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Antibiotic Resistance

Selection in the Presence of Metals and Antimicrobials

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ACTA
UNIVERSITATIS
UPSALIENSIS
UPPSALA
2018

ISSN 1651-6206
ISBN 978-91-513-0412-0
urn:nbn:se:uu:diva-356970

Dissertation presented at Uppsala University to be publicly examined in B42, BMC, Husargatan 3, Uppsala, Friday, 5 October 2018 at 09:15 for the degree of Doctor of Philosophy (Faculty of Medicine). The examination will be conducted in English. Faculty examiner: Alex O'Neill (School of Molecular and Cellular Biology, University of Leeds).

Abstract
Albrecht, L. M. 2018. Antibiotic Resistance. Selection in the Presence of Metals and Antimicrobials. *Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine* 1488. 53 pp. Uppsala: Acta Universitatis Upsaliensis. ISBN 978-91-513-0412-0.

The external environment is complex: Antibiotics, metals and antimicrobials do not exist in isolation but in mixtures. Human activities such as animal husbandry, fertilization of agricultural fields and human medicine release high amounts these compounds into the environment. The work in this thesis contributes to our understanding of how the selection of bacterial antibiotic resistance can be facilitated by the pollution by metals and antimicrobials. We show that low levels of antibiotics, metals and combinations thereof can lead to the selection of chromosomally encoded antibiotic resistance genes as well as a multidrug resistance plasmid. The underlying genetic and cellular mechanisms of selection identified relate to mutational changes in a plasmid-encoded metal resistance operon, and metal-associated increases in cellular membrane permeability. We further show that exposure to quaternary ammonium compounds can result in cross-resistance to antibiotics following genetic changes in genes related to efflux, membrane synthesis and transcription/translation. Taken together, the work in this thesis suggests that the stewardship of antibiotics should include prudent use of metals and antimicrobials.

Keywords: Antibiotic resistance, Metals, Antimicrobials, Bacterial evolution, Bacterial genetics

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ISSN 1651-6206
ISBN 978-91-513-0412-0
urn:nbn:se:uu:diva-356970 (http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-356970)

”*Science is part of the reality of living;*
it is the what, the how and the why
of everything in our experience.”
- Rachel Carson, Silent Spring

List of Papers

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

I Gullberg, E.*, **Albrecht, L.M.***, Karlsson, K., Sandegren, L., Andersson D.I. (2014) Selection of a Multidrug Resistance Plasmid by Sublethal Levels of Antibiotics and Heavy Metals. *mBIO*, 5:01918–14.

II **Albrecht, L.M.**, Sandegren, L., Andersson, D.I. Mutation in the Copper-Induced *sil* Operon Enables High-Level Silver Resistance and Silver-Facilitated Co-Selection of Multidrug Resistance Plasmid. *Manuscript.*

III **Albrecht, L.M.**, Andersson, D.I. Potentiation of the Selective Effect of Antibiotics by Metal Ions. *Manuscript.*

IV **Albrecht, L.M.**, Andersson, D.I. Cross-Resistance to Antibiotics After Exposure to Quaternary Ammonium Compounds. *Manuscript.*

Paper not included in thesis:

Knöppel, A., Knopp, M.*, **Albrecht, L.M**.*, Lundin, E.*, Lustig, Ulrika.*, Näsvall, J., Andersson, D. I. (2018) Genetic Adaptation to Growth Under Laboratory Conditions in *Escherichia Coli* and *Salmonella enterica. Front Microbiol.* 2018; 9:756.

*Authors contributed equally to this work

Contents

Abbreviations

ATPase	Adenosine triphosphatase
BC	Benzalkonium chloride
CTAB	Cetrimonium bromide
DDAB	Didodecyldimethylammonium bromide
DHFR	Dehydrofolate reductase
DHPS	Dihydropteroate synthase
DNA	Deoxyribonucleic acid
E. coli	*Escherichia coli*
EF-G	Elongation factor G
ESBL	Extended-spectrum beta-lactamase
M. tuberculosis	*Mycobacterium tuberculosis*
MCC	Minimum co-selective concentration
MIC	Minimum inhibitory concentration
MIC_{res}	MIC of a resistant strain
MIC_{susc}	MIC of a susceptible strain
mRNA	Messenger ribonucleic acid
MRSE	Methicillin-resistant *Staphylococcus aureus*
MSC	Minimum selective concentration
NPN	N-Phenyl-1-naphtylamine
PBPs	Penicillin binding proteins
QAC	Quaternary ammonium compound
qPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
RNAP	RNA polymerase
RND efflux pump	Resistance-Nodulation-Division efflux pump
VRE	Vancomycin-resistant *Enterococci*
VRSE	Vancomycin-resistant *Staphylococcus aureus*

Introduction

"*Science and everyday life cannot and should not be separated.*"
- Rosalind Franklin

Since the introduction of the first antibiotics in the late 1930s, human medicine has had to its disposal an effective therapeutic tool for the treatment of bacterial infections. Illnesses that had previously been life threatening could from then on be cured by a short course of treatment that, in addition, presented few side effects to the patient. As the field of medicine has advanced, antibiotics have allowed the routine implementation of aggressive procedures such as cancer treatments, organ transplants, surgical reconstructions and the insertion of artificial joints and teeth. Without prophylactic treatment with antibiotics, these medical procedures would carry a high risk of serious infection.

However, it has become clear that exposure to antibiotics leads to the development of resistance (Austin *et al.*, 1999). As is the case for any component or condition in a microbe's environment, an antibiotic is just another factor to which it will adapt. Thus, the ability to adapt to changing conditions, evolution in other words, ensures that the bacteria that acquire mechanisms to survive and replicate in the presence of an antibiotic will increase in abundance at the cost of bacteria that did not acquire similar mechanisms. In this manner, resistant bacterial populations arise and are able to establish themselves in the local environment, e.g. the site of infection in a patient, and in some cases even spread their mechanisms of resistance further, e.g. by mobile genetic elements to other bacterial species. In light of this, it has become evident that wise and prudent use of antibiotics is vital to ensure that medicine can continue to enjoy the benefits of antibiotic treatments in the years to come.

Further, the work in this thesis contributes to a body of evidence that suggests that the presence of other components, such as metals and antimicrobials, affects bacterial growth and adaptation in the presence of antibiotics in ways that could enhance the development and selection of antibiotic resistance. Therefore, the prudent use and stewardship of metals and antimicrobials is another factor to consider in combating the problem of clinical antibiotic resistance.

The Wonder Drugs

Antibiotics

Most commercial antibiotics are naturally produced by fungi or bacteria as part of their repertoire of secondary metabolites. Perhaps most well know is the production of penicillin by the *Penicillium* mold, but most of our commonly used antibiotics, among them streptomycin and tetracycline, are products of soil bacteria belonging to the genus *Streptomyces* (Procópio *et al.*, 2012). The role of secondary metabolites, and thus the natural function of antibiotics, is not well understood. These small molecules are produced by microbes under certain circumstances, and are not involved in the growth and reproduction of the organisms in an obvious way. A common theory is that antibiotics are exuded to combat surrounding microbes that compete for the same local resources, and there are studies showing that bacteria inhibit growth of their neighbors under certain circumstances (Cornforth and Foster, 2015). In other cases, antibiotics have been found to affect cellular transcriptional responses, and they could thereby be acting in the capacity of signaling molecules, regulating interactions and responses within bacterial communities (Goh *et al.*, 2002; Linares *et al.*, 2006). It has recently been suggested that antibiotics in fact are part of the physiological function of the producing organism by being involved in regulation of the cellular growth rate (Esnault *et al.*, 2017).

Regardless of their original purpose, antibiotics have functioned as "wonder drugs" in the treatment of human and animal infections for decades. The commercial antibiotics are divided into several different classes according to molecular structure and mechanism of action in the bacterial cell, and major classes are listed in Table 1.

Clinical Antibiotic Resistance

Clinically relevant antibiotic resistance means that a particular pathogen fails to respond to the recommended course of antibiotic treatment. Often, an alternative course of treatment can be successful, but some infections persist because the disease-causing pathogen is resistant to all drugs that can reach the target tissue at concentrations sufficient to clear the infection.

Table 1. *Important classes of antibiotics, their cellular binding site and the mechanism by which they inhibit bacterial growth.*

Class	Examples	Binding site	Mechanism of action
Aminoglycosides	Gentamicin Kanamycin Streptomycin	Ribosome	Inhibit protein synthesis
Amphenicols	Chloramphenicol	Ribosome	Inhibit protein synthesis
Ansamycins	Rifampicin	RNAP	Inhibit RNA synthesis
Beta-lactams	Carbapenems Cephalosporins Penicillins	PBPs	Inhibit cell wall synthesis
Glycopeptides	Vancomycin	Peptidoglycan	Inhibit cell wall synthesis
Lincosamides	Clindamycin	Ribosome	Inhibit protein synthesis
Lipopeptides	Daptomycin	Membrane	Disrupt cell membranes
Macrolides	Erythromycin	Ribosome	Inhibit protein synthesis
Oxazolidinones	Linezolid	Ribosome	Inhibit protein synthesis
Sulfonamides	Sulfamethoxazole	DHPS	Inhibit folic acid synthesis
Tetracyclines	Tetracycline	Ribosomes	Inhibit protein synthesis
Polymyxins	Colistin Polymyxin B	Membrane	Disrupt cell membranes
Quinolones	Ciprofloxacin Nalidixic acid	Topoisomeras-es	Inhibit DNA replication
Others	Fosfomycin	MurA	Inhibit cell wall synthesis
	Fusidic acid	EF-G	Inhibits protein synthesis
	Trimethoprim	DHFR	Inhibit folic acid synthesis

Clinical antibiotic resistance in bacteria was noted to occur already at the very beginning of the antibiotic era in the late 1930s and early 1940s. Both sulfonamide and penicillin resistance was reported within a few years after the launching of these two drug classes into therapeutic treatments. Likewise, streptomycin resistant mutants of *Mycobacterium tuberculosis* were identified in patients shortly after treatment was initiated (Medical Research council, 1948; J Davies and D Davies, 2010). Multi-resistant *M. tuberculosis* strains currently cause non-treatable tuberculosis infections. Similarly, multi-resistant *Neisseria gonorrhoeae* is an increasing problem for treatment of gonorrhea (Unemo *et al.*, 2010; Ohnishi *et al.*, 2011).

Antibiotic resistance is a major concern in the treatment of hospital-acquired, or nosocomial, infections, which typically afflict elderly or immunocompromised patients. These pathogens are opportunist; they are commonly part of the human commensal flora, but when the opportunity presents itself in an immunocompromised host, they are able to colonize vulnerable body sites and establish an infection.

Gram-positive bacteria like *Streptococcus pneumonia*, *Staphylococcus aureus* and *Enterococci* are well-documented nosocomial pathogens that have acquired resistance to several drugs. Vancomycin-resistant *Enterococci* (VRE) and methicillin-resistant *Staphylococcus aureus* (MRSA) are great concerns, and some MRSA strains further develop vancomycin resistance

(VRSA). MRSA can also cause skin infections in otherwise healthy individuals, as seen with the much-publicized community-acquired cases (Rice, 2006; Woodford *et al.*, 2009).

Gram-negative bacteria are intrinsically resistant to more antibiotics than are Gram-positives. This is due to the low permeability of the outer membrane coupled with the presence of membrane spanning efflux pumps. These two mechanisms prevent entry and promote extrusion of antibiotics respectively, decreasing intracellular concentrations. In addition, Gram-negatives, like Gram-positives, can acquire additional resistance mechanisms. *Pseudomonas aeruginosa* readily develops multi-resistance through a combination of intrinsic and acquired resistance mechanisms, and it is a major nosocomial agent, especially concerning lung infections in cystic fibrosis patients (Poole, 2011). The major nosocomial players, *Escherichia coli*, *Klebsiella pneumonia* and *Acinetobacter baumannii* cause infections in the urinary tract, lungs, bloodstream, bones and joints. They are increasingly acquiring resistance to the last-resort beta-lactam antibiotics, cephalosporins and carbapenems (Giske *et al.*, 2008).

Survival Strategies of Bacteria

Becoming Resistant

Bacterial genetic adaptation to antibiotics can be achieved through two distinct pathways: (I) by a spontaneous genetic change in the chromosome or (II) by the acquisition of a resistance-conferring gene encoded on a foreign piece of DNA through horizontal gene transfer (Figure 1).

Genetic Changes in Chromosome

A spontaneous genetic change in the chromosome could be a point mutation, insertion or deletion in a gene or regulatory element, or an amplification of a genetic segment (Figure 1A). A genetic change could have a number of consequences in the cell including disabling a gene or altering its expression level. These two events are relatively frequent since they can happen through a number of different avenues such as amino acid substitutions, deletions, insertions and amplifications that affect a gene directly or its regulators. For example, antibiotic resistance is often acquired through increased expression of an efflux pump as a consequence of a disruption in a gene that represses the expression of the pump or through an amplification of the genetic segment that encodes the pump (Piddock, 2006).

Other possible consequences could be an alteration of the conformation of the gene product or a change in the antibiotic binding site. In **Paper II** we describe the first scenario where a point mutation provides silver resistance by ostensibly changing the conformation of the sensor protein. Resistance to rifampicin is achieved by mutations in the binding site of the RNA polymerase preventing the antibiotic from binding its target (Telenti *et al.*, 1993; Campbell *et al.*, 2001; Brandis *et al.*, 2015). An altered protein conformation or a changed binding site would be less frequent events since they require specific mutations to happen in a restricted genetic region (Schaaper *et al.*, 1986; Drake, 1991; Abdulkarim and Hughes, 1996). Despite the lower frequency, these mechanisms are of greater clinical importance since the resistance levels are higher and are associated with lower fitness costs (Huseby *et al.*, 2017).

A chromosomal genetic change is stable and is inherited by the next generation. When the change provides a benefit, such as resistance during antibiotic exposure, the bacteria that possess it will survive and reproduce

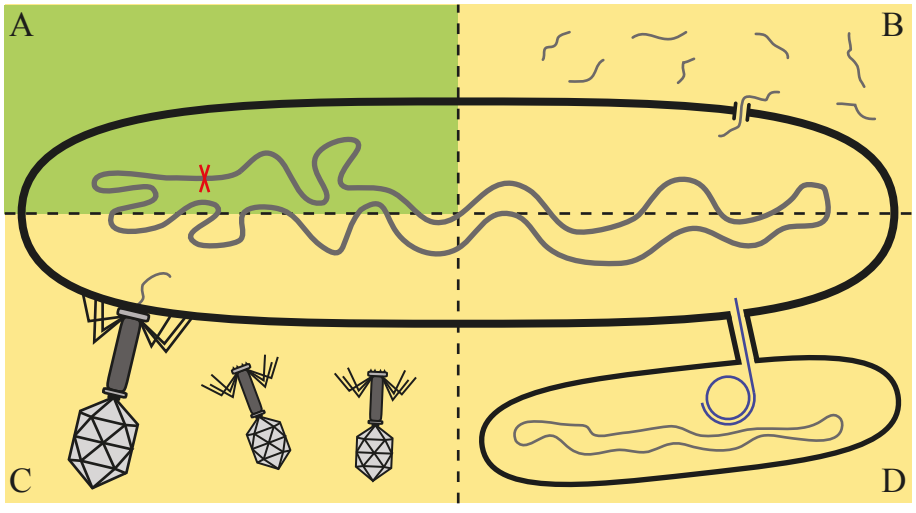

Figure 1. Mechanisms of bacterial antibiotic resistance acquisition. Antibiotic resistance can develop by internal changes in the chromosome (green) or by horizontal gene transfer (yellow). (**A**) Internal mechanisms include spontaneous genetic changes in the form of amino acid substitutions, insertions, deletions or amplifications in the bacterial chromosome. Horizontal gene transfer includes (**B**) the uptake of naked DNA from the environment (transformation), (**C**) transfer of genetic material by phage infection (transduction), and (**D**) the uptake of a mobile genetic element such as a plasmid (conjugation).

at a higher rate than their competitors, and the change gets fixed in the local bacterial population even in the absence of the antibiotic. Notable exceptions to this stability are gene amplifications that rapidly segregate in the absence of selection pressure (Sandegren and Andersson, 2009; Reams *et al.*, 2010).

Horizontal Gene Transfer

Horizontal gene transfer is a common way to acquire a resistance-conferring gene. This happens by obtaining a foreign piece of DNA by (I) the uptake of naked DNA from the environment (transformation) (Figure 1B), (II) infection by a bacterial phage (transduction) (Figure 1C) or (III) the uptake of a mobile genetic element by conjugation (Figure 1D) (Soucy *et al.*, 2015). Mobile genetic elements are pieces of DNA that, as the name suggests, can move independently of the cell. The most notable mobile genetic elements are transposons and plasmids.

A transposon is a segment of DNA that can integrate itself into the bacterial chromosome. Transposons in the Tn21 family are great drivers in the spread of antibiotic resistance because they carry an array of antibiotic resistance genes that are spread among bacteria as the transposon moves within and across species barriers (Liebert *et al.*, 1999). Transposons can also

integrate themselves into the other mobile genetic element mentioned, plasmids.

Plasmids are circular extra-chromosomal entities of DNA that contain the genes necessary for their own proliferation and often also their own intercellular transfer mechanism. They carry genes that are not essential to the growth of the cell under normal conditions but that can confer benefits to the cell in certain circumstances, such as antibiotic and metal resistance genes. Plasmids can harbor sets of resistance genes to several different antibiotics and metals making the cell multi-drug resistant. Thus, the acquisition of one plasmid can render a bacterium resistant to an array of compounds. This way, plasmids can often be of great benefit to their bacterial host cell as they provide the cell with a competitive advantage and greater reproductive success in a set environment. The drawback for the plasmid-carrying bacterium is the fitness cost, or decrease in growth rate, that a plasmid often confers on a cell. The slower growth rate will place the cell at a disadvantage in environments lacking direct selective pressure on the plasmid (Dahlberg and Chao, 2003). The presence of plasmids is to a great extent responsible for the rapid spread of clinical resistance among Gram-negative bacteria. Resistance to several classes of antibiotics, among them beta-lactams (including carbapenems), has been identified in plasmids present in *Enterobacteriaceae* isolated from clinics (Kumarasamy *et al.*, 2010), and plasmid-encoded beta-lactamases such as the extended-spectrum beta-lactamase (ESBL) CTX-M group and carbapenemases cause outbreaks of infections worldwide (Carattoli, 2009).

Plasmid pUUH239.2

The plasmid pUUH239.2 (Figure 2) is an example of an ESBL-encoding plasmid with clinical relevance. It was isolated from a *Klebsiella pneumonia* strain that caused nosocomial outbreaks among elderly and immunocompromised patients at Uppsala University Hospital between 2005 and 2007. This plasmid consists of a backbone similar to another *Klebsiella pneumonia*-associated plasmid, pKPN3, and it encodes a cassette of antibiotic resistance genes also found in plasmids in the recognized outbreak strain *E. coli* ST131 (Woodford *et al.*, 2009; Sandegren *et al.*, 2011). The gene cassette includes resistance to aminoglycosides, macrolides, sulfonamides, tetracyclines, trimethoprim and beta-lactams, including the extended spectrum beta-lactamase gene CTX-M-15 that breaks down cefotaxime. In addition, the plasmid carries genes for resistance to quaternary ammonium compounds as well as to the metals silver, copper and arsenic (Sandegren *et al.*, 2011).

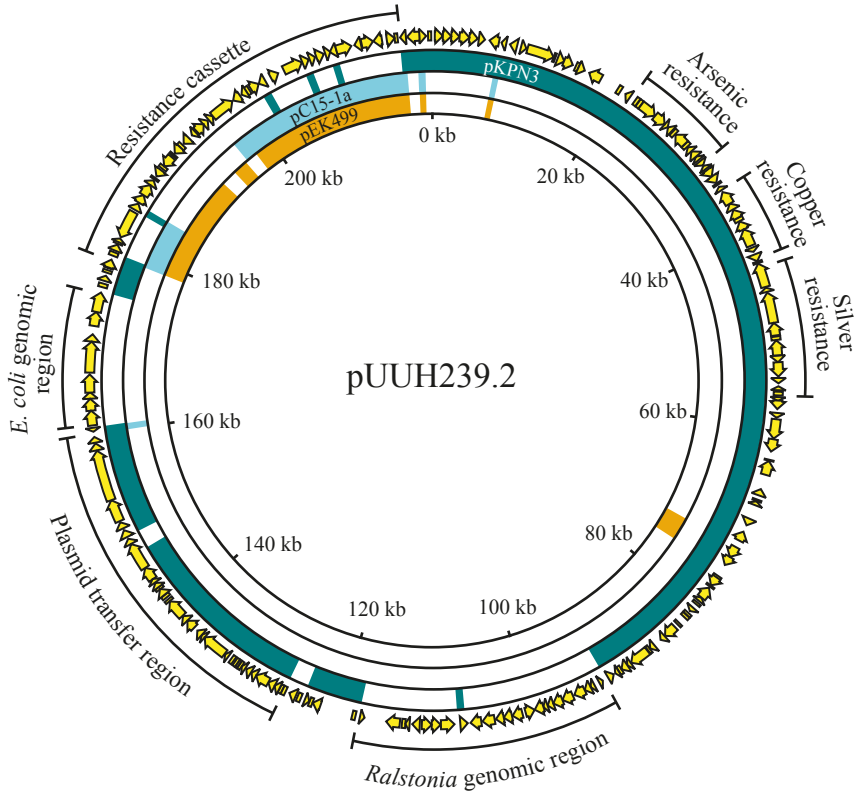

Figure 2. The multidrug resistance plasmid pUUH239.2 and alignment to pKPN3, pCP15-1a and pEK499. The backbone is similar to that of plasmid pKPN3 and includes three metal resistance operons. The extensive antibiotic resistance cassette is shared with plasmids in the infectious strain E. coli ST131.

Mechanisms of Resistance

The mechanisms of antibiotic resistance involve interfering with or preventing the action of the antibiotic molecule. The bacterial cell has a number of potential strategies at its disposal, and more than one mechanism can sometimes work against the same antibiotic (Figure 3).

A ubiquitous and general mechanism of resistance is (I) decreasing cellular concentration of the antibiotic by preventing uptake or enhancing efflux (Figure 3A). The uptake of antibiotics can for example be reduced by the deletion of membrane porins through which the antibiotics enter the cell (Nikaido, 2001; Nikaido, 2003). The unspecific efflux pump, AcrAB-TolC spans the membranes of many clinical Gram-negatives, and transports a wide array of toxic substances out of the cell including the antibiotics ciprofloxacin, tetracycline and chloramphenicol. Genetic changes that lead to overexpression of the pump increases the level of resistance (Baucheron *et al.*, 2004; Keeney *et al.*, 2007; Swick *et al.*, 2011). Another common strategy is (II) preventing the antibiotic from binding to its cellular target. This can be achieved by altering the antibiotic binding site in the target molecule (by mutation or enzyme activity) (Figure 3B) (Hooper, 2000; Brandis *et al.*, 2015), by the presence of a "protective" molecule that competes with the antibiotic for binding the target (Figure 3C) (Connell *et al.*, 2003) or by enzymatic alteration or destruction of the antibiotic molecule itself (Figure 3D) (Bonnet, 2004; Robicsek *et al.*, 2005). Both rifampicin and quinolone resistance is achieved by mutations in the cellular target, RNA polymerase and DNA gyrase respectively, that inhibit binding of the antibiotic molecule (Telenti *et al.*, 1993; Heisig and Tschorny, 1994; Piddock *et al.*, 1999; Marcusson *et al.*, 2009; Brandis *et al.*, 2015). Ribosomal protection proteins, such as TetM protect the ribosomes from tetracyclin binding (Connell *et al.*, 2003; Dönhöfer *et al.*, 2012) and topoisomerase protection proteins can confer resistance to fluoroquinolone antibiotics (Tran *et al.*, 2005; Garoff *et al.*, 2018) Resistance to the important beta-lactams is provided by destruction of the antibiotic molecule by beta-lactamases (Bonnet, 2004). Further, the cell can (III) bypass the harmful action of the antibiotic. This can be done by abandoning the use of the target molecule by finding alternative proteins to perform the same biochemical function as the target molecule (Figure 3E) or by increasing expression of the target molecule (Figure 3F). An example for the use of an alternative protein is MecA-mediated methicillin resistance in MRSA (Stapleton and Taylor, 2002) and overexpression of MurA, the target of the antibiotic fosfomycin, can confer clinical resistance levels (Couce *et al.*, 2012).

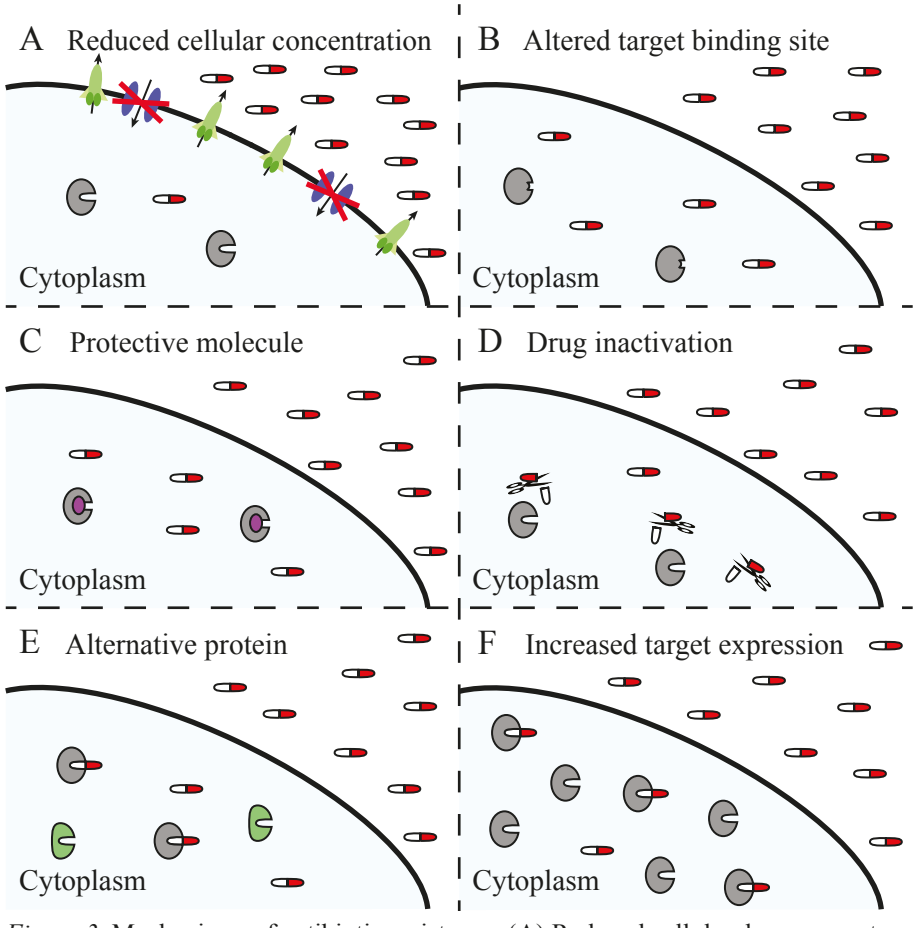

Figure 3. Mechanisms of antibiotic resistance. (**A**) Reduced cellular drug concentration by decreasing uptake or increasing efflux. (**B**) Prevention of drug binding by altering the binding site on drug target. (**C**) Prevention of drug binding by the presence of a protein that competes for the same binding site on drug target. (**D**) Prevention of drug binding by enzymatic destruction of the drug. (**E**) Bypassing the harmful effect of the drug by the presence of alternative protein to perform the same biochemical function as the drug target. (**F**) Bypassing the harmful effect of the drug by increasing the level of expression of the drug target.

Resistance and the Natural Environment

"*There are so many ways in which we are destroying the planet. And once we understand, once we care, then we have to do something.*"
- Jane Goodall

Origin of Antibiotic Resistance Genes

Despite the current problem of clinical antibiotic resistance, it has been well established that antibiotic resistance determinants originated long before the human antibiotic era. Cultured Gram-negatives and Gram-positives from a New Mexico cave that had been isolated for 4 million years collectively exhibited resistance to more than a dozen commercially available antibiotics including streptomycin, clindamycin, sulfamethoxazole as well as semi-synthetic macrolides and the last-resort drug daptomycin (Bhullar *et al.*, 2012). Similarly, 30 000 year old permafrost contained bacterial genes for resistance to vancomycin, beta-lactams and tetracycline. Interestingly, the vancomycin resistance determinant, *vanA*, resembled modern versions (D'Costa *et al.*, 2011).

These studies demonstrate the presence of a wide range of antibiotic resistance determinants before human-induced selection had any influence on bacterial evolution. Since antibiotics are themselves produced by microorganisms, this may not be surprising. But as is the case for antibiotics, the role of antibiotic resistance genes in nature is not clear. They could have evolved to interfere with signaling or to ward off toxic effects of antibiotics in the producing organism itself or in competitors. Beta-lactamases might originally be proteins involved in cell wall synthesis, the antibiotic resistance activity being an unintentional side effect (Massova and Mobashery, 1998).

The Bacterial Resistome

The collective reservoir of bacterial antibiotic resistance genes, referred to as the resistome, has most likely served as the origin of resistance determinants that have been introduced into pathogenic strains (Figure 4).

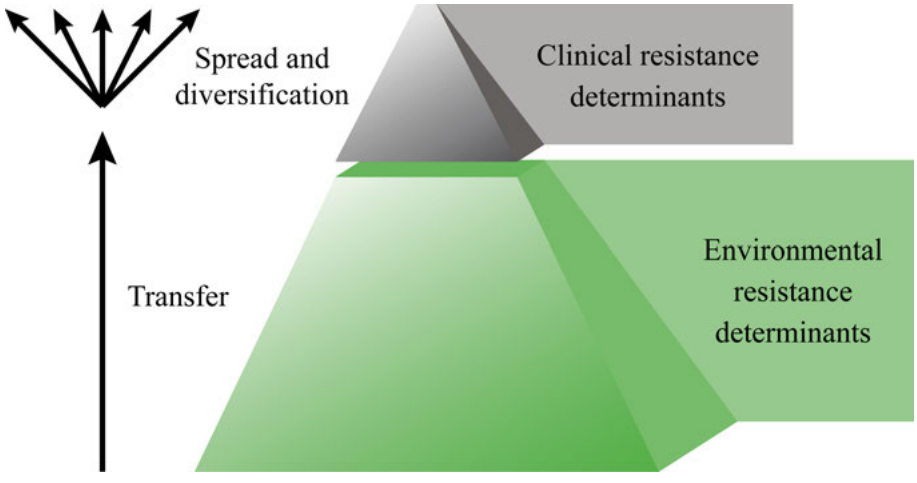

Figure 4. The variety of antibiotic resistance genes that pose problems in the clinic is the mere tip of the iceberg of the total diversity present in environmental bacteria. The genes that get transferred from environmental strains to clinical pathogens undergo a process of spread and diversification where they adapt to selection pressures specific to the clinical setting.

Studies have revealed an impressive diversity of resistance determinants in natural soils and waters. A survey of the genus *Streptomyces* in urban, agricultural and forest soil found that all 480 cultured strains were resistant to at least seven of the antibiotics tested. The antibiotics included the natural compounds vancomycin and erythromycin, their semi-synthetic derivatives and the synthetic molecules ciprofloxacin and linezolid. Some of the resistance mechanisms had never previously been described (D'Costa *et al.*, 2006). Likewise, soil bacterial DNA isolated from a Wisconsin oak savanna contained nine different aminoglycoside resistance genes, and the six acetyltransferases among them differ from already described aminoglycoside acetyltransferases (Riesenfeld *et al.*, 2004). A similar approach found beta-lactamases in relatively pristine Alaskan soil. These enzymes exhibited great diversity, falling into four distinct structural classes, and differing in sequence from previously known versions (Allen *et al.*, 2009).

It is evident that the environmental resistome harbors antibiotic resistance genes covering a much more diverse spectrum than the ones identified in the clinic (Figure 4). Some mechanisms found in nature have not been found in the clinic at all whereas others, like the aminoglycoside and vancomycin resistance determinants mentioned above, have similar functional mechanisms compared to those in clinical strains although the enzymes differ in specific sequences and functions. This indicates that clinically problematic resistance genes originated in the environmental resistome of mostly non-pathogenic strains. Upon acquisition by human pathogens, the genes would have evolved to optimally respond to the specific antibiotic stresses imposed by humans.

The Environmental Resistome and Human Pathogens

Antibiotic resistance genes are widespread and diverse in environmental bacteria, but in order to pose a clinical threat to humans they need to enter and be expressed in strains that belong to the mammalian commensal flora or that can colonize the mammalian gut, nasopharynx, lungs or urinary tract and potentially cause infection. A proposed scenario is that anthropogenic use and distribution of antibiotics create artificially high concentrations in human habitats and thus facilitate the process of gene transfer from environmental bacteria to mammalian pathogenic strains. There are a number of studies surveying resistance frequencies in commensal bacteria from wild animals that point towards a connection between resistance and exposure to humans or human activities.

In one study, fecal *E. coli* was sampled from animals in distinct groups according to density of human population within the animal's habitat. There was a clear trend where occurrence of resistance increased with human population density, and the highest frequencies were found within farm animals and pets (Skurnik, 2006). Two separate studies show that enteric bacteria from gorillas whose habitat overlapped with humans and livestock, as well as from baboons that resided near a tourist lodge, exhibited more antibiotic resistance than enteric bacteria from gorillas and baboons living in isolated areas with minimal human contact (Rolland *et al.*, 1985; Rwego *et al.*, 2008). In a similar manner, rodents living close to human populations in the UK were found to have a high prevalence of resistance to beta-lactams in their commensal *Enterobacteriaceae* (Gilliver *et al.*, 1999), whereas *Enterobacteriaceae* isolated from moose, deer and vole in remote areas of Finland harbored almost no antibiotic resistance (Österblad *et al.*, 2001).

These studies are all observational and do not show the underlying mechanisms of the observed pattern, but they do show a correlation between human contact and antibiotic resistance in animal enteric bacteria. Thus, they offer indications that (I) residual concentrations of antibiotics that are present in populated areas select for resistance in enteric strains and (II) that the resistance genes and/or resistant enteric strains spread and are shared between creatures residing within the geographical area where the selective antibiotics are present.

The resistance circulating within animal populations in this manner could originally have been selected for among human patients or in clinics and subsequently spread into the surrounding animal habitat. Alternatively, the genes could originate from the environmental resistome. As such, the "artificial" selection pressure by residual antibiotics released by human activities would have allowed resistance genes that were transferred from environmental bacteria to enteric bacteria to establish themselves within the enteric bacteria. A metagenomic study identified resistance genes in bacteria from various U.S. soils that were identical to genes encoded in human pathogenic

strains. The genes conferred resistance to beta-lactams, aminoglycosides, amphenicols, sulfonamides and tetracyclines (Forsberg *et al.*, 2012). Plasmid-borne beta-lactamases belonging to the CTX-M group have also been found on the chromosomes of the soil and water dwelling *Kluyvera* indicating that this clinically relevant resistance has environmental origins (Poirel *et al.*, 2002). Aquatic *Shewanella* have been identified as the reservoirs of the ciprofloxacin resistance gene *qnrA* as well as the oxacillinase group of beta-lactamases (Poirel *et al.*, 2004; Poirel *et al.*, 2005). Two separate studies also present evidence that the exchange of antibiotic resistance genes happened by way of mobile genetic elements such as integrons (Skurnik, 2006; Forsberg *et al.*, 2012).

Metals and Antimicrobials

Resistance to Metals

Metals are natural components in the earth's crust occurring in rocks, soil and water. Thus, bacteria are continuously exposed to metals, and some, like iron, copper and zinc, function as essential micro-nutrients in bacterial physiology by e.g. stabilizing proteins and catalyzing chemical reactions (Bruins *et al.*, 2000). However, due to the reactive nature of metals, excessive cellular concentrations are toxic. In addition, certain metals, such as silver and mercury, serve no physiological purpose and are toxic already at low concentrations. Several possible metal-associated toxic effects have been proposed. These include the interference of protein function by destruction of iron-sulfate clusters and depletion of thiols, as well as disruption of the electron transport chain (Lemire *et al.*, 2013).

Thus, metal toxicity has necessitated the development of bacterial metal resistance systems, and it is a reasonable assumption that this developed early in evolution. Phylogenetic studies of mercury and arsenic resistance markers indicate ancient origins (Jackson and Dugas, 2003; Boyd and Barkay, 2012), and mercury resistance genes have been identified in bacterial samples from permafrost reported to be between 15 000 and 40 000 years old (Mindlin *et al.*, 2005).

Metal resistance genes are not confined to the chromosomes, but also exist on mobile genetic elements making them easier to transfer between bacteria. Already in ancient times, the mercury resistance systems were encoded on transposons (Kholodii *et al.*, 2003; Mindlin *et al.*, 2005). Further, a recent, encompassing study involving a wide range of bacterial species found metal/biocide resistance mechanisms in 86% of the genomes analyzed, and arsenic, cadmium, copper and mercury resistance systems were prevalent on plasmids (Pal *et al.*, 2015).

While metals occur naturally in nature, human activities result in high amounts being released in certain locations. The anthropogenic redistribution of metals leads to increased concentrations in many environments that are also exposed to antibiotics and potential human pathogenic bacteria, such as sites for, and waste water from, animal husbandry and food production. Pollution of arsenic, cadmium, copper, mercury and zinc originate from farm animal and fish feed supplements, agricultural pesticides, natural and artificial fertilizers as well as ship hull antifouling agents (Han *et al.*, 2002; Ni-

cholson *et al.*, 2003; Dean *et al.*, 2007; Silbergeld and Nachman, 2008; Burridge *et al.*, 2010). Silver comes in direct contact to humans and human commensal flora as it is widely used as an antimicrobial agent. It is applied to wounds, bandages, commercial textiles and common household items (Silver, 2003; Dai *et al.*, 2010; Mijnendonckx *et al.*, 2013).

The *sil* Operon

The term operon refers to a collection of genes, often transcribed together on one transcription unit, that collectively perform a certain function. The operon referred to as *sil* (Figure 5) was originally identified on the plasmid pMG101.

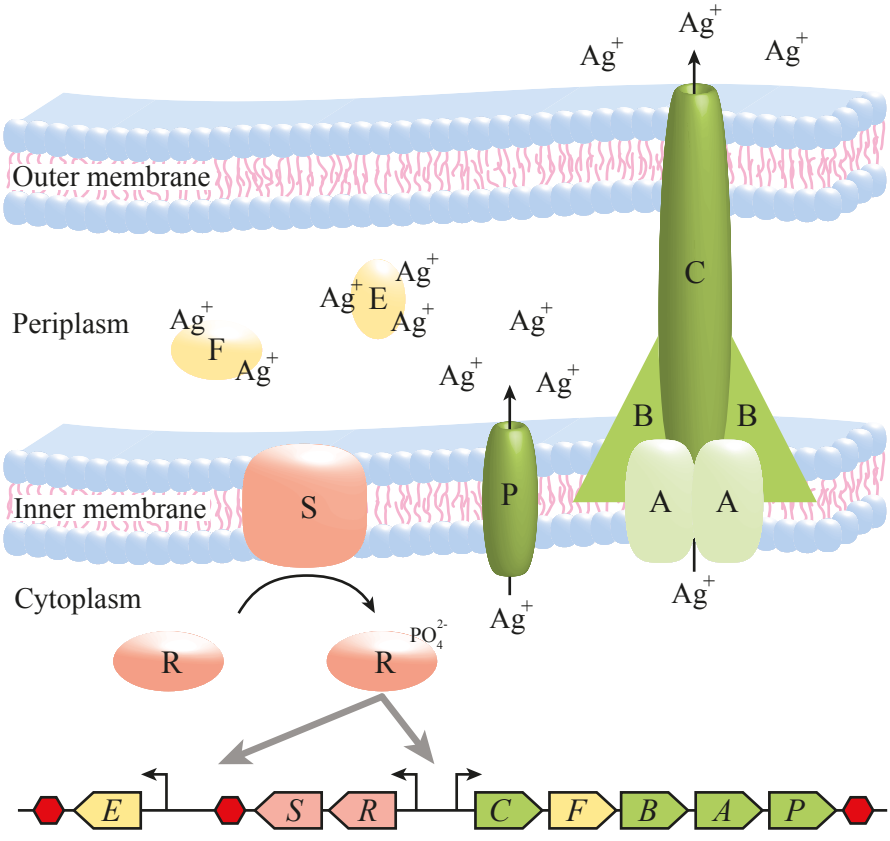

Figure 5. A schematic view of the *sil* operon and its genes as encoded on pUUH239.2. There are three separate transcription units with their own promoters (angled arrows) and terminators (red hexagons). The system includes two efflux pumps, *silCBA* and *silP* (green), two periplasmic metal binding proteins, *silF* and *silE* (yellow) and a two-component regulatory system, *silR* and *silS* (red). The sensor, SilS, spans the inner membrane and upon detecting its signal it phosphorylates the SilR response regulator, which allows initiation of transcription.

This plasmid was present in a *Salmonella enterica* serovar Typhimurium isolated from patients in a hospital burn unit where silver nitrate was used in treatment (McHugh *et al.*, 1975). The *sil* operon constitutes a stretch of eight identified genes, s*ilCFBAPRSE*, that were discovered to provide silver resistance (Gupta *et al.*, 1998; Gupta *et al.*, 1999).

The functions of the *sil* genes were ascribed based on homologies with previously known metal resistance operons. It includes two efflux pumps and two metal binding proteins, and there are three separate transcription units. The largest transcription unit, *silCFBAP*, encodes the RND efflux pump *silCBA* that spans both the inner and outer cell membranes, the inner membrane-spanning P-type ATPase efflux pump *silP* as well as the periplasmic metal binding protein *silF*. The other two transcription units are encoded facing in the opposite direction. The first unit encodes a two-component system composed of a sensor kinase, *silS*, and a response regulator, *silR*. The second unit encodes the periplasmic binding protein *silE* (Figure 5) (Gupta *et al.*, 1999; Randall *et al.*, 2015).

The *sil* operon is frequently encountered in clinical Gram-negative bacteria (Gupta *et al.*, 1998; Kremer and Hoffmann, 2012; Sütterlin *et al.*, 2014; Finley *et al.*, 2015; Sütterlin *et al.*, 2017), and on plasmids (Gupta *et al.*, 2001; Kremer and Hoffmann, 2012), among them the plasmid pUUH239.2. Furthermore, *sil* genes have been found in association with beta-lactamases (Sütterlin *et al.*, 2014) and with copper (*pco*) and arsenic (*ars*) tolerance genes (Pal *et al.*, 2015).

Co-selection of Metal and Antibiotic Resistance

Observational studies have reported co-selection involving metals and antibiotics where metal exposure was associated with increased antibiotic resistance (Berg *et al.*, 2005; Berg *et al.*, 2010). This phenomenon can occur as a result of two different mechanisms. The first mechanism involves general efflux pumps that are able to expel both metals and antibiotics. Exposure to a metal could, for example, increase expression of the pump, leading to concurrent increases in metal and antibiotic resistance (Mata *et al.*, 2000; Flach *et al.*, 2017).

The second mechanism involves two different resistance determinants (one towards the metal and the other towards the antibiotic) that both are encoded on the bacterial chromosome or on a mobile genetic element. In such a case, exposure to either the metal or the antibiotic would select for the genome in question, e.g. a transposon or a plasmid, with all its encoded resistance genes. This latter process, designated co-selection, has been observed within animals; fecal bacteria from pigs that were fed with copper-supplemented fodder showed increased copper, macrolide and glycopeptide resistance, and they carried a plasmid encoding the relevant resistance genes (Hasman and Aarestrup, 2002; Hasman *et al.*, 2006).

There is also a link between chromosomally encoded resistance to zinc and to methicillin in *Staphylococcus aureus* indicating that zinc exposure can select for MRSA strains (Cavaco *et al.*, 2010). This shows that metal exposure can have direct consequences for clinically relevant strains. Clinical relevance is further evidenced by a study that found that water-dwelling opportunistic human pathogens exposed to cadmium also demonstrated resistance to ampicillin and gentamycin (Stepanauskas *et al.*, 2006).

Antimicrobials: Quaternary Ammonium Compounds

Antimicrobials are used in a wide array of consumer products to inhibit and control bacterial growth. There is concern that the impact of these agents on microbial ecology could be one driving force in the development and spread of antibiotic resistance. One group of commonly used antimicrobials, quaternary ammonium compounds (QACs) (Figure 6), has been studied with respect to this concern.

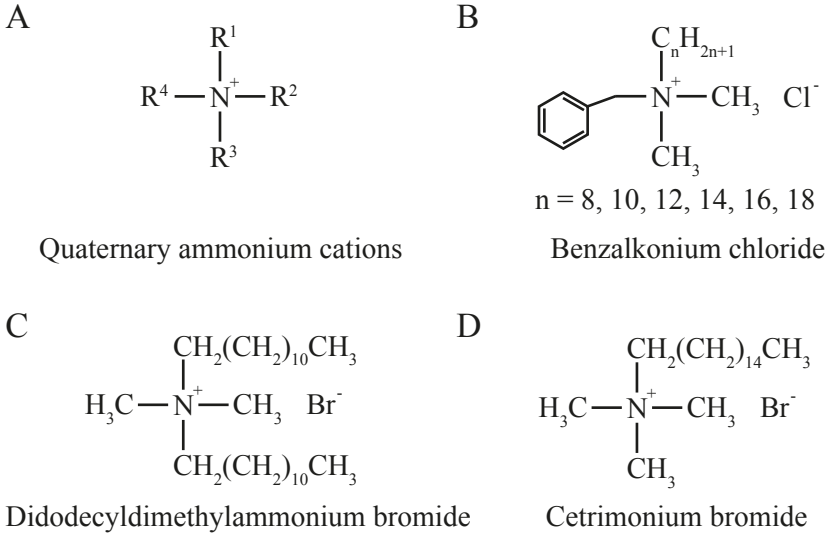

Figure 6. The molecular structures of quaternary ammonium compounds. (**A**) The general structure consisting of a central positively charged nitrogen with four side groups of which at least one will be a long hydrocarbon chain. (**B**) Benzalkonium chloride has one long hydrocarbon chain that can vary in length from eight to eighteen carbons. (**C**) Didodecyldimethylammonium bromide has two long hydrocarbon chains. (**D**) Cetrimonium bromide has one long hydrocarbon chain.

QACs have been extensively used since the mid 1940s. They are common active ingredients in commercial products for cleaning and disinfecting surfaces in households and in the food processing industry, and they are added as preservatives to personal hygiene products such as lotions, sunscreen, hair conditioners and hand sanitizers (Holah *et al.*, 2002; Buffet-Bataillon *et al.*, 2012). The basic structure of QACs consists of a positively charged central nitrogen that is bound to four separate groups (Figure 6A). The four bound groups have different structures depending on the specific QAC, but at least one of them will constitute a hydrophobic carbon chain. Cetrimonium bromide (CTAB) and benzalkonium chloride (BC) each possess one hydrophobic hydrocarbon chain, and didodecyldimethylammonium bromide (DDAB) possesses two hydrophobic hydrocarbon chains, among the four groups (Figure 6B-D). The positive charge on the nitrogen is balanced by the presence of a negatively charged chloride or bromide ion forming the QAC salt.

QACs initiate their antibacterial mechanism of action by virtue of being positively charged cations. The positive charge allows them to interact with the negatively charged phospholipids that make up the cellular outer membrane for subsequent integration of the hydrophobic hydrocarbon tail into the hydrophobic bacterial membrane core. This action disrupts membrane integrity leading to leakage of cellular components and disturbance of membrane-bound proteins (Gilbert and Moore, 2005; Ceragioli *et al.*, 2010; Ferreira *et al.*, 2011).

Resistance to Quaternary Ammonium Compounds

Membrane disruption is considered to be the main mechanism of QAC antimicrobial action, but intracellular mechanisms have been implicated at lower concentrations. Exposure to CTAB at lower levels was observed to generate production of reactive oxygen species in *E. coli* (Nakata *et al.*, 2010), and BC exposure generated changed expression levels of genes involved in oxidative stress (Moen *et al.*, 2012).

Documented QAC resistance mechanisms mainly involve efflux or changes to the cell membrane. *Salmonella* and *Pseudomonas* exposed to QACs acquired resistance associated with efflux and increased activity of the *acrAB* efflux system (Mc Cay *et al.*, 2010; Guo *et al.*, 2014; Webber *et al.*, 2015). Genes related to membranes and osmotic stress have also been implicated (Tabata *et al.*, 2003; Moen *et al.*, 2012). Additionally, resistance through biodegradation has been observed where a *Pseudomonas* strain was able to break down a QAC by its central metabolism (Takenaka *et al.*, 2007).

Bacteria that develop resistance to QACs often exhibit cross-resistance to antibiotics. Exposure to QACs have resulted in cross-resistance to ciprofloxacin, chloramphenicol, nalidixic acid, tetracycline and trimethoprim (Mc Cay *et al.*, 2010; Guo *et al.*, 2014; Webber *et al.*, 2015).

Complex Mixtures and Sub-MIC Concentrations

Selection in the Presence of Mixtures and Low Concentrations

The minimum inhibitory concentration (MIC) of an antibiotic is the lowest concentration that inhibits the growth of a bacterial strain. The MIC of a resistant strain (MIC_{res}) would thus be higher that the MIC of a susceptible strain (MIC_{susc}). It has been generally assumed that the selection of antibiotic resistant strains takes place at antibiotic concentrations that lie above the MIC_{susc}. However, in **Paper I**, **Paper II** and **Paper III** we demonstrate that selection of a resistant strain can also happen at concentrations below the MIC_{susc}, that is, at sub-MIC concentrations. Figure 7 is used to illustrate the theoretical concepts described below.

The growth rate of a bacterial strain is a key factor in its success. When competing against other strains for local nutrients, a higher growth rate equals a greater competitive edge. Thus, the growth rate is often referred to as fitness, and a decrease in growth rate would be a decrease in fitness. Genetic changes, such as a mutation, or the acquisition of a mobile genetic element, such as a plasmid, are factors that can inflict a fitness cost on a strain. However, the growth rate is highly dependent on the environment, and it is only meaningful in relative terms as being compared to potential competitors. If the mutation or plasmid provides resistance to an antibiotic, the strain possessing it would have a growth rate advantage in the presence of the antibiotic compared to a strain lacking the resistance marker.

Thus, in the absence of the antibiotic, the susceptible strain would have a higher growth rate than the resistant strain (Figure 7, green field). As the antibiotic concentration increases, the fitness cost of the resistant strain would be counterbalanced by the advantage of being resistant, and the resistant strain would grow faster and outcompete the susceptible strain (Figure 7, yellow and orange fields). The lowest antibiotic concentration at which the resistant strain outcompetes the susceptible strain is referred to as the minimum selective concentration (MSC).

In **Paper I** and **Paper II**, we show that the MSC of antibiotics and metals can lie at sub-MIC concentrations, well below the MIC_{susc} (Figure 7, orange field). In **Paper I** and **Paper III**, we show that when in the presence of mix-

tures, the resistant strain can be selected at even lower concentrations, at so called co-selective concentrations (Figure 7, yellow field). The concentration of each selective component in a mixture is referred to as the minimum co-selective concentration (MCC).

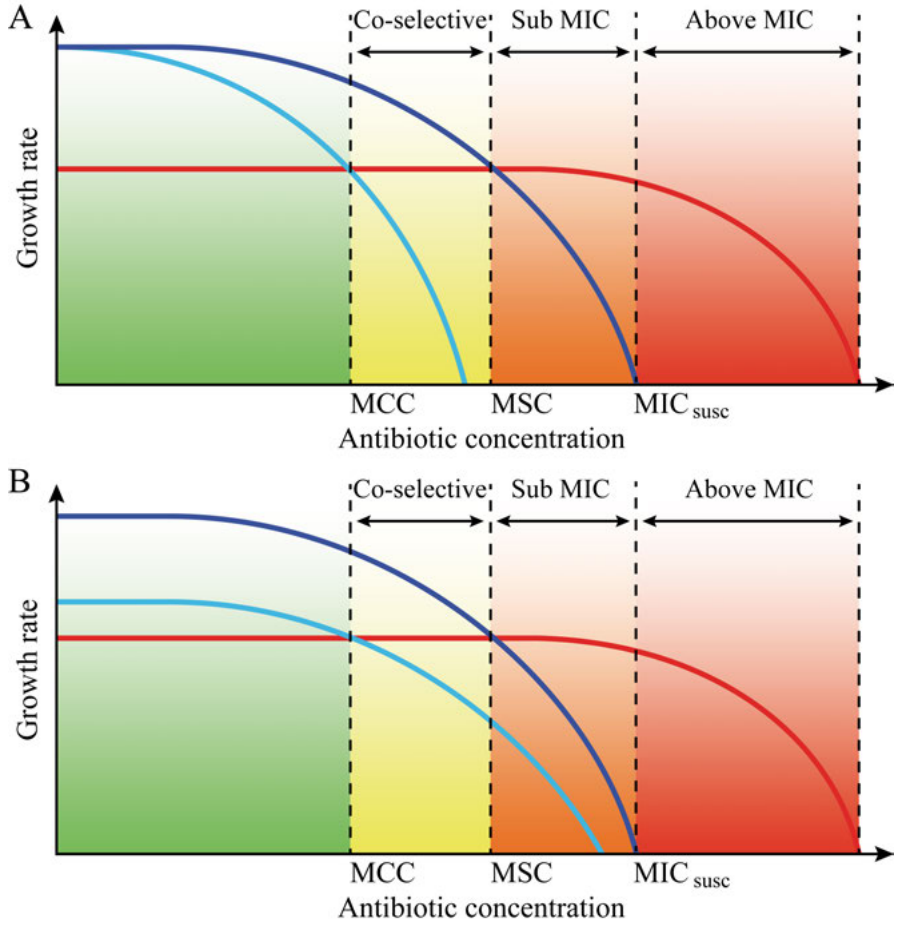

Figure 7. A theoretical overview over bacterial growth rates at increasing antibiotic concentrations. The lines represent the bacterial growth curves of a resistant strain (red), a susceptible strain (dark blue) and a susceptible strain in the presence of a mixture of antibiotics/metals (light blue). The different fields indicate a range of antibiotic/metal concentrations where the strain with the higher growth rate is selected. At the very lowest concentrations (green field), the susceptible strain (dark blue) is selected. At high concentrations (red field), and at sub-MIC concentrations (orange field), the resistant strain is selected. In mixtures of antibiotics/metals, the resistant strain is selected at even lower concentrations, referred to as the co-selective concentrations (yellow field). Selection at the co-selective concentrations can be due to (**A**) a steeper decrease in growth rate of the susceptible strain or (**B**) an initial decrease in growth rate of the susceptible strain.

Selection in the co-selective window can be explained by two distinct mechanisms. (I) The presence of a secondary compound in the mixture could increase the drug influx into the cell leading to an increased effective intracellular drug concentration. This would lead to a steeper decrease in growth rate of the susceptible strain relative to in the presence of one antibiotic/metal alone. (Figure 7A, dark blue versus light blue growth curve). (II) Alternatively, a secondary compound in the mixture could lead to a decrease in growth rate of the susceptible strain relative to the presence of one antibiotic/metal only. In this case, the steepness of growth rate decrease of the susceptible strain would not change but reach the growth rate of the resistant strain at a lower concentration due to the lower initial starting point (Figure 7B, dark blue versus light blue growth curve). The dynamics of selection in complex mixtures most likely involve a combination of these two mechanisms.

Current Investigations

Paper I: Selection of a Multidrug Resistance Plasmid by Sublethal Levels of Antibiotics and Heavy Metals

Aim: To investigate the potential for selection of a multidrug resistance plasmids in the presence of lower antibiotic and metal concentrations, as well as in the presence of mixtures of these selective components.

Background: The selection of multidrug resistance plasmids is an important factor in the spread of clinical antibiotic resistance. Selection can take place in environments where selective components, such as antibiotics and metals, are present, including the clinical setting, wastewater, wastewater treatment plants and agricultural areas (Garbarino *et al.*, 2003; Bolan *et al.*, 2003; Kümmerer, 2009). The effect of these milieus on plasmid selection is not well understood. In this paper, we determined the minimum selective concentration of various antibiotics and metals for a strain carrying the multidrug resistance plasmid pUUH239.2.

Method: The MSCs of tetracycline, trimethoprim, kanamycin, erythromycin, the two metals copper and arsenic as well as mixtures of these components were determined by competing an *E. coli* strain carrying the multidrug resistance plasmid pUUH239.2 (resistant strain) against an isogenic *E. coli* strain that lacked the plasmid (susceptible strain) in the presence of the components mentioned above. The resistant and susceptible strains were combined at a 1:1 ratio and grown in the presence of various concentrations of the antibiotic(s)/metal for up to forty generations. After each ten generations, the culture was transferred to fresh medium, and the ratio of resistant to susceptible strain was determined. A fluorescent marker, blue or yellow fluorescent protein, had been inserted in the chromosome of both strains to enable detection using flow cytometry. The ratios were used to calculate the selection coefficient of the resistant strain. The selection coefficient was plotted as a function of antibiotic(s)/metal concentration, and the point where the curve intercepts the x-axis represents the MSC.

Results: For all individual antibiotics and metals, the MSC was below the minimum inhibitory concentration for the susceptible strain The values

ranged from 2/3 below the MIC_{susc} in kanamycin to close to 140-fold below the MIC_{susc} in arsenite. Further, we observed that the presence of more than one antibiotic/metal contributed to an increase in the selection coefficient. The following combinations were tested: (I) tetracycline and arsenite, (II) tetracycline, arsenite and trimethoprim, and (III) trimethoprim and erythromycin. Adding arsenite (at the concentration of 0.5 x the MSC of arsenite) to tetracycline resulted in increased selection coefficients at all tetracycline concentrations and a subsequent 20% decrease in the MSC of tetracycline. The presence of a third component, trimethoprim, revealed a trend where each component, present at concentrations below or at its respective MSC, contributed to an increase in selection coefficient. The strongest effect was observed in setup III where synergy was apparent as the concentration of each antibiotic required for selecting the resistant strain was lower when combined with the other antibiotic than when alone.

Conclusions: Antibiotics and metals at concentrations well below the MIC_{susc} can select for a multidrug resistance plasmid. More strikingly, when present in mixtures, even lower (sub-MSC) concentrations of antibiotics and metals can contribute to the selection. This means that minute concentrations of antibiotics and metals in mixtures can contribute to the selection of multidrug resistance plasmids even if the concentration of each component alone is insufficient for selection.

Discussion: Minute concentrations of antibiotics and metals exist in many environments, and the same environments would often be contaminated with more than one component. Examples of this include wastewater, effluents from drug manufacturing plants, wastewater treatment plants, areas of livestock, poultry and fish rearing as well as agricultural fields amended with synthetic and organic fertilizers, irrigated with wastewater or exposed to pesticides. Additionally, these environments would be contaminated with human or animal feces containing potential pathogenic bacterial strains as well as the plasmids they carry. Thus, the use and distribution of antibiotics and metals is a concern in many external environments where the lower concentrations were previously assumed to be insufficient for selection of antibiotic resistance.

Paper II: Mutation in the Copper-Induced *sil* Operon Enables High-Level Silver Resistance and Silver-Facilitated Co-Selection of Multidrug Resistance Plasmid

Aim: To gain understanding of the *sil* operon, and to investigate the potential for silver-mediated selection of a multidrug resistance plasmid.

Background: The antimicrobial properties of silver are utilized in silver-impregnated bandages, creams, ointments, household products and clothing items to prevent topical wound infections and combat undesired odor (Silver, 2003; Dai *et al.*, 2010; Mijnendonckx *et al.*, 2013). In addition, *sil* genes, that have been associated with silver and copper resistance, have frequently been identified in clinical Gram-negatives (Gupta *et al.*, 1998; Kremer and Hoffmann, 2012; Sütterlin *et al.*, 2014; Finley *et al.*, 2015; Sütterlin *et al.*, 2017), and on plasmids that also encode antibiotic resistance genes (Gupta *et al.*, 2001; Kremer and Hoffmann, 2012; Pal *et al.*, 2015). These observations elicit concerns that human use of silver could be selecting for silver resistance, and even more importantly, for multidrug resistance plasmids. The *sil* genes, *silCFBAPRSE*, commonly encoded in a cluster referred to as an operon, encode two efflux pumps, two metal binding proteins and a two-component regulatory system consisting of the transmembrane sensor, *silS*, and its response regulator, *silR*. The *sil* operon would be activated through detection of a signal by SilS, and the subsequent initiation of transcription by SilR. Unexpectedly, bacteria encoding *sil* genes do not always demonstrate a resistance phenotype, and studies have revealed that a point mutation in *silS* is required for resistance to silver (Randall *et al.*, 2015; Elkrewi *et al.*, 2017).

Methods: To characterize the *sil* operon during growth in the presence of silver and copper, we compared an *E. coli* strain encoding the *sil* operon on the plasmid pUUH239.2 (sil^+ strain) to an isogenic *E. coli* strain where the operon had been removed from the plasmid using genetic engineering (sil^- strain). Resistance to copper and silver was assessed by measuring growth rate and final culture density in the presence of each metal by monitoring the culture densities during growth using light at 600 nm wavelength. The expression level of the *sil* operon in response to copper and silver was assessed by extracting mRNA from the bacterial cultures. The level of mRNA was then determined by reverse transcription to obtain DNA and subsequent quantification of the DNA using qPCR. Silver resistant mutants were selected by plating cultures of the pUUH239.2-carrying strain on petri dishes infused with silver at the concentration of 8 x MIC. Colonies that grew after incubation over night were purified by restreaking and subsequently se-

quenced. Competitions were performed between a silver resistant *silS* mutant and the susceptible wild type in the presence of silver. By measuring the ratio of resistant to susceptible strain, the selection coefficient of the resistant strain was calculated and plotted as a function of the silver concentrations. The intercept of the x-axis represents the MSC of silver.

Results: In the presence of silver, the *sil*$^{+}$ strain had no advantage over the *sil*$^{-}$ strain neither in terms of growth rate nor final culture density. In line with this observation, there was no detectible silver-induced expression of the *sil* operon. In contrast, in the presence of copper, the *sil*$^{+}$ strain had a faster growth rate and a denser final culture compared to the *sil*$^{-}$ strain, and accordingly, copper-induced expression of the *sil* operon was increased approximately 10-fold as compared to in the absence of metal. Seven individual point mutations in the sensor gene *silS* provided silver resistance demonstrated as a 125-fold increase in the MIC of silver. The silver resistant mutants exhibited inducer-independent expression of the *sil* operon at levels between a 15-fold and 45-fold increase compared to the wild type. Further, we found that the MSC of a silver resistant mutant was approximately ¼ of the MIC of the susceptible strain.

Conclusions: The *sil* operon is primarily a copper resistance system that, once expressed, also shows activity towards silver. Since silver does not induce expression, silver resistance requires a mutation that renders the operon constitutively expressed. Once silver resistance has been obtained through a *silS* mutation, the pUUH239.2 plasmid can be selected for in the presence of sub-MIC concentrations of silver.

Discussion: This study highlights how the presence of a metal can influence bacterial evolution. In this case, bacteria responded to silver exposure by redirecting the use of an already fully developed copper resistance operon towards silver detoxification by acquiring only one single point mutation. This type of redirected use of already functioning genes can be a strategy for bacteria to become resistant to not only metals but also other toxic compounds such as antibiotics. Furthermore, we show that the presence of low silver concentrations can select for a multidrug resistance plasmid. While silver might not be a crucial antimicrobial in modern medical care, and silver resistance, per se, may not be a significant threat to human health, bacterial exposure to silver nevertheless can be problematic in terms of its potential to co-select for antibiotic resistance. Since many silver infused commercial products would come in direct contact with human commensal and pathogenic bacteria when applied to skin or disposed of in municipal waste or sewage, the indiscriminate use of silver is worth reconsidering.

Paper III: Potentiation of the Selective Effect of Antibiotics by Metal Ions

Aim: To assess the ability of metals to potentiate the selective effect of antibiotics on antibiotic resistant strains.

Background: Mixtures of antibiotics and metals exist in many environments. Metals such as arsenic, cadmium, copper, mercury, and zinc are components in fish and farm animal food supplements, agricultural pesticides, natural and artificial fertilizers and ship hull antifouling agents (Han *et al.*, 2002; Nicholson *et al.*, 2003; Dean *et al.*, 2007; Silbergeld and Nachman, 2008; Burridge *et al.*, 2010). Silver is added to ointments, creams, bandages, plastics and textiles (Silver, 2003; Dai *et al.*, 2010; Mijnendonckx *et al.*, 2013). At the same time, antibiotics are distributed widely within agriculture, fish farms and human medicine. As a consequence, metals and antibiotics mix in wastewater, wastewater treatment plants, sludge from wastewater treatment plants, agricultural soils amended with sludge or manure, fields treated with pesticides, fish farms and industrial runoff. These complex environments would exert evolutionary pressures on the microbial communities that are largely unknown and difficult to predict. Here, we have studied potentiation of selection, one aspect of metal-antibiotic interactions with respect to bacterial communities. Potentiation of selection refers to the presence of a metal enhancing the selective effect of an antibiotic on an antibiotic resistant strain.

Methods: We assessed potentiation by measuring the MSC of an antibiotic on a resistant strain in the presence and in the absence of a metal. Potentiation was observed in the cases where the MSC of the antibiotic decreased in the presence of a metal. Competitions were run between a *Salmonella enterica* Typhimurium LT2 encoding a chromosomal antibiotic resistance gene and a susceptible isogenic wild type in the presence of various concentrations of an antibiotic and a constant concentration of a metal. The antibiotics used were streptomycin, rifampicin and fusidic acid, and the metals included arsenic, cadmium, copper, mercury, silver and zinc. Every possible metal-antibiotic combination was tested where the metal concentration was constant at 0.25 x MIC of the respective metal. The resistant and the susceptible strains were combined at a 1:1 ratio and grown in the presence of relevant antibiotic or metal-antibiotic combination. After each ten generations of growth, the culture was passaged into fresh medium, and the ratio of the resistant strain to the susceptible strain was measured by flow cytometry. The ratios were used to calculate the selection coefficient of the resistant strain, which was then plotted as a function of the antibiotic concentration. The point where the curve intercepts the x-axis is the MSC of the antibiotic.

Outer membrane permeability following metal exposure was measured using the compound N-Phenyl-1-naphtylamine (NPN), which fluoresces once it enters the hydrophobic environment of the bacterial cell membrane. Thus, the more permeable the membrane, the more NPN will enter and the higher the fluorescent signal will be.

Results: In certain metal-antibiotic combinations, the selective effect of the antibiotic was potentiated, whereas in others not. Silver had the larges spectrum of activity, potentiating selection of the resistant strain in the presence of all three antibiotics. The largest effect of silver was observed in rifampicin where the MSC decreased approximately 4-fold. A similar degree of potentiation was observed by cadmium in the presence of streptomycin. The selective effect of fusidic acid was potentiated by cadmium and mercury (in addition to silver), decreasing the MSC about 2-fold. The observed potentiation could be due to the metals exerting damage to the bacterial cell membrane facilitating entry of the antibiotic. Our results showed that exposure to the metals that had a potentiating effect, namely silver, cadmium and mercury, did result in the highest levels of outer membrane permeability, with the exception of copper, which was the most potent metal in this regard despite not showing any potentiating effect on the antibiotics.

Conclusions: In some metal-antibiotic combinations, the presence of the metal effectively decreases the antibiotic concentration required to select for the antibiotic resistant strain. Our results also indicate that metal-mediated outer membrane permeability is involved in the observed potentiation. However, the exact mechanism of potentiation requires further study, as metals shown to increase membrane permeability did not potentiate all antibiotics. For example, copper did not potentiate, but did result in a large increase in outer membrane permeability. This could be due to the activity of a copper defense protein that is activated during the competition assay but not during the outer membrane permeability assay.

Discussion: In mixtures, the presence of metals could be facilitating the selection of resistant strains by decreasing the antibiotic concentration required for selection. This has implications in environments where antibiotics and metals exist together, such as wastewater, effluents from drug manufacturing plants, wastewater treatment plants, areas of livestock, poultry and fish rearing as well as agricultural fields amended with synthetic and organic fertilizers, irrigated with wastewater or exposed to pesticides. Therefore, distribution and release of metals, and not only antibiotics, could be a contributing factor in the spread of antibiotic resistance.

Paper IV: Cross-Resistance to Antibiotics After Exposure to Quaternary Ammonium Compounds

Aim: To investigate the genetic mechanisms behind resistance to quaternary ammonium compounds and cross-resistance to antibiotics.

Background: Quaternary ammonium compounds (QACs) are antimicrobials used in a variety of household and personal care products to inhibit bacterial growth (Holah *et al.*, 2002; Buffet-Bataillon *et al.*, 2012). QACs contain a positively charged core and a hydrophobic tail. This structure enables them to adhere to the negatively charged bacterial cell membrane and integrate their hydrophobic tail, causing membrane damage. This causes leakage of cellular components and disruption of membrane-bound proteins, and it is thought to be the main mechanism of QAC antibacterial activity (Gilbert and Moore, 2005; Ceragioli *et al.*, 2010; Ferreira *et al.*, 2011). There is concern about QAC resistance and its link to antibiotic resistance, which has been reported in several studies (Tabata *et al.*, 2003; Mc Cay *et al.*, 2010; Moen *et al.*, 2012; Guo *et al.*, 2014; Webber *et al.*, 2015).

Methods: *E. coli* was passaged in the presence of the three common QACs, benzalkonium chloride (BC), didecyldimethylammonium bromide (DDAB) and cetrimonium bromide (CTAB). Passaging was performed by growing the bacteria in microtiter plates over night and transferring them to fresh medium every morning. There were two separate rounds of passaging. The initial round was done in the presence of a constant sub-MIC concentration. The concentration each QAC used was where the exponential phase growth rate of *E. coli* was reduced to 80% compared to in the absence of the QAC. Four independent populations were passaged in each QAC for 200 generations. For the second round of passaging, a clone was isolated from a population exposed to each QAC during the initial round of passaging, and it was divided into four new populations. The new populations continued to be passage in the same QAC, and the concentration was incrementally increased by 20% until dense cultures ceased to form. At the end of the experiment, one clone from each population was whole genome sequenced. The MICs of all QACs as well as of the antibiotics ampicillin, chloramphenicol, ciprofloxacin, erythromycin, polymyxin B, streptomycin, tetracycline and trimethoprim were measured.

Results: The genetic changes identified fell into four main categories according to cellular function of the gene involved: efflux, membranes, transcription/translation and other. In response to the sub-MIC passaging, most genetic changes were in genes related to efflux pumps: duplications of *acrAB* and *emrE* as well as mutations and insertions/deletions in genes that

regulate expression of efflux systems. Following the second round of passaging, the genetic changes were mainly in genes related to membranes or transcription/translation. Several point mutations were identified in the genes *msbA*, *lpxL* and *lpxM* that are involved in lipid A synthesis and transport (Clementz *et al.*, 1997; Raetz *et al.*, 2007; Eckford and Sharom, 2010), and there were point mutations in genes encoding RNA polymerase, DNA gyrase and ribosomal subunits. With the exception of polymyxin B, cross-resistance was observed towards all QACs and antibiotics, and most of the increases in MICs were in the range of 2- to 4-fold. There were some notable exceptions e.g. the MIC of CTAB increased up to >16-fold, the MIC of trimethoprim increased up to 12-fold after exposure to BC, and the MICs of streptomycin and chloramphenicol increased up to 11-fold and 32-fold, respectively, after exposure to DDAB.

Conclusions: Exposure to QACs, both at sub-MIC and higher concentrations, can result in cross-resistance to other QACs and antibiotics.

Discussion: The increases in resistance to CTAB, trimethoprim, streptomycin and chloramphenicol were substantial, showing that exposure to QACs can cause significant cross-resistance to antibiotics. Although the levels of increased resistance in many cases were relatively low in this experiment, any increase in resistance gives the bacterium an advantage over fully susceptible strains. This advantage can serve as a stepping-stone in the development of even higher levels of resistance. Not surprisingly, many of the genetic changes identified were related to efflux mechanisms. However, we also show changes in genes related to membranes and transcription/translation. The exact mechanisms behind QAC and antibiotic resistance provided by these genetic changes warrants further investigation.

Concluding Remarks

"*In nature, nothing exists alone.*"
- Rachel Carson, Silent Spring

The work included in this thesis shows that selection of antibiotic resistance is a process that does not happen in isolation. Rather, as is the case in all biological and ecological systems, a multitude of components interact to influence and shape the outcome.

Metal- and antimicrobial-facilitated selection of antibiotic resistance could be occurring in any location where bacteria interact with these components. Of particular interest are sites in proximity to humans or human activities that are subjected to antibiotic, metal and antimicrobial pollution. Inside a patient body is one such environment, as the patient may be exposed to antimicrobials, such as QACs or silver, while undergoing a course of antibiotics.

Various sites in the external environment are also potential hot spots for selection. Hospital and municipal wastewater contains antibiotics, QACs and metals excreted from patients and industries. They all end up and mix at wastewater treatment plants, and sludge from these plants is used to fertilize agricultural fields (Kümmerer, 2004; Zhang *et al.*, 2015). Antibiotics are widely distributed as growth promoters and prophylactics within animal husbandry, and metals are present in pig and poultry fodder, feed supplements distributed at fish farms, agricultural pesticides and artificial fertilizers. Agricultural manure and runoff therefore contain antibiotics and metals. As a consequence, agricultural fields are exposed to both antibiotics and metals when manure, wastewater for irrigation, pesticides and fertilizers are applied (Han *et al.*, 2002; Nicholson *et al.*, 2003; Dean *et al.*, 2007; Silbergeld and Nachman, 2008; Burridge *et al.*, 2010). Wastewater and agricultural areas also contain mammalian enteric bacteria that could potentially cause human infections.

Antibiotic resistant bacteria selected at the sites described above have a direct route to humans via the food chain. They could be present on fruits, vegetables and grains grown in fields exposed to antibiotics and metals. Beef, pork, poultry and fish are other components of the human food supply routinely exposed to both antibiotics and metals in the form of growth promoters and prophylactic treatments.

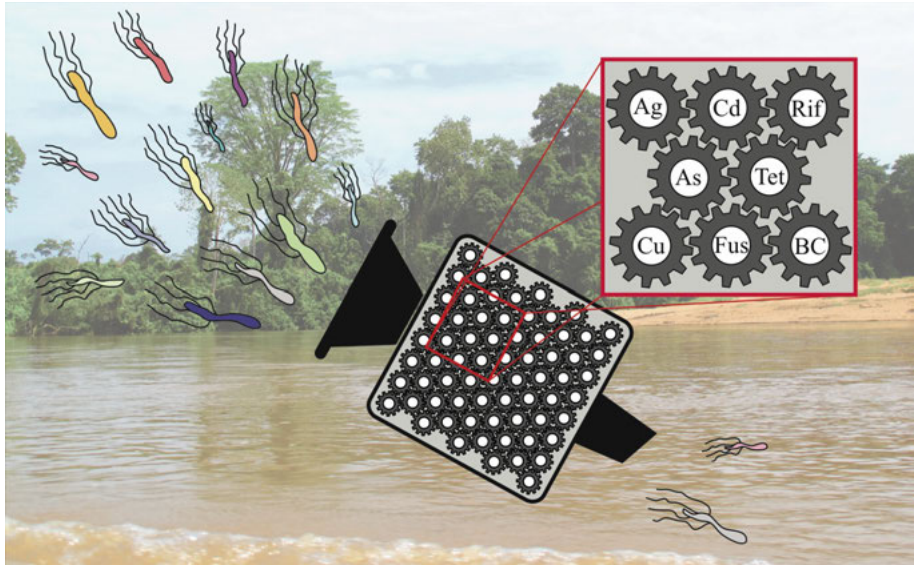

Figure 8. The complex dynamics in an environment that contain mixtures of antibiotics, metals and antimicrobials can exert selection pressures on the bacterial community that result in reduced microbial diversity in favor of a few resistant species.

Thus, antibiotics, metals and antimicrobials released by human activities can coexist with potential human pathogens in hospital and agricultural settings creating opportunities for selection and co-selection of clinically relevant antibiotic resistance (Figure 8).

In light of this, minimizing the emission of antibiotics and co-selective elements into the environment is of great importance. This would be achieved by decreased usage within human medicine, veterinary care and agriculture, as well as the implementation of effective systems for collecting runoff and sewage. Within animal husbandry, lower livestock density and more nutrient-rich fodder are methods to control infectious diseases and maintain required growth rates without the use of prophylactics and other growth promoters. Proper collection methods of waste and sewage is another vital factor, meaning that access to basic hygiene and a well functioning sewage system are necessary for collection, containment and destruction of pollutants (Pruden *et al.*; 2013). Optimal wastewater treatment processes are then required for the removal of contaminants from the effluent. Two possible technologies for this purpose are adsorption of contaminants onto activated carbon or molecule destruction by application of ozone (Dodd *et al.*; 2006; Altmann *et al.*; 2014). The implementation of such wastewater treatment procedures would greatly reduce environmental contamination.

Acknowledgements

To past and present members of the D7:3 corridor,
Thank you for creating a friendly and enjoyable work atmosphere. Many students and guest researchers have come and gone throughout the years, and graduated PhD students have moved on, but you have all contributed to good conversations and laughter in the "fika" nook, lunch room or lab in general.

My participation in the research of D7:3 would of course not have been possible without my supervisor **Dan Andersson**. Thank you for giving me the opportunity to learn, grow and evolve as a scientist and as a person. I appreciate your laid-back approach and positive attitude.

A special thank you to my co-supervisor, **Linus**. You demonstrate great patience and willingness to help, and you were instrumental in getting my project up and running when I was a fresh PhD student. Since then, you have been open for conversations and giving input about all things silver and pUUH, not to mention the quirky nature of *sil*.

Diarmaid, thank you for taking the time to provide valuable suggestions and alternative angles concerning my projects. Your thorough, meticulous and organized approach has been just what I needed sometimes, and it made it possible for me to gain new insight and understanding throughout the course of my work.

To my office mates, you have all been great people to share close working quarters with. **Lisa T**, you are always ready to lend an ear, and we have had many a fruitful conversation about science, life and family. I am hoping for many more. **Omar**, you show genuine interest in people's well being, always ready to offer support and advice. **Michael**, your dedication to science is unmistakable, I am sure you have an exciting career ahead of you. **Marlen**, **Jessica KS** and **Hava**, it has been a while since we shared an office, but you all added to the amicable atmosphere with good conversations, jokes and scientific insights.

Anna K, you are one of the most caring people I have ever met, and **Jocke**, you are always ready to lend a helping hand (and brain). I have really en-

joyed conversations, dinner parties and outings with the two of you, and hope there will be many more to come. **Karin**, you draw from a depth of experience as a scientist when confronted with (our) strange and unexplainable results, and **Ulrika**, the work you invest in keeping the lab organized and running is valuable to all of us. I always enjoy the company of the two of you around the "fika" table. Fun during "fika" is also provided by **Jenny**, your open and congenial nature brings happiness around the table, and **Isa**, not only do you provide humor and spot-on comments, but your resourcefulness during the process of starting up your rather unique project is impressive. **Marie**, with your generosity and joyful demeanor, you bring happiness to those around you. In addition, you always have a great story to share. **Linnéa**, despite your own claim that you are not good at "mingling", conversations with you always come easy, and they are genuine and interesting (and genuinely interesting). **Rachel**, I have enjoyed our chats about life, many over a cup of tea, and some over a glass of gin. **Eva**, your can-do attitude is an inspiration, as is your cheerful mood and heart-warming laugh. **Katrin**, your smile warms up the corridor, and it is always fun to hear your description of the "Bavarians". **Marius**, it has been interesting to hear your plot predictions and theories about books and movies, especially Game of Thrones. **Lionel**, **Serhiy** and **Doug**, it is always nice to chat when our paths meet at the coffee machine or "fika" table, and **Hervé**, your ability to make people laugh is invaluable, especially on days when "everything" fails in the lab. **Hind**, your stories about Sudan have been both educational and insightful, and **Dennis**, your stories after your experiences in Japan have been both insightful and hilarious. **Po-Cheng**, they way you calmly recount dramatic incidents and situations experienced in South Africa and Kenya is truly entertaining. It has been great learning about other cultures through the three of you. **Greta**, the way you organize your presentations is superb, and you make the best-looking science posters. **Erik WY**, in addition to scientific instruments, your knack for design has produced exquisite medieval clothing items. **Sha Cao**, you are efficient and meticulous in the lab, and good-natured and amiable around the "fika" table. **Fredrika** you always have a friendly smile on your face, and you are quick to offer your help. Helpfulness and friendliness are also trademarks of **Erik L**, as well as succinct science-related comments. **Arianne**, you demonstrate grit and perseverance in starting up your new project, and I predict rewarding results in the near future.

Thank you to my parents, **Peter** and **Magnhild**, who have always given me the greatest support, not only in my educational pursuits, but in being who I want to be, doing what I want to do and going where I want to go. You have provided me with the tools to navigate the labyrinth of life, and the encouragement to pull through challenges.

To my sister, **Gunnhild**, my life is enriched by your presence. Despite the geographical distance (and the confusions about calculating time zones before phone calls…), you remain one of my closest confidants. We have both been "crazy busy" with our respective lives these last few years, but now, having earned our degrees, we will be able to settle down and be like "normal" people again ;-) **Mandar**, welcome to the family! It has been great getting to know you, and I am looking forward to a tour of all the balmy beaches in your new neighborhood next time we visit.

Gerrit, my life companion and partner in crime. I am amazed by your scientific capacity, and your musings and theories have lit up many light bulbs in my head throughout the years. But much more importantly, it is a delightful adventure to share and build up a life together with you. You have stood by my side through thick and thin, always with great compassion, pragmatism and humor. Even the toughest days end with a smiles and laughter when you are around. You are a wonderful husband and an extraordinary dad.

Eidar, my favorite little explorer. It has been a joy to discover the world together with you these last two years, and to experience your display of awe and wide-eyed wonder about the most "mundane", every-day details. You are teaching me what life is all about. I am greatly looking forward to all adventures we will share in the years ahead, and a little brother will soon be joining us on those adventures :-).

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