Mechanisms and Biological Costs of Bacterial Resistance to Antimicrobial Peptides

HAVA LOFTON TOMENIUS
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Abstract


The global increasing problem of antibiotic resistance necessarily drives the pursuit and discovery of new antimicrobial agents. Antimicrobial peptides (AMPs) initially seemed like promising new drug candidates. Already members of the innate immune system, it was assumed that they would be bioactive and non-toxic. Their common trait for fundamental, non-specific mode of action also seemed likely to reduce resistance development.

In this thesis, we demonstrate the ease with which two species of pathogenic bacteria, the gram-negative Salmonella typhimurium (S. typhimurium), and the gram-positive Staphylococcus aureus (S. aureus), can gain increased tolerance and stable resistance to various AMPs. By serially passaging each bacterial species separately under increasing AMP selection pressure we observed increasing AMP tolerance. Resulting in independent bacterial lineages exposed to four different AMPs (including a two-AMP combination) that exhibited 2 to 16-fold increases in MIC. Substantial cross-resistance between the AMPs was observed. Additionally, the S. aureus mutants were found to be cross-resistant to human beta-defensins 1, 2, 3, and 4.

The LPS molecule, with mutations in the waaY, pmrB and phoP genes, was the principal target for S. typhimurium resistance development. The main target for S. aureus remained elusive. Reduced membrane potential was a common change for two of the mutants, but not for the others. All sequenced mutants had one or more mutations in various stress response pathways.

Fitness of the resistant mutants was assayed by growth rate analysis and in vitro virulence factor testing (e.g. survival response to bile, superoxide, acidic pH). Furthermore an in vivo survival/virulence test involving a mouse competition experiment (S. typhimurium) and sepsis model (S. aureus) was performed. In the absence of AMPs there was often little or no fitness reduction in the mutants. Our results suggest that AMP resistance mechanisms do not irrevocably weaken either species with regard to virulence characteristics or survival within the host.

In light of these findings, we suggest that the progression of therapeutic use of AMPs should proceed with great caution since otherwise we might select for AMP resistant mutants that are more resistant to our innate host defenses and thereby potentially more virulent.

Keywords: antimicrobial peptides, antibiotic resistance, fitness cost, Salmonella Typhimurium, Staphylococcus aureus, bile, serum, pH response, growth rate, mice, phoP, pmrB, waaY, LPS, LL-37, defensins, membrane potential

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I was born in Minnesota, but grew up in Cleburne, Texas, USA. I received my bachelor's in Biology from Southwestern Adventist University in 2000. After several years working as a lab technician in Tyler, Texas, I moved to Sweden and joined Dan Andersson’s lab to pursue a PhD.

To my family
List of Papers

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


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### Abbreviations

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<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AMP</td>
<td>Antimicrobial Peptides</td>
</tr>
<tr>
<td>Ara4N</td>
<td>4-amino-4-deoxy-l-arabinose</td>
</tr>
<tr>
<td>CA</td>
<td>Community acquired infection</td>
</tr>
<tr>
<td>CRISPR</td>
<td>Clustered regularly-interspaced short palindromic repeats</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epithelial growth factor receptor</td>
</tr>
<tr>
<td>FDA</td>
<td>United States Food and Drug Administration</td>
</tr>
<tr>
<td>HGT</td>
<td>Horizontal gene transfer</td>
</tr>
<tr>
<td>HA</td>
<td>Hospital acquired infection</td>
</tr>
<tr>
<td>IAD</td>
<td>Innovation-Amplification-Divergence</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MIC</td>
<td>Minimal inhibitory concentration</td>
</tr>
<tr>
<td>MEGA</td>
<td>Macrolide efflux genetic assembly</td>
</tr>
<tr>
<td>MRSA</td>
<td>Methicillin resistant <em>Staphylococcus aureus</em></td>
</tr>
<tr>
<td>S. aureus</td>
<td><em>Staphylococcus aureus</em></td>
</tr>
<tr>
<td>S. typhimurium</td>
<td><em>Salmonella enterica</em> serovar Typhimurium LT2</td>
</tr>
<tr>
<td>TCS</td>
<td>Two-component signaling system</td>
</tr>
<tr>
<td>TIVAR</td>
<td>Transient <em>in vivo</em> antibiotic resistance</td>
</tr>
<tr>
<td>VRSA</td>
<td>Vancomycin resistant <em>Staphylococcus aureus</em></td>
</tr>
<tr>
<td>WGH</td>
<td>Wheat germ histones</td>
</tr>
</tbody>
</table>
Introduction and Background

Antibiotic Resistance

“It is time to close the book on infectious diseases and declare the war on pestilence won!” is an infamous quote by the Surgeon General of the United States (William H. Stewart). This was the late 1960’s and at that time, most people in the world, including doctors and scientists, commonly believed that antibiotics had truly wiped-out infectious diseases (at least the ones caused by bacteria). Although discovered around 1930, (1928; Penicillin by Alexander Fleming and 1932; Sulpha Drugs by Gerhard Domagk) [1,2], it was not until after World War II that antibiotics first went into widespread use. The first report of a resistance mechanism, namely a penicillinase, (a penicillin destroying enzyme, known today as a β–lactamase), was filed even before penicillin became clinically available [3]. Soon afterwards more reports of resistance began to emerge. Scientists began to hunt for new antibiotics and to scrutinize each new discovery for ways of improvement through synthetic biochemical modifications. They were highly successful for a time and sporadic reports of resistance were not viewed as a threat. This is called the “Golden Age” of antibiotics [4]. However, bacteria have increasingly continued to develop resistance and their ability to develop resistance has been unintentionally promoted by worldwide misuse that includes over-prescribing, patient noncompliance, unregulated consumption, and additives to livestock feed and other agricultural uses (see pathogen interaction Figure 1). Even though the resistance dilemma started almost as soon as the discovery of antibiotics, it was not until recently that the governmental authorities around the world began to take this threat seriously. As even Alexander Fleming foresaw as early as 1945 and publically warned about in his Noble Lecture: “Then there is the danger that the ignorant man may easily underdose himself and by exposing his microbes to non-lethal quantities of the drug make them resistant.” In retrospect, given the power of genetic manipulation tools available today, it would have been clear and obvious (at the conception of antibiotics) that bacteria have an incredible capacity to develop resistance against antibiotics.
Antibiotic resistance, defined as any microorganism that is no longer susceptible to a given antibiotic to which it was originally sensitive, can develop through a number of mechanisms. It can occur spontaneously, through random mutation, during a selection process such as repeated or constant exposure to antibiotics in a natural setting such as soil. Streptomyces, a soil microbe, is after all the most common source of naturally occurring antibiotics. They produce and secrete their own antibiotics for protection against competing microbes. Rare mutants in the surrounding microbial populations may produce some benefit against the secreted antibiotic and allow that particular sub-population to thrive in an otherwise hostile environment. This new beneficial mutation could, over time and with more exposure to antibiotic selection pressure, be honed to produce a gene specialized to provide resistance to a specific antibiotic such as the before mentioned β-lactamases against penicillins. Or it could be maintained as a more generalized, nonspecific approach to resistance such as efflux pumps, designed to pump out various cellular toxins. Thus, the antibiotic susceptible population is reduced or eliminated and is replaced by the antibiotic resistant population. Following this rare random event, there are several strategies by which this new resistance mechanism could be shared with, or delivered to, other microbial species (i.e. horizontal gene transfer, mobile genetic elements). Anthropological efforts to decrease human disease and suffering have inadvertently accelerated this natural process, by placing pathogens (disease causing bacteria) in repeated contact with antibiotics. It is correct that antibiotics have been used successfully to treat infectious diseases, but they have also been vastly misused. A classic example of the misuse of antibiotics is antibiotic food additives given to meat and dairy producing animals. Low doses of
antibiotics have been used to complement cattle, swine, and poultry feed for decades to promote growth and prevent illness in agricultural livestock. This not only increases the risk of resistance development while in use, but it also contributes to low levels of antibiotics in the environment (for example, waste or run-off water). Even very low levels of antibiotics have been shown to select for antibiotic resistance development [5]. It is probably inevitable that even through the carefully controlled and regulated clinical use of antibiotics we would have eventually faced the threat of antibiotic resistance. However, with the proper research knowledge we likely could have prevented the current crisis: bacterial resistance against all known antibiotics.

A similar danger could face humanity again, this time with the discovery and subsequent push to develop antimicrobial peptides for clinical use against pathogenic bacteria. Antimicrobial peptides (AMPs) are already a part of the innate immune system. They are found in abundance in all living organisms and are likely very ancient host defense molecules [6]. Today many scientists and medical professionals claim that the method by which antimicrobial peptides attack bacterial membranes causes fundamental damage and thus consider it unlikely that bacteria would be able to develop resistance (See Fig. 4) [7]. It is true that known bacterial resistant mechanisms to antimicrobial peptides are rare, but they do exist [8]. This seems reminiscent of the 1945 situation with antibiotics. While anticipating the satisfaction of curing people from infectious disease (or perhaps making lots of money), scientists, medical professionals, and pharmaceutical companies are convincing themselves and others that it is impossible for bacteria to develop resistance to antimicrobial peptides.

We are just at the beginning of our understanding of how antimicrobial peptides function in the host and it is not yet wise for us to use molecules of our own immune system to combat pathogenic bacteria; wherein heavy usage could lead to selection of resistant mutants. We have little understanding of the scenario wherein we upset the balance of host defenses against microbial interactions. Besides the large enough problem of acquiring resistance, an additional risk is that we end up with a less effective innate immune system. This aspect of our particular studies is what sets it apart from the scenario of antibiotic resistance. Traditional antibiotic resistance signifies an end to the usefulness of one drug against one, or perhaps several, bacterial populations. However, if by exposing bacterial populations to increasing levels of AMP drugs, in many cases in order to combat the presence of antibiotic resistance, we could end up selecting for bacteria that might not only resist antibiotics, but also are easily capable of coping with important members of our first response to infection (i.e. AMPs/host defense peptides). This would be like the proverbial double-edge sword: pathogenic bacterial resistance to both antibiotic drugs and antimicrobial elements of our immune system. When all traditional antibiotics fail, we must remember that our immune system will literally be our last line of defense.
Preface to This Thesis Study

This study is but a small blade against the epic struggle against infectious disease. In order to examine the acquisition of bacterial resistance to antimicrobial peptides, we used a serial-passaging experiment (designed to mimic small-scale evolution). When applied over a relatively short time scale, this method has generated bacterial strains capable of tolerating much higher concentrations of antimicrobial peptide treatment than the initial parental strains. These resistant mutants were then isolated, sequenced and characterized for their level of resistance contribution, cross-resistance and fitness compared with the wild type parental strain. Our pilot study (paper I), using Salmonella Typhimurium, has shed light on the types of resistance mechanisms to be expected from constant selection pressure of antimicrobial peptides. Even from this initial study, our results indicate that further research is imperative if we are to accurately predict what would happen if antimicrobial peptides were used as drugs in the clinical setting.

A follow-up study (paper II) involved a subset of these mutants and provides crucial insight into the chief concern of whether or not antimicrobial peptide resistant mutants are in fact fit enough to survive in vivo. All of the reconstituted mutant strains selected in the previous study were assiduously tested to determine the possibility of survival in low-complexity “in vivo-like” conditions: meaning separate in vitro experiments designed to mimic hostile host conditions. This allowed us to predict which (if any) of the mutants might succumb to host immunity. For a better understanding of the fitness of these mutants the high-complexity, in vivo (mouse) infection model was ultimately tested.

The third study involved elements of the initial work with Salmonella, however; this time two important aspects were altered. The serial-passaging experiment was employed to more or less the same effect, but the media was changed to reflect an environment that more closely resembles that of the expected mammalian host. Media selection is usually a delicate choice when investigating AMPs. First off, many AMPs lose their antimicrobial activity in the presence of high levels of salt ions. Concerning this, host milieu is an added variable in the study of AMP resistance and could conceivably affect the adaptive ability of bacteria. The model organism was also changed to Staphylococcus aureus, which is considered a more likely target of any AMP drugs currently in the pipeline. Staphylococcus aureus also carries the added weight of being an organism that easily manifests resistance to multiple antibiotic treatments.
Antibiotic Resistance

Introduction
Before the 1940’s humanity existed without antibiotics. Millions of people died or lived with life-altering consequences of raging bacterial infectious diseases. Imagine infections, such as bacterial meningitis, that are severe even with treatment, killed 90% of patients. Soldiers routinely died of secondary wound infections. Minor surgery and childbirth often led to deadly, secondary infections. Common ailments such as ear and urinary tract infections were excruciating, longer lasting, and often caused complications. Sexually transmitted diseases such as syphilis and gonorrhea, were pure misery to live with and often gruesomely fatal. Even the first recipient of penicillin died (not enough penicillin prepared to finish curing him) because of a scratch he received while doing something as mundane as gardening. The discovery of antibiotics changed all of that. It must have been nearly unbelievable at the time that such non-invasive procedures such as taking just a few pills or injections could end so much suffering and death. However, here we are, some 75 years later, contemplating the possible return of such times.

So, what are antibiotics? Antibiotics are natural, synthetic, or semisynthetic chemicals used to treat bacterial infections. They have been largely discovered as part of the microbial defense system produced against competing microbes. They can be further sub-divided into families or classes, based on their mode of action. The β-lactams (penicillins, cephalosporins, and carbapenems) target peptidoglycan cross-linking to disrupt cell wall synthesis. Quinolones and sulfonamides inhibit essential enzymes. Tetracyclines, macrolides, aminoglycosides and lincosamides interfere with protein synthesis by binding/blocking the 30S or 50S ribosomal subunit. Glycopeptides also target the peptidoglycan cross-linking in a slightly different way than β-lactams. For example, vancomycin binds to the two D-alanine residues in an un-linked peptidoglycan strand and blocks the PBP (penicillin-binding-protein) binding site, thus the cell walls are not stable and can easily break apart. Additionally, vancomycin binds up the D-alanine precursors before they are incorporated into the peptidoglycan chain. This halts their inclusion into the cell wall and no more peptidoglycan is produced. While β-lactams bind to the active site of PBPs themselves, before they reach the peptidoglycan layer, so that they cannot cross-link the final step. Lipopeptides (dap-
Tomycin and polypeptide antibiotics (colistin/polymyxin B) are closest to the antimicrobial peptides (AMPs) studied in this thesis work and have a similar mode of action, namely disturbing the bacterial membrane. It is pertinent to note that various, multiple species of bacteria have managed to acquire resistance to all known antibiotic medicines.

Figure 2. Timeline. Antibiotic introduction and subsequent resistance development.

**Fitness**

The definition of fitness, in this context (microbiological), is the capacity of any given organism (bacteria) to grow, replicate, and divide in a particular environment with enough success to have a competitive advantage over those around it. This means that the genetic traits of the “more fit” organism will be passed on to future generations more successfully than any other “less fit” organisms. If there is a specific need for a specific genetic trait, in a particular environmental niche, those individuals that possess this trait will thrive and if the need is great enough, all those that do not possess it will be lost forever from the population. This trait is then said to be “fixed” in the population. This entire process is called natural selection and it acts over time and variations in the environment to “select” the organism best suited to that particular set of “selection pressures”.

Antibiotics are a great example of a selective pressure. For instance, a pathogen under the constant pressure of an antibiotic treatment, acquires a trait (let’s say by mutation) that happens to confer some level of tolerance to the antibiotic. Instead of being eliminated along with all the other individuals, this bacterium is more resistant to the antibiotic, but because of the new mutation, it is no longer able to (for example) evade macrophage engulfment and thus is no longer capable of causing infection. This mutation is benefi-
cial (to fitness) in the light of antibiotic resistance, but detrimental (to fitness) in light of host immune responses. The outcome for this mutant is probably death and rapid elimination from the host. Now let us imagine another scenario in which the new mutation gives tolerance to the antibiotic and has no detrimental effect on the survival of the organism inside the host. This organism would now be much more likely to survive in the new environmental niche (i.e. more fit) than any of its predecessors. There is also the third possibility that although it survives inside the host, it may no longer be overtly pathogenic. However, this can be overcome with time and more exposure to the same antibiotic. Eventually, the new population will acquire more beneficial mutations under continued selection pressure, and the organism will evolve. It seems likely in this scenario that reviving pathogenicity would also be a positive selection pressure for higher fitness.

This study contains many references to relative fitness. Within a given set of parameters, relative fitness is a useful statistical tool when measuring the average number of survivors (mutant genotype B) compared with the standardized wild type (genotype A).

Resistance

Antibiotic resistance is clinically defined as bacteria that are able to tolerate antibiotics past a defined concentration dose, or breakpoint. However, antibiotic resistance can be a relative term. Some bacteria can be more resistant than others to a given antibiotic, but still be considered below the breakpoint and therefore still sensitive to the antibiotic treatment. Constant exposure to this type of selection pressure results in bacteria that increasingly tolerate the antibiotic. Per chance they are not pathogenic, but possess mobile genetic elements, such as plasmids, that can carry the enhanced resistance gene (or set of genes) to other more pathogenic species. Interestingly, the mecA gene that causes the infamous methicillin resistance in \textit{methicillin resistant staphylococcus aureus} (MRSA’s) is believed to have been horizontally transferred; possibly from a mecA homolog found in \textit{Staphylococcus sub-species, sciuri} [9,10]. This commensal squirrel species is harmless to humans as a pathogen, but could be very dangerous as a reservoir of antibiotic resistance genes. In fact, it carries many antibiotic resistance genes similar to those now harbored by \textit{S. aureus} [11]. Horizontal gene transfer (HGT) takes advantage of mobile genetic elements, such as plasmids, or methods of physical contact, such as the pilus, to transfer DNA to neighboring organisms.

In hindsight, scientists should have predicted the emergence of antimicrobial resistance to antibiotics. Now that we more fully appreciate and understand bacterial resistance development, we should be careful not to repeat the mistakes made with antibiotics with new drug candidates such as AMPs. Bacteria are evolutionarily old organisms that have been interacting in the
environment for eons. There are many resistance mechanisms that have been
discovered to exist among environmental bacterial populations. In fact, this
is called, intrinsic resistance. Environmental bacteria carry intrinsic, or natu-
ral to self, resistance mechanisms [12]. These mechanisms are used against
other bacteria in the struggle for nutrients and space. In many cases, com-
mensal bacteria (normal host floral bacteria) may also harbor intrinsic re-
sistance methods for the purpose of maintaining their own benign presence
in the host. Since these bacteria are non-pathogenic and may even have a
beneficial relationship to the host organism we might not readily consider
their role in spreading resistance. As the dynamics of fitness and the spread
of resistance are discussed below, it is important to keep commensal bacteria
in mind as well [13].

Dynamics of Fitness and Resistance

The key points of fitness and resistance are highly interconnected and as
touched upon previously, the dynamics between susceptible versus resistant
classical bacterial strains depends on three main principles, 1) the capacity to acquire
resistance, 2) the presence of some selective pressure, and ultimately, 3) the
biological cost of carrying that resistance. Mutations, under non-selective
parameters, typically come with a cost. This cost may be offset by the tem-
porary benefit to the population of say- antimicrobial resistance. However,
when the selection pressure is alleviated, the cost of carrying the mutation
might reduce survival capacity for that organism thereby allowing other
more fit organisms to dominate the population [14]. An alarming scenario is
created if the cost of carrying the resistance mutation is minimal and com-
peeting organisms (or immune systems) do not quickly eliminate the new
resistant population. This is potentially acceptable if the new resistant pop-
ulation never encounters the selection pressure again, because they can then
be eventually outcompeted.

This is where the danger of overuse and abuse of antibiotics arises. When the
new resistant population encounters the same (or perhaps similar) antibiotic
it will again immediately outcompete the susceptible strain. The susceptible
population is weeded out and eventually lost. Higher doses of the antibiotic
may subdue the new resistant population for some time, but eventually the
strain will acquire another mutation (or amplify the existing beneficial muta-
tion) that will allow it to thrive in the higher concentrations. Naturally and
clinically speaking there are limits to how high the drug dose can be without
becoming toxic to the host. There is also the possibility of acquiring com-
pensatory mutations that compensate for the cost of the mutation conferring
the resistance, but without loss of the initial resistance [15].
Antibiotic Resistance Mechanisms and the Resitome

*Staphylococcus aureus*, a bacterial species that has little difficulty in developing resistance, was not surprisingly, the first documented bacterial strain with resistance to penicillin. It appeared promptly after it was introduced for widespread use [16]. Shortly afterwards, the Lederberg experiments demonstrated that antibiotic resistance mutations pre-existed the selective pressure of antibiotics- an important step in understanding the emergence of medically problematic antibiotic resistance [17].

There are four main categories of resistance mechanisms; 1) Inactivation, such as the inactivation of β–lactam drugs through the action of several variants of β–lactamase hydrolyzing enzymes (including penicillinas, carbapenmases, cephalosporinases, and extended-spectrum β–lactamases) is well documented [18-20]. 2) Reduced drug uptake through target modification is another common method and is achieved through a vast array of chemical group moieties that can be transferred to key locations of the drug target. An important clinical example is aminoglycoside chemical group substitution involving acetyltransferases, nucleotidytranferases, and phosphotransferases that can all cause the destruction of the antibiotic activity [21]. Additionally, reduced uptake can be caused by regulation of, or changes to, the porin proteins embedded in the outer-membrane, which can hamper the permeable ability of some antibiotics [22]. 3) Efflux pumps are common and their activity ranges from highly specific to generalized with regard to the expelled substrates. Highly specific efflux pumps often travel around on mobile genetic elements that can be acquired by various bacteria resulting in resistance. One example of this type of efflux pump is encoded by the macrolide efflux genetic assembly (MEGA), and is carried, generally along with other resistance determinants, by the transposon family Tn916/Tn1545 [23]. An example of an efflux pump with broader expulsion activity is the chromosomally encoded AcrAB-TolC pump of several species, notably *Escherichia coli* (*E.coli*) and *Salmonella enterica* serovar Typhimurium (*S. typhimurium*). Overexpression of this pump gives high levels of resistance to tetracycline, chloramphenicol, quinolones, bile salts and other antimicrobial agents [24]. Finally, 4) Alterations of metabolic pathways is best demonstrated through blocked folic acid synthesis in several unique pathways, ultimately resulting in resistance to trimethoprim and sulfamethoxazole [25,26]. See Figure 3.
The “Resistome”, a term coined by D’Costa et al (2006), applies to all resistance genes that are found to naturally occur in the environment [27]. The genes and subsequent mechanisms on this list continue to grow. In fact, a recent expedition into the immense Lachuguilla cave in Carlsbad, New Mexico, USA, uncovered a multitude of antibiotic resistant, non-pathogenic bacteria thriving deep underground. The Lachuguilla cave is considered to be a pristine environment, meaning it has been unaffected by human (including modern medicinal) exposure for millions of years. All four categories of resistant mechanisms were represented in their findings [28]. The sobering fact that antibiotic resistance genes exist in abundance in this isolated and pristine environment reveal that microbes have evolved resistance mechanisms for use against each other long before humans ever became involved. Furthermore, studies conducted on ice core samples and permafrost regions have also revealed the presence of resistance to antibiotics such as β-lactams, tetracyclines and vancomycin [29,30]. This knowledge is especially alarming if one places antimicrobial peptide treatment into a similar scenario. Although rare, antimicrobial peptide resistance mechanisms are well described in the literature [8,31,32]. What if bacteria possess their own resistome that directly, or even indirectly, targets antimicrobial peptides? Researchers have linked the evolutionary origin of human β-defensins to the so-called Big-
defensins of Cephalochordata [6]. If true, this means that antimicrobial peptides are an ancient microbial defense mechanism. One could speculate that microbes and AMPs have been co-evolving for a very long time and that there might actually be many highly developed resistance mechanisms already available in the microbial universe.

Two-Component Signaling Systems

As the current literature seems to be swelling with references to two-component signaling systems, (TCSs) it seems diligent to highlight them in association with AMP antibiotic (i.e. colistin, polymyxin B) resistance. Several TCSs have been implicated in AMP resistance, PhoPQ, PmrAB, SsrAB, RtsAB, RcsABCDF, and OmpR/EnvZ to name a few [33-35]. This consequence is perhaps not surprising in bacteria, especially Gram-negative bacteria such as *Salmonella* that regulate much of the outer membrane modifications via PhoPQ. Using PhoPQ as an example, we can understand the basic mechanism employed by TCSs. PhoQ is a histidine kinase that resides in the inner membrane. It is able to sense and respond to environmental cues such as low pH, Mg$^{2+}$ ion levels, and notably the presence of AMPs. This interaction leads to conformational changes that result in PhoQ autophosphorylation. The phosphate group is transferred to PhoP, which is the transcriptional regulator that controls many genes; among them are genes responsible for outer-membrane modification and thus, AMP resistance [36-38]. PhoPQ also regulates PmrAB [39]. The PmrAB system is responsible for sensing and responding to changes in Al$^{3+}$ and Fe$^{3+}$ levels and modifying the LPS accordingly [40]. In fact, these systems are so important that they often have mechanisms in place to bypass one member (for example- a compromised PmrB sensor kinase), and activate the transcriptional regulator partner (PmrA) via another pathway. In this case, a gene product called PmrD, under the control of PhoPQ can bypass PmrB and directly phosphorylate PmrA [41]. OmpR/EnvZ has been reported to transcriptionally activate ssrA and/or ssrB. These types of interactions are termed two-component regulatory cascades. It also seems that different sets of TCSs can have one or more cascade pathways [42].

Additionally, there is recent evidence for a rather elaborate TCS resistance mechanism in *Staphylococcus aureus* against nisin (a bacteriocin produced by *Lactococcus lactis*) and bacitracin (produced by *Bacillus subtilis*). Hiron *et al* discovered the novel BraSR resistance pathway. It was found to sense and respond directly to bacitracin and subsequently turn on an ABC transporter system, BraDE. The BraRS TCS also activated another ABC transporter VraDE that was found to act more indiscriminately to pump out both bacitracin and nisin [43]. Interestingly, even at very low levels of either drug, these systems were up-regulated. As was discussed above,
ronmental bacteria are quite likely to be the reservoir for many known and unknown bacterial resistance mechanisms against antibiotics. Under the appropriate conditions genetic transfer of an already existing resistance mechanism to pathogenic bacteria is assuredly happening. Curiously, since nisin is predominantly used as a food preservative, human activity is likely not the cause of *Staphylococcus aureus* ability to resist nisin. Nevertheless, it has managed to acquire, perhaps though the potential AMP resistome, an advanced system that provides resistance. Bacitracin and nisin are both cyclic, polypeptide antibiotics that have been on the market for many years. While they are structurally more complex than the more simple AMPs of the innate immune system, the parallel comparison is relevant, since other TCSs (PhoPQ and PmrAB) have been extensively shown to be important for polypeptide and AMP resistance [32,44,45]. Nisin, for example, works by electrostatic interaction to disrupt the membrane, which is a familiar mode of action that most AMP’s share [46].

Acquirement and Spread of Resistance

The acquirement of resistance can unfortunately occur in a variety of ways. It can happen stochastically through the process of random mutations. Random mutations occur during replication when the DNA polymerase makes a mistake that is not repaired, this can result in a permanent mismatch that goes on to become the template strand for further replication events after the cell divides. Other permanent mutations can occur through what is called strand slippage, a phenomenon that occasionally happens when a newly synthesized DNA strand “loops out” during replication and can lead to insertion or deletion of nucleotides [47]. These randomly generated mutations might happen to confer resistance under the selection pressure of an otherwise harmful substance, but are generally considered to be a slow process. Horizontal Gene Transfer (HGT), on the other hand, takes advantage of already developed resistance genes and can hasten the advance of resistance acquisition. It is now recognized to play a major role in spreading the resistance genes between species. HGT is the movement of genetic material, in the form of plasmids, transposons, integrons and chromosomal DNA, from one bacterium to another through transformation, conjugation or transduction. Plasmids are circular, self-replicating pieces of DNA that can be transferred between individual bacteria through the transformation process where competent bacteria take-up foreign DNA. Competent bacteria are simply, in a certain condition primed for taking up foreign DNA and this condition can vary widely among bacterial species [48]. Conjugation and transduction are now known to be the most common methods by which genetic material (i.e. plasmids harboring resistance genes in particular) is transferred. Conjugation is accomplished through cell-to-cell contact directed by the use of a hair-like
structure, called a pili, on surfaces of certain bacteria. This is used as a controlled pore for the transfer of said materials. Transduction is the process carried out by bacteriophages (viruses that infect bacteria) that can result in HGT of resistance to antibiotics such as β-lactams and fluoroquinolones. Transposons can carry resistance genes and operate by jumping around the chromosome inserting themselves via terminal inverted repeats recognized by the transposase enzyme and can often end up in plasmids. Integrons integrate themselves into foreign DNA through site-specific (attI) recombination events. They normally carry short extra DNA fragments with them, called gene-cassettes, capable of conferring resistance [49]. In fact, many resistance genes, or sets of genes called operons (or gene-cassettes) commonly end up in plasmids. These resistance plasmids may then carry the resistance to another bacterium, thus allowing for newly acquired resistance in that particular organism.

The spread of antibiotic resistance is dependent on all the naturally occurring genetic tools that bacteria process above, however human social behavior has exacerbated the issue. Hospitals are reservoirs for resistance gene transfer where pathogens, as well as non-pathogenic bacteria that could be repositories for resistance genes, are brought into close contact with each other and a gradient of concentrations of a variety of antibiotics. As if this was not bad enough, hospital staff (including doctors and nurses) routinely lax on proper hygiene compliance, which results in further spreading of antibiotic resistance. These modern healthcare aspects have all combined to hasten the spread of antibiotic resistance [50-52].

Unfortunately the antibiotic resistance selection pressure is not confined to hospitals alone. The World Health Organization reported in 2011 that many pharmaceuticals, including an extensive list of antibiotics, were found in surface water, sewage, streams and rivers. The concentrations of these antibiotics are considered far below MIC (minimal inhibitory concentration) used against pathogenic bacteria. However sub-MIC levels of antibiotics have been clearly identified as capable of selecting for and maintaining stable, de novo, resistance mutations in the laboratory setting [5]. These low levels of antibiotics in the environment are undoubtedly selecting for and enriching environmental microbes that already happen to carry resistance genes.
Antimicrobial Peptides (AMPs)

Introduction

Antimicrobial peptides (AMPs) are relatively small molecules, normally much less than 100 amino acids in length, and are found as part of the innate immune system of essentially all living organisms from bacteria to humans. It is now common to find them referenced in the literature as “host defense molecules” due to their additional role as first responders in immunomodulation. There are certain traits that are common to the majority of AMPs, such as their positive charge and amphipathicity, but other elements can vary extensively (i.e. secondary structure, size) [53,54]. The re-discovery of antimicrobial peptides came during the 1980’s after the pioneering work of Hans Boman et al (1980) [55]. The timing of this work corresponded to a rise in awareness of the threat of antibiotic resistance. Predictably there was immediate attention focused upon the discovery and subsequent drug potential interrogation of antimicrobial peptides.

Although the discussion about AMP resistance development probabilities has been raised sporadically in the literature [56], it is still widely avoided or dismissed with a symbolic shrug. This indifference indicates that it is still assumed (without much aforethought) that bacteria cannot evolve resistance to AMPs, due to the non-specific and vital method by which they attack the membranes to cause bacterial death (see Fig. 4).

However, AMPs are increasingly revealing that they are more sophisticated than previously thought. Not only are they now widely accepted to have immunomodulatory roles, but also more and more instances of intracellular targets are being reported [57-59].
Overview of Immunity

As stated earlier, for most of our existence humanity has coped without antibiotics. We relied on the defense mechanisms within our own bodies to fight off infections. The human immune system is usually quite robust and by preventing or minimizing infection, is capable of keeping the vast majority of us healthy most of the time. The mammalian immune system is divided into two sub-categories, 1) the innate, and 2) the adaptive. More focus will be given to the innate system, since AMPs belong to this category. The innate immune system, which is considered to be the first line of defense, maintains protective elements such as skin and non-specific immune cells (i.e. neutrophils and macrophages). It induces inflammation and the complement cascade and last, but not least, antimicrobial peptides and various chemokines and cytokines that signal interaction between these innate mech-
anisms and consist of links to the adaptive immune system. Organisms such as plants and insects have an innate immune system, and for this reason it is considered to be evolutionarily much older than the adaptive immune system possessed by vertebrates. The adaptive immune system takes time to build up and works by recognizing (from a memory-like fashion of a previous invasion) the present infectious organism. The adaptive response is highly specific and is normally enhanced by repeated exposure.

AMPS and Their Role in Immunity

To date there are over 100 antimicrobial peptides identified in the human body. Apart from their antimicrobial activity, they are involved in many key biological tasks; for instance, wound healing, sepsis control and promotion of tissue repair. AMPs reside in skin epithelia’s, sweat glands, tears, saliva, airway fluids, the gastrointestinal tract, and the urogenital system and are circulating around in the blood via neutrophils and other immune cells [60]. One reason (aside from the supposedly fundamental cause of death) that it is still so prevalent among researchers to take for granted the notion that bacteria are unable to evolve resistance to AMPs, is that AMPs are considered to be very ancient members of overall immunity. Thus, logically AMPs and bacteria have essentially co-evolved. Because AMP resistance mechanisms are thus far rarely discovered it could be reasoned that the evolutionary process has finely honed AMPs to win the competition for dominance in the host. This leads us to consider at least two possible, contrasting outcomes. First, that this is the case and forever will be the case and anthropological interference will have no effect. Or, secondly, we disturb the balance of this finely honed system: meaning that human activity could render part of our naturally occurring immune system useless.

In addition to the observed antimicrobial nature of AMPs, there are numerous reports on the discoveries of auxiliary immune system roles such that AMPs are now also defined as immunomodulators or even as immunoregulators. LL-37, the only human cathelicidin, is probably the most studied with respect to such immunomodulation roles. It is produced by mucosal and skin epithelial cells, and is quite possibly the first responder to infection and microbial invaders. Once up-regulated, LL-37 begins to attract neutrophils to the site of invasion. Neutrophils themselves also store the pro-peptide hCAP18 in granules which are cleaved to the much more active LL-37 when released to further control infection. This action is very important to the overall health of the individual, since deficiency in neutrophils, (a condition termed neutropenia) and thus severely decreased hCAP18 levels, are responsible for a number of health issues [61]. The measurements of LL-37 concentrations, taken at various physiologically relevant sites or situations, are technically difficult [62]. For instance, LL-37 during lung infection can be
high (between 5-25 µg/ml) and trauma patients exhibited up to 15 times higher plasma levels than uninjured volunteers (from ~0.013-0.2µg/ml) and anywhere from 42-142 µg/ml in seminal plasma from healthy donors. Saliva measured from children ranged wildly from 0.0002µg/ml to 0.165 (boys) or 0.275 µg/ml (girls) [60,63-65]. As can be seen, dramatic variation in LL-37 levels can be detected throughout the body, between individuals, with or without infection [62]. This makes it very difficult to determine what the normal biological role is for AMPs such as LL-37.

LL-37 can recruit more immune cells using a chemotaxic gradient of itself and an abundance of other chemokines and chemokine receptors that are up or down-regulated directly by LL-37 (near 50 genes in all) [62,66,67]. These so-called “chemoattractants” (ex. CCL2 and IL-8RB) stimulate the recruitment of auxiliary immune cells consisting of neutrophils, monocytes, mast cells and T-cells to the site of infection. Another vital role that should not be overlooked is LL-37’s ability to diminish the sepsis response created by LPS fragments [67]. LPS fragments from broken down (or shed) bacterial outer membrane components are endotoxic. This entire response strategy means that LL-37 helps to control chemotaxis of innate and adaptive immune cells, while concurrently minimizing the damage from the pro-inflammatory response. Incredibly, after the infection has been cleared, LL-37 has an additional influence in promoting wound healing. Vascularization and epithelialization are both promoted in damaged locations via LL-37’s role in transactivation of EGFR (Epidermal Growth Factor Receptor) considered responsible for numerous pathways leading to tissue repair [68]. Figure 5 illustrates the many roles of LL-37.
**Figure 5.** Representation of the many roles of LL-37. LL-37 is involved in all stages of the infection: from initial damage, to clearance of infection, and finally, the repair of tissue. MHC class II (major histocompatibility complex), is the antigen-processing pathway activated when phagocytized protein material gets presented to CD4+ T-cells. EGFR (Epidermal Growth Factor Receptor) is an epithelial cell-surface receptor that triggers skin regeneration.

Again, it is important to reiterate that by using such an important immune molecule as the next antimicrobial medicine, we risk offsetting the balance of our immune system. This is sure to be a mistake. Especially considering the substantial evidence that indicates disease symptoms are often worse in patients wherein AMPs are naturally absent or not produced in significant concentrations (such as Morbus Kostmann, chronic ulcers, periodontitis, and urinary tract infections) [69-72]. Conversely, an overabundance of LL-37 has been attributed to conditions such as rosacea and psoriasis [73,74]. This could be an unforeseen danger in pathogenic resistance to AMPs as well, that by altering the balance of immune molecules or by unknowingly reducing LL-37’s ability to recognize pathogens we risk compromising part of the...
immune system and in so doing we could be fashioning diseases to be more painful and more deadly.

Human defensins are many in number and therefore more complicated to discuss. They can be divided into three classes $\alpha$, $\beta$ and $\theta$, based on the spacing of the cysteine residues and the ensuing secondary structure. Although they are part of the same polypeptide family and can be attributed with many of the same functions as LL-37, it is simply easier to describe them in table format (see Table 1 below).

Table 1. *Members of the defensins group of AMPs.*

<table>
<thead>
<tr>
<th>Defensin #</th>
<th>Cell/tissue</th>
<th>Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha$-defensins (HNPs)</td>
<td>1</td>
<td>Innate immune cells:</td>
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<tr>
<td></td>
<td>2</td>
<td>Neutrophils,</td>
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<tr>
<td></td>
<td>3</td>
<td>Macrophages, and</td>
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<tr>
<td></td>
<td>4</td>
<td>NK</td>
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<td></td>
<td>5</td>
<td>Paneth, urogenital</td>
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<tr>
<td></td>
<td>6</td>
<td>epithelials</td>
</tr>
<tr>
<td>$\beta$-defensins (H$\beta$Ds)</td>
<td>1</td>
<td>Mucosal epithelials</td>
</tr>
<tr>
<td></td>
<td>2</td>
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<td>3</td>
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<td>4</td>
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<td></td>
<td>5</td>
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<td>6</td>
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<td>14</td>
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<td>23</td>
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<tr>
<td></td>
<td>26</td>
<td></td>
</tr>
<tr>
<td>$\theta$-defensin</td>
<td>1</td>
<td>Non-functional in humans</td>
</tr>
</tbody>
</table>

Characterization and Antimicrobial Activity

A paradox exists for the predominant role of AMPs. Are they indeed antimicrobial in the locations they are found in the natural setting of the host? Or, do they have a more subtle role, such as modulating/regulating the immune system? *In vitro* studies have shown, that under physiologically relevant conditions, such as high salt ion concentrations (i.e. $\text{Na}^{2+}$, $\text{Mg}^{2+}$) or acidic pH, the antimicrobial properties are generally modest. A possible explanation for this conflict seems to be host tissue rich in carbonate [75,76].

Additionally, Johansson *et al.* found that cytotoxicity was common at high concentrations of AMPs. Interestingly, LL-37 was antagonized and no
longer cytotoxic in the presence of human serum, although the cytokine and chemotactic activities were unaffected [77]. And yet, the numerous studies on the antimicrobial activities of AMPs cannot be dismissed [54]. The most logical explanation for this conundrum is that AMPs actually carry out multiple duties within the innate immune system. The range of physiological conditions in a living host can be extreme and when high concentrations of peptides will not cause cellular damage (i.e. phagolysosomes or acute inflammation) the antimicrobial activity is likely favored. In juxtaposition, if the conditions involve high salt concentration or areas where glycosaminoglycans are present (i.e. serum, specific types of wound fluid) it is more probable that the immunomodulatory role is favored [78]. Glycosaminoglycans are highly negative and most likely compete for LL-37 binding in tissues where they are found. One explanation for a host tissue to begin releasing an agent like glycosaminoglycan is that in areas where high concentrations of LL-37 would be present, such as fighting an infection in wound fluid, there might already be extensive tissue damage and reducing the toxic effect (of high levels of LL-37) to the cells might be beneficial. However, in an acute wound the necessity of fighting off the infection outweighs collateral tissue damage and glycosaminoglycans would hinder this process and are therefore not released. Thus, it appears that AMPs play multiple characters as part of an intricate performance of checks and balances during an immune response (see Figure 5 above).

PR-39 is also a cathelicidin (like the human LL-37) that is endogenous to the porcine host. It is involved in many of the same immunomodulation functions as LL-37, however; curiously the killing mechanism is altogether different. LL-37 is mainly thought to attack and subsequently devastate the bacterial membrane, while PR-39 seems to pass innocuously through the membrane to inhibit intracellular functions, namely protein synthesis [57]. Additionally, there is evidence to support a role for PR-39 in mitigating inflammation via blocking the proinflammatory signaling pathway instigator NF-κB[79,80].

Less is known about the other two peptides used during the serial passaging experiments performed in Paper I and III. Histones have fairly recently been highlighted for possessing antimicrobial capabilities, although the initial observation that histones could inhibit microbes came as early as 1931 [81]. Hirsch is usually given the credit for being the first to substantiate bactericidal activity of histones against a broader spectrum of bacterial species [82]. More recently, histones with antimicrobial activity and little cytotoxic effect have been isolated from frogs and fish [83,84]. Human Histone 4 was isolated from secretions of the sebaceous skin glands and found to be antimicrobial against *Staphylococcus aureus* and *Propionibacterium acnes* [85]. Several studies have sought to explain the exact killing mechanism of histone antimicrobial fragments. The conclusion is that there seems to be several methods and targets depending on which fragments are being assayed. For
example, Buforin II (fragment of H2A; frog) accumulates within the bacterial cell after permeabilizing the membrane, utilizing its characteristic proline-hinge, and binds to DNA and RNA to cause cell death. In contrast, a Buforin II variant lacking the proline-hinge (one amino acid substitution) could no longer penetrate the cell and instead converted to a more common AMP killing method of pore formation and membrane disruption [86,87]. To my knowledge the only previous work on Wheat Germ Histones (WGHs) was conducted by Lars-Olof Hedén and Ulf Rothman at Lund University in Sweden who kindly donated an extract of WGHs to this project. Hedén and Rothman reported that WGHs are extremely stable, broad-spectrum antimicrobials. They kill by permeabilizing the membrane, but do not cause overt lysis. They were described as being non-hemolytic and non-cytotoxic [88].

CNY100H-L is a highly modified derivative of the C3a complement antimicrobial peptide. Pasupuleti et al, produced it as part of a rational design experiment to mimic the evolution of antimicrobial peptides. CNY100H-L was the 100th peptide produced in their study and was further modified to replace a crucial Histidine with Leucine. The modifications served to increase net positive charge and hydrophobicity thereby increasing antimicrobial effect. Unfortunately with the H-L substitution the hemolytic activity also increases slightly [89].

**AMP Resistance Mechanisms**

**Bacterial Mechanisms**

As previously stated, bacterial resistance mechanisms to AMPs do exist. Although interactions between bacteria and humans have presumably been constant for a very long time, resistance mechanisms are limited. Most of the AMP resistance mechanisms discovered to date fall under the blanket term of ‘intrinsic resistance’. This is the type of resistance that one would expect from several millennia of co-adaption. It could be argued that modern pathogens are simply the ones that have evolved the best strategies for AMP resistance. The inducible resistance mechanism of LPS modifications (to either decrease the net negative charge, or reduce membrane fluidity) in some Gram-negative bacteria, or the cell-wall remodeling of some Gram-positive species (such as the blocking of peptidoglycan cross-linking in *S. aureus*) are both perfect examples of intrinsic resistance. Keeping this in mind, it is still interesting to draw comparisons between the types of resistance mechanisms that exist for antibiotics and the ones (albeit much fewer) that are known to exist for AMPs. One can clearly see that there are representative mechanisms to parallel at least three (of the four) main categories of resistance mechanisms to antibiotics. For example, drug inactivation is a common
mechanism used with many antibiotics, such as the enzymatic degradation (i.e. hydrolysis) that occurs with β-lactam antibiotics. The AMP resistant equivalent mechanism would be several known proteases, in particular PgtE in *Salmonella* and Aureolysin in *S. aureus*, both have demonstrated an ability to incapacitate LL-37 \[90,91\]. Many different bacterial species possess proteases that can break down the simpler linear structures of α-helical AMPs. It could be argued that bacterial proteases are evolutionary infants with regard to resistance mechanisms and that more specialized development into degradative enzymes similar to β-lactamases is foreseeable. The innovation, amplification and divergence (IAD) model theory of enzymatic improvement showcased in the experiments of Näsvall *et al* indicates the possibility of a weak, generalized activity being honed by selective elements (i.e. excessive AMP exposure) to produce a stronger, perhaps more specialized activity \[92\]. (See Figure 3, pg. 20)

Another common method for antibiotic resistance is simply to reduce the affinity of the antibiotic for the bacteria by modifying, for instance, the peptidoglycan layer (PG) as in the case for vancomycin. This method was born as a self-protection mechanism for the producing species *Streptomyces toyo-censis*, but unfortunately it can be transferred to other microorganisms through plasmids or other conjugative elements. It consists of a five-gene cluster, *vanS, vanR, vanH, vanA*, and *vanX*. VanSR acts as a two-component regulatory system for the detection of vancomycin, which in turn acts as an inducer and turns on VanHAX. This three-gene operon is responsible for switching the PG termini from a N-acyl-D-Ala-D-Ala to a N-acyl-D-Alanine-D-Lactate and concomitantly leads to a 1000-fold decrease in binding affinity that is measurable in the characteristic resistance phenotype \[93\]. Alarmingly, even within my own results and in mechanisms reported earlier, this general method of reduced binding affinity is prevalent among bacteria resistant to AMPs. The best-characterized example is probably the PmrAB two-component regulatory system and the genes that are directly regulated by it, *pmrE* and *pmrHFLJKLM*. These genes are responsible for lipid A biosynthesis and addition of 4-amino-4-deoxy-l-arabinose (Ara4N) to the 4’ phosphate of lipid A as well as other phosphate group modification of the inner core of the lipopolysaccharide (LPS). These modifications can provide resistance to the more simply structured peptides such as LL-37 and to that of the more structurally complex polypeptide antibiotics, like colistin or polymyxin B, by reducing the net negative charge of the outer membrane of many bacterial species \[32,94,95\]. (See Figure 3, pg. 20) Again, this indicates a potential for specialized development.

To date, these modifications have been either regulatory or induced strictly by chromosomal mutations and have not caused much alarm; however new reports of a plasmid-mediated resistance gene, called *mcr-1* (adds phosphoethanolamine to lipid A), that when expressed results in colistin resistance are beginning to create a stir of unease \[96\].
Host Response Mechanisms

Since AMPs are unique in the antibiotics field as already belonging to the host innate immune system, it is intriguing that certain host responses to bacteria can endow them with AMP evasion techniques.

One such interesting mechanism for promotion of bacterial entry and adhesion to host cells is the mammalian protein receptor, gC1q–R/p33. There have been several studies suggesting that bacteria, such as *Staphylococcus aureus* and *Listeria monocytogenes* use this receptor to “piggyback” into the host, masquerading as “self” and thereby evading elements of the innate immunity including AMPs [97,98]. In addition, or perhaps contrarily, this same receptor has been shown to have a protective effect for host cells during episodes of high levels of the specific AMPs LL-37 and β-defensin 3. The theory is that host cells bearing gC1q–R/p33 can neutralize the threat to themselves by binding up nearby AMPs, while simultaneously allowing the more distant AMPs to fight against invading pathogens [99]. Again, this brings to mind an intricate balance between mammalian host immunity and pathogenic bacteria.

Phenotypic switching is a bacterial phenomenon that has been shown to drastically induce high levels of resistance to AMPs such as polymyxin B and colistin. Kubicek-Sutherland *et al* eloquently demonstrated how the various conditions within the host can rapidly “switch-on” a resistant phenotype well beyond MIC levels considered relevant in mice. This switch, called *transcient-in vivo*-antibiotic-resistance (TIVAR), was found to be host-dependent, but worked mechanistically through the PhoPQ/PmrAB regulatory network. This study provides a nice correlation between the generalized *in vitro* results of many studies and the live host causality of induced and reversible resistance based on host milieu [39,100]. This particular transient resistance method seems to be employed by multiple bacterial species.

Disturbingly, as pointed out in the TIVAR article, there is considerable cross-resistance between host AMPs and polymyxins/colistin, which means simple interaction between the pathogens and AMPs native to the host are likely enough to kick-start TIVAR. In so doing, the transient resistance could give time for mutations to arise that give permanent resistance, such as perhaps mutations in *phoP* that give continuous expression of AMP resistance.

Biological Cost of Resistance

The biologically relevant cost is perhaps the most crucial point to be made with bacterial species that gain resistance to AMPs. Since poking holes in the membrane is considered such a fundamental disturbance to growing bacteria, many scientists cannot perceive how bacteria would be able to develop resistance to such an antimicrobial agent. The bacterial membrane is admit-
tedly deeply important to the survival and virulence of bacteria. Scientists, like Michael Zasloff, assume that bacteria would have to undergo major membrane reconstruction in order to provide resistance against AMPs and thus also assume this would bestow a heavy cost burden on the bacteria that they simply would not be able to overcome, especially during the assault of the rest of the immune system [53].

However, it is also widely accepted that mutant strains of bacteria that happen to confer some level of resistance to any given selection pressure will be able to outcompete their more fit counterparts and thrive in an environment that they were once sensitive to [14]. The levels of success that these resistant mutants have as pathogens will depend on how fit they are in vivo. Since not all bacteria have a resistance phenotype, there are clearly limitations to acquiring resistance. Partly, this is due to the severe growth impairment that some mutations bring with them. Paper I addresses the fitness of the AMP resistant mutants collected during that study using some basic laboratory techniques for estimating fitness cost, such as growth rate kinetics and in vitro competitions. However, Paper II exclusively focuses on the fitness of our AMP resistant mutants in separate experimental conditions such as oxidative stress, pH tolerance, survival in stationary phase, and growth in various minimal media all designed to mimic conditions that a bacterial invader would likely encounter in vivo. Paper II also provides strong evidence, in the form of a mouse competition experiment, that each of the single mutants are not severely compromised compared to the wild type.

AMP Drug-Development Progress

In 2009, at the beginning of this study, peptide drug development had largely slowed down. Ten years earlier there was a discouraging setback with the antimicrobial drug, Pexiganan, meaning it did not get FDA approval. The principal reason for this decision is that it was no better than current drugs on the market for the indicated illness. In addition there were many reports of AMPs exhibiting cytotoxicity to mammalian cells, which no doubt dissuaded further research and developmental progress. However recently, due to the double threat of increasing multi-resistant pathogens and the lack of leads for new antimicrobial products, interest in AMP drug development is re-emerging [101].

There has been a renewed enthusiasm and push for Pexiganan approval. This time the pharmaceutical company, Dipexium Pharmaceuticals, Inc., is pushing for not only FDA, but also approval with the European Medical Agency (EMA) as well [102].

The LL-37 derived OP-145 is one of the more advanced drug potentials. A recent report states that OP-145 exhibits strong antibacterial activity against in vivo S. aureus infections in mice and rabbits [103]. This is a dis-
quieting study in deference to our work with *S. aureus* resistance against AMPs. Not only is it an LL-37 derivative, but it is also being developed for use in internal infections such as embedded in the coating of medical device implants. This application brings OP-145 into direct contact with other host AMPs and host defense cells and mechanisms. Of course, the article plays down the prospect of resistance development and to date no study has been undertaken that would investigate the resistance development potential of OP-145. It is entirely possible that, like LL-37, this type of prolonged exposure could indeed allow bacteria to mutate and per chance acquire resistance to OP-145. Plus, through the cross-resistance we have seen arise during our experiments, it is likely that the bacteria could also develop cross-resistance to host milieu peptides such as its parental molecule LL-37.

Another AMP drug, surotomycin (a lipopeptide) is currently progressing towards drug approval. Resistant mutants were actually reported with an additional unexplained cross-resistance to nisin. However, these facts were shrugged aside as interesting, but “would not be expected” to cause a problem either for the efficacy *in vivo*, or presumably, the expectation of resistant mutants to crop up [104].

Instead of turning the peptides themselves into antimicrobial drugs, perhaps a better strategy would be to study and develop molecules that prevent bacteria from degrading AMPs. This would have a better chance of sparing resistance development to our own AMP reserves and might even boost their *in vivo* activity. Many bacterial species have proteases or elastases that work to breakdown peptides and proteins. Inhibitors molecules such as the metalloproteinase, galardin, are capable of inhibiting enzymes from several species that breakdown LL-37 [105].
Salmonella Typhimurium

When we started with this topic of research, there were few references to bacteria of any kind being used in progressive cycling experiments with the intent of mimicking natural selection (albeit in the laboratory). So with little basal knowledge to go on we selected an organism, which was familiar to us and came “pre-loaded” with all manner of useful genetic manipulation tools. *Salmonella* Typhimurium is one of the most studied organisms with regards to the overall cell cycle and comprehensive genome knowledge. Although, most AMP medicines being developed today are focused on the skin and outer mucosal areas such as the eyes, *Salmonella* Typhimurium is not found in those areas and admittedly will not soon be exposed to forthcoming AMP drugs. However, the value of working with this organism is the vast amount of information one can glean from, for example, being quickly and easily able to reconstruct messy evolved mutants with clean genetic backgrounds (comparable to a wild type). All subsequent experimental results can then be assigned to the one mutation that is different between the two strains. Invaluable information about the ease with which bacteria can develop resistance to AMPs was gained by beginning our investigations using this model organism.

Characterization and Lifestyle

*Salmonella* Typhimurium is a Gram-negative, rod-shaped bacterium that causes gastroenteritis in humans with symptoms including vomiting, abdominal cramping and diarrhea. Our lab strain (*Salmonella enterica* serovar Typhimurium LT2, referred to as *S. typhimurium* throughout this thesis) is deficient in *rpoS* production, which renders it less virulent [106]. In mice, it still competently induces a typhoid fever-like pathogenicity. The normal progression of infection and disease is contaminated food consumption, followed by colonization of the small intestine. The Peyer’s patches (lymphoid nodules) incorporated into the small intestine are colonized next and from there the pathogen gets taken up by macrophages and spreads to the lymph nodes, liver and spleen. Often, it is useful, when studying disease progression in animal models, to bypass the early stages of infection and administer the pathogen intraperitoneally, wherein they are taken up directly by macrophages. This method is commonly used when competition experiments are
desired, for example, to determine the fitness of an AMP resistant mutant to its otherwise genetically identical wild type counterpart in a live animal model. This approach gives valuable information on the likelihood for bacterial survival, albeit little information on their ability to colonize.

Resistance Mechanisms

*S. typhimurium* has three principle methods of AMP resistance mechanisms, 1) cell-surface remodeling, 2) efflux, and 3) proteases (AMP degradation).

1. Our work has added knowledge to the field of AMP resistance mechanisms for *S. typhimurium*, notable through the *waaY* mutant obtained during a serial passaging experiment. The gene product of *waaY* adds a phosphate group to the heptose II unit of the inner core of the LPS molecule. Our mutant is a frameshift mutation at an early position in the gene and is very likely nonfunctional. Since phosphate groups lend a negative charge, the overall result from our mutant is a less negative charge projected from the outer membrane. Our mutant is therefore able to resist/evade AMP killing because it is electrostatically less attractive to the positively charged AMPs. Importantly, even though disturbances to the integrity of the LPS molecule have in some instances been shown to decrease virulence and viability in the host, we demonstrated that the *waaY* mutant could survive during a mouse competition. The other two mutations (*phoP* and *pmrB*) that we selected to study from the results of the serial passaging experiment were already known to cause resistance to AMPs. It is interesting from an academic viewpoint that we were able to select both known and unknown resistance mechanisms from our laboratory evolution experiment. The PhoPQ two-component regulatory system is well known to be responsible for susceptibility/resistance to AMPs and is discussed in more detail in the next section. It regulates the PmrAB two-component system, which has also been linked many times to AMP resistance. PmrAB regulates expression of several genes that confer resistance through the biosynthesis, transfer and attachment of positively charged residues, such as phosphoethanolamine and 4-amino-4-deoxy-L-arabinose (Ara4N) to the lipid A segment of the LPS [107]. It is generally accepted that this decreases the negative charge of the bacterial outer membrane, thus decreasing the electrostatic interaction to the positively charged AMPs (See Figure 6).
2. PgtE, an outer membrane protease, is an important contributor of resistance to α-helical peptides such as LL-37 [90].

3. One efflux system, specific to AMP resistance, has been found in S. typhimurium, namely the sapABCDF operon. It appears to be less discriminant than the PgtE protease [108-110].

Figure 6. LPS Molecule. The basic structure of the LPS molecule. The mutated genes found in Paper I are designated with a star and black arrows indicate their location of interest for providing resistance to AMPs.

Dynamics of the Biological Cost of Resistance and Host Infection

Assisting S. typhimurium during host infection and pathogenicity are several notable and seemingly intrinsic AMP resistance mechanisms. Perhaps the most well documented player in this field is the PhoPQ two-component regulatory system. PhoP is normally repressed under good survival conditions, like in the presence of certain regulatory signals, such as Mg$^{2+}$ or neutral pH. Upon the reduction of Mg$^{2+}$ ions, reduction of pH, or interaction with AMPs, the repression is lifted and PhoQ (a sensor kinase) begins to autophosphorylate, subsequently delivering a phosphate group to PhoP [111,112]. PhoP then activates transcription of numerous genes involved with AMP resistance and survival within macrophages, thus considerably influencing virulence [112,113]. Actually, there are many studies linking the lack of PhoP production (loss of function mutants in phoP or phoQ) with attenuation and complete loss of virulence [38,114]. It is impart responsible for modifying lipid A and many studies have determined that phoP inactivation mutants leave the cell more susceptible to AMPs. It is quite intriguing
that we managed to acquire a phoP mutant that actually confers high levels of AMP resistance while still managing to survive a mouse competition experiment with no statistically significant difference to the wild type. In light of this information, it suggests that our phoP mutant is in fact overly expressed, as supported by the qPCR results showing up-regulation of the pagP gene (known to be up-regulated when phoP is de-repressed). The proposed method of this resistance acquisition is that phoP adds fatty-acid groups, such as palmitate, to the lipid A molecule that make the outer membrane more rigid and therefore harder to breech [115,116]. This results in a high level of resistance towards AMPs. Although, our pmrB mutant displayed only a low level of resistance to just one AMP in our study, it was quite fit during a mouse competition. This could conceivably be an intermediate-type resistance mutation. For instance, a mutant that is quite fit and maintains a low-level of AMP resistance could potentially remain in the host long enough to acquire additional mutations (compensatory) that increase the resistance while maintaining adequate fitness. A similar conclusion could be drawn from our waaY mutant as well, even though it performed worse in the mouse competition, it was still recovered at the end of the study, making it a viable candidate for compensatory mutations as well.

Clinical Relevance

AMP resistance in bacteria has likely existed for a long time. Perhaps pathogens, such as S. typhimurium, owe their ability to cause disease in higher organisms to the fact that they have evolved schemes to block or evade AMPs, which are important members of the first line of the host defense system. It is not hard to imagine that within the clinical setting pathogens could acquire more AMP resistant mechanisms while being exposed to patient commensal organisms. Because commensal organisms must constantly outmaneuver AMPs and other host defenses, it is possible that they would themselves harbor such mechanisms. After all, our understanding of classical antibiotic resistance coincides with the knowledge of acquiring horizontally transferred resistance mechanisms through commensal/environmental bacteria during exposure in a clinical setting.
Our interest in *Staphylococcus aureus* as a model organism arose after our initial study of *S. typhimurium* for three main reasons, 1) We knew we would get different mutations, 2) we knew of a more *in vivo* relevant medium that would work better for *Staphylococcus aureus*, and 3) *Staphylococcus aureus* is a more suitable model to work with in light of AMP resistance. First, in our *S. typhimurium* evolution experiments we found several important resistance mechanisms, but all were located in the Gram-negative specific LPS molecule of the outer membrane. By using a Gram-positive organism like *Staphylococcus aureus* (*S. aureus*) we could be certain to find different mutations due to the fact that Gram-positive bacteria do not possess LPS. Second, choosing the best laboratory media is always a bit tricky, but with AMP research it is critical since many AMPs are inactive at high ionic strength (for example, high NaCl). Thus, we wanted to use a medium that was closer to the expected mammalian milieu. This media had been tested with *S. typhimurium*, but unfortunately had not given desirable results. Finally, *S. aureus* is a relevant, human commensal model for AMP research, especially in the topic of resistance acquirement. Due to the common occurrence of cytotoxicity among AMPs in high concentrations, drug developers have concentrated on promoting AMPs mainly as salves or ointments for skin and eyes, wherein *Staphylococcus aureus* is a frequent inhabitant and often causes infection problems.

**Characterization and Lifestyle**

*Staphylococcus aureus* (*S. aureus*) is well-studied, Gram-positive bacteria, easily identified under a microscope due to its round, grape-cluster type appearance. *Staphylococcus epidermidis*, a close relative, can be ruled out through a mannitol fermentation test. *S. aureus* maintains a high level of research interest, for the simple reason that although considered to be part of the normal human flora of the nasal passages and skin it is also a potentially deadly pathogen [117]. It can cause infections on the skin, in the eyes, and urinary tract, not to mention more severe internal infections, such as pneumonia, osteomyelitis, endocarditis and toxic shock syndrome [118]. In many cases the disease progression is aggressive and hard to treat [119]. Why is it such a successful pathogen?
S. aureus is loaded with virulence factors [119]. Here is a partial list—just to name a few. Most routes of infection are reliant on the presence of adhesins, or cell surface receptors, that support colonization of host tissue [120]. So-called invasins, such as staphylokinase, or membrane pore forming toxins, such as α-toxin, can significantly contribute to establishing an infection. S. aureus also produces several potent factors for evading host protection responses such as proteases, coagulases, and protein A [121,122]. Intrinsic and acquired resistance to antimicrobial agents has been an issue for decades and continues to worsen. MRSA (methicillin-resistant-Staph-aureus) is a common cause of nosocomial, or hospital-acquired (HA) infections, and are increasingly becoming a risk in community-acquired (CA) infections as well [123].

Nasal carriage in normal, healthy adults is approximately thirty percent. This puts it approximal to other body parts (throat, eyes, mouth) that one would assume are easily infected, but surprisingly, most healthy individuals seldom have problematic infections. Still the lifestyle, mode of transmission and subsequent dissemination can occur in almost any way—another feature of S. aureus that makes it so successful as a pathogen.

Resistance Mechanisms

The enormous success of S. aureus as a pathogen is highlighted in the numerous occurrences of resistance mechanisms. Its variability and adaptive ability is truly astounding. S. aureus can exhibit resistance mechanisms to β–lactams, glycopeptides, quinolones, tetracyclines, aminoglycosides, erythromycin, linezolid and antimicrobial peptide antibiotics, most notably daptomycin. To describe all resistance mechanisms to S. aureus in detail is beyond the scope of this thesis. However, it is important to go through a few that are pertinent to the focus of our research results.

MRSA’s are not only infamous in the clinical world today, but also happen to be an appropriate description of our model strain. The main resistance mechanism promoting resistance to β–lactams (like methicillin) is the β–lactamase enzyme responsible for disrupting the active element of the β–lactam antibiotics, namely the β–lactam ring. This disruption inactivates the antibiotic and it can no longer bind to penicillin-binding-proteins (PBPs) to block cell synthesis, meaning that the bacteria can divide and replicate like normal [124]. Methicillin was formulated to solve β–lactam resistance by being itself resistant to β–lactamases. However, it only took about two years before a new PBP, called PBP2a, encoding by the gene meca, made an appearance [125]. Methicillin has a very low affinity for PBP2a, thus endowing a lower susceptibility to it, effectively rendering it useless for treatment of MRSA strains [126].
A glycopeptide antibiotic called vancomycin was introduced in the late 50’s and was used only in rare cases to combat MSRA’s. Unfortunately with the rise of MRSA’s and the subsequent need to use vancomycin, resistance to vancomycin was eventually selected and although still uncommon is now a concern. Daptomycin, a lipopeptide, was developed to treat vancomycin resistance S. aureus (VRSA) [127]. Interestingly, the main mechanism found to promote clinically relevant resistance to daptomycin is called, multiple-peptide-resistant-factor (mprF) and provides resistance to multiple peptides, including LL-37 and human defensins used in our study [128]. Furthermore another resistance mechanism, involving a two-component system, was described for S. aureus in a previous section (p. 21).

Dynamics of the Biological Cost of Resistance and Host Infection

There are prominent examples of AMP resistance mechanisms actually being necessary and required for S. aureus survival and dissemination during a host infection. The dlt operon protects the organism from being killed by neutrophils and neutrophil α-defensins. Mouse models of infection show dltABCD mutants with significant loss of fitness and ability to cause disease progression [129]. Similarly, loss of mprF leads to increased sensitivity to AMPs, attenuation, and rapid clearance from the host [130,131]. Aforementioned examples are intrinsic resistance mechanisms harbored by S. aureus. Arguments that new resistance mechanisms would be difficult to evolve through natural selection, based on the supposed difficulty of developing resistance to bacterial targets as fundamental as the membrane, seem outdated when one examines the recent literature. Habets et al. produced an evolution experiment (daily serial passaging) wherein they showed that resistance to Pexiglanan developed, with associated fitness cost, but was easily compensated for with further passaging [132]. Several of our own S. aureus mutants displayed no significant fitness cost in vitro or in vivo (See paper III).

Clinical Relevance

From the 1960’s until the 1990’s MRSA was mostly a hospital-acquired (HA) infection. Today a genetically unique MRSA strain termed community-acquired (CA) infection has largely taken over [123]. The dramatic decline of HA-MRSA infections is believed (instead of any change in HA-MRSA itself) to be a result of better hygiene practice, sterile techniques, and improved knowledge of infection control [123,133]. This is much more difficult to accomplish in the community setting. Moreover, it seems that given
the ever-extending known arsenal of *S. aureus* to cause serious infection, it should act as a warning not to rush at the first new potential drug that emerges. *S. aureus* already possess several intrinsic resistance mechanisms to AMPs. The methicillin resistance was acquired through horizontal gene transfer and selected for in the clinical setting. Why would it be so unlikely to occur with some other (perhaps yet undiscovered) AMP resistant mechanism?

Although not on a mobile, transferrable element, the novel *apsSRX* (AMP sensing system) was recently shown to regulate both of the resistance mechanisms mentioned above [134]. The ApsSRX system (under its other name, GraRS) has also been shown to be important for vancomycin resistance [135]. On the other hand targeting the ApsSRX system could effectively knock out *S. aureus*’s chief AMP resistance repertoire, though several others (VraGE, BraSR/DE) would likely still contribute some level of resistance to AMPs. During our own studies, we have witnessed that AMP resistant mutants with little to no fitness cost readily give rise to cross-resistance against human defensins. With all of this taken together, it currently seems highly risky to develop AMPs into antimicrobial medicines.
Present Investigations

Paper I—Mechanisms and Fitness Costs of Resistance to Antimicrobial Peptides LL-37, CNY100HL and Wheat Germ Histones

Aim
To determine whether Salmonella enterica Typhimurium LT2 (S. typhimurium) was capable of acquiring resistance to antimicrobial peptides (AMPs) during prolonged serial passaging under constant AMP selection pressure. Furthermore we wanted to elucidate the mutations and evaluate the contribution of each mutation to resistance and general fitness.

Results and Discussion
Salmonella enterica serovar Typhimurium LT2 (S. typhimurium) was the model organism used throughout the work in this paper. S. typhimurium is a pathogenic, Gram-negative bacterium that causes gastroenteritis in humans. It should be noted that the LT2 strain’s virulence level is reduced compared to other Salmonella stains. We set out to determine whether this bacterium was capable of acquiring resistance during maintained serial passaging under constant AMP selection pressure. The serial passaging experiment began with AMP concentrations that reduced the growth of the bacteria, but did not kill them. Each independent lineage (six for each peptide; plus two (-) peptide controls) was transferred daily to fresh media and peptide treatment. Periodically the AMP concentration was raised by 50%. After ~500 generations, susceptibility to the specific AMP treatment was reduced in 15/18 lineages. Lineages with the highest tolerance to increased AMP concentrations were isolated for clones and further tested for resistance and fitness levels. Time kill kinetics revealed a stark contrast in resistance between the cycled strains and the parental strain, namely at concentrations that would eliminate the parental, the cycled mutants would be either barely affected or at least coping much better. We measured the fitness level of each isolate in growth curve assays. The isolates from Wheat Germ Histones (WGH) and LL-37 treatment both suffered ~10% reduction in fitness, while the isolates treated with CNY100H-L faired worse in fitness with a reduction of ~30%.
The cycled isolates were whole genome sequenced and mutations in genes likely to confer resistance were revealed and moved with the help of phage transductions into a wild type background. Each mutant was subsequently evaluated for resistance contribution and precise fitness costs. Resistance was measured using microdilution assay for MIC, time kill kinetics and competition experiments. Single mutants were not only tested for resistance against the AMP treatment under which their parent (the original cycled mutant) was exposed, but also against all three AMPs used in this study.

The results are that two of the mutations, \textit{waaY} (phosphorylates the heptose II residue of the inner core of the LPS) and \textit{phoP} (part of a global, two-component regulatory system, PhoPQ, that regulates biosynthesis and modifications of lipid A and other LPS residues) both have a significant level of resistance to all three AMPs. This was evident in the time kill experiments. However, it became even further pronounced (especially for \textit{waaY}) during the competitions, when at concentrations well below MIC the mutant was able to outcompete each of the AMPs. The fitness cost of the \textit{waaY} mutant was negligible (~1%). The \textit{phoP} mutant was occasionally able to outcompete the wild type as well, however; consistent results were difficult to obtain probably due to the significant growth impairment caused by this mutation (~16%). A third mutation was also tested, \textit{pmrB}, a sensor kinase in the two-component regulatory system PmrAB. This regulatory system is also responsible for biosynthesis and modifications of the lipid A and other LPS residues, and additionally is regulated by the PhoPQ system. Slight, but significant, resistance was observed only against WGHs in the time kill assays. Interestingly, the fitness of the \textit{pmrB} mutant when measured by either of the growth rate kinetics showed no fitness decrease and in the competition assays it consistently outcompeted the wild type when under no peptide treatment. Our results for \textit{pmrB} do not match numerous other AMP resistant studies that have shown \textit{pmrB} to be involved in resistance. The reason for this may be that under the specific genetic environment of the originally cycled mutant this mutation might confer some resistance and a small increase in fitness, where as by itself it does very little, this phenomenon is referred to as epistasis.

\textbf{Conclusions}

In conclusion, paper I can be summarized by stating that bacteria (\textit{S. typhimurium}) can quickly evolve resistance to AMPs. The mutations that confer resistance do not necessarily come at a fitness cost. Theoretically, the high degree of cross-resistance combined with the low fitness burden is not likely to negate the affect on any bacterial population. This is especially true in the context of further AMP selection pressure (drug treatment) and/or by simple exposure to naturally occurring host defense peptides.
Paper II—Fitness of Salmonella Mutants with Resistance to Antimicrobial Peptides

Aim
To focus on the overall fitness, including the capacity for survival in a live mouse model, of the reconstituted mutants constructed in paper I.

Results and Discussion
One argument in favor of developing AMPs as pharmaceuticals is that any potential mutations would likely be severely compromised and would therefore not survive the onslaught of the immune system. Rigorous testing of each mutant under isolated conditions that they would likely face in vivo is aimed at enlightening our understanding of what it means to have a fitness defect under conditions such as starvation, oxidative stress, low pH, bile and serum exposure. The ultimate test of successful evasion of the immune system is an infection of a live model organism.

Our results are not encouraging. The LPS profiles uncover the suspicion that the waaY and pmrB have intact LPS structures that are indistinguishable from wild type. This is significant because intact LPS molecules are important for membrane stability and virulence [136]. The phoP mutant is missing about half of the O-antigen. The O-antigen has also been shown to be important for virulence, although the number of residues required seems to vary widely among different species. Numerous studies have concluded that phoP knockout strains are avirulent and it is therefore commonly known that phoP is necessary for virulence and resistance to AMP-like antimicrobials such as colistin and polymyxin B. Our Real-Time PCR results indicate that our phoP is constitutively expressed. We measured a gene known to be up-regulated by PhoP (pagP adds palmitate to lipid A). It is still difficult to predict virulence, but constitutive expression is thus supported by our mouse infection experiment, wherein the phoP mutant was only mildly impaired for growth inside the host during a competition against the wild type.

The other two single mutations are not completely eliminated from the mice either, suggesting that given enough time and selective pressure opportunities these strains could acquire compensatory mutations that cover for the fitness loss without removing the resistance [14]. Keep in mind that the biological host (whatever it may be) contains a plethora of AMPs that would generate a constant selective pressure.

Again, during growth in M9 minimal media there is no measurable loss of fitness for waaY or pmrB, but phoP has about a 20% reduction in growth rate compared to the wild type. E minimal media was also tested because this information was needed for the pH shift tolerance experiments. The same results held true for waaY and pmrB, but this time phoP was severely compromised with a reduced growth rate of ~50%. This is surprising since the phoP mutant tolerates pH shifts and low pH (performed in E) much better.
than the other two mutants and even better than wild type. Bacteria encounter pH shifts when trapped in the phagosome of immune cells and again when the phagosome fuses with the lysosome to create the phagolysosome [111]. This fusion between the lysosome and the phagosome triggers an event called the “oxidative burst” that releases a multitude of reactive oxygen species intended to kill microbes. To test whether or not our mutants were more susceptible to the oxidative burst than wild type we chose to do a radial diffusion disk assay using paraquat (a strong superoxide chemical used as an herbicide). The results were in line from what could be expected of strains with an altered or compromised LPS, which is thought to be a protective barrier during such exposures to oxidative stress. The waaY, pmrB and phoP were slightly (and progressively in the order written) more sensitive to paraquat than the wild type and predictably the triple mutant exhibits the highest sensitivity.

*Salmonella* is naturally quite tolerant to both serum and bile so we thought it would be interesting to see if any of the AMP resistant mutants exhibit less tolerance. A known serum/bile sensitive mutant, ΔwaaF (used as a control), was completely eliminated in the human serum exposure experiments, while only our phoP mutant had a noticeable drop in bacterial growth. All our AMP resistant mutants did better than ΔwaaF at tolerating bile exposure. In fact, phoP and pmrB were nearly indistinguishable from wild type.

**Conclusions**

The conclusion that can be drawn from Paper II is that the mutations isolated in the work for Paper I do not cause severely reduced *Salmonella* fitness *in vivo*. During the *in vitro* experiments each mutation generally performs slightly worse than wild type. However, the striking staying power of each of the single mutants in the mouse competition experiment reveals their true, low fitness costs.

**Paper III— Prolonged antimicrobial peptide exposure selects for *Staphylococcus aureus* resistance to human defense peptides**

**Aim**

The main points of this paper were similar to the above studies; for example, we wanted to determine if and how well *Staphylococcus aureus* (*S. aureus*) could develop resistance to AMPS. Additionally, this study also wanted to address the concern about using media that was more mammalian-like and mimics the environment wherein AMPS and bacteria would come into con-
tact. Furthermore, we tested the colonization and dissemination abilities of the resistant mutant strains in a live mouse model.

**Results and Discussion**

*S. aureus* is a much more probable target pathogen for current AMP drug developers. It is a frequent skin and mucous membrane commensal that is known to cause severe, life-threatening infection and illness. AMP drugs in development today are commonly ointments, salves, or drops (eyes and ears) that would all either directly target *S. aureus* as an infectious agent or expose commensal *S. aureus* to AMPs while targeting another type of infection. *S. aureus* is also relevant in resistance studies as it can aggressively acquire multidrug resistance and is becoming increasingly harder to treat with any type of drug.

We serially passaged *S. aureus* in periodically increasing concentrations of three different AMPs and one AMP combination in the mammalian mimic media. After less than 200 generations, stable resistance was exhibited and isolated mutants demonstrated up to 16-fold higher minimum inhibitory concentrations (MIC). When seven isolated mutants were tested for fitness (growth rate assays), in the mammalian media that they were passaged in, surprisingly only two of those seven had a significantly lower growth rate. Several of the mutants displayed significant cross-resistance to the other AMPs used in the study and more importantly became much less susceptible to human β-defensins 1-4. There is a consensus that mechanisms that are responsible for AMP resistance, in general, are expected to be acquired at the expense of virulence. We found this was not the case. All AMP resistant mutants were capable of causing invasive infection, with dissemination to the liver and kidneys, in a live mouse with no significant difference to the wild type. Dissemination was however significantly reduced in the spleen, but was not altogether eliminated, meaning that given time and further AMP selection pressure (i.e. from host), they could develop compensatory mutations that relieve the cost burden and leave the resistance intact.

**Conclusions**

This study highlights several key points. First, *S. aureus* was very quickly able to develop stable resistance to all AMPs used in this study. Second, *S. aureus* had no trouble acquiring stable resistance to a combination treatment of LL-37 and Wheat Germ Histones. Third, cross-resistance was seen among all the mutants against all the AMPs. Fourth, β-defensins 1-4 became less capable of killing our AMP resistant mutants. Fifth, the mouse infection experiment informed us that they are entirely capable of colonization, dissemination and causing invasive disease. Lastly, all of these outcomes should promote caution with regards to AMP drug development.
Concluding Remarks

Multi-drug resistant pathogens are on the rise and new therapeutic solutions are needed. Antimicrobial peptides have been attractive candidates due to their abundance and promiscuous activity against a range of microbes. The aim of this thesis work was to investigate the potential for resistance development in bacteria towards antimicrobial peptides. We have seen that both *Salmonella typhimurium* and *Staphylococcus aureus*, two important disease causing organisms, have little difficulty adapting tolerance to increasing levels of various antimicrobial peptide treatments.

Since bacteria exist in all manner of hostile environments, it should not be surprising that many are capable of developing tolerance to various harsh exposures. It is a reoccurring theme in our lab that no matter the agent used; whether it be a conventional antibiotic or unconventional antimicrobial, bacteria can and do adapt to their environment. We have expressly seen that the speed at which both species (but especially *S. aureus*) gained stable, high levels of resistance against several antimicrobial peptides is truly alarming. The necessity of further comprehensive research concerning resistance to antimicrobial peptides with a multifaceted approach to rates of emergence, genetic mechanisms and disease pathology cannot be stressed enough. The future risk we face when pathogenic bacteria become tolerant to important members of our own immune systems is too great to be taken lightly.
Future Perspectives

The most obvious follow-up work would be to ascertain the exact mechanisms behind the high level of resistance seen in the *Staphylococcus aureus* AMP resistant mutants from Paper III. Proteomics analysis has since been performed and gives tantalizing clues, but without the use of an easy genomics method for reconstructing *S. aureus* mutations into clean, wild type backgrounds, it is difficult to unravel the unexpected mutational spectrum into definitive answers about how organisms such as *S. aureus* develop resistance to antimicrobial peptides. Perhaps in the near future, a CRISPR (clustered-regularly-interspaced-short-palindromic-repeats) genetic manipulation system will be in place for *S. aureus*. Hopefully, this will allow for precious time and resources to be spent answering relevant questions about resistance development rather than having to follow endless genetic reconstruction protocols with limited success.

Another follow-up to this work would be a comprehensive sampling and testing of environmental or perhaps commensal bacteria for the presence of AMP resistant mechanisms. There are many studies being conducted to discover antibiotic resistant determinants existing in environmental bacteria; however, no studies that I know of that specifically test for resistance to antimicrobial peptides. It would be especially interesting to identify AMP resistant mechanisms born by mobile or transposable elements. As this has been confirmed as the most common method of gene exchange for the acquisition of resistance [137].
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References


A doctoral dissertation from the Faculty of Medicine, Uppsala University, is usually a summary of a number of papers. A few copies of the complete dissertation are kept at major Swedish research libraries, while the summary alone is distributed internationally through the series Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine. (Prior to January, 2005, the series was published under the title “Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine”.)