Evolution and Mechanisms of Tigecycline Resistance in *Escherichia coli*

MARIUS LINKEVIČIUS
Abstract


Antibiotic resistance is an ongoing global medical crisis and we are in great need of new antibacterial agents to combat rapidly emerging resistant pathogens. Tigecycline is one of few drugs that have been introduced into medicine during the last two decades. It is a broad-spectrum third generation tetracycline that is active against multidrug-resistant bacteria that cause complicated infections.

In this thesis I examined the development of tigecycline resistance in *Escherichia coli* and associated *in vitro* and *in vivo* fitness effects. Selections of spontaneous *E. coli* mutants revealed relatively high accumulation rates of changes in the multidrug efflux system AcrAB-TolC regulation network and in heptose biosynthesis and transport pathways important for lipopolysaccharide (LPS) synthesis. Both groups of mutations led to reduced susceptibility to tigecycline and slower growth compared to the wild-type bacteria. Additional *in vitro* fitness assays and *in vivo* competitions showed that LPS mutants were less fit than efflux mutants, providing a possible explanation for why up-regulation of multidrug efflux pumps is the main tigecycline resistance mechanism reported in clinical isolates.

Tigecycline was designed to evade the two most common tetracycline resistance mechanisms conferred by Tet proteins, efflux and ribosomal protection. However, tigecycline is a substrate for the tetracycline modifying enzyme Tet(X). Screening of Tet protein mutant libraries showed that it is possible to select Tet mutants with minimal inhibitory concentrations of tigecycline that reach clinically relevant levels. Mutations in Tet proteins that permitted a better protection from tigecycline frequently exhibited reduced activity against earlier generations of tetracyclines, except for the Tet(X) enzyme mutants, which were better at inactivating all tested tetracyclines. This is particularly worrisome because different variants of Tet(X) have recently spread to multidrug-resistant pathogens through horizontal gene transfer. Therefore, Tet(X) mutants with improved activity threaten the medical future of tetracyclines.

Multidrug resistance is easily disseminated through horizontally spreading conjugative plasmids. pUUH239.2 is an example of a successful conjugative plasmid that caused the first clonal outbreak of extended spectrum β-lactamase-producing *Klebsiella pneumoniae* in Scandinavia. This plasmid was formed after rearrangements between two different plasmid backbones and it carries resistance genes to multiple antibiotic classes, heavy metals, and detergents.

*Keywords*: Tigecycline, Bacterial resistance, Fitness, *Escherichia coli*, AcrAB, LPS, Tet proteins, Tet(A), Tet(K), Tet(M), Tet(X), tet genes, pUUH239.2

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Chance favors the prepared mind.

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List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.


II Linkevicius, M., Anderssen, JM., Sandegren, L., Andersson, DI. (2015) Fitness of *Escherichia coli* mutants with reduced susceptibility to tigecycline. *Manuscript*


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Abbreviations

C          Cytoplasmic
CTX        C-terminal helix
ERN        Efflux regulation network
ESBL       Extended spectrum β-lactamase
EUCAST     European Committee on Antimicrobial Susceptibility Testing
FAD        Flavin adenine dinucleotide (oxidized)
FDA        Food and Drug Administration
HGT        Horizontal gene transfer
IM          Inner membrane
IS          Insertion sequence
IV          Intravenous
kbp        Kilo-base pair
LPS        Lipopolysaccharide
MFS        Major facilitator superfamily
MIC        Minimal inhibitory concentration
MLST       Multilocus sequence typing
MRSA       Methicillin-resistant *Staphylococcus aureus*
MSC        Minimal selective concentration
NADP⁺      Nicotinamide adenine dinucleotide phosphate (oxidized)
NADPH      Nicotinamide adenine dinucleotide phosphate (reduced)
OM         Outer membrane
P          Periplasmic
PBP        Penicillin-binding protein
PDB        Protein Data Bank
PFGE       Pulsed-field gel electrophoresis
RND        Resistance-nodulation-division
RPP        Ribosomel protection protein
ST         Sequence type
Tet        Tetracycline specific
TGC        Tigecycline
TM         Transmembrane α-helix
Tn         Transposon
VRE        Vancomycin-resistant enterococci
Introduction

The discovery of antibiotics is one of the greatest advances in medicine of the 20th century and it has had a huge impact on the treatment of bacterial infections. However, resistance to these compounds was generally observed soon after they were introduced into clinical practice. Antibiotic resistance was acknowledged as a serious global problem during the 1990s, although it emerged as early as the 1940s. Many different factors influence the appearance of resistant bacteria. Firstly, bacteria demonstrate a huge genetic plasticity that includes many mechanisms of mutation acquisition as well as exchange of genetic material. Secondly, anthropogenic factors also have a great impact on resistance development and its dissemination. Inadequate practices of antibiotic prescriptions in hospitals, the use of antimicrobials as feed additives promoting the growth of livestock, crowding, poor nutrition, and sanitation cause development and spread of antibiotic resistance within countries. Furthermore, resistant bacteria can be promptly disseminated worldwide due to high international travelling (Salyers and Wilson, 2010).

The increasing antibiotic resistance threat triggered a response from the pharmaceutical companies. During the mid 1960s and 1970s, the main reaction was the improvement of currently available antibiotic scaffolds to expand the spectrum of activity of the antibiotics in order to try to overcome the existing resistance mechanisms. With this in mind, the new generations of β-lactams and macrolides were introduced at the end of the 1970s. This approach was effective for several decades, allowing drug developers to be one step ahead of the resistant bacteria. However, chemical modifications of existing antibiotic structures reached a limit and discoveries of new classes of antibiotics drastically slowed from the 1970s to the present day. This was a result of several factors. For instance, some previous off-patent drugs down-regulated the drug prices. Additionally, regulatory agencies implemented stricter safety barriers that slowed down the introduction of new antimicrobials into the market. Finally, the unsatisfactory results from the target-based new drug discovery screens also affected the situation of antibiotic development. Only a few new antibiotic groups from existing classes (ketolides, glycylcyclines) and only one entirely new antibiotic class (oxazolidinones) were introduced during the early 2000s (Wright, 2007).

A decreased interest in the antibiotic research area was also observed among the scientific community, where there was a general opinion that knowledge about bacterial resistance mechanisms and mobile genetic
elements transferring resistance determinants was already discovered during the 1980s and 1990s. The funding agencies did not demonstrate much support to the bacterial resistance field either. However, this perspective is rapidly changing, and it is widely recognized that at present there are a number of important questions concerning antibiotic resistance that need to be addressed. Thus, as we are running out of possible treatment options for bacterial pathogens, it is crucial to develop strategies to extend the lifespan of already existing antimicrobials. Furthermore, in order to implement well-balanced strategies to handle antibiotic resistance, it is essential to understand, describe, and predict the dynamics of its development and the resulting effects on bacterial fitness, virulence, and transmission (Martínez et al., 2007; Salyers and Wilson, 2010).

Bacterial resistance

There are two main antibiotic resistance strategies that bacteria employ to evade antibiotic activity (Figure 1).

The first one is a reduced intracellular concentration of antibiotic. This can be achieved in several different ways. For example, Gram-negative bacteria have an outer membrane (OM), which by itself is a barrier. Many hydrophilic drugs (e.g. β-lactams) enter Gram-negative cells through the OM porins, whose expression can be reduced so as to prevent the influx of the drug. Up-regulated antibiotic-specific or unspecific efflux pumps are also protecting the target by expelling the drug out of the cell. Additionally, specific enzymatic reactions can be performed to degrade (e.g. β-lactamases) or inactivate the drug through modification (e.g. aminoglycoside acetyltransferases), resulting in low amounts of active antibiotic inside the cell (Wright, 2011).

The second strategy is protection of the antibiotic target. A simple way of modifying the target is the accumulation of spontaneous mutations that weaken the interaction between the target and the antibiotic. For instance, the mutation A2058G in 23S rRNA confers resistance to macrolides in Gram-positive cocci. A more sophisticated way is to obtain an alternative version of the target, which is insensitive to the antibiotic. Penicillin-binding protein PBP2a in *Staphylococcus aureus* is resistant to methicillin, while other PBPs are not. It is also possible to acquire a new set of proteins producing a new target to which the antibiotic cannot bind. For example, in vancomycin-resistant enterococci (VRE), VanH, VanA, VanX, and VanY proteins (encoded on the conjugative transposon Tn1546) are responsible for synthesizing D-Ala-D-lactate dipeptide that replaces the common D-Ala-D-Ala used to cross-link the layers of peptidoglycan. This dipeptide is not recognized by vancomycin. Another option is to harbor target modification proteins. For example, RNA methylases methylate nucleotide
A2058 of 23S rRNA and macrolides, streptogramins, and lincosamides cannot form hydrogen bonds necessary for binding to the modified ribosome (Wright, 2011).

![Figure 1. Bacterial antibiotic resistance strategies.](image)

**Horizontal gene transfer of resistance genes**

Bacteria can become resistant by either accumulating mutations in the existing genes or they can acquire new resistance determinants from other bacteria through horizontal gene transfer (HGT). This is a much faster way to become resistant compared to the accumulation of spontaneous mutations. Thus, the spread of antibiotic resistance genes through HGT has become a serious problem, especially in clinical settings. Sharing of DNA between and within species can be accomplished by forming a direct cell-to-cell contact (conjugation), by exploiting a specific delivery vector such as a bacteriophage (transduction) or by direct uptake from the environment (transformation) (Thomas and Nielsen, 2005; Wright, 2007).

Two conjugative elements (conjugative plasmids and conjugative transposons) can be transported during conjugation. In order for a conjugative plasmid to transfer itself by conjugation, it must contain the transfer (tra) genes. Non-conjugative plasmids cannot initiate this process, but they can still be mobilized and spread with the help of conjugative...
plasmids. Conjugative transposons are usually found integrated in the bacterial chromosome, but they can also integrate in plasmids. They encode transposases and conjugation proteins flanked by insertion sequences. During conjugation they form circular intermediates that are transferred to another cell (Burrus et al., 2002; Norman et al., 2009).

Multidrug resistance (resistance to two or more antibiotic classes) has become very common in clinical settings. It can arise by expression of unspecific efflux pumps that pump out different antibiotics or by lower expression of, for example, porins that allow diffusion of various antimicrobials into the cell. However, acquisition of a plasmid that contains a set of different antibiotic resistance genes is one of the most common ways of becoming multidrug resistant. This phenotype can further be horizontally transferred to other bacteria. The easiest way for plasmids to obtain various resistance mechanisms is through the integration of transposons or integrons that carry resistance determinants (Figure 2). Integrons are genetic elements that encode integrases and have a special attachment (att) site with the promoter. The integrase of the integron can insert resistance gene cassettes in att sites, which can then be expressed (Wright, 2007).

Figure 2. Conjugative plasmids as vectors of resistance dissemination through HGT. Mobile genetic elements like transposons and integrons can be integrated in plasmids and may carry resistance determinants that can be transferred within and between bacterial species. Adapted from (Norman et al., 2009).
Bacterial fitness

Bacteria that express resistance mechanisms usually grow slower and are less virulent and less transmissible than their wild-type ancestors (Andersson and Levin, 1999; Andersson, 2006). This means that if the resistance mechanism imposes a high fitness cost, it will cause difficulties for the resistant bacteria to establish themselves in a population, especially if the environment is no longer under the same antibiotic pressure. However, bacteria have found several solutions to this problem.

First of all, resistance mechanisms can be tightly regulated and expressed only when bacteria are in an environment with antibiotics. This means that once the antibiotic pressure is removed, the expression of the resistance mechanism is repressed, reducing the cost, and resistant bacteria can compete better with sensitive ones. For example, in VRE the VanRS two-component system is responsible for switching on the transcription of vanHAXY genes only when the glycopeptide is in the environment and switching it off again in the absence of the drug (Depardieu et al., 2007).

Additionally, a strong selection pressure for fast-growing strains favors an accumulation of secondary mutations that compensate the fitness cost implemented by the resistance mechanism and restore it to the wild-type level (Figure 3). There is also a possibility of reverting the resistance mutation back to the wild-type genotype. However, the frequency of such an event is very low as there is just one way of reverting the resistance genotype. To summarize, even if the selective pressure of the antibiotics is removed, the resistant bacteria can overcome fitness costs (due to the resistance mechanism) and become as fit as the wild-type bacteria (Andersson and Hughes, 2010).

Figure 3. Resistance-imposed fitness cost. The most common way of restoring fitness defects is through accumulation of compensatory mutations. Additionally, possibilities exist to carry cost-free resistance mechanism or to revert the resistance phenotype to wild-type and re-establish the initial fitness.
In this report I will concentrate on the following aspects of antibiotic resistance and its associated fitness costs: (i) the development of tigecycline resistance through the accumulation of spontaneous mutations; (ii) the evolution of tigecycline resistance in tetracycline efflux, ribosomal protection and modification proteins; and (iii) the characterization of a conjugative plasmid involved in a clonal outbreak in a clinical setting.
Mechanisms of tigecycline resistance

Tetracyclines
The antibacterial activity of the first tetracycline antibiotic, chlortetracycline, was discovered by Benjamin Duggar in the soil bacterium *Streptomyces aureofaciens* in 1948 (Duggar, 1948). Tetracyclines are broad-spectrum drugs, effective against Gram-positive and Gram-negative bacteria as well as some eukaryotic parasites. Due to their impressive antimicrobial activity, few side effects, and the possibility of oral administration, tetracyclines became one of the first-line defense drugs combating bacterial infections. Furthermore, tetracycline antibiotics had some supplementary uses as metal chelators inhibiting inflammation, proteolysis, angiogenesis and as anti-apoptotic agents (Zakeri and Wright, 2008). The ability of chlortetracycline to promote the growth of chickens was discovered in 1949 (Stokstad and Jukes, 1949) and this encouraged the usage of tetracyclines at subtherapeutic concentrations in animal feed. The heavy use of tetracyclines in medicine and agriculture put a tremendous selective pressure for resistance in microorganisms. The first tetracycline-resistant bacteria were found in 1953 (Watanabe, 1963). At that time the development of resistance was balanced by discoveries of new drugs from natural sources. Additionally, natural scaffolds were chemically modified to produce semi-synthetic drugs with additional properties to circumvent existing resistance mechanisms and expand possible applications. Chemically modified analogues of tetracycline were also found to be promising in cancer treatment (Richards et al., 2011).

Clinical use of tetracyclines
Pathogens
Tetracycline antibiotics are not first-choice drugs to cure the most common bacterial infections due to presence of the resistance mechanisms discussed below. Nevertheless, many infections caused by species of *Borrelia, Rickettsia, Ehrlichia, Anaplasma, Neorickettsia*, and *Bartonella* that are spread by ticks or lice are treated using first- and second-generation tetracyclines. Doxycycline is a first-choice drug for the treatment of donovanosis (*Klebsiella granulomatis*), brucellosis (*Brucella* spp.), and
chlamydial urethritis (*Chlamydia trachomatis*). Both tetracycline and doxycycline are prescribed to treat cholera (*Vibrio cholerae*), while tetracycline is usually effective against *Helicobacter pylori* causing peptic ulcer disease and *Mycoplasma pneumoniae* leading to atypical bacterial pneumonia. *Propionibacterium acnes*, which resides on human skin, is one of the causes of the skin condition known as acne vulgaris. The second-generation tetracyclines, doxycycline and minocycline, are used as an antibiotic therapy to deal with this condition. These antibiotics are also used to treat methicillin-resistant *S. aureus* (MRSA) infections (Brenner and Stevens, 2012; Chopra and Roberts, 2001; Roberts, 2003).

In case of resistance to doxycycline or minocycline, tigecycline is one of the last-resort choices of drugs. It is active against serious Gram-positive (penicillin-resistant *Streptococcus pneumoniae*, MRSA, VRE) and Gram-negative (*Acinetobacter* spp., extended spectrum β-lactamase (ESBL), and carbapenemase producers) pathogens (Castanheira et al., 2008; Noskin, 2005). Tigecycline has been used in medical practice since 2005 to treat adult complicated intra-abdominal, complicated skin and skin structure infections, and community-acquired pneumonia. Additionally, this drug has been tested for hospital-acquired pneumonia, diabetic foot infections, nosocomial urinary tract infections, complicated *Clostridium difficile* diarrhea, and serious multidrug-resistant infections, but further studies are needed before any of these indications can get approval by medical authorities (Brenner and Stevens, 2012; Stein and Babinchak, 2013).

**Pharmacological aspects**

Most of the tetracyclines have both oral and intravenous formulations, except the newest medically approved member of the class, tigecycline, which is available only as an intravenous (IV) injection (Table 1) (Agwuh and MacGowan, 2006; Brenner and Stevens, 2012). The main routes of absorption after oral administration are in the stomach and small intestine. Food interferes with efficient penetration of tetracyclines into the organism, especially if it is rich in calcium, which forms complexes with tetracyclines that are difficult to absorb (Agwuh and MacGowan, 2006; Chopra and Roberts, 2001; Zhanel et al., 2004). Calcium-complexing does not seem to have a marked effect on tigecycline most likely due to its parenteral formulation (Stein and Babinchak, 2013).

The serum concentration after oral intake usually varies between 2 to 5 mg/L and four daily doses for the first-generation tetracyclines are needed to reach therapeutic concentrations. One to two doses are enough for the second-generation tetracyclines due to a longer elimination half-life (Chopra and Roberts, 2001). The serum concentration of tigecycline peaks at 0.62 mg/L after the recommended adult dose of 100 mg IV followed by 50 mg IV every 12 hours (Agwuh and MacGowan, 2006). The distribution in the
tissues and body fluids of the first- and second-generation tetracyclines is moderate, while tigecycline distributes well (Agwu and MacGowan, 2006; Chopra and Roberts, 2001; Zhanel et al., 2004). The main route of excretion is through renal filtration (Brenner and Stevens, 2012). Approximately 20% of the serum concentration is reached in sputum, which explains why tetracyclines are chosen to treat airway infections. In addition, tetracyclines are excreted with sweat and are present in sebum, explaining their therapeutic role against acne (Chopra and Roberts, 2001).

Table 1. Pharmacokinetic parameters of commonly used tetracyclines* (Brenner and Stevens, 2012).

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Formulation</th>
<th>Oral bioavailability (%)</th>
<th>Elimination half-life (h)</th>
<th>Routes of elimination</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tetracycline</td>
<td>Oral or IV</td>
<td>70</td>
<td>10</td>
<td>Renal</td>
</tr>
<tr>
<td>Doxycycline</td>
<td>Oral or IV</td>
<td>90</td>
<td>20</td>
<td>Fecal and renal</td>
</tr>
<tr>
<td>Minocycline</td>
<td>Oral or IV</td>
<td>95</td>
<td>20</td>
<td>Biliary and renal</td>
</tr>
<tr>
<td>Tigecycline</td>
<td>IV</td>
<td>NA</td>
<td>40</td>
<td>Biliary, fecal and renal</td>
</tr>
</tbody>
</table>

*All values are averaged from the study reports.
IV, intravenous; NA, not applicable.

Safety

The most common side effects reported for all tetracyclines are nausea and vomiting. It is possible that these drugs have irritating effects on the mucosa of the gastrointestinal tract (Brenner and Stevens, 2012; Chopra and Roberts, 2001; Zhanel et al., 2004). Pancreatitis is another adverse effect observed for the tetracycline class of antibiotics (Kaewpoowat and Ostrosky-Zeichner, 2014; Stein and Babinchak, 2013). Additionally, tetracycline drugs penetrate the bone tissue and cause teeth discoloration and bone growth inhibition. Therefore, the usage of these drugs should be avoided in children younger than 8 years. Hepatic and renal toxicities have been observed in patients with predisposed conditions or undergoing prolonged high dose tetracycline treatment (Brenner and Stevens, 2012; Chopra and Roberts, 2001; Kaewpoowat and Ostrosky-Zeichner, 2014; Zhanel et al., 2004).

After meta-analysis of data from clinical trials, increased mortality for the tigecycline group was reported when evaluating tigecycline and the comparative treatments. The Food and Drug Administration (FDA) of the United States evaluated the results of the trials, but no specific reasons for increased mortality were determined (Stein and Babinchak, 2013). Low plasma, low alveolar concentration and associated lower drug efficacy, inadequate dosing, and increased late toxicity were suggested as possible explanations for the observed tigecycline effects. The experts recommend choosing tigecycline therapy as a last-resort option (Kaewpoowat and Ostrosky-Zeichner, 2014).
Chemical structure

The scaffold of tetracyclines is formed by a four-fused ring system (A to D) called naphthacene (Figure 4). The whole molecule is divided into a hydrophobic upper peripheral (positions 5 to 9) and a hydrophilic lower peripheral part (positions 1 to 4a and 10 to 12a). Chemical modifications in the upper peripheral half of the structure do not affect the antibacterial activity of the molecule, while changes introduced in the lower peripheral half diminish the antibacterial properties (Chopra and Roberts, 2001; Nelson and Levy, 2011). Therefore, the minimal tetracycline molecule able to inhibit bacterial growth is 6-demethyl-6-deoxytetracycline (sancycline) (McCormick et al., 1960).

![Figure 4. Minimal pharmacophore of tetracyclines. Naphthacene core rings are designated DCBA; the dashed red line marks the margins between the upper peripheral part composed of carbons 5-9 and the lower peripheral part with carbon positions 1-4a and 10-12a.](image)

In addition to the linear fused carbocyclic six-member ring structure, the tetracycline molecule must maintain the keto-enol system (positions 11 and 12) not far from aromatic ring D. This comprises the main metal ion-chelating centre with a supplementary chelating site between positions 2 and 3 (Chopra, 2001; Nelson and Levy, 2011). Chelation of Mg$^{2+}$ ions by tetracyclines is important for their transport, regulation, and for binding to the target.

Tetracycline generations

Generally tetracyclines are divided into typical and atypical. Typical tetracyclines are the compounds that exhibit bacteriostatic activity and target bacterial protein synthesis, whereas atypical tetracycline analogs are bacteriocidal and they damage the cells through disruption of the cytoplasmic membrane (Chopra and Roberts, 2001). Typical tetracyclines can be separated into three generations (Figure 5):
I First-generation tetracyclines are natural antibiotics produced by *Streptomyces* species. The majority of them can be administered orally, but they are not fully absorbed in the body and are less lipophilic compared to later generations. This group contains 7-chlortetracycline (chlortetracycline), 5-hydroxytetracycline (oxytetracycline), and tetracycline (Agwuh and MacGowan, 2006; Chopra and Roberts, 2001).

II Second-generation tetracyclines are semi-synthetic products of chemically modified natural tetracycline scaffold. They are more lipophilic than the first-generation and have better absorption and pharmacokinetic properties. These tetracyclines are also available in an injectible form. The representatives of this group are 6-deoxy-5-hydroxytetracycline (doxycycline) and 7-dimethylamino-6-demethyl-6-deoxytetracycline (minocycline) (Agwuh and MacGowan, 2006; Chopra and Roberts, 2001).

III Third-generation tetracyclines are semi-synthetic or synthetic drugs developed to overcome the resistance mechanisms against earlier tetracyclines (Nelson and Levy, 2011).

Semi-synthetic:
- A group of compounds called glycylecyclines were generated by modifying position 9 of ring D of doxycycline, sancycline, and minocycline. These new tetracyclines were designed to evade the ribosomal protection and efflux conferred by Tet-resistance proteins (Barden et al., 1994; Sum and Petersen, 1999; Sum et al., 1994). Tigecycline is the only representative of the third-generation.
tetracyclines that has been approved for medical use (Babinchak et al., 2005; Ellis-Grosse et al., 2005; Tanaseanu et al., 2008).

- Another group of tetracyclines named aminomethylcyclines were developed by adding a 9-alkylaminomethyl side chain at ring D (Nelson and Levy, 2011). The leading compound of this group, omadacycline (PTK 0796), is about to enter phase III clinical trials for treatment of acute bacterial skin and skin structure infections as well as for community-acquired bacterial pneumonia. It is also being developed for treatment of complicated urinary tract infections (Butler et al., 2013; Pucci and Bush, 2013). Omadacycline is available as IV and oral formulations. Similarly to tigecycline, it evades Tet efflux and ribosomal protection mechanisms (Draper et al., 2014; Macone et al., 2014).

**Synthetic:**

- After a chemical synthesis pathway of tetracyclines was developed in 2005 (Charest et al., 2005), a new group of fully synthetic tetracycline analogues were synthesized: 8-azatetracyclines (Clark et al., 2011), pentacyclines (Sun et al., 2011), and fluorocyclines (Clark et al., 2012; Xiao et al., 2012). The most promising fluorocycline, which has a 9-pyrrolidinoacetamido modification of ring D, is called eravacycline (TP-434). As many third-generation tetracyclines, it overcomes tetracycline-specific efflux and ribosomal protection mechanisms (Grossman et al., 2012; Sutcliffe et al., 2013; Xiao et al., 2012). In addition to the parenteral therapy, an oral formulation is being developed. Eravacycline is enrolled in phase III clinical trials for complicated intra-abdominal and complicated urinary tract infections (Butler et al., 2013; Pucci and Bush, 2013; Sutcliffe et al., 2013).

**Tigecycline**

Tigecycline is a derivative of the second-generation tetracycline antibiotic minocycline with a 9-t-butylglycylamido group attached to the ring D of the tetracycline scaffold (Figure 6) (Petersen et al., 1999; Sum and Petersen, 1999). It binds ~5-fold to ~100-fold stronger to ribosomes than minocycline and tetracycline, respectively (Olson et al., 2006), due to the bulky side chain at position 9 (Jenner et al., 2013).
Uptake of tigecycline

In order to employ its antimicrobial activity, tigecycline has to cross membrane barriers to reach the cytoplasm of the cell. The entrance into a Gram-negative cell is more complicated because of the presence of the OM. The actual mechanism of uptake of tigecycline is not clear, but it is assumed that it follows the general tetracycline influx pathway (Figure 7).

Figure 7. Uptake of tigecycline into a Gram-negative cell. Tigecycline crosses the OM in complex with magnesium (TGC-Mg\(^{2+}\)) through the OM porins (e.g. OmpF) or slowly diffuses in an uncharged form (TGC) across the OM and subsequently through the IM into the cytoplasm.
Tetracyclines are chelating compounds and they form complexes with metal ions (Albert and Rees, 1956). Tetracycline and Mg$^{2+}$ positively charged complexes traverse the OM through the hydrophilic OM porins OmpF and OmpC (Mortimer and Piddock, 1993; Thanassi et al., 1995) because of the Donnan potential that forms due to separation of charges across the OM (Thanassi et al., 1995). In addition, as a result of lipophilic properties, the uncharged form of tetracyclines can cross the OM by diffusion. The rate of such diffusion is 100 times slower than diffusion through the inner membrane (IM) (Nikaido and Thanassi, 1993). Once in the periplasm, Mg$^{2+}$ and tetracycline complexes dissociate and form a protonated uncharged form of tetracycline, which diffuses through the IM into the cytoplasm following the pH difference between periplasm (lower pH) and cytoplasm (higher pH) (Argast and Beck, 1984; Nikaido and Thanassi, 1993; Thanassi et al., 1995).

**Tigecycline mode of action**

Tigecycline, like the rest of the typical tetracyclines, inhibits bacterial protein synthesis by binding to the tetracycline primary binding site on the 30S ribosomal subunit (Figure 8).

![Figure 8. Ribosomes are the target of tigecycline. The drug coordinates two Mg$^{2+}$ ions, binds to the A site of the 30S subunit, and inhibits protein synthesis.](image)

Its hydrophilic edge interacts with helices 31 (h31) and 34 (h34) of the 16S rRNA and physically prevents the EF-Tu-GTP-ami noacetyl-tRNA complex from binding to the A site and decoding mRNA. The interaction of tigecycline in complex with two Mg$^{2+}$ ions and the phosphate backbone of nucleotides G966, C1195, U1196, G1197, G1198, and C1054 of 16S rRNA is important for correct docking of the complex into the ribosome (Jenner et al., 2013; Schedlbauer et al., 2015). This interaction with the phosphate groups of 16S rRNA most likely is the reason for the broad activity of tigecycline. The drug binds with higher affinity to the ribosome compared with previous tetracyclines due to the structural differences of the molecule. If the 9-t-butylglycylamido group adopts a bent conformation, a stacking between the amide nitrogen atom and nucleobase C1054 is formed (Jenner et
al., 2013). If the orientation of the side chain is extended, ring D instead stacks with C1054 and both nitrogens of the 9-t-butylglycylamido group are predicted to form hydrogen bonds with that nucleobase (Schedlbauer et al., 2015). A subsequent stacking between nucleobases C1054 and U1196 is also established, leading to a stronger binding to the target. Additionally, the 9-t-butylglycylamido group creates a steric clash with the anticodon loop of acylated-tRNAs (Jenner et al., 2013; Schedlbauer et al., 2015). Therefore, the combination of stacking and steric hindrance provides higher inhibitory properties of tigecycline compared to earlier tetracyclines.

Tigecycline overcomes two main tetracycline resistance mechanisms

Clinical tetracycline resistance is mainly conferred by tet genes expressing efflux pumps or ribosomal protection proteins (Chopra and Roberts, 2001). Semi-synthetic tigecycline, like the rest of the glycylcyclines, was designed to overcome the principal resistance mechanisms of earlier tetracyclines (Figure 9).

![Figure 9. Tet resistance mechanisms inactive against tigecycline. (a) Efflux [e.g. Tet(A)]. (b) Ribosomal protection [e.g. Tet(M)].](image)

**Efflux**

The majority of Tet efflux pumps belong to the major facilitator superfamily (MFS) of secondary transporters. They are divided into 28 classes, which are subsequently separated into 7 groups according to protein phylogeny (Guillaume et al., 2004). The most widespread Tet efflux pumps belong to groups 1 [e.g. Tet(A), Tet(B), Tet(C)] and 2 [e.g. Tet(K), Tet(L)] and all of them are members of the MFS transporters (Chopra and Roberts, 2001; Thaker et al., 2010). These proteins are composed of 12-14 transmembrane α-helices (TMs) that anchor the exporters in the inner membrane. The TMs are connected by cytoplasmic (C) and periplasmic (P) loops that form
substrate entrance and exit gates. Exporters with 12 TMs share the typical MSF fold, which is composed of N and C domains containing 6 TMs each with the smallest structural and functional unit being a 3-TM repeat (Yan, 2013) (Figure 10). Two TMs (1, 4 and 7, 10) are partially amphiphilic and they contribute to the substrate channel formation together with two completely amphiphilic TMs (2, 5 and 8, 11), which also form important interdomain interactions. The last two hydrophobic TMs (3, 6 and 9, 12) play a structural role (Tamura et al., 2001). These transporters expel tetracyclines complexed with Mg$^{2+}$ in exchange for H$^+$ (Yamaguchi et al., 1990). The transport is powered by the flow of H$^+$ down the proton gradient into the cytoplasm and is most likely separated from the tetracycline export channel (Tamura et al., 2003).

![Figure 10](image-url)

Figure 10. 12-TM MSF transporter Tet(B) (Tamura et al., 2001). (a) Structural organization of Tet(B). (b) The arrangement of TMs from the periplasmic and cytoplasmic sides. N-domain (dashed line), C-domain (solid line), partially amphiphilic TMs (green), completely amphiphilic TMs (blue), hydrophobic TMs (pink).
The controlled expression of these genes is important, because Tet exporters are toxic to the cell (Guay and Rothstein, 1993) since they affect the electric potential across the IM (Eckert and Beck, 1989). Therefore, the expression is usually regulated at the transcriptional or translational level. The transcriptional repressor TetR negatively regulates expression of Tet pumps (Hinrichs et al., 1994). The repression is lifted once tetracycline in complex with Mg$^{2+}$ is present, as it binds to the repressor and disrupts its interaction with the operator region. In other cases translational attenuation (Schwarz et al., 1992) and translational reinitiation (Stasinopoulos et al., 1998) have been shown to regulate the expression of Tet pumps through the formation of different stem loop structures in mRNA.

Tet(B) from Tn10 is the best characterized Tet efflux pump (Tamura et al., 2001). Tigecycline can bind to TetR(B) and activate the expression of Tet(B) (Hirata et al., 2004), but most likely due to the bulky 9-t-butylglycyclamido moiety, the drug is not recognized by the Tet efflux transporters, as was shown for earlier glycyclcyclines (Someya et al., 1995). Nevertheless, in vitro selection of glycyclcycline resistant Tet(B) mutants revealed that amino acid substitutions G111E (TM4), W231C/G (TM7), L253F (TM8), L308S (TM10) caused reduced susceptibility to tigecycline (Guay et al., 1994; Tuckman et al., 2000). In addition, veterinary and clinical Salmonella isolates were carrying a Tet(A) variant associated with Tn1721 (Hentschke et al., 2010; Tuckman et al., 2000). This specific variant increased tigecycline minimal inhibitory concentration (MIC) compared to another variant associated with pRP1, and it was suggested that a double frame shift mutation in loop C3 was the reason for the observed effect (Tuckman et al., 2000). However, when the frame shift was reconstructed in an isogenic background, the increase of tigecycline MIC was not observed (Fyfe et al., 2013). In paper III, we used Tet(A) from Tn1721 as a starting variant for selection of mutants with high-level tigecycline resistance. The vast majority of Tet(A) mutants accumulated mutations in TM5, TM7, TM8, and TM11 leading to increased tigecycline MICs of clinical relevance.

Ribosomal protection

Tet ribosomal protection proteins (RPPs) are GTPases homologous to translation elongation factor EF-G (Margus et al., 2007). There are 12 different classes of RPPs and they are divided into three groups. Tet(O) and Tet(M) proteins are the most widespread and the best characterized proteins of group 1 (Chopra and Roberts, 2001; Nguyen et al., 2014; Thaker et al., 2010). Both RPPs dislodge tetracycline from its primary binding site in a GTP-dependent way, but the hydrolysis of the nucleotide is needed for the turnover of the RPP, rather than removal of the drug (Burdett, 1996; Trieber et al., 1998).
Tet(O) and Tet(M) cryo-electron microscopy models (Figure 11a) revealed that loop III of domain IV is directly interacting with the primary tetracycline binding site (Dönhöfer et al., 2012; Li et al., 2013). Residue P509 of loop III stacks with nucleobase C1054 and disrupts the stacking between tetracycline ring D and the nucleobase, which leads to dissociation of the drug from the active site. The correct positioning of loop III is achieved through the stabilizing intradomain interactions with loops I and II within domain IV and interdomain interactions between domain IV (loop I) and the C-terminal helix (CTX) (Figure 11b). CTX interacts with nucleotides A1492 and A1493 of h44 in 16S rRNA and fixes them in the flipped out conformation, which is observed during the binding of the EF-Tu-GTP-aminoacyl-tRNA complex in the A site. Additionally, CTX stabilizes the position of domain IV through interaction with nucleotide A1913 of H69 in 23S rRNA. Lastly, the interaction between ribosomal proteins L6, L7, and GTP-binding domain I results in GTP hydrolysis and dissociation of the Tet protein (Arenz et al., 2015; Dönhöfer et al., 2012).

Tigecycline forms stronger bonds with the ribosome and the 9-t-butylglycylamido group interferes with the correct positioning of the P509 residue and the C1054 nucleobase, which leads to diminished ribosomal protection (Arenz et al., 2015; Jenner et al., 2013). In paper III, we explored the potential of Tet(M) to acquire activity against tigecycline, which resulted in selection of mutants carrying changes in loop III of domain IV.

Figure 11. Cryo-electron microscopy model of Tet(M). (a) Tertiary organization of the Tet(M) protein [PDB 3J25 (Dönhöfer et al., 2012)]. (b) Arrangement of loops (I-III) of domain IV [PDB 3J9Y (Arenz et al., 2015)]. Intradomain and interdomain interactions are marked with black solid line arrows and black dashed line arrows, respectively.

Resistance to tigecycline

Clinical resistance to tigecycline was described a few years after its approval for use in clinical practice (Sun et al., 2013). However, the interpretation of
tigecycline resistance is complicated due to different clinical breakpoints set by the FDA: $\leq 2$ mg/L – sensitive, $\geq 8$ mg/L – resistant (FDA, 2005); and the European Committee on Antimicrobial Susceptibility Testing (EUCAST): $\leq 1$ mg/L – sensitive, $>2$ mg/L – resistant (EUCAST, 2006).

Gram-negative pathogens like Acinetobacter baumannii, Klebsiella pneumoniae, Enterobacter spp., and Bacteroides fragilis are among the main bacteria that develop tigecycline resistance (Sun et al., 2013). Other Gram-negative microbes like Pseudomonas aeruginosa, Proteus spp., Morganella spp., and Providencia spp. are intrinsically less susceptible to tigecycline. The reason for this reduced susceptibility is a constitutive expression of resistance-nodulation-division (RND) efflux proteins (e.g. AcrAB system) (Petersen et al., 1999). In addition, efflux pumps of the RND family have been associated with an in vitro increase of tigecycline MIC in K. pneumoniae (Bratu et al., 2009; Rosenblum et al., 2011; Ruzin et al., 2005), Escherichia coli (Hirata et al., 2004; Keeney et al., 2008), Salmonella enterica (Hentschke et al., 2010; Horiyama et al., 2011), Enterobacter cloacae (Hornsey et al., 2010b; Keeney et al., 2007), and Serratia marcescens (Hornsey et al., 2010a). Up-regulation of the MATE family transporter MepA was related to decreased tigecycline susceptibility in S. aureus (McAleese et al., 2005).

**AcrAB-ToIC transporter**

AcrAB-ToIC is one of the main constitutively expressed transport systems that E. coli exploits to prevent toxic compounds from accumulating in the cytoplasm (Figure 12). Contrary to the Tet transporters discussed above, AcrAB-ToIC is a three-component system that belongs to the RND family. It spans both membranes of E. coli and exports compounds from the cytoplasm and the periplasm into the surrounding medium (Li et al., 2015). The three parts of the transport system are the IM anchored AcrB, the OM protein ToIC, and the membrane fusion protein AcrA, which bridges the two other components (Du et al., 2014). A small IM protein AcrZ has been recently identified to interact with AcrB and influence the transport of certain compounds (Hobbs et al., 2012).

AcrB is a substrate/H$^+$ antipporter powered by the proton motive force (Zgurskaya and Nikaido, 1999). It is a trimer and each monomer consists of three domains: transmembrane, porter, and ToIC docking (Murakami et al., 2002). ToIC is also a trimeric protein, which forms an open tunnel inserted in the OM by the β-barrel domains. The α-helical domains also form a barrel structure protruding into the periplasm, but they are closed in the proximal part of the channel. The equatorial domains of ToIC were suggested to play a role in docking the OM protein to the IM transporter (Du et al., 2014; Koronakis et al., 2000). The last component of the transport system is AcrA. It consists of an α-helical hairpin, lipoyl domain, and β-barrel domain. The
α-helical hairpin is suggested to interact with TolC and induce opening of the channel (Mikolosko et al., 2006), while the other domains form interactions with AcrB (Symmons et al., 2009). The exact stoichiometry of AcrA in the fully assembled system is not determined, but according to the recent cryo-electron microscopy model, AcrA forms a hexameric bridge between the trimeric AcrB and TolC creating an uninterrupted conduit for the export of a wide variety of substrates (Du et al., 2014).

![Diagram](image_url)

Figure 12. AcrAB-TolC efflux system. Over-expression of this transporter is the main tigecycline resistance mechanism reported in *E. coli* in clinic.

The substrate is translocated through so called functional rotation of AcrB monomers. Each monomer acquires one of three suggested states: access, binding, and extrusion, in a rotational manner. During the access state, the substrate enters the transporter from the inner/outer leaflet of the IM or the periplasm and it is accommodated in the binding pocket during the binding state. The extrusion state follows next and the substrate is released into the channel to be transported outside the cell through TolC. The conformational changes happening during the switch between binding and extrusion states are coupled to the translocation of H⁺ from the periplasm to the cytoplasm (Murakami et al., 2006).

An extensive network of transcriptional regulators is involved in tuning the expression of the AcrAB-TolC system. AcrR is a dimeric two-domain local repressor of AcrAB that provides a loose repression resulting in constitutive production of the pump (Li et al., 2007; Ma et al., 1996). The major activators of the system are the global regulators MarA, SoxS, and Rob (Figure 13) (Alekshun and Levy, 1997). These proteins also up-regulate
the expression of \textit{micF}, which is an sRNA that inhibits the translation of the OmpF porin (Ariza et al., 1995; Chou et al., 1993; Cohen et al., 1988; Tanaka et al., 1997). MarA and SoxS have their local regulators MarR and SoxR, respectively, and they are both substrates for the Lon protease (Griffith et al., 2004). MarR is activated through specific aromatic compounds (e.g. salicylate) and in turn lifts the repression of MarA (Cohen et al., 1993; Martin and Rosner, 1995). SoxR senses superoxide and nitric oxide in the cell, which leads to up-regulation of SoxS (Greenberg et al., 1990; Nunoshiba et al., 1993; Tsaneva and Weiss, 1990). Constitutively expressed Rob protein is activated by fatty acids and bile salts (Rosenberg et al., 2003). Once active, these regulators bind to the \textit{mar} box upstream to the \textit{acrAB}, \textit{tolC}, and \textit{micF} genes and enhance their expression.

![Network of main AcrAB-TolC transcriptional regulators.](image)

Other regulators of AcrAB include repressors AcrS, H-NS, and activator SdiA (Hirakawa et al., 2008; Nishino and Yamaguchi, 2004; Rahmati et al., 2002). Additionally, the two-component system EvgAS positively regulates the whole AcrAB-TolC system, whereas PhoPQ activates only expression of the \textit{tolC} gene (Eguchi et al., 2003). Accumulation of spontaneous mutations in the regulation targets of the AcrAB-TolC network was investigated in \textbf{paper I} with the subsequent characterization of the mutants in \textbf{paper II}.

**Flavin-dependent monooxygenase Tet(X)**

Enzymatic modification or degradation are very common resistance mechanisms for \(\beta\)-lactams, aminoglycosides, and chloramphenicol. Only a few proteins modifying tetracyclines have been identified so far. Tet(X), a flavin-dependent monooxygenase, was initially found in the obligate anaerobe \textit{B. fragilis}, in transposons Tn4351 (Guiney et al., 1984; Speer and Salyers, 1988) and Tn4400 (Park and Levy, 1988). However, the resistance mechanism was not expressed in the anaerobically growing \textit{B. fragilis}, and only after transfer to aerobically cultivated \textit{E. coli} was the detoxification of tetracycline active (Guiney et al., 1984; Speer and Salyers, 1988; Yang et al., 2004).
Two orthologues of \textit{tet}(X), \textit{tet}(X1) and \textit{tet}(X2), were identified in a conjugative transposon CTnDOT found in \textit{Bacteroides thetaiotaomicron} (Whittle et al., 2001). \textit{Tet}(X1) and \textit{Tet}(X2) had 66\% and 99\% amino acid identity with \textit{Tet}(X), respectively. Only \textit{Tet}(X) and \textit{Tet}(X2) modified tetracycline antibiotics, while the truncated \textit{Tet}(X1) showed no activity (Yang et al., 2004). \textit{Tet}(X) was also reported in the soil bacterium \textit{Sphingobacterium} \textit{sp.} (Ghosh et al., 2009). Its nucleotide and amino acid sequence was 99.8\% and 99.5\% identical to \textit{Tet}(X2), respectively. This bacterium was suggested as a possible ancestor of the \textit{tet}(X) gene, because the G+C\% content of \textit{Sphingobacterium} (36\%) and \textit{tet}(X) (37\%) was closer than that of \textit{Bacteroides} (40-42\%) (Ghosh et al., 2009; Speer et al., 1991).

\textit{Tet}(X) is a monomeric enzyme, which belongs to group A of flavin-dependent monooxigenases (Huijbers et al., 2014). It is composed of flavin- and substrate-binding domains, the assembly of which is stabilized by the C-terminal \( \alpha \)-helix (Figure 14a). The flavin-binding domain adopts a Rossmann fold needed to bind the adenosine part of cofactor FAD, whereas a catalytically active isoalloxazine part of FAD is positioned close to the active site. The substrate-binding domain contains substrate docking and O\(_2\) binding pockets. Tetracyclines are positioned in the active site through the interactions of the A ring and amino acid residues Q192, R213, H234, and G236. Additional bonds are formed with O4 and N5 atoms of isoalloxazine and the lower periphery of tetracyclines (Volkers et al., 2011; 2013).

![Figure 14](image)

\textbf{Figure 14.} Flavin-dependent monooxigenase \textit{Tet}(X). (a) Tertiary organization of the enzyme [PDB 4A6N (Volkers et al., 2013)]. (b) Chemical modification by \textit{Tet}(X). The enzyme has activity against tigecycline, as it modifies the main chelation center of the tetracycline core, which is important for antibacterial activity.

\textit{Tet}(X) uses NADPH as an external electron donor to reduce FAD and activate oxygen by forming a hydroperoxyflavin intermediate. The intermediate introduces one oxygen atom of O\(_2\) into the substrate and
reduces the second one to $\text{H}_2\text{O}$, regenerating FAD. The introduction of oxygen is performed through the regioselective hydroxylation reaction at carbon 11a between rings B and C of the tetracycline scaffold (Volkers et al., 2011; Yang et al., 2004) (Figure 14b). As the addition of a hydroxyl group at this position affects the $\text{Mg}^{2+}$ chelation centre, which is important for binding to the ribosome, the product of the reaction loses most of its antibacterial activity (Moore et al., 2005). Tet(X) modifies the scaffold of all tetracyclines, and therefore tigecycline as well as in-development omadacycline and eravacycline are also sensitive to the hydroxylation by Tet(X) (Grossman et al., 2012; Moore et al., 2005; Volkers et al., 2013). Recent studies reported the occurrence of tet(X), tet(X2), and a new orthologue tet(X3) in multidrug-resistant isolates of Enterobacteriaceae and Pseudomona-daceae causing urinary tract infections in Africa (Leski et al., 2013). Additionally, the tet(X1) variant was found in tigecycline non-susceptible A. baumannii isolates in China (Deng et al., 2013). Some of these variants were present in the known mobile genetic elements Tn4351 and CTnDOT, suggesting an HGT event (Leski et al., 2013). The spread of Tet(X) into human pathogens, combined with the emergence of variants with improved activity against the third-generation tetracyclines (paper III), threatens the medical future of all tetracycline antibiotics.
pUUH239.2, an ESBL plasmid

Plasmids as platforms for resistance spread

Plasmids are independently replicating extrachromosomal DNA molecules found in Gram-negative and Gram-positive bacteria. They can carry different genes that provide bacteria with additional properties permitting a better survival in harsh environments and a higher competitiveness for the same resources in ecological niches. Resistance determinants of heavy metals, disinfectants, and antibiotics are very common components found on plasmids (Actis et al., 1999).

Several resistance genes can be present on the same plasmid or bacteria can harbor several plasmids with different resistance determinants on them. Resistance genes are usually flanked by various insertion sequences, or they are found within transposons and integrons. All of these mobile genetic elements cause rearrangements and reshuffling of resistance determinants on the plasmids, making them very dynamic platforms for multidrug resistance development (Actis et al., 1999).

Another crucial property that makes plasmids such an important issue in clinical and veterinary medicine is their ability to horizontally transfer genes between different bacterial cells and various bacterial species. For example, the NDM-1 metallo-β-lactamase has spread worldwide without any boundaries of species or plasmid families (Kumarasamy et al., 2010).

The first major outbreak of ESBL-producing *Klebsiella pneumoniae* in Scandinavia

Northern Europe is generally considered to be a region with low selective pressure and low prevalence of multidrug-resistant bacteria. However, the trend is changing with more reports being published on outbreaks of ESBL-producing *E. coli* in Scandinavia (Alsterlund et al., 2009; Fang et al., 2004; Naseer et al., 2007; Woksepp et al., 2011).

The first major outbreak of ESBL-producing multidrug-resistant *K. pneumoniae* started at Uppsala University Hospital in 2005 (Lytsy et al., 2008). The outbreak clone of *K. pneumoniae* produced the CTX-M-15 ESBL, which is one of the most widespread CTX-M ESBLs in Europe (Livermore et al., 2007). The enzyme was located on the conjugative
plasmid pUUH239.2 (Figure 15), the characterization of which is described in paper IV.

The outbreak was contained in 2007 due to increased compliance with hand disinfection and hospital clothing rules among staff, better hand hygiene routines for patients, implementation of improved cleaning procedures, and addition of a higher number of beds. All the efforts to control the outbreak resulted in the total cost of 3 million euros for Uppsala University Hospital (Ransjö et al., 2010).

Figure 15. Map of a conjugative plasmid pUUH239.2 with an enlarged antibiotic resistance cassette.
Present investigations

Paper I – Mechanisms and fitness costs of tigecycline resistance in *Escherichia coli*

*Aim*
To select tigecycline-resistant *E. coli* mutants *in vitro* and to characterize them with regards to mutation rates, resistance mechanisms, resistance level, cross-resistance, and fitness costs. Also, to compare *in vitro*-selected mutants with clinical isolates demonstrating reduced susceptibility to tigecycline.

*Results and discussion*
Spontaneous *E. coli* mutants with decreased susceptibility to tigecycline were selected *in vitro* at a rate of $10^{-8}$ to $10^{-6}$ per cell per generation. All mutants demonstrated low-level resistance to tigecycline and the clinical breakpoints set by EUCAST were not reached in our selection experiments. Mutations and genome rearrangements leading to increased tigecycline MICs were identified in efflux regulatory network genes (ERN group) and genes of the LPS core biosynthesis pathway (LPS group).

Many first-step ERN mutants acquired inactivating mutations in the Lon protease, which caused the stabilization of MarA and a consequent up-regulation of the AcrAB efflux system and down-regulation of the porin OmpF through increased expression of the non-coding RNA MicF. In addition, some first-step mutants had inactivating mutations in the local repressor AcrR, causing a lifted repression of *acrAB*. The second-step ERN mutants, with *lon* being the first inactivated target, further acquired mutations in *marR* or *acrR* to increase the expression of AcrAB even more. Therefore, ERN mutants showed increased MICs of tigecycline mainly due to the over-expression of the multidrug efflux pump AcrAB. Reduced influx of the drug through OmpF could have also played a role in the reduced susceptibility to tigecycline.

The first-step LPS mutants with low-level tigecycline resistance harbored inactivating mutations in heptose biosynthesis (*lpcA, rfaE, rfaD*) or transport (*rfaC, rfaF*) genes. Mutations in any of these genes result in impaired biosynthesis or assembly of LPS inner core leading to the deep-rough phenotype. Deep-rough mutants are more sensitive to hydrophobic compounds, have a leaky OM, significantly reduced expression of OM
proteins, including porins, and a mucoid appearance (Schnaitman and Klena, 1993). The sensitivity to novobiocin confirmed that LPS of these mutants was unstable. Although the exact entrance mechanism of tigecycline is not clear, it is assumed to follow the general tetracycline uptake pathway. Additionally, porin-independent diffusion through the OM was proposed as another port of entry of tetracyclines, especially the more lipophilic ones (Nikaido and Thanassi, 1993). The hydrophobic properties of tigecycline and the deep-rough phenotype of heptose pathway mutants would suggest that our in vitro selected strains should be more sensitive to tigecycline. However, a possible explanation for the observed reduced susceptibility could be that the extensive reduction of OM proteins would drastically impair the main mechanism of drug uptake and the porin-free diffusion would not ensure a sufficient intracellular concentration of tigecycline to inhibit translation. An interesting observation was made in the majority of second-step LPS mutants, which, in addition to the starting lpcA mutation, acquired duplications of the chromosomal region containing the acrAB genes, representing a combination of both resistance pathways.

Cross-resistance to hydrophobic antibiotics (chloramphenicol, erythromycin) was seen in ERN mutants, as these drugs are the substrates of the up-regulated multidrug resistance pump AcrAB. In contrast, increased sensitivity to these drugs was observed in LPS mutants expressing deep-rough phenotype. Lon mutants were very sensitive to SOS inducers (nitrofurantoin and ciprofloxacin), as one of the regulators responsible for the arrest of cell division (SulA) is the target of the Lon protease (Mizusawa and Gottesman, 1983). The comparison of first- and second-step mutants, especially in the ERN group, revealed gradual increases in the MICs of tetracycline, ampicillin, and cefotaxime. Kanamycin demonstrated no cross-resistance.

Our in vitro-selected mutants had an approximately 30% reduction in relative fitness compared to the wild-type strain. A more thorough evaluation of growth parameters was performed on reconstructed mutants. All three main phases of growth (lag, exponential, and stationary) were measured and relative fitness was found to be reduced compared to wild-type control in all stages. This suggests that these mutants would have difficulties establishing successful infections.

The analysis of whole genome sequenced clinical isolates, which showed similar tigecycline MICs to the in vitro-selected mutants, revealed that the same target genes were mutated in these isolates.

**Conclusion**

Tigecycline can select spontaneous low-level resistance in *E. coli* with relatively high mutation rates but with substantial fitness costs. Changes in the same target genes identified in vitro can also be found in clinical isolates with reduced susceptibility to tigecycline.
Paper II – Fitness of *Escherichia coli* mutants with reduced susceptibility to tigecycline

*Aim*
To evaluate the fitness of *E. coli* efflux and LPS mutants selected in paper I by exposing them to low pH, bile, oxidative stress, and human serum, the conditions that *E. coli* could encounter *in vivo*. To assess growth of these mutants during *in vitro* competitions, and to determine their survival within an animal host.

*Results and discussion*
In our study, the *in vitro* tests were designed to model the conditions that *E. coli* cells go through when infecting the host (reduced pH and high bile concentrations in the gastrointestinal tract) or when interacting with host immune cells (exposure to oxidative stress) and penetrating bloodstream (contact with human serum).

Both efflux and LPS reconstructed mutants behaved similar to the wild-type control at acidic and oxidative conditions. Even though the Lon protease controls the turnover of the acid resistance (GadE) and oxidative stress (SoxS) activators (Griffith et al., 2004; Heuveling et al., 2008), its role is to shut those responses down rather than induce them. Therefore, inactivation of Lon had no effect on *E. coli* cells in these conditions. Moreover, neither up-regulation of the AcrAB efflux system, nor truncated LPS seem to be important in the low pH and oxidizing environments.

When the mutants were exposed to human serum, they behaved similarly to the wild-type control with the exception of the lon mutant, which better tolerated the bactericidal effects of serum. Inactivating mutations of *lon* lead to overproduction of colanic acid (Markovitz, 1964), and a recent study showed that it can play a role in protecting *E. coli* from antibacterial serum activity (Phan et al., 2013). This could explain why we observed a 2-log difference between the viable counts in the *lon* mutant and the control.
However, a higher production of colanic acid is also associated with a deep-rough LPS phenotype (Parker et al., 1992), but we did not observe any differences when the LPS mutants were exposed to serum.

We performed in vitro competition assays to evaluate all three growth phases of the mutants more precisely. In addition, as antibiotic resistance can be selected at sub-MIC levels and such concentrations can be present during antibiotic treatment (Gullberg et al., 2011; Liu et al., 2011), we determined minimal selective concentrations (MSCs) of tigecycline for all reconstructed single efflux and LPS mutants with reduced susceptibility to tigecycline. Overall, efflux mutations had lower fitness cost compared with LPS mutations, which consequently led to low MSCs for efflux mutants. The marR mutation was the least costly and had an MSC of 3 ng/mL, which is approximately 16-fold lower than the MIC of tigecycline for the wild-type E. coli control. This finding suggests that acrAB regulator mutations are easier selected, compared with the LPS defect mutants, at drug concentrations far below the MIC of tigecycline.

To investigate mutant survival within the animal host, we performed in vivo competition experiments in two established mouse infection models, peritonitis and neutropenic mouse thigh muscle infection. The immune system of the host was compromised in the thigh muscle model due to depletion of neutrophils, while the neutrophil response was not affected in the peritonitis infection model. We did not observe a difference in competitions of the mutants and the wild-type control strain in peritoneum, whereas the competitions in the thigh muscle resulted in more than a 3-log decrease in viable counts for LPS mutants. One of the reasons for this might be the difference in neutrophil response. The detected killing of bacteria in the peritoneal cavity, where the innate immunity was functional, was mainly due to active phagocytic cells. However, the drop in LPS mutant bacterial counts in the thigh muscle with the compromised innate response was primarily because of growth rate differences.

Combinations of double and triple efflux and LPS mutations were constructed to evaluate their effects on fitness and tigecycline resistance. All combinations had lower fitness and increased tigecycline MICs compared to the corresponding single mutants in paper I. The most costly combination that also resulted in the highest level of tigecycline resistance was lon, marR. Both of these regulators stabilize the global activator MarA (Cohen et al., 1993; Griffith et al., 2004; Martin and Rosner, 1995), which in turn positively regulates the AcrAB-TolC system (Ma et al., 1995), leading to higher export of tigecycline from the cell. However, when the lpcA mutation was added to the lon, marR combination, the MIC of tigecycline for this mutant decreased and the fitness increased compared to the lon, marR mutant. It is possible that the LPS defect causes inefficient efflux through the AcrAB system due to instability of the outer membrane.
Conclusions
The majority of in vitro and in vivo test results suggest that efflux mutants are fitter than LPS mutants. This may explain why efflux is the most commonly observed mechanism of tigecycline resistance in clinical settings.

Paper III – Potential of tetracycline resistance proteins to evolve tigecycline resistance

Aim
To investigate the ability of Tet proteins to acquire and expand tigecycline resistance and to evaluate if tigecycline resistance in Tet proteins causes collateral sensitivity to earlier generation tetracyclines.

Results and discussion
In this study we evaluated the potential of Tet determinants to accumulate mutations leading to tigecycline resistance. We chose four common Tet proteins representing the main tetracycline resistance mechanisms. E. coli cells expressing Tet(A) and Tet(K) efflux pumps, Tet(M) ribosomal protection protein, and Tet(X) modification enzyme were tested for tigecycline activity. While none of the Tet proteins conferred a substantial increase in tigecycline resistance, Tet(A) and Tet(X) demonstrated a weak activity. We generated mutant libraries for all four proteins and screened them for increased tigecycline MICs.

The Tet(A) efflux pump used in our study originates from Tn1721 and is one of the most common Tet(A) variants found in clinical isolates (Fyfe et al., 2013; Hentschke et al., 2010). It is an integral protein inserted in the IM by 12 putative TMs connected by cytoplasmic and periplasmic loops that play a role in forming substrate entrance and exit gates, respectively. While the most frequently identified mutation (G300E) leading to increased resistance to tigecycline was located in the predicted periplasmic loop P5, the vast majority of mutations were spread throughout the transmembrane regions TM2, TM4, TM5, TM7, TM8, TM10, and TM11. Moreover, segments TM5, TM7, TM8, and TM11 were hotspots for mutations providing the pump with a tigecycline transport activity. It is important to emphasize that amino acid substitutions mainly accumulated in the transmembrane segments forming the channel of the pump, suggesting that remodeling of the channel architecture is important to accommodate the bulky molecule of tigecycline. Furthermore, in many cases the changes in the built channel resulted in loss of activity to export early tetracyclines.

Tet(K), an efflux protein composed of 14 predicted TMs, was evaluated next. While noticeably fewer mutants with reduced susceptibility to
tigecycline were selected during the Tet(K) library screens, the tendency was similar to the one described above. The main candidate mutation (Y58H) that was implicated in increased activity against tigecycline, was also located in the transmembrane region TM2. However, the export of tigecycline in Tet(K) mutants was modest and caused collateral sensitivity to the earlier tetracyclines.

In addition to efflux, ribosomal protection is the second common tetracycline resistance mechanism. We chose Tet(M), one of the best studied representatives of this group, for mutant selection experiments. Even though Tet(M) mutants with reduced susceptibility to tigecycline were carrying multiple amino acid changes, each of them contained mutations in the loop III of the domain IV. This domain is interacting with the active site of tetracyclines and is suggested to be responsible for the displacement of tetracycline antibiotics from the ribosomal A site (Arenz et al., 2015; Dönhöfer et al., 2012; Jenner et al., 2013). The L505 deletion, which resulted in a shortened loop III, had the highest increase in protective properties against tigecycline. However, this caused a dramatic decrease in the ability to protect the ribosome from minocycline, with almost no activity against doxycycline and tetracycline. Much milder effects were observed with amino acid substitutions S508G/R. Two more mutations outside domain IV (S310P and Q620R) increased tigecycline MICs when combined with S508G/R substitutions with mild collateral sensitivity to earlier tetracyclines. Collectively, these results suggest that it is possible to improve the ribosomal protection against tigecycline, but such improvements come with a functional trade-off leading to poorer activity against earlier tetracyclines.

The last tetracycline resistance mechanism we explored was modification by the Tet(X) enzyme, whose intrinsic activity against tigecycline was reported earlier (Moore et al., 2005) and also observed in our study. Contrary to the previous resistance mechanisms discussed above, which were affected by the substitutions at C9 of the D ring, Tet(X) interacts with the conserved ring A of the naphthacene nucleus (Volkers et al., 2011; 2013), where substitutions have a negative effect on antibacterial activity. A recent spread of the Tet(X) enzyme into clinical pathogens (Leski et al., 2013) is a worrisome trend, which can lead to compromised future of the whole class of tetracyclines. Therefore, we investigated the potential of this enzyme to develop high-level tigecycline resistance. During mutant screens, we identified four amino acid substitutions (T280A, N371T/I, N221K) that led to significant increases in the MIC of tigecycline, but only the N371T/I mutations were located near the substrate binding pocket. Other changes were localized near the predicted O2 binding pockets or parts of the enzyme far from the substrate-binding site. These results indicate that overall structural changes of the enzyme and O2 transport can play a role in improving tigecycline inactivation activity of Tet(X). Furthermore, in most
of the cases these alterations did not compromise the activity against earlier tetracyclines.

Conclusions
It is possible to select Tet protein mutants with high-level [Tet(A) and Tet(X)] and low-level [Tet(K) and Tet(M)] resistance to tigecycline. While these activity improvements come with a collateral sensitivity to the earlier tetracycline generations for Tet(A), Tet(K), and Tet(M), the Tet(X) enzyme is able to achieve this without compromising the activity against other tetracyclines. This is especially alarming as Tet(X) is spreading through the HGT and it can jeopardize clinical tetracycline antibiotic therapies.

Paper IV – Transfer of an *Escherichia coli* ST131 multiresistance cassette has created a *Klebsiella pneumoniae*-specific plasmid associated with a major nosocomial outbreak

Aim
To characterize the sequence of the multi-resistance plasmid of the *K. pneumoniae* clone from the outbreak and to determine its stability, fitness cost, and horizontal spread to other bacterial species *in vivo* and *in vitro*.

Results and discussion
Two plasmids named pUUH239.1 and pUUH239.2 were identified in the ESBL-producing *K. pneumoniae* strain from the Uppsala University Hospital outbreak. The smaller pUUH239.1 (~5 kbp) did not contain any antibiotic resistance genes, but it was found in all isolates of the *K. pneumoniae* clone tested. The larger pUUH239.2 (~221 kbp) was also detected in all tested isolates of the outbreak clone. It is a plasmid with a main backbone (~80%) that is 95%-100% identical to the plasmid pKPN3 from another strain of *K. pneumoniae*, and a resistance cassette with 100% sequence identity to the resistance cassettes of *E. coli* ST131 plasmids pEK499 and pC15-1a. The 41 kbp resistance cassette flanked by IS26 elements contains determinants conferring resistance to macrolides [mphR(A), mrx, mph(A)], chromate ions (chrA), quaternary ammonium compounds (qacEΔ1), trimethoprim (dhfrXII), aminoglycosides (aadA2), sulphonamides (sulI), β-lactams (bla<sub>TEM</sub>-1, bla<sub>OXA-1</sub>, bla<sub>CTX-M-15</sub>), aminoglycosides/fluoroquinolones [aac(6')-Ib-cr], and tetracyclines [tetA(A), tetR(A)]. Resistance to metal ions (arsenic, silver, and copper) is encoded outside the cassette. IS26 elements have played a central role not only in the rearrangement of the resistance cassette of pUUH239.2, but also in the insertion of the cassette into the
pKPN3 backbone. The incorporation of the resistance region from the well-known outbreak plasmids into the new backbone has created a novel plasmid in a new species, which spread successfully in clinical settings. It is interesting to note that _K. pneumoniae_ belonging to multilocus sequence typing (MLST) group ST16 have not been implicated in outbreaks before.

The phenotypic resistance profile of the _K. pneumoniae_ clone carrying pUUH239.2 revealed resistance to ampicillin, first- to fourth-generation cephalosporins, kanamycin, spectinomycin, erythromycin, tetracycline, trimethoprim, and sulphamethoxazole. All of these drugs had their corresponding resistance determinants present on the resistance cassette of the plasmid. In addition, reduced susceptibility to tigecycline was observed, which was associated with possible chromosomal mutations. Changes in the chromosome together with the plasmid increased MICs of quinolones.

Some patients infected by the _K. pneumoniae_ clone were also co-colonized by a second ESBL producer, _E. coli_. Pulsed-field gel electrophoresis (PFGE) patterns of plasmids from the _K. pneumoniae_ and _E. coli_ isolates were similar with some variation in the resistance cassette that was probably caused by recombination between IS26 elements. This indicates a horizontal spread of ESBL plasmid from _K. pneumoniae_ to _E. coli_ on multiple occasions in the gut of the patients. While the PFGE pattern of the chromosomes from _K. pneumoniae_ demonstrated a clonal spread, the PFGE patterns of chromosomes from _E. coli_ isolated from the double-infected patients, were different. This shows that a parallel clonal outbreak among _E. coli_ isolates did not occur. The reason for this can be the instability of the plasmid in _E. coli_ caused by the combined effects of fitness cost of plasmid carriage (4%) and relatively high segregational plasmid loss rate (~1/1000 cells per generation).

*In vitro* conjugation of pUUH239.2 to a range of Gram-negative bacteria was successful only to laboratory strains of _K. pneumoniae_ (3x10^5/donor) and _E. coli_ (5x10^-6 – 5x10^-8/donor). No transconjugants were obtained for _Citrobacter freundii, Proteus mirabilis, S. enterica_ or _P. aeruginosa_.

**Conclusion**

The integration of the resistance cassette from _E. coli_ plasmids, implicated in previous outbreaks, into the new backbone of pKPN3 plasmid resulted in a novel pUUH239.2 outbreak plasmid with higher stability in _K. pneumoniae_ than _E. coli_. The instability of the plasmid in _E. coli_ suggests that under removed selection pressure the plasmid will be rapidly lost and plasmid-free _E. coli_ cells will overtake the population.
Antibiotic resistance is a global problem, which has been accelerating since the 1940s to the current day. The armament of new antimicrobials to combat the imminent threat, however, has not kept the same pace. Therefore, we are in urgent need of novel antibacterial compounds to treat the infections caused by highly resistant bacteria. Tigecycline is one of very few modern antibiotics introduced recently into clinical practice and it has been shown to have a broad-spectrum activity against pathogenic bacteria. As tigecycline has been used clinically for a relatively short time, it is interesting to investigate the possible resistance development routes that bacteria might take to adapt to the mode of action of this antibiotic.

In paper I we explored the evolutionary capacity of E. coli to evade the antibacterial activity of tigecycline. We found that bacteria acquired spontaneous mutations in the multidrug efflux regulation and the LPS biosynthesis and assembly genes. These mutated targets resulted in reduced susceptibility to tigecycline. Furthermore, spontaneous mutations accumulated with relatively high rates and conferred a substantial fitness cost, which suggests that the mutants might have difficulties in establishing and maintaining a successful infection. To further address the fitness of the mutants with reduced susceptibility to tigecycline, in paper II we mimicked the environment E. coli would encounter in vivo. Testing of different conditions revealed that the efflux mutants were fitter compared with the LPS mutants. We also observed a similar trend during the competition experiments in mouse infection models. Therefore, it is likely that higher fitness of the efflux mutations is the reason for why up-regulation of export systems is the leading tigecycline resistance mechanism reported in clinics.

In addition to the spontaneous chromosomal changes leading to reduced susceptibility to tigecycline, in paper III we examined if tetracycline resistance genes can also evolve resistance against tigecycline, which was designed to circumvent the two most common tetracycline resistance mechanisms (efflux and ribosomal protection). Furthermore, we were interested to explore if improvements in a tetracycline modification enzyme may lead to tigecycline resistance of clinical relevance. Our results showed that it is possible to select tigecycline-resistant Tet mutants, and that in many cases such mutations result in collateral sensitivity to earlier tetracyclines. However, tigecycline resistance conferred by the Tet(X) enzyme is an exception, because mutations increasing the tigecycline resistance also
improved the activity against earlier tetracyclines. Consequentially, this particular enzyme poses a threat to the whole class of tetracycline antibiotics, especially because it has recently spread to the multidrug-resistant clinical pathogens. As Tet(X) has been found to reside in conjugative transposons, it is likely that these mobile genetic elements were used for horizontal spread of the enzyme.

Another very common way of acquiring a multidrug resistance phenotype is through mobile genetic elements like conjugative plasmids. In paper IV we characterized a multidrug-resistant plasmid pUUH239.2, which carried resistance genes to many antibiotic classes, heavy metals, and disinfectants. The rearrangements between several different plasmid backbones resulted in the formation of pUUH239.2, which successfully spread into a new bacterial host in a clinical setting. This finding demonstrates how plasmids may act like dynamic platforms for rapid dissemination of resistance determinants.

In summary, in order to establish rational and effective approaches to maintain the effectiveness of our antimicrobial arsenal and develop methods to predict future resistance evolution, we need to understand the basic mechanisms of bacterial resistance and how it spreads between bacteria as well as between hosts.
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Ir, žinoma, už didžiausią palaikymą ir skatinimą esu dėkingas savo **mamai**, tėčiui ir Domui. Be Jūsų viskas būtų daug sudėtingiau! Ačiū Jums už viską. Visus labai myliu!
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A doctoral dissertation from the Faculty of Medicine, Uppsala University, is usually a summary of a number of papers. A few copies of the complete dissertation are kept at major Swedish research libraries, while the summary alone is distributed internationally through the series Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine. (Prior to January, 2005, the series was published under the title “Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine”.)