DISSERTATIONES TECHNOLOGIAE UNIVERSITATIS TARTUENSIS

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# Studies on the antibiotic susceptibility of *Escherichia coli*

TARTU UNIVERSITY PRESS

Institue of Technology, University of Tartu, Estonia

Dissertation is accepted for the commencement of the degree of Doctor of Philosophy (Biomedical Engineering) on March 26th, 2012 by the council of the PhD study program Engineering and Technology.

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Commencement: Auditorium 121, Nooruse 1, Tartu, Estonia, at 10:15 on May 4th, 2012

Publication of this dissertation is granted by University of Tartu



ISSN 2228–0855
ISBN 978–9949–19–971–6 (trükis)
ISBN 978–9949–19–972–3 (PDF)



Tartu Ülikooli Kirjastus
www.tyk.ee
Tellimus nr. 156

# CONTENTS

# LIST OF ORIGINAL PUBLICATIONS

The current dissertation is based on the following publications referred to in the text by the Roman numbers:

I. **Luidalepp, H.**, Hallier, M., Felden, B., and Tenson, T. (2005). tmRNA decreases the bactericidal activity of aminoglycosides and the susceptibility to inhibitors of cell wall synthesis. *RNA Biol* **2**(2), 70–4.

II. Roostalu, J., Jõers, A., **Luidalepp, H.**, Kaldalu, N., and Tenson, T. (2008). Cell division in *Escherichia coli* cultures monitored at single cell resolution. *BMC Microbiol* **8**, 68.

III. **Luidalepp, H.**, Jõers, A., Kaldalu, N., and Tenson, T. (2011). Age of inoculum strongly influences persister frequency and can mask effects of mutations implicated in altered persistence. *J Bacteriol* **193**(14), 3598–605.

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My contribution to the articles is as follows:

I. I performed most of the experiments, except aminoacylation of tmRNA, analyzed all the data and participated in the writing of the paper.

II. I performed and analyzed the data of ampicillin treatment experiments and participated in the writing of the paper.

III. I performed all of the experiments, except half of the persister formation tests of single gene knock-out strains, analysed all the data and participated in the writing of the paper

# LIST OF ABBREVIATIONS

CF – cystic fibrosis
CFU – colony forming units
FACS – fluorescence activated cell sorting
FSC – forward scatter
GFP – green fluorescent protein
*hip* – high persistent
MBC – minimal bactericidal concentration
MIC – minimal inhibitory concentration
MRSA – methicillin-resistant *Staphylococcus aureus*
PCD – programmed cell death
PTC – peptidyl transferase center
ROS – reactive oxygen species
SSC – side scatter
TA – toxin antitoxin
TCA – tricarboxylic acid
tmRNA – transfer-messenger RNA

# INTRODUCTION

Antibiotics, the compounds which inhibit microbial growth or kill them, were once thought as wonder drugs closing the book of infectious diseases. Nevertheless, soon after the widespread use of antibiotics clinically relevant resistant strains appeared, and they started to spread worldwide. At first, the discovery rate of new drugs was high, and the resistant bacteria were not that big of a problem as there were plenty of new drugs or even families of drugs coming into clinical use. As time went by, less and less new drugs were identified or developed leading to the situation that there was a period of 30 years where only one entirely new family of antibiotics reached the market (Butler and Buss, 2006). As the list of effective drugs is getting scarcer and new drugs are emerging rarely, more interest has been paid to the strategies how to increase efficiency of the existing drugs.

As various physiological processes are involved in responses to antibiotics and, as antibiotics have broad impact on bacterial physiology, one strategy to potentiate existing drugs is to co-inhibit some other processes leading to higher sensitivity to the drugs. One such target is tmRNA that is conducting process called trans-translation, which releases ribosomes stalled on mRNAs (Vioque & de la Cruz, 2003, de la Cruz & Vioque, 2001, Abo *et al.*, 2002). The first part of the current thesis is concentrating on the closer examination of antibiotic sensitivity of tmRNA deficient strain of *Escherichia coli*.

Bacterial populations contain a small subpopulation of cells insensitive to most of the antibiotics. Those cells are called persisters, and they are thought to be responsible for chronic and long-term infections (Lewis, 2010). Eradicating those cells would help to make treatments with current drugs more effective. Unfortunately, the essence of persister cells is not clear, although they are known over 60 years. Therefore, the second part of the experimental section of my thesis is dedicated to describing this intriguing phenomenon in more detail. Particularly, I have been interested to find out how the age of the inoculum is affecting the frequency of persisters in the model organism *E. coli*.

# 1. REVIEW OF LITERATURE

## 1.1. Definition of antibiotics

Introduction to antibiotics should start with definition of antibiotics, but it is not as straightforward as it seems, because there are multiple definitions of antibiotics. Most common definition for antibiotics is that they are compounds that are killing microorganism or slowing down their growth at low concentrations (in other words in high dilution). Sometimes only antibacterial agents are counted as antibiotics excluding, for example, antifungal agents. Some definitions, on the other hand, include only compounds produced by microorganisms and exclude synthetic compounds as does the first definition by Selman Waksman (Waksman, 1947). Nowadays the discrimination by the origin is not well reasoned since many antibiotics used are semisynthetic and total synthesis for many natural antibiotics is possible (Tatsuta, 2008) or is even in practical use (for example, chloramphenicol (Sinhg, 2002)).

## 1.2. Classification of antibiotics

Antibiotics can be classified in different ways. Most commonly it is done by their chemical structure forming structural classes like macrolides, aminoglycosides, β-lactams etc. Mechanism of action or target is another way to classify antibiotics. Main targets for most common antibiotics are DNA, RNA, protein and cell wall synthesis, structure and function of cell membrane and folic acid synthesis. Components of biosynthetic machinery differ to some extent between eukaryotes and prokaryotes, which are making human and animal use of antibiotics, targeting previously mentioned processes, possible.

Antibiotics that belong to the same structural class mostly have the same target and mechanism of action. Aminoglycosides make an interesting exception, as they target protein synthesis in different ways. The most common aminoglycosides are causing translational misreading (Gromadski & Rodnina, 2004, Carter *et al.*, 2000). An unique member of aminoglycosides is kasugamycin, which inhibits initiation of translation and is bacteriostatic, while the other members of the class are bactericidal (Pestka, 1979).

### 1.2.1. Bactericidal and bacteriostatic antibiotics

Another way to classify antibiotics is to divide them, according to their ability to cause cell death or hamper the growth, into groups of bactericidal and bacteriostatic drugs. Although definitions of those terms seem to be straightforward and simple, the situation is actually quite complicated. Antibiotics that are bactericidal against one class of organisms do not often kill another type of bacteria.

Multiple definitions can be found in the literature for the bactericidal or bacteriostatic activity. Microbiological *in vitro* definition can be based on changes of the viability counts – in case of decrease of CFUs the drug is called bactericidal. Authors with more clinical background are often using definition based on more quantitative estimate and use a parameter called minimal bactericidal concentration (MBC). MBC is defined as the lowest concentration of the compound causing death of 99,9% bacteria during 18–24 hours of incubation (Amsterdam, 2005). This means that an antibiotic is called bactericidal, when it can cause CFU drop of at least three orders of magnitude. Antibiotics that are able to kill only 90% or 99% of the cells do not qualify as bactericidal by this rather strict definition. Some tolerant strains might not die fast enough complicating the use of this definition. This is probably the reason why sometimes lower killing yields are used for defining bactericidal activity (Lamb *et al.*, 1999).

Minimal inhibitory concentration (MIC), the lowest concentration of the compound that is inhibiting visible growth of microorganisms during 18–24 hours of incubation (Wiegand *et al.*, 2008), is another parameter used for defining the type of antibiotic activity. Particularly, MBC/MIC ratio is used for this type of estimation – bacteriostatic are antibiotics with MBC/MIC > 4 (Pankey and Sabath, 2004). Often antibiotics, classically considered being bactericidal, have MBC and MIC ratio around one (Smith, 2004).

However, the determination of MBC to MIC ratio can be problematic since the techniques of measuring those parameters have not always been standardized and some varying technical parameters can influence the outcome (Pankey and Sabath, 2004). To summarize, the definition of bactericidal or bacteriostatic activity of an agent can be applied to certain organism (or even strain) in certain testing conditions.

Characterizing antibiotics by bactericidal or bacteriostatic activity in a clinical practice takes into account *in vivo* effects of the drug. Those may not overlap with the *in vitro* results, since the pharmacodynamic/pharmacokinetic parameters are influencing the outcome. There are several cases where antibiotics having *in vitro* bactericidal activity can act *in vivo* only bacteriostatically (Menashe *et al.*, 2008) and also *vice versa* (Zurenko *et al.*, 2001).

One example of drugs with complex behavior is macrolides that are considered to be bacteriostatic; however, the situation is not so simple. Most of the macrolides are actually concentration dependently bactericidal, although the concentrations needed for killing *in vitro* are usually much higher than achievable in human plasma, meaning that in clinical use they are mostly still bacteriostatic (Zhanel *et al.*, 2001). More potent is the ketolide subfamily with the so far only marketed member telithromycin having MIC and MBC values often much lower compared to other macrolides, so that the plasma concentration is exceeding MIC or even MBC and the drug is bactericidal also *in vivo* (Muller-Serieys *et al.*, 2004, Drago *et al.*, 2005).

Beside the concentration of the drug, also the type of pathogen can influence the mode of activity of macrolides, meaning that the list of organisms killed effectively depends on the drug. For example, erythromycin is *in vitro* bactericidal against *Streptococcus pneumoniae* and *Streptococcus pyogenes*, while clarithromycin and azithromycin are bactericidal against *S. pyogenes* and *Haemophilus influenzae* and the ketolides are bactericidal against *S. pneumoniae* and *H. influenzae* (Zhanel et al., 2001, Kanatani & Guglielmo, 1994).

Another interesting fact about macrolides is that azithromycin and clarithromycin are effective against several pathogens (for example, against *H. influenzae*), although their serum concentrations do not reach the MIC value (Zhanel et al., 2001). This is due to the absorption of the drug at high levels to some tissues and fluids; in addition, azithromycin does concentrate into the white blood cells which might transport the drugs to the site of infection (Zhanel et al., 2001, Schentag & Ballow, 1991, Rodvold *et al.*, 1997).

*Pseudomonas aeruginosa* is also an example of microbe having much higher *in vitro* MIC values (measured using exponential phase cultures) than the achievable concentration in infection site (Zhanel et al., 2001, Mulet *et al.*, 2009). However, macrolides, especially azithromycin, are actively used for the treatment of chronic *P. aeruginosa* infections in cystic fibrosis (CF) patients. This is remarkable, because in the airways of the CF patients *P. aeruginosa* forms microcolonies or biofilms, i.e., formations where cells are usually less susceptible to antibiotics compared to planktonic cells. It is revealed now that azithromycin is more active (even bactericidal) against biofilm (Mulet et al., 2009) or stationary phase culture (Imamura *et al.*, 2005) compared to the exponential phase cells. There are indications that azithromycin is directly disrupting outer membrane of the stationary phase cells (Imamura et al., 2005), although the killing is strictly protein synthesis dependent (Kohler *et al.*, 2007, Klepser *et al.*, 1997, Klepser *et al.*, 1996, Athamna *et al.*, 2004). It suggests that the disruption of the outer membrane will result in increased uptake of azithromycin, which will lead to the stronger inhibition of protein synthesis and thereafter to the death of the cells.

In addition to macrolides, there are many other antibiotics that can act in some cases as bactericidal and in others as bacteriostatic. Clindamycin is a representative of lincosamide subclass of antibiotics that are considered to be bacteriostatic, but this drug has been shown to have *in vitro* bactericidal activity against some organisms (Klepser et al., 1997, Klepser et al., 1996, Athamna et al., 2004). This is the case also for linezolid (representative of oxazolidiniones) (Zurenko et al., 2001). Chloramphenicol that is considered to be a classical bacteriostatic antibiotic can also be bactericidal against some organisms (Rahal and Simberkoff, 1979). Interestingly, stationary phase cells of *H. influenzae* are more sensitive to chloramphenicol as compared to exponential phase culture (Feldman and Manning, 1983), although an opposite effect is observed with most organisms and drugs.

Chloramphenicol is bacteriostatic against most of the *E. coli* strains having MBC values 250 μg/ml or more (Rahal and Simberkoff, 1979). This is about an order of magnitude higher than concentrations regularly used *in vitro* (Sambrook *et al.*, 1989), not to mention achievable plasma concentrations (Kohanski *et al.*, 2010). Still there are *E. coli* K-1 strains with MBC values much lower (10–80 μg/ml) and comparable with the achievable plasma concentrations. The reason of this decrease in MBC value is unknown, but it might involve the specific polysaccharide capsule of these strains (Orskov *et al.*, 1977). This is supported by the observation that chloramphenicol also is bactericidal against several other bacteria causing meningitis (Robbins *et al.*, 1974, Rahal & Simberkoff, 1979), which also have capsules that can be very similar if not identical to *E. coli* K-1 (for example, *N. meningitis* group B) (Echarti *et al.*, 1983). In addition, it is possible that adaptations needed for causing meningitis are somehow involved with bactericidal activity of chloramphenicol.

The inhibitor of RNA synthesis, rifampicin, is considered bactericidal against Gram-positive bacteria but bacteriostatic against Gram-negative bacteria (Kohanski et al., 2010). Interestingly, *recA* mutant of *E. coli* is killed by rifampicin, yet the mechanism behind it is unknown (Kohanski *et al.*, 2007).

Dividing antibiotics by their ability to kill or not to kill bacteria raises the question which of the classes should be preferred for clinical use. Actually, the debate over clinical preference of bactericidal or bacteriostatic antibiotics has been going for years, and there are only some cases when clinical experiences and animal models are favoring bactericidal drugs (Pankey and Sabath, 2004). Still, there are some disadvantages of killing or specially lysing bacteria, mostly involving release of bacterial products. For example, endo- and exotoxins may be released, which can stimulate production of cytokines, which leads to harmful inflammations or even toxic shock syndrome (Pankey & Sabath, 2004, Finberg *et al.*, 2004).

### 1.2.2. Broad and narrow spectrum antibiotics

Antibiotics are divided also into a broad spectrum or narrow spectrum antibiotics depending on the variety of organisms they affect. The broad spectrum antibiotics (for example, tetracyclines and newer β-lactams) usually are potent against both Gram-positive and Gram-negative bacteria. The narrow spectrum antibiotics (for example, older penicillins and macrolides) affect only smaller group of bacteria (Acar, 1997). The difference between those antibiotic groups comes probably mostly from the cell penetration ability (for example, the outer membrane of Gram-negative bacteria can be an efficient barrier for several drugs) or low conservation of the target.

## 1.3. Effects of antibiotic combinations

Another interesting aspect about impact of antibiotics on bacteria is their effects when acting in combinations. Multidrug treatments are important in clinical use, mostly to provide broad-spectrum coverage to patients who are seriously ill and may be septicemic. In addition, multidrug combinations are used to enhance the treatment efficiency and to avoid the development of resistance; good example is tuberculosis treatment, where combination of four drugs is used (Hecht, 2005). Combining drugs is also an interesting tool for studying biological systems and interactions between different cellular processes (Tsui *et al.*, 2004, Lehar *et al.*, 2007, Lehar *et al.*, 2008, Keith *et al.*, 2005).

Combinations of drugs can be classified as antagonistic, additive or synergistic depending on their combined effect being less, equal or greater than expected sum of their individual inhibitory effects. Reasons for such effects are not often clear and can be quite complex.

The synergistic drug combinations are the one that have gained most of the attention due to the increased potency. The downside of these kinds of combinations can be the increased risk in resistance development. The antagonistic drug combinations, on the contrary, can slow down or even reverse resistance (Yeh *et al.*, 2006, Michel *et al.*, 2008, Hegreness *et al.*, 2008, Chait *et al.*, 2007). Still, those papers are theoretical or based on laboratory experiments and there are no clinical reports about this issue.

Mechanisms for achieving the synergism can be various. For example, one drug can increase uptake of another. This is the case for cell wall synthesis inhibitors that are increasing uptake of aminoglycosides by causing changes in the cell wall (Moellering and Weinberg, 1971). Other ways of causing synergistic effects are observed when the additional drug is inhibiting resistance enzymes (for example, β-lactamases) (Walsh, 2003) or efflux pumps protecting bacteria from another drug (Pages and Amaral, 2009).

Synergistic effects are often observed when combined antibiotics are inhibiting sequential reactions. One example of this kind of interactions is between trimethoprim and sulfonamides which are both inhibiting tetrahydrofolate synthesis (Walsh, 2003). The mechanism of how the synergistic outcome forms is not clear. Moreover, there are some doubts on theoretical grounds about the possibility of simple inhibition of sequential reactions to lead to synergistic effects (Webb, 1963). Recently, it has been shown that the synergy of trimethoprim and sulfonamides is much more complicated and probably involves differential inhibition of various downstream biosynthetic reactions (Nichols *et al.*, 2011).

Synergistic effects between the antibiotics inhibiting sequential reactions are also observed between classes of different cell wall syntheses inhibitors, for example, between penicillins and vancomycin or phosphomycin analogues (Pillai *et al.*, 2005).

Another interesting synergistic interaction occurs between streptogramins, especially between the representatives of streptogramin A and B groups, which are produced by organisms usually in pair ways (Pestka, 1979; Walsh, 2003). Separately they are considered to be bacteriostatic agents, but this combination is in addition to higher potency also bactericidal (Lamb et al., 1999). Drugs of both classes are binding to the region of peptidyl transferase center (PTC). The representatives of class A are inhibiting peptidyl transferase reaction directly by interfering with substrate binding at both acceptor and donor sites of the PTC. Their binding is causing conformational changes in the ribosome, this way enhancing binding of the streptogramin B into the nascent peptide exiting tunnel (Harms *et al.*, 2004), where it is inhibiting peptide chain elongation and is inducing detachment of incomplete peptide chains. Cooperatively the affinity of the drugs to the ribosome is very high and thereby leads to efficient blocking of protein synthesis.

In addition to already mentioned synergistic interactions there are many more combinations where mechanism of antibiotic action is largely or totally unknown (Yeh et al., 2006, Pillai et al., 2005).

Antagonistic interactions are also common between antibiotics but are mostly described *in vitro*. The reason for the underrepresentation of *in vivo* studies might come from the difficulties to recognize antagonism in case of complex diseases in the presence of intact immune system, which is actually playing very important role in the eradication of pathogens (Pillai *et al.*, 2005).

Antagonistic interactions have been often observed between bacteriostatic and bactericidal antibiotics. Examples involve bacteriostatic inhibitors of protein synthesis in combination with β-lactams or vancomycin. Those bactericidal antibiotics kill only growing cells but in the mentioned examples bacteriostatic drugs are causing stasis that is diminishing lethal activity of the killing drug. The antagonism between the bactericidal aminoglycosides and bacteriostatic inhibitors of protein synthesis is explained by possible inhibition of energy-dependent active uptake of aminoglycosides or just by inhibition of translation that has to be active for killing by aminoglycosides (Pillai et al., 2005).

Suppressive drug interaction is the extreme case of the antagonistic drug interactions when the combined inhibitory effect is not just smaller than the expected additive sum but even smaller than the effect of either drug alone (Pillai et al., 2005). This type of interactions occur between protein and DNA synthesis inhibitors. During DNA damage synthesis of DNA is down regulated in contrary to the production of ribosomes, which leads to the imbalance between DNA and protein content in cell. Addition of protein synthesis inhibitors can decrease the production of ribosomes and thereby restore the balance of proteins and DNA and therefore improve growth and survival of the bacteria (Bollenbach *et al.*, 2009).

## 1.4. Antibiotic killing mechanisms

Besides the inhibition of certain processes, the interaction between antibiotics and their targets can also have broader effects, which, among others, may lead to the killing of cells. The most studied drug-target interactions that are connected to cell death are inhibition of topoisomerases and cause of double stranded DNA breaks (Drlica *et al.*, 2008), inhibition of synthesis and damaging of structural integrity of cell wall (Tomasz, 1979) and protein mistranslation (Magnet and Blanchard, 2005).

### 1.4.1. DNA replication inhibitors

Modulation of DNA supercoiling is essential for DNA synthesis, transcription and cell division and it is catalyzed by topoisomerases by breaking and rejoining the DNA strand. The topoisomerase II (DNA gyrase) is thought to be responsible for reducing supercoiling during replication. The topoisomerase IV at the same time is unlinking or decatenating chromatin, but it can also reduce the supercoiling (Espeli and Marians, 2004). Those reactions are targeted by the class of antibiotics called quinolones with clinically significant subclass of fluoroquinolones. Quinolones are targeting topoisomerase II and IV and thereby causing double stranded breaks to the DNA (Sugino *et al.*, 1977, Gellert *et al.*, 1977, Drlica et al., 2008, Chen *et al.*, 1996). Quinolone preferences to the topoisomerases are varying depending on the organism. In Gram-negative bacteria, the main target is the topoisomerase II, and in Gram-positive organisms the topoisomerase IV acts as primary target (Drlica and Zhao, 1997).

The topoisomerases II and IV are heterodimeric enzymes, which work as a tetrameric complexs, where each dimeric enzyme is catalyzing formation of single-stranded break into one of the DNA strands and will stay covalently bound to the 5' end of the DNA (Morrison *et al.*, 1980, Mizuuchi *et al.*, 1980). This complex will be stalled by the quinolones as the DNA religation is inhibited and the so-called cleaved complex is formed (Heddle *et al.*, 2000). Subsequently, the replication will be stalled due to the collision of replication fork and the cleaved complex (Wentzell and Maxwell, 2000).

The double-stranded breaks are reversible at the moderate (bacteriostatic) concentrations of the drug. It means that just the inhibition of the replication or the generation of breaks cannot be lethal *per se.* Still, slow killing has been observed at the low drug concentrations over longer time period, but it is mechanistically poorly understood (Drlica *et al.*, 2008).

At the higher antibiotic concentrations bacteria will be killed faster. At the same time, the double stranded breaks are not reversible anymore, implying that the cleaved complexes dissociate (Malik *et al.*, 2006, Chen et al., 1996). As the cleaved complexes are scattered all around the chromosome, the widespread fragmentation of the chromosome will occur, and it could be the direct cause of fast death (Drlica et al., 2008). This hypothesis is supported by the observation

that when the quinolone mediated killing is blocked by the anaerobiosis or by the inhibition of translation with the chloramphenicol, the fragmentation of the chromosome is in correlation with cell death (Malik et al., 2006, Malik *et al.*, 2007).

The killing mechanism by the quinolones seems to be more complex, and there are two separate mechanism suggested for the fast killing: protein synthesis-dependent and independent cell death (Drlica et al., 2008). It is known that the inhibition of the translation by the chloramphenicol can block or reduce the killing activity of some quinolones. For example, killing of *E. coli* by nalidixic acid is stopped completely (Malik et al., 2007, Crumplin & Smith, 1975), although by norfloxacin only partially (Malik et al., 2006). At the same time, there are several newer compounds, still without conventional names, that are not blocked at all (Malik et al., 2006, Malik et al., 2007). This suggests that some antibiotics can induce both pathways.

The complexity of the killing mechanism of quinolones was shown by finding that anaerobiosis can inhibit killing by some quinolones (Malik et al., 2006, Malik et al., 2007). Later it was demonstrated that quinolones are inducing hydroxyl radicals via protein synthesis-dependent pathway, and this is needed for the killing activity (Dwyer *et al.*, 2007). Preliminary results suggest that fragmentation of the chromosome is not depending on the formation of the hydroxyl radicals, meaning that fragmentation by itself cannot be the reason of death induced through this pathway (X. Wang, unpublished results).

Induction of hydroxyl radical formation was observed also for quinolones that are thought to kill via the protein synthesis-independent pathway (Wang *et al.*, 2010b). As protein synthesis has been claimed to be essential for hydroxyl radical formation (Dwyer et al., 2007), this suggests that, in case of these drugs, also the protein synthesis dependent killing pathway is induced but could be too slow to play significant role in cell death. At the same time, addition of chloramphenicol inhibited formation of hydroxyl radicals only slightly meaning that hydroxyl radicals could be formed also by protein synthesis independent pathway.

### 1.4.2. Cell wall synthesis inhibitors

Peptidoclycan is an important structural component of bacterial cell wall, which gives strength and stability to the cell and counteracts with osmotic pressure of the cytoplasm. There are many types of antibiotics that inhibit peptidoglycan production at different stages of its synthesis, and the main classes are β-lactams and glycopeptide antibiotics (Walsh, 2003). They block the crosslinking of the peptidoglycan units that are building blocks of the cell wall. β-lactams (penicillins, carbapenems, penems, monopenems and cephalosporins) bind to the transpeptidases while glycoprotein antibiotics bind to the peptidoglycan, both classes of drugs inhibit the same crosslinking reaction.

The deadly mechanism of action of the cell wall inhibitors could be quite straitforward, as it was initially thought (Wise and Park, 1965). The idea is that during growth defective peptidoglycan will be produced, which results in weakened cell wall, that at some point does not bear the osmotic pressure and the cell will rupture. Later it was noted that penicillin can stop growth of some bacterial strains without causing lysis (Tomasz *et al.*, 1970). This finding lead to the conclusion that the bacterial lysis was more complex and can included active degradation by autolysins (Tomasz, 1979, Moreillon *et al.*, 1990, Kitano & Tomasz, 1979). At the same time, the mechanism of activation of the autolytic system remained unknown until the end of last century when several loci coding for proteins involved in activation of autolysins were described (Novak *et al.*, 1999, Novak *et al.*, 2000b, Brunskill & Bayles, 1996, Rice *et al.*, 2003, Groicher *et al.*, 2000). Activation of two of the loci is dependent of proton motive force(Groicher et al., 2000, Rice et al., 2003). This suggests that the electrophysiological state of the membranes is involved in the autolytic activation. Still, the activation mechanisms of the autolytic pathways are poorly understood (Rice & Bayles, 2008, Mitchell & Tuomanen, 2002).

In case of the autolysis-inhibiting agents or the autolysin defective strains, the non-autolytic cell death has been described (Novak et al., 2000b, Moreillon et al., 1990), although the mechanism is not clear. To make it more complicated there are some indications that SOS response might be involved in the β-lactam mediated cell death (Kohanski et al., 2010). Maybe the most interesting fact is that some β-lactams that are not very effective inducers of the lysis can cause the cell filamentation at lower concentrations (Spratt, 1975) similarly to the quinolones (Drlica et al., 2008). The filamentation could be caused by their ability to activate SOS response that might be a protective reaction as SOS deficient mutants have an increased susceptibility to these drugs (Miller *et al.*, 2004).

### 1.4.3. Drugs inducing protein mistranslation

There are very many classes of the antibiotics that inhibit translation by interfering with a wide variety of steps of the protein synthesis. Among the large number of drug families, only the aminoglycosides are broadly bactericidal (Magnet and Blanchard, 2005), although the members of other classes of the inhibitors of the protein synthesis can be bactericidal at a concentration- or species-dependent manner (Zhanel et al., 2001, Schentag & Ballow, 1991, Rodvold et al., 1997, Rahal & Simberkoff, 1979, Muller-Serieys et al., 2004, Klepser et al., 1997, Kanatani & Guglielmo, 1994, Drago et al., 2005).

Three large and well studied subfamilies of the aminoglycosides are neomycin, kanamycin and gentamycin families (Bryskier, 2005). These drugs have the same one main binding site on the ribosome. They bind to A site of the 30S subunit specifically to helix 44 of 16S RNA (Yoshizawa *et al.*, 1998, Vicens & Westhof, 2002, Carter et al., 2000). These aminoglycosides interact

with two universally conserved nucleotides A1492 and A1493. These nucleotides are important to recognize the correct codon-anticodon pairing. In case of the correct codon-anticodon base-pairing, the two nucleotides will be flipped out and will form hydrogen bonds with the minihelix that is formed between codon and anticodon regions. This way the affinity between the ribosome and the aminoacyl-tRNA will be increased, and the translation will continue at higher probability with this aminoacyl-tRNA. When the mentioned aminoglycosides are bound to the ribosome, the nucleotides A1492 and A1493 are stabilized in flipped-out conformation. In case of wrong codon-anticodon pairing, it will result in increased affinity of the wrong aminoacyl-tRNA, and the translation with the "wrong" aminoacyl-tRNA will proceed with high probability. This will result in the incorporation of the incorrect amino acids and the formation of mistranslated proteins (Carter et al., 2000).

Streptomycin, which is also bactericidal drug, is structurally a bit different as compared to the aminoglycoside subfamilies mentioned above (Bryskier, 2005). Streptomycin causes mistranslation, but it has slightly different binding site that is actually adjacent to the binding site of previously mentioned aminoglycosides (Carter et al., 2000). The mechanism how the miscoding is achieved differs from the neomycin, kanamycin and gentamycin subfamilies (Gromadski & Rodnina, 2004, Carter et al., 2000). Streptomycin causes larger conformational changes in the ribosome so that the non-cognate tRNA is stabilized and fidelity is influenced at the initial binding step of aminoacyl-tRNA as well at the proof-reading step.

While the mechanism of action of the aminoglycosides at the translational level is quite well established (Gromadski & Rodnina, 2004, Carter et al., 2000), the origin of the bactericidal effect is not so clear. It is reported that the uptake of aminoglycosides into the cytoplasm requires membrane-potential and is important for the bactericidal activity (Bryan & Kwan, 1983, Arrow & Taber, 1986). This explains why some anaerobes are less susceptible to the amino-glycosides or facultative aerobes are not sensitive at anaerobic conditions (Kogut *et al.*, 1965, Hancock, 1962). It is observed that protein synthesis also is necessary for the killing activity, as it leads to the increased uptake, which probably is a result of pore formation through the incorporation of mistranslated or truncated proteins into the membrane (Davis *et al.*, 1986). The increased uptake of the drug is thought to be irreversible, and it leads to the permanently elevated intercellular concentration of the drug (Nichols and Young, 1985). It has been proposed that the following cell death is caused by complete inhibition of protein synthesis through the elevated drug concentration (Mehta & Champney, 2002, Magnet & Blanchard, 2005) or the disruption of the membrane integrity (Bryan and Kwan, 1983). It is also proposed that the aminoglycosides competitively displace $Mg^{2+}$ and $Ca^{2+}$ ions, which link adjacent lipopolysaccharides, which results in the formation of holes in the cell envelope (Stratton, 2005). Recently additional mechanisms have been proposed, as discussed below.

### 1.4.4. Common mechanism of killing through reactive oxygen species

Recently a common mechanism involved in killing by different classes of antibiotics was described (Zabransky *et al.*, 1973, Kohanski *et al.*, 2008, Kohanski et al., 2007, Dwyer et al., 2007, Dwyer *et al.*, 2009, Davies *et al.*, 2009) (Fig. 1). The end product of this mechanism was shown to be reactive hydroxyl radical capable of damaging DNA, lipids and proteins that could be the direct cause of the cell death (Imlay, 2003). This mechanism was shown to be operational for the fluoroquinolones, aminoglycosides and β-lactams (Kohanski et al., 2007), which are the most studied bactericidal antibiotics and have different primary drug-target interactions (Tomasz, 1979, Magnet & Blanchard, 2005, Drlica et al., 2008).

The first common step toward the formation of hydroxyl radical is a production of superoxide (Dwyer et al., 2009). Superoxide is forming also from molecular oxygen through a respiratory electron transport chain also during homeostasis (Storz and Imlay, 1999) and is converted to hydrogen peroxide by superoxide dismutase. Subsequently, the hydrogen peroxide is in turn eradicated by catalases/peroxidises (Imlay, 2008). In case of antibiotic stress, the activity of the respiratory electron transport chain is increased so that the formation of superoxide is overwhelming cellular defense mechanisms (Dwyer et al., 2009).

The increased activity of the electron transport chain included stimulated NADH reduction through tricarboxylic (TCA) cycle. It has been shown that by modulating TCA cycle ability to form NADH, by removing TCA cycle key enzymes, reduces killing efficacy of drugs in correlation with the potential NADH forming ability (Kohanski et al., 2007).

An excess of superoxide can damage Fe-S clusters in proteins that leads to a formation of free ferrous iron ($Fe^{2+}$) and could include also the unbalance in iron metabolism and regulation (Dwyer et al., 2007). Ferrous iron can catalyze the formation of hydroxyl radical through Fenton reaction (Imlay *et al.*, 1988) that could lead to the already mentioned damage of DNA, lipids and proteins (Imlay, 2003).

It has been suggested that the oxidative damage death pathway might be a maladaptive response originating from oxygen free past and is now exploited by bactericidal drugs and some DNA damaging toxins (Dwyer et al., 2007). Interestingly, it has been hypothesized that the apoptosis mechanisms in eukaryotes, which have several parallels with the radical mediated cell death in bacteria, might have evolved from the same maladaptive response (Dwyer et al., 2007).

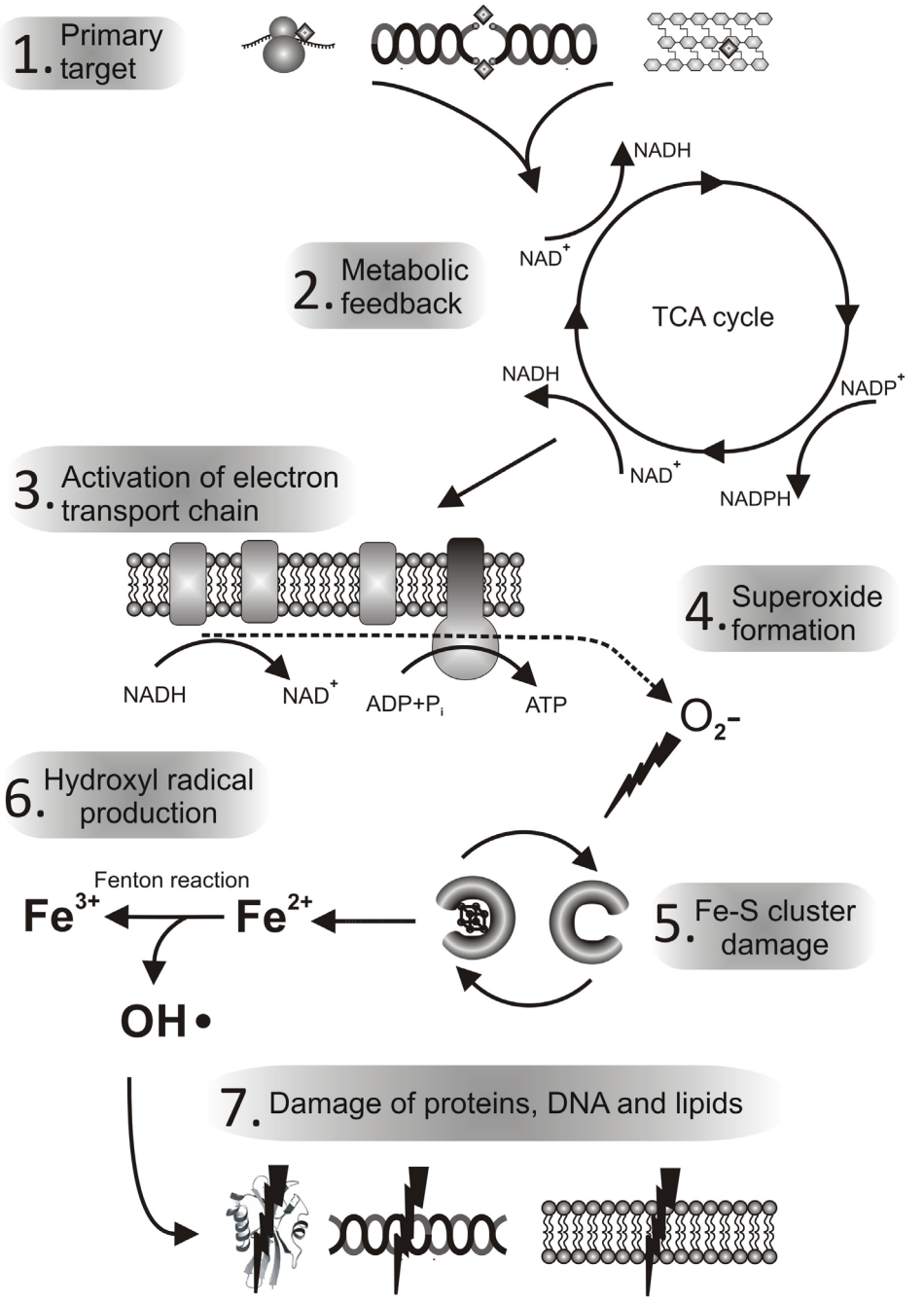


**Figure 1.** Common mechanism of cell death induced by bactericidal antibiotics (adapted from Kohanski et al., 2010).

The above-described common mechanism of the activation of the death pathway by various drugs is not very clear. The main question is how the TCA cycle and electron transport chain are activated? It has been described in some extent for the aminoglycosides (Kohanski et al., 2008). It has been shown that the incorporation of mistranslated and misfolded proteins into the membrane does induce the two-component stress response system CpxAR which activates the envelope stress response that is important for efficient killing. In addition, a redox responsive activity of another membrane associated two-component system ArcAB is crucial for the oxidative damage death pathway, and it is thought to activate the TCA cycle and respiration electron transport chain by an unknown mechanism. ArcAB is thought to be activated by CpxAR, but the exact way is waiting to be described. Interestingly, the disruption of those two-component systems reduced the killing efficacy also by the quinolones and β-lactams showing the broader role of the redox-responsive and envelope stress-responsive two-component systems in this pathway (Kohanski et al., 2008).

The existence of common death pathway is also supported by the fact that the SOS response deficient strain is more sensitive to all three classes of the bactericidal antibiotics, emphasizing the role of DNA damage by hydroxyl radicals (Kohanski et al., 2007). In addition, it has been shown that β-lactams are capable to induce the SOS response (Miller et al., 2004) as well as quinolones that directly cause the DNA damage (Kelley, 2006). It is also noteworthy that the chaperon systems are activated by all bactericidal drugs tested, although only for the aminoglycosides the primary drug-target interaction could lead to the protein misfolding. This could indicate a possible hydroxyl radical mediated protein damage (Kohanski et al., 2007).

It is important to note that the knock-out strains for all those mentioned key components are usually, at least in some extent, still sensitive to bactericidal antibiotics (Mitchell & Tuomanen, 2002, Kohanski et al., 2008, Kohanski et al., 2007, Dwyer et al., 2007). Also, when iron chelator and thiourea, a scavenger of hydroxyl radical, were added to the culture, the cell death was not prevented in all cases (Kohanski et al., 2007). Usually the cell death is attenuated or delayed, which shows that the activation of the oxidative damage pathway can be more complex than described today. It could also mean that the hydroxyl radical driven cell death is not the only death pathway and could be responsible only for the initial fast killing in the wild type cells. A good example is the fluoroquinolone norfloxacin, which induces both the protein synthesis-dependent and independent death pathways (Malik et al., 2007) while hydroxyl radicals probably contribute only to the protein synthesis-dependent pathway (Wang et al., 2010b).

In addition, there are few other aspects that have to be considered in the described common death pathway. For example, it is admitted that some of the mutant strains have slower growth rate (Kohanski et al., 2007) that might be the reason of the slower killing rate as it is expected that faster growing cells are killed more rapidly. Also, the fact that most of the antibiotics kill bacteria in

anaerobic conditions (Hecht, 2005), where the formation of reactive oxygen species (ROS) is certainly diminished, rules out exclusiveness on the ROS mediated pathway. The drug concentrations used in the studies on the ROS mediated killing pathway were relatively low, which suggests that the eminence of the ROS depending killing seen by Collins and coworkers (series of studies reviewed by Kohanski et al., 2010) to be relevant only at low drug concentrations and is overshadowed by other killing pathways at higher antibiotic concentrations.

### 1.4.5. Eagle effect

An interesting aspect of antibiotic killing is so called Eagle or paradoxical effect (Eagle and Musselman, 1948). H. Eagle was first who noted that increasing concentration of the drug over the MBC value can have paradoxical effect in the way that the number of cells killed by the treatment decreased. In case of penicillin, this effect was shown to appear among different species and strains. Later the same effect has been observed also for different penicillins, cephalosporins and also aminoglycosides (Shah, 1982). Although Eagle effect has been observed frequently, the clinical significance of this phenomenon appears to be unclear (Shah, 1982, Amsterdam, 2005).

There are several mechanistic hypotheses to explain the phenomenon of Eagle effect. For example, it has been suggested that at high concentration the inhibitors of cell wall synthesis can somehow slow down translation and prevent the cell growth, which is necessary for drug activity (Amsterdam, 2005). Another proposed hypothesis states that overall inhibition of PBP-s at high concentration is less favoring to lysis as compared to the situation at lower concentrations or autolysins, which cleave and remodel peptidoglycan in normal conditions, are less activated at high concentrations of cell wall inhibitors (Rice and Bonomo, 2005).

### 1.4.6. Toxin-antitoxin mediated killing

A toxin-antitoxin system is a set of two or more closely linked genes where one codes for a toxic protein and another for an antitoxin. The toxin-antitoxin systems are divided into three subgroups by the nature of the antitoxin. In the type I systems, the antitoxins are small antisense RNAs that repress an expression of the toxins, whereas in the type II system antitoxins are proteins that neutralize toxins through the binding. The type III systems contain a small RNA antitoxin that also inactivates toxins through the formation of complex (Yamaguchi *et al.*, 2011).

The toxin-antitoxin systems were initially identified as addiction modules in plasmids (Gerdes *et al.*, 1986) where they ensured inheritance of the plasmid by daughter cells. In the absence of plasmid the antitoxin, which is always more labile than the toxin, will be degraded faster, leading to the activation of the

toxin. Later the toxin-antitoxin modules were also found in the bacterial chromosomes where their functions are not completely understood (Yamaguchi et al., 2011).

In the context of the current thesis the type II toxin-antitoxin systems are most relevant. These toxins are usually endoribonucleases that cleave mRNAs and thereby stop translation and bacterial growth. In addition, type II toxin-antitoxin systems MazEF and ChpBK have been reported to induce bacterial killing or programmed cell death (PCD) (Kolodkin-Gal *et al.*, 2009, Aizenman *et al.*, 1996). It has to be noted that the story is contradictory as other laboratories have had difficulties to reproduce the results concerning the role of MazEF in PCD (Gerdes *et al.*, 2005). One of the oppositional observation was that MazEF is causing rather reversible stasis and is not causing cell death (Pedersen *et al.*, 2002). In return, the existence of "point of no return" was demonstrated (Amitai *et al.*, 2004), meaning that inhibition by toxin is reversible only in certain timeframe and becomes irreversible later and will lead to the cell death.

Afterwards, it was shown that MazEF mediated PCD has specific requirements. First, it is density- (Kolodkin-Gal *et al.*, 2007) and growth-phase (Hazan *et al.*, 2004) dependent and second, it is activated by a pentapeptide called extracellular death factor (EDF) (Kolodkin-Gal et al., 2007) in a strain-specific manner (Kolodkin-Gal and Engelberg-Kulka, 2008). For example, the widely used K-12 wild type strain MG1655 is not capable to produce the EDF in substantial amounts and is therefore PCD deficient.

In addition to various stressful conditions (UV, high temperature, oxidative stress) (Hazan *et al.*, 2004), some classical bacteriostatic antibiotics like spectinomycin, chloramphenicol and rifampicin have also been demonstrated to activate PCD (Sat *et al.*, 2001).

The main players in the activation of PCD are MazF toxin and EDF. The different stressful conditions that are shown to activate PCD (Hazan et al., 2004), are probably causing inhibition of translation or transcription, which will prevent expression of the antitoxin MazE resulting in to the activation of toxin MazF endoribonuclease activity. This, in turn, activates EDF production that, through the positive feedback loop, is leading to the further activation of MazF (Kolodkin-Gal and Engelberg-Kulka, 2008). It is proposed that the activation might come from direct interaction between those two molecules (Belitsky *et al.*, 2011).

The activation of MazF endoribonuclease activity leads to a massive cleavage of mRNA and to a decrease in the activity of translation (Zhang *et al.*, 2003). Nevertheless, translation is not completely shut down, and there is a set of proteins synthesized (Amitai *et al.*, 2009). Some of those proteins have been identified as "death" or "survival" proteins according to the phenotype of the corresponding knock-out strain. If the deletion increases the survival, the protein is recognized as "death" protein and *vice versa*. It is hypothesized that

synthesis of “death” or “survival” proteins is heterogeneous between different cells in the way that some cells are induced to die and others to survive.

In the case of PCD, two death pathways have identified: ROS-dependent and ROS-independent (Kolodkin-Gal *et al.*, 2008). ROS dependent pathway is observed during the treatment with translation and transcription inhibitors. These drugs act probably by bulk inhibition of protein synthesis including ROS defense proteins. ROS-independent pathway is activated by DNA damaging drugs and could work via selective activation of death proteins.

## 1.5. Bacterial responses to survive antibiotic treatment

Bacteria can survive antibiotic treatment in different ways. Subsequently, I describe three ways: resistance, persistence and antibiotic tolerance. Special emphasis goes to the persistence as the Results part of this thesis is mostly focused on persistence.

### 1.5.1. Resistance

Resistance is bacterial ability to survive and grow in the presence of antibiotics. Resistance can be accomplished in several ways and usually it involves a special resistance gene that is providing a mechanism to overcome the toxic effect of antibiotic(s).

Spread of resistance is a serious problem for healthcare system, and it has been tried to overcome the problem by introducing new antibiotics or even new classes of antibiotics, but for every new antibiotic clinically significant resistance has eventually emerged. For most of the drugs, the development of resistance has taken a year or two after the clinical introduction (Walsh, 2003, Levy & Marshall, 2004). There are also some exceptions. One example is vancomycin that was initially introduced in the 50s, but clinically significant resistance was reported almost 30 years later. The reason is that the use of vancomycin was very limited due to its toxicity and the development of new penicillins with better properties, resulting in vancomycin being used as a drug of last resort (Levin and Rozen, 2006).

When talking about resistance, intrinsic and acquired resistance should be distinguished (Walsh, 2003). Intrinsic resistance is an innate ability of a bacterial species or strain to resist a particular antibiotic. It can be driven by any of the different resistance mechanisms described below. In addition, the intrinsic resistance can be caused by some trivial reasons e.g. anaerobic bacteria are resistant to aminoglycosides because of the lack of oxidative metabolism to drive uptake (Bryan and Kwan, 1983), Gram-negative bacteria are resistant to various drugs (for example, vancomycin and streptogramins) because of their inability to penetrate outer membrane (Walsh, 2003, Leclercq & Courvalin,

1991), some bacteria have PBPs with low affinity to certain β-lactam drugs (Murray, 1990, Fontana *et al.*, 1992, Bodey, 1990). Example of intrinsic resistance is also producers of antibiotics that must have mechanisms to protect themselves from the action of the drugs they are producing (Walsh, 2003). The resistance genes of antibiotic producers have been thought to be a potential reservoir for acquired resistance (Walsh, 2003).

Multiple resistance mechanisms can reside in one single bacterium resulting in multidrug resistant bug often called "superbug". Cases when multiple resistance markers are transferring together as a single unit are not rare, making acquisition of multi-resistance very simple (Nikaido, 2009).

Resistance is achieved mainly by three groups of mechanisms – detoxifying antibiotics, altering target site and by reducing drug concentration. For every drug class most of the mechanisms have been identified, although their clinical relevance is variable.

## Antibiotic detoxification

The mechanisms for antibiotic detoxification can be divided into two larger groups: degradation and modification of the drug. Probably the best known example of drug degradation involves β-lactamases which are opening the lactam ring of the β-lactam drugs thereby making the compounds unable to bind to transpeptidases (Livermore, 1995). In addition to β-lactams, degradation of the drug can also be clinically important resistance mechanism for streptogramins (Mukhtar and Wright, 2005).

Modification of the drug, on the other hand, involves the addition of different chemical substituents. Resistance by drug modification has been shown to be clinically important for aminoglycosides (Walsh, 2003), streptogramins (Mukhtar and Wright, 2005), lincosamides (Leclercq, 2002), phenicols (Murray and Shaw, 1997) and quinolones (Robicsek *et al.*, 2006).

## Altering the drug binding site

Altering the drug target is relatively widespread mechanism of resistance and is clinically significant to most of the drug classes. This mechanism could work through mutation(s) in the gene coding the target, modification of the target via a posttranscriptional mechanism or acquiring additional, insensitive copy of the target (Walsh, 2003). An example of an easily acquired resistance by the change in the gene coding the target is a mutation of the gene coding for RNA polymerase leading to rifamycin resistance (Tupin *et al.*, 2010, Floss & Yu, 2005). This resistance is selected for so fast that rifamycins are usually co-administered with other drugs (Wallis *et al.*, 2008).

Resistance against ribosome targeting drugs can often be established by mutations in rRNA genes (Rice and Bonomo, 2005). However, this mechanism is not clinically significant as rRNAs are usually encoded in multiple operons and simultaneous selection for all rRNA genes, together with gene conversion

between different operons is not very likely (Prammananan *et al.*, 1999, Klappenbach *et al.*, 2001). Still, some pathogens like *Rickettsia prowazekii* (Andersson *et al.*, 1995) and *Mycoplasma pneumoniae* (Bercovier *et al.*, 1986) harbor single rRNA operon, and for the latter a clinically significant resistance through mutations in the 23S rRNA gene has been reported (Morozumi *et al.*, 2010). On the other hand, the mutations in ribosomal proteins can be and are clinically significant as ribosomal proteins are encoded by a single gene (Wilcox *et al.*, 2001).

Clinically significant resistance through changes in rRNA is usually achieved by the active mechanism of modifying nucleotides (Douthwaite *et al.*, 2005). For example, resistance against macrolides (erythromycin and other older generation macrolides) is achieved by methylation of 23S rRNA at nucleotide $A_{2058}$. This modification decreases the affinity also against lincosamides and B type streptogramins. This type of resistance is described as macrolide-lincosamide-streptogramin B ($MLS_B$) resistance phenotype. The methylation can be constitutive or induced in the presence of the drug (Roberts *et al.*, 1999).

Famous "superbug" MRSA (methicillin-resistant *Staphylococcus aureus*) is another example of acquired resistance (Walsh, 2003). Methicillin was introduced in 1950s to overcome penicillin resistance among Gram-positive pathogens that was achieved by hydrolysis of the drug by β-lactamases. Later, when MRSA started to spread all over the world, it was noted that in 90% of clinical isolates the resistance was not acquired through more effective β-lactamase but by additional copy of PBP2 with low β-lactam binding affinity.

## Reducing drug accumulation

Low levels of antibiotics inside the cell can be achieved by actively exporting the drug molecules out from the cell or reducing permeability of the cell envelope.

Active efflux can be clinically relevant for many different classes of antibiotics (for example, β-lactams, macrolides, A type of streptogramins, fluoroquinolones and tetracycline). Efflux pumps can be run on the proton motive force or on the energy from ATP hydrolysis, although the latter is less common (Walsh, 2003). In addition, there are also reports about $Na^+$ driven efflux pumps (Morita *et al.*, 2000, Huda *et al.*, 2001).

Every bacterium has a relatively wide variety of efflux pumps to transport out wastes and other unwanted molecules. Many of these pumps do not have strict substrate specificity (Walsh, 2003, Piddock, 2006). The result is that many of these pumps have the ability to pump out some antibiotics. For example, *P. aeruginosa* intrinsical resistance to various antibiotics originates partially from efflux pumps (Zavascki *et al.*, 2010).

Often the pumps can carry out a wide variety of drugs (named accordingly – multidrug efflux pumps) and confer multidrug resistance phenotype.

Conversely, some pumps, for example, tetracycline efflux pumps, tend to have rather narrow specificity (Walsh, 2003, Chopra & Roberts, 2001).

Reduction of permeability of the cell envelope can result from changes in composition of the outer membrane. Many hydrophobic drugs (macrolides, aminoglycosides, rifamycins etc.) are entering the cell by diffusion via outer membrane by hydrophobic interactions. Therefore, changed composition of the membrane can lead to the resistance to those drugs (Delcour, 2009).

Hydrophilic drugs (like β-lactams, also tetracyclins and fluoroquinolones), on the other hand, are exploiting porins that are functioning for transporting small molecular compounds to the cell. Resistance against those drugs can be achieved by changing porin profile in the outer membrane. This could include loss or sever reduction of the amount of porins. In addition, porin specificity could be changed by mutations (Nikaido, 2003, Delcour, 2009).

### Other mechanisms

Resistance can be achieved also by other mechanisms. For example, tetracycline resistance can be mediated by so called ribosomal protection proteins. They are structurally similar to elongation factors and can bind to the ribosomal A-site where they can with the help of energy from GTP hydrolysis figuratively wipe tetracyclines off from ribosomes (Connell *et al.*, 2003).

## 1.5.2. Persistence

Joseph Bigger reported already at the early dawn of the antibiotic era (Bigger, 1944) that penicillin does not sterilize bacterial culture. Today's researchers from resistance era would have probably thought that those non-dying cells are some sort of indication of resistance. Bigger, on the other hand, understood that the phenomenon he has encountered, is something completely different. In fact, he suggested that those penicillin insensitive cells are quiescent or dormant and therefore survive the treatment, as it was already known that penicillin can kill only actively growing cells (Hobby *et al.*, 1942).

Persisters are defined as bacterial cells that survive the treatment by bactericidal antibiotics and are capable of continuing growth after removal of the drug. This phenomenon is distinguished from resistance by the fact that after reinoculation a new heterogeneous culture will be formed, where most of the cells are again sensitive to the drug, making it a phenotypic not a genotypic trait (Lewis, 2010, Bigger, 1944). It has been assumed that persisters are a physiologically discrete subpopulation differing from the bulk of the cells that are killed slowly because they are dormant. Bacterial time-kill curves are usually having the shape of biphasic exponential decay formed from fast and slow phases (Fig. 2). Some authors have clearly pointed out that persisters are only the cells forming the slow phase (Dhar & McKinney, 2007, Balaban *et al.*, 2004). Therefore, there is a risk that when antibiotic treatment is too short the read of colony forming units (CFU) does not represent persister frequencies

correctly as it might also contain a significant number of normal, fast killed cells (at this moment not yet killed). Regular antibiotic treatment lengths in published papers are usually equal or longer than 3 hours, which is enough to show the true persister frequency in growing culture incubated in a rich medium (like LB) (Keren *et al.*, 2004a, Jõers *et al.*, 2010). However, there is a risk for inappropriate (physiologically irrelevant) persister estimation arising from the common but primitive definition of persistence. To avoid this risk it should be clearly emphasized that antibiotic treatment should be long enough, and a physiologically relevant definition for persisters should be brought up.

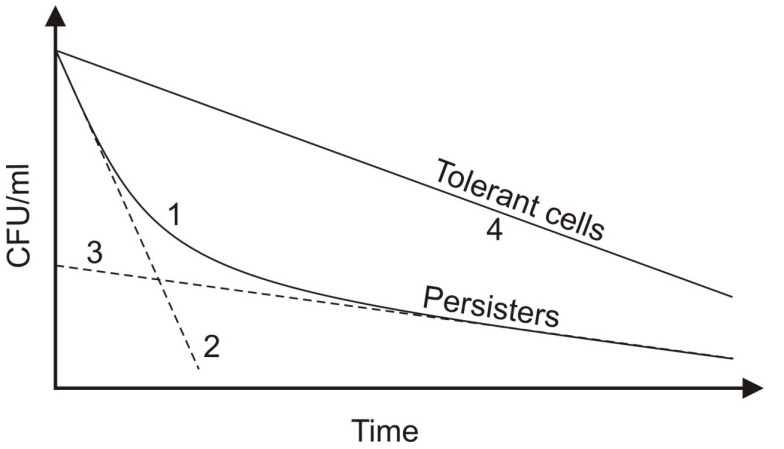


**Figure 2.** Biphasic killing of bacteria. Total population of bacteria (1) is killed by biphasic manner. Fast phase (2) is composed of normal cells and slow phase (3) is formed by persisters. Tolerant cells (4) are killed slowly.

Although persisters have been known for a long time, the nature behind this phenomenon is not clear. One reason for this is their very low frequency that is typically reported being in the range of $10^{-4}$–$10^{-6}$ (Moyed & Bertrand, 1983, Keren et al., 2004a). Their scarcity makes them very difficult to study, so that the first, more profound, series of studies were published about 40 years after the first initial report in this field by Moyed and colleagues (Scherrer & Moyed, 1988, Moyed & Broderick, 1986, Moyed & Bertrand, 1983, Black *et al.*, 1991, Black *et al.*, 1994). After Moyed, persisters came to the spotlight again in the beginning of the 21st century (Spoering & Lewis, 2001, Sinhg, 2002) and are relatively actively studied up to date.

Persisters have been reported to be found in numerous bacterial species (De Groote *et al.*, 2009). In addition to bacteria, persister-like behavior has been reported also among fungi (LaFleur *et al.*, 2006, Jabra-Rizk *et al.*, 2004) and cancer cells (Sharma *et al.*, 2010).

## Mechanism of persister formation

Mechanistic studies on persisters have been conducted mainly on *E. coli,* and most of the results described below in this paragraph accounts for the *E. coli,* and therefore the organism is mostly not mentioned. Also, some data originating from other type of organisms have been published and are mentioned here separately.

First locus to be connected with persistence was *hipBA* (Moyed and Bertrand, 1983) that codes antitoxin-toxin module where the toxin is an EF-Tu targeting kinase (Maisonneuve *et al.*, 2011). *hipBA* locus was identified when mutants with increased persistence were selected. One of the strain identified was *hipA7* (high persistent) with the persister level increased about three orders of magnitude (Moyed and Bertrand, 1983). Later, the *hipA7* strain was found to carry two point mutations in *hipA* gene (Korch *et al.*, 2003). These mutations are expected to result in weaker binding between the toxin and antitoxin (Maisonneuve et al., 2011). It is thought that the higher average free toxin level would lead to a situation where the number of cells, exceeding the threshold level of toxin causing dormancy, is increased and therefore more persisters are formed. This is supported by a study where ectopic over-expression of HipA toxin caused dormancy of single cell when the toxin concentration was over a certain threshold and the length of dormancy was depending on how long the toxin concentration over the threshold (Rotem *et al.*, 2010).

Two types of persisters have been characterized – type I and II. Classical persister assay is performed with growing culture where the inoculum is originating from overnight (stationary phase) culture. With this setup it has been shown through continuous dilution and growth cycles that persisters are eventually diluted out, meaning that they are originating from the initial stationary phase inoculum (Keren et al., 2004a). This kind of persisters are the cells that have not yet started to grow actively and are called type I persisters (Balaban *et al.*, 2004).

Type II persisters are forming during the growth, when actively growing cells are switching to slowly growing or non-growing state. In the wild type *E. coli* the level of type II persisters is even lower than the level of type I persisters. Therefore, the type II persisters are usually masked by type I persisters, making them hard to study. There is another high persistent mutant *hipQ* (Wolfson *et al.*, 1990) that has an increased level of type II persisters (Balaban et al., 2004). *HipQ* has not gained much attention, the strain is not well characterized and the mechanism behind it is not known.

Persisters are identified also in biofilms (Spoering and Lewis, 2001) and sometimes their level is measured in stationary phase culture (Ma *et al.*, 2010, Li & Zhang, 2007, Keren et al., 2004a). As those habitats reflect non-growing or slowly growing environments, care should be taken when drawing mechanistic parallels with persisters from growing cultures.

Persisters are dormant during the antibiotic treatment and are starting to grow later, after the antibiotic is removed. Bulk of the persisters are thought to

originate from stationary phase inocula, that is supported by data showing that the persister levels reflect the kinetics of awakening from dormancy (Jõers et al., 2010). Surprisingly, it was noticed (Gefen *et al.*, 2008) that in a narrow time window, right after dilution, supposedly dormant cells were also able to synthesize proteins. This suggests that complete differentiation into persister state might not happen in stationary phase, rather after inoculation into the fresh medium. Gefen and colleagues saw also that the exposure to bactericidal antibiotics during this narrow time window significantly reduced the persister level, but this result was conflicted by a later paper (Jõers et al., 2010), although the results are not directly comparable as different growth conditions and strains were used.

Although during the last 10 years several serious attempts have been made, the molecular mechanisms responsible for persister formation are largely unknown. Three reports analyzing the gene expression of *E. coli* persister cells (Shah *et al.*, 2006, Keren *et al.*, 2004b) and one in *Mycobacterium tuberculosis* (Keren *et al.*, 2011) are available. It turned out that the persister transcription profile differs from stationary and exponential phase cells (Shah et al., 2006). Large sets of genes were down regulated in persister cells including several metabolism and flagellum related genes, a response that is expected in dormant cells (Shah et al., 2006, Keren et al., 2011). A smaller set of various genes, with variable functions, were up regulated. These included different stress response related genes, which is consistent with the persister survival function but does not explain dormancy (Shah et al., 2006, Keren et al., 2011). Another outstanding set of up-regulated genes are coding for toxin-antitoxins (TAs) (Shah et al., 2006, Keren et al., 2004b, Keren et al., 2011). Many of the *E. coli* toxins are endoribonucleases (Maisonneuve et al., 2011) and HipA is protein kinase targeting EF-Tu (Schumacher *et al.*, 2009). As toxins are able to stop macromolecular synthesis, their activity could be a direct cause for stasis and dormancy. Surprisingly, only few single gene knock-out mutants of the TA systems showed lower persister frequency, and even this effect is observed in specific conditions or background (Shah et al., 2006, Kim & Wood, 2010, Keren et al., 2004b, Harrison *et al.*, 2009). The small effect of deleting TA systems was explained by a possible redundancy between different TA systems and the need for multiple knock-outs, for more significant effects, was suggested. Recently, a report of 10 TA system knock-out was published and indeed, a 200 fold drop in persister frequency was demonstrated. The reduction was progressive, depending on the number on TA systems deleted (Maisonneuve et al., 2011). Also, in *M. tuberculosis* the deletion of some toxins has been found to decrease persister level *in vitro*, but the *in vivo* significance of this effect is in question, as no effect was seen in a mouse model (Singh *et al.*, 2010).

Different screens have been run to identify genes that could change persister frequency: *E. coli* (Ma et al., 2010, Hurwitz *et al.*, 1981, Hansen *et al.*, 2008) and *P. aeruginosa* (De Groote et al., 2009) knock-out library screens, and *E.*

*coli* transposon (Li and Zhang, 2007) and over-expression (Spoering *et al.*, 2006) library screens. Indeed, numerous genes, coding various unrelated proteins, were found to influence persister level, but no clear mechanism has been elucidated from these data so far.

It has thought that the mechanism leading to persistence in the presence of various drug classes is the same. Contrary to that, it was recently reported that there is no correlation between persister levels upon treatment with ampicillin, norfloxacin or streptomycin (representatives of different drug classes) as measured in cultures of natural *E. coli* isolates (Stewart and Rozen, 2011). The problem with this report is that the drug concentrations used were much lower than usual in persister assays – the concentrations were only slightly higher than the MIC. As MIC and MBC values of different strains do not have to be rigidly connected to each other, the killing efficiencies of different drugs might have been varying resulting in diverse levels of drug-specific persisters.

In addition, it has been noted that the induction of SOS response is crucial for persister formation only when bacteria are treated with fluoroquinolones (Dorr *et al.*, 2009). It is proposed, that, in addition to the protective effect of SOS response, the synthesis of the membrane protein TisB is induced by SOS response, which could cause the loss of proton motive force and the formation of dormant cells (Dorr *et al.*, 2010).

Various conditions have been identified to induce the formation of persisters. For example, over expression of toxins and other unrelated proteins, shown to be toxic, have been reported to increase persistence (Vazquez-Laslop *et al.*, 2006, Shah et al., 2006, Korch & Hill, 2006, Kim & Wood, 2010, Keren et al., 2004b, Harrison *et al.*, 2005, Dorr et al., 2010). Also, the addition of quorum sensing molecules could increase persistence (Moker *et al.*, 2010), although the addition of spent medium to early exponential phase cells does not induce pesisters in *E. coli* and *P. aeruginosa* (K. Lewis, unpublished data). For mycobacteria, it has been shown that persister phenotype can be induced also in macrophages of zebrafish larvae. In this case, the bacterial cells have lost sensitivity to antibiotics through activation of efflux pumps and this phenomenon is associated with replicating bacterial populations (Adams *et al.*, 2011). For biofilm cells, it has been reported that antibiotic insensitivity is not caused just by the passive effects of growth arrest; it is rather mediated by active starvation responses, reducing the oxidant stress (Nguyen *et al.*, 2011).

## Ecological significance of persistence

Persisters have been suggested to be an adaption for survival in fluctuating environments (Kussell & Leibler, 2005, Kussell *et al.*, 2005). Diversity of phenotypes, used as "insurance policy" in changing environmental conditions, is established in ecology and population genetics as a bet-hedging strategy (Beaumont *et al.*, 2009). Persister state could be achieved via stochastic switching or by switching in response to sensing the changes in the environment. Responsive switching could be energetically more costly as it

would need the apparatus for sensing and reacting, and therefore, would be more favored if the environment is changing more frequently. Stochastic switching would be more favored when changes in the environment are not so frequent. In this case the switching rate would need to be adjusted to the frequency of environmental changes. In addition, catastrophic changes in the environment would favor stochastic switching as in this case there would be no time to respond (Kussell and Leibler, 2005).

It has been also proposed that persisters could simply be the outcome of senescence (Klapper *et al.*, 2007). Bacterial cell can accumulate damages, for example, oxidative damage (Fredriksson and Nystrom, 2006). It has been demonstrated that a subpopulation of stationary phase cells with lower ability to form colonies has a higher level of carbonylated (oxidatively damaged) proteins (Desnues *et al.*, 2003). This leads to the hypothesis that the persistence phenotype could, at least partially, reflect random nature of cell damage, as the cells that acquired more damage during stationary phase are resuming growth at a slower rate as compared to cells with lower level of damage. Several molecular mechanisms could be involved in senescence. For example, each bacterial cell has an old pole and a new pole that originates from last division. It has been shown that cells inheriting old pole during division, have decreased growth rate. Moreover, the decrease of growth rate of the cell, inheriting the old pole, is accumulating during the consecutive divisions (Stewart *et al.*, 2005). The cause of this effect could be asymmetric accumulation of damaged cell components (Lindner *et al.*, 2008). This effect could explain the formation of type II persisters during growth. It has to be noted that also contradictory observations have been made, where the ageing pole did not have any effect on the growth rate whatsoever (Wang *et al.*, 2010a).

## Persisters and infectious diseases

Infectious diseases are often hard to treat even when the pathogen is not resistant. Good examples are persistent or chronic infections that need prolonged treatment (for example, syphilis or tuberculosis) or reappear after recovering. Chronic infections are often accompanied by biofilms. Persisters residing in biofilms are thought to be the source of recurrence (Lewis, 2010). A proposed model suggests that antibiotics are killing most of the bacteria and the immune system eliminates bacteria escaping antibiotic treatment. Persister cells residing in biofilm will be protected from the immune system and after the drop in antibiotic concentration they could repopulate the biofilm again and cause reappearance of the infection.

Potential causality between persisters and therapy failure is supported by selection of hip mutants *in vivo*. This is the case for cystic fibrosis patients with *P. aeruginosa* infections (Mulcahy *et al.*, 2010). This infection is incurable, but periodic dosing of antibiotics, lasting years, can relieve symptoms. Eventually long lasting and periodic treatment is leading to the rise of strains with about 100 times higher persister level with unchanged MIC. Similar phenomenon has

been observed for long term oral carriage of eukaryotic *Candida albicans* suggesting wider impact of persister phenomenon and *hip* strains (Lafleur *et al.*, 2010). As the testing of persister frequencies is not common in clinical labs, it is explaining why persisters and high persister mutants are overlooked among chronic infections.

Currently persisters have been recognized as an issue in clinical microbiology and efforts are being made to develop approaches to fight persisters. For example, a chemical library has been screened and a compound reverting persister cells to antibiotic-sensitive cells, and therefore mediating their death, has been identified (Kim *et al.*, 2011). Similar effect has also been observed with certain metabolites (Allison *et al.*, 2011).

### 1.5.3. Antibiotic tolerance

Third way how bacteria circumvent lethal activity of antibiotics is antibiotic tolerance. This term was initially introduced by clinical microbiologists to describe a new type of bacterial response to antimicrobials (Tomasz et al., 1970). In 1970 new types of mutant pneumococcal strains were characterized. Those strains had unchanged MIC, but the rate of antibiotic induced killing was decreased, or even reduced to the level that they didn't lose viability at all.

Unaffected MIC makes antibiotic tolerance different from resistance. Antibiotic tolerance differs also from the persistence phenomenon, as it comprises all cells in the genetically homogeneous population, while persistence affects only small subpopulation of the cells. In addition, tolerance is heritable (genotypic) trait, as opposed to persistence that is an example of phenotypic trait (Keren et al., 2004b).

Nonetheless, during the last decade the term antibiotic tolerance and a linked term phenotypic tolerance have often been used in the meaning of persistence (Wiuff *et al.*, 2005, Spoering et al., 2006, Lewis, 2001, Hansen et al., 2008, Dhar & McKinney, 2007). One possible reason lays in the fact that the last in-depth disquisitions on antibiotic tolerance remain behind decades (Tuomanen *et al.*, 1986, Handwerger & Tomasz, 1985), although there are several more recent studies covering the topic in its original meaning (Moscoso *et al.*, 2010, Mitchell & Tuomanen, 2002, Bizzini *et al.*, 2010, Andres *et al.*, 2005) and it is also clearly stated in several handbooks (Smith, 2004, Amsterdam, 2005). There are also a few recent reports that are emphasizing the differences between those two phenomena (Levin & Rozen, 2006, Gefen & Balaban, 2009).

The best way to clarify the difference between persistence and antibiotic tolerance, in its original meaning, is on example of antibiotic kill curve. Usually antibiotic kill curves can be simplified as biphasic exponential decay (although actually it could also be multiphasic) (Fig. 2). First, the fast phase reflects killing of the major population of the cells and this killing rate is changed in case of changes in antibiotic tolerance. The second, and slower killing phase reflects persisters and usually their absolute level is affected, instead of killing

rate. The killing rate can also be changed, but for persistence this is not commonly evaluated.

Even among the clinical microbiologist the term antibiotic tolerance has problems with multiplicity of definitions (Tuomanen et al., 1986, Handwerger & Tomasz, 1985). For clinical purposes the assessment of antibiotic tolerance includes comparing the ratios of MBC to MIC. Generally ≥ 32 fold ratio has been taken as a marker for tolerant strain (Sabath *et al.*, 1977, Meylan *et al.*, 1986, Goessens *et al.*, 1984), although higher and lower ratios are also used (Handwerger and Tomasz, 1985). It is important to keep in mind that MBC value is acquired through the endpoint measurement. This means that it does not give information about the rate of killing that was originally used to define antibiotic tolerance. It has to be emphasized that the most accurate way to measure antibiotic tolerance would be through the killing curve.

Antibiotic tolerance is sometimes divided into two classes: phenotypic and genotypic tolerance (Tuomanen et al., 1986). Usually, genotypic tolerance reflects mutant strains that behave differently from reference or the wild type strain in regular growth conditions in the presence of antibiotics. The term genotypic tolerance can also be also used for comparing two or more unrelated bacterial strain.

Phenotypic tolerance characterizes growth conditions where bacteria do not respond or respond slower to antibiotic. The phenomenon was described already in 1940s (Hobby et al., 1942), although it was named much later (Tuomanen et al., 1986, Handwerger & Tomasz, 1985). A good example of phenotypic tolerance is stationary phase culture that is not sensitive to most of the antibiotics.

The antibiotic tolerance is usually observed in cases of antibiotics causing cell lysis, for example, β-lactams or glycoprotein antibiotics (e.g. vancomycin). Also, the mechanistic studies on the tolerance are mainly done with those antibiotics (Tuomanen et al., 1986, Mitchell & Tuomanen, 2002). Lysis caused by those antibiotics is conducted via autolysins that are constitutively expressed extracellular enzymes. In normal conditions their activity is controlled but they can be activated in certain conditions (for example, during treatment with the antibiotics mentioned earlier) (Mitchell and Tuomanen, 2002). Antibiotic tolerance is caused by the absence of autolytic activity and it can be achieved by the loss of autolysin expression or the inability to activate autolysins. There are several genes whose deletion diminishes autolysin expression, but their role in the regulation of expression of autolysins is not completely clear (Novak *et al.*, 2000a, Novak *et al.*, 1998, Charpentier *et al.*, 2000). At the same time, the clinical isolates of the tolerant strains are expressing autolysins, meaning that clinically important way for formation of tolerance is through the changes in the activation of autolysins. Some loci, coding for proteins involved in the activation pathway of autolysins (including a two-component regulator system), have been described (Rice et al., 2003, Novak et al., 1999, Novak et al., 2000b,

Groicher et al., 2000), but our understanding of the pathway is far from complete (Rice & Bayles, 2008, Mitchell & Tuomanen, 2002).

## 1.6. Trans-translation and antibiotic susceptibility

Various genetic determinants can influence intrinsic antibiotic susceptibility of bacteria (Keiler *et al.*, 1996), including many whose dominant function is not resistance. One such example is transfer-messengerRNA (tmRNA) that has been reported to decrease susceptibility to various inhibitors of protein synthesis (Vioque & de la Cruz, 2003, de la Cruz & Vioque, 2001, Abo et al., 2002).

tmRNA, also known as 10Sa RNA, is coded by the gene *ssrA*. It is a molecule that is directing the process called trans-translation and is highly conserved among eubacteria (Gueneau de Novoa and Williams, 2004). tmRNA is an interesting bifunctional RNA molecule combining the characteristics of tRNA and mRNA. Its tRNA like domain resembles $tRNA^{Ala}$ and can be aminoacylated with alanine from the 3' end. tmRNA also contains a reading frame, which is coding a tag added to the C terminus of nascent peptides.

Trans-translation recycles ribosomes stalled on mRNA (Keiler et al., 1996). There can be several reasons for stalling, for example, mRNA might lack stop codon at the 3' end because of truncation (Keiler et al., 1996) or improper transcription (Ivanova *et al.*, 2004). Stalling can be caused also by rare codons (Roche and Sauer, 1999) or insufficient termination (Hayes *et al.*, 2002, Collier *et al.*, 2002). In all mentioned cases, ribosomal A-site is empty which is necessary for the tmRNA to bind and function.

Trans-translation starts with the binding of the tRNA-like region of the tmRNA to the A-site of the ribosome. After the transpeptidation reaction, the problematic mRNA will be replaced with the mRNA-like region of the tmRNA, which codes short signal for proteolysis. Translation will continue and will result in the tagging of the synthesized protein (Keiler, 2008).

Initially it was suggested that the function of trans-translation is to recycle ribosomes, stalled on mRNA, to ensure translational capacity (Keiler et al., 1996). The process would also include the removal of potentially toxic truncated proteins and problematic mRNAs causing stalling. In light of recent evidence, it has been suggested that the function of trans-translation is not to maintain translational capacity of the cell but rather the release of some particular problematic ribosomes (Hayes and Keiler, 2010). For example, ribosomes that stall during co-translational protein secretion may cause problems, as the translocon complex will be degraded. Release of the stalled ribosome by tmRNA could help to prevent the degradation. In addition, it has been hypothesized that misfolded nascent peptides may cause translational stalling to give them extra time to fold properly. In case the folding fails, the potentially toxic misfolded proteins would be released from the ribosome by trans-translation system and degraded (Hayes and Keiler, 2010).

In addition to releasing stalled ribosomes, tmRNA has broader role on the cell physiology. For example, tmRNA regulates the expression of some genes (Garza-Sanchez *et al.*, 2011, Abo *et al.*, 2000). In addition, it is known that deletion of *ssrA* can change cell physiology – virulence, sporulation, cell cycle progression and stress responses can be affected (reviewed in Keiler, 2007). At the same time, the deletion of *ssrA* can also be lethal for some organisms (Huang *et al.*, 2000, Hayes & Keiler, 2010). The lethality is associated with presumably non-active variant of ArfA, a protein which was recently demonstrated to rescue *E. coli* stalled ribosomes in tmRNA-independent way (Chadani *et al.*, 2010). Similar properties have been also demonstrated for another protein called YaeJ (Chadani *et al.*, 2011).

# 2. RESULTS AND DISCUSSION

## 2.1. Objectives of the present study

There are various factors that can influence sensitivity of bacteria to antibiotics. For example, over-expression (Vazquez-Laslop et al., 2006, Li & Nikaido, 2009) or deletion (Keren et al., 2004a) of several genes can change bacterial susceptibility to different antibiotics. Inhibitors of protein synthesis have been reported to be more potent against trans-translation deficient strains (Vioque & de la Cruz, 2003, de la Cruz & Vioque, 2001, Abo et al., 2002). In addition to genetic factors, the growth conditions can have an impact on the effectiveness of antibacterial drugs (Hobby et al., 1942).

Effect of the previously mentioned factors on antibiotic susceptibility can be observed on the level of the whole population, for example, the killing rate of the whole population or growth at the presence of an antibiotic. At the same time, the effects can be subpopulation specific. One example of such sub-populations is persisters. Several genes (Spoering et al., 2006, Ma et al., 2010, Li & Zhang, 2007, Hansen et al., 2008, De Groote et al., 2009), compounds (Allison et al., 2011) and growth conditions have been shown to change persister level or susceptibility to antibiotic (Keren et al., 2004a, Jõers et al., 2010).

As there are still many unknowns in the subjects previously mentioned, the general aim of this thesis was to identify and further characterize the factors which influence the sensitivity of *E. coli* to various antibiotics. For clarity, the work is divided into following aims:

- To study further antibiotic susceptibility of tmRNA deficient *E. coli.*
- To develop a flow cytometrical assay to study cell division at the single cell level.
- To explore heterogeneity of the cell division at the single cell level through different growth stages.

## 2.2. The lack of tmRNA increases the bactericidal activity of aminoglycosides and the susceptibility to inhibitors of cell wall synthesis (I)

There are reports that tmRNA deletion results in higher sensitivity to inhibitors for protein synthesis compared to wild-type strains(Vioque & de la Cruz, 2003, de la Cruz & Vioque, 2001, Abo et al., 2002). Therefore, it has been suggested that tmRNA can recycle ribosomes that are stalled by antibiotics inhibiting protein synthesis. At the same time, as mentioned earlier, tmRNA can play a role in various aspects of bacterial physiology. This is raising the question, does the inactivation of the *ssrA* increase the sensitivity also to drugs which have other targets than ribosome?

In those few reports, describing the sensitizing effect of *ssrA* removal, only the growth inhibitions have been measured. We wanted to know, if the deletion of *ssrA* influences bactericidal activities of the drug.

To test those possibilities, we compared antibacterial effects of 14 antibiotics, 6 of them having a different target from ribosome, on the wild-type and Δ*ssrA* strains. Two approaches were used. First, the growth inhibition of the two strains was measured. Second approach, testing the possible variability on bactericidal activity, was carried out with drugs which showed differences between the wild-type and Δ*ssrA* strains.

### 2.2.1. Growth inhibition

Concentration ranges for 14 antibiotics were selected from low or no inhibition to maximum inhibition. The experiment was started with dilution of overnight or exponential phase cultures into fresh media containing an antibiotic and after 12 hours of aerobic incubation the optical density was measured. As overnight and exponential phase cultures were giving similar drug sensitivity patterns, only the results of overnight cultures are shown (I, Fig. 1 and 2).

In agreement to previous reports (Vioque & de la Cruz, 2003, de la Cruz & Vioque, 2001, Abo et al., 2002), the Δ*ssrA* was more sensitive to several inhibitors of protein synthesis as compared to wild type (I, Fig. 1). Most prominent effects were seen with kanamycin, streptomycin and erythromycin. Smaller difference was observed in the case of chloramphenicol and puromycin. No significant difference was seen with tetracycline, clindamycin and spectinomycin.

Is it possible to find parallels between mechanisms of action of ribosome targeting antibiotics and their sensitization effect of the Δ*ssrA* strain? Antibiotics that showed different activity between the test strains are inhibiting translation in various manner: erythromycin binds to the ribosome exiting tunnel and is sterically blocking the elongation of nascent peptide and is causing the drop-off of peptidyl-tRNA (Tenson *et al.*, 2003, Lovmar *et al.*, 2004); kanamycin and streptomycin are causing misreading of mRNA (Magnet and Blanchard, 2005); puromycin is a structural analog of tyrosyl-tRNA and is acting as acceptor in transpeptidation reaction thereby interfering with the binding of aminoacyl-tRNA to the A-site (Spahn and Prescott, 1996); chloramphenicol inhibits peptidyl transfer (Spahn and Prescott, 1996). Antibiotics that had equal effectiveness against the wild-type and Δ*ssrA* strain are inhibiting translocation (spectinomycin) (Magnet and Blanchard, 2005), hindering the binding of aminoacyl-tRNA to the A-site (tetracycline) (Chopra and Roberts, 2001) or blocking peptidyltransferase reaction and causing the drop-off of peptidyl-tRNA (clindamycin) (Tenson et al., 2003).

It turns out that the Δ*ssrA* strain has different sensitivity to several antibiotics with partially overlapping mechanisms. Examples are chloramphenicol and tetracycline (interfers the binding of aminoacyl-tRNA to the A-site),

erythromycin and clindamycin (drop-off of peptidyl-tRNA), chloramphenicol and clindamycin (blocks peptidyl transferase reaction). This leads to the conclusion that the molecular mechanism of protein synthesis inhibitors is not enough to predict the sensitization effect of *ssrA* deletion.

Interestingly, we saw a difference between the wild type and Δ*ssrA* strain also in the case of some antibiotics not targeting the ribosome (I, Fig. 2). The sensitization effect of *ssrA* deletion was observed for ampicillin and fosfomycin, which are both inhibitors of the cell wall synthesis, although they are blocking different steps in the process (Walsh, 2003). Inhibitors of RNA polymerase (rifampicin), DNA topoisomerase (norfloxacin and ofloxacin) and dihydrofolate reductase (trimethoprim) (Walsh, 2003) did not cause significant growth differences between the strains.

Link between the cell envelope and the ribosome targeting antibiotics could arise from the hypothesis that trans-translation system could be important for rescuing SecYEG translocon from the degradation, when translating ribosomes are stalled by antibiotics during co-translational translocation (Hayes and Keiler, 2010). It has been reported that drugs blocking translation elongation are inducing degradation of SecY and SecG that could lead to sever growth impairment or lethality (van Stelten *et al.*, 2009). The sensitization effect of *ssrA* deletion for some antibiotics could be the consequence of different selective inhibition of protein synthesis as some drugs could inhibit more effectively proteins translocated to the inner membrane than others.

Interestingly, antimicrobial effect of some protein synthesis inhibitors also operates through the cell envelope. For example, aminoglycosides are causing misreading of the mRNA during translation (Magnet and Blanchard, 2005) resulting in the formation of proteins that are potentially misfolded and are, through the exposed hydrophobic regions, prone to mislocalize into the membranes and interfere their functioning.

Trans-translation and inhibitors of the cell wall synthesis could be linked also by the fact that tmRNA is tagging SecM protein, which is a regulator of SecA expression (Collier *et al.*, 2004). SecA is an ATPase that is targeting proteins into the SecYEG translocon (Veenendaal *et al.*, 2004). It is possible that the lack of trans-translation is causing perturbations in the export of proteins from the cytoplasm, and therefore the cells are not capable to respond adequately to extracellular stresses.

Sensitization by defective trans-translational machinery could also be a result of the changed general physiology of the cell that tmRNA is known to cause, as mentioned earlier. At the same time, different antibiotics have been shown to induce various stress responses (VanBogelen & Neidhardt, 1990, Shapiro & Baneyx, 2002, Sabina *et al.*, 2003, Goh *et al.*, 2002, Bianchi & Baneyx, 1999). It has been reported that ribosome targeting antibiotics, kanamycin, streptomycin and puromycin, which caused a greater growth inhibition of Δ*ssrA* strain compared to the wild type strain (I, Fig. 1), are inducing the heat shock response (VanBogelen and Neidhardt, 1990).

Interestingly, *E. coli* Δ*ssrA* strain is also sensitive to heat shock (Komine *et al.*, 1996, Karzai *et al.*, 1999). On the other hand, *ssrA* deletion caused sensitization to erythromycin and chloramphenicol (I, Fig. 1) which are inducing cold shock response but not to tetracycline that induced the same response (VanBogelen and Neidhardt, 1990). In conclusion, it is difficult to correlate stress responses related to antibiotics with their effects on the Δ*ssrA* strain.

Recently, two proteins ArfA and YaeJ, have been described to be able to rescue stalled ribosomes independently from tmRNA (Chadani et al., 2010, Chadani et al., 2011). It is possible that those proteins can rescue stalled ribosomal complexes induced by some antibiotics but not by others. The antibiotic sensitivity pattern of the Δ*ssrA* strain might be the result of the ability of ArfA and YaeJ to compensate for the lack of tmRNA. Validating this hypothesis would need further testing.

### 2.2.2. Bactericidal activity

Since the ability to cause cell death is an important parameter of antibiotics, we measured the bacterial viability in the presence of drugs which previously showed differeces in the activity against the Δ*ssrA* and wild type strains. We wanted to test whether bactericidal antibiotics are more effective against the Δ*ssrA* strain or do bacteriostatic drugs start to behave bactericidally. We also included norfloxacin as the representative of bactericidal fluoroquinolones. The concentrations used were chosen such that caused in both strain a complete or maximum inhibition of growth.

The biggest difference in the killing efficiency between the wild type and Δ*ssrA* strain was observed for kanamycin and streptomycin (I, Fig. 3). A drop of CFUs, less than an order of magnitude, of the Δ*ssrA* strain was specific also for the bacteriostatic erythromycin. Other bacteriostatic ribosome targeting drugs did not show any significant differences between the wild type and Δ*ssrA* strain.

Norfloxacin, ampicillin and fosfomycin did not act differently on the test strains (I, Fig. 4). As the initially chosen concentration was killing very quickly, and we were afraid that the killing is too rapid to observe the differences, we also used two lower concentrations. The lowest concentration was chosen to cause only growth inhibition, while the third caused only slow killing.

Deletion of the *ssrA* caused the most prominent sensitization effect against aminoglycosides. The tmRNA defective strain showed a significant growth defect and was more prone to be killed by kanamycin and streptomycin. Those effects can be explained at the molecular level. It has been reported that even low concentrations of aminoglycosides are inducing global SsrA tagging (Abo et al., 2002). Aminoglycosides cause misreading of the mRNA during protein synthesis, which can be realized by over reading of the stop codon or frameshifting, and can lead to ribosome stalling on the 3' end of the mRNA. The SsrA-specific tagging could be the result of tmRNA activity releasing

ribosomes stalled on the 3' ends of the mRNAs. The growth defect caused by destruction of trans-translational machinery could arise from the reduced pool of free ribosomes.

In addition to releasing the ribosomes, tmRNA directs proteins, mistranslated by the ribosomes that reach the 3' end of the mRNA, into degradation. Those proteins are potentially toxic as they contain an additional C-terminal sequence that might hinder proper folding of the proteins and they could mislocalize, through exposed hydrophobic regions, into membranes. At the same time, an important factor for bactericidal activity of aminoglycosides is thought to be the accumulation of mistranslated proteins in membranes, resulting in the loss of membrane potential. The stronger bactericidal effect of aminoglycosides against the Δ*ssrA* strain could result from the increased accumulation of misfolded proteins in the membranes. This hypothesis would be interesting to test with the mutant strain coding for the protease resistant SsrA-tag to see if the accumulation of mistranslated proteins, by itself, is capable to induce cell death at lower concentrations of aminoglycosides.

The quest for novel compounds enhancing the effectiveness of current drugs has got off the ground during the last decade. We conclude, based on the current results, that tmRNA could be a potential target for those novel drugs, to potentiate several ribosome targeting drugs and cell wall inhibitors. Especially interesting would be the potentiation of aminoglycosides as their bactericidal activity is also enhanced. Another aspect about aminoglycosides is that, due to the toxicity issues, their use is restricted (Hermann, 2007). Potentiating their effectiveness would be specific only for bacteria allowing the use of lower concentrations and thereafter increase their usage in clinical practice, due to the decrease of toxicity.

## 2.3. Heterogeneity of stationary phase cultures and its impact on the frequency of persisters (II, III)

Most of the studies in the field of bacteriology have been conducted on the whole population level so that parameters have been measured as an average of the population. This kind of approaches will fail to take heterogeneity into consideration, although it is acknowledged as an important aspect of various processes, including the ones taking place in stationary phase cultures (Cormack *et al.*, 1996).

The studies on heterogeneity have so far been somewhat limited due to the technical aspects of many methodologies that often do not allow describing the processes at the single cell level. This limitation can be overcome by microscopy, specifically live-cell imaging, which enables us to follow growth, death and responses to the environment of the single cells (Stewart et al., 2005, Balaban et al., 2004). However, live-cell imaging has its own limitations. It can be time consuming and often subjective if special software is not used. In

addition, the number of cells analyzed per experiment can be rather small, making the statistical analysis complicated or even impossible. Also, the physical isolation/collection of specific cells or a subpopulation can be complicated if not possible at all. Those limitations can be at least partially overcome using high-throughput microscopy, but this equipment might not be available in every facility (Pepperkok and Ellenberg, 2006).

### 2.3.1. Tool for studying heterogeneity at the single cell level

Flow cytometry can be used for measurements of a large number of cells almost simultaneously at the single cell level with a low cost. In addition, flow cytometer is a relatively common piece of equipment. Moreover, single cells or certain subpopulations can be isolated via fluorescence activated cell sorting (FACS). Those were the reasons that lead us to the development of a flow cytometric assay that is relying on the dilution of fluorescent proteins (II, Fig. 1).

The principle of the assay is based on the stepwise dilution of the marker protein (in the current study the green fluorescent protein (GFP)) (II, Fig. 1). GFP-labeled cells, dividing at a similar rate, will form a single peak, decreasing in intensity over time on the histogram of fluorescent intensity. In case of the non-dividing cells, the intensity of the peak will stay the same. Heterogeneous population, on the other hand, would result in a more complicated histogram, for example, the mixture of non-dividing and dividing cells would generate two peaks.

We used the stable isoform of GFP (GFPmut2) (Cormack et al., 1996) which is not degraded during the timeframe of our experiments. Similar expression systems have shown that this protein is accumulating in large amounts, and it is freely diffusing in the cytoplasm (van den Bogaart *et al.*, 2007, Mullineaux *et al.*, 2006, Elowitz *et al.*, 1999). This means that after the removal of the inductor, the weakening of the signal is reporting the cell division, not affected by the stochastic dilution process, which could be the case when only a few molecules are present in the cell (Shapiro, 2003). Similarly, the process is not affected by protein degradation or aggregation.

### 2.3.2. Cell division in different growth phases

First experiments to test the method were to analyze the cell divisions of *E. coli* in different growth phases at the single cell level (II, Fig. 2). For this purpose the cells were labeled with GFP by the addition of the inducer. Before starting to collect samples for flow cytometric analysis, the inducer was removed by diluting the culture into fresh media (exponential phase culture) or the medium was replaced by conditioned medium, devoid of the inducer (other growth phases).

As expected, the cells in the exponential phase culture were dividing in a uniform manner as the reduction of GFP intensity was continuous over the whole population (II, Fig. 2A). From the reduction rate of the fluorescence intensities during the first time points we calculated the doubling time, which was approximately 25 minutes. This is in correlation with the doubling time of exponential phase culture measured by optical densities (data not shown). In the later time points the fluorescent intensities started to reach the basal level, and therefore the quantitative relationship between growth rate and GFP dilution was lost.

Generally, the stationary phase of bacterial culture has been thought to be a stage where growth of the cells is halted. This is concluded from the fact that CFUs per milliliter of the stationary phase culture and also the cell number, measured microscopically, do not change. It is also possible that in the stationary phase there is a balance between cell death and cell division, therefore keeping the cell numbers constant. So far, there was no direct method to confirm either of these hypotheses. In addition, it was not known whether all the cells enter the stationary phase at the same time, in other words, do all the cells stop growth at the same time or is it distributed in time.

Our assay enabled to address those questions. We saw that the cells, prelabeled with GFP, entered the non-dividing state (stationary phase) in a uniform manner as the GFP dilution slows down and stops, without changing the shape of the peak of the fluorescence intensity in the histogram (II, Fig. 2B). Similar experiment was repeated with the cells from deeper stationary phase. We did not see any reduction in GFP intensities, meaning that at least *E. coli* is in stationary phase, for a certain timeframe, in a non-proliferating state.

### 2.3.3. Heterogeneity upon recovery from stationary phase

Persisters are a very small subpopulation (commonly referred to as 1:10 000 – 1:100 000) of cells, known to originate mostly from stationary phase as cells which after dilution into fresh medium recover slower and therefore are insensitive to the bactericidal drugs (Lewis, 2010). We were interested to see whether our assay is sensitive enough to observe the population of non-proliferating cells after the dilution into a fresh media. To achieve this, we kept cells, prelabeled with GFP, for 24 hours in the stationary phase in LB medium and then diluted them into fresh medium. For our surprise, only a small fraction of cells started to divide, as their GFP intensity started to decrease (II, Fig. 3A). The rest of the cells retained high GFP content, meaning that they stayed dormant. As the number of cells with intermediate fluorescence remained very low we can say that the number of cells resuming growth later was low.

As the frequency of non-dividing cells was much higher than the commonly measured persister frequency we were interested to know what is behind this difference. It is likely that during the stationary phase, some cells have lost the viability but do not lyze and are measured as non-dividing cells in our assay. To

test this, we used propidium iodide staining, commonly used to differentiate dead cells from the living cells (Pin and Baranyi, 2008). We saw that an only small fraction of the whole population (both non-dividing and dividing cells) had lost their membrane integrity (II, Fig. 4). From these data we conclude that probably the massive cell death is not behind the differentiation into non-dividing and dividing subpopulations.

### 2.3.4. Persisters and the age of inoculum

As the high number of cells, unable to recover from stationary phase, was probably not a result of cell death, we decided to go more into the detail and measure the persister frequency in the same conditions (in LB medium, using inoculum from 24 hours old stationary phase culture). Using ampicillin and the plating method we determined the persister frequency to be about 10% (data not shown), which is much higher than commonly reported (Moyed & Bertrand, 1983, Keren et al., 2004a).

It is known that during the stationary phase the cells are losing viability (Siegele *et al.*, 1993, Desnues et al., 2003) and also the delay of growth resumption, upon dilution into fresh media, has been observed (Pin and Baranyi, 2008). At the same time, in the reports where persister frequencies have been measured the inoculum has been originating from 12–15 hours old cultures (Gefen et al., 2008, Balaban et al., 2004) or not much attention was paid to the age of the inoculum. In the latter case usually only the use of the overnight culture is referred (Keren et al., 2004a, Dorr et al., 2010). Most probably, the overnight culture would mean the length of the stationary phase about 12–16 hours, which is shorter than the stationary phase studied by us. We thought that observed high frequency of non-dividing and persister cells might be linked to the changes in the cultivability during the stationary phase. From this, we decided to measure the dependence of persister frequency on the length of the stationary phase.

We measured the dependence of the persister frequency on the age of inoculum, both in LB (rich medium) and in a MOPS medium supplemented with glucose (minimal medium) (III, Fig. 1). Interestingly, in both media we saw a drastic change in persister frequency as the inoculum aged. When the inoculum was taken from a short stationary phase culture, the observed persister frequencies were in the range of commonly reported values $10^{-4}$–$10^{-6}$ (Moyed & Bertrand, 1983, Keren et al., 2004a), but as the inoculum got older, the persister frequency started to increase and reached up to 10% of the total number of cells. It is noteworthy that in the LB medium the persister frequency increased in the matter of hours, but in the MOPS medium it took longer time and the time frame was measured in days.

We have demonstrated that GFP-labeled cells, which do not start to divide upon dilution into the fresh media, are insensitive to ampicillin, as they are not lyzed and are easily detectable by the high retained fluorescence (II, Fig. 6).

Therefore, we were able to follow also the number of non-dividing cells in the very same experiment with persister frequency determination. We noticed that the number of unlyzed cells at the end of the antibiotic treatment (at the moment of plating a sample of culture to determine the frequency of survivors (persisters)) shared the pattern with the persister frequency – in case the short stationary phase was used for inoculation, their level was low but increased as the inoculum aged (III, Fig. 1). The change was not identical, as the ratio of persisters to non-dividing cells changed. For example, in LB, the ratio was initially < 1:1 000 and increased up to 1:10 and it started to decrease again when inoculum from the older stationary phase culture was used.

Those experiments illustrate the fact that not all dormant cells, surviving the ampicillin treatment, are persisters, contrary to previous beliefs (Shah et al., 2006, Kussell & Leibler, 2005, Keren et al., 2004b). In these previous studies, the unlyzed or dormant cells were collected, and their gene expression profiles were analyzed. From these data the generalization for persister cells was made. In the light of current results, we can say that the gene expression profiles, analyzed earlier, describe all dormant cells not able to start growth upon dilution into the fresh medium and the persisters were forming only a small fraction from those cells. A more appropriate approach would have been to comparing the profiles of cells collected after different lengths of antibiotic treatment. The differences between consecutive profiles would have reflected the cells that had started to divide and lyzed in between collecting two consecutive samples – the cells that do fulfill the definition of persisters.

The inoculum's age-dependent increase of persister frequency, surviving the 3 hour long ampicillin treatment, could manifest itself in terms of delay in the growth resumption. Indeed, a closer look at the dynamics of the growth resumption reveals the delay. The longer the stationary phase, the longer time it takes for the cells to start growth (III, Fig. 2), a phenomenon seen also before (Pin and Baranyi, 2008).

Those are the first experiments reporting the high persister frequency of the wild type *E. coli* in growing culture. In fact, the persister frequency is comparable to the classical high persister strain *hipA7* (Keren et al., 2004a, Dorr et al., 2010). Therefore, we were interested to measure the persister levels of *hipA7* in our assay. For the *hipA7* strain we observed a dynamic persister profile when different inocula were used (III, Fig. 1). In case the inoculum originated from the short stationary phase the persister frequency was higher as compared to the wild type strain. The persister frequency was in the range reported before (Keren et al., 2004a, Dorr et al., 2010). As the inoculum aged, the persister frequency increased, but at lower rate as compared to the wild type strain, leading to the similar persister levels of the both strains when aged inocula were used.

Quite often the *hipA7* strain has been used as a model to study the persister phenomenon as it has high persister frequency which is making the collection of material and data, or measuring persister levels easier. On the other hand, *hipA7*

is a mutant strain and it is not completely clear how the higher persister rate is achieved. In addition, we do not exactly know how the mutations behind the *hipA7* strain influence the physiology of the cell and can it have an impact on the results obtained using this strain. One difference, compared to the wild type strain is known – the *hipA7* strain is cold sensitive (Scherrer and Moyed, 1988), although it could directly be linked to the binding kinetics of HipA and HipB proteins (Rotem et al., 2010). Therefore, the use of the wild type strain for studying the persister phenomenon would be more appropriate. As we have now revealed the conditions where the persister level of the wild type strain is much higher than previously observed, the use of wild type strain could be prevail more.

This kind of persister dynamic has not been observed before. As already mentioned, the commonly used inoculum in the persister assays has probably spent in stationary phase about 12–16 hours. In our assay, the inoculum that has spent similar time in stationary phase in LB medium is showing the similar persister frequencies for the wild type and *hipA7* cultures (III, Fig. 1). One difference in our setup, compared to the previous reports, is GFP labeling that we are using for observing the growth resumption. Even without the induction of GFP expression (III, Fig. S1) or using a strain not carrying plasmid for GFP expression, we observed similar results (III, Fig. 4). The second important difference is the use of filter-sterilized LB medium. We noticed that using autoclaved LB medium the growth resumption results were not reproducible. We often observed that, after the 24 hour long stationary phase, nearly all cells were able to resume growth upon dilution into fresh medium (data not shown). After we started to use the filter-sterilized medium, the reproducibility issues were gone. A possible reason for the observed phenomenon is that the autoclaving can change the composition of the medium (Padgett and Leonard, 1994; Wang and Hsiao, 1995), resulting in the changes of the growth supporting properties of the medium (Field and Lichstein, 1958; Pedrotti *et al.*, 1994). The chemical changes can depend on several factors, including the volume of the material and type of the autoclave (Wang & Hsiao, 1995, Cook *et al.*, 1989), which we probably were not able to control. So it seems that the chemical composition of the LB medium can change processes taking place during the stationary phase in LB and are changing the cultivability of the cells. This would mean that the previous studies were conducted using media where cultivability of the cells did not decrease so fast as in our LB medium. With regard to the MOPS medium, the previously unnoticed high persister frequencies of wild type *E. coli* can be explained by the fact that nobody had previously used inocula from the stationary phase culture as old as we were using to perform the persister assay.

The growth resumption dynamic of the *hipA7* strain was very similar to the wild type strain (data not shown). This is conflicting with the previous observation (Balaban et al., 2004) that the *hipA7* strain has a higher level of cells recovering slowly from dormancy as compared to wild type. As the growth

conditions (microfluidic device versus batch culture) and cell detection method (microscopy versus flow cytometry) are different the results might not be directly comparable. However, in accordance with the previous results, *hipA7* differed from the wild type in the high persister level in case the inoculum was taken from the early stationary phase (III, Fig. 1).

To test, is the observed dependence of the persister frequency on the age of the inoculum specific to ampicillin or is it common to all bactericidal drugs, we conducted the persister assays using other types of bactericidal drugs (III, Fig. 3). Persister frequencies in response to norfloxacin treatment, a representative of fluoroquinolones, were quite similar to the results with ampicillin. On the other hand, amikacin (belongs to the class of aminoglycosides) killed all cells irrespective of inoculum age. This is in correlation with previous reports showing that treatment with aminoglycosides can lead to a very low level of persisters or even sterilization of the cultures (Zuroff *et al.*, 2010, Wiuff et al., 2005, Spoering & Lewis, 2001). The effect of aminoglycosides raises an interesting possibility that the persisters could synthesize proteins. This is in correlation with previous work from Gefen and coworkers, where they observed that a small subpopulation of cells, resuming growth later as compared to the majority of the cells, was able to synthesize proteins during the small timeframe upon dilution into a fresh medium (Gefen et al., 2008). In addition, it has been demonstrated that certain metabolites, presumably also present in fresh LB medium, can promote proton motive force, possibly also in persister cells, that facilitates aminoglycoside uptake, which could result in cell death (Allison et al., 2011).

The explanation for the changes of the growth resumption and persister levels could be the ageing of the cells during stationary phase. As previously mentioned, it has been reported that during stationary phase, cells can accumulate damages that can be reflected in carbonylation of proteins as an example of oxidative damage. When cells are damaged the growth resumptions could be affected as the repair of their constituents may take additional time. Desnues and colleagues reported bimodal distribution of oxidatively damaged proteins among stationary phase cells. The higher degree of accumulated damage was associated with lower cultivability (Desnues et al., 2003). This could be a reason behind the increasing number of cells with slow growth resumption ability.

In contrast to the ageing theory, the changes we observed in the behavior of the cells could also be explained by the bet-hedging theory, generating phenotypic heterogeneity under uncertain conditions (Beaumont et al., 2009). It is possible that the expectations for the forthcoming conditions are changing during the stationary phase. Therefore, overall changes in the growth resumption ability, including formation of the cells resuming growth slowly, might be a tightly controlled process and might reflect the higher expectation of longer famine period or catastrophic events. Those explanations do not have to be mutually exclusive as the controlled formation of the dormant cells could

take advantage of the changed cultivability caused by the accumulation of damages.

### 2.3.5. Genes related to persistence

In addition to *hipA7*, there are also several deletion mutants which have changed the persister frequency (Spoering et al., 2006, Scherrer & Moyed, 1988, Ma et al., 2010, Li & Zhang, 2007, Kim & Wood, 2010, Hansen et al., 2008, Dorr et al., 2010, Dorr et al., 2009, Aizenman et al., 1996). Interestingly, the lists of the genes identified do not overlap and in the light of the current results the influence of the experimental conditions seems likely to be the cause. To test this hypothesis we chose 1–3 strains, which showed strongest reduction in persister levels, from each report and tested their persister levels in the conditions of the ageing inocula. As several previous screens (Ma et al., 2010, Hansen et al., 2008) were conducted with the Keio collection (Baba *et al.*, 2006), we initially decided to use also the Keio strains. It turned out that, in our assay conditions, several selected strains did not differ from the wild type strain at all (III Fig. S3). It did not escape our attention that those strains might have acquired secondary compensatory mutations and kanamycin resistance cassette, used to replace the target gene, could cause polar effects. On this basis, we constructed most of the strains anew (exception was *sucB* that we were not able to delete in a clean background) and removed the antibiotic resistance cassette. Indeed, there were slight changes in persister frequencies compared with the strains originating from the Keio collection (III, Fig. 4).

We identified various effects of the age of inoculum on the knockout strains and they fall into three groups. The first contains *mqsR* (toxin from a TA system) (Kim & Wood, 2010) and *phoU* (regulator of phosphate metabolism) (Li and Zhang, 2007) knockout strains, and they were behaving similar to the wild type, when the inoculum was taken from a younger stationary phase, but in case of the later time points the persister levels were more than an order of magnitude lower.

The second group contained *glpD* (enzyme in cental metabolism) (Spoering et al., 2006), *surA* (peptidyl-prolyl *cis-trans* isomerase) (Hansen et al., 2008) and *dnaJ* (chaperon) (Hansen *et al.*, 2008) knockout strains that had a lower level of persisters when the inoculum originated from the early-stationary phase cultures. In the last time point, their persister level exceeded the wild type levels.

The third group is formed by *sucB* (enzyme in TCA cycle) (Ma et al., 2010), *ygfA* (enzyme in folate biosynthesis) (Hansen et al., 2008) and *recA* (DNA recombination protein) (Dorr et al., 2009) knockout strains, as they showed a lower persister levels in every time point examined. It is important to note that the *recA* deletion strain had strong growth defects (data not shown); therefore the relevance of this strain in the context of the current work is questionable. Interestingly, the effect of the *sucB* deletion was considerably larger and therefore gained more attention from us. We asked what could be behind the

low persister phenotype. It can be achieved by very fast growth resumption that is accompanied by the low fraction of slowly recovering cells. Another possibility is that the cells, which survived the antibiotic treatment, did not survive the plating and are causing low CFU read. To test those hypotheses, we measured the persister frequencies and growth resumption in parallel (III, Fig. 5). Indeed, growth resumption of the *sucB* deletion strain was much faster as compared to wild type, and it was not significantly delayed or slowed down during the ageing of the inoculum.

The phenotype of the *sucB* knockout strain could be the result of the growth dynamics of the culture, as the maximum optical density during stationary phase is about 1/3 of the wild type (data not shown). This suggests that the *sucB* knockout strain does not use all the resources before entering into stationary phase. This assumption is supported by the preliminary results, showing that the wild type strain can resume growth in the stationary phase media of *sucB* mutant (data not shown). The ability of the *sucB* mutant to resume growth rapidly, upon dilution into the fresh medium, indicates that it could be in a relatively active state during the stationary phase, subsidized by the remaining resources. Therefore, the strain might not be properly prepared for the stationary phase, in the sense of feast and famine conditions. Indeed, this assumption is supported by the preliminary experiments, showing that *sucB* mutant is rapidly losing its viability in the spent stationary phase media of the wild type strain (data not shown). This trade-off might explain why wild type cells do not have acquired the *sucB* knockout-like properties of the rapid growth resumption, which could be an advantage after entry into the growth supporting conditions.

*sucB* codes for an enzyme, involved in the tricarboxylic acid cycle. Several other enzymes from the same pathway have previously been shown to be involved in modulating the antibiotic action (Kohanski et al., 2007). Therefore, we measured persister levels of the deletion strains of *icdA*, *mdh* and *acnB* (III, Fig. 6). Phenotype of *acnB* deletion was most similar to the *sucB* mutant with the low persister level. However, in the last time point the persister level increased and was comparable with the wild type. The persister levels of other deletion strains (*mdh*, *icdA*) were exceeding wild type at the first time point but were merging with the level of wild type later, resembling the phenotype of the *hipA7* strain (III, Fig. 1). This demonstrates that central metabolism can change the persister levels, although explaining the effect of particular genes requires further experimentation.

The patterns in the persister levels of the mutant strains give rise to a hypothesis that the difference in the persister levels, between the wild type and several knock-out strains, may arise from the changed speed of the formation of persisters during the ageing of the stationary phase. For example, this process seemed to have accelerated for *mqsR* and *phoU* mutants (III, Fig. 4 B, D), but delayed for the deletion strains of *glpD*, *surA*, *dnaJ* (III, Fig. 4 A, C and E) and *acnB* (III, Fig. 6) when compared with the wild type. Moreover, in the case of the wild type strain the persister levels are changing hand-in-hand with growth

resumption. This suggests that perhaps, in those mutant strains, the changed dynamics of the persisters is accompanied also with the delay or acceleration of growth resumption. To validate this hypothesis further, measuring of the growth resumption kinetics, together with the gene expression and/or proteome analysis, is needed to compare the wild type and mutant strains. Another outstanding mutant is the *ygfA* deletion strain (III, Fig. 4 F), as its persister dynamic is following the dynamics of the wild type (the persister levels did increase and decrease in the same rhythm), although it was lower in every time point. It would be interesting to see in what extent is the growth resumption of the affected *ygfA* deletion strain, and how it relates to the changes in persister levels.

In conclusion, I can say that we have developed a method, enabling to observe heterogeneity in bacterial cultures based on the growth differences. This method can be and already is adapted to other organisms and experimental systems to study heterogeneity (Helaine *et al.*, 2010). In addition, I have demonstrated various dynamic profiles in the persister frequencies that are influenced by the growth conditions, history of inoculum and genotype of the strain.

# CONCLUSIONS

- Deficiency of trans-translation system increases susceptibility of *E. coli* to several ribosomal targeting antibiotics and inhibitors of the cell wall synthesis, and enhances the bactericidal activity of aminoglycosides.
- The flow cytometric assay was developed to follow cell division at single cell level. Using this assay, it was demonstrated that *E. coli* is dividing uniformly through different growth stages and during stationary phase is in a non-proliferating state
- Persisters are forming only a small fraction from all non-dividing cells.
- The frequency of persisters depends on the inoculum, particularly on its age and the medium composition. As the stationary phase culture is ageing, the ability of the cells to resume growth upon dilution into fresh media is changing: growth resumption delays as the rate of growth resumption goes down and the number of cells resuming growth in a slower rate is increasing, which results higher frequency of persisters. In rich medium, these processes are much faster compared to the minimal medium.
- The persister levels of various mutants were differentially influenced by the age of the inoculum underlining great importance of the experimental details during the screening for altered persistence.

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# SUMMARY IN ESTONIAN

## *Escherichia coli* antibiootikumitundlikkust mõjutavad tegurid

Antibiootikumid on ühendid, mis tugevalt pärsivad mikroobide kasvu või surmavad neid. Esimeste antibiootikumide kasutusele võtmise ajal arvati, et need ravimid vabastavad inimkonna jäädavalt ohtlikest ning tihti surmaga lõppevatest nakkushaigustest. Peatselt hakkasid aga ilmuma teated resistentsetest bakteritüvedest, mis laialdase leviku tõttu muutsid paljud antibiootikumid kasutuks. Siiski avastati või sünteesiti esialgu pidevalt juurde mitmeid uusi antibakteriaalseid ühendeid, mis aitasid hoida resistentsete tüvede leviku kontrolli all. Peagi aga langes uute antibiootikumide turule tuleku tempo üsna märgatavalt ning nüüdseks on resistentsed tüved üle maailma tõsiseks probleemiks ja harvad ei ole juhud, kus ka nö. reservravimid on kasutud. Seetõttu on viimasel ajal otsitud viise, kuidas olemasolevate antibiootikumide toimet võimendada.

Korduvalt on näidatud, et antibiootikumidel on laialdane mõju bakteri füsioloogiale. Seetõttu on ühe võimalusena antibiootikumide mõju võimendamiseks otsitud lisaks otsesele sihtmärgile ka teisi protsesse, mille paralleelne inhibeerimine võiks tuua kaasa suurema antibiootikumi tundlikkuse. Üheks selliseks protsessiks on osutunud trans-translatsioon, mida viib läbi tmRNA ning mis seisneb mRNA-le kinnijäänud ribosoomide vabastamises.

Varem on tmRNA-d kodeeriva geeni eemaldamise mõju vaadeldud ainult valgusünteesi inhibeerivate antibiootikumide korral (Vioque & de la Cruz, 2003, de la Cruz & Vioque, 2001, Athamna et al., 2004, Abo et al., 2002). Kuna tmRNA mõjutab mitmeid erinevaid rakulisi protsesse, otsustasime uurida, kas vigase trans-translatsiooni süsteemiga *Escherichia coli* on tundlikum ka teiste sihtmärkidega antibiootikumide suhtes. Me testisime laia valikut erinevaid antibiootikume ning leidsime, et lisaks mitmetele valgusünteesi inhibeerivatele antibiootikumidele muutis tmRNA puudumine *E. coli* tundlikumaks ka rakukesta sünteesi inhibiitoritele. Lisaks kasvuinhibitsiooni mõõtmisele võrdlesime ka tapmise efektiivsust. Me leidsime, et ainult aminoglükosiidid, mis on valgusünteesi inhibiitorid, surmasid tmRNA puudulikku tüve tunduvalt efektiivsemalt kui metsiktüüpi tüve. Sellest me järeldasime, et tmRNA ja trans-translatsioon võiksid olla sihtmärk, et võimendada mitmete valgusünteesi inhibiitorite (eriti aminoglükosiidide) ning rakukestasünteesi inhibiitorite toimet.

Kõik seniuuritud bakteripopulatsioonid sisaldavad väikest hulka antibiootikumidele tundetuid baktereid, mida kutsutakse persistoriteks. Selle nähtuse puhul ei ole siiski tegemist resistentsusega, kuna persistorid on geneetiliselt idententsed ülejäänutega, mis tähendab, et tegemist on hoopis fenotüübilise muutlikkuse nähtusega. Kuna persistoreid peetakse krooniliste ja pikaajaliste infektsioonide põhjustajaks, siis nende hulga vähendamine võiks samuti aidata

muuta raviskeeme praeguste antibiootikumide puhul efektiivsemaks. Siiski on persistorite fenomeni olemus, hoolimata enam kui 60 aasta tagusest esmakirjeldusest, suurel määral selgusetu. Seetõttu keskendusin doktoritöö teises osas persistorite teket mõjutavate faktorite uurimisele.

Persitorite kohta on teada, et nad on madala metaboolse aktiivusega nn. uinunud olekus olevad bakterid (Lewis, 2010). Samas on selliste bakterite hulk populatisoonis väga madal, mistõttu on olnud neid seniste meetoditega üsna keeruline lähemalt uurida. Sellest probleemist ülesaamiseks töötasime välja bakterirakkude jagunemise jälgimiseks uudse meetodi, mis põhineb fluorestseeruva valgu lahjenemisel. Kuna rakkude detektsioon toimub voolutsütomeetri abil, siis on selle meetodi abil võimalik kiirelt mõõta paljude rakkude parameetreid. See lubab tuvastada ka väikest hulka mittejagunevaid rakke.

Järgnevalt tuvastasime, et peale pikemat statsionaarset faasi muutus rakkude kasvama hakkamise võime nii, et järjest rohkem tekkis mittejagunevaid rakke ja üldine kasvama hakkamine hilines. Persistorite hulk järgis seejuures mittekasvavate rakkude dünaamikat. Kokkuvõtvalt võib öelda, et persistorite tase sõltub olulisel määral inokulumi ajaloost, eriti just vanusest ning ka kasvatamiseks kasutatud söötmest.

Persistoreid põhjustavate mehhanismide otsingul on tuvastatud mitmeid geene, mille eemaldamine vähendab persistorite hulka. Siiski ei kattu erinevates uuringutes tuvastatud geenide nimekirjad sageli omavahel. Me näitasime, et põhjuseks võivad olla erinevused katsetingimustes. Näiteks võib mutandi persistorite tase erineda metsiktüüpi tüvest ainult teatud inokulumi vanuse korral.

# ACKNOWLEDGEMENTS

First I would like to thank my supervisor, Professor Tanel Tenson, for being patient with me and helping me to get to know the world of science. I cannot leave unmentioned all the current and former members of Tanel's lab (there are too many to name you all) for creating a friendly atmosphere, offering a helping hand when asked for or not dared to ask for. Special thanks go to Arvi Jõers and Niilo Kaldalu for sharing the enthusiasm, the ideas about persisters and bacterial heterogeneity, and also some of their labor time.

I cannot forget the other people from the 4th floor of the Institute of Technology for turning the former spooky castle into a buzzing facility. I am grateful to Johanna Roostalu for the interesting project which I inherited after she left.

I thank also Vallo "Vussvillem" Varik for the interesting philosophical discussions and constant acknowledgements about my dysgraphic craftsmanship. I am also thankful for Veiko Voolaid for the constant updates on the bicycle scene.

Riho Mõtlep and Tambet Tõnissoo will be remembered for the xdream sufferings. I, arguably expelled even from a childrens' choir, am also grateful to the award-winning musical collective "Inju KEK" and its cofounders for a chance of decade long musical self expression as a leading singer.

Finally, I would like to thank my family for the whole support during the course of my studies. Special thanks go to my sweet wife Veronica, for being a loving companion and for tolerating all my weirdness. I can also not forget my dear son Uku, always waiting me at home with a big smile.

# PUBLICATIONS

# CURRICULUM VITAE

## I. Personal particulars

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   1988–2000 Taebla Gymnasium
   2000–2004 University of Tartu, technology of transgene, BSc
   2004–2006 University of Tartu, molecular biology, MSc
   2006–… University of Tartu, PhD studies (biomedical technology)

8. Language skill: estonian, english, russian, german

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   2010–... University of Tartu – research specialist

## II. Research and development work

1. Main fields of research
   Role of trans-translation system in the antibiotic susceptibility of *E. coli*. Stationary phase heterogeneity of *E. coli*. Persisters.

2. A list of publications
   - **Luidalepp, H.**, Hallier, M., Felden, B., and Tenson, T. (2005). tmRNA decreases the bactericidal activity of aminoglycosides and the susceptibility to inhibitors of cell wall synthesis. *RNA Biol* **2**(2), 70–4.
   - Roostalu, J., Jõers, A., **Luidalepp, H.**, Kaldalu, N., and Tenson, T. (2008). Cell division in *Escherichia coli* cultures monitored at single cell resolution. *BMC Microbiol* **8,** 68.
   - **Luidalepp, H.**, Jõers, A., Kaldalu, N., and Tenson, T. (2011). Age of inoculum strongly influences persister frequency and can mask effects of mutations implicated in altered persistence. *J Bacteriol* **193**(14), 3598–605.

3. Research grants and scholarships

| | |
|---|---|
| 2010 | Kristjan Jaak's travel grant |
| 2009 | Stipendium of shire of Taebla |
| 2009 | Kristjan Jaak's travel grant |
| 2007 | FEBS fellowship |
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| 2004 | Stipendium of Estonian Students' Fund USA |

4. Other administrative and professional activities

| | |
|---|---|
| 2006– ... | member of Estonian Biochemical Sociaty |
| 2005–2006 | member of council: University of Tartu, Faculty of Biology and Geography and University of Tartu, The Institute of Molecular and Cell Biology |
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## III. Teaching work

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| | |
|---|---|
| 2006–2010 | Lecture "Chaperons and protein folding" in course "Protein biosynthesis" |

# Curriculum Vitae

## I. Üldandmed

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   1988–2000 Taebla Gümnaasium
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   2004–2006 Tartu Ülikool, molekulaarbioloogia, MSc
   2006–... Tartu Ülikool, doktoriõpe (biomeditsiini tehnoloogia)

8. Keelteoskus: eesti keel, inglise keel, vene keel, saksa keel

9. Töökogemus:
   2010–... Tartu Ülikool – molekulaarbioloogia laborant

## II. Teaduslik ja arendustegevus

1. Peamised uurimisvaldkonnad
   Transtranslatsiooni süsteemi mõju *E. coli* antibiootikumitundlikusele. *E. coli* statsionaarse faasi heterogeensus. Persistorid.

2. Publikatsioonide loetelu
   - **Luidalepp, H.**, Hallier, M., Felden, B., and Tenson, T. (2005). tmRNA decreases the bactericidal activity of aminoglycosides and the susceptibility to inhibitors of cell wall synthesis. *RNA Biol* **2**(2)**,** 70–4.
   - Roostalu, J., Jõers, A., **Luidalepp, H.**, Kaldalu, N., and Tenson, T. (2008). Cell division in *Escherichia coli* cultures monitored at single cell resolution. *BMC Microbiol* **8,** 68.
   - **Luidalepp, H.**, Jõers, A., Kaldalu, N., and Tenson, T. (2011). Age of inoculum strongly influences persister frequency and can mask effects of mutations implicated in altered persistence. *J Bacteriol* **193**(14)**,** 3598–605.

3. Saadud uurimistoetused ja stipendiumid
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   2009 Taebla valla stipendium
   2009 Kristjan Jaagu reisistipendium
   2007 FEBS'i stipendium
   2005 Artur Linnu geenitehnoloogia stipendium
   2004 Eesti Üliõpilaste Toetusfond USAs stipendium

4. Muu teaduslik organisatsiooniline ja erialane tegevus (konverentside ettekanded, osalemine erialastes seltsides, seadusloome jms.)
   2006–... Eesti Biokeemia seltsi liige
   2005–2006 Tartu Ülikooli Bioloogia-geograafiateaduskonna nõukogu liige ja Tartu Ülikooli Bioloogia-geograafiateaduskonna Molekulaar- ja rakubioloogia instituudi nõukogu liige
   2003–2004 Tartu Ülikooli Bioloogia-geograafiateaduskonna nõukogu liige ja Tartu Ülikooli Bioloogia-geograafiateaduskonna Molekulaar- ja rakubioloogia instituudi nõukogu liige

## III. Õppetöö

1. Andmed kõrgkoolis tehtud auditoorse õppetöö kohta
   2006–2012 Loeng "Chaperonid ja valkude voltumine" aines "Valkude biosüntees"

# DISSERTATIONES TECHNOLOGIAE UNIVERSITATIS TARTUENSIS

1. **Imre Mäger.** Characterization of cell-penetrating peptides: Assessment of cellular internalization kinetics, mechanisms and bioactivity. Tartu 2011, 132 p.
2. **Taavi Lehto.** Delivery of nucleic acids by cell-penetrating peptides: application in modulation of gene expression. Tartu 2011, 155 p.