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Development and Stability of Antibiotic Resistance

BY

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Abstract

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Antibiotic resistance is of current concern. Bacteria have become increasingly resistant to commonly used antibiotics and we are facing a growing resistance problem. The present thesis was aimed at studying the impact of antibiotic treatment on pathogenic bacteria as well as on the normal human microbiota, with focus on resistance development.

Among the factors that affect the appearance of acquired antibiotic resistance, the mutation frequency and biological cost of resistance are of special importance. Our work shows that the mutation frequency in clinical isolates of *Helicobacter pylori* was generally higher than for other studied bacteria such as *Enterobacteriaceae*; $\frac{1}{4}$ of the isolates displayed a mutation frequency higher than *Enterobacteriaceae* defective mismatch repair mutants and could be regarded as mutator strains.

In *H. pylori*, clarithromycin resistance confers a biological cost, as measured by decreased competitive ability of the resistant mutants in mice. In clinical isolates, this cost could be reduced, consistent with compensatory evolution stabilizing the presence of the resistant phenotype in the population. Thus, compensation is a clinically relevant phenomenon that can occur in vivo.

Furthermore, our results show that clinical use of antibiotics selects for stable resistance in the human microbiota. This is important for several reasons. First, many commensals occasionally can cause severe disease, even though they are part of the normal microbiota. Therefore, stably resistant populations increase the risk of unsuccessful treatment of such infections. Second, resistance in the normal microbiota might contribute to increased resistance development among pathogens by interspecies transfer of resistant determinants.

Keywords: antibiotic resistance, selection, mutation frequency, biological cost of resistance, compensatory evolution, *Helicobacter pylori*, normal microbiota

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IV. Sjölund M, Tano E, Blaser MJ, Andersson DI and Engstrand L, Fitness of antibiotic resistant *Staphylococcus epidermidis*. In manuscript.

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Abbreviations

AP-PCR	Arbitrary primed PCR
ATC-system	The Anatomical Therapeutic Chemical system
bp	Base pairs
CFU	Colony forming units
CI	Competition index
ClaS	Clarithromycin-susceptible
ClaR	Clarithromycin-resistant
CoNS	Coagulase-negative staphylococci
kb	Kilo base pairs
DDD	Defined daily dose
DNA	Deoxyribonucleic acid
FCS	Fetal calf serum
LB	Luria-Bertani
MIC	Minimal inhibitory concentration
MMR	Mismatch repair
MRSA	Methicillin-resistant <i>Staphylococcus aureus</i>
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
PBP	Penicillin binding protein
PBS	Phosphate buffered saline
RifR	Rifampicin-resistant
RNA	Ribonucleic acid
SmR	Streptomycin-resistant
VRE	Vancomycin-resistant enterococci

The Antibiotic Resistance Problem

Bacteria have become increasingly resistant to commonly used antibiotics, and we are facing a growing resistance problem (Finch 1998). Before the introduction of antibiotics, the hospital pathogens of major concern were *Staphylococcus aureus* and Group A-streptococci (Williams 2001). Today, however, the physicians are facing infections caused by resistant Gram-negatives, multi-resistant *S. aureus*, coagulase-negative staphylococci, pneumococci and enterococci (Baquero 1997; Williams 2001; Clark, Hershberger et al. 2003). Thus, infections that were readily cured by antibiotics in the past may today be difficult or impossible to treat. This threat has brought forward the concept of a “post-antimicrobial era”, in which some infections would no longer be susceptible to antibiotic therapy (Cohen 1992). Since antibiotics not only are important for treating specific infections, but also have a profound impact on many other aspects of medicine, such as oncology and transplantation surgery, the “post-antimicrobial era” represents a worst-case scenario similar to the pre-antibiotic era, when morbidity and mortality associated with infectious diseases were high.

Most reports suggest the emergence of resistant bacteria is the price we have to pay for an inappropriate use of antibiotics during the last decades, and that the overuse and misuse of antibiotics constitute the major force behind the appearance and spread of resistance (Levy 2001). However, development of antibiotic resistance is a multifaceted problem which is dependent on several factors (Barbosa and Levy 2000; Low 2001), including:

- (i) *The volume of drug use*
- (ii) *The rate of formation of resistant mutants*
- (iii) *The biological cost of resistance and to what extent compensatory evolution may act to reduce such a cost*

In the present thesis, each one of these factors will be addressed and their relevance in the development and spread of antibiotic resistance discussed.

THE INTRODUCTION OF ANTIBIOTICS

With the introduction of the sulphonamides in the 1930's, followed by penicillin in the 1940's, the antimicrobial era had begun. Considered one of the most important events in medical history, the discovery of antibiotics revolutionized the field of infectious diseases by giving the physicians the ability to prevent, cure and reduce transmission of certain diseases. Consequently, a significant reduction in morbidity and mortality associated with infectious disease had been achieved (Cohen 2000).

The success of penicillin in the 1940's led researchers to intensify the search for new antibiotics that could treat other bacterial diseases. Therefore, during the 1940's to the beginning of the 1970's, the development and production of antimicrobial compounds was very successful, resulting in several new classes of antibiotics. However, during the 1970's the production of antibiotics declined, and it took almost 30 years before a new class of antibiotics – the oxazolidinones (Norrby 2001; Moellering 2003) – was introduced on the market. Instead, during this period of time, the new antibiotics introduced primarily consisted of chemical modifications of already known compounds.

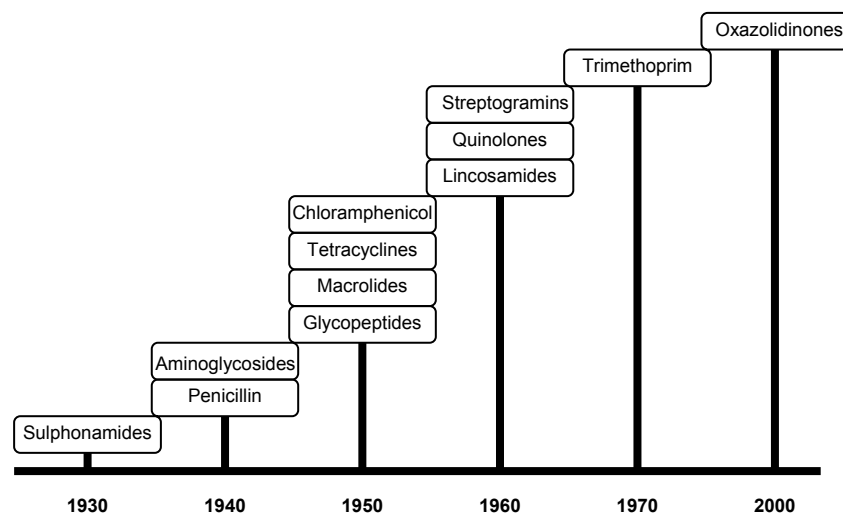


Figure 1. The introduction of different antibiotics. Adapted from Norrby and Cars, *Antibiotika- och Kemoterapi*, 2003 (Norrby 2003).

Mechanisms of Action of Antibiotics

Antibiotics can be classified in several ways. One common method of classification is by their mechanism of action against the infecting bacteria. Some antibiotics act by interfering with the synthesis of proteins and nucleic acids in the bacteria, while others attack the cell wall or disrupt the cell membrane (Green 2002). A clinically important group of antibiotics interferes with the synthesis of the peptidoglycan, the most important component of the cell wall. This group of antibiotics is called the β -lactams and can further be divided into the penicillins, cephalosporins, monobactams and carbapenems (Green 2002). Another large group of antibiotics inhibits the synthesis of various intracellular molecules, such as DNA, RNA, ribosomes and proteins. Examples of such antibiotics are rifampicin, which inhibits the RNA polymerase, and the quinolones (Blondeau 2004), which inhibit the enzymes responsible for coiling and uncoiling the DNA molecule, a process necessary for DNA replication and transcription. There are also other mechanisms; macrolides interfere with the 50S subunit of the ribosome, whereas tetracyclines affect the 30S ribosomal subunit, both inhibiting protein synthesis.

Table.1 Mechanisms of action of different antibiotic classes (Schmid 2001).

Antibiotic class	Molecular target	Examples
<i>β-lactams</i>	Cell wall; Penicillin binding proteins	Penicillins (benzylpenicillin, ampicillin, amoxycillin) Cephalosporins, (cefotaxime, ceftazidime) Carbapenems (imipenem, meropenem) Monobactams (aztreonam)
<i>Aminoglycosides</i>	30S, 50S ribosomal subunits	Gentamicin, tobramycin, amikacin, streptomycin
<i>Trimethoprim, sulphonamides</i>	Folate synthesis	Trimethoprim, sulfadiazine
<i>Quinolones</i>	Gyrase, Topoisomerase IV	Nalidixic acid, Fluoroquinolones (ciprofloxacin, norfloxacin, moxifloxacin)
<i>Macrolides</i>	50S ribosomal subunit	Erythromycin, clarithromycin, azithromycin

Table.1 continued.

Antibiotic class	Molecular target	Examples
<i>Lincosamides</i>	50S ribosomal subunit	Clindamycin
<i>Streptogramins</i>	50S ribosomal subunit	Quinopristin/Dalfopristin
<i>Tetracycline</i>	30S ribosomal subunit	Tetracycline, doxycycline
<i>Glycopeptides</i>	Cell wall peptidoglycan	Vancomycin, teicoplanin
<i>Chloramphenicol</i>	50S ribosomal subunit	Chloramphenicol
<i>Rifamycins</i>	RNA polymerase	Rifampicin
<i>Polymyxin</i>	Cell membrane	Polymyxin B, colistin
<i>Oxazolidinone</i>	50S ribosomal subunit	Linezolid

Antibiotics may also be classified as bactericidal or bacteriostatic (Stratton 2003). In general, antibiotics attacking the cell wall belong to the group of bactericidal drugs, since a defective cell wall eventually will cause the bacteria to lyse and die. Among the bacteriostatic antibiotics, macrolides, lincosamides and chloramphenicol can be mentioned. With bacteriostatic drugs, the host immune system plays an important role, helping to clear the infection once bacterial growth has subsided. For that reason, bactericidal drugs should be considered in patients that are immuno-compromised, as well as in patients with serious infections such as endocarditis and meningitis, in which cases a fast reduction of bacteria is warranted (Pankey and Sabbath 2004).

ANTIBIOTIC RESISTANCE

For many years antibiotics seemed to be winning the war against infectious disease. However, despite the successful development of several different antibiotic classes, the introduction of a new drug was almost always followed by resistance. Shortly after the introduction of penicillin, resistance was detected in *Staphylococcus aureus*, and by 1970 most *S. aureus* isolates were penicillin-resistant (Chambers 2001). In a similar manner, clinicians soon witnessed clinical failure of other antibiotics due to bacterial resistance development. For every decade to follow, bacteria resistant not only to single but multiple antibiotics have become more and more widespread (Tenover and Hughes 1996). Today, we are facing a problem of multi-resistant *Salmonella*, *Shigella*, *Campylobacter*, *Pseudomonas aeruginosa* and *Mycobacterium tuberculosis*, penicillin-resistant pneumococci, vancomycin-resistant enterococci and methicillin- and vancomycin-resistant *Staphylococcus aureus* (Hand 2000; Jones 2001; Lieberman 2003). At the same time fewer antibiotics are being produced, and it is becoming more and more apparent that a careful and prudent use of antibiotics is necessary in order to curtail the development of bacterial resistance (Levy 2001).

Mechanisms of Antibiotic Resistance

In basic terms, the increase in prevalence of antibiotic resistant bacteria during the last decades can be attributed to evolution and natural selection. All populations of organisms, including bacteria, will include variants with unusual traits, in this case the ability to withstand antibiotics. Consequently, every time a specific antibiotic is used, the antibiotic resistance trait will be positively selected, and the bacteria carrying this trait will increase in number and eventually predominate the population.

The bacterial traits of antibiotic resistance may be due to several different mechanisms (Normark and Normark 2002), including:

- (i) *a decreased uptake of the drug*
- (ii) *an increased export of the drug*
- (iii) *inactivation or modification of the drug target*
- (iv) *the introduction of a new drug resistant target*
- (v) *hydrolysis of the antibiotic*
- (vi) *modification of the drug*
- (vii) *prevention of activation of the drug*

How do the bacteria acquire these traits? First, antibiotic resistance traits are naturally occurring in the environment and have been so since long before antibiotics were introduced into human medicine (Davies 1994). One theory for the presence of antimicrobial resistance genes in the environment is that they originate from bacteria or fungi that use them as protection from antibiotics produced by other bacteria (Hawkey 2000). Another theory for the source of antibiotic resistance determinants is that certain housekeeping genes, such as sugar kinases and acetyltransferases, may have evolved to modify antibiotics, as in the case of aminoglycoside resistance (Davies 1994). Second, some bacteria are naturally resistant to certain antibiotics on account of their genetic composition. For example, *Mycoplasma* spp. is always resistant to β -lactams, since this species lacks the peptidoglycan in the cell wall. Bacteria carrying such resistant traits are designated to be *intrinsically resistant*, i.e. naturally resistant to an antibiotic without any genetic alterations. Finally, bacteria may be genetically altered to become resistant, a process called *acquired resistance* (Normark and Normark 2002).

Acquired Antibiotic Resistance

Bacteria can acquire resistance by either of two mechanisms;

- (i) *spontaneous mutation*
- (ii) *horizontal transfer*

Mutations may render the bacteria resistant by modifying the drug target (mutations in ribosomal proteins, penicillin-binding proteins etc.), by changing the uptake of the drug (mutations in a porin) or by inducing an increased efflux of the drug (mutations causing overexpression of efflux pumps) (Hooper 2001; Normark and Normark 2002).

Spontaneous mutation is dependent on the mutation rate and the presence of proofreading and repair mechanisms (Miller 1996). Some strains display an extremely high mutation rate and are called mutator strains. These bacteria are usually defective in the mismatch repair system or lack the ability of proofreading (Miller 1996; Bridges 2001). The role that mutator strains might play in generating and speeding up antibiotic resistance development is discussed further in the chapter “Rate of formation of resistant mutants”.

Horizontal transfer is a mechanism that allows bacteria to share genetic material and thereby maintain genetic diversity (Maiden 1998). It also constitutes the main mechanism for acquiring antibiotic resistance determinants. Essentially, three different processes are involved in horizontal gene transfer: conjugation, transduction and transformation (Rice 2000). Since these mechanisms can occur not only within the same but also within different species, horizontal transfer constitutes a major force behind the spread of resistance (Salyers and Amabile-Cuevas 1997; Maiden 1998). The manner

by which horizontal transfer renders bacteria resistant is primarily via the introduction of new antibiotic targets. This is commonly seen as the recruitment of new genes carried on plasmids or transposons. Thus, as opposed to spontaneous mutation, the resistance determinant is pre-existing in a reservoir and is not the direct result of antibiotic selection of mutants from within an entirely susceptible bacterial population.

A new antibiotic target could also be introduced by transformation of DNA and a subsequent recombination into the chromosome. An example of a resistance determinant that has originated from horizontal transfer and transformation, is the development of mosaic genes of penicillin-binding-proteins in *S. pneumoniae*, conferring penicillin resistance (Hakenbeck 1999). Few human pathogens have this ability; most other clinically important pathogens become penicillin-resistant due to the acquisition of genes encoding β -lactamases, which inactivate the β -lactams.

Table 2. Major bacterial pathogens and resistance patterns (Burman 2001).

Bacterial pathogen	Antibiotic resistance
<i>Escherichia coli</i>	β -lactam resistance due to plasmid-mediated β -lactamases, trimethoprim resistance, quinolone resistance due to mutations in <i>gyrA</i>
<i>Klebsiella</i>	β -lactam resistance due to plasmid-mediated β -lactamases
<i>Helicobacter pylori</i>	Macrolide and metronidazole resistance
Enterococci	Glycopeptide resistance, aminoglycoside resistance, quinolone resistance, penicillin/carbapenem resistance (mainly <i>E. faecium</i>)
<i>Staphylococcus aureus</i>	Methicillin resistance (MRSA) often combined with multidrug resistance (aminoglycosides, macrolides, tetracyclines)
<i>Streptococcus pneumoniae</i>	Penicillin resistance combined with multidrug resistance (tetracycline, macrolides, chloramphenicol)
<i>Streptococcus pyogenes</i>	Macrolide resistance, tetracycline resistance
<i>Haemophilus influenza</i>	β -lactam resistance mediated by β -lactamases or target modification
Mycobacteria	Multidrug resistance (rifampicin, isoniazid and others)

SELECTION OF ANTIBIOTIC RESISTANT BACTERIA

Several factors will have an impact on how rapidly resistance evolves in a bacterial population. Of special importance is the selective pressure associated with the volume of drug use and the rate by which bacteria may acquire and develop resistance.

The Volume of Drug Use

As previously mentioned, the increased antibiotic usage is thought to be one major force behind resistance development (Austin, Kristinsson et al. 1999). With few exceptions, resistance has evolved to all antibiotics after a couple of years of clinical use. The correlation between antibiotic use and increased resistance is well established and has been reported in several studies (Arason, Kristinsson et al. 1996; Granizo, Aguilar et al. 2000; Bronzwaer, Cars et al. 2002). For example, in Iceland, a strong correlation between antibiotic use in the community and carriage of penicillin-resistant pneumococci in children was described (Arason, Kristinsson et al. 1996), and in the Netherlands, an increased prescription of fluoroquinolones for urinary tract infections was associated with increased resistance to norfloxacin (Goettsch, van Pelt et al. 2000). The link between the use of antibiotics and the selection of resistance was further demonstrated by Asensio et al. who showed that the number of days patients received antibiotics was correlated to the risk of getting colonized or infected by methicillin-resistant *S. aureus* (MRSA) (Asensio, Guerrero et al. 1996).

Because of these observations, a decreased use of antibiotics has become one leading strategy in order to reduce and limit the further spread of resistance, although clinical evidence supporting the idea that a reduction in antibiotic use results in a reduced frequency of resistance is rather weak. Only two studies have provided support for the reversibility of antibiotic resistance: Seppälä et al. showed that a decrease in macrolide consumption in Finland led to a significant decrease in the number of macrolide resistant *Streptococcus pyogenes*, and in Iceland the incidence of penicillin-resistant pneumococci declined after a reduction of antibiotic use (Seppälä, Klaukka et al. 1997; Austin, Kristinsson et al. 1999). These data have, however, been challenged by the fact that other factors, such as clonal shifts towards more susceptibility in the bacterial population, might have caused the apparent correlation between reduced antibiotic use and decreased frequency of resistant strains (Andersson 2003). Nevertheless, it is generally agreed that the development of resistance is faster than the rate by which resistant isolates decline, and the volume of drug use is definitely an important parameter affecting the emergence of resistance.

The consumption of antibiotics varies between different countries (Cars, Mölstad et al. 2001). For example, in the European Union, the outpatient use in 1997 varied between 9 and 36.5 DDD/1000 inhabitants /day, and the countries with the highest and lowest consumption were France and the Netherlands, respectively. A relationship between antibiotic use and prevalence of resistant strains can be seen within the European Union where the amount of resistant strains follows a south to north gradient, with the southern European countries having a higher prevalence (Cars, Mölstad et al. 2001).

In Europe, antibiotic resistance development has been monitored by the European Antimicrobial Resistance Surveillance System (EARSS) since 1998, and attempts to reduce the use of antibiotics by minimizing inappropriate antibiotic use are being made in several countries (Cars, Mölstad et al. 2001; Ball, Baquero et al. 2002). A corresponding system called NARMS (National Antibiotic Resistance Monitoring System) is monitoring the emergence and spread of antibiotic resistant strains in the US and is run by the Centers for Disease Control and Prevention in Atlanta.

In order to be able to compare drug utilization data from different countries, the data need to be collected and presented in a standardized way. In 1996 the ATC/DDD system was introduced by the WHO and recommended as an international tool for presenting drug utilization statistics. In the Anatomical Therapeutic Chemical (ATC) system, drugs are divided into different groups according to their chemical, pharmacological and therapeutic properties. Thus, in the ATC system, all drugs will be given a specific code. In the DDD system, the defined daily dose (DDD) of all ATC-classified drugs is established. The defined daily dose is defined as the assumed average dose per day for a drug used for its main indication in adults and the drug consumption is often expressed as DDDs/1000 inhabitants per day or, when considering drug utilization in hospitals, DDDs per 100 bed days.

The Rate of Formation of Resistant Mutants

The rate by which spontaneous mutation and horizontal transfer render bacteria resistant will also affect the resistance development.

Mutations occur naturally in bacterial populations and can be found at a frequency of 10^{-9} – 10^{-12} per base pair replicated (Bridges 2001). In order to counteract the accumulation of mutations, bacteria have developed two main mechanisms that serve to correct mismatching nucleotides and increase the fidelity. First, the DNA-polymerase exhibits a proofreading activity, which involves a 3'- to 5' exonuclease effect that removes incorrectly paired bases in the DNA. Second, bacteria possess a post-replicative system called the mismatch-repair system (MMR), which recognizes the mismatched base, excises it from the newly synthesized strand and restores the sequence with the correct base (Miller 1996).

Some bacteria display an unusually high mutation rate due to the lack of a functional mismatch repair system. These bacteria are classified as mutator strains and may have up to a 1000-fold higher mutation rate than the wild type (Miller 1996). Such strains have been reported among natural isolates of several different pathogens: *Escherichia coli*, *Salmonella typhimurium*, *Pseudomonas aeruginosa* and *Helicobacter pylori* (see Paper I in the present thesis) (LeClerc, Li et al. 1996; Oliver, Canton et al. 2000; Björkholm, Sjölund et al. 2001). Since mutation plays a central role in the evolution of antibiotic resistance, the presence of mutators in a bacterial population could enhance the generation of resistance mutations (Giraud, Matic et al. 2002; Chopra, O'Neill et al. 2003; Miller, O'Neill et al. 2004). Evidence supporting this theory has been presented: *E. coli* mutators have been shown to develop resistance to rifampicin and ciprofloxacin up to 1000-fold faster than normal strains (Miller, O'Neill et al. 2002). It has further been shown that an *E. coli* *mutD* mutator carrying the β -lactamase TEM-1 easily can mutate to obtain the extended spectrum β -lactamase TEM-52 (Orencia, Yoon et al. 2001). This requires three successive mutations and indicates that an increased mutation rate could facilitate the emergence of resistance.

Thus, a correlation between a high bacterial mutation rate and faster resistance development has been established in laboratory media and in animal experiments (Tanabe, Kondo et al. 1999; Giraud, Matic et al. 2002; Schaaff, Reipert et al. 2002). However, whether resistance development is faster in a patient infected with a mutator strain is still unclear even though it has been addressed in a few studies. Oliver et al. showed that *P. aeruginosa* mutator strains isolated from cystic fibrosis patients were more resistant than wild type strains. Additionally, Komp-Lindgren et al. presented evidence that a high mutation rate in clinical isolates of *E. coli* strongly correlated with fluoroquinolone resistance (Oliver, Canton et al. 2000; Komp Lindgren, Karlsson et al. 2003). As part of the present thesis, we investigated whether an elevated mutation frequency was correlated to increased resistance development among commensal isolates of *E. coli*, enterococci, coagulase-negative staphylococci and α -streptococci (paper II) (Gustafsson, Sjölund et al. 2003). As opposed to the findings of Oliver et al., we did not detect a correlation between an increased mutation frequency and resistance development for four different classes of antibiotics (aminoglycosides, β -lactams, macrolides and trimethoprim-sulphamethoxazole). However, when comparing the mutation frequency of ciprofloxacin-resistant and -susceptible isolates a correlation was found for resistant *E. coli*. This is in accordance with the findings of Komp-Lindgren and indicates that a high mutation rate may play a significant role in generating resistance caused by point mutations.

Thus, mutator strains are more likely to accumulate mutations, like resistance mutations, but they are also more likely to go extinct because of acquired lethal mutations (Giraud, Radman et al. 2001). However, both theoretical and experimental data have shown that, under certain conditions, it

can be beneficial to be a mutator (Mao, Lane et al. 1997; Sniegowski, Gerish et al. 1997; Taddei, Matic et al. 1997; Taddei, Radman et al. 1997). Because of their high mutation rate, mutator strains can more easily adapt to changes in the environment (Tenaillon, Toupan et al. 1999). Thus, a mutator can reach a higher density in association with an adaptive mutation, given that the adaptive mutation is at a selective advantage (Arjan and de Visser 2002). Such hitchhiking of mutators could be one potential explanation to why mutators have been found so frequently among isolates of *P. aeruginosa*, *H. pylori*, *E. coli* and *S. typhimurium*.

The rate by which antibiotic resistance mutations are formed is further dependent on the target size, *i.e.* how many genes and base substitutions that can confer resistance (Martinez and Baquero 2000). For instance, in *E. coli* seven point mutations in the gene *gyrA* will result in fluoroquinolone resistance, whereas only three mutations in *parC* leads to resistance (Hooper 1999; Martinez and Baquero 2000). Consequently, the mutation rate for the *gyrA* gene will be higher. However, the overall mutation rate of fluoroquinolone resistance in *E. coli* is determined by the sum of all possible ways of achieving resistance.

Moreover, external conditions, such as exposure to toxic compounds, might affect the mutation rate to resistance. For instance, certain types of antibiotics (fluoroquinolones and aminoglycosides) have been shown to exhibit a mutagenic activity and may lead to increased resistance development (Ysern, Clerch et al. 1990; Ren, Rahman et al. 1999).

Horizontal gene transfer can mediate resistance by the exchange of plasmids, transposons or chromosomal DNA. Some bacteria are naturally transformable, *i.e.* can take up naked DNA very easily. Such bacteria can acquire resistance either by recruiting a new gene encoding an alternative, antibiotic-resistant target molecule, or by intragenic recombination between related genes that results in novel alleles that are mosaic genes and encode resistant proteins. The formation of resistant mosaic genes are of special importance for *S. pneumoniae* and *Neisseria* which have evolved penicillin resistance by mosaic genes encoding PBPs with low affinity for β -lactams (Hakenbeck 1999).

Transmission of Resistant Bacteria

Once resistance to an antibiotic has appeared in a bacterial population, the next question is whether the resistant bacteria will be able to survive and spread. The success of the bacteria will partly be determined by bacterial factors, that is, the bacteria's epidemic properties to colonize and be transmitted to new hosts. These bacterial properties are mainly dependent on the bacterial fitness and will be discussed further in the next chapter; Stability of resistant bacteria. Moreover, a number of non-bacterial factors may influ-

ence the dissemination of bacteria. For example, hospital hygiene and infection control measurements have played a crucial role in combating resistance and will continue to have a central role in preventing the spread of resistance in hospitals, especially in countries where multi-drug resistant bacteria such as MRSA and VRE are common (Pittet 2003).

STABILITY OF ANTIBIOTIC RESISTANT BACTERIA

The stability and maintenance of a resistant population is mainly determined by the fitness and transmission costs of resistance (Andersson and Levin 1999; Björkman and Andersson 2000). More specifically, this is dependent on the relative rates by which resistant and susceptible bacteria

- (i) *grow and die within and outside hosts*
- (ii) *are transmitted between hosts*
- (iii) *are cleared from infected hosts*

In order to predict the stability and persistence of antibiotic resistant bacteria, all these parameters would be very valuable to know. However, determining these parameters is in practice very difficult. Instead, we choose to measure the growth rate of the bacteria, in vitro and in vivo, which will reflect the rates of exponential growth of the bacteria, their resource utilization efficiencies and their mortality in the presence and absence of a host. By studying the growth rates of resistant bacteria and their competitive performance against susceptible bacteria, we can get a measure of the relative fitness of the resistant bacteria and thereby predict the stability and persistence of the resistant population (Andersson and Levin 1999).

There is no doubt that bacteria benefit from possessing an antibiotic resistance gene as long as the antibiotic is present. But what happens when the selective pressure of antibiotics is removed? If carrying a resistance gene confers a reduction in fitness of the bacteria in the absence of an antibiotic pressure, then one strategy to combat resistance development would be to stop using the antibiotic until the resistant bacteria declined to a low frequency. A number of studies have indeed presented evidence that resistant bacteria generally are less fit than their susceptible counterparts. This fitness reduction is commonly described as the biological cost of resistance and is considered as a key parameter in determining the rate of ascent and dissemination of antibiotic resistance.

The Biological Cost of Antibiotic Resistance

Data from a number of laboratory studies indicate that most resistance-conferring mutations are associated with a fitness cost in bacteria (Björkman, Samuelsson et al. 1999; Reynolds 2000; Björkholm, Sjölund et al. 2001; Nagaev, Björkman et al. 2001). This cost can be explained by the fact that the majority of resistance mutations occur in genes that have essential functions in the cell and may alter or impair the function of the target. For example, *rpoB* mutations in *E. coli* affect the rate of transcription (Reynolds 2000) and in a similar manner, mutations in the *fusA* gene in *S. typhimurium* (Björkman, Nagaev et al. 2000) and *S. aureus* (Nagaev, Björkman et al. 2001) or *rpsL* mutations in *E. coli* (Levin, Perrot et al. 2000) and *S. typhimurium* (Björkman, Nagaev et al. 2000) decrease the rate of translation, and as a consequence, the growth rate is reduced. Also, accessory elements carrying resistance genes may confer a cost, which can be related to the replication and maintenance of the elements themselves (Björkman and Andersson 2000).

Essentially, there are three ways of measuring the biological cost of resistance in bacteria:

- (i) *Retrospectively*, by studying the relationship between antibiotic use and resistance in hosts (Seppälä, Klaukka et al. 1997; Austin, Kristinsson et al. 1999)
- (ii) *Prospectively*, by measuring the rates at which humans become infected and are cleared of resistant and susceptible bacteria
- (iii) *Experimentally*, by estimating the relative rates of growth, survival, clearance and transmission of susceptible and resistant bacteria in vitro and in vivo. (Schrage, Perrot et al. 1997; Björkman, Hughes et al. 1998; Björkman, Samuelsson et al. 1999; Reynolds 2000; Björkholm, Sjölund et al. 2001; Gustafsson, Cars et al. 2003)

The majority of data indicating that antibiotic resistance is costly for the bacteria has been obtained experimentally, and is based on the relative rates of growth and competitive performance between antibiotic-susceptible and resistant bacteria. Björkman et al. showed that mutations in *rpsL*, *rpoB* and *gyrA* causing streptomycin, rifampicin and nalidixic acid resistance, respectively, all confer a cost in *S. typhimurium*, in vitro as well as in vivo (Björkman, Hughes et al. 1998). Cost-associated resistance mutations have further been described for *fusA* mutations causing fusidic acid resistance in *S. aureus* and for isoniazid-resistant *M. tuberculosis* (Li, Kelley et al. 1998).

The greatest limitation when studying the biological cost of resistance experimentally is how to interpret negative results, *i.e.* when no cost seems to

be associated with the resistance. If no cost can be detected during several different competition experiments, performed in vitro and in vivo, it is likely that the cost indeed is low or non-existent. It is, however, of great importance to perform the experiments both in vitro and in vivo since some mutations may engender a large cost in vitro but no cost in vivo and vice versa (Nagaev, Björkman et al. 2001).

In conclusion, many chromosomal resistance mutations engender a fitness burden on the bacteria. Accordingly, in the absence of an antibiotic pressure, resistant bacteria should be less competitive than their susceptible counterparts and decline to a low frequency, but that is not the case: bacteria can overcome the cost of resistance by evolving adaptations that restore the original fitness.

Compensatory Evolution

To avoid the cost associated with resistance, bacteria may adapt very fast to their newly acquired trait. The bacteria do this by genetic compensation, *i.e.* by introducing mutations that reduce the cost of maintaining the resistance gene/mutation (Levin, Perrot et al. 2000). This phenomenon has been described as compensatory evolution, and has been reported to occur in vitro (Schrag, Perrot et al. 1997; Björkman, Hughes et al. 1998; Björkman, Samuelsson et al. 1999; Levin, Perrot et al. 2000; Reynolds 2000), in laboratory animals (Björkman, Hughes et al. 1998; Nagaev, Björkman et al. 2001), and in humans (Sherman, Mdluli et al. 1996; Björkholm, Sjölund et al. 2001).

The mechanism behind compensation usually involves mutations in the active site of the target protein that will restore the efficiency of the protein back to a wild type level. The exact physiological mechanisms by which compensatory mutations restore fitness have been described in a few cases (Björkman, Samuelsson et al. 1999; Nagaev, Björkman et al. 2001). For example, the cost associated with *rpsL* mutations causing streptomycin resistance in *S. typhimurium*, could be compensated by extragenic mutations that restore the translation efficacy to wild type or nearly wild type levels (Björkman, Samuelsson et al. 1999; Nagaev, Björkman et al. 2001).

The degree to which compensation may restore the fitness varies greatly. Some mutations fully restore the fitness, whereas others only restore the fitness partially. Björkman et al. showed that the selective conditions might play an important role determining the type of compensatory mutation achieved. When selecting for compensated *rpsL* mutants in vivo, only intragenic mutations were detected, whereas selection in vitro only conferred extragenic suppressor mutations (Björkman, Hughes et al. 1998; Björkman, Samuelsson et al. 1999; Nagaev, Björkman et al. 2001).

To date, evidence for compensatory evolution occurring in bacteria isolated from humans has been presented for isoniazid-resistant *Mycobacterium*

tuberculosis (Sherman, Mdluli et al. 1996), fusidic acid-resistant *Staphylococcus aureus* (Nagaev, Björkman et al. 2001) and for clarithromycin-resistant *Helicobacter pylori* (Björkholm, Sjölund et al. 2001). To what extent compensation occurs in clinical settings and stabilizes resistant pathogens is of great medical interest. In the current thesis, we show that clinical compensation may occur for clarithromycin-resistant *Helicobacter pylori*. This was accomplished by isolating a susceptible pre-treatment strain and a resistant post-treatment strain that were clonally related. Thereafter, the fitness difference of the clinical pair was established and compared with the fitness divergence of a defined in vitro pair containing the same resistance mutation and for which the possibilities for compensation had been minimized. By showing that the fitness difference in the clinical pair was smaller than for the in vitro pair, we conclude that compensatory evolution had reduced the fitness cost (Björkholm, Sjölund et al. 2001).

An additional way of restoring the fitness is by true reversion of the resistance mutation. This mechanism, which requires a back mutation at the specific site of the resistance mutation, is not as likely to occur as compensatory mutations at other sites. This can be attributed to the higher rates of compensatory mutations relative to that of reversion, and to the presence of population bottlenecks, where the most frequent genotype is more likely to be transferred instead of the most fit (Levin, Perrot et al. 2000). Moreover, once a compensated mutant has evolved in a population, it is highly unlikely that the mutant will revert to a susceptible state. Schrag and colleagues showed that when a streptomycin-resistant *rpsL* allele was replaced by a wild type *rpsL*⁺ allele in evolved fitness-compensated *rpsL* strains, the resulting streptomycin-susceptible bacteria were less fit than wild type, uncompensated *rpsL* mutants or fitness-compensated mutants (Schrag, Perrot et al. 1997). In other words, genetic compensation establishes an adaptive valley, which makes it difficult to return to the uncompensated, streptomycin-susceptible genotype. This phenomenon is illustrated in Figure 2.

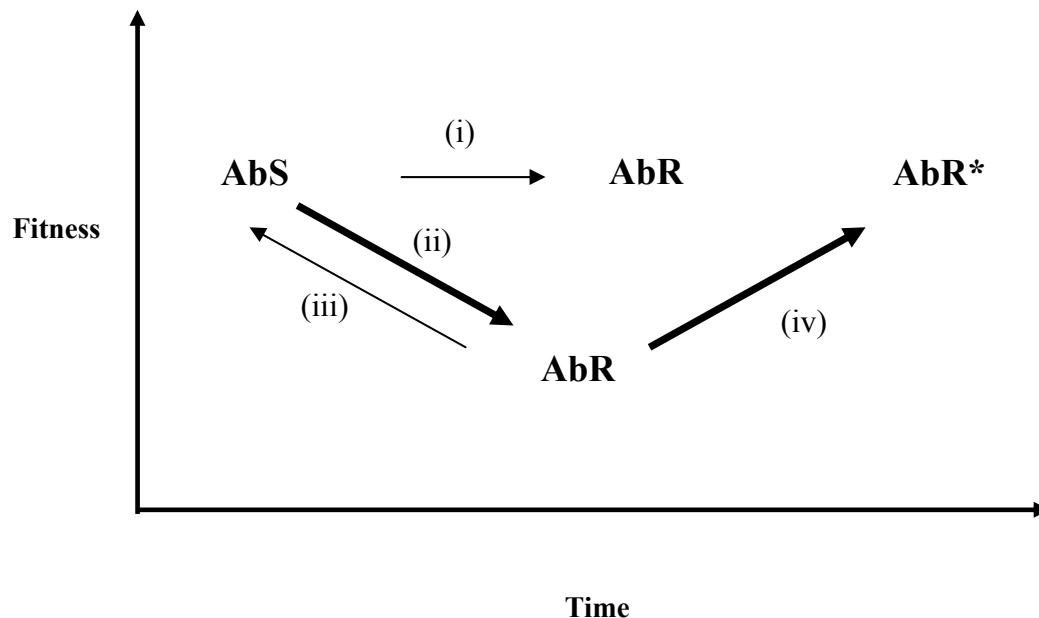


Figure 2. Stability of antibiotic resistant bacteria. (Modified after Björkman J. 2000, The biological cost of resistance). Antibiotic resistant mutants (AbR) can be as fit as (i) or less fit (ii) than the susceptible wildtype (AbS). Fitness may be restored by true reversion (iii) or by compensatory evolution (AbR*)(iv). Compensatory mutations are more likely to occur than true reversion.

FURTHER FACTORS INFLUENCING THE STABILITY OF ANTIBIOTIC RESISTANCE

While antibiotics appear to be the main force behind the selection of antibiotic resistant bacteria, they are not totally responsible for their persistence and spread. Once selected, there are several factors that will influence the stability and maintenance of resistant bacteria in the absence of antibiotics. One major mechanism has already been mentioned, compensatory evolution. Other important factors contributing to making resistance development an irreversible process are the genetic linkage between resistance genes and no-cost associated types of resistance.

Genetic Linkage of Resistance Genes

Genetic linkage between resistance genes will result in co-selection of the genes, *i.e.* the selection and spread of a certain resistance gene due to co-selection with another resistance gene. Multiple resistance genes are frequently found on plasmids and transposons, and the use of any of the antibiotics will result in selection for all the others. For example, despite a significant decrease in the use of sulphonamides in the UK from 1991 to 1999, the frequency of resistance to sulphonamides remained high (1991; 39.7%, 1999; 46.0%), due to linkage of sulphonamide resistance to other resistance genes, that were under continued selective pressure (Enne, Livermore et al. 2001). Moreover, in poultry, since *vanA* can be co-selected with *erm(B)* in *Enterococcus hirae* isolates (Borgen, Sorum et al. 2002), despite the exclusion of avoparcin from animal feed, vancomycin resistance can be maintained from the use of macrolides. This mechanism may not only obscure the relationship between antibiotic use and resistance development but will also act to stabilize resistant populations.

No-cost Associated Resistance Mutations

No-cost associated mutations will leave the resistant bacteria as fit as their susceptible counterparts and make them just as competitive. However, most of chromosomally encoded mutations conferring resistance are costly to the bacterium, although mutants conferring no measurable cost both in vitro and in vivo have been described. One example of a no-cost associated mutation is the AAA (Lys)→AGA (Arg) mutation in *rpsL* in *S. typhimurium*, which leads to streptomycin resistance.

It has been argued that no-cost mutations preferentially appear during selective conditions in vivo and that resistance mutations in clinical isolates mainly are of this type (Böttger, Springer et al. 1998; Sander, Springer et al. 2002). Using *Mycobacterium smegmatis* as a model organism, Sander et al. showed that under natural conditions (clinical isolates), strong selection pressure seems to exist for resistance mutations, which impose little or no fitness burden. They further hypothesize that costly resistance mutations acquired in vivo might only be found when a cost-neutral resistance does not exist for a given drug (Sander, Springer et al. 2002).

THE ROLE OF THE HUMAN NORMAL MICROBIOTA IN ANTIBIOTIC RESISTANCE DEVELOPMENT

Antibiotic treatment of bacterial infections not only exerts a selective pressure against the pathogen to which it is directed, but also affects the indigenous microbiota. Consequently, regardless of the indications for their use, antibiotics will likely select for resistance among the indigenous microflora, with the likelihood of resistance developing depending on factors previously addressed in this thesis; the rate and pattern of antibiotic use, the mutation rate of the bacteria, and the horizontal transfer rate of resistance genes. The effects of antibiotic treatment on the human microbiota and its role in contributing to resistance development are reviewed below.

The Human Microbiota

The human normal microbiota consists of three major bacterial ecosystems, the gastro-intestinal, the cutaneous and that of the upper respiratory tract. The bacteria of these ecosystems are essential to human health since they stimulate the immune response, aid in the digestion of food, help metabolize drugs and act as a barrier against invading pathogens. The gastro-intestinal ecosystem is the best studied and by far the most populous: the large intestine comprises 10^{14} bacteria in total, or 10^{11} bacteria/g of fecal material (Berg 1996). In the intestine, the quantitatively most important group of bacteria is the anaerobes (10^{11} - 10^{12} CFU/g), whereas enterobacteria (including *E. coli*) and enterococci constitute 0.1 to 1% of the bacterial population (10^6 - 10^8 CFU/g) (Berg 1996; Wold 2000).

During antibiotic treatment all three ecosystems will be affected, but to different extents. Depending on the spectrum of the agent, the degree of absorption and route of elimination, the effects on the flora will vary. The potential ecological effects of different antimicrobial agents on the human microbiota have been described in several studies (Brismar, Edlund et al. 1991; Stark, Adamsson et al. 1996; Adamsson, Nord et al. 1999; Edlund, Alvan et al. 2000; Edlund, Beyer et al. 2000; Matute, Schurink et al. 2002) and summarized in an article by Sullivan et al. (Sullivan, Edlund et al. 2001). One common effect noted after antibiotic treatment was alterations in the balance between anaerobic and aerobic species.

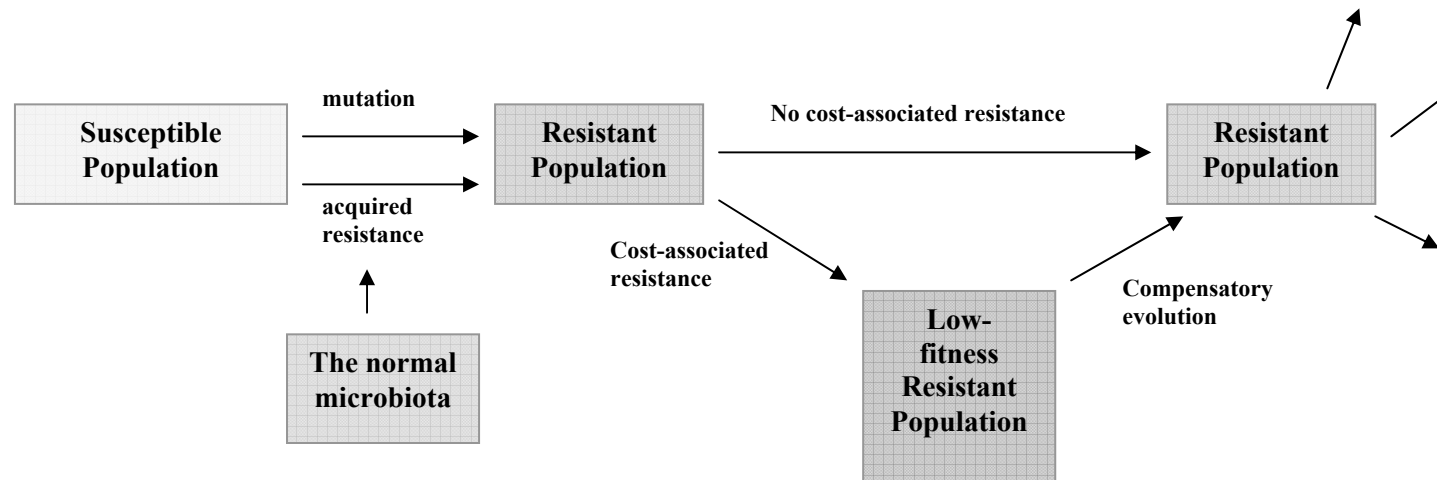
The consequences of having such an ecological imbalance in the flora might be variable. First, this may result in overgrowth of already present microorganisms such as yeasts and *Clostridium difficile*, which may lead to diarrhea or colitis. Second, an ecological imbalance may cause a reduction of

colonization resistance (Vollaard and Clasener 1994), *i.e.* the ability of the microbiota to withstand colonization of invading bacteria such as pathogens. Another possible consequence of antibiotic treatment is resistance development within the normal microbiota (Sullivan, Edlund et al. 2001).

Resistance among the normal microbiota is likely to play a very important role in resistance development of pathogenic bacteria (Andremont 2003). The tremendous number of bacteria in the microbiota allows for several different resistant mechanisms to develop, constituting a potential reservoir of resistance genes that subsequently can be transferred to other species (Courvalin 1994). For example, *tetQ*, which confers resistance to tetracycline, and *erm*-genes, which confer resistance to erythromycin, can exchange among *Bacteroides* spp and between *Bacteroides* and other species of the human colon (Salyers and Amabile-Cuevas 1997; Shoemaker, Vlamakis et al. 2001; Andremont 2003). Transfer of resistance genes probably also occurs in other ecosystems. For instance, parts of the mosaic PBP genes of *S. pneumoniae*, conferring penicillin resistance, are likely to originate from viridans streptococci, which tend to be more resistant (Dowson, Coffey et al. 1993; Bryskier 2002). Moreover, it is assumed that the *mecA* gene that renders *S. aureus* resistant to all β -lactams originates in coagulase-negative staphylococci (Wu, de Lencastre et al. 2001). Thus, gene exchange through horizontal transfer mechanisms may represent the major avenue by which pathogens acquire resistance.

Additionally, in the current thesis, we present evidence for long-term persistence of resistant enterococci and staphylococci in the normal microbiota, as a direct consequence of a one-week antibiotic therapy (Sjölund, Wreiber et al. 2003). We also show that the commensals of patients with high antibiotic use are significantly more resistant than corresponding strains isolated from control patients with no antibiotic treatment (Gustafsson, Sjölund et al. 2003). This further underlines the importance of a careful and prudent use of antibiotics. After all, the susceptible bacteria in the normal microbiota might be our best allies in combating further development and spread of antibiotic resistance and are crucial in preventing colonization by resistant pathogens. A study by Roos and colleagues in Sweden exemplified the importance of a susceptible commensal flora and how it can be used to prevent colonization by pathogenic bacteria. They showed that recolonization with viridans streptococci in children with acute otitis media had an interfering activity against the otopathogens, and that the rate of re-occurrence of acute otitis media significantly was reduced in children receiving the viridans streptococci (Roos, Hakansson et al. 2001).

In summary, a susceptible normal microbiota plays a crucial role in preventing the invasion of pathogens as well as resistant bacteria. In order to maintain the sensitivity of our microbiota and limit further spread of antibiotic resistance, the ecological effects of the administration of antibiotics have to be considered.



Selection of resistance is dependent on:

- Mutation rate and presence of mutator strains
- Rate and extent of horizontal transfer (including the impact of the human microbiota acting as a reservoir of resistance genes)
- Population size
- Selective pressure

Stability of resistance is dependent on:

- The biological cost of resistance
- Compensatory evolution
- Genetic linkage with other genes undergoing selection
- Selective pressure

Figure 3. Overview of the main factors influencing selection and stability of antibiotic resistance.

GENERAL AIM

The present thesis is focused on the selection and stability of antibiotic resistance, and is aimed at studying the impact of antibiotic treatment on pathogenic bacteria as well as the normal human microbiota. We address this by studying the mechanisms by which resistance appears, as well as by studying the biological cost and persistence of resistance.

As a model organism for pathogenic bacteria, we have chosen the peptic ulcer bacterium, *Helicobacter pylori*. This bacterium infects more than 50% of the western world's population and is associated with both a medically and economically important infection.

As major model organisms for the human normal microbiota, we have chosen enterococci and staphylococci, since both species are clinically important and common causes of nosocomial infections. We also address the effect of long-term antibiotic treatment on the human normal flora and will here, in addition to enterococci and staphylococci, include streptococci and the Gram-negative *Escherichia coli*.

SPECIFIC AIMS

The specific aims of the present investigation were:

- I.** To assess the frequency of spontaneous mutation in *H. pylori* and investigate whether mutator strains can be found among clinical isolates. An additional aim was to establish the biological cost of clarithromycin resistance in *H. pylori* and investigate whether compensatory evolution may act to reduce such a fitness cost.
- II.** To assess the importance of high antibiotic consumption as a selector for antibiotic resistance and elevated mutation frequencies.
- III.** To investigate the effect of a commonly used antibiotic treatment on the normal flora of *Enterococcus* spp. focusing on resistance development.
- IV.** To study the effect of a commonly used antibiotic treatment on the normal flora of *Staphylococcus* spp. and establish the fitness of resistant *S. epidermidis* by using an in vivo competition model.

MATERIALS AND METHODS

Bacterial Species Studied in the Present Thesis

Helicobacter pylori

Helicobacter pylori is a Gram-negative, spiral-shaped bacterium that colonizes the human gastric mucosa (Dunn, Cohen et al. 1997). About half of the world's population is carrying *H. pylori*, which makes it one of the most common bacterial infections in humans. The infection is associated with gastritis and, in a subset of individuals, also peptic ulceration. *H. pylori* is further recognized as a major risk factor for the development of gastric cancer and has since 1994 been classified as a type I (definite) carcinogen by the WHO (IARC 1994).

A triple therapy consisting of two antibiotics and an acid suppressing drug is required and recommended to clear the infection, and usually leads to complete elimination of the bacteria and to the healing of ulcers (de Boer and Tytgat 2000). Commonly used antibiotics are clarithromycin, amoxicillin, metronidazole and tetracycline. Resistance development is the major cause of treatment failure and resistance to all the above-mentioned antibiotics has been described (Dunn, Cohen et al. 1997; de Boer and Tytgat 2000).

In the first paper of this thesis, *H. pylori* was chosen as a model organism for pathogenic bacteria and used for studying the mechanisms by which resistance appears, as well as for addressing the stability of resistance.

Enterococcus spp.

The Gram-positive *Enterococcus* species are normal inhabitants of the human colon and constitute 0.1 to 1% of the gastro-intestinal flora. Over the last years, enterococci have emerged as important bacterial pathogens, causing nosocomial infections such as endocarditis and bacteraemia (Murray and Weinstock 1999). Enterococci rapidly develop resistance (Murray 1990). Soon after the introduction of penicillin, resistant clones of enterococci were

found, necessitating the use of other antibiotics to treat infections. Currently, enterococci show resistance towards chloramphenicol, erythromycin, clindamycin, tetracycline, aminoglycosides, penicillin, fluoroquinolones and vancomycin.

Enterococci were included in two papers of the present thesis (paper II and III) where the effect of antibiotic treatment on the normal flora of enterococci was assessed.

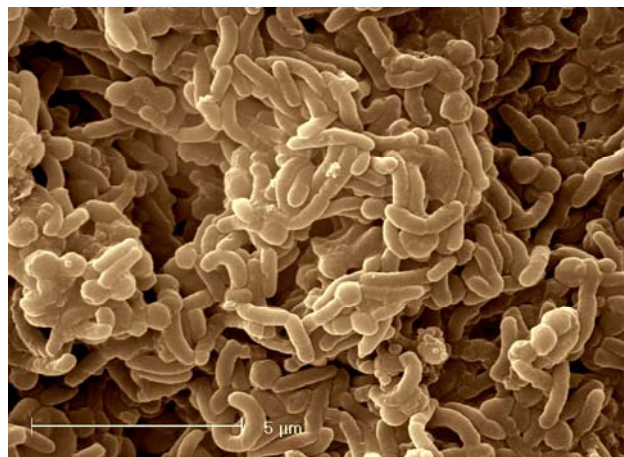


Figure 4. *Helicobacter pylori*

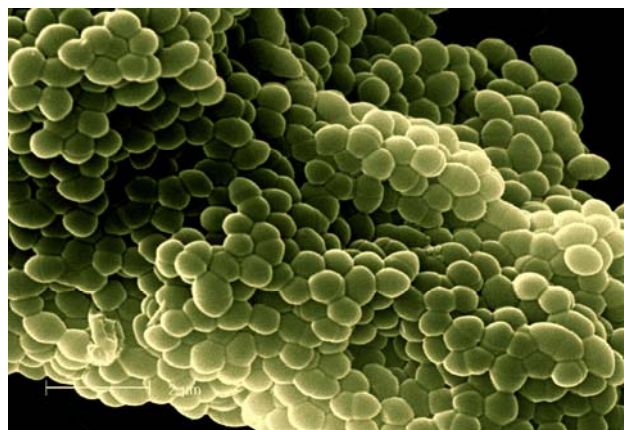


Figure 5. *Enterococcus faecalis*

Staphylococcus epidermidis

Staphylococcus epidermidis belongs to the group of coagulase-negative staphylococci (CoNS) and represents one of the most prevalent species of the human cutaneous microbiota (Kloos and Bannerman 1994). Like enterococci, the CoNS have emerged as major nosocomial pathogens, primarily causing infections associated with implanted medical devices (Huebner and Goldmann 1999). Coagulase-negative staphylococci have been reported to be the third most common causative agent of nosocomial infections and the most frequent cause of nosocomial bloodstream infections. Infections in immuno-compromised patients are especially problematic and may be difficult to treat because of acquired resistance of the bacteria. In recent years, *S. epidermidis* has become resistant to many commonly used antibiotics and may be a reservoir for antibiotic resistance genes in hospitals.

In paper IV, the effect of a commonly used antibiotic treatment on the normal flora of *S. epidermidis* was assessed, with focus on resistance development. We further used a human in vivo model to establish the fitness and stability of macrolide-resistant *S. epidermidis*.

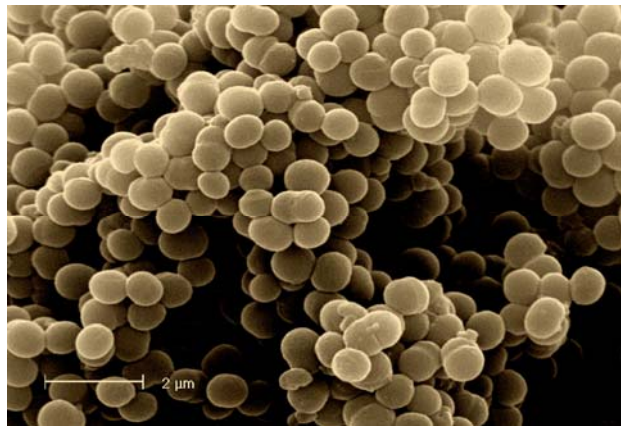


Figure 6. *Staphylococcus epidermidis*

α -streptococci

α -streptococci belong to the group of viridans streptococci, which is a heterogeneous group of α -hemolytic and non-hemolytic streptococci. The α -streptococci normally colonize the pharynx and the gastrointestinal tract but can also give rise to various infections. Most common is dental caries caused by *S. mutans* and subacute endocarditis. The majority of strains are highly susceptible to penicillin, which is the first drug of choice against streptococcal infections (Murray 1998). α -streptococci were included in paper II of the present thesis, where resistance development and mutation frequencies in the normal microbiota were studied.

Escherichia coli

Escherichia coli belongs to the family *Enterobacteriaceae*, a large and medically important group of Gram-negative bacteria. Among the genus *Escherichia*, *E. coli* is the most clinically important bacterium associated with several diseases, such as meningitis, gastroenteritis, urinary tract infections and sepsis (Murray 1998).

In paper II, in which we examined how extensive use of antibiotics affected the normal microbiota, *E. coli* was used as an indicator organism for the gastro-intestinal flora.

Bacterial Strains (I-IV)

Paper (I)

The *H. pylori* isolates used were:

- (i) Clinical isolates collected in a clinical treatment trial performed by Hultén and colleagues (Hulten, Gibreel et al. 1997). Isolates of *H. pylori* were obtained from patients before and 3 months after undergoing an anti-*Helicobacter* treatment including the macrolide clarithromycin. From this material, paired susceptible pre-treatment and resistant post-treatment isolates of the same strain were obtained.
- (ii) Clinical isolates collected in a case-control study of gastric cancer performed by Enroth et al. (Enroth, Kraaz et al. 2000).
- (iii) Reference strain 26695

Paper (II)

Isolates of *E. coli*, enterococci, α -streptococci and coagulase-negative staphylococci (CoNS) were isolated from nostril, pharynx and feces samples collected from patients at the Center of Cystic Fibrosis (n=18) and Department of Haematology (n=18), University Hospital Uppsala, Uppsala, Sweden. Strains from primary health care patients (n=30) with no antibiotic use one year prior to the study were used as controls.

Paper (III)

Enterococci were isolated from fecal samples obtained from 10 patients participating in a prospective cohort study examining the eradication of *H. pylori* in dyspeptic patients. Samples were obtained one day before treatment, immediately after, one year after, and three years after treatment. From each study patient and sample, 10 independent colonies of enterococci were isolated and verified.

Paper (IV)

Staphylococcus epidermidis was isolated from nostril samples of patients participating in a prospective cohort study examining the eradication of *H. pylori* in dyspeptic patients. Samples were collected one day before treatment, immediately after, one year after, and four years after treatment. From each study patient and sample, 10 independent colonies of *S. epidermidis* were isolated and verified by Gram staining, catalase, oxidase and mannitolose-trehalose testing.

In the competition assay, reference strain RifR35 was included (Gustafsson, Cars et al. 2003).

DNA Preparation and Polymerase Chain Reaction (I-IV)

DNA was extracted from the bacterial strains using Amplicore Respiratory Preparation Kit (Roche) or Dneasy Tissue kit (Qiagen), both according to the manufacturers' instructions. Polymerase chain reactions were performed using standard conditions and the PCR Master kit (Roche). The primers used are listed in Table 3. All amplified products were separated on a 1.5% agarose gel and visualized with ethidium bromide.

Table 3. Primers used in papers (I-IV).

Primer	Sequence	Method
AP-PCR <i>H.pylori</i>	5'-CACTCGTCGGGAATGCCCT-3'	DNA-fingerprinting
23SF (p18)	5'-AGTCGGGACCTAAGGCGAG-3'	Detection of
23SR (p21)	5'-TTCCCGCTTAGATGCTTTTCAG-3'	ClaR mutations
rpoB3	5'-GACGTGGTCCATACCTGTAC-3'	Detection of
rpoB4	5'-CAATTCATGGACCAAGCTAA-3'	RifR mutations (CoNS).
rpoB fw	5'- CCACTTAGGTAACCGTCGTA-3'	Detection of
rpoB rw	5'- TAATCAATCCAATGTTTGGTC -3'	RifR mutations (enterococci, α -streptococci).
rpsL fw	5'- TCACCAGCTTTGAACATTGG-3'	Detection of
rpsL rw	5'- CCGTATTTAGAACGGCCTTG-3'	SmR mutations
AP-PCR D11344	5'-AGTGAATTCGCGGTGGATGCCA-3'	DNA-fingerprinting
ermb1	5'-GAAAAGGTACTCAACCAAATA-3'	Erm(B) detection
ermb2	5'-AGTAACGGTACTTAAATTGTTTAC-3'	
ermc1	5'GCTAATATTGTTTAAATCGTCAATTCC3'	Erm(C) detection
ermc2	5'-GGATCAGGAAAAGGACATTT-3'	

Determination of Antibiotic Susceptibilities (II)

Antibiotic susceptibilities were determined by disk diffusion (Oxoid, Ltd., England) according to recommendations by the Swedish Reference Group for Antibiotics (SRGA) and its subcommittee on methodology (SRGA-M).

Minimal Inhibitory Concentration Determinations (MIC) (I-IV)

Minimal inhibitory concentrations were determined using the E-test (AB Biodisk, Solna, Sweden), as recommended by the Swedish Reference Group for Antibiotics (SRGA).

Mutation Frequency Determination (I, II)

The mutation frequency for *H. pylori* was determined using rifampicin resistance as a marker. For each strain, 10 µl ($<10^4$ cells) of an overnight culture was inoculated into 20 independent 1 ml cultures (Brucella broth supplemented with 5% FCS) and incubated under microaerophilic conditions at 37°C for 36-48 h. Viable count was performed on 3 of the tubes. To determine the number of resistant mutants, the whole amount of each tube was spread on GC-agar plates containing rifampicin (15 µg/µl). The plates were incubated for 5 days and colonies were counted. The mutation frequency was calculated from the median number of resistant mutants divided by the viable count.

In mutation frequency determinations, all the mutants present in a given population are measured, irrespective of whether the mutation events occurred early or late during the growth of the population. By using the median of the 20 cultures for the respective strain, the impact of any occasional jackpot cultures was minimized. In addition, to assure that the 10 µl of bacteria used to inoculate the 1 ml sample cultures was free from pre-existing resistant mutants, 30 µl of the pre-culture was plated on a selection plate. The sample cultures were used to measure the mutation frequency only if no mutants were present in these 30 µl.

In a similar manner, the mutation frequency to clarithromycin resistance was determined using selective plates containing clarithromycin at a concentration of 10 µg/µl.

In paper II, the mutation frequency to rifampicin resistance was estimated for *E. coli*, α -streptococci, CoNS and enterococci. The frequency was calculated from 10 independent cultures from each of the 3 isolates from each

patient/control, resulting in 30 cultures for each species from each patient or control. The cultures were inoculated with 10^3 bacteria, from a fresh pre-culture, in 0.4 ml Todd-Hewitt broth (Difco). The cultures were incubated over-night at 35°C giving 10^8 - 10^9 cfu/ml. The total number of cells was determined by viable count or optical density measurements. Each culture was spread on a rifampicin-containing blood agar plate (50 mg/L for *E. coli* and enterococci; 0.1mg/L for α -streptococci and CoNS). Plates were dried and incubated for 24 h and then colonies were counted. The mutation frequency for each of the 3 isolates was estimated by taking the median number of resistant mutants from the 10 cultures divided by the total number of bacterial cells. Since the three isolates of each species from each patient showed similar mutation frequencies, we calculated a geometric mean mutation frequency for the 30 cultures.

All pre-cultures were controlled for pre-existing mutants by plating 10^4 bacteria on a selective plate. If pre-existing resistant mutants were found, the culture was discarded.

The α -streptococci were also analyzed for their mutation frequency to streptomycin resistance. Five independent cultures of one strain from each patient (n=14) and control (n=17) were included. The remaining strains had to be excluded because of low bacterial density in the overnight culture or high initial MIC values to streptomycin. Todd-Hewitt broth (50 ml) was inoculated with 10^3 bacteria from a fresh broth culture and incubated over night at 35°C, in 5% CO₂. The bacteria were concentrated by centrifugation at 1400 g for 15 min, and the pellet was applied to blood agar plates containing 120 mg/L streptomycin, and colonies were counted after 24 h.

Sequencing (II)

The *rpoB* gene from 4 independent rifampicin-resistant colonies of enterococci, α -streptococci and CoNS was sequenced to verify that the resistant mutants had mutations in the *rpoB* gene. Primers for the β -subunit of *rpoB* were designed using *Streptococcus pyogenes* (GenBank accession number AJ295718) and *Staphylococcus warneri* (GenBank accession number AF325895) sequences. DNA was prepared by using the Dneasy Tissue kit (Qiagen). The PCR products were sequenced with ABI prism (Applied Biosystems, Warrington, UK) and compared to the original strains.

The *rpsL* gene from 3 independent streptomycin-resistant colonies of α -streptococci was sequenced to verify that the resistant mutants contained mutations in the *rpsL* gene. Primers were constructed from the *rpsL* gene of *S. pyogenes* (Genbank accession number AE006493).

Statistical Analysis (II)

The statistical significance of the difference in mutation frequency was determined from the geometrical mean by Mann-Whitney test in Statistical Analyzing System version 8 and was performed by Kasia Grabowska at the Swedish Institute for Infectious Disease Control. Further comparison of mutation frequency and total DDD was performed with Microsoft Excel.

Mathematical Modelling (I)

Appendix 1

In paper I, we found a relatively high frequency of hypermutable strains of *H. pylori*. The presence of such strains in a bacterial population is predicted to speed up resistance development caused by chromosomal point mutations. However, in our material we did not find a correlation between an increased mutation frequency and resistance development. Therefore, we decided to address this theoretically by constructing a mathematical model and running computer simulations in the software program Mathematica in order to determine whether a high mutation frequency is likely to increase resistance development.

By using a mathematical model describing the dynamics of an *H. pylori* population, we calculated the probability that resistant bacteria are present (and eventually become fixed) in an infected stomach when antibiotic treatment is initiated (appendix 1). With this model, we show that the likelihood of acquired resistance developing is dependent on the population size, the mutation rate and the rate by which resistant mutants grow.

In the model, we consider an antibiotic-susceptible population of bacteria (N) that is kept constant in size by the random removal of individuals at the same rate as growth occurs. In this population, resistant mutants can arise with a probability of u , which corresponds to the mutation frequency. Since there is no antibiotic pressure included in this model, the resistant mutants that appear are counterselected, *i.e.* are not as fit as wild types and not favored by natural selection. The counterselection is modelled by giving the mutants a reduced growth rate; wild type bacteria always grow with the rate 1 whereas mutants grow by the rate $1+s$, where s is the selection coefficient and always is < 0 .

By using these basic conditions, we model the upcoming and growth of resistant mutants in a population of *H. pylori*. We show that the probability of resistant mutants appearing is highly dependent on the mutation rate (u) and population size (N). In figure 5 in Appendix 1, the probability of fixation of resistant mutants (P_{fix}) is plotted as a function of uN , and the impact of

different growth rates of the mutants are shown. A realistic population size of *H. pylori* infecting a human stomach ranges from 10^7 - 10^9 bacteria, and the mutation frequency to clarithromycin resistance is approximately 10^{-9} . These numbers yield a uN value of 0.01-1, which in Figure 5 demonstrate that the mutants are close to fixation. However, if the mutation frequency is subject to a 100-fold increase, a higher uN value is achieved, which, according to the plot in Figure 5, leads to a fixation of resistant mutants. Thus, theoretical modelling suggests that a high mutation frequency will lead to an increased resistance development by increasing the probability that resistant mutants are present when treatment is initiated.

Appendix 2

A major finding of paper I was that clarithromycin resistance confers a biological cost and that this cost can be compensated for. The pre- and post-treatment pairs of *H. pylori* isolates that we used in our competition experiments were isolated 3 months apart. Assuming that compensatory mutations were selected after the resistance mutations, we estimated the succession time from susceptible → resistant → compensated bacteria in the stomach. This is described in Appendix 2 where a mathematical model is used to support our hypothesis that a step-wise selection from susceptible → resistant → compensated is possible during the time frame of 3 months.

In this model, we consider three genotypes; 0 is the susceptible wild type, 1 is the resistant and 2 is the compensated. At time 0, antibiotic is added to a fully susceptible population (n_0) and only a small number of bacteria [those which become resistant (n_1)] survive the treatment. Since the resistance is costly to carry in the absence of an antibiotic pressure, these bacteria grow slowly with a rate k_1 . During this growth, a new, compensating mutation can occur with the probability of u_2 per replication. This new variant grows with a higher rate k_2 , which always is $>k_1$.

In Appendix 2, we estimate the time it will take for the compensated mutant to appear and take over the population ($P_{i0}=1$). We present the outcome of 8 different simulations, each of which has been assigned a certain set of parameter values. For instance, we model the impact of different initial densities (n_1^0) and different growth rates (k_1) of the resistant bacteria. We also model the impact of different mutation frequencies to compensation (u_2). For most of the parameter values we use, compensation occurs and becomes fixed within 90 days. Thus, according to this model and the assumptions we use, the transition from susceptible → resistant → compensated is possible within the time frame of 3 months.

In vivo Competition Model (I)

The animal experiments were conducted using protocols approved by the Animal Studies Ethical Committee of Stockholm.

In paper I, the bacterial fitness of clarithromycin-resistant *H. pylori* strains was estimated in vivo using a mouse model. Since the human Lewis^b blood group antigen (Le^b) functions as a receptor for the bacteria's adhesins and mediates attachment to the gastric cells, a transgenic mouse model expressing Le^b-containing glycoconjugates was used to establish a chronic, high-density infection of *H. pylori*. These mice contain a human α 1,3/4 fucosyl-transferase ORF under the control of nucleotides -596 to +21 of a fatty acid binding protein gene (Falk, Bry et al. 1995).

Equal amounts of ClaS wild type and ClaR mutant of each clonally related pair were mixed and orally inoculated in a volume of 50 μ l into 6-10 week old animals. Four animals/dose/time point were used, and the experiments were performed twice. *H. pylori* were recovered by homogenization of half of the stomach. Samples were cultured on GC-agar plates containing vancomycin, polymyxin B and trimethoprim with and without 1 μ g/ml clarithromycin.

Pulsed-Field Gel Electrophoresis (PFGE) (IV)

Bacterial cells were harvested by centrifugation from 3 ml overnight cultures in brain-heart infusion broth and resuspended in 3 ml of Tris-HCl buffer, pH 7.6. The bacterial suspension (150 μ l) was mixed with 150 μ l 2% agarose (Sigma, USA) in Tris-HCl buffer and used for making the gel plugs. The plugs were incubated at 35°C overnight in 4 ml of Lysis buffer 1 (6mM Tris-HCl, pH 7.6, 1M NaCl, 100mM EDTA, pH 7.5, 0.5% Brij 58, 0.2% deoxycholate, 0.5% sodium lauryl sarcosine (Sarcosyl), 1mg/ml lysozyme (Life Technology, Invitrogen, USA) and 7 μ g/ml lysostaphin (Sigma, USA), then incubated overnight at 55°C in 4 ml of Lysis 2 buffer (1% sodium lauryl sarcosine (Sarcosyl), 0.5 M EDTA, pH 9.5, 50 μ g/ml Proteinase K (Boehringer Mannheim, Germany). The plugs were washed three times for at least 30 minutes at 35°C in 4 ml of TE-buffer. A 3 mm slice of each gel plug was incubated overnight at 25°C with *Sma*I (Life Technology, Invitrogen, USA) and buffer, then placed in the wells of a 1.0% agarose gel (Ultra pure agarose, Life Technology, Invitrogen, USA), sealed with 1.0 % agarose and put in 0.5 x TBE buffer. Electrophoresis [Gene Path Electrophoresis System (BioRad, USA)] was performed with the following conditions: 5-60 s switch interval with a voltage gradient of 6V/h at an angle of 120° for 23 h. After electrophoresis, the gel was stained with ethidium bromide for 30 min then destained in distilled water for 1 h, and the DNA visualized with a UV light

scanner (Gel doc 1000) and the restriction fragment profiles interpreted by comparison with each other, with reference *S. aureus* strain (NCTC8325), and with a lambda phage DNA standard (New England Biolabs).

Competition Assay on Human Skin (IV)

The bacterial fitness of *S. epidermidis* strains was established using a competition assay on human skin. This method involves inoculation of bacteria on the forearms of human volunteers, from which the bacteria then are sampled using a pad attached to a plastic applicator.

In paper IV, the bacteria were sampled using a pad (5x5 cm, 85% viscose and 15% polyester), moistened by a buffer (PBS pH 7.4 supplemented with 2% Tween 80 and 0.3% lecithin) and attached to an applicator. The applicator was rubbed back and forth 10 times at the site of bacterial application. The pad then was removed from the applicator, transferred to a sterile plastic bag (Seward Laboratories, London, UK) and processed together with 20 ml buffer (PBS, pH 7.4 supplemented with 2% Tween 80 and 0.3% lecithin) in a Stomacher Lab Blender 400 (Seward Laboratories, London, UK) for 1 min. To detect the recovered bacteria, the processed buffer was plated in 2 dilutions, 0.5 and 3 ml (day 1) or 5 ml (day 3) on media containing erythromycin (100 µg/ml) or rifampicin (50 µg/ml). After plates were incubated overnight at 35°C in 5% CO₂, colonies were counted and a competition ratio was calculated between each clarithromycin-resistant isolate and the reference strain. These ratios were further used to calculate the relative fitness of the two clarithromycin-resistant strains from each patient. To assess the total number of bacteria recovered from the skin, 0.5 ml of buffer was cultured on non-selective media (Columbia blood agar plates, Difco).

RESULTS AND DISCUSSION

The scientific work of the present thesis has focused on development and stability of antibiotic resistance, and the aim was to study the impact of antibiotic treatment on pathogenic bacteria as well as the normal human microbiota. We have addressed this by studying the mechanisms by which resistance appears, as well as by studying the biological cost and persistence of resistance. The major findings of this thesis are presented and discussed below.

Mutator strains are common among clinical isolates of *Helicobacter pylori* (I).

In paper I, we assessed the frequency of spontaneous mutation in the gastric pathogen *H. pylori*.

Twenty-nine clinical strains of *H. pylori* were used for mutation frequency testing. For each strain, 20 independent 1 ml cultures were set up and used for measuring the formation of spontaneous mutations. This was accomplished by using rifampicin resistance as a mutational marker and by plating the entire culture onto media containing rifampicin at a concentration of 15 µg/ml. The number of mutants was counted, and the mutation frequency was obtained by dividing the median number of mutants by the total number of bacteria in the culture.

Our results show that the mutation frequency was unusually high in *H. pylori*; 25% of all isolates displayed a similar or higher frequency than *Enterobacteriaceae* defective mismatch repair mutants. The observed frequencies varied, however, among the isolates, ranging from 10^{-5} to 10^{-8} (median 10^{-6}).

To assess whether the high mutation frequency was correlated to resistance development, the MIC values to 6 antibiotics were determined for all isolates included. However, no correlation between a high mutation frequency and the number of resistance determinants was found in this material.

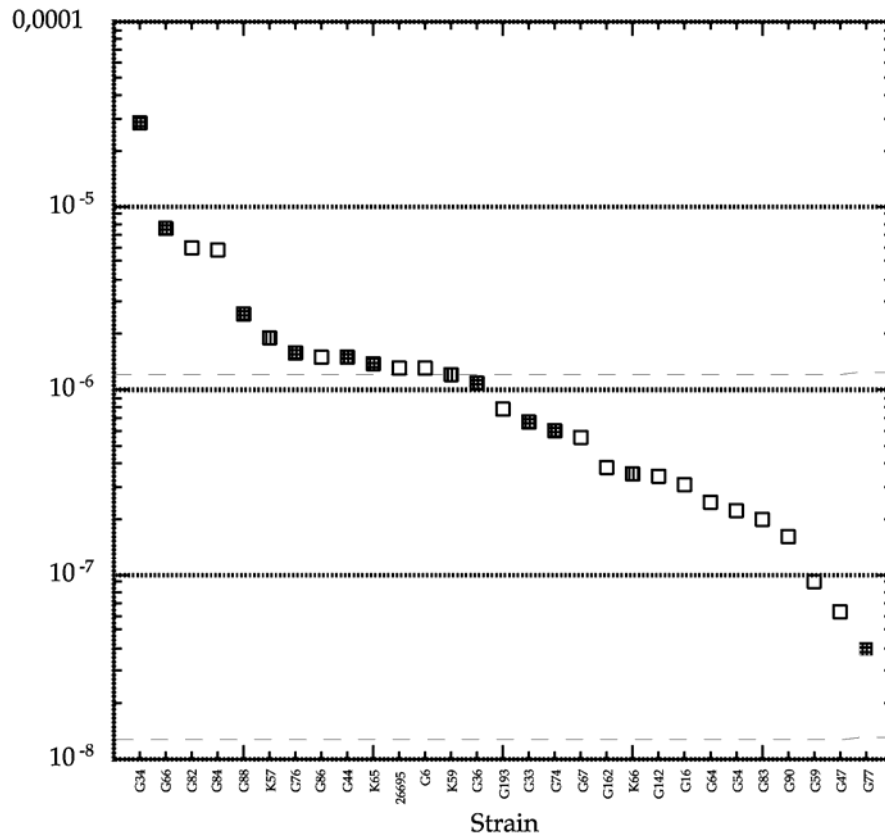


Figure 7. Mutation frequency to rifampicin resistance for 29 *H. pylori* isolates. The dotted lines indicate the approximate mutation frequencies to rifampicin resistance for wild type and mutator *Enterobacteriaceae*. Indicated are strains isolated from peptic ulcer- (stippled), non-ulcer dyspepsia (open) and gastric cancer patients (vertical lines).

The high number of hypermutable strains present among *H. pylori* clinical isolates requires explanation. One possible reason for *H. pylori* having a generally higher mutation frequency is that *H. pylori* seems to lack a functional mismatch repair system. Analysis of the two sequenced *H. pylori* genomes (J99 and 26695) revealed that the bacterium lacks some of the most important genes involved in the mismatch repair system, *mutH* and *mutL*. Furthermore, it has been suggested that *mutS* in *H. pylori* has another function than observed for *Enterobacteriaceae* (Eisen 1998). To verify this, we constructed a *mutS* knock out and tested the mutation frequency. In *Entero-*

bacteriaceae, this would confer a 1000-fold increase in mutation frequency. However, in the present material, no difference was found between wild type and knock out, which thereby supports that *mutS* has another function in *H. pylori*.

The selective pressure of the surrounding environment acting on the bacteria could also explain the high number of hypermutable isolates within this material. It has been shown both theoretically and experimentally, that a changing environment facilitates the upcoming of mutator strains; because of the high mutation rate, such strains can more easily adapt to changes than a wild type. In the case of *H. pylori* colonization, the bacteria are constantly challenged by changing pH values, the epithelial cell turnover, food digestion and the host immune response. Thus, strains that are able to adapt to these conditions are likely to be at a selective advantage.

Likewise, antibiotic use and selection of resistant mutants can enrich for bacteria with higher mutation frequencies (Mao, Lane et al. 1997). However, as mentioned previously, no clear correlation between the level of resistance and mutation frequency was found in this material, suggesting that the exposure of the bacteria to a continuously changing environment in the gastric mucosa might play a more significant role in the enrichment for mutators in *H. pylori*.

Antibiotic pressure is a selector for elevated mutation frequencies (II)

Even though under laboratory conditions (Mao, Lane et al. 1997) and in animal models (Giraud, Matic et al. 2001) selection for antibiotic resistance can rapidly enrich for mutators, it remains unclear whether clinical use of antibiotics is an important selector for strains with an increased mutation frequency. In paper II, we addressed this by examining whether high antibiotic use enriched for commensal bacteria with elevated mutation frequencies.

The rationale behind this study and the reason for choosing commensal bacteria was to examine a situation where the host environment was unaltered, and where no pathogenesis occurred. As previously mentioned, a continuously changing environment due to pathogenesis is predicted to select for mutators. Thus, to examine a situation where the environmental conditions are relatively constant and where no infectious pathogenesis occurs, we chose to study the impact of extensive antibiotic use on commensal bacteria. The resistance patterns and mutation frequencies were studied in four species: *E. coli*, enterococci, α -streptococci and CoNS.

When comparing the patients and controls, a significant difference in mutation frequency was noted for *E. coli*, enterococci and CoNS. Thus, the

patients had commensal bacteria with a 3-, 1.8-, and 1.5-fold higher mutation frequency as compared with the controls ($P = 0.0001$, 0.016 and 0.012 , respectively). For α -streptococci, no significant difference ($P = 0.74$) in mutation frequency to rifampicin resistance between the controls and patients was evident. To further assess whether α -streptococci from patients had an increased mutation frequency, we also measured the mutation frequency to streptomycin resistance for this group of bacteria. The mutation frequency was 4.7×10^{-10} and 1.5×10^{-10} for patients and controls, respectively ($P = 0.024$), suggesting that strains with an increased mutation frequency might be enriched also in the α -streptococci. Why this difference was only seen for streptomycin resistance and not rifampicin resistance is unclear, but might be related to which types of base pair substitutions cause resistance to the two antibiotics.

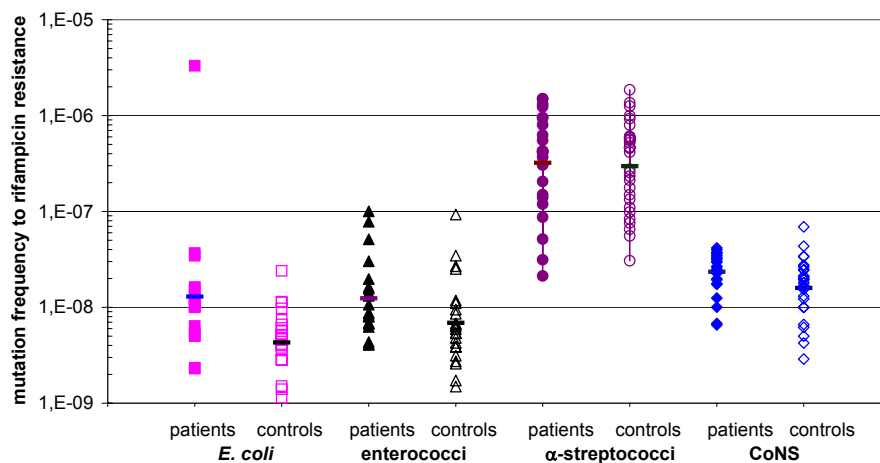


Figure 8. Mutation frequencies to rifampicin resistance of four species isolated from patients and controls. Each data point represents the mutation frequency of one bacterial species from one patient or control. The overall geometric mean mutation frequency for each bacterial species for the whole patient and control group is indicated by a line.

We further examined whether there was a correlation between the level of resistance to different antibiotic classes and the mutation frequency. For aminoglycosides, β -lactams, macrolides and trimethoprim-sulfamethoxazole, no correlation was observed. In contrast, when comparing ciprofloxacin-resistant and -susceptible isolates of *E. coli* within the patient

group, the mutation frequencies differed significantly. Thus, ciprofloxacin-resistant *E. coli* showed a higher mutation frequency than the susceptible isolates ($P = 0.036$). Because of this observation, the MICs of ciprofloxacin were determined for all strains of *E. coli* and enterococci from patients and controls. The MICs of ciprofloxacin for *E. coli* varied between 0.006 and >32 mg/L for patients and 0.004 and 0.016 mg/L for controls, and this difference was highly significant ($P = 0.002$). Similarly, the corresponding MICs of ciprofloxacin for enterococci were 0.38-32 mg/L for patients and 0.25-3 mg/L for controls ($P = 0.0006$). Furthermore, we examined whether antibiotic exposure (DDDs) was correlated with level of resistance or mutation frequency using linear regression. There was no correlation between total DDD of antibiotics and mutation frequency, nor between DDD of fluoroquinolones and ciprofloxacin resistance or mutation frequency. Finally, within each patient there was no correlation between the mutation frequencies for the different bacterial species.

Even though the increased mutation frequency in the patient group was significant, the magnitude of the increase was small. These increases could be caused by mutations of small effect in any of the processes that affect the mutation frequency in a bacterium (e.g. DNA polymerase, mismatch repair, excision repair, etc.). Unexpectedly, at least in the light of previous studies where frequencies of strong mutators were 1.2–36%, only one such mutator (an *E. coli* from the patient group) was found among the 193 commensal isolates examined. One explanation for the rarity of strong mutators could be that for these investigated bacterial species, mutators are impaired for growth in the commensal flora of hosts, thereby preventing them from increasing to a high frequency. A second potential explanation is that putative mutator genes were removed by recombination after resistance was acquired. Finally, a third possibility, and the one we find most likely, is that most of the antibiotics given to these patients are poor selectors of mutators. Thus, it is predicted that mutators are mainly enriched when the resistance is conferred by a chromosomal mutation (e.g. *gyrA*) that is formed at a higher rate in the mutator than the non-mutator. Most of the resistances in the examined bacteria result from genes that are located on horizontally transferred genetic elements (e.g. plasmids), and a mutator is not expected to increase the rate of plasmid transfer. The only exception is fluoroquinolone resistance, which is caused by sequential chromosomal mutations that confer stepwise increases in resistance. Thus, acquiring high-level fluoroquinolone resistance is predicted to enrich for mutators. Support for this idea comes from the finding that a significant correlation between resistance and increased mutation frequency was only seen for ciprofloxacin.

Clarithromycin resistance confers a biological cost in *Helicobacter pylori* but can be reduced by compensatory evolution in vivo (I)

To address whether clarithromycin resistance confers a cost in *H. pylori*, we performed competition experiments between isogenic susceptible and clarithromycin-resistant isolates. The experiment was performed both in vitro and in vivo. By using a transgenic mouse model, expressing the human blood group antigen Lewis^b on the surface of the mucus cells, we were able to establish a chronic infection in the mouse stomach. Five different clonally related pairs of clinical isolates were used, each one consisting of a susceptible pre-treatment isolate and a resistant post-treatment isolate. Further, we performed competition experiments for one pair for which the resistant isolate was isolated in vitro. In each experiment, the mice were inoculated with a mixture of the bacteria in a 1:1 ratio, and after certain time points, the CFU was determined by plating on selective and non-selective media. The competition index (CI) was calculated and the fitness associated with the resistance mutation estimated.

For two of the clinical pairs, G6-G44 and G47-G108, for unknown reasons no infection could be established in the mice. Thus, only three of the clinical pairs and the defined pair isolated in vitro were used. Comparison of the fitness in mice of the pair ClaS (67:21)-ClaR (67:21, A₂₁₄₃ => G) with the pair ClaS (G142)-ClaR (G193, A₂₁₄₃ => G) suggested that the cost of clarithromycin resistance had been reduced in the G193 mutant. The ratios of ClaS/ClaR bacteria at day 7 were 1167 and 14, respectively, indicating that most of the fitness cost of the A2143G mutation had been lost in strain G193. An even clearer example of the reduction in cost was demonstrated by the comparison between the ClaS (G34)-ClaR (G49, A₂₁₄₂ => G) and ClaS (G83)-ClaR (G162, A₂₁₄₂ => G) pairs. For the G34/G49 pair the post-treatment resistant mutant lost against the susceptible strain; the competition ratio was 337 at day 7. In contrast, in the second pair G83/G162, the post-treatment resistant mutant out-competed the susceptible pre-treatment strain; the competition ratio was 0.004 at day 7. Thus, in this case the cost of the A2142G mutations was completely eliminated in strain G162.

At least two explanations can be proposed for the different costs of an identical resistance mutation in different isolates. One possibility, as has been shown to occur both in laboratory media and in experimental animals, is that a compensatory mutation was selected after the resistance mutation occurred, resulting in a strain with increased fitness. Another possibility is that the cost depends on the genetic background of the clinical isolates in which the resistance mutation appeared. However, with respect to the stabilization of resistance mutations, it is less important whether the compensatory mutations were present before or appeared after the resistance mutation,

because in both cases the cost would be reduced and result in stabilization of the resistant clone in the population.

Assuming that compensatory mutations were selected after the resistance mutation, we estimated the succession time from susceptible → resistant → compensated bacteria in the stomach. This was accomplished by using theoretical modelling and simulations *in silico*. From this analysis, we estimated a take over time of 40-120 days, similar to the 90 days between the isolation of the pre- and post-treatment strains. Thus, by using the model and the underlying assumptions described in Appendix 2, we present calculations that are consistent with the possibility that there was a stepwise selection from susceptible → resistant → compensated mutant during the time between sampling of the pre- and post-treatment strains.

Antibiotic treatment selects for stable resistant strains in the human normal microbiota (II, III, IV)

Antimicrobial agents are known to influence the human microbiota, and the extent of disturbance depends on a variety of factors, including drug dosage, route of administration and the pharmacokinetic/dynamic properties of the agent. Even though the microbiota may return to normal rapidly after completion of a treatment, this thesis work presents evidence for long-term persistence (several years) of selected resistant commensal bacteria. Such persistence and the natural exchange of genes between bacteria make the human microbiota a reservoir of resistance genes for potential spread to pathogens.

In paper II, we show that extensive antibiotic use selects for commensals with highly increased resistance. A clear shift towards more resistance was seen among isolates from antibiotic-treated patients for virtually all types of resistances examined. For example, the frequency of ciprofloxacin resistance in enterococci and CoNS was 63% and 67%, respectively, as compared to 0% in the controls. Similarly, the frequency of erythromycin-resistant CoNS was 52% in the patient group and 0% in the controls. Resistance to tobramycin was uncommon in *E. coli* and CoNS, in spite of a rather high use of tobramycin. Finally, vancomycin and imipenem resistance was not detected at all in the patient group, but these drugs were also the least used.

The pronounced shift towards increased resistance among the patient isolates was expected. However, we cannot say whether the resistant clones were pre-existing in the individuals, selected *de novo* during treatment or acquired from the hospital environment, but it is clear that the resistant strains were enriched during treatment to eventually dominate the microflora.

In paper III, the effect of a commonly used antibiotic treatment used to eradicate *H. pylori* on the normal microbiota of enterococci was assessed. In

this study, five patients were included, all colonized with *H. pylori* and having either a duodenal or gastric ulcer for which a seven-day course of clarithromycin 250 mg b.i.d., metronidazole 400 mg b.i.d. and omeprazole 20 mg b.i.d. was indicated. Subjects who previously had been treated to eradicate *H. pylori*, or who had received antibiotic treatment within the prior 4 weeks were not included. Five other patients, who also had dyspeptic symptoms, but who did not receive any antibiotic treatment, were used as controls. During the study period, no further antibiotic treatment was initiated. In the 10 subjects, fecal samples were collected 1 day before, 3-7 days immediately after, one year, and three years after treatment. From each study patient and from each sample, 10 independent colonies were isolated on bile esculin agar, restreaked on blood agar plates and their DNA fingerprint, MIC to clarithromycin, and *erm*(B) presence determined.

Results showed that a one-week treatment of *H. pylori*, consisting of clarithromycin, metronidazole, and omeprazole, selects for highly macrolide resistant enterococci and that these can persist at least 3 years post-treatment in the absence of any further selection. Three of the five treated patients possessed highly resistant enterococci one year after the treatment was ended. In one of these patients, the resistant population persisted for three years.

Similarly, we assessed the effects of the same treatment regimen on the flora of *S. epidermidis* (paper IV). Samples from the nares of five patients were collected one day before, 3-7 days immediately after, and one and four years after treatment. From each study patient and each sample, 10 independent colonies of *S. epidermidis* were isolated on Columbia blood agar plates (Difco) and verified by Gram staining, positive catalase, negative Dnase, negative mannitolose and negative trehalose testing.

One day prior to treatment, all five patients harbored clarithromycin-susceptible (MIC <0.5 µg/ml) *S. epidermidis* among the 10 independent colonies examined. In four patients, all 10 isolates were susceptible, but in one patient, two isolates (of 10) were highly resistant due to the presence of *erm*(C). Immediately after completing treatment, four of five patients displayed high-level clarithromycin-resistant (MIC >256 µg/ml) isolates. The other isolates from this time point were either resistant with lower MIC values (16-96µg/ml) or susceptible. Highly resistant isolates still could be detected one year after treatment in four patients and in three of five patients four years post-treatment. By PCR, all highly resistant isolates harbored *erm*(C).

To explain the long-term persistence of resistance in this study, we investigated whether macrolide-resistant *S. epidermidis* changed in fitness over the 4-year study period. By performing competition experiments between isogenic clarithromycin-resistant isolates and a susceptible reference strain on the forearms of human volunteers, we provided evidence that fitness of resistant *S. epidermidis* increased. Whether this increase in fitness is due to genetic compensation of the cost associated with *erm*(C) carriage cannot be

concluded from the current data, but serves as a possible explanation to our findings. The Erm(C) methylase in *S. epidermidis* is encoded on a 2.3 kb plasmid; however, the cost of *erm(C)* to *S. epidermidis* is not known.

To summarize, antibiotic treatment affects our indigenous microbiota and can potentially give rise to long-term colonization with resistant populations. Long-term persistence of resistant enterococci and staphylococci is important for several reasons. First, both *S. epidermidis* and enterococci belong to the group of bacteria that occasionally can cause severe disease, even though they are part of the normal microbiota. Therefore, stably resistant populations increase risk of unsuccessful treatment of such infections. Second, resistance in the normal microbiota might contribute to increased resistance development among pathogens by interspecies transfer of resistant determinants. Since the population size of the normal microbiota is so large, it is possible for multiple different resistant variants to develop, which increase the risk of spread to populations of pathogenic bacteria. Persistent resistant populations in the normal microbiota further enhance the risk of transfer, especially if the selecting antibiotic is used for treatment.

CLINICAL IMPLICATIONS

In the current thesis, we present evidence that compensation of the cost of antibiotic resistance occurs in a clinical setting. Only two former reports have described such compensation *in vivo*. The implication of *in vivo* compensation is that resistant pathogens may be more difficult to get rid of than initially anticipated. Since compensatory evolution makes the resistant isolates just as fit as their susceptible counterparts, they are likely to persist and remain for a long time, even in the absence of an antibiotic pressure.

The presence of such stable resistant bacteria may confer problems for individual patients as well for the society in general. Treatment of infections caused by resistant bacteria usually takes longer than infections caused by susceptible bacteria and is thus more costly. Moreover, resistant infections have been associated with higher mortality rates in intensive care patients.

In the present thesis, we further show that clinical use of antibiotics selects for stable resistance in the human microflora. This is important for several reasons. First, many commensals occasionally can cause severe disease, even though they are part of the normal microbiota. Therefore, stable resistant populations increase the risk of unsuccessful treatment of such infections. Second, resistance in the normal microbiota might contribute to increased resistance development among pathogens by interspecies transfer of resistant determinants.

In conclusion, our results provide insight into how resistance is stabilized in a bacterial population and show that the normal microbiota may be an important reservoir of resistance genes. These findings accentuate the need for strategies to prevent resistance development. Therefore, a restrictive and more prudent use of antibiotics should continue to be encouraged together with an improved infection and transmission control. Hopefully this may serve to reduce resistance and act to prolong the lifespan of our currently available antibiotics.

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