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Identification of RseP as a receptor for hybrid bacteriocin H1 in emerging pathogen *Staphylococcus haemolyticus*.

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Biotechnology

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Til min kjære Christopher,
Takk for at du alltid er der for meg.

Ås, Juni 2020

Karolina Teresa Bartkiewicz

Abstract

Staphylococcus haemolyticus is an emerging pathogen which may transfer multi-drug resistance genes to highly virulent *Staphylococcus* species. *S. haemolyticus* is a biofilm-producing bacterium and one of the most frequent isolates of nosocomial infections associated with implanted medical devices, such as catheter. To this date there are only a few therapeutic options that can stop this bacterium, including the last resort antibiotic, vancomycin and teicoplanin, for which resistance have already been reported. This study shows that an alternative antimicrobial agent has great potential to inhibit this pathogen. Hybrid bacteriocin H1 consisting of N-terminal part of EntK1 and C-terminal part of EntEJ97, exhibits strong activity against *S. haemolyticus*. A combination of bacteriocin H1 with garvicin KS displays greater antibacterial activity against *S. haemolyticus* and is promising in inhibiting resistant mutants, which appear to be the main problem in H1 application.

The aim of this study was to reveal the nature that indicates the specificity of H1 toward *S. haemolyticus*. The Zn-dependent protease RseP in *S. haemolyticus* was established as a receptor for bacteriocin H1. This is based on the fact that the *S. haemolyticus* gene *rseP* was heterologously-expressed in naturally H1 resistant *Lactobacillus plantarum* WCFS1 and the resulting transformant became highly susceptible to H1.

RseP isolated from resistant mutants were sequenced in order to verify the nature of the bacterial resistance to H1. Interestingly, obtained data showed intact *rseP* gene in all mutants. WGS analysis was a conclusive step in this study and indicated mutation in a putative Ecs ABC transporter. Similar discoveries from previous investigations suggest the influence of Ecs transporter on RseP activity at protein level. One might speculate that Ecs protein inhibits RseP interaction with bacteriocin by blocking its activity. However, the results of this study do not allow for specific conclusions on correlation between Ecs and RseP. Further studies of between RseP and Ecs interaction with bacteriocin H1 are required.

Sammendrag

Staphylococcus haemolyticus kan overføre gener for multi-resistansen til sterkt virulente *Staphylococcus* arter. *S. haemolyticus* er en biofilm produserende bakterie og et av de hyppigste isolatene i nosokomiale infeksjoner, og er ofte forbundet med implantert medisinsk utstyr som for eksempel kateter. Til dags dato er det bare noen få terapeutiske alternativer som kan hindre denne bakterien. Dette inkluderer også bruk av antibiotika som en «last resort», selv bakterier som er resistente mot en slik behandling allerede er rapportert. Denne studien viser at et alternativt antimikrobielmiddel har stort potensiale for å hemme dette patogenet. Hybrid bakteriosin H1 består av N-terminal part av EntK1 og C-terminal part av EntEJ97 og viser sterk aktivitet mot *S. haemolyticus*. En kombinasjon av de to lederløse bakteriosinene H1 og garvisin KS utviser stort antibakteriell aktivitet mot *S. hamolyticus* og er lovende for å hemme resistente mutanter, som ser ut til å være hovedproblemet i H1 applikasjonen.

Målet med denne studien var å avsløre naturen som angir spesifisiteten til H1 mot S. haemolyticus. Den Zn-avhengige proteasen RseP i *S. haemolyticus* ble etablert som reseptor for bakteriosin H1. Detter er basert på det faktum at *S. haemolyticu*s - genet *rseP* ble heterologt uttrykt i naturlig H1-resistent *Lactobacillus plantarum* WCFS1 og den resulterende transformanten ble svært sensitiv til H1.

RseP isolert fra resistente mutanter, ble sekvensert for å verifisere naturen av bakteriell resistans mot H1 og variasjoner i fenotype ved stressrespons. Interessant nok, var *rseP* i alle mutanter. WGS analysen var et avgjørende trinn i dette arbeidet, og indikerte delesjoner i Ecs ABC transporter. Tilsvarende funn fra tidligere studier antyder en påstått effekt av Ecs transporter på RseP-aktivitet på proteinnivå. Man kan spekulere at Ecs protein hemmer RseP interaksjon med bakteriosin H1 ved å blokkere dets aktivitet. Resultatene fra denne studien tillater ikke konkrete konklusjoner om sammenheng mellom Ecs og RseP. Videre studier om interaksjonen mellom RseP og Ecs med bakteriosin H1 er nødvendig.

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List of abbreviations

aa - amino acid

ABC - ATP-binding cassette

ATP - adenosine triphosphate

BHI - Brain heart infusion

BLAST - Basic Local Alignment Search Tool

BR - broad range

CaCo - sodium cacodylate

ENA - European Nucleotide Archive

GA - glutaraldehyde

GMM - gene modified microorganisms

GRAS - generally recognized as safe

M - mutant

M.R.S. - de man, Rogosa, Sharpe

M.W - molecular weight

MIC - minimum inhibitory concentration

MRSA - methicillin resistant *Staphylococcus aureus*

MSA- multiple sequence alignment

NCBI - National Center for Biotechnology Information

NGS - Next Generation Sequencing

nt - nucleotide

O/N - overnight

OD - optical density

PA- pairwise alignment

PBS - phosphate-buffered saline

PCR - polymerase chain reaction

PEG - polyethylene glycol

PFA - paraformaldehyde

RIP - regulated intramembrane proteolysis

RPM - rounds per minute

RSEP - regulator of sigma-E protease

RT - room temperature

S.O.C. - Super Optimal Catabolite

SNP- single nucleotide polymorphisms

TEM - transmission electron microscopy

TFA - trifluoroacetic acid

Tm - melting temperature

VRE - vancomycin resistant enterococci

WGS - Whole Genome Sequencing

WT - wild type

1. Introduction

1.1. Antimicrobial agents

Antimicrobials have revolutionized medicine and improved quality of life by overcoming serious infections in humans, animals and plants. Shortly after first introduction of penicillin in clinical applications, the antibiotic saved many lives by preventing bacterial infections during World War II. Eventually, antibacterial agents became available for general public. Unfortunately, by increasing and unwise use pathogenic bacteria gain resistance against antibiotics. Resistance genes easily spread among pathogens by horizontal gene transfer (Davies and Davies, 2010, C. Lee Ventola, 2015, 2018). A study of Cassini el al. (2019) indicated that 33,000 deaths cases a year are caused by resistant bacteria infections.

Long and costly production time and low prices of antibiotics makes that antibiotics no longer are a profitable investment for pharmaceutical companies. As a consequence, more attention have been paid to search for alternative antimicrobials (Davies and Davies, 2010, Aslam et al., 2018). Many researchers have shown that naturally produced bacterial peptides known as bacteriocins have great potential to be used in food and medical applications (Cotter et al., 2013, Kumariya et al., 2019). Bacteriocins are small, ribosomally synthetized peptides. They display both broad- and narrow spectrum of antimicrobial activity against many bacterial species, including antibiotic resistant strains. These properties together with low toxicity and possibility of *in situ* production make them an attractive substitute to antibiotics. Another beneficial feature of bacteriocins is their simple biosynthesis, which makes them available for bioengineering (Cotter et al., 2013, Kumariya et al., 2019). Although the first antibacterial inhibition of bacteriocin was described in 1925 in Gram-negative *Escherichia coli* (colicin), many studies have focused on antimicrobial peptides (AMPs) from Gram-positive bacteria, especially from lactic acid bacteria (LAB). LAB bacteriocins are considered to be safe since the producer-bacteria has been used as natural food fermentation for centuries (Cotter et al., 2005, Perez et al., 2014).

1.1.1. Bacteriocin applications

Bacteriocins are naturally produced by bacteria in order to kill other bacterial species in competition for nutrition and habitat (Kumariya et al., 2019). In the food industry, bacteriocins are used in quality and safety applications, for instance as a control of pathogens in fermented food. Currently, the bacteriocin nisin has been utilized in food as biopreservative in over 50 countries (O'Sullivan et al., 2002, Shin et al., 2016). When searching for new antimicrobial

agents for food application, several principles must be acknowledged. Above all, bacteriocin-producing bacteria must be generally recognized as safe (GRAS) and cannot contribute to any risk to consumers' health. In addition, antimicrobials should have broad spectrum of inhibition, be thermally stabile, soluble and be efficient against specific food pathogen. Bacteriocins produced by lactic acid bacteria have greatest potential to approval, but they are mostly active against Gram-positive strains. In the cheese industry, *Lactococcus lactis* producing bacteriocin lacticin 3147, is used to obtain the beneficial effect in product such as flavour, quality and safety. Due to low toxicity, bacteriocins and bacteriocins-producing bacteria have also been used in preservation of meat (pediocins), pasteurized and fermented food (nisin) (O'Sullivan et al., 2002, Cotter et al., 2005, Silva et al., 2018). Antimicrobials can be applied in different manners, for instance for *in situ* production or direct addition of purified agent. Bacteriocins-producing probiotics in food may allow bacteriocin production *in vivo* when delivered to intestine, and thereby maintain healthy gut by inhibiting pathogen in gut. An important aspect off bacteriocin exploitation is that even low concentration may supress harmful organisms. This will not have a negative effect on microorganisms living in that intestinal niche in contrast to broad-spectrum antibiotics, which are known to disrupt the commensal human microbiota (O'Sullivan et al., 2002, Cotter et al., 2005, Cotter et al., 2013, Kumariya et al., 2019).

Several studies have revealed effectiveness of antimicrobial peptides in veterinary and human medicine. Experiments with animal models of infections treated *in vivo*, have demonstrated the effectiveness of bacteriocins in preventing dental diseases (Shin et al., 2016). Nisin, the most studied bacteriocin, has already been licenced to be used against mastitis infections in dairy cows (Shin et al., 2016). Bacteriocins have also been described as an alternative to antibiotics against clinically important pathogens such as *Staphylococcus aureus* and *Enterococcus faecium* (Cotter et al., 2005, Shin et al., 2016, Reinseth et al., 2019).

Many biotechnology approaches significantly contribute to the search for new and effective antimicrobials for use in clinical and food applications. Nevertheless, further development requires understanding of their structure and mode of action to ensure stability, overcome resistance and reduce toxicity. Numerous studies facilitate the comprehension of regulatory mechanisms to optimize bacteriocin production, making it more attractive for large-scale use (Cotter et al., 2005, Cotter et al., 2013, Telke et al., 2019, Mathur et al., 2017).

Bacteriocins show strong activity against target bacteria at pico- and to nanomolar concentrations, making them even more potent than some antibiotics. Additionally, many bacteriocins exhibit synergistic interaction with other antimicrobials. An example, a strong synergy of GarKS with nisin and Gram-negative antibiotic (polymyxin B) against *S. aureus*

was described by Hai Chi and Helge Holo (2018). This may diminish the likelihood of the resistance development and broaden the spectrum of activity, as well as lower the cost of production and treatment (Mathur et al., 2017). Combinatory treatment can lower the required concentration of antimicrobials to kill the target and reduce the probability of side effects caused by toxicity of antibiotics. Several laboratory investigations have demonstrated that biofilm-forming bacteria are more resistant to antimicrobials than free-living (planktonic) bacteria. Numerous researches showed that variants of nisin in combination with different antibiotics were efficient against *Staphylococcus* biofilms, for instance, a combination of nisin with penicillin effectively inhibited biofilms of *S. aureus* SA113. Nisin in synergy with ciprofloxacin was efficient in treatment of 24 hours-old MRSA biofilms. The results of bioengineered bacteriocins in combinations with other antimicrobial substance have been documented to be more effective at inhibiting biofilms than individual antimicrobials (Mathur et al., 2017).

1.2. Bacteriocins from Gram-positive bacteria

Bacteriocins are small peptides produced by bacteria. These ribosomally synthesized peptides are active against other bacteria, mostly closely related species. They play a role in rivalry between populations, eliminating each other from habitats, especially when competitors are focused on the same nutrient resource (Ovchinnikov et al., 2017, Kumariya et al., 2019). Bacteriocins are synthetized as propeptides that are modified and further exported from cell by ABC transporters. Immunity and transport proteins are regulated by the same gene cluster that encodes for bacteriocin genes. (Uzelac et al., 2013, Ovchinnikov et al., 2017, Kumariya et al., 2019).

1.2.1. Classification of bacteriocins

Bacteriocins can be categorized into three main classes based on their structure (table 1.1). Class I bacteriocins are 19-50 amino acids (contain nontypical amino acids) long peptides that are post-translationally modified. This class of bacteriocins are divided into subclasses called lantibiotics (Ia), which contain lanthionine (non-proteinogenic amino acid), labyrinthopeptins (Ib) containing labyrinthin and labionin, and the last (Ic) subclass comprises of sactiobiotics that have sulphur-α-carbon linkage (Kumariya et al., 2019). Class II bacteriocins are small, heat stabile, unmodified peptides, which are subdivided into four categories: pediocin-like peptides (IIa), two-peptides complexes (IIb), circular peptides (IIc)

and non-pediocin-like peptides (IId) which are applied in this thesis. The last of the group of bacteriocins, class III (bacteriolysins) are heat labile and lytic proteins (Kumariya et al., 2019). Several studies have focused on bacteriocins produced by lactic acid bacteria (LAB) and because this microbiota naturally inhabit the human microflora, it is generally recognized as safe (GRAS). These LAB bacteriocins have slightly different classification and they contain peptides that belong to all class I, II and III of Gram-positive bacteria (Eijsink et al., 2002) .

Table 1.1. Classification of bacteriocins from Gram-positive bacteria. Bacteriocins are categorized into three main classes that are further divided to subclasses. Class III is a collection of both Gram-positive and Gram-negative domain-type molecules (Kumariya et al., 2019).

Class	Subclass	Features	Example
I (modified)	Ia	Lantibiotics (<5 kDa peptides), containing lanthioninge bridges that gives characteristic ring structures (Cotter et al., 2005)	Nisin
	Ib	Carbacyclic lantibiotics of globular structure	Labyrinthopeptin A1
	Ic	Sactibiotics, antibiotics containing linkage between sulphur to α-carbon	Thuricin CD
II (non-modified)	IIa	Pediocins-like peptides with YGNGV-C motif at the N-terminal	Sakacin
	IIb	Two-peptides complex	Lactococcins G
	IIc	Circular bacteriocins	Garvicin ML
	IId	Non-pediocin-like leaderless peptides	LsbB, EntK1, EntEJ97, Garvicin KS
III		Bacteriolysins, large, heat-labile molecules (Cotter et al., 2005); colicins from Gram-negative bacteria are included in this class	Enterolysin A

Class I

Class I bacteriocins are synthetized as precursors with leader sequence and structural region that is post-translationally modified (PTM). Enzymatic dehydration of serine and threonine forms 2,3-dehydroalanine (Dha) and 2,3-dehydrobutyrine (Dhb). These amino acids covalently bind to the sulfhydryl from the neighbouring cysteine group and form sulphide bridges that gives the specific features of lantibiotics (figure 1.1). These unusual residues are called lanthionine (Ala-S-Ala) and β-methyllanthionine (Abu-S-Ala). Nisin, the most studied lantibiotic, is a small peptide containing 34 amino acids (aa) after modification and is produced by *Lactococcus lactis* as different natural variants. They all have broad spectrum of activity. Nisin has been approved for many applications, especially in food industry as a biopreservative.

Some lantibiotics are active against antibiotic-resistant pathogens such as methicillin-resistant *S. aureus* (MRSA) and vancomycin-resistant enterococci (VRE) (Nes et al., 2007, Cotter et al., 2013, Kumariya et al., 2019). In this study, nisin Z was used as a positive control due to its high activity against Gram-positive bacteria.

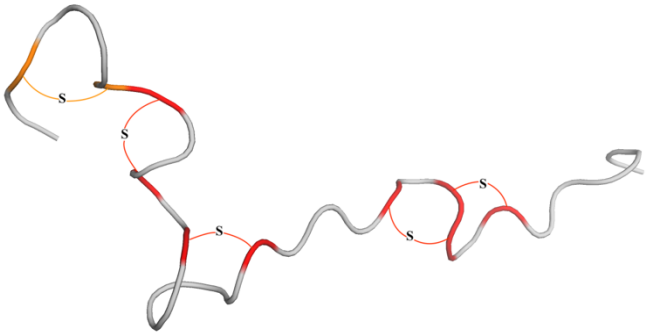


Figure 1.1. Proposed structure of the mature nisin Z. This linear structure was estimated by homology modelling Swiss-Model, based on sequence form UniProt (entry: P29559). The algorithm had predicted this structure by using NMR solution structure of the nisin-lipid II complex (PDB entry: 1wco) as template sequence with 97.06% sequence identity. Graphical presentation was performed by The PyMOL Molecular Graphics System v. 2.2.0. Sulphide bridges between Ala-Ala residues (coloured orange) create lanthionine and Abu-Ala (coloured red) form β-methyllanthionines.

Class II

Typical class II bacteriocins are small (<10 kDa), thermally stabile, cationic non-lantibiotics. Non-modified peptides in this group are very diverse and their classification is therefore difficult. Different categorization are suggested, however class II can be further divided to subclasses a-d (Cotter et al., 2005, Kumariya et al., 2019).

Class IIa

Pediocin-like peptides have narrow spectrum of activity, and they are especially active against *Listeria monocytogenes*. These bacteriocins are 36 to 49 amino acids long and contain a conserved sequence motif YGNGVxCxxxxCxVxWxxA (x can be any amino acid) at the hydrophilic and cationic N-terminal part, which enable the nonspecific binding to surface of the target cell. Those two close cysteine residues in this amino acid motif can form a disulphide bridge. It is suggested that the hydrophobic C-terminus of the peptide, with less conserved sequence, facilitates binding specificity to target cell membrane. Sakacin P (produced by *Lactobacillus sakei*) is an example of this class of bacteriocins and contains secondary structures like β-strand at the N-terminal part and an α-helix at the C-terminal part (figure 1.2) (Ingolf F.Nes et al., 2006, Kjos et al., 2011, Cotter, 2014).

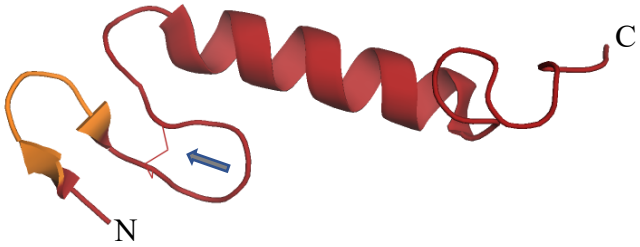


Figure 1.2. Three-dimensional structure of class IIa bacteriocin sakacin P (PDB entry: 1OG7) in lipid micelles. Graphical presentation was performed by The PyMOL Molecular Graphics System v. 2.2.0. Illustration shows the conserved motif residues (YGNGV) in orange. Disulfide is indicated by arrow on cartoon representation.

Class IIb

Class IIb bacteriocins need two peptides (α and β) to form an effective complex. These two non-pediocin- like peptides are encoded from the same operon which also includes a self-protection immunity gene. The combined activity of two peptides can arise as synergistic cooperation when one or both peptides display some activity, but together they give a better antimicrobial effect. Alternatively, peptides without activity can be enhanced by each other, for instance the two-peptide lactococcin G from *L. lactis* (Eijsink et al., 2002, Ingolf F.Nes et al., 2006).

Class IIc

Class IIc consists of circular bacteriocins, which are a result of peptide binding between the N- and C-terminal end of linear peptide. Circular bacteriocins are synthetized as precursors with leader sequence on N-termini, which are cleaved off prior to circularization. Class IIc are 58 - 70 amino acids in length after maturation. These cyclic bacteriocins show a broad spectrum of activity. The circular bacteriocins are resistant to enzymatic degradation and stabile under the influence of heat and pH. These circular bacteriocins can be further divided based on level of amino acids sequence identity. There are two separate groups c(i) with garvicin ML (figure 1.3) produced by *Lactococcus garvieae* and group c(ii) with gasericin A (Cotter et al., 2005, Gabrielsen et al., 2014).

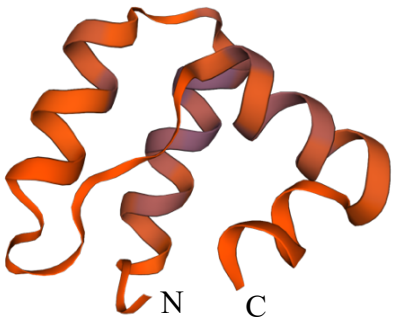


Figure 1.3. Three-dimensional structure of bacteriocin in IIc subclass. This linear structure was estimated by homology modelling Swiss-Model, based on Garvicin ML (entry: D2KC49) sequence form UniProt. The algorithm had predicted this structure by using bacteriocin AS-48 (SMTL ID: 4rgd.1) as template sequence with 35.71% sequence identity. Circularization of bacteriocin happens by peptide bond formation (not shown) between amino group at the N-terminal part and carboxyl group at the C-terminal part.

Class IId

The last subgroup of class II bacteriocins are non-pediocin-like peptides with a diverse spectrum of activity. This group differs from other non-modified classes due to the lack of the leader sequence at the N-terminal end and the presence of formylated methionine on the same end of the peptide. This formylated methionine still has no characterized function. Class IId bacteriocins have a unique regulatory system. The expression of LsbB bacteriocin in *L. lactis* has been shown to be dependent on transcription of the terminator sequence (Uzelac et al., 2015) and become active immediately after synthesis without any processing. In this way, it is important that producers without leader sequence have an immunity system that neutralize the peptide until secretion (Telke et al., 2019). Within this class of bacteriocins, there is a distinguished LsbB-like bacteriocin family with four members: LsbB, EntEJ97, EntK1, and EntQ eneterocins. These peptides have relatively conserved C-terminal region, with characteristic motif (KXXXGXXPWE) (figure 1.4). This may indicate that these bacteriocins have the same receptor binding site and that C-terminal part is responsible for receptor interaction. Especially important are the hydrophobic residues Trp^{25} and Ala^{30} in LsbB sequence (Ovchinnikov et al., 2014).

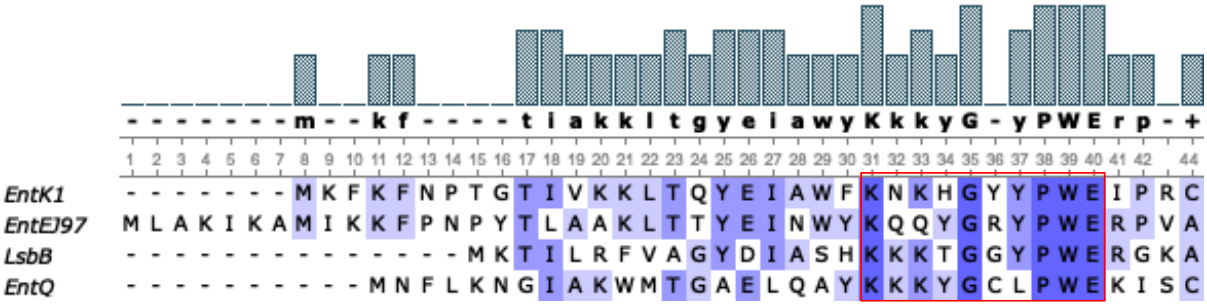


Figure 1.4. Multiple sequence alignment (MSA) of four peptides from LsbB-like bacteriocin family. Predicted by CLUSTALW alignment tool and exhibited using Unipro UGENE softaware v33.0. Consensus of identity is displayed above the sequences. Less conserved residues are coloured light violet while highly conserved amino acids are highlighted with dark violet together with KXXXGXXPWE motif at the C-terminal part (marked in red). N-terminal end vary in length and contain fewer conserved residues.

Class III

Class III consist of bacteriolysins, which are large (≥30 kDa), heat-labile proteins with antimicrobial activity. These molecules consist of domain structures with divergent functions such as transport, binding and lysis. Some of class III bacteriolysins act against both Gram-positive and Gram-negative bacteria. In addition, this class also includes bacteriocins produced by gram-negative bacteria, for example colicins from *E. coli* (Cotter et al., 2005, Kumariya et al., 2019).

1.3. Structure of bacteriocins applied in this study.

This study drew attention to class IId leaderless bacteriocins, which are non-modified peptides. They exhibit activity against several Gram-positive bacteria including pathogenic strains of staphylococci and enterococci.

1.3.1. Enterocin K1

As mentioned earlier, the enterocin K1 (EntK1) is a linear and leaderless peptide that that belongs to LsbB-like bacteriocin family. EntK1 has a broader activity spectrum than LsbB, with especially high activity against producer strains *Enterococcus faecium.* Research of Ovchinnikov et al. (2017) showed that EntK1 in combination with stress in form of high temperature can be used in treatment of antibiotic-resistant enterococci, and even VRE. EntK1is 3-4 amino acids longer than LsbB, with local folding within the peptide forming a α-helix from residue 8 to 24. The N-terminal part of EntK1 is amphiphilic and consists mostly of α-helix

structure, while the C-terminal part is unstructured in 1:1 tetrafluoroethylene (TFE) and water solution (figure 1.5). It does not form secondary structure in water (Ovchinnikov et al., 2017).

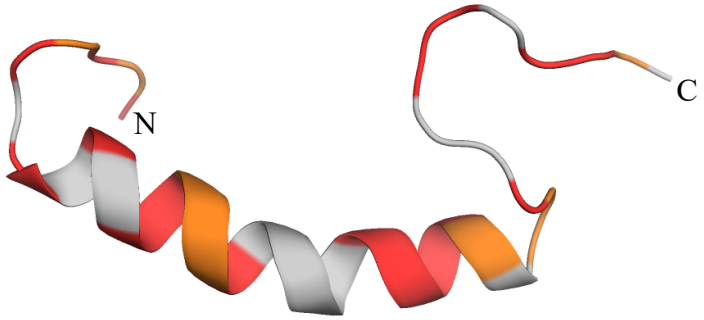


Figure 1.5. NMR Structure of EntK1(PDB entry: 5L82) in 1:1 TFE/H_2O. Represented as cartoon using the PyMOL Molecular Graphics System v. 2.2.0. The basic amino acids are coloured orange, and non-polar residue are red.

1.3.2. Enterocin EJ97

Enterocin EJ97 (EntEJ97) is a cationic bacteriocin produced by *Enterococcus faecalis* EJ97. Based on sequence identity and the fact that this is leaderless bacteriocin, EntEJ97 is classified to LsbB-like peptide family. Although it is 14 amino acids longer than LsbB, it has conserved C-terminal motif (figure 1.4) (Galvez et al., 1998, Ovchinnikov et al., 2017). EntEJ97 is sensitive to proteolytic enzymes such as trypsin and pronase, but it is stable over a wide range of temperatures and pH, as well as in organic solvents and reducing agents (Galvez et al., 1998). EntEJ97 is active against several Gram-positive bacteria, including *L. monocytogenes.* However, the producer strain is resistant to its own bacteriocin. The sensitivity of different bacteria to EntEJ97 may result from variation in surface morphology of the target cell, proteolytic activity and the aspect of peptide aggregation (Galvez et al., 1998).

1.3.4. Garvicin KS

Garvicin KS (GarKS) is a leaderless multi-peptide (three peptide), with a broad spectrum of activity, produced by *L. garvieae*. The three structural genes (*gakABC*) are close together at the same loci with the support proteins: ABC transporter and self-immunity (figure 1.6 A). GarKS have 32-34 amino acids peptides with similar properties. Each peptide displays a slight activity alone, but combined they are active against many pathogens, such as Listeria, *Staphylococcus*, *Bacillus*, *Streptococcus* and *Enterococcus* (Ovchinnikov et al., 2016). The three peptides exhibit a big contrast in highly sequence similarity at N-teminal ends and almost no sequence identity on C-terminal part of the peptides (figure 1.6 B). By replacing tryptophan W26 with alanine on GakA, Ovchinnikov et al.(2016) showed the

significance of W26 in bacterial inhibition for this single peptide and in combination with GakB and GakC.

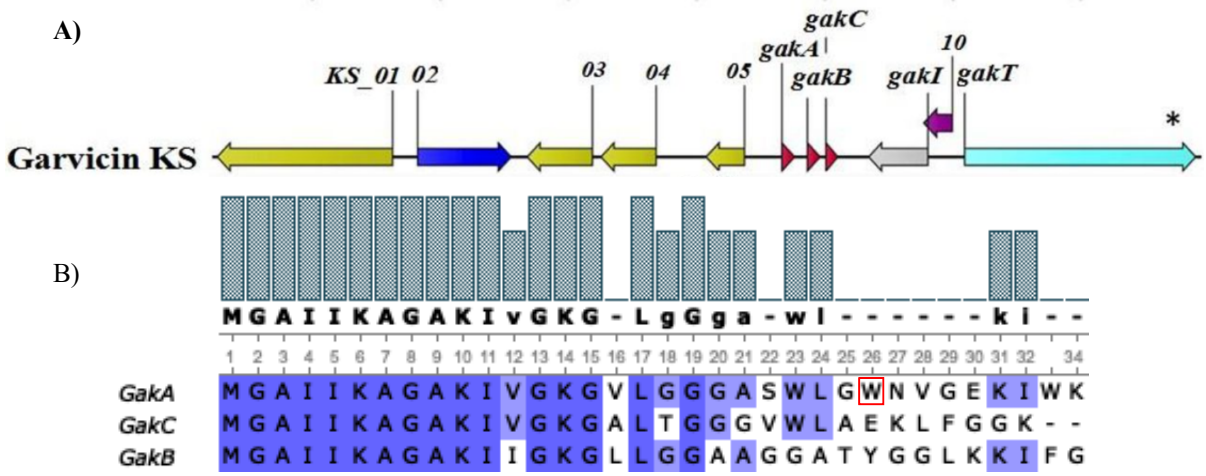


Figure 1.6 Garvicin KS. A) gene cluster consisting tructural genes (*gakA, gakB, gakC*, colored red) are located next to each other, *gakT* (blue) encoding ABC transporter, *gakI* coding for immunity protein (gray) and other genes are in the same loci in genome. Figure source: Ovchinnikov et al. (2016). B) Multiple sequence alignment of peptide GakA, GarB and GarC. Predicted by CLUSTALW alignment tool and exhibited in Unipro UGENE softaware v33.0. Percentage identity is displayed above the sequences in grey columns. Less conserved residues are coloured light violet while highly preserved amino acids are highlighted with dark violet. Tryptophan W26 is highlighted in red.

1.3.5. Hybrid bacteriocin H1 and H2

Synthetically produced hybrid bacteriocins H1 and H2 has been constructed by combing the ends of leaderless peptide EntK1 and EntEJ97 (figure 1.7) described above. H1 have N-terminal end of EntK1 and C-terminal end from EntEJ97, while H2 have N-terminal end of EntEJ97 and C-terminal end from EntK1. These peptides have a high sequence identity and the common ´KXXXGXXPWE´ motif of LsbB-like bacteriocin family (figure 1.7).

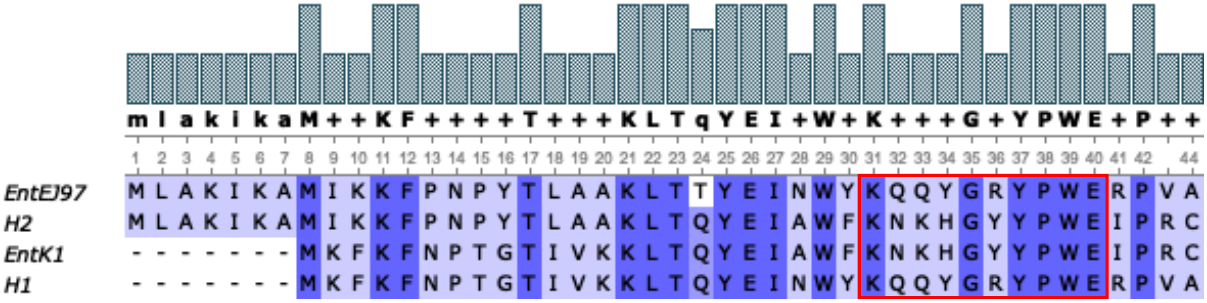


Figure 1.7. Multiple sequence alignment of four bacteriocins used in this study. Predicted by CLUSTALW and showed in Unipro UGENE softaware v33.0. Percentage identity is displayed above the sequences. Less conserved residues are coloured light violet while highly preserved amino acids are highlighted with dark violet. Conserved KXXXGXXPWE motif is highlighted in red.

1.3.6. Micrococcin P1

Micrococcin P1 (MiP1) is a thiopeptide (thiazolyl peptides) belonging to the class of antimicrobial molecules. MiP1 is active against several Gram-positive bacteria, including methicillin-resistant *S. aureus* (MRSA). MiP1 is ribosomally synthesized and post-translationally modified by *Staphylococcus equorum* WS 2733. This molecule contains sulphur and nitrogen rich heterocyclic rings. MiP1 acts by inhibiting ribosomal protein synthesis, typically the step of polypeptide elongation in bacterial cells (Degiacomi et al., 2016).

1.4. Mechanisms of action

Due to the diverse structure of bacteriocins, there are many different modes of action on target cells. There are distinguished different mechanisms of action among bacteriocins targeting Gram-positive bacteria. However, membrane permeabilization by pore formation is a common strategy for LAB bacteriocins. (Cotter, 2014, Kumariya et al., 2019).

Class I lantibiotics target lipid II, a precursor in peptidoglycan biosynthesis. Nisin binds to lipid II with high affinity and enables inhibition of peptidoglycan synthesis and cell growth. Lipid II also serves as a docking site for lantibiotics with dual mechanism of action, e.g. nisin, enhances pore formation and, consequently, membrane disruption and eventually cell death (figure 1.8) (Cotter et al., 2013, Kumariya et al., 2019).

Class II bacteriocins have cationic properties that interact with the negatively charged phospholipids and initiate the pore formation in the cell membrane (figure 1.8). This leads to the disruption of the cell envelope, causing leakage of cytosolic components (Ovchinnikov et al., 2014). This mechanism has been recorded in pediocin-like (class IIa) and circular peptides (class IIc). Nevertheless, these agents have also been shown to require a specific target molecule for antimicrobial activity. Pediocin-like bacteriocins target permease mannose-phosphotransferase (PTS), a molecule that transports sugar inside *L. monocytogenes* cells, while two-peptide bacteriocins target undecaprenyl pyrophosphate phosphatase (UppP) to inhibit peptidoglycan synthesis. Circular bacteriocins display concentration dependent activity, for example, low concentrations of garvicin ML shows requirement for binding maltose ABC transporter, while high concentrations are receptor- independent (Gabrielsen et al., 2014).

It has been determined that LsbB-like family of leaderless bacteriocins (IId) act on the target cell, by first initiate an unspecified electrostatic interaction between amphiphilic N-terminal part and negatively charged phospholipid layer on the cell surface. Then, the conserved motif residues at the C-terminal part of the peptide interact with a specific membrane receptor (figure 1.8). Researchers have demonstrated that Trp^{25} and Ala^{30} bacteriocin residues and the

distance between them have a crucial role in their antimicrobial activity because it facilitates their interaction with the bacterial membranes protein RseP (previously known as YvjB) (Ovchinnikov et al., 2014, Miljkovic et al., 2016).

Class III bacteriolysins act by hydrolysis of the cell-wall in the target cell, which differs from other bacteriocins. It is suggested that the C-terminal part function as a target recognition site while the N-terminal part performs a catalytic function. For instance, lysostaphin brakes cross-bridges of the peptidoglycan due to lysis of target cell (Cotter et al., 2005).

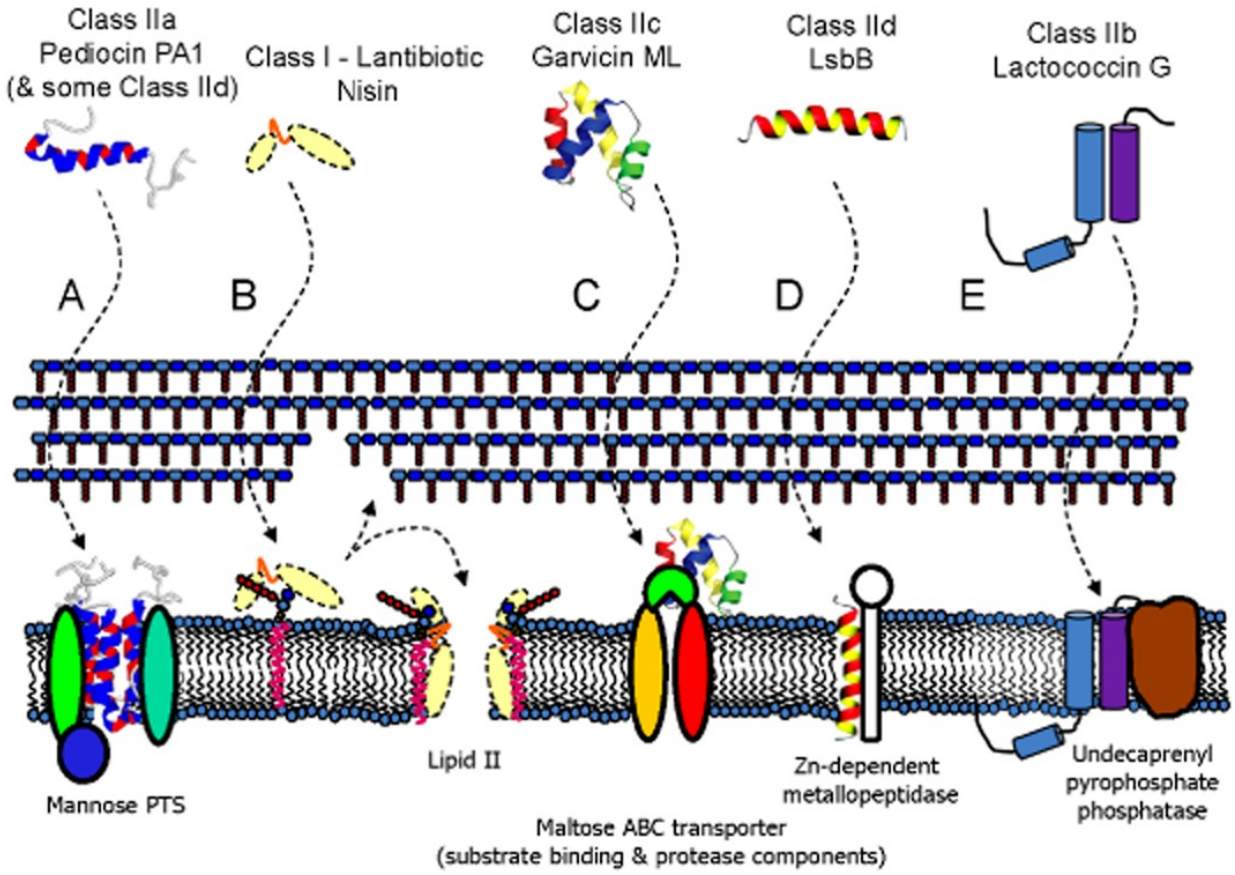


Figure 1.8. Proposed mechanisms of action of Gram-positive bacteria bacteriocins. Class I lantibiotics have two action methods, e. g. nisin, inhibits the cell wall synthesis by bunding to lipid II and it uses this molecule as a docking site, this causes membrane invasion and subsequently pore formation. Class II amphiphilic bacteriocins act by electrostatic interactions between its positively charged residues and anionic lipids that are present in the membranes of Gram-positive bacteria. Additionally, class II bacteriocins also bind to specific receptors leading to depolarization of cytoplasmic membrane and death. Figure source: Cotter et al. (2014), permission for publications obtained from author.

1.5. Resistance development

All life forms including microorganisms can adapt to changing environment. As mentioned above, frequent exposure of bacteria to antimicrobial agents caused development of resistance mechanisms against antibiotics and bacteriocins. Bacteriocins have not been commonly used in medical applications, therefore the knowledge about bacteriocin resistance in pathogenic bacteria is obtained mostly by laboratory investigation (Cotter et al., 2013, Kumariya et al., 2019).

There have been detected different processes in target bacteria that allow them to overcome the bacteriocin activity. First, because cell surface is a typical target of bacteriocins of Gram-positive bacteria, alterations in the composition and structure of the envelope may cause the bacteriocin´s inability to reach its target. For instance, resistance to nisin may be a result of reduced receptor accessibility caused by changes in cell envelope (Cotter et al., 2013).

Secondly, studies have shown that downregulation of maltose ABC transporter in *Lactococcus Lactis* bacterium cause its resistance to circular bacteriocin garvicin ML. ABC transporter is an adenosine triphosphate (ATP) driven protein, which biosynthesis is activated by presence of maltose. Higher expression of genes coding for maltose ABC transporter increase susceptibility of bacteria to garvicin ML. This may suggest that maltose ABC transporter is a bacteriocin receptor and binding to it can cause a membrane permeabilization. (Gabrielsen et al., 2012). Similar cases were detected in lipid II targeting lantibiotics and mannose phosphotransferase system (Man-PTS) targeting class II bacteriocins (Kjos et al., 2011). However, it may happen that the maltose ABC transporter plays a role of transferring bacteriocin to the target molecule inside the cell (Gabrielsen et al., 2012).

Another factor that may contribute to bacteriocin resistance is the physiological condition of the target cell. Higher membrane rigidity can affect the pore formation. For instance, decrease in D-alanine of teichoic acid in cell wall made E. faecalis and S. pneumoniae more sensitive to bacteriocins such as nisin (Eijsink et al., 2002, Kumariya et al., 2019).

Moreover, bacteriocin producing bacteria are resistant to their own bacteriocins by expression of immunity genes which are integrated in the same loci as bacteriocin structural genes. Expression of these genes in Gram-positive bacteria requires extracellular accumulation of auto-inducer peptides (pheromones). Target cells have several immunity mechanisms, which include the ABC transporter to export the bacteriocin from the membrane. Specific immunity proteins interact with bacteriocins on the extracellular side the cell. An immune mimicry has been observed as a resistant mechanism in non-bacteriocin-producing bacteria that contains immunity genes. Immune mimicry can also be described as an autoimmune response to

bacteriocin in bacteria that produce closely related peptides (Eijsink et al., 2002, Hassan et al., 2012, Cotter et al., 2013).

Finally, resistance to bacteriocin may occur by mutations in the genes encoding the specific bacteriocin receptor protein. By constructing hybrid molecules of lactococcal RseP, Miljkovic et al. (2016) showed that changes in essential amino acids in receptor lead to partial or fully resistance to LsbB bacteriocin. They demonstrated that the $Tyr^{356}Gln$ and $Ala^{353}Thr$ substitutions in mutant cells produced bigger zones of inhibition. Ovchinnikov el at. (2017) suggested that the alteration in active site residues of RseP might change protein structure and its accessibility for bacteriocins.

Identification of specific receptors is crucial for understanding the mechanisms of immunity in bacterial cells. This information can serve as a reference in bacteriocins engineering and can help to restrain resistance mechanisms among pathogens (Miljkovic et al., 2016, Perez et al., 2018).

1.6. Receptor protein for LsbB-like bacteriocin family

By performing heterologous expression, gene knockout and sequencing analysis, Uzelac et al. (2013) and Ovchinnikov el at. (2017) showed that the *rseP* gene is required for sensitivity to leaderless bacteriocin LsbB, EntK1 and EntEJ97 in lactococci and eneterococci.

RseP (regulator of sigma E, protease) is a membrane bound Zn-dependent protease that belongs to highly conserved family of M50 proteases. These proteins act in regulated intermembrane proteolysis (RIP), a process where proteins are cleaved in their transmembrane segments. The proteolysis reaction is widespread in both prokaryotes and eukaryotes (Brown et al., 2000, Hizukuri et al., 2014). Site-2-protease (S2P) is human ortholog for YvjB in *L. lactis,* SpoIVFB in *Bacillus subtilis*, YaeL in *E. coli* (renamed to RseP) and in *E. faecalis* Eep. Sequence alignment of homologous RIP proteins revealed a conserved HExxH motif at the N-terminal part embedded in hydrophobic segment. The presence of those two histidine residues coordinate the zinc ion, and a negative charge of Glu activates a water molecule that initiate the nucleophilic attack on the peptide bond (hydrolysis) (Brown et al., 2000, Feng et al., 2007, Hizukuri et al., 2014). E. coli RseP spans the membrane with four transmembrane segments. The two linked PDZ domains are at the periplasm site and β-hairpin-like loop is near the active site in TM1. The C-terminal end also contains preserved NLLPxxxLDG sequence in third transmembrane region, which is suggested to be a binding site for transmembrane substrate protein (figure 1.9)(Hizukuri et al., 2014, Akiyama et al., 2015).

The site 2 proteases are widely distributed in living organisms and have extensively developed functions in cell physiology and pathology (Frank et al., 2012). *E. coli* RseP studies described the involvement of this enzyme in the transmembrane signal transduction pathway during response to extracytoplasmic stress (Alba and Gross, 2004, Koide et al., 2008, Akiyama et al., 2015). Similar research by Varahan et al. (2013) have also shown that *E. faecalis* RseP also regulates intermembrane proteolysis of anti-sigma factor RsiV, causing the activation of sigma factor SigV during response to lysozyme. The mutants with deleted the *rseP* genes were more susceptible to stress (Varahan et al., 2013). Investigation by Saito et al. (2011) suggest that in addition to being involved in second cleavage of RseA, RseP acts in the proteolytic removal of remaining signal peptides from the membrane.

Heinrich et al. (2008) reported influence of an ATP-binding cassette (ABC) transporter (also known as EcsAB) on RseP while they implicated site-directed mutagenesis to knock-out the *ecsA* gene coding for ATP binding cassette of the transporter. Their results indicated that in absence of transporter protein the RseP is not functional and the sigma factor (σ^w) is not induced. Furthermore, deletion of *ecsAB* was suggested to block the Zn-dependent proteolysis of FtsL membrane protein responsible for cell division. Proposed explanation for that was that the failure of σ^w induction was not limited to stress response but influenced by the absence of ABC transporter. The overproduction of RseP demonstrated the induction of sigma factor in mutants without transport protein, which may prove the inhibition of RseP activity by the substrate of ABC transporter. Proposed explanation was that increased concentration of protease reduced the blocking effect on RseP (Heinrich et al., 2008).

By performing excision of genes encoding for RseP, Frank et al. (2012) found that this membrane metalloprotease in *E. faecalis* is activated during early bacterial infection, which means that this protease is important for virulence in *in vitro* model. They also observed that *rseP* expression is increased during biofilm formation, and mutations within this gene change biofilm phenotype (Frank et al., 2012). A study by Varahan et al. (2014) showed that deletion of ABC transporter has an influence on biofilms thickness and biomass which were highly reduced in mutants without transport protein. Moreover, they have revealed that both RseP and ABC transporter are involved in processing and secretion of sex pheromones in *E. faecalis* (Varahan et al., 2014), which was recently demonstrated for *S. aureus* (Schilcher et al., 2020). Furthermore, by performing the null mutations of the *ecsAB* in *Staphylococcus aureus,* Jonsson et al. (2010) revealed the importance of this protein for growth and cell wall and surface composition. In addition, they showed that transporter protein mutants were more susceptible

to various antimicrobial substances. Mutations in *ecsAB* weaken the bacteria in consequence the they developed milder infections in mice (Jonsson et al., 2010).

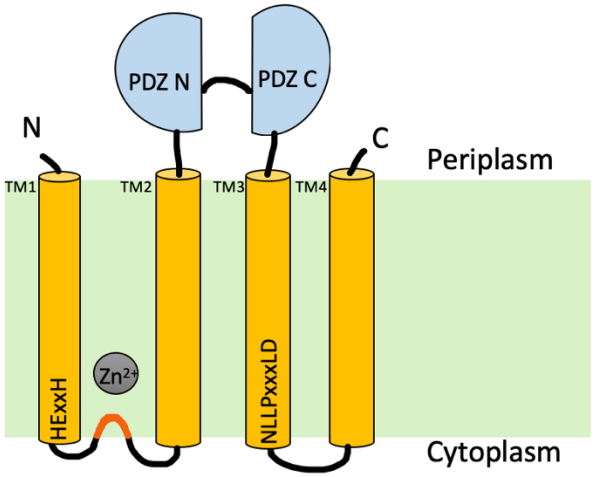


Figure 1.9. Schematic representation of *E. coli* RseP. Four transmembrane segments span the membrane. In TM1 is contained the active site HExxH which coordinates the catalytic zinc ion. In TM3 is the NLLPxxxLDG motif which function as substrate binding site supported by MRE β-loop (membrane-reentrant β-loop) (coloured orange). On the periplasmic side are two PZD domains (coloured blue) that regulate RseP protease activity.

1.6.1. Regulated intermembrane proteolysis – stress response

The RseP in *E. coli* is one of the best described intermembrane-cleaving proteases. RseP plays important regulatory role in σ^E (sigma^E) signalling system (figure 1.10). Stress in the form of high heat or pH change, denature proteins (outer-membrane porins - OMPs) which activate the DegS enzyme. DegS gives the first cleavage (site 1 proteolysis - S1P) in anti-sigma^E protein (RseA) on the periplasmic side and realises the RseB domain, which together with PZD domains from RseP were suppressing the second cleavage. Second proteolysis (site 2 proteolysis - S2P) by RseP cleaves the RseA transmembrane region, which realise of the σ^E enabling transcription of target genes important for cell survival (Hizukuri et al., 2014, Akiyama et al., 2015). As mentioned above, similar mechanisms were found in *S. subtilis* and *E. faecalis* (Varahan et al., 2013).

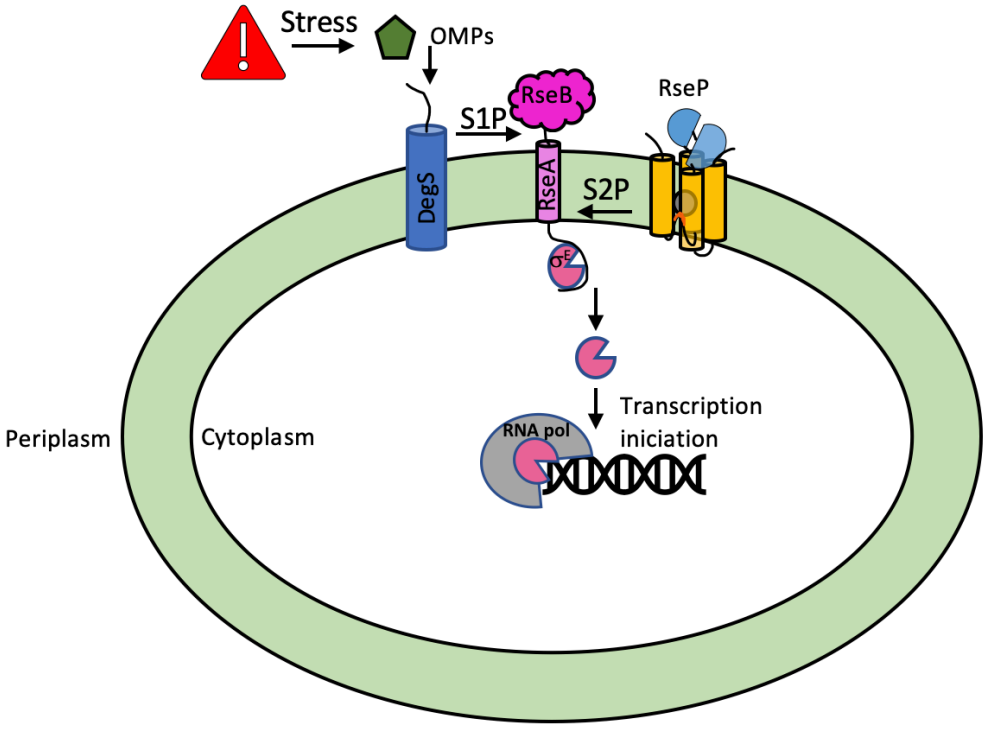


Figure 1.10. Proposed illustration for anti-sigma^{E} (RseA) proteolysis in response to extracytoplasmic stress in *E. coli*. The first cleavage (S1P) of RseA leads to cutting off RseB and makes the anti-sigma^{E} available for second proteolysis (S2P) by RseP. As a result, the sigma^{E} factor is realised into cytoplasm and together with RNA polymerase initiates expression of genes important for stress resistance.

1.7. *Staphylococcus haemolyticus*

The human body is a natural habitat for *Staphylococcus* species. They are part of the normal bacterial flora of the skin and mucosal membrane in humans. However, staphylococci cause numerous nosocomial (hospital-linked) infections which occurs by implanted medical devices, where the bacteria tend to form biofilms. Especially susceptible for infections are diabetic patients, as well as convalescents after surgery or dialysis. (Takeuchi et al., 2005, Soumya et al., 2017, Pain et al., 2019). *S. haemolyticus* causes peritonitis, ortitis, urinary tract infections, as well as septicaemia and bone and joint infections (Takeuchi et al., 2005). *S. haemolyticus* is an opportunistic coagulase-negative (CoN) pathogen and one of the top species frequently isolated from human blood. *S. haemolyticus* is classified as most antibiotic resistant of CoNS species. Although, resistance to vancomycin and teicoplanin is uncommon phenomenon for *Staphylococcus* species, *S. haemolyticus* was the first Gram-positive microbe that has been reported resistant to glycopeptide antibiotics. *S. haemolyticus* also is suggested to be a reservoir for methicillin-resistance genes (*mecA*) which may have been horizontally

transferred to *S. aureus* and converted it from sensitive to resistant bacterium (MRSA) (Czekaj et al., 2015, Pain et al., 2019). *S. aureus* is toxins- and other factors producing bacteria that cause cell damage and prevent the host from eliminating pathogen. As a consequence, already dangerous strains of S. aureus may become an even greater threat (Czekaj et al., 2015).

After whole-genome sequencing, Takeuchi et al. (2005) revealed that *S. haemolyticus* contain multiple insertion sequences (ISs) that allow regular rearrangements in genome. This genomic flexibility and frequent DNA shift may lead to diversity and adaptation of new abilities, for instance obtaining antibiotic resistance or virulence genes. Takeuchi et al. (2005) showed several open reading frames (ORFs) present in genomes of the three most important species of *Staphylococcus*: *aureus*, *epidermidis* and *haemolyticus*, many of which were required for virulence. They also found a ´*oriC* environ´ which is not homologous in these three species. That genome segment, placed downstream of replication origin, is proposed to contain species-specific genes and acting as a recombination hotspot (Takeuchi et al., 2005).

Comparative genomic analysis of *S. haemolyticus* from clinical and commensal strains uncovered that clinical isolates have several of genes associated with antibiotic resistance and biofilm formation, the most virulent factor in CoNS. Nevertheless, no typical genes used as a marker (ica loci- intracellular adhesion loci) for invasive S. epidermidis and required for biofilm production in CoNS were found. Another disclosed feature of clinical isolates was the novel Capsule Polysaccharide (CP) operons involved in human immune defence. The study of whole genomes of geographically diverse origins showed homology relationship among antibiotic resistance genes which indicates the clonal spread between countries (Pain et al., 2019, Cavanagh et al., 2014, Czekaj et al., 2015).

There are a limited number of treatment options available for *S. haemolyticus* infections, which include the mentioned glycopeptides and one of the most effective antimicrobials - linezolid that blocks the protein synthesis by binding to 23 rRNA and prevens translation process. However, cases of strain resistant to that last-chance antibiotic have been already reported in India and several European countries. The arising threat of frequent hospital infections, biofilm formation and spread of antibiotic resistance requires further investigations for new antibacterial agents (Czekaj et al., 2015).

This study examined *S. haemolyticus* species which were isolated in 1991-2005 in Norwegian hospitals and analysed by Cavanagh et al. (2014).

1.8. Aim of this study

The aim of this study was to investigate the receptor of hybrid bacteriocin H1 and analyse the potential of the peptide as a possible treatment for *S. haemolyticus* infections. This hybrid bacteriocin exhibits higher activity toward *S. haemolyticus* bacteria than the enterocins EntK1 and EntEJ97. The fact that both EntK1 and EntEJ97 recognize the same receptor protein may signify that H1 also exploits RseP as a receptor.

This thesis is based on the laboratory experiments done in following steps:

- Defining the spectrum of inhibition for hybrid bacteriocins H1, H2 and their original peptides EntK1 and EntEJ97 against 51 different strains from various genera.
- Defining MIC for H1 bacteriocin towards 20 *S.s haemolyticus* strains
- Studying the synergy of the hybrid H1 and two other antimicrobials against *S. haemolyticus* strains.
- Investigating the potential of bacteriocin H1 compared to common antibiotics.
- Generating and evaluating phenotype and genotype of resistant mutants in relation to wild types.
- Demonstration that RseP is a receptor for hybrid bacteriocin with heterologous expression of the rseP gene.

2. Materials and methods

During laboratory work with bacterial cells, it was necessary to conduct the experiments in aseptic conditions to prevent contamination of cultures or solutions. Special attention was applied to working with bacterial species that are in infection risk group 2 (Regulations on measures and limit values, appendix 2; The Norwegian Ministry of Labor and Social Affairs) and genetically modified microorganisms (GMM). Hand washing, using gloves and disinfection with ethanol were the basic routines that were practiced together with autoclaving of tools, gas burner and the correct disposal of hazardous waste.

2.1. Equipment and instruments

Equipment	Supplier
Acid-washed glass beads (<106 microns)	Sigma
Epperndorf tubes 1.5 ml	Epperndorf
Falcon Conical Centrifuge Tubes (50ml, 15 ml)	-
Hand gloves	VWR
Inoculation loops	VWR
Laboratory bottles, round (50-1000 ml)	VWR
Microtest Plate 96 Wells with lid	Sarsted
Multichannel pipette	Eppendorf Research
Petri dishes	-
PCR tubes (0.2 ml)	VWR
Pipettes	Eppendorf Research
Pipette tips	VWR
Test tubes	-
Toothpicks	-

Instruments	Supplier
Autoclave	Matachana
Digital weight	Salter
Fei Morgagni 268 Transmission electron microscope	-
Freezer (-20 ºC)	-
Freezer (-80 ºC)	Forma Scientific

Gas burner	Intergra Biosciences
Gel Doc™ EZ System	BIO-RAD
Gene Pulser™	BIO-RAD
Heraeus™ Multifuge™ X1 Centrifuges	Thermo Fisher Scientific
Heraeus Pico Microcentrifuges	Thermo Fisher Scientific
Holter laminar flow cabinet	Thermo Scientific
Horizontal electrophoresis system	BIO-RAD
Incubators	Termaks
Magnetic stirrer	Stuart
Microwave	-
MP FastPrep-24 Tissue and Cell Homogenizer	Savant
NanoDrop 2000 Specrophotometer	Nanodrop Technologies
SimpliAmp™ Thermal Cycler	Applied Biosystems™
Spectrostar Nano	BMG Labtech
Puls Controller™	BIO-RAD
Water bath	Julabo
Qubit® 2.0 Fluorometer	Life Technologies

2.2. Bacterial cultivation

Bacteria were cultivated in liquid medium or on solid agar plates. Pure cultures were obtained by sterile inoculation of single colony onto autoclaved growth medium (5 ml or 10 ml). Incubated overnight (~ 18 hours) at conditions suitable for each specific species. Most of bacteria were cultivated in BHI medium at 30°C or 37°C with agitation ~200 rpm. *L. plantarum* was cultivated in M.R.S. medium at 37°C without shaking.

2.3. Medium and agar

BHI was used as a universal culture media for cultivation of bacterial indicators and different *Staphylococcus* strains. BHI medium, agar and soft agar was made in accordance to the supplier´s protocols, then it was autoclaved for 15 minutes at 121°C. BHI agar was poured into petri dishes in the flow cabinet to keep the plates from contamination. Nutrition broth was autoclaved prior to portioning into tubes. If needed, erythromycin was added after sterilizing, in final concentration of 200 μg/ml in liquid form.

Materials:

BHI agar (Brain-Heart-Infusion):

500 ml dH_2O

7.5 g Agar powder; VMR Chemicals

18.5 g BHI; Oxoid™

BHI broth

500 ml dH_2O

18.5 g BHI; Oxoid™

BHI soft agar

100 ml dH_2O

0.8 g Agar powder; VMR Chemicals

3.7 g BHI; Oxoid™

M.R.S. medium, agar and soft agar were used for cultivation *Lactobacillus plantarum*. They were made in accordance to the supplier´s protocols, then it was autoclaved for 15 min at 121°C. M.R.S. agar was poured into petri dishes in the flow cabinet to keep the plates from contamination. If needed, erythromycin was added after sterilizing in final concentration of 10 μg/ml in liquid form.

Materials:

M.R.S. agar (De man, Rogosa, Sharpe):

26 g M.R.S.; Oxoid™

500 ml dH_2O

7.5 g Agar powder; VWR Chemicals

M.R.S broth

26 g M.R.S.; Oxoid™

500 ml dH_2O

M.R.S soft agar

5.2 g M.R.S.; Oxoid™

100 ml dH_2O

0.8 g Agar powder; VWR Chemicals

2.4. Storage of bacteria

To keep bacterial isolates preserved for extended periods of time, glycerol stocks were made and stored at -80ºC. Pure liquid culture was mixed either with 45% glycerol solution to final concentration ~ 20 %. Glycerol protects bacterial cells from crystallization in low temperatures and makes them structurally stable.

2.5. Antimicrobial agents

Bacteriocins (table 2.1) were diluted in 0.1 % trifluoroacetic (TFA) acid to a start concentration of 10 mg/ml, then further diluted with distilled water to working concentration of 1 mg/ml, 0.2 mg/ml and 0.04 mg/ml.

Table 2.1. Antimicrobial peptides used for this thesis.

Name		Sequence (5´- 3´)	Reference
Enterocin K1		MKFKFNPTGTIVKKLTQYEIAWFKNKHGYYPWEIPRC	(Ovchinnikov et al., 2017)
Enterocin EJ97		MLAKIKAMIKKFPNPYTLAAKLTTYEINWYKQQYGRYPWERPVA	(Galvez et al., 1998)
H1		MKFKFNPTGTIVKKLTQYEINWYKQQYGRYPWERPVA	-
H2		MLAKIKAMIKKFPNPYTLAAKLTQYEIAWFKNKHGYYPWEIPRC	-
Nisin Z		ITSISLCTPGCKTGALMGCNMKTATCNCSIHVSK	(Shin et al., 2016)
Micrococcin P1		SCTTCVCTCSCCTT	(Degiacomi et al., 2016)
GarvicinKS	GakA	MGAIIKAGAKIVGKGVLGGGASWLGWNVGEKIWK	(Ovchinnikov et al., 2016)
	GakB	MGAIIKAGAKIIGKGLLGGAAGGATYGGLKKIFG	
	GakC	MGAIIKAGAKIVGKGALTGGGVWLAEKLFGGK	

Table 2.2. Antibiotics used in this study

Name	Supplier
Erythromycin	Oxoid™
Ciprofloxacin	Oxoid™
Rifampicin	Oxoid™
Vancomycin	Oxoid™

Penicillin	Oxoid™
Teicoplanin	Oxoid™

2.6. Spot-on-lawn inhibitor spectrum assays

2.6.1. Bacteriocin inhibition spectrum determination

Spot-on-lawn assay was performed on 51 bacterial strains (table 2.3) of different genera with use of four different antimicrobial peptides (table 2.1) to define bacteriocin spectrum of activity. Bacteria were picked with the sterile toothpicks from the 45% glycerol stock and added to autoclaved culture test tubs with BHI (5 ml) media for overnight growth at 30ºC in incubator shaker 200 rounds per minute (rpm). This procedure was also used to performed spot-on-lawn assay for transformed *L. plantarum* (2.18). This work was done in cooperation with Mikkel Brønner (Master student, LMG lab).

Table 2.3. Bacterial indicators strains. List of bacterial strains used in this study.

Strain code	Name
LMGT2805	*Bacillus cereus*
LMGT2711	*B. cereus* ATCC 9139 B
LMGT2731	*B. cereus* 1230, Granum 11-91
LMGT4133	*Staphylococcus haemolyticus* SH1
LMGT3233	*S. simulans*
LMGT4134	*S. arlettae*
LMGT3129	*S. hominis*
LMGT3522	*S. epidermidis*
LMGT2738	*Carnobacterium divergens* NCDO 2306
LMGT2332	*C. piscicola*
LMGT3465	*Enterococcus avium*
LMGT2333	*E. faecalis*
LMGT3088	*E. faecalis*
LMGT3330	*E. faecalis* 158B.
LMGT3331	*E. faecalis* 111A.
LMGT3332	*E. faecalis* 29C.
LMGT2763	*E. faecium*
LMGT2772	*E. faecium*
LMGT2783	*E. faecium*
LMGT2876	*E. faecium*
LMGT2353	*Lactobacillus curvatus*
LMGT2355	*L. curvatus*
LMGT2352	*L. plantarum*

LMGT3125	*L. plantarum*
LMGT2361	*L. sakei*
LMGT2380	*L. sakei*
LMGT2787	*L. salivarius*
LMGT3390	*Lactococcus garvieae*
IL1403	*L. lactis*
LMGT2081	*L. lactis*
LMGT2386	*Leuconostoc gelidum*
LMGT2710	*Listeria innocua*
LMGT2785	*L. innocua*
LMGT 2813	*L. ivanovii*
LMGT2604	*L. monocytogenes*
LMGT2650	*L. monocytogenes*
LMGT2651	*L. monocytogenes*
LMGT2652	*L. monocytogenes*
LMGT2653	*L. monocytogenes*
LMGT3023	*S. aureus*
LMGT3263	*S. aureus*
LMGT3325	*S. aureus*
LMGT3326	*S. aureus*
LMGT3328	*S. aureus*
LMGT3329	*S. aureus* 992
LMGT3890	*Streptococcus dysgalactiae*
LMGT3555	*S. thermophilus* Sfi13
LMGT3912	*S. uberis*
LMGT3918	*St. uberis*
LMGT3590	*Escherichia coli* DH5α
LMGT3591	*E. coli* TG1

Procedure:

Work was performed under the sterile conditions and with use of the gas burner to prevent the samples from contamination.

1. BHI soft agar was warmed up in microwave until it was completely melted (1.5 minutes). Warmed soft agar (5 ml) was transferred to a sterile test tubes w/lid and kept on water bath (45^{o}C) until use.
2. Tubes with O/N cultures were vortex. The soft agar was inoculated with (50 µl of O/N culture). Vortexed and distributed on marked Petri plate with BHI agar, then dried on the bench for 10 minutes.

3. Previously diluted bacteriocins (3 µl) were placed on the designated spot on the agar (figure 2.1), dried on the bench for 10 minutes and incubated at 30°C for 24 hours. After 24 hours the plates were inspected and pictures were taken.

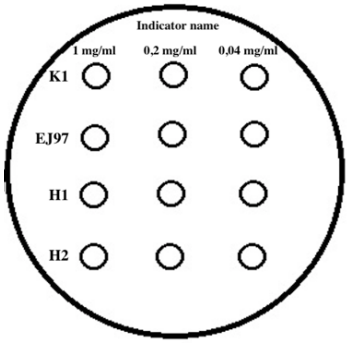


Figure 2.1. Schematic illustration of bacteriocins placement on the BHI/MRS agar plates, seen from above.

2.6.2. *S. haemolyticus* sensitivity assay

Twenty-one different *S. haemolyticus* strains (table 2.4) were provided by University Hospital of North Norway in Tromsø together with UiT The Arctic University of Norway in Tromsø.

To determine spectrum of bacterial sensitivity against antimicrobial peptides it was performed spot-on-lawn assay (for procedure see section 3.6.1) with use of four bacteriocins (table 2.1): EntK1, EntEJ97, H1, H2 (with concentration 1 mg/ml, 0.2 mg/ml and 0.04 mg/ml.

Table 2.4. *S. haemolyticus* strains isolated at University Hospital of North Norway used in this thesis.

ENA accession number	NCBI accession number	LMGT code	Isolation source
ERS066281	ERS066281.7067_4_39	4115*	Blood
ERS066282	ERS066281.7067_4_40	4114	Blood
ERS066283	ERS066281.7067_4_41	4113	Blood
ERS066284	ERS066281.7067_4_42	4112	Blood
ERS066285	ERS066281.7067_4_43	4111	Blood
ERS066286	ERS066281.7067_4_44	4110	Blood
ERS066287	ERS066281.7067_4_45	4109	Blood
ERS066288	ERS066281.7067_4_46	4108	Blood
ERS066289	ERS066281.7067_4_47	4107	Blood
ERS066290	ERS066281.7067_4_48	4106**	Blood
ERS066291	ERS066281.7067_4_49	4105*	Blood
ERS066292	ERS066281.7067_4_50	4104	Blood
ERS066293	ERS066281.7067_4_51	4103	Blood

ERS066294	ERS066281.7067_4_52	4102	Blood
ERS066295	ERS066281.7067_4_53	4101	Blood
ERS066296	ERS066281.7067_4_54	4100	Blood
ERS066297	ERS066281.7067_4_55	4099	Blood
ERS066298	ERS066281.7067_4_56	4098	Blood
ERS066299	ERS066281.7067_4_57	4097	Blood
ERS066300	ERS066281.7067_4_58	4096	Urine

* Bacterial strain where resistant colonies were isolated from bacteriocin H1 inhibition zone with 1 mg/ml concentration.

** Bacterial strain where resistant colonies were isolated from bacteriocin EntEJ97 inhibition zone with 1 mg/ml concentration.

2.6.3. Comparison of antibiotics and bacteriocin antibacterial efficiency

To compare the activity of the H1 bacteriocin with commonly used antibiotics, a spot-on-lawn assay was performed. Six of *S. haemolyticus* (table 2.4) species provided by University Hospital of North Norway, were tested against six different antibiotics (table 2.2) and bacteriocin H1 on agar plate.

Materials:

Ciprofloxacin 5 µg disks (CIP5)

Erythromycin 15 µg disks (E15)

H1 1mg/ml

H1 0.2mg/ml

O/N cultures

Penicillin G 10 units disks (P10)

Rifampicin 5 µg disks (RD5)

Teicoplanin 30 µg disks (TEC30)

Vancomycin 5 µg disks (VA5)

Procedure:

1. O/N culture (1 ml) was transferred to BHI agar plate and spread evenly over the surface with and dried at RT.
2. Antibiotics were distributed on the plate with use of disc dispenser.
3. Then, 1 mg/ml and 0.2 mg/ml of bacteriocin H1 (3 µl) was applied on designated spots on the plate (figure 2.2).

4. Incubated 24 hours at 30ºC.
5. After incubation, the pictures were taken, and zones of inhibition were examined.

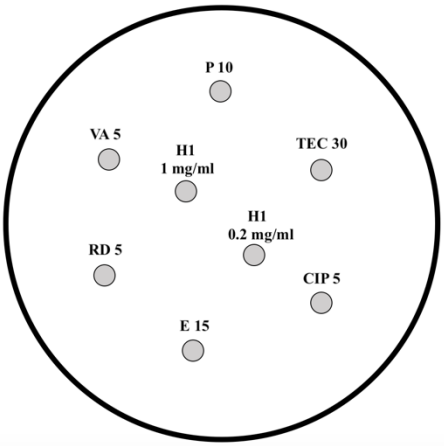


Figure 2.2. Schematic representation of reagent arrangement on agar plate for comparative efficiency of antimicrobials. Antibiotic discs were distributed with disc dispenser before addition of bacteriocin H1 in two different concentrations (1mg/ml and 0.2 mg/ml).

2.6.4. Combinatory treatment

A synergy test was performed to investigate the potential of combination of antimicrobial peptides. Initial concentrations of agents applied in this experiment were determined based on efforts of Mikkel Brønner (Master student at LMG lab). Six *S. haemolyticus* (table 2.4) strains were treated with different combinations of three bacteriocins on a microtiter plate:

- single components: H1; Garvicin KS (GarKS); Micrococcin P1 (MiP1);
- paired components: H1 + Garvicin KS (GarKS); H1 + Micrococcin P1 (MiP1); Garvicin KS (GarKS) + Micrococcin P1 (MiP1);
- all three components: Garvicin KS (GarKS) + H1 + Micrococcin P1 (MiP1).

Materials:

BHI liquid medium

Garvicin KS (1 mg/ml)

H1 (1 mg/ml)

Micrococcin P1 (1 mg/ml)

O/N culture

Procedure:

1. Bacteriocins were diluted in BHI liquid medium and mixed together in Eppendorf tube to final concentration of 100 µg/ml for bacteriocin H1 and Garvicin KS, 10 µg/ml for Micrococcin P1.
2. To all wells in columns 2-11, it was added BHI liquid medium (135 µl).
3. Next, column 12 was added BHI (150 µl) and was used as a negative control, without bacteria.
4. The formulations of bacteriocins and BHI (285 µl) were added respectively to the first wells of column 1, then they were being two-fold diluted until the column 10. Column 11 was a positive control, with no bacteriocin (figure 2.3).
5. To all wells in column 1-11, it was added bacterial culture (15µl).
6. Incubated for 24 hours at 37ºC.
7. Finally, MIC values were determined by visually by grading turbidity. Additionally, measures were done by spectrophotometer by measuring optical density (OD).

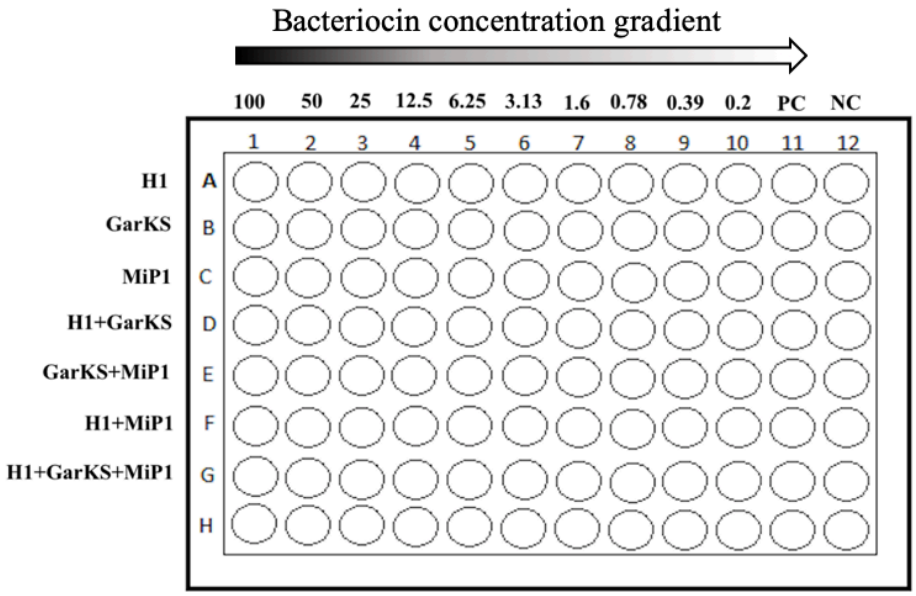


Figure 2.3. 96 wells microtiter plate illustration. Bacteriocin was two-fold diluted along the columns with highest concentration in column 1 to lowest in 10. Column 11 serve as a positive control (PC) with no bacteriocin added. Column 12 remained as a negative control (NC) with only BHI medium.

2.7. Isolation of resistant mutants

Resistant colonies of *S. haemolyticus* generated during spot-on-lawn experiment (2.6.2) were picked up with inoculation loop and streaked on BHI plates (figure 2.4) for cultivation. After 24 hours incubation at 30ºC, single colonies were picked with sterile toothpick from each

plate and transferred into test tubes with BHI media (5 ml). Liquid cultures were incubated overnight at 30°C. Glycerol stocks were made the following day to store the resistant mutants.

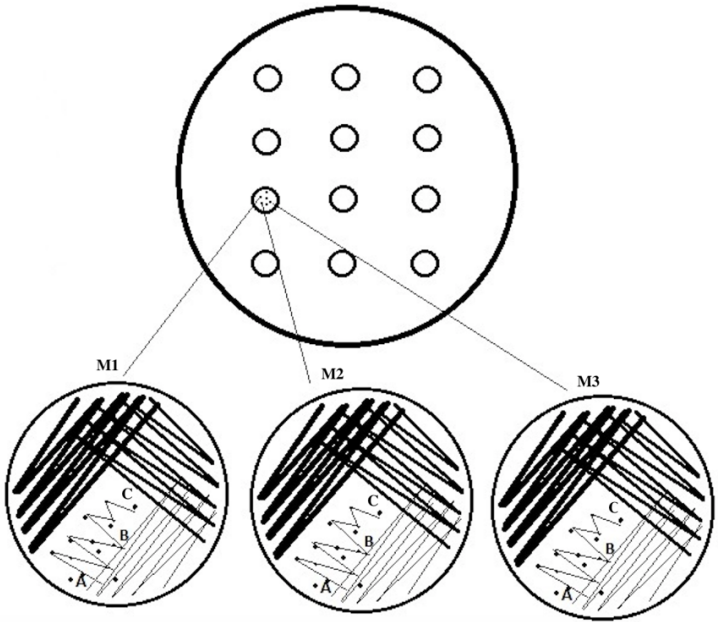


Figure 2.4. Graphical representation of the resistant mutant isolation technique. Three resistant colonies from spot-on lawn experiment were streaked on tree separate agar plates (M1, M2, M3). One colony (C) isolated from each plate was cultivated. Two colonies (A, B) from each plate were used in resistance test (2.7.1.).

2.7.1. Resistance test

This experiment was done to verify the resistant colonies against bacteriocins. Resistant colonies were isolated from LMG4105, LMG4106 and LMG4115 (2.7, figure 2.4). After incubation at 30°C overnight single colonies were picked with sterile toothpick and streaked over previously prepared plates with bacteriocins (EntK1, EntEJ97, H1, H2) stripes. The wild type of each bacteria strain was picked with sterile toothpick from the glycerol stock culture and used as a positive control (figure 2.5).

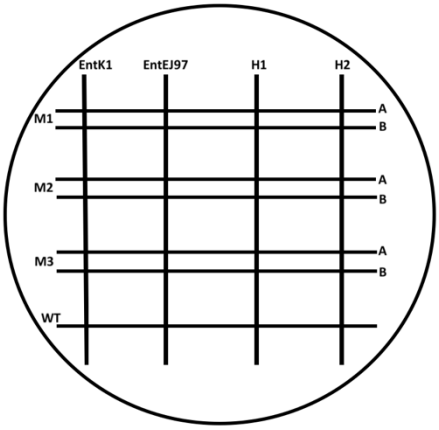


Figure 2.5. Illustration showing the resistance test method. Horizontal stretches represent the different bacterial colonies steaked on agar plate with vertical bacteriocins stretches.

2.7.2. Stress assay

To observe bacterial respond to induced stress, an experiment exposing the bacteria to high temperatures was performed. This allowed to compare the ability to growth between wild types and their resistant isolates at non-optimal conditions. The experimentation was carried out using both cultivation on solid agar and in liquid medium.

Cultivation on agar plates

Three BHI agar plates were divided into four equal quarters by two lines. Different wild type strains were streak on previously designated spot on one of the plates quarters (figure 2.6). The resistant strains were streaked beneath their wild types. Plates were incubated at three different temperatures: 37°C (as a positive control), 40°C and 45°C, for 24 and 48 hours.

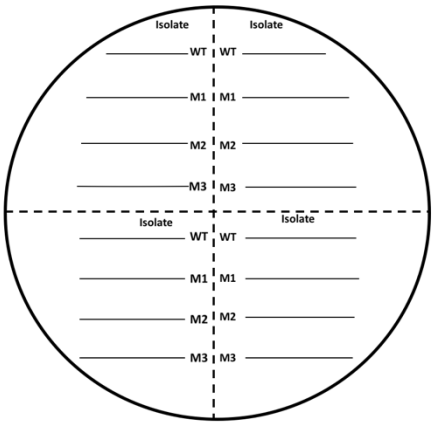


Figure 2.6. Schematic representation of bacterial arrangement on agar plate for stress assay. The resistant mutants were streaked on designated places beneath the corresponding wild types.

Cultivation in liquid medium

Materials:

BHI broth

O/N cultures

Procedure:

1. Each bacterial culture was diluted 1:200 in BHI medium and mixed well by pipetting.
2. Bacterial solutions (200 µl) were placed in a separate well on microtiter plate.
3. The plate was covered with transparent film and lid to avoid contamination during transportation.

4. Microtiter plate was incubated for 48 hours with absorption measurement at 600 nm every 30 minutes.

Separate assays were performed for each temperature treatment: 37°C, 40°C and 45°C.

2.7.3. Minimum inhibitory concentration (MIC)

To determine the lowest concentration of bacteriocin H1 that prevents bacterial growth, two-fold agent dilution was applied on microtiter plates. The absorbance of wavelength 600 and 595 nm was measured by the spectrophotometer and gave the degree of cell density in the suspension. MIC_{50} was calculated for 20 *S. haemolyticus* strains from University hospital of North Norway, isolated resistant mutants and transformed *L. plantarum* strains.

Materials:

BHI broth

H1 bacteriocin (1 mg/ml)

O/N culture

Procedure:

1. Bacteriocin H1 stock solution (1 mg/ml) was diluted in BHI medium to start concentration of 200 μg/ml.
2. BHI media (100 μl) was transferred into all 96 wells by using the multi-pipette.
3. Into all wells in the column 1 it was transferred diluted bacteriocin H1 (100 μl) so the final concentration was 50 μg/ml.
4. The serial two-fold dilution was done by mixing and taking up the solution (100 μl) from the first well and transferred to the next. It was done along all rows except the last one (12), what remained as a positive control without any bacteriocin.
5. Bacteria cultures were diluted 50 times in BHI medium (5 ml). Then the solutions were added to all wells in appropriate row for the specific strain and mixed by pipetting.
6. Microtiter plate was incubated at 37°C for 48 hours with absorption measurement at 600 or 595 nm.

The absorbance results collected for 5 hours and 24 hours incubation were used for analysis.

2.7.4. Transmission electron microscopy (TEM)

Transmission electron microscopy was applied to observe possible morphological differences between wild types and their mutants. Work was conducted in cooperation with Lene Cecilie Hermansen Senior Engineer at IPV - Imaging Centre in Ås, Sofie S. Kristensen PhD Candidate at the Norwegian University of Life Sciences and Mikkel Brønner Master student at the Norwegian University of Life Sciences. All work was done in laminar flow cabinet and sterile bench.

Materials:

0.1 M CaCo Buffer, pH 7.4

0.04 M Sodium Cacodylate (CaCo) Buffer, pH 7.4

1% OsO_4 in 0.1 M CaCo Buffer

50 % ethanol

70 % ethanol

90 % ethanol

96 % ethanol

100 % ethanol

Fixative solution (2 % glutaraldehyde; 1.25 % paraformaldehyde; 0.04 M CaCo Buffer)

Low melt agarose

LR White Resin Only Med. Garde, Electron Microscopy Sciences; VWR

O/N cultures

Phosphate-buffered saline (PBS)

Fixation

Done at the Laboratory of Microbial Gene Technology, Norwegian University of Life Sciences.

Procedure:

1. Bacterial suspension (1 ml) was transferred to Eppendorf tube and centrifuged for 10 minutes at 1.4 rpm. Supernatant was discarded.
2. Cell pallet was washed with PBS (1 ml) by pipetting a few times and centrifuged at 1.4 rpm for 10 minutes. Supernatant was removed and step was repeated.
3. Cells were resuspended with fixative solution (0.5 ml) and stored at 4^{o}C until next day.

Washing

Procedure:

1. After overnight fixation, the fixative solution from the sample was collected to a waste flask and fixed cells were added 0.04 M CaCo Buffer (0.5 ml).
2. Then, the buffer was removed and new portion (0.5 ml) was added.

Post fixation:

Done at the IPV - Imaging Centre in Ås.

Procedure:

Done by Lene Cecilie Hermansen:

1. The 0.04 M CaCo Buffer was removed from the sample just before 2 drops of Low melting agarose (warmed up until melting) was added. With help of sterile toothpick, it was created lump of agarose with bacteria.
2. After the agarose had solidified, the lump was transferred to a glass bottle and incubated in mixture of 1% OsO_4 and 0.1 M CaCo Buffer for 70 minutes at RT.

Done by Karolina T. Bartkiewicz, Sofie S. Kristensen and Mikkel Brønner:

3. The bacterial lump was washed three times with new dosage of 0.1 M CaCo buffer with 15 minutes incubation each time, on shaking platform.

Dehydration

Procedure:

1. The buffer was removed from bacteria.
2. Bacterial sample was added alcohol (5 ml) in different concentrations: 50 % ethanol, 70 % ethanol, 90 % ethanol, 96 % ethanol and 4x 100 % ethanol. Specimen was incubated 15 minutes between removing old and adding new solution (on shaking platform).

Infiltration

Procedure:

1. After removal of the last 100 % ethanol solution from the specimen, it was added a mixture (2 ml) of LR White and 100 % ethanol in ratio 1:3. Incubated overnight at RT with shaking.
2. The day after, the old mixture of resin and ethanol was removed and new one was added in ratio 2:2. Incubated overnight at RT on shaking platform.

3. Then, 2:2 solution was removed and added 3:1 with LR White and ethanol. Incubated at RT overnight (with shaking).
4. The last step of infiltration was done later the same day. Previous mixture was removed and added 100 % Resin. Incubated O/N at RT on shaking platform.

Embedding

The next day, the specimen was cut in two pieces and transferred into a capsule and coved with LR White. Specimen number written on the peace of paper was added to the resin capsule, covered with the capsule lid filled with more resin. Then, polymerised in 60ºC oven for three days.

Sectioning and staining

Done by Lene Cecilie Hermansen.

Ultramicrotomy is a process where the specimen was trimmed and sections of the sample were cut at thickness of 60 nm on a diamond knife. Then the selected slice was collected onto a grid and stored in a grid cassette. Prior to staining the specimen, the potassium permanganate was mixed with 4 % uranyl acetate and placed on the parafilm as a drop. The grid with specimen was added to the mixture and incubated for 10 minutes. Then the grid was washed in drops of water with moving the grid to another drop every 5 minutes (repeated 10 times).

Imaging

Done in cooperation with Lene Cecilie Hermansen, Sofie S. Kristensen and Mikkel Brønner. Cells were observed under the transmission electron microscopy with use of the iTEM FEI software.

2.8. Bioinformatics

To evaluate biological data, computational methods were used to analyse information taken from public databases.

Phylogeny

Phylogenetic tree was made based on the 16S rRNA sequences of the indicators species to see an overview of the evolutionary relationship of taxa and the bacteriocins activity spectrum. The phylogenetic tree was constructed using MEGA X: Molecular Evolutionary

Genetics Analysis Version 10.1.6. software alignment done by Muscle algorithm and Neighbor-Joining method for tree design.

Gene prediction

In order to amplify the gene of interest, *rseP*-specific primers had to be designed. To obtain the *rseP* sequence it was searcher for a "*rseP Staphylococcus haemolyticus*" in NCBI (National center for Biotechnology Information) gene database. The result was RIP metalloprotease RseP (NC_007168.1:c1707049-1705763 *S. haemolyticus* JCSC1435). It was then used as a reference to find same sequence in stains from University Hospital of North Norway in Tromsø (table 2.4) that had been Whole Genome Sequenced and genome assembly was submitted in ENA (European Nucleotide Archive).

2.9. DNA purification

DNA was extracted and purified to perform *rseP* amplification with gene specific primers. Target gene was further used in cloning experiment and gene sequencing. Overnight cultures of the resistant mutants and their wild types were used for DNA isolation.

Mechanical cell disruption by bead beating in FastPrep 24™ unit was combined with alkaline lysis by using the E.Z.N.A.® Plasmid DNA Mini Kit I from Omega (BIO-TEK) to obtain DNA of high purity.

Materials:

2 ml Collection Tubes
DNA Wash Buffer
Elution Buffer
HBC Buffer
HiBind® DNA Mini Columns
RNase A
Solution I
Solution II
Solution III

Procedure:

1. Bacterial culture grown overnight (1.5 ml) was transferred to the Eppendorf tube (1.5 ml) and centrifuged at 13,000 x g for 4 minutes to collect cell pallets, and supernatant was discarded.
2. The cell pallets were added Solution I with RNase A (400 µl) for resuspension. Previously added RNase A degrade cellular RNA during cell lysis.
3. Lysing Matrix B (2 ml) tube was filled with 0.4 g of 0.1 mm silica beads and added resuspended cells. The cells were disrupted in FastPrep 24™ homogenizer by multidirectional beating, two times 20 seconds with speed 6 m/s and a short break between them.
4. Tube was centrifuged for 5 minutes with maximal speed and the supernatant was transferred to a new Eppendorf tube.
5. For alkaline lysis it was added Solution II (250 µl), mixed well by turning the tube up and down a few times and incubate for 2 minutes until the lysate was clear. Lysis solution was added to solubilize the cell membrane and denature proteins as well as for breaking the hydrogen bonds between the DNA bases.
6. In the fume hood it was added hazardous Solution III (350 µl) for neutralization (hydrogen bonding between DNA bases were re-established) of the lysis reaction and by gently rotation of the tube mixing the solutions. Centrifuged for 10 minutes with 13,000 x g.
7. The supernatant with was transferred into HiBind® DNA Mini Column in Collection Tube, then centrifuged again for 1 minute with 13,000 x g.
8. Flow-though was discarded into a hazardous waste flask in flow hood, DNA was bind to the silica membrane by adding the HBC-Buffer with isopropanol (500 µl). Centrifuged for 1 minute with 13,000 x g, the flow-through was discarded and the collection tube reused.
9. The washing off undesirable cell components was done by adding DNA Wash Buffer (750 µl), centrifuged for 2 minutes and the discarded the filtrate.
10. The HiBind® DNA Mini Column was centrifuged for drying for 1 minute with 10,000 x g and transferred carefully into a new Eppendorf tube.
11. To extract the isolated DNA it was added the DNA Elution Buffer or distilled water (80 µl) into the middle of the silica membrane, incubate at room temperature for 2 minutes and centrifuged for 1 minute with 13,000 x g.

2.9.1 Isolation of gDNA for Whole Genome Sequencing (WGS)

Whole Genome Sequencing was carried out in aim to examine the entire genome of isolated mutants and their wild types. To obtain chromosomal DNA from microbial samples, complete disruption of the bacterial cells was required. Procedure was accomplished according to NucleoSpin® Genomic DNA from microorganisms User manual, page 13 (Macherey-Nagel).

2.10. DNA concentration

NanoDrop 2000 Spectrophotometer

The quantification and qualification of the isolated DNA was evaluated by the NanoDrop spectrophotometer. The concentration of amino acids as well as the sample purity ratio A260/A280 was measured after the DNA extraction or PCR product clean-up.

Procedure:

1. The pedestal was lifted and cleaned with distilled water (2 μL).
2. For blanking the measurement, it was used elution buffer or distilled water (2 μL), depending on what DNA was extracted with.
3. The pedestal was cleaned with a soft paper, sample (2 μL) was applied and the measured.
4. After use the pedestal was cleaned with distilled water, dried with paper and closed.

Qubit® Fluorometer

The Qubit® fluorometery was used for measuring the accurate concentration of the DNA in a sample by fluorometric method. Qubit was usually used for samples of very low concentration. Procedure was performed accordingly to Qubit™ dsDNA BR Assay Kit manual from Invitrogen by Thermo Fisher Scientific.

2.11. Primers design and preparation

To perform molecular cloning with In-Fusion Cloning method, special primers had to be designed to amplified *rseP* genet from *S. haemolyticus* LMG4105 (WT), *S.aureus* LMGT3023 (WT). For that purpose, the pDRAW32® 1.1.142 software was used. The 15 nucleotides long 5´end of the primer was homologous to the linearized vector. The 3´end was gene-specific and contained GC between 40–60% of the sequence, what gave melting

temperature (Tm) between 58–65°. The restriction enzyme site had to be included between those two parts.

Primers used in sanger sequencing by GATC were designed by selection of the nucleotide sequences of length 19-24 nucleotides. To obtain gene-specific oligos with good annealing efficiency the CG content should have a range of 50% then the melting temperature (Tm) was near 60°C. One of the challenges in sanger sequencing is the low quality of the first 50 nucleotides in reads. To ensure that entire *rsep* had been covered by sequencing reads, one of the primers was chosen to be approximately 100 nucleotides upstream and one 100 nucleotides downstream from the gene.

The primers (table 2.5) were shipped in stable dry form and resuspend in distilled water to 100 μM stock solution upon arrival. To obtain working solution of 10 μM, the stock solution was diluted with dH_2O in ratio 1:9. Both solutions were kept at -20°C.

Table 2.5. List of the primers used in this study.

Primer name	Sequence (5´- 3`)	Tm (°C)	Length (nt)	Annotation
SH_LMG4105_RseP_F	GGA GTA TGA TTC ATA TGA GCT ATT TAA TCA CTA TTG TCT CAT TT	63.8	44	Forward In-Fusion primer for *S. haemolyticus rseP* amplification
SH_LMG4105_RseP_R	TCG AAC CCG GGG TAC CTT ACA AGA AAT AAC GTT GTA TAT CGT TC	70.1	44	Reverse In-Fusion primer for *S. haemolyticus rseP* amplification
SH_LMG4105_RseP_Seq_F1	TTG AGT GCA CAT TTG ACT AGA C	57	22	Forward primer for *S. haemolyticus rseP* amplification and sequencing, 139 nt upstream
SH_LMG4105_RseP_Seq_R1	ACT CAA TGC TTC TGC TTC AGC	59.6	21	Reverse primer for *S. haemolyticus rseP* amplification and sequencing, 84 nt downstrem
SH_LMG4105_RseP_Seq_F2	ATC GCT CCA CGA CAT CGA C	62.4	19	Forward primer for *S. haemolyticus rseP* sequencing

SH_LMG4105_RseP_Seq_R2	GCT GCA GAC TGA ATG TCA TC	58.6	20	Reverse primer for *S. haemolyticus rseP* sequencing
SH_LMG4105_RseP_Seq_F3	GAA CGA AAC TTT GTA TAC CAT CCG	57.5	24	Forward primer for *S. haemolyticus rseP* sequencing
SH_LMG4015_RseP_Seq_R3	ATG TAC TGG CAC TAA CAA ACT G	57	22	Reverse primer for *S. haemolyticus rseP* sequencing
SH_LMG4105_RseP_Seq_R4	AAA TTC GAC CAC CAT CAA GTG C	59.6	22	Reverse primer for *S. haemolyticus rseP* sequencing
S.aureus_RseP_F	GGA GTA TGA TTC ATA TGG TGA GCT ATT TAG TTA CAA TAA TTG CAT	65.3	45	Forward In-Fusion primer for *S. aureus rseP* amplification
S.aureus_RseP_Seq_F	CGA GAG ACA GAC AAT TTG CAC A	59.6	21	Forward primer for *S.aureus rseP* sequencing
S.aureus_RseP_Seq_R	GGA TTC CAA CCA GCG AGT GAA	61	21	Forward primer for *S. aureus rseP* sequencing
SH_LMG4105_RseP_6H_R1	TCG AAC CCG GGG TAC CTT A**AT GAT GAT GAT GAT GAT G**CA AGA AAT AAC GTT GTA TAT CGT TC	73.5	62	Reverse In-Fusion primer with 6His-tag for *S. haemolyticus rseP* amplification
S.aureus_RseP_R1	TCG AAC CCG GGG TAC CTT ATA AGA AAT ATC GTC GAA TAT CAT TC	68.9	44	Reverse In-Fusion primer for *S. aureus rseP* amplification
S.aureus_RseP_6H_R1	TCG AAC CCG GGG TAC CTT A**AT GAT GAT GAT GAT GAT G**TA AGA AAT ATC GTC GAA TAT CAT TC	72.6	62	Reverse In-Fusion primer with 6His-tag for *S. aureus rseP* amplification
Myc_Rsep	TCG AAC CCG GGG TAC CTT A**CA GAT CCT CTT CTG AGA TGA GTT TTT GTT C**TA AGA AAT ATC GTC GAA TAT CAT TCC	>75	75	Reverse In-Fusion primer with Myc-tag for *S. aureus rseP* amplification

Aur_seq_R	TGT GCA AAT TGT CTG TCT CTC G	59.6	22	Reverse primer for *S. aureus rseP* sequencing

Restriction sites are underlined; 6His-tag and Myc-tag are in bold letters

2.12. PCR

Polymerase chain reaction was performed to amplify *rseP* sequence with In-Fusion primers as well as to multiply the target fragment of the template into larger amount before the sanger sequencing.

To avoid non-specific annealing, the reaction mixture was prepared on ice and the enzyme with the template DNA were added just before the thermal reaction start. The component of the biggest volume, usually distilled water, was added to the PCR tube first, so the next ingredient was diluted in it.

Materials:

5 X Q5 Reaction Buffer; Biolabs
10 mM Deoxynucleotide (dNTP) solution mix; Biolabs
Forward/Reverse primer 10 uM (table 2.5); Invitrogen™
Distilled water
Ice
Q5® High-Fidelity DNA Polymerase; Biolabs
Q5® High-Fidelity 2X Master Mix; Biolabs
Template DNA

Procedure:

1. The master mix was prepared by mixing calculated volumes of each component that was needed for all of the samples (table 2.6; 2.7).
2. Then, the required volume of master mix was distributed in previously marked PCR tubes, the gDNA and polymerase enzyme were added last.
3. The thermal cycler was programmed according to table 2.8, and the samples were placed inside before the reaction had started.
4. After the PCR reaction was finished, it was proceeded with gel electrophoresis (2.14).

Table 2.6. Components sett-up for Q5 PCR reaction.

Reaction components	Volume
5X Q5 Reaction Buffer	10 µl
10 mM dNTPs	1 µl
Q5® High-Fidelity DNA Polymerase	0.5 µl
10 µM Forward primer	2.5 µl
10 µM Revers primer	2.5 µl
dH_2O	32.5 µl
gDNA	1 µl
Total	50 µl

Table 2.7. Components sett-up for PCR reaction with ready mixed master mix.

Reaction components	Volume
10 µM Forward primer	2.5 µl
10 µM Reverse primer	2.5 µl
DNA template	1 µl
Q5® High-Fidelity 2X Master Mix	25 µl
dH_2O	19 µl
Total	50 µl

Table 2.8. Thermal cycles for Q5® High-Fidelity DNA Polymerase.

Cycle name	Temp (°C)	Time	Number of repeats
Initial denaturation	98	30 s	1
Denaturation	98	10 s	
Primer annealing	65	30 s	30
Extension	72	40 s	
Final extension	72	2 min	1
Infinity hold	4	∞	1

2.13. Colony PCR

After each transformation process it was important to determine if the target sequence had been inserted into the plasmid or the plasmid into the cell. Colony PCR is a method which verifies that with no need for plasmid purification.

Materials:

Bacterial colony after transformation

Cloning Forward/Reverse primer 10 uM (table 2.5); Invitrogen™

Distilled water

Ice

Red Taq-DNA-polymerase 2X MasterMix, 1,5 mM $MgCl_2$; VWR

Procedure:

1. A single bacterial colony was picked from the selective media with sterile toothpick and distributed on the PCR tube´s bottom.
2. Closed PCR tube was transferred into the microwave and run for 45 seconds at maximal radiation to allow cell lysis.
3. Reaction components were added to the lysed cells according to table 2.9.
4. The tube was placed in the thermal cycler machine and program was chosen according to table 2.10.
5. After the amplification had finished, the product was loaded on 1,2% agarose gel (2.14).

Table 2.9. List of the colony PCR components.

Reaction components	Volume
Red Tag 2X Master Mix	25 µl
10 µM Forward primer	1 µl
10 µM Revers primer	1 µl
dH_2O	23 µl

Table 2.10. Thermal cycles for Red Taq DNA Polymerase Master Mix.

Cycle name	Temp (°C)	Time	Number of repeats
Initial denaturation	95	2 min	1
Denaturation	95	20 s	
Primer annealing	55/60	20 s	30
Extension	72	1 min	
Final extension	72	5 min	1
Infinity hold	4	∞	1

2.14. Gel electrophoresis

Gel electrophoresis was performed to confirm target gene amplification, colony PCR and plasmid restriction digestion. Negatively charged molecules like DNA, travel to the positively charged anode when placed in the electric field. With this method it is possible to separate macromolecules of varying sizes during the migration through the agarose matrix with pores.

Materials:

1X TAE Buffer (Tris-acetate-EDTA); Thermo Scientific™
Distilled water
FastDigest Green Buffer (10X); Thermo Scientific™
Gel Loading Dye, Purple (6X); Biolabs
MetaPhorTM Agarose; Lonza
peqGREEN DNA/RNA dye; PEQLAB
Quick-Load® 1 kb DNA Ladder; Biolabs

Procedure:

1. To dissolve agarose powder (6 g) in 1X TAE buffer (500 ml) it was used autoclave (121^{o}C for 18 minutes) and stored at 55^{o}C until use.
2. The gel tray was placed on the gel caster and the clams were tighten.
3. Into melted agarose solution (60 ml) it was added peqGREEN DNA/RNA dye (2.5 μl) and mixed well. Then the mixture was poured into the gel tray and the appropriate size comb was placed on it.
4. When the agarose gel had solidified, it was moved to electrophoresis tank (with cathode above) and filled with 1X TAE Buffer until the gel was covered completely.
5. Each PCR product was added Gel Loading Dye (8 μl) to track the DNA fragment. Alternative dyes were used during vector restriction digestion and the colony PCR. Those two, contained tracking dyes suitable for direct loading of PCR product on agarose gel without addition of electrophoresis loading dyes.
6. The comb was removed and the Quick-Load® 1 kb DNA Ladder (20 μl) was loaded in the first well then followed by dyed samples.
7. After placing the safety cover, power source was run for 60 minutes at 90 V.

8. Finally, the gel was exposed to UV light in gel imaging system where the separated DNA bands were analysed.

2.15. PCR product clean-up

After separation of linearized plasmid and amplified DNA by gel electrophoresis, the visible bonds with correct size were cut out of the gel and used in dissolving and purification process. Purified DNA was then used in further experiments.

Procedure was carried out according to the instructions in the PCR Clean-up Gel extraction User manual, page 19-20 (Macherey-Nagel).

2.16. GATC sanger sequencing

Sequencing of isolated DNA from resistant mutants and corresponding wild types was performed to obtain the sequence of the *rseP* gene. Possible alteration in nucleotides could elucidate the cause for observed phenotype characteristics. Sequencing was also done for the *rseP* after each cloning procedure to confirm that correct insert was added to the plasmid.
The LightRun sequencing GATC (Eurifins GATC Biotech) was used as a sequencing service that delivered high quality reads. Barcodes were ordered before sample preparation.

Sample preparation

To achieve the best quality data, the template DNA must be at proper amount. For plasmid DNA it was used 500 ng and 250 ng for PCR fragment. The important requirement was to not use elution substance with ethylenediaminetetraacetic acid (EDTA). It binds the magnesium ion and disturbs the DNA polymerase in sequencing reaction.
Calculated amount of the template DNA was diluted with dH_2O to minimum volume of 7.5 µl in Eppendorf tube and added 10 µM appropriate primer (2.5 µl) (table 2.5). Tubes were labelled with correct barcode stickers with number corresponding to the number in lab record. Samples were kept in -4°C until dispatch.

Reads assembly and analysis

After the sequencing was completed, results were revealed ready to download on the Eurofins Genomics platform (https://www.eurofinsgenomics.eu). The file contained chromatogram, text and fasta formats. Chromatogram files were opened with SnapGene Viewer software (SnapGene®) and QIAGEN CLC Main Workbench software, to see the quality of the

reads and determine what sequence could be used for assembly. Contigs were established by CAP3, sequence assembly program available online (http://doua.prabi.fr/software/cap3), as well as by using the QIAGEN CLC Main Workbench software. The Basic Local Alignment Search Tool (BLAST) (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and the Multiple Sequence Alignment by CLUSTALW (https://www.genome.jp/tools-bin/clustalw) were implicated in sequence analysis.

2.17. Whole Genome Sequencing (WGS)

Whole Genome Sequencing using Illumina® Next Generation Sequencing (NGS) technology was performed on extracted genomic DNA from two wild types and four of adequate mutants. Sample preparation and sequencing was done by Davide Porcellato, Researcher at Faculty of Chemistry, Biotechnology and Food Science. Quality-control, reads assembly and annotation was performed by Thomas Oftedal, PhD Candidate at Faculty of Chemistry, Biotechnology and Food Science (NMBU, Ås).

Unipro UGENE software v.33.0 was used for contigs assembly, and SnapGene® Viewer version 5.0.7. together with BLAST (NCBI) and The Universal Protein Resource (UniProt) was implicated in annotation.

2.18. Heterologous expression of *rseP*

In order to confirm the importance of *rsep* gene for H1 sensitivity in *S. haemolyticus*, *rseP* was heterologously expressed in *L. plantarum* (figure 2.7) which is naturally resistant to the hybrid bacteriocin. DNA fragment was fused in pLp1261_InvS vector system that is a derivative to (Sørvig et al., 2003). Finally, propagated in One Shot® TOP10 Chemically Competent *E. coli* and expressed in *L. plantarum* WCFS1.

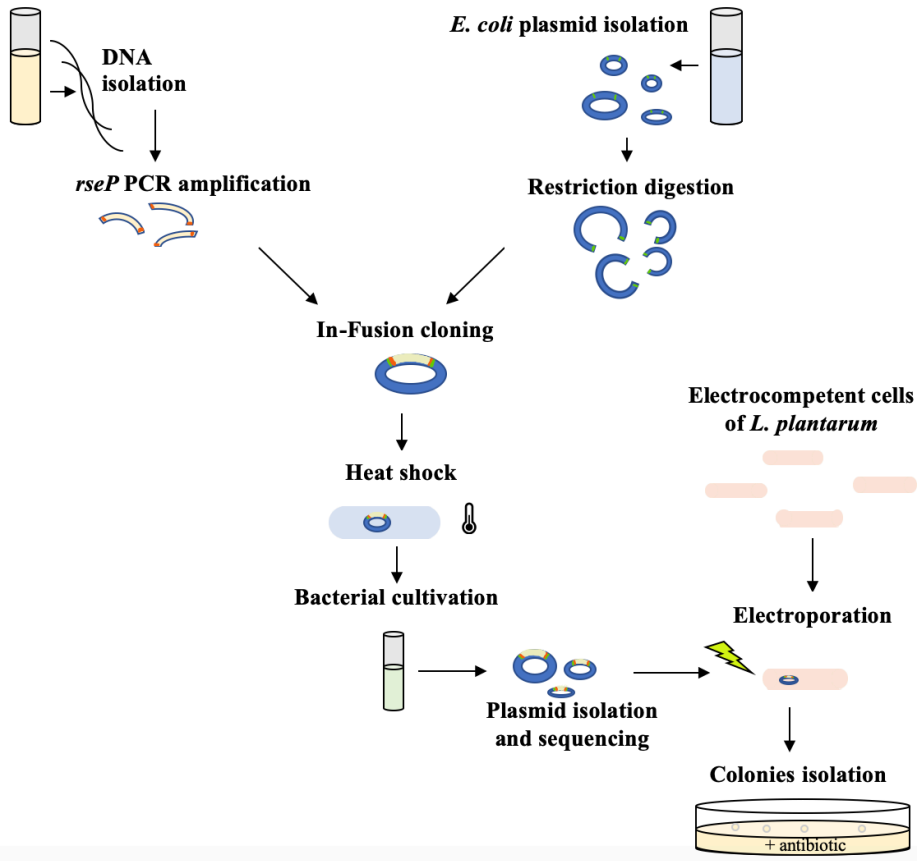


Figure 2.7. Schematic representation workflow of heterologous expression of *rseP* gene. Previously isolated DNA fragment (details in section 2.9) and amplified with In-Fusion primers (details in see section 2.12) was cloned with pLp1261_InvS vector isolated from *E. coli*. The plasmid with insert was then transformed by heat shock into TOP10 *E. coli* for propagation, prior to introduction into electrocompetent cells.

Plasmid DNA isolation

To extract pure plasmid DNA from *E. coli* cells the Nucleo Spin® Plasmid kit (Macherey-Nagel) was used. An appropriate antibiotic (erythromycin) was used in bacterial cultivation as a selection marker. Buffer A1 was added RNase A and Wash Buffer A4 was mixed with ethanol (for exact amount see the Plasmid DNA Purification User Manual NucleoSpin® Plasmid, page 14-15 (Macherey-Nagel).

Concentration of the extracted plasmid DNA was measured with previously mentioned Qubit fluorometer. Samples were stored at -20°C until use.

Vector linearization

Plasmid (pLp1261_InvS = 6428 bp) from *Es. coli* was digested with specific restriction enzymes to cut out the *InvS* gene (827 bp) and linearize plasmid. For this, Thermo Scientific™

FastDigest Restriction Enzymes Acc65I (G^GTACC) and NdeI (CA^TATG) that have the target sequences on both ends of the *InvS* gene (figure 2.8) was used.

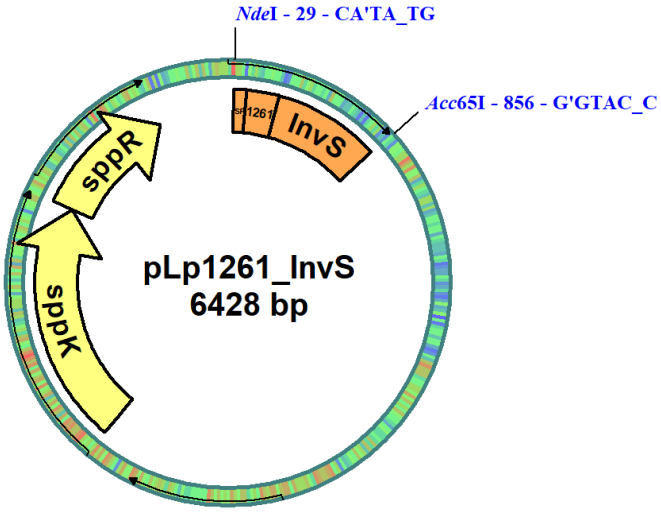


Figure 2.8. Vector pLp1261_InvS was digested with NdeI and Acc65I restriction enzymes to cut off the *InvS* gene.

Materials:

10x FastDigest Green Buffer; Thermo Scientific™

Distilled water

Extracted plasmids ´pLp1261_InvS´

FastDigest NdeI (10X); Thermo Scientific™

FastDigest Acc65I (10X); Thermo Scientific™

The reaction components were added to two Eppendorf tubes in accordance to table 2.11 and incubated for 1 hours at 37°C. Sample with no enzyme was used as a positive control.

Table 2.11. List of the plasmid restriction digestion components.

Components	With enzyme	Without enzyme
dH_2O	15 µl	20 µl
10x FastDigest Green Buffer	5 µl	5 µl
Extracted plasmid	25 µl	25 µl
NdeI	2.5 µl	-
Acc65I	2.5 µl	-
Total	50 µl	50 µl

After digestion, plasmids fragments were separated on agarose gel, extracted with PCR Clean-up Gel Extraction kit (Macherey-Nagel) and the concentration was measured with Qubit fluorometer.

In-Fusion cloning

In-Fusion is an effective way to clone an insert into a vector without any ligase. This method required a linearized vector, gene-specific primers (with 15 nucleotides homologous to the vector) and insert template. Linearization of the vector and target gene amplification has previously been described. In-Fusion cloning was conducted according to the In-Fusion® HD Cloning Kit User Manual (page 10).

Needed amount of vector and insert for cloning reaction was calculated using the molar ratio 2 (insert/vector) on https://www.takarabio.com. Calculated quantities of vector, PCR fragment and enzyme premix were added to dH_2O up to 10 µl (or 30 µl) total volume. Incubated for 15 minutes at 50ºC in a water bath, then moved on ice. Samples were stored at -20ºC if no further transformation were done the same day.

Heat shock

To perform transformation, bacterial cells should be liable to take up DNA under controlled conditions. In this study it was used One Shot® TOP10 Chemically Competent *E. coli* (Invitrogen™) cells with high efficiency transformation and plasmid propagation was used. Procedure was carried out according to producer´s protocol.

Materials:

BHI plates with erythromycin

Ice

One Shot™ TOP10 cells (one tube for one transformation); Invitrogen™

Recombinant plasmid

S.O.C. Medium; Invitrogen™

Procedure:

1. Suspension (50 µL and 100 µl) was spread separately on dry and preheated BHI plates with appropriate antibiotic, incubated O/N at 37ºC.

2. The next day, plates were inspected and colonies were isolated for further analyses. The colony PCR, plasmid isolation and sequencing were performed.
3. Glycerol stocks of pure cultures were made and stored at -80ºC.

Transformation of Electrocompetent Cells

The last step of transformation was to express *Staphylococcus rseP* in *L. plantarum* WCFS1 cells. The procedure was modified from Helge Holo and Ingolf F. Nes (Holo and Nes, 1989) transformation protocol to obtain electrocompetent cells of *L. plantarum* cells.

Electrocompetent cells preparation

Materials:

2 % Glycine; Sigma Life Science

30 % Poly ethylene glycol M.W. 1450 (PEG_{1450}); ACROS Organics™

Ice

O/N culture *L. plantarum* WCFS1

M.R.S. broth

Protocol:

1. O/N culture was serial diluted (10^{-1}- 10^{-11}) in M.R.S medium with 1% glycine.
2. Incubated overnight at 37ºC.
3. The next day, culture solution (1 ml) with previously checked OD_{600} of 2.5 ± 0.5 was added to MRS with 1% glycine (20 ml) and further grown until OD_{600} of 0.7 ± 0.07 then the culture was put on ice for 20 minutes.
4. Cells were collected by centrifugation at 5,000 x g for 10 minutes at 4ºC and supernatant was discarded.
5. Next, the cell pallets were resuspended in 5 ml ice cold 30% PEG_{1450} (freshly made) and added more 30% PEG_{1450} (20 ml).
6. Incubated on ice for 10 minutes and centrifuged again at 5,000 x g for 10 minutes at 4ºC.
7. The supernatant was removed, cells were resuspended in 30% PEG_{1450} (400 µl) before divided to smaller portions (40 µl) in Eppendorf tubes, and frozen at -80ºC for storage.

Electroporation

Materials:

Electrocompetent cells

Extracted plasmid

Gene Pulser®/MicroPulser™ Electroporation Cuvettes, 0.2 cm gap; BIO-RAD

Ice

MRS plates with erythromycin

MRSSM (MRS medium + 0.5 M sucrose + 0.1 M $MgCl_2$)

Procedure:

Prior to electroporation, the electrocompetent cells were thawed on ice. Eppendorf tubes (one for each reaction) and cuvettes were cooled on ice. The BIO-RAD Gene Pulser was set on 1.5 kV and 25 μF capacitance, while the BIO-RAD Pulse Controller was set on 400 Ω resistance.

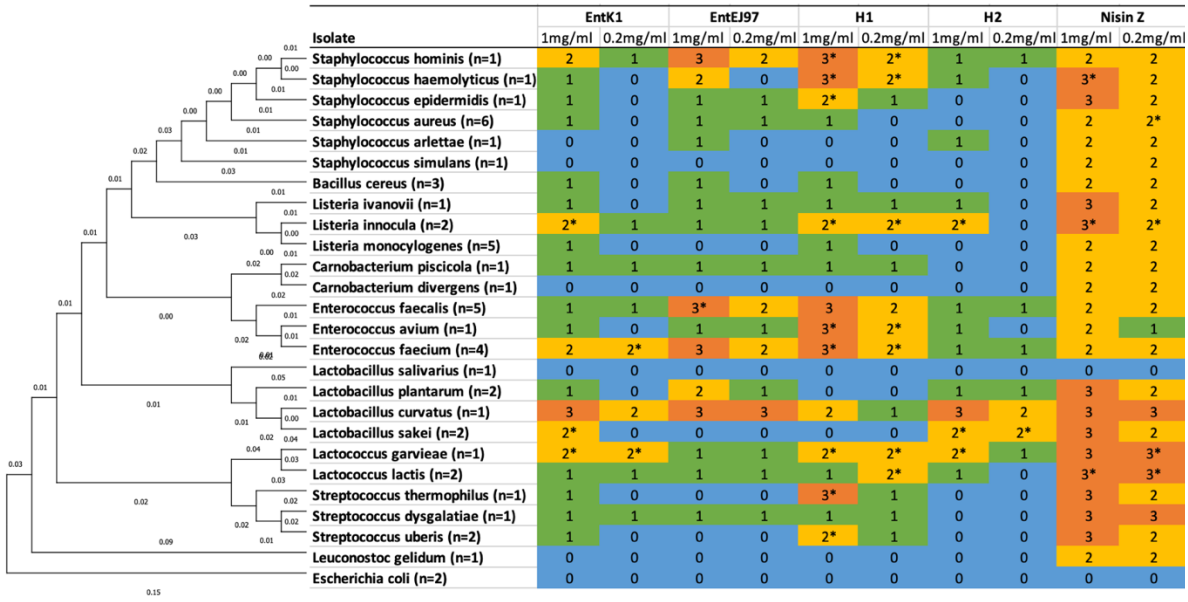
1. To vail of (40 μl) of competent cells, plasmid (5 μl) was added mixed gently and transferred to the electroporation cuvette.
2. The cuvette was placed in pulser unit, and the electroporation was carried for 1 second.
3. The cuvette was removed and added MRSSM (450 μl) prior to suspension transfer to Eppendorf tube.
4. Incubated at 37°C for 4 hours.
5. Culture was plated out on MRS agar with erythromycin in two different volumes on each plate (50 μl and 100 μl) and incubated at 37°C for two days. Remaining culture was kept at RT until next day.
6. Finally, colonies evaluated using colony PCR. Glycerol stocks were made and stored at -80°C for further analysis.

3. Results

The evaluation for *S. haemolyticus* strains from India were obtained from Mikkel Brønner (Master student), who shared his results for purpose of this research.

3.1. Spectrum of inhibition assay

Spectrum of inhibition for 51 bacterial strains (section 2.6.1) of different genera indicated various activity for bacteriocins EntK1, EntEJ97, H1 and H2 (figure 3.1). Figure 3.1 shows that Enterocin K1 and EJ97 are active against broad range of Gram-positive bacteria. However, EntEJ97 exhibit wider activity especially among *Staphylococcus* species and the producer-bacteria related enterococci. H1 shows strong activity against *Enterococcus* and several species of *Staphylococcus*, in particular the *S. haemolyticus*. Bacteriocin H2 have a much narrower spectrum of inhibition, but it displays activity mostly against Lactobacillus species almost similar to the EntK1. Nisin Z was used as a positive control due to its wide spectrum of activity for Gram-positive bacteria. Neither of the tested bacteriocins were active against Gram-negative *E. coli* species.



	EntK1		EntEJ97		H1		H2		Nisin Z	
Isolate	1mg/ml	0.2mg/ml	1mg/ml	0.2mg/ml	1mg/ml	0.2mg/ml	1mg/ml	0.2mg/ml	1mg/ml	0.2mg/ml
Staphylococcus hominis (n=1)	2	1	3	2	3*	2*	1	1	2	2
Staphylococcus haemolyticus (n=1)	1	0	2	0	3*	2*	1	0	3*	2
Staphylococcus epidermidis (n=1)	1	0	1	1	2*	1	0	0	3	2
Staphylococcus aureus (n=6)	1	0	1	1	1	0	0	0	2	2*
Staphylococcus arlettae (n=1)	0	0	1	0	0	0	1	0	2	2
Staphylococcus simulans (n=1)	0	0	0	0	0	0	0	0	2	2
Bacillus cereus (n=3)	1	0	1	0	1	0	0	0	2	2
Listeria ivanovii (n=1)	1	0	1	1	1	1	1	0	3	2
Listeria innocula (n=2)	2*	1	1	1	2*	2*	2*	0	3*	2*
Listeria monocylogenes (n=5)	1	0	0	0	1	0	0	0	2	2
Carnobacterium piscicola (n=1)	1	1	1	1	1	1	0	0	2	2
Carnobacterium divergens (n=1)	0	0	0	0	0	0	0	0	2	2
Enterococcus faecalis (n=5)	1	1	3*	2	3	2	1	1	2	2
Enterococcus avium (n=1)	1	0	1	1	3*	2*	1	0	2	1
Enterococcus faecium (n=4)	2	2*	3	2	3*	2*	1	1	2	2
Lactobacillus salivarius (n=1)	0	0	0	0	0	0	0	0	0	0
Lactobacillus plantarum (n=2)	1	0	2	1	0	0	1	1	3	2
Lactobacillus curvatus (n=1)	3	2	3	3	2	1	3	2	3	3
Lactobacillus sakei (n=2)	2*	0	0	0	0	0	2*	2*	3	2
Lactococcus garvieae (n=1)	2*	2*	1	1	2*	2*	2*	1	3	3*
Lactococcus lactis (n=2)	1	1	1	1	1	2*	1	0	3*	3*
Streptococcus thermophilus (n=1)	1	0	0	0	3*	1	0	0	3	2
Streptococcus dysgalatiae (n=1)	1	1	1	1	1	1	0	0	3	3
Streptococcus uberis (n=2)	1	0	0	0	2*	1	0	0	3	2
Leuconostoc gelidum (n=1)	0	0	0	0	0	0	0	0	2	2
Escherichia coli (n=2)	0	0	0	0	0	0	0	0	0	0

Figure 3.1. Graphical representation of spot-on-lawn spectrum of inhibition assay plotted onto the phylogenetic tree. This figure shows average scores for inhibitory activity of bacteriocin EntK1, EntEJ97, H1, H2 and nisin Z correlated to evolutionary relationship among 25 different bacterial species (obtained in cooperation with Mikkel Brønner). Observations were done after 24 hours incubation at 30°C. Nisin was used as an inhibitory control due to broad activity spectrum. Scorings system: 0= no inhibition (blue); 1= Unclear zone (green); 2= small zone (<1 cm) (yellow); 3= big zone (≥1 cm) (orange); "*" zone with resistant mutants. Complete collection of inhibition spectrum assay results can be found in appendix A1. The tree was generated by neighbor-joining algorithm.

3.1.1. Spectrum of inhibition assay for *S. haemolyticus* strains

As previously mentioned, *S. haemolyticus* is an emerging threat in hospital infections, with very few treatment options due to its antibiotic resistance (Czekaj et al., 2015). Interestingly, the spectrum of inhibition assay (section 3.1) exhibited that bacteriocin H1 is highly active against *S. haemolyticus*. This observation became the basis for further research on bacteriocin potential. S. *haemolyticus* from India were involved to compare bacteriocin efficiency against geographically diverse strains.

Sensitivity of the *S. haemolyticus* to leaderless bacteriocins EntK1, EntEJ97, H1 and H2 was analysed using spot-on-lawn experiment (section 2.6.2). Table 3.1 shows that bacterial isolates from Norway and India displayed similar sensitivity to these four peptides. Bacteriocin EJ97 and H1 have similar activity against different *S. haemolyticus* strains, however H1 generated bigger zones of inhibition than EJ97 even at lover concentrations. Both bacteriocins produced resistant mutants (table 3.1). EntK1 and H2 display nearly no inhibitory efficacy except the most susceptible *S. haemolyticus* strains, such as LMG4106, LMG4111 and LMG4112.

Table 3.1. The scoring table of bacteriocins (EntK1, EntEJ97, H1, H2) activity at different concentrations against *S. haemolyticus* strains after 24 hours incubation at 30ºC. The table shows results for strains from Norwegian hospitals and isolates from India (obtained from Mikkel Brønner). Scorings system: 0= no inhibition; 1= Unclear zone; 2= small zone (<1 cm); 3= big zone (≥1 cm), "*" zone with resistant mutants. Complete collection of results for Norwegian starins can be found in appendix A2.

Isolate (n=1)	**EntK1**		**EntEJ97**		**H1**		**H2**	
	1mg/ml	0.2mg/ml	1mg/ml	0.2mg/ml	1mg/ml	0.2mg/ml	1mg/ml	0.2mg/ml
LMG4096	0	0	2*	1	3*	2*	0	0
LMG4097	0	0	2*	1	3*	2*	0	0
LMG4098	0	0	3*	1	3*	2*	0	0
LMG4099	0	0	2*	1	3*	2*	0	0
LMG4100	0	0	2*	1	3*	2*	0	0
LMG4101	0	0	3*	1	3*	2*	0	0
LMG4102	0	0	3*	1	3*	2*	0	0
LMG4103	0	0	3*	1	3*	2*	0	0
LMG4104	0	0	2*	1	3*	2*	0	0
LMG4105•	0	0	3*	2*	3*	2*	0	0
LMG4106•	2*	0	3*	3*	3*	2*	0	0
LMG4107	0	0	2*	0	3*	2*	0	0
LMG4108	0	0	2*	1	3*	2*	0	0
LMG4109	0	0	2*	0	3*	2*	0	0

LMG4110	0	0	3*	1	3*	2*	0	0
LMG4111	1	0	3*	2*	3	3	2*	0
LMG4112	1	0	3*	2*	3*	3*	1	0
LMG4113	0	0	2*	1	3*	2*	0	0
LMG4114	0	0	2*	0	3*	2*	0	0
LMG4115•	0	0	2*	1	3*	2*	0	0
LMGT4068••	0	0	2*	1	3*	2*	0	0
LMGT4069••	0	0	2*	1	3*	2*	0	0
LMGT4070••	0	0	2*	1	3*	2*	0	0
LMGT4071••	0	0	2*	1	3*	2*	0	0
LMGT4072••	0	0	2*	1	3*	2*	0	0
LMGT4073••	0	0	2*	1	3*	2*	0	0

•Resistant mutants were isolated from this strain for further investigation of resistance mechanism

••Strains from India, measures were done by Mikkel Brønner

The two-fold dilution of bacteriocin H1 on microtiter plate (section 2.7.3) determined the minimum concentration required to inhibit growth of various *S. haemolyticus*. Table 3.2 shows that the H1 concentration 0.78 µg/ml (170 nM) prevented most Norwegian and Indian strains after 5 hours at 37ºC. After 24 hours incubation, several of the isolates became more resistant and the concentration required to inhibit growth was higher, which points at production of resistant mutants. Resistant mutants were also observed in spot-on-law experiment (table 3.1). There is no obtained data for Indian strains for 24 hours treatment.

Table 3.2. MIC_{50} (µg/ml) values for H1 against *S. haemolyticus* strains based on microtiter plate assay. The minimum concentration of bacteriocin required for bacterial growth inhibition was calculated from OD600 values measured after 5 hours and 24 hours incubation at 37ºC.

Isolate (n=1)	5h	24 h
LMG4096	0.78	1.6
LMG4097	0.78	>50
LMG4098	0.78	6.25
LMG4099	1.6	3.13
LMG4100	1.6	3.13
LMG4101	0.78	>50
LMG4102	0.78	0.78
LMG4103	0.78	1.6
LMG4104	1.6	1.6
LMG4105*	0.78	>50
LMG4106*	0.2	0.39

LMG4107	0.78	1.6
LMG4108	0.78	0.78
LMG4109	0.78	0.78
LMG4110	1.6	1.6
LMG4111	0.2	0.39
LMG4112	0.2	0.39
LMG4113	1.6	3.13
LMG4114	1.6	1.6
LMG4115*	0.78	1.6
LMGT4068**	0.39	
LMGT4069**	0.39	
LMGT4070**	0.78	
LMGT4071**	0.78	
LMGT4072**	0.78	
LMGT4073**	0.78	

* Resistant mutants were isolated from this strain for further investigations (3.3)

**Strains from India, measures were done by Mikkel Brønner

3.2. Bacteriocin H1 potential

The potential of bacteriocin H1 as a possible substitute for antibiotic in treatment of *S. haemolyticus* was determined by comparative susceptibility (section 2.6.3). The antibiotics applied in this experiment are usually used against infections of Gram-positive bacteria. The inhibition zones produced by antibiotic disk diffusion method were compared with zones produced by hybrid bacteriocin H1 in spot-on-lawn assay after 24 hours of incubation at 37°C (appendix A3). Table 3.4 shows that H1(1mg/ml) was one of the most active antimicrobial agents tested. Several of the applied antibiotics displayed strain dependent activity, while bacteriocin H1 was equally effective against all the strains. After 24 hours incubation, resistant mutants were observed for both bacteriocin H1 and antibiotics.

Table 3.4. The inhibition efficiency of 3 and 0.6 µg of H1 against *S. haemolyticus* was compared with 5 µg vancomycin (VA5), 10 units of penicillin G (P10), 30 µg teicoplanin (TEC30), 5 µg ciprofloxacin (CIP5), 15 µg erythromycin (E15) and 5 µg rifampicin (RD5) by disc diffusion method. Results were determined after 24 hours incubation at 37°C. Scorings system: 0= no inhibition; 1= Unclear zone; 2= small zone (<1 cm); 3= big zone (≥1 cm), "*" zone with resistant mutants.

Isolate (n=3)	VA5 (5 µg)	P10 (10 units)	TEC30 (30 µg)	CIP5 (5 µg)	E15 (15 µg)	RD5 (5 µg)	H1 (3 µg)	H1 (0.6 µg)
LMG4097	1	0	1	0	2*	3*	3*	1
LMG4103	2*	0	2*	3*	3*	3*	3*	2*

LMG4105	1	0	2	3*	1	3*	3*	2*
LMG4106	3	3	2	3*	1	3	3	2*
LMG4113	2	0	2	3*	3*	3*	3*	1
LMG4115	1	0	2*	0	0	3*	3*	1

To overcome the production of bacteriocin resistant mutants (results 3.1 and 3.2), combinations of bacteriocin with other agents were applied to increase the antimicrobial potency. Microtiter plate assays (section 2.6.4) have evaluated the efficiency of single and combinatory formulations with two-fold dilution series of bacteriocin H1, garvicin KS and micrococcin P1 against *S. haemolyticus* after 24 hours treatment (figure 3.2). Bacteriocin combinations show greater inhibitory efficiency than individual bacteriocins. Several strains are more susceptible to formulations containing H1. H1+GarKS exhibits highest activity for almost all isolates used in this investigation. Results in figure 3.2 indicate that bacteriocin H1 has high synergy effect in combination with other antimicrobials.

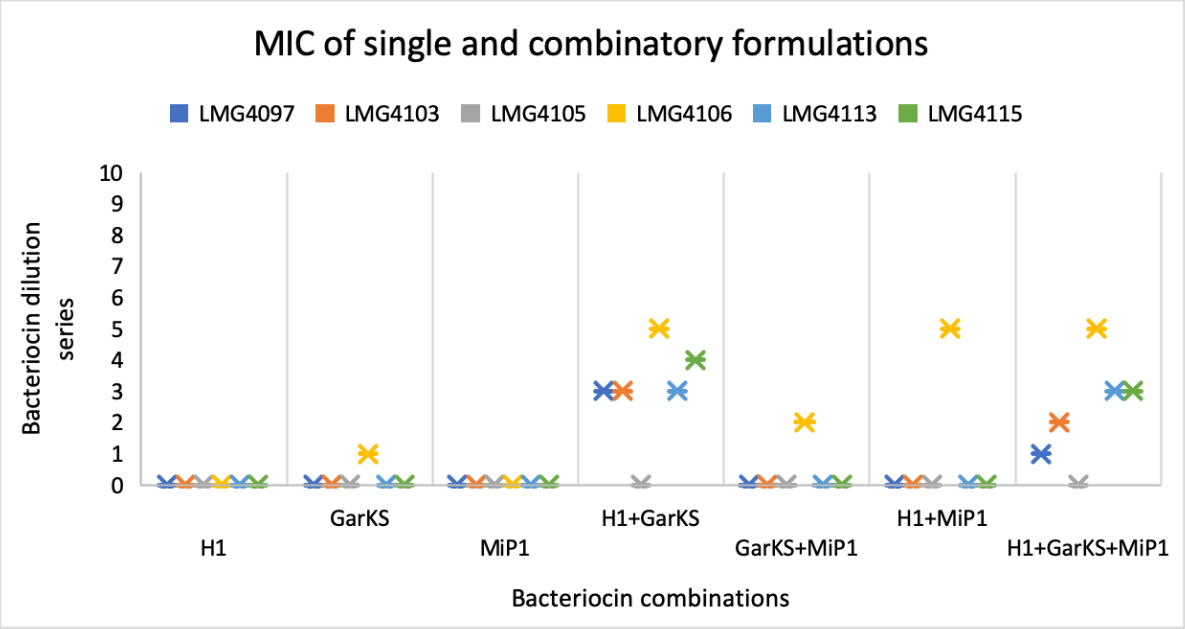


Figure 3.2. Bacteriocin combinations efficiency against *S. haemolyticus* (n=3) after 24 hours treatment at 37°C. Two-fold dilution of single agents and bacteriocin combinations determined visually. The Y-axis represents the number of dilution series of formulations required to inhibit the bacterial growth, which 1 is the inhibition at highest concentrations and corresponds to 100 µg/ml for H1, 100 µg/ml for GarKS and 10 µg/ml for MiP1. 0 indicates no inhibition at initial concentration. In contrast, the dilution series 10 represents the lowest concentrations and corresponds to 0.2 µg/ml for H1, 0.2 µg/ml for GarKS and 0.02 µg/ml for MiP1. The X-axis shows the efficiency of different bacteriocin combinations against different bacteria.

3.3. Heterologous expression of *rseP*

Heterologous expression was conducted to confirm the importance of *rseP* in *S. haemolyticus* for sensitivity of hybrid bacteriocin H1. *S. aureus* LMGT3023 was used as a negative control species for bacteriocin sensitivity due to its bacteriocin resistance.

Mapping of restriction enzymes (details in section 2.18) within ´pLp1261´ vector and primer design was conducted using pDRAW32 software (figure 3.3). Plasmids isolated from *E. coli* were digested with enzymes NdeI and Acc65I (gel picture in appendix A4) and subsequently cloned with PCR amplified *rseP* fragments from two different *S. haemolyticus* and S. *aureus*.

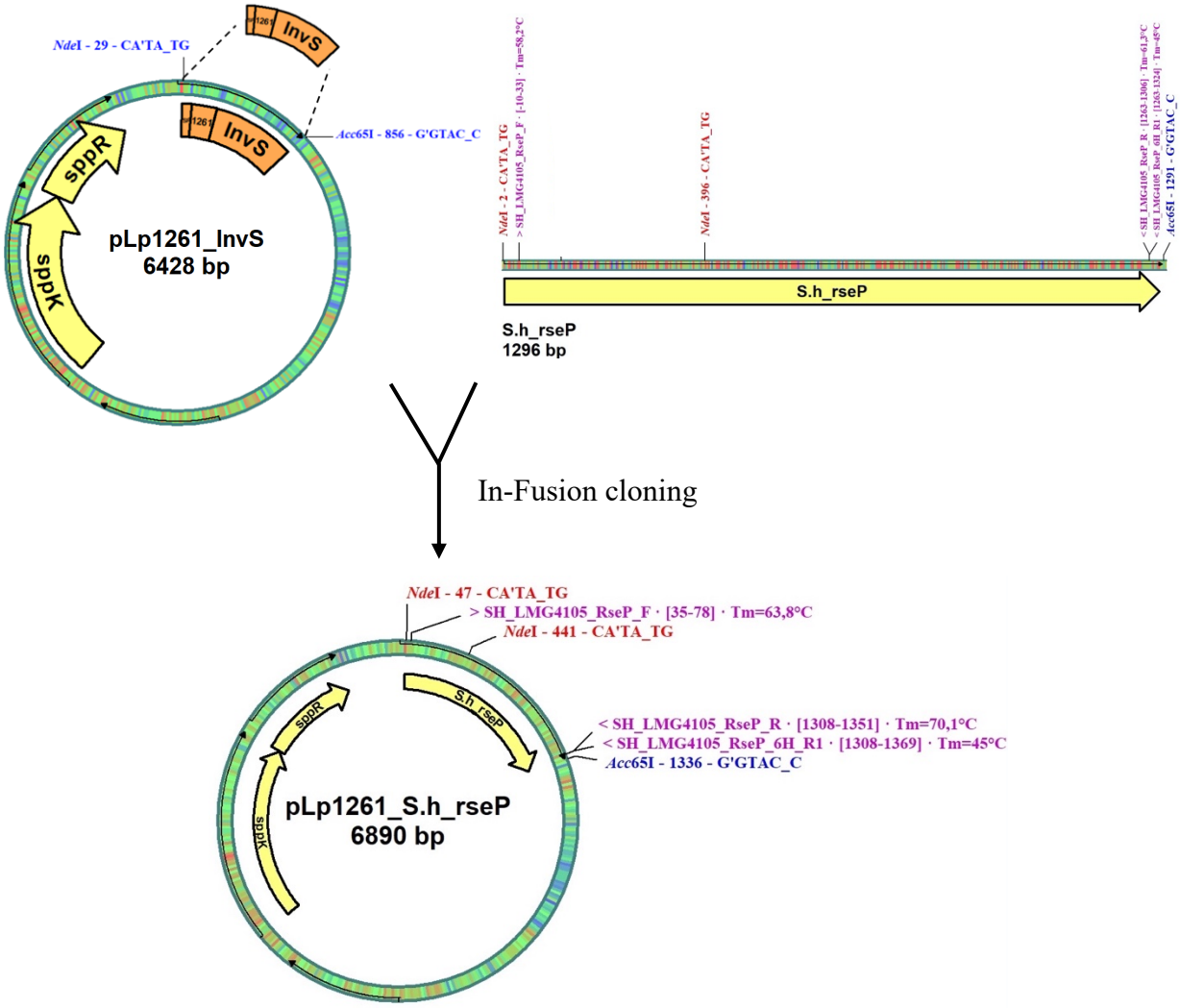


Figure 3.3. Construction of the plasmid vector pLp1261_S.h_rseP for the expression of *rseP* fragment in L. plantarum WCFS1. Restriction sites NdeI and Acc65I are colored red and blue respectively. Primers used in PCR-annealing of the *rsep* fragment are displayed in pink. Constructions were designed in pDRAW32 software. Similar strategy was applied when constructing vector pLp1261_S.au_rseP.

After In-Fusion cloning, genetic transformations of chemically competent One-Shot™ TOP10 *E. coli* cells were successfully performed. Transformations were confirmed by colony PCR, gel electrophoresis (appendix A5) and GATC sanger sequencing of the *rseP* fragments (appendix A6-7.1).

Finally, isolated plasmids were transformed into electrocompetent *L. plantarum* WCFS1 cells. Transformation results were verified by colony PCR and gel electrophoresis (figure 3.4). Based on gel pictures, it was confirmed that transformations were successfully completed for all strains.

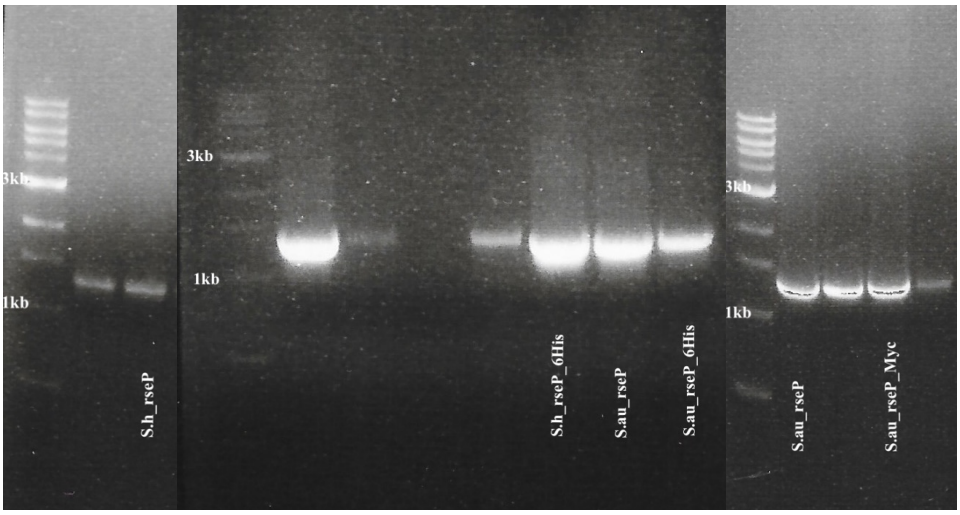


Figure 3.4. Pictures of gel electrophoresis with of colony PCR products. Expected product size was from 1278 bp S.h_rseP to 1299 bp S.au_rseP_Myc harboured in *L. plantarum*.

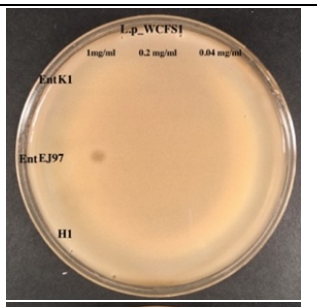
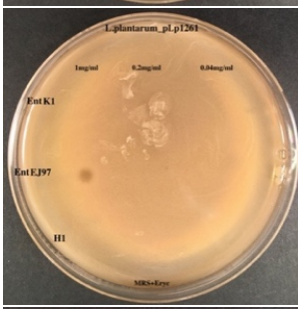
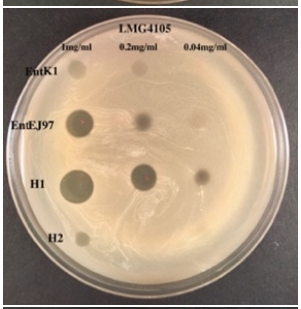
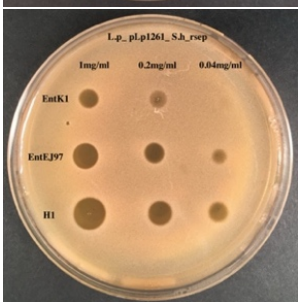
Susceptibility of transformed *L. plantarum* strains to bacteriocins EntK1, EntEJ97 and H1 was determined by spot-on-lawn assay (section 2.6). The MIC_{50} was decided with microtiter plate assay (section 2.7.3).

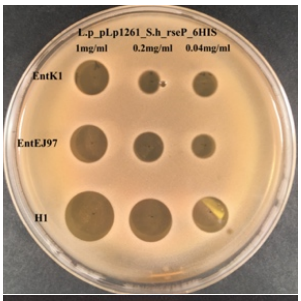
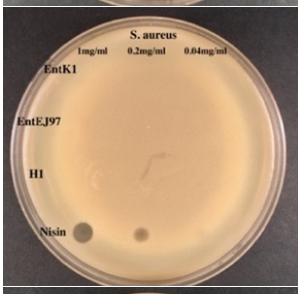
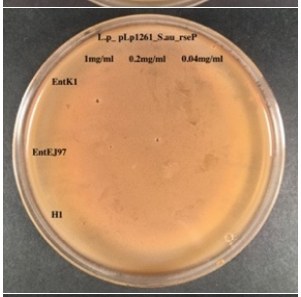
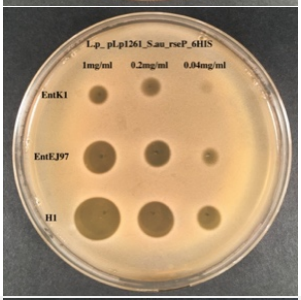
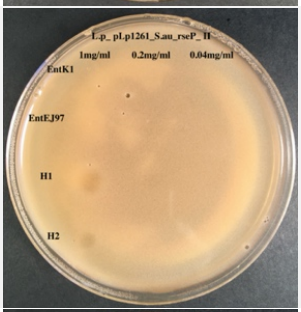
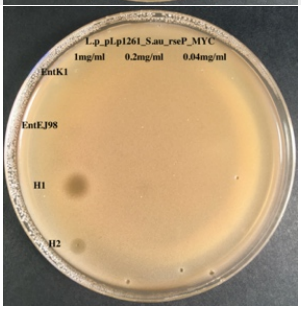
Table 3.7 shows that *L. plantarum* WCFS1 WT (A) have natural resistance to H1 and transformation with empty vector (B) did not induced sensitivity. Previously it has been showed that *S. haemolyticus* LMG4105WT displayed sensitivity towards H1 with MIC_{50} 1.6 µg/ml (C). Interestingly, L. plantarum strains harbouring *S. haemolyticus rseP* (D-E) exhibited higher levels of susceptibility to bacteriocin H1 and EntK1, EntEJ97, which indicates that *rseP* in S. haemolyticus is directly involved in bacterial sensitivity to bacteriocin H1.

Table 3.7 shows that transformed *L. plantarum* harbouring *rseP* from *S. aureus* (G, I) are completely resistant to H1, compared to wild type (F). In contrast, it was detected that the strains with 6His-tag C-terminally fused to the *rseP* fragment (H) required only 0.1 µg/ml of H1 after 5 hours and was still sensitive after 48 hours incubation.

To evaluate the 6His-tag impact on bacteriocin sensitivity, Myc-tag was fused to *rseP* from *S. aureus* (J). The newly transformed strains were resistant to bacteriocins and susceptibility similar to *S. aureus* LMG3023WT (F) and strains without tags (G, I). This showed that 6His-tag can alter bacteriocin sensitivity.

Table 3.7. Collection of bacteriocin H1 inhibition activity against transformed *L. plantarum* and wild types. Results are represented with H1 MIC_{50}=µg/ml (5 hours, 24 hours and 48 hours) and spot-on-lawn assay (24 hours) after incubation at 37ºC.

Isolate (n=3) annotation	Abbreviation	MIC_{50} 5 h	MIC_{50} 24 h	MIC_{50} 48 h	Spot-on-lawn (24 h)
A) *L. plantarum* WCFS1WT	L.p_WCFS1	>50	>50	>50	
B) *L. plantarum* + empty vector plasmid	L.p_pLp1261	>50	>50	>50	
C) *S. haemolyticus* LMG4105WT	LMG4105	1.6	>50	>50	
D) *L. plantarum* + cloned *rseP* from *S. haemolyticus*	L.p_pLp1261_S.h_rseP	0.39	1.6	3.13	

E) *L. plantarum* + cloned *rseP* +6His-tag *from S. haemolyticus*	L.p_pLp1261_S.h_rseP_6HIS	0.05	0.05	0.05	
F) *S. aureus* LMG3023WT	S. aureus	>50	>50	>50	
G) *L. plantarum* + cloned *rseP* from *S. aureus*	L.p_pLp1261_S.au_rseP	>50	>50	>50	
H) *L. plantarum* + cloned rseP+6His-tagfrom *S. aureus*	L.p_pLp1261_S.au_rseP_6HIS	0.1	0.78	1.6	
I) *L. plantarum* + cloned rseP from *S. aureus*	L.p_pLp1261_S.au_rseP_II	>50	>50	>50	
J) *L. plantarum* + cloned *rseP* +Myc-tag from *S. aureus*	L.p_pLp1261_S.au_rseP_MYC	>50	>50	>50	

3.4. Resistant mutant evaluation

Naturally produced resistant mutants were isolated from three random *S. haemolyticus* strains used in spot-on-lawn assay (section 2.6.2): A) LMG4105, B) LMG4106 and C) LMG4115 (figure 3.5). The resistant strains were induced by exposure to 1 mg/ml bacteriocin H1 and EJ97 at 30°C (figure 3.5) and were the basis for further research.

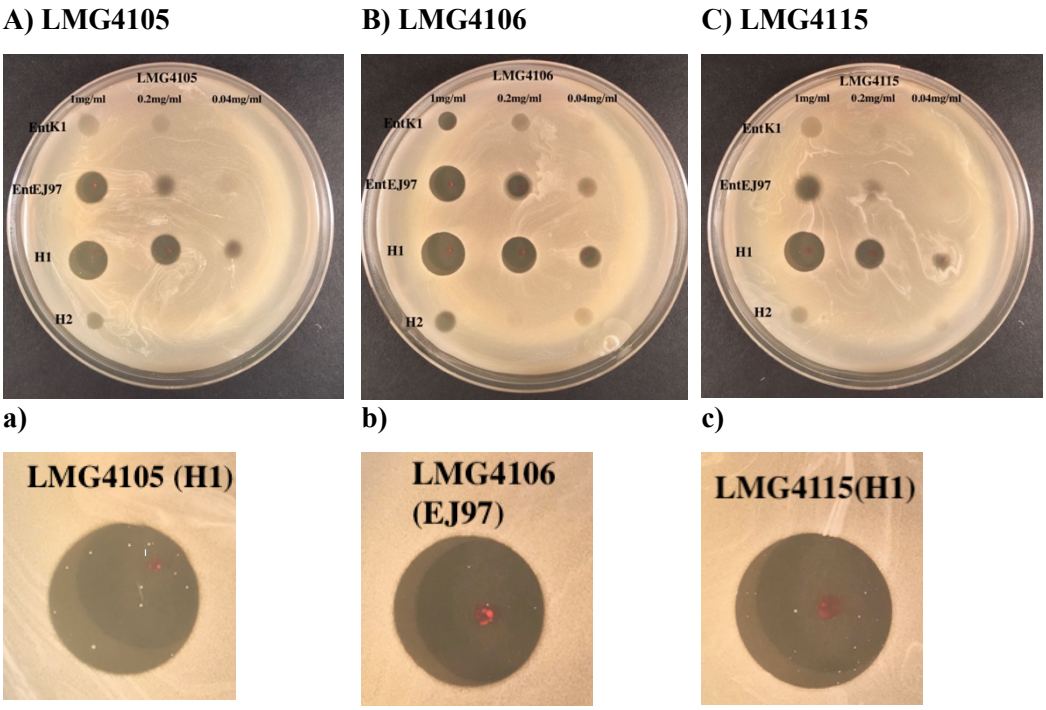


Figure 3.5. Resistant mutants were isolated from inhibition zones in bacteriocin sensitivity assay on agar plate containing indicator bacteria. Picture A, B and C shows the levels of inhibition of *S. haemolyticus* strains corresponding to bacteriocins concentration. Illustrations a, b and c display resistant mutants induced when exposed to 1 mg/ml of H1 and EntEJ97.

3.4.1. Bacteriocin resistance

The results of resistance test (section 2.7.1) confirmed resistance of the mutant strains against bacteriocin EntK1, EntEJ97, H1 and H2, while wild types showed sensitivity to EntEJ97 and H1 (pictures available in appendix A8).

The microtiter plate assay with two-fold dilution of bacteriocin H1 (section 2.7.3) have evaluated resistance level of the isolated mutants in relation to their wild types (table 3.5). The MIC_{50} values in table 3.5 shows resistance of all mutants to bacteriocin during incubation for 5 hours, 24 hours and 48 hours at 37°C. In contrast, the wild types exhibited susceptibility to bacteriocin after 5 hours incubation, however they displayed resistance after 24 and 48

hours. Values for MIC_{50} of LMG105WT, LMG4106WT and LMG4115WT from table 3.5 were slightly higher in comparison to table 3.2, which could indicate experimental errors in previous investigation.

Table 3.5. Results of MIC_{50} (ug/ml) of H1 bacteriocin against resistant isolates and their wild types measured with OD600. MIC_{50} values display the resistance of the mutants over 5 hours, 24 hours and 48 hours constant incubation at 37ºC.

Isolate (n=1)	5 h	24 h	48 h
LMG4105WT	1.6	>50	>50
LMG4105M1	>50	>50	>50
LMG4105M2	>50	>50	>50
LMG4105M3	>50	>50	>50
LMG4106WT	0.39	1.6	>50
LMG4106M1	>50	>50	>50
LMG4016M2	>50	>50	>50
LMG4115WT	0.78	>50	>50
LMG4115M1	>50	>50	>50
LMG4115M2	>50	>50	>50
LMG4115M3	>50	>50	>50

3.4.2. Stress assay

RseP is known to be involved in stress response in various bacteria and deletion of *rseP* cause sensitivity to extracytoplasmic stress (Alba and Gross, 2004, Koide et al., 2008, Varahan et al., 2013, Akiyama et al., 2015). A mutation in this Zn-dependent protease may putatively convert bacterial sensitivity to heat. Here, the stress assay was performed to demonstrate the response of isolated mutants to high temperatures (section 2.7.2). Figure 3.6 shows that after 24 hours of temperature treatment at 40ºC (illustration B) inhibition of LMG4106M1 and LMG4106M2 was observed, however extended treatment time (bottom illustration B) shows recovery of these strains. The same mutant strains were inhibited at 45ºC treatment (illustration C), in addition to a wild type LMG4106WT and other mutant LMG4105M3. Nevertheless, the inhibited strains were also partially recovered after 48 hours incubation. Isolate treatment at 37ºC was used as a positive control for bacterial growth.

Study of stress assay in liquid medium (appendix A9) shows similar results from agar plates (figure 3.6). After 24 hours incubation at 40ºC and 45ºC the most sensitive to heat were strains LMG4106 (WT, M1 and M2) and LMG4105M3. OD600 had lower values for treatment

at 45ºC than 37ºC and 40ºC, indicating that bacterial growth was inhibited during first 24 hours of incubation.

Mutants produced by *S. haemolyticus* strains from India display a phenotype similarity with the strains form Norway. Nevertheless, none of the Indian isolates were inhibited at 40ºC, which may be caused by higher heat tolerance due to the climatic origin of the bacteria. Several *S. haemolyticus* were inhibited during the first 24 hours incubation at 45ºC (appendix A10). However, after 48 hours, those inhibited cells were recovered. There were no significant differences in bacterial growth between mutants and wild types.

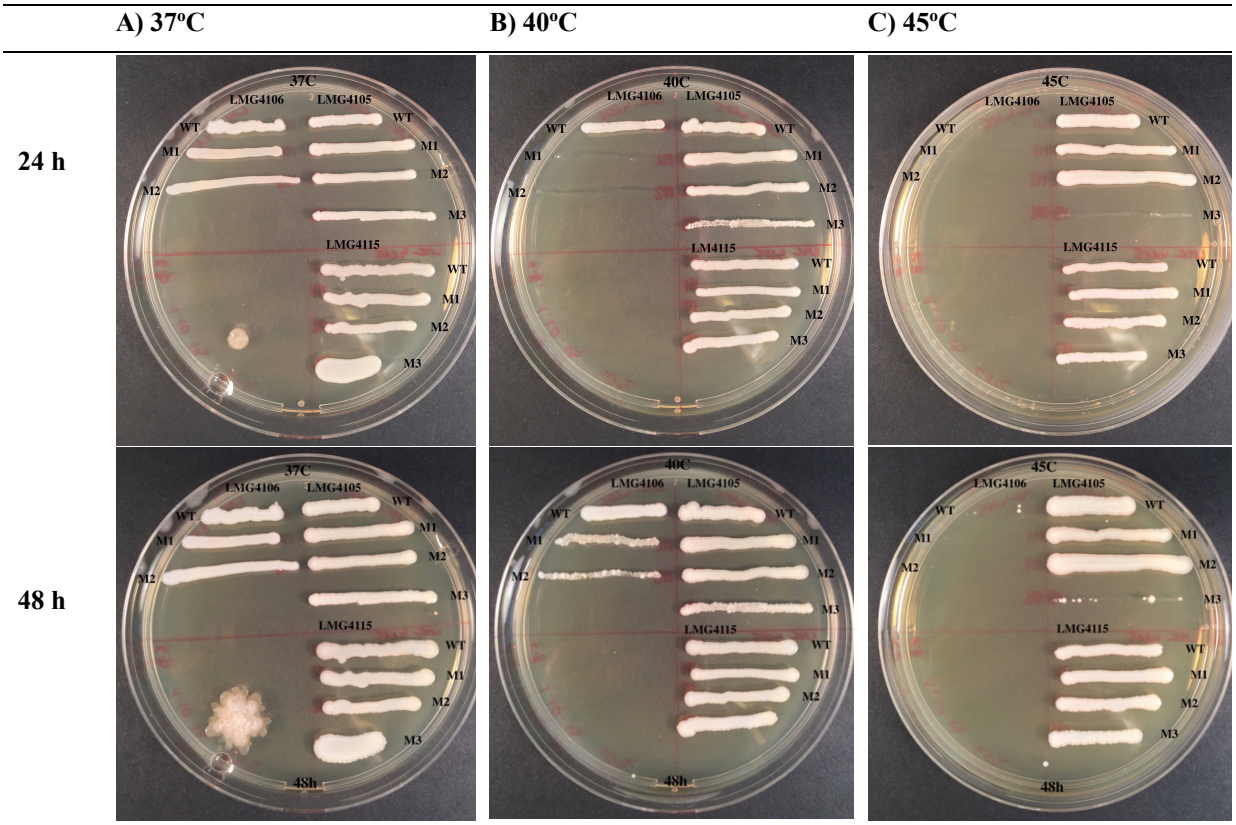


Figure 3.6. Isolated mutants (M1-3) and wild types (WT) were treated for 24 hours (top picture) and 48 hours (bottom picture) with A) 37ºC, B) 40ºC, C) 45ºC on solid media. Treatment at 37ºC was used as a positive control for bacterial growth.

3.4.3. Cell surface morphology

Change in antimicrobial susceptibility of mutants suggested that these resistant isolates might have visible variations on cell surface (Galvez et al., 1998, Jonsson et al., 2010, Cotter et al., 2013). The transmission electron microscopy (described in section 2.7.4) was used to evaluate morphology of wild types and mutants of LMG4105 and LMG4115 strains (figure 3.7). The 60 nm thick sections of bacterial specimen embedded in resin were exposed to

electron beams to visualize bacterial cells. Obtained micrographs showed no notable variation between surfaces of wild types (A, D) and corresponding mutants (B-C, E-F). Nevertheless, there was observed difference of the cells surface between LMG4105 (A-C) and LMG4115 (D-F) strains. The surfaces of LMG4105 were rough and LMG4115 were shown to be smoother. Alterations in cells wall in LMG4105M1 (B), LMG4115M1 (E) and LMG4115M2 (F) might be a consequence of sectioning or embedding error the specimen.

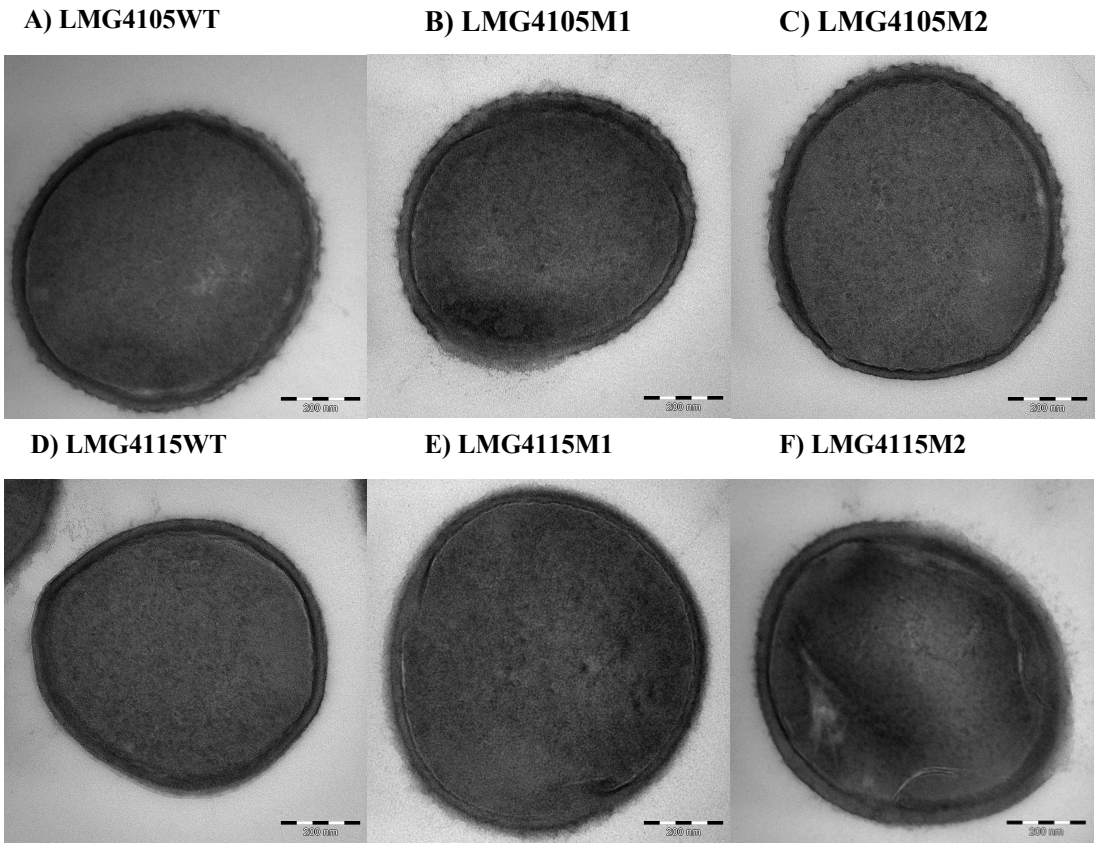


Figure 3.7. Transmission electron microscope micrographs of *S. haemolyticus* LMG4105WT (A), LMG4105M1 (B), LMG4105M2 (C), LMG4115WT (D), LMG4115M1 (E) and LMG4115M2 (F). Single cell of each strain is represented in 200 nm resolution.

3.5. Sequence analysis

Differences in phenotypes observed in resistant isolates above are similar to those reported in previous studies indicating mutation in *rseP* (Galvez et al., 1998, Varahan et al., 2013, Ovchinnikov et al., 2017). Genome sequencing was conducted to compare the variation in genotype with detected bacterial phenotypes.

GATC sequencing of *rseP* fragment

To detect variation in *rseP* genotype and compare them with the perceived trails, sequencing was performed on amplicons of mutants and subsequently aligned with corresponding wild types. Genomic DNA was extracted from all mutants isolated from the inhibition zones in figure 3.5 and their wild types for further amplification of *rseP* fragment with specific primers (section 2.9-2.15). PCR products at size of ~1511 bp (appendix A11) were purified and sequenced by GATC sanger sequencing. Reads were assembled to contig which were translated to amino acids and analysed by multiple sequence alignment (MSA) (appendix A12-15.1).

There were no mutations at protein level of the RseP in the resistant isolates LMG4105, LMG4106 and LMG4115 compared to the wild types. Sequencing of *rseP* fragment for Indian strains LMGT4068 (WT and M1) revealed no mutation within amino acids sequence (appendix A16).

Whole Genome Sequencing

The intact *rseP* gene directed the suspicion of mutations to other systems that might be responsible for characteristic phenotypes, sush as the putative influence of ABC transporter on activity of RseP metalloprotease (Heinrich et al., 2008, Frank et al., 2012, Varahan et al., 2014, Ovchinnikov et al., 2017). The whole genome sequencing was performed using Illumina Next Generation Sequencing technology by researchers at the Norwegian University of Life Sciences (section 2.17).

Analysis conducted on two mutants LMG4105M1 and LMG4105M2 showed similar variations in genome. Complete WGS annotation for variation in *S. haemolyticus* strains using the Snippy algorithm is included in appendix A17-18. Table 3.6 shows short versions of WGS annotations for LMG4105M1 and LMG4105M2. Single nucleotide polymorphisms (SNPs) were detected by alignment to the reference sequence (the wild type LMG4105WT genome) and frameshift in the coding region was revealed. In both genomes, the mutation was a result of deletion of the nucleotide at position 992 in node 30 of the reference sequence. This was further investigated by a reads mapping to a reference using Unipro UGENE v.34. Alignment of short reads with UGENE (appendix A19) illustrates reads assembly and the detected deletion in position 993. The deletion of 885T changed the coding region for putative ABC transporter permease protein. This frameshift mutation may terminate translation prematurely and consequently the protein may not be fully synthetized (pairwise alignment in appendix A20).

Table 3.6. Specifications for WGS of LMG4105 resistant mutant M1 and M2. Complete results are shown in appendix A17-18.

Isolate	Position ingenome assembly	Type of mutation	NT_Position in gene	AA_Position in protein	Effect	Product
LMG 4105M1	Node 30 p.992	deletion	855/1224	285/407	frameshift_variant c.855delT p.Phe285fs	ABC transporter permease
LMG 4105M2	Node 30 p.992	deletion	855/1224	285/407	frameshift_variant c.855delT p.Phe285fs	ABC transporter permease

WGS was performed twice on LMG4115WT, nevertheless the sequencing data was with low coverage. In consequence, the reads library was not successfully assembled. Obtained data did not show any significant mutations in the genomes of LMG4115M1 and LMG4115M2 (appendix A21-22 respectively).

4.Discussion

4.1 H1 has strong activity against S. haemolyticus

Spectrum inhibition assay of leaderless bacteriocins EntK1, EntEJ97 and their two hybrids H1 and H2, showed that H1 was most active peptide against *S. hamolyticus* (figure 3.1). The hybrid peptide H1, combining EntK1 N-terminal part and EntEJ97 C-terminal, demonstrated the widest spectrum of inhibition and was particularly active against *Staphylococcus* and *Enterococcus* species. The reverse combination of the eneterocins, hybrid H2, was least active of the four bacteriocins applied. The similar efficiency of EntEJ97 and H1 may support the postulation of Ovchinnikov et al. (2014) that LsbB-like bacteriocin family have their receptor binding part in C-terminal.

Variation of activity of the four bacteriocins may indicate that not only the highly conserved restudies of KXXXGXXPWE motif are responsible for receptor interaction. It may also be other amino acids at C-terminal. An example of such is the last alanine residue of EntEJ97 and H1 which was also described by Ovchinnikov et al. (2014) in LsbB. It was suggested to be an important residue in interaction with hydrophobic environment of the membrane or receptor molecule.

The strong activity of H1 against *S. haemolyticus* was further investigated in spot-on-lawn experiment with strains from Norway and India. The results demonstrated that geographically diverse *S. haemolyticus* were equally susceptible to H1. Microtiter plate assay showed that the effectiveness of H1 against *S. haemolyticus* decreased over time, which may indicate an increase in the number of resistant bacteria in assays.

4.2. Antimicrobial potential of H1

The comparative susceptibility of pathogenic *S. haemolyticus* to various antimicrobials showed a high inhibitory potential of H1 in an *in vitro* experiment. Common glycopeptide antibiotics (vancomycin and teicoplanin) as a last resort for life threatening infections by staphylococci (Czekaj et al., 2015) showed strain dependent activity in disc diffusion method. In contrast, bacteriocin H1 exhibited similar efficiency to all *S. haemolyticus* strains both from Norway and India (unpublished data, obtained from Mikkel Brønner). This might signify that bacteriocin resistance genes are not yet distributed among *S. haemolyticus*, whereas antibiotic resistance is already widespread in hospital isolates (Cavanagh et al., 2014).

The study of combinatory formulations of three different antimicrobials exhibited a strong synergy effect of H1 when combined with others antimicrobial, especially garvicin KS. Both bacteriocins H1 and GarKS are leaderless peptides with a great potential for inhibiting Gram-positive bacteria (Ovchinnikov et al., 2017) and may have the same mode of action. Garvicin KS have been previously described to have high synergy effect with nisin bacteriocin (Chi and Holo, 2018), however it was not yet tested with other leaderless peptide. Combination of antimicrobial agents are thought to be more potent in reducing resistance development (Mathur et al., 2017). It can be assumed that increase in the concentration of peptides that may target similar protein, could repress higher number of receptor and causes more effective inhibition of bacterial growth. This experiment showed that synergistic interaction between H1 and garvicin KS yielded antimicrobial effect against resistant mutants (data not shown). Furthermore, combination of H1 and garvicin KS has a great potential in inhibiting biofilm of *S. haemolyticus* strains (data not shown, obtained from Mikkel Brønner).

RseP is a receptor for H1

Previous researches have shown that lactococcal and enterococcal Zn-dependent protease RseP is a receptor for leaderless bacteriocin LsbB, EntK1 and EntEJ97 (Uzelac et al., 2013, Ovchinnikov et al., 2017). Here it was confirmed that RseP from *S. haemolyticus* is important for bacterial susceptibility to the hybrid bacteriocin H1. This was established by heterologous expression of *S. haemolyticus rseP* in naturally resistant *L. plantarum* WCFS1. This also was justified by expression of unsensitive *S. aureus rseP* into *L. plantarum* WCFS1.

Interestingly, this study showed that close related species of *S. haemolyticus*, such as resistant *S. aureus* with high sequence similarity in RseP (figure 4.1), may not interact with H1. By performing site directed mutagenesis, a Serbian researchers group demonstrated that a single mutation in RseP may cause loss of bacterial sensitivity (Miljkovic et al., 2016). This indicates that small differences in amino acid sequences may cause changes in protein structure. An alternative folding may produce protein that is less or not at all accessible for bacteriocin (Ovchinnikov et al., 2017).

Figure 4.1. Comparison of the amino acid sequence of S.h_RseP and S.au_RseP using CLUSTAL 2.1 multiple sequence alignment. "*" indicates positions with conserved residues; ":" indicates aa of strongly similar properties; "." indicates aa of weakly similar properties. RseP in S. haemolyticus (S.h_RseP) and S. aureus (S.au_RseP) shows high sequence identity. Active site is highlighted in red and substrate binding site in green.

An interactive protein topology visualization Protter, allowed to predict differences in the structure of S.h_RseP and S.au_RseP (figure 4.2). This prediction showed that S.au_RseP may have different protein structure on C-terminal part including substrate binding site (NLLPxxxLDG), which was located close to periplasmic site. This may hinder the intermembrane interaction with a substrate and its proteolytic activity.

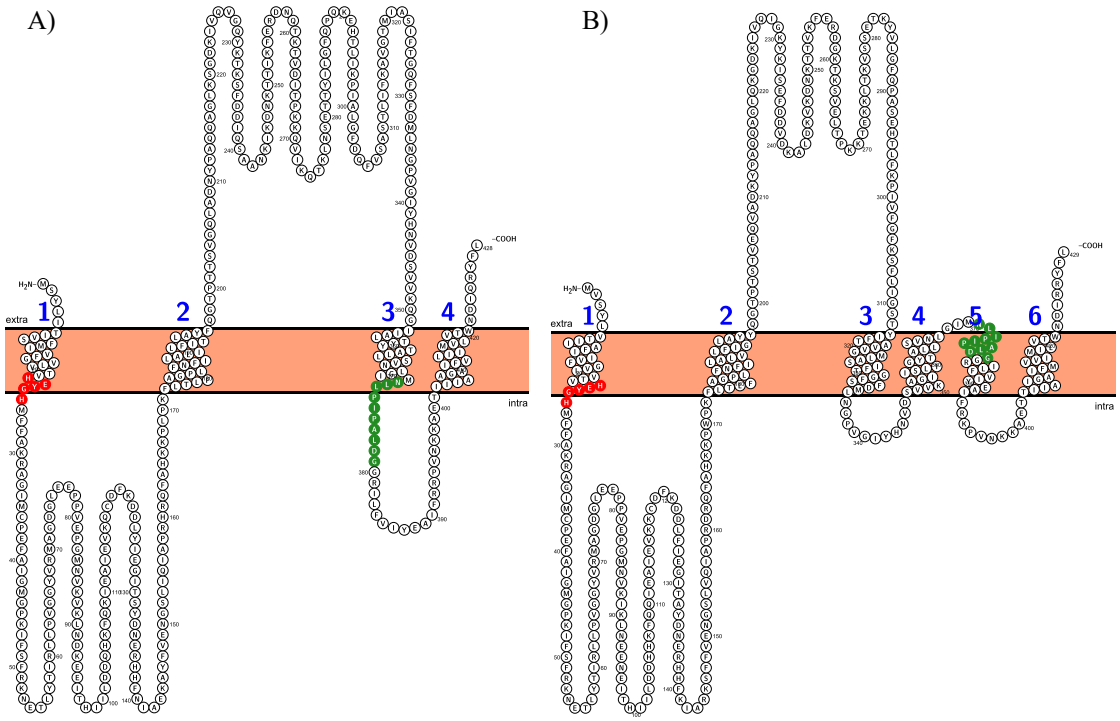


Figure 4.2. Schematic representation of predicted RseP proteins expressed in A) L.p_pLp1261_S.h_rseP and B) L.p_pLp1261_S.au_rseP. Visualization was done using Protter web-based application (http://wlab.ethz.ch/protter/start/). The active site (HExxH) is highlighted in red and substrate binding site (NLLPxxxLDG) is coloured in green. Prediction of the structure of RseP_S.a (B) exhibits that the substrate binding site might be positioned differently in the membrane, which may impede its function.

A method that would confirm the transformation of *rseP* is the detection of specific protein by western blot. The *rseP* inserts were purposely extended with tags (6His-tag and Myc-tag) sequences due to identification of expressed RseP protein in transformants cells with use of tag antibodies. Unfortunately, due to time curtailment, confirmation of the protein expression was not completed.

Nonetheless, strains with polyhistidine-tagged RseP protein exhibited higher susceptibility to applied bacteriocins. Significant facilitation of the tag in bacteriocin-receptor interaction may in result from conformational changes of RseP, thus making the active site more exposed to the bacteriocin. The positively charged tags attached to the C-terminal end of S.h_RseP and S.au_RseP receptors, might have interacted with negatively charged phospholipids of the membrane.

4.3. Evaluation of the phenotype of resistant mutants

Various experiments were performed to compare the observed phenotype of resistant mutants to wild types. Firstly, differences in bacteriocin sensitivity between mutants and wild

types were analysed. The results of microtiter plate assay and resistant test clearly showed the difference in susceptibility between mutant isolates and wild types (table 3.5 and figure A8). The mutants have high level of resistance to H1, while wild types exhibited loss of susceptibility after 24 hours of incubation, which have been previous discussed with microtiter plate assay for *S. haemolyticus* (4.1).

Secondly, bacterial growth was observed in varied temperatures. In this research it was demonstrated that stress in form of high heat has negative impact on growth of several *S. haemolyticus* strains. Assays in solid and liquid media showed that the most sensitive strains against H1 (including wild types) were inhibited during first 24 hours of exposure to elevated temperatures. Nevertheless, these susceptible bacteria reappeared during the next 24 hours of treatment on agar plates, indicating that bacterial growth is only delayed and not fully repressed. The possible explanation may be that bacteria have a supplementary stress response system that helps them to adapt to rough environment when the primary mechanism fails. Ovchinnikov et al. (2017) reported that resistant mutants appeared at 30^{o}C but not at 45^{o}C during 8 hours incubation. However, it can be speculated that extended treatment time might show similar results which were presented in this work. Interestingly, results from Indian strains showed that elevated temperatures slowed down both mutants and wild types. This may suggest that they have same response to stress.

Finally, possible morphological variations between wild types and resistant mutants were evaluated. Alterations in the composition and structure of the cell surface may result in different susceptibility to antimicrobials (Cotter et al., 2013). By performing the transmission electron microscopy with cells of *S. haemolyticus* LMG4105WT and LMG4115WT and their isolated mutants, interesting phenotypes were observed. Even though there were no significant variations in cell surface between wild types and mutants, difference in morphology between strains was clearly detected. Cell surface of strains LMG4105 were rough compared to smoother strains LMG4115. This phenomenon may have reflection in biofilm development. The biofilm assay results for LMG4105WT and LMG4115WT (data not shown, obtained through personal communication with Mikkel Brønner) indicated that the former has slightly better ability to form biofilm. It would have been tempting to isolate mutants from additional *S. haemolyticus* strains to study the morphological structure compared to ability of biofilm formation.

4.4. Genotype of the resistant mutants

The phenotypic differences between wild types and mutants of *S. haemolyticus* were clearly seen in bacteriocin susceptibility experiments. Although this observed trial was supposed to be result of alteration in Zn-dependent metalloprotease (Miljkovic et al., 2016), *rseP* in the resistant isolates were intact. Thus, the lack of RseP mutations may have the reflection in stress response, where several mutants and wild types exhibited same phenotypes.

Subsequently, WGS was performed to uncover potential genotypic variations. WGS analysis of two selected mutants LMG4105M1 and LMG4105M2 suggested the putative ABC transporter to be involved in resistance development. Frameshift mutations might have cause synthesis of unfunctional proteins.

Similar discovery was made by Ovchinnikov et al. (2017) where they observed mutations in *pptA* and *pptB* coding for PptAB (putative ABC transporter) in several *E. faecalis* susceptible to bacteriocins with intact *rseP*. By applying the amino acids sequence of the putative ABC transporter from *S. haemolyticus* LMG4105WT in the Protein Basic Local Alignment Search Tool (BLASTp), the result showed 98.77% sequence identity with the ABC transporter, EcsB family permease protein in *S. aureus* and 28.80% sequence identity with ABC transporter permease EcsB in *B. subtilis* (data not shown). Related Ecs proteins were earlier described to influence the antibacterial resistance and virulence, which will be discussed in next section.

Low reads coverage for LMG4115WT hindered successful assembly, consequently the obtained data was not sufficient for alignment with mutants. The current analysis does not show any significant variations in genome. The whole genome sequencing and reads assembly should be repeated for LMG4115WT. Furthermore, WGS should preferably be performed on the remaining strains that were resistant to H1, which eventually did not have mutations in *rseP*. Optionally, the coding region for putative EcsB in all *S. haemolyticus* strains could have been sequenced to confirm the mutations and validate the supposition that EcsB is responsible for bacterial susceptibility.

4.5. EcsAB influence resistance and virulence

WGS demonstrated mutation in putative *ecsB* gene in H1 resistant mutants. Ecs proteins are ATPase binding cassette (ABC) transporters that are associated with antibiotic resistance systems, and consist of ATP-binding and permease domains encoded by *ecsA* and *ecsB,*

respectively (Jonsson et al., 2010). It was previously been reported that a mutation in *B. subtilis ecsA* inhibits the intermembrane proteolysis of amylase precursor by blocking RseP activity. However, is was also shown that the defect in RseP activity was not regulated by the *ecsA* on transcriptional level (Heinrich et al., 2008, Jonsson et al., 2010).

On the other hand, absence of Ecs in *S. aureus* repressed genes required for cell adherence and wall synthesis, consequently altering the virulence and cell wall composition (Jonsson et al., 2010). Nonetheless, the transmission electron microscopy did not reveal visible differences in cell surface or size in H1 resistant mutants.

Furthermore, defective *ecsB* was shown to be unable to produce biofilm in *B. subtilis* (Heinrich et al., 2008, Varahan et al., 2014). Although the mutants obtained in this study have not been validated for biofilm formation, it would be very interesting to conduct such an experiment and compare the results with corresponding wild types, which are reported to be good biofilm producers (data obtained from Mikkel Brønner).

It has been proposed that the *ecsAB* mutation prevents the RseP proteolytic activity and thus it inhibits cell division and stress response (Heinrich et al., 2008). This study shows that strains with intact *rseP* and mutated *ecsB* are not entirely inhibited during high temperature treatment. Moreover, several WTs were also inhibited in similar manner to the mutated variants. This may point out that there is another "helper" system involved in the stress response, which induces the sigma factor when the RseP activity is inhibited by different factors. Such a factor may be a large amount of misfolded extracellular proteins or accumulation of EcsAB substrates. Speculation that the several high-temperature-sensitive strains were only slowed down may also indicate the time to canvass another peptidase. Varahan et al. (2014) suggested that LspA lipoprotein signal peptidase might enhance the activity of inhibited RseP in *E. faecalis* while deletion of *escAB*.

Although the data obtained in this work are not sufficient enough to draw any certain conclusions about involvement of putative EscAB in stress response and bacteriocin resistance, several investigations have been revealing similar observations. To obtain reliable results, a larger number of resistant mutants should be isolated and perform phenotypic and genotypic analysis.

4.6. Final remarks and future prospects

This thesis demonstrates that RseP in virulent *S. haemolyticus* is a receptor for the hybrid bacteriocin H1. This was proved by heterologous expression of *S. haemolyticus rseP* in

naturally resistant L. plantarum WCFS1, which became significantly sensitive to H1 after transformation. By performing various susceptibility experiments, bacteriocin showed to have a great potential as an optional treatment of this emerging pathogen *S. haemolyticus*, however appearance of resistant mutants is a big issue.

To reduce the development of mutants, combinations of H1 with different antibacterial agents were tested based on work done by Mikkel Brønner (Master student). Most resistant mutants were inhibited when applying the combination of H1 and GarKS, which both are leaderless bacteriocins and are supposed to have similar mode of action.

One of the goals of this study was to investigate the mechanism of resistance in isolated H1 resistant mutants. Results show high levels of resistance to H1 and other leaderless bacteriocins. Nonetheless, RseP, the receptor for H1, appears to be intact. WGS shows that the putative Ecs ABC transporter is a potential explanation of the resistance in mutants. It is tempting to speculate that H1 might undergo proteolysis by RseP and that EcsAB might further transport the processed peptide inside the cell to bind to the target molecule in or outside the cell. The proposed mechanism by which the ABC transporter might affect RseP activity is blocking this protease on protein level. It has been previously suggested that Ecs might remove the remaining membrane peptides (Jonsson et al., 2010). Thus, lack of export protein might cause aggregation of a large number of Ecs substrates in membrane and disruption of RseP.

Prior to rationale for use of H1 as a therapeutic against *S. haemolyticus*, further investigations are required due to explain the interaction between the receptor and the bacteriocin as well as the influence of Ecs on RseP in bacteriocin resistance. A promising method for study the properties of molecules in peptide-protein interactions can be fluorescent labelling of peptides. By using the fluorescent energy transfer (FRET), a fluorescent tag on H1 would help identify how bacteriocin acts on receptors and the cell (Thomas Oftedal, personal communication, May 2020).

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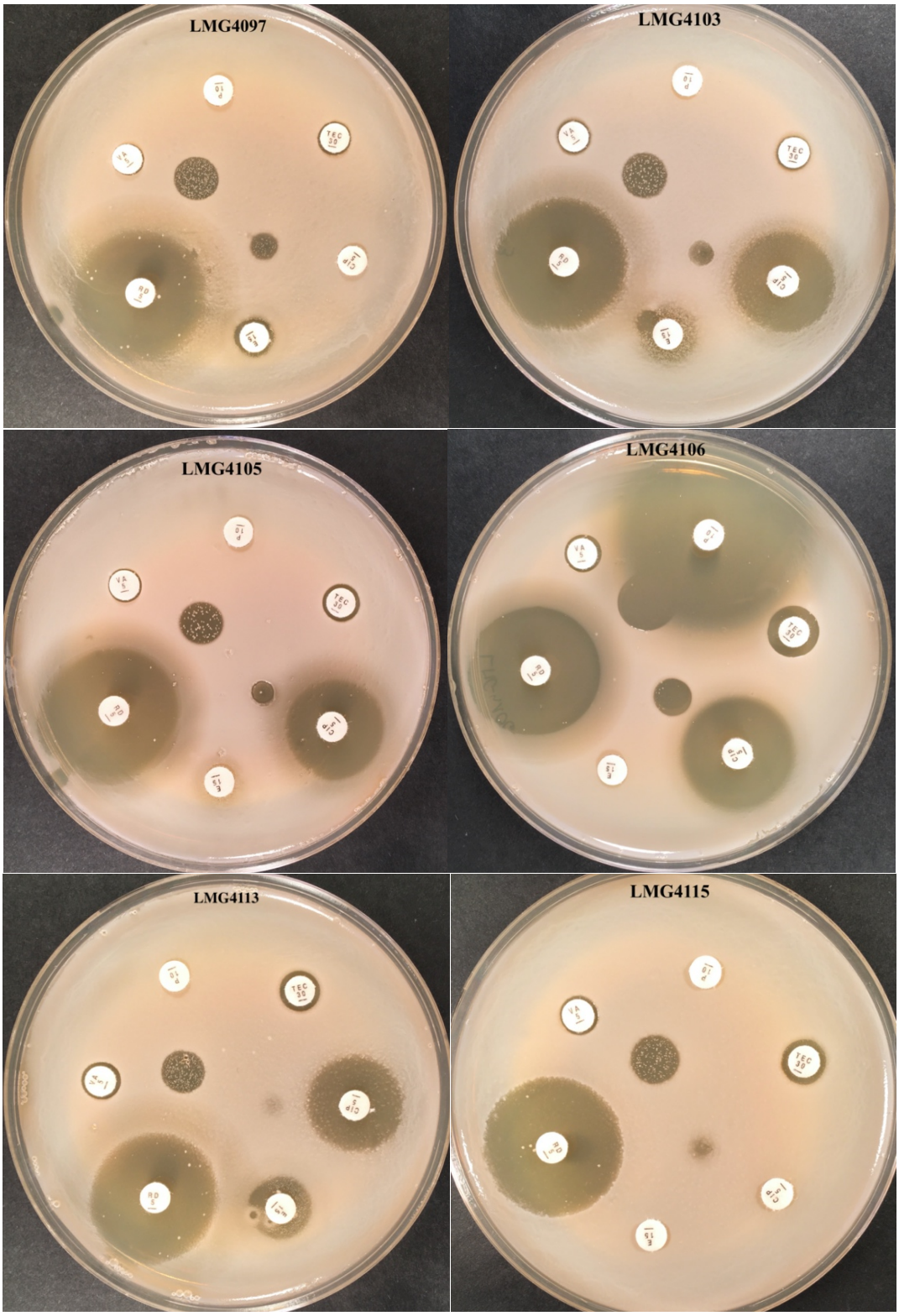
Appendix

	EntK1			EntEJ97			H1			H2			Nisin Z		
Isolate	1mg/ml	0.2mg/ml	0.04mg/ml	1mg/ml	0.2mg/ml	0.04mg/ml	1mg/ml	0.2mg/ml	0.04mg/ml	1mg/ml	0.2mg/ml	0.04mg/ml	1mg/ml	0.2mg/ml	0.04mg/ml
Staphylococcus hominis (n=1)	2	1	1	3	2	1	3*	2*	1	1	1	0	2	2	1
Staphylococcus haemolyticus (n=1)	1	0	0	2	0	0	3*	2*	1	1	0	0	3*	2	2
Staphylococcus epidermidis (n=1)	1	0	0	1	1	0	2*	1	0	0	0	0	3	2	2
Staphylococcus aureus (n=6)	1	0	0	1	1	0	1	0	0	0	0	0	2	2*	1
Staphylococcus arlettae (n=1)	0	0	0	1	0	0	0	0	0	1	0	0	2	2	1
Staphylococcus simulans (n=1)	0	0	0	0	0	0	0	0	0	0	0	0	2	2	1
Bacillus cereus (n=3)	1	0	0	1	0	0	1	0	0	0	0	0	2	2	2
Listeria ivanovii (n=1)	1	0	0	1	1	0	1	1	0	1	0	0	3	2	2
Listeria innocula (n=2)	2*	1	0	1	1	1	2*	2*	1	2*	0	0	3*	2*	2*
Listeria monocylogenes (n=5)	1	0	0	0	0	0	1	0	0	0	0	0	2	2	2*
Carnobacterium piscicola (n=1)	1	1	1	1	1	0	1	1	1	0	0	0	2	2	2*
Carnobacterium divergens (n=1)	0	0	0	0	0	0	0	0	0	0	0	0	2	2	2
Enterococcus faecalis (n=5)	1	1	0	3*	2	1	3	2	1	1	1	0	2	2	2
Enterococcus avium (n=1)	1	0	0	1	1	0	3*	2*	1	1	0	0	2	1	1
Enterococcus faecium (n=4)	2	2*	1	3	2	1	3*	2*	1	1	1	0	2	2	1
Lactobacillus salivarius (n=1)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Lactobacillus plantarum (n=2)	1	0	0	2	1	0	0	0	0	1	1	0	3	2	2*
Lactobacillus curvatus (n=1)	3	2	2*	3	3	2*	2	1	0	3	2	2*	3	3	2
Lactobacillus sakei (n=2)	2*	0	0	0	0	0	0	0	0	2*	2*	1	3	2	2
Lactococcus garvieae (n=1)	2*	2*	1	1	1	1	2*	2*	1	2*	1	1	3	3*	2
Lactococcus lactis (n=2)	1	1	0	1	1	1	1	2*	1	1	0	0	3*	3*	2
Streptococcus thermophilus (n=1)	1	0	0	0	0	0	3*	1	0	0	0	0	3	2	2
Streptococcus dysgalatiae (n=1)	1	1	0	1	1	0	1	1	0	0	0	0	3	3	2*
Streptococcus uberis (n=2)	1	0	0	0	0	0	2*	1	0	0	0	0	3	2	2
Leuconostoc gelidum (n=1)	0	0	0	0	0	0	0	0	0	0	0	0	2	2	2
Escherichia coli (n=2)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

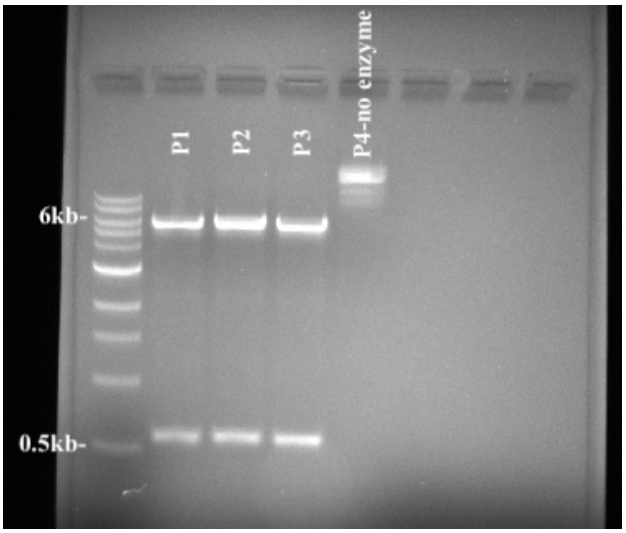
A1: Complete collection of bacteriocin spectrum of activity for 51 bacterial strains. Scorings system: 0= no inhibition; 1= Unclear zone; 2= small zone (<1 cm); 3= big zone (≥1 cm), "*" zone with resistant mutants.

	K1			EJ97			H1			H2		
Isolate	1mg/ml	0.2mg/ml	0.04mg/ml	1mg/ml	0.2mg/ml	0.04 mg/ml	1mg/ml	0.2mg/ml	0.04mg/ml	1mg/ml	0.2mg/ml	0.04mg/ml
LMG4096	0	0	0	2*	1	0	3*	2*	0	0	0	0
LMG4097	0	0	0	2*	1	0	3*	2*	0	0	0	0
LMG4098	0	0	0	3*	1	0	3*	2*	0	0	0	0
LMG4099	0	0	0	2*	1	0	3*	2*	0	0	0	0
LMG4100	0	0	0	2*	1	0	3*	2*	0	0	0	0
LMG4101	0	0	0	3*	1	0	3*	2*	0	0	0	0
LMG4102	0	0	0	3*	1	0	3*	2*	1	0	0	0
LMG4103	0	0	0	3*	1	0	3*	2*	0	0	0	0
LMG4104	0	0	0	2*	1	0	3*	2*	0	0	0	0
LMG4105	0	0	0	3*	2*	0	3*	2*	0	0	0	0
LMG4106	2*	0	0	3*	3*	0	3*	2*	2	0	0	0
LMG4107	0	0	0	2*	0	0	3*	2*	0	0	0	0
LMG4108	0	0	0	2*	1	0	3*	2*	1	0	0	0
LMG4109	0	0	0	2*	0	0	3*	2*	0	0	0	0
LMG4110	0	0	0	3*	1	0	3*	2*	1	0	0	0
LMG4111	1	0	0	3*	2*	0	3	3	1	2*	0	0
LMG4112	1	0	0	3*	2*	0	3*	3*	1	1	0	0
LMG4113	0	0	0	2*	1	1	3*	2*	0	0	0	0
LMG4114	0	0	0	2*	0	0	3*	2*	0	0	0	0
LMG4115	0	0	0	2*	1	0	3*	2*	1	0	0	0

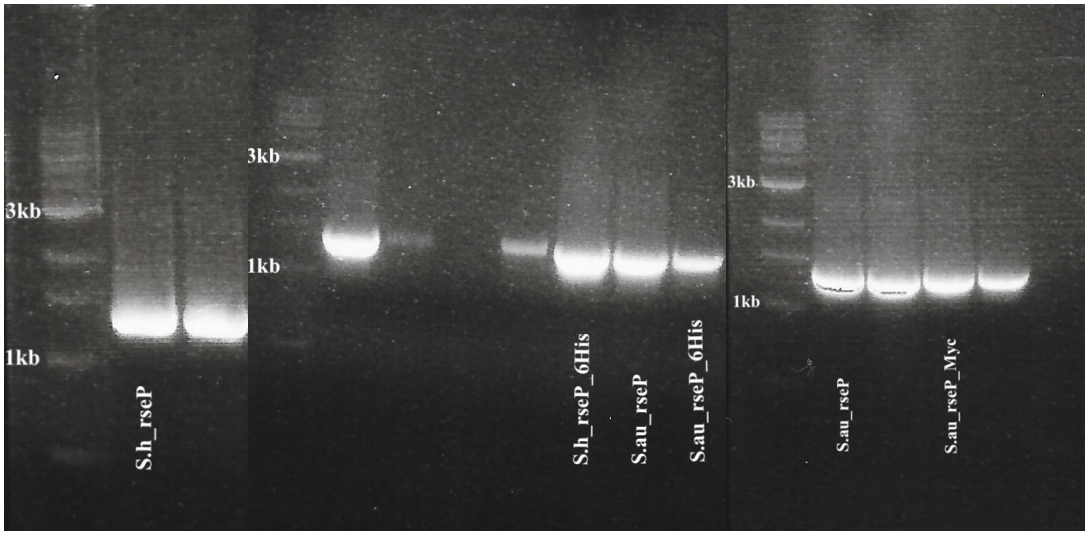
A2: Complete collection of bacteriocin spectrum of activity for 20 *S. haemolyticus* strains. Scorings system: 0= no inhibition; 1= Unclear zone; 2= small zone (<1 cm); 3= big zone (≥1 cm), "*" zone with resistant mutants.



A3: Comparative susceptibility of antimicrobials agents. Plate pictures showing zones of inhibition of *S. haemolyticus* indicators produced by various antimicrobials: 5 µg vancomycin (VA5), 10 units of penicillin G (P10), 30 µg teicoplanin (TEC30), 5 µg ciprofloxacin (CIP5), 15 µg erythromycin (E15) and 5 µg rifampicin (RD5) and 3µg and 0.6 µg H1.

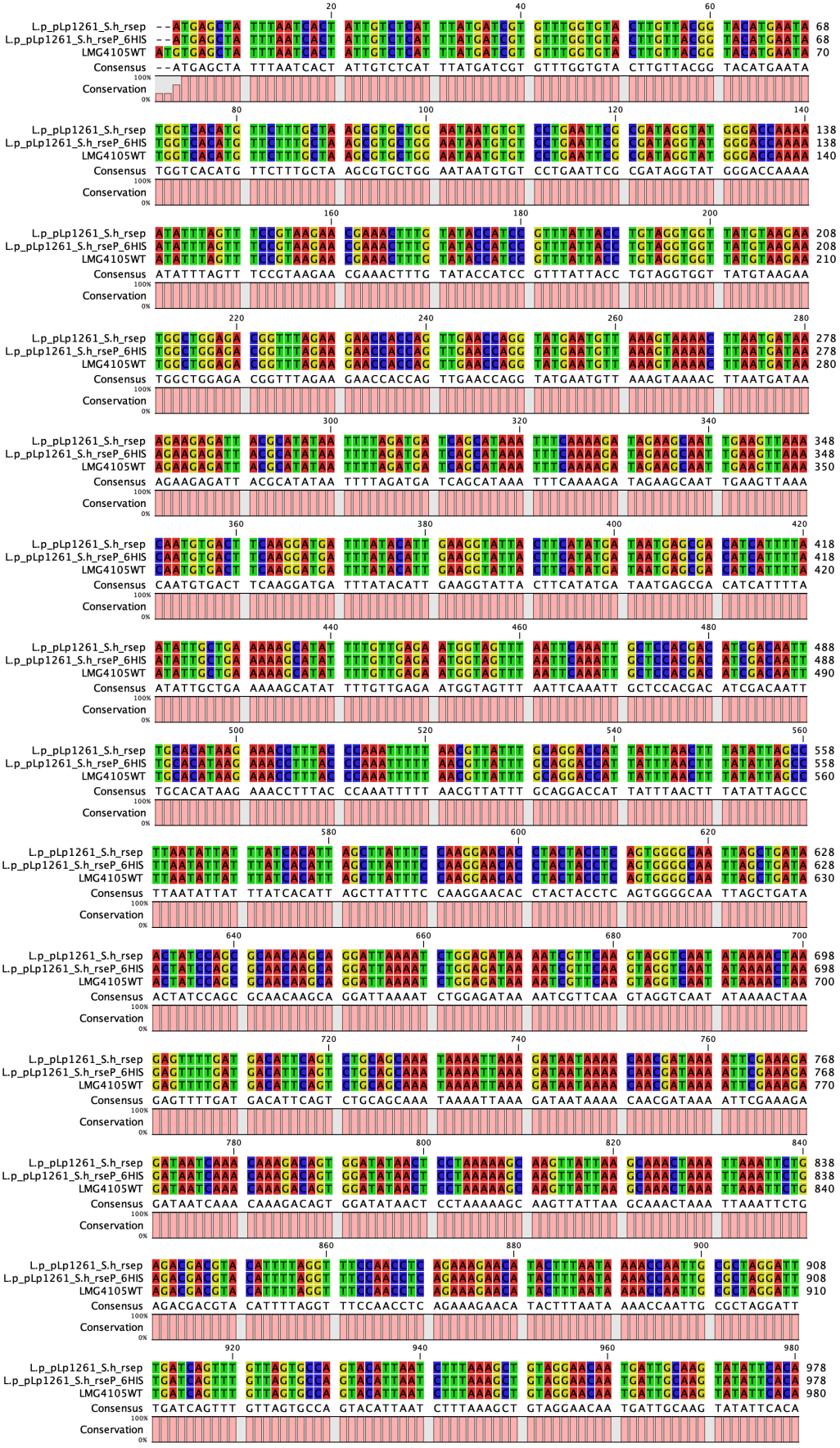


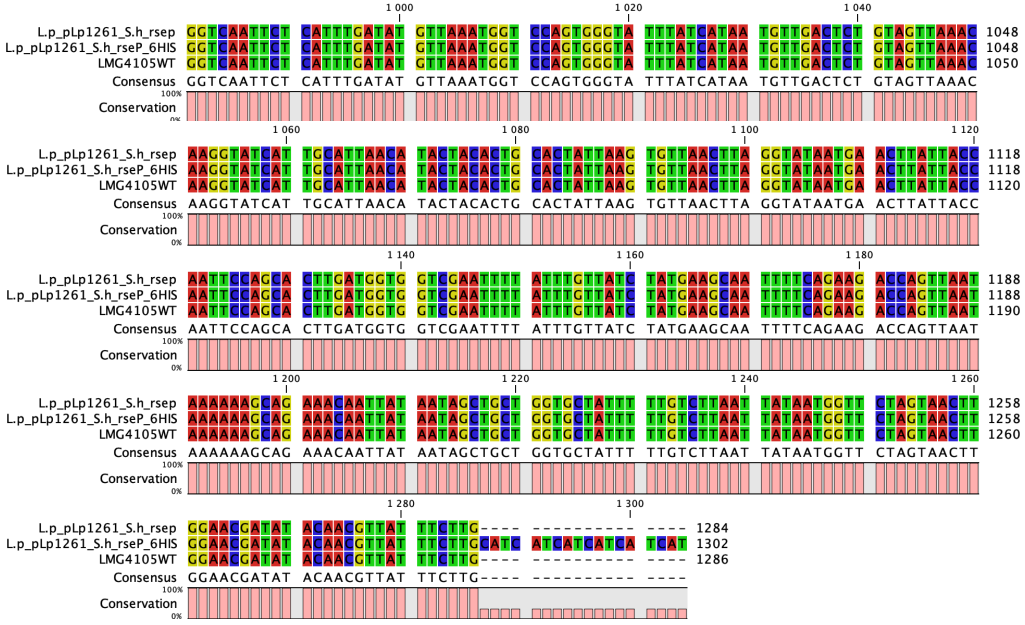
A4: Digestion of ´pLp1261_InvS´ vector. P1-3 shows linearized plasmids with size 5601 bp. P4 is a negative control and contains un-cut plasmid with size 6428 bp.



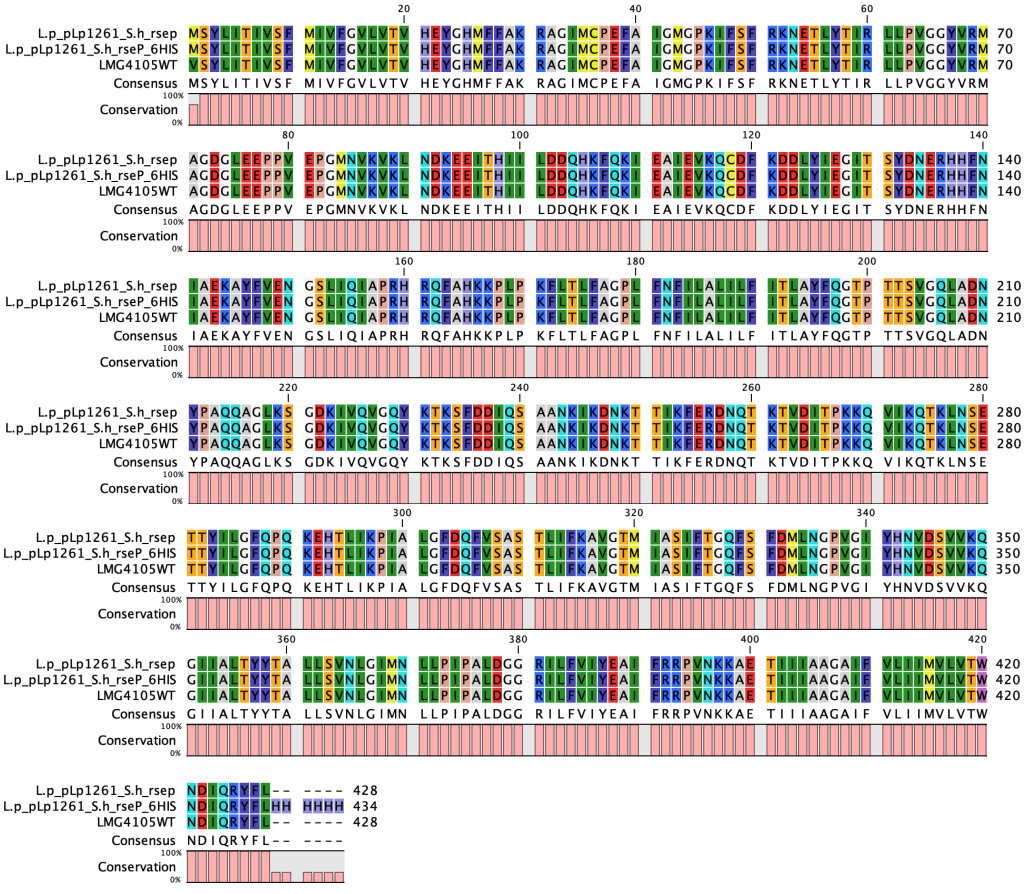
A5: Colony PCR products after transformation in TOP10 *E. coli*. Pictures show colony PCR products of transformed *rseP* fragments.

A6: MSA of transformed *L. plantarum* with *S. haemolyticus rseP*

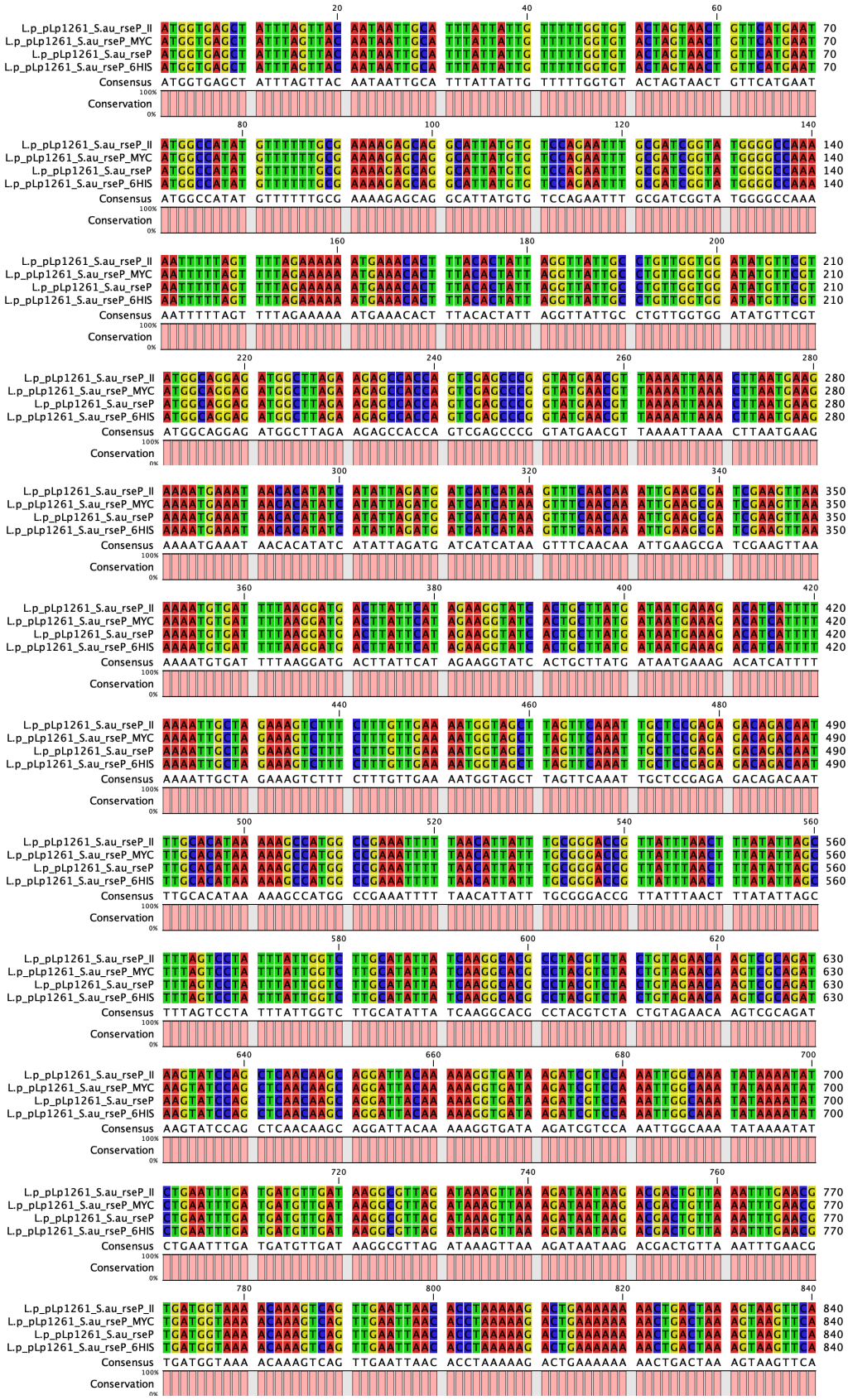


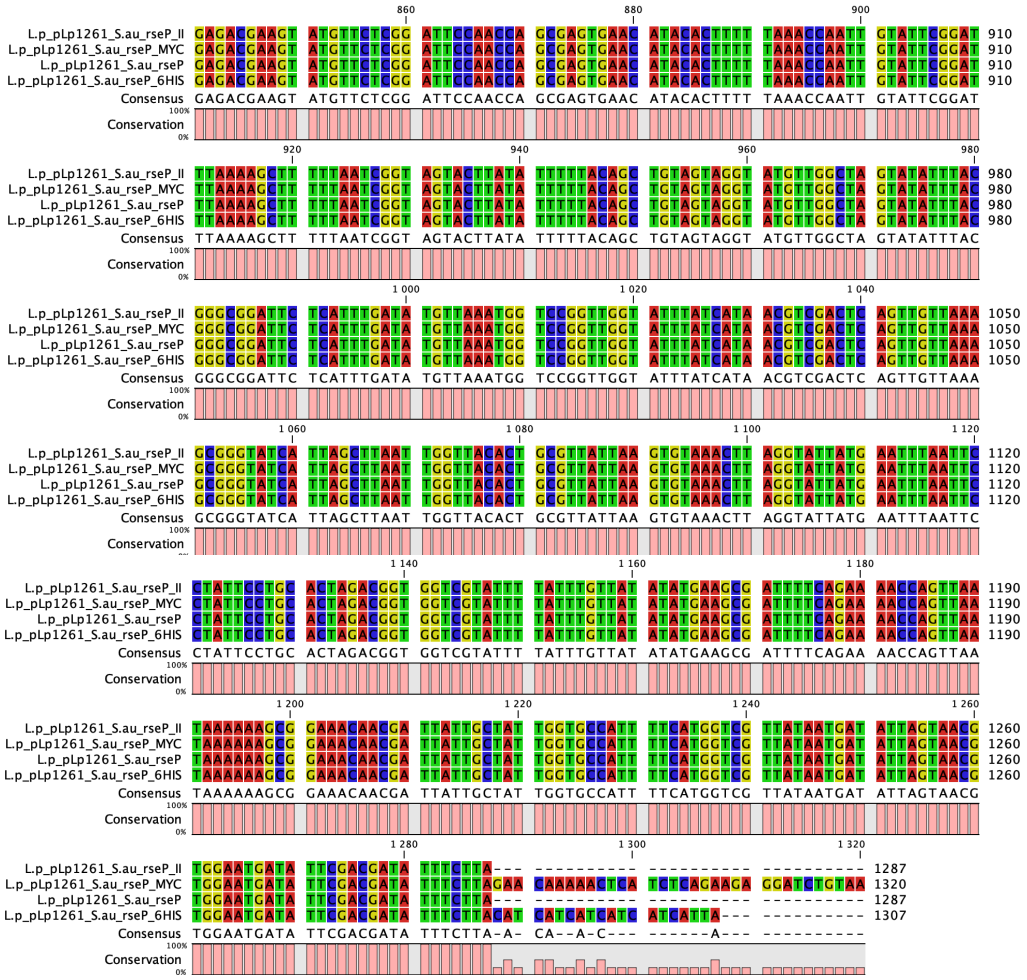


A6.1: MSA of transformed *L. plantarum* with *S. haemolyticus* RseP

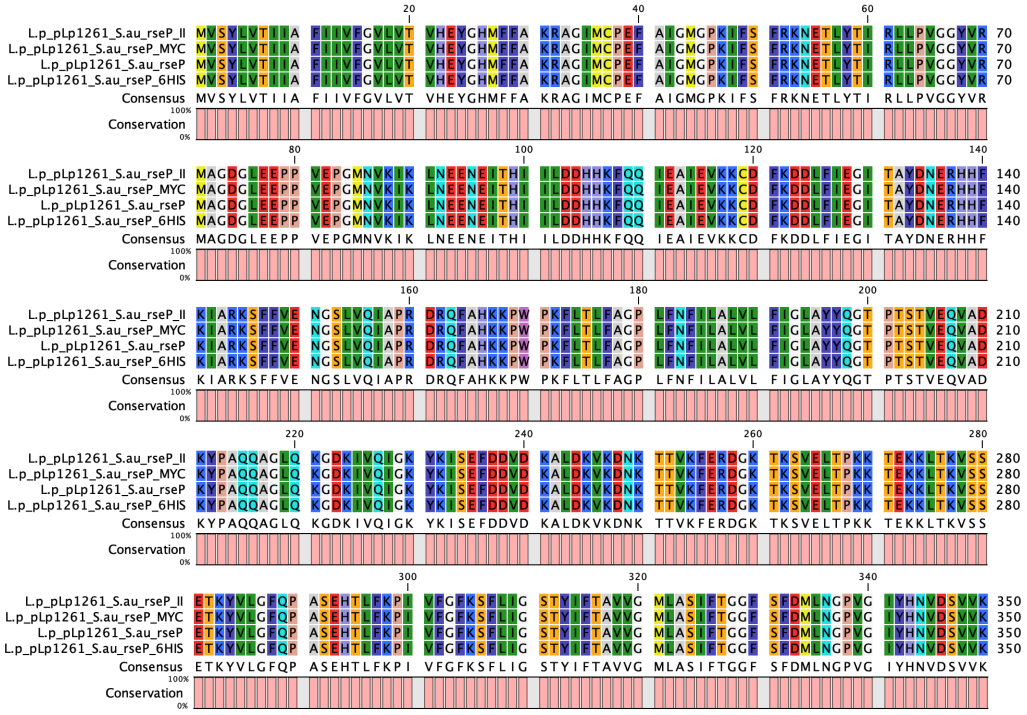


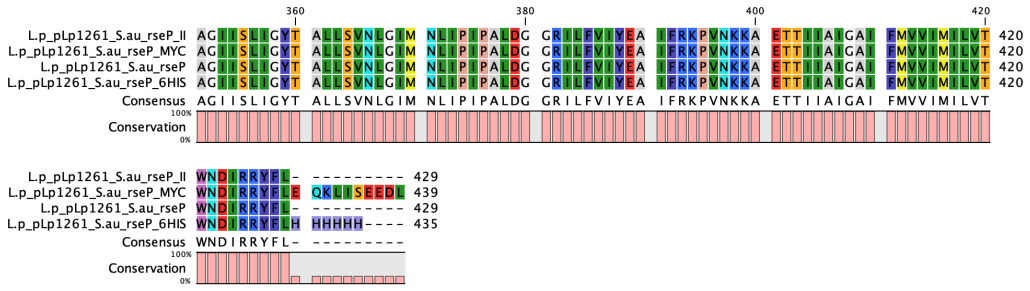
A7: MSA of transformed *L. plantarum* with *S. aureus rseP*

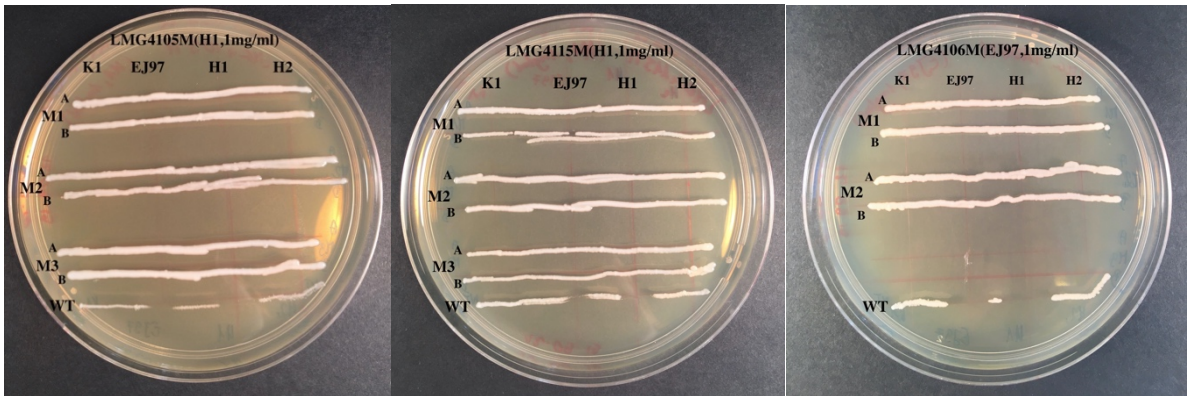




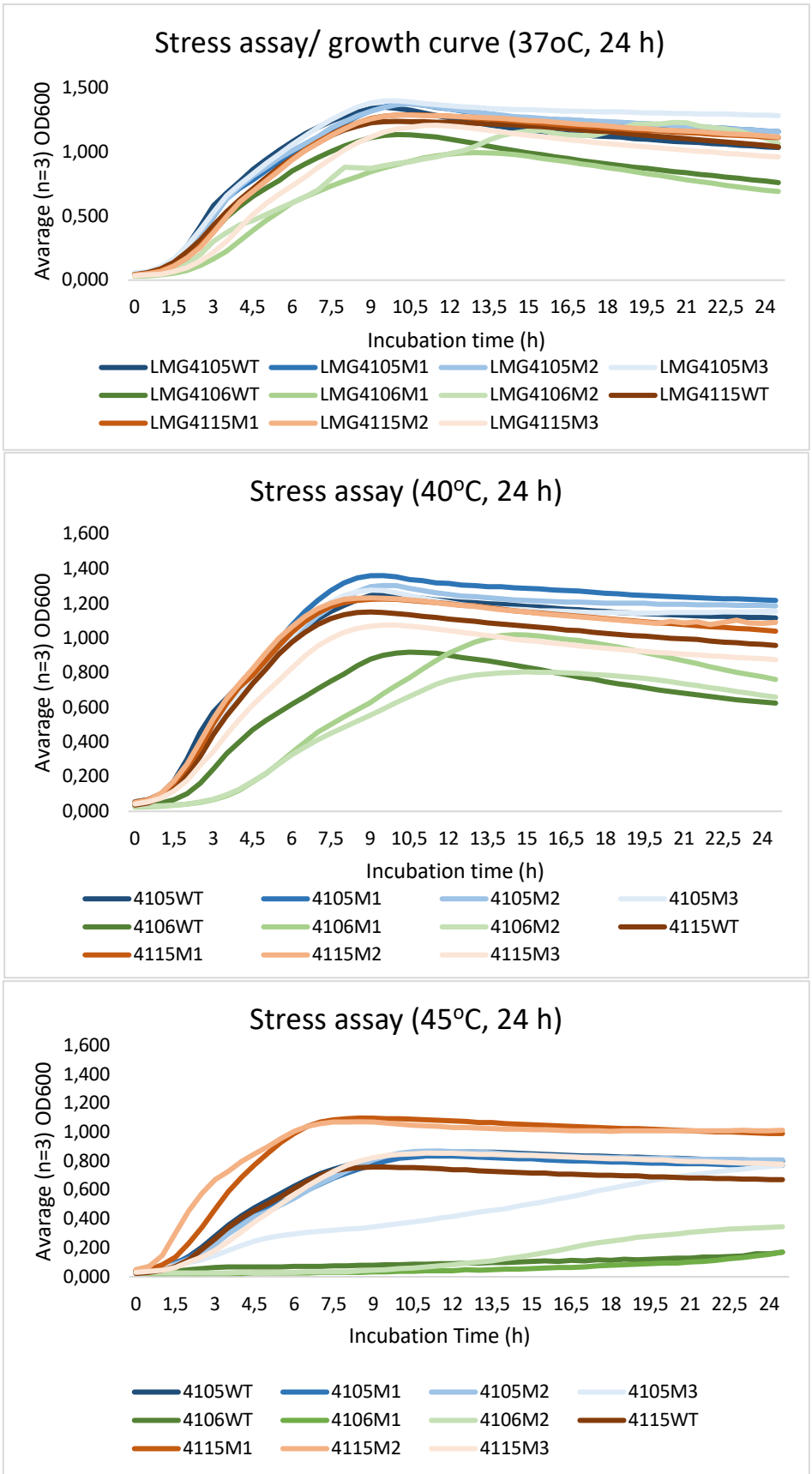
A7.1: MSA of transformed *L. plantarum* with *S. aureus* RseP



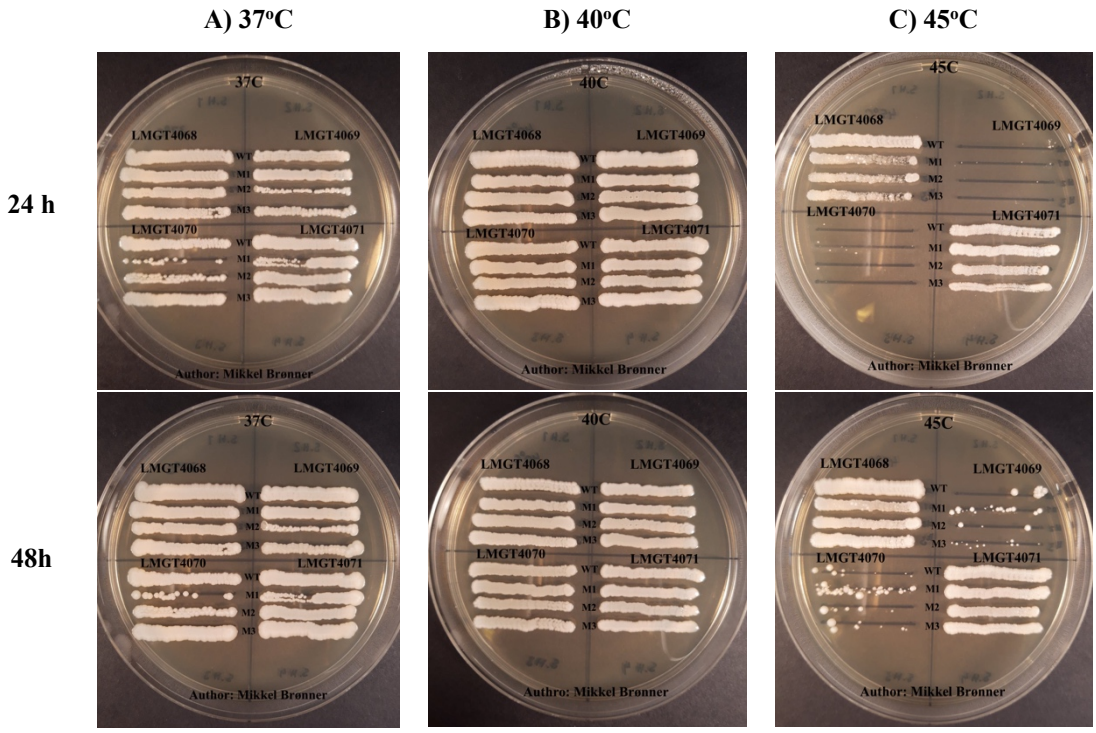




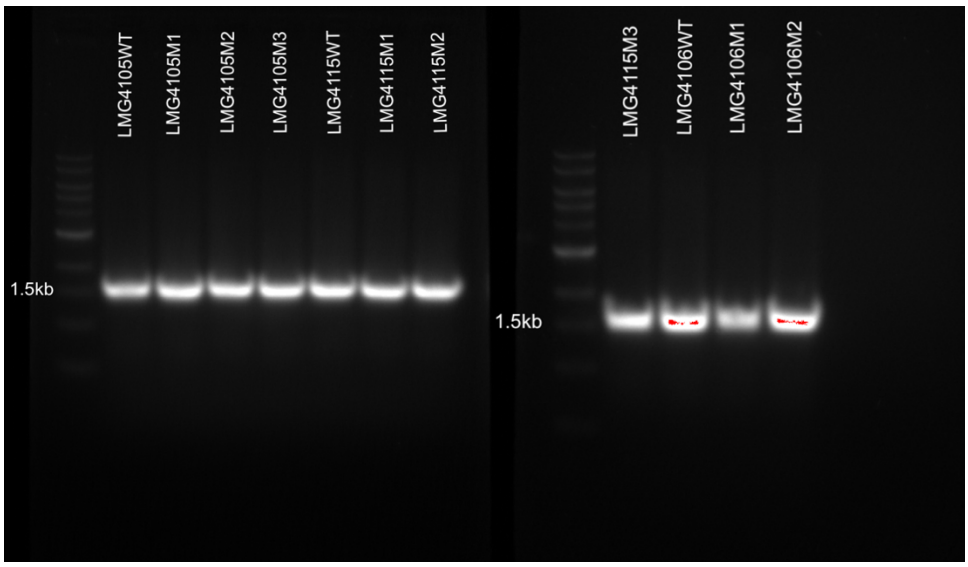
A8: Resistance test. Pictures of BHI plates with inoculated mutants and corresponding WT (bottom lines) in horizontal lines. Left: LMG4105; middle: LMG4115 and right: LMG4106. Bacteriocins (1 mg/ml) are streaked in vertical lines.



A9: Stress assays of mutants and WTs in liquid media. Upper: growth curve at 37ºC; middle: stress treatment at 40ºC; bottom: stress treatment at 45ºC.

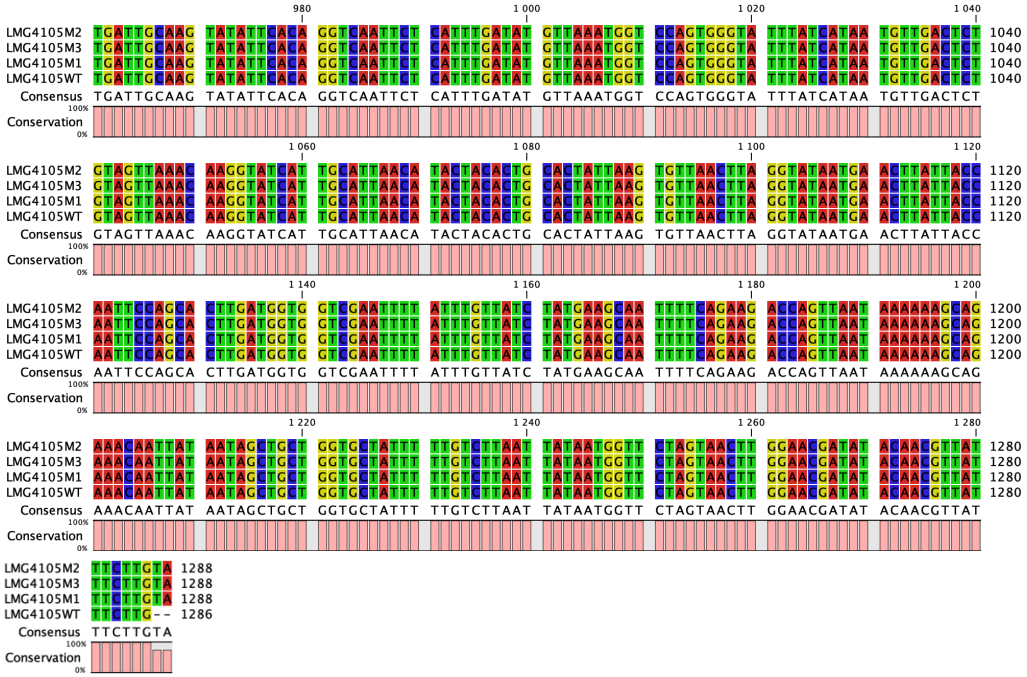


A10: Stress assay on solid media for India strains (data obtained from Mikkel Brønner). Isolated mutants (M1-3) and wild types (WT) were treated for 24 hours (top picture) and 48 hours (bottom picture) with A) 37°C, B) 40°C, C) 45°C on solid media. Treatment at 37°C was used as a positive control for bacterial growth.

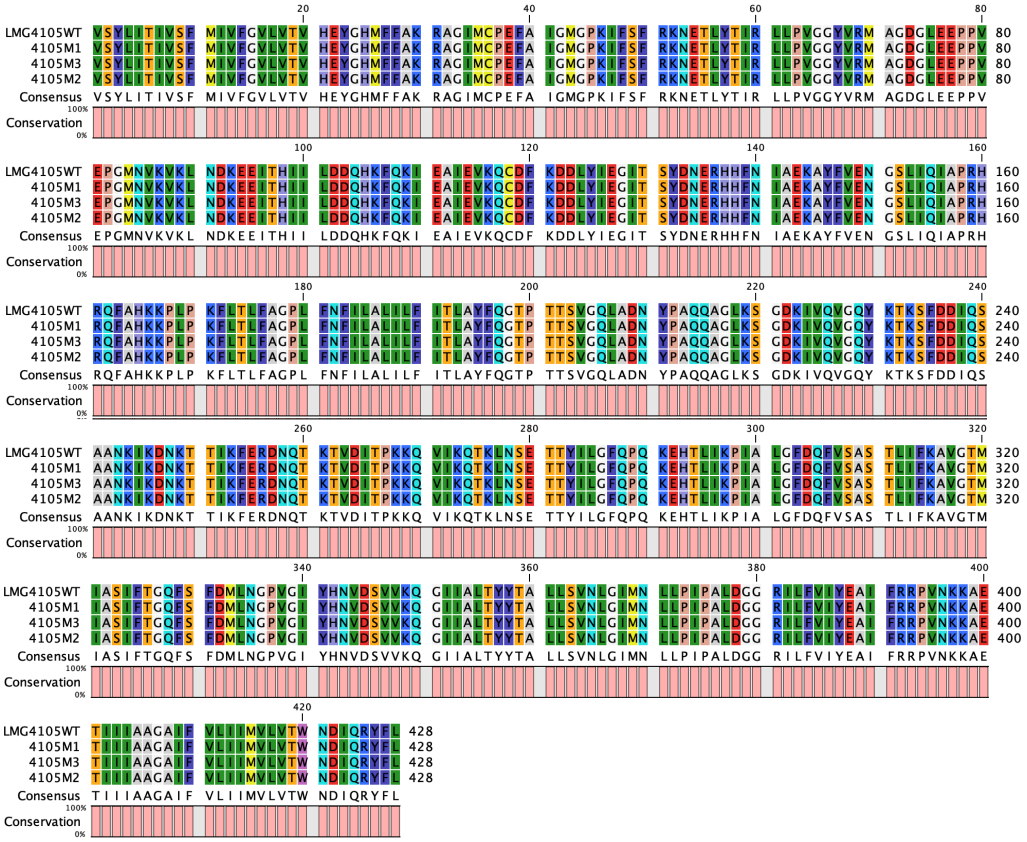


A11: Gel electrophoresis of amplified *rseP* from resistant mutant wild types. Predicted size of the PCR product is ~ 1511 bp.

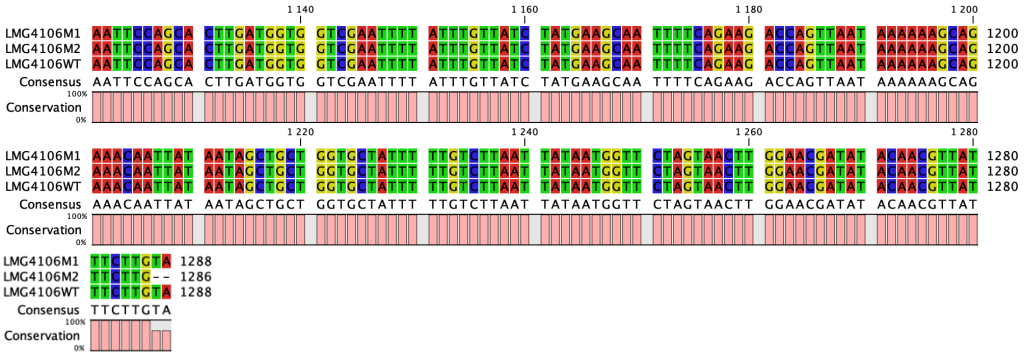
A12: MSA of *S. haemolyticus* LMG4105 *rseP*



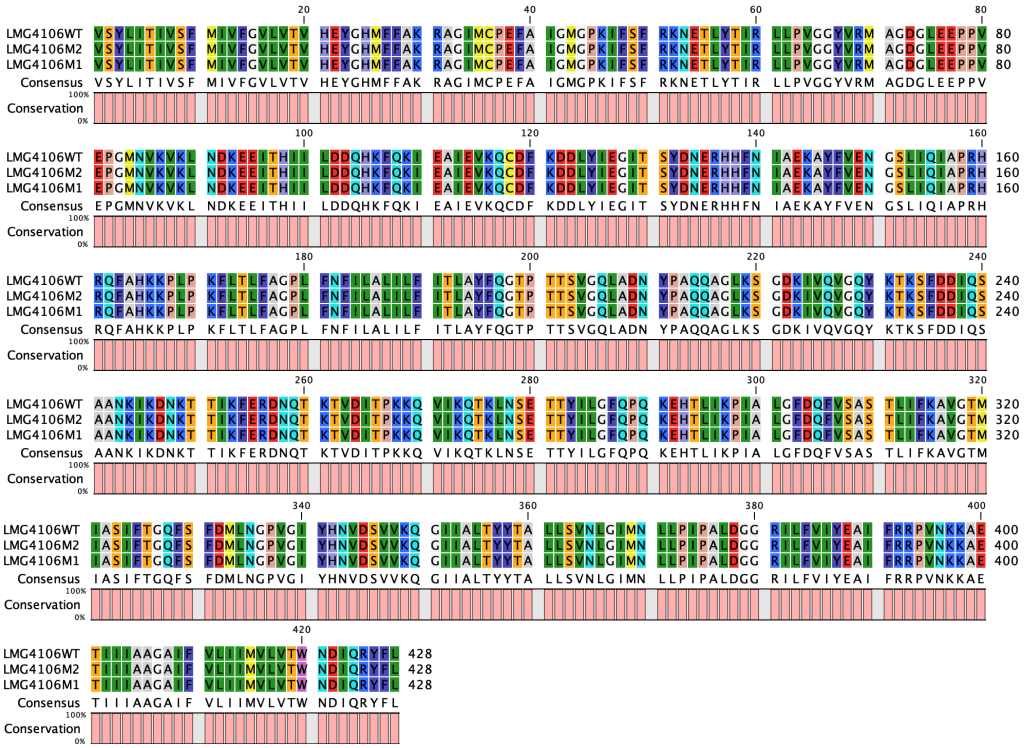
A12.1: MSA of *S. haemolyticus* LMG4105 RseP



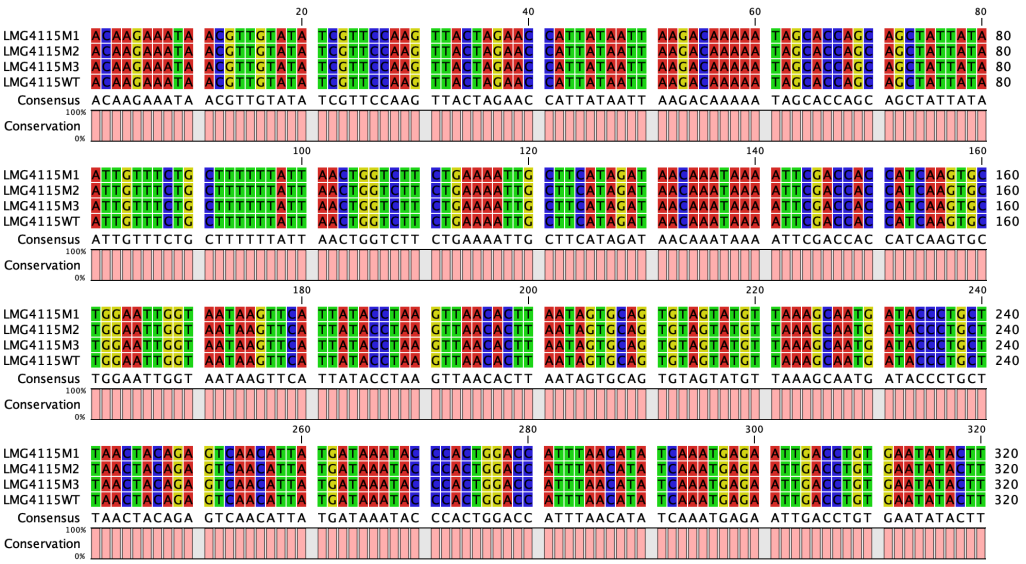
A13: MSA of *S. haemolyticus* LMG4106 *rseP*

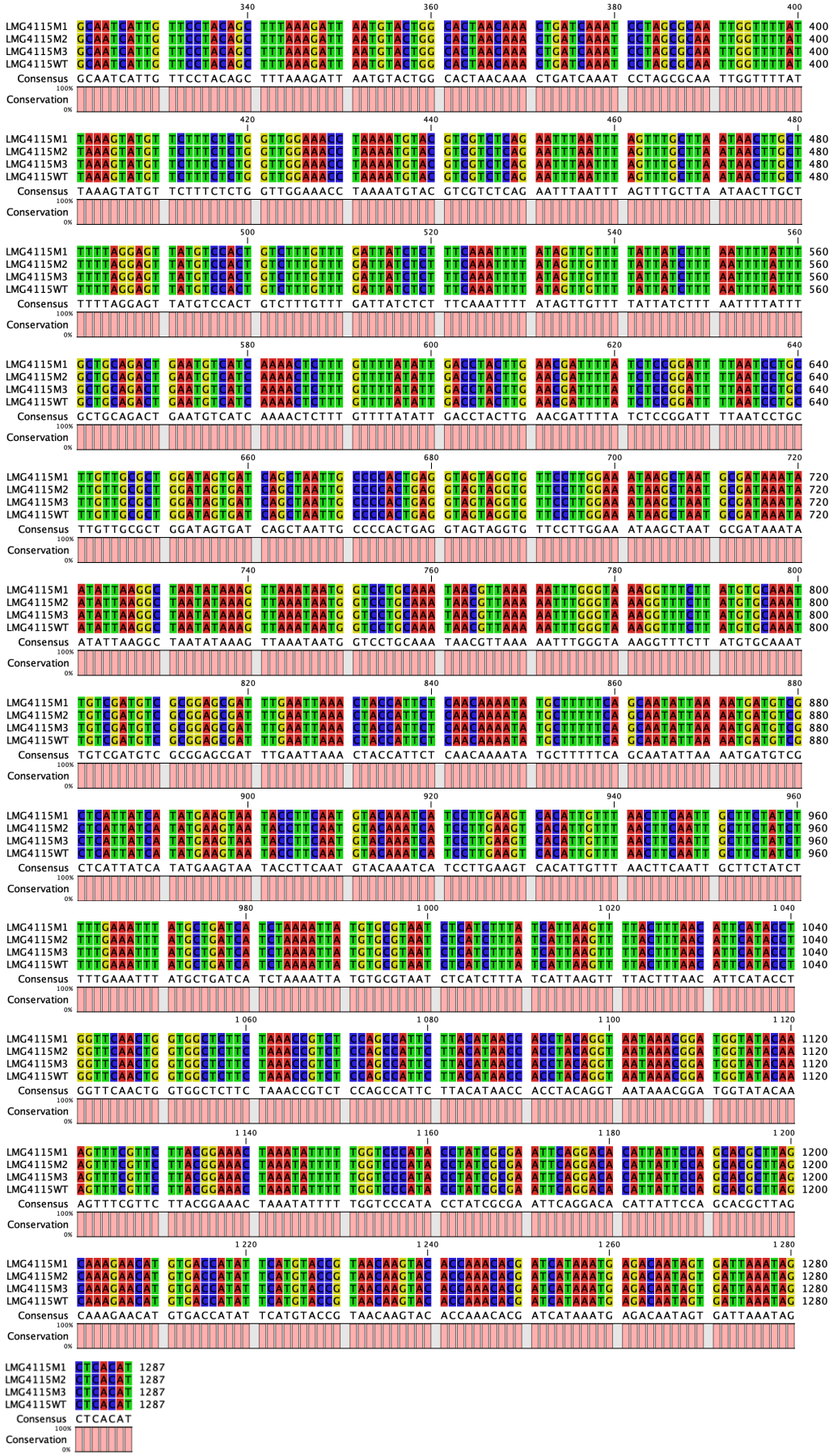


A13.1: MSA of *S. haemolyticus* LMG4106 RseP

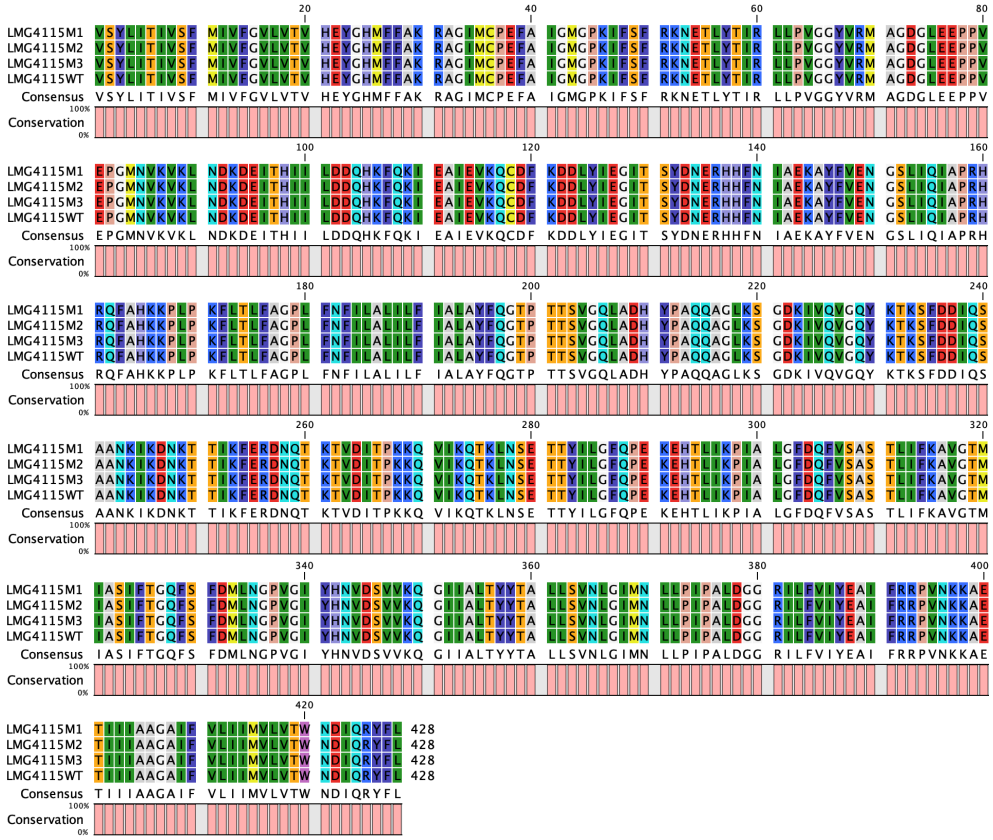


A14: MSA of *S. haemolyticus* LMG4115 *rseP*

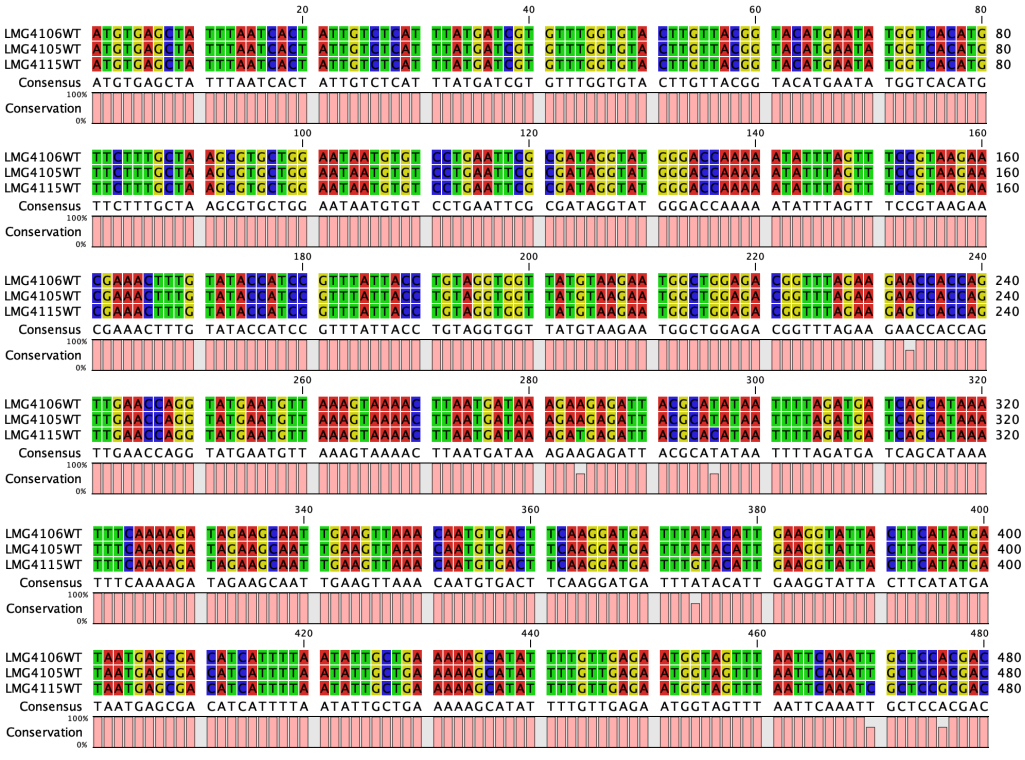



LMG4115M1
LMG4115M2
LMG4115M3
LMG4115WT
Consensus
Conservation
100%
0%

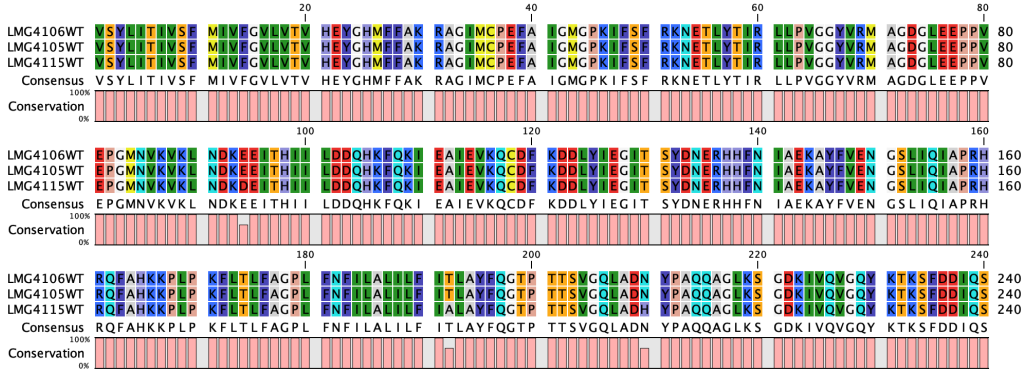
A14.1: MSA of *S. haemolyticus* LMG4115 RseP

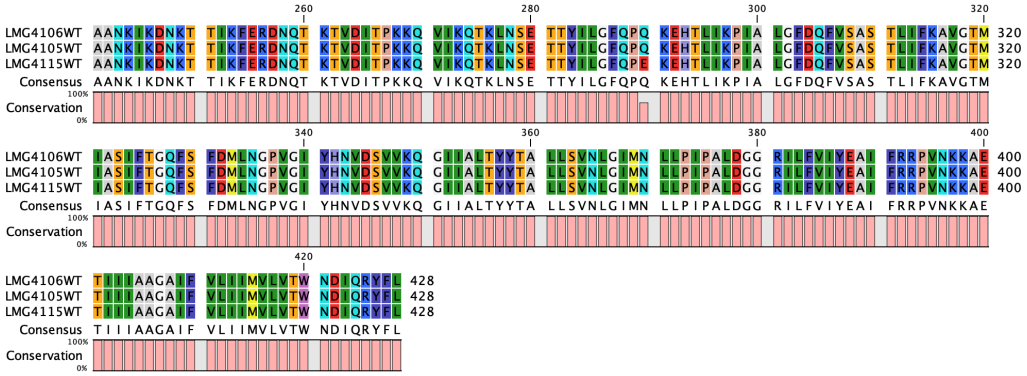


A15: MSA of WTs *S. haemolyticus rseP*

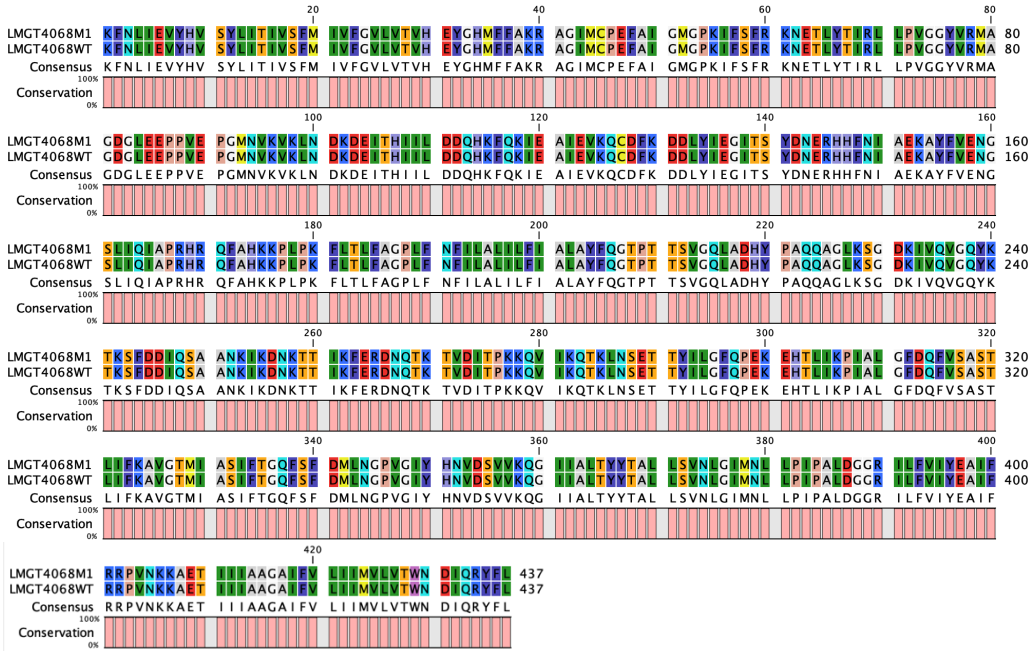


A15.1: MSA of WTs *S. haemolyticus* RseP





A16: PA of *S. haemolyticus* LMG4068 RseP



A17: WGS annotation for variant detection of *S. haemolyticus* LMG4105M1 using Snippy algorithm

CHROM	POS	TYPE	REF	ALT	EVIDENCE	FTYPE	STRAND	NT_POS	AA_POS	EFFECT	LOCUS_TAG	GENE	PRODUCT
IGLBMNHE_3	72515	snp	T	G	G:11 T:0	CDS	+	95/288	32/95	missense_variant c.95T>G p.Leu32Trp	IGLBMNHE_00367		hypothetical protein
IGLBMNHE_5	23964	snp	G	A	A:19 G:0	CDS	+	142/180	48/59	missense_variant c.142G>A p.Val48Met	IGLBMNHE_00534		hypothetical protein
IGLBMNHE_8	48722	snp	G	A	A:24 G:0	CDS	+	152/864	51/287	missense_variant c.152G>A p.Arg51Lys	IGLBMNHE_00779		hypothetical protein
IGLBMNHE_10	10815	snp	T	A	A:28 T:0	CDS	+	506/732	169/243	missense_variant c.506T>A p.Leu169His	IGLBMNHE_00883		metallophosphoesterase family protein
IGLBMNHE_11	60381	snp	C	T	T:32 C:0								
IGLBMNHE_15	22	snp	G	A	A:10 G:0								
IGLBMNHE_15	45	snp	G	A	A:15 G:0								
IGLBMNHE_21	15657	snp	C	T	T:14 C:0	CDS	-	117/171	39/56	synonymous_variant c.117G>A p.Leu39Leu	IGLBMNHE_01470		hypothetical protein
IGLBMNHE_23	27588	snp	C	T	T:18 C:0	CDS	+	248/999	83/332	missense_variant c.248C>T p.Ser83Leu	IGLBMNHE_01561		HAMP domain-containing histidine kinase
IGLBMNHE_25	22108	snp	C	T	T:18 C:0	CDS	+	30/756	10/251	synonymous_variant c.30C>T p.Thr10Thr	IGLBMNHE_01633		hypothetical protein
IGLBMNHE_25	26137	snp	T	C	C:16 T:0	CDS	-	592/2715	198/904	missense_variant c.592A>G p.Asn198Asp	IGLBMNHE_01635		hypothetical protein
IGLBMNHE_30	992	del	TA	T	T:17 TA:0	CDS	-	855/1224	285/407	frameshift_variant c.855delT p.Phe285fs	IGLBMNHE_01777		ABC transporter permease
IGLBMNHE_30	22623	complex	ATTTT	TTTTG	TTTTG:20 ATTTT:0	CDS	-	824/1455	274/484	missense_variant c.820_824delAAAATinsCAAAA p.LysMet274GlnLys	IGLBMNHE_01799		ABC transporter substrate-binding protein/permease
IGLBMNHE_31	23456	snp	C	T	T:21 C:0	CDS	+	319/339	107/112	missense_variant c.319C>T p.His107Tyr	IGLBMNHE_01849		hypothetical protein
IGLBMNHE_32	1103	snp	C	T	T:17 C:0	CDS	+	770/1053	257/350	missense_variant c.770C>T p.Thr257Ile	IGLBMNHE_01854		DNA internalization-related competence protein ComEC/Rec2
IGLBMNHE_32	26025	snp	G	A	A:11 G:0	CDS	+	1220/1806	407/601	missense_variant c.1220G>A p.Arg407Lys	IGLBMNHE_01880		DNA primase
IGLBMNHE_38	20579	snp	G	A	A:23 G:0	CDS	-	153/564	51/187	synonymous_variant c.153C>T p.Cys51Cys	IGLBMNHE_02019		hypothetical protein
IGLBMNHE_44	4868	snp	C	T	T:10 C:0								
IGLBMNHE_45	15538	snp	C	T	T:13 C:0	CDS	-	7/99	3/32	missense_variant c.7G>A p.Gly3Ser	IGLBMNHE_02132	blaI_1	penicillinase repressor BlaI
IGLBMNHE_55	7617	snp	T	G	G:13 T:0	CDS	-	174/192	58/63	synonymous_variant c.174A>C p.Arg58Arg	IGLBMNHE_02268		hypothetical protein
IGLBMNHE_85	558	snp	T	A	A:13 T:1								
IGLBMNHE_89	1336	ins	G	GTA	GTA:14 G:0								
IGLBMNHE_92	1451	snp	G	A	A:28 G:0								
IGLBMNHE_157	382	snp	T	C	C:17 T:0								

A18: WGS annotation for variant detection of *S. haemolyticus* LMG4105M2 using Snippy algorithm

CHROM	POS	TYPE	REF	ALT	EVIDENCE	FTYPE	STRAND	NT_POS	AA_POS	EFFECT	LOCUS_TAG	GENE	PRODUCT
IGLBMNHE_3	65456	snp	T	A	A:11 T:0								
IGLBMNHE_3	72515	snp	T	G	G:15 T:0	CDS	+	95/288	32/95	missense_variant c.95T>G p.Leu32Trp	IGLBMNHE_00367		hypothetical protein
IGLBMNHE_5	23964	snp	G	A	A:15 G:0	CDS	+	142/180	48/59	missense_variant c.142G>A p.Val48Met	IGLBMNHE_00534		hypothetical protein
IGLBMNHE_6	64225	snp	A	C	C:22 A:0								
IGLBMNHE_6	64322	snp	A	C	C:16 A:0								
IGLBMNHE_8	48722	snp	G	A	A:23 G:0	CDS	+	152/864	51/287	missense_variant c.152G>A p.Arg51Lys	IGLBMNHE_00779		hypothetical protein
IGLBMNHE_10	10815	snp	T	A	A:21 T:0	CDS	+	506/732	169/243	missense_variant c.506T>A p.Leu169His	IGLBMNHE_00883		metallophosphoesterase family protein
IGLBMNHE_11	60381	snp	C	T	T:28 C:0								
IGLBMNHE_15	22	snp	G	A	A:14 G:0								
IGLBMNHE_15	45	snp	G	A	A:25 G:0								
IGLBMNHE_20	34007	snp	A	T	T:12 A:0	CDS	+	57/306	19/101	missense_variant c.57A>T p.Leu19Phe	IGLBMNHE_01443		hypothetical protein
IGLBMNHE_21	15657	snp	C	T	T:20 C:0	CDS	-	117/171	39/56	synonymous_variant c.117G>A p.Leu39Leu	IGLBMNHE_01470		hypothetical protein
IGLBMNHE_23	27588	snp	C	T	T:25 C:0	CDS	+	248/999	83/332	missense_variant c.248C>T p.Ser83Leu	IGLBMNHE_01561		HAMP domain-containing histidine kinase
IGLBMNHE_25	22108	snp	C	T	T:21 C:0	CDS	+	30/756	10/251	synonymous_variant c.30C>T p.Thr10Thr	IGLBMNHE_01633		hypothetical protein
IGLBMNHE_25	26137	snp	T	C	C:24 T:0	CDS	-	592/2715	198/904	missense_variant c.592A>G p.Asn198Asp	IGLBMNHE_01635		hypothetical protein
IGLBMNHE_29	27035	snp	C	T	T:15 C:0								
IGLBMNHE_30	992	del	TA	T	T:36 TA:0	CDS	-	855/1224	285/407	frameshift_variant c.855delT p.Phe285fs	IGLBMNHE_01777		ABC transporter permease
IGLBMNHE_30	22623	complex	ATTTT	TTTTG	TTTTG:17 ATTTT:0	CDS	-	824/1455	274/484	missense_variant c.820_824delAAAATinsCAAAA p.LysMet274GlnLys	IGLBMNHE_01799		ABC transporter substrate-binding protein/permease
IGLBMNHE_31	23456	snp	C	T	T:19 C:0	CDS	+	319/339	107/112	missense_variant c.319C>T p.His107Tyr	IGLBMNHE_01849		hypothetical protein
IGLBMNHE_32	26025	snp	G	A	A:21 G:0	CDS	+	1220/1806	407/601	missense_variant c.1220G>A p.Arg407Lys	IGLBMNHE_01880		DNA primase
IGLBMNHE_38	20579	snp	G	A	A:29 G:0	CDS	-	153/564	51/187	synonymous_variant c.153C>T p.Cys51Cys	IGLBMNHE_02019		hypothetical protein
IGLBMNHE_44	4868	snp	C	T	T:16 C:0								
IGLBMNHE_46	214	snp	G	A	A:14 G:0								
IGLBMNHE_55	7617	snp	T	G	G:13 T:0	CDS	-	174/192	58/63	synonymous_variant c.174A>C p.Arg58Arg	IGLBMNHE_02268		hypothetical protein
IGLBMNHE_89	1336	ins	G	GTA	GTA:15 G:0								
IGLBMNHE_92	1451	snp	G	A	A:57 G:3								
IGLBMNHE_95	1206	snp	T	A	A:11 T:0								
IGLBMNHE_157	382	snp	T	C	C:30 T:0								

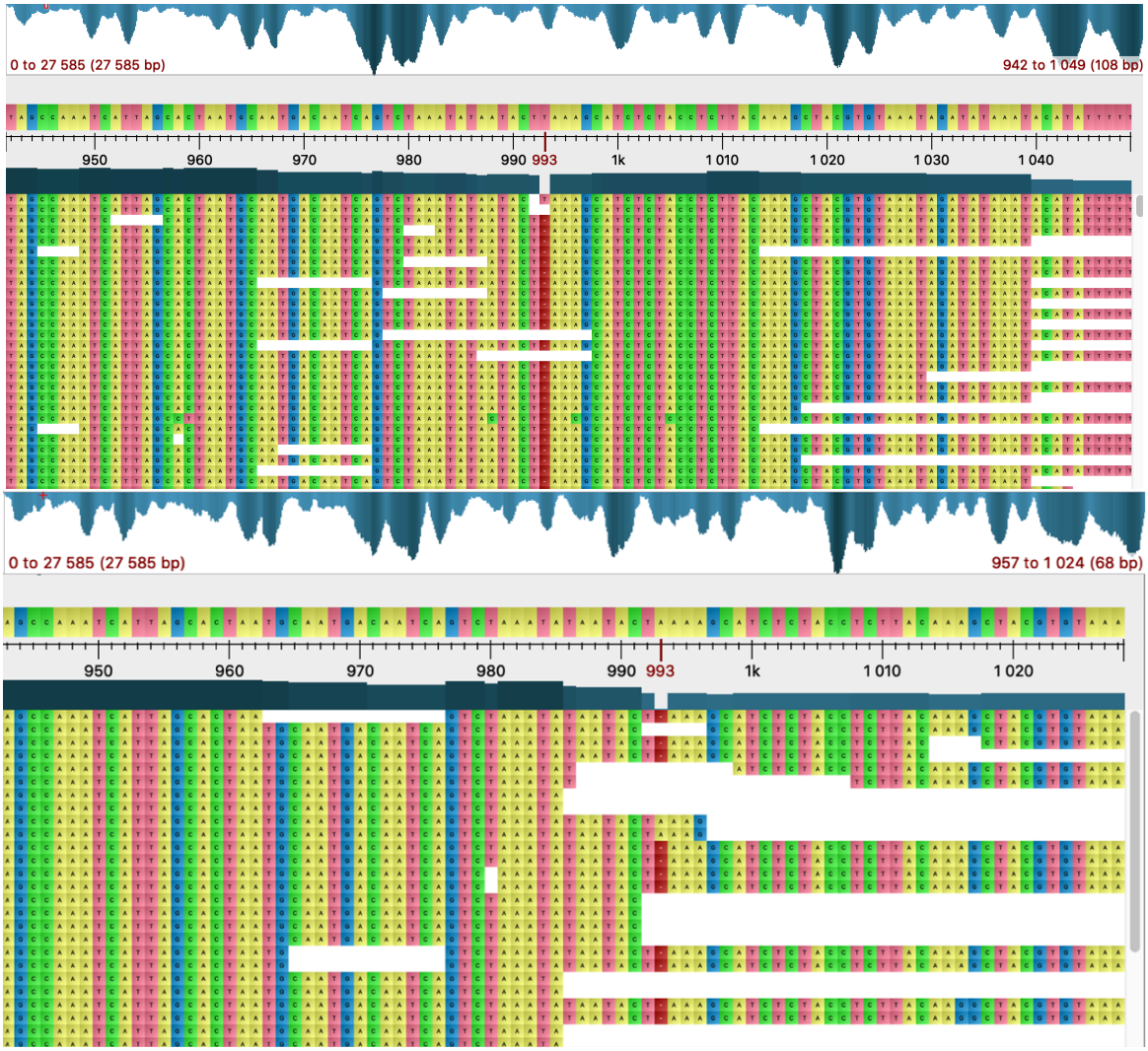
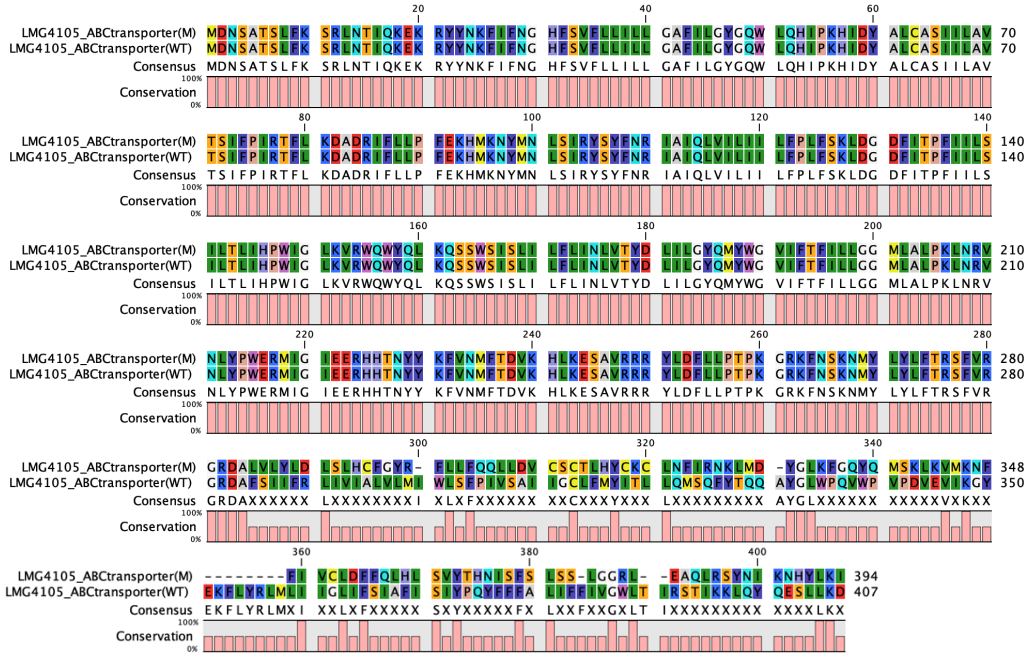


Figure A19. Screenshots of short reads mapping of LMG4105M1 (upper) and LMG4105M2 (bottom) to reference LMG4105WT represented in Unipro UGENE v34. The mutation causing frameshift was found to be in node 30 of reference sequence in both datasets. Coverage proportion is represented on the top of each figures in blue. Both libraries of the paired ends show nucleotide deletion in position 993.

A20: PA of putative LMG4105 ABC transporter permease



A21: WGS annotation for variant detection of *S. haemolyticus* LMG4115M1 using Snippy algorithm

CHROM	POS	TYPE	REF	ALT	EVIDENCE	FTYPE	STRAND	NT_POS	AA_POS	EFFECT	LOCUS_TAG	GENE	PRODUCT
MAPDOLPC_2	65953	snp	C	T	T:73 C:0	CDS	-	347/807	116/268	missense_variant c.347G>A p.Arg116Gln	MAPDOLPC_00150		lysozyme
MAPDOLPC_5	26501	snp	C	T	T:19 C:0	CDS	+	49/192	17/63	synonymous_variant c.49C>T p.Leu17Leu	MAPDOLPC_00290		hypothetical protein
MAPDOLPC_10	2937	snp	C	T	T:11 C:0	CDS	-	1/180	1/59	initiator_codon_variant c.1G>A p.Val1?	MAPDOLPC_00438		hypothetical protein
MAPDOLPC_10	2944	complex	TCCTT	ACCTC	ACCTC:11 TCCTT:0								
MAPDOLPC_10	24508	snp	C	G	G:23 C:0	CDS	+	49/3036	17/1011	missense_variant c.49C>G p.Gln17Glu	MAPDOLPC_00463		SMC family ATPase
MAPDOLPC_12	13175	snp	A	G	G:16 A:0	CDS	+	659/1011	220/336	missense_variant c.659A>G p.Asn220Ser	MAPDOLPC_00503		galactose mutarotase
MAPDOLPC_12	16357	snp	G	A	A:14 G:0								
MAPDOLPC_15	26102	complex	TTATTTT	GTAGTTG	GTAGTTG:43 TTATTTT:0								
MAPDOLPC_16	11183	snp	C	T	T:27 C:0								
MAPDOLPC_19	965	complex	AAAG	TAAA	TAAA:17 AAAG:0								
MAPDOLPC_19	975	snp	A	T	T:18 A:0								
MAPDOLPC_19	980	snp	A	T	T:17 A:0								
MAPDOLPC_19	986	complex	ATAG	CTAT	CTAT:12 ATAG:0								
MAPDOLPC_19	998	complex	GTT	TTA	TTA:12 GTT:0								
MAPDOLPC_19	1007	snp	A	T	T:13 A:0								
MAPDOLPC_19	1119	complex	GGAGAG	TGATAA	TGATAA:13 GGAGAG:0								
MAPDOLPC_19	1134	complex	AA	TC	TC:20 AA:0	CDS	+	5/741	2/246	missense_variant c.5_6delAAinsTC p.Gln2Leu	MAPDOLPC_00666		NAD-dependent protein deacylase
MAPDOLPC_19	1140	snp	G	T	T:20 G:0	CDS	+	11/741	4/246	missense_variant c.11G>T p.Trp4Leu	MAPDOLPC_00666		NAD-dependent protein deacylase
MAPDOLPC_19	1154	snp	A	G	G:23 A:0	CDS	+	25/741	9/246	missense_variant c.25A>G p.Lys9Glu	MAPDOLPC_00666		NAD-dependent protein deacylase
MAPDOLPC_21	14760	snp	A	C	C:10 A:0								
MAPDOLPC_21	17382	snp	T	A	A:25 T:0	CDS	-	18/216	6/71	missense_variant c.18A>T p.Glu6Asp	MAPDOLPC_00740		hypothetical protein
MAPDOLPC_24	20481	snp	G	A	A:29 G:0								
MAPDOLPC_31	22127	snp	A	T	T:15 A:0								
MAPDOLPC_33	16098	snp	C	A	A:16 C:0								
MAPDOLPC_37	6898	snp	A	T	T:32 A:0								
MAPDOLPC_37	14850	snp	C	T	T:64 C:0	CDS	-	1253/1761	418/586	missense_variant c.1253G>A p.Cys418Tyr	MAPDOLPC_01082		siderophore synthetase
MAPDOLPC_56	8759	snp	G	A	A:13 G:0								
MAPDOLPC_61	14726	snp	G	A	A:15 G:0								
MAPDOLPC_63	3770	snp	T	A	A:30 T:0								
MAPDOLPC_63	3776	snp	T	A	A:35 T:0								
MAPDOLPC_63	6368	snp	C	T	T:32 C:0	CDS	-	1415/1428	472/475	missense_variant c.1415G>A p.Gly472Asp	MAPDOLPC_01484		trypsin-like serine protease
MAPDOLPC_63	6384	snp	T	G	G:33 T:0	CDS	-	1399/1428	467/475	missense_variant c.1399A>C p.Ser467Arg	MAPDOLPC_01484		trypsin-like serine protease
MAPDOLPC_64	3045	snp	G	A	A:20 G:0								
MAPDOLPC_69	13199	snp	G	A	A:13 G:0								
MAPDOLPC_69	13238	ins	T	TA	TA:14 T:0								
MAPDOLPC_73	2807	snp	T	A	A:34 T:0	CDS	+	282/762	94/253	synonymous_variant c.282T>A p.Thr94Thr	MAPDOLPC_01604		VOC family protein
MAPDOLPC_76	7767	snp	A	T	T:15 A:0	CDS	-	4/795	2/264	missense_variant c.4T>A p.Cys2Ser	MAPDOLPC_01648	tagH	teichoic acids export ABC transporter ATP-binding subunit TagH

MAPDOLPC_76	11245	snp	T	C	C:24 T:0								
MAPDOLPC_80	8493	snp	C	T	T:31 C:0								
MAPDOLPC_99	8475	snp	A	T	T:20 A:0								
MAPDOLPC_99	8483	snp	A	T	T:18 A:0								
MAPDOLPC_123	1887	snp	C	A	A:48 C:0	CDS	+	502/1170	168/389	missense_variant c.502C>A p.Gln168Lys	MAPDOLPC_02046		toxic anion resistance protein
MAPDOLPC_124	6434	snp	C	T	T:34 C:0								
MAPDOLPC_125	4980	snp	C	T	T:17 C:0	CDS	+	752/1731	251/576	missense_variant c.752C>T p.Ala251Val	MAPDOLPC_02058		hypothetical protein
MAPDOLPC_147	805	snp	A	C	C:18 A:0								
MAPDOLPC_147	2794	snp	T	G	G:18 T:0	CDS	+	121/573	41/190	missense_variant c.121T>G p.Tyr41Asp	MAPDOLPC_02181		hypothetical protein
MAPDOLPC_156	87	snp	T	A	A:32 T:0	CDS	-	1698/1716	566/571	synonymous_variant c.1698A>T p.Val566Val	MAPDOLPC_02221	ptsP	phosphoenolpyruvate--protein phosphotransferase
MAPDOLPC_158	1516	snp	T	A	A:31 T:0								
MAPDOLPC_163	48	snp	G	T	T:15 G:0								
MAPDOLPC_169	2632	snp	A	C	C:12 A:0								
MAPDOLPC_190	2940	snp	C	T	T:16 C:0								
MAPDOLPC_215	103	snp	G	A	A:21 G:0								
MAPDOLPC_230	539	snp	T	G	G:16 T:0								
MAPDOLPC_230	539	snp	T	G	G:16 T:0								
MAPDOLPC_231	1472	ins	C	CA	CA:11 C:0								
MAPDOLPC_232	1255	snp	T	G	G:29 T:0	CDS	-	120/399	40/132	synonymous_variant c.120A>C p.Pro40Pro	MAPDOLPC_02440	comEA	ComE operon protein 1
MAPDOLPC_232	1303	snp	G	T	T:26 G:0	CDS	-	72/399	24/132	missense_variant c.72C>A p.His24Gln	MAPDOLPC_02440	comEA	ComE operon protein 1
MAPDOLPC_232	1348	complex	GTCATTG	ATCTTTT	ATCTTTT:23 GTCATTG:0	CDS	-	27/399	7/132	missense_variant c.21_27delCAATGACinsAAAAGAT p.Asn8Lys	MAPDOLPC_02440	comEA	ComE operon protein 1
MAPDOLPC_232	1414	snp	G	T	T:23 G:0								
MAPDOLPC_232	1426	snp	A	T	T:20 A:0								
MAPDOLPC_254	751	snp	C	T	T:11 C:0								
MAPDOLPC_279	137	snp	C	A	A:14 C:0	CDS	-	345/426	115/141	missense_variant c.345G>T p.Glu115Asp	MAPDOLPC_02474		hypothetical protein
MAPDOLPC_279	212	snp	T	C	C:15 T:0	CDS	-	270/426	90/141	synonymous_variant c.270A>G p.Ala90Ala	MAPDOLPC_02474		hypothetical protein
MAPDOLPC_283	126	snp	A	T	T:174 A:0								
MAPDOLPC_283	185	snp	T	G	G:220 T:0								
MAPDOLPC_283	238	snp	G	A	A:219 G:0								
MAPDOLPC_283	325	snp	T	G	G:219 T:0								
MAPDOLPC_284	141	snp	G	T	T:34 G:1								
MAPDOLPC_284	213	snp	G	T	T:46 G:6								
MAPDOLPC_284	291	snp	G	T	T:45 G:3								
MAPDOLPC_284	309	snp	G	T	T:35 G:3								
MAPDOLPC_284	333	snp	G	T	T:31 G:2								
MAPDOLPC_284	338	snp	A	C	C:36 A:0								
MAPDOLPC_284	351	snp	T	G	G:30 T:0								
MAPDOLPC_286	104	snp	T	G	G:26 T:1								
MAPDOLPC_286	140	snp	T	C	C:30 T:0								
MAPDOLPC_286	248	snp	T	G	G:26 T:1								
MAPDOLPC_286	287	snp	G	A	A:41 G:0								
MAPDOLPC_286	299	snp	A	G	G:35 A:1								
MAPDOLPC_286	338	complex	GGC	TGA	TGA:19 GGC:0								
MAPDOLPC_286	353	snp	A	G	G:15 A:0								
MAPDOLPC_286	358	complex	CATCT	AATCA	AATCA:11 CATCT:1								
MAPDOLPC_286	367	snp	A	C	C:14 A:0								
MAPDOLPC_286	371	snp	A	G	G:11 A:1								
MAPDOLPC_286	375	snp	A	G	G:13 A:0								

A22: WGS annotation for variant detection of *S. haemolyticus* LMG4115M2 using Snippy algorithm

CHROM	POS	TYPE	REF	ALT	EVIDENCE	FTYPE	STRAND	NT_POS	AA_POS	EFFECT	LOCUS_TAG	GENE	PRODUCT
MAPDOLPC_2	65953	snp	C	T	T:43 C:0	CDS	-	347/807	116/268	missense_variant c.347G>A p.Arg116Gln	MAPDOLPC_00150		lysozyme
MAPDOLPC_3	5644	snp	A	T	T:45 A:0								
MAPDOLPC_5	26501	snp	C	T	T:30 C:0	CDS	+	49/192	17/63	synonymous_variant c.49C>T p.Leu17Leu	MAPDOLPC_00290		hypothetical protein
MAPDOLPC_8	21436	snp	G	A	A:12 G:0								
MAPDOLPC_10	2937	snp	C	T	T:18 C:0	CDS	-	1/180	1/59	initiator_codon_variant c.1G>A p.Val1?	MAPDOLPC_00438		hypothetical protein
MAPDOLPC_10	2944	complex	TCCTT	ACCTC	ACCTC:17 TCCTT:0								
MAPDOLPC_10	24508	snp	C	G	G:36 C:0	CDS	+	49/3036	17/1011	missense_variant c.49C>G p.Gln17Glu	MAPDOLPC_00463		SMC family ATPase
MAPDOLPC_12	13175	snp	A	G	G:30 A:0	CDS	+	659/1011	220/336	missense_variant c.659A>G p.Asn220Ser	MAPDOLPC_00503		galactose mutarotase
MAPDOLPC_12	16357	snp	G	A	A:24 G:0								
MAPDOLPC_15	26102	complex	TTATTTT	GTAGTTG	GTAGTTG:28 TTATTTT:0								
MAPDOLPC_16	11183	snp	C	T	T:31 C:0								
MAPDOLPC_19	965	complex	AAAG	TAAA	TAAA:11 AAAG:0								
MAPDOLPC_19	975	snp	A	T	T:13 A:0								
MAPDOLPC_19	980	snp	A	T	T:13 A:0								
MAPDOLPC_19	986	complex	ATAG	CTAT	CTAT:13 ATAG:0								
MAPDOLPC_19	998	complex	GTT	TTA	TTA:13 GTT:0								
MAPDOLPC_19	1007	snp	A	T	T:10 A:0								
MAPDOLPC_19	1022	snp	T	C	C:10 T:0								
MAPDOLPC_19	1030	snp	A	G	G:11 A:0								
MAPDOLPC_19	1036	snp	A	T	T:11 A:0								
MAPDOLPC_19	1053	snp	A	T	T:11 A:0								
MAPDOLPC_19	1070	complex	AGC	TGT	TGT:12 AGC:0								
MAPDOLPC_19	1078	snp	A	T	T:13 A:0								
MAPDOLPC_19	1087	snp	T	A	A:13 T:0								
MAPDOLPC_19	1092	complex	ATA	TTT	TTT:11 ATA:0								
MAPDOLPC_19	1104	snp	G	A	A:14 G:0								
MAPDOLPC_19	1119	complex	GGAGAG	TGATAA	TGATAA:16 GGAGAG:0								
MAPDOLPC_19	1134	complex	AA	TC	TC:20 AA:0	CDS	+	5/741	2/246	missense_variant c.5_6delAAinsTC p.Gln2Leu	MAPDOLPC_00666		NAD-dependent protein deacylase

MAPDOLPC_19	1140	snp	G	T	T:20 G:0	CDS	+	11/741	4/246	missense_variant c.11G>T p.Trp4Leu	MAPDOLPC_00666		NAD-dependent protein deacylase
MAPDOLPC_19	1154	snp	A	G	G:23 A:0	CDS	+	25/741	9/246	missense_variant c.25A>G p.Lys9Glu	MAPDOLPC_00666		NAD-dependent protein deacylase
MAPDOLPC_21	14760	snp	A	C	C:30 A:0								
MAPDOLPC_21	17382	snp	T	A	A:48 T:0	CDS	-	18/216	6/71	missense_variant c.18A>T p.Glu6Asp	MAPDOLPC_00740		hypothetical protein
MAPDOLPC_24	20481	snp	G	A	A:48 G:0								
MAPDOLPC_31	22127	snp	A	T	T:33 A:0								
MAPDOLPC_33	16098	snp	C	A	A:26 C:0								
MAPDOLPC_37	6898	snp	A	T	T:34 A:0								
MAPDOLPC_37	14850	snp	C	T	T:74 C:0	CDS	-	1253/1761	418/586	missense_variant c.1253G>A p.Cys418Tyr	MAPDOLPC_01082		siderophore synthetase
MAPDOLPC_56	8759	snp	G	A	A:40 G:0								
MAPDOLPC_57	13800	snp	C	T	T:10 C:0								
MAPDOLPC_61	14726	snp	G	A	A:10 G:0								
MAPDOLPC_62	14100	snp	T	G	G:20 T:0								
MAPDOLPC_63	3770	snp	T	A	A:20 T:0								
MAPDOLPC_63	3776	snp	T	A	A:21 T:0								
MAPDOLPC_63	6368	snp	C	T	T:54 C:0	CDS	-	1415/1428	472/475	missense_variant c.1415G>A p.Gly472Asp	MAPDOLPC_01484		trypsin-like serine protease
MAPDOLPC_63	6384	snp	T	G	G:56 T:0	CDS	-	1399/1428	467/475	missense_variant c.1399A>C p.Ser467Arg	MAPDOLPC_01484		trypsin-like serine protease
MAPDOLPC_64	3045	snp	G	A	A:47 G:0								
MAPDOLPC_69	13199	snp	G	A	A:17 G:0								
MAPDOLPC_69	13238	ins	T	TA	TA:14 T:0								
MAPDOLPC_73	99	snp	A	T	T:15 A:0								
MAPDOLPC_73	2807	snp	T	A	A:56 T:0	CDS	+	282/762	94/253	synonymous_variant c.282T>A p.Thr94Thr	MAPDOLPC_01604		VOC family protein
MAPDOLPC_76	7767	snp	A	T	T:17 A:0	CDS	-	4/795	2/264	missense_variant c.4T>A p.Cys2Ser	MAPDOLPC_01648	tagH	teichoic acids export ABC transporter ATP-binding subunit TagH
MAPDOLPC_76	11245	snp	T	C	C:38 T:0								
MAPDOLPC_80	63	snp	G	A	A:12 G:0								
MAPDOLPC_80	8493	snp	C	T	T:48 C:0								
MAPDOLPC_87	8799	snp	G	A	A:25 G:0	CDS	+	952/1869	318/622	missense_variant c.952G>A p.Glu318Lys	MAPDOLPC_01761		hypothetical protein
MAPDOLPC_99	8475	snp	A	T	T:19 A:0								
MAPDOLPC_99	8483	snp	A	T	T:19 A:0								
MAPDOLPC_101	8447	snp	C	A	A:10 C:0								
MAPDOLPC_105	7248	snp	C	A	A:23 C:0	CDS	-	579/588	193/195	missense_variant c.579G>T p.Lys193Asn	MAPDOLPC_01920		YihA family ribosome biogenesis GTP-binding protein
MAPDOLPC_105	7260	snp	G	A	A:23 G:0	CDS	-	567/588	189/195	synonymous_variant c.567C>T p.Asp189Asp	MAPDOLPC_01920		YihA family ribosome biogenesis GTP-binding protein
MAPDOLPC_106	8022	snp	G	C	C:20 G:0								
MAPDOLPC_123	1887	snp	C	A	A:49 C:0	CDS	+	502/1170	168/389	missense_variant c.502C>A p.Gln168Lys	MAPDOLPC_02046		toxic anion resistance protein
MAPDOLPC_124	6434	snp	C	T	T:27 C:0								
MAPDOLPC_125	4980	snp	C	T	T:38 C:0	CDS	+	752/1731	251/576	missense_variant c.752C>T p.Ala251Val	MAPDOLPC_02058		hypothetical protein
MAPDOLPC_129	51	snp	T	G	G:10 T:0								
MAPDOLPC_143	3693	snp	G	A	A:31 G:0								
MAPDOLPC_147	805	snp	A	C	C:27 A:0								
MAPDOLPC_147	2794	snp	T	G	G:38 T:0	CDS	+	121/573	41/190	missense_variant c.121T>G p.Tyr41Asp	MAPDOLPC_02181		hypothetical protein
MAPDOLPC_156	48	snp	T	C	C:10 T:0								
MAPDOLPC_156	87	snp	T	A	A:19 T:0	CDS	-	1698/1716	566/571	synonymous_variant c.1698A>T p.Val566Val	MAPDOLPC_02221	ptsP	phosphoenolpyruvate--protein phosphotransferase
MAPDOLPC_158	1516	snp	T	A	A:41 T:0								
MAPDOLPC_158	1630	snp	G	A	A:13 G:0								
MAPDOLPC_158	1642	snp	G	T	T:11 G:0								
MAPDOLPC_158	4239	snp	C	T	T:22 C:0								
MAPDOLPC_166	1297	snp	C	T	T:13 C:0	CDS	-	443/1074	148/357	missense_variant c.443G>A p.Arg148Lys	MAPDOLPC_02261		hypothetical protein
MAPDOLPC_169	2632	snp	A	C	C:23 A:0								
MAPDOLPC_169	3728	snp	G	T	T:24 G:0								
MAPDOLPC_190	2940	snp	C	T	T:11 C:0								
MAPDOLPC_213	83	snp	T	G	G:12 T:0								
MAPDOLPC_230	539	snp	T	G	G:14 T:0								
MAPDOLPC_231	1472	ins	C	CA	CA:21 C:0								
MAPDOLPC_232	1255	snp	T	G	G:42 T:0	CDS	-	120/399	40/132	synonymous_variant c.120A>C p.Pro40Pro	MAPDOLPC_02440	comEA	ComE operon protein 1
MAPDOLPC_232	1303	snp	G	T	T:33 G:0	CDS	-	72/399	24/132	missense_variant c.72C>A p.His24Gln	MAPDOLPC_02440	comEA	ComE operon protein 1
MAPDOLPC_232	1348	complex	GTCATTG	ATCTTTT	ATCTTTT:33 GTCATTG:0	CDS	-	27/399	7/132	missense_variant c.21_27delCAATGACinsAAAAGAT p.Asn8Lys	MAPDOLPC_02440	comEA	ComE operon protein 1
MAPDOLPC_232	1414	snp	G	T	T:26 G:0								
MAPDOLPC_232	1426	snp	A	T	T:26 A:0								
MAPDOLPC_234	493	snp	C	T	T:15 C:0	CDS	-	22/498	8/165	missense_variant c.22G>A p.Gly8Arg	MAPDOLPC_02443		hypothetical protein
MAPDOLPC_240	953	snp	A	G	G:13 A:0								
MAPDOLPC_254	751	snp	C	T	T:18 C:0								
MAPDOLPC_283	126	snp	A	T	T:88 A:0								
MAPDOLPC_283	185	snp	T	G	G:102 T:0								
MAPDOLPC_283	238	snp	G	A	A:101 G:1								
MAPDOLPC_283	325	snp	T	G	G:99 T:0								
MAPDOLPC_284	141	snp	G	T	T:23 G:0								
MAPDOLPC_284	213	snp	G	T	T:26 G:0								
MAPDOLPC_284	291	snp	G	T	T:22 G:1								
MAPDOLPC_284	338	snp	A	C	C:15 A:0								
MAPDOLPC_284	351	snp	T	G	G:13 T:1								
MAPDOLPC_286	104	snp	T	G	G:17 T:2								
MAPDOLPC_286	140	snp	T	C	C:17 T:1								
MAPDOLPC_286	287	snp	G	A	A:21 G:1								
MAPDOLPC_286	338	complex	GGC	TGA	TGA:11 GGC:0								

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