E. COLI PERSISTER CELL SURVIVAL AND RHIZOBIA ATTACHMENT TO SOYBEAN ROOTS

BY

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THESIS ACCEPTANCE PAGE

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This thesis is approved as a creditable and independent investigation by a candidate for the master's degree and is acceptable for meeting the thesis requirements for this degree. Acceptance of this does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

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ABBREVIATIONS

Abbreviation	Definition
α	Alpha
β	Beta
bu/ac	Bushels per acre
°C	Degree Celsius
µ	Micro
M	Milli
ABC	ATP-binding cassette
Amp	Ampicillin
AmpC	Enzymes-overexpressed cephalosporinases
AST	Antibiotic susceptibility test
BNF	Biological nitrogen fixation
CDC	Center for Disease Control
Cip	Ciprofloxacin
CFU	Colony-forming unit
EPS	Exopolysaccharides
ESBLs	Extended-spectrum β-lactamases
GFP	Green fluorescent proteins
Glu-Pyr	Glucose-Pyruvate
Gly-Pyr	Glycerol-Pyruvate
Km	Kanamycin
LB	Luria Bertani
LCO	Lipo-chitooligosaccharide

PBPs	Penicillin-binding proteins
MATE	Multidrug and toxic compound extrusion
MBC	Minimum bactericidal concentration
MDK	Minimum duration of antibiotic exposure
MFS	Major facilitator superfamily
MIC	Minimum inhibitory concentration
MMB+	MMB media with 20 amino acids
N_2	Nitrogen
NH_3	Ammonia
NFR	Nod Factor Receptors
OD	Optical density
RND	Resistance-nodulation-cell division
ROS	Reactive oxygen species
RpoS	RNA polymerase, sigma S
SDM	Strongly dependent on metabolism
SMR	Small multidrug resistance
SOS	Save our soul
TA systems	Toxin-antitoxin system
TEM	Transmission electron microscopy
VBNC	Viable but non culturable
WDM	Weakly dependent on metabolism
WHO	World Health Organization

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ABSTRACT

E. COLI PERSISTER CELL SURVIVAL AND RHIZOBIA ATTACHMENT TO SOYBEAN ROOTS

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The theme of this thesis revolves around how bacteria respond and thrive during stress. Chapters 1-3 are about how bacteria deal with life-threatening antibiotics. Chapter 4 covers new research on how bacteria can move from a stressful individual lifestyle (free-living bacteria) to initiating a symbiotic relationship with a plant (a less stressful lifestyle). In Chapter 1, I briefly summarize the current state of knowledge in the field of antibiotic resistance and persistence. In Chapter 2, I add to this knowledge by providing new insights into several antibiotics' potency and exploring the antibiotic Eagle effect. In Chapter 3, I use pyruvate to study the revival of persister cells. Finally, in Chapter 4, I switch gears and briefly discuss how I optimized the initial steps of soybean germination and rhizobia culturing techniques to monitor the root-bacterial attachment.

1. CHAPTER 1. LITERATURE REVIEW.

1.1. Background and significance

The discovery of antibiotics in the 20th century was a milestone in medicine as it led to millions of lives saved [1]. However, this medical advancement is threatened by a rise in antibiotic-resistant bacteria and is a global threat to humans and animals. This is a growing problem because of the lack of new antibiotics and because some bacteria can develop antibiotic resistance quickly. For example, erythromycin was introduced in 1952 to treat *Staphylococcus aureus* infections. In less than a year, about 70% of the *S. aureus* isolated were erythromycin-resistant [1]. Antibiotic resistance is not only a problem for those already infected, but antibiotics are also used to prevent diseases, e.g. they stop bacteria from making a foothold during surgery [1]. Misuse of antibiotics in humans and animals, and poor control and prevention techniques are leading to more antibiotic-resistant bacteria [2]. In 2019, the Centers for Disease Control and Prevention (CDC) reported that more than 2.8 million antibiotic-resistant infections occur each year in the USA. This number is predicted to increase [3]. Sadly, more than 35,000 people are killed in the USA by antibiotic-resistant infections each year [4], and this number is also predicted to increase [3]. According to a collaborative study by CDC, the national cost to treat infections caused by the six major multi-resistant pathogens is about $4.6 billion. It is imperative that we understand the mechanisms that allow for antibiotic resistance so we can develop new drugs and preventative measures to save lives and reduce the economic impact on society. My goal is to study bacterial antibiotic persistence because they mutate at a high rate and are a driving force of antibiotic resistance.

1.2. Antibiotic resistance

Alexander Fleming discovered penicillin in 1928 [4], and this single antibiotic has saved more than 200 million lives [4]. Though Alexander Fleming supported using antibiotics worldwide, he also raised awareness against the misuse of antibiotics by emphasizing that these drugs should be consumed in adequate doses and only when prescribed after clinical diagnosis [4]. Antibiotics are clinically essential drugs, but they have also been effective in non-clinical aspects such as treating blight in apple and pear orchards and enhancing growth in livestock [5]. However, the rate in the development of antibiotic resistance overtakes the pace of discovery of novel drugs [6]. With a rise in the frequency of antibiotic resistance and the spread of resistance genes, the treatment and prevention of bacterial infections are becoming intricate [7].

Antibiotic resistance is a genetic change that allows them to survive or grow in the presence of antibiotics [1]. In this review, I describe the different types (1.3.) and mechanisms (1.4.) of antibiotic resistance and types of antibiotics and their targets (1.5.). I will also briefly describe what we already know about antibiotic resistance (1.6.) and persistence (1.7.).

1.3. Types of antibiotic resistance

1.3.1. Natural resistance

Natural resistance is present due to the structural characteristics of the bacteria. (they do not need to acquire this type of resistance). This type of resistance exists in the bacteria because the bacterial cell lacks the antibiotic's target site or because the antibiotic fails to reach the target site [8]. For example, *Mycoplasma*, which naturally lacks a cell wall, is not killed by β-lactam antibiotics such as penicillin; all β-lactam target the cell

wall [8]. Natural resistance in bacteria can be exhibited in two ways, either intrinsically or induced. In intrinsic resistance, the genes are always present in the organism independent of any previous antibiotic exposure and not acquired by horizontal gene transfer. In induced resistance, the expression of resistant genes is caused by exposure to an antibiotic [9, 10]. Both intrinsic resistance and induced resistance often rely on efflux pumps. However, intrinsic resistance often employs reduced outer-membrane permeability [9, 11]. Intrinsic resistance has been well-studied in, *E. coli* against macrolides, *Klebsiella spp* against ampicillin, and all Gram-negative bacteria against glycopeptides and lipopeptides [12].

1.3.2. Cross-resistance

Cross-resistance is resistance to antibiotics of the same class by a single molecular mechanism. It occurs when different antibiotics either use the same target or the same route to access the target to inhibit cellular growth or induce death [13]. One of the examples of cross-resistance is the presence of the AcrAB–TolC efflux pump; this pump can provide resistance against a range of antimicrobials including metals and dyes [14]**.**

1.3.3. Acquired resistance

Acquired resistance can occur either by receiving DNA by horizontal gene transfer through transduction (virus-mediated), transformation (take-up "naked" DNA), and conjugation (bacterial cell "mating"), or by mutations in the chromosomal DNA [12]. The transfer of genetic materials via plasmids is a common mechanism for acquiring resistant genes [10]. For example, the transfer of the *mecA* gene on a plasmid is one method of gaining resistance to MRSA (Methicillin-resistant *Staphylococcus aureus*), leading to one of the most familiar infections in humans [5].

A mutation is a change in the nucleotide sequence of the organism's DNA. Mutations that confer antibiotic resistance can often occur in specific genes such as genes encoding drug transporters, genes encoding drug targets, and genes encoding regulators controlling drug transporters [10]. Mutations can lead to changes in the transcription and translation rate [5]. In addition, mutations can alter protein structures leading to resistance [5]. Some mutations can confer resistance by enabling the bacteria to produce enzymes that inactivates the drugs, proteins that export the antibiotics outside the cell through efflux pumps, by altering the cell structure targeted by the antibiotic, or by decreasing the permeability of the antibiotic (thus less antibiotics can enter the cell and cause damage) (Fig. 1) [15]. Environmental factors can also promote resistance; resistant organisms have been discovered for all clinically used antibiotics [7]. Even though antibiotic resistance is the primary reason for the bacteria to survive antibiotic treatments, another phenomenon that is highly responsible and often underestimated as an aspect of treatment failure is antibiotic persistence [3].

1.4. Mechanisms of resistance

Here I will describe four mechanisms of resistance and their importance to persistence.

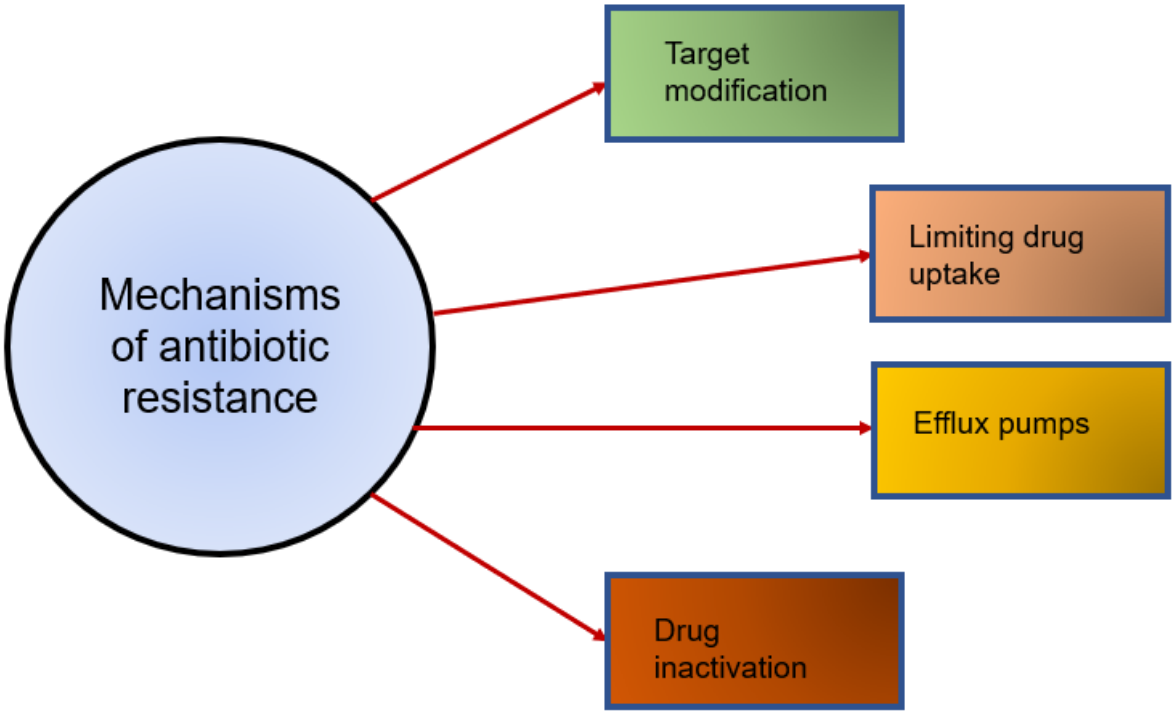


Fig. 1. Common mechanisms to acquire antibiotic resistance.

1.4.1. Drug target modification

There are several components in the bacterial cell that may be the targets of antibiotics and bacteria can enable resistance against them by modifying these target sites [12]. The modification of the penicillin-binding proteins (PBPs) in the peptidoglycan layer of the cell wall is a common mechanism which Gram-positive bacteria use to resist β-lactam drugs such as penicillin and ampicillin. They can increase the number of PBPs,

which in turn, leads to effective dilution of β-lactam. β-lactam bind irreversibly to PBPs and cannot disrupt cell wall formation; thus, increasing the number of PBP decreases the effective concentration of β-lactam. A common treatment for dealing with this type of resistance is to increase the amount of β-lactam prescribed. Alternatively, bacteria can resist β-lactam by decreasing the number of PBPs, which in turn, leads to a decrease in the binding sites. Another mechanism used by the bacteria is by changing the structure of PBPs, which hinders the binding of the β-lactam [16, 17].

Glycopeptides like vancomycin and lipopeptides (e.g. daptomycin) target the bacterial cell wall and cell membrane, respectively. Therefore, gram-negative bacteria may acquire intrinsic resistance against these antibiotics [18]. In the case of vancomycin, bacteria acquire *van* genes. These genes alter the drug binding ability by changing the structure of the peptidoglycan layer of the cell wall [9, 16]. In contrast, drugs like daptomycin can alter the charge of the cell membrane surface to positive. This is acquired due to mutations in genes such as *mprF* [19, 20]. As daptomycin requires calcium for binding, this mutation inhibits calcium-binding, thus inhibiting the drug's binding to its target [19, 20].

Aminoglycosides and oxazolidinones are antibiotics that inhibit protein synthesis. Resistance against these antibiotics is enabled by acquiring ribosomal mutations that allow alteration in the ribosomal subunits. Methylation of the ribosomal subunits is another mechanism bacteria use to survive aminoglycosides and macrolides. The methylation interferes with the binding ability of these drugs to the ribosome [21, 22].

Other drugs like fluoroquinolones such as ciprofloxacin, target nucleic acid synthesis. Resistance is enabled by mutations that lead to modifications in DNA gyrase or topoisomerase IV structures, resulting in the drugs' inability to bind to these targets [23].

1.4.2. Limiting uptake of drugs

Bacteria can have natural properties that enable them to limit the uptake of certain antibiotics. For example, hydrophilic drugs have minimal access to mycobacteria as they have an outer membrane with high lipid content [24, 25]. Also, *Mycoplasma* does not have a cell wall, making antibiotics that target the cell wall (e.g. β-lactams, glycopeptides) ineffective [26]. Bacteria belonging to enterococci, have intrinsic resistance against aminoglycosides. Like all Gram positive bacteria, they do not have an outer cell membrane, making it difficult for the polar drug molecules to penetrate through the cell wall [25].

Bacteria with large outer membranes may frequently use porin channels to uptake substances. Gram-negative bacteria usually uptake hydrophilic molecules through these porin channels [27, 28]. Changes in these porin channels can limit the uptake of certain drug molecules. There are two ways: 1) These porin channels can be modified to inhibit drug uptake by decreasing the number of porins available for the drugs to access, or 2) by altering the selectivity of these channels (caused by mutations) [24].

The formation of biofilm structures can help protect bacteria from antibiotics. Biofilms are large communities formed by bacterial colonization and may contain a wide variety of organisms in this community [29-31]. Biofilms can protect pathogens from the defensive mechanisms of the host as well as from antimicrobial agents. Drugs may fail to penetrate the thick, sticky biofilm matrix and reach their target pathogen, and therefore much higher drug concentrations might be required to target such pathogens. Another property of the biofilm is that some cells in the matrix have slowed metabolism; hence drugs that target growing cells are less effective [29-31]. A major reason biofilm can

withstand normally lethal concentration of antibiotics compared to free-living bacteria is that about 1% of the cells in a biofilm are in the antibiotic persister state [32, 33]. Later, I will describe the importance of persistence, how this state allows bacteria to survive long-term exposure to antibiotics, and how this state drives antibiotic resistance evolution.

1.4.3. Drug efflux pumps

Efflux pumps are channels that function to export toxic molecules (antibiotics) out of the cell. Some of these efflux pumps can be expressed constitutively (the genes are transcribed nearly continuously), and some can be induced by the environment [27, 34]. Bacteria can possess multiple types of efflux pumps. There are five families of efflux pumps: multidrug and toxic compound extrusion (MATE), major facilitator superfamily (MFS), ATP-binding cassette (ABC), small multidrug resistance (SMR), and the resistance-nodulation-cell division (RND) [24, 27, 35, 36].

In Gram-negative bacteria, all the five families of efflux pumps are widely distributed, with the most significant flux belonging to the RND family [27, 37]. The RND pumps in Gram-negative organisms associate with an outer membrane porin protein and a periplasmic membrane fusion protein, to export substrates out of the cell envelope [24, 27, 35, 36]. Gram-positive bacteria have intrinsic resistance as they have genes for efflux pumps encoded in their chromosomes. They use MFS and MATE pump families to efflux antibiotics belonging to fluoroquinolones [37-40]. The ABC efflux family is unique as they function by deriving energy from ATP hydrolysis. These pumps contain both an uptake and export system. Bacteria use them to transport ions, drugs, sugars, proteins, amino acids, and polysaccharides. These pumps can be found in *Vibrio cholerae* used to transport tetracycline and fluoroquinolones [24, 41, 42].

A recent study showed that the efflux pump AcrAB-TolC (from the RND family) allows

E. coli persisters to survive the fluoroquinolone delafloxacin [43]. In genetically identical bacterial populations, the genes *acrAB* and *tolC* are expressed heterogeneously, and the cells with higher expression have reduced mutation frequency and antibiotic susceptibility [44, 45]. Also, in *E. coli* populations treated with β-lactam drugs, higher expression of AcrAB-TolC promotes persister formation and reduces the antibiotic accumulation in growing cells [46]. However, we still do not know the role of other families of efflux pumps in *E. coli* persister cell formation.

1.4.4. Drug inactivation

One essential mechanism of bacteria to confer resistance is by inactivating the drug molecules. They can do this in two ways, either by degrading the drug molecule or adding a chemical group to the drug molecule that hinders or deactivates the drug. β-lactamase can hydrolyze (degrade) β-lactam antibiotics [15, 24]. Bacteria will attach chemical groups such as adenyl, acetyl, and phosphoryl groups to inactivate the antibiotic. While phosphorylation and adenylation are used to inactivate aminoglycosides, acetyls are widely used against drugs of several classes such as fluoroquinolones, aminoglycosides, and streptogramins [15, 47-49].

1.5. Different types of antibiotics and their targets

I will focus on the few particular classes of antibiotics described in Table 1. However, persisters and antibiotic-resistant bacteria have been detected in all antibiotic classes [50].

Table 1. Different classes of antibiotics and their targets.

Antibiotic class	Mode of action	Common examples
B-lactams	Inhibits cell wall synthesis	Ampicillin, penicillin
Quinolones/ Fluoroquinolones	Inhibits DNA synthesis	Ciprofloxacin
Glycopeptides	Inhibits cell wall synthesis	Vancomycin
Aminoglycosides	Inhibits protein synthesis	Gentamicin, kanamycin
Polymyxins	Changes in cell membrane permeability	Polymyxin B

1.5.1. Bacterial metabolism affects antibiotic efficacy

The efficacy of antibiotics against bacteria can be associated with the cellular metabolism in three ways [51]. First, the antibiotic inhibits or kills the bacteria by altering the metabolic state of bacteria. Second, the susceptibility of the bacteria to the antibiotic depends on the metabolic state of the bacteria. For example, non-growing *E. coli* at 4° C are largely unaffected by the addition of β-lactam antibiotics [51]; cell wall synthesis occurs when the cell is multiplying but at 4° C cells are not multiplying. Third, altering the bacterial metabolic state, can alter the antibiotic efficacy [51], and an example of this is bacterial persistence.

Persister cells are a subpopulation that survive lethal exposures to antibiotics. These cells are metabolically repressed and genetically identical to the susceptible population [52]. As antibiotics are key to treat recurring infections caused by these persister cells, it is of high importance that the metabolic state of the persister cells is considered when employing antibiotic therapies. The dependence on metabolism by the most commonly used antibiotic classes, such as aminoglycosides, β-lactams, and quinolones, reduce their efficacy against chronic and recurrent infections [53, 54]. Some examples of antibiotics that are strongly dependent on metabolism (SDM) are ampicillin and ciprofloxacin [55]. The aminoglycoside, gentamicin is a unique case, as at lower concentrations it showed properties of being strongly dependent on metabolism (SDM), while at higher concentrations it behaved like an antibiotic that is weakly dependent on metabolism (WDM) [55]. Some examples of WDM antibiotics, that retain their efficacy are colistin [56, 57] and mitomycin C [58]. These antibiotics are favorable to treat recurring infections as they are effective in different metabolic states. However, their use

in clinical applications are limited as they are often toxic to human cells at therapeutic levels [59]. The differences in the efficacy of these antibiotics in association with the bacterial metabolic state shall be taken into consideration in order to harness treatment against recalcitrant infections.

1.6. Antibiotics and their classification

Antibiotics are agents that are used to treat bacterial infections and they can be classified into numerous groups based on several criteria. Here, I will talk about the classification of antibiotics on the basis of their modes of action (Table 1).

Antibiotics are classified based of mechanism of actions such as inhibitors of protein synthesis, nucleic acid synthesis, cell wall synthesis and cell membrane function [60]. Cell wall synthesis in bacteria is inhibited by interrupting the formation of the peptidoglycan layer. B-lactams such as penicillin, exert their activity by binding to the receptors in the cell membrane known as penicillin binding proteins (PBP) [60, 61]. For instance, ampicillin binds in the PBP binding site and can form multiple hydrogen bonds with the amino acid residues such as serine and aspartic acid [61]. Protein synthesis is an essential function in cell survival and hence is an integral target of the antibiotics. Depending on their type, antibiotics can bind to either the 30S or the 50S ribosomal subunit and inhibit the cellular translation [60]. They act by interrupting with either initiation, elongation or termination steps of protein synthesis [62]. Some of the classes that can target protein synthesis are aminoglycosides, macrolides, clindamycin, tetracyclines, etc. [60]. Antibiotics targeting nucleic acid synthesis impair the activity of the enzymes responsible for DNA or RNA synthesis. Rifamycin can inhibit RNA synthesis and lead to loss in cell viability by interrupting the action of RNA polymerase

[62, 63]. DNA synthesis inhibitors like quinolones can impair the activity of enzymes such as DNA gyrase and topoisomerase IV [64] . Antibiotics like daptomycin can disrupt the function of the bacterial cell membrane by altering the membrane potential resulting in the redirection of the proteins involved in cell wall synthesis and cell division [65].

1.7. Antibiotic resistance in *E. coli*

E. coli can be a causative agent of severe infections in humans and animals [66]. *E. coli* also is an important member of the normal flora of the human gut and some other mammals [67]. Resistant *E. coli* strains are often transmitted through animals and humans either by direct contact, through the food chain, or via animal excretions [66]. Over the decades, resistant *E. coli* has been a major challenge and is a vital public health concern [66].

1.7.1. Resistance to β-lactams

Several genes allow *E. coli* to be resistant to β-lactam. β-lactamases degrade β-lactam, while repressed cephalosporinases (AmpC), extended-spectrum β-lactamases (ESBLs) and carbapenemases provide broad term resistance against β-lactams [66, 68]. *E. coli* possessing ESBLs can confer resistance against penicillin, aminopenicillin, and cephalosporin. They are of critical importance in veterinary medicine as they cause treatment failure as the incidence of these strains are increasing in food producing animals [68]. Class C β-lactamases, also known as AmpC-type enzymes can provide high level resistance to *E. coli* against cephalosporins [69]. These plasmid-encoded enzymes are categorized into different types such as DHA-, CMY- and ACC-type [70]. Majority of the animals possess the CMY-type enzyme [71, 72].

1.7.2. Resistance to quinolones/fluoroquinolones

Quinolones are antibiotics that work against DNA gyrase and most effective against Gram-negative bacteria [73]. Fluoroquinolones are quinolones derivatives and most often target topoisomerase IV and DNA gyrase. This class of antibiotics can be used to treat infections in both animals and humans. *E. coli* conferring resistance against these antibiotics possess mutations in the genes of the two drug targets, namely the DNA gyrase and topoisomerase IV [73]. In *E. coli*, DNA gyrase have two GyrA subunits, whereas topoisomerase IV has two ParC subunits. Single mutations in the gyrA gene leads to resistance against quinolones, whereas to confer resistance against fluroquinolones, mutations in gyrA and/or parC are required [73]. Apart from mutations, plasmid-encoded resistance against these drugs is common in *E. coli*. Multiple plasmid-mediated resistance mechanisms have been associated, that can prevent the quinolone from binding its target, for example, enzymatic inactivation of drugs such as ciprofloxacin and presence of active efflux pumps [74].

1.7.3. Resistance to aminoglycosides

Aminoglycosides are bactericidal antibiotics, frequently used in a combination with other antibiotics to treat severe infections such as urinary tract infections, meningitis, sepsis and pneumonia in both humans and animals [66]. As these drugs target cellular translation (Table 1), resistance against these drugs in *E. coli* can be conferred by either mutation in the 16S RNA subunit and/or mutations in the S5 and S12 ribosomal proteins [75-77]. Furthermore, the drug target can also be modified by methylation of the G1405 and A1408 residues of the 16S RNA, conferring high resistance against antibiotics like gentamicin, tobramycin and amikacin [76]. Also, the drug can be rendered

inactive by enzymatic action, thus inhibiting its binding with the target site. These modifications in *E. coli* can be done by the three different types of enzymes, namely phosphotransferases, nucleotidyltransferases and acetyltransferases [66].

1.7.4. Prevention and control of antibiotic resistance

The WHO has provided guidelines to help prevent and minimize the spread on antibiotic resistance [78]. These include:

1. Antibiotics should not be consumed unless prescribed.

2. Hands must be washed regularly, and food shall be prepared in a hygienic environment.

3. Vaccinations shall be taken when required.

4. Safe distance shall be maintained from sick people.

These guidelines were designed to minimize the speed at which antibiotic resistance developed. However, these steps will not stop the spread of resistance. A better understanding of the mechanisms that allow resistance, specifically antibiotic tolerance and persistence (discussed later), may allow better control of resistance development.

1.8. The minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) determine the susceptibility of the antibiotics

It is essential to determine the susceptibility of pathogens against antibiotics, to combat the frequent rise of resistance against these drugs. It is an important aspect of clinical microbiology laboratories in order to determine the choice of drugs for particular infections [79]. For some bacterial isolates, empirical therapy can be affective as resistance has not been identified, however, for majority of the isolates a susceptibility detection is necessary if they have mechanisms for acquired resistance [79]. Antimicrobial/antibiotic susceptibility testing (AST) can lead to the discovery of novel drugs and can also play a significant role in epidemiology [80]. New drug discovery is a goal in order to combat the rising multidrug tolerant bacterial species. Some major sources of natural drug molecules are eukaryotic microbes, prokaryotes and plants [81]. Even though a handful of diverse compounds has been synthesized [82], it is strenuous to compare between the efficacy of these molecules due to non-standardized approaches [80]. The results of antibiotic susceptibility can be either qualitative (categorized into areas of sensitive, intermediate or resistant) or quantitative conveyed as minimum inhibitory concentration (MIC) [83] and minimum bactericidal concentration (MBC). MIC is defined as the minimum antibiotic/drug concentration required to inhibit the distinguishable bacterial growth [84], whereas the MBC is defined as the minimum antibiotic/drug concentration required to sterilize 99.9% of an inoculum [85]. There are numerous techniques to detect the accurate numbers of MIC and MBC for screening the efficacy of antibiotics, and it is vital to figure out a reliable technique for understanding the potency of new drugs in human health.

1.8.1. Routinely used techniques to determine MIC and MBC

1.8.1.1. ETEST or the antibiotic gradient technique

The ETEST technique uses the principle of an antibiotic/antimicrobial gradient against the susceptible agent in an agar plate. The agar plate is inoculated with the microbe in test, and testing strip with a range of increasing antibiotic concentrations is placed onto the agar plate [30]. After the antibiotic strip is laid, the agar plate is incubated for overnight and MIC values can be determined by viewing the strips and identifying an intersection of an elliptical zone of growth inhibition within a point of the strip [36]. Whilst, the growth is inhibited in the elliptical zone, the bacterial cells adjacent to the strip could remain fully viable based on the MBC of the drug [3]. Even though this technique is not cost effective (around 2-3 dollars per strip), it is a simple, rapid, and less tenuous method to conduct an AST [34]. The technique can be used to determine MIC values for antibacterial, antimycobacterial and antifungals [35].

1.8.1.2. Agar antibiotic disc diffusion

This is one of the oldest and popular techniques for antibiotic susceptibility testing [86]. In this technique, agar plates are inoculated with a defined inoculum of the test microbe meeting the scale of 0.5 McFarland turbidity scale [87]. Commercially bought antibiotic discs (diameter of 6 mm) with known and specific antibiotic/drug concentrations are placed onto the agar surface and the plates are incubated for overnight under suitable conditions [87]. Post incubation, zone of inhibitions are observed near the discs and the diameter of the zone is measured in millimeters using a ruler [88]. The results obtained from this technique is qualitative as it is defined into categories such as sensitive, intermediate, and resistant. Hence, this technique is not reliable to achieve reliable MIC values [89]. The MIC value is read as the drug concentration that gives the largest diameter of zone of inhibition [90]. Some of the advantages of this technique is that it is very basic and simple, cost effective, small amount of reagents are required and the results are easily reproduced [91].

1.8.1.3. Agar antibiotic dilution technique

The agar dilution technique depends on the principles of diffusion. This method follows the approach of adding different concentrations of antibiotics (commonly two-fold serial dilutions) into molten agar medium and preparing petri plates with different concentrations [87]. The agar plates are later spread with a known inoculum of the test microbe. The MIC value is determined to be the minimum antibiotic concentration that starts to show a growth inhibition [80], whilst the MBC is determined to be the lowest antibiotic concentration required to see <1 colonies on the petri plates. This method is manual and is standardized by the National Committee for Clinical Laboratory Standards

[87]. This is a simple technique and less costly, however, it could be time consuming and labor-intensive.

1.8.1.4. Broth antibiotic dilution assay

There are two types of broth dilution assays known as macrodilution (in tubes containing at least 2 ml volume) and microdilution (conducted in a 96-welled microplate) [80, 87]. The dilution assay follows the principle of two-fold serial dilutions of the antibiotic incorporated in either the tubes or wells and then inoculated with a standard volume of an inoculum (microbial suspensions adjusted to the scale of 0.5 McFarland turbidity) [80, 87]. The tubes/microplate are incubated overnight to determine a growth curve relying on the optical densities. The MIC value is read as the lowest drug concentration to show a growth inhibition, whilst the MBC concentration is read as the lowest drug concentration to kill 99.9% of the initial inoculum [80, 87]. Some of the major disadvantages of microdilution assay are, it is time consuming, large amounts of reagents are required and the error rate in preparations of drug stocks with different concentrations are high [79]. The factors that can affect the accuracy of the MIC values are the inoculum size [92], the method of inoculum preparation and the incubation conditions [93]. One of the key advantages of this technique is that it provides quantitative results for determining MIC values [79]. Some of the other advantages of microdilution technique is that it allows large number of replicates at lower costs, the results are reproducible and small sample sizes required. Hence, microdilution assay is easier than macrodilution assays [94].

1.9. Antibiotic tolerance and persistence

In 1944, Joseph Bigger identified that the cells of *Staphylococcus pyogenes* grew repeatedly upon exposure to penicillin [95]. He saw that penicillin was bacteriostatic (growth inhibiting) to the 'dormant' and 'non dividing' cocci cells, indicating them as 'persisters' [95]. A subpopulation of cells that can tolerate lethal doses of antibiotic stress, by having their growth arrested or slowed down can be defined as persister cells [96]. However, distinguishing between 'persistent infections' and 'antibiotic persistence' is essential as the former indicates the infections in a host that cannot be eradicated by the host's immune system, while the latter refers to a state which is only caused by tolerance of effects of antibiotics [97]. Persister cells are genetically identical to the susceptible population and are not resistant to antibiotics and it is ambiguous why they endure antibiotic stress [98]. Persistence can be triggered in bacteria while they counter stress while infecting hosts and thus it is a medical concern as it leads to rise in antibiotic resistance [96]. Persister cells can remain sensitive to the effects of the antibiotics once regrown from the dormant state [99]. Unlike the resistant cells, persister cells fail to replicate in the presence of the antibiotics [97]. A bulk of the infections caused by bacterial biofilms are due to the formation of persister cells [100]. Whilst, the metabolic dormancy of persister cells has been illustrated, the mechanisms triggering this dormant state and the genes responsible to wake up these persister cells still remain unclear [101].

Tolerance is a phenotypic state where the tolerant cells are dividing and growing while the ongoing antibiotic treatment. They are termed as tolerant cells as their killing rate is significantly slow compared to the susceptible cells [97]. In contrast to resistance, tolerance is only true to bactericidal antibiotics [102, 103]. Both the tolerant and the

persistent population can survive lethal antibiotic treatments (Fig. 2) without an increase in the MIC of the drug [97]. While tolerance is the common ability of the population to survive long term antibiotic exposure, persistence is a phenomenon of only a subpopulation of cells [102]. Properties of tolerant cells such as reduced metabolism, reduced ATP levels and cell dormancy can also be seen in persister cells. Hence, it can be said that persister state is a notable state of tolerance, where a fraction of the population survive the antibiotic stress better than the rest of the population [97].

A quantitative metric to measure tolerance is the MDK, that can be deduced from time-kill assays, based on the idea that tolerant cells require more time to be killed when compared with the susceptible population [103]. MDK is defined as the minimum duration of antibiotic exposure required to sterilize a given bacterial population [104], at antibiotic concentrations that is significantly higher than the MIC [103]. The notion to refer the MDK as a measure for tolerance is because the death rate reaches a saturation point at very high antibiotic concentrations and thus it is practically unaffected by the increased concentrations and only depends on the duration of treatment [105].

According to studies, tolerance can be categorized into two forms, first tolerance by slow growth and second tolerance by lag. These two forms are distinguished as tolerance in slow growth arises during steady state while tolerance in lag is triggered by stress or starvation [103].

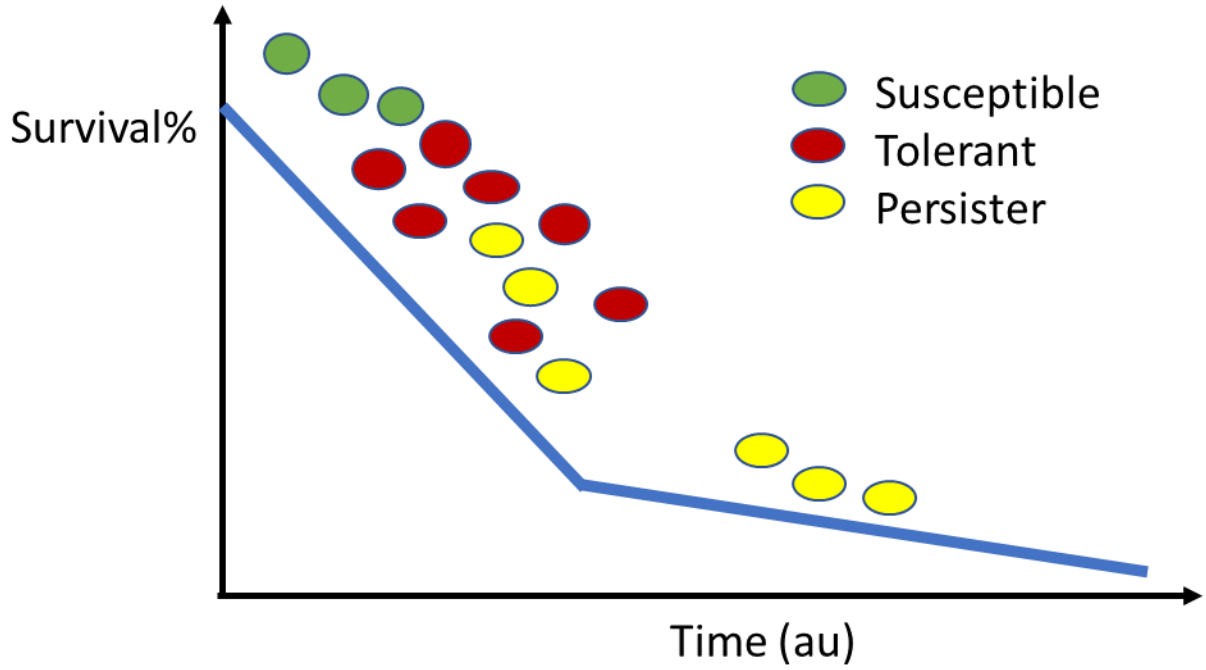


Fig. 2. Biphasic death curve: Tolerant cells die quicker than persister cells when exposed to lethal concentration of antibiotics. Persister cells do not divide, while tolerant cells can divide when exposed to antibiotic stress. Tolerant cells can survive longer at low levels of antibiotic.

1.9.1. Antibiotic persistence is a driving force of resistance

Resistant bacteria can grow and divide in the presence of antibiotics, while persistence cells are a subset of the bacterial population that can survive antibiotics by not dividing but will eventually be killed by the antibiotic (Fig. 3) [97]. As mentioned in the previous section, we distinguish persister (long-tolerance) from short-term tolerant cells

using a biphasic death curve (Fig. 2). The progeny, revived of persister cells, are susceptible to the same antibiotics as their parental population.

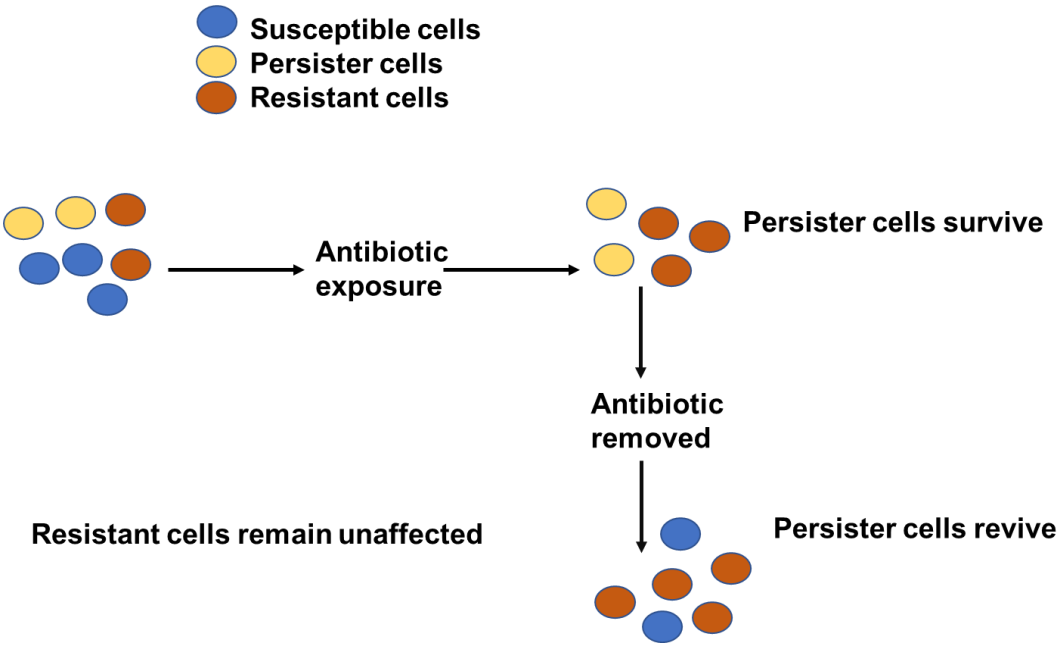


Fig. 3. Difference between susceptible cells, persister cells and resistant cells.

1.9.2. Types of persisters

Persister cells are often classified into two types though Type II is controversial. Environmental stimuli trigger the formation of Type I persisters, while Type II persisters may arise stochastically [98, 99]. Type I persistence are sometimes called triggered persisters. They are caused by stress in the bacterial population [99] such as starvation [97]. A fraction of persister cells can still be found when the stress is removed and the

culture is diluted into a fresh medium [97]. Other stresses that can cause triggered persistence are high cell number [106], immune factors, and acid stress [107]. Also, antibiotics are responsible for arresting growth in the population and triggering persistence [108].

Previous researchers proposed type II persisters (spontaneous persisters); persisters that grow slowly, divide, and form during steady-state growth [109, 110]. These cells were slow-growing before antibiotic treatment. They are predicted to develop when culture is in balanced growth, with all the parameters being constant and the exponential growth being steady [99]. Type II persisters, if they do exist, are thought to be much rarer than Type I persisters [97]. However, the existence of spontaneous persisters has been questioned because most define persisters as non-multiplying, and the experimental evidence is debated [111].

1.9.3. Mechanism related to persistence

Persister cells may be formed by diverse molecular mechanisms. They develop due to the heterogeneity in the population [112]. Qualitative changes the bacterial physiology enable the survival and revival of persister cells in a population [112]. Multiple models were proposed to be responsible for persister formation, and I cover four predicted model mechanisms below.

1.9.3.1. Stringent response

During nutrient starvation and other kinds of stress, the ubiquitous second messenger guanosine tetraphosphate (ppGpp) and the alarmone guanosine pentaphosphate (ppGpp)) act collectively in the ‘stringent response’ to maintain the metabolic homeostasis of the population [113, 114]. Upon heat shock and amino acid

starvation in *E. coli*, the (p)ppGpp RelA synthetase is activated. During carbon, fatty acid, nitrogen, and phosphate starvation, the (p)ppGpp synthetase activity of the enzyme SpoT is activated [113-115]. This (p)ppGpp network influences the cellular processes such as replication, transcription, and translation. The (p)ppGpp modifies the transcriptional events by binding the RNA polymerase. This changes the translational profile until the nutritional conditions of the population improves [116]. Bacteria that fail to produce (p)ppGpp have reduced levels of persister formation and survival upon stress [112]. Persister cell formation can be induced either by stochastic (p)ppGpp formation in the rare cells in exponential phase [117], or by environmental trigger producing the alarmone, for example in cells of biofilms or stationary phase [118].

1.9.3.2. RpoS mediated response

RpoS mediated response is a general stress response that facilitates bacterial survival in adverse conditions [112]. Sigma factor S (encoded by the *rpoS*) [119] is activated upon stress and is the driver that transitions cells from exponential to stationary phase [120]. RpoS binds with the RNA polymerase and regulates the expression of many genes, inducing *rpoS*, which increases the production of RpoS. Many stresses can trigger RpoS accumulation [120]. However, it is still unclear, whether RpoS mediated response is involved in persister cell formation or can only modulate the persister levels in a population.

1.9.3.3. TA systems

Though Toxin-antitoxin (TA) systems were once thought to be essential to persistence, recent results from our lab showed that persisters do not require TA systems [121]. However, TA system can modulate persister levels. TA modules in bacteria are

genetic elements that contain two components, a stable toxin protein and an unstable antitoxin. These genetic elements are located on the bacterial plasmids or chromosomes [122, 123]. The toxin produces inhibitory effects on cellular growth by restricting the vital cellular processes, while the antitoxin counters the lethality of the toxin by inhibiting its function [124]. There are six different types of TA systems based on the nature of the antitoxin; however [125], only type I and type II are the most studied in context with persister formation [112]. Studies on the TA modules of *E. coli* has provided the maximum information regarding their involvement in persister formation [126]. The TA modules induce latency in the bacterial population under stress conditions, such as antibiotic exposure [33]. In *E. coli*, several type II TA modules have been defined, such as the HipBA, the MazEF, the HokB/SokB, the TisB/IstR, the YafQ/DinJ and the MqsRA modules [127]. The first gene related to the formation of persister cells was the hipA toxin gene. In *E. coli*, when the expression of the hipA gene escalates, it induces the (p)ppGpp production via RelA, arresting cellular growth [128]. Like the HipBA system, the HokB/SokB TA module is also regulated by the (p)ppGpp network [108]. The SOS system (see below) activates the TisB/IstR module, TisB induces antibiotic tolerance by acting as an ion channel that diminishes the cellular proton motive force and ATP [129]. The MazF toxin halts cellular growth by breaking down the RNA and induces persister cell formation [130]. In contrast, the toxin of the YafQ/DinJ system promotes persister cell formation by reducing the levels of cellular indole [131]. As mentioned earlier, the TA modules induce persistence in a bacterial population by triggering the cells into metabolic dormancy, under various kind of stress [132].

1.9.3.4. SOS response

We still do not know whether the SOS response is essential to induce persistence, or it can only modulate persister levels. The SOS response is induced upon cellular DNA damage, permitting the cell to repair the damage and promotes survival [112]. This mechanism is an essential way for bacteria to survive under diverse stress conditions [112, 127, 133]. The SOS response involves genes that can affect DNA recombination, biofilm formation and genes conferring antibiotic resistance [134]. The two essential proteins involved in the SOS response are the DNA binding activating protein RecA and LexA, which represses transcription [135]. In *E. coli*, the RecA system positively regulates the SOS response [136]. The SOS system also induces the type I TA module TisB in *E. coli*, along with the regulation of DNA repair [129]. When *E. coli* cells are exposed to fluoroquinolones antibiotics, the SOS response activates the TisB toxin inducing persister cell formation [133, 137].

1.9.4. Resuscitation of persister cells

If antibiotic treatment is cut short, persister cells will resurrect to susceptible cells, causing recurrent infections [138]. A recent study suggested that in *E. coli*, the resuscitation of the persister cells is dependent on the cells' capacity to sense the presence of nutrients, such as the availability of single amino acids as carbon sources [101]. The amino acid alanine revived the highest levels of persister cells [101]. While studying the role of individual proteins in persister resuscitation, this study also demonstrated the role of chemotaxis in waking up the persister cells. The results showed that the alanine signal waking up the persister cells was dependent on chemotaxis signaling, in which the chemotaxis proteins Tar and Trg, along with the regulators CheY and CheA were

associated [101]. They also studied the association of the (p)ppGpp system in the revival of persister cells, and unlike what earlier studies had suggested, they concluded that the (p)ppGpp system does not contribute to the resuscitation of persister cells [101].

1.9.5. Persisters vs. Viable but non-Culturable (VBNC) cells

1.9.5.1. VBNC cells

VBNC cells were identified in *E. coli* and *V. cholerae* in 1982 [139]. It was seen that a significant portion of the cells were viable even though non-culturable on traditional agar plates [139]. VBNC is a state of hibernation and dormancy exhibited by bacteria to counter stress as long as conditions do not favor their growth again [140]. VBNCs are defined as the population that is metabolically inactive and non-culturable in media in which they grow in the absence of stress [140]. It is said that VBNCs are in an extensive state of hibernation when compared to persister cells [140]. Approximately 100 species of microbes may exhibit the VBNC phenomenon when subjected to different kinds of stress [141]. Bacteria tend to have morphological changes while they enter the VBNC state; for example, *Helicobacter pylori* and *E. coli* changes from rod shape to spherical shape [142] while *V. cholerae* becomes spherical from arc-shaped [143]. VBNCs can protect cell membrane fluidity by changing the fatty acid composition to endure harsh stressful conditions such as high/low salinity (chemical stress) or heat (physical stress) [144]. Even though the ATP content remains comparatively high, in VBNC cells, bacteria have decreased rates of respiration and macromolecule synthesis [145]. In *E. coli*, downregulation of genes involved in DNA replication, cell division, protein synthesis (membrane proteins) and genes involved in pathogenicity were observed when the cells were induced in VBNC cells [145]. The retention of pathogenicity in VBNCs differ between species. For example, in *E. coli* O157:H7, *H. pylori* and *V. parahaemolyticus*, the cells need to revive in culturability to cause diseases.

Organisms like *Legionella. Pneumophila* may remain toxic and have virulent properties as VBNC cells [146].

1.9.5.2. Resuscitation of VBNCs

Unlike the persister cells, which are in transient dormancy, VBNCs may be in a deeper state of dormancy and they fail to grow in nutrient media even after the removal of the stress [147]. The resuscitation period for the VBNCs are significantly longer, and in some bacterial species, the revival might require special treatment [148]. The requirement of longer time durations for the revival could be because, after being in profound dormancy, the VBNCs might need additional time to restore the ratio of toxin-antitoxin, to repair the damaged proteins contributing to growth and to restore the metabolic fitness of the population [149]. Even though, the mechanisms promoting the VBNCs resuscitation are unclear, studies have shown multiple factors associated with the revival response. For example, pyruvate, catalase, α-ketoglutarate and YeaZ promoting factor have been identified to be as factors that induce resuscitation [150-152]. A recent study showed that the cells of *E. coli* was induced to VBNCs by cold stress, and was later revived by pyruvate [153]. This study suggested that pyruvate is the preferred carbon source as glucose uptake is downregulated in the starving cells [153]. Other studies have shown that the VBNCs of pathogenic *Salmonella typhi* was resuscitated by tween 20 and catalase [154]. Overall, even though the underlying molecular mechanisms driving the revival of VBNCs are unclear, it is of great importance that further work is done on this aspect as it is still debatable whether VBNCs are misidentified dead cells.

1.9.5.3. Are VBNCs real? Are they persisters or dead cells?

According to a recent study, VBNCs are dead and the cells which revive from dormancy after removal of stress are the viable persister cells [155]. *E. coli* VBNCs

observed by transmission electron microscopy (TEM), lacked cytosolic components even though they had an intact cell membrane. These particles were termed as ‘cell shacks’ and they failed to resuscitate due to an absence of DNA in their cell components [156]. Also, as these cell shacks have only intact membranes and no cytosol, using DNA staining dyes such as propidium iodide isn’t a dependable tool to demonstrate the viability of these shacks [156]. Hence, it is argued that the death of non-persister cells and persister cells result into VBNCs which are indeed dead cells and TEM is a more reliable technique to study them [155].

1.10. Antibiotic Eagle effect

In the past 70 years, the antibiotic Eagle effect has been illustrated in various microorganisms while testing the bactericidal concentrations of antibiotics [157, 158]. Eagle effect was discovered by Harry Eagle in 1948, when he was doing time-kill assays of penicillin with several bacterial strains [159, 160]. During the time-kill assays, Eagle noticed a paradoxical effect on the survival percent of few bacterial strains upon treatment with antibiotic concentrations higher than the bactericidal concentrations [50]. The Eagle effect has been demonstrated in both Gram-positive bacteria, Gram-negative bacteria as well as mycobacteria. This phenomenon has been seen in these microbes when tested with a diverse range of antibiotics of different classes having different modes of actions [50]. Antibiotics like β-lactams, such as penicillin has shown Eagle effect in organisms like *Staphylococcus aureus*, Group B *Streptococcus*, Group C *Streptococcus* [159, 160], whereas antibiotics belonging to aminoglycosides (tobramycin) has shown Eagle effect in *E. coli* and *Pseudomonas aeruginosa* [157]. Moreover, quinolones like ciprofloxacin induced Eagle effect in organisms like *S. aureus*, *P. aeruginosa*,

Streptococcus pneumoniae [161] and *E. coli* [162]. Some *mycobacterium* species also showed to exhibit Eagle effect when exposed to quinolones like ciprofloxacin and moxifloxacin [163]. For instance, the MBC of moxifloxacin against one of the strains of *Mycobacterium tuberculosis* was 0.5 µg/ml, however, when treated with 8 µg/ml (16-fold higher) of the antibiotic, the killing effect declined by 1.5 logs in comparison with the control [164].

Recently, it was reported that telavancin and vancomycin, belonging to lipoglycopeptides induced Eagle effect in *Clostridium difficile*. However, the effect was not seen when *C. difficile* was exposed to other lipoglycopeptides like dalbavancin, ramoplanin and teicoplanin [165]. These findings indicate that Eagle effect is not necessarily induced by analogues from the same antibiotic class [50]. As Eagle effect has been demonstrated in numerous bacterial species, it can be a mechanism for these organisms to combat antibiotic stress [50]. Hence it is of great importance that we determine the significance of Eagle effect and illustrate how poor experimental designs to determine the MBC concentrations can lead to wrong classification of this phenomenon.

1.10.1. *E. coli* Eagle effect

Approximately 43 years ago, it was reported that antibiotics such as tobramycin and amikacin belonging to the aminoglycosides could induce paradoxical growth in *E. coli* [157]. When treated with 2-fold higher than the optimum killing concentration, the percent surviving the bactericidal effect increased. As, aminoglycosides interfere with cellular translation to show bactericidal activity, it was suggested that at higher concentrations, these drugs, stimulated the synthesis of proteins leading to the paradoxical effect [157]. In 1990, it was demonstrated that paradoxical growth was seen

in *E. coli* when exposed to high concentrations of a range of fluoroquinolones (enoxacin, norfloxacin, fleroxacin and ciprofloxacin) and nalidixic acid [162]. Additionally, it was reported that post 8 h treatment, the reduced killing effect was not seen for any of the five mentioned antibiotics [162]. At MBC, high filamentation and vacuolation was observed in the bacterial morphology, while at concentrations greater than the MBC, it was noted that the cells looked like normal rods with little filamentation [162]. Recently, it was published that ciprofloxacin can induce Eagle effect in exponential phase cultures of *E. coli*. The MIC (0.01 µg/ml) was determined by a microdilution assay, and then killing assay was performed by treating an exponential phase culture with 0.3 µg/ml (MBC) and 3 µg/ml of ciprofloxacin [166]. One of the possible reasons that could've led to this response is the interference in the RNA synthesis at concentrations higher than the bactericidal concentration [167, 168].

1.10.2. Possible mechanisms of the Eagle effect

There are only a handful of studies determining the primary mechanisms that can trigger Eagle effect [50]. It is demonstrated that a major contributing factor in inducing Eagle effect when exposed to β-lactams, is the catalyzing effect of the enzyme murein hydrolase [169]. This enzyme catalyzes the hydrolysis of cell wall components and leads to the subsequent bactericidal activity of the antibiotics [169]. As β-lactams are known to inhibit the function of the autolysin murein hydrolase, it was studied that lower antibiotic concentrations lead to more lysis [170]. It was also reported that for *Enterococcus faecalis* strains, upon treatment with high concentrations of penicillin, the killing effect reduced due to the interruption in peptidoglycan synthesis and cell growth [171]. This study also claimed that instead of the exponential phase, the Eagle effect was seen in the

lag phase [171]. Moreover, β-lactamases might play an essential role in driving the Eagle effect in some organisms. For instance, in *Proteus vulgaris*, elevated concentrations of cefmenoxime triggered reduced bactericidal activity by enhancing the production of β-lactamases, while this effect wasn't evident in presence of a β-lactamase inhibitor [172]. For antibiotics belonging to quinolones, the paradoxical growth could be triggered by the inhibition of protein synthesis. In *E. coli*, it was observed that the Eagle effect induced survival resulted from the correlation between low levels of reactive oxygen species (ROS) with that of cellular translation [173]. Likely, the antibacterial effect of moxifloxacin against *M. tuberculosis* was reduced as a result of reduced protein synthesis [164].

1.10.3. Does the Eagle effect describe persistence or tolerance?

There are several similarities between persistence and Eagle effect, such as in both these phenomena, phenotypic modifications of the bacterial cellular wall were observed. Also, for both these phenomena, a subpopulation of the cells survive lethal antibiotic stress and survival can be enhanced with an increased bacterial load [50]. When Eagle observed the paradoxical growth, he observed that with high inoculum, the bactericidal activity of penicillin was reduced [174]. Likewise, in stationary phase, persister state is implicated as a consequence of high bacterial load and reduced metabolic activity of the population [33, 175]. A reduced availability of the target for the antibiotic has been proposed as the idea behind both persistence and Eagle effect [50]. Eagle proposed that the reduced activity of the cell wall inhibitor penicillin was an outcome of reduced metabolism and reduced bacterial growth, as a result of which less targets are available for the antibiotic to act on [174]. This is also postulated in case of

bacterial persistence, where reduced cell growth (tolerance) or no cell growth al all leads to fewer targets presented for the antibiotics to be significantly effective [103]. In contrast, it has been shown that persister cells can be equally damaged with antibiotics as that off non-persisters, but persister cells survive due to the presence of DNA repair mechanism that follows antibiotic damage [176]. This suggests that even though a different set of mechanisms can contribute to the trigger of persisters, the same has not been deduced yet for Eagle effect [50].

One major difference between antibiotic persistence and Eagle effect is that, a subpopulation that displays dose-dependent persistence, is efficiently sterilized with higher doses of antibiotic [103]. However, in case of the subpopulations under Eagle effect, with higher antibiotic concentrations, the bacterial survival increases [50]. Therefore, bacterial persistence and Eagle effect might have similarities, but they have essential differences that make them two separate phenomena, and thus studies on cells exhibiting Eagle effect should be done in order to have a better knowledge about the association between both these occurrences [50].

In my second Chapter, I will discuss how the Eagle effect does not occur during persistence for *E. coli* (See chapter 2). I have yet to determine if the Eagle effect occurs during tolerance for *E. coli.*

2. CHAPTER 2. DETERMINING THE MINIMUM BACTERICIDAL CONCENTRATION OF ANTIBIOTICS AND TESTING FOR THE EXISTENCE OF AN ANTIBIOTIC PERSISTER EAGLE EFFECT IN *E. COLI.*

2.1. Abstract

Bacterial persistence is a dormant state where a very small subpopulation, is non dividing, metabolically repressed and can withstand high doses of antibiotic treatment. Likewise, Eagle effect is a phenotypic state where persister cells survive extremely high doses of antibiotics and the survival percent increases at concentrations higher than the MBC. MBC values can be determined by several techniques, however, regular techniques like broth microdilution can show inaccurate results for certain antibiotics, such as ciprofloxacin. Our study shows that broth microdilution technique is not reliable to determine the MBC values of ampicillin and ciprofloxacin. An antibiotic saturation assay conducted with high antibiotic concentrations showed that death rate remains unaffected at antibiotic concentrations higher than the MBC. Time-kill assays with ampicillin and ciprofloxacin show that at both 3 h and 24 h, the persister percent remains constant above concentrations higher than MBC. We determined that the MBC value of ampicillin and ciprofloxacin at liquid state is 20 µg/ml and 2 µg/ml, respectively. This study also suggests that the Eagle effect is not true for our strain of *E. coli* against bactericidal antibiotics such as ciprofloxacin and ampicillin.

2.2. Introduction

The rise in antibiotic resistance is a continuous global health concern despite the efforts to control and prevent it [177]. Over 70 years ago, Hobby [178] and Bigger [95] noticed that bactericidal antibiotics failed to completely sterilize a culture. Bigger

observed that a subpopulation of dormant cells survived, he named these subpopulations as persisters [95]. These bacterial persisters can resuscitate and re-establish the chronic infections when the antibiotic stress is removed [138]. Antibiotics target bacterial cells at different cellular structures and either inhibit the growth (bacteriostatic) or kill them (bactericidal) [179]. To demonstrate the susceptibility of antibiotics against bacteria, it is essential to conduct susceptibility tests that can determine the minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) accurately. Different techniques like agar dilution and broth dilution are commonly used to demonstrate the MBC concentrations based on which time-kill assays are conducted [84, 87]. The MBC is usually defined as the antibiotic concentration at which 99.9% of the population dies [180]. However, in 1948, Harry Eagle saw that the survival percent of bacteria increased at very high concentrations of the antibiotic penicillin [159]. Even though, the conditions at which both persistence and Eagle effect might occur could have similarities, these two are distinct phenomena [50]. Studies on the cells exhibiting a paradoxical growth under high antibiotic concentrations will provide transparent understanding on how the antibiotic concentrations may have an effect in classifying the Eagle effect.

In this study, we have used different antibiotic susceptibility techniques to deduce the MIC and MBC values of *E. coli* in both solid and liquid media. Our approach was to find out a standard technique to determine both the MIC and the MBC values accurately, as studies have shown how lower doses of antibiotics (lower than actual MBC) can lead to irreversible mutations and development of frequent resistance against the antibiotics [181, 182]. Our study also focuses on determining the occurrence of Eagle effect in *E.*

coli, against antibiotics of different classes. We demonstrated that the MIC and MBC values can differ for solid and liquid media, and hence techniques like broth microdilution might not be reliable to determine MBC values for time kill assays. Our work also shows that the antibiotic concentration reaches a point of saturation above the MBC and leads to no change in the persister percent.

2.2.1. Hypothesis

1. An antibiotic's MBC concentration is the point when adding more antibiotics does not lead to more death.

2. Eagle effect (Fig. 4) can be seen in *E. coli* against ampicillin and ciprofloxacin.

2.2.2. Objectives

1. Determine the MIC and MBC of ampicillin, ciprofloxacin, apramycin, and gentamicin in *E. coli*.

2. Determine whether the Eagle effect is a phenomenon of *E. coli* persister cells.

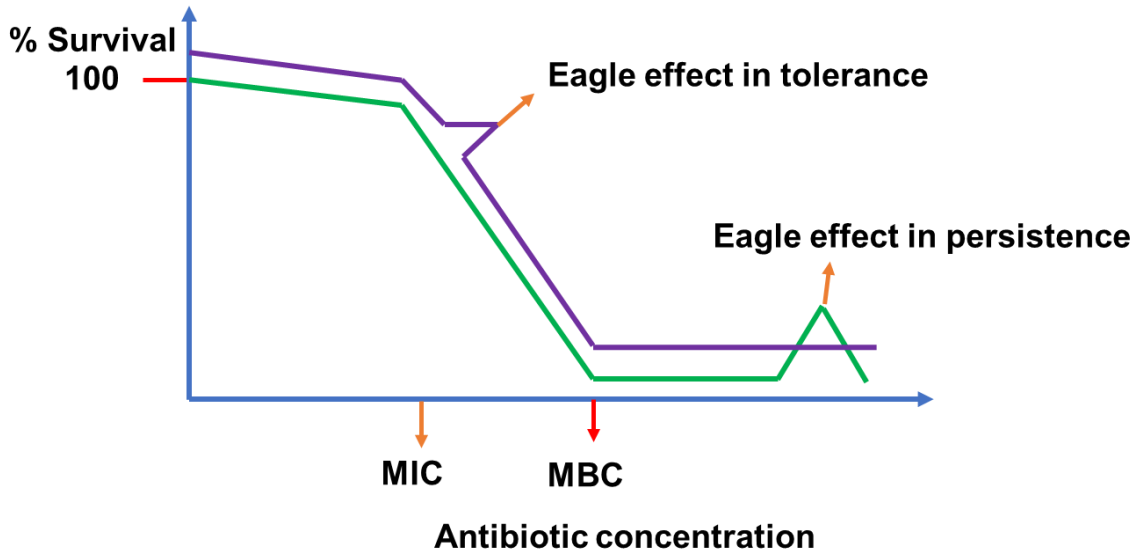


Fig. 4. Eagle effect.

2.3. Results

2.3.1. The MIC and MBC depends on the physical state of the growth medium.

The antibiotic concentration required to inhibit the growth of a population depends on the physical state of the growth medium. We used ETEST strips to determine the MIC of antibiotics (Table 2) with different modes of action against *E. coli*. The MIC of ampicillin and ciprofloxacin, determined by the strips is 1 µg/ml and 0.01 µg/ml, respectively. The MIC and MBC in solid media was further determined by agar dilution technique, by counting the colonies in selective plates (Fig. 5). An exponential-phase culture (7.6 X 10^7 CFU/ml) was plated in plates containing ampicillin of different concentrations (from low to high). The decrease in CFU/ml with an increased antibiotic concentration was observed after the incubation period (Fig. 5a). After 36 h of incubation at 37ºC, in solid, the MIC concentration was 1 µg/ml, and the MBC concentration was 10 µg/ml. For ciprofloxacin, the MIC and MBC in solid (Fig. 5b) was determined in the same way by starting with an exponential phase culture (1.8 x 10^8 CFU/ml) and after 36 h of incubation at 37 ºC, in solid, the MIC concentration was 0.005 µg/ml while the MBC concentration was 0.1 µg/ml. Furthermore, the MIC and MBC in solid media for other antibiotics such as apramycin (Fig. 5c) and gentamicin (Fig. 5d) was deduced by the agar dilution method. Our results show that, the MIC value determined by the ETEST strip of ampicillin is identical to the MIC determined by the agar dilution technique. However, for ciprofloxacin, the MIC value determined by the ETEST strip (0.01 µg/ml) is 2-fold higher than the MIC value (0.005 µg/ml) obtained by agar dilution technique.

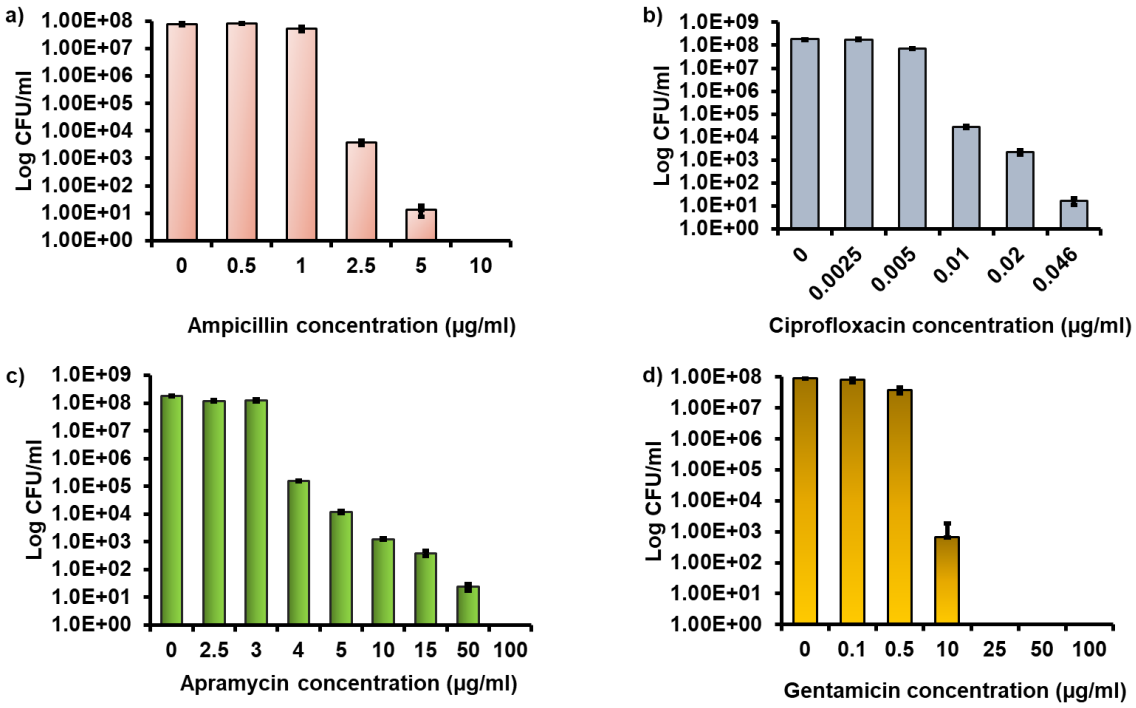


Fig. 5. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of different antibiotics for *E. coli* in solid media. **a)** ampicillin **b)** ciprofloxacin **c)** apramycin **d)** gentamicin, determined by agar dilution technique. The ETEST strips cannot be used to determine the MBC in solids.

Table 2. The MIC and MBC of antibiotics in solid media. The MIC determined by agar dilution technique and ETEST strips are same for ampicillin, while it varies by 2-fold for ciprofloxacin. The MBC in solid state cannot be determined by the strips. Agar dilution technique can be used to determine the MBC in solid state.

Antibiotic	**µg/ml**		
	MIC-Agar	**MBC-Agar**	**MIC-strips**
Ciprofloxacin	0.005	0.1	0.01
Apramycin	2.5	100	
Ampicillin	1.0	10	1.0
Gentamicin	0.5	25	

2.3.2. The MIC-MBC of antibiotics in liquid state determined by microdilution assay. The MIC-MBC determined by this technique may not be accurate for all antibiotics.

To determine whether the MIC and MBC values in liquid, were similar with the respective values in solids, we conducted a broth microdilution assay. The assay was done with an exponential phase culture to determine the growth curve (Fig. 6) at different concentrations for each of the antibiotics (ciprofloxacin, apramycin and gentamicin). For ciprofloxacin (Fig. 6a) and apramycin (Fig. 6b), based on the ODs, the MIC values were 1 µg/ml (200-fold higher than the MIC value in solid) and 15 µg/ml (6-fold higher than the MIC value in solid), respectively. For gentamicin (Fig. 6c), based on ODs, the MIC value was 2.5 µg/ml (5-fold higher than MIC value in solid). The MBC values determined for ciprofloxacin, gentamicin and apramycin was 20 µg/ml, 25 µg/ml, and 100 µg/ml, respectively (Table 3). While the MBC values determined by agar dilution and microdilution assays remain same for gentamicin and apramycin, in case of ciprofloxacin the microdilution assay resulted in a 200-fold higher value of MBC. Our results show that, the MIC values in liquid state are significantly different from that off the MIC in solid state. This raises the curiosity about the accuracy of the MBC values determined by the microdilution technique and hence to confirm the correct MIC-MBC a more precise method should be approached.

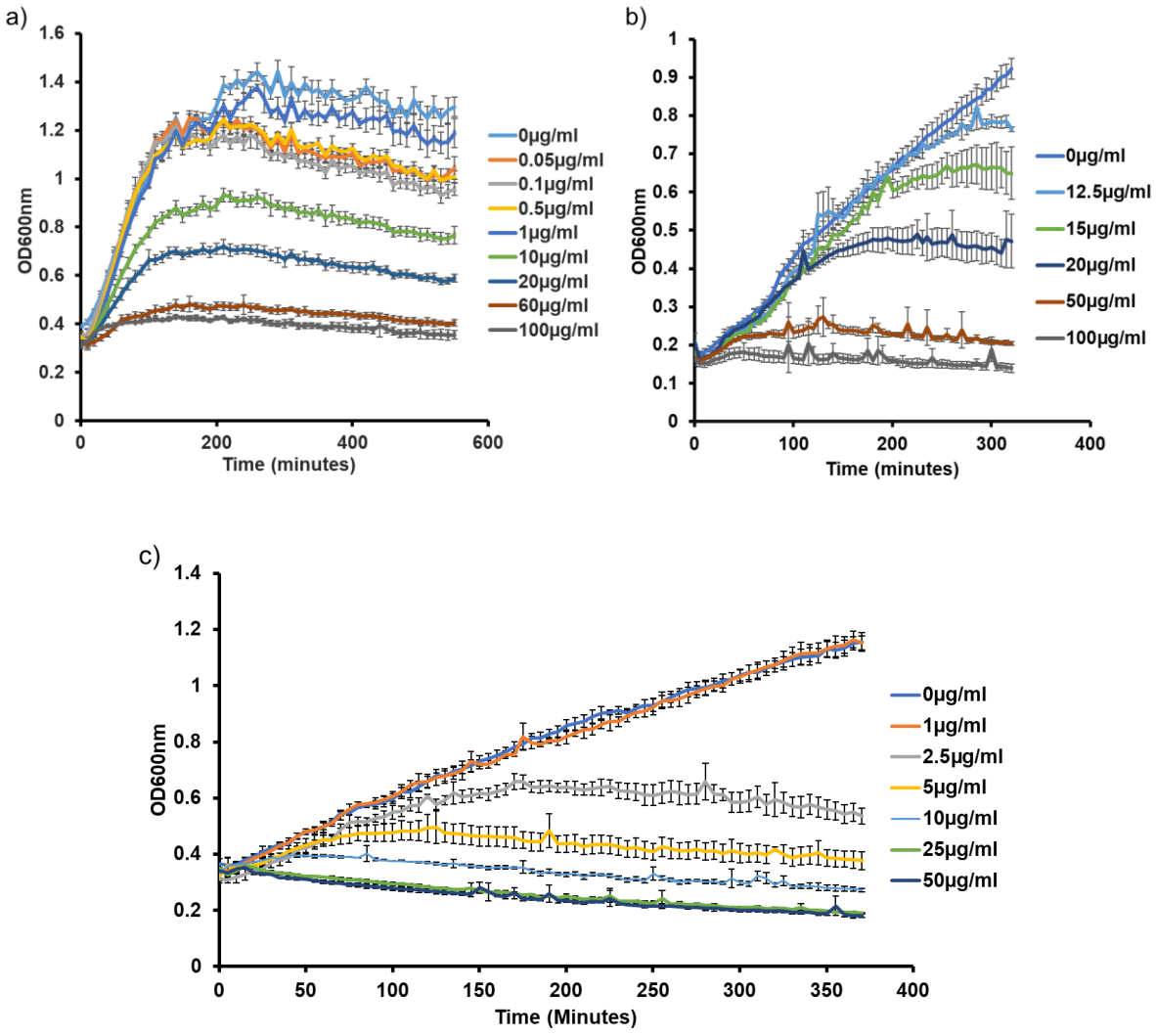


Fig. 6. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of different antibiotics for *E. coli* in liquid media. **a)** ciprofloxacin **b)** apramycin **c)** gentamicin. The MIC values in liquid are different from the MIC values in solid for all the antibiotics. The MBC value of ciprofloxacin is different in solid and in liquid state.

Table 3. The MIC and MBC of these antibiotics determined by broth microdilution assay. The MIC values in liquid state is different from the MIC values in solid state. The MBC values remained same for all antibiotics except ciprofloxacin.

Antibiotic	MIC (µg/ml)	MBC (µg/ml)
Ciprofloxacin	1	100
Apramycin	15	100
Gentamicin	2.5	25

2.3.3. The death rate of *E. coli* remains constant at concentrations above the MBC.

Even though there are multiple techniques to illustrate the MBC values of bactericidal antibiotics, our results indicate that the rule of adapting the MBC by general broth dilution technique can give false results. While, determining the MBC values in solid media by agar dilution technique, it was demonstrated that for our strain of *E. coli*, the MBC values for both ampicillin and ciprofloxacin was significantly lower (10 X and 200 X respectively) when compared with the MBC values obtained in liquids by microdilution assay. Hence, to determine the specific concentration of antibiotic at which around 99.9% of the bacterial population is sterilized, an antibiotic saturation assay (see methods) was conducted (Fig. 7). An exponential phase culture (~ $10^7/10^8$ CFU/ml) was treated with a range of different concentrations of ampicillin and ciprofloxacin (from 0 µg/ml to 1000 µg/ml) for 24 h. Cultures from each of the concentrations were removed after 3rd hour and 24th hour of treatment, washed to remove the antibiotics and plated in nutrient agar medium. The plates were incubated for 36 h-48 h and CFU/ml was counted to determine the concentration at which the minimal bactericidal effect is seen (Table 4). After 3 h treatment with ciprofloxacin, at a concentration of 0.075 µg/ml (Fig. 7a)

inhibitory effects were seen. The bactericidal effect was seen at a concentration of around 2 µg/ml, with a survival percent of 0.003%. The persister percentage remained constant with higher concentrations of ciprofloxacin and did not show any increase even at a concentration as high as 500 µg/ml. After 24 h treatment (Fig. 7b), the survival percent did not show any significant difference in between the range of 5 µg/ml to 100 µg/ml. For ampicillin, after 3 h, the survival percent remained significantly similar, in the range of 0.01%-0.03% (Fig. 7c) for concentrations ranging from 20 µg/ml to 1000 µg/ml. After 24 h treatment (Fig. 7d), the survival percent did not show any significant difference in between the range of 20 µg/ml to 1,000 µg/ml. Overall, the results of antibiotic saturation confirms that the MIC-MBC values obtained for liquids by microdilution assay was inaccurate for both ampicillin and ciprofloxacin.

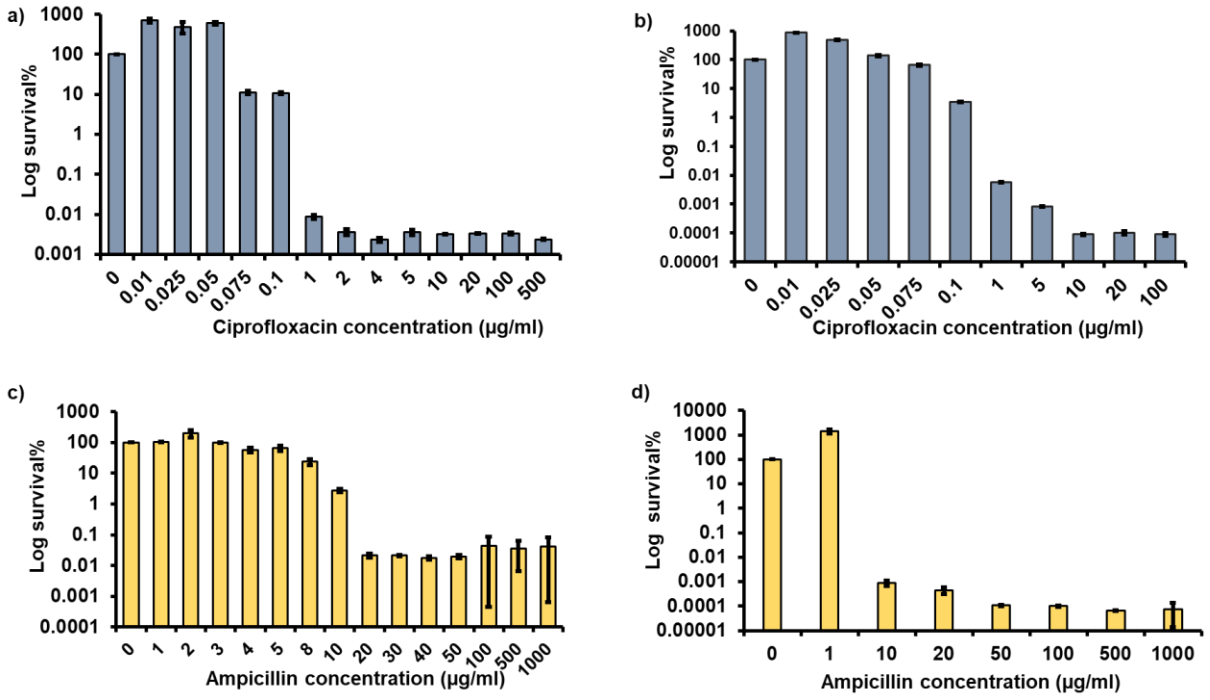


Fig. 7. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of different antibiotics for *E. coli* in liquid media determined by antibiotic saturation assay. **a)** ciprofloxacin-3 h **b)** ciprofloxacin-24 h **c)** ampicillin-3 h **d)** ampicillin-24 h. The p-value is >0.05 showing no apparent statistical differences between 20 µg/ml and 100 µg/ml in ampicillin and between 5 µg/ml and 20 µg/ml in ciprofloxacin. The MIC-MBC values determined by antibiotic saturation assay is different from the MIC-MBC values obtained by microdilution assay.

Table 4. The MIC and MBC of these antibiotics determined by antibiotic saturation assay. The MIC-MBC values determined by antibiotic saturation assay is different from the MIC-MBC values obtained by microdilution assay.

Antibiotic	MIC (µg/ml)	MBC (µg/ml)
Ampicillin	4	20
Ciprofloxacin	0.075	2

2.4. Discussion

The contribution of antibiotics was a breakthrough in modern medicine and decades later we are still dependent on them for treating infectious diseases. The rise in antibiotic resistance has become a global threat for mankind [1]. In order to reduce the misuse of antibiotics, it is critical that a standard technique is followed to determine the inhibitory and bactericidal concentrations of the commercially available antibiotics. In this study, we have illustrated that MBC values of antibiotics in *E.* coli can be determined empirically. Antibiotic susceptibility testing of pathogenic bacteria is an essential task in order to demonstrate the efficacy of these drugs and to minimize the development of antibiotic resistance against them [87]. We have successfully shown that both the MIC and MBC values for all antibiotics of different classes such as ampicillin, ciprofloxacin, apramycin and gentamicin vary in between solid media and liquid media. We have compared the MIC values determined by agar dilution technique with the MIC values obtained by using ETEST strips for ampicillin and ciprofloxacin. For each of the tested antibiotics, we have shown that MIC values measured by the above two techniques are identical for ampicillin, while it shows 2-fold variation for ciprofloxacin (Table 2).

However, when we tested the MIC values for all the four above antibiotics in liquid media, we saw significant variation between the MIC in solid state and the MIC in liquid state. Initially, we tested the MIC and MBC values in liquid media using the broth antibiotic microdilution assay and determined the values by plotting a growth curve of OD_{600nm} against time. We saw that there was 5-6-fold variation between the MIC-solid and MIC-liquid of apramycin and gentamicin, whereas there was a gigantic 200-fold variation between the MIC-solid and MIC-liquid of ciprofloxacin.

Unlike the MICs, when we compared the MBC values determined by agar dilution technique with the broth dilution technique, we saw that both apramycin and gentamicin exhibited identical MBC values (100 µg/ml and 25 µg/ml respectively) in each of the solid and liquid states. However, for ciprofloxacin, the MBC in liquid was approximately 200-fold higher when compared with the MBC in solid. Also, after doing the persister assay with ciprofloxacin, it was observed that even though a concentration of 20 µg/ml was high enough to induce persistence in 3 h. However, after generating a growth curve by broth microdilution assay, at 20 µg/ml, we could still see the curve having an upward trend instead of a flat line indicating growth. This demonstrated that microdilution assay was not a reliable technique to accurately measure the MIC and MBC values of antibiotics. In order to induce persistence in a defined bacterial population, a certain concentration of antibiotic is required (MBC), going above which, the persister levels remain unchanged. We determined this MBC value by doing an antibiotic saturation assay (ASA).

2.4.1. Antibiotic saturation assay is an ideal method to determine the appropriate MBC of antibiotics.

Bacterial persistence acts as an intermediate state before acquiring heritable resistance against antibiotics [183]. Persister cells can emerge into resistant mutants by adapting mutations due to stress response or simply by horizontal gene transfer [183]. Mutation rates leading to antibiotic resistance can also be exhibited by bactericidal antibiotic therapies. Sub-lethal doses of bactericidal antibiotics during clinical therapies, such as treatment of cystic fibrosis can lead to an increase in the mutation frequency in the infected lungs [184]. If the population is treated with sub-lethal doses of bactericidal antibiotics, it triggers the ROS production and stimulates the RpoS-mediated activation of the polymerase IV resulting into genome wide mutations [184-186]. Very low doses of antibiotics, either present naturally or used in treatments can lead to the selection of pre-existing mutants and also give rise to de novo mutations [182]. Mechanisms of antibiotic resistance comes with a fitness cost [181]. During treatment with reduced doses of antibiotics, this fitness cost allows many susceptible populations to surpass the resistant population, resulting in reduced reversibility of resistant mutations [181]. The tolerant (slow-growing) population can also survive exposure to bactericidal antibiotics [175]. The MIC of the tolerant populations resembles the MIC of the susceptible population, and the tolerant cells can survive antibiotic stress by growing very slowly [97]. In bacterial populations, tolerance play a critical role in conferring resistant population as tolerance rises quickly under the influence of already preexisting mutations, leading to the development of new partial resistant mutations in a slow growing population [187].

Using high levels of antibiotics can sterilize the tolerant population much quicker leading to lower mutation rates in the population.

In this study, we performed an antibiotic saturation assay with a range of high concentrations of antibiotics and showed that increasing antibiotic concentrations does not lead to increased death.

Eagle effect was deduced by Harry Eagle while he was working with penicillin [159]. Few studies have reported that Eagle effect can be seen in *E. coli*, induced by different classes of antibiotics like β-lactams, aminoglycosides, and quinolones [157, 162, 164, 166]. In this study, we conducted persister assays of *E. coli* with different classes of bactericidal antibiotics such as ampicillin (β-lactam) and ciprofloxacin (fluoroquinolone) (Fig. 7a-d) and deduced the survival percentage of the persister cells with an increase in antibiotic concentration. Our data elaborates two important factors about techniques commonly used to determine MIC and MBC values. Firstly, MIC and MBC values determined by microdilution assay is not standard and can illustrate inaccurate results. This is of both practical and clinical importance as wrong MBC values (lower than the correct MBC) can lead to the usage of lower than required amount of the drug to sterilize infections. This increases the chance of acquiring antibiotic tolerance against the pathogen [187], leading to a rise in mutation rates due to prolonged survival time [184]. Secondly, the persister levels do not change once the antibiotic concentration reaches a saturation point. Increasing the antibiotic concentrations by several times the MBC, does not affect the survival percent of the persister cells. Our second finding indicates that Eagle effect is not true for our strain of *E. coli* and with very high levels of antibiotics, the death rate remains unaffected.

Though, we do not claim that the findings of Harry and Musselman was wrong, when they asserted that post 3 h treatment, the increase in antibiotic concentration led to a state of paradoxical growth [159, 160], we assume that it is highly possible that they saw Eagle effect in the short-termed tolerant cells of the population (see future goals) due to the use of an incorrect technique to determine the MBC value. For some antibiotics such as ampicillin, if we assume the MBC value as 5X or 10X higher than the value of MIC, it still does not alter the persister levels as these values fall in the range at which persister levels remain constant. However, for some antibiotics like ciprofloxacin, our data shows that the MBC value is approximately 27X higher than the MIC, and in such a case, one can easily perform the time-kill assay with a wrong MBC value if they assume the MBC value to be a number that is 5X or 10X higher than the MIC. Regardless of how MIC values are generated, our work provides a new perception on the correct techniques and experimental designs to determine the MBC values. Therefore, we propose that instead of relying on regular techniques like broth dilution or agar dilution, antibiotic saturation assays should be considered regularly to establish the MBC values of the commonly used and the newly developed antibiotics.

2.4.2. The steps to accurately determine the MBC

1. Determine the MIC and MBC in solid by agar dilution technique.

2. To determine the MBC in liquid, select a range of antibiotic concentrations, that includes 2X, 4X, 6X, 10X, 20X, 50X, and 100X (the range can change depending on the antibiotics) of both the solid MIC and solid MBC and conduct a time-kill assay for 3 hours.

3. Once, the MBC in liquid is figured out, conduct an antibiotic saturation assay, with a range of antibiotic concentrations that includes the liquid MBC, and concentrations that are higher than the liquid MBC. This shall confirm the concentration from which there is no change in the persister levels.

4. To further confirm the results, conduct the time-kill assay for 24 hours. The MBC value should remain the same for both 3rd hour and 24th hour.

2.4.3. Future goals for this project

In future, our lab wants to study an antibiotic that is weakly dependent on metabolism (colistin) and antibiotic that can either behave like it is strongly dependent on metabolism or weakly dependent on metabolism, with respect to the growth conditions (gentamicin). Consequently, we desire to illustrate the presence of Eagle effect in tolerant cells (if any) upon treatment with ampicillin and ciprofloxacin.

2.5. Materials and methods

2.5.1. Microbial strains and media

Escherichia coli DH5alphaZ1 pkm24NB82 was used in this study. For growth and antibiotic survival assays, cells were cultured at 37°C in MMB+ media (25 µg/ml kanamycin (Km) with glucose or glycerol as carbon sources). All cultures were plated in Luria Bertani agar media for colony counts.

2.5.2. MIC and MBC assays

2.5.2.1. MIC-MBC with strips

A single colony or two from a pure culture plate was taken and suspended in 5 ml of Luria Bertani (LB) broth and vortexed. A sterile cotton swab was inserted into the suspension and swirled at least twice. The cotton swab was spread on a LB agar plate, by

rotating the plate 45 degrees so that the suspension is evenly spread. The suspension was allowed to dry in the plate for 10-15 minutes. A sterile forceb was used to place the antibiotic strip in the center of the plate, making sure the strip has properly stuck to the agar surface. The plate was incubated at 37 °C for 24 h and observed for zone of growth inhibition.

2.5.2.2. MIC-MBC with agar plates

For this assay, cultures were grown to stationary phase in, MMB+ media or LB media, with kanamycin (Km) then diluted 100 times to bring up the culture into exponential phase before the assay. The cultures were then serially diluted in ice cold 1.5 ml tubes before the plating. The antibiotic stocks were made by using 50 ml plastic tubes and filter (0.2 um) sterilizing the solution. Antibiotics were added to molten agar, to make plates containing different concentrations of antibiotics. The plates were made with a range of different antibiotic concentrations, spread with the serially diluted exponential phase culture, and incubated at 37°C for 36 h-48 h. The colonies were counted to determine the CFU/ml.

2.5.2.3. MIC-MBC with liquid by using the microdilution assay

For the liquid MIC-MBC test, an exponential phase culture was grown in MMB+ glucose/glycerol. Stocks of different antibiotic concentrations were made by dilution from the original stock and 30 µl were added to the wells of a 96-welled microplate containing 270 µl of the culture. The microplate was put in a plate reader, and the ODs were measured for a duration of 180-300 minutes. The ODs were used to generate a growth curve over time.

2.5.3. Persister assay

To determine the killing curves, an overnight culture was diluted by 100X in fresh MMB+ media (with 25 µg/ml Km and either with glycerol or glucose). The cultures were shaken at 37°C shaker in 250 rpm till it reached the exponential phase (OD 0.4-0.5) and then treated with the antibiotics (100 µg/ml ampicillin and 20 µg/ml ciprofloxacin). Before treatment 1 ml of the culture was removed and plated to count the CFU/ml at 0 h. After 3 h and 6 h of treatment, the cultures were washed in 1X MMB to remove the antibiotic and plated to count the CFU/ml. The experiment was done with 3 biological replicates.

2.5.4. Antibiotic saturation assay

For the antibiotic saturation assays, an exponential-phase culture was grown in MMB+ glucose media with 25 µg/ml Km. Once the culture reached an OD of 0.4-0.5, 1 ml was removed and plated to count the CFU/ml at 0 h. The culture was separated into 5 ml tubes and each tube was added with different antibiotic concentrations, from a range of low to very high concentrations (Table 5). The tubes were put at a 37 ºC shaker, at 250 rpm for 3 hours. After 3 h, 1 ml culture was removed from each of the tubes and washed with 1X MMB to remove the antibiotic. After the washing, the culture was serially diluted and plated to count CFU/ml. The tubes were kept in the shaker, and after 24 h of treatment, 1 ml of the cultures from each tube was removed and washed with 1X MMB. The cultures were serially diluted and plated to count CFU/ml. The plates were incubated at 37 ºC for 36 h-48 h. The CFU/ml was counted after the incubation period was over. The experiment was done with 3 biological replicates.

Table 5. Antibiotics and concentrations used for antibiotic saturation assay.

Antibiotic	**µg/ml**	
	Lowest	**Highest**
Ampicillin	0	1000
Ciprofloxacin	0	500

3. CHAPTER 3. PERSISTER CELLS REVIVE FASTER IN PRESENCE OF PYRUVATE.

3.1. Abstract

Bacterial cells can exhibit phenotypic states of dormancy in adverse conditions and can survive as both persister cells and VBNCs. VBNCs and persister cells can revive from the dormant state once the stress is removed. However, VBNCs might require longer time and cannot grow in traditional nutrient agar medium unlike persister cells. Persister cells can resuscitate under various conditions, specifically by sensing nutrients as carbon sources. Here, we studied the effect of the pyruvate in resuscitation of *E. coli* persister cells. This study shows that, *E. coli* persister cells do not wake up with pyruvate during an ongoing antibiotic treatment. Furthermore, this work demonstrates that pyruvate is a resuscitating element of persister cells. Upon treatment with bactericidal antibiotics, such as ampicillin and ciprofloxacin, the persister cells resuscitated with pyruvate upon the removal of the antibiotic. Pyruvate revives the persister cells quicker than that of glucose and glycerol, without affecting the growth rate of the population. To conclude the results, further work must be done on the revival of *E. coli* VBNCs, observe them under the microscope and compare it with that of persisters to deduce whether VBNCs are misidentified persister cells.

3.2. Introduction

In 1982, Colwell and colleagues discovered that VBNCs are dormant phenotypic variants of bacteria, which can tolerate harsh stress conditions [139]. They said that VBNCs were functionally viable, but failed to grow in media [139]. Hobby detected that bactericidal antibiotics could not sterilize a population completely [178], and few years

later Bigger observed that a metabolically inactive subpopulation was able to tolerate lethal antibiotic stress, which he named as persister cells [95]. Both VBNCs and persister cells can be induced by same types of stress and interestingly, these two phenomena can exist together [140]. It was reported that in a log phase culture of *E. coli*, there are approximately 100-fold more VBNCs than persister cells [188]. Persister cells can resuscitate, become functionally susceptible and cause recurrent infections [138]. VBNCs can resuscitate but still not grow in nutrient media and may take longer durations for resuscitation [150]. According to a recent study, *E. coli* VBNCs cells were induced by cold shock for a period of 120 days and then the cells were resuscitated upon addition of pyruvate [153]. The study showed upregulation of the proteome of the VBNCs and they reported that pyruvate was used as a carbon source by the VBNCs of *E. coli* during resuscitation [153]. *E. coli* persister cells can resuscitate with addition of specific carbon sources, by detecting nutrients via chemotaxis and phosphotransferase membrane proteins [101].

In this study, we investigate the resuscitation of *E. coli* persister cells by pyruvate during and post antibiotic stress. Our approach is to induce persistence with bactericidal antibiotics ampicillin and ciprofloxacin, and determine the effect of pyruvate in the persister resuscitation in presence of other carbon sources such as glucose and glycerol. The second study focuses on the controversial topic of the existence of viable but non culturable (VBNC) cells. I am assisting Tahmina Hossain in this project. The long-term goal is to determine whether VBNCs are dead or whether they are misidentified persister cells.

3.2.1. Hypothesis

1. Persister cells do not wake up with pyruvate during antibiotic stress.

2. Persister cells resuscitate quicker in the presence of pyruvate.

3.2.2. Objectives

1. Determine persister percentage of *E. coli* with ampicillin and ciprofloxacin with and without pyruvate.

2. Revive persister cells post 3 h treatment, with and without pyruvate.

3.3. Results

3.3.1. Persister cells do not resuscitate during antibiotic treatment.

The survival percentage of *E. coli* is unaffected when the population is treated with revival factors during antibiotic stress. A stationary phase culture was diluted in fresh media containing glycerol as the carbon source and treated with ampicillin, with and without the presence of 0.05% pyruvate (Fig. 8). After 3 h and 6 h treatment, a sample of the culture was removed, washed, and plated to determine the decrease in the CFU/ml from the original CFU/ml (3×10^9 CFU/ml). The survival percentage of *E. coli* after 3 h and 6 h treatment of antibiotic with and without pyruvate had a difference of < 1-fold (Fig. 8a)

To further confirm our results, we investigated the survival percentage of an exponential-phase culture (1.5×10^8 CFU/ml), this time using glucose as the carbon source and treated with ciprofloxacin, with and without the presence of 0.05% pyruvate. A sample of the culture was removed after every 1 h and washed and plated. The survival

percentage of *E. coli* after 3 h and 6 h treatment of antibiotic with and without pyruvate had a difference of approximately 1-fold (Fig. 8b). These results demonstrate that, while a susceptible population is under stress and exhibiting persistence, the persister cells do not revive during the period of stress (antibiotic treatment). Hence, we can say that pyruvate does not resuscitate the persister cells during an ongoing antibiotic treatment.

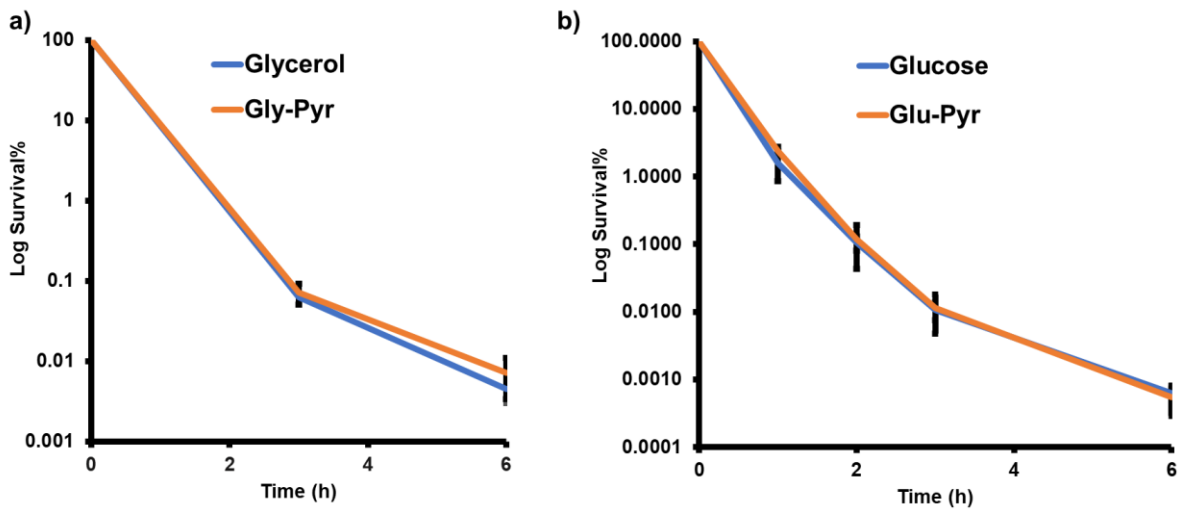


Fig. 8. Comparison of killing rates of *E. coli* **a)** glycerol and glycerol + pyruvate (Gly-Pyr). **b)** glucose and glucose+ pyruvate (Glu-Pyr). N = 3, and SEM is shown. Persister cells do not resuscitate with pyruvate during an ongoing antibiotic treatment.

3.3.2. *E. coli* persister cells resuscitate quicker in presence of pyruvate.

To determine whether *E. coli* persister cells could resuscitate faster in presence of pyruvate, an exponential phase culture (1.7 X 10^8 CFU/ml) grown in glycerol was treated with ampicillin for 3 h to induce the persister cells (Fig. 9). The percent survival was

0.04%. After 3 h treatment, two sets of media, one containing glycerol and the other one containing glycerol with 0.05% pyruvate was separately used to wake up the persister population. The results showed that the persister cells revived quicker in presence of pyruvate (Fig. 9a) than that off the revival in only glycerol without affecting the growth rate. To further confirm our results, we repeated the revival assay after inducing persistence with an antibiotic having a different mode of action such as ciprofloxacin (Fig. 9b). The results were coherent with what we saw previously with ampicillin, demonstrating persister cells resuscitated quicker when pyruvate was present. The population treated with pyruvate started reviving around 1000^{th} minute, while the population without any pyruvate treatment started reviving around 1500^{th} minute.

To determine whether, pyruvate was able to revive the persister cells quicker in presence of another carbon source glucose, we repeated the revival assay (see methods) with glucose and pyruvate (Fig. 10). Our results show that post 3 h treatment with ampicillin and ciprofloxacin, the persister cells resuscitated quicker when pyruvate was present. The difference in the revival rate with and without pyruvate was higher in ampicillin than that of ciprofloxacin. When treated with ampicillin, pyruvate started reviving the population around 750^{th} minute when compared to the onset of revival in the 1000^{th} minute for the population without pyruvate. However, upon treatment with ciprofloxacin, even though the pyruvate revived the population quicker, the revival started around the same time for both pyruvate-treated, and non-pyruvate treated population.

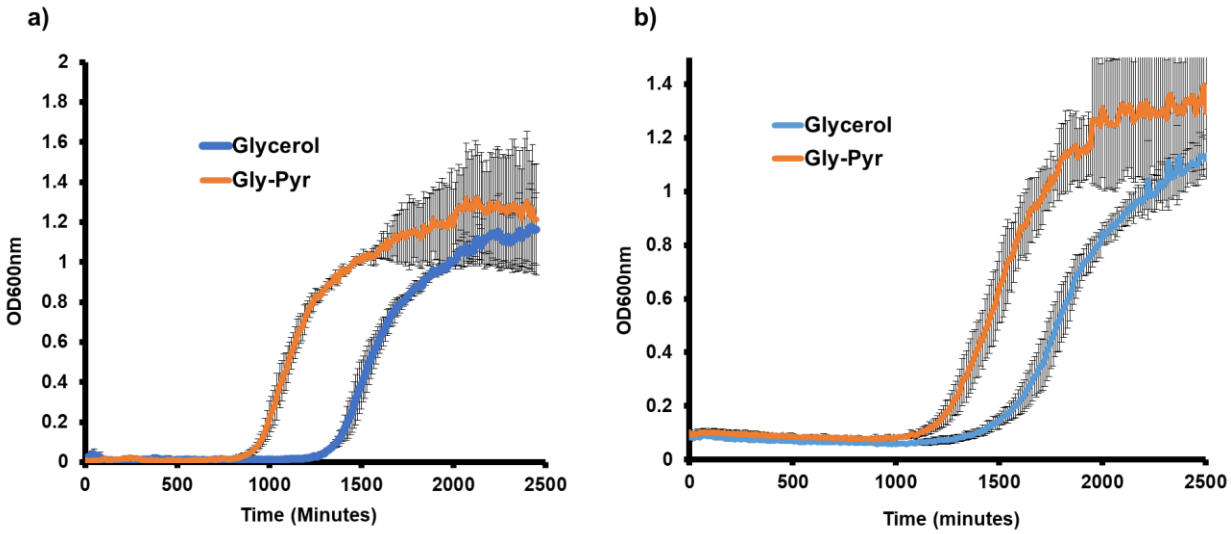


Fig. 9. Comparison of the revival of persister cells **a)** after 3 h ampicillin treatment **b)** after 3 h ciprofloxacin treatment, with glycerol and with glycerol + pyruvate (Gly-Pyr). N = 3, and SEM is shown. Pyruvate revived the persister population quicker without affecting the growth rate.

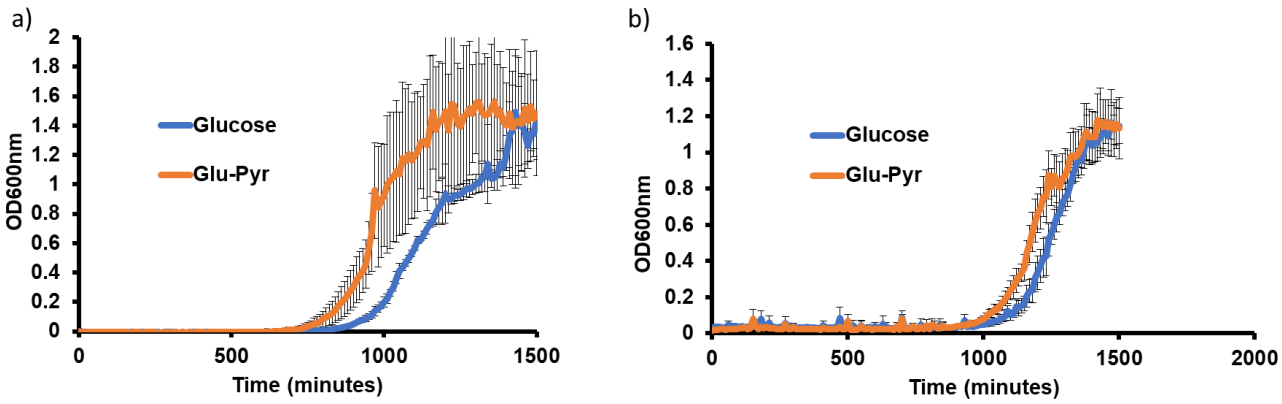


Fig. 10. Comparison of the revival of persister cells **a)** after 3 h ampicillin treatment **b)** after 3 h ciprofloxacin treatment, with glucose and with glucose + pyruvate (Glu-Pyr). N = 3, and SEM is shown. Pyruvate revived the persister population quicker, the onset of revival was faster in the ampicillin induced persister population.

3.4. Discussion

Bacteria can survive adverse environmental conditions (for e.g. effects of antibiotic) without having any mutation, by exhibiting phenotypic variation such as persister cells and viable but non-culturable (VBNC) cells [189]. Persister cells can wake up from the dormant state and grow like the susceptible population once the stress is removed [96]. VBNCs can be differentiated from persisters as they are undetectable by traditional agar plate methods while surviving in liquid culture [96]. A recent study showed that pyruvate can rapidly induce the resuscitation of the VBNCs in *E. coli* cells. According to them, only VBNCs were observed after the cells were kept at 4˚C for 120 days [153].

In this study we determined the effect of pyruvate on the resuscitation of *E. coli* persister cells. Our results show that, persister cells do not revive during the period of antibiotic stress (Fig. 8). We added ampicillin to *E. coli* cells with and without pyruvate and counted the CFU/ml post 3 h and 6 h treatment. After 3 h of ampicillin treatment, the persister levels with pyruvate was 0.012% while it was around 0.011% without pyruvate. These results show that the persister levels did not vary significantly with or without the addition of pyruvate, and remained approximately similar indicating that pyruvate does not revive persister cells during an ongoing antibiotic treatment. To verify our results, we repeated the experiment with a different antibiotic, such as ciprofloxacin and like our previous results, we saw that post 3 h treatment with ciprofloxacin, pyruvate did not change the persister percentage during the antibiotic treatment.

We further assessed the effect of pyruvate in resuscitation of *E. coli* persister cells post 3 h treatment of an exponential phase culture. The revival assay (see methods) was

conducted by inducing persistence with ampicillin using glycerol as the carbon source (Fig. 9). We saw that addition of 0.05% pyruvate in the washed cells revived them quicker without affecting the growth rate. The revival assay was repeated by treating an exponential phase culture with ciprofloxacin, and after 3 h, pyruvate revived the persister cells faster without altering the growth rate.

To assess the ability of the pyruvate in resuscitating persister cells, when induced in presence of the preferred carbon source, glucose, we repeated the same revival assay with the persister cells acquired from an exponential phase culture grown in MMB+ with glucose (Fig. 10). Firstly, we treated the culture with ampicillin for 3 h, and similarly in a second assay, we treated the culture with ciprofloxacin for 3 h, to compare the revival rates in presence of glucose with and without pyruvate (Fig. 10). The results show that, pyruvate was effective enough to revive the dormant persisters quicker when compared with glucose alone, similar to what was observed with glycerol previously. Although, with ampicillin treatment, the onset of pyruvate revival occurred approximately 200 minutes earlier than that of glucose alone, while upon ciprofloxacin treatment the revival with pyruvate started around the similar time of the onset of revival with glucose, even though the former reached the early stationary phase quicker. Regardless of the role of pyruvate in the resuscitation of VBNCs, we show that pyruvate can be a source that can lead to faster revival of persister cells when the antibiotic stress is removed.

3.4.1. Future goals for this project

In future, our lab aims to see whether VBNCs revive in the presence of pyruvate. It will be done at single cell level by diluting an exponential phase culture to 0.5 cells and reviving them with and without pyruvate. Also, another goal of this project is to

determine the similarity between the CFU/ml count on plate with the number of cells seen under the microscope, in order to deduce the existence of VBNCs for our strain of *E. coli*.

3.5. Materials and Methods

3.5.1. Microbial strains and antibiotics

Escherichia coli DH5alphaZ1 pkm24NB82 was used in this study. For growth and antibiotic survival assays, cells were cultured at 37°C in MMB+ media (25 µg/ml kanamycin with glucose or glycerol as carbon sources). All cultures were plated in Luria Bertani agar media for colony counts. The antibiotic stocks of ampicillin and ciprofloxacin were made by dissolving in water and 0.1 molar HCL respectively. The ampicillin stock (100 mg/ml) was stored at -80°C for longer use, and the ciprofloxacin stock (1 mg/ml) was stored at -20°C for future use.

3.5.2. Persister assay

To determine the killing curves, an overnight culture was diluted by 100X in fresh MMB+ media (with 25 µg/ml Km and either with glycerol or glucose). The culture was also diluted 100 X in MMB+ media (with 25 µg/ml Km and either with glycerol or glucose) and with 0.05% pyruvate in it. The cultures were shaken at 37°C shaker in 250 rpm till it reached the exponential phase (OD 0.4-0.5) and then treated with the antibiotics (100 µg/ml ampicillin and 20 µg/ml ciprofloxacin). Before treatment 1 ml of the culture was removed and plated to count the CFU/ml. After 3 h and 6 h of treatment, the cultures were washed with 1X MMB and plated to count the CFU/ml. The experiment was done with 3 biological replicates.

3.5.3. Revival assay of persister cells

An overnight culture was diluted by 100X in fresh MMB+ media (with 25 µg/ml kanamycin and either with glycerol or glucose). The cultures were shaken at 37°C shaker in 250 rpm till it reached the exponential phase (OD 0.4-0.5) and then treated with the antibiotics (100 µg/ml ampicillin and 20 µg/ml ciprofloxacin). Before treatment 1 ml of the culture was removed and plated to count the CFU/ml. After 3 h of treatment, the cultures were kept at 4°C. 1 ml of the cultures were removed, washed with 1X MMB to remove the antibiotic and plated to count the CFU/ml. The fridge culture was taken and centrifuged at 8000 x *g* for 15 minutes to concentrate the culture by 50X. The culture was then washed with 1X MMB and serially diluted in a microplate (with and without pyruvate containing media) and shaken at 300 rpm in a plate reader. The plate reader ran from 36 h-48 h to determine the growth curve based on ODs. The experiment was done in 3 biological replicates.

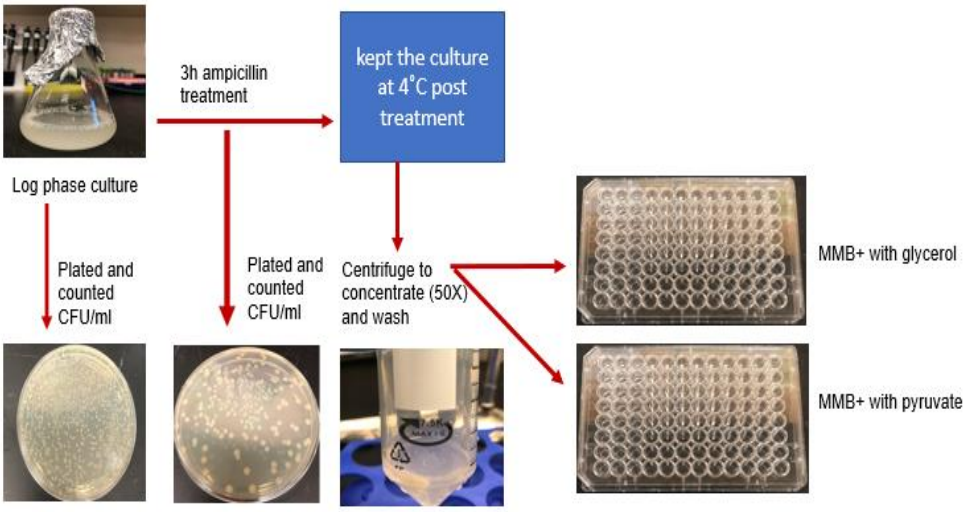


Fig. 11. Pyruvate persister revival assay. A log phase culture was treated with antibiotic. Post treatment, the persister cells were revived using different carbon sources.

4. CHAPTER 4. MONITORING ROOT-BACTERIAL ATTACHMENT AND BIOFILM FORMATION.

4.1. Abstract

Bradyrhizobium diazoefficiens USDA 110 are soil residing bacteria that can associate with legumes such as soybeans in a symbiotic relationship and fix atmospheric nitrogen in return of carbon as an energy source from the plants. *B. diazoefficiens* are poor competitors when the rhizosphere is concerned, and hence enhancing the competitive properties of the organism can increase the frequency of nitrogen fixation. The attachment of *B. diazoefficiens* to the soybean roots are facilitated by specific lectin proteins on the surface of the roots. This study focuses on how soybean root hairs can form nodulation and how root hair curling takes place upon formation of the infection thread by bacterial attachment. Our approach focuses on studying the root-bacterial attachment under a confocal microscope, by confirming the bacterial strains and morphology. Also, the future goal of this project is to monitor the attachment of different *B. diazoefficiens* USDA 110 strains and observe their affinity to beads coated with different lectin types. Furthermore, the attachment will be monitored by live imaging of soybean roots once the best affinity lectin protein is deduced.

4.2. Introduction

The United States leads the production of soybean globally, and soybean is the second largest crop in South Dakota [190]. Soybeans are legumes which have the capability to form symbiotic relationships with nitrogen-fixing soil bacteria such as *Bradyrhizobium diazoefficiens* (earlier known as *B. japonicum*) [190]. The nitrogen fixed in this way yields approximately 60 bu/ac, while in order to achieve higher yields, the

application of commercial fertilizers are required [190]. The drawback is that these fertilizers are not only expensive but can also lead to environmental pollution [191]. Due to the rise in the demands for soybeans, in both the domestic and global markets, the producer council has set a target to achieve yields of 100 bu/ac. To meet the requirements, an increased capacity in the nitrogen fixation is essential [192]. One of the major drawbacks is for root colonization, that *B. diazoefficiens* are poor competitors when compared to the indigestion populations, thus there is a substantial bottleneck during nodule occupancy [193, 194]. The long-term goal of our project is to increase the nodule occupancy by *B. diazoefficiens* that can lead to greater nitrogen fixation capacity.

4.2.1. Hypothesis

We hypothesize that modified root surfaces with engineered biomolecules (i.e., lectin proteins) will increase attachment of *B. diazoefficiens* strains, leading to a rise in nitrogen-fixing capacity.

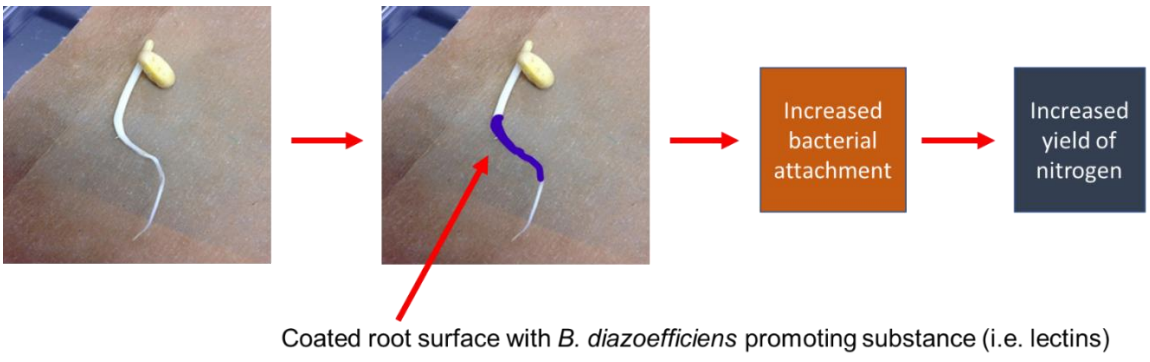


Fig. 12. Artificial coating of a soybean root with lectin proteins.

4.3. Literature review on Rhizobia-Soybean symbiosis

4.3.1. *Bradyrhizobia* species and their properties

Bradyrhizobia spp are motile, rod shaped, soil resident bacilli, with a Gram-negative cell wall [195]. They can form symbiotic relationship with leguminous plants, where the plant provides them carbohydrates from photosynthesis as a source of their energy in exchange of which the bacteria fix atmospheric nitrogen for the plants [196]. The *Bradyrhizobia* and the *Azorhizobia*, belong to the genera of Rhizobium [196]. These rhizobia bacteria can induce cell division in the root cortex of leguminous plants upon release of mitogenic signal molecules. Root-hair infection (nodulation) is one of the mechanisms by which rhizobia invades the root of the host plants [196]. *Bradyrhizobia spp* belong to the major community of soil microbes, however, unlike the rhizobia, not all soil microbes are capable of nitrogen fixation [195]. When compared to the other rhizobia spp, *Bradyrhizobia spp* are slow growing in nature and mostly require approximately 8 hours to double the population [197]. *Bradyrhizobia spp*, can show phenotypic variations when grown in different sugar sources [198]. In rhizobia-legume symbioses, the exopolysaccharides (EPS) play a substantial role in creating a successful infection thread and nodulation of the host legume [196]. The presence of different sugar sources can also alter the composition of the EPS and it is these changes that can affect the infection thread and legume nodulation of the host plant [199]. Even though, *Bradyrhizobium diazoefficiens* possess a subpolar flagellum, they can swarm on moist surfaces upon the induction of lateral flagella by L-arabinose in either liquid or semi-solid media [198]. This is of importance because, the motility of rhizobia is essential for them to compete for nodulation in the rhizosphere [200]. The successful root invasion typically depends on

several factors such as the activation of the nodulation genes and the positive chemotaxis of the bacteria towards the host exudates [201]. Rhizobia can infect hosts like pea plants, soybeans and groundnuts [196]. In this study, we are studying the root-bacterial attachment of *Bradyrhizobium diazoefficiens* (previously known as *Bradyrhizobium japonicum*) to soybean (*Glycine max*) root.

4.3.2. The process of nitrogen fixation

The chemical element nitrogen, also known as azote is essential as it contributes to the chemical composition of poisons, food, fertilizers and explosives [202]. The composition of N_2 in the atmosphere is 10^{15} tons, from which around 3 X 10^9 tons of the gas is transformed globally by the process of nitrogen cycle [203]. Around 60% of the earth's N_2 is fixed by biological processes, whereas 25% of the N_2 is chemically fixed via fertilizers [204]. Over the years, both the developing and the non-developing countries have seen a significant rise in the usage of N-fertilizers [205]. In the recent decades, the importance of nitrogen production by biological processes have decreased considerably as the food and crop production with N-fertilizers are more in demand [205]. However, as the usage of N-fertilizers are associated with extreme water pollution (release of toxic wastes in drinking water supplies) and eutrophication, the N_2 fixation by biological production is of great practical importance [206-208]. Also, even though fertilizers are applied in large doses, approximately 50% of it can be leached out [208]. This not only wastes money but energy too apart from being a severe source of water pollution [209]. The universal interest to produce N_2 biologically relies mostly on the symbiotic cooperation between rhizobia and leguminous plates, as this system impacts the nitrogen cycle quantitatively [205, 210]. Amongst the 13000 species of legumes, approximately

20% has been positively tested for N_2 fixation [205]. It has been estimated that the symbiotic relationship of legumes with rhizobia contributes to approximately 50% of the biologically fixed nitrogen entering the soil ecosystems [211]. Legumes are of ecological and agricultural importance as they play an essential role in fixing N_2 to chemical forms such as nitrates and ammonia. Additionally, it is reported that approximately 70 million tons of nitrogen is produced by legume symbioses [212]. Some of the repercussions of legume N_2 fixation is that the depletion of soil nitrogen reduces and the levels of cellular proteins in plants increase [213]. Plant growth is often hindered due to inadequate mineral nitrogen, thus the symbiotic system of the plants have evolved with various organisms [213].

4.3.3. Symbiosis between Soybeans and *Bradyrhizobium diazoefficiens*

One of the substantial difficulties of evolutionary biology is demonstrating the symbiotic associations between species [214, 215]. Such mutualistic relationship between species are universal even though it is difficult to understand the evolutionary constancy of such associations [215, 216]. If a single symbiont family associates with its specific host, then the two mutuals have a common interest that benefits each other [217, 218]. In the land based systems, rhizobia-legume symbiosis is the fundamental source of biologically fixed nitrogen [219]. Nitrogen fixed in such a way also proves to be a renewable source of nitrogen for agriculture [220]. The production of nitrogen through symbiotic association between legumes like soybeans with *Bradyrhizobium* is a cost-effective technique with adequate yield supply. This is also very substantial in pasture production and can be a strategy to improve the nitrogen nutrition of the grasses [221]. Soybean serves as the template to conduct studies on biological nitrogen fixation (BNF)

[222]. While temperature and light intensity can affect the efficiency of nitrogen fixation by soybeans [223], chemicals administered for crop protection can also have antagonistic effects on soybean BNF efficiency. One of the essential criteria to achieve higher efficiency is the selection of the specific strain of *B. diazoefficiens* [190, 224, 225]. It is also important as the inoculated strains if *B. diazoefficiens* has to survive and adapt to the soil environment in order to participate in BNF [226-228]. Soybeans are the only hosts with which *B. diazoefficiens* associate for symbiosis. The host-rhizobia interaction causes soybean root nodulation, which drives chemical reactions to reduce gaseous N_2 into ammonia (NH_3). As a result, the soybeans provide the bacteria with carbohydrates utilized by the inoculants as an energy source [222]. The EPS of the bacteria play a significant role for the initial phase of root colonization and subsequent survival of the bacteria in the soil environment [222].

4.3.4. Mechanism of root infection by rhizobia

The process of the movement of the rhizobia from the root surface into the nodulating cells is known as root infection [229]. The root-rhizobia infection is highly regulated and constitutes of three fundamental stages segregated as bacterial invasion of the root epidermis, multiplication in the root cortex and release of the bacterial cells in growing cells of nodule primordium [229]. The host-bacteria recognition takes place by a mechanism of crosstalk upon release of signals, which in return activates the root cortex cells to divide resulting in nodule organogenesis [230]. The crosstalk occurs in the rhizosphere, when the legumes such as soybeans release chemical signals (flavonoids) that can induce the rhizobia and trigger the synthesis and release of the Nod factors [231]. These Nod factors are combinations of lipo-chitooligosaccharide (LCO) with different

modifications, which is specific for every rhizobium strain enhancing the host-recognition specificity [232]. The flavonoids interact with the internal proteins of rhizobia [232]. The Nod factors are produced when the bacterial nod genes are activated. The nod genes encode enzymes that catalyzes the synthesis of the chitin backbone of the LCOs along with the acylation reaction and chemical substitutions [233]. The root epidermal cells of the host bear specific receptors NFR (Nod Factor Receptors) in their plasma membrane to bind the Nod factors [234]. The Nod factors cause root hair curling by triggering a series of complex changes inside the root hair. This forms the infection thread, the structure of which is a cellulose lined tube and bacteria travels through this tube into the root cells [235], infecting adjacent root cells. As a result, continuous cell proliferation takes place and forms the root nodule [236].

4.4. Results

4.4.1. *Bradyrhizobium diazoefficiens* USDA 110 confirmation under the microscope

We imaged two strains of *B. diazoefficiens* USDA 110 under the confocal NIKON microscope. The two strains were tagged with a green fluorescent protein (GFP) and a red fluorescent protein (mCherry). Each of these strains were confirmed to have these tagged proteins once around 1 µl of the exponential phase cultures were observed under the microscope at 100X (Fig. 13). The two strains were rod-shaped under the microscope exhibiting the general morphological characteristics of *Bradyrhizobia spp*.

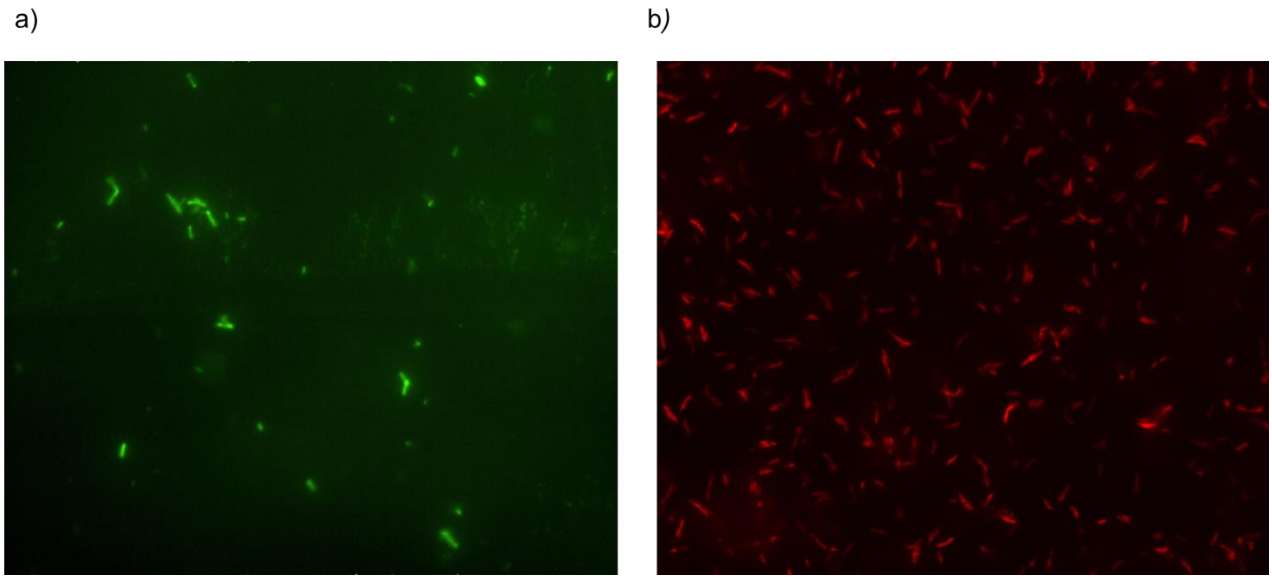


Fig. 13. *Bradyrhizobium diazoefficiens* USDA 110 **a)** GFP tagged and **b)** mCherry tagged, under the microscope at 1,000 magnifications.

4.4.2. Imaging root-bacterial interactions under the microscope

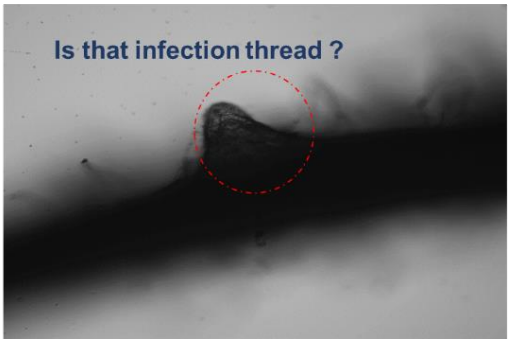
The confocal NIKON microscope was used to image the interactions of soybean root hairs with *B. diazoefficiens* USDA 110 (GFP or mCherry). The emergent root hairs of soybean were first observed under 10X without inoculation, in order to see whether the hairs had any curling or nodulation pre-inoculation. The root length was 45 mm on the 8th day of germination and under the microscope, the root hairs appeared to be thin thread like structures.

Moreover, the emergent root hairs were inoculated (see methods) with an exponential phase grown culture of *B. diazoefficiens* tagged with mCherry. A glass bottom dish (see methods) was used to image the seeds under the microscope. All the images were taken at 10X, as we tried with both 20X and 100X, in case of which the permanent focus of the microscopic lens would shift too often (after 2 h of the start of the

microscope) and would result in blurred images leading to experimental failure for multiple times. Therefore, a seed was observed for 24 h at 10X under the microscope, to see whether the permanent focus remained stable, and it was seen that the focus was stable for the entire period of imaging. Hence, after observing the root hairs pre-inoculation (Fig. 14a), the root hairs were inoculated at 8th day of germination and was observed at 10X. The images were taken at continuous time points after an interval of 60 seconds, for consecutive 7 days post inoculation, in order to see root hair curling. The focus of the setup was checked after every 6 h interval to keep it stable under the microscopic lens. Once, the images were taken, at 48th h of inoculation, a tumor like growth (nodulation) (Fig. 14b) was seen around the root hair surface. Furthermore, at 144th h of inoculation, some curled root hairs were also seen near the nodulation zone. The next goal is to determine fluorescence inside the root hair curls.

a)

b)



c)

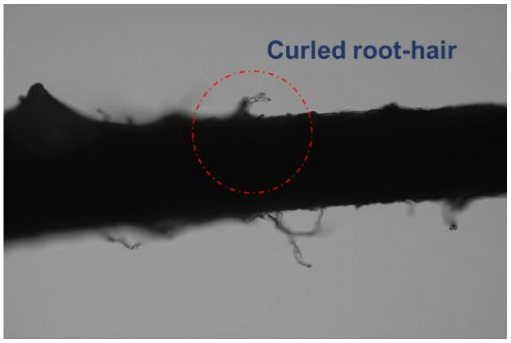


Fig. 14. Images of soybean root hairs at 10X under the microscope, **a)** soybean root hairs at 8th day of germination, pre-inoculation **b)** soybean root hairs at 48 h of inoculation (left) showing nodulation and **c)** at 144 h of inoculation (right) showing root hair curling adjacent to the nodulation zone.

4.5. Discussion

B. diazoefficiens are symbionts of soybeans in the process of biological nitrogen fixation (BNF) [222]. If the rate of the inoculants attaching to the root surfaces can be increased, then it gives a competitive advantage to the inoculants for higher nodule occupancy and biofilm formation [237]. However, the mechanism and genetic events involved in enhancing the host-bacteria attachment and colonization is unclear [237]. Also, post attachment and colonization, the changes in the host-bacterial metabolism are poorly understood. Our project focuses on bridging the gap of this knowledge and provide sufficient understanding on how the competition of the *B. diazoefficiens* for soybean attachment can be influenced and the subsequent events enhancing the bacterial colonization.

My contribution in this project has been a short one, along with my fellow lab members working on this project, I have been involved in the optimization of the germination protocol for soybean along with optimizing the culturing techniques of the *B. diazoefficiens*. Soybean germination is an easy, yet sensitive task given that it requires sufficient time and controlled environment to achieve well germinated seeds that can be used for experiments. The seeds were germinated under very controlled and sterile conditions such as in autoclaved containers and trays while using disinfectants like bleach to sterilize the seeds and remove the seed coats. We followed the standard protocol, but

after facing issues with repeated contamination and poor soybean growth we had to trouble shoot the whole process and optimize it. Soybeans were germinated in dark and in room temperature, with proper watering. It is possible that a whole batch of seeds does not germinate at all, hence, multiple batches are required to be germinated at the same time to obtain the optimum results. Furthermore, culturing of the *B. diazoefficiens* required supervision from Dr. Sen and Dr. Brozel. The organism is slow growing and required controlled conditions such as specific temperature and antibiotics to achieve the growth till exponential phase.

We have shown root hair curling of soybeans post inoculation with *B. diazoefficiens* tagged with mCherry. However, our team's goal is to determine the fluorescence in the nodulations and confirm that the bacteria indeed is contributing to root hair infection. Also, our team will focus on determining the lectin binding efficiency of the bacteria. This will be studied by coating microbeads with lectins and imaging the attachment of different strains of *B. diazoefficiens* with these microbeads. The strains will be provided to us by Dr. Brozel. Once we figure out the competency between different *B. diazoefficiens* strains, our team will focus on live imaging of the strains attaching with soybean roots.

4.5.1. Overcame challenges of this study

4.5.1.1. Contamination during soybean germination

One of the initial hurdles faced during the optimization stages was the soybean contamination during germination. The soybeans used to get contaminated with fungus often making it difficult to proceed with the imaging. After facing this issue for multiple times, several combinations of non-selective antimicrobials were used to minimize the

frequency of contamination. To figure out an optimum concentration of bleach and ethanol required to sterilize the seeds completely before germination, several concentrations of each of the reagents were used to determine the growth of the seeds faster with minimal contamination. It was seen that around 5% bleach solution followed by 5 minutes of shaking was enough to loosen the seed coats. Furthermore, 4 minutes of shaking with addition of 70% ethanol was required to completely sterilize the seeds. Once this combination started producing higher number of sterile root growth, it was used for the future germinations.

4.5.1.2. Slow growing bacteria

Bacteria usually takes 8-10 days to reach the exponential phase while growing in R2A liquid broth. Also, the frequency of contamination was significantly high, and it was strenuous to obtain an exponential phase culture with least difficulties. One technique to achieve a faster growth was to use the whole glycerol stock during a single inoculation. In order to achieve improved growth and less fungal contamination of the bacteria, we have grown a large volume of culture (50-100 ml) from the original glycerol stock, and once it reaches exponential phase, we concentrated it by centrifugation. The concentrated culture was later aliquoted in 1.5 ml tubes and stored at -80ºC for future use. However, the best way to overcome this problem is grow small volumes (5 ml) and concentrate, small volumes of inoculations led to lesser contamination.

4.6. Materials and Methods

4.6.1. Germination of soybeans

The soybean seeds were germinated following aseptic rules. The square shaped germination papers and glass beakers to soak and shake the seeds were autoclaved.

Square shaped plastic germination trays were soaked in bleach for approximately twenty minutes. After drying off the plates, the sterile germination papers were laid on the germination tray under the laminar hood to avoid contamination. Around, 8-12 seeds were taken in a small sterile beaker and shaken at 10% bleach for approximately 5 minutes. The bleach solution was rinsed off by rinsing the seeds with sterile distilled water for 6-7 times. Furthermore, 70% ethanol was added to the seeds and the seeds were shaken for about 4 minutes, after which the ethanol was rinsed off with sterile distilled water for 6-7 times. Ones the seeds were ready, they were placed horizontally on the germination papers under the laminar hood beside each other leaving enough space for the seeds to grow. Around 2 ml of sterile milliQ water was pipetted into the germination papers to soak them and provide moisture for the seeds. The germination tray (Fig. 15) was covered with aluminum foil and kept in dark for the next 7 days for the seeds to germinate. The seeds were observed every day and sterile milliQ water added in order to retain the moisture in the germination papers.

Fig. 15. A tray set up with soybean seeds prepared for germination.

The germination period usually lasts between 7-12 days. With 3-5 days (Fig. 16), the radicle of the seed comes out while in between 6-9 days the emergent root hairs show up. Once the emergent root hairs are seen, inoculation should be done with around 2 µl of an exponential phase culture.

Fig. 16. Soybeans at different stages of germination.

4.6.2. Culturing of *Bradyrhizobium diazoefficiens* USDA 110

The bacteria were cultured in R2A broth, with addition of tetracycline (25 µg/ml) and chloramphenicol (20 µg/ml). The tetracycline was used to maintain a resistant plasmid in both the GFP and mCherry tagged strains, while chloramphenicol was used to prevent contamination. The cultures were shaken at 30 °C shaker in 250 rpm till it reached the exponential phase (OD 0.4-0.5). Once the culture reached exponential phase, the cultures were concentrated by spinning for 3 minutes at 17 x *g* to prepare it for inoculating the seeds.

4.6.3. Imaging of the seeds

The seeds were imaged using inverted NIKON microscope. The seeds placed in a glass bottom dish and were inoculated with around 2 µl-3 µl of *B. diazoefficiens* USDA 110 (tagged with GFP or mCherry) at the emergent root hairs. Post inoculation, the root

was sealed at the center of the glass dish using agar pads so that the root hairs are aligned into the right position to be focused under the microscope. To retain the moisture in the set-up (Fig. 17), few drops of sterile water was pipetted around the edge of the plate, and the lid of the plate was sealed with parafilm.

Fig. 17. Soybean seed in a glass bottom dish, set-up for imaging under the microscope.

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