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Interplay between nitric oxide and antimicrobial susceptibility in *Escherichia coli*



A thesis submitted to the University of Kent Faculty of
Sciences for the degree of MSc in Microbiology.

Faculty of Sciences, School of Biosciences,

The University of Kent

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2017

Declaration

I confirm that no part of this thesis has been submitted in support of an application for any degree or qualification at either the University of Kent, any other university or higher education learning institution.

Joy Miller

16 November 2017

Abstract

Due to the emergence of widespread antibiotic resistance in bacterial pathogens, a greater understanding of bacterial responses to current drugs is required. A recent study reports that aerobic respiration is required for correct function of bactericidal antibiotics. Intriguingly, human macrophages and neutrophils produce the respiratory inhibitor nitric oxide in response to bacterial infection, so it would seem that the host immune system is impairing the ability of antibiotics to work effectively. To test this hypothesis, *Escherichia coli* strains were exposed to GSNO, a nitric oxide donor, and were subsequently exposed to antibiotics. Increasing the concentration of NO resulted in a dose-dependent attenuation in antibiotic efficacy, which is consistent with our hypothesis. Previous work in the Shepherd lab has reported the respiratory oxidase cytochrome *bd-I* as being resistant to NO, so we hypothesised that expression of this respiratory complex would sensitise *E. coli* to antibiotics when grown in the presence of NO. Experiments with cytochrome *bd-I* knockout strains and a NO donor were performed to test this hypothesis. This work highlights discrepancies between minimal lethal concentrations in the presence and absence of NO, which has obvious implications for our understanding of antibiotic efficacy during infection.

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Abbreviations

Adenosine Triphosphate	ATP
Amoxicillin	AMX
Centimeter	cm
Colony Forming Units	CFU
<i>Clostridium difficile</i>	<i>C. difficile</i>
Dihydropteroate Synthase	DHPS
<i>Escherichia coli</i>	<i>E. coli</i>
Electron Transport Chain	ETC
Extended-Spectrum β -lactamase	ESBL
Gentamycin	GM
Luria-Bertani Medium	LB
M9 Minimal Medium	M9
Methicillin-Resistant <i>Staphylococcus aureus</i>	MRSA
Microgram	μg
Mililiter	ml
Milimolar	mM
Minimum Bactericidal Concentration	MBC
Minimum Inhibitory Concentration	MIC
Molar	M
Multidrug Resistant	MDR
Nitric Oxide	NO
Nitrofurantoin	NF

Proton Motive Force	PMF
Reactive Nitrogen Species	RNS
Reactive Oxygen Species	ROS
Sequence Type 131	ST131
S-Nitrosoglutathione	GSNO
<i>Staphylococcus aureus</i>	<i>S. aureus</i>
Uropathogenic <i>E. coli</i>	UPEC
Wild type	WT

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Chapter I

Introduction

A rampant increase in antimicrobial resistance is one of the largest threats to global health. High resistance rates have been documented in both health-care and community-acquired infections around the world (World Health Organization, 2014), spurring a need for a greater understanding of antimicrobial resistance. Antimicrobial agents consist of compounds which are antibacterial, antifungal, antiparasitic, and antiviral. This work focuses on antibacterial compounds, which are comprised of antibiotics and synthetic antibacterial agents.

Antibiotics are natural chemical compounds produced by microorganisms to increase their chances of fitness over other microorganisms. These compounds can inhibit the growth of, or promote the death of, another microorganism. Antibiotic producers protect themselves from the compounds they generate by developing resistance mechanisms. Resistance is an evolutionary defence mechanism (or a combination of mechanisms) which allows a microorganism to grow in the presence of an antimicrobial (El-Baky 2016). Strategies a microbe may use include: inactivation of the antibiotic, altering an antibiotic's target, reducing the permeability of its cell to reduce uptake, pumping out the antibiotic via efflux mechanisms, or changing its biochemical pathway (Muntia and Arias 2016).

Phylogenetic reconstruction studies suggest that antibiotic resistance genes occurred in nature before the antibiotic era of the last 80 years, indicating that humans have been exposed to antibiotics for centuries (Aminov 2010). Although resistance is a natural process, incorrect use of antimicrobial treatment in humans and animals is concomitant to the acceleration of resistance rates worldwide (Dodds 2017).

1.1. Emergence of antibiotic resistance

Methicillin-Resistant *Staphylococcus aureus* (MRSA) is one of the most recognized examples of antibiotic resistance, as it is resistant to most β -lactams as well as antimicrobials from other classes. MRSA infections are common in hospitals but have also become prevalent in community-acquired infections as well as livestock. (Arede *et al.* 2012). *S. aureus* was the first organism in which multiple drug resistance became published (Jevons 1961), but unfortunately this occurrence is on the rise with several other pathogens. As mentioned previously, antibiotic resistance

is an evolutionary mechanism in which bacteria that co-reside with antibiotic-producing microorganisms developed for survival. This form of resistance became associated with intrinsic resistance, whereas the current trends seen in antimicrobial resistance are due to acquired resistance-defined as the acquisition of resistance to an antimicrobial by an organism which was previously sensitive to the drug (Munita and Arias 2016). A microorganism is considered resistant to an antimicrobial if its minimum inhibitory concentration (MIC) is higher than the breakpoint value, which is determined by a standard test and often set by a committee, such as the European Committee on Antimicrobial Susceptibility Testing (Mouton *et al.* 2011). A microorganisms' MIC is determined through *in vitro* testing in the clinical laboratory, and represents the lowest concentration of an antimicrobial that inhibits visible growth of the organism. The minimum bactericidal concentration (MBC) is determined as the lowest concentration of antimicrobial which promotes cell death based on the inability to re-culture a previously antibiotic-treated subculture (Andrews 2001).

Antibiotic use as a therapy for non-bacterial infections or prophylaxis in animal husbandry are examples of antibiotic misappropriation which lead to an increase in antibiotic resistance through selective pressure. Selective pressure occurs because bacteria are exposed to sub-inhibitory antimicrobial concentrations which allow the organisms to continue growing and select for resistance or genetic and phenotypic variability (Andersson and Hughes 2014). Due to this selective pressure, bacteria could develop genetic mutations and then transfer them to other bacteria through horizontal gene transfer, further exacerbating the problem.

Clostridium difficile is an example of an antimicrobial-resistant bacterial pathogen in which animal antibiotic treatment is a contributing factor to the spread of resistant isolates, as some human and animal isolates are closely related and interspecies transmission can occur through food. *C. difficile* resistance to tetracycline, doxycycline, and erythromycin antimicrobials has been reported in isolates collected from animals (Zidaric *et al.* 2012) while Tenover, Tickler, and Persing (2012) reported that resistance to clindamycin and moxifloxacin antimicrobials is widespread in human isolates from North America. Multidrug resistant (MDR) *C. difficile* isolates resistant to clindamycin, moxifloxacin, rifampin were also present in

isolates that they screened. *C. difficile* has been identified by the Centers for Disease Control and Prevention as one of the most urgent threats of antibiotic resistant microorganisms in the United States and was reported as the leading cause of death associated with gastroenteritis as well as one of the most prevalent health-care associated infections (Lessa *et al.* 2015).

Another striking example of resistance emerging in bacterial pathogens is *Escherichia coli* ST131, a globally disseminated sequence type that is commonly resistant to several classes of antibiotic (Croxall *et al.* 2011, Totsika *et al.* 2011). This MDR strain is observed in healthcare- and community-acquired urinary tract infections and produces a CTX-M-15 extended spectrum β -lactamase (ESBL), protecting the strain from the effects of β -lactam antimicrobials (Petty *et al.* 2014). *E. coli* EC958, a WT ST131 strain is used in this study.

1.2. Classes of antibacterial agents and modes of action

Antibacterial agents can be classified using several methods. They are typically grouped by chemical structure, overall phenotypic effect, or mode of action. **Table 1.1** lists the main classes of antibacterial agents, grouped into classes based on chemical structure, and briefly identifies each classes' bacterial target and mode of action. **Figure 1.1** depicts each class' main target in the bacterial cell. Each class is further described in the subsections following.

Antimicrobial Class	Main Target	Mode of Action
Aminoglycosides	30S ribosomal subunit	Inhibits protein synthesis
Amphenicols	50S ribosomal subunit	Inhibits protein synthesis
β -lactams	Cell wall	Inhibits cell wall synthesis
Fluroquinolones	DNA gyrase, topoisomerase IV	Inhibits DNA synthesis
Macrolides	50S ribosomal subunit	Inhibits protein synthesis
Nitrofurans	Several	Unknown
Polymixins	Cell membrane	Disrupts membrane
Sulfonamides	Folic acid pathway	Binds DHPS
Tetracyclines	30S ribosomal subunit	Inhibits protein synthesis

Table 1.1. Brief descriptions of antibacterial classes.

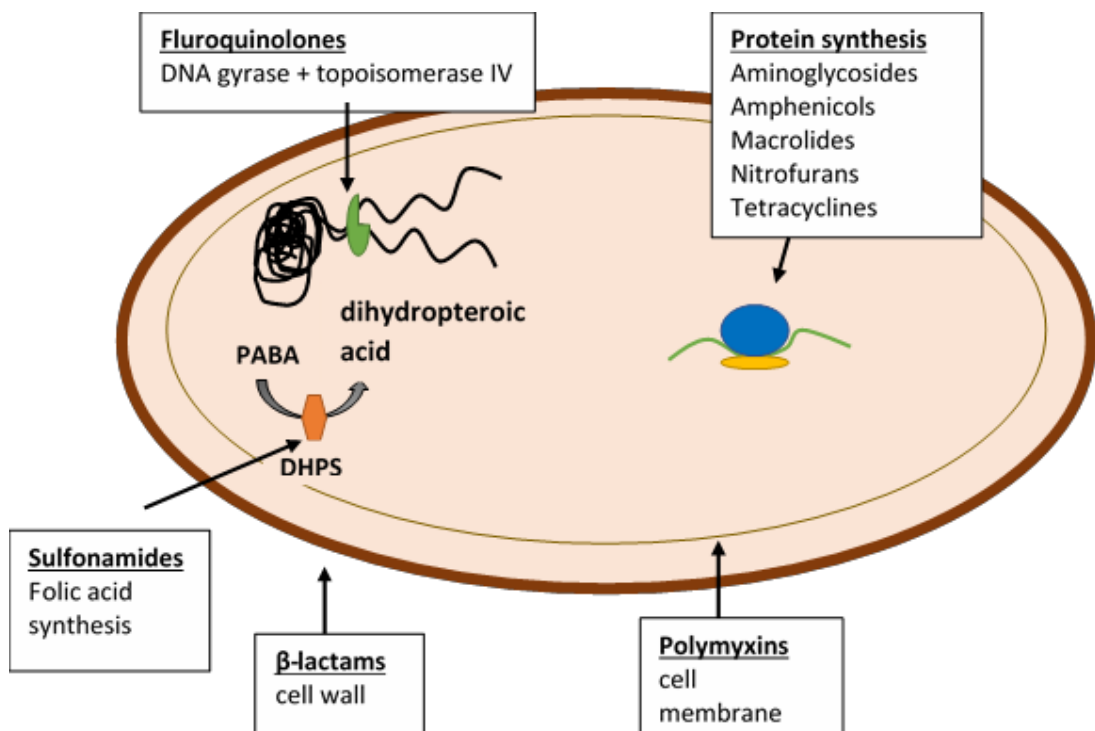


Figure 1.1. The main targets in a bacterial cell for major classes of antimicrobials.

1.2.1. Aminoglycosides

Aminoglycosides are a group of broad-spectrum bactericidal antibiotics containing amino sugars that are bonded by glycosidic linkage. Aminoglycosides were discovered when streptomycin was isolated from *Streptomyces griseus* in the 1940s (Doi Wachino, and Arakawa 2016). Over the next three decades, other antibiotics in this group were discovered and, subsequently, second-generation semi-synthetic versions were created. Common examples of aminoglycosides include kanamycins, neomycins, and gentamicins (Becker and Cooper 2013), of which gentamycin is used in this study. Streptomycin was immensely instrumental in treating tuberculosis following its initial discovery, and other antibiotics in this class were often used to treat gram-negative and some gram-positive species. They have been replaced in clinical practice in favour of other therapies, but are often still used to treat severe gram-negative bacterial infections (Galimand, Courvalin, and Lambert 2003).

Aminoglycosides act by binding selectively to 16S rRNA, part of the 30s subunit, and interrupting protein synthesis which leads to cell death (Udumula *et al.* 2012). Upon binding to 16S rRNA, aminoglycosides cause translational errors which inhibit translocation. Bacteria have developed resistance to this class by methylation or substitution of the bases which bind these agents with 16S rRNA, reducing uptake of the drug through changes to membrane permeability and efflux, and most commonly modification of enzymatic activity of the antimicrobial (Galimand, Courvalin, and Lambert 2003).

1.2.2. Amphenicols

Amphenicols are class of synthetic antimicrobials which contain a phenylpropanoid structure. Chloramphenicol is the most common drug from this class. It was first isolated as an antibiotic from *Streptomyces venezuelae* in the late 1940s and has since been chemically synthesized along with its derivatives. It is active against gram-positive and gram-negative bacteria and can be useful in treating bacterial meningitis, although it is not commonly used due to toxic side effects (Aminov 2017).

Chloramphenicol is a bacteriostatic antibiotic that inhibits protein synthesis (Allsion *et al.* 1962). It irreversibly binds to the 50S ribosomal subunit and prevents chain elongation by inhibiting peptidyltransferase (Xaplanteri *et al.* 2003). Bacterial resistance to chloramphenicol is widely spread, with the most common resistance mechanism being inactivation of the drug through acetyltransferases (Schwarz *et al.* 2004).

1.2.3. β -lactams

β -lactam antimicrobials are a large class of bacterial agents which contain a β -lactam ring as their core structure. There are several subclasses which include penicillins, cephalosporins, carbapenems, and monobactams (Aminov 2017). Penicillin was the first antibiotic of this class to be discovered. It is naturally produced by the *Penicillium sp.* and was discovered in the 1920s, followed by the discovery of cephalosporins in the 1940s from a culture of *Cephalosporium* fungus (Kardos and Demain 2011). Semisynthetic versions of penicillin were later produced to overcome resistance to penicillinase (methicillin) and to provide a broader-spectrum activity (ampicillin) (Aminov 2017). Third- and subsequent generations of β -lactam antibiotics have since been developed, of which amoxicillin is used in this study (Handsfield *et al.* 1973).

β -lactam antibiotics target bacterial cell walls. They inhibit cell wall synthesis using several mechanisms. Penicillins prevent the cross-linking of peptidoglycan by binding to the active site of transpeptidase enzymes. Transpeptidases catalyse the last step of crosslinking the peptide chains in the cell wall. As a result of this binding, transpeptidases are unable to catalyse the crosslinking, weakening and eventually degrading the cell wall. Without a cell wall, the bacterial cells lyse and cell death occurs (Yocum, Rasmussen, and Strominger 1980). Resistance to penicillins is provided by the *blaZ* and *mecA* genes which code for β -lactamase and penicillin-binding protein 2a. β -lactamases destroy β -lactam rings in penicillins, rendering the drug ineffective. Penicillin-binding protein 2a is a specific type of transpeptidase which β -lactam antimicrobials cannot inhibit (Blazques, *et al.* 2014).

1.2.4. Fluoroquinolones

Fluoroquinolones are a class of broad-spectrum synthetic antimicrobials which are derived from a quinolone called nalidixic acid (Aminov 2017). They prevent DNA gyrase from supercoiling DNA in the cell. They can also interact with topoisomerase IV, which is needed to separate DNA, thereby inhibiting cell division. (Tran and Jacoby 2002). Ciprofloxacin and norfloxacin are two common examples of this drug class.

Resistance to quinolones is encoded by the gene *qnr*. Quinolone resistance is acquired through mutations to chromosomal genes as drugs in this class are synthetic. These mutations have been found to be transferrable (Tran and Jacoby 2002). Qnr can bind to DNA gyrase and diminish its ability to bind DNA in the gyrase-DNA complex, resulting in a decrease in available targets for quinolones (Tran, Jacoby, and Hooper 2005).

1.2.5. Macrolides

Macrolides are a large class of antibiotics whose basic structure is comprised of a 14- to 16- atom lactone ring coupled with sugar moieties. The first clinical antibacterial agent discovered in this group was erythromycin, which is produced by *Saccharopolyspora erythrae* (Pavlova *et al.* 2017), and is one of the most common antibiotics of this class. Other common macrolide antibiotics include azithromycin (Retsema *et al.* 1987) and clarithromycin (Morimoto *et al.* 1987), which are chemically modified second-generation drugs with improved spectrum and potency over erythromycin.

Macrolides inhibit protein synthesis by binding to the nascent peptide exit of 23S rRNA of the large subunit on the bacterial ribosome. By binding here, they can block the translocation reaction which results in a failure of continued growth of peptide chains of certain proteins (Sothiselvam *et al.* 2016). The most common form of resistance to macrolides occurs when demethylation of the 23S rRNA at the adenine residue A2058 does not allow the antimicrobial to bind at its target site (Aminov 2017).

1.2.6. Nitrofurans

Nitrofurans are a group of synthetic drugs recognized for their antimicrobial properties in the 1940s. The exact mode of action of Nitrofurans is still unknown, but some studies have provided general targets of nitofuran activity. Reduced forms of these drugs bind to ribosomal proteins by forming highly reactive intermediates and inhibiting protein synthesis (McCalla, Reuvers, and Kaiser 1970; McOsker and Fitzpatrick 1994). It has also been demonstrated that nitrofurans cause single strand breaks in DNA (McCalla, Reuvers, and Kaiser 1971).

The most common antimicrobial in this class is Nitrofurantoin, which is used in this study. Nitrofurantoin is used to treat uropathogens, as there is a low incidence of resistance to the drug while many uropathogens are often resistant to many of the other antimicrobial classes (Lindgren *et al.* 2015).

1.2.7. Polymyxins

Polymyxins are a class of polypeptide antimicrobials which generally have a cyclic peptide and a hydrophobic tail. They were discovered in the 1940s and are produced by *Bacillus polymyxa*. They target the cell membranes of gram-negative bacteria, acting as detergents to disrupt the membrane and increase membrane permeability. The increase in permeability results in a leakage of ions and macromolecules necessary for the cell to survive (Sahala and Dixon 2008; Sader *et al.* 2015). A secondary mode of action has been suggested that polymyxins also inhibit type II NADH-quinone oxidoreductases (Deris *et al.* 2016).

Polymyxin B and polymyxin E (also known as colistin) are the most commonly used antimicrobials in this class. They are usually reserved as a last resort drug, but can be used for the treatment of MDR Enterobacteriaceae, *Acinetobacter baumannii*, and *Pseudomonas aeruginosa* (Tran *et al.* 2016). An increase in MDR strains requiring colistin treatment have resulted in an emergence of colistin resistance. It has been suggested that a possible mechanism for colistin resistance is the addition of phosphoethanolamine to lipid A in the outer membrane (Qureshi *et al.* 2015).

1.2.8. Sulfonamides

Sulfonamides are a class of synthetic antimicrobials which are derivatives of sulphanilamide, an analog of *p*-aminobenzoic acid. They target the enzyme dihydropteroate synthase (DHPS), which catalyzes a reaction that forms dihydropteroic acid. The formation of dihydropteroic acid is necessary for bacteria to synthesize folic acid. Targeting DHPS blocks folic acid synthesis in bacteria, which bacterial organisms need for survival (Skold 2000).

Sulfonamides were once a popular class used in antimicrobial treatment, therefore resistance to these drugs is common for many pathogens. Resistance is linked to mobile genetic elements, making it difficult to eliminate as integrons promote the rapid spread of antimicrobial resistance genes (Aminov 2017).

1.2.9. Tetracyclines

Tetracyclines are a class of broad-spectrum antibiotics discovered in the 1940s. The first antibiotic discovered was chlortetracycline, which is produced by *Streptomyces aureofaciens* (Aminov 2017). They inhibit protein synthesis by preventing tRNA from accessing the acceptor site in the ribosome (Chopra and Roberts 2001).

Wide-spread resistance to tetracycline exists because of extensive use of sub-therapeutic levels of these antibiotics in animal feed to promote animal growth. Tetracycline resistance commonly occurs via gene acquisition. Some of these genes code for efflux pumps, while others code for protein protecting ribosomes (Chopra and Roberts 2001). The tet(X) gene has also been shown to provide resistance as it encodes for an enzyme deactivating tetracycline molecules. To combat resistance issues, second- and third-generation tetracyclines were developed for clinical use. Third-generation tigecycline has shown to have a small level of resistance in clinical isolates and is an effective treatment for many MDR pathogens (Aminov 2017).

1.3 Distinguishing between bacteriostatic and bactericidal antimicrobials

Antibiotics can also be grouped into two categories based on their overall phenotypic effect on the microorganism; growth is inhibited by bacteriostatic antibiotics while bactericidal antibiotics elicit cell death. However, it has been argued in literature that the distinction of the *in vitro* definitions of bacteriostatic and bactericidal antibiotics are not clinically relevant, especially when treating gram-positive bacterial infections (Pankey and Sabath 2004), abdominal infections, skin infections, soft tissue infections, and pneumonia (Nemeth, Oesch, and Kuster 2015). On the other hand, Findberg *et al.* (2004) concluded that antimicrobials with *in vitro* bactericidal activity are needed to treat endocarditis and meningitis, even though bactericidal agents can have disadvantages such as inflammation from cytokines as a result of bacterial products from lysed cells. There is also the argument that bactericidal treatment is superior to bacteriostatic treatment simply because eradication of bacteria diminishes the likelihood of resistance development (Stratton 2003).

Despite clinical studies showing the distinction between bacteriostatic and bactericidal antimicrobials to be irrelevant, microbiological and molecular studies have demonstrated that these two categories have very different phenotypic effects. Levin *et al.* (2017) hypothesized that a reduction in growth due to bacteriostatic antibiotic treatment is a result of a reduced ribosome count in these cells, since the majority of bacteriostatic antibiotics mainly target translation machinery. They postulated their query on other studies which demonstrated that numbers of ribosomes in a cell are proportional to the growth rate of the bacteria, and their results supported their hypothesis. Belenky *et al.* (2015) demonstrated that treatment with different classes of bactericidal antibiotics produced similar metabolic changes which are indicative of toxic metabolic by-products building up.

1.4. The impact of bacterial respiration upon antibiotic resistance

A recent study has reported that bacterial respiration exacerbates the toxic effects of bactericidal antibiotics, and the presence of bacteriostatic agents were shown to abrogate this potentiation. In this study, bacteriostatic drugs suppressed respiration, which in turn affected bactericidal antibiotic lethality (Lobritz *et al.* 2015).

The inhibition of the respiratory chain affects antibiotic lethality in several ways. By inhibiting respiratory processes, the cell is unable to grow, which can be protective for bacteria that are being treated with antibiotics which target growth activities (Baek, Li, and Sasseti 2011), such as β -lactams targeting cell wall synthesis. Inhibition of respiration can also attenuate the effectiveness of aminoglycosides which require a threshold of PMF for the organism to uptake the drug. If respiration is inhibited, antibiotic uptake is arrested and ultimately diminishes antibiotic effectiveness (Allison, Brynildsen, and Collins 2011). Another means in which inhibition affects antibiotic lethality is that by impeding bacterial metabolism, the toxic metabolic by-products produced during antibiotic treatment are reduced (Belenky et al. 2015; Dwyer et al. 2014). This has implications for the role of the respiratory inhibitor nitric oxide during infection, which is introduced in **Section 1.6** following a brief introduction to bacterial respiration in **Section 1.5** below.

1.5. The aerobic respiratory chain of *E. coli*

During bacterial respiration, an energized state is produced by reactions resulting in electron transport. During electron transport, there is a charge separation of protons from hydroxyl ions occurring across the surface of the cytoplasmic membrane, creating an electrochemical potential and pH difference across the membrane. This potential energy state is known as the proton motive force (PMF) (Krulwich, Sachs, and Padan 2011). The energized state in the proton motive force can be conserved in the form of ATP or used for other purposes in the cell. The PMF is produced by reactions from the enzymes, proteins, and non-protein molecules of the electron transport chain (ETC) (Price and Driessen 2010).

The role of the ETC is to facilitate electron transfer from electron donor to acceptor and to initiate the process of energy conservation. The ETC is branched so that it can be adaptable under varying conditions in the environment (Steinsiek, Stage, and Bettenbrock 2014). Several enzymes are involved in the oxidation and reduction process of electron transport which include dehydrogenases, flavoproteins, iron-sulfur proteins, and cytochromes. In the ETC, electrons are transported between donors and acceptors, of which oxygen is a final electron acceptor during aerobic respiration (Henkel *et al.* 2014).

Electron carriers are arranged throughout the cytoplasmic membrane with dehydrogenases being the first stop in the chain, and during aerobic respiration the terminal oxidases are the last carriers involved. As the final electron carriers in the aerobic ETC, terminal oxidases donate electrons and protons to the terminal electron acceptor oxygen (Price and Driessen 2010). **Figure 1.2.** diagrams a general ETC.

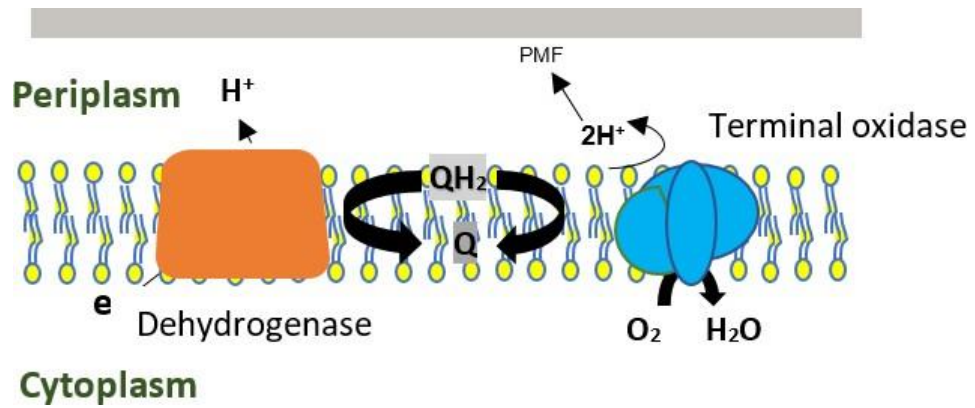


Figure 1.2. Generalized electron transport chain of *E. coli*. The ETC transfers electrons from donors to acceptors and participates in energy conservation. Dehydrogenases catalyse the removal of H^+ from metabolites and transfer electrons to the quinone pool in the cytoplasmic membrane. Quinones shuttle electrons from dehydrogenases to terminal oxidases, where the final electron acceptor is reduced. *E. coli*'s terminal oxidases also generate a PMF and Cytochrome *bo'* acts as a proton pump, while Cytochrome *bd* does not. In the figure above, O_2 is shown as the final electron acceptor, which is reduced to water.

1.5.1 The terminal oxidases of the *E. coli* respiratory chain

E. coli's aerobic respiratory chain contains two terminal quinol oxidases—cytochrome *bo'* and cytochrome *bd*. Because *E. coli* is a facultative anaerobe, it can grow under different conditions of oxygen availability, where different oxidases are expressed: cytochrome *bo'* is a low affinity oxidase and is expressed under aerobic conditions, and cytochrome *bd* is a high affinity oxidase and is expressed under microaerobic conditions (D'Mello, Hill and Poole 1995). *E. coli* terminal oxidases contain heme prosthetic groups which lose or gain a single electron in the process of oxidation or reduction. The terminal oxidases of *E. coli* differ in their reduction potential and are designated according to the types of hemes they contain (Puustinen and Wikstrom 1991). Cytochrome *bo'* is composed of the protein cluster CyoABCD; cytochrome *bd*-I is composed of CydABX. In addition, cytochrome *bd*-I is tolerant to the respiratory inhibitor nitric oxide (Mason *et al.*, 2009; Shepherd *et al.* 2016), which is encountered during infection, which has implications for the efficacy of antibiotics (introduced in Sections 1.5 and 1.6).

Bacterial terminal oxidases could potentially be promising new drug targets. Previously, the contribution that terminal oxidases make towards bacterial fitness have been described for *S. aureus* (Hammer *et al.* 2013). Also, Kalia *et al.* (2017) found that inhibition of the two terminal oxidases of *Mycobacterium tuberculosis* allowed a bacteriostatic drug to perform in a bactericidal manner. It has also been shown that the virulence of some pathogenic bacteria is dependent on cytochrome *bd* (Giuffre *et al.* 2014). Previous work in the Shepherd lab confirmed that cytochrome *bd*-I is resistant to NO (Mason *et al.* 2009), allowing bacteria to survive nitrosative stress produced by its human host. **Figure 1.3.** demonstrates the effect of NO in *E. coli*'s respiratory chain. Understanding the role of terminal oxidase expression in antibiotic resistance would further propel research towards new strategies for combatting this worldwide problem.

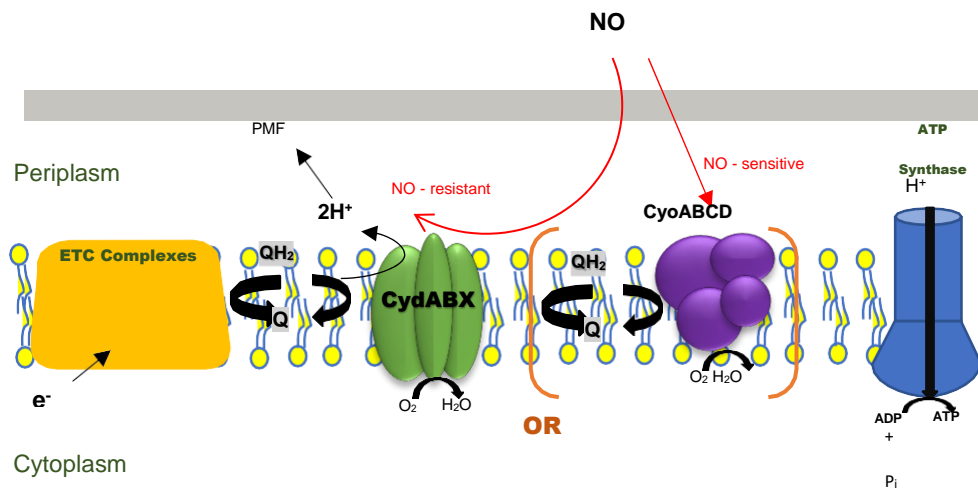


Figure 1.3. The effect of NO in *E. coli*'s respiratory chain. Cytochrome *bo'* (CyoABCD protein cluster) is the dominant oxidase expressed in high oxygen conditions and its activity is inhibited by NO. Cytochrome *bd-I* (CydABX protein cluster) is primarily expressed under low oxygen conditions and is tolerant to NO, allowing respiration to continue in the presence of NO.

1.6. *E. coli* encounters the respiratory inhibitor nitric oxide (NO) during infection

The human body has its own defence mechanisms against bacteria. During infection NO is produced endogenously by mammalian innate immune cells, mostly macrophages and neutrophils. NO is a small molecule which damages microorganisms through nitrosative damage to a wide range of cellular targets, including thiols and metal centres of bacterial proteins. A vast range of organisms are effectively targeted by NO, including pathogenic strains of *E. coli*. NO is bactericidal at high concentrations, but elicits bacteriostatic effects via inhibition of the terminal oxidases of the respiratory chain (via binding to haem cofactors) (Fang 1997; Giuffre *et al.* 2014).

1.7. Hypothesis: NO can undermine the efficacy of antibiotics

Since terminal oxidase activity has been shown to have a positive effect upon the potency of bactericidal antibiotics (Lobritz *et al.* 2015), the terminal oxidase inhibitor NO is anticipated to diminish the toxic effects of these antimicrobials. This study aims to demonstrate that NO impairs the efficacy of bactericidal antibiotics via the use of GSNO, a nitric oxide releaser. Furthermore, the expression of NO-tolerant (cytochrome *bd-I*) and NO susceptible (cytochrome *bo'*) terminal oxidases is also anticipated to impact upon the potency of antibiotics.

Chapter 2

Materials and Methods

2.1. Bacterial Strains

Table 2.1. *E. coli* strains used in this work.

Strain #	Strain Description	Source/Reference
MS2	K-12 MG1655 WT	Bachmann, 1996
MS10	ST131 EC958 WT	Totsika <i>et al.</i> , 2011
MS15	MG1655 cydAB::Cm	Shepherd Laboratory
MS52	K-12 BW25113 WT	Datsenko and Wanner, 2000
MS493	BW25113 cyoA::Kan	Baba <i>et al.</i> , 2006
MS404	MG1655 cydAB::Cm (pSU2718-cydABX)	Shepherd Laboratory

2.2. Chemicals and water

2.2.1. Purchased chemicals

All chemicals were purchased from Sigma Aldrich unless otherwise stated. Agar, tryptone, and yeast extract were purchased from Oxoid. NaCl was purchased from Fisher Scientific.

2.2.2. GSNO preparation

GSNO was prepared by dissolving 3.08g reduced glutathione and 0.69g NaNO₂ into 18 ml water. 862 µl of 36% HCl was then added (11.65M) to the solution which was covered with foil and stirred on ice for 40 minutes. 20 ml of acetone was added to the solution which stirred for another 10 minutes. The precipitate was collected by vacuum filtration while washed with five 2 ml volumes of cold water, three 10 ml volumes of acetone, and three 10 ml volumes of diethyl ether. The precipitate was dried overnight in a vacuum desiccator lined with CaCl₂. The pink powder precipitate was stored at -80°C for up to one month.

GSNO concentration was determined by dissolving the powder described above into water and measuring the absorbance at 545 nm. The equation below was used to calculate the concentration.

$$A_{545} = \text{concentration} \times \text{length} \times \epsilon$$

$\epsilon = 15.9 \text{ M}^{-1} \text{ cm}^{-1}$ at 545 nm (Mohr *et al.* 1999), length = 1 cm.

GSNO was filter sterilised using 0.22 µm Millipore filters before measuring the absorbance.

2.2.3. Water and sterilization

Distilled-deionised water was used unless otherwise stated. Milli-Q water was when greater sterility was needed. Growth media was sterilized by autoclaving at 121°C, 15 psi for 20 minutes.

2.3. Media

2.3.1. LB medium

LB broth was made by dissolving 10g NaCl, 10g tryptone, and 5g yeast extract into distilled-deionised water to make up a 1L solution. To make LB agar, 15g of dried nutrient agar was added to the solution.

2.3.2. M9 minimal medium

M9 medium was made by dissolving 200 ml 5X M9 salts, 2 ml 1M MgSO₄, 100 µl 1M CaCl₂, 20 ml 20% Glucose, and 10 ml 10% (w/v) casamino acids in water to make up to a 1L solution. 5X M9 salts contained 80.24g Na₂HPO₄·2H₂O, 15 g KH₂PO₄, 2.5 g NaCl, and 5 g NH₄Cl dissolved in water to make up a 1L solution. Casamino acids 10% (w/v) were prepared by adding dried casamino acids to water to make up a 100 ml solution, autoclaved and cooled before use.

2.4. Antibiotics

Amoxicillin (AMX) stock solutions were prepared at 1 mg/ml dissolved in DMSO. Gentamycin (GM) stock solutions were prepared at 1 mg/ml dissolved in water. Stocks of AMX and GM were stored at -20°C. Nitrofurantoin (NF) was prepared fresh for daily use at 10 mg/ml. It was dissolved in DMSO and serially diluted in M9 media to working concentrations.

2.5. Antibiotic Concentrations

E. coli cells were treated with AMX at 40 µg/ml, (5x) minimum inhibitory concentration (MIC), GM between 10 µg/ml-100 µg/ml, (5x)-(50x) MIC, and NF at 100 µg/ml (MIC value is 64 µg/ml which is bacteriostatic, so we treated with a higher dose). MIC values were based on the BSAC MIC and zone diameter breakpoint tables for Enterobacteriaceae (BSAC 2013).

2.6. Growth Conditions

E. coli overnight cultures were grown at 37°C in LB media shaking at 180 rpm in conical flasks. Cultures for time-kill assays were diluted from the overnight culture into M9 minimal medium supplemented with 0.1% casamino acids. Cells were grown at 37°C shaking at 180 rpm in conical flasks.

2.7. Time-Kill and Minimum Bactericidal Concentration Assays

Overnight samples of *E. coli* were diluted 1:100 into 10 ml of fresh M9. Each strain was grown aerobically to 10⁸ cfu/mL. Cells were aliquoted into sterile 15 mL falcon tubes and GSNO was added to cells in one of the falcon tubes and Milli-Q water was added to cells in the other falcon tube. Cells were then incubated for 30 minutes at 37°C. Cells were aliquoted into sterile 1.5 ml Eppendorf tubes and appropriate antibiotics added. At specified time points, samples were taken from each culture and serially diluted 10⁰-10⁷ into fresh M9 across a 96-well plate. Two biological repeats were included for every assay and at least six technical repeats.

Viable counts were determined by spotting samples from the dilutions described above onto LB agar. Agar plates were inverted once the spotted samples were absorbed by the agar and grown overnight at 37°C. Colonies were counted the following day and percent survival determined by comparing the cfu/ml at each time point to the initial cfu/ml. Percent survival is presented on a log₁₀ scale in all figures.

Chapter 3

Results

3.1. Nitric oxide impairs the efficacy of bactericidal antibiotics

3.1.1 Attenuation of antibiotic efficacy is NO dose-dependent in *E. coli* K-12

To investigate the efficacy of bactericidal antibiotics in the presence of NO, a well characterized *E. coli* K-12 strain, BW25113 WT, was exposed to a NO donor, S-Nitrosoglutathione (GSNO), and subsequently treated with Amoxicillin (AMX), a β -lactam bactericidal antibiotic described in **Section 1.2.3**. Cultures were grown aerobically as described in **Section 2.6** and then treated with 40 $\mu\text{g/mL}$ AMX (5x MIC), after a 30-minute exposure to GSNO. A time-kill analysis of *E. coli* K-12 is presented in **Figure 3.1** as percent survival over time, determined by comparing cfu/mL of samples taken at each time point to the cfu/mL at $t = 0$. **Figure 3.1A** tracks the GSNO dose response with AMX for *E. coli* BW25113 WT over 2 hours. Cultures were exposed to either 2mM, 5mM, 10mM or 15mM concentrations of GSNO before treatment with AMX. As the concentration of GSNO increased, the bactericidal effects of AMX decreased. **Figure 3.1B** shows the % survival for *E. coli* BW25113 WT at $t=2\text{h}$. When treated with AMX only, only 1.6 % of the cells survival. When exposed to 2mM GSNO, some alleviation was seen, as the survival rose to 2.9%. However, a pre-treatment with 15mM of GSNO provided the greatest protection from AMX, as the strain had a survival of 151.8% at $t=2\text{h}$.

To determine if the same effects could be observed in other *E. coli* K-12 strains, another well-characterized strain, *E. coli* MG1655 WT, was also treated with AMX after GSNO exposure. As 15mM GSNO was most effective in relieving AMX-mediated killing in the BW25113 WT strain, the MG1655 WT strain was exposed to a 15mM GSNO concentration over four hours to assess if the same trend was observed at later time points. Consistent with the BW25113 WT strain data, **Figure 3.1C** displays attenuation of antibiotic efficacy in *E. coli* MG1655 WT over 4 hours when treated with AMX after NO exposure. Overall, the data in **Figure 3.1 A, B,** and **C** demonstrated that NO impairs the efficacy of AMX in *E. coli* K-12.

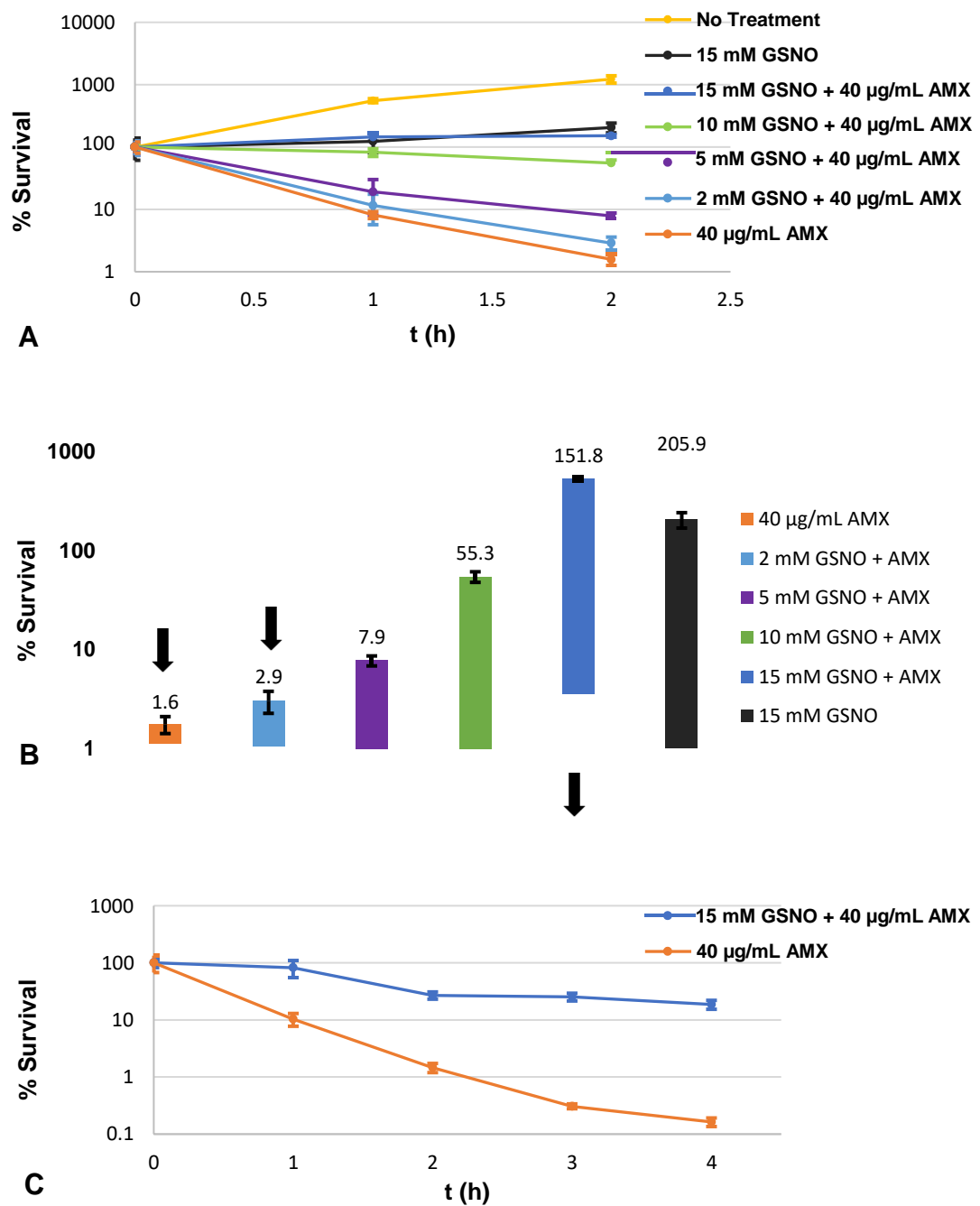


Figure 3.1 *E. coli* K-12 strains treated with AMX +/- GSNO exposure. (A) GSNO dose response of *E. coli* BW25113 WT treated with 40 µg/mL AMX. (B) % survival comparison of *E. coli* BW25113 WT GSNO dose response at t=2h. Arrows highlight differences in survival between 2mM GSNO +AMX and 15mM GNSO + AMX in comparison with AMX only treatment. (C) % survival of *E. coli* MG1655 WT treated with 40 µg/mL AMX. Error bars represent standard deviation of six repeats, including two biological repeats.

3.1.2 Antibiotic efficacy is attenuated in pathogenic *E. coli* in the presence of NO

To determine if antibiotic efficacy is also attenuated in pathogenic *E. coli* in the presence of NO, a well characterized pathogenic *E. coli* ST131 strain, EC958 WT, was treated with a bactericidal antibiotic in the presence of GSNO. Because the EC958 WT strain contains a CTX-M-15 type extended-spectrum β -lactamase (ESBL) (Petty *et al.* 2014), it is inherently resistant to AMX. Therefore, the EC958 WT strain was assessed with Gentamycin (GM), an aminoglycoside, described in **Section 1.2.3**. To compare the GM results with AMX, one of the K-12 strains, *E. coli* BW25113 WT, was treated with 10 $\mu\text{g}/\text{mL}$ of GM (5x MIC) after 30 minutes of 15mM GSNO exposure. Consistent with the AMX results, **Figure 3.2A** demonstrates that antibiotic efficacy is attenuated when *E. coli* K-12 is treated with GM after GSNO exposure. *E. coli* EC958 WT was then treated with GM after 15mM GSNO exposure. At 5x the MIC, no killing was exhibited over the 2-hour time period (data not shown). *E. coli* EC958 WT was then treated with higher concentrations of GM. **Figure 3.2B** displays results for treatment with 25 $\mu\text{g}/\text{mL}$ (12.5x MIC) and 100 $\mu\text{g}/\text{mL}$ (50x MIC) GM after GSNO exposure. Antibiotic treatment with GM was ineffective at 25 $\mu\text{g}/\text{mL}$ with the cell survival over 100% at $t=2(\text{h})$. However, when *E. coli* EC958 WT was treated 100 $\mu\text{g}/\text{mL}$ GM, cell survival was less than .001% at $t=2(\text{h})$ when treated with only the antibiotic. When pre-exposed to GSNO, cells treated with 100 $\mu\text{g}/\text{mL}$ of GM exhibited 54% survival at 2 hours (**Figure 3.2C**), demonstrating that the antibiotic efficacy was attenuated in the presence of NO.

In addition to AMX resistance, *E. coli* EC958 WT is resistant to several classes of antibiotics (Croxall *et al.* 2011, Totsika *et al.* 2011). Since treating *E. coli* ST131 was only successful at concentrations much higher than the MIC in the 2 hour experiment, the EC958 WT strain was treated with another antibiotic at a dose much closer to the MIC. Nitrofurantoin (NF), a Nitrofuran antibiotic described in **Section 1.4.5.**, was used to evaluate both the K-12 and pathogenic strains. **Figure 3.3A** and **Figure 3.3B** display results of the treatment of *E. coli* BW25113 WT (**A**) and *E. coli* EC958 WT (**B**) with 100 $\mu\text{g}/\text{mL}$ NF (>1.5x MIC) with and without GSNO exposure.

Unexpectedly, no alleviation of NF-mediated killing was observed when either strain was exposed to NO.

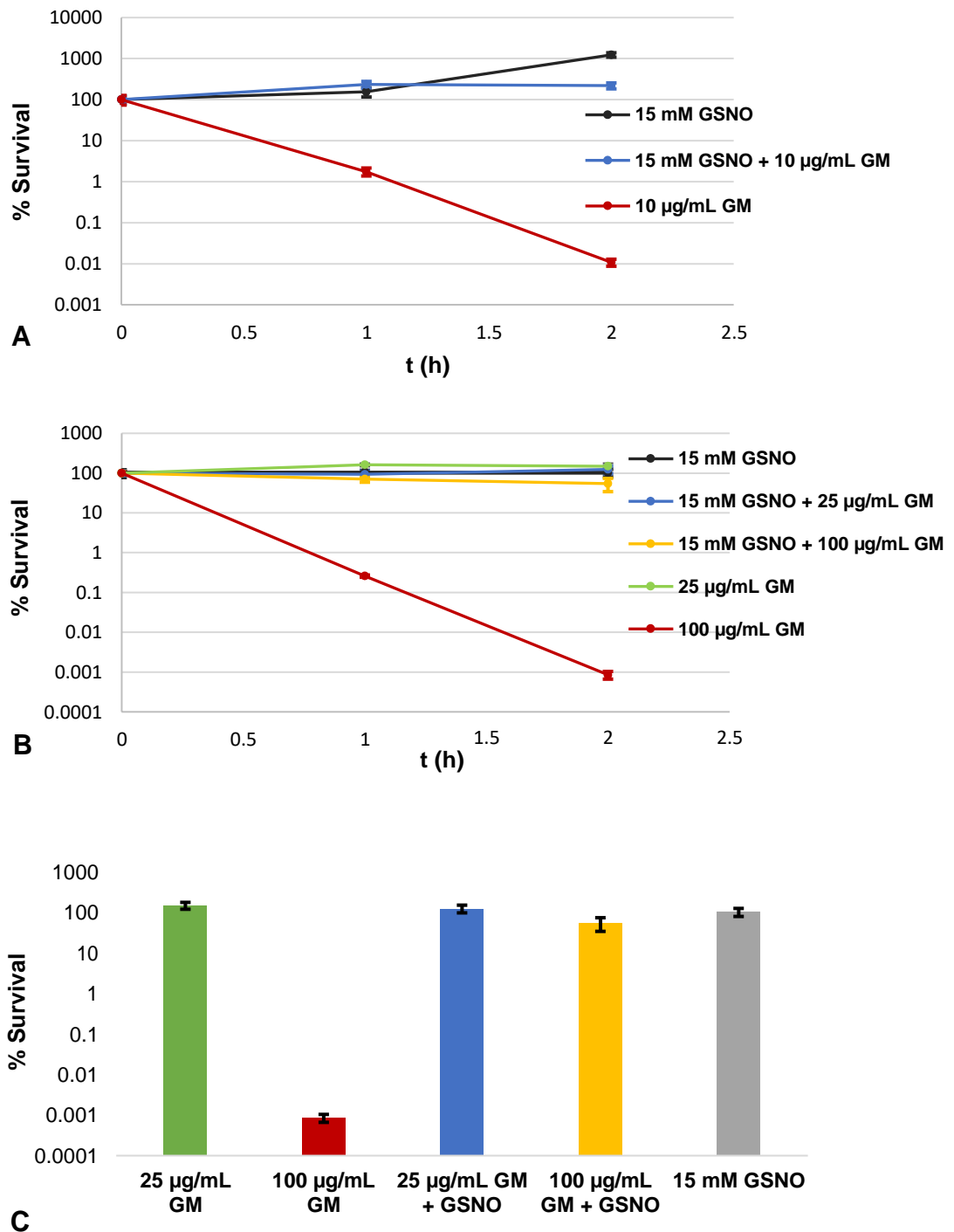


Figure 3.2. Time kill assay of *E. coli* treated with Gentamycin. (A) BW25113 WT K-12 strain treated with 10 µg/mL Gentamycin. +/- 15mM GSNO. (B) Pathogenic EC958 WT ST131 strain treated with 25 µg/mL and 100 µg/mL Gentamycin +/- 15mM GSNO. (C) % survival of EC958 WT at t=2h. Error bars represent standard deviation of six repeats, including two biological repeats.

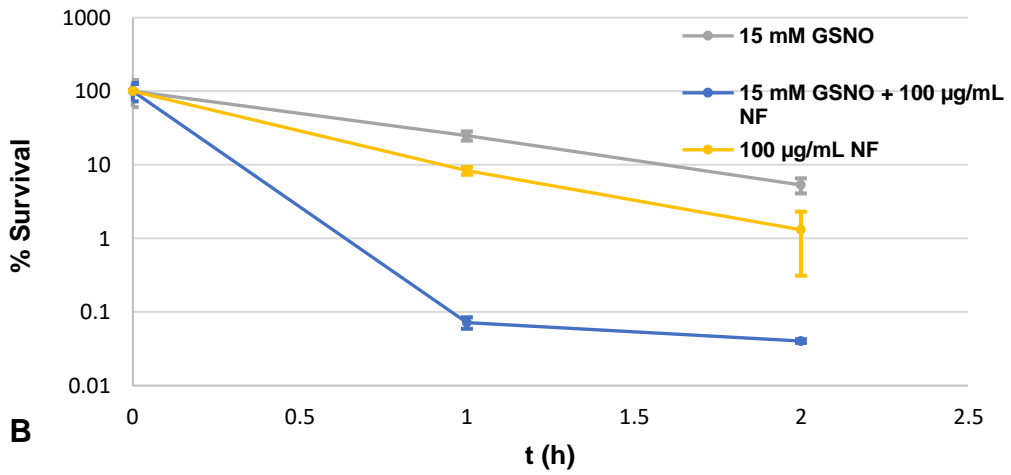
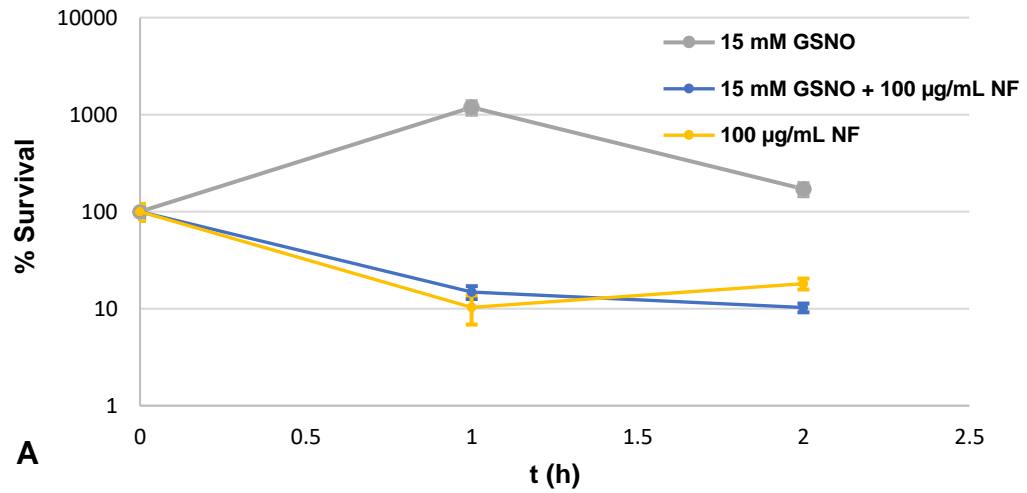


Figure 3.3 Time kill assays of *E. coli* treated with nitrofurantoin. (A) BW25113 WT and pathogenic ST131 strain (B) EC958 WT treated with Nitrofurantoin +/- 15mM GSNO. Error bars represent standard deviation of six repeats, including two biological repeats.

3.1.3 Exposure to NO increases the Minimum Bactericidal Concentration

To determine whether the minimum bactericidal concentration (MBC) is enhanced or diminished when *E. coli* is in the presence of NO, *E. coli* K-12 and pathogenic strains were treated with a range of antibiotic concentrations after exposure to 15 mM of GSNO. An increase in the MBC would result in the curve moving to the right and indicating that a higher dose of antibiotic would be required to effectively kill bacteria; a decrease in the MBC would result in the curve moving to the left and indicating that the addition of NO increased bacterial killing. Alternatively, if GSNO were to inactivate the antibiotic, it would be expected that there would be at least 100% survival of bacteria pre-exposed to GSNO at every concentration since the cells would replicate within the 90-minute incubation period. **Figure 3.4A** shows a dose response with GM +/- 15 mM GSNO for *E. coli* BW25113 WT. Cells were treated with a 1000-fold range of GM from 0.1 µg/mL to 100 µg/mL with samples taken after 90 minutes of exposure to GM. An increase can be observed in the MBC curve from 1 µg/mL to 100 µg/mL. In **Figure 3.4B** *E. coli* EC958 WT also shows attenuation of efficacy for GM between 0.5 µg/mL to 1 mg/mL in presence of NO, as well as slight resistance to the antibiotic compared to the K-12 strain.

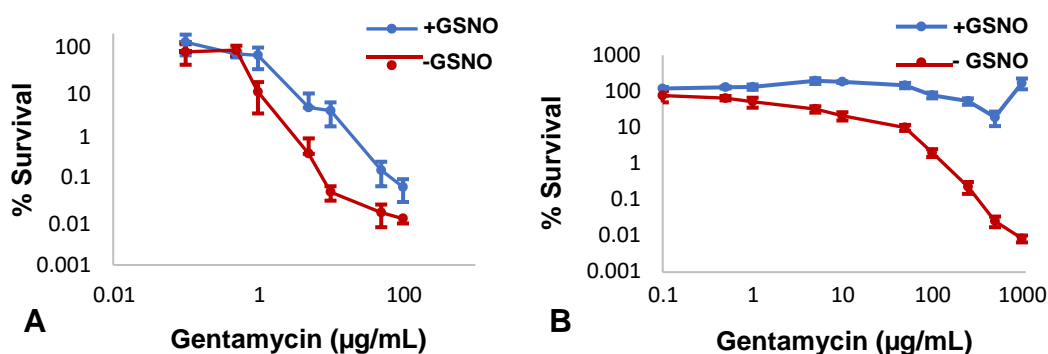


Figure 3.4 Dose response of *E. coli* (A) K-12 BW25113 WT and (B) pathogenic *E. coli* EC958 WT treated with a range of Gentamycin concentrations +/- 15mM GSNO. % survival is based on a 90-minute exposure to antibiotic and shown as a function of Gentamycin concentration. Error bars represent standard deviation of at least four repeats, including two biological repeats.

3.2 The ability of *E. coli* to respire in the presence of nitric oxide influences antibiotic efficacy

3.2.1 Attenuation of antibiotic efficacy is not as pronounced in a cytochrome *bo'* mutant as a WT strain

Since the toxic effects of bactericidal antibiotics are dependent upon bacterial respiration (Lobritz *et al.* 2015), it was hypothesised that changes in the expression of cytochromes in *E. coli*'s respiratory chain could relieve some bactericidal effects of an antibiotic. Cytochrome *bo'*, described in **Section 1.5.1**, is predominantly expressed in aerobic conditions. A deletion in the *cyo* operon is likely to make the cells more reliant on the NO-tolerant cytochrome *bd-I*, allowing *E. coli* to undergo aerobic respiration in the presence of NO and remain sensitive to NO. It was therefore anticipated that the ability of NO to decrease antibiotic efficacy would be attenuated in a cytochrome *bo'* mutant. To test this hypothesis, *E. coli* K-12 strain BW25113 $\Delta cyoA$, was treated with 40 $\mu\text{g/mL}$ of AMX after a 30-minute exposure to 15 mM GSNO. The % survival of samples taken over 2 hours is presented in **Figure 3.5**. The NO-mediated decrease in antibiotic efficacy was not as dramatic in the $\Delta cyoA$ strain (**Figure 3.5A**) compared to the *E. coli* BW25113 WT (**Figure 3.5B**). Also, the $\Delta cyoA$ strain appears to be more resistant to AMX than the WT when treated with the antibiotic alone, as the % survival is higher at both $t=1\text{h}$ (**Figure 3.5C**) and $t=2\text{h}$ (**Figure 3.5D**) compared to the WT strain, yet the % survival is not higher for the $\Delta cyoA$ cultures pre-exposed to GSNO than the WT strains.

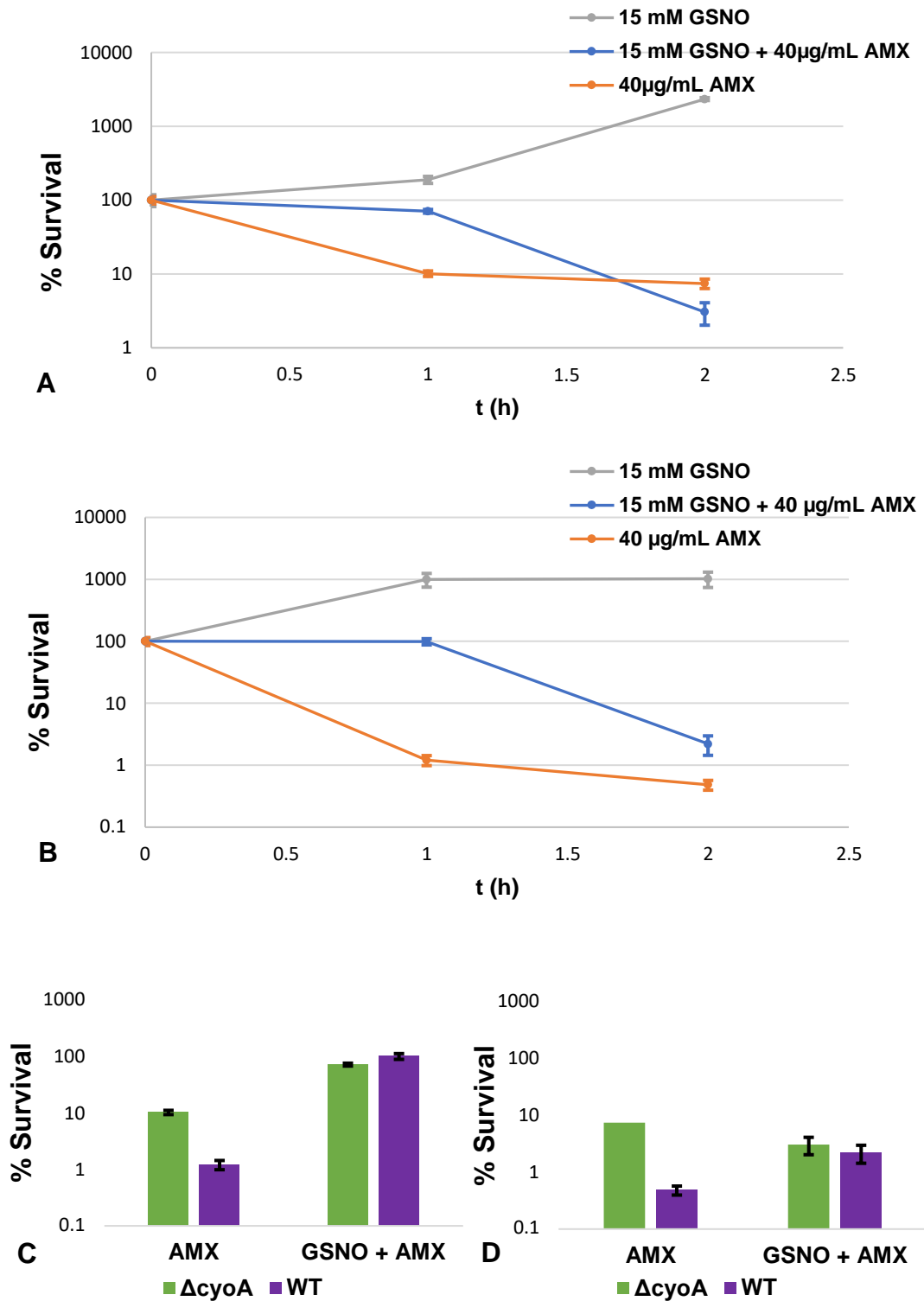


Figure 3.5 % survival of wild type and $\Delta cyoA$ *E. coli* K-12 strains in response to amoxicillin. (A) BW25113 $\Delta cyoA$ and (B) BW25113 WT treated with 40 $\mu\text{g}/\text{mL}$ AMX +/- 15mM GSNO. Comparison of % survival at t=1h (C) and (D) t=2h. Error bars represent standard deviation of 5 repeats, including two biological repeats.

3.2.2 When NO-tolerant cytochrome *bd-I* is lost, attenuation of antibiotic efficacy is more pronounced in the mutant strain than WT strain

As described in **Section 1.5.1**, cytochrome *bd-I* is a high affinity oxidase and is predominantly expressed under microaerobic conditions. Studies have shown this respiratory cytochrome to be NO-tolerant (Mason *et al.* 2009). A deletion in the *cydABX* operon would diminish the ability of the cells to respire in the presence of NO: it was anticipated that this would increase the ability of NO to alleviate antibiotic toxicity. To test this hypothesis, *E. coli* K-12 strain *E. coli* MG1655 Δ *cydAB* was treated with 40 μ g/mL of AMX in the presence of 9.5mM GSNO. % survival of *E. coli* MG1655 Δ *cydAB* treated with AMX only and pre-treated with GSNO is shown in **Figure 3.6A**. *E. coli* MG1655 WT is shown in **Figure 3.6B**. GSNO is slightly more effective in alleviating AMX-mediated killing in the Δ *cydAB* strain in comparison to the WT strain (shown clearly at t = 1h). A strain of *E. coli* MG1655 containing a cytochrome *bd-I* overexpression plasmid (pSU2718-*cydABX*) was also treated with 40 μ g/mL of AMX in the presence of 9.5mM GSNO. Because cytochrome *bd-I* is tolerant to NO, overexpression was predicted to reduce the ability of GSNO to alleviate AMX-mediated killing and AMX would be more lethal to the bacterium. In **Figure 3.6C**, the % survival of this strain in the presence of NO is lower than both the Δ *cydAB* and WT strains, further illustrated in **Figure 3.6D**.

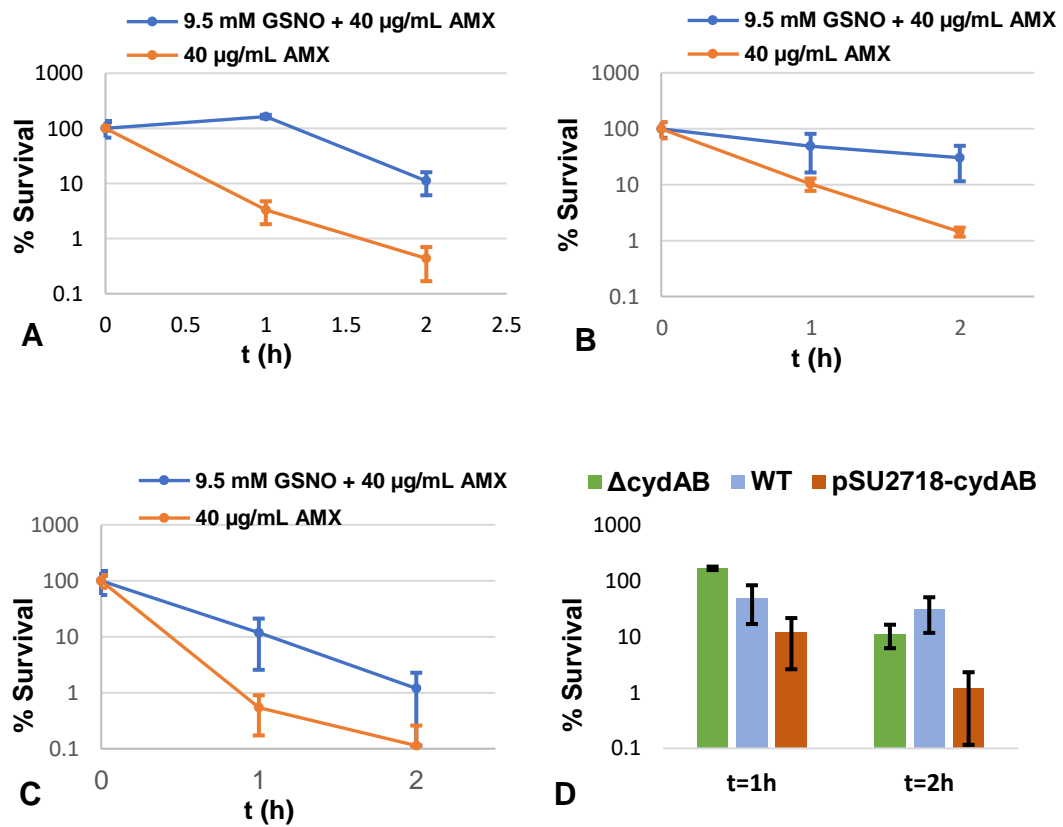


Figure 3.6 % survival of wild type and $\Delta cydAB$ *E. coli* K-12 strains in response to amoxicillin. (A) *E. coli* MG1655 $\Delta cydAB$ (B) *E. coli* MG1655 WT and (C) *E. coli* MG1655 pSU2718-*cydABX*, and overexpression of cytochrome *bd-I* (D). Comparison of three strains at $t=1\text{h}$ and $t=2\text{h}$ when treated with 40 $\mu\text{g}/\text{mL}$ in the presence of NO. Error bars represent standard deviation of at least 5 repeats, including two biological repeats.

Chapter 4

Discussion

4.1. Nitric oxide affects the potency of bactericidal antibiotics

Lobritz *et al.* (2015) observed that the stunted growth effects of bacteriostatic antibiotics were a result of a suppression in cellular respiration, while bactericidal antibiotics promoted cell death by accelerating respiration. Dwyer *et al.* (2014) and Kohanski *et al.* (2007) also found that treatment with different classes of bactericidal drugs resulted in an elevation of oxygen consumption and an induced depletion of NADH, indicating that cellular respiration was accelerated. When Lobritz *et al.* (2015) combined the antagonistic phenotypic outcomes of bacteriostatic and bactericidal drugs, they discovered that treating bacteria with a bacteriostatic antibiotic prior to bactericidal treatment stifled the deleterious effects of the bactericidal antibacterial. They concluded that the state of bacterial metabolism significantly influences antibiotic efficacy.

Several hypotheses indicate that inhibition of the respiratory chain thwarts the effectiveness of bactericidal antibiotics. Antibiotic uptake is hindered without the presence of the PMF which is generated during respiration (Allison, Brynildsen, and Collins 2011); respiration is needed for cell growth and many bactericidal antibiotics target cell growth processes (Baek, Li, and Sasseti 2011); inhibition of respiration hinders the production of toxic metabolic by-products, which are a consequence of downstream metabolic changes that occur when the antibiotic interacts with its main cellular target (Belenky *et al.* 2015).

This work employs those results and hypothesizes that since NO is a respiratory inhibitor, bacterial exposure to NO would quell the effects of bactericidal treatment.

4.1.1. Attenuation in the presence of NO is dose dependent

This study found that the attenuation of bactericidal antibiotics occurs in the presence of NO and that attenuation is dose dependent. *E. coli* K-12 was exposed to a range of GSNO concentrations varying from 2mM-15mM prior to bactericidal antibiotic treatment. When exposed to GSNO before antibiotic treatment, *E. coli* cultures had an incremental increase in % survival correlating with an increase in GSNO concentration (**Figure 3.1A**). 15mM GSNO was determined to be the most effective dose to use in the study (**Figure 3.1B**). GSNO itself has antibiotic properties; in small concentrations it is bacteriostatic and in larger concentrations bactericidal (Shairer *et al.* 2012). *E. coli* was treated with a GSNO only control to verify that the GSNO concentration itself was not killing the bacteria. At 15mM, GSNO appeared to be bacteriostatic as the % survival of the bacteria was lower than the % survival of the no treatment group, yet the % survival at t=1 and t=2 was not lower than the % survival at t=0, indicating that GSNO was stunting the growth of the culture at this concentration, but not killing the cells. Further studies could have been done with higher concentrations to determine the maximum concentration of GSNO before the treatment was determined to be bactericidal, but for this study's purposes 15mM was sufficient to demonstrate that bactericidal antibiotics are attenuated in the presence of NO (**Figures 3.1-3.2, 3.4-3.6**).

4.1.2. Attenuation occurred in both K-12 and pathogenic strains

Antibiotic attenuation occurred in both K-12 and pathogenic *E. coli* (**Figures 3.1-3.2**). The pathogenic strain, *E. coli* EC958 WT, appeared to be a bit resistant to GM, as the MBC shifts for the pathogenic strain (**Figure 3.4B**) in comparison with the WT strain (**Figure 3.4A**). One possible explanation could be that the pathogenic strain contained a subpopulation of persister cells. This could have been tested further by completing a second time-kill assay on colonies from overnight growth that were previously treated with GM (Balaban *et al* 2004). To further investigate these results, an MIC susceptibility test (Andrews 2001) could be performed on the EC958 WT strain to determine if the MIC is indeed higher in the pathogenic strain than the K-12 strain.

The EC958 strain is also resistant to AMX as well as several other antibiotics in other classes. In another study in the Shepherd lab, the EC958 strain was shown to be sensitive to Nitrofurantoin, Meropenem, and Colistin in addition to GM. The time-kill assays were repeated using NF for bactericidal treatment of the BW25113 WT and EC958 WT strains. Surprisingly, no attenuation of antibacterial treatment was observed for either strain (**Figure 3.3A-B**). Perhaps these unexpected results are due to treatment with too low of a drug concentration as NF is bacteriostatic at lower concentrations and bactericidal at higher concentrations. Lindgren *et al.* performed time-kill assays on clinical isolates of *E. coli* (2015) and found that in their isolates at least 8x the MIC was required for bactericidal effect. However, the MICs for the isolates in their study were lower than the MIC breakpoint value listed in the BSAC susceptibility table which was referenced for this study (BSAC 2013). Again, experiments to determine the MIC of NF for the BW25113 WT and EC958 strains could be performed.

4.1.3. A large discrepancy occurs in the Minimum Lethal Concentration for *E. coli* in the presence of NO

In **Figure 3.4** we observed that exposure to NO increases the MBC for both the *E. coli* K-12 and pathogenic strain when treated with GM. These results further support the observation that NO attenuates antibiotic efficacy, as bacterial killing is still present after bactericidal treatment, but at much higher concentrations. This provides evidence that GSNO is not inactivating the drug but rather has a diminishing effect

on the capabilities on the antibiotic. If GSNO were inactivating the drug, we would expect to see a % survival > 100% at each concentration, as the cells would replicate over a 90-minute incubation period. This would result in more of a straight line, rather than a curve since an increase in antibiotic concentration would not result in a decrease in % survival. In the BW25113 WT (**Figure 3.4A**) and the EC958 WT (**Figure 3.5B**) pre-exposure to GSNO resulted in a decrease in survival rates when treated with GM and is represented visually by a curved graph, demonstrating that an increase in concentration resulted in a decrease in survival. While the EC958 WT (**Figure 3.5B**) MBC is less pronounced in the presence of GSNO in comparison to the BW25113 WT (**Figure 3.5A**), a downward trend can be observed around 100 µg/mL. Further studies could be done to explore this by incubating each antibiotic with GSNO overnight and comparing the percent survival of *E. coli* treated with the GSNO/antibiotic mixture and *E. coli* treated with the antibiotic alone, as the concentration of NO would diminish over a longer period of time.

4.2 Cytochrome expression also impacts the potency of bactericidal antibiotics

Based on the results that AMX and GM were attenuated in the presence of NO, we wanted to examine if the expression of NO-tolerant cytochrome *bd-I* would curtail these effects. An *E. coli* BW25113 $\Delta cyoA$ strain was treated with 40 µg/ml AMX after exposure to 15mM GSNO. It was expected that the deletion of the *cyo* operon would result in a higher level of dependence on cytochrome *bd-I*. Since this terminal oxidase is tolerant to NO, deletion of the *cyo* operon would allow *E. coli* to respire in the presence of NO. As demonstrated in Lobritz *et al.* (2015), bactericidal activity correlated with accelerated respiration, therefore *E. coli*'s ability to respire in the presence of NO would diminish NO's ability to attenuate AMX's lethality.

The results in **Figure 3.5** are surprising because we expected that dependence on the NO tolerant cytochrome *bd-I* would allow *E. coli* to respire in the presence of NO. Consequently, it was expected that no attenuation of AMX would occur, as the bactericidal nature of the antibiotic would increase respiration, resulting in toxic effects for *E. coli*. We expected that the survival rate would be close to, if not equal to, that of AMX treatment alone. Treatment with AMX in the presence of NO did not alleviate AMX-mediated killing as dramatically as was observed with *E. coli* BW25113 WT.

We also hypothesized that a loss of cytochrome *bd-I* function would result in a greater attenuation of antibiotic efficacy than a WT strain. We tested this hypothesis by treating an *E. coli* MG1655 Δ *cydAB* mutant with AMX after GSNO exposure. An *E. coli* MG1655 strain containing an overexpression plasmid (pSU2718-*cydABX*) was also treated with AMX after GSNO exposure. It was predicted that an overexpression of cytochrome *bd-I* would reduce the attenuation of AMX, resulting in a lower percentage of survival of these mutant cells than MG1655 WT. **Figure 3.6D** clearly illustrates that a deletion in the *cydABX* operon results in the greatest attenuation of antibiotic efficacy, while an overexpression results in the greatest reduction of alleviation of bactericidal effects compared to the WT strain.

These results are very significant because bacteria are exposed to NO as a result of host mammalian immune defences. Hagan *et al.* (2010) found that in *E. coli* isolates from women with urinary tract infections, *cydA* genes were in the top 3% of genes expressed, while *cyoABCDE* gene expression differed between patient isolates. If *E. coli* were to increase its expression of *cydA* genes when presented with nitrosative stress during infection, bactericidal antibiotic treatment could be attenuated, creating a selective-pressure environment for cells which survive treatment to develop antibiotic resistant mutations. Further work is needed to provide further evidence that NO attenuates antibiotic efficacy.

4.3 Future Work

The findings in this thesis could be further substantiated by repeating the experiments presented here with cPTIO. cPTIO forms NO₂ by donating oxygen to NO, acting as a NO scavenger molecule (Yoshida *et al.* 1993). cPTIO's reaction with NO would keep NO from inhibiting respiration, and the antibiotic treated cells exposed to GSNO should have a similar survival rate to cells treated with antibiotic only. Experiments in this thesis could also be performed with another NO donating molecule in the place of GSNO. The results could be compared to that of treatment with GSNO, building a more robust set of results. Experiments should also be repeated with activated macrophages to verify if similar results are observed when *E. coli* is challenged with mammalian immune cells.

In addition to these experiments, the oxygen conditions for bacterial growth could also be varied to create microaerobic conditions at which oxygen levels are between 2-5% to mimic physiological oxygen levels. This would modify the ratio of cytochrome expression in the electron transport chain, resulting in cytochrome *bd-I* being dominantly expressed. Since cytochrome *bd-I* is tolerant to NO, aerobic respiration would continue, but the attenuation of antibiotic killing may not be as pronounced and could reflect that of the $\Delta cyoA$ respiratory mutant, as respiration of cytochrome *bo'* would be inhibited by NO. Because microaerobic conditions are closer to those in vivo, this set of experiments would be important in determining the biological importance of these findings. However, there are other mechanisms to be considered in vivo, such as the presence of flavoglobin Hmp and flavorubredoxin NorVW detoxification systems, as well as NrfA and Hcp/Hcr reductases, all of which *E. coli* and other gram-negative bacteria use to retort the effects of NO (Shepherd 2016). Therefore, additional experiments with respiratory mutants and later mutants for detoxification and reductases at varying oxygen conditions should be conducted as well to better replicate the scenario of bacterial infection in vivo.

Chapter 5

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