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Selection for Antibiotic Resistance Below Minimal inhibitory concentration in Biofilm

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Abstract

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Antibiotics are today one of the most important cornerstones in modern healthcare when it comes to treating bacterial infections. It is an asset human kind have been leaning on for the last century, but excessive and widespread misuse of antibiotics have left deep scars in the form of multi resistant pathogenic strains of bacteria that we soon will not be able to treat. A lot of research have been invested in understanding the mechanisms and spread of resistance within bacteria living in planktonic form, overlooking the fact that there are more lifestyles that causes problems. In this study, focus has been put on antibiotic resistance within bacteria living as biofilms, a lifestyle that causes problems in chronic infections and prosthetics/medical implants.

By constructing resistant mutants derived from a biofilm forming strain of Escherichia coli, the minimal selection concentration has been investigated in both planktonic and biofilm assays for Streptomycin and Ciprofloxacin. By comparing the results, it is possible to evaluate if and how the antibiotic resistance properties differ between the two lifestyles. Focus has been put on concentrations of antibiotics below the minimal inhibitory concentration with the objective to see how selection of antibiotic resistant mutants take place with the susceptible strain still growing, although with reduced growth rate.

The hope is that the results gained in this study will provide a foundation for future research regarding antibiotic resistance in biofilms, and be part of the solution to the excessive resistance problem before it is too late.

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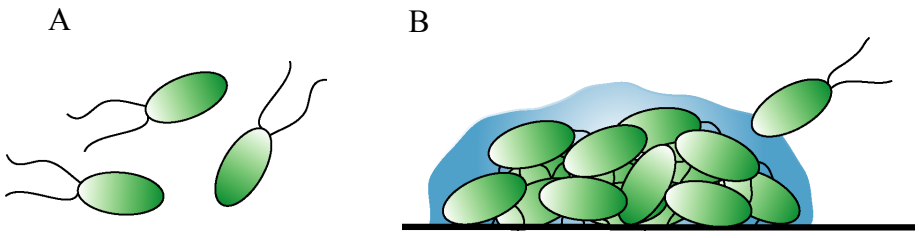
Sammanfattning

Världen som vi känner den idag står inför ett växande hot. Ett hot som på ett väldigt drastiskt och skoningslöst sätt kommer slå vår syn på hantering av bakteriella infektioner i spillror. Vi kommer behöva tänka om, anpassa oss och risken finns att många också kommer behöva sätta livet till. Antibiotika är idag en av de viktigaste hörnstenarna i den moderna sjukvården för behandling av infektioner orsakade av bakterier. Dock har, under de senaste årtiondena, den stabila grunden börjat svikta, med ökande vårdproblem relaterade till utvecklingen och spridningen av antibiotikaresistenta bakterier.

Antibiotikaresistens innebär att bakterier utvecklar en motståndskraft mot olika typer av antibiotika, vilket är ett fenomen som sker slumpmässigt under celldelningsprocessen. Dessa förändringar i arvsmassan (DNA) hos bakterier gör att de klarar högre koncentrationer av antibiotika, de blir antibiotikaresistenta. Resistens uppkommer inte bara vid antibiotikabehandling, det utvecklas även i naturen som en del av bakteriernas försvar mot andra fientliga antibiotika-producerande stammar.

Dock har år av överanvändning och missbruk av antibiotika inom sjukvård och jordbruk lett till att utvecklingen av resistens har ökat lavinartat, och det har nu gått så långt att sjukdomsframkallande bakterier är resistenta mot så många olika varianter av antibiotika att vi inte längre kan bota infektionerna som de orsakar. För att bättre kunna tackla det problem som vi skapat för oss själva så behöver vi öka kunskapen om de mekanismer som ligger bakom resistensen, samt hur den sprids inom och mellan olika populationer av bakterier.

Den största delen av den forskning som bedrivs kring antibiotikaresistens görs på bakterier som lever i planktonisk form. Det innebär att de i en lösning lever som enskilda individer och fritt rör sig runt. I detta projekt har vi studerat en annan bakteriell livsstil, biofilm. Bakterier som lever i biofilmer samlar ihop sig i täta grupper, ofta förankrade vid en yta, och bäddar tillsammans in sig i en matris av extracellulära substanser som de tillsammans bygger upp (figur 1).



Figur 1. Två bakteriella livsstilar: (A) planktonisk form där bakterierna lever som enskilda individer samt (B) biofilmer där bakterierna samlar ihop sig i täta grupper inbäddade in en matris.

Matrisen består till största delen av polysackarider, proteiner, lipider och extracellulärt DNA, och ger de inneslutna bakterierna ett skydd mot externa hot. Bland dessa externa hot har vi dessvärre den antibiotika som vi behandlar infektioner

med, som får det svårt att ta sig igenom matrisen vilket leder till att antibiotikan inte kommer åt bakterierna innanför. Därmed blir det ännu svårare att behandla infektioner som orsakas av bakterier som lever i biofilmer, de bygger upp en gemensam sköld mot omvärlden.

Det finns två begrepp som är viktigt när man håller på med antibiotika. Det första är "Minimal Inhibitory Concentration", förkortat MIC. MIC är den lägsta koncentrationen av en viss typ av antibiotika som inhiberar synlig tillväxt av en specifik bakterie. Det andra begreppet är "Minimal Selection Concentration", MSC. För att förstå vad MSC betyder så behöver även begreppet fitnesskostnad redas ut. När en bakterie blir resistent mot antibiotika innebär det att en förändring (mutation) sker i DNAt hos bakterien. Förändringen kommer med en kostnad (fitness) som försämrar tillväxten av den resistenta bakterien, att ha mutationen kostar ett visst pris som bakterien betalar i form av ökad energiåtgång. MSC är den koncentration av antibiotika där fitnesskostanden som antibiotikaresistensen leder till blir värt sitt pris, den koncentration där mutationen som leder till antibiotikaresistens blir gynnsam att ha. För koncentrationer av antibiotika lägre än MSC är mutationen energimässigt för dyr, och bakteriepopulationen som saknar den resistensgivande mutationen (vildtyps bakterien) växer snabbare och utkonkurrerar den muterade bakterierna. Ju högre antibiotika koncentrationen ökar över MSC ju mer fördelaktig blir mutationen, tills en koncentration nås då vildtypen helt slås ut, vilket motsvarar MIC för den känsliga stammen. Det behöver alltså finnas antibiotika närvarande för att antibiotikaresistens ska selekteras, vilket människan har möjliggjort genom överanvändning och spridning både vitt och brett. När man tidigare studerat resistens så har man utgått från att selektionen av resistenta mutanter endast sker när bakterierna befinner sig i koncentrationer av antibiotika över MIC, alltså när vildtypen är helt utslagen. Men på senare år har en ny teori vuxit fram, med bevis för att selektionen även sker under MIC, när vildtypen fortfarande växer, om än långsammare. Detta innebär att de miljöer där resistens utvecklas är betydligt fler än vad vi tidigare trott! Lägre koncentrationer av antibiotika kan finnas på många olika platser, så som reningsverk, jordbruk och även fritt i naturen. Möjligheten för resistens att spridas är alltså ännu större, och det är nu av ännu större vikt att antibiotikaresistens tas på allvar.

Det är i mitten av allt detta som följande projekt har passats in. En biofilmsbildande bakteri, *Escherishia coli,* har exponerats för olika antibiotika koncentrationer under MIC av ett par olika antibiotika, och utifrån den genererade data har MSC analyserats. Vi har observerat att antibiotikaresistenta bakterier även i biofilm klarar sig bättre än bakterier känsliga för antibiotika vid låga koncentrationer mycket lägre än de som avdödar de känsliga bakterierna. Förhoppningen är att de resultat som har tagits fram ska kunna bidra till att bana väg för större förståelse kring biofilm och antibiotikaresistens, samt öppna samhällets ögon för den stundande katastrof som annars kommer träffa oss. För faktum är att antibiotikaresistensen är ett problem som världen inte längre kan blunda för.

Contents

Abbreviations and acronyms

BHI	Brain Heart Infusion
CBD	Calgary Biofilm Device
CF	Cystic fibrosis
eDNA	Extracellular DNA
EPS	Extracellular Polymeric Substance
HGT	Horizontal Gene Transfer
LA	Luria–Bertani Agar
LB	Lysogeny Broth
MIC	Minimal Inhibitory Concentration
MSC	Minimal Selection Concentration
PCR	Polymerase chain reaction
S-value	Selection coefficient
Sub-MIC	Concentrations of antibiotics below the minimal inhibitory concentration
UTR	Urinary tract infection

1. Introduction

1.1 Antibiotic resistance

Our world is standing in front of a growing threat that dramatically will change the way we address and manage bacterial infections. Today, antibiotics are one of the most important cornerstones in modern healthcare for treatment of infectious diseases caused by bacteria and to enable surgical and cancer treatments. However, during the last decades, serious failures in the use of these drugs have been experienced due to the rapid development and spread of antibiotic resistance. Although resistance against antibiotics is a phenomenon that occurs naturally in bacteria for metabolic functions and/or as protection against competing bacteria (Gullberg *et al.* 2011), years of widespread and excessive use of antibiotics worldwide (Andersson & Hughes. 2014), including misuse in human/animal medicine and agriculture (Gullberg *et al*. 2011), have accelerated the development and spread of antibiotic resistance to pathogenic bacteria.

Resistance mechanisms in bacteria can be gained in two ways, by mutations or horizontal gene transfer. Mutations are changes that occurs randomly in the DNA, and sometimes these changes can lead to a higher tolerance against certain antibiotics. When exposed to said antibiotic, the cells carrying the mutation will survive and spread their resistance genes to their daughter cells.

Horizontal gene transfer occur when bacteria gain mobile genetic elements that originates from another organism and insert into their own DNA. These genetic elements are transferred in one of three different ways; conjugation, transduction or transformation (Gyles and Boerlin. 2014). Conjugation requires contact between the cells, and the genetic material is transported as plasmids between donor and recipient bacteria through a pilus (Ochman *et al.* 2000). Transduction is the process were DNA transfer into bacteria are mediated by bacterial viruses called bacteriophages. After infecting a bacterial cell, the phage might incorporate some of the bacterial DNA and transfer that to new bacteria via the next round of infection (Brussow *et al*. 2004 and Ochman *et al.* 2000). The third mechanism is transformation, were the bacteria is in a state of natural competency, a physiological state that enables the bacteria to take up DNA from its environment (Kruger and Stingl. 2011).

1.2 Minimal Inhibitory Concentration/Minimal Selection concentration

An important concept related to antibiotic resistance is the minimal inhibitory concentration (MIC), defined as the lowest concentration of a drug that inhibits visible growth of the target bacteria under specified *in vitro* conditions. In a clinical setting when treating an infection, the concept is to maintain dosing concentration

above the MIC in the affected body compartments for sufficient time to eliminate the infection. Minimal selective concentration (MSC) is also a relevant concept, defined as the lowest concentration of an antibiotic that results in the selection of resistant mutants in a population of an isogenic susceptible strain (Andersson & Hughes. 2014)

With the increasing problem of antibiotic resistance, it is clear that more focus is needed to understanding the selection of, and the mechanisms underlying, the development of resistance in bacterial strains. The leading hypothesis regarding resistance is the *mutant selective window* hypothesis, which assumes that selection of resistant mutants only occurs at antibiotic concentrations between the MIC of the susceptible wild-type and the MIC of the resistant population, as illustrated by the red window in figure 2 (Andersson & Hughes. 2014). However, a growing amount of evidence indicates that antibiotic concentrations below the MIC, so called sub inhibitory MIC (sub-MIC), enrich for resistant mutants in a population of resistant and susceptible bacteria. Sub-MIC conditions still allows the susceptible cells to grow, but with a decreased growth rate compared to the resistant strain. Thus, the resistant sub-population will expand and out-compete the susceptible strains (Wistrand-Yuen. 2018, Andersson & Hughes. 2014).

To grasp the concept of resistance, it is important to understand that a mutation that result in resistance comes with some kind of price for the bacteria, it takes energy to maintain the mutation. This price is called fitness cost, and is an estimation of how much the fitness, usually measured as reduced growth rate of the bacteria, is affected by the mutation. Chromosomal resistance mutations, in most cases, have a negative impact on the bacteria, and the effects of these genetic changes are often associated with reduction in the bacterial growth rate. Due to the fitness cost that accompanies these chromosomal resistance mutations, resistant bacteria are outcompeted by susceptible high-fitness wildtype strains in the absence of antibiotics (Knopp & Andersson. 2018). This is illustrated in the green window in figure 2. When the concentration of antibiotics increases, the antibiotic resistant mutation is a necessity for the bacteria to survive. The MSC is the lowest concentration of antibiotics were the fitness cost of the resistance mutation is balanced by the antibiotic-conferred selection for the resistant mutant.

At antibiotics concentrations below MIC (the orange window in figure 2), the antibiotics will not wipe out the susceptible strain completely, but it will disturb the competition dynamics in populations between resistant and susceptible bacteria by reducing the growth of the susceptible strain in relation to the resistant strain. According to this, the MSC becomes a function of the fitness cost, with low-cost resistance mutations selected under sub-MIC conditions (Wistrand-Yuen. 2018, Andersson & Hughes. 2019).

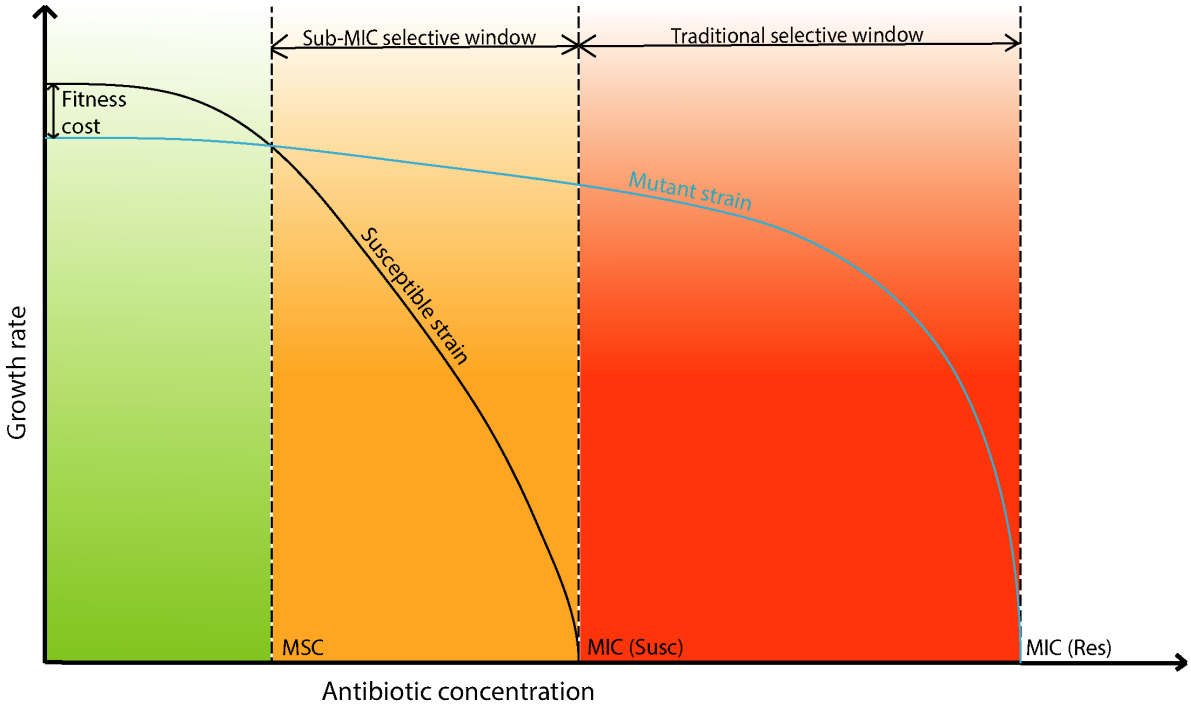


Figure 2. Illustration of the growth rates of a susceptible strain and a mutant strain as a function of antibiotic concentration. In the green window, the antibiotic selection pressure is low, leading to a faster growth for the susceptible strain compared to the resistant strain. The cost of the mutation leading to resistance is too high. In the yellow window (sub-MIC) the fitness cost of the resistance is balanced out by the antibiotic conferred selection of the resistant mutant and the growth rate of the susceptible strain is decreasing. In the red window (traditional selection window), the susceptible strain has reached its MIC_{susc} concentration and is completely wiped out. The growth rate of the mutant will decrease with increasing concentration until it reaches its MIC_{res} value. The figure is modified from Gullberg *et al* 2011.

1.3 Biofilm

The complexity of biofilms is a subject that has eluded scientists for generations. In an attempt to capture and scale the topic down to is core, O'Toole *et al* (2000) defined biofilms as communities of microorganisms that are attached to a surface. After almost 20 years, this definition still stands true, but a lot more insight has been gained since then thanks to intensive research.

Vert *et al* (2012) proposed a more comprehensive definition, defining biofilms as "aggregates of microorganisms in which cells are frequently embedded in a self-produced matrix of extracellular polymeric substance (EPS) that are adherent to each other and/or a surface". In addition to the EPS, which will be described in more detail below, another important update to the definition is that a biofilm must not necessarily be attached to a surface. Also in the absence of a substratum it is possible for mobile biofilms to form. These biofilms are termed flocs (Flemming *et al*. 2016).

Biofilms are highly diverse. They can be comprised of a single bacterial strain, but in most environments, they form communities of multiple species, attaching to a variety of biotic and abiotic surfaces (O'Toole *et al*. 2000).

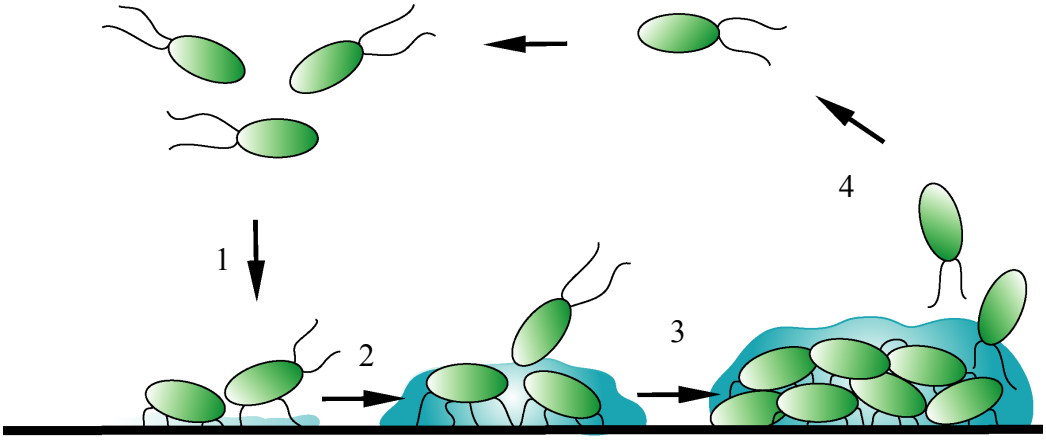


Figure 3. The general lifecycle of a biofilm contains four steps. (1) The initial attachment to a surface or to each other. (2) The formation of micro colonies. (3) Maturation of the biofilm. (4) Dispersal from the biofilm.

The way biofilms are generated differ a lot between species, but the general development cycle is the same and is considered to include four steps; (1) initial attachment to surfaces or to each other, (2) microcolony formation, (3) biofilm maturation and (4) biofilm dispersal (Tolker-Nielsen. 2015). This cycle is illustrated in figure 3. Studies indicate that the biofilm state is a stable form in the biological cycle (O'Toole *et al*. 2000), and that biofilm formation is a natural stationary phase of bacterial growth (López *et al*. 2010).

1.3.1 Biofilm initiation and formation

It is believed that biofilm formation is initiated by bacteria sensing environmental conditions that triggers them to relocate to a surface bound lifestyle (O'Toole *et al*. 2000). For bacteria to make the switch from planktonic way of living to the communal biofilm way of life, there is need for molecular changes in the bacteria (Tolker-Nielsen. 2015). These molecular mechanisms that regulate biofilm formation vary vastly between species and there may also be a great variation between strains of the same bacterium (López *et al*. 2010). Further, as mentioned before, it has been shown that initiation of biofilm formation is linked to the environment in which the bacteria resides in (O'Toole *et al*. 2000), with the same strains forming different biofilm structures depending on the surroundings (Tolker-Nielsen. 2015).

1.3.2 The biofilm matrix

Even though there are large differences some attributes are generally the same for most biofilms. Just about all biofilms are embedded in a self produced extracellular matrix which hold the cells together (López *et al*. 2010). This matrix of extracellular polymeric substances (EPS) consists mainly of water, polysaccharides, proteins, lipids

and extracellular DNA (eDNA), encasing the cells in the biofilm. Intermolecular interactions between the EPS components determine the organization of the EPS molecules in the matrix (Flemming and Wingender. 2010), mediating the formation of the biofilm architecture. The result is a spatial organization were the cells in the biofilm are clustered in microcolonies (Neu & Lawrence. 2014). Pores and channels can form in the matrix between these colonies (Karimi *et al*. 2015) facilitating the transport of liquids (Wilking *et al*. 2013). In the remaining space the ESP molecules fill the voids between the cells, defining the environment and living conditions for the microcolonies and in addition, supply mechanical stability to the biofilm (Persat *et al*. 2015). In some types of bacterial species, the matrix contains adhesive proteins. These proteins connect with each other, holding the biofilm together by interacting with proteins on neighboring cells. There are also types of cell appendages, such as pili and fimbriae, which are used to attach cells to different surfaces and to each other (López *et al*. 2010).

Overall, the nature of the matrix varies between species (López *et al*. 2010), and the formation is a dynamic process, relying on parameters such as nutrient accessibility, synthesis/secretion of extracellular material and stress (Flemming *et al*. 2016). The matrix is never considered complete, and is continuously changed and remodeled. This reshaping is driven by specific enzymes that degrade and reconfigure the matrix, which is significant for the biofilm to be able to adapt to alterations in the environment. Also, the resulting dispersal of the biofilm is important for surface relocation (Whitfield *et al*. 2015).

The matrix confers several advantages to the included bacteria, such as resource capture by passive sorption properties, which is facilitated by the sponge-like characteristic of the EPS matrix (Billings *et al*. 2015). This enables the bacterial cells to take up nutrients that are present in the water phase of the biofilm, or that is part of the substratum on which the biofilm grows (Flemming *et al*. 2016). There are many different sorbent systems with binding sites including both anionic and cationic exchangers, making it possible for many types of molecules to accumulate in the biofilm. Even suspended solid particles can be trapped and integrated into the matrix (Flemming *et al*. 2016), both organic and inorganic. This means that not only nutrients, but also unpleasant substances such as toxins, may accumulate in the biofilm (Writer *et al*. 2011). If not degraded by the cells, absorbed substances will be released to the water phase from the matrix, or be contained in the biofilm until it decays. Consequently, biofilms can act as a source of contaminants (Flemming & Levis, 2002).

1.3.3 Signaling

Communication is key when working together, which has also been proven to be true for bacteria when forming biofilms. The mechanism of cell to cell communication by chemicals is called quorum sensing, a tool controlling a large number of

developmental processes, including those related to biofilm formation (López *et al*. 2010). The molecules responsible for the signaling come from various sources, some are produced and secreted by the bacterial community, in which case the molecules are termed autoinducers. They accumulate extracellularly and the concentrations increase proportionally to the cell density. Reaching higher amounts, the autoinducers trigger signal transduction cascades, resulting in multicellular responses in the bacterial community (Camilli & Bassler. 2006). These responses do not exclusively result in biofilm formation, there are also cases were quorum-sensing negatively regulates biofilm formation, triggering transcription of genes involved in biofilm dispersal (Boles & Horswill. 2008). The types of autoinducers vary between species, but a broad distinction can be made between Gram-negative and Gram-positive species. Many Gram-negative bacteria have quorum sensing systems that responds to a class of autoinducers known as acyl homoserine lactones (AHLs), whilst Gram-positive organisms often have autoinducers in the form of peptides (López *et al*. 2010).

1.3.4 Dispersal

The last step in the biofilm-cycle is the dispersal, when the biofilm with different processes detaches from the surface. Detachment can be caused by external disturbance, internal biofilm processes, or by the release of the extracellular matrix/surface-binding proteins. Three distinguishable dispersal strategies can be specified; swarming/seeding dispersal, clumping dispersal and surface dispersal. Pursuing the first strategy, individual cells fall away from the biofilm, and is released into the surrounding. The second strategy progress as it sounds like; aggregates of cells are shed as chunks. Surface dispersal is a bit different, with the biofilm structures moving across surfaces (Hall-Stoodley *et al*. 2004).

1.3.5 Biofilms and tolerance/resistance

It is advantageous for bacteria to be in the biofilm format since it clearly increases the tolerance/resistance to exogenous substances and conditions. However, it is important to separate the benefits that will stay with the organism while detaching from the biofilm, and the benefits that only derive from the biofilm lifestyle. It is therefore of the essence to make a distinction between biofilm-associated antimicrobial tolerance and antimicrobial resistance, which can be found in free living bacteria (Ciofu *et al*. 2017). You can say that *tolerance* is specific for a biofilm, and this feature will be lost when the bacteria decompose into planktonic cells, while *resistance* is a genetic trait that will remain with the bacterial cell regardless of its lifestyle. Although both features are used to describe the increased ability of an organism to survive otherwise lethal concentrations of certain compounds, the underlying mechanisms are different (Flemming *et al*. 2016). In the subsequent part, the mechanisms generating tolerance will be discussed.

Restricted penetration through the EPS matrix is one feature of the biofilm that contributes to antimicrobial tolerance (Ciofu *et al*. 2017). For some compounds the EPS acts as a diffusion barrier preventing molecules to reach the bacteria, however this is not the case for most antibiotics. Even when substances manage to diffuse through the matrix, the EPS components may have the ability to quench the effect of the antimicrobial substances (Billings *et al*. 2015). This form of inhibition is termed diffusion-reaction inhibition (Daddi *et al*. 2012).

The slow growth rate of bacteria in biofilms is also a characteristic that makes them tolerant against certain antibiotics. Biofilms contains significant amounts of cells in the stationary phase (Amato *et al*. 2014), with low metabolic activity. This makes them non-susceptible to the group of antibiotics that targets processes that occur in growing bacteria (Ciofu *et al*. 2017). Resistance also has the ability to spread amongst cells in a biofilm. Due to multiple factors such as high cell density, gathering of mobile genetic material and increased genetic competence, biofilms are ideal environments for resistance genes to spread to individuals in a population through horizontal gene transfer (Mah *et al*. 2012). On top of that, the matrix allows an ideal environment for cell-to-cell contact, which is necessary for some types of gene transfer (Madsen *et al*. 2012).

1.3.6 Why are biofilms dangerous?

Biofilms and planktonic bacteria cause different kinds of infections, with biofilms typically involved in chronic infections, while planktonic bacteria play a key role in acute phase diseases (Maciá *et al*. 2014). Infections linked to devices were the first clinical infections associated with biofilms, and infections related to medical devices like prosthetic heart valves and intravenous catheters are still a major problem. The staphylococci (especially *Staphylococcus epidermidis* and *Staphylococcus aureus*) are the bacteria most commonly associated with device-related infections, followed by *Pseudomonas aeruginosa* (Hall-Stoodley *et al*. 2004). The staphylococci are often found in wounds and implants, and typically origin from colonies on the skin (Akiyama *et al*. 2003), making them opportunistic pathogens.

Cystic fibrosis (CF) is a good example of chronic infections caused by biofilm. CF is an autosomal recessive disease caused by a mutation in the cystic fibrosis transmembrane conductance regulator gene. Certain mutations in this gene leads to a dysfunctional electrolyte secretion and absorption. The disease is very complex, but the primary site of morbidity is the respiratory system. Chronic endobronchial bacterial infection and inflammations in the airways leads to airway hindrance, degradation of the airway epithelium and finally respiratory failure. The bacterial species responsible for the chronical infection is *P. aeruginosa*, which grows in biofilms in the lungs of CF patients. In the biofilm, there is a selection for mucoid bacterial variants with high production of the exopolysaccharide alginate and

tolerance to antibiotic therapy, making them very hard to eradicate (Hall-Stoodley *et al*. 2004).

The major problem with biofilm formation on medical implants and in chronic infections, is indeed the difficulty to eliminate them. As mentioned before, the EPS matrix makes biofilms highly tolerant against antibiotics, and the organization in close cell-to-cell fashion facilitates resistance to be spread more easily between bacteria. Studies have even shown that exposure of biofilms to antibiotics below the minimal inhibitory concentration (sub-MIC) stimulate the bacteria to adhere to eukaryotic host cells and tissues, the first step in biofilm formation, to a greater extent (Blickwede *et al*. 2005). Other studies have provided evidence that sub-MIC concentrations of antibiotics actually induce biofilm formation in some bacterial species (Hoffman *et al*. 2005). Thus, biofilms not only have a high tolerance against antibiotics, there is also a possibility that low concentrations of antibiotics actually works in their favor.

1.4 The project

1.4.1 Aim of the project

This study is part of a bigger project with the overall aim to study the MSC values of antibiotic resistant and susceptible strains for streptomycin, ciprofloxacin, rifampicin, fosfomycin, nitrofurantoin and trimethoprim for the *Escherichia coli* strain CFT073 in biofilms and planktonic growth.

MSC values for antibiotic resistant and susceptible strains are first determined in planktonic growth and based on these the sub-MIC antibiotic concentrations for the biofilm assay are chosen, with the assumption that they might be in the same range. To compare wildtype and resistant strain, CFT073 is altered, constructing two resistant mutants for each antibiotic. The mutants are isogenic, a part from the chromosomally integrated fluorescent tags YFP or dTOMATO that are used to count them using flow cytometri. This means that for each antibiotic there will be a pair of mutants with the same resistance mutation, but with different tags.

In this project, the aim was to determine the MSC values for streptomycin and ciprofloxacin, using already constructed mutants. In addition, two pairs of new mutants were constructed with resistance against fosfomycin and nitrofurantoin.

1.4.2 Urinary tract infections (UTIs) and *E. coli* CFT073

The model bacterium used in this paper is the uropathogenic *E. coli* CFT073, a pathogenic strain isolated from a woman that suffered from acute pyelonephritis (inflammation in the kidneys) (Welch RA *et al*. 2002). The urinary tract is one of the most common sites for bacterial infections, and the most common infecting species is

E. coli (Mobley MS *et al* 2009). Studies have shown that 40-50% of all women will encounter at least one symptomatic urinary tract infection during their lifetime, and for many the infection will reoccur (Forsyth VS *et al*. 2018). *E. coli* strains that causes UTIs goes under the term uropathogenic *E. coli*, and differ from commensal strains in that they carry extragenic material which encode gene products that contribute to the pathogenic properties of the bacteria ((Mobley MS *et al* 2009)).

The genome of CFT073 is 590,209 bp longer than the commonly used lab strain MG1655 4.6 Mbp), and contains genes that encodes specific fimbrial adhesins, secreted autotransporters and phase switch recombinases (Welch RA *et al* 2002). The fimbriae are important for the biofilm forming lifestyle of the strain, and in CFT073 twelve genes that presumably encodes fimbriae have been discovered (Mobley MS *et al* 2009).

1.4.3 The calgary biofilm device

In 1999 Ceri *et al* published a paper describing the Calgary Biofilm Device (CBD), a new type of technology for rapid determination of antibiotic susceptibility in bacterial biofilms. Since there often is a difference between antibiotic susceptibility for a strain in planktonic compared to biofilm growth, there was a need for a technique to perform reproducible studies on bacterial cells in biofilms exposed to antibiotics. The setup of the CDB is a two-part reaction vessel, a top component in the form of a lid with 96 pegs and a bottom component with channels. The pegs are designed to fit into the channels of the bottom component, and to fit into the wells of a standard 96-well plate. The biofilm can then be inoculated in the wells and grown on the pegs, then harvested as single replicates (Ceri H *et al*. 1999).

The device used for this paper is based on the CDB, but altered and optimized by Erik Wistrand-Yuen. One key feature that was not optimal with the CDB was the harvesting procedure. To extract the biofilm from one peg at the time, the pegs needed to be broken of the lid, a process increasing the risk of contaminations. In the adapted version, the pegs are removable, and can be directly pushed through the lid from the top, down in to a container (in this case, glass tubes). A silicone mat was added to the top of the lid to enhance the stability of the pegs. The pegs are reusable, and after washing they are constructed to be reattached to the lid. For an easy harvesting process, a rack was constructed that can hold 24 glass tubes, and a holder were the lid can be placed that fits above the rack with glass tubes. When put in position, the pegs can be pushed straight through the lid, down into the glass tubes below. The lid fit perfectly to microtiter plates (Thermo Scientific™), and the biofilm is cultivated on the pegs in separate wells.

1.4.4 Lambda red recombineering system

When working with bacteria, it is often desirable to be able to modify the chromosomal genome. There are some naturally competent bacteria, but most other bacteria, including *E. coli*, are not naturally transformable. One of the reasons for *E.*

coli not being transformable is due to the presence of intracellular exonucleases that degrades linear DNA.

Many bacteriophages encode their own homologous recombination system (Smith GR. 1988), a fact that has been used when constructing the lambda red recombineering system, which is derived from the lambda bacteriophage. The system contains three key genes: γ, β and exo whose products are called Gam, Beta and Exo encoded on a plasmid (Murphy KC. 1998).

When the plasmid is introduced to the cell, the Gam protein protects dsDNA fragments from being degraded by the host RecBCD exonuclease V, making it possible for Beta and Exo to gain access to the ends of the DNA, promoting recombination (Datsenko & Wanner. 2000). When recombination of a ssDNA, only Beta is necessary, but since the other two proteins do not have any negative effect on the host cell, the plasmid with all three genes is used for recombination of both dsDNA and ssDNA.

For this study, the lambda red genes are expressed from the plasmid pSIM5 (Datta *et al*. 2006), carrying a chloramphenicol resistance cassette. The chloramphenicol resistance cassette is used to select for the bacteria carrying the plasmid. The expression of the three genes γ, β and exo is temperature regulated, and the expression is activated with an increased temperature from 30 °C to 42 °C for 15-30 min. The bacterial strain with the correct mutation is easily cured of the plasmid after an overnight incubation at 42 °C.

1.5 The antibiotics

1.5.1 Ciprofloxacin

Ciprofloxacin belongs to the antibacterial agent class of fluoroquinolones, and is one of the most used clinical drugs. It was introduced for clinical use in 1987 (Fàbrega A *et al*. 2009). Quinolone antibiotics act by inhibiting DNA synthesis, through targeting two essential type II topoisomerases; DNA gyrase and topoisomeras IV. For ciprofloxacin, DNA gyrase is the target. It is an ATP-dependent enzyme that induce negative supercoiling, which is essential for chromosome condensation (Zahedi Z *et al*. 2018). Since DNA gyrase is only present in bacteria, it is an excellent target for antibiotics as it will not interfere with eukaryotic cells that lack the protein (Fàbrega A *et al*. 2009). The enzyme comprises of two subunits, GyrA and GyrB. In this project, resistance is established by targeting the GyrA subunit, which plays a key role in DNA breakage/reunion. It is encoded by *gyrA*, the gene that is most frequently mutated in *E. coli* strains carrying ciprofloxacin resistance (Zahedi Z *et al*. 2018. According to literature two of the most important mutations leading to a clinical resistance in *E. coli* are the amino acid mutations in codon 83 or 87 (Fàbrega A *et al*. 2009).

1.5.2 Streptomycin

Streptomycin is an aminocyclitol aminoglycoside (Springer B *et al*. 2001) that raises the frequency of missense error in the bacterial translation machinery by increasing the affinity between the ribosome and non-cognate tRNAs which interferes with the ribosomal proofreading. This will result in an accumulation of defect proteins, which is bactericidal. In addition, streptomycin also has the ability to increase the drop-off of peptidyl-tRNA from the ribosome due to a weakened P site binding of peptidyl-tRNA (Pelchovich G *et al*. 2013). In the majority of cases, resistance to streptomycin is obtained though mutations in the *rpsL* gene, which encodes the 30S ribosomal protein S12 (Andersson DI and Hughes D. 2014). This protein has a key function to maintain translational accuracy. In *E. coli*, mutations causing clinical streptomycin resistance affects codons 42 or 87 (Pelchovich G *et al*. 2013).

1.5.3 Fosfomycin

Fosfomycin is a broad spectrum, bactericidal antibiotic with a very low toxicity against eukaryotic cells. It acts by inhibit the enzyme UDP-N-acetylglucosamine-3-O-enolpyruvyl transferase (MurA), which is essential for the synthesis of peptidoglycan (Silver LL. 2017). In the cytoplasm, UDPN-acetylglucosamine-3-O-enolpyruvate, the link between the peptide and glycan parts of the peptidoglycan, is formed from UDP-N-acetylglucosamine and phosphoenolpyruvate (PEP). Fosfomycin acts as a PEP analog by covalently binding to the Cys115 residue, a key part of the active site of MurA, and thereby inhibiting the formation of the linker (Silver. 2017), preventing the bacteria from building a functioning cell wall. In *E. coli*, fosfomycin is able to enter the cell via two nutrient transporters, one of them being the hexose-6-phosphate transporter (UhpT), which is induced by extracellular glucose-6-phosphate (G6P) (Castaneda-Garcia A *et al*. 2013).

1.5.4 Nitrofurantoin

Nitrofurantoin is a part of the group Nitrofurans, compounds characterized by the presence of one or more nitro-groups on a nitroaromatic or nitro-heterocyclic backbone (Sandegren L *et al*. 2018). The specific mode of nitrofurans are complex, but by comparing resistant and susceptible strains of *E. coli*, the literature suggests that for the nitrofurans to show their antibiotic effect, they need to be activated by reducing activity that exist in the bacterial cell (Asnis RE, 1957, Asnis RE *et al*. 1952). According to McOsker and Fitzpatrick (1994), nitrofurantoin is converted by bacterial nitroreductases to electrophilic intermediates, which leads to inhibition of the citric acid cycle as well as the synthesis of DNA, RNA and protein.

Resistances against nitrofurans are caused by step-wise mutations, were there is a connection between increased resistance and decreased reductive capacity, since the nitrofurans needs the cells reducing activity to be activated. It has been shown in *E. coli* that the resistance is linked inactivation of the genes *nfsA* and *nfsB*, which encodes oxygen-insensitive nitroreductases (Sandegren L *et al*. 2018). In the paper by

Sandegren *et al* (2008), all spontaneous nitrofurantoin resistant *E. coli* mutants demonstrated first-step mutations only in *nfsA*. Most of the two-step mutants contained an additional mutation in the *nfsB* gene, suggesting that inactivation of *nfsA* followed by inactivation of *nfsB* is the main mechanism for high-level nitrofurantoin resistance in *E. coli.*

2. Methods

2.1 Strains/medium/growth conditions

All strains used in this study are derived from *Escherichia coli* CFT073. To enable single-cell tracking of the different strains during the competition assays, genes coding for a red (*dTOMATO*) or a yellow (*SYFP2*) fluorescent protein have been integrated into the chromosome, resulting in strains DA56711 and DA56709. These strains also carry the lambda red plasmid to enable transformation. All mutants are derived from these strains, which means that all constructed mutant strains come in pairs, which are isogenic except for the different florescent tags. The strains used in this paper are presented in table 1.

Table 1. A list of all the strains used in this study.

Strain	Genotype	Source of reference
DA56709	*E. coli* CFT073 *galK::kan*-J23101-*SYFP2*/pSIM5-cam	Our strain collection
DA56711	*E. coli* CFT073 *galK::kan*-J23101-*dTomato*/pSIM5-cam	Our strain collection
DA58419	*E. coli* CFT073 *galK::kan*-J23101-*SYFP2*	Our strain collection
DA58420	*E. coli* CFT073 *galK::kan*-J23101-*dTomato*	Our strain collection
DA66036	*E. coli* CFT073 *galK::kan*-J23101-*SYFP2*, *gyrA* S83F	Our strain collection
DA66037	*E. coli* CFT073 *galK::kan*-J23101-*dTomato*, *gyrA* S83F	Our strain collection
DA66038	*E. coli* CFT073 *galK::kan*-J23101-*SYFP2*, *rpsL* K42N	Our strain collection
DA66039	*E. coli* CFT073 *galK::kan*-J23101-*dTomato, rpsL* K42N	Our strain collection

The liquid and solid media used for bacterial growth were Brain Heart Infusion (BHI) broth (Oxoid Limited, UK), Lysogeny Broth (LB) no salt, LB low salt and LB agar (LA) (Sigma-Aldrich, USA). The strains were grown at 37°C if noting else is noted, planktonic growth under shaking (180 rpm) conditions and biofilm standing still in plastic boxes.

2.2 Construction of strains

To construct mutants with the desired resistance mechanisms, relevant genes on the chromosome were changed by recombination with a ssDNA oligonucleotide (sequences presented in appendix 1), homolog to the target gene, except for one changed nucleotide (nt) in the middle generating a STOP-codon. The fosfomycin resistant strain was generated by the substitution of TTA with a STOP codon (TAA) at position 5 in the gene *uhpT*. The nitrofurantoin resistant strain was supposed to be generated in the same fashion, by first substitute GAA with a STOP codon (TAA) in *nfsA* followed by a second mutation were TTA is substituted with a STOP codon (TAA) in *nfsB* (see appendix for primers and oligo). However, some complications arise, resulting in change of *nfsB* as the first mutation. This is further discussed later.

The λ red recombineering system was used as described by Datsenko & Wanner (2000) with some modifications. Two 250 ml E-flasks containing LB no salt medium with 12.5 mg/L chloramphenicol were inoculated with a 200-fold dilution of an bacterial culture grown overnight of one of the strains containing the fluorescent proteins YFP or dTOMATO (DA56711 and DA56709), meaning that all mutants are constructed as pairs.

The cultures were grown to an OD_{600} of around 0.25 under continuous shaking (150 rpm), 30 °C for approximately 1.5 hours and then transferred to a shaking 42°C water bath for 30 min. This activates the expression of the pSIM5 plasmid encoded genes *beta*, *gam* and *exo* that are necessary for the recombineering. The cultures were then put on ice for 5 minutes, transferred to 50 ml falcon tubes, spun down and the pellet was re-suspended in 12.5 ml ice cold 10% glycerol (performed three times for proper wash). The final pellet of each culture was re-suspended in 200 µl 10% glycerol, from which 50 µl were transferred to an electroporation cuvette together with 2 µl of the desired oligonucleotide (containing the mutated amino acid). The electroporation was performed using the GenePulser Xcell™ from Bio-Rad set to 1.8 kV, 2 µF and 200 ohm. The electroporated cells were recovered in 1 ml LB no salt with 12.5 mg/L chloramphenicol, shaking over night at 30 °C. The day after the bacterial cells were spread on agar plate containing 12.5 mg/L of chloramphenicol and fosfomycin or nitrofurantoin for selection of successful mutants.

To verify that the mutants carry the desired mutation, the gene of interest was amplified using PCR (cycling condition in appendix), and the product purified using the Gene jet gel extraction kit from Thermo Fischer™ according to the instructions from the producer and sent for sequencing.

2.3 Biofilm growth/extraction/cycling

The biofilm was grown on the pegs in the modified version of the Calgary Biofilm Device (Ceri *et al*. 1999) created by Erik Wikstrand-Yuen.

Over night bacterial cultures (4 biological replicates per strain) were diluted 10.000-fold (resulting in approximately 10 generations of growth per harvest cycle) and inoculated in 96 well plates, 200 µl per well. The cultures were then grown static for 24 hours at 37°C, contained in plastic containers with lids. When 24 hours had passed, and the biofilm had time to establish growth on the pegs, the peg-lid were moved to a new 96 well plate containing different concentrations of the antibiotic of interest (streptomycin or ciprofloxacin). The concentrations were chosen in relation to the MSC value determined in assays analyzing planktonic growth, with the assumption that the MSC value for the biofilm might be in the same range. With that as the starting point, values higher and lower than the presumed MSC were selected. The cultures were then grown for 24 hours at 37°C when the growth media was changed by transferring the peg-lid to fresh media with the same antibiotic concentrations as used for the previous 24 hours. After a total of 48 hours, equal to 10 generations of growth, the biofilm was harvested. The pegs were washed by being moved to a 96-well plate with 250 µl of 1xPBS per well for 3x1 minute, the PBS changed between each cycle. The pegs were then removed from the lid into glass tubes containing 600 µl BHI and vortex for 2 minutes to extract the biofilm from the pegs. 1 µl of each harvested biofilm was cycled in a new 96 well plate containing 200 µl BHI/well, a processes repeated 3 times, generating approximately 30 generations during six days. Per 10th generation, all the ratios between wild type and mutant were analyzed using flow cytometry (see competition experiments). The whole assay flow chart is illustrated in figure 4.

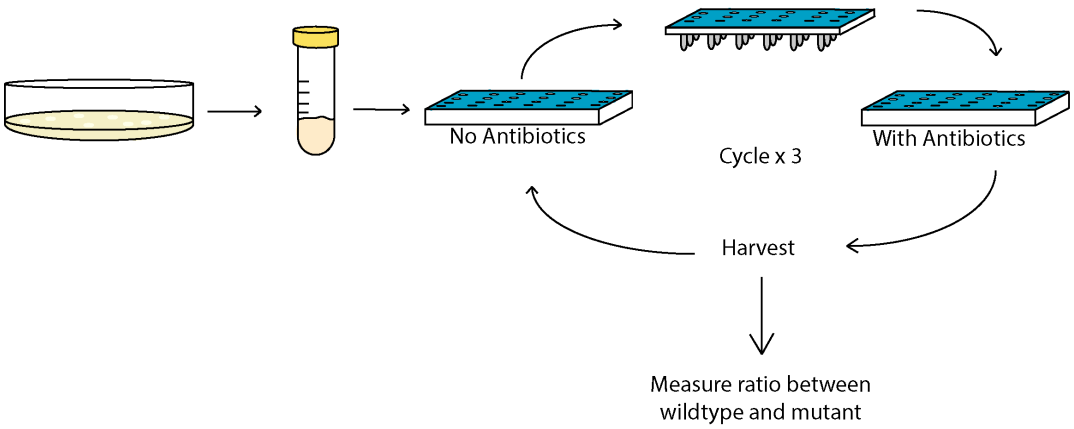


Figure 4. A graphic illustration of the workflow to generate biofilm in competition experiments

2.4 Competition experiments

To evaluate the MSC for the mutants versus the susceptible strain, competition assays were conducted on the extracted biofilm. Over night cultures grown in Brain Heart Infusion medium of the susceptible strain with either *yfp* or *dTOMATO* were mixed with the resistant mutant carrying the other marker. The mixes were prepared in both ways, meaning mixes with susceptible strain (YFP) vs. mutant strain (dTOMATO) and dyeswapped mixes with susceptible strain (dTOMATO) vs. mutant strain (YFP). The mixes were then diluted, added to the wells and grown according to the protocol above. 1:1 ratios were used for the ciprofloxacin assay, but for the streptomycin assay the ratios had to be adjusted to enable readable measurements for all 30 generations (1:4, 1:1, 4:1). For every harvested cycle, 10 µl of each biofilm were added to 140 µl PBS in a 96 well plates (Thermo Scientific™). The ratio between susceptible and mutant were then measured by flow cytometri using the MACSQuant VYB device from Miltenyi Biotec, counting 10^5 events per sample.

2.5 Calculation of MSC

The data obtained from the MACSQuant VYB measurements were analyzed using the program CompDAta (Pilekie & Wistrand-Yuen). The program plot the ratios of resistant:susceptible strain as functions of the number of generations of growth at different concentrations of antibiotic, see figure 5.

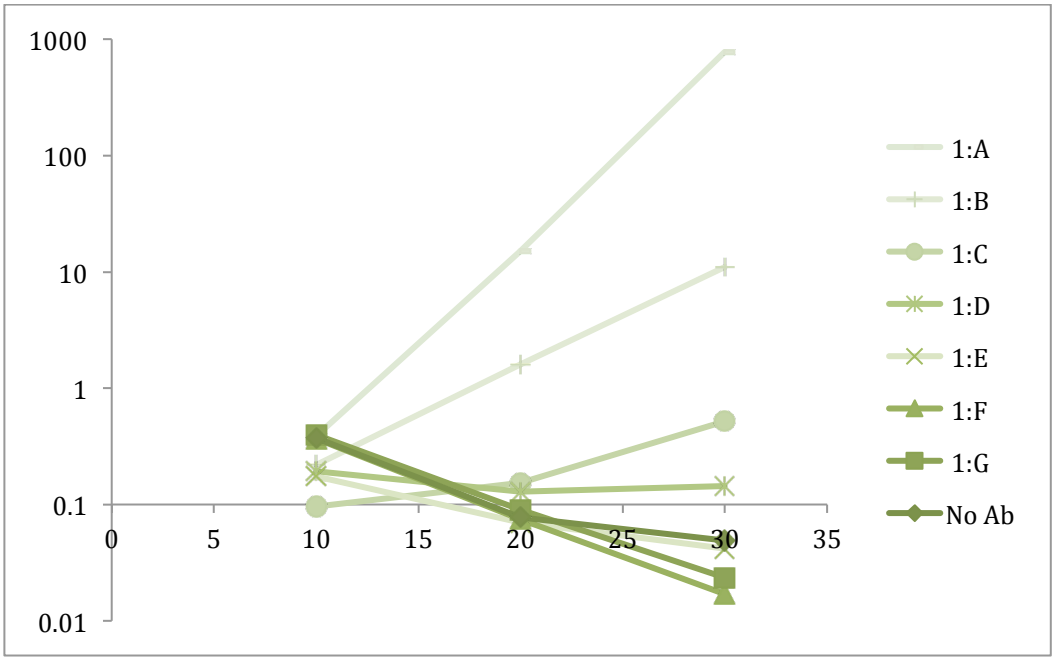


Figure 5. The ratio of susceptible:mutant strain as functions of generations of growth. Each line represents a single competition experiment performed with 4 biological replicates (4 of each pair) at one specific concentration. The slope of each line is a measure of the selection coefficient (s-value).

Each line represents a single competition experiment performed with 4 biological replicates (4 of each pair) at one specific concentration. The slope of each line is a measure of the selection coefficient (s-value). By plotting the obtained s-values as a function of antibiotic concentration, the intercept were s=0 represent the MSC value, were the fitness cost of the resistance is balanced by the antibiotic-conferred selection for the resistant mutant (Gullberg *et al*. 2011).

2.6 MIC determination

Etests determined the MIC values for the different antibiotics, used according to the instructions from the manufacture (bioMérieux, France). The tests were performed on Mueller-Hinton agar plates, incubated over night at 37°C. When testing the fosfomycin mutants, the Mueller-Hinton agar plates had an addition of glucose-6-phosphate (G6P) (125 mg/L).

3. Results

3.1 Construction of strains

All strains constructed for this project are derived from CFT073, a biofilm forming strain of *E. coli.* In this study two mutant strains were constructed, one fosfomycin resistant mutant (*uhpT*) and one nitrofurantionin resistant mutant (*nfsA/nfsB*). In both cases, the relevant genes were supposed to be disrupted by introducing an STOP codon, preventing them from being correctly translated. Since the genes are essential for the function of the antibiotics, the mutants will be resistant to different degrees.

Table 2. An overview of all the mutant strains which are included in the project.

Antibiotics	**Mutated gene**	***YFP***	***dTomato***	**Finished**	**Started**	**Planed**	**Source**
Ciprofloxacin	*gyrA*	DA66036	DA66037	✓			Fernberg
Streptomycin	*rpsL*	DA66038	DA66039	✓			Fernberg
Rifampicin	*rpoB*	DA66034	DA66035	✓			Fernberg
Fosfomycin	*uhpT*	DA66040	DA66041	✓			This paper
Nitrofurantoin	*nfsA/nfsB*	-	-		✓		This paper
Trimethoprim	-	-	-			✓	-

The strains used for the planktonic and biofilm assays in this study had already been constructed (Fernberg J. 2019). A complete overview of the strains is presented in table 2.

3.1.1 *uhpT*

The literature clearly indicates that the essential gene for fosfomycin activity is *uhpT* (Castaneda-Garcia A *et al*. 2013), and therefore this gene was disrupted by introducing a STOP codon, see above. The obtained transformants were checked for resistance by growing them on LA-plates containing 30 mg/L and 50 mg/L fosfomycin and grown for 24h at 37°C. There was visible growth on both concentrations, and clones obtained from both concentrations were selected for sequencing (primers in appendix) to confirm the introduction of the STOP codon. The analysis of the sequence showed that all mutants had acquired the desired mutation.

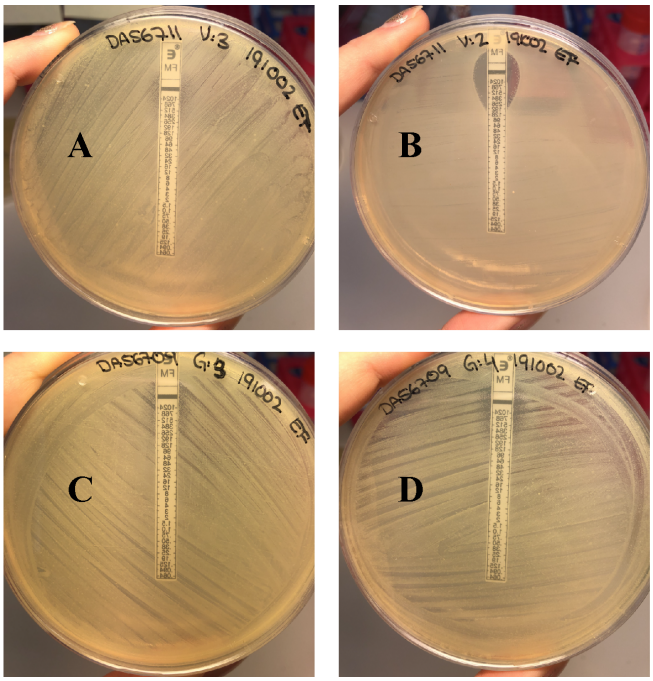


Figure 6. Etests of the fosfomycin resistant mutant strains. (A) and (B) are tests of the strains containing the *dTomato* tag. (C) and (D) are the *YFP* strains. A, C and D show the same results while B have a deviant outcome. Therefore, B was deselected.

To further validate the mutant strains the growth rate was measured at 37°C in BHI using a Bioscreen C Analyzer (Oy Growth Curves Ab Ltd, Helsinki, Finland). Since the strains should be isogenic except for the different fluorecent tags, they should have the same growth rate. Four strains with the same growth rate and matching phenotype were chosen, two with each of the tags. As a final evaluation, their MIC values were determined using Etests. As seen in figure 6, three of four strains showed the same results, with cells growing also at the highest concentration of fosfomycin. The fourth mutant strain, tagged with *dTomato*, did not show the same MIC value and was not used further (Fig 6 B).

To determine which of the two *YFP* mutants that matched the remaining *dTomato* strain, a competition assay with mixes of each *YFP* strain and the *dTomato* strain was performed. The assay started with 1:1 ratio mixes, cycling and measurements performed for 3x24 h. Since the strains should be isogenic except for the tags, which should have the same fitness cost, the 1:1 ratio should stay the same throughout the whole assay. One of the *YFP* mutants matched the *dTomato* strain better, resulting in the DA66040/DA66041 pair.

3.1.2 *nfsA/nfsB*

Previous studies have shown that to obtain a higher level of nitrofurantoin resistance, two genes need to be disrupted, both *nfsA* and *nfsB* (Sandegren L *et al*. 2018). They

concluded that development of resistance is a stepwise process, starting with mutations in *nfsA* followed by *nfsB*. Therefore, the strategy in this project was to mimic this process by a two-step recombination setup starting with *nfsA*.

The recovered transformats, and the control, were streaked on LA-plates containing 12.5 mg/L and 25 mg/L nitrofurantoin.

After 24 h incubation at 30°C small growth of colonies were observed on the higher concentration for the mutants and great amount of growth on the lower concentration. Although, for the lower concentration we also had a lot of growth on the control, implying that spontaneous mutation with nitrofurantoin resistance are able to evolve at that concentration. This might be a concern for the mutants obtained from the 12.5 mg/L plate, since there is a greater risk that they have gained additionally spontaneous mutations.

Single colonies from both concentrations were re-streaked on new plates and the nfsA gene was amplified by PCR and sequenced.

The sequencing data showed that apart from the desired mutation, all strains except for three had also acquired spontaneous mutations. It is of great importance that our mutant strains only contains the mutation that we have introduced, we want the two strains to be isogenic except for the fluorescent tags. We want to be sure that the data we obtain from the planktonic and biofilm assays are correlated only to the mutation we introduced, and not affected by any spontaneous mutation. Unfortunately, the three strains that looked promising were all marked with *YFP*, meaning that we lacked mutants tagged with *dTomato* and consequently had no complete pair with both fluorescent tags.

3.2 Planktonic growth

Initial assays were performed in planktonic growth with wild type and resistant strains to evaluate the selection pressure of sub-MIC antibiotic concentrations. From this data, an MSC value could be calculated and used as a guideline for the biofilm assay of the same mutant strain. Previous results indicate that the MSC for the mutant strains in biofilm is approximately the same as for the planktonic grown cells, and therefore we use this assumption as a starting point when choosing concentrations. Although, the MSC could still be completely different between planktonic and biofilm form. MICs for the susceptible wildtype *E. coli* CF073 have been determined by broth micro dilution (ciprofloxacin = 0.064 mg/L and streptomycin = 24 mg/L).

The competitions between susceptible strain and mutant strains were performed in tubes under shaking conditions with a range of concentrations of antibiotics. The strains were mixed at a 1:1 ratio and grown according to the layout described above. Every 10^{th} generation, the ratio between susceptible strain:mutant strain were

measured by flow cytometri, enabled by the genetically fluorescent tags, *yfp* and *dTomato*.

The assay was performed for streptomycin with each pair of wildtype and mutant *rpsL K42R*, DA66038/DA58420 and DA66039/DA58419. In the first set of experiments the ratios of susceptible and resistant strain were set to 1:1 for the whole range of concentrations. This resulted in one of the two strains being completely erased by the 30th generation in the higher and lower ends of the concentration spectrum. Therefore it was decided to start the assay with altered rations of the strains to get measurable results for all three readings. The concentrations and rations are presented in table 3.

Table 3. The concentrations of streptomycin and ratio of susceptible:mutant strain used in the planktonic assay.

[Streptomycin] (mg/L)	Times MIC_{susc}	Ratio wildtype:mutant
0	0	1:4
0.8	1:30 x MIC	1:4
2.4	1:10 x MIC	1:1
3	1:8 x MIC	1:1
4	1:6 x MIC	4:1
6	1:4 x MIC	4:1
8	1:3 x MIC	4:1

The obtained data was analyzed using CompDAta and the evaluated s-values were plotted against the different streptomycin concentrations, see figure 7. The MSC can from this graph be set to ≈ 1/8 of MIC_{susc} (3 mg/L).

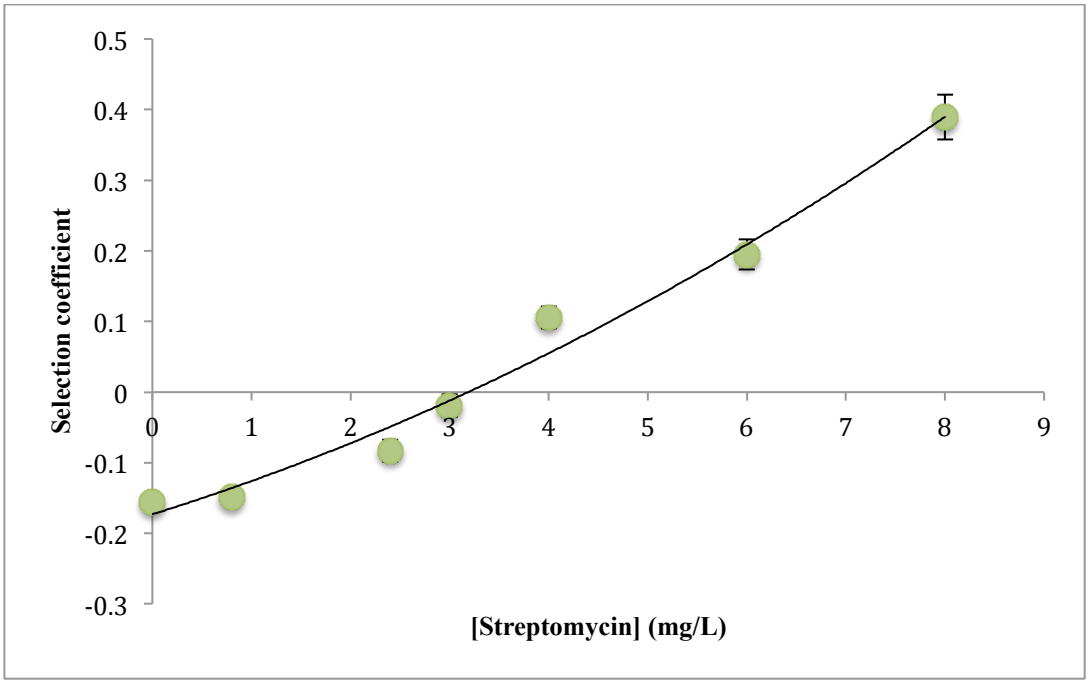


Figure 7. Planktonic assay. The s-values obtained using CompDAta as a function of the streptomycin concentrations. The MSC value can be determined to 3 mg/L (1/8 MIC).

This assay has already been preformed for *gyrA S83L* (Fernberg J. 2018), and the MCS was determined to approximately 1/200 MIC_{susc} (0.0032 mg/L).

3.3 Biofilm growth

3.3.1 Streptomycin

Since the results from the planktonic assay showed a MSC value of 1/8 MIC_{susc}, the concentrations in the biofilm assay were chosen to give data points both below and over this value. The chosen concentrations and ratios susceptible:mutant strain are presented in table 4. As for the planktonic assay, it was performed with each pair of wildtype and mutant *rpsL* K42R, DA66038/DA58420 and DA66039/DA58419. The biofilm was cycled for 3x48h and the obtained data analyzed the same way as for the planktonic data. The resulting graph showing the selection coefficient as a function of streptomycin concentration is presented in figure 8. At some concentrations after the 30^{th} generation, one of the strains had completely wiped out the other, and the ratio were therefore not readable. The conditions were No streptomycin, 1:24xMIC, 1:16xMIC and 1:2xMIC. Therefore the last measurement of these concentrations had to be removed, and is not included in the graph. The reason for this will be discussed in more detail later.

Table 4. The concentrations of streptomycin and ratio of susceptible:mutant strain used in the biofilm assay. The concentrations are chosen based on the MSC value from the planktonic assay with the same strains.

[Streptomycin] (mg/L)	Times MIC_{susc}	Ratio wildtype:mutant
0	No streptomycin	1:4
1	1:24 x MIC	1:4
1,5	1:16 x MIC	1:1
3	1:8 x MIC	1:1
6	1:4 x MIC	4:1
12	1:2 x MIC	4:1

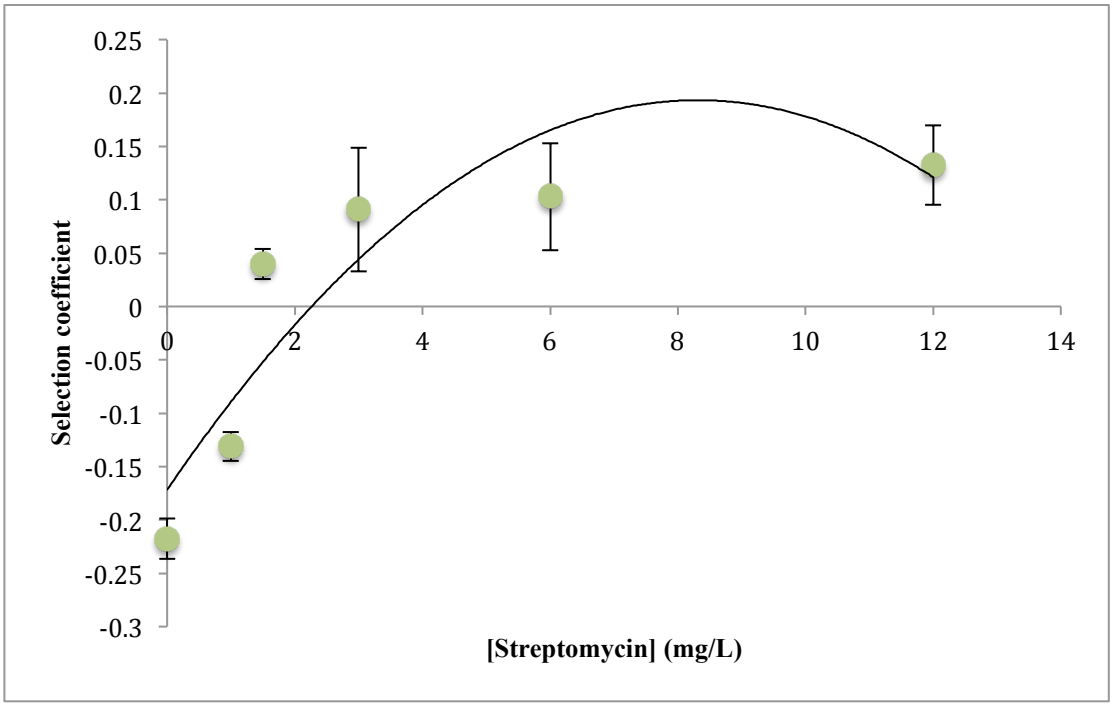


Figure 8. Biofilm assay. The s-values obtained using CompDAta as a function of the streptomycin concentrations. The data is incomplete, but the MSC could possibly be around 2.5 mg/L.

From this graph we can conclude that the MCS for the biofilm is approximately the same as for the planktonic form, around ≈ 1/8 of MIC_{susc} (3 mg/L). Although, the curve fit is not ideal, due to the higher concentrations. The setup needs to be performed again, using lower concentrations of streptomycin and even more adjusted ratios. However, preliminary results indicate that the MSC value for the planktonic and biofilm form of the strain are close to the same.

3.3.2 Ciprofloxacin

Based on previous work, we knew that MSC for *gyrA* S83L in planktonic form is approximately 1/200 MIC (0.00032 mg/L). A biofilm assay had also been performed,

and the MSC for the biofilm had been estimated to 1/120 MIC (0.00053 mg/L). The concentrations in our biofilm assay were based on this information, choosing concentrations around the estimated MSC for the biofilm, wanting to increase the resolution of the x-axis intersection. The chosen concentrations and ratios are presented in table 5.

Table 5. The concentrations of ciprofloxacin and ratio of susceptible:mutant strain used in the biofilm assay. The concentrations are chosen based on the MSC value from a biofilm assay performed with the same strains.

[Ciprofloxacin] (mg/L)	Times MIC_{susc}	Ratio wildtype:mutant
0	No Ciprofloxacin	1:1
0.0004	1:160 x MIC	1:1
0.00053	1:120 x MIC	1:1
0.00064	1:100 x MIC	1:1
0.0008	1:80 x MIC	1:1
0.001067	1:60 x MIC	1:1
0.00128	1:50 x MIC	1:1

The assay was performed with each pair of wildtype and mutant *gyrA* S83L, DA66036/DA58420 and DA66037/DA58419, executed in the same way as for streptomycin. One difference between the assays was the starting ratios between wildtype and mutant, which were possible to keep at 1:1 for all concentrations. The resulting graph showing the selection coefficient as a function of ciprofloxacin concentration is presented in figure 9.

Since the fitness cost for the *gyrA* mutation is very low, the MSC value is very low. From the derived graph it is very hard to determine with any statistical significance were the graph intercepts the axis (s=0). Additionally, in the performed measurements there were some problems with the generated data for some of the concentrations, and we had to remove some of the replicates to generate a readable graph. These factors combined prevent us from drawing any conclusions about the MSC value from this graph since the data is incomplete. This will be discussed in more depth later on.

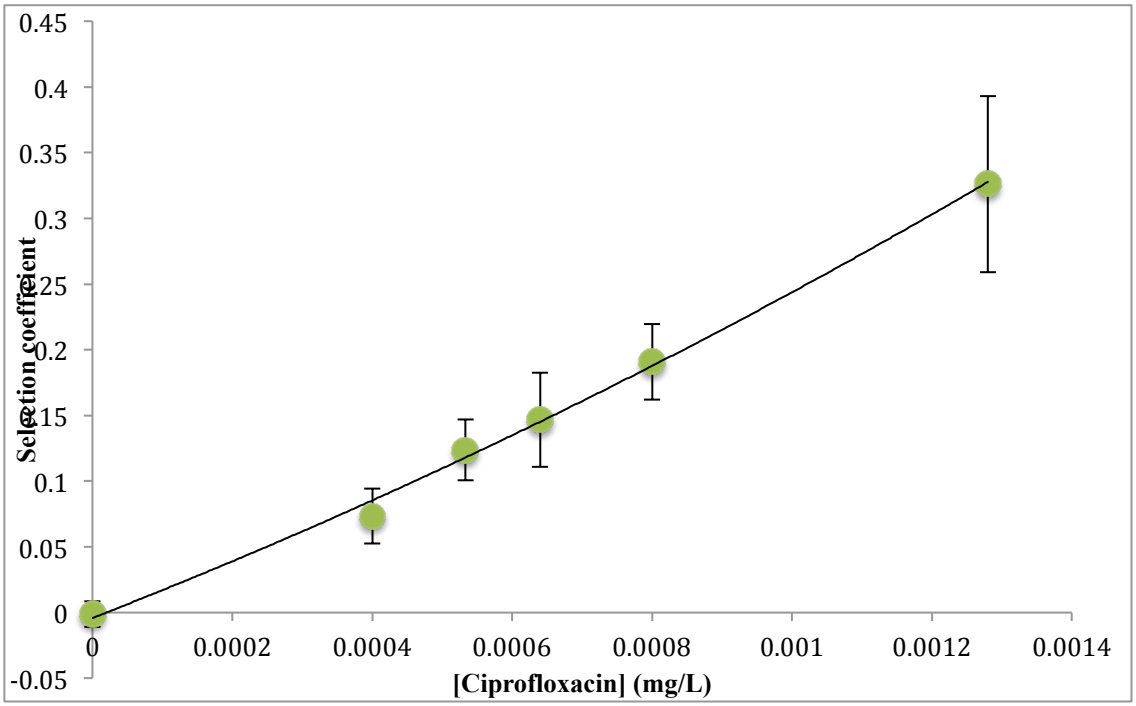


Figure 9. Biofilm assay. The s-values obtained using CompDAta as a function of the ciprofloxacin concentrations. The data is incomplete, and it is very hard to determine with any statistical significance were the graph intercepts the axis (s=0).

4. Discussion

Great amounts of research have been, and are still made on bacteria in planktonic form, to study how resistance is gained and spread among these populations. Since we have come such a long way within that field, more focus needs to be but on bacteria living as biofilms. With the high cell density, a lot of cell-to-cell contact and large amounts of shared genomic material, biofilms constitute ideal environment for the spread of antibiotic resistance via horizontal gene transfer. The way they are composed also cause them to be very protected from their environment, making them hard to get rid of. There are already today healthcare related problems with biofilm formation on medical implants and prosthetics as well as chronic infections caused by biofilms.

One factor that probably contributes to biofilms being less studied is that that they are much more complex and time consuming to grow. The planktonic cycling assays we perform in this study lasts three days, generating 30 generations of growth. The same cycling, with a total of 30 generations of biofilm growth, stretches over six days. On top of that, the cells are in need of something to attach to (in this study the pegs) and the cycling process includes an extra harvesting step. This requires more resources and more time.

There is also a possibility for large variations to be introduced between replicates since all the pegs are grown as individuals and handled in many steps. Some resistance mutations have very low fitness costs, and even the smallest of variations introduced between the replicates due to multiple steps can results in misleading data. This will be discussed more thoroughly later. There is also a bigger variety in the measurements due to the extracellular components from the EPS, which introduce background noise and makes the data harder to read. The measurements also include an increased number of cells that are stuck together due to their biofilm forming properties, and therefore cannot be separated as single data points in the cell sorting analysis.

4.1 Mutants

One of the greatest challenges with the construction of the resistant mutants was to create two isogenic strains, except for the fluorescent tags, which only contains the desired mutation and no additional spontaneous ones.

When constructing the *uhpT* mutants, we started with 27 clones that all contained the desired mutation, but after analysis of growth rate and MIC, we could see that some strains had deviant results. If the strains were isogenic, the MIC and growth rate of the strains should be the same, and when grown on agar plates their colonies should have the same phenotypes. Since this was not the case for all 27 clones, some of them must have gained extra mutations and therefore behaved differently. The final pair, DA66040/DA66041, have the same growth rate and MIC as well as the same fitness cost for the fluorescent tag and mutation. Although, to be completely sure that they are isogenic, they need to be whole genome sequenced and compared with each other as well as with the susceptible wild type.

The *nfsA/nfsB* resistant strains turned out more complex to construct, first and foremost due to the two-step mutation process. When performing the first step, it is crucial that the plasmid used for recombineering is kept during the whole process, and that we end up with a mutant strain still carrying it. The reason being that the genes needed for the recombination process are encoded on the plasmid, meaning that it is essential for the second mutation step. Besides that, it turned out to be troublesome to pinpoint the right concentration of nitrofurantoin to separate the desired mutation from spontaneous ones.

The *nfsA* mutant has an increased MIC of approximately 2xMIC. With the higher concentrations, almost no clones were recovered, in contrast to the lower concentrations at which the same amount of growth on the control (indicating room for spontaneous mutations) was obtained. When repeating this, the antibiotics concentrations should be more carefully optimized to facilitate selection only for the desired mutants.

4.2 Streptomycin

The streptomycin assays, both planktonic and biofilm, showed similar results with a MSC value around 1/8 MIC. When performing the planktonic assay with 1:1 ratios, the 30th generation was not measurable since one of the strains being completely overgrown by the other.

We adjusted the ratios between the susceptible strain and the mutant strain to be able to get readable results for all three of the time points, and this was a successful measure for the planktonic growth. For the biofilm assay, even at the altered ratios, outgrowth was still experienced at the 30th generation for some conditions (No streptomycin, 1:24xMIC, 1:16xMIC and 1:2xMIC). For future assays, it would be necessary to adjust the ratios even more.

There are also some data points in the graph (figure 8) that are misrepresentative: 12 mg/L (1:2 x MIC) and 6 mg/L (1:4 x MIC). Both of them seem to be out of order in comparison to the other measurements. For the higher concentration, it might be that the sensitive strain was wiped off to fast, resulting in the measurements performed on the 20th and 30th generation being not reliable. The lower concentration should not create a problem, but it seems unlikely that 6 mg/L and 3 mg/L have the same fitness cost. It should increase with the concentration. Although, since both concentrations have the highest standard deviation, new measurements need to be performed to draw any conclusions.

However, the preliminary results indicate that the MSC value for the biofilm assay seems to be around 2.5 mg/L, almost the same value as for the planktonic assay. This means that, potentially, the MSC values do not change between biofilm and planktonic form. Since biofilms have increased defence mechanisms against antibiotics, it is remarkable that the mutants living as biofilms have MSC values that are about the same (even possibly lower) than the planktonic cells. Repeating the assay with some optimizing modifications will hopefully prove if these preliminary results stand true.

4.3 Ciprofloxacin

Previous data executed by Fernberg. 2018 on biofilm showed that the fitness cost for *E coli* CFT073 mutant *gyrA* S83L is 2.5%. This is in line with the data from a study by Knopp and Andersson from 2018 were they determined the fitness cost of *Salmonella* Typhimurium LT2 to be below 3%. In this study we wanted to confirm the obtained MSC value by adding concentrations of ciprofloxacin over and under s=0, and get a more precise value of were the graph intercept the axis.

The assay was performed according to plan, but there were some problems evaluating the obtained data. Some of the concentrations had great variation between all of the

replicates, and some had large differences between the dye-swapps. This resulted in that data had to be excluded for us to be able to construct a graph of the s-values, meaning that no conclusions can be drawn from it. Why this happened is not clear, but one factor that probably is important is the low fitness cost of the mutation. This means that even the smallest variation between the replicates obtained at the same concentration will create a too large difference when combined since the margins are so small, and even the slightest miss-shape of the curve will have a great effect on the resulting data.

The experiments with the *gyrA* mutants were also performed in two separate assays, one for the DA66036/DA58420 and another independent assay for the dyeswapp, DA66037/DA58419. For the *rpsL* mutants, both combinations were performed at the same time. The assays being performed at different time points should not matter, but it might be an explanation to why the results between the dyeswapps differ for some of the concentrations, some variation in the way the samples were handled.

Regardless of the reason, the obtained data results in a graph were it is not possible to determine the MSC, not even after removal of the odd looking measurements. Not even the data from the three concentrations closest to the assumed MSC, see figure 9, produce a useful graph. The concentration were the graph intercepts with the axis is too low to be of statistical significance, at least in relation to the size of the variations between the measurements that the graph is based on.

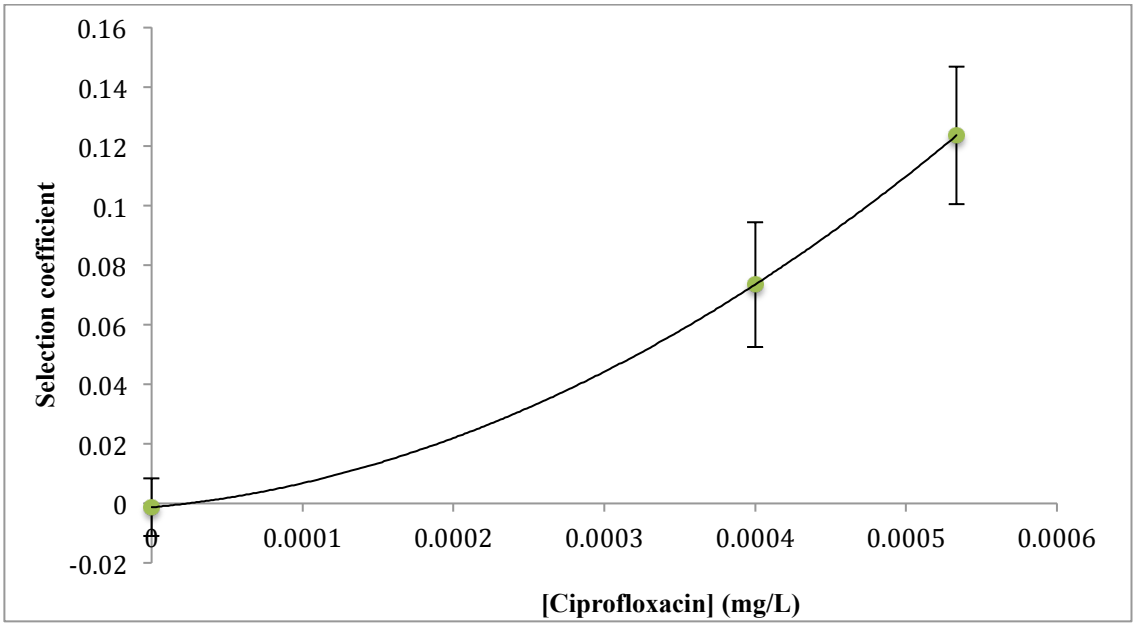


Figure 9. Biofilm assay. The s-values obtained using CompDAta as a function of the ciprofloxacin concentrations. In this graph, only the three concentrations closest to the interception point is presented.

Since the data is so inconclusive, and due to the fact that measurements had to be removed, we can only use it as a point of reference for further experiments.

5. The future

5.1 Mutant strains

During this project, we successfully constructed a pair of mutant strains resistant against fosfomycin, DA66040/DA66041. But before initiating competition assays, we want to whole genome sequence both strains to make sure that they are isogenic to the susceptible strain except for the mutation we introduced. Since it turned out to be hard to introduce a STOP-codon in *nfsA*, it might be worth trying to knock out the gene completely, and see if that gives better results.

5.2 Competition assays

5.2.1 Streptomycin

Determination of the MSC value for the streptomycin resistance mutants, DA66038/DA66039, in planktonic form was performed and measured to 3 mg/L (1/8 MIC). To enable measurements of the 30th generation, the starting ratios of susceptible:mutant strain had to be adjusted, an alteration we brought with us to the biofilm assay. Despite that, even with the altered ratios, outgrowth was still experienced at the 30th generation for some conditions and data had to be removed to produce a graph. This means that we can only draw preliminary conclusions from this experiment, and we can use the results when designing a new biofilm assay setup. The ratios must be altered even more, and new concentrations needs to be chosen closer to the estimated MSC value.

5.2.2 Ciprofloxacin

The biofilm assay performed with the ciprofloxacin resistant mutants, DA66036/DA66037, were based on results from a previously performed assay with the same mutants (Fernberg. 2019). The data generated from this study is inconclusive since some measurements needed to be removed to produce a graph and therefore it is only preliminary. Although, when performing this assay once more, these data together with the data generated by Fernberg can serve as reference points, and comparisons should definitely be made to see if there are any correlations between the different assays.

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Appendix

Table A1. Oligos and primers used in construction of mutants, PCR and sequencing reactions.

Oligo/ Primer	Sequence (5' to 3')	Description
uhpT	TTCGAGCGGAAGGTCCAGGG TCGGCTTGCGAACCTGGTTTt AGAAAGCCAGCATGGGTTAC TCCTGAAATGAATACCTGC	Oligo used for the construction of a mutation in the gene *uhpT* resulting in resistance against Fosfomycin
uhpT-F	TGGCCCGCAGATGTTAAT	Forward primer used for PCR of the gene *uhpT*
uhpT-R	TGGCAGACAGGATCAGCA	Reverse primer used for PCR of the gene *uhpT* and in the sequencing reaction
nfsA	TGTTAATAATCGCCTCACGCT GCGCTTCGGAAATGGGTTAA TCAGTGAAATGGCGAATGGA GCGATGGCCACAAATAAGT	Oligo used for the construction of a mutation in the gene *nfsA*, one of the stepwise mutations for resistance to Nitrofurantonin
nfsA-F	ACCCGGACGACCAAAAAT	Forward primer used for PCR of the gene *nfsA* and in the sequencing reaction
nfsA-R	AAGGCACAGCCCAAACAG	Reverse primer used for PCR of the gene *nfsA* and in the sequencing reaction
nfsB	TTTCACATGGAGTCTTTATGG ATATCATTTCTGTCGAGCGTC ATTCCACTAAGGCATTTGATG CCAGCAAAAAA	Oligo used for the construction of a mutation in the gene *nfsB*, one of the stepwise mutations for resistance to Nitrofurantonin
nfsB-F	GCGAGGCATCAAGCATTT	Forward primer used for PCR of the gene *nfsB* and in the sequencing reaction
nfsB-R	GAAACGCCTGGCTCTTGA	Reverse primer used for PCR of the gene *nfsB* and in the sequencing reaction

Table A2. PCR thermal cycling conditions

Step	Temperature	Time	Number of cycles
Initial denaturation	95°C	4 minutes	1
Denaturation	95°C	30 seconds	
Annealing	55°C	30 seconds	30
Extension	72°C	1 minutes	
Final extension	72°C	7 minutes	1