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Bacterial Resistance to Antimicrobial Peptides

Rates, Mechanisms and Fitness Effects

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Abstract
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The rapid emergence of bacterial resistance to antibiotics has necessitated the development of alternative treatment strategies. Antimicrobial peptides (AMPs) are important immune system components that kill microbes rapidly and have broad activity-spectra, making them promising leads for new pharmaceuticals. Although the need for novel antimicrobials is great, we also need a better understanding of the mechanisms underlying resistance development to enable design of more efficient drugs and reduce the rate of resistance development. The focus of this thesis has been to examine development of bacterial resistance to AMPs and the resulting effects on bacterial physiology. The major model organism used was *Salmonella enterica* variant Typhimurium LT2.

In Paper I, we observed that bacteria resistant to PR-39 appeared at a high rate, and that the underlying *sbmA* resistance mutations were low cost or even cost-free. Such mutants are more likely to rapidly appear in a population and, most importantly, will not disappear easily once the selective pressure is removed. In paper II, we isolated protamine-resistant *hem*- and *cydC*-mutants that had reduced growth rates and were cross-resistant to several other antimicrobials. These mutants were small colony variants (SCVs), a phenotype often associated with persistent infections. One SCV with a *hemC*-mutation reverted to faster growth when evolved in the absence of protamine. In paper III, the mechanism behind this fitness compensation was determined, and was found to occur through *hemC* gene amplification and subsequent point mutations. The study provides a novel mechanism for reversion of the SCV-phenotype and further evidence that gene amplification is a common adaptive mechanism in bacteria. In Paper IV, the antibacterial properties of cyclotides, cyclic mini-proteins from plants, were evaluated. Cycloviolacin O2 from violets was found to be bactericidal against Gram-negative bacteria. Cyclotides are very stable molecules and may be potential starting points for development of peptide antibiotics.

Keywords: antimicrobial peptides, antibiotic resistance, fitness cost, bacterial evolution, small colony variants, cyclotides

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Rule #32
Enjoy the little things

List of Papers

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

I **Pränting, M**., Negrea, A., Rhen, M., Andersson, DI. (2008) Mechanism and fitness costs of PR-39 resistance in *Salmonella enterica* serovar Typhimurium LT2. *Antimicrobial Agents and Chemotherapy*, 52(8): 2734–2741.

II **Pränting M**., Andersson DI. (2010) Mechanisms and physiological effects of protamine resistance in *Salmonella enterica* serovar Typhimurium LT2. *Journal of Antimicrobial Chemotherapy*, 65: 876-887.

III **Pränting M**., Andersson DI. Escape from growth restriction in small colony variants of *Salmonella typhimurium* by gene amplification and mutation. *Submitted manuscript*.

IV **Pränting* M**., Lööv* C., Burman R., Göransson U., Andersson DI. The cyclotide cycloviolacin O2 from *Viola odorata* has potent bactericidal activity against Gram-negative bacteria. *Journal of Antimicrobial Chemotherapy*, 65: 1964-1971.

*These authors contributed equally to this work.

Contents

Abbreviations

AMP	Antimicrobial peptide
ABC-type transporter	ATP-binding cassette transporter
ALA	Aminolevulinic acid
ATP	Adenosine 5'-triphosphate
ppGpp	Guanosine 5'-diphosphate 3'-diphosphate
CF	Cystic fibrosis
cyO2	Cycloviolacin O2
HNP-1	Human neutrophil protein 1
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
tRNA	Transfer RNA
IleRS	Isoleucyl-tRNA synthetase
MDR	Multi-drug resistant/resistance
MIC	Minimum inhibitory concentration
LPS	Lipopolysaccharide
LB	Luria Bertani broth
LA	Luria Bertani agar
PCR	Polymerase chain reaction
SCV	Small colony variant
TD-SCV	Thymidine-dependent SCV
SXT	Trimethoprim/sulfamethoxazole
aa	Amino acid
Asn	Asparagine
His	Histidine
Leu	Leucine
Pro	Proline
Ser	Serine
Thr	Threonine

Introduction

Antibiotic resistance among pathogenic bacteria is increasing at an alarming rate, and at the same time, few new antibiotics reach the market. Thus, we are facing increasing problems when treating many common bacterial infections, which make development of alternative treatment strategies very important. Antimicrobial peptides (AMPs) are small peptides found in most organisms. As well as having an immunomodulatory role, they also function in the protection against microbes. AMPs have promising therapeutic properties: they kill microbes rapidly, have broad activity-spectra and there are few reports of emerging bacterial resistance, and therefore much effort is focused on finding potential novel antibacterial drugs among AMPs.

Unspecific targets and therefore high cost of resistance are some factors that have led researchers to speculate that it will be difficult or even unlikely for bacteria to develop resistance to these peptides if used in a clinical setting (Boman, 2003; Jenssen *et al.*, 2006; Schroder, 1999; Zasloff, 2002). However, no systematic study of resistance development to AMPs has been performed and we have little knowledge of the outcome with regard to resistance development if these peptides were to be used for medicinal purposes. Mutation rates will influence resistance development, as will several other factors such as fitness costs for the bacterium and ability to compensate for such costs, areas about which we have little information today for AMPs. Another concern is that by using AMPs as pharmaceuticals, we may select for bacterial strains that are resistant also to the normal repertoire of host-defense peptides in the human body (Bell & Gouyon, 2003). Although the need for new antimicrobials is great, we also need a greater understanding of the mechanisms underlying resistance development to, hopefully, be able to design more efficient antibacterials and reduce problems associated with resistance development and spread.

In this thesis, I have examined at what rate and by which mechanisms bacteria acquired resistance to antimicrobial peptides. Further, the impact of AMP-resistance on bacterial fitness and virulence was determined as well as if and how bacteria might genetically compensate for costs associated with AMP-resistance. Knowledge about these parameters is valuable for our understanding of bacterial resistance evolution in general and may help us predict the potential risk of resistance development if AMPs are to be used in a clinical setting.

Definition of fitness and resistance

Fitness is the ability of a genotype or an organism to survive, propagate, and spread in a specific environment. Consequently, the fitness of an organism may vary depending on the environment and should therefore be determined under different conditions. There are several ways to estimate fitness in the laboratory and below follows a summary of the methods used in this thesis. A simple and common approach is to measure exponential growth rates, and this was the main assay we employed (**paper I-III**). It is quite a crude measurement since it does not take into account for example time in lag phase or survival in stationary phase, but it gives an idea of the competitive ability when bacterial growth is at its fastest. Growth rates of the mutants in **paper I** were not significantly impaired, and therefore complementary fitness determinations were performed. First, survival in stationary phase was determined. Bacterial strains were grown in rich medium and then left for one month without further supplementations, and the decline in number of viable cells was monitored. We further examined how well the mutant bacteria could compete with a wild-type strain in the test tube. Pair-wise competition between the examined strain and a genetically tagged reference strain is a more accurate way to determine fitness, as this method takes into account the whole growth cycle. Since the genes affecting fitness in a more natural environment such as a mouse host can be very different from those in the test tube, competition experiments were also carried out in mice. Very small fitness costs (<1%) that will be significant on a longer time scale will still go undetected by these methods.

Resistance is another term frequently used in this thesis, and can be defined in different ways. For example, clinical resistance refers to the ability of a bacterium to resist the lethal action of an antibiotic when used at concentrations relevant for treating infections. In this thesis, resistance is used to describe a genotype or organism that is less susceptible to an AMP or antibiotic compared to the parental wild-type or reference strain. That is, these strains have acquired mutations or horizontally transferred genes that increased their resistance. This resistance level may or may not be high enough to resist potential clinical treatment concentrations of that agent.

Antimicrobial peptides

During the 1980s, a strong interest in the field of AMPs developed. At this time, the Swedish professor Hans G. Boman and his collaborators had discovered the existence of a humoral immune defense system in Drosophila (Boman *et al.*, 1972). To isolate the effector molecules Boman moved on to study a larger insect, the cecropia moth pupae, and in 1981 the primary structure of two antimicrobial peptides (cecropin A and B) was published

(Steiner *et al.*, 1981). In the following years, the research field expanded rapidly and AMPs from several different organisms, including humans, were isolated and characterized (Ganz *et al.*, 1985; Lazarovici *et al.*, 1986; Oppenheim *et al.*, 1988; Soravia *et al.*, 1988; Zasloff, 1987). Today over a 1000 AMPs have been isolated or predicted from genetic sequences and many scientists around the world are working with different aspects of AMP-activity and function. Research areas include physiological relevance of AMPs, AMP-deficiency and the connection to different diseases, antimicrobial and immunomodulatory activities and development of peptide antimicrobials from AMPs or synthetic mimics.

Characteristics and function

AMPs are widely distributed and have been found in organisms ranging from prokaryotes to plants, insects and mammals. They are generally considered as a part of the innate immune system and rapidly increase in concentration in the host upon challenge by pathogens. AMPs are expressed in many different cell types and tissues. They are gene-encoded and can be expressed constitutively or induced upon stimulation by for example bacterial components, or both (reviewed in (Yang *et al.*, 2004)). AMPs are typically small molecules, 10-60 amino acid (aa) residues in length and most are positively charged and amphipathic, containing both hydrophilic and lipophilic parts spatially separated. There is a considerable diversity in aa-content, length and structure among AMPs (reviewed in (Brogden, 2005)). Some are enriched for certain amino acids, such as the proline and arginine rich PR-39 or the histidine-rich histatins (Agerberth *et al.*, 1991; Oppenheim *et al.*, 1988). Many peptides have been found to adopt a linear, α-helical conformation, especially when associated with membranes, while others contain cysteines and form intra-molecular disulfide bridges and β-sheets. In addition, some peptides have conserved pro-sequences, like the cathelicidins, which all contain a cathelin-like proregion but a variable AMP-domain (Zanetti *et al.*, 1995).

AMPs have been shown to have direct activity against a broad spectrum of microbes including a variety of Gram-positive and Gram-negative bacteria, fungi, viruses and protozoa (reviewed in (Jenssen *et al.*, 2006)). In recent years, it has become evident that several of these peptides also functions as modulators of both the innate and adaptive immune responses. For example, human neutrophil defensins are chemotactic for T-cells and immature dendritic cells (Yang *et al.*, 2000) and the human cathelicidin LL-37 can act on the receptor formyl peptide receptor-like 1 to chemoattract neutrophils, monocytes and T-cells (De *et al.*, 2000). AMPs have also been demonstrated to influence wound healing, induce inflammation and protect against septic chock by neutralizing LPS, to name a few examples (reviewed in (Yang *et al.*, 2004)). It is likely that AMPs exert their action by both

directly killing microbes as well as by stimulating the immune system. Since the main focus of this thesis is bacterial adaptation to the direct lethal action of AMPs, from now on peptides will mainly be discussed in terms of their *antibacterial* activities.

Mechanism of antibacterial activity

Many peptides exert their primary action on the bacterial membrane in a fast and non-specific manner. The peptides must first associate with the bacteria. Initial contact is presumably mediated by electrostatic attraction between the (most often) positively charged peptide and negatively charged molecules on the surface of bacteria, such as lipopolysaccharides (LPS) on Gram-negative bacteria and teichoic acids on Gram-positive bacteria. Binding displaces Mg^{2+} and Ca^{2+} ions from the membrane and disrupts ionic cross-bridging of negative charges, in this way de-stabilizing the outer membrane/cell wall (da Silva & Teschke, 2003; Hancock, 1997; Hancock & Chapple, 1999). This is suggested to facilitate "self-promoted uptake" of peptides with subsequent accumulation in the cytoplasmic membrane, de-stabilization/pore formation and eventually cell death.

Three models of peptide-induced membrane rupture are extensively discussed in the AMP-litterature, namely the barrel-stave, the toroidal pore and the carpet model (Figure 1) (reviewed in (Brogden, 2005)). Barrel-stave pores form as peptides perpendicularly intercalate the membrane and the hydrophobic peptide parts align with the lipid tails while the hydrophilic parts line the interior of the pore (He *et al.*, 1996). Alamethicin is one of few peptides experimentally proven to employ this mechanism (Qian *et al.*, 2008). In the more commonly observed toroidal-pore model, peptide helices insert into the membrane and cause the lipid monolayer to bend so that the lipid head groups come to form the lining of the pore together with the hydrophilic parts of the peptide while the lipid tails associates with the hydrophobic peptide regions (Ludtke *et al.*, 1996; Matsuzaki *et al.*, 1998; Yang *et al.*, 2001). In the carpet model the peptides adsorb to the membrane surface and once a sufficient amount have accumulated the membrane disintegrates into micelles in a detergent-like manner (Pouny *et al.*, 1992; Shai, 1999). Peptides also influence membrane stability in many other ways. For example, at lower peptide concentrations formation of transient pores may arise and facilitate uptake (Ludtke *et al.*, 1996). Cationic peptides can also cause permeability-increasing clustering of anionic membrane lipids, and interaction between the peptides and the polar lipid head groups can lead to lateral expansion of the membrane and relaxation of the acyl chains, resulting in membrane thinning (Figure 1) (Epand & Epand, 2009; Ludtke *et al.*, 1995; Mecke *et al.*, 2005). It is important to point out that membrane disruption is a complex event that likely involves a combination of mechanisms. Peptide properties such as length, charge and hydrophobicity,

composition of the target membrane and membrane electrical potential are examples of factors that will influence the mechanism (reviewed in (Toke, 2005)).

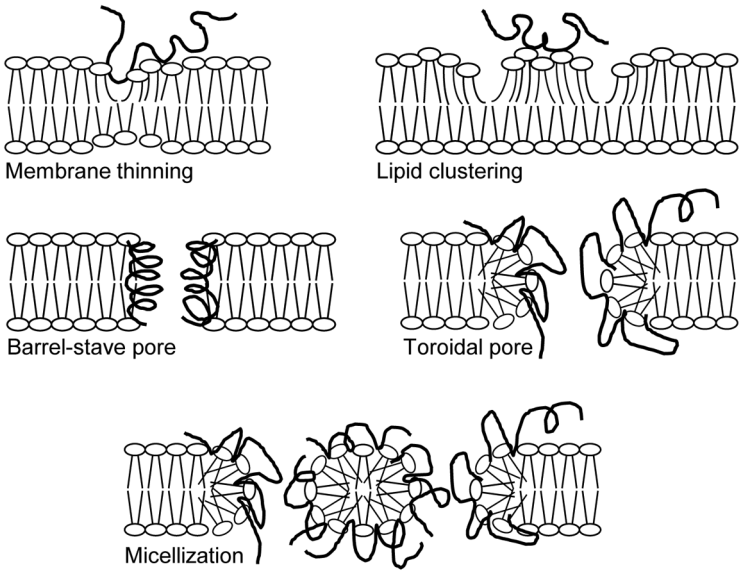

Figure 1. Membrane-permeabilizing mechanisms of AMPs.

Although the majority of AMPs seem to act on the bacterial membrane, several peptides accumulate inside bacterial cells without obvious damage to their membrane. One example is Buforin II, a linear, α-helical peptide that contains a proline-hinge that enables it to traverse the membrane and enter the cytoplasm, where it is believed to interact with DNA and RNA (Park *et al.*, 1998; Park *et al.*, 2000). Proline-rich AMPs, such as the porcine PR-39 and apidaecin from honeybees, is a large group of peptides believed to act intracellularly (reviewed in (Gennaro *et al.*, 2002)). Their final targets have in most cases not been identified. Also membrane active AMPs may have subsequent intracellular targets.

Physiological relevance of AMPs

In insects, inactivation of AMPs or AMP-regulatory genes has profound effects. For example, silencing of defensins in mosquitoes or an AMP-gene in the roundworm *Caenorhabditis elegans* results in increased sensitivity to *Staphylococcus aureus* infection and poor ability to use *Escherichia coli* as a food source without subsequent bacterial colonization, respectively (Blandin *et al.*, 2002; Roeder *et al.*). Further, immunodeficient *Drosophila* flies are extremely sensitive to microbial infection, but expression of single AMPs in these mutants can restore wild-type resistance to certain pathogens (Tzou *et*

al., 2002). But are AMPs important for the defense against microbial invasion also in mammals? Most information about the protective ability of AMPs are inferred from *in vitro* susceptibility studies and *in vivo* localization to immune cells and at surfaces exposed to microbes in the animal, but a growing body of evidence also directly confirms their relevance in living organisms. Several knock out mice models have been developed to examine the role of mice-AMPs. *Cnlp*-null mice are unable to produce CRAMP, the mouse homologue of the human cathelicidin LL-37. These mice develop more severe skin infections and are more susceptible to urinary tract infections than CRAMP-proficient mice (Chromek *et al.*, 2006; Nizet *et al.*, 2001). $MAT^{-/-}$ mice lack mature paneth cell α-defensins and are more susceptible to oral challenge with *Salmonella* than $MAT^{+/+}$ mice, and absence of β-defensin 1 allows increased bacterial colonization of the mouse bladder (Morrison *et al.*, 2002; Wilson *et al.*, 1999). In agreement with these data, expression of human AMPs in mice can increase resistance against pathogenic challenge (Bals *et al.*, 1999; Salzman *et al.*, 2003).

Additional evidence for the relevance of AMPs comes from the implication of AMP-deficiencies in several human disorders such as Crohn's disease, psoriasis and atopic dermatitis (Fellermann *et al.*, 2006; Yamasaki & Gallo, 2008). A well-described example is morbus Kostmann, a genetic disease characterized by a low number of neutrophils. Available treatment restores neutrophil levels, but patients are still prone to bacterial infections (Putsep *et al.*, 2002). It was demonstrated that patients with morbus Kostmann, as compared to healthy controls, had very low plasma and saliva concentrations of LL-37 and lacked LL-37 in their neutrophils, suggesting a role for this peptide in health. Furthermore, increased AMP resistance has been observed in clinically more invasive bacterial strains. *E. coli* isolates from patients with upper urinary tract infections were more resistant to LL-37 than those involved in disease of the lower urinary tract (Chromek *et al.*, 2006). LL-37 is synthesized and excreted in the epithelia of the urinary tract upon interaction with bacteria; hence increased LL-37-resistance may facilitate infection.

Development of peptides for medicinal purposes

Several companies are currently attempting to commercialize the use of AMPs in medicine. The peptides are pursued not only for anti-infective functions but also as immuno-stimulatory agents, anti-tumor drugs and endotoxin-neutralizing agents. To date, development of AMPs for therapeutic applications has proven difficult and as of yet no AMP-based drugs have reached the market. Many of the peptide-based drugs have failed to demonstrate efficiency in clinical trials, as exemplified by Pexiganan, a synthetic analogue of an AMP from toads that was administered in a cream for treatment of diabetic foot ulcers. Although effective in phase III clinical

trials it was not approved for use by the Food and Drugs Administration since it had no advantage over conventional antibiotics (Moore, 2003). Another example is Iseganan, a synthetic protegrin that failed to reduce oral mucositis in a phase III clinical trial, resulting in abandoned development (Donnelly *et al.*, 2003; Trotti *et al.*, 2004). Although these results are discouraging, several AMPs are still in preclinical development and in clinical trials (reviewed in (Zhang & Falla, 2010)). Some of the major issues with AMP-pharmaceuticals concern low peptide stability, costly production and pleiotropic biological effects that may result in toxicity and make it difficult to determine function and activity (Gordon *et al.*, 2005; Hancock & Sahl, 2006). These factors (among others) have restricted development and mainly topically administered AMPs have reached late stage clinical trials.

Mechanisms of bacterial resistance

Given the ability of bacteria to adapt to novel conditions and develop resistance to existing antibacterials, it seems possible that extensive use of AMPs in clinics and hospitals could select for new resistance mechanisms or lead to the dissemination of existing resistance determinants. The following observations support this hypothesis and will be discussed in more detail below. Having co-evolved with their hosts for millions of years, bacteria have already developed counteractions against the killing effects of host peptides. Whether fortuitous side activities of pathways with other purposes or evolved as a direct response to host AMPs, these mechanisms serve to protect the bacterial cells during host colonization. I refer to these mechanisms as intrinsic resistance mechanisms since they do not require additional genetic alteration of the bacteria. There are also a few examples of acquired resistance where bacteria have evolved a higher tolerance directly in response to AMPs, as compared to untreated parental strains. In addition, AMP-producing bacteria contain immunity genes for self-protection that are often located on plasmids or transposons.

Intrinsic resistance

There are numerous examples of intrinsic AMP-resistance mechanisms. Many of these have been identified by mutagenesis studies screening for hypersusceptibility to AMPs. An overview of the described mechanisms can be found in Figure 2. A common way for bacteria to increase AMP-resistance levels is by modifications that reduce the permeability or net negative charge of the cell wall or outer membrane. This presumably reduces affinity of AMPs for these structures and/or decreases interaction and uptake. Inactivation of such genes results in higher susceptibility to various AMPs and decreased virulence in mice infection models (Gunn *et al.*, 2000; Kristian *et al.*, 2003; Peschel *et al.*, 2001; Poyart *et al.*, 2003). These types of modifications have been identified in many bacterial species and are

encoded by a number of genes (reviewed in (Nizet, 2006)). To exemplify, the products of the *dltABCD* genes are responsible for addition of positively charged D-alanine to anionic teichoic acids in the cell wall of several Gram-positive bacteria (Abachin *et al.*, 2002; Peschel *et al.*, 1999; Poyart *et al.*, 2003). Another important enzyme is MprF, which mediates L-lysine modifications of phosphatidylglycerol in *S. aureus*, forming the only phospholipid with a net positive charge (Peschel *et al.*, 2001). Further, many Gram-negative bacteria modify LPS-molecules in the outer membrane with aminoarabinose (Gunn *et al.*, 2000; McCoy *et al.*, 2001; Moskowitz *et al.*, 2004). This mechanism involves several genes that are under the control of the two-component signaling pathway PmrA-PmrB. This, in turn, can be induced by the two-component regulator PhoP-PhoQ, a system that is well studied in *Salmonella*, where it is involved in sensing Mg^{2+}-concentrations in the environment and regulation of several virulence genes including AMP-resistance genes (reviewed in (Groisman, 2001)). One such gene is *pagP,* which is responsible for incorporation of an additional acyl chain in the lipid A component of LPS, resulting in decreased membrane permeability (Guo *et al.*, 1998).

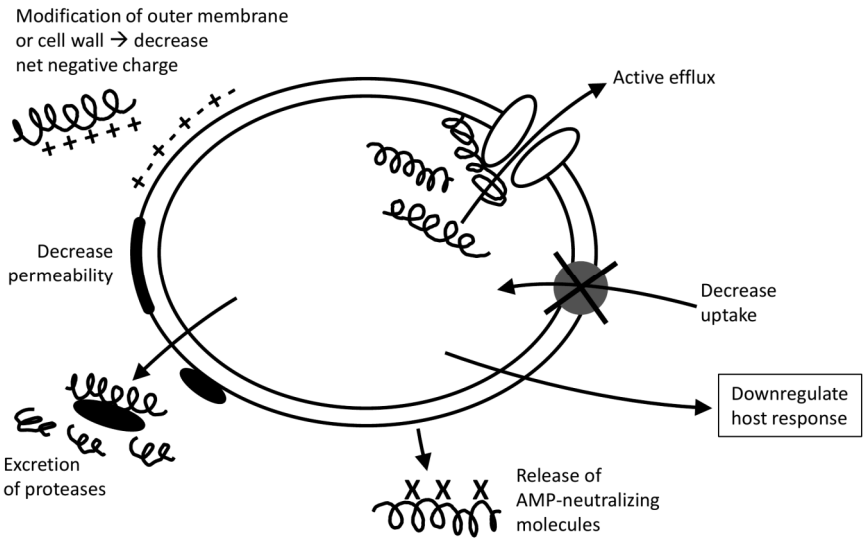

Figure 2. Intrinsic AMP-resistance mechanisms in bacteria.

Another way for bacteria to resist AMP-mediated killing is by production of proteases. For example, the *Salmonella* outer membrane protease PgtE can cleave α-helical AMPs and the metalloproteinase aureolysin from *S. aureus* can degrade the human peptide LL-37, as can proteinases of several other pathogenic bacteria (Guina *et al.*, 2000; Schmidtchen *et al.*, 2002; Sieprawska-Lupa *et al.*, 2004). Bacteria may also prevent AMPs from

reaching their target by formation of biofilms or by excretion of molecules that bind to and neutralizes the peptides (Jin *et al.*, 2004; Llobet *et al.*, 2008; Otto, 2006). Efflux systems have been associated with AMP-resistance in some cases. The Mtr-pump of *Neisseria gonorrhoeae* is an energy dependent broad-substrate transporter that can export certain peptides (Shafer *et al.*, 1998). Another example of a peptide translocase is QacA, which is encoded by a multi-drug resistance (MDR) plasmid that is frequently found in *S. aureus* strains (Kupferwasser *et al.*, 1999). In addition certain bacteria, e.g. *Shigella* species, can directly suppress expression of certain AMPs and chemokines in the host via plasmid-encoded virulence factors (Islam *et al.*, 2001; Sperandio *et al.*, 2008). The ability to resist or alter host defenses is an important virulence factor of pathogens.

Acquired resistance

A limited number of studies have attempted to examine how bacteria can acquire higher AMP-resistance than baseline-levels and how this effect physiology and virulence, and I have summarized some examples here. A few years ago, Perron *et al*. performed an important experiment in which they showed that high-level bacterial resistance to an AMP could be achieved in the laboratory (Perron *et al.*, 2006). Lineages of *Pseudomonas fluorescens* and *E. coli* were subjected to successively increasing concentrations of the synthetic magainin-analogue pexiganan, an AMP developed for pharmaceutical purposes. 22 out of 24 bacterial lineages, both mutators and non-mutators, evolved high-level resistance within 600-700 generations of growth. This resistance was stable and appeared to not affect logarithmic (log) phase growth rates of the bacteria. Unfortunately fitness was not extensively studied and the resistance determinants were not identified. In another study, Nizet and co-workers used transposon-mutagenesis and serial exposure to peptides to generate a library of Group A *Streptococcus* mutants demonstrating increased resistance to cathelicidin peptides (Nizet *et al.*, 2001). One isolated resistant clone was disrupted in a gene encoding a protein similar to transcription regulators of the GntR family. This strain was more virulent and caused lesions of larger size that also persisted longer than those of the parental strain after subcutaneous inoculation into mice. A third example is the generation of a *S. aureus* transposon-insertion mutant with increased resistance to thrombin-induced platelet microbicidal protein (tPMP) (Dhawan *et al.*, 1997). The transposon was located in the gene *snoD* (*mnhD*) that encodes a complex I NADH oxidoreductase. Disruption of this gene resulted in increased membrane fluidity, loss of transmembrane potential and increased ability of the mutant to survive in rabbits (Bayer *et al.*, 2006; Dhawan *et al.*, 1997). tPMP resistant strains have also been selected by repetitive exposure to sub-lethal concentrations of the peptide (Yeaman *et al.*, 1994). A fourth example describes resistance in *E. coli* to several proline-rich AMPs with intracellular

targets, which is mediated by reduced peptide uptake through mutations in the transporter-encoding gene *sbmA* (Mattiuzzo *et al.*, 2007). Similarly, we observed increased AMP-resistance in spontaneously formed *Salmonella typhimurium* SbmA-mutants (**paper I)**.

Resistance can be achieved by mutations that allow constitutive expression of intrinsic resistance genes. For example, certain mutations in the regulatory genes *phoP-phoQ* or *pmrA-pmrB* lead to increased resistance to cationic drugs such as protamine, polymyxins, colistin and LL-37 (Gunn & Miller, 1996; Roland *et al.*, 1993; Sun *et al.*, 2009b) and constitutive expression of phosphorylcholine, which is attached to LPS-oligosaccharides, dramatically increase resistance to LL-37 in *Haemophilus influenzae* (Lysenko *et al.*, 2000). In addition, slow growing sub-populations of bacteria commonly referred to as small colony variants (SCVs) often display an AMP-resistance phenotype (see SCV-section and **paper II** below).

AMP-producing bacteria

Bacteria themselves can produce antimicrobial peptides, which likely provide them with a competitive advantage by killing other bacteria living in the same niche. These so called bacteriocins are often more potent than AMPs of the innate immune system. To protect themselves producers carry resistance genes, usually together with the biosynthesis genes on the chromosome or on movable genetic elements such as conjugative transposons and plasmids (reviewed in (Nolan & Walsh, 2009)). These immunity proteins are usually efflux pumps, bacteriocin-sequestering enzymes or competitors for target binding (reviewed in (Chatterjee *et al.*, 2005; Cotter *et al.*, 2005). A representative example is epidermin immunity in *Staphylococcus epidermidis*. The gene cluster necessary for production and self-immunity is located on a 54-kb plasmid (Peschel & Gotz, 1996; Schnell *et al.*, 1988). Immunity is mediated (at least partly) by the EpiFEG ATP-binding cassette (ABC) transporter, which exports the peptides from the cytoplasmic membrane (Peschel & Gotz, 1996).

Nisin is a well-studied bacteriocin that is used as a food preservative. The genes encoding immunity against nisin in the producer *Lactococcus lactis* can be transformed into other bacteria where they also provide nisin-protection (Stein *et al.*, 2003). Common food-spoiling bacteria and food-borne pathogens can acquire nisin-resistance. For example, spontaneous resistance in *Listeria monocytogenes* has been estimated to occur at frequencies between 10^{-2} and 10^{-7}, depending on the strain and environmental conditions (Gravesen *et al.*, 2002). Nisin resistance mechanisms include changes in the bacterial cell membrane or cell wall, shielding of the nisin-target lipid II and excretion of inactivating enzymes (reviewed in (Chatterjee *et al.*, 2005)).

From the examples described here it is clear that bacteria may develop AMP resistance and that several AMP resistance determinants already exist in nature, which could spread if selected for.

Antibiotic resistance

Soon after the first introduction of antibiotics in the 1940s there were reports of pathogenic bacteria that could resist the lethal action of these drugs. Since then, there has been a constant struggle to develop new or modify existing antibiotics to stay one step ahead of the bacteria. Although resistance is common in environmental bacteria, it used to be less frequent among pathogenic bacteria (D'Costa *et al.*, 2006; Martinez, 2008; Wright, 2007). Today, resistant and multi-drug resistant (MDR) pathogenic strains are widespread and we are facing bigger and bigger problems when treating many common bacterial infections. At the same time, development of novel antibiotic classes has slowed dramatically and few new antibacterials have been introduced since the 1960s (reviewed in (Boucher *et al.*, 2009; Wright, 2007).

Our knowledge about bacterial resistance mechanisms and the parameters influencing antibiotic resistance development and stability is increasing, and it is clear that this is a complex problem involving several factors. When it comes to AMP-resistance, there are few examples of the physiological effects resistance have on the bacteria. Many of the same parameters that influence antibiotic resistance development will likely affect development of AMP-resistance, if these molecules are to be used as pharmaceuticals. Therefore, the next section is focused on antibiotics, common bacterial resistance mechanisms against these molecules and parameters that influence resistance development and stability.

Antibiotic targets and bacterial resistance mechanisms

There are five main targets of antibiotics: cell wall synthesis, tetrahydrofolate biosynthesis, the replication machinery, the transcription machinery and protein synthesis. Preferentially, antibiotics are designed to interfere with targets that are unique to the bacteria and therefore not harmful to human cells. Bacteria have managed to develop resistance against all antibiotics currently available for medicinal use, and there is an enormous diversity in antibiotic resistance mechanisms. Common resistance strategies include active efflux of the antibiotic from the bacterial cell, decreased cell wall/membrane permeability (by e.g. porin mutations), target alterations that reduce affinity for the antibiotic and enzymatic inactivation of the drug.

Rate of resistance development and spread

Which factors contributes to the rate of appearance and spread of resistant bacteria? As stated above, resistance is a complex problem that involves multiple parameters. These include mutation supply rates, the fitness costs of resistance, ability to disseminate resistance genes and the volume of drug usage (selective pressure). In addition to these factors, which are discussed below, it is also likely that the number of resistance genes in the environment has an impact on how fast resistance develops and disappears. For example, MDR environmental bacteria are common and may serve as a reservoir for resistance genes, as may resistant bacteria in the human commensal microflora (D'Costa *et al.*, 2006; Martinez, 2008; Sommer *et al.*, 2009). Furthermore, recent publications of whole genome sequences of bacteria have revealed a vast number of putative resistance genes that may be functioning or evolve into functioning resistance determinants (reviewed in (Wright, 2007)). This reservoir of resistance genes in the biosphere is sometimes referred to as the antibiotic resistome (D'Costa *et al.*, 2006; Wright, 2007).

Fitness costs

Not all bacteria are resistant, so there must be factors counteracting resistance development. Since antibiotics target pathways essential and optimized to bacteria, the changes that result in resistance are expected to confer a concomitant fitness cost, displayed as for example a reduced growth rate or virulence. Numerous laboratory studies have confirmed that resistance often is associated with a cost (reviewed in (Andersson & Hughes, 2010)). In some cases, this cost prevents resistant bacteria to rise in prevalence. For example, fosfomycin resistant *E. coli* can be readily selected in the laboratory, but are not observed as frequently in patients with urinary tract infections treated with this antibiotic. It has been suggested that the growth impairment conferred by fosfomycin-resistance prevents the bacteria from establishing themselves in the bladder before being flushed out (Nilsson *et al.*, 2003). This example emphasizes the importance of looking at more than one parameter when examining resistance development, i.e. a high mutation rate to resistance does not necessarily mean that an antibiotic is ineffective. High fitness costs are also positive in the sense that they should facilitate reversion of resistance in the absence of selection. It is generally assumed that once the selective pressure is removed, more fit, susceptible strains will outcompete the slower growing resistant bacteria. Unfortunately, several factors act to decrease the likelihood of reversibility, as discussed below.

Mutation rates

The combined rates of novel mutations and horizontal gene transfer (HGT) determine the rate at which resistant bacteria emerge. *De novo* chromosomal mutations can give rise to a variety of antibiotic resistance phenotypes. For example, mutations in the genes encoding ribosomal RNA or proteins may confer resistance to antibiotics that targets the ribosome, and mutations in the gene encoding DNA gyrase can decrease susceptibility to quinolones (Michael *et al.*, 2006; Roberts, 2008). Mutation rates in bacteria (per genome/replication cycle) are relatively robust and seem to have evolved to an optimal level that allows some mutations (facilitating adaptation) but not too many, since this would be detrimental to the cell (Drake, 1991; Sniegowski *et al.*, 2000). Once a beneficial mutation appears, it may increase in prevalence until present in most cells, thus becoming fixed in the population. How fast this happens will be influenced by the selective advantage of the mutation (Elena & Lenski, 2003). More than one beneficial mutation may arise independently in different bacterial cells of an otherwise homogenous population. This leads to longer fixation times as these genotypes compete against each other, a phenomenon called clonal interference (Gerrish & Lenski, 1998; Rozen *et al.*, 2002). In addition, stochastic events will influence which genotype reaches high frequency, as rare beneficial alleles may be accidentally lost (Gerrish & Lenski, 1998; Rozen *et al.*, 2002).

Occasionally, strains with an elevated mutation rate can appear and be selected for. These so called mutators are often defective in components of DNA-repair or DNA-editing pathways (reviewed in (Miller, 1996)). On a longer time scale, it is expected that a mutator phenotype will be counterselected due to the continued acquisition of deleterious mutations (de Visser, 2002; Denamur & Matic, 2006; Funchain *et al.*, 2000). However, under some circumstances being a mutator may be advantageous. Mutators are especially common in clinical samples and are enriched in harsh conditions such as those encountered during chronic infections or during antibiotic treatment (Chopra *et al.*, 2003; Denamur *et al.*, 2002; Gustafsson *et al.*, 2003; Oliver *et al.*, 2000; Roman *et al.*, 2004). Bacteria with increased mutation rates have also been selected after exposure to antibiotics both in the test tube and in mice (Giraud *et al.*, 2002; Mao *et al.*, 1997). If strong selection is applied in the form of e.g. an antibiotic, mutators are more likely to carry a mutation that will confer a selective advantage (in this case resistance), and a large selective advantage will allow the mutator gene to hitchhike along. That is, the mutator phenotype is indirectly selected for. Subsequent mutations or horizontal gene transfer that reverse the mutator phenotype may follow, resulting in a resistant strain with a normal mutation rate (Denamur *et al.*, 2000). Interestingly, results from a recent study suggest

that antibiotics themselves can act as mutagens, increasing resistance to unrelated antibiotics (Kohanski *et al.*, 2010).

Spread of antibiotic resistance

Pathogenic, antibiotic resistant bacteria can spread through e.g. infected food, water or inadequate hygienic procedures. Poor hygienic routines in hospitals and clinics and overcrowded wards are important contributors to hospital outbreaks of MDR bacteria, which often originate from one bacterial strain/resistance plasmid that spreads to many patients (Canton & Coque, 2006; Livermore & Hawkey, 2005; Maragakis *et al.*, 2008; Ransjo *et al.*, 2010). Resistance genes can spread within and between bacterial species through horizontal gene transfer (HGT). Three different processes facilitate HGT in bacteria. Transformation is the term for uptake of naked DNA into the cell and subsequent integration into the genome. The transformability of bacteria is known as competence, and varies widely between species (reviewed in (Solomon & Grossman, 1996)). Certain bacteria, for example *Streptococcus pneumoniae*, are naturally competent and efficiently take up extracellular DNA. Cells can also share DNA by cell-to-cell contact in a process called conjugation. A hair-like appendage (pilus) on one cell docks to another cell and form a bridge structure through which the DNA is passed. The third way to transfer genes is by transduction, which is mediated by viruses that infect bacteria, so called bacteriophages. Sometimes during infection, the phage particle packages some of the host DNA by mistake. When this phage infects another bacterium, the DNA may be incorporated in the genome of the new host.

Resistance genes often reside on plasmids, transposons and integrons. These elements can disseminate by HGT, and are facilitating the spread of MDR. Plasmids are circular, extrachromosomal entities that generally carry genes that are not essential for survival but confer some selective advantage, such as e.g. virulence determinants or antibiotic resistance genes (reviewed in (Frost *et al.*, 2005)). Bacteria carried plasmids also before the use of antibiotics in medicine, with the difference that they did not contain resistance genes (Datta & Hughes, 1983; Hughes & Datta, 1983). Now, plasmids are associated with most types of resistance mechanisms known. Large plasmids containing numerous resistance genes are not uncommon, and pose a serious threat to our ability to treat infections. Antibiotic genes are also often found between insertion sequences on composite transposons. Transposons carry genes that allow them to move from one genetic location to another, and after insertion into e.g. plasmids, transfer to new hosts is possible (Frost *et al.*, 2005). Resistance genes can appear as free circular DNA without promoters for transcription (so called gene cassettes). These can insert behind a promoter on a structure known as an integron via homologous recombination sites present in both elements (reviewed in (Fluit & Schmitz, 2004)). This gene acquisition can occur repeatedly, forming

"super integrons" with multiple resistance cassettes. Another genetic element associated with resistance is the conjugative transposon, which can excise from the genome and form a plasmid-like structure and promote its own transfer (Salyers *et al.*, 1995).

Selective pressure

Once resistance is acquired, selection may act to increase the frequency of the resistant clones. Natural selection results in reproduction and spread of organisms that are best adapted to a specific environment. Consequently, antibiotic resistance will be favored when antibiotics are present. Selection will be influenced by the level of exposure to the antibiotics (the selective pressure) in the patient and in the surroundings. Although antibiotics occur naturally in the environment, it is the extensive use by humans that have allowed wide spread of resistance determinants and selection of resistant and MDR pathogenic bacteria. In 2002, it was estimated that 100,000-200,000 tonnes of antibiotics are used annually over the world (Wise, 2002), and there is a substantial use and over-use of antibiotics within many areas. For example, antibiotics are used to treat animal and plant infections, promote growth of livestock and as prophylactic treatment to prevent livestock infections (Aarestrup, 2005; Mayerhofer *et al.*, 2009; Sarmah *et al.*, 2006). Apart from directly selecting for resistance, this leads to accumulation of antibiotics and resistant bacteria in the environment. Of course a major contributor is also usage within human medicine, where one problem concerns inadequate knowledge of doctors and patients. Antibiotics that are practically useless due to widespread resistance may still be prescribed, and antibiotics are also commonly given against virus infections (Reynolds & McKee, 2009; Steinman *et al.*, 2003). Many patients are not aware that antibiotics are ineffective against viruses and may pressure the doctor for a prescription. Studies have shown that patients are more content after leaving the doctor if he or she received an antibiotic prescription, whether effective against the infection in question or not (Linder & Singer, 2003; Pontes & Pontes, 2003; Welschen *et al.*, 2004). There is also the risk that patients stop taking the antibiotics prematurely when starting to feel better, which can negatively affect the outcome of treatment and/or select for resistant mutants (Kardas, 2007; Pechere *et al.*, 2007). Furthermore, in some countries doctors may economically benefit from prescribing certain pharmaceuticals (Reynolds & McKee, 2009). Another major problem is that in many countries antibiotics are available without a prescription, and these drugs may be of poor quality (Gaudiano *et al.*, 2007; Okeke & Lamikanra, 1995). A reduced antibiotic usage would likely help to limit the spread of antibiotic resistance and selection of novel resistance mechanisms, however for this to happen, stricter legislations as well as informational campaigns are necessary.

Reversibility of resistance

Will bacterial resistance disappear if we discontinue use of a particular antibiotic? A few clinical studies have tried to address this question. In two studies, one in Finland and one in Iceland, reduced antibiotic consumption was followed by a decrease in the number of resistant bacteria (Kristinsson, 1997; Seppala *et al.*, 1997). However, the cause could not be directly linked to decreased antibiotic use and was instead suggested to occur by clonal replacement of the resistant bacteria (Arason *et al.*, 2002; Kataja *et al.*, 2002). Reduced use of sulphonamides in the United Kingdom over a period of nine years did not decrease the prevalence of sulphonamide-resistant *E. coli*. Genetic linkage to other, selected resistance genes and low cost of mutations were suggested to counteract reversibility (Enne *et al.*, 2001). Similarly, a Swedish intervention study where use of trimethoprim was restricted resulted in only a small decrease of trimethoprim resistance in *E. coli* (Sundqvist *et al.*, 2010). It seems that reversibility of resistance is not as straightforward as once believed, and several processes work against reversibility, some of which are discussed below.

Co-selection of resistance genes

Co-selection can allow an antibiotic resistance mechanism to persist even if that specific antibiotic is not present, and is a major concern for the prospects of reversing resistance. One example of current interest comes from the rapid spread around the world of MDR-plasmids encoding extended spectrum beta-lactamases (Canton & Coque, 2006). These plasmids are found in Gram-negative bacteria and some confer resistance to most if not all classes of antibiotics. Any one of these is enough to select for the whole plasmid. In addition, these large plasmids may carry genes that confer resistance to metals such as copper or silver, which may be enough for selection of the plasmid in the environment. The combination of MDR-plasmids and intrinsically resistant bacteria such as *Pseudomonas aeruginosa* is disastrous as potential treatment options for such infections are quickly disappearing (Boucher *et al.*, 2009; Chopra *et al.*, 2008). Another important observation is that resistance plasmids may increase fitness of their host independent of the resistance markers they carry (Dionisio *et al.*, 2005; Enne *et al.*, 2004; Yates *et al.*, 2006). For example, two resistance plasmids in *E. coli* from the commensal flora of calves could be conjugated to new *E. coli* hosts where they conferred a competitive advantage (Yates *et al.*, 2006). Consequently, resistance genes on such plasmids may be maintained without antibiotic selection.

One resistance mechanism may confer cross-resistance to several antibiotics, further increasing the potential for co-selection. That is, several antibiotics can select for and stabilize the same resistance determinant. For example, overexpression of certain efflux pumps can provide resistance to a

wide variety of antibiotics (Nikaido, 1996). New resistance mechanisms may also evolve from existing ones. A few years ago, a variant aminoglycoside acetyltransferase was found to act not only on aminoglycosides but also on ciprofloxacin, a synthetic, structurally dissimilar antibiotic (Robicsek *et al.*, 2006).

Fitness costs and reversibility

Although most resistance mutations confer a cost for the bacterium, some mutations appear to be low cost or cost-free. Amino acid substitutions in ribosomal protein S12 can result in streptomycin resistance. In both *E. coli* and *S. typhimurium*, a K42R (lysine→arginine) substitution that confers high-level resistance appears to be cost-free. That is, no (or small) fitness effects have been observed in laboratory media and animal infection models (Bjorkman *et al.*, 1998; Enne *et al.*, 2005). Furthermore, the same substitution is the most common mutation encountered in clinical isolates of *Mycobacterium tuberculosis* (Bottger *et al.*, 1998; Sander *et al.*, 2002). Similarly, low-cost rifampicin mutations identified in the laboratory are frequently found also in clinical isolates (Billington *et al.*, 1999; O'Neill *et al.*, 2006). These studies indicate, as might be expected, that low cost mutations are those preferentially selected in a clinical setting, and may pose a problem for reversibility. Such mutants are likely to appear more rapidly in a population when antibiotics are present and, most importantly, are less likely to disappear in the absence of a selective pressure (Andersson & Hughes, 2010; Bjorkman *et al.*, 1998; Bottger *et al.*, 1998; Pym *et al.*, 2002; Ramadhan & Hegedus, 2005; Sander *et al.*, 2002).

Fitness costs are not necessarily the same in different environments. For example, certain rifampicin resistance mutations can confer a cost to bacteria when in exponential growth phase, but still accumulate in ageing bacterial colonies. Pre-existing rifampicin resistant mutants seem to have a growth advantage in this setting and can continue to grow while parental bacteria do not (Wrande *et al.*, 2008). Likewise, a *gyrA*-mutation causing ciprofloxacin resistance in *Campylobacter jejuni* was shown to increase survival in the absence of antibiotic selection in a chicken infection model, but conferred a cost when transferred to another *Campylobacter* strain (Luo *et al.*, 2005). Thus, resistance may provide a competitive advantage even in the absence of antibiotics. Furthermore, extremely slow growing antibiotic resistant mutants are also sometimes selected *in vivo* (Lannergard *et al.*, 2008; Proctor *et al.*, 2006). Evidently, slow growth may be favored in some situations. These examples emphasize the need to perform fitness determinations under several conditions, and shows that it is not always simple to infer the effect on fitness from *in vitro* studies.

Compensatory evolution

Another observation that further decrease the prospects of reversing antibiotic resistance in bacteria is that fitness costs associated with resistance can be ameliorated by second-site mutations (Bjorkman *et al.*, 1998; Levin *et al.*, 2000; Nagaev *et al.*, 2001; O'Neill *et al.*, 2006; Paulander *et al.*, 2007; Schrag & Perrot, 1996). Such compensatory mutations may partly or even completely restore bacterial fitness without affecting the resistance phenotype, and may potentially stabilize a resistant population even in the absence of an antibiotic selective pressure. Compensatory mutations can be intragenic, i.e. in the same gene as the original resistance mutation, or extragenic, in another gene. The cost associated with a certain resistance mutation can often be compensated by several different mechanisms involving both intragenic and extragenic mutational events. One example is provided by a study of compensatory evolution in a streptomycin-resistant *S. typhimurium* strain (Maisnier-Patin *et al.*, 2002). A lysine to asparagine substitution at position 42 of ribosomal protein S12 in this strain is responsible for streptomycin-resistance and causes increased translation fidelity and slow growth. Growth compensated mutants could be isolated after evolving independent lineages of the S12-mutant through population bottlenecks in LB-medium. Thirty-six different compensatory mutations were found in four different ribosomal proteins including S12. The compensatory mutations likely shifted translation fidelity of the ribosome back to a more normal ("error-prone") state, allowing faster growth. Only a few strains reverted back to the original wild-type sequence. Although resistant bacteria can revert back to wild-type sequence at low frequencies, the target for compensatory mutation is usually larger and therefore the probability for such mutations to occur is higher. Furthermore, compensatory mutations may themselves impose a fitness cost if separated from the resistance mutation (Bjorkman *et al.*, 1999; Schrag *et al.*, 1997). In this case, loss of the resistance mutation will simultaneously decrease bacterial fitness, decreasing the probability of resistance-reversion.

Another way to achieve compensation is by increasing the concentration of a gene product through gene amplifications or promoter-up mutations. An example comes from a study of bacterial resistance to mupirocin, an antibiotic that inhibits the essential enzyme isoleucyl-tRNA synthetase (IleRS) (Paulander *et al.*, 2010). Changes in this protein lead to resistance, but concomitantly reduced bacterial fitness. The bacteria could compensate for this cost by additional IleRS-changes or by increasing the levels of the defective IleRS, either through an increase in *ileS* copy number (amplification) or by increasing expression through promoter mutations, or both. The authors suggest that transient *ileS* gene amplifications, which increased both population size and copy number, increased the likelihood of subsequent rare compensatory mutations. Additionally, gene amplifications

and subsequent overproduction of a second-site gene-product has been found to ameliorate the fitness burden in actinonin-resistant *Salmonella* (Nilsson *et al.*, 2006).

The role of amplification in adaptation

Adaptation in the "Cairns system" provides an illustrative example of how amplification can facilitate adaptation. In this experimental system, a leaky frameshift mutation in *lac* prohibits bacterial growth on minimal medium with lactose (Cairns & Foster, 1991). Still, over time, revertant Lac^+ colonies accumulate on this medium, and it was originally suggested that lactose-selection in itself increase the rate of reversion by increasing the general mutation rate in the cell (Cairns & Foster, 1991; Ponder *et al.*, 2005; Rosenberg & Hastings, 2004). However, there is now compelling evidence for an amplification-facilitated evolutionary trajectory (Figure 3) (Andersson *et al.*, 1998; Roth & Andersson, 2004; Roth *et al.*, 2006). Preformed (i.e. existing before addition of bacteria to the lactose medium) duplications/amplifications of the leaky *lac*$^-$-allele in some cells is suggested to provide selective advantage to a small part of the population by allowing slow growth. This growth may enable further increase in copy number and additional growth increase. As the number of cells in the population and the number of *lac*-copies in each cell increases, so does the probability for rare mutations that restore Lac function. Once a *lac*$^+$-allele arises fitness is increased substantially, leading to released selection for the amplification since it no longer provides a growth advantage, and the amplified array can segregate back to the haploid state.

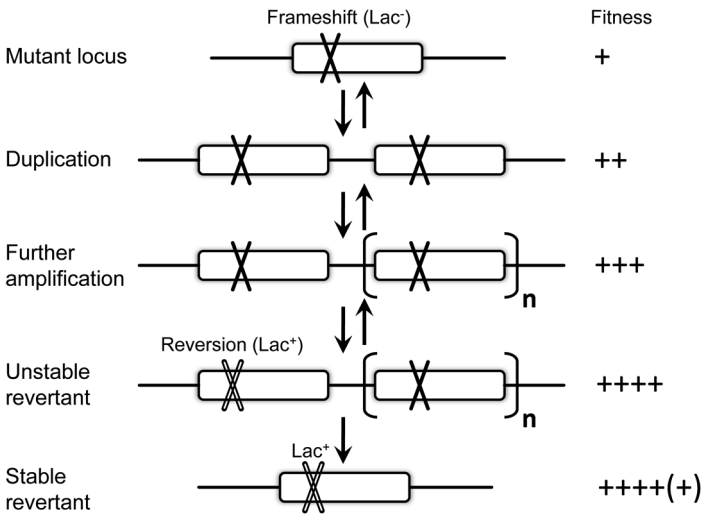

Figure 3. Amplification model

The mutation rate/base-pair/generation in *S. typhimurium* has been estimated to ~10^{-9}-10^{-11} (Hudson *et al.*, 2002), while duplications have been estimated to be present at frequencies of approximately 10^{-2}-10^{-5} depending on the genetic location (Anderson & Roth, 1981). Consequently, a cell is much more likely to carry a duplication than a point mutation. Duplications/amplifications are increasingly considered as an important driving force for adaptation and evolution. In **paper III**, we suggest that compensation of the small colony phenotype of a protamine resistant strain is facilitated by transient gene amplifications in a similar way as in the Lac-system above. Gene duplications and amplifications have also been implicated in adaptation in several other systems were growth is restricted by e.g. limited carbon sources or harmful substances such as antibiotics or heavy metals (Fogel & Welch, 1982; Newcomb *et al.*, 2005; Reams & Neidle, 2003; Sonti & Roth, 1989; Sun *et al.*, 2009a).

Small colony variants (SCVs)

As the work with this thesis progressed, a substantial part came to involve genetically distinct, slow growing subpopulations of bacteria commonly referred to as SCVs (growth of one of these strains on LA is shown in Figure 4). Such variants have been described for many different bacterial species including *P. aeruginosa*, *S. epidermidis*, *E. coli* and different *Salmonella* serovars, but *S. aureus* SCVs have been most extensively studied (Baddour *et al.*, 1990; Cano *et al.*, 2003; Colwell, 1946; Haussler *et al.*, 1999; Kahl *et al.*, 1998; Morris *et al.*, 1943; Proctor *et al.*, 1995; Proctor *et al.*, 2006; Roggenkamp *et al.*, 1998). It has been suggested that the ability to form SCVs promote survival and persistence and is important for the disease progression of intracellular pathogens (Proctor *et al.*, 2006). We and others have found these variants to be more resistant to several AMPs as compared to their parental counterparts, and it is therefore relevant to include a discussion of the characteristics and importance of SCVs here.

Characteristics of SCVs

Slow growth (it can take several days for a SCV to appear on standard laboratory agar), lack of pigmentation, inability to use many carbon sources, unusual biochemical profile and auxotrophy for hemin, menadione or thiamine are some typical SCV-characteristics (reviewed in (Proctor *et al.*, 2006)). Furthermore, the SCV-phenotype can be unstable, and the cells often exist in mixed populations together with faster growing strains that will overgrow the SCV if grown in standard laboratory media. These features make it difficult to recover and correctly identify the strains in the clinical microbiology laboratory (Proctor *et al.*, 1998; Proctor *et al.*, 1995; Spanu *et*

al., 2005). SCVs are less virulent than their faster growing parents in most instances, but can cause more severe infections and be more cytotoxic in some cases (Jonsson *et al.*, 2003; Proctor *et al.*, 2006; von Gotz *et al.*, 2004).

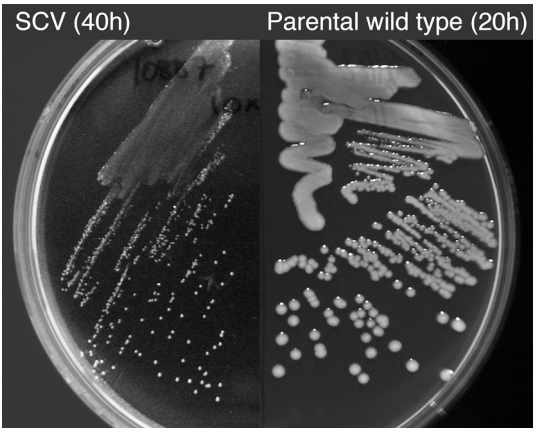

Figure 4. Growth of a *S. typhimurium* SCV (40 h incubation) and the parental wild-type strain (20 h incubation).

Another important feature of SCVs is their increased tolerance to several antibiotics, in particular aminoglycosides (reviewed in (Proctor *et al.*, 1998; Proctor *et al.*, 2006)). Susceptibility determinations with SCVs are problematic. Their slow growth will for example alter the time needed for measuring susceptibility, making it difficult to standardize the testing methods, and if faster growing variants are co-isolated with the SCVs or appear during testing, misinterpretation of the resistance profile may result. Even though a SCV appear susceptible to an antibiotic *in vitro*, it still does not guarantee successful patient treatment. SCVs are even less susceptible to antibiotics when attached to biopolymer surfaces (Chuard *et al.*, 1997), can have an increased ability to form biofilms (Haussler, 2004), are often associated with implanted devices or prosthetics (Baddour *et al.*, 1990; Roggenkamp *et al.*, 1998; Spanu *et al.*, 2005; von Eiff *et al.*, 1999) and have an increased capacity to survive within cells (Cano *et al.*, 2003; von Eiff *et al.*, 1997), in this way escaping exposure to antimicrobials. As a result, SCVs are often involved in persistent and recurrent infections that are difficult to treat (Haussler *et al.*, 1999; Kahl *et al.*, 1998; Proctor *et al.*, 1995; Roggenkamp *et al.*, 1998).

Clinical relevance of SCVs

In 1995, Proctor and co-workers described five patient cases where the causative organisms were *S. aureus* SCVs (Proctor *et al.*, 1995). Four patients had recurrent (sometimes years apart) bone and joint infections and

one patient had a sinus infection. All infections were refractory to antibiotic treatment and symptoms often persisted despite treatment with several different antibiotics over extended periods. Up until this time the connection between persistent and recurrent infection and SCVs had not been appreciated, but SCVs had been isolated from patient samples and described also before this. For example, SCVs of *Salmonella* (*Eberthella typhosa*) had been found in typhoid patients, dwarf colonies of *E. coli* in urine samples from patients with urinary tract infection and variant forms of *Neisseria gonorrhoeae* had been associated with gonococcal infection (Borderon & Horodniceanu, 1978; Morris *et al.*, 1943; Morton & Shoemaker, 1945). Today, reports have been published on various types of SCV-associated infections caused by both Gram-negative and Gram-positive bacteria. Cystic fibrosis (CF), osteomyelitis, abscesses and foreign body related infections are some examples (Abele-Horn *et al.*, 2000; Haussler *et al.*, 1999; Kahl *et al.*, 1998; Kipp *et al.*, 2003; Rolauffs *et al.*, 2002; Spanu *et al.*, 2005; Spearman *et al.*, 1996; von Eiff *et al.*, 1999), but SCVs have also been implicated in more common infections such as urinary tract infections (Tappe *et al.*, 2006).

The prevalence of SCVs in clinical specimens has been evaluated in a few studies. Small colonies were observed in 15 out of 1110 (~1%) *S. aureus* isolates from clinical material (specific sites not specified) and *N. gonorrhoeae* SCVs were associated with 2% of examined gonococcal cases (Acar *et al.*, 1978; Morton & Shoemaker, 1945). SCVs are especially frequent in patients with CF, a genetic disorder that result in abnormal respiratory secretions and severe lung infections. SCVs were observed in ~40% (33/86) of patients harboring *P. aeruginosa* and in 49% (26/53) of *S. aurues* positive CF-patients (Haussler *et al.*, 1999; Kahl *et al.*, 1998). Approximately 4% of the CF-patients from nine CF-centers in Belgium were SCV carriers (Vergison *et al.*, 2007). Only a few of these centers actively screened for these variants. Since SCVs are so difficult to recover, there is likely an underestimation in the number of reported cases.

Types of SCVs

SCVs from clinical samples are often defective in the biosynthetic pathways of heme, menadione or thymidine as determined by supplementation studies. Heme serves as a cofactor for many cytochromes and menadione is needed for production of menaquinone, both important components of the electron transport chain. Defects in these pathways result in defects in electron transport and respiration, which in turn leads to reduced levels of ATP and a decreased membrane potential, effects that can explain many of the observed SCV-characteristics (discussed in (Proctor *et al.*, 1998; Proctor *et al.*, 2006). For example, low amounts of ATP will be available for membrane biosynthesis and metabolism resulting in slow growth, defective respiration

decreases ability to utilize sugars and a reduced membrane potential has been suggested to increase resistance to aminoglycosides by means of decreased uptake (although studies in our lab indicates the possibility also of other resistance mechanisms, see below). Construction of a stable, genetically defined SCV by insertion of an erythromycin resistance cassette into *hemB* in *S. aureus*, has confirmed the characteristics observed for clinical SCVs (von Eiff *et al.*, 1997). Hemin and menadione auxotrophic mutants can be isolated from patients with e.g. osteomyelitis, wound- and implant-related infections (Abele-Horn *et al.*, 2000; Proctor *et al.*, 1995; Spearman *et al.*, 1996; von Eiff *et al.*, 1999).

Thymidine dependent SCVs (TD-SCVs) have been isolated primarily from patients with CF, but can also be found in e.g. wounds or in blood samples (Besier *et al.*, 2008b)) and are especially common in patients treated with trimethoprim/sulfamethoxazole (SXT). Many of these strains have mutations in *thyA*, encoding thymidylate synthase (Besier *et al.*, 2007; Chatterjee *et al.*, 2008). The bacteria cannot produce thymidine, which is essential for DNA synthesis, and must therefore take up thymidine from the environment to grow. This also leads to SXT-resistance by a bypass mechanism. SXT inhibits synthesis of tetrahydrofolic acid, a co-factor for thymidylate synthase, which is no longer needed. It is not fully understood how TD-SCVs can have many of the same characteristics as SCVs with heme or menadione deficiency, although it has been shown that the tricarboxylic acid cycle activity is defective and that transcription-level of several genes, including the virulence regulator *agr*, is changed in the TD-SCVs (Chatterjee *et al.*, 2007; Kahl *et al.*, 2005). Some of these strains may also have additional, unidentified mutations. CF-associated bacterial strains have been found to more often have high mutation rates than non-CF isolates, with TD-SCVs frequently having a mutator or even strong mutator phenotype (Besier *et al.*, 2008a; Ciofu *et al.*, 2005; Oliver *et al.*, 2000)

A large number of genes in the bacterial genome (in addition to those described above) encode proteins important for respiration and retaining redox-potential. That is, the potential target for formation of SCVs is very large. In **paper II**, we isolated peptide resistant SCVs of *S. typhimurium*. Most of these strains carried mutations in heme-biosynthesis genes but three strains had mutations in *cydC*, a gene important for maintaining correct redox environment in the bacterial cell.

Present investigations

Here follows a description of the model organisms and peptides studied in this work, and a summary of the main results and conclusions of the studies included in this thesis.

Model organisms

The main model organism used in this study is *Salmonella enterica* serovar Typhimurium LT2 (referred to as *S. typhimurium*). In humans, this Gram-negative, intracellular pathogen causes gastroenteritis with symptoms such as fever and diarrhea (Hohmann, 2001). The bacteria colonizes the small intestine and invade epithelial cells and M cells in the Peyer's patches, in this way traversing the intestinal epithelium (Mastroeni & Sheppard, 2004). The ability of certain *Salmonella* serovars to cause systemic disease is dependent on survival and replication within host macrophages (Fields *et al.*, 1986; Ohl & Miller, 2001). Several animal models have been developed to study interactions between the bacteria and their host, for example the murine typhoid fever model (reviewed in (Mastroeni & Sheppard, 2004)). In mice, *S. typhimurium* causes a systemic infection that resembles the severe human disease typhoid fever. The LT2 strain has a reduced level of synthesis of the *rpoS* gene product, which makes it less virulent than other strains (Swords *et al.*, 1997), but it is still capable of causing systemic disease in mice. Furthermore, its genome is fully sequenced and there are several tools available for genetic manipulation.

For cyclotide activity studies in **Paper IV**, susceptibility of three Gram-positive bacterial strains (*S. aureus*, *S. epidermidis* and *Streptococcus pyogenes*) and six Gram-negative strains (*S. typhimurium* LT2, *E. coli* MG1655, *P. aeruginosa* and three different *Klebsiella pneumoniae* strains) were examined. These strains included both laboratory strains and clinical isolates.

Model peptides

Peptides of different origin and mode of action were studied in this thesis. The main peptides examined were PR-39, protamine and a set of cyclotide-

peptides and are described in detail below. An additional set of peptides was used for cross-resistance studies: LL-37, CNY100H-L, mCRAMP, rCRAMP, lactoferricin B (fragment 4-14) and human neutrophil protein 1 (HNP-1). The origin, type and sequence of each peptide (except protamine, see below) are listed in Table 1 or Figure 5 (peptides containing disulfide bridges).

Table 1.

Peptide	Origin/Type	Sequence
CNY100H-L	Man/Synthetic	CKYILLLRRQHARAWRRGLR
mCRAMP	Mouse/Cathelicidin*	ISRLAGLLRKGGEKIGEKLKKIGQKIKNF FQKLVPQPE
rCRAMP	Rat/Cathelicidin*	RFKKISRLAGLLRKGGEKFGEKLRKIGQ KIKDFFQKLAPEIEQ
Lactoferricin B (4-14)	Cow/Fragment**	RRWQWRMKKLG
LL-37	Man/Cathelicidin*	LLGDFFRKSKEKIGKEFKRIVQRIKDFLR NLVPRTES
PR-39	Pig/Cathelicidin*	RRRPRPPYLPRPRPPPFFPPRLPPRIPPGFP PRFPPRFP

*Processed into their active form by proteolytic enzymes
**Generated by enzymatic cleavage of lactoferrin

PR-39 (paper I)

PR-39 is a linear, proline- and arginine-rich peptide that was isolated from porcine small intestine in 1991 (Agerberth *et al.*, 1991), and later from porcine neutrophils (Shi *et al.*, 1994) and wound fluid (Gallo *et al.*, 1994). It is transcribed as a prepropeptide classified as a cathelicidin, which is processed to its active form presumably by elastase (Cole *et al.*, 2001; Zaiou & Gallo, 2002). The peptide function as a broad-spectrum antibacterial through a non pore-forming mechanism, properties shared by other proline-rich peptides found in both mammals and invertebrates (Boman *et al.*, 1993; Gennaro *et al.*, 2002; Otvos, 2002; Shi *et al.*, 1996). PR-39 acts on multiple intracellular targets, including DNA and protein synthesis, possibly through induction of proteolytic activity in the bacteria (Boman *et al.*, 1993). The non-lytic effect has been confirmed by scanning electron microscopy studies that also revealed formation of elongated cells indicative of cell-division defects (Shi *et al.*, 1996). However, studies with other proline-rich peptides indicate that they may have membrane-perturbing effects under some experimental conditions (Skerlavaj *et al.*, 1990). We used PR-39 that was synthesized in its active form by EzBiolab Inc. and Innovagen AB.

Protamine (paper II and III)

Protamines are small, cationic and basic histone-like peptides that are rich in arginine residues. These peptides are found in for example sperm cells of vertebrates, where they are involved in packaging of DNA (reviewed in (Balhorn, 2007)). This packaging is thought to protect the genetic material from physical and chemical damage and influence which genes are expressed in the early embryo. Protamine sulphate from fish milt has been found to exert antimicrobial activity against a variety of organisms (Hansen & Gill, 2000; Islam *et al*, 1984; Johansen *et al.*, 1995; Kamal & Motohiro, 1986; Uyttendaele & Debevere, 1994). Mechanism of bacterial killing is not fully understood, but presumably involves initial contact through electrostatic interactions between the positively charged peptide and negative charges on the bacterial envelope, followed by a general disruption of the membrane, leakage of cellular components and/or inhibition of intracellular targets (Aspedon & Groisman, 1996; Johansen *et al.*, 1997; Stumpe & Bakker, 1997). Protamine is lacking the typical amphiphilic aa-composition of AMPs, but it has still been used successfully as a model peptide in several studies substituting for AMPs that are expensive, difficult to isolate or easily inactivated (Dhawan *et al.*, 1997; Groisman *et al.*, 1992; Sadowska *et al.*, 2002; Yeaman *et al.*, 1996). Small sequence heterogeneities among protamines make isolation and purification difficult, and as a result the protamine sulphate product contains a mixture of slightly different peptides (Chang *et al.*, 2001).

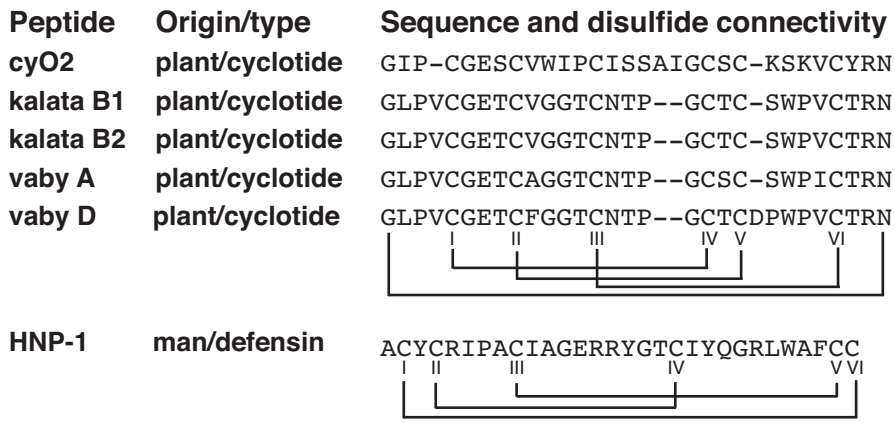

Figure 5. Origin, type and aa-sequence of disulfide containing peptides used in the study. The cysteine-residues are connected I-IV, II-V and III-VI in cyclotides and I-VI, II-IV and III-V in HNP-1.

Cyclotides (paper IV)

Cyclotides are gene-encoded mini-proteins found in abundance in plants from the Violaceae and Rubiaceae plant families (Craik *et al.*, 1999; Gruber *et al.*, 2008). The peptides form an end-to-end cyclic structure and contain several cysteine residues that form a "knot-motif" through disulfide connectivity (Figure 6a) (Craik *et al.*, 1999; Göransson & Craik, 2003; Saether *et al.*, 1995). These structural features stabilize the peptides and provide protection against thermal, proteolytic and chemical degradation (Colgrave & Craik, 2004; Ireland *et al.*, 2006). There are two cyclotide subfamilies, the Möbius and the bracelets. The Möbius peptides have a twist in the peptide backbone caused by a *cis*-proline peptide linkage, which the bracelets lack (Craik *et al.*, 1999). A diverse range of biological activities have been demonstrated for cyclotides, including antifouling, anti-HIV, anti-tumor, insecticidal and anthelmintic effects, but their main natural function has been proposed to be protection against plant pathogens (Colgrave *et al.*, 2008; Göransson *et al.*, 2004; Ireland *et al.*, 2008; Jennings *et al.*, 2001; Lindholm *et al.*, 2002). Activity has been suggested to be mediated through interaction with and destabilization of lipid membranes (Huang *et al.*, 2009; Shenkarev *et al.*, 2006; Svangård *et al.*, 2007). In **Paper IV**, we investigated the antibacterial properties of four möbius (kalata B1 and kalata B2 from *Oldenlandia affinis*; vabyA and vabyD from *Viola abyssinica*) and one bracelet cyclotide (cycloviolacin O2, cyO2 in short, isolated from *Viola odorata*, Figure 6b).

(a) (b)

Figure 6. (a) The cyclotide framework, represented by cyO2. The structure contains a β-sheet (blue arrows), a small α-helix (blue) and three disulfide bridges (yellow). (b) *Viola odorata* (sweet violet).

AMP resistant bacteria can be selected at high rates

In **paper I** and **II**, spontaneously occurring AMP-resistant mutants were selected on Iso-Sensitest agarose medium supplemented with PR-39 or

protamine sulphate, respectively. Drops with bacteria were applied onto the medium and after incubation the number of appearing colonies was counted and independent clones were isolated. The mutation rate to PR-39 resistance was calculated to 4×10^{-7}/cell/generation and the mutation rate to protamine sulphate resistance to 2×10^{-7}/cell/generation. These mutation rates are actually higher than those estimated for *S. typhimurium* for many conventional antibiotics. For example, the mutation rate in LB to rifampicin and nalidixic acid resistance has been calculated to approximately 2×10^{-8} and 4×10^{-9}/cell/generation, respectively (Koskiniemi *et al.*, 2010). The minimum inhibitory concentration (MIC) of PR-39 increased about three- to fourfold for the resistant mutants compared to the wild-type parental strain, as measured by a microdilution assay. When killing was monitored over time the resistant strains could better survive and recover from treatment also with PR-39 concentrations higher than the MIC. The level of protamine sulphate resistance varied substantially between the isolated mutants, and observed MIC-values ranged from 2 to 20 times of that needed to inhibit growth of the wild-type strain.

Mutations in *sbmA*, *hem*-genes and *cydC* cause AMP-resistance

Mutations responsible for AMP-resistance were identified using a transposon insertion method. Strains were isolated in which the resistance mutation had been replaced by wild-type sequence linked to a transposon. In all 21 PR-39 resistant mutants (**paper I**) the transposon was inserted close to the gene *sbmA*. Exact positions of the mutations were identified in ten mutants by PCR and sequencing. All ten mutations were located at different sites in *sbmA* and included aa-substitutions, small deletions and nonsense mutations. A constructed *sbmA*-deletion strain had similar PR-39 resistance level as the spontaneous mutants, and expression of SbmA from a plasmid restored PR-39-susceptibility. These results confirmed the connection between SbmA and the resistance phenotype and suggested a nearly complete or complete inactivation of the gene product in the mutants. In *E. coli*, *sbmA* encodes an inner membrane protein predicted to be part of an ABC-type transporter, and mutation in this gene has been implicated in resistance to several proline-rich AMPs (LeVier & Walker, 2001; Mattiuzzo *et al.*, 2007). By fluorescently labeling one of the peptides, Mattiuzzo *et al* showed that less peptide was internalized in an *sbmA*-mutant than in the wild-type strain (Mattiuzzo *et al.*, 2007). The *S. typhimurium* and *E. coli sbmA* genes are ~82% identical on the nucleotide level and share ~92% of the amino acids, and likely have similar function. Therefore we suggest that

the mechanism of PR-39 resistance in *S. typhimurium* is decreased uptake of the peptide into the bacterial cell by the defective SbmA-transporter.

Mutations causing protamine sulphate resistance were found in *hemA* (aa-substitutions, deletions and nonsense mutations), *hemB* (deletions), *hemC* (aa-substitutions), *hemL* (aa-substitutions, frame shift mutations) and *cydC* (nonsense mutations). The *hem*-genes are involved at different stages in synthesis of heme, the prosthetic group of many cytochromes and catalses (Figure 7). The *hemL* and *hemA* gene products are required to synthesize the intermediate compound aminolevulinic acid (ALA), while *hemB* and *hemC* are involved in subsequent steps (Elliott & Roth, 1989; Xu *et al.*, 1992). Since defects in electron transport and respiration would explain many of the characteristics of the mutants, we hypothesized that lack of heme and not any other intermediate in the biosynthesis pathway was responsible for protamine resistance. Several observations supported this hypothesis. 1) Protamine sulphate resistance mutations were found in four different heme-biosynthesis genes. 19/23 resistant strains carried *hem*-mutations at in total 14 different locations. 2) A *hemB* and *hemG* mutant from our strain collection had similar growth and protamine resistance pattern as the spontaneous protamine mutants. 3) Growth of *hemL* and *hemA* mutants could be improved by addition of ALA to the medium. In addition, growth and susceptibility of a *hemA*-mutant could be restored with extracellular hemin (after introduction of an envelope mutation that allowed hemin-uptake). 4) Inactivation of *cysG*, the first step in siroheme and cobalamin synthesis after the branch point from the heme-biosynthetic pathway (Figure 7), in the wild-type strain did not increase protamine resistance and did not affect ALA-complementation when in combination with an *hemA*-mutation.

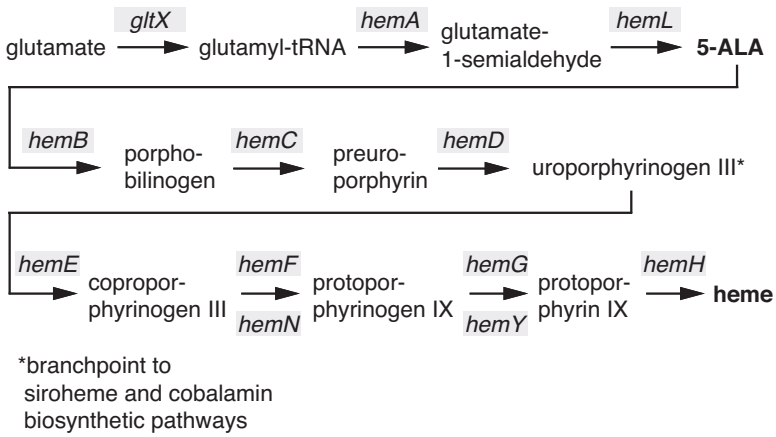

Figure 7. The heme-biosynthetic pathway in *Salmonella.*

Three mutants acquired stop codons in *cydC*. These mutants grew similarly to the *hemL*-mutants on LA-plates (not as severe growth defect as

other *hem*-mutants and less resistant). The *S. typhimurium cydC* sequence is about 75 % homologous to *cydC* in *E. coli*, where the *cydDC*-operon encodes an ABC-family transporter (Poole *et al.*, 1993). Defects in this transporter, among other things, result in incorrect assembly of cytochrome *bd* terminal oxidase and to an imbalance in the periplasmic redox environment (Pittman *et al.*, 2005; Poole *et al.*, 1993). That is, a *cydC* mutation will result in pleiotropic effects on redox homeostasis and the respiratory chain in the bacterial cell. The characteristics (discussed in more detail below) and identified mutations led us to conclude that all the protamine sulphate resistant strains were SCVs.

PR-39 and protamine resistance mutations confer cross-resistance to other AMPs and antibiotics

The ability of single bacterial mutations to confer resistance to multiple antibiotics will decrease treatment options and may allow increased selection and maintenance of resistance. Selected PR-39 and protamine sulphate resistant mutants were subjected to an array of different antibiotics and AMPs to examine possible cross-resistance. The only significant cross-resistance observed for PR-39 resistant mutants (**paper I**) was for bleomycin, for which the MIC was increased 5fold, indicating that this glycopeptide antibiotic also is imported or act via the SbmA-protein in *Salmonella*. Susceptibility to the examined AMPs (LL-37, mCRAMP, rCRAMP, CNY100H-L and protamine sulphate) was not changed significantly. This is not so surprising given that these peptides are thought to act more unspecifically on the bacterial membrane. However, studies by other research groups have identified additional peptides that are less active against *E. coli* SbmA-mutants, including Bac5(1-31) and Bac7(1-16) (bovine proline-rich peptides), apidaecin Ib (insect proline-rich peptide) and microcins B17 and J25 (bacterially produced AMPs) (Mattiuzzo *et al.*, 2007; Salomon & Farias, 1995; Yorgey *et al.*, 1994).

The slow growing SCVs in **paper II** were cross-resistant to a wider range of antibiotics and AMPs. Especially the very slow growing *hemA*, *hemB* and *hemC* mutants were less susceptible to several drugs, including gentamicin, streptomycin, colistin, the bovine-derived AMP lactoferricin B (4-14) and the human defensin HNP-1 (peptides were only tested against the *hemC*-mutant). To our knowledge, this is the first study examining both antibiotic- and AMP-susceptibility of *S. typhimurium* SCVs. The results indicate that the strains are protected against antibiotics as well as host defense peptides, which should facilitate both survival and persistence during an infection.

Fitness effects of AMP resistance mutations

To determine the biological cost of PR-39 resistance (**paper I**) we initially measured bacterial growth rates in exponential phase in Iso-Sensitest broth, and determined survival in stationary phase. To obtain a more accurate estimate of fitness, which includes effects over the full growth cycle, competition experiments between a genetically tagged wild-type strain and the resistant mutants were performed in LB and M9 medium. Two strains were mixed at a 1:1 starting ratio and were allowed to grow before passage into fresh medium. The ratio of resistant mutant to wild-type bacteria was continuously measured over 13 cycles, from which the selection coefficient and relative fitness could be estimated. A similar competition experiment (over one growth cycle) was also carried out in a mouse infection model. Surprisingly, none of the examined PR-39 resistant mutants were significantly impaired in fitness in any of the experimental set-ups. The same mutations observed to confer no or low-cost in the laboratory have been found to cause resistance also in clinically isolated antibiotic resistant bacteria (Bottger *et al.*, 1998; O'Neill *et al.*, 2006; O'Neill *et al.*, 2007; Sander *et al.*, 2002). Whether due to true no-cost mutations or acquisition of compensatory mutations in these strains, it indicates that low-cost mutations are favored in patients (Andersson & Hughes, 2010). Continuous antibiotic selection is presumably not needed for stabilization of such mutations in a population.

Contrary to the low fitness costs associated with PR-39 resistance, protamine sulphate resistance was associated with a substantial reduction in bacterial fitness *in vitro* (**paper II**). Exponential growth rates were measured in Iso-Sensitest broth supplemented with glucose, and were found to vary depending on the type of resistance mutation. The *cydC* and *hemL* mutants were more fit (relative growth rates of 0.53-0.85) than *hemA*, *hemB* and *hemC* mutants (relative growth rates of 0.27-0.39). Some strains also had significantly increased lag phases and formed tiny colonies on solid agar. The slower growing strains were generally more resistant. It is usually assumed that slow growing resistant bacteria will be replaced by susceptible, fast growing strains once selection is removed. However, the clinical importance of slow growing bacterial variants is increasingly recognized and these strains can persist in patients also in the absence of antibiotic pressure (Proctor *et al.*, 2006). It is apparent that a slow growth phenotype can be advantageous under certain conditions.

The protamine resistant phenotype can be unstable

The slow growth phenotype of some of the protamine resistant strains in **paper II** was unstable in the absence of selection, and large colonies would

occasionally appear after overnight growth in rich medium. To examine compensatory evolution in the different SCVs, several independent lineages of selected strains were cycled through population bottlenecks in unsupplemented LB. Samples were plated at regular intervals to screen for faster growing strains. Two examined mutants with aa-substitutions in *hemA* and *hemL*, respectively, were relatively stable and only a few lineages compensated within the time frame of the experiment (~40 cycles). These strains carried intragenic compensatory mutations that were in the same codon as the original mutation, resulting in either another aa-substitution or genetic reversion to the wild-type sequence. This resulted in loss of protamine resistance. No compensation was observed in a strain with a *hemB*-deletion. In contrast, compensation in all ten evolved lineages of a *hemC* mutant occurred within 70 generations of growth. The original resistance mutation in this strain causes a threonine (Thr, acc) to proline (Pro, ccc) substitution at position 241 of porphobilinogen deaminase (HemC). The *hemC* gene in five compensated mutants was sequenced with surprising results: one strain had no additional mutations in *hemC*, two strains carried additional point mutations in *hemC* and two strains had double sequence at the position of the compensatory mutation. These results indicated the presence of multiple *hemC* copies in some of the strains. Ability to revert to a faster growing phenotype once the environment is favorable may facilitate a more infectious state of SCVs.

The slow growth of a SCV can be compensated via gene amplification and subsequent mutations

In **paper III** we further investigated the mechanism of compensatory evolution in the *hemC* mutant. 18 new lineages were evolved in addition to the 10 lineages from **paper II**. Sequencing and copy number determinations revealed the following compensated variants with regard to amino acids at position 241 of HemC. Nine lineages carried one *hemC* copy in which the Pro of the parental mutant had been replaced by another amino acid (His, Leu, Ser or Asn). In three other strains, the defective *hemC*-allele encoding Pro at codon 241 was present in more than one copy. Finally, in 14 lineages there were at least two *hemC* copies, where the mutant Pro as well as a new amino acid (His, Leu, Ser, Thr or an unknown combination) was present. The end result was in most cases a strain with a *hemC* sequence different from that of the wild-type *S. typhimurium* strain.

The results lead us to hypothesize an evolutionary trajectory where the first step is amplification of the defective *hemC* copy. This increases fitness slightly and thereby growth of the population and provides a larger target for additional mutations. This increases the probability of second-site mutations

that restore protein function in one of the copies. These mutations in turn increase fitness substantially and releases selection for the amplification, which segregates back to one copy. Continued evolution of intermediately compensated strains enabled us to confirm that the steps in the suggested pathway can take place. Low fitness strains with several defective *hemC*-copies could be evolved to higher fitness and another aa at position 241 of HemC, and high fitness compensated mutants with duplications or amplifications could be evolved to lose the extra, defective gene copies.

The *hemC* gene is situated in between the two rRNA-operons *rrnA* and *rrnC* on the *Salmonella* chromosome. These provide homologies or structures that may facilitate the formation of duplications. Southern hybridization in three strains confirmed that, at least in these strains, the amplifications did not stretch beyond the *rrnA* and *rrnC* operons.

The cyclotide cyO2 is a potent antibacterial peptide

The antibacterial activity of cyclotides has only been addressed in two previous studies and with contradictory results (Gran *et al.*, 2008; Tam *et al.*, 1999). Therefore, we examined the effect of a set of cyclotides against bacteria (**paper IV**). Inhibitory effects of the different peptides were assessed with radial diffusion assays and MIC-assays, and bactericidal effect by monitoring viable counts at different time points after peptide addition. One cyclotide in particular, cyO2 from the violet *Viola odorata*, was found to inhibit growth of both *S. typhimurium* and *E. coli*, while *S. aureus* was unaffected by treatment. Although some of the other peptides also exhibited antibacterial activity, cyO2 was by far the most potent. At 12.5 μM it was bactericidal to all Gram-negative species tested, namely *S. typhimurium*, *E. coli*, *P. aeruginosa* and three different *K. pneumoniae* strains, including a MDR clinical isolate. Complete or near complete killing of *E. coli* and *P. aeruginosa* was achieved down to 1 μM and 3 μM, respectively.

The prospects of using cyclotides for agricultural and therapeutic applications are promising. Cyclotides are very resistant to heat, chemicals and enzymatic degradation (Colgrave & Craik, 2004; Ireland *et al.*, 2006), conditions that would inactivate many conventional AMPs. Several synthesis methods are available and under continuous development, and introduction of new residues into the cyclotide framework is possible (Clark *et al.*, 2006; Craik *et al.*, 2010; Gunasekera *et al.*, 2008). Perhaps production for use as antibacterial drugs may be a possibility in the future.

The charged residues in cyO2 are necessary for activity

CyO2 contains three positively charged amino acid residues, two lysines and one arginine, and one negatively charged residue, a glutamic acid, resulting in a net charge of +2. In **paper IV** we examined what effect chemical masking of these charges would have on the antibacterial activity of the peptide. All modified peptides were less active against *S. typhimurium* than unmodified cyO2, only the arginine-modified peptide retained low activity. Masking of the positive charges likely decreases attraction of the peptides to the negatively charged bacterial membrane. On the other hand, this would indicate that removing the negative charge should lead to increased activity, which was not the case. The glutamic acid residue is conserved in almost all cyclotides found to date, and masking of this residue has been observed to destabilize the cyclotide framework and cause disorder in the hydrophobic patch that interacts with membranes (Craik *et al.*, 2006; Göransson *et al.*, 2009; Plan *et al.*, 2007). This may lead to less efficient membrane interaction and decreased ability to aggregate in the membrane.

Future perspectives

Several interesting follow-up experiments emerge from the results in this thesis. For example, can we evolve higher-level PR-39 resistance in the isolated low-level resistant strains? Is it possible to evolve compensated mutants from SCVs in the presence of AMPs and if so, which are the underlying mutations? Are gene amplifications as a mean of reversing the SCV-phenotype possible also in other bacteria or for other types of mutations (apart from *hemC*)? It would be interesting to evolve more strains with an unstable SCV-phenotype and determine the mechanism of compensation and also to screen for gene amplifications in clinical strains using PCR/quantitative real time PCR. Another question is how many AMPs the SCVs can resist, and if there is a difference in resistance between different types of SCVs? That is, one could expand the cross-resistance studies to include several SCVs and AMPs of different origin and mode of action.

A couple of projects with connection to the work in this thesis have already been initiated. One question currently examined is by what mechanism *S. typhimurium* SCVs become resistant to aminoglycosides. Resistance in SCVs is presumably caused by decreased attraction to and less uptake of the aminoglycosides across the SCV membrane due to a decreased membrane potential (Baumert *et al.*, 2002; Mates *et al.*, 1982; Proctor & von Humboldt, 1998). Work in our laboratory however suggests a more complicated situation, at least for the SCVs described in this thesis. When the cryptic *Salmonella* gene *aadA* is knocked out in the SCVs, resistance to

streptomycin and spectinomycin is completely abolished. The *aadA* gene encodes a putative aminoglycoside adenyltransferase that likely inactivates these antibiotics. We suspect that the level of the alarmone ppGpp is involved in regulation of *aadA*-expression and that the *hem*-mutations (by an at present unknown mechanism) influences ppGpp levels. Slow growth *per se* does not trigger resistance, as other types of slow growing, *hem*-unrelated mutants are not aminoglycoside-resistant. The *aadA*-encoded adenyltransferase does not confer resistance to other aminoglycosides like e.g. kanamycin and gentamicin, and for these antibiotics we do not at present know the mechanism of resistance in SCVs.

In an attempt to gain a more broad knowledge about bacterial resistance development to AMPs, we have initiated a small scale cycling experiment. This study is inspired by the resistance evolution experiments performed by Perron *et al*, in which mutants with high level resistance to pexiganan evolved, but where the resistance mutations were not identified (Perron *et al.*, 2006). Individual lineages of *S. typhimurium* were grown and passaged through population bottlenecks in refined LB (low concentration of salt and cations) in progressively increasing concentrations of PR-39, LL-37, CNY100H-L and wheat germ histones (6 lineages/peptide, 8 controls without peptide). After between 300-500 generations of growth, several lineages could survive a higher peptide concentration than at the start of the experiment. Especially lineages treated with wheat germ histones increased their resistance relative to wild-type (MIC >300 μg/ml as compared to 12.5-25 μg/ml for positive controls). Although less pronounced, increases in resistance were also observed for the other peptides (two- to fivefold increases in MIC). The experimental setup allows for successive mutations all contributing to resistance. That is, the end result is heterogeneous populations with different mutations and resistance level. Therefore, a number of clones were isolated from resistant lineages and characterized in terms of growth and MIC. Whole genome sequencing (Solexa) was performed on DNA from 4 clones (1 histone, 1 CNY100H-L and 2 LL-37 resistant mutants) to identify the resistance mutations. In addition to known AMP resistance determinants such as *phoP* and *pmrB* mutations, a couple of genes not previously associated with AMP-resistance were identified. Both LL-37 and histone resistant mutants carried mutations (frame shift or deletion) in *waaY* and *waaZ* (*rfaY-Z*). These gene products are involved in modification (e.g. heptose-phosphorylation) of the core region of LPS. (Frirdich *et al.*, 2003; Yethon *et al.*, 1998). Mutations in these genes likely results in reduced number of negative charges in the bacterial membrane and thus reduced peptide interaction. In the case of histone resistance (which was much higher than for LL-37), another explanation is also plausible. Other bactericidal histone-derived peptides have been demonstrated to act intracellularly (reviewed in (Cho *et al.*, 2009)), and perhaps the effects of the *rfaY*-mutation prevents uptake of the wheat germ histones into the bacterial

cell. Two additional mutations, in *ffh* (encoding a protein involved in transport, targeting and integration of inner membrane proteins) and in *ytfN* (encoding a putative periplasmic protein), were identified in the sequencing data (in the CNY100H-L and the LL-37 resistant mutants, respectively). Resistance to AMPs also increased for some clones from the positive controls, indicating that adaptation to the media also could give protection against AMPs. In general, these clones were not as resistant as those evolved in the presence of peptides.

At present we are confirming putative mutations by PCR. The mutations will be moved into clean backgrounds to determine contribution of each mutation to the resistance phenotype. In future experiments, we will attempt to combine mutations to examine if we can produce a high level resistant strain. Other interesting aspects such as fitness effects of mutations, cross-resistance to other AMPs/antibiotics and effect of sub-MIC concentrations of antibiotics on resistance development to AMPs will also be examined.

Concluding remarks

It is difficult to foresee what will happen if we start to use AMPs extensively as antibacterial drugs. What we do know is that we are in dire need of new antibiotics that can target MDR pathogenic bacteria, and that AMPs may be successful at doing that. But we also know that bacteria are masters at adaptation and have evolved strategies to overcome the deleterious effect of all antibiotics introduced to date. Several factors including mutation rates, fitness costs of mutations, compensatory evolution, prevalence of resistance genes in the environment and ability to transfer resistance between bacteria will influence resistance development to AMPs, parameters which we have little knowledge of today. In this thesis, I have tried to address some of these issues in detail for a set of AMPs.

In **Paper I** and **II**, we observed that bacteria with lower susceptibility to AMPs appeared at a high rate and that the mutants were cross-resistant to several other antibiotics and AMPs. PR-39 resistant strains were less susceptible to bleomycin, and protamine resistant mutants were less susceptible to HNP-1, lactoferricin, colistin and several aminoglycosides.

The PR-39 mutants described in **paper I** were found to be as fit as the wild-type parental strain both in laboratory media and mice and survived equally well over extended periods in stationary phase. Such mutants are more likely to be stabilized in a population also in the absence of selection than are those with a large fitness cost. The protamine resistant mutants (**paper II**), on the other hand, had severely impaired fitness (i.e. slow *in vitro* growth rate). These mutants had the characteristics of SCVs, genetic subpopulations known to be able to cause persistent and re-occurring infections. In this case, the lowered growth rate is thought to provide a survival advantage within the host, complicating the argument that slow growing mutants will be quickly out-competed once selection is removed.

Some of the slow growing SCV-strains reverted to a faster growing phenotype when evolved in the absence of selection, a common feature of SCVs (Acar *et al.*, 1978; Haussler *et al.*, 1999; Proctor *et al.*, 1998; Roggenkamp *et al.*, 1998). In **paper III**, we identified the mechanism behind fitness compensation in one of the SCV-strains. The initial adaptive response was selection for an unstable gene amplification. Subsequently mutations appeared that restored protein function in one of the gene copies, restoring fitness further. This released selection for the amplification, which segregated back to the haploid state and even higher fitness. The study

provides a novel mechanism for reversion of the SCV-phenotype and further evidence that gene amplification is a common adaptive mechanism in bacteria. Slow growth may promote survival and persistence while reversion to faster growth facilitates infection and spread once growth conditions improve.

Cyclotides are plant-derived peptides with many properties that are advantageous from a drug development point of view, for example ability to withstand heat and proteolytic degradation. In **paper IV**, we observed that certain cyclotides have antibacterial activity. One cyclotide in particular (cycloviolacin O2) was found to be active against Gram-negative bacteria, including a MDR isolate of *K. pneumoniae*, while Gram-positive strains were less susceptible. Masking of charged peptide-residues resulted in decreased antibacterial activity.

From the results in this thesis and additional studies in the literature, it is clear that bacteria can acquire resistance against at least certain AMPs and that resistance development is a relevant threat to the efficiency of AMPs that should be carefully evaluated experimentally. Furthermore, the results indicate that the bacterial resistance mechanisms may confer cross-resistance to several AMPs, which could potentially be a very serious problem. Not only would this affect selection and stability of drug resistant mutants, but it could result in bacteria that can better withstand our own innate immune defenses, ultimately rendering us more susceptible to infection. In this sense, it will be important to weigh the benefits of new antibacterials against their propensity to induce bacterial resistance. Hopefully, some time in the future, we will understand enough about the factors that influence resistance development to enable design of more effective pharmaceuticals that will not be rendered useless as rapidly by resistance development.

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