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Exploring the link between RseP and susceptibility to the bacteriocin H1 in *Staphylococcus aureus*

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Abstract

Antimicrobial resistance is an escalating concern in today's interconnected world, with *Staphylococcus aureus* emerging as one of the primary causative pathogens. *S. aureus* is a common opportunistic pathogen found within the human skin microbiota, able to cause a wide range of infections with varying degrees of severity. With increasing numbers of deaths linked to antimicrobial resistance, the need for finding novel drugs and treatment alternatives is crucial to preventing a resurgence of historically high numbers of deaths related to infections. Bacteriocins have been investigated as an alternative to antibiotics.

Bacteriocins are a diverse group of peptides ribosomally produced by bacteria that exhibit antimicrobial effects on mostly closely related species. They differ in complexity, inhibition spectra, and size. A particularly interesting family of bacteriocins is called the LsbB-family, consisting of four sequence-related native members and some synthetically engineered ones. H1 is a hybrid LsbB-family bacteriocin built up by the N-terminal half of enterocin K1 and the C-terminal half of enterocin EJ97. All the members share the same receptor protein, RseP. RseP is a site-2 metalloprotease involved in regulating the σ^E factor in *Escherichia coli*. It has a conserved structure and is located in the cell membrane, with domains inside and outside the membrane. RseP homologs can be found across bacterial phyla and even in humans, making it a very conserved protein.

This thesis aims to investigate the prevalence of H1 susceptibility in a collection of *S. aureus* isolates and to test whether differences in susceptibility can be linked to sequence variations in *rseP*. The collection comprised 129 isolates from the Laboratory of Microbial Gene Technology (LMGT). After the initial susceptibility testing, the *rseP* gene of 34 isolates was sequenced, and clustering at 100% identity on residue level was performed to identify the number of unique RseP sequences. A correlation analysis between H1 susceptibility and RseP sequence was conducted to determine the residues most significantly associated with susceptibility. The two most significant were positions 304 and 308, with E304 and K308 positively correlated with susceptibility, while K304 and I308 were negatively associated. To investigate their importance, *rseP* from a susceptible *S. aureus* strain was cloned and expressed in a non-susceptible Δ*rseP* strain *L. lactis*. After confirming the sensitivity of the new *L. lactis* mutant towards H1, E304 and K308 were substituted for K304 and I308. However, these substitutions alone were insufficient to confer nonsusceptibility, as this clone was still susceptible to H1. This indicates that more research is needed to identify all factors affecting the susceptibility of *S. aureus* towards H1.

Sammendrag

I dagens sammenkoblede verden er antimikrobiell resistens er en voksende bekymring, der *Staphylococcus aureus* er en av de mest fremtredende patogenene. *S. aureus* er en vanlig opportunistisk patogen, funnet i menneskets hudmikrobiota, og kan forårsake et bredt spekter av infeksjoner med varierende alvorlighetsgrad. Med økende antallet dødsfall knyttet til antimikrobiell resistens, er det avgjørende å finne nye medisiner og behandlingsalternativer for å forhindre en gjenoppblomstring av historisk høye infeksjonsrelaterte dødstall. Bakteirociner har blitt undersøkt som et alternativ til antibiotika.

Bakteriociner er en meget variert gruppe av peptider, ribosomalt produsert av bakterier, med antimikrobiell effekt på hovedsakelig nært beslektede arter. De finnes i et bredt spekter av kompleksitet, inhiberingsspektra, og størrelse. En spesielt interessant familie av bakteriociner er LsbB-familien, bestående av fire sekvensrelaterte hovedmedlemmer og noen syntetisk konstruerte. H1 er en hybrid av de hovedmedlemmene, N-terminalenden av enterocin K1 og C-terminalenden av enterocin EJ97. Alle medlemmene har RseP som reseptorprotein. RseP er en posisjon-2-metallprotease som er involvert i reguleringen av σ^E-faktoren i *Escherichia coli*. Den har en bevart struktur og er lokalisert i celle-membranen med domener både på innsiden og utensiden av membranen. RseP-homologer er konservert på tvers av forskjellige bakterie phylum, til og med i mennesker, noe som gjør det til et svært bevart protein.

Denne avhandlingen har som mål å undersøke forekomsten av sensitivitet mot H1 i en samling *S. aureus* isolater og prøve å koble forskjeller i sensitivitet til sekvensvariasjoner i *rseP*. Samlingen, som bestod av 129 isolater, ble hentet fra Laboratoriet for mikrobiell genteknologi (LMGT). Etter den første sensitivitetstesten ble *rseP*-genet til 34 isolater sekvensert og gruppert på 100% identitet på residu-nivå for å identifisere antallet unike RseP-sekvenser. En korrelasjonsanalyse mellom H1-sensitivitet og RseP-sekvens ble gjennomført for å identifisere de residuene som var mest signifikante korrelert med sensitivitet mot H1. De to mest betydningsfulle ble funnet i posisjon 304 og 308, med E304 og K308 mest positivet korrelert med sensitivitet, mens K304 og I308 var negativt korrelert. For å undersøke residuenes betydning for sensitivitet mot H1, ble genet *rseP* fra en sensitiv *S. aureus* klonet og uttrykt i en ikke-sensitiv *rseP*-deletert stamme av *L. lactis.* Etter at sensitivitet mot H1 i den nye *L. lactis* mutanten var bekreftet, ble E304 og K308 ble substituert med K304 og I308. Disse substitusjonene alene var ikke nok til å fjerne sensitiviteten, siden klonen fortsatt var sensitiv mot H1. Dette indikerer at det er behov for mer forskning for å identifisere alle faktorer som påvirker sensitiviteten til *S. aureus* mot H1.

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1 Introduction

Throughout history, humans have been plagued by infectious diseases caused by various pathogenic bacteria. The absence of proper treatment options has resulted in a significant loss of life. A major breakthrough in treating common bacterial infections was the discovery of antibiotics in the early 20th century (Fleming, 1929), which have been widely used ever since (Gaynes, 2017).

In today's world, the spread of diseases is a global issue intricately linked to trade and travel (Baker et al., 2022). Despite significant advances in pharmacology and healthcare worldwide, mortality and morbidity remain substantial issues in many developing countries. The risk of spreading resistant bacteria escalates with increased globalization and fewer border restrictions. The improved access to antimicrobial drugs also leads to heightened antimicrobial resistance. Antimicrobial resistance (AMR) poses a grave threat to public health, as it could lead to a future where common bacterial infections are no longer easily treatable, potentially raising mortality rates. Thus, there is an urgent need for responsible use of antibiotics and the development of novel alternative treatment strategies. It is fundamental to address this global issue by understanding the underlying causes and mechanisms for acquiring and disseminating AMR (Holmes et al., 2016).

Antimicrobial resistance genes (ARG) often go through multiple stages when moving from environmental reservoirs to human pathogens (Ellabaan et al., 2021). This process requires a bacterium to either mutate or take a genetic element from one species and integrate it into its genome. Mobile genetic elements (MEG), such as transposons, can move a chromosomal ARG to a plasmid or phage. This process facilitates further transfer of these genes to another bacterium, a phenomenon known as horizontal gene transfer (Sommer et al., 2017). ARGs can be transferred from one cell to another through three primary mechanisms: transduction, conjugation, or transformation (Holmes et al., 2016). Transduction occurs when bacteriophages assist in DNA transfer between bacteria. DNA containing ARG can be packed into a virus particle, which again infects a new host and transfers the DNA. Conjugation involves the formation of a sex pilus, which creates a channel between two cells, enabling the transfer of plasmids. This method is particularly concerning regarding AMR. In the transformation process, some bacteria can absorb free DNA from the environment and

incorporate it into their own genome. Many of the most deadly pathogens, such as β-lactamase-producing *Enterobacteriacea*, vancomycin-resistant Enterococci, and methicillin-resistant *Staphylococcus aureus* (MRSA), are examples of bacteria that have acquired resistance by horizontal gene transfer (Hamilton & Wenlock, 2016).

Many antibiotics are produced naturally by bacteria and fungi (Holmes et al., 2016). This implies that natural resistance genes already exist in the environment. However, these natural contributors are not believed to be the main drivers behind the spreading of AMR. In the absence of external selection pressure, antibiotic-resistant bacteria and their susceptible counterparts can coexist in a state of undisturbed equilibrium within an ecosystem (Jose Luis Martinez, 2009; Jose L Martinez, 2009). For example, quinolone is a class of synthetic antibiotics, meaning natural resistance genes to them are not found in nature (Laxminarayan et al., 2013; Ruiz et al., 2012). Nevertheless, resistant strains could be isolated shortly after the introduction of most antibiotics, indicating rapid adaptation of bacteria. When exposed to antimicrobial compounds, microorganisms experience a high selection pressure to acquire resistance. The overuse of antibiotics is the primary driver of the rise in resistant bacteria due to the resulting increased selective pressure on these organisms (Aminov, 2009; Forsberg et al., 2014).

The emergence of increasingly resistant bacteria is a complex issue triggered by several problematic practices worldwide (Holmes et al., 2016). The AMR crisis is multifaced, involving sectors such as agriculture, fish farming, wastewater treatment, and human health (Holmes et al., 2016). To effectively combat the AMR crisis, it is crucial to consider the problem from a broad perspective, encompassing many aspects of society. The concept of "One Health" embodies this approach, focusing on optimizing the health of ecosystems, including the animals and humans living in them (Lancet, 2023). The One Health approach includes domestic and wild animals, plants, and crops in the broader environment, recognizing that every component within an ecosystem is interdependent and interconnected to each other. This approach to addressing the AMR crisis extends beyond just this issue, aiming also to tackle challenges related to climate change and food security. Thus, in addition to finding novel drugs and strategies to fight AMR pathogens, practicing better control of antibiotic use – a strategy known as antibiotic stewardship - in human and veterinary medicine and agriculture may contribute to mitigating the challenges of AMR in the future.

According to the World Health Organization (WHO), six primary pathogens are associated with AMR-related human fatalities (Murray et al., 2022). These are *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Streptococcus pneumoniae*, *Acinetobacter baumannii*, and *Pseudomonas aeruginosa,* mentioned in descending order according to their prevalence. These six pathogens account for over 900,000 fatalities out of a total of 1.27 million deaths attributable to antimicrobial resistance. Furthermore, these bacteria were responsible for 3.57 million of the 4.95 million deaths associated with AMR globally in 2019. The pathogen that was attributed with the highest number of deaths was methicillin-resistant *Staphylococcus aureus* (MRSA), which alone was responsible for more than 100,000 deaths.

1.1 *Staphylococcus aureus*

The discovery of *staphylococci* was made by Sir Alexander Ogston in 1880 while studying the cause of sepsis, also known as blood poisoning (Ogston, 1984). In 1884, the physician and microbiologist Anton Rosenbach isolated a strain of *Staphylococcus* and obtained a pure culture, which he named *Staphylococcus aureus* because of its yellow color (Rosenbach, 1884). *S. aureus* cells in the microscope formed what Rosenbach called grape-like clusters ('Staphylo' from Ancient Greek: a bunch of grapes) (Ogston, 1984). *S. aureus* is a Gram-positive bacterium that belongs to the phylum Bacillota (previously Firmicutes) (Oren & Garrity, 2021) and is a facultative aerobic species with optimum growth at 37°C and neutral pH (Edwards & Massey, 2011).

Surrounding the Gram-positive *S. aureus* is a thick cell wall with a porous appearance (Harris et al., 2002). The cell wall is mainly comprised of peptidoglycan, which forms a tight, multifaceted-layered grid that is able to withstand the correct osmotic pressure, and the cells do not burst due to the turgor pressure. The key proteins involved in peptidoglycan polymers are the penicillin-binding proteins (PBPs). Approximately 40% of the cell wall mass is negatively charged teichoic acids, polymers with a phosphate group. The teichoic acids can be cell membrane-associated lipoteichoic acids or covalently bound to the cell wall, wall teichoic acid. Combined, the teichoic acids and peptidoglycan comprise 90% of the weight of the cell wall, while the rest are exoproteins, peptidoglycan hydrolases, and surface proteins.

The main component of the cell membrane of *S. aureus*, like many other bacteria, is a phospholipid bilayer, which allows for the correct concentration of nutrients and waste inside the cell by acting as a selectively permeable barrier to the environment (Nikolic & Mudgil, 2023). The lipids of the *S. aureus* membrane include glycolipids, cardiolipins, monoglycosyldiacylglycerols, lysyl-phosphatidylglycerols, diglycosyldiacylglycerols, and phosphatidylglycerols. These molecules play essential roles in the membrane's stability, as well as antibiotic resistance, by altering the susceptibility to positively charged molecules and the membrane's permeability. Other components of the membrane include membrane-associated proteins, aiding in the transport of nutrients and waste products, signal transduction, and regulation.

To better distinguish between different strains of staphylococci, R. W. Fairbrother posited a new classification system that separates pathogenic and non-pathogenic staphylococci with regard to coagulase production (Fairbrother, 1940). Strains that produce coagulase are coagulase-positive staphylococci (CoPS), and those that do not are coagulase-negative (CoNS). It was known that pathogenic strains were CoPS and able to ferment mannitol. In contrast, non-pathogenic strains were CoNS and unable to ferment mannitol. *S. aureus* is a coagulase-positive (CoPS). By 1970, the number of classified staphylococci reached 10; by 2014, the number exceeded 40 (Euzeby, 1997). According to Becker et al. (Becker et al., 2014), the genus *Staphylococcus* can be divided into four main categories: animal-associated and other CoNS, human-associated CoNS, animal-associated and other CoPS, and human-associated CoPS.

S. aureus is a very common opportunistic pathogen, and it is one of the most prevalent pathogens found in hospitals and on the skin of humans (Humphreys, 2012; Lowy, 1998). An estimated 30% of people are persistent carriers of *S. aureus* (Wertheim et al., 2005). CoNS can be found in a significant proportion of bacteria living on the skin and mucous membranes of humans and animals (Grice & Segre, 2011). However, many CoPS are opportunistic pathogens capable of causing a wide range of infections, from mild skin and tissue infections to more severe infections such as endocarditis and fatal pneumonia. They are also frequently the causative agents of infections in the respiratory tract, surgical sites, or prosthetic joints (Tong et al., 2015). Furthermore, they can cause cardiovascular infections as well as nosocomial bacteremia. *S. aureus* alone is estimated to cause hundreds of thousands to millions of severe infections worldwide (Murray et al., 2022). Treatment of infections caused

by *S. aureus* is increasingly challenging due to the rise and prevalence of antibiotic resistance in this species.

S. aureus can acquire antibiotic resistance genes (ARG) by horizontal gene transfer, described in more detail above, with mobile genetic elements (MGEs) (Bitrus et al., 2018). MGEs can be bacteriophages, plasmids, pathogenicity islands (PAIs), staphylococcal cassette chromosome *mec* (SCC*mec*), or transposons (Bitrus et al., 2018; Rasheed & Hussein, 2021). Resistance to β-lactam antibiotics such as penicillin G and ampicillin is conferred by β-lactamase production or by alternative penicillin-binding proteins such as PBP2a (encoded by *mecA*) (Rasheed & Hussein, 2021). Penicillin contains a β-lactam ring that binds to the penicillin-binding proteins (PBPs) located in the cell envelope and inactivates them. Inactivation of PBPs inhibits and prevents bacterial cell wall synthesis, leading to cell lysis and death. β-lactamase (penicillinase) is encoded by the *blaZ,* which is always located on plasmids, and this resistance mechanism involves the inactivation of the b-lactam ring in penicillin. The *mecA* gene is located on a mobile chromosomal DNA fragment, which confers resistance because the encoded PBP has a low affinity of b-lactams. Strains encoding the *mecA* gene are also resistant to many β-lactamases and methicillin (MRSA). Although methicillin is no longer used in medicine, this antibiotic was resistant to β-lactamase.

Vancomycin is a glycopeptide frequently used to treat MRSA infections. It is part of the antibiotic class of glycopeptides and was discovered in 1953 (Klevens et al., 2007; Micek, 2007). Relatively recently, in 2002, resistance to vancomycin in *S. aureus* was also documented (Pray, 2008). High-level resistance to glycopeptides in *S. aureus* is primarily due to the *vanA* gene cluster, which encodes proteins that modify the binding target of vancomycin, lipid II, by the addition of D-Lac residues (Cong et al., 2020). Vancomycin is often considered a “drug of last resort” in treating MRSA infections.

1.2 Bacteriocins

With the rise of antibiotic resistance, alternative antimicrobial agents are needed. Bacteriocins are a class of antimicrobial compounds that many believe could play an important role in the fight against antibiotic resistant bacteria (Cotter et al., 2013). They exhibit low toxicity, have high potency *in vitro* and *in vivo*, can have both narrow- and broad-spectrum inhibition, and have the possibility for *in situ* production by probiotic bacteria (Cotter et al., 2013). Unlike traditional antibiotics, bacteriocins are often unmodified peptides, which allows for the possibility of synthetic bioengineering (e.g., to change the spectrum or potency of a bacteriocin). To be able to take full advantage of the great possibilities of the use of bacteriocins within biotechnological and medical technologies, it is essential to understand more about the structure and mode of action of the bacteriocins (Ovchinnikov et al., 2014).

Bacteriocins are defined as small peptides produced on the ribosome by bacteria with antimicrobial activity (Tagg et al., 1976). Both bacteria and archaea can produce bacteriocins (Cotter et al., 2005; Klaenhammer, 1993). There exist bacteriocins with great diversity in size and structure, potency and receptors, mode of action, and specific immunity mechanisms (Gillor et al., 2008). A common belief is that bacteriocins play an essential part in bacterial competition for resources or niches (Nes et al., 2007). Bacteriocins are typically between 25-70 amino acids long with amphipathic and cationic properties (Ovchinnikov et al., 2014). However, the diversity is large, and sizes range from very small bacteriocins such as microcin C7 (7 amino acids) to large (~70 kDa) bacteriocins such as pyocins are known.

Bacteriocins generally have a mechanism of action different from antibiotics and are, therefore, equally potent against antibiotic resistant strains as their susceptible counterparts (Nes, 2011). Furthermore, some bacteriocins have a potency several orders of magnitude higher than antibiotics, with minimal inhibitory concentrations 100-1000 times lower. Compared to antimicrobial peptides (AMPs) produced by eukaryotic cells, bacteriocins have a narrow inhibition spectrum (Nes et al., 1996). Typically, bacteriocins only inhibit related species or genera, but some have a broader spectrum. The high specificity of inhibition is achieved by utilizing specific molecules on the target cell as receptors that the bacteriocin exploits to inhibit or kill the cell.

Many bacteriocins are produced by lactic acid bacteria (LAB), which naturally inhabit the gastrointestinal tract and are naturally found in many food products. As such, many LABs have achieved a generally regarded as safe (GRAS) status by the Food and Drug Administration (FDA) (Nes, 2011; Perez et al., 2014). Some examples are nisin, lactococcin A, and lacticin 3147, produced by LAB found in foods. However, using LAB to produce bacteriocins in starter cultures or isolated bacteriocins (without cells) as additives in foods is very limited, even though products exist for commercial applications. This will likely facilitate and ease the regulatory hurdles faced by new antimicrobials for human or veterinary use.

Bacteriocins can be classified into four main groups (Antoshina et al., 2022). Class I and II are heat-stable bacteriocins with a molecular weight of less than 10 kDa. Class I are post-translationally modified peptides, while class II are unmodified. Class III are heat-labile peptides with a molecular weight of 10-30 kDa, and Class IV are large proteins with a molecular weight higher than 30 kDa.

Leaderless bacteriocins

Class II is divided into pediocin-like bacteriocins (IIa); two-peptide-bacteriocins (IIb); leaderless bacteriocin (IIc); and linear non-pediocin bacteriocins (IId) (Antoshina et al., 2022). Leaderless bacteriocins are a subgroup of class II and can be further divided into four families: the aureocin A53-like, enterocin L50-like, LsbB-like, and multipeptide bacteriocins. It is likely that most class II bacteriocins utilize a specific receptor to recognize target cells (Nes, 2011). Class IIa and some class IId target a membrane component of the mannose phosphotransferase system (Man-PTS). While a family of IIc bacteriocins called the LsbB family targets a zinc metalloprotease called RseP (Ovchinnikov et al., 2014), and targets that play an important role in pathogens such as staphylococci and enterococci.

1.3 LsbB family of bacteriocins

Within the class IIc bacteriocins, a small family of sequence-related leaderless peptides called LsbB exists (Oftedal, 2023; Uzelac et al., 2013). This group has four native members and some synthetic derivates. The native members are enterocin EJ97 (EntEJ97), enterocin K1 (EntK1), enterocin Q (EntQ) and LsbB. A synthetic member named H1 consists of the N-terminal half of EntK1 and the C-terminal half of EntEJ97 (Kranjec et al., 2021). They all share the same target receptor, a site-2 membrane-bound metalloprotease called RseP (Kristensen et al., 2023; Uzelac et al., 2013).

The LsbB family peptides are cationic, between 30-44 amino acids long, and contain a conserved motif KxxxGxxPWE in the C-terminal end(Antoshina et al., 2022; Ovchinnikov et al., 2014). In aqueous solutions, the bacteriocins are unstructured but form an α-helical structure when exposed to a hydrophobic environment (Ovchinnikov et al., 2017). The structures of EntK1 and LsbB have been solved in 50% TFE (trifluoroethanol), and similar α-helical structures for EntEJ97 and EntQ have been predicted.

A

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EntQ -------MN--F-LKNGIAKWMTGAELQAYKKKYGCLPWEKISC 34
LsbB --------------MKTILRFVAGYDIASHKKKTGGYPWERGKA 30
EJ97 MLAKIKAMIKKFPNPYTLAAKLTTYEINWYKQQYGRYPWERPVA 44
EntK1-------MKFKFNPTGTIVKKLTQYEIAWFKNKHGYYPWEIPRC 37
H1   -------MKFKFNPTGTIVKKLTQYEINWYKQQYGRYPWERPVA 37
                      :   ::  ::  .*:: *  ***   .
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B

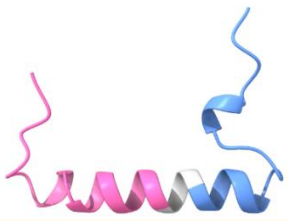

Figure 1.1. A Multiple sequence alignment of the known active members of the LsbB family. Below the MSA, the fully conserved residues are marked with an asterisk (*), groups of strongly similar properties are marked with a colon (:), and conservation of weakly similar properties are marked with a period (.). The MAS was constructed using Clustal Omega (Madeira et al., 2022). **B** Predicted structure of the hybrid bacteriocin H1 made by ColabFold (Mirdita et al., 2022) visualized in ChimeraX (Goddard et al., 2018; Meng et al., 2023; Pettersen et al., 2021). The N-terminal from K1 is shown in pink, and the C-terminal of EJ97 is in blue. The overlapping sequence (YEI) is shown as white.

Spectrum of activity

The bacteriocin EntEJ97 was discovered from *Enterococcus faecalis* EJ97, isolated from municipal wastewater, and was the founding member of this family (Gálvez et al., 1998). LsbB is produced by *Lactococcus lactis* BGMN1-5 isolated from cheese (Ovchinnikov et al., 2014). LsbB is the smallest of the LsbB bacteriocins, consisting of only 30 amino acids, and this bacteriocin also has the narrowest inhibition spectrum with potent activity only against *L.*

lactis IL1403. EntQ has bactericidal activity against some *Latilactobacillus sakei* and *E. faecium* strains and is produced by *Enterococcus faecium* L50, which was isolated from fermented Spanish sausage (Cintas et al., 2000). EntK1 was discovered by genome mining and confirmed using synthetic peptides only, where it showed activity against *L. lactis, E. faecium*, and *Enterococcus hirae* (Oftedal, 2023; Ovchinnikov et al., 2017).

1.4 RseP

RseP is a membrane protein and functions as a site-2 zinc metalloprotease (SP2). It is recognized as the target for all members of the LsbB family of bacteriocins in certain Gram-positive bacteria (Kristensen et al., 2022; Uzelac et al., 2013). This is of particular interest because of the important role of RseP in bacteria. For bacteria to adapt to a changing environment and alter their gene expression accordingly, signal transduction mechanisms must exist that can convey information across the membrane. The ability of a bacterium to sense and adapt to the environment is essential (Kristensen et al., 2023). In several species, RseP has been shown to be involved in activating extracytoplasmic function σ-factors that help the cell survive under stressful conditions. These σ-factors are normally held inactive by anti-σ factors attached to the membrane. Release and activation of the σ-factor occurs via regulated intramembrane proteolysis (RIP). In this mechanism, a signal triggers the first proteolysis of the anti-σ factor by a site-1 protease, which then permits a second cleavage of the anti-σ factor by a site-2 protease, in this case RseP.

S2Ps can be divided into four subgroups based on different topology and structure of domains (Kinch et al., 2006). All S2P have conserved catalytic site formed by the two motifs HExxH and xDG located in the transmembrane segment towards the inside of the cell marked in **Figure 1.2**. Other characterized regions and domains are the PDZ domain, the GxG motif, and the membrane-reentrant β-hairpin-like loop (β-MRE loop) (Kristensen et al., 2022). The four transmembrane helices form a channel-like structure, and the active site is located within this channel, which enables intramembrane cleavage of proteins. The PDZ domain is placed on top of the channel and it is indicated to have a size-elimination function that blocks interactions with the active site of RseP prior to S1P cleavage (Kristensen et al., 2022).

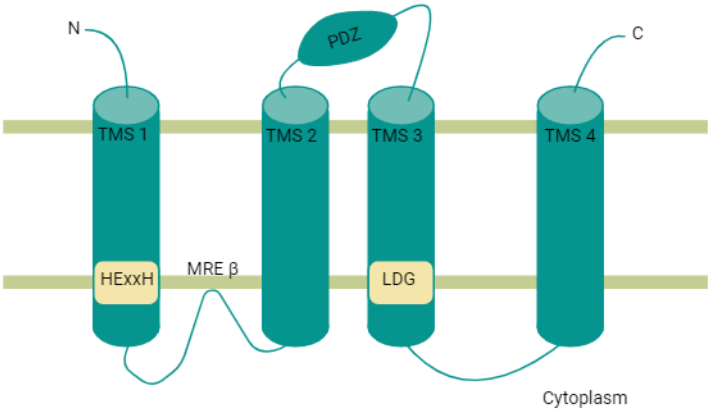

Figure 1.2. Predicted membrane topology of RseP from *S. aureus*. Figure adapted from Kristensen et al. (2022).

In *E. coli*, RseP is important for regulating the sigma E-factor and is a protease (Kanehara et al., 2001). The RIP mechanism and the role of RseP is best described in *E. coli*, where RseP cleaves the anti-σ factor RseA. This is a well-described process of a RIP cascade in bacteria (Kristensen et al., 2023). Under normal conditions, the alternative σ^E-factor is bound to the anti-σ factor RseA, located in the membrane. However, under stressful conditions, misfolded outer membrane proteins (OMPs) can accumulate in the periplasmic space. These misfolded OMPs will have several hydrophobic residues exposed on the surface, to be recognized by the PDZ domain of the site-1 protease DegS. This recognition initiates the first cleavage of RseA in the extracytoplasmic region. The subsequent cleavage of RseA by RseP releases σ^E into the cytosol, resulting in the activating of stress-response genes (Kristensen et al., 2023).

Various versions of S2P have been seen to be involved in several different processes, including pathogenicity, virulence, and survival (Kristensen et al., 2023). SpolIVFB is a S2P that regulates the transcription of σ^K involved in sporulation in *B. subtilis.* S2P has been linked to lysozyme resistance in numerous Gram-positive bacteria. In some Gram-negative bacteria, RseP is vital for optimal iron uptake. RasP (RseP) has also been recognized in *Bacillus* to regulate σ-factors involved in the translation of genes that are important in resistance to antimicrobials. All these examples highlight the importance of RseP in many bacteria. When a pathogen is establishing an infection, the ability to adapt appropriately is essential. Activation of ECF σ-factors by RseP likely plays an important role in infection by human pathogens.

In *E. faecalis,* RseP is also involved in the production of sex pheromones and is sometimes named Eep in this species (enhanced expression of pheromone)(Kristensen et al., 2023). Sex pheromones are necessary for the conjugation of some plasmids between bacterial cells, including plasmids carrying antibiotic resistance genes. The involvement of RseP in the release of sex pheromones is suggested to be via proteolysis of precursors of lipoproteins. These lipoproteins contain signal peptides that are first cleaved off by a type II signal peptidase; the leftover signal peptide is then processed by RseP, resulting in a mature pheromone that can be exported to the outside of the cell. This may be a conserved pathway for the release of sex pheromones in some Gram-positive. The involvement of RseP in the release of sex pheromones has been proposed in numerous species, such as *L. monocytogenes*, *Streptococcus*, and *S. aureus*.

RseP is well conserved in staphylococci, including *S. aureus*; however, barely anything is known about its function, role, or importance in this bacterium (Kristensen et al., 2023). In the strain *S. aureus* NCTC8325-4 *rseP* is located in the same operon as the essential gene *proS*, a prolyl-tRNA synthase (Chaudhuri et al., 2009). The A sex pheromone, cAM373_SA, has been found to require RseP for secretion in *S. aureus*; this pheromone is derived from the lipoprotein CamS (Cheng et al., 2020). Furthermore, mutants of *S. aureus* with RseP deleted showed a lower ability to adhere to human epithelial cells and lower survival when exposed to human neutrophils and host infection.

1.5 Aim of thesis

The hybrid bacteriocin H1 is a recently developed novel bacteriocin shown to be active against *S. haemolyticus* (Kranjec et al., 2021). In a previous master thesis, H1 was found to be active against 1/8 *S. aureus* strains tested, but apart from that, its activity against *S. aureus* is largely unknown (Ottesen, 2023). The aim of this work is to determine the prevalence of H1 susceptibility in a collection of *S. aureus* isolates and to test whether differences in susceptibility can be linked to sequence variations in RseP. Furthermore, this work aims to determine the potential of using the LsbB family bacteriocin H1 against *Staphylococcus aureus*.

2 Materials and methods

2.1 Strains and growth conditions

All strains used in this work are listed in **Table 2.1**, except for the 129 isolates of *S. aureus* in the LMGT collection, listed in **Appendix A**. Cultures were stored at -80 °C with 15-20% glycerol.

Table 2.1. Strains used in this experiment, along with their characteristics and references.

Strain	Characteristic	Reference
Lactococcus lactis IL1403		(Chopin et al., 1984)
Lactococcus lactis IL1403 Δ*rseP* (*yvjB)*		(Røren, 2022)
Staphylococcus aureus LMGT[1] 3255		Lab collection
Staphylococcus aureus NCTC 8325-4	NCTC 8325 Δϕ11 Δϕ12 Δϕ13	(Novick, 1967)
Staphylococcus aureus JE2	USA300 LAC Δp01 Δp03	(Fey et al., 2013)
Escherichia coli GeneHogs	F-*mcrA* Δ(*mrr-hsd*RMS-*mcr*BC) φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G *fhu*A::IS2	Invitrogen
Escherichia coli IM08B	DH10B Δ*dcm*, P_{help}-*hsdMS*, P_{N25}- *hsdS* hsdS	(Monk et al., 2015)
Escherichia coli IM08B	pLOW-dCas_aad9	(Myrbråten et al., 2022)

[1]: Laboratory of Microbial Gene Technology (LMGT)

E. coli was grown in (LB) (Oxoid) at 37 °C and 180 rpm. Tryptic soy broth (TSB) or brain heart infusion (BHI) was used for *S. aureus,* which was grown at 37 °C with shaking (180 rpm). For susceptibility testing of *S. aureus*, a protocol adapted from the disc diffusion protocol from EUCAST was used; for this protocol, Mueller-Hinton (MH) was used as medium, and plates were incubated at 35°C. *L. lactis* was cultivated at 30 °C in M17 (Oxoid) supplemented with 0.5% glucose. In *L. lactis*, erythromycin and chloramphenicol were used at 10 µg/ml for selection of plasmids pNZ8037 and pNZ9530, respectively, or at 5 µg/ml of each antibiotic when used in combination.

2.2 Antimicrobials

Bacteriocins enterocin EJ97 (EntEJ97), enterocin K1 (EntK1), H1, and LsbB were obtained as synthetic peptides from PepMic (Suzhou, China) at a purity of 95% or higher. Synthetic peptides were solubilized in Milli-Q water containing 0.1% trifluoroacetic acid (TFA). Lactococcin A (LcnA) had been purified previously from *L. lactis* subsp. *cremoris* LMG 2130 as described by Holo et al. (1991) and used as the eluted fraction in approximately 40% ethanol (Holo et al., 1991).

2.3 Primers and general PCR

All primers used in this work are listed in **Table 2.2**.

Table 2.2. Primers used in this work.

Name	Sequence 5' -3'
3255_RseP_In_F1	ATGACTTATGTTACGTATACAGC
3255_RseP_In_R2	GCACGTCACGCATCGTTGG
RseP_Oligo1	GAAAACTTGTTCTACAGTAGACGT
RseP_Oligo2	AAACACGTCTACTGTAGAACAAGT
RseP_hom_up_F	tcaggcgccattcgccattcTATAGTATGAAAGTTAGAACGCTG
RseP_hom_up_R	AATATCGTCGAATATCATTCTCGCTACACCTCGATTGTTT
RseP_hom_down_F	AAACAATCGAGGTGTAGCGAGAATGATATTCGACGATATTTCTTATAA
RseP_hom_down_R	gcgaagaggcccgcaccgatGTTGCTAATTCAATATTATCTGTGC
rseP 3255 FWD	CAGGCATGCCTGCAGGTCGAAGGAGGAAGCTTAtgagctatttagttacaataattgcatttatta
rseP 3255 REV	AAAACGACGGCCAGTGAATTttataagaaatatcgtcgaatatcattc
pCasSA_sec_Harm_R	CCCTGGCGTTACCCAAC
pCasSA_sec_Harm_F	GAGAGTGCACCATATGCGGT
rseP_SalI_F_pLOW	tatGTCGACtcgaggtgtagcgagtgagc
rseP_EcoRI_R_pLOW	aaaaGAATTCgatggcacgtcacgcatcg
pCasSA_HOM_check_F	GTGAAATACCGCACAGATGC
pCasSA_HOM_check_R	CAACTTAATCGCCTTGCAGC
pCasSA_sg_check_F	TAGGCTGCTACACCTAGC
pCasSA_sg_check_R	CTGACTCGAGCATTCTAGACC
MK610	TATCCGGAGGTGTAGCATGTC
pLOW_check_F	TGAGCAGTAACAACCTCTGC
pLOW_check_R	CAGTGAATTCGATGGCACG
3255RseP_BamHI_F	TCAGGATCCcaatcgaggtgtagcgagtgagc
3255RseP_XbaI_R	CTGTCTAGAgcacgtcacgcatcgttgg
pNZ8037_test_F	cgcgagcataataaacggc
pNZ8037_test_R	acgcctgttttaacgattatgc
3255RseP_EK_to_KI_F	ggatttAAAAGCTTCCTAATAggtagtacactaatttttacagctgtagtagg
3255RseP_EK_to_KI_R	ccTATTAGGAAGCTTTTaaatccatatacaattggtttgaagagc

In this work, polymerase chain reactions (PCR) were performed using either Phusion HF (Thermo Fisher) and RedTaq (VWR). The reactions were prepared according to the producers' protocol, with the recipes described in **Table 2.3**.

Table 2.3 Recipe for Phusion HF (Thermo Fisher) and RedTaq (VWR)

Component Phusion	**Final Concentration**	**Component RedTaq**	**Final Concentration**
Nuclease-free water		Nuclease-free water	
5X Phusion HF Buffer	1X	RedTaq 2x Master Mix	1X
10 mM dNTPs	200 µM	10 µM Forward Primer	0.5 µM
10 µM Forward Primer	0.5 µM	10 µM Reverse Primer	0.5 µM
10 µM Reverse Primer	0.5 µM	Template DNA	<250 ng
Template DNA	< 250 ng		
Phusion DNA Polymerase	1.0 units/50 µl PCR		

The general PCR temperatures used in reactions with Phusion HF and RedTaq are listed below in **Table 2.4.** PCR with Phusion (Thermo Fisher) and RedTaq (VWR) temperature steps template.**Table 2.4**.

Table 2.4. PCR with Phusion (Thermo Fisher) and RedTaq (VWR) temperature steps template.

Cycles	**Step**	**Temperature** Phusion/ RedTaq	**Time** Phusion/RedTaq
1 x	Initial denaturation	98 °C / 95 °C	30 seconds
35 x	Denaturation	98 °C / 95 °C	10 seconds
	Annealing primers*	45-72 °C / 50-65 °C	15 seconds
	Extension	72 °C	30 seconds/1 kb / 1 minute/1 kb
1x	Final extension	72 °C	300 seconds
	Hold	10 °C	

*The optimal annealing temperature for the primer pair was determined using the New England BioLabs Tm calculator.

All PCR products were purified using NucleoSpin®Gel and PCR Clean-up kit (Macherey-Nagel), and concentration was estimated using a NanoDrop 2000 Spectrophotometer (Thermo Scientific).

2.4 *S. aureus* susceptibility testing against bacteriocin H1

The protocol used to assess *S. aureus* isolates susceptibility toward H1 was adapted from the disc diffusion protocol from EUCAST (Matuschek et al., 2014). A total of 129 isolates of *Staphylococcus aureus* were streaked out on BHI agar plates and grown overnight at 37 °C (see **Table 6.1.A** in the appendix for an overview of isolates). Colonies were picked and deposited in a saline suspension (0.9% NaCl) to reach a turbidity of approximately 0.5 McFarland (at least four colonies). Suspensions were vortexed thoroughly to obtain a homogeneous suspension and subsequently (within 30 min) streaked on MH agar plates using a cotton swab and allowed to dry for around 15 min. The bacteriocin H1 was then applied at a volume of 5 µl of each of three dilutions: 1 mg/ml, 0.1 mg/ml, and 0.01 mg/ml, along with a penicillin G disc of 1 unite (0.600 micrograms) (Oxioid). The plates were incubated at 35 °C overnight (16-24 h). The diameter of zone size was measured in mm with a caliper, listed in **Table 6.1.A**. Cell suspensions were stored at -20°C after use and later used directly as a template for PCR in **section 2.5**.

2.5 PCR and sequencing of *rseP*

The *rseP* gene was amplified by PCR and sequenced from 34 isolates randomly selected from insensitive, intermediate, and sensitive isolates. PCR reactions of 50 µl were assembled as shown in **Table 2.3** using Phusion High–Fidelity DNA Polymerase (Thermo Scientific), and the primer pairs 3255_RseP_In_F1 (forward primer) and 3255_RseP_In_R2 (reverse primer), see **Table 2.2**.

Table 2.5. PCR reactions for amplification of rseP

Component	Concentration
5X Phusion HF Buffer	1X
dNTPs	200 µM
Forward Primer	0.5 µM
Reverse Primer	0.5 µM
Phusion DNA Polymerase	1 U

Thermocycling conditions used are shown in **Table 2.6**.

Table 2.6. Thermocycling conditions for amplification of rseP

Cycles	Step	Temperature	Time
1	Initial denaturation	98 °C	30 s
35	Denaturation	98 °C	10 s
	Annealing primers	60 °C	15 s
	Extension	72 °C	45 s
1	Final extension	72 °C	5 min
	Hold	10 °C	

PCR reactions were confirmed by agarose gel electrophoresis, then purified using NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel). Purified PCR products were sent for Sanger sequencing (Eurofins) with the same primer pair used in the PCR (two sequencing reactions per PCR product).

2.6 RseP sequence clustering analysis

The obtained chromatograms were mapped to the *rseP* sequence from *S. aureus* LMGT 3255, which was used as a reference using the software Snapgene (Dotmatics). All differences between the chromatogram sequence and reference were inspected manually to ensure accuracy. A consensus sequence of the two chromatograms was then assembled, and the open reading frame was translated and saved as a protein sequence. The protein sequences were clustered at a 100% identity threshold using the CD-HIT web server to identify unique RseP sequences (Fu et al., 2012; Li & Godzik, 2006). The unique RseP sequences are listed in **Appendix C**, and for an overview, see **Table 3.2**. Additional RseP sequences from the NCBI database were downloaded and clustered as a comparison. The RseP sequences from NCBI

identified according to the following search criteria were downloaded: sequence length of 428 residues, contained the words “zinc metalloprotease” in title or description, and was listed as belonging to the organism *S. aureus*.

Clustal Omega was used to construct a multiple sequence alignment and phylogenetic trees of all 34 sequenced isolates from the S. aureus collection and with cluster representatives from both the LMGT collection and NCBI, all listed in Appendix C (Madeira et al., 2022).

2.7 Residue level genotype-phenotype correlation analysis

A residue level genotype to phenotype correlation analysis was performed using SigniSite v2.1 (Jessen et al., 2013). The size of the inhibition zone produced by H1 toward each isolate was used as the phenotype parameter (in mm) (from section 2.4), and the RseP sequence (from section 2.5) as the genotype. The analysis was performed with a significance threshold α of 0.05 without multiple testing corrections.

2.8 Preparation of competent cells and transformation of *E. coli*, *S. aureus,* and *L. lactis*

2.8.1 Transformation of *E. coli* IM08B

E. coli IM08B cells were made chemically competent using a one-step procedure. A fresh overnight culture was diluted to 1/50 in LB medium, then grown at 37 °C (shaking at 180 rpm) to an OD_{600} ~ 0.4. The cells were harvested at 4500 x g for 10 min at 4 °C, then resuspended in 1/10 volume TSS buffer (10% PEG800, 5% DMSO, 50 mM $MgCl_2$ at pH 6.5). Competent cells were stored at -80 °C until use.

Chemically competent *E. coli* IM08B was thawed on ice, and then further incubated on ice with a ligation mixture or purified plasmid for 30 min, followed by a heat shock at 42 °C for 45 seconds. After two minutes at room temperature, 500 µl SOC medium was added (see **6.4** Appendix D), and cells were then incubated at 37 °C for 30 min with shaking before plating on LB with the selective antibiotic. Plates were incubated at 37 °C until visible colonies were present.

2.8.2 Electroporation of *S. aureus*

An overnight culture of *S. aureus* was diluted 1:100 in BHI and grown at 37 °C (shaking at 180 rpm) to an OD_{600} ~ 0.4. Cells were harvested by centrifugation at 4000 x g for 10 min at 4 °C and washed in 25 ml ice-cold Milli-Q water twice, using the same conditions for centrifugation. Cells were then washed in 25 ml ice-cold 10% glycerol three times. The pellet was resuspended in 2 ml ice-cold 10% glycerol with 0.5 M sucrose and stored at –80 °C until use.

S. aureus was transformed by electroporation using 3 µl of plasmid per 50 µL electrocompetent cells. A 1 mm electroporation cuvette was used in a Gene Pulser (Bio-Rad) attached to a Pulse Controller (Bio-Rad) with the settings 2.1 kV, 100 ohms, and 25 µF. Immediately following the electric pulse, 700 µl of ice-cold TSB with 0.5 M sucrose was added. The mix was incubated at 37 °C with shaking (180 rpm) for 2 hours and then plated on TSB agar with antibiotics for selection. Plates were incubated at 37 °C until visible colonies were present.

2.8.3 Electroporation of *L. lactis*

Electrocompetent *L. lactis* was prepared according to Holo and Nes (Holo & Nes, 1989, 1995). An overnight culture of *L. lactis* was diluted 1:100 in SGM17 (see **6.4** Appendix D) containing 1%, 1.5%, 1.8%, or 2% of glycine (Sigma-Aldrich) and incubated overnight at 30 °C. The highest glycine concentration, giving an OD600 of between 0.2 and 0.7, was harvested (5000 x g, 10 min, 4°C). The cell pellet was washed twice in 20 ml ice-cold 10% glycerol with 0.5 M sucrose. After washing, the pellet was resuspended in 1/100 volumes of ice-cold 10% glycerol with 0.5 M sucrose and stored at –80 °C until use.

L. lactis was transformed using 2 µl of ligation mixture per 40 µl of electrocompetent cells. Electroporation was done in a 2 mm cuvette with the settings 2.0 kV, 25 µF, and 200 Ω using a Gene Pulser (Bio-Rad) with a Pulse Controller (Bio-Rad). Immediately following the electric pulse, 960 µl ice-cold SGM17MC was added (SGM17 supplemented with 20 mM magnesium chloride and 2 mM calcium chloride) and then incubated for 2 hours at 30 °C before plating on GM17 with selective antibiotics. Plates were incubated at 30 °C until colonies were visible (1-2 days).

2.9 Cloning

2.9.1 Plasmids used in cloning experiments and

Table 2.7. Plasmids used in cloning experiments and heterologous expression.

Name	Characteristics	Reference
pCasSA-mod	Kan^R Tetracylin indacable Cas9 (tetR)	Lab collection
pLOW	Low copy number shuttle vector for IPTG-inducible expression of proteins in *S. aureus*, Amp^R (*E. coli*), Ery^R (*S. aureus*)	(Liew et al., 2011)
pNZ8037	Nisin induced, Cam^R	(de Ruyter et al., 1996)
pNZ9530	NisRK, Ery^R	(Kleerebezem et al., 1997)

2.9.2 Manipulation of pCasSA-mod for deleting *rseP* in *S. aureus*

This protocol used for cloning in pCasSA-mod was adapted from Chen et al. (Chen et al., 2017). The sgRNA sequence designed to target *rseP* was constructed from two oligos, RseP_Oligo1 and RseP_Oligo2 (**Table 2.2**). The two oligos were phosphorylated using T4 Polynucleotide Kinase (NEB) in T4 DNA Ligase buffer (NEB) at 37 °C for 1 hour. The oligos were annealed by heating to 95 °C for 3 min with 50 mM NaCl and allowing it to cool slowly at room temperature. Annealed oligos were diluted 1:10 in Milli-Q water.

Annealed oligos were cloned into the pCasSA-mod plasmid using Golden Gate assembly. A 10 µl reaction was assembled with 120 ng plasmid, 20 fmol annealed oligos, T4 DNA ligase (NEB), and 10 U BsaI-HFv2 (NEB). The following thermocycling conditions were used: 25 cycles of 37 °C 2 min and 16 °C 5 minutes. Followed by 50 °C 5 min, then 80°C 15 minutes.

Golden gate assembly mix was added directly (2 µl) to 50 µl chemically competent *E. coli* IM08B for transformation, see section 2.8.1. Transformants were selected using 30 µg/ml kanamycin. Transformants were verified by colony PCR and sequencing using primers pCasSA_sg_check_F and pCasSA_sg_check_R (**Table 2.2**). The correct plasmid was isolated from the transformant using E.Z.N.A.® Plasmid DNA Mini Kit (Omega Bio-Tek) and linearized with restriction enzymes BglI (NEB) and PvuI-HF (NEB) in NEB Buffer 3.1 and incubated overnight at 37 °C. The linearized plasmid was purified using NucleoSpin ® Gel and PCR Clean-up kit (Macherey-Nagel).

Primers amplifying 1000 bp up- and downstream for *rseP* were designed using the web program Benchling. The upstream homology was amplified using primers RseP_hom_up_F and RseP_hom_up_R, and the oligo downstream was made using the primers pairs RseP_hom_down_F and RseP_hom_down_R (**Table 2.2**). Both were amplified using Phusion DNA polymerase (ThermoFisher) in 50 µl reactions per the provided protocol. The PCR products were purified using NucleoSpin ® Gel and PCR Clean-up kit (Macherey-Nagel).

Linearized vector and homology arms were mixed in a molar ratio of 1:3:3 using 100 ng vector to a final volume of 2.5 µl with 2.5 µl of GeneArt Gibson Assembly HiFi Master Mix. Gibson assembly reaction mixture was incubated for 15 min at 50 °C. Chemically competent *E. coli* IM08B was transformed with the Gibson assembly mixture and selected on LB agar containing 30 µg/ml kanamycin. Transformants were checked with colony PCR using primers pCasSA_HOM_check_ F and pCasSA_HOM_check_R (**Table 2.2**.) and sent to sequencing at Eurofins. No transformants with both correct homology arms were detected, and the construction of the knock-out strain of *S. aureus* NCTC8325-4 was unsuccessful, due to the unsuccessful construction of pCasSA-mod.

2.9.3 Manipulation of pLOWCas9_aad9

The plasmid pLOW-dCas_aad9 was purified from *Escherichia coli* IM08B pLOW-dCas_aad9 using E.N.Z.A. ® Plasmid DNA Mini Kit (Omega Bio-Tek). Genomic DNA from *S. aureus* LMGT 3255 purified with GenElute ™ Bacterial genomic DNA Kit (Sigma-Aldrich). The purified DNA from *S. aureus* LMGT 3255 was used as a template for PCR to amplify the *rseP* gene using primers primers rseP_SalI_F_pLOW and rseP_EcoRI_R_pLOW (**Table 2.2**). PCR was performed using RedTaq (VWR) according to the provided protocol. The annealing temperature was 57 °C, and the extension time was 2 min. The PCR product was purified from 0,8% TAE agarose gel with NucleoSpin ® Gel and PCR Clean-up kit (Macherey-Nagel) and 30 µl elution buffer. The concentration was estimated with a NanoDrop 2000 Spectrophotometer (Thermo Scientific).

The purified PCR product and the pLOWCas9_aad9 were digested with FastDigest SalI (Thermo Scientific) and FastDigest EcoRI (Thermo Scientific) in FastDigest buffer (Thermo Scientific) at 37°C for 30 min. The digested PCR fragment was purified using the NucleoSpin ® Gel and PCR Clean-up kit (Macherey-Nagel). The linearized vector was first separated on a

0.8% TAE agarose gel, and the vector backbone was extracted from the gel and purified with the NucleoSpin ® Gel and PCR Clean-up kit (Macherey-Nagel). The concentration was estimated using the NanoDrop 2000 Spectrophotometer (Thermo Scientific).

The PCR product of *rseP* was ligated into the pLOW backbone with T4 DNA Ligase (NEB) following the protocol from the manufacturer, except that 100 ng of plasmid and 55 ng of insert were used. The ligation mix was incubated at 16 °C for 18 hours, then heat inactivated by incubating at 65 °C for 10 min. *E. coli* IM08B was transformed with the ligation mix as described in section 2.8.1, an plated out on LB agar containing 100 µg/ml ampicillin was used to select for transformants, which was incubated at 37°C until visible colonies were present. Colonies were confirmed by colony PCR with the primers pLOW_check_F and pLOW_check_R (**Table 2.2**). The plasmid from a positive transformant (expected fragment size by colony PCR) was purified using the E.N.Z.A. ® Plasmid DNA Mini Kit (Omega Bio-Tek). The concentration was estimated with a NanoDrop 2000 Spectrophotometer (Thermo Scientific).

S. aureus NCTC 8325-4 was transformed with the plasmid by electroporation as described in section 2.8.2 and plated out on TSB agar with 5 µg/ml erythromycin. When colonies were visible, transformants were picked for colony PCR with RedTaq (WVR) and primers pLOW_check_R and MK610 (**Table 2.2**). Prior to PCR, cells were first resuspended in MilliQ water and heated at 94°C for 5 min. For the PCR an annealing temperature of 57°C and extension time of 120 s was used. The PCR product was purified with NucleoSpin ® Gel and PCR Clean-up kit (Macherey-Nagel) and 30 µl elution buffer, and the concentration was estimated with a NanoDrop 2000 Spectrophotometer (Thermo Scientific). Purified PCR products were sent for Sanger sequencing (Eurofins) with the same primer pair used in the PCR (two sequencing reactions per PCR product). No correct transformants were identified.

2.10 Heterologous expression of *rseP* from *S. aureus* in *L. lactis*

Genomic DNA from *S. aureus* LMGT 3255 from section 2.9.3 was used as a template in a PCR with primers 3255RseP_BamHI_F and 3255RseP_XbaI_R (**Table 2.2**) amplify *rseP*. Phusion DNA polymerase (Thermo Fisher) was used for PCR following the recommended protocol provided by the manufacturer. An annealing temperature of 57 °C and an extension

time of 120 s was used. The size of the PCR product was confirmed on 0.8% TAE agarose gel and purified with Macherey-Nagel NucleoSpin® Gel and PCR Clean-up kit. The consternation was estimated with a NanoDrop 2000 Spectrophotometer (Thermo Scientific).

The lactococcal expression vector pNZ8037 and the PCR product were digested with FastDigest BamHI and FastDigest XbaI in FastDigest buffer (Thermo Scientific). Digests were incubated at 37°C for 30 minutes. Both reactions were purified using Macherey-Nagel NucleoSpin® Gel and PCR Clean-up kit. A ligation mix with the linearized vector pNZ8037 and the PCR product was prepared as described in section 2.9.3. *L. lactis* IL1403 Δ*rseP* was transformed with 2 µl of the ligation mixture as described in section 2.8.3 and plated on GM17 plates with 5 µg/ml chloramphenicol and incubated at 30 °C overnight.

Colony PCR with primers pNZ8037_test_F and pNZ8037_test_R (**Table 2.2**) was performed to confirm transformants in 50 µl PCR reactions using RedTaq DNA Polymerase (VWR). Colonies were first picked and deposited in Milli-Q water, then heated to 95 °C for 5 min. The annealing temperature was set to 51 °C and the extension time to 1 min and 45 s. Transformants were grown overnight at 30 °C in GM17 with 10 µg/ml chloramphenicol.

Plasmids were purified using the E.N.Z.A ® Plasmid DNA Mini Kit (Omega Bio-Tek) according to the provided protocol with one modification. Solution I was supplemented with 4 mg/ml lysozyme (Sigma-Aldrich) and incubated at 37 °C for 30 minutes before proceeding with the protocol. The plasmid was sequenced using Sanger sequencing (Eurofins) to ensure the correct insert. The transformant with correct plasmid *L. lactis* pNZ8037-*rseP*$_{3255}$ was made electrocompetent and transformed again as described in section 2.8.3 with the plasmid pNZ9530 to allow for inducible expression from the pNZ8037 vector. After transformation, selection and growth was done in GM17 with 5 µg/ml of erythromycin and 5 µg/ml chloramphenicol.

2.10.1 Targeted mutagenesis of RseP by Overlap extension PCR

A PCR fragment was constructed of the *rseP* gene from *S. aureus* LMGT 3255 containing two amino acid substitutionsE304K and K308I. The changes were introduced with the primers and overlap extension PCR. To make the first fragment for the overlap PCR, primers 3255RseP_BamHI_F and 3255RseP_EK_to_KI_R were used, and to make the second

fragment primers 3255RseP_XbaI_R and 3255RseP_EK_to_KI_F were used (**Table 2.2**). Genomic DNA from section 2.9.3 was used as the template in a PCR reaction with Phusion DNA polymerase (Thermo Scientific). The PCR was prepared according to the protocol from the manufacturer: annealing at 63 °C and an extension time of 30 s. The fragments were separated on 0.8% TAE agarose gel and purified using Macherey-Nagel NucleoSpin® Gel and PCR Clean-up kit.

The two fragments were mixed in equimolar amounts in a new PCR with the outer primers 3255RseP_BamHI_F and 3255RseP_XbaI_R (**Table 2.2**). Thermocycling conditions included an initial denaturation at 98 °C for 30 s, followed by 10 cycles of denaturation (98 °C, 15 s), annealing (51 °C, 10 s), and extension (72 °C, 30 s). Then, 35 cycles of denaturation at 98 °C for 10 s, annealing at 71 °C for 10 s, and extension at 72 °C for 45 s. With a final extension at 72 °C for 7 min.

The PCR product verified by gel electrophoresis on a 0.8% TAE agarose gel and purified from the gel with a Macherey-Nagel NucleoSpin® Gel and PCR Clean-up kit. The restriction enzymes FastDigest BamHI and FastDigest XbaI (Thermo Scientific) were mixed with purified PCR product and the pNZ8037 vector in separate reactions according as described previously and incubated for 30 min at 37 °C. The two reactions were purified using the Macherey-Nagel NucleoSpin® Gel and PCR Clean-up kit.

A ligation mix of the digested vector pNZ8037 and insert was set up as described in section 2.9.3 and electroporated into *L. lactis* IL1403Δ*rse*P as described in section 2.3.3, and plated on GM17 plates with 5 µg/ml chloramphenicol and incubated at 30 °C o/n. The electroporation was repeated and electroporated into *L. lactis* IL1403Δ*rse*P already containing pNZ9530, selected with 5 µg/ml erythromycin.

2.11 Heterologous expression of $RseP_{3255}$ and H1 susceptibility testing

L. lactis IL1403 Δ*rseP* and *L. lactis* IL1403 with both pNZ9530 and pNZ8037 or pNZ8037*rseP*$_{3255}$ were grown overnight in GM17 containing 5 µg/ml chloramphenicol and 5 µg/ml erythromycin at 37 °C. The culture was then diluted in GM17 with antibiotics (2 % inoculum) and incubated for four hours at 30 °C. Nisin was added to a final concentration of 0.5 ng/ml for induction of recombinant RseP expression. After induction, cultures were incubated for 1 h at 30 °C. Cultures were then diluted 50-fold in GM17 soft agar (0.8% agar) with 0.5 ng/ml nisin and antibiotics and poured over GM17 plates also containing nisin at 0.5 ng/ml and antibiotics. 5 µl with H1 at a concentration of 1 mg/ml, 0.1 mg/ml, and 0.01 mg/ml was spotted on the plate. The plate was incubated overnight at 30 °C.

3 Results

3.1 Most isolates of *S. aureus* in the LMGT-collection are susceptible to H1

The bacteriocin H1 is an engineered novel bacteriocin constructed by fusing the N-terminal half of EntK1 to the C-terminal half of EntEJ97 (Kranjec et al., 2021). Initial sensitivity testing of H1 against a large panel of bacterial species showed that H1 was particularly active against *S. haemolyticus*, while no activity was observed against *S. aureus*. However, one isolate of *S. aureus* designated LMGT 3255 was later found to be very sensitive to the hybrid bacteriocin (n = 8) (Ottesen, 2023). To gain a better understanding of the activity of H1 against *S. aureus*, sensitivity testing was performed on an extensive collection of 129 isolates of *S. aureus* belonging to the LMGT (Laboratory of Microbial Gene Technology) collection. Using a spot-on-lawn assay, the size of the inhibition zone produced by H1 against each isolate was measured. The isolates were classified according to the characteristics of the inhibition zones. Of the isolates tested, 97 (75%) were sensitive to the bacteriocin with an inhibition zone larger than 6 mm (**Table 3.1**). Only 26 isolates (20%) were insensitive to the bacteriocin in this collection, with no zone of inhibition or a non-transparent zone of inhibition. The remaining 6 isolates were considered to have an intermediate susceptibility (inhibition zone larger than 1 mm but below 6 mm). For all the isolates' inhibition zone to H1, see **Appendix A**.

Table 3.1. Distribution of H1 sensitivity in *S. aureus* isolates from the LMGT collection. The bacteriocin H1 was used at 1 mg/ml, and 5 µl was spotted on each plate. It is the diameter of the inhibition zone listed in mm.

Inhibition zone (mm)	Isolates
0	26
1	1
2	1
3	1
4	1
5	2
6	6
7	17
8	28
9	29
10	15
>10	2
Total:	129

It should be noted that the insensitive isolates of *S. aureus*, included the most common laboratory strains used, such as NCTC 8325-4, RN4220, SH1000, and COL. The only sensitive among the well-characterized laboratory strains was *S. aureus* N315.

3.2 Sensitivity to H1 in *S. aureus is* correlated with sequence variations in *rseP*

The LsbB bacteriocin family, from which H1 is derived, is known to exploit the zinc metalloprotease RseP as its target receptor, and binding of H1 to RseP is required for its antimicrobial activity (Kranjec et al., 2021). It has been shown for EntK1 and EntEJ97 that sequence variations in RseP are important for sensitivity in enterococci. To examine if differences in RseP between the *S. aureus* isolates could explain the differences in susceptibility towards H1, the *rseP* gene was sequenced (using Sanger sequencing) from 34 randomly selected insensitive, intermediate, and sensitive isolates.

Sequence cluster analysis of the obtained sequences revealed 9 unique RseP sequences among the 34 isolates (clustered at 100% protein sequence identity), as shown in **Table 3.2.** Representative sequence from each cluster can be found in **Appendix C**. Grouping of isolates with the same RseP sequence (clusters), showed similar sensitivity towards H1 within each group. The exception was strain N315, which was less sensitive than the other members of the same cluster **(Table 3.2**). For the rest, isolates within the same group (cluster) varied in sensitivity by no more than 3 mm (difference in inhibition zone diameter).

Table 3.2. Cluster analysis of the amino acid sequence from 34 isolates. Clustering was performed with cd-hit at an identity threshold of 1 (100%). The H1 zone was from 5 µL at the concentration of 1 mg/ml.

Cluster	Isolate	H1 zone (mm)
Cluster 1	3041	6*
Cluster 2	MU50	10
	3222	10
	N315	4
	3263	8
	3264	8
	3265	10
Cluster 3	3043	6
Cluster 4	3225	0
	3227	0
	RN 4220	0
	COL	0
	3258	0
	3273	0
	NCTC 8325	0
Cluster 5	A70	10
	3255	10
	3259	7
	3272	8
	3275	7
	3277	8
	3305	7
	3306	9
	3898	7
	3956	8
	3959	9
Cluster 6	3256	10
	3257	8
	3260	8
	3274	9
Cluster 7	3261	5*
	3262	6*
Cluster 8	3271	5*
Cluster 9	3276	8

Isolates with a 4-digit number are from the LMGT collection (Laboratory of Microbial Gene Technology).
*: The zone is fuzzy and with an unclear edge (see **Figure 3.1**).

To illustrate what is defined as a sensitive cluster, one representative from each cluster went through a new susceptibility assay, as described in section 2.4. **Figure 3.1** shows the sensitivity of each cluster. Clusters 2, 5, 6, and 9 have zones with full clearing (no growth) and a sharp edge. They are the most sensitive and have the biggest zones, as shown in **Table 3.2**. Although spontaneous resistant mutants occasionally can be seen as single colonies

within the inhibition zones (e.g., cluster 5 at 1 mg/ml). However, these are relatively rare and not expected to grow in the entire zone. For this reason, isolates with diffuse inhibition zones were considered intermediate sensitive. The inhibition zones from clusters 1, 7, and 8 showed visible growth in the zone and with fuzzy edges (note that the growth within the zones is difficult to judge based on the images in **Figure 3.1**). Clusters 1, 7, and 8 were diffuse and unclear and therefore considered intermediate sensitive. In cluster 3, the zone contained many more mutants, but the zone is clear of any growth when looking away from these mutants and has a sharper edge than the insensitive clusters. This cluster was also considered intermediate sensitive. Cluster 4 appears to be completely insensitive, with almost no visible zone in 1 mg/ml, and was the only cluster being insensitive to H1.

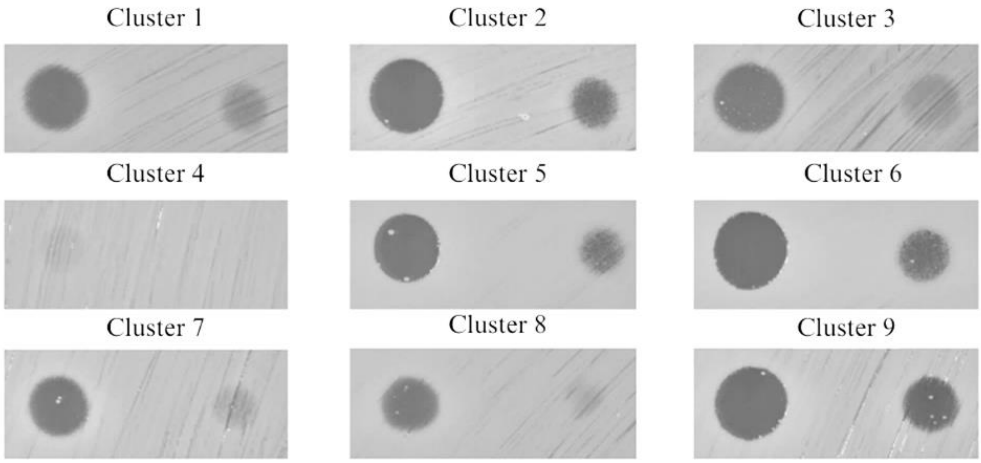

Figure 3.1. Inhibition zones from H1 at 1 mg/ml (left) and 0.1 mg/ml (right) against one representative isolate from each cluster. Although not easily visible in the image, inhibition zones for clusters 1, 7, and 8 were diffuse/unclear and therefore grouped as intermediate sensitive.

A multiple sequence alignment of all 34 RseP sequences from the clusters was used to construct a phylogenetic tree **Figure 3.2.** The insensitive and sensitive clusters are clearly separated, indicating that insensitive isolates are more distantly related with more sequence differences.

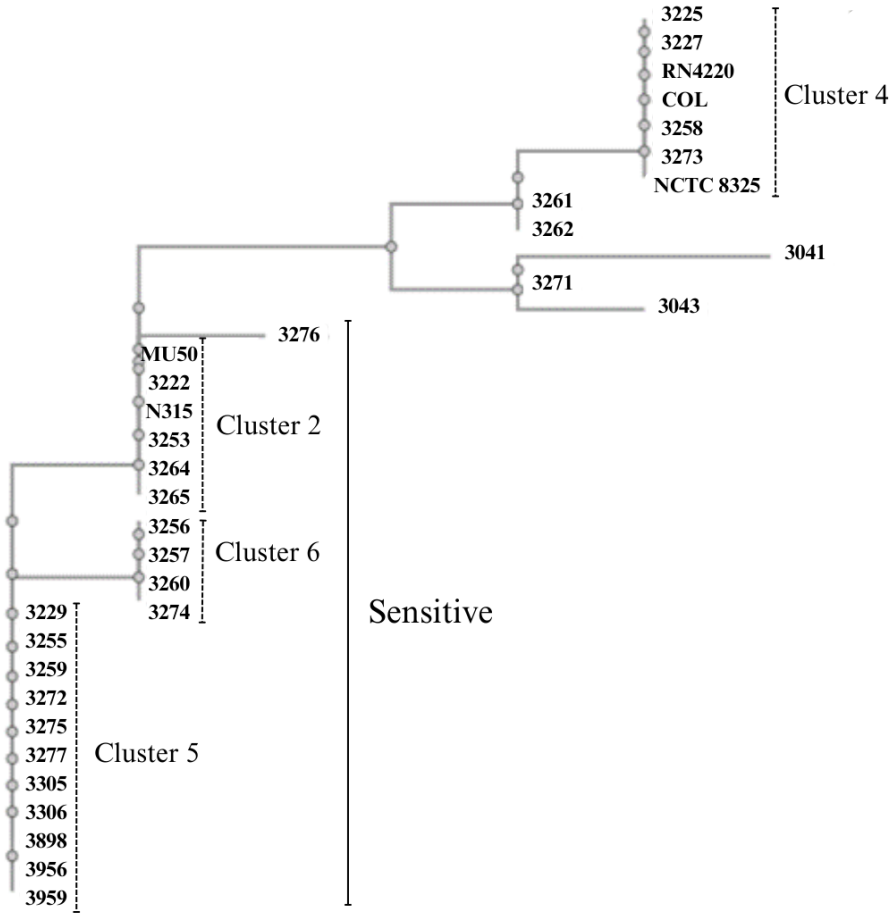

Figure 3.2. Phylogenetic tree of the 34 RseP sequences, constructed using Clustal Omega (Madeira et al., 2022). All 4-digits refer to an isolate in the LMGT collection. Only the clusters with more than two representatives are marked with a dotted line, and only the sensitive ones are marked with a solid line.

A search in the NCBI database was conducted to investigate how representative the LMGT strain selection is compared to the sequenced *S. aureus* genomes in the database. 5950 sequenced site-2-metalloproteases in *S. aureus* were downloaded. Along with the 34 sequenced *rseP* from the LMGT collection, a new clustering of nearly 6,000 sequences was performed. This cluster analysis gave 122 clusters (122 unique RseP sequences) at 100% protein sequence identity level. This was 113 more than the number found within the sequences only from the LMGT collection. The cluster analysis also reveals that 64% of the 122 clusters only contain one sequence, and 88% have below 10 sequences within the cluster. This indicates that the database contains many unique sequences.

Table 3.3. Overview of the three largest clusters from the NCBI sequences and LMGT sequences.

Cluster	Number of sequences[I]	Fraction of total sequences[I]	Number of LMGT-sequences	Fraction of LMGT-sequences
Cluster 1[II]	1	0 %	1	3 %
Cluster 2	682	11 %	6	18 %
Cluster 3[II]	1	0 %	1	3 %
Cluster 4[II]	7	0 %	7	21 %
Cluster 5	570	10 %	11	32 %
Cluster 6	115	2 %	4	12 %
Cluster 7[II]	2	0 %	2	6 %
Cluster 8[II]	1	0 %	1	3 %
Cluster 9[II]	1	0 %	1	3 %
Cluster 10	2435	41 %	-	-
Cluster 11	316	5 %	-	-
Cluster 18	1496	25 %	-	-
Total	5984		34	

[I]: NCBI-sequences and LMGT-sequences. [II]: only in the LMGT collection.

The five largest clusters contain over 90% of the 6000 sequences (clusters 2, 5, 10, 11, and 18). Therefore, examining the biggest clusters may be a better representation. It was also noted that clusters 1, 3, 4, 7, 8, and 9 (all containing one or a few isolates) are unique to the LMGT collection. The analysis showed, however, that three of the five main clusters (10, 11, 18) were not represented in the LMGT collection used here. Clusters 2, 5, and 6 contain both isolates than only the LMGT isolates. The total number of isolates in these clusters adds up to 1380, containing 23% of the total number of the 5984 sequences. The isolates in these three clusters are expected to be sensitive to H1 because they have the same *rseP* as the most sensitive clusters.

A phylogenetic tree constructed of an MSA with cluster representatives from the 11 first clusters and number 18 (**Figure 3.3)**. These comprise 9 from the LMGT collection and the three largest from the NCBI collection not found in the LMGT collection. (clusters 10, 11, and 18, **Table 3.3**). This analysis was performed to identify whether the most common *rseP* in the NCBI database not represented in the LMGT dataset is closely related to any of the LMGT sequences. Here, it was found that cluster 10 and cluster 18 clustered close to cluster 4 and 7, while cluster 11 was closely related to cluster 3 sequences. Thus, all of these not-tested sequences were most closely related to non-sensitive RseP-clusters.

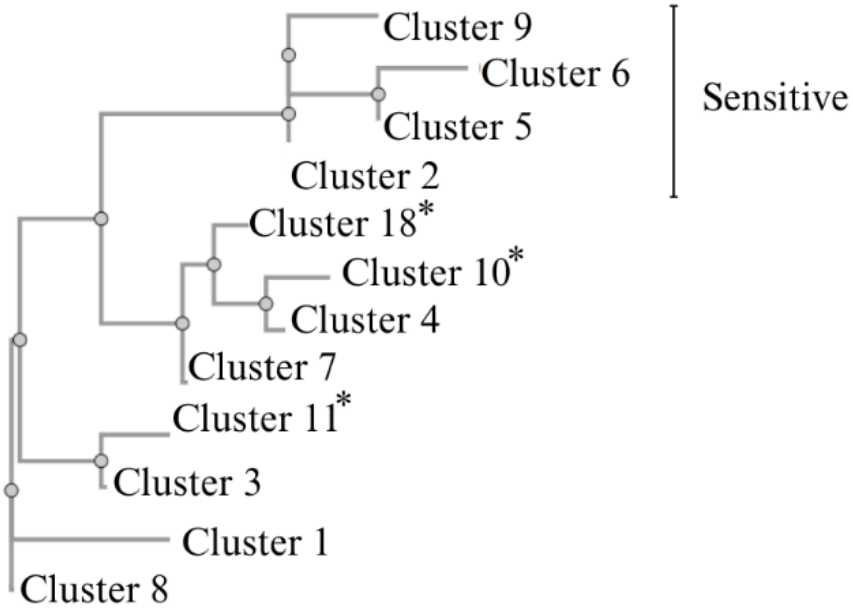

Figure 3.3. A phylogenetic tree of clusters 1-11 and 18, constructed with Clustal Omega (Madeira et al., 2022). *Sensitivity has not been determined experimentally. Only the sensitive clusters are marked with a solid line.

3.3 Specific residues are correlated with H1 susceptibility in *S. aureus*

Next, the specific amino acid differences between clusters 1-9 were studied in more detail. Differences in the RseP sequence were found in 11 positions between all 9 clusters (**Figure 3.4)**. For positions 188, 218, 233, and 257, variable residues are only found in one cluster, while the rest are identical. For positions 272, 293, 301, 304, 305, 308, and 316, on the other hand, the same variation of residue can be found in more than one cluster, but they are still either one of two residues.

```
          ▼          ▼          ▼          ▼          ▼          ▼       ▼  ▼▼  ▼       ▼
Clu1  186 ALVLFIG  216 AGLQKG  231 KIFEFD  255 ERDGKT  270 TERKLT  291 SEHTLFKPIVYGFKNFLIGSTLIFSAVV
Clu2  186 ALVLFIG  216 AGLQKG  231 KISEFD  255 ERDGKT  270 TEKKLT  291 SEHTLFKPIVFGFKSFLIGSTLIFTAVV
Clu3  186 ALVLFIG  216 AGIQKG  231 KISEFD  255 ERDGKT  270 TERKLT  291 SEHTLFKPIVYGFKSFLIGSTLIFSAVV
Clu4  186 ALVLFIG  216 AGLQKG  231 KISEFD  255 ERDGKT  270 TEKKLT  291 SEHTLFKPIVFGFKSFLIGSTLIFTAVV
Clu5  186 ALVLFIG  216 AGLQKG  231 KISEFD  255 ERDGKT  270 TERKLT  291 SERTLFKPIVYGFESFLKGSTLIFTAVV
Clu6  186 ALVLFIG  216 AGLQKG  231 KISEFD  255 ERNGKT  270 TERKLT  291 SERTLFKPIVYGFESFLKGSTLIFTAVV
Clu7  186 ALVLFIG  216 AGLQKG  231 KISEFD  255 ERDGKT  270 TERKLT  291 SEHTLFKPIVFGFKSFLIGSTLIFTAVV
Clu8  186 ALVLFIG  216 AGLQKG  231 KISEFD  255 ERDGKT  270 TERKLT  291 SEHTLFKPIVYGFKSFLIGSTLIFSAVV
Clu9  186 ALDLFIG  216 AGLQKG  231 KISEFD  255 ERDGKT  270 TERKLT  291 SEHTLFKPIVYGFESFLKGSTLIFTAVV
```

Figure 3.4. Sequence variations in RseP among the 9 clusters, MSA constructed with Clustal Omega (Madeira et al., 2022). Variable residue positions are indicated with a triangle above, and a bold letter in the sequence.

To investigate which residues were most important in correlating if an isolate was susceptible to H1 or not, a statistical analysis was performed using a program called SigniSite (Jessen et al., 2013). A significance threshold α of 0.05 with no corrections was chosen. This program identified which residues correlate with a phenotype (expressed as a numeric value), positive correlation is given a high Z-score. The phenotype in this context is the size of the inhibition zones of H1 (see **Table 3.2**). In this analysis, positions 304 and 308 were recognized as most significant. In addition, positions 272, 293, and 301 were also identified as significantly correlated with sensitivity.

Figure 3.5. Amino acid residues on the positive y-axis are associated with strong phenotype values (large zones), and residues on the negative y-axis with weak phenotype values (small zones), i.e., residues above the z=0.0 line have a z-score larger than zero and are thus predominant. Blu residues are positively charged, black residues are non-polar, green are polar, and red are negatively charged.

In **Figure 3.6**, the residues and their positions identified by the SigniSite analysis as mist significant are highlighted in the superimposition of predicted structures of RseP from *S. aureus* LMGT 3225 (cluster 4, insensitive, in green), and *S. aureus* LMGT 3255 (cluster 5, sensitive, in blue).

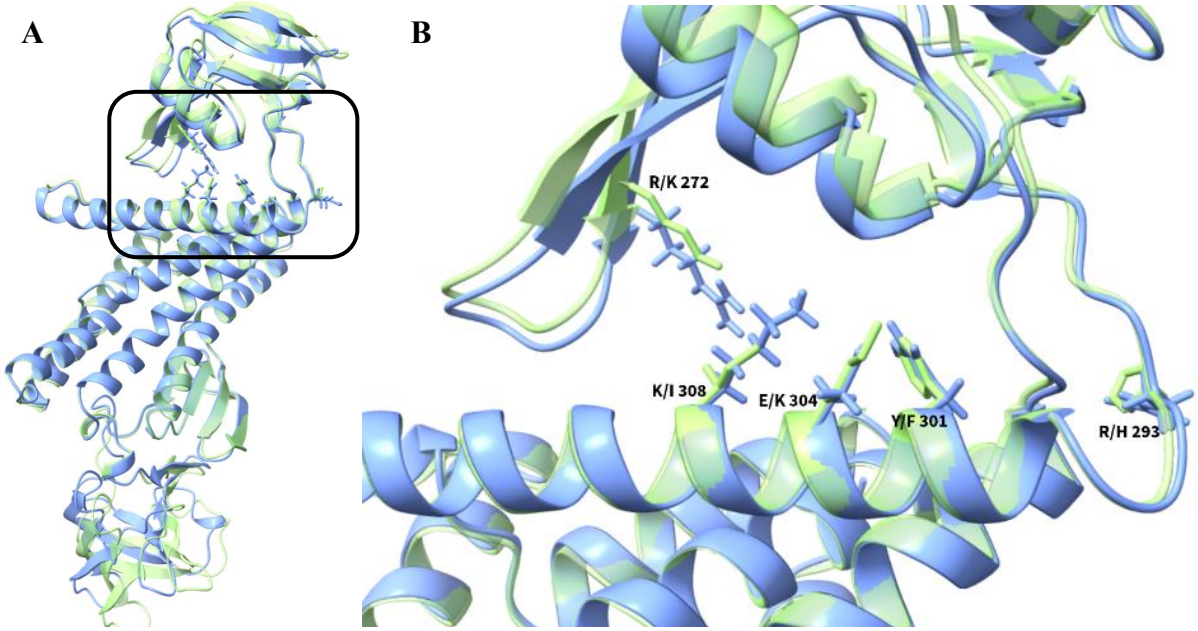

Figure 3.6. A predicted structure of RseP from3225, in green, and 3255 in blue made separately by ColabFold (Mirdita et al., 2022), superimposed and visualized in ChimeraX (Goddard et al., 2018; Meng et al., 2023; Pettersen et al., 2021). **A**: shows the whole RseP, and the black square indicates the same residue on the zoomed-inn part to visualize the different residues better. **B:** The residues in positions 272, 293, 301, 304, and 308 are shown with side chains to illustrate the differences between the two proteins aligned on top of each other.

3.4 Attempts to delete *rseP* and heterologous expression in *S. aureus* NCTC8325-4

To verify further experimentally whether the identified residues (Fig. 3.5 and 3.6) were determinants for sensitivity to H1, the aim was to alter these two residues from a sensitive RseP cluster sequence to see whether this also alters the phenotype from sensitive to not sensitive. Specifically, it was decided to change E to K in position 304 and K to I in position 308, because the four sensitive clusters, 2, 5, 6, and 9, see **Figure 3.1**, from **Figure 3.4** all had E (glutamate) in position 304 and K (lysine) in position 308. All the others had K (lysine) in position 304 and I (isoleucine) in position 308.

Because transformation in most of the *S. aureus* isolates from the LMGT collection is difficult, it was decided to use the easily transformable lab strain NCTC8325-4. *S. aureus* NCTC8325-4 is not sensitive to H1. To avoid interference with the native *rseP*, it was attempted to make a deletion of *rseP* in *S. aureus* using CRISPR/Cas9-system in plasmid pCasSA-mod (see section 2.9.2). However, construction of the appropriate plasmid needed for this deletion could not be made. Next, it was attempted to heterologous express an alternative *rseP* from plasmid pLOW (see section 2.9.3) also in *S. aureus* NCTC8325-4. After many attempts to construct the correct pLOW-plasmid, no successful variants were detected in *S. aureus*. While the insert appeared correct after cloning in *E. coli* IM08B, only truncated plasmids were found after transformation into *S. aureus*. Therefore, it was decided to move on to use *L. lactis* IL1403Δ*rseP* as a heterologous host for the expression of *rseP* variants.

3.5 Heterologous expression of *S. aureus rseP* in *L. lactis*

To investigate the effect of altering E to K in position 304 and K to I in position 308 in RseP, a strain without a native copy of *rseP* was important to use to be able to interpret the zone size correctly. A strain of *Lactococcus lactis* IL1403 Δ*rseP* was therefore used (unpublished).

The *rseP* gene from *S. aureus* LMGT3255 (cluster 5), was cloned into plasmid pNZ8037 downstream of a nisin-inducible promoter. The plasmid was transformed into an *L. lactis* IL1403Δ*rseP*, along with the plasmid pNZ9530. The pNZ9530 encodes for the regulatory genes *nisR* and *nisK* and allows for a nisin-induction to regulate the inserted gene, *rseP*. *An L. lactis* IL1403 and *L. lactis* IL1403ΔrseP, both harbouring the empty pNZ8037 vector, were included as a control.

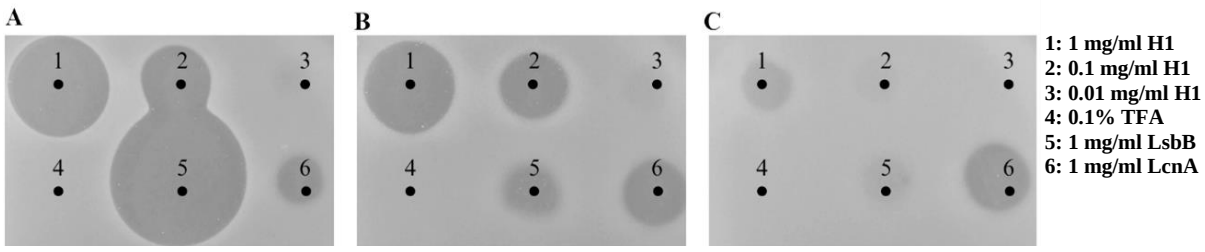

Figure 3.7. Nisin induction test of the plasmid constructed with *rseP* from *S. aur*eus LMGT 3255 in *L. lactis*. Adding 10 µL in spots 1-6, specified on the right. A: *L. lactis* IL1403 WT with pNZ9530 and pNZ8037, B: *L. lactis* IL1403Δ*rseP* with pNZ9530 and pNZ8037-*rseP*$_{3255}$. C: *L. lactis* IL1403 Δ*rseP* with pNZ9530 and pNZ8037

Figure 3.7 shows that expression of *rseP* from *S. aureus* LMGT3255 induces sensitivity to H1. While an diffuse inhibition zone is only visible at the 1 mg/ml H1 in the empty vector control (**Figure 3.7**C), clear growth inhibition is observed both at 1 mg/ml and at 0.1 mg/mL H1 upon expression of *rseP*$_{3255}$ (**Figure 3.7**B). It can also be noted that H1 is equally active against the *L. lactis* IL1403 wild type (**Figure 3.7**A). Furthermore, a number of controls were included in the assay. As expected, the solvent TFA did not cause any growth inhibition. Furthermore, it is evident that the WT-*rseP* is much more sensitive to the bacteriocin LsbB (in point 5) compared to the *rseP*-deletion strain. This is as expected since LsbB has been reported to target *L. lactis* RseP as a receptor specifically. The small and diffuse zone in point 1 (**Figure 3.7**C) can be an unspecific interaction because the zone is much smaller and diffuse. Finally, the non-related bacteriocin lactococcin A, whose mechanism is independent of RseP was also included. As expected, inhibition was observed in all three strains.

Based on the most significant residues for the genotype-phenotype correlation analysis in **Figure 3.5**, overlap extension PCR was performed to change of residue E to K in position 304 and K to I in position 308 of *rseP*$_{3255}$ in plasmid pNZ8037. This new plasmid was called pNZ8037- *rseP*$_{EKKI}$. The phenotype of this new plasmid was compared to pNZ8037-*rseP* $_{3255}$, IL1403Δ*rseP* and IL1403with pNZ9530 and empty pNZ8037.

Similar type of bacteriocin sensitivity test as above was done, except that the volume of the bacteriocin drop was reduced from 10 to 5 µl.

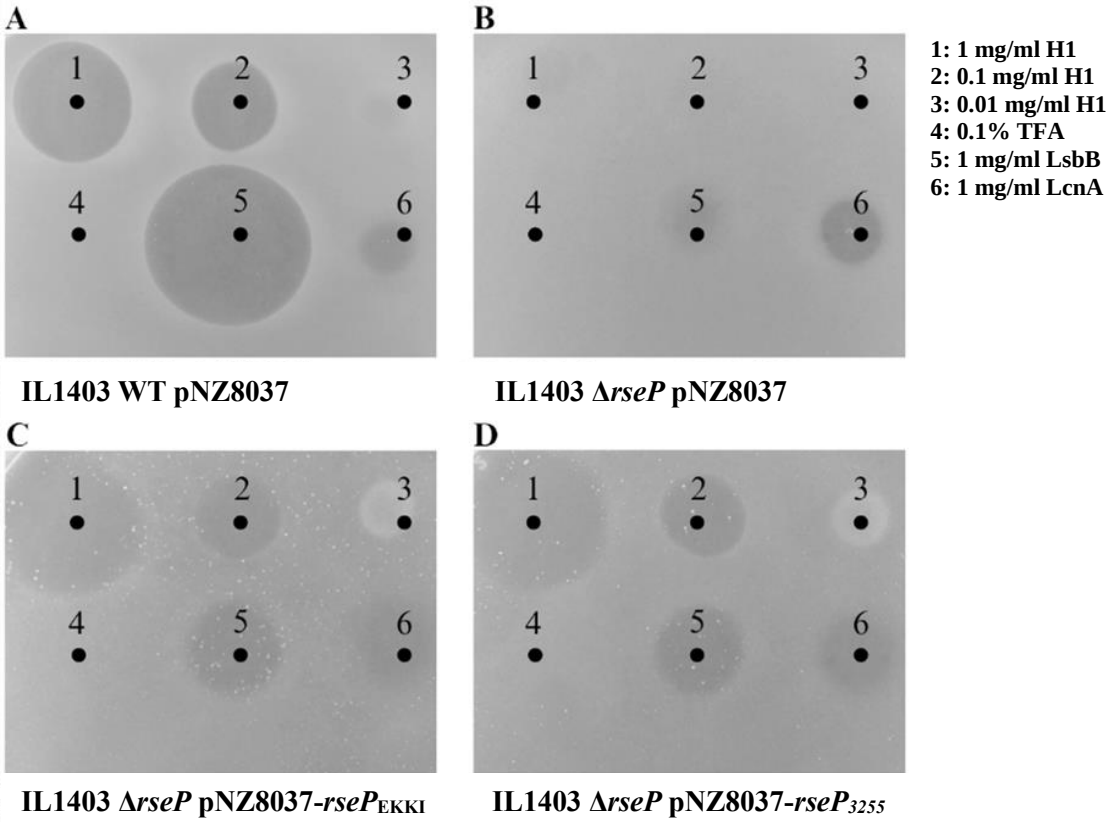

Figure 3.8. Nisin induction test after altering EK to KI. Adding 5 µL in spot 1-6. A: *L. lactis* IL1403 WT with pNZ9530 and pNZ8037, B: *L. lactis* IL1403 Δ*rseP* with pNZ9530 and pNZ8037. C: *L. lactis* IL1403 Δ*rseP* with pNZ9530 and pNZ8037-*rseP*$_{EKKI}$. D: *L. lactis* IL1403 Δ*rseP* with pNZ9530 and pNZ8037-*rseP*$_{3255}$.

For wildtype IL1403, there are still large zones in points 1, 2, and 5 (**Figure 3.8**A), the same as in **Figure 3.7**When *rseP* variants are expressed from a vector (**Figure 3.8** C and D), the growth on the plate was much poorer compared to wild-type (with natural regulation of rseP expression) and empty vector (**Figure 3.8** A and B). The growth appeared as single colonies (**Figure 3.8** C and D), as opposed to the smooth lawn of growth in the soft agar (**Figure 3.8** A and B). Unexpectedly, mutating E to K in position 304 and K to I in position 308 in *rseP* from *S. aureus* 3255 did not seem to change the sensitivity of the strain (**Figure 3.8** C). They both display a large inhibition zone in point 1 and a clearly defined zone in point 2. The white zone in spot 3 in **C** and **D** is absent in **A** and **B** (**Figure 3.8**). This has not been present in the previous tests; maybe it is an unspecific reaction because of the poor growth. The same controls as in **Figure 3.7** were included in **Figure 3.8**. The small zone in point 1 in **B** is absent in **Figure 3.8** compared to **Figure 3.7**, indicating that this interaction was unspecific. The zone in point 6, with lactococcin A, was also much smaller and more diffuse in **C** and **D**, which may come from the poor growth. The zone in pot 6 was smaller for all in **Figure 3.8** compared to **Figure 3.7** because the volume of added bacteriocin was reduced.

4 Discussion

H1 is a synthetically engineered hybrid of the N-terminal of K1 and the C-terminal of EJ97 (Kranjec et al., 2021). RseP is a site-2 protease and a highly conserved protein with numerous homologs in a wide variety of species. RseP is essential for binding LsbB-like bacteriocins, such as H1, and acts as the target receptor in the bacterial cell membrane for these bacteriocins (Kristensen et al., 2022). By uncovering which residues are involved in the interaction between H1 and RseP, novel bacteriocins with even broader spectra than EJ97 and H1 can be designed. In this work, the link between H1 sensitivity and RseP sequence variations was examined in *S. aureus*.

4.1 Methods for determining sensitivity to bacteriocin H1

The protocol for determining the prevalence of susceptibility to H1 in the LMGT collection of *S. aureus* was a "spot-on-lawn" assay, which was set up by adapting the protocol from the EUCAST disc diffusion assay (Matuschek et al., 2014). This was because one disc of penicillin G was used, but the result will not be used for any other purpose than comparing the zone size between isolates in this project.

To obtain comparable zone sizes between the isolates, equal molecular diffusion of identical molecules was assumed between agar plates, and therefore all the plates were prepared in the same manner. Obtaining the approximate turbidity of 0.5 McFarland from four colonies is a key step in the methodology (see Methods section 142.4), and obtaining a homogeneous saline suspension may be the most varying factor in this part of the experiment. Not all the isolates had colonies of the same size, but to account for smaller colonies, at least four or more were added to obtain the desired turbidity. The cotton swab used was intended to absorb equal amounts, but this can also be a factor requiring improvement. A more accurate approach could involve using a micropipette to extract an equal amount of each suspension and then streak out on a plate.

An alternative to the "spot-on-lawn" assay could be the minimum inhibition concentration (MIC) assays. MIC assays were also conducted to provide an additional quantitative measure of sensitivity. However, the optical density (OD) measurements were taken prematurely, making it difficult to interpret the results because the OD had not yet reached a sufficient

value. Another issue was that the bacteriocin H1 appeared to precipitate at high concentrations. This issue could potentially be resolved by pipetting more while mixing in the bacteriocin. Furthermore, when OD was measured every 20 minutes for a duration of 20 hours, a mutant often emerged, leading to growth from the mutant in the well. The results from MIC assays were consequently excluded from the thesis.

4.2 The prevalence of susceptibility towards H1 among *S. aureus* isolates in the LMGT collection

In this work, a collection of 129 *S. aureus* isolates were tested for their susceptibility to the bacteriocin H1. In the susceptibility prevalence mapping assay, 75% of the isolates had an inhibition zone of 6 mm or larger after spotting 5 µl of H1 at a concentration of 1 mg/ml and were considered susceptible or sensitive. While 20% of the strains were classified as non-sensitive, 5 % had a zone below 6 mm and were considered intermediate sensitive. This high fraction of strains being sensitive to H1 was surprising, given that in an initial assay with 8 different strains of *S. aureus*, only one strain (12.5%) was found to be susceptible (Ottesen, 2023). This finding indicates that a significant fraction of *S. aureus* isolates are sensitive to H1, which underscores the promising potential of H1 as a potential future treatment alternative against *S. aureus*. It should be noted that H1 sensitive strain include both penicillin-susceptible and non-susceptible strains, which is not surprising given that H1 has a completely different antimicrobial mechanism compared to penicillin. 22% of the H1 sensitive strains were penicillin resistant. See **Appendix A** and **Appendix B**.

However, it should be noted that the LMGT collection utilized as a source of isolates in this study is primarily comprised of isolates of animal origin. After analysis of the NCBI genome database, this collection appears to lack common RseP representatives compared to collections found in public databases. When clustering RseP sequences from the NCBI search with the LMGT collection, 122 clusters were identified. Among these, 64% of the clusters were comprised of a single sequence, and over 90% of the 5984 sequences could be found in the 5 most prominent clusters. Based on this clustering, 23% of the sequences from NCBI were identical to RseP variants in the LMGT collection, found in clusters 2, 5, and 6. Notably, many of the clusters from the LMGT collection remained without corresponding isolates from the NCBI search. This can indicate that the isolates from the LMGT collection potentially

diverge from the NCBI database. It should also be noted that the sequences from NCBI may not accurately represent the true biological diversity of *S. aureus*; it may present a skewed picture focusing on isolates of human clinical origin. While a more comprehensive phylogenetic analysis is required to draw more robust conclusions regarding the phylogeny, it seems reasonable to assume that the collection used here only represents a subset of *S. aureus* isolates (see further discussion on RseP sequences and correlation to H1 sensitivity below).

4.3 Correlation between H1 sensitivity and sequence of the RseP receptor

The susceptibility prevalence assay showed large differences in H1 sensitivity between isolates. The LsbB-group of bacteriocins, such as H1, use RseP as a target receptor in sensitive cells (Kranjec et al., 2021). Therefore, it was hypothesized that differences in sensitivity could result from variation in the RseP sequence. By sequencing the *rseP* gene of 34 randomly selected isolates with variable sensitivity, 9 different clusters of RseP sequence were found in the LMGT collection.

The phenotype-genotype association analysis was done based on the H1 sensitivity and the RseP sequence of 34 randomly selected isolates with varying sensitivity. All the isolates that showed no inhibition zone towards H1 had the same RseP sequence found in cluster 4, indicating that a specific sequence can be associated with insensitivity toward H1. In contrast, isolates demonstrating an intermediate inhibition zone have unique RseP sequences and are separated into 4 clusters, clusters 1, 3, 7, and 8, suggesting that slight variations in RseP may lead to unspecific interaction and a varying degree of sensitivity. Furthermore, isolates with an inhibition zone of 7 mm and above were grouped in 4 different clusters, clusters 2, 5, 6, and 9, with multiple isolates within all clusters except 9.

It should be noted that this work did not assess the variation in expression of the *rseP* gene between the isolates. Thus, when comparing the zone sizes and correlating this to RseP sequences, it was assumed that the expression and regulation of RseP are similar between the different isolates. The promotor region of the isolates could be compared to examine this assumption further, but this region was not sequenced. The regulation of *rseP* is not well studied, and how *rseP* expression is affected by different environmental factors and strain

backgrounds is unknown. This aspect highlights a potential limitation in our investigation, the regulation of RseP could significantly affect the susceptibility to H1 in the different strains. Regulation is further discussed in section 4.4.

In the SigniSite analysis, depicted in **Figure 3.5**, the phenotype (numeric value of inhibition zone) correlated with all the residues in the sequence. The program performs a test for each position where there is a difference in at least one of the sequences. The SigniSite program allows for a Bonferroni correction, which reduces the chance of making a type 1 error, to reject the null hypothesis falsely. If the number of observations increases, the probability of assuming that a residue is important for the phenotype also increases, when it might only be a coincidence. With more sequences, a correction is needed to eliminate wrong assumptions of significance. It was chosen to keep the figure without correction because of the low number of sequences.

A strength of SigniSite is that it is very easy to use and demands a very clear numeric value of phenotype, which this experiment allowed for. It also simplifies the work because no subgrouping is necessary. On the other hand, a weakness of the input was that all 34 sequences were included, but as demonstrated by the clustering at 100% residue level, not all clusters had an equal number of representatives. A different approach could be to only look at the four largest' clusters, namely clusters 2, 4, 5, and 6, to eliminate the background of the residues where only one sequence is different. A different model could be made if only one representative from each cluster with the average zone size from that cluster maybe. This approach may also give a satisfactory impression of the importance of the sequences of the clusters with only one representative. However, this reduces the number of sequences, reducing the statistical power of the analysis.

With all 34 sequences included, from **Figure 3.5**, the three most significant residues putatively affecting sensitivity to H1 are located in positions 301- 308. According to the predicted structure of RseP, **Figure 3.6**, these residues are located on the transverse helix membrane surface toward the periplasmic space. The local charge and biochemical properties of the residues in this area in a membrane environment have not been investigated and can be challenging to determine. Notably, by predicting interactions between RseP and H1, the bacteriocin appears to interact with RseP in the region where the residues strongest correlated with H1 susceptibility are found (**Figure 4.1**).

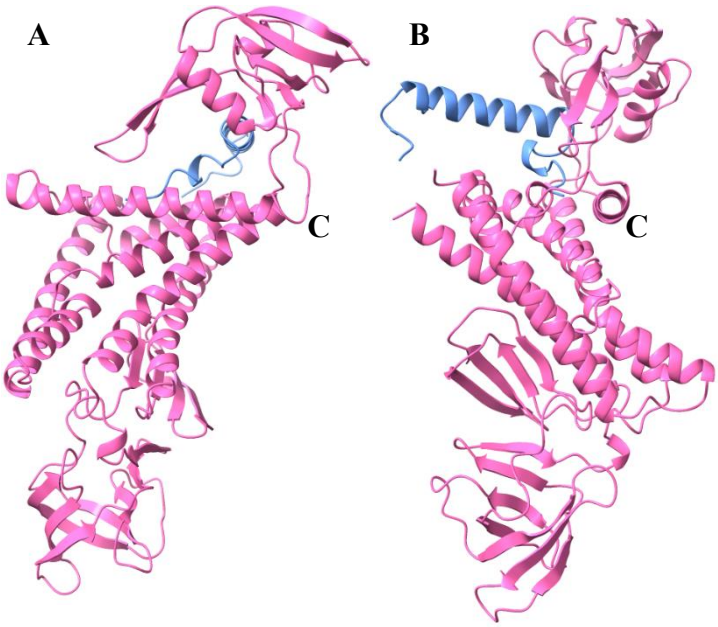

Figure 4.1. Predicted structure made with ColabFold of RseP (in pink) with interaction with H1 (in blue), illustrated in ChimeraX (Goddard et al., 2018; Meng et al., 2023; Mirdita et al., 2022; Pettersen et al., 2021). **A:** RseP in the same position as in **Figure 3.6**. **B**: is a 90° rotation of the same model as in **A**. **C:** marks the helix transverse of membrane, where residues 301-308 is located, both in **A** and **B**.

The residues in positions 301, 304, and 308 have the highest z-scores, indicating that they are the most important ones for H1 sensitivity, see **Figure 3.5**. In position 301 the polar tyrosine (Y) or non-polar phenylalanine (F) can be found. Either negatively charged glutamic acid (E) can be found in position 304 or positively charged lysine (K). In position 308, positively charged lysine (K) or the non-polar isoleucine (I) can be found. The bacteriocin H1 is small and positively charged (for sequence, see Figure 1.1), indicating a more favourable interaction with the polar and charged residues (YEK) in comparison with the nonpolar and positively charged ones (FKI). It is important to remember that this is a predicted interaction and not an experimentally determined one, and the mode of action of H1 is not determinant. The C-terminal end of EntK1 has been shown to interact with RseP (Ovchinnikov et al., 2017). Even though the C-terminal end of H1 comes from EntEJ97, all the LsbB-family bacteriocins are sequence created with a conserved motif at the C-terminal end with KxxxGxxPWE (Ovchinnikov et al., 2014). For a sequence comparison of some of the members in the LsbB-family, see **Figure 1.1**. When designing the mutagenesis experiment, only positions 304 and 308 were chosen to focus on because these showed the largest z-scores from the SigniSite analysis. In addition, as mentioned before, the sensitive clusters 2, 5, 6, and 9 all had E304 and K308, while the others had K304 and I308.

Another notable residue in **Figure 3.5** is in position 272, with either arginine (R) or lysine, (K), where both can potentially be positively charged. This position is oriented inwards toward the predicted H1 binding site, see **Figure 4.1**. It could be speculated that arginine is associated with sensitivity towards H1 because it has a longer side group and contains three nitrogen groups, so the charge potentially can make H1 interact in a more favourable/stable position with the other residues on the transverse helix also are positively correlated with sensitivity (YEK), while lysine only has one nitrogen and a shorter side group. It may not have such an important effect it the other residues on the helix are negatively correlated sensitivity (FKI)

The least significant observation in **Figure 3.5**, with the lowest z-score, is arginine (R) or histidine (H) in position 293. When compared to **Figure 3.4,** it can be observed that only clusters 5 and 6 contain R, and all the other clusters contain H in position 293. Clusters 5 and 6 contain the most sequences and they all have large zones. Conversely, clusters 2 and 9 are also susceptible to H1, but they have H in position 293, as all the other strains of the less sensitive and non-sensitive clusters. When investigating where the significant residues are located on the surface of RseP (**Figure 3.6**), position 293 is located prior to the cross-helix and pointing away from the predicted interaction surface between H1 and RseP (**Figure 4.1**). It should be noted that the significance of this residue is low, and when the SingiSite analysis was performed again with Bonferroni correction, the 293 position was not included as significant. Therefore, a correction to the analysis in **Figure 3.6** might be justifiable, even though the number of sequences is 34.

A phylogenetic analysis was made from an MSA using Clusta Omega (Madeira et al., 2022), **Figure 3.2**, and it grouped the sensitive clusters together, as well as the ones with a not clear zone or no zone (marked in the figure as not sensitive) together. This, along with the analysis correlation of residue position to the numeric phenotype value of the inhibition zone to H1 (**Figure 3.5**), can support the hypothesis that RseP sequences can directly affect an isolate's susceptibility to H1.

A new MSA and phylogenetic tree was made (**Figure 3.3**), which also contained the three largest clusters from the NCBI clustering (clusters 10, 11, and 18). This was done to investigate whether the most common RseP sequences from the NCBI database are closest

related to the sensitive clusters or the non-sensitive clusters. For an alignment of insensitive cluster 4, sensitive cluster 5 and clusters 10, 11 and 18 see appendix 3. As shown in **Figure 3.3**, clusters 10, 11, and 18 are placed closest to cluster 4, which was insensitive to H1. Because clusters 10 and 18 have been grouped with cluster 4, and they have many of the residues negatively correlated with the inhibition zone (xxFKI) (see **Figure 3.5** and appendix 3), they are suspected not to be sensitive to H1. Indicating that the most prevalent strains from the NCBI database may not be susceptible to H1. Cluster 11 is more unique, with positive and negative residues correlated with the inhibition zone (RxYKI) (see **Figure 3.5** and appendix 3). It is grouped close to cluster 3 and is suspected to have an intermediate sensitivity to H1, like cluster 3.

4.4 Heterologous expression of *rseP*

To corroborate the correlation between RseP-sequence and H1 sensitivity, heterologous expression, and mutation of RseP were necessary. Ovchinnikov et al. tried in 2017 to heterologous express the gene *rseP* from *E. faecalis* LMG3358 in the distantly related *Streptococcus pneumoniae* SPH131 (Ovchinnikov et al., 2017). *S. pneumoniae* was not sensitive to the bacteriocins EntK1, EntEJ97, or LsbB. Post constructing an *rseP* knockout clone in *S. pneumoniae* and cloning an inducible variant of the enterococcal *rseP* on the chromosome, in that. After induction of *rseP*, the strain became sensitive to the bacteriocins. These results indicated that the enterococcal *rseP*, which replaced the native *rseP* in *S. pneumoniae*, is involved in sensitivity to EntEJ97 and EntK1 directly (Ovchinnikov et al., 2017).

This work tried a similar experiment but with *S. aureus* NCTC8325-4 as the non-sensitive host and *rseP* from the sensitive *S. aureus* LMGT3255. The construction of the knockout clone was unsuccessful. No *E. coli* would take up a Golden gate assembled plasmid pCasSA-mod with both homology arms.

Although an *rseP* deletion mutant was not obtained, the pLOW-$rseP_{3255}$ was transformed into *S. aureus* NCTC8325-4. However, no transformants with the correct insert were found. Liew et al., whom designed the pLOW-plasmid, investigated whether the IPTG-regulated promotor in the plasmid (P-*spac)* showed a titratable gene expression when the concentration of IPTG was altered (Liew et al., 2011). In the assay used, with the *bgaB* gene from *Bacillus*

stearothermophilus. They found, however, almost no variance of expression in response to the varying concentrations of IPTG, even though, *lacI*, was also expressed from the pLOW plasmid. This suggests that the concentration of the repressor *lacI* was too low to repress P-*spac* effectively in the uninduced condition. It is therefore possible that the introduction of pLOW-*rseP*$_{3255}$ resulted in immediate overexpression of *rseP*, which normally occurs when inducing the plasmid, that may be toxic to the cell, resulting in alterations of the plasmid, because the strain still had its native copy of *rseP* in place.

rseP is located upstream in the same operon as the essential gene *proS*, (prolyl-tRNA synthase) (Chaudhuri et al., 2009). No other promotor regions at an appropriate distance have been identified in connection to *rseP*; it is therefore assumed that *rseP* and *proS* are expressed together. The amount of protein in the cell membrane is challenging to calculate. It is dependent on many factors, including mRNA stability and protein turnover. If the expression of the native copy of *rseP* normally is low, an excess amount can be toxic to the cell. Therefore, it is probable that the *S. aureus* cells with both chromosomal native *rseP* and a plasmid carrying *rseP* could only grow with a truncated version of the *rseP* in the plasmid.

The assumption that an overexpression of *rseP* is toxic to the cell was also supported by the nisin induction test in *L. lactis.* Initially, the nisin induction experiment was set up with different concentrations of nisin, resulting in different *rseP* expression levels, to identify the lowest induction concentration. Both 0.1 and 0.5 ng/ml of nisin showed equal growth, but at 1 ng/ml, the growth was much poorer. At a concentration of 3 or 5 ng/ml, the growth was very poor, indicating that the induction of *rseP* was too high and became toxic for the cell (results not shown). Supporting the argument that too high induction, yielding too high expression of *rseP,* resulted in poor growth, indicating that it may be toxic to the cell to overexpress *rseP*, also in *L. lactis*.

4.5 Mutating key residues in RseP did not abolish H1 sensitivity upon heterologous expression in *L. lactis*

Due to the unsuccessful construction of the knockout mutant in *S. aureus*, an alternative approach was adopted. For further experimentation, a strain of *L. lactis* IL1403 Δ*rseP* with a spontaneous mutation inducing a stop codon in the second position was selected. This mutation effectively resulted in a strain without its native copy of *rseP.*

The nisin induction test in *L. lactis* (**Figure 3.7**) was set up with IL1403 WT containing pNZ8037 (A), IL1403 Δ*rseP* with pNZ8037-$rseP_{3255}$ (B) and IL1403 Δ*rseP* also containing pNZ8037 (C), where all contain pNZ9530 for nisin induction. It revealed differences between the wildtype strain (A) and the knockout strain (C), both with the empty vector pNZ8037. These differences suggest that H1 has an effect on the native *rseP* of *L. lactis*. The zone is larger and clearer in the knockout strain with pNZ8037-$rseP_{3255}$ (B) when compared with the knockout strain containing an empty vector(C). Consequently, the zones can be interpreted more accurately when only one copy, either chromosomal or plasmid bound is present.

It was hypothesized that altering E to K in position 304 and K to I in position 308 would result in a change in the zone size in **Figure 3.8**. However, no difference between the *L. lactis* in pNZ8037-$rseP_{EKKI}$ (C) and pNZ8037-$rseP_{3255}$ (D) was observed. This indicates that more factors beyond the RseP sequences play a role in determining the susceptibility to H1 in *S. aureus*. The *resP* fragment originates from an *S. aureus* strain, and it is possible that some physicochemical or biochemical differences in the membrane environment between *S. aureus* and *L. lactis* may explain why the mutant remains sensitive. Another factor can be that only altering the two residues in positions 304 and 308 did not change the local environment enough to avoid binding H1.

Alternatively, the *L. lactis* harbouring pNZ8037-$rseP_{EKKI}$ remains sensitive due to a higher expression level of *rseP* compared to the native expression in *S. aureus*. The induction of gene expression from a plasmid may result in an expression level that is too high compared to the native expression. The presence of an excess of insensitive variants of RseP proteins in the membrane could potentially induce unspecific interactions, contributing to the observed sensitivity. To assess the importance of the expression level of *rseP*, the inhibition zone to H1 from a deletion mutant carrying *rseP* on an inducible vector can be compared with the inhibition zone to H1 from the native strain of the *rseP*-insert. The deletion mutant is preferably not sensitive to H1, and the *rseP* comes from a sensitive strain. These zones should be equal if *rseP* is expressed in equal amounts in both, but if the mutant with *rseP* on a plasmid has a larger zone, *rseP* is expressed in a larger amount than normally. If that is the case, this needs to be considered when comparing zone sizes from native insensitive strains to sensitive altered on plasmids. The nisin test with *L. lactis* should ideally have included a

control of *S. aureus* LMGT3255, to compare the zone size of the native expression to the expression from the plasmid.

It is also possible that *L. lactis* has poorer fitness; as previously mentioned, high induction of *rseP* has resulted in poorer growth compared to the empty vectors (**Figure 3.8**). A more effective experimental setup might have involved taking a non-sensitive *S. aureus,* knocking out the native copy of *rseP,* and inserting an *S. aureus*-compatible one. However, this experiment was unsuccessful (see discussion above).

4.6 Conclusions and future perspectives

This work found that many of the *S. aureus* isolates from the LMGT collection were susceptible to H1 (75%). The sequence of RseP is well conserved, with a few large groups of identical sequences, and some unique ones, as seen when clustering sequences from the LMGT collection with the NCBI database. When comparing the sequence of RseP to susceptibility, it is prominent that those who share the same RseP also have similar sensitivity towards H1. When investigating the most significant residues in this experiment and altering them from sensitive E304 and K308 to K304 and I308, no change in zone size was visible. This may not be a result of wrong interpretations of earlier results but may come from the fact that RseP from *S. aureus* was expressed in *L. lactis.* Little is known about the regulation and the native expression in these species in comparison with each other, leaving much room for interpretation and further work. However, it is important to note that these correlations are observational and further experimental validations is necessary to confirm the role of RseP sequence variation in determining the sensitivity of the strain.

So, could H1 be used as a treatment option in the future? Further work should include strains of human isolates to investigate whether RseP in *S. aureus* found on humans differs from those found on animals or the environment. If there is a difference and H1 is more active against *rseP* found in bovine or animal isolates, it can be used as a treatment for them. Suppose a comprehensive understanding of what decides the interaction of H1 and RseP is achieved; then this information can be used in prediction tools to design novel bacteriocins with an even broader spectrum of inhibition than H1 and EJ97. The absence of an N-terminal leader makes the LsbB family of bacteriocins ideal for bioengineering and in vitro synthesis. The activity of the LsbB family of bacteriocins may not only kill important human pathogens

but also bind to and inhibit an important protease. Simultaneously, mutants that acquire resistance against these bacteriocins will likely have reduced virulence and survival. Novel bacteriocins with an even broader spectrum can be designed to combat pathogens resistant to current drugs, antibiotics, and treatments. This potential for designing novel drugs with a significant impact in treating antimicrobial resistance pathogens, especially considering the conserved nature of the receptor protein, is a promising avenue for future research. The potential of using H1 as a novel drug against pathogenic strains of *S. aureus* needs more work to unlock the full potential within treatment, discovering synergies and novel treatment strategies.

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6 Appendix

6.1 Appendix A

Table 6.1.A. Sensitivity data 129 *S. aureus* isolates from the LMGT-collection, inhibition zone towards H1 diameter in mm.

Isolate	Zone	Isolate	Zon	Isolate	Zone
S. aureus LMGT 1682	0	*S. aureus* LMGT 3315	8	*S. aureus* LMGT 3938	9
S. aureus LMGT 3041	7	*S. aureus* LMGT 3316	8	*S. aureus* LMGT 3939	6
S. aureus MU50	10	*S. aureus* LMGT 3317	8	*S. aureus* LMGT 3940	9
S. aureus LMGT 3043	7	*S. aureus* LMGT 3318	3	*S. aureus* LMGT 3942	10
S. aureus LMGT 3222	10	*S. aureus* LMGT 3319	8	*S. aureus* LMGT 3944	7
S. aureus LMGT 3223	9	*S. aureus* LMGT 3320	5	*S. aureus* LMGT 3949	8
S. aureus LMGT 3224	10	*S. aureus* LMGT 3321	6	*S. aureus* LMGT 3951	1
S. aureus LMGT 3225	0	*S. aureus* LMGT 3322	5	*S. aureus* LMGT 3954	9
S. aureus LMGT 3226	0	*S. aureus* LMGT 3323	10	*S. aureus* LMGT 3955	8
S. aureus LMGT 3227	0	*S. aureus* LMGT 3324	8	*S. aureus* LMGT 3956	8
S. aureus RN 4220	0	*S. aureus* LMGT 3325	0	*S. aureus* LMGT 3957	10
S. aureus A70	10	*S. aureus* LMGT 3326	9	*S. aureus* LMGT 3959	9
S. aureus COL	0	*S. aureus* LMGT 3327	2	*S. aureus* LMGT 3960	8
S. aureus N315	4	*S. aureus* LMGT 3328	9	*S. aureus* LMGT 3962	9
S. aureus LMGT 3255	10	*S. aureus* LMGT 3329	7	*S. aureus* LMGT 3964	10
S. aureus LMGT 3256	10	*S. aureus* LMGT 3409	11	*S. aureus* LMGT 3967	9
S. aureus LMGT 3257	8	*S. aureus* LMGT 3410	9	*S. aureus* LMGT 3969	8
S. aureus LMGT 3258	0	*S. aureus* LMGT 3598	11	*S. aureus* LMGT 3970	7
S. aureus LMGT 3259	7	*S. aureus* LMGT 3756	0	*S. aureus* LMGT 3974	7
S. aureus LMGT 3260	8	*S. aureus* LMGT 3757	0	*S. aureus* LMGT 3975	8
S. aureus LMGT 3261	0	*S. aureus* LMGT 3891	7	*S. aureus* LMGT 3977	8
S. aureus LMGT 3262	0	*S. aureus* LMGT 3892	7	*S. aureus* LMGT 3979	10
S. aureus LMGT 3263	8	*S. aureus* LMGT 3894	9	*S. aureus* LMGT 3981	9
S. aureus LMGT 3264	8	*S. aureus* LMGT 3895	8	*S. aureus* LMGT 3982	8
S. aureus LMGT 3265	10	*S. aureus* LMGT 3896	8	*S. aureus* LMGT 3985	9
S. aureus LMGT 3266	9	*S. aureus* LMGT 3897	9	*S. aureus* LMGT 3988	8
S. aureus LMGT 3271	0	*S. aureus* LMGT 3898	7	*S. aureus* LMGT 4076	7
S. aureus LMGT 3272	8	*S. aureus* LMGT 3901	8	*S. aureus* LMGT 4077	9
S. aureus LMGT 3273	0	*S. aureus* LMGT 3902	9	*S. aureus* LMGT 4078	10
S. aureus LMGT 3274	9	*S. aureus* LMGT 3905	8	*S. aureus* LMGT 4079	0
S. aureus LMGT 3275	7	*S. aureus* LMGT 3907	8	*S. aureus* LMGT 4080	0
S. aureus LMGT 3276	8	*S. aureus* LMGT 3908	8	*S. aureus* LMGT 4081	0
S. aureus LMGT 3277	8	*S. aureus* LMGT 3909	9	*S. aureus* LMGT 4082	0
S. aureus NCTC 8325	0	*S. aureus* LMGT 3910	10	*S. aureus* LMGT 4083	0
S. aureus NCTC8325-4	0	*S. aureus* LMGT 3913	8	*S. aureus* LMGT 4084	0
S. aureus LMGT 3304	9	*S. aureus* LMGT 3914	6	*S. aureus* LMGT 4085	7
S. aureus LMGT 3305	7	*S. aureus* LMGT 3917	8	*S. aureus* LMGT 4086	7
S. aureus LMGT 3306	9	*S. aureus* LMGT 3924	9	*S. aureus* LMGT 4087	6
S. aureus LMGT 3308	9	*S. aureus* LMGT 3926	9	*S. aureus* LMGT 4088	9
S. aureus LMGT 3309	9	*S. aureus* LMGT 3928	9	*S. aureus* LMGT 4089	0
S. aureus LMGT 3310	10	*S. aureus* LMGT 3930	7	*S. aureus* LMGT 4090	9
S. aureus LMGT 3311	7	*S. aureus* LMGT 3931	7	*S. aureus* LMGT 4091	0
S. aureus LMGT 3314	6	*S. aureus* LMGT 3933	9	*S. aureus* LMGT 4160	0

6.2 Appendix B

Table 6.2.B. The 129 *S. aureus* isolates from the LMGT collection with the inhibition zone from penicillin G (1 unite)

Isolate	Zone	Isolate	Zon	Isolate	Zone
S. aureus LMGT 1682	0	*S. aureus* LMGT 3315	30	*S. aureus* LMGT 3938	29
S. aureus LMGT 3041	36	*S. aureus* LMGT 3316	35	*S. aureus* LMGT 3939	25
S. aureus MU50	0	*S. aureus* LMGT 3317	28	*S. aureus* LMGT 3940	25
S. aureus LMGT 3043	8	*S. aureus* LMGT 3318	31	*S. aureus* LMGT 3942	27
S. aureus LMGT 3222	39	*S. aureus* LMGT 3319	30	*S. aureus* LMGT 3944	31
S. aureus LMGT 3223	39	*S. aureus* LMGT 3320	34	*S. aureus* LMGT 3949	29
S. aureus LMGT 3224	39	*S. aureus* LMGT 3321	33	*S. aureus* LMGT 3951	28
S. aureus LMGT 3225	22	*S. aureus* LMGT 3322	35	*S. aureus* LMGT 3954	28
S. aureus LMGT 3226	35	*S. aureus* LMGT 3323	10	*S. aureus* LMGT 3955	28
S. aureus LMGT 3227	20	*S. aureus* LMGT 3324	10	*S. aureus* LMGT 3956	26
S. aureus RN 4220	26	*S. aureus* LMGT 3325	10	*S. aureus* LMGT 3957	29
S. aureus A70	27	*S. aureus* LMGT 3326	9	*S. aureus* LMGT 3959	29
S. aureus COL	0	*S. aureus* LMGT 3327	8	*S. aureus* LMGT 3960	28
S. aureus N315	6	*S. aureus* LMGT 3328	35	*S. aureus* LMGT 3962	27
S. aureus LMGT 3255	11	*S. aureus* LMGT 3329	31	*S. aureus* LMGT 3964	31
S. aureus LMGT 3256	8	*S. aureus* LMGT 3409	16	*S. aureus* LMGT 3967	28
S. aureus LMGT 3257	14	*S. aureus* LMGT 3410	0	*S. aureus* LMGT 3969	31
S. aureus LMGT 3258	14	*S. aureus* LMGT 3598	17	*S. aureus* LMGT 3970	25
S. aureus LMGT 3259	9	*S. aureus* LMGT 3756	28	*S. aureus* LMGT 3974	28
S. aureus LMGT 3260	11	*S. aureus* LMGT 3757	26	*S. aureus* LMGT 3975	27
S. aureus LMGT 3261	11	*S. aureus* LMGT 3891	26	*S. aureus* LMGT 3977	20
S. aureus LMGT 3262	0	*S. aureus* LMGT 3892	26	*S. aureus* LMGT 3979	26
S. aureus LMGT 3263	6	*S. aureus* LMGT 3894	26	*S. aureus* LMGT 3981	30
S. aureus LMGT 3264	0	*S. aureus* LMGT 3895	29	*S. aureus* LMGT 3982	28
S. aureus LMGT 3265	0	*S. aureus* LMGT 3896	26	*S. aureus* LMGT 3985	31
S. aureus LMGT 3266	0	*S. aureus* LMGT 3897	27	*S. aureus* LMGT 3988	27
S. aureus LMGT 3271	25	*S. aureus* LMGT 3898	25	*S. aureus* LMGT 4076	26
S. aureus LMGT 3272	0	*S. aureus* LMGT 3901	28	*S. aureus* LMGT 4077	8
S. aureus LMGT 3273	5	*S. aureus* LMGT 3902	28	*S. aureus* LMGT 4078	26
S. aureus LMGT 3274	16	*S. aureus* LMGT 3905	26	*S. aureus* LMGT 4079	5
S. aureus LMGT 3275	8	*S. aureus* LMGT 3907	30	*S. aureus* LMGT 4080	0
S. aureus LMGT 3276	25	*S. aureus* LMGT 3908	27	*S. aureus* LMGT 4081	0
S. aureus LMGT 3277	26	*S. aureus* LMGT 3909	30	*S. aureus* LMGT 4082	27
S. aureus NCTC 8325	25	*S. aureus* LMGT 3910	6	*S. aureus* LMGT 4083	0
S. aureus NCTC8325-4	31	*S. aureus* LMGT 3913	28	*S. aureus* LMGT 4084	6
S. aureus LMGT 3304	25	*S. aureus* LMGT 3914	30	*S. aureus* LMGT 4085	10
S. aureus LMGT 3305	25	*S. aureus* LMGT 3917	30	*S. aureus* LMGT 4086	7
S. aureus LMGT 3306	25	*S. aureus* LMGT 3924	25	*S. aureus* LMGT 4087	14
S. aureus LMGT 3308	24	*S. aureus* LMGT 3926	30	*S. aureus* LMGT 4088	0
S. aureus LMGT 3309	28	*S. aureus* LMGT 3928	30	*S. aureus* LMGT 4089	5
S. aureus LMGT 3310	28	*S. aureus* LMGT 3930	25	*S. aureus* LMGT 4090	0
S. aureus LMGT 3311	31	*S. aureus* LMGT 3931	28	*S. aureus* LMGT 4091	5
S. aureus LMGT 3314	32	*S. aureus* LMGT 3933	27	*S. aureus* LMGT 4160	0

6.3 Appendix C

Sequence of RseP from the 9 LMGT clusters and 10, 11, and 18 from the NCBI database.

>Cluster 1
MSYLVTIIAFIIVFGVLVTVHEYGHMFFAKRAGIMCPEFAIGMGPKIFSFRKNETLYTIRLLPVGGYVRM
AGDGLEEPPVEPGMNVKIKLNEENEITHIILDDHHKFQQIEAIEVKKCDFKDDLFIEGITAYDNERHHFKI
ARKSFFVENGSLVQIAPRDRQFAHKKPWPKFLTLFAGPLFNFILALVLFIGLAYYQGTPTSTVEQVADKY
PAQQAGLQKGDKIVQIGKYKIFEFDDVDKALDKVKDNKTTVKFERDGKTKSVELTPKKTERKLTKVSS
ETKYVLGFQPASEHTLFKPIVYGFKNFLIGSTLIFSAVVGMLASIFTGGFSFDMLNGPVGIYHNVDSVVK
AGIISLIGYTALLSVNLGIMNLIPIPALDGGRILFVIYEAIFRKPVNKKAETTIIAIGAIFMVVIMILVTWNDI
RRYFL
>Cluster 2
MSYLVTIIAFIIVFGVLVTVHEYGHMFFAKRAGIMCPEFAIGMGPKIFSFRKNETLYTIRLLPVGGYVRM
AGDGLEEPPVEPGMNVKIKLNEENEITHIILDDHHKFQQIEAIEVKKCDFKDDLFIEGITAYDNERHHFKI
ARKSFFVENGSLVQIAPRDRQFAHKKPWPKFLTLFAGPLFNFILALVLFIGLAYYQGTPTSTVEQVADKY
PAQQAGLQKGDKIVQIGKYKISEFDDVDKALDKVKDNKTTVKFERDGKTKSVELTPKKTERKLTKVSS
ETKYVLGFQPASEHTLFKPIVYGFESFLKGSTLIFTAVVGMLASIFTGGFSFDMLNGPVGIYHNVDSVVK
AGIISLIGYTALLSVNLGIMNLIPIPALDGGRILFVIYEAIFRKPVNKKAETTIIAIGAIFMVVIMILVTWNDI
RRYFL
>Cluster 3
MSYLVTIIAFIIVFGVLVTVHEYGHMFFAKRAGIMCPEFAIGMGPKIFSFRKNETLYTIRLLPVGGYVRM
AGDGLEEPPVEPGMNVKIKLNEENEITHIILDDHHKFQQIEAIEVKKCDFKDDLFIEGITAYDNERHHFKI
ARKSFFVENGSLVQIAPRDRQFAHKKPWPKFLTLFAGPLFNFILALVLFIGLAYYQGTPTSTVEQVADKY
PAQQAGIQKGDKIVQIGKYKISEFDDVDKALDKVKDNKTTVKFERDGKTKSVELTPKKTERKLTKVSSE
TKYVLGFQPASEHTLFKPIVYGFKSFLIGSTLIFSAVVGMLASIFTGGFSFDMLNGPVGIYHNVDSVVKA
GIISLIGYTALLSVNLGIMNLIPIPALDGGRILFVIYEAIFRKPVNKKAETTIIAIGAIFMVVIMILVTWNDIR
RYFL
>Cluster 4
MSYLVTIIAFIIVFGVLVTVHEYGHMFFAKRAGIMCPEFAIGMGPKIFSFRKNETLYTIRLLPVGGYVRM
AGDGLEEPPVEPGMNVKIKLNEENEITHIILDDHHKFQQIEAIEVKKCDFKDDLFIEGITAYDNERHHFKI
ARKSFFVENGSLVQIAPRDRQFAHKKPWPKFLTLFAGPLFNFILALVLFIGLAYYQGTPTSTVEQVADKY
PAQQAGLQKGDKIVQIGKYKISEFDDVDKALDKVKDNKTTVKFERDGKTKSVELTPKKTEKKLTKVSS
ETKYVLGFQPASEHTLFKPIVFGFKSFLIGSTLIFTAVVGMLASIFTGGFSFDMLNGPVGIYHNVDSVVKA
GIISLIGYTALLSVNLGIMNLIPIPALDGGRILFVIYEAIFRKPVNKKAETTIIAIGAIFMVVIMILVTWNDIR
RYFL
>Cluster 5
MSYLVTIIAFIIVFGVLVTVHEYGHMFFAKRAGIMCPEFAIGMGPKIFSFRKNETLYTIRLLPVGGYVRM
AGDGLEEPPVEPGMNVKIKLNEENEITHIILDDHHKFQQIEAIEVKKCDFKDDLFIEGITAYDNERHHFKI
ARKSFFVENGSLVQIAPRDRQFAHKKPWPKFLTLFAGPLFNFILALVLFIGLAYYQGTPTSTVEQVADKY
PAQQAGLQKGDKIVQIGKYKISEFDDVDKALDKVKDNKTTVKFERDGKTKSVELTPKKTERKLTKVSS
ETKYVLGFQPASERTLFKPIVYGFESFLKGSTLIFTAVVGMLASIFTGGFSFDMLNGPVGIYHNVDSVVK
AGIISLIGYTALLSVNLGIMNLIPIPALDGGRILFVIYEAIFRKPVNKKAETTIIAIGAIFMVVIMILVTWNDI
RRYFL
>Cluster 6
MSYLVTIIAFIIVFGVLVTVHEYGHMFFAKRAGIMCPEFAIGMGPKIFSFRKNETLYTIRLLPVGGYVRM
AGDGLEEPPVEPGMNVKIKLNEENEITHIILDDHHKFQQIEAIEVKKCDFKDDLFIEGITAYDNERHHFKI
ARKSFFVENGSLVQIAPRDRQFAHKKPWPKFLTLFAGPLFNFILALVLFIGLAYYQGTPTSTVEQVADKY
PAQQAGLQKGDKIVQIGKYKISEFDDVDKALDKVKDNKTTVKFERNGKTKSVELTPKKTERKLTKVSS
ETKYVLGFQPASERTLFKPIVYGFESFLKGSTLIFTAVVGMLASIFTGGFSFDMLNGPVGIYHNVDSVVK
AGIISLIGYTALLSVNLGIMNLIPIPALDGGRILFVIYEAIFRKPVNKKAETTIIAIGAIFMVVIMILVTWNDI
RRYFL
>Cluster 7
MSYLVTIIAFIIVFGVLVTVHEYGHMFFAKRAGIMCPEFAIGMGPKIFSFRKNETLYTIRLLPVGGYVRM
AGDGLEEPPVEPGMNVKIKLNEENEITHIILDDHHKFQQIEAIEVKKCDFKDDLFIEGITAYDNERHHFKI
ARKSFFVENGSLVQIAPRDRQFAHKKPWPKFLTLFAGPLFNFILALVLFIGLAYYQGTPTSTVEQVADKY
PAQQAGLQKGDKIVQIGKYKISEFDDVDKALDKVKDNKTTVKFERDGKTKSVELTPKKTERKLTKVSS
ETKYVLGFQPASEHTLFKPIVFGFKSFLIGSTLIFTAVVGMLASIFTGGFSFDMLNGPVGIYHNVDSVVKA
GIISLIGYTALLSVNLGIMNLIPIPALDGGRILFVIYEAIFRKPVNKKAETTIIAIGAIFMVVIMILVTWNDIR
RYFL

>Cluster 8
MSYLVTIIAFIIVFGVLVTVHEYGHMFFAKRAGIMCPEFAIGMGPKIFSFRKNETLYTIRLLPVGGYVRM
AGDGLEEPPVEPGMNVKIKLNEENEITHIILDDHHKFQQIEAIEVKKCDFKDDLFIEGITAYDNERHHFKI
ARKSFFVENGSLVQIAPRDRQFAHKKPWPKFLTLFAGPLFNFILALVLFIGLAYYQGTPTSTVEQVADKY
PAQQAGLQKGDKIVQIGKYKISEFDDVDKALDKVKDNKTTVKFERDGKTKSVELTPKKTERKLTKVSS
ETKYVLGFQPASEHTLFKPIVYGFKSFLIGSTLIFSAVVGMLASIFTGGFSFDMLNGPVGIYHNVDSVVKA
GIISLIGYTALLSVNLGIMNLIPIPALDGGRILFVIYEAIFRKPVNKKAETTIIAIGAIFMVVIMILVTWNDIR
RYFL
>Cluster 9
MSYLVTIIAFIIVFGVLVTVHEYGHMFFAKRAGIMCPEFAIGMGPKIFSFRKNETLYTIRLLPVGGYVRM
AGDGLEEPPVEPGMNVKIKLNEENEITHIILDDHHKFQQIEAIEVKKCDFKDDLFIEGITAYDNERHHFKI
ARKSFFVENGSLVQIAPRDRQFAHKKPWPKFLTLFAGPLFNFILALDLFIGLAYYQGTPTSTVEQVADKY
PAQQAGLQKGDKIVQIGKYKISEFDDVDKALDKVKDNKTTVKFERDGKTKSVELTPKKTERKLTKVSS
ETKYVLGFQPASEHTLFKPIVYGFESFLKGSTLIFTAVVGMLASIFTGGFSFDMLNGPVGIYHNVDSVVK
AGIISLIGYTALLSVNLGIMNLIPIPALDGGRILFVIYEAIFRKPVNKKAETTIIAIGAIFMVVIMILVTWNDI
RRYFL
>Cluster 10
MSYLVTIIAFIIVFGVLVTVHEYGHMFFAKRAGIMCPEFAIGMGPKIFSFRKNETLYTIRLLPVGGYVRM
AGDGLEEPPVEPGMNVKIKLNEENEITHIILDDHHKFQQIEAIEVKKCDFKDDLFIEGITAYDNERHHFKI
ARKSFFVENGSLVQIAPRDRQFAHKKPWPKFLTLFAGPLFNFILALVLFIGLAYYQGTPTSTVEQVADKY
PAQQAGLQKGDKIVQIGKYKISEFDDVDKALDKVKDNKTTVKFERDGKTKSVELTPKKTEKKLTKVSS
ETKYVLGFQPASEHTLFKPIVFGFKSFLIGSTYIFTAVVGMLASIFTGGFSFDMLNGPVGIYHNVDSVVKA
GIISLIGYTALLSVNLGIMNLIPIPALDGGRILFVIYEAIFRKPVNKKAETTIIAIGAIFMVVIMILVTWNDIR
RYFL
>Cluster 11
MSYLVTIIAFIIVFGVLVTVHEYGHMFFAKRAGIMCPEFAIGMGPKIFSFRKNETLYTIRLLPVGGYVRM
AGDGLEEPPVEPGMNVKIKLNEENEITHIILDDHHKFQQIEAIEVKKCDFKDDLFIEGITAYDNERHHFKI
ARKSFFVENGSLVQIAPRDRQFAHKKPWPKFLTLFAGPLFNFILALVLFIGLAYYQGTPTSTVEQVADKY
PAQQAGIQKGDKIVQIGKYKISEFDDVDKALDKVKDNKTTVKFERDGKTKSVELTPKKTERKLTKVSSE
TKYVLGFQPASEHTLFKPIVYGFKSFLIGSTYIFSAVVGMLASIFTGGFSFDMLNGPVGIYHNVDSVVKA
GIISLIGYTALLSVNLGIMNLIPIPALDGGRILFVIYEAIFRKPVNKKAETTIIAIGAIFMVVIMILVTWNDIR
RYFL
>Cluster 18
MSYLVTIIAFIIVFGVLVTVHEYGHMFFAKRAGIMCPEFAIGMGPKIFSFRKNETLYTIRLLPVGGYVRM
AGDGLEEPPVEPGMNVKIKLNEENEITHIILDDHHKFQQIEAIEVKKCDFKDDLFIEGITAYDNERHHFKI
ARKSFFVENGSLVQIAPRDRQFAHKKPWPKFLTLFAGPLFNFILALVLFIGLAYYQGTPTSTVEQVADKY
PAQQAGLQKGDKIVQIGKYKISEFDDVDKALDKVKDNKTTVKFERDGKTKSVELTPKKTERKLTKVSS
ETKYVLGFQPASEHTLFKPIVFGFKSFLIGSTYIFTAVVGMLASIFTGGFSFDMLNGPVGIYHNVDSVVKA
GIISLIGYTALLSVNLGIMNLIPIPALDGGRILFVIYEAIFRKPVNKKAETTIIAIGAIFMVVIMILVTWNDIR
RYFL

Alignment of cluster 4, 5, 10, 11 and 18

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          ▼          ▼          ▼          ▼          ▼       ▼  ▼▼  ▼   ▼  ▼
 Clu4  216 AGLQKG  231 KISEFD  255 ERDGKT  270 TEKKLT  291 SEHTLFKPIVFGFKSFLIGSTLIFTAVV
 Clu5  216 AGLQKG  231 KISEFD  255 ERDGKT  270 TERKLT  291 SERTLFKPIVYGFESFLKGSTLIFTAVV
Clu10  216 AGLQKG  231 KISEFD  255 ERNGKT  270 TEKKLT  291 SEHTLFKPIVFGFKSFLIGSTTIFTAVV
Clu11  216 AGIQKG  231 KISEFD  255 ERDGKT  270 TERKLT  291 SERTLFKPIVYGFKSFLIGSTTIFSAVV
Clu18  216 AGLQKG  231 KISEFD  255 ERDGKT  270 TERKLT  291 SERTLFKPIVYGFKSFLIGSTTIFTAVV
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6.4 Appendix D

TAE-buffer: Trizma Base 242g, glacial acetic acid 57,1 ml, 0.5 M EDTA pH8.0 100 mL, tot 1000mL.

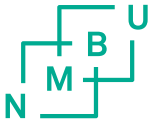
SOC(Super Optimal broth with Catabolite repression): 2% Tryptone (Oxoid), 0.5% Yeast Extract (Oxoid), 0.05% NaCl (VWR, BDH Chemicals), 2.5 mM KCl (VWR, BDH Chemicals), 10 mM $MgCl_2$ (Sigma-Aldrich), 20 mM glucose (Matuschek et al., 2014)

Glycine (Sigma-Aldrich)

SGM17: M17 (Oxoid) with 0.5M sucrose (Sigma-Aldrich), and 0.5% glucose (Sigma-Aldrich)

SGM17MC: SGM17 with 20 mM $MgCl_2$ (Sigma-Aldrich) and 2 mM $CaCl_2$ (Merck KGaA)

LB- medium: Tryptone (Oxoid) 10 g, yeast extract (Oxoid) 5 g, NaCl (VWR, BDH Chemicals) 10 g.



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