Study of two-peptide bacteriocins and their immunity genes from Lactobacillus

Studie av to-peptid bakteriosiner og deres immunitetsgener fra Lactobacillus

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Forord

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Erik Rasmussen

Contents

Abstract

Genome mining was conducted on putative genes from the bacteriocin producing *pln* locus in *Lactobacillus plantarum*. The bacteriocins plantaricin NC8 and the putative plantaricin J51 were tested for activity among many different indicator strains, related to the producing strains. Plantaricin J51 was established as a novel two-peptide bacteriocin. A narrow spectrum of inhibition was observed for both bacteriocins, which is typical for class IIb bacteriocins.

Among the strains sensitive for plantaricin NC8, *L. sakei* NCDO 2714 and *L. plantarum* 2-1 were transformed with the putative immunity gene *plNC8c* (which is assumed to confer immunity to plantaricin NC8) and heterologously expressed. Soft agar assays and microtiter plate assays of transformed strains revealed an increase in immunity to plantaricin NC8. *plNC8c* was thus established as a novel bacteriocin immunity gene. No cross-immunity was observed. *L. plantarum* DSM 20174 was transformed with the putative immunity gene J51-*Orf5* (which is assumed to confer immunity to plantaricin J51) and heterologously expressed. Soft agar assays and microtiter plate assays however were too ambiguous for any conclusions to be drawn. Nevertheless it is very likely that J51-*Orf5* functions as an immunity gene.

Protein expression studies were conducted on *L. sakei* NCDO 2714 expressing the PLNC8C immunity protein with an N-terminal FLAG™-tag. The immunity protein was purified using immunoprecipitation with an ANTI-FLAG™ antibody bound to a resin complex. Detection levels were low, suggesting a low expression level. No receptor was identified, although this study suggests that disulfide bridge complex formation might be involved in immunity function.

Further sequence analyses revealed a “contaminant” open reading frame present in the *plNC8c* immunity gene which might explain high backgrounds and low presence of the protein in purification and detection steps. These finding do however not interfere with the function of the immunity gene.

Sammendrag

"Genome mining" ble utført på antatte gener fra det bakteriosin produserende *pln* locuset i *Lactobacillus plantarum*. Bakteriosinene plantaricin NC8 og det antatte plantaricin J51 ble testet for aktivitet blant en rekke ulike indikatorstammer, som alle er beslektet med de produserende stammene. Plantaricin J51 ble etablert som et nytt to-peptid bakteriosin. Et smalt spekter av hemming ble observert for begge bakteriosinene, noe som er typisk for klasse IIb bakteriosiner.

Blant stammene som var sensitive for plantaricin NC8, ble *L. sakei* NCDO 2714 og *L. plantarum* 2-1 transformert med det antatte immunitetsgenet *plNC8c* og uttrykt heterologt. Dette genet er antatt å gi immunitet til plantaricin NC8.. Mykagaranalyser og mikrotiterplateanalyser av de transformerte stammene avslørte en økning i immunitet for plantaricin NC8. *plNC8c* ble derfor etablert som et nytt bakteriosin immunitetsgen. Ingen kryssimmunitet ble observert. *L. plantarum* DSM 20174 ble transformert med det antatte immunitetsgenet J51-*Orf5* (som er antatt å gi immunitet til plantaricin J51) og uttrykt heterologt. Myk agar analyser og mikrotiterplateanalyser var for diffuse til å trekke en konklusjon. Uansett er det meget sannsynlig at J51-*Orf5* gir immunitet.

Proteinuttrykkstudier ble utført på *L. sakei* NCDO 2714 som uttrykker PLNC8C immunitetsproteinet med en N-terminal FLAG™-tag. Immunitetsproteinet ble renset ved hjelp av immunopresipitering med et ANTI-FLAG™ antistoff bundet til et resinkompleks. Deteksjonsnivået var lavt, noe som tyder på et lavt uttrykk av proteinet. Ingen reseptor ble identifisert, men denne studien antyder at opprettelsen av et disulfidbro kompleks er involvert i immunitetsfunksjonen.

Videre sekvensanalyser avslørte en "kontaminerende" åpen leseramme tilstede i *plNC8c* immunitetsgenet som kanskje kan forklare høy bakgrunn og lav tilstedeværelse av proteinet i rensing- og deteksjonsstegene. Dette funnet fører ikke til forstyrrelser for funksjonen av immunitetsgenet.

Abbreviations

aa	Amino acid(s)
bp	base pair(s)
dH_2O	Distilled water
dsDNA	double stranded DNA
DTT	Dithiothretiol
EDTA	Ethylenediaminetetraacetic acid
g	gram(s)
LAB	Lactic Acid Bacteria
min	Minute(s)
ON	Over Night
PTM	Post Translational Modifications
rpm	Revolutions per minute
RT	Room Temperature
sec	Second(s)
ssDNA	single stranded DNA
T_m	Melting temperature[1]

[1] As defined by Santa Lucia (1998)

1 Introduction

1.1 Lactic Acid Bacteria (LAB)

Lactic acid bacteria are Gram-positive bacteria belonging to the low G+C group in the phylum *Frimicutes* (Sneath et al. 1986). This phylum contains three classes, in which LABs are found in the class *Bacilli* (sometimes also referred to as *Firmibacteria*).

LABs are characterized by their ability to produce lactic acid as one of the major end-products of the carbohydrate fermentation pathway (Felis & Dellaglio 2007).They usually lack cytochromes, and are thus dependent on carbohydrate fermentation to fuel their substrate-level phosphorylation. LABs are also usually non-motile (meaning they do not possess flagella) and do not form endospores (Sneath et al. 1986). LABs belong to the order *Lactobacillales* which has many genera *including Enterococcus, Lactococcus, Lactobacillus, Leuconostoc, Streptococcus* and *Pediococcus* (Wiley et al. 2010).

1.1.1 The genus Lactobacillus

The bacteria belonging to the genus *Lactobacillus* are non-motile and non-sporulating rods or coccobacilli (Tannock 2004). They have complex nutritional needs, which are reflected in their natural habitat. They are usually found in carbohydrate rich environments like in plants, food, spoiled food and in the intestine of humans and animals (Hammes & Vogel 1995). In the large intestine, however, simple carbohydrates are not available for fermentation, so the lactobacilli residing there are dependent on other microorganisms to degrade complex carbohydrates (like plant materials) and make them available to the lactobacilli (Balows et al. 1992). Although they are present in the gut microflora of various animals, very few of them are pathogenic (Claesson

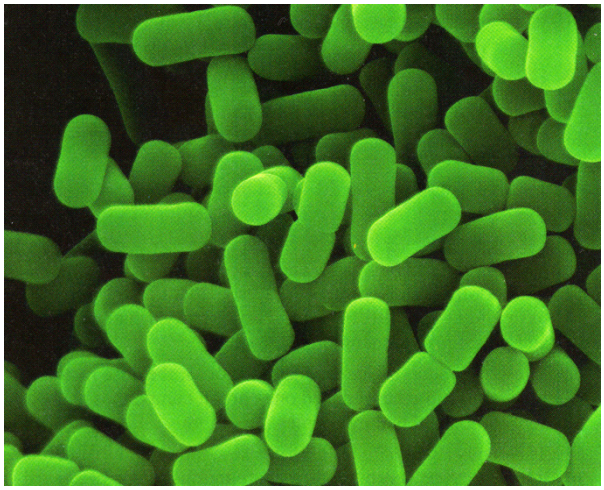

Figure 1.1 *Lactobacillus plantarum* as viewed through an electron microscope (Wiley et al. 2010). The bacteria has been artificially colored.

et al. 2007). Over 100 species are known (per February 2007), and this makes the genus one of the largest groups of LABs (Felis & Dellaglio 2007).

Lactobacilli are widely used in the food industry in production of foods that require fermentation (Tannock 2004). Most of these products are dairy products like yoghurt and different cheeses, but also in other products like pickles, sauerkraut and salami, can lactobacilli be found in the starter cultures. Because lactobacilli are found as starter culture in food, lactobacilli and their secreted products are classified as GRAS (Generally Recognized as Safe) by the US Food and Drug Administration (Guarner & Schaafsma 1998). This has led to a tremendous increase in the use of lactobacilli in different probiotic dietary supplements. Humankind may have passively benefited from lactobacilli since the dawn of fermented food production, as many lactobacilli secrete compounds that can inhibit the growth of unwanted pathogens. However, lactobacilli are also involved in food spoilage, and care must therefore be taken not to introduce food-spoiling lactobacilli, while preserving the beneficiary flora during food fermentation.

Lactobacilli may ferment glucose homofermentatively (more than 85% of the fermentation products is lactic acid), using the Embden-Meyerhof pathway, or heterofermentatively (producing lactic acid, carbon dioxide, ethanol and/or acetic acid in equimolar amounts), using the phosphoketolase pathway (Figure 1.2) (Tannock 2004). This ensures that their environment is slightly acidic, which is reflected in the optimum pH range for lactobacilli, which is also slightly acidic (between pH 4.5 and 6.4). This means that lactobacilli cannot rapidly colonize any new habitats directly, but must hold out until the pH is within acceptable limits. The lactate secreted by lactobacilli is one of many substances that they secrete which creates an environment that is unfavorable to other bacteria. This gives them a competitive advantage over other bacteria which have a more efficient energy metabolism (i.e. oxidative phosphorylation instead of substrate-level phosphorylation). Therefore the lactobacilli can out-compete unwanted bacteria in the gut, and this property is also considered as an important probiotic characteristic (Tortora et al. 2007). Another compound which lactobacilli secrete in order to gain an advantage over other bacteria is small antimicrobial compounds called bacteriocins.

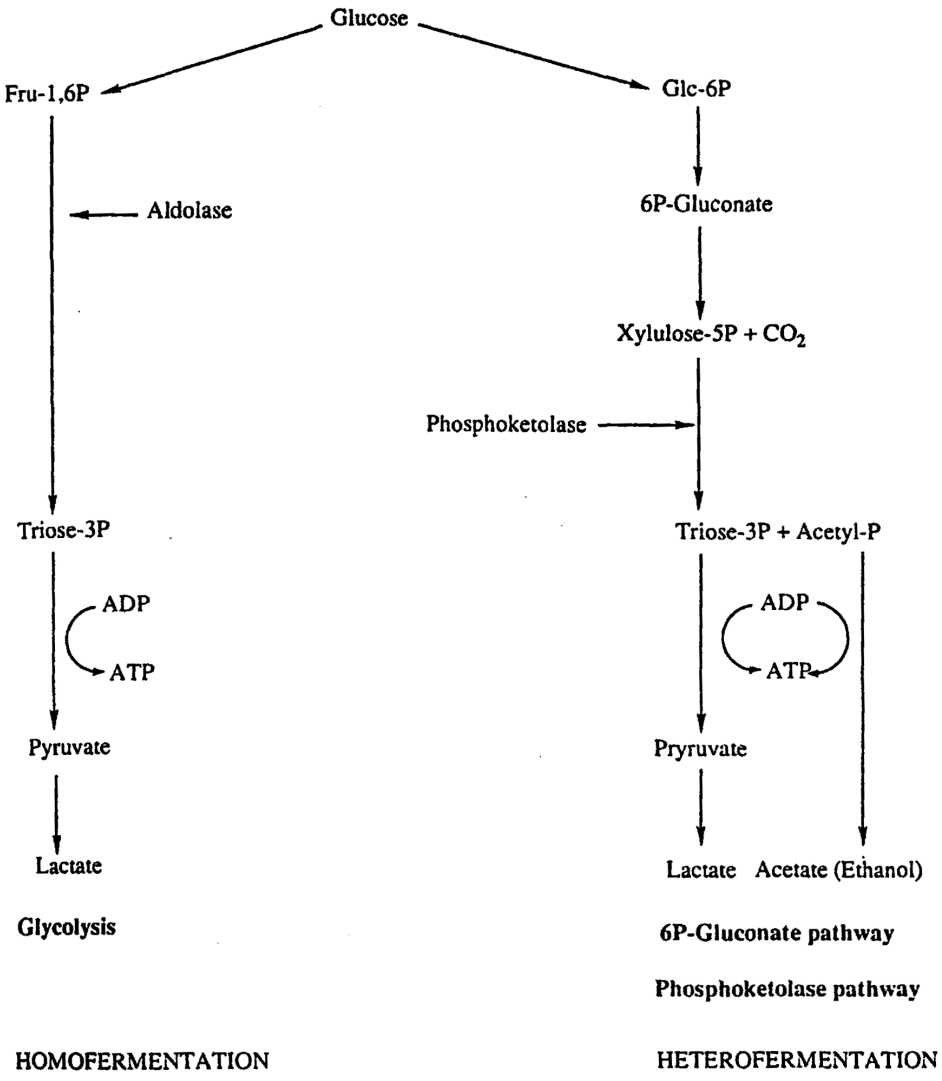

Figure 1.2 Overview of different fermentation pathways used by LABs (Adams & Moss 2008). Lactobacilli usually employ only one of the pathways, although some can utilize both.

1.1.2 The genus *Pediococcus*

The bacteria belonging to the genus *Pediococcus* are non-motile cocci (Carr et al. 2002). They ferment glucose homofermentatively (using the Emden-Meyerhof pathway) and they tolerate a lower pH than several lactobacilli (below pH 4.5). Not many species belonging to this genus are known, compared to the well-studied genus *Lactobacilli* (Felis & Dellaglio 2007). The species within this genus all lack catalase and they range from aerobic to microaerophilic (Carr et al. 2002). Pediococci also have complex nutritional needs, which is also reflected in their natural habitats which includes plants, dead plant material and foods. Some of the species are also used in the production of fermented foods like pickles, olives, sauerkraut, bacon and some fermented sausages. However a few of the species are also

known to be food spoilers, like *P. damnosis* which is a known contaminant in beer, causing a cloudy appearance and acid-like taste (Rainbow 1975). Some pediococi are also known to cause swelling of cucumbers during pickles production (Carr et al. 2002). Like lactobacilli, some pediococci are known to produce substances that have a strong antimicrobial effect. One of these substances is a bacteriocin called pediocin PA-1 (Mathys et al. 2007).

1.2 Bacteriocins

Bacteriocins are small antimicrobial peptides that can be produced by a vast range of bacteria. Both Gram-negative and Gram-positive bacteria produce bacteriocins, but only the latter will be discussed here (Jack & Jung 2000). Bacteriocins produced by Gram-positive bacteria share many of the same attributes as the ones produced by Gram-negative bacteria (Duquesne et al. 2007).

Bacteriocins are gene encoded, meaning that most of the information present in the mature peptides can be found in just one gene (Cotter et al. 2005). Some bacteriocins are post-translationally modified, and some bacteriocins require two peptides to exert antimicrobial activity, thus also requiring information from more than one gene (Nissen-Meyer et al. 1992; Skaugen et al. 1994). This is unlike other antibiotics, which is usually a product formed by many different enzymes, each modifying parts of the substrate, leading up to the formation of the finished antibiotic compound (Kieslich 1986). This requires the information from several genes. Immunity for both bacteriocins and antibiotics is usually encoded by a single gene which is present in the producing strain (Diep et al. 2009).

Most bacteriocins have a narrower field of inhibition than antibiotics. Bacteriocins are often active against antibiotic-resistant pathogenic bacteria, and could therefore be used in clinical treatments of infections (Kruszewska et al. 2004). For example, bacteriocins could be useful for treatment of infections caused by nosocomial antibiotic-resistant bacteria, as antibiotic resistance is becoming an increasing problem in hospital environments. *Clostridium difficile* is an example of such a bacterium, which is becoming ever more resistant to antibiotics, but still remains sensitive to some bacteriocins produced by LABs (Rea et al. 2010). The bacteriocin thuricin CD, which has a very narrow spectrum of inhibition, has been shown to specifically eliminate *C. difficile*, without a large impact on the indigenous bacterial community in the gastrointestinal tract (Rea et al. 2011). Thuricin CD may therefore be a

viable alternative in the treatment of *C. difficile*- associated intestinal diseases, as the current method by using broad-spectrum antibiotics often leads to recurring infections and problems with antibiotic resistance (Aslam et al. 2005).

The typical mode of action used by bacteriocins to kill bacterial cells is to permeabilize the cell membrane (Nes et al. 2007). This is probably in most cases achieved by the binding of the bacteriocin to a receptor on the cell membrane, which in turn creates a pore in the membrane, allowing the free flux of ions and other small molecules (like K^+, PO_4^{3-}, H^+) over the membrane. This eliminates the proton motive force, effectively killing the cells. Pore size, stability and selectivity of transferable molecules vary between different bacteriocins (Eijsink et al. 2002). Not all bacteriocins bind to a receptor in the cell membrane. Nisin binds to lipid II, a part of the bacterial cell wall synthesis machinery situated in the cell wall (Breukink & de Kruijff 2006). Upon binding of nisin, pores containing both nisin and lipid II are formed (Hsu et al. 2004). Independently of pore formation, cell wall synthesis is also blocked upon binding of nisin to lipid II (Hasper et al. 2006). Although bacteriocins have been studied in great detail, only a few receptors and how bacteriocins interact with them have been studied in detail.

1.2.1 Classification of bacteriocins from LABs

The LAB bacteriocins constitute a large and heterogeneous group of bacteriocins. They vary in their peptide size, post translation modifications, chemical stability and mode of action. It is therefore practical to employ a classification scheme to further group the different bacteriocins. Although the bacteriocins are usually given names deriving from the producing strain, this does not imply that bacteriocins from taxonomically close strains, or even bacteriocins produced from the same strain have the same properties.

Constructing a classification scheme for the LAB bacteriocins is not a straightforward task. This has led to the use of several classification schemes (Cotter et al. 2005; Nes et al. 2007). The classification from Nes et al. (2007) will be used here, as it one of the most used classification schemes, while also building upon one of the first broadly accepted classification schemes (Figure 1.3) (Klaenhammer 1993).

Class I (Lantibiotics) bacteriocins consist of post translationally modified bacteriocins, which are characterized by several ring structures known as lanthionine or β-methyllanthionine,

giving the name to this class (Bonelli et al. 2006). The lantibiotics may also contain other post translationally modified amino acids (like D-alanine) (Skaugen et al. 1994). The Class III (Large, heat-labile) bacteriocins consist of antimicrobial proteins rather than peptides, but are still included in the classification. The unmodified cyclic bacteriocins belong to class IV (Cyclic bacteriocins) (Nes et al. 2007). Class II, which consist of non-modified, heat-stable bacteriocins, is the largest and most studied group, and these bacteriocins are discussed in section 1.2.2.

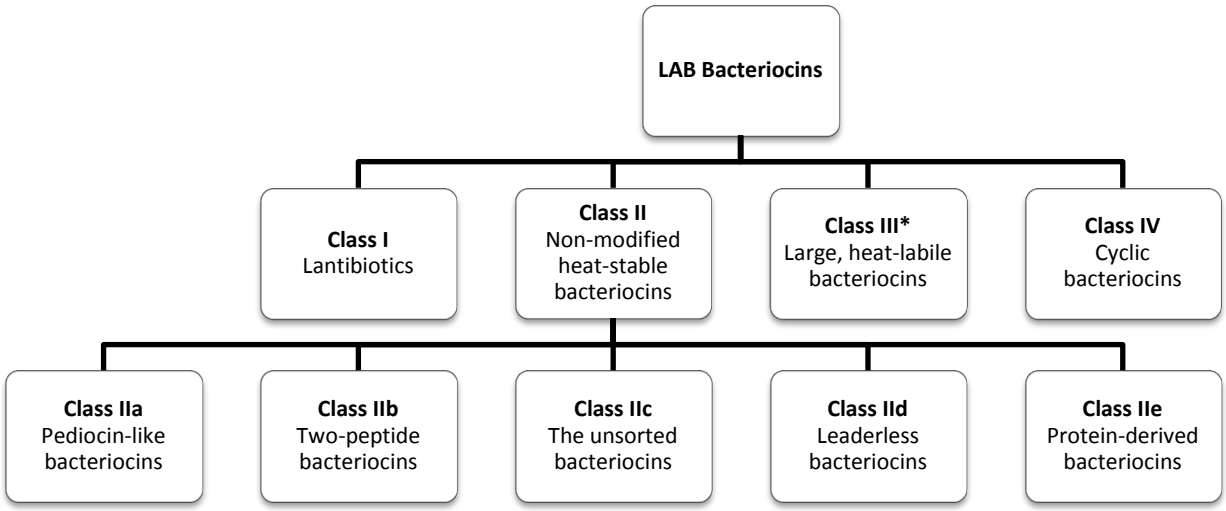

Figure 1.3 Classification of LAB Bacteriocins according to Nes et al. (2007). *Class III consists of proteins rather than peptides, however, this group is still included within the classification scheme.

1.2.2 Non-modified, heat-stable bacteriocins (Class II)

Members of this class are divided into several subclasses. Class IIa (pediocin-like) bacteriocins are characterized by an N-terminal consensus motif YGNGVxCxxxxCxVxWxxA (where x is any amino acid, and the underlined aa are fully conserved). These bacteriocins seems to contain two distinct domains; one highly conserved, cationic and hydrophilic N-terminal region, and one less conserved and more hydrophobic C-terminal region (Nissen-Meyer et al. 2009). Structures have been resolved for four Class IIa bacteriocins; sakacin P, leucocin A, curvacin A and carnobacteriocin B2 (Fregeau Gallagher et al. 1997; Haugen et al.

2005; Uteng et al. 2003; Wang et al. 1999). This subclass has again been divided into several subgroups based on the amino acid sequence of the bacteriocins (Nissen-Meyer et al. 2009). Over 35 bacteriocins belonging to Class IIa are known. Pediocin PA-1 is an example of a Class IIa bacteriocin (belonging to subgroup 1) (Nissen-Meyer et al. 2009). It is also shown to have a strong activity against the food borne pathogen *Listeria monocytogenes* (Rodriguez et al. 2002). What is peculiar about this bacteriocin is that it forms a disulfide bridge in its tertiary structure, which is necessary for the bacteriocin to display any microbial activity. Thus subjecting the bacteriocin to denaturing conditions (like Dithiothretiol or 2-mercaptoethanol) will abolish the antimicrobial activity of the peptide. The receptor for Class IIa bacteriocins have been determined to be a specific part of the Mannose Phosphotransferase System (Kjos et al. 2009; Kjos et al. 2010a). The exact mechanism is not known, but it is suggested that the bacteriocin binds to and induces a conformational change in the man-PTS IIC protein, which creates a pore in the target membrane (Kjos 2011).

The bacteriocins belonging to class IIb require the combined action of two different peptides for optimal activity, and these are discussed in section 1.2.3. Class IId consists of bacteriocins lacking any leader sequences. Eight bacteriocins belonging to class IId are known (Nes et al. 2007). Although the peptides lack the leader sequence in which other bacteriocins are dependant on for secretion, the class IId bacteriocins seem to utilize the same dedicated transport mechanism as other class II bacteriocins (The ABC transporter, discussed in section 1.2.4). Class IIe consists of peptides derived from degradation of larger proteins. Only four bacteriocins belonging to this subclass are known. The best studied class IIe bacteriocin is Propionicin F. This bacteriocin has 43 residues, which are processed from a protein containing 255 residues (Brede et al. 2004). The processing involves both N-terminal and C-terminal processing of the protein in order to yield the mature bacteriocin peptide. The non-modified heat-stable bacteriocins that do not fit any other subclass are grouped in class IIc (the unsorted bacteriocins) (Nes et al. 2007). Lactococcin A is the best studied class IIc bacteriocin. It has a very narrow range of inhibition, and its potency also varies between sensitive strains (Holo et al. 1991). It also lacks the characteristic N-terminal consensus motif typical of class IIa bacteriocins, and is therefore grouped in class IIc. Lactococcin A utilizes the same receptor as class IIa bacteriocins, which is the mannose PTS (Diep et al. 2007).

1.2.3 Two-peptide bacteriocins (Class IIb)

The bacteriocins belonging to subclass IIb differ from other class II bacteriocins in that they require the synergistic activity of two peptides to exert antimicrobial activity (Oppegard et al. 2007). Although some of the peptides can show antimicrobial activity individually, this is greatly increased by addition of the complementary peptide (Jimenez-Diaz et al. 1995). Together, the two complementary peptides are active at the nanomolar to picomolar range (Nes et al. 2007). Even though the two peptides functions as one bacteriocin unit, the single peptides are very similar to the one-peptide bacteriocins from Class IIa (Pediocin-like bacteriocins) in the physical sense. They both contain amphiphilic and hydrophobic regions and they are mostly cationic. The genes encoding the two different peptides are also closely associated genetically, being encoded in the same operon (Diep et al. 2009). Over 17 different class IIb bacteriocins have been characterized (Oppegard 2010).

Three-dimensional structures of some class IIb bacteriocins have been elucidated. Among them are the well-studied lactococcin G and the plantaricins EF and JK (Fimland et al. 2008; Rogne et al. 2008; Rogne et al. 2009). See Figure 1.5 for an overview of peptides constituting plantaricin JK. These peptides are unstructured in aqueous solution, but become structured in membrane mimicking environments. As shown in Figure 1.5, the structures are dominated by a defined central α-helical region (pink color) flanked by flexible regions (white color) in the ends. No three-dimensional structures have been determined for any peptide heterodimers, but a direct physical interaction between the complementary peptide, when exerting microbial activity has been shown (Hauge et al. 1999). More specifically, the peptides constituting Lactococcin G (LcnG-α and LcnG-β) are proposed to interact with each other through certain parts of the α-helical domains in each peptide (Oppegard et al. 2008). This interaction only occurs in membrane mimicking environments (see Figure 1.4).

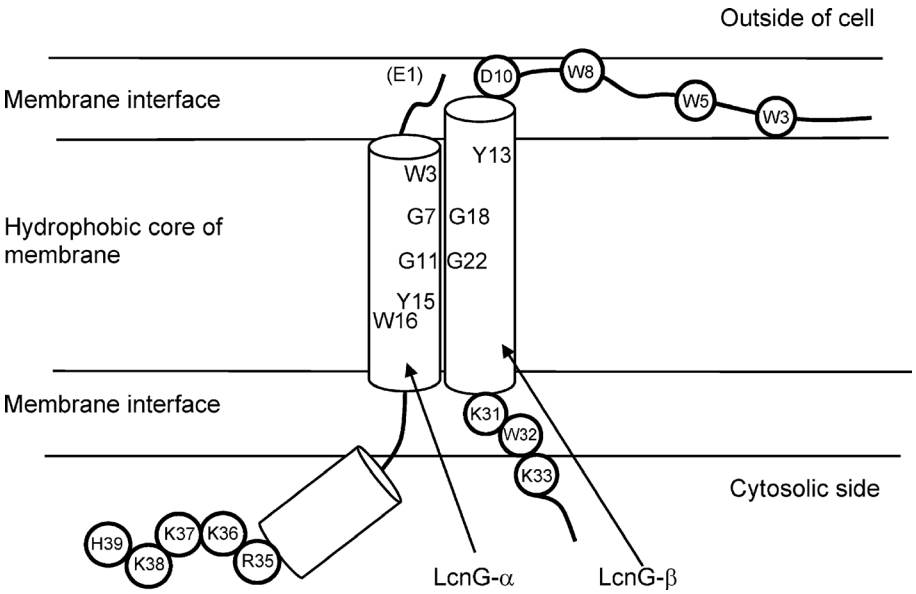

Figure 1.4 Schematic overview of proposed structure of Lactococcin G when exerting anti-microbial activity (Oppegard et al. 2008). The two peptides interact through the GxxxG-motifs found in the α-helical regions of both peptides.

The inhibitory spectrum of class IIb bacteriocins is very narrow; plantaricins EF and JK are only active against a few strains of *Lactobacillus* and *Pediococcus* as this study also shows (Anderssen et al. 1998).

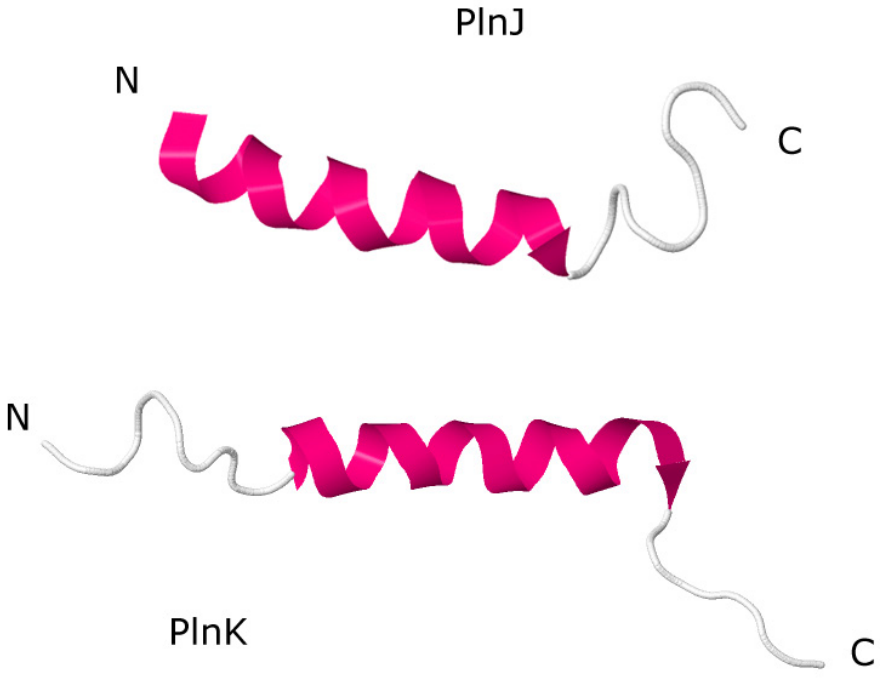

Figure 1.5 Three-dimensional structures of individual peptides constituting plantaricin JK. N- and C-termini are indicated. Structures visualized using FirstGlance in Jmol (http://firstglance.jmol.org/).

Similar to other class II bacteriocins, class IIb bacteriocins kill target cells by pore formation. However, the molecular mechanism underlying this process is completely unknown, and no target receptor for any class IIb bacteriocins have been identified.

1.2.4 The bacteriocin producing plantaricin locus

Plantaricins EF and JK are both translated from the same locus called the plantaricin locus in select *L. plantarum* strains (Diep et al. 2009). Five different loci, from different strains of *L. plantarum* have been studied so far (C11, WCSF1, NC8, J51 and J23), revealing a mosaic composition of genes between the different strains. See Figure 1.6.

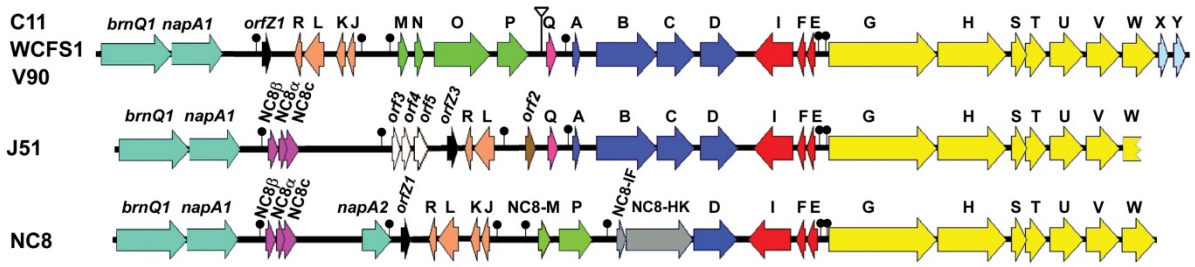

Figure 1.6 Overview of the mosaic plantaricin locus adapted from Diep et al. (2009). Lollipops indicate promoter sites. *brnQ1* and *napA1* are not part of the *pln* locus (only shown as upper boundaries). Lower ends of NC8 and J51 and *pln* loci are not completely sequenced. *pln* loci sequence from J23 is not shown.

As also observable from Figure 1.6, the single peptides constituting plantaricin EF and JK are transcribed together with their cognate immunity genes (*plnI* and *plnL* respectively) from the same operons (Nes et al. 1995). The class II bacteriocins usually contain a so called double glycine leader sequence at the N-terminal of the peptide. This sequence has the consensus motif LSxxELxxIxGG (where x is any amino acid) where the second last glycine is fully conserved (Nes et al. 1996). This leader sequence suppresses the activity of the peptide while inside the producing cell. It also serves as a recognition motif for the ATP-Binding Cassette (ABC) transporter on which the peptide binds and is secreted out of the cell (Havarstein et al. 1995). The leader sequence is cleaved off during translocation over the membrane by dephosphorylating a bound ATP molecule, using the energy to secrete the peptide. The dedicated ABC transporter thus contains a proteolytic N-terminal domain, which acts as a protease when removing the leader sequence. The ABC transporter (*plnG*) requires an accessory protein (*plnH*) in order to secrete the bacteriocin peptides, however,

the mechanism of interaction between the accessory protein and the ABC transporter remains unknown.

Although some bacteriocin production systems have been reported to be constituently active, many lactobacilli employ a tight regulation of bacteriocin production to avoid wasting energy when bacteriocin production is not needed (Eijsink et al. 2002). The mechanism behind the regulation is based on a so called three-component regulatory system, which is a quorum sensing based system (Nes et al. 1996). The three components consist of a secreted inducer peptide, a transmembrane histidine kinase, and a response regulator (usually a transcription factor). The inducer peptide (*plnA*) is translated and exported to the outside of the cell, with the help of the same ABC transporter used to secrete bacteriocin peptides. When the concentration of inducer reaches a certain threshold, it binds to the histidine kinase (*plnB*), triggering a cascade reaction chain that eventually leads to the phosphorylation and activation of the response regulator (*plnC* and *plnD*) inside the cell. This response regulator specifically binds to select promoter areas as homodimers and activate transcription of target genes (Risoen et al. 1998). *L. plantarum* NC8 does not contain the same histidne kinase (*plnB*), response regulator (*plnC*) and induction factor (*plnA*) as the other studied strains. Instead the NC8 strain relies on a different histidine kinase (*NC8-HK*) which is situated in the same area where the *plnB* gene would be (Maldonado-Barragan et al. 2009). The response regulator gene *plnC* is also absent in strain NC8, suggesting that only *plnD* is needed. The induction factor *plnA* is also replace with another gene, *NC8-IF*, which serves the same function. The bacterial population uses this three component system to communicate and regulate the production of bacteriocins, as the high concentration of inducer peptide needed to surpass the threshold level, is only achieved at high cell densities.

The precise mechanism on how many of the immunity proteins confer immunity to the producing host is not known. As the immunity genes have a conserved location, many putative immunity proteins have been discovered. These proteins vary greatly in sequence length, composition and structure, suggesting a vast repertoire of different immunity mechanisms. Recently, the immunity genes conferring immunity for plantaricins EF and JK (*plnI* and *plnL* respectively) have been studied, showing that these immunity genes belong to the Abi protein family (Kjos et al. 2010b). The Abi protein family has been characterized in eukaryotes, but any activity or molecular function has not been deduced in bacteria. The Abi

proteins functions as zinc dependent proteases in eukaryotes, specifically targeting the C-terminal sequence CAAX (where A and X are any aliphatic and any residue respectively) (Pei & Grishin 2001). Certain residues in the conserved motifs seem to be important in maintaining bacteriocin immunity, although any proteolytic activity by the bacterial immunity proteins has not been shown. However, several putative genes with a CAAX protease signature are present in the *pln* locus (*plnP, plnT, plnU, plnV and plnW*), but any function for these genes have not been deduced (Diep et al. 2009). The precise mechanism behind the immunity function has yet to be unraveled.

As observable from Figure 1.6 several putative genes with unknown function (like *plnN* and *plnO*), bacteriocin genes and their immunity genes have yet to be experimentally verified. This represents a new issue as several genomes from *Lactobacillus* are being sequenced, but experimental data confirming the authenticity of the putative genes is missing. Since many bacteriocin related operons contain conserved sequences, they are easy to assign to novel sequences. In order to verify the function of such genetic elements, the approach of searching sequenced genomes for putative bacteriocin elements, and then expressing and verifying them experimentally, has proven successful in previous studies (Begley et al. 2009).

The two-peptide bacteriocin of the plantaricin locus, plantaricin NC8 has been characterized (Maldonado et al. 2003). The putative immunity gene (*plNC8c*) however, remains to be experimentally determined. The *plNC8c* gene is believed to harbor three transmembrane helices which are necessary for membrane insertion. The J51 operon containing the putative plantaricin J51 and its immunity gene (J51-*Orf3*, J51-*Orf4* and J51-*Orf5* respectively) is even more unexplored, as none of the ORFs have been determined experimentally (Navarro et al. 2008).

1.3 Aim of this study

The focus of this study will be to examine the putative immunity genes *plNC8c* and J51-*Orf5* and the putative plantaricin J51 genes (J51-*Orf3* and J51-*Orf4*).

- Examine the sensitivity of plantaricin NC8 and the putative plantaricin J51 among different strains.
- Express the respective putative immunity genes in sensitive strains and examine if sensitivity is altered.
- Examine if the putative immunity genes are being expressed and look for possible receptor candidates.

2 Materials

2.1 Bacterial strains

Name	LMGT number	Growth Temperature (°C)	Comments[1]
Escherichia coli		37	
▪ DH5α®			Used for cloning
▪ DH5α® pMG36e	2702		Contains pMG36e, used as control plasmid
▪ B276			Contains p363, used as vector in cloning
Lactobacillus acidophilus		37	
▪ NCDO 1748	2303		
▪ ATCC 3456	2712		
L. amylophilus NCDO 2503	2300	30	
L. amylovorus NCDO 2657	2301	30	
L. casei NCDO 2743	2324	30	
L. cellobiosus NCDO 927	2306	30	
L. coryneformis NCDO 2741	2309	37	
L. curvatus		30	
▪ NCDO 2739	2310		
▪ CTC 435	2371		
▪ 89	2355B		
▪ BCS35	3291		
L. delbrueckii NCDO 213	2331	37	
L. fermentum ATCC 9338 V	2716	37	
L. helveticus ATCC 15009	2761	37	
L. plantarum		37	
▪ 965	2003		
▪ R	2352		
▪ DSM 20174	2378		Used for heterologous expression
▪ AA23	2389		
▪ ACA-DC 289	3160		
▪ 2-1	3176		Used for heterologous expression
▪ 2-2	3177		
▪ ACA-DC 3333	3215		
▪ ACA-DC 3341	3216		
▪ ACA-DC 3342	3217		
▪ NC8			Used to isolate *plNC8c* gDNA
L. rhamnosus GG	3087	37	

L. salivarius 83	3494	37	
L. sakei		30	
▪ MK326			Expresses part of sakacin 23K
▪ MK327			Expresses part of sakacin 23K
▪ 2F13	3147		
▪ 23K	3051		
▪ 64F	3052		
▪ NCDO 2714	2313		Used for heterologous expression
▪ CTC A4	2373		
▪ 460	2376		
▪ DSM 20017	2377		
▪ 706	2334		
▪ 45	2340		
▪ 77	2360		
L. viridesence NCDO 1655	2314	37	
Lactococcus lactis		30	
▪ IL1403	B244		Contains p369, used as control in SDS-PAGE
▪ MG1363	2144		
Leuconostoc mesenteroides Y105	3081	30	
Pediococcus acidilactici	2351	30	Produces pediocin PA-1
P. pentosaceus NCDO 990	2315	30	

[1] All strains without specific comments were used in the screening for bacteriocin sensitive strains.

2.2 Equipment and instruments

Type	**Model(s)**	**Manufacturer**
Agarose gel vessels		Amersham Biosciences
Autoclave	SC 500	Matachana
Balance	XF-3200	Salter
Balance	ER-182A	Salter-And
Balance	CP2202S	Sartorius
Centrifuge	5804R	Eppendorf
Centrifuge, large	J2-21M/E	Beckmann
Centrifuge, large	RC6	Sorvall
Centrifuge, tabletop	541D	Eppendorf
Centrifuge, tabletop, refrigerated	Biofuge Fresco	Heraeus
DNA Sequencer	ABI Prism 377	Applied Biosystems™
Electroporation apparatus	Pulse Controller	Bio Rad®
Electroporation apparatus	Gene Pulser™	Bio Rad®
FastPrep™	FP 120	BIO 101
Film	Amersham Hyperfilm™ ECL	GE Healthcare
Freezer	-20°C Freezer	Whirlpool

Freezer	-86 °C Freezer	Forma Scientific
French press cell	French® Pressure Cell	Aminco®
Fume hood		Kebo Healthcare Systems
Gas burner	Fireboy and flameboy	Integra Biosciences
Gel doc	Benchtop UV	UVP
Ice machine	KF85	Porkka
Incubator		Termaks
Incubator	Series 25	New Brunswick Scientific Co. Inc
Microtiter plate scanner	Multiskan® Ascent	Labsystem
Microwave oven	M182DN and M172N	Samsung
Nanodrop Spectrophotometer	ND-1000	NanoDrop®
pH Meter	MP 220	Mettler Toledo
Plate colony counter	Colony Counter	Gallenkamp
Power Supply	PS 250 and PS 500XT	Hoefer Scientific Instruments
Power Supply	Power Pac 300	Bio Rad®
Refrigerator	Grand Cooler	Bosch
Rotary Mixer	Multi Bio RS-24	Biosan
SDS-PAGE equipment		Bio Rad®
Shaker, large	KL-2	Edmund Bühler
Shaker, small	PS-3D	Grant Instruments
Spectrophotometer	Colorimeter 252	Ciba-Corning
Sterile benches	TL 2472	Holten
Sterile benches		KEBO Production
Thermocycler	MyCycler™ and S1000™	Bio rad
Thermocycler	PTC-100™	MJ Research, Inc.
Thermocycler	Mastercycler gradient	Eppendorf
Vortex mixer	G-560E	Sicentific Industries
Vortex mixer	MS2 Minishaker	IKA®
Water bath	6A	Julabo
Water bath, 55°C	TW20	Julabo
Water System	Milli-Q	Millipore
Western blot chamber	Hypercasette™	Amersham Life Science
Western blotting system	Mini Trans-Blot System	Bio Rad®

2.3 Kits

Kit	**Supplier**
ABI PRISM® BigDye® Terminator v3.1 Cycle Sequencing Kit	Applied Biosystems
E.Z.N.A Plasmid Mini kit I	Omega bio-tek
ECL Plus™ Western Blotting Detection Reagents	Amersham Biosciences
Nucleospin® Extract II	Macherey-Nagel
QIAGEN Plasmid Midi Kit	Qiagen

2.4 Chemicals

Name of chemical	Supplier
Acetic Acid (glacial)	Merck
Acrylamide/Bis-acrylamide (37.5:1) 40%	Serva
Ammonium Persulfate	Shelton Scientific
Ammonium Sulfate	Merck
ANTI-FLAG® M2 Affinity gel	Sigma®
Anti-FLAG® M2 Antibody	Sigma®
Bacto Agar	Saveen Werner AB
2-merkaptoethanol (β-merkaptoethanol)	Merck
Bovine Serum Albumin (BSA)	New England Biolabs
Bromophenol blue	usb®
Calcium chloride ($CaCl_2$)	Merck
Citric acid Monohydrate	Merck
Development Solution G 153	AGFA
Disodium carbonate (Na_2CO_3)	Merck
Disodiumhydrogenphosphate 2-hydrate ($Na_2HPO_4 \cdot 2H_2O$)	VWR International
Dithiothretiol (DTT)	Invitrogen™
dNTPs (dATP, dCTP, dTTP)	TaKaRa
Dodecyl Sulfate Sodium Salt (SDS)	Merck
Erythromycin	Sigma®
Ethanol	Kemetyl
Ethidium bromide	Sigma®
Fixation Solution G 354	AGFA
Formaldehyde – 37% (v/v)	Sigma-Aldrich®
Glass beads, acid washed ≤106µm	Sigma®
Glucose Monohydrate	Merck
Glycerol	Sigma®
Glycine	Merck
Hydrochloric acid fuming 37%(HCl)	Merck
Isopropanol	Arcus
M17 Broth	Oxoid
Magnesium chloride hexahydrate ($MgCl_2 \cdot 6H_2O$)	Merck
Methanol	VWR International
Monopotassium phosphate (KH_2PO_4)	VWR International
MRS (de mann, Rogosa, Sharpe) Broth	Oxoid
Polyethylene Glycol 1500 (PEG_{1500})	Merck
Potassium Chloride	Merck
Silver Nitrate ($AgNO_3$)	Sigma-Aldrich®
Skim milk powder	Oxoid
Sodium Chloride (NaCl)	Merck
Sodium Hydroxide pellets (NaOH)	Sigma-Aldrich®
Sucrose	Merck
TEMED	Bio Rad®
Tritiplex III	Merck

Triton X-100	Sigma®
Trizma® Base (Tris-base)	Sigma®
Trizma® hydrochloride (Tris-HCl)	Sigma®
Tryptone	Oxoid
Tween-20	Sigma®
Ultrapure™ Agarose	Invitrogen™
Yeast Extract	Oxoid

2.5 Buffers used

GYT (Glycerol Yeast Extract Tryptone) contains: 10% (v/v) glycerol, 0.125% (w/v) yeast extract and 0.25% (w/v) tryptone.

GTE (Glucose TE) contains: 50nM glucose, 50mM Tris-HCl pH 8.0 and 10mM EDTA pH 8.0. The solution was stored at 4°C.

Loading Buffer (6x) for agarose gel electrophoresis contains: 40% (w/v) sucrose, 5mM EDTA and a few grains of bromophenol blue.

PBS-T (Phosphate Buffered Saline - Tween) contains: 137mM NaCl, 26.8µM KCl, 10.14mM Na_2HPO_4, 1.76mM KH_2PO_4 and 0.5% (v/v) Tween-20. The pH was adjusted to 7.4.

SDS Sample Buffer (2x) contains: 10mM Tris-HCl pH 6.8, 4% (w/v) SDS, 20% (v/v) glycerol and a few grains of bromophenol blue. When appropriate, 10% (v/v) β-merkaptoethanol was added.

TAE (Tris Acetic EDTA) **buffer** contains: 40mM Tris-base, 1µM EDTA pH 8.0 and 1.1% (v/v) acetic acid.

TBS (Tris Buffered Saline) **buffer** (10x): 80g NaCl, 2g KCl and 30g Tris-base was mixed with 1L dH_2O. pH was adjusted to 7.4 with HCl, and the TBS was then autoclaved and stored at 4°C.

TE (Tris EDTA) **buffer** (0.1x) contains: 1mM Tris-HCl pH 8.0 and 100µM EDTA pH 8.0.

TES (TE with salt) **buffer**: 10mM Tris-HCl pH 7.5, 100mM NaCl and 1mM EDTA pH 8.0.

Transfer buffer for Western blotting contains: 192mM glycine, 19mM Tris-base and 10% (v/v) methanol. The solution was stored at 4°C.

Running buffer (5x) for SDS-PAGE contains: 192mM glycine, 25mM Tris-base and 10% (v/v) methanol.

2.6 Growth media

GM17 (Glucose Minimal 17) **medium**: 37,25g of M17 Broth (Oxoid) per liter of dH_2O was dissolved and autoclaved for 15 min.

LB (Lysogeny broth[2]) **medium**: 10g of Tryptone, 5g of Yeast Extract and 10g of NaCl per liter of dH_2O were dissolved and autoclaved for 20 min.

LA (Lysogeni Agar): 15g of Bacto Agar was added per liter of LB medium prior to autoclaving

MRS medium: 52g of MRS broth (Oxoid) per liter of dH_2O was dissolved and then autoclaved for 15 min.

MRS-agar: 15g of Bacto Agar (Saveen Wener AB) was added per liter of MRS media prior to autoclaving.

MRS-soft agar: 8g of Bacto Agar (Saveen Wener AB) was added per liter of MRS media prior to autoclaving.

MRSSM (MRS Sucrose Medium)**:** 10mM $MgCl_2 \cdot 6H_2O$ and 0.5M Sucrose were added to MRS medium.

2x MRS: 104g of MRS broth (Oxoid) per liter of dH_2O was dissolved and then autoclaved for 15 min.

SOC (Super Optimal broth with Catabolite repression) medium: 20g of Tryptone and 5g of Yeast Extract were dissolved per liter of dH_2O. In addition, 10µM NaCl and 2.5µM KCl were added before the solution was autoclaved for 20 min. Afterwards 20µm Magnesium Chloride and 20µm glucose were added before the solution was sterile filtrated.

[2] Nomenclature defined in postscript in article by Bertani (2004)

2.7 Enzymes[3]

Enzyme	Buffer	Supplier
DyNAzyme™ II DNA polymerase	10x Optimized DyNAzyme™ Buffer	Finnzymes
HindIII	Buffer 2	New England BioLabs
Lysozyme		Sigma
Mutanolysin		Sigma
Phusion® polymerase	5x Phusion® Buffer	Finnzymes
T4 DNA ligase	10x Ligase Buffer	New England BioLabs
XbaI	Buffer 4	New England BioLabs
XhoI	Buffer 4 and Buffer 2	New England BioLabs
DNAse I		Qiagen

2.8 Standards

Standard	Supplier
1kb DNA Ladder	Invitrogen
Prestained Protein Marker, Broad Range #P7708S	New England BioLabs
Prestained Protein Marker, Broad Range #P7702S	New England BioLabs

2.9 Synthetic Peptides[4]

Name	Sequence	Reference
PLNC8α	DLTTKLWSSWGY YLGKKARWNL KHPYVQF	Maldonado et al. (2003)
PLNC8β	SVPTSVYTL GIKILWSAYK HRKTIEKSFN KGFYH	
PlnJ51-Orf3	SNNKFWTWAGYTYENWRISSRRAFNLRQRKNTMTHH	Navarro et al. (2008)
PlnJ51-Orf4	IWQWIVGGLGFLAGDAWSHSDQISSGIKKRKKKGYGY	
PlnJ	GAWKNFWSSLRKGFYDGEAGRAIRR	Anderssen et al. (1998)
PlnK	RRSKRNGIGYAIGYAFGAVERAVLGGSRDYNK	
PlnE	FNRGGYNFGKSVRHVVDAIGSVAGIRGILKSIR	
PlnF	VFHAYSARGVRNNYKSAVGPADWVISAVRGFIHGs	

[3] Nomenclature as defined by Roberts et al. (2003)

[4] All peptides were ordered from Genscript with a purity of >90%

3 Methods

3.1 Cultivation and storage of bacterial strains

3.1.1 Cultivation of bacterial strains

Lactobacilli, leuconostocs and pediococci were grown in MRS medium at temperatures at 30°C or 37°C without shaking. Erythromycin resistant (em^r) strains were grown with 10µg/ml or 5µg/ml erythromycin in the medium.

E. coli DH5α® strains were grown in LB medium at 37°C. Liquid cultures were grown with moderate shaking (250rpm). Em^r strains were grown with 300µg/ml erythromycin in the medium.

Lactococcus lactis strains were grown in GM17 medium at 30°C. Em^r strains were grown with 10µg/ml erythromycin in the medium.

3.1.2 Storage of bacterial strains

All bacterial strains were stored in 15% (v/v) glycerol stocks. The stocks were prepared by adding sterile glycerol to a final concentration of 15% (v/v) to overnight bacterial culture in a cryo tube (Nalgene), and then stored at -80°C. Glycerol was added to prevent cell bursting when storing samples at -80°C.

3.2 Ammonium sulfate precipitation

As the sakacin 23K bacteriocin was not obtained as synthetic peptides, the α- and β-peptides were extracted from the two producing strains *L. sakei* MK 326 and *L. sakei* MK 327 respectively (Kjos et al. 2010b). The bacteriocin pediocin PA-1 was obtained using the same method from *Pediococcus acidilactici* (Nieto Lozano et al. 1992).

1. 100ml cultures were grown overnight and then centrifuged at 7000 × g for 10 min.
2. The supernatants were then removed and cooled at 4°C, and 30g of ammonium sulfate was then added to each solution.
3. The solutions were then incubated at 4°C for 45 min with moderate shaking.
4. Afterwards the solutions were centrifuged at 20 000 × g for 30 min. at 4°C.

5. The supernatants were then decanted and the pellets were dissolved in 2ml dH_2O for a final concentration of 50x.
6. The solutions were then incubated for 10 min at 70°C to kill any remaining bacterial cells.
7. The peptide concentrates were then stored at -20°C.

3.3 Soft agar assay

Soft agar assays were carried out to qualitatively assay the bacteriocin sensitivity among different strains. All assays were carried out by diluting ON cultures approximately 100-fold in MRS soft agar (30µl of ON culture in 4ml of MRS soft agar) and immediately plate out the solution on MRS agar plates. After air drying the plates for a minimum of 5 min, 1-2 µl of each bacteriocin (in a 1:1 ratio of each peptide when combined[5]) were spotted. A spot with dH_2O was used as a control. The plates were then incubated at 30°C or 37°C over night. The next day the plates were taken out of the incubator and the diameter of any zones of inhibition was measured.

3.3.1 Wild-type soft agar assays

Soft agar assays of wild-type strains were performed in order to screen for sensitive strains and to establish an initial degree of sensitivity. Assays were prepared as described in section 3.3 above with 2µl of each bacteriocin spotted on.

3.3.2 Soft agar assays of transformed cells

Soft agar assays were performed on transformed cells in order to investigate if sensitivity to the complementary bacteriocin was changed. Assays were prepared as in section 3.3 above with the addition of 10µg/ml or 5µg/ml erythromycin in both the MRS soft agar and the MRS plates, with 2µl of 10-fold dilutions of NC8 and J51 bacteriocins spotted on.

3.3.3 Soft agar assays of transformed cells in presence of DTT

Transformed cells with FLAG™*plNC8c* where grown in the presence of DTT in order to investigate if expression of the immunity gene would be changed. The soft agar assays were performed as described by Cesselin et al. (2009). Different concentrations of DTT were added to the soft agar of each plate along with 10µg/ml erythromycin and immediately

[5] The usage of a 1:1 peptide ratio is discussed in section 5.1.

plated out on MRS plates containing 10µg/ml erythromycin. Bacteriocins were spotted on as in section 3.3 with pediocin PA-1 as a control. All plates were incubated anaerobically at 30°C.

3.4 Microtiter plate assays

A microtiter plate assay was performed on both wild type and transformant clones in order to quantify the sensitivity. This was done using a microtiter plate. All of the applied bacteriocins were mixed in a 1:1 ratio.

ON cultures of indicator strains were diluted 50- to 100-fold in MRS medium. 150µl of these dilutions were added to 2-fold dilution series of each bacteriocin (diluted in a total of 50µl MRS medium). Wells without bacteriocin were used as growth controls, and wells containing only MRS medium were used as negative controls. The plates were incubated at 30°C or 37°C until the growth in positive control wells had reached an OD_{620} of 0.3-0.5. The plates were shaken at 960 rpm for 10 sec. before reading to compensate for any uneven distribution of cells within the wells.

3.4.1 Wild type microtiter plate assays

Strains who displayed sensitivity to either PLNC8 or the putative plantaricin J51 were selected for microtiter plate assaying in order to quantify the sensitivity. Both PLNC8 and the putative plantaricin J51 were used, along with PlnJK and sakacin 23K. The MIC assay was carried out as in section 3.4.

3.4.2 Microtiter plate assays of transformant clones

Strains that had been transformed with the putative *plNC8c* or J51-*Orf5* bacteriocin immunity genes were set up for microtiter plate assaying in order to quantify the sensitivity. Both plantaricin NC8 and the putative plantaricin J51 was used (regardless of cloned putative immunity gene), along with plantaricin JK. The microtiter plate assay was carried out as in section 3.4 with 10µg/ml or 5µg/ml erythromycin in the MRS medium.

3.5 Isolation and purification of genomic DNA and plasmids

There are several ways to extract and purify DNA from bacterial cells. The choices of lysis and purification techniques are dependent on a number of factors. The cell wall (Gram-positive or Gram- negative) type of DNA (genomic or plasmid) and downstream application of the DNA (screening, cloning etc.) are all factors which must be taken into consideration when determining a strategy for lysis and DNA purification.

Commercially available kits were used to extract plasmid DNA from bacterial cells. Since these kits are optimized for *E. coli* (which is a Gram-negative bacteria (Krieg & Holt 1984)), some modifications were made in order to obtain sufficient amounts of plasmid DNA from lactobacilli.

Two different plasmid preparation methods were applied: MIDIprep and miniprep. The methods differ in the amount and purity of the DNA eluate. While MIDIprep gives a larger amount of plasmid DNA, it also requires a larger volume of bacterial culture from which the DNA should be isolated from. The DNA is also precipitated during MIDIprep, which frees the DNA of other polluting agents. Therefore the MIDIprep method was chosen when large amounts of high quality plasmid DNA were needed for subsequent cloning applications. When the high fidelity of the MIDIprep technique was not needed (such as in PCR for colony screening), the less time consuming miniprep method was chosen.

3.5.1 Isolation of genomic DNA

Genomic DNA was isolated from *Lactobacillus plantarum* NC8 using a mechanical lysis method called Fastprep. This method involves subjecting the cells to a powerful shear force by shaking them in the presence of different sized glass beads. The beads will penetrate and fragment the cell wall and membrane of bacterial cells. As the beads will also fragment the chromosomal DNA, a kit for isolating plasmids can be employed to further purify the total genomic DNA lysate.

1. *L. plantarum* NC8 was grown according to section 3.1.1 and harvested by centrifugation at 16 000 × g for 1 min at 4°C.
2. The pellet was resuspended in buffer 1 from the E.Z.N.A Plasmid Minikit I and mixed with 0.5g of glass beads (≤106µm) in a 2ml FastPrep tube.

3. The tube was shaken twice at 4m/s for 20 sec in a Fastprep machine and then centrifuged at 16 000 × g for 2 min at 4°C (to spin down beads and unwanted cell debris).
4. The supernatant was then collected and the remaining pellet was again resuspended in buffer 1 and step 3 was repeated two more times.
5. The E.Z.N.A Plasmid Minikit I protocol was then resumed from step 4 in the E.Z.N.A miniprep protocol (See Appendix 1).
6. The concentration of DNA eluate was measured on a Nanodrop spectrophotometer, and 5 µl of the eluate was analyzed on an agarose gel according to section 3.7.

3.5.2 Isolation of plasmids for cloning and sequencing (MIDIprep)

Plasmids where isolated from *E. coli* using the QIAGEN Plasmid Midi Kit according to the manufacturer (QIAGEN 2005). This isolation protocol exploits the slightly different denaturation and renaturation properties of chromosomal and plasmid DNA. Bacterial cell pellets were resuspended in a Tris EDTA buffer (Buffer P1) containing RNAseA (to quickly degrade RNA when the cells have been lysed) and lysed by addition of a NaOH-SDS solution (Buffer P2). A sodium acetate containing solution (Buffer P3) was added to neutralize the solution and to shift the solution to a high-salt binding condition. Plasmids will renature quickly under such conditions, while larger chromosomal DNA will aggregate and precipitate along with SDS-protein complexes and high molecular weight RNA. The supernatant containing plasmid DNA was then transferred to a silica-gel based anion-exchange column that readily binds DNA under high-salt conditions. Other macromolecules present in the supernatant will not bind to the column and was washed away using a high-salt based solution (Buffer QC). The DNA was eluted from the column using an isopropanol based buffer (Buffer QF). The DNA was further precipitated using isopropanol, then ethanol. The DNA pellet was then air-dried before resuspending it in dH_2O. The concentration of the DNA was measured using a Nanodrop Spectrophotometer.

3.5.3 Isolation of plasmids for colony screening (Miniprep)

Plasmids from *E. coli* were isolated using the E.Z.N.A Plasmid Minikit I according to the manufacturer (Omega-bio-tek 2009). When isolating plasmids from lactobacilli some modifications to the manufacturers' protocol were made. Bacterial cell pellets were washed with TES buffer (to remove medium and adjust pH) and resuspended in ice-cold GTE buffer

containing 5mg/ml Lysozyme, 15U/ml Mutanolysin and 100µg/ml RNAse A. This suspension was incubated at 37°C until the suspension became sticky when adding Solution 2 from the E.Z.N.A Plasmid Minikit I (indication of cell lysis, usually 10-50 min). The E.Z.N.A Plasmid Minikit I Spin Protocol was then resumed from Step 4 (See Appendix 1). This protocol follows the same trend as the MIDIprep protocol (section 3.5.2). The cell suspension is lysed using a NaOH-SDS solution (Solution II) and a sodium acetate solution is added to neutralize the solution again and shift the solution to a high-salt binding condition. Plasmid and highly fragmented chromosomal DNA will renature under these conditions, and large chromosomal DNA and proteins will precipitate. The supernatant (containing plasmid DNA) was then transferred to a silica-gel based column that readily binds the DNA, leaving other macromolecules prone to elution with a wash buffer (Buffer HE). The DNA was then washed using an ethanol based solution (DNA Wash Buffer). The DNA was eluted using the included elution buffer and the concentration and quality were measured using a Nanodrop spectrophotometer.

3.6 Polymerase Chain Reaction (PCR)

PCR is a basic and widely used technique in molecular biology (Sambrook & Russel 2001a). As such, there are many different variations of this technique, but most of them are used to amplify fragments of DNA. Typical components of a PCR are: a template (usually DNA), a pair of oligonucleotide primers, a thermostable DNA polymerase and dNTPs. The reaction is carried out in cycles of different temperatures to promote different activities. A cycle typically starts with (i) denaturing of the dsDNA at high temperature, (ii) annealing of the primers to the denatured ssDNA strands at a lower temperature, and (iii) extension of complementary DNA from the primers catalyzed by the thermostable DNA polymerase at its optimum temperature. Each step in the cycle is fine tuned for each application, as is the amount of total cycles.

The choice of DNA polymerase was dependent on the downstream application of the amplified DNA fragments. When maximum fidelity was required, such as in cloning, an enzyme with good processivity and proofreading capabilities like the Phusion® DNA polymerase was used. In less sensitive applications, like in colony screening, a more basic (and cheaper) DNA polymerase could be utilized. The DyNAzyme™ II DNA polymerase, which

lacks the 3' → 5' proofreading capability of the Phusion® enzyme, was used for colony screening.

3.6.1 PCR for amplification of *plNC8c*

Genomic DNA containing the putative *plNC8c* bacteriocin immunity gene was isolated from *Lactobacillus plantarum* NC8 according to section 3.5.1.

Two different 50µl reactions were set up using the Phusion® DNA polymerase (Finnzymes) system and the manufactures instructions as guidelines. One reaction was set up with mk303 as the forward primer, and the other reaction with the mk304 forward primer. The reverse primer mk305 was used in both reactions. The primers each contain a restriction site as an overhang used in the subsequent cloning procedure; mk305 contains an XhoI site, mk303 contains an XbaI site, while mk304 contains a HindIII site. For the primer sequences see Table 3.8.

The PCR mixtures were set up as described in Table 3.1 below.

Table 3.1 PCR-mixture of *plNC8c* gDNA.

Solution	Volume (µl)
5x buffer	10
Forward-primer (100µM)	0.5
Reverse-primer (100µM)	0.5
dNTPs (10µM of each nucleotide)	1
Phusion® DNA Polymerase (2U/µl)	0.5
Template gDNA (477ng/µl)	0.5
dH_2O	37
Total	50

The reactions where performed in a thermocycler following the program in Table 3.2 below

Table 3.2 PCR-program for plNC8c amplification.

Step	Temperature (°C)	Time	
1	95	5 min	Initial denaturing
2	95	30 sec	Denaturing
3	60	30 sec	Annealing
4	72	30 sec	Extension
5	30 cycles of step 2-4		
6	72	7 min	Final extension

5µl of the PCR products were then analyzed on an agarose gel with a 1kb ladder for size comparison according to section 3.7.

3.6.2 Touchdown PCR for amplification of J51-*Orf5*

Genomic DNA containing J51 immunity gene was obtained as a pellet from Professor Carmen Torres, Universidad de La Rioja, Spain. This DNA pellet was dissolved in dH_2O and the concentration was measured with a Nanodrop spectrophotometer.

Reaction mixtures of 50µl were set up using the Phusion® DNA polymerase system. Two different reactions were set up; one with mk320 as the forward primer, and the other with mk321 as the forward primer. mk322 was used as reverse primer in both reactions. The mk322 primer contains an XhoI restriction site as overhang, while mk320 contains an XbaI site and mk321 a HindIII site used in the subsequent cloning. For the primer sequences see Table 3.8.

Since a strong primer-dimer band was present in the agarose gels after running the same protocol as for the *plNC8c* PCR amplification, a different approach was taken.

A so called touchdown PCR was implemented with some modifications (R.H.Don et al. 1991). This type of PCR is useful for optimizing reactions with unknown annealing temperatures, or otherwise complex or difficult PCRs. This technique is less time consuming than having to empirically determine the source(s) of error(s) for each reaction. Touchdown PCR takes advantage of the exponential feature of the PCR. The annealing temperature is usually set a few degrees higher than the calculated T_m (calculated T_m for the primers were 60°C), and then lowered some degrees after 5 cycles until a "touchdown" of 55°C when 30 cycles are carried out. This will greatly increase the advantage towards the specific product. See Table 3.4 below for a description of the program used on the thermocycler.

A Q-solution (Qiagen) was also added to the PCR mixture in order to increase the amount of DNA output. The reactions were set up according to Table 3.3 below.

Table 3.3 PCR mixture for J51-*Orf5* gDNA.

Solution	**Volume (µl)**
5x buffer	10
Q-solution	0.5
Forward-primer (100µM)	0.5
Reverse-primer (100µM)	0.5
dNTPs (10µM of each nucleotide)	1
Phusion® DNA Polymerase (2U/µl)	0.5
Template gDNA (40ng/µl)	0.5
dH_2O	36.5
Total	50

The reactions were performed in a thermocycler following the program in Table 3.4 below

Table 3.4 PCR-program for J51-*Orf5* amplification.

Step	**Temperature (°C)**	**Time**	
1	95	5 min	Initial denaturing
2	95	30 sec	Denaturing
3	62	30 sec	Annealing
4	72	30 sec	Extension
5	5 cycles of step 2-4		
6	95	30 sec	Denaturing
7	58	30 sec	Annealing
8	72	30 sec	Extension
9	5 cycles of step 6-8		
10	95	30 sec	Denaturing
11	55	30 sec	Annealing
12	72	30 sec	Extension
13	30 cycles of step 9-11		
14	72	7 min	Final extension

5µl of the PCR products were then analyzed on an agarose gel with a 1kb ladder for size comparison according to section 3.7.

3.6.3 PCR for colony screening

After cloning and transformation, a simple PCR test was used to check if any transformed *E. coli* or lactobacilli contained the desired insert. If the desired insert was present in cells from tested colonies, this insert would be amplified by PCR and produce a strong band when subjected to an agarose gel electrophoresis.

Plasmids were isolated according to section 3.5.3 and the DNA concentration of the eluate was measured using a Nanodrop spectrophotometer. The same primer pairs as in the initial amplification were used (i.e. mk303 and mk305 or mk304 and mk305 for the *plNC8c* constructs, and mk320 and mk322 or mk321 and mk322 for the J51-*Orf5* constructs. The reactions were set up according to Table 3.5 below.

Table 3.5 PCR-mixture of plasmid DNA for colony screening.

Solution[1]	**Volume (µl)**
10x DyNAzyme™ buffer	2
Forward-primer (10µM)	1
Reverse-primer (10µM)	1
dNTPs (10µM of each nucleotide)	0.5
DyNAzyme™ II polymerase (2U/µl)	0.2
Template plasmid DNA (approximately 100ng)	2 - 12
dH_2O	3.3 -13.3
Total	20

[1] 0.5 µl solution Q was added to the reaction for screening of J51 clones.

The reactions where performed in a thermocycler following the program in Table 3.6 for the *plNC8c* clones and Table 3.7 (Touchdown PCR) for the J51-*Orf5* clones.

Table 3.6 PCR program for *plNC8c* colony screening.

Step	**Temperature (°C)**	**Time**	
1	95	5 min	Initial denaturing
2	95	30 sec	Denaturing
3	60	30 sec	Annealing
4	72	45 sec	Extension
5	30 cycles of step 2-4		
6	72	7 min	Final extension

Table 3.7 PCR-program for J51-*Orf5* colony screening.

Step	Temperature (°C)	Time	
1	95	5 min	Initial denaturing
2	95	30 sec	Denaturing
3	62	30 sec	Annealing
4	72	45 sec	Extension
5	5 cycles of step 2-4		
6	95	30 sec	Denaturing
7	58	30 sec	Annealing
8	72	45 sec	Extension
9	5 cycles of step 6-8		
10	95	30 sec	Denaturing
11	55	30 sec	Annealing
12	72	45 sec	Extension
13	30 cycles of step 9-11		
14	72	7 min	Final extension

10µl of the PCR products were then analyzed on an agarose gel with a 1kb ladder for size comparison according to section 3.7.

Table 3.8 PCR primers.

Name	Sequence (5'→ 3')[1]	Complementing restriction enzyme
mk303	ACGTTCTAGAAGTTATGGAGCTCTTGGGGA	XbaI
mk304	ACGTAAGCTTATGGAGCTCTTGGGGATATTA	HindIII
mk305	ACGTCTCGAGATCGATTCTATGAATAAACCGG	XhoI
mk320	ACGTTCTAGATTTGTTCTTTTAATGTTTCGGGT	XbaI
mk321	ACGTAAGCTTATGTTTCGGGTAGATAAAGACA	HindIII
mk322	ACGTCTCGAGATAAACTCTCATTAGTGCTGCA	XhoI

[1] Restriction sites are underlined

3.7 Agarose gel electrophoresis

Agarose gel electrophoresis was used to inspect and separate DNA. Agarose forms a network of pores in the gel-state which allows for a separation of the negatively charged DNA fragments by size when an electric current is applied. Smaller fragments will migrate more easily than larger ones through the pores. The fluorescent dye and intercalating agent ethidium bromide was added to 1% (w/v) TAE-agarose gels and DNA samples were mixed with loading buffer prior to application. An electric current of approximately 75V was applied during the electrophoresis of the gels. The fragments were visualized under ultra violet light (λ = 312nm).

3.8 Plasmid construction

The pMG36e plasmid was used as an expression vector (Guchte et al. 1989). This plasmid contains an erythromycin resistance gene (*ermC*) useful for selection of cells containing the plasmid. It also has another functional gene, *repA* which governs the replication of the plasmid. The multiple cloning site (MCS) is located downstream of a constituently active promoter called P32 which will lead to any insert being transcribed at a relative high level (Venema 1993).

When constructing plasmids with *plNC8c* and J51-*Orf5* genes, a derivate of the pMG36e plasmid called p363 was used (Diep et al. 2007). The p363 contains the *flciA* gene inserted in the MCS of pMG36e. This gene has a so called FLAG™ encoding sequence just downstream of the promoter area. The FLAG™ sequence (MDYKDDDDK) is a highly hydrophilic sequence stretch that will usually not fold into the protein (Hopp et al. 1988). This sequence will be fused to the N-terminal of the translated gene resulting in a tag that makes it possible to detect and purify the protein. By using restriction enzymes which cuts either upstream or downstream of the FLAG™ sequence, one can easily construct *plNC8c* and J51-*Orf5* genes both with and without a FLAG™-tag in their respective translated products.

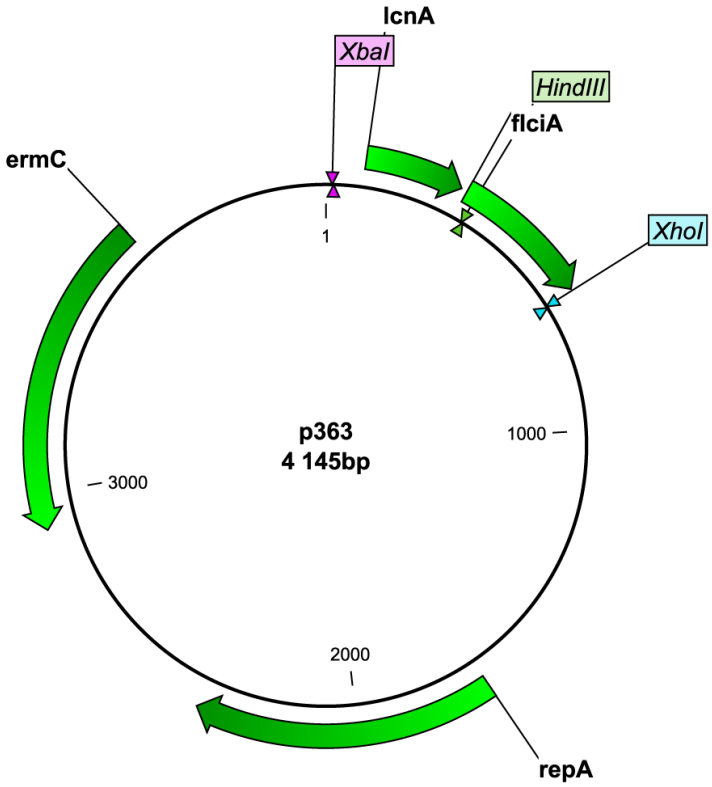

Figure 3.1 Overview of the p363 plasmid, a pMG36e derivate (Guchte et al. 1989). Genes are indicated in green with arrows showing direction of transcription. Restriction sites are shown as hourglass figures.

Before the genes can be inserted into the plasmids, the plasmids need to be cut open, so the inserts have free ends to ligate to. This is done by using restriction enzymes that cleave the plasmid and linearize it. These restriction enzymes do not cut the dsDNA vertically, but rather generate a so called “sticky end” with an overhang at the opposite strand at each fragment (see example in Figure 3.). This overhang also reduces the possibility for the plasmid to religate to itself after being cut. Since the p363 plasmid already had an insert between the two restriction sites, a smaller band should appear when subjecting the restriction reaction to an agarose gel electrophoresis. This would serve as a control that the plasmids had been cut correctly.

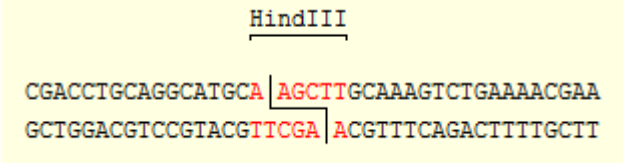

Figure 3.2 Cutting example of the restriction enzyme HindIII with the recognition site marked in red, and actual cut by full lines (Old et al. 1975)

Before initiation of the restriction reaction, the PCR products need to be free of pollutants that can disturb the restriction reaction. The PCR solutions from section 3.6.1 and 3.6.2 were purified using the Macherey-Nagel Nucleospin® Extract II kit according to the manufacturer (Macherey-Nagel 2010). This protocol is based on purifying DNA on a silica membrane based column, which adsorbs DNA under high salt conditions, leaving contaminants like salts and soluble macromolecular complexes vulnerable to elution. Two volumes of Buffer NT were added per 1 volume of PCR solution, and the mixed solution was loaded on NucleoSpin® Extract II Column. An ethanol based buffer (Buffer NT3) was added to elute contaminants. DNA was eluted by addition of a slightly alkaline buffer (Buffer NE) and the DNA concentration of the eluate was measured using a Nanodrop spectrophotometer.

3.8.1 Restriction reactions

A total of eight restriction reactions were set up, four with the plasmid vectors and four with the inserts according to Table 3.9 below.

Table 3.9 Restriction reactions.

	***pINC8c* restriction reactions**				**J51-*Orf5* restriction reactions**			
	Wild type gene		**With FLAG™- tag**		**Wild type gene**		**With FLAG™- tag**	
	Vector	**Insert**	**Vector**	**Insert**	**Vector**	**Insert**	**Vector**	**Insert**
DNA	3µl (625ng/µl)	9µl (132ng/µl)	3µl (625ng/µl)	9µl (164ng/µl)	3µl (625ng/µl)	14µl (40ng/µl)	3µl (625ng/µl)	14 µl (43ng/µl)
10x buffer	Buffer 4 (2µl)		Buffer 2 (2µl)		Buffer 4 (2µl)		Buffer 2 (2µl)	
BSA	2µl (1mg/ml)							
Enzyme 1	1µl XbaI (20U/µl)		1µl HindIII (20U/µl)		1µl XbaI (20U/µl)		1µl HindIII(20U/µl)	
Enzyme 2	1µl XhoI (20U/µl)							
dH_2O	11µl	5µl	11µl	5µl	11µl	0 µl	11µl	0 µl
Total	20 µl							

The reactions were incubated in a water bath at 37°C for 2h. After 1h, 1µl of CIP (Calf Intestinal alkaline Phosphatase) and 2µl of Buffer 3 was added to each of the vector reactions. This was done to prevent self-religation of the vector.

The whole volumes of the reactions were analyzed in an agarose gel as described in section 3.7. The visualized bands were then cut out of the gel and the mass was measured using a balance.

3.8.2 Gel clean up

As the samples from section 3.8.1 contained heavy amounts of agarose and ethidium bromide that would hinder subsequent ligation reactions, these contaminants needed to be removed. This was done using the same kit as described in section 3.1.1, as this kit is also designed to purify DNA from agarose gels. In order to dissolve the agarose, 200µl Buffer NT per 100mg of gel was added, and the samples were heated at 50°C for 5-10min and vortexed every 2-3min until the gel was completely dissolved. The rest of the procedure is identical as the one described in section 3.1.1. The concentrations of the eluted DNA samples were measured using a Nanodrop spectrophotometer.

3.8.3 Ligation and drop-dialysis

The inserts and vectors were now ready to be ligated using an enzyme which fuses two DNA fragments by making a covalent bond between the 3' hydroxyl ends of one nucleotide, with the 5' phosphate end of another nucleotide (Sambrook & Russel 2001b). The T4 DNA ligase (isolated from bacteriophage T4, New England Biolabs) was used in this study. As plasmids without inserts are unwanted, a molar ratio of 1:5 of plasmid versus insert was chosen to maximize insertion of inserts into plasmids. Four reactions were set up according to Table 3.10 below.

Table 3.10 Ligation reaction mixes.

	plNC8c		**J51-*Orf5***	
Constructs	**Wildtype**	**FLAG™-tag**	**Wildtype**	**FLAG™-tag**
Insert	4µl (23ng/µl)	4µl (11ng/µl)	6µl (21ng/µl)	6µl (22ng/µl)
Vector	1µl (55g/µl)	1µl (29ng/µl)	2µl (39ng/µl)	2µl (31ng/µl)
Buffer	2µl T4 DNA ligase buffer			
Ligase	1µl T4 DNA ligase (400U/µl)			
dH_2O	12µl		9µl	
Total	20µl			

The solutions were incubated on a thermocycler programmed to run at 16°C for 16h, then 65°C for 20min (to deactivate the ligase).

As any salts present in the ligation mixes may cause an overflow of electricity and kill cells or even destroy equipment during electroporation, it is important to remove salts after ligation. This was done by drop dialysis. The samples were spotted on a filter with 0.025µm pores, floating on top of 0.1x TE-buffer and incubated for at least 1h.

3.9 Preparation and transformation of cells

As direct transformation of ligation reactions into lactobacilli is very difficult, *E. coli* DH5α® cells were chosen as intermediate hosts. *E. coli* DH5α® have a much higher transformation rate than lactobacilli, and the generation time is shorter, ensuring that more plasmids can be harvested from the same volume of culture (Aukrust et al. 1995; Tortora et al. 2007).

There are several different transformation methods for the transformation of bacteria, but most of them work by the same principle of weakening the cell envelope, and thereby allowing the influx of foreign DNA (Sambrook & Russel 2001b). Transformation by electroporation works by subjecting the cells to an electrical pulse of high voltage (over one thousand Volts) in a suspension of DNA, which creates water filled pores in the membrane big enough to let the foreign DNA (which is negatively charged) pass into the cells (Neumann et al. 1982). Before the cells can be transformed, they need to be prepared for electroporation by removing extracellular polysaccharides and weaken the cell wall. Electroporation was chosen as the method of transformation for both *E. coli* and *Lactobacillus* although the protocols differ slightly from each other.

3.9.1 Preparation of electro competent *E. coli* DH5α® cells

E. coli DH5α® cells were prepared according to Ausbel (1995) with some minor modifications.

1. 100ml of LB medium was inoculated with 1ml ON culture of *E. coli* DH5α ®cells and grown according to section 3.1.1 to an OD_{600} of 0.4 – 0.5.
2. The culture was then chilled on ice for 30 min.
3. Cells where then harvested by centrifuging for 10 min at 7000 × g at 4°C and the supernatant was removed.

4. The pellet was washed four times with 50ml of ice-cold 10% (v/v) glycerol solution.
5. The final cell pellet was then resuspended in in ice-cold GYT medium to a final volume of 500µl.

Cells where either transformed immediately according to section 3.9.2, or stored in smaller aliquots at -80°C.

3.9.2 Transformation of ligation mixes in electrocompetent *E. coli* DH5α®

Ligation mixes from section 3.8.3 were transformed into *E. coli* DH5α® cells according to Ausbel (1995).

1. 20µl of electrocompetent cells (from section 3.9.1) was transferred to an ice-cold 1mm cuvette placed in the electroporation apparatus.
2. The electric pulse was delivered with the following settings: voltage 1.7kV, resistance 200 Ω, and capacitance 25µF.
3. 1ml of ice-cold SOC medium was added immediately to the cuvette after electroporation.
4. The cells where resuspended and transferred to eppendorf tubes and incubated at 37°C for 1h with moderate shaking.
5. 100µl and 200µl aliquots where then plated out on LA plates containing 300µg/ml erythromycin and grown according to section 3.1.1 for one to two days. A 10-5 fold dilution plated out on medium not containing antibiotics was used as a transformation efficiency control.

At least five colonies from each construct where picked with a sterile toothpick and placed in 5ml of LB medium with 300µg/ml of erythromycin and grown ON. Plasmids where then isolated from each of the colonies according to section 3.5.3 and checked for inserts using PCR screening as in section 3.6.3. Colonies revealed to have a positive PCR product where stored as in section 3.1.2 and a high-yield plasmid isolation was performed according to section 3.5.2. The isolated plasmids were checked for correct insert by sequencing as in section 3.10.

3.9.3 Preparation of electro competent *Lactobacillus* cells

Lactobacillus strains sensitive for bacteriocins NC8 and J51 where prepared for electroporation according to (Aukrust et al. 1995). These cells where transformed with plasmids containing *plNC8c* or J51-*Orf5* isolated from *E. coli* for heterologous expression of the respective genes. A glycine gradient was used to find the highest glycine concentration which still allowed optimal growth of cells.

1. 5ml of culture was grown ON and reinnoculated in 5ml cultures containing 1%, 1.3%, 1.5%, 1.8%, 2% and 2.5% (v/v) glycine in MRS medium and grown ON.
2. Cells where then diluted to an OD_{600} of 0.1 in 50ml MRS cultures containing 1%, 1.5% and 2% (v/v) glycine and grown to an OD_{600} of 0.4.
3. Cells where then harvested by centrifugation at 3000 × g at 4°C for 10 min.
4. The supernatant was then removed, and cells were washed with 12.5 ml of ice-cold TES buffer and centrifuged as in step 3 above.
5. The supernatant was then discarded and the pellet was washed with 50ml of 1mM $MgCl_2$ and centrifuged as in step 3 above.
6. The supernatant was then removed and the pellet was washed in 25ml of 30% (w/v) PEG_{1500} and centrifuged at 6000 × g at 4°C for 10 min.
7. The supernatant was then removed and the pellet was resuspended in 500µl of 30% (w/v) PEG_{1500}.

Cells where either transformed immediately according to section 3.9.4, or stored in smaller aliquots at -80°C.

3.9.4 Transformation of plasmids in bacteriocin sensitive lactobacilli

Plasmids isolated from *E. coli* were transformed in lactobacilli. The pMG36e plasmid isolated without insert from *E. coli* DH5α® pMG36e was used as a control. Lactobacilli where transformed according to Aukrust et al. (1995).

1. 5µl of plasmid DNA was added to 40µl of competent cell suspension (from section 3.9.3) and mixed gently.
2. The solution was then transferred to an ice-cold 2mm cuvette placed in the electroporation apparatus
3. The electric pulse was delivered with the following settings: voltage 1.5kV, resistance

400 Ω and capacitance.

4. 960µl of ice-cold MRSSM medium was added immediately to the cuvettes after electroporation.
5. The cells where resuspended and transferred to culture tubes and incubated at 30°C for 4h.
6. 250µl and 755µl aliquots where plated out on MRS agar plates and grown according to section 3.1.1 for one to three days.

At least five colonies from each construct where picked with a sterile toothpick and placed in 5ml of MRS medium with 10µg/ml or 5µg/ml of erythromycin and grown ON. Plasmids where then isolated from each of the colonies according to section 3.5.3 and checked for inserts using PCR screening as in section 3.6.3. Colonies revealed to have a positive PCR product where stored as in section 3.1.2.

3.10 DNA sequencing

Plasmids isolated from transformed *E. coli* DH5α® cells where double checked for insert using DNA sequencing. This was done using a variant of the so called chain-terminating method (Sanger et al. 1977). A PCR like reaction was set up with only one primer for each reaction (forward or reverse) with fluorescently labeled dideoxynucleotides (ddNTPs) in shortage (compared to unlabeled dideoxynucleotides). This will terminate the elongation whenever a dideoxynucleotide is incorporated into the DNA fragment by the polymerase. Each 3' end of the newly synthesized strand will therefore be fluorescently labeled. Different labels are used for different ddNTPs and since there is only one primer for each reaction, fragments of different sizes will be produced. These fragments will migrate through a gel-filled capillary tube based on size (same principle as agarose gel electrophoresis explained in section 3.7). The fragments can then be read by a laser at the end of the tube which determines the sequence of the DNA sample based on the incorporated fluorescent dye (Swerdlow & Gesteland 1990).

3.10.1 DNA sequencing reaction

The DNA sequencing reaction is in principle much the same as the PCR with minor differences explained in section 3.10 above. Two reactions were set up for each clone already displaying a positive PCR product when checked for insert (as in section 3.6.3).One reaction was set up using the forward primer, and one with the reverse. Each reaction was set up according to Table 3.11 with one of the corresponding primers from Table 3.8.

Table 3.11 DNA sequencing reaction mixes.

Solutions	Volume (µl)
Premix	2
Sequencing buffer	3
Primer (1µM)	3.2
Plasmid DNA (approximately 200ng)	0.5 - 2
dH_2O	11.3 - 9.8
Total	20

The reactions were performed on a thermocycler following the program in Table 3.12 below.

Table 3.12 Sequencing reaction program.

Step	Temperature (°C)	Time	
1	96	1 min	Initial denaturing
2	95	10 sec	Denaturing
3	50	5 sec	Annealing
4	60	4 min	Extension
5	25 cycles of step 2-4		

The reactions where then precipitated as describes in section 3.10.2 below.

3.10.2 Precipitation of DNA sequencing reaction products

Before the reactions can be read, they need to be free of pollutants like primers and unused fluorescent ddNTPs which can disturb the reading of the sequence by the laser. The principle behind this DNA precipitation method is much the same as the principle behind the precipitation in MIDIprep (see section 3.5.2).

1. 2µl of 125mM EDTA was added to each of the 20µl sequence reactions along with 2µl of 3M Sodium Acetate. The solutions where then transferred from the PCR tubes to 1.5ml Eppendorf tubes.

2. 52µl of 96% (v/v) ethanol was then added to each tube and mixed by invertion 5 times.
3. The solutions were then incubated for 15 min at room temperature.
4. The tubes were then centrifuged at 16 000 × g for 30 min at 4°C.
5. The supernatant was then discarded and 70µl of 70% (v/v) Ethanol was then added to each tube for washing.
6. Tubes were then centrifuged at 16 000 × g for 10 min.
7. The supernatant was then discarded and the pellet was dried under a desk lamp for 5-10 min and then stored at -20°C.

The pellets where dissolved and applied to the ABI Prism 377 DNA Sequencer and read by the machine.[6] The chromatograms were curated using the BioEdit software by removing poor sequence regions in the ends (Hall 1999). Each complementary forward and reverse sequence were combined using the CAP Contig Assembly Program within the BioEdit software suite (Huang 1992). The assembled contigs were aligned with their respective reference sequences using the sequence alignment feature of the blastn software hosted on NCBIs webpage (Zhang et al. 2000).

3.11 Cell lysis and protein purification

In order to study protein expression in the transformed *Lactobacillus* strains, the protein contents of the cells needs to be extracted. Several techniques exist, among them are FastPrep (a modification of the protocol described in section 3.5.1) and French press, which is more suited for large scale protein extraction. The French press method also employs a mechanical lysis strategy, but by the way of subjecting cells to a sudden pressure drop from 31 026 kPa to 100 kPa causing the cells to burst open, releasing the protein content.

As the subsequent immunoblotting techniques is best suitable for FLAG™-tag species of *plNC8c*[7], only *L. sakei* transformed with FLAG™-*plNC8c* and *L. plantarum* 2-1 transformed with FLAG™ plNC8c were cultured. The respective strains cloned with the empty plasmid pMG36e was used as negative controls, and the *Lactococcus lactis* IL1403 strain containing a

[6] The DNA sequencer was operated by lab-technician Zhian Salehian.
[7] No strains sensitive to J51 where able to be transformed with constructs containing FLAG™J51-*Orf5*

FLAG™-tagged *flciA* gene was used as a positive control (Diep et al. 2007). The *flciA* gene is roughly the same size as the FLAG™-*plNC8c* gene when expressed (12.3kD and 11.5kD respectively).

3.11.1 Fast-prep

The Fast-prep protocol for protein extraction is very similar to the fast-prep protocol for DNA extraction (as explained in section 3.5.1) although some modifications were performed, as the E.Z.N.A. plasmid minikit I protocol was not followed.

1. 40ml cultures were harvested by centrifugation at 7 200 × g for 3 min at 4°C.
2. The supernatant was then discarded and the pellet was dissolved in 1ml ice-cold TBS buffer and centrifuged at 16 000 × g for 1 min at 4°C.
3. The supernatant was then discarded and the pellet was resuspended in 1ml of ice-cold TBS buffer and transferred to a FastPrep tube.
4. 0.8g of glass beads (≤106µm) were added to the tubes.
5. The tubes were shaken twice at 6m/s for 30 sec at 4°C.
6. The tubes where then centrifuged at 16 000 × g for 2 min at 4°C.
7. The supernatant was transferred to a separate tube and 350µl of ice-cold TBS buffer was added to the FastPrep tubes.
8. The FastPrep tubes were shaken and centrifuged again as in steps 5 and 6.
9. The supernatant was transferred to their respective tubes as in step 7 and the protein concentration was measured on a Nandrop spectrophotometer.

3.11.2 French-press

French press lysis was carried out according to Nissen-Meyer et al. (1993).

1. A 2L culture of each strain was harvested by centrifugation at 10 600 × g for 10 min at 4°C when OD_{600} was between 0.4 and 0.9.
2. The supernatant was discarded and the pellet was resuspended in 80ml ice-cold TBS buffer and centrifuged at 6000 × for 10 min at 4°C.
3. The pellet was washed again as described in step 2.
4. Each pellet was resuspended in 40ml ice-cold TBS buffer and passed through a French Pressure cell at 31 026 kPa.
5. 40µl of DNAse I (10mg/ml) was added to the suspension, and the suspension was

passed through the French pressure cell again at 31 026 kPa.[8]

6. The suspensions where centrifuged at 6000 × g for 20 min at 4°C.
7. The supernatant was then split into 8ml aliquots and the concentration was measured on a Nanodrop spectrophotometer.
8. The aliquots where stored at -80°C

3.11.3 Protein purification by resin binding

The FLAG™ proteins where purified using the ANTI-FLAG™ M2 Affinity gel which contains ANTI-FLAG™ M2 antibodies covalently attached to agarose, and eluted using a competitive binding strategy by the use of 3X FLAG™ peptides (Einhauer & Jungbauer 2001).

As the ANTI-FLAG™ M2 resin is stored in a glycerol solution, the glycerol needs to be removed before use.

1. 40µl of resin solution was used for each sample.
2. 1ml of ice-cold TBS was added to the resin solution and mixed carefully by invertion. The tube was centrifuged at 11 000 × g for 5 sec and incubated on ice for 2 min.
3. The supernatant (containing glycerol) was then removed.
4. The washing steps (step 2-3) where then repeated 3 times.
5. The volume was then adjusted to 100µl per sample by addition of ice-cold TBS buffer
6. 24mg of protein sample (dissolved in ice-cold TBS buffer to a total of 900µl)and 100µl of the resin solution from the previous step was added to each sample along with 100µl of 10% Triton X-100 (to avoid unspecific binding to the resin).[9]
7. The resin containing samples were then mixed on a rotor at 8 rpm over night at 4°C.
8. The next day the resin samples were washed by centrifugation at 11 000 × g for 5 sec and incubated on ice for 20 minutes.
9. The supernatant was then removed and 1ml of ice-cold TBS was added.
10. The washing steps (step 8-9) were then repeated 4 times.
11. 3µl of 3x FLAG™-tag stock (5mg/ml) was mixed with 100µl of ice-cold TBS buffer and added to each resin-sample solution from the previous step. The samples were mixed

[8] The French pressure cell was washed with water, dH_2O and ice-cold TBS buffer between each different sample
[9] Different detergents at different concentrations were also tested without yielding any better results (data not shown)

on a rotor at 8 rpm for 30 min at 4°C. The tubes were flicked every 10 min.

12. The samples were then centrifuged at 11 000 × g for 10 sec and incubated on ice for 2 min.
13. 100µl of the supernatant, containing the eluted proteins, was then transferred to a new tube and stored at -20°C.

3.12 SDS-PAGE, staining and Western blotting

SDS-PAGE was performed to analyze the purified protein fractions from section 3.11.3. The proteins were visualized using unspecific staining (Coomassie and Silver staining) or blotted onto a membrane for immunodetection.

3.12.1 SDS-PAGE

SDS-PAGE was used to separate proteins according to size. This technique is somewhat similar to the agarose gel electrophoresis used to separate DNA (explained in section 3.7). Proteins are denatured by heating prior to electrophoresis. The proteins are subjected to an electrical field in a gel-based mesh network containing negatively charged SDS molecules, which coats the protein fragments giving them an equal mass to charge ratio upon which proteins are separated.

1. Samples were mixed with an equal volume of 2x SDS-Sample buffer (both with and without 2-merkaptoethanol where appropriate) and boiled for 3-5 min at 100°C before being applied to the polyacrylamide gel.
2. SDS-PAGE was performed according to (Laemmli 1970) with a 4% stacking gel[10] and a 12% or 15% separation gel[11]. Prestained Protein Markers were used to determine relative sizes of the observable bands along with protein samples from *Lactococcus lactis* IL1403 transformed with *flciA* which was used as a positive control.
3. Electrophoresis was performed at 100V in a 1x running buffer until the sample dye had exited the gel.

[10] Stacking gel contains: 4% acrylamid/bis-acrylamid (40/0.8), 0.125M Tris-HCl (pH 6.8), 0.1% (w/v) SDS, 0.05% (w/v) APS, 0.1% (v/v) TEMED and 0.1% (w/v) bromophenol blue

[11] Separation gel contains: 15% or 12% acrylamid/bis-acrylamid (40/0.8), 0.375M Tris-HCl (pH 8.8), 0.1% (w/v) SDS, 0.05% (w/v) APS and 0.05% (v/v) TEMED.

3.12.2 Coomassie staining

Coomassie staining was performed to visualize proteins separated by SDS-PAGE. This dye binds proteins unspecifically, however the mechanisms behind the dye-protein binding is not well understood.

1. The gel was submerged in a staining solution[12] containing the Commassie Brilliant Blue G-250 dye and heated to boiling point in a microwave oven to increase the staining rate. The gel-solution was incubated for 30 min with moderate shaking.
2. The gel was washed once with dH_2O to remove unbound dye, and then submerged in a destaining solution[13] and heated to boiling point in a microwave oven (to increase the destaining rate). The gel-solution was then incubated for 2h with moderate shaking with a small piece of paper tissue in the solution, to further increase the destaining rate.

3.12.3 Silver Staining

Silver staining is another method to visualize proteins in a polyacrylamide gel. The method is based on the binding of Ag^{2+}-ions to amino acid side chains (Merril et al. 1981). These ions are then reduced, producing visible silver stained bands. The silver staining method is 10-100 fold more sensitive than staining with coomassie brilliant blue (depending on which protocol is used).

1. The proteins in the gel were fixed by submerging the gel in a 100ml solution containing 50% (v/v) methanol and 10% (v/v) acetic acid, which was then heated in a microwave oven at 800W for 1 min, before shaking at room temperature for 15 min.
2. The solution was then exchanged with 100ml dH_2O for washing. The gel-water solution was heated in a microwave oven at 800W for 1 min, and then shaken for 15 in room temperature.
3. The water was then poured off and a 100ml dH_2O solution containing 5µg/ml DTT, to prevent reformation of disulfide bonds, was added to the gel. The gel-DTT solution was then heated in a microwave oven at 800W for 1 min, and then shaken at room temperature for 15 min.

[12] Staining solution contains: 0.2% (w/v) Coomassie Brilliant Blue R-250, 50% (v/v) methanol and 7.5% (v/v) acetic acid.
[13] Destaining solution contains: 5% (v/v) methanol and 7% (v/v) acetic acid.

4. The solution was then removed and a dH_2O solution containing 0.2% (w/v) silver nitrate was added, and then shaken at room temperature for 15 min.
5. The silver nitrate solution was then removed and the gel was washed twice with 100ml of dH_2O and then twice with 50ml developer solution, which contains 0.3% (w/v) disodium carbonate.
6. For development 0.037% (v/v) formaldehyde was added to 100ml developer solution, poured on gel and shaken until bands were clearly visible.
7. To stop the developing reaction, 5ml of 2.3M citric acid (to a final concentration of 110mM) was added and then shaken for an additional 10 min.
8. The solution was then removed, and the gel was washed several times with dH_2O (to minimize yellow background). When necessary, 100ml of 5% (v/v) glycerol was added for storage.

3.12.4 Western blotting

Western blotting was performed to specifically detect the FLAG™-tagged PLNC8c immunity proteins. The proteins separated by SDS-PAGE were transferred to polyvinylidene membrane by electro-blotting. The proteins were visualized by a series of continuous steps starting with the addition of ANTI-FLAG™ M2 antibodies which binds specifically to the FLAG™-tag containing proteins. Then a secondary antibody (which binds specifically to the first antibody) with the conjugated enzyme horseradish peroxidase (HRP) was added. This enzyme catalyzes a reaction with the ECL detection reagents resulting in chemiluminescence that can be visualized by exposure to a film.

Western blotting was performed as described by Straume et al. (2006).

1. SDS-PAGE separated proteins were electroblotted onto a polyvinylidene membrane using the Mini Trans-Blot System for 1h at 100V in cold transferbuffer.
2. The membrane was then incubated in 5% (w/v) skimmed milk powder dissolved in PBS-T for 1h with gentle shaking to prevent unspecific binding of antibodies to the membrane.
3. The membrane was washed for 10 min with 30ml PBS-T with gentle shaking before incubating the membrane with the primary antibody ANTI-FLAG™ M2 in PBS-T (1:4000) for 1h with gentle shaking.

4. The membrane was again washed 3 × 10 min with 30ml PBS-T with gentle shaking before incubating the membrane with the secondary HRP-linked antibody Anti-Mouse Ig in PBS-T (1:4000) for 1h with gentle shaking.
5. The membrane was washed again 3 × 10 min with 30ml PBS-T with gentle shaking to remove any unbound antibodies and the PBS-T solution was removed.
6. 1ml of ECL Plus Solution A and 25µl of ECL Plus Solution B was mixed, brought to room temperature and applied on the membrane. A film was exposed to the membrane for 15-20 min and developed for 10 sec in a development solution and fixed for 10 sec in a fixation solution. The film was washed in water before and after the fixation and finally air-dried.

4 Results

4.1 Bacteriocin sensitivity assays

Assays were performed on a wide variety of indicator strains in order to investigate the spectrum of inhibition by plantaricin NC8 and the putative plantaricin J51. Having several strains that are sensitive for each of the bacteriocins is also preferable when the putative immunity proteins are to be expressed heterologuously, as not all strains are easily transformable. Soft agar assays were performed first in order to quickly screen many strains for sensitivity. The sensitivity of any sensitive strains was then further quantified using a minimum inhibitory concentration assay.

4.1.1 Soft agar assays of wild type strains

Soft agar assays were performed using separate peptides, and then mixed in order to investigate if the individual peptides showed any anti-microbial effect on their own. This was also done to investigate if the two complementary peptides showed synergistic activity when combined, which is typical for two-peptide bacteriocins.

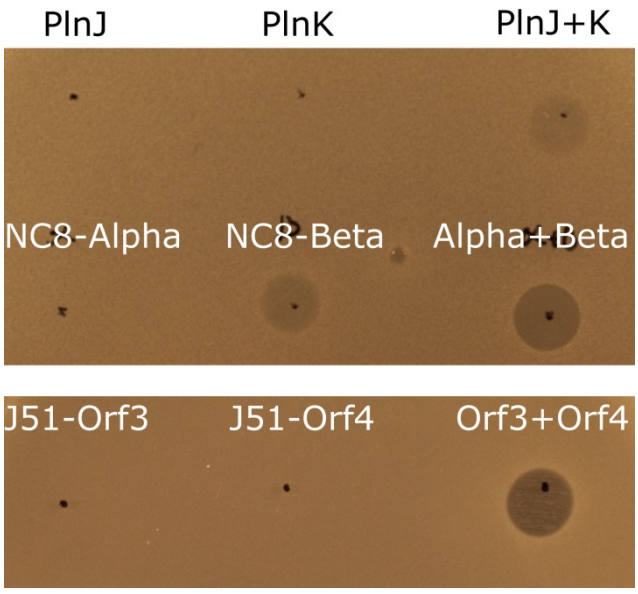

Figure 4.1 Soft agar assay of plantaricin JK (top row, 50ng of each single peptide) and plantaricin NC8 (middle row, 50ng of each single peptide) using *Lactobacillus sakei* NCDO 2714 as indicator. Bottom row is putative plantaricin J51 (200ng of each single peptide) using *L. plantarum* DSM 20174 as indicator.

As the soft agar assays indicated that the peptides needed the complementary peptides for optimal anti-microbial effect, the rest of the screening was performed using both complementary peptides (in a 1:1 ratio).

Table 4.1 Overview of soft agar assay of indicator strains to different bacteriocins.[1]

Indicator strains	LMGT number	PlnEF	PlnJK	PLNC8	Putative PlnJ51
Lactobacillus acidophilus					
▪ NCDO 1748	2303	-	-	-	-
▪ ATCC 3456	2712	-	-	-	-
L. amylophilus NCDO 2503	2300	-	-	-	-
L. amylovorus NCDO 2657	2301	-	-	-	-
L. casei NCDO 2743	2324	-	-	++	-
L. cellobiosus NCDO 927	2306	-	-	-	-
L. coryneformis NCDO 2741	2309	-	++	-	-
L. curvatus					
▪ NCDO 2739	2310	-	-	-	-
▪ CTC 435	2371	-	-	+	-
▪ 89	2355B	+	-	-	-
▪ BCS35	3291	-	-	-	-
L. delbrueckii NCDO 213	2331	-	-	-	-
L. helveticus ATCC 15009	2761	-	-	-	-
L. fermentum ATCC 9338 V	2716	-	-	++	+
L. plantarum					
▪ 965	2003	+	++	+	++
▪ R	2352	-	++	-	-
▪ DSM 20174	2378	-	++	-	+
▪ AA23	2389	-	++	-	-
▪ ACA-DC 289	3160	-	+	-	-
▪ 2-1	3176	-	-	++	-
▪ 2-2	3177	-	-	-	-
▪ ACA-DC 3333	3215	-	+	-	-
▪ ACA-DC 3341	3216	-	-	-	-
▪ ACA-DC 3342	3217	-	-	-	-
L. rhamnosus GG	3087	-	-	-	-
L. salivarius 83	3494	-	-	-	-
L. sakei					
▪ 2F13	3147	-	-	-	-
▪ 23K	3051	-	-	-	-
▪ 64F	3052	-	-	-	-
▪ NCDO 2714	2313	+	+	++	+
▪ CTC A4	2373	-	-	-	-
▪ 460	2376	-	-	-	-
▪ DSM 20017	2377	-	-	-	-
▪ 706	2334	-	-	-	-
▪ 45	2340	-	-	-	-
▪ 77	2360	-	-	-	-
L. viridesence NCDO 1655	2314	-	++	-	-
Lactococcus lactis MG1363	2144	-	-	-	-
Leuconostoc mesenteroides Y105	3081	-	-	-	-
Pediococcus pentosaceus NCDO 990	2315	-	-	-	-

[1] "–" indicates no visible sensitivity. "+ " indicates slight inhibition with diffuse zones up to 5mm in diameter. "++" indicates inhibition with clear zones up to 6mm in diameter.

Only a few strains showed sensitivity to the bacteriocins plantaricin NC8 and the putative plantaricin J51. The sensitivity of these strains was then further characterized using a mictrotiter plate assay to determine minimum inhibitory concentration (MIC).

4.1.2 Microtiter plate assays

Microtiter plate assays were performed to investigate the potency of plantaricin NC8 and the putative plantaricin J51, and if there were any differences between them. By setting up MIC assays of each strain, one could also calculate the minimum concentration of each bacteriocin (peptides mixed in a 1:1 ratio) necessary to inhibit growth by at least 50% (MIC_{50}) in ng/µl. This assay is more informative than the qualitative method of soft agar assay. Each experiment was performed at least twice, but the results were similar, so only values from one of the experiments are shown here.

Table 4.2 MIC_{50} values of the bacteriocin toward selected strains.

Indicator strain	**MIC_{50} in ng/µl[2]**		
	PlnJK	**PLNC8**	**Putative plantaricin J51**
Lactobacillus casei NCDO 2743	0.5000	0.0078	1
L. fermentum ATCC 9338 V	-	0.0156	0.1250
L. plantarum 2-1	-	0.0039	-
L. plantarum 965	0.0039	-	0.0078
L. plantarum AA23	0.0625	1	0.2500
L. plantarum DSM 20174	0.0156	-	0.1250
L. sakei NCDO 2714	-	0.0039	-

[2] "-" indicates no detectable inhibition

The microtiter plate assays performed on sensitive wild type strains clearly indicate an anti-microbial effect of the putative plantaricin J51, although not as potent as the plantaricins NC8 and JK. The plantaricins NC8 and JK are active at nano to picomolar range, while the putative plantaricin J51 seems to be 10-100 times less potent. The potency of each bacteriocin also varies between the strains, with *L. plantarum* 965 being at least 100 times more sensitive for plantaricin JK and the putative plantaricin J51 than rest of the strains.

4.2 Function of putative bacteriocin immunity proteins

In order to study the function of the putative immunity proteins for the bacteriocins plantaricin NC8 and the putative plantaricin J51, their cognate immunity genes were cloned and heterologously expressed in some of the bacteriocin sensitive strains. In total three strains were transformed. *L. sakei* NCDO 2714 and *L. plantarum* 2-1 was transformed with *plNC8c* and FLAG™-*plNC8c*. *L. plantarum* DSM 20174 was transformed with J51-*Orf5*.[14]

The same assays as performed in section 4.1 was performed again in order to investigate if sensitivity to any of the bacteriocins had been changed, and if the previously sensitive strains would become immune by the expression of one of the putative immunity genes. Soft agar assays were performed first to get an initial picture of any change in sensitivity. Microtiter plate assays were performed afterwards to quantify the changed sensitivity levels, and the values calculated were used to measure the increase in resistance compared to controls.

As some of the strains contained immunity proteins with FLAG™-sequences (used for later detection and purification of the putative immunity proteins, see section 4.3); assays were also performed on strains harboring the FLAG™-sequence and strains without the FLAG™ sequence. This was done to investigate if the FLAG™ - sequence would interfere with the function of the putative immunity gene, leading to differences in resistance between genes with an upstream FLAG™-sequence and genes without the FLAG™-sequence.

4.2.1 Soft agar assays of transformed strains

Soft agar assays were first performed to get an overview in change of sensitivity. Both strains with and without a FLAG™-sequence were used, and wild type strains without the cloned genes were used as controls. Since the used strains are also sensitive to plantarcin JK, it was included as well to investigate if the putative immunity genes could confer cross-immunity to plantaricin JK as well.

[14] A transformed strain with FLAG™-J51-*Orf5* was not achieved.

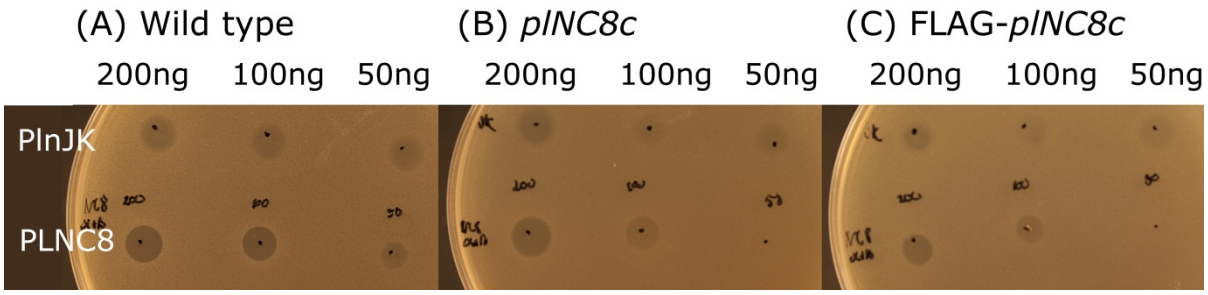

Figure 4.2 Soft agar assay of *L. sakei* NCDO 2714. (A) Wild type strain. (B) Strain transformed with *plNC8c*. (C) Strain transformed with FLAG™-*plNC8c*. Top row of spots is plantaricin JK, bottom row is plantaricin NC8. Concentrations are from left to right: 200ng, 100ng and 50ng in all rows of their respective bacteriocins.

The soft agar assay of the transformed *L. sakei* NCDO 2714 strains did indicate an increased immunity for plantaricin NC8. Any differences in sensitivity between FLAG™ and non-FLAG™ constructs were not observed. Cross-immunity was also not observed for plantaricin JK.

A soft agar assay comparing wild type and transformed strains of *L. plantarum* 2-1 was also performed in order to investigate if the immunity showed any difference between strains.

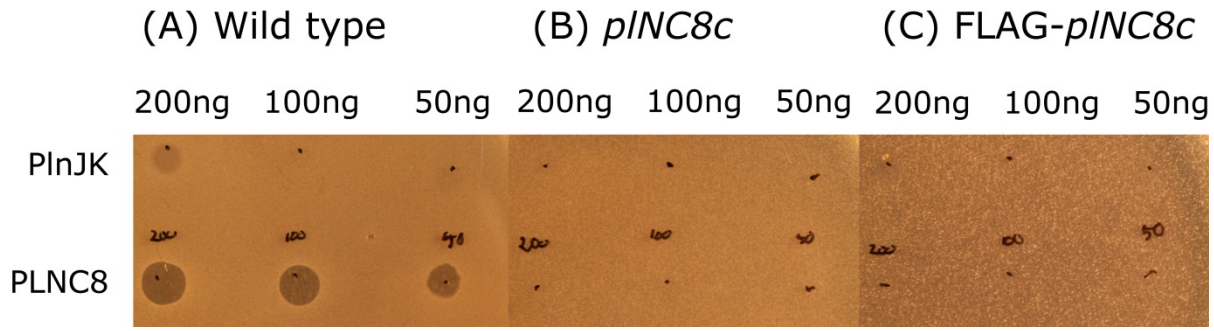

Figure 4.3 Soft agar assay of *L. plantarum* 2-1. (A) Wild type strain (B) Strain transformed with *plNC8c*. (C) Strain transformed with FLAG™ *plNC8c*. Top row of spots is plantaricin JK, bottom row is plantaricin NC8. Concentrations are from left to right: 200ng, 100ng and 50ng in all rows of their respective bacteriocins.

The soft agar assay of the transformed *L. plantarum* 2-1 strains indicates that the immunity function of the *plNC8c* gene is not strain specific. No variance between FLAG™ and non-FLAG™ constructs were detected. Although it might seem that there are no zones of inhibition in the transformed strain when subjected to plantaricin JK, this is not true. Zones were observed, but these were hard to capture on camera, partly due to the poor and "pelagic" growth of the transformed strains. This indicates that *plNC8c* does not confer cross-immunity to plantaricin JK in this strain as well.

4.2.2 Microtiter plate assays of transformed strains

Microtiter plate assays of transformed strains were performed in order to quantify difference in sensitivity between strains harboring the putative immunity genes and those that do not. Strains transformed with the empty plasmid pMG36e were used as controls.

L. sakei NCDO 2714 expressing *plNC8c* had an eight fold increase in MIC_{50} compared to the empty pMG36e control. *L. plantarum* 2-1 expressing *plNC8c* had over a 192 fold increase in MIC_{50}, as sensitivity was only detected in the empty pMG36e controls.

4.3 Protein expression studies

In order to study the expression of the putative immunity gene *plNC8c*, protein purification combined with SDS-PAGE and different subsequent visualization techniques were employed. This was done to investigate if the putative immunity gene is expressed, and also obtain information on possible receptors which may co-purify with the immunity protein. A previous study that has utilized such an approach has proven successful in revealing new information about bacteriocin/immunity receptors, binding and mode of action (Diep et al. 2007). As the bacteriocin receptor is not known for class IIb bacteriocins, its investigation seems warranted. Since the FLAG™-epitope did not interfere with the immunity function of the putative immunity gene, it can be used to detect the presence of the putative immunity protein (e.g. via binding of an antibody with some sort of reporter), or to directly purify the putative immunity protein (e.g. by immunoprecipitation via coupling of the antibodies to a solid substrate).

A separate culture of *L. sakei* NCDO 2714 transformed with FLAG™- *plNC8c* with the addition of 62,5ng/ml of plantaricin NC8 was also set up. This was done to hopefully increase the expression of the putative immunity gene. *L. sakei* NCDO 2714 transformed with the empty control plasmid pMG36e was also used along with *Lactococcus lactis* IL1403 harboring the gene *flciA* which was used as a positive control (Diep et al. 2007). The putative immunity protein J51-*Orf5* was not studied during this part of the study, as FLAG™-J51-*Orf5* transformants were not achieved. For an overview of the abbreviated names used in each gel-figure see Table 4.3.

Table 4.3 Overview of abbreviated names used in describing samples in upcoming SDS-PAGE analyses.

Strain	Construct	Presence of bacteriocin	Shortened name
L. sakei NCDO 2714	Empty pMG36e plasmid	No	e
	pMG36e with FLAG™-*plNC8c*	No	-B
	pMG36e with FLAG™-*plNC8c*	Yes	+B
Lactococcus lactis IL 1403	pMG36e with *flciA*	No	B244

4.3.1 Visualization of non-purified versus purified protein extracts

First off, unpurified protein extracts from transformed *L. sakei* NCDO2714 strains were run on 15% acrylamid/bis-acrylamid (40/0.8) separating polyacrylamide gels. These gels were visualized using both Coomassie blue staining and immunoblotted using the Western blot technique. The reducing chemical 2-mercaptoethanol was not added to the sample buffers used, in order to preserve any disulfide bridges present in the samples. The western blots revealed very little specific protein present, while the coomassie stained gels indicated huge amounts of background. Therefore a purification procedure was employed to hopefully obtain more specific protein products, while minimizing background proteins. Purified and non-purified extracts were run again on the same gels and Western blots and the results can be seen in Figure 4.4 and Figure 4.5 below.

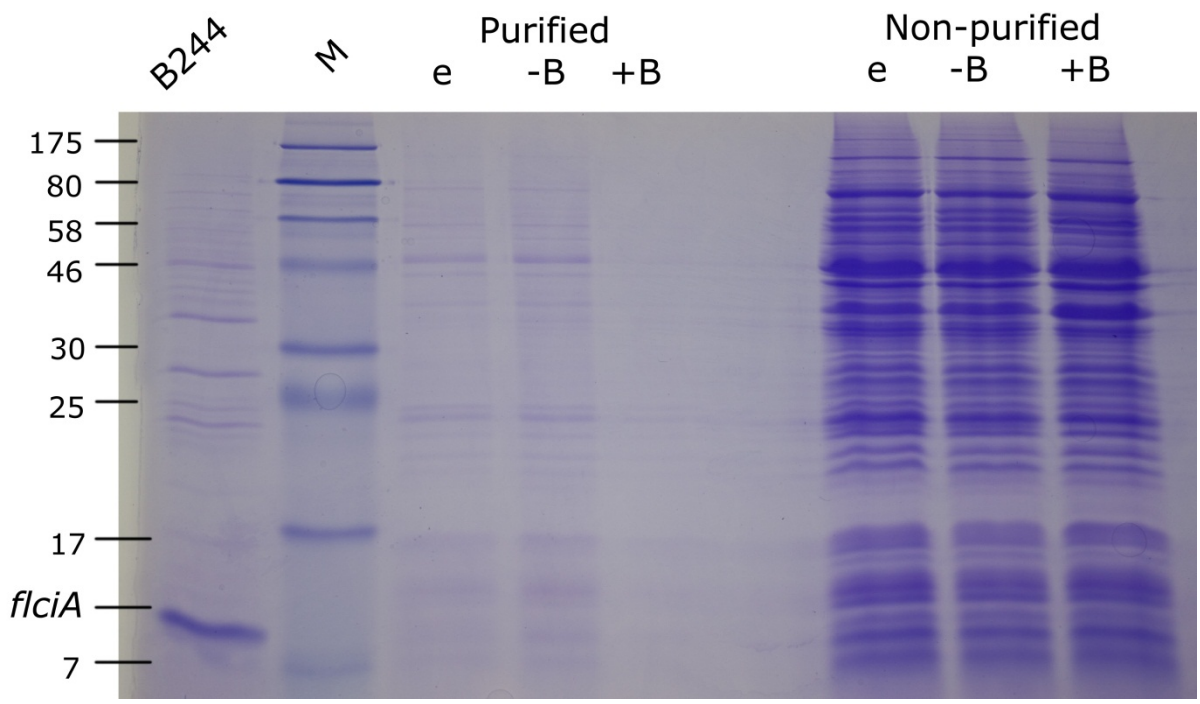

Figure 4.4 Coomassie staining of *L. sakei* NCDO 2714 strains. (Lane 1) Purified B244. (L2) Size Marker. (L3) Purified e. (L4) Purified -B. (L5) Purified +B. (L6) Gap. (L7) Non-purified e. (L8) Non-purified -B. (L9) Non-purified +B. Sizes displayed on the left are in kDa. Approximately 50µg of non-purified protein fractions were applied on gel.

Lactococcus lactis B244 expresses the gene *flciA* (which contains a FLAG™-tag) and is approximately the same size as the FLAG™-*plNC8* when expressed (12.3kD and 11.5kD respectively). The B244 strain was therefore included as a control.

The coomassie stained gel revealed a very low grade of purification. A purified amount of *plNC8c* as large as the purified amount of *flciA* was desired. This was not the case, so a Western blot of the same duplicate gel was also performed, in order to hopefully visualize the expressed *plNC8c* gene.

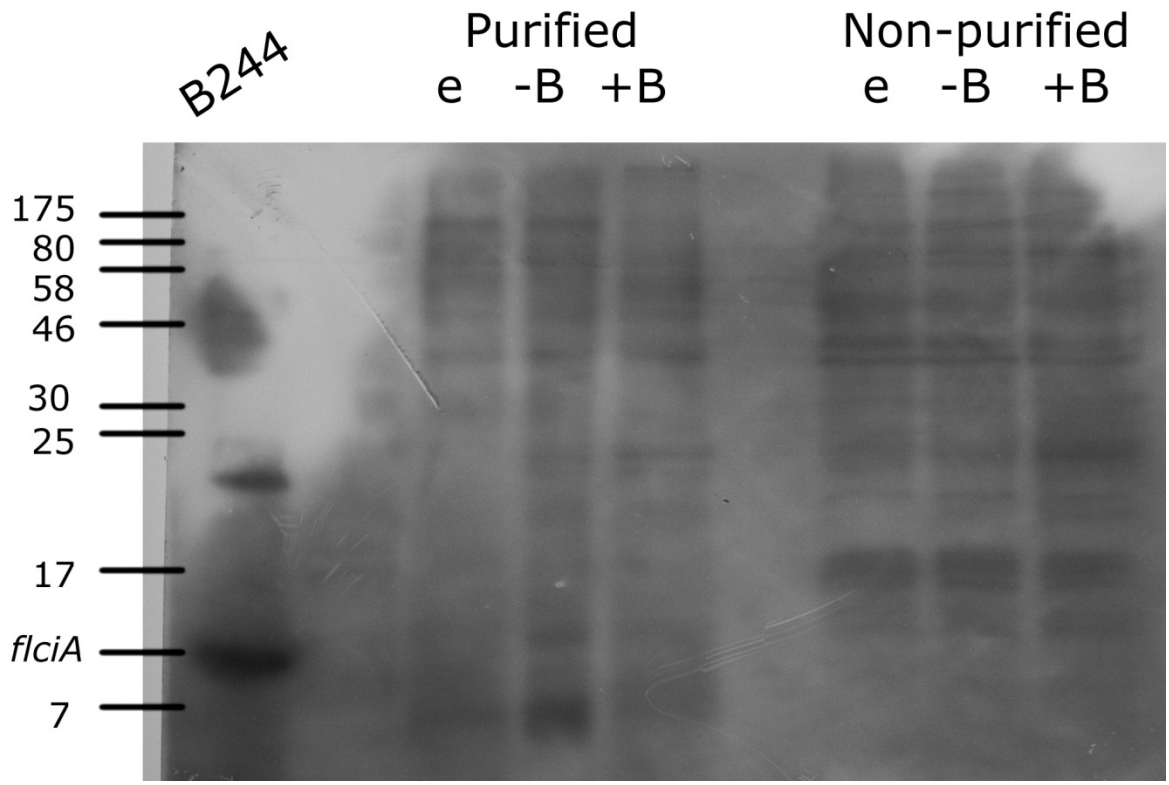

Figure 4.5 Western blot of *L. sakei* NCDO 2714 strains. (Lane 1) Purified B244. (L2) Size Marker, not visible. (L3) Purified e. (L4) Purified -B. (L5) Purified +B. (L6) Gap. (L7) Non-purified e. (L8) Non-purified -B. (L9) Non-purified +B. Sizes shown on the left are in kDa estimated from original membrane. Approximately 100µg of non-purified protein fractions were applied on gel.

A lot of bands were present in Figure 4.4 and Figure 4.5 in the non-purified fractions. The Western blot in Figure 4.5 also suggests that the expression of the putative immunity protein is very low, as no distinct bands were observed in the expected size range.

In a previous work by Diep et al. (2007), bacteriocin receptors were identified by co-purification with the bacteriocin immunity protein and the bacteriocin itself. Such receptors could in those studies be identified as additional protein bands when comparing the protein profiles of strains with and without the FLAG™-tagged immunity protein. In order to investigate if similar results could be obtained for plantaricin NC8, a silver staining procedure of purified fractions from *L. sakei* NCDO 2714 was performed.

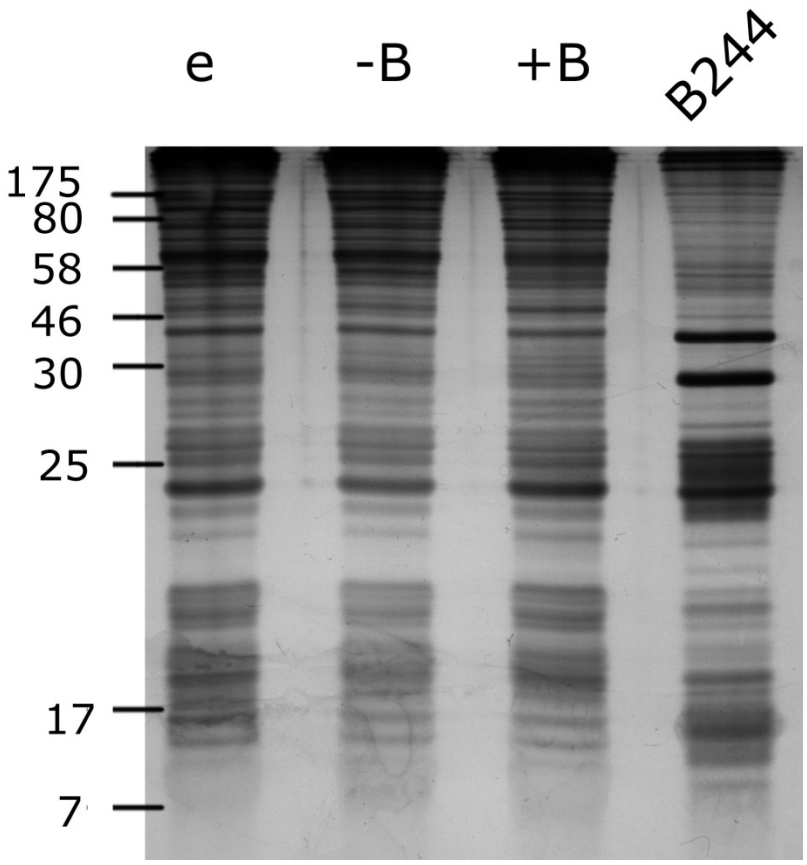

Figure 4.6 Silver staining of purified samples from *L. sakei* NCDO 2714 strains[15]. (Lane 1) e. (Lane 2) -B. (Lane 3) +B. (Lane 4) B244.

The samples showed very few differences between them and no receptor candidate band could be identified. Another SDS-PAGE was performed using a 12% concentration of acrylamid/bis-acrylamid (40/0.8). The decrease of acrylamid/bis-acrylamid was done to get a better separation of the large bands present in Figure 4.6. The samples were also prepared differently using the FastPrep method (see section 3.11.1).

[15] Resin purification, SDS-PAGE and silver staining were performed by Senior Engineer Linda Godager

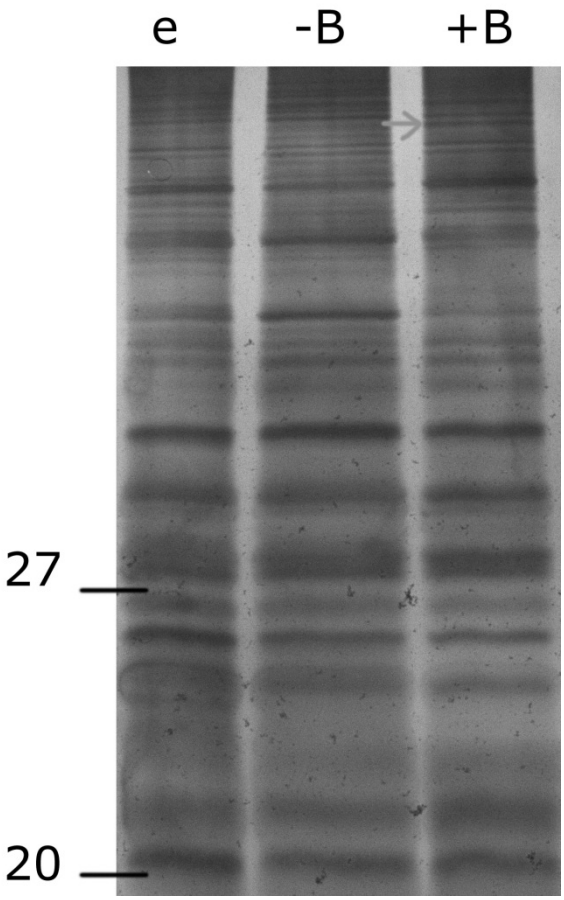

Figure 4.7 Silver stained gel of purified samples from *L. sakei* NCDO 2714 strains. (Lane 1) e. (Lane 2) –B. (Lane 3) +B. Sizes on the left are indicated in kDa. Only part of marker was present in gel after completion of staining. Arrow indicates band cut out for MALDI-TOF analysis.

The second silver stained gels yielded better separation of the high molecular weight protein bands, but still little new information. As the expressed FLAG™-*plNC8c* immunity gene is 11.5 kDa, it is not visible in this gel.

By a detailed analysis of the gel, an additional high molecular weight protein band was identified in the strain expressing the immunity protein with addition of bacteriocin (+B). In an attempt to identify this band the SDS-PAGE was performed again and stained according to Blum et al. (1987). The band indicated by a grey arrow in Figure 4.7 was excised from the gel and MALDI-TOF analysis was performed on the fragment according to Shevchenko et al. (1996)[16]. However, no results were obtained from the MALDI-TOF analysis as not enough proteins were present.

[16] Trypsination was performed by Senior Engineer Morten Skaugen

A soft agar assay with spots of resin-putative immunity protein complex was also performed, since previous studies by Diep et al. (2007) suggests that the bacteriocin can be co-purified along with the immunity protein. However, no anti-microbial activity was detected in this study.

4.3.2 Investigation of immunity protein complex formation

Suggesting that the FLAG™-*plNC8c* gene product may form a complex due to the presence of two cysteine residues present in the protein, Western blot analyses were performed with purified fractions both with and without 2-mercaptoethanol in the SDS-Sample buffer. 2-mercaptoethanol is known to reduce and thereby break disulfide bonds.

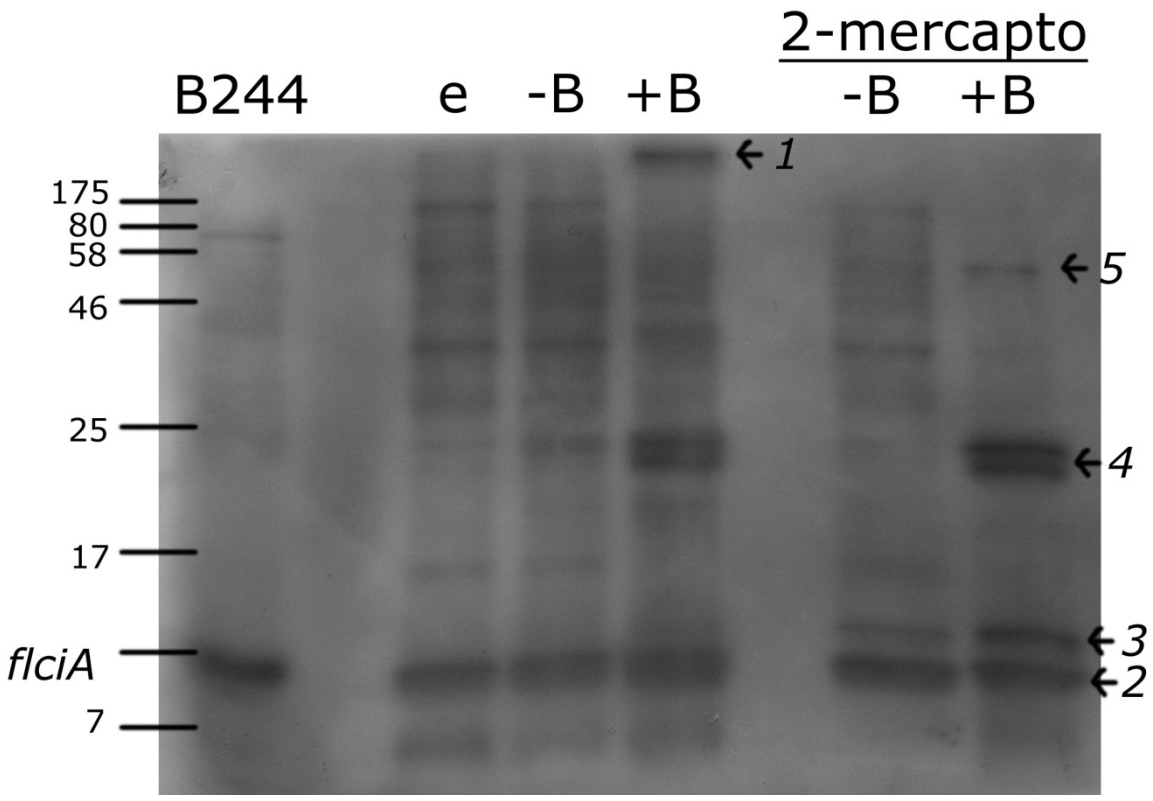

Figure 4.8 Western blot of *L. sakei* NCDO 2714 strains. Lanes 1-5 are samples without 2-mercaptoethanol. Lanes 7 and 8 are samples with 2-mercaptoethanol. (Lane 1) B244. (L2) Size marker, not visible. (L3) e. (L4) -B. (L5) +B. (L6) Gap (L7) -B. (L8) +B. (Arrow *1*) Possible complex formation. (Arrow *2*) Unknown, discussed in section 5.4. (Arrow *3*) Possible PLN8C protein. (Arrow *4*) Receptor candidates. (Arrow *5*) Unknown, discussed in section 5.4.

Figure 4.8 clearly demonstrates a change in band composition between samples with added 2-mercaptoethanol and without 2-mercaptoethanol. The experiment was performed twice and similar results were found. The results from one of the experiments are shown in Figure 4.8 . Sample B+ without added 2-mercatprothanol shows a high molecular weight band (Arrow *1*), that is not present in the sample with added 2-mercaptoethanol (2-mercapto +B). A strong band present in all samples (also in the empty plasmid strain) matching the size of *flciA* is visible at arrow *2* (discussed in section 5.4). Therefore the bands shown by arrow *3* are more likely to be the immunity protein. Possible receptor-immunity candidates are shown by arrow *4*.

As the Western blot from Figure 4.8 suggested that the putative immunity protein forms a complex (arrow *1* in sample +B with and without 2-mercaptoethanol), a soft agar of *L. sakei* NCDO 2714 transformed with FLAG™-*plNC8c* as indicator in the presence of DTT was also set up. DTT is a reducing agent which breaks any disulfide bonds. This was done to investigate if the complex was necessary for the immunity function, as the immunity protein contains two cysteine residues (see Figure 5.2). Pediocin PA-1 was used as control as its function is dependent on the formation of a disulfide bridge.

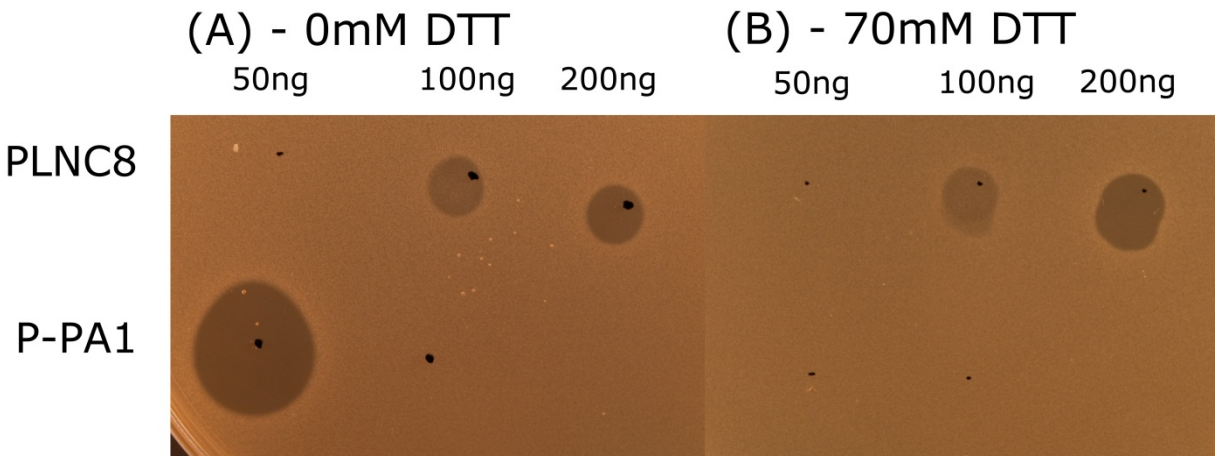

Figure 4.9 Soft agar assay of *L. sakei* NCDO 1714 transformed with FLAG™-*plNC8c*. (A) Without any DTT in Soft agar. (B) With 70mM DTT in soft agar. Top row of spots is plantaricin NC8. From left to right: 50ng, 100ng and 200ng. Bottom row lefthand corner is Pediocin PA-1 2µl of 50x concentrate. Bottom right spot is control spot with dH_2O.

The soft agar assay indicates that the complex formation might not be necessary for the immunity function of *plNC8c*. Pediocin PA-1 does not exert any anti-microbial activity in the presence of DTT, indicating that the cysteine residues have been reduced, and no disulfide bridges are present. The sensitivity for plantaricin NC8 is not increased, which indicates that a disulfide bridge is not necessary to maintain immunity against plantaricin NC8. If complex formation and disulfide bridge establishment is critical for maintaining immunity, larger zones of inhibition would be expected in samples with added DTT (Figure 4.9 (B)).

5 Discussion

5.1 Peptide ratio

Before discussing the main results, the decision to use a 1:1 ratio of each of the bacteriocin peptides needs to be discussed.

Maldonado et al. (2003) suggest that a ratio of 1:16 of NC8α- versus NC8β-peptide is the most optimal ratio for plantaricin NC8. These findings are highly debatable as a 1:16 ratio seems implausible from a genetical point of view, as both peptides are likely to be translated from a single polycistronic mRNA strand. Only one indicator strain was used in that study, which cannot rule out strain specific optimal ratios. Unfortunately that indicator strain (*Lactobacillus plantarum* 128/2) is not available in our collection. This could be the reason why Maldonado et al. (2003) did not emphasize the strong skewness in the optimal ratio of complementary peptides in their article.

Another comparable finding is the characterization of Lactococcin G. This two-peptide bacteriocin was first suggested to have an optimal molar ratio of approximately 1:8 of LcnG-α versus LcnG-β (Nissen-Meyer et al. 1992). However, further studies indicated that a 1:1 molar ratio was more probable, based on the fact that both peptides are transcribed from the same promoter (Moll et al. 1996). The 1:1 peptide ratio of the peptides comprising Lactiococcin G seems to be used in recent studies (Oppegard 2010).

There have also been a lot of studies on the plantaricins EF and JK. The initial studies suggested a 1:1 ratio of each of the complementary peptides as the optimal ratio for anti-microbial effect (Anderssen et al. 1998). Follow up studies also strengthened that notion (Hauge et al. 1999). This 1:1 ratio is also suggested as the optimal ratio for other class IIb bacteriocins like Salivaricin P and Lactococcin Q (O'Shea et al. 2010; Zendo et al. 2006).

There is one striking difference between previously mentioned studies of molar ratios between class IIb bacteriocins and this study. In this study synthetic bacteriocins are used almost exclusively. Although the manufacturer states that the delivered peptides are purified to over 90%, the grade of purity has been shown to vary considerably from statements for other peptides used in other studies in the lab (personal communication).

Even if the purified single peptide is 90% pure, there is still a chance that the some parts of the remaining 10% could be contaminated by the complementary peptide, as the sequences from most two-peptide plantaricins are not totally dissimilar (Diep et al. 2009). However this type of contamination would be easy to detect as the single peptide solution would probably display anti-microbial effects without the addition of the complementary peptide. To investigate the grade of purity among synthetic peptides a MALDI-TOF analysis could be employed. This analysis could reveal the grade of purity and could maybe determine species of any present contaminants.

Construction of so called isobolograms to determine optimal molar ratio of anti-microbial activity for plantaricin NC8 and the putative plantaricin J51 were also attempted (Berenbaum 1981). This was done by using soft agar assays to get initial indication of ratios, before setting up a microtiter plate assay using serial dilutions of each peptide in separate axes. But replicating the results proved difficult, so these results were omitted from this study. A 1:1 molar ratio of the peptides was therefore used throughout this study.

5.2 Bacteriocin sensitivity assays

5.2.1 Soft agar assays of wild type strains

In Figure 4.1 the synergistically anti-microbial effect of plantaricin JK is clearly demonstrated. As single peptides, none of the spots seem to indicate any inhibition. But when they are combined, a zone of inhibition is seen around the spot, although somewhat faint. The same is true for plantaricin NC8, but here an even clearer zone of inhibition is observed around the spot containing the two complementary peptides. However a distinct zone is also apparent in the spot containing only the β-peptide. This could stem from a few reasons. The β-peptide could display anti-microbial activity on its own, which is somewhat coherent to the findings of Maldonado et al (2003). It seems unlikely that the β-peptide is contaminated by the complementary α-peptide (or a similar peptide), as the single peptides constituting plantaricin JK and the putative plantaricin J51 does not seem to exert any anti-microbial effect on their own.

Another interesting finding from Figure 4.1 is the zones of inhibition detected when the indicator is exposed to both peptides making up the putative plantaricin J51 (J51-*Orf3* and

J51-*Orf4*). This is the first time the antimicrobial activity of plantaricin J51 has been demonstrated. No inhibition is detected among the single peptides, indicating that both peptides are necessary to exert anti-microbial activity. This strongly implies that the putative plantaricin J51 has an anti-microbial effect which is very reminiscent of a typical two-peptide bacteriocin. This, together with previously determined genetical information deducted by Navarro et al. (2008), suggest that the function of these two genes has now been uncovered, and their status as putative genes should be removed. A renaming of the two ORFs should also be carried out. Some fall pits do exist in assigning a function to these two putative genes prematurely; these include impure peptides and other causes which have been discussed above (see section 5.1).

Looking over to Table 4.1, the narrow spectrum of anti-microbial activity typical for class IIb bacteriosins is apparent. Plantaricin JK which is known to be produced in several *L. plantarum* strains, seems to inhibit growth mostly in closely related strains and species (Diep et al. 2009). The rest of the bacteriocins (plantaricins EF, NC8 and the putative plantaricin J51) seem to have a much narrower field of inhibition.

5.2.2 Microtiter plate assays of wild type strains

The microtiter plate assays from Table 4.2 seems to follow the same trend as the soft agar assays in Table 4.1 (and discussed in section 5.2.1). Plantaricin NC8 seems to be very potent, with MIC_{50} values in the nano- to picomolar range (except for *L. plantarum* AA-23). The plantaricin J51 does not seem to be that potent, with MIC_{50} values usually in the micro- to nanomolar range (with the exception of *L. casei* NCDO 2743). This could be due to different purities in the synthetic peptide solutions (discussed in section 5.1) or simply that plantaricin J51 is just not as potent as the plantaricins JK and NC8.

The MIC_{50} values for plantaricins JK and J51 with *L. casei* NCDO 2743 as an indicator might seem extreme. But the MIC assays of this strain showed a steady inhibition of growth with increasing concentrations of bacteriocin.

The method of quantitating the MIC_{50} values using a microtiter plate setup is not perfectly accurate. Although reproducibility is fairly good, it can sometimes deviate from previous results. Also, only whole wells were used when calculating MIC_{50} values (e.g. 2 was used

instead of 2.5). This is why some of the bacteriocins have a MIC_{50} value identical to other bacteriocins right down to the smallest decimal.

This is one of the first large scale investigation of sensitivity to plantaricin NC8 (and plantaricin J51) conducted to this date, as far as the author knows. This will certainly aid in the understanding of the limits of application in which these bacteriocins can be used, and it could help to detect a possible receptor for each of the bacteriocins.

5.3 Function of putative bacteriocin immunity proteins

5.3.1 Soft agar assays of transformed strains

In Figure 4.2 clear zones of inhibition are seen in the plate with the wild type strain (A), and the strain transformed with *plNC8c* (B) shows reduced sensitivity as expected. This is easiest to observe in the spot containing 50ng of plantaricin NC8, as this zone of inhibition completely disappears in the transformed strains. The strain containing a FLAG™ sequence upstream of the *plNC8c* gene (C), have almost the exact same zones of inhibition as the transformed strain without a FLAG™ sequence (B). This indicates that the FLAG™ sequence in the N-terminal part does not interfere with the immunity from the *plNC8c*. This plate assay also strongly implies that the putative immunity gene *plNC8c* is in fact an immunity gene. Together with the genetic information deducted by Maldonado et al. (2003) suggest that function of this putative gene is detected, and its status as a putative gene should be removed.

From Figure 4.2 zones of inhibition when subjected to plantaricin JK are detected among all the different plates with the indicator *L. sakei* NCDO 2714, suggesting that the immunity gene *plNC8c* does not confer cross-immunity to plantaricin JK. This indicates that *plNC8c* specifically confers immunity to plantaricin NC8, and such specificity is a common feature of bacteriocin immunity proteins (Cotter et al. 2005). The reason for this specificity is unknown, but it could indicate that the bacteriocins recognize different receptors.

The soft agar assay of the second strain transformed with *plNC8c* (*L. plantarum* 2-1), shown in Figure 4.3 seems to echo the results from *L. sakei* NCDO 2714 in Figure 4.2. This indicates that the function of the *plNC8c* gene is not strain specific. However, the transformed *L. plantarum* 2-1 strains ((B and (C) in Figure 4.3) seems to be more immune when exposed to

plantaricin NC8, compared to transformed strains of *L. sakei* NCDO 2714 ((B and (C) in Figure 4.2).This could be an indication that the expression level of the immunity protein could vary between the two strains, or the immunity-receptor interaction could be different between the two strains.

Although it seems that there are no zones of inhibition in L. plantarum 2-1 transformants ((B) and (C) in Figure 4.3) when exposed to plantaricin JK compared to the wild type strain, this is not true. There are zones of inhibition but this is not very clear from the photographs in Figure 4.3. This is also due to the "pelagic" growth sometimes observed in both liquid cultures and solid media of *L. plantarum* 2-1.

5.3.2 Microtiter plate assays of transformed strains

The microtiter plate assays results seem to follow the trend from the soft agar assays in Figure 4.2 and Figure 4.3. There is a huge difference in sensitivity between the two strains transformed with *plNC8c*. This could mean that another factor is also somehow involved in the immunity for plantaricin NC8, and this factor is somehow not present in *L. sakei* NCDO 2714. The sensitivity for the transformed strains of *L. plantarum* 2-1 is in fact so low that no sensitivity was detected at all compared to strains harboring the empty pMG36e plasmid. This is a bit peculiar as the sensitivity of the wild type strains of *L. sakei* NCDO 2714 and *L. plantarum* 2-1 does not differ as much.

Only one clone transformed with J51-*Orf5* was achieved, as the other clones sensitive to plantaricin J51 were not able to be successfully transformed. Some differences in sensitivity were observed between wild type strains and strains transformed with J51-*Orf5*; however these results were not easily reproducible (results not shown). Thus, we cannot conclude that J51-*Orf5* functions as an immunity protein for plantaricin J51, although this seems very likely.

5.4 Protein Expression studies

In order to study the expression of the *plNC8c* gene, SDS-PAGE with different subsequent visualization techniques were employed. The initial coomassie staining (as shown in Figure 4.4) shows few bands in the purified samples, and no clear band with the expressed PLNC8C immunity protein, suggesting that the protein expression level is very low. This is a bit

peculiar, as the *flciA* gene which is used as a control (and is situated in the same type of vector) is much more easily detected. Therefore the Western blotting technique was employed, in hope to better visualize specific bands containing FLAG™ sequences. A silver staining method was used for a more sensitive unspecific staining.

All of the Western blots and silver stained gels (Figure 4.5, Figure 4.6, Figure 4.7 and Figure 4.8) showed an extremely huge background. This is probably due to a poor or suboptimal purification process. In order to improve this, an assay of different detergents used in step 5 (in section 3.11.3) in the resin binding purification was set up. Solutions ranging from 1-5% (v/v) of Triton X-100 and Tween -20 were set up, and the samples were visualized on a silver stained polyacrylamide gel (results not shown). However the sample containing 1% (v/v) Triton X-100, as used before, seemed to give the lowest background (results not shown). Thus the use of 1% (v/v) Triton X-100 was continued.

Another reason for the poor purification of the FLAG™-tagged immunity protein could be that it was expressed at too low levels. The constitutive expression vector used in this study, pMG36e with the promoter P32, is optimized for expression in *Lactococcus lactis*, but has been used for protein expression also in *Lactobacillus* species (Diep et al. 2007; Straume et al. 2007). The P32 promoter might not be optimal for this study, resulting in insufficient expression levels.

Also potentially problematic, sequence analyses of the cloned genes used in this study revealed a start codon (ATG) upstream of the inserted genes (*plNC8c* and J51-*Orf5*). This start codon was also present in constructs containing a FLAG™ sequence. However, no stop codon was present upstream of the start codon of the inserted genes. Further sequence analyses also revealed that a strong Shine Dalgarno sequence, which is necessary for optimal ribosomal binding to the transcript, was also present upstream of the “contaminant” ORF. This would probably lead to two species of each of the downstream genes; one original gene (the immunity gene with or without a FLAG™ tag, depending on construct) and one gene with an upstream amino acid sequence of about 18 to 28 aa, depending on construct (see Figure 5.1).

Figure 5.1 Overview of partial sequence of the pMG36e plasmid containing the FLAG™-*pINC8c*. Translated sequence marked in red, FLAG™-tag marked in green, start codon marked in purple, and Shine-Dalgarno sequence is marked in orange. Restriction sites are indicated by names of the respective restriction enzymes.

The presence of two proteins both carrying a FLAG™-tag would definitely impact the purification of the protein extracts. Both proteins would probably be purified, resulting in a lower concentration of the "true" protein product. This would also lead to a much higher background. Since the "contaminant" protein is a bit larger than the FLAG™-PLNC8C protein, it should be situated in the same band, or directly above the band containing the FLAG™-PLNC8C immunity protein. These could be the bands in Figure 4.8 marked by arrows *2* and *3* ("contaminant protein" and FLAG™-PLNC8C respectively).

All in all the extra amino acid sequence present upstream of the FLAG™-tag does not alter the function of the downstream immunity proteins. The qualitative data of this study remains the same, however some minor deviations in quantification of sensitivity might arise. The "contaminant" protein will however impede the purification process, making it more difficult to purify and detect the immunity protein in polyacrylamide gels and Western blots.

Nevertheless, although the background from the purification was high and the protein band corresponding to the immunity protein was hard to identify, the immunity observed in the tranformant strains indicate that the immunity protein indeed was expressed. These strains were therefore used in an attempt to identify the receptors and investigate the immunity function. Previous studies by Diep et al. (2007) indicated that the receptor as well as the bacteriocin could be co-purified along with the immunity protein. Soft agar assays with spots of resin-immunity complex did however not show any anti-microbial activity. This could indicate that the concentration of bacteriocins in the immunity-resin complex is so low that

anti-microbial activity is not detectable, or that the bacteriocin does not form a complex with the immunity protein or another protein bound to the immunity protein. An attempt to identify potential bacteriocin receptors by co-purifications were done by comparing silver stained gels (Figure 4.6 and Figure 4.7) of purified samples from strains with and without the FLAG™-tagged immunity protein (e and –B/+B respectively). However, there were still almost no obvious differences in the three samples.

It was speculated that the putative immunity protein could form a complex with another protein, via a disulfide bridge, due to the two cysteine residues present in the putative immunity protein. Therefore further Western blot analyses was performed with one set of samples containing 2-mercaptoethanol and one set of samples without 2-mercaptoehanol. An interesting observation is the strong band present at the very top of lane 5 (>175 kDa, indicated by arrow *1*) in Figure 4.8. This is a protein sample containing the FLAG™-*plNC8c* with added bacteriocin (in vivo) but without 2-mercaptoethanol. This strong band is however not present at the very top in lane 8, which is the same sample just with added 2-mercatpoethanol. Instead two other bands have appeared, one band with a large protein size (46-58 kDa indicated by arrow *5*), and one band present in the 10-15kDa area, around the same size as the expressed *flciA* gene (indicated by arrow *3*). This same band (in the 10-15 kDa area) is also present in the other sample containing 2-mercaptoethanol (-B). This could indicate that the immunity protein forms a complex with another large protein, via a disulfide bond, which is broken when 2-mercaptoethanol is added. This large molecular weight band could however not be identified in silver stained polyacrylamide gels (see Figure 4.6 and Figure 4.7). This is mostly because the silver staining process is unspecific, which stains all proteins, and can then potentially mask strong bands observed in Western blots.

Also observed in the Western blot in Figure 4.8 are two other interesting strong bands that appear in the 17 - 25 kDa area (indicated by arrow *4*) in samples with added bacteriocin (+B). This could be an interaction between the immunity gene and another protein, but this interaction does not seem to be affected by the addition of 2-mercaptoethanol. The two second lowest bands (residing just above the two bands with similar size as the expressed *flciA* gene) in the two samples containing 2-mercaptoethanol could very well be the expressed FLAG™-*plNC8c* gene. However the signal is very faint for a standard Western blot, and a lot of background noise is apparent.

As Western blots indicate some sort of a disulfide bridge mediated complex formation, between the *plNC8c* gene and another protein, a soft agar assay with an indicator in the presence of DTT was set up. Since the PLNC8C immunity protein contains two cysteine residues (see Figure 5.2), a disulfide bridge formation could be very likely. The indicator strain is the same strain used in the Western blot analyses (*L. sakei* NCDO 2714 FLAG™-*plNC8c*). Pediocin PA-1, a bacteriocin which has two internal disulfide bridges essential for the anti-microbial activity, was used as a control. Figure 4.9 indicates that the complex observed in the Western blot in Figure 4.8, might not be necessary for maintaining the immunity function of the *plNC8c* gene, since any disulfide bridges seems to have been completely reduced. Peciocin PA-1 does not exert any anti-microbial activity, owing to the reduction of its cysteine residues, while sensitivity for plantarcin NC8 has not increased (which would be expected if disulfide bridge formation was critical for maintaining the immunity function). However further studies should be carried out to clarify this, as the design of that soft agar assay may not be optimal (DTT should also be present in the agar plates themselves, and a maximum DTT concentration tolerance should be established).

```
>gi|27544862|gb|AAO18428.1| putative immunity protein [Lactobacillus
plantarum]
MELLGILSWQESTLEFKAPICSILGVFSVINKFFLKDINFWGWLIVFVFSVKAAVNIDMSSLDWIVQTVF
TIVSILGICHVLFHKKSFQK
```

Figure 5.2 Amino acid sequence of PLNC8 in FASTA format showing the presence of two cysteine residues (Maldonado et al. 2004). Cysteine residues indicated in red.

5.5 Conclusions

- Planaricin J51 was established as a novel two-peptide bacteriocin
- Plantaricins J51 and NC8 have a narrow spectrum of inhibition
- PLNC8C confers specific immunity to plantaricin NC8
- Any receptor protein was not identified, but this study suggest that a disulfide bridge mediated complex formation might be involved in the immunity function

5.6 Future work

As the “contaminant” protein probably has impacted purification and visualization of the immunity protein, steps should be taken to eliminate this “contamination”. The immunity genes should be cloned into another vector which is free from any contaminating elements, preferably with a stop codon just upstream of the inserted gene. The choice of vector should also be changed as the pMG36e plasmid is optimized for *Lactococcus* (Guchte et al. 1989). Another vector (like pSIP) should be utilized which is more suitable for *Lactobacillus plantarum* and *L. sakei* (Sorvig et al. 2005).

The change of vector should simplify the purification and visualization of the expressed immunity genes. MIC assays of transformed strains should be repeated, although huge deviations from this study are not expected.

Hopefully more specific proteins in Western blots and silver stained polyacrylamide gels should be present, which might also indicate a good receptor candidate. This receptor candidate should be excised from the gel and analyzed using MALDI-TOF to determine its identity.

Another approach to possibly detect and investigate the receptor and immunity function might be to generate bacteriocin resistant mutant strains and analyze these. This might also give a clue to the mode of action.

Bacteriocin sensitivity assays should also be performed on known pathogenic strains (like *Listeria monocytogenes*) in order to investigate if the plantaricins NC8 and J51 does exert any anti-microbial effect. Although it seems unlikely, any detected sensitivity could be of important use in combating the diseases caused by these microorganisms.

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Appendix 1

Plasmid Mini Kit I Spin Protocol

All centrifugation should be preformed at room temperature unless otherwise noted. For low copy plasmids refer to page 16. This protocol is designed to isolate plasmid from E. coli grown in a 1-5 mL LB culture.

User Supplied Equipment:

- 96-100% Ethanol (Do not use denatured alcohol)
- Microcentrifuge capable of 15,000 x g
- 1.5 ml or 2 ml Centrifuge Tubes
- Appropriate Centrifuge Tube for step **1**

Things to do before starting:

- Preheat Elution Buffer to 70°C if Plasmid DNA is >10kb
- Prepare DNA Wash Buffer and Solution 1 according to directions on page 6

1. Isolate a single colony from a freshly streaked selective plate, and inoculate a culture of 1- 5 ml LB medium containing the appropriate selective antibiotic. Incubate for ~ 12-16 hr at 37°C with vigorous shaking (~ 300 rpm). Use a 10-20 ml culture tube or a flask with a volume of at least 4 times the volume of the culture. It is strongly recommended that an endA negative strain of E.coli be used for routine plasmid isolation. Examples of such strains include DH5a® and JM109®.

2. Decant or Pellet bacterial cells by centrifugation at 10,000 x g for 1 min at room temperature.

3. Resuspend the bacterial pellet by adding **250 µl of Solution I/RNase A solution**, and vortexing (or pipetting up and down). Complete re-suspension (no visible cell clumps) of cell pellet is vital for obtaining good yields. Transfer suspension into a new 1.5 ml microcentrifuge tube.

4. **Add 250 µl of Solution II** and gently mix by inverting and rotating tube several times to obtain a clear lysate. A 2-3 minute incubation may be necessary. Avoid vigorous mixing as this will shear chromosomal DNA and lower plasmid purity.

   Note: ***Do not allow the lysis reaction to proceed more than 5 min.***
   (Store Solution II tightly capped when not in use to avoid acidification of Solution II from CO_2 in the air.)

5. **Add 350 µl of Solution III** and mix immediately by inverting several times until a flocculent white precipitate forms.

   Note: It is vital that the solution is mixed thoroughly and immediately after the addition of Solution III to avoid localized precipitation.

6. Centrifuge at 13,000 x g for 10 min at room temperature. A compact white pellet will form. Promptly proceed to the next step.

7. Prepare a **HiBind DNA Mini Column** by placing into a 2 mL collection tube. **Add 100 µl of Equilibration Buffer.** Centrifuge at 13,000 x g for 30-60 seconds. Discard the flow-through liquid and place the HiBind DNA Mini Column back into the same collection tube.

8. **Add the cleared supernatant from step 6** by CAREFULLY aspirating it into the HiBind DNA Mini Column. Ensure that the pellet is not disturbed and that no cellular debris has carried over into the HiBind DNA Mini Column. Centrifuge at 13,000 x g for 1 min at room temperature to completely pass lysate through the HiBind DNA Miniprep Column. Discard the flow-through liquid and place the HiBind DNA Mini Column back into the same collection tube.

9. **Add 500 µl of HB Buffer** and centrifuge at 13,000 x g for 30 to 60 seconds at room temperature to wash the HiBind DNA Mini Column. Discard the flow-through liquid and place the HiBind DNA Mini Column back into the same collection tube.

This step ensures that residual protein contaminations are removed, thus ensuring high quality DNA that will be suitable for downstream applications.

10. **Add 700 µl of DNA Wash Buffer** (diluted with absolute ethanol) and centrifuge at 13,000 x g for 30 to 60 seconds at room temperature to wash the HiBind DNA Column. Discard the flow-through liquid and place the HiBind DNA Mini Column back into the same collection tube.

NOTE: DNA Wash Buffer Concentrate must be diluted with absolute ethanol before use. See label for directions. If refrigerated, DNA Wash Buffer must be brought to room temperature before use.

11. **OPTIONAL:** Repeat wash step 10 with another 700 µl of DNA Wash Buffer (diluted with absolute ethanol).

12. Centrifuge the empty HiBind Mini Column at 13,000 x g for 2 min to dry the column

IMPORTANT: *Do not skip this step - it is critical for good yields*

13. Place the HiBind DNA Mini Column into a new/clean 1.5 ml microcentrifuge tube(not supplied). Depending on desire concentration of final product, **add 30-100 µl of Elution Buffer** (10 mM Tris-HCl, pH 8.5) or sterile deionized water directly onto the center of the column matrix. Incubate at room temperature for 1 minute. Centrifuge for at 13,000 x g for 1 min to elute DNA. An optional second elution will yield any residual DNA, though at a lower concentration.