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Mechanisms of adaptation to the fitness cost of antibiotic resistance

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”På sina resor han förnam,
Hur väl Försynens nåd reglerat,
Som floder öfverallt placerat
Der stora Städer stryka fram.”

Dumboms Lefverne
Johan Henrik Kellgren, 1791

Abstract

The dissemination and persistence of antibiotic resistance, is not only depending on the volume of drugs used but also on the resistance mechanisms effect of bacterial fitness (reproductive ability). Fitness is a multifactorial parameter that is comprised of the relative growth rate of the resistant pathogen in the host and in the environment, as well as the clearance and transmission rates compared to susceptible strains. The focus of this thesis has been to determine (i) the connection between the mechanisms of resistance and their effect on growth in vitro and in a host-model, and (ii) how different adaptive mechanisms can partly or fully reverse the deleterious effects of resistance mutations.

More specifically the fitness effect of mutational resistance towards the translational inhibitors mupirocin and streptomycin, targeting the isoleucyl–tRNA synthetase (IleRS) and the ribosomal 30S subunit, respectively were investigated using *Salmonella typhimurium* as a model organism. These studies showed that a fitness cost was associated with the resistance mutations and that suppression of their deleterious effect could be achieved by both intragenic and extragenic compensatory events. Three compensatory mechanisms that could restore fitness were identified, (i) intragenic mutations in the target protein (IleRS), (ii) extragenic (i.e. outside the target protein) mutations in the ribosomal proteins of the 30S and 50S subunits and (iii) extragenic compensatory events increasing the expression of the target protein (IleRS). The mechanism behind the resistant and compensatory mutations effect on respective target protein could be determined by in vivo and in vitro kinetical measurements of ribosomal translation (rate and accuracy) and the IleRS aminoacylation activity. The impact on activity for the resistant and compensatory mutations was shown to correlate with their effect on growth rate. However, since the fitness impact of the resistance mutations has been seen to vary between different in vitro conditions and between in vitro and in vivo conditions, we investigated and validated the nematode *Caenorhabditis elegans* (*C. elegans*) as an in vivo model for determining the fitness effects of resistance against several classes of antibiotics (including mupirocin and streptomycin). The fitness impact of the resistance mutations measured in *C. elegans,* correlated well with what had been detected in the mouse model of typhoid fever. It is worth noting that for all resistant strains, relative fitness in the two host-models was lower compared to fitness measured in the Luria Bertani broth laboratory medium.

In conclusion, we have shown how resistance and compensatory mechanisms can at the protein and cellular level affect the stability of resistance in different in vitro and in vivo models.

List of Publications

This thesis is based on the following papers, which will be referred to by their Roman numerals.

I Paulander, W*., Pennhag, A*., Andersson, D.I., and Maisnier-Patin, S. (2007)
Caenorhabditis elegans as a model to determine fitness of antibiotic-resistant *Salmonella enterica* serovar Typhimurium.
Antimicrob Agents Chemother 51: 766-769.

II Maisnier-Patin, S., Paulander, W., Pennhag, A., and Andersson, D.I. (2007)
Compensatory evolution reveals functional interactions between ribosomal proteins S12, L14 and L19.
J Mol Biol 366: 207-215.

III Paulander, W., Maisnier-Patin, S., and Andersson, D.I. (2007)
Multiple mechanisms to ameliorate the fitness burden of mupirocin resistance in *Salmonella typhimurium*.
Mol Microbiol 64: 1038-1048.

* These authors contributed equally to this work.

Contents

List of abbreviations

aa	Amino acid
aa-AMP	Aminonacyl adenylate
aaRS	Aminoacyl-tRNA synthetase
ATP	Adenosine 5'-triphosphate
bp	Base pair
CP1	Connective polypeptide 1 domain
DDD	Defined daily dose
DNA	Deoxyribonucleic acid
GISA	Glycopeptide intermediate sensitivity *Staphylococcus aureus*
Ile	L-isoleucine
IleRS	Isoleucyl–tRNA synthetase (protein)
ileS	Isoleucyl–tRNA synthetase (gene)
IPTG	Isopropyl-beta-D-thiogalactopyranoside
LB	Luria-Bertani broth
MIC	Minimal inhibitory concentration
MRSA	Methicillin resistant *Staphylococcus aureus*
PBP	Penicillin binding protein
ppGpp	Guanosine-tetraphosphate
ram	Ribosomal ambiguity
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
rpsL	S12 (gene)
tRNA	Transfer RNA

1 Introduction

1.1 Antibiotic resistance

1.1.1 Introduction

The first antibiotic was discoverd in 1929, when the Scottish bacteriologist Alexander Fleming by chance observed the antibacterial activity towards *Staphylococcus aureus* (*S. aureus*) of a penicillium mould that had contaminated his culture dishes. As Pasteur later commented on the event, "chance favours the prepared mind". When Fleming in 1945 was awarded the Nobel Prize, he somewhat prophetically warned that the misuse of penicillin would lead to resistance (Ligon, 2004). Only a few years later, over 50% of the *S. aureus* strains isolates were no longer susceptible to penicillin (Alanis, 2005). In the following years, new antibiotic classes were introduced, but towards all of them resistance mechanisms developed (Levy and Marshall, 2004).

1.1.2 Antibiotic resistance impact on society

It has been estimated that antibiotics have extended the average lifespan of US citizens with ten years, as a comparison, curing all cancers would extend the average life span with three years (Burman and Olsson-Liljequist, 2001). So the question is, what impact antibiotic resistance has had on society?

There are surprisingly few studies on increased mortality and economic cost of antibiotic resistance. The few studies that do exist mostly concern methicillin resistant *S. aureus* (MRSA) infections in hospitals. The increased mortality in the US due to MRSA has been reported to triple (from 8% to 21%) compared to susceptible strains (Rubin *et al.*, 1999). In Europe, it is estimated that annual excess mortality in MRSA is approximately 1300 deaths (van der Bruggen *et al.*, 2005). A recent report from South East Asia on antibiotic resistance related deaths estimated that neonatal sepsis caused 300000 excess deaths, due to the failure of empirical antibiotic therapy (Zaidi *et al.*, 2005; React., 2007). The studies on MRSA infections, have pointed out the increased length of the hospitalisation as the major cost, only 2-4% of the increased cost is related to the altered antimicrobial therapy (Nathwani, 2003). The average prolongation of the hospital stay has been approximated to two weeks. Calculations on what this would mean on a national scale, if 5% of all cures were troubled by bacterial resistance have been estimated to be equal that of all nosocomial infections taken together in Sweden (Burman and Olsson-Liljequist, 2001). At the community level, the economic cost of antibiotic resistance is however very hard to estimate, no study has in a satisfying way determined this. The unrecognized costs from the estimated 150 million annual antibiotic prescriptions in the US, have been approximated to in between $1 to $30 billion per year (Phelps, 1989).

Although antibiotic resistance has a considerable and undesirable economical cost, the dramatic effect is the large morbidity and mortality in the developing world. Moreover, the trend of increasing resistance to last resort compounds in the developed world, in combination with the slow introduction of new antibiotics, will in the coming years most likely have a large

impact on resistance related morbidity/mortality in the developed world if no dramatic changes in usage or development of new compounds appear.

1.1.3 Targets for antibiotics

More than 15 different antibiotic classes have been developed towards targets that are essential for physiological and/or metabolic functions (Table 1). The two major targets for the antibacterial drugs are the cell wall and protein synthesis. The cell wall is a unique target for prokaryotes (Table 1). Whereas the inhibition of protein synthesis is directed towards highly conserved structures that can also be found in eukaryotic cells, such as in mitochondria (Poehlsgaard and Douthwaite, 2005) and the isoleucyl-tRNA synthetases (Hughes and Mellows, 1978); small differences in the structures still allow for the selectivity of the antibiotics.

In the last 37 years only one new class of structurally unique antibiotics has been developed, the oxazolidonones (Linezolid) (Alekshun and Levy, 2007), which targets the prokaryotic 50S ribosomal subunit (Bozdogan and Appelbaum, 2004). Disappointingly, resistance towards Linezolid could be observed already a year after its clinical introduction (Gonzales *et al.*, 2001). The problems in recent years have been to find new molecules that either target unique prokaryotic structures, or the fine structural differences between highly conserved targets. This has led to a development of antibiotics, directed towards improving already existing molecules, which use similar mechanisms as the earlier derivates to attack the bacteria (Coates *et al.*, 2002). As a result minor modifications in already existing resistance mechanisms is enough to cause resistance to the newly introduced compounds. An example of this is the TEM-1 and SHV-1 ß-lactamase evolution towards third-generation cephalosporins (Davies, 1994; Majiduddin and Palzkill, 2003; Paterson and Bonomo, 2005). The third-generation cephalosporins were designed not to be hydrolysed by the TEM-1 and SHV-1 ß-lactamases, however mutations that alter the amino acid (aa) configuration around the active site of these beta-lactamases extend the spectrum of antibiotics susceptible to hydrolysis (Paterson and Bonomo, 2005). The fast development of resistance towards newly introduced and existing compounds places a large pressure on developing and delivering new antibiotics at a high rate, preferably towards new targets. Paradoxically because of the fast resistance development towards newly introduced antibiotics, a lot of companies are downsizing their development (Coates *et al.*, 2002).

Table 1. Summary of the most common classes of antibiotics and their target.

Target	Antibiotic
Protein synthesis	Aminoglycosides (Streptomycin); Chloramphenicol; Lincomycins; Macrolides; Mupirocin; Oxazolidonones (Linezolid); Tetracyclines.
Cell wall synthesis	Penicillins; Cephalosporins; Carbapenems; Monobactams; Glycopeptides
DNA synthesis	Quinolones; Fluoroquinolones; Metronidazole
RNA synthesis	Rifampicin
Tetrahydrofolate synthesis	Sulfonamides; Trimethoprim

1.1.4 Resistance mechanisms and transfer of resistance

The three major antibiotic resistance mechanisms are, active efflux by pumps, enzymatic modification of the antibiotic molecule and antibiotic target protein alterations. The dispersal of these resistance mechanisms can either be achieved by lateral transfer from mother to daughter cell, or by horizontal transfer that can occur between the same bacterial species, between different divisions (from gram-positive to gram-negative bacteria) and between prokaryotic and eukaryotic domains (Table 2).

Distinct bacterial species can differ substantially in mechanism and time required to develop resistance towards the same antibiotic. An example of this is the ß-lactam resistance development in the three gram positive species *S. aureus*, *Streptococcus pneumoniae* (*S. pneumoniae*) and *Streptococcus pyogenes* (*S. pyogenes*), which may either be conferred by ß-lactamases or by modifications of the penicillin binding proteins (PBPs). Penicillin resistant *S. aureus* strains could be isolated in hospitals two years after its introduction in 1940. The high rate of resistance development could be attributed to the selection on pre-existing ß-lactamases, hydrolysing the penicillin molecules (Fuda *et al.*, 2005). In the case of *S. pneumoniae* it took approximately thirty years to develop resistance towards penicillin. When resistance finally developed, it was due to alterations in one or more of the PBPs decreasing the affinity for the drug (Mouz *et al.*, 1998). The alterations in the PBPs were mediated by DNA uptake via transformation from related commensal streptococcal species already resistant towards penicillin (Hakenbeck *et al.*, 1999) creating mosaic PBPs with regions diverging up to 10% in aa sequence compared to the sensitive PBPs (Ferroni and Berche, 2001). In *S. pyogenes* on the other hand no clinical resistance towards penicillin has developed despite more than 60 years of usage (Macris *et al.*, 1998). In vitro selection of low-level resistance has however been achieved, by stepwise accumulation of resistance mutations in the PBPs (Gutmann and Tomasz, 1982). This is similar to what was discoverd in vitro for *S. pneumoniae* before resistance was clinically detected (Hakenbeck, 1999). The slow resistance increase by mutation accumulation is however not enough to achieve clinically significant resistance levels. The important difference between the two species, is that *S. pyogenes* has barriers for horizontal transfer such as extracellular DNAases and phage encoded restriction enzymes whereas *S. pneumoniae* is naturally competent for DNA transformation (Table 2). This example highlights the importance for three of the major factors driving resistance development, (i) pre-existing resistance mechanism, (ii) horizontal transfer of resistance determinants (Table 2) and (iii) mutational events conferring resistance.

The pre-existing resistance mechanisms are expected. Considering that microorganisms produce most of the clinically relevant antibiotics, to survive the antibiotic producers as well as their competitors most harbor resistance elements for protection (Wright, 2007; Yanagisawa and Kawakami, 2003). This suggests that many of the resistance mechanisms already existed before the introduction of antibiotics into clinical practice, and that it is the intensive antibiotic pressure that has selected for the transfer of the pre-existing resistance determinants (Davies, 1994; Palumbi, 2001; Wright, 2007). The mobilization of resistance determinants onto conjugative plasmids and transposons, has been shown to occur after the clinical introduction of antibiotics (Datta and Hughes, 1983; Hughes and Datta, 1983). A survey of soil dwelling *Streptomyces* species, showed that the isolates on average were resistant to eight different antibiotics, which included novel mechanisms that have not been detected in clinical isolates (D'Costa *et al.*, 2006). The transfer of resistance determinants from *Streptomyces* and other soil dwelling organisms has been suggested to be the cause of glycopeptide resistance and aminoglycoside-modifying kinases in clinically relevant organisms (D'Costa *et al.*, 2006; Davies, 1994; Guardabassi *et al.*, 2005). Other potential reservoirs for horizontal transfer of

resistance determinants is the human intestine, where it has been shown that resistant commensals can remain for years after the treatment has ended (Sjölund *et al.*, 2003). It is believed that horizontal transfer of resistant determinants from commensals to bacteria transiently passing through the colon or the nasopharyngeal (*S. pneumoniae* PBPs) plays an important role in the transmission of resistance (De Lencastre and Tomasz, 2002; Licht and Wilcks, 2006; Salyers *et al.*, 2004; Sundin and Bender, 1996).

Table 2. Summary of the natural DNA exchange processes. Adapted from (Mazel, 2001).

Procedure	Mechanism	Host Range	Role in Antibiotic Resistance	Efficiency
Transduction	Bacteriophage/Cell	Closely related species	Never demonstrated	High
Conjugation	Cell/Cell	Very broad	Important for most species	Varying, some very efficient
Transformation	DNA/Cell	Very broad	Important in *Neisseria* & *Streptococcus* species	Varying efficiency

The generation of non-transferable resistance, which arises primarily by chromosomal mutational events in the target gene for the antibiotic or by the deregulation of cellular processes mainly depends on two factors, (i) population size and (ii) mutation rate (Gerrish and Lenski, 1998; Nilsson *et al.*, 2004). Pathogens are believed to go through frequent population bottlenecks (reduction in population size) during transmission from host to host, reducing the genetic variation and thereby the capacity for generating adaptive mutations towards rapid changes in their environment, such as the exposure to antibiotics or host defenses (Andersson, 2003; Giraud *et al.*, 2002; Nilsson *et al.*, 2004). To increase the adaptive capacity, it has been suggested that elevated mutation rates would be selected. Highly increased mutations rates (≈500 times) usually due to defective DNA repair systems, have been detected in several bacterial pathogens at frequencies from around 1% (Denamur *et al.*, 2002; Björkholm *et al.*, 2001; LeClerc *et al.*, 1996) to as high as 30% in *Pseudomonas aeruginosa* isolates from cystic fibrosis lungs (Oliver *et al.*, 2000). However, besides the benefits of generating adaptive mutations, there is a cost linked to having an increased mutation rate since more deleterious mutations than beneficial or neutral mutations will be generated. In *Escherichia coli* K12 (*E. coli*) the rate of deleterious mutations have been estimated to 2-8 x 10^{-4} /genome/ generation versus 2 x 10^{-9} for beneficial mutations (Denamur and Matic, 2006). This means that there is a much larger risk for a disadvantageous mutational event, than for an advantageous event to occur. An explanation for the accumulation of mutators despite the deleterious effects of high mutation rates is that the first order selection is on the beneficial mutations that are generated and not on the mechanism for the generation of the adaptive mutations (hitchhiking effect) (Boe *et al.*, 2000; de Visser, 2002; Giraud *et al.*, 2001a; Giraud *et al.*, 2001b). This means that if sequential selection for several adaptive

mutations is required, as would be the case of antibiotic resistance, the chance for mutators to hitchhike along to a high frequency with the resistance mutations increases for each adaptive mutation required (de Visser, 2002). This could suggest that the large fraction (≈25%) of weak mutators (≈10-20 times increase in mutation rate) found in clinical isolates are selected because they deliver adaptive mutations at a higher rate, but generate less mutations with deleterious effects than isolates with high mutation rate in the long term perspective (Baquero *et al.*, 2004; de Visser, 2002; Matic *et al.*, 1997). In vitro studies have shown a beneficial effect of weak mutators towards the selection for sequential adaptation to increasing antibiotic concentrations (Orlen and Hughes, 2006).

If not adaptive, most mutations have a deleterious effect due to the random disruption of protein functions and cellular regulation. A similar effect can be seen for resistance mutations having deleterious effects on the whole cells fitness when the antibiotic is no longer present in the growth environment. This aspect of antibiotic resistance is discussed in the next chapter.

1.2 Biological cost of resistance and compensatory evolution

1.2.1 Biological cost of resistance

In an environment containing antibiotics, possession of the corresponding resistance gene confers a benefit to the organism. However, in a drug free environment, resistant genotypes might have a lower fitness (reproductive ability) than their sensitive counterparts. The reproductive ability is affected by several parameters, such as the relative rates at which susceptible and resistant bacteria grow in their hosts and in the environment, and how this affects clearance and transmission rates (Andersson and Levin, 1999; Björkman and Andersson, 2000). The only parameters that have been thoroughly examined, are the resistance mutations' impact on in vivo and in vitro relative growth rates. Aspects such as transmission and clearance rates are yet to be assessed.

A detrimental effect on growth rate has been described for several chromosomal resistance mutations, mainly due to their disruption of the target proteins' normal functions (Andersson, 2006; Björkman *et al.*, 2000; Nilsson *et al.*, 2006; Reynolds, 2000; Rozen *et al.*, 2007). Fitness cost associated with uptake and carriage of a R-plasmid carrying resistance gene has also been described. This could be related to the cost of expressing the resistance determinants and synthesising additional nucleic acids and proteins for maintaining the plasmid, imposing an energetic burden on the cell (Holloway *et al.*, 2007; Lenski, 1998; Zund and Lebek, 1980). However, transposon acquisition and resistance expression, seem to confer a small or negligible fitness impact (Elena *et al.*, 1998; Enne *et al.*, 2005). Only in a few cases has a large cost been detected, this depending more on where the insertion occurs in the genome, rather than the expression of the resistance determinant itself (Elena *et al.*, 1998). The fitness cost conferred by resistance determinants is not unique to bacteria, it has been observed for many different organisms towards lethal selection pressures. The most studied examples are the antiviral resistance mutations in the human immunodeficiency virus type 1 (HIV-1). Many of the resistance mutations reduce the virus ability to invade and propagate in human cells (Maisnier-Patin and Andersson, 2004). Another example is resistance towards insecticides such as diazinon in the Australian sheep blowflies, which results in delayed development and reduced survival ability (Lenski, 1998). Thus, biological costs due to

"escape" mutations towards a lethal selection pressure are in many cases universally observed for prokaryotes, eukaryotes and viruses.

1.2.2 Reversibility of antibiotic resistance

The correlation between volume of drugs used and resistance development (Figure 1) together with the observation that resistance determinants often confer a fitness cost has led to the question: is the development of resistance reversible? (Andersson, 2003; Austin *et al.*, 1999; Bronzwaer *et al.*, 2002)

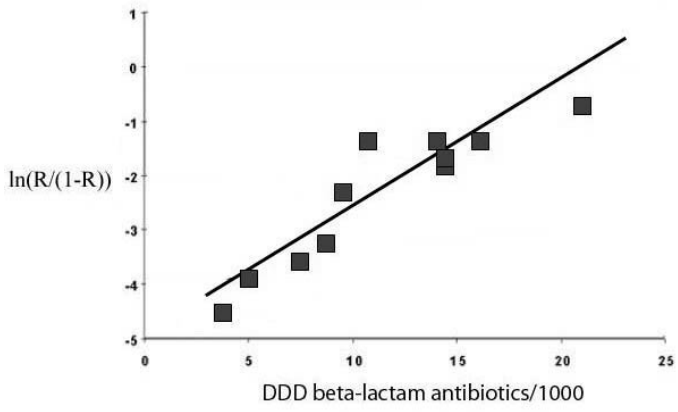

Figure 1: Adapted from (Bronzwaer *et al.*, 2002). Studies of ß-lactam antibiotics resistance and the level of ß-lactam consumption in 11 European countries, showed a correlation between volume of drug use and frequency of resistance. Represented as the log odds of resistance towards penicillin among invasive isolates of *S. pneumoniae*, regressed against outpatient sales of beta lactam antibiotics DDD = Defined Daily Dose, each square represents one country.

If the selective pressure of the antibiotic is removed (stop of usage), the resistant clones are expected to become out-competed by sensitive ones (Lenski, 1998). A few clinical studies have tried this approach to diminish resistance on a national scale. In Iceland, reduced penicillin consumption to curb the increase of a penicillin-resistant pneumococci clone, resulted in a drop in overall incidence of resistance strains from 20% in 1993, to 12% in 1997 (Arason *et al.*, 2002; Kristinsson, 1997). A similar approach was taken against macrolide resistant group A streptococci in Finland, where a decline from 16.5% in 1992 to 8.6% in 1996 could be seen (Seppala *et al.*, 1997). The results from these studies looked promising. However, later studies showed that other factors probably contributed to the rise and decline of the resistant clones. The most important factor was clonal shifts due to little or no heard immunity towards an invading serotype, which coincided with the reduction in antibiotic usage. This seems to have been a decisive factor for the outcome of these trials and probably not the reduction in antibiotic resistance (Arason *et al.*, 2002). A study in the UK, on sulphonamide resistance in *E. coli* that are not being affected by clonal shifts, showed no reduction in resistance frequency despite a decrease in prescriptions from 320000 per year in

1991 to 7000 in 1999 (Enne *et al.*, 2001). The genetic linkage to other resistance determinants seems to have been a contributing factor that stabilised the resistance at continued high levels; other yet undetermined factors such as compensatory events could also have played a role.

1.2.3 Compensation of fitness cost conferred by resistance determinants

The fitness cost that chromosomal resistance mutations and plasmids often confer to the cells, makes them less competitive than sensitive strains in an antibiotic-free environment (Figure 2, $\underline{R}^{R1}$). Counteracting this is selection for a decreased cost of resistance. This can be accomplished by reversion (Figure 2, $\underline{R}^{R1}\rightarrow\underline{S}$) of the resistance mutation or acquisition of a second site compensatory mutation, restoring fitness close or equal to the sensitive strains level (Figure 2, $\underline{R}^{R1}\rightarrow\underline{RC}$). The likelihood for reversion, compensation or extinction, is affected by factors such as bacterial population size, target for compensation, fitness cost of the resistance mutation, level of compensation and intensity of antibiotic pressure (Björkman and Andersson, 2000). One can conclude from several in vitro investigations that under most conditions compensation rather then reversion is a more frequent event. This is because the mutational target for compensation (RC) is larger than for reversion (S) (Maisnier-Patin *et al.*, 2002; Maisnier-Patin and Andersson, 2004). It has been estimated that the average number of compensatory mutations per deleterious (i.e. resistance) mutation is >11 (Poon *et al.*, 2005). Not only intragenic events can restore fitness. It has been approximated that around 30% of the compensatory events are extragenic, further enhancing the likelihood for compensation over reversion (Figure 3) (Poon *et al.*, 2005). For plasmids, compensatory events can take place either on the chromosome or on the plasmid it self (Bouma and Lenski, 1988; Dahlberg and Chao, 2003).

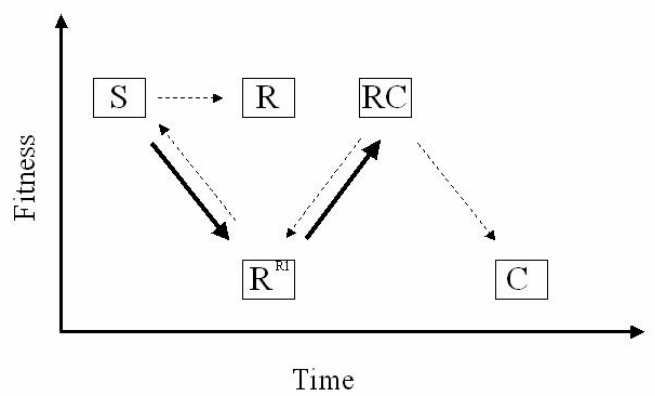

Figure 2: Compensation is a more common mechanism to ameliorate the fitness costs of resistance mutations than reversion. Fitness on the Y-axis takes into account the following parameters: growth rate, virulence, survival and transmission. S= Antibiotic sensitive, R= Antibiotic resistant with no fitness cost, $\underline{R}^{R1}$= Antibiotic resistant with fitness cost, RC= Antibiotic resistant strain that have gained fitness to various degrees via a compensatory event and C= Antibiotic sensitive with compensatory mutation conferring a fitness cost.

This has the effect, that even compensatory mutations (RC) that do not fully restore fitness (Figure 2) will be favored over reversion under most circumstances where the genetic diversity is restricted. Restricted genetic diversity (bottlenecks) is believed to occur frequently for pathogens during transmission from host to host (Levin *et al.*, 2000). Reversion only becomes a likely option when the population is large enough to allow all genetic variants to compete, selection will then favor the fittest variant; i.e. most often the revertants.

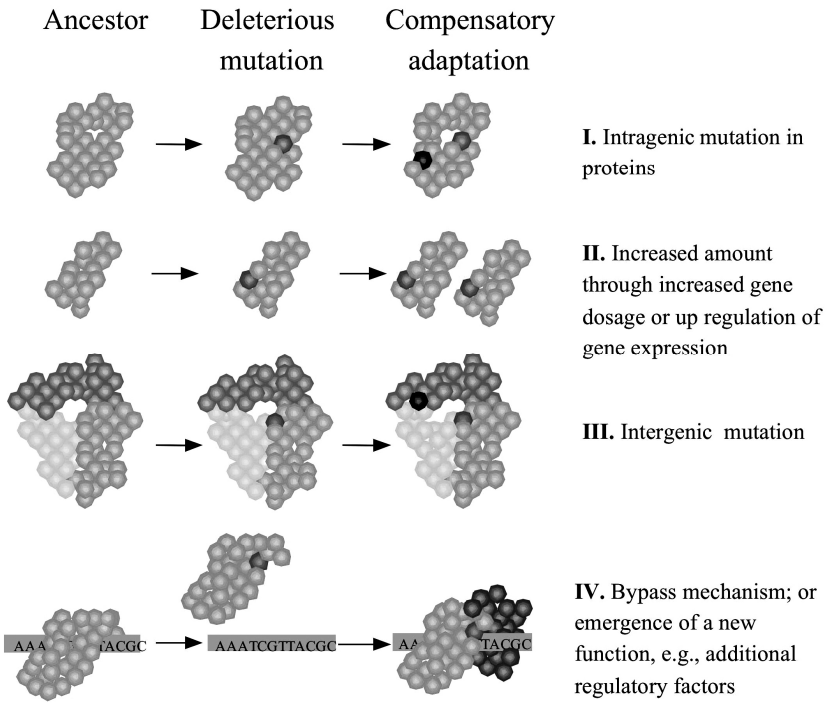

Figure 3: Different types of compensatory mechanisms adapted from (Maisnier-Patin and Andersson, 2004). **I)** Mechanisms of intragenic compensation are described in paper II and III. **II)** Mechanisms of compensation by increased gene expression as described in paper III. **III)** Intergenic compensation in a multi subunit complex as described in paper II. **IV)** Bypass mechanism compensating for the cost of isoniazid resistance in *Mycobacterium tuberculosis* (Sherman *et al.*, 1996)

1.2.4 Low cost resistance mutations and compensation in vivo

Clinically it seems like many of the chromosomal resistance mutations that are selected for, are low cost or no cost resistance mutations (se 1.4.4 for further details) (Gagneux *et al.*, 2006; Hughes and Andersson, 2001; O'Neill *et al.*, 2006). There are however cases where in addition to the resistance mutation, mutational changes were found in the resistance gene or elsewhere on the chromosome suggesting that compensation had occurred. For instance, this has been observed for fusidic acid resistance in *S. aureus* (Nagaev *et al.*, 2001), clarithromycin

resistance in *Helicobacter pylori* (Björkholm *et al.*, 2001) and isoniazid in *Mycobacterium tuberculosis* (*M. tuberculosis*) (Sherman *et al.*, 1996).

From the perspective of reversibility of antibiotic resistance, compensation represents a big problem. This is because compensatory mutations most often do not reduce resistance levels or affect the resistance mechanism when restoring fitness. There are however exceptions where the resistance level is lowered during compensation of the resistant related fitness cost (paper III; Nilsson *et al.*, 2006). This influences the time required to reduce the population of resistant bacteria, that is inversely proportional to the cost of resistance. If the fitness cost of a resistance mutation is reduced to 1% by compensatory events, it will take 20 times longer to eliminate the compensated population (by the take over from a sensitive clone) than it would have taken for the uncompensated resistant population with 20% lower fitness (Lenski, 1998). This in combination with the epistatic interactions between the resistance and compensatory mutation results in a fitness cost for the loss of the resistance mutation (Figure 2, RC→C). When separated from the resistant mutation, the compensatory mutation itself often confers a biological cost to the organism. Making the loss of resistance a highly unlikely event even when the volume of drugs is reduced.

Compensation for plasmid-conferred resistance cost and its stabilisation has been suggested for tetracycline and streptomycin resistance (Lenski, 1998; Smith, 1975; Sundin and Bender, 1996). An interesting clinical example is the development of ampicillin resistance in *Neisseria gonorrhoeae* The R-plasmid encoding for β-lactamases was initially unstable, but stabilised within a few months after resistant strains were first isolated. Plasmid stability has been seen to be strongly affected by small growth rate differences, the stabilisation of the plasmid is therefore believed to have been caused by compensation for the cost associated with carrying the plasmid (Lenski, 1998). This has however not been confirmed experimentally. It has been suggested that if a substantial reduction in ampicillin consumption would have been undertaken while the plasmid was still unstable, a reversion of the resistance development might have occurred (Lenski, 1998).

There is however an intrinsic caveat of assessing the fitness cost of resistant and compensated strains under just a few selected environmental conditions. Just because no cost is detected in one environment does not mean that it does not exist under other environmental conditions. The divergent patterns of resistance fixation and compensatory mutational accumulation between in vivo and in vitro experiments have shown this (Björkman *et al.*, 1998; Björkman *et al.*, 1999; Björkman *et al.*, 2000). Such observations make it important to assess resistance and compensatory mutations under several different in vitro conditions, especially in minimal medium with poor carbon sources and to develop new accessible in vivo models (chapter 1.3 New model for assessing in vivo fitness cost), for obtaining a more accurate picture on the effect and stability of resistance mutations.

1.3 New model for assessing in vivo fitness cost

1.3.1 Introduction

To be able to predict antibiotic resistance development, in vitro studies have been conveniently used to assess how the acquisition of resistance determinants influences the cells' fitness, and how potential fitness costs can be ameliorated by compensatory evolution. However in vitro and in vivo (mice) experiments have shown differences in mutation spectrum and fitness effects (Björkman *et al.*, 2000; Bull and Levin, 2000; Nagaev *et al.*, 2001) indicating that resistance development and persistence of pathogenic bacteria should be assessed under conditions that are as realistic as possible. Although mammalian host models such as mice and rats have been used for a long time to characterize bacterial virulence factors, advances have also been done in understanding the molecular basis of host-pathogen interactions through the use of plants or genetically tractable invertebrates such as *Caenorhabditis elegans* (*C. elegans*). Studies performed in *C. elegans* have shown that many genes known to be required for Gram-negative and Gram-positive mammalian pathogenesis are needed for pathogenesis in the nematode (Aballay and Ausubel, 2002; Couillault and Ewbank, 2002; Labrousse *et al.*, 2000).

1.3.2 *Caenorhabditis elegans*

C. elegans is a non-parasitic soil-living nematode (roundworm) that feeds on microbial blooms found in decaying matter (Sifri *et al.*, 2005). The requirements for its survival are a humid environment, ambient temperature, aerobic conditions and bacteria as food (Hope, 1999). The adult worm is only 1mm in length, making it easy to study under laboratory conditions, since it can be kept in Petri dishes or grown in liquid culture feeding on OP50, an auxotrophic *E. coli* strain.

C. elegans has been used as a model organism for a wide number of applications such as development studies, neurobiology, aging, innate immunity and as a model for bacterial pathogenesis (Sifri *et al.*, 2005). The genome sequencing revealed a 97-megabase genome, organized in six chromosomes and with more than 20,000 genes annotated. The anatomy of the worm is quite simple, the gut extends out from the pharynx, and the intestine running through the whole worm consists of only 20 polyploid epithelial cells (Figure 4). Through muscular contraction the pharynx functions as a pump, generating a pressure that concentrates the food and forces into the intestine (Avery and Shtonda, 2003). There are two types of sexual forms, male and hermaphrodite. The worm goes through four postembryonic larva stages (L1-L4) before becoming an adult. The average life cycle at 20ºC is 3.5 days from an egg to an egg-laying adult.

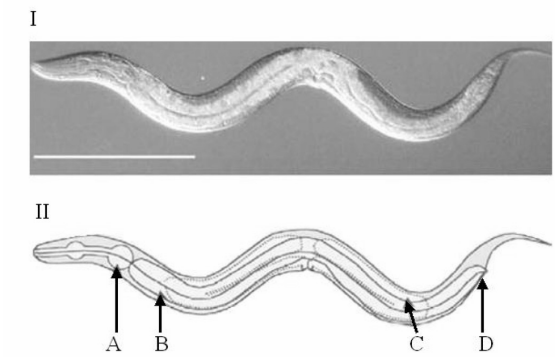

Figure 4: The anatomy of the nematode, A = Pharynx, B = Intestine, C = Gonad, D = Anus. The scale bar corresponds to approximately 250μm. Adapted from (Ewbank, 2002).

1.3.3 Immune defence

C. elegans relies on physical barriers and innate immunity for protection against pathogens, it has no adaptive immune defence. The first barrier is a tough multi-layered collagenous cuticle that surrounds the worm and protects it from bacterial penetration. This restricts the entry points for potential pathogens to the mouth and anus of the nematode. In the pharynx, a grinder consisting out of three cuticle disks mechanically disrupts the digested microbes before they reach the intestine (Figure 4). If any bacteria should survive the grinder, the rhythmic peristaltic movement of the intestine results in that ingested material is continually defecated, making colonisation of the intestine very difficult (Avery and Shtonda, 2003). In response to bacteria that have managed to colonise the nematode intestine, an inducible innate immune response is activated to inhibit the growth of pathogens or to counteract their effect on host cells (Gravato-Nobre and Hodgkin, 2005). The inducible immune response shares some of the conserved signaling pathways and effector molecules of the innate immunity in flies and mammals (Sifri *et al.*, 2005). It has been shown that genes encoding for lysozyme and lipases are induced after infections towards the gram-negative bacteria *Serratia marcescens* (Mallo *et al.*, 2002). Other groups of effector molecules with antibacterial activity that have been identified, are defensin-like antimicrobial peptides (Kato *et al.*, 2002) and amoebapores (Banyai and Patthy, 1998). These conserved features of *C. elegans* make it a good model for certain stages of prokaryotic interactions with eukaryotic cells.

1.3.4 *C. elegans* a as model organism to determine bacterial virulence

Studies performed in *C. elegans* have shown that many genes known to be required for Gram-negative and Gram-positive mammalian pathogenesis are needed for pathogenesis in the nematode (Aballay and Ausubel, 2002; Diard *et al.*, 2007; Garsin *et al.*, 2001; Kurz *et al.*, 2003; Labrousse *et al.*, 2000; Sifri *et al.*, 2003; Tenor *et al.*, 2004). Infectivity and viability loss of *C. elegans* occur either by fast killing due to the production of toxic factors, which do not require any colonization of the nematode (Mahajan-Miklos *et al.*, 1999) or by slow killing that is mediated by the establishment of a persistent infection in the gut, or by forming a biofilm over the pharyngeal opening of the nematode, thereby inhibiting food uptake (Darby *et*

al., 2002). Several of the genes needed for full virulence of *Salmonella typhimurium* (*S. typhimurium*) in mammals, are also required for pathogenesis in *C. elegans* (Aballay *et al.*, 2000; Ernst *et al.*, 2001; Labrousse *et al.*, 2000; Tenor *et al.*, 2004). These include the genes *fur-1*, *ompR* and *rpoS* involved in acid tolerance, the PhoP/PhoQ signal transduction system regulating enzymes involved in LPS modification and resistance to the host antimicrobial peptides. Also the genes located in the pathogenicity island-1 (SPI-1), which encodes a type III secretory system and some effector proteins are needed for full virulence. This means that invasion and proliferation of *S. typhimurium* in the host intestine to some extent depend on mechanisms that are common to the nematode and mammals.

1.4 Translational inhibitors

1.4.1 Introduction

In *E. coli* growing at a high rate more than 50% of the cell mass consists out of ribosomes and associated factors (Noller and Nomura, 1996). This underlines the importance of the translational machinery for the cell and its potential as a target for antibiotics; it is therefore not surprising that half of the known antibiotics interact with the ribosome or its associated factors (Wirmer and Westhof, 2006).

1.4.2 Translational inhibitors

Translational inhibitors mostly target the rRNA-rich surfaces of the ribosomal 30S and 50S subunits (Poehlsgaard and Douthwaite, 2005). The antibiotics binding to the 30S subunit are mostly interfering with the decoding of the incoming mRNAs (Yonath, 2005). Function of the 50S subunit, is inhibited by antibiotics directed towards three major functions: GTP hydrolysis, formation of peptide bonds and the channelling of peptides through the subunit tunnel (Poehlsgaard and Douthwaite, 2005; Yonath, 2005). Besides their clinical importance, a lot of information on translation has been gained by studying the effects of antibiotics inhibiting different steps of the translational pathway. I will focus on two translational inhibitors, mupirocin and streptomycin, targeting different steps (aminoacylation and decoding of mRNA) of translation.

1.4.3 Isoleucyl–tRNA synthetase (IleRS)

The fidelity of the translational process is dependent on the accuracy of two processes: codon anticodon recognition at the ribosome and the aminoacyl-tRNAs synthesis (Ibba and Soll, 2000). There are 20 different aminoacyl-tRNA synthetases (aaRSs) (one for each aa), divided into two classes (I and II), comprised of ten members each. This sub-division is based on distinct signature motifs of the ATP binding domains' structural architecture (Eriani *et al.*, 1990; O'Donoghue and Luthey-Schulten, 2003). The class I synthetases that IleRS belongs to, have a catalytic domain displaying a ATP binding Rossman fold with two conserved aa signature motifs HIGH and KMSKS. For a majority of aaRSs the first step (Figure 5 A) is to "activate" the aa by forming an aminonacyl adenylate (aa-AMP).

In the second step (Figure 5 B) aa-AMP is loaded on to the 3´end of the tRNA molecule forming an aminonacyl-tRNA complex (aa-tRNA).

$$aaRS + aa + ATP \rightarrow aaRS \cdot aa\text{-}AMP + PP_i \quad (A)$$

$$aaRS \cdot aa\text{-}AMP + tRNA \rightarrow IleRS + aa\text{-}tRNA \quad (B)$$

Figure 5: The two steps (A and B) of the aminoacylation reaction.

The charging of the aa with high specificity to its cognate tRNA is crucial for the fidelity of translation. Aminoacyl-tRNA synthetases can separate aa with small differences in the structure such as isoleucine and valine differing from each other with only a methyl group. Two different pathways of pre and post transfer editing are utilised to achieve fidelity, where the pre transfer-editing pathway is responsible for 70-90% of all editing processes (Fukunaga *et al.*, 2004; Fukunaga and Yokoyama, 2006; Hendrickson *et al.*, 2002; Schmidt and Schimmel, 1994). The pre and post transfer editing sites are located in the structural domain connective polypeptide 1 (CP1), a large insertion in the active site of the Rossman fold domain (Schmidt and Schimmel, 1995). The close location of the two sub-sites suggests that they are overlapping (Fukunaga and Yokoyama, 2006). The importance of the editing process is highlighted for editing-deficient IleRS mutants, where severe inhibition of growth can be seen (Bacher *et al.*, 2005).

1.4.4 Ribosome

The ribosome is a large ribonucleoprotein complex, which consists of two major sub-units. In prokaryotes, these subunits are designated 30S and 50S, and together they make up the 70S ribosome. The 30S subunit is the smaller of the two subunits and is composed of the 16S RNA chain together with 20-21 different proteins of varying size. The larger 50S subunit consists of 31-35 different proteins together with the 5S and 23S RNA chains. The complex process of translation has been studied in great detail. For simplicity only elongation is summarized here.

The elongation of a polypeptide is a stepwise process. The ternary complex composed of the aa-tRNA together with elongation factor TU (EF-Tu) and GTP is delivered to the A (aminonacyl) site of the ribosome. At the A site, the codon-anticodon interaction between tRNA and mRNA takes place. This interaction is monitored at the decoding site of the 30S subunit interface with the end of the 16S rRNA helix 44. The cognate tRNA-mRNA interactions trigger the hydrolysis of the GTP molecule of the ternary complex and the aa-tRNA that is bound in the ribosomal decoding site. The 3´end of the tRNA molecule with the bound aa is made available for peptide bond formation in the peptidyl transferase center (PCT) of the 50S subunit. In the following step, the advance of the ribosome along the mRNA is mediated by elongation factor G (EF-G) in complex with GTP. At the end of translocation, deacylated tRNA is in the E (exit) site, peptidyl tRNA is in the P site, and EF-G-GPD dissociates from the ribosome. This cycle is repeated for the incorporation of each aa.

1.5 Mupirocin resistance

1.5.1 Mechanism of mupirocin inhibition

Mupirocin (pseudomonic acid A) is a topical antibiotic used for controlling methicillin resistant *S. aureus* (MRSA) and beta-haemolytic streptococci in skin infections (impetigo) and in the pre-surgery elimination of *S. aureus* nasal colonization (Casewell and Hill, 1987; Cookson, 1998; Edlich *et al.*, 2005; Mori *et al.*, 2005; Watanabe *et al.*, 2001). Mupirocin usage is restricted for topical applications, since in serum a rapid hydrolysis of the ester linkage between the nucleus and nonanoic acid side-chain (Figure 6A, I), forming an inactive monic acid molecule (Sutherland *et al.*, 1985). Mupirocin exerts a bacteriostatic effect by binding to the isoleucyl–tRNA synthetase (IleRS), inhibiting the two-step aminoacylation reaction that covalently links L-isoleucine (Ile) to its cognate tRNA (Figure 5), resulting in the disruption of protein synthesis (O'Donoghue and Luthey-Schulten, 2003; Schimmel and Soll, 1979). A more detailed description of mupirocins' mode of action comes from the tryptic digestion of IleRS when ATP or mupirocin is bound, together with data from the crystal structures of IleRS in complex with the antibiotic (Nakama *et al.*, 2001; Silvian *et al.*, 1999; Yanagisawa *et al.*, 1994). These experiments show that mupirocin acts as an analogue of Ile-AMP, blocking the binding of the activated aa to the catalytic cleft of the Rossmann fold domain of IleRS (section 1.4.3 for further details) (O'Donoghue and Luthey-Schulten, 2003).
The nonanoic acid tail (Figure 6A, I) is positioned along the conserved KMSKS motif stabilising the mupirocin molecule within IleRS (Hurdle *et al.*, 2005; Nakama *et al.*, 2001), explaining the reduced inhibitory capacity of monic acid after mupirocin has been hydrolysed. The nonanoic acid tails interaction is characteristic of mupirocin, and in contrast to the molecules other moieties do not bear any resemblance to Ile-AMP. The moieties C-12 to C-14 and C-17 (Figure 6A, III) that resemble the hydrophobic side chain of Ile are recognized by the IleRS Ile-specific pocket (Nakama *et al.*, 2001). The pyran ring and the C1 to C3 (Figure 6A, II) functionally mimic the interactions of the ribose and adenine ring of Ile-AMP (Figure 6B) (Nakama *et al.*, 2001).

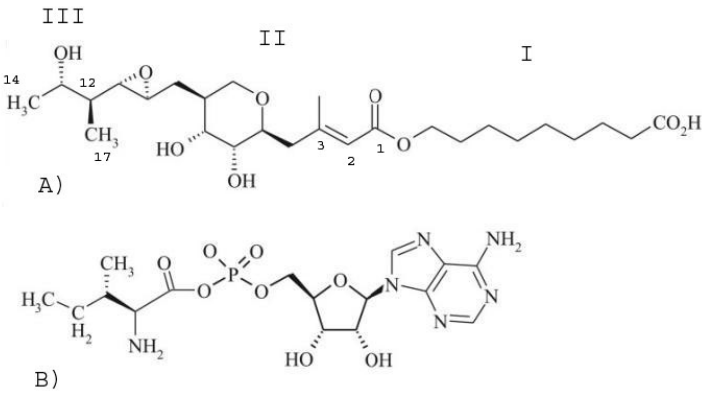

Figure 6: Structure of mupirocin (A) and isoleucyl adenylate (B): I) nonanoic acid tail, II) the pyran ring and the C1 to C3, III) C-12 to C-14 and C-17.

1.5.2 Mupirocin resistance mechanisms and fitness impact

Shortly after the introduction of mupirocin into clinical practice, high-level resistance (>700 µg/ml) could be observed (Rahman *et al.*, 1987). Resistance in *S. aureus* is defined at two levels, one high > 500 µg/ml and one low level 8-256 µg/ml (Antonio *et al.*, 2002; Gilbart *et al.*, 1993). High-level resistance is conferred by the uptake of the plasmid pOX301, encoding for the *mupA* gene that expresses a secondary isoleucyl-tRNA synthetase (IleRS2), which only shares 30% aa identity with the chromosomal *S. aureus ileS* (Hodgson *et al.*, 1994). No studies have been performed to assess the fitness cost of pOX301 uptake, and *mupA* expression, that potentially could interfere with the aminoacylation process (Schmidt and Schimmel, 1993).

The mechanism conferring low-level resistance in *S. aureus* was first characterized in *E. coli* (Yanagisawa *et al.*, 1994). A mutation located in close proximity to the conserved KMSKS region was shown to confer resistance. For clinical low-level resistant *S. aureus* isolates, two mutations located close to the KMSKS domain predominate (Antonio *et al.*, 2002) lowering the affinity for the antibiotic due to alterations in the conserved ATP binding site (Hurdle *et al.*, 2004). However, it seems like these low level resistant mutations in *S. aureus* confer no fitness cost (Hurdle *et al.*, 2004).

1.5.3 Clinical situation

Studies of the clinical resistance situation for mupirocin give a mixed picture (Lobbedez *et al.*, 2004; Watanabe *et al.*, 2001; Yun *et al.*, 2003). In the US the frequency of high-level resistant isolates ranges from 3% among coagulase negative Staphylococci to 0.3-0.4% in MRSA (Guay, 2003). However, these frequencies can vary considerably depending on the study. An investigation of the resistance pattern in South Korea showed that 11% of the isolated strains from long-term care facilities exhibited mupirocin resistance, where the distribution between high and low level resistance was approximately at a 50/50 ratio (Yoo *et al.*, 2006). Investigations from a burn unit collection where the mupirocin selective pressure is high, showed that 10% of the strains exhibit high-level mupirocin resistance (Guay, 2003). Low-level resistance isolates that for a long time were thought not to have an important role in treatment failures, because of the high mupirocin concentration at the site of application, have been reported to cause therapeutic and eradication failures (Decousser *et al.*, 2003). This is probably because of the low penetration ability of mupirocin into deeper skin layers and across intact skin (less than 0.24%).

Risk factors of resistance development are the long treatment periods that can be lasting for years, together with the routine decontamination regimes against glycopeptide intermediate sensitivity *S. aureus* (GISA) strains in many hospitals creating a strong selection pressure for resistance development.

1.6 Streptomycin resistance

1.6.1 Mechanism of streptomycin inhibition

Streptomycin (Figure 7) produced by *Actinomyces griseus* was discovered in 1944, it was the first antibiotic that could be used to treat *M. tuberculosis* infections (Schatz *et al.*, 1944).

Streptomycin acts in a concentration dependent manner on aerobically growing bacteria, where its uptake and inhibitory action proceeds in three consecutive steps (i-iii) (Taber *et al.*, 1987; Vakulenko and Mobashery, 2003).

(i) The first step is energy independent, where the positively charged streptomycin molecule binds to the negatively charged cell wall.

(ii) The second step requires a threshold transmembrane potential for an energy dependent small uptake of streptomycin across the cell wall (Bryan and Van Den Elzen, 1977). This results in streptomycin binding to the phosphate backbone of 16S rRNA at four places together with additional non-covalent interactions with the S12 protein (Carter *et al.*, 2000). The binding of streptomycin locks the ribosome in a ribosomal ambiguity (ram) like state due to restrictions of the 30S subunits conformational flexibility, thereby stabilising a partial domain closure. (Carter *et al.*, 2000; Gromadski and Rodnina, 2004). This mediates a suboptimal GTPase activation rate, facilitating both cognate and near-cognate aa-tRNA at the ribosomal A site to transmit a signal that triggers the hydrolysis of the GTP molecule by EF-Tu at the 50S subunit, causing a loss in selectivity and a dramatic increase in the ribosomal error rate (Gromadski and Rodnina, 2004; Ogle and Ramakrishnan, 2005).

(iii) The increased error rate in protein synthesis leads to the incorporation of misfolded proteins into the cell wall, conferrering a large energy dependent increase in streptomycin uptake due to the loss of membrane integrity. This large influx leads to saturation of all ribosomes, resulting in cell death.

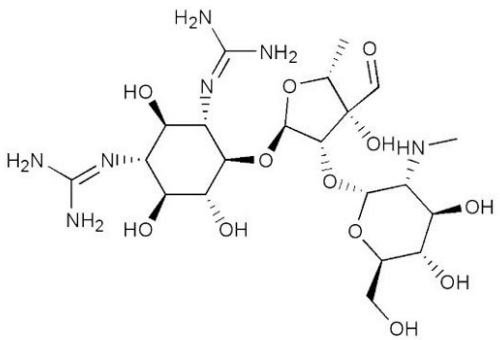

Figure 7: Structure of streptomycin

1.6.2 Resistance mechanisms

Resistance towards streptomycin was recorded only a few years after its clinical introduction (Klein and Kimmelman, 1946; Price *et al.*, 1947). Ribosomaly conferred resistance is mediated by mutations in *rpsL* (S12 protein) and/or in *rrn* (16S rRNA). Generally, resistance

does not give any reduction in streptomycin's binding affinity, instead it compensates for the effects of the ram like state by reducing conformational flexibility of the 30S subunit (Ogle and Ramakrishnan, 2005). The *rpsL* resistance mutations and the 16S rRNA mutations confer a restrictive phenotype in the absence of streptomycin. The restrictive phenotype is caused by the destabilisation of the ribosomal domain closure, creating an increased energy barrier to its formation (Ogle and Ramakrishnan, 2005). This results in a decrease of the GTP hydrolysis rate, due to the destabilisation of the interactions at the ribosomal A site (contacts between S12 and rRNA in the closed form), increasing the activation energy for achieving closed conformation and thereby lowering the translation rate.

There are however exceptions. The non-restrictive *rpsL* mutation K42R (*E. coli* numeration) confers resistance by disrupting streptomycin's affinity for the 30S subunit but it does not destabilise the ribosomal domain closure. The translation accuracy and rate thereby remain normal (Carter *et al.*, 2000). Non-ribosomal mechanisms can also mediated streptomycin resistance. Examples of this are efflux mechanisms and enzymatic modifications of streptomycin by aminoglycoside phosphotransferases and nucleotidyltransferases resulting in reduced ribosomal binding affinity for streptomycin (Llano-Sotelo *et al.*, 2002).

1.6.3 Compensatory mechanisms

Most of the streptomycin resistance mutations in 16S rRNA and S12 increase accuracy and decrease elongation rates, thereby resulting in a reduction in fitness. This reduction in fitness can however be ameliorated by second site compensatory mutations counteracting the effects of the resistance mutations. Compensatory mutations were first isolated as suppressors of streptomycin-dependent phenotypes. These mutations were referred to as ram mutations, due to the fact that when separated from the restrictive mutant they increased the read-through of nonsense codons, resulting in decreased translational accuracy (Rosset and Gorini, 1969). The first ram mutations were identified in small subunit proteins S4 and S5, after them mutations in the large subunit proteins L7/L12, 16S RNA and in EF-Tu have also been identified to confer a ram phenotype (Andersson *et al.*, 1982; Björkman *et al.*, 1999; Deusser *et al.*, 1970; Kirsebom and Isaksson, 1985; Rosset and Gorini, 1969; Zuurmond *et al.*, 1998). An investigation of how the phenotypic effects of a S12 restrictive mutant could be suppressed, revealed that 35 different aa substitutions at 23 different locations in the proteins S4, S5, S12 and L19 could restore fitness (Maisnier-Patin *et al.*, 2002).

The ram mutations act in an antagonistic way to the hyper accurate resistance mutations, restoring the ribosomal kinetics (accuracy and rate of translation) towards that of the sensitive strains (Andersson *et al.*, 1982; Kurland *et al.*, 1996; Rosset and Gorini, 1969). For the ram mutations in S4 and S5, this is achieved by disrupting the interface (a number of salt bridges) between the two proteins in the open form of the 30S subunit (A-site empty). This increases the probability of near-cognate tRNA to induce a transition from the open to the closed form of the 30S (domain closure), resulting in decreased accuracy that antagonistically compensates for the hyper-accuracy mutations. Most ram mutations act antagonistically to the restrictive resistance mutations destabilisation of the ribosomal domain closure, by decreasing the energy barrier for its formation.

The fitness effects of the ram mutations and the restrictive mutations have mostly been assessed in rich growth media. Environmental isolates have been shown to have a different ribosomal kinetic profile and on average lower growth rates than the laboratory derived strains (Mikkola and Kurland, 1992). When environmental *E. coli* isolates with varying growth rates were adapted for 280 generations in a glucose-limited chemostat, they all increased their

growth rates and their ribosomes became kinetically indistinguishable from the lab strains ribosomes (Mikkola and Kurland, 1992). The ribosomal kinetic profile and the mutations that are selected for are presumably optimised for growth in a rich growth environment, however it is still unclear if the kinetic profiles are optimal in other growth environments. Interestingly, ram mutants and wt strains have shown to induce higher levels of heat shock proteins than hyper accurate mutants, due to increased carbonylation of misfolded and inaccurate proteins, an effect that is further enhanced during starvation conditions, affecting the cells survival ability (Fredriksson *et al.*, 2006). This finding suggests that the selection on ribosomal phenotypes might be highly environment dependent.

1.6.4 Clinical situation

Today streptomycin is primarily used as a component of multidrug treatment regimes for *M. tuberculosis*, especially against multi-resistant isolates. Treatment should be directed against dividing cells in the induction phase since streptomycin does not penetrate the mammalian cells and is therefore not efficient towards intracellular bacteria (Di Perri and Bonora, 2004). Since no horizontal transfer of plasmids/transposons encoding for inactivating enzymes occurs in *M. tuberculosis*, the principal resistance mechanisms are mutational alterations in the ribosomal *rpsL* and *rrn* genes conferring high-level resistance. Clinically 47% of the isolates have single substitutions in *rpsL*: The most frequent *rpsL* mutation gives the substitution K42R that is found in 36% of the isolates followed by K87R that is found in 8% of the isolates (Hughes and Andersson, 2001). The resistance mutation K42R is especially interesting. At the corresponding position in *S. typhimurium* this mutation appears to confer no fitness cost in vivo (Björkman *et al.*, 1998), which could explain why it is so prevalent in *M. tuberculosis*. About 17% of the isolated lineages have single nucleotide substitutions in *rrn*. *M. tuberculosis* has only one copy of the 16S rRNA gene, allowing for the expression of the resistance phenotype due to a single mutation. This would not be possible in *E. coli*, considering that it has seven copies of the 16S rRNA gene, conferring that a single mutation has only a limited impact on resistance (Hughes and Andersson, 2001; Martinez and Baquero, 2000). For a third of the clinical isolates that have a low-level streptomycin resistance, no resistance mechanism had been identified until recently. But, it seems like acquisition of low-level streptomycin resistance is conferred by mutations in the rRNA methyltransferase *gidB* leading to a loss of 16S rRNA methylation (Okamoto *et al.*, 2007). Mutations in the *gidB* confer no fitness cost to the organism and are often found in clinical isolates in combination with *rpsL* and *rrn* in double and triple mutations, and it seems to be an underlying mechanism for high-level resistance development (Okamoto *et al.*, 2007). For bacterial species that can acquire DNA by horizontal uptake, the predominant resistance mechanism is conferred by enzymatic modifications of streptomycin by aminoglycoside phosphotransferases and nucleotidyltransferases carried on plasmids and transposomes (see section 1.2.3 for a further discussion of stability) (Hughes and Andersson, 2001; Sundin and Bender, 1996).

2 Present investigation

2.1 Aims

General aim

The general aim is to describe and understand the factors influencing the long-term persistence of antibiotic resistance at a molecular level and to convey these findings into clinical and antibiotic discovery related settings.

Specific aims

The specific aims with this thesis are to:

i) Determine how the resistance and compensatory mechanisms towards translational inhibitors affect the target proteins enzyme kinetics and cellular fitness.

ii) Develop and establish *C. elegans* as an in vivo model for assessing the fitness effects of resistance.

2.1 Results and discussion

The following presentations summarize the main findings of the individual papers included in this thesis. A more detailed methodological and graphic description of the results can be found in the original papers.

2.2.1 Paper I: *Caenorhabditis elegans* as a model to determine fitness of antibiotic-resistant *Salmonella enterica* serovar Typhimurium

In paper I, we wanted to establish the nematode *C. elegans* as a host model for determining the fitness effect of antibiotic resistance in *S. typhimurium*. The rational behind using *C. elegans* as a model, is that *S. typhimurium* colonizes the gut of the invertebrate causing a persistent infection. In combination with this, many genes known to be required for Gram-negative and Gram-positive mammalian pathogenesis are also needed for pathogenesis in the nematode (Sifri *et al.*, 2005). The need to assess the fitness impact of resistance mutations in vivo and not solely rely on in vitro results, is due to the discrepancies that have been observed between results obtained in vitro and in vivo models such as the mouse model of typhoid fever (Björkman *et al.*, 1998; Björkman *et al.*, 2000). This prompted us to investigate if *C. elegans* a low cost and simple host-model could be suitable for assessing the fitness effect of resistance mutations.

We wanted to compare how *S. typhimurium* fitness correlated between the *C. elegans* virulence model and the murine typhoid fever model (Björkman *et al.*, 1996). In the murine infection model, a total of 10^3-10^5 bacteria are injected intraperitoneally at a 1:1 (sensitive: resistant) ratio and recovered after 3 to 5 days by harvesting the liver and spleen of the mice. In *C. elegans* two different approaches were undertaken to determine the efficiency of the model. The first approach used was the plate-killing assay, which is the standard model for determining bacterial pathogenicity in *C. elegans*. The survival of the nematodes is monitored as a function of time, giving a TD50 value (time of death for 50% of the nematodes) for each infecting pathogen. The results obtained, showed that the streptomycin (JB127) and mupirocin (JB1855) resistant bacteria, were as virulent as the sensitive parental strain (JB124) (Figure 1, paper I). In contrast, the fusidic acid (JB393) and actinonin (DA8325) resistant mutants had a killing rate similar to the nonpathogenic *E. coli* OP50 (Figure 1, paper I), indicating a reduction in virulence. An explanation for the differences in virulence came from the infection dynamic experiments (Figure 2, paper I). When following the growth of bacteria in the nematode, a similar increase in bacterial number for the sensitive strain (JB124) as for the resistant strain JB127 was seen, which may explain the resistant strains' high level of virulence. The strain DA8325 on the other hand, did not increase in bacterial numbers indicating that it was unable to colonize the nematode, providing an explanation for its low virulence in this assay. This suggests that an impaired ability to colonize the nematode's digestive tract could explain the lower virulence of the resistant mutants. The inability to achieve a high resolution between strains in the plate killing assay, i.e. obtaining only two grouping variables "virulent" and "non virulent" lead us to develop a new infection model.

The newly developed model is a competition assay, where the nematodes were infected for several hours with a 1:1 mixture of the antibiotic-resistant and the wild-type strain, allowing the competing strains to colonize the nematode's gut. The infected worms were then placed onto a bacterial lawn of only *E. coli* OP50 (see 1.3.2 for more detailed information). Subsequent multiplication and establishment of the bacterial populations in the gut were not

affected by possible residual growth of the *Salmonella* strains on the agar plates, thereby only reflecting the growth of the bacteria inside the worm. While the measurements of the killing rate of the worms did not distinguish small changes in fitness, the competition assays could successfully assess the relative fitness of the different bacterial strains. We found that mutants resistant to streptomycin (*rpsL* mutation), rifampicin (*rpoB* mutation), nalidixic acid (*gyrA* mutation), fusidic acid (*fusA* mutation), mupirocin (*ileS* mutation) and actinonin (*folD* or *fmt* mutation) were less fit than the susceptible parental strain during growth in the worms (Figure 3, paper I). These findings corroborated the decreased fitness seen for antibiotic-resistant mutants when competed in the standard mouse model (Table 1, paper I). It is worth noting that for all resistant strains, relative fitness in the two host-models was lower compared to fitness measured in the Luria Bertani broth laboratory medium (Table 1, paper I).

In conclusion, our results show that the infectivity of the antibiotic-resistant mutants in the competition assay correlated well with their ability to multiply in mice. An example of this is provided by strain JB127, which in the plate-killing assay had a TD50 comparable to the susceptible strain, whereas in the competition assay it showed a significant 50% reduction in fitness that coincided with the results from the mice model. This raises the question of which *S. typhimurium* growth/virulence functions are required for colonization and multiplication in the intestine of *C. elegans* as well as for growth and virulence in mammals. The identification of virulence factors that are important in both mammals and *C. elegans* can plausibly be explained by conserved interactions between bacteria and eukaryotic cells, the innate immunity systems and/or similarities in the actual growth environment (e.g. nutrient levels). However, the lack of correlation between mice and *C. elegans* can be explained by the absence of professional phagocytic cells and the adaptive immune responses. It seems that the use of *C. elegans* is restricted to model certain stages between mammalian and bacterial interactions (Diard *et al.*, 2007). *C. elegans* should not be considered as a replacement of mice but as a supplement. The competition assays should be used for a first step screening of mutant library for virulence factors or resistance mutations, where selected candidates at a second stage are tested in a mammalian model system. A similar approach has been tried for antibiotic discovery. A large throughput screening with *Enterococcus faecalis* persistently infecting *C. elegans*, revealed several new compounds with a strict in vivo activity that otherwise by standard in vitro test methods would have been disregarded (Moy *et al.*, 2006). In addition, an enhanced in vivo effective dose, compared to the in vitro MIC levels could be observed for several compounds.

2.2.2 Paper II: Compensatory evolution reveals functional interactions between ribosomal proteins S12, L14 and L19

In paper II, we wanted to investigate how compensatory mutations in the 50S ribosomal subunit protein L19 affected translation and cellular fitness. The aa substitutions in L19 were identified when the ribosome was saturated with mutations compensating for the hyper accurate ribosomal phenotype of the streptomycin resistance mutation K42N in the ribosomal protein S12 (*rpsL*) (Maisnier-Patin *et al.*, 2002). No function had earlier been ascribed to L19. However, the breakthrough in the understanding of the role of L19 in translation was achieved with the high-resolution structures of the 30S and 50S structures (Ban *et al.*, 2000; Wimberly *et al.*, 2000) together with the structure of the 70S in complex with tRNA and mRNA (Selmer

et al., 2006), suggesting that the 16 S rRNA helix 44 extensive contacts with the 50S subunit, could possibly link L19 to the A-site of the S12 protein (see section 1.4.4).

To get a better understanding of L19s effect on translation and how that affects fitness, the following factors (i-iv) were investigated.
(i) In the *rpsL*^{R} background (in combination with the resistance mutation K42N), all four L19 mutations increased the fitness from 0.76 ($\underline{R}^{R1}$) to between 0.84-0.97 (Figure 2, $\underline{R}^{R1}\rightarrow\underline{RC}$, section 1.2.4) in relative fitness.
(ii) To be able to determine the specific effect of the compensatory mutations, the four L19 mutations were separated from the resistance mutation, by transferring them into a streptomycin sensitive *rpsL*^{wt} background (WT allele). The three L19 aa substitutions at position 40, decreased fitness with up to 25% in the *rpsL*^{wt} background (Figure 2, $\underline{RC}\rightarrow\underline{C}$, section 1.2.4), whereas the fourth L19 aa substitution G104A did not confer any deleterious fitness effect (Figure 1, paper II). Altered ribosomal kinetics, have been associated with a fitness decrease for mutations compensating for the restrictive effects of streptomycin resistance, such as the S4 and S5 ram mutants (see section 1.6.3 for further details).
(iii) To investigate the mechanism behind the fitness decrease, for the three aa substitutions at position 40 in L19, their perturbation of translation was tested. The in vivo elongation rates were determined by measuring the time required for β-galactosidase production after IPTG induction, for the sensitive, resistant, compensated (RC) and the three L19 aa substitutions at position 40 (C). The restrictive streptomycin resistant strain, showed a 24% decrease in elongation rate compared to the sensitive strain. The L19 substitutions Q40R and Q40H did not show any effect on translation rates, while the Q40L substitution increased the rate with 18% (Table 1, paper II). The L19 mutations in the *rpsL*^{R} background (RC) restored the relative elongation rates to between 0.9-1.1 of the sensitive strain. To determine the effect of the L19 mutations (C) on translation fidelity, the nonsense suppression level was measured. In contrast to the restrictive effect of the streptomycin resistance mutation, that decreased the nonsense suppression level (0.32), the L19 mutants increased nonsense suppression three to eight fold as compared to the wild type strain (Table 1, paper II). This is similar to the effect that has been seen for the S4 and S5 ram mutants
(iv) To model the L19 mutants' effect on ribosomal interactions, the X-ray crystal structures of the *E. coli* ribosome were used (Schuwirth *et al.*, 2005). Interactions between the 30S and 50S subunits are mostly of a RNA-RNA character. L19 is one of the few 50S subunit proteins that are in contact with the 30S subunit. More precisely the L19 residues 107-113 interact with the 16S rRNA helix 44 of the 30S subunit by forming a B6 inter subunit-bridge. This area of the 30S subunit has earlier been shown to have effects on translation, a mutation in a helix 44 residue participating in the formation of the B6 bridge, confers a hypo-accurate ribosomal phenotype (Fig 2, paper II). However, the L19 aa substitution G104A located close to the B6 bridge, did not confer any measurable effect on translation rate or accuracy (in the *rpsL*^{wt} background). The L19 aa Q40, is part of the B8 bridge formed with helix 14 (Fig 2, paper II). The aa changes at position 40 are believed to affect the interactions between helix 14 and/or 44 via the B8 bridge, causing dynamic conformational changes that modulate translation fidelity.

In conclusion, our results show that all four L19 mutations can compensate for the fitness effects of the restrictive K42N mutation in S12. The L19 compensatory mutations at aa position Q40, suppress this phenotype by increasing the ribosomal ambiguity and elongation rate. They thereby act antagonistically and cause a destabilisation of the ribosomal closed

form, resulting in a decreased energy barrier for its formation. The compensatory aa substitution G104A however, does not have any effect on translation rate and accuracy in a *rpsL*^{wt} background. One question is how G104A still can have an antagonistic effect on the restrictive streptomycin resistance mutation in the *rpsL*^{R} background? Lack of antagonism, has also been observed for compensatory mutations in *rspD* (S4), which when separated from the restrictive resistance mutation (K42N) confers streptomycin resistance and a hyper accurate phenotype, typically associated with the resistance mutation itself (Björkman *et al.*, 1999). These findings make the commonly accepted notion, about resistance and compensatory mutations acting antagonistically to restore a pseudo-wild-type-ribosomal phenotype, much more complex. This study shows how useful compensatory evolution can be for identifying novel protein functions and interactions in the ribonucleoprotein complex. The intersubunit bridges effect on the decoding step is a good example of the novel functions increasing the understanding of the tRNA movement relative to the two subunits.

2.2.3 Paper III: Multiple mechanisms to ameliorate the fitness burden of mupirocin resistance in *Salmonella typhimurium*

In paper III, we wanted to investigate the impact of mupirocin resistance on IleRS activity and cellular fitness and how a reduction in activity/fitness can be compensated for. The rational for looking at chromosomally encoded mupirocin resistance in *S. typhimurium*, when clinically most cases of treatment failure are associated with plasmid encoded resistance conferred by a novel *ileS* (*mupA*) in *S. aureus* (see sections 1.5.2-1.5.3 for more detailed information), were the following. First the investigation of the mutational resistance mechanism aimed to obtain a fuller understanding of how and why no high level chromosomal resistance had been isolated clinically, thereby making it possible to assess the risk for future resistance development. Second, because of the highly conserved nature of the synthetases the usage of the genetically more amenable bacteria *S. typhimurium*, instead of the clinically relevant *S. aureus*, we could extrapolate the results obtained with *S. typhimurium* to *S. aureus*.

We isolated four different mupirocin resistance mutations (W443R, H594Y, F596L and WV630-631L), located in the Ile-AMP/mupirocin binding pocket of the aminoacylation site, at similar locations to where *S. aureus* resistance mutations had earlier been identified (Figure 1, paper III). All four mutations conferred a mupirocin minimal inhibitory concentration (MIC) >1024 μg ml^{-1}. The long time required for the resistance mutants to appear upon isolation (4-8 days), was a result of the fitness reduction the resistance mutations conferred. The growth rates of the resistant strains in liquid growth medium {Luria-Bertani (LB)} ranged from 0.24-0.60 in relative fitness and between 0.13-0.24 in LB containing 100 μg ml^{-1} of mupirocin (Table 1, paper III). The resistance mutations H594Y and F596L had the smallest fitness impact, whereas the mutant W443R grew slowly in both LB and in LB + mupirocin (Figure 2, paper III). The substantially reduced growth rates in presence of sub MIC (>10x lower) of mupirocin, suggested that the resistance mutations had shifted the binding affinity from mupirocin towards Ile-AMP (compared to the sensitive IleRS) but not abolished it. However, the overall Ile-AMP affinity for the resistant compared to the sensitive IleRS had been lowered, resulting in decreased fitness also in the absence of mupirocin.

This led us to investigate if the fitness cost conferred by the resistance mutations could be reduced by compensatory evolution. Altogether fifty independent lineages from the four different resistant strains were serially passaged in the presence or absence of mupirocin (25

lineages under each condition) with a bottleneck of 10^6 cells. Compensated mutants generally appeared faster in LB than in LB + mupirocin (80-360 generations versus 260-520 generations). For the 25 lineages that compensated in LB, seven maintained the parental strains MIC (>1024 μg ml^{-1}), whereas 18 lineages showed a substantial reduction in MIC. All the 25 lineages that evolved in the presence of mupirocin, kept the parental strains MIC. When grown in absence of mupirocin a weak negative correlation between increased MIC and fitness could be seen, while with mupirocin present in the growth medium, a positive correlation between fitness and increased MIC could be detected (Figure 3, paper III). All 50 lineages, improved their fitness compared to the resistant parental strains. Fitness was however higher in LB compared to LB+ mupirocin (Figure 2, paper III).

The spectrum of compensatory mutations differed substantially between the two growth environments (absence or presence of mupirocin). In LB, 22/25 lineages compensated intragenically (i.e. in *ileS*). For the lineages that compensated with mupirocin present, 12/25 compensated intragenically, the other 3+13 lineages restored fitness by extragenic events. The intragenic compensatory mutations clustered into two locations on IleRS, the CP1 domain and the Rossmann fold domain (see 1.4.3 for further details). There was no difference in location of the compensatory mutations when comparing the two compensatory environments. However, the differences in ratio between extragenic/intragenic compensatory events in the different growth media probably reflected a difference in intragenic compensatory target size for the respective media. Thus, when mupirocin is present, only compensatory mutations that keep the affinity for mupirocin low, while restoring the aminoacylation rate will be selected for. In LB however, there is no selection pressure for maintaining a low mupirocin affinity (18/25 lineages lowered their MIC levels) only the restoration of aminoacylation rate is selected for, resulting in a larger mutational target that could potentially compensate for the resistance related fitness cost.

To assess the effect of the compensatory mutations on the aminoacylation reaction, in vitro aminoacylation kinetics were performed. In the aminoacylation reactions, tRNA or ATP concentrations were varied and the aminonacyl-tRNA formation was measured to determine the respective K_m and V_{max} values. The tRNA titration showed that the kinetic parameter that best correlated with the variation in fitness, was the overall catalytic rate $k_{cat\ (tRNA)}$. The saturating tRNA concentrations used for the in vitro aminoacylation reaction (4-8 μM) were lower than the $tRNA^{Ile}$ concentrations that are found intracellulary (11-25 μM). This implies that in vivo, IleRS is saturated with $tRNA^{Ile}$ and that only differences in $k_{cat\ (tRNA)}$ may affect the fitness of the strains. The ATP titrations showed that the resistance mutations lowered the ATP affinity $K_{m\ (ATP)}$ approximately twenty fold compared to sensitive IleRS, and that the compensatory mutations restored the ATP affinity to the level of the sensitive IleRS.

Extragenic compensation for the lowered catalytic rate $k_{cat\ (tRNA)}$ that is associated with the resistance mutations can be achieved either through amplification of the *ileS* gene (7/16 lineages) or by *ileS* promoter up mutations (6/16 lineages) (Paulander *et al*., unpublished data), both mechanisms result in increased expression levels of *ileS*. Kinetically, it is possible to compensate for the decreased aminoacylation rate by increasing the IleRS level, since the intracellular tRNA concentration is well above saturation level. The expression and amplification levels from the extragenically compensated mutants correlated well, with the exception for one strain where *ileS* expression levels were four times higher than the amplification levels. However, when sequencing the promoter region of that strain, one out of the two *ileS* copies contained a promoter mutation that could explain the increased expression levels (Paulander *et al*., unpublished data). The size of the amplicons has been determined to approximately 40-120 kb, implying that each chromosome contains ≈10% extra DNA

(Paulander *et al*., unpublished data). Although we don't yet have data on the fitness cost of carrying the amplified array, it is tempting to speculate that the *ileS* amplifications could be an intermediate stage before fixation of an intragenic compensatory mutation or a promoter up mutation.

In conclusion, we have shown that chromosomally conferred mupirocin resistance caused a large fitness cost that could be compensated for by three different mechanisms: intragenic compensatory mutations in the (i) Rossman fold domain and the (ii) CP1 domain of IleRS or by extragenic compensatory events causing (iii) increased gene expression through *ileS* amplifications or promoter up mutations. The in vitro aminoacylation kinetics showed that the fitness of the susceptible, resistant and compensated lineages correlated with the overall catalytic rate $k_{cat\ (tRNA)}$. However, considering that the growth rate measurements and the overall catalytic rate ($k_{cat\ (tRNA)}$) did not fully correlate, the receding difference can be explained with an effect on protein stability of the resistance and compensatory mutations (DePristo *et al.*, 2005) and/or indirect downstream effects on fitness. A potential indirect effect on fitness due to the lowered aminoacylation rate, could come from increased levels of deacetylated $tRNA^{Ile}$, activating the stringent factor RelA that via (p)ppGpp production indirectly controls *rpoS* expression levels (σ^S). The increased σ^S levels redirects the RNA polymerase from genes normally involved in exponential growth (ribosomal genes and stable RNAs), towards genes involved in maintenance and survival in the stationary phase, consequently reducing the growth rate.

The one step resistance mutations isolated in *Salmonella*, show the potential for clinical resistance development in *S. aureus* with an MIC level >100µg/ml (relating the resistance increase seen for *Salmonella* to *S. aureus*). So why have not resistant mutants with "intermediate" or "high" MIC levels been isolated? The most likely explanation is that the large fitness cost associated with the resistance mutations makes these strains unviable. However, the long incubation time required for the resistant mutants to appear on agar plates could also result in that they are missed during routine isolation procedures. As we have showed, the resistance related fitness cost could be compensated for by at least three different mechanisms. The relatively high compensatory mutation rate ($2x10^{-9}$- $6x10^{-9}$) indicates that compensation could happen within an infected individual. Clinically, circumstantial evidence for the occurrence of compensation has been detected in low-level resistant strains carrying up to four additional mutations in *ileS* besides the resistance mutation (Antonio *et al.*, 2002; Yang *et al.*, 2006). Interestingly, several of these additional mutations are located in the IleRS CP1 domain where we have identified a number of compensatory mutations. Considering that in the absence of mupirocin many compensatory mutations (18/23) lower the MIC levels, the clinical isolates with "additional" mutations in *ileS* could represent a mix of resistance mutations increasing the MIC level and compensatory mutations lowering the MIC level but restoring the aminoacylation rate.

Several interesting questions have arisen from this project and remain to be investigated. One of these questions is how the compensatory mutations in the CP1 editing domain can restore the aminoacylation rate. Potentially by answering this, the so far unresolved link between the aminoacylation and the editing reactions could be determined. Another interesting question that also remains to be answered is the determination of the fitness cost for carrying the *ileS* amplifications and if they function as an intermediate mechanism for fixation of compensatory mutations (intragenic or extragenic promoter mutations).

2.3 Concluding remarks

To be able to comprehend and predict the long-term persistence of antibiotic resistance, the evolutionary impact of the strong selective pressure that antibiotic usage confers needs to be investigated on the level of: (i) protein activity, (ii) the individual cell and (iii) the bacterial population. The evolutionary success of various resistance mechanisms is strongly related to their impact on the resistant organisms' reproductive ability. This ability is affected by several parameters, such as the relative rates at which susceptible and resistant organisms grow in their hosts and in the environment, and how this affects the clearance and transmission rates. Several of theses parameters can be and are in much need to be determined experimentally. This thesis has focused on determining the impact of resistance and compensatory mutations on in vitro and in vivo growth rates and to link these effects to alterations at the protein level so that a mechanistic understanding of the fitness effects can be obtained.

Many chromosomally encoded resistance mechanisms and horizontally acquired resistance determinants have been shown to confer a fitness cost. In the case of the chromosomally conferred resistance toward streptomycin and mupirocin (paper II and III), their fitness effect can be explained by aa substitutions either directly in the active site (IleRS Rossman fold) or indirectly by more distant structural changes affecting the active site (30S subunit A site). These disruptions of the active sites decrease the efficiency of important functions (the ribosomal translation and IleRS aminoacylation activities) therefore having a large impact on the cellular fitness. However, the resistance mutations impact on fitness can vary between different in vitro and in vivo environments (paper I). This is most likely due to secondary fitness effects of the resistance mutations on downstream cellular activities that are affected by the altered enzymatic efficiency of the "resistant proteins". An example of this is the fitness cost conferred by the fusidic acid resistant mutations in EF-G that directly effect translation but also perturbs the ppGpp accumulation. This is known to have downstream effects on *rpoS* expression levels and the expression levels of virulence and ribosomal genes that confer environment specific fitness effects. Thus, it is very important to test the fitness effect of resistance mutations in many different in vitro and in vivo environments to get an understanding of the resistance mechanisms' stability (long-term persistence). In an antibiotic-free environment where there is no selection for resistance, the stability of the resistance mechanism is inversely proportional to the cost of resistance. The stability is also affected by a selection for reduced fitness cost of the antibiotic resistance. It has been shown that compensatory mechanisms can counteract the deleterious fitness effects of the resistance determinants, thereby stabilising the resistance in the bacterial population (paper II and III). Under most circumstances, fitness is restored without any reduction in MIC levels. There are however exceptions, the mupirocin resistant strains that were compensated in the absence of the antibiotic 18/25 lineages showed decreased MIC levels (paper III). Further stabilisation of resistance in the bacterial population results from the epistatic interactions between the resistance and compensatory mutations, since a lost of the resistance mutation would confer a fitness cost (paper II).

The clinical implications of the resistance determinants fitness cost have been restricted so far to a few studies on the cycling between different antibiotics in hospital settings and on a national scale (described in 1.2.2). This concept is based on the idea that the fitness cost of the resistance determinants will cause a strong selection against the resistant strains after the selective pressure of the antibiotic is removed, causing them to be out-competed by sensitive

strains. If successful, such a strategy would make the withdrawn antibiotic effective for treatment again. However, the cycling between antibiotics in hospital settings is only possible if an alternative therapy exists, which is not the case for all pathogens. Preferably, the antibiotics subjected to the cycling, should also to as large extent possible be restricted to drugs that are only administrated clinically. The reason for this is that the basal resistance level that will be encountered in the hospital is a reflection of the level of resistance in the community. Any drop in resistance that is seen in the hospital, can to a large extent be attributed to a "dilution out effect", i.e. patients infected with resistant strains or carriers of strains resistant towards the current antibiotic therapy will leave the hospital. The new patients coming in will have a resistance level that corresponds to the resistance situation in the community.

The reasons why the antibiotic cycling have not been a successful approach, might be because of the stability of resistance in the absence of antibiotic selection in the community and in hospital settings, which is influenced by several factors. One being that many resistance determinants are often encoded on resistance cassette, suggesting that selection for one of them will in directly select for all of the resistance determinants present in that cassette (section 1.2.2, sulphonamide resistance in *E. coli*). The stability of resistance is also influenced by the selection for a reduction of the resistance-associated fitness cost, either by compensation or by selection of low or no cost resistance mutations. Thus, to maintain the efficacy of our existing and future antibiotics for as long as possible we need to use antibiotics in a prudent manner since the possibilities for reversion of resistance are highly improbable.

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4 References

(1998) Genome sequence of the nematode *C. elegans*: a platform for investigating biology. *Science* **282**: 2012-2018.

(2007) React, Action on antibiotic resistance http://www.react-group.org.

Aballay, A., Yorgey, P., and Ausubel, F.M. (2000) *Salmonella typhimurium* proliferates and establishes a persistent infection in the intestine of *Caenorhabditis elegans*. *Curr Biol* **10**: 1539-1542.

Aballay, A., and Ausubel, F.M. (2002) *Caenorhabditis elegans* as a host for the study of host-pathogen interactions. *Curr Opin Microbiol* **5**: 97-101.

Alanis, A.J. (2005) Resistance to antibiotics: are we in the post-antibiotic era? *Arch Med Res* **36**: 697-705.

Alekshun, M.N., and Levy, S.B. (2007) Molecular mechanisms of antibacterial multidrug resistance. *Cell* **128**: 1037-1050.

Andersson, D.I., Bohman, K., Isaksson, L.A., and Kurland, C.G. (1982) Translation rates and misreading characteristics of *rpsD* mutants in *Escherichia coli*. *Mol Gen Genet* **187**: 467-472.

Andersson, D.I., and Levin, B.R. (1999) The biological cost of antibiotic resistance. *Curr Opin Microbiol* **2**: 489-493.

Andersson, D.I. (2003) Persistence of antibiotic resistant bacteria. *Curr Opin Microbiol* **6**: 452-456.

Andersson, D.I. (2006) The biological cost of mutational antibiotic resistance: any practical conclusions? *Curr Opin Microbiol* **9**: 461-465.

Antonio, M., McFerran, N., and Pallen, M.J. (2002) Mutations affecting the Rossman fold of isoleucyl-tRNA synthetase are correlated with low-level mupirocin resistance in *Staphylococcus aureus*. *Antimicrob Agents Chemother* **46**: 438-442.

Arason, V.A., Gunnlaugsson, A., Sigurdsson, J.A., Erlendsdottir, H., Gudmundsson, S., and Kristinsson, K.G. (2002) Clonal spread of resistant pneumococci despite diminished antimicrobial use. *Microb Drug Resist* **8**: 187-192.

Austin, D.J., Kristinsson, K.G., and Anderson, R.M. (1999) The relationship between the volume of antimicrobial consumption in human communities and the frequency of resistance. *Proc Natl Acad Sci U S A* **96**: 1152-1156.

Avery, L., and Shtonda, B.B. (2003) Food transport in the *C. elegans* pharynx. *J Exp Biol* **206**: 2441-2457.

Bacher, J.M., de Crecy-Lagard, V., and Schimmel, P.R. (2005) Inhibited cell growth and protein functional changes from an editing-defective tRNA synthetase. *Proc Natl Acad Sci U S A* **102**: 1697-1701.

Ban, N., Nissen, P., Hansen, J., Moore, P.B., and Steitz, T.A. (2000) The complete atomic structure of the large ribosomal subunit at 2.4 A resolution. *Science* **289**: 905-920.

Banyai, L., and Patthy, L. (1998) Amoebapore homologs of *Caenorhabditis elegans*. *Biochim Biophys Acta* **1429**: 259-264.

Baquero, M.R., Nilsson, A.I., Turrientes Mdel, C., Sandvang, D., Galan, J.C., Martinez, J.L., Frimodt-Moller, N., Baquero, F., and Andersson, D.I. (2004) Polymorphic mutation frequencies in *Escherichia coli*: emergence of weak mutators in clinical isolates. *J Bacteriol* **186**: 5538-5542.

Björkholm, B., Sjolund, M., Falk, P.G., Berg, O.G., Engstrand, L., and Andersson, D.I. (2001) Mutation frequency and biological cost of antibiotic resistance in *Helicobacter pylori*. *Proc Natl Acad Sci U S A* **98**: 14607-14612.

Björkman, J., Rhen, M., and Anderson, D.I. (1996) *Salmonella typhimurium* cob mutants are not hyper-virulent. *FEMS Microbiol Lett* **139**: 121-126.

Björkman, J., Hughes, D., and Andersson, D.I. (1998) Virulence of antibiotic-resistant *Salmonella typhimurium*. *Proc Natl Acad Sci U S A* **95**: 3949-3953.

Björkman, J., Samuelsson, P., Andersson, D.I., and Hughes, D. (1999) Novel ribosomal mutations affecting translational accuracy, antibiotic resistance and virulence of *Salmonella typhimurium*. *Mol Microbiol* **31**: 53-58.

Björkman, J., and Andersson, D.I. (2000) The cost of antibiotic resistance from a bacterial perspective. *Drug Resist Updat* **3**: 237-245.

Björkman, J., Nagaev, I., Berg, O.G., Hughes, D., and Andersson, D.I. (2000) Effects of environment on compensatory mutations to ameliorate costs of antibiotic resistance. *Science* **287**: 1479-1482.

Boe, L., Danielsen, M., Knudsen, S., Petersen, J.B., Maymann, J., and Jensen, P.R. (2000) The frequency of mutators in populations of *Escherichia coli*. *Mutat Res* **448**: 47-55.

Bouma, J.E., and Lenski, R.E. (1988) Evolution of a bacteria/plasmid association. *Nature* **335**: 351-352.

Bozdogan, B., and Appelbaum, P.C. (2004) Oxazolidinones: activity, mode of action, and mechanism of resistance. *Int J Antimicrob Agents* **23**: 113-119.

Bronzwaer, S.L., Cars, O., Buchholz, U., Molstad, S., Goettsch, W., Veldhuijzen, I.K., Kool, J.L., Sprenger, M.J., and Degener, J.E. (2002) A European study on the relationship between antimicrobial use and antimicrobial resistance. *Emerg Infect Dis* **8**: 278-282.

Bryan, L.E., and Van Den Elzen, H.M. (1977) Effects of membrane-energy mutations and cations on streptomycin and gentamicin accumulation by bacteria: a model for entry of streptomycin and gentamicin in susceptible and resistant bacteria. *Antimicrob Agents Chemother* **12**: 163-177.

Bull, J., and Levin, B. (2000) Perspectives: microbiology. Mice are not furry petri dishes. *Science* **287**: 1409-1410.

Burman, L., G., and Olsson-Liljequist, B. (2001) A Global Perspective on Bacterial Infections, Antibiotic Usage, and the Antibiotic Resistance Problem. *In Antibiotic Development and Resistance*, Hughes, D. and Andersson, D, I. (eds) Tayllor and Francis, London and New York, pp 1-21.

Carter, A.P., Clemons, W.M., Brodersen, D.E., Morgan-Warren, R.J., Wimberly, B.T., and Ramakrishnan, V. (2000) Functional insights from the structure of the 30S ribosomal subunit and its interactions with antibiotics. *Nature* **407**: 340-348.

Casewell, M.W., and Hill, R.L. (1987) Mupirocin ('pseudomonic acid')--a promising new topical antimicrobial agent. *J Antimicrob Chemother* **19**: 1-5.

Coates, A., Hu, Y., Bax, R., and Page, C. (2002) The future challenges facing the development of new antimicrobial drugs. *Nat Rev Drug Discov* **1**: 895-910.

Cookson, B.D. (1998) The emergence of mupirocin resistance: a challenge to infection control and antibiotic prescribing practice. *J Antimicrob Chemother* **41**: 11-18.

Couillault, C., and Ewbank, J.J. (2002) Diverse bacteria are pathogens of *Caenorhabditis elegans*. *Infect Immun* **70**: 4705-4707.

D'Costa, V.M., McGrann, K.M., Hughes, D.W., and Wright, G.D. (2006) Sampling the antibiotic resistome. *Science* **311**: 374-377.

Dahlberg, C., and Chao, L. (2003) Amelioration of the cost of conjugative plasmid carriage in *Eschericha coli* K12. *Genetics* **165**: 1641-1649.

Darby, C., Hsu, J.W., Ghori, N., and Falkow, S. (2002) *Caenorhabditis elegans*: plague bacteria biofilm blocks food intake. *Nature* **417**: 243-244.

Datta, N., and Hughes, V.M. (1983) Plasmids of the same Inc groups in Enterobacteria before and after the medical use of antibiotics. *Nature* **306**: 616-617.

Davies, J. (1994) Inactivation of antibiotics and the dissemination of resistance genes. *Science* **264**: 375-382.

De Lencastre, H., and Tomasz, A. (2002) From ecological reservoir to disease: the nasopharynx, day-care centres and drug-resistant clones of *Streptococcus pneumoniae*. *J Antimicrob Chemother* **50 Suppl S2**: 75-81.

De Visser, J.A. (2002) The fate of microbial mutators. *Microbiology* **148**: 1247-1252.

Decousser, J.W., Pina, P., Ghnassia, J.C., Bedos, J.P., and Allouch, P.Y. (2003) First report of clinical and microbiological failure in the eradication of glycopeptide-intermediate methicillin-resistant *Staphylococcus aureus* carriage by mupirocin. *Eur J Clin Microbiol Infect Dis* **22**: 318-319.

Denamur, E., Bonacorsi, S., Giraud, A., Duriez, P., Hilali, F., Amorin, C., Bingen, E., Andremont, A., Picard, B., Taddei, F., and Matic, I. (2002) High frequency of mutator strains among human uropathogenic *Escherichia coli* isolates. *J Bacteriol* **184**: 605-609.

Denamur, E., and Matic, I. (2006) Evolution of mutation rates in bacteria. *Mol Microbiol* **60**: 820-827.

DePristo, M.A., Weinreich, D.M., and Hartl, D.L. (2005) Missense meanderings in sequence space: a biophysical view of protein evolution. *Nat Rev Genet* **6**: 678-687.

Deusser, E., Stoffler, G., and Wittmann, H.G. (1970) Ribosomal proteins. XVI. Altered S4 proteins in *Escherichia coli* revertants from streptomycin dependence to independence. *Mol Gen Genet* **109**: 298-302.

Di Perri, G., and Bonora, S. (2004) Which agents should we use for the treatment of multidrug-resistant *Mycobacterium tuberculosis*? *J Antimicrob Chemother* **54**: 593-602.

Diard, M., Baeriswyl, S., Clermont, O., Gouriou, S., Picard, B., Taddei, F., Denamur, E., and Matic, I. (2007) *Caenorhabditis elegans* as a simple model to study phenotypic and genetic virulence determinants of extraintestinal pathogenic *Escherichia coli*. *Microbes Infect* **9**: 214-223.

Edlich, R.F., Winters, K.L., Britt, L.D., and Long, W.B., 3rd (2005) Bacterial diseases of the skin. *J Long Term Eff Med Implants* **15**: 499-510.

Elena, S.F., Ekunwe, L., Hajela, N., Oden, S.A., and Lenski, R.E. (1998) Distribution of fitness effects caused by random insertion mutations in *Escherichia coli*. *Genetica* **102-103**: 349-358.

Enne, V.I., Livermore, D.M., Stephens, P., and Hall, L.M. (2001) Persistence of sulphonamide resistance in *Escherichia coli* in the UK despite national prescribing restriction. *Lancet* **357**: 1325-1328.

Enne, V.I., Delsol, A.A., Davis, G.R., Hayward, S.L., Roe, J.M., and Bennett, P.M. (2005) Assessment of the fitness impacts on *Escherichia coli* of acquisition of antibiotic resistance genes encoded by different types of genetic element. *J Antimicrob Chemother* **56**: 544-551.

Eriani, G., Delarue, M., Poch, O., Gangloff, J., and Moras, D. (1990) Partition of tRNA synthetases into two classes based on mutually exclusive sets of sequence motifs. *Nature* **347**: 203-206.

Ernst, R.K., Guina, T., and Miller, S.I. (2001) *Salmonella typhimurium* outer membrane remodeling: role in resistance to host innate immunity. *Microbes Infect* **3**: 1327-1334.

Ewbank, J.J. (2002) Tackling both sides of the host-pathogen equation with *Caenorhabditis elegans*. *Microbes Infect* **4**: 247-256.

Ferroni, A., and Berche, P. (2001) Alterations to penicillin-binding proteins 1A, 2B and 2X amongst penicillin-resistant clinical isolates of *Streptococcus pneumoniae* serotype 23F from the nasopharyngeal flora of children. *J Med Microbiol* **50**: 828-832.

Fredriksson, A., Ballesteros, M., Dukan, S., and Nystrom, T. (2006) Induction of the heat shock regulon in response to increased mistranslation requires oxidative modification of the malformed proteins. *Mol Microbiol* **59**: 350-359.

Fuda, C.C., Fisher, J.F., and Mobashery, S. (2005) Beta-lactam resistance in *Staphylococcus aureus*: the adaptive resistance of a plastic genome. *Cell Mol Life Sci* **62**: 2617-2633.

Fukunaga, R., Fukai, S., Ishitani, R., Nureki, O., and Yokoyama, S. (2004) Crystal structures of the CP1 domain from *Thermus thermophilus* isoleucyl-tRNA synthetase and its complex with L-valine. *J Biol Chem* **279**: 8396-8402.

Fukunaga, R., and Yokoyama, S. (2006) Structural basis for substrate recognition by the editing domain of isoleucyl-tRNA synthetase. *J Mol Biol* **359**: 901-912.

Gagneux, S., Long, C.D., Small, P.M., Van, T., Schoolnik, G.K., and Bohannan, B.J. (2006) The competitive cost of antibiotic resistance in *Mycobacterium tuberculosis*. *Science* **312**: 1944-1946.

Garsin, D.A., Sifri, C.D., Mylonakis, E., Qin, X., Singh, K.V., Murray, B.E., Calderwood, S.B., and Ausubel, F.M. (2001) A simple model host for identifying Gram-positive virulence factors. *Proc Natl Acad Sci U S A* **98**: 10892-10897.

Gerrish, P.J., and Lenski, R.E. (1998) The fate of competing beneficial mutations in an asexual population. *Genetica* **102-103**: 127-144.

Gilbart, J., Perry, C.R., and Slocombe, B. (1993) High-level mupirocin resistance in *Staphylococcus aureus*: evidence for two distinct isoleucyl-tRNA synthetases. *Antimicrob Agents Chemother* **37**: 32-38.

Giraud, A., Matic, I., Tenaillon, O., Clara, A., Radman, M., Fons, M., and Taddei, F. (2001a) Costs and benefits of high mutation rates: adaptive evolution of bacteria in the mouse gut. *Science* **291**: 2606-2608.

Giraud, A., Radman, M., Matic, I., and Taddei, F. (2001b) The rise and fall of mutator bacteria. *Curr Opin Microbiol* **4**: 582-585.

Giraud, A., Matic, I., Radman, M., Fons, M., and Taddei, F. (2002) Mutator bacteria as a risk factor in treatment of infectious diseases. *Antimicrob Agents Chemother* **46**: 863-865.

Gonzales, R.D., Schreckenberger, P.C., Graham, M.B., Kelkar, S., DenBesten, K., and Quinn, J.P. (2001) Infections due to vancomycin-resistant *Enterococcus faecium* resistant to linezolid. *Lancet* **357**: 1179.

Gravato-Nobre, M.J., and Hodgkin, J. (2005) *Caenorhabditis elegans* as a model for innate immunity to pathogens. *Cell Microbiol* **7**: 741-751.

Gromadski, K.B., and Rodnina, M.V. (2004) Streptomycin interferes with conformational coupling between codon recognition and GTPase activation on the ribosome. *Nat Struct Mol Biol* **11**: 316-322.

Guardabassi, L., Perichon, B., van Heijenoort, J., Blanot, D., and Courvalin, P. (2005) Glycopeptide resistance vanA operons in *Paenibacillus* strains isolated from soil. *Antimicrob Agents Chemother* **49**: 4227-4233.

Guay, D.R. (2003) Treatment of bacterial skin and skin structure infections. *Expert Opin Pharmacother* **4**: 1259-1275.

Gutmann, L., and Tomasz, A. (1982) Penicillin-resistant and penicillin-tolerant mutants of group A Streptococci. *Antimicrob Agents Chemother* **22**: 128-136.

Hakenbeck, R. (1999) beta-lactam-resistant *Streptococcus pneumoniae*: epidemiology and evolutionary mechanism. *Chemotherapy* **45**: 83-94.

Hakenbeck, R., Grebe, T., Zahner, D., and Stock, J.B. (1999) beta-lactam resistance in *Streptococcus pneumoniae*: penicillin-binding proteins and non-penicillin-binding proteins. *Mol Microbiol* **33**: 673-678.

Hendrickson, T.L., Nomanbhoy, T.K., de Crecy-Lagard, V., Fukai, S., Nureki, O., Yokoyama, S., and Schimmel, P. (2002) Mutational separation of two pathways for editing by a class I tRNA synthetase. *Mol Cell* **9**: 353-362.

Hodgson, J.E., Curnock, S.P., Dyke, K.G., Morris, R., Sylvester, D.R., and Gross, M.S. (1994) Molecular characterization of the gene encoding high-level mupirocin resistance in *Staphylococcus aureus* J2870. *Antimicrob Agents Chemother* **38**: 1205-1208.

Holloway, A.K., Palzkill, T., and Bull, J.J. (2007) Experimental evolution of gene duplicates in a bacterial plasmid model. *J Mol Evol* **64**: 215-222.

Hope, I., A. (1999) Background on *Caenorhabditis elegans,* Hope, I, A. (ed). *C.elegans a Practical Approach* . Oxford university Press, pp1-15.

Hughes, D., and Andersson, D.I. (2001) Target Mutations Mediating Antibiotic Resistance. *In Antibiotic Development and Resistance,* Hughes, D. and Andersson, D, I. (eds). Tayllor and Francis, London and New York, pp 23-37.

Hughes, J., and Mellows, G. (1978) Inhibition of isoleucyl-transfer ribonucleic acid synthetase in *Escherichia coli* by pseudomonic acid. *Biochem J* **176**: 305-318.

Hughes, V.M., and Datta, N. (1983) Conjugative plasmids in bacteria of the 'pre-antibiotic' era. *Nature* **302**: 725-726.

Hurdle, J.G., O'Neill, A.J., Ingham, E., Fishwick, C., and Chopra, I. (2004) Analysis of mupirocin resistance and fitness in *Staphylococcus aureus* by molecular genetic and structural modeling techniques. *Antimicrob Agents Chemother* **48**: 4366-4376.

Hurdle, J.G., O'Neill, A.J., and Chopra, I. (2005) Prospects for aminoacyl-tRNA synthetase inhibitors as new antimicrobial agents. *Antimicrob Agents Chemother* **49**: 4821-4833.

Ibba, M., and Soll, D. (2000) Aminoacyl-tRNA synthesis. *Annu Rev Biochem* **69**: 617-650.

Kato, Y., Aizawa, T., Hoshino, H., Kawano, K., Nitta, K., and Zhang, H. (2002) abf-1 and abf-2, ASABF-type antimicrobial peptide genes in *Caenorhabditis elegans*. *Biochem J* **361**: 221-230.

Kirsebom, L.A., and Isaksson, L.A. (1985) Involvement of ribosomal protein L7/L12 in control of translational accuracy. *Proc Natl Acad Sci U S A* **82**: 717-721.

Klein, M., and Kimmelman, L.J. (1946) The Role of Spontaneous Variants in the Acquisition of Streptomycin Resistance by the Shigellae. *J Bacteriol* **52**: 471-479.

Kristinsson, K.G. (1997) Effect of antimicrobial use and other risk factors on antimicrobial resistance in pneumococci. *Microb Drug Resist* **3**: 117-123.

Kurland, C.G., Hughes, D., and Ehrenberg, M. (1996) Limitations of translational accuracy. . *Escherichia coli and Salmonella Cellular and Molecular Biology, Neidhardt, F C. (ED)* 1,Neidhardt, F C. (EDS), Washington, DC: American Society for Microbiology Press: 979-1004.

Kurz, C.L., Chauvet, S., Andres, E., Aurouze, M., Vallet, I., Michel, G.P., Uh, M., Celli, J., Filloux, A., De Bentzmann, S., Steinmetz, I., Hoffmann, J.A., Finlay, B.B., Gorvel, J.P., Ferrandon, D., and Ewbank, J.J. (2003) Virulence factors of the human opportunistic pathogen *Serratia marcescens* identified by in vivo screening. *Embo J* **22**: 1451-1460.

Labrousse, A., Chauvet, S., Couillault, C., Kurz, C.L., and Ewbank, J.J. (2000) *Caenorhabditis elegans* is a model host for *Salmonella typhimurium*. *Curr Biol* **10**: 1543-1545.

LeClerc, J.E., Li, B., Payne, W.L., and Cebula, T.A. (1996) High mutation frequencies among *Escherichia coli* and *Salmonella pathogens*. *Science* **274**: 1208-1211.

Lenski, R.E. (1998) Bacterial evolution and the cost of antibiotic resistance. *Int Microbiol* **1**: 265-270.

Levin, B.R., Perrot, V., and Walker, N. (2000) Compensatory mutations, antibiotic resistance and the population genetics of adaptive evolution in bacteria. *Genetics* **154**: 985-997.

Levy, S.B., and Marshall, B. (2004) Antibacterial resistance worldwide: causes, challenges and responses. *Nat Med* **10**: S122-129.

Licht, T.R., and Wilcks, A. (2006) Conjugative gene transfer in the gastrointestinal environment. *Adv Appl Microbiol* **58**: 77-95.

Ligon, B.L. (2004) Sir Alexander Fleming: Scottish researcher who discovered penicillin. *Semin Pediatr Infect Dis* **15**: 58-64.

Llano-Sotelo, B., Azucena, E.F., Jr., Kotra, L.P., Mobashery, S., and Chow, C.S. (2002) Aminoglycosides modified by resistance enzymes display diminished binding to the bacterial ribosomal aminoacyl-tRNA site. *Chem Biol* **9**: 455-463.

Lobbedez, T., Gardam, M., Dedier, H., Burdzy, D., Chu, M., Izatt, S., Bargman, J.M., Jassal, S.V., Vas, S., Brunton, J., and Oreopoulos, D.G. (2004) Routine use of mupirocin at the peritoneal catheter exit site and mupirocin resistance: still low after 7 years. *Nephrol Dial Transplant* **19**: 3140-3143.

Macris, M.H., Hartman, N., Murray, B., Klein, R.F., Roberts, R.B., Kaplan, E.L., Horn, D., and Zabriskie, J.B. (1998) Studies of the continuing susceptibility of group A streptococcal strains to penicillin during eight decades. *Pediatr Infect Dis J* **17**: 377-381.

Mahajan-Miklos, S., Tan, M.W., Rahme, L.G., and Ausubel, F.M. (1999) Molecular mechanisms of bacterial virulence elucidated using a *Pseudomonas aeruginosa-Caenorhabditis elegans* pathogenesis model. *Cell* **96**: 47-56.

Maisnier-Patin, S., Berg, O.G., Liljas, L., and Andersson, D.I. (2002) Compensatory adaptation to the deleterious effect of antibiotic resistance in *Salmonella typhimurium*. *Mol Microbiol* **46**: 355-366.

Maisnier-Patin, S., and Andersson, D.I. (2004) Adaptation to the deleterious effects of antimicrobial drug resistance mutations by compensatory evolution. *Res Microbiol* **155**: 360-369.

Majiduddin, F.K., and Palzkill, T. (2003) An analysis of why highly similar enzymes evolve differently. *Genetics* **163**: 457-466.

Mallo, G.V., Kurz, C.L., Couillault, C., Pujol, N., Granjeaud, S., Kohara, Y., and Ewbank, J.J. (2002) Inducible antibacterial defense system in *C. elegans*. *Curr Biol* **12**: 1209-1214.

Martinez, J.L., and Baquero, F. (2000) Mutation frequencies and antibiotic resistance. *Antimicrob Agents Chemother* **44**: 1771-1777.

Matic, I., Radman, M., Taddei, F., Picard, B., Doit, C., Bingen, E., Denamur, E., and Elion, J. (1997) Highly variable mutation rates in commensal and pathogenic *Escherichia coli*. *Science* **277**: 1833-1834.

Mazel, D. (2001) Bacterial Genetics and Antibiotic Resistance Dissemination. *In Antibiotic Development and Resistance,* Hughes, D. and Andersson, D, I. (eds). Tayllor and Francis, London and New York, pp 91-105.

Mikkola, R., and Kurland, C.G. (1992) Selection of laboratory wild-type phenotype from natural isolates of *Escherichia coli* in chemostats. *Mol Biol Evol* **9**: 394-402.

Mori, N., Hitomi, S., Nakajima, J., Okuzumi, K., Murakami, A., and Kimura, S. (2005) Unselective use of intranasal mupirocin ointment for controlling propagation of methicillin-resistant *Staphylococcus aureus* in a thoracic surgery ward. *J Infect Chemother* **11**: 231-233.

Mouz, N., Gordon, E., Di Guilmi, A.M., Petit, I., Petillot, Y., Dupont, Y., Hakenbeck, R., Vernet, T., and Dideberg, O. (1998) Identification of a structural determinant for resistance to beta-lactam antibiotics in Gram-positive bacteria. *Proc Natl Acad Sci U S A* **95**: 13403-13406.

Moy, T.I., Ball, A.R., Anklesaria, Z., Casadei, G., Lewis, K., and Ausubel, F.M. (2006) Identification of novel antimicrobials using a live-animal infection model. *Proc Natl Acad Sci U S A* **103**: 10414-10419.

Nagaev, I., Bjorkman, J., Andersson, D.I., and Hughes, D. (2001) Biological cost and compensatory evolution in fusidic acid-resistant *Staphylococcus aureus*. *Mol Microbiol* **40**: 433-439.

Nakama, T., Nureki, O., and Yokoyama, S. (2001) Structural basis for the recognition of isoleucyl-adenylate and an antibiotic, mupirocin, by isoleucyl-tRNA synthetase. *J Biol Chem* **276**: 47387-47393.

Nathwani, D. (2003) Impact of methicillin-resistant *Staphylococcus aureus* infections on key health economic outcomes: does reducing the length of hospital stay matter? *J Antimicrob Chemother* **51 Suppl 2**: ii37-44.

Nilsson, A.I., Kugelberg, E., Berg, O.G., and Andersson, D.I. (2004) Experimental adaptation of *Salmonella typhimurium* to mice. *Genetics* **168**: 1119-1130.

Nilsson, A.I., Zorzet, A., Kanth, A., Dahlstrom, S., Berg, O.G., and Andersson, D.I. (2006) Reducing the fitness cost of antibiotic resistance by amplification of initiator tRNA genes. *Proc Natl Acad Sci U S A* **103**: 6976-6981.

Noller, H.F., and Nomura, M. (1996) Ribosomes. *Escherichia coli and Salmonella Cellular and molecular biology* , Neidhardt, F C. (ED) 1,Neidhardt**,** F C. (EDS), Washington, DC: American Society for Microbiology Pre**ss**: 167-186.

O'Donoghue, P., and Luthey-Schulten, Z. (2003) On the evolution of structure in aminoacyl-tRNA synthetases. *Microbiol Mol Biol Rev* **67**: 550-573.

O'Neill, A.J., Huovinen, T., Fishwick, C.W., and Chopra, I. (2006) Molecular genetic and structural modeling studies of *Staphylococcus aureus* RNA polymerase and the fitness of rifampin resistance genotypes in relation to clinical prevalence. *Antimicrob Agents Chemother* **50**: 298-309.

Ogle, J.M., and Ramakrishnan, V. (2005) Structural insights into translational fidelity. *Annu Rev Biochem* **74**: 129-177.

Okamoto, S., Tamaru, A., Nakajima, C., Nishimura, K., Tanaka, Y., Tokuyama, S., Suzuki, Y., and Ochi, K. (2007) Loss of a conserved 7-methylguanosine modification in 16S rRNA confers low-level streptomycin resistance in bacteria. *Mol Microbiol* **63**: 1096-1106.

Oliver, A., Canton, R., Campo, P., Baquero, F., and Blazquez, J. (2000) High frequency of hypermutable *Pseudomonas aeruginosa* in cystic fibrosis lung infection. *Science* **288**: 1251-1254.

Orlen, H., and Hughes, D. (2006) Weak mutators can drive the evolution of fluoroquinolone resistance in *Escherichia coli*. *Antimicrob Agents Chemother* **50**: 3454-3456.

Palumbi, S.R. (2001) Humans as the world's greatest evolutionary force. *Science* **293**: 1786-1790.

Paterson, D.L., and Bonomo, R.A. (2005) Extended-spectrum beta-lactamases: a clinical update. *Clin Microbiol Rev* **18**: 657-686.

Phelps, C.E. (1989) Bug/drug resistance. Sometimes less is more. *Med Care* **27**: 194-203.

Poehlsgaard, J., and Douthwaite, S. (2005) The bacterial ribosome as a target for antibiotics. *Nat Rev Microbiol* **3**: 870-881.

Poon, A., Davis, B.H., and Chao, L. (2005) The coupon collector and the suppressor mutation: estimating the number of compensatory mutations by maximum likelihood. *Genetics* **170**: 1323-1332.

Price, C.W., Randall, W.A., Chandler, V.L., and Reedy, R.J. (1947) Observations on the in Vivo and in Vitro Development of Bacterial Resistance to Streptomycin. *J Bacteriol* **53**: 481-488.

Rahman, M., Nobel, W.C., and Cookson, B.D. (1987) Mupirocin-resistant *Staphylococcus aureus*. *Lancet* **2**: 387-388.

Reynolds, M.G. (2000) Compensatory evolution in rifampin-resistant *Escherichia coli*. *Genetics* **156**: 1471-1481.

Rosset, R., and Gorini, L. (1969) A ribosomal ambiguity mutation. *J Mol Biol* **39**: 95-112.

Rozen, D.E., McGee, L., Levin, B.R., and Klugman, K.P. (2007) Fitness costs of fluoroquinolone resistance in *Streptococcus pneumoniae*. *Antimicrob Agents Chemother* **51**: 412-416.

Rubin, R.J., Harrington, C.A., Poon, A., Dietrich, K., Greene, J.A., and Moiduddin, A. (1999) The economic impact of *Staphylococcus aureus* infection in New York City hospitals. *Emerg Infect Dis* **5**: 9-17.

Salyers, A.A., Gupta, A., and Wang, Y. (2004) Human intestinal bacteria as reservoirs for antibiotic resistance genes. *Trends Microbiol* **12**: 412-416.

Schatz, A., Bugie, E., and Waksman, S.A. (1944) Streptomycin, a Substance Exhibiting Antibiotic Activity against Gram-Positive and Gram-Negative Bacteria. *Proc Soc Exp Bio Med* **55**: 244-248.

Schimmel, P.R., and Soll, D. (1979) Aminoacyl-tRNA synthetases: general features and recognition of transfer RNAs. *Annu Rev Biochem* **48**: 601-648.

Schmidt, E., and Schimmel, P. (1993) Dominant lethality by expression of a catalytically inactive class I tRNA synthetase. *Proc Natl Acad Sci U S A* **90**: 6919-6923.

Schmidt, E., and Schimmel, P. (1994) Mutational isolation of a sieve for editing in a transfer RNA synthetase. *Science* **264**: 265-267.

Schmidt, E., and Schimmel, P. (1995) Residues in a class I tRNA synthetase which determine selectivity of amino acid recognition in the context of tRNA. *Biochemistry* **34**: 11204-11210.

Schuwirth, B.S., Borovinskaya, M.A., Hau, C.W., Zhang, W., Vila-Sanjurjo, A., Holton, J.M., and Cate, J.H. (2005) Structures of the bacterial ribosome at 3.5 A resolution. *Science* **310**: 827-834.

Selmer, M., Dunham, C.M., Murphy, F.V.t., Weixlbaumer, A., Petry, S., Kelley, A.C., Weir, J.R., and Ramakrishnan, V. (2006) Structure of the 70S ribosome complexed with mRNA and tRNA. *Science* **313**: 1935-1942.

Seppala, H., Klaukka, T., Vuopio-Varkila, J., Muotiala, A., Helenius, H., Lager, K., and Huovinen, P. (1997) The effect of changes in the consumption of macrolide antibiotics on erythromycin resistance in group A streptococci in Finland. Finnish Study Group for Antimicrobial Resistance. *N Engl J Med* **337**: 441-446.

Sherman, D.R., Mdluli, K., Hickey, M.J., Arain, T.M., Morris, S.L., Barry, C.E., 3rd, and Stover, C.K. (1996) Compensatory *ahpC* gene expression in isoniazid-resistant *Mycobacterium tuberculosis*. *Science* **272**: 1641-1643.

Sifri, C.D., Begun, J., Ausubel, F.M., and Calderwood, S.B. (2003) *Caenorhabditis elegans* as a model host for *Staphylococcus aureus* pathogenesis. *Infect Immun* **71**: 2208-2217.

Sifri, C.D., Begun, J., and Ausubel, F.M. (2005) The worm has turned--microbial virulence modeled in *Caenorhabditis elegans*. *Trends Microbiol* **13**: 119-127.

Silvian, L.F., Wang, J., and Steitz, T.A. (1999) Insights into editing from an ile-tRNA synthetase structure with tRNAile and mupirocin. *Science* **285**: 1074-1077.

Sjölund, M., Wreiber, K., Andersson, D.I., Blaser, M.J., and Engstrand, L. (2003) Long-term persistence of resistant Enterococcus species after antibiotics to eradicate *Helicobacter pylori*. *Ann Intern Med* **139**: 483-487.

Smith, H.W. (1975) Persistence of tetracycline resistance in pig *E. coli*. *Nature* **258**: 628-630.

Sundin, G.W., and Bender, C.L. (1996) Dissemination of the *strA-strB* streptomycin-resistance genes among commensal and pathogenic bacteria from humans, animals, and plants. *Mol Ecol* **5**: 133-143.

Sutherland, R., Beale, A.S., Boon, R.J., Griffin, K.E., Slocombe, B., Stokes, D.H., and White, A.R. (1985) Antibacterial activity of ticarcillin in the presence of clavulanate potassium. *Am J Med* **79**: 13-24.

Taber, H.W., Mueller, J.P., Miller, P.F., and Arrow, A.S. (1987) Bacterial uptake of aminoglycoside antibiotics. *Microbiol Rev* **51**: 439-457.

Tenor, J.L., McCormick, B.A., Ausubel, F.M., and Aballay, A. (2004) *Caenorhabditis elegans*-based screen identifies Salmonella virulence factors required for conserved host-pathogen interactions. *Curr Biol* **14**: 1018-1024.

Vakulenko, S.B., and Mobashery, S. (2003) Versatility of aminoglycosides and prospects for their future. *Clin Microbiol Rev* **16**: 430-450.

van der Bruggen, J., T., Jansen, W., T, M., and Fluit, A., C. (2005) Resistance: a sensitive issue *Report from the Strategic council on Resistance in Europe (SCORE).*

Watanabe, H., Masaki, H., Asoh, N., Watanabe, K., Oishi, K., Furumoto, A., Kobayashi, S., Sato, A., and Nagatake, T. (2001) Emergence and spread of low-level mupirocin resistance in methicillin-resistant *Staphylococcus aureus* isolated from a community hospital in Japan. *J Hosp Infect* **47**: 294-300.

Wimberly, B.T., Brodersen, D.E., Clemons, W.M., Jr., Morgan-Warren, R.J., Carter, A.P., Vonrhein, C., Hartsch, T., and Ramakrishnan, V. (2000) Structure of the 30S ribosomal subunit. *Nature* **407**: 327-339.

Wirmer, J., and Westhof, E. (2006) Molecular contacts between antibiotics and the 30S ribosomal particle. *Methods Enzymol* **415**: 180-202.

Wright, G.D. (2007) The antibiotic resistome: the nexus of chemical and genetic diversity. *Nat Rev Microbiol* **5**: 175-186.

Yanagisawa, T., Lee, J.T., Wu, H.C., and Kawakami, M. (1994) Relationship of protein structure of isoleucyl-tRNA synthetase with pseudomonic acid resistance of *Escherichia coli*. A proposed mode of action of pseudomonic acid as an inhibitor of isoleucyl-tRNA synthetase. *J Biol Chem* **269**: 24304-24309.

Yanagisawa, T., and Kawakami, M. (2003) How does *Pseudomonas fluorescens* avoid suicide from its antibiotic pseudomonic acid?: Evidence for two evolutionarily distinct isoleucyl-tRNA synthetases conferring self-defense. *J Biol Chem* **278**: 25887-25894.

Yang, J.A., Park, D.W., Sohn, J.W., Yang, I.S., Kim, K.H., and Kim, M.J. (2006) Molecular analysis of isoleucyl-tRNA synthetase mutations in clinical isolates of methicillin-resistant *Staphylococcus aureus* with low-level mupirocin resistance. *J Korean Med Sci* **21**: 827-832.

Yonath, A. (2005) Antibiotics targeting ribosomes: resistance, selectivity, synergism and cellular regulation. *Annu Rev Biochem* **74**: 649-679.

Yoo, J.I., Shin, E.S., Cha, J.O., Lee, J.K., Jung, Y.H., Lee, K.M., Kim, B.S., and Lee, Y.S. (2006) Clonal dissemination and *mupA* gene polymorphism of mupirocin-resistant *Staphylococcus aureus* isolates from long-term-care facilities in South Korea. *Antimicrob Agents Chemother* **50**: 365-367.

Yun, H.J., Lee, S.W., Yoon, G.M., Kim, S.Y., Choi, S., Lee, Y.S., Choi, E.C., and Kim, S. (2003) Prevalence and mechanisms of low- and high-level mupirocin resistance in staphylococci isolated from a Korean hospital. *J Antimicrob Chemother* **51**: 619-623.

Zaidi, A.K., Huskins, W.C., Thaver, D., Bhutta, Z.A., Abbas, Z., and Goldmann, D.A. (2005) Hospital-acquired neonatal infections in developing countries. *Lancet* **365**: 1175-1188.

Zund, P., and Lebek, G. (1980) Generation time-prolonging R plasmids: correlation between increases in the generation time of *Escherichia coli* caused by R plasmids and their molecular size. *Plasmid* **3**: 65-69.

Zuurmond, A.M., Zeef, L.A., and Kraal, B. (1998) A kirromycin-resistant EF-Tu species reverses streptomycin dependence of *Escherichia coli* strains mutated in ribosomal protein S12. *Microbiology* **144 (Pt 12)**: 3309-3316.