Characterization of the effects of ‘old’ nitrofuran antibiotics on Gram-negative bacteria.

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Abstract

Nitrofurans are “old” antibacterials that are regaining popularity over the past decade due to the low prevalence of resistance amongst formidable Gram-negative bacteria. According to reports by the WHO, *Escherichia coli* and *Pseudomonas aeruginosa* are on the list of critical pathogens for which antibiotics are urgently needed because of the emergence and widespread dissemination of antibiotic resistance. *E. coli* and *P. aeruginosa* are Gram-negative bacteria that cause various diseases, such as uncomplicated urinary tract infections and chronic infections. Furazolidone, nitrofurantoin, and nitrofurazone are three nitrofuran antibiotics currently being investigated for their utility in treating infections caused by these pathogens. Nitrofurans are prodrugs that require activation through reduction by bacterial enzymes. In *E. coli*, three oxidoreductases, NfsA, NfsB, and AhpF, were shown to activate nitrofurans. Nevertheless, in the absence of these three enzymes, nitrofurans can still *kill E. coli*, albeit at an increased concentration, suggesting that additional oxidoreductases were reducing furazolidone from a prodrug to its active form. Due to the failure to identify additional nitrofuran-activating enzymes using mutagenic screens, it was hypothesised that the putative oxidoreductases must be essential for *E. coli* growth. Using a bioinformatic approach, I identified 18 essential oxidoreductases as candidates for nitrofuran-activating enzymes. These were investigated via overexpression from a high-copy-number plasmid in the *E. coli* triple mutant *ΔnfsA ΔnfsB ΔahpF*. Among those enzymes, five oxidoreductases, FolD, CydA, HemA, HemG, and MurB, decreased nitrofurantoin MIC when overexpressed and are, therefore, candidates for the nitrofuran-activating factors in *E. coli*. Furazolidone and two other nitrofurans were also investigated for antivirulence activity in *P. aeruginosa* PAO1 to determine whether its potency was more pronounced than the previously studied nitrofurazone. Effect on swimming and swarming motilities, biofilm formation, and pyocyanin production were assessed. Despite being non-inhibitory to the *P. aeruginosa* PAO1 growth at concentrations that are lethal to *E. coli*, furazolidone was found to be more potent than nitrofurazone and was capable of limiting biofilm formation and pyocyanin production. Overall, the results show the value of reviving “old” antibiotics as they can be used to treat *E. coli* infections even in the “resistant” mutants lacking three known activation enzymes and prove effective as antivirulence compounds in *P. aeruginosa*.

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Abbreviations

%	Percentage
°C	Degrees Celsius
µg	Microgram
µg/mL	Microgram per millimetre
µL	Microlitre
AMR	Antimicrobial resistance
AR	Antibiotic resistance
BHI	Brain heart infusion
$CaCl_2$	Calcium chloride
CAMH	Cation adjusted Mueller-Hinton
CDC	Centre for Disease Control
CF	Cystic fibrosis
CFTR	Cystic fibrosis transmembrane regulator
CFU	Colony forming units
CHL	Chloramphenicol
cP	Centipoise
CV	Crystal violet
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ESBLs	Extended spectrum beta-lactamases
FZ	Furazolidone
IPTG	Isopropyl β-d-1-thiogalactopyranoside
KCl	Potassium chloride
kDa	Kilodaltons
LB	Luria Bertani
MBIC	Minimal biofilm inhibitory concentration
MDR	Multidrug resistant

mg/mL	Milligram per millimetre
$MgCl_2$	Magnesium chloride
$MgSO_4$	Magnesium sulfate
MIC	Minimal inhibitory concentration
mL	Millilitre
mm	Millimetre
mM	Millimolar
NaCl	Sodium chloride
NFZ	Nitrofurazone
NIT	Nitrofurantoin
nm	Nanometre
PBS	Phosphate-buffered saline
PQS	Pseudomonas quinolone signal
QS	Quorum sensing
RNA	Ribonucleic acid
Rpm	Revolutions per minute
RT	Room temperature
S	Svedberg
SOC	Super Optimal broth with Catabolite repression
TraDIS	Transposon-directed insertion-site sequencing
USA	United States of America
USD	United States Dollar
uUTIs	Uncomplicated urinary tract infections
v/v	Volume per volume
w/v	Weight per volume
WHO	World Health Organization

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Chapter 1- General Introduction

1.1. The era of antibiotic resistance

The advent and discovery of antibiotics were concomitant with advancements in modern medicine, specifically in preventing and treating bacterial infections while supporting the intricacies of medical procedures (Aminov, 2010; Hutchings et al., 2019). For example, antibiotics are given to patients after invasive medical procedures to ensure that bacterial infections do not manifest in indwelling medical implants (Donlan, 2001; VanEpps & Younger, 2016). They also play an essential role in managing diseases in chronically ill patients and the immunocompromised, such as cystic fibrosis patients (Fetar et al., 2011; Ryan et al., 2019). Besides human use as a medicine, antibiotics are frequently used in the veterinary sector to treat and prevent bacterial infections in animals and animal husbandry as growth promoters (Olaru et al., 2020). Despite their utility in every aspect of human healthcare, antibiotic use is currently threatened by a phenomenon known as antibiotic resistance (AR). Some scientists describe the current situation as a regression of pre-antibiotic discovery.

Antibiotic resistance arises when bacteria develop or acquire a mechanism to resist and survive antibiotic action, rendering antibiotic therapies ineffective (Ventola, 2015a). Sub-populations of bacteria may become resistant to several classes of bactericidal antibiotics and, in the case of some Gram-negative pathogens, are classified as multidrug-resistant (MDR) (Shultis et al., 2022). Globally, increased trends in MDR pathogens coincide with an increased likelihood of morbidity and mortality and incur tremendous financial burdens to the hospital sector regarding treating these illnesses (Provenzani et al., 2020; Shultis et al., 2022). Naghavi and colleagues conducted one of the most comprehensive global studies in 2019 and estimated that 4.95 million persons died from illnesses where AR bacteria played a contributory role (Thompson, 2022). Of this total, six of the most notorious AR bacteria were directly responsible for approximately 1.27 million deaths (Thompson, 2022). For example, AR bacteria play an indirect role in mortality when the deaths due to drug-resistant species were compared with a scenario where vaccination or other measures prevented the infection (Murray et al., 2022). The direct impact of AR was contrastingly attributable to deaths due to drug-resistant bacterial species compared with lives lost if the bacterial species was replaced with a drug-sensitive one (Murray et al., 2022; Thompson, 2022). In the critical list of the six most deadly pathogens

implicated in these deaths, four Gram-negative bacteria were present (Thompson, 2022). *E. coli* topped the list as the most lethal pathogen with nearly 800,000 reported deaths, and *P. aeruginosa* was the least culpable, with approximately 300,000 deaths (Thompson, 2022). Increased treatment costs also accompany the alarming mortality rates caused by AR. Some reports forecast that AR pathogens will incur approximately $300 billion to $1 trillion by 2050 worldwide (Thompson, 2022; Ventola, 2015a). These projected costs are expected to increase globally by nearly $2 billion per annum (Thompson, 2022). The CDC estimates that despite the already expensive treatment methods deployed, an additional $1400 would be incurred to treat each patient that presents with AR in the USA (Thompson, 2022). The additional costs stem from extended hospital stays, lengthier recuperation periods, and the failed administration of first-line and second-line antibiotics (Dadgostar, 2019). Failed first-line therapeutics often trigger the usage of more toxic and expensive antibiotics that often potentiate an increase in disability and other chronic issues (Ventola, 2015a).

Multiple causes have led to the severe current threat of antibiotic resistance. Regulation of antibiotic usage is one of the crucial factors. Surveillance of countries with lenient and dysregulated antibiotic usage shows very high incidences of antibiotic resistance compared to those where antibiotic stewardship programs follow strict regulatory guidelines (Teuber, 2001). Inappropriately prescribed antibiotics exacerbate AR through selective pressures. Some studies have shown that prescribed antibiotics are often incorrect in 30 % - 80 % of all cases because the pathogen is often unknown during treatment (Cook & Wright, 2022; Paterson et al., 2016). In some countries, antibiotics are readily available over the counter, often leading to overuse. This practice is often detrimental to the effectiveness of antibiotics as self-diagnosis often results in the administration of subinhibitory antibiotic concentrations that promote genetic mutations, increased virulence, and overall resistance (Ventola, 2015a). These alterations render first-line antibiotics ineffective and contribute to AR dissemination on a global scale. Another contributing factor to AR is in the animal husbandry industry, where antibiotics are used as prophylactics and growth promoters (Cheng et al., 2014). At sublethal concentrations within their gastrointestinal tracts, these animals provide a niche that promotes selective pressures for antibiotic resistance in bacteria residing in their gut (Cheng et al., 2014; van Hoek et al., 2011). Despite all these problems, the most impactful issue stems from the reduced supply of novel antibiotics currently available for therapeutic use.

Globally, antibiotic development is considered a terrible investment venture because these drugs are not as profitable as those that treat non-communicable and chronic diseases such as

diabetes and hypertension (Jung et al., 2020; McKenna, 2020). Economic and regulatory obstacles imposed on antibiotic-developing companies have staggered the incentive and rate of novel antibiotic discovery (Ventola, 2015a). Cost analyses of novel antibiotics were estimated to bring in only about $50 million in revenue (Ventola, 2015a). The profitability of antibiotic discovery for the pharmaceutical industry is forecast to remain marginal because of the low cost of the medications compared to others, such as those used in cancer chemotherapy (Ventola, 2015b). Medicines for chronic conditions are deemed the most profitable and a more worthwhile investment. The Infectious Diseases Society of America reports that very few compounds have made it to phases 2 and 3 of development since agents are considered unacceptable in treating extensively resistant Gram-negative bacteria (Ventola, 2015a). In instances where a few antibiotics were approved as human therapeutics, infectious disease specialists and microbiologists advise the controlled use of novel antibiotics as part of antibiotic stewardship programs (Spellberg et al., 2008). Thus, the newly marketed antibiotics remain reserved for extreme cases of MDR pathogens due to concerns over promoting drug resistance. Using older marketed antibiotics while the newer ones are reserved leads to reduced investment returns and discourages ventures in this specific drug discovery direction (Ventola, 2015a). Also, when novel antibiotics are marketed promptly by the relevant authorities, pharmaceutical companies risk limiting profits if AR develops (Ventola, 2015a). Despite all these hurdles pharmaceutical companies face, the urgency still remains for discovering novel antibiotics. Specialists describe the current antibiotic-resistance situation as being comparable to the pre-antibiotic era, whereby morbidity and mortality rates were staggering from low-risk infections.

1.2. Mechanisms of antibiotic resistance

Bacteria can resist antibiotics through various mechanisms, including inherent properties and acquired genetic mutations. In Gram-negative bacteria, such as *Escherichia coli* and *Pseudomonas aeruginosa*, the outer membrane is a robust permeability barrier that restricts antibiotic ingress into the cell cytosol and prevents the building up of lethal concentrations inside bacterial cells (Marshall et al., 2020). This inherent property is further enhanced by the presence of multiple efflux pumps that expel the xenobiotic compounds out of the cells (Blair et al., 2014). Bacteria also gain AR by possessing antibiotic-degrading or modifying enzymes that change the physiological function of the drug targets to such a degree that it causes drug

inactivation (Liu et al., 2018; Tamez-Torres et al., 2020; Yusuf et al., 2021). The classic example of the *tet* gene confers resistance to tetracycline by encoding efflux pumps that exude the antibiotic from the cell cytosol of Gram-negative bacteria (Munita & Arias, 2016). Other modifying enzymes include hydrolases, of which β-lactamases are the most common enzymes capable of inactivating penicillins, monobactams, cephalosporins, and carbapenems (Liu et al., 2018). The widespread deactivation of several classes of antibiotics by this group of enzymes remains highly alarming as it relates to disease treatment. Currently, there are four known diverse types of extended-spectrum beta-lactamases (ESBLs), all of which facilitate the dissemination of isolates resistant to a wide range of β-lactam antibiotics (Liu et al., 2018; Wiedemann, 1983). Another group of enzymes known as transferases is the largest and most diverse group of antibiotic-modifying enzymes. Aminoglycoside antibiotics such as kanamycin are most vulnerable to modification as these large molecules contain several exposed functional groups (Liu et al., 2018). Other examples include plasmid-mediated resistance, as seen in the *mcr-1* gene, which confers colistin resistance, despite this antibiotic being a last-resort drug (Johura et al., 2020). This gene has garnered the attention of microbiologists because of its high transferability and global dissemination in many Gram-negative bacteria (Johura et al., 2020; Liu et al., 2018). Interestingly, several variants of the *mcr-1* gene exist, highlighting pathogens' genetic adaptability in response to selective pressures exerted by antibiotic use (Liu et al., 2018). Several other resistance mechanisms have been characterized; however, microbiologists believe that many other mechanisms remain elusive. Currently, numerous efforts aim to understand the mechanism of AR dissemination, with a particular interest in the distribution of resistance globally.

1.3. Mechanisms of antibiotic resistance dissemination

Once exposed to antibiotics, relatively high selection pressures are exerted on bacteria, which often results in the emergence of antibiotic resistance. This AR can then be spread through several mechanisms, rendering the dissemination of AR inevitable within bacterial populations (von Wintersdorff et al., 2016). Persister cells often circumvent the effects of antibiotics as a natural Darwinian evolution process. Over several generations, these cells can obtain AR genes through conjugation, transduction, and transformation.

Conjugation provides the most substantial route of dissemination whereby the antibiotic resistance determinants are carried on a conjugative plasmid that can be transferred from the donor cells to the recipient cells in a sophisticated manner (von Wintersdorff et al., 2016). This is the most likely mechanism of AR genes because it provides a more secure mode of transmission from the environment and is more efficient than transformation (Cabezon et al., 2017; von Wintersdorff et al., 2016). For example, the small plasmid pBP1 is disseminated worldwide and confers resistance to 30 % of all streptomycin-resistant *E. coli* (Wiedemann, 1983). This plasmid is found in the human bowel flora and was classified as an ecologically adapted source of resistance (van Treeck et al., 1981; Wiedemann, 1983). Studies are currently being carried out whereby conjugation inhibition is being considered to reduce the conference of antibiotic resistance genes amongst bacterial species (Cabezon et al., 2017; Lopatkin et al., 2017).

The transformation of genetic material by bacterial cells involves the uptake of extracellular DNA from the environment, followed by its integration into the recipient's genome (Prudhomme et al., 2006; von Wintersdorff et al., 2016). Recircularization of plasmid DNA is necessary for integration into bacterial cells when obtained from the environment (von Wintersdorff et al., 2016). Strikingly, studies have highlighted that exposure to antibiotics can improve the competence of bacteria to not only select for resistant strains but also to promote the transformation of AR genes (Charpentier et al., 2011; von Wintersdorff et al., 2016).

While bacteria can siphon genetic material from the environment using pili and adhesins, bacteriophages can also play a critical role in supplying AR genes through specialized and generalized transduction (Colavecchio et al., 2017; Torres-Barceló, 2018). Bacteriophages can mobilize AR genes, and this has been observed whereby they encode β-lactamases found in *E. coli* and act as carriers for the transfer of antibiotic-resistant plasmids in methicillin-resistant *Staphylococcus aureus* (von Wintersdorff et al., 2016). Some bacteriophages are reported to have a broad host range that varies between species, thereby increasing the range of bacteria impacted by this form of AR dissemination (von Wintersdorff et al., 2016).

Currently, research focuses on these AR dissemination mechanisms and possible methods that can be employed to combat the increasing magnitude of AR. Human pathogens continue to present with AR, which threatens public health on a global scale. The dissemination of resistance genes and human activities exacerbate global AR at unprecedented rates. As the

demand for antibiotics increases, the search for said therapeutics increases, with interests focused on alternative methods that do not include discovering *de novo* drugs.

1.4. Mainstream approaches for finding new antibiotics

Current drug development ventures employed by pharmaceutical companies are incapable of supplying the healthcare sector with novel antibiotics; thus, there is a need for alternative approaches to meet demands on a global scale. For this reason, several approaches are explored for their utility in curbing the current stagnation in antibiotic discovery and the increased emergence of antibiotic resistance. Two examples investigated in this study include the revival and repurposing of "old" drugs and the use of antivirulent agents.

Drug repurposing approaches have the potential to provide therapies in a fraction of the time, and reviving "old" antibiotics is one of the most promising tactics. In this instance, "old" antibiotics refer to those developed long ago and abandoned due to concerns over their toxicity in human and veterinary medicine. The resistance prevalence of these antibiotics is extremely low, thus making them good candidates for drug reprofiling (Pushpakom et al., 2019). Using "old" drugs remains an attractive proposition because it involves using de-risked compounds with potentially shorter development timelines and costs since the drug is sufficiently safe to warrant its use in human and veterinary medicine (Pushpakom et al., 2019). Drug reprofiling is an opportunistic strategy that uses approved or investigational drugs such as nitrofurans and colistin (Pushpakom et al., 2019). The main benefit of using this drug discovery method is the reduced likelihood of failure. Where the risk of toxicity is a concern, molecular modifications of antibiotics such as nitrofurans can reduce side effects such as nephrotoxicity and toxic metabolite production in mammalian tissues (Pushpakom et al., 2019). Drug reprofiling also has the potential to inhibit virulence factors despite being non-inhibitory to the growth of some pathogens.

The decline in the commercial development of new antibiotics has created a window of opportunity to utilize antivirulence agents. These agents inhibit the production of virulence factors that promote disease pathogenesis but are neither bacteriostatic nor bactericidal to the pathogen (Khodaverdian et al., 2013). Virulence factors increase bacterial formidability, which favors disease progression. In most instances, antivirulent agents are non-inhibitory to bacterial survival; thus, it is feasible that resistance to these agents will not meaningfully impede drug

efficacy in the same manner as bactericidal antibiotics. The logic behind utilizing antivirulent compounds is that selective pressures between the pathogen and host are created, thereby increasing the clearance of the pathogen by innate immunity (Buroni & Chiarelli, 2020). Hampering the virulence phenotypes of pathogens such as *P. aeruginosa* in diseased CF lungs creates an avenue of an increased likelihood of clearance by the innate immune system (Totsika, 2017). Currently, several antivirulence agents are being investigated for their utility in reducing infection progression (Baldelli et al., 2020; Khodaverdian et al., 2013). The potential for using nitrofuran antibiotics and diflunisal as antivirulence compounds was studied, and these agents were found to be capable of inhibiting *P. aeruginosa* and *Staphylococcus aureus,* respectively (Baldelli et al., 2020; Khodaverdian et al., 2013). Interestingly, these antivirulence compounds were both found to inhibit the quorum sensing regulon in these pathogens, which regulates many virulence phenotypes and concomitantly increases bacterial pathogenicity (Chen et al., 2022; Khodaverdian et al., 2013).

There are other ways in which new antibiotic regimes can be developed to combat the upsurge in antibiotic resistance. Firstly, combination therapies can employ old antibiotics to synergistically elicit a bactericidal effect on the pathogen (Le et al., 2020). An *in vitro* study using a combination therapy of furazolidone and sodium deoxycholate offers potential as an antibiotic therapy to treat Gram-negative bacteria (Le et al., 2020). Phage therapy is another method of renewed interest since the decline in the effectiveness of antibiotics. Bioengineered phages and lytic phage proteins show utility as treatment options for MDR pathogens (Lin et al., 2017). Phage therapy also has the potential to be supplemented with antibiotics for infections that are difficult to treat (Lin et al., 2017). These putative methods are being investigated for their utility in treating formidable Gram-negative bacteria to circumvent the dwindling effectiveness of antibiotics.

1.5. Introduction of 5-nitrofuran antibiotics

Synthetic “old” 5-nitrofurans are a class of pro-drug antibiotics initially discovered in the 1950s and used for treating diseases ranging from uUTIs to cancer (Zuma et al., 2019). The defining structure of these pro-drugs is the furan ring attached to the nitro groups. Pro-drugs are biologically inert compounds that must be converted into pharmacologically active compounds after metabolic activity (Wu, 2009). In *E. coli,* nitrofuran-reducing enzymes facilitate pro-drug

activation: the nitroreductases NfsA, NfsB, and AhpF (Le et al., 2019). The utility and popularity of some pro-drugs have since declined, despite the low emergence of antibiotic resistance due to nephrotoxicity and toxic metabolite detection, especially in food products such as livestock and aquaculture ("Scientific Opinion on nitrofurans and their metabolites in food," 2015). Several jurisdictions, including the European Union, Canada, and The United States of America, have banned some of these drugs in food production but continue their use in human medicine (Zuma et al., 2019). The discovery of *de novo* and less toxic antibiotics also reduced the need for 5-nitrofuran antibiotics in healthcare, except when used as last resort drugs due to failed treatments stemming from AR (Bongers et al., 2021). Nonetheless, this class of antibiotics contains three pro-drugs: furazolidone, nitrofurantoin, and nitrofurazone, which are offered as treatment options for microbial infections in the European Union, the US, Canada, and Brazil ("Scientific Opinion on nitrofurans and their metabolites in food," 2015; Zuma et al., 2019).

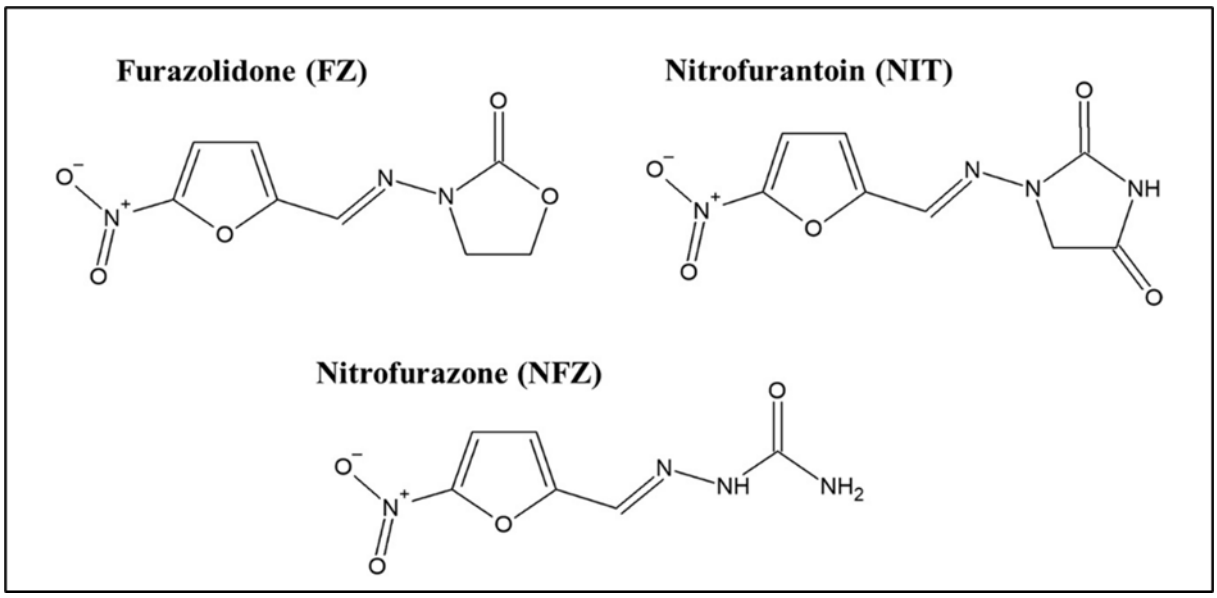

Figure 1. Molecular structures of 5-nitrofuran antibiotics used in this study.

This figure was taken with permission from Le et al. (2019).

1.6. Introduction of nitrofurantoin

Nitrofurantoin is a first-line antibiotic commonly used to treat uUTIs (Komp Lindgren et al., 2015). Despite its approval and widespread use in human medicine over the past 60 years, nitrofurantoin remains one of the most effective antibiotics for treating *E. coli*. The continued general use is primarily because the rate of microbial resistance to the drug is low. Comparably there is a reassessment of utilizing more recently discovered antibiotics such as β-lactams as first-line therapy options for treating uUTIs because of increased antibiotic resistance and the evolution of ESBLs (Komp Lindgren et al., 2015). When taken orally, nitrofurantoin reaches optimum therapeutic concentrations in the lower urinary tract and does not cause dysbiosis of the gastrointestinal tract (Squadrito & del Portal, 2022). The exact mechanism of the bactericidal effect of nitrofurantoin is not entirely understood; however, researchers suggest that nitroreductases reduce the nitro group of the compound to activate the reactive intermediates. The intermediates then bind to the ribosomes, affecting the production of DNA, RNA, and other metabolic enzymes (Komp Lindgren et al., 2015). The mechanism of this antibiotic is unique, mainly because the drug is metabolized and then activated to infer a bactericidal effect on the pathogen.

1.7. Introduction of furazolidone

Furazolidone is a broad-spectrum nitrofuran antibiotic used for treating diarrhea (Zhang et al., 2022). Despite its utility in treating *Helicobacter pylori* and *E. coli* infections, the use of furazolidone is considerably diminished because of toxic metabolites formation once nitroreductases reduce and activate the prodrug (De Angelis et al., 1999; Zhang et al., 2022). Consequently, furazolidone was written off as an outdated clinical drug as the primary producers halted production, and eventually, the drug became unavailable in the western world (Mohammadi et al., 2017). Ultimately, the leading producer rescinded its Food and Drug Administration approval to occupy the market because of the decrease in drug popularity and the potential adverse effects in both human and animal healthcare (Mohammadi et al., 2017).

1.8. Introduction of nitrofurazone

Nitrofurazone is a broad-spectrum, nitroaromatic antibiotic used as a topical treatment for burns and skin infections due to skin grafts (Ryan et al., 2011). Like other 5-nitrofurans, nitrofurazone is a potent antibiotic activated by nitroreductases in bacterial cells. In *E. coli*, the mechanism of nitroreduction is carried out by the genes nfsA and nfsB and the recently characterized ahpF (Le et al., 2019; Ryan et al., 2011). *P. aeruginosa*, however, has a group of enzymes known as azoreductases, which are homologs that perform a similar function of reducing 5-nitrofuran antibiotics (Crescente et al., 2016). Several bacterial species in the human gut contain azoreductases; a group of NAD(P)H-dependent flavoenzymes of great utility in drug metabolism in *P. aeruginosa* and *E. coli* (Crescente et al., 2016; Ryan et al., 2011). Three azoreductases, paAzoR1, paAzoR2, and paAzoR3, were recently characterized in *P. aeruginosa*, wherein the mechanism of nitroreduction was identified, and results suggest that they might be capable of reducing nitrofurazone (Crescente et al., 2016).

1.9. General Hypotheses and Aims

There is an unprecedented need for additional antibiotics on the global market as antibiotic resistance rates continue to increase exponentially. Infections that were once easily treated are causing an increase in mortality and morbidity worldwide, especially those caused by Gram-negative pathogens. In addition, the current cost of treating Gram-negative infections has increased globally since more rigorous treatment regimens are needed to hamper disease progression and limit mortality rates. As the treatment options dwindle, the drug discovery pipeline cannot sustain the demand for novel antibiotics. The quickest way to regain the ability to treat infections with MDR Gram-negative bacteria is to revive “old” antibiotics to which there is not much resistance. One such group of antibiotics, 5-nitrofurans, can be used to treat Gram-negative infections by inhibiting bacterial growth or virulence, thereby slowing pathogenesis.

Gram-negative bacteria have surprisingly made headway in procuring resistance to modern antibiotics such as β-lactams, causing clinicians to revert to using 5-nitrofurans. 5-nitrofurans are “old” antibiotics currently being investigated and repurposed for treating Gram-negative

infections in human medicine. Despite being in existence since the 1950s, a low resistance rate in Gram-negative bacteria towards 5-nitrofurans warrants investigation into their utility in an already waning market of antibiotics.

Chapter 2- Search for novel nitrofuran-activating enzymes in *Escherichia coli*

2.1. Introduction of *Escherichia coli*

Escherichia coli is the most prevalent bacterial species found within the gastrointestinal tract of mammals (Martinson & Walk, 2020). This commensal organism is often harmless but medically significant as the aetiological agent of many diseases. On most occasions, *E. coli* passes through the gastrointestinal tract of humans without causing disease; however, in some instances, this bacterium can be pathogenic and result in the development of diseases such as urinary tract infections, gastrointestinal conditions, and neonatal meningitis (Aguilar-Santelises et al., 2020; Hirakawa et al., 2021). This pathogen can be difficult to treat because of many modes of antibiotic resistance acquired through vertical and horizontal gene transfer and intrinsic properties such as membrane permeability, which impedes the movement of some antibiotics into the cell (Oliveira & Reygaert, 2021; Pacios et al., 2020). These characteristics make *E. coli* a very formidable pathogen in the clinical setting and very difficult to treat, given the limited antibiotic options present on the market. The frivolous misuse of antibiotics has exacerbated the dissemination of AR in bacterial populations in human medicine (Lehman & Grabowicz, 2019).

2.2. Clinical relevance of *E. coli* and current problems faced in the hospital setting

Antibiotic-resistant *E. coli* poses a severe threat whereby it is more costly to treat patients with infections from strains that are resistant to many classes of antibiotics (Kettani Halabi et al., 2021). In the hospital setting, *E. coli* is known for causing many infections, of which urinary tract infections are one of the most common in geriatric patients, patients with medically implanted catheters, and pregnant women (Abou-Dobara et al., 2010; Chagneau et al., 2021). Persistent relapses of MDR bacterial infections pose serious health risks and could substantially reduce the life expectancy of individuals with nosocomial and community-acquired conditions.

Globally, *E. coli* has an increasing trend in drug resistance to clinically relevant antibiotic classes (Nascimento et al., 2021). The highest antibiotic resistance rates are in Asian and African countries (Kang & Song, 2013; Thompson, 2022). In contrast, European countries such as Sweden and Denmark have the lowest, a consequence of the banned use of growth-promoting antibiotics in animal husbandry (Ibrahim et al., 2012; Kornfalt Isberg et al., 2019). Despite several countries enacting laws to limit the liberal use of antibiotics, the upward trend in AMR continues globally, a cause for concern given the increase in infections and the costs associated with these infections annually. Last-resort drugs are deployed in dire situations that lower drug efficacy and fuel antibiotic resistance, as seen in MDR pathogens (Dadashi et al., 2021).

2.3. Resistance mechanisms utilized by *E. coli* to subvert the effects of antibiotics

E. coli is one of the most problematic pathogens in modern-day history, primarily due to the evolution of many resistance mechanisms. Such mechanisms include degrading enzymes that render drugs ineffective upon entry into the cells. One mechanism denoted as active defenses described by Li et al. (2019) involves procuring active protective measures such as increased expressions of drug efflux pumps to protect bacterial cells from antibiotics. Another active defense mechanism employs outer membrane vesicles in some bacterial species, which confers protection from membrane-active antibiotics such as ampicillin (Kulkarni et al., 2015; Lee et al., 2007). *E. coli* also harbor the ability to carry integrons pertinent to the evolution of bacterial genomes via the acquisition of AR genes through horizontal gene transfer (Rowe-Magnus & Mazel, 2002). These structures ensure that multidrug-resistant genes are inherited by progeny and increase with exposure to different classes of antibiotics (Farahat et al., 2021). Selective pressures have influenced these genetic adaptations, all derived from the haphazard usage of antibiotics (Rowe-Magnus & Mazel, 2002).

2.4. Epidemiology of uncomplicated urinary tract infections caused by *E. coli*

Previous reports and investigations identified *E. coli* as the most common cause of uncomplicated urinary tract infections (uUTIs). Approximately 80 % of all globally reported cases are caused by this pathogen (Huang et al., 2022; Klein & Hultgren, 2020). The individual risks associated with uUTIs depend on various factors such as age, gender, and comorbidities; however, different lifestyles could increase the likelihood of this condition (Klein & Hultgren, 2020). Non-pregnant women often present with uUTIs, whereby bacteria colonize the bladder mucosa and remain confined to the lower part of the urinary tract (Baerheim, 2001; Flores-Mireles et al., 2015). Frequent micturition and dysuria are common symptoms associated with uUTIs, also known as uncomplicated cystitis (Czajkowski et al., 2021). In women, the contamination of the periurethral surface because of its proximity to the rectum and an intrinsically short urethra further exacerbates the likelihood of this condition (Baerheim, 2001). *E. coli* often ingress into the urethra and eventually locomotes into the bladder, where colonization and uncomplicated cystitis occurs (Flores-Mireles et al., 2015). Localized infections of the lower bladder are among the most frequent outpatient illnesses reported, with approximately 150 million individuals being affected annually (Chagneau et al., 2021). Despite the high number of reported cases, the prevalence of uUTIs remains unknown because the condition is often under-reported and requires no antibiotic treatment in otherwise healthy individuals (Nordstrom et al., 2013). Usually, the first-choice antibiotics for uUTIs are trimethoprim or nitrofurantoin (Schmiemann et al., 2012; Wright et al., 1999). However, many patients opt for self-treatments using alternative approaches to the conventional methods used for uncomplicated cystitis by incorporating natural remedies into their diets, such as cranberry juice, garlic, and horseradish (Das, 2020; Foxman & Buxton, 2013). The under-reported cases of uUTIs challenge clinicians because the strains of *E. coli* responsible for the illness might resist conventional treatments such as beta-lactams (Foxman & Buxton, 2013; Kettani Halabi et al., 2021). Moreover, disease progression because of delayed treatment and prognosis might lead to bacterial ingression into the kidneys and other parts of the urinary system (Flores-Mireles et al., 2015). In this stage of disease progression, changes in conventional therapeutics are necessary to reduce the likelihood of damage to the bladder mucosa and other severe conditions such as renal failure (Flores-Mireles et al., 2015; Foxman & Buxton, 2013).

Despite the inability to pinpoint the incidence of uUTIs globally, data collection can highlight the hotspots wherein the prevalence is of particular concern. Developing and third-world countries are hotspots from which antibiotic-resistant *E. coli* originate and spread globally through migration and international travel. Deemed as multifaceted by Bokhary et al. (2021), AR involves the movement of multiple links, such as humans and animals, and plays an inherently important role in spreading global AR. One transmission mode involves medical tourism, in which sick individuals travel to other countries for affordable healthcare (Langford & Schwartz, 2018). Patients seeking medical attention outside their home countries are usually gravely ill and most likely to harbor AR pathogens, primarily if they originate from developing and third-world countries (Bokhary et al., 2021). Medical tourism reportedly cost approximately USD 37 billion globally in 2019, with 14 million Americans traveling annually to Canada for medical assistance (Johnston et al., 2011; Langford & Schwartz, 2018). Despite the usefulness of medical tourism in procuring treatment for non-emergency healthcare, there is a risk of disease transmission and the dissemination of AR bacteria by the visiting patients (Johnston et al., 2011). Historically, globalization is a known contributor to the movement of AR and disease dissemination. Screening for certain illnesses is often required before entering some territories, such as New Zealand. The most notable screening involves chest x-rays for tuberculosis (Alvarez et al., 2011). While this effectively controls the entry of some diseases and bacteria, others deemed less pathogenic are overlooked, despite having the status as multidrug-resistant. Identifying and controlling pathogens such as *E. coli* often go unchecked compared to more challenging pathogens such as *Mycobacterium tuberculosis*. Nonetheless, the growing incidence of resistant *E. coli* infections and the lack of novel antibiotics highlight the urgency of discovering *de novo* antibiotics. It is imperative that measures are implemented to mitigate the transmission of non-traditional MDR bacteria whilst *de novo* treatment methods are discovered.

2.5. Addressing increased antibiotic resistance in *E. coli*

The unprecedented increase in AR has garnered the attention of clinicians worldwide. Many antibiotics were once convincingly capable of reducing morbidity and mortality rates in the clinical environment because of their role in disease management. Currently, *E. coli* is one of the most problematic pathogens for which previously effective antibiotics no longer work in

managing disease and infection. The effectiveness and lethality of first-line antibiotics used for treating Gram-negative infections have diminished due to AR. Of particular concern is the reduced efficacy of β-lactam antibiotics for treating *E. coli* infections (Kettani Halabi et al., 2021). The cause for alarm and a dire need for alternative antibiotics results from the discovery of extended-spectrum beta-lactamase (ESBL) enzymes capable of hydrolyzing the active ingredient in β-lactam antibiotics (Livermore, 1995). The advent and use of new antibiotics exert selective pressures on bacteria such as *E. coli*, resulting in the selection of new variants of β-lactamases specific to the newly developed drugs within this particular antibiotic class (Bradford, 2001). ESBLs inactivate β-lactam antibiotics and render the drugs ineffective against Gram-negative bacilli such as *E. coli* (Kettani Halabi et al., 2021). ESBL-producing bacteria such as *E. coli* can hydrolyze third-generation cephalosporins and resist other antibiotic classes (Kallen et al., 2006). As such, the collective consensus for drug discovery is imminent, and repurposing old antibiotics might prove to be the quickest and most cost-effective method of quickly circumventing the adaptive nature of *E. coli* towards first-line antibiotics.

2.6. Hypothesis and Aims- *E. coli* experiments

Based on previous experiments by Le et al. (2019), AhpF was found to be an additional nitroreductase responsible for activating 5-nitrofurans. However, the antibacterial activity of furazolidone (FZ) was still found to be significant after the deletion of this gene in the *ΔnfsA ΔnfsB E. coli* mutant (Le et al., 2019).

Therefore, I hypothesized that novel and unidentified oxidoreductases were responsible for substantial antibacterial activity in the triple mutant *ΔnfsA ΔnfsB ΔahpF E. coli*. As such, 18 essential genes encoding oxidoreductases in *E. coli* were identified based on genomic analyses and overexpressed in the *ΔnfsA ΔnfsB ΔahpF* triple mutant to produce newly complemented strains. MIC analyses were then carried out to determine whether any of the potential candidate genes resulted in increased sensitivity (decrease of MIC) to 5-nitrofurans. By identifying the oxidoreductases that activate 5-nitrofurans, I would be able to explain the mechanism behind the antibacterial activity of nitrofurans in the absence of the three known activating enzymes, NfsA, NfsB, and AhpF.

Chapter 3- Investigations of the effect of "old" nitrofurans on antivirulence properties in *Pseudomonas aeruginosa* PAO1

3.1. Introduction of *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a gram-negative bacterium of major concern on the WHO list of critical organisms for which antibiotics are urgently needed (Bhatia et al., 2021). It is an opportunistic and ubiquitous pathogen that exhibits several virulence phenotypes after colonization of the sessile, planktonic cell into rigid aggregates called biofilms. The sessile biofilm growth stage of this formidable pathogen is often challenging to treat, and highly resistant strains can evade the effects of several classes of antibiotics (Schleheck et al., 2009; Valentini & Filloux, 2016; Wijesinghe et al., 2019). *P. aeruginosa* is known to thrive in immunocompromised individuals such as cystic fibrosis patients with lung infections and chronic wound victims, including those harboring burn wounds and diabetic ulcers (Batoni et al., 2021; Bhatia et al., 2021). *P. aeruginosa* can cause several infections throughout bodily systems and is often responsible for increased morbidity and obstinate infections, as seen in cystic fibrosis patients (Reece et al., 2021). Despite the approval of a few classes of antibiotics in the past decade, trends show an unprecedented increase in resistance in this pathogen, which will eventually overcome the bactericidal effect of current antibiotics on the market (Ibrahim et al., 2020; Reza et al., 2019). The prevalence and growing incidence of MDR *P. aeruginosa* infections have not been parallel to novel and suitable antibiotic discoveries required to treat diseases caused by this pathogen (Siwakoti et al., 2018). Some therapeutics are still valuable for treating pseudomonal infections globally; however, clinical trends indicate these drugs' waning utility.

3.2. Clinical relevance of *P. aeruginosa* and current problems faced in the hospital setting

Classed as one of the most notorious human pathogens, *P. aeruginosa* is highly adaptable and capable of colonizing extremely harsh environments and mammalian tissues, often in immunosuppressed patients (Acosta et al., 2020; Atkin et al., 2018; Baldelli et al., 2020). *P. aeruginosa* is one of the most difficult-to-treat pathogens in healthcare settings because its various intrinsic properties enable it to bypass the effects of different classes of antibiotics (Reza et al., 2019). The main intrinsic property involves the formation of aggregates of planktonic bacterial cells to create a highly resistant biofilm whereby toxic metabolites and enzymes protect and support the bacterial niche (Baldelli et al., 2020). Moreover, increased levels of antibiotic resistance and natural evolution mechanisms enable *P. aeruginosa* to nullify the waning choices of antibiotics currently on the market. *P. aeruginosa* is relevant because of its devastating effect on cystic fibrosis (CF) patients and the accelerated decline of the lung function of individuals diagnosed with this genetic disorder (Webb et al., 2019). Recalcitrant medical lesions often caused by *P. aeruginosa* are characteristic of burn wound victims in the hospital setting. These bacterial aggregates remain persistent in mammalian tissues by inhibiting antibiotic and sterilant infiltration (Reza et al., 2019). These infections are often fulminant, and the increase in AR within bacterial species further exacerbates the recovery period in individuals with chronic wounds of this nature. Research has described CF lungs as chronically inflamed and slow in response to antibiotic treatment (Bhagirath et al., 2016). Treatment is often complex because multiple factors allow this pathogen to bypass current antibiotic regimes. Presently, studies aim to procure novel antivirulence compounds, which, despite being ineffective at killing *P. aeruginosa* cells, could limit the virulence phenotypes and lessen the severity of infections.

3.3. Mode of resistance to antibiotics used by *P. aeruginosa*

Multidrug-resistant *P. aeruginosa* is remarkably equipped to resist antibiotics in healthcare due to many virulence phenotypes (Pang et al., 2019). On a global scale, the incidence of untreatable chronic infections caused by *P. aeruginosa* in CF and chronic wound patients is a cause for concern. *P. aeruginosa* has been studied extensively and employs both intrinsic and

acquired virulence phenotypes to circumvent the effects of many classes of antibiotics (Alonso et al., 2020). Lung disease remains the highest cause of morbidity and mortality in CF patients, and *P. aeruginosa* is often the dominant pathogen in approximately 80 % of all CF patients (Moreau-Marquis et al., 2008). The lungs of CF patients provide an ideal environment that selects for slow-growing *P. aeruginosa* hypermutators which acquire beneficial traits and elevated fitness peaks against antibiotics (Bhagirath et al., 2016; La Rosa et al., 2021). This form of adaptive evolution emulated by *P. aeruginosa* is studied extensively, resulting in the discovery of elevated virulence phenotypes such as persistence, metabolic adaptation, and upregulation of preexisting virulence phenotypes needed for the incessant colonization of the CF lung (La Rosa et al., 2021; Moreau-Marquis et al., 2008). The biofilm structural aggregates of *P. aeruginosa* remain one of the most rigid and challenging-to-eradicate once established in the lungs (Moreau-Marquis et al., 2008). The inherent pulmonary environment of CF patients provides the ideal niche required for this pathogen to flourish into organized biofilms despite the lungs being deemed a harsh environment with little nutrition and constant surveillance by cells of the immune system (Lin & Kazmierczak, 2017; Schleheck et al., 2009). Interestingly, despite the low concentration of nutrition present in the lungs, the persistent inflammation of the lungs and byproducts of the immune system cells of the pulmonary airways provide *P. aeruginosa* with growth factors needed to flourish in the cystic fibrosis lung (Lin & Kazmierczak, 2017; Reece et al., 2021). This cascade of events switches on molecular components and genes that illicit a stronger immune response, often resulting in chronic inflammation of the lungs (Huffnagle et al., 2017; Lin & Kazmierczak, 2017).

3.4. Relevance of *P. aeruginosa* in cystic fibrosis lung infections

P. aeruginosa causes pulmonary infections in CF patients, often involving multiple concatenated factors, recalcitrant and persistent diseases, and increased mortality rates (Acosta et al., 2020; Adler & Liou, 2016). Globally, approximately 100 000 persons are diagnosed with CF, of which the majority are of Caucasian descent (Adler & Liou, 2016; Reece et al., 2021; Scotet et al., 2020). CF is a condition in which there are mutations of the cystic fibrosis transmembrane conductance regulator (CFTR) gene, often causing a disruption in the transport of chloride ions across the mucosal cell membrane channels (Hubble et al., 2019; Reece et al., 2021). Lung function remains hampered throughout the entire lifespan of CF patients

chronically infected with *P. aeruginosa* due to severely compromised lung architecture (Lin & Kazmierczak, 2017). Research has identified over 2000 CFTR gene mutations; however, 127 affect cellular function and phenotype, disrupt regular mucous membranes, and each range in disease severity (Veit et al., 2016). Five classes of CFTR gene mutations are recognized based on the nature of disruption to the CFTR (Rowntree & Harris, 2003). Most CF patients are diagnosed with the condition from a young age, with a life expectancy of 50 years old (Scotet et al., 2020). Previous reports identified this condition as one of infancy since mortality rates were extremely high; however, many CF patients have increased life expectancies in recent years (Stephenson et al., 2015). CF patients' incidence rate and prevalence have recently declined due to advancements in neonatal screening and treatment modalities (Bhagirath et al., 2016; Ratjen et al., 2009; Sawicki et al., 2013). Despite the staggering decrease in CF case numbers, multidrug-resistant strains of pulmonary pathogens and more complex genetic variants of the autosomal recessive trait complicates the disease complexity (Bhagirath et al., 2016). Disease complexity is exacerbated when accumulated sticky mucus traps dirt and promotes biofilm formation in the lungs, causing the impairment of mucociliary action secondary to hyperviscous mucus accumulation (Acosta et al., 2020; Bhagirath et al., 2016). Thick and sticky mucus is characteristic of CF lungs, which acts as a nidus in which opportunistic pathogens such as *P. aeruginosa* colonize airways and hinder clearance of the lungs once chronic colonization is established (Acosta et al., 2020). Moreover, *P. aeruginosa* creates dysbiosis in the pulmonary environment, thereby promoting its self-proliferation by outcompeting other pathogens that might be present (Adler & Liou, 2016; Reece et al., 2021).

The virulent nature of *P. aeruginosa* often requires antibiotic combination therapy to treat lung infections, frequently resulting in prolonged exposure to drugs that promote adaptive resistance as a natural evolutionary mechanism (Hubble et al., 2019). Consequently, pulmonary airway inflammation occurs, and lung airways collapse if left untreated. Most CF patients endure persistent lung infections because of dormant *P. aeruginosa* cells that can evade treatment regimes because of robust virulence factors (Reece et al., 2021). The multifaceted and highly adaptable nature of *P. aeruginosa* allows this pathogen to flourish in environments that are often inhospitable to others. Some studies have suggested that *P. aeruginosa* can remain in the airways of CF patients for up to 30 years while concomitantly altering its virulence phenotypes to withstand the effects of antibiotic regimens (La Rosa et al., 2021; Reece et al., 2021). Chronic lung colonization by *P. aeruginosa* markedly reduces the lifespan of CF patients by approximately ten years and shows the importance of discovering new treatment regimens

(Durda-Masny et al., 2021; Hubble et al., 2019; Reece et al., 2021). Pseudomonal infections undermine many medical and clinical advancements within immunocompromised patients, fuelling disproportionate disease progression, quality of life, and cost of treatment globally.

3.5. Relevance of *P. aeruginosa* in chronic wound infections

P. aeruginosa infections are also notoriously difficult to treat in chronic wounds (Frykberg & Banks, 2015). Chronic wounds are any wound that persists after a month of prolonged treatment and standard care (Frykberg & Banks, 2015). These persistent injuries are often found in the lower extremities in humans and can be secondary to poor circulation, diabetes, pressure ulcers, neuropathy, and arterial disease (Frykberg & Banks, 2015; Ji et al., 2014; Szczepanowski et al., 2022). Burn victims can also present with wounds confined to other parts of the body, depending on the area of impact (Sun et al., 2008; Szczepanowski et al., 2022). Elderly patients often present with chronic wounds secondary to medical predispositions or non-communicable diseases; however, persons of all ages may present with similar injuries in the clinical setting. *P. aeruginosa* often hampers the epithelial integrity in chronic wound victims and, in so doing, reduces the likelihood of speedy recoveries, especially in immunocompromised patients (Ruffin & Brochiero, 2019). *P. aeruginosa* also employs multiple mechanisms to combat and evade host immune responses and antibiotic treatment regimens in chronic wound patients (Frykberg & Banks, 2015). The epithelial layers of the skin have an increased propensity to infections by *P. aeruginosa*, with breaches of the skin providing an access point to nutrients (Ruffin & Brochiero, 2019). In chronic wounds, *P. aeruginosa* possesses structural components such as the polar flagellum, type IV pilus, and lipopolysaccharides as a means of movement via twitching, bacterial adhesion to epithelial cells and tissues, and a trigger for the human immune response, respectively (Huszczynski et al., 2019). The large 6.3 million base pairs genome of *P. aeruginosa* is critical in its pathogenicity as it encodes for a cell density-dependent quorum sensing (QS) regulon that regulates pigment production and biofilm architecture (Stover et al., 2000; Winstanley & Fothergill, 2009). These virulence phenotypes play a critical role in the colonization and persistence of the bacteria in chronic wounds (Winstanley & Fothergill, 2009). Diabetic foot ulcers are classic examples of a complicated pathological state whereby inadequate treatment of the pathogen can result in amputations (Ji et al., 2014).

3.6. Clinically relevant antibiotics used in *P. aeruginosa* infections

Since the 1980s, empirical combination therapies containing aminoglycosides have been the gold standard treatment options for pseudomonal infections of chronic wounds and chronic lung infections of CF patients (Poole, 2005). Pharmacodynamics convincingly demonstrates that aminoglycosides bind to the negatively charged lipopolysaccharides of the gram-negative bacterial cell membranes before entering the cell cytosol to disrupt protein synthesis (Bulitta et al., 2015; Poole, 2005). This bactericidal effect involves the antibiotic binding to the 30S ribosomal subunits (Ratjen et al., 2009; Schurek et al., 2008). Monotherapies such as tobramycin, gentamycin, and amikacin are the first-line treatment options that utilize protein synthesis interference to remedy pseudomonal infections (Pang et al., 2019; Ratjen et al., 2009). Schurek et al. (2008) have suggested that tobramycin elicits a pleiotropic effect on bacterial cells that causes misreadings during translation and concurrently disrupts protein synthesis. The compartmentalized structure of the lungs is difficult to reach via intravenous drug administration; thus, nebulization of antibiotics such as tobramycin into infected lungs of CF patients ensures precision in delivery while averting exposure to undesired systems (Bulitta et al., 2015; Ratjen et al., 2009; Thorn et al., 2021). The challenge of treating MDR *P. aeruginosa* using monotherapies is averted by using combination therapies to suppress physiologically compromised CF lungs.

In recent years, the outlook for treating *P. aeruginosa* infections using aminoglycosides has changed because clinicians recognize that the highly versatile genome may confer resistance to this class of antibiotics in the near future (Tümmler, 2019). Aminoglycosides elicit several bacterial cell effects; however, an increasing global trend shows the emergence of antibiotic resistance within this drug class, with worrisome cases presenting in CF patients treated with tobramycin (Halfon et al., 2019; La Rosa et al., 2021). In a study conducted as early as 1996, researchers found that aminoglycoside antibiotics elicit adaptive resistance in *P. aeruginosa* (Barclay et al., 1996). Since then, several studies have confirmed that resistance to tobramycin is slowly disseminating throughout the pseudomonal genus (Barclay et al., 1996; Valenza et al., 2010). The large and versatile mutational resistome of *P. aeruginosa* offsets chromosomal mutations within the bacterial genome and facilitates the striking ability to withstand the

bactericidal effects of many classes of antipseudomonal drugs (Cortes-Lara et al., 2021; Lopez-Causape et al., 2018; Martin et al., 2018).

Other categories of drugs have convincingly demonstrated effectiveness in treating pseudomonal infections. Table 1 highlights some mainstream antibiotics currently used for treating *P. aeruginosa* infections in immunosuppressed individuals. Three other drug classes, cephalosporins, polymyxins, and beta-lactams, are useful therapeutics for chronic *P. aeruginosa* diseases. One member of the polymyxin group is colistin, a last-resort drug used in individuals with MDR *P. aeruginosa* (Azimi & Lari, 2019). The nephrotoxicity and neurotoxicity exerted on patients post-colistin administration renders it a last-resort choice in healthcare (Azimi & Lari, 2019; Zhang et al., 2021). Despite identifying a few MDR *P. aeruginosa* to colistin, recent research has shown that resistance to this drug is concomitant with poor biofilm production, a virulence phenotype that directly coincides with increased resistance and gravely affects the lethality of antibiotics (Azimi & Lari, 2019). As such, there is potential in using synergistic combination therapies with colistin for managing MDR strains. Often, chronically infected pulmonary airways of CF patients harbor heterogeneity of *P. aeruginosa* variants (Bhagirath et al., 2016; Poole, 2005). The emergence of these MDR strains has been documented and isolated since the 1970s, and their adaptive resistance continues to undermine several classes of antibiotics (Bassetti et al., 2018; Poole, 2005).

3.7. Virulence factors that are quintessential in *P. aeruginosa* colonization

3.7.1. Motility

Despite having a single polar flagellum, *P. aeruginosa* can propagate through aqueous solutions of differing viscosities via three mechanisms, two of which utilize other cellular appendages for movement (Kohler et al., 2000). The type IV pilus facilitates twitching motility in this pathogen (Kohler et al., 2000). CF lungs are characterized by increased mucus viscoelasticity due to decreased water availability, accumulated cell debris, DNA, and other mucosal components (Rubin & Thornton, 2018; Tomaiuolo et al., 2014). Mucostasis is a permanent condition of the lungs of CF patients in which sputum viscosity is $14 – 110 \times 10^3$ cP compared to healthy lungs where sputum viscosity is $12 – 15 \times 10^3$ cP (Hill et al., 2018; Lai et

al., 2009). The permanence of the environment within CF lungs does not limit chronic colonization of *P. aeruginosa* since three modes of locomotion can be used to maneuver through the pulmonary airways. The three types of locomotion identified are twitching, swarming, and swimming, and the movement of choice often depends on the viscosity of the mucus within the CF lung (Kohler et al., 2000; Vallet et al., 2004). In chronic wounds, locomotion is a phenotypic cue essential for the chronic colonization of *P. aeruginosa* cells as they penetrate the fascia and subcutaneous layers of bodily tissues (Kim et al., 2015). Once planktonic cells settle in nutrient-rich tissues, cell density cues in *P. aeruginosa* switch gene expressions often activated from the quorum-sensing regulon to initiate biofilm formation (Vallet et al., 2004). This organized community of cells is protected from external influences such as antibiotics and sterilants and decreases the susceptibility of individual cells to a much higher magnitude (De Kievit, 2009).

Table 1. Antibiotics relevant in treating P. aeruginosa infections.

Antibiotic	Class of drug	Mechanism of action	Genes responsible for resistance	Source
Colistin	Polymyxin	Inner membrane lysis and leakage of cell cytosol	Plasmid-mediated *mcr-1*	(El-Sayed Ahmed et al., 2020)
Ceftazidime	Cephalosporin	Perforation of the cell wall by inhibiting enzymes responsible for cell wall synthesis	Plasmid-mediated bla_{KPC-3}	(Rodríguez-Zulueta et al., 2013; Shirley, 2018)
Gentamicin	Aminoglycoside	Inhibits bacterial protein synthesis by binding to the 70S complex	*aadB*	(Hamidian et al., 2012)
Imipenem	Beta-lactam	Inactivates penicillin-binding proteins, causing cell wall lysis or impedes cell wall formation	Mutation of *oprD*	(Li et al., 2012)
Tobramycin	Aminoglycoside	Inhibits bacterial protein synthesis by binding to the 70S complex	*aadB*	(Hamidian et al., 2012)
Neomycin	Aminoglycoside	Inhibits bacterial protein synthesis by binding to the 70S complex	*aph(3')-IIb*	(Zeng & Jin, 2003)

3.7.2. Biofilm Production

The colonization of planktonic *P. aeruginosa* promotes biofilm formation since it is a physiological perturbation that hampers the reepithelialization of chronic wounds (Frykberg & Banks, 2015). Biofilms are complex aggregates in which cells have additional protection against antibiotics and sterilants and confer enhanced protection against human immune defenses (Vallet et al., 2004). Biofilm formation is necessary for successfully colonizing tissues and bodily systems and facilitates disease progression after chronic colonization (Vallet et al., 2004). The *mvaT* gene is an essential regulatory component that works closely with the quorum sensing (QS) regulon to confer synchronized functions within planktonic cells (De Kievit et al., 2001; Vallet et al., 2004). The QS regulon in *P. aeruginosa* is also influential in pigment production, which retards the colonization of other bacteria and promotes colonization in the CF lungs and chronic wounds (El-Fouly et al., 2015).

3.7.3. Pigment Production

P. aeruginosa exhibits considerable versatility in producing virulence factors, of which pigment production is one of the most characteristic features of this formidable pathogen. The distinctive green color of the sputum from CF patients and the green exudates of chronic wounds often indicate secondary metabolites produced by *P. aeruginosa* (Winstanley & Fothergill, 2009). Global interwoven regulatory systems control the expression and production of these secondary metabolites, such as pyocyanin and pyoverdine, which are pivotal in colonizing various niduses (Baldelli et al., 2020; Winstanley & Fothergill, 2009). *P. aeruginosa* produces copious amounts of pyocyanin and pyoverdine in the lungs of CF patients (Lau et al., 2004). Pyoverdine is a quintessential siderophore that functions as a signaling molecule to regulate the production of enterotoxins, lipases, exotoxins, and proteolytic enzymes (Wretlind & Pavlovskis, 1983). In contrast, pyocyanin is a blue-green compound whose function is not fully understood (El-Fouly et al., 2015). Research has suggested that it is relevant as an antimicrobial agent and plays a vital role in robust biofilm formation by promoting the release of extracellular DNA (Das et al., 2016). Collectively, these pigments increase the formidability of *P. aeruginosa* in disease pathogenesis and severely hinder recovery times and rates after the onset of chronic colonization.

3.7.4. Other important virulence phenotypes of *P. aeruginosa*

Several other virulence factors are intrinsically present and encoded in the genome of *P. aeruginosa*. Figure 1 outlines the major virulence phenotypes for pathogenicity in this formidable pathogen. Research is underway to identify "old" repurposed antibiotics and their utility in treating pseudomonal infections, focusing on the virulence factors shown in Figure 1. These investigations evaluate the utility of repurposing "old" classes of antibiotics, such as the 5-nitrofurans, despite being non-inhibitory to growth. These analyses rely on the ability of 5-nitrofurans to disrupt and limit the virulence phenotypes of *P. aeruginosa* to curtail pathogenesis while another drug performs the killing action.

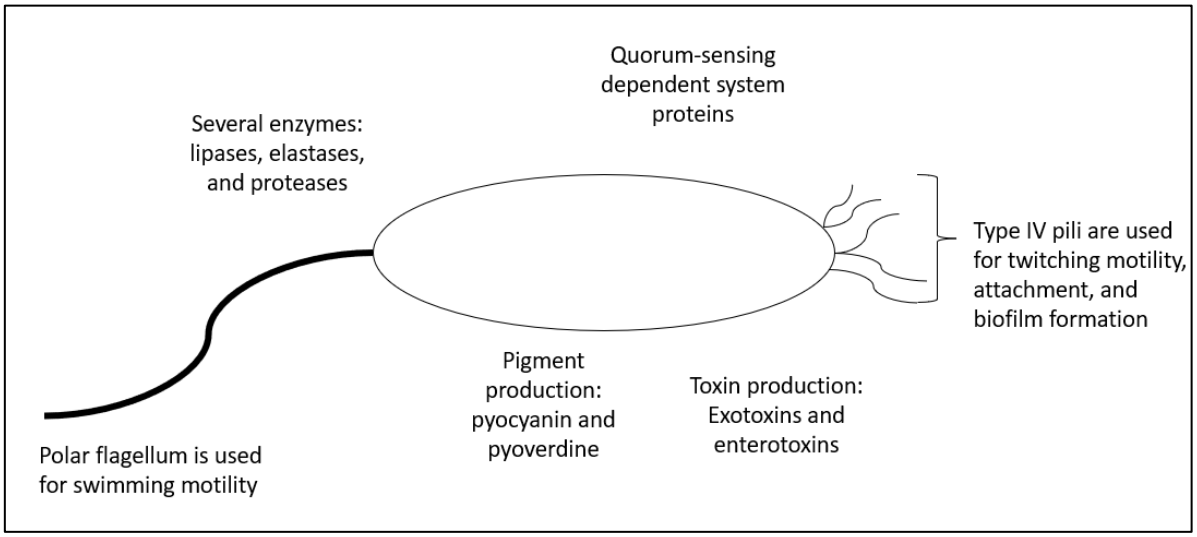

Figure 2. Some significant virulence factors of *P. aeruginosa* that contribute to its formidability and opportunistic nature as a human pathogen.

3.8. Hypothesis and Aims- *P. aeruginosa* experiments

P. aeruginosa remains one of the most formidable pathogens for which antibiotic treatments are waning in the clinical setting. Given the morbidity and mortality rates associated with CF lung and chronic wound patients, the slow development of novel antibiotics for treating pseudomonal infections is concerning. Repurposing "old" antibiotics are one way to have more options for treating pseudomonal infections. Research conducted by Baldelli et al. (2020) showed that nitrofurazone could inhibit some of the virulence factors highlighted in Figure 1 despite being non-inhibitory to the growth of *P. aeruginosa* PAO1. These include pyocyanin, rhamnolipid and biofilm production, and swarming motility. It is, however, unknown whether the highly effective furazolidone can inhibit virulence phenotypes despite not being inhibitory to the growth of *P. aeruginosa*. Given the antibacterial activity of furazolidone, I hypothesized that it would demonstrate virulence-inhibitory activities, although the effectiveness relative to nitrofurazone remains unknown.

The study aimed to determine whether furazolidone could reduce the virulence phenotypes of *P. aeruginosa*, despite being non-inhibitory to the growth. The study also sought to compare the effectiveness of furazolidone against the other nitrofurans: nitrofurazone and nitrofurantoin, to determine which of the three is most effective at limiting virulence phenotypes in the model organism *P. aeruginosa* PAO1.

Chapter 4- Materials and Methods for *E. coli* Experiments

Escherichia coli experiments- Search for novel nitrofuran-activating enzymes

4.1. *E. coli* strains and antibiotics

All the bacterial strains and plasmids used in this study are listed in Table 1. The growth media were prepared according to the manufacturer's guidelines. All *E. coli* strains were streaked on a 2 × YT (BD Difco^{TM}, USA) agar plate and incubated overnight at 37 °C for single colonies. Overnight cultures were prepared by inoculating one single colony into 5 mL of the 2 × YT broth, followed by incubation at 37 °C with 180-*rpm* shaking. Bacterial culture stocks for the newly made strains in this study were prepared by adding dimethylsulfoxide (DMSO) to fresh overnight cultures to reach a final concentration of 7 % v/v, after which the stocks were stored at – 80 °C. All the antibiotics used in this study are listed in Table 2.

4.2. Plasmid extraction

Plasmids were extracted by alkaline lysis using the ChargeSwitch®-Pro Plasmid Miniprep kit (Invitrogen, USA) according to the manufacturer's instructions and stored at – 20 °C. The cells were pelleted from a 5 mL overnight culture and resuspended in 250 µL of resuspension buffer until there were no visible cell clumps. Lysis buffer (250 µL) was added to the resuspended solution and gently inverted for thorough mixing. The mixture was then incubated at room temperature for 5 min, and then 250 µL precipitation buffer was mixed in until a white precipitate was formed. The mixture was then centrifuged for 10 min, and the supernatant was transferred into a Chargeswitch®-Pro MiniPrep Column inserted into a collection tube. The column and tube were centrifuged, and the filtrate was discarded. The column was reinserted into the tube, and 750 mL Wash Buffer 1 was added to the column and centrifuged for 60 sec.

The filtrate was discarded, the column reinserted, and 250 µL Wash Buffer 2 was pipetted into the column. The column was centrifuged for 60 sec, and the filtrate was discarded. The column was subsequently inserted into an elution tube, and 100 µL of elution buffer was added to the column. The column/tube was centrifuged for 60 sec, and the eluate containing the DNA was stored at -20 °C until transformation.

Table 2. Strains of *E. coli* used throughout this study.

Strain	Genotype	Source
BW25113	rrnB3 *ΔlacZ4787* hsdR514 *Δ(araBAD)*567 *Δ(rhaBAD)*568 rph-1	(Baba et al., 2006b)
K2506	BW25113 *ΔnfsA ΔnsfB ΔahpF*	(Le et al., 2019)
K2511	BW25113 *ΔnfsA ΔnfsB ΔahpF* pCA24N::*ahpF*	(Le et al., 2019)
K2609	BW25113 *ΔnfsA ΔnfsB ΔahpF* pCA24N::*cydA*	This study
K26[10]	BW25113 *ΔnfsA ΔnfsB ΔahpF* pCA24N::*ribD*	This study
K2616	BW25113 *ΔnfsA ΔnfsB ΔahpF* pCA24N::*gapA*	This study
K2617	BW25113 *ΔnfsA ΔnfsB ΔahpF* pCA24N::*gpsA*	This study
K2618	BW25113 *ΔnfsA ΔnfsB ΔahpF* pCA24N::*nrdB*	This study
K2619	BW25113 *ΔnfsA ΔnfsB ΔahpF* pCA24N	This study
K2620	BW25113 *ΔnfsA ΔnfsB* Δ*ahpF* pCA24N::*dapB*	This study
K2621	BW25113 *ΔnfsA ΔnfsB* Δ*ahpF* pCA24N::*folA*	This study
K2622	BW25113 *ΔnfsA ΔnfsB* Δ*ahpF* pCA24N::*ispH*	This study
K2623	BW25113 *ΔnfsA ΔnfsB* Δ*ahpF* pCA24N::*asd*	This study
K2624	BW25113 *ΔnfsA ΔnfsB* Δ*ahpF* pCA24N::*dxr*	This study
K2625	BW25113 *ΔnfsA ΔnfsB* Δ*ahpF* pCA24N::*fabG*	This study
K2626	BW25113 *ΔnfsA ΔnfsB* Δ*ahpF* pCA24N::*fabI*	This study
K2627	BW25113 *ΔnfsA ΔnfsB* Δ*ahpF* pCA24N::*folD*	This study
K2628	BW25113 *ΔnfsA ΔnfsB* Δ*ahpF* pCA24N::*hemA*	This study
K2629	BW25113 *ΔnfsA ΔnfsB* Δ*ahpF* pCA24N::*hemG*	This study
K2630	BW25113 *ΔnfsA ΔnfsB* Δ*ahpF* pCA24N::*ispG*	This study
K2631	BW25113 *ΔnfsA ΔnfsB* Δ*ahpF* pCA24N::*murB*	This study
K2632	BW25113 *ΔnfsA ΔnfsB* Δ*ahpF* pCA24N::*nrdA*	This study

Table 3. List of antibiotics used in this study.

Antibiotic	Supplier	Stock concentration	Solvent	Storage
Furazolidone (FZ)	Goldbio	10 mg/mL	DMSO	–20 °C
Nitrofurantoin (NIT)	Goldbio	20 mg/mL	DMSO	–20 °C
Nitrofurazone (NFZ)	MedChemExpress	10 mg/mL	DMSO	–20 °C
Chloramphenicol (CHL)	Sigma	50 mg/mL	DMSO	–20 °C

4.3. Preparation of chemically competent *E. coli* cells

Chemically competent cells were prepared using previously described protocols with some modifications (Green & Rogers, 2013). First, 100 µL of the overnight culture was diluted 100-fold with fresh 2 × YT medium to a total volume of 100 mL and incubated at 37 °C with 180-*rpm* shaking until reaching an optical density at 600 nm ($OD_{600\ nm}$) ranging between 0.15 and 0.20. The culture was then aliquoted into sterile 50 mL Falcon tubes and cooled on ice for 20 min, after which they were centrifuged at 4000 × *g* (ThermoFisher Scientific Heraeus Labofuge 400R, USA) for 10 min at 4 °C. From this point, it was critical to handle the sample on ice or in the cold room set at 4 °C. The supernatant was then decanted, and the pellet was resuspended in 20 mL of sterile, cold 0.1 M $CaCl_2$, before being centrifuged at 4 °C at 4000 × *g* for 5 min. The supernatant was discarded by aspiration. The pellet was resuspended in 2 mL of 10 % v/v glycerol, 0.1 M $CaCl_2$, and then aliquoted, 50 µL each, into pre-cooled, sterile Eppendorf tubes. These chemically competent cells were stored at – 80 °C until use.

4.4. Transformation of chemically competent *E. coli* cells

The extract of the empty pCA24N plasmid or the derivative containing a gene encoding a candidate nitroreductase (5 µL) was dispensed into an Eppendorf tube containing 50 µL of chemically competent *E. coli* cells. The tube was placed on ice for 30 min before being placed in a 42 °C water bath for 2 min to heat shock the cells. The tube was then immediately placed

on ice for 2 min. Following that, 1 mL of SOC medium (2 % w/v Bacto Tryptone (Difco™), 0.5 % w/v yeast extract (Difco™), 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 10 mM glucose) was added to the transformation mixture and was incubated at 37 °C with 200-*rpm* shaking for 30 min. The mixture was then 10-fold serially diluted with SOC, and 10 µL of each dilution was inoculated onto selective agar plates containing 30 µg/mL CHL to select for the transformant carrying the CHL resistance marker carried on the pCA24N plasmids. The agar plates were allowed to dry and then incubated overnight in the inverted position at 37 °C. Isolated, single colonies were used to prepare stock cultures of the new transformants.

4.5. Broth microdilution antibiotic susceptibility assay

The minimum inhibitory concentration (MIC) for nitrofurantoin and furazolidone were determined using an antibiotic susceptibility broth microdilution assay (Wiegand et al., 2008). Briefly, fresh overnight cultures were prepared in 5 mL of cation-adjusted Mueller-Hinton (CAMH) medium. On the day of the experiment, a 100-fold dilution of the overnight culture was made with fresh CAMH medium and incubated at 37 °C for 1.5 h with shaking at 180-*rpm*. After incubation, the $OD_{600\,nm}$ of the exponentially growing cell culture would be between 0.1 - 0.4. The cell culture was then adjusted with fresh CAMH to 1.0×10^6 colony forming units (CFU) per mL, 2-fold concentrate cell density required for the assay. The assay was performed in a flat-bottomed, 384-well microtiter plate (Corning Incorporated, USA), in which each well contained 50 µL of 5 x 10^5 *E. coli* CFU/mL and a concentration of 128, 64, 32, and 0 µg/mL of NIT or 64, 32, 16, and 0 µg/mL FZ. Each drug concentration was repeated in triplicate. The controls used for this experiment included three vehicle control wells containing 1.0 % v/v DMSO (positive control) and three sterility control wells per strain of bacteria tested (negative control). The 384-well microplate was incubated in a MultiSkan ™ GO Microplate Spectrophotometer at 37 °C for 20 h, where the $OD_{600\,nm}$ was recorded every hour. The MIC is defined by the minimal antibiotic concentration at which bacteria did not grow.

Chapter 5- Materials and Methods for *P. aeruginosa* Experiments

Pseudomonas aeruginosa experiments- Investigation of the effect of "old" nitrofurans on antivirulence properties

5.1. *P. aeruginosa* bacterial strains and antibiotics

Pseudomonas aeruginosa PAO1 cell stocks were stored at – 80 °C in 20 % v/v glycerol. Fresh streaks were made onto 1 % Luria Bertani (LB) agar, and isolated colonies were inoculated in 5 mL of fresh LB medium for overnight culture preparation. The overnight cultures were incubated at 37 °C with shaking at 180-*rpm*.

5.2. Swimming and swarming motility assays

The swimming motility assay was performed as previously described with some modifications (Ha et al., 2014). A sterilized toothpick was dipped into an overnight culture of *P. aeruginosa* PAO1 and was stab-inoculated in the center of an LB soft agar plate (0.3 % w/v agar) containing 20 µg/mL of nitrofuran drugs or 0.001 % v/v DMSO as vehicle controls. Each treatment was performed in three biological replicates. A medium-only control was included where the agar plate did not contain any additional agents. It was important not to touch the toothpick to the bottom of the Petri dish. The Petri dishes were then sealed with parafilm (Bemis Flexible Packaging, USA) and statically incubated upward for 18 h at 37 °C. The swimming zones were recorded by measuring the diameter of the growth.

With some modifications, the swarming motility assay was performed as described by Yeung et al. (2009). This was done by spotting 1 µL of the *P. aeruginosa* PAO1 overnight culture onto Brain Heart Infusion (BHI) agar plates (BD Difco^{TM}, USA) (0.5 % w/v agar) containing 20 µg/mL of nitrofuran drugs or 0.001 % v/v DMSO as vehicle controls. A medium-only

control was included where the agar plate did not contain any additional agents. Three biological replicates were performed for each treatment. The plates were statically incubated in the upright position at 37 °C for 18 h, and the swarming region diameter was measured and imaged.

5.3. Biofilm inhibition assay

The biofilm inhibition assay was performed as previously described by Merritt et al. (2005) with some modifications. From an overnight culture, a 100-fold dilution was made with fresh LB medium to a total volume of 20 mL, which was then incubated at 37 °C with shaking at 200-*rpm* for 2 h to reach the exponential phase ($OD_{600\ nm}$ ~ 0.13- 0.15). The cell cultures were adjusted with fresh LB medium to reach a concentration of 1.0×10^6 CFU/mL, 2-fold concentrate cell density required for the assay. The assay was performed in a flat-bottomed 96-well microtiter plate (polystyrene; Jet Biofil, USA); each well contained 150 µL of 5×10^5 CFU/mL of *P. aeruginosa* PAO1 at the exponential phase and FZ at 40, 20, 10, 5, 2.5, 1.25, or 0.625 µg/mL. Vehicle controls that contained 0.5 % v/v DMSO instead of the antibiotic were also included. Eight replicates were prepared for each treatment. The plate was incubated statically at 37 °C for 24 h. After incubation, the $OD_{600\ nm}$ of the culture was recorded, and the planktonic cells in the wells were discarded. The microtiter plate was then washed three times with 250 µL of phosphate-buffered saline (PBS) per well. Afterward, the biofilms formed in the wells were stained with 200 µL of 1 % w/v crystal violet per well at RT for 30 min. The plate was then rinsed three times with 250 µL of sterile water per well. The plate was then allowed to dry at RT for 30 min, and 250 µL of 95 % v/v ethanol was added to each well to dissolve the crystal violet for 10 min. The amount of solubilized crystal violet, which indicates the amount of biofilm biomass, was quantified by measuring light absorbance at 595 nm using a Thermoskan spectrophotometer. The $OD_{595\ nm}$ was normalized to the $OD_{600\ nm}$ of the planktonic cell density.

5.4. Pyocyanin Assay

A qualitative assay was conducted to deduce whether pyocyanin production was affected using varying concentrations of FZ. Similarly to the manner in which the biofilm formation assay was performed, a 100-fold dilution was made from an overnight culture of *P. aeruginosa* PAO1 using fresh LB medium to achieve a total volume of 20 mL. The freshly made dilution was incubated at 37 °C with shaking at 200-*rpm* for 2 h to reach the exponential phase ($OD_{600\ nm}$ ~ 0.13- 0.15). The cell cultures were adjusted with fresh LB medium to reach a concentration of 1.0×10^6 CFU/mL, 2-fold concentrate cell density required for the assay. The assay was performed in a flat-bottomed 96-well microtiter plate (polystyrene, Jet Biofil); each well contained 150 µL of 5 x 10^5 CFU/mL of *P. aeruginosa* PAO1 at the exponential growth phase and FZ at 40, 20, 10, 5, 2.5, 1.25, or 0.625 µg/mL. Vehicle controls that contained 0.5 % v/v DMSO instead of the antibiotic were also included. Eight replicates were prepared for each treatment. The plate was incubated statically at 37 °C for 24 h and then imaged for results.

Chapter 6- Results

6.1. Search for novel *E. coli* nitrofuran-activating enzymes

NfsA, NfsB, and AhpF have been shown to be redox enzymes in *E. coli* that reduce and thereby activate nitrofurans. In the triple mutant lacking all three enzymes, the MIC of the nitrofuran antibacterial furazolidone for *E. coli* increases from 2.3 μg/mL to 48 μg/mL. Nevertheless, concentrations of furazolidone above the higher MIC are bactericidal to the mutant. The antibacterial effect of furazolidone in this triple mutant has led to the hypothesis that other oxidoreductases in *E. coli*, besides NfsA, NfsB, and AhpF, can activate nitrofurans. Given that overexpression of AhpF from a plasmid significantly decreases the MIC of the triple mutant, this approach was used to screen potential nitrofuran-activating enzymes (Le et al., 2019). The candidate enzymes and their properties are described in Table 4.

Table 4. Properties of the 18 essential oxidoreductases hypothesized to have nitrofuran activating activity in the study.

Protein	Properties and functions
CydA	**Cytochrome *bd*-I subunit 1-** This terminal oxidase contains the heme b_{558} component of cytochrome *bd*-I. This protein is the site of ubiquinol oxidation in the periplasmic space
RibD	**5-amino-6-(5-phosphoribosylamino)uracil reductase-** This bifunctional enzyme catalyzes the deamination and reduction steps to synthesize 5-amino-6- (D-ribitylamno)uracil from GTP as part of the riboflavin biosynthesis pathway.
GapA	**Glyceraldehyde-3-phosphate dehydrogenase A-** This enzyme catalyzes the oxidative phosphorylation of glyceraldehyde 3-phosphate to 1,3-bisphosphoglycerate using NAD^+ and phosphate during glycolysis and gluconeogenesis.
GpsA	**Glycerol-3-phosphate dehydrogenase-** This enzyme is involved in glycerophospholipid metabolism, where it catalyzes the NAD(P)H-dependent reduction of the glycolytic intermediate dihydroxyacetone-phosphate to produce the precursor glycerol-3-phosphate.
NrdB	**Ribonucleoside-diphosphate reductase 1, β subunit dimer-** This enzyme has a B2 protein that contains a tyrosyl radical-dinuclear iron center. It is suggested that it functions to initiate catalysis by long-range electron transfer.
DapB	**4-Hydroxy-tetrahydrodipicolinate reductase-** This enzyme catalyzes the conversion of dihydrodipicolinate to tetrahydrodipicolinate as a step in lysine biosynthesis.
FolA	**Dihydrofolate reductase-** This enzyme catalyzes the reduction of dihydrofolate to tetrahydrofolate and is essential for the biosynthesis of proteins and nucleic acids.
IspH	**1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate reductase-** This enzyme is involved in the final step of the methylerythritol phosphate pathway, where it generates isopentyl pyrophosphate and dimethylallyl pyrophosphate.
Asd	**Aspartate-semialdehyde dehydrogenase-** This class B hydrogenase carries out the middle step in the homoserine biosynthesis pathway to generate L-aspartate-semialdehyde.
Dxr	**1-Deoxy-D-xylulose 5-phosphate reductoisomerase-** This enzyme is essential in the isoprenoid biosynthesis pathway, where it catalyzes the conversion of 1-deoxy-D-xylulose 5-phosphate into the dedicated MEP pathway intermediate 2-C-methyl-D-erythritol-4-phosphate. It is involved in the first committed step of this pathway.
FabG	**3-Oxoacyl-[acyl-carrier-protein] reductase-** This enzyme is a member of the short-chain dehydrogenase family. It functions in every fatty acid elongation cycle by catalyzing the NADPH-dependent reduction of 3-oxoacyl-[ACP] intermediates in the prokaryotic fatty acid biosynthesis pathway.
FabI	**Enoyl-[acyl-carrier-protein] reductase-** This enzyme is essential for the catalysis step in fatty acid biosynthesis, where the 2,3- double bond is reduced in the elongating fatty acid moiety.
FolD	**5,10-methylene-tetrahydrofolate dehydrogenase-** This protein is involved in the tetrahydrofolate interconversion pathway. This process is necessary for many functions within the cell, such as methionine, purine, and amino acid biosynthesis.
HemA	**Glutamyl-tRNA reductase-** This enzyme catalyzes the NADPH-dependent reduction of glutamyl-tRNA to glutamate 1-semialdehyde. It is essential for the first committed step in the heme biosynthetic pathway.
HemG	**Protoporphyrinogen oxidase-** This enzyme belongs to the flavodoxin family of proteins and catalyzes the oxidation of protoporphyrinogen IX to protoporphyrin IX.
IspG	**(*E*)-4-hydroxy-3-methylbut-2-enyl-diphosphate synthase (flavodoxin)-** This enzyme converts 2C-methyl-D-erythritol 2,4-cyclodiphosphate into 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate in the mevalonate-independent MEP pathway.
MurB	**UDP-*N*-acetylenolpyruvoylglucosamine reductase-** This enzyme is essential for growth by catalyzing the second committed step in peptidoglycan biosynthesis.
NrdA	**Ribonucleoside-diphosphate reductase 1, α subunit dimer-** This enzyme provides the precursors necessary for DNA synthesis. It catalyzes the biosynthesis of deoxyribonucleotides from the corresponding ribonucleotides.

6.2. Criteria for choosing 18 essential oxidoreductases

E. coli is an extensively studied pathogen; however, several characteristics remain unknown and unexplained regarding its metabolism. One such mechanism is the activation of prodrugs such as 5-nitrofurans. Previous experiments by Le and coworkers sought to uncover the mechanism behind decreased MICs of *E. coli* when challenged with FZ, where a novel oxidoreductase AhpF was found (Le et al., 2019). Despite this discovery, the activity of FZ is still detectable in the triple mutant K2511 (*ΔnfsA ΔnfsB ΔahpF),* suggesting that other yet undiscovered oxidoreductases may exist in *E. coli*. These putative enzymes have not been identified in the genetic screen that identified *ahpF*; hence it was hypothesized that these putative oxidoreductases were essential for *E. coli* survival, and hence the mutants could not have been selected for. Based on the decrease in the FZ MIC in the wild-type strain overexpressing AhpF, it was hypothesized that novel oxidoreductases, if they activate 5-nitrofurans, may decrease MIC when overexpressed. A search of the Enzyme Commission Class 1 enzymes (Oxidoreductases) in the EcoCyc *E. coli* K12 database was used to retrieve putative oxidoreductase-encoding genes. This search retrieved 303 putative protein candidates that encode oxidoreductases in *E. coli*. The premise for denoting these genes as essential was that they could not be knocked out by Lambda Red mediated recombineering. These genes were further analyzed by focusing on the essential genes within the group of 303 oxidoreductases that were identified. The Keio single-gene knockout library was accessed in tandem with the TraDIS experiment (Baba et al., 2006a; Yasir et al., 2020). Using this approach 18 candidate genes were chosen to test their capacity to activate nitrofurans (Figure 3).

The TraDIS experiment is an overexpression and re-expression method that was developed to overcome the disruption of essential genes via experimentation with said essential genes (Yasir et al., 2020). This high-throughput sequencing technique was developed to assay large libraries of isogenic transposon mutants to gain insight into gene function, essentiality, and interactions (Yasir et al., 2020). High-throughput sequencing methods have proven useful for revealing diversity between bacterial genomes (Jason et al., 2015). While several methods currently exist, they range in formidability, costs, and specialties. Despite the numerous techniques presently being used for sequencing, they were all incapable of directly pinpointing bacterial cell survival and how genetic variation might impact cell fitness levels (Yasir et al., 2020). Due to this gap in the sequencing pipeline, TraDIS was developed to utilize fragmented genomic DNA and a specific polymerase chain reaction amplification of transposon-containing fragments to

selectively enrich transposon-flanking sequences (Barquist et al., 2016). TraDIS encompasses the creation of a mutant library containing randomly inserted transposons into bacterial strains of interest (Yasir et al., 2020). This experiment aimed to create a bacterial population whereby each cell carries a single transposon insertion in the genome. From this mutant library, the bacteria are assessed phenotypically and genotypically (Barquist et al., 2016; Blanchard et al., 2015). This method allows the study of gene disruption and phenotype. In so doing, bacterial gene disruptions can be assessed when these cells are grown under selective growth conditions of interest (such as in the presence of antibiotics), leading to some mutants being more susceptible to the conditions than others (van Opijnen & Camilli, 2013; Yasir et al., 2020).

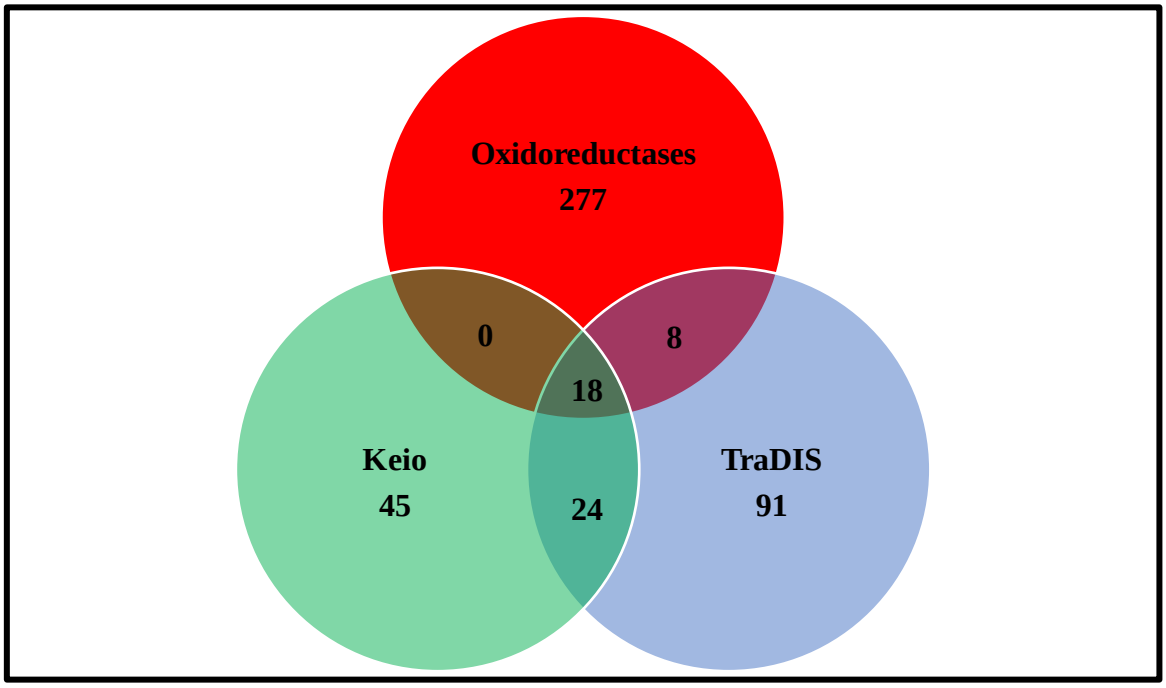

Figure 3. Venn diagram of databases that were searched to identify plausible essential oxidoreductases in *E. coli*.

The genes were retrieved based on the absence of the respective deletion mutants from the Keio collection (green), conditional lethality from transposon directed insertion-site sequencing (TraDIS) experiment (blue), and the list of oxidoreductases from the EcoCyc database (red). The overlap of all three diagrams was deemed essential, and the genes within the section were chosen for further analyses.

6.3. Initial screening of 18 candidate strains

The MICs for 5-nitrofuran antibiotics were determined using broth microdilution assays for the *E. coli* candidate strains, each overexpressing one of the 18 essential oxidoreductase proteins, hypothesized to have nitrofuran-activating activity (Table 4) (Wiegand et al., 2008). The rationale was that if an oxidoreductase contributed to the activation of nitrofurans, its overexpression would lead to a higher rate of 5-nitrofuran activation and thereby decrease the MICs of the 5-nitrofuran antibiotics. The recently characterized K2511 strain (Δ*nfsA* Δ*nfsB* Δ*ahpF*) triple mutant complemented by a pCA24N plasmid expressing a known nitroreductase protein AhpF and the K2619 strain (Δ*nfsA* Δ*nfsB* Δ*ahpF*) triple mutant transformed with the pCA24N empty vector only) were included as a positive control and negative control, respectively. Altogether, they made up 20 *E. coli* strains to be tested.

In the antibiotic susceptibility broth microdilution assays, I challenged *E. coli* cultures with nitrofurantoin at the concentrations of 128, 64, 32, and 16 µg/mL and furazolidone at the concentrations of 64, 32, 16, and 8 µg/mL with the addition of 0.1 mM IPTG to induce the expression of the candidate proteins (Table 4, Appendix 1-4). The bacterial growth curve was monitored by taking the readings of the $OD_{600\ nm}$ every hour for 20 h.

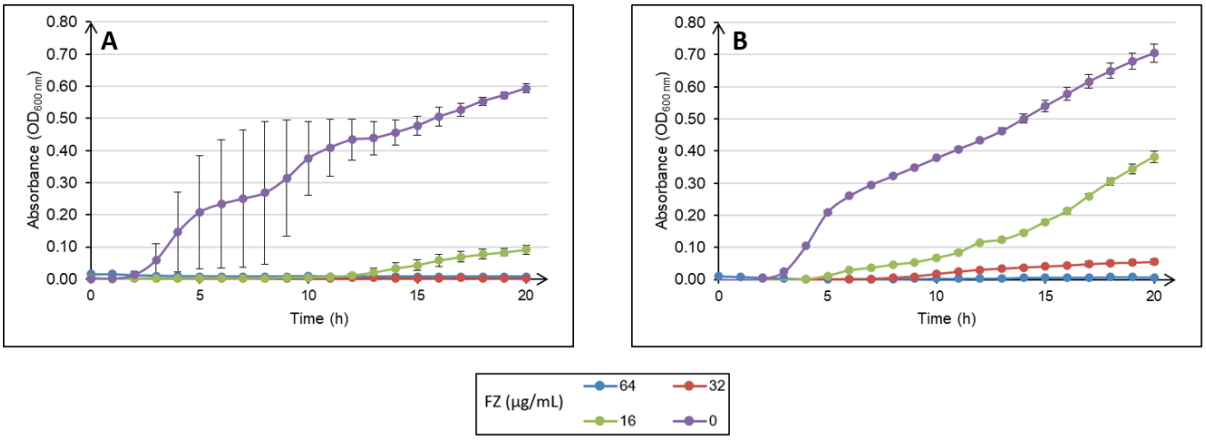

Figure 4. Determination of minimum inhibitory concentration for furazolidone of *E. coli* strains K2511 and K2619 complemented with the candidate nitroreductase-encoding gene and the empty pCA24N plasmid, respectively.

MIC testing at varying concentrations of furazolidone for *E. coli* K2506 triple mutants transformed with (A) pCA24N::*ahpF* (K2511) - positive control, (B) empty pCA24N (K2619) - negative control. Each plotted value represents the average of three replicates, and the error bars represent the standard deviation from the mean.

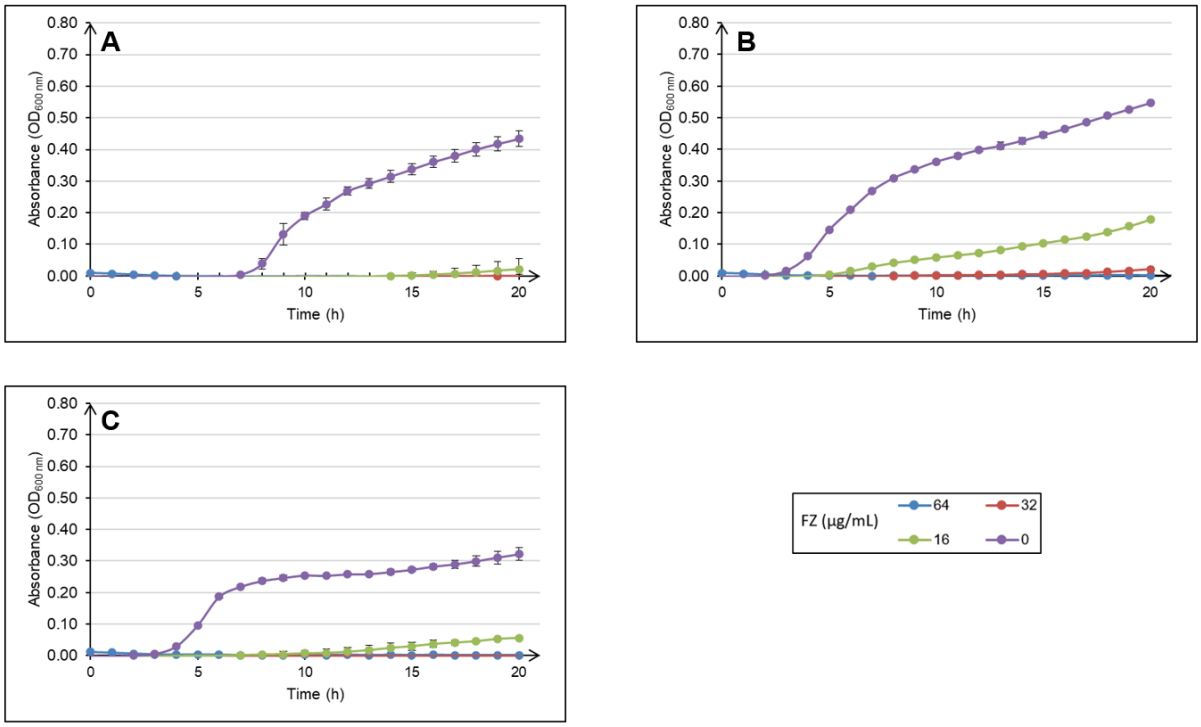

Figure 5. Determination of minimum inhibitory concentration for furazolidone of *E. coli* strains K2609, K2627, and K2628 complemented with the candidate nitroreductase-encoding genes.

MIC testing at varying concentrations of furazolidone for *E. coli* K2506 triple mutants transformed with (A) pCA24N::*cydA* (K2609), (B) pCA24N::*folD* (K2627), (C) pCA24N::*hemA* (K2628). Each plotted value represents the average of three replicates, and the error bars represent the standard deviation from the mean.

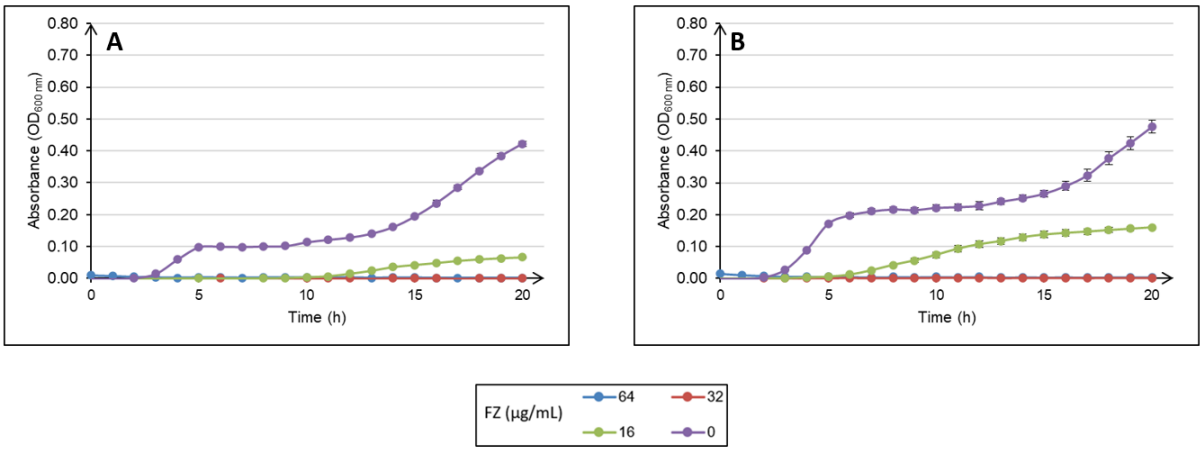

Figure 6. Determination of minimum inhibitory concentration for furazolidone of *E. coli* strains K2629 and K2631 complemented with the candidate nitroreductase-encoding genes.

MIC testing at varying concentrations of furazolidone for *E. coli* K2506 triple mutants transformed with (A) pCA24N::*hemG* (K2629) and (B) pCA24N::*murB* (K2631). Each plotted value represents the average of three replicates, and the error bars represent the standard deviation from the mean.

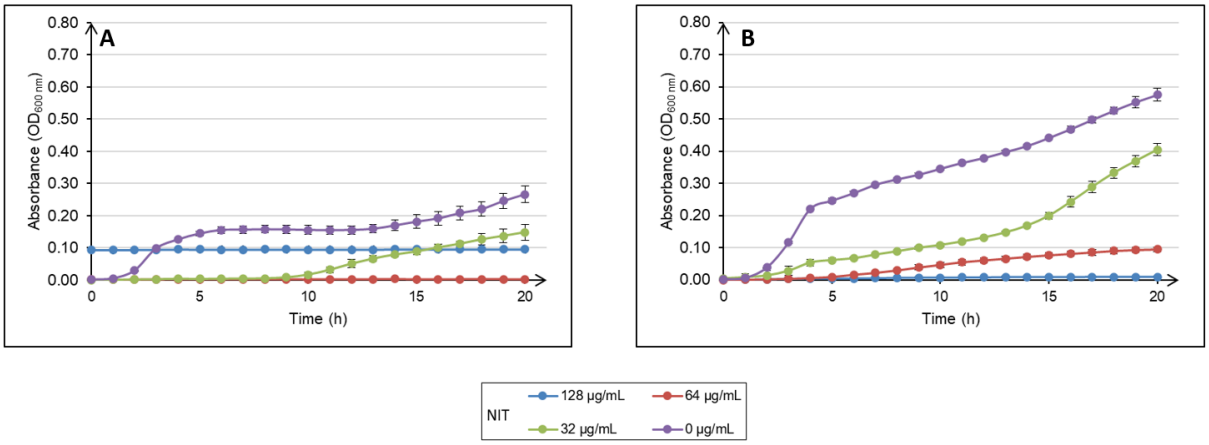

Figure 7. Determination of minimum inhibitory concentration for nitrofurantoin of *E. coli* strains K2511 and K2619 complemented with the candidate nitroreductase-encoding gene and the empty pCA24N plasmid, respectively.

MIC testing at varying concentrations of nitrofurantoin for *E. coli* K2506 triple mutants transformed with (A) pCA24N::*ahpF* (K2511)- positive control, (B) empty pCA24N (K2619)- negative control. Each plotted value represents the average of three replicates, and the error bars represent the standard deviation from the mean.

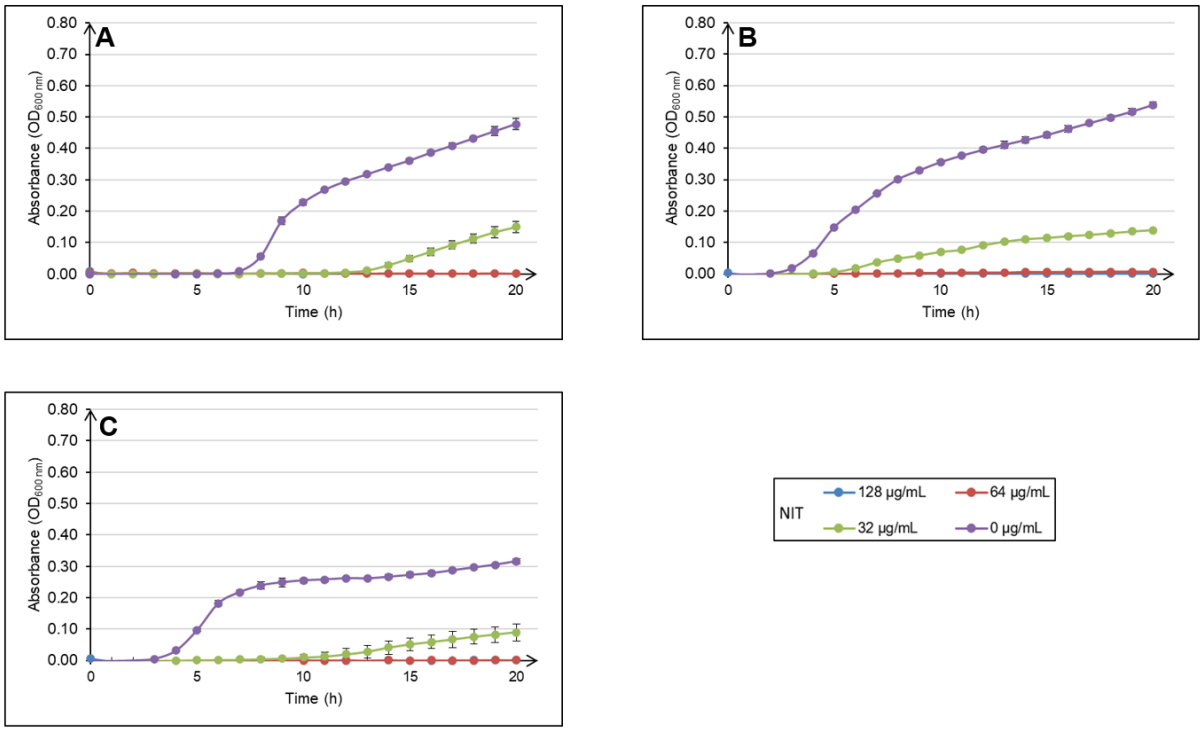

Figure 8. Determination of minimum inhibitory concentration for nitrofurantoin of *E. coli* strains K2609, K2627, and K2628 complemented with the candidate nitroreductase-encoding genes.

MIC testing at varying concentrations of nitrofurantoin for *E. coli* K2506 triple mutants transformed with (A) pCA24N::*cydA* (K2609), (B) pCA24N::*folD* (K2627), (C) pCA24N::*hemA* (K2628). Each plotted value represents the average of three replicates, and the error bars represent the standard deviation from the mean.

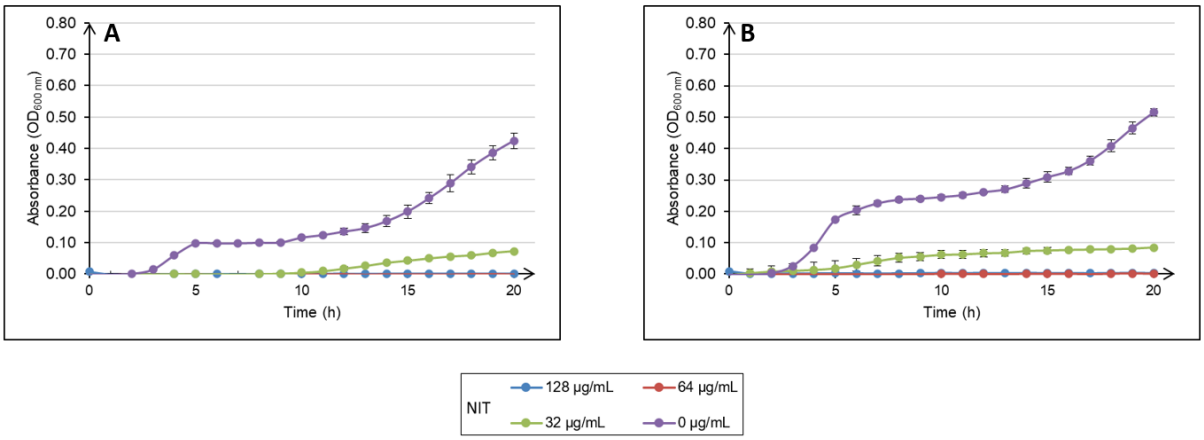

Figure 9. Determination of minimum inhibitory concentration for nitrofurantoin of *E. coli* strains K2629 and K2631 complemented with the candidate nitroreductase-encoding genes.

MIC testing at varying concentrations of nitrofurantoin for *E. coli* K2506 triple mutants transformed with (A) pCA24N::*hemG* (K2629) and (B) pCA24N::*murB* (K2631). Each plotted value represents the average of three replicates, and the error bars represent the standard deviation from the mean.

6.4. Testing MIC of shortlisted candidates by overexpression of plasmid-expressed proteins

The initial screening of 18 triple mutants, each complemented with a gene of interest, revealed five candidate strains containing pCA24N::*cydA* (K2609), pCA24N::*folD* (K2627), pCA24N::*hemA* (K2628), pCA24N::*hemG* (K2629), pCA24N::*murB* (K2631) which had decreased MICs after being challenged with furazolidone and nitrofurantoin. If the five shortlisted candidate proteins indeed activate nitrofuran to enhance its antibacterial effect, I reasoned that overexpression of these proteins by induction with a higher IPTG concentration would further increase the nitrofuran activation effect. I performed the broth microdilution assay again for those five *E. coli* strains, but this time the IPTG concentration was increased to 1.0 mM, with a wider range of nitrofuran concentrations.

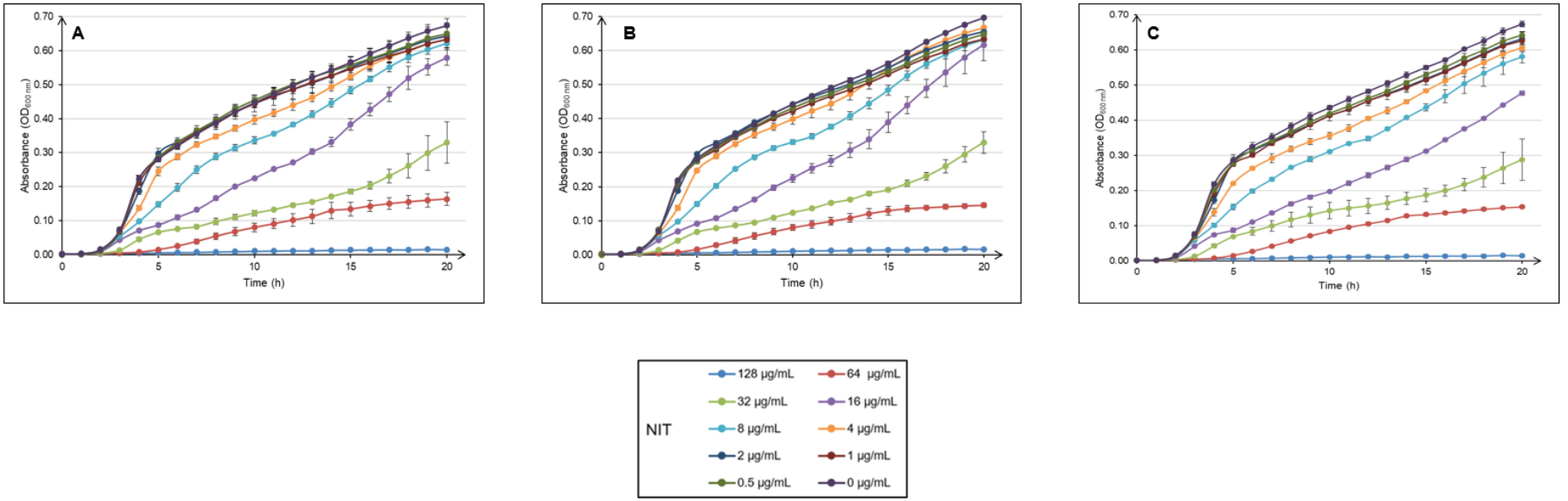

Figure 10. MIC plot of triple mutant *E. coli* K2619 (empty pCA24N) challenged with varying concentrations of NIT for 20 h of incubation at 37 °C in the presence of IPTG at A) 0 mM IPTG, B) 0.1 mM IPTG, C) 1.0 mM IPTG.

Each plotted value represents the average of three replicates, and the error bars represent the standard deviation from the mean.

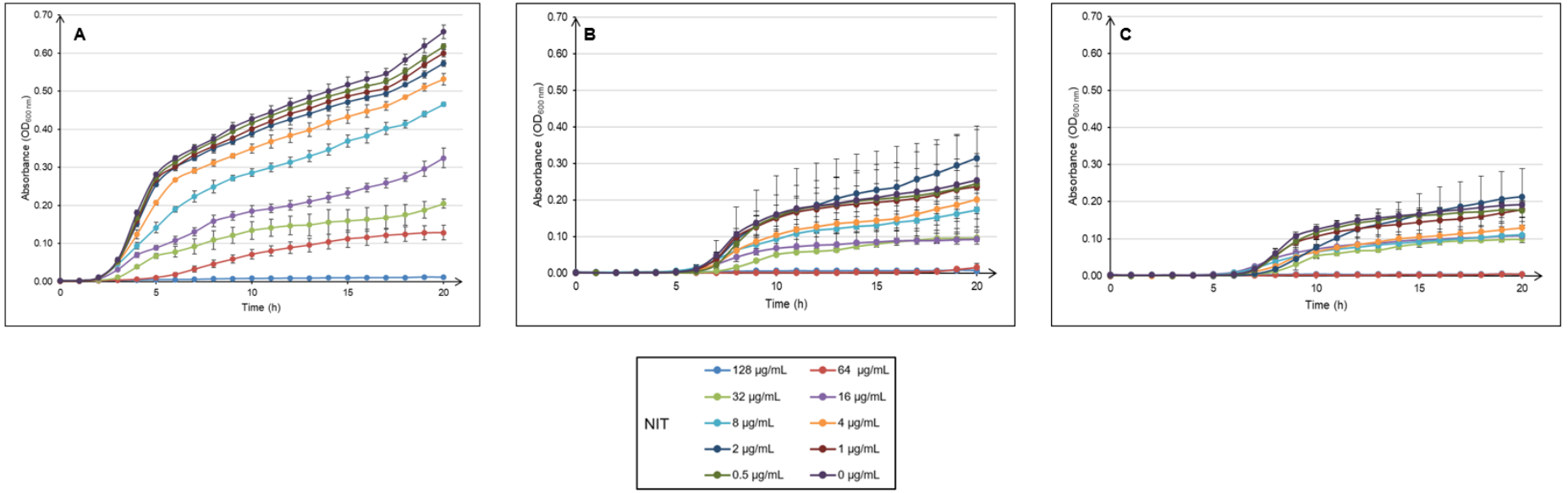

Figure 11. MIC plot of triple mutant *E. coli* K2609 (pCA24N::cydA) challenged with varying concentrations of NIT for 20 h of incubation at 37 °C in the presence of IPTG at A) 0 mM IPTG, B) 0.1 mM IPTG, C) 1.0 mM IPTG.

Each plotted value represents the average of three replicates, and the error bars represent the standard deviation from the mean.

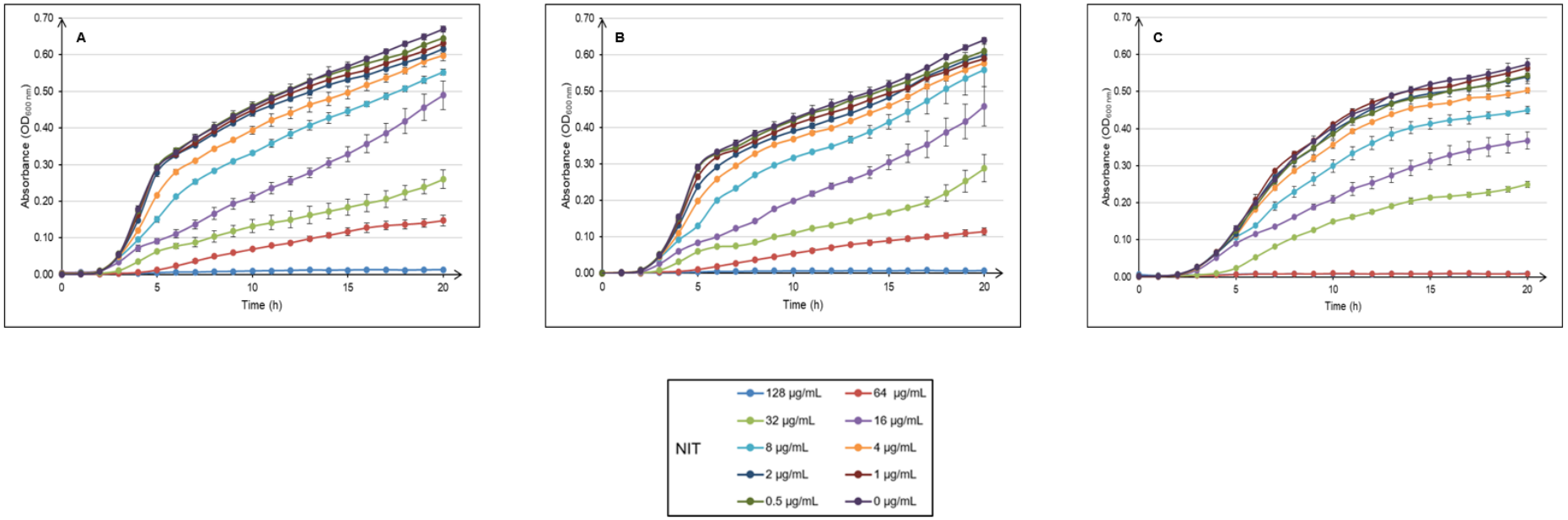

Figure 12. MIC plot of triple mutant *E. coli* K2627 (pCA24N::*folD*) challenged with varying concentrations of NIT for 20 h of incubation at 37 °C in the presence of IPTG at A) 0 mM IPTG, B) 0.1 mM IPTG, C) 1.0 mM IPTG.

Each plotted value represents the average of three replicates, and the error bars represent the standard deviation from the mean.

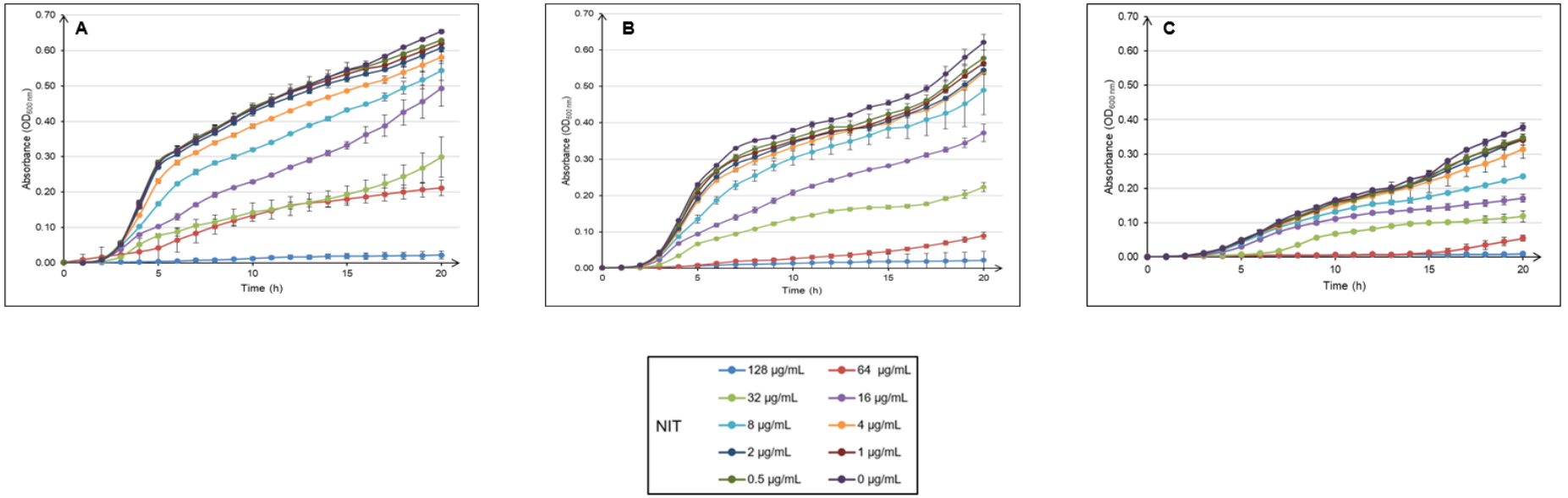

Figure 13. MIC plot of triple mutant *E. coli* K2628 (pCA24N::hemA) challenged with varying concentrations of NIT for 20 h of incubation at 37 °C in the presence of IPTG at A) 0 mM IPTG, B) 0.1 mM IPTG, C) 1.0 mM IPTG.

Each plotted value represents the average of three replicates, and the error bars represent the standard deviation from the mean.

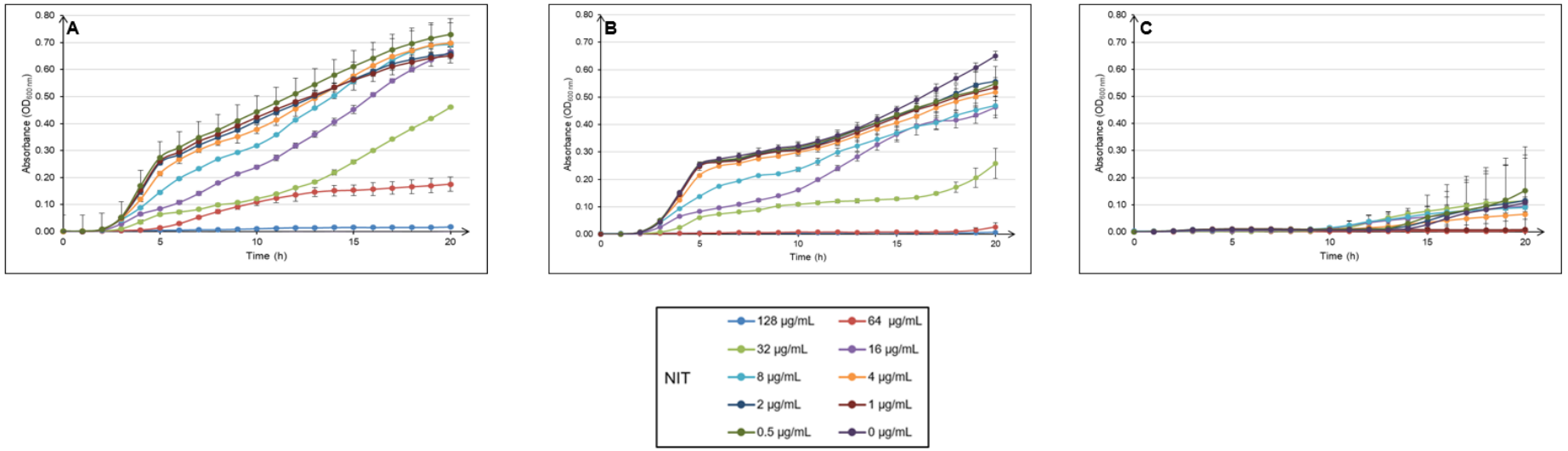

Figure 14. MIC plot of triple mutant *E. coli* K2629 (pCA24N::hemG) challenged with varying concentrations of NIT for 20 h of incubation at 37 °C in the presence of IPTG at A) 0 mM IPTG, B) 0.1 mM IPTG, C) 1.0 mM IPTG.

Each plotted value represents the average of three replicates, and the error bars represent the standard deviation from the mean.

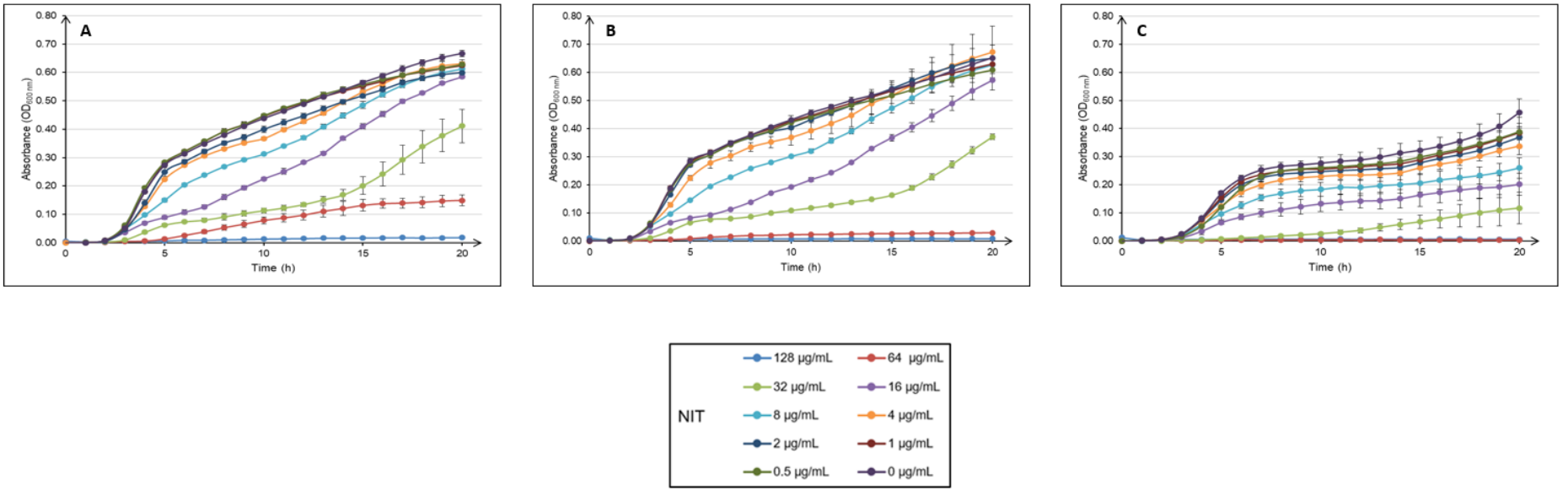

Figure 15. MIC plot of triple mutant *E. coli* K2631 (pCA24N::murB) challenged with varying concentrations of NIT for 20 h of incubation at 37 °C in the presence of IPTG at A) 0 mM IPTG, B) 0.1 mM IPTG, C) 1.0 mM IPTG.

Each plotted value represents the average of three replicates and, the error bars represent the standard deviation from the mean.

6.5. Conclusions from *E. coli* experiments

The 18 candidate essential oxidoreductases in *E. coli* were selected to be tested for a potential nitrofuran-activating activity by examining the MIC of the nitrofurans upon overexpression of these proteins in the *E. coli* Δ*nfsA* Δ*nfsB* Δ*ahpF* triple mutant. I found five candidate proteins, FolD, CydA, HemA, HemG, and MurB, showed a significant effect on increasing the sensitivity of the tested *E. coli* strains to nitrofuran drugs and, therefore could be nitrofuran-activating enzymes.

Chapter 7- Results

P. aeruginosa continues to be the leading cause of infections in chronic wounds and is the primary aetiological agent responsible for chronic lung disease in cystic fibrosis patients. Its impact is profound throughout the medical setting, mainly because of its ability to withstand the effects of several classes of antibiotics. With stagnation in the antibiotic discovery pipeline, research has now shifted to identifying antibiotics that could subdue this formidable pathogen's virulence phenotypes, despite not being inhibitory to the growth. Once identified, these compounds can then be repurposed to treat pseudomonas infections. Previous research conducted by Baldelli et al. (2020) showed the utility of nitrofurazone, a nitrofuran, for inhibiting some virulence phenotypes of *P. aeruginosa*. As such, I endeavored to test the effect of other nitrofurans on virulence phenotypes. The virulence factors were tested by challenging *P. aeruginosa* PAO1 with nitrofurantoin, furazolidone, and nitrofurazone to evaluate and compare the anti-virulence properties of swarming and swimming motility, biofilm production, and pigment production.

7.1. Effect of nitrofurans on motility of *Pseudomonas aeruginosa*

P. aeruginosa uses three modes of locomotion. Propulsion through the mucus in CF lungs depends on viscosity, with swimming used at a lower density and swarming at a higher density. With this in mind, soft agar plates of different densities were used to induce different types of movements utilized by *P. aeruginosa* by emulating the viscosity of mucus found in chronically infected CF lungs. For investigations into the effect of nitrofurans on swimming motility, 0.3 % w/v soft agar plates were used, and *P. aeruginosa* PAO1 cultures were challenged with furazolidone, nitrofurazone, and nitrofurantoin at 20 µg/mL. The effect of nitrofurans on swarming was examined in 0.5 % w/v soft agar plates containing 20 µg/mL furazolidone, nitrofurazone, and nitrofurantoin. Vehicle controls containing all components apart from nitrofurans were also used for each mode of locomotion investigated.

7.1.1. Swimming assay

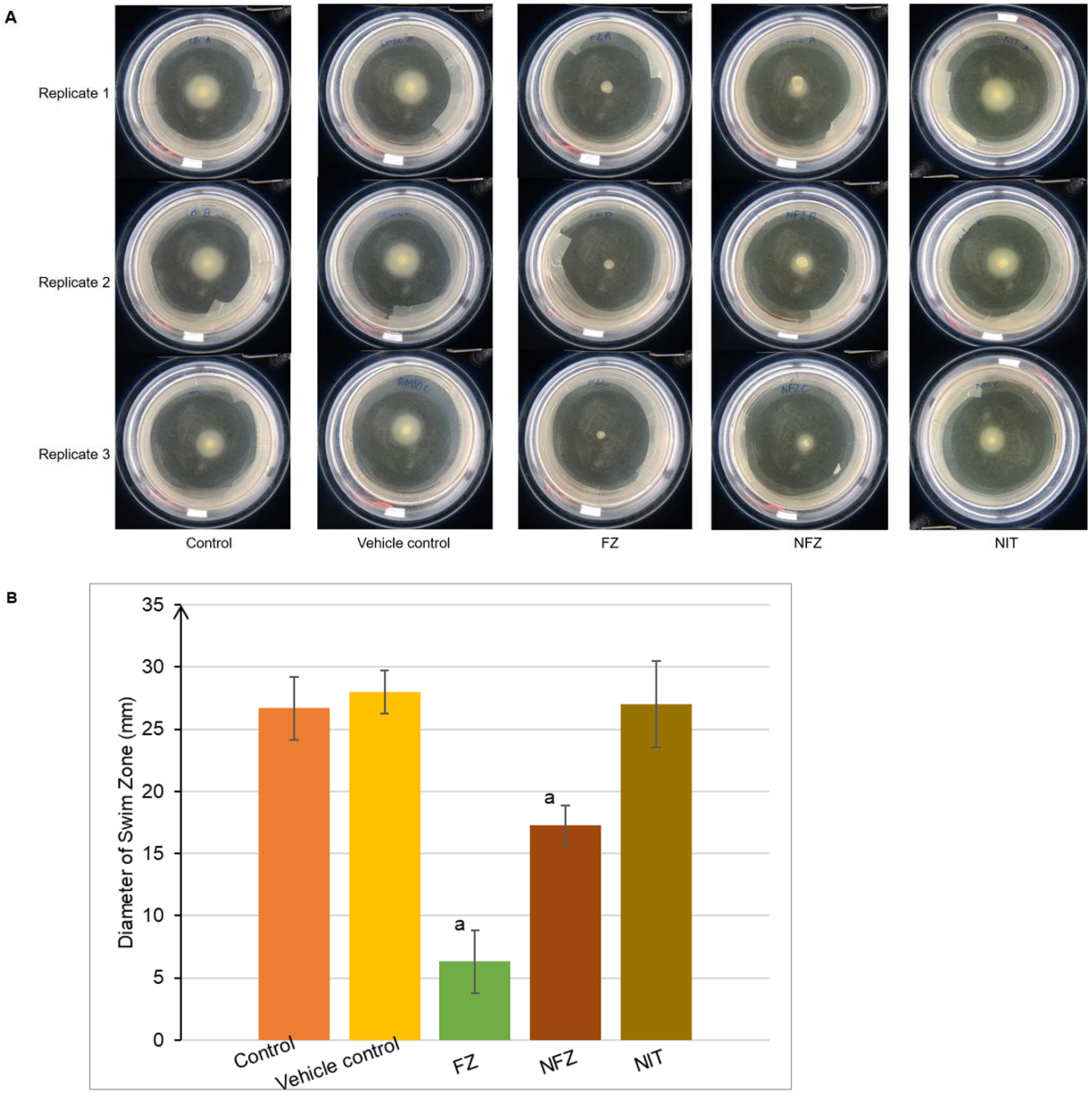

Figure 16. Swimming assay of *P. aeruginosa* PAO1 on 0.3 % LB agar.

(A) Swimming on the soft agar plate. Plates were incubated statically for 18 h at 37 °C. The control was LB agar without any agents, and the vehicle control contained 0.002 % (w/v) dimethyl sulfoxide (DMSO). All nitrofurans used were at a concentration of 20 µg/mL. Three independent biological replicates were tested for each agent. (B) Plot of swim zone diameters of different treatments. The values represent the average of three independent biological replicates, and error bars show the standard deviation from the mean of all three biological replicates. Statistical significance relative to the control is indicated by letter: a, $p < 0.05$ (ANOVA).

7.1.2. Swarming assay

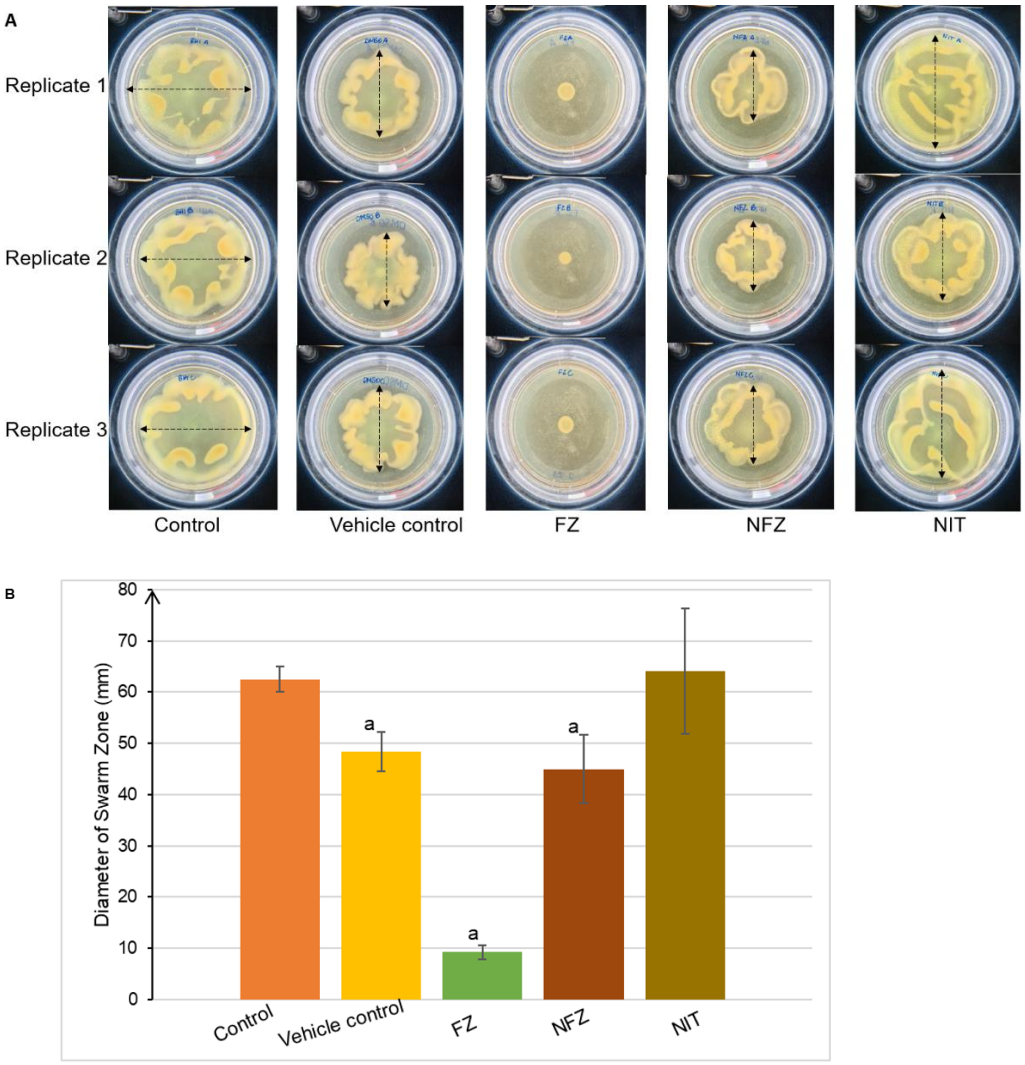

Figure 17. Motility assay of *P. aeruginosa* PAO1 shows swarming on 0.5 % brain heart infusion agar.

Plates were incubated statically for 18 h at 30 °C. The control was Luria Bertani agar without any agents, and the vehicle control contained 0.002 % (w/v) dimethyl sulfoxide (DMSO). All nitrofurans used were at a concentration of 20 µg/mL. Three independent biological replicates were tested for each agent. Double-ended arrows show regions where swarm diameters were measured when the zones were uneven. (B) Plot of swarm diameters of the different treatments. The values represent the average of three independent biological replicates, and error bars show the standard deviation from the mean of all three biological replicates. Statistical significance relative to the control is indicated by letters: a, $p < 0.05$ (ANOVA).

From these preliminary results, I found that furazolidone was the most effective at reducing the production or function of virulence factors of *P. aeruginosa* PAO1. The potency of furazolidone seemed to be more profound when compared to nitrofurazone; thus, subsequent experiments focused on varying concentrations of furazolidone.

7.2. Biofilm inhibition assay

Biofilm formation is essential for chronic lung disease of CF patients and for establishing chronic wound infections. Biofilms are an important virulence factor that allows sessile bacterial cells to circumvent the effects of antibiotics. With this in mind, *P. aeruginosa* PAO1 was challenged with varying concentrations of furazolidone to investigate the impact of the antibiotic on biofilm formation. The cells were first allowed to grow for 24 h in the presence of furazolidone and then subjected to crystal violet staining. The planktonic density of *P. aeruginosa* PAO1 was recorded after the 24 h incubation period to quantify the biofilm growth after comparing the solubilized biofilm after staining with 1 % w/v crystal violet.

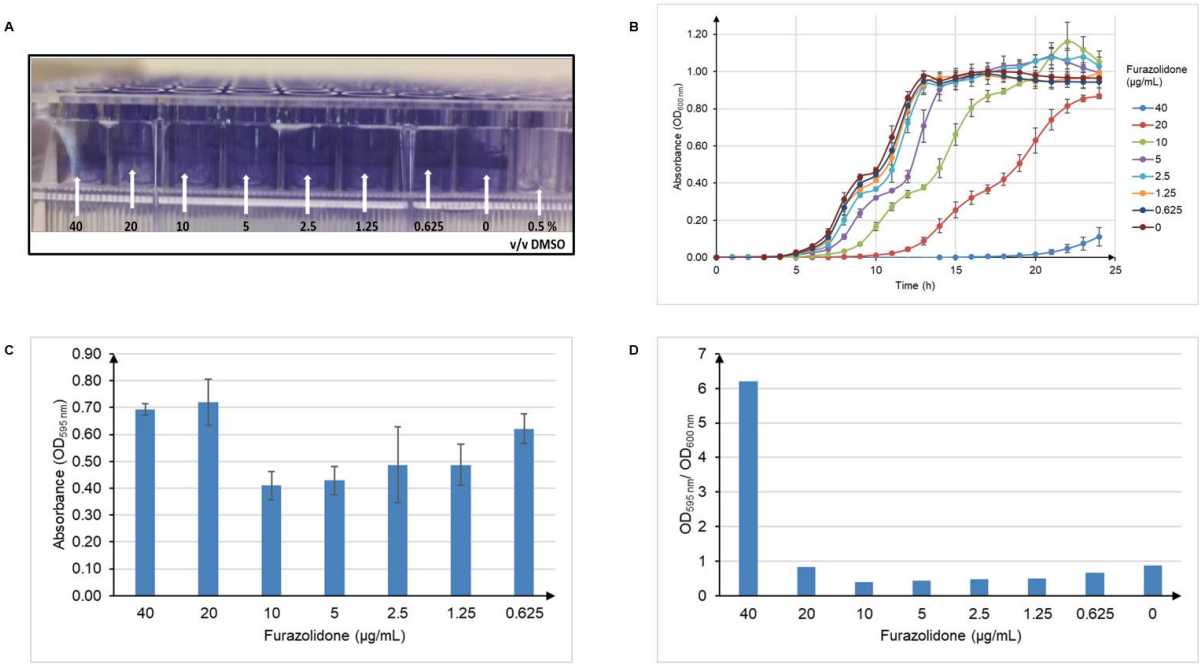

Figure 18. Effect of furazolidone on *P. aeruginosa* PAO1 biofilm formation.

(A) The image of biofilm formation as rings (indicated by white arrows) on the wall of microplate wells upon exposure to different concentrations of furazolidone (0 to 40 µg/mL). The biofilms were visualized by crystal violet staining. (B) The $OD_{600\ nm}$ of the *P. aeruginosa* PAO1 cultures in the presence of furazolidone over the period of 24 h at 37 °C, after which the biofilm formation was measured. (C) The $OD_{595\ nm}$ represents the biofilm formation for each furazolidone treatment. (D) The $OD_{595\ nm}$/ $OD_{600\ nm}$ values represent the biofilm formation normalized by the cell density. Each treatment was done in eight replicates, and the error bars indicate the standard deviation from the mean of all eight biological replicates.

7.3. Pyocyanin production

Pyocyanin is a pigment produced by *P. aeruginosa* PAO1, which is essential for colonizing CF lungs. This toxic metabolite promotes and increases the pathogen's fitness through its antibacterial properties (Velsor et al., 2006). Thus, this metabolite functions to subdue the growth of other competitor pathogens and also enables the *P. aeruginosa* PAO1 to evade reactive oxygen species produced by cells of the immune system constantly surveilling the pulmonary airways. As such, a qualitative assessment of pyocyanin production was required to deduce whether varying concentrations of FZ could influence production levels.

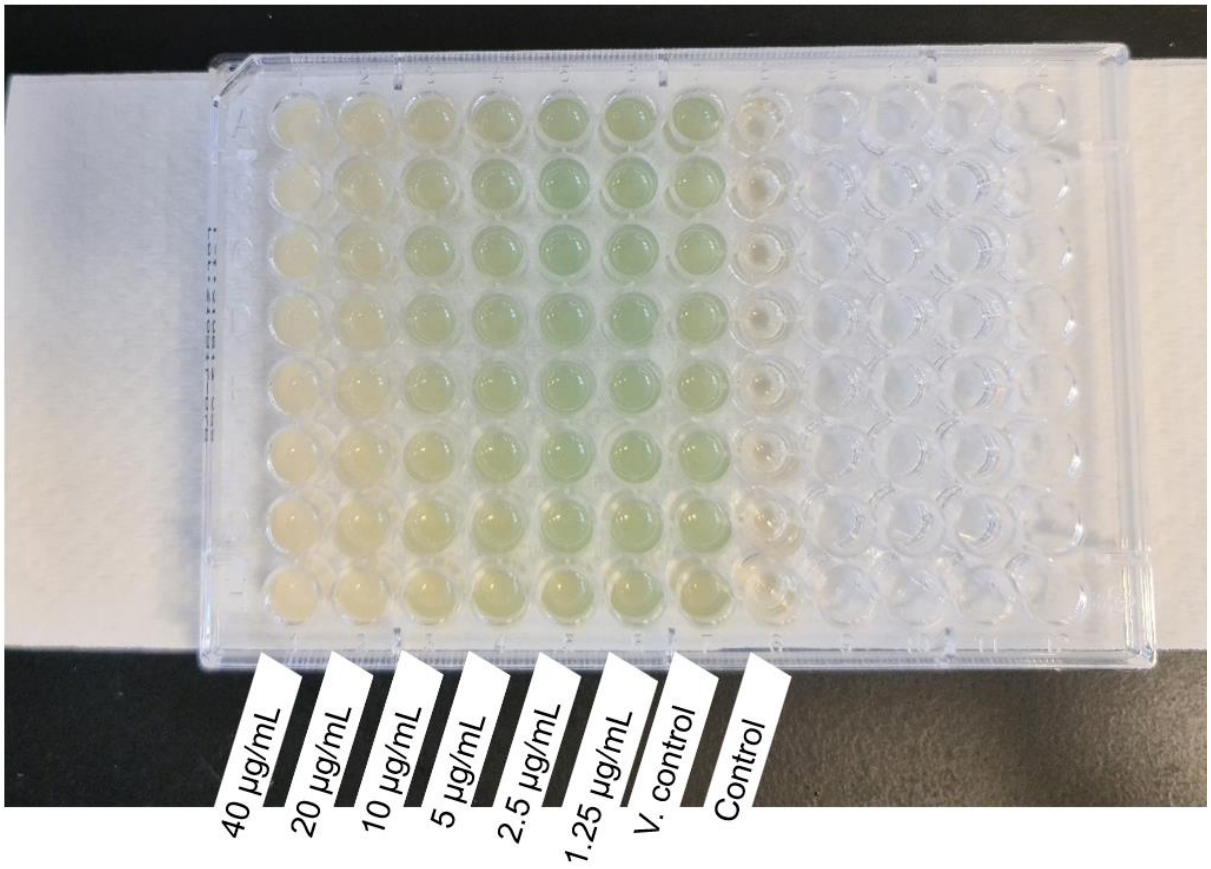

Figure 19. Visual of the 24-hour *P. aeruginosa* PAO1 cultures showing the production of the blue-green pigment pyocyanin.

The cultures were challenged with varying concentrations of furazolidone ranging from 40 µg/mL to 1.25 µg/mL. The cultures were grown in LB medium containing decreasing concentrations of furazolidone, as indicated. Eight replicates were made for each furazolidone concentration tested. V. control refers to the vehicle control containing 0.5 % v/v DMSO.

7.3. Conclusion of results from *P. aeruginosa* PAO1 experiments

The initial hypothesis was tested to determine whether the effect of furazolidone on *P. aeruginosa* PAO1 was more potent than nitrofurazone. The results indicate that furazolidone can inhibit some of this formidable pathogen's most pertinent virulence factors. As shown in Figures 17 and 18, furazolidone was found to be a better inhibitor of swimming and swarming when compared to nitrofurazone which indicates that the potency of this 5-nitrofuran is superior to that which was previously experimented by Baldelli et al. (2020). The biofilm inhibition assay shows that 10 µg/mL furazolidone was the minimum biofilm inhibitory concentration (MBIC). Beyond this MBIC, results suggest that a stimulus promotes biofilm formation. Visual analysis of pyocyanin production, as seen in Figure 20, indicates that furazolidone could also inhibit pigment production. This preliminary work sets the premise to investigate the effect on other virulence phenotypes of *P. aeruginosa* PAO1 when challenged with furazolidone in parallel to nitrofurazone.

Chapter 8- Discussion

Gram-negative bacteria are one of the most problematic groups of pathogens causing human disease because of the emergence of antibiotic resistance within bacterial populations. The waning availability of antibiotics and the costs associated with treating MDR Gram-negative infections are a cause for concern in healthcare. Of particular concern are infections caused by *E. coli*, whereby uUTIs are challenging to treat, and *P. aeruginosa*, the etiological agent responsible for chronic illnesses in immunosuppressed individuals such as CF patients and those with chronic wounds. The urgency caused by these pathogens has shifted the focus of drug discovery from acquiring *de novo* drugs to repurposing approved drugs typically used for treating conditions other than bacterial infections or reactivating "old" antibacterials for treating MDR infections. In so doing, the time required for drug approval and clinical trials is shortened and, in some cases, obviated. One such group of antibiotics named 5-nitrofurans is an “old” class of antibiotics discontinued within the clinical setting because of concerns regarding the carcinogenic nature and within the agricultural applications due to toxic metabolites found in livestock tissues. Their rescindment on the market was also secondary to the subsequent discovery and introduction of newer antibiotics with lower toxicity. Despite this, furazolidone is still used in human medicine to treat *E. coli* infections and remains one of the most commonly used antibiotics used for treating uUTIs.

This thesis explores the potential of the drug furazolidone and the prospect of discovering additional oxidoreductases in *E. coli* that can activate this prodrug into its active form. It also aims to set the groundwork for using FZ as an antivirulence drug to curtail pseudomonas infections, with a targeted focus on respiratory tract infections in CF patients and chronic wounds in immunocompromised individuals.

8.1. Summary of findings from the search for novel 5-nitrofuran-activating oxidoreductases in *E. coli*

This research aimed to identify putative oxidoreductases, from which 18 were singly overexpressed in the *E. coli ΔnfsA ΔnfsB ΔahpF* triple mutant. Previous research carried out by Le et al. (2019) led to the discovery of the novel nitrofuran-activating enzyme responsible for the decrease in the MIC of an *E. coli* mutant Δ*nfsA* Δ*nfsB*. Thus, experiments from this research were focused on discovering additional novel nitroreductase-activating enzymes. I discovered five putative genes from the study that showed a decrease in the MIC when overexpressed in the *ΔnfsA ΔnfsB ΔahpF E. coli* triple mutant.

Since the nitrofuran activation enzyme within the triple mutant lacking NfsA, NfsB and AhpF was elusive at the start of this thesis work, functional genomics was utilized to identify the 18 essential oxidoreductases as potential candidates for nitrofuran-activating enzymes (Figure 3).

After identifying 18 essential oxidoreductases as candidates for nitrofuran-activating enzymes, furazolidone and nitrofurantoin were used to challenge the *E. coli* strains overexpressing corresponding proteins from a high-copy-number expression vector containing an IPTG-inducible promoter. A strain transformed with the "empty" vector (pCA24N) was used as a negative control, and a strain expressing AhpF, an oxidoreductase reported to decrease MIC to nitrofurans when overexpressed, was used as a negative control (Figure 4A and Figure 4B). This analysis revealed five candidate genes whose overexpression resulted in a decrease in the nitrofuran MIC: CydA, FolD, HemA, HemG, and MurB (Figures 5 and 6).

The CydA protein is part of the CydABX complex in the cytoplasmic membrane and catalyzes the terminal electron transfer in respiration. The cydA subunit has a molecular mass of 58.2 kDa and is part of an enzyme complex that oxidizes ubiquinol to ubiquinone (Hoeser et al., 2014). The electrons generated from this oxidation then reduce oxygen to water and concurrently generate a proton motive force, which is required for cellular functions and processes (Hoeser et al., 2014; Kashyap et al., 2022). The overexpression of CydA in *E. coli* K2609 by induction with 0.1 mM and 1.0 mM IPTG (Figure 11) reduced the nitrofurantoin MIC relative to the negative control (from 128 to 64 µg/mL: Figure 10). CydA overexpression also somewhat suppressed the growth of the culture (Figure 11C). this result was expected, given the metabolic function of the protein and the essential role it plays in generating the

proton motive force in *E. coli*. Bacterial species such as *E. coli* are known to possess multiple cytochrome oxidase operons, which are expected to impart growth stresses and help with overall survival (Vanorsdel et al., 2013). The overexpression of this essential protein was expected to increase the generation of the proton motive force; however, this would also facilitate a notable difference in the MIC, given that these oxidoreductases activate 5-nitrofuran antibiotics.

The FolD protein is a 62 kDa, bifunctional 5,10-methylene-tetrahydrofolate dehydrogenase cyclohydrolase (Shen et al., 1999). It functions in the biosynthesis of tetrahydrofolate and purines. It is further involved in the supply of the formyl group for the initiator $tRNA^{fMet}$ (D'Ari & Rabinowitz, 1991; Shen et al., 1999). The overexpression of the FolD protein was expected to confer increased susceptibility to NIT due to its redox activity. No decrease in the MIC was detected at 0.1 mM IPTG, however, at 1.0 mM IPTG, there was an MIC decrease from 128 µg/mL to 64 µg/mL (Figure 12). The growth curve of *E. coli* K2627 (pCA24N::*folD*, Figure 12C) was less affected by IPTG than was *E. coli* K2609 (pCA24N::*cydA*, Figure 11C).

The next candidate was HemA, a 45 kDa redox enzyme involved in the synthesis of heme in *E. coli* (Verderber et al., 1997). Overexpression of the HemA protein at 0.1 mM and 1.0 mM IPTG resulted in growth inhibition of > 90 % at 64 µg/mL NIT up to 15 h of growth, hence the MIC was decreased at that timepoint (Figure 13). Research by Wang et al. (1999) revealed that HemA activity is inversely proportional to heme production, which suggests that this protein might act as rate limiting in the production of this important compound. Increased expression of the HemA protein might have thus elicited feedback inhibition on the end product heme, which would inadvertently affect other Hem proteins (HemL, HemB, HemC, HemG, etc) later on in this pathway (Wang et al., 1999). Some growth at those IPTG concentrations was observed between 15 and 20 h of incubation, resulting in an MIC of 128 µg/mL at the 20 h time point. Overall growth (in the absence of NIT) was somewhat inhibited at 1 mM IPTG (Figure 13C), with the maximum OD about 30 % lower than the one attained by the cultures grown in the absence of or at 0.1 mM IPTG (Figure 13A).

The fourth candidate tested was HemG protein, a 22 kDa oxidoreductase in the heme biosynthesis pathway that catalyzes the oxidation of protoporphyrinogen IX to protoporphyrin IX, which contains an FMN prosthetic group (Boynton et al., 2009b). Overexpression of the HemG protein at 0.1 mM IPTG resulted in the MIC decrease from 128 to 64 µg/mL. At 1.0 mM IPTG, the culture growth of *E. coli* K2629 (pCA24N::*hemG*, Figure 14) was inhibited

strongly even in the absence of NIT, with the culture reaching an OD of only 0.1 after 20 h of incubation, compared to 0.7 for the uninduced culture. It is possible that the HemG overexpression results in depletion of FMN that is otherwise required for many essential redox reactions, resulting in growth inhibition (Boynton et al., 2009a).

The final candidate gene *murB* decreased the MIC from 128 µg/mL to 64 µg/mL when overexpressed at 0.1 mM and 1.0 mM IPTG (Figure 15). The MurB protein is a 38 kDa UDP-N-acetylenolpyruvoylglucosamine reductase required for the second committed step in peptidoglycan synthesis (Benson et al., 1995; Pucci et al., 1992). From the results in Figure 15, there was no visibly significant change in the uninduced mutant growth dynamics (Figure 15A) compared to those induced with 0.1 mM IPTG (Figure 15B). The overexpression of the protein using 1.0 mM IPTG (Figure 15C) resulted in a growth inhibition of approximately 50 % compared to the 0.1 mM IPTG-induced cultures (Figure 15B). *murB* is a known essential gene in *E. coli* and other bacteria such as *Streptococcus pneumoniae* and *Bacillus subtilis* have been proven to have homologs, which makes the gene a suitable target for antibiotics (Sylvester et al., 2001). Similarly to the flavoprotein NfsA, murB adopts a ping pong bi-bi double competitive substrate inhibition mechanism, suggestive that this mechanism could be utilized during the activation of nitrofurans (Le et al., 2019; Sylvester et al., 2001). It is yet to be determined though whether this mechanism is indeed utilized for the activation of 5-nitrofuran prodrugs. *Salmonella typhimurium* and *E. coli* possess the murB protein that is approximately 82% similar and which is considered to be extremely low for the two closely related pathogens, however, their active sites active sites are very similar, thus the precedence exists to investigate whether the this mechanism is conserved between these two organisms (Sylvester et al., 2001). Taken together, the results suggest that bacterial growth and cell wall synthesis were inhibited in the cell culture where murB was overexpressed, and corroborates the assumption that murB might be a suitable oxidoreductase which activates 5-nitrofurans.

In this study, the susceptibility of the five putative oxidoreductase-containing mutants to nitrofurantoin resulted in changes between 50 % and 90 %, a trend that has been studied in other Gram-negative bacteria like *Klebsiella pneumoniae* (Osei Sekyere, 2018). These results are in keeping with the literature, which explains that ESBL-producing *E. coli* were sensitive to NIT by 57 % (Osei Sekyere, 2018). *K. pneumoniae* is a Gram-negative pathogen that has the potential to cause uUTIs (Gołębiewska et al., 2019). *K. pneumoniae* is an ESBL-producing bacterium; however, unlike in *E. coli* infections, NIT was found to be relatively unreliable for actively treating uUTIs caused by the pathogen (Tulara, 2018). *K. pneumoniae* strains, which

carried mutations about the chromosomal genes nfsA, nfsB, and ribE were responsible for conferring high resistance levels to NIT (Gołębiewska et al., 2019). This study illustrates that these nitroreductases can dictate changes to the MIC, and experiments conducted by H. Wykes (unpublished) confirmed that the ribE gene was indeed a plausible candidate for exploring its utility in decreased MIC levels in the *E. coli ΔnfsA ΔnfsB ΔahpF* triple mutant. The nfsA and nfsB oxidoreductases have been previously characterized and shown to be instrumental in the activation of the NIT prodrug. Thus it would be interesting to investigate how complementation and mutagenesis experiments with the ribE gene could affect the MIC of the mutant. Importantly though, the genomics analyses carried out did not include the ribE gene in the overlapping Venn diagrams, and as such there might be utility in investigating other genes which are not part of the initial 18 candidate genes for their prospect as nitrofuran-activating oxidoreductases.

8.1.1. Limitations of the *E. coli* experiment

Overexpression screens may sometimes fail at identifying putative and unknown drug targets; however, there is utility in this method because it can be a systematic method of identifying simple inhibition and complex mechanisms of drug actions (Moriya, 2015; Palmer & Kishony, 2014). Overexpression screens rely on the mechanism whereby resistance is conferred based on targeted or structural components of the specific drug; thus, drug resistance is thought to evolve by overexpressing the drug target (Palmer & Kishony, 2014). Another useful method utilizes screens for overexpression mutants to determine drug resistance. Many experimental practices use this hypothesis to determine the susceptibility or resistance of bacteria to antibiotics. The reasoning behind this theory is that the number of copies of the target would influence the total target activity to the required levels for cell viability and growth (Moriya, 2015). As a result, higher drug concentrations are required to reduce the total target activity for cell viability and growth when there are more copies of the target (Palmer & Kishony, 2014).

In contrast, the opposite would apply to underexpression susceptibility screens. Despite this logical thinking, these hypotheses do not always conform to all drugs (Palmer & Kishony, 2014). This challenge is known to exist in the pharmaceutical industry regarding drug discovery (Silver, 2011). Thus, evidence highlights that resistance is not reliant on a target gene since many targets can influence drug efficacy (Palmer & Kishony, 2014). Despite this, it is

safe to assume that overexpression screens which use drugs such as polymyxins and DNA intercalators are more precise since they target specific structures such as lipopolysaccharides and DNA, respectively (Emmerich et al., 2021; Olaitan et al., 2014; Waring, 1991).

As such, it would be difficult to ascertain whether the changes in MIC resulted from the other four putative genes combined with an overexpressed gene or whether one gene was solely responsible for the differences in MIC recorded throughout the research.

8.1.2. Conclusions

In summary, evaluation of the MIC change in the triple mutant lacking known nitrofuran-activating oxidoreductases when each of the 18 essential redox enzymes are overexpressed identified five candidate enzymes that caused a decrease in MIC. Further analyses of the MICs in the strains containing the inducibly-expressed five candidate oxidoreductases were performed to confirm the effect on the nitrofurantoin MIC in comparison to the negative control that contained the "empty" vector. Among the five candidates, all apart from HemA decreased the NIT MIC when overexpressed at 1 mM IPTG, while at 0.1 mM IPTG FolD, CydA, HemG, and MurB lowered the MIC from 128 to 64 µg/mL, similarly to overexpression of the known nitrofuran-reducing enzyme, AhpF.

8.1.3. Future Directions

Identification of five essential oxidoreductases FolD, CydA, HemA, HemG, and MurB that decrease the NIT MIC when overexpressed indicates that in the triple mutant lacking NfsA, NfsB, and AhpF, one or more of these enzymes may be responsible for activating nitroreductases. To confirm the enzymatic activities of these enzymes, they should be purified, and their activity should be directly demonstrated in enzymatic assays.

At the start of this thesis, it was assumed that non-essential proteins are not involved in the nitrofuran activation, given that only AhpF mutations were isolated in a published mutation screen for nitrofuran-resistant mutants of the double *nfsA nfsB* deletion mutant (Le et al., 2019). However, nitrofuran-resistant mutants of a dozen additional genes have been identified in a recent screen (H. Wykes, unpublished), suggesting that growth conditions or varying selective pressures can influence the type of mutations that can result in an increase in tolerance to nitrofurans. It is, therefore, possible that additional oxidoreductases which are not essential are involved in the activation of nitrofurans. This experiment could be extended to screen other oxidoreductases identified in *E. coli* (Figure 3). Alternatively, the knock-out mutations of the non-essential oxidoreductases could be introduced into the triple mutant by P1 generalized transduction, and their MIC determined to test their effect on the nitrofuran susceptibility and/or activation.

8.2. Summary of findings of the effect of 5-nitrofurans on antivirulence phenotypes of *P. aeruginosa*

P. aeruginosa remains the most common pathogen associated with morbidity and mortality in CF patients and is also instrumental in recalcitrant infections in patients with chronic wounds. This formidable pathogen remains one of the most expensive to treat in the intensive care unit at an estimated cost of USD $54 081 compared to susceptible strains, which cost $22 116 (Morales et al., 2012). The increased emergence of MDR *P. aeruginosa* is frequently implicated in failed medical procedures and hinders medical advancements such as using ventilators for CF patients. CF patients often need ventilators as their lungs remain chronically inflamed due to *P. aeruginosa* infections (Davis & di Sant'Agnese, 1978). The multidrug resistance in *P. aeruginosa* is inherently present; however, the pathogen can also acquire and harbor multiple resistance determinants, which are instrumental in influencing its pathogenicity. The virulence factors employed by *P. aeruginosa* are numerous and influence disease severity in immunosuppressed patients. Studies targeting these virulence factors are underway, and "old" antibiotics are used to challenge this pathogen since the bactericidal effect of modern antibiotics is waning against *P. aeruginosa* infections. Previous studies by Baldelli et al. (2020) indicated that the FDA-approved antibacterial, NFZ, showed antivirulence properties against *P. aeruginosa*. This study elucidated that NFZ could suppress pathways that regulate the PqSE protein and, inadvertently, biofilm formation and pyocyanin production (Baldelli et al., 2020). Thus, in this thesis, the analysis of the effect of nitrofurans on model organism *P. aeruginosa* PAO1 was expanded to include FZ to determine its potency and to compare the results with the previously investigated NFZ.

8.2.1. Effect of nitrofurans on the *P. aeruginosa* PAO1 motility

Motility remains one of the most instrumental virulence factors of *P. aeruginosa* as it is required to establish chronic infections, biofilm formation, and QS-mediated pigment production. In this study, two forms of motility were investigated by manipulating the viscosity of LB and BHI agar while supplemented with the agents of interest. Translocation in *P. aeruginosa* is facilitated by the external appendages wherein the polar flagella are responsible for swimming and the type IV pilus for swarming. Another mode of locomotion, twitching, is

initiated by the type IV pilus and could be investigated using 1 % w/v LB agar. In this study, twitching motility was not tested; because the assays aimed to mimic conditions in the human body. However, testing twitching motility in the future could ascertain whether the results conform with those attained in this study, wherein FZ can influence all three modes of pseudomonal locomotion.

Swimming motility

The swimming motility assay showed a significant change in the swim diameters of the three nitrofuran antibiotics tested, as seen in Figure 16. The stark difference in the swim zones of the FZ and NFZ plates (Figure 16A) indicates that FZ is more potent at limiting swimming propulsion on 0.3 % w/v soft LB agar. As previously suggested, the Pseudomonas Quinolone Signal (PQS) that induces Quorum Sensing (QS) system is responsible for the expression of many virulence factors, including swarming and swimming motilities (Baldelli et al., 2020; Shrout et al., 2006). Recent experiments conducted by Chen et al. (2022) reveal that despite an upregulation in the expression of the genes responsible for swimming motility, there was a decrease in the diameter of swim zones of *P. aeruginosa* PAO1 when challenged with FZ. These contradictory results possibly infer that low bacterial populations of cells impacted by subinhibitory concentrations of furazolidone are only capable of promoting the expression of some of the 46 motility-dependent genes of a small section of the bacterial population (Chen et al., 2022; Tremblay & Déziel, 2010). These subinhibitory concentrations were able to inhibit the rest of the bacterial population and hamper the expression of the motility-dependent genes in the rest of the culture (Chen et al., 2022). In agreement with the study by Chen and coworkers, my results confirmed that FZ is indeed capable of reducing the two main modes of translocation of *P. aeruginosa* PAO1. The effect of FZ on *P. aeruginosa* PAO1 on swimming motility is more potent when compared to NFZ as seen in Figure 16B.

Swarming motility

Similarly, the swarming assay corroborated the experiments Chen and coworkers carried out, highlighting that FZ has antivirulence properties against *P. aeruginosa* PAO1, as seen in Figure 17. In many instances, population behaviors such as those employed through swarming require

global regulators, which encode surfactants for better movement throughout viscous environments (Rütschlin & Böttcher, 2020). Extreme disruption of the QS regulon by FZ possibly modulates bacterial migration and disrupts regulatory genes *rhlR*, *lasR*, *pqsR*, and their downstream functional genes (Chen et al., 2022). Gene dysregulation could hinder producing essential surfactants like rhamnolipids, which are necessary for locomotion (Caiazza et al., 2005; Wilhelm et al., 2007). Future work could target the relative expression of genes that encode surfactants needed for chronic colonization.

Biofilm formation and pyocyanin production

P. aeruginosa is notoriously known for producing robust and organized biofilms. These intricately formed structures harbor a community of bacteria that are remarkably resistant to therapeutics. Several studies have suggested biofilm cells are more tolerant to bactericidal antibiotics than planktonic cells within the same culture. With this in mind, a *P. aeruginosa* PAO1 single-species biofilm formation assay was conducted in the presence of FZ at varying concentrations. The effect of FZ on biofilm formation was bi-modal when analyzed in a dose-response manner, with the biofilm density being lowest at 10 and 5 µg/mL of FZ but higher at concentrations above and below (Figure 18). A paradoxical increased growth in the biofilm is shown at the two highest concentrations of FZ, 20 µg/mL and 40 µg/mL, which I reasoned to be because of the Eagle effect, which remains poorly understood (Prasetyoputri et al., 2019). In contrast to the biofilm growth, the dose response of planktonic cells to the increased concentrations of FZ was as expected, with the cell density being the lowest at 40 µg/mL (Figure 18).

Once the planktonic cells were emptied from the microtiter plate, the stained biofilms were subjected to homogenous resolubilization in 1 % w/v crystal violet stain and then measured spectrophotometrically (Figure 18C). The $OD_{595\ nm}$ of the resolubilized cells showed no significant difference between the two highest concentrations tested (column 1- 40 µg/mL FZ and column 2- 20 µg/mL FZ; Figure 18A) and the vehicle control (column 8; Figure 18A). However, the biofilm density along the two-fold serially decreased FZ concentrations from 10 µg/mL to 0.625 µg/mL significantly differed from the two highest concentrations and the vehicle control. These results suggest that the MBIC and biofilm growth are not hampered beyond 10 µg/mL. The planktonic growth curve in Figure 18B determines that the MIC of the

planktonic *P. aeruginosa* PAO1, when challenged with FZ, is slightly higher than the maximum FZ 40 µg/mL tested based on the growth patterns on the planktonic curve. A comparison of the planktonic cell culture (Figure 18B) with the resolubilized biofilm (Figure 18C) revealed that biofilm formation was most robust at 40 µg/mL FZ. This finding is consistent with the reasoning behind the Eagle effect since we found that biofilm formation was markedly increased beyond the suggestive MIC of planktonic cells from the graph which was inferenced to be above 40 µg/mL based on the graph and in keeping with results from experiments by Chen and colleagues (Chen et al., 2022). The Eagle effect is a phenomenon whereby some microorganisms, such as bacteria, when exposed to concentrations above the optimal bactericidal drug concentration (OBDC), have paradoxically improved survival rates in comparison to the OBDC due to a decrease in the rate of cell death (Prasetyoputri et al., 2019). The mechanism behind this phenomenon remains elusive; however, I hypothesized that at higher concentrations than the optimal bactericidal drug concentration, some genes are activated in *P. aeruginosa* PAO1, which is known to be a highly adaptable and metabolically versatile pathogen. However, the testing of this hypothesis to elucidate if *P. aeruginosa* PAO1 can survive beyond the MIC inferred from the graph in Figure 18B remains outstanding.

Interestingly, the cultures in the microtiter plate showed change in the coloration, with the blue green color fading with increased FZ concentrations, an indication of lowered pyocyanin production at higher concentrations. From a visual analysis, as shown in Figure 19, pyocyanin production was most noticeable from right to left (column 8 to column 3). Visual examination of the plate indicated that 20 µg/mL FZ inhibited the production of the blue-green pigment. The pigment production remains to be assessed using a spectrophotometer to elucidate whether pyocyanin production is completely inhibited at that 20 µg/mL FZ.

8.2.2. Limitations of *P. aeruginosa* PAO1 experiments

A key limitation of the biofilm formation assay would be the Edge effect, in which the wells on the perimeter of the plate would lose moisture more rapidly than those in the center (Mansoury et al., 2021). The drying effect of these walls would affect the biofilm densities along the edges and significantly dissuade the results, making the outer biofilm rings more distinct. One way to remedy this would be to place tissues soaked in sterile distilled water into

the incubation chamber to reduce water lost through evaporation or to seal the plate with parafilm.

8.2.3. Conclusions

It is evident from the results obtained in the preliminary experiments that furazolidone is a more potent antivirulence antibiotic in comparison to nitrofurazone. Results from the experiments reveal that some of the most relevant virulence phenotypes of *P. aeruginosa* PAO1 can be inhibited by furazolidone. These findings corroborate with published work and extend the analyses of the 5-nitrofurans' effect and thus set the premise to conduct more experiments.

8.2.4. Future directions

The inhibitory effect of FZ on the virulence factors of *P. aeruginosa* indicates the potential of nitrofurans for use in therapeutic applications. It will be necessary to conduct further investigations to understand the molecular mechanisms of the FZ effect within pre-clinical studies that would indicate use in treatments of pseudomonal infections. Despite being non-inhibitory to the growth at concentrations suitable for human administration, this work demonstrates that sub-lethal concentrations of FZ can reduce the production of virulence factors and, thereby, the pathogenicity of *P. aeruginosa*. Thus, FZ is a promising antivirulence candidate capable of combatting the MDR *P. aeruginosa* in a globally difficult time where AR continues to emerge, and novel antibiotic discoveries are waning.

QS inhibition and metabolite production

Future work using FZ could target virulence factors such as pyocyanin and pyoverdine formation to quantify the observations in this thesis (Figure 19). Pyoverdine and rhamnolipid production are virulence factors that are pertinent to the chronic colonization of *P. aeruginosa* (Pham et al., 2019). The quorum sensing regulon is one of the most influential components of the *P. aeruginosa* genome that directly regulates gene expression of many virulence factors. The transcriptional factors disrupted when *P. aeruginosa* is challenged with FZ are not entirely

understood or known; thus, the mechanism of action and the specific genes involved remain elusive.

QS inhibition and biofilm production

Biofilms are intrinsically more difficult to eradicate than planktonic cells once they are chronically established in CF lungs and chronic wounds. The QS regulon plays an intricate role in biofilm formation; however, whether FZ can eradicate biofilms is still unknown (Zhou et al., 2020). Investigations into biofilm eradication can be assessed using *ex vivo* porcine skin models with established biofilms and whether the anti-virulence properties of FZ are more potent when compared to NFZ (Phillips et al., 2015). Recalcitrant infections are characteristic of CF lungs because it is difficult to completely rid the CF lungs of this organized community of cells. With this in mind, developing a therapeutic which could disperse cells or break biofilms into smaller structures could prove to be helpful in allowing other drugs to penetrate and kill the individual planktonic cells.

Combination therapy

Combination therapies often involve an amalgamation of different therapeutic compounds for treating medical conditions. Combination therapies often contain two or more synergistic compounds which confer enhanced efficacy compared to monotherapy. The utilization of combination therapies lessens the likelihood of antibiotic resistance in formidable pathogens such as *P. aeruginosa*, as seen in β-lactam and quinolone combinations. With this in mind, synergistic combinations involving furazolidone and other anti-pseudomonal drugs, such as tobramycin, could be investigated in checkboard assays to assess the MIC of the pathogen against various drug combinations.

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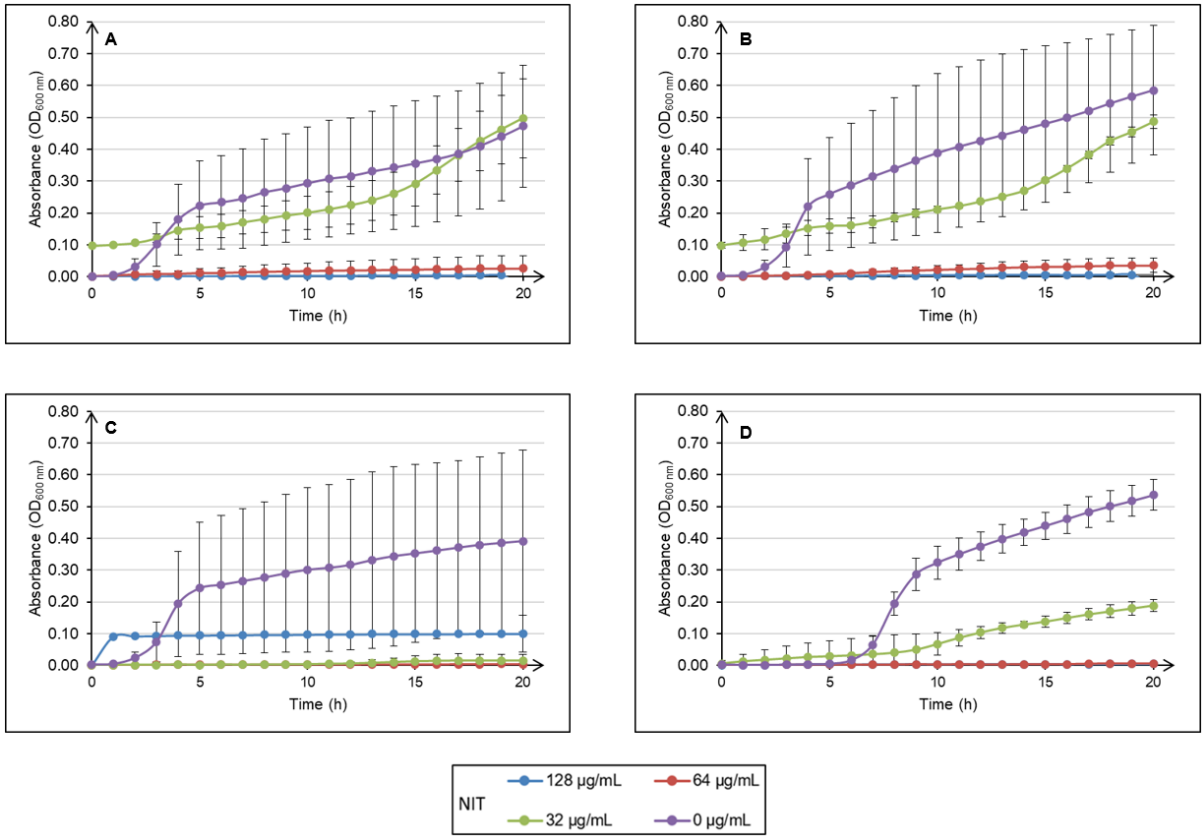
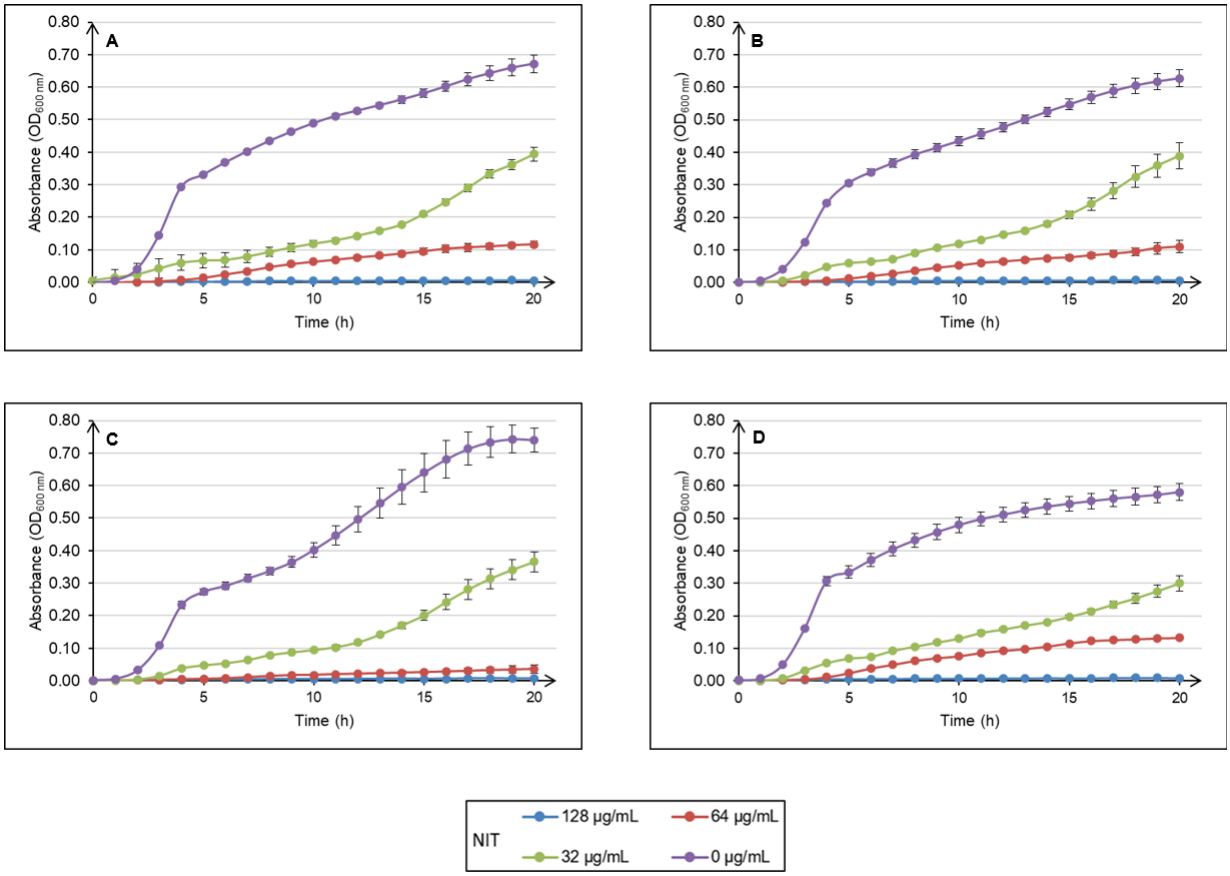
Appendix 1- MIC plots of K2610, K2616, K2617, and K2618 with varying concentrations of NIT



Determination of minimum inhibitory concentration for nitrofurantoin of *E. coli* strains K2610, K2616, K2617, and K2618 complemented with the candidate nitroreductase-encoding genes.

MIC testing at varying concentrations of nitrofurantoin for *E. coli* K2506 triple mutants transformed with (A) pCA24N::*cydA* (K2609), (B) pCA24N::*folD* (K2627), (C) pCA24N::*hemA* (K2628). Each plotted value represents the average of three replicates, and the error bars represent the standard deviation from the mean.

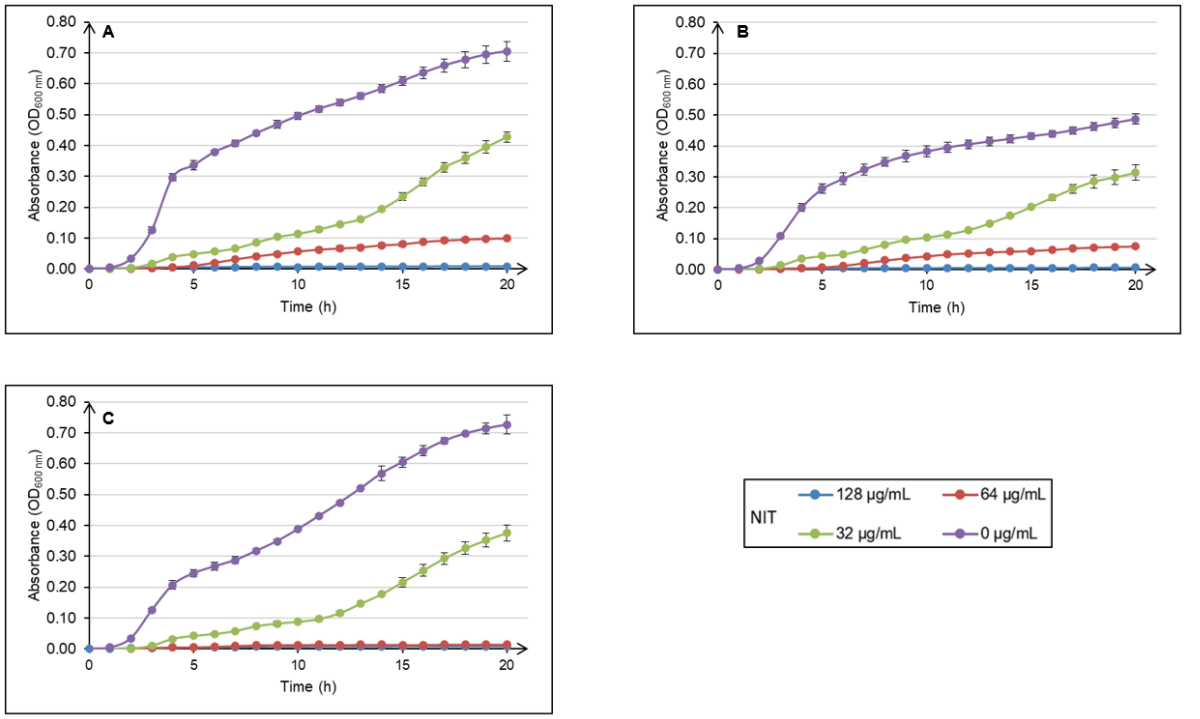
Appendix 2- MIC plots of K2620, K2621, K2622, and K2623 with varying concentrations of NIT



Determination of minimum inhibitory concentration for nitrofurantoin of *E. coli* strains K2620, K2621, K2622, and K2623 complemented with the candidate nitroreductase-encoding genes.

MIC testing at varying concentrations of nitrofurantoin for *E. coli* K2506 triple mutants transformed with (A) pCA24N::*dapB* (K2620), (B) pCA24N::*folA* (K2621), (C) pCA24N::*ispH* (K2622) and (D) pCA24N::*asd* (K2623). Each plotted value represents the average of three replicates, and the error bars represent the standard deviation from the mean.

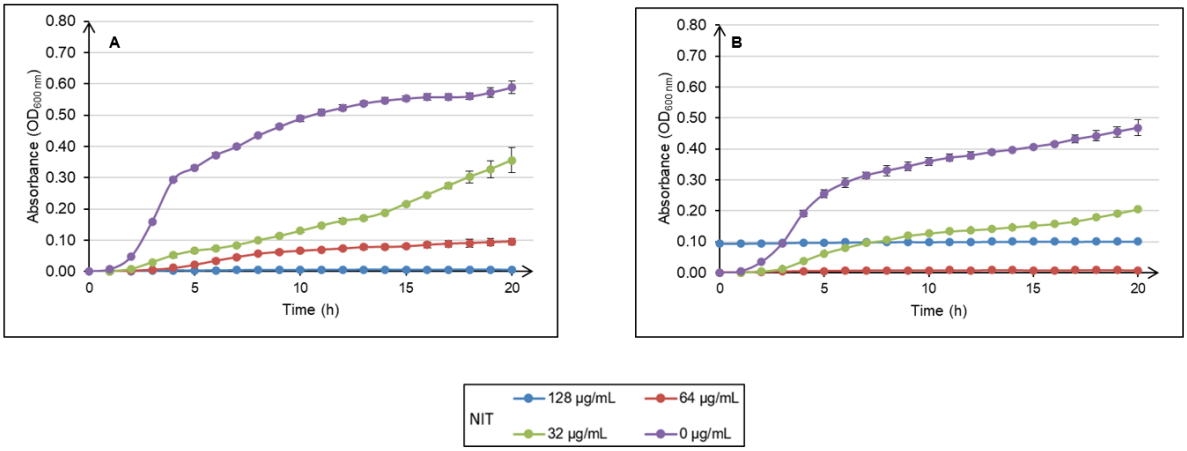
Appendix 3- MIC plots of K2624, K2625, and K2626 with varying concentrations of NIT



Determination of minimum inhibitory concentration for nitrofurantoin of *E. coli* strains K2624, K2625, and K2626 complemented with the candidate nitroreductase-encoding genes.

MIC testing at varying concentrations of nitrofurantoin for *E. coli* K2506 triple mutants transformed with (A) pCA24N::*dxr* (K2624), (B) pCA24N::*fabG* (K2625), and (C) pCA24N::fabI (K2626). Each plotted value represents the average of three replicates, and the error bars represent the standard deviation from the mean.

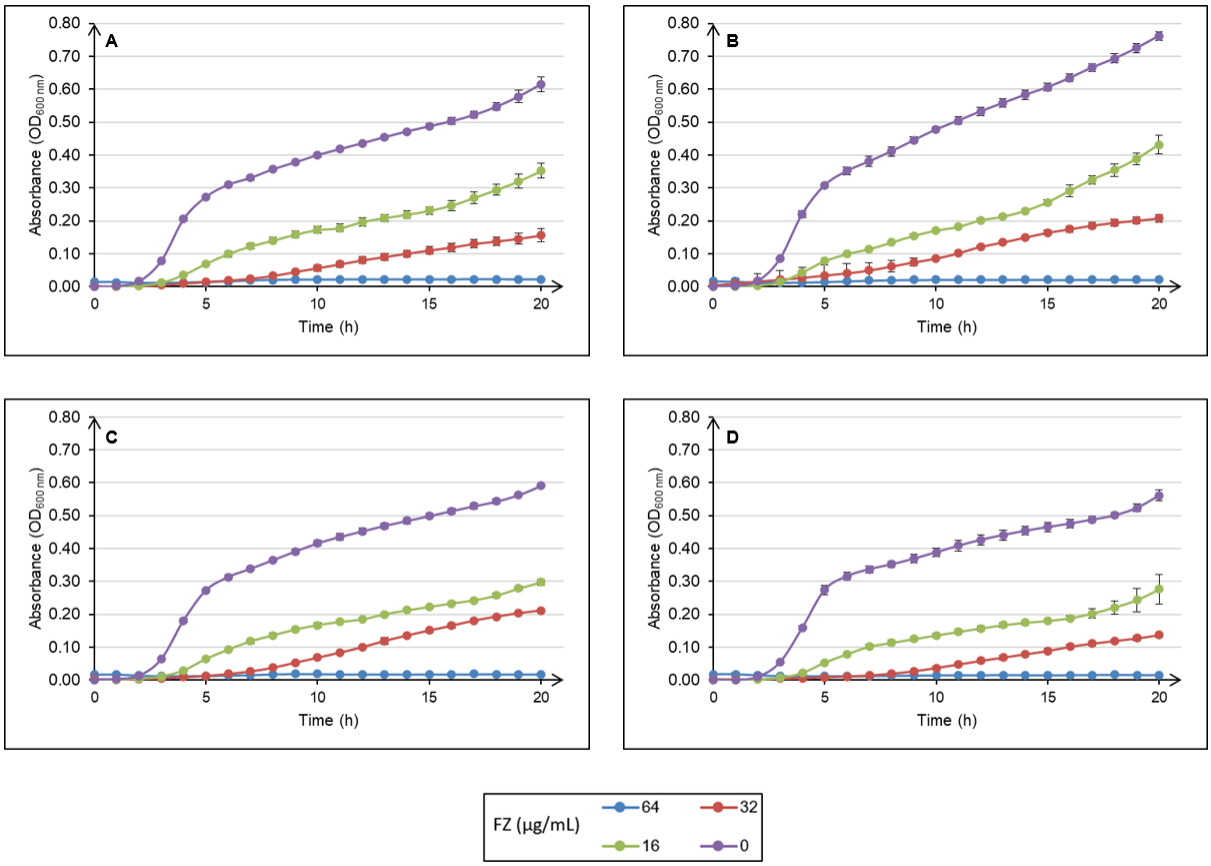
Appendix 4- MIC plots of K2630 and K2632 with varying concentrations of NIT



Determination of minimum inhibitory concentration for nitrofurantoin of *E. coli* strains K2630 and K2632 complemented with the candidate nitroreductase-encoding genes.

MIC testing at varying concentrations of nitrofurantoin for *E. coli* K2506 triple mutants transformed with (A) pCA24N::*ispG* (K2630) and (B) pCA24N::*nrdA* (K2632). Each plotted value represents the average of three replicates, and the error bars represent the standard deviation from the mean.

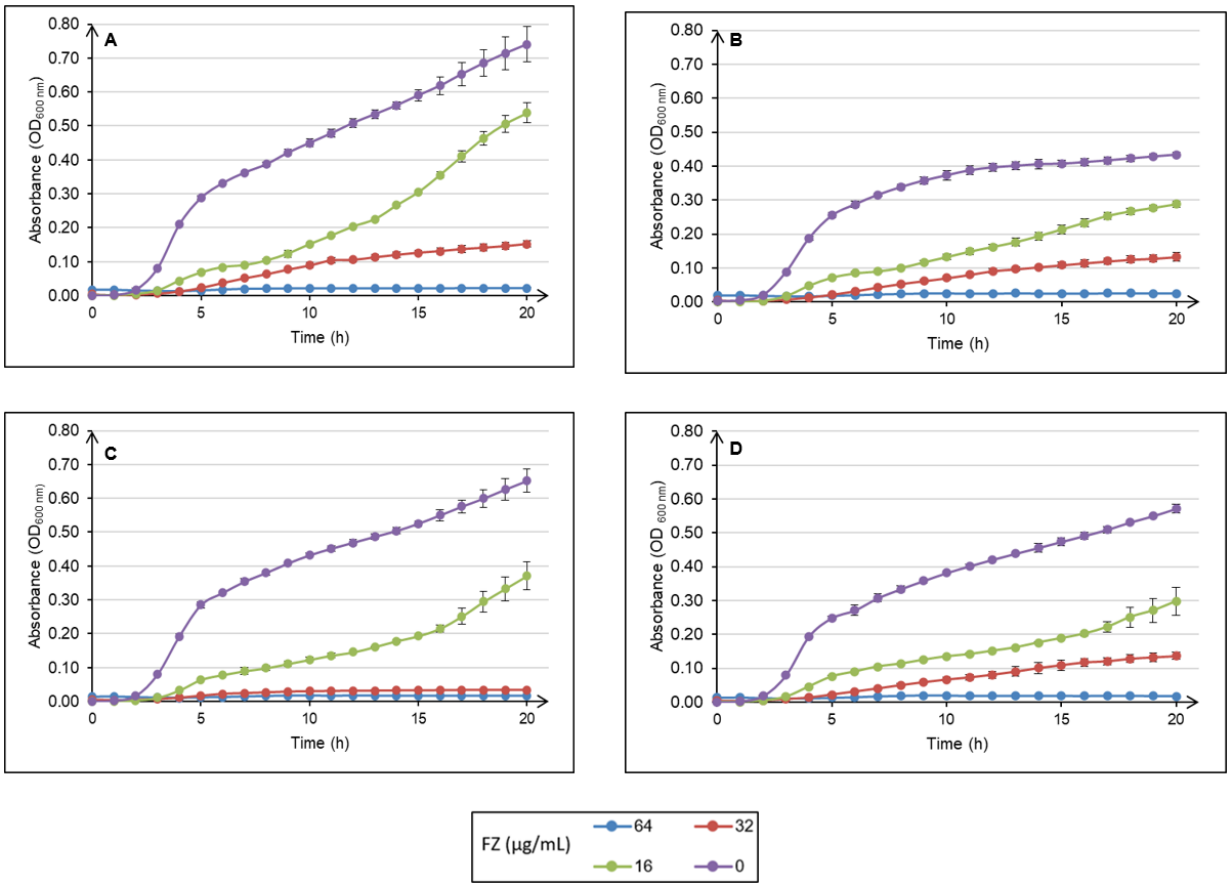
Appendix 5- MIC plots of K2610, K2616, K2617, and K2618 with varying concentrations of FZ



Determination of minimum inhibitory concentration for furazolidone of *E. coli* strains K2610, K2616, K2617, and K2618 complemented with the candidate nitroreductase-encoding genes.

MIC testing at varying concentrations of furazolidone for *E. coli* K2506 triple mutants transformed with (A) pCA24N::*ribD* (K2610), (B) pCA24N::*gapA* (K2616), (C) pCA24N::*gpsA* (K2617) and pCA24N::*nrdA* (K2618). Each plotted value represents the average of three replicates, and the error bars represent the standard deviation from the mean.

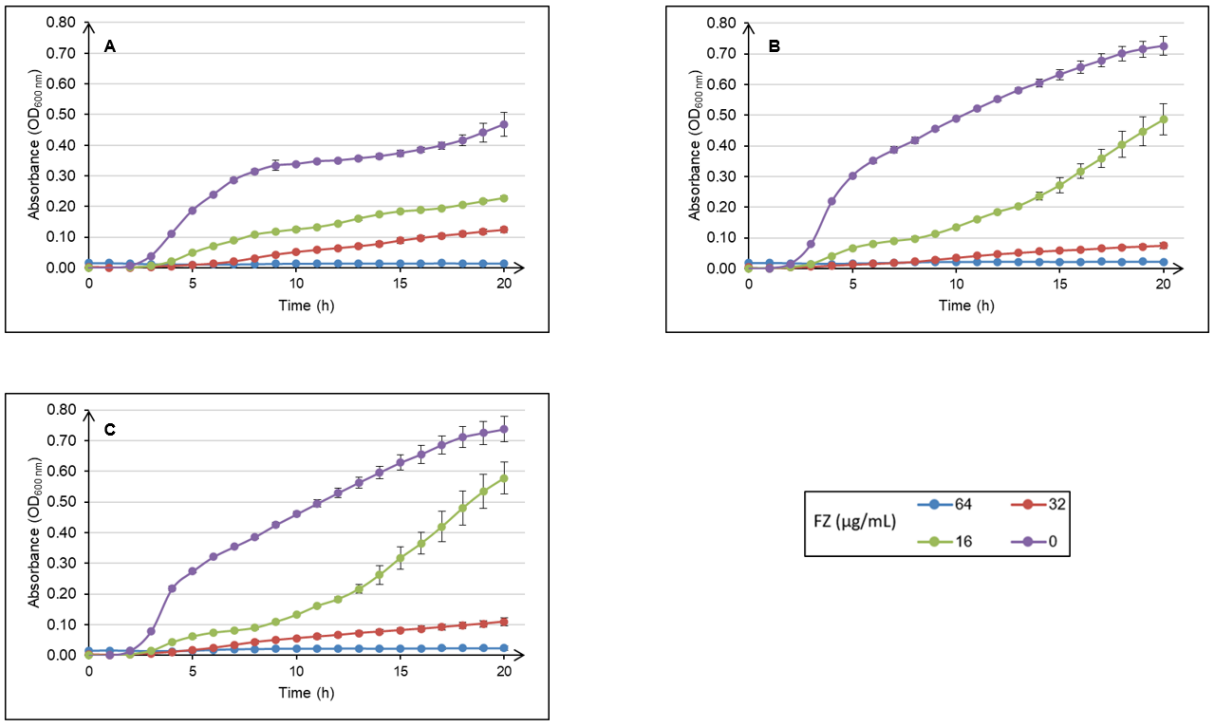
Appendix 6- MIC plots of K2620, K2621, K2622, and K2623 with varying concentrations of FZ



Determination of minimum inhibitory concentration for furazolidone of *E. coli* strains K2620, K2621, and K2622 complemented with the candidate nitroreductase-encoding genes.

MIC testing at varying concentrations of furazolidone for *E. coli* K2506 triple mutants transformed with (A) pCA24N::*dapB* (K2620), (B) pCA24N::*folA* (K2621), and (C) pCA24N::*ispH* (K2622) and pCA24N::*asd* (K2623). Each plotted value represents the average of three replicates, and the error bars represent the standard deviation from the mean.

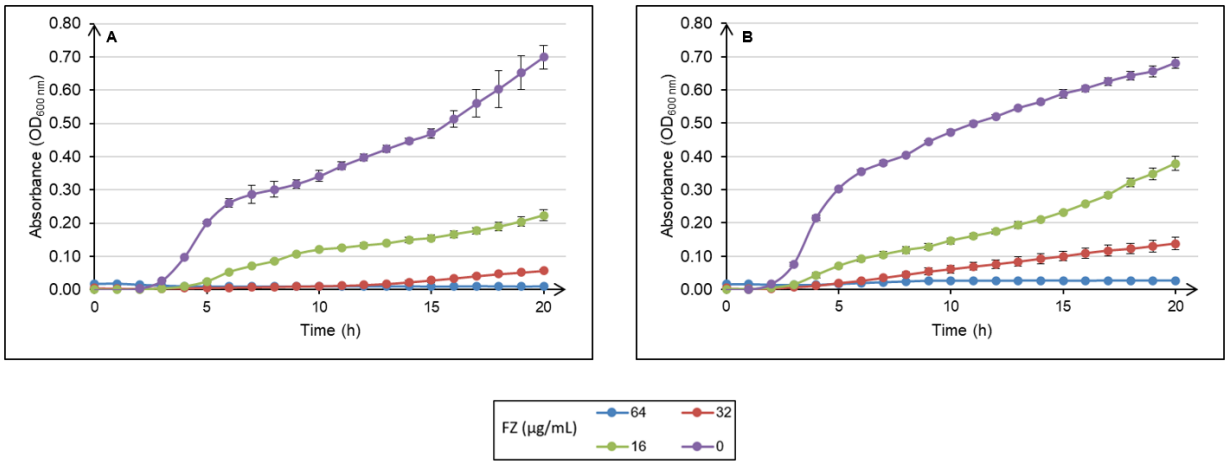
Appendix 7- MIC plots of K2624, K2625, and K2626 with varying concentrations of FZ



Determination of minimum inhibitory concentration for furazolidone of *E. coli* strains K2624, K2625, and K2626 complemented with the candidate nitroreductase-encoding genes.

MIC testing at varying concentrations of furazolidone for *E. coli* K2506 triple mutants transformed with (A) pCA24N::*dxr* (K2624), (B) pCA24N::*fabG* (K2625), and (C) pCA24N::*fabI* (K2626). Each plotted value represents the average of three replicates, and the error bars represent the standard deviation from the mean.

Appendix 8- MIC plots of K2630 and K2632 with varying concentrations of FZ



Determination of minimum inhibitory concentration for furazolidone of *E. coli* strains K2630 and K2632 complemented with the candidate nitroreductase-encoding genes.

MIC testing at varying concentrations of furazolidone for *E. coli* K2506 triple mutants transformed with (A) pCA24N::*ispG* (K2630) and (B) pCA24N::*nrdA* (K2632). Each plotted value represents the average of three replicates, and the error bars represent the standard deviation from the mean.