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Effect of Escherichia coli growth activity on its susceptibility to Azithromycin

Bachelor's Thesis (12 ECTS)

Curriculum Science and Technology

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Abstract:

Due to the gradual decline in antibiotics discovery and the escalating emergence of antibiotic resistance, treating bacterial infections have become more challenging. This study examines the effect of bacterial physiology on the efficacy of Azithromycin, using fluorescent proteins as tools to track antibiotic inhibition and bacterial growth stage. We found that there is an inverse correlation between bacterial rate of growth and the efficacy of azithromycin to inhibit protein synthesis.

Keywords:

Antibiotics, macrolides, azithromycin, bacterial physiology, fluorescent bioreporters

CERCS: B230 Microbiology, bacteriology, virology, mycology; T490 Biotechnology

Escherichia coli rakkude kasvuaktiivsuse mõju nende tundlikkusele asitromütsiini suhtes

Lühikokkuvõte:

Seoses sellega, et uusi antibiootikume tuleb kasutusele üha vahem, samal aj antibiootikumiresistentsuse kasvab, on bakteriaalsete infektsioonide ravi muutunud aina keerulisemaks. Käesolevad bakalaureusetöös uuritakse seoseid bakterite füsioloogia ja azitromütsiini efektiivsuse vahel. Eksperimentaalses töös kasutatakse fluorestseeruvaid bioreportereid et jälgida bakteriaalset valgusünteesi ja bakterite jagunemist. Töö käigus leiti negatiivne seos bakterite kasvukiiruse ja valgusünteesi inhibeerimis efektiivsuse vahel azitromütsiini poolt.

Võtmesõnad:

Antibiootikumid, makroliidid, aztromütsiin, bakterite füsioloogia, fluorestseeruvad bioreporterid

CERCS: B230 Mikrobioloogia, bakterioloogia, viroloogia, mükoloogia; T490 Biotehnoloogia

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TERMS, ABBREVIATIONS AND NOTATIONS

AZM – Azithromycin

BFP – Blue fluorescent protein

CAM – Chloramphenicol

CLR – Clarithromycin

Dox – Doxycycline

FP – Fluorescent protein

MBC – minimum bactericidal concentration - the lowest concentration that exhibits 99.9% reduction in the number of viable bacteria

MIC – minimum inhibitory concentration of drug that can completely inhibit bacterial growth in the test environment

noAB – no antibiotic

RFU – relative fluorescence units

SSC – side scatter

TimerRed - TIMERbac red fluorophore

TIMER$_{bac}$ / TIMER - Fluorescent timer – protein that changes colour with time

INTRODUCTION

Antibiotics are a vital class of medications that have saved countless lives and revolutionized modern medicine. They are used to treat bacterial infections in humans and animals and work by either killing the bacteria or preventing them from growing, thus allowing the body's natural defence to eliminate the infection. However, due to the overuse of antibiotics over the years, antibiotic resistance has emerged, making treating the previously viable infections more complicated. The discovery of novel antibiotics is, therefore, imperative, but exploring the pharmacological properties of available antibiotics is also a viable strategy to optimize their use and potentially repurpose them for new applications.

This thesis provides literature overview about the mechanism of action of macrolides and azithromycin, antibiotic resistance, and tolerance, and the bioreporters used in the experimental section. The experimental part of the thesis studies the correlation between bacterial growth activity of *Escherichia coli* and the antibiotic inhibition efficacy of Azithromycin, a broad-spectrum macrolide antibiotic. A series of experiments were carried out to examine 4 reporter plasmids, appropriate reporter plasmid was used. Using BFP and $TIMER_{bac}$ protein as bioreporters, antibiotic action and bacterial grow phase were measured using flow cytometry.

1 LITERATURE REVIEW

1.1 Antibiotics

Antibiotics are chemical agents that are used in bacterial infection treatments thanks to their ability to inhibit bacteria's growth or to kill them.

Antibiotics are typically classified as bactericidal or bacteriostatic. Bactericidal antibiotics can 'kill' bacteria, while the latter inhibits the microbes' growth. However, the definition is not so straightforward. To accurately categorise antibiotics, minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) are taken into consideration. The MIC is the lowest concentration of a drug at which a visible inhibition of bacterial growth after overnight incubation is observed. To determine the MIC, the antibiotic concentration varies within two-fold dilution step up and down from 1mg/L (Andrews, 2001). The samples that exhibit no growth are then incubated for 18-24 hours in an antibiotic-free media to further determine the number of bacteria that are still viable. MBC is then determined as the lowest concentration that exhibits 99.9% reduction in the number of viable bacteria (Pankey and Sabath, 2004).

Together with MBCs and MICs, another important measure is the minimum duration for killing (MDK), which is the time that takes to kill certain percent of the targeted culture since its exposure to antimicrobial treatment.

The MIC metric is usually used to quantify resistance of bacteria which is the ability of microorganisms to grow and bypass the high concentration of an antibiotic. Resistance is usually genetically inherited. The MIC for resistant strain is significantly higher than for the susceptible strain (Brauner *et al.*, 2016).

While there exist many methods to determine antibiotic properties and efficacy, MIC is by far the most used parameter when it comes to pharmacological selection of drugs and dosage. Even though MIC provides insights on the kinetics of the drug action, it overlooks the complex interactions between the administered drug, the infection site, and the pathogen. The actual concentration needed for successful antimicrobial treatment depend on the dosage, tissues distribution, exposure time and other factors such as the infecting agents, pH, nutrients supply and the host response (Mueller, de la Peña and Derendorf, 2004).

Since the infamous discovery of Penicillin G in 1928 by Alexander Fleming, there have been many major breakthroughs in antimicrobial studies (Gould, 2016). Besides targeting bacterial

cell-wall synthesis, nowadays, antibiotics with different mechanisms are available. Such mechanisms vary from interfering protein synthesis, depolarizing the cell membrane to inhibiting bacterial metabolic pathways. In this study, the focus is put on macrolide antibiotics, an antibiotic class that targets the bacterial ribosome to inhibit protein synthesis.

1.1.1 Translation and protein synthesis - good target for antibiotics

Translation is a process in which mRNA is translated into polypeptide. The process is carried out by ribosomes which can decode mRNA to synthesise proteins. Ribosomes in *E. coli* consist of 30S small subunit and 50S large subunit. The initiation of translation requires the binding of mRNA and initiator tRNA to the small ribosomal subunit. 50S large subunit then joins in the complex, forming a functional ribosome for polypeptide synthesis. There are three binding sites for tRNA on a ribosome, such sites are P (peptidyl), A (aminoacyl), and E (exit) sites. Elongation of amino acid chain proceeds as the initiator methionyl tRNA is bound to P-site, following by binding of an aminoacyl tRNA to A-site, a peptide bond is formed between the first and the second tRNA. This formation is catalysed by the ribosomal large subunit. After the initiation of elongation, translocation takes place. In this process, ribosome moves along the mRNA by three nucleotides, positioning the next codon in an empty A-site while moving the tRNAs to P-site and E-site respectively. This elongation continues until a stop codon is reached. Cellular release factors catalyse the hydrolysis between the tRNA and the polypeptide chain at P-site to release the complete polypeptide from the ribosome. (Cooper, 2000)

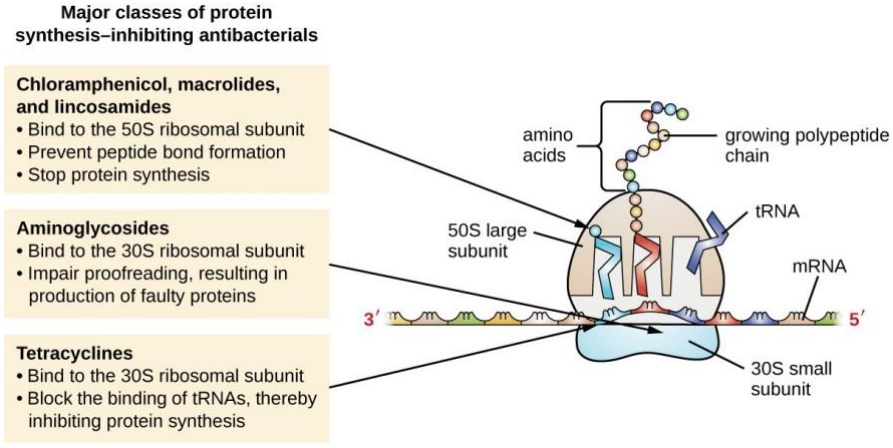

Figure 1. Classes of ribosomal-targeting antibiotics. [1]

[1] Classes of ribosomal-targeting antibiotics. Illustration adapted from https://www.coursehero.com/study-guides/microbiology/mechanisms-of-antibacterial-drugs/ - accessed on May 2023.

Given that translation and protein synthesis is a highly complex process which involves multiple steps and elements, while being a crucial process to cellular growth and survival, it is to be expected that this process is a popular target for antibiotic action in the bacterial cell (Figure 1). Almost all well-known compounds focus on the elongation cycle, and this includes every major class of antibiotics that have clinical importance. These antibiotic classes consist of aminoglycosides, chloramphenicols, fusidic acids, lincosamides, macrolides, oxazolidinones, streptogramins, and tetracyclines. Although the ribosome is a large structure, there are only a limited number of sites where antibiotics can bind and function. Antibiotic binding sites on the 30S subunit are located close together along the path taken by mRNA and tRNAs. Such antibiotics that bind to the 30S subunit tend to inhibit the translation initiation and elongation process. On the other hand, macrolides and other antibiotics bind to specific sites within the ribosomal exit tunnel, which is positioned near the Peptidyl-transferase centre (PTC) on the 50S ribosomal subunit, preventing the elongation of the most nascent chains (Wilson, 2014).

1.1.2 Mechanism of translation inhibition by ribosome-targeting antibiotics

Macrolides is a class of antibiotics whose catalogue begins with the discovery of Erythromycin in the 1950s. These antimicrobial agents bind to the ribosomal large subunit and target the protein synthesis process. Due to their broad-spectrum activity against numerous Gram-positive bacteria, they have been widely used in treatment and are considered to be an important class of antibiotics (Dinos, 2017; Vázquez-Laslop and Mankin, 2018).

The mechanism of macrolides involves binding to a specific site within the nascent polypeptide exit tunnel (NPET) of the ribosome, a tunnel through which the synthesized protein leaves the ribosome. While previously believed to inhibit protein synthesis by blocking the entire tunnel, preventing the exit of the synthesized polypeptide and therefore globally inhibit every translation, recent studies have shown that macrolides selectively target certain mRNA sequences, known as **macrolide arrest motifs** (MAMs) (Vázquez-Laslop and Mankin, 2018; Beckert *et al.*, 2021). In the event that MAMs are absent in a protein, the presence of macrolides would not have any impact on its translation (Kannan, Vázquez-Laslop and Mankin, 2012). The MAMs targeted by macrolides can vary, and those with a more extensive range of MAMs will impact the translation of a greater number of proteins. Macrolides generally interfere with translation by preventing the ribosome from catalysing peptide bond formation between the MAM residues (Vázquez-Laslop and Mankin, 2018).

Like other macrolides, Azithromycin inhibits bacterial protein synthesis by blocking the peptide exit channel on the ribosomal large subunit and interfering with translocation process during translation (Champney and Burdine, 1998). Azithromycin's increased basicity comes from an additional nitrogen in its 15-membered aglycone ring, this property allows Azithromycin to work more efficiently against Gram-negative bacteria in comparison to erythromycin from which azithromycin was derived (Dinos, Michelinaki and Kalpaxis, 2001). Another derivative of erythromycin is clarithromycin, like azithromycin, clarithromycin also binds to ribosomal 50S subunit and interferes with elongation of nascent polypeptide chain. However, clarithromycin is a more powerful inhibitor than azithromycin (Champney and Burdine, 1998).

Chloramphenicol is another broad-spectrum antimicrobial agent that belongs to the chloramphenicol antibiotic class. The agent works similarly to azithromycin as it also interferes with the elongation process during protein synthesis (Figure 1). However, in contrast to macrolides, chloramphenicol works by targeting the ribosomal A-site, directly interfere with the substrate binding(Schlünzen *et al.*, 2001). The antibiotic interacts with the 23S rRNA in the ribosomal 50S subunit and inhibits peptidyl transferase activity, preventing formation of peptide bond (Jardetzky, 1963; Schlünzen *et al.*, 2001).

When tested in standard laboratory condition Azithromycin generally has an MIC from 8-16 µg/ml for gram-negative bacteria. At the same time in humans during Azithromycin treatment the drug serum levels rase only to 0.5 µg/ml (Foulds, Shepard and Johnson, 1990). However, the drug is used in treatment of typhoid fever caused by *Salmonella typhi* (Butler *et al.*, 2001).It suggests that MIC testing in laboratory does not mimic well conditions of drug treatment in patient (Hinnu *et al.*, 2022)

1.1.3 Antibiotic Resistance and tolerance

With the excessive use of antibiotics since their discovery, some bacteria strains have developed the ability to resist antibiotic attack and survive. Antibiotic resistance refers to the genetic capability of microorganisms to proliferate even when exposed to substantially high concentration of an antibiotic, regardless of the treatment's length. This ability is quantified and characterised by the increase of minimum inhibitory concentration (MIC) (Figure 2). Antibiotic resistant strategies involve genetic mutations that change the antibiotic's target, enzymes that

directly neutralise the antibiotic, and the triggering of efflux pumps that eject the antibiotic from the cell (Brauner et al., 2016).

In response to Azithromycin, bacteria employ two primary methods of resistance. The first involves altering the antibiotic's target or binding site through the methylation of essential rRNA nucleotides or mutation of certain ribosomal components. The second method is through the activation of efflux pumps, which reduces the accumulation of the antibiotic within the bacterial cell (Wilson, 2014).

Tolerance, on the other hand, refers to the capability of microorganisms to survive brief exposure to high antibiotic concentrations without a change in the MIC (Figure 2), irrespective of its genetic heritability. There are two primary forms of tolerance: tolerance through slow growth and tolerance by lag. In the case of slow growth tolerance, the reduced growth rate enhances tolerance to various antibiotics. This form can be inherited if the bacteria naturally grow slowly, or it can be non-inherited if the bacteria grow slowly due to adverse conditions. As of the tolerance by lag, the tolerance occurs when slow growth is a result of an extended lag phase – the period needed for bacteria to adjust to a new environment before growth begins. This type of tolerance typically arises when the antibiotic treatment duration is less than the time it takes for the bacteria's growth to halt (Brauner *et al.*, 2016). Non-growing and slow-growing bacteria are usually more tolerant, because most antibiotics inhibit more efficiently when bacteria are highly active (Tuomanen *et al.*, 1986; Eng *et al.*, 1991). When a subpopulation of tolerant bacteria survives the period of antibiotic treatment much better, this subpopulation is said to be persistent to the antibiotic (Balaban *et al.*, 2019).

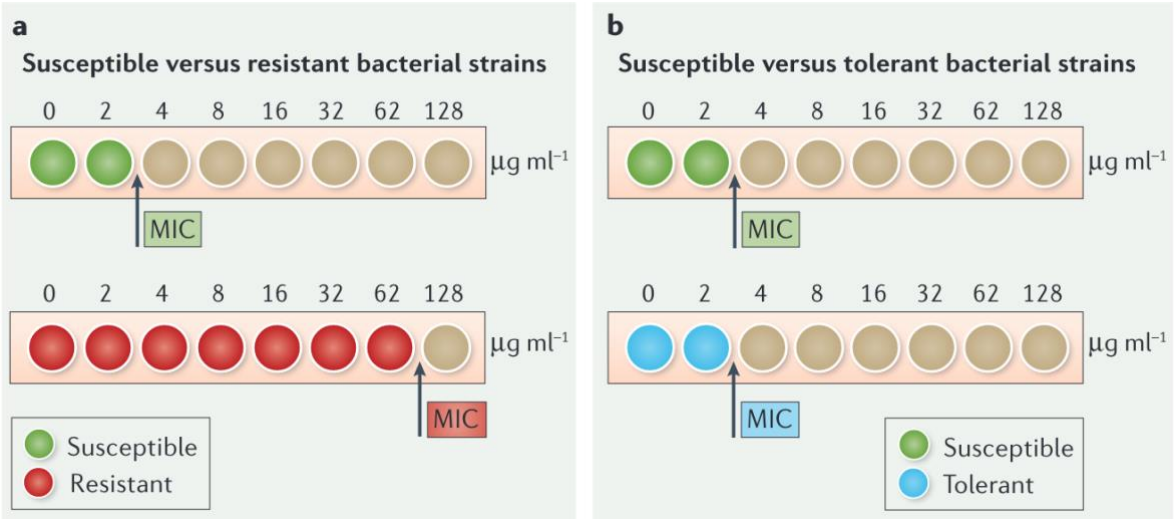

Figure 2. The MICs of drugs in resistant and tolerant strain vs susceptible strain. Coloured wells show bacteria growth. **(a)** Resistant bacteria have significantly higher MIC than the

susceptible strain. **(b)** Tolerant strain shares the same MIC with the susceptible strain. (Brauner et al., 2016).

During treatment, antibiotic resistance causes many failures, leading to increased mortality rate. When there is a viable solution, antibiotic resistant cases require significantly longer treatment time and complicated framework (Aslam *et al.*, 2018). For tolerant bacteria, when antibiotic treatment used in the infection whose growth is slow, treatment will be less effective, less killing will be observed. Treatment for tolerant infection caused by extended lag time often brings in no effective result, as the duration of lag phase could evolve to match the treatment duration (Brauner *et al.*, 2016)

1.2 Bioreporters

In laboratory grown bacterial culture, fluorescent proteins (FPs) can serve as a valuable genetic marker for tracking bacterial growth and assessing the potency of antibiotics (Schlechter *et al.*, 2021). FPs can be fused to protein targets in a 1:1 ratio, which makes them an ideal marker for quantitative measurements. Since the first discovery of green fluorescence protein (GFP) in the 1990s, a broad range of FP variants covering almost the entire visible spectrum has been developed and optimized. These variants include a diverse set of intrinsically fluorescent proteins derived from various sources, each with their own distinct colours ranging from blue (400-nm excitation/450-nm emission) to far red (600-nm excitation/630-nm emission) (Thorn, 2017). Figure 3 – C shows excitation and emission spectra for several popular FP. Most FPs are composed of a chromophore, which is responsible for the fluorescence, and a protein matrix that protects the chromophore and determines the protein's specific properties, such as colour and brightness. When FPs are excited by light at a specific wavelength, usually in the ultraviolet or blue range, the energy from the light is absorbed by the chromophore, causing it to transition to an excited state. The excited chromophore then relaxes back to its ground state by releasing energy in the form of a photon, or light particle, at a longer wavelength, resulting in fluorescence. By measuring this emission, researchers can monitor and quantify the expression of fluorescently tagged proteins, with technique such as flowcytometry, to study bacterial growth and bacterial susceptibility to the antimicrobial drugs (Thorn, 2017)

Bioreporters refer to genetic constructs that produce measurable signals in response to specific triggers or environmental conditions (Hansen *et al.*, 2001; Leveau and Lindow, 2001). These

measurable signals can be emission of light or a change in fluorescent colour. Bioreporters are versatile, and are useful for, studies of cellular physiology, biosensing, environment monitoring and countless other applications.

$TIMER_{bac}$ is fluorescent-protein based bioreporter which facilitates the monitoring and tracking of changes and growth in bacterial populations. This ability allows for the precise and quantitative detection of bacterial growth and changes over time. The protein is expressed constitutively and undergoes a complex maturation process that involves the emergence of green fluorophores followed by the delayed formation of red fluorophores. As a result, the fluorescence colour of the bacteria gradually transitions from green to red over time (Figure 3). This unique property allows researchers to track the growth and changes of bacterial populations in real-time with high precision and accuracy.

In cells that are not actively growing, both green and orange TIMER molecules can accumulate, with green molecules maturing more quickly than orange molecules. However, in dividing cells, the fast-maturing green molecules become more prevalent due to the dilution of the slow-maturing orange molecules during cell division before they can mature completely. This results in a higher proportion of green TIMER molecules in dividing cells. (Claudi *et al.*, 2014)

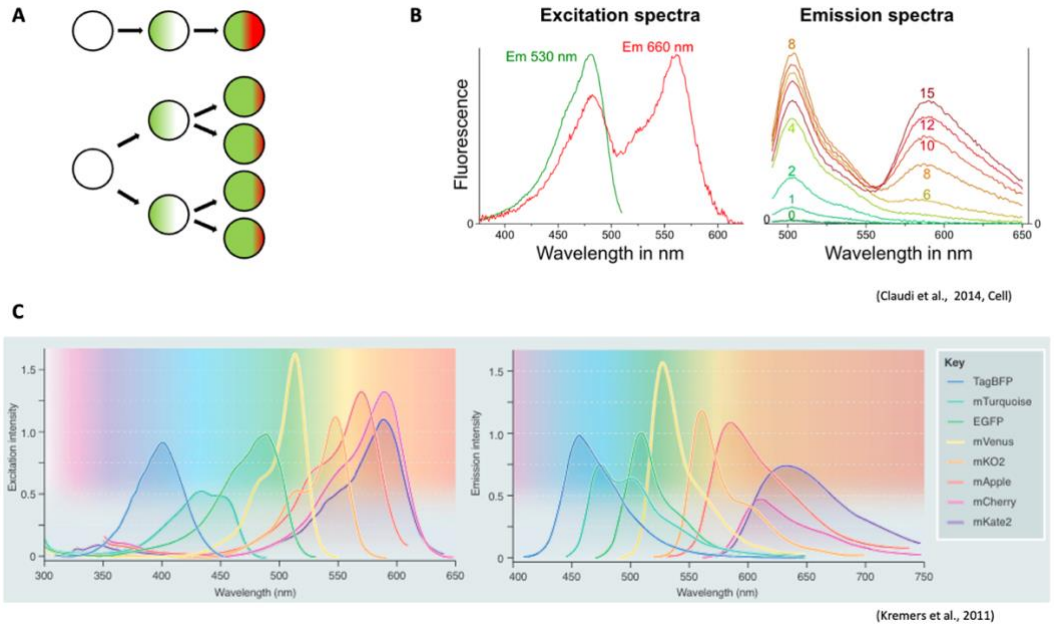

Figure 3. TIMERbac as bioreporters and their characteristics. (**A)** Non-dividing and dormant cells have both fluorophores, dividing cells have more green fluorophores. **(B)** Excitation and emission spectra of the red and green fluorophores (Claudi et al., 2014). **(C)** Excitation and emission spectra of different fluorescent proteins (Kremers et al., 2011).

2 AIM OF THE THESIS

This thesis aims to study the effect of *Escherichia coli* physiology on its susceptibility to Azithromycin.

This aim is broken down into specific aims and action:

- To test and find the bioreporter that is most optimal for the study.
- To test the MICs of the antibiotics used for the targeted strain.
- To study how bacterial growth activity corelate with Azithromycin's efficiency in protein inhibition by using BFP and $TIMER_{bac}$ proteins as bioreporters. BFP expression implies the efficacy of Azithromycin action as the antibiotic inhibit protein synthesis process, and TIMER fluorescent proteins monitor bacterial growth and cell division.

3 EXPERIMENTAL PART

3.1 Materials

3.1.1 Strains

The experiments were done using the MG1655 strain of *Escherichia coli* without and with plasmids described in 3.1.4 and Table 1.

Table 1. Strains and their respective plasmids.

Strain	Plasmid
BFP	pSC101-Timer-PtetA-BFP
BFP-OVA	pSC101-Timer-PtetA-BFP-OVA
R-BFP	pSC101-Timer-TetR-PtetA-BFP
R-BFP-OVA	pSC101-Timer-TetR-PtetA-BFP-OVA

3.1.2 Media

BD Difco^{TM} Lennox LB broth was used for the bacteria's inoculation, MG1655 competent cells and for the transformation of strains.

The standard BD Difco^{TM} Mueller Hinton Broth (MHB) was used for the determination of MIC. The media was prepared according to manufacturer Thermo Fisher Scientific instruction.

3.1.3 Antibiotics

For the selection of plasmid-carrying cells Ampicillin (at final concentration 100 µg/ml) was used.

Three antibiotics, azithromycin, clarithromycin, and chloramphenicol were used to study the effect of the antibiotics against the *E. coli* used (Table 2)

Table 2. Antibiotic stock solutions used in the study.

Antibiotic (abbreviation)	Concentration of stock solution	Solvent	Manufacturer, product number
Clarithromycin (CLR)	32 mg/ml	H_2O	Carbosynth, AC204691501
Azithromycin (AZM)	32 mg/ml	H_2O	Carbosynth, AD296571701
Chloramphenicol (CAM)	25 mg/ml	96% EtOH	AppliChem, A6435

3.1.4 Plasmids and primers

Table 3. Plasmids and primers used in the study

Plasmid	**Characters**	**Source**
pSC101-Timer-PtetA-BFP-DegTag	pSC101-based template plasmid with ampicillin resistance (ampR). This plasmid expresses BFP constitutively and has a degradation tag.	Mariliis Hinnu
pSC101-Timer-PtetA-BFP-noDeg (constitutive)	Constitutive expression of BFP	Mariliis Hinnu
pSC101-Timer-TetR-PtetA-BFP-DegTag	TetR regulated expression of BFP with degradation tag	Mariliis Hinnu

pSC101-Timer-TetR-PtetA-BFP-noDeg	TetR regulated expression of BFP without degradation tag	Mariliis Hinnu
Primers		
OPC-039	5' – GCCCTTTCGTCTTCACCTCG – 3' Binds 176 bp upstream of BFP start codon	Ordered from Metabion
Seq_rev_P2	3' – CGTTAACCAGGCCACTACGTTT – 5' Binds 406 bp downstream of BFP start codon	Ordered from Metabion

3.2 Methods

3.2.1 Preparing competent cells

A single *E. coli* colony was aerobically incubated in 3ml of LB overnight at 37°C, 220 rpm with Orbit Safe Incubator. 100x dilution of the culture was made by inoculating 100 ml of the overnight culture in 10 ml of LB in a 250ml flask. This solution is then incubated with the above-mentioned condition for 2 hours, optical density at 600nm (OD_{600}) reached about 0.2 – 0.3 (mid-exponential growth phase). This culture is then centrifuged for 10 minutes at 3000g-s and 4°C. The cells were kept on ice throughout the process. The supernatant was discarded, and cells were resuspended in 5ml cold 100mM $CaCl_2$. 1ml of the cell suspension was then centrifuged for 5 minutes at 2500g-s and 4°C. The supernatant was discarded, and once again, the cells were resuspended in 1ml of cold 100mM $CaCl_2$. This suspension was then centrifuged again under the same setting, supernatant was discarded, and cell were taken up in 100ml of cold 100mM $CaCl_2$. Final competent cells were kept on ice for 20 minutes.

3.2.2 Transformation, plasmid purification

2µl of DNA was added to 100µl of the competent cells. The solution was chilled on ice for 30 minutes, followed by heat shock at 42℃ for 45 seconds and then returned to ice for 5 minutes. The culture was incubated in 900µl LB at 37℃ in an incubator with moderate shaking. This culture was then centrifuged for 30 seconds at maximum speed. 900µl of the medium was discarded; the pellet was then resuspended with 100µl of media left in the tube and plated on LB plates that contain 100µg/ml ampicillin. Plated cells were incubated overnight at 37℃. Plasmids were extracted from overnight culture using FavorPrep Plasmid Extraction Minikit, DNA concentrations are presented in *Table 4*.

Table 4. DNA concentrations measured by NanoDrop

Plasmid	**DNA concentration (ng/µl)**
pSC101-Timer-PtetA-BFP	65.7
pSC101-Timer-PtetA-BFP-OVA	5.6
pSC101-Timer-TetR-PtetA-BFP	61.0
pSC101-Timer-TetR-PtetA-BFP-OVA	51.6

3.2.3 Preparation of DMSO stock

DMSO stocks of MG1655 strains that carry the reporter plasmids were prepared. A single colony was inoculated in 3ml of LB and incubated overnight at 37℃. The culture was then diluted 100 folds in fresh LB medium. The diluted culture was incubated aerobically in a shaker at 37℃ until an optical density at 600 nm (OD_{600}) of 0.8 was achieved. DMSO stocks of 8% were prepared on ice and distributed into PCR tubes. The aliquots were stored at −80℃.

3.2.4 MIC measurement

Bacteria cells were inoculated in 3ml of LB and incubated at 37℃ overnight. The culture was then diluted in MHB to OD_{600} 0.1 corresponding to approximately 10^8 CFU/ml. From that culture additional 100x dilution was made before inoculating bacteria.

Serial twofold dilutions were prepared on a 96-well plate to achieve a final gradient of antibiotic concentrations (µg/ml): 0.5 to 32 for AZM and CAM, 2 to 128 for CLR. The plate layout scheme is shown in **Figure 4**

		1	2	3	4	5	6	7	8	9	10	11	12
	AZM/CAM con. (ug/ml)	32	16	8	4	2	1	0.5	0	0	0	0	0
	CLR con. (ug/ml)	128	64	32	16	8	4	2	0	0	0	0	0
A	-	-	-	-	-	-	-	-	-	-	-	-	-
B	AZM								-	-	-	-	-
C	AZM								-	-	-	-	-
D	CLR								-	-	-	-	-
E	CLR								-	-	-	-	-
F	CAM								-	-	-	-	-
G	CAM								-	-	-	-	-
H	-	-	-	-	-	-	-	-	-	-	-	-	-

Figure 4. MIC microtiter plate layout scheme and antibiotic concentrations, dashed areas are filled with only MHB.

100 µl of bacterial culture was then added to the microtiter plate containing media and antibiotics at respective concentrations. The plate was sealed with parafilm, placed in a zip-block bag, and incubated at 37°C for 20 hours. Optical density at 600 nm of each well was measured by the BioTek Synergy H1 microplate reader.

3.2.5 Overnight plate reader experiment

100x dilution of the DMSO stocks was made in MHB. These suspensions were kept on ice.

In a 96-well microplate, 50 µl of MHB was added to each well, from column 7 to column 12, Doxycycline (Dox) 0.1ug/ml was added. AZM and CAM were tested against the four strains, BPF, BFP-OVA, R-BFP and R-BFP-OVA, at the concentrations from 0 to 4 (µl/ml) after serial 2-fold dilutions. The scheme of the experiment is shown in Figure 5 below.

		1	2	3	4	5	6	7	8	9	10	11	12
BFP	**A**	media						media					
BFP-OVA	**B**	media						media					
R-BFB	**C**	media			AZI			media			AZI		
R-BFB-OVA	**D**	media	4	2	1	0.5	0	media					
BFP	**E**	media						media					
BFP-OVA	**F**	media						media					
R-BFB	**G**	media			CAM			media			CAM		
R-BFB-OVA	**H**	media						media					

Figure 5: Overnight plate reader experiment scheme

The plate was placed in the BioTek Synergy H1 microplate reader, and measurements were taken at 15-minute interval for 17 hours. Bacterial growth was monitored by measuring absorbance at 600nm, expression of BFP was monitored by measuring fluorescence (excitation at 400 nm and emission collected at 450 nm)

3.2.6 Sequencing

Polymerase chain reaction (PCR) was performed on the DMSO stocks of *E. coli* carrying the plasmids pSC101-Timer-PtetA-BFP and pSC101-Timer-PtetA-BFP-OVA, using 5x HOT FIREPol EvaGreen qPCR Supermix.

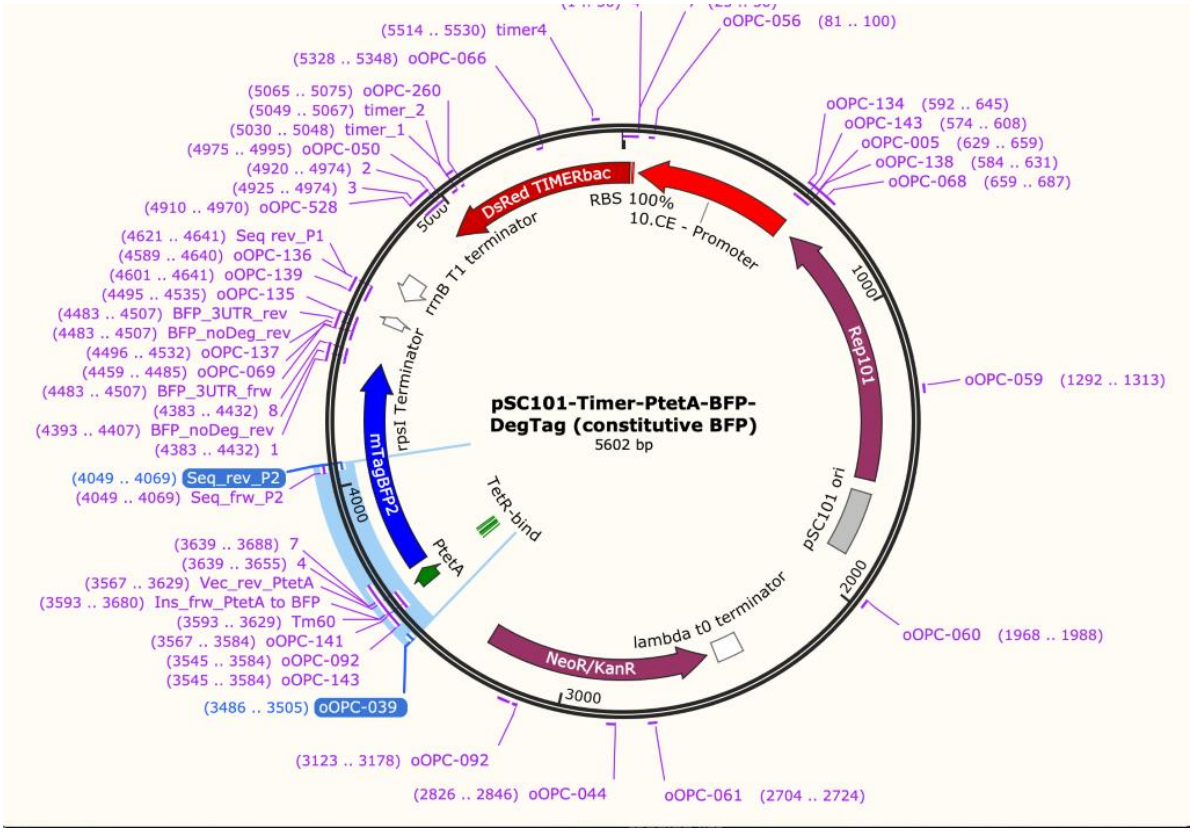

Figure 6. Sequencing region of plasmid map is shown in the blue highlighted region, in between Seq_rev_P2 and oOPC-039

For amplification of region of interest following primers were used: forward primer, OPC-039 and reverse primer, Seq_rev_P2.

The PCR products were sent to sequenced using Sager sequencing technique at University of Tartu's Institute of Genomics.

3.2.7 Time-killed curve experiment

30ul of bacterial DMSO stock is inoculated in 3ml LB and incubated overnight at 37℃ in a shaker. Overnight culture is diluted 1000x in 10ml MHB media. The dilution is then distributed into eight Eppendorf tubes and incubated at 37℃ (T0). After 1-2 hours (T1), AZM, CLR and CAM were added to achieve a final concentration 128 (μg/ml). Samples were taken at initial time point (T0), after 1-2 hours (T1 timepoint), after next 2h hours of growing in the presence or absence of antibiotics (T2 timepoint) and after 24 hours (T3 timepoint). The duration of between timepoints might vary between experiments. Viable cell numbers were estimated via colony counts by serial 10-fold dilution in PBS and plating on LB agar.

3.2.8. Flow cytometry:

Flow cytometry was done using the Attune NxT flow cytometer.

To prepare for flowcytometry measurement, 10x dilution of cell suspension was done in PBS. Measurements were taken at similar timepoints as mentioned above. Red fluorescence of TIMER was detected at channel YL2 (Excitation with 561 nm laser and emission collected at 605 to 625 nm range. Blue fluorescence of BFP protein was detected at VL1 channel (excitation with 405 nm laser and emission collected at 415 to 465 nm range)

3.3 Results

3.3.1 MICs measurement of studied antibiotics

To establish antibiotic concentrations that inhibit the growth of *E. coli* cells, MIC assay in 96 –well format was performed. Determined MICs of AZM, CLR and CAM for the BFP-OVA strain are documented in the Table 5 below. This measurement was collected from at least two parallels from 2 experiment repeats.

Table 5. MICs for the BFP-OVA strain

	AZM	CLR	CAM
MIC (μg/ml)	2	8	1

3.3.2 Overnight plate reader experiment to compare the reporter plasmids

To evaluate how the reporters respond to Azithromycin, the *E. coli* cells carrying respective reporters, BFP, OVA-BFP, R-BFP, R-BFP-OVA, were incubated in different concentration of antibiotics: 0 μg/ml, 0.5 μg/ml, 1 μg/ml, 2 μg/ml, and 4 μg/ml in a 96-well microplate over the course of 17 hours. Their reading (optical density at 600 nm showing bacterial growth and BFP fluorescence) was taken every 15 minutes by a microplate reader.

In the regulated strains, R-BFP and R-BFP-OVA, the expression of BFP is supposed to be induced by the presence of Dox. In the samples with 0 μg/ml concentration of antibiotic, after 17 hours, little to no expression of BFP in R-BFP and R-BFP-OVA was detected when there is no presence of Dox (Figure 7), indicating that the plasmids work as anticipated. Simultaneously, the constitutive strains exhibit noticeably reduced BFP expression in the presence of Dox, suggesting that Dox might inhibit some of the protein expression.

	-Dox	+Dox
BFP	31181	15808
BFP-OVA	56992	47693
R-BFP	529	31167
R-BFP-OVA	574	24083

Figure 7. Expression of BFP in from the 4 reporter plasmids after 17 hours. In the samples without antibiotic, the plasmids were incubated in the media either with or without Dox.

Over the course of 17 hours, during exposure to various concentrations of Azithromycin the strain that carries BFP-OVA expresses BFP significantly higher, almost two-fold, than its counterpart without OVA tag (Figure 8 – A, C). In the inducible strains, R-BFP and R-BFP-OVA, there is lower level of expression in the strain that carries OVA tag (Figure 8 – B, D). Inhibition of AZM starts already at concentration of 0.5 μg/ml. With most plasmids, the concentrations of effective inhibition do not follow a specific order, 2 μg/ml AZM might inhibits more efficiently than its concentration at 4 μg/ml in the regulated strains. Only in the constitutive BFP-OVA, inhibition efficacy is observed to be inversely proportional to AZM concentration (Figure 8 – C).

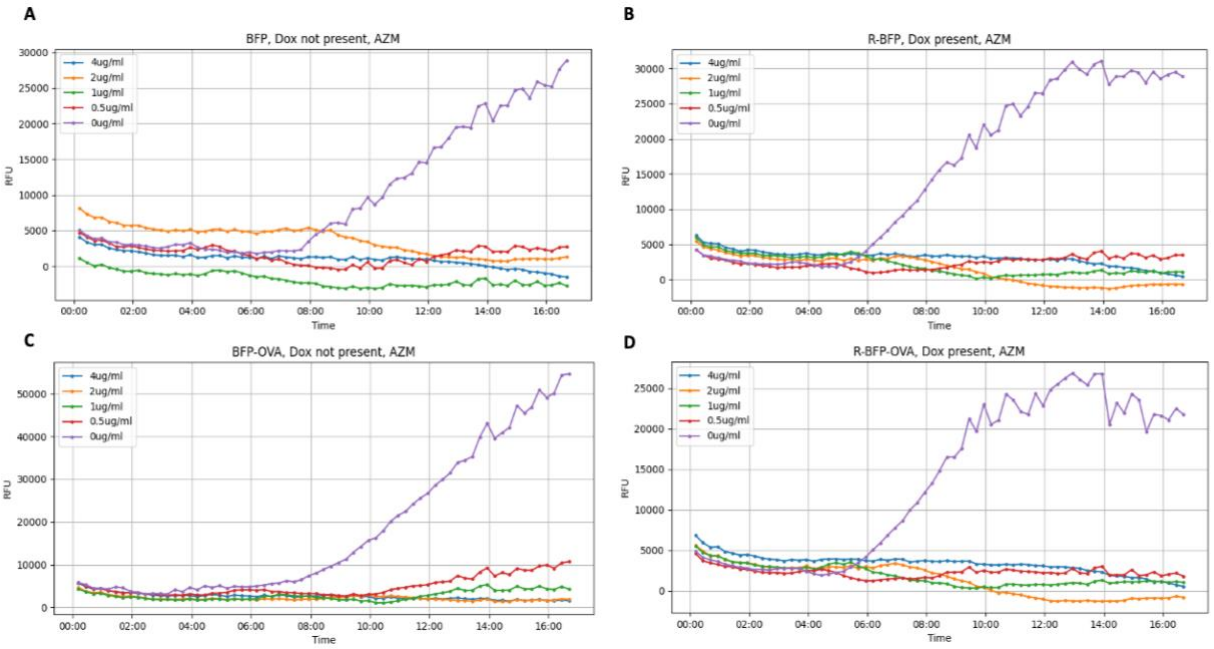

Figure 8: Expression of BFP from the 4 reporter plasmids. BFP expressions over 17 hours in various antibiotic concentrations. The bacterial strains carrying reporter - **(A)** Constitutive BFP, **(B)** R-BFP induced by the presence of Dox 0.1 μg/ml, **(C)** Constitutive BFP-OVA, and **(D)** R-BFP-OVA induced by Dox 0.1 μg/ml.

All the reporters work as anticipated, the OVA-BFP and BFP plasmids constitutively express BFP all the time, while their regulated counterparts require the presence of an inducer to express BFP, in this case, Dox was used. There was a significant decrease in BFP expression from the constitutive strains in the samples with Dox, suggesting that the inducer might inhibit some BFP expression. OVA-BFP is the most sensitive reporter to AZM as there is a clear inverse proportional relationship between protein expression and AZM concentration.

3.3.3 Sequencing

Despite OVA-BFP being deemed the most appropriate plasmid for the study after comparison of the plasmids, its significantly higher BPF expression in comparison to the constitutive strain without the tag raises a question. To confirm the integrity of the plasmids, PCR products of the strains that constitutively express BFP with and without OVA tag were prepared, the promoter regions and part of its coding sequence were sequenced. After sequences alignment, there is no major mutation in the constitutive BFP strain, a deletion of adenine before the promoter region was found. In the BFP-OVA strain, there is also deletion of adenine and another mutation – substitutions of a thymine by a guanine in the promoter region. The sequencing result is summed up in the Table 6 and their alignment is visualised in Figure 9 below.

Table 6: Sequencing result from sequences alignment

Mutation	BFP	OVA-BFP
Missing an adenine 18bp upstream of –35 box of RNA Polymerase binding sit	yes	yes
A guanine mutation in the 18 bp between the -10 and -35 regions in the promoter	no	yes

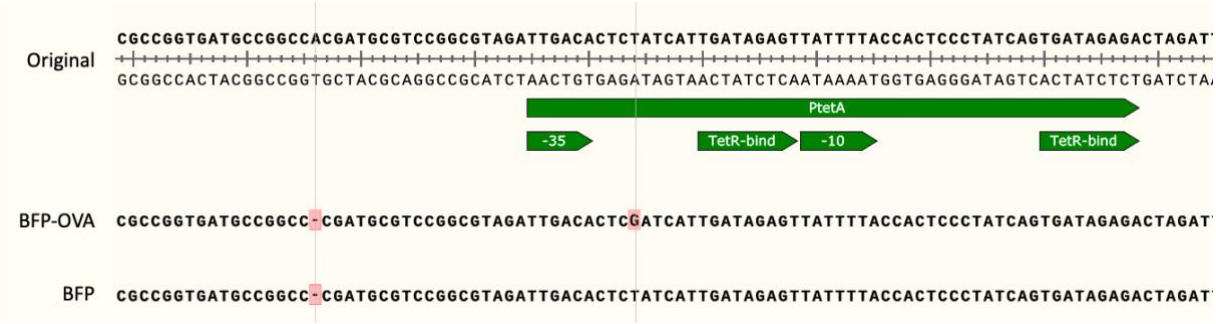

Figure 9. Sequences alignments of constitutive plasmids with and without OVA tag, BFP and BFP-OVA respectively.

3.3.4 Antibiotic time-killed experiment and FACS data

Using flow cytometry, the interaction between individual bacterial cells and AZM was monitored. In the general experimental setup, antibiotics were added 2 hours after diluting the

overnight culture of OVA-BFP strain into fresh MHB media. Samples were collected at four specific time-points: initially after dilution (T0 – 0 hour), immediately after the addition of antibiotics (T1), 2 hours after incubation with or without antibiotics (T2), and 22 hours later (T3), with a concentration of 128 μg/ml. Depending on the experiment, duration of time between T0 and T1 varied. Experiment 2 allows bacteria 1 hour shorter in time in their lag phase to adapt before adding AZM, summary of the set-up and bacterial growth/killing is presented on Figure 10.

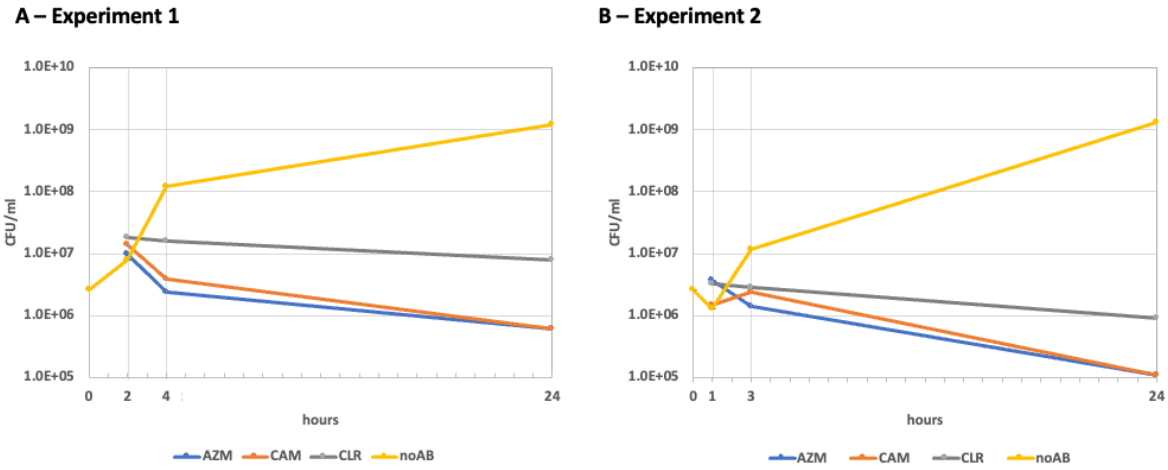

Figure 10. Time-kill curve. CFU-s of bacterial cultures grown at 128 μg/ml of azithromycin (AZM), chloramphenicol (CAM) and clarithromycin (CLR) over 24 hours.

From each time-point viable bacterial numbers were determined by CFU plating (Figure 10). At the same time all samples were analysed also by flow cytometry to see how bacteria respond to antibiotics.

The red fluorescence signal of $TIMER_{bac}$ protein (TimerRed) together with side scatter of 488 nm laser light can be used to estimate the growth of bacteria. When bacteria are actively dividing, TIMER red fluorophore does not have time to accumulate due to its slow maturation rate. When bacteria are actively growing, there is a decrease in TimerRed signal and increase in the side scatter (SSC) signal.

Based on that, it is possible to divide bacteria into 3 categories: stationary phase cells (high TimerRed and low SSC); active cells (high TimerRed and high SSC) and dividing cells (low TimerRed and high SSC).

Figure 11 shows experiment 1 with more active growth at T1 than in experiment 2, under the condition that the two experiments have only one difference which is the duration of grow.

In experiment 1, at time-point 1 (T1) : 2 hours of growing in the new media, there is an increase in side scatter level, bacteria are getting active. 2 hours later, at time-point T2, there is a significant decrease in TimerRed level, signifying that bacteria are now actively dividing after 4 hours in the new media. Similarly, to experiment 1, in experiment 2, bacteria after 1 hour (time-point T1) of adaptation have started being active. At time-point T2 – 2 hours after T1, there is little decrease in TimerRed expression in relation to two hours earlier.

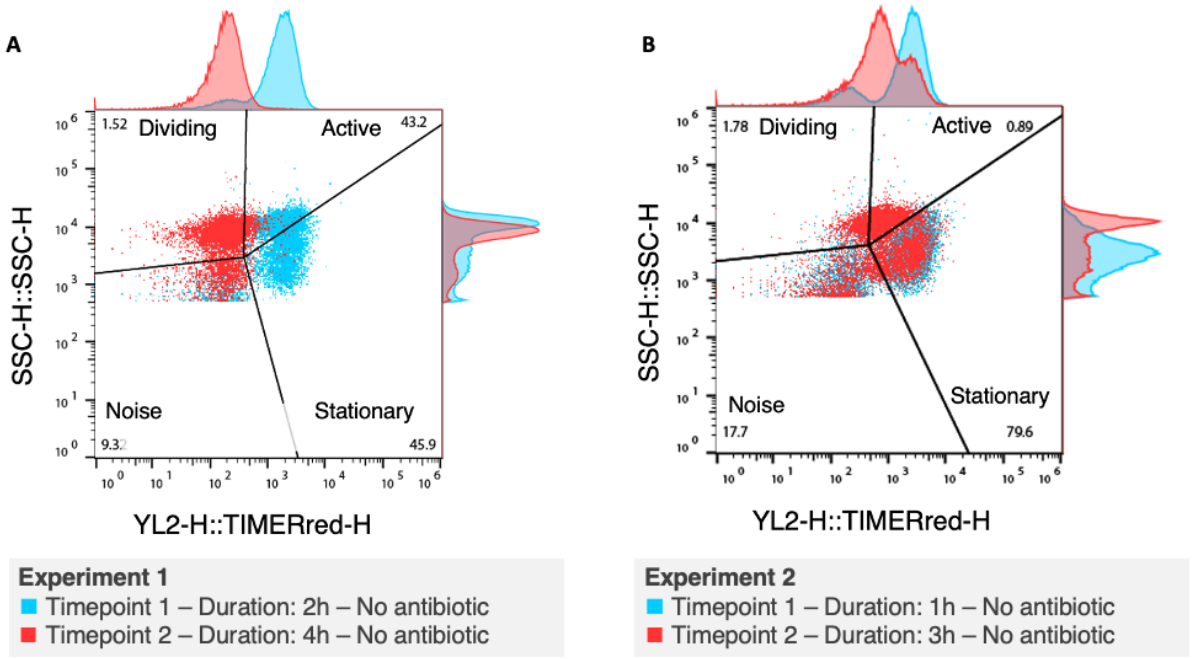

Figure 11. Expression of TimerRed fluorophore in the samples without AZM. Red fluorescent signal of $TIMER_{bac}$ protein (YL2-H::TIMERred-H) versus side scatter level (SSC-H::SSC-H) in **(A)** experiment 1 where bacteria had more time to adapt and grow than in **(B)** experiment 2

Distribution of bacteria into these three categories, stationary phase; active, and dividing cells, is shown for control and antibiotic-exposed conditions on Figure 12. In the experiment 1 where bacteria had more time to adapt and grow between the measurement time-points, more cells are actively dividing across samples with and without antibiotics compared to experiment 2 (Figure 12).

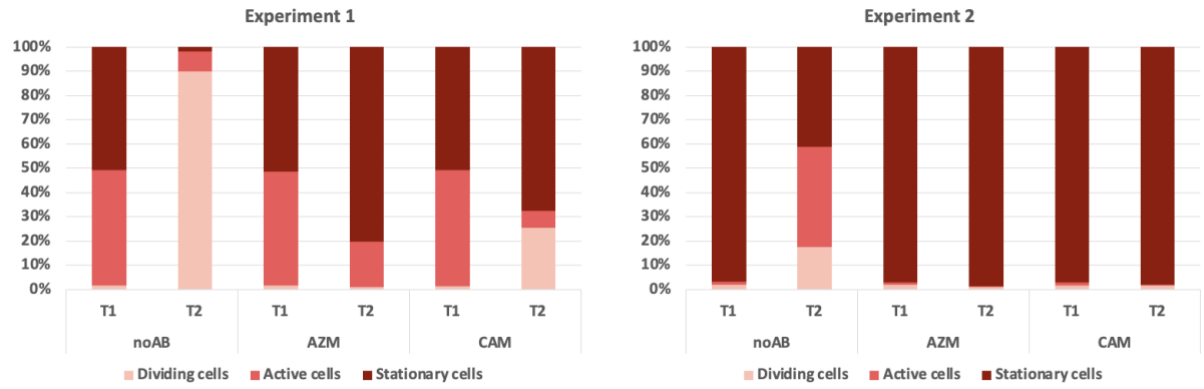

Figure 12. Cells at different growth state in sample without antibiotics, with AZM, and CAM.

As BFP expression level can be an indicator for the action of AZM on bacteria then next bacterial samples are analysed based on their blue fluorescence levels. In experiment 1, the samples where after AZM were added at time-point T1, BFP level increases at time-point T2 (Figure 13 – A), showing that AZM did not inhibit translation efficiently. While in experiment 2, bacteria had less time to adapt and grow before the addition of AZM, the expression of BFP decreases after two hours of adding the antibiotic (at time-point T2) (Figure 13 – A). This suggests that when bacteria are not actively growing, AZM targets and inhibits the protein synthesis more efficiently.

Samples with CAM added show inhibition in both experiments. However, stronger inhibition was observed when growth is highly active in experiment 1, and in experiment 2 where bacteria are not as active, CAM effect is less visible than in the first experiment, only a small decrease in BFP level was observed (Figure 13 – B). CAM, therefore, works more efficiently when bacteria are more actively growing.

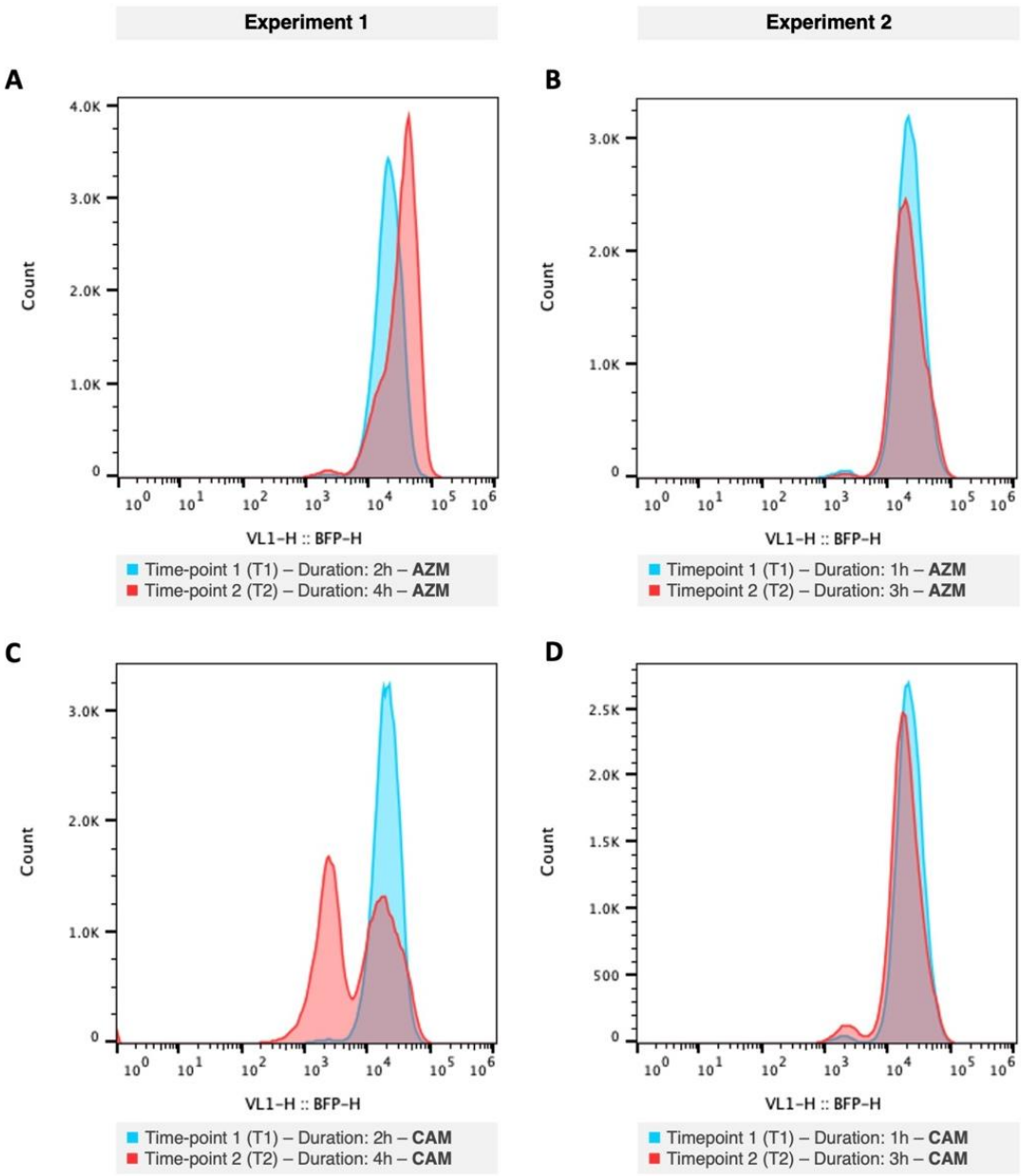

Figure 13: Histogram of BFP expression. **(A – B)** – Expression level of BPF in the samples with the addition of AZM. **(A)** In experiment 1, where AZM was added 2 hours after diluting bacteria to new media, BFP expression increases at time T2, no inhibition is observed. While in **(B)** experiment 2, inhibition takes place and a decrease in BFP expression was observed. **(C – D)** – Expression level of BPF in the samples with CAM. Inhibition is observed in both experiments, however, experiment 1 **(C)** has higher efficacy than experiment 2 **(D)**.

3.4 DISCUSSION

The aim of this study was to better understand the effect of Azithromycin on gram-negative bacteria like *E. coli*. For that, reporter-strain enabling to monitor both bacterial cell division activity as well as protein synthesis activity was used.

Regardless of antibiotic presence, the levels of BFP expressed from the constructs with OVA tag is expected to be lower in the 17-hour-grown culture than in those without the tag, as OVA-peptide may destabilises the expression of fluorescent protein (Bumann, 2001). In the inducible strains, this is true as R-BFP-OVA produced less blue fluorescent than R-BFP (Figure 7). However, it was observed in this study that constitutive plasmid with OVA tag expressed the highest BFP level (Figure 7). This anomality raised questions and thus the constitutive plasmids were sequenced. The sequencing result of the plasmid come out with a guanine mutation in the 18 bp between the -10 and -35 box. This is along the binding regions of RNA polymerase and sigma factors (Paget, 2015). It is suspected that this mutation increases the expression level of BFP at extent that the destabilizing effect of OVA tag is not detectable.

For many antibiotics, the bacterial growth rate and antimicrobial efficacy have long been known to have a direct proportional relationship. This means that bacteria are more susceptible to antibiotics when they are growing exponentially, as many cellular processes are at their peak activity (Tuomanen *et al.*, 1986; Eng *et al.*, 1991). Hindering the rate of growth can also lead to increase tolerance to many antibiotics (Brauner *et al.*, 2016). However, our study suggests an opposite view, slow rate of growth aids the action AZM, higher inhibition in the samples with less growth was observed (Figure 13). Findings from several earlier studies have also spotted this anomality, that with high growth rate, *E. coli* growth inhibition by roxithromycin decreases (Dinos *et al.*, 2003; Greulich *et al.*, 2015). A recent publication presents a possible explanation for this phenomenon (Łapińska *et al.*, 2022), demonstrating that fast growing cells exhibit a high abundance of ribosomes, some ribosomes manage to escape the binding of macrolides, enabling synthesis of proteins that carry out essential cellular processes, including efflux pumps, which is a major strategy in bacterial resistance to macrolides (Wilson, 2014; Brauner *et al.*, 2016).

With the use of antibiotic concentration at 128 (µg/ml), after 24 hours of exposure, both of our experiments end with similar growth inhibition and killing efficacy (Figure 10). However, on a closer look at earlier time-points, different variants of growth affect the efficacy of the drug (Figure 13). It is worth noting that it is not always important to reduce growth, but how bacteria interact with the host environment and their physiology are also essential. Macrolides have been

proven to be effective within bacterial biofilm infection, they specifically targets the inner centre of the colony more efficiently (Wu *et al.*, 2015; Łapińska *et al.*, 2022).

Results presented in this thesis together with literature data may add more understanding about the disparities between azithromycin's high MICs (usually 8 – 16 μg/ml) in vitro and its efficacy in treating typhoid fever by *S. typhi* with much smaller serum levels (<1μg/ml) (Butler *et al.*, 1999, 2001). In comparison to growing in an ideal and nutrition-dense environment like lab growth media, bacteria tend to have harder time to grow in human mucus (Conway and Cohen, 2015; Furter *et al.*, 2019).

In general, this study is in agreement with several collective publications that propose and support the unconventional idea that bacterial slow growth might aid antimicrobial efficacy of antibiotics, specifically azithromycin. On a wider outlook, azithromycin might be useful to treat slow-grow bacteria that usually require prolonged treatment. Further study of interaction between macrolides and other gram-negative bacteria, specifically the *Enterobacteriaceae* family, might bring in more useful insights to improve currently available treatment plans and inspire new strategies to overcome challenges in resolving bacterial infection.

SUMMARY

Better understanding of already available antibiotics allows us to optimise repurpose their use in treatment. This is especially important nowadays when discovering new drug is a labour-intensive process and is not always easy. With antibiotic resistance is an escalating problem, this becomes an even more realistic approach. In this study, we aim to gain better understanding on the effect of azithromycin on gram-negative bacteria like *E. coli.*

A 17-hour experiment was carried out to find the most appropriate reporter strain for this study. Constitutive strains, BFP and OVA-BFP, together with the inducible ones, R-BFP and B-BFP-OVA were tested with different concentrations of azithromycin. Inhibition was shown across the strains, however, the most strain that has the most stable and sensitive result was BFP-OVA.

MIC assay in 96 – well format was performed to determine MICs of azithromycin, clarithromycin, and chloramphenicol for the BFP-OVA strain, which are 2 (μg/ml), 8 (μg/ml), and 1 (μg/ml) respectively.

To study how bacterial growth activity corelate with Azithromycin's efficiency in protein inhibition, the interaction between individual bacterial cells and AZM was quantitatively studied by flow cytometry. BFP and $TIMER_{bac}$ proteins were used as bioreporters, they enable the monitoring of bacterial growth and antibiotic inhibition simultaneously. Two experiments were carried out in which experiment 1 allows bacteria more time to adapt and grow than experiment 2 before the addition of antibiotic, more active growth and less BFP protein synthesis inhibition by azithromycin was observed in experiment 1 than in experiment 2.

It is possible to conclude that azithromycin works more efficiently when bacteria are in their stationary phase.

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