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# *Escherichia coli* biofilm resistance to β-lactam antibiotics

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# Declaration of Authorhip

I Elli Amanatidou hereby declare that this thesis and the work presented in it is entirely my own. Where I have consulted the work of others, this is always clearly stated.

Signed:

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# Abstract

Biofilms have been implicated in recurring nosocomial infections, often associated with high mortality rates. Crucially, they have also been shown to be highly tolerant to commonly used antibiotics. In this work I investigated how the biofilm lifestyle protected bacterial cells from antibiotics. I engineered an environmental *Escherichia coli* isolate to express a clinically relevant $bla_{\text{CTX-M-14}}$ gene which confers resistance to β-lactam antibiotics, including third generation cephalosporins. β-lactamases are considered to be potential social traits, i.e. public goods whose advantages are shared between bacterial cells. I used the resulting experimental system to test three central hypotheses: (a) social interactions between bacterial strains are enhanced in biofilms compared to planktonic cultures, and cooperation is facilitated when strain segregation is permitted, (b) the biofilm structure, including the matrix and cell localisation, affects biofilm tolerance & (c) the physiological state of bacteria in biofilms influences antibiotic tolerance.

I found that biofilms facilitate cheating but the dynamics of this behaviour did not always agree with theoretical work on social evolution; in particular, cooperative cells did not benefit from high frequencies as was expected. Additionally, I used Optical Coherence tomography (OCT) and fluorescence microscopy to investigate the colony biofilm structure and strain localisation within the biofilms, and observed distinctive responses to antibiotic treatment. This was the first time colony biofilms were observed it their entity using microscopy. Finally, I concluded that the metabolic rates of cells did affect antibiotic tolerance but a straightforward correlation between metabolic rates and growth rates could not be established. Taken together, my results suggest that both the sociality of β-lactamase expression and the intrinsic tolerance associated with the biofilm lifestyle may be significant factors in *E. coli* biofilm resistance to β-lactam antibiotics. This has implications for the development of antimicrobial strategies against Gram-negative bacteria.

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"Houston we've had a problem", no more.

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# Acronyms

**AHVLA** Animal Health and Veterinary Laboratories Agency

**AIC** Akaike Information Criterion

**ANOVA** Analysis Of Variance

**ATP** Adenosine triphosphate

**CAT** Chloramphenicol resistance gene

**CFU** Colony Forming Units

**CLSM** Confocal Laser Scanning Microscopy

**CV** Crystal Violet

**DF** Dillusion Factor

**EDTA** Ethylenediaminetetraacetic Acid

**EGFP** Enhanced Green Fluorescent Protein

**EPS** Extracellular Polymeric Substances

**ESBL** Extended Spectrum Beta Lactamases

**GFP** Green Fluorescent Protein

**HF** High Fidelity

**HR** High Resistant

**IPTG** Isopropyl-beta-D-thiogalactopyranoside

**LB** Lysogeny Broth

**LR** Low Resistant

**MDK** Minimum Duration of Killing

**MIC** Minimum Inhibition Concentration

**MRSA** Methicillin Resistant Staphyloccoccus aureus

**NA** Numerical Aperture

**NHS** National Health Service

**OCT** Optical Coherence Tomography

**OD** Optical Density

**PBS** Phosphate Buffered Saline

**PCR** Polymerase Chain Reaction

**PMT** Photomultiplier

**QS** Quorum Sensing

**RI** Refractive Index

**SE** Standard Error

**TEM** Transmission Electron Microscope

**UK** United Kingdom

**UV** Ultraviolet

**WT** Wild Type

# Chapter 1

# Introduction

Increasing bacterial antibiotic resistance has become a great concern in the last decade. A recent report from the United States [CDC, 2013] estimates that more than two million Americans are infected by antibiotic resistant strains every year, 23,000 of whom die. Increased resistance has been reported in nosocomial and community settings [Bean et al., 2008]. Some of the most important threats include the multi β-lactam resistant *Enterobacteriaceae*, such as *Escherichia coli* and *Klebsiella spp*; the number of deaths attributed to this family of bacteria is 1700 per year [CDC, 2013]. According to a European wide survey on healthcare-associated infections in hospitals, a third of in hospital environments were reported to be resistant to third generation cephalosporins with *Klebsiella pneumoniae* and *E. coli* showing the highest resistance levels [ECDC, 2013]. Other resistant *Enterobacteriaceae* reported in this study included *Enterobacter spp* and *Proteus spp* but levels of resistance did not exceed 5% in those cases. An increase in the resistance of *Escherichia coli* to β-lactam antibiotics [HPA, 2007] and to third generation cephalosporins more specifically [WHO, 2014] have also been reported. One of the main reasons for the increased antibiotic resistance is considered to be the overuse of antibiotics [ECDC, 2010]. According to the UK's NHS data, β-lactams are the most commonly prescribed antibiotics [NHS, 2010]. A

case study of treating necrotizing fasciitis (flesh-eating disease) caused by *Enterobacteriaceae*, carrying the $bla_{\text{CTX-M-15}}$ gene, highlights the difficulty and dangers involved in the spread of antibiotic resistance seen worldwide [Soleimanian et al., 2011].

Bacteria often develop or acquire tools that confer inherited antibiotic resistance, i.e. resistance mechanisms that can be passed on to daughter cells. Spontaneous mutations subsequently selected for (vertical evolution), or horizontal gene transfer (transformation, conjugation & transduction) are the main mechanisms of such resistance [Tenover, 2006]. Genes acquired through horizontal transfer may also mutate, thus enriching the arsenal of antibiotic resistance tools. The spread of the genes coding for Extended Spectrum Beta Lactamases (ESBLs) is a good example of this latter mechanism [Woodford & Ellington, 2007]. ESBLs comprise of β-lactamases produced by any of the *TEM* [Perilli et al., 2002], *SHV* [Kliebe et al., 1985] and CTX-M [Cottell et al., 2011] genes and they are capable of inactivating penicillins and third generation cephalosporins. Of the ESBL genes, CTX-M is the one that has become of increasing concern over the last few decades as its variants have been isolated more frequently from patients in hospitals and in community settings, especially in Europe [Cantón & Coque, 2006; Livermore et al., 2007; Woodford et al., 2004]. It is believed that the CTX-M genes were mobilised from the chromosome of *Kluyvera spp* on plasmids and then spread to other species via horizontal gene transfer [Humeniuk et al., 2002]. At the same time, mutations extended the spectrum of CTX-M genes further [Hall & Knowles, 1976; Kliebe et al., 1985] as multiple variants of these genes have been identified [Cantón & Coque, 2006]. An additional resistance mechanism is believed to be the appearance of phenotypic variants due to epigenetic inherited changes of gene expression [Adam et al., 2008], including drug indifference and persistence. "Drug indifference" refers to whole bacterial populations that are tolerant to antibiotics, whereas "persisters" is a term attributed to the proportion of cells that exhibit tolerance to antibiotics in a given population [Brauner et al., 2016].

Another phenotypic mechanism of antibiotic resistance and the focus of this

thesis, is biofilm formation. Biofilms are complex multicellular bacterial structures, embedded in a self-produced extracellular matrix [Costerton, 1999; Flemming et al., 2007]. A biofilm infection manifests itself by reoccurrence after antibiotic treatment [Davey & O'Toole, 2000] and the onset of the symptoms might begin months or even years after infection [Fux et al., 2003]. Generally, biofilms have been reported to be more resistant to antibiotics compared to their planktonic counterparts (see section 1.2). In fact, biofilm antibiotic tolerance can be up to 1000 fold higher than the planktonic cells they originate from [Ceri et al., 1999; Nickel et al., 1985]. As biofilm infections need to be treated repeatedly and they often require the removal of any infected foreign bodies (e.g valves, implants), they contribute significantly to hospitalisation costs [Römling & Balsalobre, 2012].

Biofilms are commonly formed by pathogenic bacteria; indeed it is believed that more than 60% of nosocomial infections in the developed world are due to biofilm formation [Davey & O'Toole, 2000; Fux et al., 2003]. They have been implicated in dental diseases and infected medical transplants [Wilson, 2001]. Biofilms are the leading cause of endocarditis, a disease that has a 70% mortality rate [Davey & O'Toole, 2000]. Additionally, *Pseudomonas aeruginosa* biofilms found in the lungs of patients suffering with cystic fibrosis have been associated with health deterioration and death [Emerson et al., 2002; Gibson et al., 2003; Schaedel et al., 2002]. More specifically, *Pseudomonas* lung infection of such patients results in the destruction of lung tissue, the subsequent inadequate respiration and ultimately death [Frederiksen et al., 1999]. *E. coli* biofilms are commonly implicated in urinary tract infections in women [Soto et al., 2006].

In the subsequent paragraphs I will summarise the unique characteristics associated with the biofilm lifestyle and how they may be implicated in antibiotic resistance. A statement of the main aims of this work will complete this chapter.

## 1.1 The biofilm lifestyle

The typical biofilm definition includes the bacterial structure with microcolonies and water channels embedded in a cell-produced extracellular matrix and attached to a surface [de Beer & Stoodley, 1995; Flemming et al., 2007; Maeyama et al., 2004; Stewart et al., 2009]. However, attachment is not necessary for a bacterial aggregate to be called a biofilm [Flemming & Wingender, 2010] and not all biofilms are so complex; many are simple, flat and compact [Schuster & Markx, 2013]. Finally, biofilms can be also formed by fungi and algae [Palmer et al., 2006]. Neu & Lawrence [2010] defined biofilms on the basis of either structure or function. The structural definition includes the basic biofilm components of microbial cells (bacteria, fungi, archaea, protozoa or algae), the extracellular matrix (EPS) and an "interface" where the biofilm forms. Attachment to a solid surface is not considered necessary. The functional definition is intended to describe the effect of the microbial interactions in the biofilm on the overall function of the structure. It is believed that the formation of biofilms is a process that enables microbes to survive by sharing nutrients, resisting environmental dangers and sharing of genes through horizontal transfer [Davey & O'Toole, 2000]. This section is dedicated to summarising the importance of the matrix and 3D structure to the biofilm lifestyle.

### 1.1.1 The matrix

Most of the biofilm is made up of the matrix, which accounts for more than 90% of its dry mass. It is constructed by the matrix-embedded bacteria and made of extracellular polymeric substances (EPS), including polysaccharides, nucleic acids, proteins and lipids [Branda et al., 2005; Flemming & Wingender, 2010]. However, water is the main component of the matrix and in fact, of the biofilm as a whole [Vasudevan, 2014]. The formation and composition of the EPS depends on the producing species and is affected by environmental factors such as

temperature [Gancel & Novel, 1994; Gandhi et al., 1998], nutrient availability [Gancel & Novel, 1994; Gandhi et al., 1998; Looijesteijn et al., 1999] and shear forces [Stoodley et al., 1999].

The functions of the matrix are multiple and essential for the lifestyle and the survival of the biofilm. The EPS is responsible for the adhesion onto surfaces, the mechanical stability [Mayer et al., 1999], immobilisation and protection against UV radiation (UV can be used to disinfect surfaces or water), pH shifts, osmotic shock, desiccation, biocides, antibiotics and the host immune system. Additionally, it serves as a nutrient source and maintains the availability of lysed cell components such as DNA that can be used for horizontal gene transfer [Davey & O'Toole, 2000; Flemming & Wingender, 2010].

Confocal Laser Scanning Microscopy (CLSM) has shown that the biofilm structure consists of microbial pockets, heterogeneously distributed and separated by water channels [Hope & Wilson, 2006; Korber et al., 1994; Lawrence et al., 1991; Wimpenny & Colasanti, 1997]. The water channels serve as a means to exchange nutrients and metabolites between the microbial cell clusters [de Beer & Stoodley, 1995; Stoodley et al., 1994]. At the same time, they function as a waste removal system [Davey & O'Toole, 2000]. The architecture and the mechanical stability of a biofilm are strongly affected by the composition of the EPS, as for instance by the types of polysaccharides, the DNA and the presence of non-enzymatic proteins, and by the chemical interactions between its anionic and cationic components [Flemming & Wingender, 2010; López et al., 2010].

### 1.1.2 The effect of biofilm architecture on embedded microorganisms

The EPS is the main factor affecting the compartmentalisation of the biofilms [Sutherland, 2001*a*,*b*]. The extracellular environment of each microbial pocket is the surrounding EPS and the water channels that constitute the means of commu-

nication and nutrient transport between the cell clusters or pockets. Therefore, each pocket constitutes its own microniche [Costerton et al., 1994].

#### 1.1.2.1 Chemical and physiological gradients and different bacterial phenotypes

The biofilm architecture results in the creation of a number of chemical gradients, e.g. oxygen, nutrient and pH gradients. These in turn result in different physiological gradients with differences in respiration rates, nutrient availability and protein synthesis, within the same biofilm [de Beer et al., 1994; Drenkard, 2003; Tolker-Nielsen & Molin, 2000; Wimpenny & Kinniment, 1995; Xu et al., 2000]. The microorganisms located near the outer layers of the biofilm have better access to oxygen and nutrients and they therefore exhibit faster growth rates than their counterparts located in deeper layers of the biofilm [Drenkard, 2003]. Similarly, those microorganisms that grow near the bulk fluid interface are more viable [Xu et al., 2000]. Thus, different bacterial phenotypes, e.g. slow and fast growing, can be observed within a biofilm, even when the microorganisms are separated by a distance of as little as 10 μm [Xu et al., 2000]. This is true for multi-species as well as single-species biofilms [Stewart, 2002].

#### 1.1.2.2 Sociality

Social behaviours in nature are defined as those that affect the reproductive success of the actor and another individual [Davies et al., 2012]. When those who benefit from the behaviour include close relatives, the actor gains an indirect benefit which add to her own reproductive success Hamilton [1964*a*,*b*].

Social traits in bacteria include sharing of resources and cooperation against threats [Foster, 2011]. The production of iron-scavenging molecules and their exploitation by non-producers is a well studied such trait [Griffin et al., 2004].

Some studies have reported that the production of β-lactamases is a social trait [Dugatkin et al., 2005; Medaney et al., 2015; Perlin et al., 2009]. β-lactamases are located mainly in the periplasm of Gram-negative bacteria or they are excreted in Gram-positives [Livermore & Woodford, 2006; Minsky et al., 1986; Wilke et al., 2005]. Irrespective of where they are localised, β-lactamases clear the antibiotic in their immediate environment, thus potentially allowing nearby susceptible bacteria to survive. Therefore the enzyme is utilised as a "public good" available to producers and non-producers alike.

The biofilm architecture with its bacterial microniches is more likely to support microbial interactions, cooperative or otherwise, between metabolically different species [Foster, 2010; Mitri et al., 2011]. The biofilm structure has been shown to affect social dynamics due to limitations imposed on the public good diffusivity Kümmerli et al. [2009]; Zhou et al. [2014]. Stewart & Costerton [2001] suggested approaching the issue of biofilms in general and of biofilm resistance to antibiotics in particular, as a defence mechanism of a multicellular organism rather than one of individual organisms and this is the approach taken in this work.

## 1.2 Current theories on the reduced antibiotic susceptibility of biofilms

It is a commonly shared view within the research community that biofilms show increased resistance to antibiotics and biocides [Bordi & de Bentzmann, 2011; Donlan & Costerton, 2002; Olson et al., 2002; Prashanth et al., 2007; Prosser La Tourette et al., 1987; Smith, 2005; Tresse et al., 1995]. Higher antibiotic resistance in biofilms by 10 - 1000 fold has been reported [Cerca et al., 2005; Mah et al., 2003]. Even bacteria that are normally susceptible to antibiotics may develop tolerance when in biofilms [Stewart & Costerton, 2001]. However, the biofilm-forming bacteria do not always show increased antibiotic tolerance out-

side the biofilm context [Lewis, 2007]. It is generally believed that this increased tolerance is not a result of the inherited resistance mechanisms used by planktonic bacteria [Stewart & Costerton, 2001]. The only exception to this might be the up-regulation of efflux pump mechanisms in some bacterial biofilms formed by some *Pseudomonas* and *E.coli* strains [Drenkard, 2003; Hancock & Klemm, 2007]. In other cases, researchers have suggested alternative mechanisms instead, as discussed in the following subsections. Four theories regarding the mechanism of tolerance in biofilms are discussed below: limited antibiotic penetration through the matrix, antibiotic inactivation by β-lactamases, social interactions and physiological state.

### 1.2.1 Limited antibiotic penetration

One of the most popular theories on why biofilms appear to be more tolerant to antibiotics is that the biofilm structure and matrix inhibit the penetration of those substances. This could be achieved by slower diffusion of the antibiotic within the matrix, enzymatic inactivation of the applied antibiotic and/or removal of the antibiotic through the water channels. Diffusion and matrix effects as well as antibiotic inactivation by β-lactamases are discussed below.

#### 1.2.1.1 Diffusion and matrix effects

An obvious explanation of the increased tolerance to antibiotics exhibited by biofilms seems to be the presence of the matrix. The matrix may hinder the diffusion of antimicrobial substances into the deeper layers of the biofilm either by acting as a physical barrier or by binding the molecules. In fact, the thickness of the matrix has been associated with limiting the penetration of leukocytes in *Staphylococcus aureus* biofilms [Fux et al., 2003; Leid et al., 2002]. However, theoretical studies on the diffusion and absorption (e.g. binding of antibiotics to periplasmic glucans [Mah et al., 2003]) of several reactive and non-reactive

substances through biofilms indicated that although absorption may hinder antibiotic penetration, it does not sufficiently explain resistance [Stewart, 2002]. The action of the antibiotics rifampin, ampicillin and ciprofloxacin, as visualised with the aid of electron microscopy in two studies [Zahller & Stewart, 2002; Zheng & Stewart, 2002], also showed that in most cases antibiotics were able to reach the cell clusters within the biofilms, but not to kill them. The case was different for ampicillin in biofilms of β-lactamase producing strains. These experiments are described in detail below.

#### 1.2.1.2 Inactivation of β-lactam antibiotics by β-lactamases

Anderl et al. [2000] investigated the penetration of ampicillin in wild type *Klebsiella pneumomiae* biofilms. This team of researchers developed biofilms on polycarbonate filter membranes and then exposed them to 5000 μg/ml antibiotic concentration, 10 times the Minimum Inhibition Concentration (MIC) as measured in planktonic cultures of the same species. β-lactamase producing *K. pneumonia* prevented ampicillin from penetrating the biofilm while the antibiotic penetrated the β-lactamase deficient biofilms. On the other hand, similar experiments with ciprofloxacin showed that this type of antibiotic penetrated the biofilms in less than 20 min. The authors attributed their results to inactivation of ampicillin by β-lactamase enzymes in the surface layers of the biofilms at a rate higher than the diffusion rate. The two *Klebsiella* strains were shown to have similar growth rates and ciprofloxacin susceptibilities as well as biofilm structures, as seen under the microscope, which means that those factors did not account for the differences seen.

Zahller & Stewart [2002] investigated the penetration of ampicillin & ciprofloxacin in *Klebsiella pneumoniae* colony biofilms grown on polycarbonate filter membranes. The biofilms were grown using the method described by Anderl et al. [2000]. The biofilms were then fixed, stained and cut before examination under the transmission electron microscope (TEM). Each biofilm was sampled at three

locations, namely, near the air interface, in the middle of the biofilm and near the filter membrane. These workers found that ciprofloxacin did affect the cells near the air interface, as these were shown elongated in the TEM images (cell elongation is caused by the antibiotic interference with the chromosome segregation) and their density was reduced. No elongation was observed in the other two locations. Biofilms treated with ampicillin were greatly affected by the antibiotic when the biofilm former was not a β-lactamase producer. Bloated and lysed cells were evident at all three locations, although some areas in the middle of the biofilm appeared unaffected.

The scenario was completely different when the biofilms formed by a beta-lactamase producing strain were examined; TEM images did not show any evidence of antibiotic action in this case anywhere in the biofilm. The authors hypothesised that the antibiotic was hydrolysed by β-lactamases at the outer layer of the biofilm at a rate faster than its diffusion rate thus preventing its penetration into the biofilm. Giwercman et al. [1991] showed that β-lactamases in *Pseudomonas aeruginosa* biofilms are inducible by β-lactam antibiotics (imipenem and piperacillin) and that the production of the enzymes was higher with higher doses of antibiotics. Similarly, the average β-lactamase activity developed in the presence of carbenicillin was 1.5x higher in *Pseudomonas aeruginosa* biofilms than in planktonic cells [Davies & Geesey, 1995]. These results indicate that β-lactamases when present do play an active role in increasing the biofilm resistance.

### 1.2.2 Tolerance due to physiological variation

It has been proposed that the presence of nutrient, pH and oxygen gradients in the biofilm that result in different bacterial growth levels may be one of the factors contributing to the increased antibiotic tolerance of these structures [Stewart & Franklin, 2008; Walters et al., 2003], as antibiotics such as β-lactams have been found to be effective against rapidly growing bacteria only [Tuomanen et al., 1986]. Ito et al. [2009] also found an association between growth rate and antibi-

otic efficiency by showing that antibiotics (ampicillin, kanamycin and ofloxacin) were less effective against planktonic *E. coli* cultures growing without glucose compared to those growing on glucose. In order to test the effect of growth rate in *E. coli* biofilms on antibiotic resistance, these researchers developed mature biofilms in flow cells and then removed the surface layers of the biofilms by air flushing (to reduce any effects of limited penetration and heterogeneous physiological stages). When kanamycin and ofloxacin were applied to those cells, consisting of the inner layers of the biofilms, regrowth of the biofilm was inhibited. Treatment with ampicillin though did not inhibit regrowth after the antibiotic was removed from the growth medium; thick biofilms were formed again after 72 h. The resistance to ampicillin shown by Ito et al. [2009] and attributed to persisters was explained as the result of the low metabolic activity of this type of cells. In essence, since β-lactam antibiotics inhibit cell wall synthesis and there is no such synthesis in persisters, the antibiotics are ineffective. This theory was further supported by the results shown with the use of ofloxacin, which killed the bacterial cells.

Zheng & Stewart [2002] also used the Anderl et al. [2000] system coupled with TEM in order to investigate the penetration of rifampin in *Staphylococcus aureus* biofilms. The workers concluded that although the biofilms were again protected, this was not due to limited penetration of the antibiotic as the cell densities and frequencies were reduced especially near the biofilm-air interface. Also, the cell wall was found to be thicker after treatment with antibiotics in all locations and thickening of the cell wall is considered to be a common reaction to antibiotic treatment in *Staphylococci*. In this case the authors suggested that the biofilm was protected due to the presence of slow-growing bacteria in the deeper layers of the biofilm.

Contrary to the common belief that biofilms are more resistant to antibiotics than planktonic bacteria, Spoering & Lewis [2001] reported that this is not actually the case when comparing planktonic stationary phase bacteria and biofilms. In fact it was the stationary phase bacteria that appeared to be more resistant than biofilms to a range of antibiotics (ofloxacin, tobramycin, carbenicillin and

peracetic acid). The authors attributed the results seen to the presence of persisters in stationary phase, biofilms and exponential phase bacteria in order of decreasing abundance.

## 1.3 Aims and objectives

The overall aim of this project was to investigate the mechanisms of resistance to β-lactam antibiotics in *E. coli* biofilms using a single experimental system in an attempt to eliminate variations on the results due to system differences. An environmental natural biofilm-forming isolate was used in order to exclude artefacts of biofilm formation by laboratory strains that may not apply to naturally occurring ones.

β-lactamases were approached as a social trait when investigating the effect of producers on the biofilm population under antibiotic stress. Novel methods were developed to allow for the visualisation of the cells in the whole depth of the colocy biofilms using confocal microscopy and Optical Coherence Tomography (OCT). Furthermore, susceptible biofilm tolerance to antibiotics was investigated using a new approach designed with consideration for the physiological state of the cells in the biofilms.

After engineering the system in order to express a clinically relevant β-lactamase gene (Chapter 2), the following factors were investigated for their contribution to biofilm resistance:

- The production of β-lactamases and their use as a public good, i.e the extent of protection offered to susceptible bacteria in biofilms (Chapter 3)
- The interactions between the antibiotic and the biofilm structure, i.e. the matrix and the spatial mass distribution (Chapter 4)

- The metabolic activity in biofilms and its relationship to antibiotic tolerance (Chapter 5)

A general discussion on the results obtained and their relevance to current research and real life applications is provided in Chapter 6.

# Chapter 2

# Strain selection and engineering

The aim of the experiments described in this section was to select a wild type (WT) *Escherichia coli* strain that possesses the ability to form biofilms and subsequently to genetically manipulate this strain, in order to create a pair of visually identifiable, isogenic bacteria that would be positive or negative for the production of an Extended Spectrum Beta Lactamase (ESBL), coded for by the $bla_{\text{CTX-M-14}}$. The WT strains screened for biofilm formation properties were previously isolated from UK farms in Surrey and Berkshire in Spring-Summer 2012 [Medaney, 2014].

## 2.1 Introduction

### 2.1.1 Selection of a β-lactamase enzyme

β-lactamases are a diverse group of enzymes that are divided into four classes (A, B, C & D) on the basis of their aminoacid motifs, or in three groups based on their functionality (Group 1-cephalosporinases, Group 2 - serine β-lactamases & Group

3 - metallo-β- lactamases) [Bush & Jacoby, 2010]. The Extended Spectrum Beta Lactamases (ESBLs) form a heterogeneous group of enzymes that belong to class A, Group 2 and can be divided into nine further clusters [Bonnet, 2004]. They are most commonly defined as the β-lactamases that can hydrolyse penicillins, the three first generations of cephalosporins and aztreonam [Paterson & Bonomo, 2005]. At least 26 bacterial species carry these genes but they are particularly prevalent in *E. coli* [Zhao & Hu, 2013].

The ESBL gene used here was the $bla_{\text{CTX-M-14}}$ gene, which belongs to the CTX-M-9 group [Bonnet, 2004; Cantón & Coque, 2006]. CTX-M-14 β-lactamases are, along with CTX-M-15, the most widespread type of ESBLs in the world [Dhanji et al., 2012]. Cottell et al. [2011] identified the $bla_{\text{CTX-M-14}}$ gene on plasmid pCT, which was isolated from an *E. coli* strain on a cattle farm in the UK. The prevalence of CTX-M-9 group β-lactamases in the UK in 2004 was reported to be 4.1% in hospital isolates [Woodford et al., 2004]. CTX-M-14 carrying plasmids were detected in 19% of human clinical isolates of *E. coli* in 2012 [Dhanji et al., 2012]. This indicates an increase in prevalence of approximately 5-fold within 8 years (2004 to 2012).

### 2.1.2 Selection of a reporter gene

The use of fluorescence in biology and in biofilm research in particular has been proven to be of great value (see for example Almeida et al. [2011]; O'Connell et al. [2006]; Park et al. [2011]). Several important characteristics of the fluorescent proteins for experimental work, include brightness, photostability, toxicity and environmental sensitivity [Shaner et al., 2005]. In general, brightness is an inherent property of the given protein. Photostability is determined by the light source and intensity but the mechanisms are not well understood at present. The ideal maturation rate of the protein is faster rather than slower, in order for the final signal to be high and stable [Zeiss, 2014]. Additionally, the chosen proteins have to be optimised for expression in the chosen biological system.

### 2.1.3 Challenges related to the use of recombinant proteins during genetic engineering of cells

Working with recombinant proteins presents several challenges, the most important ones being the potential loss of expression, the correct protein folding after translation and the potential toxicity for the host cell. Several protein delivery systems are currently in use, one of them being the incorporation of the gene of interest onto a plasmid. This offers the advantage of a relatively easy genetic manipulation, as well as a high gene dose, especially when the plasmid-carrier is a high-copy plasmid [Palomares et al., 2004; Rosano & Ceccarelli, 2014]. However, maintaining the plasmid imposes a metabolic burden on the host bacterial cells, which increases as the size of the plasmid, the size of the insert, the expression levels and the protein toxicity increase. This metabolic load results in lower growth rates that carry the risk of the faster-growing, plasmid-free cells overtaking the population [Baneyx, 1999; Palomares et al., 2004]. Environmental factors such as nutrient availability and growth temperature can have an impact on the plasmid copy number and as a result, on the plasmid stability [Palomares et al., 2004].

When the gene of interest is incorporated directly into the host's chromosome, which is another commonly used delivery system, the stability issues described above can be eliminated [Baneyx, 1999]. However, the process can be very time-consuming and the production rate of the protein of interest low, as fewer copies of the gene are usually present in this case.

With regard to how environmental factors more specifically affect protein expression and plasmid stability, Broedel et al. [2001] investigated the effect of growth media on the expression of a range of recombinant proteins in *E. coli* cells, including Green Fluorescent Protein (GFP). The authors concluded that there is no ideal medium for the expression of all proteins but instead that each protein showed better yields in a different medium. However, LB was not the best medium in any one case. For flow systems in particular, buffered media were believed to

be more effective for high protein yields. In addition, nutrient limited growth conditions can affect plasmid stability [Kumar et al., 1991]. Hebisch et al. [2013] found that the *E. coli* strain chosen as an expression system, and more specifically the growth rate and the duration of the lag phase, plays an important role in the maturation of GFP and mCherry fluorescent proteins. These researchers concluded that any correlation of fluorescent protein expression with the bacterial growth rate and lag-time is not consistent across the spectrum of fluorescent proteins and cannot be generalised. The role of growth rate in the gene expression in bacteria was also investigated by Klumpp et al. [2009] who modeled the effect of growth rate (in limited media) on the level of constitutively expressed proteins and found that the protein concentration was decreased at faster growth, although the number of protein copies per cell increased. The authors argued that this was a result of the increased cell volume Klumpp et al. [2009].

Finally, another issue that needs to be considered is the cost to the bacterial cell of unneeded proteins, such as fluorescent proteins. Shachrai et al. [2010] found that during the early exponential phase the cost of unneeded proteins (GFP or lac operon) was high, but that this cost decreased substantially during what the authors named as, exponential phase 2, even as the protein production increased. *E. coli* broth cultures in minimal and rich media were used for this study. Bacteria experienced phase 2 between 12.5 and 17.5 h of growth in minimal media, while in rich media (supplemented with amino acids) the reduced cost lasted for longer, beyond the 17.5 h. These workers also found that the increased protein cost during phase 1 was associated with a high ribosome production rate which at this stage, was a growth-limiting factor. In phase 2 ribosome and protein production balanced out and the protein production cost decreased.

In conclusion, a system expressing a recombinant protein, and more specifically a fluorescent protein, should have a number of characteristics as listed below, in order to be reliable:

- The proteins chosen should be suitable for expression in the desired biolog-

ical system

- The expression of the fluorescence should be stable for a given period of time, defined by the desired length of the experiments
- The stability of the expression should be consistent throughout all sets of experiments undertaken

## 2.2 Materials & Methods

### 2.2.1 Media and antibiotics

Lysogeny Broth (LB) (Lennox), Terrific broth and 2% (w/v) LB agar plates were prepared according to the manufacturer's instructions (Sigma L3022; Sigma T0918; Oxoid LP0011). Working solutions of 20 μg/ml chloramphenicol (chl), 100 μg/ml ampicillin sodium salt (amp) and 8 μg/ml cefotaxime (cef) were used for the preparation of antibiotic-containing media. Ofloxacine (ofl) for the Minimum Inhibition Concentration (MIC) assay was from Sigma (O8757). Phosphate Buffered Saline (PBS; Sigma-Aldrich PBS tablet, Cat no P4417-100AT) was used for serial dilutions. 1 mM Isopropyl-beta-D-thiogalactopyranoside (IPTG)(Apollo, BIMB1008) was used for the recovery of transformants and was added to all media to induce the IPTG-inducible lac promoter of the fluorescent genes.

### 2.2.2 Screening for biofilm formers

**Bacterial strains**. Fourty-eight (48) Wild Type (WT) *E. coli* strains were recovered from glycerol stocks and screened for their ability to form biofilms, in comparison to a positive control (*E. coli*, Nissle 1917) and a negative control (*E. coli* DH10β). *E.coli* Nissle 1917 was isolated from Mutaflor capsules containing

$2.5x10^9$ bacterial cells (capsules bought from Linda Versand Apotheke in Germany). The capsule was cut with a flame sterilized scalpel and the contents released in 10 ml PBS. The solution was vortexed, serially diluted in PBS and plated. A colony was picked after a 24 h incubation at 37 °C and used to make glycerol stocks for the positive control.

**Screening protocol**. Individual bacterial colonies from agar LB plates were incubated in LB broth at 37 °C with agitation at 180 rpm. After 24 h, 5 or 1 µl of inoculum was transferred into a 24-well (first two assays) or 96-well plates containing 1 ml or 180 µl of LB respectively. The plates were incubated for 24 h at 37 °C, with agitation (120 rpm). A slightly altered procedure was followed after the second round of screening due to a relatively high variability observed in (OD) readings between replicates of the same sample. Inoculated well-plates were initially incubated overnight (16 h) at 37 °C with agitation and then left in the incubator for another 24 h without agitation in order to allow for any biofilms formed to stabilize. After removal of the LB medium with pipetting, each well was washed gently with PBS in order to remove loosely attached cells that were not part of the biofilm. The PBS was then removed and the cells that were attached to the walls and the bottom of the wells were stained with 1 ml or 180 µl (equal to the initial broth volume) of 0.5% (w/v) Crystal Violet (CV) solution (in PBS) and left to incubate at room temperature for 20 min. The dye was washed three times with PBS solution (volume equal to CV solution used) in order to remove any excess dye that was not staining biofilm cells. The remaining dye was subsequently solubilised with a 20% acetone/ 80% ethanol solution in order to detach any biofilm from the walls of the well plate and measure the Optical Density (OD) of the solution. The sample OD was measured at 600nm wavelength, using a SpectraMax 190 absorbance microplate reader (Molecular Devices). The samples were mixed by shaking for 5 sec before reading. The experiments were performed in a stepwise manner: the best biofilm formers from step 1 were compared to new strains in step 2 until the final step, where the pre-selected best biofilm formers were also compared against the positive control.

### 2.2.3 Plasmid construction and bacterial transformations

**Bacterial strains and plasmids**. DH10beta was used for cloning. The pCT plasmid (described by Cottell et al. [2011]), carrying the $bla_{\text{CTX-M-14}}$ gene was provided by the Animal Health and Veterinary Laboratories Agency (AHVLA, Weybridge, UK). Plasmid pTopo carrying the dif-CAT (chloramphenicol gene) cassette was provided by Cobra Biomanufacturing [Bloor & Cranenburgh, 2006]. The fluorescent vectors pAmCyan and pE2-Crimson, optimised for bacterial expression and recommended for propagation in *E. coli*, were bought from Clontech (Cat no 632440 and 632553 respectively). The properties of the fluorescent proteins carried by these vectors can be found in Table 2.1. The commercial vector plasmid maps can be seen in Fig. 2.1.

**Table 2.1:** Properties of the fluorescent proteins used in experiments

| Vector | pE2-Crimson | pAmCyan |
|---|---|---|
| Origin of replication | pUC | pUC |
| Selective gene | Ampicillin | Ampicillin |
| Fluorescent protein Promoter | lacZ | lacZ |
| Fluorescent Protein | E2-Crimson | AmCyan 1 |
| Protein Origin | DsRed-Express2 | *Anemonia majano* |
| Excitation max | 611 nm | 458 nm |
| Emission max | 646 nm | 489 nm |
| Extinction coefficient | 126000 $Mol^{-1}$ $cm^{-1}$ | 39000 $Mol^{-1}$ $cm^{-1}$ |
| Brightness (Relative to EGFP) | 180 | |

**Obtaining the vector/insert constructs**. The antibiotic resistance genes ($bla_{\text{CTX-M-14}}$ and CAT) and the vectors were amplified by PCR with *Sac*I sites at both ends. The Clontech fluorescent vectors were used as the backbone with all their original elements intact, except for part of the ampicillin resistance gene ($Amp^r$) which was replaced by the CTX-M-14 or CAT gene. For further details on PCR (primers, cycles, reaction components) see Appendix A. All PCR products were confirmed with gel electrophoresis.

**Digestions and ligations**. All PCR products (inserts and vectors) were

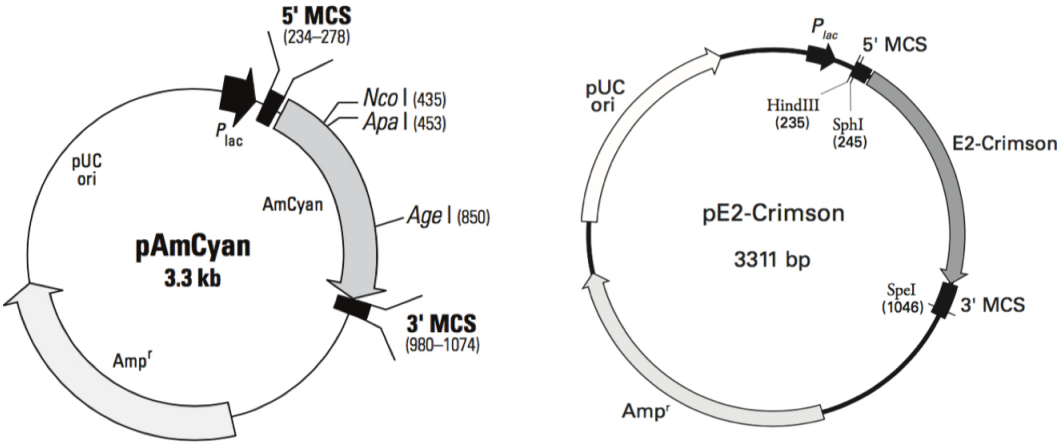


**Figure 2.1:** Backbone of the Clontech plasmids (taken from www.clontech.com)

cleaned up with the Promega Wizard SV Gel and PCR Clean-Up System (Cat no A9281) before digesting with enzyme *Sac*I-HF, chosen because this restriction site not present in any of the vectors or inserts. Typical digestion volumes were 50 µl (to avoid star activity), containing 0.5 -1 µg DNA. The reactions were incubated at 37 °C for 30 min to 1.5 h. The vector was dephosphorylated with an alkaline phosphatase that was added into the vector digestion reaction after the first 30 min and the whole reaction was incubated at 37 °C for another 15 min. The digestions were cleaned up, before proceeding to ligations, with the same Promega clean-up system mentioned above. The typical ligation volume was 20 µl and the vector:insert ratio was calculated by formula 2.1. Typical vector:insert ratios were 1:2, 1:3, 1:5, or 1:6. The ligations were incubated at room temperature for a time period of 2 h to 4 h and then inactivated as per the enzyme manufacturer's instructions. All enzymes used can be found in Table 2.2. PCR primers and cycle conditions can be found in Appendix A.

$$ng\ of\ insert = \frac{ng\ of\ vector * insert\ size\ in\ kb}{vector\ size\ in\ kb} * (\frac{insert}{vector}\ desired\ ratio) \quad (2.1)$$

**Table 2.2:** Enzymes used for the PCRs, digestions, dephosphorylations and ligations

| Enzyme | Provider | Function |
|---|---|---|
| Q5 high fidelity polymerase (M0491S) | NEB | PCR (resistance genes and fluorescent vector) |
| OneTaq hot start high fidelity polymerase (M0493S) | NEB | Colony PCR |
| SacI-HF restriction enzyme (R3156S) | NEB | Sticky ends digestion |
| T4 DNA ligase (M0202S) | NEB | Sticky ends ligation |
| Antarctic phosphatase (M0289S) | NEB | Cyan and crimson dephosphorylation |
| TSAP (M9910) | Promega | Cyan and crimson dephosphorylation |

**Plasmid purifications and bacterial transformations**. Plasmid pCT was isolated using the Qiagen Highspeed midi plasmid kit (Qiagen, UK, Cat no 12643). Plasmid pTopo and the subsequently engineered plasmids (cyan or crimson with $bla_{\text{CTX-M-14}}$ or CAT) were isolated with the Qiagen Highspeed mini plasmid kit (Qiagen UK, Cat no 27104 ). The engineered plasmids were transformed into DH10β-cells with electroporation using the high efficiency electrotransformation protocol for *Escherichia coli*, suggested by Bio-Rad[1]. The transformed wild strains were recovered on IPTG-containing LB plates with the appropriate antibiotic selector. PCR was used used for the confirmation of the transformants.

### 2.2.4 Sequencing constructed plasmids

The insertion was confirmed by sequencing. The constructed, cloned plasmids were purified from *E.coli* DH10β with the Qiagen Highspeed mini plasmid kit (Qiagen UK, Cat no 27104 ) and sent to Eurofins Genomics for sequencing. The sequencing primers used can be found in Appendix A.

[1]available at http://www.bio-rad.com/webroot/web/pdf/lsr/literature/4006174B.pdf, accessed 14 Sep 2013

### 2.2.5 Plasmid stability and fluorescent protein expression

**Drastic strain selection**. Five ml LB were inoculated with a colony of the transformant of interest and incubated overnight (for approximately 17 h) at 37 °C in the presence of the relevant selective antibiotic. Five µl of this starting culture were transferred into fresh LB (no antibiotic) for another overnight culture for 3 passages (n=2). The cultures were sampled at the starting point (0 h) (after the first growth cycle in antibiotic containing media) and at 24 h, 48 h & 72 h thereafter. For the assessment of plasmid stability, part of the culture at each sampling point was serially diluted and plated out (3 replicate plates per dilution factor) on selective antibiotic containing media with the spread method. Those strains that grew better on antibiotic containing plates were selected for further stability assessments.

**Improving fluorescence expression**. Starting cultures grown in Terrific broth for 17 h or 24 h were used to inoculate 5 ml LB broth tubes (time point 0). A passage assay was performed as before to assess the fluorescence expression under these conditions. The cultures were sampled at 24 h and 48 h, serially diluted and plated on LB and on antibiotic containing plates (chl or cef) using the spread method. Alternatively, cultures were plated on LB and colonies picked with sterile toothpicks and transferred to antibiotic containing plates.

For the estimation of the fluorescent protein expression the equation 2.2 was used.

$$fluor.\,expression = \frac{no\,of\,fluorescent\,colon.\,on\,LB_{+\text{antib.}}}{no\,of\,white\,colon.\,on\,LB_{+\text{antib.}} + no\,of\,fluorescent\,colon.\,on\,LB_{+\text{antib.}}} \quad (2.2)$$

### 2.2.6 Growth kinetics

Cultures of 11-CrimCTXM, 11-CyanCAT and WT CC11-1 grown for 24 h in 5 ml Terrific broth, with cefotaxime or chloramphenicol as needed for selection, were diluted in PBS to an $OD_{600}$ = 0.05 and 20 μl were used for the inoculation of a 96-well plate containing 180 μLB broth in each well (5 replicates per strain). Non-inoculated LB containing wells were used as controls. The plate was then placed in the SpectraMax at 37 °C and the growth was monitored for 24 h. An $OD_{600}$ measurement was taken every 5 min after a 3 sec agitation.

### 2.2.7 Fitness cost assay

Starting cultures were prepared as for the growth kinetics experiments and mixed at different proportions. Mixtures included 11CrimCTXM - WT (RW), 11CyanCAT -WT (YW) and 11CrimCTXM-11CyanCAT (RY) pairs (x3). A portion of 100 μl of each mixture were inoculated in 900 μl LB broth in 24-well plates and incubated at 37 °C for 24 h with agitation (120 rpm). After the 24 h incubation period the cultures were serially diluted and plated, using the droplet method, on LB and antibiotic containing plates. Colonies were counted 24 h later. The proportion of fluorescent colonies was estimated by *[fluorescent colonies on LB(-ant)/ total colonies on LB(-ant)]* and was confirmed by counts on antibiotic containing plates: *[total colonies on LB(+ant)/ total colonies on LB(-ant)]* for higher accuracy. Relative fitness was calculated using Equation 2.3 [Ross-Gillespie et al., 2007], where *x2* equals the proportion of the given strain in the final point of measurement and *x1* the proportion in the initial point of measurement.

$$Relative\ Fitness = \frac{x2*(1-x1)}{x1*(1-x2)} \tag{2.3}$$

### 2.2.8 Minimum Inhibition Concentration (MIC) assays

For the MIC assays, starting cultures of strains CC11-1 (WT), 11-CyanCAT and 11-CrimCTXM were prepared by inoculating 5 ml Terrific Broth as before. The cultures were grown at 37 °C for 24 h. After this incubation time $OD_{600}$ was measured with the Nanodrop and adjusted by dilution, if necessary, in order to obtain cultures of similar OD. Five μl of the diluted starting culture was then inoculated (x2) into 175 μl LB plus ampicillin, cefotaxime, chloramphenicol or ofloxacin at 6 final concentrations (4861, 607, 76, 9.5, 1.2 or 0 μg/ml) in a 96-well plate. The plate was placed in the Spectramax190 plate reader (Molecular Devices) and the growth was monitored for 24 h. A measurement was taken every 5 min, after a 5 sec agitation of the plate. The MIC was estimated based on the OD measurements and the visual growth in the wells at the end of the 24 h but the growth curves used as a check control to ensure that growth did not occur at any point at those concentration where no visual growth was seen at the final point.

### 2.2.9 Biofilm formation

To test whether the best biofilm formers identified by the crystal violet assay grew on polycarbonate membranes, overnight LB bacterial cultures were diluted to $OD_{600}$= 0.05. Filter membranes (Thermofisher, 11342885), placed on LB agar plates with sterile forceps were inoculated, in the centre, with 5 μl of the diluted culture. The colony biofilms were incubated at 37 °C for 48 h. The membranes were transferred to new plates after 24 h to ensure nutrient availability [Merritt et al., 2011].

### 2.2.10 Statistical analysis

Statistical analyses were conducted in R [R Core Team, 2013]. Linear regression analysis or one-way ANOVA were used for the biofilm screening results and linear regression for the fitness cost results. Models were checked by inspecting residual plots.

## 2.3 Results

### 2.3.1 Screening for biofilm formation

The strains that appeared to be the best biofilm formers during the first 5 rounds of screening (on the basis of $OD_{600}$ mean) were compared with each other and against the positive and negative controls in a shortlisting final screening round. Three 96-well plates containing three replicates of each strain were prepared for the final shortlisting. The higher the OD reading the more cells present in the well and the better the biofilm forming ability of the strain. As can be seen in Figure 2.2, OC3-1, COW63, CC11-1, OC2-8 and OC2-1 showed higher or equal ability to the positive control *E. coli* Nissle to form biofilms and were selected as the best biofilm formers. The results of the first 5 assays, as well as a figure with all the results combined can be seen in Appendix B.

A two-way ANOVA test with identity of the well-plate as a parameter was, run on the final selective design and showed that plate contributed to variation in biofilm formation [$F_{(19,142)}$=23.04, $p<0.05$]. The strain identity was marginally significant in explaining variation in biofilm formation [$F_{(19,142)}$=1.64, $p=0.06$]. In order to investigate further the differences between the selected strain and the positive and negative controls linear regression analysis was performed as well.

The linear regression analysis confirmed that selection of strains based on ODs (Fig. 2.2) was valid, as strains Cow63 (t=1.862, p=0.065), OC2-1 (t=2.240, p=0.027), CC11-1 (t=1.658, p=0.09), OC2-8 (t=2.501, p=0.014) & OC3-1 (t=2.122, p=0.036) were indeed the best biofilm formers compared to the standard laboratory strain *E. coli* DH10β (negative control). The overall regression model was not sufficient to explain the differences in OD seen [$F_{(17,144)}$=1.255, adjusted $R^2$= 0.03, p=0.23], However, it should be noted that the strains used in this last assay were pre-selected as potentially good biofilm formers and that large differences between the strains were therefore not expected. When a linear regression model was run on all the data from all the six assays, CC11-1 was found to be a significantly better biofilm former than the negative control (t=2.054, p=0.04) and not significantly worse than the positive control (t=-0.22, p=0.17). This time the model did not improve much on the ability to explain the variation within the data, compared to the aforementioned analysis run on the final assay, but the variance explained was significant ($F_{(48,689)}$=3.93, adjusted $R^2$=0.16, p <0.001).

Subsequently, strain COW63 was found to be inherently resistant to ampicillin (100 μg/ml) and cefotaxime (8 μg /ml) and it was therefore dropped. The remaining strains OC2-8, OC2-1, OC3-1 and CC11-1, along with the controls Nissle and DH10B were inoculated on polycarbonate membranes on LB agar plates. By visual observation it was noted that all strains developed biofilms on the membranes but differences in biofilm thickness were obvious. More specifically, most strains developed thick, concentrated biofilms with the exception of DH10B and OC2-1 which developed thin, spread biofilms. For this reason strain OC2-1 was also dropped. As a result, strains OC2-8, OC3-1 and CC11-1 were used for transformations with the constructed plasmids.

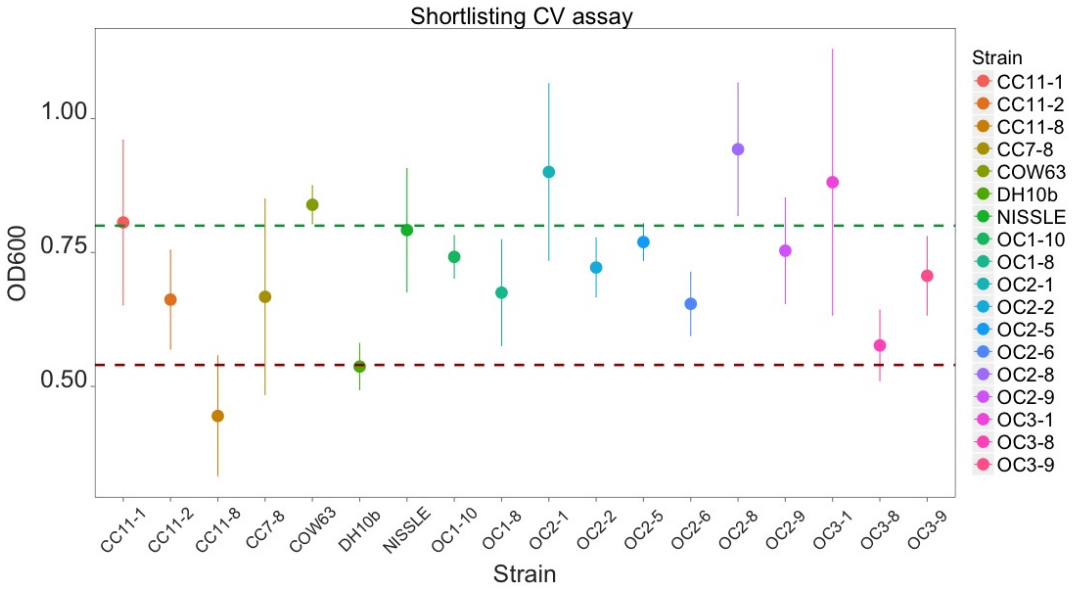


**Figure 2.2:** Mean OD+/-SE of the WT strain cultures after Crystal Violet (CV) staining, during the final shortlisting assay. The dashed red line represents the mean OD value for the negative control *E. coli* DH10β and the green dashed line represents the mean OD value for the positive control *E. coli* Nissle.

### 2.3.2 Plasmid construction

All PCRs were successfully optimised to amplify the desired products (details in Appendix A). The $bla_{\text{CTX-M-14}}$ and CAT genes were obtained with *Sac*I restrictions sites added to both sides for sticky end ligations. PCR products of pAmCyan and pE2-Crimson vectors without part of the $\text{Amp}^{\text{r}}$ gene and with *Sac*I restriction sites added were also obtained (Fig. 2.4). In total, four plasmids were created with the sequence of the original plasmid as shown in Figure 2.1 but with an insertion replacing the removed part of the $\text{Amp}^{\text{r}}$ gene; the insertion was either the $bla_{\text{CTX-M-14}}$ gene or the CAT gene (Fig. 2.3).

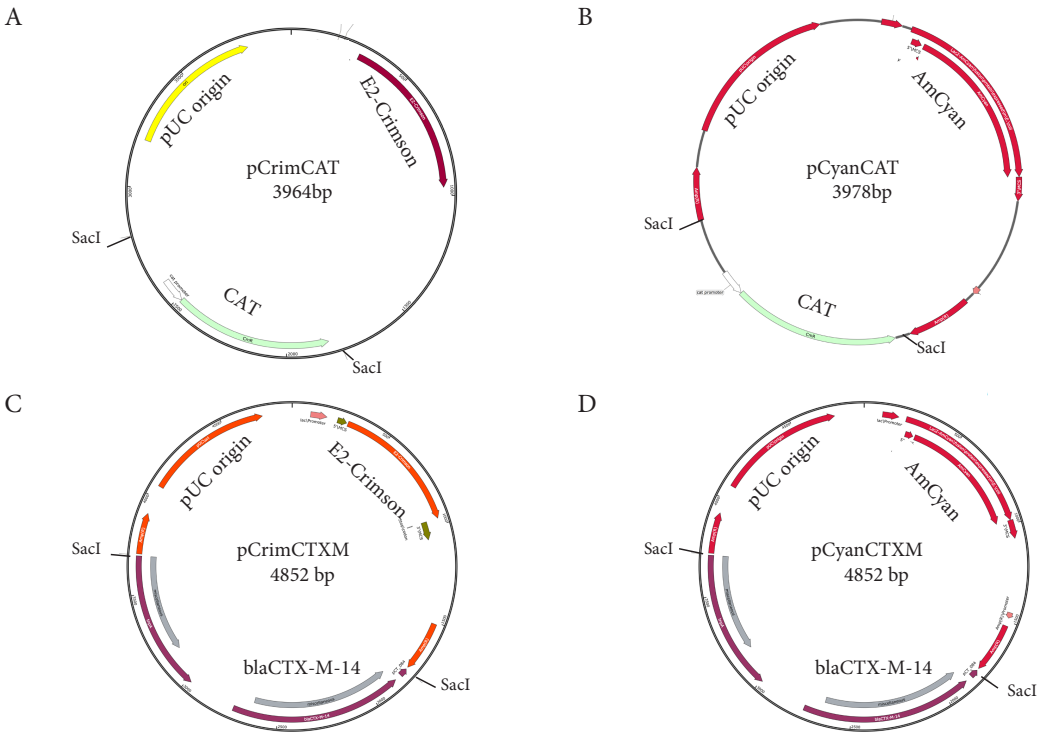


**Figure 2.3:** Expected sequence maps of constructed plasmids. Expected plasmid sequences were generated with Serial cloner and map made in Snapgene Viewer 3.2 (snapgene.com). Plasmid expected sizes, origin of replication, fluorescent proteins (E2-Crimxon, AmCyan) and inserted genes (CAT, $bla_{\text{CTX-M-14}}$) are visible.

### 2.3.3 Bacterial transformations

The newly created plasmids were initially transformed into DH10β strains and purified from this strain. The purified plasmids were subsequently used for the transformation of the wild type strains. The results of the transformations are shown in table 2.3. However, CC11-1 plus pCrimCTXM and the CC11-1 plus pCyanCTXM transformants were not obtained immediately but rather after repeated efforts that were eventually successful.

**Table 2.3:** Results of wild strain transformations

| Strain/Plasmid | Cyan+CTX-M | Cyan+CAT | Crimson+CTX-M | Crimson+CAT |
|---|---|---|---|---|
| **OC3-1** | positive | positive | positive | positive |
| **OC2-8** | positive | positive | positive | positive |
| **CC11-1** | negative | positive | negative | positive |

### 2.3.4 Plasmid stability and fluorescence expression

The stability of the plasmids pCyanCTXM and the pCyanCAT in the transformed WT strains was assessed by a passage assay as described above, only on the basis of the number of colonies formed on antibiotic containing plates. During this assay it became evident that the fluorescence was not always expressed as white colonies appeared on the antibiotic plates (Fig. 2.5A). Colony PCR targeting the fluorescence gene showed that the gene was present in most cases (Fig.2.6). Strain CC11-1 appeared to exhibit the highest expression of fluorescence up to and including day 3. Growth was variable but again only three transformants survived up to the final day of the assay,namely OC3-1 with pCrimCAT, CC11-1 with pCyanCAT and OC2-8 with pCrimCTXM (Fig 2.5B).

These results drove further attempts to obtain the CC11-1 mutants with pCrimCTXM. Additionally, the issue of plasmid carrying cells that did not express the fluorescence needed to be resolved. Starting cultures of CC11-1 with

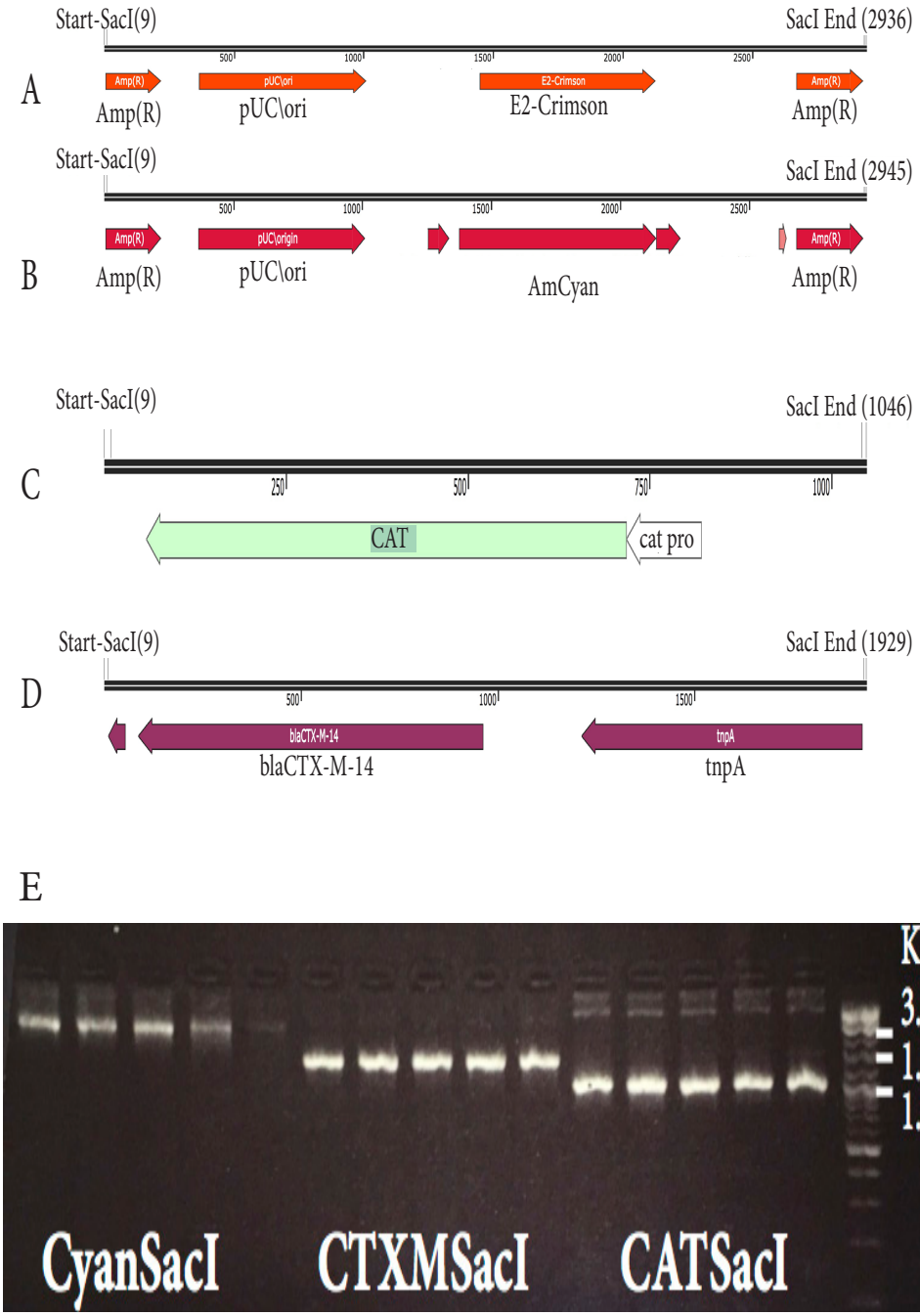


**Figure 2.4:** Sequences maps of components used to constructed the 4 plasmids: (A)CrimSacI, (B) CyanSaci, (C) CAT-SacI, (D) CTXM-SacI. Made in Snapgene 3.2.1(snapgene.com). (E) PCR products used for plasmid construction. From the left: 5 copies of CyanSacI (2930 bp), 5 copies of CTXMSacI (1463bp) and 5 copies of CATSacI (1026bp). The ladder is a NEB 2-log ladder (Cat no N3200S)

pCyanCAT (11CyanCAT) or pCrimCTXM (11CrimCTXM) were grown in Terrific broth plus the relevant antibiotic for either 17 or 24 h and the expression of fluorescence was assessed by counting the proportion of fluorescent cells on antibiotic plates. Figure 2.7 shows that growing a starting culture in Terrific broth improved the expression of the protein. The duration of the starting culture also had an influence with 24 h starting cultures resulting in less variability. Therefore 24 h starting cultures in Terrific broth were used for all the following experiments.

### 2.3.5 Plasmid fitness cost

The cost of carrying the constructed plasmids (pCrimCTX-M & pCyanCAT) was measured for strains 11CyanCAT (Y) and 11CrimCTXM (R) by competing them with each other or with the WT (W) at different starting proportions. pCyanCAT was less costly for its host when co-cultured with the pCrimCTXM carrier strain but the fitness of the plasmid carriers was lower than that of the WT in both cases. Additionally, the fitness of both plasmid carriers was frequency dependent, decreasing at higher relative abundance (Fig. 2.8). Linear regression analysis showed that all factors considered (initial proportion of plasmid carrier, plasmid type and competitor in the culture) contributed significantly to the overall variation in fitness seen ($F_{(4,29)}$=14.67, adjusted $R^2$=0.62, p<0.0001). pCyanCAT carrier was significantly fitter (t=4.517, p<0.0001) than pCrimCTXM carrier. Any samples where the plasmid carrier did not grow either at the first or second sampling points, were removed from the analysis because relative fitness could not be calculated.

### 2.3.6 Growth Kinetics

As can be seen in Fig. 2.9 the WT had a growth advantage over the engineered strains. In addition the strain carrying pCyanCAT grew better than the one

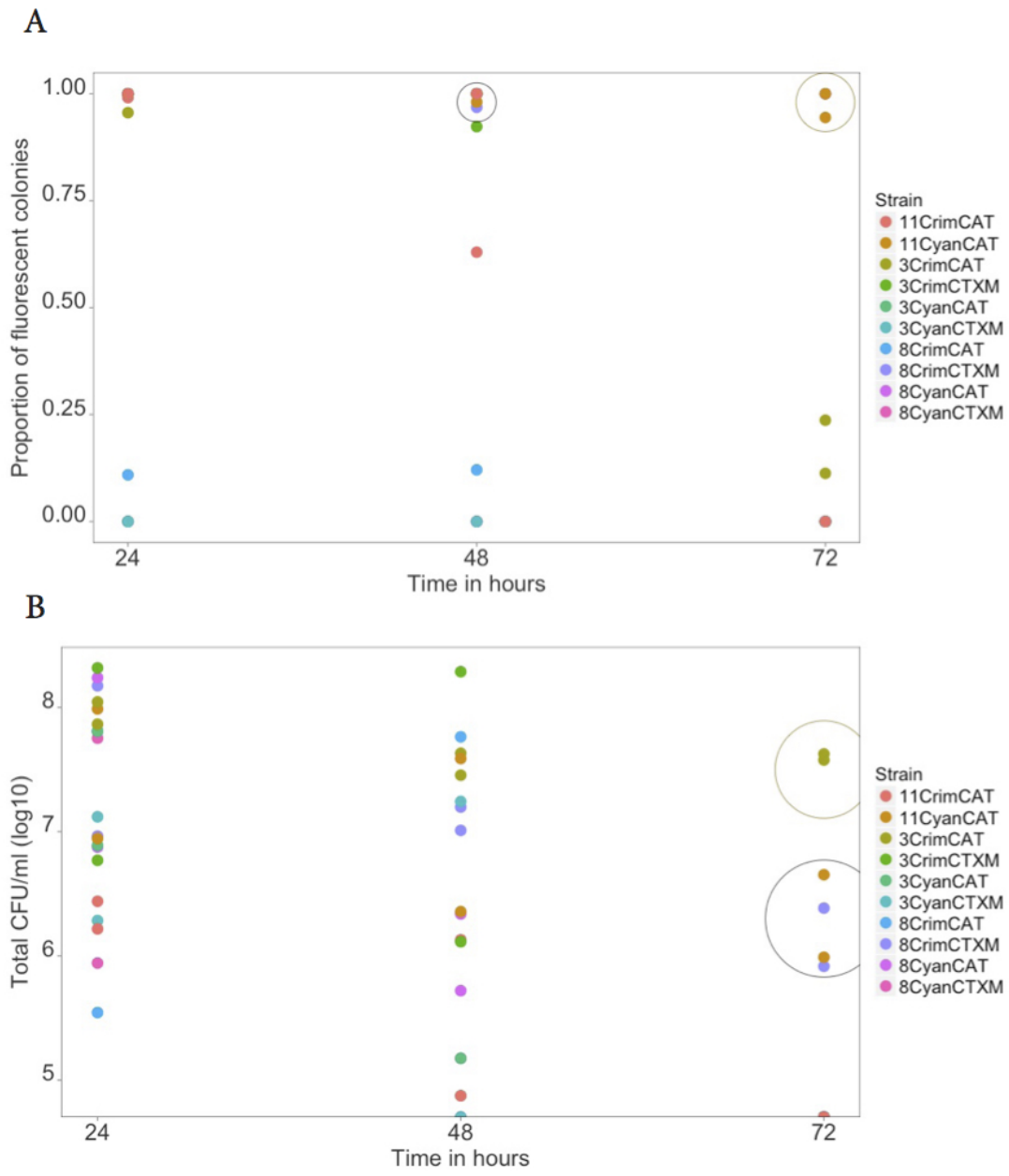


**Figure 2.5:** (A) Proportion of fluorescent cells on antibiotic containing plates over 3 days-drastic stability assay. The circles highlight the strains that were associated with better fluorescence expression in days 2 and 3. Strain 11CyanCAT exhibited the best expression of fluorescence until day 3. (B) Growth on antibiotic containing plates over 3 days, indicative of plasmid retention. The circles highlights strains 3CrimCAT, 11CyanCAT and 8CrimCTXM which grew up to day 3.

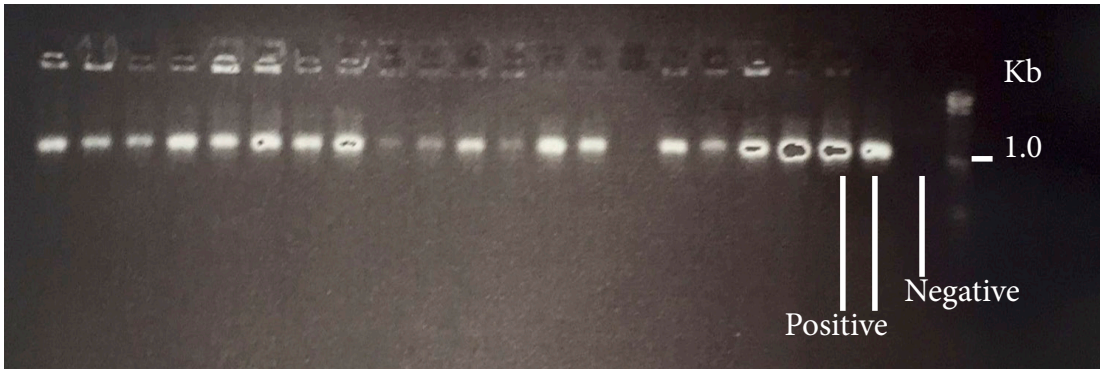


**Figure 2.6:** Colony PCR products for the detection of the fluorescence gene in white colonies recovered on antibiotic containing plates (chl) from strain11CyanCAT in this case. From the left: 19 colonies, 18 of which were positive for the fluorescence gene, 2 positive controls (fluorescent 11CyanCAT colonies) and a negative control (CC11-1 WT). The expected product size was 1073bp. The ladder is a NEB 2-log ladder (Cat no N3200S).

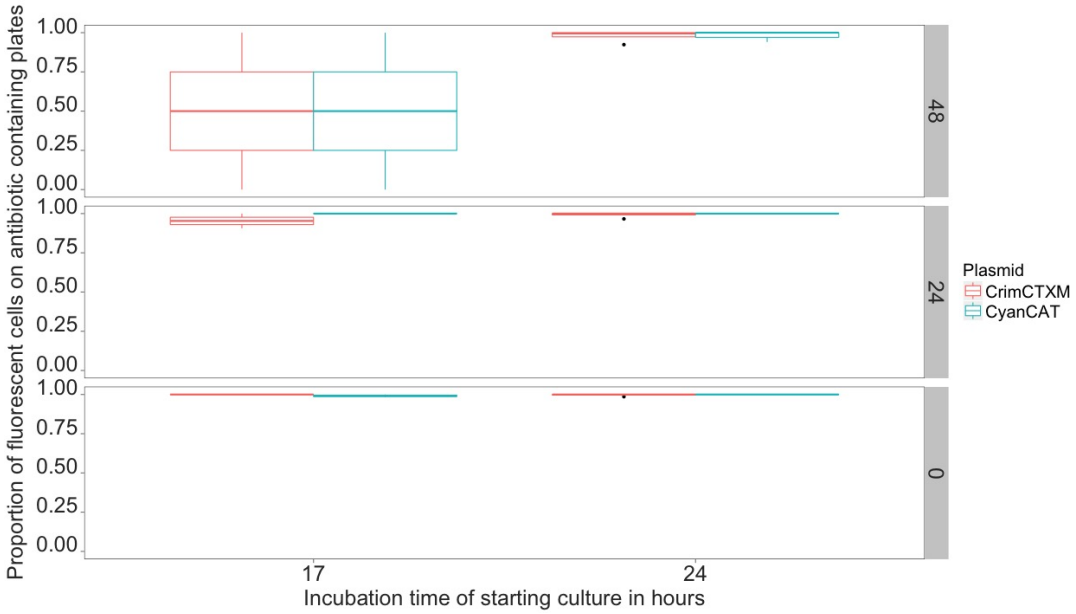


**Figure 2.7:** Fluorescence expression in 11CyanCAT and 11CrimCTXM strains (n $\geq$ 2) in varying starting culture conditions. Each of the gray boxes shows a sampling timepoint (0, 24, 48 h).

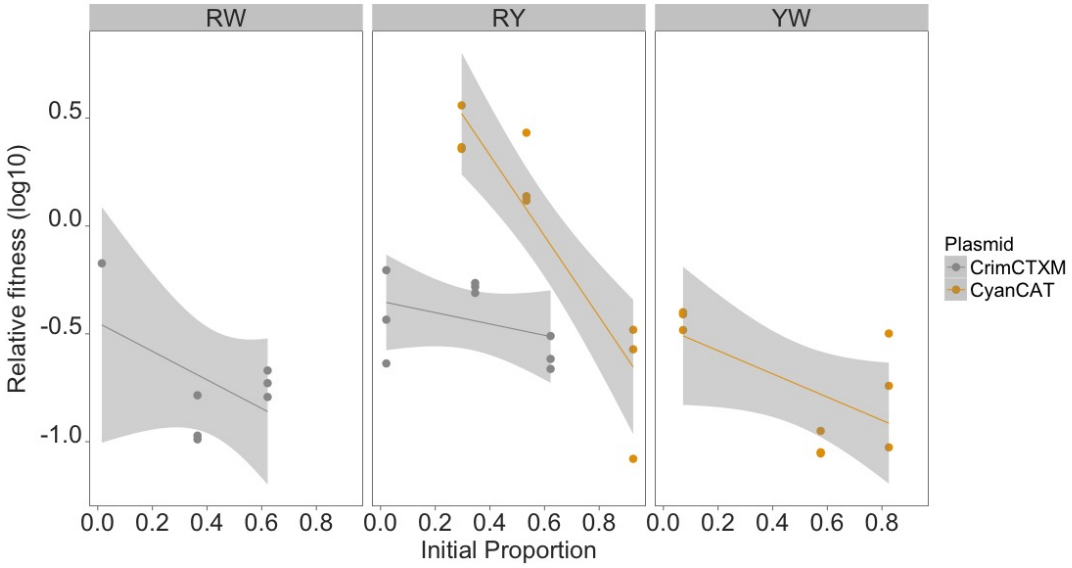


**Figure 2.8:** Relative fitness of plasmids pCyanCAT (Y) & pCrimCTXM (R) in CC11-1 background when competing with the WT (W). pCyanCAT carrier was significantly fitter compared to pCrimCTXM carrier when competing each other. Both plasmid carriers where less fit than the WT. Additionally, fitness was frequency dependent with plasmid carriers being less fit when abundant. The lines represent linear regression fit with SE.

carrying the pCrimCTXM. A detailed analysis of the first 2 h of growth showed that strain 11CrimCTXM remained in lag phase for longer (Fig. 2.10).

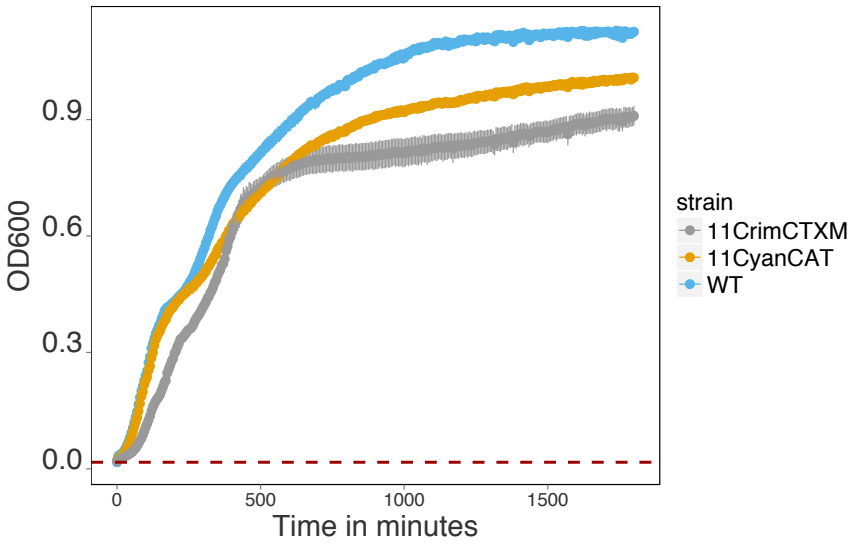


**Figure 2.9:** Growth kinetic curves of the WT and the engineered CC11-1 strain with plasmids pCrimCTXM, pCyanCAT in LB. The points represent the mean of 5 OD measurements with +/-1 SE bars

**MIC assays**. Fig. 2.11 shows that both the WT strain and the engineered susceptible strain shared the same patterns of resistance to β-lactams, i.e they both died at about 9 μg/ml ampicillin and at less than 1 μg/ml cefotaxime. Refining MIC assays showed that they could not survive concentrations of more than 3 μg/ml. The strain carrying the β-lactamase gene was resistant to ampicillin and cef in concentrations as high as 607 μg/ml (refining MIC assays showed that it can survive concentrations up to approximately 3.7 mg/ml ampicillin). All strains showed the same susceptibility to ofloxacin, as expected, as they all had the same genetic background.

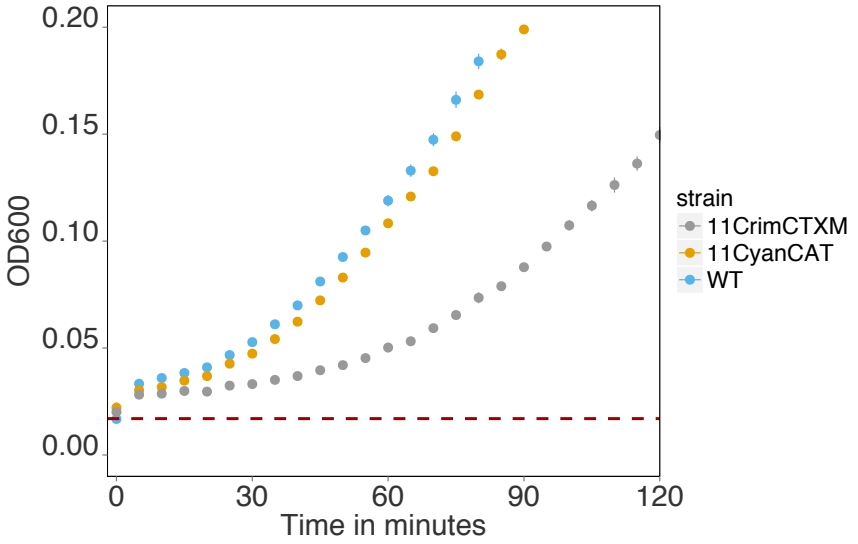


**Figure 2.10:** Zoom in the lag phase of the growth kinetic curves of the WT and the engineered CC11-1 strain with plasmids pCrimCTXM, pCyanCAT in LB

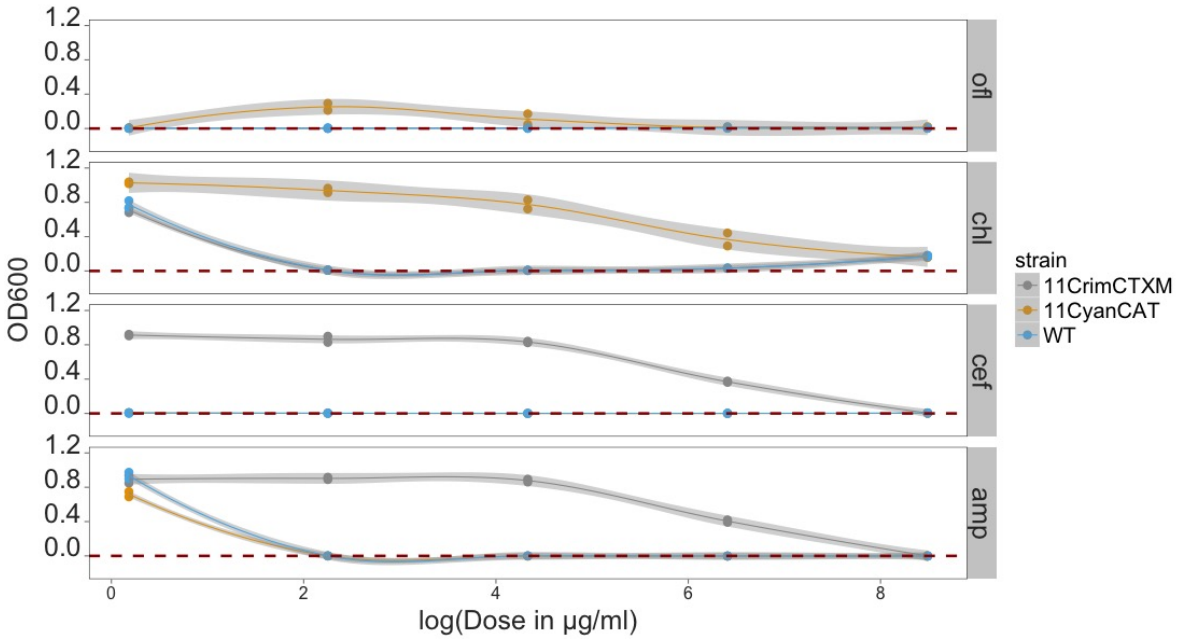


**Figure 2.11:** The results of the MIC assays for ofloxacin (ofl), chloramphenicol (chl), cefotaxime (cef) and ampicillin (amp) at different doses. All 3 strains survived a dose of 1.2 µg/ml of all antibiotics tested but only the ones carrying the respective resistance genes survived higher doses of chl, cef and amp. Resistant strains did not survive at concentrations of 4.8 mg/ml. The log scale on the X axis facilitates the readability of the graph.

## 2.4 Discussion

The aim of the set of experiments described here was to engineer a pair of isogenic WT *E. coli* strains that would be natural biofilm formers, with one of them being resistant to β-lactam antibiotics. Such a pair was successfully obtained with the aid of engineered fluorescent plasmids, one carrying an ESBL gene of clinical interest ($bla_{\text{CTX-M-14}}$) and the other resistance to chloramphenicol as a selection marker. The fluorescence expression was optimised following literature leads (see Section 2.1) pointing towards the manipulation of environmental factors for the stabilisation of fluorescence expression. As LB did not perform well in expression experiments [Broedel et al., 2001] and nutrient rich media were found to be better for expression [Kumar et al., 1991], Terrific broth was tested here. Shachrai et al. [2010] showed that longer incubation times may result in better fluorescence expression, so an incubation time of 24 h was also tested. This combination of environmental conditions for the starting cultures sufficed to improve the expression of the fluorescence and non-fluorescent colonies on antibiotic media were not observed in any of the subsequent experiments described elsewhere in this report.

Plasmids pCrimCTXM and pCyanCAT, which were used for the final selected pair in CC11-1 genetic background, were both costly to their hosts as competition with the WT showed. pCyanCAT carrier (11CyanCAT) performed better when competing with the pCrimCTX-M carrier (11CrimCTXM), indicating that the production of the ESBL carrying plasmid was more costly and this was also confirmed by statistical analysis. Additionally, the cost of the plasmids was frequency dependent with cost being reduced as the initial proportion of plasmid carrier decreased in both cases. The fitness cost results correspond to the growth kinetic results showing that 11CrimCTXM required a longer lag phase.

Better growth of bacterial cells carrying cooperative genes when they are abundant have been observed [Raymond et al., 2012] which seems to be the case also here with the ESBL-producing strain. However, the pattern was the same for the chloramphenicol-resistant strain as well, although chloramphenicol resistance

is not considered to be cooperative. The higher cost of pCrimCTXM compared to pCyanCAT may be a consequence of the larger insertion in this plasmid (1463 bp) compared to the insertion in the CyanCAT plasmid (1026 bp).

Finally, MIC assays confirmed that the chosen WT biofilm former did not possess an inherent resistance to any antibiotics used in any of the experiments performed as part of this work. It also aided the decision on suitable antibiotic doses in subsequent experiments as discussed in relevant chapters.

In conclusion, a reliable system of a WT biofilm forming isogenic strains was developed and optimised. One of the strains expressed a potentially cooperative trait (resistance to β-lactams) which was used for further investigations in the sociality of these enzymes and their role in biofilm antibiotic resistance in subsequent experiments, described in the following chapters.

# Chapter 3

# The sociality of β-lactamases in biofilms

## 3.1 Introduction

This chapter gives an overview of the social behaviour theory as applied to microorganisms followed by a description of the aims set for this part of the work. The methodology, the experimental results and an overall discussion complete the chapter.

### 3.1.1 The concepts of social behaviour and social evolution

"A behaviour is social if it has fitness consequences for both the individual that performs that behaviour (the actor) and another individual (the recipient)" where fitness is measured as the reproductive success of the individuals involved [Davies

et al., 2012].

William Hamilton, the evolutionary biologist who shaped the field of behavioural ecology as we know it today, categorised social behaviours according to whether they are beneficial or costly to the actor and the recipient as mutually beneficial, altruistic, selfish or spiteful [Hamilton, 1964*a*,*b*]. Mutually beneficial and altruistic behaviours are termed collectively as "cooperation". The first refers to the case when both the actor and recipient benefit from the behaviour in question and the latter to the case when the recipient benefits to the cost of the actor. Selfish behaviour benefits the actor to the detriment of the recipient and spiteful is harmful to both individuals involved. Kin selection as defined by Hamilton [1964*a*,*b*] in his early work, has been the main explanatory mechanism for cooperation as an evolutionarily stable strategy compatible with Darwin's natural selection theory [Darwin, 1872]. According to the kin selection theory, the fitness of an individual is the sum of direct fitness and indirect fitness, where direct fitness refers to own offspring and indirect to offspring of relatives. The sum is termed inclusive fitness. Therefore, genetic relatedness favours cooperation as it increases indirect fitness. However, examples of altruism among genetically non-related individuals have also been observed in nature [Davies et al., 2012].

As theory explains, a critical component of cooperation is the beneficial effect of an act on another individual. In order for it to be examined through the prism of evolution, though, it has to be selected for by natural selection because of this beneficial effect [Foster, 2010; West, Griffin & Gardner, 2007*b*], at least partially. For the purposes of the work discussed in this chapter, the explanations for cooperative behaviour as reviewed and categorised by West, Griffin & Gardner [2007*a*,*b*] will be assumed. This classification includes direct benefits, which are meant to explain mutually beneficial behaviours and indirect benefits to explain altruistic behaviours, although those two general categories are not mutually exclusive (Figure 3.1).

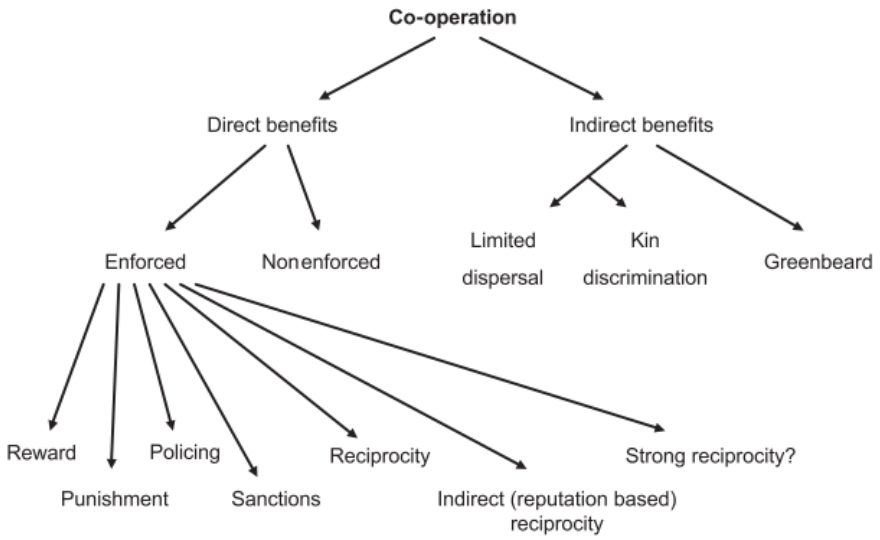


**Figure 3.1:** Classification of current explanations for cooperative behaviour. Image taken from West, Griffin & Gardner [2007*b*]

### 3.1.2 Social behaviour in microbial life

Although social behaviour is usually considered to be a privilege of organisms more sophisticated than the humble bacteria, those have not been overlooked by scientists - Darwin's contemporaries and beyond [Foster, 2010]. It is now widely accepted that microbes lead social lives and share social traits [Foster, 2011; Strassmann et al., 2011]. Social traits in microbes include the production of secreted products such as siderophores [Griffin et al., 2004], β-lactamases [Dugatkin et al., 2005; Medaney et al., 2015; Perlin et al., 2009], toxins and their antidotes [Gardner et al., 2004; Raymond et al., 2012], quorum sensing molecules [Diggle et al., 2007; Fuqua et al., 1994; Miller & Bassler, 2001; Sandoz et al., 2007] and biofilm development [Nadell et al., 2009; Oliveira et al., 2015]. These type of products are termed "public goods" [Davies et al., 2012; Raymond et al., 2012]. Bacteria compete for resources and they cooperate to increase chances of survival and in doing so they employ several strategies (reviewed by Foster [2010]; Hibbing et al. [2010]; West et al. [2006]).

Kin selection is a favoured evolutionary theory that often applies to microbes [Foster & Xavier, 2007; Griffin et al., 2004; MacLean, 2008; Strassmann et al., 2011; West, Diggle, Buckling, Gardner & Griffin, 2007], especially in the case of secreted products [Nadell et al., 2009]. In microorganisms, kin selection may work via limited dispersal of cells which increases relatedness in a local level and therefore the secreted public good benefits only the producers that are located nearby [Griffin et al., 2004; Smith, 2001], or via kin discrimination whereby cells direct the benefit of the public good towards their kins by preventing other species/clones from exploiting it. For example, *E. coli* produces bacteriocins that are toxic to cells that do not carry the antitoxin gene [Riley & Gordon, 1999]. The production of a public good can also have direct benefits to the producer in which case in may be produced in a mutualistic relationship with other species or when the expression of the gene is enforced in the population (policing) [West et al., 2006]. An example of the later is the case of nitrogen fixing rhizobia bacteria that are cut out of oxygen supply by the plant if they stop fix nitrogen [Kiers et al., 2003].

Bacteria, therefore, are not solitary organisms but they have been found to exhibit social behaviours, such as altruism. Nevertheless, the current assumption in pathogen risk analysis is that each bacterial cell acts as an independent agent of disease when this may not be the case [Cornforth et al., 2015]. Social traits in bacteria may be a viable target for new drug development, by, for example, introducing a drug susceptible non-pathogenic cheater strain in a resistant pathogenic population that employs a social strategy to survive antibiotics [Brown, West, Diggle & Griffin, 2009]. However, such an approach may not be effective if resistant bacteria have the capacity to re-emerge in a population dominated by cheats [Harrison, 2013; Raymond et al., 2012] or if cheating is restricted to a tiny subset of bacteria, such as persisters [Medaney, 2014]. It is therefore important to better understand bacterial sociality in order to develop new antimicrobial strategies.

### 3.1.3 The case of β-lactamases as a public good

β-lactamases are normally found in the periplasm of Gram-negative bacteria or extracellularly in Gram-positives [Livermore & Woodford, 2006; Minsky et al., 1986; Wilke et al., 2005]. β-lactamases are thought to be potential public goods [West, Diggle, Buckling, Gardner & Griffin, 2007] and this can be true even when the enzymes are not excreted from the cell, as even when cell-bound they can detoxify their immediate environment from antibiotics allowing the growth of otherwise susceptible bacteria.

Competition experiments between susceptible and antibiotic resistance strains of the same or different species have shown that β-lactamase-producing *Escherichia coli* can protect susceptible strains, without gene transfer, essentially arguing that the production of the enzyme is a public good in bacterial communities [Dugatkin et al., 2005; Perlin et al., 2009]. Medaney et al. [2015] showed that β-lactamases can be exploited by slow-metabolising cells, appearing on agar plates only after the antibiotic had been cleared by β-lactamase producing bacteria. Yurtsev et al. [2013] mixed isogenic *Escherichia coli* strains, one of which expressed a plasmid-carried β-lactamase enzyme gene and exposed them to several antibiotic doses up to 200 μg/ml. They found that the proportion of β-lactamase producing bacteria retained in the population equilibrated at approximately 25% at an ampicillin dose of 100 μg/ml. This equilibrium proportion turned out to be dependant on the ratio of antibiotic concentration/ initial total cell density. Additionally, it has been suggested that at high antibiotic concentrations, when resistant bacteria are killed, the lysis of the resistant cells may be enough /to protect susceptible cells due to the release of β-lactamases [Sykes & Matthew, 1976]. Therefore, it could be argued that in addition to living cells clearing the antibiotic in their surrounding area, dead cells also offer cell-bound β-lactamases as public-goods.

Bagge et al. [2004] showed that the expression of β-lactamases in biofilms depended on the type of β-lactam antibiotic applied and the dose. A low dose of imipenem, a carbapenem, induced the expression of β-lactamases in the periph-

ery of the biofilm, although cells were metabolically active in the centre as well. A high dose induced the whole biofilm. The third generation cephalosporin ceftazidime induced enzyme expression the periphery only irrespective of the dose.

### 3.1.4 Biofilms and sociality

Well-mixed bacterial populations in broth have been shown to express cooperative traits [Dugatkin et al., 2005; Griffin et al., 2004] but the spatial structure observed in biofilms may alter the dynamics of cooperation, as producers may get advantageous access to any self-produced public goods due to diffusion limitations and local interactions [Drescher et al., 2014; Kümmerli et al., 2009]. Zhou et al. [2014] found that spatial structure limited availability of quorum sensing related public goods to producers only. Kümmerli et al. [2009] showed that increasing viscosity in the media limited cell dispersal and public good diffusion in favour of the producers' fitness. Limited public good diffusion, limited cell dispersal, i.e. high cell density, and microcolony expansion limited competition and favoured cooperation in a model by Dobay et al. [2014].

Foster & Bell [2012] proposed that competition rather than cooperation is the dominant force driving evolution in biofilms. Different strains usually evolve to occupy separate niches in the biofilm, potentially as a result of division of labour [Kim et al., 2016; Vlamakis et al., 2008] and they may compete for the best spots [Kim et al., 2014]. However, cooperative behaviour may also be promoted in biofilms [Foster, 2010; Kreft, 2004] and the presence of cheaters, cells non-producing Quorum Sensing (QS) signal in this case, is likely to reduce biofilm viability [Popat et al., 2012]. Spatial segregation of the different populations, resulting from expansion, is more likely to promote cooperation [Seminara et al., 2012; Van Dyken et al., 2013] when well-mixed populations result in competition [Griffin et al., 2004; Nadell et al., 2016] as the cooperators are separated.

## 3.2 Aims and Hypotheses

As discussed above, published work suggests that less cheating is facilitated in biofilms. This is a result of strain segregation due to the seclusion of cooperators into local patches where they tend to find themselves next to relatives. Additionally, the diffusion of public goods is expected to be restricted due to spatial structure limitations. As theory suggests, any interactions are expected to be dependent on the frequency of cooperators/ cheaters as well as on whether the two groups are mixed or segregated. Additionally, higher antibiotic doses are expected to favour the cooperators. On this basis the following aims and hypothesis were formulated.

**Aim**: To investigate whether β-lactamases are utilised as public goods in biofilms and the conditions that facilitate the enzyme sociality. More specifically, I hypothesised that:

**Hypothesis 1**: Growth in a biofilm mode does not favour cheating, when strain mixing is not imposed upon the population, due to strain segregation.

**Hypothesis 2**: Population mixing results in higher susceptible fitness and therefore broth cultures and mixed and re-stablished biofilms facilitate more cheating.

**Hypothesis 3**: Higher cooperator frequencies in the founding population and higher antibiotic doses select for cooperators both in broth and in biofilms.

The frequencies of β-lactamase producing (resistant) bacteria, the fitness of non-producers (susceptible) and the total population size in mixed *Escherichia coli* biofilms under different antibiotic doses were used for inferences on β-lactamase sociality when examining the aforementioned hypotheses.

The resistant engineered *E. coli* strain created in Chapter 2, along with the wild type bacteria (WT) were co-cultured and compared to control mono-cultures as explained in section 3.3 below. The effects of starting proportions of resistant cells and antibiotic dose were measured in broth and biofilms and compared in terms of final resistant proportion, fitness and total bacterial abundance.

## 3.3 Materials & Methods

**Bacterial strains**. *Escherichia coli* CC11-1(WT, susceptible) and the constructed 11-CrimCTXM (resistant) strains were used for experiments in this chapter.

**Media and antibiotics**. Terrific broth (Sigma T0918) and LB (Lennox) and 2% (w/v) LB agar plates were prepared according to the manufacturer's instructions (Sigma L3022; Oxoid LP0011). 1 mM IPTG was added to the LB agar plates and the LB broth. Starting cultures were prepared in 5 ml Terrific broth containing 8 μg/ml cefotaxime for the resistant strain or no selection agents for the susceptible strain. The ampicillin used was purchased from Sigma (A0166). Sigma-Aldrich PBS tablet (P4417-100AT) was used for the biofilm disruption and for serial dilutions. The inoculum was prepared by diluting the starting cultures to a cell density of approximately $10^7$ CFU/ml and mixing as appropriate when required.

### 3.3.1 Sociality in ageing, undisrupted biofilms

Polycarbonate membranes on LB agar plates were inoculated with 5μl of either resistant, susceptible or a mixture of the two bacteria. The biofilms were grown on LB plates for 24 h and were then transferred to new LB or LB containing ampicillin [1000 μg/ml] plates for 3 days. The membranes were transferred on

new plates every day to prevent any changes due to nutrient limitations. Each day 3 biofilms per treatment (ampicillin dose) per biofilm type (resistant, susceptible, mixtures) were disrupted by vortexing in 10 ml PBS and plated out with the droplet method.

### 3.3.2 Sociality in mixed and re-established biofilms (biofilm passage)

Polycarbonate filter membranes (Thermofisher 11342885) on LB agar plates were inoculated with 5 µl inoculum of either susceptible, resistant or a mixture of the two strains. The biofilms were left to grow for 24 h before they were transferred to new plates with ampicillin of 0, 500, 1000, 2000 µg/ml for another 24 h. At this stage the biofilms were disrupted by vortexing in 10 ml PBS. The disrupted biofilms were used to inoculate new membranes and this process was repeated for a total of 5 passages. The numbers of resistant and susceptible cells were also counted at each stage. The mixed cultures were defined as above.

### 3.3.3 Broth passage

LB-containing (Sigma L3022) 24-well plates (900 µl) with ampicillin (Sigma A0166) at a concentration of either 0, 100 or 1000 µg/ml were inoculated with 100 µl of a susceptible, resistant or a mixed culture. A sample of each well was serially diluted and plated out 24 h later and the bacterial colonies of each type (resistant and susceptible) were counted. A portion of 100 µl of the 24 h cultures was used as an inoculum for new cultures.

The mixed cultures were defined as High Resistant (HR) or Low Resistant (LR) on the basis of the resistant proportion in the inoculum with a mean proportion of 0.49+/- 0.02 SE in HR , and 0.02+/-0.01 SE in LR. The Resistant

mean was 0.9+/- 0.09 SE due to plasmid loss in some of the starting cultures.

### 3.3.4 β-lactamase activity assay

The activity of β-lactamases produced in broth and in biofilms was assessed by a nitrocefin assay:

**Broth.** Cultures were grown as before (3.3.3). After 24 h they were centrifuged at 4600 rpm for 15 min. The supernatant was used for measuring the activity of the extracellular β-lactamases and the pellet was stored at $-20\,^{\circ}$C until processing. For measuring the intracellular activity the pellet was resuspended in 500 µl PBS and incubated at $37\,^{\circ}$C with 1 mg/ml lysozyme and 2 mM EDTA for 1 h. The lysate was used for measurements without spinning in order to capture any β-lactamases attached to cell membranes. For the measurements 2 µl of the sample was mixed with 198 µl and 20 µg nitrocefin in 96-well plates and placed in a spectrophotometer for up to 18 h. For the Low Resistant and Susceptible cultures grown on LB 20 µl sample was mixed with 180 µl PBS due to expected low enzyme activity.

**Biofilms.** Biofilms were grown as before (3.3.2) and after a 24 h exposure to 1000 µg/ml ampicillin they were disrupted in 5 ml PBS and centrifuged at 4600 rpm for 15 min. The supernatant was used to measure extracellular activity and the pellet was stored at $-20\,^{\circ}$C until processing. For measuring the intracellular activity the pellet was resuspended in 5 ml PBS and incubated at $37\,^{\circ}$C with 1 mg/ml lysozyme 2 mM EDTA for 1 h. For the measurements 180 µl of the sample was mixed with 20 µg nitrocefin in 96-well plates and placed in a spectrophotometer for up to 18 h. The $OD_{486}$ results were corrected for reaction volume before processing.

### 3.3.5 Statistical analysis

Statistical analysis was performed with R Core Team [2013]. Package *nlme* [Pinheiro et al., 2016] was used for the linear mixed effects models. Fixed and random effect residuals were checked for normality and homoscedasticity by plotting. Residuals appeared to be sufficiently normal for the practical purposes of analysis. A top-down model building strategy was used in all cases. The best model fit was chosen on the basis of the Akaike Information Criterion (AIC) and the effect on the model assumptions, i.e normality & homoscedasticity of residuals. After the significance of fixed variables was checked, insignificant terms were removed by model simplification. ANOVA comparisons of simplified models were performed in addition to AIC tests. Further details about the the fitted models are provided in the relevant sections.

## 3.4 Results

### 3.4.1 Population dynamics in ageing, undisrupted biofilms

Mixed strain biofilms and single strain biofilms were grown on LB for 24h before being exposed to ampicillin for several days, undisrupted. Control biofilms were allowed to grow on LB for the same amount of time. Biofilms were sampled daily and the total cell density, as well as the proportion of resistant cells was measured.

#### 3.4.1.1 Viability of susceptible cells in ageing biofilms

The generations of bacteria after 24 h of exposure to 1000 µg/ml ampicillin was calculated in mixed and mono-culture biofilms. As can be seen in Fig. 3.2

susceptible cells in mixed cultures grew better at higher proportions of resistant bacteria in the founding populations. This was the case both in the presence in absence of the antibiotic. Notably, a low proportion of resistant cells in the initial population did not allow for the susceptibles to grow in the presence of ampicillin. Moreover, susceptible cell numbers reduced in exposed, mono-culture biofilms, where β-lactamase producers were absent. Growth in this graph was measured as number of susceptible generations, where *generations = [log(no of susceptible cells at the final point) - log( number of cells at the initial point)]/log2.*

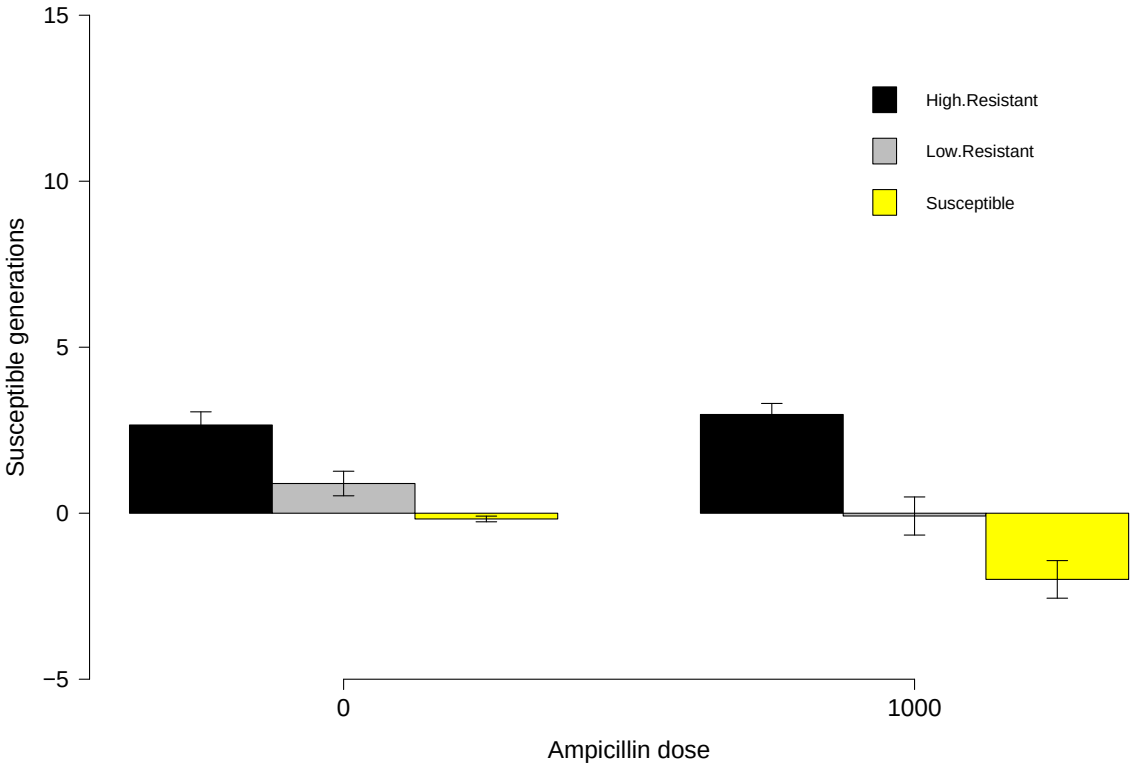


**Figure 3.2:** The generations of susceptible bacteria in ageing biofilms after 1 day of exposure to 1000 μg/ml ampicillin compared to the controls growing in the absence of the antibiotic.

After a 3-day exposure to ampicillin, the relative fitness of the susceptible cells in HR biofilms remained similar to what it was in the absence of the antibiotic. In exposed LR biofilms though, the susceptible fitness decreased. The decrease was more profound in exposed biofilms compared to unexposed ones. Additionally,

plasmid loss in R biofilms resulting in susceptibles gaining in fitness (Fig. 3.3).

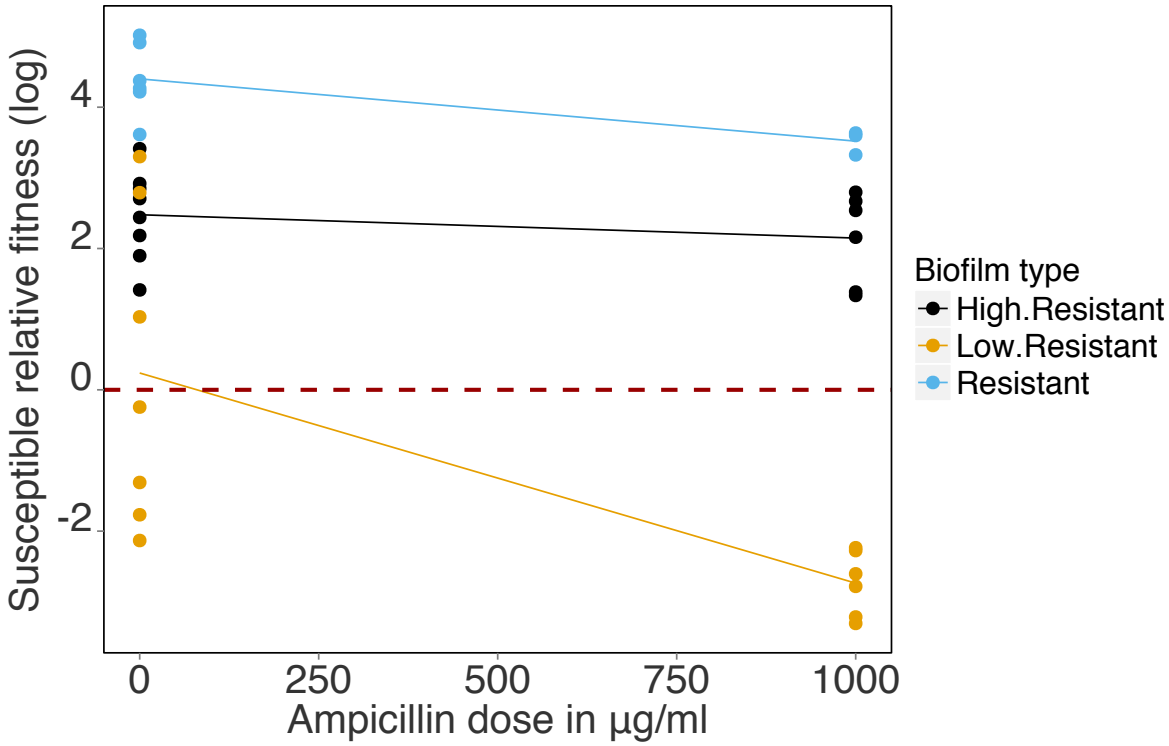


**Figure 3.3:** Relative susceptible fitness in ageing biofilms over time at 0 or 1000 µg/ml ampicillin. Each colour represents a different resistant proportion in the inoculum The Y axis is in a log scale where 0 equals $10^0$.

#### 3.4.1.2 Proportion of resistant cells in ageing biofilms

In line with the results so far, the proportion of resistant bacteria in undisrupted biofilms after a 3-day exposure decreased when they were abundant (R), remained relatively stable in HR biofilms and increased slightly in LR biofilms. In the absence of the antibiotic resistant cells showed a similar pattern in most cases but they tended to be eliminated when initially available in low numbers (LR) (Fig. 3.4).

The initial resistant proportion was the most significant factor affecting the

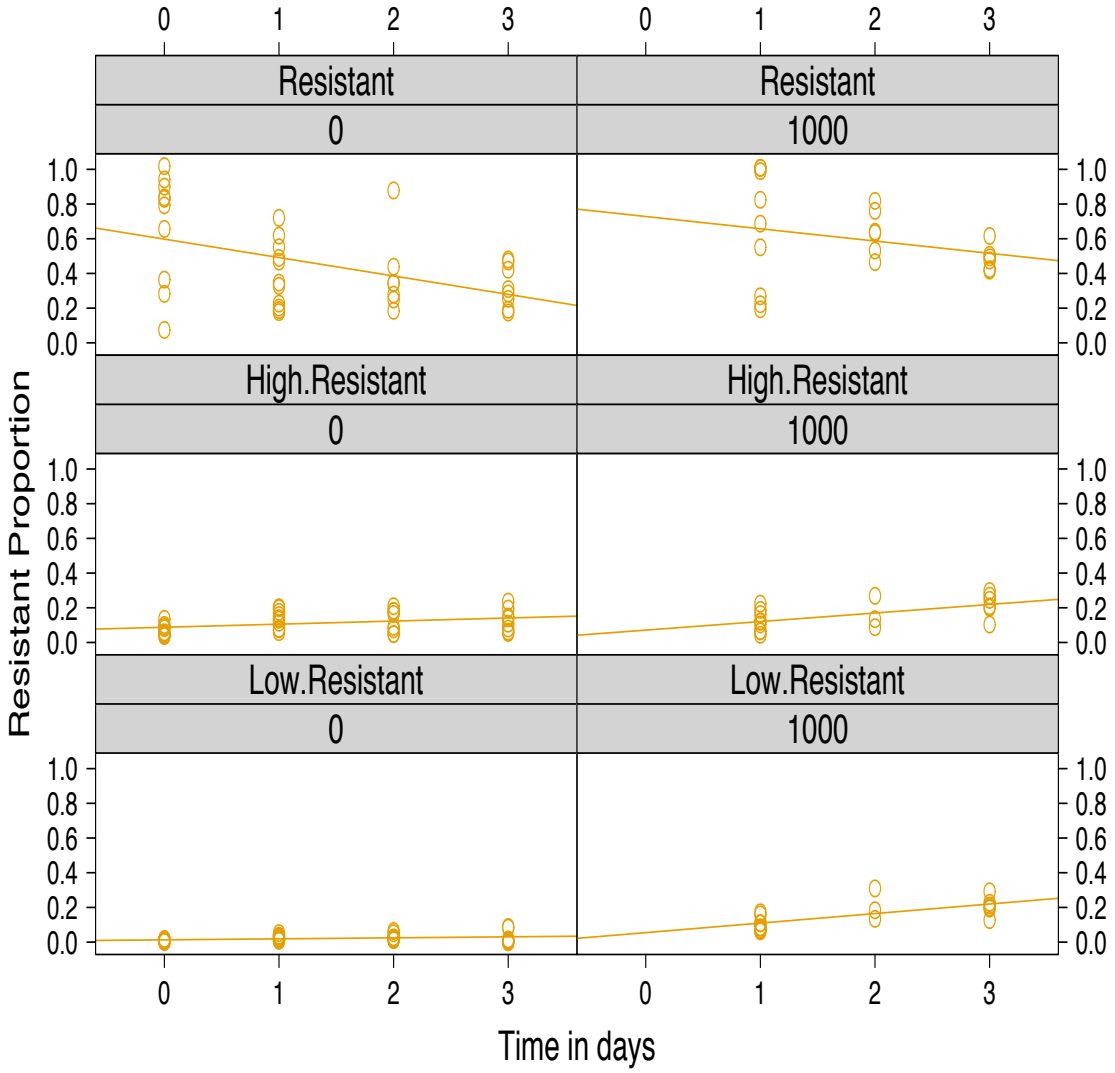


**Figure 3.4:** The proportion of resistant bacteria retained in ageing biofilms when growing in the absence and in the presence of ampicillin at an ampicillin dose of either 0 or 1000 µg/ml.

final proportions. Time affected the proportions on the basis of initial proportions and so did antibiotic dose. The model explained approximately 50% of the variance in final resistant proportions [$F(3,162)=55.53$, $p<0.0001$]. Table 3.1 shows the process of model reduction with AIC values.

#### 3.4.1.3 Culture viability in ageing biofilms as measured by cell abundance

In parallel, the total population size of exposed biofilms increased over time in all cases but in the exposed mono-culture susceptible biofilms, as can be seen in Fig. 3.5. The population size in unexposed biofilms also increased in all cases.

**Table 3.1:** Model fitting for the change in the frequency of resistant cells in the ageing biofilms assay. Model 5 was the best fit model. DF: degrees of freedom

| Mod | Term removed | AIC | DF |
|---|---|---|---|
| 1 | Full model | -84.34059 | 9 |
| 2 | dose | -86.16205 | 8 |
| 3 | time | -87.67751 | 7 |
| 4 | Initial prop:time:dose | -88.84336 | 6 |
| 5 | Initial prop:dose | -90.25276 | 5 |

### 3.4.2 Population dynamics in mixed and re-established biofilms & broth cultures

In order to test the effect of mixing and structure disturbance on population dynamics in biofilms, mixed strain biofilms and single strain biofilms were grown on LB for 24 h before being exposed to ampicillin and disrupted again in a continuing cycle. A broth passage assay was also performed in order to compare the dynamics in the two modes of growth.

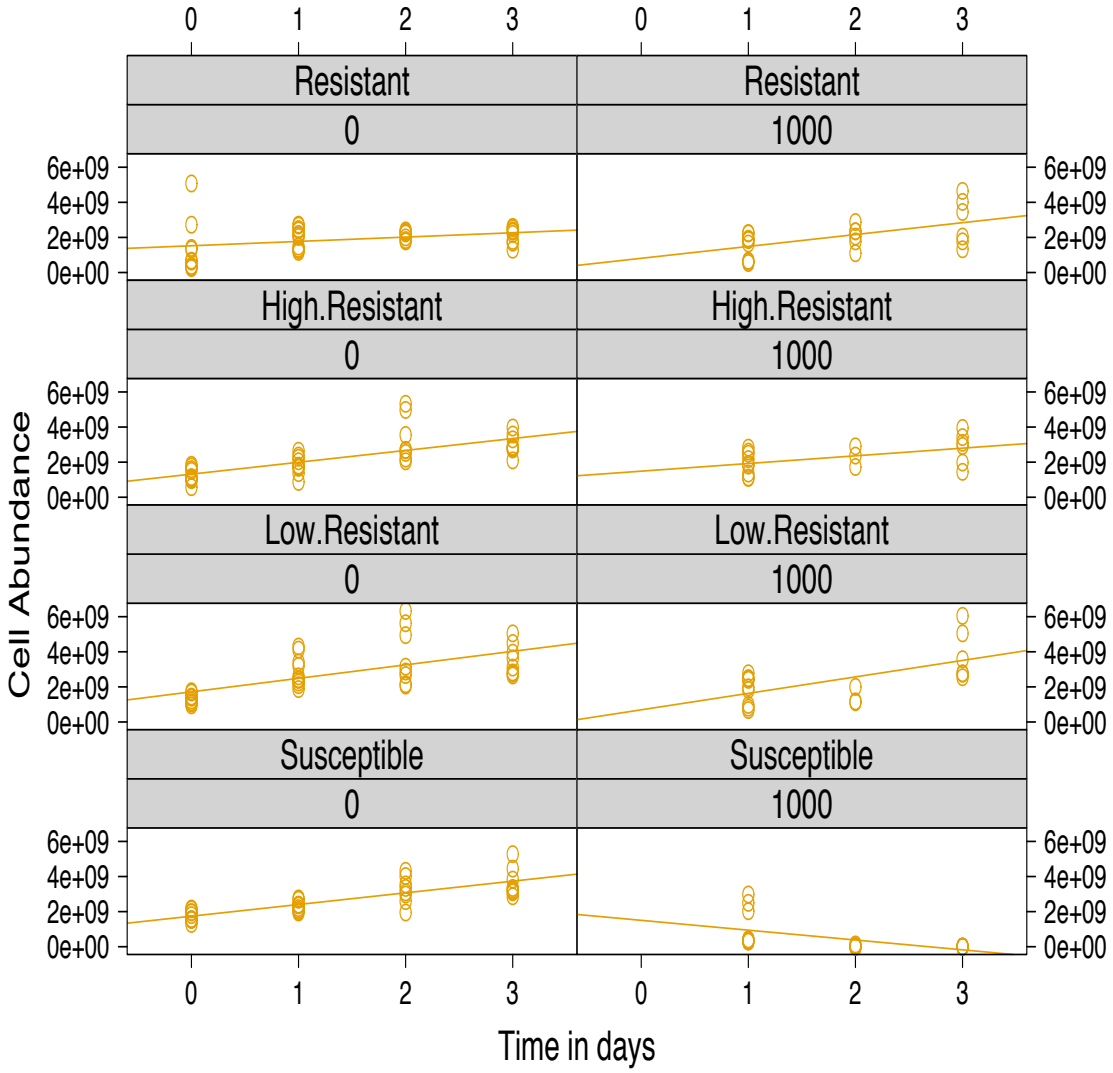


**Figure 3.5:** Cell abundance in ageing biofilms when growing in the absence and in the presence of ampicillin (1000 µg/ml).

#### 3.4.2.1 Viability of susceptible cells during the passage assays

As can be seen in Fig. 3.6 the growth of susceptible cells after one passage cycle in biofilms was favoured more than in the undisrupted biofilms discussed above. Once more, in HR biofilms susceptible cells grew better than in LR or pure susceptible biofilms. Susceptible growth also depended on the ampicillin dose with better growth in lower doses in LR and S biofilms. In HR biofilms where producers were more in numbers, the dose did not seem to affect the susceptible growth.

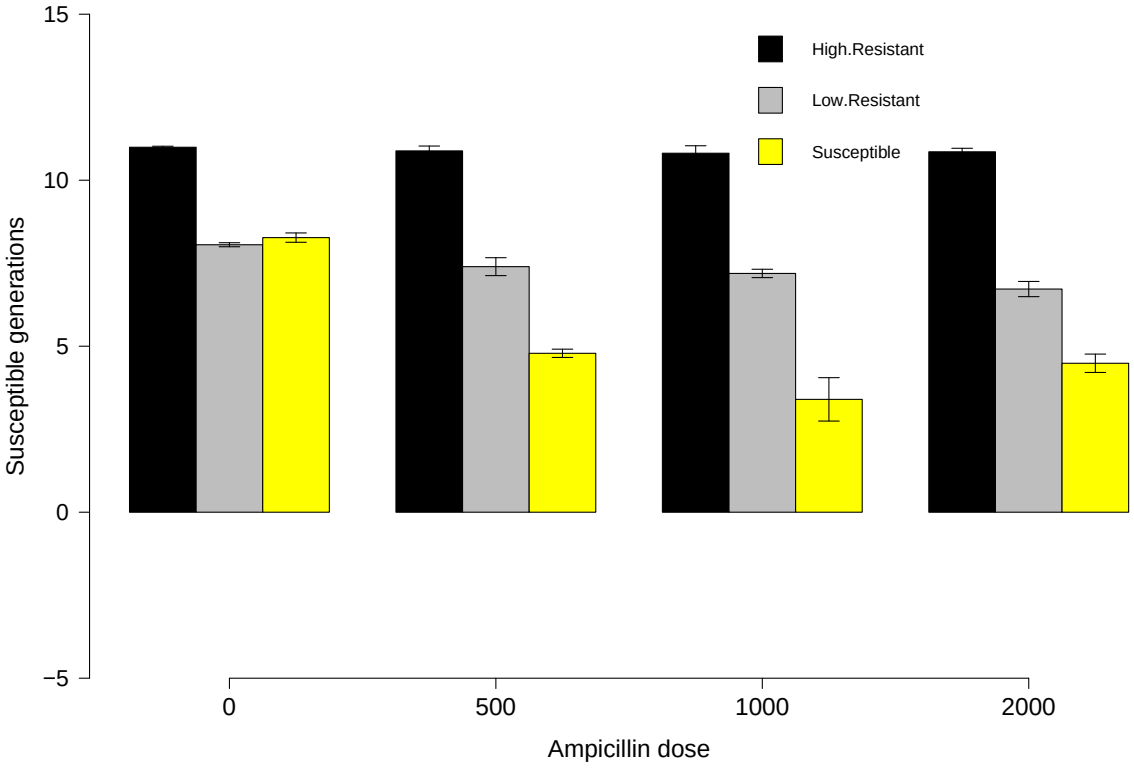


**Figure 3.6:** The mean generations of susceptible bacteria in mixed and re-established biofilms after 1 cycle. Ampicillin doses are presented in μg/ml. The error bar represents 1 SE.

In the case of broth cultures, the susceptible cells were more profoundly affected by the antibiotic. Purely susceptible cultures did not grow in the presence

of the antibiotic and at the highest dose the susceptible cells in LR biofilms were reduced in numbers. In this case as well susceptible cells did better in HR biofilms were resistant cells in the founding population were abundant (Fig. 3.7).

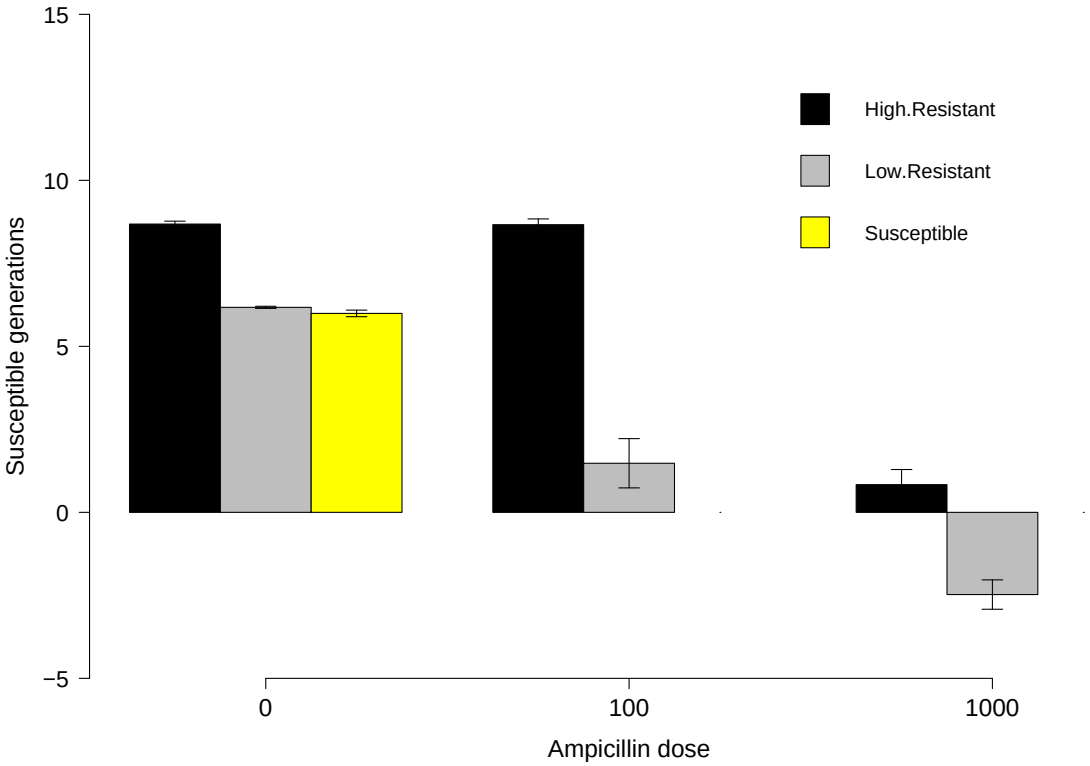


**Figure 3.7:** The mean generations of susceptible bacteria in broth after 1 passage cycle. Ampicillin doses are presented in μg/ml. The error bar represents 1 SE.

The relative fitness of the susceptible cells in mixed HR and LR biofilms or broth cultures, after 5 passage cycles, can be seen in Fig. 3.8. Higher fitness was achieved in exposed biofilms compared to exposed broth cultures and in HR biofilms in all cases. An antibiotic dose of 1000 μg/ml had a bigger effect on susceptible fitness in broth cultures compared to biofilms.

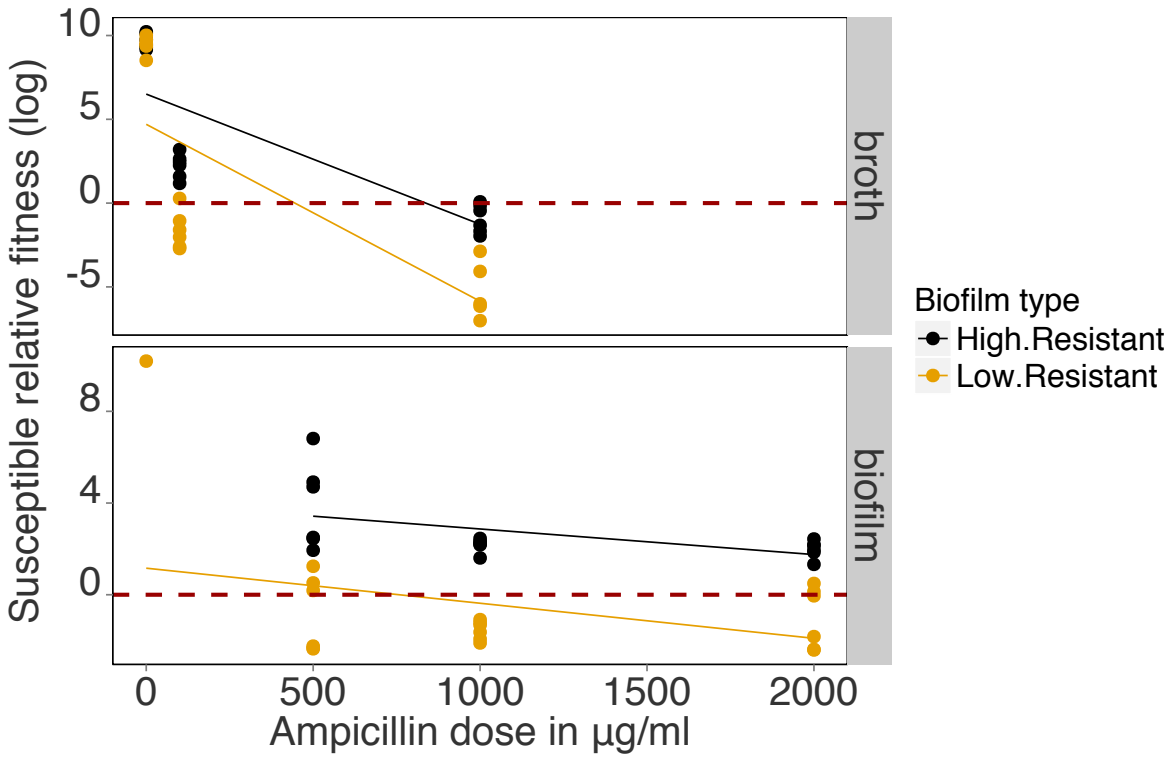


**Figure 3.8:** Relative susceptible fitness in broth and in mixed and re-established biofilms after 5 passage cycles at different antibiotic doses. Each colour represents a different resistant proportion in the inoculum The Y axis is in a log scale where 0 equals $10^0$.

#### 3.4.2.2 Proportion of resistant cells during the passage assays

The proportion of resistant cells surviving under these conditions tended to decrease overtime until reaching a similar proportion irrespective of the initial proportion in the inoculum (Fig. 3.9) after 5 passages. This convergence point depended on the ampicillin dose and the mode of growth (Fig. 3.11). The proportion converged in biofilms at 0.08+/-0.005, 0.14+/-0.01 and 0.18+/- 0.05 at 500, 1000 and 2000 μg/ml ampicillin respectively. When no antibiotic was present in the media the proportion of resistant bacteria was reduced to nearly zero in all types (R, HR, LR, S) of biofilms (Fig. 3.9).

For modelling the change in the proportion of resistant bacteria in the biofilm passage assays, the response variable was modelled against time, antibiotic dose, initial resistant proportion and experimental block (results from 2 independent assays were pulled together for this analysis). Lineage was used as a random intercept with or without time as a random slope. Different variance-covariance structures were tested for a more accurate representation of the correlation between the residuals. As can be seen in Table 3.2 the model with time as a random slope and a compound symmetry variance-covariance structure was the best fit according to the AIC (baseline model). This model was then simplified by removing insignificant fixed effects one by one and checking the AIC against the previous model and against the baseline model. ANOVA comparison did not show significant differences between the simplified models and therefore the decision for the best fit was based on the AIC as shown in the table.

Initial resistant proportion ($\beta$= 0.81, SE=0.07, p=0.0) and antibiotic dose ($\beta$= 0.0006, SE=0.00001, p=0.0001) had a significant effect on the resistant proportion in biofilms. Time was also significant ($\beta$= 0.021, SE=0.01, p=0.07). Time affected differently the proportion of resistant bacteria depending on the initial proportions ($\beta$=- 0.18, SE=0.02, p=0.0). The interaction between initial proportion, dose and time was also significant ($\beta$= 0.00001, SE=0.000005, p=0.007).

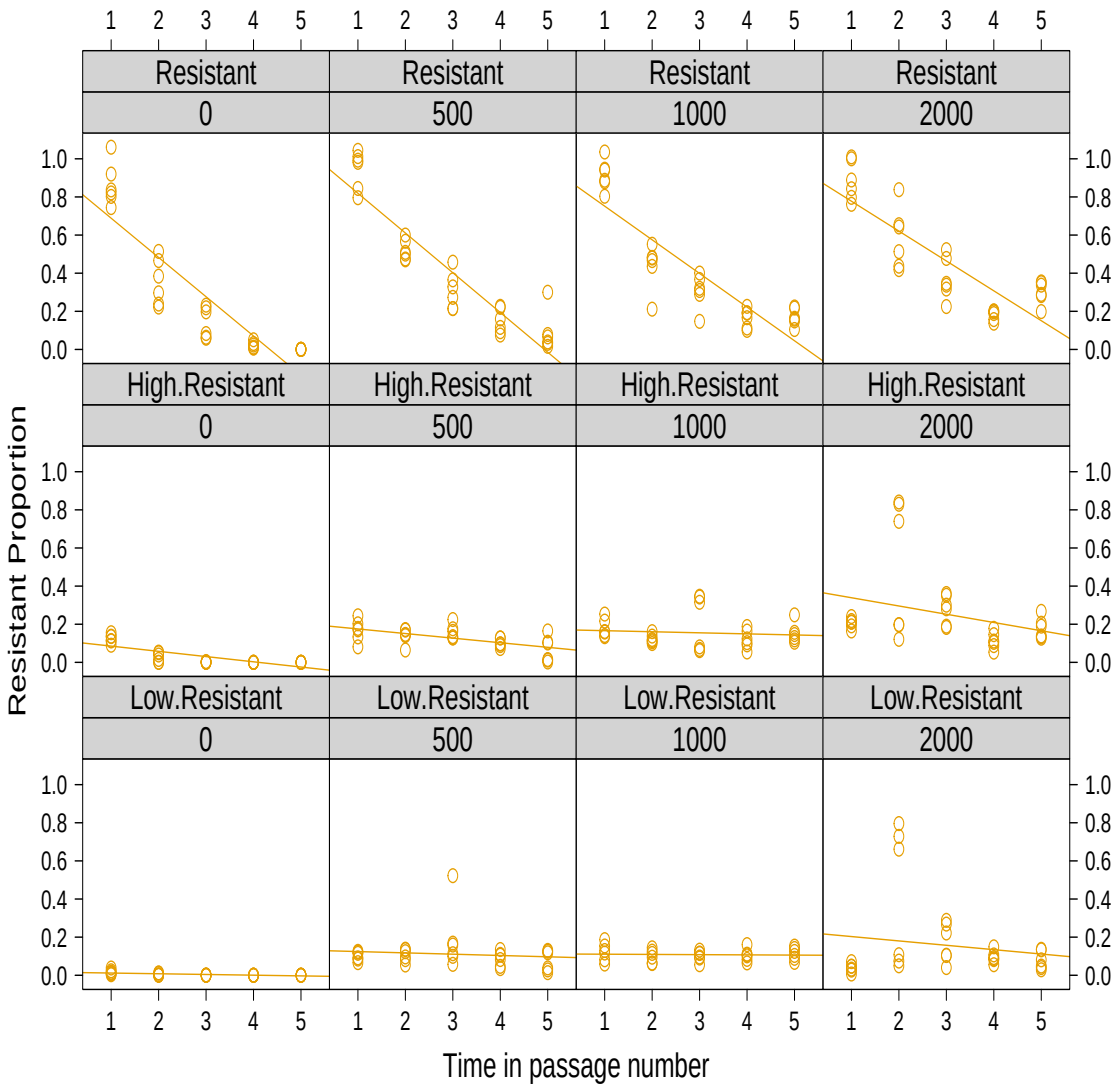


**Figure 3.9:** The proportion of resistant bacteria retained in biofilms during a 5 day passage at different antibiotic doses (0, 500, 1000, 2000 μg/ml). The biofilm types indicate the proportion of resistant bacteria in the inoculum, with High.Resistant mean =0.49+/- 0.02 SE, Low.Resistant mean=0.02+/-0.01 SE and Resistant mean=0.9+/- 0.09 SE.

**Table 3.2:** Model fitting for the change in the frequency of resistant cells in the biofilm passage assay. Model 6 was chosen as the most suitable model based on the AIC values and it was simplified (models 9-12). Model 11 was the best fit model. I: intercept, S: slope, DF: degrees of freedom

| Mod | Random effects | Variance-covariance | Term removed | AIC | DF |
|---|---|---|---|---|---|
| 1 | I: lineage | default | | -337.5511 | 11 |
| 2 | S:time, I:lineage | default | | -367.2016 | 13 |
| 3 | I: lineage | compound symmetry | | -335.5511 | 12 |
| 4 | I: lineage | diagonal | | -335.5495 | 12 |
| 5 | I: lineage | first order autoregression | | -338.5754 | 12 |
| 6 | S:time, I:lineage | compound symmetry | | -388.5025 | 14 |
| 7 | S:time, I:lineage | diagonal | | -335.5495 | 12 |
| 8 | S:time, I:lineage | first order autoregression | | -370.7132 | 14 |
| 9 | S:time, I:lineage | compound symmetry | blocks | -389.9835 | 13 |
| 10 | S:time, I:lineage | compound symmetry | initial prop:dose | -390.8539 | 12 |
| 11 | S:time, I:lineage | compound symmetry | time:dose | -392.8079 | 11 |
| 12 | S:time, I:lineage | compound symmetry | time | -391.4999 | 10 |

In broth cultures the proportion of resistance cells decreased over time in R biofilms irrespective of the dose. In HR and LR biofilms exposed to the highest dose the proportion of resistant cells was increased and then retained over time. A convergence was noticed in this case as well (Fig. 3.11). The proportion of convergence in broth was 0.16+/-0.008 and 0.79+/-0.016 at 100 and 1000 μg/ml respectively. In unexposed cultures resistant cells were eliminated over time (Fig. 3.10).

For modelling the change in the proportion of resistant bacteria in the broth passage assays, the response variable was modelled against time, antibiotic dose, initial resistant proportion and experimental block. Lineage was used as a random intercept with or without time as a random slope. Different variance-covariance structures were tested as for the biofilm data. As can be seen in Table 3.3 the model with time as a random slope and no within group correlation (default) variance-covariance structure was the best fit according to the AIC. This model was then simplified by removing insignificant fixed effects one by one and checking the AIC against the previous model and against the baseline model. Again

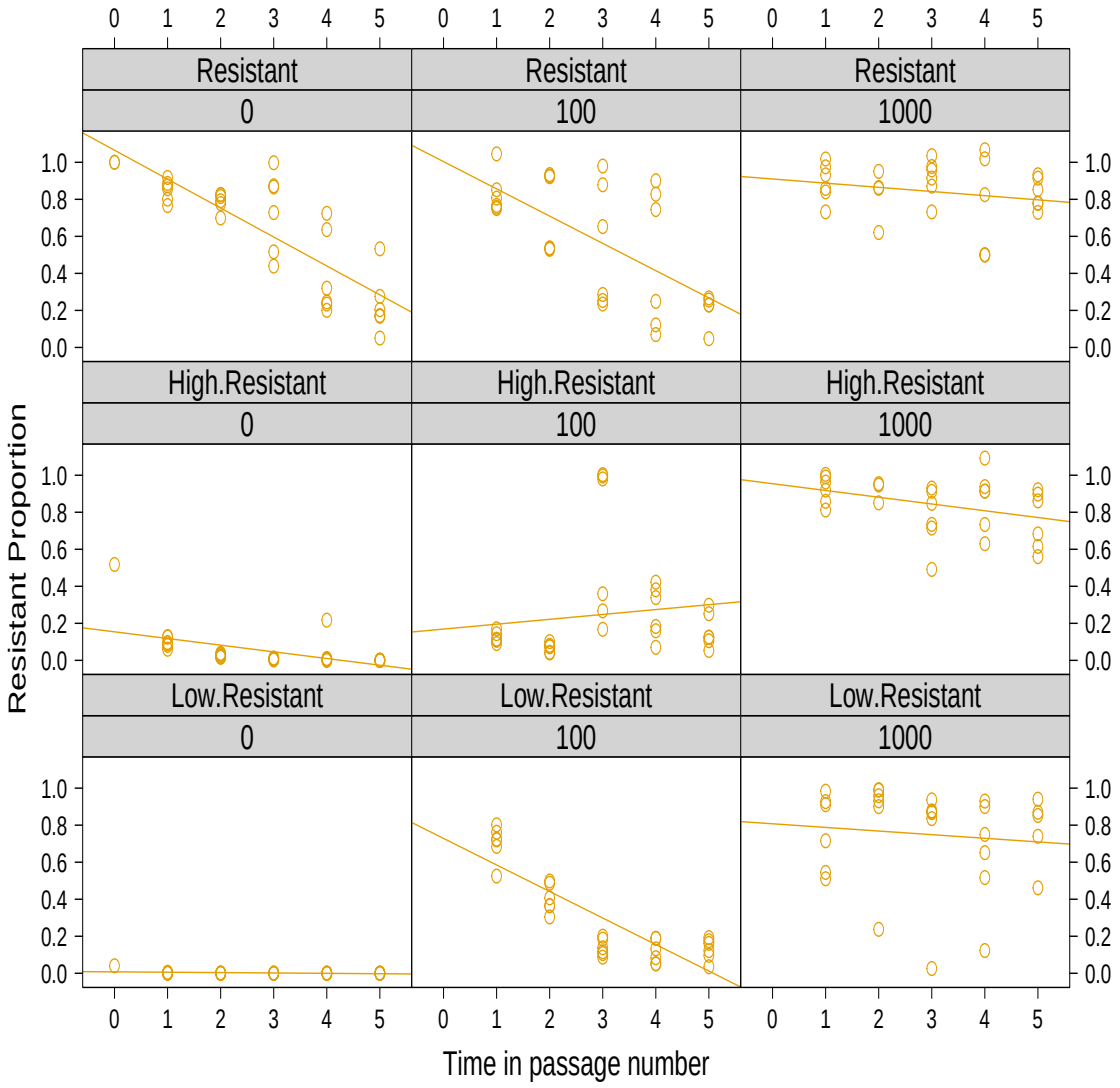


**Figure 3.10:** The proportion of resistant bacteria retained in broth during a 5 day passage at different antibiotic doses (0, 100, 1000 µg/ml). The biofilm types indicate the proportion of resistant bacteria in the inoculum, with High.Resistant mean =0.49+/- 0.02 SE, Low.Resistant mean=0.02+/-0.01 SE and Resistant mean=0.9+/- 0.09 SE.

ANOVA comparison did not show significant differences between the simplified models and therefore the decision was based on the AIC as shown in the table.

Initial resistant proportion (β= 0.68, SE=0.12, p=0.0) and antibiotic dose (β=0.0007, SE=0.00007, p=0.0) had a significant effect on the resistant proportion in biofilms. Time also had significant effect (β= -0.03, SE=0.02, p=0.06). Time affected differently the proportion of resistant bacteria depending on the initial proportions (β= -0.08, SE=0.03, p=0.003) and the interaction between initial proportion, dose and time was also significant (β= 0.00008, SE=0.00003, p=0.008).

**Table 3.3:** Model fitting for the change in the frequency of resistant cells in the broth passage assay. Model 1 was chosen as the most suitable model based on the AIC values and it was simplified (models 3-6). Model 5 was the best fit model. I: intercept, S: slope, DF: degrees of freedom

| Mod | Random effects | Variance-covariance | Term removed | AIC | DF |
|---|---|---|---|---|---|
| 1 | S:time, I:lineage | default | | -50.20969 | 13 |
| 2 | I: lineage | default | | -40.34369 | 11 |
| 3 | S:time, I:lineage | compound symmetry | | -48.20972 | 14 |
| 4 | S:time, I:lineage | diagonal | | -38.34369 | 12 |
| 5 | S:time, I:lineage | compound symmetry | time:dose | -52.18319 | 12 |
| 6 | S:time, I:lineage | compound symmetry | blocks | -51.26655 | 11 |

The effect of the antibiotic dose on the proportion of resistant bacteria at the convergence point can be seen in Figure 3.11. For an ampicillin dose of 1000 μg/ml the proportion of resistant bacteria required to maintain a stable total population as the convergence point in broth was 79% whereas in biofilm it was 14%.

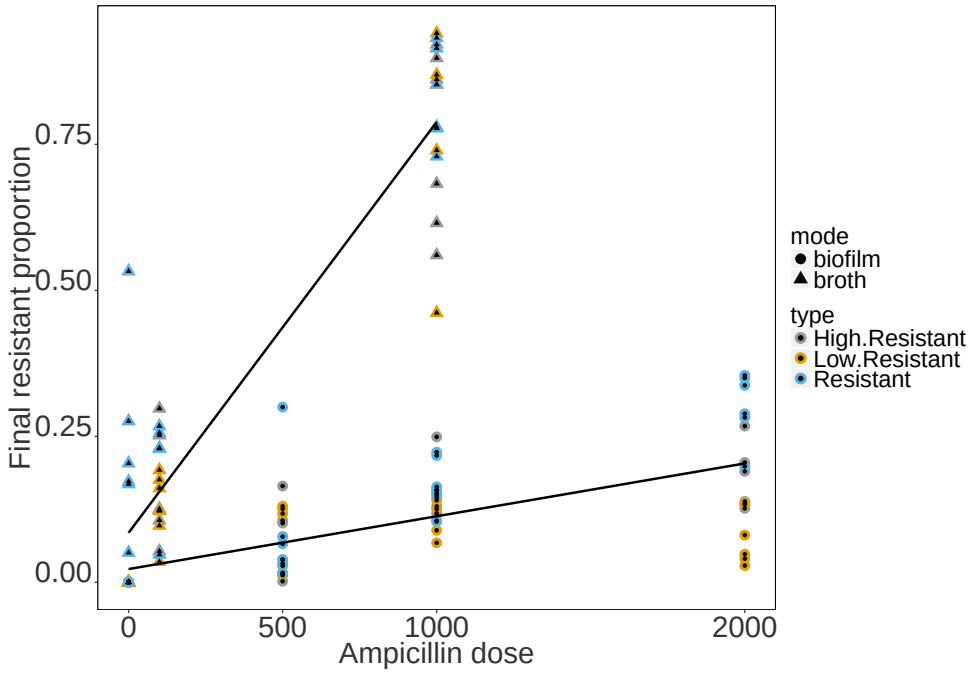


**Figure 3.11:** The change in the convergence point (passage point 5) proportion as affected by the antibiotic dose (µg/ml).

#### 3.4.2.3 Culture viability during the passage assays as measured by cell abundance

The resistant proportion at the convergence point reflected the amount of resistant bacteria required to maintain the total cell density at a level similar to the one in ampicillin free populations as can be seen in Figures 3.12 & 3.13. The total cell density remained similar under all antibiotic doses tested when resistant bacteria were present in the culture, irrespective of the starting proportion. When the population consisted of susceptible bacteria only, a 10-fold decrease in total cell density was observed in biofilms whereas population death occurred in broth cultures (Figures 3.12 & 3.13). In the case of the ageing biofilms the total cell abundance increased, when during the passage it remained stable (Fig. 3.5 & 3.12).

### 3.4.3 β-lactamase activity in broth and biofilms

The average β-lactamase activity per resistant cell in broth and biofilms was assessed according to the level of nitrocefin hydrolysis achieved after 10 min of reaction. As it can be seen in Fig 3.14A, more extracellular enzyme was present in broth cultures. In biofilms more intracellular enzyme was recorded in those biofilms exposed to ampicillin whereas when no antibiotic was present the levels were more variable. The overall enzymatic activity was higher in biofilms. The variation of enzyme activity was higher in biofilms but this can probably attributed to the varying levels of resistant bacteria present compared to exposed broth cultures that had reached similar proportions at the time of the assay (Fig. 3.14C).

When the different types of biofilms were looked at more closely (Fig. 3.14B) it appeared that in biofilms the level of intracellular enzyme was always higher than the extracellular but also, the less resistant bacteria present the more enzyme per cell was produced. In broth cultures exposed to ampicillin nearly all the produced

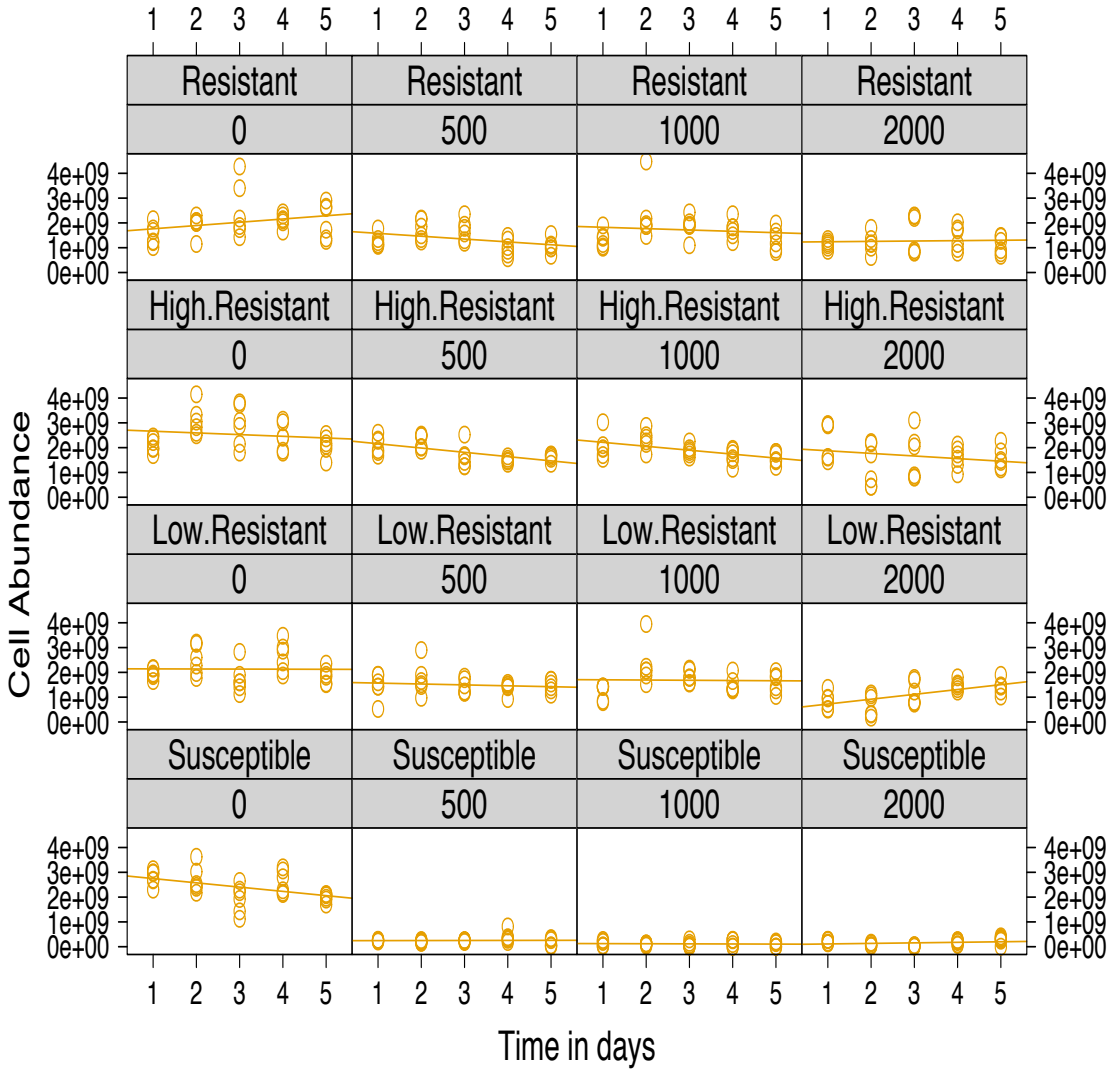


**Figure 3.12:** Cell abundance in disrupted and re-established biofilms over time at different antibiotic doses (0, 500, 1000, 2000 µg/ml).

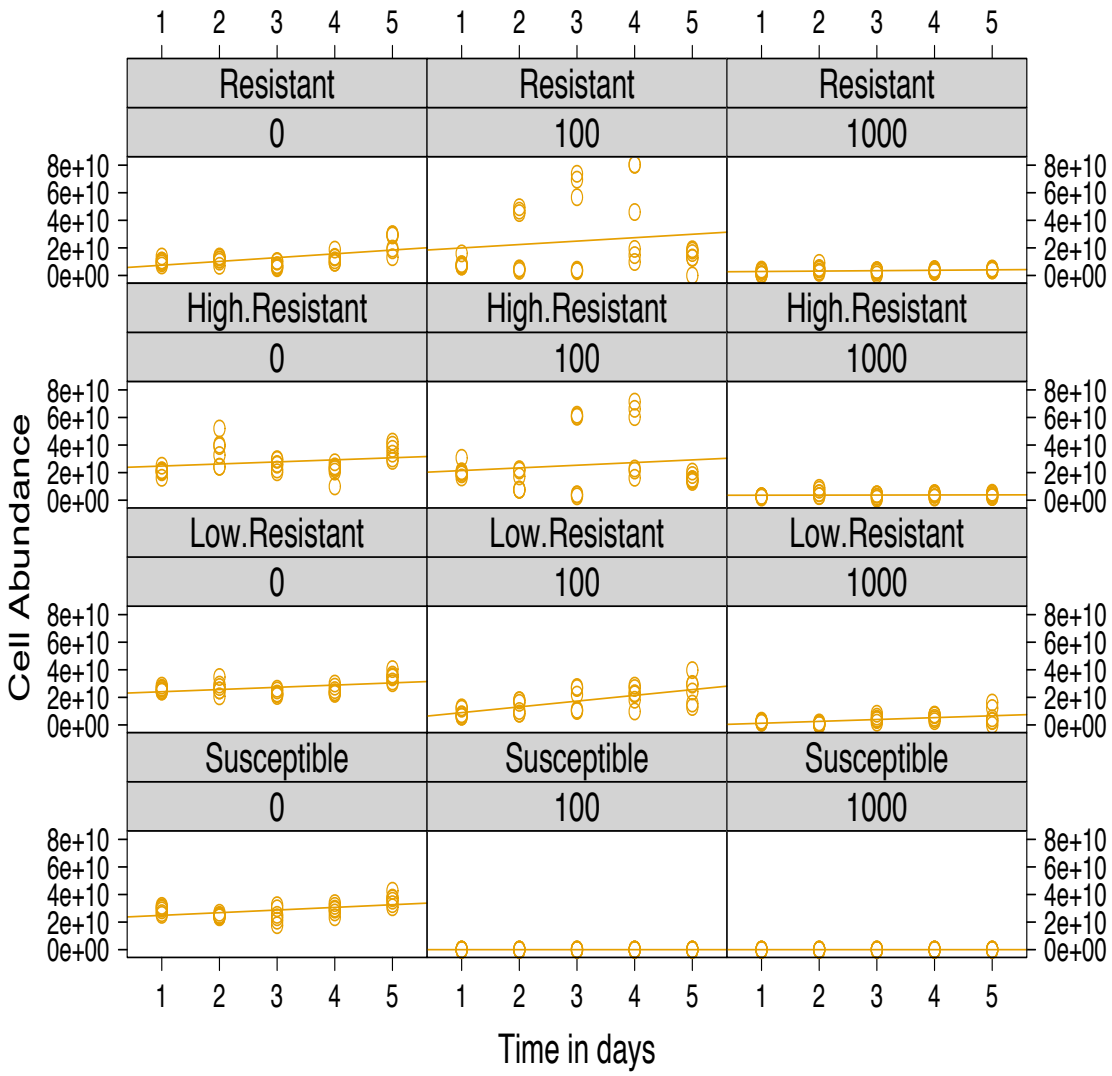


**Figure 3.13:** Cell abundance in broth over time (0, 100, 1000 µg/ml).

enzyme was extracellular. Enzyme production in unexposed broth cultures was generally very low.

### 3.4.4 Discussion

A static biofilm system with no liquid interface, carrying a non-conjugative, high copy plasmid was used in this work. The social trait, i.e the β-lactamase $bla_{\text{CTX-M-14}}$ gene which confers resistance to extended spectrum β-lactam antibiotics was carried on this plasmid. β-lactamase carrying plasmids have been found to inhibit biofilm formation [Gallant et al., 2005], although in other occasions the opposite effect was observed [Neupane et al., 2016; Teodósio et al., 2012]. As seen in cell abundance and biofilm growth results here and in Chapter 4 no plasmid and/or resistance gene inhibitory effect was observed on biofilm formation.

Biofilms offer an ideal environment for microbial interactions due to high cell density and proximity [Stewart & Franklin, 2008]. Kin selection theory, as applied to biofilms is mainly concerned with spatial localisation and public good or cell diffusion as seen in section 3.1.4. In short, proximity to relatives and limited public good diffusion or cell dispersal is expected to favour the cooperative trait. Therefore, cell segregation leads to cooperation when cell mixing favours competition.

The main social traits usually studied in a biofilm context are the production of EPS [Kim et al., 2016; Seminara et al., 2012; Vlamakis et al., 2008], Quorum Sensing (QS) [Popat et al., 2012; Zhou et al., 2014] and nutrient uptake and growth dynamics [Kim et al., 2016; Liu et al., 2015; Ren et al., 2015]. Here, I set out to understand social interactions in relation to the production of β-lactamases, an enzyme that has been characterised as potentially social before (Section 3.1.3). In order to test how biofilms responded to the antibiotic when resistant bacteria were present and when they were not, different types of biofilms, defined by the proportion of resistant bacteria in the founding population, were grown for 24 h

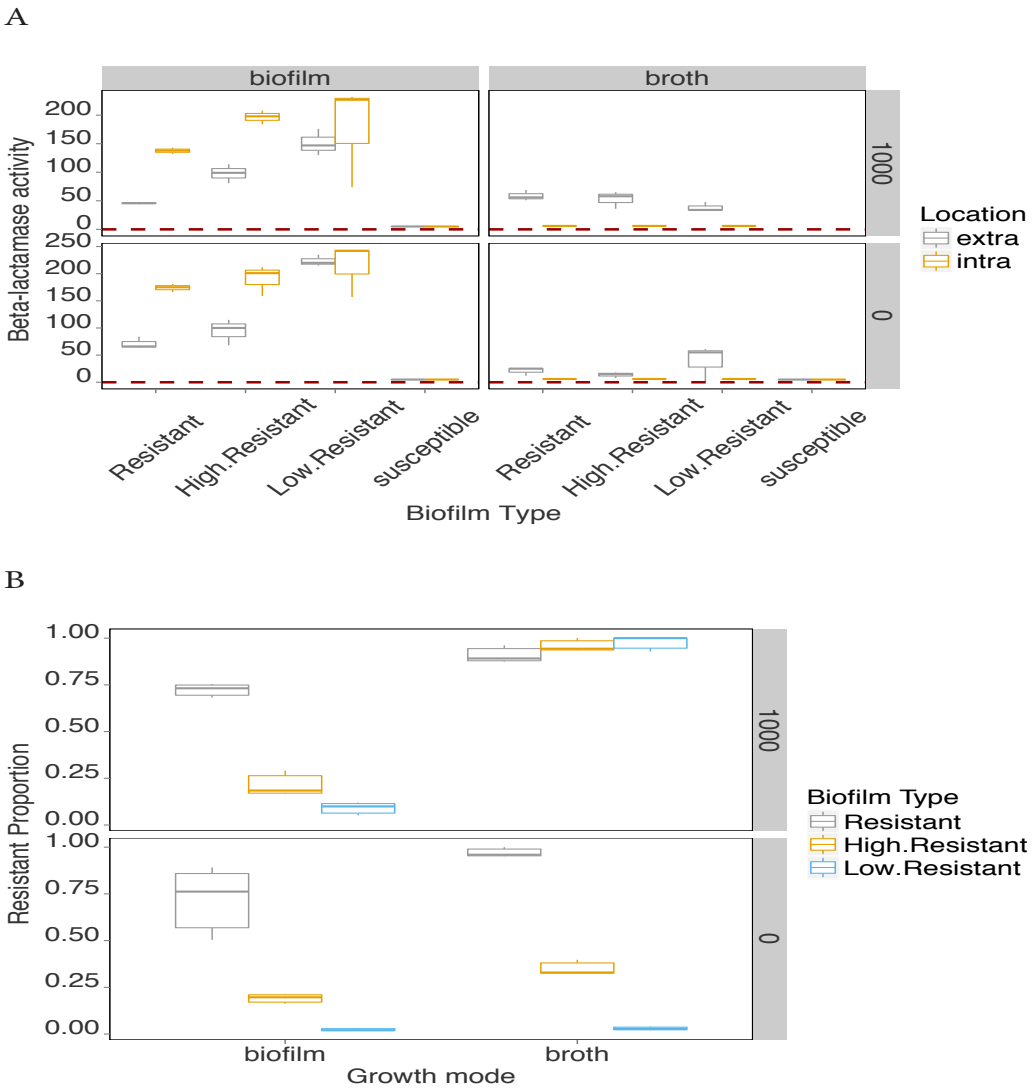


**Figure 3.14:** β-lactamase activity plots, showing the level of extracellular and intracellular activity per cell, per antibiotic dose (0, 1 mg/ml ampicillin) in broth and biofilms, 10 min after the initiation of the reaction, corrected for reaction volume. (A) levels of intracellular and extracellular activity in broth and biofilms by type.The red dashed lined indicates zero activity (B) Proportion of resistant bacteria in broth and biofilms at the time of measurement.

before exposed to different antibiotic doses. Two experimental set ups allowed for the biofilm architecture to be considered as a contributing factor to resistance and to bacterial interactions.

#### 3.4.4.1 Growth in biofilms facilitates cheating only when the producers are abundant in the founding population

In the first set of experiments, biofilms were exposed to ampicillin for 3 days and sampled daily. Figure 3.4 shows that under these conditions, when the biofilm structure was maintained during the exposure, the proportion of resistant bacteria was higher in the presence of ampicillin compared to the passage. Proportions measured after a 3 day exposure were 22% in HR, 21% in LR and 49% in R biofilms. Resistant cooperator cells were only maintained or increased when the biofilms were under antibiotic stress, although the decrease in cooperator numbers appeared to be slower than in the passage. In LB, 32% resistant cells remained in R biofilms, 13% in HR and 2% in LR. Compared to survival of the cooperators in broth, these results show that plasmid carrying cells were maintained in biofilms for longer in agreement with results reported in the literature [Madsen et al., 2013]. Susceptible cells in mixed biofilms were able to benefit during exposure only when the producers were abundant in the founding population 3.2. After 3 days of exposure, the susceptible relative fitness was not affected by ampicillin in R and HR biofilms (Fig. 3.3). However, it declined in LR, exposed biofilms.

Cell abundance was lower than in the controls in all cases, indicating that the continuous exposure stressed the biofilms even in the presence of resistant cells. Fully susceptible biofilms died after the 3 day exposure. They did survive a two day exposure but the cell number reduced 100-fold after 48 h of exposure compared to what it was after 24 h.

#### 3.4.4.2 Population mixing favours cheaters both in broth and in biofilms. Cheating is higher in biofilms but reduces as antibiotic dose increases

In the second set of experiments the biofilms were disrupted after exposure to ampicillin and the disrupted population was used to establish new biofilms that were treated in the same way for a total of 5 passages. During this experiment, the structure was disrupted and the population dynamics of the newly established biofilms at each step provided insight into the evolution of strain interactions into the biofilms. As can be seen in Figure 3.9, the proportion of resistant bacteria in biofilms, where they were initially abundant decreased over time under all antibiotic doses tested. The proportions converged at approximately 8% at 500, 14% at 1000 μg/ml ampicillin, and at 18% at 2000 μg/ml. When antibiotics were not present in the medium, resistant cells were effectively eliminated, indicating that antibiotic pressure was required for plasmid maintenance in the population. The total cell abundance was not affected by the antibiotic when resistant cells were present (Fig. 3.12). WT, susceptible biofilms showed a 10-fold decrease in cell abundance but were still able to survive the treatments.

Looking at the relative fitness of susceptible bacteria in broth and biofilms (Fig. 3.9) shows that susceptible cells tended to have a higher fitness when the resistant bacteria were abundant in both modes of growth which is in good agreement with the literature [Raymond et al., 2012]. Biofilms allowed for better growth of susceptible cells under antibiotic pressure compared to broth. Increasing antibiotic dose reduced susceptible fitness (Fig. 3.8) but it remained positive under all doses studied.

These results indicate that maximal cell viability, as measured by cell abundance in the antibiotic treated biofilms compared to controls, did not require a fully resistant population of bacteria in biofilms. A very small proportion of resistant cells appeared to be enough to maintain viability at levels similar to growth without antibiotic, something that fully susceptible biofilms did not achieve. In

broth cultures, the proportion of resistant bacteria converged to approximately 80% in the presence of 1000 μg/ml ampicillin (Fig. 3.10). This was about 6 times higher than in biofilms. Therefore, although the production of β-lactamases confers protection to susceptible bacteria in broth and in biofilms, the effect was higher in biofilms. Additionally, maximal population viability was not achieved in broth cultures exposed to ampicillin even in the presence of resistant cells(Fig. 3.13). Under no antibiotic pressure, the plasmid carrying cells were eliminated in broth as well. The proportion of cooperators maintained depended on the antibiotic dose and the mode of growth as can be seen in Fig. 3.11.

#### 3.4.4.3 Patterns of β-lactamase production differ in broth and biofilms

Finally, the average production of β-lactamases per cell was measured in broth and biofilms. Given, that the fitness of cheating cells was higher in biofilms, one would expect that the public good was more readily available. Therefore, it was surprising to see that more intracellular β-lactamases were recorded in biofilms compared to broth. However, the overall β-lactamase activity was higher in biofilms (Fig. 3.14A) and this suggests that the localisation of the enzyme in or outside the cells did not affect its potential as a public good. Although, production of β-lactamases in response to antibiotics has been found to be higher in biofilms compared to planktonic cells before [Giwercman et al., 1991] the phenomenon has not been explained. In the present experiments, it could indicate that the constant diffusion of the antibiotic in the biofilms from the agar does not allow the cells to permanently detoxicate the environment and therefore they are required to produce the enzyme in high quantities. However, enzyme production was also high in unexposed biofilms.

Another potential explanation is that the high cell density in biofilms and close proximity to neighbours may trigger more enzyme production. High cell density has been shown to activate antibiotic production mechanisms before [Bainton et al., 1992] and to protect bacteria against antibiotics [Butler et al., 2010; Udekwu

et al., 2009]. Since the intracellular enzyme also has a social effect and the assumption is that the cooperators are surrounded by relatives, they do not need to excrete the public good and risk its diffusion in the biofilm.

In broth nearly all the enzyme produced was extracellular. In this case the population is expected to be well-mixed and with no spatial structure, so the cooperators are more likely to encounter cheaters and that would reduce their fitness. However, the increased fitness of the cooperators under these conditions is not in agreement with theory.

The proportion of resistant bacteria present in the cultures at the time of measurement (Fig. 3.14C) affected the β-lactamase production per cell with more enzyme produced by cells when they were rare, suggesting a potential mechanism that allows the same viability of biofilms with different levels of resistant cells present. This result indicates high cooperation levels once more, and expected proximity to neighbours. The antibiotic exposure increased β-lactamase production per cell in broth but it did not affect the total enzyme production in biofilms.

#### 3.4.4.4 Conclusion

In conclusion, biofilms that grow undisrupted favour the growth of the cooperators. In contrast to what theory predicts though,the cooperative strain increased in numbers when rare and decreased when abundant. When mixing was imposed on the population cheaters were favoured and the effect was again higher when the cooperators were abundant. In this case cheating depended on the antibiotic dose, with higher doses having a limiting effect. A higher proportion of resistant cells in the founding population also favoured the cheaters. Growing in broth made cheating more difficult under high antibiotic doses. Finally, the production of β-lactamases appeared to be mostly intracellular in biofilms but extracellular in broth.

# Chapter 4

# The biofilm structure

## 4.1 Introduction

This chapter is about the visualisation of the biofilm structure and its response to antibiotic treatment in conjunction with social interactions. An introduction to the theory behind the development of the specific hypothesis is followed by the description of the experimental work. The results of the experiments and future directions are discussed towards the end of the chapter.

### 4.1.1 Antibiotic penetration into the biofilm

As discussed in section 1.2.1 it has been proposed that the biofilm matrix may be responsible for the increased antibiotic tolerance of bacterial biofilms either due to total prevention of penetration or due to reduced diffusion associated with the biofilm matrix. Indeed, *Pseudomonas* biofilms have been shown to be highly tolerant to aminoglycosides and β-lactams (tobramycin, piperacillin) on several occasions [Anwar et al., 1989; Hoyle et al., 1992]. In most cases, however, al-

though the presence of the matrix was suggested as a possible reason for this increased tolerance, a link between the matrix and the reduced antibiotic penetration could not be established. Such links were supported on some occasions, e.g. tobramycin has been shown to bind to matrix components after those were isolated from *Pseudomonas* biofilms. Nevertheless, this binding did not result in a large enough reduction of the diffusion coefficient to protect the cells under roughly physiological conditions [Nichols et al., 1988]. The extracellular DNA in the matrix of *Pseudomonas* biofilms has been associated with increased tolerance to cationic antimicrobial peptides via the induction of a resistance operon but had no effect on β-lactam (ceftazidime) and fluoroquinolone resistance (ciprofloxacin) [Mulcahy et al., 2008]. However, aminoglycosides, β-lactams and fluoroquinolones have all been shown to penetrate *Pseudomonas* biofilms grown on transwell plates without difficulty [Shigeta et al., 1997].

These observations have led to a second hypothesis, that any reduction in antibiotic diffusion rates caused by the biofilm structure may result in cell protection just by allowing for extra adaptation time [Jefferson et al., 2005]. This was supported by experimental data showing that fluorescent vancomycin added to the liquid on top of biofilms reached the bottom layers of the biofilms with a delay of approximately 60 min. This was determined by the detection of fluorescent cells where vancomycin had penetrated. In broth cultures, fluorescent cells were detected within 5 min from the application of the antibiotic. A third hypothesis discussed is that although antibiotics do surmount the matrix barrier they may be removed by the water channels. If this is the case they may not be able to reach their target in bacterial cells [Costerton et al., 1994; Walters et al., 2003]. This hypothesis has been disproved for rifampin in *Staphylococcus* biofilms [Zheng & Stewart, 2002].

Oubekka et al. [2012] answered several of the discussed questions, with the use of confocal microscopy and a fluorescent antibiotic. They showed that vancomycin penetrated *Staphylococcus* biofilms within less than 8 min and actually reached the bacterial cells, as fluorescence was concentrated around the bacterial cell walls. However, no reduction in cell viability was observed. Moreover, 50% of the

antibiotic was found to be bioavailable (free to diffuse in the biofilm) indicating that it was not its binding reaction with biofilm components that was responsible for biofilm tolerance, although binding probably occurred.

Finally, the hypothesis that bacteria on the biofilm surface hydrolyse certain antibiotics, thus protecting cells inside the biofilm has also been investigated. Nichols et al. [1989] included β-lactamase action at the biofilm-antibiotic interface into a model to measure protection due to antibiotic (tobramycin and cefsulodin) hydrolysis and showed that although the matrix itself did not constitute a protection barrier, when hydrolysing enzymes were present the antibiotic concentration reaching the centre of bacterial aggregates was reduced compared to the concentration in the surrounding broth. Anderl et al. [2000] also showed that in the case of the β-lactam ampicillin, the antibiotic was able to penetrate β-lactamase deficient *Klebsiella pneumoniae* but not the β-lactamase producing strain, suggesting that the penetration of active antibiotic molecules in the biofilm was limited.

### 4.1.2 Spatial structure

Biofilms are commonly defined as structured bacterial communities with microcolonies and water channels, attached to surfaces and embedded in a self-produced extracellular matrix [de Beer & Stoodley, 1995; Flemming et al., 2007; Maeyama et al., 2004; Stewart et al., 2009]. These biofilm components form the basis of what is known as the biofilm architecture or 3D structure. However, attachment is not necessary for a bacterial aggregate to be called a biofilm [Flemming & Wingender, 2010] and not all biofilms are so complex; many are simple, flat and compact [Schuster & Markx, 2013]. Finally, biofilms can be also formed by fungi and algae [Palmer et al., 2006].

The biofilm structure can be affected by several factors including genetics, cell interactions and the environment [Schuster & Markx, 2013]. Conversely, the biofilm structure may influence all of the aforementioned factors forming a

complex network of interactions. For example, the structure changes the oxygen and nutrient availability within the biofilm [de Beer et al., 1994], resulting in physiological heterogeneity [Stewart & Franklin, 2008] and metabolic stratification [Teal et al., 2006; Tolker-Nielsen & Molin, 2000]. It is often the case that biofilm heterogeneity is referred to as biofilm structure [Beyenal, Lewandowski & Harkin, 2004]. For the purposes of this chapter, biofilm structure refers to the biofilm architecture, i.e to the biomass distribution. Textural parameters describing heterogeneity, when used, are mentioned by name as defined in section 4.1.4.

Biofilm structure also affects cell-cell interactions; highly structured, repeatable patterns have been observed when biofilms were formed with more than one strain, that were attributed to the division of labour within the community [Kim et al., 2016]. Cells on the biofilm periphery were found to be responsible for the oscillations in *Bacillus* biofilm growth, by limiting nutrient resources for cells in the middle. At the same time the periphery offered protection to external attacks by hydrogen peroxide [Liu et al., 2015]. High cell densities in the inoculum were found to influence bacterial competition in experiments by van Gestel et al. [2014]. At high initial cell densities the EPS-producers (cooperators) were at a disadvantage compared to non-producers (cheaters) as the cheaters outcompeted them.

The role of the biofilm structure in antibiotic tolerance is not well understood, although several studies have implicated some of the biofilm components as factors contributing to tolerance. For example it has been hypothesised that antibiotics may be directed out of the biofilms by entering the water channels [Costerton et al., 1994; Walters et al., 2003]. In addition to the mechanical properties of the biofilms that may be contributing to increased antibiotic tolerance, the structured environment seems to influence this tolerance in more diverse ways, for example by affecting the diffusion of molecules within the structure. In this way, spatial structure is likely to interfere with quorum sensing by limiting access to quorum signal molecules to producers only. In this way cheaters are prevented from exploiting the public good [Zhou et al., 2014]. It then follows, that if dif-

fusion of public good molecules is restricted due to spatial structure, antibiotic hydrolysing enzymes with a public good status, such as β-lactamases, may also be limited in certain niches within the biofilm. Spatial structure has also been found to allow for the maintenance of cooperative plasmids, that may be carrying antibiotic resistance genes, rather than those that do not carry the cooperative trait, i.e. cheaters [Mc Ginty et al., 2010; Xavier & Foster, 2007].

Detailed studies delved further into biofilm structure. It appears that, in biofilm grown in reactors, cell density is higher at the bottom layers and porosiy higher at the top; Most of the living bacteria were also recorded at the top layers [Zhang & Bishop, 1994]. The distribution of the bacterial biomass and its association with the EPS was also studied by Staudt et al. [2004]. More bacteria and EPS were found in the lower levels of the biofilms grown in rotating reactors, compared to the top and the EPS was not always localised around the cells.

### 4.1.3 Microscopy in biofilm research

#### 4.1.3.1 Confocal Laser Scanning Microscopy (CLSM)

CLSM has been an invaluable tool in microbiological studies [Neu & Lawrence, 2014]. The utilisation of fluorescent molecules as a staining aid or as expressed by microbial genes after genetic manipulation, in combination with CLSM and its variations [Neu et al., 2010] have found applications to the study of bacteria, fungi and viruses alike [Neu & Lawrence, 2014]. Biofilm development, studied by time-lapse or real-time imaging [Palmer et al., 2006], and architecture [Lawrence et al., 1991; Weissbrodt et al., 2013], the composition of the biofilm matrix [Neu & Lawrence, 1999], diffusion[de Beer & Stoodley, 1995], as well as, microbial interactions have been extensively studied by the use of CLSM [Neu & Lawrence, 2015].

#### 4.1.3.2 Optical Coherence Tomography

Optical Coherence Tomography (OCT) has been extensively used as a non-invasive medical diagnosis procedure in areas such as ophthalmology, histopathology and in vascular imaging [Blatter et al., 2012; Fujimoto et al., 2000]. Additionally, it has recently been used in the study of biofilms [Wagner et al., 2010; Xi et al., 2006]. OCT works by measuring the coherence of light pulses that reflect onto a reference mirror placed at a known distance from the sample and pulses that reflect onto the sample. Both signals return back to the same detector and the difference in time in reaching the detector is used to "read" the structure [Fercher et al., 2010].

### 4.1.4 Biofilm quantification using microscopy images

Biofilm quantification has been a major issue of discussion in the literature resulting in the development of sophisticated software [Beyenal, Lewandowski & Harkin, 2004; Heydorn et al., 2000; Xavier et al., 2003] that allow for the calculation of several quantitative parameters defining a biofilm. Biofilm quantification parameters can be defined as textural or areal-volumetric, describing heterogeneity and morphology respectively [Beyenal, Donovan, Lewandowski & Harkin, 2004; Yang et al., 2000; Zielinski et al., 2012]. Areal parameters include average run length, aspect ratio, diffusion distance, thickness and porosity. Textural parameters include entropy, energy (these two parameters are not identical to those used in thermodynamics), homogeneity and roughness [Beyenal, Lewandowski & Harkin, 2004; Zielinski et al., 2012]. All measurements are based on pixel and voxel counting after thresholding, the latter being the most critical choice in image quantification [Yang et al., 2001]. The textural and morphological parameters that were utilised for the characterisation of the biofilms tested in this chapter are summarised below:

**Areal Porosity**: the ratio of void pixels to the total number of pixels. It

can be expressed as a percentage [Beyenal, Lewandowski & Harkin, 2004] and it provides a measure of the void area within the biofilm image.

**Roughness**: a dimensionless coefficient calculated on the basis of biofilm thickness measurements within the image. It provides a measure of biofilm heterogeneity [Heydorn et al., 2000].

## 4.2 Aims and hypotheses

The biofilm structure with its matrix, microcolonies and water channels is an important aspect of the biofilm lifestyle. In this chapter I aim to investigate the biofilm structure in the model system used for the experiments and understand any potential effect of this structure on β-lactam tolerance and vice versa. The antibiotic interactions with biofilm components and the interactions between resistant and susceptible cell were investigated. A new bacterial system consisting of the engineered environmental *E. coli* strain described in Chapter 2, with a natural ability to form thick biofilms was used. Within this context the hypotheses below were investigated:

**Hypothesis 1**: The biofilm matrix prevents the antibiotic from penetrating into the biofilm

**Hypothesis 2**: β-lactamase producing bacteria prevent the antibiotic from reaching its target in the cells

**Hypothesis 3**: Structural changes in the biofilm as a response to the antibiotic may contribute to its increased antibiotic tolerance

## 4.3 Materials & Methods

### 4.3.1 Biofilm cultivation methods

Black polycarbonate filter membranes with pore size 0.2 μm (Sterlitech, PCTB 0225100), on LB agar plates containing 1 mM IPTG, were inoculated with 5 μl starting culture of resistant (red), susceptible (green) or mixed biofilms diluted to a cell density of approximately $10^7$ CFU/ml and incubated at 37 °C for 24 h. Subsequently, 24 h biofilms were transferred to new LB plates or 1 mg/ml ampicillin containing plates as required by the protocol. The biofilm types used in these experiments were defined on the basis of the resistant proportion in the inoculum as described in Table 4.1. The proportions of resistant cells at the final point of imaging for 48 h biofilms on LB and 24 h biofilms exposed to ampicillin for 24 h are shown in Fig 4.1.

**Table 4.1:** Definition of biofilm types according to the proportion of resistant cells in the inoculum. SE refers to standard error.

| Type | Proportion | SE |
|---|---|---|
| Resistant | 0.92 | 0.3 |
| High Resistant | 0.61 | 0.7 |
| Low Resistant | 0.03 | 0.005 |
| Susceptible | 0.0 | na |

The microscopes and the related settings used in this study are shown in Table 4.3. The inverted microscope was only used for the lectin screening. The upright microscope was used in all other experiments.

### 4.3.2 Visualisation of the lower layers of the biofilms

In order to visualise the deeper layers of the biofilms the membranes with colony biofilms on top were transferred from agar plates on microscopy slides using

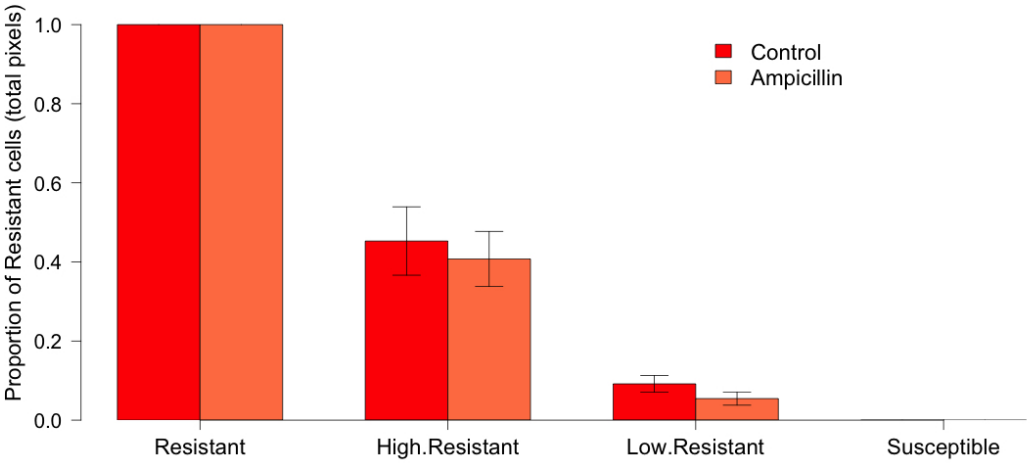


**Figure 4.1:** Resistant (red) proportions in biofilms grown on LB for 48 h (control on the graph) or on LB for 24 h and then on 1000 μg/ml ampicillin (ampicillin on the graph), as measured by pixel counting using ConAn (see Section 4.3.7). Mean proportions per colony per type are shown on this graph. The error bars represent +/- 1 SE. Any cells that may had lost the fluorescence at the time of imaging could not be detected and considered for these calculations.

forceps and were left to dry out for about 40 min at room temperature. After this time the colonies detached from the membrane. With the use of toothpicks they were turned upside down for imaging. The membranes were also imaged after the colony was removed for detection of any remnants of the colony biofilm, however none was found.

### 4.3.3 Antibiotic diffusion through the biofilm matrix

Five μl of Bodipy FL penicillin (Invitrogen, B-3233) at a concentration of 1 mg/ml was added on top of biofilms (R, HR, LR, S) grown on LB agar plates for 24 h. Sequential scanning was used when green cells and antibiotic coexisted in the biofilm in order to avoid cross-talk. Single images of the diffusion stages and 3D images of the colonies before (5 random spots per colony) and after full diffusion (10 random spots per colony over 120 min after the application of the antibiotic) were recorded. The total time until the whole volume of the antibiotic penetrated in the biofilm was measured by detecting the light reflection coming from the surface of the antibiotic solution droplet with confocal reflection microscopy (see Fig. 4.9), as well as the green signal of the antibiotic. When the antibiotic was first added it was difficult to detect the biofilm surface due to the volume of the liquid and the surface disturbance and this is why the droplet surface was followed. Cell and antibiotic mean signal before and after penetration were calculated on the basis of signal volume ($\mu m^3$).

### 4.3.4 The effect of ampicillin on spatial biofilm structure

Biofilms were grown as before on black polycarbonate membranes on LB plates for 24 h. At this point they were transferred either on new LB plates or LB plates containing 1 mg/ml ampicillin for another 24 h. Biofilm dimensions, volumetric and textural properties, as well as strain localisation and interactions were

**Table 4.2:** Stains for imaging white cells. All products mentioned in the table were purchased from Molecular Probes, Invitrogen unless otherwise specified.

| Stain | Target | Colour | Emission Max (nm) | Excitation Max (nm) | Product Code |
|---|---|---|---|---|---|
| Syto 9 | Nucleic acids | Green | 485 | 498 | S-34854 |
| Syto 9 | Nucleic acids | Green | 485 | 498 | S-34854 |
| Syto 60 | Nucleic acids | Red | 652 | 678 | S11342 |
| Sybr Green | Nucleic acids | Green | 495 | 520 | S-7567 |
| FM4 - 64 | Membrane | Red | 515 | 640 | T3166 |
| Sypro Red | Proteins | Red | 550 | 630 | S-6654 |
| Propidium Iodine | Dead cells | Red | 535 | 617 | L-7012 |
| CellTracker CMTPX | Cells | Red | 577 | 602 | C34552 |
| Rhodamine 6G chloride | Mitochondria | Green | 515 | 585 | R-634 |
| Acridine Orange | Nucleic Acids | Green, Red | 502 | 526 | A3568 |
| Mitotracker Green FM | Mitochondria | Green | 490 | 530 | M7514 |
| RedoxSensor | Mitochondria - Lysosomes | Red | 540 | 600 | R-14060 |
| Yeast Vacuole Marker | Yeast vacuoles | Green, Blue | 460 | 500 | Y7531 |
| FUN 1 Cell Stain | Yeast Cytoplasm - Vacuoles | green,red,yellow | | | F7030 |
| Calcofluor White | Fungi | Blue | 370 | 420 | F-6259, Sigma |
| JC1 | Mitochondrial Membrane Potential | Green | 498 | 583 | T3168 |

measured at two different ampicillin doses (0, 1000 μg/ml) both in monoculture and mixed biofilms. Images of the top layers ($\sim$ 30-90 μm) of the biofilms were acquired and analysed.

### 4.3.5 Confirmation of void existence

A number of stains (Table 4.2) were employed in order to detect any cells that may had lost the fluorescence carrying plasmids. This was in order to confirm that what seen as voids in the CLSM datasets were not areas filled with colourless cells that could not be detected by fluorescence microscopy. All stains were tested with broth cultures of WT cells (in terrific broth) in addition to the 48 h R, S, or mixed biofilms. For the staining, 10 μl of 1000x stock dilution were added underneath the membrane and the stain was left to diffuse from the bottom of the biofilms (membrane side) toward the top (biofilm-air interface). This method of diffusion was chosen in order to minimise the disturbance of the structure that a flow from the top could cause. Images were acquired 2 h after staining.

**Table 4.3:** Specifications and settings of the microscopes used in this study

| | **Leica SP5X upright** | **Leica SP1 Inverted** |
|---|---|---|
| **Light source** | White light laser | fixed laser lines of 488, 561 and 633 nm |
| **Lens** | Air, 63x, NA 0.7 | Water, 63x, NA 1.2 |
| **Image size** | 123x123 µm | 158x158 µm |
| **Resolution** | 1024x1024 | 512x512 |
| **pixel size** | 120.25x120.25 nm | 310x310 nm |
| **Step size** | 1 µm | 0.5 µm |
| **speed** | 400 Hz | Medium |
| **Pinhole** | Airy 1 | Airy 1 |
| **E2-Crimson excitation** | 611 nm-80% | na |
| **E2-Crimson emission** | 625-725 nm | na |
| **AmCyan excitation** | 475 nm-100% | na |
| **AmCyan emission** | 490-550 nm | na |
| **Bodipy FL penicillin excitation** | 504 nm-80% | na |
| **Bodipy FL penicillin emission** | 515-580 nm | na |

### 4.3.6 Screening for glycoconjugate-binding probes (fluorescent lectins)

*Escherichia coli*-CC11-1 WT biofilms were grown in 96 well-plates with hydrophilic or hydrophobic surfaces. Terrific broth (200 µl) was inoculated with 2 µl starting culture, grown for 22 h at 37 °C, with agitation at 180 rpm. The inoculated plates were incubated at 37 °C for 24 h, with agitation at 120 rpm. After the incubation time the medium was removed by pipetting and replaced with 100 µl of 100 times diluted lectin stock solution chosen from a list of 72 lectins as listed in Appendix C. A different lectin was added to each well. The lectin was left to incubate at room temperature for 1 h before it was removed by careful pipetting or absorption. The samples were then rinsed twice with 200 µl water. The inverted Leica SP1 microscope was used for this part of the experiment (see Table 4.3).

### 4.3.7 Biofilm quantification with OCT

Biofilms were grown on polycarbonate filter membranes as described in section 4.3.1 for 24 h and then transferred to new LB or ampicillin containing plates as described in section 4.3.4. The biofilms were imaged with a ThorLabs Ganymede BNG-912-J OCT imaging system, running ThorImage 4.2 software. A Refractive Index (RI) of 1.38 was used as this has been reported to be the RI of *E. coli* cells [Liu et al., 2013]. Biofilm thickness was measured at 5 locations within each biofilm and averaged. The maximum biofilm diameter on the membrane side was also recorded. Three replicates per condition (biofilm type, antibiotic dose, biofilm age) were measured.

### 4.3.8 CLSM image processing and quantification

**Software used for Image quantification**. JImageAnalyser 1.4, an in-house version of ImageJ at UFZ Magdeburg, was used for manual thresholding. The same software was also used for the quantification of the antibiotic diffusion images. ConAn 1.3 (BioCom GbR, Germany) was used for the quantification of biofilm textural and volumetric parameters (porosity and roughness), after thresholding and background correction were applied. Imaris 8.1.2 (Imaris, Biplane AG, available at http://bitplane.com) was used for thresholding and for producing rendered images for presentation purposes.

Automatic thresholding methods are generally considered more robust and reproducible and are therefore desirable [Xavier et al., 2003; Yang et al., 2001]. The automatic thresholding options offered by ConAn (Auto, Objective Threshold Selection, Otsu, SMy) were tested in this case but they resulted in noisy images and they were not preferred. Manual thresholding often gives results more representative of the real observations, as seen under the microscope, which was the case with the datasets obtained for these experiments. The thresholds used were obtained by averaging the thresholds of all spots in the same colony. Each

**Table 4.4:** Total Number of Images analysed, broken down per experiment. Biofilm types include R, HR, LR and S. The number of colonies refers to the number of replicates per biofilm type. The number of spots refer to the the spots imaged per individual biofilm. In the case of the diffusion experiment 5 spots were imaged before and 10 after the application of the antibiotic. The treatments refer to biofilms growth either on LB plates only or ampicillin containing plates after 24 h on LB. In the case of the top structure visualisation 5 replicates were used for the resistant and mixed types and 4 for the susceptible types.

| Experiment | Biofilm Types | No of Colonies | No of spots | No of Treatments | Total |
|---|---|---|---|---|---|
| **Diffusion** | 4 | 5 | 15 | 1 | 300 |
| **Structure - top** | 3 | 5 | 5 | 2 | 150 |
| **Structure - top** | 1 | 4 | 5 | 2 | 40 |
| **Structure-bottom** | 4 | 3 | 3 | 2 | 72 |
| **White cells** | 4 | 3 | 3 | 2 | 72 |

channel was thresholded separately. Theses averages were used for quantifying all the images coming from the respective colony. Whenever applicable (i.e. when thresholds were similar) average thresholds per biofilm type/ antibiotic dose were used.

**Image quantity**. A total of 632 confocal datasets were recorded and used for further analysis and quantification as shown in Table 4.4.

## 4.4 Results

### 4.4.1 3D biofilm architecture

CLSM imaging of the top layers of the biofilms revealed that cell clusters of resistant and susceptible bacteria were separated by voids (Figure 4.2). Resistant and susceptible clusters were clearly visible, but intertwining, in the mixed biofilms. It was also evident that at least in the case of the mixed biofilms the void coverage increased over time. Finally, resistant cells in the LR biofilms that were exposed to ampicillin (Fig. 4.2K) appeared to form more distinct clusters compared to the same biofilm type on LB (Fig. 4.2C).

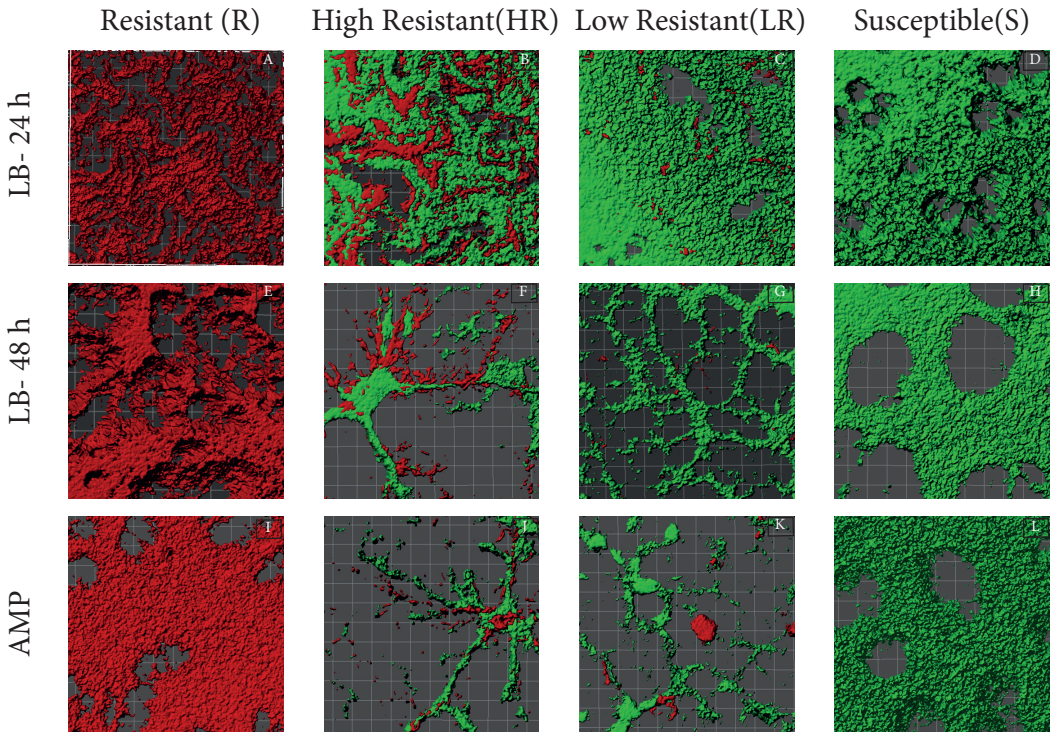


**Figure 4.2:** The structure of the top layers of colony biofilms; The top row shows biofilms grown on LB plates, for 24 h, without antibiotic. The second row shows biofilms grown on LB plates, for 48 h, without antibiotic. The bottom row shows biofilms grown on LB for 24 h and then exposed to 1 mg/ml ampicillin for another 24 h; Susceptible cells are illustrated as green and resistant as red. Imaris Isosurfaces were used to enhance image presentation. Grid size equals 10 μm.

In order to observe the biofilm structure at the bottom layers where the laser could not penetrate, dried out biofilms were used. Drying preserved the structure of the biofilms with the microcolonies and location of the voids, remaining clearly visible. When looking at the biofilms from the bottom we observed that in mixed biofilms the very bottom layer of the biofilm was covered by resistant (red) cells irrespective of the presence of antibiotic in the agar plates or the proportion of resistant cells in the inoculum. Additionally, the bottom layers of all biofilm types appeared to be less porous than the top layers shown in Figure 4.2. Dried biofilms overall appeared to be denser than the top layers of the biofilms that were imaged without any manipulation, however, this may be an artefact of the water removal. Figure 4.3 shows top, bottom and side views of dried biofilms.

**Verification of void existence**. CLSM revealed a biofilm structure consisting of cell clusters and voids. Given that the plasmid, carrying the fluorescent protein gene, could be naturally lost in both biofilms and broth cultures, it was essential to establish that those non-fluorescent cells were not taking up the space that looked like voids in the datasets. Staining with rhodamine confirmed that, indeed, the structure consisted of cells clusters and voids (Figure 4.4). The signal from the stain came from within the cell clusters.

### 4.4.2 Quantification of the biofilm growth and structure

**Biofilm growth.** Total colony biofilm depth and diameter were measured with OCT and the results are presented in Figures 4.5 and 4.6 respectively. Biofilms grown on LB increased their thickness by about 70% [63-81%] over a period of 1 day (24 h to 48 h biofilms). Similarly, they also grew in diameter by about 40% [22-56%]. Two-day old biofilms grown on LB or 24 h biofilms exposed to ampicillin for 24 h, grew more when both strains were present. Exposure to ampicillin did not affect the growth of resistant or the mixed biofilms, irrespective of the proportion of resistant cells present. However, the exposed susceptible biofilms did not grow either in thickness or diameter. Additionally, R and HR biofilms on

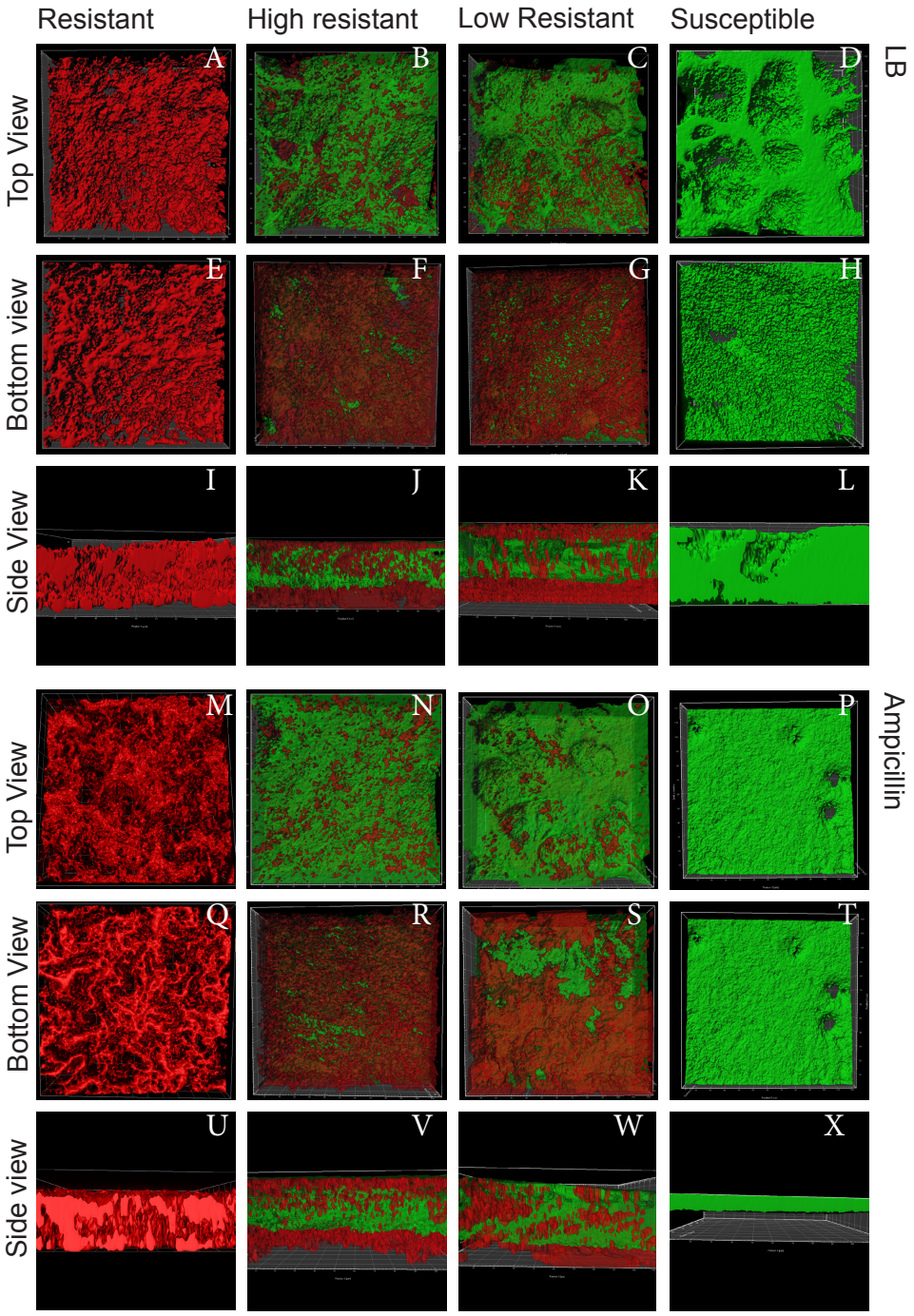


**Figure 4.3:** Images of dried biofilms. Top three rows show biofilms grown on LB and bottom three rows biofilms grown on ampicillin. Each column represents a different biofilm type (R, HR, LR, S). Imaris Isosurfaces were used to enhance image presentation. The scale bar is equal to 10 μm.

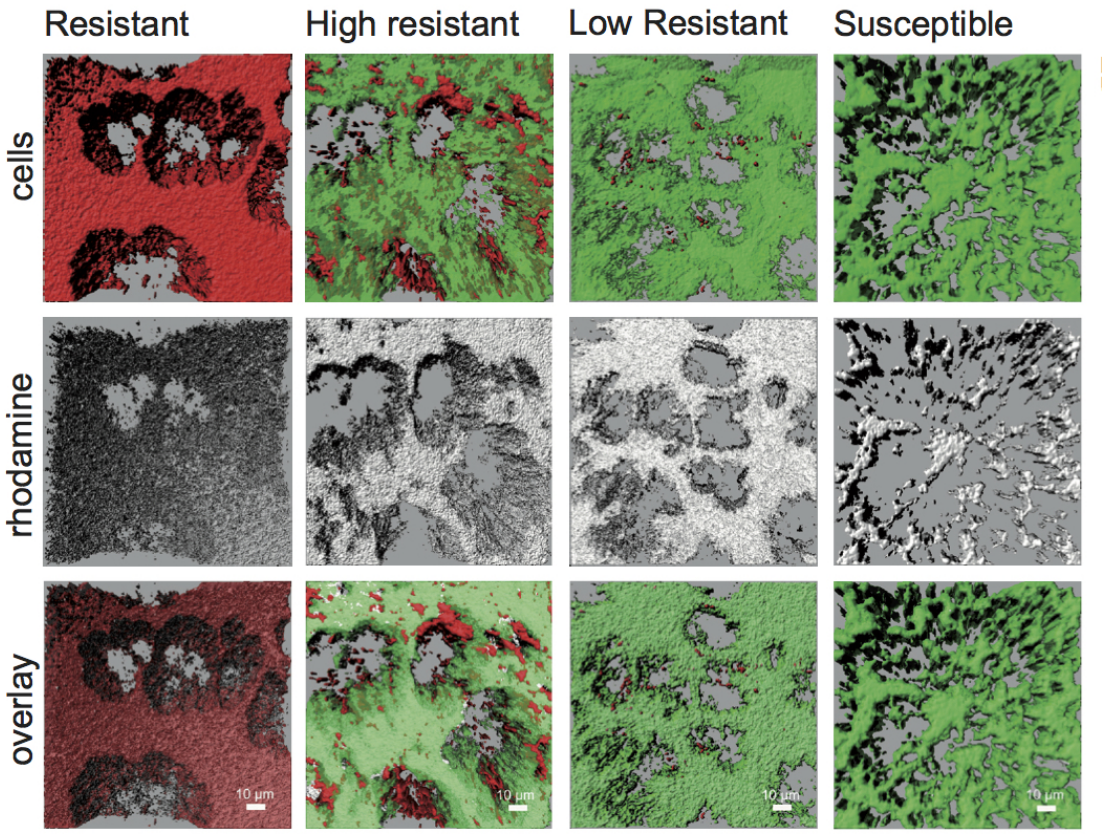


**Figure 4.4:** Biofilms grown on LB for 48 h were stained with rhodamine to confirm the existence of the voids. Signal from the cell (susceptible and resistant) channels, rhodamine channel and an overlay of all 3 channels are shown in this image. The scale bars equals 10 µm.

ampicillin seemed to grow marginally more than on LB, something that was not true for the LR biofilms.

Average thickness in μm was as follows: R, 24 h on LB 133.7+/-4.0 SE; R 48 h on LB 241+/-9.7 SE; R, exposed 249.9+/-4.5 SE; HR, 24 h on LB 165+/-5.5 SE; HR, 48 h on LB 294+/-9 SE; HR, exposed 306 +/-7.6 SE; LR, 24 h on LB 175+/-4.3 SE; LR, 48 h on LB 322.5+/-7 SE; LR, exposed 292.3 +/-17.4 SE; S 24 h on LB 173.5+/-8.2 SE; S, 48 h on LB 282.1+/-28.8 SE; S, exposed 167.3+/-6.1 SE.

Average diameter in mm was as follows: R, 24 h on LB 3.4+/-0.19 SE; R 48 h on LB 4.1+/-0.2 SE; R, exposed 4.6+/-0.06 SE; HR, 24 h on LB 3.2+/-0.17 SE; HR, 48 h on LB 4.4+/-0.05 SE; HR, exposed 4.7 +/-0.07 SE; LR, 24 h on LB 3.1+/-0.1 SE; LR, 48 h on LB 4.7+/-0.1 SE; LR, exposed 4.4 +/-0.2 SE; S 24 h on LB 3.5+/-0.1 SE; S, 48 h on LB4.7+/-0.1 SE; S, exposed 3.3+/-0.1 SE.

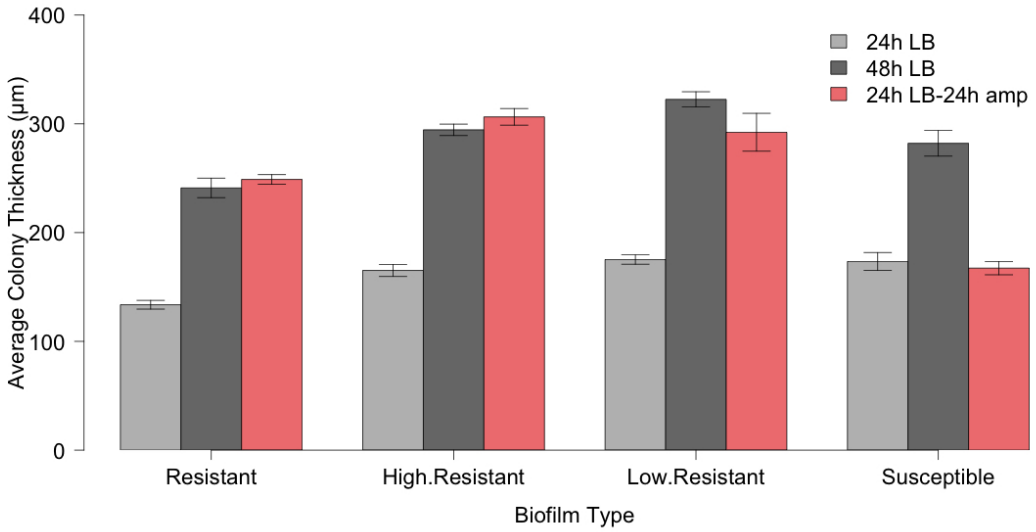


**Figure 4.5:** Biofilm thickness as measured in μm with OCT for 1 and 2-day old biofilms grown on LB or 24 h biofilms exposed to 1000 mg/ml ampicillin as noted in the legend. The error bars represent +/- 1 SE.

**Textural and volumetric parameters**. The biofilms grown for the exper-

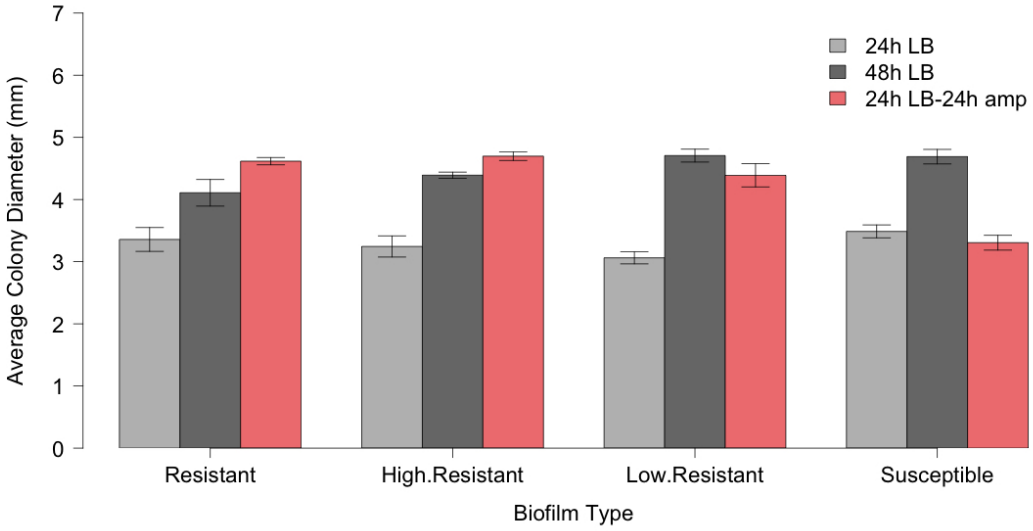


**Figure 4.6:** Biofilm diameter as measured in mm with OCT for 1-day or 2-day old biofilms grown on LB or 1 mg/ml ampicillin as noted in the legend. The error bars represent +/- 1 SE.

iments described here, were too thick for the laser to penetrate in full depth. Therefore, datasets of the intact, fully hydrated, top layers (20 - 90 μm) were initially recorded. In order to be able to record datasets of the bottom layers, the biofilms were dried out. Since the dried and stained biofilms were manipulated with unknown effects on the biofilm areal and textural characteristics, this analysis was based on the datasets of the top layers, that were imaged without the application of any pre-processing treatment. Therefore the results cannot necessarily be directly extrapolated to the whole depth of the biofilm structure.

As it can be seen in Figure 4.2 a characteristic of the these biofilm structures was the presence of voids, of different sizes and frequencies. Therefore, it seemed appropriate to quantify biofilm porosity and compare how it changed between biofilms over time. Figure 4.7 shows the mean porosity per biofilm type. Changes in resistant biofilm porosity did not seem to be dramatic over 24 h for biofilms grown on LB. However, it seemed to reduce slightly in biofilms exposed to ampicillin. The porosity of mixed biofilms, on the other hand, increased up to a percentage of more than 90% in one day and exposure to ampicillin did not

seem to affect this. In the case of the susceptible biofilms, porosity increased over 24 h when grown on LB but decreased when exposed. In fact, exposed, susceptible biofilms exhibited the lowest porosity (0.65+/-0.03 SE) of all types on the second day.

Average porosity was as follows:R, 24 h on LB 0.78+/-0.02 SE; R 48 h on LB 0.8+/-0.02 SE; R, exposed 0.75+/-0.03 SE; HR, 24 h on LB 0.73+/-0.01 SE; HR, 48 h on LB 0.98+/-0.002 SE; HR, exposed 0.97 +/-0.007 SE; LR, 24 h on LB 0.54+/-0.04 SE; LR, 48 h on LB 0.96+/-0.008 SE; LR, exposed 0.96 +/-0.01 SE; S 24 h on LB 0.72+/-0.07 SE; S, 48 h on LB0.86+/-0.02 SE; S, exposed 0.65+/-0.03 SE.

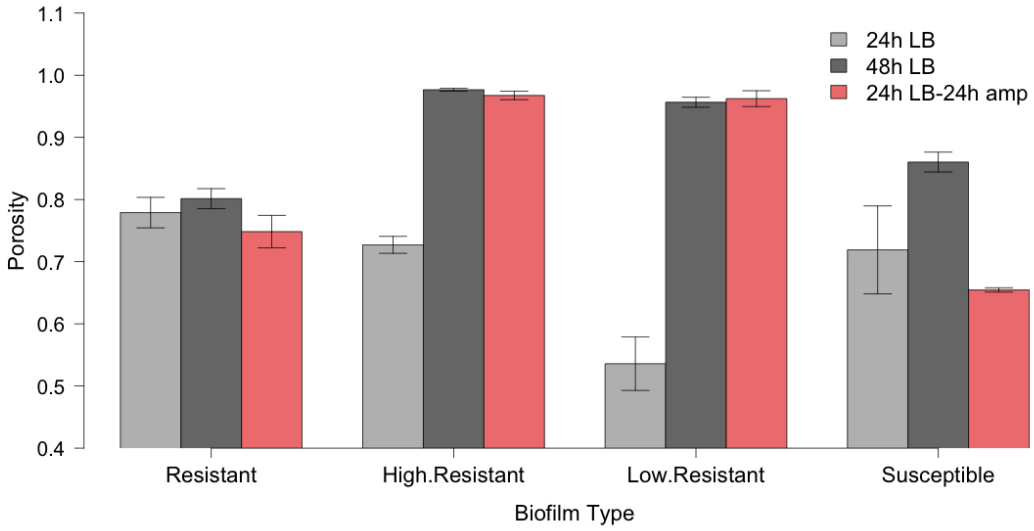


**Figure 4.7:** Biofilm porosity in the top layers of colony biofilms grown on LB for 24h (grey bars) or 48 h (brown bars) or grown on LB for 24 h and then exposed to ampicillin for another 24 h (green bars).The error bars represent +/- 1 SE.

All biofilm types, started off with a similar roughness after 24 h of growth (Fig. 4.8). Resistant biofilms retained a similar roughness under all conditions studied (day 1, day 2, exposure to ampicillin). Mixed biofilms exhibited an increased roughness from 24 h to 48 h, both in the presence and absence of the antibiotic. Susceptible biofilms on the other hand showed increased roughness over 24 h only

when they were not exposed to ampicillin.

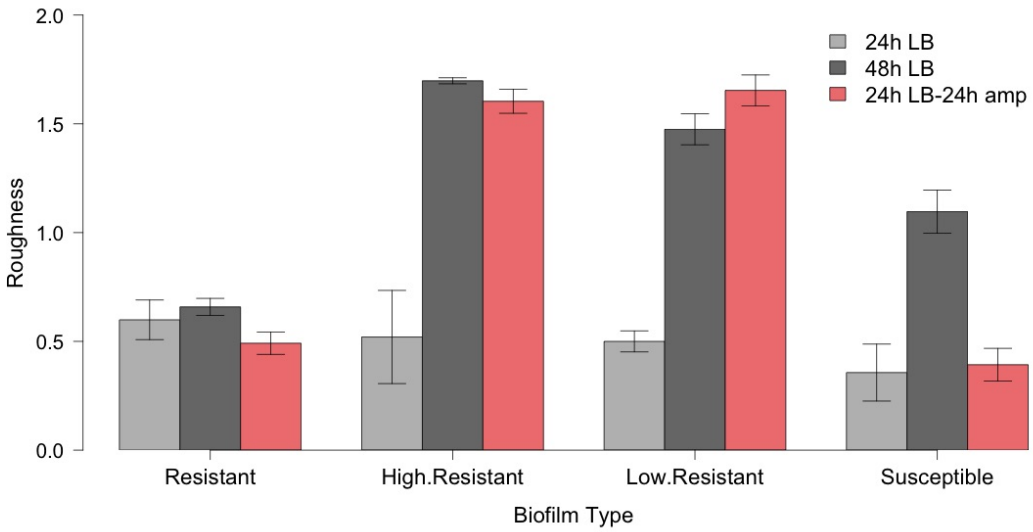


**Figure 4.8:** Biofilm roughness in the top layers of colony biofilms grown on LB (black bars) or ampicillin containing plates (grey bars). The error bars represent +/- 1 SE.

### 4.4.3 Antibiotic diffusion

Penicillin could fully penetrate the biofilm matrix when applied as a droplet of aqueous solution on top of the colony at a concentration of 1.0 mg/ml. The antibiotic overcame the matrix barrier and fully diffused into the biofilm. The route of the antibiotic in a resistant and a susceptible biofilm can be seen in Figure 4.9. Figure 4.10 shows the difference in the mean fluorescence signal (pixel volume $\mu m^3$) coming from resistant and susceptible cells after the antibiotic had fully diffused into the biofilm compared to the signal recorded before the application of the antibiotic. The shape and size of the microcolonies was altered after the antibiotic had penetrated and as it diffused towards lower levels. It can also be seen that the antibiotic was detected in the voids and in the cell clusters. In the susceptible biofilms (T1) it can be seen that bacteria detached from the colonies and were suspended in the liquid before the antibiotic had reached the colony

surface. To calculate the difference the following equation was used:

$$signal\ difference\ = \frac{pixel\ volume\ before}{total\ volume\ before} - \frac{pixel\ volume\ after}{total\ volume\ after} \tag{4.1}$$

where pixel volume ($\mu m^3$) refers to the signal coming from either green or red cells or the antibiotic and total volume refers to the sum of all pixel volume in a given image (red, green and antibiotic).

As it can be seen in Figure 4.10 more antibiotic was detected in susceptible biofilms compared to all other types. At the same time susceptible cells in susceptible biofilms appear to be affected more than both resistant and susceptible cells in all other types as less cell signal was detected after the antibiotic penetration. These images represent the antibiotic effects in the top layers of the biofilm.

Additionally, the antibiotic penetration time was measured with full penetration defined by the point when the whole antibiotic solution was in the biofilm. As can be seen in Figure 4.11 the more resistant cells in a biofilm, the more time was required for full penetration. Mean diffusion times (in min) for each biofilm type were as follows; R: 65.2+/-1.5, HR: 50.6+/-5.9, LR: 34.0+/-3.1, S: 38.2+/-2.2.

## 4.5 Discussion

### 4.5.1 Antibiotic diffusion

Diffusion of fluorescent penicillin in all four types of biofilms grown for the purposes of these experiments (R, HR, LR, S) showed that the matrix did not prevent

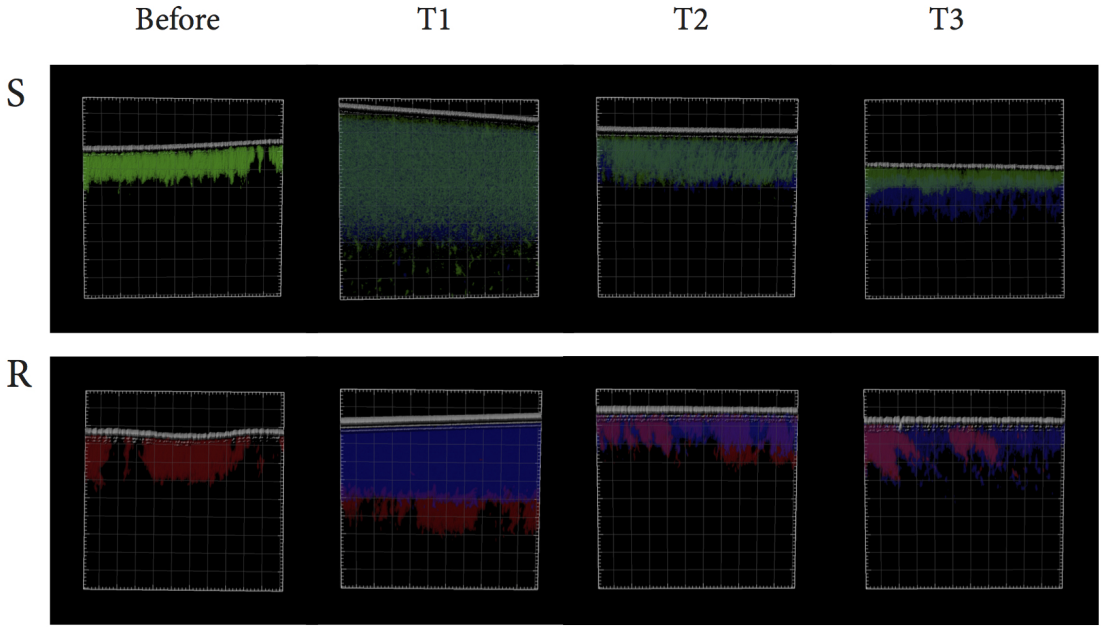


**Figure 4.9:** Penicillin diffusion in a susceptible (S) and resistant (R) biofilm until full penetration. Green represents susceptible cells, red resistant cells and blue the antibiotic. Pink or purple indicate that both antibiotic and cell signal was detected in those areas. The grey line is the surface reflection of the antibiotic at time point T1 or the colony surface at all other time points. Imaris isosurfaces were used for these images, with transparency 65-80%. The grid size is 10 μm.

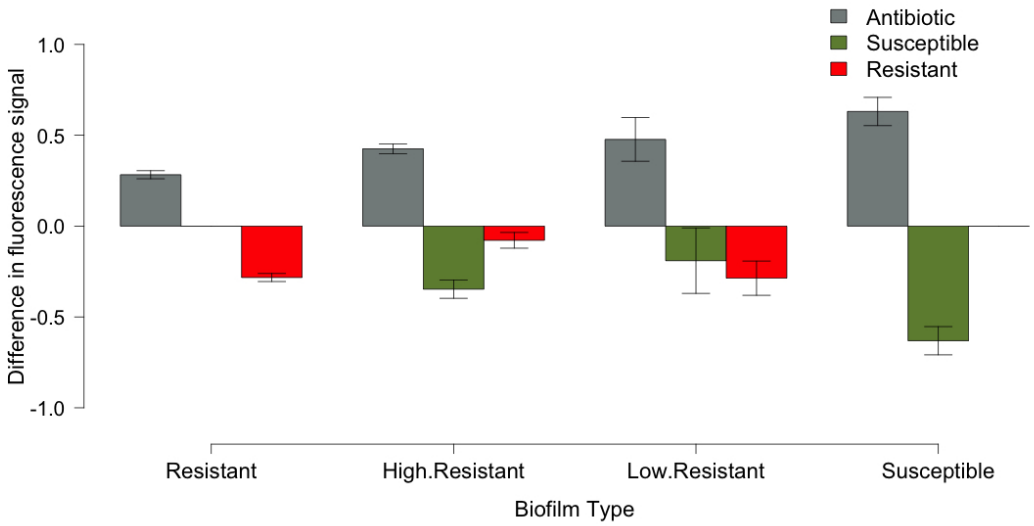


**Figure 4.10:** Difference in cell and antibiotic signal in four biofilm types before and after the application of 1 mg/ml ampicillin on top of the biofilm. Green is the signal coming from susceptible cells and red from resistant. The error bars represent +/- 1 SE

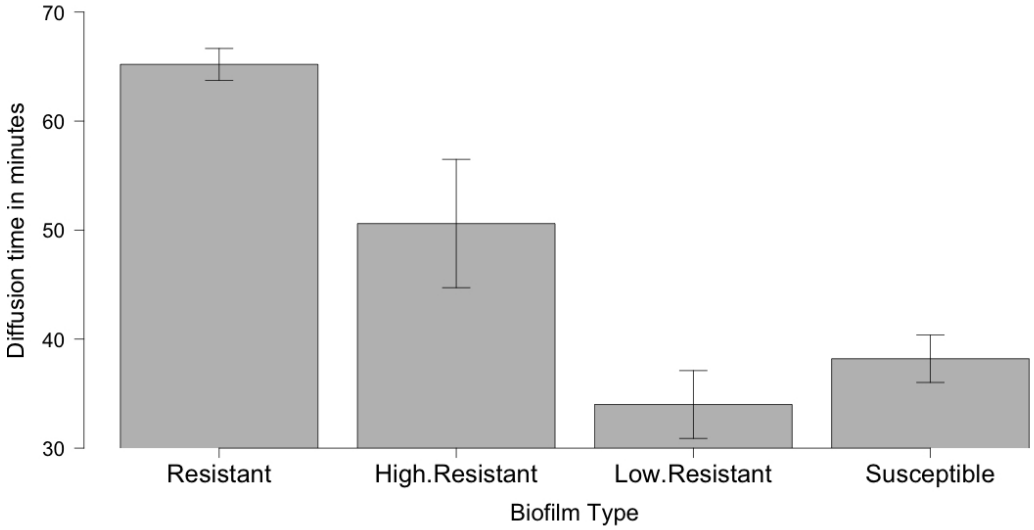


**Figure 4.11:** Mean antibiotic diffusion time (minutes) in 24 h biofilms grown on LB. The error bars represent +/- 1 SE.

the antibiotic penetration (Figures 4.9, 4.10, 4.11). Antibiotic traits on the agar plates, underneath the polycarbonate membrane confirmed that the penetration was full, along the whole depth of the biofilms. However, the antibiotic did not fully penetrate for several minutes (Fig. 4.11). This effect may be partially due to the ability of the matrix to repel liquids as has been shown in the case of *Bacillus subtilis* biofilms [Kovács et al., 2012]. When pure water droplets were added on top of resistant or susceptible biofilms, the time needed for the water to fully penetrate was similar for both biofilm types (40+/-1.7 and 44.3+/4.7 min respectively).

In addition to this potential matrix effect though, the production of β-lactamases also appeared to influence penetration time. In fact the time it took for the antibiotic solution to fully diffuse into the biofilms depended on the proportion of the resistant cells present with more time required to diffuse in resistant biofilms and less time in low resistant or susceptible biofilms. The fluorescent penicillin used in this study carried a negative charge (one acidic group) which would increase further by hydrolysis (two acidic groups). Since the matrix is mostly anionic [Costerton et al., 1987; Mulcahy et al., 2008; Nichols et al., 1988], the more negatively charged hydrolysed penicillin would be expected to show reduced ability to penetrate the biofilm, which tallies with the results presented here, i.e more time was required to fully penetrate R and HR biofilms, presumably due to the production of more β-lactamases.

The antibiotic signal detected in the top layers of the biofilms over the course of two hours after full penetration (Fig. 4.10) was the highest in the case of susceptible biofilms and the lowest in resistant biofilms, i.e, when the antibiotic needed less time to penetrate susceptible biofilms it also lingered in the top layers for longer. This indicates that the diffusion of the hydrolysed negative charged molecule once in the biofilm, accelerates compared to the intact molecule (in susceptible biofilms), although the mechanism is not understood. Since the antibiotic lingered around the cells for longer in susceptible biofilms, targeting more of them in the process, this could account for the high reduction in the signal coming from the susceptible cells in S biofilms. Fig. 4.10 also suggests that the

signal coming from susceptible cells in mixed biofilms was not reduced as much as in S biofilms. This latter observation could indicate a protection effect due to the production of β-lactamases by the resistant cells. Reduced fluorescence signal coming from the cells was probably due to cell lysis resulting from the action of the antibiotic and subsequent protein degradation.

If the reduction of cell signal after antibiotic treatment is due to cell death and lysis, this result indicates that in mixed biofilms susceptible cells did benefit by the production of β-lactamases and this was despite the higher levels of intracellular enzyme seen in Chapter 3. The biofilms used in this case were 24 h biofilms that generally showed less porosity (Fig. 4.7) and less heterogeneity (Fig. 4.8). This observation supports the conclusion that the main factor affecting diffusion in this case was the production of β-lactamases rather than other structural characteristics.

In conclusion, the results presented here refute hypotheses 1 and 2; neither the matrix nor β-lactamases prevented the antibiotic from penetrating into the biofilm. Additionally, the antibiotic seemed to be able to reach its target in both resistant and susceptible cells. Nevertheless, the production of β-lactamases did confer protection to both resistant and susceptible cells, when present. Biofilm growth and structure are discussed next.

### 4.5.2 Biofilm growth

All types of biofilms tested here increased substantially in thickness and diameter over 48 h (Fig. 4.5 and 4.6). This correlates well with temporal thickness measurements elsewhere in the literature where it has been observed that biofilm thickness increases over time [Dervaux et al., 2014; Larimer et al., 2016]. This finding also corresponds to visual observations of colony biofilms on agar plates. Colony biofilms on agar were visually larger when growing on LB over time in all cases (R, HR, LR and S biofilms).

When mature biofilms (24 h old) were exposed to ampicillin for 24 h their thickness and diameter increased in a similar manner to biofilms grown on LB only whenever resistant bacteria were present. Ampicillin did not affect growth in those cases. On the contrary, in the case of the susceptible biofilms ampicillin impeded growth. The biofilms did not increase in size (thickness and diameter), again consistent with visual observations on plates. As shown in Chapter 3, CFU counts from susceptible biofilms exposed to ampicillin, were approximately 10 times lower than in all other cases. This indicates that susceptible bacteria in exposed biofilms experience death, which in turn results in no further increase of the biofilm size. Even though bacteria died under these conditions, and in fact no CFUs were recovered from susceptible biofilms after 3 days of exposure (see Chapter 5), the biofilm remained visible on the membranes. This result seems to be in agreement with other reports indicating that the maintenance of biofilm structure, after treatment with antibiotic, does not depend on cell viability. At least not in terms of the viscoelastic properties that were measured by these researchers [Zrelli et al., 2013].

Overall, growth measurements indicate that under normal conditions, all biofilm types grow in thickness and diameter. After a 24 h of exposure to a high dose of ampicillin, biofilm growth was not affected in the presence of resistant cells. In purely susceptible biofilms though, no change in growth was observed, probably due to cell death taking place. The results reported here validate OCT as a method capable of measuring bacterial growth in the case of the colony biofilms. To the best of our knowledge this is the first time this methodology has been used with this type of biofilms.

### 4.5.3 Biofilm structure: texture and volume

Confocal imaging showed that colony biofilms were made of cell clusters separated by voids. The existence of the voids was confirmed with cell staining which revealed that the voids were not filled with non-fluorescent cells (Fig. 4.3). How-

ever, other biofilm components such as extracellular polysaccharides, proteins or DNA may still be present in those areas. Attempts to stain and visualise those components with glycoconjugate binding lectins were not successful as no binding occurred. A list of stains used for this can be found in Appendix C.

OCT showed that in most cases biofilm volume did not seem to be affected by ampicillin, after a 24 h exposure. Changes to the internal structure were investigated next. In order to test if this was the case, biofilm porosity (Fig. 4.7), was the first parameter to measure on the basis of visual observations in the recorded datasets (Fig 4.2). The results indicate that monoculture biofilms were generally less porous than mixed biofilms. Porosity increased over time for all types grown on LB. With regard to exposed biofilms, porosity was not affected in mixed biofilms; after a 24 h exposure it increased to a level similar to that on LB. However, porosity was actually reduced in the case of single-strain biofilms, with susceptible, exposed biofilms being the less porous type. Increased porosity could indicate that the cell microcolonies reduce in size over time or that as the biofilm grows, the newly created space is taken up by voids or other biofilm components that were not visible by CLSM.

Based on biofilm roughness measurements, the heterogeneity of mixed biofilms increased with time and the antibiotic did not affect this. This heterogeneity may be a result of strain social interactions. Resistant biofilms however, tended to retain a relatively low heterogeneity. In the case of the susceptible biofilms, the antibiotic did affect the biofilm heterogeneity preventing it from increasing over time, as it would happen if no antibiotic was present (Fig 4.8).

In conclusion, all biofilms studied here, where resistant cells were present, increased in size over 2 days in a similar manner both in the presence and in the absence of the antibiotic. However, the internal structure of the different biofilm types did differ substantially. Mixed biofilms, where resistant and susceptible bacteria grew together, had a higher porosity and heterogeneity and the antibiotic did not affect this trend. Additionally, the microcolony size tended to be smaller on day 2 compared to what it was on day 1 and this may explain the

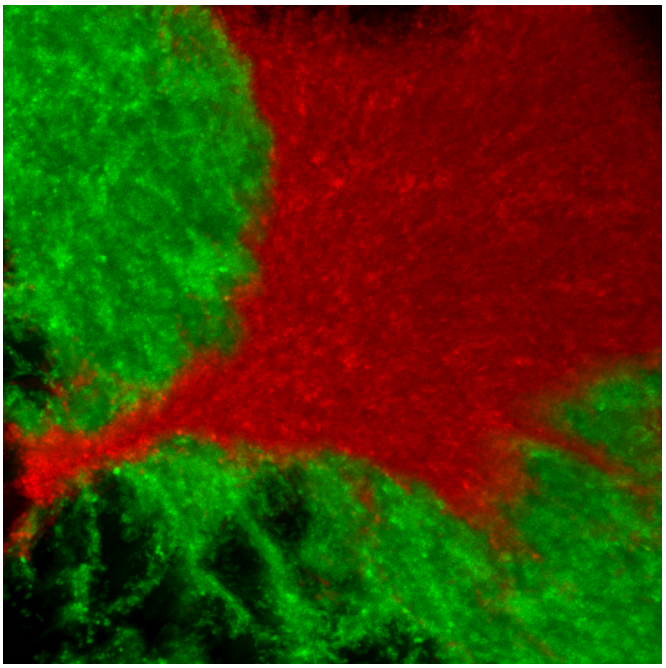

**Figure 4.12:** Part of the periphery of a HR biofilm grown on LB for 48 h (Maximum intensity Projection).

higher porosity measurements. Resistant biofilms showed a more stable porosity and heterogeneity over time (day 1 and day 2) and across treatments (with or without antibiotic). Finally, the susceptible biofilms appear to be more porous and heterogeneous on day 2 compared to day 1 when grown on LB, but when exposed to ampicillin, they did not grow in size, or heterogeneity. Additionally, their porosity decreased. Using modelling, Picioreanu et al. [1999] suggested that a porous structure is a result of stress on the biofilm and if this is the case then decreasing porosity may be a biofilm response to stress.

### 4.5.4 Microbial interactions and cell localisation

In terms of susceptible and resistant cell localisation in the biofilm, CLSM images of the bottom layers of the biofilms showed that in mixed biofilms the resistant cells covered the bottom layer of the biofilm and that was the case whether

ampicillin was added to the medium or not (Fig. 4.3). It was also noted that resistant cells generally occupied areas with direct access to the voids, surrounding the susceptible cells under all growth conditions. de Beer et al. [1994] showed that oxygen was more available in the voids compared to the middle of cell clusters, therefore it may be the case that the resistant bacteria in this case required more resources (nutrients, oxygen) for growth. This may be related to the higher cost of the plasmid they carried (see Chapter 2). Alternatively, the resistant bacteria in mixed biofilms, may be outcompeted by the susceptible ones and pushed to the edges. This theory could be supported by the fact that the periphery of the colony biofilms was mainly occupied by resistant bacteria (Fig. 4.12). Resistant bacteria pushed to the periphery are also visible on agar plates. It was also observed that in the case of LR biofilms in the presence of ampicillin, the resistant cells, appeared to form more distinct microcolonies, separated from the susceptible ones. This arrangement may allow them to cooperate for a more efficient production of the public good (β-lactamases) in order to protect themselves from the antibiotic.

In conclusion, imaging of the top layers of the biofilms showed that mixed biofilms were more porous than single-strain ones and this was not affected by the antibiotic. However, susceptible biofilms on ampicillin were less porous suggesting that changes in porosity may be a strategy employed by bacteria in biofilms to cope with the antibiotic. For example in a denser structure, the liquid phase will be limited and thus diffusivity of molecules will be impeded [Hamdi, 1995; Stewart, 2003]. Another interesting observation was that resistant cells in mixed biofilms tended to form more distinct clusters in the presence of the antibiotic, something that did not happen under normal growth conditions (Fig. 4.2). This offers some partial answers to the third hypothesis investigated here, although further work would be required for the full influence of structure on antibiotic tolerance to be revealed.

### 4.5.5 Summary and future directions

The effect of β-lactam antibiotics on biofilms in the presence or absence of resistant bacteria was investigated here. Neither the matrix nor β-lactamases prevented the antibiotic from entering into the biofilms. However, the presence of β-lactamase producing cells did affect the time required for full antibiotic penetration, as well as the amount of the antibiotic detected in the top layers of the biofilm after full penetration. Although this result settles the question of whether limited penetration of antimicrobial substances is responsible for the observed increased antibiotic tolerance exhibited by biofilms, it still raises the question of what is the mechanism by which β-lactamase producers achieve the discussed effects.

Furthermore, it has been seen here that susceptible biofilms exhibit some internal structural changes, as a response to the antibiotic. Whether these changes are universal or restricted to the experimental system used for this study remains to be confirmed. It would also be of interest to investigate those structural characteristics in response to other antimicrobials and with more controls such as non-toxic fluorophores or even molecules beneficial for the bacteria. Understanding the genetic mechanisms and identifying the elements of bacterial interactions influencing those changes would also be of great interest.

# Chapter 5

# Bacteria in Biofilms: The Sleeping Beauties of the microcosm?

## 5.1 Introduction

Discussions about the metabolic state of bacteria that "persist" after antibiotic treatments, despite their genetic susceptibility, are ongoing. In this chapter, I aim to shed light on the role of metabolism in biofilm antibiotic tolerance. A review of the literature is followed by the description of the methodology used, the results and a discussion.

### 5.1.1 Metabolism and antibiotic tolerance

The term "persisters" was first coined by Joseph Bigger in 1944 to describe *Staphylococci* cells that survived penicillin treatment in broth cultures [Bigger, 1944]. After testing the level of persisters under different conditions, Bigger deduced that persisters must be non-dividing, dormant, cells that penicillin could not kill owing to its mode of action, i.e because of its ability to target dividing cells only. He also distinguished this newly identified bacterial state from inherently resistant cells, such as the β-lactamase producing bacteria that had been discovered a few years earlier [Abraham & Chain, 1940]. After interest in persisters revived, 30 years ago [Moyed & Bertrand, 1983], a lot of effort was directed towards understanding the genetic makeup [Amato et al., 2013; Pu et al., 2016; Rocco et al., 2013; Wang & Wood, 2011] and biological significance [Dhar & McKinney, 2007; Kussell et al., 2005; Lewis, 2001] of these cells. It has become clear that persistence is not a heritable trait [Balaban et al., 2004; Bhargava et al., 2014; Jayaraman, 2008; Keren, Kaldalu, Spoering, Wang & Lewis, 2004] but is believed to be controlled epigenetically instead [Smits et al., 2006; Zhang, 2014].

Although persisters are generally acknowledged to be a subpopulation of cells that can survive antibiotic treatment, without possessing any inherent resistance mechanisms [Kint et al., 2012; Singh et al., 2009], the actual nature of persisters has troubled scientists in the last few decades. The notion that persisters are actually dormant and Bigger's definition of dormancy [Bigger, 1944], have both been challenged. Kwan et al. [2013] and Shah et al. [2006] confirmed that translation levels in persister cells are low and different to expression patterns in both exponential and stationary phase cells, in good agreement with the dormancy hypothesis. Yet, Pu et al. [2016] showed that persister metabolism is rather more complex than that, as they appear to be actively resistant to antibiotics by overexpressing efflux pumps, which resulted in less accumulation of β-lactam antibiotics in the cell. Meanwhile, the definition of dormancy was expanded to include slow-metabolising cells when Balaban et al. [2004] identified two distinct populations of persisters in *E. coli*: Type I and Type II persisters. Type I refers to

a proportion of non-growing cells generated in the stationary phase without any applied stress conditions (e.g. antibiotics) and it is proportional to the number of stationary cells in the inoculum. If supplied with fresh nutrients, these Type I persisters start to grow after an extended lag phase. These persisters do not depend on the total population size. Type II persisters, on the other hand, are slow-growing cells continuously produced in the culture in a stochastic manner. Their numbers depend on the total population size and they are not related to the stationary phase cells. In all cases persistence is considered to be a trait that applies to a subpopulation of cells rather than to the population as a whole.

The terms "tolerance" or "drug indifference" have been used to describe whole bacterial populations that are less sensitive to antibiotics [Handwerger & Tomasz, 1985; Jayaraman, 2008]. Two types of tolerance were proposed by Brauner et al. [2016]: tolerance by slow growth and lag tolerance. The first was intended to describe tolerance seen in stationary phase or drug induced tolerance related to slow metabolic rates. The latter was intended for the tolerance that lag phase bacteria may experience while adapting to new environments. In this view persistence becomes a special case of tolerance, with only a proportion of cells exhibiting the reduced sensitivity trait.

The extent of persistence to various antibiotics, has been reported to vary from 0.001% to 0.1% [Keren, Kaldalu, Spoering, Wang & Lewis, 2004; Kwan et al., 2013; Lewis, 2008; Mulcahy et al., 2010; Orman & Brynildsen, 2013*a*; Spoering & Lewis, 2001; Wood et al., 2013] of the population but generally no more than 2% [Shah et al., 2006], although, 100% tolerance to ampicillin in stationary cells has also been reported [Keren, Kaldalu, Spoering, Wang & Lewis, 2004]. These proportions have been found to be dependent on the growth phase of the culture, with exponential growth showing the least tolerance to antibiotics (lowest proportions of survivors recorded) and stationary cells the highest, even compared to biofilm-released cells [Spoering & Lewis, 2001]. Tolerance to ampicillin or tobramycin has been found to be higher compared to ofloxacin [Keren, Kaldalu, Spoering, Wang & Lewis, 2004; Spoering & Lewis, 2001] or sometimes the other way round, with 0.07% persistence to ampicillin and 0.15% to ofloxacin in pre-

determined low-metabolisers [Orman & Brynildsen, 2013*a*]. Antibiotics used in those experiments did not normally exceed 100 μg/ml for ofloxacin [Kwan et al., 2013; Mulcahy et al., 2010; Orman & Brynildsen, 2013*a*] or 600 μg/ml for β-lactams / 200 μg/ml ampicillin. In all cases, tolerance was measured after an antibiotic challenge which was performed in nutrient rich media and for 3-48 h.

Although the leading hypothesis has been that persisters are a subpopulation of non-growing cells [Balaban et al., 2004; Keren, Kaldalu, Spoering, Wang & Lewis, 2004; Lewis, 2001] that appear mainly at the stationary phase [Balaban et al., 2004] or cells in deep dormancy [Balaban et al., 2004; Keren, Kaldalu, Spoering, Wang & Lewis, 2004; Lewis, 2007] normally present in the population [Shah et al., 2006], this has been challenged as new data become available. Orman & Brynildsen [2013*a*] argued that persisters are not necessarily dormant as growing cells also produced persisters to antibiotics, and Wakamoto et al. [2013] showed that persisters cells were actually metabolising cells. These studies led to counterarguments about the validity of the methodologies used [Wood, 2016] but further evidence by Pu et al. [2016] (discussed above) came to support the hypothesis that persisters are a heterogeneous group of cells and the underlying mechanism of their ability to tolerate antibiotics may not be metabolism-related. Furthermore, the concept of persister heterogeneity was enriched by differences in their gene expression patterns [Allison et al., 2011; Gefen & Balaban, 2009; Kint et al., 2012; Orman & Brynildsen, 2013*a*], formation triggers [Amato et al., 2013; Gefen & Balaban, 2009; Kussell et al., 2005; Ma et al., 2010; Orman & Brynildsen, 2013*a*; Tashiro et al., 2012] and tolerance patterns to different stressors, i.e tolerating a certain stressor does not necessarily imply similar tolerance against another [Allison et al., 2011; Gefen & Balaban, 2009; Kint et al., 2012; Orman & Brynildsen, 2013*a*]. Nonetheless, multi-drug persisters have also been reported [Willenborg et al., 2014; Wiuff et al., 2005]). Zhang [2014] suggested a yin-yang model; persisters are constantly lurking within a growing population while, at the same time a portion of the persisters population reverts back to active growth in a linked, continuous manner. Jõers et al. [2010] showed that persister frequency follows an awakening kinetic curve that depends on the growth conditions.

### 5.1.2 Persisters in biofilms

The inherent physiological heterogeneity observed in biofilms, due to nutrient [Anderl et al., 2003; Anwar et al., 1992; Serra & Hengge, 2014; Stewart & Franklin, 2008], oxygen [Huang et al., 1995; Serra & Hengge, 2014; Stewart & Franklin, 2008; Walters et al., 2003], waste product and quorum sensing molecule [Serra & Hengge, 2014] gradients, results in the production of several phenotypic variants that exhibit different metabolic activities. Cells located near the nutrient/oxygen interface metabolise at higher rates, whereas cells located further in the biofilm resemble stationary phase cells [Anwar et al., 1992; Serra & Hengge, 2014; Wentland et al., 1996; Williamson et al., 2012]. Huang et al. [1995] measured the respiratory activity in *Klebsiella* biofilms treated with a disinfectant and found that after the treatment, respiration was higher in the deeper layers of the biofilm, near the substrate, indicating that those layers may be characterised by lower metabolic rates. Williamson et al. [2012] found that mRNA production in deeper layers was low, indicating low metabolic activity. Ito et al. [2009] also showed that deeper layers in mature biofilms resisted β-lactam treatment but did not resist aminoglycoside and fluoroquinolone treatment pointing to metabolism as a candidate explanation for the increased biofilm tolerance seen in the literature. They also found that metabolic activity genes were down-regulated in mature biofilms.

It is no wonder that this wealth of metabolic rates seen in biofilms has been explored as a potential mechanism of resistance to antibiotics. It has been proposed several times that the main mechanism of antibiotic tolerance seen in biofilms is the presence of non-metabolising or slow-metabolising cells [Balaban et al., 2004; Brooun et al., 2000; Lewis, 2001, 2007; Singh et al., 2009; Williamson et al., 2012]. Persisters in a biofilm could survive antibiotic treatments due to their slow metabolism and re-establish an infection after the antibiotic is withdrawn, since the biofilm matrix would presumably prevent the host immune system from reaching the cells [Lewis, 2007]. Tashiro et al. [2012] found a link between persister levels and cell density, which could support the hypothesis that more persisters may be found in biofilms. Nevertheless, when biofilm cell tolerance was compared

to stationary cell tolerance, it was found to be similar, if not lower in the former, when exposure to antibiotic was short (6 h) [Anderl et al., 2003; Spoering & Lewis, 2001]. When exposure was 48 h long, biofilm cells survived better than stationary [Singh et al., 2009]. However, it should be noted that in all these comparison studies, biofilm persisters were considered to be all the cells that survived antibiotic treatment in an intact biofilm, i.e exposed biofilms were disrupted and colonies counted. Surviving cells counted in this way could have been protected by several tolerance mechanisms active in the intact biofilms. If persisters are hypothesised to be slow or non-metabolising cells then their isolation method needs to be improved to accurately measure their metabolic state (more on isolation methods in section 5.1.5).

### 5.1.3 Persistence as a social trait

The evolutionary roots of persistence, as a result of slow metabolic rates, have also been investigated. Persisters, as slow metabolising cells, experience the direct benefit of surviving catastrophic evens at the cost of not growing; persisters need up to 13 hours more to start growing, from broth cultures on LB plates, compared to regularly diving cells [Balaban et al., 2004]. At the same time, they benefit their counterparts by not competing for resources. This effect is expected to be increased within clonal, i.e. genetically related populations [Gardner et al., 2007]. Not only the formation of persisters can thus be considered as a social trait, but in a study by Möker et al. [2010] their production was also increased as a response to a quorum sensing-related molecules in *Pseudomonas aeruginosa* cultures. Medaney et al. [2015] showed that cells appearing on agar plates later than normal were able to exploit the protection from β-lactams, offered by β-lactamase producing bacteria, better than growing susceptible cells. These cells were presumed to be dormant, only appearing when β-lactamase producers had detoxified the environment from the antibiotics. In summary, slow-metabolism in bacteria is likely to be a social trait per se but it can also respond to and exploit other social traits; a triangle of interactions that can be studied further as a

potential tool in combating bacterial infections. Understanding the social aspect of bacterial metabolism may allow for a more targeted approach. For example, at the moment persister production is viewed as a partially random effect when it could be a regulated response to other factors related to bacterial competition and cooperation. With the use of a mathematical model subsequently confirmed in vivo Ackermann et al. [2008] showed that a phenotypic variability within a population of cooperators may be a survival strategy; since cooperation is costly and may lead to self-destruction, if a proportion of the population does not express the phenotype they may benefit from the self-sacrifice of the cells that expressed it and died. If this is the case, the results of Medaney et al. [2015] could be further supported as evidence for a persistence-sociality link. Ackermann et al. [2008] found that such a mechanism would be more effective in structured environments.

### 5.1.4 Significance of persisters in health and disease

Persisters have been found in clinical isolates of *Staphylococci* [Bigger, 1944], *E. coli* [Marcusson et al., 2005], *Mycobacterium tuberculosis* [Wallis et al., 1999] and *Pseudomonas aeruginosa* [Mulcahy et al., 2010], which makes the issue a relevant medical problem rather than merely a laboratory phenomenon. Cells that can tolerate antibiotic treatment and revive after the end of the course can have implications in treatment of infections, especially in cases when biofilms are also involved. Persisters need to be considered when developing antibiotics and when deciding the time course of treatments [Gefen & Balaban, 2009; Lewis, 2013; Mulcahy et al., 2010]. They can also be targeted for new treatment strategies [Gefen & Balaban, 2009; Rogers et al., 2012]. A protein that may be essential in the maintenance of the slow-metabolic state, when relevant, may be a viable drug target option [Lewis, 2010*a*]. Considering metabolic states other than exponentially growing cells during drug development may also be a matter of regulatory change [Lewis, 2007].

### 5.1.5 Methods to measure persisters in biofilms

It can be argued that the "observer effect" in quantum physics whereby the result of an observation can be influenced by the act of observing it [Scully et al., 1991] can be equally true in the case of persisters. If the current consensus that persisters are non or slow-metabolising cells is true, then by their very nature, persisters are difficult to isolate; any sample manipulation may affect the physiological state of the cells by either "waking them up", i.e inducing growth or inducing further persister formation. Nevertheless, several methodologies have been developed to study these cells. The most commonly used methodology for persister isolation is exposing the culture of interest to the antibiotic of interest then enumerating the surviving cells [Anderl et al., 2003; Jõers et al., 2010; Keren, Shah, Spoering, Kaldalu & Lewis, 2004; Moyed & Bertrand, 1983; Singh et al., 2009; Spoering & Lewis, 2001; Stewart, 2002]. When the interest does not lie in persistence to specific antibiotics a lytic antibiotic such as a β-lactam is used [Jõers et al., 2010; Keren, Shah, Spoering, Kaldalu & Lewis, 2004; Moyed & Bertrand, 1983; Stewart, 2002], presumably in an attempt to target growing cells. These approaches are sensible when the population of interest is a planktonic culture. However, the case of biofilms is a lot more complicated. As has been discussed elsewhere in this report, several mechanisms of antibiotic resistance have been and are being investigated, many of which appear to be valid contributors to the increased tolerance seen in biofilms. When a biofilm is disrupted after a treatment, be it an antibiotic or any other stressor, what mechanism contributed to cell survival cannot be readily deduced.

The dose killing curve method has been used in some cases as an extension to the enumeration method discussed above [Brooun et al., 2000; Singh et al., 2009; Spoering & Lewis, 2001]. The initial idea behind this method was that increasing doses of antibiotic would kill increasing numbers of growing cells. But if persisters are present in the population, the curve will plateau at some concentration [Brooun et al., 2000; Lewis, 2008; Singh et al., 2009; Spoering & Lewis, 2001]. A time-dependent bimodal killing curve is also perceived as an indication

of persisters [Balaban et al., 2004]. On this basis a Minimum Duration of Killing (MDK) curve has been proposed as a method to detect persisters. The method measures the time needed to kill the persister proportion of the population at an antibiotic concentration higher than the MIC [Brauner et al., 2016].

A novel method to isolate persisters was developed by Shah et al. [2006] using an unstable GFP protein with a promoter that is expressed only during active cell growth. During normal growth, the fluorescence intensity is expected to increase and stabilise. The researchers were able to sort *E.coli* cells according to their metabolic activity, using a cell sorter. Roostalu et al. [2008] also used weakening GFP signal, coming from a non-degradable protein, to measure cell division. In a similar spirit, Orman & Brynildsen [2013*a*] used a fluorescent protein (mCherry) to sort cells by metabolic activity. The different groups were then exposed to antibiotics for 5 h and the survivors, which were considered to be slow-metabolising persisters, were counted. In this way, it was possible to decide which metabolic group produced more persisters. Levin-Reisman et al. [2010] developed a technique to monitor delayed growth on agar plates by periodically obtaining and analysing images. This was achieved by a computer controlled system of an array of scanners and relays. To identify type I and type II persisters Keren, Kaldalu, Spoering, Wang & Lewis [2004]; Willenborg et al. [2014] diluted the investigated cultures in fresh medium and incubated them in a passage assay. At certain time points the cultures were exposed to antibiotics and enumerated. It was expected that in this way type I persisters would be gradually eliminated due to dilution effects, whereas type II persisters would remain unaltered.

In order to measure metabolic activity per se Virta et al. [1998] used a luciferase assay (ATP reaction), Orman & Brynildsen [2013*a*] used a reductase activity assay and Orman & Brynildsen [2013*b*] tetrazolium assays. Orman et al. [2015] developed an aminoglycoside potentiation assay, which relies on carbon metabolism and CFU counts in order to measure the metabolism of predetermined persister populations. Once again, all these methods may provide fairly accurate results on the metabolism of planktonic cells but in order to be used for biofilms, the biofilms need to be disrupted first. Therefore, in such a context they

should be used with caution. Wentland et al. [1996] used fluorescence staining or RNA and DNA to define growth rates in biofilms, on the basis that RNA/DNA ratios correlate with growth.

Based on the above it is evident that although a lot of progress has been made in isolating and measuring persisters, this is often down in an indirect way due to the technical difficulties associated with their isolation. Furthermore, a direct measurement of persisters in biofilms is a technical challenge that is still to be met. For the purposes of this work all planktonic or biofilm-released cells that survived an antibiotic challenge were defined as tolerant without a prerequisite for slow metabolic rates. Although, it was not possible to apply any methodology to accurately measure cell growth in biofilms in situ, every effort was made to maintain the metabolic state of bacteria close to what it was before they were released from the biofilm. The protocols were also developed with the aim to exclude biofilm structure-related resistance mechanisms that could have led to survival.

## 5.2 Aims and hypotheses

The aim of the work described in this chapter was to measure tolerance in biofilms and establish any potential relationship to metabolic activity. The hypotheses tested in this chapter are listed below:

**Hypothesis 1**. As biofilms age, tolerance levels increase and this is due to decreasing metabolic rates.

**Hypothesis 2**. Biofilm tolerance levels are higher compared to planktonic cultures

**Hypothesis 3**. Biofilms exposed to antibiotics exhibit higher levels of tol-

erance compared to non-stressed biofilms, as antibiotic-stress survivors would be expected to be enriched for persisters.

## 5.3 Materials & Methods

### 5.3.1 Strain and media

**Bacterial strains**. Wild Type (WT) *Escherichia coli* CC11-1 was used for all experiments in this chapter.

**Media and antibiotics**. Terrific broth (Sigma T0918), LB (Lennox) broth and 2% (w/v) LB agar plates were prepared according to the manufacturer's instructions (Sigma L3022; Oxoid LP0011). Starting cultures were prepared by inoculating 5 ml Terrific broth with a WT colony and incubating at 37 °C for 22 h, with agitation at 180rpm. Sigma-Aldrich PBS tablets (Cat no P4417-100AT) was used for the biofilm disruption and serial dilutions. The ampicillin and ofloxacin used were purchased from Sigma (Cat No A0166 and O8757 respectively).

### 5.3.2 Tolerance in biofilm-released cells

Biofilms were grown on polycarbonate membranes as before for 24 h and they were transferred to LB, or 1 mg/ml ampicillin plates for up to 3 days. Each day samples were disrupted in 10 ml PBS, centrifuged, resuspended in 10 ml PBS and split in equal portions. Either 1 mg/ml ampicillin or 1 mg/ml ofloxacin were added to two of the portions. A sample in PBS without antibiotics was used as a control to estimate natural changes in the population during the incubation time. Samples were incubated at room temperature for 2 h. After this time the samples were centrifuged again at 4600 rpm for 10 min, resuspended in equal amounts

of PBS, serially diluted and plated out using the droplet method. As this was an attempt to capture the metabolic state of the bacteria in the biofilms before disruption, a 2 h exposure was chosen in order to eliminate any effects on the metabolic state of the cells that longer incubation times could have. Additionally, 2 h were considered to be adequate time to inhibit growth of susceptible cells, as growth monitored during the MIC assays described in Chapter 2 did not indicate growth ability during the experiment.

### 5.3.3 Tolerance in stationary planktonic cells

In order to test persister levels in stationary phase broth cultures, 4.5 ml LB inoculated with 500 µl diluted starting culture and incubated for 24 h at 37 °C. After 24 h the cultures were used to inoculate new LB media in the same manner and the rest was split in equal portions and centrifuged at 4600 rpm for 10 min. The pellets were resuspended in PBS or PBS plus 1 mg/ml ampicillin or ofloxacin. The samples were incubated at room temperature for 2 h. After this time they were centrifuged again, resuspended in PBS and plated out. The process was repeated over 2 days.

### 5.3.4 Measuring metabolism in biofilms

#### 5.3.4.1 Awakening biofilm-released tolerant cells

Polycarbonate filter membranes were inoculated with 5 µl starting culture as before and incubated at 37 °C for 24 h. The 24 h biofilms were transferred to new LB plates for 1 to 3 days. Each day 3 biofilms were disrupted in 10 ml LB (instead of PBS) and 20 µl were taken out, serially diluted in PBS and plated for colony counting. This served as the baseline count of cells. The remaining solution of disrupted biofilms was split in equal portions and ampicillin (1 mg/ml)

was added to one of them. The samples were incubated at 37 °C for 2 h, with agitation at 180 rpm. After this second incubation, the samples were centrifuged at 4600 rpm for 10 minutes, resuspended in PBS, serially diluted and plated out on LB plates. When the experiment was repeated an ofloxacin treatment of 1 mg/ml was also added.

#### 5.3.4.2 Measuring ATP production in biofilm-released cells

**ATP measurements**. Biofilms were inoculated as before and were grown on LB plates. At 5, 10, 17, 24, 48 and 72 h of growth they were disrupted in 10 ml PBS. 100 µl of the disrupted samples plus 100 µl BacTiter Glo (Promega, Cat No G8230) reagent were mixed in 96-well plates. 100 µl PBS mixed with 100 µl reagent was used as a control. Endpoint luminescence measurements of the reagent reaction with the ATP produced by the bacteria were taken at high PMT (photomultiplier) voltage for higher sensitivity in detecting low concentrations of ATP and medium precision with a spectrofluorometer(SpectraMax Gemini XS, Molecular Devices, Workingham, UK).

**Measuring ATP in exposed biofilm-released cells**. 5, 10, 17, 24, 48 and 72 h old biofilms were exposed to 1 mg/ml ampicillin for 24 h and ATP production was measured in the same way as above.

**ATP curve**. ATP, 100 mM solution, purchased from ThermoScientific (Cat No R0441) was used to produce an ATP curve. The protocol suggested by Promega in the BacTiterGlo manual was used. Briefly, 1 M ATP was prepared in PBS (100 µl of 1 µM ATP solution contains $10^{10}$ moles ATP) and diluted in 10-fold serial dilutions in PBS (1 M to 10 pM; 100 µl volume contained $10^{10}$ to $10^{15}$ moles of ATP). 100 µl of the ATP dilutions were added to a 96 well plate and mixed with 100 µl of BacTiter-Glo reagent. The 96-well plate was placed in the luminescence reader, mixed for 5 seconds and left to incubate for 1 min. Luminescence was recorded. PBS plus reagent was used as a control.

### 5.3.5 Statistical analysis

Statistical analyses were conducted in R [R Core Team, 2013]. Linear regression analysis was used to model the log tolerance ratio in ageing biofilms, stationary broth cells or pre-exposed biofilms against age, antibiotic type and exposure time or passage time, as appropriate. Log Tolerance Ratio was defined as the natural logarithm of the ratio: *[number of antibiotic survivors]/[number of cells before antibiotic challenge]*. $10^5$ cells were added to the CFU counts before calculating the log in order to be able to include zero CFU counts in the analysis. Residual plots were used for checking model assumption of residual normality and homoscedasticity.

## 5.4 Results

### 5.4.1 Building an ATP curve

As can be seen in Figure 5.1, the luminescence signal corresponded very well to increases in ATP concentration. The regression line generated here was used to calculate ATP concentrations in biofilm and broth samples, where luminescence was measured.

### 5.4.2 Tolerance to antibiotic fluctuates and increases as biofilm ages

Figure 5.2A shows how biofilm-released cells responded to antibiotic treatment as the biofilms aged. Tolerance was measured on the basis of colony forming units and represents the proportion of cells that survived the 2 h antibiotic challenge

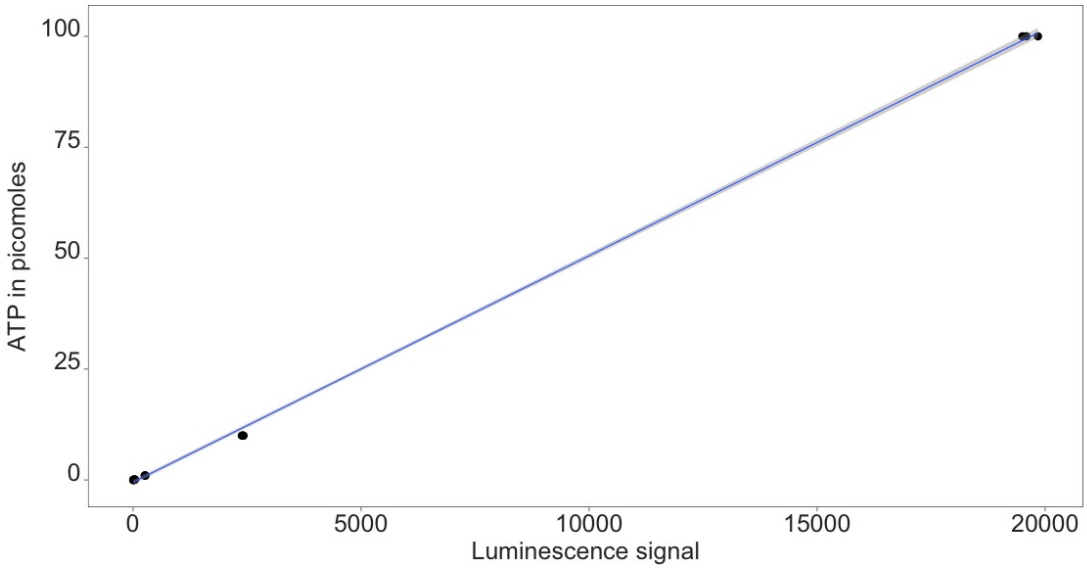


**Figure 5.1:** The ATP curve used to estimate ATP production in biofilm and broth samples, based on luminescence measurements. The regression equation represented by the line in the graph was *[ATP]= 5.105220e-15[Luminescence signal] - 5.100973e-13 with* $R^2$ *= 0.9995, p<0.0001*

compared to the number of cells that were present before the challenge. An oscillation in the first 48 h and subsequent stabilisation of tolerance is visible for both classes of antibiotics used in these experiments. At all ages cells were significantly less tolerant to ofloxacin compared to ampicillin (t=-8.394, p<0.0001). In a linear regression model here both time and antibiotic type contributed significantly to explaining variation [$F_{(2,45)}$=41.18, p<0.0001, $R^2$ adjusted=0.63]. These tolerance patterns corresponded to an increasing number of CFUs that appeared to stabilise after 24 h (Figure 5.2C), and a metabolic rate that increased sharply from 5 to 10 h and then decreased until it reached stability (Fig. 5.2B). Within the first 24 h of growth, growth rates did not correspond to ATP production very accurately, as CFUs kept increasing even when ATP production was reduced.

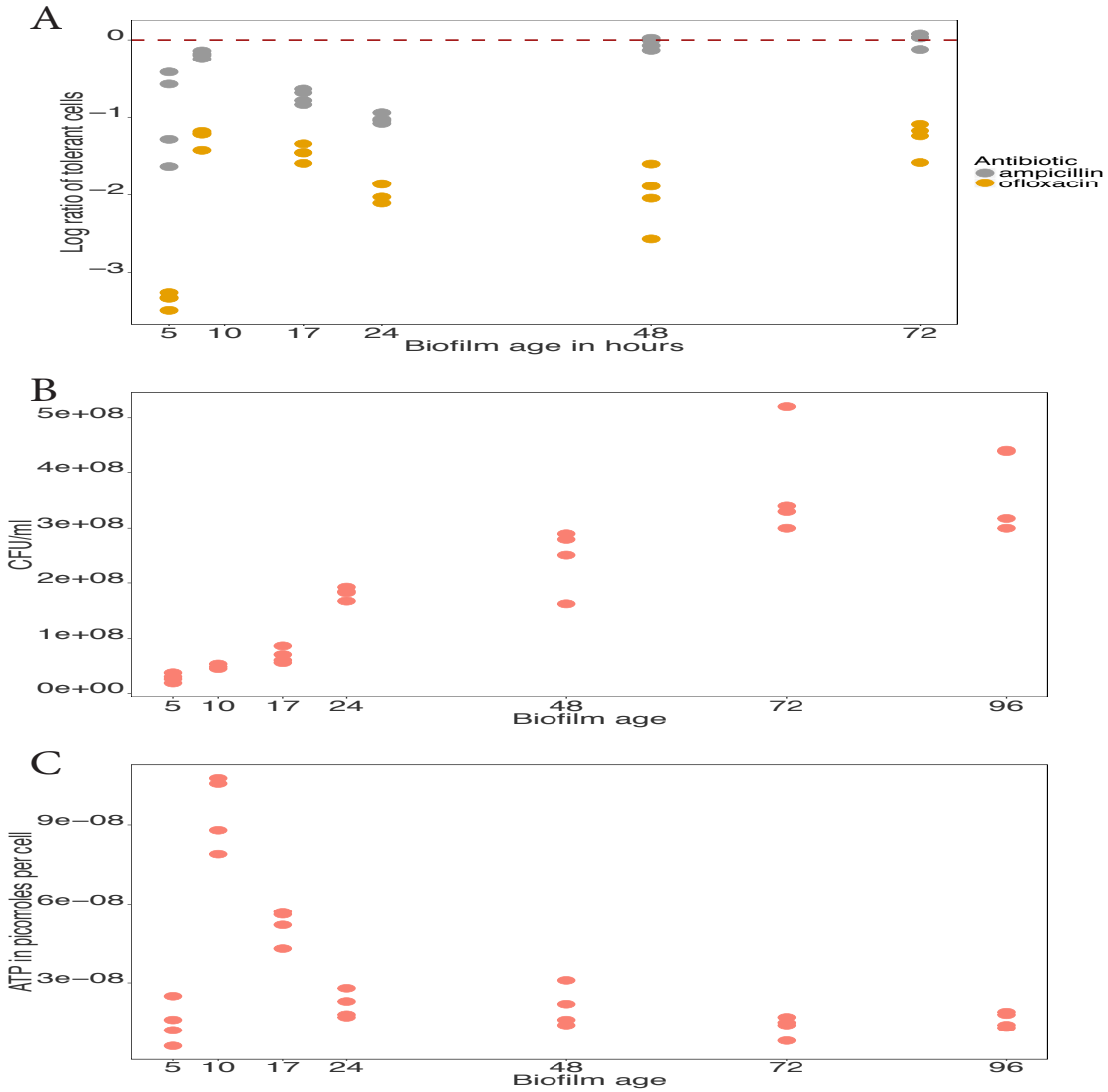


**Figure 5.2:** (A) Survival rates at 1 mg/ml ampicillin or ofloxacin. Survival increased as the biofilms aged. The same trend was observed for both classes of antibiotics. The dashed red line indicates 100% survival.Each colour represents a different resistant proportion in the inoculum The Y axis is in a log scale where 0 equals $10^0$. (B) Colony Forming Units (CFUs) increased as biofilm aged and reached a plateau after 24 h. (C) ATP production per cell was generally higher in younger biofilms and stabilised in mature biofilms, older than 24 h.

### 5.4.3 Stationary cultures are more tolerant to antibiotics compared to biofilm-released cells

Tolerance in 24 h stationary broth cultures (Fig. 5.3) was higher than in 24 h biofilms (Fig. 5.2A); 68% for ampicillin and 37% for ofloxacin, on average, at 24 h and increased even further after one passage (48 h) to 100% for both ampicillin and ofloxacin. The respective values for 24 h biofilms were approximately 10% for ampicillin and 1% for ofloxacin. For 48 h biofilms it was 90% for ampicillin and 1% for ofloxacin. Tolerance to ampicillin and ofloxacin in stationary broth cells were not significantly different at both time points (t=-0.879, p=0.39). The model [$F_{(2,29)}$=19.96, p<0.0001, $R^2$-adjusted= 0.55] explained 55% of the variation seen with time being a significant factor (t=6.256, p<0.0001).

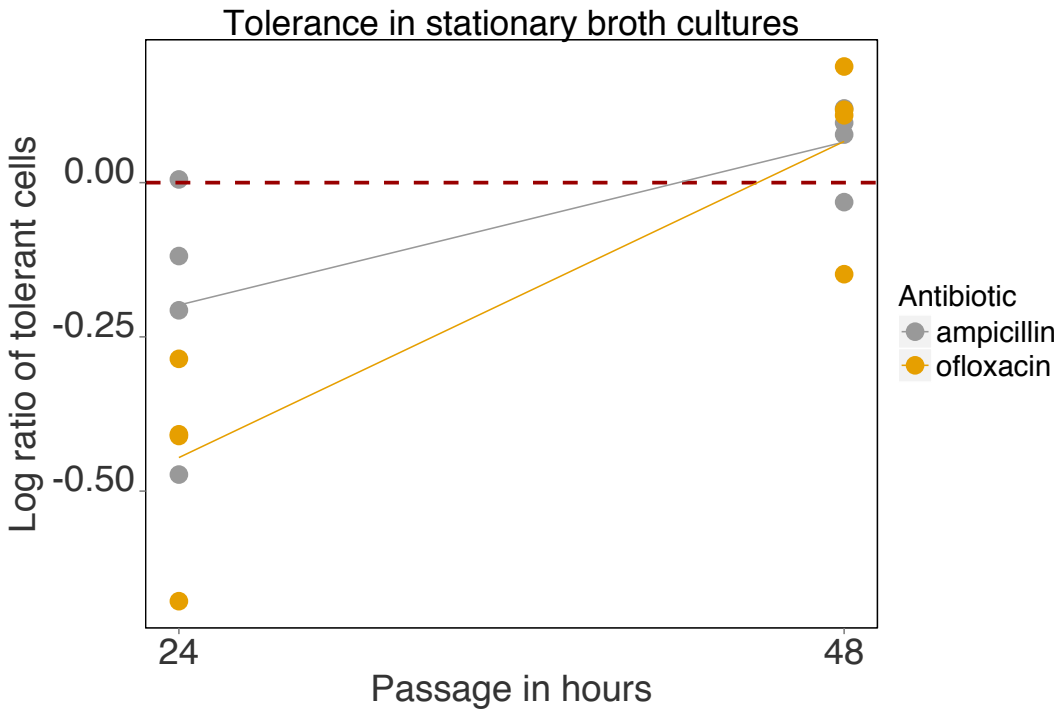


**Figure 5.3:** Stationary cell tolerance to ampicillin and ofloxacin. Tolerance was measured in a 24 h culture and in a subsequent passage culture (48 h). Each colour represents a different resistant proportion in the inoculum The Y axis is in a log scale where 0 equals $10^0$.

### 5.4.4 Ampicillin exposed biofilm-released cells show higher tolerance to antibiotics although long-term exposed biofilms gradually die

I predicted that if tolerance was important for survival in biofilms, biofilm-released cells that had survived exposure to antibiotics would be enriched for tolerant cells (Hypothesis 3). This hypothesis was supported in that, in a different set of experiments, where tolerance of pre-exposed biofilm-released cells to ampicillin and ofloxacin was measured, it was found to increase over time for ampicillin but remained relatively stable for ofloxacin (Fig. 5.4C). The number of cells in exposed biofilms decreased over time until they were killed after a 72 h exposure (Figure 5.4D). Cells from pre-exposed biofilms were more tolerant to ampicillin than from unexposed biofilms although, this was not significant (t=-1.504, p=0.14) in a linear regression model ($F_{(3,56)}$=30.13, $R^2$-adjusted=0.67, p<0.0001). The effect of time was not significant either (t=1.308, p=0.19) but ofloxacin tolerance was significantly lower than ampicillin (t=-9.310, p<0.0001) in the same model.

ATP production was measured in biofilms of different ages that were exposed to ampicillin for 24 h. Fig.5.4A shows that the exposed biofilms appeared to metabolise more rapidly as they aged (older than 24 h), in contrast to slowing metabolism of the unexposed biofilm. It should be noted that 5 h old biofilms exposed to ampicillin did not survive the treatment. At the same time the number of cells surviving exposure increased over time and stabilised after 24 h (Fig.5.4B). In biofilms that were not exposed to ampicillin the ATP production per cell decreased in older biofilms and CFU/ml increased, as seen in Fig. 5.2. The unexposed biofilm data are the same in the two figures but each figure is meant to discuss a different hypothesis. Unexposed older biofilms survived ampicillin treatment better than younger ones, as the number of cells dying decreased. However, unexpectedly their metabolic rates did not seem to slow down as a result of the ampicillin induced stress in pre-exposed biofilms. On the contrary, they appeared to be higher than in unexposed biofilms from 24 h old biofilms upwards.

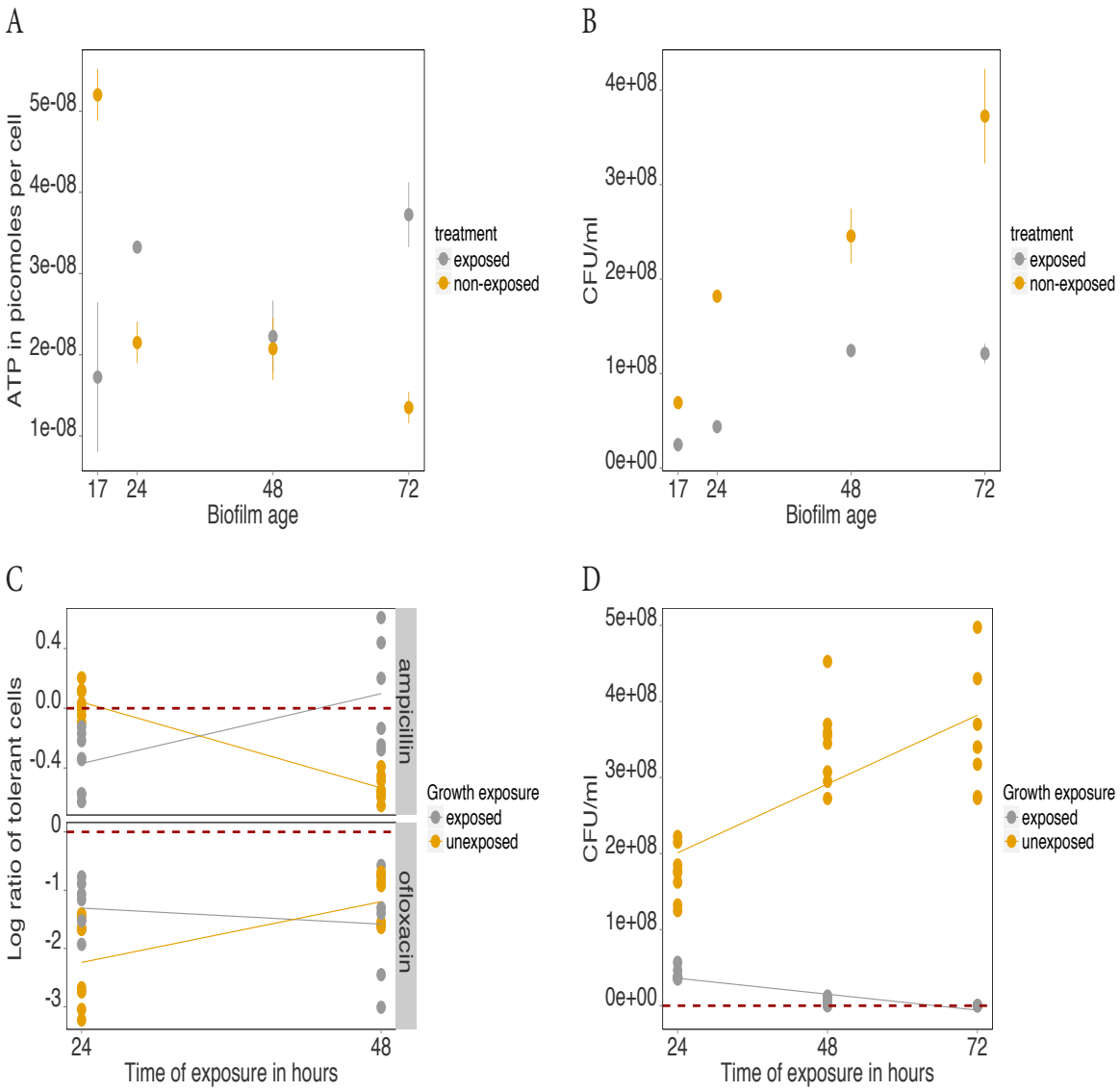


**Figure 5.4:** (A) Mean+/- SE ATP production in biofilms exposed to ampicillin for 24 h compared to unexposed controls (B) CFU/ml +/- SE in exposed biofilms compared to unexposed controls (C) Tolerance to ampicillin and ofloxacin in biofilm-released cells when the intact biofilms had been exposed to ampicillin for up to 72 h.Each colour represents a different resistant proportion in the inoculum. Where the Y axis is in a log scale where 0 equals $10^0$. (D) CFU/ml in biofilms exposed to ampicillin for up tp 72 h.

### 5.4.5 Biofilm-released cells in nutient rich media show a lower tolerance to antibiotics

In order to verify that metabolic rates were indeed involved in the results described thus far, biofilms were disrupted in LB and incubated in nutrient rich media, instead of being disrupted in PBS at room temperature. Figure 5.5B shows that under these conditions the cells grew in numbers compared to the disruption point but growth rates decreased as biofilms aged. At the same time the proportion of cells that tolerated the antibiotic treatment this time decreased substantially (Fig.5.5A).

## 5.5 Discussion

Tolerance and persistence are terms used interchangeably in the literature although they do not always describe the same condition. In this report survivors of a 2 h antibiotic challenge were described as tolerant and it was left as an open question whether they are also persisters, i.e. whether they represented only a small proportion of the population, in an attempt to abide to recent definitions found in published work (section 5.1.1) and avoid further confusion. However, the word persisters was used when referring to work of others. Slow metabolic rates were not included in the definition but instead it was the aim of this work to determine if tolerant cells were in fact slow metabolisers.

In the experiments described in this chapter unusually high doses of antibiotics were used (nearly 1000 times higher than the MIC) to test tolerance. Since the main aim of this work was to investigate tolerance in biofilms rather than in broth, the antibiotic concentration was chosen with the biofilm physiology in mind, i.e. lower doses were not challenging enough for biofilms as they had been previously found to survive doses as high as 6 mg/ml for 24 h (data not shown). Additionally, biofilms were disrupted in PBS, as well as in growth-promoting media and the

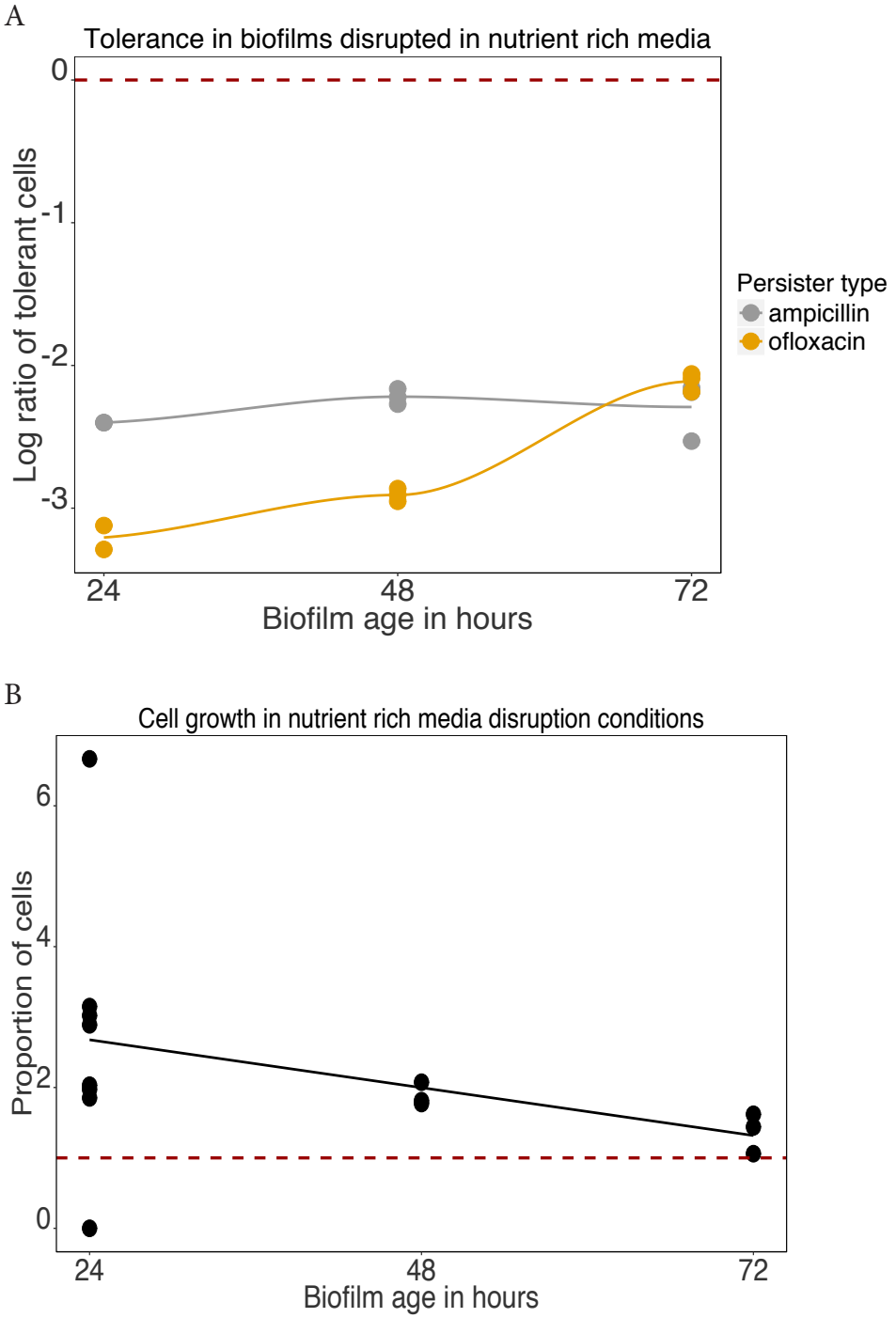


**Figure 5.5:** (A) Antibiotic tolerance decreased substantially compared to what was seen in Fig. 5.2A.Each colour represents a different resistant proportion in the inoculum Where the Y axis is in a log scale where 0 equals $10^0$. (B) Proportion of cells compared to numbers at the disruption point; cell growth under these conditions is evident

antibiotic challenge was kept relatively short. These precautionary measures were taken in order to ensure that the metabolism of the biofilm-released cells remained similar to what it was in the biofilms, as far as this could be achieved with the disruptive methods used. Disruption in growth-promoting media was used as a control. It was therefore assumed that the biofilm-released cells retained a metabolism similar to what it was before the biofilm disruption. Also, although the method could not capture biofilm metabolism in situ, disruption eliminated other reasons that bacteria can survive for when in biofilms, leaving metabolism as the main candidate. Brooun et al. [2000] tested resistance of biofilm-**bound** cells in growth medium and biofilm-**released** cells in PBS and found the tolerance to be similar. This result validates the assumption that disruption in PBS retains the metabolic state of the cells. However, the results reported in this paper were not in survival ratios and could not be used as a comparison.

Interestingly, even at such a high dose, the levels of tolerance were extremely high in biofilms, fluctuating with biofilm age (Fig. 5.2A). In fact tolerance corresponded to a proportion of ampicillin-tolerant cells equal to 15% for 5 h old biofilms up to 100% in 48 h and 72 h old biofilms. Ofloxacin tolerance started at 7% in 8 h old biofilms (5 h old biofilms did not survive ofloxacin treatment) and did not exceed this level in older biofilms. These figures are higher than those reported by other researchers, probably reflecting the differences in the methodology used.

To make my methodology more comparable to what was seen in the literature and to check on the metabolic state of the biofilm-released cells, biofilms were also disrupted and incubated in nutient rich media. Under these circumstances any low-metabolising cells were expected to initiate growth and results were strikingly different. The biofilm-released cells showed low survival during the antibiotic challenge in high nutrient media (Fig. 5.5), with ampicillin tolerance levels in this figure corresponding to 0.2% for 24 h biofilms and up to 0.5% for 48 h and 72 h biofilms (ofloxacin tolerance was 0.04% for 24 h biofilms up to 0.6% in 72 h biofilms). Tolerance measured in this way was more in line with levels reported elsewhere in the literature for biofilms not older than 24 h. When

nutient rich media were used, cells started growing (Fig.5.5B). The substantial differences in tolerance between bacteria in PBS and in high nutrient broth indicate that physiology responds very quickly to nutrients and that this substantially affects tolerance. It is plausible that the different assays reflect different modes of antibiotic tolerance that exist in biofilms. The PBS-challenged cells had high levels of tolerance but the results from the LB-challenged assays indicate that only a small proportion of these were fully dormant persisters.

Stationary broth cultures were more tolerant than biofilm-released cells as has been reported before [Anderl et al., 2003; Brooun et al., 2000; Spoering & Lewis, 2001]. Stationary cells after 1 passage, where cells from the 24 h time point were diluted in fresh broth media and incubated for another 24 h (48 h time point), not only survived both ampicillin and ofloxacin treatments, at concentrations that planktonic bacteria cannot normally survive, but they appeared to have grown as well (Fig. 5.3) (it should be noted, that substantial cell death occurred in the PBS controls after the 2 h incubation at 48 h). Even at 24 h 68% of stationary cells survived the ampicillin treatment and 37% the ofloxacin treatment. Cells in the control were not affected by the incubation at this time point. Again, this level of tolerance was beyond anything that was found in the literature using the nutrient rich media method of disruption. Stationary cells though, were a lot more tolerant than biofilm-released cells using this method too. In order to test for genetic resistance due to random mutations, some of the colonies recovered from the antibiotic challenge were re-streaked onto antibiotic containing agar plates and they did not grow. The same control was also used for biofilm-released cells.

Increased tolerance in pre-exposed biofilms was consistent with the expectation that under antibiotic stress, metabolism slows down and therefore, higher tolerance to antibiotics should be expected [Dörr et al., 2010]. Firstly, biofilms survived the ampicillin in the growth medium for 2 days before they were finally eliminated (Fig. 5.4D). This was a far higher survival rate compared to planktonic cells that could not even survive a dose of 9 μg/ml in the MIC assay. Brauner et al. [2016] suggested that if longer exposures to the same antibiotic dose are effective in killing a population, this should be considered as an indication for

the presence of slow-metabolising persisters. If this is true and given that a 72 h exposure was required to kill biofilms, this could be consistent with the hypothesis that biofilm-bound cells are slow-metabolisers. Intact biofilms exposed to 1 mg/ml ofloxacin survived for 24h only (data not shown). Figure 5.4C illustrates that 48 h pre-exposed biofilm-released cells show increased ampicillin tolerance (100%) compared to 48 h biofilms that had not been pre-exposed to ampicillin (30%). Ofloxacin tolerance in 48 h exposed biofilm-released cells was about 3%, similar to 24 h and 48 h unexposed biofilms. In 24 h exposed biofilms, ofloxacin tolerance was about 10%.

Thus far, results suggested that biofilm-bound cells exhibit reduced metabolic rates as biofilm released cells were extremely tolerant to antibiotics. Similarly, stationary state cells were also very tolerant to both antibiotics tested. Disruption in LB and incubation in nutrient rich media precipitated growth and resulted in a very substantial decrease in tolerance, corroborating the hypothesis that cells in biofilms are "sleeping beauties". Furthermore, pre-exposed biofilms exhibited an increased tolerance, consistent with hypothesis 3.

Although the results discussed above appear to be in good agreement with what others have concluded, slow metabolism was only assumed as the reason behind the patterns seen. In order to get a measure of metabolic activity, ATP production was measured in biofilm-released cells. Metabolic activity in biofilms younger than 24 h old showed an increase and then a subsequent decrease in metabolic rates (Fig. 5.2C) which stabilised for biofilms up to 96 h old. The oscillation in metabolic rates seen in younger biofilms may be a result of the biofilm growth patterns seen by Liu et al. [2015]. These researchers observed a periodic reduction in biofilm expansion after it had reached a certain size, owing to nitrogen limitations which stopped the peripheral cells from growing in an oscillating manner. Peripheral cells were found to experience glutamine limitations. Since, both glutamate and ammonium are needed for the production of glutamine and glutamate was provided in the medium, it was ammonium that was in shortage for the cells in the periphery. This was found to be associated with the reliance of peripheral cells to ammonium produced by the internal cells in the

biofilm. Ammonium production internally was in turn limited when glutamate was consumed by the peripheral cells and did not reach the internal layers. This co-dependence of peripheral and internal cells in the biofilm appeared to be a mechanism of social conflict resolution.

Older biofilms showed a stable, decreased average metabolic rate compared to young biofilms. Tolerance levels (Fig. 5.2A) in older biofilms were consistent with the slow-metabolism hypothesis but this was not the case for younger biofilms where increases in tolerance corresponded to higher metabolic rates and higher growth rates as measured by CFUs (Fig. 5.2B). This result was unexpected and it would require further investigation. The discrepancy may have been due to the use of mean ATP production per cell as an indication of metabolic rates. Using this measure assumes that all cells metabolise equally. Research suggests that this may not be strictly true [Ito et al., 2009; Stewart & Franklin, 2008; Wentland et al., 1996]. Also, metabolic rates as measured by ATP may not be a good indicator of growth rates, at least not in young biofilms, as can be seen by comparing Figures 5.2A & B; CFUs kept increasing even when ATP decreased in biofilms younger than 24 h.

When ATP was measured in pre-exposed biofilms the results were surprising as generally higher metabolic rates were observed in 24 h old biofilms and older, compared to unexposed biofilms (Fig. 5.4A). Therefore, the underlying hypothesis that cells in stressed biofilms would not be metabolising, as much, could not be validated by this experiment. Ampicillin tolerance in pre-exposed 24 h biofilms was slightly lower than in unexposed biofilms but increased in 48 h old biofilms. Metabolism in 48 h biofilms in this case could not be mapped to 5.4A though as the treatment was different.

Two types of antibiotics were used in this study: the β-lactam ampicillin and the fluoroquinolone ofloxacin. β-lactams are believed to be more effective against actively diving cells [Tuomanen et al., 1986] as they target the cell wall synthesis process [Tomasz, 1979] whereas ofloxacin interferes with DNA synthesis [Sato et al., 1986] and can kill non-growing, as well as growing cells [Tanaka et

al., 1992]. It is also particularly active against *E. coli* [Sato et al., 1986]. It was expected that ofloxacin would be more effective in killing cells that could be tolerant to ampicillin due to their slow metabolic state. Indeed ofloxacin was more effective in killing cells but the killing pattern followed that of ampicillin and that of metabolic rates in an almost identical manner in biofilms grown on LB (Fig. 5.2A &C). Stationary cells showed a very high level of tolerance to this antibiotic as well; a lot higher than biofilms. In the case of biofilms pre-exposed to ampicillin, ofloxacin tolerance did not change much over time (Fig. 5.4), when tolerance to ampicillin increased.

In conclusion, the majority of results presented here support the idea of slow-metabolising cells in biofilms behaving like sleeping beauties. However, the high metabolic rates seen in biofilm-released cells coming from biofilms that were previously exposed to ampicillin are not consistent with the hypothesis that intact biofilms exposed to antibiotics survive the treatment due to reduced metabolism. Additionally, metabolic rates in young biofilms did not validate the metabolism hypothesis either. These observations in conjunction with reports indicating that persisters may actually be actively metabolising cells, highlight the need for methods more accurate measuring metabolic rates in bacteria without affecting those rates in the process. Additionally, a definition of active metabolism and an establish link of such metabolism to growth rates are required in order to be able to use tolerance to β-lactams as an indicator of slow growth.

# Chapter 6

# General Discussion

Microbes play an important role in our lives and this should not come as a surprise since they have cospeciated with humanoids since 15 million years ago [Moeller et al., 2016]. Their influence in the function of our human bodies has become evident in the last few years. For example gut bacteria have been associated with mood alterations in mice [Bravo et al., 2011], potentially by modulating the production of neurotransmitters [Strandwitz et al., 2016]. Bacterial community make up has been associated with the training of our immune system [Mazmanian et al., 2005].

Bacteria are also social beings, living in communities [Costerton et al., 1994], communicating [Losick & Kaiser, 1997; Miller & Bassler, 2001], cooperating and competing with each other [Foster, 2010; Griffin et al., 2004]. These bacterial interactions are likely to play a role in human health and disease. Bacterial interactions have not been incorporated in our current risk assessments of pathogenicity, although they may be significant [Cornforth et al., 2015]. Social traits can be used for new drug development, with the "Trojan Horse" strategy being a discussed option [Brown, West, Diggle & Griffin, 2009]. Nonetheless, such a strategy would rely on the resistant cooperators not bouncing back in the cheater dominated

population via evolutionary selection [Harrison, 2013; Raymond et al., 2012].

In this work, bacterial interactions were studied in the context of biofilm tolerance to antibiotics. Biofilms are very common in nature [Costerton, 1999] and they are believed to be ideal for the expression of bacterial sociality [Foster, 2010]. Furthermore, they are highly tolerant to antibiotics, at a time when increased antibiotic resistance has reached alarming levels [ECDC, 2013; EFSA, 2016]. The experimental system chosen comprised of an environmental *E. coli* strain with a natural ability to form thick biofilms, engineered to express the clinically relevant $bla_{\text{CTX-M-14}}$ gene (Chapter 2). This gene confers resistance to β-lactam antibiotics by coding for the production of an antibiotic hydrolysing enzyme which is considered to be a public good, i.e a social trait [Dugatkin et al., 2005; Yurtsev et al., 2013]. The sociality of β-lactamases in biofilms along with the interaction of the antibiotic with the biofilm structure and the bacterial metabolic state were the main themes investigated.

## 6.1 The sociality of β-lactamases

In Chapter 3 the sociality of β-lactamases was examined in biofilm and planktonic cultures using competition experiments between β-lactamase-producing (cooperators) bacteria and non-producers (cheaters). The central hypothesis was that social cheating, i.e exploitation of β-lactamases by non-producers is enabled in biofilms more so than in planktonic cultures due to high cell density and proximity to relatives, as predicted by evolutionary theory. Additionally, in the case of biofilms theory predicts that population mixing favours social cheating but population segregation favours cooperation (Section 3.1.4).

The experimental results indicated that in biofilms the cheaters maintained a higher fitness in undisrupted biofilms when they were present at low or intermediate frequencies in the starting culture which is what theory predicts [Ross-

Gillespie et al., 2007] and what other researchers reported for other social traits. Additionally, the intact biofilm structure allowed for more cheating than disrupted and re-established structures but only when initial frequencies of cheaters were low or intermediate. When cheaters were present in high frequencies in the starting culture, the fitness loss in undisrupted biofilms was higher than in disrupted and re-established ones. Cheating was disfavoured in broth cultures, in the presence of ampicillin. The proportion of cooperators required for maximal biofilm viability, i.e. for total cell abundance to reach levels equal to unexposed biofilms, was no more than 14%, when in planktonic cultures this was 6 times higher and with no maximal viability achieved. These results support the hypothesis that biofilms form a fertile ground for cooperation and social conflict between bacterial strains but cooperation is negative frequency dependent for the cooperators which does not agree with theoretical models and other experimental work in biofilms, looking at other social traits.

Another interesting observation was that β-lactamase production in biofilms was mostly intracellular when in broth it was extracellular instead. This may indicate that strains were segregated in the biofilm, as theory predicts for cooperative behaviours, and diffusion of the public good was not required in biofilms but it was in broth were strains were mixed. Also, the amount of enzyme produced in biofilms was not affected by the antibiotic, when in broth production was minimal when no antibiotic was present in the media. Additionally, when the cooperators were rare they produced more enzyme per cell on average in biofilms.

When considering targeting bacterial sociality for treatment of infection, two questions need to be asked: (a) is it going to be effective? and (b) is it going to be evolutionarily stable? In order for a social intervention to be successful the interactions between the strains or species used and their effect on virulence need to be well-understood. For example, it has been suggested that clonal infections are more potent because the pathogens are more closely related and they cooperate instead of competing, as would happen in a mixed-clone infection [Brown, Fredrik & Taddei, 2009; Diggle, 2010; Harrison et al., 2006]. Kinship may only be an effective way to reduce virulence at an intermediate frequency though [Gardner et

al., 2004]. With regards to the second question posed, in order to effectively treat a pathogen that expresses a cooperative trait, the treatment has to permanently select for cheats. However, as discussed above, the cooperator:cheater balance has not been shown to be supportive of the elimination of the cooperative trait in a natural experimental setting [Raymond et al., 2012]. A potentially successful strategy was used by Ross-Gillespie et al. [2014] who found that quenching extracellular siderophores in a *Pseudomonas* culture reduced the virulence of the strain without evolutionary restoration of the trait. A theoretical model in support of this strategy where a public good is targeted instead of a vital mechanism for cell survival was developed by Pepper [2012] and showed that such a strategy can be evolutionary stable. It has also been argued that the cost of expressing a trait may not be the only factor affecting its potential extinction. Virulence, spatial heterogeneity as well as host susceptibility were among the factors identified to influence population dynamics, concluding that species coexistence, as seen in natural systems, is an alternative scenario [Colijn et al., 2010].

When evolutionary theory is considered as an antimicrobial strategy, it is normally assumed that the pathogen-target is the resistant-cooperator but research has showed that antibiotic resistance genes are present in the natural gut flora [Lu et al., 2014]. In such a case, the resistant beneficial bacteria may be outcompeted by a susceptible cheat. One then has to ask whether the cooperator or the cheater needs to be targeted in the given infection and the answer to this question can change the strategy dramatically.

On the basis of the results discussed above, it would appear that if it is the pathogenic cheats that need to be targeted, then the biofilms need to be maintained undisrupted in order to maintain the structure that benefits cooperators more than mixing. Additionally, high antibiotic doses need to be used. But this would only work if the cooperators were low in the first place, i.e in later stages of infection. If on the other hand, the target was a population of resistant cooperators that were causing the infection, then the situation would be more complicated. Increasing the antibiotic dose in this case, would benefit the pathogenic cooperators, unless the biofilm was disrupted. This would mainly

work if the cooperators were present in high frequency, as this would allow for the establishment of the susceptible cheater. This suggests that when the pathogens are resistant cooperators, which is commonly the case, increasing the antibiotic dose would not help to eliminate the infection, but introducing a susceptible strain at a later stage of infection when the cooperators are high in frequency could work as a strategy. Targeting the public good itself as suggested by others may then be also be an option for infections caused by cooperative pathogens. If such a strategy was chosen, then cooperators would have to be present at a low frequency and the biofilm would have to be disrupted. In this way the cooperators would be disfavoured and more extracellular enzyme would be produced, i.e. easier target. This strategy could be combined with an antibiotic treatment targeting virulence [Ternent et al., 2015]. According to Colijn et al. [2010] though any of these strategies would need to be tested in a natural system in order to identify other potential parameters affecting the dynamics.

## 6.2 The effect of biofilm structure on antibiotic tolerance

In Chapter 4 antibiotic tolerance was investigated in relation to the biofilm structure, with the aid of CLSM and OCT. Three aspects of the biofilm structure were considered for this part of the work; the structure as a barrier to the antibiotic penetration, mechanical responses to antibiotic treatment and strain localisation in mixed biofilms. The hypotheses that formed the core of the experimental design in this part were: a) the antibiotic is prevented from reaching its target in the cell by the biofilm matrix. When resistant bacteria are present in the biofilms, the hydrolysis of the antibiotic forms an additional obstacle, protecting the cells, b) antibiotic treatment affects the bacterial populations, changing their localisation dynamics and c) structural changes to the antibiotic contribute to the increased tolerance seen.

The results showed clearly that the antibiotic could penetrate the biofilm; neither the matrix nor β-lactamases stopped it from reaching its target in the cell wall. However, the penetration time increased with the proportion of resistant bacteria present. The fluorescence signal coming from the cells was reduced after the antibiotic treatment but the effect was higher in the case of fully susceptible cells where the cell signal decreased more than in all other cases. This result indicated that the antibiotic is not prevented from reaching its target in the cell and it affects all populations present, even if they are β-lactamase producers. However, when producers are present non-producers can be affected less, therefore the antibiotic clearance appeared to be a social trait.

In terms of strain localisation, I observed that resistant cells in mixed biofilms occupied the bottom layers of the biofilms, near the nutrient source. The two strains were segregated with resistant cells usually surrounding the susceptible ones, which occupied a larger area and were higher in numbers. This segregation suggested a cooperative behaviour if the utilisation of β-lactamases as a public good follows the same rules as other social traits such as EPS production [Kim et al., 2016]. A potential response to the antibiotic was noticed in the case of Low Resistant (LR) biofilms, i.e. where the proportion of resistant bacteria in the founding population was less than 10% (this number did not increase substantially during the experiment). In LR biofilms resistant cells formed more distinct clusters, compared to LR biofilms that had not been exposed to ampicillin. This may be the result of the antibiotic affecting susceptible cells locally, as well as resistant cells that happened to be scattered and isolated, leaving behind a stronger cluster of resistant cells.

Finally, fully susceptible biofilms, exposed to ampicillin showed a lower porosity compared to unexposed susceptible biofilms. Mixed exposed and unexposed biofilms showed higher porosity than single-strain biofilms. If low porosity is indeed an indication of a stressed biofilm [Picioreanu et al., 1999], this could explain the observed change in these experiments. Other properties of the biofilm could be investigated using image analysis, that could provide clues for further research in how bacterial interactions may affect the biofilm structure and the other way

round. For instance, the direction of biofilm growth and heterogeneity can be used as evidence for competition or cooperation within the biofilms; maybe only one of the populations grows in a certain direction and not the others and this may be a response to certain environmental conditions.

To the best of my knowledge this is the first time that the structure of colony biofilms has been recorded in full depth. When colony biofilms are investigated, the top layers only can normally be recorded with CLSM due to technical difficulties related to light penetration. Drying the biofilm allowed us to overcome the difficulty and the addition of OCT provided further information about growth in colony biofilms.

## 6.3 The effect of metabolic rates on antibiotic tolerance

In Chapter 5 the physiological state of the cells was looked at. I hypothesised that as biofilms age they become more tolerant to antibiotics and this corresponds to lower metabolic rates. Additionally, if low metabolic rates is a mechanism of tolerance, then cells originating from biofilms pre-exposed to antibiotics would show higher tolerance levels. ATP appears to reduce when dormancy mechanisms are triggered and has been suggested as the phenotypic switch that induces dormancy [Lewis, 2010*b*]. I found that both mean ATP production and antibiotic tolerance to β-lactam ampicillin and fluoroquinolone ofloxacin fluctuated in biofilms younger than 24 h old. Subsequently, mean ATP production reduced and stabilised over time and antibiotic tolerance increased and stabilised in a corresponding pattern. Furthermore, biofilm cells that survived antibiotic treatment were indeed more tolerant to the new antibiotic challenge, consistent with the initial hypothesis. However, ATP production in pre-exposed biofilm cells was in fact higher than in non-exposed ones.

Tolerance levels seen in these experiments were significantly higher than what was found in the literature (less than 10% tolerance), i.e. more than 50% and often 100% of cells tolerated both antibiotics, reflecting the differences to the methodology used. In order to retain the metabolic state of the cells as similar as possible to what it was before biofilm disruption, the methodology chosen deviated from the work of others who used ideal growth conditions to measure tolerance at. The antibiotic challenge was kept relatively short (2 h) and during this time the cells were incubated at room temperature in no nutrient containing media. In this way the metabolic state of the cells was kept unchanged throughout the experiments as indicated but colony counting in controls were no antibiotic was present. In most cases, the number of cells recovered was the same as at the point of disruption. When ideal growth conditions were used for incubation during the antibiotic challenge, the results obtained were more in line with results typically reported elsewhere, but the controls showed that the cells exhibited growth under these conditions. Therefore, the extent of tolerance in biofilm-released cells related to slow metabolic rates cannot be captured when cells are prompted to grow. The cells surviving in this way, which is a proportion of less than 1%, may be cells in deep dormancy or there may be a different, yet unknown, mechanism involved.

The mean ATP production per cell in young biofilm oscillated until it stabilised after 24 h. This is an indication of a differing gene regulation pattern in immature biofilms. Mempin et al. [2013] found that extracellular ATP is produced in planktonic bacterial cultures and that this ATP was higher in the exponential phase and minimised at the stationary phase. The ATP production followed a curve pattern with a peak at the transition from the exponential to the stationary phase. These researchers found that the ATP depletion in stationary cells was related to the ATP consumption by the cells which enhanced survival. If this is the case, we can speculate that in young biofilms, as the population grows, more ATP is produced until there is enough to sustain the population and as the cell abundance stabilises the production drops. Since ATP production did not affect tolerance negatively, this suggests that metabolism, as measured by mean ATP production per cell, may not be the only mechanism involved in tolerance. Of

course not all cells metabolise at the same rate in a biofilm, but given the extremely high levels of tolerance seen in these experiments, one could assume that a generalisation regarding metabolic rates for the biggest part of the population may not be unjustified. This does not rule out the possibility that a very small percentage of the population is indeed in a state of dormancy, unaffected by all the changes discussed.

In conclusion, it is critical that a methodology to measure growth and metabolic rates in situ, in biofilms is developed in order to get a better understanding of how metabolism may be implicated in antibiotic resistance. Additionally, it would be interesting to compare persister levels in cooperative populations against non-cooperative ones in order to confirm whether the suggestion that persistence may be an insurance against the elimination of self-destructive cooperators in a population Ackermann et al. [2008]

## 6.4 Conclusion

Intracellular β-lactamases can be exploited as public goods, since they detoxify their immediate environment. Indeed, cheating was highly enabled in the experimental system used here. However, given that the biofilms tested were not homogeneous, i.e the resistant and susceptible populations were not perfectly mixed, and the production of the public good was mostly intracellular, the mechanism of sociality needs to be investigated further. It has been reported elsewhere that nutrient availability promotes the seclusion of cooperators within their own group but under nutrient-stress conditions cooperators are outcompeted [Mitri et al., 2011]. Lineage segregation is also believed to promote cooperation as well-mixed populations allow for better exploitation of public goods [Nadell et al., 2010], which may be related to the resistant cell clustering we observed in antibiotic-stressed biofilms when the resistant phenotype was rare.

Another line for investigations could be directed towards the changes in the mechanical properties of the biofilms as a response to mixed populations and to antibiotic stress. Porosity was a parameter evidently affected in my experiments. Stress appeared to decrease porosity when mixed population biofilms appeared to form more porous biofilms irrespective of antibiotic stress conditions. These results indicate that the use of space in biofilms is affected both by social interactions and environmental conditions.

Finally, the study of metabolism in biofilms would benefit greatly from the development of a methodology that could accurately measure metabolic rates in situ. However, what could constitute a reliable indicator of growth in relation to metabolic rates would also benefit from further work. My results indicate that cells in mature biofilms are slow metabolisers and highly tolerant to antibiotics, however, it is not clear whether this correlation is true in younger biofilms where high metabolic rates correlated with high tolerance as well. Although, this discrepancy may just be due to the use of ATP as an indicator of growth.

The problem of increased antibiotic resistance is an evolutionary problem and it should approached as such. However, caution is required when changing the bacterial population balance in natural systems. For instance, Zipperer et al. [2016] recently discovered a novel antibiotic-producing bacterial strain which is a natural inhabitant of the human nose that, when present, prevented *Staphylococci* infections. This is a good example of bacterial warfare indicating that bacterial sociality can have a positive, as well as negative effect on human health. Interventions cannot be a blunt instrument but, if used wisely, this can be a promising area for the development of new therapeutic strategies.

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*Bibliography style used : "agsm" of the Harvard family*

# Appendix A

# PCR primers and cycle conditions

Table 1: All primers used for cloning. Homology in primer sequences are shown in red.

| Purpose | Primers | Sequence | Function | Product size | Template |
|---|---|---|---|---|---|
| Cloning components | CATSacIF1 (1226) | 5′ – AAAAGAGCTCGGCGTAGCACCAGGCGTT | CAT gene (sticky ends) – forward primer | 1026bp | pTOPOdif |
| | CATSacIR1(2251) | 5′ – AAAAGAGCTCGGCCGCCAGTGTGATGGA | CAT gene (Sticky ends) – reverse primer | | |
| | CTXSacIF1(69329) | 5′- AAAAGAGCTCACGGTCTGCGTTGTCGGG | CTX-M gene (sticky ends) – forward primer | 1463bp | pCT |
| | CTXSacIR1(71242) | 5′ – AAAAGAGCTCCTGCAAACGGTGCTGCGG | CTX-M gene (Sticky ends) – reverse primer | | |
| | CyanSacF4(2177) | 5′- AAAAGAGCTCTTCTGCGCTCGGCCCTTC | Cyan – amp out Forward primer | 2930bp | pAmCyan/ pE2-Crimson |
| | CyanSacR4(1781) | 5′ – AAAAGAGCTCTGCCCGGCGTCAATACGG | Cyan – amp out Reverse primer | | |
| PCR Confirmation of white colonies | Cyanlac F1 (72) | 5′ – CCCGACTGGAAAGCGGGCAG | Fluorescence gene forward primer | 1073bp | pAmCyan/ pE2-Crimson |
| | Cyanlac R1 (1144) | 5′ – ACCGTCTCCGGGAGCTGCAT | Fluorescence gene reverse primer | | |

Table 2: PCR conditions for CTXM +SacI sites and Cyan (amp out)+SacI sites/Crimson(amp out)+SacI sites withQ5 High Fidelity polymerase and CTXMSacI F1/R2 and CyanSacI F4/R4 primers respectively

| Step | Temperature | Time |
|---|---|---|
| Initial denaturation | 98 | 1min |
| Denaturation | 98 | 10sec |
| Annealing | 70 | 30sec |
| Extension | 72 | 40 sec |
| Repeat steps 2-4 for 34 times | | |
| Final extension | 72 | 2min |
| Incubation | 10 | Forever |

Table 3: PCR conditions for CAT+SacI sites with Q5 High Fidelity Polymerase and CATSacI F1/R1 primers

| Step | Temperature | Time |
|---|---|---|
| Initial denaturation | 98 | 1min |
| Denaturation | 98 | 10sec |
| Annealing | 71 | 30sec |
| Extension | 72 | 40 sec |
| Repeat steps 2-4 for 34 times | | |
| Final extension | 72 | 2min |
| Incubation | 10 | Forever |

Table 4: Conditions for colony PCR to confirm Cyan+CTXM DH10β transformants with One Taq Hot Start Polymerase and CTXMSacI F1/R1 primers

| Step | Temperature | Time |
|---|---|---|
| Initial denaturation | 94 | 5min |
| Denaturation | 94 | 30sec |
| Annealing | 70 | 30sec |
| Extension | 72 | 1.30 min |
| Repeat steps 2-4 for 34 times | | |
| Final extension | 72 | 5 min |
| Incubation | 10 | Forever |

Table 5:Colony PCR for confirmation of Cyan+Cat DH10β transfromants with One Taq Hot Start Polymerase and CATSacI F1/R1 primers

| Step | Temperature | Time |
|---|---|---|
| Initial denaturation | 94 | 5min |
| Denaturation | 94 | 30sec |
| Annealing | 71 | 30sec |
| Extension | 72 | 1 min |
| Repeat steps 2-4 for 34 times | | |
| Final extension | 72 | 5 min |
| Incubation | 10 | Forever |

Table 6: Colony PCR for confirmation of Cyan+CAT DH10β white colonies from plasmid stability assay with OneTaq Hot Start Polymerase and CyanLac F1/R1 primers

| Step | Temperature | Time |
|---|---|---|
| Initial denaturation | 94 | 5min |
| Denaturation | 94 | 30sec |
| Annealing | 60 | 30sec |
| Extension | 68 | 1.10 min |
| Repeat steps 2-4 for 34 times | | |
| Final extension | 68 | 5 min |
| Incubation | 10 | Forever |

Table 7: Typical PCR reaction for a final volume of 25µl per reaction (double quantities were used for a final reaction volume of 50µl)

| Components | Quantities (µl) | |
|---|---|---|
| DNA | 1.0 | 1.0 |
| dNTPs [10mM] | 0.5 | 0.5 |
| Forward primer [10µM) | 1.25 | 1.25 |
| Reverse primer [10µM) | 1.25 | 1.25 |
| DNA Polymerase (Q5) | 0.25 | 0.25 |
| Polymerase buffer | 5.0 | 5.0 |
| Polymerase enhancer | - | 5.0 |
| Water | 15.75 | 10.75 |

Table 8: Typical colony PCR reaction with OneTaq polymerase for a final reaction volume of 50µl

| Components | Quantities (µl) |
|---|---|
| DNA | 0.0 |
| dNTPs [10mM] | 1.0 |
| Forward primer [10µM) | 1.0 |
| Reverse primer [10µM) | 1.0 |
| DNA Polymerase (OneTaq) | 0.25 |
| Polymerase buffer | 10.0 |
| Polymerase enhancer | 5.0 |
| Water | 31.75 |

Table 9:Primers used for the sequencing of the constructed plasmids

| Primer | Sequence | Plasmid |
|---|---|---|
| pCrimCTXM-3819R | GAT ACG GGA GGG CTT ACC A | CrimCTXM & CyanCTXM |
| pCrimCTXM-1319F | GGC CTC GTG ATA CGC CTA TT | CrimCTXM & CyanCTXM |
| pCrimAT-2870R | CCG GCT CCA GAT TTA TCA GCA | CrimCAT |
| pCrimCAT-1376F | ACG TCA GGT GGC ACT TTT CG | CrimCAT |
| pCyanCAT-2833R | GAT ACG GGA GGG CTT ACC A | CyanCAT |
| pCyanCAT-1333F | GGC CTC GTG ATA CGC CTA TT | CyanCAT |

# Appendix B

# Crystal violet assays

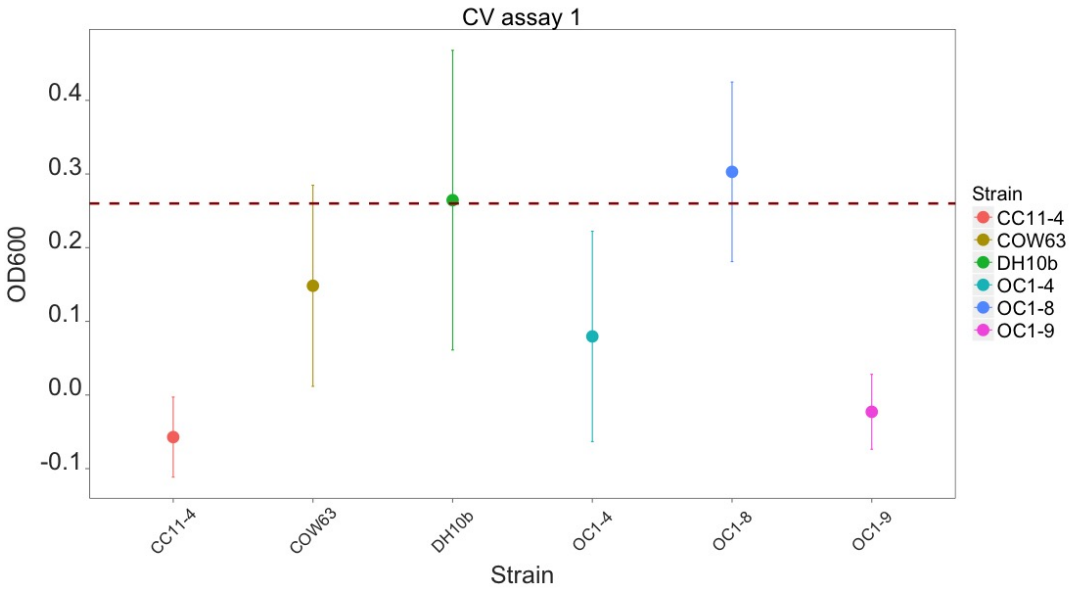


**Figure B.1:** CV assay 1

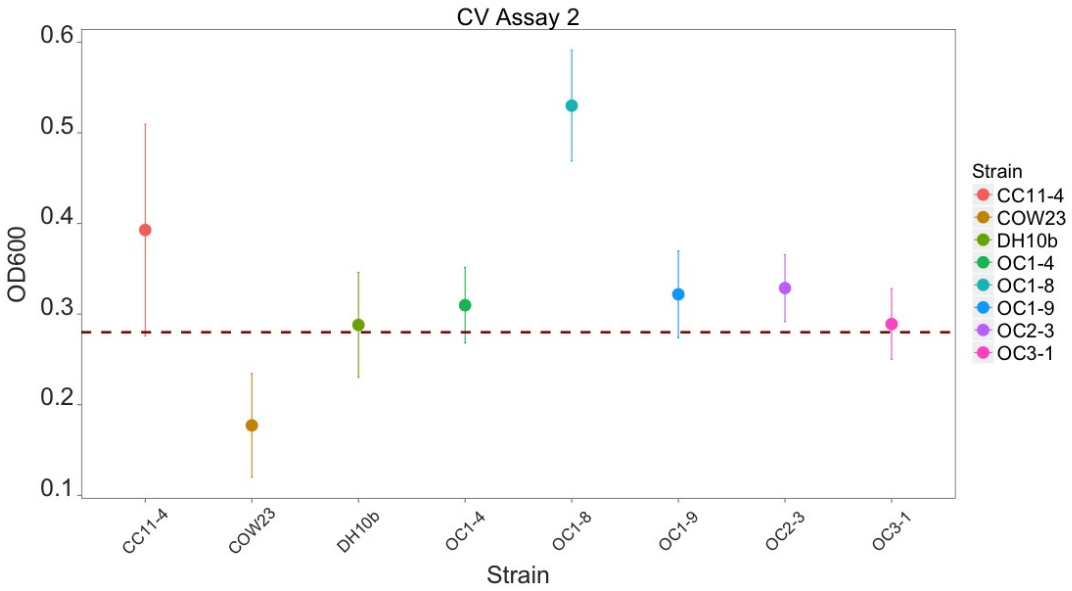


**Figure B.2:** CV assay 2

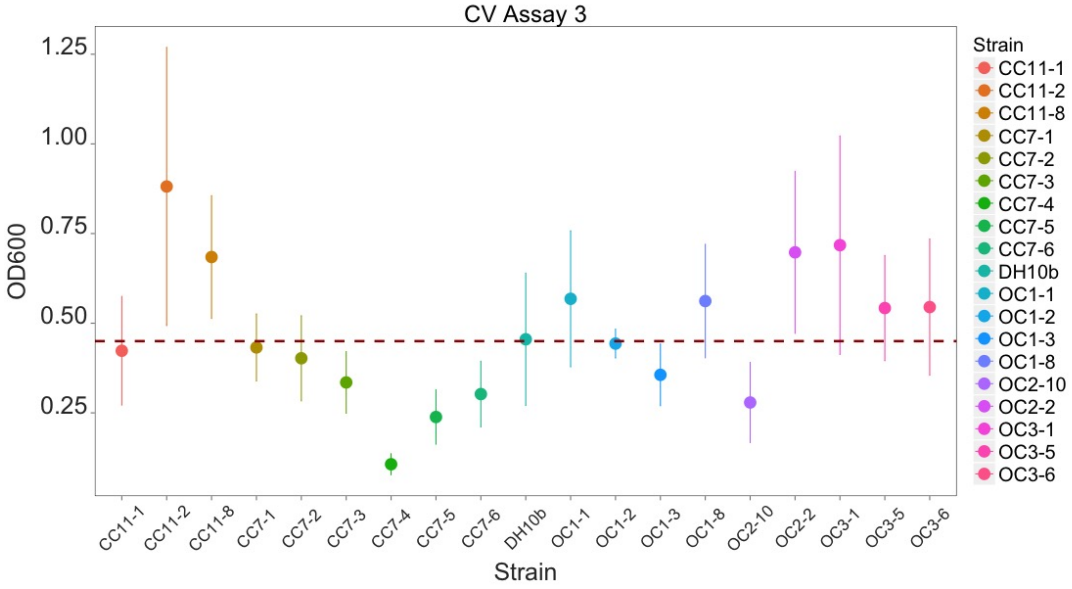


**Figure B.3:** CV assay 3

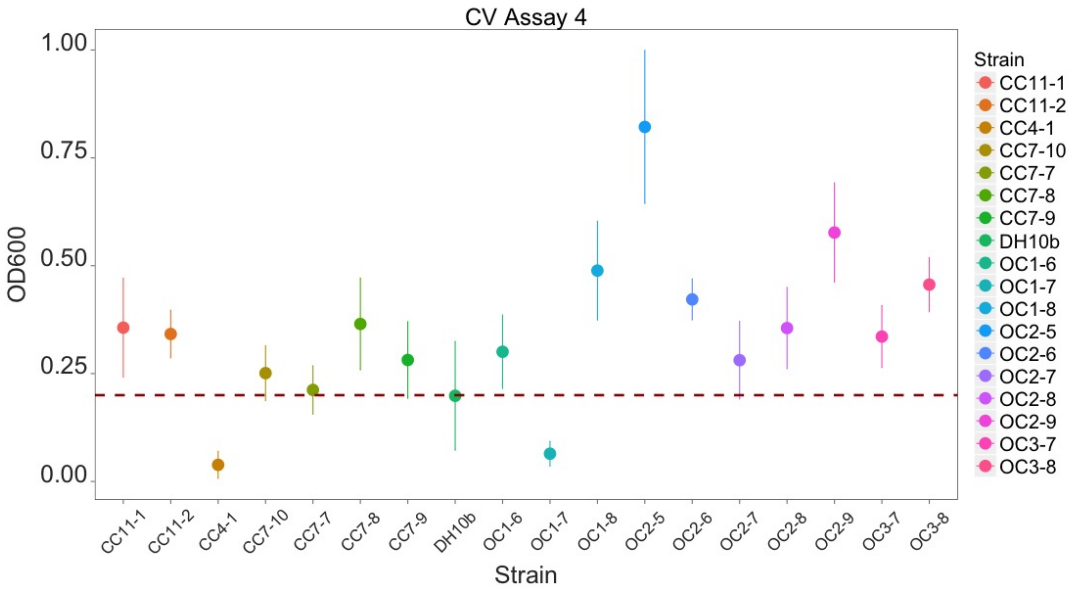


**Figure B.4:** CV assay 4

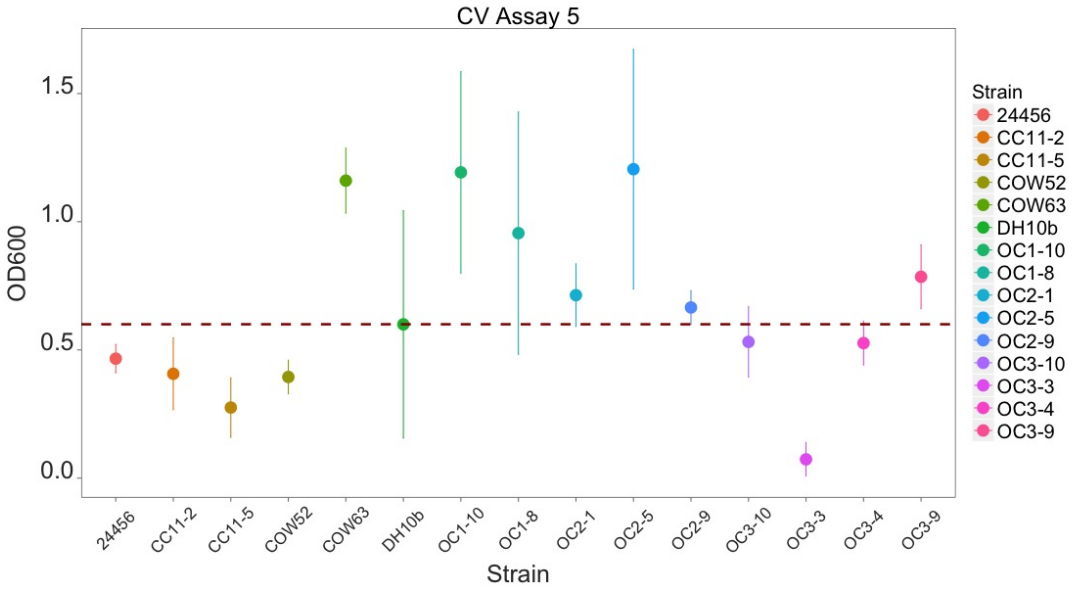


**Figure B.5:** CV assay 5

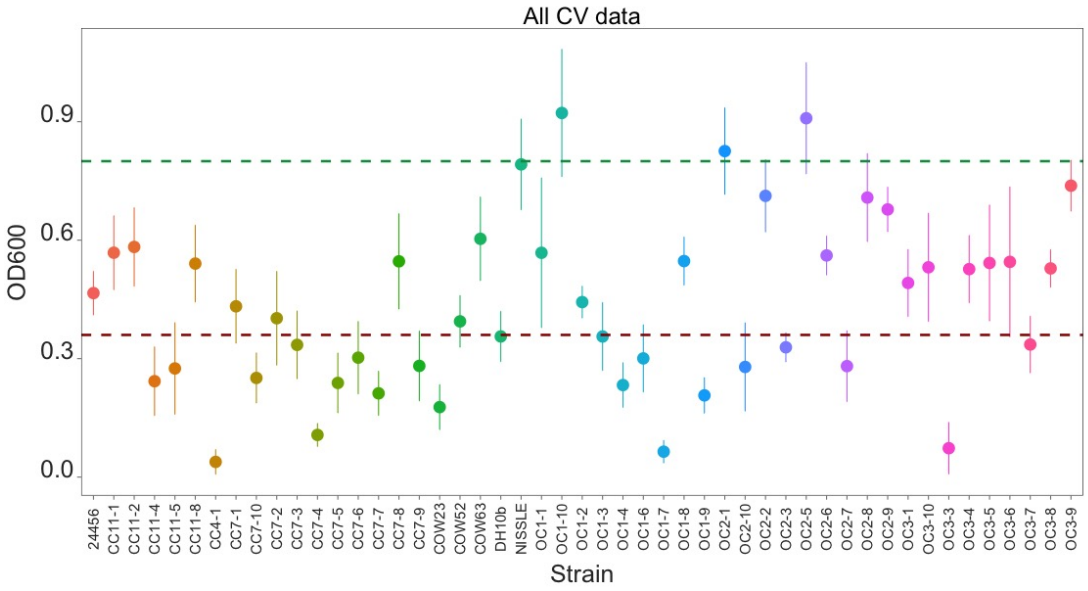


**Figure B.6:** All assays

# Appendix C

# Lectin Screening

| | **FITC conjugated Lectins** | | |
|---|---|---|---|
| 1 | **AAA**_Anguilla anguilla - FITC | F-4901 | Sanbio |
| 2 | **ABA**_Agaricus bisporus - FITC | F-5001 | Sanbio |
| 3 | **ACA**_Amaranthus caudatus FITC | F-8201 | Sanbio |
| 4 | **AIA**_Artocarpus integrifolia - FITC | F-6301 | Sanbio |
| 5 | **AMA_**Arum maculatum FITC | F-8012 | Sanbio |
| 6 | **ASA**_Allium sativum - FITC | F-8007 | Sanbio |
| 7 | **BDA**_Bryonia dioica - FITC | F-8002 | Sanbio |
| 8 | **BPA**_Bauhinia purpurea - FITC | F-2501 | Sanbio |
| 9 | **CA_**Colchicum autumnale - FITC | F-8004 | Sanbio |
| 10 | **CAA**_Caragana aborescens - FITC | F-6701 | Sanbio |
| 11 | **Calsepa**_Calystega sepiem - FITC | F-8011 | Sanbio |
| 12 | **ConA**_Canavalia ensiformis - FITC | F-1104 | Sanbio |
| 13 | **CPA**_Cicer arietinum - FITC | F-6601 | Sanbio |
| 14 | **CSA**_Cytisus scoparius - FITC | F-3201 | Sanbio |
| 15 | **DBA**_Dolichos biflorus - FITC | L-9533 | Sigma |
| 16 | **DGL**_Dioclea grandiflora - FITC | F-9002 | Sanbio |
| 17 | **DSA**_Datura stramonium - FITC | F-5701 | Sanbio |
| 18 | **ECA**_Erythrina cristagalli - FITC | F-5901 | Sanbio |
| 19 | **EEA**_Euonymus europaeus - FITC | F-4201 | Sanbio |
| 20 | **GHA**_Glechoma lederacea - FITC | F-9003 | Sanbio |
| 21 | **GNA**_Galanthus nivalis - FITC | F-7401 | Sanbio |
| 22 | **GS-I**_Griffonia simplicifolia - FITC | L-9381 | Sigma |
| 23 | **HAA**_Helix aspersa - FITC | F-3801 | Sanbio |
| 24 | **HHA**_Hippeastrum hybrid (Amaryllis) - FITC | F-8008 | Sanbio |
| 25 | **HMA**_Homarus americanus - FITC | F-6201 | Sanbio |
| 26 | **HPA**_Helix pomatia - FITC | L-1034 | Sigma |
| 27 | **IRA**_Iris hybrid - FITC | F-8010 | Sanbio |
| 28 | **LAA_**Laburnum alpinum - FITC | F-5301 | Sanbio |
| 29 | **LAL**_Laburnum anagyroides - FITC | F-8101 | Sanbio |
| 30 | **LBA**_Phaseolus lunatus - FITC | F-1701 | Sanbio |
| 31 | **LcH**_Lens culinaris - FITC | F-1401 | Sanbio |
| 32 | **LEA**_Lycopersicon esculentum - FITC | L-0401 | Sigma |
| 33 | **LFA_**Limax flavus - FITC | F-5101 | Sanbio |
| 34 | **Lotus**_Tetragonolobus purpurea - FITC | L-5644 | Sigma |
| 35 | **LPA**_Limulus polyphemus - FITC | F-1501 | Sanbio |
| 36 | **MAA**_Maackia amurensis - FITC | F-7801 | Sanbio |
| 37 | **MNA-G_**Morniga G - FITC | F-9005 | Sanbio |
| 38 | **MOA_**Marasmium oreades - FITC | F-9001 | Sanbio |
| 39 | **MPA**_Maclura pomifera - FITC | F-3901 | Sanbio |
| 40 | **NPA**_Narcissus pseudonarcissus - FITC | F-8006 | Sanbio |
| 41 | **PHA-E**_Phaseolus vulgaris - FITC | F-1802 | Sanbio |
| 42 | **PHA-L**_Phaseolus vulgaris - FITC | F-1801 | Sanbio |
| 43 | **PMA_**Polygonatum multiflorum FITC | F-8009 | Sanbio |
| 44 | **PNA_**Arachis hypogaea FITC | F-2301 | Sanbio |
| 45 | **PSA_**Pisum sativum - FITC | L-0770 | Sigma |
| 46 | **PSL**_Polyporus squamosus - FITC | F-9006 | Sanbio |
| 47 | **PTA_**Psophocarpus tetragonolobus - FITC | L-3264 | Sigma |
| 48 | **PWA**_Phytolacca americana - FITC | F-1901 | Sanbio |
| 49 | **RPA**_Robinia pseudoaccacia - FITC | F-4101 | Sanbio |
| 50 | **SBA_**Glycine max - FITC | F-1301 | Sanbio |
| 51 | **SJA**_Sophora japonica - FITC | F-2901 | Sanbio |
| 52 | **SNA**_Sambucus nigra - FITC | F-6802 | Sanbio |

| | | | |
|---|---|---|---|
| 53 | **STA_** Solanum tub - FITC | F-4701 | Sanbio |
| 54 | **TKA**_Trichosanthes kirilowii - FITC | F-5601 | Sanbio |
| 55 | **TL**_Tulipa sp. - FITC | F-8001 | Sanbio |
| 56 | **UDA**_Urtica dioica - FITC | F-8005 | Sanbio |
| 57 | **UEA-I**_Ulex europaeus - FITC | L-9006 | Sigma |
| 58 | **VFA_**Vicia faba - FITC | F-4801 | Sanbio |
| 59 | **VGA_**Vicia graminea FITC | F-4400 | Sanbio |
| 60 | **VRA**_Vigna radiata - FITC | F-6501 | Sanbio |
| 61 | **VVA_**Vicia villosa - FITC | F-4601 | Sanbio |
| 62 | **WFA**_Wisteria floribunda - FITC | F-3101 | Sanbio |
| 63 | **WGA_**Triticum vulgaris - FITC | L-4895 | Sigma |
| | **Alexa488 labeled lectins** | | |
| 1 | **AAL**_Aleuria aurantia lectin | | Vector |
| 2 | **CCA**_Cancer antennarius crude | L-7200 | Sanbio |
| 3 | **Co**_Codium fragile | L-2638 | Sigma |
| 4 | **IAA**_Iberis amara | L-3300 | Sanbio |
| 5 | **MIA**_Mangifera indica crude | L-6000 | Sanbio |
| 6 | **PAA_**Perseau americana crude | L-6100 | Sanbio |
| 7 | **PA-I**_Pseudomonas aeruginosa | L-9895 | Sigma |
| 8 | **PPA**_Ptilota plumosa | L-9260 | Sigma |
| 9 | **RTA_**Trifolia repens crude | L-5800 | Sanbio |