repertoire of responses to fluctuating environmental conditions (Martinez *et al.* 2009). TCSs are involved in survival,

physiology, virulence, and antibiotic resistance in pathogenic bacteria (Barrett *et al.* 1998; Stephenson and Hoch 2002; Rasko *et al.* 2008). TCSs include multistep phosphorelay cascades that are arranged in a complex array of networking circuits, which can detect and respond to different environmental stimuli and regulate large numbers of genes (Stock *et al.* 2000; Mascher *et al.* 2006; Gao and Stock 2009). TCSs in *E. coli* regulate the gene expression of many genes. For example, the EnvZ-OmpR TCS regulates expression of the outer membrane porin proteins OmpC and OmpF in response to osmotic stress and other environmental signals. While both OmpC and OmpF require OmpR for high-level expression, the extent to which each porin is expressed depends on the phosphorylation state of OmpR. At high-osmolarity conditions OmpR is phosphorylated by the sensor kinase EnvZ, resulting in increased transcription of *ompC* and repression of *ompF* (Aiba and Mizuno 1990; Cai and Inouye 2002). At low-osmolarity conditions, the expression pattern reverses. As porins serve as a gateway for many beneficial compounds to passively diffuse into the periplasm, small toxic compounds, including many antibiotics, such as β -lactams and other hydrophilic antibiotics (tetracycline, chloramphenicol, and fluoroquinolones) can also use these channels to gain entry into the cell. Thus, a decrease in porin-mediated permeability caused by mutational inactivation of porins or alteration of the channel properties, can increase resistance to many different antibiotics, although often at a cost (Barrett *et al.* 1998; Nikaido 2003; Hirakawa *et al.* 2003; Fajardo *et al.* 2008; Bem *et al.* 2015). For example, removal of OmpC and OmpF in *E. coli* can cause a 20% reduction in exponential growth rate (Knopp and Andersson 2015). Similarly, in *P. aeruginosa*, the PhoP-PhoQ TCS affects susceptibility to antibiotics (aminoglycosides, antimicrobial

peptides, and polymyxin B) as well as virulence by modifying the LPS layer (Macfarlane *et al.* 2000; Gooderham and Hancock 2009). Thus, investigating bacterial TCS and their contributions to intrinsic resistance will help to understand the capacity of bacteria to evade antibiotics, and may help to define novel targets whose inactivation can influence bacterial drug susceptibility.

In acquired resistance, a formerly susceptible microbe can become resistant either by incorporating new genetic material or as a result of mutations (Martinez *et al.* 2009). Acquired resistance is typically the primary concern when considering clinical breakthrough AMR. Acquired resistance can evolve through the accumulation of *de novo* mutations, typically in chromosomal genes, or via horizontal gene transfer (HGT) of resistance genes. Mutation driven resistance can render the bacterium resistant by modifying the antibiotic target, as is the case for FQ resistance mutations in *gyrA*, which codes for the A subunit of DNA gyrase (Bagel *et al.* 1999; Piddock 1999), or rifampicin resistance mutations in *rpoB*, which codes for the β - subunit of RNA polymerase (Campbell *et al.* 2001). Resistance can also be achieved by reducing uptake; mutations that reduce expression of, or that alter the structure of membrane porins, cause resistance by this mechanism (for example, *marR* causing activation of efflux) (Nikaido 1989). Similarly, increased drug efflux can also lead to resistance - mutations leading to overexpression of the AcrAB-TolC efflux pump in *E. coli* confer resistance to FQs, phenicols, and some β -lactams (Nikaido 1996; Komp-Lindgren *et al.* 2003).

The general known acquired resistance mechanisms are:

1. Reduced influx of the antibiotic. Antibiotic molecules need to cross the cell envelope to reach sufficient intracellular concentrations. They can enter the cell via outer membrane porins (OMPs) but chromosomal mutations in the genes regulating OMPs (for example, OmpF in *E. coli*) can prevent or reduce the entry of the antibiotic into the cell (Nikaido 2003; Fernández and Hancock 2012). Resistance mediated by this mechanism is often less specific and effective against multiple antibiotics.
2. Increased efflux of the antibiotic. Bacterial MDR pumps transport various structurally unrelated compounds out of the cell. Amino acid alterations, deletions or insertions in genes (e.g. the *mar* operon and the AcrR repressor) regulating MDR pump expression (e.g. AcrAB-TolC pump in *E. coli*) can increase the efflux of antibiotics, such as ciprofloxacin, tetracycline and chloramphenicol out of the cell (Poole 2005). Some genetic changes can also lead to overexpression of the pump, increasing the level of resistance (Baucheron *et al.* 2004; Swick *et al.* 2011). In *E. coli*, the OmpF influx and AcrAB-TolC efflux systems are co-regulated by the global transcriptional regulator MarA.
3. Target modification. The antibiotic target binding site can be altered by mutation, diminishing the affinity of the antibiotic towards the target. This resistance mechanism typically arises by chromosomal mutations. For example, mutations in the target encoding gene *gyrA* can increase resistance to ciprofloxacin (Heisig and Tschorny 1994; Piddock *et al.* 1999; Marcusson *et al.* 2009). In *M. tuberculosis*, a

single point mutation in *rpoB* causes rifampicin resistance (Garibyan *et al.* 2003; Lambert 2005).

4. Modification or inactivation of the antibiotic. Some bacteria produce antibiotic modifying enzymes that inactivate/modify antibiotics in ways that reduce their toxicity. The aminoglycoside-modifying enzymes can N-acetylate (acetyltransferases), phosphorylate (phosphotransferases), or adenylate (nucleotidyltransferases) the aminoglycoside molecule and thus inactivate the antibiotic target binding (Smith and Baker 2002; Wright 2005). Resistance to β -lactams such as penicillin and cephalosporin is conferred by catalyzing the cleavage and inactivation of their β -lactam ring mediated by β -lactamases (Poole 2004; Alekshun and Levy 2007).

Resistance by HGT often involves the acquisition of external genetic material that have evolved in other species and that are easily transferred between different organisms. The notable transferable mobile genetic elements consist of plasmids, transposons, insertion sequences, phages, and chromosome cassettes (Simillie *et al.* 2010; Stokes and Gillings 2011). In methicillin resistant *S. aureus* (MRSA), for example, the acquisition and expression of the *mecA* gene (coding the penicillin-binding protein-2a) on its chromosomal DNA spreads resistance to β -lactam antibiotics. All MRSA strains contain the staphylococcal cassette chromosome (SCC*mec*), which harbours the *mecA* gene and is surrounded by mobile genetic elements Tn554 and IS431 (Malachowa and DeLeo 2010). The foreign piece of DNA can be acquired by the mechanisms of transformation (uptake of DNA from the surroundings), transduction (transfer of resistance genes via

bacteriophage), or conjugation (transfer of plasmids with resistance genes from one bacterium to another via a pilus) (Ochman *et al.* 2000; Popa and Dagan 2011).

1.4 Fluoroquinolones (FQs)

The FQs are among the most important and frequently prescribed synthetic antibiotics. They have broad-spectrum activity, good pharmacokinetic and pharmacodynamic properties, and rapid bactericidal activity. Nalidixic acid (NAL) was the first quinolone to be discovered in 1962 as an impurity generated during the manufacturing of the anti-malarial drug chloroquine, and was found to have antibacterial activity (Leshner *et al.* 1962; Mitscher 2005). NAL was initially used for the treatment of urinary tract infections (UTIs) caused by Gram-negative bacteria. However, limited clinical use, high toxicity, high minimum inhibitory concentration (MIC), and rapid resistance evolution spurred to the development of improved quinolones by modifications to the core nucleus (Emmerson and Jones 2003). Subsequently, in the 1970's, the additions of a 6-fluoro modification, and a 7-piperazine ring resulted in improved derivatives of NAL (Wolfson and Hooper 1985; Appelbaum and Hunter 2000) (Figure 1.2). The addition of fluorine increased drug penetration and drug target potency (Andersson and MacGowan 2003; Mitscher 2005). Following generations of FQs generated an improved spectrum of activity and pharmacokinetic properties. Examples include 2nd generation ciprofloxacin (CIP), 3rd generation levofloxacin (LEVO), and fourth-generation moxifloxacin (MOX). Some of the novel FQs currently in clinical trials, showing superior antimicrobial

activity, include avarofloxacin, delafloxacin, finafloxacin, zabofloxacin, and nemonoxacin (Guan *et al.* 2013; Kocsis *et al.* 2016).

Figure 1.2 The quinolone core nucleus and the other structural modifications in the development of nalidixic acid, ciprofloxacin, and levofloxacin.

CIP, or 1-cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-7-(1-piperazinyl)-3-quinoline-carboxylic acid, is a second-generation FQ. It is a clinically relevant antibiotic and is considered to be a highly prioritized important antimicrobial (WHO 2017). It is one of the most successful and widely used antibiotics with improved spectrum, bioavailability, and broad-spectrum activity against most Gram-negative (*Enterobacteriaceae*,

Haemophilus influenza, *Neisseria* spp., *P. aeruginosa*) and some Gram-positive (*S. aureus*, *Streptococcus pneumonia*) bacterial pathogens. CIP is used to treat a wide range of bacterial infections including UTIs, gastrointestinal infections, respiratory tract infections, sexually transmitted diseases, and skin or bone related infections (Wolfson and Hooper 1985; Ball 2000; Guan *et al.* 2013).

FQs are DNA damaging agents that act by targeting two essential bacterial enzymes, DNA gyrase and DNA topoisomerase IV (Topo IV). Both target enzymes are type II topoisomerases that control the superhelicity of DNA to maintain a functional DNA structure (Figure 1.3) (Schoeffler and Berger 2008; Pommier *et al.* 2010). DNA gyrase is an ATP-dependent tetrameric protein composed of two GyrA and two GyrB subunits (A₂B₂), encoded by the genes *gyrA* and *gyrB*, respectively (Hooper and Jacoby 2015). DNA gyrase introduces negative, and relaxes positive supercoils generated by the separation of the strands during DNA replication ahead of the enzyme helicase. Gyrase catalyzes a double stranded break in the supercoiled DNA, passages another strand of the supercoiled DNA through it, and finally re-seals the broken DNA strands (Hooper 1999; Jacoby 2005). GyrA is involved in the double-strand breakage and reunion reaction, whereas GyrB is responsible for the ATPase activity of the enzyme. Topo IV is also a tetrameric protein (C₂E₂), encoded by the genes *parC* and *parE* respectively. Topo IV relaxes both positive and negative supercoils and is primarily responsible for decatenation of interlinked daughter chromosomes at the end of replication process prior to cell division (Drlica and Zhao 1997; Hooper 2001; Schoeffler and Berger 2008; Pommier *et al.* 2010).

Figure 1.3 Type II topoisomerases in *E. coli*. (A) DNA gyrase (relaxes supercoils during DNA replication), and (B) topoisomerase IV (de-catenates daughter chromosomes before segregation process).

FQs form a complex with their target enzymes and the DNA in front of the replication fork. Type II topoisomerases bind to DNA crossovers and cleave one of the double-stranded DNA segments but remain attached to the 5' ends of the DNA so that the double-stranded DNA break is bridged. Subsequently, the enzyme associates with the intact double-stranded DNA segment and transfers it through the break, untangling the supercoil or catenation. Once the strand passage is completed, the double-stranded DNA break is re-ligated (Hooper 1999; Drlica *et al.* 2008; Schoeffler and Berger 2008). Consequently, the binding of FQs to the DNA-enzyme complex blocks the enzyme on the DNA and prevents resealing of the DNA strands, leading to the formation of lethal double-stranded DNA breaks (Drlica and Zhao 1997). The impasse created by FQs

inhibits the movement of the replication fork and advancement of DNA polymerase resulting in cell death (Willmott *et al.* 1994; Hiasa *et al.* 1996).

The extensive use of FQs in clinical and veterinary medicine has resulted in the rapid emergence of resistance. Clinically relevant resistance to FQs is often due to the combined effects of multiple mutations. In *E. coli*, mutations are almost always present in *gyrA*. Additional mutations in *gyrB*, *parC* and/or *parE* may be present, along with mutations in *marR*, *acrR* or *soxR* that alter efflux and influx. Thus, the complete resistance towards FQs in *E. coli* can be acquired through three main mechanisms: chromosomal mutations (single nucleotide substitution) that confer resistance to FQs by reducing drug-target binding, by reducing the intracellular concentration of the drug (by increased efflux), or downregulation of outer membrane porins (decreased influx through the membranes) (Figure 1.4) (Blair *et al.* 2014).

Figure 1.4 Resistance mechanisms to FQ (CIP) in *E. coli* involve: (1) downregulation of outer membrane proteins (OMP) (such as, porins *ompF* and *ompC*), (2) *de novo* mutations in DNA gyrase (*gyrA/B*) and topoisomerase IV (*parC/E*), and (3) upregulation of AcrAB TolC efflux pump.

Mutations in the target genes *gyrA*, *gyrB*, *parC*, and *parE* often contribute to the FQ^R phenotype (Deguchi *et al.* 1997; Komp Lindgren *et al.* 2003; Marcusson *et al.* 2009). The most frequently identified mutations in clinical isolates are located in a small part of *gyrA* (in particular amino acid residues S83 and D87) and *parC* (in particular amino acid residues S80 and E84), known as the quinolone resistance-determining region (QRDR). The QRDR corresponds to residues 67-106 of GyrA and residues 63-102 of ParC (Oram and Fisher 1991; Everett *et al.* 1996; Heisig 1996; Piddock 1999; Friedman *et al.* 2001; Komp Lindgren *et al.* 2003; Hopkins *et al.* 2005; Hooper and Jacoby 2015).

Mutations in *gyrB* are less frequent and the most studied are the D426N and K447E, displaying decreased susceptibility to NAL, CIP, and norfloxacin (Ince and Hooper 2003).

The most common mutations that appear frequently in clinically resistant isolates and increase levels of resistance above clinical breakpoints are combinations of *gyrA* S83L, *gyrA* D87N, *parC* S80I and *parC* E84K/A/V/G, and although less frequent, mutations in *gyrB* and *parE* are also observed (Vila *et al.* 1996; Drlica and Zhau 1997; Piddock 1999; Marcusson *et al.* 2009; Hooper and Jacoby 2015; Huseby *et al.* 2017; Basra *et al.* 2018). These combinations of mutations are associated with higher MIC gains, but reduced fitness costs, which possibly can explain their prevalence in clinical settings.

Mutations in FQ^R clinical strains coding for protein repressors that regulate drug efflux are also often present. AcrAB-TolC is the major tripartite multidrug efflux pump associated with FQ resistance in *E. coli* (Aleksun and Levy 1997; Komp Lindgren *et al.* 2003; Marcusson *et al.* 2009; Li *et al.* 2015; Huseby *et al.* 2017). AcrAB-TolC is a multi-component efflux transporter, the expression of which can be negatively regulated by the global regulatory genes of MarR, or the local repressors AcrR and SoxR (Komp Lindgren *et al.* 2003; Marcusson *et al.* 2009). Any inactivating mutation that downregulates the function of regulatory genes (*marR*, *acrR*, *soxR*) increases the expression of AcrAB-TolC, resulting in decreased intracellular concentrations of FQs and other antibiotics, such as chloramphenicol or tetracycline (Webber and Piddock 2003; Everett *et al.* 1996; Fernández and Hancock 2012). Efflux-related mutations cause a smaller gain in

resistance phenotype than target mutations, but they hold a larger mutational target size. Notably, efflux mutations in combination with the target mutations can increase the MIC substantially (Marcusson *et al.* 2009). MarR is the repressor of the MarRAB operon and controls the intracellular level of the global transcriptional regulator MarA and MarB (Figure 1.5) (Alekshun and Levy 1997; Hooper 1999). MarR represses transcription of *marRAB* by binding as a dimer, within the *mar* operator, preventing binding of the RNA polymerase. MarR is a 144 amino acid protein folded into α -helices and β -sheets and consists of a dimerization domain and a DNA-binding domain (Alekshun *et al.* 2001). The N- and C-terminal regions (amino acids 10-21 and 123-144) form the dimerization domain and intertwine with the corresponding regions in the other subunit of the dimer. The DNA-binding domain (amino acids 55-100) is composed of a winged-helix DNA binding domain ($\alpha 2$ - $\beta 1$ - $\alpha 3$ - $\alpha 4$ - $\beta 2$ -W(wing)1- $\beta 3$) (Fig 2.2 of chapter 2 of this thesis).

The resistance gain associated with decreased porin production is small relative to other resistance mechanisms. This could be because FQs can also directly cross the outer membrane by diffusion through the phospholipid bilayer, and are thus not completely dependent on outer membrane porins for entry (Cohen *et al.* 1989; Nikaido and Thanassi 1993; Cramariuc *et al.* 2012). *E. coli* has two major barrel-shaped outer membrane porins, OmpF and OmpC (Silver 2011; Li *et al.* 2015). CIP and other small hydrophilic molecules are able to enter through these channels. Therefore, the downregulation of porin expression due to mutations that reduce drug influx can also contribute to FQ resistance (Fernández and Hancock 2012). Inactivating mutations in *marR* (also discussed in chapter 2 of this thesis) can reduce the expression of the outer membrane porin OmpF, causing a decrease in FQ influx.

Figure 1.5 Schematic representation of MarRAB operon in *E. coli*.

Resistance to FQs is also associated with the presence of several genes acquired by HGT (Guan *et al.* 2013). These include plasmid-mediated quinolone resistance (PMQR) genes, which are now frequently reported in clinical isolates (Piddock 2014; Rodriguez-Martinez 2016). PMQR genes work by conferring target protection, drug modification, or drug efflux. Qnr proteins, a member of the pentapeptide repeat family, protect the target enzymes DNA gyrase and topoisomerase IV from FQ binding (Martínez-Martínez *et al.* 1998; Tran *et al.* 2005; Munita and Arias 2016). The efficacy of FQs is further reduced by the emergence of the plasmid-borne bifunctional aminoglycoside and FQ resistance gene *aac-(6')-Ib-cr* (Ramirez and Tolmasky 2010). This gene, which classically encodes aminoglycoside *N*-acetyltransferase, differs by only two base pairs to modify FQs through acetylation. The acetylation reduces the antibacterial activity of FQs (specifically CIP), generating high MIC values (Robicsek *et al.* 2006a, b; Park *et al.* 2008). Recently, another CIP-modifying enzyme was found. A plasmid-borne gene named *crpP* isolated from a *P. aeruginosa* isolate increases CIP resistance by phosphorylation of the drug's carboxyl group (Chávez-Jacobo *et al.* 2018).

A novel FQ inner membrane efflux pump QepA (quinolone efflux pump A) is flanked together with other antibiotic resistance determinants and through HGT can be expressed in the recipient cell (Périchon *et al.* 2007; Yamane *et al.* 2007). Several variants of QepA can be found in clinical isolates of *E. coli*: QepA2, QepA3, and QepA4 that differ from Qep1 by a few nucleotide changes. (Hooper and Jacoby 2015; Wang *et al.* 2017).

1.5 Persistence of AMR

The fitness costs or benefits associated with AMR mutations can influence the persistence, spread and emergence of antibiotic resistance within a bacterial population (Andersson and Levin 1999; Cohen *et al.* 2003; Andersson and Hughes 2010; Vogwill and MacLean 2015; Melnyk *et al.* 2015). In the presence of antibiotics, resistant mutants have an added fitness advantage over their susceptible counterparts. However, it is generally accepted that resistance is costly, and resistant bacteria suffer a metabolic burden in the absence of antibiotic that affects phenotypic traits, such as reductions in growth rate, survival, efficiency and stability relative to their sensitive genotypes (Andersson and Levin 1999; Reynolds 2000; Andersson 2006; Andersson and Hughes 2010). The fitness impairment is the repercussion of antibiotic mechanisms of action, since antibiotics target essential cellular enzymes in the bacterial cell and acquired resistance mutations often impair the processes mediated by these enzymes. For example, in *E. coli*, mutations in the RNA-polymerase β -subunit encoded by *rpoB*, which is the target of rifampicin (RIF), can confer a high-level of resistance but reduced transcriptional efficiency and eventually the growth rate (Reynolds 2000; O'Neill 2006;

Brandis and Hughes 2013). Likewise, mutations in the target of streptomycin (STR), i.e. the ribosomal protein S12 encoded by *rpsL*, can have adverse effects in the form of decreased translational elongation rates in *E. coli* and *S. typhimurium* (Björkman *et al.* 1999; Levin *et al.* 2000). Some other examples where target alteration mutations confer a reduction in biological fitness are fusidic acid resistance caused by *fusA* mutations (Macvanin *et al.* 2003; 2004) or accumulation of FQ resistance mutations in DNA gyrase (Komp Lindgren *et al.* 2005; Marcusson *et al.* 2009). Similarly, resistance conferred by efflux-regulating mutations and porin-deficient mutants (OmpC and OmpF in *E. coli*) are also associated with a disorder in bacterial physiology, such as reduction in the exponential growth rates or reduced virulence in case of pathogenic organism (Andersson and Levin 1999; Maisnier-Patin and Andersson 2004; Du Toit 2017; Olivares *et al.* 2017).

Naively, it would be anticipated that scaling back of antibiotic selective pressure will favor selection against resistance, raising the possibility of reduction in resistance frequencies among bacterial population. But not all resistance mutations bear a cost, and such low, or no cost mutations are likely to persist and provide comparable fitness levels to resistant bacteria as their susceptible counterparts in the absence of antibiotic selection pressure (Björkman *et al.* 2000; Gottesman *et al.* 2009; Enne *et al.* 2010; Andersson and Hughes 2010; Sundqvist *et al.* 2010). For example, some RIF^R *rpoB* and STR^R *rpsL* mutations do not impose a fitness cost (Marcusson *et al.* 2009; Brandis *et al.* 2014).

Resistant clones that suffer a cost also often restore their fitness, and thus are not rapidly outcompeted by susceptible strains in antibiotic-free environments. This can

occur via a genetic reversion to the wild-type state, which is unlikely since only one single nucleotide polymorphism (SNP) will cause the reversion and that will always cause loss of resistance. A more likely consequence is that resistant mutants acquire additional fitness-compensatory mutations while still maintaining resistance in the population (Handel *et al.* 2006; Andersson and Hughes 2010). The compensatory mutations have a larger mutational target size than reversion and can occur in the same gene that causes the resistance phenotype (intragenic compensation), or in other genes (extragenic compensation) causing a restoration of the functionality of the protein targeted by the antibiotic, and stabilizing the resistant mutants in the population (Schrage *et al.* 1997; Björkman *et al.* 2000; Reynolds 2000; Nagaev *et al.* 2001; Maisnier-Patin and Andersson 2004; Marcusson *et al.* 2009; Hall *et al.*, 2010; Andersson and Hughes 2010). For example, low-fitness STR^R *rpsL* mutants compensate the deleterious effects of protein elongation rates by intragenic compensatory mutations, as well as via mutations in *rpsD* or *rpsE* (Schrage *et al.* 1997; Björkman *et al.* 1998; 1999). Likewise, the fitness defects caused by *rpoB* mutations in RIF^R mutants of *Mycobacterium* and *Salmonella* are ameliorated by second-site mutations in *rpoB* itself (Reynolds 2000), or by other mutations in *rpoA*, or *rpoC* (Comas *et al.* 2011). Studies have also shown that strains having multiple resistance mutations are fitter than expected given the fitness of strains that carry only one mutation (Trindale *et al.* 2009; Borrell *et al.* 2013; Durão *et al.* 2015; Vogwill *et al.* 2016).

Some other factors can also influence bacterial fitness while still maintaining resistance, such as epistasis interactions among different resistance elements (Weinreich *et al.* 2006; Rozen *et al.* 2007; Trindade *et al.* 2009; Silva *et al.* 2011; Wong 2017), or

selection of resistance mutations by unrelated stressors, such as the environment or genetic background (Trindade *et al.* 2009; 2012; Rodríguez-Verdugo *et al.* 2013).

Epistasis, or genetic interactions, refers to the influence of genetic background on the fitness effect of a mutation. Thus, the effect of a mutation in a gene can vary depending on the presence/absence of mutations elsewhere in the genome (Cordell 2002; Weinrich *et al.* 2005; Lehner 2011). AMR mutations and associated compensatory mutations may interact epistatically, as compensatory mutations can be beneficial in the presence of AMR mutation but not otherwise. For example, reduced growth rate due to an increase in the elongation time among STR^R mutations in *rpsL* can be restored by compensatory mutations in *rpsL*. However, in a wild-type background, both resistant and compensatory mutations could be costly (Maisnier-Patin *et al.* 2002; Sander *et al.* 2002). In addition, the phenotypic effect of a mutation depends on the genetic context or strain in which it has appeared. For example, the fitness effects of *rpoB* mutations in different strains of *Pseudomonas* exhibit strain-dependent fitness effects (Vogwill *et al.* 2016). Moreover, some studies (Rozen *et al.* 2007; Trindade *et al.* 2009; Silva *et al.* 2011) have also demonstrated pervasive epistasis via *in vitro* competition assays in the absence of antibiotics, where the same resistance allele may have different selective effects across genetic backgrounds.

1.5.1 *Estimating fitness*

Due to importance of fitness in determining the persistence of resistance, there is substantial interest in estimating the fitness effects of resistance mutations. Fitness can be measured in several ways, by comparing the relative maximum exponential growth rate, whereby a strain with reduced doubling time compared to another strain will be considered less fit. Doubling times are calculated using optical density measurements of a growing culture. The present studies in this thesis use exponential growth rate as a proxy for bacterial fitness. Competition assays are another method for fitness estimation, whereby two or more isogenically related susceptible and resistant strains are co-cultured and a fraction of the culture is transferred into fresh medium periodically. Using different genetic markers, the relative frequencies of the strains are quantified at set time. Phenotypic traits can also be used as fitness proxies. These assays assess the strain's capability to withstand different stresses, such as salt, heat, osmolarity or acid conditions (Nagaev *et al.* 2001; Paulander *et al.* 2009; Hall *et al.* 2011).

1.5.2 *Implications for the control of AMR*

The economic and human cost of AMR is accelerating uncontrollably worldwide. To control the global unwanted impact of AMR, aggressive efforts are urgently required, such as improving global surveillance data of AMR, finding new antibiotics, reducing antibiotic use in food production, and facilitating research trials. In recent times, the WHO (WHO report 2017) has recommended scaling down the unnecessary use of

antibiotics in animals. This step will help to preserve the effectiveness of medically important antibiotics in food producing animals and to control the emergence of resistance. Interestingly, as investigated by Enne *et al.* 2010, efforts to reduce the prevalence of resistance by restricting the antibiotic use in both animal and human populations has had mixed outcomes. In some cases, reduced antibiotic prescriptions lead to reduced prevalence of resistance but not in others. For example, in *S. pneumoniae* the prevalence was reduced after reduction of penicillin, however a persistent quinolone resistant *E. coli* was found remotely where quinolones were not prescribed (Austin *et al.* 1999; Enne *et al.* 2001; Enne 2010; Pallecchi *et al.* 2012). On a similar note, a recent systematic review and meta-analysis by Tang *et al.* (2017) found that interventions that restrict the use of antibiotics in food-producing animals can reduce the prevalence of AMR bacteria by 39%. Such policy decisions that harmonize regulation will certainly help positively in restricting AMR and should be the focus of future research. Overall, these strategies assume that resistance is costly, which is supported by the results obtained in chapter 2 of this thesis.

1.6 Current investigations

While the primary mechanisms of mutation-driven resistance are understood for most drugs, there are important research gaps in understanding the full spectrum of resistance mutations and their pleiotropic effects even for well-studied antibiotics. Thus, I have begun my thesis with the aim of understanding the rate and consequences of chromosomal resistance mutations in order to investigate their evolutionary fates.

Chapter 2 is the first study that attempts to generate a full catalogue of quinolone chromosomal resistance mutations and their pleiotropic effects. Quinolones are an important synthetic class of antibiotics that are used widely in clinical treatments. I have identified a number of resistance mutations in regions of the known contributors of quinolone resistance (DNA gyrase subunits A and B, and the MDR regulator Mar). I observed variation in resistance levels between mutations, widespread cross-resistance between quinolones, significant costs of resistance among resistant isolates. These findings have implications for public health strategies, chiefly in the context of antibiotic selection.

Chapter 3 is an attempt to understand the genetic network underpinning the function of DNA gyrase, and to take advantage of novel vulnerabilities in quinolone resistant *gyrA* S83L mutants. Using a synthetic genetic small RNA screen, I have identified genes whose inhibition increases susceptibility to quinolones in *gyrA* S83L mutants of *E. coli*. These genes are thus potential targets for restoring quinolone susceptibility in the clinic, either through the use of sRNA or small molecule inhibitors. More broadly, I show that sRNA screens can be a powerful tool for investigating genetic networks in bacteria.

While high-level acquired resistance can result from one or a few mutations, a broader network underlies intrinsic resistance. Intrinsic resistance in part results from the impermeability of the bacterial cell wall - for example, the outer membrane of Gram-negative bacteria serves as a permeability barrier to many antibiotics, and constitutive expression of membrane spanning efflux pumps can reduce the intracellular antibiotic

concentration to sub-inhibitory levels (Cox and Wright 2013). In other cases, intrinsic resistance can result from the lack of a particular drug target, or from the formation of biofilms or spores. In the last decade, genome-scale screens have identified many additional redundant constellations of genes and proteins that contribute to intrinsic resistance to antibiotics. For example, the analysis of comprehensive mutant libraries from *E. coli* (Tamae *et al.* 2008; Liu *et al.* 2010) and *P. aeruginosa* (Breidenstein *et al.* 2008; Fajardo *et al.* 2008) has demonstrated that many genes contribute to intrinsic resistance. Two-component signal transduction systems (TCSs), which are involved in bacterial responses to many stresses, have emerged as important players in intrinsic resistance.

In **Chapter 4**, I examine the involvement of *E. coli*'s TCSs in responding to multiple stresses. To do so, I investigate the impact of multiple stressors, including antibiotics, on eight TCS mutants of *E. coli*. I examine the response of each mutant to a variety of environmental stress conditions in combination with four antibiotics from different classes. This study broadens our understanding of the mechanisms by which *E. coli*'s responds to external stressors, including antibiotics.

Chapter 5 is the concluding chapter that briefly summarizes the principal findings of this thesis. I finish with **Chapter 6**, discussing the future perspectives in light of prevalence of AMR .

Chapter 2

The mutational landscape of quinolone resistance in *Escherichia coli*

2.1 Abstract

The evolution of antibiotic resistance is influenced by a variety of factors, including the availability of resistance mutations, and the pleiotropic effects of such mutations. Here, we isolate and characterize chromosomal quinolone resistance mutations in *Escherichia coli*, in order to gain a systematic understanding of the rate and consequences of resistance to this important class of drugs. We isolated over fifty spontaneous resistance mutants on nalidixic acid, ciprofloxacin, and levofloxacin. This set of mutants includes known resistance mutations in *gyrA*, *gyrB*, and *marR*, as well as two novel *gyrB* mutations. We find that, for most mutations, resistance tends to be higher to nalidixic acid than relative to the other two drugs. Resistance mutations had deleterious impacts on one or more growth parameters, suggesting that quinolone resistance mutations are generally costly. Our findings suggest that the prevalence of specific *gyrA* alleles amongst clinical isolates are driven by high levels of resistance, at no more cost than other resistance alleles.

2.2 Introduction

The increasing prevalence of antimicrobial resistance (AMR) has become an urgent public health problem worldwide. For example, *Escherichia coli* resistance to ciprofloxacin, the antimicrobial most purchased by hospitals in Canada between 2008-2014 (PHAC 2015-2016), rose to 26.7% in 2015 from 21.6% in 2009 (CARA 2015). The present AMR crisis has been attributed to the misuse and overuse of antibiotics, as well as the scarcity of novel drug development (Piddock 2012; Bartlett *et al.* 2013; Gross 2013; Gould and Bal 2013; Viswanathan 2014). Given the rapid increase in the prevalence of resistance, an understanding of the principles underlying resistance evolution is vital.

Adaptation, of which the evolution of AMR is a prime example, is driven by the interplay between mutation, selection, and demographic processes like drift. Mutation determines the rate at which beneficial variants are introduced into a population, while selection and demography govern the fates of these variants. Thus, in understanding the evolution of AMR, we are interested in both mutation and selection. We expect, for example, that higher mutation rates will generally lead to a more rapid evolution of resistance. The spread of a given mutation will then be influenced by its selective consequences, including its effect on resistance, and on its pleiotropic effects, such as fitness in the absence of antibiotic, and collateral sensitivity or cross resistance to other antibiotics.

2.2.1 Mutation

The rate of mutation to resistance is given by the product of population size (N), overall mutation rate (μ), and the fraction (f) of mutations that are beneficial (i.e., those that grant resistance). Thus, resistance mutations will appear more frequently for larger populations, or for populations with higher mutation rates. Up to a point, this dependence on $N\mu$ leads to an increase in the rate of adaptation; however, as $N\mu$ approaches 1, a population is no longer limited by mutational input, but instead by competition between competing mutations (e.g., Gerrish and Lenski 1998; Wilke 2004).

Relevantly here, the fraction of mutations granting resistance almost certainly differs between different antibiotics; for some antibiotics, there will be a greater availability of resistance mutations. Variation in the availability of resistance mutations may reflect differences in the number of genetic loci that can contribute to resistance. For example, resistance to trimethoprim is largely conferred by mutations in a single gene encoding dihydrofolate reductase (DHFR) (Toprak *et al.* 2011), while resistance to chloramphenicol and streptomycin can be conferred by mutations in a number of different genes (Toprak *et al.* 2011; Winkler and Kao 2012). Moreover, the number of individual mutations conferring resistance may differ between genes: while a wide range of loss-of-function lesions in the transcriptional regulator *marR* will grant multi-drug resistance (Alekshun *et al.* 2000), only a handful of mutations in *gyrA* confer quinolone resistance (Ruiz 2003; Hooper 1995). Thus, even given equal population size and overall mutation rates, we expect different rates of evolution to resistance to different antibiotics due to differences in the mutational target size.

At a population level, mutation can be quantified by mutation rates or the mutation frequencies. The mutation rate is an estimate of the probability of a mutation occurring in an organism or gene during each generation or per cell division, whereas mutation frequency is simply the proportion of mutant bacteria present in a culture. The number of mutated cells in a culture depends on when the mutation occurred during the growth of the bacterial population. If a mutation occurs early in the growth of the culture, the number of mutants will be higher than those that arise later during growth. These parameters cause the frequency to vary greatly, making the frequency method an inaccurate measurement of mutation. As such, mutation rate is the preferable parameter to estimate (Rosche and Foster 2000).

A number of methods have been developed for calculating the rate of mutation; all of these methods measure the probable number of mutations per generation in a culture (Rosche and Foster 2000). The two broadly used methods are mutation accumulation assays and fluctuation assays. In a mutation accumulation assay the population is propagated through recurrent bottlenecks, presumably of a single cell, such that all mutations are nearly neutral, and the effect of selection is mitigated. Sequencing can then be used to quantify the number of mutations accumulated (Kibota and Lynch 1996; Garcia-Dorado and Gallego 2003).

Fluctuation assays are based on a model of clonal expansion of mutant clones as described by Luria and Delbrück (1943) from bacteriophage T1 sensitivity to resistance. In this method, the distribution of number of mutants is determined in parallel cultures and mutation rate is estimated from this distribution (Figure 2.1). Luria and Delbrück

calculated the mutation rates of *E. coli* from a phage sensitive phenotype to a phage resistant phenotype. *E. coli* cultures exposed to the bacteriophage become clear at first because of cell lysis, but become turbid after prolonged incubation, since some T1 phage-resistant cells would survive. T1^R mutations occurring early in the experiment, prior to the exposure of the phage, would be present in higher frequencies than T1^R variants arising during viral exposure. Therefore, the frequencies of the resistant variants differ between independent cultures, depending on when during growth the mutations have taken place. As the number of mutant cells per culture varies due to the timing of mutation events, Luria and Delbrück argued that the distribution of mutant colonies did not follow a Poisson distribution and resistant mutants arise independently in the culture, even before selection on bacteriophage. However, the number of mutations that arise in each culture per generation follows a Poisson distribution.

Figure 2.1 The fluctuation assay. Each of the three cultures has two mutational events during growth; however, the number of mutant cells varies depending on when during growth the mutations have taken place.

Several statistical methods have been developed for analyzing the results of fluctuation assays. Fluctuation assays provide the distribution of the numbers of mutants per culture, r , which is used to calculate m , the number of mutations per culture. When m is divided by number of cells, N_t , it provides the mutation rate μ . Although the distribution of mutants is not Poisson, the distribution of the number of mutations is a Poisson parameter. Many methods are used to calculate m , each having its pros and cons, depending on the particular experimental conditions. The p_0 method is suitable when the number of mutational events in a culture is low; this method estimates the mutation rate from the fraction of plates on which no colonies are observed, and thus does not use all of the data. The classical Lea and Coulson (LC) (1949) is the method of the median. This method assumes that if the median number of mutants is large enough, then most

mutations occur earlier. The major drawbacks of this method are that it uses little information from the fluctuation assay, and it is sensitive to any variation in the assumptions, e.g., phenotypic lag, which will result in reduced precision. The most accurate method for estimating m is the Ma-Sandri-Sarkar maximum likelihood method (MSS-MLE), which has become the standard method for calculating mutation rates. This method is valid for any range of the expected number of m , and 95% confidence intervals can be measured by an empirically determined set of equations. Moreover, it also calculates mutation rate from the entire dataset unlike the LC and p_0 methods (Ma *et al.* 1992; Rosche and Foster 2000). Mutation rate per cell is then calculated as $\mu = m/N$, where N is the mean number of cells per culture, or approximately the number of cell divisions per culture (Sarkar *et al.* 1992; Hall *et al.* 2009).

2.2.2 Selection

Once a resistance mutation has arisen in a natural population, its persistence and spread may be affected by its pleiotropic effects, including its fitness costs and effects on resistance to other drugs. (Andersson and Levin 1999; Andersson and MacGowan 2003; Pál *et al.* 2015; Vogwill and MacLean 2015). In the presence of antibiotic, a resistant bacterium has a clear advantage compared to susceptible genotypes. However, in an antibiotic-free environment, a resistance mutation may impose a burden, for example through reduced growth rates relative to sensitive strains (Reynolds 2000; Levin *et al.* 2000; Tenover 2006; Hall *et al.* 2011). However, while resistance mutations are often costly, not all resistance mutations bear a cost, and such cost-free mutations are likely to

persist (Komp-Lindgren *et al.* 2003; Luo *et al.* 2005; Zhang *et al.* 2006; Rozen *et al.* 2007). Melnyk *et al.* (2015) conducted a meta-analysis including 179 single chromosomal mutations conferring resistance to 16 antibiotics from 8 bacterial species. They reported 8 no-cost mutations, with variable costs of resistance depending on antibiotic class and the species assayed.

Increased resistance to one antibiotic may be accompanied by increased cross-resistance to other antibiotics. Cross-resistance is often observed between members of the same class of antibiotic. For example, all quinolones target the essential enzymes DNA gyrase and Topoisomerase IV (Topo IV), whose subunits are encoded by the *gyrA/B* and *parC/E* genes, respectively. Resistance to quinolones can be conferred by point mutations affecting specific portions of GyrA and ParC, known as the quinolone resistance-determining regions (QRDR) (Horowitz and Wang 1987; Yoshida *et al.* 1990; Hooper 1999; Ruiz 2003). Changes at amino acid positions 83 and 87 of *gyrA* lead to a significant loss in quinolone susceptibility. Moreover, known resistance mutations in *gyrB* mutations affect amino acid positions 426 and 464, sites that interact with the bound quinolone molecule close to the QRDR of GyrA (Sullivan *et al.* 1995; Lu *et al.* 1999; Friedman *et al.* 2001). Correspondingly, resistance mutations in *gyrA*, confer resistance across quinolones. Moreover, *gyrB* mutations affecting positions 426 and 464 interact with the bound quinolone molecule close to the QRDR of GyrA, and are known to confer resistance to particular quinolones (Yoshida *et al.* 1991; Heddle and Maxwell 2002; Weigel *et al.* 2002).

parC mutations are additionally associated with resistance to quinolones (Vila *et al.* 1996; Heisig 1996). Topo IV acts as a secondary target to quinolones in Gram negatives and *parC* mutations are found along with the primary *gyrA* mutations among clinical isolates (Huseby *et al.* 2017; Basra *et al.* 2018). Resistance mutations in the primary target (DNA gyrase) precede those in the secondary target, in a stepwise selection for high-level resistance under prolonged exposure of increasing drug concentrations. In a multistep mutational pattern, particular sets of allele combination of *gyrA* S83L, D87N, and *parC* S80I genotype tend to be seen in clinical *E. coli* isolates at higher frequencies, evolving a high-level clinical resistance (Everett *et al.* 1996; Bagel *et al.* 1999; Komp Lindgren *et al.* 2003; Baudry-Simner *et al.* 2012; Johnson *et al.* 2013; Betitra *et al.* 2014).

Cross-resistance can also occur between drug classes - for example, *marR* mutations selected by quinolones also confer resistance to phenicols, tetracyclines, and rifampicin (Hooper 1999; Alekshun and Levy 1999a; Alekshun and Levy 1999b). Mutations in *marR*, which encodes a negative regulator of the *marRAB* operon, render the repressor function inactive, resulting in increased efflux and reduced permeability (Cohen *et al.* 1993; Hooper 1995; Alekshun and Levy 1997; Alekshun *et al.* 2001; Randall and Woodward 2002). Clinical strains of *E. coli* resistant to quinolones typically harbor both drug target mutations and efflux pump regulatory mutations (Hooper 1999; Komp Lindgren *et al.* 2003; Hooper and Jacoby 2015).

By contrast, in collateral sensitivity or negative cross-resistance, acquisition of resistance to one antibiotic may grant sensitivity to other antibiotics. For example,

resistance to aminoglycosides in *E. coli* can be conferred by electron transport chain mutations that reduce proton-motive force (PMF). This decrease in PMF negatively affects the activity of multi-drug efflux pumps, such as AcrAB-TolC, granting hypersensitivity to many other antibiotics (Imamovic and Sommer 2013; Lázár *et al.* 2013).

Here, we assess mutation rates, levels of resistance, and pleiotropic effects for chromosomal mutations conferring resistance to different quinolones, in an effort to understand the full set of parameters contributing to the origin, spread, and persistence of quinolone resistance. Quinolones were first used clinically in the 1960's and have undergone multiple rounds of development. The first-generation quinolone, nalidixic acid (NAL), possesses a limited spectrum of activity, but fluorination of the core structure generated the so-called 2nd generation quinolones like ciprofloxacin (CIP). Further overall structural developments resulted in 3rd-generation drugs such as levofloxacin (LEVO). We predict that, while broad mechanisms of resistance will be shared between quinolones, resistance mutations will differ in their effects towards different antibiotics owing to differences in antibiotic penetration and/or structural configuration.

2.3 Materials and methods

2.3.1 Bacterial strains and media

The *E. coli* laboratory strain K-12 (MG1655) was used for all experiments. Lysogeny broth (LB) (10 g/l tryptone, 5 g/l yeast extract, 10 g/l NaCl per litre; Bishop) was used for agar and broth cultures.

2.3.2 Quinolone susceptibility assays

Minimum inhibitory concentration (MIC) values for the ancestral strain and for antibiotic-resistant mutants were determined for NAL, CIP, and LEVO (Sigma-Aldrich) using a 96 well plate assay in triplicate. Antibiotic concentrations started at 10µg/ml, 1µg/ml, and 8µg/ml for NAL, CIP and LEVO, respectively, and were diluted in a two-fold series and dispensed with 125µl/well of LB into 96-well plates. The 96 well plates were incubated overnight at 30 C, with shaking at 150 rpm. The MIC was defined as the lowest concentration of antibiotic for which 90% growth inhibition was visibly observed after overnight culture.

2.3.3 Fluctuation analysis and estimation of mutation rates

Mutation rates to resistance were determined using fluctuation assays (Luria and Delbrück 1943; Foster 2006). A single colony of *E. coli* MG1655 was grown overnight in liquid LB at 37 C, with shaking at 150 rpm. Fresh 200µl cultures were inoculated with ~100 cells each. Each independent culture was then grown to saturation overnight. The final number of cells, N_t was estimated from plate counts on LB without antibiotic. Selective plates were supplemented with antibiotics (NAL, CIP or LEVO) at a concentration of 1xMIC or 2xMIC. 30 replicate populations were plated for each antibiotic at each concentration. The observed number of mutants, r , was then counted for each replicate.

For estimating the number of mutational events m , the MSS maximum-likelihood method was used (Ma *et al.* 1992). This method is based on a recursive algorithm for estimating the Luria-Delbrück distribution for a given number of mutational events (Luria and Delbrück 1943). This method is valid over the entire range of values of m (Sarkar *et al.* 1992; Hall *et al.* 2009). The mutation rate per cell per generation, μ , is calculated as m divided by the total number of bacteria plated on selective plates (N_t) (Zheng 1999; Rosche and Foster 2000; Foster 2006). The detailed terminology and equations are provided in supplementary Table A2.1 and Box A2.1.

2.3.4 PCR amplification and sequencing of candidate genes

Targeted sequencing of the known resistance loci *gyrA* and *marR* was carried out in order to identify potential resistance mutations. To ensure independence, a single mutant colony was picked from every plate from the fluctuation assay on which there was growth, and inoculated overnight in LB broth without antibiotic. DNA was then extracted using the EZ-10 spin column bacterial DNA miniprep kit (Bio Basic) and PCR was used to amplify the QRDR of *gyrA* (Gyrase forward - 5'GTAAAACGACGGCCAGTGATGAGCGAC3', Gyrase reverse - 5'CGGTACGGTAAGCTTCTTC3') and the entire *marR* gene (MarR-forward 5'GTAAAACGACGGCCAGTGGTCAATTCA3', MarR reverse - 5'TCTGGACATCGTCATACCTC3'). PCR amplicons were sent to Genome Quebec for Sanger sequencing. Mutations in these particular regions were compared with the wild type MG1655 strain of *E. coli*.

2.3.5 Whole-genome sequencing

Whole-genome sequencing (WGS) was carried out to identify potential resistance mutations in clones for which mutations were observed in neither *gyrA* nor *marR*. Sequencing libraries were prepared using the Nextera XT kit (Illumina), and sequencing was carried out on the MiSeq platform using paired-end 300bp reads. Raw reads were processed using Trimmomatic-0.35 (Bolger *et al.* 2014), allowing for a minimum Phred-scaled quality score of 20 for leading and trailing bases, truncating reads once average

quality dropped below 20 in a 4bp sliding window, and dropping all reads of length less than 36. Read quality was assessed using FastQC

(<https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>).

Reference-based assembly was carried out using Bowtie-2 (Langmead *et al.* 2009), with *E. coli* K-12 (MG1655; NC_00913.2) as the reference genome. SNPs were called using Samtools (Li *et al.* 2009) and SNP effects were inferred using SNPeff (Cingolani *et al.* 2012).

2.3.6 24-hour growth curve analysis

Fitness in the absence of antibiotic was estimated using 24-hour growth curves for each single colony isolate. Growth curves were obtained in triplicate in 96-well plates, inoculated at a 1:100 dilution from overnight cultures. OD₆₀₀ was measured on a Biotek ELx808 plate reader every 37 minutes for 24 hours, incubating at 37 C with 30 seconds of shaking every 5 minutes. Three growth parameters, lag time, maximum growth rate and optical density at stationary phase after 24 hours, were estimated using the program GrowthRates (Hall *et al.* 2013).

2.4 Results and Discussion

2.4.1 Estimation of mutation rates

We estimated mutation rates to resistance for each of the three quinolones NAL, CIP, and LEVO using fluctuation assays (Luria and Delbrück 1943). The probability of a mutational event per cell per generation was estimated at 1X and 2X MIC where MIC values were 10 µg/ml, 15 ng/ml, and 31 ng/ml for NAL, CIP and LEVO, respectively. The number of observed mutants (r) on each of 30 selective plates was used to estimate mutation rates to NAL^R, CIP^R, and LEVO^R using the MSS maximum likelihood method (Sarkar *et al.* 1992; Hall *et al.* 2009). At 1x and 2xMIC, mutation rates differed between antibiotics, with lower rates to NAL^R than to CIP^R or LEVO^R (CIP>LEVO>NAL), presumably because fewer resistance mutations are available for NAL that can grant sufficiently high levels of resistance (Supp Table A2.2, Figure 2.2).

Figure 2.2 Spontaneous mutation rate per 10⁸ cells to quinolone resistance among *E. coli* K-12 (MG1655). The mutation rate was estimated from 30 independent cultures at 1X and 2X MIC concentration. Error bars represent 95% confidence intervals. Note that no colonies were obtained at 2xMIC for NAL.

2.4.2 Identification of resistance mutations

We obtained a total of 56 spontaneous quinolone resistant mutants from the fluctuation assays (one from each plate), and identified putative resistance mutations in 50 of these (Table 2.1, Supp Table A2.3). Targeted Sanger sequencing identified 36 *gyrA* mutants, 9 *marR* mutants, and 1 double *gyrA/marR* mutant. We performed WGS on remaining of the 10 resistant mutants and identified an additional 4 *gyrB* mutants. No mutations were identified in rest of the 6 resistant mutants, as WGS might not be able to detect big deletions or duplications. We note that *gyrA/gyrB* or *marR/gyrB* double mutants will be undetected by our approach. However, given that only one *gyrA/marR* double mutant was detected, the frequency of double mutants is probably low. In *gyrA*, mutations were observed at nucleotides encoding amino acid positions 67, 81, 83, and 87 (Figure 2.3A), consistent with previous findings that the GyrA QRDR spans amino acid 67 to 107 (Horowitz and Wang 1987; Yoshida *et al.* 1990; Heisig and Tschorny 1994; Hopkins *et al.* 2005). The most common alterations in the *gyrA* QRDR region were S83L (n=18) and D87G/N/Y (n=11). These mutations were found in mutants isolated against all three drugs, presumably because these positions are located within the positively charged region close to the DNA-enzyme binding site (Hooper 1999). Amino acids 83 and 87 are located near the active site of DNA gyrase, along with the tyrosine-122 residue that interacts with the broken DNA strand following cleavage (Oram and Fisher 1991; Maxwell 1992; Everett *et al.* 1996; Heisig 1996; Hopkins *et al.* 2005; Komp-Lindgren *et al.* 2005; Marcusson *et al.* 2009; Moon *et al.* 2010). The α helix-4 region is particularly essential to quinolone binding and the substitution to leucine at position 83 makes the

vicinity of α helix-4 of the gyrase less electron rich, crippling gyrase-quinolone binding (Hallett and Maxwell 1991; Maxwell 1992; Lu *et al.* 1999). Mutations at position 87 and 81 also perturb the alignment of the α helix-4 structure.

marR mutations were obtained only in LEVO^R isolates. These point, frameshift, and missense mutations were dispersed throughout the gene, as expected given that the Mar phenotype can arise from any loss-of-function mutation (Figure 2.3C). MarR consists of two domains, one N-C terminal domain and a helix-turn-helix (HTH) DNA binding domain. The closely packed hydrophobic core and intermolecular hydrogen bonds stabilize the N-terminus (residues 10-21) of one subunit and the C-terminus (residues 123-144) of the second subunit, holding the dimer together. Some of the mutations reported in this study belong to the oligomerization dimer domain of MarR, such as those at positions 10, 27, and 33 of one terminal subunit, and positions 123 and 126 of the other equivalent subunit. The rest of the MarR protein is linked via antiparallel helices emerging out from each of the subunits, encompassing the DNA binding domain (residues 55-100) (Alekshun *et al.* 2000; Alekshun *et al.* 2001). Mutations at positions 64 and 69 fall in the HTH DNA binding motif. Other mutations reported here at position 42 and 102 residue at α and β sheets of MarR, which are essential for the interaction between the two antiparallel strands of HTH DNA binding domain (Alekshun *et al.* 2001). We note that previous work has identified *marR* mutants (R77H, R94C, and Δ 72-82) during laboratory adaptation of *E. coli* to CIP (Wong and Seguin 2015). A possible explanation for our lack of *marR* mutants on CIP may be that *marR* mutants were growing slower on NAL and CIP than were *gyrA* or *gyrB* mutants.

WGS revealed *gyrB* mutations in four isolates for which no *gyrA* or *marR* mutations were detected by Sanger sequencing (Figure 2.3B). H281L, S464F, and L509Q mutations were found in strains isolated on CIP, and a D426N mutation was found in a strain isolated on NAL. The GyrB enzyme consists of two domains, an N-terminal domain (amino acid 2-393) that incorporates the ATPase catalytic site, and a C-terminal domain (amino acid 394-804) that interacts with GyrA (Reece and Maxwell 1991; Brino *et al.* 2000). D426N and S464F have been previously reported to confer quinolone resistance (Nakamura *et al.* 1989; Yoshida *et al.* 1991; Vila *et al.* 1996; Hopkins *et al.* 2005; Komp-Lindgren *et al.* 2005). Both of these mutations are part of the QRDR of GyrB and cause conformational changes in the structure of the gyrase subunits (Weigel *et al.* 2002; Hopkins *et al.* 2005). The *gyrB* D426N mutation has been reported before along with a mutation at position L447, both of which provide a neutral vicinity, owing to their respective opposite charges. Both of these residues are suggested to be part of a quinolone-binding pocket (Yoshida *et al.* 1991; Gensberg *et al.* 1995; Hooper 1999; Heddle and Maxwell 2002).

Interestingly, the H281L and L509G mutations have not been previously reported to confer quinolone resistance. These two novel mutations are located outside the GyrB QRDR. Position 281 is located in the transducer region of GyrB, which forms a cavity just large enough to facilitate the transfer of the trapped double-stranded DNA through the DNA gate in the presence of ATP (Kampranis *et al.* 1999; Champoux 2001; Sissi and Palumbo 2010; Stanger *et al.* 2014). Position 509 is within the TOPRIM domain of GyrB, part of the catalytic DNA cleavage-rejoining complex along with the GyrA winged helix domain (Sissi and Palumbo 2010; Gubaev and Klostermeier 2014).

Table 2.1 MIC fold-increase and mutations in *gyrA*, *gyrB*, and *marR* among NAL^R, CIP^R, and LEVO^R mutants of *E. coli* K-12 (MG1655).

Antibiotic	MIC WT (ug/ml)	MIC fold-change of mutants			Resistance mutation			Total no. of NAL ^R , CIP ^R , and LEVO ^R mutants	No. of <i>gyrA</i> , <i>gyrB</i> , and <i>marR</i> mutants
		NAL	CIP	LEVO	<i>gyrA</i>	<i>gyrB</i>	<i>marR</i>		
Nalidixic acid	10							11	
		64			A67S				2
		256			S83L				6
		128			D87G				2
		64				D426N			1
Ciprofloxacin	0.015							24	
			64		G81C/D				3
			128		S83L				4
			32		S83W				2
			64		D87Y/G/N				7
			32			H281L			1
			32			S464Y			1
			16			L509G			1
Levofloxacin	0.031							21	
				16	G81C				1
				16	S83L				8
				16	D87G				1
				16	D87G		R27P		1
				16			E10stop		1
				16			L33R		1
				8			Q42E		1
				8			L64fs		1
				8			G69E		1
				8			T102S		1
				8			Q117stop		1
				4			L123S		1
				8			N126fs		1

Figure 2.3 The domain structures of gyrase A, B, and MarR. Mutations obtained in this study are indicated in bold above. Panel A: Arrangement of GyrA. This subunit of DNA gyrase consists of the breakage-reunion (BRD) domain, and the quinolone resistance determining region (QRDR-A) site. Panel B: Arrangement of GyrB, with the ATPase, Transducer (221-392), and Toprim (418-533) regions. The QRDR-B is shown within the Toprim domain of GyrB (Stanger *et al.* 2014). Panel C. MarR domain structure, comprising four helices (H) and three β -sheets (B). H3 and H4 (57-80) are the recognition and DNA binding motifs containing H-T-H motifs and the β -sheet winged structure. H1, H5, and H6 are associated with dimerization (Sulavik *et al.* 1995; Alekshun *et al.* 2001; Finn *et al.* 2016; Uniport Consortium 2018).

2.4.3 Direct responses to selection

An increase in fitness in the selective environment is referred to as the direct response to selection; here, the direct response to selection is measured by an increase in MIC towards the drug on which a mutant was selected. We found substantial variation between drugs in the magnitude of the direct response. Mutants isolated on NAL showed a stronger direct response to selection than did mutants isolated on CIP or LEVO, with a mean increase of 256-fold MIC towards NAL. Mutants isolated on CIP and LEVO showed mean increases of 64-fold and 16-fold towards CIP and LEVO, respectively (Figure 2.4).

Among resistance mutations, *gyrA* mutations consistently showed higher levels of resistance than *gyrB* or *marR*, regardless of the antibiotic they were isolated on, which impacts the variation in MIC values significantly (Table 2.2). Furthermore, within each gene, the level of resistance varied by mutation. In the case of *gyrA*, the S83L mutation conferred higher resistance among all the isolates compared to other mutational sites of *gyrA* (87, 81 or 67). This suggests that the widespread occurrence of the S83L mutation amongst clinical isolates is due to the high level of resistance (Supp Figure A2.1).

Figure 2.4 Direct responses to selection. Changes in MIC for resistant mutants towards the drug on which they were selected: NAL (A), CIP (B), and LEVO (C). The boxplot presents the median, first, and third quartiles, with whiskers showing either the maximum (minimum) value or 1.5 times the interquartile range of the data, whichever is smaller (larger). The points (open circles) indicate the outliers.

Table 2.2 Two-way analysis of variance (ANOVA) for the effects of antibiotic and gene on levels of resistance.

Factor	F-value	P-value
Gene	10.00	0.00025*
Antibiotic	39.30	1.34e-10*
Gene*Antibiotic	2.83	0.09

2.4.4 Cross resistance between quinolones

Cross-resistance between quinolones was widespread: all of the resistant mutants isolated on one quinolone displayed increased resistance, in varying degrees, to the other two quinolones. Nonetheless, different quinolones were not equally affected by the resistance mutations (Figure 2.5). Overall, mutants were more resistant towards NAL than they were towards CIP or LEVO. Among CIP and LEVO, mutations showed smaller gains in resistance on LEVO. Nevertheless, significant correlations between levels of resistance for CIP and LEVO (Pearson's $r = 0.64$, $t = 6.2$, $P = 8.433\text{e-}08$), NAL and CIP ($r = 0.47$, $t = 3.93$, $P = 0.0002$), and NAL and LEVO ($r = 0.38$, $t = 3.07$, $P = 0.0032$) suggest a closer relationship between the newer quinolones. This variation in resistance among quinolones can be explained by the intrinsic structural drug differences between older and newer quinolone classes. NAL is devoid of any cyclic derivatives whereas CIP and LEVO have substituents at positions C-6, C-7, and C-8 that offer greater

spectrum/potency of activity. Thus, the modified quinolone substituents likely reduce resistance levels by increasing the affinity for GyrA, and by stabilizing the quinolone-DNA complex (Domagala 1994; Tillotson 1996; Zhao *et al.* 1998; Dong *et al.* 1999; Fukuda and Hiramatsu 1999; Jorgensen *et al.* 1999; Lu *et al.* 1999; Pestova *et al.* 2000; Barnard and Maxwell 2001; Peterson 2001; Sanders 2001; Zhao and Drlica 2001; Michot *et al.* 2005; Becnel *et al.* 2009; Morgan-Linnell *et al.* 2009; Azéma *et al.* 2011).

These trends are also evident for specific loci. *gyrA* mutants showed the highest gain in resistance (average 128XMIC) on NAL in comparison with CIP (32XMIC) or LEVO (16XMIC). *gyrB* mutants also displayed a higher increase in resistance to NAL (64XMIC), but the same increase on CIP and LEVO (16XMIC). The novel H281L and L509G mutations gained similar increases with CIP and LEVO, at 32XMIC and 16XMIC respectively. On NAL, H281L gained 64XMIC whereas L509G gained similar increase in resistance as with CIP or LEVO, i.e. 16XMIC. Meanwhile, *marR* mutants did not show as high increases in MICs, with increases of 32X, 16X, and 8XMIC on NAL, CIP and LEVO.

Figure 2.5 Cross-resistance between resistant mutants. Fold-increase in MIC of resistant mutants isolated on NAL (A), CIP (B), and LEVO (C) against all three antibiotics. The boxplot presents the median, first, and third quartiles, with whiskers showing either the maximum (minimum) value or 1.5 times the interquartile range of the data, whichever is smaller (larger). The points (open circles) indicate the outliers.

2.4.5 Cost of resistance

The persistence of resistance in the absence of antibiotic is determined in part by the fitness costs associated with resistance mutations (Björkman *et al.* 2000; Gagneux *et al.* 2006; Andersson and Hughes 2010). No-cost mutations may contribute to the persistence of resistance mutations in the absence of antibiotic. We measured three fitness components in the absence of antibiotic for our set of quinolone resistant mutants: maximum growth rate (V_{max}), density at stationary phase (Max OD), and length of lag phase. Resistant mutants were consistently found to be costly, exhibiting significant differences in V_{max} , Max OD, and lag time compared to their drug-susceptible ancestor MG1655 (Figure 2.6, Table 2.3) (Supp Figure A2.2 A, B, C). Thus, overall, we observe significant costs of resistance for quinolone resistance mutations, consistent with previous studies (Bagel *et al.* 1999; Barnard and Maxwell 2001; Kugelberg *et al.* 2005; MacLean and Vogwill 2014; Melnyk *et al.* 2015).

Figure 2.6 Cost of resistance of quinolone resistant mutants. The fitness components measured are growth rate, cell density, and lag time between *gyrA*, *gyrB*, and *marR* resistance mutations. All the fitness components are compared to control *E. coli* K-12 (MG1655). The boxplot presents the median, first, and third quartiles, with whiskers showing either the maximum (minimum) value or 1.5 times the interquartile range of the data, whichever is smaller (larger). The points (open circles) above or below the whiskers indicate the outliers.

Table 2.3 Effects of resistance mutations on growth parameters.

Factor	Post hoc (Tukey HSD) comparisons with <i>E. coli</i> K-12 MG1655					
	Growth rate (OD ₆₀₀ /minute)		Length of lag time (minutes)		Cell density at stationary phase (OD ₆₀₀)	
	Mean	P-value	Mean	P-value	Mean	P-value
<i>gyrA</i> - MG1655	-0.032	0.0000011	33.4	<2.0e-16	-0.55	<2.0e-16
<i>gyrB</i> - MG1655	-0.033	0.0000191	28.9	<2.0e-16	-0.52	<2.0e-16
<i>marR</i> - MG1655	-0.029	0.0000173	32.3	<2.0e-16	-0.53	<2.0e-16

Some studies have reported that mutations granting higher levels of resistance impose higher costs (Melnik *et al.* 2015). However, we fail to find a significant relationship between MIC and any fitness component. No correlations were found between MIC and growth rate ($P = 0.42$, $\tau = 0.08$), length of lag phase ($P = 0.82$, $\tau = 0.02$) or cell density ($P = 0.12$, $\tau = 0.15$) (Figure 2.7). We note that *gyrA* mutations confer no greater costs than other resistance mutations (*gyrB*, *marR*). Moreover, amongst the handful of mutations in *gyrA* (S83, D87, G81) that can confer high level resistance (Heisig and Tschorny 1994; Heisig 1996), a few prominent alleles of *gyrA* tend to be found in *E. coli* clinical isolates (Everett *et al.* 1996; Komp-Lindgren 2003; Betitra *et al.* 2014; Basra *et al.* 2018). That these mutations confer high levels of resistance, but are no more costly than other *gyrA* mutations, could help to explain the prevalence of specific *gyrA* mutations amongst clinical isolates.

Figure 2.7 No correlation between level of resistance (fold-change in MIC) and growth rate, cell density or length of lag phase for all mutants.

2.5 Conclusions and Perspectives

Quinolones target DNA gyrase and topoisomerase IV. Resistance against them is achieved by target alteration and/or through efflux and permeability associated mutations. We find that 50/56 spontaneous mutants obtained through fluctuation assays were resistant through mutations in the known resistance conferring genes – *gyrA*(36), *gyrB*(4), *marR*(9), and *gyrA/marR*(1). This finding suggests that there are few other quinolone resistance mutations available in *E. coli* K-12; this is somewhat surprising, given that selection experiments in *P. aeruginosa* have identified novel resistance mutations (Wong *et al.* 2012). We find significant costs of resistance, and differences in the mutational supply rate among resistant isolates. Notably, we find *gyrA* mutations conferred higher resistance, without greater fitness cost, than other mutations. This finding may explain the prevalence of *gyrA* mutations in clinical samples. We also find variation in cross-resistance amongst quinolone resistant isolates, implying that different resistance mutations respond differently to quinolone variants. Thus, antibiotic variants may have different implications for the evolution of resistance. Optimally, we should choose an antibiotic for which resistance is costly, and where single mutations have relatively small effect, as was the case here for levofloxacin.

Chapter 3

An sRNA screen for reversal of quinolone resistance in *Escherichia coli*

3.1 Abstract

In light of the rising prevalence of antimicrobial resistance (AMR) and the slow pace of new antimicrobial development, there has been increasing interest in the development of adjuvants that improve or restore the effectiveness of existing drugs. Here, we use a novel small RNA (sRNA) screening approach to identify genes whose knockdown increases ciprofloxacin (CIP) sensitivity in a resistant strain of *Escherichia coli*. 5000 constructs were initially screened on a *gyrA* S83L background, ultimately leading to 30 validated genes whose disruption reduces CIP resistance. This set includes genes involved in DNA replication, repair, recombination, efflux, and other regulatory systems. Our findings increase understanding of the functional interactions of DNA gyrase, and may aid in the development of new therapeutic approaches for combating AMR.

3.2 Introduction

The rapid evolution of antimicrobial resistance (AMR) among bacterial pathogens and the arrested development of new antibiotics have driven the search for novel approaches to counteract the resistance crisis (Levy and Marshall 2004; Spellberg 2004; Payne 2007; Davies and Davies 2010). Recently, re-sensitization of resistant strains to existing antibiotics has emerged as a promising strategy (Wright 2000; Bassetti *et al.* 2008). Compounds that reduce bacterial resistance, and that thereby restore the effectiveness of existing drugs, are a promising variety of antibiotic adjuvant (Wright 2016; González-Bello 2017). Certain β -lactamase inhibitors, for example, restore susceptibility to cephalosporins by inhibiting degradative enzymes (extended-spectrum β -lactamases, ESBLs) that are often responsible for resistance (Drawz and Bonomo 2010; King *et al.* 2014).

Here, we use a bacterial small RNA (sRNA) screen to identify genes whose knockdown re-sensitizes DNA gyrase-mediated resistance to the fluoroquinolone antibiotic ciprofloxacin (CIP). CIP is a synthetic antibiotic used globally for the treatment of many bacterial infections (Hooper 1999; Anderson 2004; Bolon 2011); high-level resistance is typically conferred by mutations in the *gyrA* gene, which encodes one subunit of DNA gyrase, the primary target of quinolones (Drlica and Zhao 1997; Walsh 2000). The S83L substitution in the GyrA subunit confers a high-level of CIP resistance in *E. coli* (Bagel 1999; Bhatnagar and Wong submitted). We reasoned that CIP

susceptibility might be restored in *gyrA* mutants by disrupting genes involved in the function of DNA gyrase, or by altering cell permeability to CIP.

Bacterial sRNAs are widespread, non-coding RNA molecules. They are typically 50-300 nucleotides in size, trans-encoded, with distinct stem loops as secondary structures (Argaman *et al.* 2001; Gottesman 2004; Vogel and Sharma 2005, Sharma and Vogel 2009; Yoo *et al.* 2013). They play a prominent role in bacterial physiology by controlling gene expression post-transcriptionally. Each sRNA consists of two important regions. One is the recognition region that regulates sRNA-mRNA base-pairing through antisense short complementary base-pairing with the 5' untranslated region (UTR) or translation initiation region (TIR), and the other is the scaffold (Hfq) region that stabilizes sRNA-mRNA base-pairing (Møller *et al.* 2002; Zhang *et al.* 2002; Storz *et al.* 2004; Jousselin *et al.* 2009; Vogel and Luisi 2011; Holmquist and Vogel 2013; Vazquez-Anderson and Contreras 2013; Lee and Moon 2018; Lee *et al.* 2019). Binding of sRNA to mRNA targets can reduce gene expression by inhibiting translation or promoting mRNA degradation.

In eukaryotes, RNA interference (RNAi) is used extensively for studies of gene function. RNAi mediated gene silencing through short interfering (siRNA) and short hairpin (shRNA) RNAs has become a mainstay in cancer research and is a recognized basis of target validation and drug development (Silva *et al.* 2005, 2008; Schlabach *et al.* 2008; Scholl *et al.* 2009). In prokaryotes, comparable use of sRNA as a genetic tool is promising, but still needs to be explored further (Nakashima *et al.* 2006; Man *et al.* 2011; Meng *et al.* 2012; Sharma *et al.* 2011, 2013). Sharma *et al.* (2011, 2013) have exploited

the modular nature of natural bacterial sRNAs and with a fused fluorescent protein (GFP) as reporter, successfully isolated and characterized three artificial sRNAs. These artificial sRNAs post-transcriptionally repressed the endogenous gene expression of *ompF* (encoding an outer-membrane porin), *fliC* (a motility gene), and *recA* (involved in DNA repair) in *E. coli*. *recA* downregulation subsequently led to increased susceptibility to CIP. Similarly, Man *et al.* (2011) also designed artificial sRNAs for specific gene silencing in bacteria. Their synthesized sRNAs downregulated the expression of exogenous (EGFP) and endogenous (*uidA*) genes in *E. coli*.

In the context of AMR, Lee *et al.* (2011) demonstrated the potential of sRNA screens for the reversal of antibiotic resistance. They conducted a genetic screen using antisense RNA interference conditional expression against 245 essential genes for reversal of AMR in methicillin-resistant *S. aureus* (MRSA) to restore β -lactam activity. The library was expressed on wild-type as well as resistant MRSA strains (COL and USA300) under partially inducing conditions. They found 45 genes whose knockdown reduced resistance to at least one of seventeen clinically relevant antibiotics, including those involved in cell division (*ftsZ* and *ftsA*), protein secretion (*spsB*), teichoic acid biosynthesis (*tarL*), and signal transduction. Notably, since these are essential genes, they would be missed in any knockout-driven screening approach, highlighting a key benefit of sRNA screens.

In this study, we randomized the antisense sequences of three naturally occurring sRNAs to generate an sRNA expression library with the potential to target diverse mRNA transcripts. We identified a number of sRNA sequences that reduce quinolone

resistance on a *gyrA* S83L background. Further bioinformatic and functional analyses confirmed several genes whose down-regulation reduces resistance levels, and that may thus be promising adjuvant targets.

3.3 Materials and Methods

3.3.1 Bacterial strains, media, and plasmid construction

One shot Top10 *E. coli* (Invitrogen, F- mcrA Δ (mrr-hsdRMS-mcrBC), j80lacZ Δ M15, Δ lacX74 recA1 araD139 Δ (ara leu)7697, galU, galK, rpsL (StrR), endA1, nupG) chemically competent cells were used for the development of randomization methods and for vector maintenance. For the sRNA screen, a quinolone resistant derivative of *E. coli* K-12 (MG1655) was generated by gene gorging (Herring 2003). Briefly, a fragment of *gyrA* encoding the S83L substitution (via a TCG->TTG mutation) and an I-SceI restriction site was generated by megaprimer PCR (Herring 2003) and cloned into the PCR2.1 vector using TOPO cloning (Invitrogen). This donor plasmid was co-transformed into *E. coli* K-12 (MG1655) along with the mutagenesis plasmid pACBSR. I-SceI endonuclease and λ -red functions encoded on pACBSR were then induced with arabinose. Potential mutants were plated on LB, and replica plated to LB+50 μ g/ml kanamycin, LB+25 μ g/ml chloramphenicol, or LB+25ng/ml ciprofloxacin to identify clones that had incorporated the S83L substitution and lost the donor and mutagenesis plasmids. Successful mutagenesis was confirmed by Sanger sequencing.

Cultures were grown in Lysogeny broth/agar cultures (LB) (10 g/l tryptone, 5 g/l yeast extract, 10 g/l NaCl; Bishop) at 37 C throughout this study. LB media supplemented with 100 μ g/ml ampicillin (Sigma-Aldrich) was used for plasmid

maintenance. Susceptibility assays were performed using CIP (Sigma-Aldrich) at 100ng/ml.

3.3.2 Randomized library construction

The small RNA expression vectors pBad-DsrA, MicF, and Spot42 (Sharma *et al.* 2011; kindly donated by Yohei Yokobayashi, Okinawa Institute of Science and Technology) were used as templates for polymerase chain reaction (PCR) reactions. Randomized artificial sRNAs were constructed by incorporating random sequences in the antisense domains of sRNAs using PCR primers with degenerate bases. Platinum Pfx polymerase (Invitrogen) was used to PCR amplify the whole plasmid with a common reverse primer Sartrev containing 20 degenerate bases and an sRNA specific forward primer consisting of 10 degenerate bases for randomizing the antisense domain. The complete list of oligonucleotides used in this study is provided in supplementary Table B3.1.

The PCR amplicon was purified (Bio Basic) and digested using the *DpnI* enzyme (New England Biolabs) for 2 hours at 37 C to cleave the methylated template parental DNA. Digested plasmids were phosphorylated using T4 polynucleotide kinase (New England Biolabs) for 20 minutes at 37 C. After phosphorylation, the linear DNA was self-ligated using T4 DNA ligase (New England Biolabs) and incubated at 25 C overnight.

The consistency of the randomization protocol was first verified in Top10 cells. Subsequently, the randomized sRNA library was introduced into an *E. coli gyrA* S83L mutant by transforming chemically competent cells with the self-ligated PCR amplicons.

Transformations were plated on LB agar supplemented with ampicillin, single colonies were picked, grown overnight at 37 C at 150 rpm with 100µg/ml ampicillin, and glycerol stocks were prepared in a 96 well plate for further screening. Controls in each of the 96 well plates included *gyrA* S83L carrying empty sRNA plasmid and untransformed *E. coli* MG1655. Libraries were constructed separately for each of the three expression vectors, consisting of ~5000 transformants in total. ~40 plasmids from the randomized library (roughly equal numbers from each plasmid backbone) were extracted and sent for Sanger sequencing to confirm randomization.

3.3.3 Ciprofloxacin sensitivity screen

In order to identify sRNA constructs that increased CIP sensitivity, 24 hour growth curves in CIP-containing media were assayed for ~5000 *gyrA* S83L mutant clones bearing randomized constructs. Cultures were inoculated at a 1:100 dilution from glycerol stock cultures and grown overnight at 37 C, 150 rpm in LB with 100µg/ml ampicillin for sRNA plasmid maintenance. For 24-hour growth curves, the overnight cultures were diluted (1:100) in LB supplemented with 100ng/mL CIP, 0.5mM arabinose, and 100µg/ml ampicillin. Arabinose is required to induce expression of the sRNA construct. 100 ng/mL CIP was chosen as an intermediate concentration between the minimum inhibitory concentration (MIC) of the WT (30 ng/mL) and the S83L mutant (600 ng/mL). The OD₆₀₀ of each culture was measured on a Biotek ELx808 plate reader every 37 minutes for 24 hours, incubating at 37 C with 30 seconds of shaking every 5

minutes. Two growth parameters, lag time, and maximum growth rate, were estimated using the program GrowthRates (Hall *et al.* 2013).

Clones showing growth repression in the primary CIP sensitivity screen were selected for secondary screening in the presence and absence of CIP (100ng/ml) using similar experimental methods as described above. Clones whose growth was repressed in the presence of CIP, but not in LB alone, were selected for further analysis.

3.3.4 Target identification

In order to identify potential target mRNAs, sRNA vectors were extracted from CIP-sensitive clones and the sRNA region was Sanger sequenced. Putative mRNA targets were identified for the randomized sRNA sequences using the online software packages IntaRNA (Mann *et al.* 2017), Target RNA (Kery *et al.* 2014), and RNA predator (Eggenhofer *et al.* 2011). These web based programs predict hybridizations between two RNA molecules, and provide a graphical overview of the sRNA-mRNA binding interactions (Tjaden 2008; Smith *et al.* 2010; Eggenhofer *et al.* 2011). The resulting target genes were identified and selected for experimental validation.

3.3.5 Validation for selected targets

Candidate genes whose knockdown may induce CIP sensitization were validated experimentally by constructing double mutants, in which the *gyrA* S83L mutation was combined with knockout mutations of the computationally predicted target genes. The knockout mutants were selected from the Keio mutant collection (Baba *et al.* 2006), wherein a kanamycin resistance cassette was used to replace sequences of non-essential genes. The *gyrA* S83L mutation was introduced by oligonucleotide-mediated recombineering (Ellis *et al.* 2001) into the Keio deletion mutants, to construct each double mutant. A mobilizable derivative of pMA7SacB was used (Lennen *et al.* 2016), in which an arabinose-inducible promoter (P_{BAD}) controls expression of the β subunit of λ -Red recombinase and the *E. coli* Dam methylase. Dam methylase induction has been shown to increase mutagenesis efficiencies in *E. coli* by transiently interfering with the DNA mismatch repair system (Lennen *et al.* 2016). The plasmid was introduced into recipient strains by conjugation using a donor *E. coli* strain (WM3064) that is auxotrophic to diaminopimelic acid (DAP) (Dehio and Meyer 1997; Saltikov and Newman 2003). Recipients (Keio mutants) were cultured in LB broth supplemented with 30 μ g/ml kanamycin and the donor was cultured in LB supplemented with 0.3 mM DAP and 100 μ g/ml ampicillin (for Red plasmid maintenance). Mating mixtures were spotted on LB agar with 0.3 mM DAP and incubated overnight. Exconjugates were selected with 100 μ g/ml ampicillin on LB lacking DAP, to prevent growth of donors. λ -Red recombination was performed on Keio mutants harboring the Red plasmid as previously described (Lennen *et al.* 2016) using an oligonucleotide encoding the *gyrA* (S83L)

mutation (5'-

AACGCAGCGAGAATGGCTGCGCCATGCGGACGATCGTGTCATAGACCGCCAA

GTCACCATGGGGATGGTATTTACCGATTACGTCACCAA-3'). Transformants

harboring the *gyrA* mutation were selected by plating on LB supplemented with 30 µg/ml

CIP and 30 µg/ml kanamycin. The strains were cured of the λ Red plasmid by *sacB*

counterselection by streaking on LB agar containing 5% sucrose. The *gyrA* gene of each

putative double mutant was PCR amplified and Sanger sequenced to verify the presence

of the S83L mutation (PCR primers: Gyrase forward -

5'GTAAAACGACGGCCAGTGATGAGCGAC3', Gyrase reverse -

5'CGGTACGGTAAGCTTCTTC3').

3.3.6 Ciprofloxacin susceptibility assay

Minimum inhibitory concentrations (MIC) for CIP were determined for each of the *gyrA*

S83L/ Keio knockout double mutants using a 96 well plate assay. Antibiotic

concentrations were started at 125ng/ml, 4ug/ml, and 8ug/ml for Keio single knockout

mutants, double mutants (Keio single knock out mutant + *gyrA* S83L), and controls

(*cybC* only and *cybC* + *gyrA* S83L) respectively, and were diluted in a two-fold series

and dispensed with 125µl/well of LB into 96-well plates. The 96-well plates were

incubated overnight at 37 C, with shaking at 150 rpm. The MIC was defined as the lowest

concentration of antibiotic for which 90% growth inhibition was visibly observed after

overnight culture.

3.4 Results

3.4.1 Randomized sRNA library construction

We generated a library of artificial sRNAs by randomizing the antisense domain sequences of three native *E. coli* sRNAs: DsrA, MicF, and Spot42. We transformed the sRNA library into an *E. coli gyrA* S83L mutant and collected a total of ~5000 clones, each bearing a plasmid with an artificial sRNA construct (Figure 3.1A, B). We arbitrarily isolated 40 clones from the plasmid library and sequenced the sRNA regions to check for the incorporation of randomized sequences. The sequencing results confirmed the presence of unique randomized sequences in each of the engineered sRNA constructs (example sequences are shown in Table 3.1).

Table 3.1 Randomized sequences of artificial sRNAs constructs. ~10-30 randomized bases (highlighted) were incorporated upstream of the scaffold regions of DsrA, MicF, and Spot42 by PCR amplification of source vectors lacking the antisense regions (“empty” vectors). All the sRNAs start from vector-derived sequence of 5’ACTCGAG (red).

Scaffold	Randomized region
Wild-type DsrA sequence	actcgagcaatttttaagtcttctgcttaag actcgagttgtacctgctttcgatacgactttcatcaatttttaagtcttctgcttaag
Example randomized DsrA	actcgagttctctcgctggactgaacgtggagctggaatttttaagtcttctgcttaag actcgaggaatcggaccatgataccactgagtttaatttttaagtcttctgcttaag
Wild-type MicF sequence	actcgagcgtcattcatttctgaatgtctg actcgagtccccttcacgggtgcaacgggccaatggcgtcattcatttctgaatgtctg
Example randomized MicF	actcgagctatacagatcatttacaccccgatcacccgtcattcatttctgaatgtctg actcgaggtagtagactgcgtcattcatttctgaatgtctg
Wild-type Spot42 sequence	actcgagatttggtgaatatttagccgc
Example randomized Spot42	actcgaggaggggggatttggtgaatatttagccgc actcgagccgtatagaaccacatctgcctgggggggatttggtgaatatttagccgc

Figure 3.1 A schematic representation for the construction of the randomized artificial sRNA library. (A) The antisense domain is randomized with ~10-30 degenerate bases. The randomized bases were incorporated upstream of the scaffold regions of DsrA, MicF, and Spot42 by PCR amplification of source vectors lacking the antisense regions (“empty” sRNA). (B) The subsequent randomized product is transformed into a *gyrA* S83L mutant to obtain a randomized artificial library, with each clone bearing a novel sRNA construct.

3.4.2 Ciprofloxacin sensitivity screening

To identify clones with increased susceptibility towards CIP, all ~5000 *gyrA* S83L clones bearing sRNA constructs were grown in LB supplemented with 100ng/mL CIP and arabinose to induce sRNA expression. The MIC of the *gyrA* S83L mutant is 600ng/ml, so we reasoned that incubation at this sub-MIC concentration of CIP would be a good indicator for changes in sensitivity. Controls included wild-type *E. coli* MG1655 grown in LB without CIP (median growth rate: 0.09 OD₆₀₀ /minute and length of lag phase: 71 minutes), and a *gyrA* S83L mutant bearing a non-randomized plasmid grown in LB+CIP (median growth rate: 0.08 OD₆₀₀ /minute and length of lag phase: 250 minutes) (Figure 3.2). We selected a total of 528 clones exhibiting substantial reductions in growth rate and/or increased lag phase (outliers from Figure 3.2). From the individual sRNA scaffolds, we selected 199 clones from DsrA (median growth rate: 0.08 OD₆₀₀ /minute and length of lag phase: 395 minutes), 231 clones from MicF (median growth rate: 0.08 OD₆₀₀/minute and length of lag phase: 393 minutes), and 98 clones from Spot42 (median growth rate: 0.08 OD₆₀₀/minute and length of lag phase: 275 minutes).

We repeated the growth curves of the 528 putatively CIP susceptible clones in the presence and absence of CIP (Figure 3.3, Supp Figure B3.1). This secondary screen was performed in order to verify the CIP-sensitive phenotypes and to determine whether or not the growth deficiencies were specific to CIP; a growth deficit in LB alone would indicate a general fitness effect of the sRNA, rather than reversal of CIP resistance. From this secondary screen, we selected 48 clones showing little or no growth inhibition in LB without CIP (Mean growth rate: 0.085 OD₆₀₀/minute and length of lag phase: 478

minutes), but whose growth was inhibited in LB supplemented with CIP (Mean growth rate: 0.071 OD₆₀₀/minute and length of lag phase: 824 minutes).

Figure 3.2 Boxplot distributions of CIP-sensitivity phenotypes for ~5000 *gyrA* S83L mutants harbouring randomized sRNA plasmid constructs. The growth rate (A) and lag time (B) distributions are depicted for clones cultured in LB with 600 ng/ml CIP. *E. coli* MG1655 grown without CIP, and *gyrA* S83L mutants bearing empty sRNA plasmids grown with CIP, were used as controls. The boxplot presents the median, first, and third quartiles, with whiskers showing either the maximum (minimum) value or 1.5 times the interquartile range of the data, whichever is smaller (larger). The outliers (528 clones) selected for further investigation have substantial reductions in growth rate and/or increased lag phase.

Figure 3.3 Effects of sRNA expression on growth rate (OD₆₀₀/minute) and length of lag phase (minutes) with and without CIP. The 48 selected constructs showing repression in growth in the presence of CIP are indicated in blue.

3.4.3 Identification of target genes

We sequenced the randomized antisense regions of the sRNA plasmids isolated from the 48 clones exhibiting growth defects specific to the presence of CIP. Candidate target mRNAs were identified using the online tools IntaRNA (Mann *et al.* 2017), Target RNA (Kery *et al.* 2014), and RNA predator (Eggenhofer *et al.* 2011). These packages predict target mRNA sites in the *E. coli* MG1655 transcriptome with the potential for complementary sRNA interactions. We detected complementary hits for 31/48 randomized sequences (an example of complementarity between an artificial sRNA and its predicted target gene (*sbmC*) is shown in Supp Figure B3.2). Since these tools identify complementary matches between short sequences, several sequences were matched to multiple targets, resulting in a total of 222 potential gene targets (Supp Table B3.2). Candidate targets included genes involved in DNA repair and recombination (*recC*, *recD*, *recJ*, *sbmC*), the SOS response and error prone replication (*umuC*, *xseA*), transcriptional regulation (*yeeY*, *mtlR*), cell wall division and assembly (*zipA*, *minC*), transport and efflux (*emrB*, *tolQ*), two-component regulatory systems (*cpxA*, *cpxR*, *ompR*, *cheB*), and chaperone proteins (*hybB*, *dnaK*).

3.4.4 Experimental validation of targets

We carried out validation experiments for 36 genes that encoded potential mRNA targets (Supp Table B3.3). Since CIP primarily interacts with DNA gyrase, we focused on putative target genes related to gyrase and its functions in DNA replication, repair and

recombination. Additional genes were chosen because of their roles in efflux transport systems, two-component regulatory systems, transcriptional regulators and as chaperone proteins (Table 3.2). For each candidate target gene, the corresponding knockout mutant was obtained from the Keio single gene knockout collection (Baba *et al.* 2006). The *gyrA* S83L mutation was then transferred into the Keio clone by λ -Red mutagenesis, generating a double mutant.

To determine which candidate target genes conferred increased sensitivity to CIP on the *gyrA* S83L background, we obtained the MICs of each double mutant and the corresponding single mutants (Supp Table B3.4). Knockout of a pseudogene, *cybC*, was used as a control for the effect of the kanamycin cassette present in the Keio clones. The MIC values of a number of double mutants showed reduced susceptibility when compared to the *cybC*+S83L (MIC=1000ng/ml) double mutant. Importantly, for 30 genes, the reduction in CIP resistance was more pronounced on the *gyrA* S83L background than on the WT background; these genes fall below the 1:1 line on Figure 3.4. Inactivation of these genes thus leads to substantial reductions in *gyrA*-mediated CIP resistance. Some of the mutants falling into this category include *xseA*, *tus* (double mutant MIC reduction of 16-fold), *sbmC* (double mutant MIC reduction of 8-fold), *tolQ* and *tolC* (double mutant MIC reduction of 16-fold).

Table 3.2 36 genes selected for experimental validation, encoding predicted sRNA targets associated with reductions in *gyrA* S83L mediated CIP resistance. (The locus tags indicate to the accession number, obtained from EcoCyc database collection (EcoCyc database: Keseler *et al.* 2017)).

S.NO.	Target gene	Function	Category	Locus tag	Reference
1	<i>recC</i>	DNA helicase, ATP-dependent dsDNA/ssDNA exonuclease	DNA replication, repair and recombination	b2822	Persky and Lovett (2008)
2	<i>recD</i>	Exonuclease V (RecBCD complex) alpha chain	DNA replication, repair and recombination	b2819	Persky and Lovett (2008)
3	<i>recJ</i>	ssDNA exonuclease; 5' 3' specific	DNA replication, repair and recombination	b2892	Han <i>et al.</i> (2006)
4	<i>rnt</i>	Ribonuclease T (RNase T)	DNA replication, repair and recombination	b1652	Viswanathan <i>et al.</i> 1999)
5	<i>rpoN</i>	RNA polymerase sigma 54 (sigma N) factor	DNA replication, repair and recombination	b3202	Zhang <i>et al.</i> (2016)
6	<i>smrB</i>	Putative DNA endonuclease	DNA replication, repair and recombination	b2331	Keseler <i>et al.</i> (2016)
7	<i>sbmC</i>	DNA gyrase inhibitor	DNA replication, repair and recombination	b2009	Nakanishi <i>et al.</i> (1998)
8	<i>tus</i>	Inhibitor of replication at Ter; DNA binding protein	DNA replication, repair and recombination	b1610	Hill <i>et al.</i> 1989
9	<i>topB</i>	DNA topoisomerase III	DNA replication, repair and recombination	b1763	DiGate <i>et al.</i> (1988)
10	<i>umuC</i>	DNA polymerase V; subunit C	DNA replication, repair and recombination	b1184	Tang <i>et al.</i> (1999)
11	<i>uvrD</i>	DNA-dependent ATPase I and helicase II	DNA replication, repair and recombination	b3813	Matson and Robertson (2006)
12	<i>xseA</i>	Exonuclease VII large subunit	DNA replication, repair and recombination	b2509	Vales <i>et al.</i> (1982)
13	<i>dnaQ</i>	DNA polymerase III epsilon subunit	DNA replication, repair and recombination	b0215	Echols <i>et al.</i> (1983)
14	<i>hldD</i>	DNA helicase IV	DNA replication, repair and recombination	b0962	Mendonca <i>et al.</i> (1993)

15	<i>priB</i>	Primosomal protein N	DNA replication, repair and recombination	b4201	Heller and Mariani (2005)
16	<i>priC</i>	Primosomal replication protein N	DNA replication, repair and recombination	b0467	Heller and Mariani (2005)
17	<i>rnr</i>	Exoribonuclease R; RNase R	DNA replication, repair and recombination	b4179	Cheng and Deutscher (2002)
18	<i>uvrA</i>	ATPase and DNA damage recognition protein of nucleotide excision repair	DNA replication, repair and recombination	b4058	Wagner <i>et al.</i> (2009)
19	<i>yeeY</i>	Predicted DNA binding transcriptional regulator	Transcriptional regulators	b2015	Keseler <i>et al.</i> (2016)
20	<i>mtlR</i>	Transcriptional repressor (mannitol)	Transcriptional regulators	b3601	Figge <i>et al.</i> (1994)
21	<i>ydaG</i>	Uncharacterized protein	Uncharacterized protein	b1355	Keseler <i>et al.</i> (2016)
22	<i>ydaV</i>	Uncharacterized protein (predicted ATP binding protein)	Uncharacterized protein	b1360	Hidese <i>et al.</i> (2014)
23	<i>ompR</i>	Response regulator with EnvZ	Two component regulatory system	b3405	Cai and Inouye (2002)
24	<i>cheB</i>	Fused chemotaxis regulator protein	Two component regulatory system	b1883	Baker <i>et al.</i> (2006)
25	<i>cpxA</i>	Sensory histidine kinase with CpxR	Two component regulatory system	b3911	Bury-Mone' <i>et al.</i> (2009)
26	<i>cpxR</i>	Response regulator with CpxA	Two component regulatory system	b3912	Batchelor <i>et al.</i> (2005)
27	<i>dnaK</i>	Chaperone Hsp70; co-chaperone with DnaJ	Chaperons proteins	b0014	Mayer <i>et al.</i> (2000)
28	<i>hybB</i>	Predicted hydrogenase 2 cytochrome b type component	Chaperons proteins	b2995	Menon <i>et al.</i> (1994)
29	<i>emrB</i>	Multidrug efflux system protein	Transport, Efflux system	b2686	Lomovskaya <i>et al.</i> (1995)
30	<i>tolA</i>	Membrane anchored protein in TolA-TolQ-TolR complex	Transport, Efflux system	b0739	Bernadac <i>et al.</i> (1998)
31	<i>tolQ</i>	Membrane spanning protein in TolA-TolQ-TolR complex	Transport, Efflux system	b0737	Bernadac <i>et al.</i> (1998)
32	<i>tolC</i>	AcrAB-TolC multidrug efflux transport system	Transport, Efflux system	b3035	Bernadac <i>et al.</i> (1998)
33	<i>minC</i>	Septum site determining protein, inhibitor of FtsZ ring polymerization	Cell division	b1176	Pichoff, and Lutkenhaus (2001)
34	<i>zipA</i>	FtsZ stabilizer	Cell division	b2412	Hale and de Boer (2002)
35	<i>proC</i>	Pyrroline-5-carboxylate reductase NAD(P)-binding	Catalytic, biosynthetic pathways	b0386	Deutch <i>et al.</i> (1982)
36	<i>pyrB</i>	Catalytic subunit	Catalytic, biosynthetic pathways	b4245	Donahue and Turnbough (1994)

Figure 3.4 Fold change in MIC values for 36 knockout mutants on wild-type and *gyrA* S83L backgrounds. The 30/36 mutants that fall below the 1:1 line show reduction in CIP resistance on the *gyrA* S83L background but not on the wild-type background.

3.5 Discussion

We carried out an sRNA screen to identify genes whose knockdown restores quinolone sensitivity in *E. coli*. Following an initial screen of ~5000 sRNA-bearing clones, and secondary screening of over 500 clones, we found 30 genes whose disruption increases sensitivity of a *gyrA* S83L mutant by 2-fold or more. These genes have known functions in DNA repair and replication, drug efflux, and transcriptional regulation. Our findings expand knowledge of the genetic interaction network of the essential gene *gyrA*, and provide potential targets for the development of antibiotic adjuvants to restore sensitivity in quinolone resistant pathogens.

Chemical-genetic sensitivity screens have largely used knockout approaches, whereby a library of knockout mutants is screened for sensitivity or resistance to an antibiotic at sub-lethal concentrations (e.g., Tamae *et al.* 2008, reported 140, (approximately 3%) out of 4000 Keio mutants against 7 antibiotics); Gomez and Neyfakh 2006; Breidenstein *et al.* 2008; Fajardo *et al.* 2008; Liu *et al.* 2010). Typically, such screens are carried out on a wild-type, antibiotic susceptible background, so they are not well-suited to identifying genes whose knockdown reverses resistance. Nonetheless, Tamae *et al.* (2008) did show that knockouts of five genes recovered from a sensitivity screen (*recC*, *recA*, *fis*, *xseA*, *tolC*; all but *fis* were also found here) did reduce CIP resistance on a *gyrA* mutant background. sRNA screens offer a powerful means for identifying genetic-background specific effects, since sRNA libraries can be readily

generated in any transformable strain (Sharma *et al.* 2011, 2013; Lee *et al.* 2011). Moreover, inducible sRNA constructs can be used for studying gene functions of essential genes as well as non-essential genes (Rodrigo *et al.* 2012), which is not possible with knockouts. Our sRNA screen identified several candidate essential genes (*gyrA* and *parC*, encoding the targets of CIP) whose knockdown reduced growth in CIP. The randomization approach that we have adopted here (see also Sharma *et al.* 2011, 2013) is particularly promising, since it does not require targeted cloning of gene-specific sRNAs.

Direct use of sRNAs as therapeutics would require the efficient expression of a synthetic sRNA construct inside the target bacterial cell. This would require a competent delivery system, such as phage or a conjugative plasmid system. An engineered-phage approach illustrated by Lu and Collins (2009) expressed the LexA and OmpF proteins from phage M13. This resulted in reduction in antibiotic resistance evolution in mice when injected with engineered-phages. Thus, a comparable approach could be a plausible delivery system for combating resistant bacteria. An alternative strategy would be to identify small molecules that inhibit the targets identified here. For example, we showed that inhibition of *sbmC* reduces CIP resistance by 2-fold, and SbmC is a known target of the peptide antibiotic Microcin B17 (Baquero *et al.* 1995; Collin *et al.* 2011). Thus, sRNA-mediated target discovery may be an efficient option for generating prospects for novel therapeutics.

Chapter 4

Two-component regulatory networks as determinants of stress responses and antimicrobial resistance in *Escherichia coli*

4.1 Abstract

Two-component signal transduction systems (TCS) serve as stimulus-response mechanisms that allow bacteria to monitor diverse environmental cues. The typical bacterial TCS consists of a transmembrane sensor kinase and a cytoplasmic response regulator, regulating physiological responses to a variety of environmental parameters, including phenotypic resistance due to various stressors. The present study aims to elucidate the physiological basis of bacterial tolerance in response to different envelope stresses using well elucidated TCSs that act as global regulators of envelope stress tolerance, as well as antibiotic resistance. Here, I have characterized the responses of eight mutants from four TCSs of *Escherichia coli* to combinations of external stressors and antibiotic challenges. I identified TCS mutants exhibiting different phenotypic

changes in response to stresses, antibiotics and stressor+antibiotic combinations. While the stressor-only conditions generally slowed growth rates of the TCS mutants, increased growth rates were observed under some stressor+antibiotic combination. Similarly, TCS mutants showed gain in resistance in the presence of stressor, with reductions in resistance levels in its absence. Overall, this work broadens the understanding of the mechanisms by which *E. coli* responds to the external physiological environment, including antibiotics.

4.2 Introduction

The tolerance of bacterial pathogens to fluctuating environmental challenges is based on their rapid repertoire of responses to distinct environments. Environmental stresses that perturb bacterial cell envelope integrity can impact its growth and survival (Poole *et al.* 2012). It is of great interest, therefore, to understand bacterial survival strategies in the landscape of membrane stress. In both Gram-positive and Gram-negative bacteria, two-component signal transduction systems (TCSs) play a pivotal role in regulating diverse protective stress responses (Stock *et al.* 1989; Hoch and Silhavy 1995; 2000; Gao and Stock 2009). Recognition of specific environmental cues/signals that perturb the cell envelope's integrity, and interpretation of this information through modulating transcriptional responses, is the essence of such TCSs (Stock *et al.* 2000; Mascher *et al.* 2006; Laub *et al.* 2007). TCSs have been shown to respond to a variety of environmental signals, such as osmolarity, antibiotics, and nutrient availability (Parkinson 1993; Hoch and Silhavy 1995; Mizuno 1997; Oshima *et al.* 2002; Krell *et al.* 2010). TCSs regulate a range of activities, such as sporulation, biofilm formation, chemotaxis, nutrient utilization, virulence to antimicrobial susceptibility, and resistance (Mizuno 1997; Stock *et al.* 2000).

TCSs are comprised of two protein modules: a transmembrane receptor or sensor histidine protein kinase (HK) protein, and its cognate, intracellular signalling receiver known as a response regulator (RR) (Stock *et al.* 2000). The various HKs and RRs constitute a complex signal transduction system, culminating in the regulation of gene

expression. Concisely, after sensing an external stimulus (e.g., ligand binding) the HK undergoes a conformational change and as a result auto-phosphorylates at a conserved histidine (H) residue using of the γ -phosphate of ATP. Subsequently, the signal information as a phosphoryl moiety is transferred to a conserved aspartate residue of a cognate RR, triggering the expression of a large number of target genes (Stock *et al.* 2000; Gao and Stock 2009; Zschiedrich *et al.* 2016) (Figure 4.1).

Figure 4.1 Schematic of a stereotypical two-component signal transduction system's (TCS) phosphorylation cascade. The sensor domain responds to a stimulus and uses ATP to autophosphorylate at conserved histidine (H) residue, located at the kinase core domain. The phosphoryl group (P) is transferred to a conserved aspartate (D) residue of its cognate response regulator, which subsequently triggers the output response.

In *Escherichia coli*, ~30 TCSs have been identified by experimental evidence and protein sequence similarity. For comparison, this is roughly half the number of TCSs found in *Pseudomonas aeruginosa* (Rodrigue *et al.* 2000; Gooderham and Hancock 2009). Mizuno *et al.* (1997) used the sequences of OmpR, NarL, NtrC, and CheY as probes to explore *E. coli* TCSs with similar open reading frames. The members of each TCS family show strong amino acid conservation of domains involved in signal transduction, reflecting functional similarities (Supp Figure C4.1). For example, in the OmpR family of TCS, the phosphotransfer, ATP-domain, and regulator domain have maintained common sequences and structure (Gross *et al.* 1989; Hoch 2000).

In Gram-negative bacteria, many TCSs respond to envelope stresses triggered by different stressors. In the context of antimicrobial resistance (AMR) TCSs act as global regulators of AMR as they optimize bacterial metabolism based on their surrounding environmental challenges (such as envelope stress responses) and thereby impact levels of resistance (Tamae *et al.* 2008; Fajardo *et al.* 2008; Martinez *et al.* 2009; Dam *et al.* 2018). Given that antimicrobial compounds target components of the cell envelope or penetrate the cell envelope to gain entry to their respective targets, it is not surprising that membrane permeability impacts antimicrobial susceptibility (Hirakawa *et al.* 2003; Pagès *et al.* 2008; Fernandez and Hancock 2012; Poole 2014). TCSs also act as regulators, and thus participate in regulating the expression of many genes leading to effects on efflux pumps, cell envelope components, and antibiotic-degrading enzymes (Lee *et al.* 2009). TCSs modulate antibiotic susceptibility through their global regulatory phosphorelay networks that regulate bacterial physiology while adjusting to different environmental cues. Here, I study several well elucidated TCSs that are global regulators of envelope

stress and that impact antimicrobial susceptibility in *E. coli*: PhoQ-PhoP, EnvZ-OmpR, CpxA-CpxR, and BaeS-BaeR .

The PhoP-PhoQ (Pho) TCS is linked to virulence and cationic antimicrobial peptide (CAMP) resistance in several *Enterobacteriaceae*, *P. aeruginosa*, and *Salmonella typhimurium* (Kato and Groisman 2008; Gooderham and Hancock 2009). The Pho system facilitates the response to limiting magnesium concentrations and affects antimicrobial susceptibility against aminoglycosides and membrane disrupting CAMPs (e.g., polymyxin B and colistin) (Macfarlane *et al.* 2000; Cheng *et al.* 2010; Zschiedrich *et al.* 2016). This is primarily mediated by regulating the genes involved in the production of lipid A of the bacterial lipopolysaccharide (LPS) layer (Macfarlane *et al.* 2000; Gooderham and Hancock 2009; Needham and Trent 2013; Dalebroux and Miller 2013; Ram and Goulian 2013). The Pho system also regulates other features of bacterial metabolism, such as glycogen metabolism of *E. coli* (Montero *et al.* 2009) as well as several other genes involved in mediating bacterial metabolism (Santos-Beneit 2015).

Another relevant TCS is EnvZ-OmpR, widely found in *E. coli* and other Gram-negative bacteria. This system responds to changes of medium osmolarity and plays a role in iron homeostasis, synthesis of flagella, and amino acid metabolism (Forst and Roberts 1994; Cai and Inouye 2002). The EnvZ-OmpR phosphorelay system regulates these bacterial physiological effects, in part, through transcriptional regulation of two major outer membrane proteins, OmpF and OmpC (Forst and Roberts 1994; Yamamoto *et al.* 2005; Yoshida *et al.* 2006). As porins are the gateway for the entry of small β -lactams and other hydrophilic antibiotics, such as tetracycline, chloramphenicol and

fluoroquinolones, a decrease in porin-mediated permeability caused by any loss-of-function/regulatory mutations in the porins confers high level resistance to antibiotics (Nikaido 2003; Doumith *et al.* 2009; Källman *et al.* 2009).

Similarly, in *E. coli* and other Gram negative organisms, the CpxR-CpxA (Cpx) system contributes to multidrug resistance (MDR) (Hu *et al.* 2011; Audrain *et al.* 2013; Kurabayashi *et al.* 2014; Surmann *et al.* 2016). The Cpx system is predominantly involved in the envelope stress maintenance system and responds to a variety of environmental stresses including bactericidal antibiotics that generate misfolded envelope proteins and impact the cell envelope adversely (Raffa and Raivio 2002; Ruiz and Silhavy 2005; Raivio 2014; Delhaye *et al.* 2016). In *E. coli*, the Cpx system upregulates the transcription of the *marRAB* operon. This operon activates TolC-dependent tripartite MDR efflux transporters, which in turn facilitates resistance to aminoglycosides, β -lactams, novobiocin, and CAMPs (Audrain *et al.* 2013; Mahoney and Silhavy 2013; Raivio 2014; Weatherspoon-Griffin *et al.* 2014; Delhaye *et al.* 2016). Girgis *et al.* (2009) in their transposon-mutagenized library of *E. coli* determined the involvement of Cpx system in governing intrinsic antibiotic susceptibility.

The BaeR-BaeS (Bae) TCS also contributes to MDR by modulating the expression of multidrug transporters in *E. coli* (Nishino and Yamaguchi 2001; Baranova and Nikaido 2002; Nishino and Yamaguchi 2002; Leblanc *et al.* 2011). The Bae system responds to envelope stress and is known to confer resistance to novobiocin and β -lactams by upregulating Bae-dependent *mdtABCD* and *acrD* MDR efflux transporters (Baranova and Nikaido 2002; Hirakawa *et al.* 2003; Bury-Moné *et al.* 2009; Leblanc *et*

al. 2011; Rosner and Martin 2013). Lin *et al.* (2013) identified the Bae system in *Acetobacter baumannii*, and showed that *baeSR* mutants are susceptible to tigecycline. The Bae system also confers resistance to variety of other antimicrobials, including aminoglycosides, tetracycline, rifampicin, and fluoroquinolones (Hu *et al.* 2011; Guerrero *et al.* 2013).

In this study, I have selected eight mutants from four TCSs (Pho, EnvZ-OmpR, Cpx, and Bae) that are the global regulators of stress tolerance and antimicrobial resistance in *E. coli*. I have obtained TCS mutants from the Keio single-gene knockout mutant collection and exposed them to a stand-alone stressor (NaCl and MgCl₂) and in a combination of stressor plus antibiotic conditions. These clinically relevant antibiotics belong to different antibiotic classes- ciprofloxacin (CIP), ceftazidime (CEF), tetracycline (TET), and chloramphenicol (CHM). In normal metabolic regulation, upon sensing environmental stress, TCSs get activated. Therefore, if a particular TCS is involved in the response to a particular stress condition, presumably we can expect a TCS mutant to display a decreased growth phenotype in the presence of that particular stress condition. Similarly, in a stressor+antibiotic encounter, TCS mutants are expected to show a different growth phenotype, providing direct evidence that these mutants play an important role in the tolerance of stressful conditions, as well as antibiotics. Accordingly, the profiling of *E. coli* responses to envelope stresses has expanded our understanding of intricate control systems in elucidating the potential link between complex TCS, and their impact on growth upon exposure to a particular antibiotic.

4.3 Materials and Methods

4.3.1 *E. coli* strains

Eight mutants from four TCSs (Pho, EnvZ-OmpR, Cpx, and Bae) were selected from the Keio single-gene knockout collection (Baba *et al.* 2006). The Keio collection was derived from the wild-type strain K-12 BW25113 (*lacI⁺rrnB_{T14} ΔlacZ_{WJ16} hsdR514 ΔaraBAD_{AH33} ΔrhaBAD_{LD78}*). Each of the 3985 strains in the Keio collection carries a complete deletion of a different gene with a kanamycin resistance cassette replacing the gene of interest. Throughout this experiment, I have used a knockout of *cybC*, which is a pseudogene, as a control for the effect of the kanamycin resistance cassette present in the Keio clones.

4.3.2 Media and culture conditions

M9 minimal media (M9 MM) (composition per liter (Bioshop): 6.78g Na₂HPO₄, 3.00g KH₂PO₄, 0.50g NaCl, 1.00g NH₄Cl, 0.24g MgSO₄, 0.01g CaCl₂, 8.00g glucose as the carbon source, 16g agar, pH 7) was used for agar and broth cultures. When needed, the growth medium was supplemented with inducers and antibiotics. The antibiotics ciprofloxacin, ceftazidime, tetracycline, and chloramphenicol were used (Sigma-Aldrich) in this study at their minimum inhibitory concentrations (MIC) (Andrews 2001): ciprofloxacin (0.015μg/ml), ceftazidime (0.06μg/ml), tetracycline (1μg/ml), and

chloramphenicol (2µg/ml). The chemicals that were used as inducers in this study and their concentrations are: MgCl₂ (200µM, Bioshop) and NaCl (0.5M, Bioshop) (Supp Table C4.1).

4.3.3 The growth of TCS mutant strains in M9 MM +/- stress conditions on solid medium

Each of the TCS mutants was transferred from the frozen glycerol Keio collection stock into 5ml of M9 MM with 0.8% (w/v) glucose as the sole carbon source and grown overnight on a rotary shaker at 37 C, 150 rpm (optimal growth conditions). The overnight culture was inoculated into a fresh 150ul of M9 MM in a 96-well plate at a 1:500 dilution and grown overnight on a rotary shaker at 37 C, 150 rpm. The high dilution minimizes nutrient leftovers from the overnight culture.

For colony size measurements the overnight cultures were used and spotted in triplicate on rectangular plates (single well with lid (86x128 mm), Thermo fisher scientific) having M9 MM with 0.8% glucose and 1.5% agar (using a pinning tool (V&P scientific Inc.(408FP6))). Where required, the stressor +/- antibiotic were pre-added into the solid media at the same concentrations as mentioned above. All combinations of stressor and antibiotic were used, including M9 MM alone, M9 MM + stressor, M9 MM + antibiotic, and M9 MM + stressor + antibiotic. Overnight cultures were spotted at the centre of the plates in order to avoid errors in colony quantification, since colonies on the periphery are oversized compared to interior colonies. All plates were then incubated at

37 C overnight. High resolution images of each plate were obtained using the ProtoCOL 3 instrument (Synbiosis, MD, USA) with an ultra-HD 5 megapixel scientific grade camera. Colony size was measured using two automated image analysis software packages, available online, SGA tools and the R package gitter image (<http://bioconductor.org/biocLite.R>). These tools quantify growth by obtaining an image of the plate and measuring pixel counts of the colony within a defined boundary.

4.3.4 24-hour growth curve analysis

The growth of each TCS mutant in broth culture was estimated using 24-hour growth curves using three independent replicates. Growth curves were obtained in 96-well plates, inoculated at a 1:100 dilution from overnight cultures in a 96-well plate with fresh M9 MM. Again, all four possible combinations of stressor and antibiotic were used, including M9 MM + no antibiotic, M9 MM + stressor, M9 MM + antibiotic, and M9 MM + stressor + antibiotic. Absorbance (OD₆₀₀) was measured on a Biotek ELx808 plate reader every 37 minutes for 24 hours, incubating at 37 C with 30 seconds of shaking every 5 minutes. Two growth parameters, lag time, and maximum growth rate after 24 hours, were estimated using the program GrowthRates (Hall *et al.* 2013).

4.3.5 Antibiotic susceptibility assay

Minimum inhibitory concentration (MIC) values were determined for all of the TCS mutants involved in this study using +/- stressor conditions in a 96 well plate assay.

Antibiotic concentrations were diluted in a two-fold series and dispensed with 125µl/well of M9 MM into 96-well plates. The 96 well plates were incubated overnight at 30 C, with shaking at 150 rpm. The MIC was defined as the lowest concentration of antibiotic for which 90% growth inhibition was visibly observed after overnight culture. The MIC value was determined in triplicates with similar results.

4.4 Results and Discussion

I systematically examined four TCSs that are involved in *E. coli*'s response to envelope stresses, such as NaCl and MgCl₂, and their impact on growth upon confronting a particular antibiotic. These TCSs are also involved in conferring intrinsic resistance to *E. coli* due to the low permeability of its outer membrane, regulating expression of efflux pumps and porins. To study these TCSs, I used knockout mutants from the Keio single gene knockout collection (Baba *et al.* 2006). For each mutant, I measured (1) growth rate parameters in M9 MM under four treatments (a) M9 MM as control (b) MM with a stressor (c) MM + antibiotic (d) MM + stressor + antibiotic, and (2) antibiotic susceptibility under stressor+/-antibiotic conditions. I have chosen stressors (sodium and magnesium) to match known functions of the TCS genes; some of the stressors are common to more than one TCS mutant. I have chosen four antibiotics from different drug classes with different modes of antimicrobial action (CIP, CEF, TET, and CHM) (Supp Table C4.2). As a control, a pseudogene *cybC* is used under all the growth parameters. Overall, each of the eight different TCS mutants exhibited compromised growth rates in comparison to *cybC* while growing in M9 MM without any treatments (no stressor, no antibiotic) (Anova: $F = 57.59$, $P = 6.06 \times 10^{-8}$ ***). The different responses of TCS mutants to different treatments, i.e. antibiotics and stressor+antibiotic, combinations are discussed below.

4.4.1 The impact of stressors on TCS mutants

Each of the mutants performed worse with stressor than without stressor, whereas the control *cybC* does not (Figures 4.2-4.5, blue and red lines under no drug) (Table 4.1).

This finding includes *cpxA* and *cpxR*, *envZ*, *ompR*, *baeS*, *baeR* under a sodium stressor and *phoQ*, *phoP* in the presence of magnesium. These TCSs are known to contribute to membrane architecture, suggesting a plausible explanation for decline in growth under the selected stressor conditions.

Table 4.1 A two-way analysis of variance (ANOVA) for the effects of stressor and mutants. (Stars indicate significance: *** $p < 0.001$).

Factor	F-value	P-value
Mutant	274	2.05e-05 ***
Stressor	274	4.76e-08 ***
Mutant * Stressor	0.183	0.987

4.4.2 The impact of antibiotic on TCS mutants

To investigate the role of these TCS genes in antibiotic susceptibility in *E. coli*, I selected four clinically relevant antibiotics (CIP, CEF, TET, and CHM) from different drug classes. I measured the growth rates of TCS mutants at the MIC of each drug. Overall, I observed that each of the mutants showed significantly reduced growth in the presence of any of the four antibiotics tested, compared to the control *cybC* knockout and irrespective of the presence of the stressor (Figures 4.2-4.5 red line) (Table 4.2). Thus, each of the antibiotics impacted growth rates of all the mutants significantly indicating a decreased tolerance to these antibiotics. This is not surprising given the involvement of these genes in efflux systems, porin-mediated permeability, and LPS modification.

Table 4.2 A two-way analysis of variance (ANOVA) for the effects of antibiotic and mutants. (Stars indicate significance: *** $p < 0.001$).

Antibiotic	F-value	P-value
CIP	337.4	<2e-16***
CEF	229.9	<2e-16***
TET	184.1	1.16e-15 ***
CHM	266.9	<2e-16 ***

4.4.3 The response of stressor+antibiotic combinations on TCS mutants

Each of the TCS mutants tested here showed significantly compromised growth rates compared to the control *cybC* knockout in a stressor+antibiotic combination (Table 4.3). As each of the TCSs are activated and regulate a set of different genes to cope with the environmental stresses, a TCS mutant is expected to confer impairment in its assigned function.

In the presence of stressor+antibiotic, *envZ* and *ompR* exhibited lower growth rates in comparison to the antibiotic-only treatment. Surprisingly, the mutants *baeS*, *baeR*, *cpxA*, *cpxR*, *phoQ*, and *phoP* showed increased growth rates compared to the antibiotic-only treatment (compare the blue and red lines in Figures 4.2-4.5). Intriguingly, this suggests that the presence of stressor in a medium can increase a mutant's tolerance to antibiotics in some mutants. One possible explanation for the improved tolerance of some TCS mutants under antibiotic+stress conditions is that some of the regulated genes are common between different TCSs. For example, the Cpx and Bae systems can regulate the expression of the same genes in relieving envelope stress, triggered by limited magnesium concentrations. These genes are *mdtABCD* (a multidrug efflux pump), *spy* (a periplasmic protein), and *acrD* (a predicted membrane facilitator) (Raffa and Raivio 2002; Baranova and Nikaido 2002; Zhau *et al.* 2003; Bury-Moné *et al.* 2009; Leblanc *et al.* 2011). Because of this redundancy, I speculate that the Bae system is upregulating these genes in Cpx mutants, and vice versa. By contrast, EnvZ-OmpR is the main regulatory system that controls permeability of the outer membrane by regulating the two major porins of *E. coli* (OmpC and OmpF). The extent to which each porin is expressed

predominantly depends on the phosphorylation state of OmpR when set by EnvZ. OmpR can be cross-phosphorylated by other TCSs (such as Cpx or Bae), but in that case only negligible phosphorylation of OmpR is observed (Skerker *et al.* 2005; Siryaporn and Goulian 2008; Bury-Moné *et al.* 2009). This lack of redundancy may therefore explain the decreased growth rates observed in *envZ* and *ompR* mutants under stressor+antibiotic conditions.

Table 4.3 Effect of mutants on the growth rate (OD₆₀₀/minute) parameter. (Stars indicate significance: *p < 0.05, **p < 0.01, and ***p < 0.001).

Factor	Post hoc (Tukey HSD) comparisons of mutants with control <i>cybC</i> under stressor+antibiotic treatment	
	Growth rate (OD ₆₀₀ /minute)	
	F value	P- value
<i>cybC-phoQ</i>	16.97	0.00208 **
<i>cybC-phoP</i>	6.051	0.0337 *
<i>cybC-envZ</i>	53.72	2.51e-05***
<i>cybC-ompR</i>	11.07	0.00766**
<i>cybC-cpxA</i>	6.976	0.0247 *
<i>cybC-cpxR</i>	23.73	0.00065 ***
<i>cybC-baeS</i>	9.505	0.0116 *
<i>cybC-baeR</i>	7.408	0.0215 *

Figure 4.2 Growth rate of *baeS* and *baeR* mutants in the presence and absence of stressor (NaCl). The red line represents no stressor and blue with stressor condition. The *cybC* control can be seen without stressor (black line) and with stressor (green line). (Each point represents the average of three independent biological replicates).

Figure 4.3 Growth rate of *cpxA* and *cpxR* mutants in the presence and absence of stressor (NaCl). The red line represents no stressor and blue with stressor condition. The *cybC* control can be seen without stressor (black line) and with stressor (green line). (Each point represents the average of three independent biological replicates).

Figure 4.4 Growth rate of *phoQ* and *phoP* mutants in the presence and absence of stressor (MgCl_2). The red line represents no stressor and blue with stressor condition. The *cybC* control can be seen without stressor (black line) and with stressor (green line). (Each point represents the average of three independent biological replicates)

Figure 4.5 Growth rate of *envZ* and *ompR* mutants in the presence and absence of stressor (NaCl). The red line represents no stressor and blue with stressor condition. The *cybC* control can be seen without stressor (black line) and with stressor (green line). (Each point represents the average of three independent biological replicates).

4.4.4 Antibiotic susceptibility

I quantified the MICs of all the eight mutants (*baeS*, *baeR*, *cpxA*, *cpxR*, *phoQ*, *phoP*, *envZ*, and *ompR*) against all four antibiotics, with and without stressor. Overall, mutants in the presence of stressor showed either equal or increased resistance levels of 1-3 fold compared to the no-stressor condition, while comparable differences were not seen for the *cybC* mutant control (Figure 4.6 A, B, C, D). Two mechanisms may underlie this result. First, improved resistance levels may be induced by the envelope stress response through TCS-mutant independent mechanisms. Second, while experiencing a stressful encounter, different TCSs may operate as a proxy for one another, with many of the same genes regulated by these different systems. For example, the Cpx and Bae TCSs have synergistic effects in response to envelope stress (Raffa and Raivio 2002; Dorel *et al.* 2006; Rosner and Martin 2013). The Pho system is also linked to other TCSs (such as Rcs or Evg TCSs) that can form a cross-regulatory network and are implicated in a coordinated synthesis/modification of cell envelope through the activation of common transporters, such as MgtA, MgtB, MgrB (magnesium transporters) and SafA (a transmembrane protein) in response to the status of external magnesium (Kato *et al.* 1999; Groisman *et al.* 2001; Hagiwara *et al.* 2004; Eguchi *et al.* 2007; Moon and Gottesman 2009). However, I observed that *envZ* and *ompR* mutants showed decreased resistance levels for the four antibiotics tested, suggesting that EnvZ-OmpR is essential for regulating and maintaining bacterial envelope stress tolerance and porin synthesis.

In the absence of stressor, each of the TCS mutants showed a reduction in levels of resistance to all four antibiotics, ranging from 2-4 fold, whereas no changes were

observed for the *cybC* knockout control (Figure 4.6 A, B, C, D and Supp Table C4.3). A 2-fold decrease in the MICs of CIP and CEF and a 4-fold decrease in the MIC of TET and CHM can be observed for both *envZ* and *ompR* mutants compared with that of the control *cybC* (Figure 4.6B). Other mutants, such as *cpxR* and *cpxA*, also showed increased sensitivity to the antibiotics tested (Figure 4.6C). These results are in agreement with other studies describing the effects of these mutants on intrinsic resistance to various antibiotics. Tamae *et al.* (2008) and Liu *et al.* (2010) also found in genome-wide sensitivity screens that mutants of *envZ* and *ompR* were susceptible to one or more of the antibiotics tested.

Figure 4.6A Log₂ MIC change of *phoQ*, *phoP* TCS mutants under stressor (MgCl₂) +/- antibiotic conditions compared to control *cybC*.

Figure 4.6B Log₂ MIC change of *envZ*, *ompR* TCS mutants under stressor (NaCl) +/- antibiotic conditions compared to control *cybC*.

Figure 4.6C Log₂ MIC change of *cpxR*, *cpxA* TCS mutants under stressor (NaCl) +/- antibiotic conditions compared to control *cybC*.

Figure 4.6D Log₂ MIC change of *baeR*, *baeS* TCS mutants under stressor (NaCl) +/- antibiotic conditions compared to control *cybC*.

I carried out agar growth assays under similar conditions as the liquid growth assays, and results were broadly similar to the liquid growth assay (Supp Table C4.4, C4.5, Supp Figure C4.2, C4.3). As observed here, colony sizes varied significantly among all the treatments compared to control.

4.5 Conclusions and Perspectives

TCSs are one of the predominant signaling regulatory families that are used by a bacterium to rapidly cope with changes in its environment. TCSs can impact bacterial performance considerably. The current study is an effort to gain more insight into the role of *E. coli* TCSs in conferring phenotypic resistance upon confronting metabolic stresses. I identified eight mutants from four TCSs, which upon exposure to a combination of stresses +/- antibiotic could show different growth phenotypic responses. As such, in the present study I found that *envZ* and *ompR* mutants showed increased sensitivity to all four antibiotics tested, and to the presence of stress+antibiotic combination as well. The other six mutants showed different phenotypic responses in +/- stress and/or antibiotic conditions. This study illustrates that the efficacy of TCSs is likely to depend on the environment it confronts, which suggests that the presence of stress in a medium can actually increase or decrease growth capacity as well as tolerance to antibiotics of some TCS mutants.

Bacterial tolerance pathways are upregulated to increase survival upon confronting a stressful encounter, and when these responses impact antibiotic activity, they have the potential to contribute to resistance. These transient, reversible resistance gains are linked to the physiological state of a bacterium upon confronting different habitats or stressors. For example, Allison *et al.* (2011) took advantage of bacterial metabolic states to increase susceptibility to aminoglycosides by combating persister cells by priming their metabolism. Similarly, biofilm-forming microorganisms display

different metabolic states, where actively growing cells are killed by antibiotics and the fitness of remaining persister cells is dependent on nutrient availability, regulated by TCSs (Corona and Martinez 2013; Olivares *et al.* 2013; Zhang *et al.* 2013).

TCSs contribute to stress regulation associated with clinically relevant AMR, for example cell envelope stress-responsive TCS targets associated with efflux systems and LPS modification in Gram-negative organisms (Fraud and Poole 2010; Poole 2012; Poole *et al.* 2014). Due to their effects on AMR, virulence, and survival, TCSs have emerged as potential targets for therapeutic intervention. Lee *et al.* (2009) showed that the inactivation of the AmgRS envelope stress TCS in *P. aeruginosa* increases susceptibility to aminoglycosides in clinical settings. Similarly, Marchand *et al.* (2004) showed that the inactivation of *adeRS* resulted in improved aminoglycoside effectiveness in *Acinetobacter baumannii*, and inactivation of the SOS response that is dependent upon oxidative stress renders cells more susceptible to fluoroquinolones by decreasing fluoroquinolone stimulated persister formation by *E. coli* (Dörr *et al.* 2009). Many synthetic compounds are also explored that can inhibit TCSs, affecting bacterial growth and virulence (Barrett *et al.* 1998; Barrett and Hoch 1998; Macielag and Goldschmidt 2000; Stephenson and Hoch 2002; Gotoh *et al.* 2010; Harris *et al.* 2013). Rasko *et al.* (2008) have reported a small molecule that inhibits the sensor HK QseC in *Salmonella typhimurium*, lowering the virulence of several pathogens in animal studies without killing the bacterial cells. Watanabe *et al.* (2012) discovered a novel antibacterial Singermycin B, that targets the *walkR* TCS to inhibit bacterial growth in MDR bacteria including methicillin-resistant *S. aureus*.

Despite a growing appreciation of the importance of TCSs to bacterial physiology, a complete understanding of TCS function, and how the TCS network integrates signals and stimuli in an orderly manner, has yet to be achieved. TCSs are not just linear on/off switches, but there is emerging concern about the complexity of the bacterial signaling networks (Olivera *et al.* 2010; Capra and Laub 2012; Jung *et al.* 2012; Guckes *et al.* 2013; Salazar and Laub 2015; Breland *et al.* 2017). These complexities include cross-interactions between closely related TCSs in a way that can be beneficial for the bacteria, such as a gain in antimicrobial resistance (Kraxenberger *et al.* 2012; Fried *et al.* 2013; Behr *et al.* 2014; Guckes *et al.* 2017; Urano *et al.* 2017; Wei *et al.* 2017). Therefore, cross regulation may pose a problem as any particular set of genes regulated by a specific TCS could also be under the control of other systems, and thus the effect of inhibiting a given component may not be observed if the functions of this system can be complemented by other systems. This present study also highlights one aspect of TCS, i.e. how bacteria respond to stresses that are encountered through environmental changes. In order to recognize the complete potential of TCS as stress responses and AMR determinants, more insight is required.

Chapter 5

Concluding Remarks

Antimicrobial resistance (AMR) is one of the most pressing issues of recent times. Multi-drug resistance in bacterial pathogens not only causes harm by jeopardizing medical treatments but also imposes a major economic burden. A growing gap between clinical need and antibiotic innovation has aggravated the crisis. Therefore, it is becoming increasingly challenging to find new efficacious antibiotics and novel therapeutic strategies to combat the accelerating resistance problem. This thesis provides and expands our understanding of both the evolution of AMR and approaches to counteract the resistance problem once it has arisen. In this section, I will discuss the significance of the findings presented in this work within the context of tackling the problems of AMR.

In *chapter 2* of my thesis, I aim to achieve comprehensive insights about the rates, molecular bases, and pleiotropic consequences of resistance evolution. I obtained 56 spontaneous resistance mutants using fluctuation analysis across three generations of quinolones in *Escherichia coli*. I found 36 *gyrA*, 4 *gyrB*, 9 *marR*, and 1 double *gyrA/marR* mutants, with two novel *gyrB* mutations. Overall, *gyrA* mutations conferred higher resistance, without greater fitness cost than other mutations, granting a selective advantage. I found widespread cross-resistance amongst quinolone resistant isolates, implying that different resistance mutations respond differently to quinolone variants.

The results obtained in this chapter have implications for public health strategies, notably in antibiotic selection. The precise predictions about the rate and trajectories of resistance evolution towards new and old generations of antibiotics provide possibilities to take appropriate measures for the use of antimicrobials. In addition, as costs of resistance drive the evolution and persistence of resistance genes, a quantitative understanding of these costs and their effects is useful for optimizing treatment regimens and in managing resistance. As suggested in the current study of quinolones, the selection of levofloxacin would be ideal, since resistance is costly and single mutations have considerably smaller effects.

While it is imperative to develop new antimicrobials, improvements in antibiotic adjuvants are also essential. New strategies to preserve already existing antibiotics, for example using adjuvants, is one promising contribution. The efficacy of β -lactamase inhibitors over the past decades demonstrates the effectiveness of adjuvant concept (Drawz and Bonomo 2010; King *et al.* 2014). To help counteract the resistance problem, in **chapter 3**, I have used a novel approach for genetic screens in *E. coli*. Using a small RNA (sRNA) screen I have identified potential drug targets for the re-sensitization of quinolone resistant *E. coli*. Screening of ~5000 sRNA constructs on a *gyrA* mutant background led to the identification of 30 auxiliary drug targets. These genes are involved in DNA replication, repair and recombination (18), transport and efflux system (4), two-component regulatory system (4), transcriptional regulators (2), chaperone proteins (2), cell division (2) catalytic, biosynthetic pathways (2), and uncharacterized proteins (2). The repression of these genes reduced resistance to the fluoroquinolone ciprofloxacin in a *gyrA* S83L mutant. This screening approach has the potential to

establish a promising future for combating AMR and can be used for various already existing antibiotics and different microorganisms.

Finally, I examined the involvement of *E. coli*'s two-component signal transduction systems (TCS) in countering multiple stresses in **chapter 4**. I used eight TCS mutants from the Keio single gene knock-out collection. I examined the growth phenotype of each mutated TCS to different environmental stress conditions in combination with four antibiotics from different classes. Overall, the mutants showed different phenotypic changes in response to different treatment regimens. The study indicates the involvement of TCSs in governing critical biological parameters of *E. coli*, ranging from regulating environmental stresses to intrinsic resistance. Managing *E. coli*'s ability to utilize TCSs should prove a worthy consideration as therapeutic targets.

Recent work has revealed the constellations of genes involved in phenotypic resistance with several of these genes contributing to bacterial intrinsic resistance, and also encode key elements of bacterial physiological states (Fajardo *et al.* 2008; Tamae *et al.* 2008; Martinez and Rojo 2011). The current study of TCSs provides a link to the physiological state of bacteria and to the responses that microorganisms receive when confronted to different stresses including antibiotics. Thus, this knowledge will be relevant in providing a better understanding of molecular mechanisms of resistance and contribute towards new adjunctive alternatives to improve the efficacy of antibiotic therapy programs.

In conclusion, to combat AMR, we need to understand the evolutionary trajectories that underlie the acquisition of antibiotic resistance mutations and their

pleiotropic aftermaths, which are the critical drivers of the evolution of AMR (Chapter 2), how bacterial fitness depend on the environment it lives on (Chapter 4) and, to counteract the problem we need novel methods, such as genetic screens that can identify targets that can restore the effectiveness of already existing antibiotics (Chapter 3). This research work will lead us to understand the evolution and genetic complexity of resistance and will be helpful in contributing to the knowledge of antibiotic resistance evolution in future studies.

Chapter 6

Future Perspectives

The widespread use of antibiotics has increased the evolutionary selection pressure for the emergence of antimicrobial resistance (AMR). The projects presented in this thesis seek to expand our understanding of the genetic basis for the evolution of resistance, and also involve novel concepts to circumvent the resistance problem once it has arisen.

The knowledge to predict evolutionary trajectories plays an integral part in the drug-development program that can make better pre-informed decisions. This type of knowledge is crucial in evaluating the prospects of the emergence of resistance to novel drugs and managing AMR in the future. This includes the mutation supply rate, level of resistance, identification of resistance mutations, and their pleiotropic aftermaths (addressed in chapter 2 of this thesis). Currently there is limited quantitative data available that restricts efforts to predict the trajectory of resistance evolution, especially in relevant environments (clinical and natural). More insight is required to better predict the evolution of AMR. This useful knowledge comprises information about epistatic interactions and compensatory evolution, co-selection and horizontal gene transfer, and population bottlenecks and clonal interference. Thus, using additional risk-assessment steps, it may impart better predictions to determine the success of a drug candidate in clinical development.

The limited supply of antimicrobials has prompted the search for alternative approaches that can potentiate the effects of existing antibiotics. Investigating novel drug targets that exist in bacterial pathogens through interactive chemical-genetic sensitivity screens has gained a lot of interest. In a high-throughput approach using small RNA (sRNA) (addressed in chapter 3 of this thesis), I identified *Escherichia coli* genes that could be targeted to reverse quinolone resistance. In the next steps, we require a competent delivery system for efficient expression of synthetic sRNA constructs. Phage therapy or conjugative plasmids might be used as a successful sRNA delivery system (Lu and Collins 2009; Rastogi *et al.* 2018).

Antibiotic tolerance plays a critical role in bacterial survival, which has largely been an underappreciated area. The metabolic state of a bacterium depends on the environment where it lives, which in turn impacts antibiotic tolerance. The tolerance conferring effects are induced by environmental stresses and genetic factors that are translated into physiological responses. This is mediated largely by two-component signal transduction systems (TCS) in bacteria (addressed in chapter 4 of this thesis), which mediate signaling through the envelope stress response. Further steps require an accurate understanding of the clinical manifestation of antibiotic tolerance and the contribution of TCSs. TCSs are one of the promising combination therapy approaches that can be explored further to gain high-quality drug targets for potentiation of known antibacterial drugs.

To control or reduce the AMR evolution we need further effective strategies to improve our antimicrobials. Our knowledge of various mechanisms involved in

underpinning AMR has expanded in recent years. Researchers have also recognized challenges (Payne *et al.* 2007; Tommasi *et al.* 2015) that constrain efforts to restrict AMR. Some of the effective strategies include, (i) Potentiation approaches. High-throughput random transposon mutagenesis libraries in *Pseudomonas aeruginosa* and *E. coli* led to the identification of many genes responsible for intrinsic resistance to different classes of antibiotics (Breidenstein *et al.* 2008; Fajardo *et al.* 2008; Tamae *et al.* 2008; Liu *et al.* 2010). This has led the way for potential drug combinations whereby one agent can inhibit intrinsic resistance mechanisms to enhance the activities of other antibiotics. For example, inactivation of non-essential *E. coli* genes, such as *fabI*, *recQ*, *sapC*, and *dacA* can greatly potentiate the activity of already existing drugs, including rifampicin, aminoglycosides, triclosan, and some β -lactams (Liu *et al.* 2010; Blake and Neill 2013; Cox *et al.* 2014). (ii) Active uptake approaches. By taking advantage of membrane transporters, antibiotic influx can be increased. For example, fosfomycin uses bacterial sugar transporters to enter the cells (Czaplewski *et al.* 2016). (iii) Cycling of antibiotics. We can prolong the life span of already existing antibiotics. For example, a fluoroquinolone plus a macrolide or a β -lactam plus an aminoglycoside or tetracycline (with different modes of action). This type of approach has been used in the past for treatment of cancer infection, however a comprehensive pharmacodynamic matching is essential, such as standardized predetermined concentrations of antibiotics for the maximum synergy (Bergstrom *et al.* 2004; Masterton 2005; Davies and Davies 2010). Therefore, this type of system does not provide a long-term solution, but certainly reduces the selection pressures for resistance. (iv) Combinatorial approaches. Attempts have been made to inhibit or bypass resistance mechanisms in pathogens, especially with

β -lactam antibiotics. For example, Clavulanic acid or natural products, such as Aspergillomarasmine A (AMA) inhibits β -lactamases enzymes and is used in combination with β -lactam antibiotics (Reading and Cole 1977; King *et al.* 2014). Also, collateral sensitivity or cross-resistance strategies are encouraging options in a combination therapy (Imamovic and Sommer 2013; Pál *et al.* 2015; Lázár *et al.* 2015).

(v) Non-conventional approaches. There are some alternative approaches to address bacterial infections, such as phage therapy. For example, Sahota *et al.* 2015 used phages directly inhaled into the lungs against *P. aeruginosa* to treat cystic fibrosis. Also, Nale *et al.* 2015 used a phage mixture in the gastrointestinal tract for the treatment of *Clostridium difficile* infection. Restoring the patient's microbiome (usually in *C. difficile* infection) is another non-conventional approach. Gerding *et al.* 2015 used nontoxinogenic *C. difficile* that can outcompete the infecting toxic *C. difficile*, helping to re-establish the host microbiome. Monoclonal antibodies can also be used as an alternative to antibiotics. Antibodies have already been successfully used in inflammatory diseases treatment (Czaplewski *et al.* 2016; Pegu *et al.* 2017; Bagnoli and Payne 2017). DiGiandomenico *et al.* (2014) showed a bispecific monoclonal antibody to target virulence factor (PcrV) and persistence factor (PsI) in *P. aeruginosa*.

All of these mentioned innovative approaches face challenges to be successfully used, such as efficacy and robustness in clinical development path or cost of development. But, investing in these collaborative measures can significantly advance our ability to control AMR. In addition, these efforts will only be successful if taken as a comprehensible international effort (O'Neill 2014; Boeckel *et al.* 2014). In essence, for

our future, we have to use the best of available technologies, knowledge, and expertise to circumvent the resistance crisis and develop next generation antimicrobials.

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Appendix A

Appendix to Chapter 1

Table A2.1 Terminology used for the estimation of mutation rates. Based on MSS-likelihood method (Sarkar *et al.* 1992).

Term	Definition
m	No. of mutational events per culture
μ	Mutation rate; probability of mutation per cell per division or generation
N	Number of cells
N_0	Initial number of cells in a culture
N_t	Final number of cells in a culture
r	Observed number of mutants in a culture
f	Mutant fraction or frequency (r/N)
C	No. of cultures in experiment
p_0	Proportion of cultures without mutants
p_r	Proportion of cultures with r mutants

Box A2.1 Equations used in for the estimation of mutation rates (Based on the MSS-likelihood method).

The values of N_t and the probability of m were generated by observing r mutants on selective medium, p_r (Equation 1).

$$p_0 = e^{-m}; \quad p_r = \frac{m^r}{r!} \sum_{i=0}^{r-1} \frac{p_i}{(r-i+1)}$$

Equation 1. Probability function to measure ‘r’ mutants; p_r to estimate the initial value of ‘m’

The value of m was calculated by the multiplying all the p_r for each observed value of r and analyzed until the likelihood function reaches its maximum (Equation 2) (Ma *et al.* 1992). The mutation rate ‘M’ was calculated by following equation, $M = m/N_t$ (Sarkar *et al.* 1992; Hall *et al.* 2009).

$$f(r | m) = \prod_{i=1}^C f(r_i | m)$$

Equation 2. MSS likelihood function with product of p_r for each observed value of r , among all the parallel cultures.

Table A2.2 Spontaneous mutation rate, with 95% confidence interval in *E. coli* K-12 (MG1655) supplemented with different quinolone antibiotics.

<i>E. coli</i> K-12 (MG1655) with selective antibiotic	Antibiotic concentration (µg/ml) at 1XMIC and 2XMIC	Mutation 'm'	Mutation rate (M) per 10 ⁸ cells	95% Confidence interval (Upper difference)	95% Confidence interval (Lower difference)
Nalidixic acid (1XMIC)	11.11	0.368	0.76	0.49	0.39
Ciprofloxacin (1XMIC)	0.015	1.158	2.41	1.06	0.91
Ciprofloxacin (2XMIC)	0.030	0.293	0.61	0.42	0.33
Levofloxacin (1XMIC)	0.0312	1.067	2.22	1.01	0.86
Levofloxacin (2XMIC)	0.0625	0.174	0.36	0.29	0.22

Table A2.3 MIC fold-change and resistance mutations in *gyrA*, *gyrB*, and *marR* among 56 NAL^R, CIP^R, and LEVO^R mutants of *E. coli* K-12 (MG1655).

Mutant	Resistance mutations	MIC to NAL (fold change)	Mutant	Resistance mutations	MIC to CIP (fold change)	Mutant	Resistance mutations	MIC to LEVO (fold change)
NAL1	D87G (<i>gyrA</i>)	128	CIP1		16	LEVO1	S83L (<i>gyrA</i>)	16
NAL2	S83L (<i>gyrA</i>)	128	CIP2	L509G (<i>gyrB</i>)	16	LEVO2	L33R (<i>marR</i>)	16
NAL3	D426N (<i>gyrB</i>)	64	CIP3	S83W (<i>gyrA</i>)	32	LEVO3	T102S (<i>marR</i>)	8
NAL4	A67S (<i>gyrA</i>)	64	CIP4	S83W (<i>gyrA</i>)	32	LEVO4	Q117stop (<i>marR</i>)	8
NAL5	S83L (<i>gyrA</i>)	256	CIP5	D87N (<i>gyrA</i>)	32	LEVO5	N126fs (<i>marR</i>)	8
NAL6	D87G (<i>gyrA</i>)	128	CIP6	S83L (<i>gyrA</i>)	64	LEVO6	D87G (<i>gyrA</i>)	16
NAL7	S83L (<i>gyrA</i>)	256	CIP7	H281L (<i>gyrB</i>)	32	LEVO7	L123S (<i>marR</i>)	4
NAL8	S83L (<i>gyrA</i>)	256	CIP8	D87Y (<i>gyrA</i>)	64	LEVO8	E10stop (<i>marR</i>)	16
NAL9	S83L (<i>gyrA</i>)	256	CIP9	G81C (<i>gyrA</i>)	64	LEVO9	S83L (<i>gyrA</i>)	8
NAL10	A67S (<i>gyrA</i>)	64	CIP10	D87Y (<i>gyrA</i>)	32	LEVO10	G69E (<i>marR</i>)	8
NAL11	S83L (<i>gyrA</i>)	256	CIP11	D87N (<i>gyrA</i>)	64	LEVO11	S83L (<i>gyrA</i>)	16
			CIP12	S464Y (<i>gyrB</i>)	32	LEVO12	L64fs (<i>marR</i>)	8
			CIP13	D87Y (<i>gyrA</i>)	64	LEVO13	S83L (<i>gyrA</i>)	4
			CIP14	G81D (<i>gyrA</i>)	128	LEVO14	Q42E (<i>marR</i>)	8
			CIP15		64	LEVO15	S83L (<i>gyrA</i>)	8
			CIP16	G81D (<i>gyrA</i>)	128	LEVO16	S83L (<i>gyrA</i>)	8
			CIP17	D87Y (<i>gyrA</i>)	32	LEVO17	S83L (<i>gyrA</i>)	16
			CIP18		64	LEVO18	S83L (<i>gyrA</i>)	16
			CIP19		64	LEVO19	D87G, R27P (<i>gyrA</i> , <i>marR</i>)	16
			CIP20	D87G (<i>gyrA</i>)	64	LEVO20	G81C (<i>gyrA</i>)	16
			CIP21	S83L (<i>gyrA</i>)	64	LEVO21		8
			CIP22	S83L (<i>gyrA</i>)	128			
			CIP23	S83L (<i>gyrA</i>)	16			
			CIP24		64			

Figure A2.1 Comparison between mean fold-change in MIC values for different resistance mutations in *gyrA*, *gyrB*, and *marR* regions. Resistance mutations isolated as a direct selection on NAL (A), CIP (B), and LEVO (C).

A. *gyrA* mutations

B. *gyrB* mutations

C. *marR* mutations

Figure A2.2 Variation in costs of resistance - growth rate, cell density, and lag phase between *gyrA* (A), *gyrB* (B), and *marR* (C) resistance mutations. All the fitness components are compared to control *E. coli* K-12 (MG1655). The boxplots present the median, first, and third quartiles, with whiskers showing either the maximum (minimum) value or 1.5 times the interquartile range of the data, whichever is smaller (larger).

Appendix B

Appendix to Chapter 2

Table B3.1 List of oligonucleotides used in this study.

Name	Sequence
pBad_fwd	ATGCCATAGCATTTTTATCC
pBad_rev	GATTTAATCTGTATCAGG
DsrA_fwd	AATTTTTTAAGTGCTTCTTGCTTAAG
DsrA_fwd_randomized	N10-AATTTTTTAAGTGCTTCTTGCTTAAG
MicF_fwd	CGTCATTCATTTCTGAATGTCTG
MicF_fwd_randomized	N10-CGTCATTCATTTCTGAATGTCTG
Spot42_fwd	ATTTGGCTGAATATTTTAGCCGC
Spot42_fwd_randomized	N10-ATTTGGCTGAATATTTTAGCCGC
Sart_rev	N20-CTCGAGTATGGAGAAACAGTAGAGAG

Table B3.2 The list of preliminary (222) gene targets. All the gene targets identified from the 31 sRNA randomized sequences, and their putative mRNA complementarity hits. The hit matches are based on IntaRNA, Target RNA, and RNA predator matches (Mann *et al.* 2017; Kery *et al.* 2014; Eggenhofer *et al.* 2011).

S.No	Native Scaffold	Randomized Sequence	Target gene hit and their respective function
1	DsrA	ttctctcgctcggactgaacgtgga gctgg	<i>uvrC</i> ATPase and DNA damage recognition protein of nucleotide excision repair excinuclease UvrABC <i>exoD</i> Exonuclease V (RecBCD complex); beta subunit <i>topB</i> DNA topoisomerase III <i>emrA</i> Multidrug efflux system <i>ampC</i> Penicillin-binding protein beta-lactamase intrinsically weak <i>seqA</i> Negative modulator of initiation of replication <i>tolC</i> Transport channel <i>rpoN</i> RNA polymerase sigma 54 (sigma N) factor <i>recO</i> Gap repair protein <i>minC</i> FtsZ ring <i>umuD</i> Translesion error-prone DNA polymerase V subunit RecA-activated auto-protease
2	DsrA	ttgtacctgcttgcgatacgacttgc atc	<i>mtlR</i> Mannitol operon repressor <i>tolA</i> Membrane anchored protein in TolA-TolQ-TolR complex
3	DsrA	gaattcggaccatgataccactga gttt	<i>ydhK</i> Putative efflux protein (PET) component of YdhJK Efflux pump <i>yoeB</i> Toxin of the YoeB-YefM toxin-antitoxin system <i>kdpA</i> Potassium translocating ATPase subunit A <i>yhhX</i> Putative oxidoreductase <i>zipA</i> FtsZ stabilizer <i>mutH</i> Methyl-directed mismatch repair protein <i>recJ</i> ssDNA exonuclease; 5' 3' specific <i>rpoC</i> RNA polymerase beta prime subunit <i>rpoN</i> RNA polymerase sigma 54 (sigma N) factor

			<i>yeeY</i> Predicted DNA binding transcriptional regulator <i>polA</i> 5' to 3' DNA polymerase and 3' to 5'/5' to 3' exonuclease
4	DsrA	gagaccgtgac	<i>zipA</i> FtsZ stabilizer <i>helD</i> DNA helicase IV <i>ygfI</i> Putative DNA-binding transcriptional regulator <i>rpoH</i> RNA polymerase sigma 32 (sigma H) factor <i>cho</i> or <i>ydjQ</i> Endonuclease of nucleotide excision repair
5	DsrA	tcctaaggctttatctctga	<i>dut</i> Deoxyuridinetriphosphatase <i>rssB</i> PcnB-degradosome interaction factor response regulator <i>dcuS</i> Sensory histidine kinase in two-component regulatory system with DcuR regulator of anaerobic fumarate respiration <i>evgA</i> Response regulator in two-component regulatory system with EvgS <i>rpoE</i> RNA polymerase sigma E factor <i>rpoN</i> RNA polymerase sigma 54 (sigma N) factor <i>dnaA</i> Chromosomal replication initiator protein DnaA DNA-binding transcriptional dual regulator <i>recB</i> Exonuclease V (RecBCD complex) beta subunit <i>dksA</i> DNA binding transcriptional regulator of rRNA transcription; DnaK suppressor protein
6	DsrA	ccccacactcccacattcaNNctgtg	<i>recB</i> Exonuclease V (RecBCD complex) beta subunit <i>cheY</i> Chemotaxis regulator transmitting signal to flagellar motor component <i>djlA</i> Membrane-anchored DnaK co-chaperone DNA-binding protein <i>yacG</i> DNA gyrase inhibitor <i>recQ</i> ATP-dependent DNA helicase <i>rpoD</i> RNA polymerase sigma 70 (sigma D) factor <i>ompR</i> Response regulator in two-component regulatory system with EnvZ <i>umuC</i> DNA polymerase V; subunit C
7	DsrA	ctgtctctgtac	<i>ydaG</i> Putative DNA endonuclease <i>dnaC</i> DNA biosynthesis protein <i>recC</i> Exonuclease V (RecBCD complex); gamma chain <i>holD</i> DNA polymerase III; psi subunit <i>rpoE</i> RNA polymerase; sigma 24 (sigma E) factor

8	DsrA	tcctaaggctttatctc	<i>dnaA</i> Chromosomal replication initiator protein DnaA DNA-binding transcriptional dual regulator <i>rpoN</i> RNA polymerase sigma 54 (sigma N) factor <i>minC</i> Inhibitor of FtsZ ring polymerization <i>rpoE</i> RNA polymerase sigma E factor <i>recB</i> Exonuclease V (RecBCD complex) beta subunit <i>rssB</i> PenB-degradosome interaction factor response regulator <i>mdtA</i> Multidrug efflux system; subunit A
9	DsrA	cggcgaaatgttggtg	<i>ypdC</i> Putative DNA-binding protein <i>rho</i> Transcription termination factor <i>ydaV</i> Rac prophage; predicted DNA replication protein <i>dnaX</i> DNA polymerase III/DNA elongation factor III, tau and gamma subunits <i>ligA</i> DNA ligase; NAD(+) dependent <i>dnaB</i> Replicative DNA helicase
10	DsrA	cgccccggaacgccgcgtgttat cgcat	<i>holE</i> DNA polymerase III theta subunit <i>yacG</i> DNA gyrase inhibitor <i>ftsQ</i> Division assembly protein; membrane anchored protein involved in growth of wall at septum <i>cpxA</i> Sensory histidine kinase in two component regulatory system with CpxR
11	DsrA	acttcattgagacagaacg	<i>yjaA</i> Stress-induced protein <i>kdpE</i> Response regulator in two-component regulatory system with KdpD <i>ttdB</i> L-tartrate dehydratase beta subunit <i>pinQ</i> Qin prophage putative site-specific recombinase <i>proC</i> Pyrroline-5-carboxylate reductase NAD(P)-binding <i>pepD</i> Aminoacyl-histidine dipeptidase (peptidase D) <i>atoC</i> Fused response regulator of Ato operon in two-component system with AtoS: response regulator/sigma54 interaction protein <i>topA</i> DNA topoisomerase I; omega subunit <i>hybC</i> Hydrogenase 2 large subunit
12	DsrA	caactggtgaa	<i>smrB</i> Putative DNA endonuclease <i>atoC</i> Fused response regulator of ato operon in two-component system with AtoS: response regulator/sigma54 interaction protein <i>xthA</i> Exonuclease III

			<i>ypdA</i> Predicted sensory kinase in two component system with YpdB <i>rbbA</i> Fused ribosome associated ATPase ATP binding protein/ATP binding protein/predicted membrane protein <i>uvrC</i> ATPase and DNA damage recognition protein of nucleotide excision repair excinuclease UvrABC <i>cho</i> or <i>ydjQ</i> Endonuclease of nucleotide excision repair <i>zapB</i> Septal ring assembly factor; stimulates cell division <i>polA</i> 5' to 3' DNA polymerase and 3' to 5'/5' to 3' exonuclease
13	MicF	tacaacccttgccgagaagctagt gttat	<i>seqA</i> Negative modulator of initiation of replication <i>recE</i> Rac prophage exonuclease VIII 5' to 3' specific dsDNA exonuclease <i>zapC</i> FtsZ stabilizer <i>smf</i> DNA recombination-mediator A family protein <i>ruvC</i> Component of RuvABC endonuclease
14	MicF	tccccttcacgggtgcaacgggc caatgg	<i>uhpA</i> Response regulator in two-component regulatory system with UhpB <i>sbmC</i> DNA gyrase inhibitor <i>yffQ</i> Uncharacterized protein <i>yafG</i> CP4-6 prophage conserved protein <i>hybC</i> Hydrogenase 2 large subunit <i>yacG</i> DNA gyrase inhibitor <i>priA</i> Primosome factor n' (replication factor Y) <i>minD</i> Membrane ATPase of the MinC MinD MinE system <i>recT</i> Rac prophage; recombination and repair protein <i>rpoD</i> RNA polymerase; sigma 70 (sigma D) factor <i>dnaE</i> DNA polymerase III alpha subunit
15	MicF	gcagatctccctagatgcctagtta ggtg	<i>rpoE</i> RNA polymerase sigma E factor <i>priB</i> Primosomal protein N <i>zapC</i> FtsZ stabilizer
16	MicF	tctcacccgatgtataaacgtc ccaat	<i>yhiM</i> Acid resistance protein inner membrane <i>ftsK</i> DNA translocase at septal ring sorting daughter chromosomes
17	MicF	aactgccagtgactacgctgtta ggag	<i>ftsY</i> Signal Recognition Particle (SRP) receptor

			<i>emrB</i> Multidrug efflux system protein <i>polB</i> DNA polymerase II <i>dnaN</i> DNA polymerase III; beta subunit
18	MicF	ctcataaactccgtctccaggatta gggt	<i>recD</i> Exonuclease V (RecBCD complex) alpha chain <i>arfA</i> Alternate ribosome-rescue factor A <i>uvrY</i> Response regulator in two-component regulatory system with BarA <i>yneJ</i> Putative DNA-binding transcriptional regulator <i>envY</i> Porin thermoregulatory transcriptional activator <i>glnG</i> Fused DNA binding response regulator in two component regulatory system with GlnL response regulator/sigma54 interaction protein <i>narX</i> Sensory histidine kinase in two component regulatory system with NarL <i>recE</i> Rac prophage exonuclease VIII 5' to 3' specific dsDNA exonuclease <i>recJ</i> ssDNA exonuclease; 5' 3' specific <i>rep</i> DNA helicase and single-stranded DNA-dependent ATPase <i>priB</i> Primosomal protein N
19	MicF	gacactaaatacgaatgggtggat ttact	<i>rep</i> DNA helicase and single-stranded DNA-dependent ATPase <i>pyrB</i> Aspartate carbamoyltransferase catalytic subunit <i>dnaX</i> DNA polymerase III/DNA elongation factor III, tau and gamma subunits <i>uvrC</i> Excinuclease UvrABC endonuclease subunit <i>holC</i> DNA polymerase III chi subunit <i>dnaA</i> Chromosomal replication initiator protein DnaA DNA-binding transcriptional dual regulator <i>uvrB</i> Excision nuclease of nucleotide excision repair DNA damage recognition component <i>rsgA</i> Ribosome small subunit dependent GTPase A <i>sbcC</i> Exonuclease; dsDNA; ATP dependent <i>rep</i> DNA helicase and single-stranded DNA-dependent ATPase <i>cpxA</i> Sensory histidine kinase in two component regulatory system with CpxR
20	MicF	accagggcggtg	<i>dnaT</i> DNA biosynthesis protein (primosomal protein I) <i>cheA</i> Fused chemotactic sensory histidine kinase in two-component regulatory system with CheB and CheY: sensory histidine kinase/signal sensing protein <i>recD</i> Exonuclease V (RecBCD complex) alpha chain

			<i>rpoC</i> RNA polymerase beta prime subunit <i>priC</i> Primosomal replication protein N" <i>holD</i> DNA polymerase III psi subunit <i>dinB</i> DNA polymerase IV <i>sbcC</i> Exonuclease; dsDNA; ATP dependent <i>recE</i> Rac prophage exonuclease VIII 5' to 3' specific dsDNA exonuclease
21	MicF	gacactaaatacgaatgggtggat ttact	<i>zapC</i> FtsZ stabilizer <i>recE</i> Rac prophage exonuclease VIII 5' to 3' specific dsDNA exonuclease <i>tus</i> Inhibitor of replication at Ter; DNA binding protein <i>dnaX</i> DNA polymerase III/DNA elongation factor III; tau and gamma subunits <i>sbcC</i> Exonuclease; dsDNA; ATP dependent <i>rep</i> DNA helicase and single-stranded DNA-dependent ATPase
22	MicF	cagttctttagcagcgacagcg tgaca	<i>ompR</i> Response regulator in two-component regulatory system with EnvZ <i>yfcJ</i> Putative arabinose efflux transporter <i>rpoB</i> RNA polymerase beta subunit <i>cheB</i> Fused chemotaxis regulator protein glutamate methylesterase in two component regulatory system with CheA <i>barA</i> Hybrid sensory histidine kinase; in two component regulatory system with UvrY <i>uvrA</i> ATPase and DNA damage recognition protein of nucleotide excision repair excinuclease UvrABC <i>dnaT</i> DNA biosynthesis protein (primosomal protein I) <i>torT</i> Periplasmic sensory protein associated with the TorRS two-component regulatory system <i>recD</i> Exonuclease V (RecBCD complex) alpha chain <i>arcA</i> DNA binding response regulator in two component regulatory system with ArcB or CpxA <i>rep</i> DNA helicase and single-stranded DNA-dependent ATPase <i>rnt</i> Ribonuclease T (RNase T) <i>mutL</i> Methyl directed mismatch repair protein <i>ftsK</i> DNA translocase at septal ring sorting daughter chromosomes
23	MicF	atatacatggagttt	<i>dnaT</i> DNA biosynthesis protein (primosomal protein I) <i>rpoN</i> RNA polymerase sigma 54 (sigma N) factor <i>recE</i> Rac prophage exonuclease VIII 5' to 3' specific dsDNA exonuclease

			<i>barA</i> Hybrid sensory histidine kinase; in two component regulatory system with UvrY <i>rpoB</i> RNA polymerase beta subunit <i>uvrD</i> DNA-dependent ATPase I and helicase II <i>dnaT</i> DNA biosynthesis protein (primosomal protein I)
24	MicF	ctaccagaaccgttacgtctaaacaat	<i>tolQ</i> Membrane spanning protein in TolA-TolQ-TolR complex <i>sapB</i> Antimicrobial peptide transport ABC transporter permease <i>dnaQ</i> DNA polymerase III epsilon subunit <i>rpoZ</i> RNA polymerase omega subunit <i>ftsA</i> ATP-binding cell division FtsK recruitment protein <i>torL</i> Response regulator inhibitor for tor operon
25	MicF	gctatcatcattaactttattattac	<i>seqA</i> Negative modulator of initiation of replication <i>obgE</i> GTPase involved in cell partitioning and DNA repair <i>yddM</i> Putative DNA-binding transcriptional regulator <i>ydiE</i> Putative transporter. putative MFS sugar transporter membrane protein <i>recA</i> DNA strand exchange and recombination protein with protease and nuclease activity <i>rapA</i> RNA polymerase associated helicase protein (ATPase and RNA polymerase recycling factor) <i>recB</i> exonuclease V (RecBCD complex) beta subunit <i>dcuS</i> Sensory histidine kinase in two-component regulatory system with DcuR regulator of anaerobic fumarate respiration <i>cpxR</i> Response regulator in two-component regulatory system with CpxA
26	MicF	accctacgaacagtgcaat	<i>yihP</i> Putative 23-dihydroxypropane-1-sulphonate exporter membrane protein <i>mdtC</i> Multidrug efflux system subunit C <i>torT</i> Periplasmic sensory protein associated with the TorRS two-component regulatory system <i>yehW</i> Putative ABC transporter permease <i>rnr</i> Exoribonuclease R; RNase R <i>uvrB</i> Excision nuclease of nucleotide excision repair DNA damage recognition component
27	MicF	ttaaggaaaaatgtttNNNNggctcgt	<i>ftsK</i> DNA translocase at septal ring sorting daughter chromosomes <i>ygbL</i> Putative class II aldolase <i>cydD</i> Glutathione/cysteine ABC transporter export permease/ATPase

			<i>holE</i> DNA polymerase III theta subunit <i>holB</i> DNA polymerase III; delta prime subunit <i>rapA</i> RNA polymerase associated helicase protein (ATPase and RNA polymerase recycling factor)
28	MicF	ggtagtagactg	<i>hybC</i> Hydrogenase 2 large subunit <i>priA</i> Primosome factor n' (replication factor Y) <i>xseA</i> Exonuclease VII large subunit <i>topB</i> DNA topoisomerase III <i>rpoH</i> RNA polymerase sigma 32 (sigma H) factor <i>rpoB</i> RNA polymerase beta subunit <i>xthA</i> Exonuclease III <i>hybB</i> Predicted hydrogenase 2 cytochrome b type component
29	MicF	ctatacagatcatttacaccc	<i>rpoH</i> RNA polymerase sigma 32 (sigma H) factor <i>uvrD</i> DNA-dependent ATPase I and helicase II <i>uvrC</i> Excinuclease UvrABC endonuclease subunit <i>cpxR</i> Response regulator in two-component regulatory system with CpxA <i>fic</i> Stationary phase protein; cell division <i>ftsP</i> Septal ring component that protects the divisome from stress; multicopy suppressor of ftsI(Ts) <i>uvrY</i> Response regulator in two-component regulatory system with BarA <i>ftsQ</i> Division assembly protein; membrane anchored protein involved in growth of wall at septum
30	Spot42	ccgtatagaaccacatctgcctgg ggggg	<i>yhjX</i> Pyruvate-inducible inner membrane protein putative transporter <i>polA</i> 5' to 3' DNA polymerase and 3' to 5'/5' to 3' exonuclease <i>uvrB</i> Excision nuclease of nucleotide excision repair DNA damage recognition component <i>dnaT</i> DNA biosynthesis protein (primosomal protein I) <i>umuD</i> Translesion error-prone DNA polymerase V subunit RecA-activated auto-protease <i>dnaK</i> Chaperone Hsp70; co chaperone with DnaJ
31	Spot42	ggagggggggg	<i>dnaT</i> DNA biosynthesis protein (primosomal protein I) <i>torS</i> Hybrid sensory histidine kinase in two component regulatory system with TorR <i>phoP</i> DNA binding response regulator in two component regulatory system with PhoQ <i>narQ</i> Sensory histidine kinase in two component regulatory system with NarP (NarL)

Table B3.3 The preliminary 74 candidate genes based on their function among 31 artificial sequences of sRNA and their hybridization with target mRNA. Targeted sequencing identified 36/74 mutants (bold) having the *gyrA* S83L mutation successfully incorporated.

S.NO.	Target gene	Function
1	<i>emrA</i>	Multidrug efflux system
2	<i>fic</i>	Stationary phase protein, cell division
3	<i>rep</i>	DNA helicase and single-stranded DNA-dependent ATPase
4	<i>recB</i>	Exonuclease V (RecBCD complex) beta subunit
5	<i>recC</i>	Exonuclease V (RecBCD complex); gamma chain
6	<i>recD</i>	Exonuclease V (RecBCD complex) alpha chain
7	<i>recE</i>	Exonuclease VIII 5' to 3' specific dsDNA exonuclease
8	<i>recJ</i>	ssDNA exonuclease; 5' 3' specific
9	<i>recO</i>	Gap repair protein
10	<i>recQ</i>	ATP-dependent DNA helicase
11	<i>recT</i>	Recombination and repair protein
12	<i>rho</i>	Transcription termination factor
13	<i>rnt</i>	Ribonuclease T (RNase T)
14	<i>rpoC</i>	RNA polymerase beta prime subunit
15	<i>rpoD</i>	RNA polymerase sigma 70 (sigma D) factor
16	<i>rpoN</i>	RNA polymerase sigma 54 (sigma N) factor
17	<i>ruvC</i>	Component of RuvABC resolvase endonuclease
18	<i>yacG</i>	DNA gyrase inhibitor
19	<i>ypdC</i>	Putative DNA-binding protein
20	<i>smrB</i>	Putative DNA endonuclease
21	<i>smf</i>	DNA recombination-mediator A family protein
22	<i>seqA</i>	Negative modulator of initiation of replication
23	<i>sbmC</i>	DNA gyrase inhibitor
24	<i>sbcC</i>	Exonuclease; dsDNA; ATP dependent
25	<i>tus</i>	Inhibitor of replication at Ter; DNA binding protein
26	<i>torT</i>	Periplasmic sensory protein associated with the TorRS two-component regulatory system

27	<i>tolA</i>	Membrane anchored protein in TolA-TolQ-TolR complex
28	<i>tolQ</i>	Membrane spanning protein in TolA-TolQ-TolR complex
29	<i>tolC</i>	Transport channel
30	<i>topB</i>	DNA topoisomerase III
31	<i>umuC</i>	DNA polymerase V; subunit C
32	<i>uvrD</i>	DNA-dependent ATPase I and helicase II
33	<i>uvrC</i>	Excinuclease UvrABC endonuclease subunit
34	<i>xthA</i>	Exonuclease III
35	<i>xseA</i>	Exonuclease VII large subunit
36	<i>ypdA</i>	Predicted sensory kinase in two component system with YpdB
37	<i>yeeY</i>	Predicted DNA binding transcriptional regulator
38	<i>ydaG</i>	Putative DNA endonuclease
39	<i>ydaV</i>	Predicted DNA replication protein
40	<i>yddM</i>	Putative DNA-binding transcriptional regulator
41	<i>zipA</i>	FtsZ stabilizer
42	<i>ampC</i>	Penicillin-binding protein beta-lactamase intrinsically weak
43	<i>cho</i>	Endonuclease of nucleotide excision repair
44	<i>cheB</i>	Fused chemotaxis regulator protein glutamate methylesterase in two component regulatory system with CheA
45	<i>cpxA</i>	Sensory histidine kinase in two component regulatory system with CpxR
46	<i>cpxR</i>	Response regulator in two-component regulatory system with CpxA
47	<i>dnaK</i>	Chaperone Hsp70; co chaperone with DnaJ
48	<i>dnaQ</i>	DNA polymerase III epsilon subunit
49	<i>dnaT</i>	DNA biosynthesis protein (primosomal protein I)
50	<i>dinB</i>	DNA polymerase IV
51	<i>emrB</i>	Multidrug efflux system protein
52	<i>hybB</i>	Predicted hydrogenase 2 cytochrome b type component
53	<i>holE</i>	DNA polymerase III theta subunit
54	<i>holC</i>	DNA polymerase III chi subunit
55	<i>holD</i>	DNA polymerase III; psi subunit
56	<i>helD</i>	DNA helicase IV
57	<i>hybC</i>	Hydrogenase 2 large subunit
58	<i>mdtA</i>	Multidrug efflux system; subunit A
59	<i>mdtC</i>	Multidrug efflux system subunit C
60	<i>minC</i>	Inhibitor of FtsZ ring polymerization
61	<i>mtlR</i>	Mannitol operon repressor
62	<i>mutL</i>	Methyl directed mismatch repair protein

63	<i>ompR</i>	Response regulator in two-component regulatory system with EnvZ
64	<i>pinQ</i>	Qin prophage putative site-specific recombinase
65	<i>polB</i>	DNA polymerase II
66	<i>priA</i>	Primosome factor n' (replication factor Y)
67	<i>priB</i>	Primosomal protein N
68	<i>priC</i>	Primosomal replication protein N''
69	<i>proC</i>	Pyrroline-5-carboxylate reductase NAD(P)-binding
70	<i>pyrB</i>	Aspartate carbamoyltransferase catalytic subunit
71	<i>rbbA</i>	Fused ribosome associated ATPase ATP binding protein/ATP binding protein/predicted membrane protein
72	<i>rnr</i>	Exoribonuclease R; RNase R
73	<i>rsgA</i>	Ribosome small subunit dependent GTPase A
74	<i>uvrA</i>	ATPase and DNA damage recognition protein of nucleotide excision repair excinuclease UvrABC

Table B3.4 MIC values (ng/ml) of single (Keio knockout mutant) and double mutant (Keio knockout mutant + *gyrA* S83L mutation) towards Ciprofloxacin.

Target gene	Keio knockout mutant MIC	Double mutant strain MIC (Keio knockout mutant + <i>gyrA</i> S83L mutation)	Effect of the Keio mutation vs. <i>cybC</i>	Effect on S83L background	Effect on WT background	Effect on S83L background
<i>cybC</i> (Control)	31	1000	1	1	0	0
<i>recC</i>	15	500	0.5	0.5	-1	-1
<i>recD</i>	31	500	1	0.5	0	-1
<i>recJ</i>	31	500	1	0.5	0	-1
<i>rnt</i>	31	500	1	0.5	0	-1
<i>rpoN</i>	31	1000	1	1	0	0
<i>smrB</i>	31	500	1	0.5	0	-1
<i>sbmC</i>	15	125	0.5	0.125	-1	-3
<i>tus</i>	7.8	62.5	0.25	0.0625	-2	-4
<i>topB</i>	31	500	1	0.5	0	-1
<i>umuC</i>	31	500	1	0.5	0	-1
<i>uvrD</i>	15	125	0.5	0.125	-1	-3
<i>xseA</i>	7.8	62.5	0.25	0.0625	-2	-4
<i>dnaQ</i>	31	500	1	0.5	0	-1
<i>helD</i>	31	500	1	0.5	0	-1
<i>priB</i>	31	500	1	0.5	0	-1
<i>priC</i>	31	500	1	0.5	0	-1
<i>rnr</i>	15	125	0.5	0.125	-1	-3
<i>uvrA</i>	15	125	0.5	0.125	-1	-3
<i>yeeY</i>	31	500	1	0.5	0	-1
<i>mtlR</i>	31	500	1	0.5	0	-1
<i>ydaG</i>	31	500	1	0.5	0	-1
<i>ydaV</i>	31	500	1	0.5	0	-1
<i>ompR</i>	15	500	0.5	0.5	-1	-1
<i>cheB</i>	15	125	0.5	0.125	-1	-3
<i>cpxA</i>	31	500	1	0.5	0	-1
<i>cpxR</i>	31	500	1	0.5	0	-1
<i>dnaK</i>	31	500	1	0.5	0	-1

<i>hybB</i>	31	500	1	0.5	0	-1
<i>emrB</i>	31	125	1	0.125	0	-3
<i>tolA</i>	31	500	1	0.5	0	-1
<i>tolQ</i>	7.8	62.5	0.25	0.0625	-2	-4
<i>tolC</i>	15	62.5	0.25	0.0625	-2	-4
<i>minC</i>	15	500	0.5	0.5	-1	-1
<i>zipA</i>	31	1000	1	1	0	0
<i>proC</i>	15	500	0.5	0.5	-1	-1
<i>pyrB</i>	31	500	1	0.5	0	-1

Figure B3.1 A boxplot distribution of 528 randomized plasmid constructs growing in the presence and absence of CIP. The growth parameters tested here are (A) growth rate (OD₆₀₀/minute) and (B) length of lag phase (minutes). Non-randomized plasmid growing in the presence of CIP is used as a control. The boxplots present the median, first, and third quartiles, with whiskers showing either the maximum (minimum) value or 1.5 times the interquartile range of the data, whichever is smaller (larger).

Figure B3.2 A model predicted by IntaRNA, showing the complementary base-pairing between artificial sRNA sequence and its target mRNA hit (Mann *et al.* 2017). The mRNA target is identified as *sbmC*, which is a DNA gyrase inhibitor gene. The complementary base-pairs and positions are indicated in the figure.

Appendix C

Appendix to Chapter 3

Table C4.1 The list of stressors and their concentrations used in this study.

TCS	Stressor	Concentration	References
Pho	MgCl ₂	200uM	Montero <i>et al.</i> 2009; Ram and Gaulian 2013
EnvZ-OmpR	NaCl	0.5M	Mascher 2006; Siryaporn and Goulian 2008
Cpx	NaCl	0.5M	Bury-Moné 2009; Delhayé 2016
Bae	NaCl	0.5M	Raffa and Raivio, 2002; Baranova 2002; Bury-Moné 2009

Table C4.2 The antibiotics used in this study and their mode of action.

Antibiotic	Class	Mode of action	References
Ceftazidime	Third-generation cephalosporin	Bactericidal. Interfere with bacterial cell wall synthesis and prevents cross-linking of peptidoglycan	Hayes and Orr 1983
Ciprofloxacin	Second generation fluoroquinolone	Bactericidal. Inhibit enzymes DNA gyrase and topoisomerase IV, which are essential for bacterial DNA replication, recombination, repair, and transcription	LeBel 1988
Tetracycline	Tetracyclines	Bacteriostatic. Binds reversibly to the bacterial 30S ribosomal subunit and making the ribosomal site inaccessible for incoming aminoacyl tRNA	Chopra and Roberts 2001
Chloramphenicol	Phenicol	Bacteriostatic. Interferes with bacterial protein synthesis.	Allison <i>et al.</i> 1962

Table C4.3 MIC values (ug/ml) of TCS mutants under stressor +/- antibiotic conditions.
(The MIC assay was performed in triplicates with similar results).

Mutant +/- Stressor	CIP	CEF	TET	CHM
<i>cybC</i> -Stressor	0.015	0.06	1	2
<i>cybC</i> + stressor (NaCl)	0.015	0.06	1	2
<i>cybC</i> + stressor (MgCl ₂)	0.015	0.06	1	2
<i>phoQ</i> +stressor	0.015	0.125	2	4
<i>phoQ</i> -stressor	0.0075	0.015	0.125	0.25
<i>phoP</i> +stressor	0.031	0.125	2	4
<i>phoP</i> -stressor	0.0075	0.015	0.125	0.5
<i>envZ</i> +stressor	0.031	0.031	0.5	0.5
<i>envZ</i> -stressor	0.0037	0.015	0.0625	0.125
<i>ompR</i> +stressor	0.031	0.125	0.5	1
<i>ompR</i> -stressor	0.0037	0.015	0.0625	0.125
<i>cpxR</i> +stressor	0.015	0.25	2	4
<i>cpxR</i> -stressor	0.0075	0.031	0.0075	0.0075
<i>cpxA</i> +stressor	0.031	0.125	1	4
<i>cpxA</i> -stressor	0.0075	0.125	0.0075	0.0075
<i>baeS</i> +stressor	0.031	0.25	2	4
<i>baeS</i> -stressor	0.0075	0.015	0.031	0.015
<i>baeR</i> +stressor	0.031	0.5	2	4
<i>baeR</i> -stressor	0.0075	0.015	0.015	0.031

Table C4.4 Normalized colony size measurement for eight mutants compared to *cybC* on solid media under various experimental treatments. (Each value represents the average measurement of three independently obtained normalized values for each of the mutants).

Media (stressor+/-antibiotic)	Mutant normalized colony size								
	<i>cybC</i>	<i>baeS</i>	<i>baeR</i>	<i>cpxA</i>	<i>cpxR</i>	<i>phoQ</i>	<i>phoP</i>	<i>envZ</i>	<i>ompR</i>
M9 MM	1.96078	1.46685	1.62367	1.50108	1.30515	1.31109	1.54409	1.78252	1.9344
M9MM+NaCl	1.8848	1.35939	1.22135	1.24019	0.96509	X	X	1.72534	1.82534
M9MM+MgCl ₂	1.86216	X	X	X	X	1.03005	1.01651	X	X
M9MM+CIP	1.62013	0.02171	0.48431	0.52069	0.7713	0.61499	0.36078	0.89668	0.86933
M9MM+CEF	1.7705	0.62872	0.66601	0.96078	0.88786	0.90878	0.81564	1.29549	1.66537
M9MM+TET	1.61107	0.53866	0.37668	0.69576	0.60208	0.43666	0.31144	1.12696	1.15737
M9 MM+CHM	1.06001	0.81899	0.85856	1.3487	0.70793	0.95608	0.74267	1.58049	1.96078
M9MM+CIP+NaCl	1.73427	1.0765	1.15495	1.06656	0.87118	X	X	0.37632	0.21836
M9MM+CEF+NaCl	1.76641	1.19183	1.32562	1.11561	1.39772	X	X	0.67201	0.77201
M9MM+TET+NaCl	1.09607	1.27137	1.16077	1.26457	1.06434	X	X	0.6495	0.6495
M9MM+CHM+NaCl	1.73609	1.07932	1.3881	1.16078	0.91582	X	X	0.56968	0.79239
M9MM+CIP+ MgCl ₂	1.74177	X	X	X	X	0.41048	1.3516	X	X
M9MM+CEF+ MgCl ₂	1.06291	X	X	X	X	1.16078	1.23997	X	X
M9MM+TET+ MgCl ₂	0.92565	X	X	X	X	0.78968	0.93113	X	X
M9MM+CHM+ MgCl ₂	1.00179	X	X	X	X	0.91726	1.08668	X	X

Table C4.5 A two-way analysis of variance (ANOVA) for the effects of different treatments between control and mutants.

Treatment	<i>cybC-phoQ</i>	<i>cybC-phoP</i>	<i>cybC-envZ</i>	<i>cybC-ompR</i>	<i>cybC-cpxA</i>	<i>cybC-cpxR</i>	<i>cybC-baeS</i>	<i>cybC-baeR</i>
	F P values	F P values	F P values	F P values	F P values	F P values	F P values	F P values
M9 MM	60.43 0.01*	20.06 0.04*	6.35 0.128	0.131 0.75	25 0.036*	61 0.015*	31 0.03*	22 0.04*
M9MM+NaCl	X	X	5.17 0.151	0.72 0.484	60 0.01*	171 0.005**	36 0.02*	64 0.01*
M9MM+MgCl ₂	10 0.009*	111 0.008**	X	X	X	X	X	X
M9MM+CIP	202 0.004**	317 0.003**	135 0.007**	145 0.006**	242 0.004**	181 0.005**	1070 0.0009***	305 0.003**
M9MM+CEF	148 0.006**	182 0.005**	5 0.017*	118 0.003**	131 0.007**	155 0.006**	260 0.003**	244 0.004**
M9MM+TET	230 0.004**	287 0.003**	29 0.032*	24.9 0.03*	133 0.007**	165 0.006**	189 0.005**	257 0.003**
M9 MM+ CHM	28 0.01*	20 0.04*	54 0.017*	128 0.007**	16 0.05.	24 0.03*	23 0.04*	18 0.05.
M9MM+CIP+NaCl	X	X	316 0.003**	401 0.002**	64 0.01*	149 0.006**	20 0.04*	14 0.06.
M9MM+CEF+NaCl	X	X	240 0.004**	198 0.005**	84 0.011*	14.4 0.06	66 0.014*	23 0.04*
M9MM+TET+ NaCl	X	X	40 0.023*	40 0.02*	5.6 0.14	0.20 0.69	6.13 0.13	1.8 0.45
M9MM+CHM+ NaCl	X	X	227 0.004**	141 0.006**	45 0.02*	134 0.007**	62 0.015*	66 0.014*
M9MM+CIP+ MgCl ₂	303 0.003**	26 0.04*	X	X	X	X	X	X
M9MM+CEF+ MgCl ₂	1.9 0.30	6.2 0.12	X	X	X	X	X	X
M9MM+TET+ MgCl ₂	0.25 0.6	0.06 0.94	X	X	X	X	X	X
M9MM+CHM+ MgCl ₂	6.8 0.12	1.4 0.35	X	X	X	X	X	X

Figure C4.1 Structural features and domain organization of sensor (HK) and response regulator (RR) proteins of *E. coli* TCS (EnvZ-OmpR, Cpx, and Pho). Region I consist of conserved histidine residue (His), region II is the conserved asparagine, and region III contains glycine-rich residues. The RR consists of N-terminal receiver domain (I) with conserved aspartate (Asp) residue and the other homologous C-terminal output domains (II) for transcriptional regulation. The numbers on the right shows the total number of amino acid residues. (The domain structure and sequences are inspired from Stock *et al.* 1989; Mizuno *et al.* 1997; Finn *et al.* 2016; Keseler *et al.* 2017)

Figure C4.2 Boxplot of averaged normalized colony-size scores comparing various growth treatments (M9 MM and stressor $\pm$ -antibiotic) of control *cybC* versus eight mutants. The boxplot present the median, first, and third quartiles, with whiskers showing either the maximum (minimum) value or 1.5 times the interquartile range of the data, whichever is smaller (larger).

Figure C4.3. A representative image for phenotypic quantification of colony size variation between control *cybC* and Keio knockout mutant *envZ* under various treatments (M9 MM, M9MM+stressor, and stressor +/- antibiotic).