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Collateral sensitivity in clinical *Escherichia coli* isolates resistant to ciprofloxacin and/or mecillinam

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// ABSTRACT

Background

The escalating emergence of AMR is a growing public health concern, and a result of the use and misuse of antibiotics since its introduction. To prevent the development of resistance and preserve the efficacy of antimicrobial agents, new treatment strategies is of utmost importance. One possible approach may be to take advantage of collateral sensitivity, a phenomenon where bacteria acquiring resistance to one antimicrobial drug simultaneously became more sensitive to others. Our aim in this project is to investigate the generality of collateral sensitivity in clinical urinary tract isolates of *E. coli*. In addition, we wanted to investigate effects of the mutations on biofilm forming ability of the resistant mutants.

Methods

In this study we generated mecillinam and/or ciprofloxacin resistant mutants in clinical isolates of *E. coli.* The generated mutants were further characterized by determining IC_{90} values to investigate how the selection for resistance may have affected their susceptibility to six other antimicrobials. The results were eventually displayed in heatmaps. Biofilm forming ability of the wild-type strains and resistant isolates was investigated using a standard microtiter plate assay, detecting biofilm by crystal violet staining.

Results

Our results show that the resistant isolates of ciprofloxacin and mecillinam demonstrated different collateral sensitivity and cross-resistance effects in clinical isolates of *E. coli*. The isolates being resistant to both antibiotics tend to be largely dominated by the cross-resistance effects seen for the isolates resistant to ciprofloxacin alone. Our results also show the resistant isolates generally are not producing much biofilms

Conclusion

Based on our *in vitro* results, we suggest that mecillinam is an applicable drug to use in the first-line treatment of UTIs. We also suggest that ciprofloxacin should be used prudently due to the risk of resistance development to several other drugs.

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ABBREVATIONS

AI	Autoinducer
AMR	Antimicrobial resistance
AST	Antimicrobial susceptibility testing
AZT	Azithromycin
BHI	Brain heart infusion
CFU	Colony forming units
CHL	Chlormaphenicol
CIP	Ciprofloxacin
CR	Cross-resistance
CS	Collateral sensitivity
dH_2O	Destilled water
DNA	Deoxyribonucleic acid
E. ccli	*Escherichia coli*
e.g.	For example
EPS	Extracellular polymeric substances
E-test	Epsilometer-test
EtOH	Ethanol
EUCAST	European Committee on Antimicrobial Susceptibility Testing
ExPEC	Extra-intestinal pathogenic *Escherichia coli*
GEN	Genatmicin
HCl	Hydrochloric acid
IC_{90}	Inhibition concentration 90
IPEC	Intestinal pathogenic *Escherichia coli*
LB	Luria Broth
LBA	Luria Broth agar
M9	Minimal salt
MALDI-TOF	Matrix Assisted Laser Desorption Ionization Time-of-Flight
MEC	Mecillinam
MH	Mueller Hinton

MHA II	Mueller Hinton agar II
MIC	Minimum inhibitory concentration
NaCl	Sodium chloride
NAG	*N*-acetyl glucosamine
NAM	*N*-acetyl muramic acid
NorPD	Norwegian Prescription Database
PBP 2	Penicillin binding protein 2
QRDR	Quinolone resistance determining regions
QS	Quorum sensing
R	Resistance
RNA	Ribonucleic acid
Rpm	Rotations per minute
S	Sensitive
TFA	Trifluoroacetic acid
THF	Tetrahydrofolate
TMP	Trimethoprim
TSB	Tryptic soy broth
TSBglu	Tryptic soy broth with 1% glucose
UNN	University Hospital of Northern Norway
UPEC	Uropathogenic *Escherichia coli*
UTI	Urinary tract infection
WT	Wild type

1 INTRODUCTION

1.1 Preface

The discovery of penicillin by Alexander Fleming in 1928 has been recognized as one of the greatest scientific achievements in the late 19th century. The introduction of antimicrobials represented a revolution in the field of medicine, as countless lives have been saved from a variety of infections [2]. Despite knowledge about antimicrobial resistance (AMR) since the dawn of the antibiotic era an escalating emergence of resistant bacteria, coupled with a diminished interest in the discovery of novel drugs, has made "superbugs" a threat to public health worldwide [3].

The rapid emergence of AMR is an inevitable outcome of antimicrobial use and misuse in health-care and agriculture, as well as the release of antimicrobials into the environment. Bacteria either possess innate characteristics making them resistant to certain antibiotics, or initially susceptible bacteria may develop different survival mechanisms to resist selective antimicrobial pressure [4]. The evolution and spread of multidrug-resistant bacteria are associated with increased mortality and morbidity, attributed to an estimated 25 000 deaths and at least €1,5 billion in extra health care costs annually in Europe alone [5]. Moreover, in addition to the limiting management of infectious disease, the burden of resistant bacteria will also have a detrimental effects on medical procedures such as chemotherapy, surgery, haemodialysis and organ transplantations, since antimicrobial therapy is a prerequisite for the success of these procedures [5].

Despite the urgent need for new drugs, several factors have led to a flagging interest in antibiotic development by the pharmaceutical industry [3]. While awaiting the development of novel treatment options, implementing antimicrobial stewardship programs focused on prudent the use of currently available antibiotics is of importance to prolong the life-span of remaining effective agents [6]. In 2013 Imamovic and Sommer proposed a novel drug cycling program focusing on collateral sensitivity as a promising treatment strategy to combat AMR [1]. Although this phenomenon, where bacteria acquiring resistance to one antimicrobial

also become more susceptible to others, was first described back in 1952 [7], it has been largely disregarded within microbiology until recently.

In this project, the generality of collateral sensitivity (CS) and cross-resistance (CR) profiles will be investigated in clinical *Escherichia coli* isolates with laboratory-selected resistant to mecillinam and ciprofloxacin, as well isolates resistant to both antimicrobials. Additionally, we investigate if the mechanisms involved in the observed resistance in the mutants affect the ability of the isolates to form microbial biofilms.

1.2 Antibacterial agents

Antibacterial agents, more commonly referred to as *antibiotics*, are a group of chemical substances used to treat bacterial infections. The great success of these drugs is owed much to their ability to selectively target bacterial cells, either by inhibiting growth of the microorganisms (bacteriostatic) or by killing them (bactericidal), while having minimal effects on the host cells and tissues. Further, these agents can be categorized depending on the range of bacterial species that they are effective against; traditional broad-spectrum agents usually act against a wide range of bacteria of both Gram-negatives and Gram-positives, whereas narrow-spectrum agents only target specific types bacteria.

Most antimicrobials can be classified according to their principal mode of action. The four main groups interfere with one of the following essential processes in bacteria; cell wall synthesis, protein synthesis, nucleic acid synthesis or folate synthesis.

1.2.1 Drug target - Cell wall

The bacterial cell wall is composed of the polymer peptidoglycan. Peptidoglycan is present only in species belonging to the domain of *Bacteria*, making the cell wall an important antimicrobial target [8]. The peptidoglycan cell wall is found in both Gram-negative and Gram-positive bacteria, protecting the bacterial cell against osmotic pressure that can lead to cell lysis [8].

The late steps in cell wall formation involve the cytoplasmic synthesis of building blocks composed of *N*-acetyl muramic acid (NAM) linked to *N*-acetyl glucosamine (NAG) with an attached pentapeptide side chain [8, 9]. Penicillin binding proteins (PBPs), perform extensive cross-linking of the peptide side chains, thereby producing the mature, lattice-like peptidoglycan layer [8, 9]. β-lactams (e.g. mecillinam) are presumably the most well-known cell wall inhibitors. These antimicrobials interact directly with different types of PBPs, thus preventing the bacterial cell from maintaining its shape and osmotic stability [9]. Vancomycin, a glycopeptide antimicrobial, is another cell wall inhibitor that, although binds to a target different from the β-lactams, blocks the same step by producing steric hindrance to transpeptidase action [9].

1.2.2 Drug target - Protein synthesis

The vast majority of proteins are either catalytic (enzymes) or structural proteins, both are vital components for the bacterial cell and essential for cellular function. Ribosomes synthesize proteins by building chains of amino acids in specific sequences [8].

The prokaryotic ribosome consists of the 30S and 50S ribosomal subunits and is structurally different from the eukaryote ribosome [8]. Several classes of antimicrobials agents interfere with bacterial protein synthesis by targeting one or both of the subunits [10]. Aminoglycosides exhibit a bactericidal effect by binding to the 30S subunit, causing misreading of the genetic code [10]. Tetracycline also binds to the 30S subunit, but since its binding is transient, it exhibits bacteriostatic effect [10]. Macrolides bind to the 50S subunit and can have either a bacteriostatic and bactericidal effect, while chloramphenicol binds reversibly to the 50S unit and has a bacteriostatic effect [10].

1.2.3 Drug target - Nucleic acid synthesis

Nucleic acids, which include DNA and RNA, are genetic material that is essential in all living organisms. Antimicrobials can interfere with specific processes in synthesis of nucleic acid at several levels. Fluoroquinolones (e.g. ciprofloxacin) interfere with type 2 topoisomerases, inhibiting DNA-synthesis [9]. Another example are rifamycins, which specifically inhibit RNA-synthesis [10].

1.2.4 Drug target - Folate synthesis

Tetrahydrofolate (THF) is a cofactor involved in the synthesis of DNA, RNA and bacterial cell wall proteins [10]. The cellular requirement of folates is universal, but prokaryotes differs from mammalian cells in that they commonly lack a transport system for the uptake of folic acid from the environment. The bacterial necessity to synthesize folates makes it an applicable target for antimicrobials [11]. Trimethoprim and sulphonamides are antimicrobials inhibit different enzymes in the biosynthesis of THF and….,respectively [10].

1.3 Antimicrobial resistance

Shortly after the introduction and widespread use of antibiotics in the 1940s, there were reports of microbial evolution in response to antimicrobial exposure, where the bacteria were able to resist the inhibitory effects of the newly discovered drugs. At the end of Alexander Flemings Nobel Lecture in 1945, he mentions the danger of antimicrobial misuse- *"It is not difficult to make microbes resistant to penicillin in the laboratory by exposing them to concentrations not sufficient to kill them, and the same thing has occasionally happened in the body"* [12]. Despite warnings, from both Fleming and other scientists, throughout history the inappropriate use of antimicrobials has accelerated bacterial evolution. and made Antimicrobial resistance more frequently observed, which complicates the treatment of infections [3].

1.3.1 Mechanisms of antimicrobial resistance in bacteria

Antimicrobial resistance (AMR) can be broadly divided into intrinsic resistance and acquired resistance. Intrinsic resistance is a naturally occurring trait due to biological characteristics of a microorganism, that make all strains of that species likewise resistant to specific antibiotics [13]. Alternatively, acquired resistance may arise in an initially susceptible bacterium, either through the acquisition of new genetic material or through mutations to the chromosomal DNA [13]. As microbes adapt to their environment random mutations may give them new abilities, such as enzymatic modification of antimicrobials, alteration of drug target site, impermeability reducing drug uptake and increased efflux pump expression, which can

reduce the effect of antimicrobial agents [4]. More specific resistance mechanisms will be described below for the model organism used in this study, *E. coli.*

1.3.1.1 Mutations

A mutation is caused by a change in the nucleotide sequence of the DNA in an organism's genome. Thus, a mutant will differ from its parental strain in its genotype, the sequence of the genome [8]. Depending on the mutation, the observable characteristics of a mutant, its phenotype, may or may not differ from its parent. There are several types of mutations (discussed below) and their phenotypic effects are highly variable, being neutral, beneficial or harmful [8]. Mutations can either be induced or spontaneous; induced mutations arising after for example exposure of mutagens, while spontaneous mutations occur without external intervention, usually from rare errors made by polymerases during DNA replication that are not repaired [8].

Point mutations are mutations that affect one single base pair. These changes can occur by single nucleotide base substitutions in the DNA [8]. Further, these mutations can be divided into two subtypes describing the type of base substitution; transition and transversion. Transitions are mutations in which one purine base (A or G) substitutes for another purine, or one pyrimidine base (C or T) substitutes for another pyrimidine [8]. For transversions, a purine base substitutes for a pyrimidine base or vice versa [8]. Point mutations can also arise by the loss or gain of a single base pair (deletions or insertions), which lead to a shift in the reading frame that will affect the amino acid sequence, likely resulting in alteration of the protein function [8]. Deletions and insertions may involve a single base-pair, or result in the loss or gain of hundreds or even thousands of base pairs [8].

There are several functional consequences of point mutations. A silent mutation is defined as a mutation that does not affect the phenotype of the cell [8]. Because a silent mutation does not lead to alteration in the amino acid sequence, these mutations are also referred to as synonymous mutations [8]. When a codon for one amino acid is replaced by a codon for another amino acid, it's called missense mutations [8]**.** Finally, a nonsense mutation is where the codon for one amino acid is replaced by a translation termination (stop) codon [8]. Because both missense and nonsense mutations change the protein sequence, they are referred to as non-synonymous mutations[8].

1.3.1.2 Vertical and horizontal gene transfer

Vertical gene transfer is the transmission of genes from a bacterium to its daughter cells during DNA replication and cellular division. AMR mechanisms that are on the genome are transferred vertically [8]. However, microorganisms may also acquire resistance genes by horizontal gene transfer (HTG); transformation, transduction and conjugation [8].

Transformation is a genetic process where the lysis of a donor cell causes the release of free DNA, which can be taken up by a competent recipient [8]. The recipient may then incorporate the foreign DNA into its own genome. This may include AMR genes, making the recipient cell resistant as well [8].

Transduction involves the transfer of genes by a bacteriophage from one cell to another [8]. When a bacteriophage infects a bacterial cell, the enzymes responsible for packaging viral DNA into the bacteriophage protein capsid sometimes package part of the host DNA as well [8]. Once the bacteriophage host cell is lysed, the bacteriophages can inject their packaged genetic material into a new bacterial cell, thus potentially spreading resistance genes [8].

Conjugation is a mechanism of genetic transfer mediated by cell-to-cell contact through a pilus [8]. The bacterial donor cells transfer copies of mobile genetic elements, such as plasmids and transposons, to the recipient, which is then able to maintain them [8].

1.3.2 Fitness – the cost of antimicrobial resistance

Although the development or acquisition of antimicrobial resistance mechanisms constitutes an advantage in favour of the microorganisms, these genetic changes usually cause a reduction in growth rate, competitive ability and/or virulence, referred to as a fitness cost [14]. Due to the complexity of factors influencing fitness, the precise mechanisms behind this phenomenon are not fully understood. However, studies show that the cost of resistance is highly variable. Since the genetic mechanisms of resistance often affect important biological functions in the cell, they may play a significant role on the fitness of the cell. It is also suggested that resistance obtained by chromosomal mutations tends to carry a much larger cost than resistance acquired from plasmids [14].

1.4 The Norwegian National Strategy Against Antibiotic Resistance 2015-2020

From 2005-2012 an increased use of antibiotics in Norway was observed. However, the report *Drug Consumption in Norway 2012-2016* from March 2017, shows a steady decline in the use of antimicrobials [15]. In order to continue this trend, in June 2015 the Norwegian Government established a national strategy against AMR that will hopefully reduce the unnecessary consumption of antibiotics by 30% by 2020, as measured in DDD/1000 inhabitant/day, as compared to the 2012 consumption. The major goal of this initiative is to be among the top three countries in Europe with the lowest use of antibiotics in humans [16].

In healthcare, around 80% of all antibiotic prescriptions occur in general practice. Due to the high volume of antibiotic prescribed in this sector, a great potential exists to reduce the consumption of antibiotics, especially when treating respiratory infections in children and acute uncomplicated UTIs in women. In hospitals, representing 9% of all prescriptions, the increased use of broad spectrum antibiotics appears to be the biggest challenge, as changes in resistance patterns cannot explain this rise alone [16].

Interventions described in the strategy against AMR focus on various preventative measures such as vaccination of the population and hygiene improvements, as well as different efforts to better educate prescribers and the general public about antibiotics, which may lead to more appropriate use of these drugs. Some of the actions steps mentioned in the strategy address issues that are well known to pharmacists. The first involves the mismatch between antibiotic packaging and recommended duration of treatment for several antibiotics with marketing authorization in Norway. Pivmecillinam (Pivmecillinam®, Selexid®) for example, is only used for the treatment of UTIs and, is according to statistics from the Norwegian Prescription Database (NorPD) the antibiotic with the second highest numbers of users Norway in 2015 [17]. When a standard Pivmecillinam treatment of 200 mg 3 times daily for 3 days is prescribed, the smallest package of 20 tablets must be dispensed, which result an additional 11 tablets being provided to the user. The consequence is the potential for over- and misuse of the drug [16]. A similar problem involves the lack of antibiotic drug formulations, especially in the treatment of respiratory infections in children. The unpleasant taste of penicillin-mixture formulations may complicate the administration of the

drug and, due to the lack of good alternatives on the market, may lead to the prescription of broad-spectrum antibiotics with a better taste. Although narrow-spectrum penicillin is first line treatment for respiratory infections, statistics from NorPD shows that 30-50% of children are using broad spectrum antibiotics to treat these infections[16] [18]. The last issue concerns the 1 year validity of antibiotic prescriptions, which is the same as for other prescribed drugs. According to the Institute of Public Health, a large number of prescriptions may be dispensed from the pharmacies much later after it was prescribed to patients having acute infections [16].

1.5 *Escherichia coli*

The bacterial species *Escherichia coli* is a rod-shaped, motile Gram-negative in the *Enterobacteriaceae* family [19], and the model organism in this study. This facultative anaerobe play a predominant role in the intestinal microbiota of humans and other mammals, usually deriving mutual benefits to its host [19, 20]. However, *E. coli* is a highly versatile species, providing the potential to cause a broad spectrum diseases of varying severity [20]. Among the most common infections caused by *E. coli* are urinary tract infections (UTIs), diarrheal diseases and bloodstream infections [20].

The ability of *E. coli* to overcome host defenses and colonize in different niches is due to the acquisition of specific virulence factors, resulting in pathogenicity. Depending on their site of infection, pathogenic *E. coli* strains can be categorized as intestinal pathogenic *E. coli* (IPEC) or extraintestinal pathogenic *E. coli* (ExPEC), and are further subdivided into several distinct pathotypes [20]. These are groups of strains that among other characteristics, such as phylogenetic background and disease manifestation, exhibit unique combinations of certain virulence traits. Example of such virulence attributes are specific adherence factors and toxins [20].

1.6 Biofilms in *E. coli*

Bacterial cells may be capable of anchoring to a variety of materials, aggregate, and form clusters of colonies embedded in an adhesive matrix secreted by the cells themselves. This

mode of bacterial life is termed a "biofilm". Biofilm formation allows bacterial cells to remain in a convenient niche where they live in close proximity with each other, thus improving their likelihood of survival (Brock).

E. coli biofilms consist of a secreted matrix of polysaccharides called extracellular polymeric substances (EPS) [21]. The biofilms in *E. coli* mainly consists of three exopolysaccharides: colonic acid, cellulose and β-1,6-N-acetyl-D-glucosamine polymer (PGA) [21]. The cell-to-cell communication within the biofilm occurs via the chemical signaling pathway known as quorum sensing (QS); a process where microbes secrete autoinducer (AI) substances to the extracellular environment until the required high density is achieved and, thereafter, factors affecting biofilm formation and maturation are upregulated [21].

Biofilm formation provides protection to the bacteria against several factors such as host-defense mechanisms, phagocytosis, biocides and hydrodynamic shear forces, as well as increasing the bacterial tolerance to antimicrobial agents, hampering their efficacy [21, 22]. Besides being the main causative agent for recurrent urinary tract infections, *E.coli* biofilms are also associated with infections related to indwelling medical device-related infectivity, such as urethral and intravascular catheters, prosthetic joints and shunts and grafts, which can potentially leading to more serious infections [21]. It is estimated that 65% of human bacterial infections involve biofilm formation [23]

1.7 Urinary tract infections

The urinary tract includes the kidneys, ureters, urinary bladder and the urethra. This organ system exhibits various factors that contribute to maintain a sterile environment. These defense properties include mechanistic factors, such as regular bladder emptying and urine flow, as well as the chemical-defense components of epithelia and, upon bacterial invasion, epithelial shedding and influx of effector immune cells [24, 25].

However, host defense mechanisms are frequently outcompeted by microbial invasion from the exterior environment. UTIs arise when microorganisms, especially bacteria, manage to colonize within the urinary tract and are among the most common infections in the world [26]. UTIs are estimated to affect 130-175 million people annually [26]. These infections are

overrepresented among women due to the anatomic proximity of bowel flora to the short urethra. Clinically, UTIs are classified both by the location of the infection in the lower (cystitis) or upper (pyelonephritis) urinary tract, and as uncomplicated or complicated. Renal disease, diabetes and catheterization are some of the risk factors that may complicate the treatment of UTIs, increasing the cost of treatment, morbidity and mortality [27].

1.7.1 Uropathogenic *E. coli*

Although UTIs can be caused by several different pathogens, uropathogenic *E. coli* (UPEC) strains are the most predominant causative agent, and are responsible for 70-95% of community acquired UTIs [28]. This pathotype harbors a range of diverse virulence factors that contribute to the colonization of the urinary tract, thus causing infection.

Adhesins, such as Type 1-fimbriae and P fimbriae, are assembled on the surface of the bacteria and adhere to receptors of the host urothelial cells. These virulence factors are probably the most important determinant in the pathogenicity of UPEC [28, 29]. UPEC may also secrete toxins that cause damage of the epithelial cells, such as hemolysin, cytotoxic necrotizing factor 1, and autotransporter toxins [29]. Other virulence factors are flagella-mediated motility, evading host defenses and creating biofilm-like structures of intracellular bacterial communities [29].

1.7.2 Treatment of UTIs

According to Norwegian guidelines from the Directorate of Health, the recommended first-line treatment of acute complicated and complicated cases of cystitis are trimethoprim, nitrofurantoin and pivmecillinam [30]. Depending on the severity of the infection, whether the infection is uncomplicated or complicated, the duration of treatment may vary from 1-3 days or 5-7 days. Ciprofloxacin and amoxicillin may be suitable drugs of choice in complicated cystitis where resistance to conventional treatments is observed [30].

According to statistics from the NorPD, pivmecillinam is the most frequently prescribed antibiotic for the treatment of UTIs in Norway [17]. The statistics also indicate, as mentioned above, a considerably higher infection rate in women than men (shown in **Table 1**).

Table 1. Antimicrobials dispensed from pharmacies in Norway. Data from the Norwegian Prescription Database from 2011-2015, showing anyone who has had at least one of the listed antibiotics dispensed from a Norwegian pharmacy per year.

	NUMBER OF INDIVIDUALS					
Drug	**2011**	**2012**	**2013**	**2014**	**2015**	**Women (%)**
Mecillinam	189 598	193 934	200 464	203 619	200 771	**87**
Trimethoprim	83 347	80 744	76 689	76 400	72 115	**85**
Nitrofurantoin	36 784	36 250	36 821	36 878	36 514	**85**

1.7.3 The ECO·SENS study – *E. coli* and UTIs

The ECO·SENS studies were conducted in periods between 1999-2000 and 2008-2009, and investigated the prevalence of AMR and antimicrobial susceptibility among frequently used antimicrobials in uropathogenic *E. coli* isolates from urinary tract infections (UTIs) in women [31, 32]. The isolates used in this study are derived from the ECO-SENS collections.

The first ECO-SENS study investigated samples from 16 European countries and Canada [31]. The second study (ECO·SENS II) tested isolates from only five selected countries; Austria, Greece, Portugal, Sweden and the UK, representing different geographical areas in Europe suggested to have more or less frequent antimicrobial resistance [32].

ECO·SENS II confirmed the findings from the first study; where *E. coli* was the most prevalent uropathogen in all of countries. Additionally they found that the consumption and emergence of multidrug-resistant bacteria was higher in Southern Europe [32]. Lastly they observed a continuing high prevalence of resistance in community uropathogenic *E. coli* to ampicillin, sulfamethoxazole, trimethoprim and sulfamethoxazole/trimethoprim combination, while resistance-levels to amoxicillin/clauvanic acid, mecillinam, fosfomycin and nitrofurantoin did not show significant increase as compared to the first ECO-SENS study [32]. For instance, the prevalence of ciprofloxacin resistance increased from 1,1% (1999-2000) to 3,9% (2008-2009), while mecillinam resistance only increased from 0,9% to 1,6% [32].

1.8 Antibacterial agents of interest

Due to the central roles of mecillinam and ciprofloxacin in the treatment of uncomplicated and complicated UTIs respectively, these two drugs were chosen as the main focus in our study. Their mechanisms of action and resistance are described in further detail below.

1.8.1 Mecillinam

Mecillinam, an amdinopenicillin discovered in the 1970s, is an extended-spectrum β-lactam antimicrobial showing high specificity to penicillin-binding protein 2 (PBP2) in the Gram-negative cell wall [33]. Due to its potent antibacterial activity against *Enterobacteriaceae,* especially *E. coli*, the drug is mainly used in the treatment of uncomplicated UTIs. The extensive long-term use in Scandinavian countries shows that mecillinam is a well-tolerated drug with a beneficial resistance profile [33].

Because of the poor gastrointestinal absorption of mecillinam, the prodrug pivmecillinam was introduced to make the drug appropriate for oral administration [34] Enhanced lipophilicity and increased bioavailability was possible by introducing an extended double-ester that masked the polar carboxyl group in mecillinam [35]. This improved the ability of the drug to cross hydrophobic cell membranes in the gastrointestinal tract. Once it enters the bloodstream, it undergoes enzymatic hydrolysis by ubiquitous esterases that release the active antibiotic in the body (**Figure 1**) [35].Following absorption, high concentrations of active mecillinam are excreted in the urine, which according to studies may reach several hundred mg/litre during treatment [36]. When it reaches its target site, mecillinam binds to and inhibits the transpeptidase activity of PBP2, prevents the cell wall elongation of rod-shaped cells, leading to cell death [37].

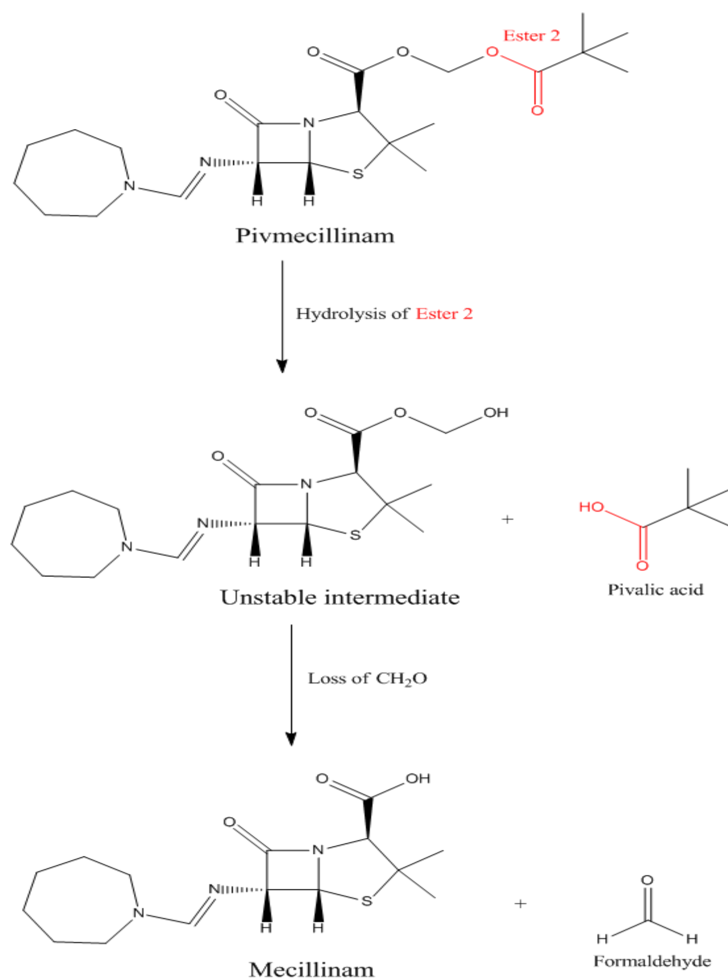

Figure 1: Mechanism of pivmecillinam (prodrug) to the active drug mecillinam.

1.8.1.1 Mechanisms of resistance to MEC in *E. coli*

Antimicrobial resistance to mecillinam can arise by DNA mutations in a wide range of genes; there are at least 38 mutational targets known [37]. These genes are involved in various cellular functions in bacteria such as the respiratory chain, cysteine biosynthesis, tRNA synthesis, the ribosome and pyrophosphate metabolism [37].

Since mecillinam is a β-lactam antimicrobial, it can be inactivated by β-lactamase enzymes produced by bacteria. These enzymes are hydrolyzing the amide bond in the β-lactam ring [38]. The loss or functional change in the outer membrane porins can also result in mecillinam resistance. In *E. coli*, changes in porins such as OmpC, OmpF and PhoE can lead to resistance to β-lactams [39]. Mecillinam resistance may also arise due to modification of

drug target site, PBP2, which is responsible for the elongation of rod-shaped cells. For example, some of these mutations are causing elevation of the signal for the stringent response, ppGpp, which may render the target of PBP2 [37]. At last, the over-expression of efflux-pumps that are pumping the drugs out of the cell can arise, hampering the effect of the drug [40]. Example of such pumps are AcrAB-TolC and AcrAD-TolC [40].

1.8.2 Ciprofloxacin

Ciprofloxacin, a member of the quinolone class of antimicrobial agents, is a broad-spectrum antimicrobial that has bactericidal activity against both Gram-negative and Gram-positive bacteria [41]. As a second generation quinolone, developed in 1981, it differs from the first generation quinolones by the introduction of fluorine, piperazine and a cyclopropyl substituent in its structure **(Figure 2),** leading to improved cellular uptake and broad-spectrum activity, but also reduction of adverse effects [42].

Ciprofloxacin interferes with two type 2 topoisomerases, both of which are essential for bacterial DNA replication; DNA gyrase and topoisomerase IV. Both enzymes are composed of subunits, encoded by *gyraA* and *gyrB* (for DNA gyrase) and by *parC* and *parE* (for topoisomerase IV) [43]. Ciprofloxacin interacts with these enzymes resulting inhibiting their activity at the DNA cleavage stage and preventing DNA strands from rejoining after DNA or RNA synthesis, which will eventually lead to cell death [43, 44]. The main quinolone targets are generally different in Gram-negative and Gram-positive bacteria, where for Gram-negative bacteria DNA gyrase is the main target, in Gram-positives it is the topoisomerase IV [45].

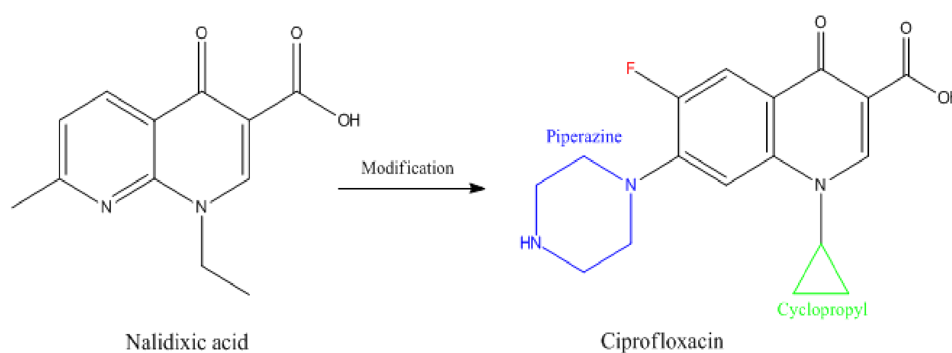

Figure 2: Chemical structures of nalidixic acid and ciprofloxacin

1.8.2.1 Mechanism of resistance to CIP in E. coli

Antimicrobial resistance to ciprofloxacin can arise via a combination of mechanisms, such as gene mutations causing alterations of the drug target enzymes, decreased membrane permeability and/or the production of protein that protect the drug. Ciprofloxacin resistance is acquired chromosomal mutations or plasmids [46].

Mutations in the genes encoding the two enzyme targets, DNA gyrase and topoisomerase IV, are frequently observed in target-mediated ciprofloxacin resistance. The major mutational target in *E. coli* is the DNA gyrase, either *gyrA* or *gyrB*. However, mutations in *gyrA* are more commonly observed [43, 45]. These mutations emerge in a DNA-sequence known as the quinolone resistance-determining region (QRDR) [43, 45]. In addition, mutations can be observed in the subunits *parC* and *parE* of topoisomerase IV, but mutations to *parE* seem to have negligible effect [45].

Ciprofloxacin resistance can also be acquired by changes in the membrane permeability. This can occur due to a decreased expression of porins, such as *ompF which* regulate influx, as well as the overexpression of efflux pumps, for example in SoxRS, regulating both *ompF* and the levels of certain efflux pumps [45]. Another example is chromosomal mutations in the MarRAB regulon that can lead to the expression of efflux pump systems, such as AcrAB-TolC. Mutations affecting the *marR* gene induce the constitutive expression of *acrAB* and *tolC*, leading to the efflux of multiple, diverse drug classes and the development of a multi-resistance phenotype [43, 45]. Ciprofloxacin resistance can also be acquired from plasmids. The *qnr* gene encodes a protective protein that prevents ciprofloxacin from binding to its target [47].

1.9 Important definitions in describing antimicrobial susceptibility

The performance of antimicrobial susceptibility testing (AST) is one of the most important tasks in clinical microbiology laboratories. Various techniques of *in vitro* susceptibility testing can be used to assess the susceptibility of microorganisms to certain antimicrobials, which is critical to inform the appropriate therapy choice in the case of bacterial infection [48].

There are different ways to perform AST, but the most well-known methods include broth dilution and antimicrobial diffusion assays. The broth dilution method, representing the gold standard of AST, is usually performed by adding bacterial inoculum to a 96 well plate containing a predetermined concentration of antimicrobials diluted in MH-broth [48]. Antimicrobial diffusion assays are performed by applying a gradient strip or a disk, impregnated with the antimicrobial at known concentrations, to an inoculated agar plate. After incubation, the zone of inhibition can be used to determine the susceptibility of the microorganisms [48].

AST is an essential part of this project. The gradient strip method was used to determine the minimum inhibitory concentration (MIC), defined as the lowest concentration of an antimicrobial drug required to inhibit the visible growth of a microorganism, thus confirming resistance to ciprofloxacin and/or mecillinam. The microbroth dilution method was used to determine the 90% inhibition value (IC_{90}) and describe collateral sensitivity and collateral resistance networks for the isolates.

1.9.1 EUCAST – Clinical breakpoints

To determine whether antimicrobial therapy will be successful or not, AST results require interpretive criteria to define the tested isolates as susceptible, intermediate or resistant. The European Committee on Antimicrobial Susceptibility testing (EUCAST), established in 1997, publishing clinical breakpoints with yearly updates to standardize the breakpoints across Europe [49]. These breakpoints are also used to confirm resistance in the mutants generated in this study.

1.10 Collateral sensitivity – a new strategy to combat AMR

Improvement of antibiotic treatment strategies is of utmost importance to prevent the development of resistance and preserve the efficacy of antimicrobial agents. One possible approach may be to take advantage of a side-effect in the evolution of antimicrobial resistance development, also known as collateral sensitivity (CS) [1].

Szybalski and Bryson first describes this phenomenon in a report investigating cross-resistance (CR). In addition to their expected observations of CR, they also observed the reverse phenomenon where bacteria acquiring resistance to one antimicrobial drug simultaneously became more sensitive to others, which they named CS [7]. Further investigations of CS have been absent until recently; when in 2013 Imamovic and Sommer published a similar study investigating changes in susceptibility due to AMR development [1]. They evolved resistance in *E. coli* to 23 clinically-relevant drugs and mapped the resulting network of CS and CR changes to each drug. Based on reciprocal CS the authors proposed a cycling scheme - collateral sensitivity cycling. The idea behind this drug cycling is to suppress the emergence of resistant bacteria and be able to reintroduce antimicrobials in the cycle, thereby increasing their life-span [1].

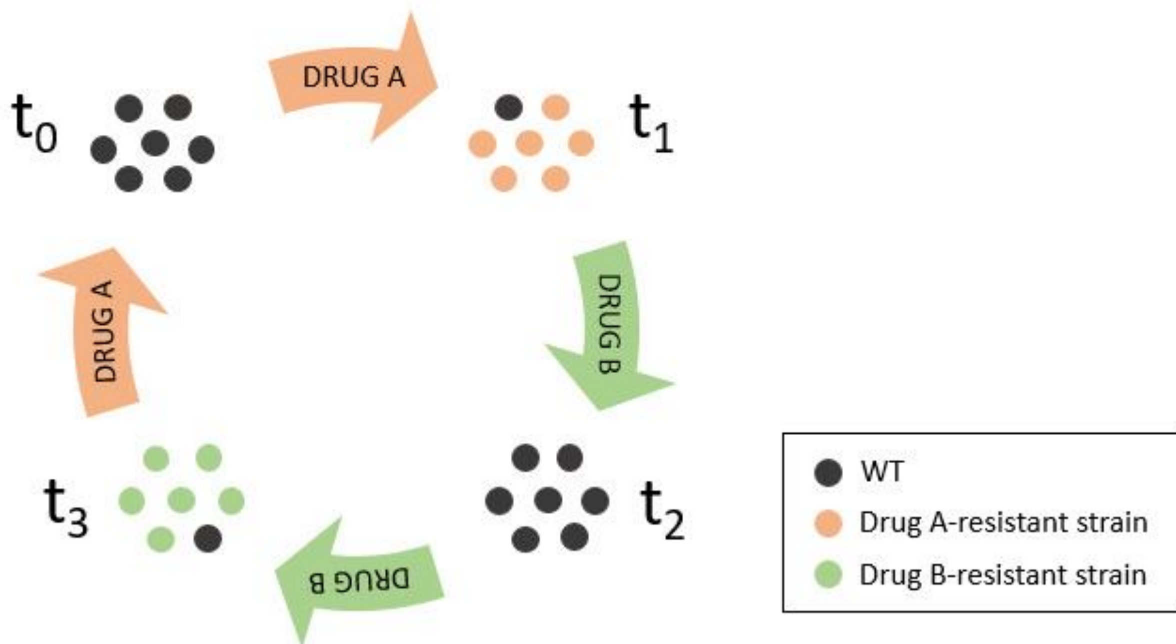

Figure 3: Collateral drug cycling. Model describing the main idea of collateral drug cycling. Adapted and modified from: [1]

A schematic of collateral sensitivity cycling is shown in **Figure 3**. At the start (t_0), a sensitive, pathogenic WT cell population (black circles) is exposed to treatment with drug A. After a given time of exposure, the population will develop resistance (orange circles) and the drug becomes ineffective (t_1). Following resistance development to drug A, the bacteria become collaterally sensitive to drug B. By switching treatment to drug B, the resistant bacteria are

killed at a higher rate than the WT population, who's sensitivity to drug A and B has not changed (t_2). Resistance to drug B (green circles) will then emerge (t_3), and treatment is then switched back to the initial treatment of drug A, to which drug B-resistant cells show collaterally sensitivity. The main idea is that the strain will be recycled back to its original sensitive phenotype.

1.10.1 Research focus of the MicroPop Research Group

In the MicroPop research group, collateral sensitivity profiles have previously been mapped for ten *E. coli* isolates resistant to mecillinam or ciprofloxacin, as shown in **Figure 4** and **Figure 5** (Podnecky et al., unpublished results). The red color represents increases in resistance, while the blue color represents increases in sensitivity.

For the ciprofloxacin-resistant mutants CR effects were more frequent than CS. However, CS observed in most isolates to the aminoglycoside gentamicin, as well as fosfomycin. Another interesting observation is the variation between the strains. While K56-78 has a lot of cross resistance, K56-2 strain show almost no changes in susceptibility at all.

In the mecillinam resistant mutants, there are much less trends and changes in susceptibility being observed. However, more collateral sensitivity and neutrals are seen compared to the ciprofloxacin mutants. The exception is for azithromycin, where almost all of the strains shows collateral sensitivity.

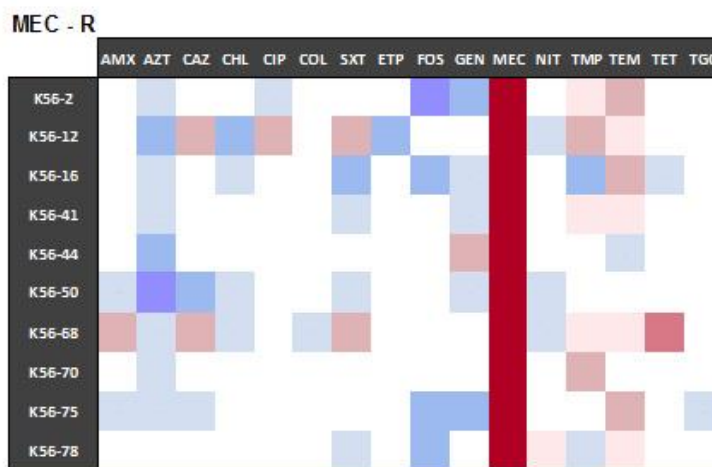

Figure 4: Collateral sensitivity network for mecillinam resistant isolates from the MicroPop research group.

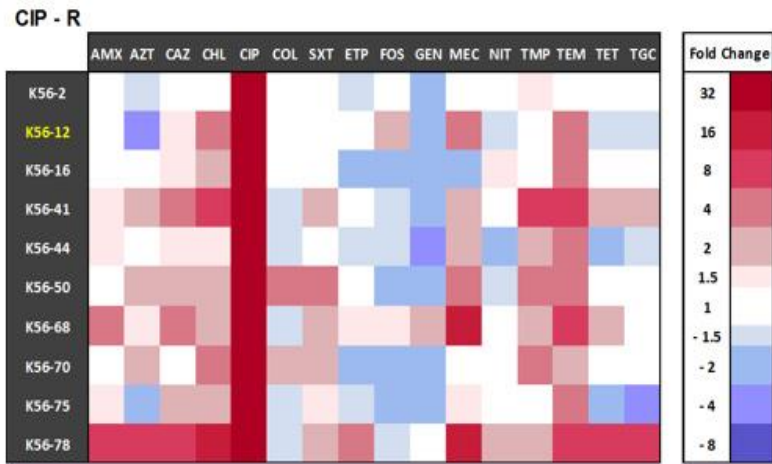

Figure 5: Collateral sensitivity network for ciprofloxacin resistant isolates from the MicroPop research group.

2 AIMS AND HYPOTHESIS

2.1 Aims

Our aim in this project is to investigate the generality of collateral sensitivity and collateral resistance in clinical urinary tract isolates of *E. coli* made resistant to mecillinam and/or ciprofloxacin, two important drugs in the treatment of UTIs. The results are mapped in heatmaps and compared to earlier observations in the MicroPop research group and the literature. In addition, we will investigate effects of the mutations on biofilm forming ability of the resistant mutants.

2.2. Hypothesis

Our main hypothesis is that collateral sensitivity networks exist on a population level. We hypothesize that we will see more cross-resistance in ciprofloxacin resistant mutants than collateral sensitivity. For the mecillinam resistant mutants we hypothesize to see more collateral sensitivity. For the isolates evolved resistant to both drugs, we hypothesize that the presence of more than one resistance determinant will affect the CS network. Additionally, we hypothesize that some of the resistance mechanisms might interfere with the biofilm forming abilities of the mutants.

3 MATERIALS AND METHODS

3.1 Bacterial strains

The bacterial strains used for generating MEC- and/or CIP-resistant mutants are listed in **Table 2**. Clinical isolates of *E. coli* from the ECO·SENS strain collection are used in this study. The chosen isolates are pansusceptible, meaning that they are not resistant to commonly tested antimicrobials for *E. coli* (ECO-SENS) and are plasmid-free isolates [32]. Additionally, the control strains ATCC 25922 and ATCC 35984 were used for AST and the biofilm assay, respectively.

Table 2. *E. coli* UTI isolates used in this study

Strains	Sequence type	Phylogroup	Country of origin	Year
K56-22	73	B2	Sweden	2000
K56-23	73	B2	Sweden	2000
K56-24	73	B2	Sweden	2000
K56-25	73	B2	Sweden	2000
K56-26	73	B2	Sweden	2000
K56-29	73	B2	Sweden	2000
K56-30	1161	B2	Sweden	2000
K56-35	73	B2	UK	2000
K56-38	73	B2	UK	2000
K56-46	73	B2	Greece	2007-2008
K56-63	135	B2	Sweden	2007-2008
K56-67	141	B2	Sweden	2007-2008
K56-73	73	B2	UK	2007-2008
K56-76	976	B2	UK	2007-2008
K56-77	1236	B2	UK	2007-2008
K56-80	141	B2	UK	2007-2008

3.2 Growth media

The use of growth media is essential to facilitate good environmental and nutritional growth conditions for the bacteria. Cultivation of bacteria were accomplished in both liquid- and solid media in this project.

3.2.1 Luria-Bertani (LB) medium

Luria-Bertani (LB) broth is a nutritionally rich media commonly used for the growth and maintenance of *E. coli* strains. Bacteria were cultivated in either liquid or solid media prepared in the MicroPop laboratory as specified by the manufacturer.

LB broth:

Mix 20 g LB broth (BD Difco ™, Miller) with 800 ml dH_2O and autoclave at 121°C. The medium is cooled and then stored at 4°C.

LB agar (LBA):

Mix 20 g LB broth (BD Difco ™, Miller) and 12 g Select agar (Sigma-Aldrich) with 800 mL dH_2O and autoclave at 121°C. The medium is cooled to 50-60°C and poured into sterile petri dishes. The agar plates are drying overnight and stored at 4°C.

3.2.2 Mueller-Hinton (MH) medium

Cation-adjusted Mueller-Hinton II (MH) broth is a growth medium that is widely used for AST. MH agar plates were prepared in the laboratory as specified by the manufacturer, while MH broth was obtained from the University Hospital of Northern Norway (UNN) in Tromsø.

MH II agar (MHA):

Mix 30,4 g of MH II agar (Sigma-Aldrich) with 800 mL dH_2O and autoclave at 121°C. The medium is cooled to 50-60°C and poured into sterile petri dishes. The agar plates are left to solidify overnight and stored at 4°C.

3.2.3 Tryptic Soy Broth (TSB) medium and TSB with glucose

Tryptic soy broth is a nutrition rich medium used for the determination of biofilm formation in this project. TSB and TSB with 1% glucose were obtained from the University Hospital of Northern Norway (UNN) in Tromsø.

3.2.4 Other solutions and reagents

0,85% saline:

Add 0,65 g of sodium chloride (≥99,5%, Fluka) into 80 ml dH_2O. Autoclave at 121°C and store the solution at room temperature.

80% glycerol:

Add 54 ml of a glycerol solution (86-89% purity, T) (Sigma-Aldrich) into a 100 ml graduated cylinder and adjust with dH_2O to 100 ml in total volume. Autoclave at 121°C and store the solution at room temperature.

70% ethanol:

Add 70 ml of 96% to 30 ml of dH_2O.

Matrix Solution for MALDI-TOF:

Stock solution: add 475 µl of MilliQ-water into a tube, then add 500 µl acetonitrile and 25 µl trifluoracetic acid. Mix vortexing. The solution is then aliquoted into 1,5 ml tubes and store cool.

Add 250 µl of the stock solution to a HCCA-tube. Vortex for 1 minute until the solution is completely transparent. Protect from light during storage.

TFA for MALDI-TOF:

Add 50 µl of dH_2O and 200 µl of 100% TFA in an Eppendorf tube. Shake for 1 minute.

3.3 Bacterial cultivation

Different techniques were used to cultivate bacteria in this study. Depending on the purpose for the cultivation, bacteria were either cultured in liquid or solid medium, as described below.

3.3.1 Overnight cultures

Overnight cultures were made by inoculating a pure isolated colony of bacteria from an agar plate into 5 ml of LB. The solution is then incubated under conditions that will support growth, 37°C for *E. coli,* shaking at approximately 225 rpm.

3.3.2 Plating techniques on solid medium

Three different plating methods were used to achieve desired growth of bacteria on solid medium. This was done in a petri dish containing an appropriate growth medium with agar as a solidifying agent.

3.3.2.1 Streak for isolation technique

The 3-zone streaking technique is used to achieve the growth of pure isolated colonies. By spreading the bacterial inoculum across different areas of the plate, single colonies are observed at end of the streak were the bacteria is spread thinly.

Start by using a sterile loop and pick the inoculum from an agar plate, liquid medium or a freeze stock culture. Then streak the inoculum in zone 1 by moving the loop back and forth, in a zigzag-pattern, down the first third of the agar plate. Following the initial streak, use a new sterile loop and streak the next third of the plate by dragging a few lines from zone 1 into zone 2. Continue with the same loop and drag a few lines from zone 2 to zone 3. Incubate the plates for 16-20 hours overnight at 37°C.

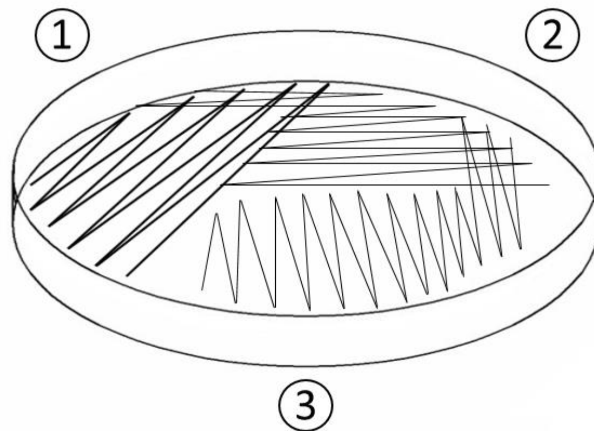

Figure 6: Demonstration of streak for isolation technique.

3.3.2.2 Spread plating

Plating with sterile glass beads is an easy technique to achieve evenly spreading of a bacterial liquid culture on the entire growth surface of an agar plate. Around 20 sterile glass beads are poured onto the plate together with the liquid culture. Shake the plate(s) in a horizontal plane until the inoculum is completely absorbed. The glass beads are then collected into a waste container for decontamination and sterilization when the plate is dry.

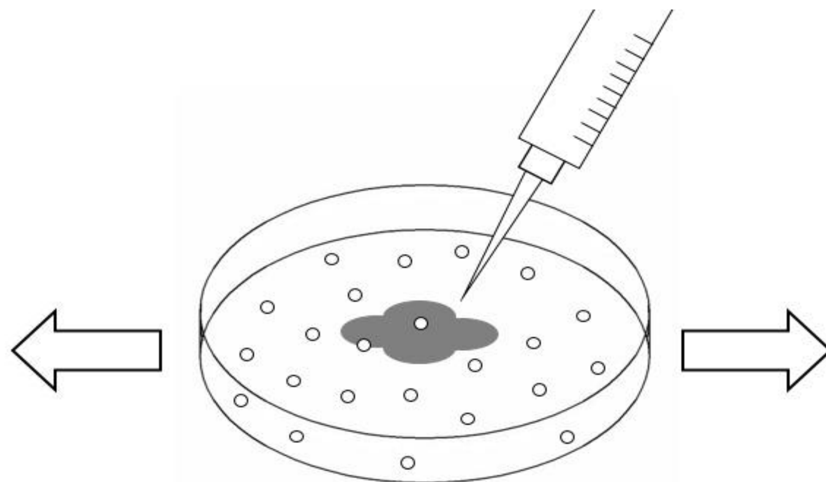

Figure 7: Demonstration of spread plating technique.

3.3.2.3 Swab plating

This technique is performed by using an electrically driven rotator that spins with a constant speed to achieve uniform plating of the inoculum on agar plates.

Start by putting a sterile cotton swab in a 0,5 McFarland solution, and removing excess liquid by pressing the swab on the inside of the glass tube. Place the agar plate on the rotator. Start inoculation by placing the swab gently on the edge of the plate, and then slowly move the swab into the centre. Then, twist so that a new side of the swab is in contact with the agar and start moving the slowly back to the initial point.

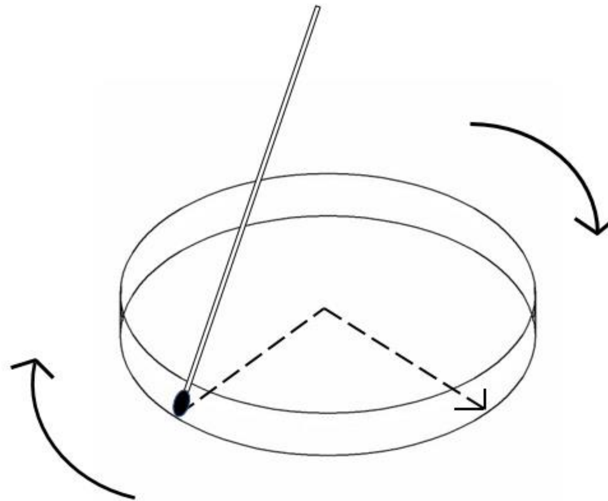

Figure 8: Demonstration of swab plating technique.

3.4 Other standard microbiology techniques

3.4.1 McFarland standard

McFarland standards are made by adjusting the turbidity of bacterial cells in a suspension, ensuring that the number of bacteria will be within a certain range. A 0,5 McFarland suspension ($6x10^8$ CFU/ml), used for AST in this project, was prepared by suspending a small number of isolated colonies from an agar plate into a glass tube containing 0,85% NaCl. The correct turbidity is determined by measuring the optical density of the suspension in a calibrated densitometer.

3.4.2 Storage in freeze stock cultures

A freeze stock culture (stored at -80°C) is made to allow long-term storage of bacterial strains. The freeze stock is prepared by adding 250 µl of 80% glycerol as a cryoprotecting agent together with 750 µl of an overnight culture into freeze tubes.

3.5 Generation of antimicrobial resistant mutants

Antimicrobial resistant isolates of *E. coli* with reduced susceptibility to ciprofloxacin or mecillinam were generated by static selection under antimicrobial pressure. The protocol was adapted for each of the two drugs to acquire clinically resistant mutants for each antimicrobial. However, while the MEC mutants are generated in the laboratory after one single step, the CIP mutants needed to be exposed to a gradually increasing antimicrobial concentration before reaching clinically resistant levels.

Moreover, in addition to the selection of mutants with either resistant to mecillinam or ciprofloxacin resistance alone, clinically resistant isolates made resistant to both antimicrobials were also generated. This procedure was performed by using the same methods as for the single-mutants. Using the same strains, the double-mutants were acquired by making MEC mutants resistant to ciprofloxacin, as well as CIP mutants made resistant to mecillinam.

All the mutants generated were used for the IC_{90}-testing to investigate CS and CR to specific antimicrobial agents, described more in detail in **Section 3.8.**

3.5.1 Mecillinam resistant mutants

3.5.1.1 Mecillinam stock solution

Mecillinam stock solutions were made to a final concentration of 1 mg/ml to make MHA MEC plates. To prepare the solution, 100 mg of mecillinam powder (Sigma-Aldrich) was dissolved in 100 ml of ddH_2O. The suspension was sterile filtered using a 0,2 µM filter unit, and then 4 ml volumes was aliquoted into cryovials and stored at -25°C.

3.5.1.2 Preparation of mecillinam selective plates

Selective plates containing 16 µg/ml mecillinam were used to acquire resistant mutants from wild-type strains. However, when making CIP resistant mutants resistant to mecillinam, plates containing 8 µg/ml were also used.

As specified by the manufacturer, a solution of 400 ml MH II agar were prepared by mixing 15,2 g of MHA II with 400 ml of dH_2O, before autoclaving at 121°C. The medium was then cooled to 50-60°C and mecillinam stock solution was added into the MH broth to the desired final concentration (**Table 3**). The agar plates were then poured and left to solidify overnight. Plates were stored at 4°C for maximum of one week.

Table 3. MEC selective plates

MHA MEC plate	**MEC_8**	**MEC_{16}**
Concentration of MEC Stock (mg/ml)	1	1
Volume of MEC added to 400 ml of MH agar (ml)	3,2	6,4

3.5.1.3 Method – Static antimicrobial resistance selection

Preparation of selection inoculum:

1. Grow isolate(s) of interest by scraping a small sample of a freeze stock and streak for isolation on LB plate(s) (see **Section 3.3.2.1**). Incubate overnight at 37°C.
2. Pick an isolated colony and inoculate it into 5 ml LB media in a sterile tube. Incubate the overnight culture at 37°C with shaking at 225 rpm.

Selecting mecillinam resistant mutants using selective plates:

3. 100 µl of the overnight culture is plated with glass beads onto MHA-Mec_{16} plates (see **Section 3.3.2.2**).
4. 100 µl of the overnight culture is also added into the first well of a sterile 96-well plate and serially diluted (1:10 dilutions) in 0,85% saline to a final dilution factor of 10^{-6}/10^{-7} as shown in **Table 3.** Each dilution is mixed well by pipetting 10-20 times. Then 100 µl of the 6th and 7th dilutions are plated with glass beads to achieve countable colonies on non-selective LBA plates.
5. After overnight incubation, or up to 48 hours, visible colonies on selective MHA-MEC_{16} plates and non-selective LB plates are counted to determine the mutation frequency rate (see **Section 3.5.3**).

Purification and storage of mecillinam resistant mutants:

6. Pick a single colony from the selective plate and streak for isolation on a new MHA MEC_{16} plate. Incubate overnight at 37°C.
   I. If there are no single colonies, touch a selection of colonies and streak on a new selective plate to achieve single colonies.
   II. Using one plate per colony, streak at least 3 colonies of different appearances for isolation. Make note of phenotypes.
7. Pick well-isolated single colonies using a sterile loop and inoculate into 5 ml of LB. Incubate overnight at 37°C shaking 225 rpm.
8. Make freeze stocks of the overnight culture in 20% glycerol and store at -80°C.

3.5.2 Ciprofloxacin resistant mutants

3.5.2.1 Ciprofloxacin stock solution

Ciprofloxacin stock solutions were made to a final concentration of 25 mg/ml. The stock was used to make MHA CIP plates and overnight cultures. The solution was prepared by dissolving 50 mg of the ciprofloxacin powder in 2 ml of 0,1 HCl. The suspension was filter sterilized using a 0,2 µM filter unit, aliquoted into sterile tubes and stored at -25°C.

3.5.2.2 Preparation of ciprofloxacin selective plates

To achieve bacterial strains resistant to ciprofloxacin, selective media of different concentrations were used.

As specified by the manufacturer, a solution of 400 ml MH II agar were prepared by mixing 15,2 g of MHA II with 400 ml of dH_2O, before autoclaving at 121°C. The medium was then cooled to 50-60°C and appropriate volumes of the ciprofloxacin stock solution was added into the MH broth to the desired final concentration (**Table 4**). The agar plates were poured the agar plates and left to solidify overnight. The plates were stored at 4°C for a maximum one week.

Table 4. Volume of CIP added in 400 ml MH II to make selective plates of various concentrations.

MHA CIP plate	$CIP_{0,016}$	$CIP_{0,032}$	$CIP_{0,064}$	$CIP_{0,128}$	$CIP_{0,25}$	$CIP_{0,5}$	CIP_{1}	CIP_{2}
Concentration of CIP Stock (µg/ml)	0,016	0,032	0,064	0,128	0,25	0,5	1	2
Volume of CIP added to 400 ml of MH agar (µl)	0,256	0,512	1,024	2,048	4	8	16	32

3.5.2.3 Method – Stepwise static antimicrobial selection

Preparation of selection inoculum:

1. Grow isolate(s) of interest by scraping a small sample of a freeze stock and streak for isolation (see **Section 3.3.2.**1) on LB plate(s). Incubate overnight at 37°C.
2. Pick an isolated colony and inoculate it into 25 ml LB media in a sterile 100 ml flask. Incubate the overnight culture at 37°C with shaking at 150 rpm.

Selecting ciprofloxacin resistant mutants using selective plates:

3. Pipet 10 ml of the overnight culture into a sterile 15 ml conical tube and pellet the culture by centrifuging at 4000 rpm for 10 minutes at room temperature (20°C). Decant the supernatant into a waste bottle and resuspend the cell pellet in 1 ml of LB medium by pipetting thoroughly so that the dilutions and cell concentration calculations will be accurate. Pipet 100 µl of the resuspended pellet onto MHA CIP selective plates and spread the inoculum with glass beads (see **Section 3.3.2.2**).
4. 100 µl of the resuspended pellet was also added into the first well of a sterile 96-well plate and serially diluted in saline to a final dilution factor of $10^{-7}/10^{-8}$ as shown in Table. Mix well by pipetting 10-20 times. 100 µl of the 7th and 8th dilutions were plated with glass beads to achieve countable colonies on non-selective LBA plates. Selective- and non-selective plates are incubated overnight at 37°C.
5. After overnight incubation, or up to 48 hours, visible colonies on selective MHA-CIP plates and non-selective LBA plates are counted to determine the mutation frequency rate (see **Section 3.5.3**).

6. If growth is not present on selective plates above the clinical breakpoint, a mixture of colonies from the highest concentration plate with growth is used to inoculate a new flask with 25 ml MH medium containing the corresponding CIP concentration.

The process is then repeated from step 3 to 6 with increasing concentrations of CIP selection until growth on MHA with CIP at 1 or 2 µg/ml is observed.

Purification and storage of ciprofloxacin resistant mutants:

7. Pick a single colony from MHA CIP_1 or CIP_2 and streak for isolation on a new MHA CIP_2 plate. Incubate overnight at 37°C.
   III. If there are no single colonies, touch a selection of colonies and streak for isolation on a new selective plate to achieve single colonies.
   IV. Using one plate per colony, streak at least 3 colonies of different appearances for isolation to have a selection of isolates. Make note of phenotypes.
8. Pick well-isolated single colonies using a sterile loop and inoculate them into 5 ml of LB. Incubate overnight at 37°C shaking 225 rpm.
9. Make freeze stocks of the overnight culture in 20% glycerol and store at -80°C.

3.5.3 Mutation frequency

To determine the total number of bacteria plated from the overnight culture or the re-suspended pellet, the dilution factor is multiplied with the number of counted colonies on the non-selective dilution plate:

$$Total\ bacteria\ plated\ (CFU) = dilution\ factor\ 10x\ x\ counted\ colonies\ (\frac{CFU}{100\mu l})$$

The mutation frequency can then be calculated:

$$\text{Mutation frequency} = \frac{colony\ count\ on\ selective\ plate}{Total\ bacteria\ plated}$$

3.6 Identification of *E. coli* by MALDI-TOF

Matrix Assisted Laser Desorption Ionization Time-of-Flight (MALDI-TOF) mass spectrometry (MS) is a versatile analytical technique used to detect and characterize organic molecules [50]. This identification system has become a useful tool within microbiology in recent years due to its rapid, sensitive and inexpensive identification of different microorganisms.

In MALDI, the sample to be analysed is shot with laser pulses causing thermal desorption and ionization of highly abundant microbial proteins. Singly protonated ions are accelerated, based on their mass-to-charge ratio (m/z), into a time-of-flight (TOF) mass analyser. A detector, measuring the time a particle require to travel the length of the flight tube, generates a characteristic mass spectrum of the sample. The MALDI Biotyper software compares the resulting mass spectrum to a known database of different microorganisms, thus identifying the bacterial species by its "fingerprint". In this way, we can confirm that the selected mutants in this study are truly *E. coli* [50].

3.7.1 Cleaning the 96-well target plate

1. Place the target plate into an empty petri dish and add enough 70% EtOH to submerge the plate. Incubate for 5 minutes.
2. Rinse the plate with distilled water and wipe with absorbent tissue moistened with 70% EtOH.
3. Rinse again with distilled water and dry the plate on absorbent tissue.
4. Add 100 µl of 80% TFA onto the target plate and clean every well with absorbent tissue.
5. Finish by cleaning the target plate by rinsing with distilled water.

3.7.2 Direct transfer of bacterial colonies to target plate

1. Resistant mutants are struck for isolation on LBA plates (see **Section 3.3.2.**1) and incubated at 37°C overnight.
2. Using a wooden stick, a single isolated colony is picked from the LBA plate and transferred to a selected well on the target plate. The bacteria sample is spread evenly within the well creating a thin layer.

3. When all samples are applied to the target plate, then 1 µl of matrix are added to each well. When dried matrix crystallizes, the inoculated target plate is ready to be analyzed.

3.7.3 Analysis and results

- A score over 2.000 represents accurate identification at the species level.
- A score between 1.700-1.999 indicates a non-reliable identification of species, but accurate identification at the genus level.
- A score under 1.700 indicates no reliable identification.

3.7 Minimum inhibitory concentration testing by diffusion strips

To confirm antimicrobial resistance in the generated mutants, antimicrobial gradient diffusion strips were used to determine MIC values. These strips are made of porous paper impregnated with a predefined concentration gradient of antibiotic on one side, across 15 two-fold dilutions, while the other side has a MIC scale in µg/ml and a code representing the antimicrobial drug for use during result interpretation.

When the MIC-test strip is applied on an inoculated agar plate, the antibiotic immediately diffuses out into the agar. After incubation for 16-20 hours, either no inhibition or an elliptical inhibition zone centred along the strip will be observed. The MIC value is read directly from the scale at the edge of the elliptical inhibition zone. With any antimicrobial, the growth on one side of the strip growth may be higher than the other side; the highest value is used as the MIC.

Method:

1. Streak the mutants of interest for isolation onto non-selective MHA plates (see **Section 3.3.2.1**) and incubate overnight at 37°C.
2. Use a sterile cotton swab to pick up a small amount of the isolated colonies from the MHA plate. Twist the swab around on the plate to achieve an even and thin layer of bacteria on the swab.

3. Suspend the colonies in a glass tube containing 0.85% NaCl to an optical density of 0.5 McFarland. Adjust the suspension by adding more bacteria or saline if necessary.
4. Within 15 minutes, put a new cotton swab into the suspension, remove excess liquid by pressing it to the inside of the glass tube and inoculate the agar plate using the swabbing for confluency method (see **Section 3.3.2.3**). Let the plate dry for maximum 15 minutes.
5. Sterilize tweezers using 70% EtOH and a gas flame, then apply the MIC-strip to the agar plate. Gently press the strip to the agar without moving it after application.
6. Put the plate(s) in the incubator within 15 minutes after the strip is applied. Incubate the plate(s) at 37°C for 16-20 hours.

The MICs are interpreted using the EUCAST clinical breakpoint, shown in **Table 5**.

Table 5. EUCAST Clinical Breakpoints.

	Ciprofloxacin	Mecillinam
EUCAST Clinical breakpoints	Sensitive (S) ≤ 0,5 Resistant (R) > 1	Sensitive (S) ≤ 8 Resistant (R) > 8

3.8 IC_{90} antimicrobial susceptibility testing

The inhibition concentration-90 (IC_{90}) value, defined as the lowest concentration of the drug that inhibits at least 90% of the growth of a tested isolate, is used to determine the antimicrobial susceptibilities. Changes in the susceptibility (collateral sensitivity) or resistance (cross-resistance) were investigated with 6 selected antimicrobials (Table) by initially performing a 2-fold dilution, followed by a 1,5-fold dilution based testing to achieve a more precise result.

Table 6. Antimicrobial agents used in this study.

Antimicrobial	Antimicrobial class	Antimicrobial target
Azithromycin	Macrolide	Protein synthesis (50S ribosome unit)
Chloramphenicol	Other class	Protein synthesis (50S ribosome unit)
Ciprofloxacin	Fluoroquinolone	DNA-replication (DNA gyrase and topoisomerase IV)

Gentamicin	Aminoglycoside	Protein synthesis (30S and 50S ribosome subunits)
Mecillinam	Extended spectrum β-lactam	Cell wall synthesis (PBP-2)
Trimethoprim	Antifolate	Folic acid synthesis (DHFR)

Procedure:

1. Isolates of interest, as well as an *E. coli* control strain (ATCC 25922), were struck for isolation on LBA plates (see **Section 3.3.2.1**) and incubated at 37°C overnight.
2. The 96-well plate(s) are prepared by loading MH broth into the wells, depending on which dilutions series being tested, as shown in **Table 7** and **Table 8**.

Table 7. 2-fold dilution

	1	2	3	4	5	6	7	8	9	10	11	12
A	100 µL		100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL
B	100 µL		100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL
C	100 µL		100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL
D	100 µL		100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL
E	100 µL		100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL
F	100 µL		100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL
G	100 µL		100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL
H												

Table 8. 1,5-fold dilution

	1	2	3	4	5	6	7	8	9	10	11	12
A	100 µL		50 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL
B	100 µL		50 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL
C	100 µL		50 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL
D	100 µL		50 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL
E	100 µL		50 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL
F	100 µL		50 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL
G	100 µL		50 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL
H												

3. The working stock of the antimicrobial is diluted in MH broth to 2x the highest tested concentration. To ensure that correct antimicrobial stock solution is made, the specific MIC-testing guidelines and/or manufacturer's guidelines are followed.
4. For 2-fold dilutions, 200 µl of the antimicrobial working stock is added in column 2. For 1,5-dilutions, 200 µl of the antimicrobial working stock is added in column 2, as well as 150 µl into column 3.

5. For 2-fold, 100 µl is taken from column 2 to column 3, and then mixed by pipetting 10-15 times. The drug is serially diluted across the plate, and 100 µl of the mixture is discarded at the end from column 11.

   For 1,5-fold, 100 µl is taken from column 2 to column 4, and then mixed by pipetting 10-15 times. Continue to mix and serially dilute the antibiotic in every second column (even numbered columns), and discard 100 µl of the mixture in the end from column 10. Furthermore, 100 µl is taken from column 3 to column 5, and then mixed by pipetting 10-15 times. Continue to mix and serially dilute the antibiotic in every second column (odd numbered columns), and discard 100 µl of the mixture in the end of column 11.

6. Prepare a 0,5 McFarland in 0,85% sterile saline from each strain by picking up a few isolated colonies from the LBA plates.
7. The 0,5 McFarland is diluted 1:1000 by adding 5 µl into 4,995 ml MH broth and mixed by inverting the tube.
8. Finally, 100 µl of the diluted McFarland is added into wells in columns 1-11.
9. The 96-well plate is incubated at 37°C for 18 hours with shaking at 700 rpm.
10. After 18 hours of incubation the OD_{600nm} was measured in a plate reader.

The OD_{600nm} measurements of the sample with different antimicrobial concentrations, as well as the positive and negative controls, were used to calculate the IC_{90}:

$$\% \, inhibition = \left(1 - \frac{(\text{OD600 drug } treated - \text{ OD600 negative control})}{(\text{OD600 positive control} - \text{ OD600 negative control})}\right) x \; 100$$

Table 9. Equipment used in the IC_{90} assay.

Equipment	**Producer**
Microplater Shaker TiMix 5 control	Edmund Büler GmbH
VersaMax ELISA Microplate Reader	Molecular devices

3.9 Determination of biofilm formation

Procedure:

1. Streak the strains of interest onto MHA plates and incubate overnight at 37°C.
2. Inoculate a single colony from the MHA plates into 5 ml TSB and incubate overnight at 37°C with shaking at 220 rpm.
3. The overnight culture is diluted 1:100 in fresh TSB supplemented with 1% glucose, adding 30 µl culture into 3 ml TSB with 1% glucose.
4. For each strain, 150 µl of the bacterial suspension is added into a column of a 96-well polystyrene tissue culture plate (Thermo Fisher Scientific), including positive control strain RP62A and negative blank control.

The plate is incubated for 24 hours at 37°C without shaking.

5. Remove the liquid cultures by pipetting and wash the plate gently by pipetting distilled water in and out of the wells three times.
6. Leave the plate at room temperature overnight or in an incubator at 55°C for one 1 hour to dry the plate.
7. Add 200 µl of crystal violet in each well to stain the biofilm. Incubate for 5 minutes.
8. Wash the wells gently with tap water in the sink.
9. Add 200 µl of EtOH in each well and measure the OD of the adherent biofilm in a plate reader at 570 nm.
10. These experiments must be done in a minimum three parallels.

Calculations:

11. Before calculating the average value its necessary to avoid the effect that extreme low or extreme high observations will have on the average. The outliers are therefore removed from the data. The average value is calculated for each parallel:

$$Average = \frac{\mathrm{X1} + \mathrm{X2} + \mathrm{X3} \ldots \mathrm{Xn}}{\mathrm{N}}$$

Then the average value of all parallels, as well as the standard deviation (STD), is calculated.

4 EXPERIMENTAL RESULTS

In this study, we wanted to investigate CS and CR effects in clinical *E. coli* strains from UTIs of different genetic backgrounds. Isolates clinically resistant to mecillinam and ciprofloxacin, two important drugs in the treatment of UTIs, were generated. Additionally, we wanted to see whether the mutations can affect the biofilm forming ability of the resistant mutants.

4.1 Isolation of clinically resistant mecillinam isolates

In this project, mecillinam resistant isolates were generated from eight clinical strains selected from the ECO-SENS collections. The aim was to obtain resistant isolates with MICs above 8 µg/ml, the clinical breakpoint for mecillinam. Visible growth on selective plates with 16 µg/ml were observed after one single selection step for all the strains, after incubation for one or two days. From each strain, resistant isolates were generated and purified and 3-5 single colonies were stored.

To determine MIC values and confirm clinical resistance to mecillinam, antimicrobial gradient diffusion strips were used. The isolates that were successfully purified and classified as clinically resistant, are listed in **Table 10.** The MIC value was interpreted by reading the concentration directly of the strip. The MIC interpretation was sometimes challenging for the mecillinam mutants, as spontaneous mutants with even higher mecillinam MICs were frequently observed within the zone of inhibition. MIC determination for ciprofloxacin was also performed to detect any cross-resistance to this drug.

Table 10: *E. coli* strains successfully purified and made clinically resistant to mecillinam.

Strain	Mutation frequency	Isolates	MIC CIP (µg/ml)	MIC MEC (µg/ml)
K56-25	$3,07 x 10^{-8}$	**III**	0,064	32
		IV	0,064	32
K56-30	$3,24 x 10^{-8}$	IV	0,016	32
K56-35	$2,05 x 10^{-8}$	V	0,032	8-12
K56-67	$3,24 x 10^{-8}$	II	0,016	32

K56-73	2,38x10^{-8}	**I**	0,032	≥256
		II		16
		III		64
K56-76	2,15x10^{-7}	I	0,016	8
K56-77	9,5x10^{-8}	I	0,023	24
K56-80	1,06x10^{-7}	III	0,012	24-32

As well as generating mecillinam resistant isolates by static selection, some strains from the ECO-SENS collection were already selected for mecillinam resistant mutants in the MicroPop laboratory, but had to be confirmed clinically resistant by MIC-testing and *E. coli* by MALDI-TOF. These isolates, displayed in **Table 11**, was also used in the project.

Table 11: MEC resistant isolates generated in the MicroPop research group.

Parental strain	MIC CIP (µg/ml)	MIC MEC (µg/ml)
K56-22	0,032	48
K56-26	0,023	24
K56-29	0,047	64
K56-38	0,012	16
K56-46	0,023	12-16
K56-63	0,047	≥256

These were generated in the MicroPop group by Elizabeth Aarag Fredheim.

During the static selection process, the K56-49, K56-51 and K56-61 strains stood out from the rest. Generating mecillinam resistant mutants with these strains was attempted twice, however the colonies had variable morphologies both in size and shape, and a homogenous colony phenotype was not achieved in any of the strains after trying to purify them. These strains were therefore excluded from the project. Similar observations of different morphological types and sizes were also seen in other strains, but typically one or more of the resulting mutants were successfully purified. In addition, slow growing and contaminated isolates were also observed, and the isolates were then excluded.

The mutation frequency rate was the highest for strain K56-73 (2,15x10^{-7}), and the lowest for strain K56-35 (2,05x10^{-8}). The highest MICs above the clinical breakpoint were for strains

K56-63 and K56-73 which were over the detection limit of the strip (≥256 µg/ml), and the lowest for strain K56-76 (8 µg/ml), respectively. All isolates were identified as *E. coli* by MALDI-TOF, except from one case where several isolates derived from strain K56-51 were identified as *Stapholycoccus saprophyticus.* These were obviously contaminants and were excluded from the study.

4.2 Isolation of clinically resistant ciprofloxacin isolates

In addition to the mecillinam mutants, ciprofloxacin resistant isolates were generated from six strains selected from the ECO-SENS collections. By exposing the strains to a progressively increasing antimicrobial concentration, resistant isolates with MIC above 1 µg/ml, the clinical breakpoint for ciprofloxacin, were obtained. Resistant isolates were generated requiring 2-3 antimicrobial selection steps and multiple mutants were purified for each strain (3-5 single colonies).

As was done for the mecillinam mutants, antimicrobial gradient diffusion strips were used to determine MIC values and confirm resistance to ciprofloxacin. The isolates that were purified and successfully classified as clinically resistant are listed in **Table 12.** MIC determination for mecillinam was also performed to detect any cross-resistance to this drug.

Table 12: ***E. coli*** **strains successfully purified and made clinically resistant to ciprofloxacin.**

Strain	Mutation frequency			Isolates	MIC MEC (µg/ml)	MIC CIP (µg/ml)
	Step 1	Step 2	Step 3			
K56-23	$5,85x10^{-11}$	$3,39x10^{-10}$		I	0,125	12-16
K56-23				II	0,125	24
K56-23				**III**	0,094	32
K56-23				IV	0,125	12
K56-23				V	0,064	24
K56-24	$5,65x10^{-11}$	$1,03x10^{-8}$	ND	I	0,19	6-8
K56-24				II	0,19	6
K56-24				II	0,094	6
K56-24				**IV**	0,094	8
K56-25	$2,89x10^{-11}$	$2,1x10^{-9}$	ND	I	0,094	6-8

K56-25				**II**	0,125	8
K56-25				III	0,125	6
K56-25				IV	0,125	8
K56-26	$3,4x10^{-10}$	$2,55x10^{-10}$	$8x10^{-9}$	**II**	NR	12
K56-26				IIII	8-12	8
K56-29	$2,73x10^{-9}$	$5,9x10^{-10}$	ND	I	0,25	32
K56-29				**II**	0,19	32
K56-29				III	0,19	32
K56-29				IV	0,19	32
K56-38	$8,29x10^{-11}$	$9,67x10^{-9}$	ND	II	1	6
K56-38				**IV**	0,25	8

ND: Mutation frequency not determined due to dense growth on selection plate.

Two ciprofloxacin resistant isolates from the MicroPop laboratory were also used in the project, listed in **Table 13.**

Table 13: Isolates generated by a previous master student Chon Kit Lam in the MicroPop research group.

Strain	MIC MEC (μg/ml)	MIC CIP (μg/ml)
K56-22	0,25	8-12
K56-30	0,125	12-16

As was seen with some mecillinam mutants, different phenotypes were observed in some of the ciprofloxacin mutants. These isolates were either successfully purified or excluded from the project. However, this issue was not seen as frequently as for the mecillinam mutants. In some cases the mutation frequencies could not be determined due to dense growth covering the selective plates.

The highest ciprofloxacin MICs above the clinical breakpoint were observed for strains K56-29 and K56-23 (32 μg/ml), and the lowest was for strain K56-38 (6 μg/ml). All of the isolates were identified as *E. coli* by MALDI-TOF.

4.3 Isolation of clinically resistant ciprofloxacin and mecillinam isolates

We wanted to investigate if the presence of two resistance determinants, as well as the order of which these mutants were generated, would have an impact on CS and CR profiles. A selection of isolates resistant to ciprofloxacin or mecillinam were used to generate these double mutants. The same methods as for selection of resistance in single mutants was used to select for the secondary resistance trait. The aim was to achieve clinical isolates resistant to both mecillinam and ciprofloxacin above their clinical breakpoints.

4.3.1 Mecillinam resistant isolates selected for ciprofloxacin resistance

Five MEC mutants were chosen and selected on ciprofloxacin to obtain resistant isolates. Between one to five resulting isolates were selected and purified for each strain (**Table 14**), and they were further tested using MIC gradient diffusion strips.

Table 14: Clinically resistant mecillinam isolates successfully purified and made clinically resistant to ciprofloxacin.

Mecillinam isolate	Mutation frequency			Isolates	MIC MEC (µg/ml)	MIC CIP (µg/ml)
	Step 1	Step 2	Step 3			
K56-22 II	ND	$2,47x10^{-9}$	$1,1x10^{-9}$	**I**	≥256	8
				II	≥256	6-8
				III	≥256	32
				IV	≥256	8-12
				V	≥256	32
K56-25 III	$1,13x10^{-9}$	$3,29x10^{-9}$		I	≥256	4
				II	≥256	3
				III	≥256	6
				IV	≥256	3
K56-26 II	$6,67x10^{-10}$	ND		**I**	ND	3
K56-30 IV	$1,62x10^{-10}$	$3,7x10^{-10}$			≥256	6

ND: MIC not determined.

Although the process of selecting mecillinam resistant isolates with ciprofloxacin didn't require more selection steps than for the single ciprofloxacin resistant isolates, it was generally more challenging than using the WT strains. The selective plates usually had to be

incubated for two days due to slow growth, and the colonies were often very small. Variable phenotypes were frequently observed, and purification and separation attempts were performed without success for some of the strains. Interestingly, one isolate, K56-29 MEC, lost its resistance to mecillinam after becoming resistant to ciprofloxacin.

An interesting observation is the high MIC values for mecillinam, being ≥256 µg/ml for all the isolates. Such high MIC values has just been observed in two of the mecillinam mutants generated. The MICs for CIP are more variable, ranging from 3 up to 32. All the strains were identified as *E. coli* by MALDI-TOF.

4.3.2 Ciprofloxacin resistant isolates selected for mecillinam resistance

Five CIP mutants were selected on mecillinam to obtain them resistant isolates. The mutants were struck on plates with concentration of 8 µg/ml and 16 µg/ml of mecillinam. Three to five colonies with different phenotypes were isolated and purified for each strain, and they were further tested using MIC gradient diffusion strips. The isolates that were successfully purified and found to be resistant above the clinical breakpoints are listed in the **Table 15** below.

Table 15: Clinically resistant ciprofloxacin isolates successfully purified and made clinically resistant to mecillinam.

Ciprofloxacin isolate	Mutation frequency	Number of isolates	MIC CIP (µg/ml)	MIC MEC (µg/ml)
K56-22 I	ND	**I**	3	ND
K56-25 II	$4,7 \times 10^{-8}$	I	4	≥256
		II	6	≥256
K56-26 II	$3,92 \times 10^{-8}$	**I**	8	≥256
		II	8-12	≥256
		III	3	≥256
		IV	4	≥256
K56-30 I	ND	I	4	≥256
		II	12	≥256
		III	12	≥256
		IV	8	≥256

All the mutants had to be incubated for two days as the colonies appeared extremely small after only one day of incubation. At day two, the colonies were still rather small on both MEC_8 and MEC_{16} plates. For two of the isolates, K56-22 CIP and K56-30 CIP, the plates were fully covered in colonies, thus mutation frequencies could not be determined.

For these double mutants, a high MIC value above the limit of detection ≥256 µg/ml for mecillinam was observed for all the isolates except K56-22, which was difficult to interpret. The MICs for ciprofloxacin are above the clinical breakpoint and range between 3 to 12.

4.4 IC_{90} determination and assembly of collateral susceptibility networks

The generated mutants and their WT parental strains were further characterized by determining IC_{90} values to investigate how the selection for resistance may have affected their susceptibility to six other antimicrobials; AZT, CHL, CIP, GEN, MEC and TMP. These were chosen based on previous results to investigate if trends observed for other isolates could be confirmed. The drugs were chosen to test trends of increased sensitivity, increased resistance as well as no change in susceptibility following generation of mutants resistant to either CIP or MEC.

An initial 2-fold dilution scheme was used to increase the range of concentrations tested and improve the probability of determining the IC_{90} This initial testing was followed by a final IC_{90} determination using a 1,5-fold dilution to achieve more precise values. The OD_{600} was measured after 18 hours and after 42 hours for some slow-growing mutants. Results from the 1,5-fold experiment were used to calculate fold changes by comparing the resistant isolates to their WT parental strains. The resulting fold changes are presented in following heatmaps where they are illustrated by colors of varying intensities. Decreases in susceptibility indicating a fold change equal to or above 1,5 are highlighted in red (CR), and for simplicity described as positive fold-changes. An increase in susceptibility or a fold change equal to or less than -1,5 fold are highlighted in blue (CS) and is for simplicity described as negative fold-changes. Isolates that did not show a relevant change in susceptibility are displayed in white.

4.4.1 Collateral sensitivity profiles of mecillinam resistant isolates

The mecillinam resistant isolates all displayed reduced susceptibility above the clinical breakpoint for mecillinam and 64-341 fold increases in IC_{90} (**Figure 9**). Further analyses of the collateral networks revealed that CS or no change in susceptibility are the dominant trends among these mutants. More specifically, CS was observed in all of the mecilinam resistant isolates towards AZT and GEN; the largest increase in susceptibility was observed in K56-22 towards AZT with a fold range of -3,33. However, the exceptions are isolate K56-29, which shows CR to GEN and isolate K56-30 that has no change in its susceptibility to AZT. Towards CIP, most isolates showed no change in susceptibility.

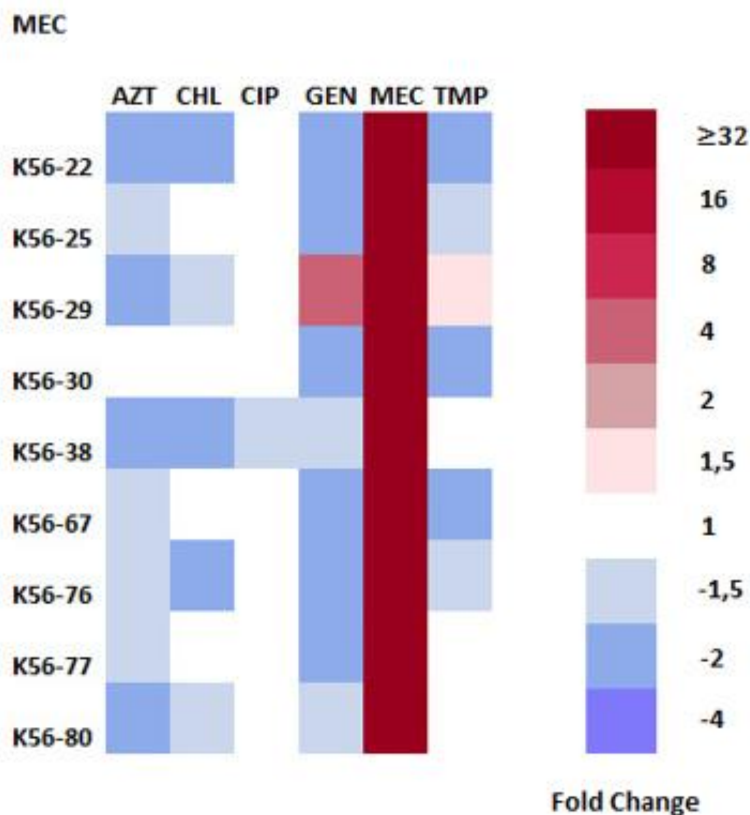

Figure 9: Collateral sensitivity network of mecillinam resistant mutants. Heatmap describing fold changes for collateral sensitivity (CS) and cross-resistance (CR) in nine mecillinam resistant isolates compared to their WT parental strains.

Some of the generated mecillinam mutants are not shown in this heatmap, as they were excluded during the IC_{90} experiment. The K56-73 and K56-63 isolates were extremely slow growing, even after 42 hours, so accurate IC_{90} values based on OD measurements could not be accurately determined. Interestingly, these two strains were also the ones with the

highest MIC-values of ≥256 in the gradient-strip diffusion test. Another strain, K56-22, was also slow growing, but was characterized by IC_{90} testing, as the OD_{600} of the positive control ended above 0,3. Finally, isolate K56-35 was excluded as it was found have an IC_{90} result for mecilinam that was below the clinical breakpoint.

4.4.2 Collateral sensitivity profiles of ciprofloxacin resistant isolates

As expected, all the ciprofloxacin resistant isolates showed reduced susceptibility above the clinical breakpoint for ciprofloxacin with 342-12,288 fold increases in the IC_{90}. However, one of the isolates, K56-25, was included in this heatmap although it was slow growing. As the positive control did not increase that much after 42 hours, the 18-hour value has been used.

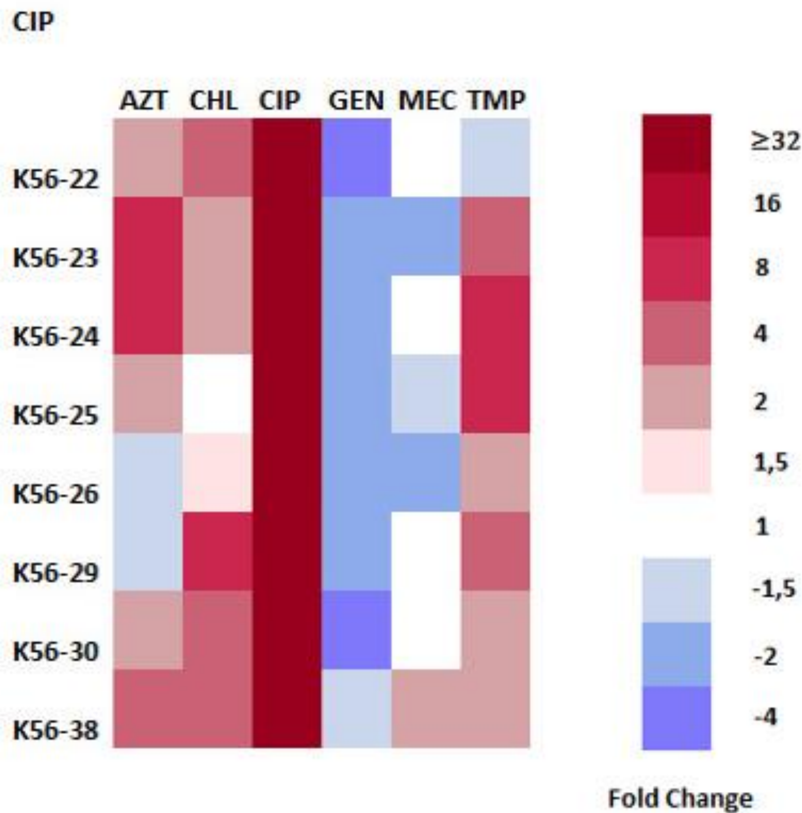

Figure 10: Collateral sensitivity network of ciprofloxacin resistant mutants. Heatmap describing fold changes for collateral sensitivity (CS) and cross-resistance (CR) in eight ciprofloxacin resistant isolates compared to their WT parental strains.

Analyses of the collateral networks show that a decrease in susceptibility is commonly observed in the mutants, especially to CHL and TMP. Most isolates also show CR towards

AZT. For MEC only one isolate shows displayed CR, while the others has no change or an increase in sensitivity. However, GEN stands out from the other drugs, as CS is present in all the isolates with fold changes ranging between -1,5 to -4 and an average value of -2,44.

4.4.3 Collateral sensitivity profiles of isolates resistant to both ciprofloxacin and mecillinam

The ciprofloxacin and mecillinam resistant isolates all displayed reduced susceptibility above the clinical breakpoint (256-512 and 42,7-256 fold increases in IC_{90}, respectively). However, two of the generated double mutants, K56-25 M/C and K56-26 C/M, were excluded in this heatmap as the IC_{90} testing revealed that they are not clinically resistant to mecillinam. In addition, the isolates that were observed as slow growing among the single mutants, K56-22 and K56-25, were also slow growing as double mutants.

Analyses of the collateral networks show that the CR effects seen for the CIP mutants to a certain degree dominate the effects caused by MEC resistance. Another interesting observation is that CS to GEN was observed in all isolates with fold changes ranging from -2,63 to -5,26, a trend that was also observed in the isolates resistant to CIP or MEC individually.

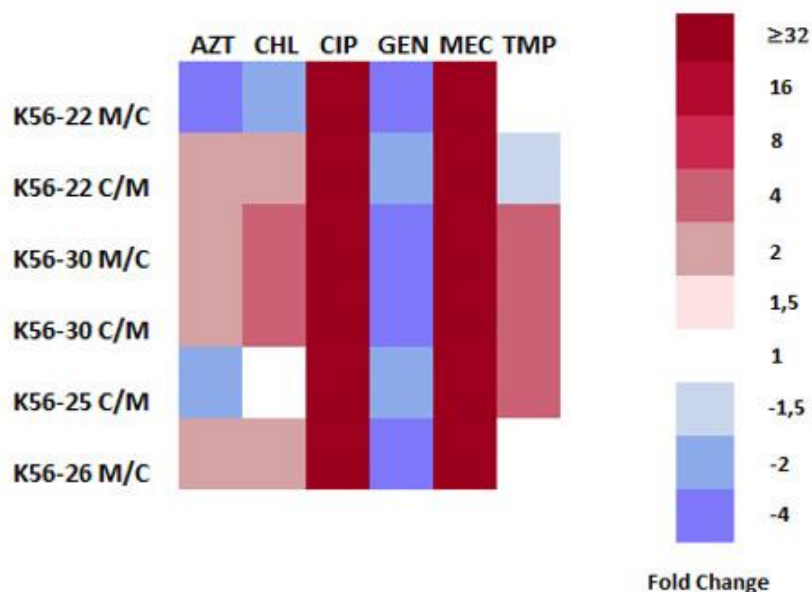

Figure 11: Collateral sensitivity network of ciprofloxacin and mecillinam resistant mutants. Heatmap describing fold changes for collateral sensitivity (CS) and cross-resistance (CR) in ciprofloxacin and mecillinam resistant isolates compared to their WT parental strains.

4.5 Biofilm formation

4.5.1 Optimization of biofilm essay on *E. coli*

To decide the most suitable growth media to be used for further investigation of biofilm formation in clinical resistant *E. coli* isolates, five WT strains and RP62A were tested with six different growth media; LB, MH, TSB with 1% glucose (TSBglu), TSB, BHI and M9.

The average biofilm formation, shown in the diagram below, indicates there was no major difference in the amount of biofilm being formed in the different *E. coli* strains. However, there were much greater biofilm formation observed in the control strain RP62A tested in TSBglu than the other growth media; this growth media was therefore selected for further use in the project.

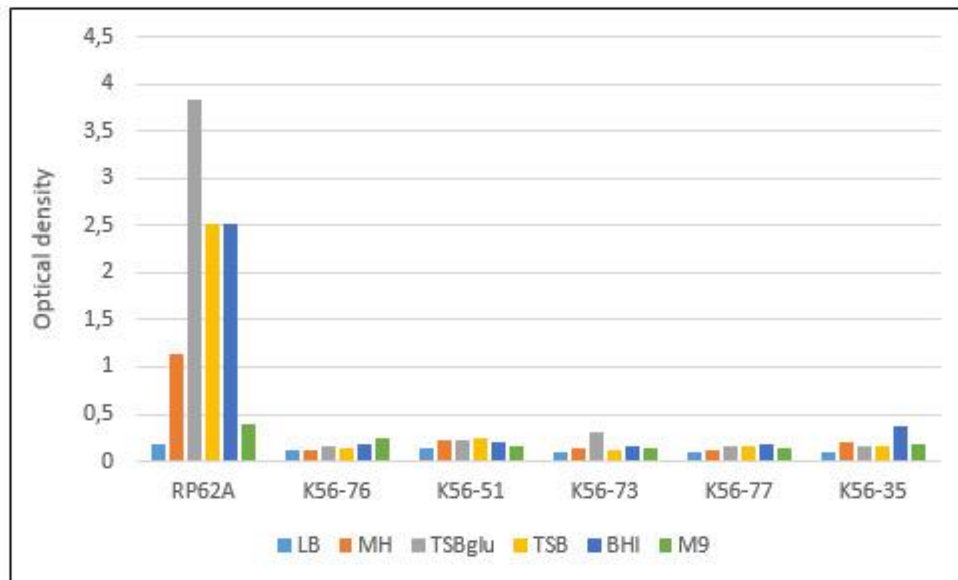

Figure 12: Biofilm formation in control strain RP62A and *E. coli* WT strains. Diagram displaying biofilm formation of *E. coli* WT strains and control strain in different growth medium.

4.5.2 Determination of biofilm formation in clinical resistant isolates

The isolates confirmed to be resistant to ciprofloxacin, mecillinam or ciprofloxacin and mecillinam (double mutants) were tested to investigate if the mutations that were selected under antibiotic pressure influence biofilm formation. Double mutants that were confirmed

to in fact only be resistant to one drug were also included in the analysis. The different isolates, their WT parental strains and a control strain were tested, and the OD_{570} was measured. Three replicates were performed and the average value of these replicates was calculated.

The results show that biofilm formation is low for all the mutants (**Figure 13**). While the control strain usually has an OD_{570} value between 3,7-4, the highest observed value in the mutants is 0,56 in K-56 25 M/C double mutant which is only resistant to CIP, and the lowest measurement of 0,07 was from the K56-38 CIP isolate.

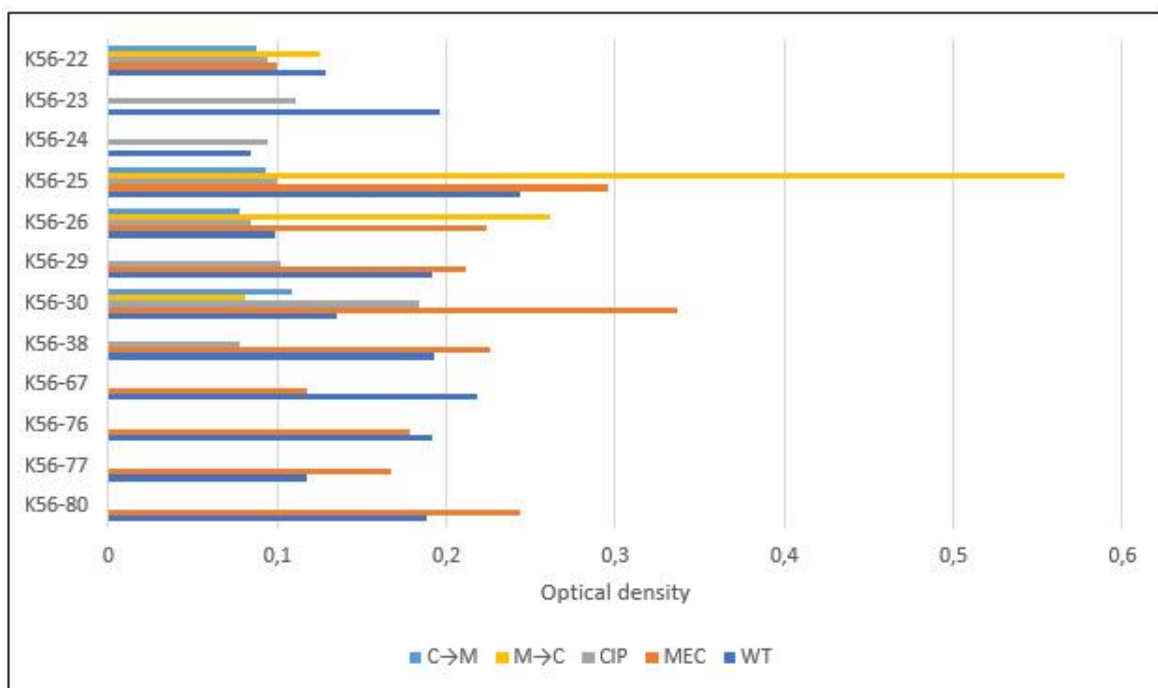

Figure 13: Biofilm formation in clinically resistant isolates. Diagram displaying biofilm formation in the different mutants generated in TSB with 1% glucose.

There is no clear trend in the biofilm formation of the antibiotic resistant mutants compared to their WT. Four of the twelve WTs produce more biofilm than all their mutants. Seven of the ten mecillinam mutants also produce more biofilm than their respective WTs. For the CIP mutants, only two out of the eight are forming more biofilm than their WTs. However, the MEC mutants generally have a slightly higher value than the CIP mutants of the same strain, with the exception of K56-25 M/C.

5 DISCUSSION

The escalating emergence of AMR is a growing public health concern. The acceleration of bacterial evolution causing resistance to antibiotics is an inevitable outcome of the use and misuse of antibiotics since its introduction in the 1940s [51]. As the development of new drugs has stagnated, there is a need to investigate novel strategies that can contribute to extend the efficacy of antimicrobial agents. One approach may be to achieve a better understanding of the phenomena of CS, a side-effect in the evolution of AMR. As proposed by Imamovic and Sommer, reciprocal CS can be applicable in drug cycling, suppressing the emergence of resistant bacteria [1]. The main focus in this study is to further investigate these phenomena by evolving resistance in clinical strains of *E. coli* from UTIs, and to investigate the biofilm forming abilities of the isolates.

5.1 CS and CR effects

Our results show that the resistant isolates of CIP and MEC demonstrated different CS and CR effects in clinical isolates of *E. coli*. CS was frequently observed in the MEC resistant mutants in comparison to CR, which was only shown in one MEC resistant isolate for GEN and TMP. In contrast, CR more frequently appeared than CS in the CIP mutants. CR was seen in most isolates for TMP and CHL. Also for AZT this effect is dominating. These results are consistent with an ongoing study in the MicroPop research group based on single resistance determinants in clinical E. coli strains (Podnecky et al, unpublished results).

The effect of more than one resistance determinant on collateral networks is currently unknown. To the best of my knowledge, we here present the first data on collateral networks of clinical *E. coli* strains resistant to both CIP and MEC. Interestingly, it looks like the isolates being resistant to both CIP and MEC tend to be largely dominated by the CR effects seen for the isolates resistant to CIP alone. In this study, we have not investigated the underlying mutations causing resistance in our isolates. In MEC resistant isolates, there are numerous genes that may confer resistance to the drug, making it difficult to predict what kind of mutations that have occurred in our mutants [37]. However, it is known that mutations in the drug target for the CIP mutants frequently occur, as well as genes involved

in the regulation of efflux pumps. This may be an explanation for the high CR interactions observed when CIP develops resistance [45]. The CR effects was especially clear in isolate K56-30 where it is seen to all drugs except from GEN. Moreover, these isolates were not affected by the order of which they were generated, as the network appear to be identical. However, the order of which the isolates were generated may have influenced the CS and CR profiles for the isolates derived from strain K56-22. In the MEC resistant isolate selected for CIP resistance, CS are the dominating effect observed. For the other isolate, a combination of CR and CS was seen. In two of the isolates, K56-25 and -26, we were not able to generate clinically resistant isolates to CIP and MEC in both orders for comparison. However, for these isolates a combination of CS and CR effects was seen. Taken together, these results strongly suggest that collateral networks identified in strains resistant to single antimicrobials needs to be interpreted with caution. It is clear that certain combinations of resistance determinants may affect these networks. It is therefore necessary to further explore CS and CR effects beyond single resistance determinants.

An important overall observation in our results is the CS effects observed to the aminoglycoside GEN. Except for one MEC resistant isolate, all the other clinically resistant isolates displayed CS effects to this drug. The MicroPop research group did a similar study where they investigated and displayed CS and CR effects in a heatmap by using IC_{90} assays. When comparing our results to their findings, we can see similar pattern of CS and CR profiles in CIP and MEC resistant isolates (see **Section 1.9.1**). The observation that CR effects more frequently occur in CIP mutants, as well as a strong tendency of CS to GEN, are consistent with our results. For the MEC resistant isolates, the MicroPop group observed that an increase or no change in susceptibility to different drugs are dominating the network, as well as that CS appear more frequent than the CR effects seen for the CIP mutants. Their results also show that CS frequently occur to AZT in MEC resistant isolates, like our results. Half of their MEC resistant isolates displayed CS to gentamicin.

5.1.1 Comparison to previous studies

In Imamovic and Sommers study from 2013, they evolved resistance to single drugs from one laboratory strain of *E. coli* against 23 clinically relevant drugs spanning 11 distinct classes

of antibiotics. CS and CR were determined by IC_{90} assays and they displayed their results in a heatmap [1]. Like our findings, they observed CR patterns to AZT, CHL and TMP in their CIP resistant isolates. However, CS in GEN is not described in their results. As Imamovic and Sommer did not evolve isolates resistant to MEC, we are comparing our results for the MEC resistant isolates to β-lactams in their study. They included five drugs from this class of antibiotics. Unlike our results, Imamovic and Sommer frequently observed CR patterns in β-lactam resistant isolates. CR was seen to CIP, CHL, AZT and TMP in all the five drugs included, which is distinctly different from our results. In addition, CS to GEN was seen in cefuroxime only.

Lazar published a similar study in 2013, short time after Imamovic and Sommer. They evolved resistance to single drugs, using 12 antimicrobials, in a single ancestral clone of *E. coli* and mapped networks of CS interactions to a number of agents. Additionally, they tried to explain the underlying mechanisms of the CS they observed [52]. They did not include MEC in the study, thus we compare our results for the MEC resistant isolates to the drugs having the equivalent mechanism of action as MEC, the cell-wall inhibitors. Like for our results, they observed CS effects to GEN in resistant isolates of both CIP and the cell-wall inhibitors from the adaption to low antibiotic concentrations [52]. In a similar study published by Lazar a in 2014, they focused on networks of CR interactions by generating *E. coli* populations from one single ancestral clone resistant to 1 of 12 antibiotics in total [53]. Here they observed CR in the CIP resistant isolates to TMP. For the cell-wall inhibitors, CR interactions were observed to TMP, CIP and CHL [53].

In conclusion, this report and ongoing activitiy in MicroPop research group highlight the need for addressing collateral networks in large collections of clinical strains to identify conserved patterns that may inform future treatment protocols. Previous published studies investigating CS and CR are focusing on single laboratory strains. Investigating clinical isolates of diverse genetic background is a strength in our study. These strains exhibit variations of susceptibility and give us a broader insight in CS and CR profiles on a population level. However, our findings are based on *in vitro* results, and a subject that remains to be explored is if CS can be used in clinical setting. Further *in vivo* studies have to be performed to answer this knowledge gap.

5.1.2 Clinical relevance of our findings

Our findings may be helpful in future treatment strategies to select against AMR in clinical isolates being resistant to CIP and/or MEC, two important drugs in the treatment of UTIs. Our results suggest that MEC is a suitable drug of choice in the treatment of UTIs, consistent with current guidelines in Norway. CR towards the other drugs tested were not frequently observed, and CS effects of this drug may be taken advantage of in collateral drug cycling. Additionally, long term use of this antibiotic has shown that resistance to MEC is rare *in vivo,* despite that resistance is easily obtained in the laboratory [37]. Thulin and co-workers recently suggest that this probably is an effect of the physiology and mechanistic host defences such as bladder emptying and urine flow, making it difficult for the bacteria to maintain and develop resistance in the bladder [37]. The ECO·SENS studies also show that the level of resistance to MEC is low, as the prevalence in Europe was 1,6% in 2008-2009 [32].

Our results also show that treatment with CIP may lead to CR to several other drugs, making it a less suitable choice in the treatment of UTIs. In contrast to MEC, this antibiotic is extensively used worldwide in the treatment of several Gram-positive and Gram-negative infections, and resistance to CIP and other fluoroquinolones occur at an increasing rate in several bacterial species [46]. This is confirmed by the ECO·SENS studies, as the prevalence of ciprofloxacin resistance increased from 1,1% (1999-2000) to 3,9% (2008-2009) [32].

An important finding in our study is the CS effects observed in all the resistant isolates towards the aminoglycoside GEN, an important broad spectrum antibiotic used in the treatment of a wide range of infections worldwide [54]. This drug is frequently used in hospital acquired infections of multidrug-resistant Gram-negatives [55]. Aminoglycosides are also an interesting class of antibiotics as previous studies show a wide range of CS effects to other antibiotics when resistance develops in these drugs [1, 52]. The theory behind Imamovi and Sommer’s drug cycling is based on reciprocal CS. In order to test if their cycling will work, we would need to evolve resistance to GEN and then find CS towards CIP.

5.2 Biofilm formation in clinical resistant *E. coli* isolates

One goal in this study was to investigate whether the mutations occurring in the clinically resistant *E. coli* isolates affect their ability to produce biofilms. Before determining biofilm formation in the resistant isolates, an optimization experiment was performed to decide the most suitable growth media to be used further in the experiment, as the biofilm formation can be variable in different growth media/culture conditions [56]. There was no significant difference between the different media tested, but TSBglu were chosen due to a greater biofilm formation observed in the control strain RP62A.

Our results show that the selected mutants and their WT parental strains are generally producing little biofilm compared to the positive control, *Staphylococcus epidermidis* RP62A, a well-known strong biofilm producer. Four of the twelve WTs produce more biofilm than all of their respective mutants. For the CIP mutants, only two out of the eight are forming more biofilm than their WTs. However, seven of the ten MEC mutants were shown to produce slightly more biofilm than their WT parental strains.

However, different methods could also have been tried in this experiment, as this may influence the production of biofilm. Methods such as flow cell, tube test, Calgary biofilm device, congo red agar and biofilm reactor are examples

5.3 Challenges and limitations in our study

5.3.1 Limitations in the IC90 assay

By comparing the results from the 2-fold dilution and the 1,5-fold dilution, the results usually matched each other well. However, the results were not always consistent, as different data were observed between the two experiments (see **Appendix F**). Using CIP resistant isolates as an example, the IC_{90} in the 2-fold dilutions sometimes appeared to be higher (>16) than determined in the experiment using the 1.5-fold dilution. This is likely a result of human error during the experiment. The 2-fold and 1,5-fold dilution experiments were only performed once. The exception was for some of the 1,5-fold dilutions that had to be repeated due to errors in the 2-fold experiment, as the IC_{90} was not achieved within the

predicted range. In order to ensure correct IC_{90} values and achieve more precise results, the experiments should be performed more than once.

One observation in the IC_{90} assay was the appearance of slow growing strains. For the MEC mutants, the highest MICs above the clinical breakpoint were for strains K56-63 and K56-76 (≥256 µg/ml). In liquid MH medium, MEC mutant K56-73 did not grow at all, whereas clear growth was observed in LB after overnight incubation. This indicates that MH II broth lack some kind of nutrient essential for this isolate. MEC mutant K56-63 was extremely slow growing, having an OD_{600} of 0,27 in the positive control after 42 hours of incubation. These MEC resistant isolates were excluded from analysis. Thulin had similar observations when measuring growth rate of MEC mutants in MH medium, where the relative growth rates ranged from 0,25-0,7 [37]. They suggest that this may be due to reduced growth rate associated with severe fitness costs, which is frequently observed when MEC resistance is acquired in the laboratory [37].

Reduced growth rates were also seen in MEC mutant K56-22 and CIP mutant K56-25, as well as their generated double mutants. These resistant isolates were characterized by IC_{90} testing, as the OD_{600} of the positive control ended above 0,3. However, including these mutants may have influenced the result as its not completely precise, and has probably underestimated the IC_{90} value.

5.3.2 Challenges and limitations in generating and confirming clinical resistance

A challenge in the confirmation of clinically MEC mutants, are spontaneous mutations frequently being observed in the zone of inhibition when interpreting MIC values by gradient diffusion strips. Many mutations can lead to MEC resistance [37]. This is the likely cause of the problem of purifying MEC mutants, as and also the reason for the observed spontaneous mutants observed when using E-test.

However, another interesting observation for the clinical isolates being resistant to both CIP and MEC, is the high MIC-values observed in all the isolates towards MEC by performing E-test, which were over the detection limit of the gradient diffusion strip (≥256 µg/ml) for all isolates except one. As described above, a possible explanation for these high values may be challenges of interpreting the correct value due to spontaneous mutations.

6 CONCLUSION AND FUTURE ASPECTS

We conclude that the favourable CS and CR profile in MEC resistant isolates possibly makes it an applicable drug to use in the first-line treatment of UTIs. Our results also confirm that CR is frequently observed in CIP resistant isolates, indicating that this drug should be used prudently due to the risk of resistance development to several other drugs. These suggestions are supported by the results from the MicroPop research group, showing similar patterns of CS and CR as in this study. Furthermore, our findings in the isolates resistant to both CIP and MEC show that the CR profile in CIP resistant mutants are able to dominate over the CS effects in MEC resistant mutants. This is an unnerving observation, considering the fact that most clinical *E. coli* isolates are resistant to several drugs. This might indicate that collateral sensitivity based drug cycling will not have the desired effects of steering bacteria on an evolutionary trajectory towards increased antimicrobial sensitivity in real life settings *in vivo*. Also, the gap in knowledge on how transferrable resistance determinants might affect collateral sensitivity networks, leaves a considerable amount of research still left to determine if collateral sensitivity cycling can work. However, the CS effects to GEN, seen in all the resistant isolates generated in this study, except for one MEC mutant, suggests that GEN may have some promise to be used as a part of collateral drug cycling to other drugs showing reciprocal CS.

There are several unanswered questions regarding collateral susceptibility changes that should be further investigated. First of all, there is a need to perform future studies on various bacterial species of diverse genetic backgrounds to achieve a broader insight in these effects on a population level. There is also a need to achieve more knowledge of the underlying mechanism(s) of CS in order to get a better understanding of this phenomenon. There should also be performed studies in order to achieve a better understanding of how single-determinant data will be affected in strains being resistant to more than one drug. There is also a need to investigate whether CS can be used in clinical setting. Further *in vitro* and *vivo* studies have to be performed to answer this knowledge gap.

When determining biofilm formation, we can observe that our resistant isolates generally are not producing much biofilms, which is a positive observation due to their ability to complicate the treatment of infections. However, considerable more in vitro studies, testing the isolates with different methods is needed to determine this. We did not observe any highly significant trends towards collateral changes in biofilm forming ability following the resistance mutations. However, the observation of a trend towards more biofilm-accumulation in the MEC resistant mutants warrants further studies.

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8 APPENDIX

Appendix A: Results from static selection of mecillinam resistant mutants.

Parental isolate	CFU/ml inoculum	CFU/ml mutants	Mutation frequency	Isolated mutants	MIC CIP (µg/ml)	MIC MEC (µg/ml)
K56-25	$2,18x10^{9}$	67	$3,07x10^{-8}$	III	0,064	32
				IV	0,064	32
K56-30	$7,1x10^{8}$	23	$3,24x10^{-8}$	IV	0,016	32
K56-35	$2,49x10^{9}$	51	$2,05x10^{-8}$	V	0,032	8-12
K56-67	$3,77x10^{9}$	147	$3,9x10^{-8}$	II	0,016	32
K56-73 I	$2,6x10^{9}$	62	$2,38x10^{-8}$	I	0,032	≥256
K56-73 II				II		16
K56-73 III				III		64
K56-76	$4,63x10^{9}$	997	$2,15x10^{-7}$	I	0,016	8
K56-77	$1,46x10^{9}$	139	$9,5x10^{-8}$	I	0,023	24
K56-80	$3,75x10^{9}$	396	$1,06x10^{-7}$	III	0,012	24-32

Appendix B: Results from static selection of ciprofloxacin resistant mutants.

Strain	Mutation frequency			Number of isolates	MIC MEC (µg/ml)	MIC CIP (µg/ml)
	Step 1	Step 2	Step 3			
K56-23	$5,85x10^{-11}$	$3,39x10^{-10}$		I	0,125	12-16
K56-23				II	0,125	24
K56-23				**III**	0,094	32
K56-23				IV	0,125	12
K56-23				V	0,064	24
K56-24	$5,65x10^{-11}$	$1,03x10^{-8}$	ND	I	0,19	6-8
K56-24				II	0,19	6
K56-24				II	0,094	6
K56-24				**IV**	0,094	8
K56-25	$2,89x10^{-11}$	$2,1x10^{-9}$	ND	I	0,094	6-8
K56-25				**II**	0,125	8

K56-25				III	0,125	6
K56-25				IV	0,125	8
K56-26	$3,4x10^{-10}$	$2,55x10^{-10}$	$8x10^{-9}$	**II**	NR	12
K56-26				IIII	8-12	8
K56-29	$2,73x10^{-9}$	$5,9x10^{-10}$	ND	I	0,25	32
K56-29				**II**	0,19	32
K56-29				III	0,19	32
K56-29				IV	0,19	32
K56-38	$8,29x10^{-11}$	$9,67x10^{-9}$	ND	II	1	6
K56-38				**IV**	0,25	8

ND: Mutation frequency not determined due to dense growth on selection plate.

Appendix C: Results from static selection of mecillinam mutants made ciprofloxacin resistant.

Mecillinam isolate	**Mutation frequency**			**Number of isolates**	**MIC MEC (µg/ml)**	**MIC CIP (µg/ml)**
	Step 1	**Step 2**	**Step 3**			
K56-22 II	ND	$2,47x10^{-9}$	$1,1x10^{-9}$	**I**	≥256	8
				II	≥256	6-8
				III	≥256	32
				IV	≥256	8-12
				V	≥256	32
K56-25 III	$1,13x10^{-9}$	$3,29x10^{-9}$		I	≥256	4
				II	≥256	3
				III	≥256	6
				IV	≥256	3
K56-26 II	$6,67x10^{-10}$	ND		**I**	ND	3
K56-30 IV	$1,62x10^{-10}$	$3,7x10^{-10}$			≥256	6

ND: MIC not determined.

Appendix D: Results from static selection of ciprofloxacin mutants made mecillinam resistant.

Parental isolate	Mutation frequency	Isolated mutants	MIC CIP (µg/ml)	MIC MEC (µg/ml)
K56-22 I	ND	**I**	3	ND
K56-25 II	$4{,}7x10^{-8}$	I	4	≥256
		II	6	≥256
K56-26 II	$3{,}92x10^{-8}$	**I**	8	≥256
		II	8-12	≥256
		III	3	≥256
		IV	4	≥256
K56-30 I	ND	I	4	≥256
		II	12	≥256
		III	12	≥256
		IV	8	≥256

Appendix E: Example of MALDI-TOF results for identification of *E. coli*

Analyte name	Analyte ID	Organism (best match)	Score value	Organism (second best match)	Score value
A1	1	*Escherichia coli*	2,364	*Escherichia coli*	2,322
D1	1	*Escherichia coli*	2,408	*Escherichia coli*	2,314
A8	8	*Staphylococcus saprophyticus*	2,173	*Staphylococcus saprophyticus*	2,03

Appendix F: IC90 determination. Tested against six different antimicrobials on parental WT and their mutants.

Azithromycin

Strains	Highest concentration 2-fold	Highest concentration 1,5-fold	IC90 2-fold	IC90 1,5-fold	Fold change	
					IC90 2-fold	IC90 1,5-fold
K56-22 WT	12 µg/ml	24 µg/ml	3	2,25		
K56-22 MEC	12 µg/ml	6 µg/ml	0,75	0,75	0,25	0,33
K56-22 CIP	12 µg/ml	24 µg/ml	3	4,5	1	2
K56-22 M→C	12 µg/ml	1,5 µg/ml	0,1875	0,375	0,0625	0,167
K56-22 C→M	12 µg/ml	24 µg/ml	3	4,5	1	2
K56-23 WT	12 µg/ml	12 µg/ml	1,5	1,5		
K56-23 CIP	12 µg/ml	32 µg/ml	3	12	2	8
K56-24 WT	12 µg/ml	3 µg/ml	1,5	1,5		
K56-24 CIP	12 µg/ml	32 µg/ml	0,75	12	0,5	8
K56-25 WT	12 µg/ml	16 µg/ml	3	2		
K56-25 MEC	12 µg/ml	16 µg/ml	3	1,5	1	0,75
K56-25 CIP	12 µg/ml	24 µg/ml	6	6	2	3
K56-25 M→C	12 µg/ml	12 µg/ml	1,5	2,25	0,5	1,125
K56-25 C→M	12 µg/ml	16 µg/ml	3	0,75	1	0,375
K56-26 WT	12 µg/ml	8 µg/ml	1,5	1,5		
K56-26 MEC	12 µg/ml	6 µg/ml	0,75	1,5	0,5	1
K56-26 CIP	12 µg/ml	6 µg/ml	0,75	1,125	0,5	0,75
K56-26 M→C	12 µg/ml	16 µg/ml	3	4	2	2,67
K56-26 C→M	12 µg/ml	2 µg/ml	0,1875	1	0,125	0,67
K56-29 WT	12 µg/ml	12 µg/ml	3	2,25		
K56-29 MEC	12 µg/ml	24 µg/ml	3	1,125	1	0,5
K56-29 CIP	12 µg/ml	32 µg/ml	1,5	1,5	0,5	0,67
K56-30 WT	12 µg/ml	12 µg/ml	1,5	1,5		
K56-30 MEC	12 µg/ml	6 µg/ml	0,75	1,5	0,5	1
K56-30 CIP	12 µg/ml	48 µg/ml	6	4,5	4	3
K56-30 M→C	12 µg/ml	12 µg/ml	1,5	4,5	1	3
K56-30 C→M	12 µg/ml	24 µg/ml	3	4,5	2	3

K56-35 WT	12 µg/ml	ND	1,5	ND		
K56-35 MEC	12 µg/ml	ND	0,375	ND		
K56-38 WT	12 µg/ml	24 µg/ml	3	3		
K56-38 MEC	12 µg/ml	12 µg/ml	1,5	1,5	0,5	0,5
K56-38 CIP	12 µg/ml	48 µg/ml	>6	12	-	4
K56-63 WT	12 µg/ml	ND	3	ND		
K56-63 MEC	12 µg/ml	ND	0,375	ND		
K56-67 WT	12 µg/ml	12 µg/ml	1,5	1,5		
K56-67 MEC	12 µg/ml	12 µg/ml	1,5	1,125	1	0,75
K56-73 WT	12 µg/ml	ND	1,5	ND		
K56-73 MEC	12 µg/ml	ND	ND	ND		
K56-76 WT	12 µg/ml	12 µg/ml	1,5	1,5		
K56-76 MEC	12 µg/ml	4 µg/ml	0,375	1	0,25	0,67
K56-77 WT	12 µg/ml	12 µg/ml	1,5	1,5		
K56-77 MEC	12 µg/ml	6 µg/ml	0,75	1,125	0,5	0,75
K56-80 WT	12 µg/ml	12 µg/ml	1,5	2,25		
K56-80 MEC	12 µg/ml	6 µg/ml	1,5	1,125	1	0,5

Chloramphenicol

Strains	Highest concentration 2-fold	Highest concentration 1,5-fold	IC90 2-fold	IC90 1,5-fold	Fold change	
					IC90 2-fold	IC90 1,5-fold
K56-22 WT	16 µg/ml	32 µg/ml	4	4		
K56-22 MEC	16 µg/ml	16 µg/ml	2	2	0,5	0,5
K56-22 CIP	16 µg/ml	64 µg/ml	>8	24	-	6
K56-22 M→C	16 µg/ml	32 µg/ml	4	2	1	0,5
K56-22 C→M	16 µg/ml	64 µg/ml	>8	12	-	3
K56-23 WT	16 µg/ml	32 µg/ml	4	4		
K56-23 CIP	16 µg/ml	64 µg/ml	8	12	2	3
K56-24 WT	16 µg/ml	16 µg/ml	2	3		
K56-24 CIP	16 µg/ml	32 µg/ml	4	6	2	2

K56-25 WT	16 µg/ml	32 µg/ml	4	6		
K56-25 MEC	16 µg/ml	32 µg/ml	4	6	1	1
K56-25 CIP	16 µg/ml	64 µg/ml	8	6	2	1
K56-25 M→C	16 µg/ml	32 µg/ml	4	6	1	1
K56-25 C→M	16 µg/ml	64 µg/ml	8	6	2	1
K56-26 WT	16 µg/ml	32 µg/ml	4	4		
K56-26 MEC	16 µg/ml	32 µg/ml	4	3	1	0,75
K56-26 CIP	16 µg/ml	32 µg/ml	4	6	1	1,5
K56-26 M→C	16 µg/ml	64 µg/ml	8	8	2	2
K56-26 C→M	16 µg/ml	64 µg/ml	8	6	2	1,5
K56-29 WT	16 µg/ml	32 µg/ml	4	4		
K56-29 MEC	16 µg/ml	32 µg/ml	4	3	1	0,75
K56-29 CIP	16 µg/ml	64 µg/ml	>8	32	-	8
K56-30 WT	16 µg/ml	16 µg/ml	2	4		
K56-30 MEC	16 µg/ml	16 µg/ml	2	4	1	1
K56-30 CIP	16 µg/ml	128 µg/ml	>8	24	-	6
K56-30 M→C	16 µg/ml	64 µg/ml	8	16	4	4
K56-30 C→M	16 µg/ml	128 µg/ml	>8	16	-	4
K56-35 WT	16 µg/ml	ND	0,03125	ND		
K56-35 MEC	16 µg/ml	ND	2	ND		
K56-38 WT	16 µg/ml	32 µg/ml	4	6		
K56-38 MEC	16 µg/ml	32 µg/ml	4	3	1	0,5
K56-38 CIP	16 µg/ml	64 µg/ml	>8	24	-	4
K56-63 WT	16 µg/ml	ND	4	ND		
K56-63 MEC	16 µg/ml	ND	4	ND		
K56-67 WT	16 µg/ml	16 µg/ml	2	2		
K56-67 MEC	16 µg/ml	16 µg/ml	2	2	1	1
K56-73 WT	16 µg/ml	ND	2	ND		
K56-73 MEC	16 µg/ml	ND	ND	ND		
K56-76 WT	16 µg/ml	32 µg/ml	4	3		
K56-76 MEC	16 µg/ml	16 µg/ml	2	1,5	0,5	0,5
K56-77 WT	16 µg/ml	32 µg/ml	4	3		
K56-77 MEC	16 µg/ml	16 µg/ml	2	3	0,5	1
K56-80 WT	16 µg/ml	32 µg/ml	4	3		
K56-80 MEC	16 µg/ml	32 µg/ml	4	2	1	0,67

Cirpofloxacin

Strains	Highest concentration 2-fold	Highest concentration 1,5-fold	IC90 2-fold	IC90 1,5-fold	Fold change	
					IC90 2-fold	IC90 1,5-fold
K56-22 WT	0,125 µg/ml	0,0625 µg/ml	0,0078125	0,00585938		
K56-22 MEC	0,125 µg/ml	0,0625 µg/ml	0,0078125	0,00585938	1	1
K56-22 CIP	32 µg/ml	32 µg/ml	>16	3	-	511,9
K56-22 M→C	32 µg/ml	64 µg/ml	>16	2	-	341,3
K56-22 C→M	32 µg/ml	48 µg/ml	>16	1,5	-	255,9
K56-23 WT	0,125 µg/ml	0,0625 µg/ml	0,0078125	0,0078125		
K56-23 CIP	32 µg/ml	192 µg/ml	>16	48	-	6144
K56-24 WT	0,125 µg/ml	0,0625 µg/ml	0,00390625	0,00292969		
K56-24 CIP	32 µg/ml	192 µg/ml	>16	36	-	12287,9
K56-25 WT	0,125 µg/ml	0,0625 µg/ml	0,0078125	0,0078125		
K56-25 MEC	0,125 µg/ml	0,03125 µg/ml	0,00390625	0,00585938	0,5	0,75
K56-25 CIP	32 µg/ml	64 µg/ml	>16	3	-	384
K56-25 M→C	32 µg/ml	32 µg/ml	>16	1	-	128
K56-25 C→M	32 µg/ml	64 µg/ml	>16	3	-	384
K56-26 WT	0,125 µg/ml	0,0625 µg/ml	0,0078125	0,0078125		
K56-26 MEC	0,125 µg/ml	0,03125 µg/ml	0,00390625	0,00390625	0,5	0,5
K56-26 CIP	32 µg/ml	64 µg/ml	>16	3	-	384
K56-26 M→C	32 µg/ml	32 µg/ml	>16	1,5	-	192
K56-26 C→M	32 µg/ml	64 µg/ml	>16	3	-	384
K56-29 WT	0,125 µg/ml	0,0625 µg/ml	0,0078125	0,00585939		
K56-29 MEC	0,125 µg/ml	0,03125 µg/ml	0,015625	0,0078125	2	1,33
K56-29 CIP	32 µg/ml	64 µg/ml	>16	8	-	1365,3
K56-30 WT	0,125 µg/ml	0,0625 µg/ml	0,0078125	0,00585938		
K56-30 MEC	0,125 µg/ml	0,0625 µg/ml	0,0078125	0,00585938	1	1
K56-30 CIP	32 µg/ml	32 µg/ml	2	2	256	341,3
K56-30 M→C	32 µg/ml	32 µg/ml	2	3	256	511,9
K56-30 C→M	32 µg/ml	32 µg/ml	4	2	512	341,3
K56-35 WT	0,125 µg/ml	0,03125 µg/ml	0,00390625	0,00585938		
K56-35 MEC	0,125 µg/ml	0,03125 µg/ml	0,00390625	0,00390625		
K56-38 WT	0,125 µg/ml	0,0625 µg/ml	0,0078125	0,00585938		
K56-38 MEC	0,125 µg/ml	0,03125 µg/ml	0,00390625	0,00390625	0,5	0,61
K56-38 CIP	32 µg/ml	192 µg/ml	>32	72	-	12287,9

K56-63 WT	0,125 µg/ml	ND	0,015625	ND		
K56-63 MEC	0,125 µg/ml	ND	0,015625	ND		
K56-67 WT	0,125 µg/ml	0,0625 µg/ml	0,0078125	0,00390625		
K56-67 MEC	0,125 µg/ml	0,03125 µg/ml	0,0078125	0,00292969	1	0,75
K56-73 WT	0,125 µg/ml	ND	ND	ND		
K56-73 MEC	0,125 µg/ml	ND	ND	ND		
K56-76 WT	0,125 µg/ml	0,25 µg/ml	0,015625	0,0078125		
K56-76 MEC	0,125 µg/ml	0,25 µg/ml	0,015625	0,0078125	1	1
K56-77 WT	0,125 µg/ml	0,25 µg/ml	0,015625	0,0078125		
K56-77 MEC	0,125 µg/ml	0,125 µg/ml	0,0078125	0,00585938	0,5	0,75
K56-80 WT	0,125 µg/ml	0,125 µg/ml	0,015625	0,00585938		
K56-80 MEC	0,125 µg/ml	0,0625 µg/ml	0,0078125	0,00585938	0,5	1

Gentamicin

Strains	Highest concentration 2-fold	Highest concentration 1,5-fold	IC90 2-fold	IC90 1,5-fold	Fold change	
					IC90 2-fold	IC90 1,5-fold
K56-22 WT	2 µg/ml	4 µg/ml	0,25	0,5		
K56-22 MEC	2 µg/ml	2 µg/ml	0,25	0,1875	1	0,375
K56-22 CIP	2 µg/ml	2 µg/ml	0,25	0,125	1	0,25
K56-22 M→C	2 µg/ml	1 µg/ml	0,125	0,125	0,5	0,25
K56-22 C→M	2 µg/ml	1 µg/ml	0,125	0,1875	0,5	0,375
K56-23 WT	2 µg/ml	4 µg/ml	0,5	0,375		
K56-23 CIP	2 µg/ml	2 µg/ml	0,25	0,1875	0,5	0,5
K56-24 WT	2 µg/ml	4 µg/ml	0,5	0,375		
K56-24 CIP	2 µg/ml	2 µg/ml	0,25	0,1875	0,5	0,5
K56-25 WT	2 µg/ml	8 µg/ml	1	0,5		
K56-25 MEC	2 µg/ml	2 µg/ml	0,25	0,1875	0,25	0,375
K56-25 CIP	2 µg/ml	2 µg/ml	0,25	0,1875	0,25	0,375
K56-25 M→C	2 µg/ml	2 µg/ml	0,25	0,125	0,25	0,25
K56-25 C→M	2 µg/ml	2 µg/ml	0,25	0,1875	0,25	0,375

K56-26 WT	2 µg/ml	4 µg/ml	0,5	0,375		
K56-26 MEC	2 µg/ml	2 µg/ml	0,25	0,1875	0,5	0,5
K56-26 CIP	2 µg/ml	1 µg/ml	0,125	0,125	0,25	0,33
K56-26 M→C	2 µg/ml	1 µg/ml	0,0625	0,09375	0,125	0,25
K56-26 C→M	2 µg/ml	2 µg/ml	0,25	0,1875	0,5	0,5
K56-29 WT	2 µg/ml	4 µg/ml	0,5	0,375		
K56-29 MEC	2 µg/ml	8 µg/ml	>1	1,5	-	4
K56-29 CIP	2 µg/ml	2 µg/ml	0,125	0,125	0,25	0,33
K56-30 WT	2 µg/ml	4 µg/ml	1	0,25		
K56-30 MEC	2 µg/ml	2 µg/ml	0,25	0,125	0,25	0,5
K56-30 CIP	2 µg/ml	1 µg/ml	0,25	0,0625	0,25	0,25
K56-30 M→C	2 µg/ml	1 µg/ml	0,125	0,046875	0,125	0,1875
K56-30 C→M	2 µg/ml	1 µg/ml	0,125	0,046875	0,125	0,1875
K56-35 WT	2 µg/ml	ND	0,5	ND		
K56-35 MEC	2 µg/ml	ND	0,125	ND		
K56-38 WT	6 µg/ml	6 µg/ml	0,5	0,25		
K56-38 MEC	6 µg/ml 6 µg/ml	6 µg/ml	0,25	0,1875	0,5	0,75
K56-38 CIP	6 µg/ml	2 µg/ml	0,25	0,1875	0,5	0,75
K56-63 WT	6 µg/ml	ND	0,5	ND		
K56-63 MEC	6 µg/ml	ND	1	ND		
K56-67 WT	6 µg/ml	6 µg/ml	0,5	0,25		
K56-67 MEC	6 µg/ml	2 µg/ml	0,25	0,125	0,5	0,5
K56-73 WT	6 µg/ml	ND	0,5	ND		
K56-73 MEC	6 µg/ml	ND	0,0625	ND		
K56-76 WT	6 µg/ml	6 µg/ml	0,5	0,375		
K56-76 MEC	6 µg/ml	2 µg/ml	0,25	0,1875	0,5	0,5
K56-77 WT	6 µg/ml	6 µg/ml	0,5	0,375		
K56-77 MEC	6 µg/ml 6 µg/ml	2 µg/ml	0,25	0,1875	0,5	0,5
K56-80 WT	6 µg/ml	8 µg/ml	1	0,25		
K56-80 MEC	6 µg/ml	2 µg/ml	0,25	0,1875	0,25	0,75

Mecillinam

Strains	Highest concentration 2-fold	Highest concentration 1,5-fold	IC90 2-fold	IC90 1,5-fold	Fold change	
					IC90 2-fold	IC90 1,5-fold

K56-22 WT	1 µg/ml	6 µg/ml	0,25	0,1875		
K56-22 MEC	64 µg/ml	256 µg/ml	32	48	128	256
K56-22 CIP	1 µg/ml	1 µg/ml	0,125	0,1875	0,5	1
K56-22 M→C	64 µg/ml	256 µg/ml	>32	48	-	256
K56-22 C→M	64 µg/ml	64 µg/ml	8	12	32	64
K56-23 WT	1 µg/ml	1 µg/ml	0,125	0,125		
K56-23 CIP	1 µg/ml	0,5 µg/ml	0,0625	0,0625	0,5	0,5
K56-24 WT	1 µg/ml	1 µg/ml	0,125	0,09375		
K56-24 CIP	1 µg/ml	0,5 µg/ml	0,0625	0,125	0,5	1,33
K56-25 WT	1 µg/ml	6 µg/ml	0,25	0,1875		
K56-25 MEC	64 µg/ml	128 µg/ml	16	12	64	64
K56-25 CIP	1 µg/ml	6 µg/ml	0,25	0,125	1	0,67
K56-25 M→C	64 µg/ml	1 µg/ml	0,125	0,375	0,5	2
K56-25 C→M	64 µg/ml	64 µg/ml	8	8	32	42,67
K56-26 WT	1 µg/ml	6 µg/ml	0,125	0,1875		
K56-26 MEC	64 µg/ml	32 µg/ml	4	4	32	21,33
K56-26 CIP	1 µg/ml	1 µg/ml	0,0625	0,0625	0,5	0,33
K56-26 M→C	64 µg/ml	6 µg/ml	0,5	24	4	128
K56-26 C→M	64 µg/ml	6 µg/ml	0,5	0,1875	4	1
K56-29 WT	1 µg/ml	6 µg/ml	0,125	0,1875		
K56-29 MEC	64 µg/ml	128 µg/ml	>32	64	-	341,33
K56-29 CIP	1 µg/ml	2 µg/ml	0,25	0,1875	2	1
K56-30 WT	1 µg/ml	6 µg/ml	0,25	0,1875		
K56-30 MEC	64 µg/ml	256 µg/ml	32	24	128	128
K56-30 CIP	1 µg/ml	6 µg/ml	0,5	0,25	2	1,33
K56-30 M→C	64 µg/ml	256 µg/ml	32	16	128	85,33
K56-30 C→M	64 µg/ml	256 µg/ml	32	24	128	1
K56-35 WT	1 µg/ml	0,5 µg/ml	0,03125	0,0625		
K56-35 MEC	64 µg/ml	6 µg/ml	0,25	1		
K56-38 WT	1 µg/ml	6 µg/ml	0,25	0,1875		
K56-38 MEC	64 µg/ml	256 µg/ml	32	24	128	128
K56-38 CIP	1 µg/ml	4 µg/ml	0,5	0,5	2	2,67
K56-63 WT	1 µg/ml	ND	0,25	ND		
K56-63 MEC	64 µg/ml	ND	>32	ND		
K56-67 WT	1 µg/ml	1 µg/ml	0,125	0,125		
K56-67 MEC	64 µg/ml	256 µg/ml	32	16	256	128

K56-73 WT	1 µg/ml	ND	0,125	ND		
K56-73 MEC	64 µg/ml	ND	ND	ND		
K56-76 WT	1 µg/ml	6 µg/ml	0,25	0,1875		
K56-76 MEC	64 µg/ml	256 µg/ml	32	24	128	128
K56-77 WT	1 µg/ml	1 µg/ml	0,125	0,125		
K56-77 MEC	64 µg/ml	256 µg/ml	32	8	256	64
K56-80 WT	1 µg/ml	6 µg/ml	0,25	0,125		
K56-80 MEC	64 µg/ml	128 µg/ml	16	16	64	128

Trimethoprim

Strains	Highest concentration 2-fold	Highest concentration 1,5-fold	IC90 2-fold	IC90 1,5-fold	Fold change	
					IC90 2-fold	IC90 1,5-fold
K56-22 WT	6 µg/ml	6 µg/ml	0,375	0,375		
K56-22 MEC	6 µg/ml	1,5 µg/ml	0,1875	0,140625	0,5	0,375
K56-22 CIP	6 µg/ml	6 µg/ml	0,375	0,28125	1	0,75
K56-22 M→C	6 µg/ml	6 µg/ml	0,375	0,375	1	1
K56-22 C→M	6 µg/ml	3 µg/ml	0,375	0,28125	1	0,75
K56-23 WT	6 µg/ml	6 µg/ml	0,375	0,375		
K56-23 CIP	6 µg/ml	24 µg/ml	3	2,25	8	6
K56-24 WT	6 µg/ml	1,5 µg/ml	0,1875	0,140625		
K56-24 CIP	6 µg/ml	12 µg/ml	1,5	1,5	8	10,67
K56-25 WT	6 µg/ml	6 µg/ml	0,375	0,375		
K56-25 MEC	6 µg/ml	6 µg/ml	0,375	0,28125	1	0,75
K56-25 CIP	6 µg/ml	16 µg/ml	>3	3	-	8
K56-25 M→C	6 µg/ml	12 µg/ml	1,5	1,5	4	4
K56-25 C→M	6 µg/ml	12 µg/ml	1,5	1,5	4	4
K56-26 WT	6 µg/ml	6 µg/ml	0,375	0,375		
K56-26 MEC	6 µg/ml	6 µg/ml	0,75	0,5	2	1,33
K56-26 CIP	6 µg/ml	6 µg/ml	0,75	1	2	2,67
K56-26 M→C	6 µg/ml	6 µg/ml	0,75	0,5	2	1,33
K56-26 C→M	6 µg/ml	16 µg/ml	3	1,5	8	4

K56-29 WT	6 µg/ml	6 µg/ml	0,375	0,375		
K56-29 MEC	6 µg/ml	6 µg/ml	0,75	0,5625	2	1,5
K56-29 CIP	6 µg/ml	16 µg/ml	3	2	8	5,33
K56-30 WT	6 µg/ml	6 µg/ml	0,375	0,375		
K56-30 MEC	6 µg/ml	6 µg/ml	0,1875	0,1875	0,5	0,5
K56-30 CIP	6 µg/ml	12 µg/ml	1,5	1,125	4	3
K56-30 M→C	6 µg/ml	12 µg/ml	1,5	1,5	4	4
K56-30 C→M	6 µg/ml	12 µg/ml	1,5	2,25	4	6
K56-35 WT	6 µg/ml	ND	0,1875	ND		
K56-35 MEC	6 µg/ml	ND	0,1875	ND		
K56-38 WT	6 µg/ml	3 µg/ml	0,375	0,28125		
K56-38 MEC	6 µg/ml	2 µg/ml	0,1875	0,25	0,5	0,89
K56-38 CIP	6 µg/ml	6 µg/ml	0,75	0,75	2	2,67
K56-63 WT	6 µg/ml	ND	0,75	ND		
K56-63 MEC	6 µg/ml	ND	1,5	ND		
K56-67 WT	6 µg/ml	1,5 µg/ml	0,1875	0,1875		
K56-67 MEC	6 µg/ml	1 µg/ml	0,09375	0,09375	0,5	0,5
K56-73 WT	6 µg/ml	ND	0,1875	ND		
K56-73 MEC	6 µg/ml	ND	3	ND		
K56-76 WT	6 µg/ml	3 µg/ml	0,375	0,375		
K56-76 MEC	6 µg/ml	3 µg/ml	0,375	0,28125	1	0,75
K56-77 WT	6 µg/ml	3 µg/ml	0,375	0,28125		
K56-77 MEC	6 µg/ml	3 µg/ml	0,375	0,28125	1	1
K56-80 WT	6 µg/ml	1,5 µg/ml	0,1875	0,28125		
K56-80 MEC	6 µg/ml	3 µg/ml	0,375	0,375	2	1,33

Appendix G: Biofilm formation

		Average value		
Strain	**Parallel 1**	**Parallel 2**	**Parallel 3**	**Average**

K56-22 WT	0,1094	0,1404	0,1364	0,1288
K56-22 MEC	0,0985	0,0956	0,1045	0,0995
K56-22 CIP	0,0978	0,0956	0,0905	0,0947
K56-22 M→C	0,1217	0,1247	0,1302	0,1255
K56-22 C→M	0,0929	0,0883	0,0826	0,088
K56-23 WT	0,15	0,2801	0,1576	0,195925
K56-23 CIP	0,1045	0,123	0,1042	0,1106
K56-24 WT	0,0775	0,0922	0,0829	0,0842
K56-24 CIP	0,0915	0,1084	0,0837	0,0945
K56-25 WT	0,2081	0,2489	0,2753	0,2441
K56-25 MEC	0,2866	0,3037	0,2977	0,296
K56-25 CIP	0,0726	0,1163	0,1089	0,0993
K56-25 M→C	0,3448	0,7685	0,5826	0,5653
K56-25 C→M	0,0809	0,0989	0,1003	0,0934
K56-26 WT	0,095	0,0929	0,1092	0,099
K56-26 MEC	0,1529	0,2497	0,2691	0,2239
K56-26 CIP	0,0857	0,0758	0,0923	0,0846
K56-26 M→C	0,2509	0,2735	0,2599	0,2614
K56-26 C→M	0,0797	0,0765	0,0779	0,0781
K56-29 WT	0,1487	0,1657	0,2593	0,1912
K56-29 MEC	0,1568	0,2237	0,2552	0,2119
K56-29 CIP	0,0865	0,105575	0,1151	0,1024
K56-30 WT	0,1338	0,1629	0,1090	0,1353
K56-30 MEC	0,2797	0,4287	0,3016	0,3367
K56-30 CIP	0,1004	0,1847	0,2656	0,1836
K56-30 M→C	0,0858	0,082	0,0761	0,0813
K56-30 C→M	0,1144	0,1216	0,0893	0,1085
K56-38 WT	0,1490	0,2569	0,1735	0,1931
K56-38 MEC	0,2323	0,2369	0,2070	0,2254
K56-38 CIP	0,0775	0,0753	0,0793	0,0774
K56-67 WT	0,1678	0,301	0,1875	0,2186
K56-67 MEC	0,1374	0,113	0,1018	0,1173

K56-76 WT	0,1724	0,1997	0,2016	0,1913
K56-76 MEC	0,2061	0,1901	0,1383	0,1782
K56-77 WT	0,1217	0,1199	0,1104	0,1173
K56-77 MEC	0,1814	0,1678	0,1537	0,1676
K56-80 WT	0,1344	0,2781	0,1535	0,1887
K56-80 MEC	0,2905	0,2433	0,1960	0,2433

APPENDIX