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The importance of the two- and three component systems WalRK and VraTSR for antibiotic resistance and phage susceptibility in *Staphylococcus aureus*

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Preface

This master thesis project has been carried out at the Department of Veterinary and Animal Sciences at University of Copenhagen. ***So first – I would like to say thank you to my supervisors at University of Copenhagen for welcoming me and allowing me to perform my master thesis project in such an inspiring and challenging environment!***

Anaëlle Fait, thank you for being the best supervisor I ever could have imagined! For answering all my nervous questions in the lab, for discussing all the interesting and confusing results, and for always saving my day with food when I forgot my lunch in Malmö…
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Jack Abrahamsson
Malmö, June 2022

Abstract

Staphylococcus aureus is a human commensal bacterium, living primarily on the skin and in the nose. It is also a pathogen able to cause a wide range of diseases, including food poisoning, infection of wounds but also more serious infections such as bacteremia and endocarditis. Methicillin-resistant *Staphylococcus aureus* (MRSA) is resistant to virtually every ß-lactam antibiotic, making infections difficult to treat. Vancomycin is a last-resort antibiotic used for treating MRSA infections; however, increasing reports have been made about resistance development towards vancomycin.

S. aureus strains with decreased susceptibility to vancomycin are called vancomycin-intermediate *Staphylococcus aureus* (VISA). While ß-lactam resistance often arises by acquisition of a gene encoding for an alternative penicillin binding protein (PBP), PBP2a, vancomycin resistance develops through accumulation of mutations in a variety of genes, in particular genes related to the cell wall. These mutations often lead to changed gene expression. For instance, the three-component system VraTSR, involved in regulating the cell wall stress response system is important in VISA, and genes encoding the two-component system WalRK, involved in cell wall maintenance and homeostasis. Furthermore, bacteriophages are viruses that adsorb to the bacterial cell wall in their initial infection stage. Changes in the regulatory systems WalRK and VraTSR might affect the cell wall and consequently phage susceptibility. In this study, we assessed how upregulating individual components of these regulatory systems affected the susceptibility of MRSA strain JE2 to vancomycin and ß-lactam antibiotics, as well as susceptibility to phage infection. Additionally, differences in the response to overexpression of these genes in JE2 compared to JE2-derived VISA strains were investigated.

Results showed that overexpression of single genes affected the different resistances in *S. aureus*. For instance, *vraR* increased the minimal inhibitory concentration (MIC) of both oxacillin and vancomycin. Furthermore, epistasis seemed to be important since overexpression of several genes had opposite effects in JE2 and vancomycin-adapted JE2 with regards to bacteriophage susceptibility. This suggests prediction of the effect of single genes on susceptibility to vancomycin, ß-lactam antibiotics and phages is difficult, and further research is needed to gain a deeper understanding of resistance mechanisms in VISA. That knowledge is of importance if vancomycin is still going to be an effective treatment for MRSA infections in the future.

Sammanfattning

Staphylococcus aureus är en kommensal bakterie, vars främsta nisch i människokroppen är huden och näsan. Det är också en patogen förmögen att orsaka ett stort antal infektioner, bland annat matförgiftning, infekterade sår men också allvarligare infektioner som blodförgiftning och hjärtmuskelinflammation. Meticillin-resistenta *Staphylococcus aureus* (MRSA) är resistenta mot praktiskt taget alla ß-laktamantibiotika, vilket gör infektionerna svåra att behandla. Vankomycin är ett av få läkemedel som har verkan mot MRSA-infektioner, dock har rapporter om resistensutveckling mot vankomycin ökat på senare tid.

S. aureus med minskad känslighet mot vankomycin kallas för vankomycin-intermediära *Staphylococcus aureus* (VISA). Medan resistens mot ß-laktamantibiotikum ofta uppkommer genom att bakterierna förvärvar en gen som kodar för ett alternativt penicillinbindande protein (PBP), PBP2a, så utvecklas vankomycinresistens genom ackumulerade mutationer i olika gener, särskilt relaterade till cellväggen. Dessa mutationer leder ofta till förändrat genuttryck. Bland de viktigaste generna i VISA är de som kodar för trekomponentsystemet VraTSR, involverat i ett svarssystem mot cellväggstress i *S. aureus*, samt gener som kodar för tvåkomponentsystemet WalRK, involverat i cellväggsunderhåll och homeostas. Fortsättningsvis är bakteriofager virus som adsorberar till den bakteriella cellväggen i deras första infektionssteg. Förändringar i de regulatoriska systemen WalRK och VraTSR kan eventuellt påverka cellväggen på ett sätt som påverkar möjligheten för fager att infektera bakterierna.

I den här studien undersökte vi hur en uppreglering av individuella komponenter från de regulatoriska systemen påverkade MRSA-bakteriestammen JE2:s mottaglighet mot vankomycin, ß-laktamantibiotikum, och bakteriofaginfektioner. Tilläggsvis undersöktes skillnader i av att uppreglera dessa gener i JE2 jämfört med JE2-deriverade VISA-stammar.

Resultat från den här studien visade att genom att överuttrycka enskilda gener så kunde de olika resistenserna påverkas i *S. aureus*. Exempelvis, genen *vraR* ökade den minimala inhibitoriska koncentrationen (MIC) av både oxacillin och vankomycin i flertalet bakteriestammar. Fortsättningsvis verkade epistas, interaktionen gener emellan, vara av vikt eftersom överuttryck av flertalet gener hade motsatt effekt i bakteriestammen JE2 gentemot vankomycinanpassade JE2-stammar med avseende på mottagligheten mot bakteriofaginfektioner. Detta implicerar att det är svårt att förutse effekten av enskilda gener på mottagligheten mot vankomycin, ß-laktamantibiotika och bakteriofager, och fortsatt forskning är nödvändig för att förstärka kunskapen och förståelsen för resistensmekanismerna i VISA. Den kunskapen är av vikt om vankomycin ska kunna vara ett effektivt läkemedel för behandling av MRSA-infektioner även i framtiden.

Nu eller aldrig är det dags att vinna kampen om antibiotika mot bakterierna!

Antibiotika – för många ses det som mirakelläkemedlet som kan bota nästan alla sjukdomar som orsakas av bakterier. Men det är också något de flesta av oss tar för givet. På senare år har det allt oftare pratats om den postantibiotiska eran, och forskare börjar bli oroliga att vanliga infektioner som tidigare har kunnat behandlats med antibiotika kan bli livshotande i framtiden. Därför är det väldigt viktigt att förstå varför bakterier blir resistenta så att vi kan tillverka nya läkemedel som kan bota infektioner och kanske lura bakterierna så vi kan behandla dem lätt och snabbt även i framtiden!

Bilden ovanför visar en forskare som håller upp en så kallad petriskål som det växer bakterier i, kanske är de resistenta mot antibiotika?

Människokroppen är hem åt 100 triljoner bakterier! Det betyder att i våra kroppar bor ungefär tio gånger fler bakterier än vad vi har egna celler. De allra flesta bakterierna sitter inte bara still i kroppen och rullar tummarna, utan de jobbar hårt. Bakterier i våra tarmar bryter ner maten vi äter och tillverkar vitaminer och andra ämnen vi behöver för att överleva och må bra, och på huden lever en massa bakterier som vaktar mot elaka mikrober som vill ta sig in i kroppen. Ibland kan det hända att vi får ett sår och då kan de göra att såret blir svullet och varigt. Eller så kan vi råka äta mat med bakterier i och de kan då ta sig in i magen och ge oss matförgiftning.

Som tur är fungerar oftast vårt immunförsvar bra och kan ta hand om de elaka bakterierna, men ibland måste vi få lite hjälp på traven av läkemedel – antibiotika! De flesta av oss har någon gång i livet druckit äcklig hostmedicin eller ätit tabletter när vi har en öroninflammation. Oftast är läkaren väldigt noga med att säga att man måste äta upp alla tabletter och inte sluta när man känner sig frisk. Det är nämligen så att vissa bakterier kan överleva om man inte äter så mycket antibiotika som läkaren skriver ut. Om man har otur kan de bakterierna lära sig att överleva medicinen, och nästa gång vi blir sjuka hjälper inte antibiotika längre – de har blivit resistenta!

För att undersöka hur bakterierna kan bli resistenta mot antibiotika har de i det här projektet blivit genmodifierade. Det betyder att en gen, alltså en liten bit DNA, har stoppats in i bakterien på ett laboratorium, och sedan har bakterien fått växa med en massa god mat och i ett varmt och skönt rum där det är 37°C varmt. Men i maten har också lite antibiotika gömts, och efter att bakterierna har varit inne i det varma rummet i 24 timmar så lever vissa, men andra har dött. Vissa bakterier som har fått en gen har lyckats förändra sig lite och experimenten visade att de överlevde bättre än de bakterier som inte hade den genen. De kanske har fått en lite tjockare cellvägg, vilket är som bakteriernas hud, som gör att de klarar sig bättre mot antibiotikan, men det går tyvärr inte att veta med säkerhet. Genom att göra fler sådana här undersökningar kan vi få reda på vilka gener som gör att bakterierna blir resistenta mot antibiotika, och kanske lyckas hitta nya mediciner som biter på de elaka bakterierna!

List of abbreviations

ALE – Adaptive Laboratory Evolution

CFU – Colony Forming Unit

LTA – Lipoteichoic Acid

MIC – Minimum Inhibitory Concentrations

MRSA – Methicillin Resistant *Staphylococcus aureus*

PBP – Penicillin Binding Protein

PFU – Plaque Forming Unit

VISA – Vancomycin Intermediate *Staphylococcus aureus*

WTA – Wall Teichoic Acid

TABLE OF CONTENTS

1. Introduction

Antibiotic resistance development is an increasing problem around the world, making it more and more difficult to treat bacterial infections. Extensive research about resistance mechanisms is continuously performed to develop new pharmaceuticals as well as get a better understanding of how to treat bacterial infections more efficiently. *Staphylococcus aureus (S. aureus)* is a human commensal with the primary niches being the skin and the nose. However, it is also a pathogen, causing many different infections, for example infected wounds and endocarditis (Tong, Davis, Eichenberger, Holland, & Fowler, 2015). *S. aureus* has developed resistance to various antibiotics throughout the years, including ß-lactam antibiotics such as different types of penicillin. *S. aureus* that has acquired the gene *mecA* becomes resistant to virtually all ß-lactam antibiotics and is called methicillin-resistant *Staphylococcus aureus* (MRSA) (Wielders, Fluit, Brisse, Verhoef, & Schmitz, 2002). A last-resort antibiotic used for treating MRSA infections is vancomycin, a glycopeptide antibiotic. Vancomycin targets the cell wall of *S. aureus* by binding to a precursor for peptidoglycan, the main component of the *S. aureus* cell wall. Therefore, vancomycin has another target than ß-lactam antibiotics which target the enzyme synthesizing the peptidoglycan, although they are both bactericidal by disrupting the cell wall (Hu, Peng, & Rao, 2016). In recent years, reports of vancomycin resistance in the clinics have been more frequent. By acquiring mutations in several different genes, primarily connected to cell wall biosynthesis and regulation, decreasing susceptibility to vancomycin can lead to so-called vancomycin-intermediate *Staphylococcus aureus* (VISA) (Gardete et al., 2012).

In this project, the effect of overexpressing genes from the cell wall regulatory systems WalRK and VraTSR will be investigated, in one MRSA strain and two different VISA strains. The two VISA strains were chosen due to their distinct evolutionary pathways of adapting to vancomycin from the same MRSA ancestor. One strain obtained a higher resistance to ß-lactam antibiotics while the other had a higher ß-lactam antibiotic susceptibility after adapting to vancomycin. Therefore, the effect of overexpressing genes from the regulatory systems will be evaluated in the three chosen strains, to see if the genetic background due to vancomycin adaptation will affect the response to overexpressing cell wall regulatory proteins. The effect of each gene on susceptibility to ß-lactam antibiotics (oxacillin), vancomycin as well as

bacteriophages will be analysed. Bacteriophages are viruses infecting bacteria, many phages infecting *S. aureus* are lytic and eventually kills the bacteria. Previous findings show that infecting MRSA with a lytic phage caused a change in gene expression of genes related to the cell wall stress response, among others *vraR* (Fernández et al., 2017). Additionally, the exact cell wall changes due to changing the expression of the regulatory systems VraTSR and WalRK when adapting to vancomycin are not fully understood (Gardete et al., 2012). Taken together, the previous findings made it interesting to investigate whether upregulation of genes from the regulatory systems VraTSR and WalRK would cause changes to the cell wall of *S. aureus* in such a way that phage adsorption would be changed. Furthermore, phage therapy is increasingly researched due the fact that phages in many cases are host specific. In the clinical setting, phage therapy would thereby be able to more specifically treat an infection caused by a specific bacterium, as compared to broad-spectrum antibiotics (Azam & Tanji, 2019).

Hopefully, the obtained results can be used to gain more knowledge about what genes are important for resistance development in *S. aureus*. Furthermore, this knowledge can be of interest when further investigating the resistance mechanisms and interactions with bacteriophages in VISA. Additionally, the obtained knowledge about the effect of cell wall regulatory systems on phage susceptibility could hopefully be used in further research regarding phage therapy and similar topics.

The outline of this report will be presented in chronological order. First, a theoretical background will be given. Here, information about *Staphylococcus aureus*, resistance mechanisms towards ß-lactam antibiotics and vancomycin, as well as an introduction to bacteriophages will be provided. Next, the materials and methods used will be presented, in a detailed format. In the end, results from the different antibiotic resistance analyses as well as phage susceptibility assays will be presented, followed by a discussion about the observations and the possible molecular explanations behind them.

1.1 Aim

The aim of this project was to increase the knowledge about what role the regulatory systems VraTSR and WalRK play in *Staphylococcus aureus* resistance to vancomycin, ß-lactam antibiotics as well as bacteriophages. Vancomycin resistance development in *Staphylococcus aureus* is a complex process, often involving stepwise mutations and changes in expression levels of several proteins. For instance, the cell wall stress response three-component system VraTSR and the cell wall maintenance two-component system WalRK have been shown to be involved in vancomycin resistance (Dai et al., 2017; Hu et al., 2016).

To assess the role of a specific gene, several different methods can be used, for example a gene deletion followed by phenotypic analysis, or to overexpress the gene. In this study, due to time limitations, it was chosen to overexpress the seven different genes from the two- and three component systems WalRK and VraTSR individually. The overexpressions were made in JE2, an MRSA strain that has not been exposed to vancomycin before, as well as two vancomycin-intermediate *Staphylococcus aureus* (VISA) strains, that already have developed a level of resistance to vancomycin.

Then, the minimal inhibitory concentrations of vancomycin and the ß-lactam antibiotic oxacillin were tested. The reason for testing the susceptibility to oxacillin, a ß-lactam antibiotic, even though the three strains have an MRSA strain as their ancestor and thereby should be resistant to ß-lactam antibiotics, is that vancomycin adaptation was shown to lead to changes in oxacillin susceptibility (Fait, 2021). Therefore, we were interested to investigate whether further changes in expression levels of the cell wall regulatory systems VraTSR and WalRK would restore or further change the oxacillin resistance compared to the MRSA-ancestor. Furthermore, susceptibility to infection by four different bacteriophages was studied.

The project objective is to answer the following research questions:

- Do changes in expression of walRK and vraTSR affect antibiotic resistance?
- Do changes in expression of walRK and vraTSR affect susceptibility to phage infection?
- Does overexpression of walRK and vraTSR lead to different effects in a vancomycin-naïve strain compared to vancomycin-adapted ones?

2. Theoretical background

2.1 *Staphylococcus aureus*

Staphylococcus aureus (*S. aureus*), is a Gram-positive bacterium, that long has been one of the most common sources of serious infections in human beings (Krismer, Weidenmaier, Zipperer, & Peschel, 2017). One of the attributes of *S. aureus* pathogenicity is the ability to cause a wide range of infections, for example infections in skin and soft tissue, food poisoning, endocarditis and sepsis, as well as biofilm production in medical devices (J. Foster, 2002). However, *S. aureus* is also a commensal bacterium, present in around 30% of the human population, primarily on the skin and in the nose (Tong et al., 2015).

Of concern is the emergence of strains that are resistant to different types of antibiotics. For example, the first article about *S. aureus* being resistant to the antibiotic methicillin was published in 1961. Then, methicillin had only been used for treating infections during one year, and cases of resistant *S. aureus* strains were already reported in hospitals (Jevons, 1961). Nowadays, these strains are commonly known as methicillin-resistant *Staphylococcus aureus* or MRSA. Today, many MRSA isolates are found to be resistant to virtually all types of antibiotic classes (Enright et al., 2002). This makes it difficult to treat infections caused by MRSA, as well as finding new types of antibiotics to treat infections with.

Already in 1944, it was reported that *Staphylococcus aureus* gained resistance to penicillins by utilizing a protein that could neutralize the effect of the antibiotic, termed penicillinases (Kirby, 1944). Since then, many new antibiotics, including penicillins with different side groups resistant to penicillinases, have been introduced to the market. However, *S. aureus* strains have developed resistance to most of these antibiotics as well, notably by acquiring mutations in genes encoding proteins involved in the cell wall machinery (Lowy, 2003). Therefore, the next section will address the cell wall of *S. aureus*, before moving on to Section 2.3 where *S. aureus* resistance to ß-lactams and vancomycin, both cell-wall targeting antibiotics, will be described.

2.2 The cell wall of *S. aureus*

2.2.1 Peptidoglycan

The peptidoglycan is an important polymer forming the cell wall of Gram-positive bacteria, and in part responsible for cell wall rigidity (Schleifer & Kandler, 1972). The peptidoglycan is composed of two main subunits, the amino sugars N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc). The precursors UDP-MurNAc are synthesized in the cytoplasm. A pentapeptide chain is assembled onto each MurNAc subunit which subsequently binds to a membrane-embedded undecaprenyl pyrophosphate, resulting in the formation of Lipid I. A GlcNAc residue is glycosylated onto Lipid I, forming lipid II. A flippase enzyme MurJ transfers Lipid II to the periplasmic side of the bacterial cell membrane. There, several molecules of Lipid II are linked together by enzymes performing transglycosylation forming the glycan strands. Cross-links are formed between the glycan strands in a process called transpeptidation. The cross-links work to increase the rigidity of the cell wall as well as creating a mesh-like structure depending on the extent of cross-linking (Shaku, Ealand, Matlhabe, Lala, & Kana, 2020). A schematic overview of the above-described process is found in Figure 2.1 below.

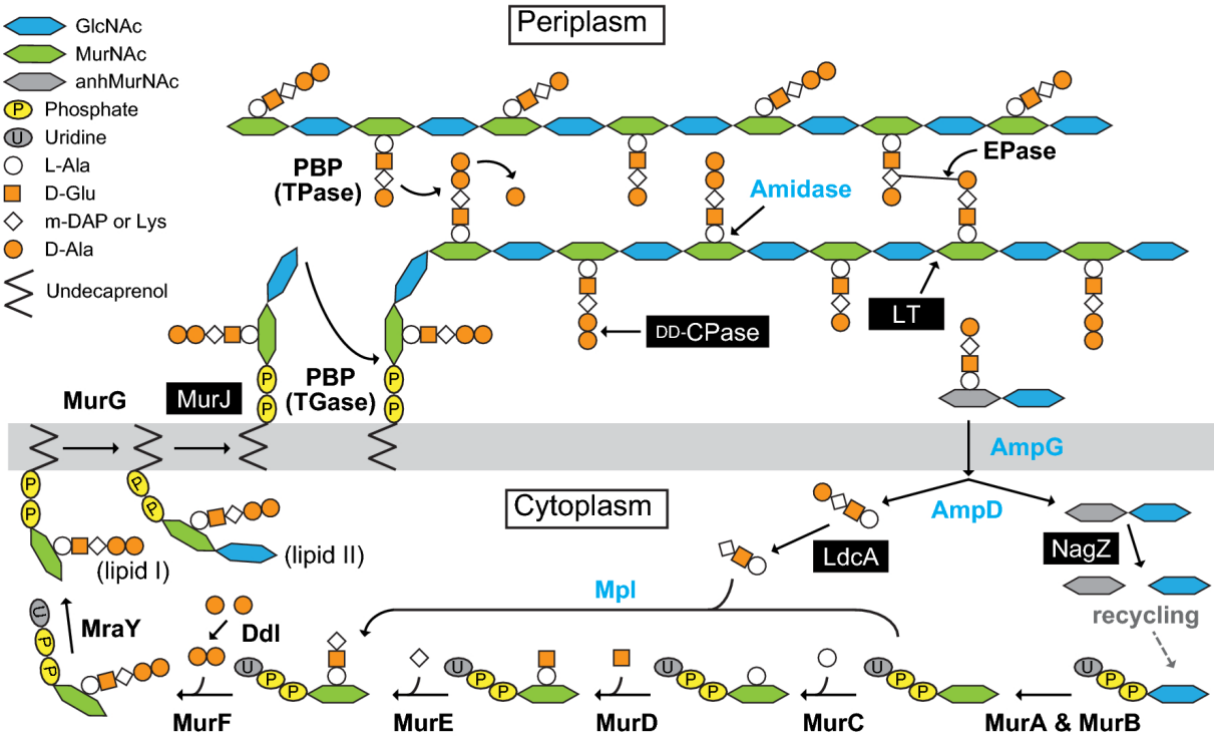


Figure 2.1. An overview of the peptidoglycan biosynthesis. On the bottom is shown the cytoplasmic side of the cell membrane, where Lipid II synthesis takes place. The flippase (MurJ) is responsible for transferring the Lipid II to the periplasmic side, where penicillin binding proteins (PBP) can synthesize polymeric peptidoglycan. GlcNAc is shown in blue and MurNAc is shown in green. Picture obtained from (Utsunomiya et al., 2021)

Recently developed technologies such as small amplitude atomic force microscopy (AFM) has been used to further investigate the cell wall of *S. aureus* on molecular nano-level. The cell wall was found to be approximately 20 nm thick but consisting of different fibres of peptidoglycan. In the inner parts of the cell wall, the glycan chains were found to be more densely packed, while the outer layers seem to be more loosely packed. The cross-linking of the peptidoglycan creates a mesh-like organization of the cell wall. The outer layers of the cell wall has a higher water content than the inner parts, giving it gel-like properties. (Pasquina-Lemonche *et al*., 2020).

2.2.2 Teichoic acids

The cell wall of *S. aureus* has several different molecules apart from peptidoglycan. Phospholipids, surface proteins, polysaccharides, especially capsular polysaccharides present in a capsule around the peptidoglycan, as well as teichoic acids (TAs) are components making up a large portion of the cell wall in *S. aureus* (Rajagopal & Walker, 2017). The TAs have been assigned functions such as binding to proteins and receptors present in the environment and partly controlling the synthesis of cell wall proteins and enzymes. Furthermore, since peptidoglycan is neutral in charge, the TAs also play a physical role in the defence against positively charged molecules by working as repellents, protecting the cell wall. The TAs was discovered many decades ago, but their complete function has not been completely elucidated (Xia, Kohler, & Peschel, 2010).

The teichoic acids exist in two different forms, called wall teichoic acid (WTA) and lipoteichoic acid (LTA). Both WTAs and LTAs consist of several ribitol-phosphate (Rho-P) units linked together, and the WTAs are connected to the GlcNAc in the cell wall via covalent bonds. On the other hand, the LTAs differ from the WTAs in the sense that they are connected to the cell membrane rather than the cell wall, by another form of bond, namely a glycolipid bond. This can be observed in Figure 2.2 below.

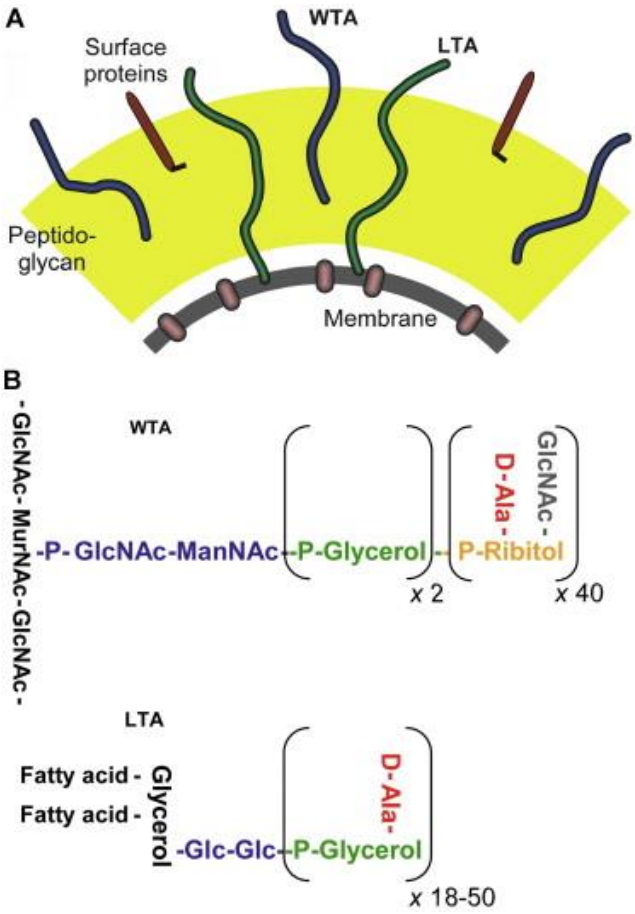


Figure 2.2. Overview of the structure of the cell wall of *S. aureus.* Pictured in A) is the peptidoglycan, cell membrane and the anchor point of WTA and LTA. In B) the bonds and structures of the WTAs and LTAs are presented. Reprinted from (Guoqing Xia et al., 2010).

The distinct role of LTA and WTA in *S. aureus* is not completely understood, but both seem to be important for cell viability. This was shown by Oku et al. (2009) who demonstrated that deleting both the gene encoding WTA (*tagO)* and the gene encoding LTA (*tarM*) lead to cell death. Although LTAs and WTAs have different functions in *S. aureus,* the presence of either WTA or LTA was sufficient for cell survival (Guoqing Xia et al., 2010).

2.3 Antibiotic resistance in *S. aureus*

2.3.1 Methicillin Resistant Staphylococcus aureus

Penicillins are a type of ß-lactam antibiotic that function by binding to the active site of so-called penicillin-binding proteins (PBPs), which are enzymes responsible for glycan polymerization and cross-linking. This binding leads to the inactivation of the PBP hence inhibition of cell wall synthesis, which results in cell death (Shaku *et al.*, 2020).

Methicillin was introduced as an alternative to other ß-lactam antibiotics since it was considered more effective and less prone to resistance development. However, resistance against methicillin in *S. aureus* in the clinic was reported already one year after its introduction (Jevons, 1961). Resistance to methicillin is due to a gene called *mecA*, that encodes a type of PBP called type 2A. This particular protein provides *S. aureus* resistance to most ß-lactam antibiotics and is today spread amongst most of the methicillin resistant *Staphylococcus aureus* (MRSA) strains. The *mecA* gene is located in the Staphylococcal Cassette Chromosome mec (SCCmec), a mobile genetic element, and can therefore be spread between bacteria. Studies have shown that horizontal gene transfer is one of the leading causes of resistance spreading in the *S. aureus* population (Gould et al., 2012; Wielders et al., 2002).

2.3.2 Vancomycin Resistance in Staphylococcus aureus

Vancomycin is one of the few available antibiotics available for treating patients that are infected with MRSA. Even though not as widespread as the resistance towards ß-lactam antibiotics, vancomycin resistant *S. aureus* is an increasing concern for the future. Vancomycin is a glycopeptide molecule that binds to the peptidoglycan precursor lipid II. Cell wall biosynthesis is stopped and the bacterial cell will eventually die from the absence of a functioning cell wall, making vancomycin a bactericidal antibiotic (Howden, Davies, Johnson, Stinear, & Grayson, 2010).

S. aureus can be classified depending on their level of susceptibility to vancomycin; vancomycin susceptible *S. aureus* (VSSA), vancomycin resistant *S. aureus* (VRSA), vancomycin intermediate *S. aureus* (VISA), and heterogenous VISA (hVISA) (Howden et al., 2010). These different classes will be described next.

VSSA and VRSA

According to the Clinical and Laboratory Standards Institute, if a *S. aureus* strain shows a vancomycin minimal inhibitory concentration (MIC) of ≤2 mg/L, it can be classified as susceptible to vancomycin (VSSA). The MIC test for vancomycin resistance in *S. aureus* is in short carried out by diluting the antibiotic and bacteria in broth and growing it for 24 hours at 37°C, then determining the lowest concentration that kills all bacteria, which is then determined to be the MIC. Opposite to VSSA, *S. aureus* strains with a vancomycin MIC ≥16 mg/L are considered resistant and are called VRSA (CLSI, 2019; Howden et al., 2010).

VISA and hVISA

If the broth microdilution method described above shows a resistance to vancomycin in the range 4-8 mg/L, the bacteria are determined to be intermediately resistant, thereby called VISA. However, even if an *S. aureus* isolate tests susceptible using the CLSI standard broth method, a risk remains that it is in fact VISA. Studies have shown that approximately one per hundred thousand or one per million bacterial cells can be VISA in a population that has been determined susceptible to vancomycin. However, cell counts in broth MIC assays range in the ten thousand colony forming units (CFUs) and VISA subpopulations can therefore remain undetected. This phenomenon is called heterogenous VISA (hVISA) (Howden *et al*., 2010). The gold standard for detection of VISA and hVISA is to perform a Population Analysis Profile (PAP). In essence, overnight cultures of *S. aureus* are diluted and plated on vancomycin concentrations ranging between 0.5-4 mg/L. After 48h incubation, CFUs are counted and plotted against the vancomycin concentrations, and the area under the curve (AUC) is calculated. The hVISA strain Mu3 is used as reference. The AUC ratios to Mu3 can be used to distinguish hVISA in a *S. aureus* population (Wootton et al., 2001).

2.4 Genes of importance for vancomycin resistance

Decreased susceptibility to vancomycin is complex due to mutations and differences in expression of several different genes. For example, comparative genomics have shown that several regulatory systems related to the cell wall can be mutated or present changes in expression levels in VISA compared to MRSA ancestors (Hu et al., 2016). For example, the cell wall stress response system VraTSR and the essential cell wall regulatory system WalRK have been shown to be affected in VISA. These systems are shown in Figure 2.3 and will be described further in section 2.4.1 and 2.4.2. Even though these two systems have three genes each (shown in Figure 2.3), the accepted nomenclature for these systems in literature is that VraTSR is a three-component system (Boyle-Vavra, Yin, Jo, Montgomery, & Daum, 2013; Hu et al., 2016) and that WalRK is a two-component system (Dubrac, Bisicchia, Devine, & Msadek, 2008; Gajdiss et al., 2020) even though WalH is the regulator of the WalRK system like VraT is the regulator in the VraTSR system.

A suggestion for this difference is that the *walRK* operon is essential for *S. aureus* survival, while deletion of *walH* only lead to downregulation of WalRK but not cell death, thereby WalH has not been as strongly linked to WalRK as VraT has been to VraSR (Gajdiss et al., 2020). Therefore, in this study it was chosen to keep the nomenclature of calling VraTSR a three-component system and WalRK a two-component system, not to confuse with the previously published literature.

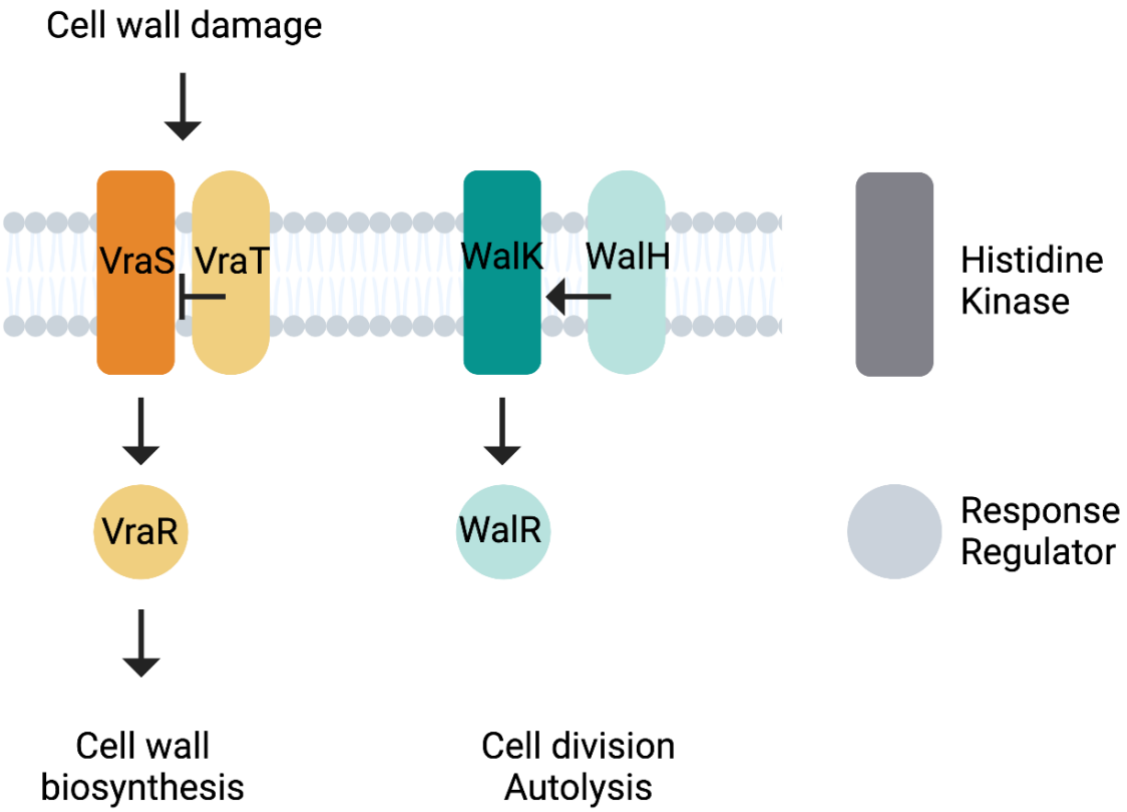


Figure 2.3. Illustration of the three- and two-component systems VraTSR and WalRK. The figure depicts a cell membrane, with a lipid bilayer shown in the background. Membrane associated proteins are spanning across the membrane, for example VraS and VraT. To the right, the shapes are described, and rectangles symbolise histidine kinases where circles represent response regulators. Ovals depict regulators of the systems. Flat headed arrows show negative regulators, while proteins having an activating effect are shown with pointed arrows. The overall cause of induction is shown above the regulatory systems, while the generalized effects are shown below.

2.4.1 The three-component system vraTSR

One important antibiotic resistance regulator in *S. aureus* is the VraTSR regulatory system. This is a three-component system responsible for sensing cell wall stress. This can be of particular interest for antibiotics targeting the cell wall. For example, upregulation of this system leads to decreased susceptibility towards several antimicrobial agents targeting the cell wall (McCallum, Berger-Bächi, & Senn, 2010). VraS is activated when the cell wall is experiencing stress and subsequently phosphorylates the VraR regulator (Figure 2.3). The VraR regulator in turn controls several genes important for the cellular response to cell wall stress (McCallum *et al*., 2010). VraT is the regulator of VraSR. It is a negative regulator, meaning that increased expression levels of *vraT* downregulates *vraSR* expression (Hu et al., 2016).

The operon *vraSR* is important both for vancomycin resistance and to avoid detection by the human immune system. This was shown by studying a MRSA strain type USA300, similar to strain JE2 which is used in this thesis work. The strain was passaged with vancomycin to obtain a VISA strain. By using whole genome sequencing, different mutations and genetic differences in VraT and *vraSR* were found. These mutations could be linked to changes in these proteins and thereby correlated with increased vancomycin tolerance, as well as decreased virulence. The authors suggestion for the loss of virulence is an immune-evading mechanism. The protein VraT in combination with the operon *vraSR* is called an on/off switch, due to the observation that overexpression of VraT in the VISA strain increased the activity of the *vraSR* regulon. When the gene *vraS* was mutated, the function of the response regulatory VraR was lost, and the strain became susceptible to vancomycin and regained several virulence factors (Gardete *et al*., 2012).

Indeed, the VraSR system is important for regulating virulence during skin infection. This was shown by creating a 3D skin model using keratinocyte fibroblasts for infection by a MRSA USA300 clinical isolate. A loss-of-function mutation in *vraR* resulted in a 90% decrease in infection rate in the 3D skin, as well as smaller lesions and less apoptosis, indicating that the genes encoded by *vraSR* are important virulence factors for Community Associated-MRSA (CA-MRSA) strains during vancomycin adaptation (Barua, Yang, Huang, & Ip, 2021).

By deleting *vraS* and *vraR*, Jo, Montgomery, Yin, Boyle-Vavra, and Daum (2011) showed that oxacillin treatment was improved compared to an unmutated MRSA strain. The researchers showed that the *in vitro* oxacillin MIC decreased from 32 to 8 µg/mL, a four-fold decrease, and that *in vivo* skin lesions and experimental pneumonia were more easily cleared with oxacillin. This showed that the *vraSR* operon is important for both resistance towards oxacillin as well as the virulence of MRSA (Jo *et al*., 2011).

When the whole *vraTSR* operon is deleted, the resistance towards several ß-lactam antibiotics decreases. This was shown by using mouse models, treating mice infected by MRSA strains with ß-lactam antibiotics *in vivo*, when the *vraTSR* operon was deleted. These findings imply that VraTSR is important for resistance towards several antimicrobial compounds, not just vancomycin. The researchers also showed that VraT is of importance for the resistance mechanism in MRSA, both towards methicillin but also by negatively regulating the activity of the *vraSR* operon (Figure 2.3) (Boyle-Vavra et al., 2013).

Boyle-Vavra, Yin, and Daum (2006) showed that deletion of the gene *vraS* leads to a loss of oxacillin resistance in MRSA, while replacement of the *vraS* gene rescued oxacillin resistance. The VraTSR three-component system regulates several different genes, including *mecA* coding for penicillin-binding protein 2a (PBP2a), a protein important for ß-lactam resistance. These results indicate that VraTSR is important for ß-lactam resistance in MRSA (Boyle-Vavra et al., 2006). Although not shown in Figure 2.3, *vraU* is often up- or downregulated in VISA, but the exact mechanism or function of the protein VraU has not been shown (Boyle-Vavra et al., 2013).

2.4.2 The two-component system walRK

The WalRK system has been shown to be an important gene regulator, controlling at least 9 genes important for cell wall metabolism in *S. aureus*. WalK is a histidine kinase, while WalR is the response regulator of the system, similarly to VraS and VraR described in section 2.4.1. One of the main pathways where WalRK plays a role is in the positive control of autolysins, for example AtlA, and peptidases, such as LytM (Dubrac, Boneca, Poupel, & Msadek, 2007).

AtlA is an enzymatic protein, that is split into two parts with separate functions. This was discovered by Bose, Lehman, Fey, and Bayles (2012), who mutated both parts of the AtlA protein separately and saw different phenotypic changes in *S. aureus*.

Mutations in the aminidase part of the murein hydrolase AtlA caused greater changes in cell clumping and cell wall degradation compared to mutations in the glucosaminidase. This indicated that the aminidase part of the murein hydrolase AtlA is primarily responsible for its autolytic activity.

Furthermore, the role of the glycyl-glycyl endopeptidase LytM was studied by Delaune et al. (2011). This enzyme functions as a peptidoglycan hydrolase. The researchers showed that when deleting the genes of the WalRK system, the *S. aureus* cells died, meaning that the genes *walR* and *walK* are essential for cell viability. However, when overexpressing *lytM* in the Δ*walK*/Δ*walR* mutants, the cells survived. Therefore, the hydrolysis of the cross-links in peptidoglycan is important for cell survival. If *walRK* operon is deleted, there is no regulator for cell wall autolysis (Delaune et al., 2011).

Taken together, some of the studies so far have shown that the *walRK* operon plays an important role in the maintenance of the cell wall, by regulating enzymes that either participate in lysis or hydrolysis of peptidoglycan cross-links. A hypothesis is that if this system is downregulated, the bacterial cells would have less control over the autolysins and peptidases, while overexpression of the same regulon would cause the opposite effect.

How can this be of importance for the resistance towards antibiotics? WalK acts by sensing the levels of Lipid II in the cell. The Lipid II levels are an indication of the cell wall synthesis since it is a component of peptidoglycan. When bacteria are treated with antibiotics, the levels of Lipid II can be affected, thereby increasing, or decreasing, the level of expression of *walK*, leading to a control of cell wall hydrolysis by *S. aureus*. For example, ß-lactam antibiotics partly act by hindering the usage of Lipid II by the penicillin binding proteins, increasing the levels of Lipid II in the cell. This is sensed by WalK, which thereby can be activated since higher levels of Lipid II indicates an excess amount of cell wall produced, hence a need for regulation. On the other hand, vancomycin leads to a lower amount of Lipid II in the cell, since vancomycin binds Lipid II, more specifically a part of it called the D-Ala-D-Ala moiety. Thereby, the expression levels of *walK* is probably decreased since the cell senses the need for building and cross-linking peptidoglycan (Dubrac et al., 2008).

Just like the VraTSR three-component system, there are regulators of the WalRK system, one being WalH, encoded by the gene *walH* (Figure 2.3). WalK does not function without the activation by WalH. Additionally, when *walH* was mutated in *S. aureus,* it caused less effect of the WalK histidine kinase. This indicated that WalH is a positive regulator, or activator of the *walRK* two-component system, and furthermore that overexpression of *walRK* lead to an increase in resistance to vancomycin (Cameron, Jiang, Kostoulias, Foxwell, & Peleg, 2016). Additionally, Gajdiss et al. (2020) showed that WalH, together with another regulator WalI, regulates the WalRK system by phosphorylation.

2.5 Bacteriophages

Since adaptation to vancomycin has been shown to cause changes to the cell wall of *S. aureus*, vancomycin adaptation could affect the susceptibility to bacteriophages. Bacteriophages are viruses infecting bacteria, and the viral particles adsorb to the cell wall to enter the host cell. In the following section, the different bacteriophages infecting *S. aureus* as well as their mechanism of infection will be described.

2.5.1 Background

Bacteriophages are parasites living inside bacterial cells, hijacking the host cell metabolism to produce more viral particles. Phages that are in the lytic phase takes over the host cell, producing enough viruses until the cell lyses and releases the bacteriophages in the environment. There are phages that are purely lytic, and their only way of infecting and replicating is by using the host metabolism to replicate. However, other phages are so called temperate. These can enter a lysogenic cycle, meaning that their genome can exist inside the host cell in the form of a prophage, which can either be integrated into the chromosome of the bacteria, or in a plasmid. If the prophage is not induced, the viral genome with replicate along with the host DNA, and the daughter cells will contain the bacteriophage DNA after cell division. Sometimes, a lysogenic to lytic switch can happen. The temperate phage starts replicating, finally lysing the host cell and releasing phages into the surroundings. The lysogenic to lytic switch can be both spontaneous but also induced. For example, when the bacterial cell DNA is exposed to damage, the SOS response can activate and induce transcription of the bacteriophage DNA (Howard-Varona, Hargreaves, Abedon, & Sullivan, 2017), (Azulay *et al.*, 2022).

According to Xia and Wolz (2014), the phages infecting *S. aureus* can be divided into 11 serogroups, the most prominent ones presented in Figure 2.4 below. The three families they belong to are *Siphoviridae*, *Myoviridae* and *Podoviridae*. As can be seen in Figure 2.4, the different serogroups vary in their tail length, tail width, capsid head form and size. As will be described later in section 2.5.3, the modes of adsorption to the host cells and modes of infection with regards to lytic or lysogenic infections also differ among the serogroups.

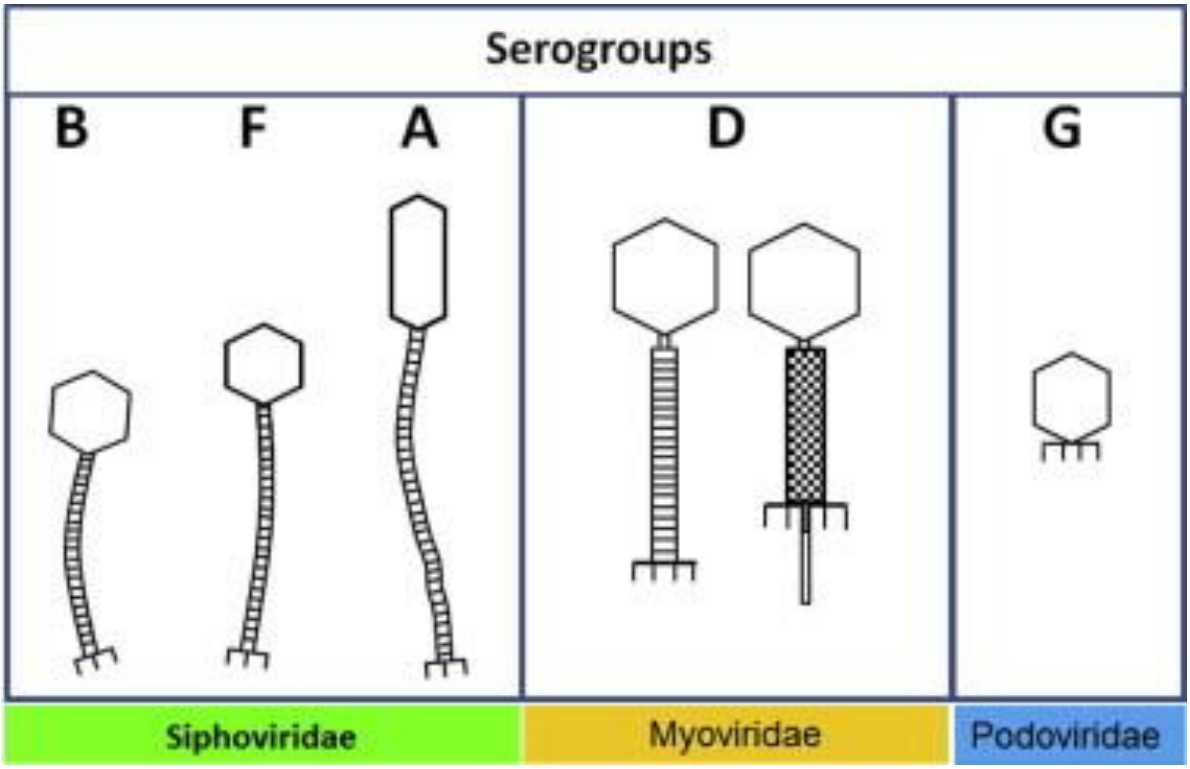


Figure 2.4. Depiction and illustration of different bacteriophage classes. Shown are the *Siphoviridare* (Class B, F and A), *Myoviridare* (Class D) and *Podoviridare* (Class G).

2.5.2 What strains of phages infect S. aureus?

Four different phage species were used in this study and will be described in the following section: φIPLA-RODI (RODI), Stab20, Stab21 and φ11.

- φIPLA-RODI

According to González *et al*. (2017), RODI belongs to the family of Myoviruses. The phage was isolated from sewage water and was found to lyse biofilms produced by *Staphylococci*. RODI was furthermore determined to belong to the subgroup Kayviruses (Gutiérrez *et al*., 2015).

- Stab20

The bacteriophage Stab20 was isolated from *Staphylococcus xylosus* by Oduor, Kadija, Nyachieo, Mureithi, and Skurnik (2020). It was determined to belong to the family of *Myroviridae*, especially the subfamily *Twortviridae*. In this subfamily, it was placed in the genus *Kayvirus,* like RODI. The researcher's bioprospecting for new *Staphylococcal* bacteriophages found that Stab20 could infect 40 of 41 *S. aureus* strains they tested for, showing a broad host range. This fact increased the interest for the phages with regards to for example future phage therapy as a complement to antibiotic treatment of bacterial infections.

- Stab21

Like Stab20, the phage Stab21 was isolated by Oduor *et al*. (2020). It was determined to be a close relative of Stab20, as it also belongs to the genus *Kayvirus* in the subgroup *Twortviridae,* in the group *Myoviridae*. Stab21 was found to be slightly bigger than Stab20, with regards to both capsid head size, the length and width of the tail but smaller with regards to the base plate.

- φ11

Unlike RODI, Stab20 and Stab21, the bacteriophage φ11 belongs to the family of *Siphoviruses.* More specifically, it belongs to the B serotype that can be seen in Figure 2.4 above. Unlike the three lytic phages RODI, Stab20 and Stab21, φ11 is a temperate phage (Xia & Wolz, 2014).

2.5.3 Mechanisms of adsorption to S. aureus

The four phages described in section 2.5.2 have different ways of adsorbing to the cell wall of *S. aureus* in order to infect the cells. The complete mechanism of adsorption and infection is not completely understood; however, important components of the cell wall and the differences between families of phages will be described in the following section. The primary mechanism for adsorption of the *Siphoviruses* seems to be the WTAs. More specifically, the α-GlcNAc residues decorating the WTAs are important for the phage entry into the host cells. This was shown by G. Xia *et al*. (2010) by deleting the gene *tarM,* which is the gene responsible for adding the α-GlcNAc residues to the WTAs. By deleting this gene, the φ11 were no longer able to adsorb to the cell wall of the bacteria (G. Xia *et al*., 2010).

Additionally, it has been shown that it is the WTAs and not the LTAs that are needed for the adsorption of *Siphoviruses*, including φ11, to the cell wall of Staphylococci. This was shown by knocking out the gene *ltaS*, which encodes the protein participating in synthesizing LTAs. All the tested phages could still infect the LTA-deficient *S. aureus* strains, indicating that LTAs are not important for infection by *Siphoviruses* (Xia et al., 2011).

Furthermore, Xia et al. (2011) could confirm that the WTAs are of importance for phage infection, by deleting the gene *tagO*, making the bacteria unable to produce any WTAs. Upon deletion of *tagO*, no phages could infect the bacteria, while plaques appeared on the complemented strain. Additionally, the researchers confirmed that serotype B of the *Siphoviruses* adsorb to the α-GlcNAc residues by deleting *tarM*, while *Myoviruses*, for example those of serogroup D (Figure 2.4), could still infect the *tarM* mutants. Therefore, they concluded that it is likely that serogroup D of the *Myoviruses* attach to the backbone of the WTAs (Xia et al., 2011).

2.5.4 How do bacteriophages infect S. aureus?

Studying the adsorption efficiency of the phage φ11 to the cell wall of *S. aureus*, Li *et al*. (2016) could show that a protein in the tail of the phage, located in the baseplate, was important for adsorption to the host cell. The protein is called Gp45. To analyze the binding site in the cell wall of Gp45, the researchers created knockout mutants, deleting both *tarM* and *tarS*, as well as *tagO*, to check whether adsorption occurred even if WTAs are not present. Deleting both *tarM* and *tarS*, leading to the absence of α- and β-GlcNAc residues on the WTAs, made φ11 unable to infect *S. aureus*. Knocking out only *tarM* or *tarS* did not cause the same effect. This demonstrated that GlcNAc residues on the WTAs are important for φ11 adsorption, but that both α- and β residues are sufficient for adsorption on their own (Li *et al*., 2016).

In 1971, it was shown that teichoic acids, as well as murein, is important for phage infection in *S. aureus*. Especially, the *O-Acetyl* group at residue position 6 in murein was found to be important for the phage adsorption to the cell wall. However, it could not be concluded if murein was important for the stability of the wall teichoic acids, or if the *O-Acetyl* group itself serves as a receptor binding domain for the bacteriophages (Shaw & Chatterjee, 1971).

When the bacteriophage attaches to the cell wall, the DNA is inserted into the host cell. The mechanism of injection has been suggested by Grayson and Molineux (2007) to be osmotic pressure. Due to the densely packed DNA in the bacteriophages, the osmotic pressure works to absorb the viral DNA into the cytoplasm of the host cell. After the replication of the viruses, two main proteins seem to function to lyse the host cell – endolysin and holin. These proteins have been shown to exist in *Podoviridae*, *Myoviridare* and *Siphoviridare* as well. However, in the latter, the endolysin seem to differ compared to the other two phage endolysins, in the sense that it has activity in D-alanyl-glycyl endopeptidase and not only the N-acetylmuramyl-L-alanyl endopeptidase (Navarre, Ton-That, Faull, & Schneewind, 1999; Xia & Wolz, 2014)

Furthermore, Chhibber, Kaur, and Kaur (2014) showed that bivalent cations, especially calcium ions, seem to be important for the adsorption and infection of bacteriophages in MRSA. The hypothesis is that since many *S. aureus* phages attach to the negative backbone of the WTAs, positive cations in the media help with neutralizing the charges to avoid repelling the phages, Therefore, adding more cations is thought to further simplify primary adsorption of the phages to the bacterial cell wall (Chhibber et al., 2014). More specifically, the researchers saw a hundred-fold increase in the number of phage particles produced. Furthermore, when the agar used for phage infection was supplemented with 5 mM calcium ions, the time needed to reach 50% phage adsorption was decreased from 20 min to 3 min. This indicated that calcium increases infection efficiency both with regards to time and number of plaques formed.

2.6 Background for the current thesis

This thesis takes basis in the work presented in the PhD Thesis **Vancomycin adaptive strategies in *Staphylococcus aureus*** by Anaëlle Fait, PhD from University of Copenhagen. In the following section, the background information required to understand the concept of this thesis as well as the origin of the *S. aureus* strains used in the laboratory work will be presented.

2.6.1 S. aureus USA300 strain JE2

USA300 are the most prominent community associated MRSA (CA-MRSA) strains found in the United States. It has been found that many of the USA300 isolates differ by few nucleotide polymorphisms, suggesting that the isolates are closely related and have not radically changed since the spread of MRSA (Kennedy *et al*., 2008).

The strain used for studying antimicrobial resistance and bacteriophage susceptibility in this thesis is called JE2, and is a plasmid cured USA300 strain presented in an article by Fey *et al.* (2013). This strain has been extensively characterized, for example by whole genome sequencing, making it a suitable strain for comparing effects of evolution experiments and gene overexpression (Diep *et al*., 2006).

2.6.2 Adaptive Laboratory Evolution

The VISA strains used in this project was obtained from an adaptive laboratory evolution (ALE), performed by Anaëlle Fait (Fait, 2021). To investigate the development of vancomycin resistance in *S. aureus*, particularly the evolution of MRSA to VISA, 10 different cultures of the MRSA USA300 strain JE2 was adapted to vancomycin. This was done by growing 10 separate lineages in TSB with increasing concentrations of vancomycin. 30 exposure cycles were performed, where one exposure cycle is a two-step process in short meaning transferring the bacteria to fresh media with a subMIC concentration, the highest concentration where growth was previously observed, constantly leading to the bacteria adapting to higher vancomycin concentration (Figure 2.5). The ALE gave rise to different phenotypes between the VISA strains, both regarding the vancomycin MIC, as well as the doubling time, colony morphology and blood hemolysis. The ten JE2-derived VISA strains were called L1, L2, L3, …, L10.

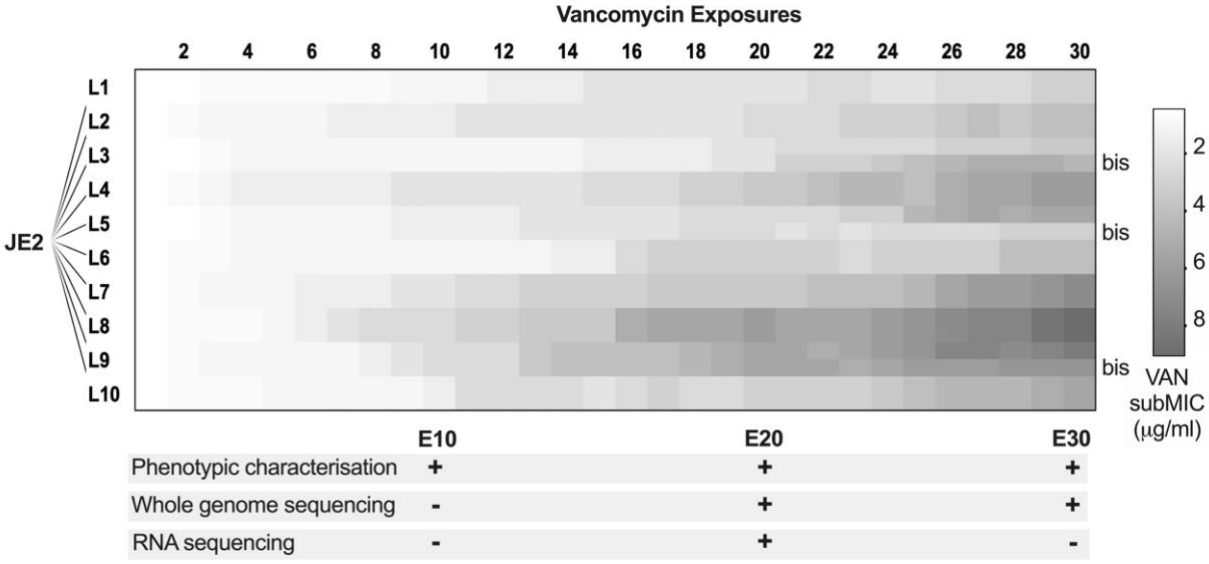


Figure 2.5. An overview of the ALE experiment developing VISA strains (L1-L10) from the common ancestor JE2. Shown are the ten different lineages, L1-L10, and their vancomycin subMIC in µg/mL. The colours represent different concentrations, and the scale is shown to the right. Shown are the vancomycin subMICs for the 30 exposure cycles. Finally, on the bottom is shown a table of at what time points phenotypic characterization, whole genome sequencing and RNA sequencing were performed. The figure was borrowed and edited with permission from Anaëlle Fait (Fait, 2021).

2.6.3. VISA strains

The VISA strains that will be used in this thesis are obtained from Exposure cycle 20 (E20) as shown in Figure 2.5 above. The reason for choosing the VISA strains from E20 was partly because there is more knowledge about these strains; RNA sequencing was performed for all of them. Therefore, it was thought that interesting results could be compared to the RNA sequencing, possibly explaining the observations. Furthermore, in the adaptive laboratory evolution performed by Fait (2021), some of the strains were divided at E20, leading to two separate VISA lineages at E30, depicted "bis" to the right of the table in Figure 2.5 above. Taking L9 as an example, it exists in two variants at E30 (L9_E30bis). Therefore, it was decided easier to analyse L9_E20 rather than deciding which of the L9_E30bis lineages to choose from.

2.6.4 Strains chosen for the current thesis

Due to limited time of the current master thesis project, not all VISA strains could be analysed. Therefore, the background about the VISA strains from Fait (2021) was used to choose relevant isolates. Firstly, the susceptibility of the 10 VISA strains to different antibiotics was analysed. Oxacillin was chosen as the representative for β-lactam antibiotics, and looking at Figure 2.6 below, adaptation to vancomycin leads to a collateral increase in the oxacillin MIC for the VISA strains L2, L4 and L7, while the other isolates showed a decrease in oxacillin MIC compared to the VSSA ancestor strain JE2. We chose L4 and L9 as representatives of these two distinct evolutionary routes.

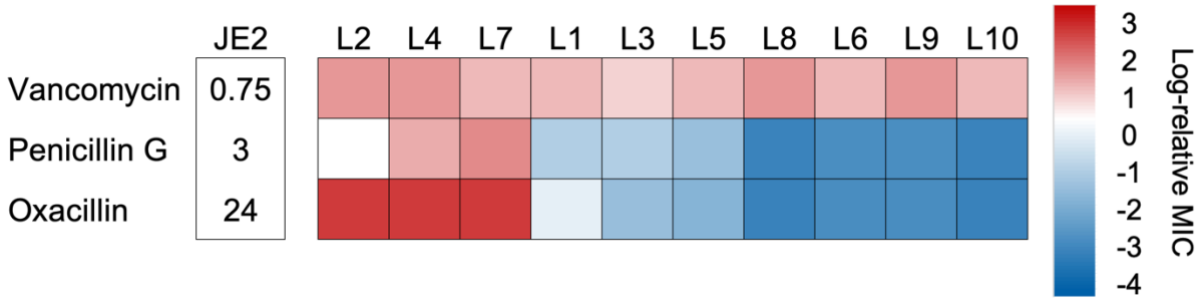


Figure 2.6. The log-relative MIC values of the 10 VISA strains at E20 to vancomycin, penicillin G and oxacillin. To the right is shown log scale colour code. Borrowed with permission from Anaëlle Fait (Fait, 2021).

2.6.5 Adaptation to vancomycin caused mutations and overexpression

During whole genome sequencing analysis of the JE2-derived VISA isolates, Fait (2021) could see that several genes harboured mutations in more than one of the independently evolved VISA isolates. The *walK* mutation was correlated with an increased oxacillin MIC for L4, L7 and L1. In L8, L9 and L10, *vraT* was mutated, in combination with upregulations of the VraR regulon. That upregulation in particular was associated with a decrease in oxacillin MIC in those VISA isolates. Of particular interest in this project, L4 presented a mutation in *walK*, represented by the locus tag SAUSA300_RS00110 in Figure 2.7 below, while L9 had a mutation in *vraT*. These results can be seen in Figure 2.7 below where mutations in L9 are marked orange and mutations in L4 are marked green.

Gene	Locus	Lineages mutated		
vraT	SAUSA300_RS10195	L8	L9	L10
/	SAUSA300_RS04225	L1	L5	
pitR	SAUSA300_RS03480	L4		
pitA	SAUSA300_RS03485	L4	L8	
ssaA	SAUSA300_RS12415	L3	L5	L10
autolysin	SAUSA300_RS09395	L3	L6	
walK	SAUSA300_RS00110	L4	L7	L1
walR	SAUSA300_RS00105	L8		
walH	SAUSA300_RS00115	L8		
rpoB	SAUSA300_RS02820	L6		
rpoC	SAUSA300_RS02825	L2	L9	
rpoD	SAUSA300_RS08300	L2		
pdhC	SAUSA300_RS05360	L6		
pdhA	SAUSA300_RS05350	L5		
pdhD	SAUSA300_RS05365	L8		
puuR	SAUSA300_RS05375	L5		
pykA	SAUSA300_RS08975	L4	L7	
apt	SAUSA300_RS08670	L1	L10	
rseP	SAUSA300_RS06255	L2	L6	
prsA	SAUSA300_RS09800	L3	L9	
tarF	SAUSA300_RS01325	L8	L9	

Figure 2.7. Mutations observed in the different VISA strains. Mutated genes that were presented in more than one VISA strain are presented in a list. In orange and green are the mutated genes of interest for the chosen representative VISA strains L4 and L9. The table has been borrowed and modified with permission from Anaëlle Fait (Fait, 2021).

Furthermore, looking at the RNA sequencing data, it could be seen that when adapting to vancomycin, the different strains had up- or downregulation of certain genes. In Figure 2.8 below, the gene expression levels for the different VISA strains are shown, where the numbers represent the relative expression levels compared to JE2, and the colours show the fold change. Red means increased expression while blue represents a decrease in expression levels compared to JE2 (Fait, 2021).

		Lineages, E20	L1	L2	L4	L7	L3	L5	L6	L8	L9	L10
Gene name	**Old locus tag**	**Locus tag**										
Two-component systems	SAUSA300_	SAUSA300_RS										
vraS	_1866	_RS10190	0.78	1.01	0.78	0.99	2.48	1.40	1.07	1.69	2.22	5.25
vraR	_1865	_RS10185	0.76	0.90	0.71	0.98	2.27	1.24	1.00	1.63	1.85	4.86
graS	_0646	_RS03465	0.77	1.06	1.05	1.14	0.93	0.82	0.91	0.95	1.20	0.89
graR	_0645	_RS03460	0.93	1.28	1.10	1.22	0.98	0.85	1.14	1.16	1.22	0.91
walR	_0020	_RS00105	0.95	1.45	1.13	1.12	1.17	0.97	1.36	1.14	1.15	0.91
walK	_0021	_RS00110	0.88	1.47	1.09	1.11	1.12	0.88	1.16	1.04	1.20	0.93
walH	_0022	_RS00115	0.86	1.32	1.03	1.06	1.09	0.79	0.98	0.98	1.26	0.97

Figure 2.8. Gene expression levels in the 10 VISA strains. In red are genes more expressed than JE2, and in blue are genes less expressed than JE2. Borrowed and modified with permission from Anaëlle Fait (2021).

In Table 2.1 is shown a summary of information about the representative VISA strains L4 and L9 as well as their ancestor JE2.

Table 2.1. Summary of information about JE2 and the JE2-derived VISA strains L4 and L9. *vancomycin MIC and oxacillin MIC in mg/L, as well as the mutations and changes in gene expressions caused when becoming VISA from JE2.*

Strain	Vancomycin MIC (mg/L)	Oxacillin MIC (mg/L)	Mutations	Gene expression
JE2	0.75	24	-	-
L4	2	256	*walK*	-
L9	2	0.5	*vraT*	⇑ VraR regulon

3. Materials and methods

3.1 Plasmid expression system

3.1.1 Choosing the plasmid expression system

To investigate the effect of the genes *vraR, vraT, vraS, vraU, walK, walR* and *walH* on vancomycin and oxacillin resistance, as well as bacteriophage susceptibility, plasmids were produced and transformed into *S. aureus.*

The first question raised was what vector to use for overexpression. There were two different plasmid expression systems available; pSK9067 and pRAB-lacZ, presented in Figure 3.1 and Figure 3.2 respectively.

The plasmid pSK9067 has a P_{spac} promoter, which is inducible by isopropyl β-D-1-thiogalactopyranoside (IPTG). Additionally, in *S. aureus* the selective antibiotic is erythromycin, at a concentration of 10 µg/mL. The pSK9067 has a gene insert, encoding for the green fluorescent protein GFP. This gene sequence will be cut out using restriction enzymes (SalI, BamHI, SmaI or EcoRI) when inserting a gene of interest for overexpression, as presented in Figure 3.1 below (Brzoska & Firth, 2013).

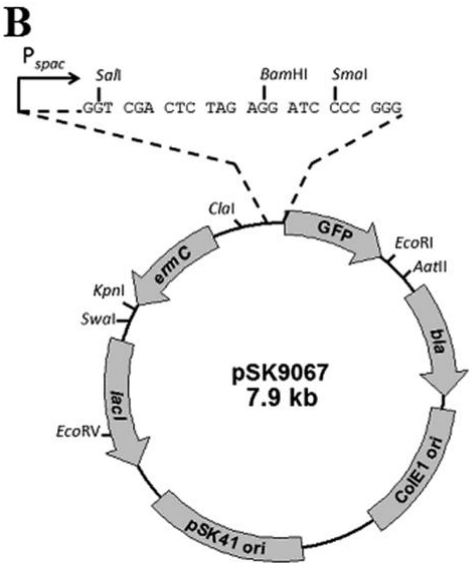


Figure 3.1. An illustration of the plasmid pSK9067. The plasmid is inducible by IPTG. Picture borrowed and edited from Brzoska and Firth (2013).

On the contrary, the pRAB-lacZ plasmid expression system with the promoter $P_{xyl/tet}$, is inducible by anhydrotetracycline (AHT). This can be observed to the left of Figure 3.2 below, where AHT induction leads to stronger expression of protein at 25.1 kDa. The selective antibiotic of the pRAB-lacZ plasmid is chloramphenicol at a concentration of 10 µg/mL (Helle *et al*., 2011).

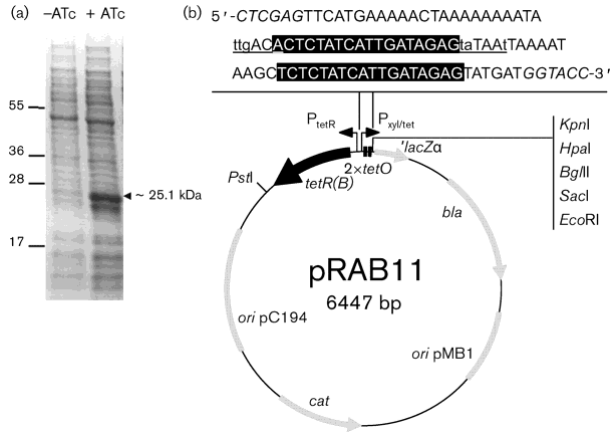


Figure 3.2. An illustration of the pRAB11-lacZ. The plasmid is inducible by anhydrotetracycline, as visualized from the SDS-PAGE bands to the left of the figure. (Figure borrowed from Helle *et al*. (2011)

3.1.2 Concentration of inducer for the two expression systems

The first step of choosing the expression system was to check the effect of the different inducers on the bacterial growth. IPTG was earlier described to have a maximum induction at 800 µM, but even at 400 µM, the activity of the expression system was already more than 200 times higher than when the system was uninduced (Brzoska & Firth, 2013). Therefore, 400 µM IPTG was chosen as the concentration for the pSK9067 plasmid.

The concentration determination test of anhydrotetracycline hydrochloride (AHT) was performed in biological duplicates. The suitable AHT concentration was determined for two transformants, one called JE2 Ø, transformed with the empty pRAB-lacZ plasmid, and one called JE2 *vraR*, where JE2 was transformed with the pRAB-lacZ plasmid with the gene *vraR* inserted downstream of the P_{spac} promoter.

The analysis was performed in a Bioscreen C machine, using a Honeycomb plate with 100 wells, as illustrated in Figure 3.3 below. To each well, 100 µL of bacteria were added, along with 100 µL tryptic soy broth (TSB) supplemented with 10 µg/mL of the selective antibiotic for the pRAB-lacZ plasmid: chloramphenicol. A range of concentrations of AHT was added, from 0-200 ng/mL. For a detailed description of how the dilutions and preparation of bacterial cultures were performed, see Section 3.6.1 below. Additionally, the experiment was performed using technical duplicates, each of the bacterial cultures from the biological duplicates were tested in two different rows to account for technical errors.

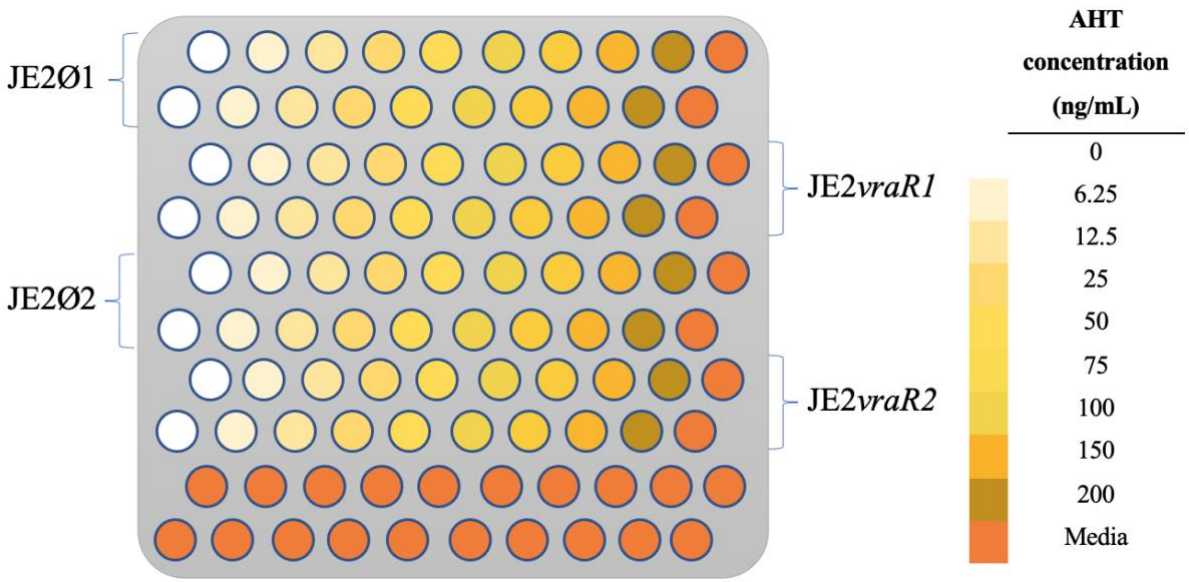


Figure 3.3. A schematic drawing of the Bioscreen C plate used for the AHT toxicity testing. To the right, a concentration scale is shown, ranging from 0-200 ng/mL AHT. To the white wells, no AHT was added, however 100 µL TSB-CAM10 buffer and 100 µl of bacterial dilution. To the dark orange wells depicted 'Media', 200 µL of TSB-CAM10 buffer was added, to ensure that evaporation and shaking in the Bioscreen did not affect the measurements of the other samples. Drawing: Jack Abrahamsson.

The results of the Bioscreen measurements are presented in Appendix A. It was not possible to find a concentration which did not affect the growth of the bacteria, while still producing a sufficient gene expression. Therefore, it was decided to proceed with the plasmid expression system pSK9067 in the further overexpression studies.

3.2 Overexpression plasmid design

3.2.1 Designing primers for plasmid production

Primers for the genes of interest for producing plasmids were designed. For example, recombinant strains were created with plasmids containing the genes *walK, walR* and *walH*. First, the gene sequences were looked up by using the databases KEGG and AureoWiki, looking for the *S. aureus* strain USA300_ FPR3757. Then, the whole genome of *S. aureus* USA300_ FPR3757 was looked up on NCBI Nucleotide and saved in FASTA format. The FASTA file was uploaded to the database Galaxy, to easier visualize only the nucleotide sequence. A part of the gene sequence for *walK, walR* and *walH* were then copied and searched for in the genome, to find the position in the genome. Then, some 50 nucleotides before and after the actual gene sequences were chosen and copied into a document, used to design the primer sequences. A start codon and a stop codon were found, and then a part of the ShineDalgarno sequence AGGAGGUAA was looked for around 8 nucleotides before the start codon. Then, a primer that had a melting temperature of 59-61°C was chosen, that has a C or a G in the 3' end to ensure sufficient annealing during PCR reactions. The primers designed for overexpressing the genes *walK, walR* and *walH* are presented in Table 3.1 below.

Table 3.1. Primer design for the genes walK, walR and walH. *To the left is shown the name of the primer, consisting of the gene name, the direction where FW means forward and RV means reverse, as well as the restriction enzymes chosen for inserting the primers into the plasmids. To the right is shown the primer sequence in the 5' to 3' direction, including the ShineDalgarno sequences.*

Name	5'-3' Sequence
walK-FW_Sal1	GATACAGGATCCGAGTAGAGGTCGAAACGAATG
walK-RV_EcoRI	GATACAGAATTCGCCACTTCATTCGTTTCGACC
walR-FW_BamHI	GATACAGGATCCGAAAAGAGGTTTATGCAAATGG
walR-RV_EcoRI	GATACAGAATTCGCCACTTCATTCGTTTCGACC
walH-FW_BamHI	GATACAGGATCCGAATAATAAGGAGCATATTAAATCTGTC
walH-RV_EcoRI	GATACAGAATTCGTCTTTGTCAGTTTCCAGTTC

3.2.2 PCR reactions for plasmid production

The designed primers were diluted in the desired volume of MilliQ water to reach a concentration of 100 pmol/µL. Then, 10 µl of that solution was mixed with 90 µl of MilliQ water to reach a concentration of 10 µM, which is desired for the PCR reaction.

For a 20 µl PCR reaction, the following reagents were mixed in the order of large to small volume: 12.4 µL of MilliQ water, 4 µL of 5x Phusion HF buffer, 1 µL of bacterial DNA, 1 µl forward primer, 1 µl reverse primer, 0.4 µL of 10mM dNTPs and 0.2 µL of polymerase (DreamTaq Green DNA Polymerase (5 U/µL), (ThermoFisher Scientific)). If two PCR reactions were to be prepared, a master mix containing about 2.5 times the earlier described volumes were prepared in an Eppendorf tube, and 18 µL was pipetted into each of the two PCR tubes. Then, the forward and reverse primer (1 µL of each) for the respective reactions were added.

After that, the tubes were run in a PCR Thermocycler (Thermo Fisher Scientific) with different settings depending on the gene length and annealing temperature determined by the primer melting temperatures as well as according to the instructions specific to the polymerase used.

3.2.3 Producing plasmids for transformation.

i) Digestion

Inserts

The following was mixed: 10 µL gene insert, 6 µL MQ water (nuclease free), 2 µL 10x FastDigest buffer from ThermoScientific, 1 µL BamHI and 1 µL EcoRI. The mixture was incubated for 20 minutes at 37°C without shaking.

Plasmid (pSK9067)

The following was mixed: 13 µL MQ water (nuclease free), 2 µL 10 FastDigest buffer from ThermoScientific, 2 µL plasmid (pSK), 1 µL Fast AP thermosensitive alkaline phosphatase from ThermoScientific, 1 µL BamHI and 1 µL EcoRI. The mixture was incubated for 15 minutes at 37°C without shaking.

ii) Purification

The digested gene inserts (20 µL) and the pSK plasmid (20 µL) was purified using a ThermoScientific GeneJet PCR purification kit.

iii) Ligation

The T4 DNA ligase from ThermoScientific was used. The protocol states that the following should be mixed: 20-100 ng vector DNA, 1:1 to 5:1 molar ratio of insert over vector, 2 µL 10x T4 DNA ligase buffer, 1 U T4 ligase and add nuclease free MQ water up to 20 µL. Therefore, it was decided to mix 2 µL 10x T4 DNA ligase buffer, 2 µL pSK vector DNA, 6 µL gene inserts, 1 µL ligase and 9 µL nuclease free MQ water. All the mixing was performed on ice. Then, the mixture was incubated for 20 minutes in a 22°C incubator without shaking. After incubation, the ligated plasmids were put on ice before the transformation of *E. coli* IMO8.

3.2.4 Plasmid design

After digestion, purification and ligation, the plasmids were stored at -20°C until they were to be used for transformation. The final plasmid design in shown in Figure 3.4 below, where the restriction sites and position of gene insert is marked.

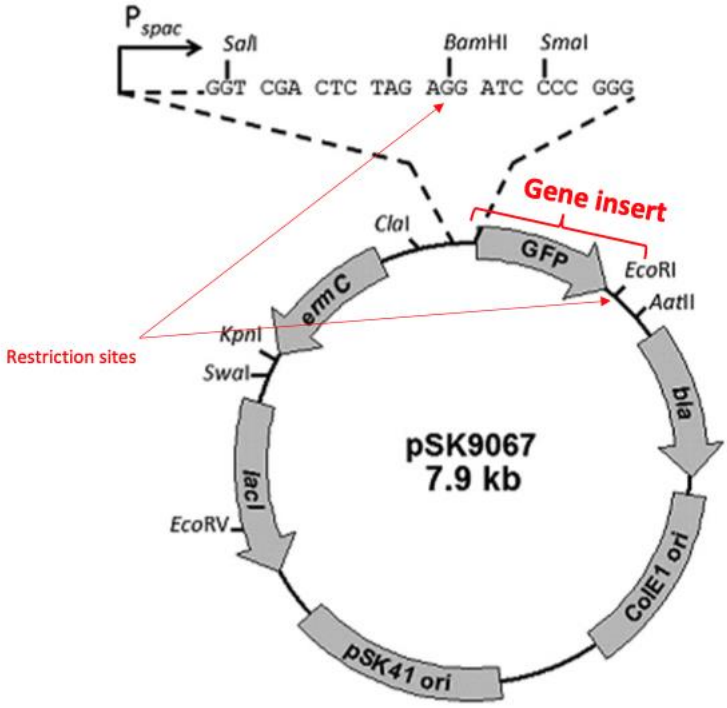


Figure 3.4. The final design of the plasmid pSK9067. With red arrows, the restriction sites chosen are shown, while a red bracket is showing the position of the gene inserts. Figure borrowed and edited from Brzoska and Firth (2013).

3.3 Making competent cells

3.3.1 Growing strains for plasmid production

i) E. coli

E. coli strain IM08 was used for producing plasmids. The strain was grown on agar plates containing TSA and 100 µg/mL ampicillin. After single colonies appeared, a single colony was inoculated in 5 mL Luria-Bertani broth (LB) medium (Sezonov, Joseleau-Petit, & D'Ari, 2007), containing 100 µg/mL ampicillin, after which it was grown over night in a shaking incubator at 37°C. The next morning, 1 mL of the inoculum was transferred to 100 mL of LB, after which it was grown in a shaking incubator at 37°C for 2 hours.

ii) *S. aureus*

The *S. aureus* strains that the goal is to transform were grown on agar plates containing TSA and 10 µg/mL erythromycin. After single colonies appeared, a single colony was inoculated in 10 mL TSB medium, containing 10 µg/mL erythromycin, after which it was grown over night in a shaking incubator at 37°C.

3.3.2 Treatment of bacteria to make the cells competent

i) Calcium chloride competent cells

The *E. coli* strain IMO8 were chilled on ice for 10 minutes after the 2-hour incubation. All the following steps were performed on ice. The tubes containing the bacteria were centrifuged at 6000rpm for 3 minutes, and the supernatant was thrown away. The supernatant was dissolved in 0.1 M $CaCl_2$ that had been stored on ice, a volume of 10 mL. The suspended cells were incubated for 20 minutes on ice, and then they were centrifuged again at 6000rpm for 3 minutes. The supernatant was once again thrown away, and the cell pellet was dissolved in a solution of 0.1 M $CaCl_2$ containing 15% glycerol, a volume of 5 mL. The cells were aliquoted into Eppendorf tubes and stored at -80°C.

ii) Electroporation

The electroporation of the *S. aureus* strains that were going to be transformed was performed according to the materials and methods section in an article by Monk et. al (2012).

The cultures that had been growing overnight (see 3.3.1.) were diluted 100 times to measure the optical density using a spectrophotometer at 578 nm. Then, the cells were diluted in TSB that had been warmed in a 37°C incubator for 1 h, according to the $OD_{578\ nm}$ measurement to an OD of 0.5. Thereafter, the cells were incubated in a shaking incubator at 37°C for 30 minutes and then put on ice for 10 minutes. Five centrifugations at 4000G for 10 minutes were performed, where the cell pellet was resuspended in 50 mL ice cold water twice, and then in 5 mL, 2 mL and 250 µL of glycerol at a concentration of 10% (wt/vol) that had been kept in the freezer. 50 µL of the solution were aliquoted in separate 1.5 mL Eppendorf tubes, which was then stored at -80°C.

3.4 Transformations

3.4.1 Transformation of E. coli.

i) Calcium chloride competent cells

10 µL of the plasmid containing the inserts was mixed with 100 µL competent *E. coli* IMO8. The 110 µL of the plasmid – bacterial solution was mixed with 900 µL of Luria-Bertani agar (LA) (Sezonov et al., 2007) to obtain 1010 mL solution, which was transferred to a new tube. The bacteria were incubated at 37°C in a shaking incubator for 30 minutes. After that, the cells were plated on LA plates containing 100 µg/mL ampicillin and grown overnight in a 37°C incubator.

ii) Colony PCR

After the *E. coli* IMO8 cells had been grown at 37°C overnight, colony PCR was performed to confirm that the bacteria contained the construct (plasmid with gene insert). 7 µL nuclease free MQ water was mixed with 10 µL Taq polymerase solution and 1 µL of forward and reverse primer for the pSK9067 plasmid respectively. 1 single overnight colony was picked, streaked on an LA plate containing 100 µg/mL ampicillin, then the loop was dispersed in the PCR mixture. A PCR program was run using times calculated for the separate genes inserted into the pSK plasmid, using an Applied Biosystems ProFlex PCR cycler from Thermo Fisher.

The LAamp plates were put in a 37°C incubator overnight to check for growth. For the colony PCR, the DreamTaq DNA polymerase was used, in addition to the bacterial colony DNA, as well as forward and reverse primers for the pSK9067 plasmid. The forward and reverse primers are flanking the plasmid, making the DNA polymerase only polymerizing the DNA that is inserted. When the PCR was finished, it was run through a gel electrophoresis to visualize if the PCR products are of the expected size, which is the size of the inserts, as well as which colonies had the right plasmid. These colonies were to be used going forward, trying to insert the plasmid into *S. aureus* CC8 strains.

3.4.2 Transformation of S. aureus

i) Electroporation

The *S. aureus* strains to be transformed with the designed plasmid were electroporated in order to take up the plasmid. First, the *E. coli* containing the plasmids of interest were treated. To obtain the *walK*, *walR* and *walH* plasmids from the *E. coli* strain IMO8, the overnight cultures were centrifuged using an Eppendorf centrifuge at 8000G for 5 minutes in room temperature. The supernatant was discarded, and the cell pellet was resuspended and purified according the instructions provided in the Thermo Scientific GeneJet plasmid purification kit.

Then, the competent *S. aureus* strains of interest were prepared for electroporation. This was done by centrifuging the competent cell freezer stocks that had been kept at -80°C, discarding the supernatant. The cell pellet was resuspended in a solution of 50 µL 500 µM sucrose and 10% glycerol, and then it was mixed with 10 µL of plasmid and transferred to a BioRad electroporation cuvette. A BioRad electroporation machine was used at 21 kV to electroporate the cells. Directly after electroporation, 660 µL of TSB and 330 µL of filter sterilized 1.5 M sucrose. The whole volume was transferred into an incubation tube and put in a shaking incubator at 37°C for one hour. Since the pSK9067 plasmid has erythromycin as selective marker in *S. aureus*, the cells were plated on TSA plates containing 10 µg/mL of erythromycin.

ii) Colony PCR

The same methodology for the PCR reaction as described in Section 3.4.1 i) was followed when picking *S. aureus* colonies. When a single colony was picked, it was streaked on a TSAerm10 plate and incubated at 37°C. If the colony PCR was successful, meaning if the picked colony contained the gene insert of interest, an overnight culture was created by inoculating the bacteria in 1 mL TSA containing 10 µg/mL erythromycin in a 15 mL Falcon tube, and grown overnight shaking at 37°C. The next day, a freezer stock of the selected clone was made by mixing 300 µL 50% glycerol with 700 µL overnight culture in a Cryo tube, and put in the freezer at -80°C.

3.5 Handling of bacterial stocks and cultures

3.5.1 Inoculations

Colonies were inoculated and grown overnight in tryptic soy broth (TSB). This was performed by adding 3 mL of TSB into each glass tube for inoculation. In each of the tubes, a single colony was picked from an agar plate with a plastic loop and added to the glass tube. Then, the glass tubes were put in a shaking incubator at 37°C overnight, unless otherwise stated.

3.5.2 Growth conditions during inoculation

Strains containing a plasmid were grown together with antibiotics, to ensure only bacteria containing the desired plasmid grows. For example, when growing strain 3 and 5 (see Table 1), erythromycin was added (see introduction) according to Calculation 3.1, based on Equation 3.1. 3 µL of erythromycin was added to each glass tube containing 3 mL TSB, before inoculation. For the wild type strains, for example JE2, no plasmid is added and thereby there is no antibiotic selection pressure. Therefore, no erythromycin was added to tubes containing wild type strains, as well as the control tubes.

3.5.3 Freezing of cultures for later use

Strains were saved in glycerol stocks at -80°C. Glycerol and water were mixed to a glycerol concentration of 50% v/v. 300 µl of glycerol solution was mixed with 700 µl bacteria grown in TSB in Cryotubes, after which the tubes were put in the freezer at -80°C.

Equation 3.1: Method for calculating the volume (V_1) of a chemical to add to a final volume, with a desired concentration (c_2), when the initial concentration (c_1) is known.

$$c_1 \times V_1 = c_2 \times V_2 \Longrightarrow V_1 = \frac{c_2 \times V_2}{c_1}$$

Calculation 3.1: Calculating the amount of the antibiotic compound erythromycin to add to 6 mL of TSB, enough to fill two glass tubes before inoculation with bacteria.

$$V_{erythromycin,start} = \frac{c_{erythromycin,final} \times V_{final}}{c_{erythromycin,start}} = \frac{10\ \mu g/mL \times 6000 \mu L}{10000\ \mu g/mL} = 6\ \mu L\ erythromycin$$

3.6 Minimal Inhibitory Concentration analyses

A part of this project consisted of testing the Minimal Inhibitory Concentration (MIC) of different antibiotics on the different overexpressing recombinant strains created, as described in section 3.1-3.4. The two antibiotics of interest was vancomycin as well as oxacillin. As described in section 3.5 above, erythromycin was used as the selective antibiotic for the selection of strains containing a plasmid of interest. The reason why a normally multi-resistant MRSA strain can be selected through erythromycin will be described next. Many MRSA clinical isolates have gained resistance to erythromycin as well, even though the resistance mechanism differs in that erythromycin is a macrolide, inhibiting the protein synthesis of the bacteria. Resistance to erythromycin is gained through primarily three different genes; *ermA, ermB* and *ermC* (Nicola, McDougal, Biddle, & Tenover, 1998). Performing whole genome sequencing of the USA300 MRSA strain being the ancestor for JE2 used in this study, resistance to erythromycin was confirmed through localization of the *ermC* gene in a plasmid called p03. This plasmid was completely removed, giving rise to JE2 being susceptible to erythromycin again. Therefore, plasmids containing *ermC* can be used during overexpression experiments so that erythromycin can select only the bacteria containing the plasmid (Fey et al., 2013).

The MIC tests for oxacillin and vancomycin MIC were performed with two different methods; using the Bioscreen to obtain $OD_{600\ nm}$ measurements used for creating growth curves to subsequently analyze the growth of the bacteria with oxacillin over time. Secondly using broth microdilutions in 96 well plates for both vancomycin and oxacillin. These methods will be described in the following section.

3.6.1 MIC tests in 96 well plates

Oxacillin MIC test using microtiter plates

To perform a Minimum Inhibitory Concentration (MIC) test for oxacillin, a 96 well microtiter plate was used, as described in Figure 3.5 and Table 3.1 below, according to Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2019). Each well was to contain 100 µL of liquid. Since Column 1 should only contain TSB-erythromycin as a negative control, 100 µl media was added. Column 2 should contain bacteria and no oxacillin, therefore 50 µl of bacterial dilution was pipetted into each of the wells, and 50 µl of TSB-erythromycin was added. For the rest of the wells, the ones not containing IPTG (row A-D) was filled with 50 µl bacterial dilution, 25 µl oxacillin, and 25 µl extra TSB-erythromycin solution. To the wells containing IPTG (row E-H), 50 µl bacterial dilution, 25 µl oxacillin, and 25 µl IPTG was added. Since a serial dilution was performed, the 12^{th} row should have the highest concentration, in this case 128 µg/mL. However, since 25 µL of oxacillin is added and the final volume in the well is 100 µL, the oxacillin is diluted 4 times. Therefore, Equation 3.1 was used to calculate the desired concentration (512 µg/mL) and thereby wanted volume of oxacillin stock solution of the concentration 4 mg/mL (77 µL). The same methodology was used to perform the IPTG dilution, to a final concentration of 1 mg/mL or 400 µM, so 4 mg/mL was added into each well.

To determine the MIC, the plates were added to a 37°C incubator for 24 hours, after they were visually inspected for growth. The MIC was determined from the lowest concentration where no growth was observed.

Figure 3.5 below is showing a schematic drawing of the 96 well plate used for determining the oxacillin MIC. Increasingly stronger green color means a higher oxacillin concentration, whereas the white and yellow columns (1 and 2) depict only media and bacterial dilutions respectively, used as a negative and positive control.

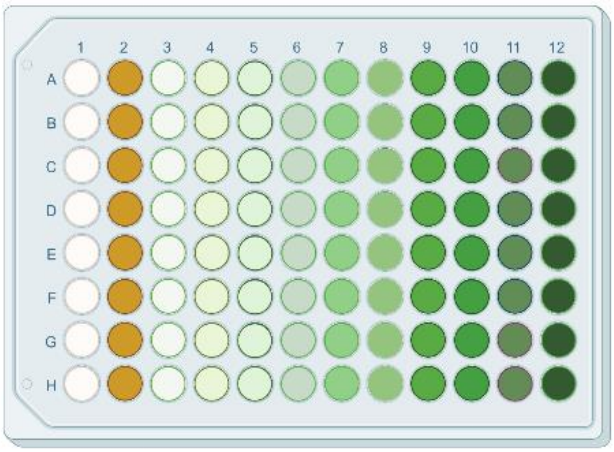


Figure 3.5. A 96 well microtiter plate used for testing oxacillin MIC. Column 1 and 2 has only media and bacterial dilutions added respectively and is used as negative and positive controls. Column 3-12 has increasing concentrations of oxacillin respectively, ranging from 0.25-128 µg/mL, using two-fold serial dilutions. Row A, B, E and F has JE2Ø with IPTG added in rows E and F, while row C, D, G and H has JE2 *vraR* with IPTG added in rows G and H. *Created with BioRender.com*

When more than one strain was to be tested the same day, it was decided to fill some plates with 25 µL oxacillin, 50 µL bacterial dilutions and 25 µL TSB-erm10 or 25 µL IPTG respectively. In these cases, it was possible to use a multichannel pipette to fill all the wells at once. Additionally, in further MIC experiments using the 96 well plates, no bacteria or oxacillin was added to row A and H, since there is a known issue with evaporation from the wells at the edges and corners of 96 well-plates. (Mansoury, Hamed, Karmustaji, Al Hannan, & Safrany, 2021)

McFarland test for determining bacterial dilutions

Before the bacteria could be added to the microtiter plate for oxacillin MIC tests or to the Bioscreen Honeycomb plates, colonies were diluted to a McFarland standard of 0.5 using a Nephelometer. First, the machine was calibrated using the accompanied McFarland standard solution, which corresponds to a McFarland unit of 0.5. Around 5 mL of saline solution (FK) was added to glass tubes, and around 3-4 colonies that were to be tested for MIC were picked from the respective agar plates with a plastic loop, then dispersed in the saline solution. The glass tubed were vortexed and then the Nephelometer was used to determine the McFarland concentration. If the result was not in the right range, the bacterial saline solution was either more diluted or more bacterial colonies were added. Then, the solutions were diluted 100 times in TSB, by for example mixing 4 mL TSB with 40 µL of bacterial saline solution (CLSI, 2019). In all experiments, the bacteria were further diluted twice into the Honeycomb or 96 well plates.

3.6.2 Bioscreen MIC tests

Antibiotic stock preparations for using the Bioscreen C

The machine Bioscreen C was used extensively during the first 3 weeks of this project, in order to perform MIC analyses of different antibiotics. One of the aims was to obtain growth curves, for example when the plasmid pRAB-lacZ was to be used, with the inducer AHT. This compound is known to be toxic (Lutz & Bujard, 1997) in some concentrations, why an AHT toxicity test was performed, as described in Section 3.1.2 above.

To make sure only the bacteria containing the plasmid of interest would survive and grow in the Bioscreen measurements, a selective antibiotic marker was used. For the plasmid pSK9067 (inducible by IPTG), erythromycin was used as the selective marker, at a concentration of 10 µg/mL. The erythromycin stock solution was 10 mg/mL, meaning that a 1000 times dilution was needed to reach the concentration of 10 µg/mL. For most MIC testing experiments, 40 mL of TSB-buffer was needed, so 40 µL of erythromycin was added to 39,960 µL of TSB. This solution will from here on be called TSB-erm10. For the plasmid pRAB-lacZ (inducible by AHT), chloramphenicol was used as the selective marker, at a concentration of 10 µg/mL. This solution will from here on be called TSB-cam10.

Vancomycin MIC using Bioscreen C

To perform the vancomycin MIC test, no dilution series was made. Instead, a stock consisting of 5 mL of 16 µg/mL vancomycin in TSB-erm10 was made. All concentrations prepared needed to be 4 times higher than what was wanted in the Bioscreen, since 50 µL vancomycin solution was to be added in each well containing 200 µL fluid in total.

A schematic drawing of the Bioscreen plate is shown in Figure 3.6 below. The experiments were performed in technical duplicates, meaning that the strains JE2Ø and JE2*vraR* were pipetted into two rows instead of just one, to minimize the technical errors. Additionally, the experiments were performed with and without IPTG, meaning that 8 rows were used in total. To the last two rows, only TSB-erm10 was added as a negative control to ensure no contamination.

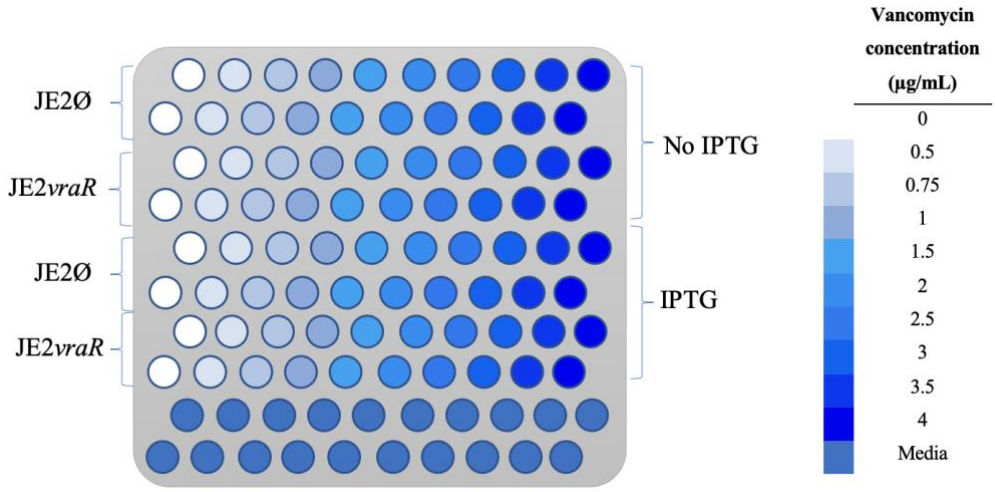


Figure 3.6. A schematic drawing of the Bioscreen C plate used for the vancomycin MIC tests. To the right, a concentration scale is shown, ranging from 0-4 µg/mL vancomycin. To the white wells, no vancomycin was added, however 100 µL TSB-ERM10 buffer and 100 µl of bacterial dilution. The IPTG concentration used was 400 µM. Drawing: Jack Abrahamsson.

Oxacillin MIC using Bioscreen C

The oxacillin MIC tests were performed using serial dilutions. A stock of 1000 µL TSB-erm10 with a concentration of 128 µg/mL was prepared. This concentration is 4 times higher than what is needed in the Bioscreen plate, since only 50 µL is added to each 200 µL well, and thereby the oxacillin will be diluted 4 times. Then, 500 µL of TSB-erm10 was added into 8 new Eppendorf tubes, and 500 µL of each previous solution was added stepwise to get a two-fold serial dilution.

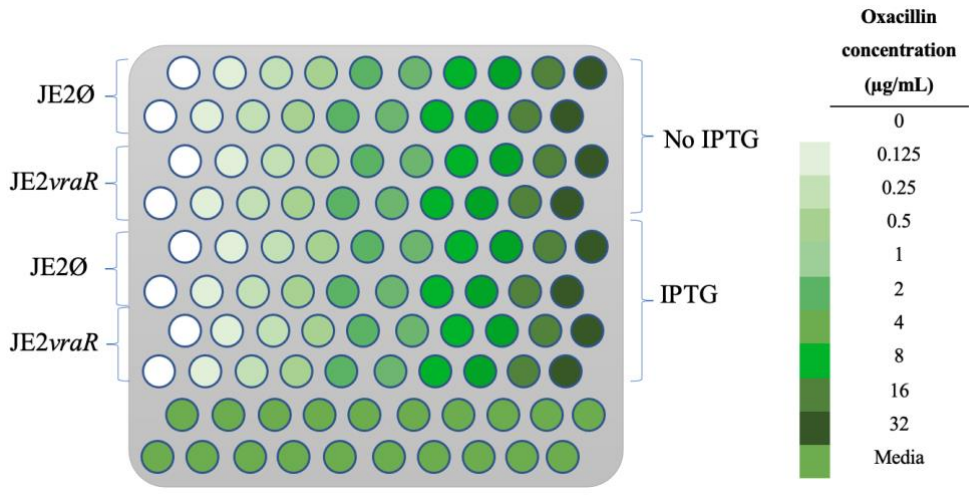


Figure 3.7. A schematic drawing of the Bioscreen C plate used for the oxacillin MIC tests. To the right, a concentration scale is shown, ranging from 0-32 µg/mL oxacillin. To the white wells, no oxacillin was added, however 100 µL TSB-ERM10 buffer and 100 µl of bacterial dilution. The IPTG concentration used was 400 µM. Drawing: Jack Abrahamsson.

3.7 Bacteriophage susceptibility testing

3.7.1 Media preparation

Two different types of agars were used for the plaque analysis of phages, phage base agar (PB) and phage top agar (PTA). Additionally, phages were diluted using phage buffer (PHB).

i) Phage top agar

PTA was made by weighing out 4 g of Thermo Scientific™ Oxoid™ Nutrient Broth n°2 and 0.7 g agar, then mixing it with 200 mL distilled water. Lastly, the mixture was autoclaved.

ii) Phage base

PB was made according to the protocol for PTA but weighing out double the amount of agar, 1.4 g per 200 mL distilled water.

iii) Phage buffer

500 mL PHB was made by mixing 10 mL of 2.5M Tris-HCl with a pH of 8.0, with 2 mL $CaCl_2$ with a concentration of 1M, as well as 5 mL of 0.1M $MgSO_4$ and 2.95 g NaCl. Then, distilled water was added to adjust the volume. The PHB was aliquoted into five 100 mL bottles.

3.7.2 Phage stock preparation

i) Lytic phages

Stocks of the lytic phages RODI, Stab20 and Stab21 were prepared. First, 5 mL of overnight culture of the phage susceptible *S. aureus* strain 8325-4 as well as *S. xylosus* were made. The next day, the $OD_{600\ nm}$ of the bacteria was measured, and the overnight culture was diluted to an $OD_{600\ nm}$ of 0.05. Then, the bacteria were grown at 37°C shaking, until the $OD_{600\ nm}$ had reached 0.15. Then, the cells were spun down in a centrifuge at 4°C, 4000 rpm for 10 minutes. The supernatant was discarded, while the bacterial pellet was resuspended in equal amounts of Phage buffer and TSB. Then, 250 µL of phage lysate was added per 5 mL of liquid, and then put in an incubator at 30°C, shaking at 80 rpm until the cells had lysed. If the bacterial culture had not lysed after 3-4 hours, the Falcon tubes were put on the bench overnight. After complete lysis was observed, the phage stock was filtered through a 0.22 µm filter.

ii) Temperate phages

A stock of the temperate phage φ11 was prepared in a similar way compared to the lytic phages as described in section 3.7.2i). When the 8325-4 strain had reached an $OD_{600\ nm}$ of 0.15, the solution was supplemented with 2 µg/mL mitomycin C. Then, the stock was incubated and filtered as described in section 3.7.2i).

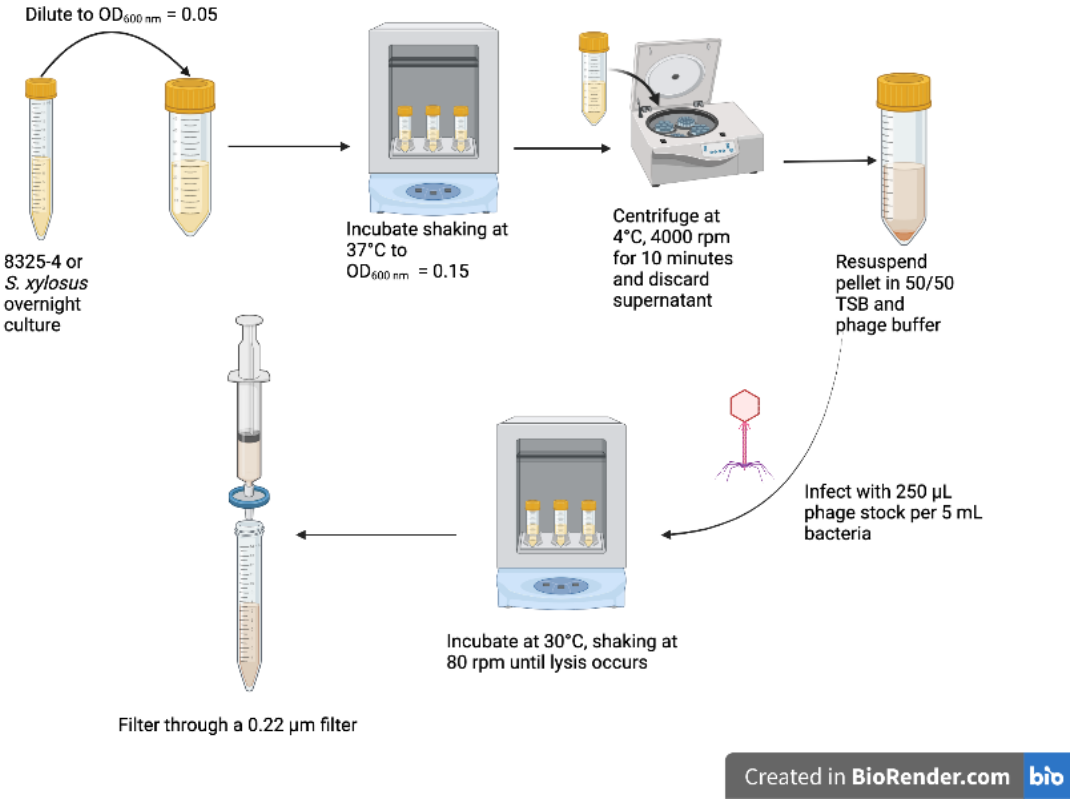


Figure 3.8. Phage lysate stock production. Depending on what phages were used, either 8325-4 or *S. xylosus* were used. Filtered phage stock lysates were stored at 4°C at all times. *Created with BioRender.com.*

3.7.3 Preparation for phage plaque assays

To determine the bacteriophage susceptibility of the different overexpressing recombinant strains, the method phage plaque infection was chosen. The reason is that a ten-fold serial dilution of the four different phages of interest; RODI, Stab20, Stab21 and φ11 could be spotted on the same lawn of bacteria on a square Petri dish.

Production of plates for phage plaque assay

Phage base was melted in the microwave until all agar was completely dissolved. Before pouring the plates, 10 mM of $CaCl_2$ solution was added. Then, the plates were poured and dried in a fume hood.

To produce the top layer where phages were to be spotted, phage top agar was used. First, the bacteria of interest were grown overnight in TSB. At the start of the experiment, the bacteria were diluted to an $OD_{600\ nm}$ of 0.01, and then grew shaking in a 37°C incubator to an $OD_{600\ nm}$ of 0.35. The bacteria that had grown to an $OD_{600\ nm}$ of more than 0.4 were diluted back to 0.35 again with TSB. PTA was melted in the microwave and cooled down to approximately 50°C. Then, 330 µL of bacteria were mixed with 10 mL of PTA that had been supplemented with 10 mM $CaCl_2$. The bacterial agar solution was poured on top of the PB layer and the plates were dried for at least 10 minutes in a fume hood.

Phage dilutions

To test the concentration of the phage stocks produced according to Figure 3.8, the stocks were diluted according to a ten-fold serial dilution setup, presented in Figure 3.9 below. The initial phage stock was diluted 10 times in PHB by mixing 50 µL of phage stock with 450 µL PHB in a 1.5 mL Eppendorf tube, then vortexing well. 50 µL was subsequently transferred from each tube to a new tube, creating a dilution series from 10^1 to 10^7 dilution.

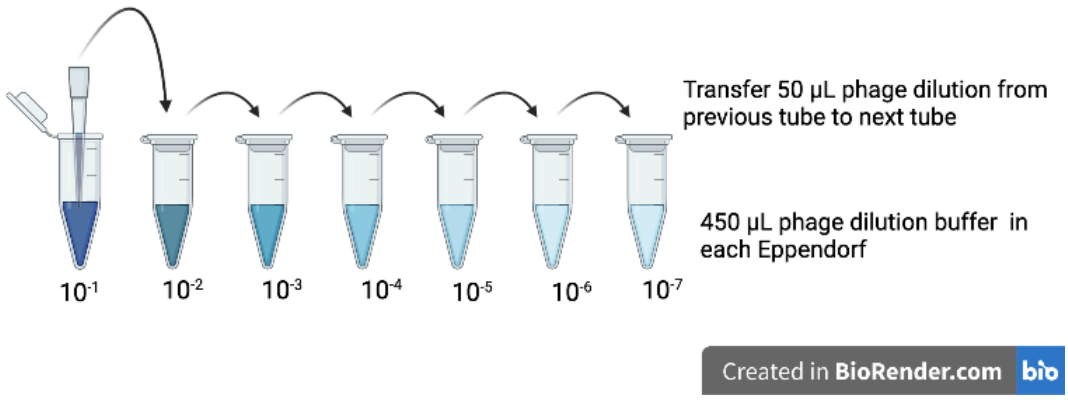


Figure 3.9. The setup for serial dilution of phage stocks. The 10^{-1} dilution was created by mixing 450 µL of PHB with 50 µL of the concentrated phage lysate. *Created with BioRender.com*

3.7.4. Phage plaque assay to determine PFU of phage stocks

The phage lysates were tittered by determining the plaque forming units (PFUs) of the stock solutions. The PFU assay was done in several rounds, to test the PFU of the lytic phages RODI, Stab20 and Stab21 on both *S. xylosus* and 8325-4.

Additionally, the temperate phage φ11 was tested on 8325-4 (an *S. aureus* strain), since the natural host of the phage φ11 is *S. aureus*. The setup of the PFU assay for the lytic phages is presented in Figure 3.10 below. The same method was used when testing the PFU of the temperate phage φ11. Two different phage lysates had been produced for the three lytic phages, why each of the phages have two dilution series on the square plate.

Each of the phage dilutions were spotted on the dried bacterial lawn according to Figure 3.10 below. 10 µL of each dilution were pipetted onto the bacterial lawn.

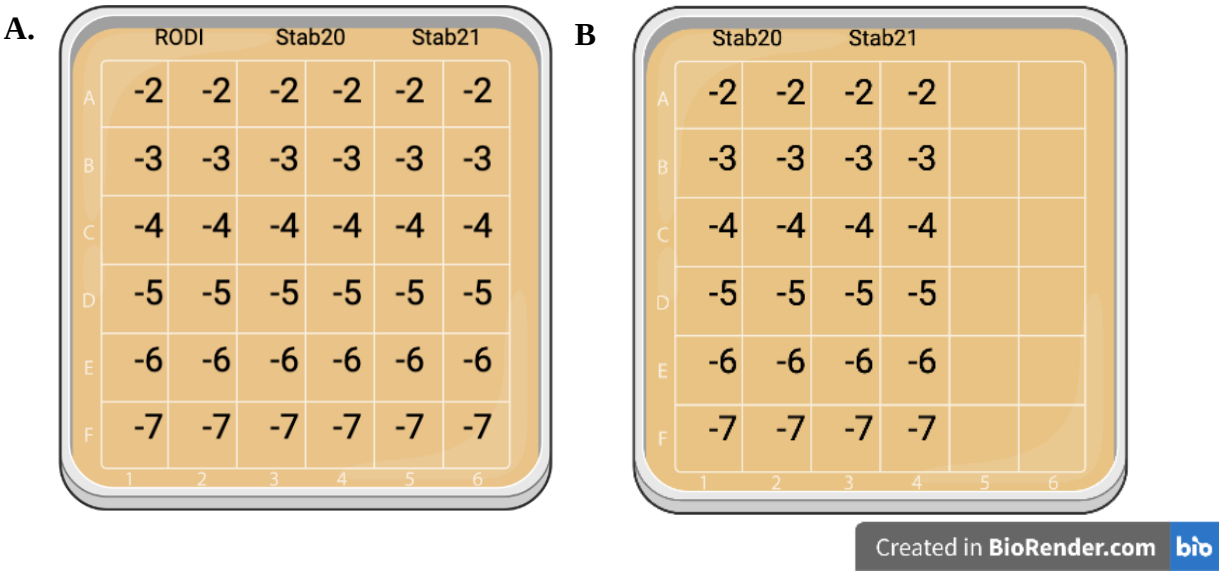


Figure 3.10. Setup for determining the PFU of the lytic phage stocks produced according to section 3.7.2.

A. Setup of the phage plaque assay for determining the PFU of the lytic phages RODI, Stab20 and Stab21 on the *S. aureus* strain 8325-4. For each of the phages, two different lysates were available. The numbers stand for the 10^x dilution factor of phage solution spotted onto the bacterial lawn.

B. Setup of the phage plaque assay for determining the PFU of the lytic phages Stab20 and Stab21 on *S. xylosus*. The numbers stand for the 10^x dilution factor of phage solution spotted onto the bacterial lawn. Figures created with Biorender.com.

The result of the first phage plaque assay will be presented next since the result was used to determine the dilutions of the four different phages for testing phage susceptibility on the overexpressing recombinant strains.

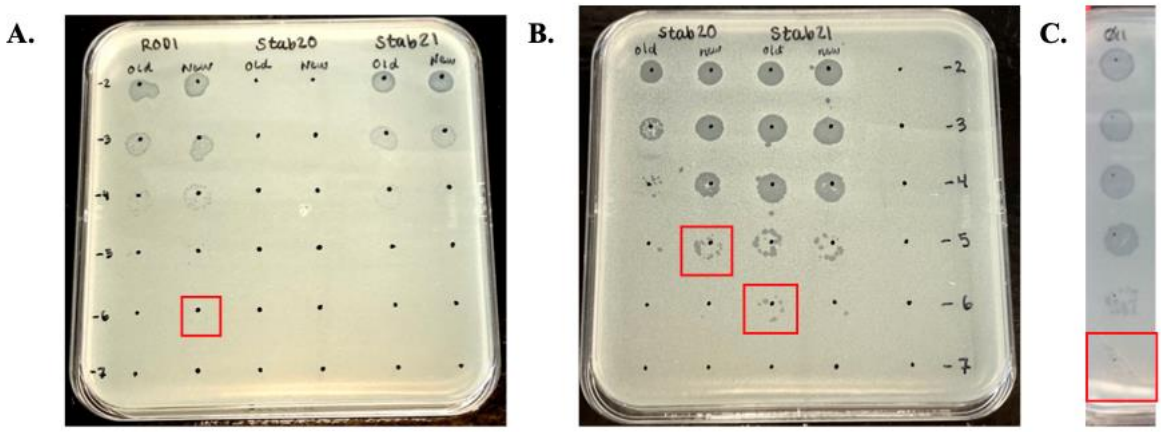


Figure 3.11. Results from the PFU determination phage plaque assay.

A. The three lytic phages RODI, Stab20 and Stab21 were tested on the *S. aureus* strain 8325-4. Two different phage stocks for each phage, called old and new, were tested for PFUs. In the red square is the highest RODI dilution where plaques were visible, being 1,000,000 times dilution. Photo: Jack Abrahamsson.

B. Two different phage stocks, called old and new, of the two lytic phages Stab20 and Stab21 were tested on *S. xylosus*. Marked by two red squares are the highest dilutions where countable plaques are observed, for Stab20 its 100,000 times dilution and for Stab21 its 1,000,000 times dilution. Photo: Jack Abrahamsson.

C. A cut picture from the phage plaque assay using the temperate phage φ11. The highest dilution where plaques are countable are marked with a red square (symbolizing the 10^7 dilution). Photo obtained from: Anaëlle Fait.

In Figure 3.11 **A.** above, it is visible that the **New** RODI stock showed plaques at a 10^6 dilution. Additionally, from Figure 3.11 **B.** it can be determined that for Stab20, the **New** lysate had countable plaques at a 10^5 dilution, while Stab21 had countable plaques at a 10^6 dilution for the **Old** lysate. In Figure 3.11 **C.**, it is visible that the 10^7 dilution created plaques. The PFU/mL was calculated according to Equation 3.2 below.

Equation 3.2: Calculation of PFU/mL from a phage plaque assay.

$$\frac{PFU}{mL} = \frac{Number\ of\ countable\ plaques}{Volume\ of\ phage\ solution \times Dilution\ factor}$$

For RODI, 2 plaques were visible. For Stab20, 15 plaques were counted. For Stab21, 10 plaques were counted. Since the volume of phage solution used to spot was 10 µL, and the dilution

factors were 10^{-6}, 10^{-5} and 10^{-6} for RODI, Stab20 and Stab21 respectively, Equation 3.2 could be used to calculate the PFU/mL for each of the phage lysates. Furthermore, Equation 3.2 was also used to calculate the PFU/mL for the temperate phage φ11, which showed 5 plaques. The result is presented in Table 3.2 below.

Table 3.2. The PFU/mL for four different phages. *The PFU/mL is calculated from the number of PFUs on the bacterial strains which are susceptible to the phage in question. For RODI and φ11, it is 8325-4, while Stab20 and Stab21 infects S. xylosus.*

	Bacteriophages			
	RODI	**Stab20**	**Stab21**	**φ11**
PFU/mL	$2 * 10^8$	$1.5 * 10^8$	10^9	$5*10^9$

According to Table 3.2, it was decided that the same dilutions, between 10^2 and 10^7 could be used. However, since it was visible from Figure 3.11 **A.** that Stab20 could not infect 8325-4, a *S. aureus* strain, it was decided to spot the ten times diluted phage stock for all four phages as well. This was done to catch an eventual increase in susceptibility in the *S. aureus* recombinant strains. The final setup of the phage plaque assay that was used for the rest of the phage experiments is presented in Figure 3.12 below.

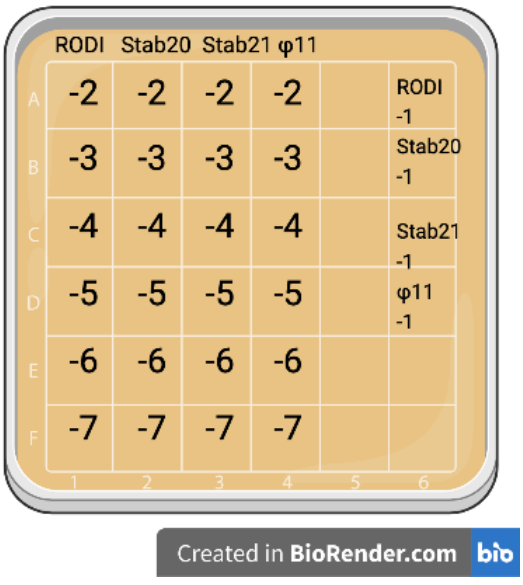


Figure 3.12. The final setup of the phage plaque assay. The setup was used for testing the bacteriophage susceptibility of all the overexpressing strains. The results are presented in Result section 4.3. The numbers stand for the 10^x dilution factor of phage solution spotted onto the bacterial lawn. *Created with BioRender.com.*

3.7.5. Phage plaque assay to test phage susceptibility

It was decided to use the dilution of bacteriophages presented in Figure 3.12 for the susceptibility testing of the overexpressing recombinant strains. Each bacterial strain, for example JE2 *Ø* and JE2 *vraR*, was tested with and without IPTG to determine the effect of inducing protein expression. Therefore, plates were performed in different ways.

For the tests without IPTG, the square petri dishes were prepared according to section 3.7.3, with the exception that 10 µg/mL erythromycin was added when the strains grew overnight. Additionally, the same concentration of erythromycin was added to the PTA. When making the plates for testing the effect of overexpression, 400 µM IPTG was added to the PB before pouring the plates. Additionally, 400 µM IPTG was added to the PTA when mixing it with the bacteria. Lastly, there was a difference when growing the bacteria in the incubator before making the plates. When the strains that should be tested for overexpression were diluted to $OD_{600\ nm}$ = 0.01, the media was supplemented with 400 µM IPTG to induce protein production already before growing to mid-log phase. This difference is illustrated in Figure 3.13 below.

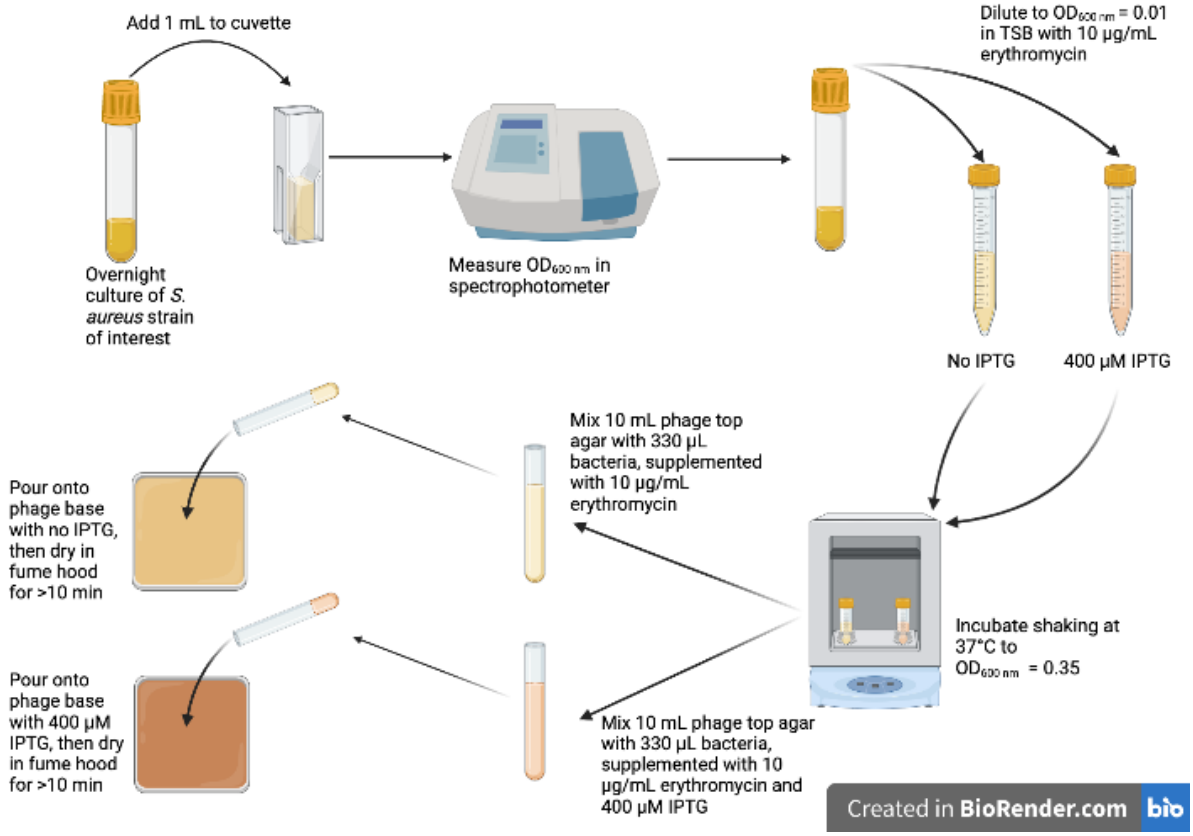


Figure 3.13. An illustration of the production of plates for phage plaque assay. An overnight culture of the strain of interest is diluted to $OD_{600\ nm}$ = 0.01 in TSB+erm10, which is done in two subcultures, one without IPTG and one with 400 µM IPTG. Then, the subcultures are grown to mid-log phage, around $OD_{600\ nm}$ = 0.35. Finally, bacteria and melted PTA is mixed and poured onto PB plates, then dried in a fume hood.

The phage dilutions were spotted onto the dried plates according to the setup presented in Figure 3.12. The phage plaque assay was performed in biological triplicates, and the results are presented in Result section 4.3.

4. Results

In the following section, the results obtained from laboratory experiments performed between January and May 2022 will be presented. This section will be divided into three main parts; firstly, the results from the transformation assays will be shown in Section 4.1, followed by the analyses of antibiotic resistance in Section 4.2, and lastly the studies of bacteriophage susceptibility will be presented in Section 4.3.

4.1 Transformations

According to the transformation protocols presented in Materials and methods Section 3.4, the different combinations of genes and *S. aureus* strains are presented in Table 4.1 below. As can be seen, JE2 as well as the two VISA strains L4 and L9 could be transformed with plasmids cloned with all 7 genes of interest as well as the empty plasmid pSK9067_Ø. These 24 recombinant strains were used for all subsequent experiments regarding antibiotic resistance and bacteriophage susceptibility. Transformation of the VISA strains L3 and L6 with plasmids carrying the genes *walK, walR* and *walH* was not successful. Additionally, transformation of the VISA strain L10 had earlier been unsuccessful as well, therefore it was not considered for transformation in this thesis.

Table 4.1. Results of transforming plasmids in *S. aureus*. The six different *S. aureus* strains JE2, E20-L3, L4, L6, L9 and L10 were transformed with the genes *walK, walR, walH, vraR, vraS, vraT, vraU* and a plasmid without gene insert, called Ø (containing GFP). "√" represents a successful transformation, "**X**" represents an unsuccessful transformation, and "-" means that the transformation was not tried. The plasmid used for the transformations was psK9067.

	S. aureus strain					
Gene	JE2	L3	L4	L6	L9	L10
walK	√	**X**	√	**X**	√	**X**
walR	√	**X**	√	**X**	√	**X**
walH	√	**X**	√	**X**	√	**X**
vraR	√	-	√	-	√	**X**
vraS	√	-	√	-	√	**X**
vraT	√	-	√	-	√	**X**
vraU	√	-	√	-	√	**X**
Ø (GFP)	√	-	√	-	√	**X**

4.2 Antibiotic Resistance

The effect of overexpressing genes from the two- and three component systems WalRK and VraTSR in three genetic backgrounds on two different types of antibiotics; oxacillin (a β-lactam) and vancomycin, was tested. The results are presented in Section 4.2.1 and 4.2.2 respectively.

4.2.1 β-lactam antibiotic susceptibility

Oxacillin was chosen as a representative antibiotic for the β-lactam antibiotics. The oxacillin MIC testing was performed in biological duplicates for all 8 genes in the three genetic backgrounds using broth microdilution.

Broth microdilution oxacillin MIC tests were performed in biological duplicates. The results from the two replicates is shown in Table 4.2 and 4.3 below, the oxacillin MIC is presented in mg/L without IPTG as well as using 400 µM IPTG. The blue color represents an increase in MIC due to overexpression. Overexpression of *vraR* increased the oxacillin MIC fourfold in JE2, while *vraS* overexpression doubled the MIC. In red is shown the genes which had a more than four-fold decreased MIC compared to the control strain JE2 Ø even without IPTG. For example, *vraU* induced a two-fold increase in oxacillin MIC when overexpressed, but still the MIC was two times lower than the control strain JE2 Ø. For the genes having a lower oxacillin MIC even without IPTG from the start, *vraT*, *walK*, *walR* and *walH*, the observed fold change

was not considered completely reliable, which is the reason for not coloring it in blue but in grey instead.

Table 4.2. The effect of overexpressing genes in JE2 on oxacillin susceptibility. *The stronger the blue color – the higher the increase in MIC value due to overexpression. The stronger the red color – the higher the decrease in MIC value due to overexpression. Red numbers symbolize outliers. Grey color depicts values that are neither increased not decreased compared to JE2 Ø.*

	JE2 Ø	*vraR*	*vraS*	*vraT*	*vraU*	*walK*	*walR*	*walH*
No IPTG	16	16	16	8	4	8	8	8
IPTG	16	64	32	16	8	8	16	16
Fold change	-	**4**	2	2	2	-	2	2

Table 4.3. The effect of overexpressing genes in JE2 on oxacillin susceptibility. *The stronger the blue color – the higher the increase in MIC value due to overexpression. The stronger the red color – the higher the decrease in MIC value due to overexpression. Red numbers symbolize outliers. Grey color depicts values that are neither increased not decreased compared to JE2 Ø.*

	JE2 Ø	*vraR*	*vraS*	*vraT*	*vraU*	*walK*	*walR*	*walH*
No IPTG	16	32	16	16	8	8	8	8
IPTG	16	128	32	16	8	8	16	16
Fold change	-	**4**	2	-	-	-	2	2

Subsequently, the broth microdilution MIC test for oxacillin was performed for the VISA strains L4 and L9. The results for L4 are presented in Table 4.4 and 4.5 below for two separate biological replicates. The *walK*, *walR* and *walH* overexpression recombinant strains had lower oxacillin MIC when tested in the absence of IPTG compared to the control strain (L4 Ø). This behavior, observed by a 16 times lower MIC compared to the control strain, was highlighted using a red font.

The *vraR* and *vraT* constructs in L4 showed a reproducible change in regard to oxacillin MIC, where *vraR* overexpression increased the MIC fourfold and *vraT* twofold.

Table 4.4. The first biological replicate showing the effect of overexpressing genes in L4 on oxacillin susceptibility. *The numbers presented in the table are the MIC values in mg/L. 400 µM IPTG was used for overexpression. The blue and red colors represent increased or decreased MIC values respectively. Red numbers symbolize outliers.*

	L4 Ø	*vraR*	*vraS*	*vraT*	*vraU*	*walK*	*walR*	*walH*
No IPTG	32	64	32	32	32	2	2	2
IPTG	32	256	64	64	64	2	4	4
Fold change	-	4	2	2	2	-	2	2

As can be seen in Table 4.5, several of the MIC values are lower without IPTG as compared to L4 Ø, containing the same expression system with GFP as gene insert. These values are marked with red numbers in the table.

Table 4.5. The second biological replicate showing the effect of overexpressing genes in L4 on oxacillin susceptibility. *The numbers presented in the table are the MIC values in mg/L. 400 µM IPTG was used for overexpression. The blue and red colors represent increased or decreased MIC values respectively. Red numbers symbolize outliers.*

	L4 Ø	*vraR*	*vraS*	*vraT*	*vraU*	*walK*	*walR*	*walH*
No IPTG	32	32	8	32	8	2	4	4
IPTG	32	128	32	64	32	2	8	8
Fold change	-	4	4	2	4	-	2	2

MIC values for the eight L9 recombinant strains are presented in Table 4.6 below. The only gene that repeatedly showed an effect on oxacillin MIC during overexpression was *walK*. Overexpressing this gene in L9 caused a twofold decrease in oxacillin MIC.

Table 4.6. The first biological replicate showing the effect of overexpressing genes in L9 on oxacillin susceptibility. *The numbers presented in the table are the MIC values in mg/L. 400 µM IPTG was used for overexpression. The blue and red colors represent increased or decreased MIC values respectively.*

	L9 Ø	*vraR*	*vraS*	*vraT*	*vraU*	*walK*	*walR*	*walH*
No IPTG	1	1	1	2	1	1	1	1
IPTG	1	2	1	2	2	0.5	0.5	0.5
Fold change	1	2	1	1	2	0.5	0.5	0.5

The second biological replicate of the MIC values for the eight L9 recombinant strains are presented in Table 4.7 below.

Table 4.7. The second biological replicate showing the effect of overexpressing genes in L9 on oxacillin susceptibility. *The numbers presented in the table are the MIC values in mg/L. 400 µM IPTG was used for overexpression. The blue and red colors represent increased or decreased MIC values respectively.*

	L9 Ø	*vraR*	*vraS*	*vraT*	*vraU*	*walK*	*walR*	*walH*
No IPTG	1	1	1	1	1	2	4	4
IPTG	1	1	0.5	1	1	1	4	4
Fold change	1	1	0.5	1	1	0.5	1	1

From Table 4.2, 4.3, 4.4 and 4.5, it can be seen that overexpression of *vraR* has the greatest effect on oxacillin MIC compared to the other genes, with a 4-fold increase. To further investigate the effect of *vraR* overexpression in MRSA, OD_{600nm} measurements were performed according to Figure 3.7 in the protocol presented in Section 3.6.2. The strains used were JE2 overexpressing *vraR* as well as JE2 Ø as a negative control.

In Figure 4.1 below, the results from oxacillin MIC from OD_{600nm} measurements are presented. Figure 4.1 **A.** and **B.** are showing the first and second biological replicate respectively. The values presented are depicting the OD values after 24 hours. The stronger the green color, the higher the OD value. The MIC value was determined by as the lowest oxacillin concentration that inhibited growth fully. TSB gives an OD reading of 0.1 as seen in the bottom rows, so the measurements for JE2 showing 0.1 means that the bacteria did not grow. For example, the oxacillin MIC for JE2 *vraR* increased from 4 mg/L to 16 mg/L when IPTG was added. 4 mg/L and 16 mg/L are the lowest concentrations of oxacillin that are completely bactericidal with and without IPTG respectively.

A.

		OXACILLIN (mg/L)									
		Control	0.125	0.25	0.5	1	2	4	8	16	32
JE2 Ø	No IPTG	1.6	1.0	1.1	0.5	0.1	0.1	0.1	0.1	0.1	0.1
JE2 *vraR*	No IPTG	1.4	1.2	1.2	0.5	0.4	0.5	0.1	0.1	0.1	0.1
JE2 Ø	IPTG	1.4	1.1	1.2	0.3	0.2	0.1	0.1	0.1	0.1	0.1
JE2 *vraR*	IPTG	1.6	1.2	1.2	1.3	0.5	0.3	0.3	0.3	0.1	0.1
TSB		0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1

B.

		OXACILLIN (mg/L)									
		Control	0.125	0.25	0.5	1	2	4	8	16	32
JE2 Ø	No IPTG	1.6	0.8	0.9	0.3	0.2	0.2	0.1	0.1	0.1	0.1
JE2 *vraR*	No IPTG	1.7	1.1	1.1	0.8	1.0	0.2	0.1	0.1	0.1	0.1
JE2 Ø	IPTG	1.6	1.0	0.6	0.1	0.1	0.1	0.1	0.1	0.1	0.1
JE2 *vraR*	IPTG	1.6	1.2	1.2	1.3	1.1	1.1	0.7	0.6	0.1	0.1
TSB		0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1

Figure 4.1. Results from oxacillin MIC experiment in Bioscreen OD reader. The values represent the OD measurements generated after 24 hours. The stronger the green color, the higher the OD value. JE2 Ø and JE2 *vraR* was analyzed with 400 µM IPTG and without IPTG. TSB was added as a negative control, and one column depicted "Control" is showing the OD values for the wells where only bacteria were added as a positive control for growth. **A.** depicts the results from the first biological replicate, while **B.** symbolizes the second biological replicate.

Growth curves were performed in a Bioscreen C OD_{600nm} reader to check whether overexpression with IPTG affected the growth of these strains. In Figure 4.2, the growth curves for an oxacillin MIC experiment using JE2 Ø, the negative control, are presented. The blue curves show the growth curves where no oxacillin is added and could be used as a reference to check whether IPTG affects growth or not.

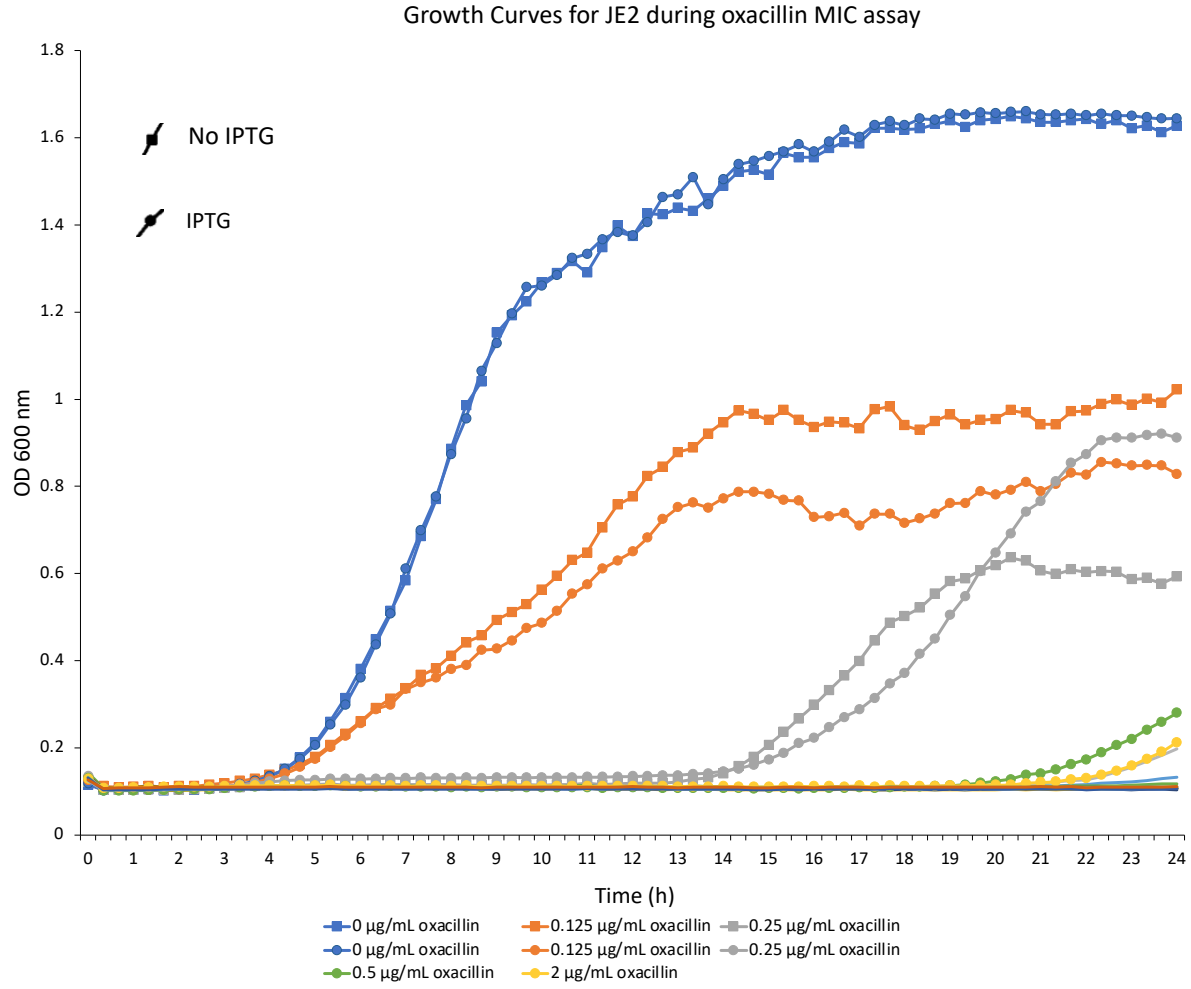


Figure 4.2. Comparison of the growth curves of the strain JE2 Ø during oxacillin MIC experiments. The comparison was made to see if IPTG had any effect on the growth of the bacteria. The blue graphs represent 0 µg/mL oxacillin, the orange graphs 0.125 µg/mL and the grey graphs symbolizes 0.25 µg/mL. Squared lines

represent growth curves of JE2 Ø that has been growing without IPTG, while circled lines represent growth curves of JE2 Ø that has been growing with IPTG.

Additionally, growth curves were obtained for all the experiments performed using the Bioscreen C, for example the results presented in Figure 4.1 **A.** and **B.** These growth curves showed that IPTG did not impair the growth of either JE2 Ø nor JE2 overexpressing *vraR*. Furthermore, in addition to increasing the final OD_{600nm}, *vraR* overexpression in JE2 improved the overall growth compared to JE2 Ø. These growth curves for all the oxacillin MIC experiments are presented in Appendix A, Figure A1 A. and B. for JE2 Ø and Figure A2 A. and B. for JE2 *vraR*.

In Materials and methods Section 3.1.2, the choice of expression system and thereby inducer was discussed. In Figure 4.3 **A.** and **B.**, the growth curves from two AHT toxicity experiments using JE2 Ø are presented.

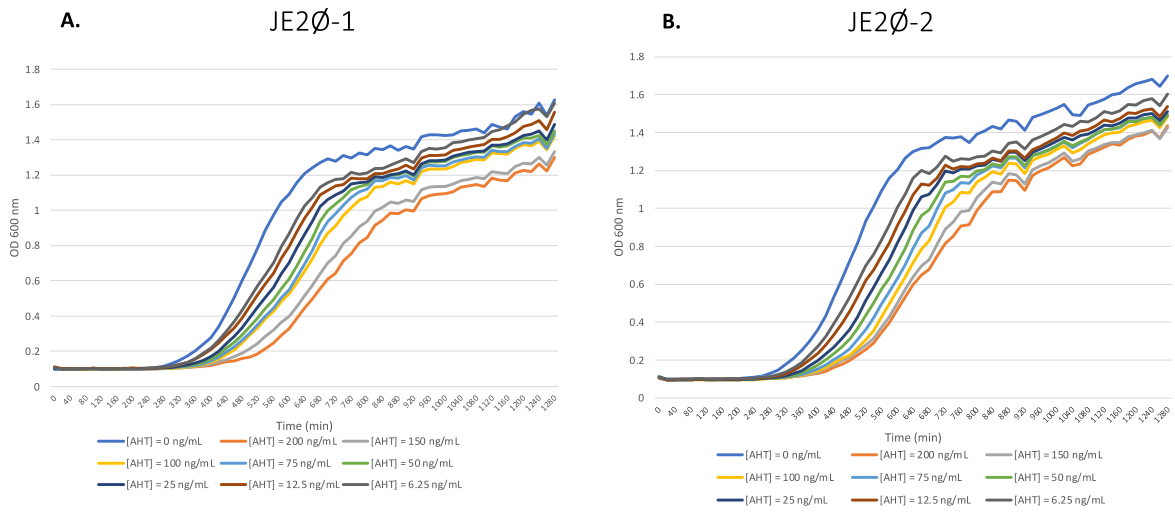


Figure 4.3. Growth curves of JE2 Ø exposed to different AHT concentrations. A. The first biological replicate. B. The second biological replicate. On the x-axis the time is shown in minutes, while the y-axis shows the OD 600 nm measurements.

From Figure 4.3 **A.**, however the two highest concentrations of AHT, 150 ng/mL and 200 ng/mL caused a significant decrease in OD_{600nm} after 24 hours. Therefore, an AHT concentration between 25-100 ng/mL was decided to be an optimal inducer concentration, decreasing the impaired growth while still having sufficient induction of the promoters.

Furthermore, the same analysis was made for JE2 overexpressing *vraR*, and these growth curves are presented in Figure 4.4 **A.** and **B.**

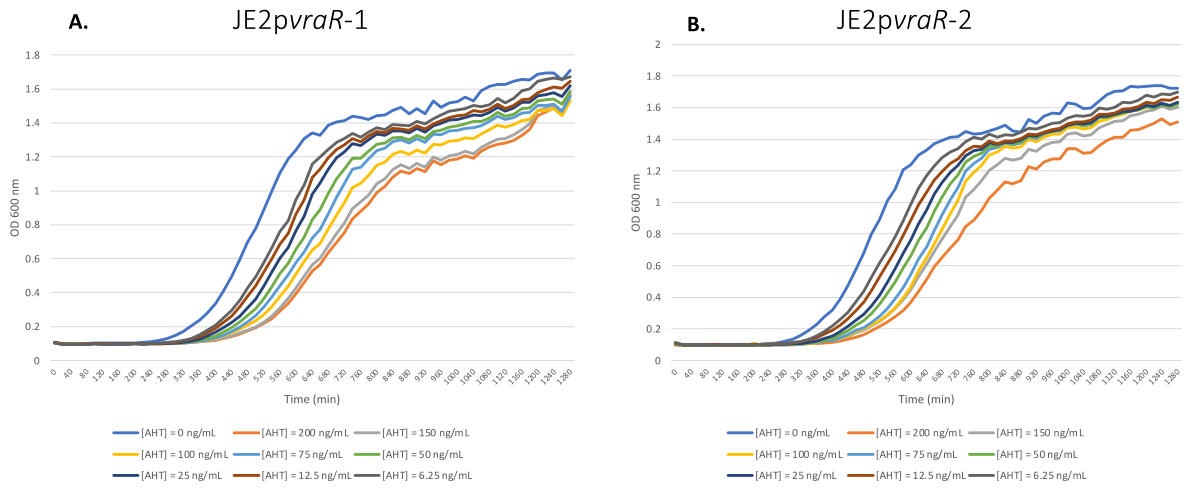


Figure 4.4. Growth curves of JE2 *vraR* exposed to different AHT concentrations. A. The first biological replicate. B. The second biological replicate. On the x-axis the time is shown in minutes, while the y-axis shows the OD 600 nm measurements.

The same pattern as for JE2 Ø, presented in Figure 4.3 **A.** and **B.** could be observed for JE2 overexpressing *vraR*. The higher the AHT concentrations, the lower the OD during exponential phage, indicating impaired growth due to AHT. Therefore, it was determined that the earlier concentration range, 25, 50 and 100 ng/mL could be suitable for testing the effect of overexpression of *vraR* on oxacillin resistance.

However, performing these analyses according to the protocol presented in Materials and methods Section 3.6.2, adding oxacillin and AHT together affected growth, no matter what concentration or strain (JE2 *vraR* or the control strain JE2 Ø). The MIC values from these analyses are presented in Appendix B, as maximum OD values after 24 hours in Table B1, B2, B3 and B3, as well as the growth curves corresponding to these analyses in Figure C1 A. and B. and Figure C2 A. and B in Appendix C.

4.2.2 Vancomycin susceptibility

To assess the importance of the two- and three component systems WalRK and VraTSR on vancomycin susceptibility, MIC tests using broth microdilution in 96 well plates were performed. Firstly, the vancomycin MIC for the 7 JE2 recombinant strains as well as the control

JE2 Ø was analyzed, and the results are presented in Figure 4.5. In this graph, the MIC values in mg/L are shown on the y-axis, while the different genes are presented on the x-axis.
The results without and with 400 µM IPTG are shown in light grey and dark grey respectively. It is recommended to perform these analyses several more times, preferably confirming them with methods such as *E-tests* as well (CLSI, 2019). For example, further analyses could hopefully explain the decreases in vancomycin MIC even without IPTG observed for some of the genes in Figure 4.5 below.

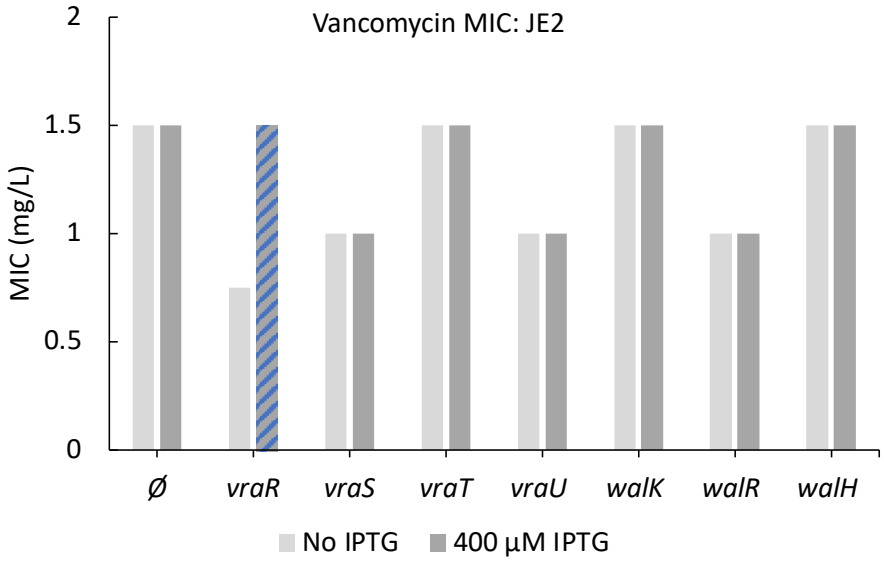


Figure 4.5. Results from vancomycin MIC analysis of JE2. In light grey are the MIC values in mg/L without IPTG, and in dark grey are the MIC values when 400 µM IPTG was added. Genes that caused a change in MIC are shown with a striped bar, blue indicating increased resistance and red increased susceptibility. The stronger the red or blue color, the bigger the change in vancomycin increments.

Overexpression of *vraR* led to a twofold increase in vancomycin MIC (Figure 4.5). Induction of the other constructs had no effect on vancomycin MIC. To further analyse the reproducibility of these results, further analyses are recommended.

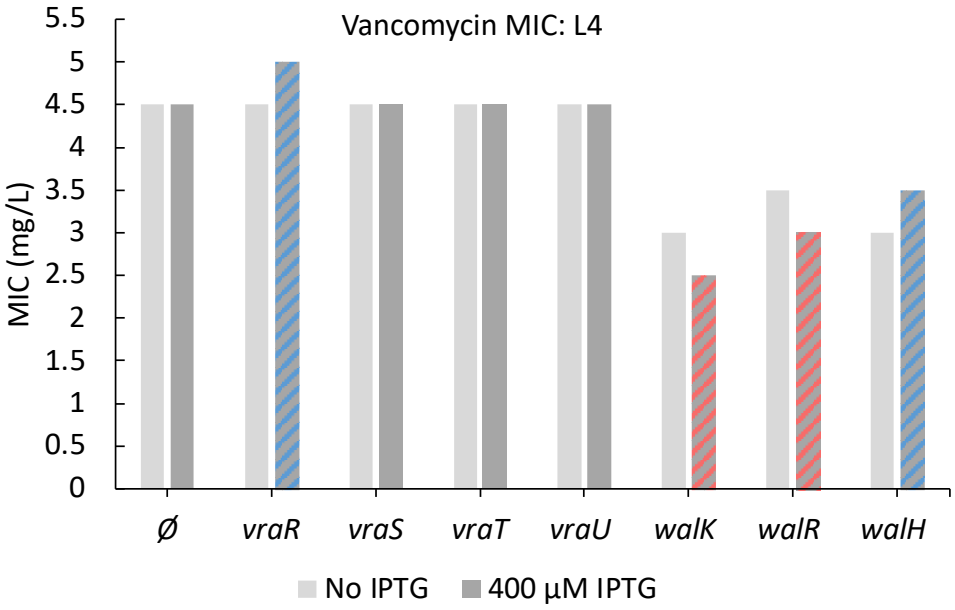


Figure 4.6. Vancomycin MIC for L4. The bar chart shows the vancomycin MIC in mg/L for the 7 gene inserts *vraR*, *vraS*, *vraT*, *vraU*, *walK*, *walR* and *walH* as well as the control Ø.

In L4, and compared to JE2, overexpression of *vraR* only had a minor effect on vancomycin MIC with an increase from 4.5 mg/L to 5 mg/L (Figure 4.6). As in JE2, overexpression of *vraS*, *vraT* and *vraU* did not affect vancomycin MIC. However, without induction, the MICs for *walK*, *walR* and *walH* constructs was decreased, suggesting the plasmids impair the vancomycin susceptibility or overall fitness of the strains. Induction of these systems lead to minor changes in vancomycin MIC. To confirm these results, further analyses are recommended.

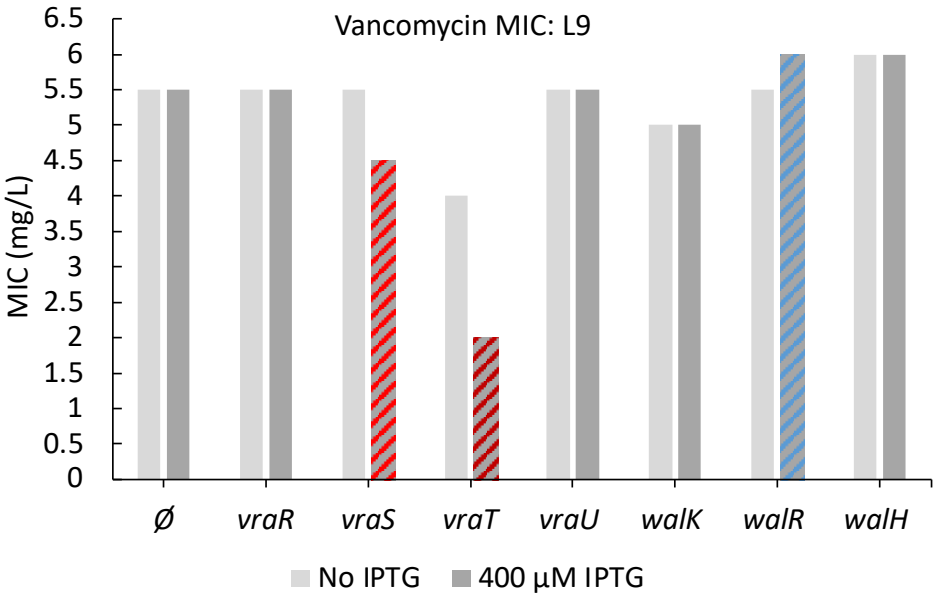


Figure 4.7. Vancomycin MIC for L9. Shown in light grey are the MIC values without IPTG while dark grey symbolizes the results with 400 µM IPTG added during the experiment. The red striped bars show the genes that has a lower MIC value compared to without IPTG, and the darker the red the bigger the change in MIC. In blue on the other hand are the genes that lead to an increase in vancomycin MIC.

Strikingly, and contrary to JE2 and L4, overexpression of *vraR* in L9 did not affect vancomycin MIC (Figure 4.7). However, overexpression of *vraS* and *vraT* decreased vancomycin MIC from 5.5mg/L to 4.5mg/L and 4mg/L to 2mg/L respectively. No changes were observed during overexpression of the other constructs, except for a slight increase in vancomycin MIC when overexpressing *walR*.

To verify the effect of overexpressing *vraR* on vancomycin MIC in MRSA, Bioscreen C runs were performed in biological duplicates. The result is presented in Table 4.8. From this table, it can be seen that *vraR* overexpression increased the vancomycin MIC slightly, from 1.5 mg/L to 2 mg/L, looking at the $OD_{600\ nm}$ after 24 hours for JE2 *vraR* with and without IPTG.

Table 4.8. Results from vancomycin MIC experiment in Bioscreen C OD reader. *The values represent the OD measurements generated after 24 hours. The stronger the blue color, the higher the OD value. JE2 Ø and JE2 vraR was analyzed with 400 µM IPTG and without IPTG. TSB was added as a negative control, and one column depicted "Control" is showing the OD values for wells where bacteria were added as a growth control.*

		VANCOMYCIN (mg/L)									
		Control	0.5	0.75	1	1.5	2	2.5	3	3.5	4
Ø	No IPTG	1.7	1.6	1.6	0.8	0.1	0.1	0.1	0.1	0.1	0.1
vraR		1.7	1.7	1.6	0.8	0.1	0.1	0.1	0.1	0.1	0.1
Ø	IPTG	1.7	1.7	1.6	0.4	0.1	0.1	0.1	0.1	0.1	0.1
vraR		1.6	1.6	1.6	1.6	1.4	0.1	0.1	0.1	0.1	0.1
TSB		0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1

To check whether the growth of JE2 overexpressing *vraR* was impacted even though the final OD value was not increased by more than 0.5 mg/L, growth curves were plotted from the $OD_{600\ nm}$ values generated from the Bioscreen C run. Growth curves are presented in Figure 4.8 below. Eight out of twenty growth curves are shown in Figure 4.8. For the full raw data with all the growth curves, see Figure D1 in Appendix D.

What can be seen in Figure 4.8 is that the growth curves are similar for JE2 *vraR* with and without IPTG and in the absence of vancomycin. However, growth is impaired when overexpressing *vraR* in the presence of 0.75 mg/L vancomycin, compared to the non-induced strain. The difference is even larger when comparing the growth curves with and without IPTG at 1 mg/L vancomycin, where overexpression of *vraR* led to a steeper growth curve although they both reach approximately the same OD after 24 hours. Finally, there is growth at 1.5 mg/L when overexpressing *vraR* but when IPTG is not added, there is no measurable growth. The most obvious observation in Figure 4.8, is the higher the vancomycin concentration, the bigger the lag phase. As an example, being exposed to 1 mg/L vancomycin, it takes almost 10 hours for JE2 *vraR* to start growing with 0 µM IPTG.

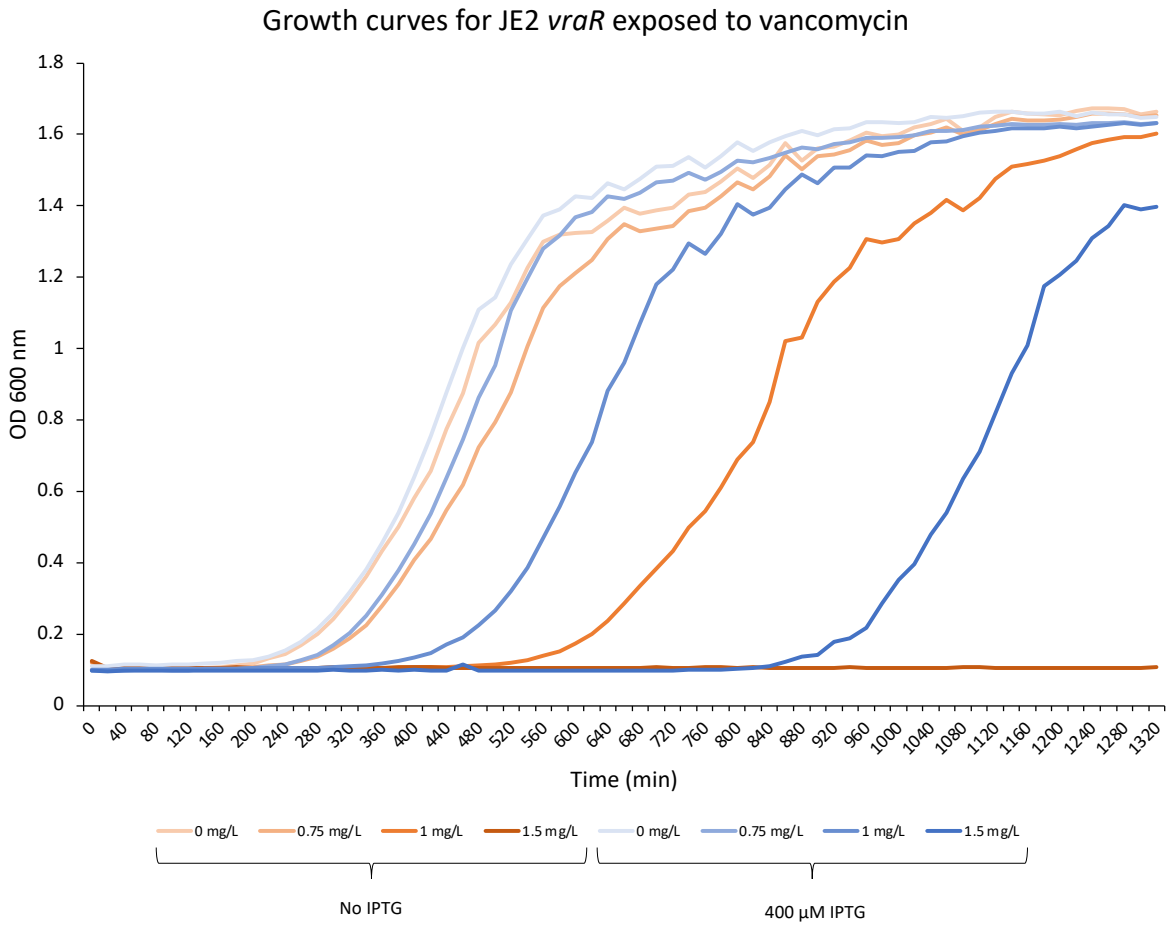


Figure 4.8. Growth curves for JE2 *vraR* with and without IPTG. On the y-axis is the OD values at 600 nm wavelength. The x-axis shows the time in minutes, the experiment was run for 24 hours. In orange are the growth curves for JE2 *vraR* that has grown without IPTG in the growth media. In blue are the growth curves for JE2 *vraR* where the media was supplemented with 400 µM IPTG.

4.3 Bacteriophage Susceptibility

The four phages described earlier, namely the three lytic phages RODI, Stab20 and Stab21 as well as the lysogenic phage φ11, were spotted onto bacterial lawns. The strains analysed were the 24 overexpressing recombinant strains, 7 in each genetic background (MRSA strain JE2 and VISA strains L4 and L9) as well as one control for each genetic background. Plaque forming units were counted for each strain without induction, indicating the initial susceptibility of the strains to the phages. These results were compared to PFU counts during induction with IPTG, to study the effect of gene overexpression of the two- and three component systems *walRK* and *vraTSR* on resistance to phage infection.

4.3.1 Overview of phage plaque assay results

In the following section, three tables will be presented. In these tables, the results from all 144 plates, performed in biological triplicates, will be presented. The phage plaque assays were performed according to the method described in Section 3.7.5. Table 4.9 shows the results for JE2 phage susceptibility, while Table 4.10 and 4.11 show the results for the VISA strains L4 and L9 respectively. These tables are meant to function as an overview and comparison between genes and to the wild type MRSA strain JE2. For a more detailed graphical illustration of the effect of overexpressing each gene on the number of plaques formed, see Section 4.3.2.

Table 4.9. A compilation of the results from the phage plaque assay of the S. aureus strain JE2. *The three lytic phages RODI, Stab20 and Stab21 as well as the lysogenic phage φ11 were analysed in biological triplicates, indicated by the three rows for each phage. The numbers in the table indicate the highest dilution where plaques can be visually detected, for example -5 depicts a dilution of 10^5. The leftmost column in grey is showing the susceptibility of wild type JE2 to the four phages. Each of the 7 overexpression recombinant strains as well as the control strain JE2 Ø were tested with and without 400 µM IPTG. Red colour depicts a statistically verified increased phage infection in the sense that a higher dilution induces plaques. Likewise, blue colour shows the opposite, where a lower dilution was able to induce plaques. The stronger the red or blue colour, the higher the fold change of phage dilution that induced plaques. White colour depicts results that was not statistically verified.*

	JE2	JE2 Ø	JE2 Ø IPTG	vraR	vraR IPTG	vraS	vraS IPTG	vraT	vraT IPTG	vraU	vraU IPTG	walK	walK IPTG	walR	walR IPTG	walH	walH IPTG
RODI	-5	-5	-5	-4	-5	-5	-6	-4	-3	-5	-4	-4	-5	-5	-6	-4	-2
RODI	-5	-5	-5	-5	-6	-5	-6	-5	-4	-6	-5	-5	-6	-5	-6	-5	-4
RODI	-5	-5	-5	-5	-6	-5	-6	-5	-4	-6	-5	-5	-5	-5	-6	-5	-4
Stab20	0	0	0	0	0	0	0	0	0	0	0	0	-1	0	-1	0	0
Stab20	0	0	0	0	-1	0	0	0	0	0	0	0	-1	0	-1	0	0
Stab20	0	0	0	0	0	0	-1	0	0	0	0	0	-1	0	-1	0	0
Stab21	-5	-5	-5	-4	-5	-6	-6	-5	-3	-5	-3	-4	-5	-5	-6	-5	-3
Stab21	-5	-5	-5	-5	-4	-5	-5	-6	-4	-6	-4	-4	-5	-4	-5	-5	-4
Stab21	-5	-5	-5	-5	-4	-5	-5	-6	-4	-6	-4	-5	-5	-4	-5	-5	-4
ϕ11	-7	-7	-7	-6	-7	-6	-7	-7	-7	-7	-6	-7	-7	-7	-7	-7	-6
ϕ11	-7	-7	-7	-6	-7	-7	-7	-7	-7	-6	-7	-7	-7	-7	-7	-7	-6
ϕ11	-7	-7	-7	-7	-7	-7	-7	-7	-7	-6	-7	-7	-7	-7	-7	-7	-6

Table 4.10. A compilation of the results from the phage plaque assay of the VISA strain L4. *The three lytic phages RODI, Stab20 and Stab21 as well as the lysogenic phage φ11 were analysed in biological triplicates, indicated by the three rows for each phage. The numbers in the table indicate the highest dilution where plaques can be visually detected, for example -5 depicts a dilution of 10^5. The leftmost column in grey is showing the susceptibility of wild type JE2 to the four phages. Each of the 7 overexpression recombinant strains as well as the control strain L4 Ø were tested with and without 400 µM IPTG. Red colour depicts a statistically verified increased phage infection in the sense that a higher dilution induces plaques. Likewise, blue colour shows the opposite, where a lower dilution was able to induce plaques. The stronger the red or blue colour, the higher the fold change of phage dilution that induced plaques. White colour depicts results that was not statistically verified.*

	JE2	L4 Ø	L4 Ø IPTG	vraR	vraR IPTG	vraS	vraS IPTG	vraT	vraT IPTG	vraU	vraU IPTG	walK	walK IPTG	walR	walR IPTG	walH	walH IPTG
RODI	-5	-3	-3	-3	-4	-4	-3	-3	-4	-3	-5	-4	-2	-4	-3	-4	-3
RODI	-5	-4	-4	-3	-4	-4	-3	-3	-5	-3	-5	-5	-4	-5	-4	-5	-4
RODI	-5	-4	-4	-4	-5	-5	-4	-3	-5	-4	-5	-5	-4	-4	-4	-5	-4
Stab20	0	-2	-2	-1	-2	-3	-2	-1	-2	-1	-3	-4	-3	-4	-3	-4	-3
Stab20	0	-1	-1	-1	-2	-3	-2	-2	-3	0	-3	-4	-5	-5	-4	-5	-4
Stab20	0	-1	-1	-2	-3	-3	-2	-2	-3	-1	-3	-4	-5	-5	-4	-5	-4
Stab21	-5	-3	-3	-2	-3	-4	-3	-3	-4	-2	-5	-4	-3	-4	-3	-4	-3
Stab21	-5	-3	-3	-3	-4	-4	-3	-3	-4	-2	-5	-5	-4	-4	-5	-5	-4
Stab21	-5	-3	-3	-3	-4	-4	-3	-3	-4	-4	-4	-5	-4	-4	-5	-5	-4
ϕ11	-7	-6	-6	-2	-3	-6	-4	-5	-6	-3	-6	-6	-5	-7	-6	-7	-6
ϕ11	-7	-6	-6	-5	-4	-6	-4	-5	-6	-3	-6	-7	-6	-7	-7	-7	-6
ϕ11	-7	-6	-6	-5	-6	-7	-5	-6	-7	-5	-7	-7	-6	-7	-7	-7	-7

Table 4.11. A compilation of the results from the phage plaque assay of the VISA strain L9. *The three lytic phages RODI, Stab20 and Stab21 as well as the lysogenic phage φ11 were analysed in biological triplicates, indicated by the three rows for each phage. The numbers in the table indicate the highest dilution where plaques can be visually detected, for example -5 depicts a dilution of 10^5. The leftmost column in grey is showing the susceptibility of wild type JE2 to the four phages. Each of the 7 overexpression recombinant strains as well as the control strain L9 Ø were tested with and without 400 µM IPTG. Red colour depicts a statistically verified increased phage infection in the sense that a higher dilution induces plaques. Likewise, blue colour shows the opposite, where a lower dilution was able to induce plaques. The stronger the red or blue colour, the higher the fold change of phage dilution that induced plaques. White colour depicts results that was not statistically verified.*

	JE2	L9 Ø	L9 Ø IPTG	vraR	vraR IPTG	vraS	vraS IPTG	vraT	vraT IPTG	vraU	vraU IPTG	walK	walK IPTG	walR	walR IPTG	walH	walH IPTG
RODI	-5	-2	-2	-4	-4	-3	-2	-2	-5	-4	-5	-3	-4	-2	-4	-3	-5
RODI	-5	-3	-3	-4	-3	-3	-2	-3	-5	-3	-5	-5	-4	-4	-5	-3	-4
RODI	-5	-2	-2	-4	-4	-3	-2	-3	-5	-4	-5	-4	-4	-3	-5	-3	-5
Stab20	0	0	0	0	-1	-2	-1	0	0	0	0	0	0	0	0	0	-1
Stab20	0	0	0	0	0	-2	-1	0	0	0	0	0	0	0	0	0	-1
Stab20	0	0	0	0	-1	-2	-1	0	0	0	0	0	0	0	0	0	-1
Stab21	-5	-3	-3	-4	-5	-3	-2	-2	-5	-3	-4	-3	-4	-2	-3	-4	-5
Stab21	-5	-4	-4	-3	-4	-3	-2	-3	-5	-3	-4	-3	-4	-4	-5	-3	-4
Stab21	-5	-3	-3	-4	-4	-3	-2	-3	-5	-3	-4	-3	-4	-3	-4	-4	-5
ϕ11	-7	-6	-6	-6	-6	-7	-5	-5	-7	-6	-7	-6	-7	-6	-7	-6	-7
ϕ11	-7	-7	-7	-7	-6	-7	-5	-5	-7	-6	-7	-7	-6	-7	-6	-6	-7
ϕ11	-7	-6	-6	-6	-6	-7	-5	-6	-7	-6	-7	-6	-7	-7	-7	-6	-7

4.3.2 Effect of overexpressing single genes on phage susceptibility

The bar charts below show the effect of overexpressing the 7 individual genes from the two- and three component systems WalRK and VraTSR on phage infection in the 3 different genetic backgrounds. As an example, the *vraT* overexpression is visualized in Figure 4.9. On the x-axis are the four different phages RODI, Stab20, Stab21 and φ11, for the three *S. aureus* strains JE2, L4 and L9. On the y-axis is the fold change of the number of plaque-forming units that overexpression of each individual gene produced. For example, in Figure 4.9, overexpressing *vraT* in JE2 decreased the number of PFUs for RODI by 10 times, Stab21 PFU counts were decreased 100 times, while no effect in infective ability was observed for the three other phages. This is also visualized by a number on each of the bars, where 0 shows the absence of effect on phage infectivity during induction by IPTG, 10 depicts a tenfold increase, and -10 (negative) depicts a tenfold decrease in the number of PFUs counted during induction compared to non-induced conditions. Induction of *vraT* expression caused a tenfold increase in PFU counts for all four phages in L4. Strikingly, *vraT* overexpression decreased the number of Stab21 PFU by 100-fold in JE2, while it increased the number of RODI and Stab21 PFU by a 100-fold in L9.

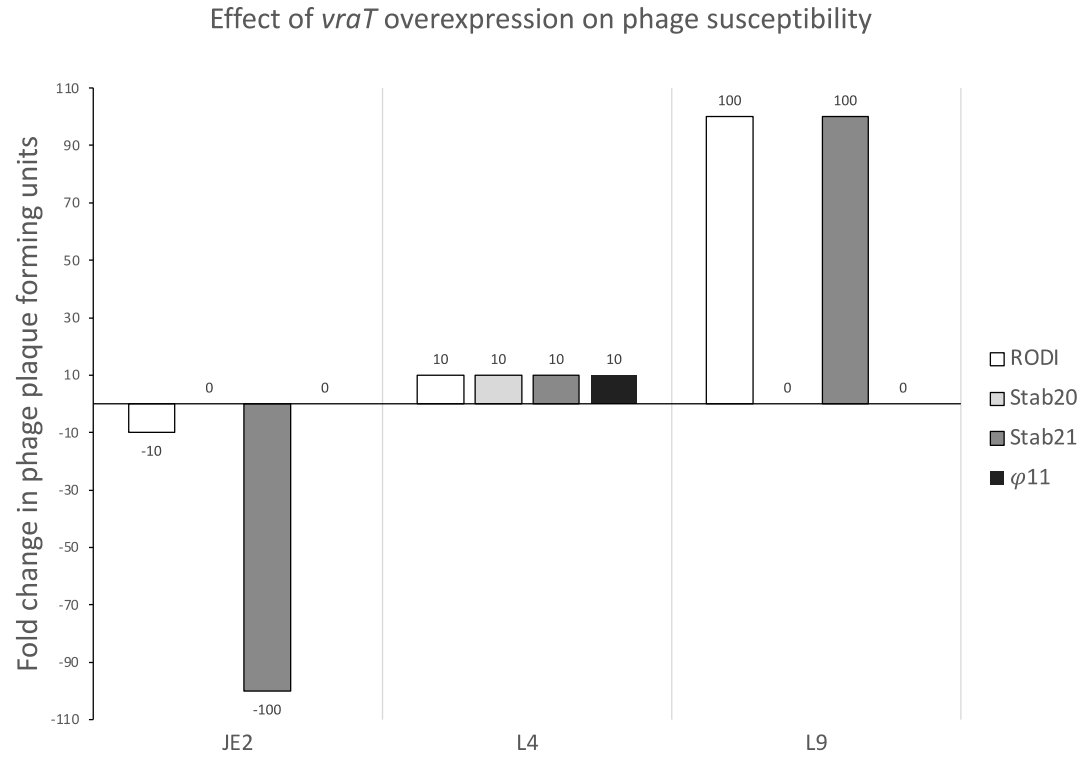


Figure 4.9. Results from phage plaque assay when overexpressing the gene *vraT*. Bar charts showing the fold change in the number of plaque forming units during overexpressing. The magnitude represents the increase (positive values) or decrease (negative values) in the number of PFUs when adding 400 µM during the phage plaque assay.

In Figure 4.10 below, in the left figure is shown the effect of *vraR* overexpression on phage susceptibility. There were only small increases in PFU counts when overexpressing *vraR*, affecting only RODI in JE2 but all phages in L4. To the right is the effect of overexpressing *vraS*. The biggest effect was with φ11, where there was a 100-fold decrease in PFUs in L4 and L9 compared to the control, while no effect was seen for JE2.

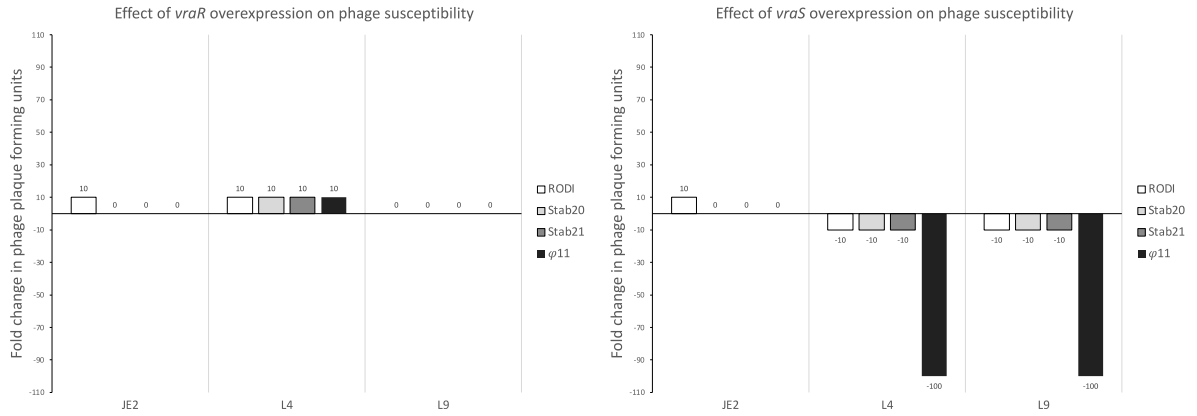


Figure 4.10. Results from phage plaque assay. Bar charts showing the fold change in the number of plaque forming units when overexpressing a gene. The magnitude represents the increase (positive values) or decrease (negative values) in the number of PFUs when adding 400 µM during the phage plaque assay. **Left:** Effect of *vraR* overexpression. **Right:** Effect of *vraS* overexpression.

In the left part of Figure 4.11, PFU counts for all phages were 100 times higher in L4 when *vraU* is overexpressed. On the other hand, RODI and Stab21 formed 10-fold and 100-fold less PFUs in JE2 during *vraU* overexpression. In the right part of Figure 4.11, during *walR* overexpression in L9, infection by RODI was increased by 100-fold. On the other hand, phage susceptibility in L4 was not affected at all, and only slight increases in PFU counts were seen for JE2.

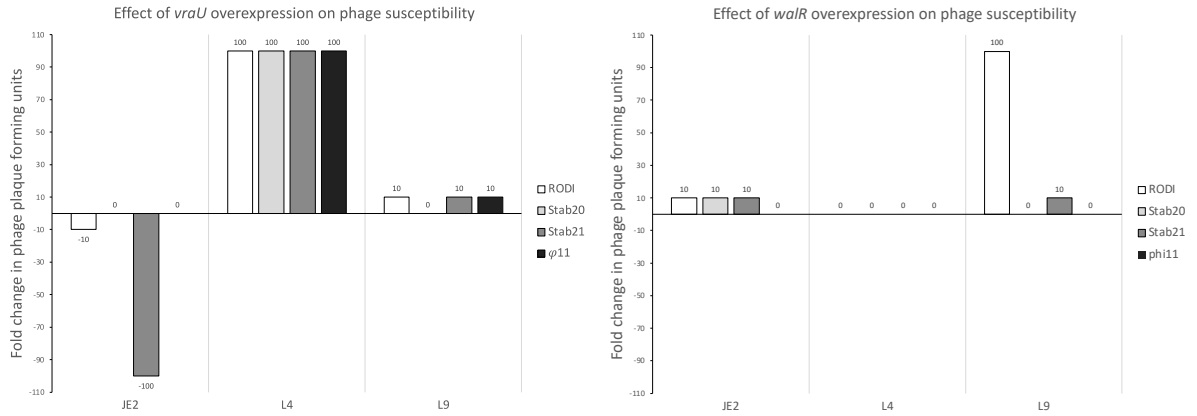


Figure 4.11. Results from phage plaque assay. Bar charts showing the fold change in the number of plaque forming units when overexpressing a gene. The magnitude represents the increase (positive values) or decrease (negative values) in the number of PFUs when adding 400 µM during the phage plaque assay. **Left:** Effect of *vraU* overexpression. **Right:** Effect of *walR* overexpression.

Lastly, *walK* and *walH* overexpression results are shown in the left and right part of Figure 4.12 respectively. Overexpression of *walK* only affected infection by Stab20 in JE2 and by Stab21 in L9, with a 10-fold increase in PFU. On the other hand, *walH* overexpression had the same effect on all four phages in JE2 and L9; however, the number of PFUs decreased by tenfold in the former and increased by tenfold in the latter.

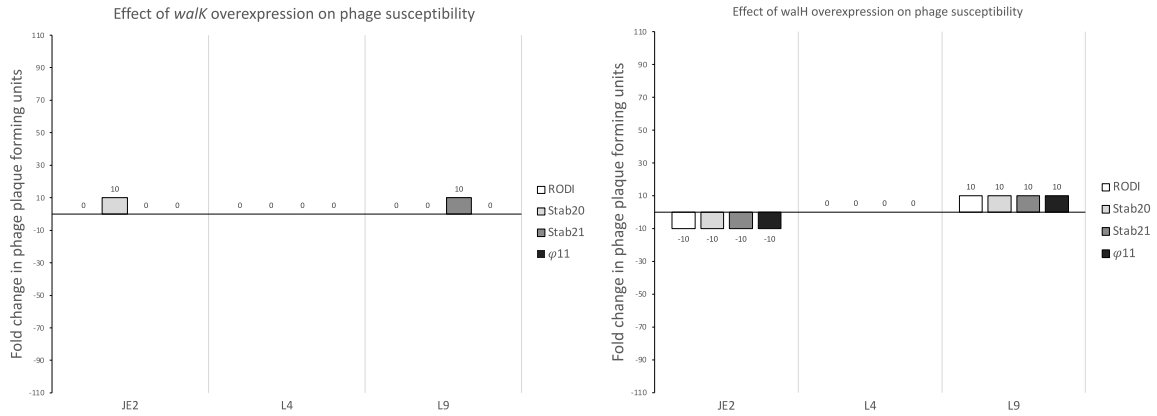
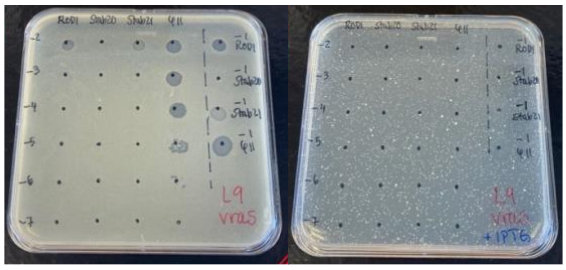


Figure 4.12. Results from phage plaque assay. Bar charts showing the fold change in the number of plaque forming units when overexpressing a gene. The magnitude represents the increase (positive values) or decrease (negative values) in the number of PFUs when adding 400 µM during the phage plaque assay. **Left:** Effect of *walK* overexpression. **Right:** Effect of *walH* overexpression.

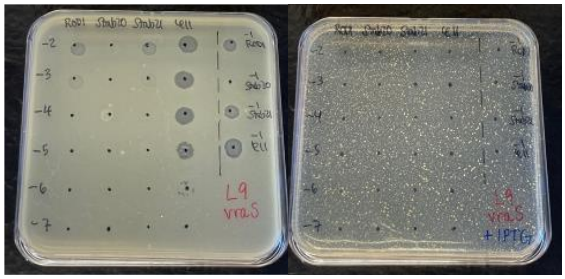
4.3.3 Effect of the gene vraS on S. aureus growth

During the analysis of the plates from the phage plaque assay, it was noticed that when inducing the expression of the gene *vraS* using 400 µM IPTG in the *S. aureus* strain L9, the growth was severely impaired. This can be seen in Figure 4.13 below. The left plates show the uninduced strains while the right plates show the induced. Figure 4.13 **A.**, **B.** and **C.** show the three biological replicates respectively. As can be seen from the plates on the right-hand side of the figure is that the growth is not uniform and does not create a whole bacterial lane as compared to the uninduced L9 *vraS.* It is therefore very difficult to distinguish at what dilutions phage plaques are formed.

A.



B.



C.

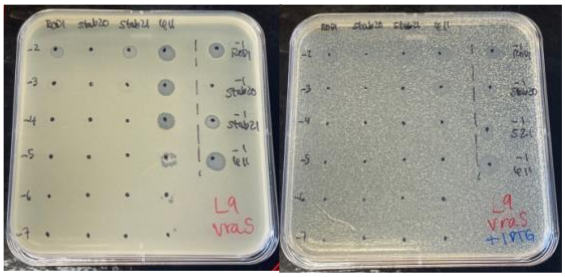


Figure 4.13. Plates from the phage plaque assay analysing the gene *vraS* in the VISA strain L9. The four phages RODI, Stab20, Stab21 and φ11 were analysed in dilutions ranging from 10^{-1} to 10^{-7}. **A.** Biological replicate one. Left plate: No IPTG added. Right plate: 400 µM IPTG. **B.** Biological replicate two. Left plate: No IPTG added. Right plate: 400 µM IPTG. **C.** Biological replicate three. Left plate: No IPTG added. Right plate: 400 µM IPTG.

Due to the results from overexpressing *vraS* in L9, it was decided to culture all the 24 overexpressing recombinant strains with and without IPTG in the Bioscreen C for 24 hours. From the OD measurements, growth curves were made. These are presented in Figure E3 in Appendix E. The growth curve for L9 *vraS* showed gravely impaired growth for L9 *vraS* with IPTG, which was expected from looking at the plates from the phage plaque assay.

During the analysis of the growth curves, it was discovered that the growth curves for the recombinant L4 *walK*, *walR* and *walH* did not correlate with the other growth curves, making it hard to compare with the control L4 Ø. The effect of this discovery will be further assessed in the Discussion section below.

5. Discussion

To discuss and analyze the effect of the separate genes from the two- and three-component systems, it is important to first get an overview of what the results are really showing, and what can be concluded from them. Then, it is possible to dig deeper into the possible explanations and molecular events that may affect the antibiotic resistance and bacteriophage susceptibility.

Considering the results presented in Section 4, it is obvious that the regulatory two- and three-component system WalRK and VraTSR are of importance in the resistance towards vancomycin and oxacillin and the susceptibility to bacteriophages. Even though the mechanism behind the resistance towards antibiotics and phages may differ, some genes seem to affect *Staphylococcus aureus* in a way that changes the susceptibility towards them both.

5.1 Transformations

5.1.1 Transformation with the plasmid pSK-9067

The VISA strains L4 and L9, as well as the MRSA strain JE2 were all successfully transformed with plasmids containing the genes *vraR, vraS, vraT, vraU, walK, walR* and *walH,* as well as the plasmid containing GFP as a gene insert, '*Ø*', used as a control strain. This can be observed in Table 4.1.

Why were L4 and L9 chosen as representative strains? Firstly, these strains are vancomycin-adapted derivatives of JE2. They adapted to vancomycin via two opposite pathways: increased oxacillin MIC in L4 (256mg/L) and decreased oxacillin MIC in L9 (0.5 mg/L) (Table 2.1), while the oxacillin MIC for JE2 is 24 mg/L. The reason why the MIC results presented in this thesis are slightly different compared to those shown in Table 2.1, adapted from (Fait, 2021), could be that the methods used for determining the MIC differ. In Table 2.1, the results are based primarily on *E-tests* (CLSI, 2019), a method able to detect even small changes in the resistance to antibiotics. Broth microdilution is based on growing the bacteria in liquid medium, and after 24 hours visually inspecting for growth, making it subject to interpretation of the results. Additionally, bacteria can behave differently on solid medium and in liquid medium, which has been shown by comparing *E-tests* with broth microdilution analyzing antibiotic susceptibility in clinical isolates of *S. aureus* (Sader, Rhomberg, & Jones, 2009).

However, Table 2.1 still show the distinct pathways of vancomycin adaptations in the two VISA strains, and the results from this thesis work highlight fold changes – which are the interesting effect, rather than obtaining the exact same MIC values as before. It is kept in mind that different mediums and different growth conditions (e.g., solid/liquid) can affect the exact MIC values.

Next, L4 and L9 were deemed good representatives for each pathway, with seemingly different genetic routes of adaptation, since their mutations are different, leading to different phenotypes, although still very closely related with terms of genetic background. Additionally, we have compared the effect of overexpression VraTSR and WalRK in JE2 with L4 and L9. Since the VISA strains have adapted to vancomycin by acquiring a set of mutations, probably rewiring the strains, and especially via these systems. Then we can hypothesize that overexpression of VraTSR and WalRK would lead to different effects at the phenotypic level. This could be increasing an effect that is already present in JE2, or even giving a different effect. The fact that the effect of these overexpressions could be modulated by mutations in L4 and L9 would highlight the role of epistasis in this study, which will be discussed later.

5.1.2 Effect of plasmid transformation on growth S. aureus strains

Transforming JE2, and the two VISA strains L4 and L9 with the 8 constructed plasmids containing the genes of interest in most cases did not seem to affect the growth of the strains. However, as described in Section 4.3.3, overexpressing *vraS* using IPTG severely impaired the growth of L9, which can also be observed as a decrease in doubling time in Figure E3 in Appendix E. However, it is not completely clear whether it is the gene *vraS* that has this effect on the growth L9, and therefore, a further discussion about this particular gene will take place in Section 5.4.

The growth of the overexpressing strains L4 *walK*, L4 *walR* and L4 *walH* seemed to be affected by the plasmid pSK-9067 even though no IPTG was added (Figure E2 in Appendix E). Additionally, these results were also reflected when looking at Table 4.4 and 4.5, where the oxacillin MIC was 8-16 times lower from the start compared to the control strain L4 Ø. Furthermore, this phenomenon was observed when performing broth microdilution to test the vancomycin MIC of L4. In Figure 4.6, the vancomycin MIC was 2-3 increments lower in the presence of vancomycin even without IPTG compared to the control strain L4 Ø.

Taken together, it would be difficult to compare the results from the strains L4 *walK*, L4 *walR*, L4 *walH* and L9 *vraS* with previous results and results from the studies presented in this thesis, since it cannot be concluded whether the effects seen are due to plasmid burden, leaky expression from the plasmids affecting the growth of the strains, or something else. The differences are much bigger for these genes, for example L4 *walK* had a 16 times lower oxacillin MIC compared to L4 Ø, while the biggest difference in JE2 was for JE2 *walK* with a two-fold decrease of oxacillin MIC without IPTG compared to the control strain JE2 Ø.

Therefore, regards the remarkably lowered MIC values in some of the L4 strains will be considered when analyzing the results, and less credibility will be given to the results of the recombinant L4 *walK*, L4 *walR* and L4 *walH*. A suggestion for why the WalRK system as well as the WalH regulator affects growth in L4 more, could be that *walK* is mutated in L4 (Figure 2.7). If the promoters are leaky, or that there is an endogenous molecule in *S. aureus* causing a continuous expression of the gene inserted in the plasmid, possibly the WalRK system would cause a bigger impact on L4. This would be the result of the fact that the mutation in *walK* was statistically proven to relate to an increased oxacillin MIC compared to its ancestor JE2. Overexpressing WalRK and WalH, regulating genes involved in autolysis, this could possibly explain the decreased oxacillin MIC even without IPTG, since the effect of the *walK* mutation causing an increased oxacillin MIC is reversed by overexpression. To know for sure why this observation is made in L4, further studies are recommended.

5.2 Antibiotic resistance

In this section, the effect of overexpressing genes from the two- and three-component systems *walRK* and *vraTSR* on the susceptibility towards β-lactams and vancomycin in different genetic backgrounds will be addressed.

5.2.1 β-lactam antibiotic resistance

Upregulation of single genes from the three-component system *vraTSR*, involved in the cell wall stress response in *S. aureus*, can increase the oxacillin MIC in the MRSA strain JE2. Overexpressing *vraR* and *vraS* caused a fourfold and twofold increased oxacillin MIC respectively (Table 4.2, 4.3, Figure 4.1 A. and B.).

These results correlate with previous reports that have shown that VraSR are important for oxacillin susceptibility. When *S. aureus* is exposed to cell wall damaging β-lactam antibiotics such as oxacillin, upregulation of the VraSR system can be induced, regulating the levels of penicillin-binding protein 2a that decreases oxacillin susceptibility (Boyle-Vavra *et al*., 2006). The VraSR regulatory system is regulating 46 genes that are important for the cell wall function and stress response (Dai *et al*., 2017). VraR is the response regulator of this system, so increased expression of VraR would lead to downstream effects and regulation of genes improving the response to damage from oxacillin.

VraT and VraU overexpression did not affect the oxacillin susceptibility of JE2 (Table 4.2, 4.3). VraT is a negative regulator of the VraSR system (Gardete et al., 2012; Hu et al., 2016). A hypothesis was that overexpressing VraT would negatively regulate VraSR and have the reverse effect compared to VraSR overexpression due to less reactivity to oxacillin stress. However, a decrease in oxacillin MIC due to overexpressing VraT was not observed. VraU expression levels have been shown to be affected in VISA strains compared to MRSA, since it is a part of the *vraUTSR* operon. Making a nonpolar deletion in *vraU* have been reported not affect the expression levels of VraS, but deleting VraT completely removed the activity of VraS. VraU is therefore not considered to be required for VraSR activation (Boyle-Vavra et al., 2013). Indeed, in line with previous results, VraU did not affect the oxacillin MIC in this study (Table 4.2, 4.3).

Next, we wondered how different genetic backgrounds would affect β-lactam resistance when overexpressing the vancomycin resistance related genes. Therefore, the same two- and three component systems were overexpressed in the VISA strains L4 and L9, which from the start had a 3 times higher and 3 times lower oxacillin MIC value compared to JE2 respectively, as shown in Figure 2.6.

The only two genes that when overexpressed caused changed in oxacillin MIC in L4 were *vraR* and *vraT*. As in JE2, overexpression of *vraR* increased oxacillin MIC fourfold, while overexpression of *vraT* increased the oxacillin MIC twofold. Overexpression of *walK*, *walR* and *walH* all affected the oxacillin MIC in L4, however, the MIC was already decreased compared to L4 Ø even without IPTG treatment. As discussed in Section 5.1.2, the results regarding L4 *walK*, *walR* and *walH* cannot be regarded as conclusive.

Interestingly, overexpression of the gene *vraT* increased the oxacillin MIC two-fold in L4, while there was no effect when overexpressing the same gene in JE2. Since VraT is the negative regulator of VraSR (Boyle-Vavra et al., 2013), it was hypothesized that the gene would decrease the oxacillin MIC due to downregulation of cell wall stress response genes. In L4, the opposite was observed, with overexpression of VraT causing an increase in oxacillin MIC. This suggests the importance of epistasis in the response to gene overexpression, essentially the interplay between different genes. Indeed, overexpression of the same gene in a vancomycin-naïve background (JE2) had a different effect than when overexpressed in a vancomycin-adapted derivative (L4). L4 differs from JE2 by only 7 mutations (Fait, 2021). Overexpression of *vraR* in L4 also caused an increase in oxacillin MIC, further proving its importance in the response to oxacillin.

Contrary to the other genetic backgrounds, overexpression of neither of the genes *vraR, vraS,* nor *vraT* affected the oxacillin MIC in L9. In Section 2.6, it is described that the genes *vraS* and *vraR* are expressed 2.22 and 1.85 times more in L9 compared to JE2 from the start. One suggestion as to why overexpressing the VraTSR system had no effect on L9 could be that the system is already functioning at a higher level. Therefore, overexpressing genes from this system has no increased effect on regulating the downstream cell wall stress response genes.

On the other hand, overexpression of *walK* decreased the oxacillin MIC twofold in L9. What could be the reason for the lowered oxacillin resistance when overexpressing the gene *walK*? The essential WalRK system have been assigned the role of regulating cell wall maintenance and play a role in cell division (Dubrac et al., 2008). Lowered expression levels of WalRK have been shown to cause changes to the cell wall, for example, decreased production of peptidoglycan. However, increased peptidoglycan cross-linking was observed due to a decrease in *walRK* expression (Delaune et al., 2011; Dubrac et al., 2007). Increased expression of *walRK* is therefore thought to cause a decrease in cross-linking and possibly a decrease in cell wall thickness, which could affect oxacillin susceptibility. Possibly, this could explain the observation of a two-fold decreased oxacillin MIC when overexpressing the histidine kinase WalK in L9.

5.2.2 Effect of inducers on cell viability and growth in β-lactam antibiotic resistance assays

Using the pRAB-lacZ expression system, the same results were not observed compared to the pSK9067 plasmid. Firstly, *vraR* overexpression did not increase the oxacillin MIC (Table B1, B3 and B4, Appendix B) as was seen using the pSK9067-IPTG expression system. This went against the hypothesis based on earlier reports of the importance of VraSR for oxacillin susceptibility (Boyle-Vavra et al., 2006). Furthermore, the growth of JE2 Ø was affected as well as can be seen by a decrease in the doubling time (Figure C1, Appendix C). This phenomenon was observed at both 25 ng/mL and 50 ng/mL, for both JE2 Ø and JE2 *vraR*, and led us to wonder if there could be a possible synergistic effect between the inducer AHT and oxacillin. However, this would be interesting to further investigate in future projects. A discussion about AHT as an inducer will take place below, as well as in Section 5.4.

AHT as an inducer

To decide whether to use AHT as an inducer, growth curves were constructed and analyzed from OD measurements using the Bioscreen C. Looking at the growth curves (Figure 4.3 and 4.4 **A.** and **B.**), it could be observed that AHT indeed affected growth of JE2 Ø by slightly shifting the growth curves. However, the affect was not so dramatic, and we therefore chose to perform oxacillin MIC experiments by performing OD measurements in the Bioscreen C with the expression system pRAB-lacZ and AHT. The AHT concentrations 25, 50 and 100 ng/mL were used, and the results are presented as Table B1, B2, B3 and B4 in Appendix B and growth curves in Figure C1 and C2 Appendix C.

The final OD was lowered when JE2 Ø and JE2 overexpressing *vraR* grew with both AHT and oxacillin (Table B1, B2, B3 and B4). The only exception to this rule is Table B2 showing OD measurements using 50 ng/mL AHT. There, the final OD for JE2 *vraR* was higher with AHT, but this was not confirmed in any other trials. Taken together, we were curious if AHT in fact affected growth, even at the lower concentrations chosen. Indeed, AHT decreased the doubling time of JE2 Ø at both 25 ng/mL and 50 ng/mL even when oxacillin was not added (Figure A1, Appendix A). The same phenomenon was observed for JE2 *vraR*. Furthermore, even though the bacteria exposed to AHT reach a higher OD after 24 hours, the growth is highly affected when AHT and oxacillin is added at the same time.

To conclude, AHT was not considered a reliable inducer in this study. This is based on the observation that AHT affected the doubling time in the control strain JE2 Ø, but also that the growth rate was lowered for the approximately 16-17 first hours, when AHT and oxacillin were added simultaneously. Next, we wondered how IPTG would perform as an inducer using the expression system pSK-9067.

IPTG as an inducer

Overexpressing the gene *vraR* in JE2 increased the MIC fourfold (Figure 4.1 **A.** and **B.**). This was concluded since the cells exposed to 400 µM IPTG grew at a four times higher concentration of oxacillin. When growth curves were constructed from OD measurements, the final OD and thereby the cell density, was not increased when overexpressing VraR (Figure C2, Appendix C). These results indicate that most cells do not survive at a higher extent due to overexpressing the gene *vraR,* but that the cells that survive can tolerate a higher oxacillin concentration. OD measurements from the Bioscreen C is interesting since the machine allows for shaking during incubation to minimize the risk of clump formation in the microtiter plate wells. In theory, this could give more accurate readings and thereby analysis of cell growth. Furthermore, the growth curves provide more data about the fitness of the cells.

Growth curves showing JE2 overexpressing the gene *vraR* showed steeper exponential phases when IPTG was added (Figure C2 A. and B. in Appendix C). This indicated that the fitness of the bacteria is increased due to VraR overexpression. This could once again be due to VraR being the response regulator in the VraTSR three-component system regulating the response towards stress from cell wall damage, for example oxacillin (Boyle-Vavra et al., 2006).

IPTG did not appear to affect the growth of *S. aureus,* at least not to the same extent as AHT. The exponential growth phase for JE2 Ø was not impaired when exposed to 400 µM IPTG compared to only media (Figure 4.2). In combination with the measurable effects of gene expression, for example as seen in Table 4.2 and 4.3, IPTG was deemed a suitable inducer for this study.

5.2.2 Vancomycin resistance

Since earlier studies have shown that the VraTSR and WalRK systems are upregulated in VISA strains (Gardete *et al.*, 2012), we were interested in the effect of overexpressing the 7 genes from these regulatory systems in JE2 as well as the JE2-derived VISA strains L4 and L9.

In JE2, our studies showed that the only gene overexpression affecting the susceptibility to vancomycin is *vraR*. More specifically, VraR overexpression increased the vancomycin MIC two-fold in JE2 (Figure 4.5). The *vraSR* operon has been described as a type of switch that can be turned on or off depending on the circumstances (Gardete et al., 2012). The VraTSR system regulates the activity of other genes that synthesize and thickens the cell wall, thereby decreasing the susceptibility to several antibiotics damaging the cell wall, for example vancomycin (Dai et al., 2017). Since VraR is the response regulator of the VraTSR regulatory system, overexpressing it is thought to affect the susceptibility to vancomycin, which we indeed observed (Figure 4.5).

Therefore, we also wondered how overexpressing genes from the three-component system VraTSR and the two-component system WalRK would affect the susceptibility to vancomycin in strains already adapted to vancomycin, namely L4 and L9.

As discussed earlier, L4 *walK*, *walR* and *walH* will not be discussed. Therefore, the only overexpression causing a changed vancomycin MIC in L4 was the gene *vraR*. Overexpressing VraR caused a slight increase in vancomycin MIC, from 4.5 to 5 mg/L. This effect is not as large as in JE2, but still shows the importance of the response regulator VraR for the decreased susceptibility to cell wall damaging antibiotics.

In L9, overexpressing the gene *vraS* decreased the vancomycin MIC from 5.5 to 4.5 mg/L, while overexpressing the gene *vraT* decreased the vancomycin MIC two-fold (Figure 4.7). However, as discussed in Section 5.1, the overexpressing L9 *vraS* has impaired growth when exposed to IPTG, so it cannot be concluded whether the decrease in vancomycin MIC is due to poorer growth or an actual change in the bacterial cell that affects the tolerance to vancomycin. Overexpression of *vraT* did not affect the vancomycin MIC in JE2, but in L9 the effect was a two-fold increase. One suggestion for the difference could be that L9 presents a mutation in *vraT*, acquired during adaptation of JE2 to vancomycin (Section 2.6). The VISA strain may have evolved alternative ways of coping with cell wall stress than with the VraTSR system. Therefore, overexpressing the regulator and activator of the system, VraT, (Boyle-Vavra et al., 2013; Hu et al., 2016) would upregulate a redundant system that costs energy to the cell. This in turn could reduce the fitness of the bacterial cell, and maybe even affect the tolerance to cell wall stressors such as vancomycin, which would explain the decrease in the MIC.

Another explanation could be the earlier described fact that VraT is the negative regulator of the *vraSR* operon, and overexpressing VraT could thereby cause a downregulation of VraSR, and decreased response to cell wall stress (Dai et al., 2017). However, it is surprising that this would only happen in L9 and not in JE2, something that would need further analysis.

On the other hand, gene overexpression of *walR* increased the vancomycin MIC of L9 from 5.5 mg/L to 6.0 mg/L in L9. Earlier studies have investigated the role of the WalRK system in VISA and showed that mutations in *walK* lead to reduced resistance to vancomycin, due to for example a thinner cell wall. The explanation for the increased vancomycin susceptibility was due to increased phosphorylation of WalR, which is the response regulator and should sense the cell wall stress and thereby regulate the *walRK* system (Hu et al., 2016). Maybe, the case for L9 could be an opposite scenario? Overexpressing the gene *walR*, encoding the response regulator, could prepare the bacterial cells for cell wall stresses. When L9 overexpressing the gene *walR* was exposed to vancomycin, possibly the larger number of response regulator WalR proteins could react stronger to the cell wall stress, thereby increasing the vancomycin MIC. This is something that would have to be investigated further.

Finally, we were interested in analyzing the role of the response regulator VraR in vancomycin tolerance further. This was done by measuring the OD when growing JE2 Ø and JE2 *vraR* in the Bioscreen for 24 hours. The results showed that there is no increase in the final OD when expression of the gene *vraR* was induced by 400 µM IPTG (Table 4.8). However, when constructing growth curves from the OD data, the exponential phase is steeper for the VraR overexpressing strains, implying a better fitness or ability to handle cell wall stress when exposed to vancomycin (Figure 4.8). However, the overall cell density, therefore survival, was not significantly improved, indicated by the fact that the OD values were not higher after 24 hours.

5.3 Bacteriophage susceptibility

A summary of the results presented in Section 4.3 is presented in Table 5.1 below. In short, it was shown that overexpressing the response regulators WalH and VraT caused the opposite effect in the VISA strains L4 and L9 compared to their ancestor JE2. The most prominent effect was seen comparing JE2 and L9 when overexpressing VraT. Overexpressing the histidine kinase VraS caused the only increase in resistance to phages among the VISA strains. Upregulations of the response regulators WalR and VraR as well as the histidine kinase WalK had the same effects on phage susceptibility in JE2 and VISA. This compilation table presented in Table 5.1 is meant to guide the reader through the discussion, making it easier to observe the differences caused by overexpression compared to the result Section 4.3.

Table 5.1. Compilation of the most important findings from the phage plaque assays. The function of the protein encoded by the gene, as well as the gene name is presented. Then, the effect of overexpressing that gene on the susceptibility to phages is shown for JE2, L4 and L9 respectively. The colors represent a fold change in the number of plaque forming units as **x10**, **x100**, **0**, **/10**, **/100**. The cells in the table are divided are when different effects were observed for different phages. Lastly, a short description of the main effect of overexpressing that gene is described.

		Effect on phage susceptibility			
Function	**Gene name**	**JE2**	**L4**	**L9**	**Main effect of overexpressing the gene**
Regulators	*walH*				Opposite effect in JE2 and VISA
	vraT				Opposite effect in JE2 and VISA
Histidine Kinases	*walK*				Increased susceptibility in JE2 and L9
	vraS				Decr. phage susc. in L4 (the only gene!)
Response Regulators	*walR*				Same effect in JE2 and L9
	vraR				Same effect in JE2 and L4

5.3.1 Discussion about the effect of overexpressing single genes on phage susceptibility

First, the effect of overexpressing genes from the two- and three component systems WalRK and VraTSR on phage susceptibility in JE2, will be addressed. Then, in section 5.3.2, the effect of epistasis and genetic background will be discussed, where comparisons with the JE2-derived VISA strains L4 and L9 will be made.

RODI

The ability of the lytic phage RODI to infect JE2 was affected during overexpression of all genes tested except for *walK* (Table 4.10, Figure 4.12 Left). One striking difference was that the regulator of both regulatory systems, VraT and WalH, decreased the susceptibility towards RODI by 10 times (Figure 4.9, Figure 4.12 Right). Opposite, the response regulators encoded by *vraR* and *walR*, as well as the histidine kinase encoded by *vraS*, increased the susceptibility to RODI tenfold (Table 4.10). One possible reason for this difference could be that WalH is the regulator or activator of the WalRK system. Deleting this gene caused an overexpression of the WalRK system and an increase in vancomycin resistance in several different Gram-positive bacterial species, often leading to the belief that the role of WalH is the same in *S. aureus* (Dubrac et al., 2008). However, several studies have shown that WalH seem to have a role in binding to the histidine kinase WalK, facilitating its activation (Cameron et al., 2016; Gajdiss et al., 2020). Thereby, a hypothesis is that overexpressing WalH could possibly increase the activation of the WalK histidine kinase, which would cause a change in regulation of genes involved in cell wall homeostasis. It is possible that this change increases susceptibility to infection by the phage RODI.

Overexpression of the gene *vraR* caused 10 times more PFUs by RODI compared with the uninduced JE2 (Figure 4.10 Left). Interestingly, exposure to RODI caused a downregulation of several different regulatory genes in *S. aureus*, amongst others *vraR* (Fernández *et al.*, 2017). It seems logical that overexpressing a gene that in wild type MRSA would be downregulated upon exposure to phage RODI as a possible defense mechanism, would cause an increased infection rate. Inducing expression of the VraSR regulator VraT caused a tenfold decrease in the number of PFUs. Earlier studies about antibiotic resistance have shown that deleting *vraT* increases susceptibility to oxacillin and other β-lactam antibiotics, indicating that VraT is a negative regulator of the VraSR two-component system (Boyle-Vavra et al., 2013). This observation correlates with the earlier hypothesis that overexpressing WalH, which is the

regulator of the WalRK system, would decrease the susceptibility to phage infection. Therefore, there is need for further studies to confirm the reason for the increase in resistance towards RODI infection.

Stab20

Stab20 is interesting since it is completely unable to infect JE2, both WT JE2 and the control strain JE2 Ø (with and without 400 µM IPTG) were completely unaffected by Stab20 (Table 4.10). However, when overexpressing WalK and WalR, plaques were visually observed at a tenfold dilution of the stock solution (*walK*: Figure 4.11 Right. *walR:* Figure 4.12 Left).

It is difficult to know the exact cause of the changed phage susceptibility. Even though the role of the WalRK two-component system in the cell wall homeostasis has been proven, it is not known exactly what changes happen in JE2 when overexpressing the genes *walK* and *walR*. To know more, microscopy, RNA sequencing or RT-qPCR could be used to investigate cell wall changes possibly affecting primary or secondary adsorption of phages, or even an intracellular change causing secondary effects.

One possible theory about the increased infection by Stab20 is the fact that myoviruses attach to the WTAs, recognizing the anionic backbone (Xia et al., 2011). Furthermore, *S. aureus* has a protein called Staphylococcal Protein A in the cell wall, which is thought to bind to neutralizing antibodies, thereby avoiding the human innate immune system (Labrie, Samson, & Moineau, 2010). Protein A is also thought to help in blocking bacteriophages reaching their target by hiding the receptors they bind to. Furthermore, during stress, autolysins and peptide hydrolases can sometimes be more active and cleave off the GlcNAc side chains, thereby increasing the level of Protein A in the environment. This is also thought to be a defense mechanism against the immune system (Becker, Frankel, Schneewind, & Missiakas, 2014). Although very speculatory, since the proteins encoded by *walR* and *walK* are the response regulator and histidine kinase in a regulatory system handling among other things the autolysins and hydrolases maintaining the cell wall homeostasis, overexpressing the genes may affect the cell wall in a way that decreases the resistance to Stab20. One suggestion is that an increased level of autolysins and glycopeptide hydrolases that cleave off for example Protein A, thereby removing a part of the defense against myoviruses. This is something that would have to be investigated further.

Stab21

Regarding the phage Stab21, one of the most striking observations is that increased levels of VraT and VraU, the regulators of the VraSR regulatory system, increased the resistance to Stab21 one hundred times (Figure 4.9 and 4.11 Left). Furthermore, overexpression of the genes *walR* and *walH* caused a tenfold increase and decrease in Stab21 infection respectively (Figure 4.11 Right, Figure 4.12 Right). Reports of the two lytic phages Stab20 and Stab21 were made at the same time, since they were discovered in sewage water in Albania (Oduor et al., 2020). However, the phages RODI and Stab21 seemed much more similar in their infection patterns of JE2, both with and without overexpressing the regulatory genes. Therefore, a similarity search was made in NCBI BLAST, to find out how closely related the three myophages are.

Surprisingly, by doing the BLAST search and creating a phylogenetic tree, visualized in Figure 5.1, RODI and Stab20 are more closely related. However, there is only a 0.03 mismatch between Stab21 and RODI, indicating a high similarity between these phages' nucleotide sequences as well.

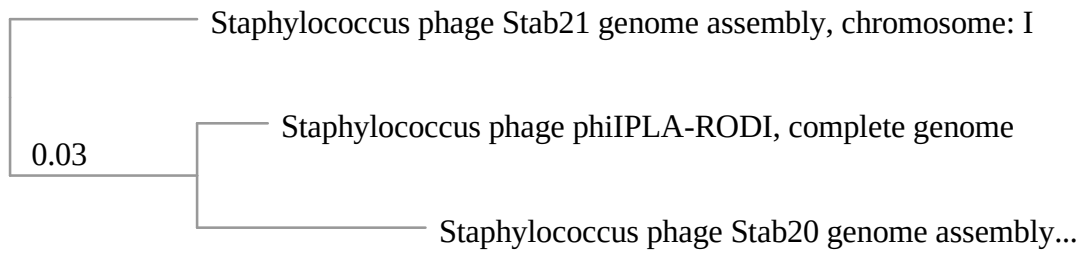
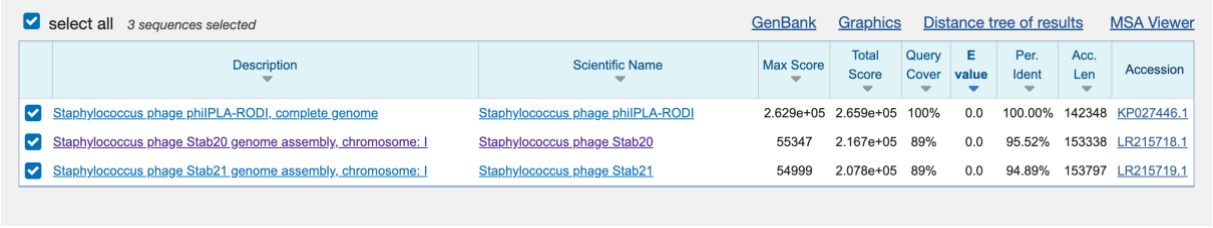


Figure 5.1. Phylogenetic tree of the nucleotide similarities of the phages Stab21, RODI and Stab20. Created in NCBI BLAST.

https://www.ncbi.nlm.nih.gov/blast/treeview/treeView.cgi?request=page&blastRID=872N1Y8K114&queryID=gb|KP027446.1|&entrezLim=&ex=&exl=&exh=&ns=100

Furtermore, it was shown that the percentage of identity between the three phages was high, 95.52% between RODI and Stab20, while its 94.89% between RODI and Stab21, as seen in Figure 5.2 below. Therefore, the similarity between infection properties of RODI and Stab21 cannot be explained by their similarity in nucleotide sequences but has to be investigated further.


select all 3 sequences selected GenBank Graphics Distance tree of results MSA Viewer

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Staphylococcus phage phiIPLA-RODI, complete genome	Staphylococcus phage phiIPLA-RODI	2.629e+05	2.659e+05	100%	0.0	100.00%	142348	KP027446.1
Staphylococcus phage Stab20 genome assembly, chromosome: I	Staphylococcus phage Stab20	55347	2.167e+05	89%	0.0	95.52%	153338	LR215718.1
Staphylococcus phage Stab21 genome assembly, chromosome: I	Staphylococcus phage Stab21	54999	2.078e+05	89%	0.0	94.89%	153797	LR215719.1

Figure 5.2. Nucleotide sequence analysis of RODI, Stab20 and Stab21. The program used was NCBI BLAST. https://www.ncbi.nlm.nih.gov/blast/treeview/treeView.cgi?request=page&blastRID=872N1Y8K114&queryID=gb|KP027446.1|&entrezLim=&ex=&exl=&exh=&ns=100

φ11

Compared to RODI, the ability of φ11 to infect JE2 was only affected by overexpressing one single gene: *walH* (Figure 4.12 Right). Partly, an explanation for this could be the fact that the infection rate of φ11 is very high for JE2, the stock solution is diluted to 10^{-7} and still induces visible plaques. Since the PFU of the φ11 stock solution was $5*10^{-9}$, a dilution of -7 log units means that there are around 500 viruses able to infect the bacteria. Therefore, the phages are so diluted it could be difficult to detect the effect of any resistance development due to overexpressing a single gene using the plaque assay. To be completely certain about the effect of each single gene, each dilution of phage would have to be tested on separate plates. This was considered too difficult in this project, due to time limitations and the number of combinations of genes and strains (144). However, it was still noticed that WalH, the regulator of the WalRK system, caused a 10 times increased resistance towards infection by φ11, just like for RODI. Therefore, this could be an additional indication of the importance of the two-component system WalRK for phage infection since an increase in regulation of this system increases the resistance towards the phages in JE2.

5.3.2 Effect of genetic background, addressing the VISA strains L4 and L9

L4

To repeat, L4 is a JE2-derived VISA strain with small downregulations in the expression levels of VraS and VraR compared to JE2 system, but primarily a *walK* mutation (Figure 2.7 and Figure 2.8). Unlike JE2, overexpressing individual genes in L4 changed the susceptibility to each of the four phages tested. Increased levels of VraR and VraT caused an increase in infection tenfold by all phages (Figure 4.9 and 4.10 Left), while VraU increased the infection hundred times in all phages (4.11 Left). Upregulations of the gene *vraS* caused an increase in resistance, tenfold for RODI, Stab20 and Stab21, and hundredfold for φ11 (Figure 4.10 Right). This is an interesting observation, possibly indicating a higher plasticity of the VISA strain L4 and ability to adapt to different gene expression levels compared to its ancestor JE2.

One important difference between L4 and JE2 is that Stab20 can infect even the control strain L4 Ø. Therefore, it seems like the genetic background already has adaptations making L4 more susceptible to Stab20 infection. However, WT L4 has a higher resistance to the three other phages compared to JE2. It seems like some adaptations made by L4 when becoming VISA from its ancestor JE2, for instance the mutations in *walK* have affected the bacterial cell in a way that made it more resistant to RODI, Stab21 and φ11. However, as seen in Table 4.10, overexpressing the genes *vraR, vraT* and *vraU* made L4 more susceptible to phages, while *vraS* made L4 even more resistant to all four phages. Possibly, this could be an indication of that the histidine kinase VraS is of importance for resistance to phages, at least decreasing the susceptibility to phage infection when overexpressed from an already downregulated level. The role of the cell wall stress response system VraTSR in phage susceptibility would have to be further researched.

L9

L9 is a JE2-derived VISA strain characterized by for example upregulations of the VraSR regulatory system and mutations in *vraT*. Overexpressing VraT caused a hundredfold increase in visible PFUs for the lytic phages RODI and Stab21 (Figure 4.9). Obviously, this protein, the regulator of the cell wall stress response system is affecting L9 a lot. A suggestion for the observation is the following: overexpressing a gene that is mutated, that normally is regulating a system that is already overexpressed when becoming a VISA strain, could possibly be redundant for L9. Then, overexpressing this protein could become costly for the bacterial cell. Whether it is reasonable that this solely would cause such a dramatic increase in the susceptibility to lytic phages is difficult to speculate in.

L9 Ø is more resistant to most phages from the start than JE2. Stab20 was still unable to infect L9 just like in JE2. However, overexpressing *walH,* encoding the regulator of the cell wall regulatory two-component system WalRK, made Stab20 able to infect L9 at a tenfold dilution (Figure 4.12 Right). More specifically, *walH* overexpression made L9 more susceptible to infection by all four phages, which is the complete opposite than what was observed in JE2. This was also true for the other genes encoding regulators of the VraSR system, *vraT* and *vraU*. When overexpressed, the effect was an increased resistance to phage infection in JE2 but increased susceptibility in L9. Speculatory, this could possibly be an indication of the effect of epistasis, that the VISA strains have adapted to using the regulatory systems VraTSR and WalRK in different ways than their genetic ancestor JE2.

Therefore, the effect of overexpressing genes regulating these systems cannot be predicted on beforehand. Consequently, it would be very interesting to further investigate the transcription levels of different proteins, for example by RT-qPCR, to get a better understanding of the molecular mechanisms affecting the susceptibility to phage infection in MRSA compared to different VISA strains.

5.4 Limitations and future research

5.4.1 Limitations

During the first two weeks of the laboratory work, oxacillin and vancomycin MIC experiments were performed using the JE2 strains JE2Ø and JE2 *vraR*. Experiments were performed using both the plasmid pSK9067, with a promoter inducible by IPTG, as well as the plasmid pRAB-lacZ, with a promoter inducible by AHT. The pSK9067 plasmid contains the *tetR* gene, which is coding the protein acting as the repressor for a promoter, namely $P_{xyl/tet}$. Additionally, the plasmid contains a gene for a protein acting as repressor of the promoter P_{spac}. The repressor gene is *lacI* (Brzoska & Firth, 2013). According to an article by Zhang et. Al (2000), the *xyl/tet* promoter systems are leaky, meaning that the genes can be transcribed even in the absence of an added promoter. The reason for leaky promoters can be that the organism has an endogenous protein that can act as a promoter, or a heterologous transactivator. (Akmammedov, Geigges, & Paro, 2017; Zhang *et al*., 2000)

Therefore, one of the reasons for the interest of using the pRAB-lacZ plasmid is the fact that it does not contain a gene that will be transcribed during promoter leakage (see Figure 3.2). pSK9067 contains a gene for GFP (see Figure 3.1) so in strain JE2Ø, that contains an "empty" pSK9067 plasmid without gene inserts, the GFP protein could be expressed continuously. Interestingly, what was seen in the results (Table B1, B2, B3 and B4 and Figure C1), the growth of the JE2Ø containing the pRAB-lacZ plasmid and induced by AHT was more affected compared to the growth of the JE2Ø strains containing the pSK9067 plasmid induced by IPTG. This indicates that the cells may benefit more from expressing a gene instead of having a completely empty plasmid. Therefore, the JE2Ø containing the plasmid pSK9067 was considered a better control for growth, and it was an additional reason for deciding to from there on using the pSK9067 plasmid.

The inducer anhydrotetracycline (AHT) is a derivative of tetracycline. However, since it binds much stronger to the same sites as its precursor, it is less toxic to the bacterial cells compared to tetracycline. This is partly due to the lower concentrations applied. Additionally, AHT is considered to be a good inducer since it can be used for several different promoters, for example the strong $P_{LtetO-1}$ promoter (Lutz & Bujard, 1997).

On the contrary, a recent study by Wenzel *et al.* (2021) showed that both tetracycline and its derivative anhydrotetracycline seem to affect the cell membranes, by a separate method compared to the earlier proposed protein translation effects. By using transmission electron microscopy (TEM), the researchers showed, by staining the membranes with Nile red, that both tetracycline and AHT caused membrane invaginations. However, these were not the result of the protein translation inhibition that is the normal antimicrobial acitivity of tetracycline compounds, but a completely new pathway. Since there is not much research performed in this area, it may be so that there is a synergistic effect between AHT and oxacillin, explaining the effect on the growth seen in Figures C1 and C2 when oxacillin is added together with AHT. Since oxacillin is a ß-lactam antibiotic targeting the cell wall, it would be interesting for future research to investigate the potential of a synergistic effect by oxacillin or other cell wall targeting antibiotics with the inducer anhydrotetracycline or other tetracycline derivative compounds (Wenzel *et al*., 2021).

No expression system is perfect, and the solution is to find a system that can function to an extent that the observations can be explained and compared to a control. In this thesis, IPTG was chosen over AHT for those particular reasons.

Limits to the range of strains available

In some cases, the transformation did not work since no colonies appeared on the TSA plates. There are several possible explanations for this, one of these being unsuccessful transformation, that the plasmids were not inserted into the cells. This error can have several sources, for example that the cells are not competent, due to errors during the making of the competent cells, or that the electroporation did not work. It could also be so that the bacterial cells are not able to be transformed. *S. aureus* is known for being notoriously difficult to transform (I. Monk & Foster, 2012).

In addition to the above described limitation, the strains L3, L6 and L10 that was not possible to transform at all are not completely characterized. It could be that these strains have acquired mutations or changes during ALE that makes them difficult or impossible to transform. The conclusions in this thesis could be even more supported if more strains had been available. On the other hand, it brings even more layers of data about the strains into the equation, possibly making clear conclusions even more difficult to draw.

In those cases that no colonies were visible on the TSA plates after transformation, the remaining 900 µL of cells that were not plated after electroporation were spun down in microcentrifuge tubes according to the protocol by Grosser & Richardson (2016). However, instead of 1 minute spinning as stated in the protocol, the cells were spun down at 3000rpm for 4 minutes. Then, all supernatant but 100 µL was discarded. The cell pellet was resuspended in the 100 µL remaining supernatant, which then was plated on a TSA plate with 10 µg/mL erythromycin. In most cases, colonies were obtained for the strains that were not positive using the regular transformation protocol by Monk, et.al (2012). (Grosser & Richardson, 2016; I. R. Monk, Shah, Xu, Tan, & Foster, 2012)

Laboratory setting limits the complete understanding

Since this experiment has been performed in the laboratory setting, it is not certain that the same phenomena would be observed in nature or even *in vivo*, since that are more complex environments. Additionally, in this study only one gene is overexpressed at the same time, and then it is more difficult to draw conclusions about interactions between genes. For example, it maybe could be a possibility to investigate the effects of overexpressing two genes at the same time using two different plasmid expression systems and thereby inducers.

5.4.3 Future research

The results presented in Section 4.3.3 show a big effect on the bacterial lawn of L9 with a plasmid containing the gene *vraS,* when induced by IPTG (Figure 4.13). Due to time limitations, this is an interesting result that there unfortunately have not been time for investigating further. What could explain the differences, for example that L9 overexpressing VraT gets a decreased vancomycin MIC but there is no effect in JE2? The effect of epistasis and genetic background have been touched upon, but it would certainly be interesting to further investigate the importance of this phenomenon.

A possible future study, which also is a limitation to this project, is to further investigate the actual molecular mechanisms behind the phenomena observed. For example, to what extent is for example VraR expressed? With the obtained results, it is possible to say that overexpressing *vraR* leads to a fourfold increase in oxacillin MIC in JE2 and L4, and no effect in L9. However, the reason why this is observed is not possible to know, it is only possible to speculate. It would be interesting to perform for example RT-qPCR to get a picture of the expression levels of different proteins.

Studies have shown that VraR is upregulated when VISA are being exposed to vancomycin. At the same time, VraR is binding to the promoter of an *agr* quorum sensing system (Dai et al., 2017). It would be interesting to look at this phenomenon in the overexpressing VISA strains presented in this thesis project. Possibly, it could even be possible to investigate if this interaction has effects on phage susceptibility.

Lastly, as described in Section 1.1 Aim, it was chosen to evaluate the effect of each single gene from the regulatory systems VraTSR and WalRK by overexpression. This has its limitations, for example plasmid burden or leaky promoters, adding several different new parameters to address when analyzing the results (Helle et al., 2011). In future studies, it would be interesting to choose the genes that in this study seemed important and interesting, for example *vraR* for oxacillin susceptibility and *vraT* and *walH* for phage susceptibility and perform deletion mutations. These studies are thought to take more time, but possibly the results will be clearer due to less added parameters.

6. Conclusions

- Overexpression of single genes from the two- and three-component systems WalRK and VraTSR changes phage susceptibility. However, the mechanism or effect of overexpression on *S. aureus* is not known. For example, there could be an effect on primary or secondary adsorption or intracellular mechanisms affecting the response to phage infection.
- The response regulator encoded by *vraR*, and the histidine kinase encoded by *vraS* are affecting the vancomycin and oxacillin resistance in JE2 and L4, by increasing the MIC. Contrary, these genes had no effect on antibiotic susceptibility in L9. Possibly, the three-component system vraTSR may already overexpressed to a maximum level in L9.
- Even in the cases where the final cell density was not higher when overexpressing *vraR*, the growth of the surviving cells was better during exposure to both oxacillin and vancomycin. This further confirmed the importance of the VraR regulated cell wall stress response system in increasing the fitness of the cells and improving the response to cell wall damaging antibiotics.
- Overexpression of several genes has the opposite effects in JE2 compared to VISA strains regarding phage susceptibility. This suggests a genetic rewiring of the VISA strains – possibly they have made adaptations to using the regulatory systems in a different way? Shows that epistasis is important, the effect of single genes is dependent on the genetic background, other mutations, and protein expression levels.
- The VISA strain L4 seems to have a higher plasticity compared to the JE2 and L9, since all four bacteriophages are affected by overexpressing single genes from the system vraTSR, and to higher extent in L4.
- The expression system pSK9067 affects the growth of some *S. aureus* strains. Nevertheless, the exact mechanism behind this effect is not known, but it could be the result of a leaky promoter or plasmid burden.

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Appendices

Appendix A. Growth curves for oxacillin MIC using AHT as inducer

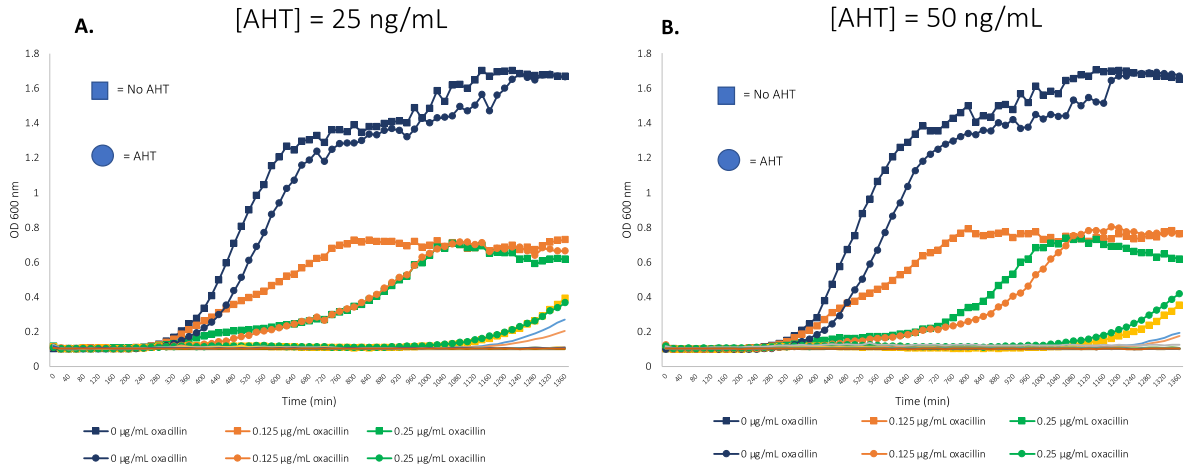

Figure A1. Strain JE2-Ø growth curves with and without AHT from an oxacillin MIC experiment.

A. JE2 Ø with 0 and 25 ng/mL added from the first biological replicate.

B. JE2 Ø with 0 and 50 ng/mL AHT added.

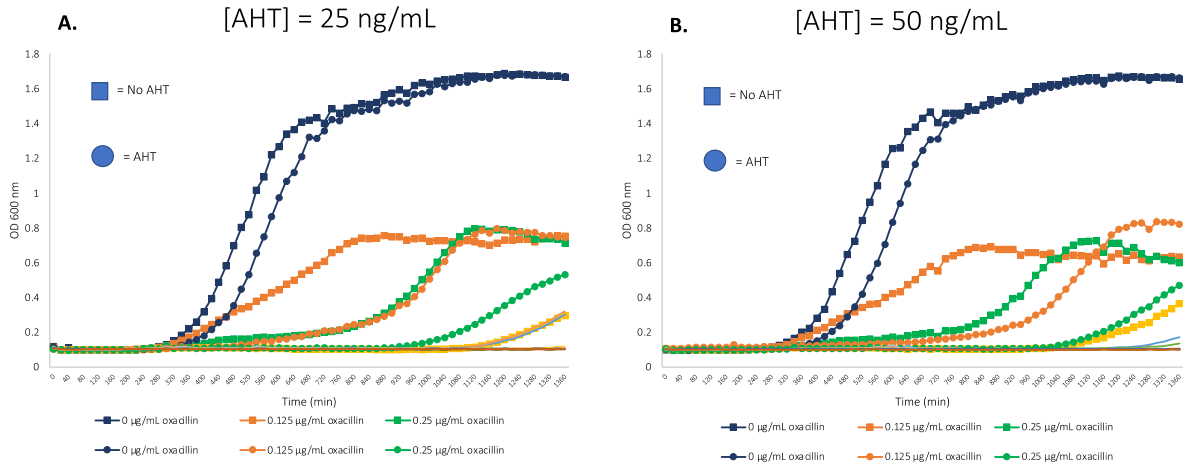

Figure A2. Strain JE2 *vraR* growth curves with and without AHT from an oxacillin MIC experiment.

A. JE2 *vraR* with 0 and 25 ng/mL added from the first biological replicate.

B. JE2 *vraR* with 0 and 50 ng/mL AHT added.

Appendix B. Oxacillin MIC using AHT as inducer

Table B1. Oxacillin MIC with 25 ng/mL AHT. The darker the green color, the higher the final OD at 600 nm after 24 hours.

		OXACILLIN MIC 25 ng/mL AHT									
		Control	0.125	0.25	0.5	1	2	4	8	16	32
JE2 Ø	No AHT	1.7	0.7	0.6	0.4	0.3	0.1	0.1	0.1	0.1	0.1
JE2 *vraR*		1.7	0.8	0.7	0.3	0.3	0.1	0.1	0.1	0.1	0.1
JE2 Ø	AHT	1.7	0.7	0.4	0.2	0.1	0.1	0.1	0.1	0.1	0.1
JE2 *vraR*		1.7	0.7	0.5	0.3	0.1	0.1	0.1	0.1	0.1	0.1
Only media		0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1

Table B2. Oxacillin MIC with 50 ng/mL AHT. The results are from the first biological replicate. The darker the green color, the higher the final OD at 600 nm after 24 hours.

		OXACILLIN MIC 50 ng/mL AHT									
		Control	0.125	0.25	0.5	1	2	4	8	16	32
JE2 Ø	No AHT	1.5	0.8	0.7	0.5	0.1	0.1	0.1	0.1	0.1	0.1
JE2 *vraR*		1.7	0.9	1.0	0.5	0.1	0.1	0.1	0.1	0.1	0.1
JE2 Ø	AHT	1.7	0.9	0.5	0.1	0.1	0.1	0.1	0.1	0.2	0.1
JE2 *vraR*		1.2	1.0	0.8	0.2	0.3	0.1	0.1	0.1	0.1	0.1
Only media		0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1

Table B3. Oxacillin MIC with 50 ng/mL AHT.The results are from the second biological replicate. The darker the green color, the higher the final OD at 600 nm after 24 hours.

		OXACILLIN MIC 50 ng/mL AHT Second experiment									
		Control	0.125	0.25	0.5	1	2	4	8	16	32
JE2 Ø	No AHT	1.7	0.8	0.6	0.4	0.2	0.1	0.1	0.1	0.1	0.1
JE2 *vraR*		1.7	0.6	0.6	0.4	0.2	0.1	0.1	0.1	0.1	0.1
JE2 Ø	AHT	1.7	0.8	0.4	0.2	0.1	0.1	0.1	0.1	0.1	0.1
JE2 *vraR*		1.7	0.8	0.5	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Only media		0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1

Table B4. Oxacillin MIC with 100 ng/mL AHT. The darker the green color, the higher the final OD at 600 nm after 24 hours.

		OXACILLIN MIC 100 ng/mL AHT									
		Control	0.125	0.25	0.5	1	2	4	8	16	32
JE2 Ø	No AHT	1.3	0.7	0.7	0.3	0.1	0.1	0.1	0.1	0.1	0.1
JE2 *vraR*		1.6	0.8	0.8	0.1	0.1	0.1	0.1	0.1	0.1	0.1
JE2 Ø	AHT	1.7	1.0	0.2	0.1	0.1	0.1	0.1	0.1	0.1	0.1
JE2 *vraR*		1.3	1.0	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Only media		0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1

Appendix C. Oxacillin growth curves.

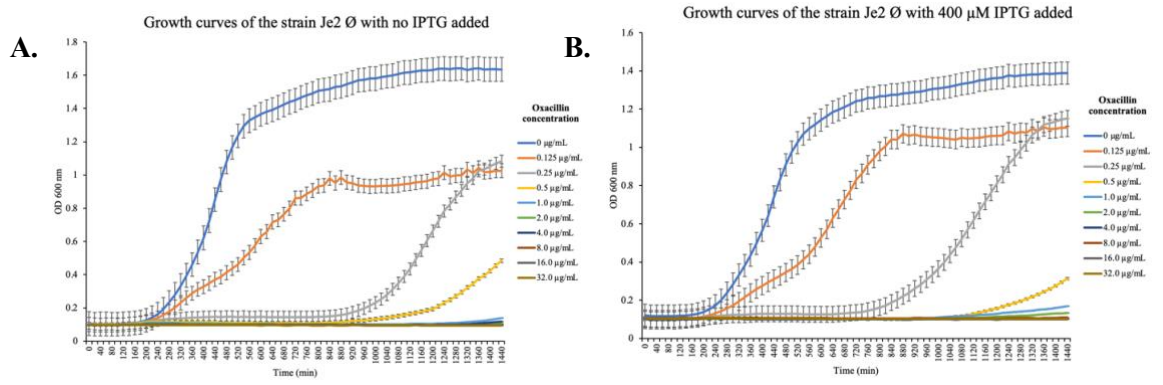

Figure C1. Strain JE2-Ø growth curves with and without IPTG from an oxacillin MIC experiment.

A. JE2 Ø with no IPTG added.

B. JE2 Ø with 400 µM IPTG added.

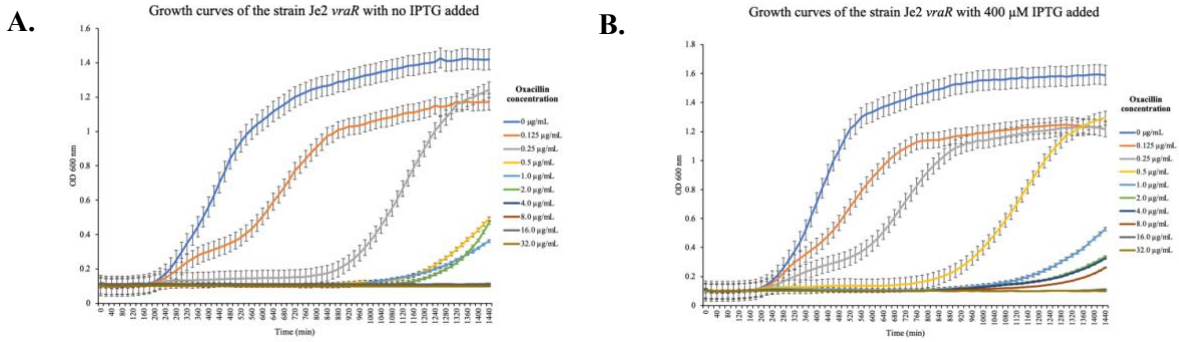

Figure C2. Strain JE2-*vraR* growth curves with and without IPTG from an oxacillin MIC experiment.

A. JE2 *vraR* with no IPTG added.

B. JE2 *vraR* with 400 µM IPTG added.

Appendix D. Vancomycin MIC

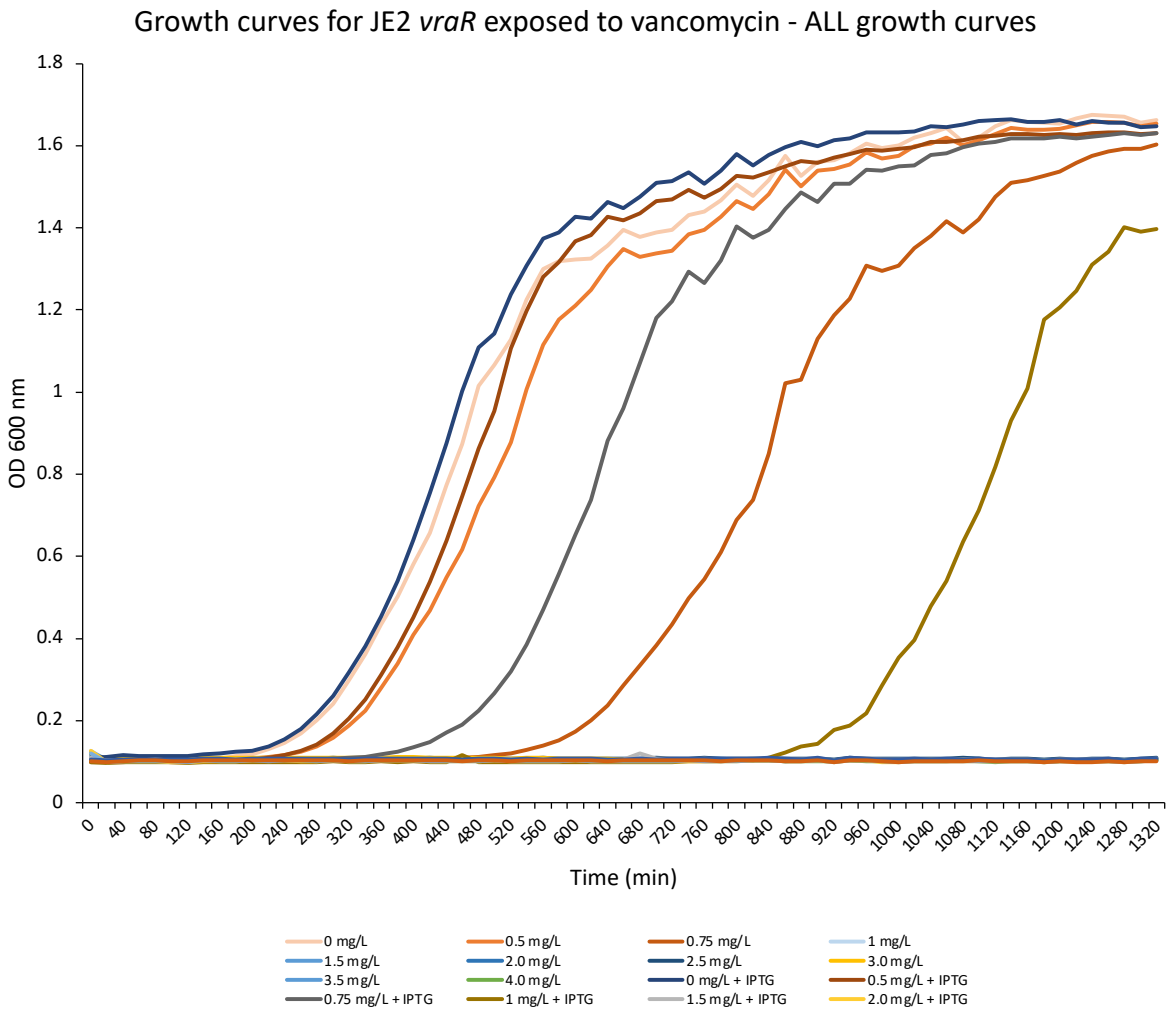

Figure D1. Strain JE2 *vraR* growth curves with and without IPTG from vancomycin MIC experiment in the Bioscreen C. Shown are the growth curves for all the tested vancomycin concentrations, ranging from 0-4.0 mg/L, with and without IPTG.

Appendix E. Growth curves for overexpressing recombinant strains

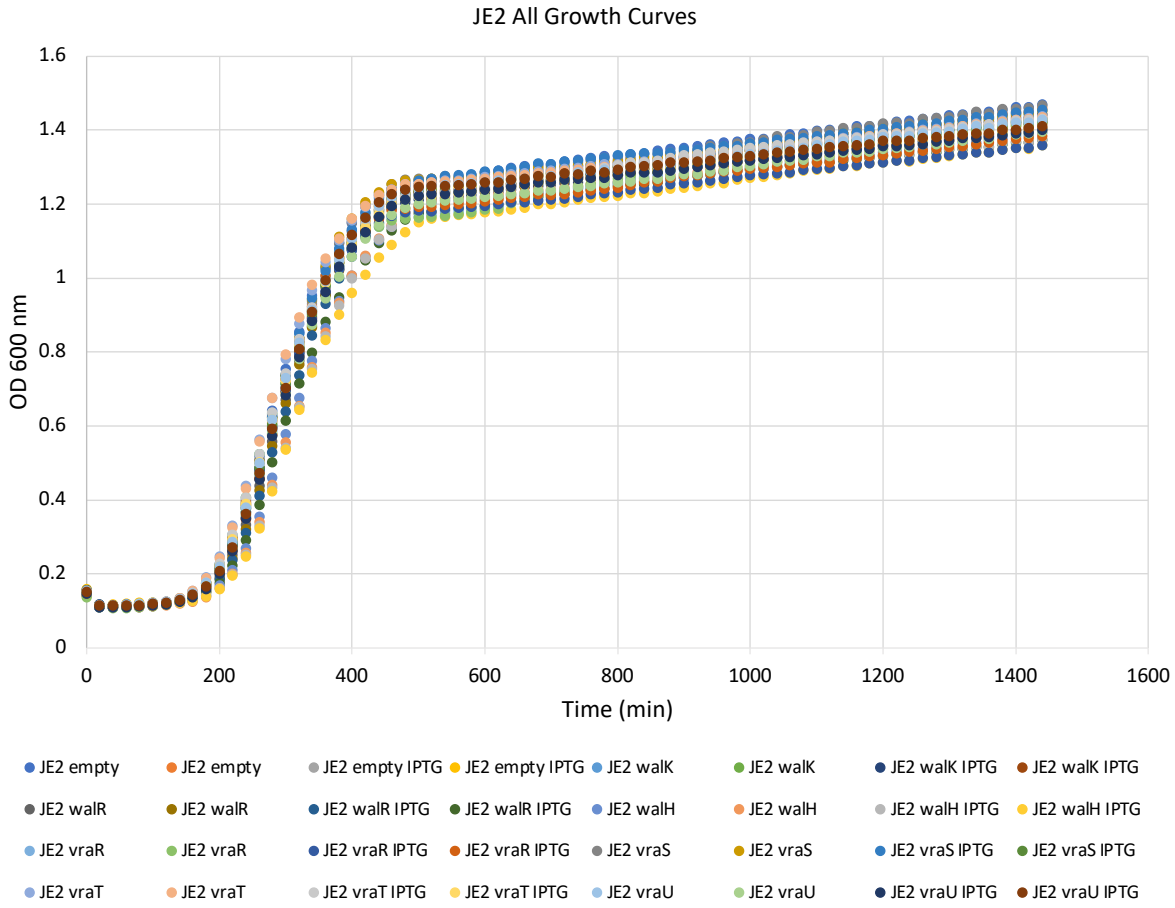

Figure E1. Strain JE2 growth curves with and without IPTG for all 7 seven overexpressing recombinant strains and the control strain Ø from a Bioscreen C measurement. Shown are the growth curves for all the strains in technical duplicates with and without 400 µM IPTG.

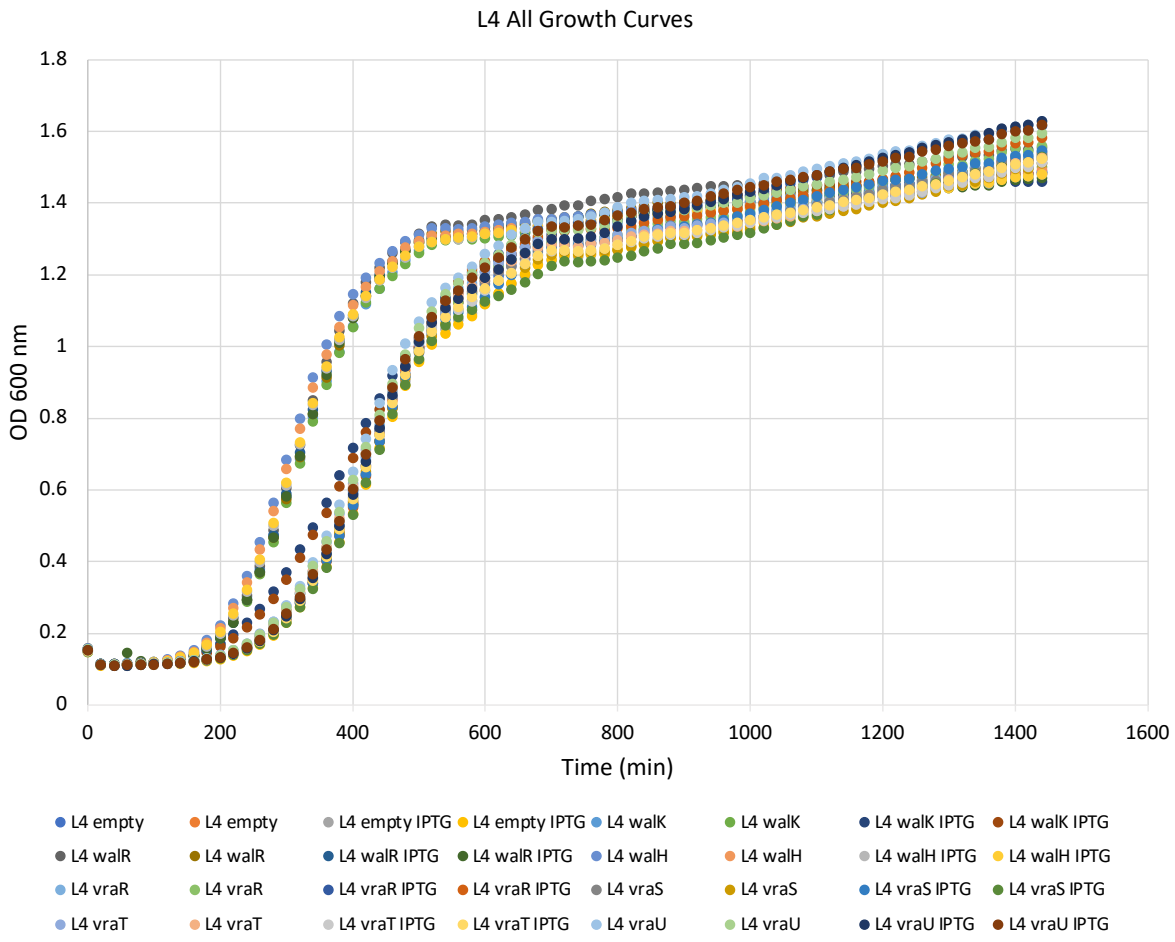

Figure E2. Strain L4 growth curves with and without IPTG for all 7 seven overexpressing recombinant strains and the control strain Ø from a Bioscreen C measurement. Shown are the growth curves for all the strains in technical duplicates with and without 400 µM IPTG.

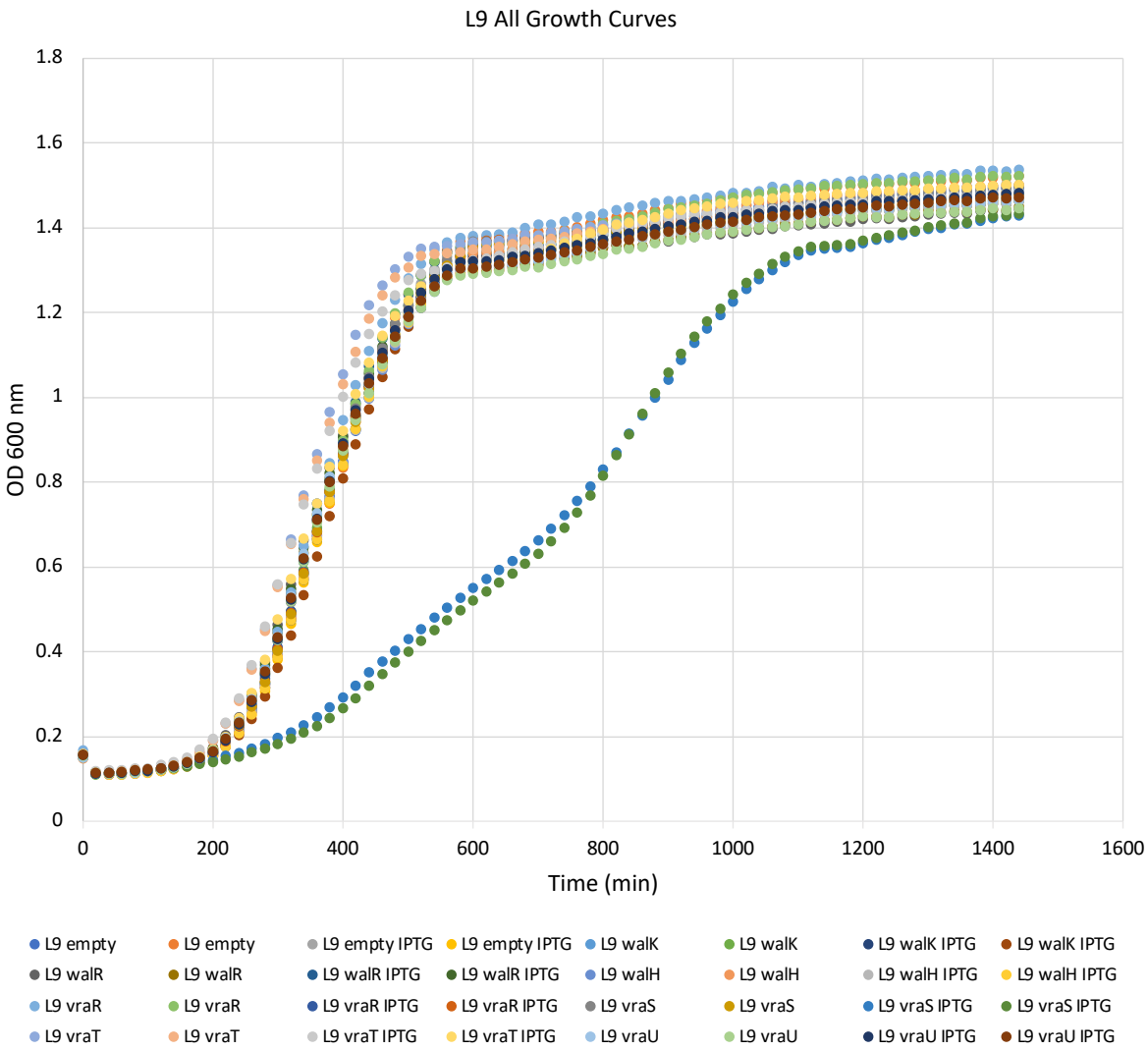

Figure E3. Strain L9 growth curves with and without IPTG for all 7 seven overexpressing recombinant strains and the control strain Ø from a Bioscreen C measurement. Shown are the growth curves for all the strains in technical duplicates with and without 400 µM IPTG.