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"Construction of a Heterologous Expression Vector for Plantaricin F, One of the Peptides Constituting the Two-Peptide Bacteriocin Plantaricin EF"

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Abstract

Certain species of lactic acid bacteria produce and secrete bacteriocins, which are ribosomally synthesized antimicrobial peptides. These peptides recognize and kill target cells by rendering their membrane permeable for various small molecules. There has been an increased interest in lactic acid bacteria bacteriocins because of their potential use as food additives and pharmaceuticals. Plantaricin EF is a two-peptide bacteriocin produced by the lactic acid bacteria *Lactobacillus plantarum* C11. The two peptides constituting this bacteriocin are called Plantaricin E (PlnE) and Plantaricin F (PlnF). For optimal antimicrobial effect, the two peptides have to be present in equal molar amounts. Circular dichroism studies suggest that the peptides interact physically with each other upon contact with target membranes. The inter-peptide interactions between PlnE and PlnF are thought to be mediated by GxxxG motifs, which are located in their amphiphilic α-helical region. GxxxG motifs are known to confer helix-helix interactions between membrane-inserted polypeptides. In addition, tyrosine and tryptophan residues tend to be prominent in trans-membrane proteins, especially in the parts of proteins exposed to the interface region of the membrane. It is believed that these aromatic residues enhance stability because of interactions with membrane-lipids in the interface region.

To study the importance of GxxxG motifs in helix-helix interactions between PlnE and PlnF, the glycine-residues in PlnF have been altered by *in vitro* site-directed mutagenesis. The tyrosine and tryptophan residues in PlnF were also altered by *in vitro* site-directed mutagenesis, in order to investigate how PlnF will orient itself in target cell membranes. In order to do this, the gene encoding PlnF, *plnF,* and the gene encoding its cognate immunity protein, *plnI,* have been connected to the sakacin P leader-sequence, and cloned into the shuttle-vector pLPV111. The vector was transformed into the lactic acid bacteria *Lactobacillus sake* Lb790 containing the plasmid pSAK20. pLPV111 and pSAK20 are part of a heterologous expression system designed for expression of the many different bacteriocin. By using this expression system, PlnF is expressed separate from PlnE. This makes it easier to isolate and purify PlnF, as well as constructing and purifying the mutant version of PlnF, for subsequent structure and function analysis.

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1. Introduction

1.1 Antimicrobial Peptides

All living animals have the need to protect themselves against the invading, pathogenic organisms they are exposed to every day. These are organisms such as enveloped viruses (i.e. HIV and herpes virus), bacteria, fungi and parasites, but also cancer cells [1]. Several different defense-mechanisms have evolved, and antimicrobial peptides (AMPs) are important members of the host defense system. These small, cationic, ribosomally synthesized peptides are produced in many different tissue and cell types in various invertebrate, plant and animal species [2; 3; 4]. Especially organs that are more prone to invading pathogens, such as the eyes, ears, mouth, esophagus, lungs, stomach, bone marrow and testicles produce antimicrobial peptides [5]. Since invasion of a single bacterium can result in an infection in 24 hours, given a 50 min doubling time [1], the body is dependent on the innate immune system, which can respond rapidly to the infection. It has been suggested that antimicrobial peptides should be included as a part of the innate immune system [1]. Even though they are not as specific as the immunoglobins of the adaptive immune system, they can be produced up to 100 times faster and also diffuse much faster, and with lower cost of energy [5; 6; 7]. It is further evident that a single animal can produce more than 20 different antimicrobial peptides [1], for instance cows can produce as many as 36 different antimicrobial peptides of various classes and variants [8].

Generally speaking, antimicrobial peptides consist of 12-50 amino acid residues, 50% or more of these residues are hydrophobic [1; 9]. The peptides often have a net positive charge of +2 to +7, and low proportion of neutral polar and negatively charged amino acids, reflecting their ability to interact with negatively charged target membranes [10; 9; 1].

1.2 Bacteriocins

Bacteriocins are ribosomally synthesized antimicrobial peptides produced by bacteria, that often, but not always, target and kill bacteria related to the bacteriocin-producing strain [11; 10]. Most bacteria produce only one bacteriocin, but some species can produce several different bacteriocins [12]. Although descriptions of substances with bacteriocin-like activity first came in the late 19th century by Babes [13], the first documentation of the nature of a bacteriocin came in 1925 when Gratia demonstrated that an *Escherichia coli* (*E. coli*)-produced substance, termed colicin, had the ability to inhibit growth of other *E. coli* strains[14]. Since then, many antimicrobial peptides have been identified and characterized in most bacterial strains, both Gram negative and Gram positive bacteria, and the collective term "bacteriocins", which was originally coined for antimicrobial antibiotic proteins of the colicin type [15], was extended to also include non-colicin antimicrobial peptides produced by Gram positive bacteria [16].

Most bacteriocins produced by the Gram negative bacteria are large proteins (>20 kDa) [10] that range in size from about 200 to 800 amino acid residues [12]. The most extensively studied bacteriocins produced by Gram negative bacteria are by far the *E. coli*-produced colicins. Killing-modes of colicins range from membrane pore formation to nuclease activity against DNA, rRNA and tRNA in target cells [12]. Bacteriocins produced by Gram positive bacteria are most often peptides smaller than 6 kDa [10]. These bacteriocins are more abundant and diverse than the bacteriocins produced by Gram negative bacteria, and they have a broader activity spectrum (i.e. kill a greater variety of bacteria-species) [16; 11]. One of the key differences between the bacteriocins produced by Gram positive and Gram negative bacteria is that bacteriocin-production in Gram positive bacteria is not lethal to the producing cell, as apposed to what is the case for production in Gram negative bacteria. Whereas Gram negative cells generally rely on cell-lysis for release of bacteriocins, Gram positive bacteria have evolved transport systems to release their bacteriocins into the environment, the transport system consisting of either a bacteriocin-specific transporter or a *sec*-dependent pathway [17; 18; 12]. Moreover, Gram positive bacteria produce an immunity protein that protects bacteriocin-producing bacteria from being killed by their own bacteriocin [19].

In recent years, there has been a rapid spread of multi-resistant pathogenic bacteria due to the use of broad-spectrum antibiotics. Bacteriocins may provide a solution to this problem because of their specificity towards target cells and different killing-mechanism.

There has in recent years especially been much interest in and research on the bacteriocins produced by the Gram positive lactic acid bacteria (LAB), because these bacteria are non-pathogenic and naturally inhabit the gut of humans and animals. LAB and the bacteriocins they produce are therefore considered safe for eukaryotes, and have thus a potential as biopreservatives in food and feed. The LAB-produced bacteriocin nisin has already been used as a food preservative for 30 years [20].

The remainder of this introduction will focus on the LAB bacteriocins (defined in section 1.3), and especially class IIb LAB bacteriocins, which plantaricin EF is categorized as.

1.3 Classification of LAB Bacteriocins

LAB bacteriocins are divided into three different classes based on their structure and function (Fig. 1.1) [21]. The class I bacteriocins are the post-translationally modified peptide bacteriocins, class II bacteriocins are the unmodified peptide bacteriocins, while class III bacteriocins are the large, heat-labile protein bacteriocins.

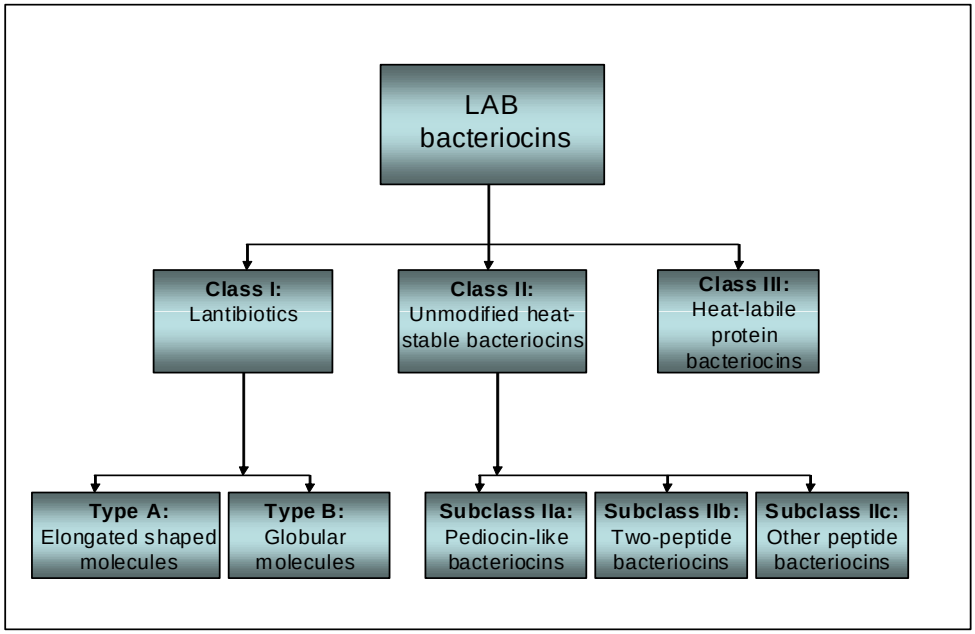

Fig. 1.1: Classification of LAB bacteriocins. Class I bacteriocins are the lantibiotics, which are divided into two subclasses based on their structure. The class II bacteriocins are the unmodified, heat-stable peptide bacteriocins, and these are divided into three subclasses. The third class of bacteriocins are the heat-labile protein bacteriocins.

1.3.1 Class I Bacteriocins

Class I bacteriocins, also known as lantibiotics, contain the unusual post-translationally modified residues lanthionine, 3-methyllanthionine and other modified residues, such as dehydroalanine and dehydrobutyrine, none of which are known to exist in other proteins or peptides [22]. Lanthionine and 3-methyllanthionine are synthesized in a two-step mechanism where serine and threonine residues, respectively, are dehydrated and form thioether bonds with -SH groups in cysteine residues [23]. The lantibiotics range in size from 19 to 38 amino acids, and the percentage of residues involved in modifications are from 24% to 47% [24].

The class I bacteriocins are further divided into type A and B, which are the elongated and globular peptides, respectively [25]. The elongated type A peptides are amphipathic molecules with molecular masses ranging from 2164 Da to 3488 Da, and with two to seven net positive charges [11]. These peptides kill target cells by permeabilizing their membranes by pore-formation [25; 24; 26]. The globular, smaller type B peptides have molecular masses from about 1950 Da to 2050 Da, and have either a low net positive charge, no net charge or a net negative charge [11; 26]. Their mode of action is inhibition of enzymatic activity [24; 26].

1.3.2 Class II Bacteriocins

The class II bacteriocins consist of peptides without modified residues [21]. The class is further divided into three subclasses; subclass IIa, IIb and IIc. Within subclass IIa are the pediocin-like bacteriocins, which have antilisterial activity, and most share 40 to 60 % sequence similarity, including a highly conserved 14-residue motif in their N-terminal region, which also contains two conserved cysteins that form a disulfide bridge [27; 28]. Subclass IIb contains the two-peptide bacteriocins, all of which consist of two different peptides, both of which must be present in equal molar amounts to obtain an optimal antimicrobial effect [29]. The last subclass is IIc, which contains one-peptide bacteriocins that share no sequence-similarity to the pediocin-like bacteriocins [30].

1.3.3 Class III Bacteriocins

The bacteriocins that belong to class III are probably the least studied class of the LAB bacteriocins. This class consist of large (>20 kDa), heat-labile antimicrobial proteins [11].

1.4 Class IIb Bacteriocins

About 15 class IIb two-peptide bacteriocins are known, and these are listed in Table 1.1. This study deals with the class IIb two-peptide bacteriocin plantaricin EF, and the class IIb bacteriocins, and particularly plantaracin EF, will consequently be discussed in more detail.

Table 1.1: Overview of genetically and biochemically characterized class IIb bacteriocins.

Bacteriocin[1]	Producer	Reference
Lactococcin G	*Lactococcus lactis* LMGT2081	29
Enterocin 1071 [2]	*Enterococcus faecalis* FAIR-E 309	38
	Enterococcus faecalis BFE 1071	39, 108
Lactococcin Q	*Lactococcus lactis* QU 4	96
Plantaricin EF	*Lactobacillus plantarum* C11	31, 54
Plantaricin JK	*Lactobacillus plantarum* C11	31, 54
Plantaricin S	*Lactobacillus plantarum* LCP010	33, 97
Plantaricin NC8	*Lactobacillus plantarum* NC8	98
Lactacin F	*Lactobacillus johnsonii* VPI11088	99, 100
Brochocin C	*Brochothrix campestris* ATCC 43754	34
Thermophilin 13	*Streptococcus thermophilus* Sfi13	32
ABP-118	*Lactobacillus salivarius* subsp. *salivarius* UCC118	101
Mutacin IV	*Streptococcus mutans* UA140	102
Lactococcin MN	*Lactococcus lactis* 9B4	35
Lactocin 705	*Lactobacillus casei* CRL 705	103
Leucocin H[3]	*Leuconostoc* MF215B	104
Salivaricin P[4]	*Lactobacillus salivarius*	105

[1]Enterocin L50 has two peptides [106], but is not not included in the table, as it is not considered a bona fide two-peptide bacteriocin, since the sequences of the two peptides are similar, and the peptides individually exerts antimicrobial activity. Lactococcin MMT24 [107] is not included in the table since its sequence is unknown and therefore might be identical to any of the other above-mentioned two-peptide bacteriocins.

[2]Refer to Franz *et al.* [38] for the correct sequence of enterocin 1071.

[3]Leucocin H has only been partially sequenced and may contain modified amino acid residues, and might thus belong to the class I bacteriocins (the lantibiotics).

[4]Salvacin P is almost identical in amino acid sequence to ABP-118, except for two residues.

1.4.1 Biosynthesis and Secretion of Class IIb Bacteriocins

For all two-peptide bacteriocins, the two structural genes encoding each of the two peptides are found next to each other in the same operon, which indicates that the two peptides are produced in approximately equal amounts [30; 31]. In addition, the gene encoding the immunity protein is also found in the same operon as the bacteriocins' structural genes [30]. The immunity protein protects the bacteria from being killed by their own bacteriocin. Although it is unclear by what mechanism the immunity protein protects cells, it is suggested that it involves an interaction with membrane components [30]. This interaction with membrane components is based on structure predictions of a number of putative immunity proteins of two-peptide bacteriocins, which all appear to contain transmembrane helices, such as the immunity proteins of thermophilin 13 [32], plantaricin S [33], brochocin C [34], lactococcin MN [35], lactococcin G [36] and plantaricin EF and JK [31].

The two peptides that constitute all two-peptide bacteriocins characterized so far are synthesized with a 15-30 residue double-glycine-type leader sequence at the N-terminal end [19; 30]. For transport of the bacteriocins out of cells, an ABC-transporter and an accessory-protein are needed. The ABC-transporter resides in the membrane and cleaves of the N-terminal leader-sequence as it transports the peptides to the outside [30]. Studies involving the lactococcin G ABC transporter show that its N-terminal region contains the recognition- and cleavage-site for the lactococcin G leader-sequence, and that the leader is cleaved off at the C-terminal side of the double-glycine motif [37]. Thus, the ABC transporter appears to exert proteolytic activity at the same time as it transports the bacteriocin out of cells. The exact role of the accessory protein is unclear, but it seems to be required for secretion of bacteriocins [28; 30]. The genes encoding the ABC transporter and the accessory protein are in some cases found on the same operon as the bacteriocin structural genes and the gene encoding the immunity protein, as for lactococcin G [36], in other cases on a different, but nearby, operon, as for enterocin 1071 [38; 39] and plantaricin EF and JK [31].

1.4.2 Regulation of the Biosynthesis of Class IIb Bacteriocins

It has been evident for some years that bacteriocin production in Gram positive bacteria is, in some cases, regulated in a cell-density dependent manner, through a so-called three-component regulatory pathway (sometimes referred to as a two-component regulatory pathway) [19; 40] (Fig. 1.2). The genes encoding all three components are located on the same operon, and are simultaneously transcribed. The three components in this pathway are i) an induction factor, ii) a sensor histidine protein kinase and iii) a response regulator [19; 30; 40].

The induction factor is a pheromone peptide, which is produced with an N-terminal double glycine-type leader, and is processed and secreted by the same ABC-transporter that transports bacteriocins out of cells [40]. The synthesis of these pheromone peptides seems to be an autoregulatory process, where the production of the peptide itself is increased upon activation of the sensor histidine kinase [41; 40].

The sensor histidine protein kinase is a membrane-bound protein that is activated upon binding of the peptide pheromone, which occurs when a certain threshold concentration of the peptide pheromone is attained [40]. The activation of the kinase involves autophosphorylation of a conserved histidine residue in the cytoplasmic domain of the kinase. The phosphate-group is subsequently transferred to an aspartate residue in the receiver domain of the response regulator, which is a cytoplasmic protein. This leads to a conformational change in the response regulator, causing it, in most cases, to bind to specific regions of the target promoter, causing transcriptional activation of genes involved in the bacteriocin production and the transport of bacteriocins out of the cell [42; 40].

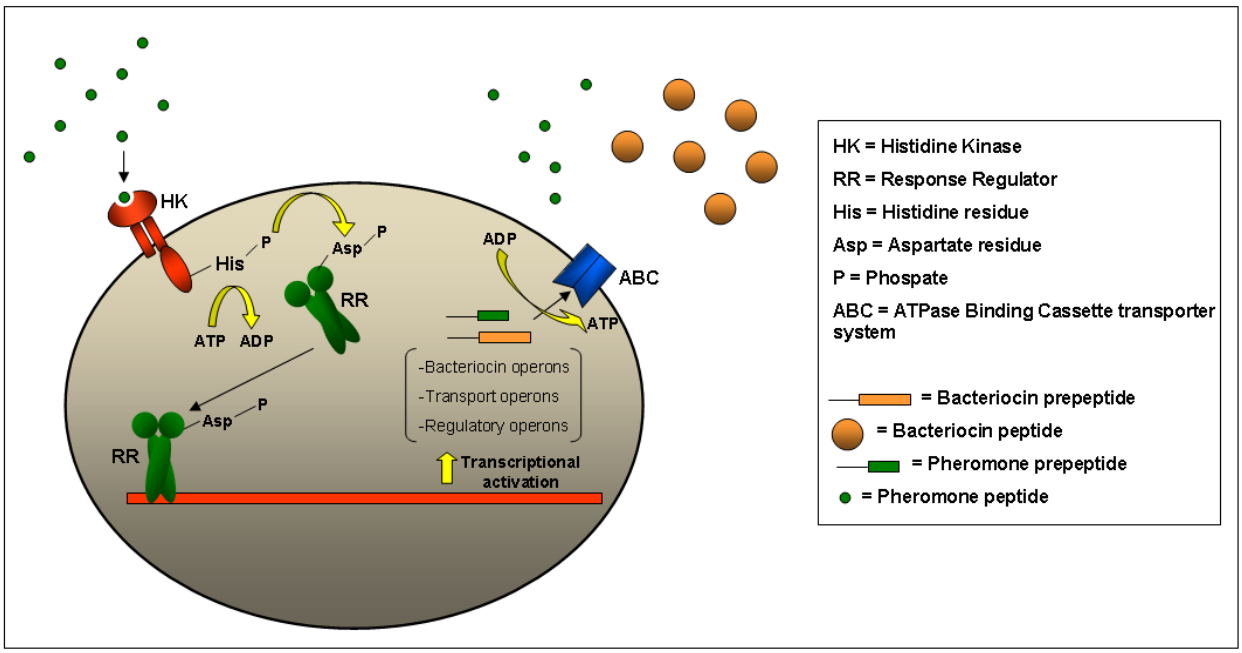

Fig. 1.2: Schematic overview of the three-component regulatory pathway that regulates biosynthesis of some two-peptide bacterocins (such as plantaricin EF) in Gram positive bacteria. Binding of the pheromone peptide to the membrane-bound histidine kinase leads to activation of the kinase, which again leads to activation and subsequent binding of a response regulator to the target promoter. After transcriptional activation of the bacteriocin operon, the bacteriocin is produced and transported out of the cell [40].

1.4.3 Structure and Mode of Action of Class IIb Bacteriocins

Sequence analysis of class IIb bacteriocins shows that both peptides constituting the bacteriocins are cationic, and contain putative amphiphilic α-helices and/or hydrophobic regions [30]. The net positive charge of the peptides presumably facilitates interaction with the negatively charged membrane of target cells, whereas the amphiphilic or hydrophobic segments facilitates penetration into the membrane [28]. Structural studies by circular dichroism (CD) spectroscopy of some class IIb bacteriocins have shown that these peptides are unstructured and have low α-helical content in water, but achieve a higher α-helical content when exposed to more membrane-like environments, such as micelles and negatively charged liposomes. This has been shown to be the case for the two peptides (termed LcnG-α and LcnG-β) constituting the class IIb bacteriocin lactococcin G [43]. It was also shown that increased α-helical structuring is obtained when LcnG-α and LcnG-β interact with each other in the presence of membrane-like entities, such as liposomes [43]. This inter-peptide interaction and structuring presumably occurs when the peptides come in contact with the target

membrane, but before they get fully embedded in the membrane [44]. By treating cells with one of the peptides followed by extensive washing, and then adding the complementary peptide, growth inhibition was observed. When mixing cells treated with one of the peptides with cells treated with the complementary peptide, no lactococcin G activity was found [45]. This indicates that the individual peptides have the ability to interact stably with target membranes, but with no individual bactericidal effect, and that the peptides are not able to diffuse once bound to the membrane. When added to cells simultaneously, LcnG-α and LcnG-β show bactericidal effect at picomolar concentrations [29]. A similar scenario is found for the two peptides constituting the two-peptide bacteriocin plantaricin JK [46; 47].

Although the mode of action for class IIb bacteriocins is not entirely understood, two general mechanisms have been described; the "barrel-stave" model (Fig. 1.3), and the "carpet" model (Fig. 1.4) [48; 49]. In the "barrel-stave" model (Fig. 1.3), the peptides associate and form trans-membrane pores, with the hydrophobic surfaces of the α-helices facing the hydrophobic core of the lipid bilayer, and the hydrophilic surfaces of the helices facing inwards, making an aqueous pore [49]. According to the second model, the "carpet" model (Fig. 1.4), the peptides do not insert into the lipid bilayer, but rather bind onto the membrane of target cells, and cover it in a carpet-like manner. High local concentrations of peptides can lead to disruption of the membrane [49]. There have been speculations about whether or not there is a target membrane-located receptor molecule involved in binding of two-peptide bacteriocins. This could be the basis for the different target cell specificity of these bacteriocins.

After insertion into the membrane, the various bacteriocins show some specificity regarding which molecules they conduct across the membrane [30]. Lactococcin G has been shown to make the target membrane permeable for potassium ions, but also other monovalent cations [50], whereas plantaricin JK leads to anion-leakage in target cells [46; 47]. In all cases, the consequences are detrimental for the target cells, as the proton motive force is destroyed.

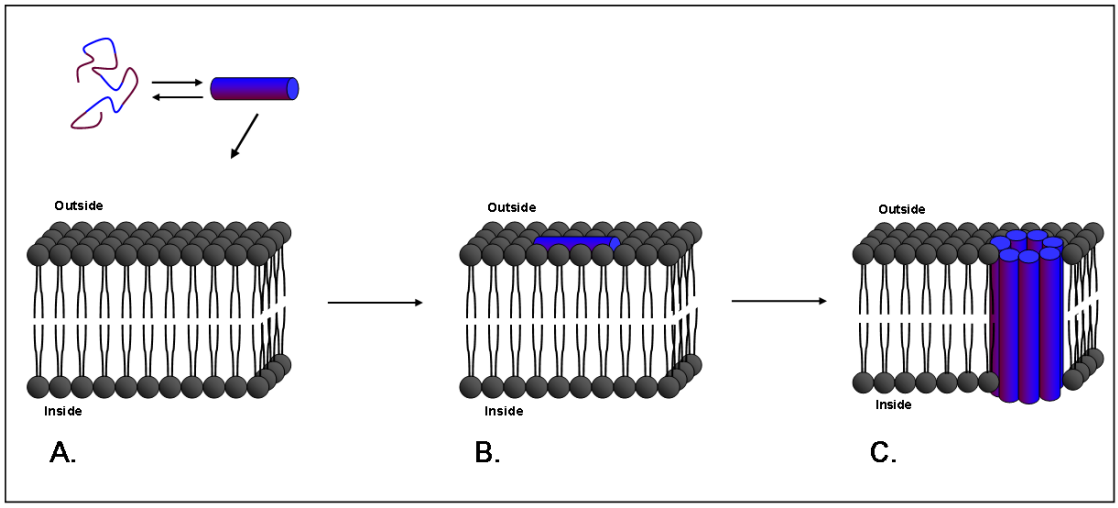

Fig. 1.3: Illustration of the "barrel-stave" model for permeabilization of target membranes by antimicrobial peptides. The antimicrobial peptides adopt α-helical structures when they come in contact with a membrane-like environment **(A)**, and the hydrophobic regions (marked in purple) interact with the membrane surface, while the hydrophilic regions (marked in blue) face the solvent **(B)**. When a threshold concentration of peptides is reached, the peptides associate and form trans-membrane pores, with the hydrophobic surfaces facing outside, and the hydrophilic surfaces facing inwards, making an aqueous pore, which support leakage **(C)** [49].

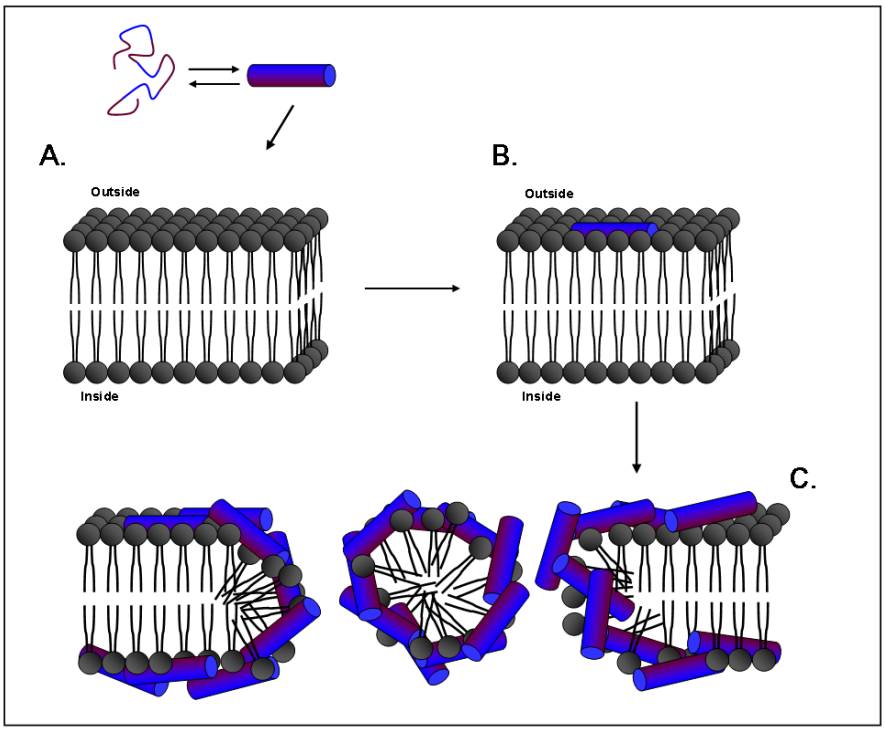

Fig. 1.4: Illustration of the "carpet" model for permeabilization of target membranes by antimicrobial peptides. The unstructured antimicrobial peptides form α-helices when they come in contact with a membrane-like environment **(A)**. The antimicrobial peptide binds to the membrane with the hydrophobic regions (blue) interacting with the phospholipid bilayer, and the hydrophilic part (blue) facing the solvent **(B)**. A high local concentration of peptides leads to permeabilization of the membrane **(C)** [49].

1.4.4 Plantaricin EF

Plantaricin EF is a two-peptide bacteriocin produced by the lactic acid bacteria *Lactobacillus plantarum* C11. The bacteriocin consists of the two peptides termed PlnE and PlnF. Indications of bacteriocin production by *L. plantarum* C11 were first reported in 1990 [51]. Sequencing of the gene encoding the plantaricin-inducing factor plantaricin A (PlnA)[1], *plnA,* together with its operon, *plnABCD*, was reported in 1993 [52; 53]. The first characterization of the PlnE and PlnF structural genes was reported in 1996, when four new PlnA-induced operons were identified by Northern blot and DNA sequencing [31; 40] (Fig. 1.5). Two of the PlnA-induced operons, *plnEFI* and *plnJKLR,* encode two two-peptide bacteriocins (plantaricin EF and plantaricin JK, respectively) and their cognate immunity proteins (plantaricin I and plantaricin L, respectively) [31; 54]. A third PlnA-induced operon, *plnMNOP*, was by analogy to other operons that encode functional bacteriocins thought to encode a typical bacteriocin-like peptide (PlnN) and cognate immunity proteins (PlnM and PlnP) [31], but the mature PlnN peptide showed no bactericidal activity against indicator strains [54]. A fourth PlnA-induced operon, *plnGHSTUV,* encodes an ABC transporter (PlnG) and an accessory protein (PlnH) that are involved in processing and transport of double-glycine-leader type pre-peptides [31].

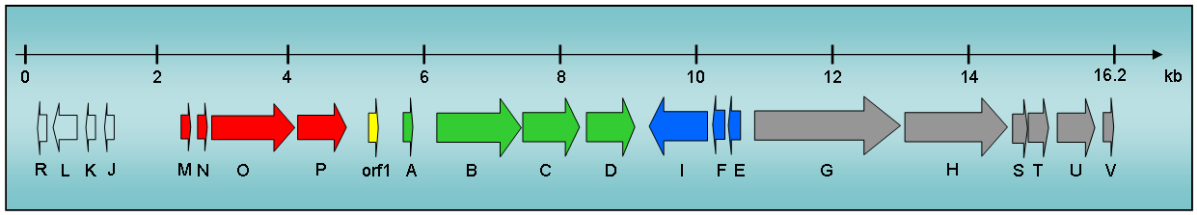

Fig. 1.5: Genetic map of the 16,139 bp pln-locus in *L. plantarum* C11. Northern blot and DNA sequencing show 22 ORFs organized into the five PlnA-induced operons in *Lactobacillus plantarum* C11, including the *plnABCD* operon, which encodes PlnA [31]. The *plnJKLR* operon encodes the two-peptide bacteriocin plantaricin JK and the immunity protein PlnL. The *plnMNOP* operon encodes peptides with unknown function. The genes encoding the two-peptide bacteriocin plantaricin EF and its immunity protein PlnI, are in the *plnEFI* operon. *Orf1* shows no similarity to other known proteins when homology searches are performed.

[1] Plantaricin A is the peptide pheromone of the three component regulatory pathway that triggers the production of plantaricin EF.

Transcription of all five PlnA-induced operons[2] in *L. plantarum* C11 has been shown to be activated during the exponential growth phase, and down-regulated before the stationary phase [55]. All of the operons contain a -35 nucleotide region, and a preceding, conserved pair of direct repeats [31; 56], and it has been further demonstrated that these repeats serve as binding sites for both a transcriptional activator and a repressor, encoded by *plnC* and *plnD*, respectively [57; 58]. This transcriptional regulation varies between the promoters, regarding both time and strength, even though all five promoters share an overall similarity [59].

PlnE and PlnF are co-transcribed from the same operon, *plnEFI*, together with the cognate immunity protein, PlnI. The bacteriocins structural genes *plnE* and *plnF* encode two pre-peptides, each with an N-terminal double-glycine leader type (Fig. 1.6) [31].

Fig. 1.6: Amino acid sequence of the PlnE and PlnF pre-peptides. The N-terminal leader sequence is shown in blue, with the two glycine-residues shown in green. The sequence of the mature peptide is shown in black, with glycine-residues in the GxxxG-motifs shown in red.

Production of PlnE and PlnF, together with PlnA, PlnJ, PlnK and PlnN, were detected by amino acid sequencing and mass spectrometry in 1998 [54]. Amino acid sequencing showed that the mature PlnE consists of 33 amino acid residues, while the mature PlnF consist of 34 amino acid residues. PlnE has a molecular mass of 3703 Da and isoelectric point of 12.1 (mature peptide), while PlnF has a molecular mass of 3545 Da and isoelectric point of 10.7 (mature peptide), as determined by amino acid sequencing and mass spectrometry [54; 31].

The two peptides, PlnE and PlnF, have to be present in equal amounts to obtain optimal antibacterial activity. Although PlnF (but not PlnE) alone exerts some antimicrobial activity, the activity increases at least 10^3 times when the two peptides are combined [46; 54].

[2] The five PlnA-induced operons are *plnABCD, plnEFI, plnJKLR, plnMNOP* and *plnGHSTUV*.

An Edmundson α-helical wheel display of their amino acid sequences (Fig. 1.7) shows that both peptides contain segments that can potentially form amphiphilic α-helical regions [54; 60].

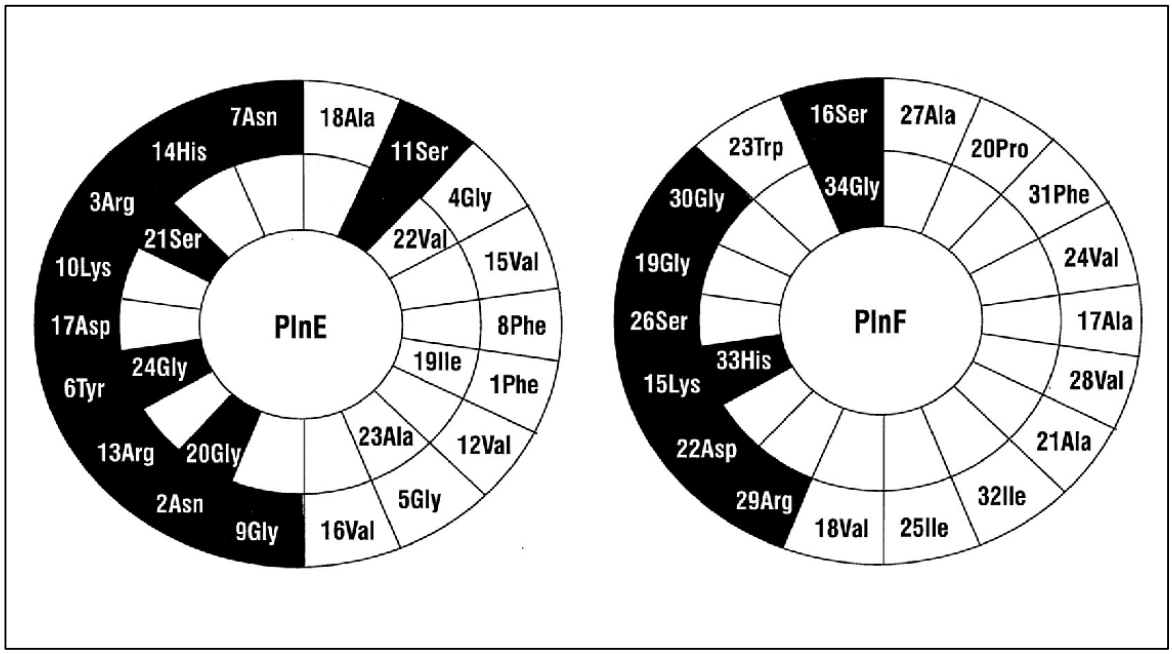

Fig. 1.7: The Edmundson α-helical wheel representation of PlnE and PlnF. Hydrophilic residues are colored black, and hydrophobic residues are colored white [54]. Figure from Anderssen *et al.* [54].

As mentioned previously (section 1.4.3), such amphiphilic regions are often associated with pore-forming toxins which insert themselves in cell membranes through a "barrel-stave"-mechanism [48; 49]. In fact, circular dichroism (CD) studies of PlnE and PlnF, both combined and individually, show that both peptides goes from being unstructured and having a low α-helical content in aqueous solution, to adapting a mainly α-helical structure in the presence of more membrane-like environments, like trifluoroethanol (TFE) or dodecylphosphocholine (DPC) [46]. CD measurements of the peptides in the presence of anionic 1,2-dioleoyl-sn-glycero-3-[phosphor-rac-(1-glycerol)] (DOPG) liposomes show that premixing the peptides before adding the liposomes gives a higher calculated α-helical content than mixing liposomes containing one peptide with liposomes containing the complementary peptide [46]. This suggests that formation of α-helices enables the PlnE and PlnF to interact with and permeabilize target membranes.

Mode of action studies reveal that plantaricin EF causes leakage of monovalent cations, namely protons (H^+), rubidium (Rb^+) and choline ions from target cells. The efflux of these cations causes dissipation of the trans-membrane pH gradient (ΔpH) and the membrane potential (Δψ), leading to inhibition of metabolism and subsequent growth inhibition [47].

The three-dimensional structures of PlnE and PlnF in the presence of DPC-micelles were determined by NMR spectrometry in 2008 [61] (Fig. 1.8 and 1.9, respectively). PlnE consists of two α-helical regions, one from residue 10 to 21, and the other from 25 to 31. Both helices are amphiphilic. The calculated structure of PlnF indicates an α-helical region stretching from residue 7 to 32, and around Pro20 the helix has a kink of 38±7 degrees, giving the helix more flexibility around this position. This α-helix has an amphiphilic C-terminal part, and a polar N-terminal part [61].

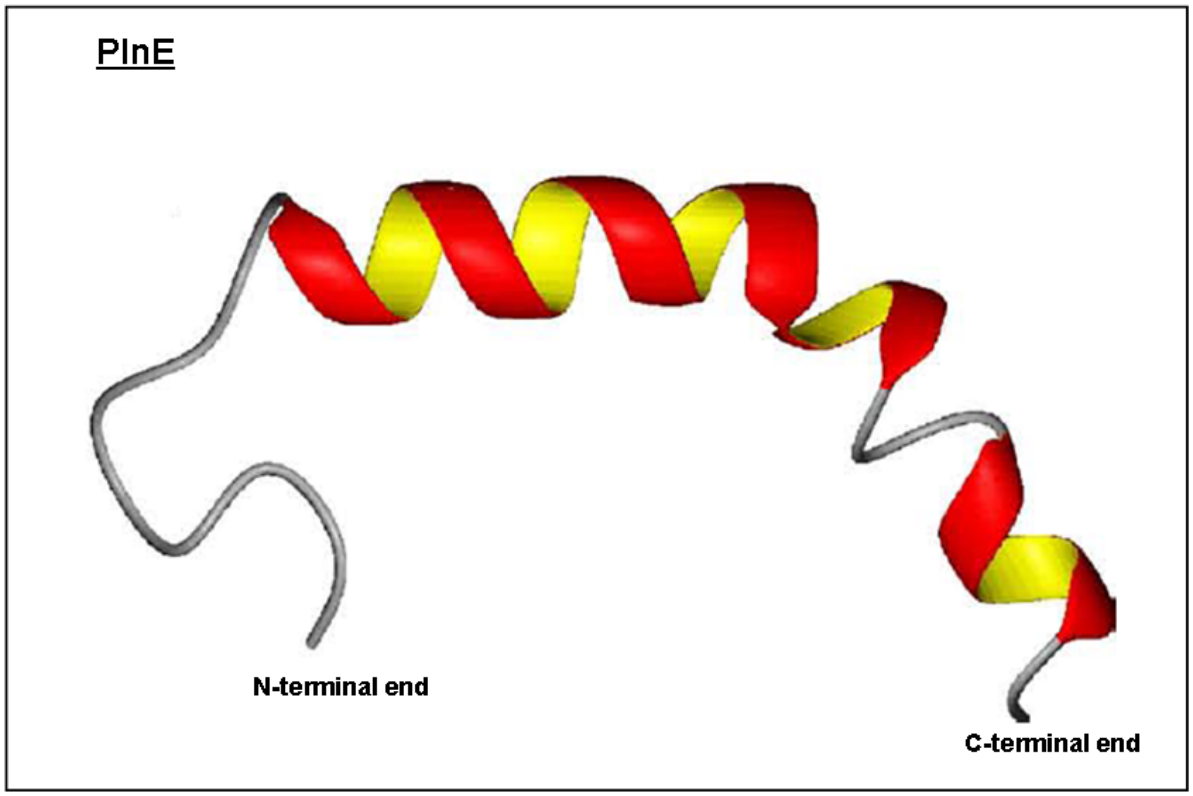

Fig. 1.8: Cartoon-drawing of the three-dimensional structure of PlnE. The peptide contains two α-helical regions, from residue 10 to 21, and from residue 25 to 31. Figure from Fimland *et al.* [61].

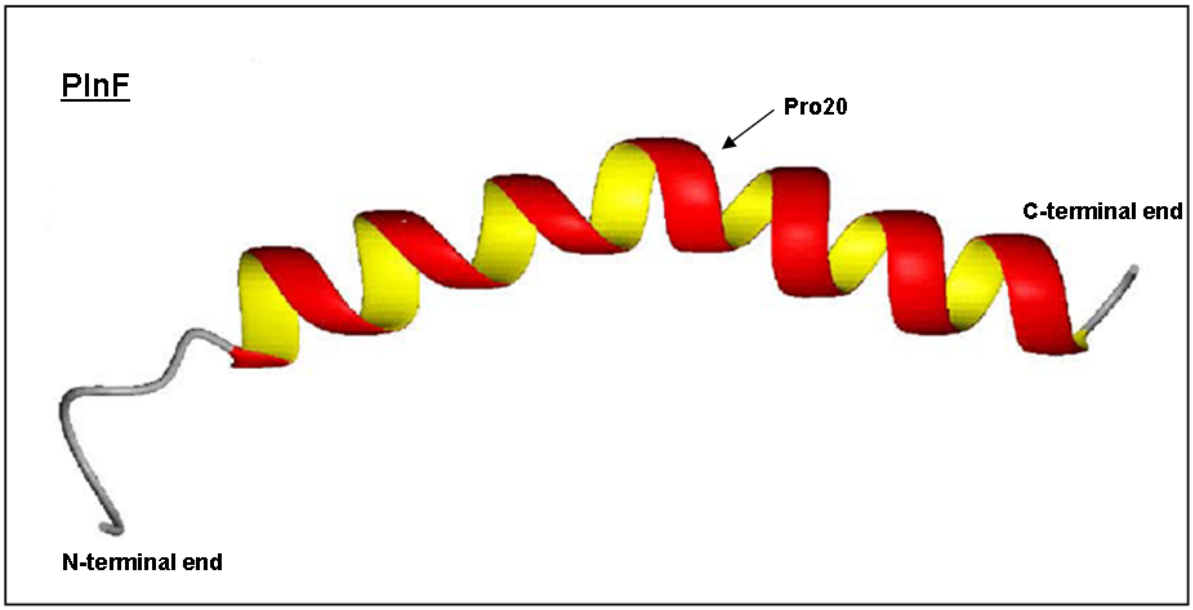

Fig. 1.9: Cartoon-drawing of the three-dimensional structure of PlnF, which forms one α-helical region from residue 7 to 32. Pro20 creates a kink in the helix. Figure from Fimland *et al.* [61].

1.4.5 GxxxG Motifs

GxxxG motifs consist of two glycine-residues separated by any three amino acid residues, a sequence shown by computational analysis to occur more frequently in trans-membrane helices than is expected from a random occurrence [62; 63]. If the GxxxG motif is found in an α-helix, the two glycine residues are approximately on the same side of the helix. The glycine residues may be substituted by other small amino acids, such as serine or alanine, and one may then speak of (small)xxx(small) motifs [64; 65]. These motifs are known to stabilize helix-helix interaction in trans-membrane proteins, by providing a framework for dimerization [64]. It is suggested that the glycine-residues affect the dimerization of α-helices by providing a surface for packaging of other amino acid residues at the interface, allowing for close helix proximity, which is the basis for van der Waals interactions between trans-membrane helices [66]. The occurrence of (small)xxx(small) motifs (where (small) can be glycine, alanine or serine) also minimizes steric hindrance of the helix backbone, because of the size of glycine, alanine and serine. This again is believed to enable Cα-H···O hydrogen bonds to form across the helix backbones, stabilizing the interaction between the helices [65; 67]. While the different GxxxG motifs confer α-helical interactions, the different motif sequence variants also promote specific associations, because of the topology of the interface

[64]. It has, for example, been shown that neither of PlnE nor PlnF functions together with either of the two peptides in the other two-peptide bacteriocins lactococcin G or plantaricin JK [54].

Mutational studies [68; 69] and NMR-studies [70] of the peptides of the two-peptide bacteriocin lactococcin G suggest that the peptides interact through their GxxxG motifs in a parallel orientation and in a staggered fashion relative to each other [70].

Both PlnE and PlnF contain GxxxG motifs. PlnE has two motifs, one from residue 5 to 9, and the other from residue 20 to 24. Only one GxxxG motif exists in PlnF, spanning from residue 30 to 34. It has not been determined how the two peptides interact, but the most likely pairing seems to be between the 20 to 24 residue GxxxG motif in PlnE and the 30 to 34 residue motif in PlnF in a parallel orientation [61]. It is also suggested that the interaction bringing the helices together may not be between both GxxxG motifs, but between a flat region (residue 16 to 20) in PlnF and the 20 to 24 residue GxxxG motif in PlnE, since it has been shown that GxxxG motifs alone do not confer helix dimerization [61; 71].

1.4.6 Tyrosine and Tryptophan Residues

Tyrosine (Tyr) and tryptophan (Trp) are aromatic residues that are prominent in trans-membrane proteins, and especially in the interface regions of membranes. In fact, all trans-membrane proteins with known three-dimensional structure share this preference for Tyr and, especially, Trp residues in these regions [72]. The non-polar, aromatic side chains of Tyr and Trp tend to be buried within the hydrophobic part of the interface, and these two residues presumably enhance stability due to interface interactions with lipids [73; 74; 75; 76].
Mutational analysis of Trp and Tyr in the two-peptide bacteriocin lactococcin G revealed a somewhat surprising tolerance for different residues replacing Trp and Tyr, suggesting that the C- and N-terminal ends of the lactococcin G peptides are flexible, and not embedded in a strict hydrophobic or hydrophilic environment [69].

PlnF contains two Tyr residues, in position 5 and 14 (Y5 and Y14) and one Trp residue, in position 23 (W23, Fig. 1.6. bottom).

2. Aim of the Study

Two-peptide bacteriocins consist of two different peptides which have to be present in equal molar amount to exert optimal antimicrobial effect. The peptides are thought to interact with each other through helix-helix interactions, and it is evident that many two-peptide bacteriocins contain one or more GxxxG motifs. One could therefore postulate that these GxxxG motifs are involved in the helix-helix interaction between complementary peptides. To investigate the importance of GxxxG motifs in the interaction between Plantaricin E (PlnE) and Plantaricin F (PlnF), mutational studies of GxxxG motifs in PlnF (and PlnE) are necessary. In addition, tyrosine and tryptophan residues are prominent in trans-membrane proteins, especially in the interface region. These residues are thought to interact with membrane-lipids, and thereby confer stability to the membrane-embedded protein. By performing mutational analysis of these GxxxG motifs and tyrosine and tryptophan residues, it is possible to gain insight into the structure-function relationship of PlnE and PlnF.

The main aim of this study was to make a heterologous expression system for PlnF, using the two plasmids pSAK20 and a pLPV111-derivate. The pLPV111-derivate is constructed by connecting the gene encoding PlnF, *plnF,* and the gene encoding the immunity protein, *plnI,* to the leader-sequence of the bacteriocin sakacin P. pSAK20 contains all the genes necessary for production and transport of PlnF out of the cell. In wild type plantaricin EF-producers, PlnE and PlnF are produced in approximately equal amounts. Since PlnE and PlnF have similar biochemical properties, they are difficult to separate from each other based on these properties. The heterologous expression system makes it easier to purify the peptides, and to introduce point mutations.

In order to assay the antimicrobial activity of mutant PlnF and wild type PlnE, and compare it to the activity of wild type plantaricin EF, the GxxxG motif and the tyrosine and tryptophan residues in PlnF were altered to other amino acid residues with different biochemical properties. This was done by *in vitro* site-directed mutagenesis.

3. Methods

3.1 Gene Technological Methods

3.1.1 Polymerase Chain Reaction (PCR)

PCR is a method used for amplification of a DNA sequence without the use of living organisms [77]. The principle behind PCR is the construction of two primers that anneal complementary to the ends of the desired sequence, which is then amplified in successive cycles by a heat-stable DNA polymerase, in a 5' to 3' direction (the amplified products are also used as templates in successive rounds of PCR). The result is several orders of magnitude amplification of DNA [77].

Each PCR cycle consists of three steps: denaturation of DNA, annealing of primers, and elongation by a DNA polymerase. The time and temperature required for each step may vary, depending on several factors. The cycling is preceded by an initial step called "hot-start", which denatures the DNA template, yielding single stranded DNA. A final elongation step is performed after the thermal cycling, to ensure complete elongation of newly synthesized PCR products. The number of cycles may vary.

A typical PCR reaction mix requires several components. The template to be amplified may be several genes, a single gene, parts of a gene, or a non-coding region. Two oligonucleotide primers are designed, that anneal to the 3' end and 5' end of the DNA fragment to be amplified. In order to design the primers, the sequence at the end of the fragment, which is to be amplified, must be known. The four deoxynucleside triphosphates are the building blocks from which a new DNA strand is synthesized. Heat-stable DNA polymerases are responsible for the elongation of the new strand. A divalent cation is added to the reaction mix, which functions as a co-factor for the polymerase.

The DNA polymerases used in this project were the *Pfu* DNA polymerase (Fermentas) and the *Taq* DNA polymerase (Fermentas). The *Taq* polymerase is less expensive than *Pfu* polymerase, but lacks 3' to 5' exonuclease proofreading activity. In addition it makes DNA products with 3' adenine (A) overhangs. This makes the polymerase useful in TA-cloning, as

the products subsequently can be ligated into vectors with 3' thymine (T) overhangs [78]. The *Pfu* polymerase, on the other hand, has 3' to 5' proofreading activity, and therefore has a lower random mutation rate than *Taq* polymerase.

PCR can be used in both the Megaprimer method [79] and for introduction of site-directed mutations in DNA, both of which were used in this thesis. The Megaprimer method was used to connect the sakacin P leader-sequence to the gene encoding PlnF. Point-mutations were constructed according to the protocol in The QuikChange® Site-Directed Mutagenesis kit [80].

3.1.2 The Megaprimer Method

This method is performed according to the principles described in Sambrook's "Molecular cloning" [79]. In this method, three oligonucleotide primers are used to perform two successive rounds of PCR. In the first round, two of the primers are used to synthesize a PCR product, which is used as a Megaprimer in the second round of PCR, together with a third oligonucleotide primer. The method is often used to connect a smaller DNA fragment to a bigger fragment.

In this project, the method was used to connect the sakacin P leader sequence to the gene encoding PlnF (Fig. 3.1). The templates used were the plasmid pLT100α for the sakacin P leader-sequence amplification, and genomic DNA from *L. plantarum* C11 for amplification of the genes encoding PlnF and the immunity protein.

In the first round of PCR (Fig. 3.1), the primers PlnFA and SakPB (see appendix 6.3.1 for sequences) was used to amplify the sakacin P leader sequence. PlnFA consists of two parts. One part anneals to the end of the leader-sequence in the first reaction, thus serving the primer-role. The other part does not anneal to the template (pLT100α), and exists as a non-complementary "tail" in the first round, but anneals to the start of the PlnF gene in the template (*L. plantarum* C11 genomic DNA) in the second round of PCR. PlnEFimm was the other primer used in this round. After the two PCR rounds, a DNA fragment originating from two different templates is generated.

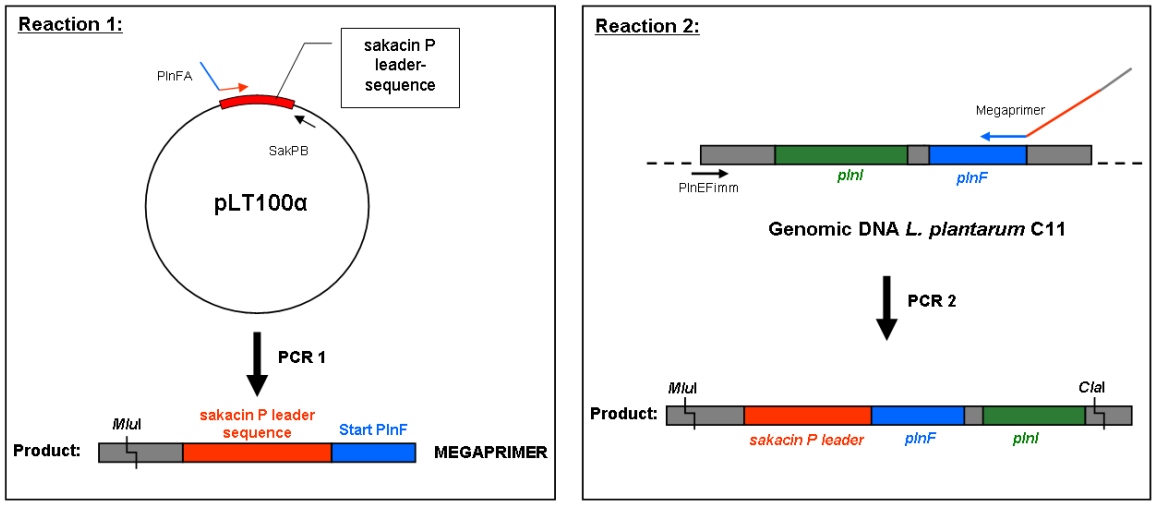

Fig. 3.1: Illustration of the connection of sakacin P leader-sequence to the genes encoding PlnF and PlnI by use of the Megaprimer method. The sakacin P leader-sequence is amplified in a first round of PCR with the primers PlnFA and SakPB. The template is the pLT100α plasmid. PlnFA consists of two parts; one part that is complementary and anneals to pLT100α, and a "tail" consisting of the start of *plnF*, which does not anneal to pLT100α. The PCR leads to the synthesis of a 334 bp megaprimer. In the second round of PCR, genomic DNA from *L. plantarum* C11 is used as template. The Megaprimer anneals to the start of the *plnF*-gene. The other primer, PlnEFimm, anneals to the end of the *plnI*-gene. After the second round of PCR, the final product is a 1437 bp fragment consisting of the sakacin P leader-sequence, and the genes encoding PlnF and PlnI.

Reaction Conditions

When the *Pfu* DNA polymerase was used in the first round of PCR, the reaction conditions were as suggested by the *Pfu* DNA polymerase protocol (Fermentas), and are listed below:

1-5 µl DNA template (5-50 ng)
1.25 µl 20 µM SakPB
1.25 µl 20 µM PlnFA
5 µl 10X *Pfu* buffer without $MgSO_4$
6 µl 3 mM $MgSO_4$
2 µl 5 mM dNTP (1.25 mM each)
dH_2O to a final volume of 50 µl
1 µl 2.5 U/µl *Pfu* DNA Polymerase

Taq DNA polymerase (Fermentas) was used in the second round of PCR to create a final PCR product with 3' A overhangs. This was done in order to sub-clone the final PCR product into a pGEM® T-Easy Vector, by the use of TA-cloning, for restriction digestion. The reason it was decided to use TA-cloning in this project will be explained more thoroughly in the results-section.

The reaction mix for *Taq* DNA polymerase was the same as for *Pfu* polymerase, but with a few alterations, these being:

1-5 µl DNA template (5-50 ng)
1.25 µl megaprimer (50-200 ng)
1.25 µl 20 µM PlnEFimm
5 µl 10X *Taq* Polymerase buffer
4 µl 25 mM $MgCl_2$
2 µl 5 mM dNTP (1.25 mM each)
dH_2O to a final volume of 50 µl
0.5 µl 5 U/µl *Taq* DNA Polymerase

After the initial "hot-start", the DNA polymerase was added to the mix. This preceding step is necessary for inactivation of proteases and denaturation of the template DNA strands.

The PCR was carried out using a PTC-200 Peltier Thermal cycler (MJ research), with the following program (Table 3.1):

Table 3.1: Parameters for the PCR-program used in construction of the expression vector containing the genes coding for PlnF and PlnI.

Segment	Number of cycles	Temperature		Time
1	1	95 ºC	Hot-start	7 min
2	30	95 ºC	Denaturation	45 sec
		50 ºC	Annealing	45 sec
		72 ºC	Elongation	2 min per 1 kb for *Pfu* 1 min per 2 kb for *Taq*
3	1	72 ºC	Final Elongation	10 min

3.1.3 QuikChange® Site-Directed Mutagenesis

General Information

QuikChange® Site-Directed Mutagenesis is a four-step procedure (Fig. 3.2), and is used for introduction of point mutations, switching of amino acids, and deletion or insertion of amino acids residues [80]. The method uses a double stranded DNA vector and two oligonucleotide primers containing the desired mutation. The primers are complementary to each other and to the double stranded template DNA. After denaturation of the vector DNA, the mutagenic primers anneal complementary to opposite strands of the vector. Elongation by *Pfu* DNA polymerase results in a mutated plasmid with staggered nicks. After the thermal cycling, the PCR product is treated with *Dpn*I endonuclease. *Dpn*I has the target sequence 5'-$G^{m6}ATC$-3', and is used to digest hemimethylated and methylated parental DNA, leaving the non-methylated, newly synthesized DNA intact for further use. After *Dpn*I treatment, the plasmids are transformed into competent *E. coli* DH5α, where the cells DNA repair machinery seals the nick.

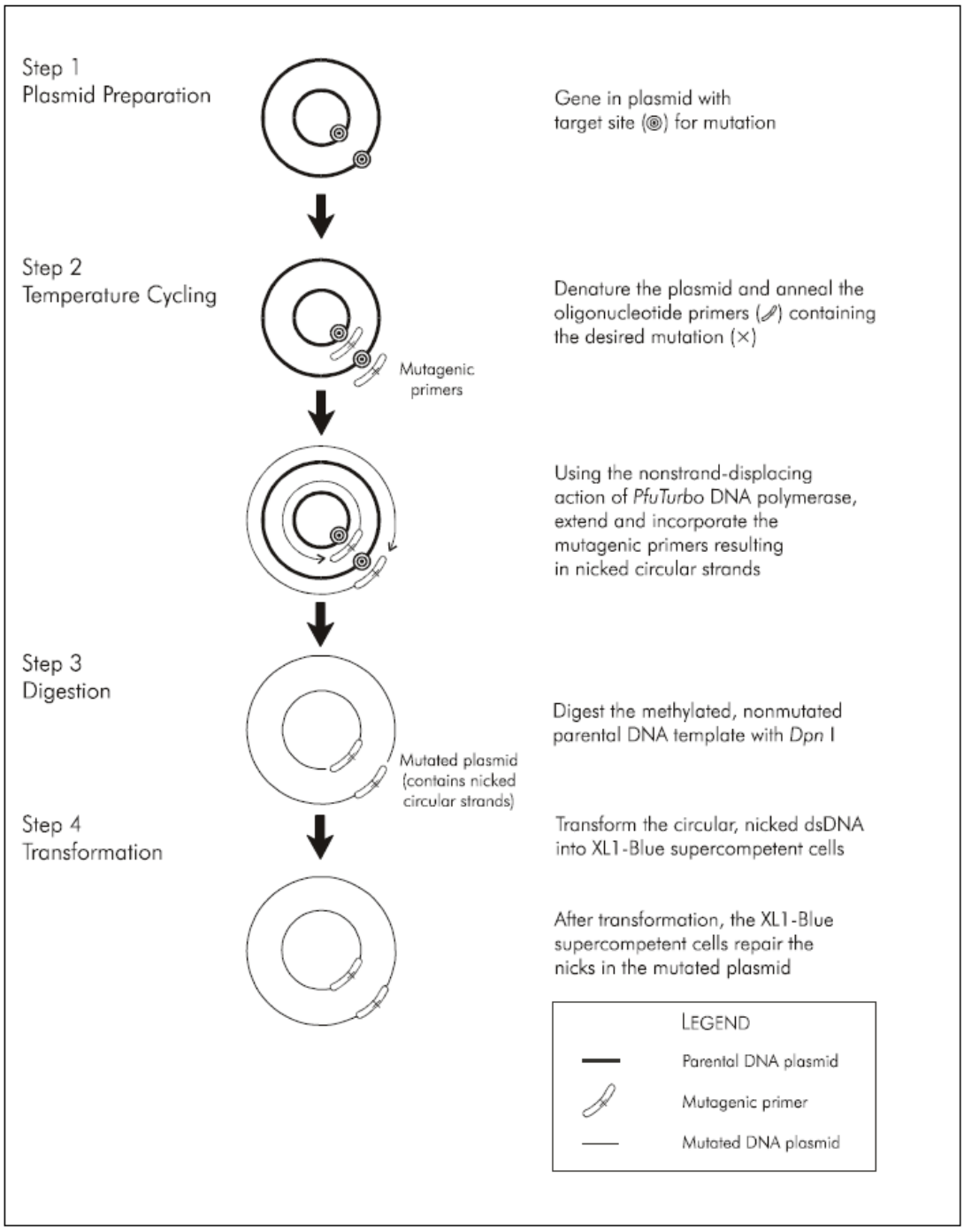

Fig. 3.2: An illustration of the four steps of the QuikChange® Site-Directed Mutagenesis protocol. In step one the plasmid is isolated for the mutagenesis reaction. Step two involves the mutagenic PCR. The third step is digestion of the template with *Dpn*I, leaving only the mutated PCR products. The final step is transformation of the mutated plasmid. Figure from http://www.stratagene.com/manuals/200516.pdf (25.05.2009)

Reaction Conditions

The sequences of the primers used to introduce the desired mutation into *plnF* are listed in section 6.3.2.

All the work was carried out as suggested by the QuikChange® Site-Directed Mutagenesis protocol, but with small alterations to the reaction mix, which was as follows:

1-5 µl DNA template (5-50 ng)
1.25 µl forward primer (125 ng)
1.25 µl reverse primer (125 ng)
5 µl 10X *Pfu* buffer without $MgSO_4$
6 µl 3 mM $MgSO_4$
2 µl 5 mM dNTP (1.25 mM each)
dH_2O to a final volume of 50 µl
1 µl 2.5 U/µl *Pfu* DNA polymerase

The mutagenic PCR reactions were performed using a PTC-200 Peltier Thermal cycler (MJ research), with the following program (Table 3.2):

Table 3.2: PCR parameters used for introducing point mutations.

Segment	Number of cycles	Temperature		Time
1	1	95 ºC	Hot-start	7 min
2	16	95 ºC	Denaturation	45 sec
		50 ºC	Annealing	45 sec
		72 ºC	Elongation	11 min
3	1	72 ºC	Final Elongation	10 min

After the PCR, 1 µl of the 10 U/µl restriction enzyme *Dpn*I (Fermentas) was added to the reaction mix, and incubated for 1 hr at 37 °C.

3.1.4 Plasmid and DNA Isolation

Plasmids were isolated using the Nucleospin® Plasmid Kit from Machery-Nagel, according to the standard protocol. An additional step was added for plasmid isolation from Gram positive lactic acid bacteria. After resuspension of the cell pellet in the resuspension buffer A1, 15 µl 40 mg/ml lysozyme was added to the solution, and incubated at 37 ºC for 10-15 min. The lysozyme degrades the cell walls in the Gram positive bacteria.

Genomic DNA from *L. plantarum* C11 was isolated using the DNeasy® Tissue Kit (QIAGEN), according to the standard protocol for Gram positive bacteria.

3.1.5 Agarose Gel Electrophoresis

This method is used to separate DNA fragments based on their size [79]. The purpose for this may be to purify PCR-products and restriction enzyme digests, and to control the size of PCR-products and plasmids. Agarose is a polysaccharide, which after boiling with Tris-actetat-EDTA (TAE) buffer and subsequent cooling forms a porous matrix. The density of the matrix can be varied by using different concentrations of agarose. The DNA samples are added to wells in the gel. By applying an electrical field to the gel, the DNA, which is negatively charged at neutral pH, will migrate toward the positively charged catode. Ethidium bromide is added to the agarose before it forms a gel. The ethidium bromide is a DNA intercalating agent, which fluoresces orange when exposed to ultraviolet light.

In this project, agarose (Sigma Aldrich) was added to 50 ml 1X TAE buffer (see appendix section 5.5) to a concentration of 1.0%, and sizing was done using either the 100 bp DNA Ladder Plus or the 1 kb DNA Ladder (Fermentas) as a standard, depending on the size of the DNA fragments to be separated. A Hoefer HE33 Mini Horizontal Submarine unit (Pharmacia Biotech-Amersham) was used together with an EPS600 (Pharmacia Biotech-Amersham) as a source of electricity. The electrophoresis conditions used were voltage of 70 V, electric current of 80 mA, and the gel was run for approximately 1 hr.

For purification of the DNA bands from the TAE agarose gel, the "GFX™ PCR DNA and Gel Band Purification Kit" (GE Healthcare) was used. In the final elution step dH_2O was used instead of the supplied elution buffer, and an elution volume of 50 µl was used.

3.1.6 Restriction Digestion and Vector Dephosphorylation

Restriction digestion is performed using restriction enzymes. These enzymes are endonucleases isolated from bacteria and archaea that recognize specific DNA sequences, called restriction sites [79]. The sites are between 4 and 8 nucleotides, and most often palindromic. The restriction sites are cleaved by the enzyme, giving rise to either "blunt ends", which is when both strands are cut at the same place, or "sticky ends", which is when the two strands are cut at different positions [79]. When digesting two DNA fragments with the same restriction enzyme, identical cleavage sites at the end of the fragments are produced. If the two ends are "sticky" they can subsequently be ligated together by baseparing. Cut DNA fragments with "blunt ends" will ligate unspecifically with other "blunt end" DNA fragments. Restriction enzymes are grouped into three different types, but only type II is suitable for molecular cloning [81]. Under the appropriate conditions, type II restriction enzymes are highly specific and will always cut at the same position within the restriction site. Type I and III, on the other hand, are not sequence specific enough to be used for cloning.

Different enzymes function optimally in the buffer and buffer concentration specified by the producer. If a double digest is to be performed, a buffer which both enzymes function in should be used. The total amount of restriction enzyme in a digest should never be over 10% (v/v), since glycerol can inhibit the enzyme activity.

Initially, the restriction enzymes *Cla*I and *Mlu*I (Promega) were used in this project for uni-directional cloning, but after experiencing some trouble with digestion with *Cla*I, the restriction enzyme *Xba*I (Fermentas) was used instead. This will be discussed more thoroughly in the results-section.

Reaction Conditions

Restriction digests were carried out as suggested by the protocols given by Promega and Fermentas, but with slight alterations, this being the amount of plasmid or PCR product being used. *Cla*I (Promega) has 100% activity in buffer C, while *Mlu*I (Promega) has 100% activity in buffer D. *Xba*I (Fermentas) functions most optimally in 1X Buffer Tango™.

35-40 µl 50-300 ng/µl plasmid or PCR product

5 µl enzyme buffer (as specified by the producer)

0.5 µl 10 mg/ml acetylated BSA (for *Cla*I and *Mlu*I digestion)

dH_2O up to a total volume of 50 µl

1.5 µl of each 10 U/µl restriction enzyme

The digestion mix was incubated for 2 hrs at 37 ºC.

The restriction digested vector was dephosphorylated at its 5' ends using Calf intestinal alkaline phosphatase (CIAP) (Fermentas). The removal of 5' end phosphate-groups inhibits re-circularization and re-ligation of the vector. After addition of 1 µl of 1 U/µl CIAP, the digestion mix was incubated at 37 °C for 30 min. The enzyme was inactivated at 85 °C for 15 min.

3.1.7 Ligation

Ligation of DNA fragments into vectors is performed by the use of DNA ligases. These enzymes have the ability to link together two double stranded DNA fragments with complementary "sticky ends" or "blunt ends", by catalyzing the formation of covalent 5'- 3' phosphodiester-bonds [79]. ATP is required as a co-factor for the reaction.

Reaction Conditions

The ligation reactions were performed according to the T4 DNA Ligase protocol from Fermentas [82]. Two ligation reactions were performed in this project. First, after unsuccessfully trying to ligate the digested PCR products into the plasmid pLPV111, the PCR product was ligated into the pGEM® T-Easy Vector by TA-cloning (Fig. 3.5). After digestion of the pGEM® T-Easy Vector containing the PCR product, the insert was ligated into the plasmid pLPV111, which was cut with the same restriction enzymes (Fig. 3.6) The concentration of both vector and insert was measured with a Nanodrop ND-1000, and ligations in 3:1, 1:1 and 1:3 insert to plasmid ratios were performed.

5-10 µl (50-400 ng) linear plasmid DNA
Insert DNA in 3:1, 1:1 and 1:3 ratios to plasmid DNA
2 µl 10X buffer for T4 DNA Ligase
0.5 µl 10 mM ATP
dH_2O up to 20 µl total volum
0.5 µl 5 U/µl T4 DNA Ligase

The ligation reactions were incubated for 2 hrs at room temperature.

The ligated product was an *E. coli-Lactobacillus* shuttle-vector, termed pPlnF100.

3.1.8 DNA Sequencing

The newly constructed plasmids were submitted to a sequencing service for sequencing by the chain-termination method of Sanger [83], in order to verify that the DNA sequence was correct.
The sequencing in this project was done by the ABI-lab at CEES, using an ABI 3730 High-throughput capillary electrophoresis sequencer.

3.1.9 Plasmids

pSAK20

pSAK20 (Fig. 3.4) is a 11.2 kb pVS2-derived plasmid [84], and contains the genes necessary for transcription of the sakacin A structural gene, and the genes coding for the proteins needed to export and process pre-sakacin A. The genes coding for the structural gene of sakacin A and its immunity protein has been removed. The plasmid also contains a cassette for chloramphenicol-resistance (*cat*) [85].

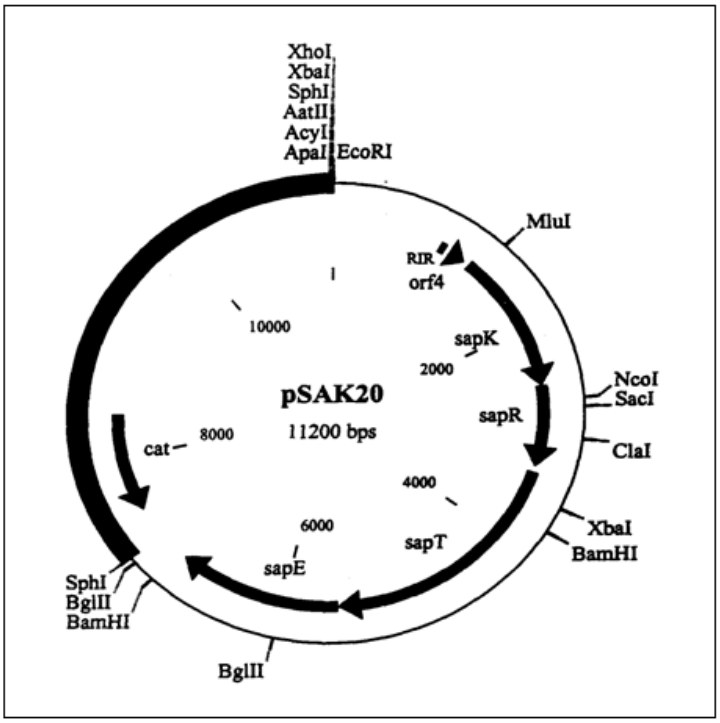

Fig. 3.4: Plasmid chart of pSAK20. *cat* is a marker for chloramphenicol-resistance. *orf4* encodes the sakacin A peptide pheromone, *sapK* encodes a histidine kinase, and *sapR* encodes a response regulator, together constituting a three-component regulatory system [30]. *sapT* encodes an ABC transporter, and *sapE* encodes an accessory factor. Figure from Axelsson *et al.* [84].

pGEM® T-Easy Vector

pGEM® T-Easy Vector (Fig. 3.5) is a 3015 bp plasmid from Promega, which is prepared by EcoRV restriction digest and addition of 3' terminal thymidine to both ends [86]. This allows for higher efficiency of ligation of PCR products generated by DNA polymerases which add 3'-end single deoxyadenosines. The plasmid contains a marker for ampicillin-resistance, and a l*acZ*-gene with a multiple cloning site (MCS) within the coding region of the β-galactosidase enzyme. This allows for blue-white selection.

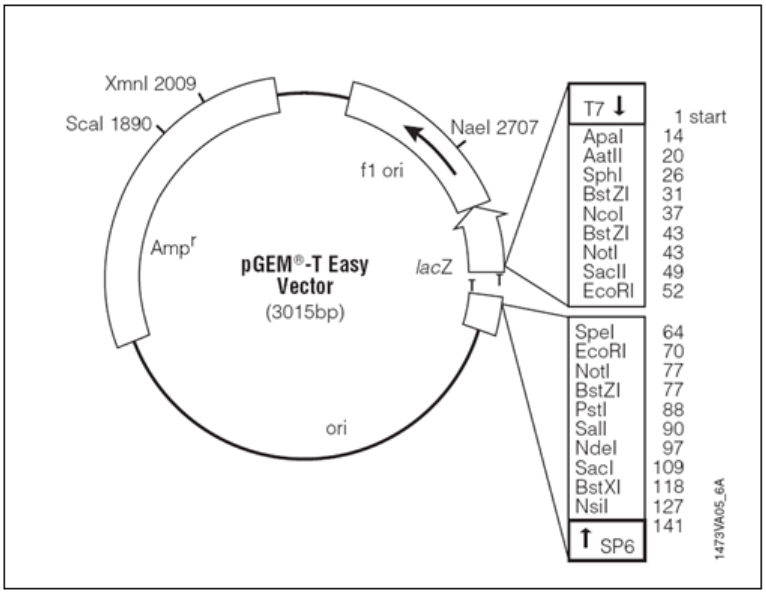

Fig. 3.5: Plasmid chart of the pGEM® T-Easy Vector. *Amp*^{r} is a marker for ampicillin resistance. The gene coding for the β-galactosidase enzyme (*lacZ*) contains a polylinker region, allowing for blue-white selection. Figure from Promega [86].

pLPV111

pLPV111 (Fig 3.6) is a pGEM-7Zf(+) (Promega) derivative plasmid. It is an *E. coli-Lactobacillus* shuttle-vector, with a p256 replicon for replication in *Lactobacillus*, and a replication origo (ori) for replication in *E. coli.* The ampicillin-resistance gene has been replaced by the erythromycin-resistance gene (*ermB*) from the *Lactobacillus reuteri*-plasmid pLUL631. The lacZ-polylinker region comes from pGEM-7Zf(+).

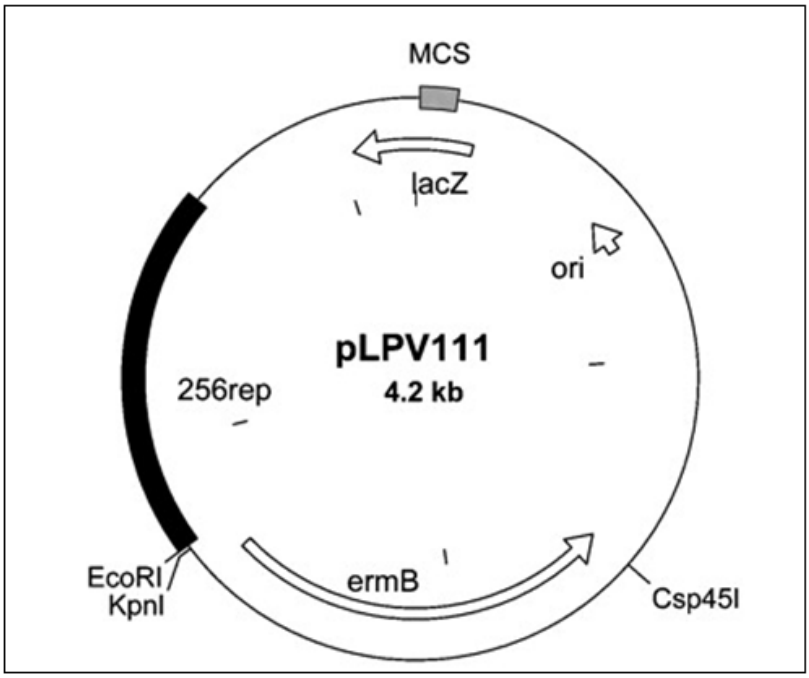

Fig. 3.6: Plasmid chart of the pLPV111 shuttle vector. The vector contains a marker for erythromycin restistance (*ermB*), and the gene encoding the β-galactosidase enzyme *(lacZ*), which enables blue-white selection of colonies. Figure from Sørvig *et al.* [87].

pLT100α

pLT100α is a pLPV111 derived plasmid constructed for expression of LcnG-α and its cognate immunity protein, LcnGimm, and with the sakacin P leader-sequence (Fig. 3.7). The plasmid was constructed by ligating the sakacin P leader-sequence and the genes encoding LcnG-α and the lactococcin G immunity protein into the shuttle-vector pLPV111. The plasmid contains the gene encoding erythromycin-resistance (*ermB*), and a replication origo for replication in both *E. coli* and *Lactobacillus,* ori and 256rep, respectively.

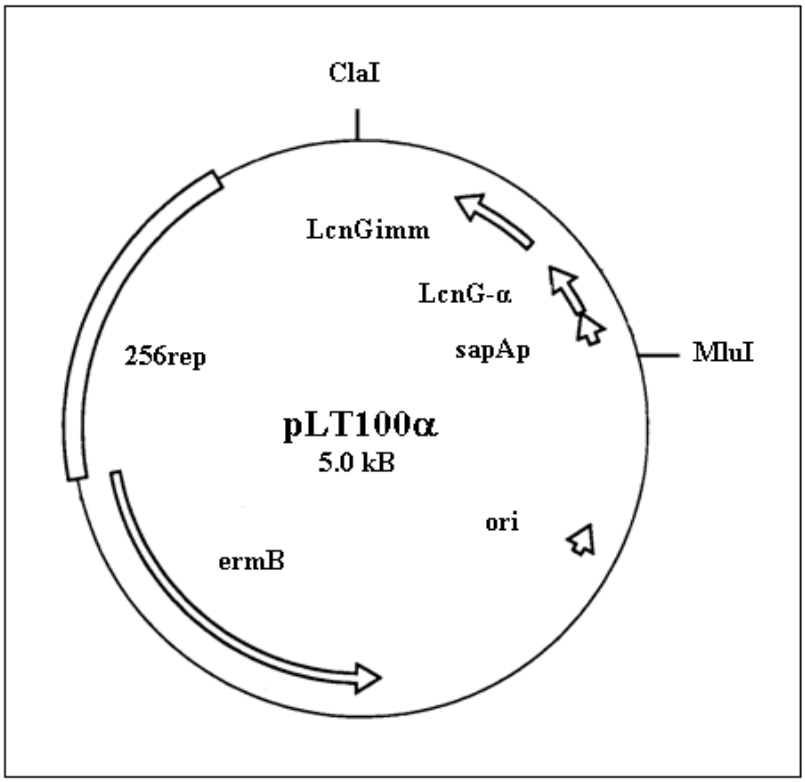

Fig. 3.7: Plasmid chart of the pLPV111-derived plasmid pLT100α. The plasmid contains a marker for erythromycin-resistance (*ermB*), and the gene coding for LcnG-α, one of the peptides constituting the two-peptide bacteriocin Lactococcin G. In addition it contains the gene encoding the lactococcin G immunity protein. Figure adapted from Oppegård [88].

3.1.10 Bacteriocin Expression

3.1.10.1 Heterologous Bacteriocin Expression in *Lactobacillus sake*

A system for heterologous expression of LAB bacteriocins class II has been described by Axelsson *et al.* [84]. This system is based on the expression system of the class IIa pediocin-like bacteriocin sakacin A. Two divergently described operons are needed for sakacin A production and transport out of the cell. One operon contains the sakacin A structural gene (*sapA*) and the structural gene encoding the immunity protein (*saiA*). The other operon

contains three genes encoding a three-component regulatory system necessary for transcriptional activation of the sakacin A structural gene and the immunity protein gene; *orf4* encodes a peptide pheromone, *sapK* encodes a histidine kinase, and *sapR*, which encodes a response regulator. This operon also contains the gene coding for the ABC-transporter, *sapT,* which is necessary for transport of sakacin A out of the cell, and an accessory factor, *sapE.* It has been shown that the two operons can be separated and placed on different plasmids, and still have transcription activation of the sakacin A promoter *in trans* [85; 84]. By replacing the sakacin A structural gene and immunity protein gene with the gene encoding the bacteriocin of interest and its cognate immunity gene, and transforming them into a bacteriocin-deficient strain, it is possible to get an equal or higher production of the bacteriocin than in the wild type strains [84].

The heterologous expression system was used in this project, with the plasmid pSAK20 (Fig. 3.4) containing the *orf4sapKRTE* operon, and also a chloramphenicol-resistance cassette (*cat*). The other plasmid is a pLPV111-derivate, pPlnF100 (Fig. 3.3), containing the second operon, but with the sakacin A structural gene (*sapA*) and the gene encoding its cognate immunity protein (*saiA*) having been replaced by the PlnF structural gene (*plnF)* and the gene coding for its immunity protein (*plnI*). Both genes constitute an operon, and are placed under control of the sakacin A promoter. The *plnF*-gene encodes a precursor peptide with the N-terminal sakacin P leader sequence, which is cleaved off during transport out of the cell. It has been experimentally shown that the sakacin A leader-sequence and the sakacin P leader-sequences are equally efficiently recognized and processed by the sakacin A ABC-transporter [84]. pLT100α contains the sakacin P leader-sequence, and was used as template in this project for construction of pPlnF100. Several bacteriocins with sakacin P leader-sequences have previously been successfully produced and purified from cells expressing only the sakacin A ABC-transporter, including wild type and mutant lactococcin G and enterocin 1071 peptides [88]. The plasmid, pPlnF100, also contains a marker gene coding for erythromycin-restistance (*ermB).* pPlnF100 is a shuttle vector that can be replicated in both *E. coli* and *Lactobacillus.* In this project, the plasmids were transformed into the bacteriocin-deficient strain *L. sake* Lb790.

3.1.10.2 Bacterial Strains and Growth Conditions

E. coli DH5α was used for plasmid cloning and isolation. The cells were grown in LB medium at 37 °C at 225-250 rpm. Erythromycin was added to a final concentration of 150 μg/ml for selection (pPlnF100 contains the gene encoding erythromycin resistance). For selection of *E. coli* DH5α containing the pGEM® T-Easy Vector, 100 μg/ml ampicillin was added to the medium. The medium was solidified by adding 1.5% agar (w/v) for cell growth on plates. *E. coli* DH5α was grown on LB-plates containing 150 μg/ml erythromycin for selection pPlnF100, or 100 μg/ml ampicillin for selection of pGEM® T-Easy Vector, and incubated at 37 °C overnight.

Lactobacillus sake Lb790/pSAK20 was used for bacteriocin production, and was grown in MRS medium at 30 °C without agitation. Both chloramphenicol and erythromycin were added to a final concentration of 10 μg/ml for selection (pPlnF100 contains the erythromycin resistance gene, and pSAK20 contains the chloramphenicol resistance cassette).
For growth of *L. sake* Lb790/pSAK20/pPlnF100, MRS-plates containing 10 μg/ml chloramphenicol and 10 μg/ml erythromycin were used for selection of pSAK20 and pPlnF100, respectively. The cells were incubated at 30 °C for 2 to 3 days.
Lactobacillus plantarum C11 genomic DNA was used as a template in the second round of megaprimer PCR for amplification of the genes encoding PlnF and its immunity protein. The cells were grown in MRS medium at 30 °C without agitation.
Lactobacillus viridiscens NCDO 1655 (LMGT2314) was used as an indicator strain, and the cells were incubated in MRS and grown overnight at 30 °C without agitation.

3.1.11 Preparation of Competent Cells and Cell Transformation

3.1.11.1 Preparation of Chemical Competent *E. coli* DH5α-cells

A 0.5 ml *E. coli* DH5α overnight culture was used to inoculate 25 ml of fresh LB-medium, and incubated at 37 °C (225-250 rpm) until the optical density at 600 nm reached 0.3. The cells were then put on ice to stop growth, and immediately harvested by centrifugation at 5000 rpm and 4 °C for 10 min in a JA-10 rotor (Beckman). After discarding the supernatant, the cell pellet was resuspended in 5 ml 0.1 M $CaCl_2$ (4 °C). After cooling the

cells on ice for 5 min, they were again centrifugated at 5000 rpm and 4 °C for 10 min, and the supernatant was discarded. The cell pellet was resuspended in 1 ml 0.1 M $CaCl_2$ (4 °C) containing 10% (v/v) glycerol. After 15 min cooling on ice, the competent cells were divided into 100 µl aliquots and stored at –80 °C.

3.1.11.2 Transformation of Competent *E. coli* DH5α-cells

The cells were transformed with pPlnF100 and the mutated plasmids according to the QuikChange® Site-Directed Mutagenesis protocol [80]. After thawing the cells on ice, 1 µl *Dpn*I-treated DNA was added to the 50 µl cell-aliquots. After 30 min incubation on ice, the transformation reaction was exposed to a 90 sec heat pulse (42 °C) and 2 min incubation on ice. The cells were then transferred to 0.5 ml pre-heated (42 °C) LB-medium, and cultured with agitation (225-250 rpm) at 37 °C for 1 hr. Cell volumes of 100-200 µl were then plated onto LB-plates containing 100 µg/ml ampicillin when selecting for cells containing the pGEM® T-Easy Vector (Fig. 3.5), and 150 µg/ml erythromycin when selecting for pPlnF100 plasmids (Fig. 3.6).

3.1.11.3 Preparation of Electrocompetent *L. sake* Lb790/pSAK20

The cells were prepared basically according to the procedure suggested by Aukrust *et al.* [89]:

A 25 ml preculture of *L. sake* Lb790/pSAK20 was used to inoculate 100 ml fresh MRS medium containing 2.0-2.5% (w/v) glycine, to an OD_{600} of 0.1. Chloramphenicol was added to a final concentration of 10 µg/ml for selective pressure. The culture was incubated at 30 °C until an OD_{600} of 0.5-0.6 was reached, and the cells were then harvested by centrifugation at 5000 rpm at 4 °C for 7 min. The cells were resuspended in 100 ml ice cold 1 mM $MgCl_2$, and pelleted by centrifugation at 5000 rpm at 4 °C for 7 min. The supernatant was discarded, and the pellet was resuspended in 100 ml 30% (w/v) polyethylene glycol (PEG). The cells were once more pelleted by centrifugation, this time at 7000 rpm at 4 °C for 10 min. The supernatant was discarded, and the cells were resuspended in 1 ml 30% (w/v) PEG, and used to make 50 µl aliquots of competent cells.

3.1.11.4 Transformation of Electrocompetent *L. sake* Lb790/pSAK20

After thawing the cells on ice, 2 µl of plasmids were added to 50 µl of competent *L. sake* Lb790/pSAK20 cells. The mix was then transferred to an ice cold electroporation cuvet (2 mm electrode gap). The electric pulse was delivered with voltage of 1.5 kV, capacitance of 25 µF and resistance of 400 Ω. The time constant varied between 9.0 and 9.5 ms.
Directly after electroporation, 1 ml of MRSSM (see appendix) was added to the cells, and transferred to a 1.5 ml microcentrifuge tube. The cells were incubated for 2 hrs at 30 °C. Then, 100-200 µl of the transformation mix were spread on MRS agar plates with 10 µg/ml of chloramphenicol and 10 µg/ml erythromycin. The MRS plates were incubated at 30 °C for 2-3 days.

3.2 Bacteriocin Isolation and Purification

3.2.1 Chromatography

Many procedures for isolation and purification of LAB bacteriocins exist, and most of them are based on the use of column chromatography or ammonium sulphate precipitation or combinations of these [90].
Proteins are often isolated and purified by the use of column chromatography, as described in Sheehan's "Physical Biochemistry: Principles and Applications" [91]. The proteins are in a liquid mobile phase, which is passed through the chromatography column. The column contains a matrix of porous material, and is referred to as the stationary phase. Since different proteins have different properties, such as size, charge, hydrophobicity, and isoelectric point, the proteins can be separated on a column based on these characteristics. As the mixture passes through the stationary phase, the different proteins get retarded in the matrix, and will elute at different times.

A simple two-step procedure for purification of pediocin-like bacteriocins and other cationic peptides has been developed by Uteng *et al.* [92].
This procedure is based on two chromatography steps; the first being ion exchange chromatography, which separates peptides based on their net charge. The other step is reverse phase chromatography, which separates peptides based on their hydrophobic properties.

Isolation and purification of PlnF was performed basically according to the Uteng *et al.* procedure [92].

3.2.1.1 Ion Exchange Chromatography

An ion exchange chromatography column separates proteins based on their net charge, which can either be positive or negative. A column matrix with positively charged ligands will bind negatively charged peptides, and is called an anion exchange column. A column matrix with negatively charged ligands will bind positively charged proteins, and is called a cation exchange column [91]. Since different proteins also have different net isoelectric points, it is possible to manipulate a proteins binding ability to the column by varying the pH. Proteins with the same charge as the column or no net charge will not bind. By increasing the salt concentration in the solvent that passes through the column, the proteins that bind to the column will be eluted.

Since both PlnE and PlnF are positively charged at neutral pH, the cation exchanger SP-Sepharose Fastflow from GE Healthcare was used. The SP-Sepharose Fastflow matrix has negatively charged sulfopropyl groups.

Parameters

A 10 ml overnight culture of bacteriocin-producing *L. sake* Lb790 cells grown at 30 ºC was added to 1 liter MRS medium, and grown to stationary phase. The cells were pelleted by 6000 rpm for 10 min in a JA-10 rotor (Beckman). The supernatant was then applied to a cation exchange column, which contained 6 ml SP-Sepharose Fastflow (GE Healthcare) equilibrated with a 20 mM phosphate buffer (buffer A, pH~5.7). The column was then washed with 100 ml buffer A to remove any unspecifically bound material. The bacteriocin was then eluted with 40 ml of a 20 mM phosphate buffer with 1 M NaCl (buffer B). Finally, 5 ml 2-propanol was added to the 40 ml bacteriocin fraction, and the mixture was sterile-filtrated through a 0.2 μm filter.

3.2.1.2 Reverse Phase Chromatography

As well as their characteristic surface charge, peptides also differ in respect to their net hydrophobicity. Most proteins will possess regions that are more or less hydrophobic. These regions will depend on the primary structure of the proteins.

In reverse phase chromatography, according to Sheehan [91], hydrophobic groups have been immobilised on the stationary phase, while the proteins are added to the mobile phase, which is a polar solvent. When the mobile phase is added to the column, the proteins will bind to the stationary phase, due to hydrophobic interactions. By decreasing the polarity of the solvent, the peptides bound to the stationary phase will be eluted. Polar peptides will elute first, because of weak hydrophobic interactions. Non-polar peptides with stronger hydrophobic interactions will elute later.

Parameters

The reverse phase column used in this project was a Resource RPC column (3 ml) from GE Healthcare, consisting of polystyrene/divinylbenzene. It was connected to the Äkta system (GE Healthcare). The Äkta chromatography system was originally developed for protein purification, and is now widely used for preparative purposes of bioactive proteins. The system was used in this project for bacteriocin purification.

Before running the sample the column was equilibrated with 5% 2-propanol containing 0.1% trifluoroacetic acid (TFA). The eluted bacteriocins from the cation exchange column were applied to the Resource RPC column on the Äkta system (GE Healthcare). The bacteriocin was eluted with a 15-60% linear gradient of 2-propanol with 0.1% TFA. Fractionation of eluate was based on UV absorption at 214 nm and 280 nm. The flowrate was 2.00 ml/min.

3.3 Quantification Methods

3.3.1 Bacteriocin Activity Assay

The bacteriocin activity of the unmodified PlnF together with synthetic PlnE was quantified using an 8 x 12 well ELISA microtiterplate-based system. First, 50 µl of MRS-medium were added to all the wells. A total volume of 50 µl of PlnF (45 µl) and synthetic PlnE (5 µl) was then added to each well in the first column, so the total volume was 100 µl in all 8 wells in the first column. The MRS and bacteriocins were mixed by pipetting, and then 50 µl was transferred over to the adjacent wells in the second column. The content in these wells were mixed again by pipetting, and 50 µl was transferred to the adjacent wells in the third column. This procedure was continued throughout the plate. After this serial-dilution, each column contained half the amount of bacteriocin as the wells in the previous column. The indicator strain used in this project was *Lactobacillus viridescens* NCDO 1655 (LMGT2314). A stationary culture of *L. viridescens* NCDO 1655 was diluted 1:50-1:200 before being added to the microtiter plates, 150 µl in each well. The plates were incubated at 30 °C for 4-14 hours (depending on the dilution of the indicator strain). After incubation, growth inhibition of the *L. viridescens* NCDO 1655 was measured spectrophotometrically at 600 nm with a microtiter plate reader (MR700 Microplate Reader, Dynatech). In those wells with a high bacteriocin-concentration the indicator-cells did not grow.

3.3.2 MALDI-TOF Mass Spectrometry

MALDI-TOF is a mass spectrometry method (Fig. 3.8), which enables accurate molecular weight determination of peptides and proteins in mixtures, and uses the principles described in "Physical Biochemistry: Principles and Applications" [91].

MALDI stands for matrix-assisted laser desorption ionisation, and is a method used for ionisation. This method is based on bombarding the sample with pulses of high-energy UV radiation. The sample is held co-crystallized in a matrix, which has a lower concentration of the sample molecules than the matrix molecules. The purpose of the matrix is to separate the sample molecules, absorb the laser radiation which may otherwise break down the sample structure, and initiate ionisation of the sample. When the sample is exposed to the high-energy laser, the matrix and sample molecules are vaporised to gas-phase in a jet. In gas-phase, the

sample molecules get ionised by the excited matrix molecules, producing dominantly single-charged ions, but also some multi-charged ions.

MALDI uses a TOF (time-of-flight) analyser, which measures the time required for each ion to travel to the detector. This analyser accelerates the ions down a tube called the drift tube, which is free of magnetic field. The time required for each ion to travel through the drift tube and arrive at the detector is measured, and an *m/z* (mass to charge) value is calculated.

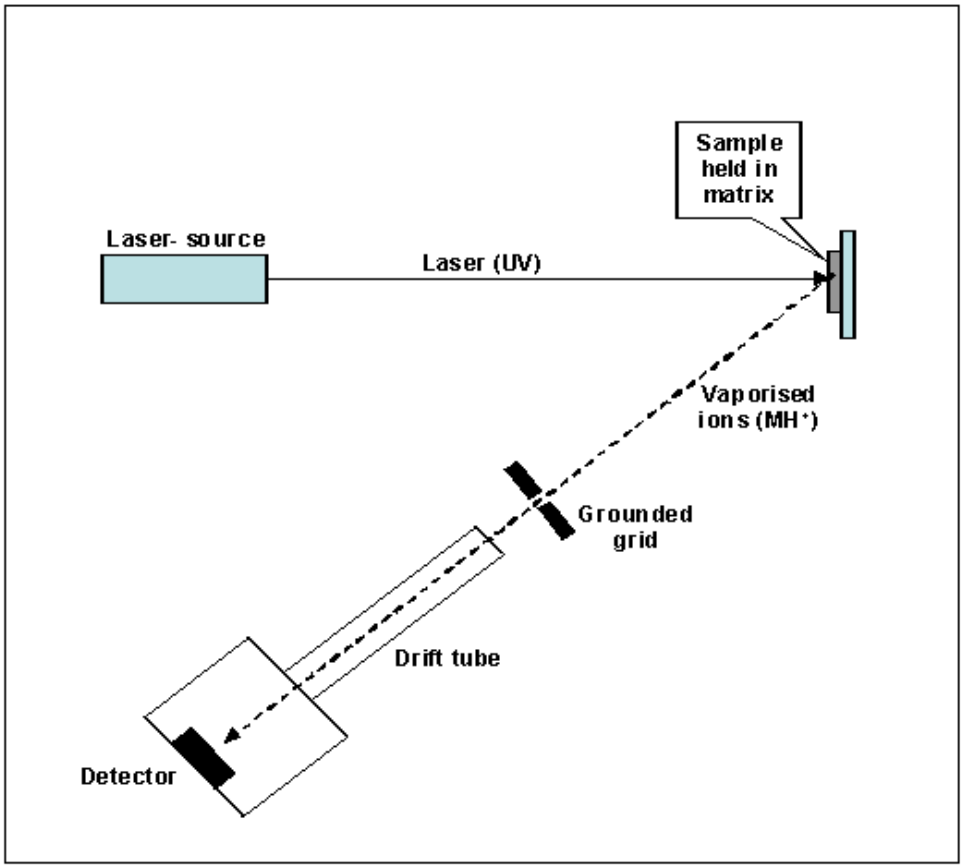

Fig. 3.8: Simplified scheme of MALDI-TOF mass spectrometry. The sample to be analysed is held co-crystallized in the matrix, which is bombarded with high-energy UV laser. This causes the sample-molecules and matrix-molecules to vaporise. The sample-molecules get ionised by the matrix-molecules, and sample-ions are accelerated through the drift tube to the detector, where the time-of-flight is measured, and the m/z-ratio is determined.

The identity of PlnF was verified by determination of the molecular mass using a MALDI-TOF spectrometer. The work was performed by Morten Skaugen at the MS/Proteomics Core Facility at the Department of Chemistry and Biotechnology, Norwegian University of Life Sciences, Ås.

4. Results

4.1 Construction of pPlnF100

A new expression plasmid, pPlnF100, was constructed for production of PlnF, and for easier construction and purification of PlnF-mutants. pPlnF100 is a derivate of the pLPV111 plasmid (section 3.1.9), and was constructed with an upstream sakacin P leader-sequence, for heterologous expression together with the pSAK20 plasmid (section 3.1.9). The gene encoding the plantaricin EF immunity protein, *plnI*, was also introduced into pPlnF100, since it may be necessary for protection of the producer against PlnF. The genes encoding PlnF and PlnI were placed under the control of the sakacin A promoter.

4.1.1 Synthesis of Insert by PCR

Connection of the sakacin P leader-sequence to the DNA sequence coding for PlnF was done using the Megaprimer method (section 3.1.2). The ~350 bp PCR product (containing the sakacin P leader-sequence) from the first round of PCR (Fig. 4.1 A) was purified, and used as a Megaprimer in the second round of PCR, thereby connecting the sakacin P leader-sequence to the genes encoding PlnF and PlnI. The ~1450 bp product from the second round of PCR was likewise run on agarose gel for separation and verification, and purified (Fig. 4.1 B).

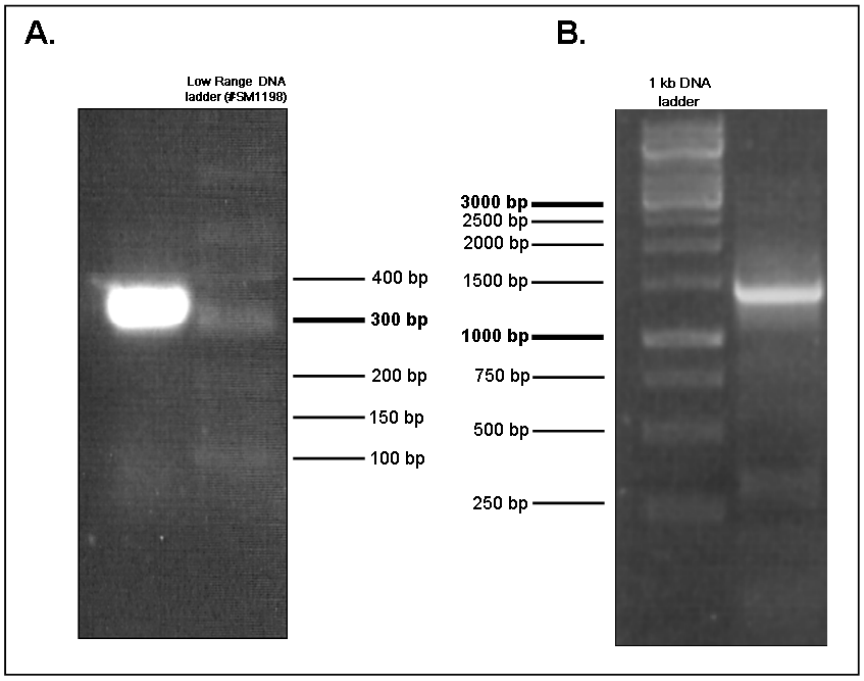

Fig. 4.1: Separation and verification of PCR products by agarose gel electrophoresis. (A) The first round of PCR led to synthesis of a ~350 bp product. This product was subsequently used as a Megaprimer in the second round of PCR, which led to the synthesis of a ~1450 bp product **(B)**.

The purified ~1450 bp PCR product contained the sakacin P leader-sequence, the *plnF*-gene, the *plnI*-gene, and the restriction sites *Cla*I and *Mlu*I, as illustrated in Fig. 4.2.
Sequencing of the PCR product confirmed the correct DNA sequence of the sakacin P leader-sequence, the *plnF*-gene, the *plnI*-gene, and the restriction sites.

Final PCR product

5′-acactttatgcttccggctcgtatgttgtgtggaattgtgagcggataacaatttca
cacaggaaacagctatgaccatgattacgccaagctatttaggtgacactatagaatact
caagctatgcatccaacgcgtgaattcccctgtttaggaatgatttctgtaggcttcaag
aagttatgccacgtgatcaaagaaatcttgttatacttcactcgtacaaaaaataataac
agaggagattcttagtt**atg**gaaaagtttattgaattatctttaaaagaagtaacagcaa
ttacaggtggagttttccatgcctatagcgcgcgtggcgttcggaataattataaaagtg
ctgttgggcctgccgattgggtcattagcgctgtccgaggattcatccacgga**tag**ttca
agccatcaagtttaagcactataagaaagcactcgatttatgactgggcctgcagtgctc
agccttttagtttatatgggggaattttaact**atg**ccattaataaaagtaataatcac
agctatttactacctaatagtgctcttttaatgaatcctttgactgatattatgggaat
aaaagacggacccttcaattcatattaactgaatctattatattaatcgcgataatcat
tctgaataggcgatatgttaagcaacctatccactggctacccgttaatatcatgtcaat
attaaagaagaatagtctgcctttgagcctagcaataatatttctacttattttctttcg
aaaccacatgtatcaatttttaatctcattattactttcgttgattgtagcgataactga
agaatacgcttttcgtggcatgatattcagaacgcttttagcactaaaccttaaaaattt
gccacattgcaggccaccatcgcttcgatgatgactgcttcgcttatttttgcggctatg
catttggtcacctttttatcacagcccgtatggtcagtattgtgtcagttctctatgttat
tgggttaggtattttacttgcagcaatctatttaaaaaccggaagcttactggctgccat
tagtgtccattggttgatagatttttctagtttctattctcaacgtattgacccaactca
atcacccattaacgggccaatggaagctcttctaaaggggctctttctaaatattttatt
cattggcattgctacgtttatcctatcctctaagcattggaagttattgagtatcctaaa
tattgaagataaaatagacgag**taa**tcatccattctgaattcctataaattattgtttgc
agcactacaatttcaattatctctgaagttttgggttacgctaagagctagtcatatcgc
taactaaatttcacgtcttaacgctatgctaatatacttacggatcgattactgtgc-3′

Fig. 4.2: The coding sequence of the final PCR product. The nucleotide sequence of the sakacin P leader-sequence (red) is followed by the nucleotide sequence of PlnF (blue) and PlnI (green). The *Mlu*I restriction site (5'-acgcgt-3') is shown underlined in purple, and the *Cla*I restriction site (5'-atcgat-3') is shown underlined in brown. The start-codon (atg) and stop-codons (tag for *plnF* and taa for *plnI*, respectively) are shown in bold. The sakacin A promoter region is located between the *Mlu*I restriction site and the sakacin P leader-sequence.

4.1.2 Ligation of Insert Into pLPV111

In order to ligate the ~1450 bp PCR product into the shuttle-vector pLPV111, the product was double-digested with the restriction enzymes *Cla*I and *Mlu*I in buffer D (Promega) according to the protocol. The restriction enzyme *Cla*I has 75-100% activity in buffer D, while *Mlu*I has 100% activity in the same buffer (according to the Promega protocol). The restriction site *Mlu*I is 133 bp from the 5' terminal end on the PCR product, while the *Cla*I restriction site is 10 bp from the 3' terminal end, as illustrated in Fig. 4.2, which in theory should result in a 1294 bp product after the double-digestion. The digestion reaction resulted in a ~1300 bp product, which was separated and verified by agarose gel electrophoresis (Fig. 4.3 A), and subsequently purified from the gel.

The pLPV111-derived plasmid pLT100α (Fig. 3.7) was also double-digested with the restriction enzymes *Cla*I and *Mlu*I in buffer D. This digestion reaction resulted in an ~800 bp fragment being excised from pLT100α. The excised fragment was equivalent in size to the sakacin P leader-sequence, the gene encoding LcnG-α, and the gene encoding the LcnG immunity protein, thereby resulting in the ~4200 bp linearized pLPV111 plasmid (Fig. 4.3 B).

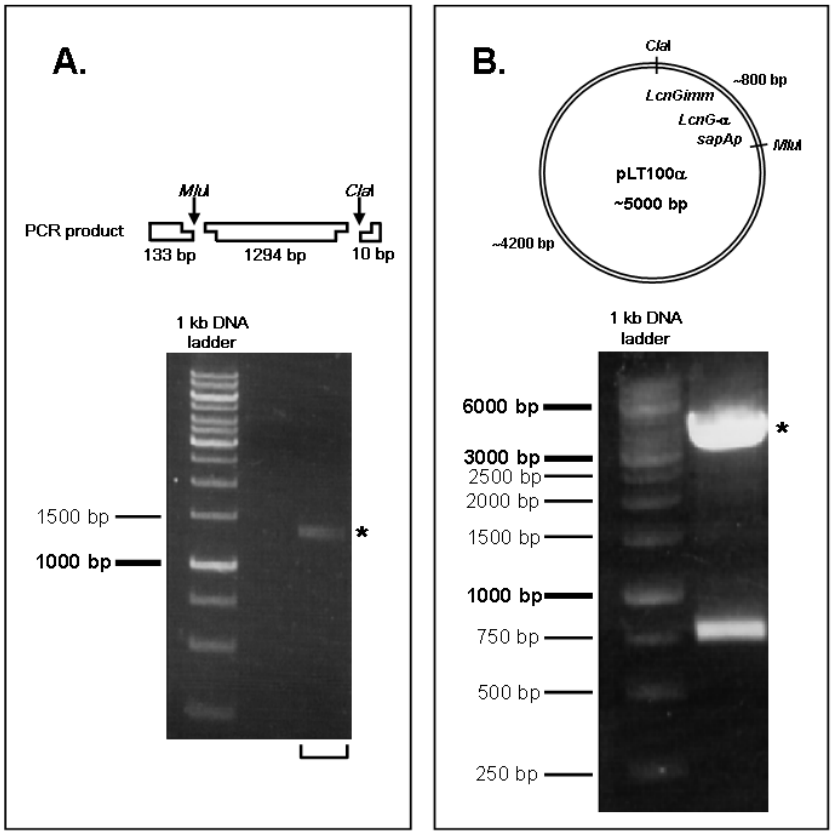

Fig. 4.3: Digestion reaction of the 1437 bp PCR product and the pLT100α plasmid with restriction enzymes *Cla*I and *Mlu*I. **(A)** Restriction digest of the final PCR products led to a ~1300 bp product. **(B)** Restriction digest of pLT100α led to an ~800 bp fragment being excised, resulting in the ~4200 bp linearized plasmid pLPV111.

Ligation reactions in 3:1, 1:1 and 1:3 ratios of insert to pLPV111 were performed, according to the T4 DNA Ligase (Fermentas) protocol (section 3.1.7). A volume of 1 µl of each of the three reaction mixtures was transformed into *E. coli* DH5α according to the QuikChange® Site-Directed Mutagenesis protocol [80], and plated onto LB plates with erythromycin for selection of successfully transformed cells. The transformation resulted in several successful transformants, and single colonies were used to inoculate fresh LB medium containing erythromycin. The cultures were grown overnight before obtaining plasmids. Plasmids from each culture were sent to sequencing for verification of the pPlnF100 construct. Sequencing results showed that the linearized pLPV111 plasmid had re-ligated. pLPV111 should not be able re-ligate, because its ends had been cleaved with different restriction enzymes, thus resulting in non-complementary overhangs [79]. In order to inhibit re-ligation of pLPV111, the linearized plasmid was treated with calf intestinal alkaline phosphatase (CIAP) (Fermentas) to remove the 5' phosphate groups.

After treatment of the linearized pLPV111 plasmid with CIAP, new ligation reactions in 3:1, 1:1 and 1:3 insert to pLPV111 ratios were performed, and 1 µl of each of the three reaction mixtures was transformed into *E. coli* DH5α, and plated onto LB plates containing erythromycin for selection of successfully transformed cells. The transformation resulted in no successfully transformed cells. The positive control (*E. coli* DH5α transformed with pTL100α) resulted in several colonies, while the negative control (competent *E. coli* DH5α transformed without plasmid) gave no transformants. *E. coli* DH5α cells were transformed once more, this time with 4 µl of the reactions mixtures, as suggested by the QuikChange® Site-Directed Mutagenesis protocol [80], and plated onto LB plates with erythromycin for selection of successfully transformed cells. No colonies were observed despite the increased amount of DNA used in the transformation. The positive control (*E. coli* DH5α transformed with pTL100α) gave successful transformants.

After the unsuccessful ligation reactions, it was decided to sub-clone the product into a pGEM® T-Easy Vector. This was done based on the fact that the *Cla*I restriction site is about 10 bp from the 3'-end of the final PCR product (see Fig. 4.2). Most restriction enzymes fail to cleave their recognition sequences if they are located near the end of DNA fragments, especially DNA fragments created by PCR [79]. Failure to get the properly cut DNA insert may be the cause of the unsuccessful ligation reactions.

4.1.3 Sub-cloning of the PCR Product Into pGEM® T-Easy Vector

Sub-cloning of the PCR product into a pGEM® T-Easy Vector was done using the principles of TA-cloning. In order to do this, the second round of PCR was performed again, using *Taq* DNA polymerase (Fermentas) instead of *Pfu* DNA polymerase, in order to create 3' end A-overhangs on the product. The 3' A-overhangs will basepare with the 3' T-overhangs of the pGEM® T-Easy Vector, thereby ligating the PCR product into the vector. After running the second round of PCR with *Taq* DNA polymerase, ligation reactions in 3:1, 1:1 and 1:3 insert to vector amounts were performed. In the first round of transformation into *E. coli* DH5α cells, 1 µl of each of the three reaction mixtures was used. This resulted in no successful transformants. A second round of ligation reactions was performed, and this time the reactions were incubated at 4 ºC overnight. The reaction products were then transformed into *E. coli* DH5α cells, using 4 µl instead of 1 µl. This resulted in 3 colonies, which were used to inoculate overnight cultures from which plasmids were obtained (Fig. 4.4). The plasmids were sequenced for verification of the constructs, and the plasmid obtained from one of the three cultures had the correct construct, and was subsequently used further in this project.

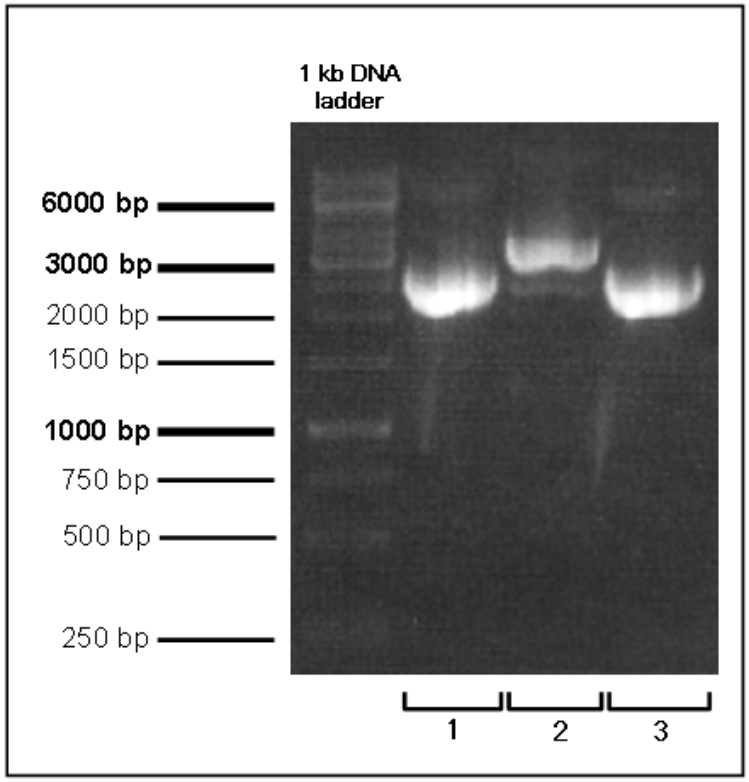

Fig. 4.4: Plasmid minipreps (1-3) from *E. coli* DH5α transformed with PCR product and pGEM® T-Easy Vector ligation mixes. All 3 minipreps contained isolated plasmid.

In order to obtain the DNA fragment of interest, the pGEM® T-Easy Vector construct (i.e. pGEM® T-Easy Vector with insert) was double-digested with restriction enzymes *Cla*I and *Mlu*I. The products of the digestion reaction were applied to an agarose gel for separation and verification of the excised fragment from the vector construct (Fig. 4.5).

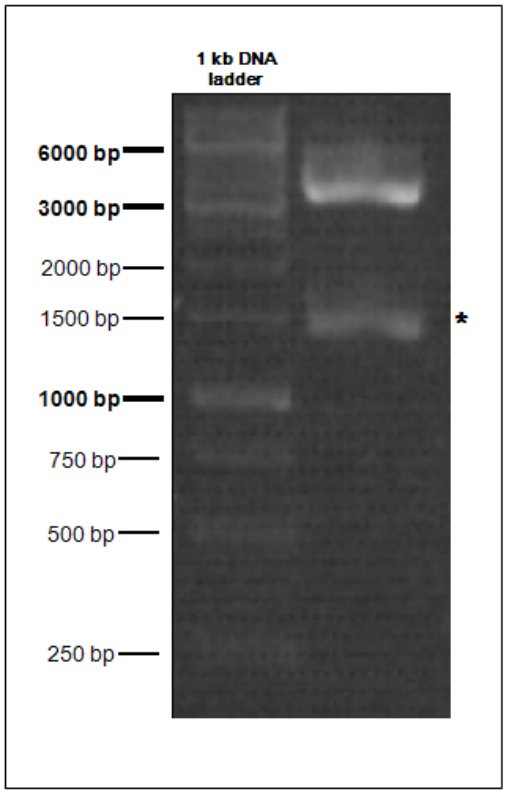

Fig. 4.5: (A) *Cla*I and *Mlu*I double-restriction digest of the pGEM® T-Easy Vector construct.

The ~1300 bp fragment (marked with obelix in Fig. 4.5) was purified from the gel, and ligation reactions in 3:1 and 1:1 insert to plasmid ratios were performed. The products of both ligation reactions were transformed into *E. coli* DH5α, and plated onto LB plates containing erythromycin for selection of successfully transformed cells. The transformation resulted in no successful transformants, while the positive control (*E. coli* DH5α transformed with pLT100α) resulted in several colonies.

The ligation efficiency was examined by performing PCR amplification over the putative insert with the pLPV111-specific primers SekF and SekR (Fig. 4.6 A). No products were amplified in the PCR reaction (Fig. 4.6 B), indicating that the ligation reaction was not successful.

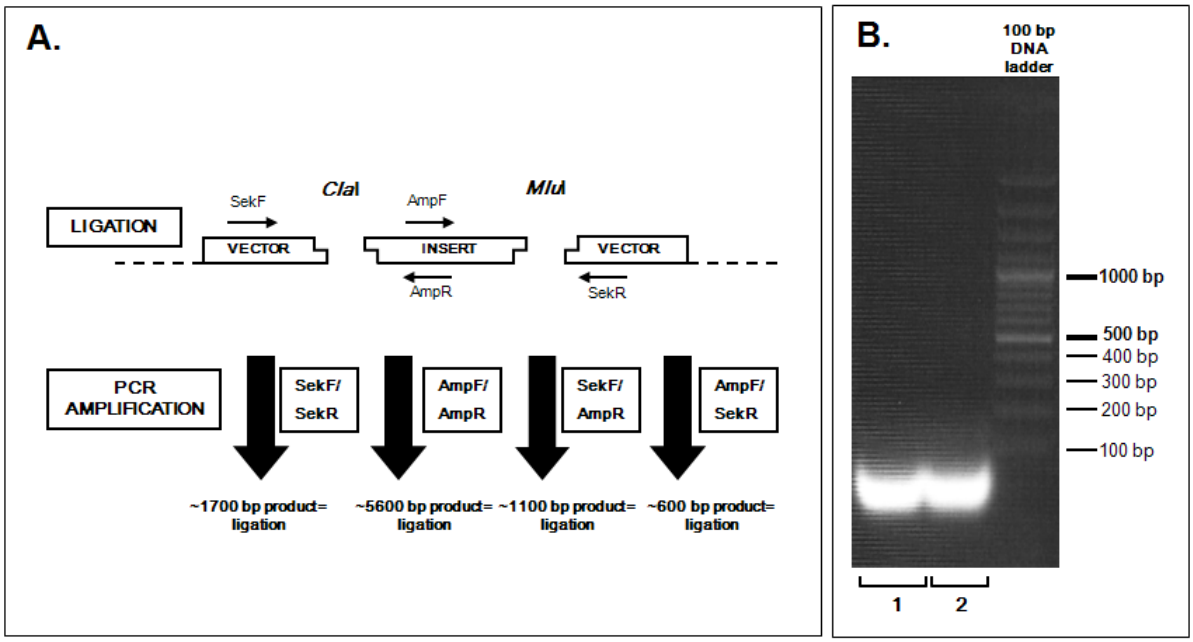

Fig. 4.6: (A) Illustration of PCR amplification of ligation reactions with primer pairs SekF/SekR, AmpF/AmpR, SekF/AmpR and AmpF/SekR. If the ligations were successful, the PCR performed with AmpF/AmpR would result in amplification of the entire plasmid construct. PCR with SekF/SekR would result in a PCR product of ~1700 bp if the ligation had been successful. PCR with primer pairs SekF/AmpR and AmpF/SekR was also performed to further confirm if the ligation reactions had been successful. **(B)** PCR amplification of insert and pLPV111 3:1 and 1:1 ligation reactions with primer pairs SekF/SekR resulted in no product.

After examining the polylinker region of pGEM® T-Easy Vector, it was found that the pGEM® T-Easy Vector contains a *Mlu*I restriction site in the polylinker region, ~65 bp downstream of the *Cla*I restriction site in the insert (not shown on pGEM® T-Easy Vector chart, Fig. 3.5). This was in addition to the *Mlu*I restriction site in the insert. The double digestion of the pGEM® T-Easy Vector construct with *Mlu*I and *Cla*I in buffer D may have only resulted in cleavage of the *Mlu*I sites, and not the *Cla*I site. This again would have resulted in a ~1350 bp fragment with *Mlu*I "sticky ends" in both ends, and may explain failure to ligate with the *Cla*I and *Mlu*I double digested pLPV111 plasmid.

In order to ensure that the pGEM® T-Easy Vector construct was cleaved properly with *Cla*I, sequential digest of the plasmid was performed instead, starting with *Cla*I in buffer C (100% activity), which led to a linearization of the vector construct (Fig. 4.7 A). The linearized construct was purified, and followed by digestion with *Mlu*I in buffer D (Fig. 4.7 B), resulting in a ~1300 bp fragment being cut out.

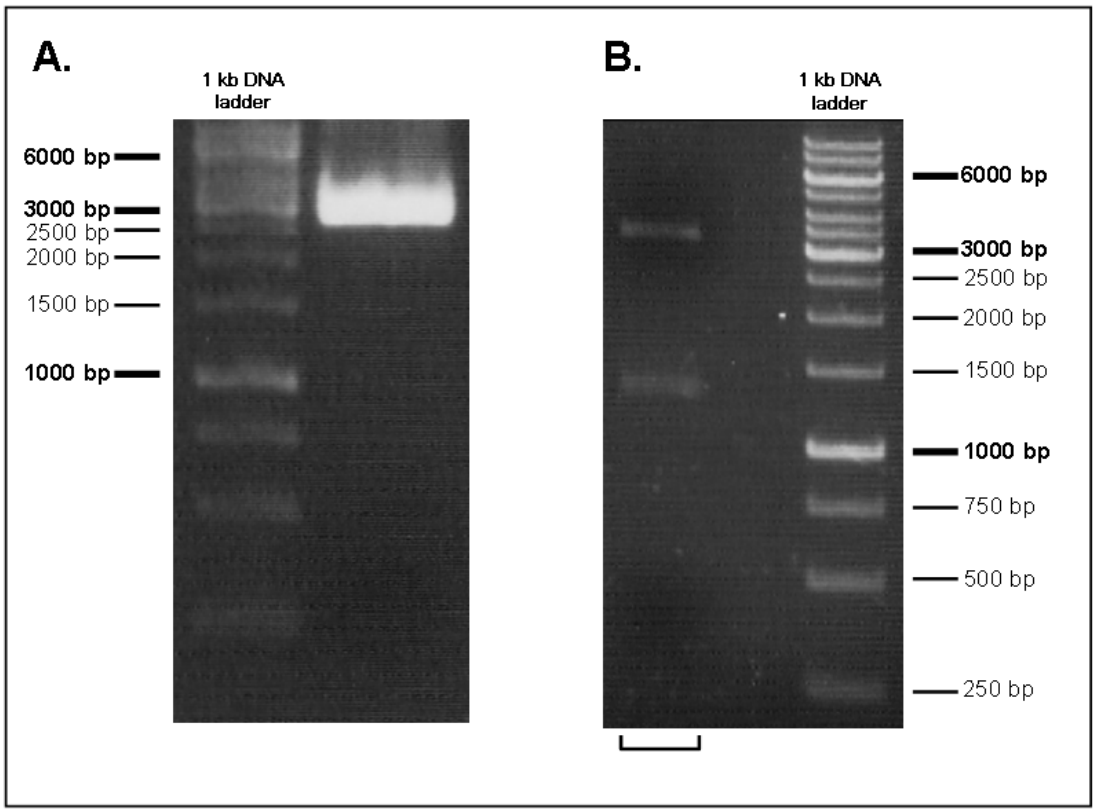

Fig. 4.7: Sequential restriction digest of the pGEM® T-Easy Vector construct with *Cla*I and *Mlu*I. (A) Restriction digest with *Cla*I of the pGEM® T-Easy Vector construct resulting in a linearized vector construct. (**B)** Subsequent restriction digest of the construct with *Mlu*I resulted in a ~1300 bp fragment being excised.

The ~1300 bp fragment was purified, and new 3:1 and 1:1 ligation reactions were performed with the insert and pLPV111. The products of the ligation reactions were transformed into *E. coli* DH5α, and plated onto LB plates with erythromycin for selection of successfully transformed cells. The transformation gave no successful transformants. Again, the positive control (*E. coli* DH5α transformed with pLT100α) gave several colonies.

PCR amplification of the ligation reactions was performed with primers AmpF and AmpR (see section 6.3.1 for sequences). These two primers anneal to the same site in the insert, but on separate strands, resulting in forward synthesis by AmpF, and reverse synthesis by AmpR, giving amplification of plasmid with insert if the ligation reactions were successful. This would increase the concentration of possible ligation products, which again would increase transformation efficiency into *E. coli* DH5α. If the ligation reactions were unsuccessful, the PCR would result in no products (Fig. 4.6 A). PCR with primer pairs AmpF/SekR and SekF/AmpR were also performed.

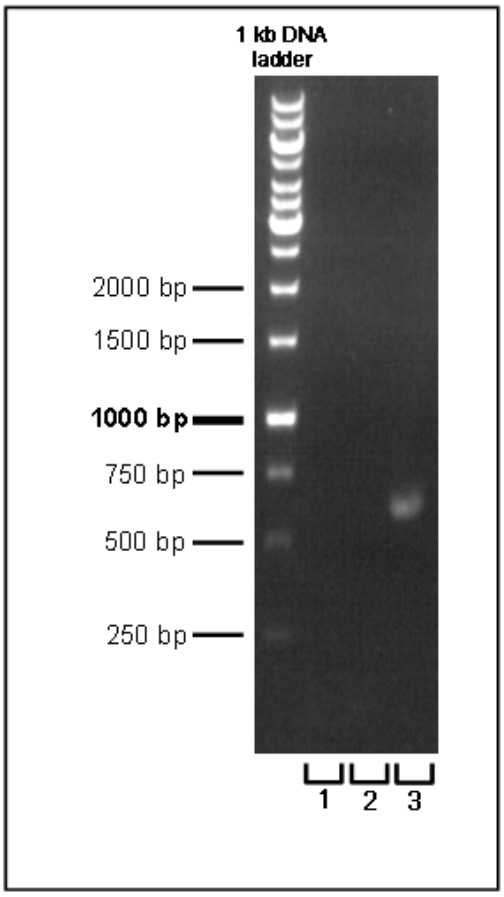

Fig. 4.8: PCR amplification of pLPV111 and insert ligation reactions with primer pairs AmpF/AmpR (lane 1), SekF/AmpR (lane 2) and AmpF/SekR (lane 3). Only PCR amplification with primer pairs AmpF/SekR resulted in a product.

The PCR amplification with AmpF/AmpR resulted in no products. The amplification with AmpF/SekR resulted in a ~550 bp product, and amplification with SekF/AmpR resulted in no products (Fig 4.8), i.e. ligation over the *Mlu*I site seemed to have been successful, but not over the *Cla*I site.

A more thorough examination of the sequence of the pGEM® T-Easy Vector construct revealed that the *Cla*I restriction site (5'-atcgat-3') in the insert overlapped with a DNA adenine methylase (*dam*) site (5'-gatc-3'). The *dam* methylase attaches a methyl group to the N^6 atom of the adenine in the recognition sequence [79], thereby preventing *Cla*I from cleaving in the same site. The first restriction digest with *Cla*I would then have been unsuccessful, leaving an intact plasmid or possibly unspecific cleaved plasmid, since the plasmid appear to have been linearized (Fig. 4.6 A), while the second restriction digest with *Mlu*I would cut at both *Mlu*I sites, excising a fragment ~65 bp longer than expected.

This might be an explanation as to why the ligation reactions had been unsuccessful, and it was decided to change the *Cla*I restriction site into an *Xba*I restriction site (5'-tctaga-3') by *in vitro* site-directed mutagenesis.

4.1.3 Mutagenesis of *Cla*I Restriction Site to *Xba*I Restriction Site

The forward and the reverse mutagenic primers PlnEFXbaIF and PlnEFXbaIR, respectively (see section 6.3.1 for sequences), were used in PCR to introduce three point mutations in and adjacent to the *Cla*I restriction site in the pGEM® T-Easy Vector construct, creating an *Xba*I restriction site. After *Dpn*I-treatment of the PCR products, 1 µl of the DNA was transformed into *E. coli* DH5α, and plated onto LB plates containing ampicillin for positive selection. Several colonies appeared on the plates, and some of the colonies were used to inoculate overnight cultures from which plasmids were isolated and purified. The *Xba*I restriction site introduced into the pGEM® T-Easy Vector construct was verified by DNA sequencing.

The DNA fragment was amplified *in vitro* with PCR using primers SakP2 and Sp6 (see section 6.3.1 for sequences of primers), so as to obtain a higher concentration of the fragment (Fig. 4.9 A, lane 1). The ~1500 bp PCR product contained the sakacin P leader-sequence, the *plnF*-gene, the *plnI*-gene, one *Xba*I restriction site, and two *Mlu*I restriction sites.

The ~1500 bp PCR product was purified, then sequentially digested, first with *Xba*I, resulting in a ~1400 bp product (Fig. 4.9 B, lane 2). The product was purified, then cleaved with *Mlu*I for 6 hrs at room temperature, in order to ensure that the product was properly digested. The *Mlu*I digest resulted in a ~1300 bp PCR product (Fig. 4.9 C) with a *Mlu*I overhang at the 5' end and an *Xba*I overhang at the 3' end, as illustrated in Fig. 4.9 A.

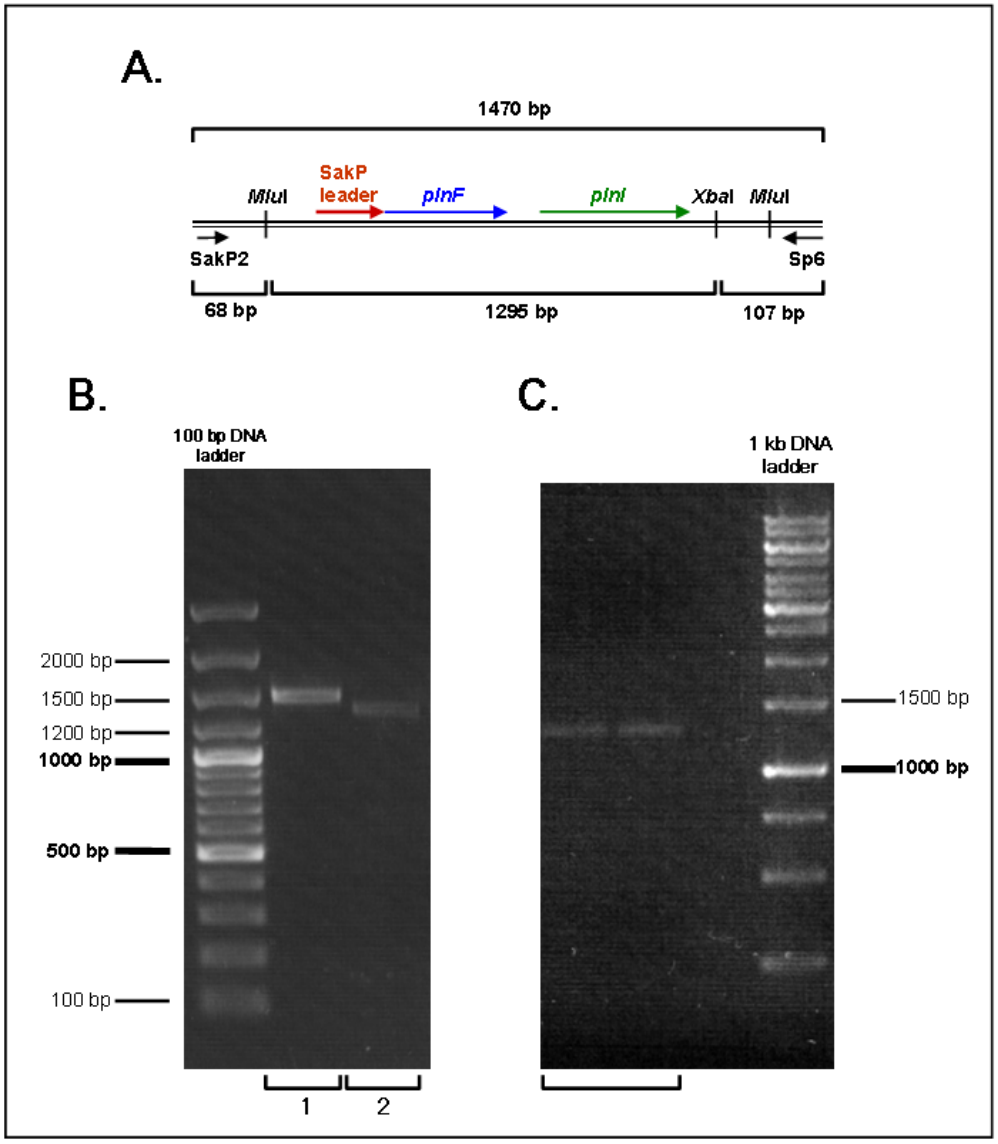

Fig. 4.9: Sequential restriction digests of the fragment containing the sakacin P leader-sequence, *plnF*-gene and *plnI*-gene. (A) Organization of the 1470 bp long amplified PCR product. **(B)** The ~1500 bp PCR product (lane 1) was digested with *Xba*I (lane 2). **(C)** *Mlu*I digestion (6 hrs) of *Xba*I digested DNA resulted in a ~1300 bp product.

The plasmid pLT100α contains two *Xba*I restriction sites, and one *Mlu*I restriction site (Fig. 4.10 A). One of the *Xba*I restriction sites in pLT100α is located in the polylinker region downstream of the gene encoding the lactococcin G immunity protein, the other site is located in the sequence coding for LcnG-α (Fig. 4.10 A). After an *Xba*I restriction digest, a ~550 bp DNA fragment was excised from pLT100α (Fig. 4.10 B). *Mlu*I restriction digest of the remaining plasmid led to a ~250 bp fragment being excised, thus resulting in the ~4200 bp linearized pLPV111 plasmid (Fig. 4.10 C, lane 3).

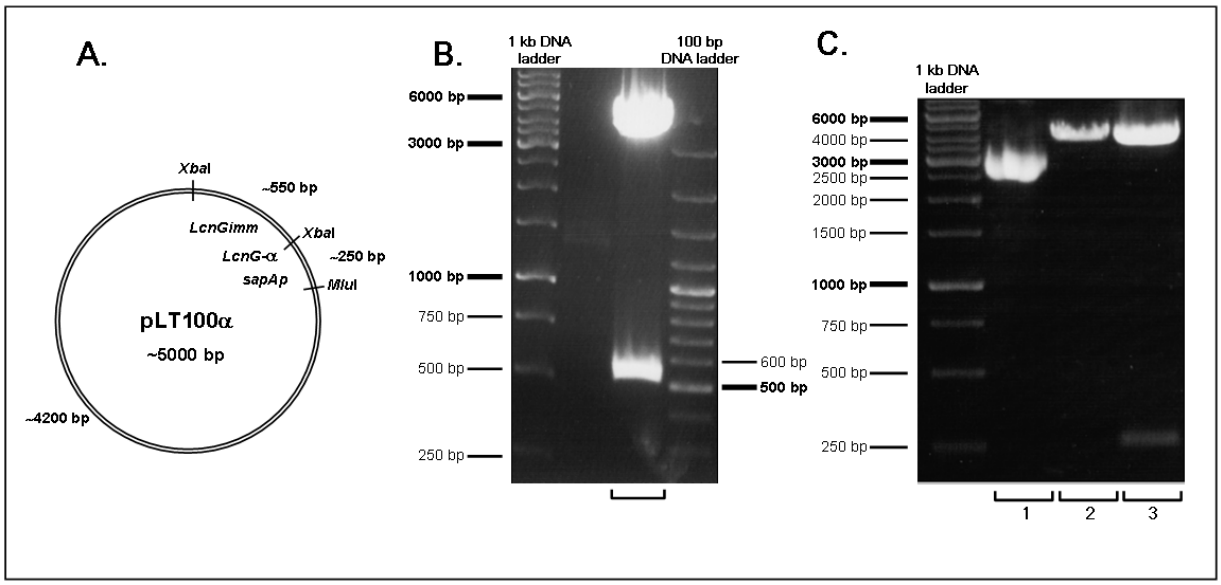

Fig. 4.10: Sequential restriction digest of pLT100α with *Xba*I and *Mlu*I. (A) Organization of *Xba*I and *Mlu*I restriction sites in pLT100α. **(B)** Restriction digest of pLT100α with *Xba*I resulted in a ~550 bp DNA fragment being excised. **(C)** Further restriction digest with *Mlu*I led to a ~250 bp DNA fragment being excised (lane 3), resulting in the pLPV111 plasmid with a cleaved *Xba*I site at the 3' end and a cleaved *Mlu*I site at the 5' end. Undigested pLT100α plasmid in lane 1 and *Xba*I digested pLT100α in lane 2, for comparison.

Ligation reactions of the insert and pLPV111 were performed in 3:1 and 1:1 ratios, and the products were transformed into *E. coli* DH5α, and plated onto LB plates containing erythromycin for selection of successfully transformed cells. The transformation gave no colonies. The positive control (*E. coli* DH5α transformed with pLT100α) resulted in colonies. PCR amplification over the two ligation sites was performed on the 3:1 ligation reaction to investigate if the insert was ligated into pLPV111 (Fig. 4.11 A). A ~1100 bp PCR product with primer pairs SekF/AmpR and a ~600 bp product with primer pairs AmpF/SekR would give an indication if the ligation reactions were successful.

The PCR amplification results verified that the insert was ligated into pLVP111 (Fig. 4.11 A), and it was decided to transform the ligation reactions into "XL-10 Gold® Ultracompetent Cells" (Stratagene) for higher transformation efficiency. The transformation was performed according to the "XL-10 Gold® Ultracompetent Cells" Protocol (Stratagene). The cells were plated onto LB plates containing erythromycin for selection of successfully transformed cells. The transformation resulted in 12 colonies, which were inoculated in fresh LB medium containing erythromycin. Of the 12 colonies, 8 had grown over the course of the

next day. These 8 cultures were minipreped, and each miniprep was run on agarose gel to verify the presence of a plasmid (Fig. 4.11 B).

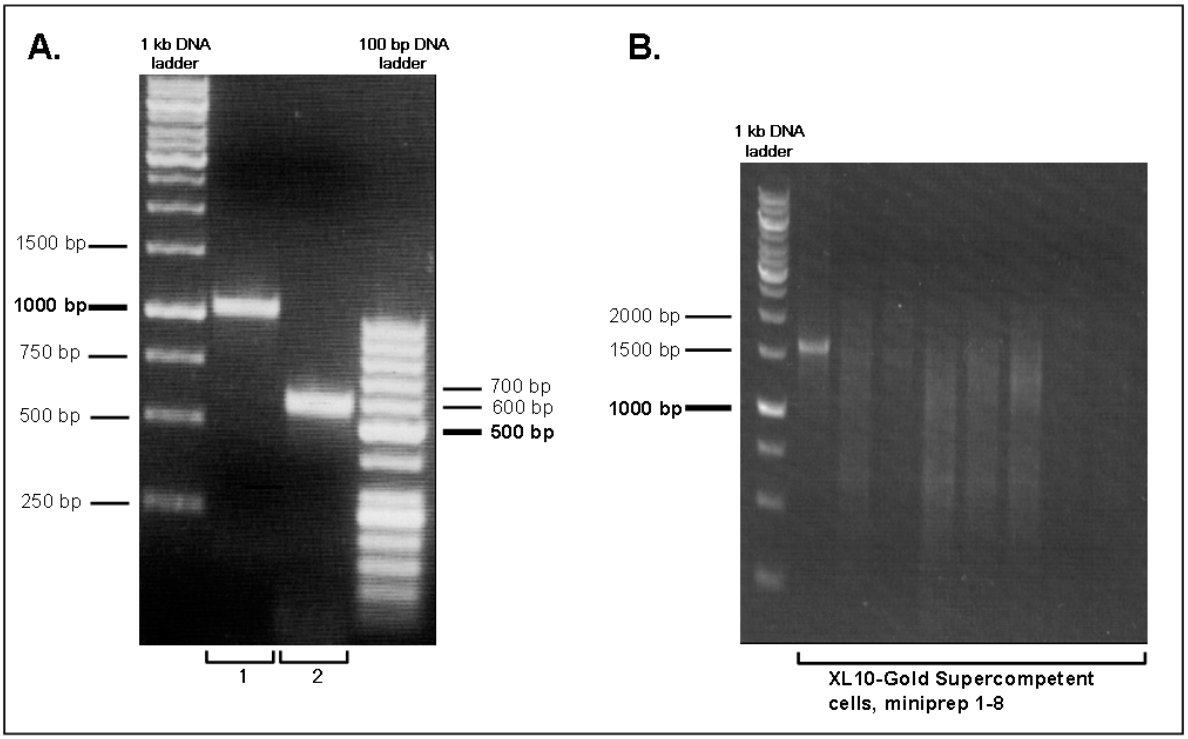

Fig. 4.11: (A) PCR amplification of the 3:1 ligation reaction with primers SekF and AmpR (lane 1) and SekR and AmpF (lane 2) resulted in PCR products of ~1100 bp and ~600 bp, respectively. The size of the PCR products indicates that the ligation reaction had been successful. **(B)** Plasmid miniprep 1-8 from XL-10 Gold® Ultracompetent Cells transformed with ligation reactions.

The plasmid minipreps showed that only one of the colonies contained a plasmid. The plasmid miniprep was sent to sequencing to verify the correct plasmid. Sequencing showed that the insert had been successfully ligated into pLPV111, but with two point mutations, one being upstream of the -35 box in the sakacin A promoter-region, the other being in the PlnI-encoding region, changing an isoleucine to a valine, which is a conservative mutation. The resulting plasmid is a 5.65 kB shuttle vector, termed pPlnF100 (Fig. 4.12).

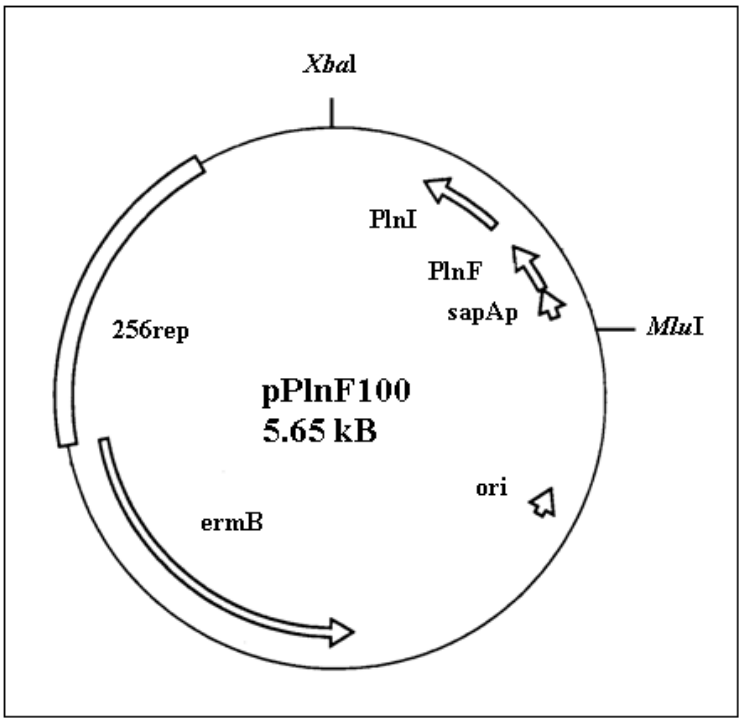

Fig. 4.12: Plasmid chart of the pPlnF100 shuttle vector. *ermB* is a marker for erythromycin-resistance. *sapAp* is the sakacin A promoter, while *plnF* and *plnI* encodes PlnF and its cognate immunity protein, respectively. The plasmid contains replication origos for replication in both *E. coli* and *Lactobacillus*, *ori* and *256rep*, respectively.

4.2 Purification and Analysis of PlnF

After construction and verification of pPlnF100, the plasmid was transformed into the bacteriocin negative strain *L. sake* Lb790/pSAK20, as described in section 3.1.11.4. Selection of successfully transformed cells was performed on MRS-plates containing erythromycin and chloramphenicol. Single colonies were used to prepare -80 ºC stocks of the cell cultures.

4.2.1 Cation Exchange Chromatography

A 10 ml pre-culture of *L. sake* Lb790/pSAK20/pPlnF100 was used to inoculate 1 liter of MRS as described in section 3.2.1.1. After incubation overnight at 30 ºC, the cell-culture was spun down, and the supernatant was applied to a cation-exchange chromatography column, and eluted with buffer B (20 mM phosphate buffer with 1 M NaCl, pH~5.7) (see

section 6.4). Sample volumes of 1 ml were taken from each step of the cation-exchange chromatography (i.e. supernatant, flow-through, wash and eluate).

The obtained samples were tested in a bacteriocin activity assay combined with synthetic PlnE, using *L. viridescens* NCDO 1655 as an indicator strain (diluted 1:200 and grown for 14 hrs). Synthetic PlnE was added in excess so that the purified PlnF was the limiting factor, and the antimicrobial activity was measured. This reflected the amount of purified PlnF in each step. Since synthetic PlnE was added to an excess, and PlnE alone has no activity, it was assumed that all of the purified PlnF was used, and on the basis of this assumption the degree of purified PlnF was calculated. Samples not combined with synthetic PlnE, and synthetic PlnE alone were used as negative controls, while synthetic PlnF combined with synthetic PlnE was used as positive control. None of samples alone nor synthetic PlnE alone gave any antimicrobial activity, whereas the combination of synthetic PlnF and synthetic PlnE gave growth inhibition in all the wells.

Table 4.1 displays the purification fractions for PlnF using cation exchange chromatography. The table also summarizes the bacteriocin activity in the different fractions obtained combined with an excess of synthetic PlnE.

Table 4.1: Purification of PlnF by the use of cation exchange chromatography. The bacteriocin was eluted from the cation exchange column with buffer B.

Fraction	Volume (ml)	Bacteriocin Activity [a)]	Yield (%)[b)]
I Culture Supernatant	1000	30	100
II Flow-through	1000	***nd**	***nd**
III Wash (buffer A)	100	15	5
IV Eluate (buffer B)	40	15	2

a) Bacteriocin activity is the fold dilution of the bacteriocin fraction that gives 50% growth inhibition of the indicator strain *L. viridescens* NCDO 1655.
b) Yield is the bacteriocin activity in the fraction divided by the bacteriocin activity of the supernatant and multiplied by the fold volume reduction.
* The abbreviation **nd** means that there was no detection of bacteriocin activity in the fraction.

As shown in Table 4.1, the 1000 ml culture supernatant (fraction I) gave growth inhibition up to a 30-fold dilution, indicating a production of PlnF by *L. sake* Lb790/pSAK20/pPlnF100, although the production is low. The 1000 ml flowthrough fraction gave no growth inhibition of the indicator cells, and this implies that PlnF in the

supernatant had bound to the column material. The fraction obtained upon washing the cation exchange column with 100 ml buffer A (20 mM phosphate buffer, pH~5.7) gave growth inhibition up to a 15-fold dilution. This indicates that some PlnF was lost during the washing step. Fraction IV was the 40 ml eluted from the column with buffer B. This eluate gave growth inhibition up to a 15-fold dilution, which indicated elution of PlnF from the column, although in small amounts, as indicated by the 2% yield of PlnF. It was postulated that the bacteriocins bind so strongly to the cation exchange column, that they could not be eluted by using buffer B.

In order to obtain more PlnF upon elution from the cation exchange column, it was decided to purify PlnF on a cation exchange column once more, this time using buffer B containing 6 M guanidine hydrochloride (GuHCl). In addition to disrupting ionic interactions, GuHCl also disrupts hydrophobic interactions, such as protein aggregates, and might therefore give higher yield.

The cation exchange chromatography was performed as before, this time eluting with buffer B containing 6 M GuHCl. Sample volumes of 1 ml were collected from each step in the chromatography of PlnF. Also, each of these fractions was combined with an excess of synthetic PlnE and tested in a bacteriocin activity assay. The control with synthetic PlnE alone gave no inhibition at all, and the control with synthetic PlnF combined with synthetic PlnE gave growth inhibition in all the wells. The results are summarized in Table 4.2.

Table 4.2: Purification of PlnF by the use of cation exchange chromatography. The bacteriocin was eluted from the cation exchange column with buffer B containing 6 M GuHCl.

Fraction	Volume (ml)	Bacteriocin Activity [a)]	Yield (%)[b)]
I Culture Supernatant	1000	65	100
II Flow-through	1000	15	25
III Wash (buffer A)	100	10	1.3
IV Eluate (buffer B with 6 M GuHCl)	40	130	8

a) Bacteriocin activity is the fold dilution of the bacteriocin fraction that gives 50% growth inhibition of the indicator strain *L. viridescens* NCDO 1655.
b) Yield is the bacteriocin activity in the fraction divided by the bacteriocin activity of the supernatant and multiplied by the fold volume reduction.

As Table 4.2 shows, the 1000 ml culture supernatant combined with an excess of synthetic PlnE gave growth inhibition up to a 65-fold dilution. The supernatant alone gave growth inhibition up to a 15-fold dilution (not shown). This indicates production of PlnF by *L. sake* Lb790/pSAK20/pPlnF100. The 1000 ml flowthrough had bacteriocin activity up to a 15-fold dilution when combined with PlnE, the same as for the flowthrough alone (not shown). This might be due to antibiotics residuals in the media. The fraction obtained upon washing the column with buffer A gave growth inhibition up to a 10-fold dilution when combined with PlnE. No growth inhibition was observed when the wash-fraction was tested alone. This indicated that only minor amounts of PlnF were washed out with buffer A (1.3% PlnF was lost). The 40 ml fraction obtained when eluting PlnF with buffer B containing 6 M GuHCl gave growth inhibition up to a 130-fold dilution when combined with PlnE. These results indicates that PlnF was present in the eluate, but at a low concentration. By comparing Tables 4.1 and 4.2, it is evident that elution with buffer B containing 6 M GuHCl gives a higher yield of PlnF than elution with buffer B.

4.2.2 Reverse Phase Chromatography

The 40 ml eluate from the cation exchange column eluted with buffer B was added 5 ml 2-propanol and applied to a reverse phase chromatography column. Several peaks were detected on the optical density profile upon linear elution with 2-propanol/0.1% TFA (Fig. 4.13). The sample was fractionated after peaks obtained at 214 nm and 280 nm. PlnF was expected to elute between 30% and 50% 2-propanol, based on elution of other two-peptide bacteriocins, such as lactococcin G (LcnG-α and LcnG-β) [88].

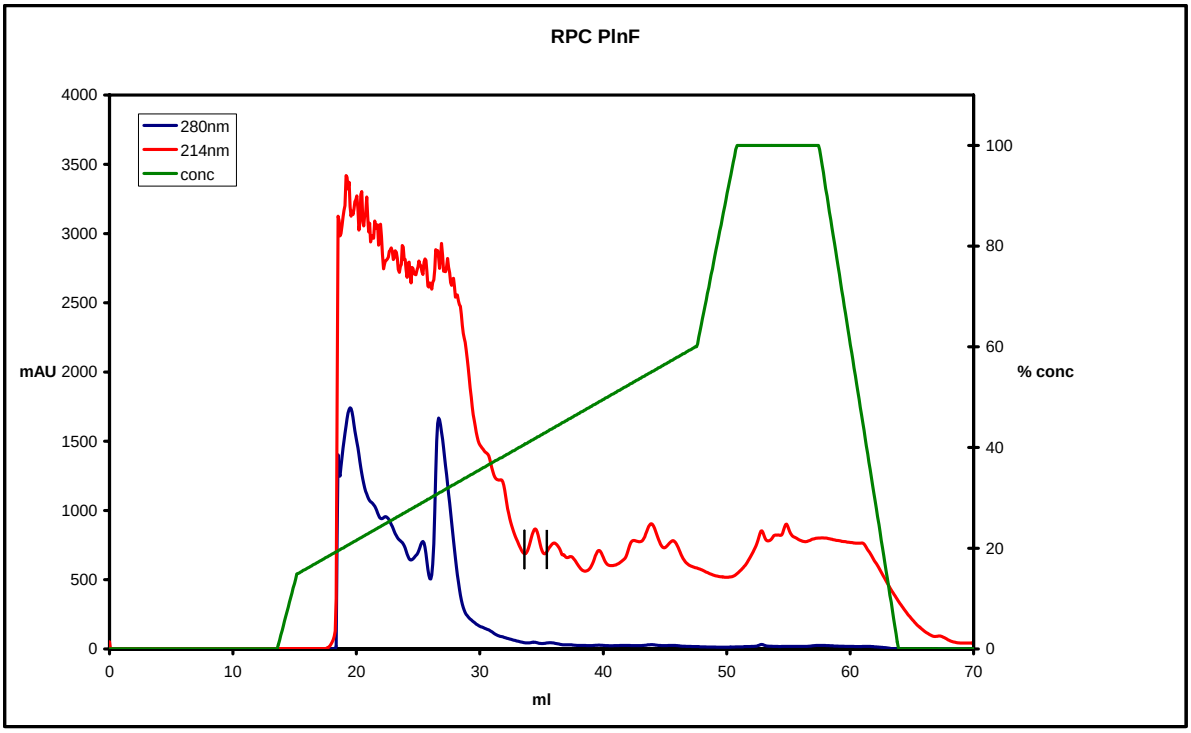

Fig. 4.13: Chromatogram of PlnF obtained by reverse phase chromatography using the Äkta system. PlnF eluted at about 45% 2-propanol. The identity of the peptide was verified by bacteriocin activity assay, and by determining the mass with MALDI-TOF MS.

Several of the fractions obtained were tested in bacteriocin activity assays combined with synthetic PlnE, using *L. viridescens* NCDO 1655 as an indicator strain (diluted 1:200 and grown for 14 hrs). Fractions not combined with synthetic PlnE, and an excess of synthetic PlnE alone were used as negative controls, and synthetic PlnF combined with synthetic PlnE was used as positive control. Synthetic PlnE alone gave no growth inhibition at all, while synthetic PlnF combined with synthetic PlnE gave growth inhibition in all wells.

The fraction marked in Fig. 4.13 combined with an excess of synthetic PlnE gave growth inhibition up to a 500-fold dilution, while the fraction alone gave no growth inhibition. The fraction was analysed by mass spectrometry for verification of the presence of PlnF, and gave a mass of 3544 Da (Fig. 4.14), which is in good agreement with the theoretical mass of PlnF (3545 Da) [47]. The peptide eluted at 45% 2-propanol in the reverse phase chromatography.

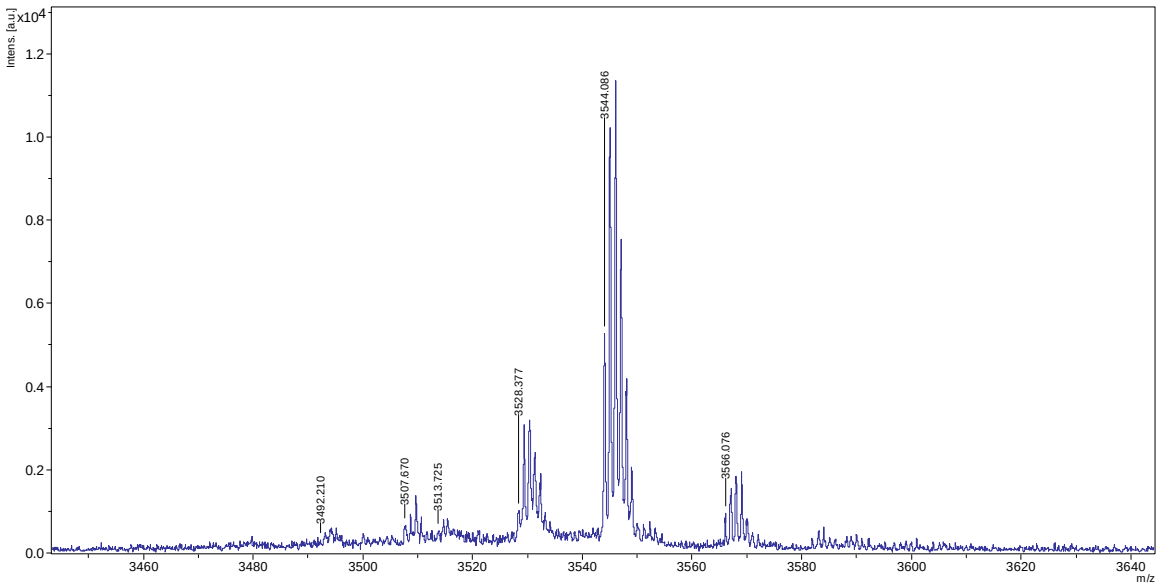

Fig. 4.14: Mass spectrum of PlnF. The peak of 3544 Da is in good consistence with PlnF's theoretical mass; 3545 Da.

Because of the low activity in the supernatant, it was postulated that the positively charged bacteriocins might bind to the negatively charged membrane surface of the producer cell, as has been shown to be the case for plantaricin C19 [93]. In order to investigate this, the pelleted cells were resuspended in 1 M NaCl. High salt concentration will disrupt any electrostatic interactions between the bacteriocins and cell membranes. The resuspended cells were pelleted once more, and the culture supernatant from the cells was tested for bacteriocin activity by combining it with an excess of synthetic PlnE. The indicator strain used was *L. viridescens* NCDO 1655 (diluted 1:200 and grown for 14 hrs). The supernatant not combined with synthetic PlnE, synthetic PlnE alone, and 1 M NaCl were used as negative controls. Synthetic PlnE combined with synthetic PlnF was used as positive control.

The culture supernatant from the cells resuspended in NaCl gave no growth inhibition. Neither of the negative controls gave growth inhibition, while the positive control gave growth inhibition in all wells. These results indicate that PlnF most likely does not bind to the membrane of the producer cells.

4.3 Construction of PlnF Mutants

To study the importance of GxxxG motifs in helix-helix interaction between the two complementary peptides of plantaricin EF, point mutations were introduced in the GxxxG motif in PlnF. Fig. 4.15 displays the nucleotide and amino acid sequences of PlnF. The glycine residues in the GxxxG motif are marked in green. Both glycines in the motif were exchanged with leucine, serine, alanine, lysine and glutamine.

Similarly, in order to examine to what extent tyrosine and tryptophan residues affect the stability of PlnF in the membrane, its two tyrosine residues were exchanged with tryptophan, arginine, leucine and phenylalanine, and the tryptophan residue was exchanged with tyrosine, arginine, leucine and phenylalanine. The two tyrosine residues are marked in blue, and the tryptophan residue in red, in Fig. 4.15.

PlnF

```
atggaaaagtttattgaattatctttaaaagaagtaacagcaattacaggtgga|
M  E  K  F  I  E  L  S  L  K  E  V  T  A  I  T  G  G
            5                          14                          23
gttttccatgcctatagcgcgcgtggcgttcggaataattataaaagtgctgttgggcctgccgattgggtcatt
V  F  H  A  Y  S  A  R  G  V  R  N  N  Y  K  S  A  V  G  P  A  D  W  V  I
            30          34
agcgctgtccgaggattcatccacgga|tagttcaagccatcaagt
S  A  V  R  G  F  I  H  G   STOP
```

Fig. 4.15: Nucleotide sequence and amino acid sequence of the N-terminal sakacin P leader-sequence (first row) followed by the nucleotide sequence and amino acid sequence of the mature PlnF. The GxxxG motif ranges from residue 30 to 34 (green), the tyrosine residues are in position 5 and 14 (blue), and the tryptophan residue is in position 23 (red).

The mutants were termed according to the amino acid residue that has been exchanged, followed by the new amino acid residue. If for instance the glycine residue in position 30 was exchanged with an alanine, the mutant was termed G30A.

A total of 21 mutants were constructed by the use of *in vitro* site-directed mutagenesis, and verified by DNA sequencing, as described in section 3.1.3 (Table 4.3). The mutations were introduced by PCR, by the use of forward and reverse mutagenic primers. The names and sequences of these primers are listed in section 5.4.2. *Dpn*I was used to digest methylated, parental DNA and to screen for unmethylated, newly synthesized DNA containing the mutations. The *Dpn*I-treated DNA was then used to transform *E. coli* DH5α, and the successfully transformed cells were selected on LB plates containing erythromycin. Single colonies of the transformed cells were used to inoculate overnight cultures. The cultures were subsequently plasmid minipreped, and the minipreps were sequenced for verification of the desired mutations. All 21 mutants were verified by DNA sequencing (results not shown).

Table 4.3: Construction, transformation and verification of pPlnF100 mutants.

Mutants	PCR Products Observed on Gel	PCR Products Transformed Into *E. coli DH5α*	Plasmid Isolation and Verification by Sequencing
GxxxG Motif			
G30L	+	+	+
G30S	+	+	+
G30A	+	+	+
G30K	+	+	+
G30Q	+	+	+
G34L	+	+	+
G34S	+	+	+
G34A	+	+	+
G34K	+	+	+
G34Q	+	+	+
Tryptophan Residue			
W23Y	+	+	+
W23R	+	+	+
W23L	+	+	+
W23F	+	+	+
Tyrosine Residues			
Y5W	+	+	+
Y5R	+	+	+
Y5L	+	+	+
Y5F	+	+	+
Y14W	+	+	*-
Y14R	+	+	+
Y14L	+	+	+
Y14F	+	+	+
Number of Mutants	**22**	**22**	**21**

***- indicates that the mutant plasmid has not been verified by DNA sequencing.**

5. Discussion

Most of the time used for this project was spent constructing the pPlnF100 plasmid for heterologous expression of PlnF and its mutants. The pPlnF100 plasmid contains the sakacin P leader-sequence and the genes encoding PlnF and its immunity protein. This expression system allows for individual and increased expression of both the bacteriocin and its mutants, as opposed to what would be the case in the natural producer of PlnF, *Lactobacillus plantarum* C11. This again will make it easier to purify and isolate the mutant peptides. The peptides will subsequently be used in structure-function analysis of plantaricin EF, and to gain insight into the importance of the GxxxG motif and the tryptophan and tyrosine residues.

5.1 Construction of pPlnF100

There were some obstacles associated with the construction of pPlnF100, mainly due to the restriction digests and subsequent ligation reactions. These obstacles were overcome by modifying some of the methods used for construction of the pPlnF100 plasmid, as described in section 4.1. After sub-cloning of the PCR product into the pGEM® T-Easy Vector, changing the *Cla*I restriction site in the PCR product into an *Xba*I restriction site, and performing sequential digest of the pGEM® T-Easy Vector construct with *Xba*I and *Mlu*I, the insert was successfully ligated into pLPV111, thereby completing the construction of the pPlnF100 plasmid. As mentioned in section 4.1, the plasmid contains two point mutations, one upstream of the -35 box in the sakacin A promoter-region, the other in the PlnI-encoding region, changing an isoleucine to a valine. Since none of these mutations are located in the *plnF*-gene, and the mutation located in the *plnI*-gene is a conservative mutation, it was decided to use the pPlnF100 plasmid with the mutations further in this project.

5.2 Purification of PlnF

pPlnF100 was transformed into *L. sake* Lb790/pSAK20 for production of PlnF, as described in section 4.2. The bacteriocin was subsequently purified and isolated using cation exchange chromatography and reverse phase chromatography, and its identity was verified using mass spectrometry and bacteriocin activity assay.

The bacteriocin activity in the culture supernatant in combination with synthetic PlnE indicated production of PlnF, although a low production. If the problem with low yield of PlnF is due to low production, this will most likely propagate to the production of the mutants as well, which might cause problems with their purification and quantification. It was postulated that the low production of PlnF might be a result of either of the two point mutations in pPlnF100. The point mutation in the immunity protein could possibly have been so deleterious that the protein could no longer serve its function to the producer cell, thereby resulting in the cell being killed by its own bacteriocin and not being able to produce PlnF. However, cultures of the producer cells *L. sake* Lb790/pSAK20/pPlnF100 were grown to the stationary phase, so the mutation is most likely neutral (as expected considering the fact that the mutation is conservative; changing an isoleucine to a valine), maintaining the function of the immunity protein. The mutation upstream of the sakacin A promoter region might result in down-regulation of transcription of PlnF. Although the mutation is not located in the core promoter (i.e. the -10 box or the -35 box), the mutation is situated in the -59 to -38 region, a region known as the UP (upstream) element. This element increases promoter activity when located upstream of the -35 box [94], and the mutation upstream of the sakacin A promoter might therefore be a possible reason for the low production of PlnF. PlnF was produced with a sakacin P leader-sequence in order for the sakacin A ABC-transporter to recognize and transport the peptide out of the cell. Even though the ABC-transporter recognizes and transports peptides with sakacin A leader-sequences, it has been experimentally shown that the ABC-transporter also recognizes peptides with sakacin P leader-sequences [84]. This has indeed been shown to be the case for both LcnG-α and LcnG-β [88]. The sakacin A leader-sequence and the sakacin P leader-sequence share ~50% amino acid sequence identity (refer to Mathiesen *et al.* for sequences [95]), and this is evidently similar enough for the ABC-transporter to recognize the sakacin P leader-sequence [88]. One might, however, speculate that the amino acid composition of the bacteriocin itself plays a part in the recognition and transport by the sakacin A ABC-transporter. It is known that production

of various bacteriocins with identical sakacin P leader sequence vary considerably. A heterologous expression system identical to the one used in this project was constructed for production of each of the two peptides PlnJ and PlnK, which constitute the two-peptide bacteriocin plantaricin JK. The genes encoding PlnJ and PlnK are preceded by a sakacin A promoter, and the peptides are synthesized with a sakacin P leader-sequence. Attempts to purify and isolate the two individually expressed peptides have been performed, but both peptides seem to be expressed at a low level, making their isolation difficult (personal conversations with C. Oppegård and P.Rogne).

Although the production of PlnF seemed to be quite low, optimalization of the purification procedure is needed, as indicated by the low yield from the cation exchange chromatography (Tables 4.1 and 4.2). When testing the activity of the buffer B eluate from the cation exchange column, only a 2% yield was obtained (Table 4.1). The activity obtained upon eluting the column with buffer B containing 6 M GuHCl resulted in 8% yield (Table 4.2). It is apparent that elution of the column with 6 M GuHCl gives a higher yield of PlnF than elution with buffer B alone, but 8% yield is still lower than would be expected, as compared to purification of other bacteriocins, such as LcnG-α and LcnG-β [88]. If the bacteriocin had not bound to the column when applying the culture supernatant, a high yield would have been detected in the flowthrough. Similarly, if PlnF had been washed out from the column with buffer A, a high yield would have been detected in the wash-fraction. This was not the case for either of the purification steps (as given in Tables 4.1 and 4.2). This indicated that PlnF was bound so strongly to the column that neither buffer B nor 6 M GuHCl were able to elute the bacteriocin. Because the problem in the purification step of PlnF apparently is associated with the cation exchange chromatography, possible solutions might be to use stronger elution buffers or different ligands in the cation exchange column, or possibly a different purification step altogether. Purification of the class IIa pediocin-like bacteriocin enterocin P has proven to be somewhat difficult, as the peptide seems to bind irreversibly to all tested column material (personal conversation with H. S. Haugen).

The fraction obtained from the reverse phase column resulted in growth inhibition up to a 500-fold dilution (section 4.2.2), giving a 1.6% yield. However, because the eluate applied onto the reverse phase column gave a 2% yield (Table 4.1), only about ~20% of PlnF in the eluate from the cation exchange column was lost during reverse phase chromatography.

5.3 Constructions of the Mutants

Constructions of the mutant versions of pPlnF100 went according to the protocol, and all the mutated versions were obtained, except for Y14W.

The residues chosen for substitution of the glycines in the GxxxG motif were serine, alanine, leucine, lysine and glutamine. The reason for choosing serine and alanine was that both these residues are small, and substitution of the glycines with any of these will most likely not affect helix-helix interaction with PlnE. Leucine was chosen because it is a large, hydrophobic residue and lysine and glutamine were chosen because they are large, hydrophilic residues. Substitution of the glycine residues with any of these three large residues is expected to be detrimental to the helix-helix interaction between PlnE and PlnF, resulting in inactivation of plantaricin EF. By replacing the glycine residues with both large hydrophobic and large hydrophilic residues, one might also gain insight into if the hydrophobicity of the residues in these positions are of significance.

The tryptophan and tyrosine residues in PlnF presumably enhance stability due to membrane interface interactions with lipids in the target membrane. In order to investigate this, it was decided to substitute the tryptophan residue with tyrosine, phenylalanine, arginine and leucine residues. Tyrosine and phenylalanine are aromatic residues, and substitution of tryptophan with either of these residues is believed to not affect the positioning of the peptide in the membrane. Arginine is a positively charged, hydrophilic residue, which is thought to position itself in the more hydrophilic membrane surface. Leucine, on the other hand, is a hydrophobic residue, and is thought to position itself in the more hydrophobic interior of the membrane. Substitution of tryptophan with any of these two residues will most likely affect the positioning of PlnF in the membrane. Likewise, by substituting the aromatic tyrosines with either of the two other aromatic residues tryptophan and phenylalanine, the positioning of PlnF in the membrane will not be affected. By changing the tyrosines with either leucine (hydrophobic residue) or arginine (hydrophilic residue), it is believed that these residues will interact either with the interior or the exterior of the membrane, respectively.

5.4 Future Aspects

On the basis of the pPlnF100 plasmid and its mutant versions constructed in this project, structure-function analysis of PlnF can now be performed. The analysis may give further insight into the importance of GxxxG motifs and tryptophan and tyrosine residues in plantaricin EF.

Another future aspect is, as previously mentioned, optimalization of the purification procedure of PlnF. A higher yield of PlnF may be obtained by altering one or several of the parameters in the cation exchange chromatography, or by using other purification methods altogether.

6 Appendix

6.1 Abbreviations

ABC	ATP-binding cassette
CD	circular dichroism
DNA	deoxyribonucleic acid
DOPG	1,2-dioleoyl-sn-glycero-3-[phosphor-rac-(1-glycerol)]
DPC	dodecylphosphocholine
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
L. sake	*Lactobacillus sake*
LAB	lactic acid bacteria
LB	Luria-Bertani
LcnG	lactococcin G
LMGT	Laboratory of Microbial Gene Technology
MALDI	matrix-assisted laser desorbtion ionization
MCS	multiple cloning site
MRS	de Man-Rogosa-Sharpe
MS	mass spectrometry
NCDO	National Collection of Dairy Organisms
NMR	nuclear magnetic resonance
OD	optical density
PCR	polymerase chain reaction
PEG	polyethylene glycol
PlnE	plantaricin E
PlnF	plantaricin F
PlnI	plantaricin I
PlnJ	plantaricin J
PlnK	plantaricin K
TFA	trifluoroacetic acid
TFE	trifluoroethanol
TOF	time-of-flight
UV	ultra violet
(v/v)	(volume/volume)
(w/v)	(weight/volume)

Single and Three Letter Abbreviation for the Amino Acids

A	Ala	Alanine
C	Cys	Cysteine
D	Asp	Aspartic acid
E	Glu	Glutamic acid
F	Phe	Phenylalanine
G	Gly	Glycine
H	His	Histidine
I	Ile	Isoleucine
K	Lys	Lysine
L	Leu	Leucine
M	Met	Methionine
N	Asn	Asparagine
P	Pro	Proline
Q	Gln	Glutamine
R	Arg	Arginine
S	Ser	Serine
T	Thr	Threonine
V	Val	Valine
W	Trp	Tryptophan
Y	Tyr	Tyrosine

Abbreviations for the Triphosphate Nucleotides

(d)ATP	(deoxy) adenosine triphosphate
(d)CTP	(deoxy) cytidine triphosphate
(d)GTP	(deoxy) guanosine triphosphate
dTTP	deoxythymidine triphosphate
UTP	uridine triphosphate
dNTP	deoxynucleotid triphosphate

Single letter abbreviations for the Bases

A	Adenine
C	Cytosine
G	Guanine
T	Thymine
U	Uracil

6.2 Chemicals and Equipment

Chemicals	**Manufacturer**
Agar	Merck
Agarose	Merck
Ampicillin	CALBIOCHEM
100 bp DNA ladder plus	Fermentas
$CaCl_2$	Merck
Chloramphenicol	Sigma
dNTPs (dATP, dCTP, dGTP, dTTP)	Amersham Biosciences
Dpn I restriction enzyme	New England Biolabs
Erythromycin	Sigma
Ethidium bromide	Amersham Biosciences
Ethylenediaminetetraacetic acid (EDTA)	Sigma
Glucose	Sigma
Glycerol	VWR
Glycine	Sigma
1 kb DNA ladder	Fermentas
6xLoading Dye Solution	Fermentas
$MgCl_2$	AppliChem
MRS broth	OXOID
Mutagenic primers	Eurogentec S.A.
M17 broth	OXOID
NaCl	VWR
NaH_2PO_4	J. T. Baker

Na_2HPO_4	Merck
Peptone	Merck
Pfu DNA polymerase buffer	Fermentas
Pfu DNA polymerase	Fermentas
Polyethylene glycol (PEG)	Merck
2-propanol	Merck
SakP2 (endprimer)	GIBCO BRL
SakPC (sequencing primer)	GIBCO BRL
SP-Sepharose Fast Flow	GE Healthcare
Taq DNA polymerase buffer	Fermentas
Taq DNA polymerase	Fermentas
Trifluoroacetic acid (TFA)	Sigma
Tris base	Angus Buffers and Biochemicals
Tween 80 (polyoxyethylenesorbitan monooleate)	Sigma
Yeast Extract	Merck

Kits	**Manufacturer**
Nucleospin® Plasmid Kit	Machery-Nagel
GFX PCR DNA Purification Kit	GE Healthcare
DNeasy® Tissue Kit	QIAGEN

Equipment	Manufacturer
ÄKTA purifier system	GE Healthcare
Electroporation cuvettes (2 mm gap)	Genetronics
Gene Pulser electroporator	BIO-RAD
JA-10 Beckman rotor	Beckman
JA-14 Beckman rotor	Beckman
NanoDrop ND-1000	NanoDrop Technologies
0.20 µm non-pyrogenic sterile filter	Sarstedt
PCR cycler PTC-200	MJ Research
Quartz cuvettes, 0.1 cm	Hellma
Quartz cuvettes, 1 cm	Hellma
Resource RPC column (3 ml)	GE Healthcare
MR700 Microplate Reader	Dynatech

6.3 Primers

6.3.1 PCR Primers

Primer*	**Sequence****
SakPB:	5′-acactttatgcttccggctcgtatgttgtgt-3′
PlnFA:	5′-ccacgcgcgctataggcatggaaaactccacctgt aattgctgttacttc-3′
PlnEFim:	5′-cgacagtaatcgatcgtaaagtatattagcatagc-3′
Sp6:	5′-tatttaggtgacactatag-3′
T7:	5′-taatacgactcactataggg-3′
SakP2:	5′-agctatgaccatgattacgccaag-3′
AmpF:	5′-gctgtgattattacttttattaatgg-3′
AmpR:	5′-ccattaataaaagtaataatcacagc-3′
PlnEFXbaIF:	5′–cgacagttctagatcgtaaagtatattagcatagc-3′
PlnEFXbalR:	5′-gctatgctaatatactttacgatctagaactgtgc-3′

6.3.2 Mutagenic Primers

Primer*	**Sequence****
GxxxG-motifs	
PlnF(G30L)F:	5′-cattagcgctgtccgaTTattcatccacg-3′
PlnF(G30L)R:	5′-cgtggatgaatAAtcggacagcgctaatg-3′
PlnF(G30S)F:	5′-cattagcgctgtccgaAgTttcatccacg-3′
PlnF(G30S)R:	5′-cgtggatgaaAcTtcggacagcgctaatg-3′
PlnF(G30A)F:	5′-cattagcgctgtccgagCattcatccacg-3′
PlnF(G30A)R:	5′-cgtggatgaatGctcggacagcgctaatg-3′
PlnF(G30K)F:	5′-cattagcgctgtccgaAAattcatccacg-3′
PlnF(G30K)R:	5′-cgtggatgaatTTtcggacagcgctaatg-3′
PlnF(G30Q)F:	5′-cattagcgctgtccgaCAattcatccacg-3′
PlnF(G30Q)R:	5′-cgtggatgaatTGtcggacagcgctaatg-3′
PlnF(G34L)F:	5′-ggattcatccacTTatagttcaagccatc-3′
PlnF(G34L)R:	5′-gatggcttgaactatAAgtggatgaatcc-3′
PlnF(G34S)F:	5′-ggattcatccacAgTtagttcaagccatc-3′
PlnF(G34S)R:	5′-gatggcttgaactaAcTgtggatgaatcc-3′

PlnF(G34A)F:	5′-ggattcatccacgCatagttcaagccatc-3′
PlnF(G34A)R:	5′-gatggcttgaactatGcgtggatgaatcc-3′
PlnF(G34K)F:	5′-ggattcatccacAAatagttcaagccatc-3′
PlnF(G34K)R:	5′-gatggcttgaactatTTgtggatgaatcc-3′
PlnF(G34Q)F:	5′-ggattcatccacCAatagttcaagccatc-3′
PlnF(G34Q)R:	5′-gatggcttgaactatTGgtggatgaatcc-3′

Trp/Tyr-residues

PlnF(Y5W)F:	5′-ggagttttccatgcctGGagcgcgcgtgg-3′
PlnF(Y5W)R:	5′-ccacgcgcgctCCaggcatggaaaactcc-3′
PlnF(Y5R)F:	5′-ggagttttccatgccAGAagcgcgcgtgg-3′
PlnF(Y5R)R:	5′-ccacgcgcgctTCTggcatggaaaactcc-3′
PlnF(Y5L)F:	5′-ggagttttccatgcctTAagcgcgcgtgg-3′
PlnF(Y5L)R:	5′-ccacgcgcgctTAaggcatggaaaactcc-3′
PlnF(Y5F)F:	5′-ggagttttccatgcctTtagcgcgcgtgg-3′
PlnE(Y5F)R:	5′-ccacgcgcgctaAaggcatggaaaactcc-3′

PlnF(Y14W)F:	5′-cgttcggaataattGGaaaagtgctgttgg-3′
PlnF(Y14W)R:	5′-ccaacagcacttttCCaattattccgaacg-3′
PlnF(Y14R)F:	5′-cgttcggaataatAGAaaaagtgctgttgg-3′
PlnF(Y14R)R:	5′-ccaacagcacttttTCTattattccgaacg-3′
PlnF(Y14L)F:	5′-cgttcggaataattTAaaaagtgctgttgg-3′
PlnF(Y14L)R:	5′-ccaacagcacttttTAaattattccgaacg-3′
PlnF(Y14F)F:	5′-cgttcggaataattTtaaaagtgctgttgg-3′
PlnE(Y14F)R:	5′-ccaacagcacttttaAaattattccgaacg-3′

PlnF(W23Y)F:	5′-gttgggcctgccgattATgtcattagcgctg-3′
PlnF(W23Y)R:	5′-cagcgctaatgacATaatcggcaggcccaac-3′
PlnF(W23R)F:	5′-gttgggcctgccgatAgAgtcattagcgctg-3′
PlnF(W23R)R:	5′-cagcgctaatgacTcTatcggcaggcccaac-3′
PlnF(W23L)F:	5′-gttgggcctgccgattTAgtcattagcgctg-3′
PlnF(W23L)R:	5′-cagcgctaatgacTAaatcggcaggcccaac-3′
PlnF(W23F)F:	5′-gttgggcctgccgattTTgtcattagcgctg-3′
PlnE(W23F)R:	5′-cagcgctaatgacAAaatcggcaggcccaac-3′

*F=forward primer, R=reverse primer

**Mutagenic positions are in capital letters. The amino acid codon of the new amino acid is marked in green.

6.4 Media, Buffers and Agarose Gels

Media

LB-medium (0.5 l):

5 g NaCl
5 g peptone
2.5 g yeast extract

Dissolved in dH_2O to 500 ml.
Autoclaved at 121 ºC for 20 min.

LB-plates (0.5 l):

5 g NaCl
5 g peptone
2.5 g yest extract
10 g agar

Dissolved in dH_2O to 500 ml.
Autoclaved at 121 ºC for 20 min.
After cooled down to ~60 ºC erythromycin was added to a final concentration of 150 µg/ml.

GM17-medium (0.5 l):

18.6 g M17 broth

Dissolved in dH_2O to 500 ml.
Autoclaved at 112 ºC for 10 min.
Addition of Tween 80 to a final concentration of 0.1 % (v/v) and glucose to a final concentration of 0.4 % (w/v).

MRS- medium (0.5 l):

26 g MRS broth

Dissolved inn dH_2O to 500 ml.
Autoclaved at 112 ºC for 110 min.

MRS-plates (0.5 l):

26 g MRS broth
7.5 g agar

Dissolved in dH_2O to 500 ml.
Autoclaved at 112 ºC for 10 min.
After cooled down to ~60 ºC both erythromycin and chloramphenicol was added to a final concentration of 10 µg/ml.

MRSSM-medium (0.11 l):

5.2 g MRS broth
17.1 g sucrose
2 g $MgCl_2$

Dissolved in dH2O to 100 ml.
Sterilized by filtrating through 0.2 µm non-pyrogenic sterile filters.

Buffers

Buffer A (1 l):

2.48 g NaH_2PO_4
0.26 g Na_2HPO_4

Dissolved in dH_2O to 1000 ml.
Autoclaved at 121 ºC for 20 min.

Buffer B (1 l):

2.48 g NaH_2PO_4
0.26 g Na_2HPO_4
58.44 g NaCl

Dissolved in dH_2O to 1000 ml.
Autoclaved at 121 ºC for 20 min.

Buffer B with 6 M guanidine hydrochloride (100 ml)

0.25 g NaH_2PO_4
0.026 g Na_2HPO_4
5.84 g NaCl
56.12 g GuHCl

Dissolved in dH_2O to 100 ml.
Autoclaved at 121 ºC for 20 min.

Agarose gel

50 x TAE-buffer (1 l):

254 g Tris base
57.1 ml glacial acetic acid
100 ml 0.5 M EDTA (pH 8)
dH2O to 1 l

Autoclaved at 121 ºC for 20 min.

1 % agarose gel:

0.4 g agarose
50 ml 1 x TAE

Heated in a microwave until agarose was dissolved.
After cooled down to ~60 ºC 3 µl ethidium bromide was added.

7 Reference List

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