Three Steps to Antibiotic Resistance?

The Development of Tigecycline Resistance in the Gram-Negative Bacteria Escherichia coli and Salmonella typhimurium

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ABSTRACT

In the light of increasing antimicrobial resistance and especially the growing prevalence of infections caused by multiresistant gram-negative bacteria in recent years, the introduction of tigecycline in 2005 to the repertoire of ammunition was a welcomed addition.

Tigecycline (TGC) belongs to a new class of antimicrobials, the semisynthetic glycylcyclines. It is a derivate of minocycline with additional side chain attached to the 9’ carbon on the tetracycline four-ringed skeleton. TGC has in vitro potency towards many important clinical multiresistant pathogens ranging gram-positive to gram-negative bacteria. Additionally, tigecycline is the only drug alongside colistin that can be used against the recently emerged New Delhi metallo-β-lactamase-1 (NDM-1) possessing strains of Escherichia coli and Klebsiella pneumoniae.

Tigecycline can evade the common tetracycline resistance mechanisms; active efflux and ribosomal protection mediated by tet-genes. This study was initiated to investigate the development of resistance towards tigecycline in gram-negative bacteria.

The wild-type (wt) strains of Escherichia coli and Salmonella typhimurium were exposed to increasing concentrations of tigecycline, mutants were collected from the Müeller-Hinton plates and the susceptibility to the antibiotic was determined by minimum inhibitory concentration (MIC). Mutation frequencies were calculated using the Lea & Coulson median method. Altogether three selection steps were conducted and the growth rates of the mutants were measured and compared to the parental strains to evaluate possible fitness cost of the decreased drug susceptibility. Cross-resistance was tested for tetracycline and chloramphenicol with Etests.

The results show that clinical breakpoints of resistance to tigecycline can be achieved only in three steps. There was no correlation between the mutation frequencies with increasing TGC concentrations. The fitness of the mutants decreased <10% in the first step and for the second and third step mutants the reduction of fitness was higher, ranging from 10-55% compared to the wt. Higher tigecycline MIC values compared to wt were observed in both bacteria for tetracycline and chloramphenicol after TGC exposure.
1. INTRODUCTION

“Nowadays people know the price of everything, and the value of nothing”  
Oscar Wilde, The Picture of Dorian Gray.

Bacteria are masters of survival skills. The ability to cope in hostile environments culminates with species that endure highly acidic environments or ones that tolerate thousand times more radiation than humans do. Rapid growth of bacteria facilitates rapid evolution, and adaptation to different environments can be seen only after few generations of growth under selective pressure. Therefore, resistance is a natural outcome of the use of antimicrobials and we cannot prevent it from happening. We can, however, try to minimize the selection for resistant strains by rationalizing the use of antibiotics by carefully selecting the drug of choice, assessing the dosage duration and trying to adhere people to the treatment (Martinez and Silley 2010).

Unfortunately, the uncontrolled use of these drugs in the past decades has created a situation where the treatment of infection has become more difficult due to the increasing number of resistant bacteria. The emergence of the commensal gram-negative bacteria, which can arise from patient’s own microbial flora, like Klebsiella, Pseudomonas, Acinetobacter and Burkholderia to the stage of resistance aside with common pathogens, are a growing problem. These bacteria can possess multidrug tolerance or resistance to begin with (Hawkey and Finch 2006). A good example of multiresistannce is a human gut bacteria Pseudomonas spp., which has extremely potent natural efflux systems to pump different antibiotics out of the cell. This situation combined with the lack of new antimicrobials released to the market poses a major threat to global health care, increasing mortality and morbidity (Cars and Nordberg 2005).

The focus of this project was to elucidate the evolution of resistance towards tigecycline. Tigecycline is the first glycyclcline class antimicrobial and a third generation tetracycline. The well-studied gram-negative organisms Escherichia coli and Salmonella enterica serovar Typhimurium LT2 were chosen to investigate the rise of resistance to tigecycline. These bacteria are also easy to culture; they grow fast and additionally have well-established systems and tools to conduct various genetic experiments.
1.1 Antimicrobials and resistance mechanisms

The action of an antibiotic can be bacteriostatic, meaning that it will prevent the bacteria from dividing, or it can be bacteriocidal, thus killing the bacteria. There are numerous antibiotic classes, which are divided into groups based on their target, chemical structure and their spectrum of activity (Kohanski et al. 2010).

Correspondingly, there are numerous ways for bacteria to avoid the killing mechanisms of the antimicrobials. Bacteria can produce enzymes that degrade the compound; such is the case in the resistance toward β-lactams, which attack the bacterial cell wall. Drugs like tetracyclines and macrolides are expelled from the bacteria via efflux pumps before they reach their ribosomal target and inhibit the protein synthesis (Table 1).

### Table 1. Five different antimicrobial classes, some of the mechanisms for the resistance and an example of how the resistance is conferred (Table adapted from Kohanski et al. 2010 and Murray et al. 2009).

<table>
<thead>
<tr>
<th>Antibiotic Class</th>
<th>Mechanism of Resistance</th>
<th>Main example (a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-lactams</td>
<td>Enzymatic inactivation</td>
<td>β-lactamase production</td>
</tr>
<tr>
<td>Disrupts the cell wall</td>
<td>Target modification</td>
<td>Mutations to PBP genes</td>
</tr>
<tr>
<td></td>
<td>Active efflux</td>
<td>Upregulation of efflux pumps</td>
</tr>
<tr>
<td></td>
<td>Membrane impermeability</td>
<td>Downregulation of porins</td>
</tr>
<tr>
<td>Aminoglycosides</td>
<td>Enzymatic inactivation</td>
<td>ANT, APH and AAC production</td>
</tr>
<tr>
<td>Inhibition of protein synthesis</td>
<td>Target alteration</td>
<td>mutations to proteins S12 and S5</td>
</tr>
<tr>
<td></td>
<td>Target modification</td>
<td>Acquisition of 16S rRNA methylases</td>
</tr>
<tr>
<td></td>
<td>Membrane impermeability</td>
<td>Defective active transport across the cytoplasmic membrane</td>
</tr>
<tr>
<td>Macrolides</td>
<td>Target modification</td>
<td>Acquisition of 23S rRNA methylases</td>
</tr>
<tr>
<td>Inhibition of protein synthesis</td>
<td>Active efflux</td>
<td>Upregulation of efflux pumps</td>
</tr>
<tr>
<td></td>
<td>Target alteration</td>
<td>23S mutations</td>
</tr>
<tr>
<td></td>
<td>Enzymatic inactivation</td>
<td>Production of esterases and phosphotransferases</td>
</tr>
<tr>
<td>Quinolones</td>
<td>Active efflux</td>
<td>Upregulation of efflux pumps</td>
</tr>
<tr>
<td>Inhibits nucleic acid synthesis</td>
<td>Target alteration</td>
<td>Mutations in Topo II enzymes</td>
</tr>
<tr>
<td></td>
<td>Enzymatic inactivation</td>
<td>Acquisition of acetyltransferase</td>
</tr>
<tr>
<td>Tetraacyclines</td>
<td>Active efflux</td>
<td>Upregulation of efflux pumps</td>
</tr>
<tr>
<td>Inhibition of protein synthesis</td>
<td>Target protection</td>
<td>tet(O) tet(M) and tet(S) acquisition</td>
</tr>
<tr>
<td></td>
<td>Enzymatic inactivation</td>
<td>tet(X)</td>
</tr>
</tbody>
</table>

(a) PBP = penicillin binding proteins; ANT = aminoglycoside nucleotidytransferase; APH = aminoglycoside phosphotransferase; AAC = aminoglycoside acetyltransferase

Further, the resistance mechanisms can be divided into intrinsic resistance and acquired resistance mechanisms. When a bacterium has an intrinsic resistance it means that it has the mechanism naturally encoded in its chromosome. For example, *Pseudomonas aeruginosa*, a clinically important gram-negative pathogen, has potent intrinsic efflux pumps, which can expel a number of different antibiotics making treatment very difficult (Dean et al. 2003). These efflux pumps belong to the nodulation resistance division (NRD) family and they confer antibiotic resistance in many gram-negative bacteria and have a wide range of substrates, not only antibiotics (Piddock 2006).
In contrast, acquired resistance is often the result of horizontal gene transfer (HGT) when a moving element such as a plasmid that carries resistance to one or more antimicrobials is introduced to the new host bacterium. Plasmids are highly dynamic in nature. By shuffling their gene content and acquiring new genes from the environment, they can be a combination of many circulating plasmids. These mosaic-like plasmids that carry multi-resistance are found to be behind in the increasing numbers of clinical outbreaks and e.g. the outbreak of ESBL-producing *Klebsiella pneumoniae* in Uppsala University Hospital in 2005-2008 was caused by such a plasmid (Sandegren, unpublished).

New mechanisms for resistance are emerging; the cases of carbapenem resistant New Delhi metallo-β-lactamase-1 (NDM-1) possessing strains of *Escherichia coli* and *Klebsiella pneumoniae* being a recent example (Kumarasamy et al. 2010).

**1.2 Mutations, selection and fitness cost**

Mutations arise spontaneously in bacterial populations creating versatility in the genome. This in turn can give an advantage to the organism in changing environments. When the population is exposed to selective conditions e.g. an antibiotic, the cells with pre-existing mutations that are beneficial in that environment may be selected and they will outgrow the ones without the mutation (Snyder and Champress 2007).

Point mutations are non-synonymous or synonymous changes in a single nucleotide, which either alter an amino acid or cause no change in the in the protein, respectively. Frameshift mutations, which e.g. can be a result of a insertion or a deletion of a nucleotide, can change the ribosomal reading frame and may lead to non-functional protein or cause a complete stop in the protein translation. In addition to aforementioned mechanisms, repetitive sequences within the genome can promote for inversions, where a stretch of nucleotides is inverted 180°, and deletions where a part of the sequence is lost due to errors during DNA replication and repair (Snyder and Champress 2007).

One of the most common mutations found in the bacterial chromosomes and plasmids are gene duplications/amplifications (GDA). GDAs are relatively easy to acquire, but also to loose due to intrinsic instability. If there is no selection pressure to maintain the duplicated region it may be lost during normal growth. Conversely, if the duplication gives higher fitness in certain environments it may be amplified and further enriched in the bacterial population (Sandegren and Andersson 2009). It has been estimated that at least 10% of the bacteria growing in non-selective conditions have duplications in their genomes. This resulting variation from the GDAs is suggested to be in the frontline mediating adaptive responses to novel conditions and acting as a bridge for more permanent and stable genetic responses such as point mutations. Therefore, GDAs are believed to be of special importance in the development of antibiotic resistance (Andersson and Hughes 2009).
On many occasions the mutations that result in decreased susceptibility to antibiotics do not come without a cost. This fitness cost in bacteria can be observed as longer generation times or reduced virulence making the transmission from one host to another harder due to metabolic changes in the cell. However, bacteria can ameliorate the biological cost by acquiring compensatory mutations that restore the fitness without losing the resistance (Shulz zur Wiesch et al. 2010; Nagaev et al. 2001). These compensatory mutations can be e.g. point mutations, but a role of GDAs has also been detected in experiments with actinonin (deformylase inhibitor) resistant *S. typhimurium* LT2 isolates, where the severely reduced fitness was alleviated by amplification of the genes for initiator tRNA (Nilsson et al. 2006).

### 1.3 Mutation rates and mutation frequencies

Mutations are stochastic in nature, yet the *mutation rate* is not constant in all parts of the genome. Studies have shown that *Haemophilus influenzae* and *Neisseria meningitides* (Denamur and Matic 2006) have hypermutability loci with high mutation rates. Conversely, genes that are close to the origin of replication (ori) of the chromosome have lower rates of mutation compared to those further away (Denamur and Matic 2006).

The fidelity of DNA replication is dependent on various mismatch repair systems and of the accuracy of the DNA polymerase. In *E. coli* the normal mutation rate is one mistake in $10^{10}$ nucleotides after DNA replication, but mutations in methyl-directed mismatch-repair (MMR) systems, like in the genes encoding for proteins MutL and MutS, can increase the mutation rate 100-1000 fold (Denamur and Matic 2006). The stress-responsive DNA polymerases V (*umuCD*) and IV (*dinB*) have also been demonstrated to increase the mutation rate transiently e.g. rifampicin resistance rates in *S. typhimurium* under starvation (Martinez and Baquero 2000).

Mutations are not always beneficial, but can be deleterious or neutral, meaning that the fate of the mutation is to be counter-selected and possibly disappear from the population or it has no effect on the host, respectively. It is also expected that all bacterial populations include mismatch repair-deficient individuals, and by chance they can have mutations that confer resistance to an antibiotic and therefore have a selective advantage when being exposed to the drug. (Denamur and Matic 2006).

The strains that harbour these mismatch-repair mutations are called mutators and they are often found in clinical samples, especially in chronic infections when bacteria have been exposed to the drug for longer periods of time. *Pseudomonas aeruginosa* that causes persistent infections in cystic fibrosis patients and *Helicobacter pylori* in patients with chronic gastritis are bacteria that have been associated with hypermutability (Björkholm et al. 2001).
Besides fitness cost, *mutation frequency* is of importance when trying to foresee the development of resistance (Björkholm *et al.* 2001). The difference between mutation frequency and rate is that while the rate is an overall number of mutation events in the whole genome independent of time and environment, the frequency is the occurrence of a mutation in a specific time under specific conditions in one cell (Martinez and Baquero 2000). This can be used in experimental evolution studies exposing bacteria to antimicrobials and counting the fraction that survived the treatment. Baquero *et al.* (2004) have divided bacteria into the following categories based on mutation frequencies after exposure to rifampicin: hypomutable $\geq 8 \times 10^{-9}$; normutable $8 \times 10^{-9}$; weak hypermutable $4 \times 10^{-8} \leq 4 \times 10^{-7}$ and strong hypermutable $\geq 4 \times 10^{-7}$.

### 1.4 Minimum inhibitory concentration (MIC) and mutant prevention concentration (MPC)

One important way to assess the antibiotic susceptibility of a bacterium is the determination of a minimum inhibitory concentration (MIC). It is the lowest concentration of an antimicrobial that will inhibit the visible growth of the tested organism and this method is used routinely in clinical laboratories to determine the best possible antimicrobial therapy. The SIR system was developed to categorize microorganisms based on their susceptibility (S), intermediate (I) or resistance (R) when predisposed to an antimicrobial. Clinically, resistance (R) means that the concentration to inhibit the growth of the microbe is higher than the safe drug concentration in the body and therefore clearance cannot be achieved. When the MIC is in intermediate (I) level, there is a high risk that the chosen drug therapy will fail. In addition, MIC testing is a vital part in evaluation of new drugs and their potency toward pathogens and these results are used to verify the breakpoints for antimicrobials (Andrews, 2001).

It is important to emphasize that genetic resistance does not mean the same as clinical resistance, which is based on the SIR system. Genetic resistance through obtained mutations gives the means for the bacteria to become resistant, but it does not directly nor necessarily confer clinical resistance. It is has been seen that accumulation of low-level resistance mutations eventually can lead to clinical resistance (Martinez *et al.* 2007).

Mutant prevention concentration (MPC) is another way to measure the *in vitro* susceptibility. MPC is the concentration that inhibits the bacterial growth plating higher numbers of bacteria $\sim 10^5$-$10^{10}$ cfu, closer to what is found in real infections, and therefore presents a more realistic environment than in the MIC testing, where the standardized inoculum is $\sim 10^5$cfu. In theory, this concentration should prevent the rise of single-step resistant mutants.

It has been proposed that these two measures MIC and MPC could be used to determine the mutant selection window (MSW), bordered by these two values and this represents the range of antibiotic concentration where the selection for resistant
bacteria may occur. This method could also be used when trying to predict and prevent the evolution of resistance, which should be a parallel goal with curing the infection itself (Blondeau 2009; Drlika 2003).

1.5 Tetracyclines

One of the most important classes of antibiotics is the tetracyclines. Tetracycline belongs to the group of antimicrobials that mediate their bacteriostatic effect via the 30S subunit of the ribosome. The mechanism is to block the aminoacyl-tRNA binding to the A-site, preventing the codon-anticodon interaction and hence halting protein synthesis (Kohanski et al. 2010).

The first-generation tetracyclines, isolated in the soil bacteria actinomycetes, were introduced to the clinical field in the 1940’s. They were cheap, had no severe side effects and could be administered orally. This resulted in extensive and widespread use in human and animal healthcare as well as in agriculture, and in 1953 the first resistant bacterium was discovered. Alarmingly, various resistance mechanisms emerged at the same rate as new derivates were introduced (Pankey 2005; Thaker et al. 2010). The second-generation tetracyclines, semi-synthetic minocycline and doxycycline were released in the 1960’s. In 2005, the first third generation tetracycline, tigecycline was released to relieve the growing pressure of clinical resistance of many important pathogens.

In studies of bacteria in natural environments, the tetracycline resistance is widespread and common; possible resistance determinants e.g. in the soil microbes are found even for the new classes of tetracyclines such as minocycline and tigecycline (Thaker et al. 2010).

The main resistance mechanisms to tetracycline are active efflux and ribosomal protection through numerous tet-genes, yet inactivation of the drug can be mediated via a tet(X) encoded enzyme (Moore et al. 2005).

1.6 Tigecycline

Tigecycline, TGC (previously known as GAR-936) has in vitro potency towards many aerobic or anaerobic gram-negative and gram-positive pathogens. Clinically, the most important ones are methicillin-resistant Staphylococcus aureus (MRSA) and penicillin-resistant Streptococcus pneumoniae (PRSP), vancomycin-resistant enterococci (VRE) and extended-spectrum β-lactamase-producing (ESBL) strains of Escherichia coli and Klebsiella pneumoniae (Townsend et al. 2006). Tigecycline is also the only drug alongside colistin that can be used in the treatment of infections caused by the New Delhi NDM-1 strains of Escherichia coli and Klebsiella pneumoniae (Kumarasamy et al. 2010).
The clinical breakpoints (EUCAST) for *Enterobactericeae* (*Escherichia coli* and *Salmonella typhimurium*) are $S \leq 1$ and $R \geq 2$ mg/L. Using SIR classification this means that strains with tigecycline MIC values of 1 mg/L or under are regarded as susceptible (S) and clinically resistant (R) strains have a value of 2 mg/L or over. The intermediate (I) strains are between these two values.

### 1.6.1 Characteristics and indications of use

Tigecycline belongs to a new class of semisynthetic antimicrobials, the glyyclcyclines. It is a derivate of minocycline with additional side chain attached the 9’ carbon on the tetracycline four-ringed skeleton (Fig. 1) (Townsend *et al.* 2006).

![Image](image1.png)

*Figure 1. The chemical structures of tetracycline (left) and tigecycline (right). The main structural differences in tigecycline are marked with red circles.*

The action of tigecycline is based on inhibiting translation by binding to the 30S ribosomal subunit’s A-site and blocking the aminoacyl-tRNA binding to the ribosome. This action is shared with other tetracyclines. Tigecycline’s efficacy of inhibiting protein synthesis is 3-fold higher than minocycline and 20-fold higher than tetracycline. Tigecycline’s potency towards microbes that are tetracycline resistant is mediated through the bulky side chain, which makes the drug bind with higher affinity to its target. The additional interactions of the drug on the ribosome’s H34 and H18 nucleotides, which are not observed with tetracycline or minocycline, are presumed to increase the binding affinity (Olson *et al.* 2006). These characteristics give TGC the ability to evade the common tetracycline resistance mechanisms; the active efflux via outer membrane pumps and the protection of the ribosome (Magalhães de Silva and Nunes Salgado 2010; Bergeron *et al.* 1996).

Tigecycline is approved as a treatment for complicated intra-abdominal and skin and soft tissue infections worldwide. Additionally, TGC is used in US also for medicating community-acquired pneumonia. TGC is administered only intravenously; in healthy individuals the maximum concentration in serum reaches 0.85-1 mg/L and is extensively taken up into tissues: the concentrations found in e.g. alveolar cells were of 78-fold compared to serum levels. The side effects are similar to other tetracyclines and include nausea, vomiting and diarrhoea, additionally colouring of the teeth and bones can occur (Peterson 2008; Pankey 2005).
Due to the increasing numbers of multiresistant bacterial strains like *Acinetobacter baumannii*, the clinicians have been prescribing tigecycline with off-label indications, such as ventilator-associated pneumonia (VAP). A study in Spain by Conde-Estévez et al. (2010) showed that almost one third of the prescribed tigecycline was off-label and it was given to patients in the intensive care unit with severe conditions as a rescue therapy in combination with other antimicrobials.

### 1.6.2 Resistance to tigecycline

There are bacteria that are inherently resistant to tigecycline. *Pseudomonas aeruginosa* has efflux pumps, belonging to the resistance nodulation division (RND) family, that expel the drug efficiently from the cell (Dean et al. 2003). Other bacteria that have reduced susceptibility to TGC belong to the groups of *Proteus* spp., *Morganella* spp. and *Providencia* spp. Alongside *Pseudomonas* spp., these bacteria have not received breakpoints from EUCAST (http://www.eucast.org).

In clinical isolates, several mutations have been implicated as the cause of reduced susceptibility to TGC. The upregulation of AcrAB efflux pumps and mutations in its global regulators *ramR* and *marA* confer resistance in *Salmonella typhimurium* (Abouzeed et al. 2008; Horiyama et al. 2010), *Enterobacter cloacae* (Hornsey et al. 2010), *Escherichia coli* (Keeney et al. 2008), *Morganella morganii* (Ruzin et al. 2005) and *Klebsiella pneumoniae* (Hentschke et al. 2010). The non-susceptibility to TGC in clinical isolates of *Acinetobacter baumannii* was traced to the overexpression of the *adeB* gene, which encodes the adeABC efflux pumps (Sun et al. 2010). In a clinical isolate of *Serratia marcescens* the upregulation of SdeXY-HasF efflux pumps conferred to TGC resistance (Hornsey et al. 2010a). All the aforementioned efflux pumps belong to the RND-family of transporters.

Structural and experimental studies have revealed that TGC can be enzymatically degraded by tet(X). Fortunately, there has not been any reported cases of tet(X) mediated tetracycline resistance in clinical isolates; it is found mostly in soil bacteria that exhibit a range of multidrug resistance. Although a homolog of the tet(X) gene has been found in the human gut microbe *Bacteroides thetaiotaomicron*; additional roles of human gut microbiota as a reservoir of resistance genes is under scrutiny (Volkers et al. 2011; Moore et al. 2005; Salyers et al. 2004).

Cross-resistance to tigecycline has been reported in cases when ciprofloxacin has been used for treatment against *Enterobacter cloacae* (Hornsey et al. 2010b). The resistance was possibly conferred via AcrAB efflux pumps, for which both tigecycline and ciprofloxacin are substrates.
1.7 The aims of this project

The broad antimicrobial spectrum of tigecycline makes it appealing in the situation where the options for treatment of multidrug-resistant pathogens are narrowing down. There are reports of clinical isolates that have become less susceptible or resistant to tigecycline but so far there are no released studies about how fast this occurs. Therefore this project was initiated to find out how fast clinical breakpoints can be reached in the gram-negative bacteria *E. coli* and *S. typhimurium*. Other points of interest were to test the fitness cost of resistance mutations for the bacteria and if cross-resistance with tetracycline and chloramphenicol occurs after tigecycline exposure.

2. RESULTS

2.1 Isolation of mutants and mutant prevention concentration (MPC)

The tigecycline MICs for the wt bacteria were determined before the first selection step to assess a baseline from which to calculate the increasing TGC concentrations for the first selection step. The growth of *E. coli* wt MG1655 was inhibited at the concentration of 0.047 mg/L, whereas for wt *S. typhimurium* it was 0.064 mg/L.

I isolated several *E. coli* and *S. typhimurium* mutants from Müeller-Hinton agar (MHA) plates with elevating concentrations of TGC. The isolation was done in step-wise manner; the mutants from the previous selection steps were parental strains for the next experiment. Furthermore, the selective concentrations for each step depended upon the varying MIC values of the selected parental strains. Only the mutant colonies that grew on the original selective concentration, when re-streaked onto same concentration, were saved for further investigations.

The wt *E. coli* MG1655 (DA5438) was the parental strain for the first step selection. Altogether 34 *E. coli* mutant strains were recovered from selective plates with TGC concentration of 0.118 mg/L.

For the second selection *E. coli* mutant strains, with different TGC susceptibility, were selected as parental strains. Mutant strain DA19140 derivates grew on 0.25 and 0.5 mg/L concentrations; 12 and 10 mutant strains were recovered respectively. The derivates from DA19153 grew on TGC concentration of 0.38 mg/L and 10 mutant strains were picked. DA19165 derivates grew on 0.5 mg/L and 9 mutant strains were obtained from these plates.

The parental strains for the third selection step were *E. coli* mutants DA20587 and DA20578. DA20587 derivates grew on 1.5 and 3 mg/L TGC concentrations and 2 and 8 mutant strains were obtained. DA20578 derivates grew on 1 and 4 mg/L TGC concentrations; 5 and 4 mutant strains were obtained, respectively.
Altogether, 94 mutant *E. coli* strains were isolated in three selection steps (Table 2). During experiments, as the concentration of TGC increased the number of recovered mutant colonies decreased, which is reflected in the number of obtained of mutant strains.

**Table 2. The number of *E. coli* mutants from three selection steps and selective concentrations of TGC.**

<table>
<thead>
<tr>
<th>Parental strain</th>
<th>No. of obtained mutant strains</th>
<th>Selective TGC conc. mg/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt <em>E. coli</em> MG1655</td>
<td>34*</td>
<td>0.188</td>
</tr>
<tr>
<td>DA19140</td>
<td>12</td>
<td>0.25</td>
</tr>
<tr>
<td>DA19140</td>
<td>10</td>
<td>0.5</td>
</tr>
<tr>
<td>DA19153</td>
<td>10</td>
<td>0.38</td>
</tr>
<tr>
<td>DA19165</td>
<td>9</td>
<td>0.5</td>
</tr>
<tr>
<td>DA20587</td>
<td>2</td>
<td>1.5</td>
</tr>
<tr>
<td>DA20587</td>
<td>8</td>
<td>3</td>
</tr>
<tr>
<td>DA20578</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>DA20578</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td><strong>TOTAL:</strong></td>
<td><strong>94</strong></td>
<td></td>
</tr>
</tbody>
</table>

*Forty subcultures were plated on TGC supplemented MHA plates for the first step selection for *E. coli* MG1655.

The wt *S. typhimurium* LT2 (DA6192) was the parental strain for the first step selection, from which 20 mutant strains were obtained from plates containing 0.256 mg/L of TGC.

*S. typhimurium* mutants DA19180, DA19189 and DA19195 were chosen as parental strains for the second selection step. The derivates of DA19180 grew on TGC concentration of 0.76 mg/L; the selection yielded 10 mutant strains. DA19189 derivates were isolated from plates containing 0.5 mg/L of TGC and 10 representatives were picked. DA19195 derivates grew on plates containing 0.76 mg/L of the drug and 10 strains were purified and saved.

For the third selection step for *S. typhimurium*, the parental strains were DA20778 and DA20785. DA20778 derivates grew on TGC concentration of 3 mg/L and 10 mutant strains were obtained. DA20785 derivates were isolated from 4 mg/L plates and 10 mutants were collected.

Seventy *S. typhimurium* mutants were collected in three selection steps (Table 3). In contrast to *E. coli* mutants, *S. typhimurium* mutants grew more stable on higher drug concentrations and isolates could be recovered in all the subcultures.
Table 3. The number of *S. typhimurium* mutants from the three selection steps and the selective concentrations of TGC.

<table>
<thead>
<tr>
<th>Parental strain</th>
<th>No. of obtained mutant strains</th>
<th>Selective TGC conc. mg/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt <em>S. typhimurium</em> LT2</td>
<td>20</td>
<td>0.256</td>
</tr>
<tr>
<td>DA19180</td>
<td>10</td>
<td>0.76</td>
</tr>
<tr>
<td>DA19189</td>
<td>10</td>
<td>0.5</td>
</tr>
<tr>
<td>DA19195</td>
<td>10</td>
<td>0.76</td>
</tr>
<tr>
<td>DA20778</td>
<td>10</td>
<td>3</td>
</tr>
<tr>
<td>DA20785</td>
<td>10</td>
<td>4</td>
</tr>
<tr>
<td><strong>TOTAL:</strong></td>
<td><strong>70</strong></td>
<td></td>
</tr>
</tbody>
</table>

The mutant prevention concentration (MPC), the concentration that no visible colonies could be seen when inoculating the plates with higher cell densities, was for *E. coli* 0.752 mg/L and for *S. typhimurium* 1.024 mg/L. Both of these values were 16-times higher compared to the concentrations, which inhibit the growth of the wt bacteria.

The morphology of the colonies on MH plates changed from the wt in both bacteria. Out of the 94 *E. coli* mutants that were obtained, approximately 62% grew to a smaller colony size compared to the wt and 27% had a mucoid phenotype. In the 70 *S. typhimurium* mutants that were collected, 60% grew in smaller colonies than the wild type and 24% had a mucoid phenotype.

### 2.2 Mutation frequencies

The mutation frequencies were calculated after each selection step by dividing the median mutant colony number with the viable count (the number of plated bacteria).

The *E. coli* mutation frequencies to TGC (Fig. 2) were at the highest being 1.73 x 10^{-6}, observed with the second-step mutant DA19140 and lowest 7.47 x 10^{-9}, in the second-step mutant DA19165.

The mutation frequencies for *Salmonella typhimurium* derived mutants (Fig. 3) were at highest 2.53 x 10^{-6} in the second step mutants from DA19189, and at the lowest 3.52 x 10^{-7} in the third step mutants from DA20785.

To test the possible connection between the mutation frequency and the TGC concentration, these variables were tested with the Pearson correlation coefficient. The correlation coefficient for *E. coli* was r= -0.19 and for *S. typhimurium* r=0.02 (data not shown).
Figure 2. The mutation frequencies of 1st, 2nd and 3rd step mutants derived from wt Escherichia coli MG1655. The 1st step mutants were isolated from plates containing 0.188 mg/L of TGC. The 2nd step mutants from DA19140 were isolated from TGC concentrations of 0.25 and 0.5 mg/L. The 2nd step mutants from DA19153 were isolated from 0.38 mg/L TGC plates. The 2nd step mutants from DA19165 were isolated from 0.5 mg/L TGC plates. The 3rd step mutants from DA20587 were isolated from 1.5 and 3 mg/L TGC plates. The 3rd step mutants from DA20578 were isolated from 1 and 2 mg/L TGC plates.

Figure 3. The mutation frequencies of 1st, 2nd and 3rd step mutants derived from wt Salmonella typhimurium LT2. The 1st step mutants were isolated from plates containing 0.256 mg/L of TGC. The 2nd step mutants from DA19180, DA19189 and DA19195 were isolated from 0.76 mg/L; 0.5 mg/L and 0.76 mg/L TGC plates, respectively. The 3rd step mutants from DA20776 and DA20785 were isolated from 3 mg/L and 4 mg/L TGC plates, respectively.
2.3 Determination of minimum inhibitory concentrations (MIC)

To assess how three consecutive TGC exposures affected the MIC values, I determined MIC values of TGC for the mutants from the selection steps with Etests. The TGC concentration that inhibited the growth of wt strain *E. coli* MG1655 was 0.047 mg/L and for wt *S. typhimurium* LT2 slightly higher; 0.064 mg/L.

First step *E. coli* mutant MIC values ranged from 0.094 to 0.25 mg/L (Table 4). The MIC values for the second step mutants ranged from 0.19 to 0.75 mg/L. For the third step mutants, tigecycline MICs were between 0.75-2 mg/L, thereby reaching the clinical breakpoint of resistance (R≥2). The increase from the wt levels to the highest MIC value of a 3rd step mutant was 42-fold.

The first step mutant MICs for *S. typhimurium* ranged from 0.125 to 0.5 mg/L (Table 4). The second step mutant MICs were between 0.38-1.5 mg/L and for third step mutants the MIC values ranged from 0.75 to 3 mg/L, hence clearly exceeding the clinical breakpoint for *Enterobactericeae*. The increase from the wt MIC value to that for the 3rd step mutant was 46-fold at highest.

### Table 4. The MIC range in *E. coli* and *S. typhimurium* mutants and increase compared to wt in three selection steps

<table>
<thead>
<tr>
<th>Sample</th>
<th>Selection step</th>
<th>1st</th>
<th>2nd</th>
<th>3rd</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em> mutants</td>
<td>MIC range mg/L</td>
<td>0.094-0.25</td>
<td>0.19-0.75</td>
<td>0.75-2</td>
</tr>
<tr>
<td></td>
<td>Increase*</td>
<td>2-5-fold</td>
<td>4-15-fold</td>
<td>16-42-fold</td>
</tr>
<tr>
<td><em>S. typhimurium</em> mutants</td>
<td>MIC range mg/L</td>
<td>0.125-0.5</td>
<td>0.38-1.5</td>
<td>0.75-3</td>
</tr>
<tr>
<td></td>
<td>Increase**</td>
<td>2-8-fold</td>
<td>6-23-fold</td>
<td>11-46-fold</td>
</tr>
</tbody>
</table>

* compared to *E. coli* wt TGC MIC 0.047 mg/L
** compared to *S. typhimurium* wt TGC MIC 0.064 mg/L

2.4 Measuring the fitness of the mutants with Bioscreen

To determine the fitness cost of the mutations that confer lower susceptibility towards TGC, I measured the growth rate of 10 mutants from each selection step and compared them to the wt strains. The wild type has the relative fitness value of 1. Unfortunately, the experiments with *E. coli* mutants failed; the wt *E. coli* generation time was too long (27 min) when compared to the previous experiments (19 min) and many of the R-values in Kaleidagraph, which indicate the reliability of the data, were too low and hence unreliable. Therefore, only results from *S. typhimurium* mutants are presented below.
The relative fitness of the first-step mutants of *S. typhimurium* was not greatly reduced in nine mutants ranging from 0.896 to 0.995, meaning a reduction of ≤10% in the growth rate. The exception was the strain DA19198 with a value as low as 0.650 (reduction of 35% compared to the wt) (Fig. 4). The second-step mutant fitness ranged from 0.632 to 0.901 and the relative growth rate was decreased by 10-40% depending on the strain (Fig. 5). The relative fitness for the 3rd step mutants showed larger differences compared to the 1st and 2nd step mutants. Values ranged from 0.449-0.863 or 15-55% decrease in fitness depending of the strains compared to wt. The mutant strains DA21770 and DA21777 had a higher fitness value than the parental strain DA20785, but did not reach the wt value of 1 (Fig. 6).

To test the correlation between the fitness and MIC values, these variables were tested with Pearson correlation (r). The first step mutants had a positive correlation coefficient r= 0.62; Second step mutants a negative r= -0.08 and third step mutants a negative r=-0.62.

![Figure 4](image.png)

*Figure 4. The relative fitness of ten 1st step selection mutants compared to wt Salmonella typhimurium LT2.*
Figure 5. The relative fitness of ten 2nd step selection mutants compared to wt S. typhimurium LT2 and parental 1st step mutants. DA19180 is the parental strain for mutants DA20778, DA20782 and DA20785. DA19189 is the parental strain for mutants DA20789, DA20793 and DA20794 and DA19195 is the parental strain for mutants DA20800, DA20803 and DA20806.

Figure 6. The relative fitness of ten 3rd step selection mutants compared to wt S. typhimurium LT2 and parental 2nd step mutants. DA20778 is the parental strain for mutants DA21760, DA21762, DA21763, DA21765 and DA21767. DA20785 is the parental strain for mutants DA21768, DA21770, DA21772, DA21775 and DA21777.

2.5 Cross-resistance testing

Five mutant strains from each three selection steps of both bacteria, with varying tigecycline MIC values, were tested for cross-resistance against tetracycline (TC) and chloramphenicol (CL) with Etests. The MIC values of the antibiotics for the wt strains of E. coli and S. typhimurium LT2 were used for comparison of results.
The results show (Table 5) that for tetracycline and *E. coli*, the MIC values for the mutants from all the three selection steps were the same (strains DA19140 and DA20561) or higher than of the wild type, ranging 0.75-3 mg/L, wild type having the MIC value of 0.75 mg/L. Tetracycline MIC values for mutant strains derived from wt *E. coli* after tigecycline exposure had increased 4-fold at the highest.

With wt *E. coli*, chloramphenicol had the MIC of 3-4 mg/L. The mutant MIC values of CL ranged from 1-24 mg/L. Interestingly, the growth of the strains DA19153, DA19172, DA20585 and DA20578 were inhibited at lower concentrations compared to the wt (< 3 mg/L). The increased MIC values were at highest 8-fold compared to wt.

**Table 5.** *The MICs for cross-resistance to tetracycline (TC) and chloramphenicol (CL) in E. coli mutants after tigecycline exposure.*

| Bacterium | Strain | Antibiotic | TGC | TC | CL*
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>WT Eco MG1655</td>
<td>DA5438</td>
<td>0.047</td>
<td>0.75</td>
<td>3; 4</td>
<td></td>
</tr>
<tr>
<td>1st step mutant</td>
<td>DA19140</td>
<td>0.125</td>
<td>0.75</td>
<td>12; 8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>DA19153</td>
<td>0.19</td>
<td>1.5</td>
<td>1.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>DA19165</td>
<td>0.25</td>
<td>1</td>
<td>12; 12</td>
<td></td>
</tr>
<tr>
<td></td>
<td>DA19172</td>
<td>0.19</td>
<td>1</td>
<td>1.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>DA19197</td>
<td>0.094</td>
<td>1.5</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>2nd step mutant</td>
<td>DA20561</td>
<td>0.125</td>
<td>0.75</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>DA20566</td>
<td>0.5</td>
<td>3</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td></td>
<td>DA20585</td>
<td>0.19</td>
<td>1.5</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>DA20578</td>
<td>0.5</td>
<td>1.5</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>DA20587</td>
<td>0.5</td>
<td>2</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td>3rd step mutant</td>
<td>DA21749</td>
<td>0.5</td>
<td>1</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>DA21753</td>
<td>0.75</td>
<td>1.5</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>DA21743</td>
<td>1.5</td>
<td>2</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td></td>
<td>DA21745</td>
<td>1</td>
<td>1.5</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td></td>
<td>DA21757</td>
<td>1.5</td>
<td>2</td>
<td>12</td>
<td></td>
</tr>
</tbody>
</table>

* for chloramphenicol some strains were tested twice

With *S. typhimurium* mutants (Table 6) the tetracycline MIC values were elevated, ranging from 1-1.5 mg/L compared to the wt 0.5 mg/L. The strain DA19191 was an exception, since the MIC value for TC was lower than wt; 0.25 mg/L. The maximum increase in the MIC values was 4-fold compared to wt; excluding the strain DA19191.

The MIC values for chloramphenicol and *S. typhimurium* mutants were of the same as wt 2 mg/L; strain DA19191, or higher, ranging from 3-12 mg/L. The highest MIC value was 6-fold higher compared to wt.
Table 6. The MICs for cross-resistance to tetracycline (TC) and chloramphenicol (CL) in S. typhimurium mutants after tigecycline exposure.

<table>
<thead>
<tr>
<th>Bacterium</th>
<th>Strain</th>
<th>TGC</th>
<th>TC</th>
<th>CL</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT Sty LT2</td>
<td>DA 6192</td>
<td>0.064</td>
<td>0.5</td>
<td>2</td>
</tr>
<tr>
<td>1st Step Mutant</td>
<td>DA19180</td>
<td>0.19</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>DA19189</td>
<td>0.25</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>DA19195</td>
<td>0.38</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>DA19191</td>
<td>0.25</td>
<td>0.25</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>DA19198</td>
<td>0.125</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>2nd step mutant</td>
<td>DA20778</td>
<td>1.5</td>
<td>1</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>DA20785</td>
<td>1</td>
<td>0.75</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>DA20794</td>
<td>1</td>
<td>1.5</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>DA20800</td>
<td>0.5</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>DA20806</td>
<td>0.75</td>
<td>1</td>
<td>8</td>
</tr>
<tr>
<td>3rd step mutant</td>
<td>DA21759</td>
<td>1</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>DA21763</td>
<td>2</td>
<td>2</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>DA21770</td>
<td>1</td>
<td>1.5</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>DA21771</td>
<td>2</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>DA21772</td>
<td>3</td>
<td>2</td>
<td>12</td>
</tr>
</tbody>
</table>

3. DISCUSSION

Studies of *E. coli* and *Staphylococcus aureus* resistance to fluoroquinolones have revealed that resistance can arise in a stepwise fashion when the bacteria are exposed to increasing levels of the drug. The increase in MICs in those studies was 4- to 8-fold in each step (Sanders 2001). The results in this study implicate that clinical resistance to tigecycline can be achieved in a similar manner for *E. coli* and *S. typhimurium*. In each selection step the tigecycline MIC increased (Table 4) reaching the clinical breakpoint of $\geq 2$ mg/L for *Enterobactericeae* in three steps with elevating concentrations of TGC. At highest the MICs for the third-step mutants were in *E. coli* 42-fold and in *S. typhimurium* 46-fold compared to wt values.

The MIC values varied greatly for both bacteria, which implicates different physiological responses and mutational events in different genes. This phenomenon is well known with fluoroquinolone resistance (Sanders 2001) and produces different MIC profiles from the same parental strain. Additionally, the values here suggest only low-level resistance mutations in each step since no high level resistance mutants were obtained. It has been proposed that mutations that render low-level resistance and are gradually accumulated in the genome are the major problem behind multidrug resistant bacteria (Drlica *et al.* 2008).
There was a noticeable difference in the recovery rates of mutants of *E. coli* to *S. typhimurium*. The number of *E. coli* mutants decreased as the TGC concentration increased, and mutants could not be collected in all the subcultures. In contrast, *S. typhimurium* mutants produced colonies even in higher drug concentrations and all the subcultures are presented in the final collection of mutant strains. This difference in numbers is presented in tables 2 and 3. If the reduced susceptibility is mediated by intrinsic efflux (AcrAB), more efficient pumps and the differences in the metabolic pathways and regulators in *S. typhimurium* could be the cause of this divergence. Partly this is supported by the wt tigecycline MIC value being already higher in *S. typhimurium* (0.064 mg/L) than in *E. coli* (0.047 mg/L).

Bacterial capsules and slime layers are a physiological response to external stress and there are numbers of genes that can be involved in the formation of this protective layer. These genes can be part of signalling pathways, genes coding for outer membrane proteins and quorum sensing molecules (Tenorio et al. 2003) and the formation of biofilms has been combined with increased antibiotic resistance. Therefore, it was interesting to notice that despite that the wild type strains did not have a mucoid phenotype, 27% of *E. coli* and 24% of *S. typhimurium* offsprings exhibited excessive production of polysaccharides. This mucoid phenotype was detected both on LA and Müller-Hinton plates and did not revert back to wt when the selection was relaxed. This suggests that this phenotype is not transient by nature (outcome of GDA) but more stable genetic solution, although the role of GDAs cannot be ruled out completely since they can be lost during growth and are considered to be facilitators to permanent adaptation (Andersson and Hughes 2009).

The mutant prevention concentration (MPC) was for the wt *E. coli* 0.752 mg/L and for wt *S. typhimurium* 1.024 mg/L. The maximum serum concentration of TGC is 0.85-1 mg/L (Pankey 2005) and is readily taken up into tissues. If TGC is prescribed for medicating complicated intra-abdominal and skin and soft tissue infections, this concentration, at least in theory, should be enough to prevent the emergence of single-step mutants. This can explain that there are still relatively low numbers of clinical isolates that have reduced susceptibility to TGC; a study in UK showed that only 2.4% of the 150 clinical isolates, which consisted of both gram-negative and positive bacteria were confirmed to be resistant and 1.4% of intermediate level (Hope et al. 2010). The situation may change, if TGC is being prescribed to off-label indications as well, since the off-label prescription of tigecycline is increasing due to rising numbers of multiresistant bacteria (Conde-Estévez et al. 2010).

The result from the MPC study here can only be regarded as suggestive, because the total amount of bacteria on the TGC plates did not exceed ~10^10 cells, which is considered to be the minimum when determining MPC. By plating fewer bacteria, we risk that we may miss very rare mutations (mutation frequency of 10^-10), which in turn may contribute to the decreased antibiotic susceptibility of the bacterium.

The Pearson correlation coefficient (r) is used to detect associations between two variables. This value can range from +1 to -1; the positive value indicates that the
values increase together and a negative correlation suggests that one value increases when the other one decreases. A strong correlation is +1 or -1, which implicates a perfect linear relation between values (Machin et al. 2007).

There was no strong correlation between the concentration of TGC and the mutation frequency in neither bacterium; in *E. coli* *r*=-0.19 and *S. typhimurium* *r*=0.02. Similar data has been presented when *E. coli* strains were subjected to rifampicin (Denamur et al. 2005). The TGC mutants exhibited a range of different mutation frequencies but the majority of mutation frequencies with TGC were in the range of $4 \times 10^{-8} \leq f \leq 4 \times 10^{-7}$, hence they are classified as weak mutators. These results are consistent with the polymorphic mutation frequencies observed in clinical *E. coli* isolates (Baquero et al. 2004).

Intermediate mutation frequencies may be preferred in nature, since the accumulation of deleterious mutations in hypermutable strains ($f \geq 4 \times 10^{-7}$) tend to make the bacteria less fit and they can be outcompeted in nonselective environments by bacteria with more stable genomes (Martinez and Baquero 2000). In a study by Baquero et al. (2004) they presented the possibility that intermediate mutation frequencies can accommodate to multidrug resistance in *E. coli*. By gradually accumulating mutations that confer low resistance to various drugs without a significant fitness cost, the bacteria will have the selective advantage when exposed to antimicrobials, and most likely will outcompete the bacteria, which do not have these pre-existing mutations in their genomes.

The results from fitness experiments with *S. typhimurium* showed decreased growth rates in the mutants, especially when 2nd and 3rd selection steps mutants were analyzed; reduction as high as 55% was observed. This severe decline in fitness demonstrates that TGC resistance comes with a fitness cost. The correlation of the third step mutant fitness reduction to elevating TGC concentration was not strong (*r*=-0.62), but it can be observed. Interestingly, two third step mutants, DA21770 and DA21777, had a higher relative fitness value than the parental strain DA20785. This increase in fitness can be the result of compensatory mutations, which restore the fitness without loosing the resistance. Indeed, the tigecycline MIC for these two mutants were 1 and 2 mg/L, whereas the parental strain was inhibited at the drug concentration of 1 mg/L.

Unfortunately, there are no results of the fitness cost for *E. coli*, but we can assume similar profiles as in *S. typhimurium*, since the deficient growth could be seen also on the LA plates. In general, *E. coli* mutants were more affected by the higher concentrations of TGC than *S. typhimurium* mutants, growing in very small colonies or not producing offsprings at all from the plated subcultures. The colony size decreased in 62% of all the *E. coli* mutants and in 60% of the *S. typhimurium* mutants after TGC exposure.

Cross-resistance to tetracycline and chloramphenicol did occur in both bacteria. Since tetracycline has the same target as tigecycline (the ribosome’s 30S subunit) this can be expected. There was no dramatic increase in the MIC levels; only 2- and 4-fold at highest, compared to wt in *E. coli* and *S. typhimurium* respectively.
With chloramphenicol, which binds to the 50S subunit and prevents the peptide bond formation of nascent protein, the results for *E. coli* mutants in table 5 show three interesting things. Firstly, there is no existing data that supports the cross-resistance to CL after TGC exposure. Yet it is evident that the MIC values are higher than the wt strain (3-4 mg/L) and over the breakpoint for *Enterobactericeae* (S≤8; R>8). Secondly, the increased susceptibility to CL (<3-4 mg/L) was observed in strains DA19153, DA19172, DA20585 and DA20578. Studies with *E. coli* and CL confirm that low-level resistance can be mediated through overexpressed efflux pumps (AcrAB) and MIC values range 16-32 mg/L (Cattoir 2010). Thirdly, this data indicates that several different mutations give TGC resistance, which is reflected into the cross-resistance profile of the mutant strains.

There was a smaller variation in *S. typhimurium* mutants in the MIC values of chloramphenicol compared to *E. coli*. Only strains DA20806, DA21763, DA21770 were at breakpoint (>8 mg/L) or over DA21772 (12 mg/L). This may indicate different mutation targets compared to *E. coli*.

The role of intrinsic efflux pumps in TGC resistance have been discussed here and also in many other studies (Abouzeed et al. 2008; Horiyama et al. 2010; Hornsey et al. 2010; Keeney et al. 2008; Ruzin et al. 2005; Hentschke et al. 2010; Sun et al. 2010; Hornsey et al. 2010a). These pumps belong to the RND family of transporters and have a wide range of substrates. They are not specific transporters for any drug and therefore high-level resistance is not achieved by this mechanism. A study by Rajendran et al. (2010) with *Burkholderia*, a gram-negative bacteria, which can have tigecycline MICs of 8-32 mg/L, showed that the potency of TGC can be restored by combining the treatment with efflux pump inhibitor (EPI). This study is supporting the role of efflux pumps in TGC resistance.

One has to consider that the *in vitro* experiments do not completely reflect the situation *in vivo*. The depletion of nutrients is a factor that may increase the mutation frequency e.g. via stress-responsive DNA polymerases (*umuCD* and *dinB*) that are notorious of making errors in DNA replication (Martinez and Baquero 2000). Additionally, the fitness of the mutants *in vitro* can differ greatly when compared to *in vivo*, since some genes are activated only when inside the host organism. Tetracycline compounds can modulate the immune system of the host, and recent study by Dunston *et al*. (2011) showed that tigecycline was the most potent among tetracyclines to inhibit the iNOS (inducible nitric oxide synthase) production in macrophages, thus limiting the inflammatory response. Many drugs are also degraded in the host and sometimes resulting in products that have no antimicrobial activity but are far from being inert. Palmer *et al*. (2010) presented that tetracycline decay products can actually reverse the selection of resistant bacteria and favour the selection of sensitive bacteria. They also hypothesized that this interplay between the drug, its degradation products and the host immune system can explain the widely encountered co-existence of resistant and sensitive bacteria in natural environments.
It is difficult to predict the evolution of resistance, which is not only depending on the bacteria per se, but on human factors, which contribute greatly to the outcome of resistance and its dissemination (Martinez et al. 2007; Drlika et al. 2008). Therefore efforts have been taken to establish methodological guidelines helping to evaluate the possible development of resistance to a new drug before it is introduced to clinical use. The experimental approaches as presented in this study are valuable in increasing the knowledge of how the emergence of antibiotic resistance occurs.

4. FUTURE PERSPECTIVES

The results indicate that clinical resistance towards TGC can be established experimentally, but it may not be as straightforward in living organisms; the host immune system being one major contributor. Even from this very limited study it can be seen how the bacteria respond to toxic environments; producing polysaccharide layers, limiting the growth rate and colony size by reducing the intake of nutrients. Needless to say, the signalling pathways behind these changes are often redundant and complex. Naturally, these phenotypic changes need to be confirmed genetically and this has already been initiated. The possible role of GDAs is also intriguing. Additional studies of cross-resistance are needed, since the exposure to TGC did change the MICs of chloramphenicol and tetracycline in both bacteria. How high TGC resistance can be achieved experimentally and how great is the fitness cost in even more resistant bacteria? Additionally, serial passage experiments of compensatory evolution ameliorating the fitness cost of TGC resistance could be of additional interest, since we already saw two third step mutants with higher fitness than the parental strain.

5. ACKNOWLEDGEMENTS

I want to thank professor Dan I. Andersson for giving me the opportunity to work with this project and I pay my respects to the diligent group that he has. Thank you for the help and the experience.

To Pontus. My friend. I would not be here in Sweden, if it weren’t for you. Thank you for supporting me through the hard times. I wish all the best for you in Finland! To My Sisters, Emilia, Kukka-Maria, Nina and Katja. I love you all. Even if we are apart I get comfort of knowing that you are there. Pinkki and Göbelios. My beloved "Sylvester and Tweety". My heart rejoices watching you two crazy buggers!!! My sweet Elkin. You have seen the best of me and the worst of me. Thank you for understanding and loving me. We make a great team! Kling Kling Kling!

This thesis is dedicated to my Father and Papu, whom both I lost during my Masters Degree studies, and to my Mother. It is hard without you guys, but I know that you are there among the stars and looking over me.
6. MATERIALS AND METHODS

6.1 Organisms used in this project

The wild-type (wt) parental strains used for the experiments were *Escherichia coli* MG1655 (DA5438) and *Salmonella enterica* serovar Typhimurium LT2 (DA6192). The mutants were isolated from Müller-Hinton agar (MHA) plates after selection steps and they are designated by their DA number. All the strains were stored in –80°C in DMSO.

6.2 Tigecycline

Tigecycline (TGC) was diluted into 70% ethanol according to the manufacturer’s guidelines to yield a concentration of 10,000 mg/L. A stock solution (1000 mg/L) was prepared (70% EtOH) and kept in -23°C. Since TGC is a relative to photosensitive tetracycline it was kept in darkness at all times to prevent the degradation by light (Hope et al. 2005).

6.3 Growth conditions and viable counts

For both bacteria the overnight (ON) cultures were grown to ~$10^9$ cfu/ml, in Müller-Hinton broth (MHB) at 37°C in shaking, if not mentioned otherwise.

All the plates supplemented with tigecycline were incubated in darkness and the antibiotic was always added in fresh (<12 h) MHA to reduce the risk of degradation by oxygen. The MHA plates were incubated at 37°C.

For viable counts, Luria Agar (LA) plates were used and the ON cultures (~$10^9$ cfu/ml) of bacteria were diluted to yield 100 and 10 colonies/plate and 100 µl of these dilutions were plated (three plates each dilution/strain) and they were incubated overnight at 37°C if not mentioned otherwise. The number of colonies was counted on each plate and the average number of colony forming units cfu/ml for each strain was used in mutation frequency calculations.

6.4 Isolation of tigecycline-resistant mutants and determination of mutant prevention concentration

Three selection steps were conducted. In each step the selected strains for both bacteria were grown to ~$10^9$ cfu/ml in MHB, and further divided into subcultures inoculating 1 ml of MHB with $10^4$ of the original ON culture: 20 subcultures/strain in the 1st selection step; 10 subcultures in the 2nd and 3rd selection step. These
individual cultures were incubated overnight at 37°C to \( \sim 10^9 \) cfu/ml and then pipetted undiluted on fresh MHA plates with elevating concentrations of tigecycline (TGC). The resulting number of bacteria pipetted was \( \sim 10^8 \) cells/plate.

Before the mutant selection, the MIC values of the wild type strains were determined to assess the susceptibility of the bacteria prior to TGC exposure. This value was used to calculate the 2-64 -fold concentrations for TGC selective plates.

The wild type *E. coli* was used as the parental strain for the first step selection. The TGC concentrations were 0.188; 0.752; and 3.008 mg/L and for wild type *S. typhimurium* LT2 the values were 0.256; 1.024 and 4.096 mg/L. The selective concentrations were 4; 16 and 64-fold the MIC value of the wt. The sample volume/plate was 100 \( \mu \)l in 4-fold and 400 \( \mu \)l in the 16 and 64-fold TGC concentration.

Three strains from both bacteria from the 1st step were selected as parental strains for the 2nd step selection. The TGC concentrations for *E. coli* mutants were as follows: DA19140: 0.25; 0.5; 1 and 2 mg/L; DA19153: 0.38; 0.76; 1.52 and 3.04 mg/L; DA 19165: 0.5; 1; 2 and 4 mg/L. The concentrations for *S. typhimurium*: DA19180: 0.38; 0.76; 1.52 and 3.04 mg/L; DA19189: 0.5; 1; 2 and 4 mg/L and for DA19195: 0.76; 1.52; 3.04 and 6.08 mg/L. The selective concentrations were 2, 4, 8 and 16-fold the MIC value of the corresponding parental MIC. Sample volume for 2 and 4-fold concentrations was 100 \( \mu \)l and for 8 and 16-fold concentrations 400 \( \mu \)l.

For the 3rd step, two mutant strains from the 2nd step were selected from each bacterium to be the parental strains. The TGC concentrations were for *E. coli* mutants DA20587: 1.5; 3; 6 and 12 mg/L; DA20578: 1; 2; 4 and 8 mg/L. For *S. typhimurium* mutants DA20778: 3; 6; 12 and 24 mg/L and for DA20785: 2; 4; 8; and 16 mg/L. The selective concentrations were 2, 4, 8 and 16-fold the MIC value of the corresponding parental MIC. Sample volume for 2 and 4-fold concentrations was 100 \( \mu \)l and for 8 and 16-fold concentrations 400 \( \mu \)l.

In each step all the antibiotic-supplemented plates were incubated for 36-40 hours at 37°C. After incubation only plates with distinguishable mutant colonies were counted; plates with growth covering completely the surface were discarded from further study. After incubation a randomly chosen mutant colony was picked and streaked onto fresh TGC plate corresponding to the concentration where they were isolated from to keep the selection pressure on. Only the colonies that grew on the TGC supplemented re-streak plate were chosen for further studies.

The mutant prevention concentration (MPC) was determined in the 1st selection step with wt strains and the actual MPC was the first concentration where there were no visible colonies after incubation time. The original method described by Blondeau (2009) was adapted and instead of inoculating \( 10^5-10^{10} \) cells/plate, a higher volume (400 \( \mu \)l) of the original ON culture was plated on the 16x and 64x MIC concentrations.
6.5 Calculation of mutation frequencies

Mutation frequencies were calculated using the Lea & Coulson method by dividing the median number of mutant colonies on the antibiotic supplemented plate by the total amount of plated bacteria (viable count) that was in the original ON culture (Rosche and Foster 2000).

6.6 Minimum inhibitory concentration (MIC) determinations with Etests

The bacteria were grown overnight at 37°C on LA plates, one colony was picked per plate and resuspended into 1 ml of 0.9 % NaCl. The suspension was swabbed onto MH agar plates and the Etest® (AB Biodisk, Sweden) strips were added. The plates were incubated for 16-20 hours in 37°C and then read from the intersection of the bacterial growth against the strip. As a quality control the wild-type strains were tested every time alongside the mutants.

6.7 Cross-resistance testing with Etests

Five mutant strains with different TGC sensitivity from the 1st, 2nd and 3rd step selections were tested for cross-resistance for tetracycline (TC) and chloramphenicol (CL) with Etests. The experiments were done as described in 6.6.

6.8 Measuring the fitness of the mutants

For exponential growth-rate measurements, 1 ml of MH broth was inoculated with one colony of bacteria from an LA-plate. The inoculants were grown overnight in 37°C to ~10^9 cfu/ml and then diluted to 10^3 cfu/ml into MH broth. The diluted samples were pipetted onto honeycomb well plates, 300 µl in quadruplets and measured for 24 hours, every four minutes in 600 nm in 37°C, in continuous shaking with a BioscreenC spectrophotometer (Labsystems). MH broth was used as a negative control and as a blank. The mean blank value was subtracted from the sample values. The growth curves were generated with Kaleidagraph using the values 0.02-0.08 to capture the exponential growth phase. The relative generation time of the mutants were calculated and compared to that of a wild-type strain, wt having the value of 1.
REFERENCES


