Master's thesis

NTNU
Norwegian University of Science and Technology
Faculty of Natural Sciences
Department of Biotechnology and Food Science

Sigrid Ruyter Smolan

Studies of antibiotic tolerance in *E. coli* K12 MG1655 and the influence of the *rpoS* and *relA* stress response systems

Mechanisms of tolerance toward ampicillin, streptomycin and ciprofloxacin

Master's thesis in Biotechnology (MBIOT5)
Supervisor: Per Bruheim
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Biotechnology

Submission date: May 2020

Supervisor: Per Bruheim, IBT

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Preface

This project was carried out at the Department of Biotechnology and Food Science at the Norwegian University of Science and Technology (NTNU) in Trondheim, and represents the final part of my Master thesis in Biotechnology.

Acknowledgements

I would first and foremost like to thank my main supervisor Per Bruheim, for his guidance and excellent feedback through my master thesis work. His supervision and interesting discussions about my results during the final weeks of my project has taught me a lot. He has been a great motivator for me, and I am very grateful for having had the opportunity to work with such an inspiring professor, who also shares my enthusiasm for beer brewing. I would also like to thank my co-supervisor Lilja Brekke Thorfinnsdottir, for invaluable knowledge and guidance through my laboratory work, and for always being available for any help I might need. Their guidance and support has been beyond my expectations, and I'm incredibly grateful to them for letting me take part in such an exciting and important research topic in my thesis.

I would also like to thank all the MicPhys group members for guidance and the great company. We made the truly amazing K3 Amber Ale together, and it was equally entertaining brewing it and tasting it together.

Finally, I would like to thank my parents and friends, especially my fiddle squad, for endless support and in keeping me motivated, especially over the past two years.

Abstract

Multi drug resistance (MDR) is a rising problem worldwide, with infections by antimicrobial resistant bacteria causing approximately 700 000 deaths annually today. In order to handle this crisis, new antibiotics with novel mechanisms of action needs to be explored, as well as new treatment regimes. However, the fast development of resistance and tolerance toward both antibiotics already in use and novel antibiotics poses a serious challenge. Antibiotic tolerance, the ability of microorganisms to survive transient exposure to high concentrations of a drug that would otherwise be lethal, is the main focus point of this thesis. As natural antibiotic producers, most B*acteria* have some inherent degree of tolerance. Tolerance may also develop in sub-populations as a first stage of defence while preparing for the development of resistance, and in that way facilitate the evolution of MDR. It is also a phenomenon which is closely related to persistence, associated with decreased growth rates and dormancy. A range of interrelated stress response systems in *Bacteria* may contribute to the development of tolerance, and the RpoS system and the general stress response, and the *relA* (p)ppGpp network are especially important.

The main aim of this project was to understand the role of stress systems in antibiotic tolerance responses. The effects of three classes of antibiotics were explored with a wild type *Escherichia coli* K12 MG1655 strain and two knockouts Δ*rpoS* and Δ*relA*. As the knockout strains were new to the laboratory, experiments visualising their growth rate and pattern were performed in other to determine if they were directly comparable to the wild type. Minimal inhibitory concentrations (MICs) with bacteria inoculated in M9 minimal media, were determined for the three strains with three classes of antibiotics. The results were further used to examine tolerance responses in bacterial cultures in two cultivation systems with continuous read outs of metabolic status. Preparatory studies were performed with a well-plate reader, in order to better establish optimal conditions for high throughput microbioreactor cultivations. Samples of the three *E. coli* strains were treated a range of sub- and above-MIC concentrations of each of the three antibiotics, which were added in the middle of the exponential phase.

In general, ciprofloxacin was found to be the most effective antibiotic against all three strains, followed by ampicillin and streptomycin. Furthermore, the knockout strains were significantly more tolerant toward all the antibiotics than the wild type, and especially Δ*rpoS*. The antibiotic responses were also seen to vary greatly with variations in growth phase status, which became especially evident in the high-resolution cultivations. It became clear during the course of these studies that a strict protocol is needed in other to determine MICs and characterise tolerance responses more accurately. The observations made in this study will aid the further exploration of the development of antibiotic tolerance in advanced cultivation technology (fermenters). Of particular interest is the distinctions of antibiotic responses between actively growing cells and dormant and persistent cultures.

Sammendrag

Forekomst av bakterier med multiresistens mot antibiotika er et økende problem over hele verden og infeksjoner forårsaket av disse organismene fører til ca. 700 000 dødsfall i året. Den raske utviklingen av resistens og toleranse mot allerede eksisterende antibiotikatyper representerer en alvorlig utfordring for folkehelsen. For å motvirke denne negative utviklingen, må nye typer medisiner med nye virkningsmekanismer utvikles i tillegg til nye behandlingsformer. Antibiotikatoleranse, altså mikroorganismers evne til å overleve forbigående eksponering for høye konsentrasjoner av et legemiddel, som ellers ville være dødelig, er hovedfokuset for denne oppgaven. Som naturlige antibiotikaprodusenter har de fleste *Bakterier* en viss iboende grad av toleranse. Denne fenotypen kan også utvikles i sub-populasjoner som et første forsvar mens bakteriene forbereder seg på utviklingen av resistens. På denne måten bidrar toleranse til å legge til rette for utviklingen av multiresistens. Toleranse er også et fenomen som er nært knyttet til forekomsten av persistere, med reduserte vekstrater og en tilstand av dvale. En rekke iboende stressresponssystemer i bakterier kan bidra til utviklingen av toleranse, og RpoS-systemet og *relA*-nettverket (p)ppGpp er spesielt viktige.

Hovedmålet med dette prosjektet har vært å øke forståelsen av stressresponssystemenes rolle i utviklingen av antibiotikatoleranse. Effekten av tre klasser av antibiotika ble derfor utforsket; henholdsvis med en villtype *Escherichia coli* K12 MG1655 og to de to knockout stammene Δ*rpoS* og Δ*relA*. Ettersom knockoutene var nye i laboratoriet, ble det først utført eksperimenter for å sammenligne vekstrater og vekstmønster mellom disse og villtypen. Minste hemmende konsentrasjoner (MIC) av 3 typer antibiotika ble bestemt for de tre bakteriestammene inokulert i M9 medium. Resultatene ble videre brukt til å undersøke toleranseresponser i to kultiveringssystemer og med kontinuerlige avlesninger av metabolsk status. Forberedende studier ble utført med en brønnplate-leser for å etablere gunstige forhold for senere mikrobioreaktor forsøk. De tre *E. coli*-stammene ble behandlet med en rekke konsentrasjoner av antibiotika over og under deres respektive MIC (tilsatt midt i eksponentiell fase).

Generelt ble ciprofloxacin observert til å være den mest effektive av antibiotikaene mot alle tre *E. coli*-stammene, etterfulgt av ampicillin og streptomycin. Videre var knockoutene betydelig mer tolerante mot alle tre antibiotikaene enn villtypen, og spesielt Δ*rpoS*. Responsen varierte også mye med variasjoner i vekstfasestatus. Det ble klart i løpet av disse studiene at en streng eksperimentell protokoll er nødvendig for å kunne sammenligne MIC mellom de ulike forsøkene og for de ulike bakteriestammene, og videre for å kunne karakterisere toleranseresponser nøyaktig. Denne studien har bidratt med ny kunnskap som vil hjelpe i den videre utforskning av antibiotikatoleranse ved bruk av avansert dyrkingsteknologi (fermentorer). Av særlig interesse er forskjellene mellom antibiotikaresponser i aktivt voksende celler og persistere og vedvarende inaktive kulturer.

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Abbreviations

6-APA	6-aminopenicillanic acid
AMP	Ampicillin
AMR	Antimicrobial resistance
ATP	Adenosine-5'-triphosphate
CDC	Centre for Disease Control
CFU	Colony forming units
CIP	Ciprofloxacin
CTD	C-terminal domain
dH_2O	Distilled water
DNA	Deoxyribonucleic acid
DO	Dissolved oxygen
E. coli	*Escherichia coli*
EtOH	Ethanol
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FHI	Norwegian Institute for Public Health
GDP	Guanosine-5'-diphosphate
GMO	Gene modified organisms
GTP	Guanosine-5'-triphosphate
HCl	Hydrochloric acid
KO	Knockout
LB	Lysogeny Broth
M9	Minimal salt media
M9-hbc	M9 with high biomass capacity
Mar	Multiple antibiotic resistance
MDR	Multi drug resistance
MIC	Minimal inhibitory concentration
MQ-H_2O	Milli-Q®-water
NaOH	Sodium hydroxide
NTD	N-terminal domain
NTU	Nephelometric turbidity units
OD	Optical density

ON	Overnight (culture)
PBP	Penicillin-binding proteins
(p)ppGpp	guanosine-3′-diphosphate-5′-triphosphate / guanosine-3′,5′-bisphosphate
QRDR	Quinolone Resistance-Determining Region
QS	Quorum Sensing
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
rpm	Rotations per minute
SP	Stationary Phase
STR	Streptomycin
TA	Toxin Antitoxin
TLS	Translation Synthesis
tRNA	transcriptional RNA
UV	Ultraviolet
WT	Wild Type

1 Introduction

1.1 The challenge of antibiotic use

Infections by multi drug resistant (MDR) bacteria represent one of the major challenges for human health worldwide. A review report [1] estimated that by the year 2050, 10 million lives a year will be at risk due to the rise of drug resistant infections. Today, approximately 700 000 people die of infections caused by antimicrobial resistant (AMR) bacteria annually, and of those approximately 50 000 cases occur in Europe [2, 3]. There is therefore an urgent need for new solutions in order to slow down the rise of drug resistance. If antibiotics lose their effectiveness, there is a substantial risk that key medical procedures could become too dangerous to perform [3]. While awaiting the development of new treatment options, a moderate or sparing use of currently available antibiotics is essential to prolonging the lifespan of remaining effective agents [4]. Most of the direct, as well as much of the indirect impact of AMR will fall on low and middle-income countries. It is also speculated that the AMR-problem could impact the degree of mortalities in people with bacterial pneumonia during Covid-19 infection. One factor which may have contributed to the much more severe corona-situation in Italy and Spain compared to Germany, is the difference in use of antibiotics, both in agriculture and in medicine.

Bacteria can possess innate resistance to certain antibiotics, or such survival mechanisms could be triggered by environmental stress, which again will impact the management of infectious diseases [5]. A serious challenge today is the fast development of tolerance and resistance to new antibiotics [6]. AMR can be widely spread and established in the population already at the early stages of introduction of the antibiotic to the clinical market. One solution could be production of novel antibiotics with a mechanism of action that makes development of resistance extremely challenging for the bacteria. Alternatively, antibiotics should be used together with other agents that inhibit the bacteria's ability to develop resistance. A central resistance-mechanism is the translation synthesis (TLS) which plays a critical part of the bacterial stress response. In this mechanism, error-free DNA polymerases are replaced with error-prone versions, which results in increased mutagenesis frequencies [7]. Another recently realized fact is that sub-populations of bacterial cultures can become tolerant as a first stage of defence while preparing for development of resistance.

1.2 The use of antibiotics globally and in Norway

Despite efforts to encourage less use of antibiotics, an international team of researchers found a 65 % rise in worldwide consumption from 2000 to 2015. This sharp increase was driven almost entirely by the use in poorer nations [1]. Under the Infectious Disease Control Act, the Norwegian Institute for Public Health (FHI, [8]) is responsible for monitoring infectious diseases, as well as the use of antibiotics in Norway and assisting in the international monitoring thereof. Compared with other European nations, Norway has a low overall use of antibiotics (Figure 1.1), in particular because the use of these drugs as growth enhancers in food production is illegal [8]. According to the FHI report, the country has since 2012 seen a reduction in antibiotic use of approximately 20 %.

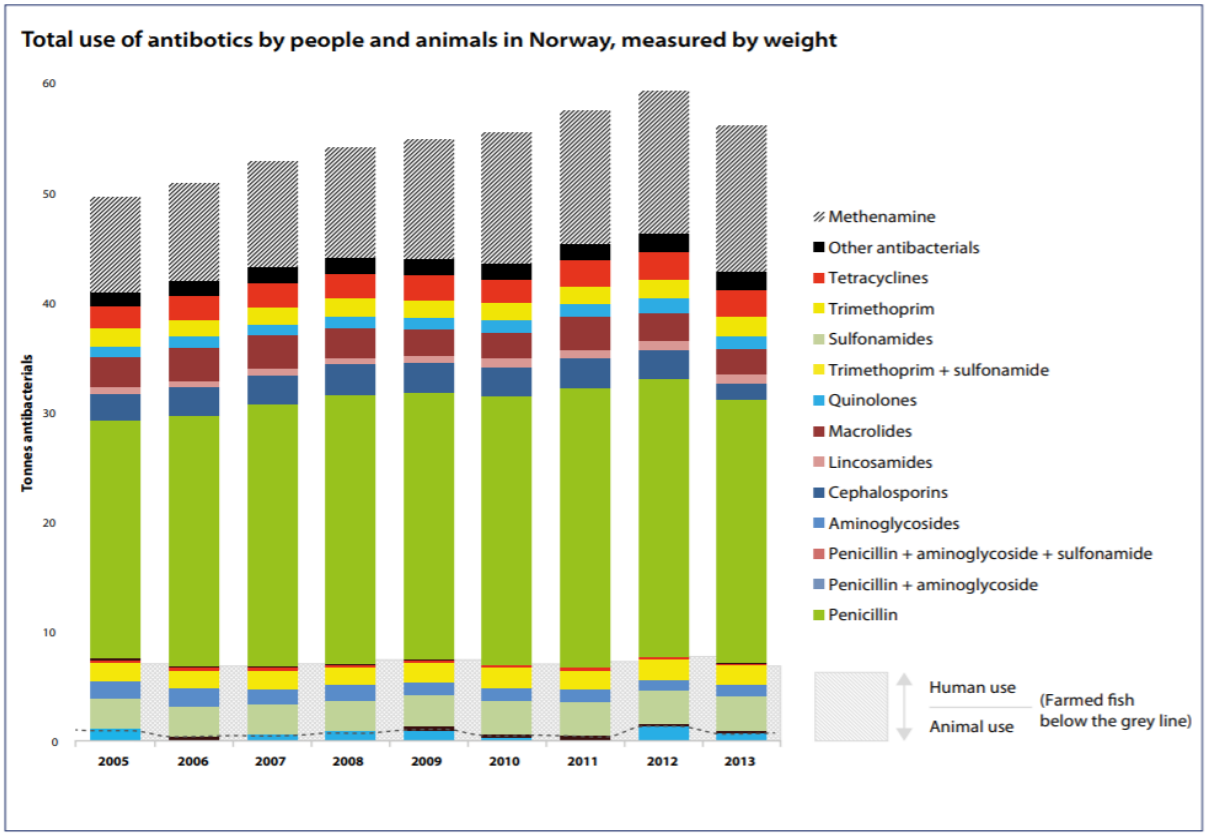


Figure 1.1: Total use of antibiotics in Norway
from the report from the Norwegian Ministries, National Strategy against Antibiotic Resistance 2015–2020, [8]

Antibiotic resistance is a naturally occurring property of a large number of bacterial species. However, over the past 10-20 years a sharp increase in the occurrence of antibiotic resistant bacteria has been observed in many different ecological niches. There are many reasons for this, but increased antibiotic use, combined with exposure to other resistance-driving substances, such as disinfectants, biocides and heavy metals,

probably has major significance. We still lack sufficient information about whether the use of preservatives in food and cosmetics leads to changes in our normal bacterial flora or contributes to the development of resistance. Ultimately, a major aim for the future is to reduce the global use of antibiotics. In Norway, the aim is an annual reduction of 3 % [8].

1.3 Antimicrobial agents

Over the last 80 years, the use of penicillin has saved countless lives from a variety of bacterial infections. The discovery of this antibiotic drug by Alexander Fleming in 1928, represented a revolution in the field of medicine, and is still recognized as one of the greatest medical achievements in the 19th century [9]. Antibacterial agents, often referred to as antibiotics, are a group of substances produced by microorganisms that targets other microorganisms and either inhibits their growth (bacteriostatic) or kills them (bactericidal), while having minimal effects on the host cells and tissues. Natural antibiotics are produced by a variety of filamentous fungi and bacteria (mostly *Actionbacteria*). These drugs are produced by large-scale industrial fermentations for clinical use, or they can be artificially modified into semi-synthetic drugs with increased efficacy [10].

Antibiotics is also separated into broad-spectrum drugs, which are effective against a relatively wide range of both Gram-negative and Gram-positive bacteria, while narrow-spectrum agents only target specific types of bacteria. For example, penicillin G is only effective against Gram-positive bacteria, as the Gram-negative are naturally resistant in being impermeable to the drug. Furthermore, most antimicrobials are classified according to their principal mode of action, interfering with essential life process in bacteria. Important targets are cell wall synthesis, protein synthesis, nucleic acid synthesis or folate synthesis [11, 12].

1.3.1 Ampicillin

Ampicillin is a broad-spectrum, semi-synthetic penicillin with bactericidal activity, which inhibits cell wall synthesis. The penicillins are a class of β-lactam antibiotics, which contains a four-membered heterocyclic β-lactam ring [13]. More than half of the antibiotics in use today are β-lactams. The compound 6-aminopenicillanic acid (6-APA)

makes up the basic structure for all penicillins and consists of a thiazolidine ring with a condensed β-lactam ring carrying varying sidechains in the C6-position. Semi-synthetic penicillins are made by chemical modifications of 6-APA. They are the most clinically effective penicillins, as they are active against both gram-negative and -positive bacteria. Ampicillin only differs from benzylpenicillin by the presence of an amino group (Figure 1.2) which enables the antibiotic to pass through the pores of the outer bacterial membrane [13].

The cell wall of both gram-negative and -positive bacteria are rich in peptidoglycans, which protects the bacteria against osmotic pressure and lysis. Transpeptidase enzymes perform extensive cross-linking of two glycan-linked peptide chains in peptidoglycans, thereby producing a mature, lattice-like layer of the cell wall in a process called transpeptidation. All β-lactam antibiotics inhibit the bacterial cell wall synthesis by interrupting this essential mechanism [14]. Ampicillins will bind to and inactivate these enzymes, thus called penicillin-binding proteins (PBP), while in the inner bacterial cell wall. This inhibition is irreversible, but cell wall synthesis still continues without cross-linking the peptidoglycan chains, which is essential for structural strength. Furthermore, the PBP-antibiotic complex stimulates release of autolysins which digests the remaining cell wall. This will eventually lead to cell lysis due to differences in osmotic pressure across the membrane.

β-lactams are highly selective and non-toxic to hosts, as the bacterial cell wall and its synthesis is unique. This is mostly because peptidoglycans are only found in bacterial cell walls. Ampicillin is stable against hydrolysis by a variety of beta-lactamases and is therefore used against a range of infections. Ampicillin can be combined with β-lactamase inhibitors to target resistant microorganisms [15].

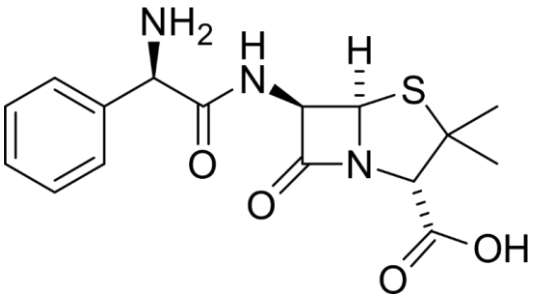


Figure 1.2: Chemical structure of ampicillin

1.3.2 Streptomycin

Streptomycin is an antibiotic that is produced naturally by the soil bacteria *Streptomyces griseus*, and is active against most gram-negative bacteria. STR was the first of the aminoglycoside antibiotics to be discovered (1944) [16], a class of drugs which targets the bacterial protein synthesis [17]. It is also the second most important antibiotic discovered after penicillin. Aminoglycosides consists of a carbohydrate structure with basic amine groups, which gives the molecules a positive charge at pH 7,4 (Figure 1.3). This in turn contributes to the antibiotics' absorption across the bacterial cell membranes in an energy-dependent reaction sequence. The drugs will accumulate to relatively high concentrations inside the bacterial cells, where they bind to ribosomes to inhibit protein synthesis [18].

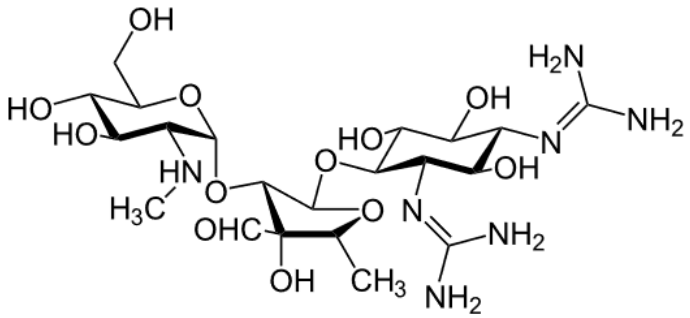


Figure 1.3: Chemical structure of streptomycin

The absorption process occurs via pores, which appear through rearrangements of components in the cell membranes. These conformational changes are caused by the drugs' amine groups reacting with negatively charged groups on lipopolysaccharides in the outer cell membrane, which displaces calcium and magnesium ions. Once inside the cells, STR bind specifically to the 30S subunits on the bacterial chromosomes. This will inhibit the organelles' movement along mRNA molecules, thus preventing translation of the mRNA triplet code. As a consequence, protein synthesis is halted, or it may be terminated completely, resulting in shortened proteins. This can further lead to a "feed-back" process where the cell permeability to the drug increases. Other aminoglycosides bind to the 50S subunit.

The bacterial 70S ribosomes are composed of a 30S subunit, which binds mRNA-molecules and initiates protein synthesis, and a 50S subunit which binds to tRNA, catalysing the elongation of the polypeptide chain. Most of the aminoglycosides are

specific to one phylogenetic type of ribosomes, and the selectivity of STR is caused by the structural difference of mammalian and bacterial ribosomes, which has a large effect on binding affinity [19]. The human 80S ribosomes are larger than their bacterial counterparts and consists of a 60S and a 40S subunit. STR can also inhibit the ribosomes of mitochondria and chloroplasts in *Eukarya*, as they have similar ribosomes to those in *Bacteria* (70S).

Less than 4 % of the antibiotics used today are aminoglycosides. They are used clinically against gram-negative *Bacteria*. Aminoglycosides which disrupts the same step in protein synthesis can still vary greatly in their mechanisms. Streptomycin was the first effective antibiotic used in tuberculosis treatment. It was later replaced by other drugs, because of serious side effects (neurotoxicity and nephrotoxicity). As bacterial resistance to the aminoglycosides develops quite quickly, they are primarily used as reserve antibiotics [20]. The prokaryotic ribosome consists of the 30S and 50S ribosomal subunits and is structurally different from the eukaryote ribosome [21]. Antimicrobials can bind to the small 16S rRNA of the 30S subunit of the bacterial ribosome, interfering with the binding of formyl-methionyl-tRNA to the 30S subunit leading to codon misreading and inhibition of protein synthesis and ultimately death of microbial cells.

1.3.3 Ciprofloxacin

Ciprofloxacin is a synthetic fluoroquinolone antibiotic, with a broad spectrum of antimicrobial activity. A bicyclic ring system consisting of a pyridone ring with a carboxylic acid makes up the basic structure for the quinolones and fluoroquinolones (Figure 1.4), which inhibit transcription and translation of bacterial DNA [22]. Fluoroquinolones inhibit the enzymes topoisomerase II (DNA gyrase) in gram-negative bacteria and topoisomerase IV in gram-positive bacteria. These two enzymes have the same function, except that DNA gyrase works in reverse. DNA gyrase inserts two negative supercoils into DNA at a time, by making double-stranded breaks. The double helix is then passed through the breaks, which are then resealed.

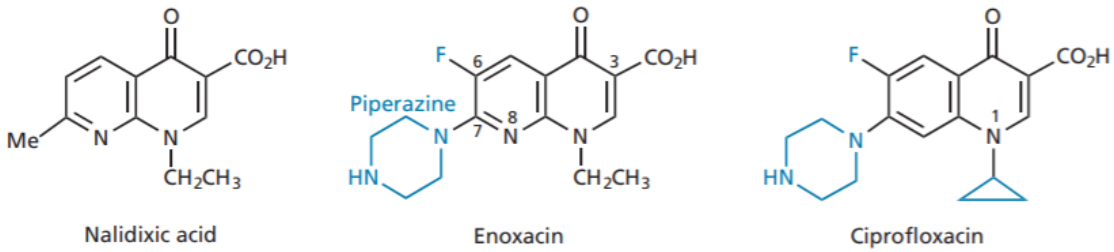


Figure 1.4: Chemical structure of Quinolones and fluoroquinolones

Quinolones blocks the final stage of the DNA gyrase-catalysed reaction by stabilizing the DNA-topoisomerase and creating a ternary complex. The binding seat for CIP only appears once the DNA-strands are cut, whereupon the drugs are bound in a stacking motive. This binding prevents the supercoiling of DNA which is required for its packaging in the bacterial cell. By preventing DNA synthesis, the drugs further inhibit cell division. A 1000-fold selectivity is observed for bacterial cells over human cells [23].

CIP is a second-generation derivative of nalidixic acid (the first of the quinolone antibiotics), which means that it differs from this agent by the addition of a fluorine-, piperazine- and a cyclopropyl substituent. These modifications, introduced with CIP, lead to the broad spectrum of these drugs, as well as improved uptake in host cells and a reduction of adverse effects [24].

The quinolones are especially effective against UTIs, as well as infections which are somewhat resistant against other agents. Ciprofloxacin, which is the most routinely used to treat UTIs, but has also been used against anthrax. It is somewhat more effective against gram-negative bacteria compared with the gram-positive. CIP is also more soluble than the first-generation quinolones, which means it can be used clinically in blood and tissues. Fluoroquinolones are also widely used for treatment and prevention of respiratory diseases in the beef and poultry industries. CIP specifically, has been widely used for more than 20 years, which may have contributed to the development of the resistance in poultry [20].

1.4 Antimicrobial Resistance

Antimicrobial resistance (AMR) is a natural phenomenon where a bacteria change their capacity to survive the action of antimicrobial drugs [20]. There are four main mechanisms through which bacteria become resistance to antibiotics (Figure 1.5):

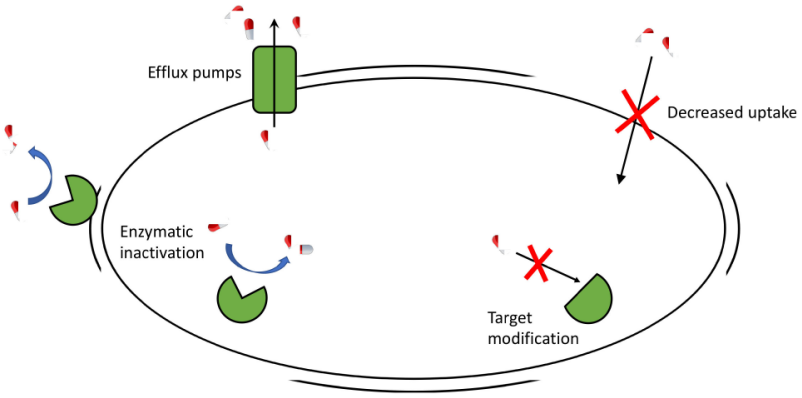


Figure 1.5: Illustration of the different mechanisms of antibiotic resistance.
Antibiotics as red and white pills, target proteins in green, illustration from [25]

- The bacteria may lack the membrane structure that the drug targets (prevents the binding of the antibiotic).
- Alter the antibiotic to inactive form (an existing bacterial enzyme is modified to interact with an antibiotic in order to make them inactive towards bacteria).
- Organism may modify target of the antibiotic – usually mutations
- Drug extrusion by efflux pumps (pumping out the antibiotics)

1.4.1 Resistance towards ampicillin

Different bacteria have varying susceptibility for the ampicillins. Some species/strains are vulnerable (streptococci), some are resistant (*Pseudomonas aeruginosa*) or resistance may develop in prolonged exposure to the antibiotic (*S. aureus)*. β-lactamases are the most effective defence against penicillins and are produced by some microorganisms. They are quite similar in structure to the transpeptidases, as they mutated from these enzymes. They function by hydrolysing the β-lactam ring, rendering the drug inactive [26], (Figure 1.6). Very effective reaction: 1000 molecules per second.

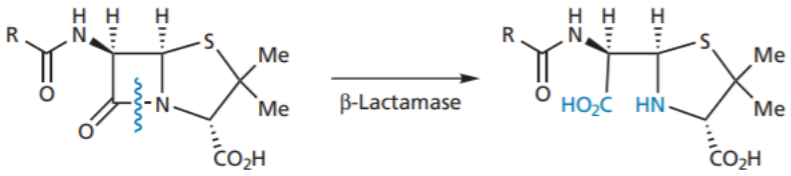


Figure 1.6: β-lactamase deactivation of penicillin

Most gram-negative, and many gram-positive bacteria produce different types of β-lactamases. Varying affinity towards different β-lactam-structures could contribute to varying degrees of resistance. Also, gram-negative cell wall – the outer layer is impenetrable to penicillins, which can contribute to resistance for some bacteria [27]. Low affinity of the transpeptidases to AMP is a phenomenon in for instance enterococci and pneumococci. Some gram-negative bacteria are also capable of conduction an efflux-process of the penicillins.

1.4.2 Resistance towards streptomycin

Streptomycin is an aminoglycoside, and the most common type of aminoglycoside resistance is enzymatic modification. Another mechanism of resistance towards streptomycin is that ribosomal protein S12 interacts with 16S rRNA, where streptomycin binds [28]. Data indicates that amino acid changes in S12 lead to an alteration or destabilization of this structure, and thereby affects the binding of streptomycin to the ribosome. Some of these mutations lead to streptomycin resistance. Streptomycin itself can also increase errors in protein synthesis.

1.4.3 Resistance towards ciprofloxacin

Resistance to fluoroquinolones are usually dependent on several factors and may occur due to several different mechanisms [29] [30, 31]. Some of the most common ways include gene mutations, alterations of the drug by modifying enzymes, an increase in the production of multidrug-resistance (MDR) efflux pumps or production of proteins that protects the antibiotic target. Mutations resulting in resistance are chromosomal and usually occurs in the genes coding for the main targets of the fluoroquinolones, that is DNA Gyrase and topoisomerase IV (*gyrA*, *gyrB*, *parC*, and *parE). Mutations occurs in a DNA-sequence called the* quinolone resistance-determining region (QRDR) of these genes which results in amino acid substitutions in the consequent protein product. This further affects the affinity of the drug toward the enzyme, which ultimately leads to resistance. CIP-resistance may also occur due to changes in the cell's permeability to the drug.

Gram-negative bacteria present an added hindrance to hydrophilic drugs like CIP, which has an intracellular target. A downregulation of porins in the outer membrane,

which allows CIP to pass, will increase the MIC of the drug. This type of mechanism is quite common in fluoroquinolone-resistant species.

1.5 Comparing Tolerance, Resistance and Persisters

Other mechanisms than resistance helps bacteria to survive during antibiotic exposure [6]. For instance, when nongrowing or slow-growing bacteria survives antibiotics treatment (when active growth is needed for killing), then they have developed tolerance [32]. A nongrowing subpopulation that survives the antibiotic treatment are called "persisters", this phenomenon often underlies treatment failure [33]. Tolerance and persistence may evolve rapidly under repeated exposure to an antibiotic. Figure 1.7 illustrates some mechanisms which may lead to tolerance, or to persister formation.

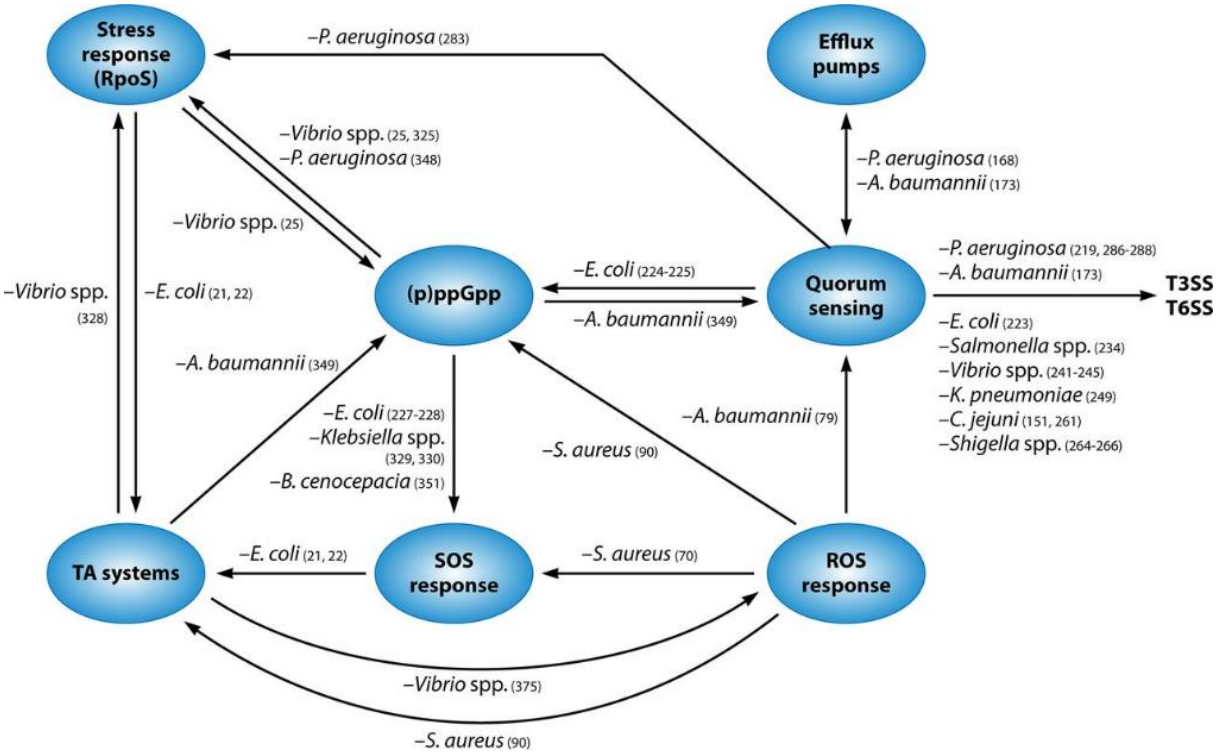


Figure 1.7: Links between different mechanisms of tolerance and persistence
(picture from[34]).

1.6 Mechanisms of antibiotic tolerance

Tolerance was defined by [35], as the ability of bacterial cells to survive a transient exposure to antibiotics that would otherwise be lethal. There exists a range of molecular

mechanisms in Bacteria which are involved in defence and survival and may lead to the development of tolerance or persister cells. All of these systems have been shown to be interrelated in various ways, and in some way may lead to different types of tolerance. For example, tolerance to β-lactams may occur when bacteria grow slowly [36]. Tolerance is used to generally describe the ability, whether inherited or not, of microorganisms to survive transient exposure to high concentrations of an antibiotic without a change in MIC, which is achieved by slowing down bacterial process [37]. However, tolerance is poorly characterized, owing to the lack of a similar quantitative method as MIC, which may lead to an error in classification of tolerant strains and resistant strain. Already in 1944, it was observed that bacteria could survive extensive use of antibiotics without developing resistance mutations [38]. Tolerance may be acquired through genetic modification or environmental conditions; poor growth conditions have been shown to increase tolerance to several classes of antibiotics. Importantly, a longer exposure to an antibiotic rather than a higher concentration is required to produce the same degree of killing. The two mechanisms related to tolerance which are most relevant to this thesis are the RpoS system and the general stress response, and the *relA* (p)ppGpp network.

1.7 Escherichia coli (*E. coli*)

E. coli are a diverse group of gram-negative, rod-shaped bacteria which are naturally found in the environment and in the intestines of all living animals (Figure 1.8). *E. coli* received their name from Dr. Theodor Escherich, who first discovered them in 1885 [39]. Most *E. coli*-strains are harmless, while some can be detrimental to human and animal health. Some examples of afflictions caused by *E. coli* are diarrhoea, urinary tract infections, respiratory illness and pneumonia. The bacteria are most commonly transmitted to humans through intake of contaminated raw or undercooked meat, raw vegetables or drinking water. In addition, people can be infected by direct contact with other persons and animals carrying pathogenic *E. coli*, or their faeces, as well as by bacteria from bathing water [40].

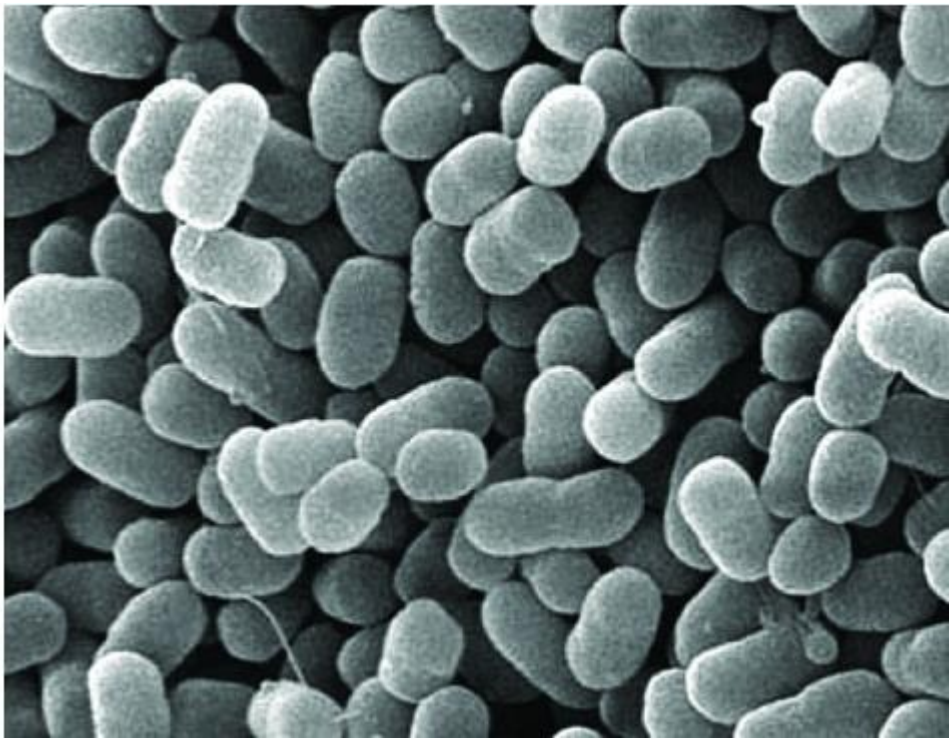


Figure 1.8: Image of rod-shaped *E. coli* bacteria , photo from Lumen microbiology [41]

According to a statistical analyses from the Norwegian Institute of Public Health surveillance report 2016-2019 [42] the occurrence of *E. coli* enteritis has increased dramatically in Norway since 2017 (Figure 1.9). However, it must be taken into consideration that the reporting system may have improved significantly during the recent years. However, the Norwegian survey is consistent with the trend observed for other countries. According to an article in the New York Times [43]; this is the case for the US, as official figures from Centre for Disease Control (CDC) [44] shows that the number of infections has risen by more than a third since 2013. Also, it was postulated that by 2020 two thirds of gram-negative bloodstream infections are caused by *E. coli*. In order to turn this negative trend, it is essential with research on both new types of antimicrobial reagents for treatment as well as the best use of already existing alternatives of antibiotics.

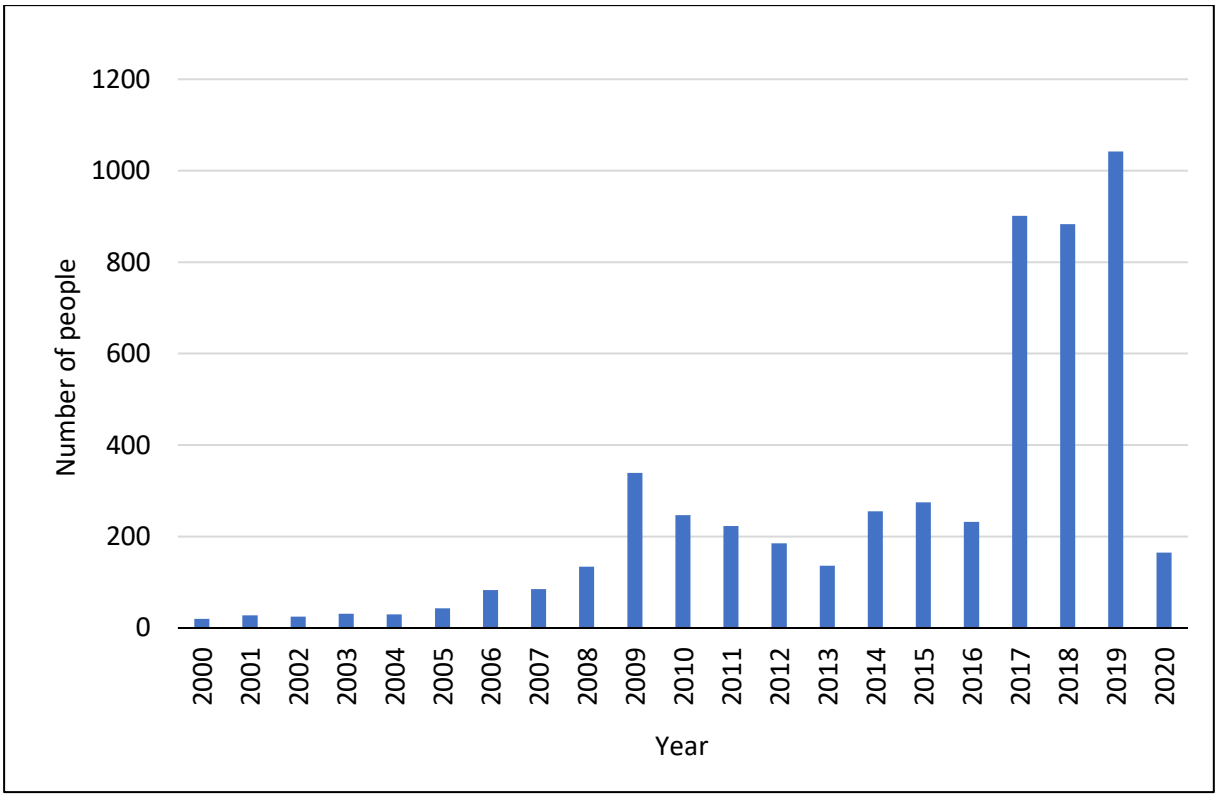


Figure 1.9: Reported occurrence of E. coli enteritis, Norway
Number of people reported infected with *E. coli* enteritis in Norway from year 2000 to March 2020 according to statistics from NIPH Surveillance, http://www.msis.no/[42]

1.8 *E. coli* strains for laboratory research

Most *E. coli* strains used for research purposes are descended from only two individual isolates, extracted from a patient in 1920. These are the B- and K12-strains, where the latter eventually led to the common laboratory strains, which is used in this master thesis [45]. Most of the commonly used bacteria also exist in different mutated forms, which has either occurred naturally due to evolution, or by directed genetic modifications (GMO). GMO mutants have knock-out and/or knock-in genes, or they are created by CRISPR/Cas-9 technology [46]. In this thesis two mutant strains of *E. coli* K12 MG1655, Δ*rpoS* and Δ*relA,* were used in addition to the wild type. The mutants had respectively the *rpoS* and the *relA* genes knocked out, both of which are important factors for the stress response in bacteria.

1.9 Stress response systems in *E. coli*, with focus on the function of the *rpoS* and *relA* genes

When bacterial populations are exposed to environmental stressors such as for instance extreme temperature, UV radiation, osmotic shock or nutrient deprivation [47, 48] the cells must be able to adapt their physiology in order to survive. In *E. coli*, one of the most important factors to initiate protection mechanisms is RpoS. The *rpoS* gene, which is highly conserved, encodes a sigma factor which is involved in the regulation of many stress response genes in *E. coli*. *rpoS* directs the transcription of as much as 10 % of the *E. coli* genome and thereby serves as the central regulator of the general protective response [49]. Studies have shown that unfavourable growth conditions triggers increased abundance of the RNA polymerase sigma factor RpoS [49]. It has also been shown that the RpoS regulon is required for nutrient scavenging, pH homeostasis and protection from oxidative stress [50]. It is important with knowledge on how *E. coli* cope with suboptimal conditions. RpoS and the genes it controls are conserved among many gram-negative bacteria, and studies of the role of RpoS in *E. coli* are likely to give valuable information regarding adaptive physiology of relevance also for other bacteria. Knock-out studies are highly valuable in this sense and will give valuable information of the function of Δ*rpoS* when compared to wild type *E. coli* with the intact RpoS sigma factor.

Several studies have suggested that bacterial cells sense the actions of antibiotics as a form of environmental stress and it has been suggested that RpoS responses may thus influence the development of antibiotic resistance in bacteria, and the resistance response in bacteria may vary for different types of antibiotics [51]. The study by Hirsch et al. [51] showed that osmotic shock increased antibiotic susceptibility in both the *ΔrpoS* and WT strains, regardless of RpoS dependency. Another more recent study did however show that loss of σs renders bacteria more susceptible to several stressors, including killing by gentamicin, consistent with the fact that knock down of the action of the RpoS protein (as well as the products of several other genes that it regulates) enhances the efficacy of antibiotics in combating bacterial growth [52]. Given the generality of the RpoS functionality, the effect is likely to be general for also other bacteria. Thus, measures to inhibit the activity of proteins like RpoS that controls the bacterial stress response, may represent a promising new tool that leads to improved treatment for bacterial infections with antibiotics, which is one of the topics of this thesis.

Furthermore, studies have shown that *relA* gene mutation in *E. coli* leads to changes in many key cellular processes, such as amino acid and nucleotide biosynthesis, lipid metabolism, transport, transcription and translation processes, as well as stress response systems [53]. The *relA* gene product can be divided into two domains, both functionally and physically. These are the N-terminal domain (NTD) which is responsible for (p)ppGpp synthesis, and the C-terminal domain (CTD) which is responsible for regulating r*elA* activity [54]. Bacteria adapt to a lack of nutrients and other environmental stresses by accumulation of guanosine-3′-diphosphate-5′-triphosphate (pppGpp) and guanosine-3′,5′-bisphosphate (ppGpp), collectively referred to as (p)ppGpp. These nucleotides are synthesized by enzymatic phosphorylation of GDP and GTP to ppGpp and pppGpp, respectively, using ATP as a phosphate donor [55].

1.9.1 The *rpoS* and *relA* responses depend on the growth phases of bacteria

It is shown that the response of a bacterial cells to external stimuli depends on which phase they are in, either exponential, stationary or long-term stationary phases [56]. Bacteria grown in closed cultures and during an experiment where no nutrients are added and no waste is removed, follow a reproducible growth pattern referred to as the growth curve (Figure 1.10). The number of cells defines the biomass density, often measured with OD_{600} and when biomass density (live cells) is plotted against time four distinct growth phases occur: lag phase, log phase, SP and decline phase (death). When the bacteria are inoculated in fresh medium, they do not immediately reproduce, and the bacterial biomass remains constant. This period, called the lag phase, is when the cells are metabolically active and increase only in cell size. The cells in lag phase synthesize enzymes and factors needed for cell division and population growth in the new environment. The population then enters the log phase where the cells grow in a logarithmic manner, and each cell generation occurs in the same time interval as the previous one. The log phase continues until nutrients are depleted or toxic products accumulate.

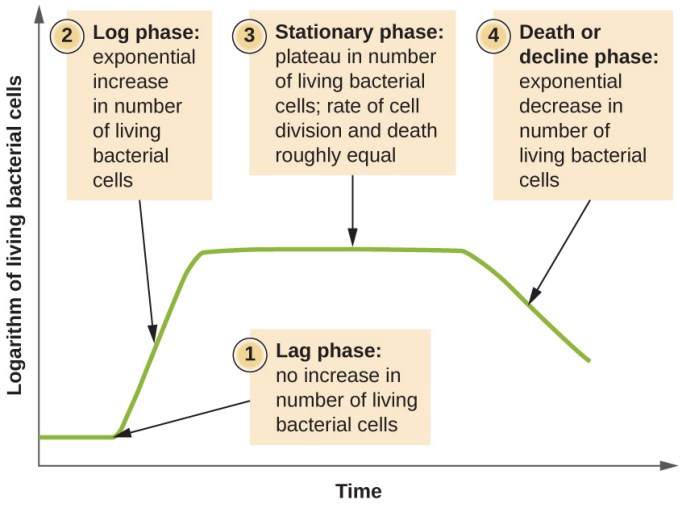


Figure 1.10: Illustration of the different growth phases of bacteria
(illustration from Lumen microbiology [41]

The transition from exponential phase to stationary phase (SP) is accompanied by morphological and physiological changes resulting in a nondividing, multiple-stress-resistant state (Figure 1.11). The progression of the exponential phase also depends on the nutrient composition of the growth media. In SP the cells become spherical and smaller with a highly cross-linked cell wall, and the membrane fluidity is reduced. The cells activate survival mechanisms by reprogramming gene expression patterns to adapt to potential stressors. This includes a dramatic increase of RpoS abundance [56]. The transcriptional regulation has been characterized of *rpoS* as cells enter SP. The mechanism involves Fis, a DNA-binding protein which acts as a transcription factor. Fis is itself growth-phase regulated in an inverse relationship to RpoS. The Fis protein is undetectable in SP but rapidly increases upon dilution into fresh medium. A strong Fis binding site near the major *rpoS* promoter (PrpoS) is required for this regulation. Fis likely binds to this site specifically during exponential growth, resulting in repression of *rpoS* transcription. As cells enter SP, Fis disappears, and *rpoS* transcription increases. As a consequence, the cells that enter SP, change from prioritising growth towards prioritising synthesis of amino acids in order to promote survival until nutrient conditions improve.

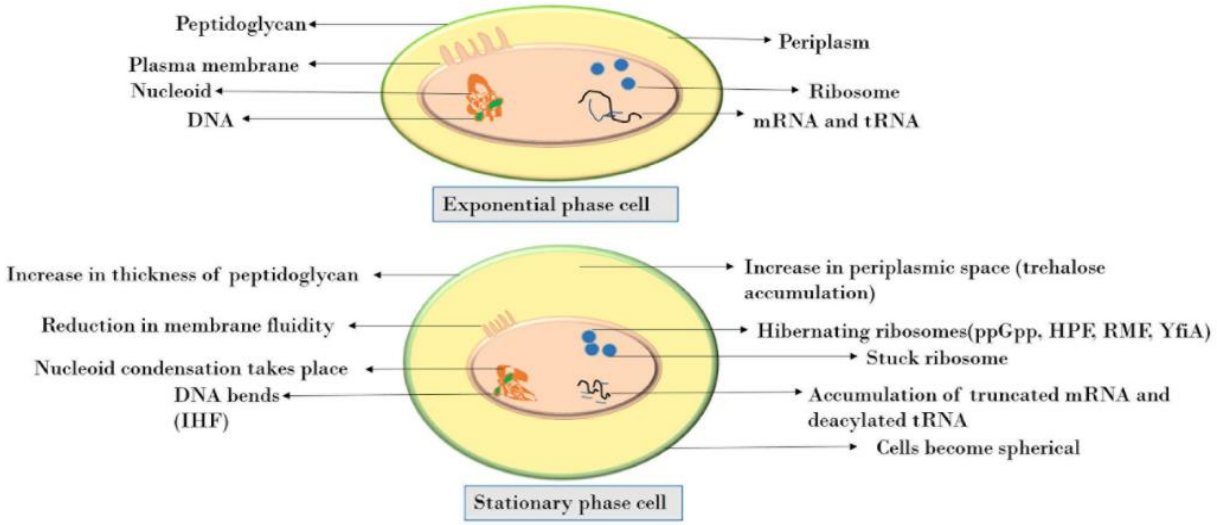


Figure 1.11: Exponential vs stationary phase
Comparison of molecular and cellular changes in exponential vs stationary phase (figure from [56], including increased thickness of the peptidoglycan layer, nucleoid condensation for DNA protection, condensation of the cytoplasm and decreased protein synthesis. The 70S ribosomes are converted into inactive 100S ribosome dimers (translational level, a process termed ribosome hibernation which is thought to be a mechanism to fine-tune the translation process according to environmental conditions). 16S rRNA fragmentation attenuate the activity of 30S ribosomal subunit and thereby protein synthesis. With limited nutrient availability, accumulation of truncated mRNA and deacylated tRNA occurs. As a result of the morphological, metabolic, transcriptional, or translational alterations, the SP cells become resistant to several external stressors.

Many bacteria (*Clostridia, Bacillus)* has the ability to form resistant spores as a consequence of starvation in SP. Non-optimal growth conditions may also lead to the formation of biofilms in many bacterial species including in some *E. coli* strains. Biofilm bacteria are physiologically similar to stationary phase bacteria, however persisters can be induced during SP in biofilms and as a consequence of stress. It is also shown that these persistent cells could go into exponential growth phase by the activation of ppGpp due to the stress of sub-lethal antibiotic concentrations and thereby be a reason behind relapsing infections and is a major cause of drug resistance [57].

1.9.2 Oxidative tolerance – the reactive oxygen species (ROS) response

It has been hypothesised that antibiotics may generate lethal ROS stress in *Bacteria*. However, all microbials have defensive mechanisms to avoid poisoning by endogenous levels of ROS, since increased intracellular levels of ROS may damage enzyme function, DNA and growth [58]. ROS have important functions as signal molecules (superoxide radicals (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radicals (HO^-)) and in regulation of homeostasis and are produced by the cells in oxygen metabolism reactions. However, they are also capable of unrestricted oxidation of cellular components, which

may lead to oxidative stress if not balanced by the action of antioxidant enzymes. Two examples are the superoxide dismutase (SOD) and catalases, which have been shown to act as protective enzymes in drug-tolerant mutants of *E. coli*. The ROS response is induced by several different types of signallers in different *Bacteria*, like (p)ppGpp, and the sigma factor. *RpoS* is also involved in regulating oxidative stress mechanisms in some species, the KatG enzyme is indirectly controlled by RpoS through its regulation of OxyR, a transcriptional regulator with a key role in the response to oxidative stress in bacteria and *RpoS* also regulates other proteins involved in the response to oxidative stress, even though they have not been fully characterized.

Some studies have suggested that salicylate induce ROS production in *E. coli,* and this again have a protective effect against lethal doses of antibiotics by inducing bacterial tolerance (persistence). The mechanisms behind this are not fully understood [59]. In addition, when hydrogen peroxide was given directly as a source of ROS to *Bacteria* it protected against a lethal dose of ofloxacin [60].

1.9.3 Energy metabolism and efflux pumps

Various metabolic pathways in *Bacteria* may also have an effect on tolerance mechanisms. However, the efflux pump systems may be the most important to mention. These are protein complexes through which the bacteria pump substances out of the cells. They may either be used to eliminate toxic species from the bacteria, or they contribute to upholding the balance of essential compounds [34].

Efflux systems are shown to have an important function in the development of persister cells in *E. coli* strains. When exposed to an antibiotic, the cell will be able to pump the drugs out of the cells to keep the drug levels low inside the cells, which is essential for survival. The same systems are found to contribute to tolerance and/or resistance, as the resulting low intracellular drug concentrations may cause an increase in the corresponding MIC values. The efflux pumps of the AcrAB system are shown to be especially important in "combination" with creating *E. coli* mutants with multiple-antibiotic-resistance (Mar). Regulation of efflux pump systems are affected by several different signalling mechanisms, for example related to the ROS response or quorum sensing.

1.9.4 The SOS response

The SOS response system is responsible for repairing damage done to the genetic material of bacteria. It is important for survival during stress and is thus related to other stress defence systems. As well as consisting of genes related to DNA repair, the SOS system also involves genes which control other cellular responses, pathogenesis, antibiotic resistance and biofilm formation [34]. The constituent proteins may vary between bacteria, but the activator RecA and the repressor LexA are important in all SOS systems. During the SOS process in *E. coli*, the production of the TisB toxin (TA module, type I) is induced. An upregulation of this substance has been shown to promote persister formation when the bacteria is exposed to for example ciprofloxacin.

1.9.5 Quorum sensing systems related to persistence

Quorum sensing (QS) determines the collective expression of several genes for a population. This includes genes that regulate phenotypes relating to virulence, production of toxins, different types of motility, biofilm formation as well as "competitive ability against competitors". QS will thus further affect a bacterial population's ability to adapt to its environment. *E. coli* populations are able to produce biofilms inside epithelial cells in the bladder, and this is the main culprit behind UTIs. As the sensitivity towards certain antibiotics is determined by the bacterial growth rate, biofilm formation is an excellent way of avoiding the effects of the drugs. This is for example shown to be the case for ciprofloxacin. Furthermore, in a biofilm state, limited nutrients will be available to the populations. This has in turn been shown to lead to an increase in the production of (p)ppGpp, which may affect the bacteria's tolerance levels toward multiple drugs [34].

1.9.6 Toxin-antitoxin (TA) modules

TAs are small genetic systems which code for a toxin and a corresponding antitoxin. The antitoxin is an unstable molecule which is able to inhibit its toxin. When triggered, for example by the SOS system or by the (p)ppGpp network, TA-modules will drive bacterial populations into persister formation.

1.10 Impact of thesis work

Antibiotics play essential roles in treating bacterial infections. Failure of antibiotic treatment may cause detrimental consequences for the hosts that is infected, irrespectively if it is a human, an animal or a plant. Bacteria that are genetically resistant to antibiotics or are have increased resistance of other reasons (as for instance specific growth conditions or interactions with other bacterial strains), can render bacterial cells insensitive to the effects of antibiotics and thereby lead to failure of treatment. Understanding how bacteria can evade antibiotics, and how antibiotics can be used in a more effective manner is important both from a scientific as well as from an applied perspective.

2 Aims

The main aim of this project was to examine tolerance mechanisms in response to three different classes of antibiotics, as well as the role of stress systems in *E. coli*, specifically the *rpoS* system and *relA* network.

A first goal was therefore to compare growth rates and growth patterns, as well as minimal inhibitory values (MIC) for the WT *E. coli* K12 MG1655 and the two knockout strains Δ*rpoS* and Δ*relA*.

A further aim was to compare tolerance mechanisms for these bacterial strains and antibiotics in a well-plate format and in a high-throughput microbioreactor system.

3 Materials and Methods

3.1 Bacterial Strains

The bacterial strains used in this thesis was the wild type (WT) *E. coli* K12 MG1655, as well as two knock-out strains: Δ*rpoS* and Δ*relA*. The knock-out strains were prepared by Dr. James Booth, Oslo University Hospital, University of Oslo. Freeze stocks of bacteria in glycerol were stored at -40 °C. The fresh ON-cultures were made from a new vial for each experiment.

3.2 Chemicals and Media

3.2.1 Media

M9

In this thesis, all experiments were performed with one of three versions of the 1X M9-media, which is a defined mineral media with glucose as the carbon source. The 1X M9 was made by mixing five different stock solutions into sterile MQ-H_2O according to table 3.1. All the stock solutions were autoclaved and stored in room temperature, until mixing in the 1X M9, except for the trace element solution which was premade. The calcium chloride was added first, to prevent precipitation.

Table 3.1: Composition of 1X M9-media
This recipe was adjusted from a protocol by Helmholz Center Munich.

Stock solution	Components	Concentration	Supplier	Product number
M9 salt solution (10X)	Na_2HPO_4	33.7 mM	Sigma-Aldrich	S9390-500G
	KH_2PO_4	22.0 mM	Sigma-Aldrich	P5655-500G
	NaCl	8.55 mM	VWR Chemicals	27810.295
	NH_4Cl	9.35 mM	Sigma-Aldrich	A9434-500G, Lot #BCBT8951
20 % glucose	Glucose	0.4 %	VWR Chemicals	101176K
1 M $MgSO_4$	$MgSO_4$-$7H_2O$	1 mM	Sigma-Aldrich	M5921
1 M $CaCl_2$	$CaCl_2$-$2H_2O$	0.3 mM	Sigma-Aldrich	223506-500G
Trace element solution	*See* Table 3.2	1X		

The 10X M9 was made by dissolving salts (Table 3.1) in MQ-H_2O. The pH was adjusted to 7,2 with a NaOH-solution (1M), which was made by dissolving NaOH-salts (Sigma-Aldrich, 71687-500G) in to MQ-H_2O on ice.

The trace element solution was made by my supervisor, Lilja B. Thorfinnsdottir, and contained the following components:

Table 3.2: Composition of trace element solution

Compound	Concentration (g/L)	Supplier	Product Number
$Fe(SO_4)$-$(H_2O)_7$	10	Sigma-Aldrich	F8633
$Zn(SO_4)(H_2O)_7$	2,25	Sigma-Aldrich	Z0251
$CaCl_2(H_2O)_2$	2	Sigma-Aldrich	223506
$Cu(SO_4)(H_2O)_5$	1	Sigma-Aldrich	C8027
$Mn(Cl2)(H_2O)4$	0,38	Sigma-Aldrich	M5005
H_3BO_3	0,14	Sigma-Aldrich	B6768
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	0,1	Merck	1011820250

Altered 1X M9 media

Two alternative versions of the 1X M9 media, which was formulated by Lilja, were used in this thesis. The M9 with high biomass capacity (M9-hbc) was created in order to give higher biomass in the BioLector. The media consisted of a new trace element solution and 1,0 % glucose in the working 1X M9-hbc solution, instead of 0,4 %.

The second altered version of the 1X M9 media was mainly used by other members in the lab, but also for the media testing experiment of this thesis, which is described in chapter 4.2.2. This modified version of the 1X M9-media was added a co-solution to a concentration of 0,1 mg/ml in the working solution, consisting of $CoCl_2$-H_2O (50 mg/ml stock solution Sigma-Aldrich, C8661).

A version of the regular 1X M9 and the modified version with the amino acids isoleucine and valine added, was also prepared. Stock solutions (2 mg/ml) of the two amino acids were made by dissolution in sterile MQ-H_2O, followed by sterile filtration. The stocks were added to the working solutions in a final concentration of 0,04 mg/ml.

LB-medium

Lysogeny broth (LB) medium was used for making agar plates, as well as in the media testing experiment described in chapter 4.2.2. This is a nutritionally rich media and the most commonly used for cultivating bacteria. The LB-media was prepared by mixing the components of table 3.3 in distilled water. It was autoclaved and stored at room temperature before usage.

Table 3.3: Composition of LB-media

Components	Concentration in working solution (g/L)	Supplier	Product number
Peptone from meat	10	Sigma-Aldrich	Enzymatic digest 70175-500G
Yeast extract	5	Sigma-Aldrich	92144-500G-F
NaCl	5	VWR Chemicals	27810.295
Oxoid™ Agar bacteriological	15	Thermo Fisher Scientific	LP0011

3.2.2 Antibiotics

Three types of antibiotics were used in this thesis, all of which are bactericidal.

Table 3.4: Antibiotics used in this thesis

Antibiotics	Mode of Action	Type of ab	Supplier	Product Number
Ampicillin	Inhibition of cell wall synthesis	β-lactam	Sigma-Aldrich	A9393
Streptomycin	Protein synthesis inhibitor	Aminoglycoside	Sigma-Aldrich	S6501
Ciprofloxacin	Inhibition of DNA gyrase	Fluoroquinolone	Sigma-Aldrich	17850

Ampicillin

A stock solution of 5 mg/mL ampicillin was made by dissolving powdered antibiotics in sterile MQ-H_2O and vortexed to completely dissolved. The solution was aliquoted into sterile Eppendorf tubes and stored at -20 °C.

Ciprofloxacin

A stock solution of 5 mg/mL ciprofloxacin was made by dissolving powdered antibiotics in a 0,1 M HCl-solution. The HCl-solution was made by adding 37 % HCl (missing product number) to sterile MQ-H_2O. When the antibiotic was dissolved completely, the solution was sterile filtrated, before aliquoting into Eppendorf tubes (100 µL). The stock solutions were stored at -20 °C.

Streptomycin

A stock solution of 25 mg/mL was made by dissolving powdered antibiotics in sterile MQ-H_2O and vortexed to completely dissolved. The solution was then sterile filtrated, before being aliquoted into Eppendorf tubes (100 µL), stored at -20 °C.

3.3 Cultivation

The bacteria to be used in each experiment was cultivated overnight in 1X M9-solution. The over-night cultures were made by mixing 100 µL bacteria freeze stock into 100 mL of media. The cultures were then incubated at 37 °C and shaking (200 rpm) for approximately 16 hours.

3.4 Experiments

3.4.1 MIC

Principle

Minimum inhibitory concentrations (MIC) are considered the "gold standard" for determining the susceptibility of organisms to antimicrobials [61]. It is defined as the minimum concentration of an antibiotic that is required to prevent net growth of the culture. The antibiotic concentrations used for determining MIC is accepted to be in doubling dilution steps down from 1 mg/L.

This parameter is determined experimentally by exposing bacterial populations to increasing concentrations of an antibiotic in a standardized growth medium. The MIC value of that specific substance will be the resulting minimal concentration where visible growth is not detected, usually after 16-20 hours of incubation.

Resistance is used to describe the inherited ability of microorganisms to grow at high concentrations of an antibiotic, and is quantified by MIC. A higher MIC-value indicates a higher level of resistance to the particular substance. By performing a series of MIC-experiments, it is possible to compare level of resistance between strains or between different antibiotics for one strain. MIC has two major limitations; it is not informative for bacterial populations that are tolerant and not resistant. Furthermore, MIC can vary due to differences in experimental conditions.

Procedure

MIC was determined for the three *E. coli* K12 MG1655 strains in 1X M9-medium in 96-well plates using a Tecan Plate Reader. The antibiotic solutions were prepared in round-bottom 96-well plates, by dilution in MQ-H_2O. The inoculums were prepared from ON-cultures (~16 h), by dilution in 1X M9 to OD_{600}=0,1.

The components were added to flat-bottom 96-well plates, with the inoculum concentration at 50 %. The type and concentrations of antibiotics were adjusted for each individual experiment, and are given in the corresponding figures in chapter 4.1. The plate was incubated in the plate reader with temperature control (37 °C) and shaking (510 rpm). Absorbance was read at 600 nm every 60 minutes for ~24 h.

3.4.2 Plate Counts

The concentration of colony forming units (CFU) was calculated for the WT in the two initial experiments. An ON culture was diluted to OD_{600}=0,01, which was further used to make a dilution series with NaOH (1M) (10^{-1} – 10^{-6}). The four highest concentrations were plated out on agar plates in triplets, which were incubated at 37 °C for approximately 24 hours. The resulting colonies were counted for each plate and the CFU/mL was calculated for the triplets. The NaOH-solution was made by dissolving NaOH-salts in MQ-H_2O on ice.

3.4.3 Testing of dilutions of the *E. coli* ON-culture for growth in the well plate format

The growth patterns for a series of six different dilutions of *E. coli* ON-cultures were determined by use of the Tecan plate reader and a flat-bottomed 96-well plate. The aim of this procedure was to determine the optimal dilution for use in later experiments, described in chapter 3.4.6, in order to achieve appropriate timing for antibiotic addition.

The dilution series (1:10 000, 1:5 000, 1:1 000, 1:500, 1:200, 1:100) was made by inoculating volumes of an ON-culture in prewarmed 1X M9-media and added to the experimental plate in triplicates. The plate was then incubated in the Tecan plate reader with temperature control (37 °) and shaking (510 rpm). The absorbance was read at 600 nm every 30 minutes for approximately 24 hours. The OD_{600} was also measured for the excess samples using a spectrophotometer.

3.4.4 Comparison of Growth Patterns for three *E. coli* strains

As the knock-out strains were new to the lab, we wished to perform experiments to determine their growth pattern, in order to decide if they were comparable to the WT.

E. coli survival mechanisms during stress has been shown to be dependent on many factors, including growth rate. It has for instance been shown to decrease during the transition from exponential phase to SP. Furthermore, the growth rate is an important aspect in the control of the *rpoS*-gene as well as other stress responses in *E. coli*, and thus inpacts important aspects of *E. coli* physiology [62].

The results were used to create growth curves for each of the three strains and determine their generation time. Each strain was inoculated into 1X M9-medium in shake flasks. Measurements of the three cultures were done immediately, and then every hour for six hours. The WT-strain and Δ*rpoS* showed very similar growth rates, while Δ*relA* had OD-values that was far lower at the same timepoints. The experiment was therefore repeated for this strain, where measurements was done every hour for nine hours.

3.4.5 Testing of Growth Media optimal for *E. coli* K12 MG1655 Δ*relA* cultivation

In order to determine the best growth medium for the knock-out strain Δ*relA*, an experiment was performed where ON-cultures with inoculum from freeze stock or lag phase ON-cultures were tested in 7 different media compositions. The types of media being tested were 1X M9, 1X M9-hbc, the altered 1X M9 and LB-medium. In addition, a version of each of the M9 media with added amino acids (isoleucine, valine) were tested. OD_{600}-measurements were recorded for each of the ON-cultures at the beginning of the experiment as well as after approximately 16 hours. The experiment did not give satisfying results and will have to be repeated.

3.4.6 Testing of Growth Rate of *E. coli* with low doses of antibiotics in the well plate format

Principle

The aim of this experiment series was to determine how low doses of antibiotics influence the growth rate in the three *E. coli* strains in M9 medium, using the Tecan plate reader.

Procedure

ON *E. coli* cultures were diluted (1:200) with prewarmed 1X M9-solution, before being added to a 96-well flat-bottom plate. The plate was then incubated in the Tecan

plate reader with temperature control (37 °C) and shaking (510 rpm), with OD-measurements (600 nm) every 30 minutes. This continued until the absorbance value reached approximately 0,25 in the plate reader, after 5-6 hours. This is equivalent to a value of 0,35 in the spectrophotometer in a 1 ml cuvette. At this point, prepared prewarmed antibiotics (25 µL) was added. The concentrations used in each experiment are indicated in the figure legends. The plate was then returned to the plate reader, with the same conditions. The experiments were terminated after approximately 24 hours of measurements, including the time before addition of antibiotics. Similar experiments were performed for both the knock-out strains Δ*rpoS* and Δ*relA*.

3.4.7 Studies of Antibiotic Tolerance in Microbioreactor Cultivations

Principle

Similar experiments to those described in chapter 3.4.6 were performed using the BioLector Pro, which is a microbioreactor system. In this thesis, only the 48-well Flowerplates MTP-48-BOH2 (m2p labs) were used. The BioLector is used to perform high-throughput fermentations together with online monitoring of bacterial biomass, pH, and DO (oxygen saturation), in order to gain more information about the cell metabolism and growth patterns. The aim of this experiment series was to gain a better understanding of the growth pattern and fermentation parameters of the three *E. coli* strains when exposed to low doses of antibiotics.

The microtiter plates consist of flower shaped wells that acts similarly as baffles in shake flasks, so as to increase mixing and gas/liquid mass transfer. The plates also contain non-invasive optical sensors which gives online monitoring of biomass, pH, DO and fluorescence. There is also the option of controlled pH and feeding rates. This was not used in this thesis but may however be used for future experiments.

Biomass is measured by a light scattering at 620 nm with detection of back scatter from bacterial cells in the wells. The intensity of collected scattered light gives a signal that is correlated to the biomass concentration. A higher value signal corresponds to a larger concentration. The system is dependent on a number of factors: frequency, filling volume in the wells, type of plate, shape and size of the microorganisms as well as media composition. Biomass values are given in NTUs (nephelometric turbidity units) where 200 NTU (which is the minimal detection value in the BioLector Pro) corresponds to an OD_{600} of 0,11 with the AMCO CLEAR® TURBIDITY STANDARD (25 °C, 100 µL, 800 rpm).

When using the Flowerplate MTP-48-BOH2, the DO-optode will emit a fluorescent signal at 775 nm when excited by light at 625 nm. The signal is quenched by oxygen in the solution, and the degree of quenching is correlated to the concentration of dissolved oxygen. The resulting values are given as percentages, with an accuracy of ± 5 %.

The pH optode contains two fluorescent dyes, one which is pH-dependent and the other is a reference. A low pH (lower concentration of hydronium ions) will result in a lower fluorescent signal from the pH-dye, which when compared to the reference gives a measurable phase shift.

Procedure

In this experiment series, we were interested in measurements of biomass, DO and pH (Filters: pH(LG1)(221), DO(RF)(228), Biomass(201)), with initial analyses being performed with the WT-strain (*E. coli* K12 MG1655) in M9-medium. The initial experiments were done with a total volume of 810 µl (~2 % inoculum), where the plates were prepared by adding medium and bacteria to the test wells, before covering it with a gas permeable sealing foil with an evaporation reducing layer. The programme used consisted of temperature (37 °C) and humidity control (85 %), and continuous shaking at 1400 rpm. The later experiments in this series were done with 1X M9-hbc as the incubation media in the BioLector, as well as a larger total volume (1000 µl), so shaking had to be reduced to 1300 rpm. Gain 5 was used for all experiments, and the cycle time was 5 minutes.

Similar experiments were later performed for the knock-out strain Δ*rpoS*. Future experiments will have to be done for the knock-out strain Δ*relA*, after repeating the process described in chapter 3.4.6.

4 Results

4.1 MIC

Several experiments were performed to determine the minimal inhibitory concentrations (MICs) of three *E. coli* K12 MG1655 strains (WT, Δ*rpoS*, Δ*relA*), with three types of antibiotics (AMP, STR, CIP). Prior to the MIC analysis, the WT was grown in an ON-culture with M9-medium until they reached SP. The bacteria were then incubated in fresh medium, with antibiotics, in the Tecan Plate Reader for approximately 20 hours. The OD_{600} was automatically read every hour.

4.1.1 Wild Type

The concentrations of antibiotics used for the initial MIC-determination for the WT *E. coli* in this thesis were based on corresponding literature values.

Ampicillin

Three parallel experiments were performed, with concentrations of ampicillin (AMP) increasing from 0,39 µg/ml to 100 µg/m. In each of the analysis the lag phase lasted for approximately 10-13 hours in the controls, which were not added any antibiotics. Two representative figures with the four lowest doses used are shown below (Figure 4.1).

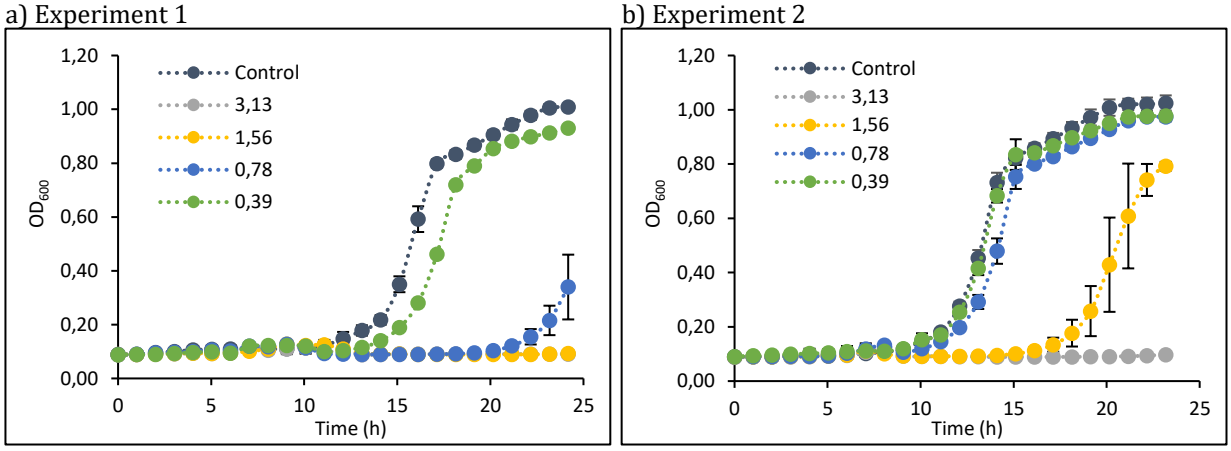


Figure 4.1: MIC for the WT treated with ampicillin
Measured absorptions by a Tecan plate reader, in samples of *E. coli* K12 MG1655 in M9 media with added ampicillin (0,39–100 µg/ml) for two representative experiments. All the experiments consisted of nine concentrations in addition to the controls. However, for simplicity only the four lowest doses are shown here. The controls were not added ampicillin. The absorbance was measured during a period of 25 hours and each datapoint represents the average of three replicates ±standard deviation. The figure with experiment 3 is shown in the appendix (Figure A.1).

Similar growth patterns between the three experiments were observed for most of the concentrations. However, the samples added 0,78 µg/ml AMP showed significant variation between replicates. This is because the dose is around the threshold where a large effect of the treatment is observed. The two second lowest concentrations used (0,39 µg/mL and 0,78 µg/mL) resulted in delayed growth with lag phases lasting for 16-22 hours. No visible growth was observed for concentrations above 0,78 µg/ml in the first and the third experiment (Figure 4.1a, A.1) and above 1,56 µg/ml in the second (Figure 4.1.b). Consequently, the MIC was determined to be in the range of 1,56 < MIC < 3,13 µg/ml ampicillin for the *E. coli* WT, as this interval was shown to contain the lowest concentration where no visible growth was observed.

Streptomycin

Three parallel experiments were performed, with concentrations of streptomycin (STR) increasing from 0,02 µg/ml to 5µg/ml. Overall, the lag phase was observed to last approximately 10-13 hours in samples with an uninhibited growth pattern. This is the case for the controls as well as samples with STR-concentrations below 0,313 µg/ml in all three experiments (Figures 4.2, A.2).

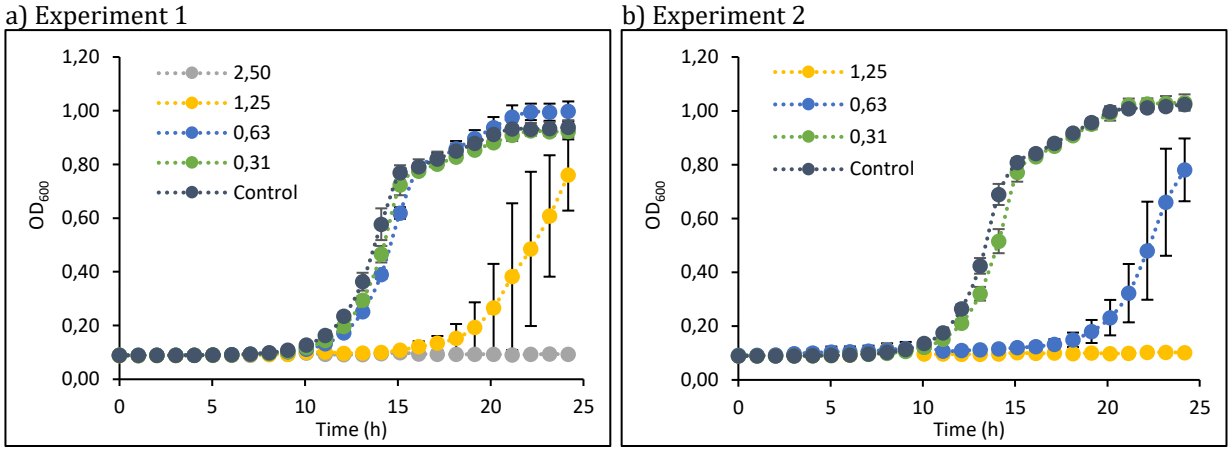


Figure 4.2: MIC for the WT treated with streptomycin
Measured absorptions in a Tecan plate reader, in samples of *E. coli* K12 MG1655 in M9 media with added streptomycin (0,0195–5 µg/ml) for two representative experiments. All the experiments consisted of nine concentrations in addition to the controls. However, for simplicity only the relevant doses are shown here. The controls were not added antibiotics. Each datapoint represents the average of three replicates ±standard deviation. The figure with experiment 3 is shown in the appendix (Figure A.2).

Wells with concentrations of 0,625 µg/ml resulted in growth after a long lag phase (~18-22 hours) in the two final experiments (Figure 4.2b, A2), and those of 1,25 µg/ml in the first (Figure 4.2.a). The great variation between experiments for these samples is seen

because the concentrations 0,625–1,25 µg/ml are in the range of the threshold for STR-dose which result in a large inhibitory effect on the bacteria. No visible growth was observed for doses above these values. Thus, the MIC for the WT treated with STR was determined to be 1,25 µg/ml < MIC < 2,50 µg/ml. There were relatively large standard deviations between parallels in the samples with delayed growth, which indicates some variability between these samples.

Ciprofloxacin

Three parallel experiments were performed, with ciprofloxacin-concentrations (CIP) increasing from 0,001 µg/ml to 2,5 µg/ml. The results show that the lag phase lasted for approximately 10 hours in the controls, as well as samples with similar growth patterns (Figure 4.3, A.3).

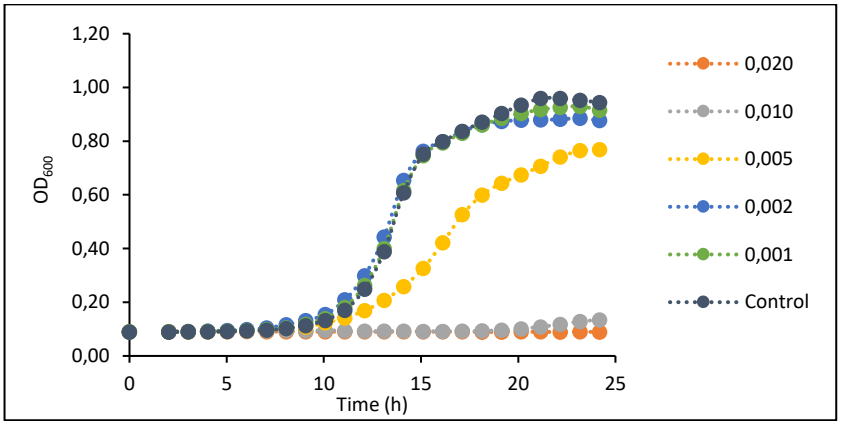


Figure 4.3: MIC for the WT treated with CIP – Experiment #3
MIC determination by measured absorptions in a Tecan plate reader, with samples of *E. coli* K12 MG1655 in M9 media with added ciprofloxacin (0,001–2,5 µg/ml) for one representative experiment. All the experiments consisted of nine concentrations in addition to the controls. However, for simplicity only the relevant doses are shown here. The controls were not added CIP. Each datapoint represents the average of three replicates ±standard deviation. Figures with experiment 1-2 are shown in the appendix (Figure A.3).

In experiment 2 (Figure A.3.b) it was further observed that the samples added the lowest dose of CIP (0,01 µg/ml) gave a delayed lag phase with some bacterial growth appearing after approximately 20 hours. However, all concentrations above 0,01 µg/ml resulted in cell death. Milder CIP-doses were therefore used in the third experiment (Figure 4.3), with results showing growth patterns like those of the controls, for samples with 0,002 µg/ml and less. A slight inhibition, although with equal lag phases to the controls, was observed for the samples added 0,005 µg/ml CIP. This may indicate that MIC for the WT treated with CIP is near 0,01 µg/ml, while the threshold for what constitutes a

large inhibitory effect lies in the range of 0,01–0,05 µg/ml. However, the analysis should be repeated with more doses (0,001–0,010 µg/ml) for a more accurate determination.

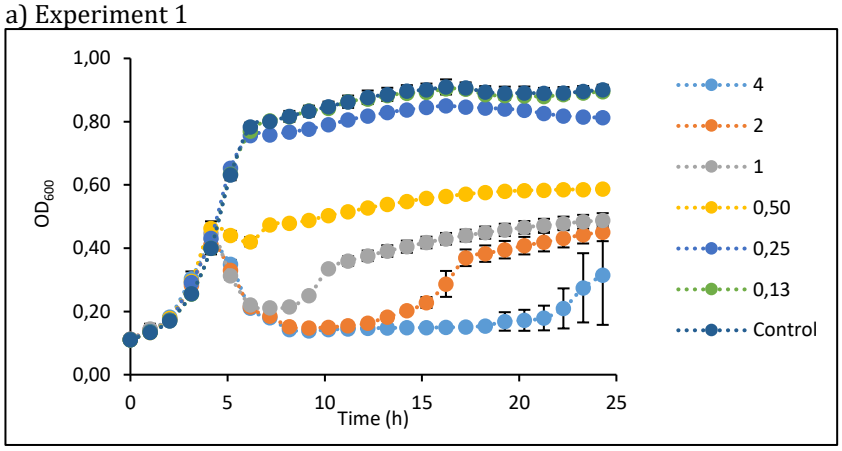
4.1.2 ΔrpoS

Two-three experiments were set up to determine the MIC for the *E. coli* knockout strain Δ*rpoS* with three antibiotics (AMP, STR, CIP).

Ampicillin

The AMP-concentrations used (0,125–8 µg/ml) in this analysis series were based on the results obtained for the WT, where MIC was found to be 1,56 < MIC < 3,13 µg/ml. No clear lag phase was observed in the experiments, and the same 5-hour initial rapid growth occurred for all samples with AMP-doses ≤ 4µg/ml (Figure 4.4).

a) Experiment 1



b) Experiment 2

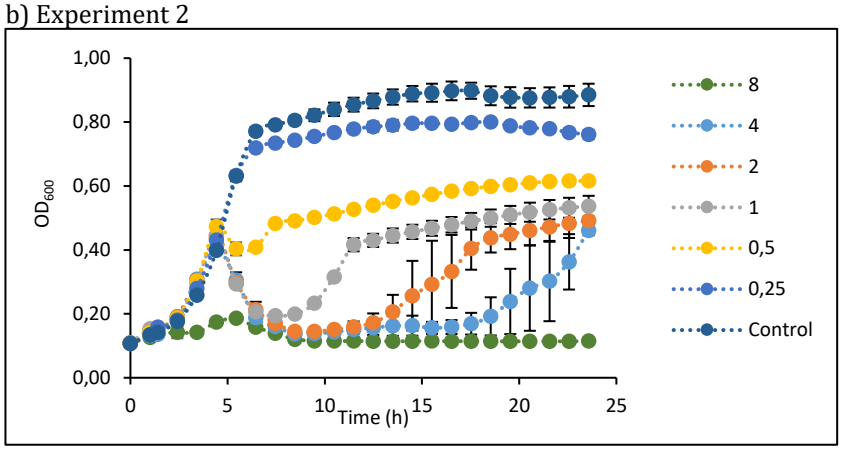


Figure 4.4: MIC for Δ*rpoS* treated with AMP
MIC determination by measured absorptions in a Tecan plate reader, with samples of the *E. coli* K12 Δ*rpoS* knockout strain in M9 media with added AMP (a: 0,13–4 µg/ml; b: 0,25–8 µg/ml). The controls have not been added antibiotics. Each datapoint represents the average of three replicates ±standard deviation.

Samples with AMP-doses ≤0,25 µg/ml showed a very similar growth pattern to the controls, which reached SP after approximately six hours. At four hours, samples with

higher AMP-concentrations (≥1 µg/ml) showed a pronounced drop in absorbance indicating cell death by lysis. Higher concentrations resulted in a longer recovery time for the bacteria, as visualized by the gradual increase in OD. The samples added 0,5 µg/ml AMP showed the same tendency, although to a much smaller degree. Here, the inhibited growth is significantly smaller than for the other samples, as well as only lasting 3 hours, instead of 7+. All samples seemed to eventually reach toward an SP with much lower bacterial cell numbers than the controls. The second experiment showed very similar results as the first. However, the higher AMP-concentration of 8 µg/ml gave diminish cell growth and death after approximately 10 hours (Figure 4.4.b). Variability between triplicates can be seen for the samples added 2 and 4 µg/ml AMP, the latter which was also observed for the first experiment. This indicates that 4 µg/ml AMP is around the threshold of what results in a severe inhibitory effect on the bacteria. Ultimately, MIC was determined to lie in the rage of 4-8 µg/ml, but probably closer to 8 µg/ml.

Streptomycin

The STR-concentrations used in these experiments (0,04–5 µg/ml) were based on the results for the WT, where MIC was determined to be between 1,25–2,50 µg/ml STR. In the two initial analysis only the highest doses of 5 µg/ml completely inhibited growth (Figure A.4). A third test was therefore set up, with concentrations ranging from 0,25 (the 2. highest dose used in exp. 1, 2) to 5 µg/ml. All the samples in this third analysis showed the same initial growth for approximately 3 hours, whereupon the bacteria added the highest STR-levels (4,5–5 µg/ml) resulted in a complete inhibition of growth (Figure 4.5).

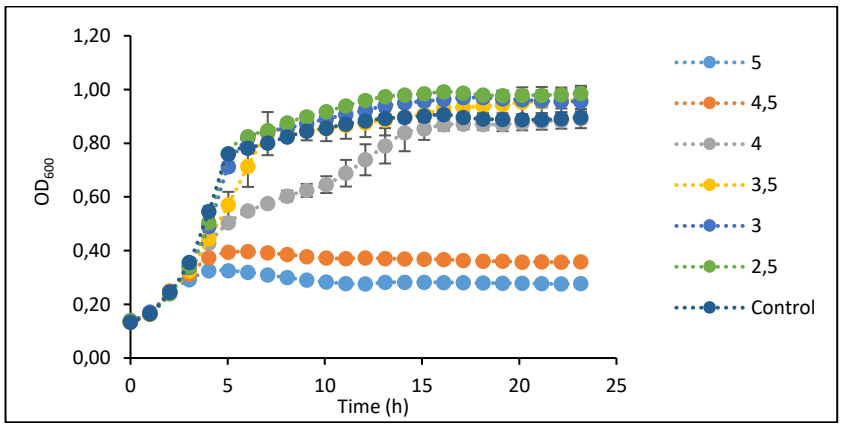


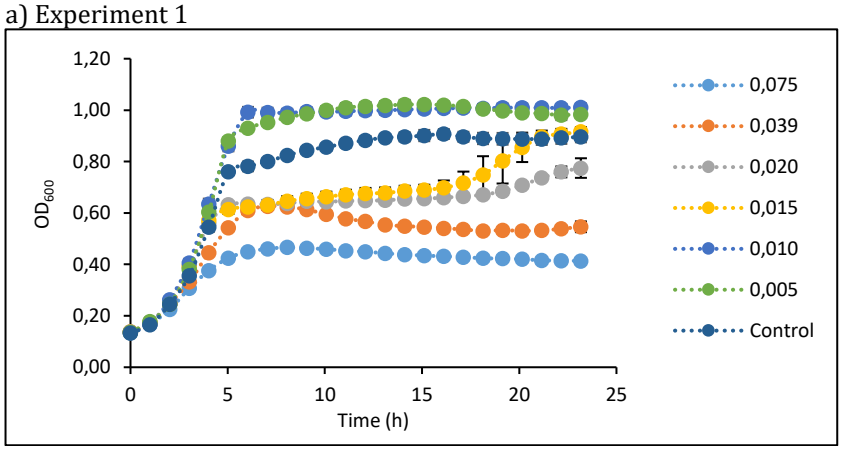
Figure 4.5: MIC for Δ*rpoS* treated with STR– Experiment #3
MIC determination by measured absorptions in a Tecan plate reader, with samples of the *E. coli* K12 Δ*rpoS* knock out strain in M9 media with added STR (0,04–5 µg/ml), for one representative experiment. The controls have not been added antibiotics. Each datapoint represents the average of three replicates ±standard deviation. Figures for experiment 1 and 2 are shown in the appendix (Figure A.4).

Bacteria with STR-doses ≤3,5 showed a very similar growth pattern to the controls, which reached SP after approximately 5 hours. Only the wells with 4 µg/ml STR, showed a deviating growth pattern, where the bacteria started growing more slowly than the controls at 5h. However, these samples reached SP after approximately 16 hours, with similar population density. The STR-MIC for the Δ*rpoS* strain was therefore determined to be 4,5–5 µg/ml, which is a quite similar to the WT-result (1,25 ≤ MIC ≤ 2,50 µg/ml).

Ciprofloxacin

The CIP-concentrations used (0,002–0,2 µg/ml) were based on the results gained for the WT, where MIC was determined to be ~0,01 µg/ml. In two experiments, the samples with CIP-doses of 0,010 µg/ml and less showed similar growth patterns to the controls, which reached SP after approximately 5 hours (Figures 4.6.a, A.5). However, the treated samples reached a higher biomass level in SP.

a) Experiment 1



b) Experiment 2

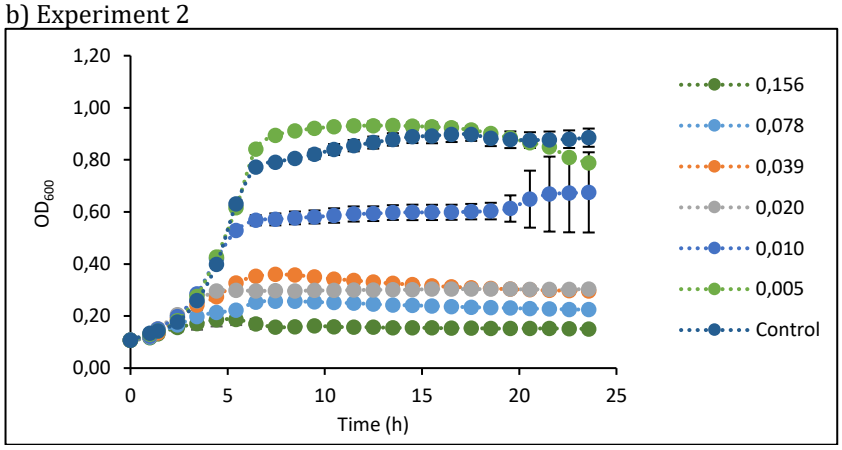


Figure 4.6: MIC for Δ*rpoS* treated with CIP
MIC determination by measured absorptions in a Tecan plate reader, with samples of the *E. coli* K12 Δ*rpoS* knock out strain in M9 media with added CIP (0,02–0,2 µg/ml), for two representative experiments. The controls have not been added antibiotics. Each datapoint represents the average of three replicates ±standard deviation. The figure for experiment 3 is shown in the appendix (Figure A.5).

In the third analysis this dose (0,010 µg/ml) resulted in the same exponential growth for the first 6 hours, after which the growth reached a plateau at OD=0,68 (Figure 4.6.b). The cell density remained stable at this level for approximately 14 hours, before some of the samples in the triplicate showed increased growth. The bacteria added CIP-doses of 0,02 µg/ml and higher showed a delayed growth, but reached SP after approximately 8 hours, with cell densities around 1/3 of the controls. Concentrations above 0,078 µg/ml inhibited bacterial growth completely. Based on these results the MIC for Δ*rpoS* with ciprofloxacin was determined to be in the range of 0,08 to 0,16 µg/ml, compared to the WT (MIC ~ 0,01 µg/ml).

4.1.3 ΔrelA

These experiments were set up to determine the MIC for the *E. coli* knockout strain Δ*relA* with three antibiotics (AMP, STR, CIP).

Ampicillin

The AMP-concentrations used (0,25–8 µg/ml) were based on the results gained for the WT (1,56 < MIC < 3.13 µg/ml). The results showed a very similar growth pattern for the controls and the samples added the lowest AMP-concentrations (0,25 µg/ml), although with a slightly lower cell density for the latter (Figures 4.7, A.6).

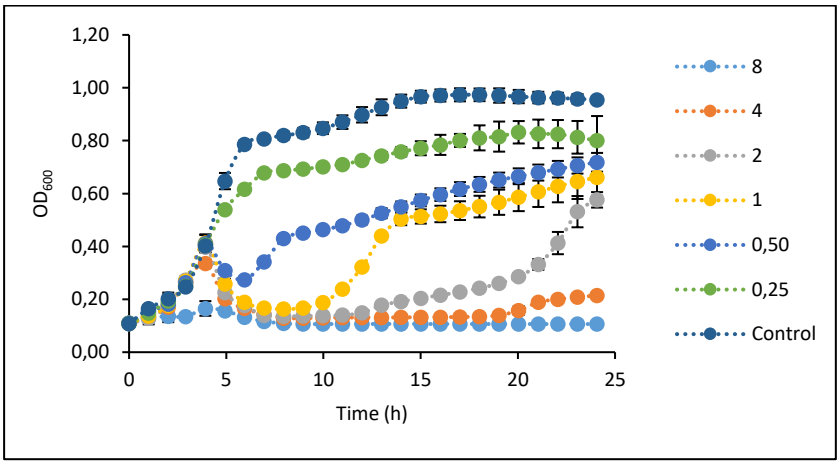


Figure 4.7: MIC for Δ*relA* treated with AMP – Experiment #1
MIC determination by measured absorptions in a Tecan plate reader, with samples of the *E. coli* K12 Δ*relA* knock out strain in M9 media with added AMP (0,25–8 µg/ml) for one representative experiment. The controls have not been added antibiotics. Each datapoint represents the average of three replicates ±standard deviation. The figures for experiment 2 and 3 are shown in the appendix (Figure A.6).

Furthermore, the results are very similar to those for Δ*rpoS* treated with AMP, with the same spectre of responses, but with different doses (Figure 4.4). In this case, the

threshold for a large effect seem to lie between 2 – 4 µg/ml, and the AMP-concentration of 8 µg/ml inhibited bacterial growth completely. The MIC for Δ*relA* treated with AMP was therefore determined to be in the range of 4-8 µg/ml.

Streptomycin

The STR-concentrations used (0,16 – 5 µg/ml) in these experiments were based on the results gained for the WT (1,25 ≤ MIC ≤ 2,50 µg/ml). The results showed that only the highest STR-dose of 5 µg/ml led to inhibition of bacterial growth (Figures 4.8, A.7).

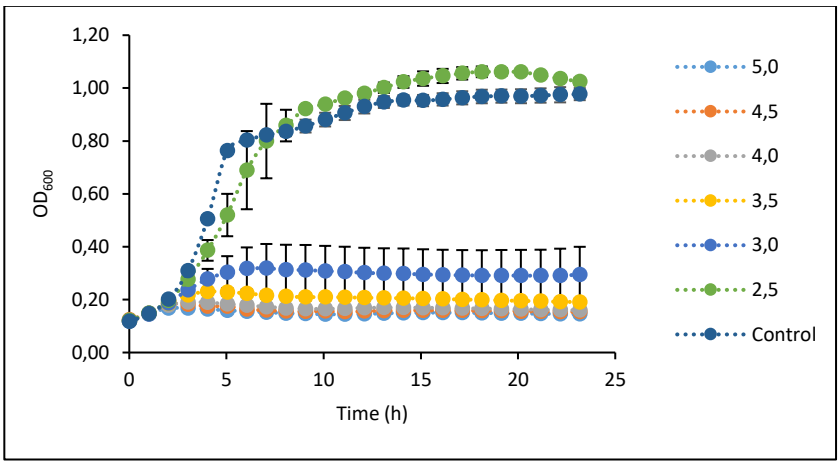


Figure 4.8: MIC for Δ*relA* treated with STR – Experiment #3
MIC determination by measured absorptions in a Tecan plate reader, with samples of the *E. coli* K12 Δ*relA* knock out strain in M9 media with added STR (0,16–5 µg/ml), for one representative experiment. The controls have not been added antibiotics. Each datapoint represents the average of three replicates ±standard deviation. The figures for experiment 1 and 2 are shown in the appendix (Figure A.7).

The second highest concentration (2,5 µg/ml) had a somewhat delayed growth but reached the same bacterial density as the controls in the first experiment (Figure A.7.a). However, in the second analysis, this concentration caused an almost complete inhibition (Figure A.7.b). A third experiment was therefore performed, where STR-concentrations between 2,5 and 5 µg/ml were tested. The results of the third experiment show that only concentrations of 3,5 µg/ml and above led to an almost complete inhibition of bacterial growth, with no regrowth during the analysis time (Figure 4.8). Samples added 3 µg/ml STR showed varying levels of inhibition for the three triplicates, while those added 2,5 µg/ml STR showed a similar growth pattern to the controls. Based on these observations, the MIC for the Δ*relA* mutant treated with STR was determined to be between 3,5-4 µg/ml.

Ciprofloxacin

The CIP-concentrations used (0,005–0,16 µg/ml) in these experiments were based on the results gained for the WT (MIC ~ 0,01 µg/ml). In two experiments, the lowest CIP-dose (0,005 µg/ml) showed a growth pattern very similar to the controls, which reached SP after approximately 5 hours (Figure 4.9, A.8.a). However, in the third experiment, a diminished growth was observed for these samples after 10 hours (Figure A.8.b). The same can be seen for the final hours in the first experiment, although to a much lesser extent (Figure 4.9). Samples added CIP-doses of 0,01 µg/ml and higher resulted in inhibition of cell growth to a varying degree (OD: 0,15-0,33), reaching SP after 5 hours. Based on these results the MIC for the Δ*relA* strain treated with ciprofloxacin was determined to be in the range of 0,04 to 0,08 µg/ml.

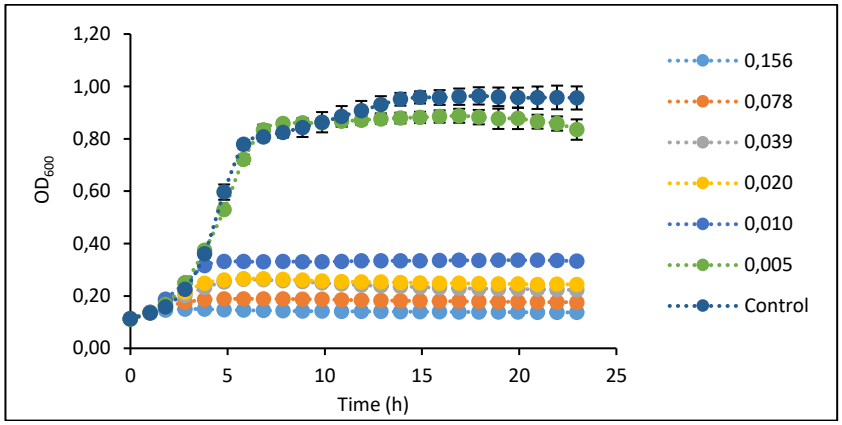


Figure 4.9: MIC for Δ*relA* treated with CIP – Experiment #1
MIC determination by measured absorptions in a Tecan plate reader, with samples of the *E. coli* K12 Δ*relA* knock out strain in M9 media with added CIP (0,005–0,16 µg/ml), for one representative experiment. The controls have not been added antibiotics. Each datapoint represents the average of three replicates ±standard deviation. The figures for experiment 2 and 3 are shown in the appendix (Figure A.8).

4.1.4 Summary of MIC-results

When comparing all the results from the MIC-experiments, ciprofloxacin is shown to be the most effective antibiotic against all three *E. coli* strains, followed by streptomycin and ampicillin (Table 4.1). AMP and STR were almost equally effective on the two knockout strains, while AMP was the least effective on the WT.

Table 4.1: Determined MIC for three *E. coli* strains with three types of antibiotics

	Antibiotics (µg/ml)		
	Ampicillin	Streptomycin	Ciprofloxacin
E. coli K12 MG1655 – WT	1,56 < MIC < 3,13	1,25 < MIC < 2,5	~ 0,01
E. coli K12 MG1655 – ΔrpoS	4,0 < MIC < 8,0	4,5 < MIC < 5,0	0,08 < MIC < 0,16
E. coli K12 MG1655 – ΔrelA	4,0 < MIC < 8,0	3,5 < MIC < 4,0	0,04 < MIC < 0,08

The OD_{600}-measurements performed in the spectrophotometer for the WT ON-cultures were very consistent, ranging from 0,499–0,505 in 1:5-dilutions. That is, the actual OD_{600} of the WT was 2,5 for every experiment. However, the cell density of the ON-cultures for the knockout strains, ΔrpoS and ΔrelA, were quite a bit lower at the onset of each experiment. In 1:5-dilutions, OD_{600}-measurements for ΔrpoS varied between 0,325–0,377, giving actual values of 1,6–1,8. Equally, the OD_{600}-measurements of 1:5-dilutions of ON-ΔrelA were in the range of 0,204–0,342, which gives actual values of 1,0–1,7. These results are compared to the corresponding values that would be achieved in the Tecan Plate reader in Table 4.2. A correlation plot between the spectrophotometer and the TPR from a different experiment is shown in the appendix (Figure B.1-2). When compared to the growth curves for the controls in the MIC-experiments, it's seen that a stable SP isn't reached until an OD_{600} of ~1 is observed (Figures 4.1-9). This may further indicate that the knockout strains were not actually in SP when they were used in the MIC-experiments, which may in turn explain the absence of a proper lag phase, as shown in the corresponding figures. As MICs are supposed to be determined from incubations with the bacteria first entering lag phase, the results for the two knockout strains might not be seen as proper MIC-analysis, and the experiments should be repeated.

Table 4.2: Comparison of OD_{600}-measurements in ON-cultures used for MIC-determination
Comparison between OD_{600}-values gained by measurements done in a spectrophotometer (S) at the onset of MIC-experiments, and the would-be corresponding values from the Tecan Plate Reader. The standard deviation is calculated a representative three ON-cultures, consisting of the max, min and middle value found for each of the three strains.

	OD_{600} (S)	OD_{600} (TPL)	Standard deviation (OD_{600} (S))
E. coli K12 MG1655 – WT	2,5	~1,10	0,3 %
E. coli K12 MG1655 – Δ*rpoS*	1,6 – 1,8	~0,75 – 0,83	2,6 %
E. coli K12 MG1655 – Δ*relA*	1,0 – 1,7	~0,51 – 0,79	9,8 %

4.2 Studies of antibiotic tolerance in the well plate format

The following chapters describe various experiments relating to tolerance studies performed in the well plate format with the three *E. coli* K12 MG1655 strains (WT, Δ*rpoS*, Δ*relA*) and with three types of antibiotics (AMP, STR, CIP). The preparatory experiments were performed in order to establish optimal conditions for the said assays, which were themselves set up for the same purpose, but in preparations for studies of tolerance in a high resolution microbioreactor system (chapter 4.3).

Tolerance is often characterised as the ability of a bacterial population to survive a transient exposure to stressors, even at concentration exceeding the MIC. Antibiotic tolerance is further separated into two cases, which are tolerance by slow growth and tolerance by lag. Tolerance is often associated with slow growth rate and reduced metabolism. Different classes of antibiotics target different essential cellular processes (Brauner et al., 2016) and may therefore affect tolerance differently.

4.2.1 Comparison of Growth Patterns for three *E. coli strains*

As the two *E. coli* knockout strains Δ*rpoS* and Δ*relA* were new to the laboratory, these experiments were performed to determine their growth rate. This was done in order to see whether results for the following tolerance-experiments would be comparable for the three strains.

E. coli survival mechanisms during stress has been shown to be dependent on many factors, including growth rate. It has for instance been shown to decrease during the transition from exponential phase to SP. Furthermore, the growth rate is an important aspect in the control of the *rpoS*-gene as well as other stress responses in *E. coli*, and thus impacts important aspects of *E. coli* physiology (Lindquist et al, 2014).

Based on the measurements done with a spectrophotometer (Figures 4.10-11), the growth pattern and generation time for the three *E. coli* strains were calculated (Table 4.3). The Δ*rpoS* strain showed similar results to the WT. However, the Δ*relA* strain seemed to have a moderately higher generation time, as well as a somewhat lower growth rate.

Table 4.3: Growth rate and generation time for three strains of *E. coli*

Strain	Growth rate (h-1)	Generation time (min)
E. coli K12 MG1655 – WT	0,5866	70,90
E. coli K12 MG1655 – Δ*rpoS*	0,5796	71,75
E. coli K12 MG1655 – Δ*relA*	0,5234	79,46

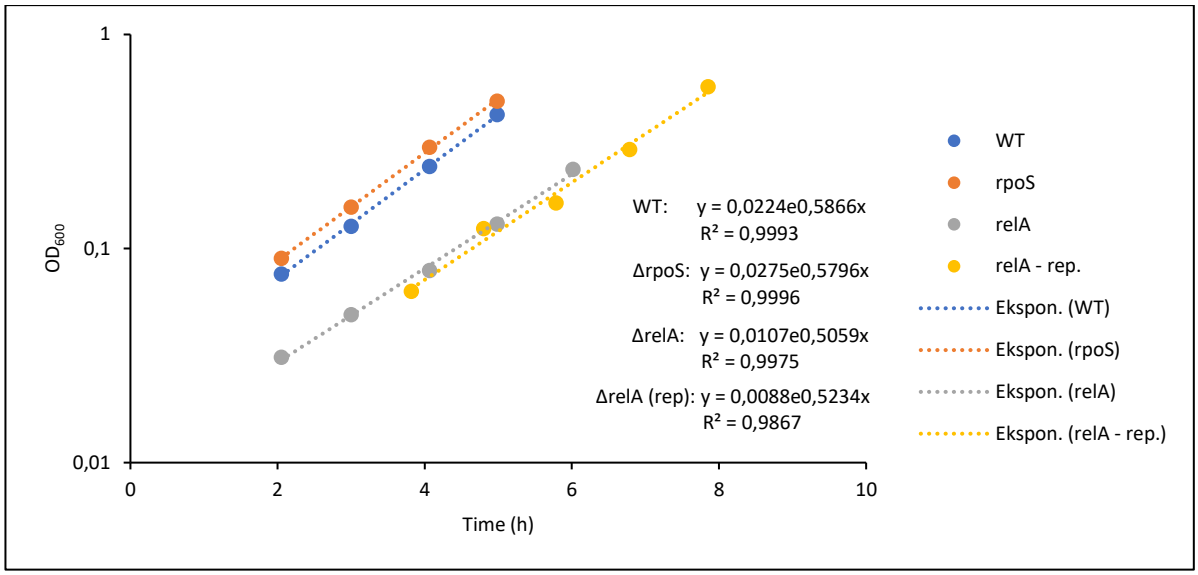


Figure 4.10: Growth rate for the three *E. coli* strains, exponential phase
OD_{600}-measurements of the three *E. coli* strains over time, done in a 1 ml cuvette and the spectrophotometer. The graph only shows the exponential phase, with OD-measurements on a logarithmic scale, which gave the basis for the calculations of generation time (Table 4.3).

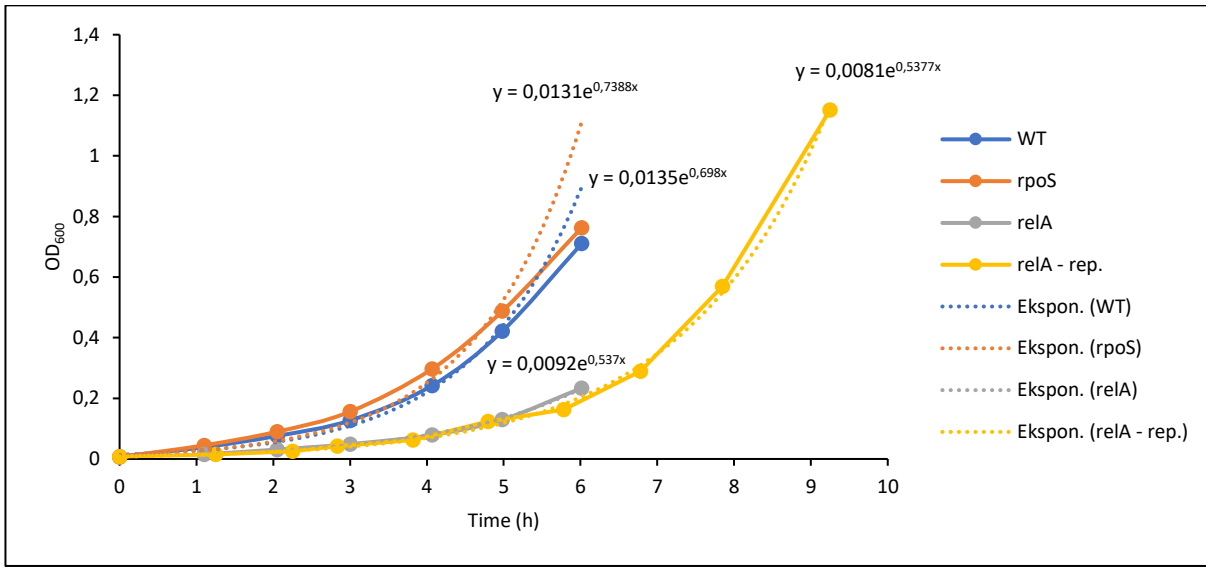


Figure 4.11: Growth curves for the three *E. coli* strains
OD_{600}-measurements of the three *E. coli* strains over time, done in a 1 ml cuvette and the spectrophotometer.

4.2.2 Testing of Growth Media optimal for *E. coli* K12 MG1655 Δ*relA* cultivation

This experiment was performed in an attempt to compare generation time and growth curves between the knockout strain *E. coli* K12 MG1655 Δ*relA* and the WT from different sources, and in different kinds of growth media. The types of media being tested were 1X M9, the altered 1X M9 and LB-medium. In addition, a version of each of the M9 media with added amino acids (isoleucine, valine) were tested. Unfortunately, the

analysis did not give clear, satisfying results and should be repeated. However, the knockout strain seemed to grow more slowly in most of the tested media, especially when transferred directly from the freeze stock. The combination of the altered M9 media and the addition of the amino acids also seemed to give a growth boost to all the bacteria tested, compared to the other M9-media.

4.2.3 Testing of inoculum dilution for optimal transition into stationary phase

This experiment was performed in order to determine the optimal dilution of ON-cultures for use in tolerance experiments, to achieve an appropriate timing for antibiotic addition (Figure 4.12). Thus, we wanted to identify a dilution where the exponential phase started after approximately five hours, since the drugs were to be added in the beginning of this phase. Based on the results, we determined that the 1:200-dilution best fitted our requirements. It was also observed that the growth rate was equal in the exponential phase for all the dilutions, while the time spent in lag phase varied. This proves that the solutions were independent of cell density but varied with growth conditions. That is; the solutions with a lower dilution will eat through the nutrients in the media faster, reaching the exponential phase faster.

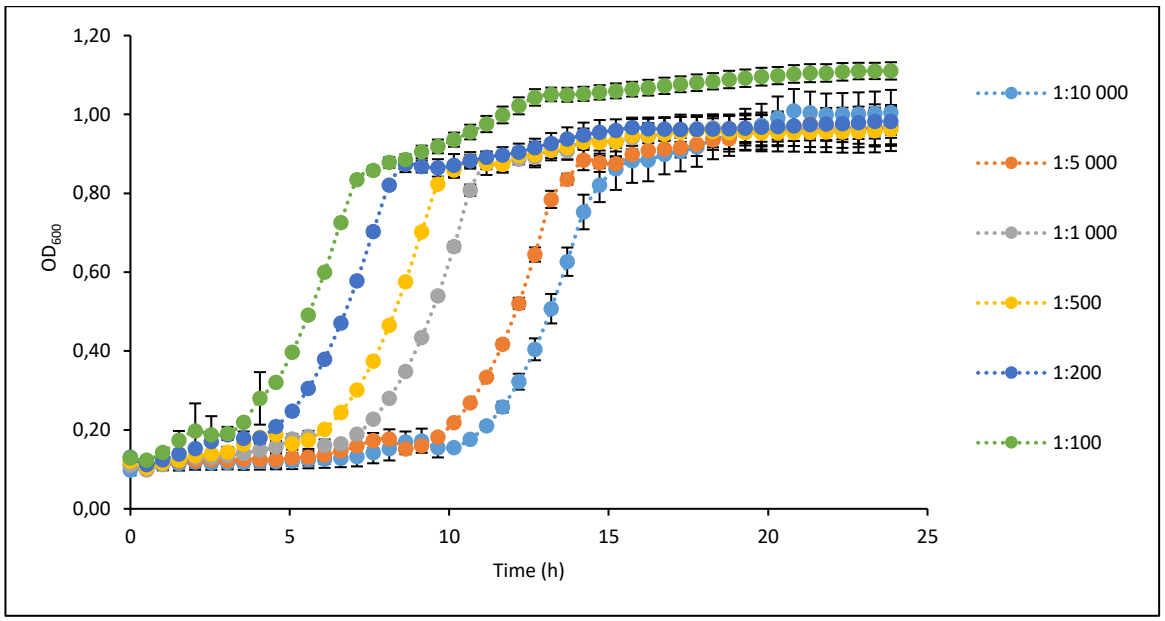


Figure 4.12: Growth rate for different dilutions of WT-ON-cultures
Testing of growth rate in various dilutions of an ON-culture of *E. coli* K12 MG1655 in M9 culture medium. The measurements were done by incubation in a Tecan plate reader for 25 hours.

4.2.4 Tolerance experiments in the well plate format

Several experiment series (consisting of 3 experiments each) were set up to determine the antibiotic tolerance for each of the three *E. coli* K12 MG1655 strains (WT, Δ*rpoS*, Δ*relA*) in M9 culture media, with each of the three antibiotics (AMP, CIP, STR). In order to achieve optimal timing of the analysis, 1:200-dilutions of all the ON-cultures were used, and the antibiotics were added after approximately 5 hours, early in exponential phase for the bacteria (OD_{600}~0,5; corresponding to OD_{600}~0,98 in the spectrophotometer). Different sets of concentrations were tested in each individual experiment in the three series, with the aim of determining the appropriate concentrations as exactly as possible. All the concentrations used are given in the figures corresponding to each analysis series.

WT treated with Ampicillin

Three experiments were set up with the WT and ampicillin (AMP), with AMP-concentrations in the range of 0,25 –8 µg/ml. A similar growth pattern to the controls was observed for samples of 4 µg/ml and less, going into SP after approximately 10 hours. However, all AMP-doses above 0,25 µg/ml, showed signs of inhibition, resulting in bacterial populations stabilizing at slightly lower ODs than the controls (Figure C.1).

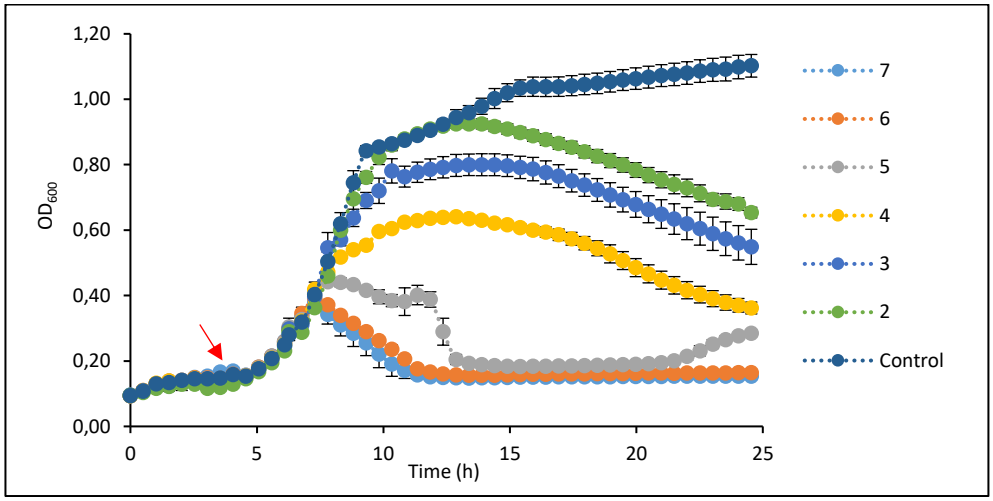


Figure 4.13: Tolerance experiment in the well-plate format for the WT and AMP – Experiment #3
One representative tolerance experiment with the WT *E. coli* K12 MG1655 in M9 culture media and ampicillin (2–7 µg/ml), by measured absorptions in a Tecan plate reader at 600 nm. The red arrow illustrates when the antibiotics were added, and the controls were not added AMP. The OD_{600} was measured every 30 minutes and each datapoint represent the average of three replicates ±standard deviation. The figures with experiment 1 and 2 are shown in the appendix (Figure C.1).

The AMP-concentrations of 6-8 µg/mL resulted in a sharp decrease in bacterial density approximately 2,5 hours after addition, probably due to cell lysis. The samples

added 5 µg/mL AMP mark a threshold for a significant response, showing a slower decrease in density than samples treated with higher doses. Also, these bacterial populations experienced a short uptick in growth after approximately 9 hours, before death occurred. All the triplicates showed the same pattern at these timepoints, but with some variation. These samples also have a period of regrowth after 22 hours. Lower AMP-concentrations resulted in growth patterns with similar exponential phases in the first hours after antibiotic addition, followed by a gradual reduction in bacterial density in a dose dependent manner. When compared to the corresponding MIC-results (1,56 ≤ MIC ≤ 3,12), these results indicate that both the growth phase and exposure time influences the stress responses of the WT strain, with different antibiotic levels triggering slightly different mechanisms. Furthermore, the bacteria's tolerance toward AMP seem to increase when they are exposed in the exponential phase, rather than the lag phase.

WT treated with Streptomycin

Four experiments were set up with the WT and streptomycin, with STR-doses ranging from 0,1–20 µg/ml. The initial experiments showed that all concentrations lower than 6,4 µg/ml resulted in a similar growth pattern to the controls (Figure C.2). A third analysis was therefore repeated with significantly higher doses. The consequent results showed that samples added STR-concentrations of 10-20 µg/ml continued to grow in line with the controls for the approximate first two hours after STR-addition (Figure 4.14).

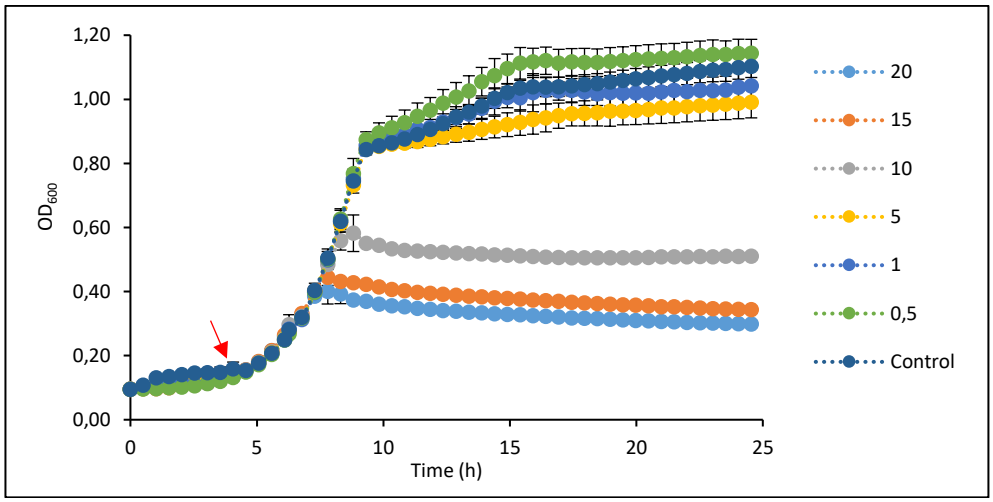


Figure 4.14: Tolerance experiment in the well-plate format with the WT and STR – Experiment #3
One representative tolerance experiment with the WT *E. coli* K12 MG165 in M9 culture media and streptomycin (0,5–20 µg/ml), by measured absorptions in a Tecan plate reader at 600 nm. The red arrow illustrates when the antibiotics were added, and the controls were not added STR. The OD_{600} was measured every 30 minutes and each datapoint represent the average of three replicates ±standard deviation. The figures showing experiment 1 and 2 are shown in the appendix (Figure C.2).

However, at 7-9,5 hours into the experiment, the bacteria went into SP with a relatively stable OD, varying between 0,3-0,5 depending on the STR-concentration. This indicates that death occurred at these time-points. Compared to the corresponding obtained MIC-values (MIC > 1,25 µg/mL STR), these results seem to indicate that the bacteria are tolerant up to a certain level, and also that they are able to survive in exponential phase for a limited amount of time after antibiotic addition.

WT treated with Ciprofloxacin

Three experiments were set up with the WT and ciprofloxacin (CIP), with CIP-concentrations ranging from 0,001–2 µg/ml. The initial experiments showed that all concentrations from 0,08 and below resulted in similar growth patterns to the controls, with no visible inhibition (Figure C.3). The third experiment resulted in a spectre of growth patterns quite similar to the results gained for the WT treated with STR (Figure 4.14). Concentrations of 0,5–2 µg/mL was shown to inhibit growth 2–3,5 hours after antibiotic addition, with populations showing a relatively stable concentration-dependent OD (Figure 4.15).

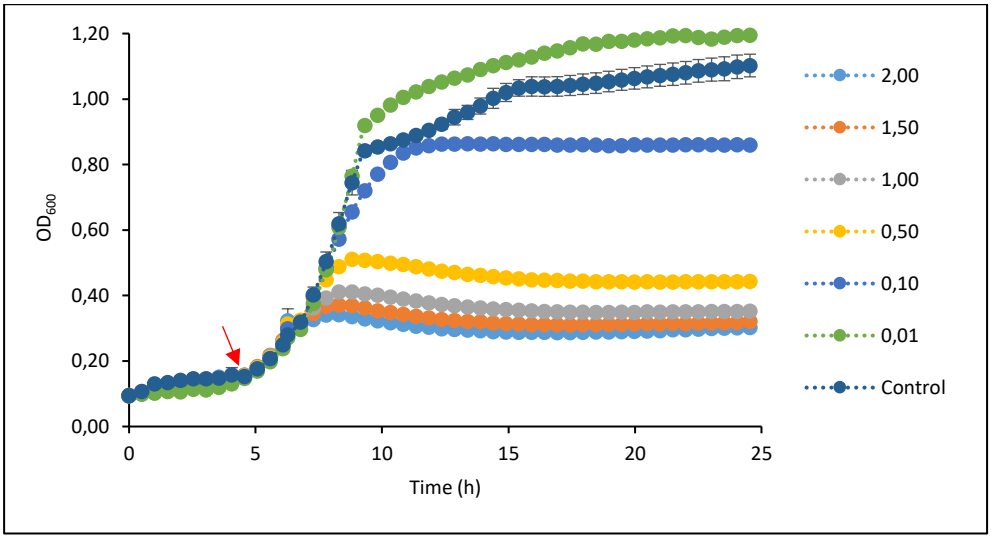


Figure 4.15: Tolerance experiment in the well-plate format with the WT and CIP – Experiment #3
One representative tolerance experiment with the WT *E. coli* K12 MG1655 in M9 culture media and ciprofloxacin (0,01–2,0 µg/ml), by measured absorptions in a Tecan plate reader at 600 nm. The red arrow illustrates when the antibiotics were added, and the controls were not added CIP. The OD_{600} was measured every 30 minutes and each datapoint represent the average of three replicates ±standard deviation. The figures with experiment 1 and 2 are shown in the appendix (Figure C.3).

The concentrations in the range of 1–2 µg/mL were the lowest doses that most effectively inhibited bacterial growth in the WT. The samples added 0,1 µg/mL CIP also

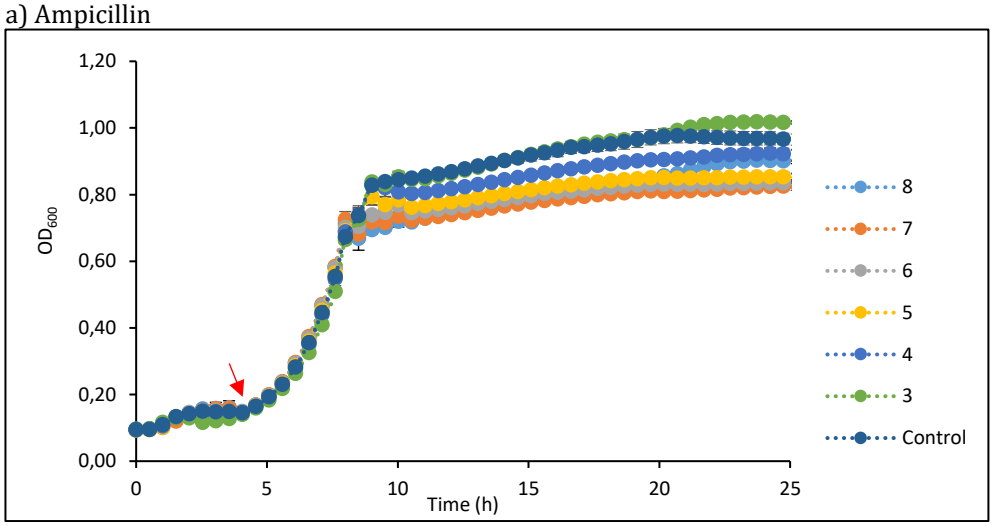
seems to be affected slightly, as the bacteria reached SP faster than the controls, and also stabilized at a lower OD-value. However, the response is much less severe than for the samples treated with 0,5 µg/ml CIP. These results seem to indicate that death occurred in samples for concentrations above 0,5 µg/ml CIP, after approximately 2 hours which is the same timepoints as for WT treated with STR (Figure 4.14). Compared to the obtained MIC-values for the WT treated with CIP (0,01), these results seem to indicate that the bacteria are tolerant up to a certain level, like seen for the STR-analysis.

Tolerance for Δ*rpoS* with AMP, STR and CIP

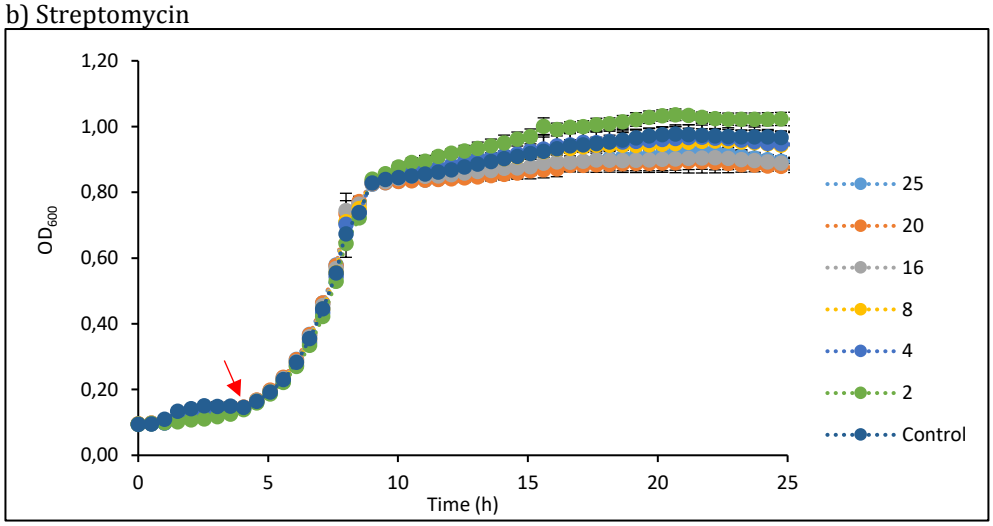
One experiment was performed with the Δ*rpoS* strain with each of the three antibiotics. The results gained in tolerance experiments with the WT was used as the basis for choosing concentrations for each of the antibiotics in this series, which is given in the figures corresponding to each analysis. The concentration range used for ampicillin-treatment (3–8 µg/ml) of the Δ*rpoS* was much the same as for the WT (0,13–8 µg/ml, Figures 4.13, C.1). The amount of streptomycin (2–25 µg/ml) and ciprofloxacin used covered a slightly higher range than what was used for the WT (STR: 0,04 – 5 µg/ml, Figures 4.14, C.2; CIP: 0,002–0,2 µg/ml, Figures 4.15, C.3).

No significant difference in growth pattern was observed for any samples treated with AMP and STR compared to the controls, which indicates a higher tolerance for these antibiotics in the Δ*rpoS* strain compared to the WT (Figure 4.16.a-b). The bacterial samples treated with CIP were seen to have a very similar exponential phase to the controls, and they went into SP at approximately the same time (Figure 4.16.c). However, the CIP-treated bacterial populations had an overall lower cell density in SP, ranging from OD_{600} = 0,6–0,8 compared to the controls (OD_{600} = 1). This indicates a moderately higher tolerance to CIP in the ΔrpoS mutant compared to the WT.

a) Ampicillin



b) Streptomycin



c) Ciprofloxacin

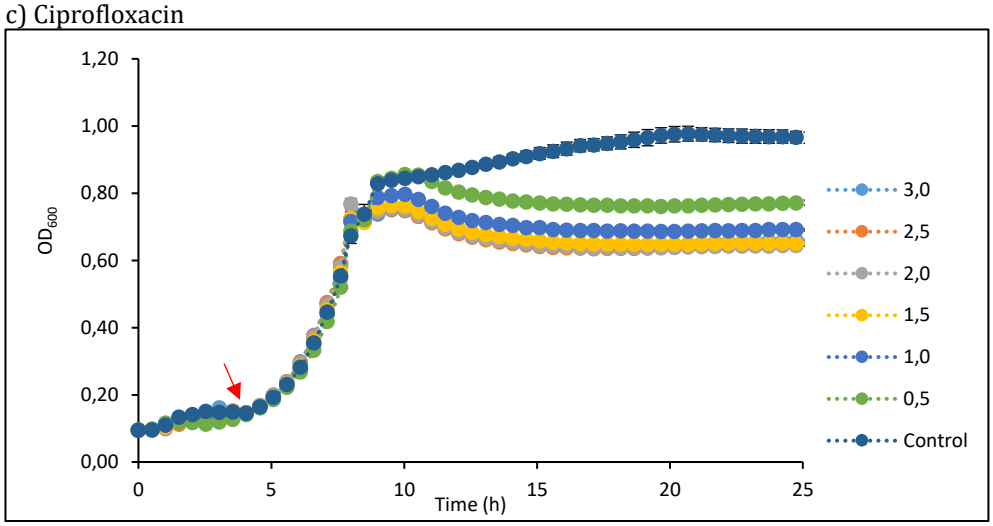


Figure 4.16: Tolerance experiments in the well-plate format with Δ*rpoS* and AMP, STR, CIP
Tolerance experiment with the knockout *E. coli* K12 MG1655 Δ*rpoS* in M9 culture media and

a) Ampicillin (3 – 8 µg/ml)
b) Streptomycin (2 – 25 µg/ml)
c) Ciprofloxacin (0,5 – 3,0 µg/ml)

by measured absorptions in a Tecan plate reader at 600 nm. The red arrow illustrates when the antibiotics were added to the wells, except for the control samples. The OD_{600} was measured every 30 minutes and each datapoint represent the average of three replicates ±standard deviation.

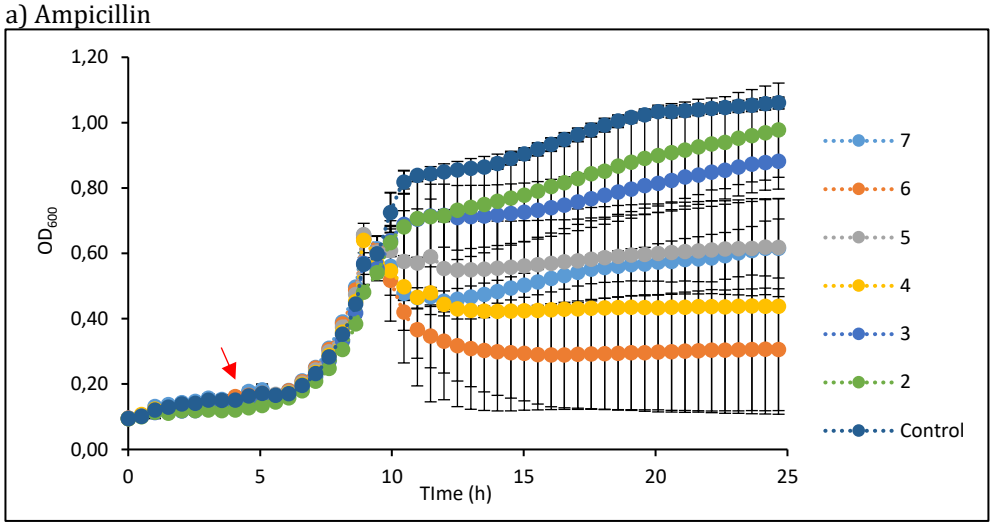
Tolerance for Δ*relA* with AMP, STR and CIP

One experiment was performed with the Δ*relA* strain with each of the three antibiotics. The amount of ampicillin (2–7 μg/ml), streptomycin (1–20 μg/ml) and ciprofloxacin (0,01–2 μg/ml) used in this analysis series was much the same as for the WT (AMP: 0,13–8 μg/ml, Figures 4.13, C.1; STR: 0,04–5 μg/ml, Figures 4.14, C.2; CIP: 0,002–0,2 μg/ml, Figures 4.15, C.3). The experiments showed similar growth patterns for all the antibiotics, varying with the doses used.

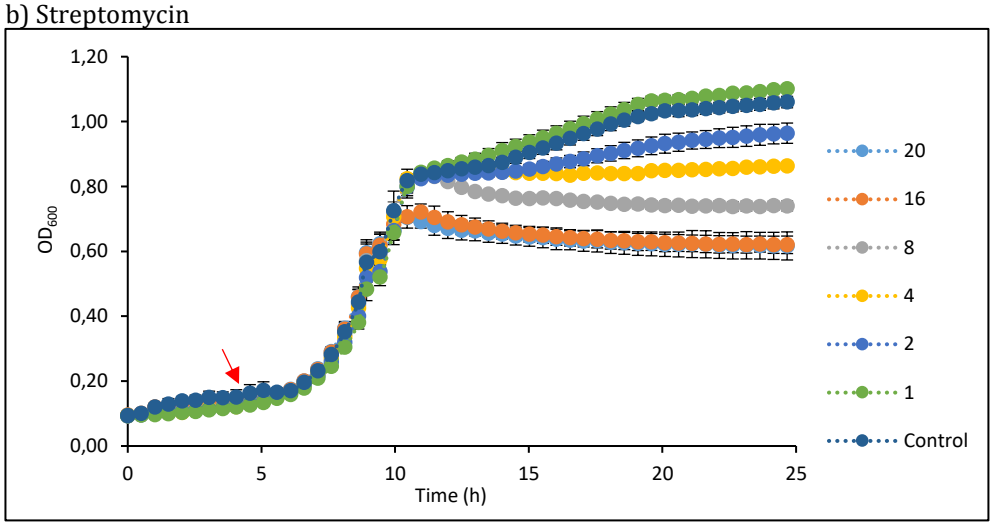
The same growth pattern was observed for all bacterial samples for the first 9-10 hours, which includes the lag and exponential phase (Figure 4.17). However, the controls in the AMP-experiment had a slightly longer exponential phase, reaching a higher OD, which continued to rise through SP. The wells which were added 2 and 3 μg/ml AMP also showed a continued increase in bacterial biomass in SP, although with a lower OD than the controls (Figure 4.17.a). The standard deviation was also relatively low for these samples (compared to very low for the controls), with a ~0,2 point variation between the samples with the lowest and highest OD. Concentrations of 4–7 μg/ml AMP caused a slight decrease in growth after 9 hours, followed by an uptick before stabilizing. However, the triplicate treated with 7 μg/ml was the only one among these samples with a relatively low standard deviation. The error was relatively high for the 5 μg/ml- (0,76; 0,67; 0,43) and 6μg/ml-triplicates (0,53; 0,20; 0,19) where death was observed for the latter in two wells. However, the highest level of error was observed for the 4 μg/ml-triplicate (0,16; 0,33; 0,81), where one sample resulted in almost uninhibited growth and another caused almost completely inhibited growth. Ultimately, it's difficult to say anything concrete about the most effective concentrations in evaluating the AMP-tolerance of Δ*relA* based solely on these results.

The results for STR and CIP show that the two highest concentrations of both antibiotics (16–10 μg/ml STR, 1,5–2,0 μg/ml CIP) resulted in the bacteria entering SP sooner than the controls, and stabilizing at a lower OD (Figure 4.17.b-c). The rest of the samples entered SP at approximately the same time as the controls but with an overall lower cell density. The samples progressed through SP in a dose-dependent manner, where those treated with the lowest doses continued to increase (1-2 μg/ml STR, 0,01 μg/ml CIP), while those treated with higher concentrations stabilized after a slight decrease in biomass (4-8 μg/ml STR, 0,10-1 μg/ml CIP). The standard error for the STR- and CIP-experiments were very low compared to those for the AMP-analysis.

a) Ampicillin



b) Streptomycin



c) Ciprofloxacin

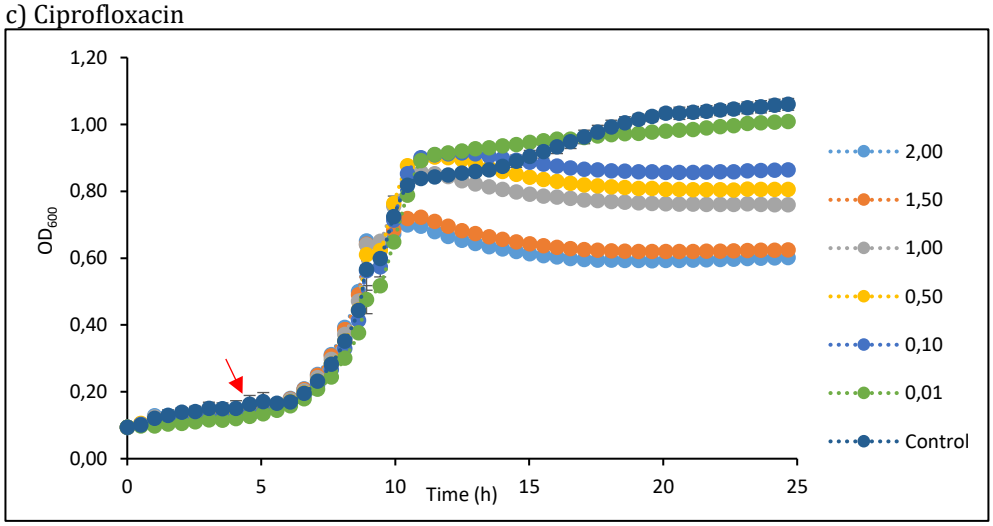


Figure 4.17: Tolerance experiments in the well-plate format with Δ*relA* and AMP, STR, CIP
Tolerance experiments with the knockout *E. coli* K12 MG1655 Δ*relA* in M9 culture media and

a) Ampicillin (2 – 7 µg/ml)
b) Streptomycin (1 – 20 µg/ml)
c) Ciprofloxacin (0,01 – 2,0 µg/ml)

by measured absorptions in a Tecan plate reader at 600 nm. The red arrow illustrates when the antibiotics were added to the wells, except for the control samples. The OD_{600} was measured every 30 minutes and each datapoint represent the average of three replicates ±standard deviation.

4.2.5 Comparison of tolerance-results

When comparing the results for the tolerance experiments performed in the well-plate format, ciprofloxacin is shown to be the most effective antibiotic used in this study against all three strains, followed by ampicillin and streptomycin (Table 4.4). This agrees well with the results in respect to CIP, which was shown to have the lowest MIC value for each of the strains. However, the tolerance results for STR shows that a significantly higher dose is required in order to procure a response, both compared to the numbers for ampicillin and to the MIC values for STR.

Table 4.4: Comparison of tolerance results

E. coli K12 MG1655 strain		**Ampicillin**	**Streptomycin**	**Ciprofloxacin**
WT	MIC	1,56 < MIC < 3,13	1,25 < MIC < 2,5	~ 0,01
	Significant response	≥ 5 µg/ml	≥ 10 µg/ml	≥ 0,5 µg/ml
	Death	≥ 6 µg/ml	≥ 15 µg/ml	≥ 1 µg/ml
Δ*rpoS*	MIC	4 < MIC < 8	4,5 < MIC < 5	0,08 < MIC < 0,16
	Significant response	Inconclusive	Inconclusive	Inconclusive
	Death	> 8	> 25	> 3
Δ*relA*	MIC	4 < MIC < 8	3,5 < MIC < 4	0,04 < MIC < 0,08
	Significant response	≥ 4 µg/ml	≥ 16 µg/ml	≥ 1,5 µg/ml
	Death	Inconclusive	Inconclusive	Inconclusive

Furthermore, the results show that the knockout strains were significantly more tolerant toward all the antibiotics than the wild type, and especially Δ*rpoS*. However, this may also be affected by a difference in growth rates between the strains, as was seen in the MIC experiments.

4.3 Microbioreactor Cultivations

These experiment series were performed with the BioLector Pro, in order to gain a better understanding of the growth patterns of the three *E. coli* K12 MG1655 strains (WT, Δ*rpoS*, Δ*relA*) when exposed to low doses of each of the three antibiotics (AMP, STR, CIP). The antibiotics were added in the middle of the exponential growth phase, after approximately 7 hours. This can be observed by a drop in the DO-curves due to the pause in shaking as the well-plate was taken out of the BioLector and is not a consequence of variations in cell density.

The concentrations used were based on the results obtained from the tolerance experiments performed in the well plate format. However, higher doses were used for each of the antibiotics to compensate for the higher bacterial biomass in the BioLector. Different ranges of concentrations were tested in each individual experiment for each antibiotic, with the aim of determining the concentrations leading to tolerance as exactly as possible. This was the first time the BioLector was used in the laboratory for these kinds of experiments, so a period with some "trial and error" was necessary.

4.3.1 Initial experiments performed with WT *E. coli* in M9 culture media

The initial experiments were performed with the WT and each of the three antibiotics in M9 media (Figures D.1-3). However, this resulted in very poor curves for biomass and DO. Although a difference between the controls and the samples treated with antibiotics was clearly visible, these experiments resulted in biomass curves with quite a lot of "noise". It turned out a higher biomass was needed in order to get a better resolution for this instrument. For this reason, all further experiments were performed with an altered M9 media with high biomass capacity.

4.3.2 Experiments performed with WT *E. coli* K12 MG1655 in M9-hbc

All the experiments presented in this section gave results with biomass curves showing a clear distinction between the lag phase and SP, but also with much less noise compared to the analysis performed with regular M9 media. Similar trends were observed for all the pH-curves, indicating that differences in growth had a small effect on pH. This also proved that the pH-changes did not interrupt the experiments, which was the main reason for including this test. For this reason, two representative pH-curves are shown for the first analysis described (with AMP), while the rest are provided in the appendix (Figures E.1-5).

Ampicillin

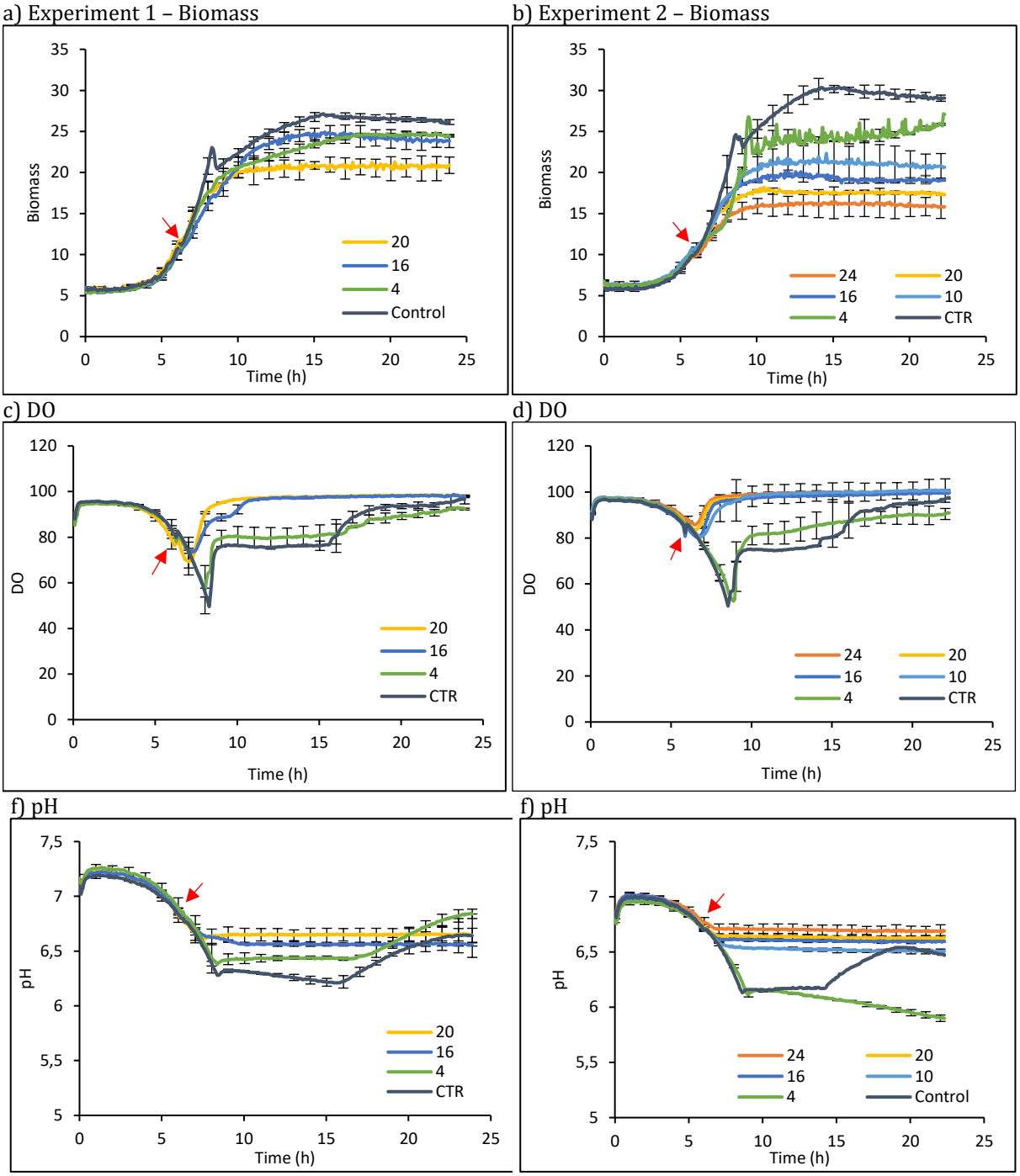


Figure 4.18: Tolerance experiments in microbioreactor cultivations with the WT and AMP
Tolerance experiments with the WT *E. coli* K12 MG1655 in M9-hbc culture media and ampicillin (4–24 µg/ml), by measurements performed in a BioLector Pro. The curves represent a-b) biomass, c-d) DO and e-f) pH for two experiments. The red arrow illustrates the time when the antibiotics were added, while the controls were not added AMP. Each datapoint represent the average of two replicates ±standard deviation shown for each hour of the experiment, unless otherwise specified.

Two experiments were performed with the *E. coli* WT and ampicillin, with AMP-concentrations in the range of 4–24 µg/ml. A clear response in the biomass curves was observed for all samples almost immediately after AMP-addition, with the antibiotic causing a slightly reduced growth rate in a dose-dependent manner (Figures 7.18.a-b). They also varied in the timepoints of transition into SP and the final biomass levels, indicating differences in growth inhibition. A sudden decrease in biomass near this transition is also observed for the controls, which is most likely due to exhausted glucose resources. This is not seen for the treated samples as they do not reach the same level of metabolic activity. However, the trend might have occurred in the samples added 4 µg/ml AMP in the second experiment, but it's difficult to say for certain due to the apparent noise in the SP of the biomass curve. 24 µg/ml AMP was the concentration used which inhibited growth the most, with treated samples stabilizing at a biomass of ~16, compared to the controls at ~30. The small differences between equal samples in the two experiments might be due to the slight deviation in timing of the antibiotic addition.

The DO-graphs support the observations from the biomass-data, but also provide a lot more information. The controls are shown to have the largest reduction in DO corresponding to the biomass peak by the transition into SP (Figure 4.18.c-d). This agrees with a higher utilisation of oxygen during growth. Only a slight drop in DO is observed for samples with higher AMP-doses (≥ 10 µg/ml) with values stabilizing at ~100 % saturation as the bacteria go into SP. The fact that the lower dose of 10 µg/ml had such a large effect on the populations was not as evident from the biomass-data. The DO-response seems to be almost identical for samples treated with 10 and 16 µg/ml AMP, although a clear difference is seen between their biomasses. The inhibitory effect is also seen for the DO-data earlier than the corresponding biomass-curves, with a turning point happening already after 40 minutes (24 µg/ml-samples). The eventual stepwise DO-increase of the untreated controls illustrates a decline in metabolic activity as the cultures reach nutrient limitations and enters SP. The same trend is shown to some extent for the samples treated with the lowest AMP-dose (4 µg/ml), although with a higher degree of variation.

The results show an initial decrease in pH as growth proceeds. When the bacteria stop growing, the pH is seen to stabilize at different levels, with the untreated controls reaching the lowest values (pH ~6±0,2) (Figure 4.18.e-f). The pH in samples with significantly inhibited growth is shown to stabilize in a dose-dependent manner almost immediately after antibiotic addition. In the controls, as well as samples with only slightly

inhibited growth (4 µg/ml AMP), a slight increase in pH is observed after ~15 hours in the first experiment (Figure 4.18.e). However, the results varied in in the second analysis, and an outlier was removed for the controls. The cause of the pH-decrease is nitrogen consumption, which is reduced with the metabolic activity for inhibited populations.

Streptomycin

Two experiments were performed with the *E. coli* WT and streptomycin (STR), with STR-concentrations in the range of 1–25 µg/ml, which was almost equal to that used for the analysis done in the well plate format (0,1–20 µg/ml).

The resultant biomass-curves for the controls were similar to those observed in the experiments performed with AMP (Figure 4.18), with a slight drop near the end of the exponential phase, before increasing into a stable SP (Figure 4.19.a). This trend was also observed for the rest of the samples, in the first experiment, indicating that they all ran out of glucose. However, the treated samples stabilized at lower biomass levels in SP than the controls. In the second experiment this was also seen for samples treated with STR-doses of 10 µg/ml and less, as well as the controls (Figure 4.19.b). However, 10 µg/ml clearly resulted in a slower growth rate after AMP-addition. Concentrations of 20-25 µg/ml STR caused an almost immediate response, with biomass levels stabilizing approximately 1 hour after addition of the antibiotics. Thus, these doses most likely killed the bacteria. The 15 µg/ml STR-dose also inhibited growth in the samples at the same time, but with biomass still increasing steadily to a level of ~24 (~84 % of controls) after ~21 hours. This indicates that the growth rate was greatly impaired, but the populations survived. There was relatively little variation between duplicates in the biomass-curves, but as the difference between the two experiments are so large, it's difficult to say anything conclusive about the results. There was also an outlier for the 10 µg/ml-samples in experiment 1, which was removed from the graphs (Figures 7.19.a, E.1.a).

The DO-data shows very little variation between samples in the first experiment compared to the second. When taken into account the huge effect which lower antibiotic doses had on oxygen saturation for the AMP-experiments, this probably means that all the data from the first experiment is unreliable. A large standard error both in DO and pH was also observed for the 5 µg/ml-samples in the second experiment, so the outlier was removed from the graphs (Figure 4.19.d, E.1.b). The second analysis resulted in DO-graphs consisting in the same patterns as described previously (Figure 4.18). The controls

and the two samples with the lowest STR-doses had the largest drop in DO, corresponding to an exhaust of glucose resources (Figure 4.19.d), followed by the stepwise increase to almost 100 % saturation. The two highest STR-concentrations (15–25 µg/ml) are shown to have the greatest effect, resulting in complete oxygen saturation almost immediately. The DO stabilized at ~ 85 % for the 15 µg/ml-samples, which supports the observations of a slower growth rate in the biomass data. The results for the pH of the samples show the same trends as described for ampicillin (Figure 4.18.e-f), with samples treated with ≥ 15 µg/ml showing the greatest effects (Figure E.1).

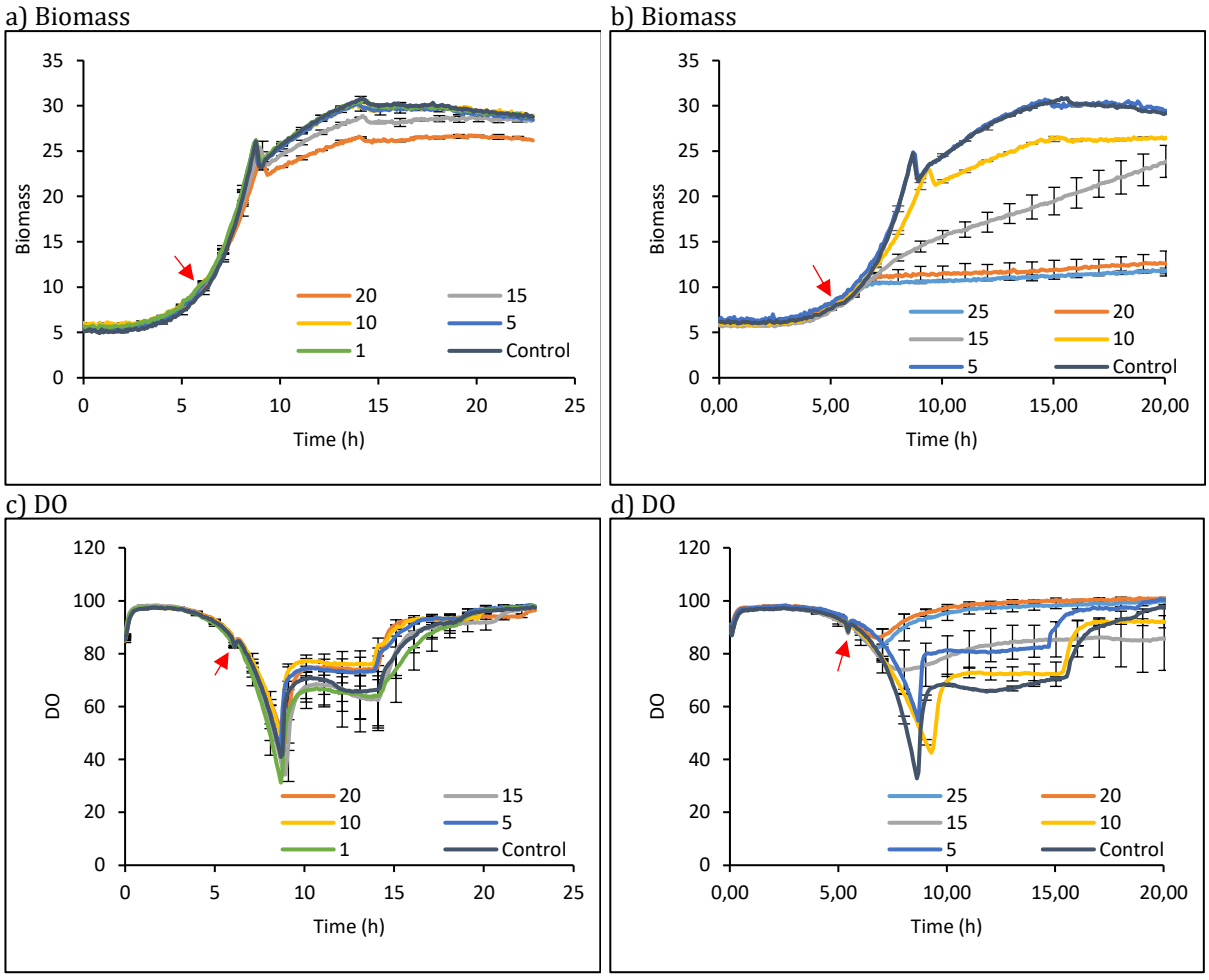


Figure 4.19: Tolerance experiments in microbioreactor cultivations with the WT and STR
Tolerance experiments with the WT *E. coli* K12 MG1655 in M9-hbc culture media and streptomycin (1–25 µg/ml), by measurements performed in a BioLector Pro. The curves represent a-b) biomass and c-d) DO for two experiments. The red arrow illustrates the time when the antibiotics were added, while the controls were not added STR. Each datapoint represent the average of two replicates ±standard deviation shown for each hour of the experiment, unless otherwise specified.

Ciprofloxacin

Three experiments were performed with the *E. coli* WT and ciprofloxacin, with CIP-concentrations in the range of 0,01–15 µg/ml. In the initial experiments, a rather large concentration range was used, which showed that all doses of 1 µg/ml CIP and above completely inhibited growth. In the third experiment, the resultant curves for the controls and the sample added det lowest CIP-concentration (0,01 µg/ml) agreed well with earlier analysis, with the small drop in biomass by the end of the exponential phase (Figure 4.18-19). The rest of the tested CIP-doses clearly inhibited bacterial growth, with concentrations of 0,5–1 µg/ml seemingly causing death at different timepoints. Doses of 0,05-0,10 µg/ml seem to cause approximately the same response by a slowed growth rate (Figure 4.20.a). The biomass curves had much less variability between duplicates than the DO- and pH-curves.

When it comes to the DO-curves, the standard deviation is very large for all the samples except those added 0,05 and 1 µg/ml (Figure 4.20.b). These two duplicates gave results as expected, with the highest concentration showing the smallest drop in DO. However, for the remaining results, the variation is quite large among duplicates, and they do not follow a clear pattern corresponding to the level of CIP-concentrations. The results for the pH of the samples show the same trends as described for earlier BioLector experiments (Figure E.2). The large variability in the 0,10 µg/ml-samples are due to one of the duplicates dropping to ~4,95 (~1,5 points lower than the rest of the samples).

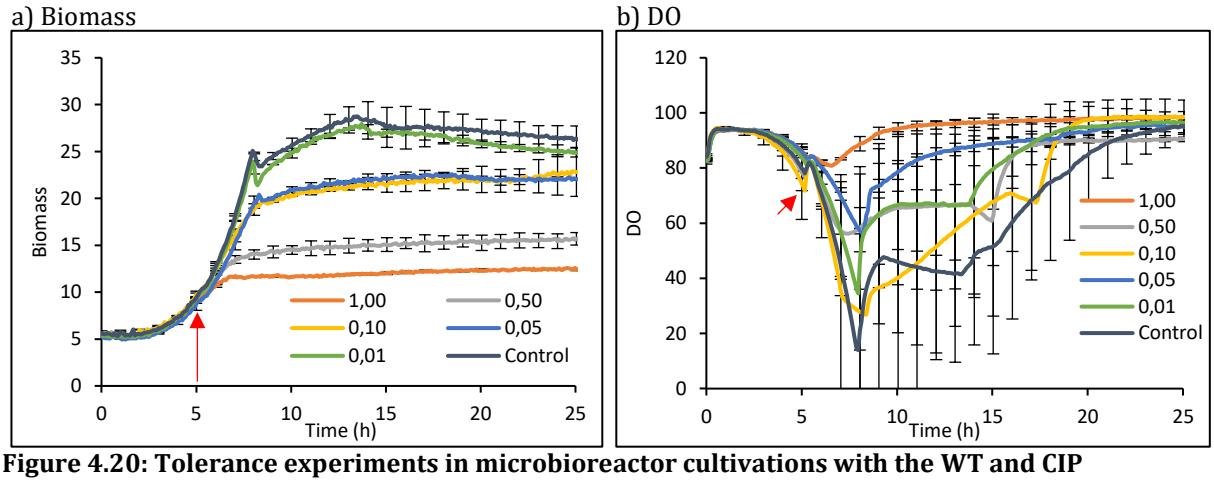


Figure 4.20: Tolerance experiments in microbioreactor cultivations with the WT and CIP
Tolerance experiment with the WT *E. coli* K12 MG1655 in M9-hbc culture media and ciprofloxacin (0,01–1 µg/ml), by measurements performed in a BioLector Pro. The curves represent a) biomass and b) DO. The red arrow illustrates the time when the antibiotics were added, while the controls were not added CIP. Each datapoint represent the average of two replicates ±standard deviation shown for each hour of the experiment.

4.3.3 ΔrpoS

Two experiments each were performed with the *E. coli* knockout strain and ampicillin (5–60 µg/ml AMP) and ciprofloxacin (CIP), while one analysis was done with streptomycin (10–50 µg/ml STR). Based on the results gained for the WT with the high-resolution tolerance experiments, a rather large concentration range was used for all the antibiotics in treatment of the Δ*rpoS* strain. This was also done because of the observation of an increased tolerance in Δ*rpoS* compared to the WT in the performed analysis in the well-plate format. The results displayed a spectre of responses which is already described for experiments with the WT, but higher doses were required of each of the antibiotics in order for an inhibitory response to occur.

In the experiments performed with the WT and AMP, concentrations above 20 µg/ml caused a significant inhibition-response (Figure 4.18). In the analysis with Δ*rpoS*, this same dose led to mixed results. In the first experiment, the bacteria were clearly affected, but to a rather low extent (Figure 4.21.a). This was also the case for one of the parallels in the second experiment, while the other showed a much more prominent effect, with a stable biomass of ~16-19 through SP (Figure 4.21.b). For this reason, and to show the variance of the results, only the more inhibited parallel is shown. The doses of 40–60 µg/ml AMP were more effective, where the latter seemed to kill the bacteria almost immediately (Figure 4.21.b, d). This is observed both from the biomass and the DO-data, where the latter show a clear distinction between samples treated with ≤20 µg/ml AMP, and those treated with ≥40 µg/ml AMP. The DO-curves show a significant response for all AMP-treatments, however with a rather large variance for the controls and 10 µg/ml-samples in the first experiment (Figure 4.21.c-d). The pH of all treated samples stabilized almost immediately after antibiotic addition in both experiments (except the least affected 20 µg/ml-parallel in exp. 2) (Figure E.3). Altogether, these results show a higher tolerance toward AMP in the Δ*rpoS* strain compared to the WT. However, further experiments should be performed to determine these qualities more accurately.

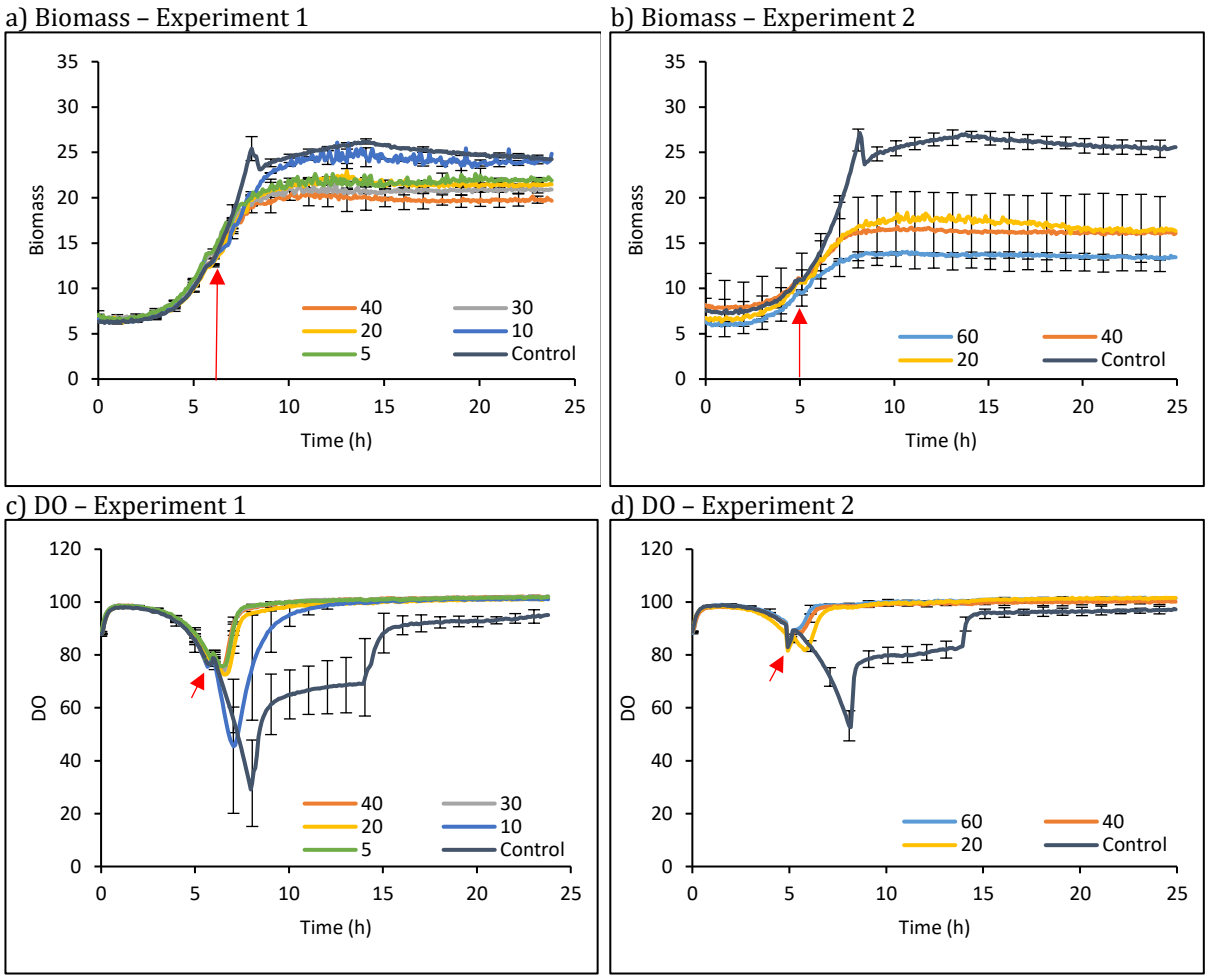


Figure 4.21: Tolerance experiments in microbioreactor cultivations with Δ*rpoS* and AMP
Tolerance experiments with the knockout *E. coli* K12 MG1655 Δ*rpoS* strain in M9-hbc culture media and ampicillin (5–60 µg/ml), by measurements performed in a BioLector Pro. The curves represent a-b) biomass and c-d) DO for two experiments. The red arrow illustrates the time when the antibiotics were added, while the controls were not added AMP. Each datapoint represent the average of two replicates ±standard deviation shown for each hour of the experiment, unless otherwise specified.

The results for Δ*rpoS* treated with streptomycin show that concentrations of 40 and 50 µg/ml STR clearly resulted in inhibition of bacterial growth, showing an abruptly slowed growth rate in the biomass curves approximately an hour after antibiotic addition (Figure 4.22.a). However, biomass levels continued to rise, but with an approximately 2-fold (32 %) reduction in cell density for the latter samples compared to the controls. The DO-curves show the responses from the bacteria in a dose-dependent manner (Figure 4.22.b), with relatively low standard error for all samples, except for the controls in the exponential phase. It seems from these results that the highest STR-doses used did not in fact kill the bacteria, as a larger large decrease in dissolved oxygen is observed even for these samples. Compared to the controls, where concentrations ≥20 µg/ml STR killed the treated bacteria, this indicates a much higher tolerance toward the antibiotic.

Experiments should be repeated with higher concentrations, to more accurately determine the dose-dependent response. The samples added 30 µg/ml STR seemed to be somewhat affected, not reaching the same level of metabolic activity as the controls, as the break in biomass due to glucose shortage is not seen. They also showed a gradual biomass increase after approximately 15 hours, above the rest of the samples. The largest effects on pH is seen for the samples treated with the three highest STR-concentrations, where the pH continued to drop to ~5,3 for the 30 µg/ml-samples and ~5,5 for the 40- and 50 µg/ml-samples (Figure E.4).

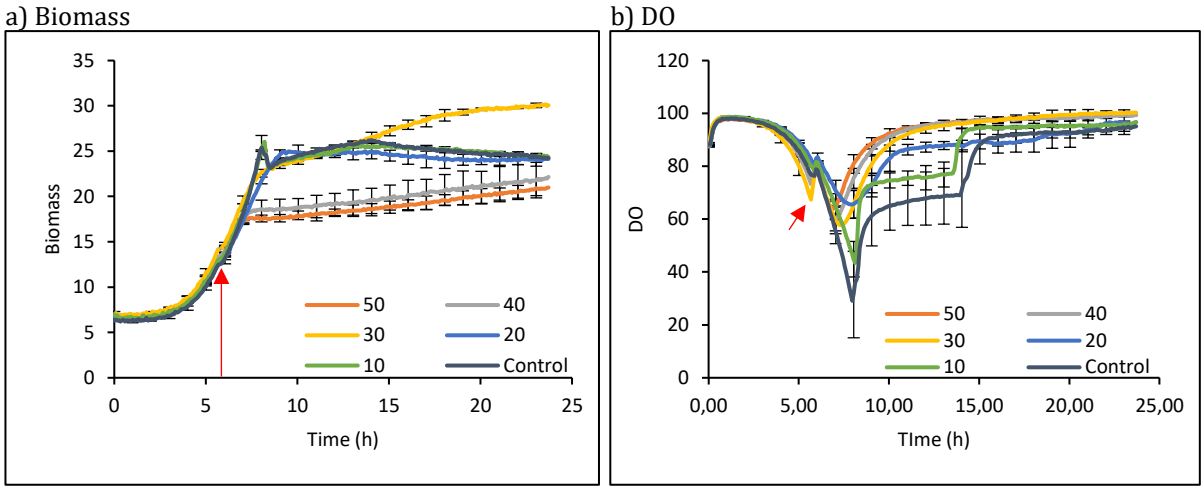


Figure 4.22: Tolerance experiments in microbioreactor cultivations with Δ*rpoS* and STR
Tolerance experiment with the knockout *E. coli* K12 MG1655 Δ*rpoS* strain in M9-hbc culture media and streptomycin (10–50 µg/ml), by measurements performed in a BioLector Pro. The curves represent a) biomass and b) DO. The red arrow illustrates the time when the antibiotics were added, while the controls were not added STR. Each datapoint represent the average of two replicates ±standard deviation shown for each hour of the experiment.

The results for Δ*rpoS* and ciprofloxacin show an inhibitory response for all the treated samples, in a clear dose-dependent manner for all three test parameters (Figures 4.23, E.5). The 5- and 10 µg/ml-doses led to an almost immediate cell death after antibiotic addition, with the biomass stabilizing at ~14 (~58 % of controls) (Figure 4.23.a-b). There is also a significant difference between the samples treated with 5 and 1 µg/ml, where the latter was the lowest concentration which clearly resulted in death in the WT in similar experiments. The DO-curves for both the WT (Figure 4.20.b) and Δ*rpoS* (4.23.c-d) show an immediate response from the samples treated with the highest CIP-doses, but the antibiotic seem to affect the biomass of Δ*rpoS* sooner. However, this could also be because of slight differences in growth rate at the time of antibiotic addition.

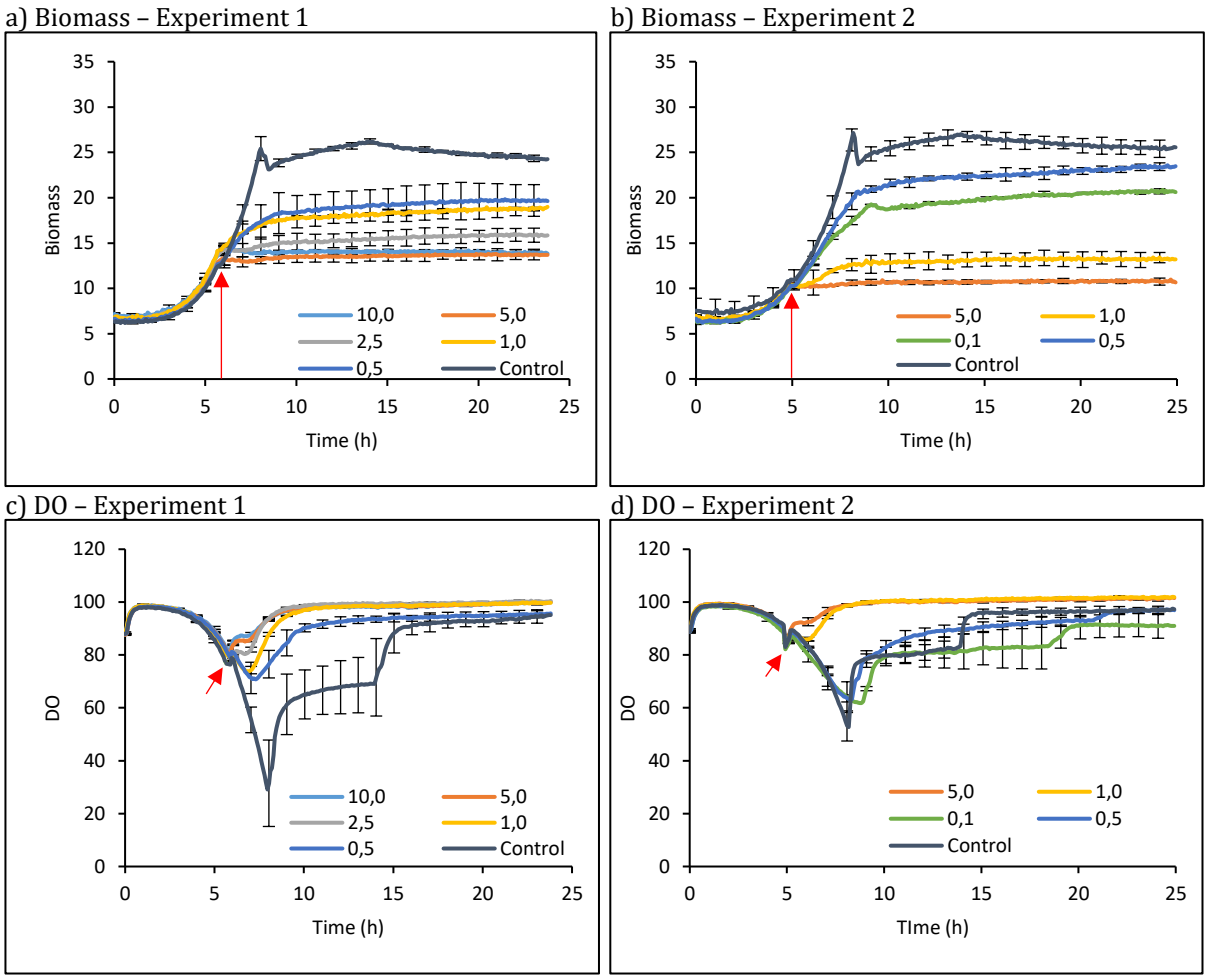


Figure 4.23: Tolerance experiments in microbioreactor cultivations ΔrpoS and CIP
Tolerance experiments with the knockout *E. coli* K12 MG1655 Δ*rpoS* strain in M9-hbc culture media and ciprofloxacin (0,1–10 µg/ml), by measurements performed in a BioLector Pro. The curves represent a-b) biomass and c-d) DO for two experiments. The red arrow illustrates the time when the antibiotics were added, while the controls were not added STR. Each datapoint represent the average of two replicates ±standard deviation shown for each hour of the experiment.

5 Discussion

5.1 MICs of *E. coli* WT for ampicillin, streptomycin and ciprofloxacin

Ciprofloxacin was the most effective antibiotic to inhibit the growth of the WT *E. coli* in my experiments, followed by ampicillin and streptomycin (Table 4.1). The determined MICs agreed relatively well with those reported by the European committee on antimicrobial susceptibility testing (EUCAST [63]), although being slightly in the lower range. EUCAST values for different *E. coli* WT strains, which is based on thousands of clinical observations, shows that MIC values for AMP varies in the range of 1-8 µg/ml, 0,008-0,125 µg/ml for CIP and 1-256 µg/ml for STR. Comparatively, MIC values for the *E. coli* K12 MG1655-WT in my study was determined to be 0,78-1,56 µg/ml for AMP (Figure 4.1), 1,25-2,50 µg/ml for STR (Figure 4.2) and 0,01-0,02 µg/ml for CIP (Figure 4.3). The EUCAST MIC values for STR had a very broad range, but according to a study by Dhanoa et al., 2015 [64], the MIC for *E. coli* K12 WT is 2 µg/ml for STR, which agrees very well with my findings.

5.2 MICs of Δ*relA* and Δ*rpoS* for ampicillin, streptomycin and ciprofloxacin, compared to WT

The determined MICs for ampicillin of the two *E. coli* knockout strains Δ*relA* and Δ*rpoS* were approximately 4-fold higher than what was found for the WT (Table 4.1), which indicates a reduced sensitivity for the antibiotic. The genes *rpoS* and *relA* have important functions in protecting bacteria from stress and nutrient deficiencies both during growth and in the stationary phase (SP), so an increased susceptibility toward AMP was expected by removing these protective mechanisms [51, 55]. This has also been shown in several studies, contrary to what was seen for my results (Figures 4.1, 4.4, 4.7). For example, in one study the Δ*rpoS* strain was shown to be about 20 times more susceptible to ampicillin than the WT strain [65]. It has also been suggested that removal of the *relA* gene damages the intrinsic defence mechanisms to antibiotic stress in the cell, and thereby its ability to grow [66]. In a study by Firminio et al., 2014 [67], a 2-times lower MIC for the Δ*relA* strain treated with AMP was found compared to the WT. Furthermore, the determined MIC for Δ*relA* treated with AMP in my study was in the same range as in

theirs (8 µg/ml, Figure 4.7). However, the MIC for the WT in my study (1,56 µg/ml < MIC < 3,13 µg/ml) was significantly lower than in theirs (16 µg/ml).

On the other hand, several studies have found higher MICs in the KO-Δ*rpoS* and -Δ*relA* strains than in the *E. coli* WT [67-70], which supports my observations (Figures 4.4–9). In these papers, the authors discussed whether the differences in growth rates and/or growth phase between the strains in their experiments could have caused the unexpected higher MICs in the mutants. Higher growth rates lead to denser biomasses in a culture, which would result in a higher diluting effect on ampicillin, thereby causing an increase in survivability. In fact, a difference in growth rate between the three strains were observed in my experiments (Figure 4.10–11). Although the three strains seem to have quite similar generation times (Table 4.3), especially the WT and Δ*rpoS*, Δ*relA* in particular displayed a tendency for a longer lag phase (Figure 4.11). The KO-strains also had lower cell densities in the ON-cultures at the onset of the MIC experiments, compared to the WT (Table 4.2). Differences in growth status between the strains in the MIC experiments in my study, could be an important factor influencing the results. While the WT was observed to have a ~10-hour lag phase (Figures 4.1-3), early exponential growth was observed for the two knockout strains within 5 hours (Figures 4.4-9). This was probably because the KOs had not reached a stable SP after 16 hours of incubation, in contrast to the WT. That is, they were most likely still growing at the onset of the MIC experiments, which would have affected the results in that they would probably have continued in their exponential growth. This is especially true for Δ*relA*, which at times was proven quite difficult to cultivate (chapter 4.2.2).

The impact of growth phases and/or growth rate for determination of MIC was also discussed in a study by Varic et al., 2016 [71], where a higher MIC with AMP was observed for Δ*relA* than for the WT. Thus, the study concluded that the loss of Δ*relA* functionality increased *E. coli* tolerance toward this antibiotic during growth resumption in fresh media. However, the results were based on experiments with rapid bacterial growth in a nutrient (aminoacidic) enriched medium and not in M9 as in my study. Altogether, their data underscore the crucial role of media composition and growth conditions for studies of antibiotic sensitivities and determination of MIC.

However, in my study, a higher MIC for the Δ*relA* and Δ*rpoS* than for the WT was found for streptomycin and ciprofloxacin, as well as for ampicillin. These differences cannot be explained by the dilution effect, since STR and CIP work by different

mechanisms than AMP. That is, AMP affects the integrity of the bacterial cells while STR and CIP interrupt protein synthesis and DNA replication respectively. Other factors must therefore have played a part in the differing responses of the three strains, probably in combination with the dilution effect of ampicillin.

One possible explanation, which hold for all three antibiotics, is related to the balance of nutrient availability and accumulation of waste products, which affects the length of the exponential growth phase [72]. Once nutrients in the medium are exhausted, the bacterial cultures enter SP. In accordance with the authors' findings, this would in my study have caused the Δ*relA* and Δ*rpoS* knockouts to have reached higher stress levels in the ON-cultures, at earlier timepoints than the WT. Thus, they would have transitioned into SP with lower biomasses, which would have affected the MIC experiments. Furthermore, studies have shown a correlation between the timing of entry into SP in ON-cultures, with growth resumption when inoculated into fresh media [73]. In periods of famine, bacteria need to slow down their metabolism until nutrients are in abundance again. The authors described that the last *E. coli* strain to do this in transitioning into SP, were the first to recover in response to new medium. It was hypothesised that bacterial cells can remember their status at the entrance into SP, which determines the timing of their regrowth in new media. The renewed availability of nutrients allows the starved bacteria to transition to exponential growth after a short lag phase, and it's possible that this happened faster in my experiments for the two knockouts due to a more serious lack of nutrients than in the WT.

5.3 Experiences from the MIC experiments and further investigations

Both the hypothesis described in chapter 5.2 needs to be examined further in new experiments in order to determine MICs more accurately. For example, since Δ*relA* and Δ*rpoS* show different growth rates than the WT in my experiments, the MICs of these strains may not be directly comparable. In fact, the values found for the KOs should not be considered as true MICs, as this would have been a factor affecting the increased antibiotic resistance observed for these strains. From the observations made, one can assume that the determination of MICs measured *in vitro* can vary greatly according to several differences in experimental conditions. My preliminary analysis indicate that

variances in compositions of culture media, number of cells at the time of inoculation, strain type, pre-treatment of the strains (i.e. freeze stock vs. ON-culture) before regrowth in fresh media as well as timing of the experiment and inoculation conditions may influence the MIC results. It was for example discussed by Brauner et al., 2016 [37] that MIC determinations has major limitations, as it is not informative of bacterial strains that are tolerant rather than resistant. Furthermore, examinations of determining conditions related to the assay should be done. For example, a comparison of the behaviour of each of the strains cultivated with different media compositions (like described in chapter 3.4.5) would be useful. It should also be considered whether an analysis of improvements could be done in cultivations in ON-cultures.

5.4 *E. coli* WT responds to sublethal concentrations of ampicillin, streptomycin and ciprofloxacin by an increased lag phase in MIC experiments

As expected, sub-MIC doses of all the antibiotics lead to an increased lag time before re-growth occurred in my studies (Figures 4.1-3). In treated samples the mentioned phase lasted for 17 or more hours, as opposed to approximately 10 hours for all the controls. According to a study by (Fridman et al., 2012, [74]), this is due to an innate tolerance capacity of bacteria, involving several defence mechanisms (described in chapter 1.9). The prolonged lag phase is way for the bacteria to gain time in upregulating their protective mechanisms before transitioning into a state of active growth. Current standard clinical assays measure MICs of an antibiotic to identify resistance, but it's equally important to test for tolerance. It's also important to avoid sub-MIC treatments, which may aide the development of tolerance.

5.5 The response of the knockout strains to sublethal concentrations of ampicillin, streptomycin and ciprofloxacin in MIC experiments

When the knockout strains were incubated with sublethal concentrations of antibiotics, the bacteria responded differently to ampicillin than to ciprofloxacin and streptomycin (Figure 4.4-9). Sub-MIC doses of AMP caused, after an initial exponential growth phase, a significant drop in biomass followed by regrowth with a slower rate than

the controls (Figure 4.4, 4.7). This is most likely due to an increase in tolerance to antibiotic treatment over time. Similar results have been observed in other studies, which claim that the first initial death of bacteria may be explained on the basis that β-lactam drugs interfere with synthesis of the cell wall. This leads to loss of structural strength in the cells and eventual lysis, which causes the drop in biomass. The impact of this inhibition also varies with the antibiotic dose, as seen in my studies (Figure 4.4, 4.7). A possible hypothesis for the regrowth observed was discussed by Mathieu et al., 2016 [75]. They saw that the genes coding for membrane synthesis were increased when bacteria were cultivated in sub-MIC doses of AMP, meaning that cells induce mechanisms to repair the induced damage to the cell wall. Adaptations in amino acid catabolism, the translation apparatus and energy metabolism were also observed, as well as the upregulation of many genes related to protection from oxidative stress. Mathieu et al., 2016 [75] concluded that exposure to sublethal doses of ampicillin had hormetic effects; i.e., treated cells acquired increased tolerance to ampicillin in agreement with the observations in my study.

Ciprofloxacin and streptomycin cause different responses in the KO strains than ampicillin. Effective doses are seen to either slow down the growth rate or kill the bacteria at different time points in the experiments (Figures 4.5-6, 4.8-9). These responses differ from those caused by ampicillin, as CIP and STR do not cause lysis, and it is not possible to separate between live and dead cells in the well plate format. However, Mathieu et al., 2016 [15] found that the adaptive stress response mechanisms, with changes in amino acid catabolism, the translation apparatus and energy metabolism due to sub-lethal treatment with ampicillin were similar for other types of antibiotics. That is, it was found to be independent of the mechanism of action of the specific antibiotics and was more of a general way for the bacteria to adjust to antibiotic stress.

5.6 Measurement of tolerance of *E. coli* strains for ampicillin, streptomycin and ciprofloxacin depends on the method of analysis

The three *E. coli* strains were shown to be somewhat tolerant toward ampicillin above their respective MICs (Figure 4.13, 4.16.a, 4.17.a). They also seem to have a higher level of tolerance in their non-growing phases. For example, all the used AMP-doses in the

well plate experiments with the WT caused an eventual decrease in biomass (Figure 4.13). This relates to the mode of action of β-lactam drugs, as tolerance is seen to develop in almost all the cells in a population, when exposed to AMP in the lag phase. This is because AMP is un-capable of killing non-growing cells, so the bacteria will have time to upregulate defence mechanisms in the lag phase and continue to do so through the exponential phase. However, when exposed at a time when the cells are dividing, this won't be the case. A possible explanation for the eventual decrease in the biomass is the balance between the number of cells and the antibiotic concentration. At the beginning of exponential phase, when the antibiotic is introduced, the cells divide faster than the rate of AMP inhibition. However, as the bacteria run out of nutrients, the cells will near the transition into SP, and the balance tips toward AMP-lysis. A threshold is seen between doses leading to the mechanism just described and those that cause immediate death. This might be caused by death in the majority of the bacteria, will tolerance develop in sub-populations (Figure 4.13).

A different kind of response is seen for streptomycin and ciprofloxacin, as these antibiotics do not lysate bacterial cells (4.16.b-c, 4.17.b-c). In my studies, the most effective doses most likely kill the bacteria at different timepoints. However, further experiments with more concentrations will have to be performed to confirm this, as well as determining a threshold between doses causing death, and those leading to the development of tolerance. The same goes for the KO strains, as insufficient concentration ranges were used in my study.

5.7 Increased tolerance of Δ*relA* and Δ*rpoS* for ampicillin, streptomycin and ciprofloxacin compared to WT, and possible mechanisms

The knockout strains Δ*relA* and Δ*rpoS* should be incapable of expressing *rpoS* and *relA* dependent genes, and are therefore expected to have lower tolerance for each of the antibiotics compared to the WT. However, the opposite was found in my thesis (Table 4.4), which reason cannot be determined reliably from my experiments, since no detailed analyses of metabolome or transcriptome were performed.

Other authors that have experienced similar results as in my study have suggested some explanations for the increased antibiotic tolerance of knockouts compared to the

WT [76]. For instance, it was speculated that downstream genes that are normally suppressed by RpoS are relieved from repression in the knockouts. This further results in an increased expression of genes that contribute to reduced susceptibility toward antibiotics, which includes genes that are normally supressed. Possible phenotypic adaptations in the Δ*rpoS* mutant may also occur; including changes in the permeability of the outer membrane and an increased expression of drug efflux pumps, in order to prevent antibiotic accumulation. Examples of the various tolerance mechanisms described in chapter 1.9, have been shown for the Δ*rpoS* strain. One study explained that a delayed susceptibility toward antibiotics is caused by upregulation of the genes coding for a ciprofloxacin efflux pump, mexAB-oprM [77, 78]. Altered susceptibility of target enzymes such as DNA gyrase and topoisomerase may be another explanation [76]. In a study by Whiteley et al., 2000 [79]an increased antibiotic tolerance in Δ*rpoS* was toward tropomycin was demonstrated, through the formation of thicker biofilms than in the WT [76]. The energy metabolism in Δ*rpoS* was also affected, as it adapts from utilising carbohydrates to the upregulation and β-oxidation of fatty acids [80]. It has also been shown that sublethal concentrations of antibiotics can induce relA independent tolerance to β-Lactams [66].

5.8 High-throughput tolerance experiments

As the BioLector was first used in our laboratory for the kinds of experiments described here, in connection with my thesis, some period of trial and error, as well as uncertainty with the method due to lack of not fully optimised protocols has to be considered. This was probably a contributing factor to the sometimes-high degree of variation in my results (Figures 4.18-23). Further analysis will have to be performed, for more accurate determination of responses toward the three antibiotics for all three strains. Furthermore, the same conclusion as was made for the MIC experiments holds here; that is, a strict and accurate protocol is needed for better reproducibility of the results. This is especially true for the precise timing of antibiotic addition, which is seen to affect the resultant response. This effect is seen for analysis done with the WT and both AMP and STR, as well as Δ*rpoS* with AMP and CIP, where the bacteria had been in the exponential phase slightly longer in the first experiments than in the second (Figure 4.18–19, 4.21, 4.23). That is, a somewhat higher biomass was observed for these experiments

upon antibiotic addition. The same seems to be the case for the WT treated with CIP, and Δ*rpoS* treated with STR, but further analysis will have to be performed to confirm (Figures 4.20, 4.22). This difference has a clear effect on the results, where earlier addition of the antibiotics causes an earlier response. To accurately define tolerance for each strain with each of the antibiotics, a more precise control of this factor will have to be considered.

The most interesting observations made from these results though, is probably the biomass versus their corresponding DO curves (Figures 4.18-23). In general, especially for the experiments where antibiotics were added later, a response in the level of dissolved oxygen was seen before a change in cell density occurred. For the most effective doses, a change in DO was seen either immediately or within approximately 40 minutes, while a significant change in biomass usually occurred after around two hours or later. The distinctions between doses marking a threshold in the transition between dominant response mechanisms is also seen clearer in the DO-data than in the biomass-data. For example, in the second experiment with the WT and ampicillin, there seems to be almost equal "distancing" between the final biomasses in SP of each of the antibiotic doses (Figure 4.18.b). However, when looking at the corresponding DO-curve, all doses including and above 10 μg/ml seem to cause almost the same effect (Figure 4.18.d). That is, there is an almost immediate increase in dissolved oxygen for all of the samples threated with these concentrations, while the DO-curve for the 4 μg/ml only differs slightly from that of the controls. This could indicate that the level of oxygen uptake by the cells is one of the first mechanisms affected by the presence of antibiotics, which may lead to further inhibition of the metabolism machinery in the bacteria, which eventually leads to a decrease in growth rate.

6 Conclusion

The *E. coli* K12 MG1655 WT used in this study seemed to be slightly on the sensitive side, as MICs in the lower range was found compared to corresponding values given by EUCAST (Table 5.1). However, a higher antibiotic tolerance was found in the knockout strains Δ*rpoS* and Δ*relA* compared to the WT, contrary to what was expected. This was especially true for Δ*rpoS*. Ciprofloxacin, a fluoroquinolone, was found to be the overall most effective antibiotic used in this study for all three strains, followed by ampicillin and streptomycin. Tolerance responses was also shown to greatly depend on the bacteria's growth status when the antibiotics were added, which was especially evident in the microbioreactor cultivations. Another interesting observation made in these experiments was the generally faster effect on dissolved oxygen, before a change in biomass levels occurred. Further research using strict protocols will have to be performed in order to gain more accuracy and reproducibility for the experiments.

Table 5.1: Overview of MIC and tolerance results
MICs are given in concentration ranges, as some variation usually will occur. Further experiments will have to be performed to determine more accurately which doses of each antibiotic causes a significant tolerance response (s. r.) in each of the three *E. coli* strains, as well as the related mechanisms.

E. coli K12 MG1655 strain		**Ampicillin**	**Streptomycin**	**Ciprofloxacin**
WT	MIC	1,56 < MIC < 3,13	1,25 < MIC < 2,5	~ 0,01
	Well-plate exp. (s.r.)	~ 5 µg/ml	~ 10 µg/ml	~ 0,5 µg/ml
	Well-plate exp. (death)	≥ 6 µg/ml	≥ 15 µg/ml	≥ 1 µg/ml
	High-throughput exp. (s.r)	~ 4 µg/ml	~ 15 µg/ml	~ 0,05 µg/ml
	High-throughput exp. (death)	≥ 20 µg/ml	≥ 20 µg/ml	≥ 1,0 µg/ml
Δ*rpoS*	MIC	4 < MIC < 8	4,5 < MIC < 5	0,08 < MIC < 0,16
	Well-plate exp. (s.r.)	Inconclusive	Inconclusive	Inconclusive
	Well-plate exp. (death)	> 8 µg/ml	> 25 µg/ml	> 3 µg/ml
	High-throughput exp. (s.r)	20 – 40 µg/ml	~ 40 µg/ml	~ 0,5 µg/ml
	High-throughput exp. (death)	≥ 60 µg/ml	Inconclusive	≥ 1,0 µg/ml
Δ*relA*	MIC	4 < MIC < 8	3,5 < MIC < 4	0,04 < MIC < 0,08
	Well-plate exp. (s.r.)	≥ 4 µg/ml	≥ 16 µg/ml	≥ 1,5 µg/ml
	Well-plate exp. (death)	Inconclusive	Inconclusive	Inconclusive

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Appendix A: Additional Figures – MIC

WT

a) Experiment #3

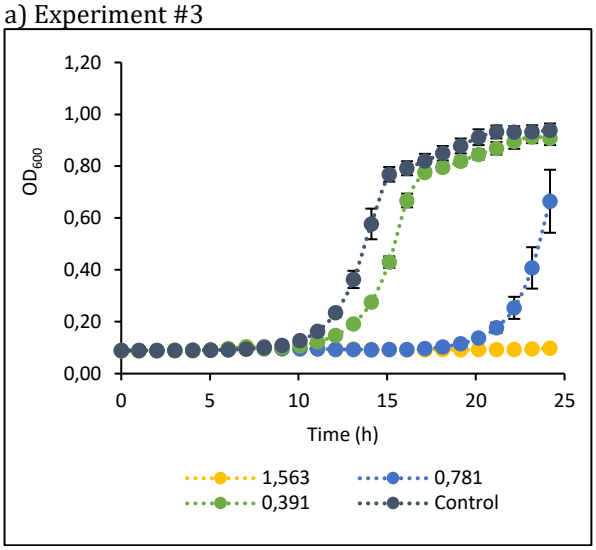


Figure A.1: MIC with the WT and AMP
– Corresponding to Figure 4.1

b) Experiment #3

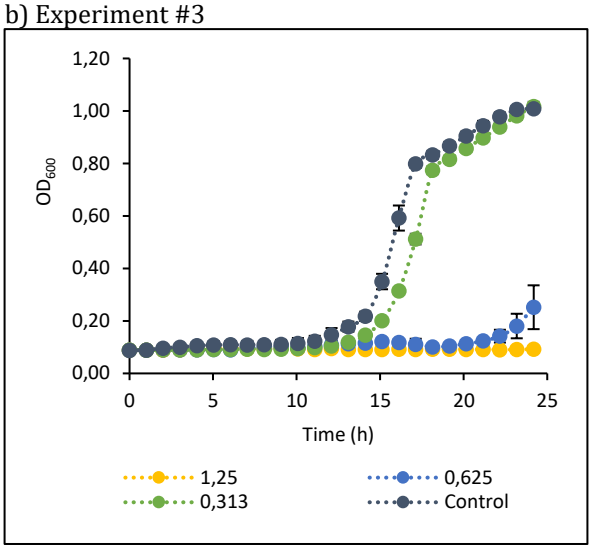
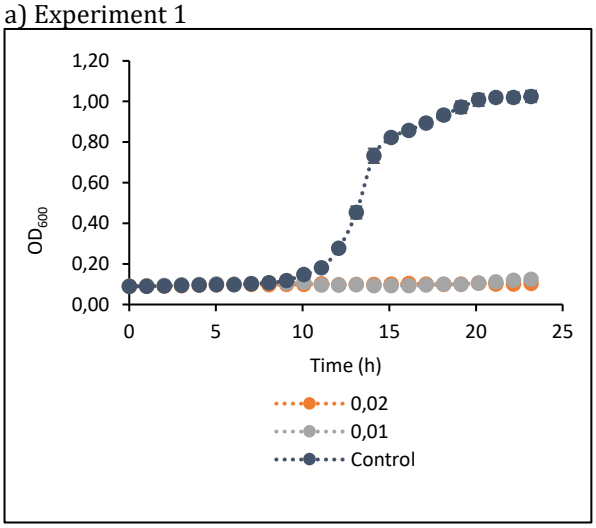


Figure A.2: MIC with the WT and STR
– Corresponding to Figure 4.2

a) Experiment 1



b) Experiment 2

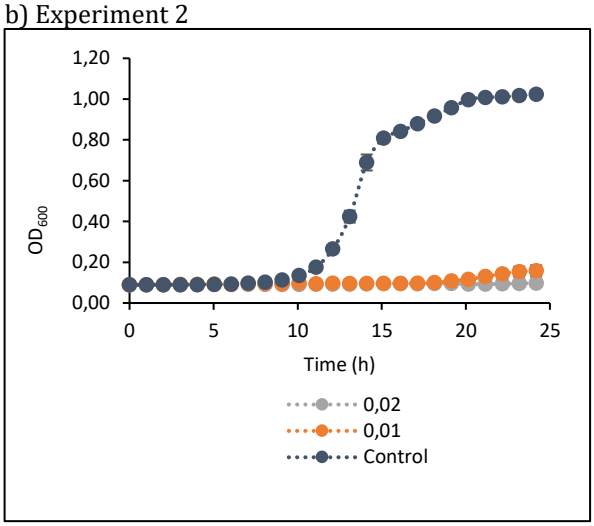
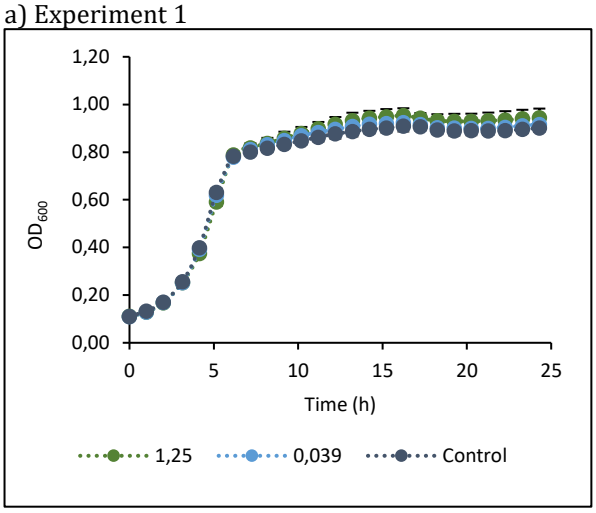


Figure A.3: MIC with the WT and CIP
– Corresponding to Figure 4.3

ΔrpoS

a) Experiment 1



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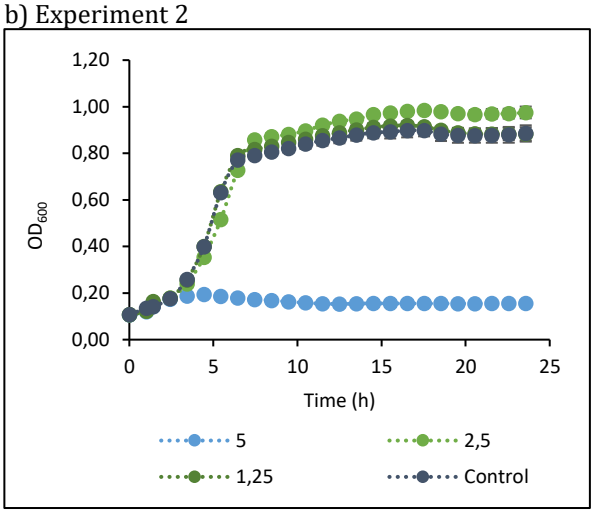


Figure A.4: MIC with Δ*rpoS* and STR
– Corresponding to Figure 4.5

a) Experiment 3

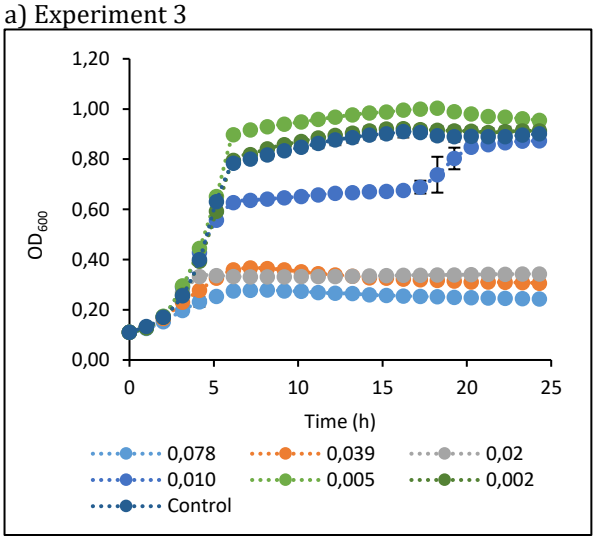


Figure A.5: MIC with Δ*rpoS* and CIP
– Corresponding to Figure 4.6

ΔrelA

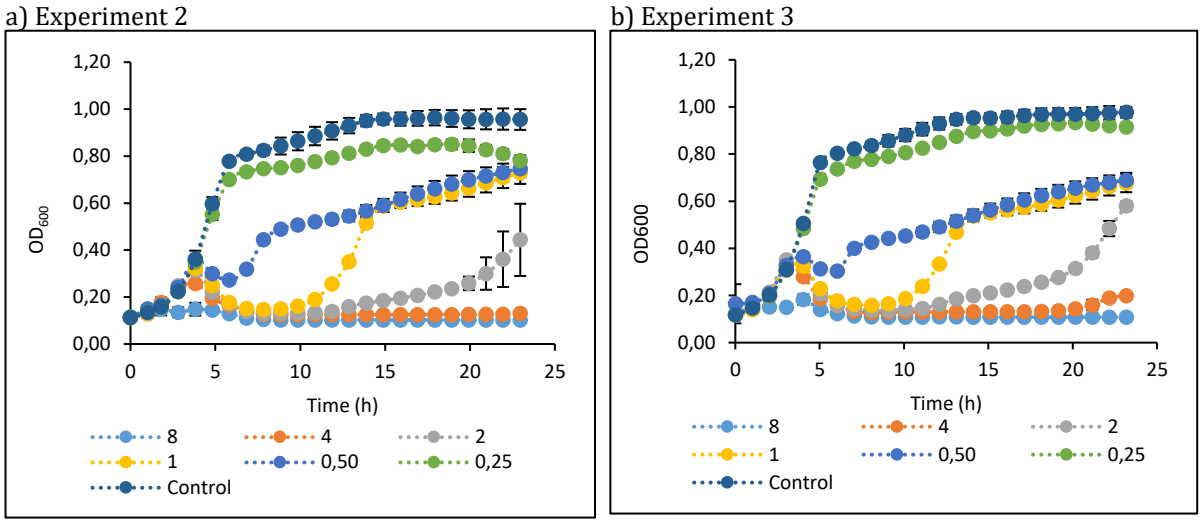


Figure A.6: MIC with Δ*relA* and AMP
– Corresponding to Figure 4.7

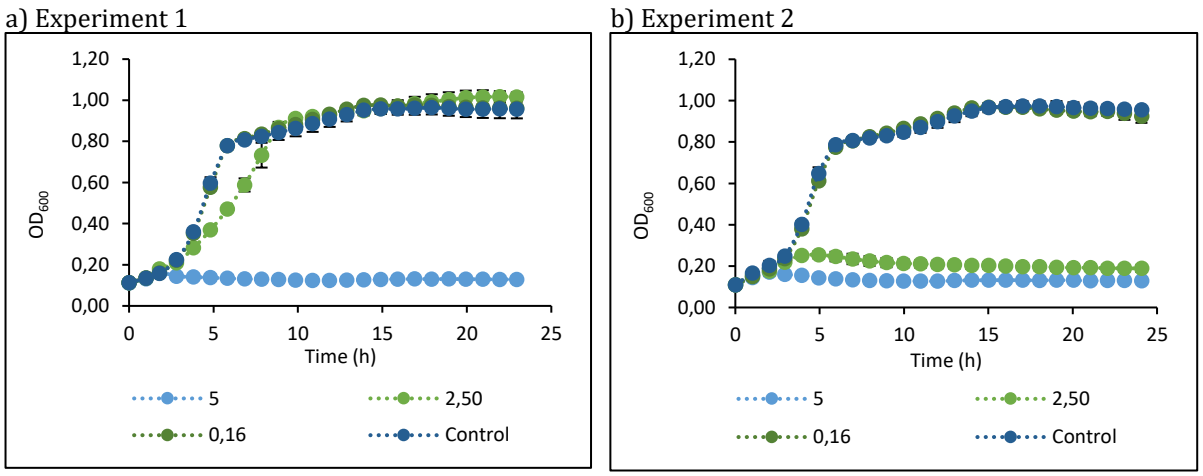


Figure A.7: MIC with Δ*relA* and STR
– Corresponding to Figure 4.8

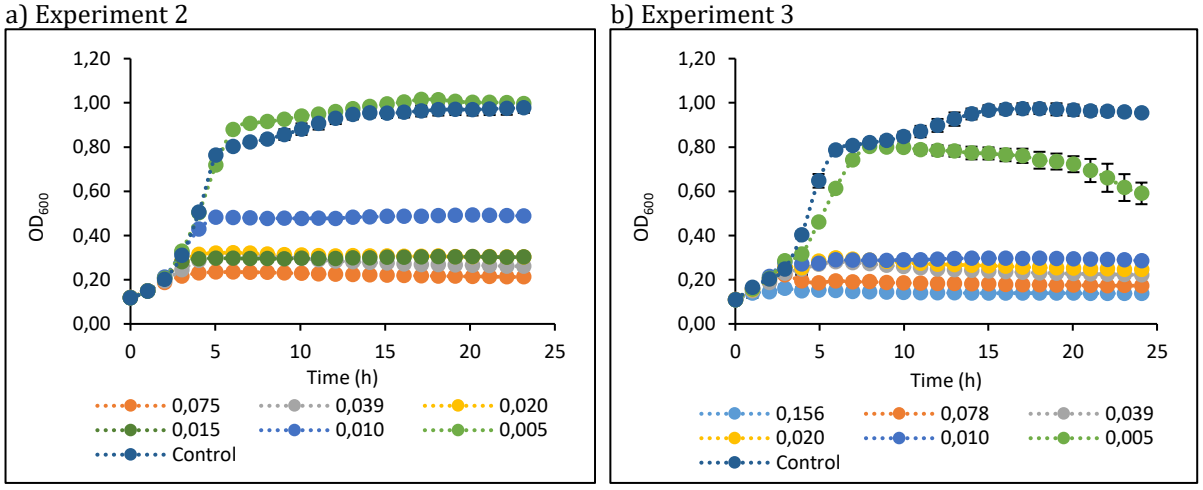


Figure A.8: MIC with Δ*relA* and CIP
– Corresponding to Figure 4.9

Appendix B: Correlation plot – spectrophotometer vs TPR

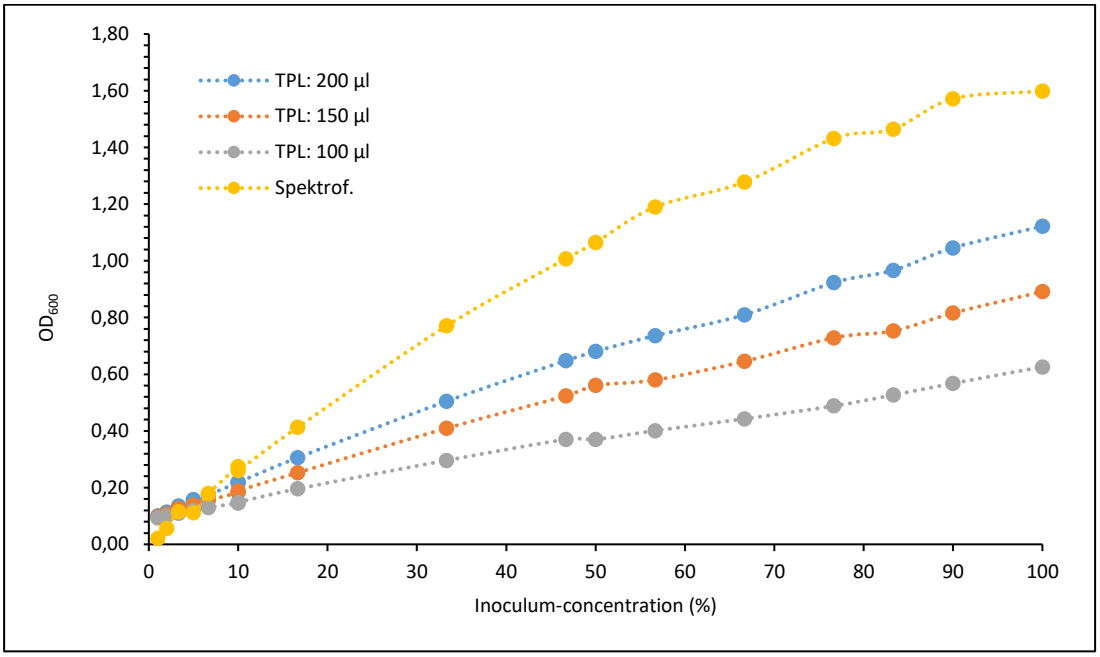


Figure B.1: Correlation Plot
The OD_{600} was measured for a range of inoculum-concentrations in 1 ml cuvettes in the spectrophotometer, as well as three different volumes in 96-well plates in the Tecan plate reader.

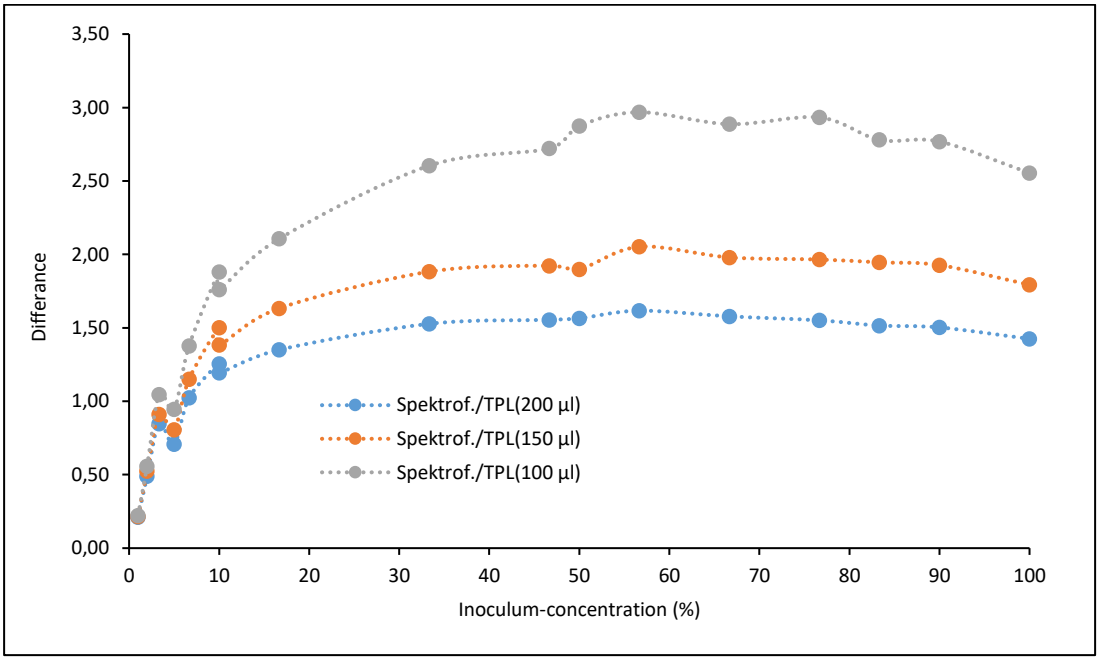


Figure B.2: Correlation Plot
This plot is based on the measurements displayed in Figure B.1.

Appendix C: Additional Figures – Tolerance experiments with the WT in the well-plate format

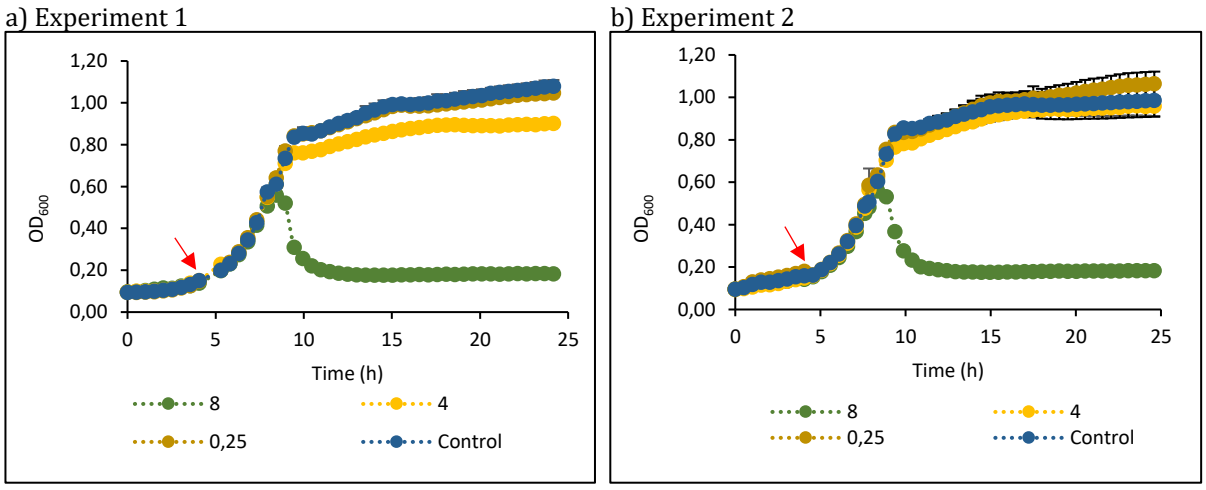


Figure C.1: Tolerance exp. with the WT and AMP – Corresponding to Figure 4.13

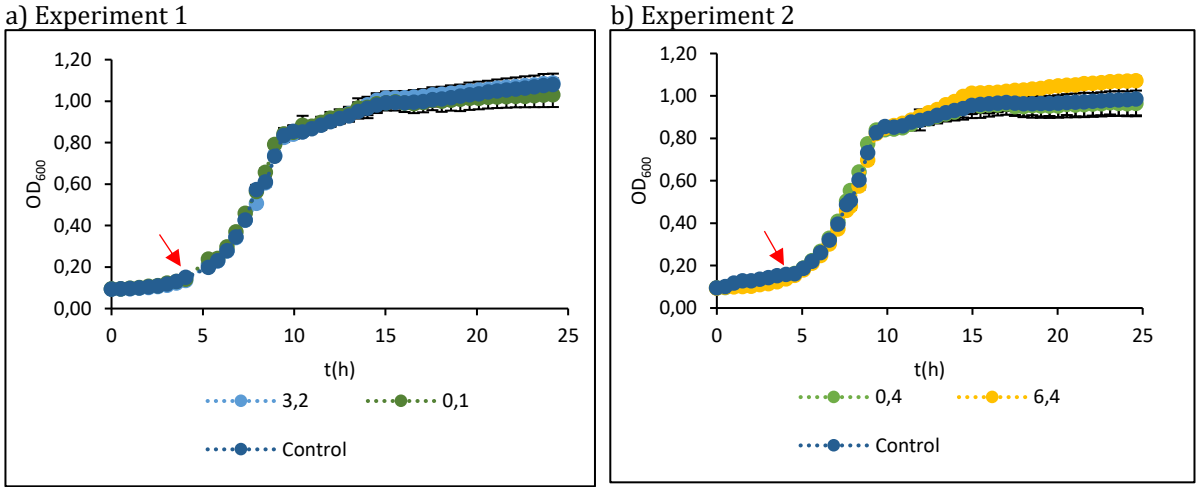


Figure C.2: Tolerance exp. with the WT and STR – Corresponding to Figure 4.14

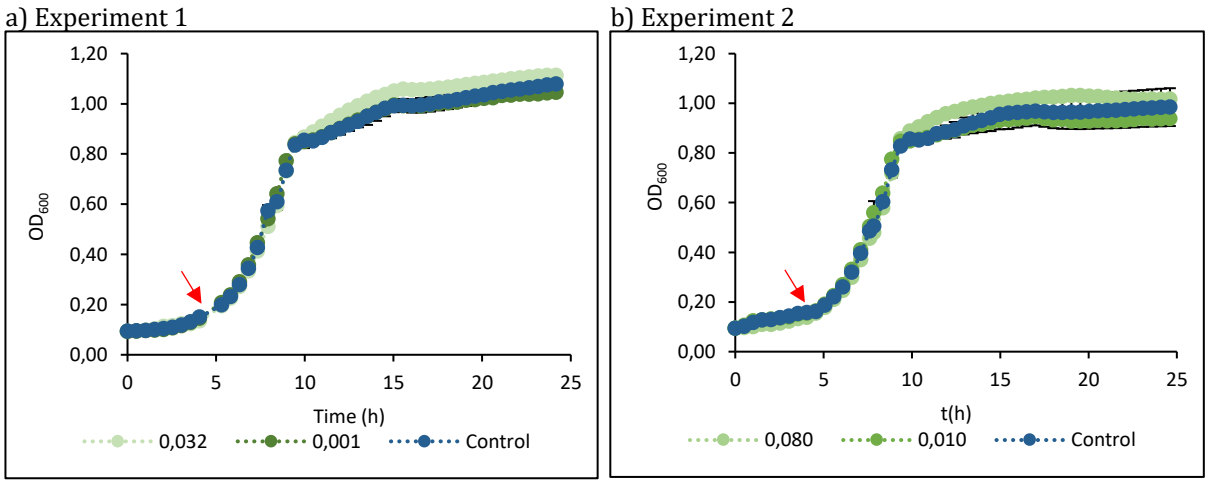


Figure C.3: Tolerance exp. with the WT and CIP – Corresponding to Figure 4.15

Appendix D: Initial experiments performed with WT *E. coli* in M9 culture media in Microbioreactor Cultivations

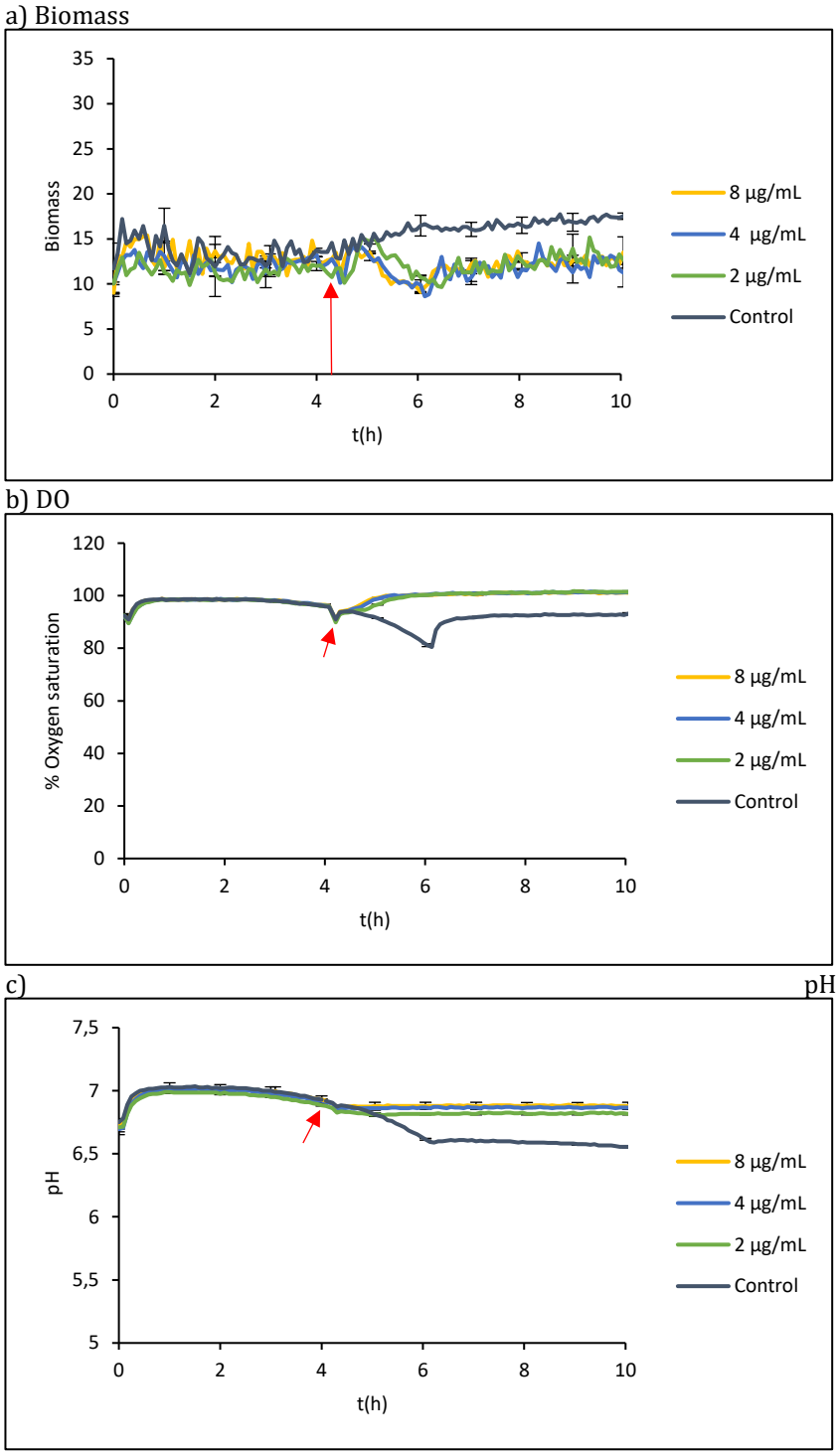
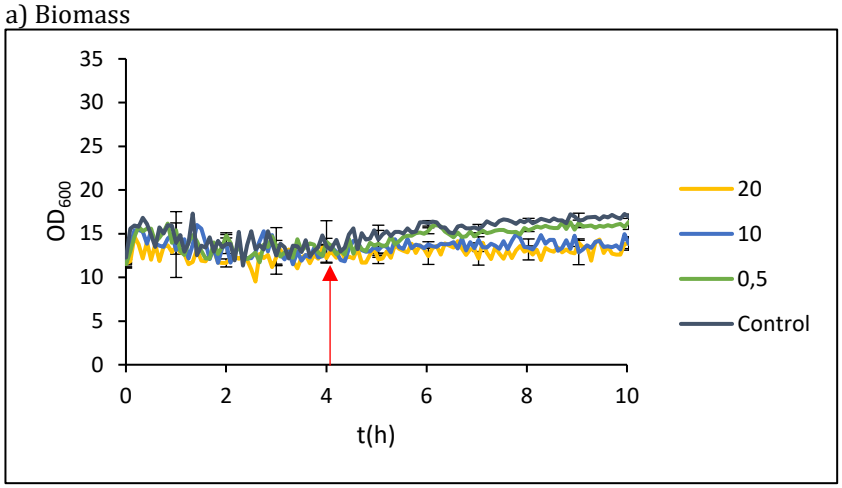
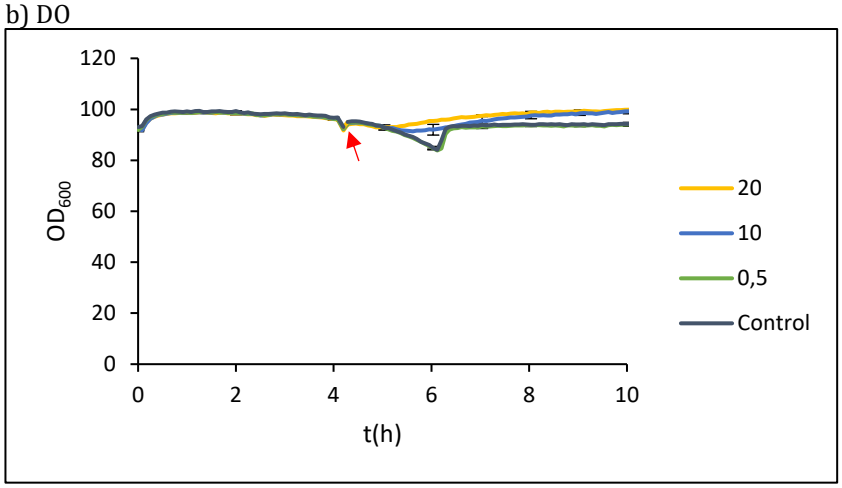


Figure D.1: Microbioreactor cultivations with the WT in M9 media, treated with AMP
Tolerance experiments with *E. coli* K12 MG1655 wild type and ampicillin treatments of 2, 4 and 8 µg/ml and a control group not given ampicillin. Red mark illustrates when the antibiotic was added in the middle of the exponential growth phase. The graphs represent a) biomass b) DO and c) pH. Each datapoint represent the average of three replicates ±standard deviation shown for each hour.

a) Biomass



b) DO



c) pH

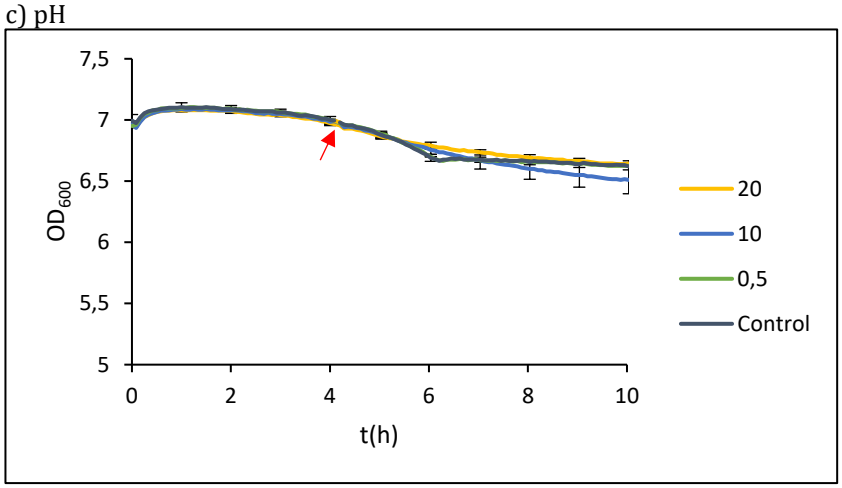
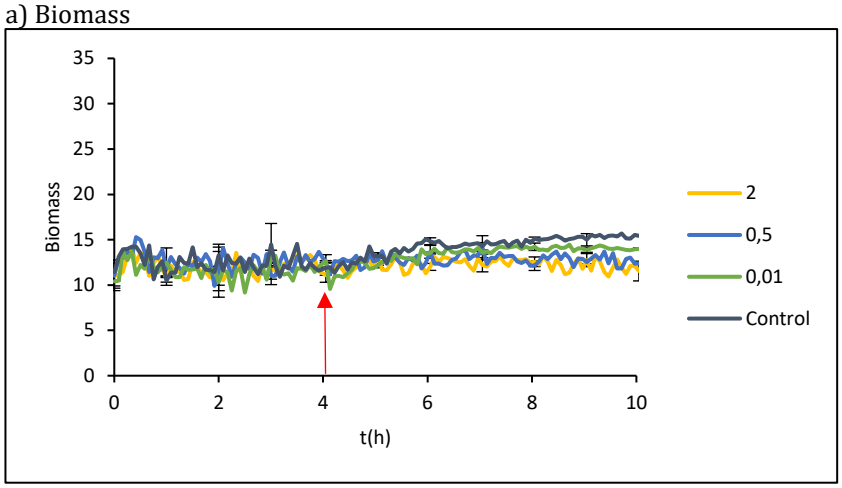
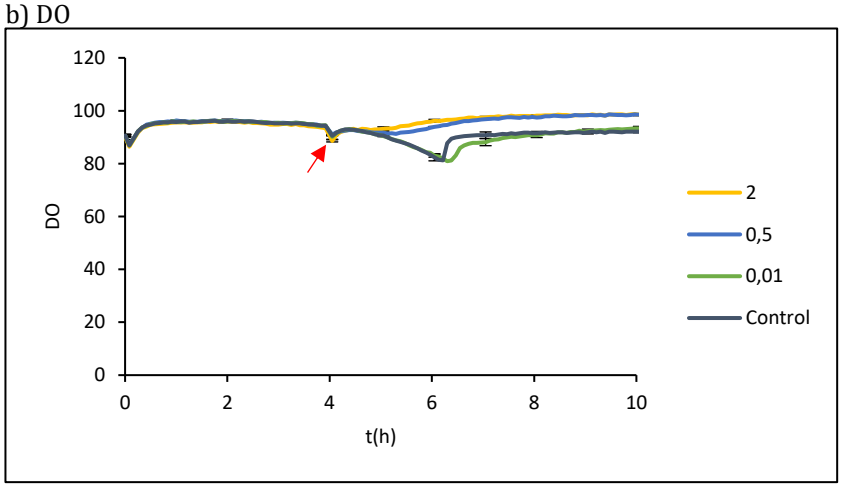


Figure D.2: Microbioreactor cultivations with the WT in M9 media, treated with STR
Tolerance experiments with the *E. coli* K12 MG1655 WT and streptomycin treatments of 0,5, 10 and 20 µg/ml and a control group not given streptomycin. The Red mark illustrates when the antibiotic was added in the middle of the exponential growth phase. The graphs represent a) biomass b) DO and c) pH. Each datapoint represent the average of three replicates ±standard deviation shown for each hour.

a) Biomass



b) DO



c) pH

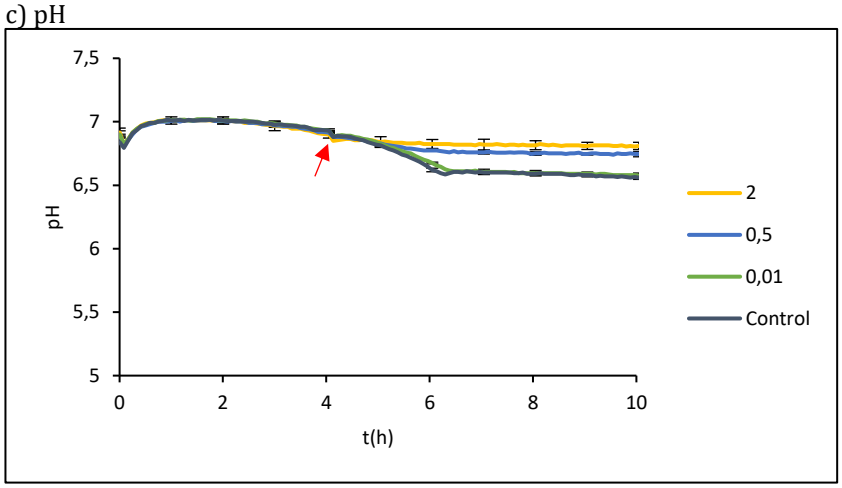


Figure D.3: Microbioreactor cultivations with the WT in M9 media, treated with CIP
Tolerance experiments with *E. coli* K12 MG1655 WT and ampicillin treatments of 2, 4 and 8 µg/ml and a control group not given CIP. The red mark illustrates when the antibiotic was added in the middle of the exponential growth phase. The graphs represent a) biomass b) DO and c) pH. Each datapoint represent the average of three replicates ±standard deviation shown for each hour.

Appendix E: pH-graphs from microbioreactor cultivations

WT

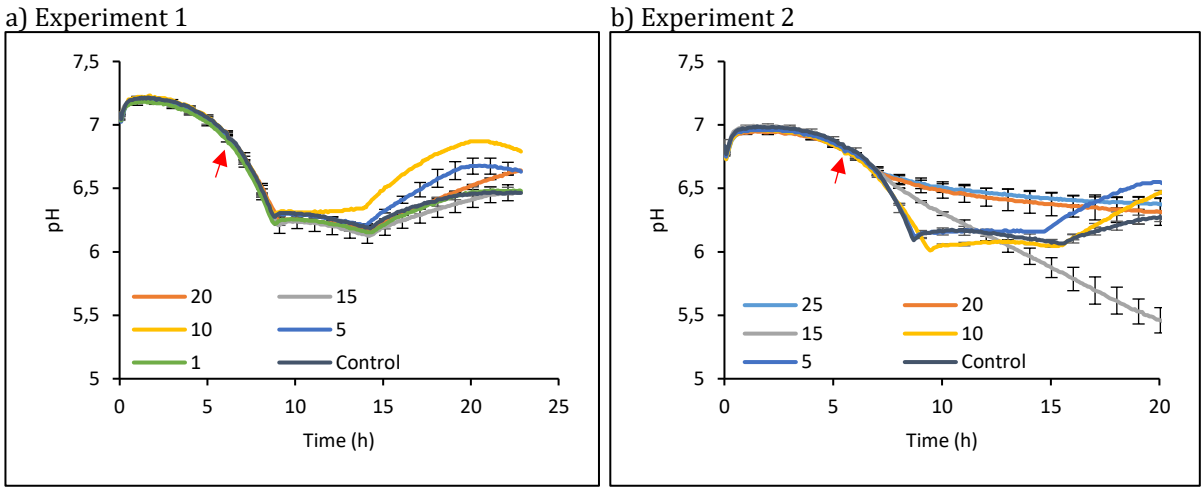


Figure E.1: Tolerance experiments in microbioreactor cultivations with the WT treated with STR – Corresponding to Figure 4.19

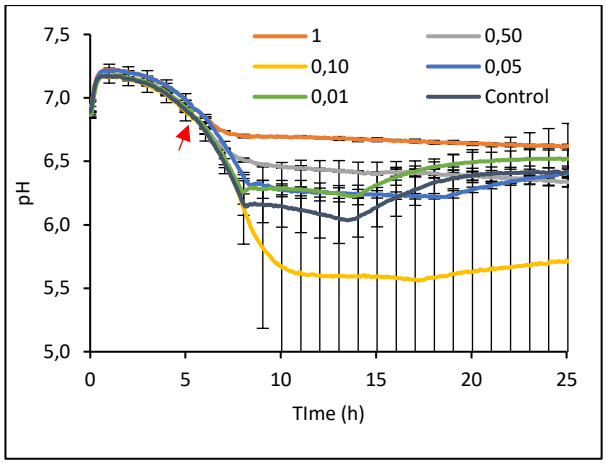
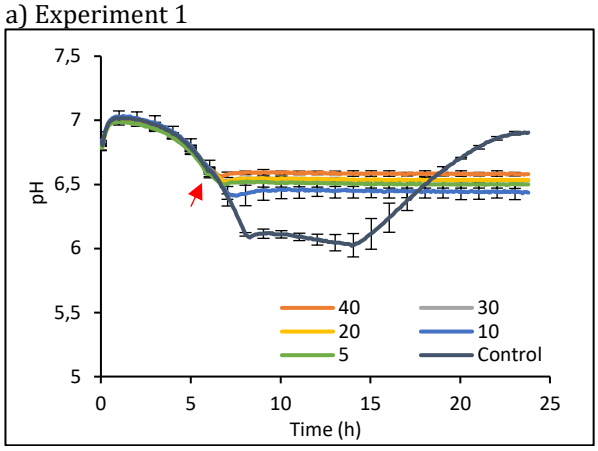


Figure E.2: Tolerance experiments in microbioreactor cultivations with the WT treated with CIP – Corresponding to Figure 4.20

ΔrpoS

a) Experiment 1



b) Experiment 2

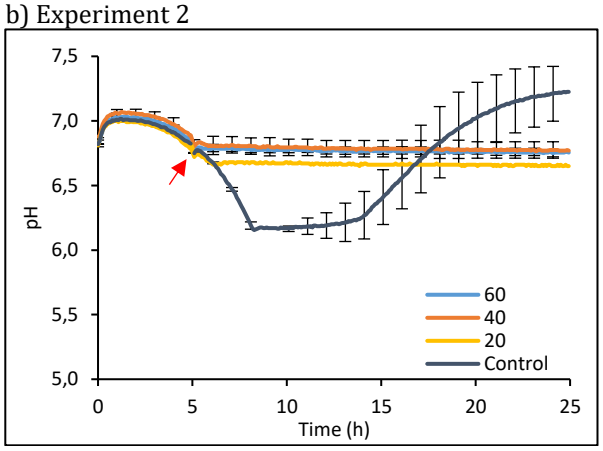


Figure E.3: pH in tolerance experiments in microbioreactor cultivations with Δ*rpoS* treated with AMP

– Corresponding to Figure 4.21

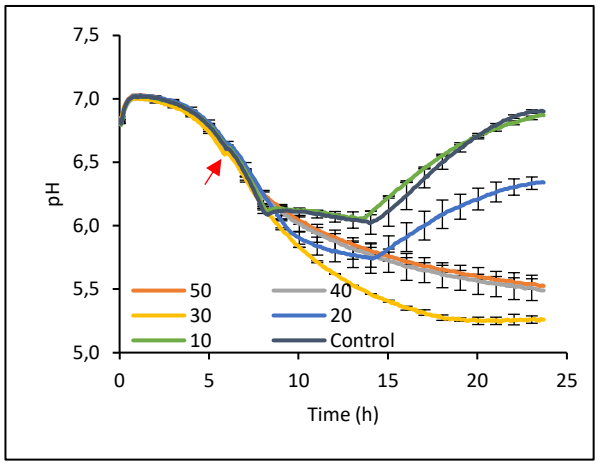
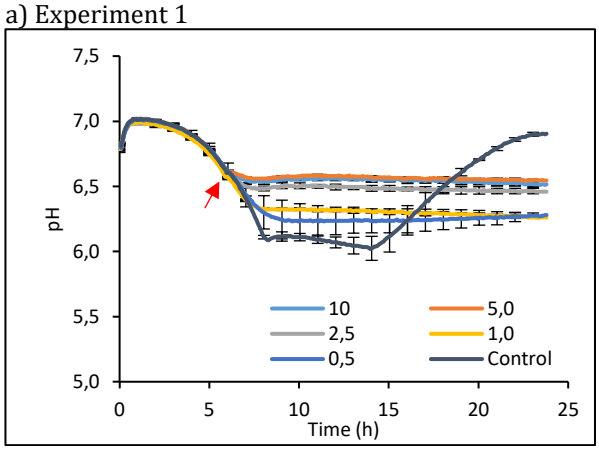


Figure E.4: pH in tolerance experiments in microbioreactor cultivations with Δ*rpoS* treated with STR

– Corresponding to Figure 4.22

a) Experiment 1



b) Experiment 2

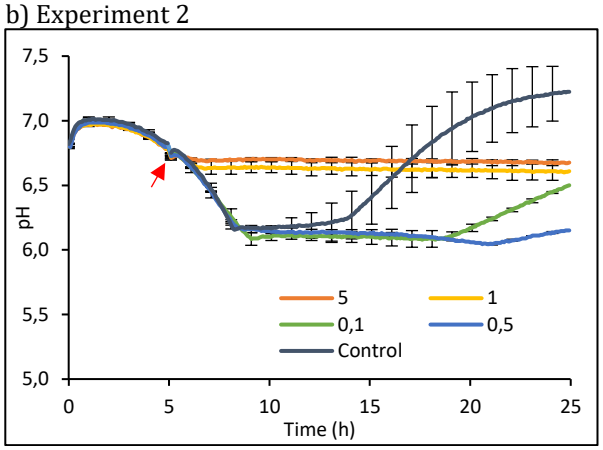
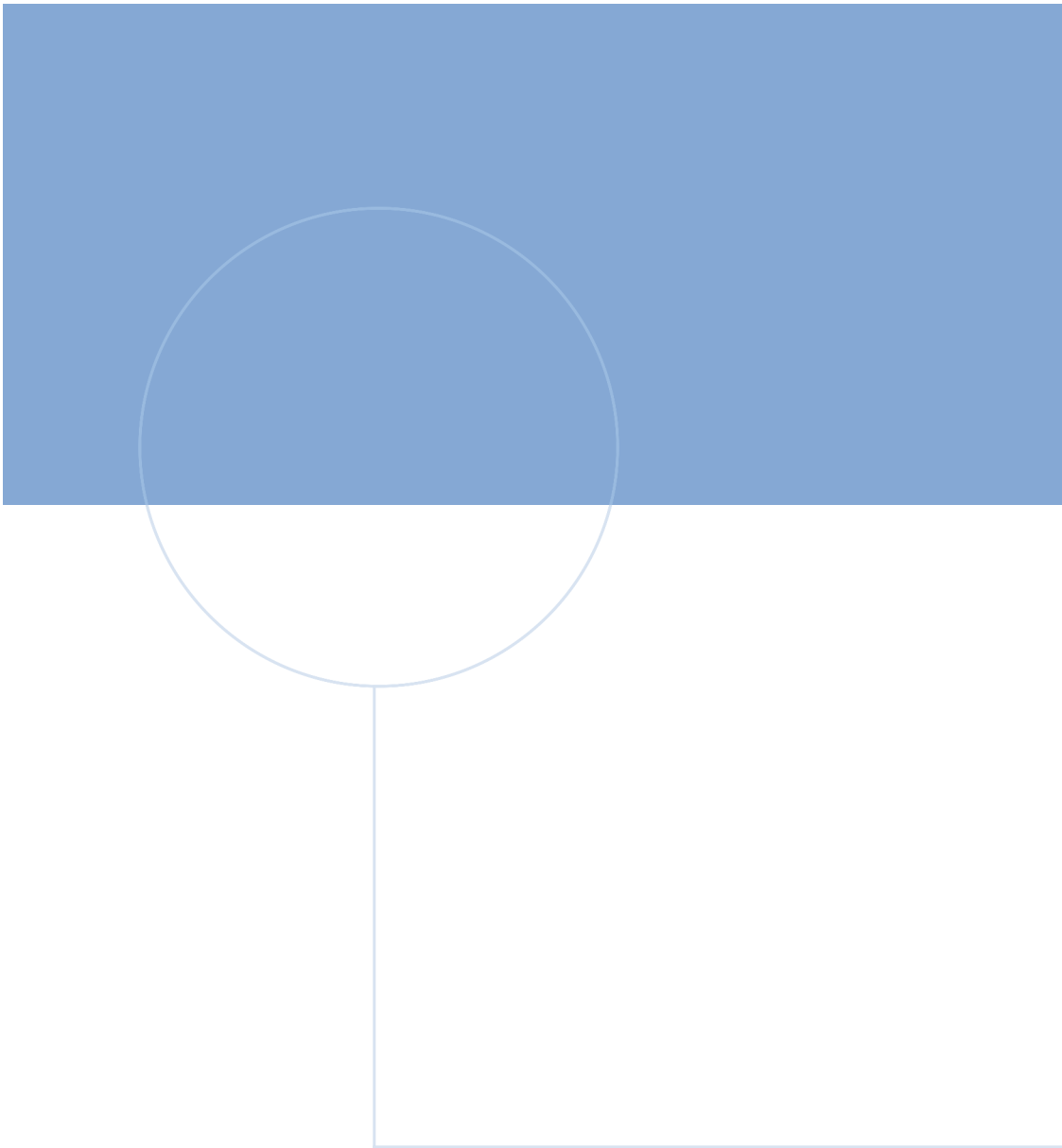


Figure E.5: pH in tolerance experiments in microbioreactor cultivations with Δ*rpoS* treated with CIP

– Corresponding to Figure 4.23



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