Vancomycin heteroresistance
in
Staphylococcus aureus

Bachelor thesis

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Abstract

Bacterial heteroresistance is the presence of small amounts of bacterial cells within a population that have an increased resistance to antibiotics (*i.e.* an increased MIC). When exposed to an antibiotic these cells have a competitive advantage and are more likely to survive and give rise to a more resistant population. This can be especially problematic in clinical infections, where it is thought to be a cause of treatment failure and has been associated with increased patient mortality. Heteroresistance is seen as an intermediate stage between antibiotic susceptibility and full antibiotic resistance.
Amongst the different types of heteroresistances that can occur, heteroresistance against vancomycin by Methicillin-Resistant *Staphylococcus aureus* (MRSA) is perhaps the most studied. This report aims to create an overview of some of the mechanisms that may lead to vancomycin heteroresistance in this so-called Vancomycin-Intermediate *S. aureus* (VISA) and to compare these to known mechanisms behind heteroresistance against other antibiotics.
It was found that mutations in different regulatory genes are especially common in VISA strains. These mutations affect growth rate, cell wall production and expression of virulence factors.
In contrast, heteroresistance to other classes of antibiotics is commonly reported to be caused by mutations in genes encoding single structural proteins. This shows that the mechanisms that lead to an increased antibiotic resistance depend on the mechanism of action of the antibiotic and that there is no 'one' way in which heteroresistance develops. Nonetheless, any type of heteroresistance may develop through improper antibiotic use and the development of heteroresistance can be greatly limited by application of the right antibiotics in high enough doses.

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Chapter 1. Introduction

1.1 Introduction

Bacterial resistance to antibiotics (antimicrobial resistance) is a problem of increasing size and concern. Infections caused by bacteria resistant to one or multiple antibiotics are more often lethal, and require more resources to treat, than infections by fully susceptible bacteria (World Health Organisation [WHO], 2014). Additionally, the extensive use of antibiotics around the world, both for healthcare and for agriculture, has caused a rise in the occurrence of antimicrobial resistance in human pathogens (Madigan et al., 2012). Consequently, antimicrobial resistance is seen by many as a major threat to human wellbeing in the 21th century (Walker *et al*., 2009; WHO, 2014; Cassir, Rolain & Brouqui, 2014).

An example of the effects of antimicrobial resistance on human health is the widespread occurrence of methicillin-resistant *Staphylococcus aureus* (MRSA). Infections by *S. aureus* were, in the past, often fully treatable with β-lactam antibiotics. However, *S. aureus* strains that had evolved to be resistant to penicillin, methicillin and other β-lactam antibiotics began appearing in hospitals between 1940 and 1970 and are common today, with the share of MRSA in *S. aureus* infections being reported to be over 80% in some regions (including the U.S.) as of 2014 (Madigan et al., 2012; WHO, 2014).

1.2 Heteroresistance

The problem of antimicrobial resistance is further enhanced by the occurrence of heteroresistance. Heteroresistance is generally defined as the presence of small amounts of antibiotic-resistant cells in any population of bacteria that is otherwise fully susceptible to this antibiotic. These cells may be either fully resistant, or simply less susceptible to the antibiotic's effect (Falagas *et al*., 2008; El-Halfawy & Valvano, 2015). This small fraction of cells is more likely to survive a treatment with antibiotics and may, once the competition by non-resistant cells has been eliminated, give rise to a new, fully antibiotic-resistant infection.

The amount of heteroresistant cells within a population differs between bacterial species, but is in the order of one cell per 10^5 - 10^6 cells (Falagas *et al*., 2008; Satola *et al*., 2011). However, this number quickly rises after repeated antibiotic exposure (McAleese *et al*., 2008).

This increased antimicrobial resistance may be the result of genetic variation between cells, giving some cells an intrinsic antibiotic resistance even before being exposed to the antibiotic. It may also emerge gradually under antibiotic selective pressure (Falagas *et al*., 2008; Satola *et al*., 2011).

Heteroresistance is a somewhat poorly characterised phenomenon, with different studies and institutes using different definitions and detection methods. Because of this, the occurrence and clinical impact of heteroresistance is relatively unknown (Falagas *et al*., 2008; El-Halfawy & Valvano, 2015). Several studies suggest that heteroresistance is a common cause of the recurrence of infections in hospital patients (Bert *et al*., 2003; Lin *et al*., 2012) and heteroresistance has been linked to increased mortality rates in multiple studies (Bert *et al*., 2003; Lamberghini *et al*., 2011; Campanile *et al*., 2011; Lin *et al*., 2012). Other studies, however, claim that its impact is overstated (Khosrovaneh *et al*., 2004) or that heteroresistance does not affect patient outcome (Adam *et al*., 2012; Park *et al*., 2012). These contrasting views may perhaps be partially attributed to differences in criteria and detection methods (El-Halfawy & Valvano, 2015).

Another contributor to the limited knowledge about heteroresistance is that the occurrence of heteroresistance in patients may go unnoticed (and be without consequence) as a functional immune system may be able to eliminate any remaining heteroresistant cells after an administered antibiotic has eliminated the majority of an infection. In immunocompromised patients, however, heteroresistant cells may pose a much larger threat (Aguilar, Giménez & Barberán, 2009). To complicate matters, it is sometimes difficult to determine the cause of the recurrence of an infection after it has occurred.

The exact definition of heteroresistance, and the criteria used to determine whether an isolate is heteroresistant, vary between studies. Recently, El-Halfawy and Falvano (2015) proposed that *'an isolate can be considered heteroresistant when the lowest antibiotic concentration giving maximum growth inhibition is greater than 8-fold higher than the highest noninhibitory concentration. An 8-fold difference may be regarded as intermediate heteroresistance, while a smaller difference denotes a homogeneous response to the antibiotic.'* This demonstrates the characteristics of heteroresistant cells: in a homogeneously susceptible population, a small increase of antibiotic concentration may inhibit or kill the entire population. In contrast, a much higher antibiotic concentration (greater than 8 times the highest noninhibitory concentration) may be required to completely inhibit or kill a population containing heteroresistant cells.

Heteroresistance has been observed in many different bacterial species, but only in a limited number of antibiotics. The majority of the reports of heteroresistance concern decreased susceptibility against glycopeptide and β-lactam antibiotics, which may mean either that heteroresistance only occurs in these antibiotics, or that past research has simply focused specifically on these antibiotics. The occurrence of heteroresistance has perhaps been the most reported, and most studied, in *S. aureus*, but examples of other pathogenic bacteria in which heteroresistance has been reported are *Burkholderia cenocepacia* (El-Halfawy & Valvano, 2013), *Enterococcus faecium* (Khan *et al*., 2008), *Acinetobacter baumannii* (Li *et al*., 2006), *Klebsiella pneumoniae* (Pournaras *et al*., 2010), *Escherichia. coli* (Baquero, Vicente & Perez-Diaz, 1985), and *Streptococcus pneumoniae* (Morand & Muhlemann, 2007). Heteroresistance appears to occur more often in gram-positive bacteria than in gram-negative bacteria (El-Halfawy & Valvano, 2015), but this may also be attributed to the fact that the majority of research on heteroresistance is done on antibiotics that are mainly effective against gram-positive bacteria.

1.3 Difference between heteroresistance and persistence

A phenomenon with characteristics and consequences similar to those of heteroresistance is that *Persistence*: upon exposure of a bacterial population to a bactericidal antibiotic, the initial rate of bacterial death is fast, but may slow down as time passes. This indicates the presence of bacterial cells (the persister cells) that are less susceptible than the others are. However, unlike heteroresistant cells, these cells have not acquired a genetic antibiotic resistance: when they are isolated, cultured and re-exposed to the antibiotic they are still susceptible. Instead, persistence is caused by different factors such as the cellular growth rate at the moment of exposure (Gefen & Balaban, 2008). Nonetheless, different studies use different definitions of both heteroresistance and persistence, so the distinction between the two is not always clear.

1.4 Heteroresistance against vancomycin in methicillin-resistant *S. aureus*

Perhaps the most discussed example of heteroresistance and its implications for human healthcare is the emergence of heteroresistance against vancomycin in methicillin-resistant *S. aureus* (MRSA). Vancomycin is a glycopeptide antibiotic that is commonly used as last-resort treatment in MRSA infections. The frequency at which this so-called heterogeneous vancomycin-intermediate *S. aureus* (hVISA or VISA) is found in patients with an *S. aureus* infection differs between studies. Studies report occurrences of 4.2%, 11.5% and 37.7% in bacteraemia patients (Lin *et al*., 2011; van Hal *et al*., 2011; Park *et al*., 2012), 27% in *S. aureus* liver transplant infections (Bert *et al*., 2003), and 19.5% in endocarditis (Campanile *et al*., 2011). Note that these numbers originate from individual studies and may not be representative averages; it is likely that the frequency at which hVISA occurs varies greatly between hospitals and countries.

A distinguishment is made between hospital-acquired hVISA and community-acquired hVISA. In the past, hVISA was almost exclusively acquired inside hospitals, but nowadays it is also commonly community-acquired. This is considered evidence of the increasing prevalence of hVISA across the world (Campanile *et al*., 2011; Madigan *et al*., 2012).
Heteroresistance is generally seen as an intermediate state between susceptibility and full resistance to an antibiotic. As vancomycin is often used as a last-resort treatment against bacteria (such as MRSA) that are not susceptible to other antibiotics, the rise of hVISA is certainly worrisome.

1.5 Aims
Although much has been written about the clinical significance of heteroresistance in general and heteroresistant vancomycin-intermediate *S. aureus* (hVISA) in specific, and much is known about the mechanisms behind resistance to vancomycin in bacteria, relatively little has been done to connect these two subjects. What causes certain cells in a population to have an increased resistance and why do only a few cells become resistant? What are common mutations in heteroresistant cells? Is random genetic variability between cells the only cause of heteroresistance, or is there also evidence of nongenetic or epigenetic processes being involved? Are these processes antibiotic-specific, or does heteroresistance against all antibiotics emerge through the same 'general' mechanisms? And, based on these questions, are there ways in which the emergence of heteroresistance can be limited?

This report aims to review the available data concerning these subjects and to create an overview of the mechanisms that may lead to heterogeneity in heritable vancomycin-resistance within a homogeneous bacterial population. Additionally, this report aims to compare this to some of the mechanisms behind heteroresistance against other well-studied antibiotics, in order to obtain some knowledge of to what extent the mechanisms that lead to heteroresistance are generalised across different bacteria and different antibiotics, and to what extent the emergence of heteroresistance is antibiotic-specific. It is hoped that this may yield some insight as to how the development of heteroresistance in hospital patients may be prevented.

To this end, the following research questions are formulated:
1. *What is the mechanism of action of vancomycin and by what mutations or mechanisms may a gram-positive bacterium obtain resistance against vancomycin?*
2. *Which of these mutations or mechanisms may likely cause a difference in vancomycin-resistance between individual cells within a homogenous population of gram-positive bacteria?*
3. *Is the occurrence of heteroresistance in vancomycin-susceptible bacterial populations solely caused by 'random' mutations in bacterial DNA, or are there also any epigenetic or non-genetic processes involved?*
4. *Are the mechanisms that lead to vancomycin heteroresistance vancomycin-specific, or are similar processes (e.g. mutations or misexpression of specific genes) involved in the occurrence of heteroresistance against other antibiotics?*

In *Chapter 1*, some of the basics of heteroresistance have been outlined. *Chapter 2* will start by briefly reviewing the method of action of vancomycin (*section 2.1*), followed by a discussion of some of the mechanisms that lead to vancomycin heteroresistance (*section 2.1 - 2.5*). After that, vancomycin heteroresistance is compared to heteroresistance against some other well-studied antibiotics (*section 2.6*). Finally, *Chapter 3* will revisit the research questions formulated above and discuss some of the implications of heteroresistance for the clinical use of antibiotics.

Chapter 2. Mechanisms of vancomycin heteroresistance

2.1 Vancomycin

Vancomycin is a glycopeptide antibiotic that blocks the construction of the cell wall and is often used for the treatment of infections caused by gram-positive bacteria that are immune to other antibiotics. Vancomycin binds to the free carboxyl end of the D-alanyl-D-alanine residues on the ends of the peptide chains of the peptidoglycan precursor molecules, forming stable complexes by means of hydrogen bonds (Perkins, 1968; Scholar & Pratt, 2000; Madigan *et al*., 2012). By doing so, it sterically hinders the process of transglycosylation, one of the final steps in cell wall construction (Watanakunakorn, 1984; Reynolds, 1989; Madigan *et al*., 2012). A schematic overview of the binding site and mechanism of action of vancomycin is shown in *Figure 1*. The molecular structure of vancomycin is shown in *Figure 2.* Vancomycin cannot penetrate the cytoplasmic membrane and can therefore only bind the peptidoglycan precursors after they have been transported out of the cell. Therefore, only the transglycosylation reaction is blocked. This causes an accumulation of precursor molecules and intermediates in the cytoplasm (Reynolds, 1989).
Figure 3 on page 8 shows how D-alanyl-D-alanine is bound by a glycopeptide antibiotic. Three different hydrogen bonds can be formed, resulting in the formation of a strong complex (Reynolds, 1989).

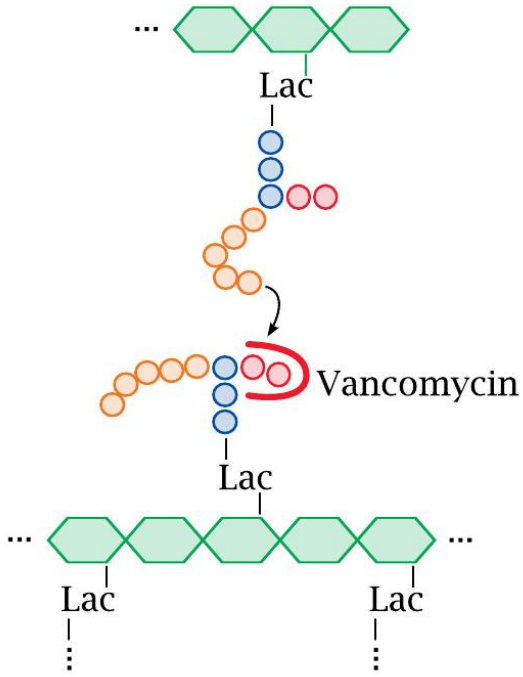

Figure 1 **- Binding of vancomycin to peptidoglycan**
A schematic overview of the location of binding and method of action of vancomycin. Vancomycin (the red line) binds to D-alanyl-D-alanine (red circles) of the peptidoglycan peptide chains, effectively blocking the process of transglycosylation (not shown here) through steric hindrance. The *N*-acetylglucosamine/*N*-acetylmuramic acid 'backbone' of the cell wall is depicted by the green hexagons.
Source: University of Waterloo, http://watcut.uwaterloo.ca/.

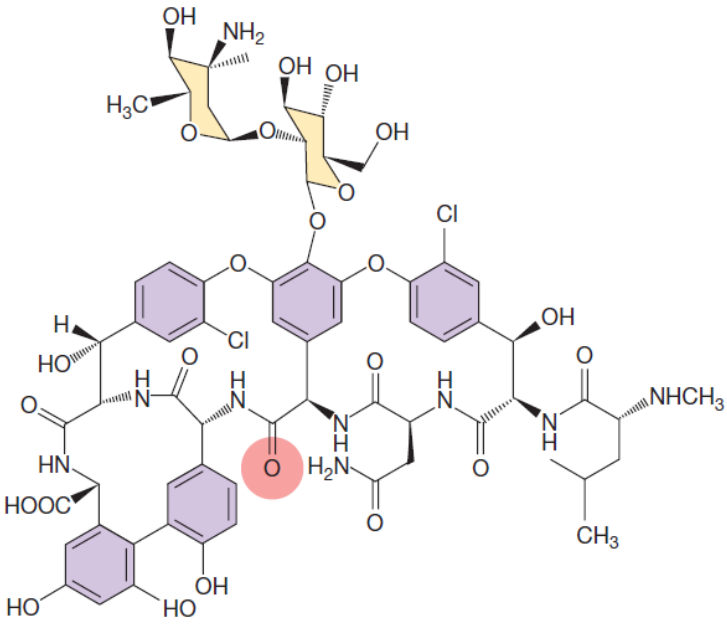

Figure 2 **- Vancomycin**
Structure of vancomycin. The structure of other glycopeptide antibiotics is comparable. The red carbonyl group is one of the binding sites between vancomycin and D-alanyl-D-alanine (see *Fig. 3*) and this group may be replaced by a methylene or primary ketimine to counter some forms of vancomycin resistance.
Source: Madigan *et al*., 2012.

2.2 Heteroresistance against vancomycin

Bacteria can use several mechanisms to obtain resistance to an antibiotic. Examples include alterations of transport proteins so that the antibiotic is no longer transported in the cell, active degradation of the antibiotic, or alteration of the structure of the antibiotic target (Madigan *et al.*, 2012). The latter is commonly used by bacteria that have acquired full vancomycin-resistance: these bacteria are able to sense the presence of the antibiotic and consequently alter the structure of the peptidoglycan peptide chains, replacing D-alanyl-D-alanine by D-alanyl-D-lactose. As a result the binding effectiveness between vancomycin and peptidoglycan is reduced 1000-fold, and the minimum inhibitory concentration (MIC) is equally increased (Koteva *et al.*, 2010; Xie *et al.*, 2011). Bacteria that are susceptible to vancomycin (and in which heteroresistance occurs) do not have such resistance mechanisms or the corresponding resistance genes, so it is unlikely that such complex mechanisms are the cause of the variation in vancomycin susceptibility observed in heteroresistant *S. aureus*. Instead, it is expected that variations in resistance are caused by more commonplace occurrences: genetic variability, random mutations, or instability in bacterial DNA due to long-time infections (El-Halfawy & Valvano, 2015).

An 'easy' way to obtain some degree of antibiotic resistance would be through a 'lucky' mutation in the DNA encoding the target of the antibiotic. Based on the mechanism of action of vancomycin (*section 2.1*), one may expect that such a mutation in DNA regions encoding transglycosylase or the peptidoglycan peptide chain structure may sometimes confer resistance to vancomycin. However, a key characteristic of vancomycin is that in works on the substrate of an enzyme instead of the enzyme itself. This makes it less likely that vancomycin resistance is obtained through a mutation in the enzyme-encoding DNA (Koteva *et al.*, 2010). Concerning this, Reynolds (1989) writes that "*if a different or mutant transglycosylase enzyme could function in the presence of a glycopeptide antibiotic (perhaps resulting from a change in conformation so that the enzyme was not obstructed by the glycopeptide/D-ala-D-ala complex), the next reaction in peptidoglycan biosynthesis would also be blocked: a transpeptidation reaction in which newly synthesized nascent peptidoglycan is transferred to the existing mature peptidoglycan*". In this case, the transpeptidation reaction would not take place and an instable cell wall without crosslinks would be formed. This way, vancomycin would effectively achieve the same effect as a β-lactam antibiotic, albeit via a different mechanism (Reynolds, 1989; Madigan *et al.*, 2012). This implies that a change in transglycosylase structure is not a likely cause of vancomycin heteroresistance in *S. aureus*.

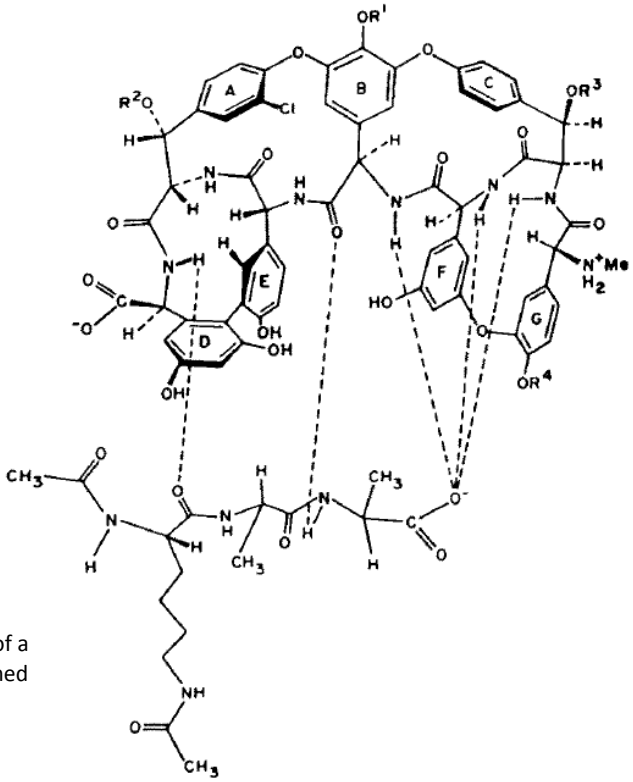

Figure 3 **- Binding between glycopeptide and D-Ala-D-Ala**
Schematic overview of the interactions between a glycopeptide antibiotic and the D-alanyl-D-alanine ending of a peptide chain. Three different hydrogen bonds can be formed at a time, resulting in the formation of a stable complex. Source: Reynolds, 1989.

It is reported that differences in vancomycin susceptibility are present in *S. aureus* populations that have not previously been exposed to the antibiotic, but are also developed step-wise over the course of longer infections and extended vancomycin exposure. If any cells in an infectious population have a small, inherent advantage in vancomycin resistance this may be further enhanced by selective vancomycin pressure. In other words, use of any antibiotic as treatment against bacterial infection implicitly results in selection for antibiotic resistance (Smith *et al*., 2008; El-halfawy & Valvano, 2015). Such an acquired resistance is not always stable and may sometimes be lost when the cells are cultured in antibiotic-free medium for several generations (Pournaras *et al*., 2010; El-halfawy & Valvano, 2015). Cases of the acquired heteroresistance being permanent have also been reported (Søgaard, 1985; El-halfawy & Valvano, 2013).

A number of studies have attempted to discover some of the mechanisms behind vancomycin heteroresistance in *S. aureus*. Several studies have tried to identify genes that are commonly expressed in VISA by examining the expression patterns of these cells and, whenever possible, comparing these to the gene expression patterns of the vancomycin-susceptible parent strains.
A general tendency in the results obtained by these studies is that different VISA strains, including closely related ones, may have greatly different expression patterns. Additionally, VISA strains are commonly found to have mutations in regulator genes that affect the expression of wide sets of genes. These include *pbp*, encoding Penicillin-binding protein, *agr*, involved in the regulation of virulence factors, and *vraRS* and *walRK*, involved in cell-wall synthesis (El-halfawy & Valvano, 2015). Two of these, *agr* and *vraRS*, and the studies concerning them, are further discussed below.

Howden *et al*. (2008) looked at the transcriptome of five different VISA strains in attempt to find mechanisms leading to vancomycin resistance. They report that *'Although there were some consistent transcriptional changes found, there were very few consistent changes across all pairs, suggesting that multiple transcriptional pathways lead to low-level vancomycin resistance in S. aureus*'. Decreased expression of the *agr* regulatory system was found in three of the five investigated strains. They report that decreased vancomycin susceptibility in *S. aureus* is commonly accompanied by changes in capsule production and decreased production of Protein A (a cell surface protein that is recognized as antigen by the human immune system). Although this may not directly confer antibiotic resistance, it does make the cells less 'visible' to the human immune response and thus more likely to cause long-term infections and may provide the cells with some degree of advantage during antibiotic treatment.
A study by McAleese *et al*. (2008) looked at the changes in gene expression in *S. aureus* strains isolated from a single patient undergoing vancomycin treatment. Isolates were obtained over the course of the treatment, allowing the researchers to track expression changes over the course of antibiotic exposure. Although all isolates originated from the same parent strain, heterogeneity in gene expression quickly developed over the course of the treatment. Some of the isolates showed a gradual decrease in vancomycin susceptibility, whereas others were still fully susceptible at the end of the treatment. Gene expression patterns were found to differ between isolates. Characteristics that were observed to accompany increased vancomycin resistance were slower growth, reduced *agr* functionality, reduced peptidoglycan cross-linkage, reduced expression of several genes involved in cell-wall production (and, consequently, reduced cell-wall production), and reduced expression of a large number of housekeeping genes.
The same study also tracked changes in gene expression that occurred when the vancomycin-susceptible parent strain was exposed to a high level of vancomycin, and these changes were compared to the gene expression patterns of the heteroresistant strains. Of the 72 genes that showed a greater than 2-fold expression increase during vancomycin exposure, 27 were found to have a permanently altered expression in the isolated vancomycin-resistant *S. aureus* strains.
15 of these were under control of *vraRS* (Kuroda *et al*., 2003; McAleese *et al*., 2008).

2.3 Dysfunction of *agr* affects quorum sensing and biofilm production

The *agr* locus encodes several transcripts that control the expression of multiple virulence factors, including quorum sensing, toxins, hemolysins, and biofilm production (Abdelnour *et al*., 1993; Novick, 2003; Cheung *et al*., 2011). The *agr* system appears to control the expression of different genes required throughout different stages of infection: as the infectious cell population reaches a certain density, *agr* downregulates the expression of surface-binding proteins and upregulates the production of a large number of exoenzymes and toxins (Cheung *et al*., 2011). Dysfunction of *agr* may result in decreased quorum sensing functionality and as a result a decreased expression of virulence factors (Howden *et al*., 2008). Additionally, *agr* Dysfunction is thought to increase biofilm production (Harigaya *et al*., 2011) and decrease autolysis (Sakoulas *et al*., 2005).
This implies that *agr* dysfunction results in a trade-off between increased antibiotic resistance (through biofilm production(Madigan *et al*., 2012)) and decreased virulence. In agreement with this, several studies report the observation that the emergence of antibiotic resistance in VISA is paired with reduced virulence (McCallum *et al*., 2006; McAleese *et al*., 2008; Peleg *et al*., 2009; van Hal *et al*., 2011).

Amongst the earliest studies that report a relationship between decreased *agr* functionality and vancomycin heteroresistance in *S. aureus* are two studies by Soukalas *et al*. (2002 and 2005). The studies report observing a decrease in vancomycin susceptibility in loss-of-function *agr* mutants, but that the effect of such a mutation differs between strains. In one of the investigated strains, loss of *agr* function went paired with the loss of hemolysin production and thus a decrease in virulence. The study also notes that disruption of *agr* may increase the ability of *S. aureus* to form biofilms on medical apparatus, thus increasing its ability to spread within a hospital. The authors propose that loss of *agr* function '*may be an early step in the development of vancomycin resistance*'.
Further studies by Howden *et al*. (2008), Tsuji *et al*. (2009), Cafiso *et al*. (2012), and Viedma *et al*. (2014) all report a relation between *agr* dysfunction and reduced vancomycin susceptibility in *S. aureus*. A study by Harigaya *et al*. (2011) provides some indication of the occurrence of *agr* dysfunction in vancomycin heteroresistance: looking at 220 *S. aureus* strains isolated from hospital patients (of which 99 (45%) were MRSA and 12 (5.5%) were considered hVISA), dysfunction of *agr* was found in 58% of hVISA strains, but in only 21% of MRSA strains and 10% of vancomycin-susceptible strains.

2.4 Cell-wall biosynthesis and *vraRS*

Increased cell-wall biosynthesis, under regulation of *vraRS*, is linked to vancomycin heteroresistance in several studies, including those by Howden *et al.* (2008) and McAleese *et al.* (2008) mentioned above, and studies by Kuroda *et al*. (2003), Boyle-Vavra *et al*. (2012), and Alexander *et al*. (2014). *Vancomycin Resistance Associated S* and *-R* (*vraSR*) is a two-component regulatory system that regulates several important steps of cell-wall synthesis in *S. aureus*, including expression of the *murZ* gene responsible for synthesis of murein precursor molecules and the *pbp2* and *sgtB* genes responsible for peptidoglycan polymerisation (Kuroda *et al*., 2003). *vraSR* is upregulated when *S. aureus* is exposed to antibiotics that target the cell wall such as β-lactam and glycopeptide antibiotics, and is thought to increase resistance to these antibiotics (Boyle-Vavra *et al*., 2012). It has been shown that MRSA cells with a deletion of the *vraRS* operon have their susceptibility to β-lactam (partially) restored (Jo *et al*., 2011).

The functionality of *vraRS* and its possible involvement in vancomycin heteroresistance was first suggested in two studies by Kuroda, Kuwahara-Arai and Hiramatsu (2000) and Kuroda *et al*. (2003). The first study reported a permanent overexpression of *vraRS* and thickened cell walls in isolated VISA strains, and found that experimental overexpression of *vraRS* in vancomycin-susceptible *S. aureus* strains increased vancomycin susceptibility.

The second study showed that exposure of *S. aureus* to vancomycin or other cell-wall affecting antibiotics resulted in the expression of 139 genes. 46 of these failed to be induced in mutants with a dysfunctional *vraRS* gene. Additionally, knockout of *vraRS* greatly increased the cells susceptibility to vancomycin. This indicates that *vraRS* is important in defense against antimicrobials and suggests that mutations in *vraRS* may greatly affect vancomycin resistance.
The study also found that the expression of several stress-response proteins was under control of *vraSR*. This includes the osmoprotectant transporters ProP and OpuD, which are considered important in protecting the cell wall from rupturing when cell wall synthesis is inhibited by antibiotics (MacMillan *et al*., 1999). Mutations in *vraRS* and, consequently, thickened cell walls have since been found in VISA strains by multiple studies (McAleese *et al*., 2008; Alexander *et al*., 2014).

2.5 Epigenetic factors influencing heteroresistance
The previous sections have discussed some of the genes commonly mutated in heteroresistant cells. It is possible, however, that epigenetic mechanisms may also play a part in the emergence of heteroresistance. The existence of non-genetic differences and specialisation between cells has been observed in different cellular mechanisms. Such intercellular differences in gene expression and protein production may, in theory, also provide some cells with an advantage in antibiotic resistance (El-Halfawy & Valvano, 2015).

There are very few studies that have investigated the contribution of epigenetics to heteroresistance. A number of studies suggest that heteroresistance emerges through epigenetic mechanisms when cells are repeatedly exposed to low, or increasing, antibiotic concentrations. These studies report that more and more cells survive after repeated antibiotic exposure, and that the amount of cells that survive is higher than can be attributed to mutations in DNA. Additionally, the rapid reversion of susceptibility that is observed when resistant cells are cultured in absence of antibiotics may be evidence of an epigenetic process (Adam *et al*., 2008; Motta, Cluzen & Aldana, 2015; Sorg & Veening, 2015). However, this last assumption could be questioned: multiple studies report that mutations that lead to heteroresistance also commonly lead to slower metabolism or cell growth (McAleese *et al*., 2008; El-Halfawy & Valvano, 2015). If this is the case, it follows that there is a large selectional pressure *against* heteroresistant cells in the absence of antibiotics, and that these mutations are thus quickly lost when they are no longer advantageous.

The most compelling evidence for the contribution of epigenetic mechanisms to heteroresistance is provided by a study by Sorg and Veening (2015), using *Streptococcus pneumoniae*. In addition to the observations mentioned above, they report having sequenced the DNA of isolated heteroresistant cells and their parent strains and finding no significant mutations. Additionally, any mutations that *were* present were still present after the bacteria had reversed to their antibiotic-susceptible state. This shows that the occurrence of mutations is not necessarily required for a cell to acquire a heritable antibiotic resistance.

2.6 Comparison to other antibiotics

Sections 2.3 and 2.4 discuss the influence of mutations in regulator genes on vancomycin resistance. How does this compare to heteroresistance against other antibiotics? Are the same genes involved? It is possible that resistances against different antibiotics that target the same part of the cell arise through similar mechanisms. Glycopeptide antibiotics (including vancomycin) affect cell wall synthesis in a similar fashion to β-lactam antibiotics so it may be expected that there is some overlap between the mechanisms behind their resistance (Madigan *et al*., 2013).

Heteroresistance to β*-Lactam antibiotics*

Mutations in regulatory genes have been reported to be one of the factors leading to β-lactam heteroresistance (El-Halfawy & Valvano, 2015). However, studies on vancomycin heteroresistance focus almost exclusively on the influence of these regulatory genes, whereas there is more attention for the effects of individual genes in studies concerning β-lactam heteroresistance. More specifically, heteroresistance against β-lactam antibiotics is commonly linked to increased expression of individual proteins such as Penicillin-binding protein, B-lactamases, or porins (Chambers, Hartman & Tomasz, 1985; Medeiros, 1997; Pournaras *et al*., 2010; Plata *et al*., 2013).
Some mechanisms linked to vancomycin heteroresistance are also linked to β-lactam heteroresistance. Heteroresistance against β-Lactam antibiotics has also been linked to slower cell growth (Hung *et al*., 2012) and *agr* dysfunction has been shown to be involved in the acquisition of oxacillin resistance in *S. aureus* (Plata *et al*., 2009).

A difference between β-lactam and glycopeptide antibiotics is that β-lactam antibiotics have to pass through cell wall poreins before they can function, whereas the larger glycopeptide antibiotics do not (Stephan *et al*., 2004; Danilchanka *et al*., 2008). This opens up multiple resistance mechanisms that are not available against glycopeptides, such as variations in the amount of poreins the cell produces or easier active degradation (Medeiros, 1997). This would explain why different mechanisms of heteroresistance are reported for β-lactam antibiotics than for glycopeptides.

Heteroresistance to other antibiotics

Relatively few studies concerning heteroresistance to other antibiotics than β-lactams and glycopeptides have been done. Heteroresistance to protein synthesis inhibitors such as erythromycin, the aminoglycoside antibiotics (including streptomycin), or tetracycline antibiotics has not yet been reported.

Heteroresistance to Colistin, an antimicrobial peptide used as last line of treatment against several multi-drug resistant bacteria, has been reported in several studies. Colistin interacts with lipopolysaccharide of the outer membrane, and mutations that disrupt the synthesis of the binding site of colostin provide increased colistin resistance (Moffatt *et al*., 2010).
Reduced susceptibility to the synthetic quinolone antibiotics is relatively easily obtained by bacteria: single point mutations in the genes encoding DNA gyrases or topoisomerases (the target molecules of quinolone antibiotics) are sometimes enough to reduce the binding efficiency of the quinolones and thus increase quinolone resistance (Angelakis *et al*., 2008; Huang *et al*., 2010; Chakravorty *et al*., 2011).

The above shows that heteroresistance to different types of antibiotics may occur, and that mutations are involved in all types of heteroresistance, but that the nature of the mutations that increase the bacterial resistance is very dependent on the mechanism of action of the antibiotic.

Chapter 3. Discussion

3.1 Discussion

The goals of this study were to create an overview of some of the common mechanisms behind heterogeneity in vancomycin resistance within bacterial populations, and to gain some indication of whether the mechanisms behind the emergence of heteroresistance in bacterial populations are universal (*i.e.* the same for all antibiotics) or antibiotic-specific. It was hoped that based on this some recommendation could be made concerning how the development heteroresistance can be prevented through proper antibiotics use.

To this end, some of the factors that are most commonly reported to play a part in the emergence of vancomycin heteroresistance in *S. aureus* have been discussed in *Chapter 2*. It has proven impossible to analyse, and list, all the different gene mutations reported to be involved in the occurrence hVISA, but the examples that have been given still provide an overview of how mutations in regulatory genes can affect many cellular processes, including antibiotic resistance. It has also been shown that there is no 'one' process or mutation that leads to heteroresistance, but that there are many different ways in which a cell may gain a competitive advantage in the presence of an antibiotic. This also holds true for different antibiotics: the mutations that may lead to heteroresistance against an antibiotic depend heavily on the mechanism of action on the antibiotic.

In spite of that, some 'universal' factors of heteroresistance do appear to exist. Heteroresistance to several different antibiotics has been linked to slower cell growth (McAleese *et al*., 2008; Cui *et al*., 2010; Hung *et al*., 2012; Wang *et al*., 2014). It is still unclear whether a decrease in growth rate directly increases a cell's antibiotic resistance or is merely a secondary effect of other resistance-enhancing changes in the cellular metabolism. Some studies suggest that slower growth may be especially advantageous in the presence of antibiotics that target cell-wall synthesis (McAleese *et al*., 2008; Wang *et al*., 2014).
Another common factor is that when heteroresistant cells are isolated and cultured in the absence of antibiotics, they commonly revert to a susceptible state after a few generations. This phenomenon has been observed with different antibiotics (Adam *et al*., 2008; Pournaras *et al*., 2010; Motta, Cluzen & Aldana, 2015; Sorg & Veening, 2015). This indicates that mutations that lead to heteroresistance generally are disadvantageous in absence of antibiotics and are thus quickly lost from the population. One of the reasons for this may be the aforementioned slower growth, as slower-growing cells will naturally divide less than 'normal' cells.
This last phenomenon is also seen as evidence of involvement of an epigenetic process in the development of heteroresistance. So far, the exact contribution of epigenetic mechanisms to heteroresistance remains unclear. The studies that look at the involvement of epigenetics are limited in number, especially in comparison to the studies that report gene mutations as causes of heteroresistance. So far, no mechanisms for epigenetic factors behind heteroresistance have been proposed. However, epigenetic differences between bacterial cells are known to occur and it is certainly possible that these influence the susceptibility of individual cells. More research on this subject may prove fruitful in the future.

It is noteworthy that there have been little or no reports of heteroresistance to protein synthesis inhibitors such as erythromycin, the aminoglycoside antibiotics, or tetracycline antibiotics. It is unknown whether this is because heteroresistance to these antibiotics does not occur, or simply because researchers have thus far concentrated their efforts on other antibiotics.
It seems unlikely that there is no genetic mutation (either in a structural protein-encoding gene or in a regulator gene) that may provide a cell with an increased resistance to these antibiotics. Since these antibiotics have to be taken up into the cell before they can function (Madigan *et al*., 2013), it is possible to conceive of alterations in the cell membrane or the responsible poreins that may hinder their uptake and thus increase resistance. 'Normal' resistance to some of these antibiotics (such as the tetracyclines) has long since been relatively common, so it is more likely that the research of heteroresistance to these antibiotics has simply not had a high priority in the past.

3.2 Future prospects

The occurrence of heteroresistance and its contribution to the emergence of full antibiotic resistance in infectous bacteria has serious clinical implications. Heteroresistance is seen as an intermediate state between antibiotic susceptibility and full antibiotic resistance, and those bacteria that are now commonly heteroresistant to an antibiotic may be the first to be fully resistant in the future. The capacity of cells to increase their minimum inhibitory concentration (MIC) is especially evident during prolonged antibiotic exposure (McAleese *et al*., 2008), so resistance is likely to emerge in extended infections.

The occurrence of genetic mutations in bacterial cells cannot be prevented, and bacteria will always be able to acquire resistance against antibiotics. However, careful and considerate use of antibiotics will go a long way towards preventing antibiotic (hetero)resistance.
It has been suggested that application of certain bacteriostatic antibiotics, or the use of too low antibiotic concentrations, creates an environment in which the development of (hetero)resistance is more likely to occur. In these cases, the metabolism and growth of the exposed cells is slowed, but has not stopped and cell populations remain largely constant. In such an environment any cells that have a slight advantage in antibiotic resistance are deprived of any competition by other (and perhaps otherwise fitter) cells and have free reign to develop their resistance (Drlica, 2003; Sorg & Veening, 2015). This is especially likely to occur in extended infections or in long treatments with relatively low antibiotic concentrations. It follows that (hetero)resistance is less likely to develop when antibiotics are prescribed in concentrations that are above the upper limit of the so-called 'mutant selection window', or the concentration that is bound to eliminate any cells with a slightly increased resistance as well as the 'normal' cells (Drlica, 2003). Concerning this, Sorg and Veening (2015) suggest that antibiotics that cause lethal cell damage *before* slowing cell growth (and thus before giving heteroresistant cells a competitive advantage) may contribute to preventing the development of antibiotic resistance.
Another way to slow the emergence of antibiotic resistance is by using combinations of antibiotics that complement eachothers effects. This idea is far from new, but may gain greater importance in the light of heteroresistance. Vancomycin, for example, has been shown to work well in combination with daptomycin, gentamicin and linezolid (Tsuji & Rybak, 2006).

In an age in which antimicrobial resistance is becoming an ever more pressing problem it is almost redundant to state that more research on the subject may be useful. Nonetheless, the problems of antimicrobial resistance have yet to be solved. Based on what has been discovered about heteroresistance so far it has become clear that the mechanisms behind heteroresistance are numerous and that there is no 'one' mechanism that can be prevented or blocked to prevent heteroresistance from occurring. It may therefore be useful to concentrate future efforts on finding ways of maximising antibiotic effectiveness and to discovering how the impact of heteroresistance can be kept to a minimum and development of full antibiotic resistance can be prevented.

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