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Mapping the binding sites of the bacteriocin LsbB

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Acknowledgement

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Abstract

LsbB is a class II leaderless bacteriocin targeting only lactococcal cells. It uses the Zn-dependent metallopeptidase RseP (YvjB) as a receptor. RseP has a conserved catalytic site, HExxH, at the N-terminal, which is common for the Zn-dependent metallopeptidases. There is evidence indicating that the binding site of LsbB is located at the C-terminal part of RseP. Another bacteriocin EJ97, related to LsbB but produced by enterococcal strains, also targets the same receptor. This bacteriocin has a broader antimicrobial spectrum, including both lactococcal and enterococcal cells. The aim of this study was to identify the bacteriocin binding sites for LsbB, as well as to examine whether the proteolytic site of RseP is important for the receptor function. In addition, it was of interest to identify which part of RseP is responsible for the binding of LsbB to only lactococcal cells, and not enterococcal cells.

To examine whether the proteolytic site is involved in receptor function, point mutations were created, where the conserved residues were changed to alanine. Heterologous expression was performed, and the altered *rseP* genes were expressed in the heterologous host *Streptococcus pneumoniae*. The results showed that changes in the active sites, especially when all of the active site residues were changed, made the strains a lot more resistant to LsbB than the strain expressing wild type lactococcal *rseP*. To evaluate which part of RseP is responsible for lactococcal cells' specific sensitivity to LsbB, hybrids were created, where parts of the lactococcal RseP were replaced with the corresponding part from the enterococcal protein. Both enterococcal and lactococcal RseP consists of four transmembrane helices, and the hybrids were made so that they contained different combinations of the lactococcal and the enterococcal helices. The sensitivity to LsbB was checked for all of the hybrid RseP, and the results showed that the second and the third helices needed to be lactococcal for the strains to be sensitive to LsbB. The strains where the second and the third helices were not lactococcal became totally resistant. Altogether, it seems like the third transmembrane helix is important for the first binding of LsbB, whilst the second helix, as well as the catalytic site, are important for the formation of the complex that leads to destruction of the cell membrane. The study revealed some interesting results, however the details of the binding and destruction of the cells by LsbB need to be studied further. A thorough understanding of the bacteriocins, their receptors and their mode of action, is important to develop these molecules into useful and safe application.

Sammendrag

LsbB tilhører klasse II lederløse bakteriosiner, som kun angriper laktokokker. Det bruker den zink-avhengige metallopeptidasen RseP (YvjB) som reseptor. RseP har et konservert katalytisk sete, HExxH, på N-terminalen, som er felles for alle zink-avhengige metallopeptidaser. Det er bevis som indikerer at bindingssetet til LsbB er lokalisert på C-terminal enden til RseP. Et annet bakteriocin EJ97, som er beslektet med LsbB, men produsert av enterokker, bruker også RseP som reseptor for å angripe celler. Dette bakteriosinet har et bredere antimikrobielt spektrum, som inkluderer både laktokokker og enterokokker. Målet med denne oppgaven var å identifisere bindingssetet til LsbB, i tillegg til å evaluere om det konserverte proteolytiske setet til RseP er viktig for funksjonen som reseptor. I tillegg var det av interesse å identifisere hvilken del av RseP som gjør at LsbB kun binder spesifikt til laktokokker og ikke enterokokker.

For å finne ut om det proteolytiske setet er involvert i funksjonen som reseptor, ble det gjort punktmutasjoner, der de konserverte residuene ble endret til alanin. Det ble så gjort heterolog uttrykkelse, der de endrete *rseP* genene ble uttrykt i den heterologe verten *Streptococcus pneumoniae*. Resultatene viste at forandringene i det aktive setet, spesielt når alle residuene i det aktive setet ble endret, gjorde at stammene ble mye mer resistente til LsbB enn stammen som uttrykte villtype laktokokk *rseP*. For å evaluere hvilken del av RseP som er ansvarlig for laktokokkenes spesifikke sensitivitet til LsbB, ble det laget hybrider. I disse hybridene ble deler av laktokokk RseP erstattet med de korresponderende delene av enterokokk RseP. Både enterokokk og laktokokk RseP består av fire transmembranhelixer, og hybridene ble laget så de inneholdt ulike kombinasjoner av laktokokke og enterokokke helixer. Sensitiviteten til LsbB ble sjekket for alle RseP-hybridene, og resultatene viste at den andre og den tredje helixen trengte å være fra *Lactococcus lactis* for å være sensitive til LsbB. Stammene der den andre og den tredje helixen ikke var fra *L. lactis* ble totalt resistente. Det ser ut til at den tredje helixen er viktig for den første bindingen av LsbB, mens den andre helixen, i tillegg til det proteolytiske setet, er viktig for formeringen av komplekset som fører til ødeleggelse av cellemembranen. Dette studiet avslørte interessante resultater, men detaljene om binding og ødeleggelse av cellene av LsbB trenger å bli studert videre. En grundig forståelse av bakteriosiner, deres reseptorer og deres virkemåte er viktig for å kunne utvikle disse molekylene for en nyttig og trygg anvendelse.

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1. Introduction

1.1 Antibiotic resistance

Antibiotic resistant bacteria are an emerging worldwide problem. This crisis is caused by the overuse and inappropriate prescribing of antibiotics, the extensive use in agriculture and the lack of few new antibiotics or alternatives to antibiotics (Ventola 2015). During the last three decades, only two new classes of antibiotics have reached the market; oxazolidinones (linezolid) and cyclic lipopeptides (daptomycin) (Gupta & Nayak 2014). Numbers from Centers of Disease Control and Prevention (CDC) show that in the United States there are at least 23.000 deaths caused by antibiotic resistance each year, where most of the deaths are caused by Methicillin resistant *Staphylococcus aureus* (MRSA) and *Streptococcus pneumoniae* (Centers for Disease Control and Prevention, 2013). Only in the USA, the costs of treating patients with antibiotic resistant bacteria are estimated to be 55 billion USD each year, although the number can be even higher (Smith & Coast 2013).

The first antibiotics were utilized in the 1940s, and have since then been widely used to target pathogens to prevent infectious diseases. Penicillin was the first antibiotic discovered, and it was done by Alexander Fleming in 1928. Penicillin was put into large-scale production in the early 1940s, treating bacterial infections during World War II (Ventola 2015). However, shortly after, bacteria developed resistance to penicillin, and urge to discover new antibiotic arouse. New antibiotics got discovered and were clinically used, yet, bacteria got resistant to these antibiotics as well. Figure 1.1 shows a timeline when the different antibiotics were introduced, and when the bacteria developed resistance to them.

There are different suggestions on how to overcome the problem of antibiotic resistant bacteria, which includes public education and knowledge of antibiotic resistance, control of the use of antibiotics, developing new antibiotics, investigation and research on old antibiotics to see if there could be an affective combination of different antibiotics (Bush et al. 2011). Another way is to develop alternatives to antibiotics, where the antimicrobial peptides bacteriocins can be a good choice.

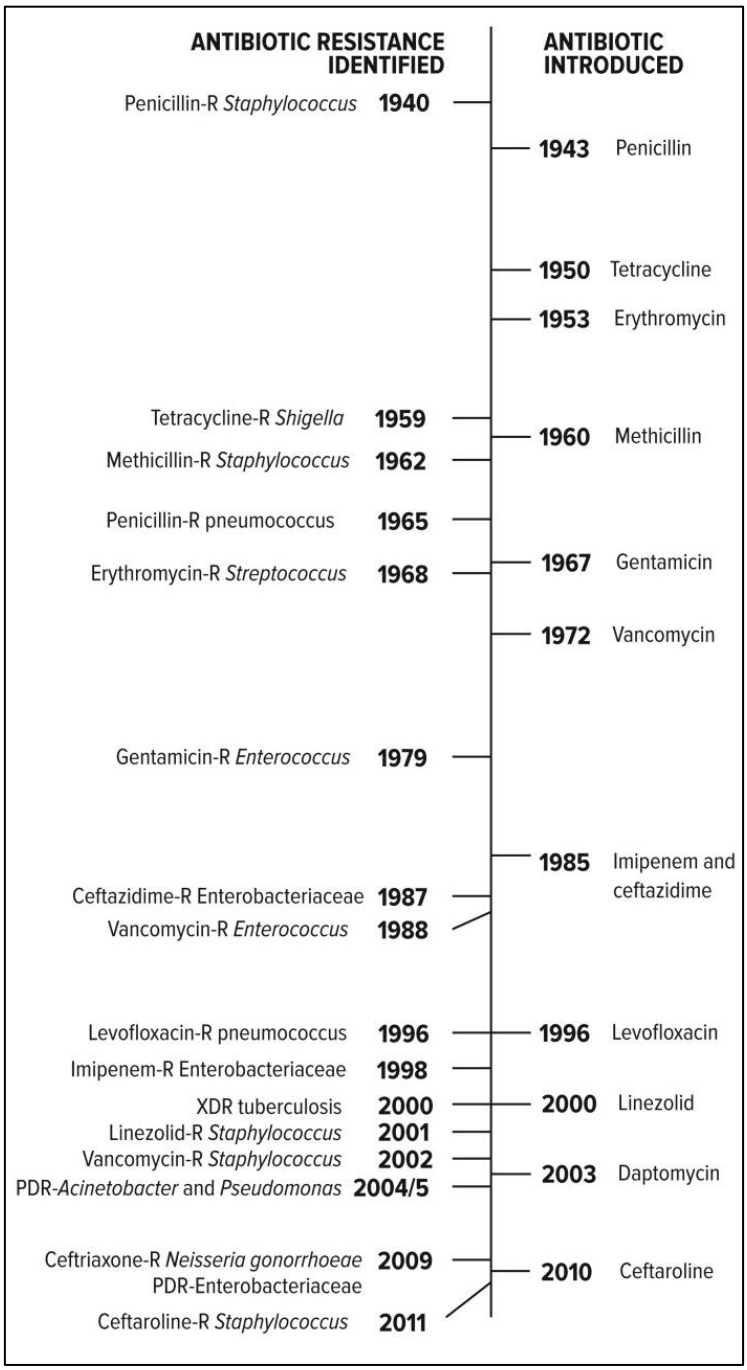

Figure 1.1. A timeline showing when the different antibiotics were introduced, and when the bacteria developed resistance. R = resistant; PDR = pan-drug-resistant, meaning resistant to all agents in all antimicrobial categories; XDR = extensively drug resistant, meaning resistant to at least one agent in all but two or more antimicrobial categories (Magiorakos et al. 2012). Penicillin was in limited use before it was put in large-scale production, explaining the early resistant bacteria. Figure adapted from Ventola (2015).

1.2 Bacteriocins

Bacteriocins are small antimicrobial peptides produced by bacteria to kill closely related bacteria. They do that for competition of food and niche. Unlike antibiotics, that are produced as secondary metabolite, bacteriocins are synthesized ribosomally (Cleveland et al. 2001). Bacteriocins contain between 25 and 70 residues, and are often cationic, amphiphilic and membrane permeabilizing peptides (Nissen-Meyer & Nes 1997). Their activity spectrum can vary from one species to several genera, but they appear to have a very specific activity.

Bacteriocins have for a long time been used as a food preservative, where nisin is the most widely used. Nisin is used in for example cottage cheese, skimmed milk, ricotta cheese, lean beaf and Kimchi, and has shown to kill *Listeria monocytogenes*, *Bacillus cereus* and *Brochothrix thermosphacta* among others. There have been performed many studies, where food has been inoculated with bacteriocin-producing bacteria to prove bacteriocin inhibiting influence on food spoilage and pathogenic species. One example is a study on the count of *L. monocytogenes* in Manchego cheese. Here it was shown that the count of *L .monocytogenes* Ohio decreased by 6 log units after 7 days when 1% of an *Enterococcus faecalis* culture was added, which produce the bacteriocin enterocin (Nunez et al. 1997). The same thing was discovered when a salami sausage was inoculated with the bacteriocin producing *Lactobacillus plantarum*. The amount of *L. monocytogenes* decreased significantly when inoculated with *L. plantarum* (Campanini et al. 1993). The fact that bacteriocins have been used in food industry for a long time, ensures that it is safe, and therefore, it should also be considered for clinical purposes.

1.2.1 Classification

Antimicrobial peptides are produced by both Gram-negative and Gram-positive bacteria. The first studies were conducted on peptides produced by gram-negative species, mostly *Escherichia coli*. Those peptides were classified as microcins and colicins, according to their origin and size. Colicins, coming from *E.coli* being more than 10 kDa, and microcins coming from other Gram-negative bacteria and being less than 10 kDa (Oscariz & Pisabarro 2001). For a long time colicins were the best studied antimicrobial peptides, however bacteriocins produced by lactic acid bacteria are gaining more interest nowadays. The main focus of this thesis is connected with bacteriocins produced by Gram-positive bacteria, therefore colicins

and microcins will not be discussed in more details.

There have been many ways to classify bacteriocins since they first got discovered, and it is not an easy task due to their various features. Bacteriocins from Gram-positive bacteria vary a lot in molecular size, structure, producer organism, post translational modifications (PTMs), inhibition spectrum, physical properties, etc. These differences make it difficult to find a system that all bacteriocins can fit in to. More bacteriocins are also being discovered, and the classification is an ongoing process. Table 1.1 shows one of many ways to classify bacteriocins (Nes et al. 2007b).

Table 1.1. The classification of bacteriocins. The bacteriocins derived from Gram positive bacteria can be divided into four main classes, which consist of several subclasses. The antimicrobial peptides produced by Gram negative bacteria can be divided into microcins and colicins (Nes et al. 2007a; Oscariz & Pisabarro 2001).

Producer bacteria	**Term**	**Class**	**Subclass**	**Description**	**Example**
Gram negative	Microcins			> 10 kDa	
	Colicins			< 10 kDa	
Gram positive	Bacteriocins	I	Type A	Linear structure	Nisin (Whitehead 1933)
			Type B	Globular structure	Mercacidin (Chatterjee et al. 1992)
			Type C	Two-component	Lacticin 3147 (Ryan et al. 1996)
		II	a	Pediocin-like motif	Pediocin A (Daeschel & Klaenhammer 1985)
			b	Two-peptide	Lactococcin G (Nissen-Meyer et al. 1992)
			c	Linear non-pediocin-like	Lactococcin A (Holo et al. 1991)
			d	Leaderless bacteriocins	LsbB (Gajic et al. 2003)
			e	Larger protein-derived bacteriocins	Propionicin F (Brede et al. 2004)

		III		Large, heat-labile	Dysgalacticin (Tagg & Wong 1983)
		IV		Cyclic	Enterocin AS-48 (Samyn et al. 1994)

Class I bacteriocins

Class I bacteriocins, also called lantibiotics, are small peptides (<5 kDa), that are synthesized as inactive prepeptides (Perez et al. 2014a). They are activated by post-translational modifications (PTMs). The extensive PTMs lead to the formation of thioether bridges (C-S-C) that produce lanthionine and methyllanthionine rings (McAuliffe et al. 2001). They also consist of the unsaturated amino acids 2,3 dehydroalanine (Dha) and 2,3 dehydrobutyrine (Dhb). Those unusual structures are characteristic for the lantibiotics. (Asaduzzaman & Sonomoto 2009) (figure 1.2). Lantibiotics can be divided into type A, type B and type C , which are linear, globular and two component, respectively (Rea et al. 2011) . Nisin is the most studied lantibiotic (fig 1.2).

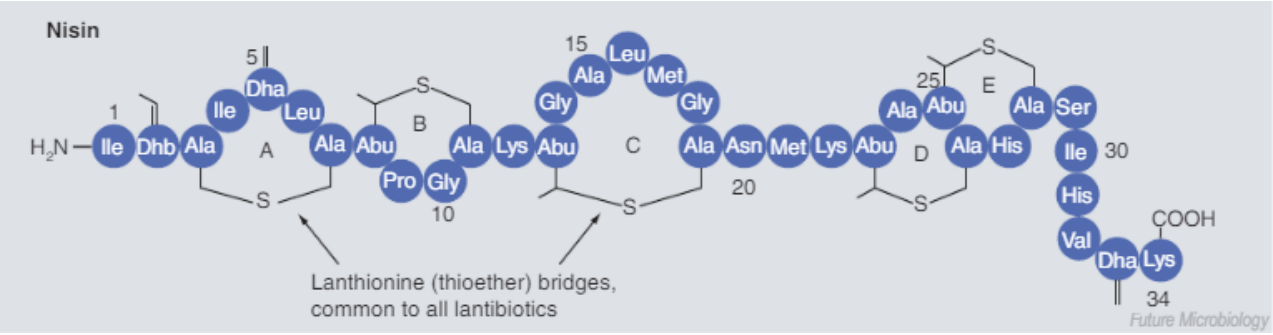

Figure 1.2. The structure of nisin. The five characteristic lanthionine rings are shown (A-E), and the arrows point to the thioether bridges (Martin & Breukink 2007). Ala-S-Ala is lanthionine, Abu-S-Ala is 3-methyllanthionine, Dha is dehydroalanine and Dhb dehydrobutyrine. Figure adapted from Martin & Breukink (2007).

Class II bacteriocins

Class II bacteriocins are small (<10 kDa) heat-stable and do not contain lanthionine (Perez et al. 2014a). They include a diverse group peptides, and do not undergo any PTMs, which makes them simpler in structure than the lantibiotics. Class II bacteriocins can be further divided into 5 different subgroups (a-e) (Nes et al. 2007b).

Class IIa bacteriocins are called pediocin-like bacteriocins, and this group is the largest and most studied subgroup of bacteriocins (Ennahar et al. 1999). They consist of 37-49 amino acids, and normally have an amino acid sequence similarity of about 40%. The pediocin-like bacteriocins have a charged N-terminal end that is very conserved, and include the YGNGVX motif (Drider et al. 2006; Nes et al. 2007b). The N-terminal end forms a β-sheet that is stabilized by a disulphide bridge. The C-terminal part folds into one or two α helices, and some of bacteriocins also form a disulphide bridge at this end to stabilize the loop structure. This loop broadens the target cell specificity, as well as enhances the specific activity and heat-stability (Fimland et al. 2000; Nes et al. 2007b).

Class IIb consists of two-peptide bacteriocins. It means that they consist of two different peptides, and both are required in equal amount to obtain sufficient activity (Nissen-Meyer et al. 2009). This is also in line with their genetics; 1) the genes encoding the bacteriocins are always at the same operon; 2) there is only one gene for immunity, which is also located in the same operon. About all of the class II bacteriocins have the GxxxG motif, that is responsible for helix-helix interactions. The structures of the two-peptide bacteriocins are often, if not always, represented by a helix-helix structure (figure 1.3) (Rogne et al. 2008).

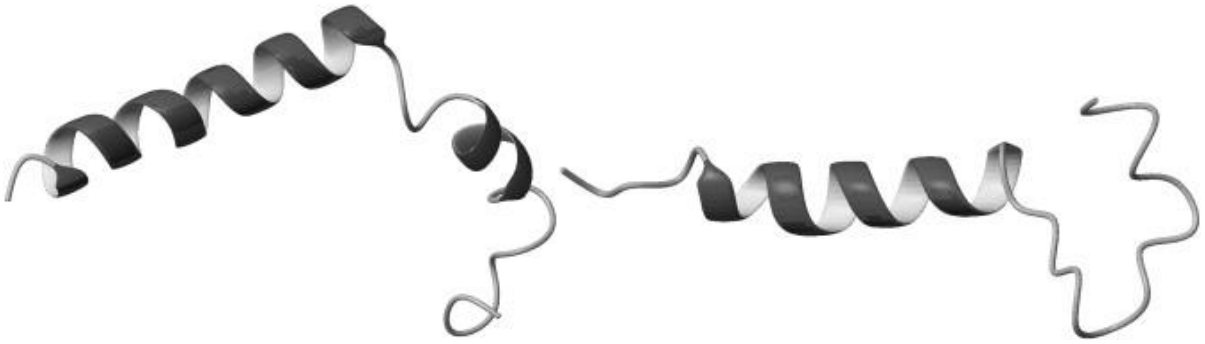

Figure 1.3. The structure of the two-peptide bacteriocin lactococcin G. Lactococcin G consist of lactococcin G-α (to the left) and lactococcin G-β (to the right). Figure adapted from Rogne et al. (2008).

Class IIc consists of unsorted bacteriocins, and includes bacteriocins that do not fit into any other of the classes (Nes et al. 2007b). Some of these bacteriocins share similarities, but not enough to be classified in another subgroup (Eijsink et al. 2002). These bacteriocins are linear, non-pediocin like and non-lantibiotic bacteriocins.

Class IId consists of leaderless bacteriocins, which means they are produced without the leader sequence. Normally, bacteriocins are produced as pre-peptides with a leader sequence, whose function is to prevent the bacteriocins from being active inside the producer, as well as being a recognition signal for the transportation (Nes et al. 1996). Because of lack of this leader sequence, class IId bacteriocins are believed to have an unique and complex biosynthetic mechanism, that is still not fully understood (Perez et al. 2014a). They don't undergo any PTMs and they are exported with formyl methionine at their N-terminals, something that distinguishes them from other bacteriocins (Liu et al. 2011; Ovchinnikov 2016). The simple structure, as well as their broad antimicrobial spectrum that a lot of them have, make them interesting for potential commercial use (Perez et al. 2014a). Two families of leaderless bacteriocins, Enterocin L50 family and Aureocin A53 family, could be defined until recently. There have now been defined two new families: LsbB and AurA70 (Ovchinnikov et al. 2014; Ovchinnikov et al. 2016). The LsbB family will be discussed in more detail later.

Class IIe consists of larger, protein-derived bacteriocins, which means they are produced by degradation of larger peptides (Nes et al. 2007b).

Class III bacteriocins are large and heat-labile. The classification and the naming are discussed, due to their lytic enzymatic activity, and are therefore considered to be renamed bacteriolysins (Rea et al. 2011).

Class IV bacteriocins are circular bacteriocins, and are characterized by their N-to-C terminal covalent linkage, which provides the circular backbone (figure 1.4) (Gabrielsen et al. 2014). This class is also suggested to be a subgroup of the class II bacteriocins, but the genetic apparatus needed for their synthesis is different from the other class II bacteriocins, and is therefore classed in a separate group (Nes et al. 2006). They are produced as linear peptides, and their N-terminal leader-sequence is cleaved off during maturation (Gabrielsen et al. 2014). The size ranges between 58-70 amino acids, and these bacteriocins are very stable compared to the non-cyclic bacteriocins. Because of their stability for high temperature, chemical treatments and degradation by proteases, circular bacteriocins are very interesting in terms of food preservatives, as well as clinical use (Perez et al. 2014b; van Belkum et al.

2011). However more knowledge is still required for application purposes.

1.2.2 Genetics of bacteriocins

The genes encoding bacteriocins are usually located in one or two operons, and the operon contains of at least four genes to ensure the function of a bacteriocin. These genes are normally located at plasmids or at moveable genetic elements (Nes et al. 2007b).The four essential genes include:

1) a structural gene encoding the prebacteriocin, which contains an N-terminal leader sequence (double-glycine leader). This structure function is to prevent the bacteriocin from being active inside the producing bacteria, and it also serves as a signal that can be recognized by the transporter system.

2) an immunity conferring gene that is always located next to the structural gene and within the same transcription unit,

3) an ABC-transporter that can transport the bacteriocin to the external environment and at the same time remove the leader sequence,

4) an accessory protein that is essential for the externalization of the bacteriocin, but where the specific role is unknown (Nes et al. 1996). In addition to the four essential genes, there are also some findings of regulatory genes (figure 1.5).

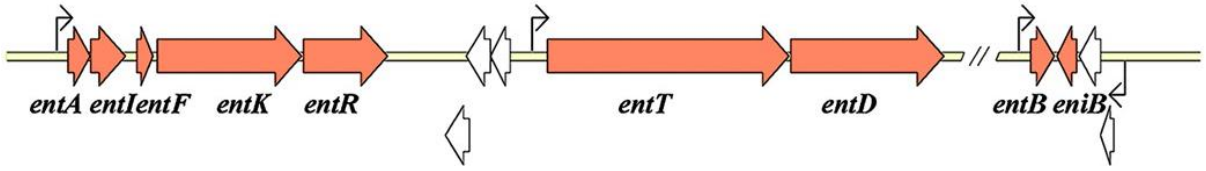

Figure 1.5. The organization of the bacteriocins enterocin A and B operon. Enterocin A operon consists of two operons. The first operon includes *entA* encoding the bacteriocin, the immunity gene (*entI*), the peptide pheromone gene (*entF*), the histidine protein kinase gene (*entK*) and the DNA binding activator, the response regulator (*entR*). The second operon encodes the ABC-transporter (*entT*) and it accessory protein (*entD*), that are responsible for the secretion of the bacteriocin and the pheromone. The enterocin B includes two operons; the first encodes the bacteriocin (*entB*) and the second contains the immunity gene (*eniB*). It is believed that the production of enterocin B is regulated by *entFKR* and that the transport is mediated by *entT* and *entD*, like enterocin A. The arrows demonstrate the regulated promoters, while the open arrows show the ORFs of unknown function (Nes et al. 2007a). Figure adapted from Nes et al. (2007a).

1.2.2.1 Biosynthesis and its regulation

The production of several bacteriocins are controlled by a three-component system that is quorom-sensing based (Nes et al. 2007b). This system consists of three co-transcribed genes: induction factor (IF, pheromone), histidin kinase (HK, sensor protein) and response regulator (RR, DNA-binding effector protein). The signal pheromone (IF) is always expressed at a low level. The amount of the pheromone (IF) will increase, either with the number of cells, or due to environmental factors/changes. It will reach a threshold, and eventually accumulate extracellularly. This will in turn activate the histidine-kinase (HK), which leads to a series of phosphorylations, and eventually phosphorylation of the response regulator (RR). The response regulator will then be capable of binding to promoters and activate transcription of a set of genes involved in the bacteriocin production (Figure 1.6) (Nes et al. 2007b). Normally, the pheromone is bacteriocin-like, or it can be the bacteriocin itself. The plantaricin A system has its own pheromone that regulates the bacteriocin production, while the peptides nisin and subtilin serve as both the pheromone and as the bacteriocin (Kleerebezem et al. 1997; Nes et al. 2007b).

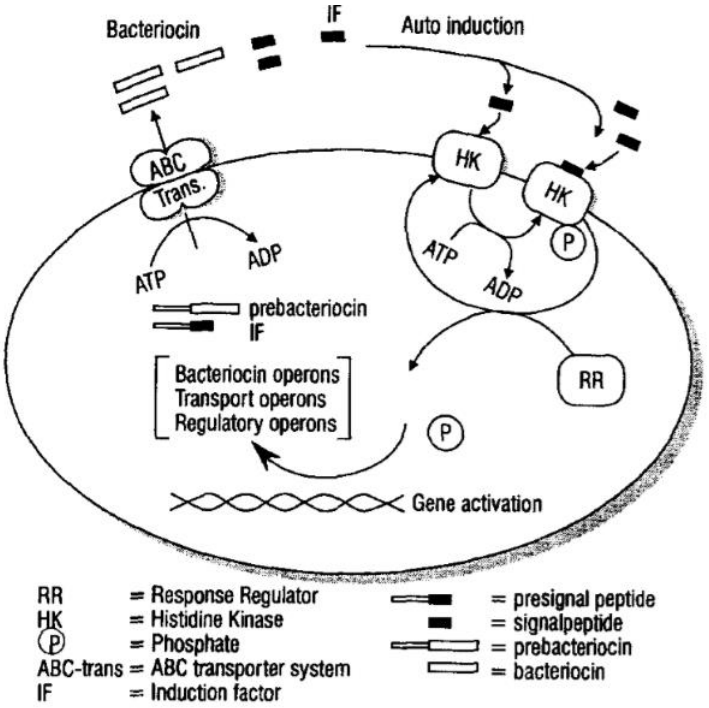

Figure 1.6. Regulation of the bacteriocin production. HK is sensing the IF, resulting in phosphorylated RR. The phosphorylated RR leads to the transcription of the operons involved in the production of bacteriocin. The presignal peptide and the prebacteriocin are translocated outside the cell, and maturated by the ABC-transporter system. The signal peptide will bind to the HK, resulting in an auto induction. Figure adapted from Nes et al. (1996).

1.2.2.2 Activation

Most bacteriocins are produced as inactive pre-peptides. This pre-peptide consists of an N-terminal leader sequence, whose role is to keep it inactive while inside the producer, and to lead the peptide to maturation and to the transportation proteins (Perez et al. 2014a). This leader sequences were for a long time only recognized as a double-glycine type of leader, while now it is known that some bacteriocins also have sec-dependent leaders (Nes et al. 1996). The bacteriocins with the double-glycine leader, are secreted by the ATP-binding cassette (ABC) transporter. The gene encoding the ABC-transporter is normally located in the same operon as the bacteriocins or close by this operon (Nes et al. 1996). In addition to secrete the bacteriocins, ABC-transporter is also proven to cleave off the leader sequence of the bacteriocin (Havarstein et al. 1995). The bacteriocins with the sec-type of leader are transported through the general secretion pathway, which is the major route of protein translocation (de Keyzer et al. 2003). During this translocation through the membrane, the signal sequence is also removed (de Keyzer et al. 2003).

1.2.2.3 Immunity

Bacteria are protected against their own bacteriocin(s). The operon encoding the bacteriocin also includes an immunity-gene and is in most cases co-regulated with the bacteriocin structural genes (Hassan et al. 2012). This immunity-protein protects the producer cell by different mechanisms, depending on the producer/bacteriocin. This mechanism can include shielding of the receptor, prevention of pore formation by binding directly to the bacteriocin, or it could block the pores (Moll et al. 1999).

1.2.3 Mode of action

Bacteriocins differ a lot, and so do their target. They may aim to perturb transcription, translation, replication and biosynthesis of the cell wall (Oscariz & Pisabarro 2001). However, most of the bacteriocins create a pore in the membrane, which leads to leakage of nutrients and metabolites, ATP depletion and destroying the proton motive force (PMF) (Eijsink et al. 2002). The size and the stability of the pore differs in different bacteriocins (Eijsink et al. 2002). There are several types of pore-formation described for antimicrobial peptides: the barrel-stave model, the wedge model, the toroidal pore model, the carpet model and the aggregate channel model (Snyder & Worobo 2014). Not all of them are identified as models

used by the bacteriocins, but some of them are, as described later.

The lantibiotics mechanism of activity includes binding and insertion of the bacteriocins into the bacterial membrane (pore-forming), and the use of a receptor or docking molecule to have a more specific and structure-based activity (Asaduzzaman & Sonomoto 2009). The pore-forming activity, happens according to the barrel-stave or the wedge model (Asaduzzaman & Sonomoto 2009). The barrel-stave mechanism starts with the bacteriocin binding to the membrane, leading to an assemblage into a pre-aggregate of monomers (Asaduzzaman & Sonomoto 2009). This results in a water-filled pore (Abee et al. 1995). When they follow the wedge model, the lantibiotics bind parallel to the membrane, making a local strain, which will bend the membrane in a way that the bacteriocins will make a pore together with the lipid molecules (Asaduzzaman & Sonomoto 2009). Lantibiotics usually create large, non-specific pores (Moll et al. 1999) (figure 1.7 a).

In most cases, the binding of lantibiotics to the membrane happens due to electrostatic interactions, however nisin has a unique way of making pores. It is proven that nisin uses lipid II as a docking molecule, where lipid II is not only the receptor, but also a part of the pore that is formed (Breukink et al. 2003). This is the case when the concentration of the bacteriocin is at nano-molar concentration (figure 1.7 b.) (Héchard & Sahl 2002). Lipid II can also be a target for lantibiotics, not only a docking molecule. Lipid II is essential for the biosynthesis of the cell wall, and is targeted by a lot of antibiotics. It is now shown that lipid II is also targeted by lantibiotics, which interferes with the peptidoglucan biosynthesis (Asaduzzaman & Sonomoto 2009) (figure 1.7 c).

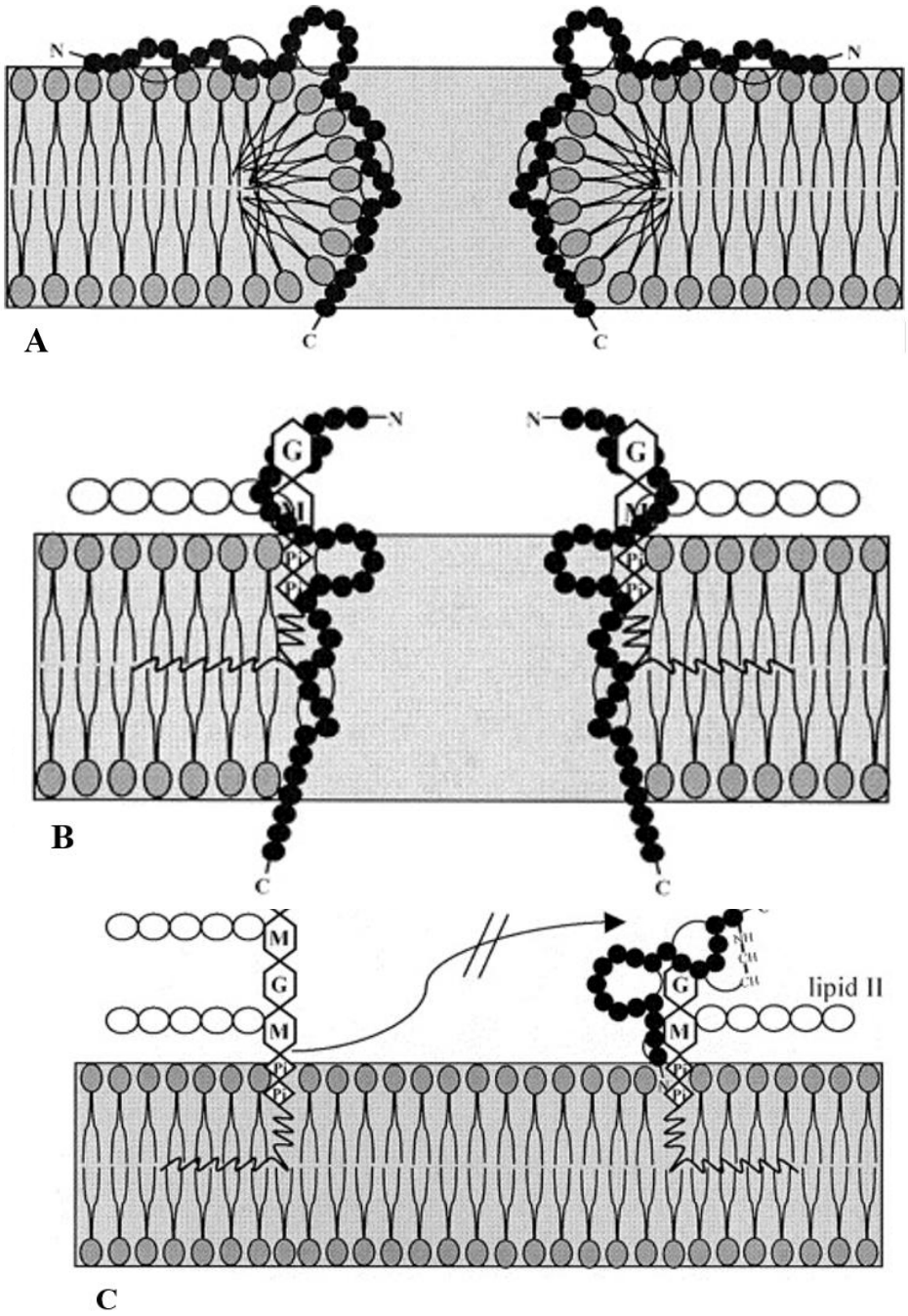

Figure 1.7. A) At micro-molar concentration, the type-A lantibiotics form a pore, here demonstrated as a wedge-like model. B) at nanomolar-concentration nisin and epidermin (Type-A lantibiotics) use lipid II as docking molecule, and form a pore. C) Binding of lantibiotics to lipid II, resulting in the destruction of the peptidoglucan synthesis. Figure adapted from Héchard & Sahl (2002).

Class II bacteriocins are also dependent on anionic phospholipids for the initial membrane interaction (Moll et al. 1999). When they form pores, they seem to make a bundle of α-helices peptides. They can either do the barrel-stave model or the carpet-like model (Moll et al.

1999). There have been proposed two different models for how class IIa bacteriocins works (Héchard & Sahl 2002). The first mechanism is dependent on a target-protein on the surface of the target cell. It has been proved that mannose phosphotransferase system (man-PTS) can function as these surface target (Diep et al. 2007; Kjos et al. 2010). The other model suggests that the bacteriocins interact with the cytoplasmic membrane, resulting in pore formation or disruption of the membrane. This mechanism seem to be independent of a receptor, and is instead possible due to electrostatic and/or hydrophobic interactions with the membrane (Héchard & Sahl 2002).

The class IIb bacteriocins seem to be dependent on specific receptors on the target cell to be active. They will also form hydrophilic pores, leading to membrane permeabilization. Class IIc bacteriocins are involved in membrane permeabilization, pheromone activity and inhibition of septum formation (Héchard & Sahl 2002). Because of the bacteriocins' narrow activity spectrum, it is believed that all the bacteriocins work in a receptor-mediated manner, although the receptors are not yet identified for all of them. However, there are some receptors identified, and there will be more to come.

1.2.3.1 Receptor recognition

There has been proved that bacteriocins use specific receptors on the target bacteria to be active. As previously mentioned, it has for a long time been known that lantibiotics use lipid II both as a receptor and a target, and that some class II bacteriocins use man-PTS as their receptor. More receptors have been identified, and the bacteriocins target them in a very specific manner (figure 1.8) (Cotter 2014). Identifying more receptors for more bacteriocins is important to get a better understanding of the mechanism, which is of big interest to make them available for commercial use.

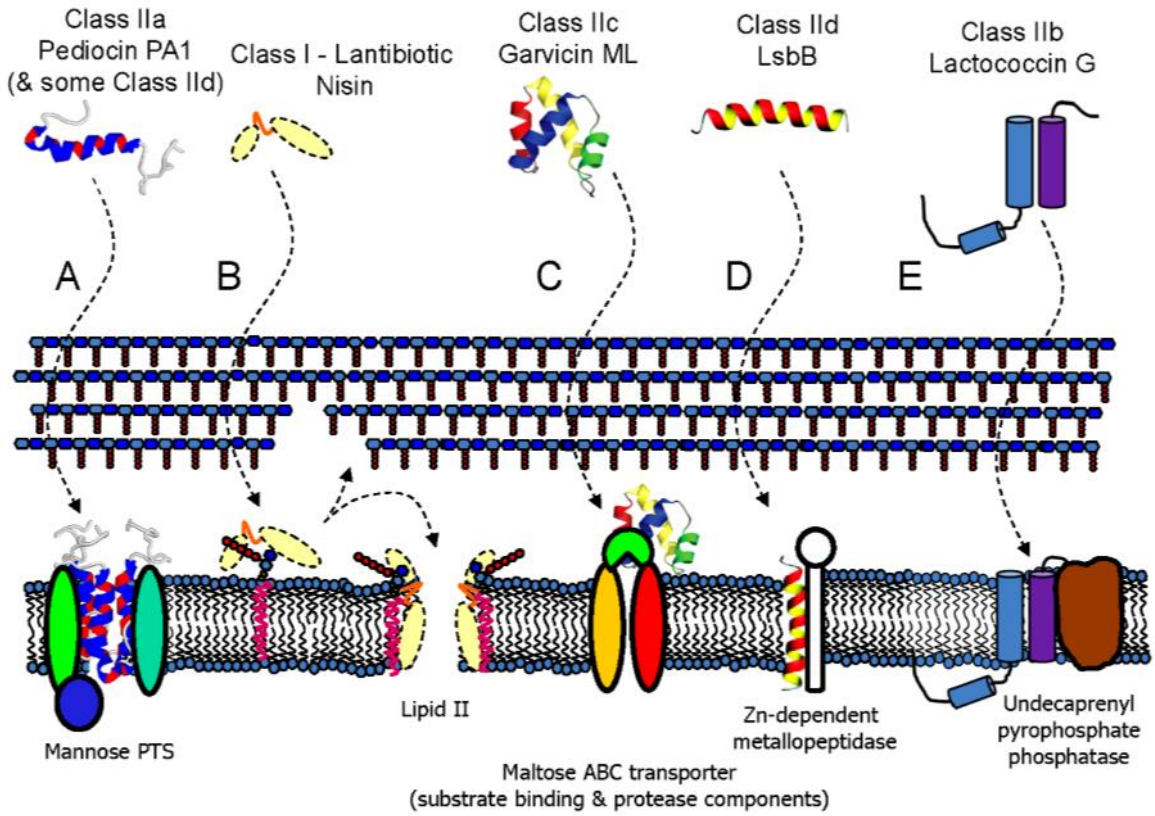

Figure 1.8. The different mode of actions and receptors for different bacteriocins. A) Class IIa and some class IId use mannose PTS as a receptor to form a pore. B) Class I, the lantibiotics, use lipid II as both a receptor and a docking molecule. C) The class IIc bacteriocin Garvicin ML uses the maltose ABC transporter as a receptor. D) The class IId bacteriocin LsbB uses the Zn-dependent metallopeptidase as a receptor, while the class IIb bacteriocin Lactococcin G (E) uses the uppP as the receptor (Cotter 2014). Figure adapted from Cotter (2014)

1.2.3.2 Known receptors

<u>The man-PTS as receptor for class IIc lactococcin A and the class IIa pediocin-like bacteriocins</u>

The mannose phosphotransferase system (man-PTS) functions as a receptor for different bacteriocins, including lactococcin A and class IIa bacteriocins. The man-PTS consists of four structural domains; IIA, IIB, IIC and IID. IIA and IIB are normally represented by one protein (IIAB) located in the cytoplasm, and IIC and IID form a complex that is located in the membrane (Kjos et al. 2010). Lactococcin A is a class IIc bacteriocin, targeting only lactococcal cells. Diep et al. (2007) proved that lactococcin A uses the proteins IIC and IID of the man-PTS as the receptor on target cells. It was shown by deletion of the Man-PTS operon, that created a resistant mutant. By expressing the different components of the Man-PTS alone

or as pairs, it was shown that the sensitivity to lactococcin A was re-established when the genes encoding IIC and IID components were expressed together (Diep et al. 2007). Class IIa bacteriocins also use the man-PTS as a receptor, however, in a different manner. Kjos et al. (2010) found a region of 40 amino acids in an extracellular loop of the IIC protein that was responsible for the bacteriocin activity. There was created hybrids consisting of different combination of a sensitive and a resistant strain to map the region involved in the bacteriocin activity, and then site-directed mutagenesis was performed to narrow the region. Thus, there was demonstrated that class IIa bacteriocins target a region of 40 amino acids in the extracellular loop of the IIC protein, whilst lactococcin A require parts from both the IIC and IID protein for the specific recognition on target cells (Diep et al. 2007; Kjos et al. 2010).

The Maltose ABC transporter as receptor for the circular bacteriocin Garvicin ML

In 2012 (Gabrielsen et al. 2012) discovered that the maltose ABC transporter functioned as a receptor for the circular bacteriocin Garvicin ML (figure 1.8 C). This class IV bacteriocin is produced by *Lactococcus garvieae* DCC43. It mainly kills *L. garvieae* and *Lactococcus lactis*, however, other strains like *Lactobacillus*, *Pediococcus* and *Streptococcus* are also shown to be highly or moderately sensitive (Gabrielsen et al. 2012). The identification started with the investigation of resistant mutants of *L. lactis* IL 1403. A 13,5 kb chromosomal deletion was detected, and among the 12 ORFs identified in this region, were the *malEFG* genes. The *malEFG* genes encode the membrane-bound maltose ABC transporter. When these three genes were reintroduced into the resistant mutants, they recovered their sensitivity to Garvicin ML, showing that the maltose ABC transporter has an essential role for the activity of this bacteriocin.

YvjB as the receptor for the leaderless bacteriocin LsbB

In 2013 (Uzelac et al. 2013) found that the Zn-dependent metallopeptidase YvjB (RseP) is the target for the leaderless class II bacteriocin LsbB, which is produced by the *L. lactis* subsp. *lactis* BGMN1-5 (figure 1.8 D). A cosmid library of the sensitive strain BGMN1-596 was made, and cloned into resistant mutants of BGMN1-596. A cosmid that had a 40-kb insert, restored the sensitivity. Further investigation identified a 1,9 kb fragment that was sufficient to regain the sensitivity, and this fragment contained the gene *yvjB* (*rseP*). *YvjB* encodes a Zn-dependent membrane-bound metallopeptidase, and seemed to be the receptor for the bacteriocin LsbB. Further investigation was conducted to build up on this hypothesis,

and it was found that: 1) all of the resistant mutants had mutations in the *yvjB* gene, 2) when the gene was removed from LsbB sensitive strains, these strains got resistant, 3) when heterologous expression of the *yvjB* gene was performed in different resistant hosts, *Lactobacillus paracasei* and *E. faecalis*, they both became sensitive to LsbB.

UppP as the receptor for the two-peptide bacteriocin lactococcin G, and enterocin 1071

In 2014 Kjos et al. proved that the undecaprenyl pyrophosphate phosphatase (uppP) functions as a receptor for the two-peptide (class IIb) bacteriocin lactococcin G, as well as for the enterocin 1071 (figure 1.8 E). The method Kjos et al. (2014) used to identify the receptor, was novel, and it is a method that could be widely used for further receptor identifications (figure 1.9). It starts with whole genome sequencing, and comparison of the resistant bacteria with the sensitive bacteria. Then, the differences are found, in this case: the *uppP*. As a next step, the uppP was expressed in the naturally resistant *S. pneumoniae*. After the heterologous expression, the *S. pneumoniae* became sensitive, proving that the uppP was identified as the receptor (figure 1.9) (Kjos et al. 2014).

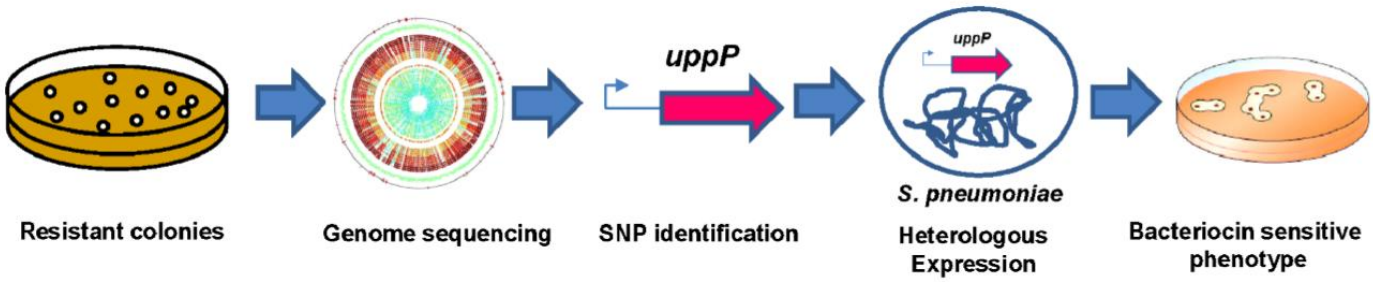

Figure 1.9. The workflow on how to identify the receptors for bacteriocins. Starts with creating resistant bacteria and their genomes sequencing, then identification of the differences between the resistant and the sensitive bacteria. At last expression of the identified protein in a naturally resistant bacteria, to see if it confers sensitivity (Cotter 2014). Figure adapted from Cotter (2014).

1.2.3.3 The site-2 protease RseP

RseP is a Zn-dependent metallopeptidase, and as previously mentioned: it works as the receptor for the bacteriocin LsbB. LsbB is a bacteriocin belonging to the class II leaderless bacteriocins, produced by *L. lactis* subsp. Lactis BGMN1-5. LsbB consists of 30 residues and targets only *L. lactis* cells (Gajic et al. 2003). LsbB uses a receptor, namely the Zn-dependent membrane-bound metallopeptidase YvjB, also called RseP (Uzelac et al. 2013). Ovchinnikov

et al. (2014) used CD and NMR spectroscopy to define the structure of LsbB, that could be defined into three functional parts: a) an N-terminal consisting of an amphiphilic α-helix; b) a small middle region containing basic amino acids; c) the C-terminal part, which is unstructured in water and in organic solvents (figure 1.10) (Ovchinnikov et al. 2014). The receptor binding site is located at the C-terminal of the bacteriocin, where tryptophan residue at position 25 is crucial for the antimicrobial activity (Ovchinnikov et al. 2014). LsbB uses the Zn-dependent metallopeptidase YvjB (RseP) as a receptor (Uzelac et al. 2013).

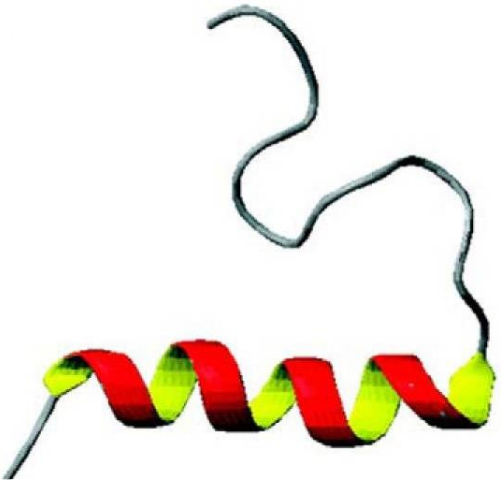

Figure 1.10. The structure of the bacteriocin LsbB. The N-terminal part consists of an α-helix, while the C-terminal is unstructured. Figure adapted from Ovchinnikov et al. (2014).

RseP has a proteolytic site, HExxH, which is conserved in all Zn-dependent metallopeptidases (Jongeneel et al. 1989). RseP consists of four transmembrane helices and the active site is located in the first one (figure 1.11). The two histidines in this conserved site stabilize/bind the zinc atom, and the glutamate is responsible for the catalytic activity (Rawlings et al. 2014). RseP has orthologous genes in various species; RasP in *Bacillus subtilis*, EeP in *E. faecalis* and RseP in *E. coli*.

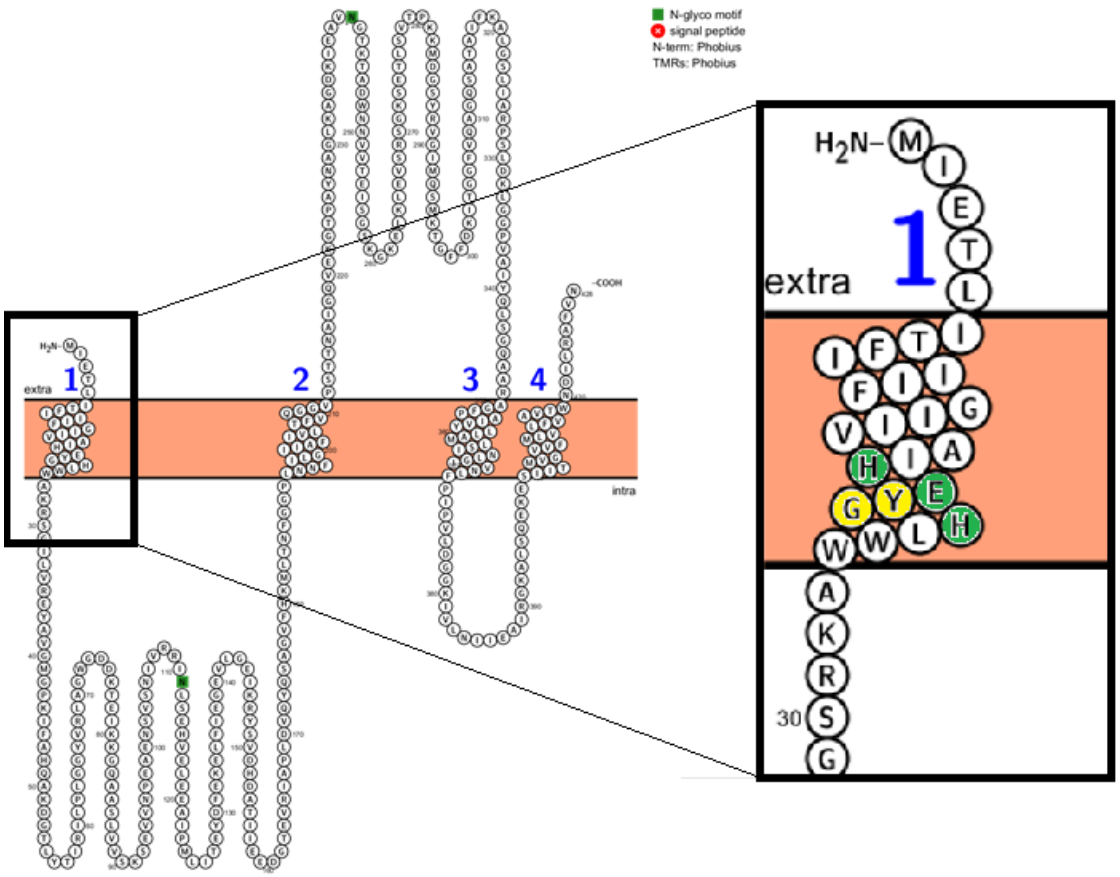

Figure 1.11. Shows the structure of YvjB/RseP, which consists of four transmembrane helices. The first helix is zoomed in, to show the active site. HE--H are marked in green, where the two residues in between (--) are marked in yellow. The picture was created using Protter (http://wlab.ethz.ch/protter/start/).

In *E. coli*, RseP is involved in stress response. RseP is a site 2-protease (S2P), and it cleaves the membrane-spanning region of the target, in a process called regulated intramembrane proteolysis (RIP) (Barchinger & Ades 2013). RseP is involved in the regulation of σ^E, which is an alternative transcription factor. σ^E activity is mainly controlled by RseA and RseB. RseA has a helical structure that is located between the conserved sites of σ^E, which are responsible for promoter recognition. RseB is bound to RseA at the periplasmic site. To release σ^E, RseA needs a first cleavage, performed by DegS, and a second cleavage, performed by RseP. When there is no stress, the outer membrane porins (OMP) are properly folded. When the cell is exposed to stress, the folding of OMP is not efficient, and the unfolded OMP will bind to PDZ domain of DegS. DegS gets activated and an unknown signal releases RseB from RseA. DegS can now cleave the periplasmic part of RseA. This leads to the activation of RseP, that

cleaves the transmembrane part of the RseA. Now, part of the RseA that is still bound to σ^E, can move to the cytoplasm. Here, ClpXP degrades the rest of the RseA, and σ^E is free to bind to core RNAP. The genes in its regulon are transcribed, and the system will reset (figure 1.12) (Barchinger & Ades 2013).

RseP has a PDZ-domain located at the central periplasmic part. This PDZ-domain is proved to function as a negative control on the degradation of RseA (Kanehara et al. 2003). The PDZ-domain is bound to RseA, preventing uncontrollable cleavage of RseA. Only when stress signals are received, and DegS gets activated to cleave off the cytoplasmic part of the RseA, so it is possible to be further degraded by RseP (Kanehara et al. 2003).

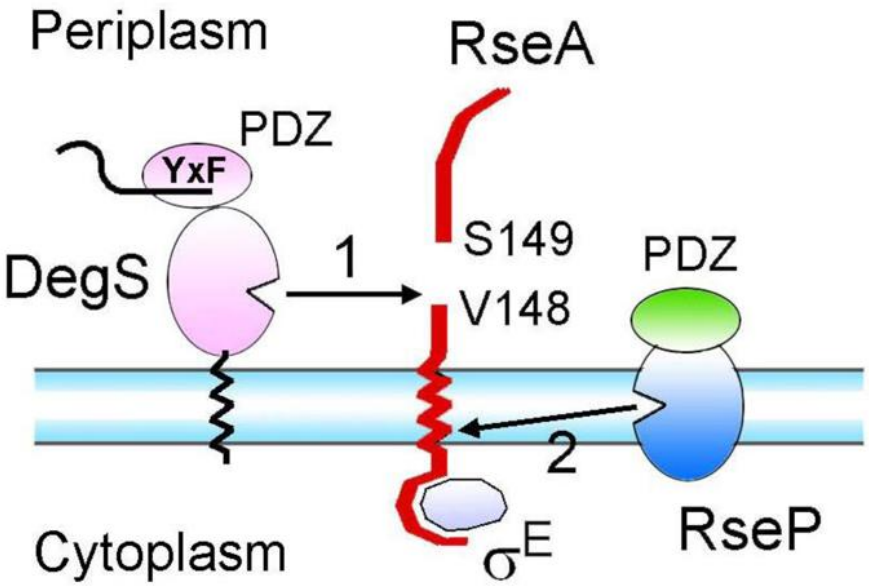

Figure 1.12. Part of the catalytic cascade happening during stress response (Li et al. 2009). The unfolded OMP bind to the PDZ domain of DegS, activating DegS, that performs the first cleavage of RseA. RseP get activated, and conducts the second cleavage. This will eventually lead to activation of the *RNAP* genes. Figure adapted from Li et al. (2009).

1.3 The aim of this study

The main goal of this study was to identify which part of RseP is responsible for binding of the bacteriosin LsbB. The work was divided into two parts. The first part was performed to reveal if the conserved catalytic site at the N-terminal of RseP, HExxH, was involved in the binding of LsbB. The second part included creating hybrids of the lactococcal and the enterococcal RseP, to identify which part is important for the specific binding of LsbB to only lactococcal cells.

The main tasks in this thesis included:

1. Perform site-directed mutagenesis at the conserved catalytic sites at the N-terminal to assess its relevance in receptor function. The conserved residues were changed to alanine.
2. Create hybrids that contained different combinations of the enterococcal and the lactococcal RseP.
3. The mutated RsePs and the hybrid RsePs were transformed into the naturally resistant *S. pneumoniae*, to verify their function as a receptor for LsbB.
4. Purify the protein by immune-precipitation and perform Western blot analysis to demonstrate the presence of the protein.

2. Materials

2.1 Growth media and agar

TH (Todd Hewitt)

- TH growth medium: 18,2 g TH for 500 ml dH_2O
- TH agar: TH medium + 15,4 agar/liter

2.1 Strains

Table 2.1. The strains utilized in this thesis.

***Streptococcus pneumonia* strains**	**Description**	**Source**
1522	*S. pneumonia*e, contains ComRS system, and Janus cassette is located behind P_{comX}.	(Berg et al. 2011)
ds221	1552, but Janus is replaced with enterococcal *rseP*	(Ovchinnikov et al. 2017)
OK1	1522 where Janus is replaced with lactococcal *rseP*.	This thesis
lmh1	1522, where Janus is replaced with lactococcal *rseP*, where H19>A	This thesis
lmh2	1522, where Janus is replaced with lactococcal *rseP*, where E20>A	This thesis
lmh3	1522, where Janus is replaced with lactococcal *rseP*, where H23>A	This thesis
lmh4	1522, where Janus is replaced with lactococcal *rseP*, where W25>A	This thesis
lmh5	1522, where Janus is replaced with lactococcal *rseP*, where HExxH>AAxxA	This thesis
lmh6	1522, where Janus is replaced with *rseP*, where the first three helices are lactococcal, the last one is enterococcal	This thesis
lmh7	1522, where Janus is replaced with *rseP*, where the first two helices are lactococcal, the last two are enterococcal	This thesis
lmh8	1522, where Janus is replaced with *rseP*, where the first helix is lactococcal, the last three are enterococcal	This thesis
lmh9	1522, where Janus is replaced with *rseP*, where the first three helices are enterococcal, the last one is	This thesis

	lactococcal	
lmh10	1522, where Janus is replaced with *rseP*, where the first two helices are enterococcal, the last two are lactococcal	This thesis
lmh11	1522, where Janus is replaced with *rseP*, first one helix is enterococcal, the last three are lactococcal	This thesis

2.2 Peptides

Table 2.2. The bacteriocin and other peptides used in this thesis. Ordered from PepMic.

Peptide	**Sequence**
LsbB	MKTILRFVAGYDIASHKKKTGGYPWERGKA
CSP	EMRLSKFFRDFILQRKK
ComS	LPYFAGCL

2.3 Enzymes and antibiotics

Table 2.3. The enzymes and antibiotic utilized in this thesis

Enzyme/antibiotic	**Supplier**
Phusion DNA polymerase	New England Biolabs
Taq DNA polymerase	New England Biolabs
Lysozym	Sigma
Deoxycholate	-
Streptomycin	-

2.4 Laboratory Equipment

Equipment	Supplier
96 wells PCR plates	VWR
Acid washed glassbeads (<106 µM)	Sigma
Comb	Bio-Rad
Cryo-tubes, 2 ml	Sarstedt
Culture tubes, 10 ml	-
Eppendorf tubes 2 ml	Eppendorf

Falcontubes (15 ml, 50 ml)	Greiner Bio-One
FastPrep tubes	MP Biomedicals
Gel-electrophoresis equipment (rack, molding form, comb)	Bio-Rad
Glass bottles 500 ml, 250 ml, 1L and 2L	-
Gloves	VWR
Membrane for protein blotting	Bio-Rad
Microtiter 96-well plates	Sarstedt
Multi channel pipette	Thermo Scientific
Parafilm	Bermis
PCR-tubes 0,2 ml	-
Petri dishes	-
Pipettes	Eppendorf
Pipette tips	VWR
Plastic loops	Sarstedt
Scalpel knife	Swann Morton
Tooth sticks	-
Tweezer	-
Whatman paper	-

2.5 Chemicals

Chemicals	Supplier
1,4-Dithiothreitol (DTT)	Sigma
10xTaq buffer	New England Biolabs
5x Phusion® HF buffer	Life technologies
Acryl/Bis™ 29:1	VWR® (Life science)
Agarose	Life technologies
APS	Merck
Bromophenol blue	Sigma
Comassie Brilliant Blue G-250	Bio-Rad
di-Sodium hydrogen phosphate dodecahydrate (Na_2HPO_4)	Merck
dH2O	-
ECL™ Peroxidase labelled anti-mouse antibody	GE Healthcare
Ethanol	Arcus
Glycerol	Merck
Glycine	Merck
Methanol	Sigma
$MgCl_2$ (50µM)	Life technologies
Monopotassium phosphate (KH_2PO_4)	Sigma
NaCl	Merck
PeqGreen	PeqLab
Potassium chloride	Merck
SDS	Merck

Skimmed milk powder	-
TEMED	Merck
Tris-base	Sigma
Tween-20	Sigma

2.6 Instruments

Instruments	Supplier
Autoclave	Matachana
Azure c400 GelDoc	AH Diagnostics
Eppendorf centrifuge	Eppendorf
Digital weight	Salter
Electrophoresis electricity supply	Bio-Rad
FastPreP-24™	MP Biomedicals
Freezer (-80 °C)	Forma Scientific
Gas burner	Intergra Biosciences
GelViewer	Bio-Rad
Heating block	Stuart Scientific
Incubator (37 °C)	Fermaks
Microtiter plate scanner	InterMed
NanoDrop ND-1000	Nanodrop Technologie
PCR machine	Bio-Rad
Spectrophotometer	Pharmacia
Sterile bench w/fume hood	Holten Laminaire
Table centrifuge	Tamro
Vortex	Scientific Industries
Water bath	Julabo

2.7 Solutions

2x SDS sample buffer
- 0,125 M Tris-HCl, pH 6.8
- 4% SDS
- 0,2 M DTT
- 20 % Glycerol
- 0,01% Bromophenol blue

5xRunning buffer
- 15 g/l Tris Base
- 72 g/l glycine
- 5 g/l SDS

PBS, pH 7.3
- 1,4 M NaCl
- 27 mM KCl
- 101 mM Na_2HPO_4
- 18 mM KH_2PO_4

1xTBS, pH 7,5
- 50 mM Tris base
- 150 mM NaCl

Transferbuffer
- 25 mM Tris-HCl, pH 8.3
- 192 mM Glycine
- 20 % MeOH

Blockingbuffer
- 1xPBS
- 0,05 % Tween-20
- 5 % non-fat dry milk

Washing buffer
- 1x PBS
- 0,05% Tween-20

50x TAE-buffer
- 242g/l Tris base
- 57,1 mL/l acetic acid
- 100 ml/l 0,5M EDTA pH 8,0

6x Loading buffer (20ml)
- 8g sucrose
- 200µl 0,5M EDTA
- bromophenol blue

2.8 DNA standards

DNA standards	Supplier
1kb ladder	New England Biolabs
Deoxynucleotides	Life technologies

2.9 Primers

Table 2.4. The different primers used in this thesis.

Primer	Sequence (5'→ 3')	Application
khb31	ATAACAAATCCAGTAGCTTTGG	Forward primer upstream of the *rseP* gene.
khb34	CTAAAAGAGTATAGGTTCCGATG	Reverse primer downstream of the *rseP* gene.
L1-P1	GGTATTATTGTCGCTATC**GCT**GAATATGGCCATCTTTGGTGGGC	Forward primer for making lmh1
L2-P2	GCCCACCAAAGATGGCCATATTC**AGC**GATAGCGACAATAATACC	Reverse primer for making lmh1
L3-P1	GGTATTATTGTCGCTATCCAT**GCA**TATGGCCATCTTTGGTGGGC	Forward primer for making lmh2
L4-P2	GCCCACCAAAGATGGCCATA**TGC**ATGGATAGCGACAATAATACC	Reverse primer for making lmh2
L5-P1	GTCGCTATCCATGAATATGGC**GCT**CTTTGGTGGGCAAAACGTTC	Forward primer for making lmh3
L6-P2	GAACGTTTTGCCCACCAAAG**AGC**GCCATATTCATGGATAGCGAC	Reverse primer for making lmh3
L7-P1	ATCCATGAATATGGCCATCTT**GCT**TGGGCAAAACGTTCAGGAAT	Forward primer for making lmh4
L8-P2	ATTCCTGAACGTTTTGCCCA**AGC**AAGATGGCCATATTCATGGAT	Reverse primer for making lmh4
L9-P1	GGTATTATTGTCGCTATC**GCTGCA**TATGGC**GCC**CTTTGGTGGGC	Forward primer for making lmh5
L10-P2	GCCCACCAAAG**GGC**GCCATA**TGCAGC**GATAGCGACAATAATACC	Reverse primer for making lmh5
L11	CGGTTTTGACTCTATCTCGC	Forward primer for sequencing *rseP*
L12	GTTATTCTGTTGATCATGATGC	Forward primer for sequencing *rseP*
L13	GTTCAAGCAGGCCAATCAGC	Forward primer for sequencing *rseP*
L14-P1	CCAGTACTTGATGGTGGAAAAATTGTCTTAAACATTATTGAAGGTGTACG	Forward primer for making lmh6
L15-P2	CGTACACCTTCAATAATGTTTAAGACAATTTTTCCACCATCAAGTACTGG	Reverse primer for making lmh6
L16-P1	GATTGCACGACCGAGTCTTGACAAACTAGGTGGGCCAGTC	Forward primer for making lmh7
L17-P2	GACTGGCCCACCTAGTTTGTCAAGACTCGGTCGTGCAATC	Reverse primer for making lmh7
L18-P1	CAGAAGTTCGGATTGCACCACTTGACGTTCAATTTCAATCAGC	Forward primer for making lmh8
L19-P2	GCTGATTGAAATTGAACGTCAAGTGGTGCAATCCGAACTTCTG	Reverse primer for making lmh8
L20-P1	GATGGCGGGAAAATTGTCTTAAATATTATTGAAGCAATTCGTGGC	Forward primer for making lmh9
L21-P2	GCCACGAATTGCTTCAATAATATTTAAGACAATTTTCCCGCCATC	Reverse primer for making lmh9
L22-P1	CGGCTCACTATTCACAGGCTCGAGTCTTGATAAACTTGGTGGC	Forward primer for making lmh10

L23-P2	GCCACCAAGTTTATCAAGACTCGAGCCTGTGAATAGTGAGCCG	Reverse primer for making lmh10
L24-P1	GGTGCGGATTGCGCCACTTGATGTTCAATATCAATCAGCCGG	Forward primer for making lmh11
L25-P2	CCGGCTGATTGATATTGAACATCAAGTGGCGCAATCCGCACC	Reverse primer for making lmh11
L26-P1	CGTTATTCTGTTGATCATGATGC	Forward primer for sequencing lactococcal part
L27-P2	GCATCATGATCAACAGAATAACG	Reverse primer for sequencing lactococcal part
L28-P1	CTTTGATAAGATTACAGGTGGC	Forward primer for sequencing lactococcal part 2
L29-P2	GCCACCTGTAATCTTATCAAAG	Reverse primer for sequencing lactococcal part 2
L30-P1	CGTTTATAAAGTTGACCATGATGC	Forward primer for sequencing enterococcal part
L31-P2	GCATCATGGTCAACTTTATAAACG	Reverse primer for sequencing enterococcal part
L32-P1	GCACTCGGCTCACTATTCAC	Forward primer for sequencing enterococcal part 2
L33-P2	GTGAATAGTGAGCCGAGTGC	Reverse primer for sequencing enterococcal part 2

2.10 Software

Software	Supplier
Geneious R 9.0.2	Biomatters Ltd.
NanoDrop 3.0.0	Thermo Scientific

2.11 Kits

Nucleospin® PCR Clean-up Gel Extraction kit — **Macherey-Nagel**

Binding Buffer NTI
Wash Buffer NT3
Elution Buffer
NucleoSpin® Gel and PCR Clean-up Columns (yellow rings)
Collection Tubes

Clarity ™ Western ECL Substrate — **Bio-Rad**

Luminol/enhancer solution
Peroxide solution

3. Methods

3.1 Scheme of work progression

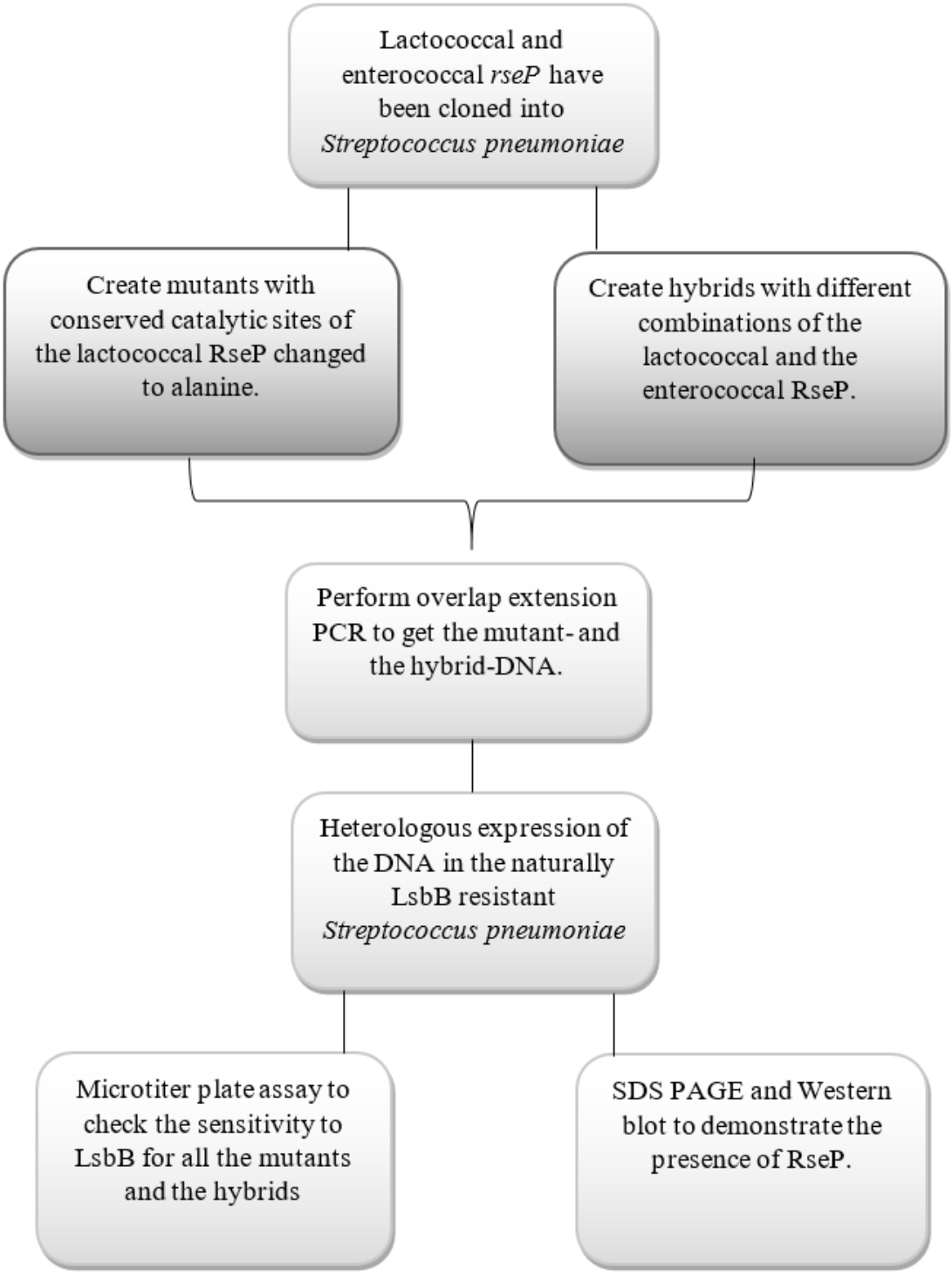

Figure 3.1. Overview of the steps performed in this thesis.

3.2 General methods in microbiology

3.2.1 Preparation of bacterial growth media

Bacteria need specific media to grow, and the media and agars were prepared according to the manufacturers' guidance. To sterilize the media and agars, they were autoclaved at 121°C for 1 hour. The liquid media was used for bacterial growth, either from frozen stocks or from single colonies. To prepare agar, agar powder was added to the media. After autoclaving, the agar was cooled down to 55 °C, and poured onto petri dishes (sterile), to prepare plates for bacteria streaking. The media and the agar were stored at room temperature, while the petri dishes were stored at 4°C.

3.2.2 Bacteria streaking

Bacteria streaking was performed to obtain pure cultures. Bacteria from frozen stocks were streaked out on agar plates with sterile plastic loops, in a pattern that diluted the bacteria into single colonies. The plates were incubated at 37°C over night (ON). Single colonies were picked up by sterile toothpicks to grow pure cultures.

3.2.3 Bacterial inoculation and cultivation

Bacteria from frozen stocks or from single colonies were grown in autoclaved liquid media (TH) at 37°C for 6-10 hours until OD_{550} of 0,3-0,4. Sterile plastic loops or sterile toothpicks were used to transfer the bacteria to the medium. When working with *S. pneumoniae*, it is important to be aware that these bacteria will autolyze if grown too long. This means that the bacteria need to be checked regularly, and can't be left ON.

3.2.4 Long term storage of cultures (glycerol stock)

Cells of *S. pneumoniae* were grown in TH till the OD_{550} was about 0,3. 1 ml of the culture was transferred to a 2 ml cryo-tube, and glycerol was added to a concentration of around 20%. The tube was vortexed and kept at -80°C. The glycerol was added to prevent formation of ice-crystals, that could have led to cell destruction at low temperatures.

3.2.5 Working stock

Working stocks were made to save time when bacterial cultures were needed for experiment. *S. pneumoniae* cells were grown from frozen stock in 3 ml TH at 37°C in a falcon tube until the OD_{550} was around 0,3. Glycerol was added to a concentration of 15%. The falcon tubes were vortexed, and kept at -20°C. When the cells were needed, the falcon tubes were centrifuged at 6000 rpm for 10 minutes, and the supernatant was thrown away. The cell pellet was resuspended in the TH broth, and grown until the working OD_{550} was reached. This would take shorter time than growing cultures from the glycerol stock.

3.2.6 Microtiter plate assay

Microtiter plate assay was performed to check the sensitivity to LsbB for all the mutants and hybrids, and then compare it with the wild type. This made it possible to define the minimum inhibitory concentration of LsbB that reduced the growth at least 50% (MIC_{50} value).

Procedure:

- 100 µl of TH broth + ComS were added in all wells using a multichannel pipette. The final concentration of ComS was 2µM.
- 95 µl of TH broth were added to all the wells in column 1.
- 5 µl of LsbB (2mg/ml) was added in all the wells in column 1.
- The first wells were mixed 5 times, before 100 µl were transferred to the next column. This was repeated until well 11. Instead of transferring 100 µl to well 12, the 100 µl was discarded. This resulted in a 2-fold dilution series of LsbB, from column 1-11. Well 12 did not have any bacteriocin, and worked as a negative control.
- 100 µl of 25 times diluted cells (diluted with TH broth), were added to all of the wells. (resulted in 50 times diluted cells).
- The plate was incubated in the reader ON, and was read every 30 minutes. Readable results were gotten after about 3-4 hours.

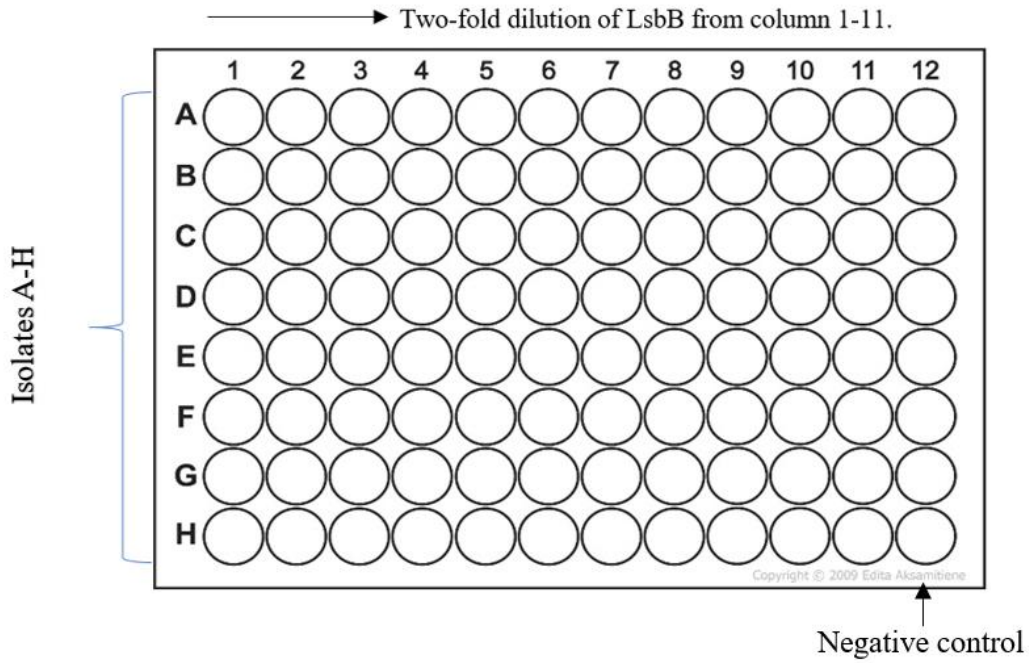

Figure 3.2. 96-well microtiter plate used to check the sensitivity to LsbB. A two-fold dilution of LsbB was made along the wells of each row from 1-11, leaving 12th row as a negative control. Using these results, MIC_{50}-values were estimated.

3.2.7 Transformation into *Streptococcus pneumoniae*

Heterologous expression was performed for all of the mutants and the hybrids. *S pneumoniae* is naturally resistant to LsbB, therefore introducing the LsbB receptor gene (*rseP*) with the different changes into *S. pneumoniae* will allow to verify the activity of LsbB to all the mutants as well as lactococcal-enterococcal receptor hybrids. *S. pneumoniae* is competent for natural genetic transformation, meaning that they can take up exogenous DNA. The *S. pneumoniae* strain used in this thesis has a Janus cassette which can be replaced by homologous recombination. In front of this Janus cassette is an inducible promoter, ComX, which makes it possible to control the expression of the inserted gene. This inducible titratable system was introduced in *S. pneumoniae* by (Berg et al. 2011), and the system is taken from *Streptococcus thermophilus*.

The mechanism where *S. pneumoniae* has the ability to take up exogenous DNA and incorporate it into its' genome, is called «competence for transformation» (Fontaine et al. 2010). Several related species in the phylogenetic mitis group, as well as *Streptococcus mutans* and all of the members of the Anginosus phylogenetic group is known to be

competent for natural genetic transformation (Berg et al. 2012). Some strains of pathogenic bacteria have this mechanism as part of their virulence factor (Fontaine et al. 2010; Li et al. 2008). The ComABCDE system responsible for exogenous DNA uptake in *S. pneumoniae* consist of the gene *comC*, which encodes the competence-stimulating-peptide (CSP), *comAB*, encoding its secretion and processing apparatus, *comD*, its transmembrane receptor, and *comE*, the cognate response regulator (Berg et al. 2012). *comX* encodes the alternative sigma factor ComX, that controls the transcription of the late competence genes, and is directly involved in the uptake and incorporation of exogenous DNA. The induction pathway is also controlled by some accessory mechanism, and in *S. pneumoniae*, these include ComW, HtrA, Clp proteins, the CiaRH- two-component system and the Ser-Thr protein kinase (fig. 3.3A) (Berg et al. 2012). *S. thermofilus* differs from other members of the Mitis and Anginosus group, in that it has a different competence induction pathway; the ComRS system (Fontaine et al. 2010).

The ComRS system starts with the production of pre-ComS, resulting in the pheromone ComS secretion out of the cell. The system also includes an Ami-transporter, that transports ComS inside the cell, and ComR, activated by ComS, leading to transcription of more ComS and ComX. ComX regulates the late competence genes (figure 3.3B) (Berg et al. 2011).

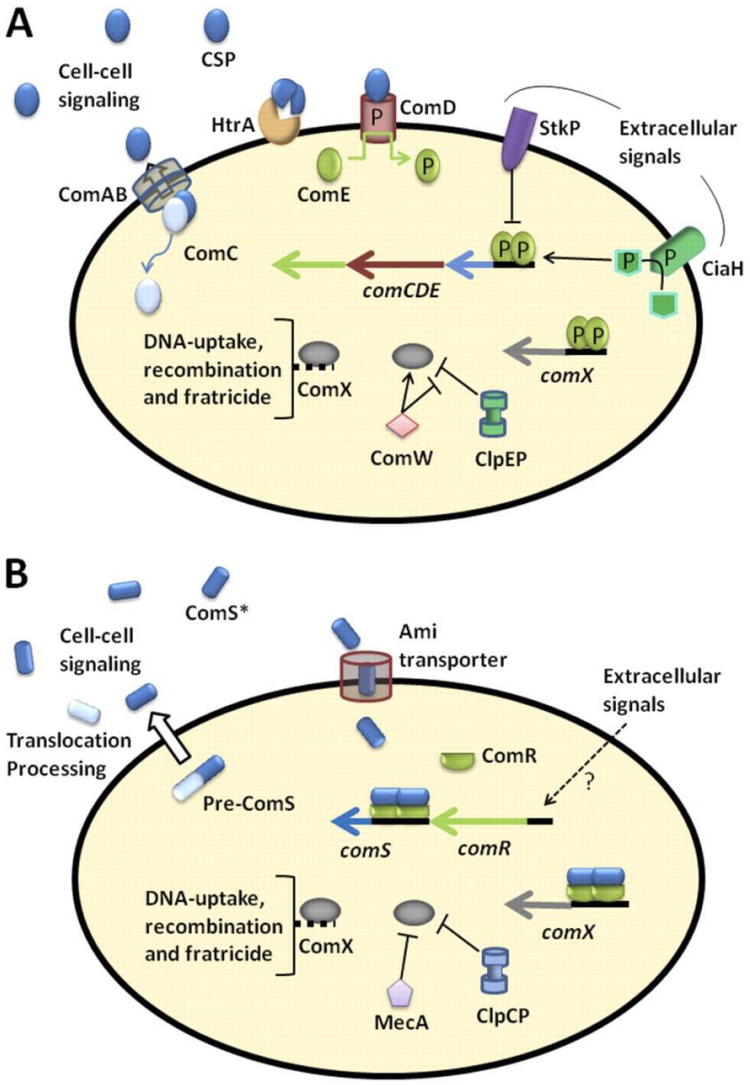

Figure 3.3. An illustration of the mechanisms controlling the natural transformation in *S. pneumonia*e (A) and *S. thermofilus* (B). (A) Extracellular signals are sensed by CiaRH and StkP, controlling the expression of comCDE. These genes encode CSP, its receptor ComD and its' response regulator ComE. When CSP accumulates outside the cell, ComD will phosphorylate ComE, resulting in more expression of comCDE and the CSP transporter ComAB. This will also activate transcription of comX, comW, and comM, among other genes. *comX* encodes the alternative sigma factor ComX, controlling the late stage of competence. ComW works as stimulation of ComX as well as protection of ComX from ClpEP protease. ComM protects the cell against CbpD, a late competent gene that is a part of disruption of incompetent cells. (B) *comS* encodes Pre-ComS, which is secreted by an unidentified transporter that is also responsible for the maturation of Pre-ComS to the pheromone ComS. The extracellular ComS is then transported into the cell by the Ami transporter. Whilst inside the cell, it activates the transcription of ComR, a transcriptional regulator. ComR binds to the promoter region of *comS* and *comX*, resulting in more ComS and transcription of the late competence genes. ClpC and MecA prevent the accumulation of ComX when the conditions are suboptimal. Figure adapted from Berg et al. (2012).

The *S. pneumoniae* strains that are used in this thesis have both of the previously mentioned systems in their genome, and they are used for different purposes. The ComABCDE system is used for the uptake of foreign DNA. By adding CSP to the environment, it is possible to provoke the cells to get competent, and they are able to take up the DNA. In addition to the ComABCDE system, which is naturally a part of the *S. pneumoniae* genome, a ComRS system was introduced to develop a regulated, titratable gene depletion system (Berg et al. 2011). When adding ComS to the environment, transcription of ComR will be activated, resulting in binding of ComR to the EComX site on P_{ComX} promoter. This again will result in transcription of the gene located downstream of the P_{ComX} promoter (figure 3.4). The strain used in this thesis, *S. pneumoniae* 1522, has a Janus cassette located downstream of the P_{comX} promoter. The Janus cassette contains kanamycin resistance marker, which selects for its acquisition, and a counter selectable $rpsL^+$ marker. This marker is based on a common spontaneous bacterial streptomycin resistance mutation in the rpsL gene. This mutation is recessive, which means that the $rpsL^+$ provided by presence of Janus cassette will give a streptomycin-sensitive phenotype. Therefore, the 1522 strain is kanamycin resistant (Kn^R) and streptomycin sensitive (Sm^S). When the Janus cassette is replaced by exogenous DNA, the situation is inverted and the strain becomes streptomycin resistant and kanamycin sensitive.

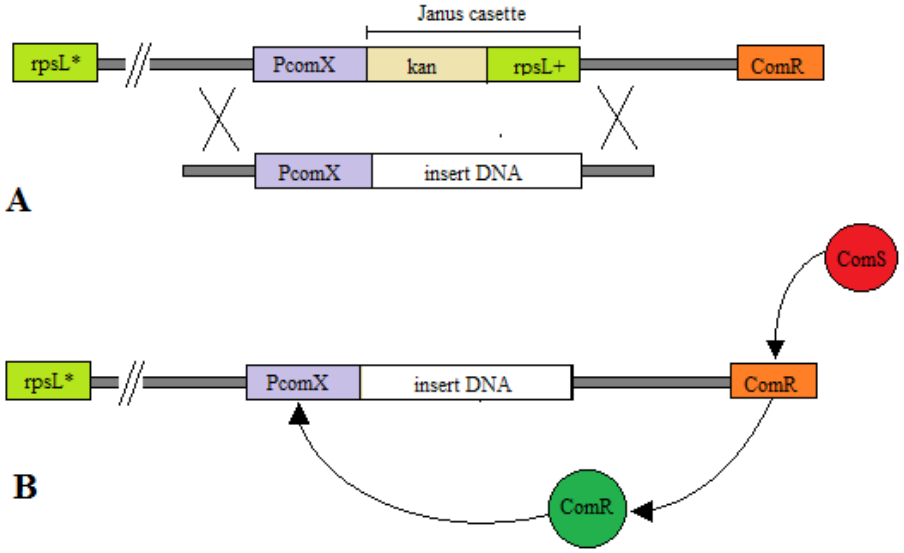

Figure 3.4. A) Replacement of the Janus cassette with the DNA of interest. The Janus cassette contains the kanamycin resistant (kan) gene, and $rpsL^+$-allele, which together with the rpsL* outside the Janus cassette, give a streptomycin sensitive phenotype. B) The transformation construct, and how the workflow of the induction system works. ComS is added and affects *comR*, leading to transcription of ComR. ComR binds to the EComX site on P_{ComX} promoter, resulting in transcription of the insert DNA.

Procedure:

- For each mutation, a volume of 0,5 ml TH-broth was needed. It was five mutations plus one negative and one positive control, making seven samples altogether. Therefore, 3,5 ml + 2ml of TH was needed, to make sure that the volume was enough for seven samples. The strain 1522 was grown in 5,5 ml TH in a falcon tube.

- There were six hybrids, plus one negative and one positive control, meaning that 4 ml+2ml TH needed to be prepared. 1522-cells were grown in 6 ml TH in a falcon tube.

- The 1522-cells were grown till OD was between 0,05- 0,1

- Eppendorf tubes were prepared, one tube for each mutation/hybrid, where 1,25 µl CSP was added in each tube

- At least 100 ng DNA from each mutation/hybrid was added to separate tubes (the DNA concentration was around 20 µl for each sample, so I added at least 5 µl in each tube).

- 0,5 ml of the 1522 culture was added in each of the Eppendorf tubes. The samples were mixed by turning the tubes upside down a few times.

- The Eppendorf tubes were incubated for 2 hours at 37°C.

- Petri dishes with TH agar with streptomycin 200 µg/ml were prepared in advance. After two hours of incubation, 30 µl of the cultures were plated out on the petri dishes, one petri dish for each mutation/hybrid. The petri dishes were put in an anaerobic jar with a gas packet, and were incubated at 37 °C ON.

- The next day, falcon tubes with 2 ml TH broth + streptomycin at a final concentration of 200 µg/ml were prepared.

- 10 colonies from each mutation/hybrid were picked up with sterile toothpicks, and put in a tube with TH + streptomycin.

- A PCR-screening was done for all the colonies. A forward primer inside the *rseP*-gene (L12) and the reverse primer khb34 downstream of the Janus cassette were used to see in which samples the transformation was successful.

- The samples where the transformation was successful were grown to an OD of around 0,3, and frozen stocks were made.

3.3 DNA-methods

3.3.1 Designing primers

Primers used in this thesis were designed for different applications: site directed mutagenesis in the lactococcal *rseP*, hybrids consisting of enterococcal and lactococcal *rseP*, and for PCR-product sequencing. Sequences of the two *rseP* genes from *E. faecalis* and *L. lactis* were already known, due to earlier sequencing. Beforehand of this thesis, the Janus cassette was replaced with the lactococcal *rseP*, creating strain OK1, and the enterococcal *rseP*, creating strain ds221 Ovchinnikov et al. (2017) These strains and their sequences were used when designing suitable primers for the right purpose. When designing primers, there are some criteria that have to be considered:

- The melting temperature should be between 60 and 66 °C. The melting temperature is calculated with equation:
  $T_M = 2(A+T) + 4(G+C)$
- The length of the primers should be between 18 and 30 nucleotides
- The primers should contain about equal amounts of the different nucleotides, where the C+G amount should be between 40-60%.
- The 3’ end should be G or C to get a strong binding. Otherwise, the priority of nucleotides is as follows: C>G>T>A. The 3’ end should at least not contain stretches of A and T.
- The primers should not be complementary to each other (to prevent primer dimers) or themselves (to prevent creation hairpins).

All the primers were ordered from Invitrogen. When receiving the primers, dH_2O was added to make a stock solution (0,1 mM). The primers were diluted to a concentration of 10µM before use. They were kept at -20 °C.

3.3.2 Directed mutagenesis

Site directed mutagenesis is a procedure used to make changes in the double-stranded DNA, and it was performed in this thesis. To do the mutagenesis, overlap extension PCR is performed, and it can be used for introducing insertions, deletions and substitutions (Heckman & Pease 2007). In this thesis, it was used for creating substitutions and hybrids. The overlap extension PCR is performed in two steps, where the first step produces two overlapping fragments. In the second step, these two fragments are combined and used as template. Primers to amplify the whole gene were used to make a full-length fragment with the mutation or with the hybrid on both DNA strands. Primers need to be designed to construct the two overlapping fragments.

Selected residues constituting the active site of RseP, were substituted with alanine by site-directed mutagenesis. This was done to verify the role of the active sites for the LsbB binding. The active site contains three conserved residues: H19-E20-x-x-H23, and five different mutants were made. The first three mutants had the three conserved residues changed one by one, the fourth mutation had a change outside the active site (to work as a control), while the fifth mutant had all the conserved residues changed. Figure 3.5 demonstrates the primer designing. For each mutation, there was designed a primer-pair: one forward and one reverse with the same mutation. This makes five primer-pairs and ten primers altogether. To make the two overlapping fragments, the forward primer khb31 (upstream of the *rseP*) and the designed reverse primer were used to make a 1 kb fragment, and the reverse primer khb34 (downstream of the *rseP*) and the designed forward primer were used to make a 2 kb fragment. Sequence of all the primers are shown in table 2.4.

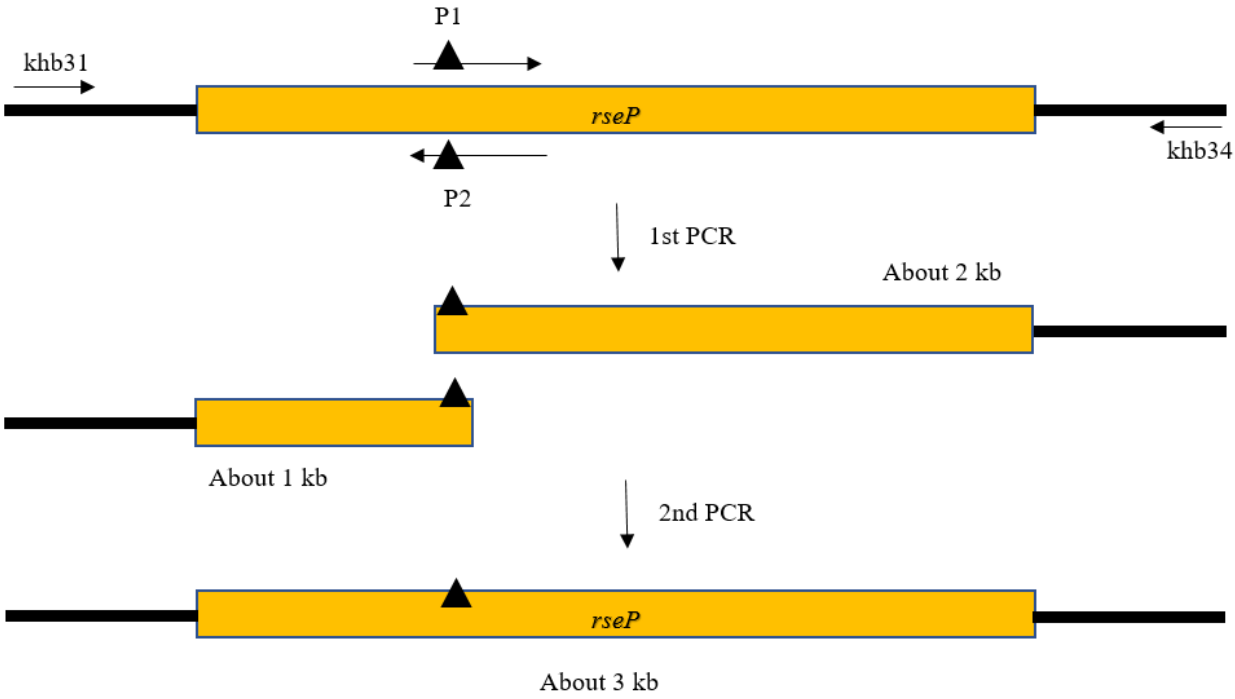

Figure 3.5. The work-flow for the site-directed mutagenesis. The horizontal arrows are primers, where P1 and P2 are the primers designed to make the mutation. The triangles demonstrate the mutations.

When designing primers for the hybrids, the four transmembrane helices were the main focus. The bacteriocin LsbB uses RseP as receptor, and kills only lactococcal cells. The bacteriocin EntEJ97, produced by enterococcal strains, kills both *L. lactis* and *E. faecalis*, also by targeting RseP. Therefore, it was of interest to identify which part of the *L. lactis* RseP that is important for binding of LsbB, the enterococcal RseP does not contain. Thus, by making combination of the lactococcal and the enterococcal *rseP*, it is possible to identify which part of the RseP makes *Enterococcus* resistant and *Lactococcus* sensitive to LsbB. Six primer pairs were designed, and all of the primers had one part complementary to lactococcal *rseP* and one part to the enterococcal *rseP*. The primers were designed in the way that the reverse primer used to amplify the lactococcal *rseP*, is used as a forward primer to amplify the enterococcal part and visa versa (figure 3.6).

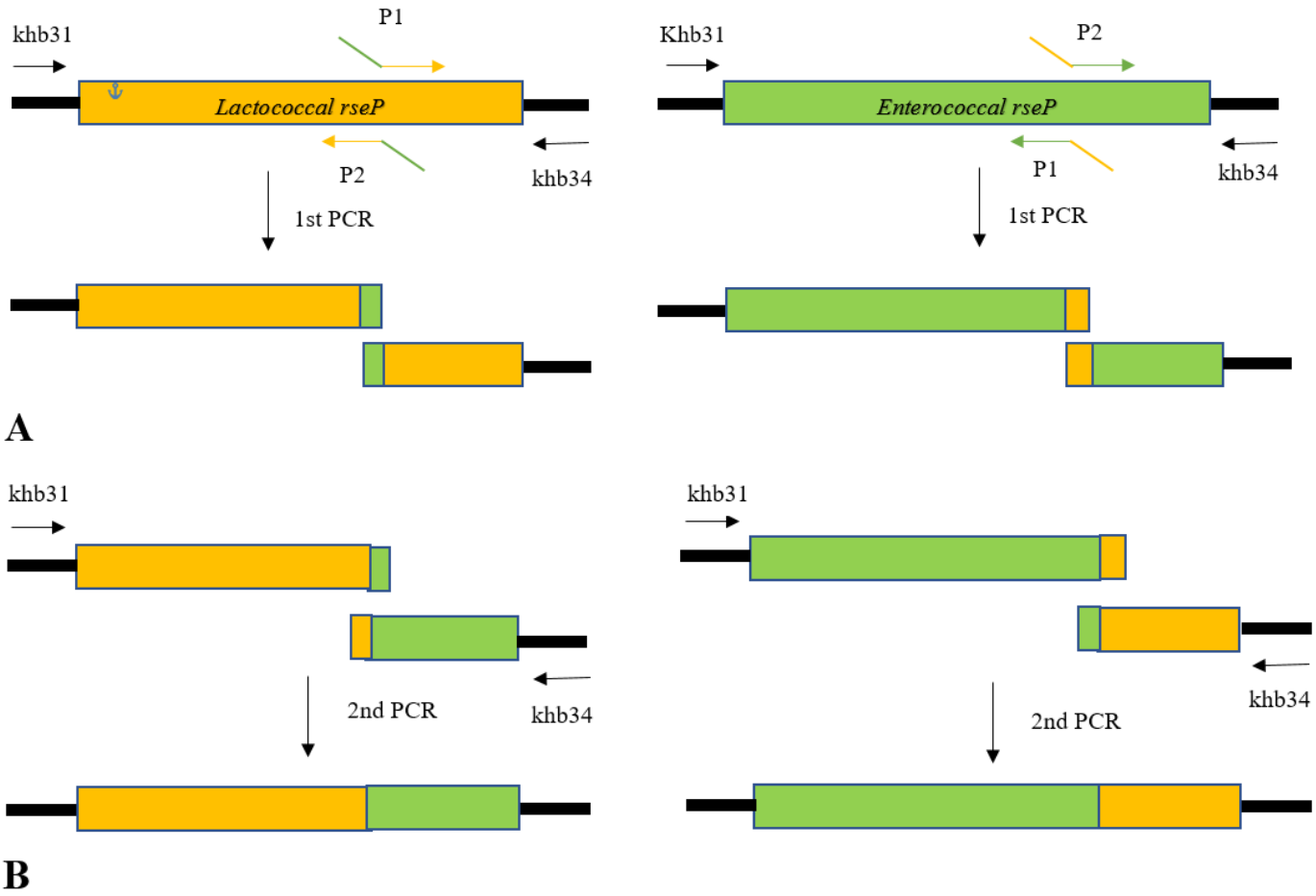

Figure 3.6. A) The first step in the overlap PCR. This step results in two fragments of the lactococcal *rseP*, with short overlapping parts from the enterococcal *rseP*, and two fragments of the enterococcal *rseP*, with short overlapping parts from the lactococcal *rseP*. Primer khb31 and the reverse designed primers (P2/P1) are used to get half of the fragments, whilst primer khb34 and the forward designed primers (P1/P2) are used to get the other half of the fragments. B) The second step PCR. The fragments with the lactococcal part are combined with the fragments containing the enterococcal part, to get a full length fragment.

3.3.3 Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) is used to amplify fragments of DNA. In this thesis, PCR was run for different purposes: to construct fragments with point-mutations in the lactococcal *rseP* and create fragments for their transformation into *S. pneumoniae*, to construct fragments resulting in hybrids of the enterococcal and lactococcal *rseP* and to create fragments for their transformation into *S. pneumoniae*, and to check if the transformations were successful. To make the fragments for transformation, overlap-extension PCR was performed.

Procedure:

The whole procedure was performed on ice.

- A mastermix was made according to the tables listed under depending on the reaction that was run. The total volume made of the mastermix depended on how many PCR-reactions that,were going to be run. The volume was always adjusted up one or to samples, in case of pipetting inaccuracy or other mistakes.
- 0,2 ml PCR tubes were marked, and the right amount of mastermix, and template were transferred to them. The total volume was 50 µl or 25µl depending on the reaction.
- The tubes were spun down, to remove bubbles that could disturb the reaction.
- The programs used on the PCR machine varied depending on the reaction that was conducted (table 3.1).

Table 3.1. Different programs and the different mastermixes used for the different PCRs performed in this thesis.

Procedure for the first step PCR constructing the two overlapping fragments.				
Mastermix		**Program**		
Component	Volume (µl)	Temperature	Time	Cycles
5x Phusion buffer	10	98 °C	2 min	1
dNTP	1	98 °C	30 sec	30
Forward primer 1 (10µM)	2,5	60 °C	30 sec	30
Reverse primer 2 (10µM)	2,5	72 °C	1 min 30 sec	30
Enzyme Phusion	0,25	72 °C	5 min	1
Template	1	4 °C	∞	
Water	32,5			
Total volume	50			

Procedure for the second step PCR to amplify the whole *rseP* gene.				
Mastermix		**Program**		
Component	Volume (µl)	Temperature	Time	Cycles
5x Phusion buffer	10	98 °C	2 min	1
dNTP	1	98 °C	30 sec	30
Primer 31 (10µM)	2,5	58 °C	30 sec	30
Primer 34 (10µM)	2,5	72 °C	1 min 30 sec	30
Enzyme Phusion	0,5	72 °C	5 min	1
Template	1 of each fragment	4 °C	∞	
MgCl	2			
Water	30,5			
Total volume	50			

Procedure for the check if the transformation was successful.				
Mastermix		**Program**		
Component	Volume (µl)	Temperature	Time	Cycles
OneTaq buffer	5	94 °C	30 min	1
dNTP	0,5	94 °C	30 sec	30
Primer L12 (10µM)	0,5	58 °C	30 sec	30
Primer 34 (10µM)	0,5	68 °C	2 min	30
OneTaq polymerase	0,125	72 °C	5 min	1
Template	1 of each fragment	4 °C	∞	
Water	18,375			
Total volume	25			

3.3.4 Agarose gel electrophoresis

Agarose gel electrophoresis was run to check if the PCR products had the right size and to check if the purification of PCR/gel-products was successful. DNA is negatively charged, and by using electric force, it will move towards the positive pole. It will be separated by size, since the smaller molecules will move faster than the large ones. The percentage of gel depends on the sizes of the fragments that are meant to be separated; the larger fragments, the lower percentage. In this thesis, the fragments that were between 1kb and 3kb, and 0,8% agarose gel was used. Peq-Green was added to the gel to label the DNA, and when exposing the gel to the UV-light, the DNA was visualized. By adding a ladder, which contains DNA of known sizes, the size of the separated bands can easily be estimated.

Procedure:

- 0,8 % agarose gel was made by adding 0,8 grams of agarose per 100 ml 1xTAE buffer, and boiled in the microwave until dissolved. Peq-Green dye was added, 2 µl per 50 ml gel. The gel was kept in water bath at 50 °C for further use.
- The liquid agarose gel was poured into a gel moulding form with a comb to make wells. The gel was left for 20 minutes to solidify.
- The gel was placed in the electrophoresis vessel, and the vessel was filled with 1xTAE buffer, so that the whole gel was covered.
- The comb was removed, and ladder (1kb) and samples with loading buffer were loaded into the wells
- The lid was put on the vessel, and plugged into the power source. The voltage was set as 80 V, and the running time varied depending on what size the product had, and how well separated the products should be. For 3kb fragments, the gel was run for about 1 hour to obtain sufficient separation.
- The gel was removed from the vessel, and was exposed to UV-light, to visualize the products.

3.3.5 PCR-product/gel-electrophoresis clean up

The products after the agarose gel electrophoresis were cut and purified, to get clean and non-contaminated DNA-samples. When sending samples for sequencing, the clean-up was done right from the PCR-product (if the products looked clean when run on the gel, meaning there were no unspecific bands). The clean-up was done with NuceloSpin® Gel and PCR Clean-up kit.

Procedure:

- The gel was run according to the procedure described above. There was always one well between each sample, to avoid cross-contamination.
- The gel was exposed to UV-light, and the desired bands were cut using scalpels. The cutting had to be fast, and the UV-light must be turned off in between the samples, because UV-light damages DNA.
- The cut gel-piece was put in an Eppendorf tube of known weight.
- The Eppendorf tube with the gel-piece was again weighted, to find out the size of the gel.
- The purification was done using NuceloSpin® Gel and PCR Clean-up kit. The procedure was performed according to the manufacturers' recommendations:
    - 200µl of NTI3 (binding buffer) was added per 100 mg gel (or twice the amount of the liquid PCR product).
    - It was heated at 50 °C for maximum 10 minutes, for the gel to dissolve (this step was skipped if the PCR product was purified).
    - The samples were transferred to the column and centrifuged at 11.000 xg for 30 sec.
    - The liquid was discarded, and 600 µl of NTI buffer (washing buffer) was added.
    - It was centrifuged at 11.000 xg for 30 sec.
    - The NTI buffer was discarded, and the washing step was repeated one more

time.

- After the NTI buffer was discarded the second time, the columns were centrifuged at 11.000 xg for 1 min, for the columns to dry.
- The liquid was discarded and the column was put into new Eppendorf tubes.
- The NE-buffer (Elution buffer) was preheated to 70 °C. 15 µl of the NE-buffer was added to each column. The tubes were incubated for 5 minutes, and centrifuged at 11.000 xg for 1 minute.
- The elution step was repeated one more time.
- Now there will be 30 µl of DNA in each Eppendorf tube.

3.3.6 Quantification of nuclei acids

The DNA-concentration and the purity of the DNA were measured using NanoDrop ND-100 after the gel/PCR product clean-up. The instrument was blanked with the elution buffer, before adding 2µl of the sample on the pedestal for measurement. The NanoDrop measured the absorption at 260 nm, which is the wavelength DNA has the maximum absorbance. The concentration of DNA was given in ng/µl. The purity of the DNA was established as a 260/280 ratio and 260/230 ratio absorbance. The 260/280 ratio should be between 1,8 and 2,0, where lower ratios indicate the presence of protein or phenols in the sample and higher values indicate the presence of RNA. The 260/230 ratio is commonly between 2,0 and 2,2, and lower values indicate presence of carbohydrates and/or phenol in the sample.

3.3.7 Sequencing of *rseP*

Sequencing of the mutated *rseP* was performed to confirm that the sequences were correct, and GATC Biotech (http://www.gatc-biotech.com) was the company providing sequencing services. This company conducts Sanger sequencing, and the SUPREMErun sequencing service was used. To sequence the *rseP* gene, three forward primers (one upstream, one in the middle and one at the end of the gene), and three reverse primers (one downstream, one in the middle and one in the beginning of the gene) were used to get good coverage of the whole gene. Sanger sequencing can give good result for about 1000 nucleotides, and the *rseP* gene is

around 3000 kb. Therefore, three forward and three reverse primers were used to make sure that all parts were equally well sequenced. To prepare samples for sequencing, the purified PCR-products were diluted to the right concentration (20-100 ng/µl). In each Eppendorf tube, there was 5 µl of a primer (5µM) and 5 µl of DNA. GATC barcodes were attached to the tubes, to make it possible to identify the different samples when the sequencing data were available in the GATC database.

3.4 Protein methods

3.4.1 Protein lysate

Protein lysate was obtained by lysing the cells mechanically using FastPrep Homogenizer. Different methods to isolate proteins were tried, including boiling the cells with SDS-sample buffer and lysozyme, however, The FasPrep Homogenizer gave the best result. The FastPrep lyse the cells by shaking the FastPrep tubes with acid washed glass beads. The protein lysate was conducted on all of the five mutants, all of the six hybrids and for OK1 as a positive control. Samples with and without added ComS were prepared, to identify the difference in expression of the *rseP* gene.

Procedure:

- The cells were grown in 50 ml TH in falcon tubes to an OD_{550} of 0,05.
- The cultures were split in two, and ComS was added to one of the cultures (2µM final concentration).
- The cells were grown to an OD_{550} of 0,4.
- The cultures were centrifuged at 6000x at 4 °C for 10 minutes.
- The cell pellet was resuspended in 600µl 1xTBS buffer
- The cells and TBS buffer were transferred to FastPrep tubes, with 0,5g of glass beads.
- The FastPrep was run for 20 seconds at 4 m/s, then paused for 5 minutes, and then repeated two more times.

- The FastPrep tubes were centrifuged at 5000x for 5 minutes. The supernatant was transferred to Eppendorf tubes.

3.4.2 Quantification of proteins

The amount of proteins was measured with NanoDrop at 280 nm. At 280 nm the amino acids tryptophan, tyrosine and to some extent cysteine have absorption. Therefore, using NanoDrop to measure, one can estimate protein amount based on average frequency of these amino acids. The NanoDrop was blanked with 1xTBS buffer. 2 µl of the supernatant from the protein isolation was added to the pedestal of the NanoDrop. The amount of protein is given in mg/ml.

3.4.3 SDS-PAGE

SDS-PAGE is SDS-Polyacrylamid gel electrophoresis. Electrophoresis is a term used when charged molecules move as a result of an electric charge, and then get separated. The proteins move to the electrode with opposite charge. Proteins have different shapes, sizes and electric charge, which can affect the separation. Electrophoresis that is performed in acrylamide gel is called polyacrylamide gel electrophoresis (PAGE), and separates proteins by size. There are different types of PAGE, which includes Blue Native (BN) PAGE, Zymogram PAGE, Discontinuous PAGE and SDS-PAGE (Bio-Rad n.d.-a). Here, SDS-PAGE was used. Sodium dodecyl sulfate (SDS) is a denaturing agent, that denatures the proteins fully, resulting in a long and rod-shaped structure of the protein (figure 3.7). SDS also binds to the protein noncovanently, and give the proteins a negative charge which is consistent with the protein's mass. This leads to the movement of the proteins to the positive pole, separated by mass.

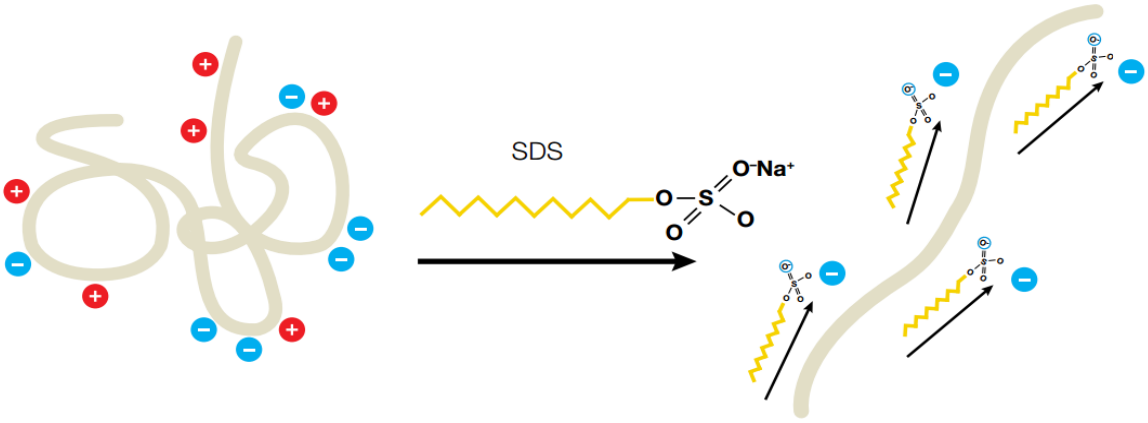

Figure 3.7. SDS binds to the protein, resulting in denaturation, and a negative charge. The protein will move to the positive pole, and will be separated by size. Picture adapted from Bio-rad (n. d.-a).

Table 3.2. Amounts of the different components for 10% resolving gel and the 4% stacking gel preparation.

Percent gel	**dH_2O**	**Acrylamide**	**Gel buffer***	**10% SDS**	**10% APS**	**TEMED**
10 %	4,85 ml	2,5 ml	2,5 ml	100 µl	50 µl	5,0 µl
4 %	3,20 ml	0,5 ml	1,25 ml	50 µl	25 µl	5,0 µl

* Resolving gel (10%): 1,5 M Tris pH=8.8

* Stacking gel (4%): 0,5 M Tris pH=6,8

Procedure:

- The glass plates were assembled in a gel caster.
- dH_2O, acrylamide, gel buffer and SDS were mixed together, according to table 3.2.
- Before APS and TEMED were added, it was important that everything was ready to apply. When APS and TEMED are added, the polymerising starts.
- When everything was mixed, 3,2 ml of the resolving gel was added to the precasted glass plates.

- The stacking gel was added on top of separating gel, until the glass was full.
- The comb was put in between the glass plates.
- Gel was left for 45 minutes to ensure total polymerisation.

Running the gel

- The comb was removed, and the electrophoresis cell was assembled.
- The running buffer was added, so that it filled the inner and the outer buffer chambers.
- 100 µg of proteins were applied to each well in the gel, and run on 150 V for 1-1 ½ hour.
- After the electrophoresis, the gel cassettes were opened, and the gel was transferred to a box with Comassie Brilliant Blue G-250.
- It was heated in a microwave to the boiling point, and then incubated with shaking for 30 minutes.
- The Comassie was poured off, and the gel was washed with dH_2O.
- The destaining solution was added and heated in a microwave to the boiling point.
- It was incubated over night with shaking, with a small piece of paper tissue that would pull in the dye.

3.3.4 Western blot

Western blot is a procedure that allows to detect a specific protein. The procedure starts with separating the proteins by electrophoresis, before the proteins are transferred to a membrane (Mahmood & Yang 2012). The membrane is incubated with a blocking solution to block the unoccupied places on the membrane. As a next step, primary antibody, that is specific to the protein of interest, is applied. Then the membrane is incubated with a secondary antibody which binds to the first antibody (Bio-Rad n.d.-b). The secondary antibody is conjugated to an enzyme or radioactive isotopes, that makes it possible to detect the protein, using fluorescent, colorimetric or chemiluminescent detection (Thermo Fisher Scientific 2016) (figure 3.8). The primary antibody used here, was received from a research group in Serbia

(Miljkovic et al. 2016). The strain *L. lactis* subsp. *lactis* BGMN1-5 was used to make the primary antibody, which consisted of two separate antibodies that binds to different parts of RseP. The secondary antibody used, was anti-mouse IgG.

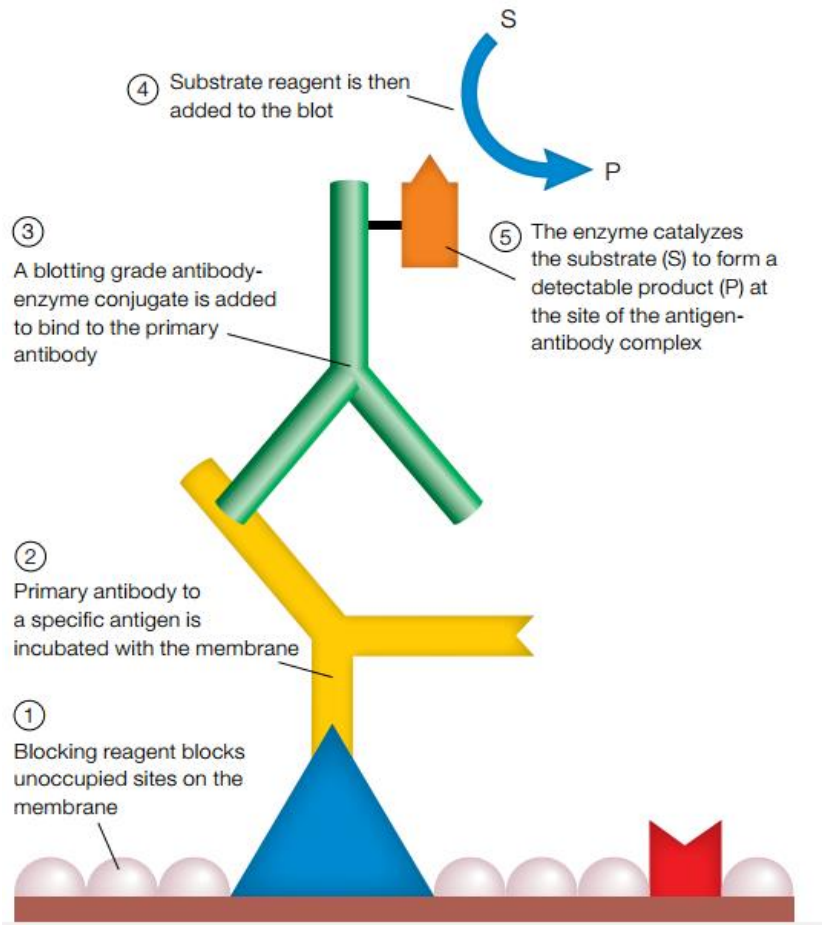

Figure 3.8. A schematic illustration of the western blot procedure. Picture adapted from Bio-Rad (n.d.-b).

Procedure:

- 100µg proteins were added to each well, and SDS-PAGE was run, as described above, 150V for around 45 minutes.
- The gel cassettes were opened, and the stacking gel was cut away.
- The gel was put in a box with transfer buffer, together with four Whatman papers (the same size as the gel) and two sponges.

- A membrane was cut to the same size as the gel. The membrane should not be contaminated, so a tweezer must always be used when handling the membrane.
- The membrane was put in methanol for 30 seconds to get activated. Then, it was put in the transfer buffer with the other components.
- When all the components were wet, they were put together as follows:
  1. The black side of the blotting cassette was put in the box with transfer buffer.
  2. One sponge was placed on top of the blotting cassette.
  3. Two Whatman papers were placed on top of the sponge.
  4. The gel was put on top on the Whatman papers and air bubbles were removed.
  5. The membrane was placed on top of the gel.
  6. The last two Whatman papers were placed on top of the membrane and air bubbles were rolled out.
  7. The last sponge was put on top, and the cassette was locked.
- The cassette was put in the buffer chamber (black towards black).
- The chamber was filled with transfer buffer and ice blocks were put in to keep it cold.
- The buffer chamber was placed in icewater, and a magnet was put in the chamber for stirring.
- The transfer was run at 90V for 1 hour.
- After the transfer was complete, the membrane was put in blocking buffer. It is important that the side of the membrane that contains the proteins is faced upwards.
- The membrane was incubated in blocking buffer with shaking for 15 minutes, before it was incubated at 4 °C over night.

First antibody binding:

- The next day: The blocking buffer was removed
- 3,33 µl of the Yvjb first antibody (included two antibodies, binding different parts of the RseP) was added to 10 ml of 5% skimmed milk diluted in TBST (1:3000 dilution)
- This solution was poured over the membrane, and incubated for an hour at shaking.

- The membrane was washed with washing buffer (TBST), and left for 15 minutes with shaking.
- The washing buffer was discarded, and the washing step was repeated three more times.

Anti-mouse IgG binding

- 1µl anti-mouse IgG was added to 10 ml of 5% skimmed milk diluted in TBST (1:10000 dilution).
- The solution was poured over the membrane, and incubated with shaking for 1 hour.
- The skimmed milk with anti-mouse IgG was discarded.
- The membrane was washed with washing buffer on shaking for 15 minutes, and then thrown away.
- The washing step was repeated three more times.

Detection

- The two detection-solutions were mixed well (1:1).
- It was poured over the membrane and incubated for 1 minute.
- A picture was taken by using Azure c400 GelDoc.

4. Results

4.1 Mutants

4.1.1 Site directed mutagenesis

Mutations at the catalytic sites in the lactococcal *rseP* were introduced to verify the importance of these sites in the binding of the bacteriocin LsbB. Five different mutants with point mutations were created by performing overlap extension PCR. The active site of RseP contains three conserved residues. In the first three mutants, these residues have been changed one by one, the fourth mutant had a residue changed outside the active site, while the fifth mutant had changed all three of the conserved sites (figure 4.1). The first step PCR resulted in two fragments for each mutation: one of 1 kb and one of 2 kb (figure 4.2). The two fragments with the same mutation had overlapping parts. The bands obtained from the first step PCR, were cut and purified using the NuceloSpin® Gel and PCR Clean-up kit. The purified products were used as a template in the second step PCR, where the two overlapping fragments with the same mutation, were combined and fused. Primers outside the gene were used (khb31 and khb34) to amplify the whole gene, resulting in a 3 kb fragment (figure 4.3). These products where cut from the gel, and purified using NuceloSpin® Gel and PCR Clean-up kit. The resulting PCR-products were used for transformation into *S. pneumoniae*.

WT: IIIFGIIVAIHEYGHLWWAKR
Mutation 1: IIIFGIIVAIAEYGHLWWAKR
Mutation 2: IIIFGIIVAIHAYGHLWWAKR
Mutation 3: IIIFGIIVAIHEYGALWWAKR
Mutation 4: IIIFGIIVAIHEYGHLAWAKR
Mutation 5: IIIFGIIVAIAAYGALWWAKR

Figure 4.1. The sequence of the wild type (WT) and the five different mutations. The catalytic residues are marked in green, while the residues that were mutated to alanine are marked in red.

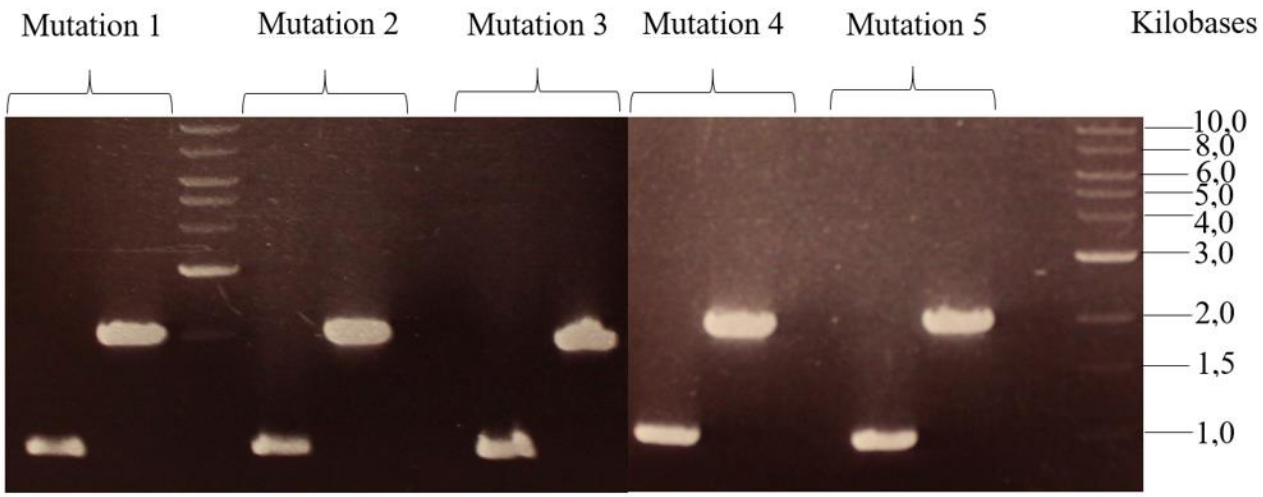

Figure 4.2. Fragments obtained from the first step PCR. For each mutation, there were two overlapping products: one at 1 kb and one at 2 kb.

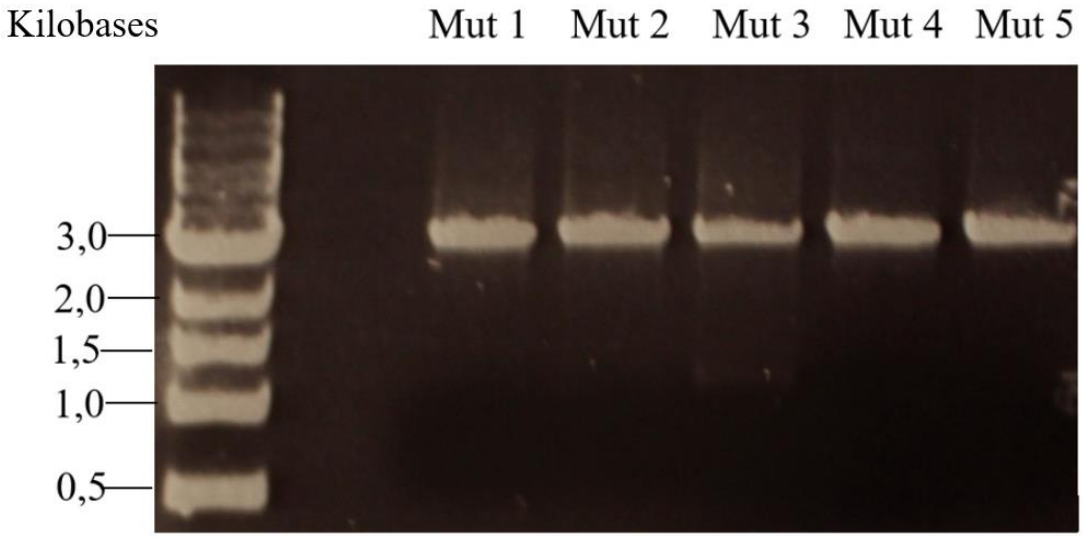

Figure 4.3. Fragments from the first step PCR (1 kb and 2kb) were merged in the second step PCR, and gave a final product of 3.

4.1.2 Heterologous expression in *Streptococcus pneumoniae*

After the site directed mutagenesis, the transformation construct was transformed into *S. pneumoniae,* in order to verify level of resistance to LsbB for different mutations. The Janus cassette present in strain 1522 was replaced with the mutated *rseP* and expressed by the ComS induction system. One transformation was done for each mutation. The whole transformation process was conducted according to the procedure described in section 3.2.7, with the inserts created according to the procedure described in section 3.3.2. To select for the transformants, streptomycin was added to the agar. When the Janus cassette is successfully

replaced with the mutated *rseP* gene, the phenotype changes from streptomycin sensitive to streptomycin resistant. This will result in growth of the transformants on the streptomycin plates. However, *S. pneumoniae* also has the ability to become spontaneously resistant. This means that it is expected to have some colonies on the negative control plate as well. Therefore, to evaluate if the transformation was successful, there should be at least twice as many colonies on the mutant-plate, compared to the negative control. A good example on how the plates should look like, is presented in figure 4.4.

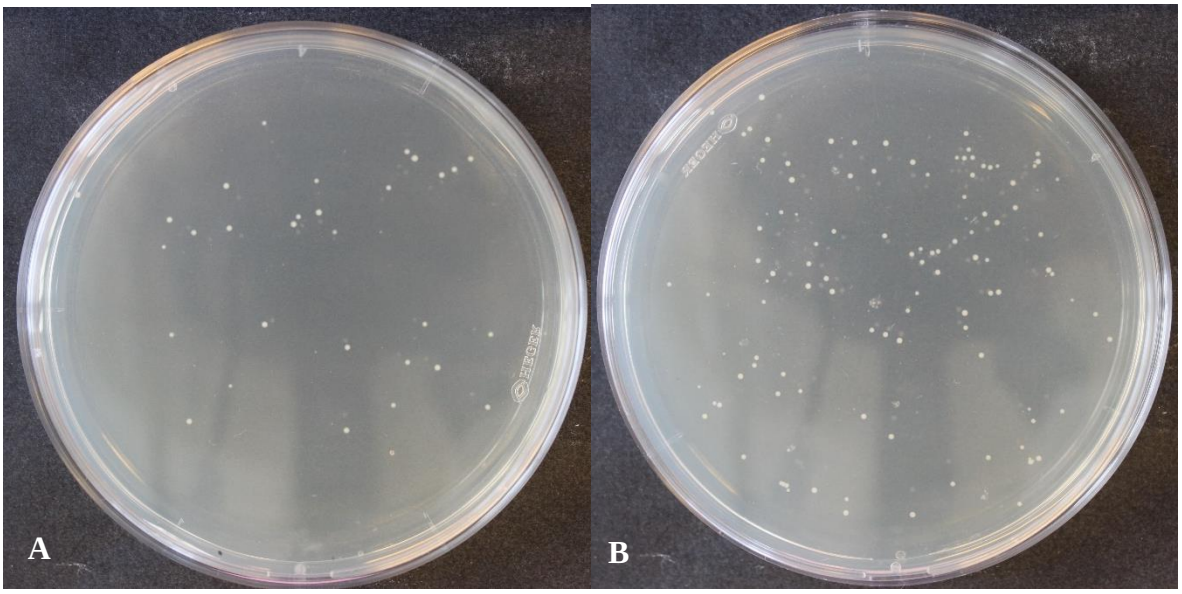

Figure 4.4. The plates with the colonies after transformation, negative control (A) and the mutation 1 (B), demonstrate that the plate with the transformants had at least twice as many colonies compared to the negative control.

To verify if the transformation was successful, ten colonies were randomly picked from the plates and a PCR was performed. A forward primer inside the *rseP* gene (L12) was combined with the reverse primer khb34, and a fragment of 1,6 kb was expected. The results demonstrated that at least one transformant for each mutation had successfully inserted the mutated *rseP*. Figure 4.5 shows the results for mutation 2, which has 4 positive reactions. The mutants were confirmed by sequencing. One mutant from each mutation was chosen to be analysed further.

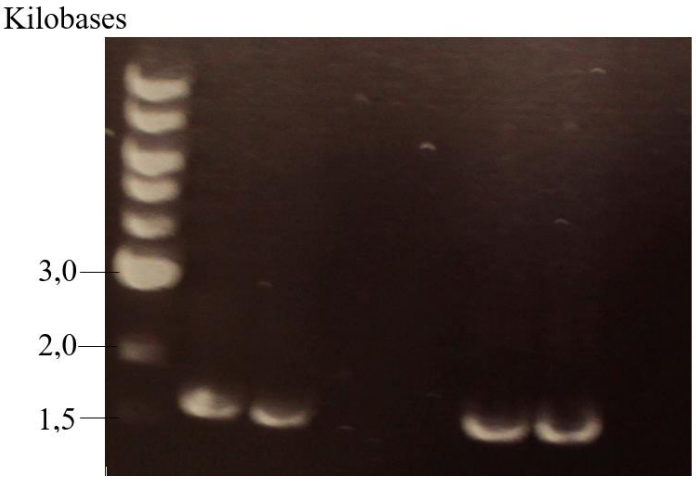

Figure 4.5. A colony-PCR was performed to confirm that the *rseP* gene was successfully transformed into *S. pneumoniae*. The picture shows gel after electrophoresis of the PCR product. The expected size of the band was 1,6 kb, and the picture shows that for mutation 2, the *rseP* had been successfully inserted in four of the colonies.

4.1.3 Microtiter plate assay

Microtiter plate assay with two-fold dilutions of LsbB was performed to estimate the bacteriocin resistance levels of the mutants. The results show that the strains expressing RseP where the residues in the conserved site was substituted with alanine (H19-E20-x-x-H23), are more resistant than the strain expressing the WT RseP (OK1). The strain expressing the RseP with substitution outside the active site (W25>A), is as sensitive to LsbB as the strain expressing the WT RseP (OK1). The MIC_{50} values for all of the mutants was calculated, and are shown in table 4.1.

Table 4.1. MIC_{50} values of resistance levels for the mutants, calculated based on the results from the microtiter plate assay.

Isolate	**Mutation**	**Mic_{50} (ng/ml)**
OK1	WT *rseP*	< 3
1522	No *rseP*	> 50000
lmh1	H19>A	1560
lmh2	E20>A	3125
lmh3	H23>A	6250
lmh4	W25>A	< 3
lmh5	HExxH>AAxxA	50000

4.1.4 SDS-PAGE

SDS-PAGE and western blot were performed to show that *rseP* was expressed and present in the cells. It was run for all of the mutants, as well as for OK1 and for *L. lactis* subsp. *lactis* BGMN1-5. OK1 was used as a positive control. The *L. lactis* subsp. *lactis* BGMN1-5 was the strain used for the YvjB- antibodies preparation (Miljkovic et al. 2016), therefore it was also included as a positive control. Before SDS-PAGE was run, protein samples needed to be prepared. All the mutants and OK1 had two samples: one with added ComS to induce the production of *rseP*, and one control without inducer peptide. The *L. lactis* strain was the strain that the cloned *rseP* gene originated from, and the protein was therefore expressed from the native promoter. Thus, only one sample was prepared for the *L. lactis* cells. After cell-destruction, the amount of protein was measured, to equalize the samples when running SDS-PAGE.

While performing the SDS-PAGE, about 100 µg of proteins from both the induced and the uninduced samples were loaded. The gel coloured with comassie staining demonstrates that the amount of proteins was approximately the same for all of the samples, except from the sample from *L. lactis* (figure 4.6). There is no visible difference between the induced and the uninduced samples. This makes it hard to tell whether RseP is present, based on comassie

stained gel. Therefore, by performing western blot, the RseP can be specifically visualised. Because the amount of protein in all samples was approximately the same, it was possible to compare visualised RseP from different samples.

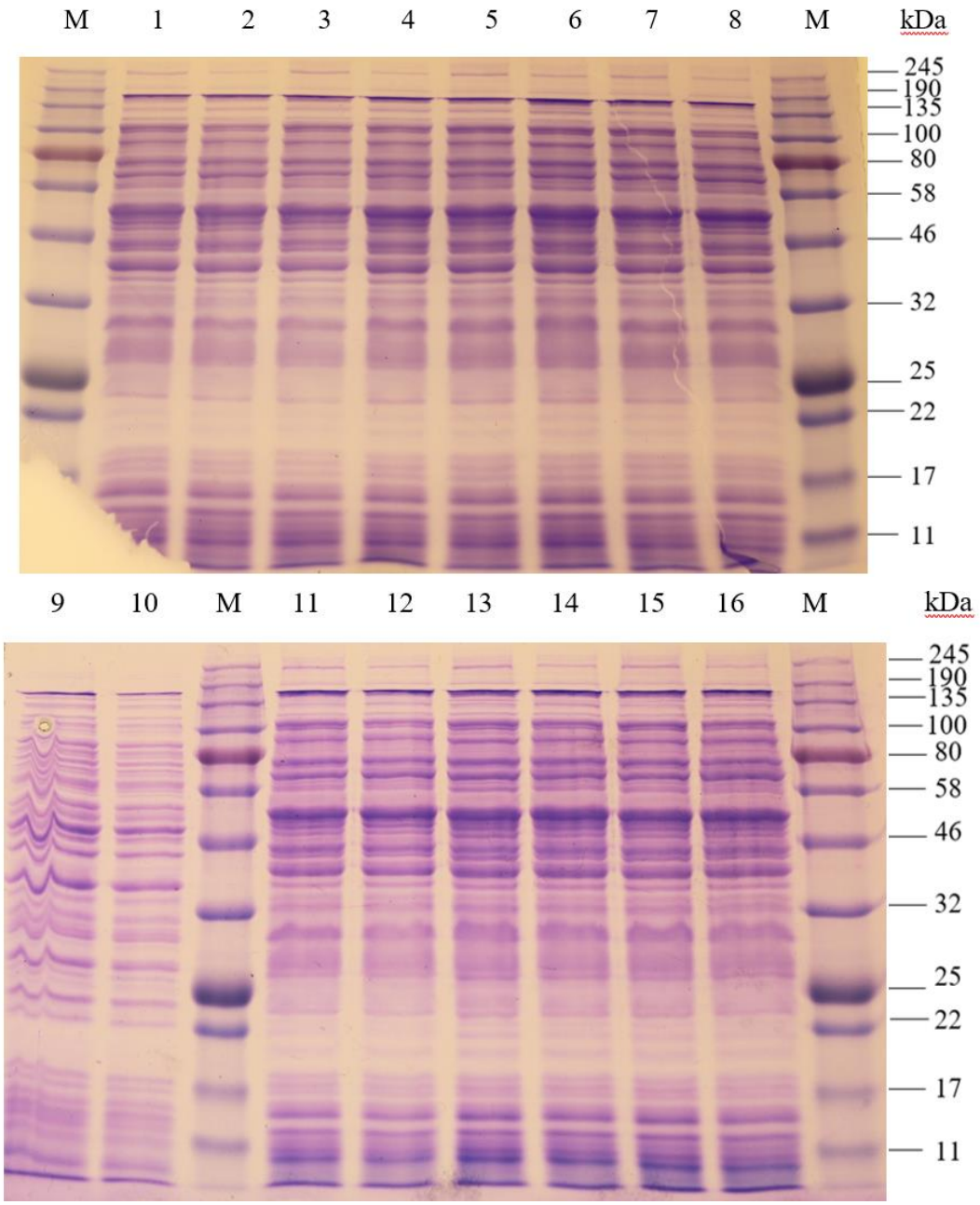

Figure 4.6. The gel after electrophoresis and comassie colouring. M) Marker; 1) OK1; 2) OK1+ComS; 3) Mutation 1; 4) Mutation 1+ComS, 5) Mutation 2; 6) Mutation 2+ComS, 7) Mutation 3; 8) Mutation 3+ComS; 9) L. lactis 100 µg; 10) L. lactis 50 µg; 11) OK1; 12) OK1+ComS; 13) Mutation 4; 14) Mutation 4+ComS; 15) Mutation 5; 16) Mutation 5+ComS.

4.1.5 Western blot

After the SDS-PAGE, western blot was performed to prove the presence of RseP in the cells. The proteins from the gel were transferred to the membrane, and the membrane was incubated with YvjB-antibodies. After incubation with the secondary antibody detection solutions were added to visualize the proteins. RseP has a mass of 46 kDa. The results show big bands at the right size for all the induced samples, however no bands are shown for the uninduced samples, meaning that the expression of RseP did not reach a detectable level without ComS (figure 4.7). There seem to be no difference in the expression between the positive control OK1 and the different mutations. When looking at the samples from *L. lactis*, a very small band at the right size is visible when 100 µg proteins were added, however the band is very thin. In addition, there are some bands between 22 and 25 kDa in all of the induced samples.

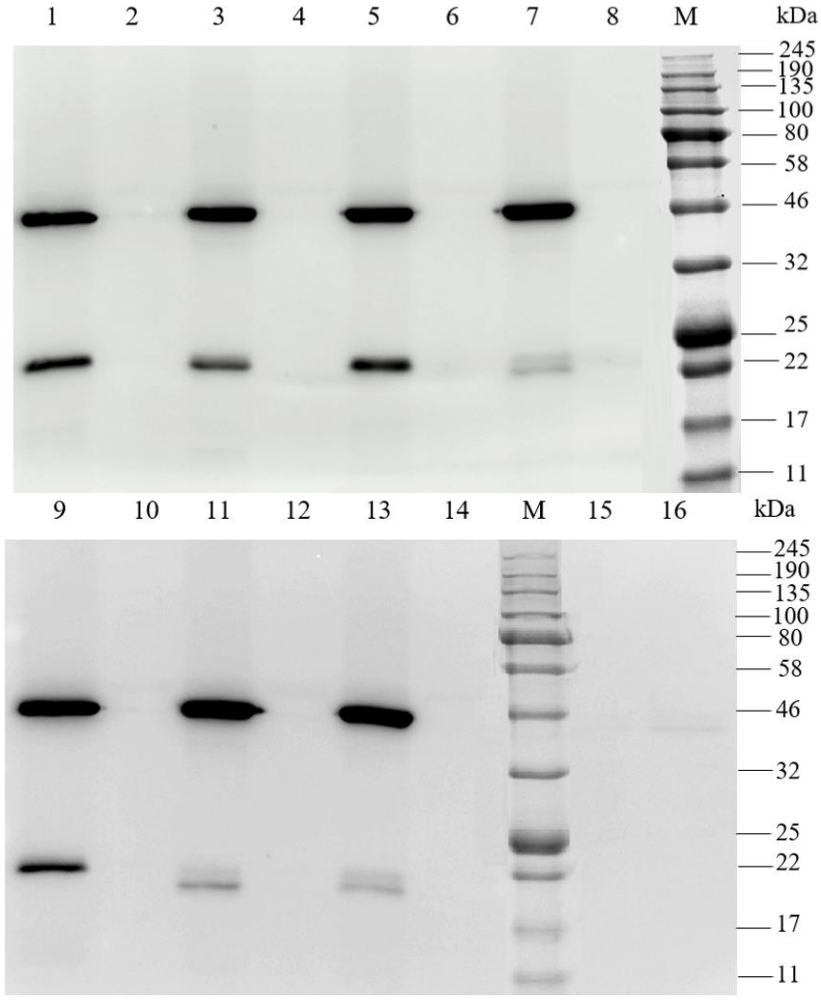

Figure 4.7. Image of the membranes after performing western blot. M) Marker; 1) Mutation 3 + ComS; 2) Mutation 3; 3) Mutation 2+ComS; 4) Mutation 2; 5) Mutation 1+ComS; 6) Mutation 1; 7) OK1+ComS; 8) OK1; 9) Mutation 5+ComS; 10) Mutation 5; 11) Mutation 4+ComS; 12) Mutation 4; 13) OK1+ComS; 14) OK1; 15) *L. lactis* 50 µg; 16) *L. lactis* 100µg

4.2 Hybrids

4.2.1 Site directed mutagenesis

Six hybrids consisting of different parts from the lactococcal and enterococcal *rseP* were made to identify which part of the *rseP* makes the *Lactococcus* strains sensitive to LsbB, and the *Enterococcus* strains resistant to LsbB. These hybrids were made by overlap extension PCR. The first step PCR constructed fragments of lactococcal *rseP* with a short stretch complementary to the enterococcal *rseP*, in addition to fragments of enterococcal *rseP* with a short stretch complementary to the lactococcal *rseP*. For more information about the hybrids, see section 3.3.2. The sizes of the fragments varied, but ranged from about 1 kb to about 2 kb (figure 4.9). The bands obtained from the first step PCR, were cut and purified using the NuceloSpin® Gel and PCR Clean-up kit. The purified products were used in the second step PCR. The first step PCR created 12 fragments, and in the second step PCR, fragments were paired, resulting in 6 different hybrids. Primers outside the gene were used (khb31 and khb34) to amplify the whole gene, resulting in a 3 kb fragment (figure 4.10). The first hybrid contained the three first helices from *Lactococcus*, and the last one helix from *Enterococcus*. The second hybrid consisted of the two first helices from *Lactococcus* and the last two from *Enterococcus*, and the third hybrid contained the first helix form *Lactococcus* and the last three from *Enterococcus*. Three more hybrids were made, where the situation was converted (figure 4.8) These products were cut from the gel, and purified using NucleoSpin® Gel and PCR Clean-up kit. The resulting PCR-products were used to do transformation into *S. pneumoniae*.

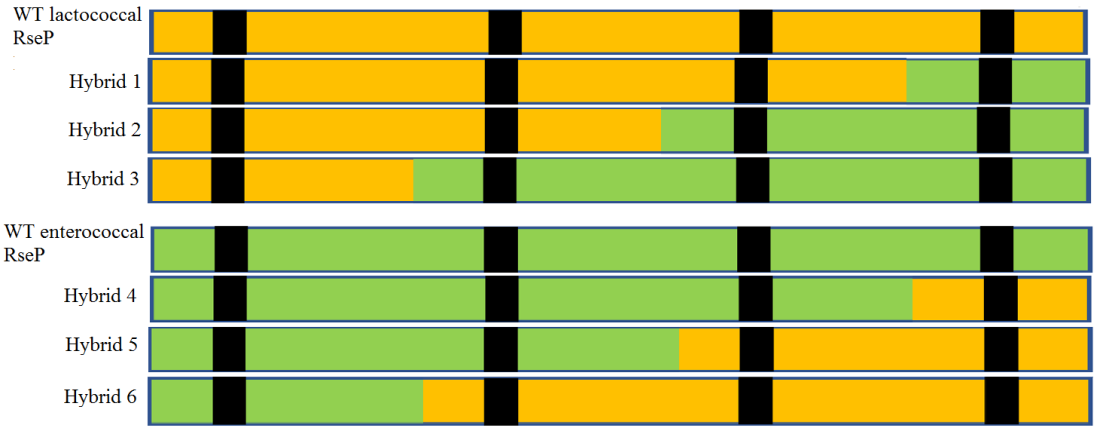

Figure 4.8. The different hybrids. The yellow bricks are lactococcal RseP, while the green bricks are enterococcal RseP. The black boxes inside the bricks demonstrate the transmembrane helices. The hybrids are different combinations of the lactococcal and the enterococcal RseP.

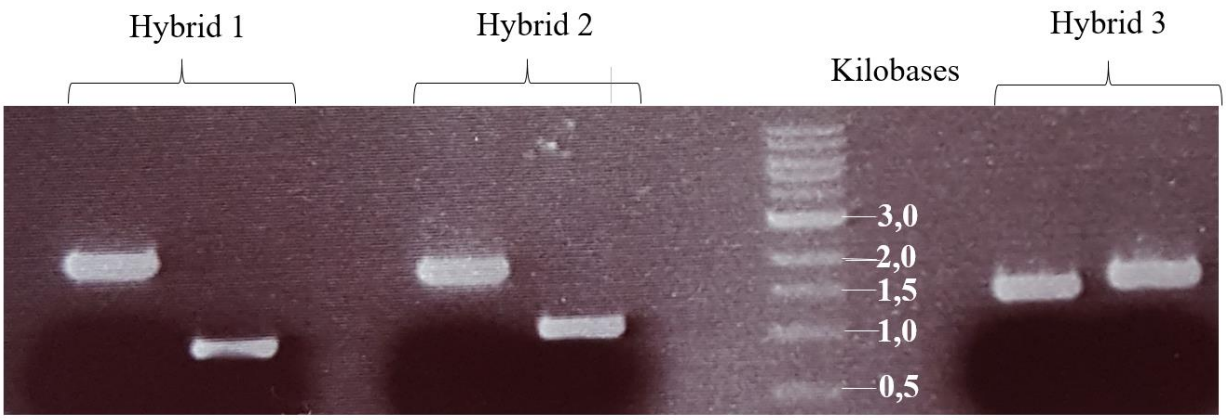

Figure 4.9. Fragments obtained for hybrid 1, 2 and 3 from the first step PCR. For each hybrid, there were two overlapping products. The sizes varied depending on the hybrid, and they ranged from around 1 kb to around 2 kb.

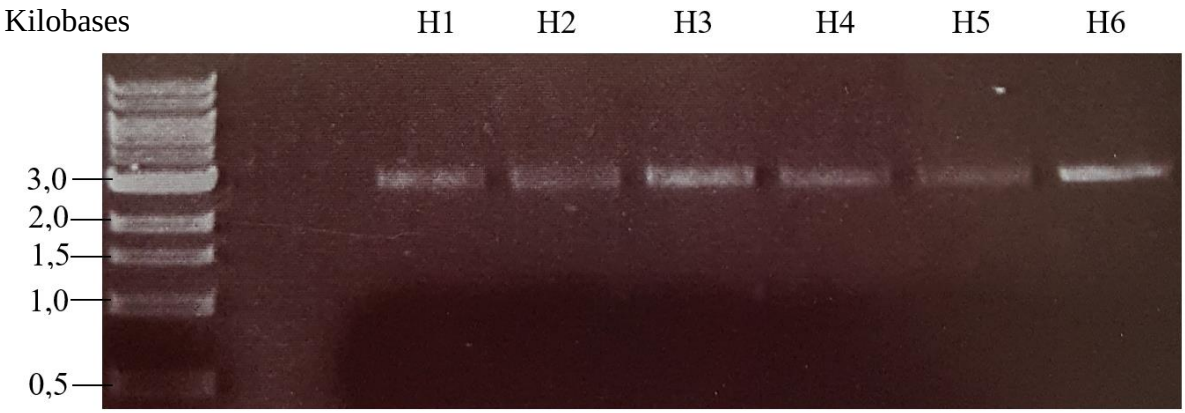

Figure 4.10. Fragments from the first step PCR were merged in the second step PCR, and gave a final product of 3 kb.

4.2.2 Heterologous expression in *Streptococcus pneumoniae*

After the site directed mutagenesis, the transformation construct was transformed into *S. pneumoniae*, in order to verify level of resistance to LsbB for different hybrids. One transformation was performed for each hybrid. See section 3.3.2 and section 3.2.7 for information about how the constructs were made, and how the transformation was performed. As mentioned previously, to evaluate if the transformation was successful, there should be at least twice as many colonies on the mutant-plate, compared to the negative control. Figure 4.11 shows an example of a successful transformation.

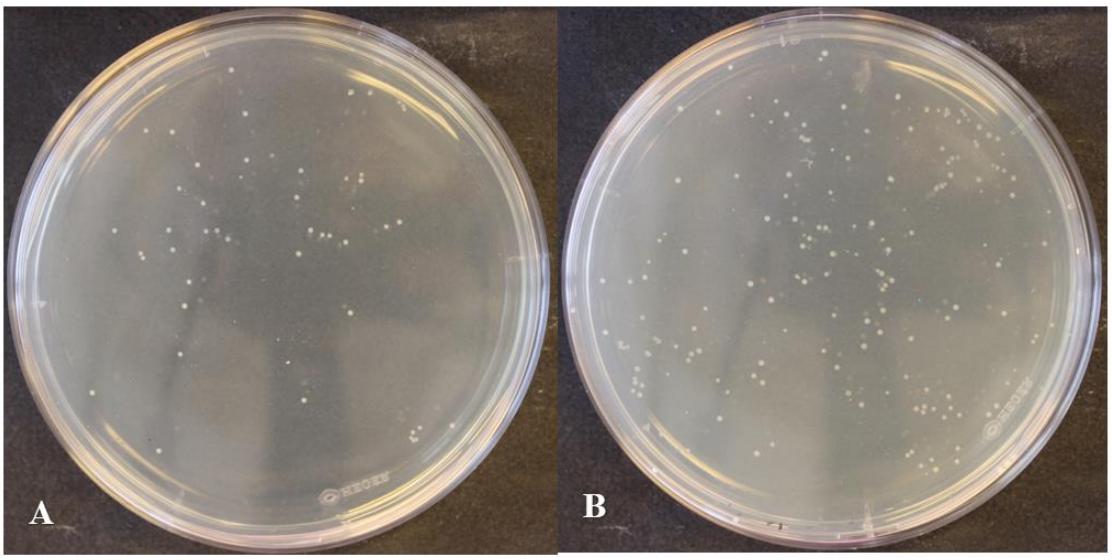

Figure 4.11. The plates with colonies after transformation: negative control (A) and the hybrid 1 (B) demonstrates that the plate with the transformants had at least twice as many colonies compared to the negative control.

Ten colonies from each plate were picked and colony PCR was performed to verify if the transformation was successful. A forward primer inside the *rseP* gene (L12) was combined with the reverse primer outside the gene (khb34), and a fragment of 1,6 kb was expected. There was at least one transformant for each hybrid that had successfully inserted the wanted fragment (figure 4.12). The sequence of the hybrids was confirmed by sequencing. One transformant for each hybrid was chosen to be analysed further.

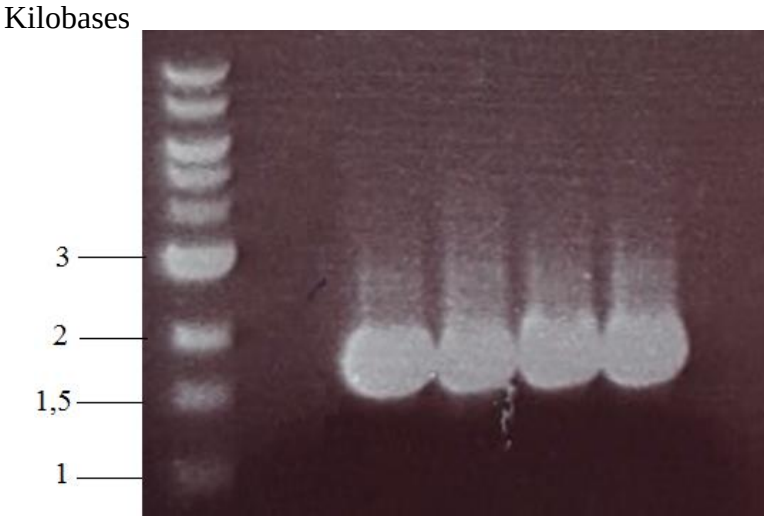

Figure 4.12. Colony-PCR was performed to confirm that the *rseP* gene was successfully transformed into *S. pneumoniae*. The picture shows gel after electrophoresis of the PCR product of hybrid 1. The expected size of the band was 1,6 kb, and the *rseP* had been successfully inserted in four out of ten colonies.

4.2.3 Microtiter plate assay

Microtiter plate assay with two-fold dilutions of LsbB was performed to estimate the bacteriocin resistance levels of the hybrids. Hybrid 1 (lmh6) and hybrid 6 (lmh 11) show sensitivity to LsbB, while the others are all resistant to LsbB. The MIC_{50} values for all of the hybrids was calculated, and is shown in table 4.2.

Table 4.2**.** MIC_{50} values of resistance levels for the hybrids, calculated based on the results from the microtiter plate assay.

Isolate	Changes	MIC_{50} (ng/ml)
OK1	WT *rseP*	< 3
1522	No *rseP*	> 50000
lmh6	First three helices are lactococcal, the last one is enterococcal	< 3
lmh7	First two helices are lactococcal, the last two are enterococcal	> 50000
lmh8	First one helix is lactococcal, the last three are enterococcal	> 50000
lmh9	First three helices are enterococcal, the last one is lactococcal	> 50000
lmh10	First two helices are enterococcal, the last two are lactococcal	> 50000
lmh11	First one helix is enterococcal, the last three are lactococcal	< 3

4.2.4 SDS PAGE

SDS-PAGE and western blot were performed to show that *rseP* was expressed and present in the cells. It was run for all of the six hybrids, as well as for OK1. OK1 was used as a positive control. Protein samples were prepared beforehand of the SDS-PAGE. Two samples were prepared for all the hybrids and OK1: one with added ComS to induce the production of RseP and one control without inducer peptide. After cell destruction, the amount of protein was measured, to equalize the samples when running SDS-PAGE.

When running the SDS-PAGE, about 100 µg of proteins from both the induced and the uninduced samples were loaded. Figure 4.13 show the comassie staining of the gels. The amount of protein looks to be approximately the same for all of the samples. There is no visible difference between the induced and the uninduced samples, therefore, it is difficult to tell whether RseP is present, based on comassie stained gel. Western blot will specifically visualise the RseP.

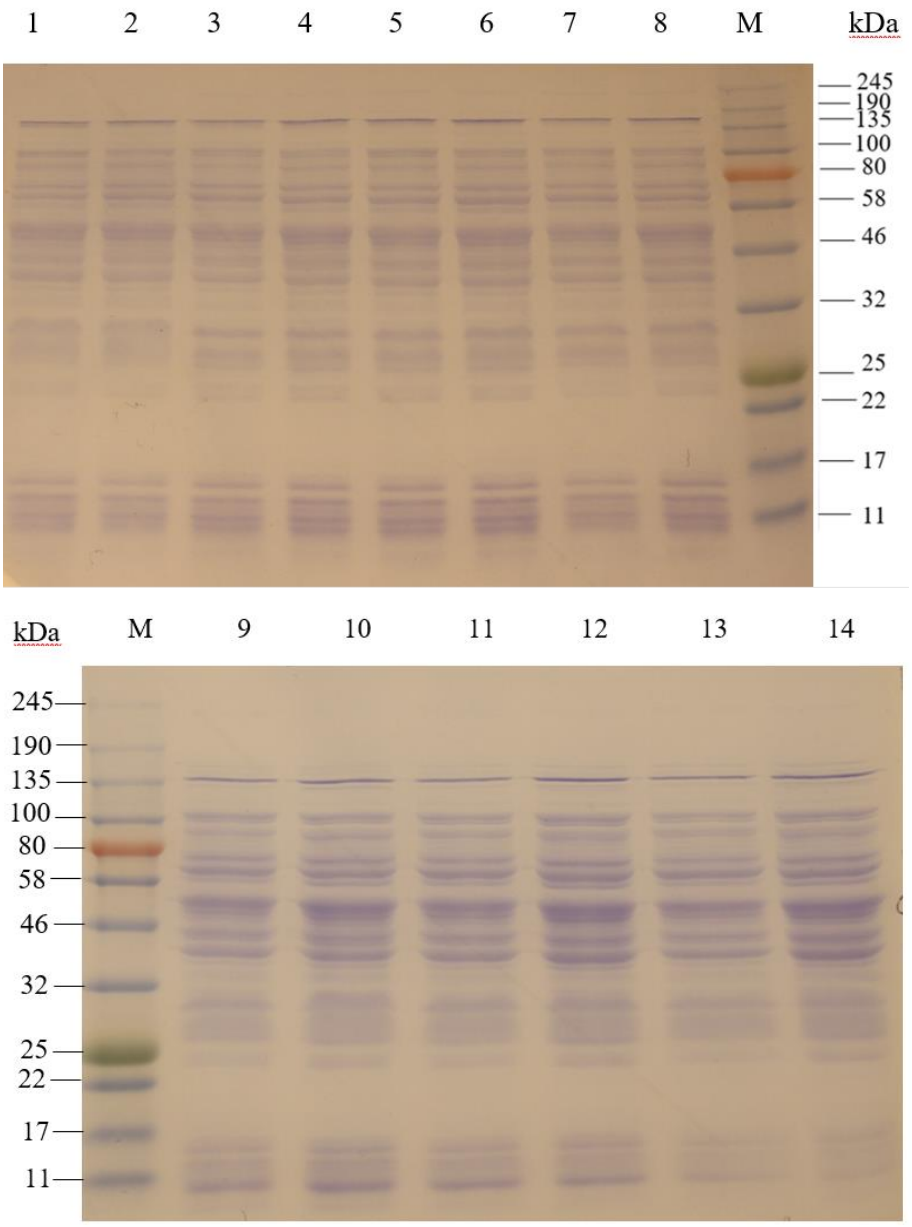

Figure 4.13. The comassie stained gel after electrophoresis. M) Marker; 1) OK1; 2) OK1+ComS; 3) Hybrid 1; 4) Hybrid 1+ComS; 5) Hybrid 2; 6) Hybrid 2+ComS; 7) Hybrid 3; 8) Hybrid 3+ComS; 9) Hybrid 4; 10) Hybrid 4+ComS; 11) Hybrid 5; 12) Hybrid 5+ComS; 13) H6; 14) Hybrid 6+ComS

4.2.5 Western blot

After the SDS-PAGE, western blot was performed to prove the presence of RseP in the cells. The proteins from the gel were transferred to the membrane, and the membrane was incubated with YvjB-antibodies. After incubation with the secondary antibody, detection solutions were added to visualize the proteins. RseP has a mass of 46 kDa. The results show big bands at the

right size for all the induced samples, while no bands are shown for the uninduced samples (figure 4.14). The results also show bands between 22 and 25 kDa for the induced samples. Two samples have thinner bands than the others; hybrid 4 and hybrid 5. Except from that, there seem to be no difference in the expression between the positive control OK1 and the different hybrids.

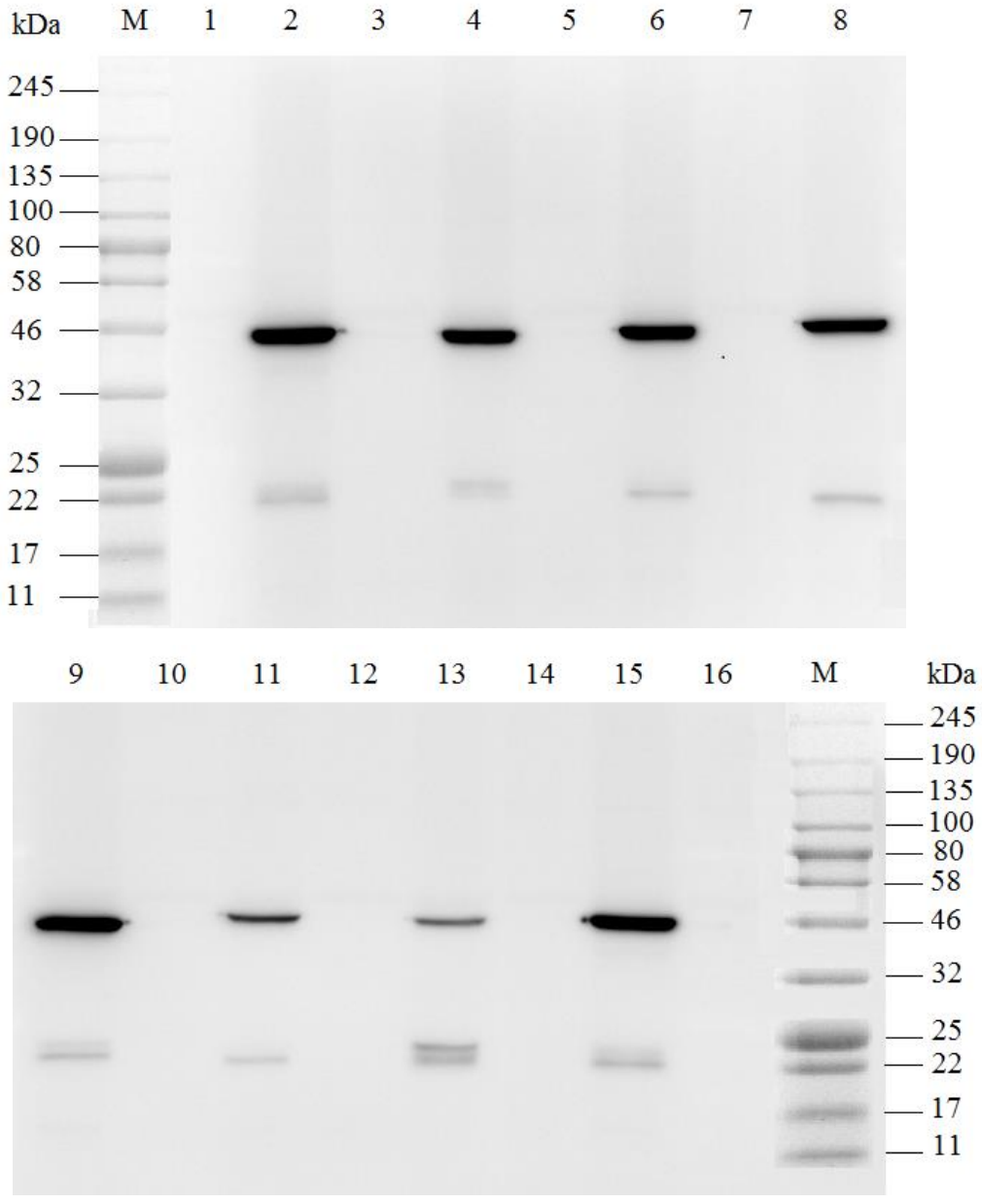

Figure 4.14. Image of the membranes after performing western blo. M) Marker; 1) OK1; 2) OK1+ComS; 3) Hybrid 1; 4) Hybrid 1+ComS; 5) Hybrid 2; 6) Hybrid 2+ComS; 7) Hybrid 3; 8) Hybrid 3+ComS; 9) Hybrid 6+ComS; 10) Hybrid 6; 11) Hybrid 5+ComS; 12) Hybrid 5; 13) Hybrid 4+ComS; 14) Hybrid 4; 15) Hybrid 3+ComS; 16) Hybrid

5. Discussion

5.1 Sensitivity to LsbB and bacteriocin binding

The site-2 proteases, including RseP, have the conserved catalytic site HExxH, that is located in the first transmembrane helix of RseP. By changing these residues in the lactococcal RseP, one by one and altogether, and then assessing them for LsbB sensitivity, it was shown that the mutants became a lot more resistant than the strain expressing the wild type RseP. This was especially true when all three conserved residues were changed concomitantly. A strain with the mutation outside the active site was also created as control, and was as sensitive to LsbB as the wild type (OK1). These results indicate that the RseP active site is important for the activity of LsbB bacteriocin. However, the fact that none of the mutants with the changed residues inside the active site got totally resistant, means that most probably there are also other parts of the RseP that are important for the LsbB binding.

The results achieved here, have also been proven in another study. Ovchinnikov et. al (2016) created analogous substitutions in the enterococcal RseP, to see how it would affect the sensitivity to the bacteriocins EntEJ97 and EntK1, that also use RseP as the receptor. That was the first time where it has been proven that the active site is important for the activity of a bacteriocin. Results from the study on enterococcal RseP are in agreement with results obtained in this thesis, indicating that the catalytic site of RseP is important for the activity of bacteriocins that utilize RseP as a receptor. The details of the interaction, and the role of the catalytic site of RseP in the bacteriocin binding, are not yet known. None of the mutants became totally resistant, therefore it can be speculated that the active site of RseP is only partly involved in the bacteriocin activity. A similar study was performed by Biswas and Biswas (2014), where they proved that the membrane bound protein LsrS exhibits a receptor-like function for the lantibiotic Smb. The conserved proteolytic-site residues were replaced to examine how it would affect activity of the bacteriocin. The mutations had no effect on the activity of Smb, meaning that the active site is not important for the binding of the bacteriocin (Biswas & Biswas 2014). Both RseP and LsrS are membrane bound proteins, that have proteolytic function. Because of that, one could think that they should interact with their respective bacteriocins in the similar matter. However, that is not the case. The proteolytic site of RseP is different from the catalytic site of LsrR, and the structure of the proteins is also different (Biswas & Biswas 2014). The catalytic site of RseP is located in the first

transmembrane helix, close to the cytoplasmic side. Therefore, it can be speculated that the catalytic site of RseP is not involved in the first binding of LsbB, however, it could be involved in the formation of a complex that leads to destruction of the membrane. LsrS consists of six transmembrane helices, where the catalytic site includes two glutamic acids (E) and one histidine (figure 5.1) (Biswas & Biswas 2014). The two glutamic acids are located at the cytoplasmic side of the fourth helix and the histidine is located in the middle of the fifth helix. Not much is known about the binding of the bacteriocin to this receptor, only that the active site is not involved. Therefore, even though both RseP and LsrS are intramembrane proteases, they have different structures and they contain different active sites, which are located at different places in the receptor. This can explain the differences in the involvement of the active site in the bacteriocin binding.

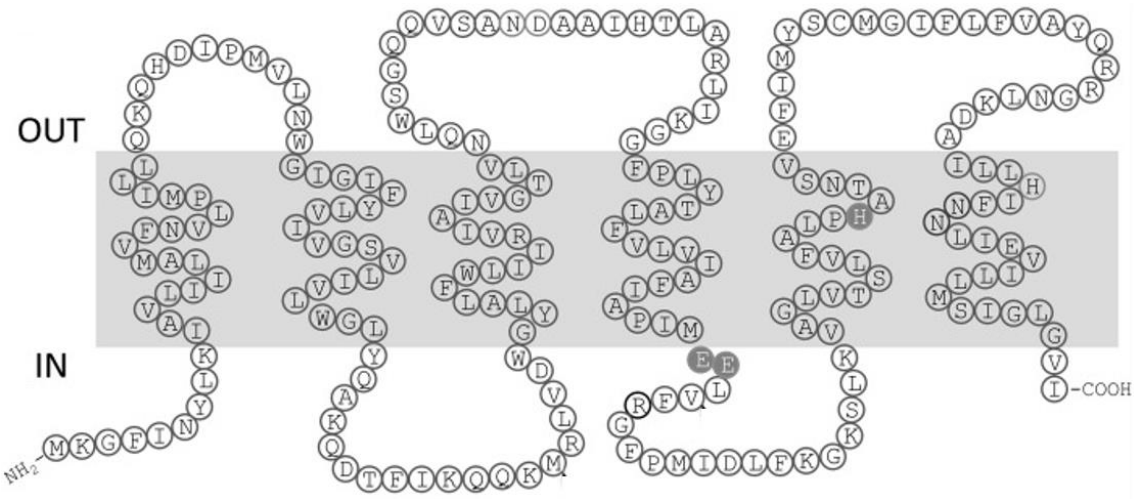

Figure 5.1. The structure of LsrS. It consists of six transmembrane helices, and the active site is marked in dark grey. Figure adapted from Biswas & Biswas (2014).

The bacteriocin LsbB produced by lactococcal cells, uses the same receptor as the bacteriocins EntEJ97 and EntK1, namely RseP. LsbB only kills lactococcal cells, whilst EntEJ97 and EntK1 have a broader activity spectrum, including both lactococcal and enterococcal cells among others (Ovchinnikov et al. 2017) . Hybrids of the lactococcal and enterococcal RseP were created to identify which part of the RseP is responsible for enterococcal cells becoming resistant to LsbB. The results show that the clone expressing RseP consisting of first three (out of four) lactococcal helices and the last enterococcal helix, was as sensitive to the LsbB bacteriocin as the strain expressing the wild type lactococcal

RseP. The same result was obtained for the clone expressing RseP consisting of first enterococcal helix and last three lactococcal helices. All the other hybrids were totally resistant to LsbB. These results indicate that both the second and the third transmembrane helices of the lactococcal RseP, as well as their flanking regions, are important for the bacteriocin LsbB to bind.

A recent study performed by Milijkovic et al. (2016) showed that LsbB interacts with the third transmembrane helix of the receptor RseP. It has been found that Tyr^{356} and Ala^{353}, located in the third helix, are essential for bacteriocin binding. When these two residues were substituted, cells became totally resistant to LsbB. There were also found semi-resistant mutants. These mutants had mutation in Gly^{188}, which is located in the second transmembrane helix. Our results demonstrate that when the third lactococcal helix is removed, the activity of LsbB is totally gone. However, according to our results, the third helix alone is not sufficient to regain sensitivity to LsbB. Thus, when the second helix is lactococcal as well, the strain becomes sensitive. Therefore, it seems like both the second and the third transmembrane helices are important for the activity of LsbB. Exactly how LsbB targets the receptor is not yet known.

Miljkovic et al. (2016) suggested that the first binding happens within the third transmembrane helix, and the second helix is important for the formation of complex and for the damage of the cell membrane. This theory is based on the fact that Tyr^{356} and Ala^{353} are located at the beginning of the third helix, meaning close to the outside of the surface of the membrane. Therefore, this region is available for bacteriocin binding. However, Gly^{188} is located at the end of the second helix, meaning close to the cytoplasmic side. This indicates that it can have an important role in complex formation and membrane damage. Results displayed in this thesis also indicate that the third helix is the most important, because the strains became totally resistant when the third lactococcal helix was replaced with the enterococcal one. It is most likely that the first binding of LsbB happens within the third transmembrane helix of RseP. However, it seems like the LsbB also needs the second transmembrane helix to be lactococcal to be able to utilize RseP as a receptor. Therefore, one can speculate that the third helix of RseP serves as docking part for LsbB, and the second helix is utilized in the next step as an anchor for pore formation.

5.2 Protein expression

SDS-PAGE and western blot were performed to see if *rseP* was expressed in all of the mutants and hybrids. Comassie staining of the SDS-PAGE gels revealed that whole cell protein profiles for all the samples, both uninduced control strains and RseP expression induced strains looked the same (figure 4.6 and 4.13). However, western blot analysis showed differences in the expression of the RseP protein for the induced and the uninduced samples. The high production of RseP resulted in antibodies binding and good visualization of the protein. The induced samples had very thick bands at about 46 kDa, meaning that RseP is well expressed when ComS is added as an induction peptide. 46 kDa is the calculated size for the RseP protein. For the uninduced samples, no bands are shown in the western blot, meaning that the expression of RseP did not reach a detectable level without ComS, and the expression of *rseP* is too low for the antibodies to bind. This was as expected. The samples that were uninduced are not sensitive to LsbB, meaning that when the RseP is not expressed LsbB doesn't have a receptor to target. The samples from *L. lactis* only showed a small band at the right size. This can be explained by the fact that these cells are not inducible, and the production of RseP from the native promoter is not high. The results from the western blot prove that the differences in the sensitivity of LsbB for the mutants and hybrids can be explained by the changes in *rseP* gene, and not because of differences in the protein expression level.

The western blot shows thinner bands for lmh9 (hybrid 4) and lmh10 (hybrid 5), than for the others. The antibodies used for the primary binding to the protein, were created using strain *L. lactis* subsp. *lactis* BGMN1-5. The antibodies consist of two separate antibodies that binds to different parts of RseP; from 80^{th} to 185^{th} amino acids and 225^{th} to 300^{th} amino acids (Miljkovic et al. 2016). These regions are located between the first and the second transmembrane helices and between the second and the third transmembrane helices, respectively. Lmh9 contains the three first transmembrane helices from *E. feacalis*, and the last fourth from *L. lactis*. Lmh10 contains the two first transmembrane helices from *E. feacalis*, and the last two helices from *L. lactis*. Even though the two last transmembrane helices are lactococcal for hybrid five, the lactococcal part starts from amino acid 327. This means that both lmh9 and lmh10 are enterococcal in parts where the antibodies bind. When aligning the enterococcal and the lactococcal RseP, there are differences in the amino acids sequence where the antibodies bind (figure 5.2). For instance, there is a big stretch around

amino acid 100 of the lactococcal RseP, that is not present in the enterococcal RseP. However, there are also parts that are similar, meaning that the antibodies still have parts where they can bind. It also looks like it's sufficient that one out of the two regions where the antibodies bind are lactococcal to get good visualization of the protein. Lmh8 (hybrid 3) only has the first region lactococcal, whilst lmh11 (hybrid 6) only has the second region lactococcal. However, they still have as thick bands as the other hybrids, and as the other mutants. It is difficult to say exactly which part of the hybrids is responsible for binding, however, it is shown in this thesis that the antibodies bind even though the lactococcal parts are missing.

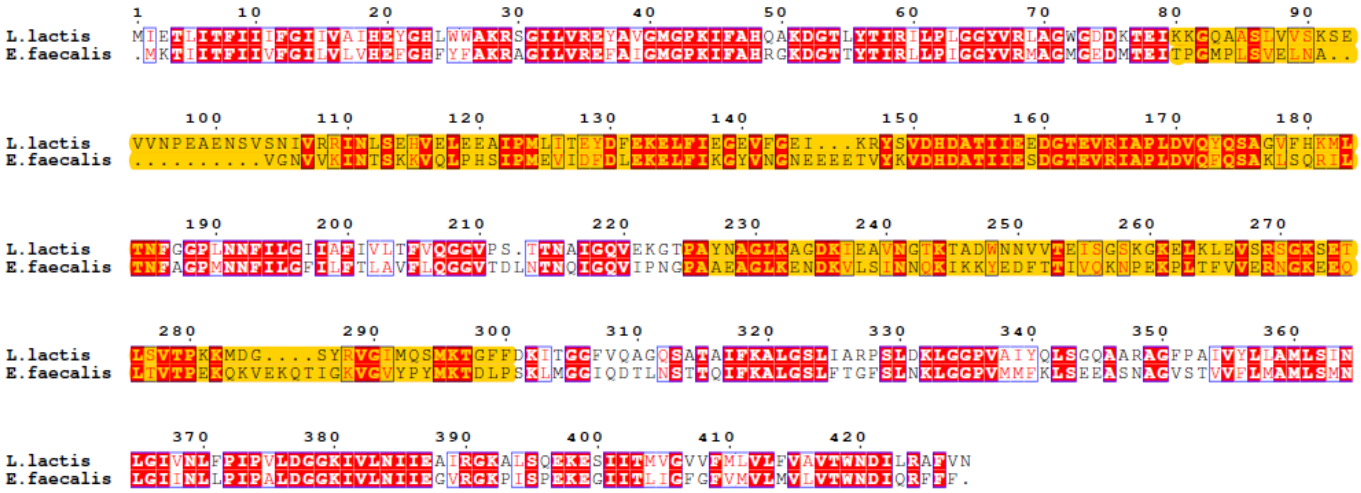

Figure 5.2. Alignment of the amino acid sequence of RsePs from *L. lactis* and *E. faecalis*. The parts where the antibodies bind are marked in yellow; 80th-185th amino acids and 225th-300th amino acids (Robert & Gouet 2014).

In addition to thick bands at 46 kDa, the western blot also showed bands between 22 and 25 kDa for all of the induced samples. At that size, they are weaker for some of the samples than for the others. The western blot performed for OK1 and lmh1-lmh5 showed that the strain expressing the WT *rseP* (OK1) in addition to lmh4, had a weaker band at that size, than the other samples. Comparing that with the sensitivity to LsbB, these two strains are the only strains that are sensitive to LsbB. Exactly why OK1 and lmh4 have thinner bands at this size compared to the more resistant strains are unknown, but it could have a connection with the sensitivity to LsbB. However, when looking at the results from the western blot performed for the hybrids (lmh6-lmh11), they all show a weak band at the lower size. If the sensitivity to LsbB had a connection with the weaker bands, lmh7-lmh10 should have had a stronger band at this size. Therefore, it looks like it was just a coincidence that lmh4 and OK1 had weaker

bands than the other mutants. This weak bands can then be a degradation product of RseP, or just some background noise.

5.3 Protein measurements

On the commassie stained SDS-PAGE gel the well with *L. lactis* proteins seems to show less protein than the other samples, although the added protein amount should have been the same (figure 4.6). This well contains proteins from a different species than the other samples that are from *S. pneumoniae*. It is known that *S. pneumoniae* lyse easily, meaning that it is easier to extract the protein. However, the FastPrep Homogenizer should easily lyse *L. lactis* cells as well. The differences could then be explained by the measurement of proteins after the extraction. NanoDrop measures the absorption at 280 nm, which is the wavelength where the amino acids tryptophan, tyrosine and to some extent cysteine absorb. The concentration of proteins is therefore only an estimate based on the average frequency of these amino acids. The content of these amino acids could differ a lot between the different species, resulting in different absorption at 280 nm. This will again lead to different concentration of proteins, although the amount in principle could have been the same. This method is not very precise, meaning that the amount of protein could be a lot different than the instrument measures.

5.4 Heterologous expression

Heterologous expression was performed in this thesis to place altered *rseP* genes into *S. pneumoniae*. The strain used for that purpose (1522), was created by Berg et al. (2011) and Ovchinnikov et al. (2016). Berg et. al (2011) created a tightly regulated, titratable depletion system that had minimal effect on the normal cellular function. The ComRS system from *S. thermofilus* was inserted into *S. pneumoniae*, which consisted of the transcriptional activator ComR, its inducer ComS and the P_{comX} promoter, controlling expression of Janus cassette placed under regulation of that promoter. The Janus cassette includes genes that make it possible to select for both its presence and its absence. As previously mentioned, *S. pneumoniae* is competent for genetic transformation, so by adding CSP (competence stimulating peptide) it can take up exogenous DNA and incorporate it into its genome by homologous recombination (figure 5.3). This system is relatively easy; it makes it possible to incorporate a gene of interest, in addition to control the expression of an inserted gene.

S. pneumoniae has a gene homologous to the lactococcal *rseP*, therefore, this gene was removed from the genome to create the strain used in this thesis (Ovchinnikov et al. 2017). This was performed to avoid potential background noise. The DNA constructs created in this thesis had the same flanking regions as the Janus cassette. This means that when the insert DNA is taken up by the cells, it replaces the Janus cassette, due to homologous recombination (figure 5.3). When the Janus cassette is removed, the gene responsible for streptomycin sensitivity is also gone, meaning that it is possible to select for the cells where the transformation has happened, by adding streptomycin to the growth media. *S. pneumoniae* is naturally resistant to LsbB, which makes it easy to verify if the RseP that was incorporated works as a receptor for LsbB or not. If the RseP can function as a receptor, the strain will become sensitive to LsbB, and if the RseP does not function as a receptor, or functions only partly, the strain will remain resistant, or become partly resistant. Therefore, the homologous expression performed in *S. pneumoniae* was a good choice, that made the procedure easy and effective.

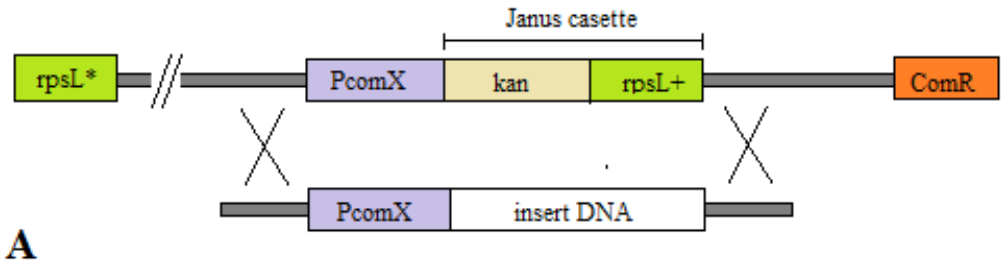

Figure 5.3. Replacement of the Janus cassette with the DNA of interest. The Janus cassette contains the kanamycin resistant (kan) gene, and rpsL^{+}-allele, which together with the rpsL* outside the Janus cassette, give a streptomycin sensitive phenotype.

5.5 Concluding remarks

This thesis aimed to identify which part of the lactococcal receptor RseP is important for binding and activity of the bacteriocin LsbB. It was proven that the catalytic site was partly involved in the activity of the bacteriocin. It was also shown that the second and the third transmembrane helices, as well as their flanking regions of *L. lactis* were important for the bacteriocin LsbB to bind and destruct the cell membrane. These results, in addition to other studies, indicate that the third helix is important for the binding of LsbB, and the second helix,

as well as the catalytic site, could be involved in the destruction of the cells.

Heterologous expression was performed in this thesis, where the altered *rseP* genes were placed into *S. pneumoniae*. The *S. pneumoniae* strain used for this purpose, had a depletion system that allowed controlled expression of the *rseP* genes (Berg et al. 2011).The expression of the different *rseP* genes was proven with western blot.

Antibiotic resistant bacteria are an emerging problem, which needs to be solved. A way to solve it, is to identify alternatives to antibiotics, and the bacteriocins could be one. However, before bacteriocins can be safely used in medicine as drugs or as food preservatives, it is important to have more knowledge about their genetics, functions and possible problems that could emerge with their application. The details of how the bacteriocin LsbB targets RseP, was not solved in this thesis. However, the study is relevant in understanding the relationship between bacteriocins and their receptors, which contributes to overall knowledge of bacteriocins as future possible alternatives to antibiotics.

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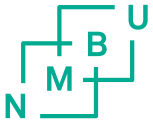

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