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Co-Immunoprecipitation with the Multi-Peptide Bacteriocin GarKS Immunity Protein

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Abstract

Garvicin KS (GarKS) is a bacteriocin recently discovered in *Lactococcus garvieae* belonging to a novel group of multi-peptide bacteriocins, whose receptor and mode of action is unknown. In this thesis heterologous expression of the immunity gene in *Lactococcus lactis* and *Staphylococcus aureus* was shown to provide immunity to GarKS in both species. In addition, the garvicin KS immunity protein (GakI) was shown to confer immunity also to aureocin A70 in *L. lactis*. Variants of GakI fused with a FLAG-tag did not change the normal function of this immunity protein.

The thesis further describes the successful optimization for co-immunoprecipitation (Co-IP) of GakI. A washing buffer using the combination of the detergent IGEPAL CA-630 at 1% and 0.575 M NaCl was developed. Using this buffer the immunity protein was shown to co-precipitate with three proteins at approximately 25, 27 and 52 kDa. Immunoprecipitate was analyzed by mass spectrometry but the three aforementioned proteins remains to be identified.

Genomic analysis of GarKS mutants was performed and revealed mutations in *ythA* and *cesR*, both likely involved in a general stress response against agents that perturb the cytoplasmic membrane. Further, proteomic analysis identified a high abundance of proteins involved in this response; YthC, YneH and FtsH in cells producing GakI and exposed to GarKS. Together they provide valuable insights into two little-understood general stress response systems in lactococci.

Sammendrag

Garvicin KS (GarKS) er et bakteriosin nylig oppdaget i *Lactococcus garvieae* som tilhører en særegen gruppe av fler-peptid bakteriosiner med en reseptor og virkemåte som er ukjent. Heterologt uttrykt immunitetsgen i *Lactococcus lactis* og *Staphylococcus aureus* ble vist å gi immunitet i begge arter med en økning i minimum inhiberingskonsentrasjon på henholdsvis 130 og 14-ganger . Garvicin KS immunitetsproteinet (GakI) ga også immunitet mot aureocin A70 i *L. lactis* med en 97-gangers økning eller mer. GakI med fusjonert FLAG-tag hadde liknende aktivitet.

I denne oppgaven fremstilles en optimaliert protokoll for ko-immunoutfelling av GakI. En vanskeprotokoll basert på en kombinasjon av det overflateaktive stoffet IGEPAL CA-630 på 1% og 0.575 M NaCl ble utviklet. Ved bruk av denne kombinasjonen ble tre proteiner ko-immunoutfelt med GakI med estimerte masser på 25, 27 og 52 kDa. Analyse ved hjelp av massespektrometri ble utført på immunoutfellingsprøvene, men proteinene lot seg ikke identifisere.

Helgenomanalyse av mutanter for GarKS ble utført og viste mutasjoner i *ythA* og *cesR*, som begge høyst sannsynlig er en del av et generelt stress respons system mot stoffer som forstyrrer cellemembranen. Videre proteomanalyse av kulturer som produserer GakI utsatt for GarKS viste en høy mengde YthC, YneH og FtsH, som alle er en del av denne responsen. Sammen gir dataene en innsikt i to stress respons systemer i laktokokker som er lite forstått.

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Abbreviations

Co-IP	Co-immunoprecipitation
Psp	Phage shock protein
ces	Cell envelope stress
ECF	Extracytoplasmic function
PG	Peptidoglycan
LAB	Lactic acid bacteria
GakI	Garvicin KS immunity protein
ABC	ATP-binding cassette
HK	Histidine kinase
RR	Response regulator
TCS	Two-component (regulatory) system
CM	Cytoplasmic membrane
PAGE	Polyacrylamide gel electrophoresis
MIC	Minimum inhibitory concentration
CM	Cytoplasmic membrane
MS	Mass spectrometry/mass spectrometric

1 Introduction

1.1 General Introduction

Bacteria are by far the most successful organism on earth, living on most of its surface and at the extremes. This success is, without doubt, due to their ability to adapt to their surrounding environment. A striking example is the observation that a motile bacterium will run and tumble towards a nutrient gradient, known as chemotaxis, detecting concentrations as low as 3 nM of ligand in the case of *Escherichia coli* [1]. However, most processes involved in adaption – maintaining homeostasis – are far more subtle. The bacterium must respond to changes in osmotic pressure, temperature, salt, acid, moisture and nutrients. Information from outside the cell must be relayed across the cell membrane in order to induce a response. The cell respond with changes in gene expression.

Bacterial gene expression is initiated by the RNA polymerase protein complex (RNAP holoenzyme), a sigma (σ) subunit of the holoenzyme recognize promoter sequences for initiation of RNA synthesis. Bacteria have one primary "housekeeping" σ factor regulating the expression of essential genes during exponential growth, an example being σ^{70} of *E. coli*. As a response to external signals or changing conditions, many bacteria have extracytoplasmic function (ECF) σ factors that are released and compete with σ^{70} for RNAP. ECF σ-factors are normally sequestered by an anti-sigma factor, inducing conditions cause the ECF to release from the anti-σ factor, e.g. by proteolytic cleavage. The collection of genes regulated by the ECF sigma factor (its regulon) act as a response to the inducing signal. Some species have a few ECF σ factors with 7 for *E. coli* and *Bacillus subtilis*, or many with over 50 for *Streptomyces coelicolor* [2]. In addition

to ECFs, other transmembrane signaling systems known as one-/two-component systems are also present that regulate a set of genes (regulon). Some species, however, have no known ECF sigma factors, this appear to be the case for *Lactococcus lactis* subsp. *cremoris* MG1363 and possibly *Lactococcus lactis* subsp. *lactis* IL1403 [3]. No function has been proposed for the only ECF sigma factor of *L. lactis* IL1403 (SigX), which is not functional in *L. lactis* MG1363 [3]. These rely on one/two-component systems for sensing the environment and each other.

Bacteria in nature live together in complex communities competing for the same resources and niches (”niche space”). As a result, bacteria have evolved intricate systems for communication and warfare that are used to mount coordinated attacks against target populations. Antimicrobial compounds are synthesized by one population that specifically inhibits another. This has the advantage of securing resources for itself and genetic relatives. In some cases antimicrobial compounds are produced and secreted in a cell-density dependent manner, called quorum sensing [4]. Inducing peptides (IP) are produced and secreted to the surroundings, inducers can include the antimicrobial itself, e.g. nisin. The IP is sensed by other members of the population and activate expression of antimicrobial compounds only when a certain threshold of inducer is reached.

Of special interest is a class of antimicrobial compounds called bacteriocins, as they are ribosomally synthesized and potent at very low concentrations. Ribosomal products are easily manipulated by standard techniques of molecular biology. Although the first bacteriocins discovered and characterized were the colicins of *E. coli*, in this thesis the term will be used exclusively for those produced by Gram-positive (G+) bacteria. A change in interest to bacteriocins of G+ bacteria was driven in large part by financial incentives and because many bacteriocins of G- bacteria were lacking in many useful characteristics for applied science.

In contrast, bacteriocins from lactic acid bacteria (LAB) used in the dairy industry are promising and many governments already approve their commercial use, e.g. nisin as a food preservative (classified as GRAS by the FDA, Generally Recognized As Safe [5]). Bacteriocins are encoded by structural genes that may be post-translationally modified, but does not involve metabolites or secondary metabolites like with antibiotics. Bacteriocins are assumed to be safer for human consumption due to their abundance in much of our food and their proteinaceous nature.

A novel new bacteriocin with antimicrobial activity against human pathogens and food-spoilage bacteria was recently isolated from *Lactococcus garvieae* [6]. Understanding the mode of action of this bacteriocin will assist in designing real-life applications for this and other bacteriocins. A common theme with bacteriocins is that - while antibiotics interact with enzymes of target cells in a specific manner - such that minor alterations in the structure of the enzyme can lead to antibiotic resistance, bacteriocins largely target components within the cytoplasmic membrane of the cell envelope.

1.2 The Gram-Positive Cell Envelope

All bacteria are enclosed by a barrier that separate the internal cytoplasm from the surroundings. Most bacteria form two distinct groups with respect to how the cell envelope is structured; (i) a thick (30-100 nm) layer of peptidoglycan (PG) anchored to a cell membrane, forming the Gram-positive group [7]. (ii) A thin layer of PG anchored to the inside of an outer membrane in a periplasmic space formed by an inner membrane; the Gram-negative group. Being the interface to the exter-

nal environment, the cell envelope serves a multitude of essential functions for the cell. The cell envelope provides shape and structure, a turgor pressure, physical protection and facilitate transport of nutrients into, and waste out of the cell [8].

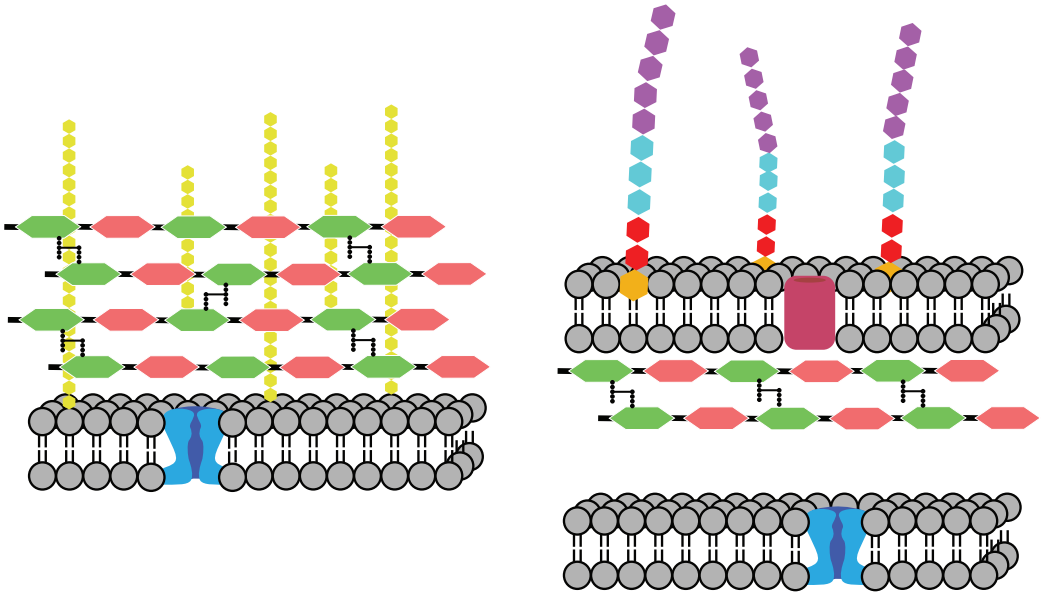

Figure 1.1: Schematic representation of the fundamental structural difference between the gram-positive (left) and the gram-negative cell envelope (right). Peptidoglycan is represented with NAM (N-acetylmuramic acid) in green and NAG (N-acetylglucosamine) in red. Lipoteichoic acid in G+ is illustrated as yellow hexagons originating from the lipid bilayer, or from NAM as wall teichoic acid. An integral membrane protein is in blue, while the protein in red depict a porin or ion-channel in the outer membrane.

Located in the membrane are numerous transport systems. Ion-channels and aquaporins maintain homeostasis in the cytoplasm, including mechanosensitive channels that can respond rapidly to sudden osmotic changes [9]. A conserved family of ATP-binding cassette (ABC) transporters are ubiquitous in both eukaryotes and prokaryotes, ABC-transporters couple the hydrolysis of ATP to the translocation of specific substrates. Many dedicated ABC-transporters for bacteriocins also have a dual function as a protease, the bacteriocin is translated as a precursor peptide with a ”double-glycine” motif which is recognized by the ABC-transporter and cleaved off concomitant with secretion [10]. However, for complex substrates

like proteins/peptides transporters of this family are usually specific for only one substrate [11]. Bacteria do not encode a transporter for every secreted protein or peptide, rather a general pathway of export is used. The protein/peptide is synthesized with an N-terminal signal sequence for recognition by either a sec pathway (sec: secretory) or tat (twin-arginine translocation) pathway for secretion. Proteins exported by the two pathways are said to be sec-dependent or tat-dependent, respectively. Tat-dependent proteins are translocated across the membrane in their folded state, while sec-dependent proteins are exported unfolded. *L. lactis* lack a Tat-pathway, thus the majority of secreted proteins are sec-dependent [12, 13]. The bacteriocin lactococcin 972 of *L. lactis* has been shown to be sec-dependent, though this is not usual for bacteriocins [14]. Another transport mechanism using phosphoenolpyruvate (PEP) is found in bacteria, this method of transport is especially implicated in the transport of sugars. A series of phosphorylations starting from PEP hydrolysis phosphorylates a sugar (e.g. glucose, mannose) with concomitant import, this system is also known as a phosphotransferase system due to the phosphorylation steps (e.g. mannose phosphotransferase/Man-PTS). In addition, gram-positive bacteria have sortases, and certain have an injectosome or a Type VII secretion system, but are beyond the scope of this thesis [15]. Different secretion systems are more common in G- bacteria, as they have two lipid bilayers, a periplasm and only a thin layer of peptidoglycan.

Peptidoglycan is the primary structural component of the bacterial cell wall. PG is a polymer of alternating N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM) monomers covalently linked at the β-1,4 position into long strands. Multiple layers of strands are cross-linked by a characteristic peptide to a structure resembling woven sheets. PG biosynthesis begin in the cytosol from the lipid II precursor molecule.

The Lipid II precursor is a NAG-NAM unit with the attached peptide linker, this "cargo" is anchored to the membrane by a lipid carrier undecaprenyl diphosphate (UPP). A flippase translocate the lipid II precursor across the membrane where it extends the PG polymer in a transglycosylation. PG strands are joined together by the peptide linker attached to NAG in a transpeptidation reaction, the amino acid composition, degree of cross-linking and length is often characteristic to a species. In *L. lactis* the structure is the pentapeptide Ala-Glu-Lys-Ala-Ala, linked to an adjacent pentapeptide from the Lys residue to the fourth Ala via a D-Asp [8]. The class of proteins catalyzing the transpeptidation have an active site that recognize the Ala-Ala moiety and are known as the penicillin-binding-proteins (PBP). Penicillin and its derivatives act as a D-alanyl-D-alanine analog but bind irreversibly, thus inactivating the transpeptidase. Lipid II molecules are continuously recycled to keep pace with cell wall synthesis and remodeling [16]. Following delivery of the "cargo" across the membrane by the lipid carrier, UPP is dephosphorylated by the UppP phosphatase to UP, which can re-enter the cycle to again be loaded with cargo [16]. In fact, UP is the carrier for both peptidoglycan synthesis and wall/lipo-teichoic acid synthesis [17]. UP availability in the cell is also the limiting factor in their biosynthesis [16].

Covalently linked to the PG are cell wall teichoic acids (WTA) that form strands extending beyond the cell, linked to the plasma membrane are lipoteichoic acids (LTA) that are embedded in the PG layer. Teichoic acids are typically repeating units of glycerol-phosphate, with the number of repeats and type of substitutions varying between species (se Figure 1.10 B). In addition to WTAs and LTAs, certain G+ bacteria are coated in an external polysaccharide (exopolysaccharide), which are loosely associated with the membrane (secreted) or covalently attached (CWPS; cell wall polysaccharide). Teichoic acids give the bacterial cell wall a negative charge and are involved in binding essential cations, recruitment and sta-

bilization of surface proteins, cell division and elongation [8].

1.3 Bacteriocins of Gram-positive Bacteria

Numerous strategies has developed in bacteria for the biosynthesis of bacteriocins, some are translated as inactive precursor peptides containing an N-terminal leader sequence while others are translated directly as the active bacteriocin. A precursor peptide subsequently undergo a process of maturation where the leader peptide is cleaved off with concomitant export that requires co-expression of a dedicated protease/transporter. The leader sequence serve at least two important functions, (i) recognition by enzymes involved in the processing and export, and (ii) keeping the bacteriocin inactive intracellularly. Leaderless bacteriocins are in their active form as translated and will necessarily require more stringent immunity, the benefit to the cell is a reduced energy cost associated with production. In contrast, some bacteria synthesize heavily modified bacteriocins that require numerous protein complexes to produce. Modified bacteriocins are more resistant to some proteases and harsh environments, and allow for more structural diversity. The high diversity of bacteriocins make classification difficult, but multiple classification schemes have been proposed.

Classification schemes of LAB/G+ bacteriocins used today are based on that proposed by Klaenhammer [18], he suggested four classes based on the structure and physical properties of the bacteriocins. Bacteriocins that are post-translationally modified, small (<5 kDa) and contain atypical amino acids, e.g. lanthionine, methyllanthionine and dehydro- amino acids were designated as Class I ("the lantibiotics"). Class II bacteriocins were differentiated from Class I by being un-

modified, heat-stable and larger (<10 kDa), this class was further subdivided: anti-Listerial pediocin-like subclass IIa. Two-component bacteriocins IIb. Cyclic peptides IIc, and linear non-pediocin-like class IId. Class III are large (>30 kDa), heat-labile, and often enzymatic (now bacteriolysins). Class IV was assigned for bacteriocins requiring a lipid or carbohydrate for activity. The latter two classes are beyond the scope of this work and will not be discussed further. There is no agreement on the classification of bacteriocins and numerous schemes have been proposed since. In addition, the recent discovery of multi-peptide bacteriocins calls for an expansion of current schemes. An updated classification adapted from Bali et al. [19] and Chi [20] will be presented here.

Class I bacteriocins follow the classification by Klaenhammer [18] and contain the lantibiotics, from "lanthionine-containing peptide antibiotics". Lanthionine is an unusual amino acid where two alanine residues have a thioether bond between their β-carbons, the presence of this residue or methyllantionine is the unifying characteristic of the Class I lantibiotics. Other characteristic residues that are added post-translationally are the dehydroalanine (Dha) and dehydrobutyrine (Dhb).

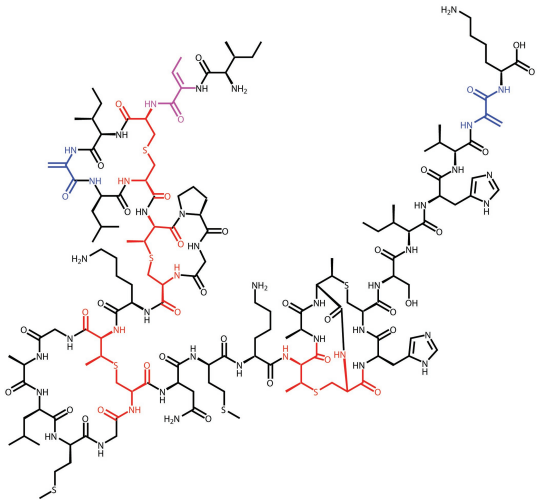

Figure 1.2: Structure of Nisin A, modified residues are colored. Dhb (pink), (β)-Lanthionine (red), and Dha (blue).

Lantibiotic biosynthesis is a costly process for the cell, as it requires multiple proteins for the modifications, regulation and export. As an example, the nisin gene cluster encode 11 proteins on three operons (*nisABTCIP*, *nisRK*, *nisFEG*); nisin precursor peptide NisA, NisB and NisC for post-translational modifications, a transporter or translocator NisT, NisP for cleavage of the leader peptide and NisR and NisK as biosynthetic regulators. Immunity mechanism involves the *nisI* and *nisFEG* products, NisI provides immunity by sequestering intracellular nisin before it reaches the cytoplasmic membrane and NisFEG by the active transport of nisin to the extracellular space [21]. NisR and NisK constitute a two-component system involved in the tight regulation of nisin biosynthesis, this system recognize nisin and activates expression of the other two operons, *nisRK* is expressed constitutively [22, 23]. Nisin and many other lantibiotics (e.g. mersacidin, epidermin, plantaricin C, lacticin 3147) inhibit peptidoglycan synthesis by interaction with the lipid II precursor as an initial anchor that forms a complex facilitating membrane pore formation.

Thiazolylopeptides (thiopeptides) are a class of >100 compounds believed to be antibiotics until very recently, as they are highly modified peptides, cyclical and containing the unusual heterocyclic thiazolyl moiety (see Figure 1.3). However, the biosynthesis of thiopeptides characterized to date originate from structural genes that are translated on the ribosome and hence bacteriocins [24, 25, 26, 27]. In addition, their biosynthesis does not appear to involve secondary metabolites [24]. This might not hold true for all thiopeptides. The best characterized members of this class are the Micrococcins P1 and P2, encoded by a 159 nt structural gene *tcl* (tcl; thiocillin) on a thiocillin gene cluster of 24 genes in *B. cereus* ATCC 14579 [24]. From the 52 amino acid propeptide, fourteen residues at the C-terminal undergo 13 post-translational modifications by at least six accessory proteins, including dehydratases homologous to those found in lantibiotic gene clusters [28]. Thiopeptides with 26-membered macrocycles like P1 and thiostrepton kill sensitive cells by binding to the ribosomal protein L11 and blocking proper interactions with elongation factor G (EF-G) causing inhibition of translation [29]. Thiopeptides with the larger 29-membered rings bind EF-Tu, blocking its interaction with tRNA (aminoacyl-tRNA) [30]. A novel target for a bacteriocin that demonstrate the unusual nature of this class. Producing cells encode an alternate variant of L11 that can substitute for the normal copy, this variant provides immunity against the bacteriocin as it does not bind the Micrococcin [29].

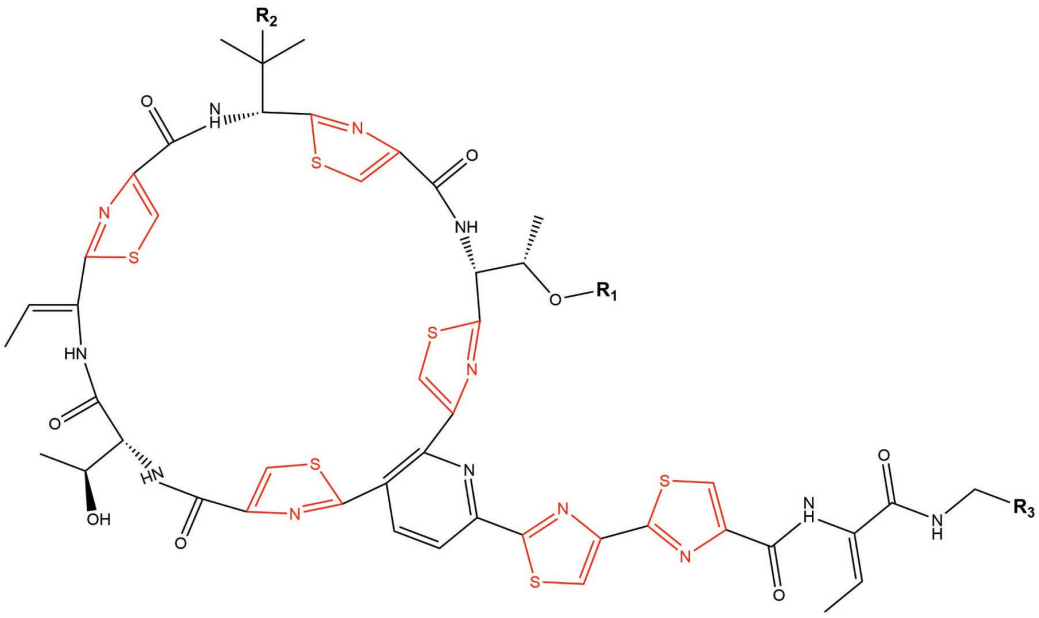

Figure 1.3: Base structure for micrococcin P1 and P2, thiocillin I-IV and YM-26618(3/4), each having a different combination of -H, -CH_3, -OH, acetyl or -$CH_2CH(OH)CH_3$ as substituents marked in bold (R). Micrococcin P1: R1 = H, R2 = H, R3 = -$CH_2CH(OH)CH_3$. The thiazole rings are indicated in red. The 26-membered macrocycle structure has been emphasized.

Class II is a large group that contain all the small unmodified bacteriocins, though some may have disulfide bridges or a circular structure. In this work the first two subdivisions for class II bacteriocins mentioned earlier are retained, but Iic will be the circular bacteriocins. The classification is further expanded with subdivision IId; non-pediocin single linear peptides, and IIe; multi-peptide bacteriocins.

Class IIa are the "pediocin-like" bacteriocins after the first and best studied member of this division pediocin PA-1, as they all share the same YGNGVXCX(4)C motif at the N-terminal end (see Figure 1.4). Bacteriocins in this group are between 37 and 55 amino acids long. Another unifying characteristic is their activity against *Listeria*. All pediocin-like bacteriocins are believed to form α-helices that embeds into target membranes, causing permeabilization, loss of the proton gradient and cell death. The producing cell expresses the bacteriocin structural

genes along with a cognate immunity protein, ABC transporter and in some cases an accessory protein. Immunity prevents disruption of the cell membrane and cell death of the producer. Enterocin P, sakacin A, pediocin PA-1 and penocin A of this class has been shown to target the mannose phosphotransferase (Man-PTS) system, Man-PTS is likely the target of most members of this class [31, 32]. A potential mechanism of action is described in more detail in the section on Class IId bacteriocins below. Structural studies of the carnobacteriocin B2, enterocin A and pediocin PA-1 immunity proteins reveal a four-helix bundle, with a disordered C-terminal. The flexible C-terminal arm is thought to be involved in immunity specificity [33].

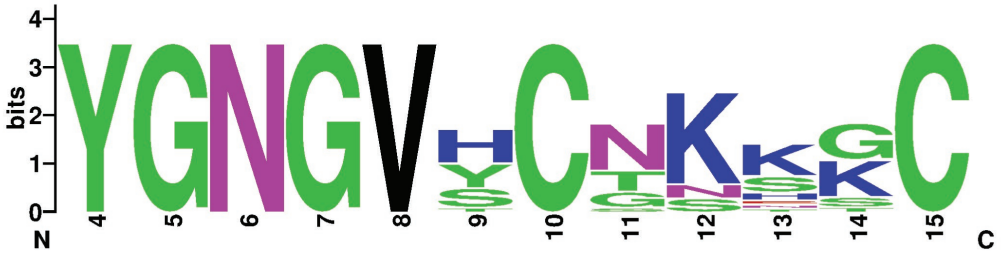

Figure 1.4: Sequence logo constructed from a multiple sequence alignment of Class IIa bacteriocins as classified by Bactibase. Acidocin A was excluded from the alignment as it has a YGNTGV sequence, Bavaricin A was excluded as multiple amino acids were uncertain.

The pediocin-like bacteriocins are synthesized with an N-terminal leader sequence containing a so-called "double-glycine" motif that is recognized by a dedicated ABC-transporter, with a few exceptions. Proteolytic cleavage near the double-glycine motif forms the mature peptide, and is coupled to the ABC-transporter for export. Bacteriocins without a dedicated transporter have a sec-type leader sequence, and are exported in a sec-dependent manner.

For pediocins like PA-1 with more than two cysteines, an accessory protein is necessary for forming the disulfide bonds as multiple combinations are possible [34]. Without the accessory protein the disulfide bonds are formed at random, giving a majority of structures with no antimicrobial activity. In this sense, the accessory protein has a chaperone-like function in that it helps ensure the correct structure (”folding”).

Class IIb members are the two-component bacteriocins that require two different peptides for optimal activity. When assayed separately, peptides of this class show inhibitory concentrations orders of magnitude higher than if assayed together. Peptides from different bacteriocins can complement each other if their sequence identity is high, e.g. enterocin 1071, lactococcin Q and lactococcin G [35]. This strongly support the notion that they act together as a single unit, further supported by the existence of only one cognate immunity protein. However, interaction studies have failed to see interactions of the two peptides in aqueous solution [36]. These bacteriocins are believed to assemble into an antibacterial unit upon interaction with the hydrophobic environment of the cell membrane and receptor components [36].

As was the case with one-peptide bacteriocins, two-peptide bacteriocins are translated with an N-terminal leader sequence. A double-glycine motif is recognized by a dedicated ABC-transporter and simultaneously cleaved and exported as the mature peptide. The leader sequence is cleaved at the C-terminal side of the double-glycine motif. They also tend to be cationic, hydrophobic/amphiphilic and of similar length.

The mode of action of Class IIb is the permeabilization of the cell membrane. Cells exposed to bacteriocins of this class have been shown to leak cations in a

specific manner, suggesting the formation of highly ordered pores/channels [36]. This does not, however, explain the narrow inhibitory spectrum of this class. Lactococcin G is only active against *Lactococcus*, and Plantaricin EF and JK only against some species of *Lactobacillus* and *Pediococcus* [37, 38].

A membrane protein UppP which is involved in the biosynthesis of peptidoglycan is important for Lactococcin G sensitivity [39]. This protein is involved in the dephosphorylation of undecaprenyl diphosphate (bactoprenol), a Lipid II precursor molecule [39]. A putative amino acid transporter of the APC superfamily is implicated in Plantaricin JK sensitivity [40]. Sensitivity of target cells to Plantaricin EF involves CorC, a membrane-bound Mg/Co efflux protein [41]. Recognition of lactococcin G and enterocin 1071 by their cognate immunity proteins depends on cellular components [42]. Initial interaction of the bacteriocin unit with specific domains of membrane proteins appear to be important for pore-formation. This is very likely to be the case also with other members of the class. Surface components on target membranes that may act as receptors have been elusive for most bacteriocins. Common to all Class IIb bacteriocins studied so far is a GX(3)G sequence motif that appear once or more in the amino acid sequence. The motif is thought to facilitate helix-helix interactions of the peptides within in the target membrane [43].

Immunity mechanisms for Class IIb bacteriocins are not well understood, but a metalloprotease of the CBPB family (pfam: PF02517) is implicated in immunity for some of the bacteriocins [44]. CPBP for "CAAX proteases and bacteriocin-processing enzymes", as they recognize a CAAX motif on target proteins in eukaryotes and are involved in bacteriocins in prokaryotes (previously Abi family). Class IIb bacteriocin loci that encode a protein of the CBPB family includes the plantaricins EF and JK, and sakacin 23K. Atypical of bacteriocin immunity, the

CPBP protein from these loci provide cross-immunity to the other bacteriocins. The mechanism of immunity is believed to involve proteolytic degradation of the bacteriocin itself, or by cleavage of parts of the receptor as to make it unrecognizable by the bacteriocin [44].

Class IIc bacteriocins are covalently linked at their amino acid chain, often at the termini, and thus form circular structures. They are hydrophobic, heat-stable and particularly resilient towards certain proteases. Circularization of the bacteriocin likely require accessory proteins, as this has been shown for garvicin ML. An accessory protein is probably also required by other members of this class, as AS-48, circularin A and carnocyclin A require at least 4 to 5 genes for biosynthesis and secretion [45]. At least nine bacteriocins of this subdivision are known, of which seven are from lactic acid bacteria. The structure has been determined for carnocylin A, which is a four-helix bundle. Most intriguing is the immunity protein for gassericin A, a small (53 aa), highly cationic and hydrophobic protein [46]. Nothing is known about how this protein confer immunity. Sensitivity to the circular bacteriocin garvicin ML involve interactions with a maltose ABC transporter [47].

Class IId are like those of Class IIa, but with no sequence similarity to the pediocins. This class has traditionally contained bacteriocins that did not belong in the other classes (not pediocin-like), and are therefore a heterogeneous group. Some studies have proposed subdivisions of this class separating the leaderless bacteriocins from those with a sec-type signal sequence (leader) and a division for sec-independent leader sequence (e.g. lactococcin A, B, M) using a dedicated ABC transporter. The mode of action and mechanism of immunity has been thoroughly studied for lactococcin A, and will be explained in more detail.

Lactococcin A (LcnA) is a narrow-spectrum bacteriocin with activity only against other lactococci, synthesized as a 75 amino acids propeptide with an N-terminal leader sequence. Removal of the leader sequence at a double-glycine motif give the mature 54 amino acid bacteriocin that is exported by a dedicated ABC transporter and accessory protein. As is the case with many other bacteriocins, LcnA kills target cells by permeabilization of the cytoplasmic membrane, this action is prevented in the native producer by a Lactococcin A immunity protein (LciA). Diep et al. [32] reasoned that the immunity protein had to interact (strongly) with the receptor in order to prevent the action of the bacteriocin. By fusing an epitope to the immunity protein LciA coupled with affinity purification, they were successful in co-purifying components of the receptor, a technique called co-immunoprecipitation. Purified together with LciA were two components of the mannose phosphotransferase system (Man-PTS IIC and IID), however, Man-PTS components co-purified only in the presence of the bacteriocin. This experiment showed convincingly that the Man-PTS is a receptor for lactococcin A, and that a LcnA-LciA-receptor complex protects the cell from the action of the bacteriocin (see Figure 1.5). Man-PTS is also the receptor for Garvicin Q, a bacteriocin with a broader inhibition spectrum that includes *Enterococcus* and *L. monocytogenes* [48]. Lactococcin Z also use the same Man-PTS receptor and is closely related to LcnA, but they share no cross-immunity [49]. This suggests that the bacteriocins target different domains on the receptor, a possible explanation for the strain specificity of many bacteriocins [49].

Three other bacteriocins of this class have a known receptor, namely enterocin K1 (EntK1), enterocin EJ97 (EntEJ97) and LsbB, as screening of resistant mutants for these bacteriocins all have mutations in the same (homologous) gene encoding a membrane-bound zinc-dependent metallopeptidase (RseP, Eep, YvjB) [50, 51]. The three bacteriocins share the motif KX(3)GX(2)PWE, and EntK1 and LsbB

have a similar structure; all having an amphiphilic α-helix at the N-terminal half with a more disordered C-terminal end [50].

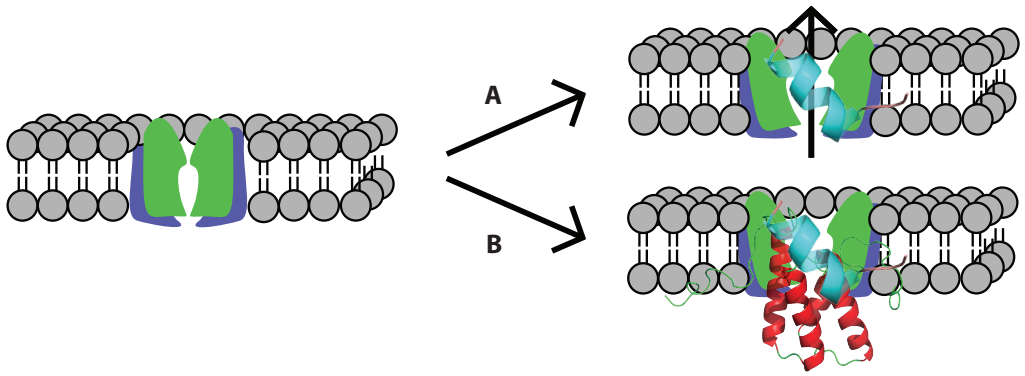

Figure 1.5: Proposed mechanism of immunity to lactococcin A. (A) LcnA binds Man-PTS (IIC in green, IID in red) inducing leakage of cytosolic contents. (B) Presence of LciA with LcnA prevents leakage by the formation of a ternary complex.

Lactococcin 972 (Lcn972) is a bacteriocin known to interact with Lipid II and to prevent septum formation in lactococci. The 66 aa bacteriocin has a structure of sandwiched antiparallell β-sheets and is known to act as a homodimer [52]. As is common with bacteriocins, Lcn972 has a very narrow inhibitory spectrum, yet Lipid II is universal in bacteria. Receptors responsible for this specificity have not been identified, but Lcn972 has been shown to activate a two-component cell envelope stress response (ces) system in *L. lactis* MG1363 [53].

Class IIe contain all multi-peptide bacteriocions, e.g. three or more different peptides are necessary for optimal activity. Only five bacteriocins of this class are recognized to date; the three-peptide bacteriocins garvicin KS (GarKS), cereucin V (CerV) and cereucin X (CerX) and the four-peptide aureocin A70 (AurA70) and cereucin H (CerH) [6]. However, CerH could be considered a three-component

bacteriocin as one of the peptides appear to be redundant with respect to antimicrobial activity [6]. Prior to the discovery of garvicin KS, AurA70 was the only multi-component bacteriocin that had been characterized. CerV, CerX and CerH were discovered in the *B. cereus* genome by sequence identity searches of the GarKS peptides against sequence databases. Unsurprisingly, GarKS share a high sequence identity with Cer-peptides, but also AurA70 share a high identity with the other multi-peptide bacteriocins (See Figure 1.6). Peptides of this class are relatively small at 26-34 aa, has a high hydrophobic character and are cationic (charge of 2-5 at physiological pH). Nothing is known about the mode of action or mechanism of immunity of this class of bacteriocins. GarKS and its cognate immunity protein GakI are central to this thesis and will be covered more in-depth below.

```
AurA      MGKLAIKAGKIIG----GGIASALGWAAGEKAVGK   31
GakA      MGAIIKAGAKIVGKGVLGGGASWLGWNVGEKIWK-   34
CevA      MGAVVKGGLKIIG----GTAASWLGWEAGTRIWK-   30
CehB      MGALVKGGLKLIG----GTAASWLGWEAGERVWK-   30
          ** :   . *::*    *  ** *** .* :

CehC      MGAIIKGGLKLV----GGGAAGGFTYGGLKKIFG    30
GakB      MGAIIKAGAKIIGKGLLGGAAGGATYGGLKKIFG    34
AurB      ----MGAVAKFLGKAALGGAAGGATYAGLKKIFG    30
CevB      ----MGAAVKMLGKAFAGGVAGGATYGGLKKIFG    30
              : .  *::     **.*** **.*******

CevC      MGAVVKGALKIIGGGAASGGAVYGLERIFGR-      31
AurD      MGAVIKVGAKVIGWGAASGAGLYGLEKIFKK-      31
GakC      MGAIIKAGAKIVGKGALTGGGVWLAEKLFGGK      32
CehD      MGAIIKGAAKVLGKGAATGGVIYGLEKLFR--      30
          ***::* . *::* ** :*. ::  *::*
```

Figure 1.6: Multiple sequence alignment of Class IIe bacteriocins most similar to the GarKS peptides. Cereucin X has been excluded from the alignment, as it appears more distantly related than the others.

1.4 Garvicin KS

GarKS was discovered by Ovchinnikov et al. [6] from the screening of a large collection of over 1800 isolates of lactic acid bacteria collected from farms in Kosovo. The authors describe the screening for broad-spectrum bacteriocin producers by using distantly related bacteria as indicators, followed by a second round of indicator strains of known bacteriocin producers. Ten samples of *Lactoccocus garvieae* met the criteria and were investigated further. By amino acid sequencing, MS-analysis and bioinformatics, the bacteriocin was revealed as a three-peptide bacteriocin encoded by the structural genes *gakA*, *gakB* and *gakC* encoding peptides of of length 34, 34 and 32 amino acids respectively. The function of GarKS is confirmed by chemical synthesis and MIC assays, which found the highest antimicrobial activity at equimolar concentrations.

The gak locus also contain an immunity protein GakI with homology to the AurI immunity protein, and a protein of the ABC-transporter family. The putative transporter GakT contain the conserved MdlB domain; an ABC-type multidrug transport system, ATPase and permease (defense mechanism).

The individual peptides of GarKS share significant sequence identity at the N-terminal (see Figure 1.7). The high sequence identity at the N-terminal of each peptide likely serve as a signal sequence recognized by the cognate transporter. No further processing of the peptides occurs following translation, all are leaderless. Amino acid substitution of the tryptophan residue at position 26 in GakA with alanine abolishes the antimicrobial activity of both the GakA peptide alone and the GarKS combination.

Table 1.1: Examples of bacteriocins from gram-positive bacteria according to the classification presented, including some physicochemical properties of the peptides. Peptides of multi-peptide bacteriocins are separated alphabetically, e.g. α / β, or A / B / C / D

Classification	Example	Length (aa)	Charge (pH 7)[1]	% Hydrophobic residues[2]	Producer
Class I					
	Nisin A	34	2.9	32	*L. lactis* subsp. *lactis*
	Pep5	34	7.8	32	*Staphylococcus epidermidis*
	Lacticin 3147	30 / 29	-1.2 / 1.8	30 / 38	*L. lactis* subsp. *lactis*
Thiazolylopeptides					
	Micrococcin P1	14	-0.4	7	*Bacillus cereus* ATCC 14579
	Micrococcin P2		-		
	Thiostrepton		-		
Class IIa					
	Pediocin PA-1	44	3	25	*Pediococcus acidilactici*
	Sakacin P	43	2.1	30	*Lactobacillus sakei*
	Enterocin A	47	3.8	30	*E. faecium* [54]
Class IIb					
	Lactococcin G	39 / 35	4.1 / 4	33 / 43	*L. lactis* subsp. *lactis*
	Plantaricin EF	33 / 34	5.1 / 3.2	39 / 47	*Lactobacillus plantarum* [55]
	Lactococcin Q	39 / 34	5.1 / 4	36 / 41	*L. lactis*
Class IIc					
	Carnocyclin A	60	4	53	*Carnobacterium piscicola*
	Garvicin ML	60	5	60	*Lactococcus garvieae*
	Enterocin AS-48	70	6	53	*Enterococcus faecalis*
Class IId					
	Lactococcin A	54	1.3	37	*L. lactis* subsp. *cremoris*
	Lactococcin 972	66	4.4	26	*L. lactis* subsp. *lactis*
	Enterocin EJ97	44	6	48	*Enterococcus faecalis*
Class IIe					
	Garvicin KS	34 / 34 / 32	4 / 5 / 4	50 / 47 / 50	*Lactococcus garvieae*
	Aureocin A70	31 / 30 / 31 / 31	4 / 4 / 4.1 / 4	52 / 53 / 42 / 52	*Staphylococcus aureus*
	Cereucin H	26 / 30 / 30 / 30	2 / 2 / 4 / 4	50 / 50 / 42 / 50	*Bacillus cereus*

[1] Charges are calculated based on the sequence and not structure, modified peptides might differ.
[2] Hydrophobic residues are Phe, Ile, Leu, Met, Val, Try, Ala and Pro.

```
GakB        MGAIIKAGAKIIGKGLLGGAAGGATYGGLKKIFG    34
GakA        MGAIIKAGAKIVGKGVLGGGASWLGWNVGEKIWK    34
GakC        MGAIIKAGAKIVGKGALTGGGVWLAEKLFGGK--    32
            ***********:*** * *..
```

Figure 1.7: Multiple sequence alignment of the three GarKS peptides. The first 21 residues are highly conserved between all three peptides. (Clustal Omega).

The immunity protein GakI is 142 aa (16.9 kDa), cationic (charge of 6 at physiological pH), hydrophobic (56% hydrophobic residues) and predicted to have four transmembrane helices (see Figure 1.8). Interestingly, a four-helix bundle is a common structural motif among bacteriocin immunity proteins. The immunity proteins for enterocin A, carnobacteriocin B2, mundticin KS, pyogenecin, pediocin PP-1 and lactococcin A are all a four-helix bundle (see Figure 1.9) [56, 57, 58, 59, 60, 61]. The significance of this structural motif is not known.

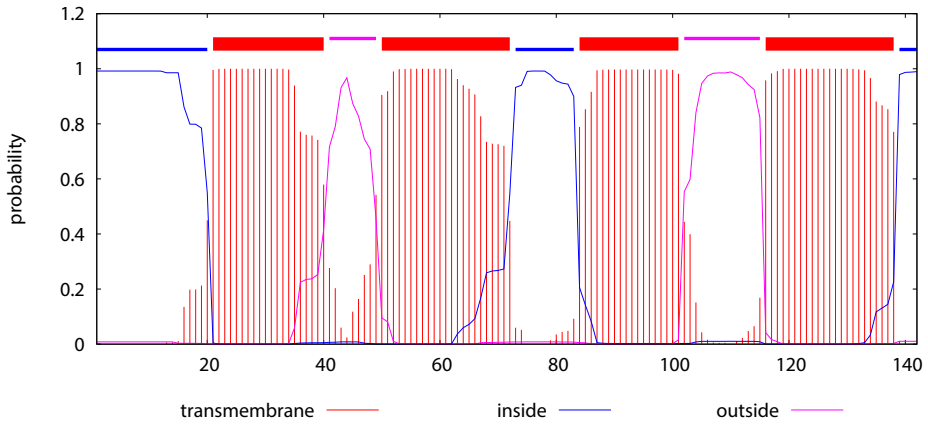

Figure 1.8: Predicted transmembrane topology of GakI, red bars indicate a transmembrane helix (TMHMM Server v. 2.0, Jpred4).

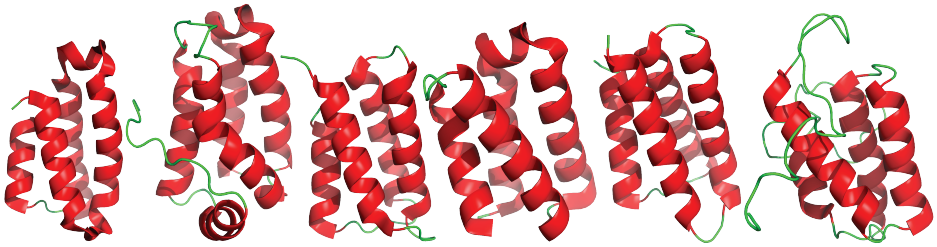

Figure 1.9: Immunity proteins for which the structure has been resolved. Immunity protein for (from left to right) enterocin A, carnobacteriocin B2, mundticin KS, pyogenecin (SPy2152), pediocin PP-1 and lactococcin A. (PDB ID: 2BL7, 1TDP, 2ZRR, 2FU2, 2IP6, 5LFI)

Whole-genome sequencing of *L. lactis* IL1403 mutants with reduced sensitivity to GarKS have mutations in *ythA*; encoding a PspC-domain containing protein (phage shock protein C) [20]. The *ythA* gene is annotated as being part of a *ythCBA* operon in *L. lactis* by ProOpDB and shown to be up-regulated during stress conditions [62, 63]. Further, *ythCBA* is part of, and regulated by, a cell envelope stress two-component regulatory system CesSR [53]. Such regulatory systems are frequently involved in adaption to antimicrobial resistance, often by modifying the cell wall [64].

1.5 Cell Wall Modifications in Antimicrobial Resistance

The negative charge on the bacterial cell surface due to the presence of teichoic acids is exploited by bacteriocins that are cationic, as the positively charged peptide will more easily adsorb to the oppositely charged cell surface [65]. However, the bacterial cell wall is a highly dynamic structure that undergo continuous remodeling and modifications. Enzymes involved in cell wall modifications alter the physicochemical properties of the cell surface, such changes are frequently involved in adaption to antimicrobial resistance [66]. This is well exemplified in *L.*

lactis resistant to the cationic bacteriocin nisin, overexpression of cell wall modifying enzymes is central in the mechanism of resistance to this antimicrobial [67]. Lipoteichoic acids are modified by the addition of D-alanine by DltC, a product of the *dltABCD* operon, which is up-regulated in nisin resistant mutants (see Figure 1.10 B). The D-alanylation provides a positive charge to the teichoic acids, allowing for tighter packing of the cell wall and possibly trap cationic bacteriocins before reaching the cytoplasmic membrane [65, 68]. The native producer of the bacteriocin bactofencin A encode a DltB homologue as an immunity protein. DltB translocates D-alanine across the membrane for incorporation into TA [69].

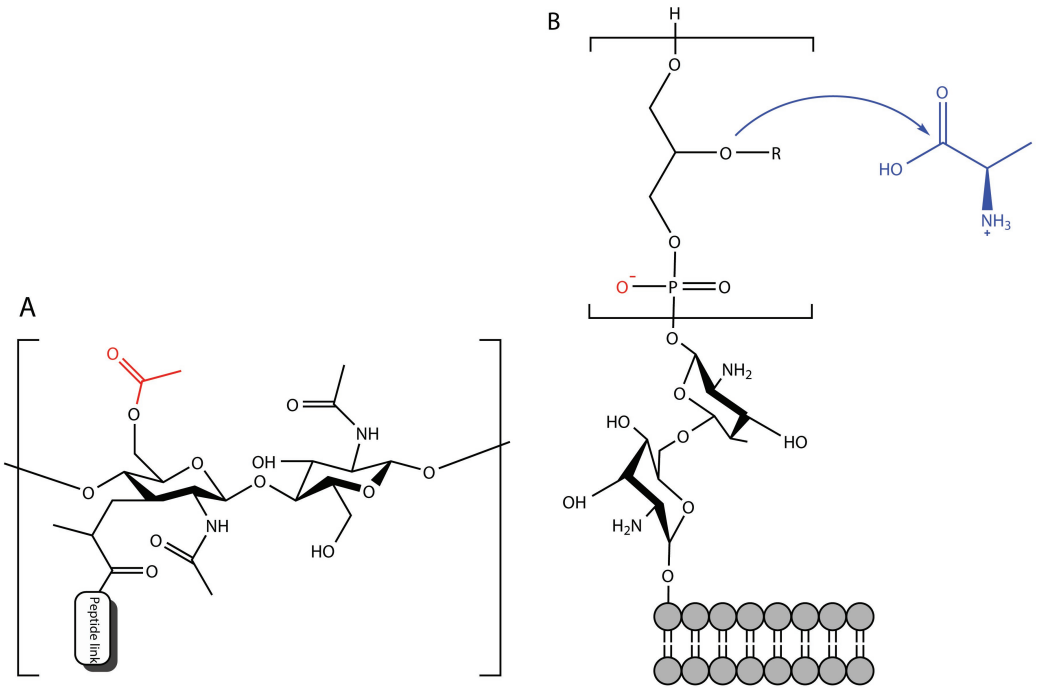

Figure 1.10: (A) Structure of a NAG-NAM unit showing the C-6 O-acetylation in red. (B) General structure of LTA in Lactococci with the negative charge on the phosphate in red, D-alanylation step shows in blue. The positive charge on the primary amine of the D-alanine partially cancel the positive charge on nearby phosphate, this contributes to reduced electrostatic repulsion.

Another modification associated with increased tolerance to nisin and other antimicrobials is the O-acetylation of peptidoglycan, catalyzed by OatA (YvhB in *L. lactis* [70]). This enzyme catalyze the addition of an acetyl to the C-6 hydroxyl group of NAM (see Figure 1.10 A) [71]. The mechanism by which O-acetylation protects the cell from nisin is not known, but is believed to improve the general integrity of the membrane [72]. OatA is particularly involved in the resistance to lysozyme, an enzyme which is part of the innate immune system of animals. Lysozyme catalyze the hydrolysis of PG, O-acetylated PG is less prone to hydrolysis by lysozyme due to steric hindrance [73]. Unmodified PG of gram-positive bacteria is otherwise relatively permeable to small molecules ($<$25 kDa), such that antimicrobial compounds like bacteriocins can reach the cell membrane [74]. Many of the aforementioned cell wall modifying enzymes are positively regulated by two-component systems that detect stress conditions.

1.6 The CesSR Two-Component System

A two-component signal transduction system is comprised of a sensor histidine kinase (HK) and response regulator (RR) pair; the sensor histidine kinase has an input domain that interacts with a signal to trigger autophosphorylation of its transmitter domain [75]. Phosphorylation of the transmitter domain activates the HK, which then preferentially phosphorylates a cognate RR. The typical response regulator is a multidomain protein with a receiver domain that is phosphorylated and an effector domain; a DNA binding transcription activator or repressor. The result of activation is the modulation of genes required by the bacterium to respond to the input signal. A majority of sensor histidine kinases are membrane proteins with the input domain exposed to extracytoplasmic space, allowing the

cell to relay information from the environment into the cell. Many bacteriocins are produced in response to an inducing factor sensed by a cognate two-component regulatory system.

It is believed that most sensor histidine kinases function as homodimers, though little is known about the mechanism by which the signal is transmitted across the cytoplasmic membrane [76]. A piston-like mechanism is believed to be involved in the HKs CitA and DcuS, a citrate sensor in *Klebsiella pneumonia* and a four-carbon dicarboxylate sensor in *E. coli* respectively. In this mechanism one of the transmembrane helices is pulled towards the periplasmic side in the ligand-bound state, exposing residues that could be involved in autophosphorylation and subsequent phosphotransfer [77, 78]. A response regulator in its activated state binds to DNA in the promoter region and recruit RNA polymerase, but also RNA-binding, protein-binding and enzymatic response regulators have been characterized [79].

O'Connell-Motherway et al. [80] identified six two-component systems in *L. lactis* MG1363 that the authors named System A-F. System D would later be implicated in the cellular response to Lcn927, as the putative histidine kinase KinD and response regulator RrD were up-regulated in response to the bacteriocin [53]. All the genes regulated by this TCS encode membrane proteins and proteins likely involved in the biogenesis, modification and maintenance of the cell wall and membrane proteins. TCS-D was renamed CesSR (Cell Envelope Stress Sensor / Regulator) due to this purported role.

The sensor histidine kinase CesS contains an ATPase domain and a dimerization/phosphoacceptor domain (pfam: PF2518, PF07730), the same two domains are found in HKs of other well-characterized stress response related TCSs [81, 82]. LiaS and VraS of *B. subtilis* and *S. aureus* respectively share the same pre-

dicted transmembrane topology and 31% sequence identity with CesS. CesR contain a LuxR-type DNA-binding helix-turn-helix motif and a response regulator receiver domain (pfam: PF00196, PF00072). CesR share 52% sequence identity with LiaR and VraR, the RRs of the aforementioned HKs.

One of the highest up-regulated operons upon exposure to Lcn927 was *llmg2164-2163*, encoding close homologs of the *L. lactis* IL1403 YthC and YthA at 91% and 98% sequence identity respectively. Llmg2164 and YthC both contain the same DUF4097 (DUF: domain of unknown function) conserved domain, YthA and Llm2164 both have the conserved PspC-domain.

1.7 The Phage Shock Protein Response

A phage shock protein was first described in *E. coli* by Brissette et al. [83], where they observed a very high expression of a 25 kDa protein caused by a filamentous phage infection. This protein would become one of the most abundant in the cell after prolonged expression of the phage integral membrane protein IV (pIV). This protein was named Psp, for phage shock protein. Mutated forms of pIV that are cytoplasmic did not induce expression of *psp*, but extreme osmotic and heat shock did as well as high concentrations of ethanol. Later characterization of the *psp* would reveal *psp* as the first gene, denoted *pspA*, of a larger operon of 4 genes; *pspA-E*. Another two genes *pspF* and *pspG* were later shown to be involved in the same system, but transcribed from separate promoters.

Deletion mutants of *pspA* have a constitutively activated Psp-response even in the absence of inducing conditions, while either *pspB* or *pspC* deletions significantly reduce the natural psp-response [84, 83, 85]. Taken together, the findings

suggest cooperative activation by PspB and PspC, while PspA negatively regulates the response. Constitutive activation of the Psp-system in the absence of an inducing signal required a transcription activator, which was identified as PspF. Protein-protein interactions of the psp-proteins was determined by Adams et al. [86] that demonstrated an interaction between PspA and PspB, and between PspA and PspC, but the PspA-PspB interaction could only be detected when all Psp-proteins were overexpressed.

The model of the Psp-system that emerged in *E. coli* was one of an inner membrane (IM) repair system, where perturbations in the IM are countered by recruitment of PspA (see Figure 1.7). PspBC complexes are likely sensors responsible for detecting and recruiting PspA, possibly by conformational changes induced by a loss of IM integrity (e.g. changes in curvature or thickness) [87]. There is also evidence that the PspA localizes spontaneously to areas of the membrane with induced stress like the deleterious effects of pore-forming protein complexes in the IM (e.g. secretins) [87]. Structural studies on PspA show that it likely exist as a large >1 MDa complex (36-mer) that is capable of reducing proton leakage across a membrane directly [88, 89].

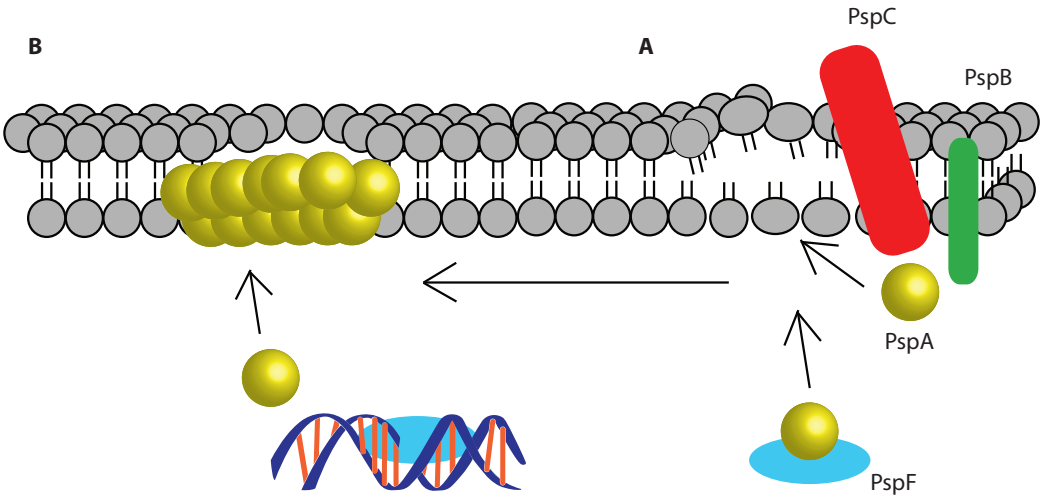

Figure 1.11: Schematic of a working model of the Psp-system in *E. coli*. (A) PspA disassociates from the inhibitor PspF upon perturbation of the membrane, the stress is also sensed by PspBC which ”donates” or recruits PspA to the site. (B) PspA oligomerizes in the site of CM stress to prevent leakage of ions and loss of the membrane potential. PspF positively regulates the Psp-operon once disassociated, producing more PspA. Adapted from McDonald et al. [87].

Screening of *Yersinia enterocolitica* mutants with reduced virulence in mice lead to the discovery of a Psp-system homologous to that in *E. coli* [90]. The reduced virulence was attributed to a growth deficiency in cells expressing the Type III secretion system without an intact psp-response [90]. A connection between the Psp-system and virulence reinvigorated interest in the system and its homologs, homology searches found that psp genes are widely conserved. Of most interest to *L. lactis* and the CesSR is the LiaFSR TCS of the gram-positive *Bacillus subtilis*, where the TCS appears to coordinate a Psp-like response (Lia: Lipid II-cycle interfering antibiotics system). In this system LiaF act as a negative regulator by preventing phosphorylation of LiaR by LiaS in the absence of inducing signals [91]. In *B. subtilis* the putative sensor LiaS activates the response regulator LiaR that in turn, induces expression of a *liaIH* operon, encoding *liaH* – a *pspA* homologue [91]. Similarly, CesSR activation in *Lactococcus* induce expression of a Psp-system [53]. However, the psp protein Llmg2163 induced in *Lactocco-*

cus has a PspC domain. The protein with a PspC domain in *B. subtilis* is YvlC, part of a *yvlABCD* operon encoding small hydrophobic transmembrane proteins and a large putative cytosolic protein YvlB with the same conserved domain as llmg2164. Yvl proteins are regulated by the cell envelope stress responsive ECF σ^W.

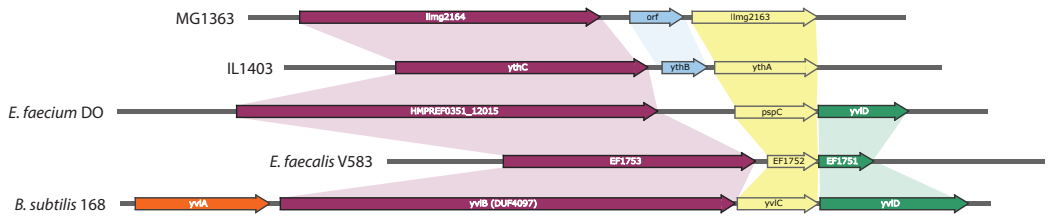

Figure 1.12: Putative psp locus of some select species/strains. Proteins containing a DUF4097 (PF13349) are colored in purple, PspC-proteins (PF4024) are in yellow and blue. Open reading frames in green encode proteins of a Mycobacterial 4 TMS phage holin superfamily (PF04020). Genes are not drawn to scale, e.g. YvlB: 365 aa and EF1753: 533 aa.

The psp-system described in *L. lactis* MG1363 are the *llmg2163-2164* genes, *llmg2163* encoding a 154 aa protein predicted to have a single transmembrane region and a PspC domain. Llmg2164 is 371 aa with a YvlB and adhesin-like conserved domain (COG3595, pfam DUF4097). The two genes are near identical in *L. lactis* IL1403 and named *ythA* and *ythC* respectively, but a third gene *ythB* is annotated preceding *ythC*; also having a PspC domain. Nothing is known about the structure and function of the psp-proteins in *L. lactis*, they are up-regulated in phage infection and osmotic shock just like those in *E. coli*, starvation, overproduction of membrane proteins and upon exposure to some cell wall active antimicrobials like nisin [63, 53, 67, 92].

1.8 Aim of Thesis

Garvicin KS (GarKS) is a new bacteriocin with promising application in the control of microorganisms, potentially from food preservation to clinical treatments. Bacteriocins like GarKS could be an important addition to our pharmaceutical arsenal in an era of increasing antibiotics resistance. A strategy is to use bacteriocins in combination, or in addition to antibiotics, to simultaneously target multiple components of the bacterial cell. Understanding the target and mode of action of GarKS will aid in devising such strategies for this bacteriocin. *Lactococcus lactis* IL1403 is a well-characterized lactic acid bacterium (LAB) with a high sensitivity towards GarKS. In addition, the full genome and proteome is publically available and extensively annotated - making it an ideal candidate for studying GarKS.

GarKS belong to a small group of only five multi-peptide bacteriocins, where the presence of all peptides are required for optimal activity. Three peptides of high sequence similarity constitute GarKS (GakA-C), only thirteen residues at the C-terminal show appreciable variation. The mode of action is unknown, but believed to be receptor-mediated. Receptors for antimicrobial agents are commonly identified by the mutations unique to resistant mutants. However, all spontaneous mutants to GarKS generated so far have mutations in genes encoding a PspC-homologue. An unlikely candidate for the receptor of GarKS.

Production of bacteriocins by the bacterial cell requires at least two other components, an immunity protein that protects the native producer and a transporter that exports the peptide. The transporter and immunity protein for GarKS has both been identified and termed GakT and GakI respectively [6]. In the majority of cases the mechanism of immunity is unknown, however for some immunity

proteins the mechanism involves strong association with the receptor only in the presence of the bacteriocin [32]. By producing a tagged variant of GakI in *L. lactis* IL1403 while exposing the cultures to GarKS, any strongly associated proteins will be subject to co-immunoprecipitation.

Co-immunoprecipitation (Co-IP) is a powerful technique for resolving protein-protein interactions *in vivo*, a tag is fused to a protein which acts as a bait. When the bait is immunoprecipitated, any strongly interacting or bound proteins will co-precipitate. Analysis of co-precipitating proteins will help identify the receptor of this bacteriocin. Co-IP is a direct approach that has been used with success previously in identifying the receptor of the bacteriocin lactococcin A using the immunity protein as bait. By immunoprecipitation of the GarKS immunity protein, the aim is to identify the receptor of this bacteriocin.

2 Materials and Methods

2.1 Strains and Growth Conditions

L. lactis IL1403 and its derivatives were grown in M17 broth supplemented with 0.5% (v/w) glucose (GM17), *E. coli* was grown in LB-medium (Oxoid Ltd., UK) while *S. aureus* was propagated in BHI growth medium (Oxoid Ltd., UK). The appropriate antibiotic (see Table 2.1) was added to the medium prior to inoculation at a final concentration of 10, 100 and 5 μg/mL for *L. lactis*, *E. coli* and *S. aureus* respectively. Cultures of *E. coli* and *S. aureus* were incubated at 37°C on a shaker, *L. lactis* was incubated at 30°C. 1% of inoculum of ON (overnight) culture was used in all experiments.

A FLAG-tag has been genetically fused to the N-terminal of *gakI* in the strain FI6, and C-terminal in FI8.
The fused sequence 5'-GACTACAAAGACGATGACGACAAG-3' is translated to the FLAG-tag N-DYKDDDDK-C.

Native *gakI* and N-terminal FLAG-tagged *gakI* was expressed in *Staphylococcus aureus* HG001, in this work named HG23 and HG6 respectively. Correspondingly, *gakI* and FLAG-gakI were expressed in *S. aureus* RN4220; RN23 and HG6 respectively. Negative controls expressing the green fluorescent protein (GFP) was used for both *S. aureus* strains, HG-GFP as a negative control for HG001 and RN-GFP for RN4220 (see Table 2.1).

2.2 Cloning of Immunity Gene in *S. aureus*

The native *gakI* gene and N-terminal FLAG-tagged *gakI* was amplified from clone 23 and FI6 by PCR with primers in the combination listed in Table 2.1 containing SalI and EcoRI restriction sites. Resulting amplicons were purified from agarose gel using a PCR Clean-Up Kit (Macherey-Nagel Co., Düren, Germany), digestion of the amplicons were performed in 20 µL reactions, 0.7-1.9 µg amplicon with 1 µL SalI-HF and 1 µL EcoRI-HF (New England Biolabs, Beverly, MA) in CutSmart buffer. Digestion of the pLOW vector was performed similarly, 10 µL of pLOW was used. Digestion mixtures were incubated at 37°C for 45 min, digestion fragments of the expected size were purified from an agarose gel with a PCR Clean-Up Kit (Macherey-Nagel Co., Düren, Germany). Amplicon digests were ligated to the pLOW vector in 20 µL reactions at 16°C ON with T4 Ligase in T4 DNA Ligase buffer (New England Biolabs, Beverly, MA) using 3 µL of purified vector and 10 µL of amplicon digests. Ligation mixtures were transformed into *E. coli* IM08B [93] by calcium chloride heat-shock transformation.

Heat-shock transformation was performed as follows: Aliquots of 50 µL chemically competent cells were incubated on ice with 10 µL of ligation reaction mixture followed by 45 seconds of incubation at 42°C. 250 µL of LB-medium was swiftly added, and incubation continued at 37°C for an hour. Cells were plated on LB-agar containing 100 µg/mL ampicillin, colonies were screened by PCR and correct transformants verified by sequencing. Plasmids were isolated from transformants using EZNA Plasmid Mini Kit (Omega Bio-Tek, Doraville, CA) for transformation into *S. aureus* HG001 and RN4220.

Electrocompetent *S. aureus* had kindly been prepared by M. Kjos as follows; ON culture was diluted 1/10 in BHI (Oxoid Ltd., UK) and incubated at 37°C with

shaking for 30 minutes before being put on ice for 10 minutes. Cells were then washed in a 1:1 volume of cold $_{d}H_2O$ twice, collected by centrifugation between washes at 4°C and 4000 × g for 10 minutes. Cells were washed further in cold, sterile 10% glycerol three times, first in a volume of 1/10, then 1/25 and lastly 1/200 of the volume of the cell suspension. Aliquots of 50 µL was stored at -80°C for later electroporation.

Electrocompetent cells were thawed on ice for 5 minutes, followed by 5 minutes at room temperature. Cells were collected by centrifugation at 5000 × g for 1 minute and resuspended in twice the volume 10% glycerol with 500 mM sucrose. Electroporation was performed at 21 kV/cm, 100 Ω and 25 µF, using 50 µL of cell suspension with 0.4-0.7 µg of plasmid DNA. Transformed cells were swiftly added to 950 mL of TSB (Oxoid Ltd., UK) supplemented with 500 mM sucrose and incubated for 2 hours at 37°C before being plated on BHI containing 5 µg/mL erythromycin. Transformants were verified by colony PCR.

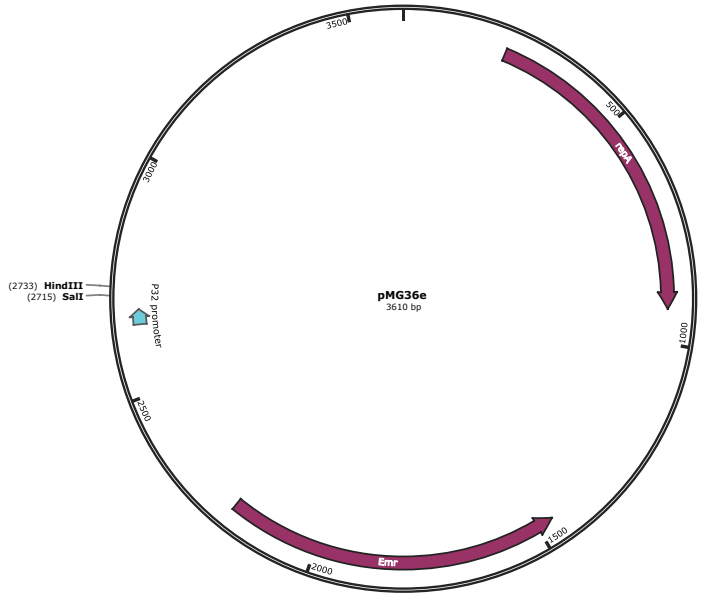

Figure 2.1: Physical map of the pMG36e vector for expression in lactococci. Emr: erythromycin resistance gene. *repA*: gene essential for replication. P32: constitutive promoter.

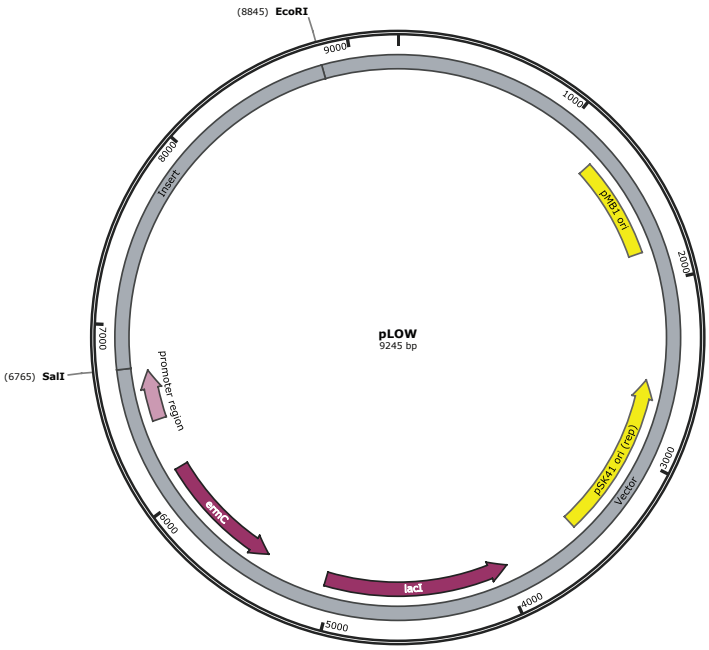

Figure 2.2: Staphylococcal shuttle and expression vector pLOW. ermC: Erythromycin resistance protein / Macrolide-lincosamide-streptogramin B resistance protein. *lacI*: lac repressor. pMB1: *E. coli*-specific origin of replication (origin of replicatoin *ori*). pSK41: staphylococcal ori.

Table 2.1: Strains, plasmids and primers used in this work.

Strain, Plasmid or Primer	Name in this work/clone	Description
Strain		
L. lactis subsp. *lactis* IL1403-pMG36e		*L. lactis* IL1403 with the plasmid pMG36e
	B100	Plasmid with no insert (empty)
	23	Native *gakI* gene
	FI6	N-terminal FLAG-tagged *gakI*
	FI8	C-terminal FLAG-tagged *gakI*
S. aureus HG001-pLOW		
	HG-GFP	Green fluorescent protein (GFP) gene
	HG23	Native *gakI* gene
	HG6	N-terminal FLAG-tagged *gakI*
S. aureus RN4220-pLOW		Restriction-deficient derivative of strain NCTC8325
	RN-GFP	GFP gene
	RN23	Native *gakI* gene
	RN6	N-terminal FLAG-tagged *gakI*
E. coli IM08B[2]		Δdcm, Ω(P_{help}-*hsdMS* P_{N25}-*hsdS*) (CC8) [93]
Plasmids		
pMG36e		Constitutive promoter P32, Ery^R
pLOW		IPTG-inducible promoter[1], Ery^R, Amp^R
Primers (5'-3')		
ATAAGCAAAAGGCAGCTGATCTC		Sequencing of pMG36e insert
GGTCGATCGAATTCGGTCCTC		Sequencing of pMG36e insert
TTGGTTGATAATGAACTGTGCT		PCR-screening and sequencing of pLOW insert
CTGCAGGTCGACCAATAAAACTAGGAGGAAATTTAAATGAAAAAAATAAACGATGAAAGAA		*gakI* amplicon from 23, SalI site (forward)
ATGAGAATTCATACTATTTTATCCATTTTACCCACAA		*gakI* amplicon from 23 and FI6, EcoRI site (reverse)
CTGCAGGTCGACCAATAAAACTAGGAGGAAATTTAAATGGACTACAAAGACGATGAC		*gakI amplicon* from FI6, SalI site (forward)

[1] Isopropyl β-D-1-thiogalactopyranoside.
[2] Intermediate cloning host. CC8: clonal complex 8. Ω: chromosomal insertion.

2.3 Minimum Inhibitory Concentration Assay

MIC assays were all performed by a serial twofold dilution of the antimicrobial agent in a 96-well microtiter plate, indicator strain was added to give a final 1/50 dilution of ON culture per well. Optical density (OD) at 600 nm was measured after a given time period or continuously on a SPECTROstar Nano® (BMG Labtech, Germany) microplate reader. Garvicin KS and aureocin A70 was obtained synthetically (Pepmic Co., Ltd., China) and prepared as a stock by dissolving 10 mg of each peptide together in 0.1% TFA to 10 mL, giving a concentration of 1 mg/mL per peptide (1 mg/mL). The ratio of each peptide constituting the bacteriocin is approximately 1 to 1 (e.g. 1:1:1 for GarKS and 1:1:1:1 for AurA70).

2.4 Co-Immunoprecipitation

Lysates used for Co-IP experiments were obtained from clones carrying *gakI* incubated for 7 hours in the presence of 0, 0.25 and 1 μg/mL GarKS for *L. lactis* IL1403 and 0 and 15 μg/mL for HG001. An empty vector or an untagged immunity protein was used as A controL for *L. lactis* IL1403, while a GFP-expressing clone was used for HG001. Samples obtained from the respective cultures are named with the concentration appended, e.g. FI6 1, HG6 15 (see Table 2.1).

Cells were harvested by centrifugation at 10,000 RPM (Avanti J-26 XP, JA-14 rotor, Beckman Coulter, Inc., Palo Alto, CA, USA) for 10 min, the cell pellet was washed once in cold TBS (50 mM Tris, 150 mM NaCl, pH 7.4) and stored at -80°C. The cell pellet was thawed on ice and thoroughly resuspended in cold TBS before being lysed using a French press (Aminco 20K cell FA-073, SLM Instru-

ments, Urbana, IL). The French press was used at an applied pressure of 15000 PSI and each sample was pressed three times and a flow rate of approximately 1.5 mL/min. The pressure cell was pre-chilled to 4°C and all work was performed on ice and in pre-chilled rotors. Lysate was subsequently clarified by centrifugation at 16000 × g for 15 min, total protein concentration was measured on a NanoDrop 2000 (Thermo Fisher Scientific, Wilmington, DE, USA) at 280 nm, aliquots of 10 mg of protein was stored at -80°C for use in Co-IP.

Clarified lysate was incubated overnight with Anti-FLAG M2 Affinity Gel (Sigma, St Louis, MO, USA) in TBS containing 1% IGEPAL CA-630 (Sigma, St Louis, MO, USA) and 0.575 M sodium chloride. Resin was subsequently washed three times in the same buffer, each time for 15 minutes on a rotator at 4°C and centrifugation at 5000 × g for 30 s. Bait protein was eluted from the resin by 3X FLAG-peptide (Sigma, St Louis, MO, USA) with vigorous shaking for 30 minutes, eluate was collected by centrifugation at 8000 × g for 30 s and analyzed by SDS-PAGE and silver staining.

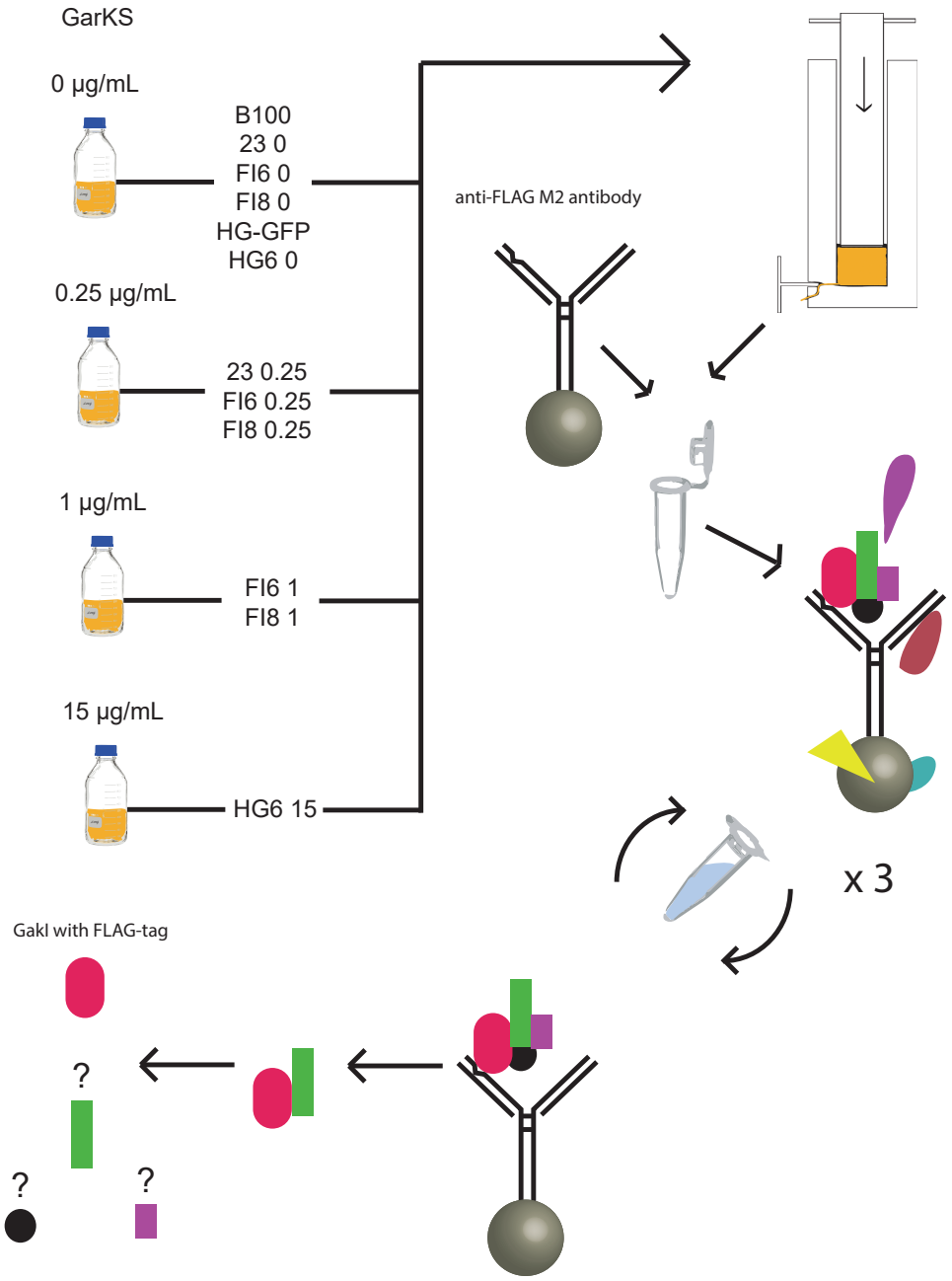

Figure 2.3: Workflow diagram of co-immunoprecipitation. Cultures were harvested following incubation in the presence of 0, 0.25, 1 and 15 µg/mL of GarKS as depicted. Lysates were incubated with the anti-FLAG M2 antibody resin, washed and eluted. Unknown proteins (?) represent proteins interacting with GakI. See Table 2.1 for a description of names.

2.5 Co-IP optimization

Co-IP was performed as described above, but the type of detergent or salt concentration as the only variable. Washing duration and method was fixed to 15 minutes each on a rotator (medium speed). The detergents Tween 20, 80 and CHAPS were also tested (data not shown).

2.6 SDS-PAGE and Silver Staining

Samples were analyzed by SDS-PAGE using Mini-Protean TGX gels (Bio-Rad Laboratories, USA) according to the recommendations of the manufacturer. Samples were boiled for 3 minutes in a reducing sample buffer (62.5 mM Tris, pH 6.8, 2% SDS, 10% (v/v) glycerol, 0.002% bromophenol blue and 5% β-mercaptoethanol) prior to loading on gel, then run at a constant voltage of 100 V.

A rapid silver staining technique using a microwave was used to visualize proteins in the sample, the three steps prior to impregnation was accelerated by microwaving the solution for 1 minute at a power of 900 W prior to each step. The protocol is summarized in Table 2.2.

Table 2.2: Silver staining protocol. All solutions were prepared in Milli-Q water ($_dH_2O$).

Step	Solution	Time
Fixation	10% AcOH, 50% MeOH	15 min
Rinse	$_dH_2O$	15 min
Enhance	5 µg/mL DTT	10 min
Impregnation	0.2% $AgNO_3$	15 min
Rinse	$_dH_2O$	2×20 s
Rinse	3% Na_2CO_3	2×20 s
Develop	0.1% HCOH, 3% Na_2CO_3	- ($\approx$ 1 min)[1]
Stop	0.2 M citric acid	15 min

AcOH: acetic acid, MeOH: methanol, DTT: dithiothreitol
dH2O: pure water ($>$ 10 MΩ/cm).
[1] Stop when desired intensity is reached.

2.7 Genomic Analysis of GarKS Mutants

Six mutants of *L. lactis* IL1403 with reduced sensitivity to GarKS together with a wild type had been sequenced previously (Norwegian Sequencing Centre, Norway). Sequencing service had been performed on an Illumina MiSeq with a read length of 300 bp. The wild type genome had been assembled, and all reads had been quality filtered using CLC Genomics Workbench (CLC bio, Denmark).

Contigs assembled for the wild type was annotated with prokka, using a custom database built from the Uniprot reference genome of *L. lactis* IL1403 [94]. Variant calling of mutants was performed using snippy [95]. Variants were verified by mapping the reads back to the variant with the bowtie2 algorithm [96]. Readmaps were inspected with Integrative Genomics Viewer [97].

2.8 MS Sample Preparation and Analysis

Immunoprecipitate was run 10 mm into a standard SDS-PAGE gel, stained by Coomassie Brilliant Blue R-250 (Sigma-Aldrich, St Louis, USA) and destained overnight . Further sample preparation, mass spectrometric analysis and database searches were performed by Morten Skaugen. The gel piece was excised and subjected to in-gel reduction, alkylation and trypsin digestion essentially as described by Shevchenko et al. [98]. Samples were purified and desalted using STAGE microcolumns (C18 membrane, Sigma 6683-U) and analyzed by LC-MS/MS on a Q Exactive Quadrupole-Orbitrap Mass Spectrometer (Thermo Fisher Scientific, USA). Spectral data was searched against a database of the *L. lactis* IL1403 proteome using the MASCOT search engine (Matrix Science). The bait protein GakI

with N and C-terminal FLAG-tag was included in the database, as well as common contaminants.

Identification of anti-FLAG M2 antibody in samples 23 and FI6 1 was performed by manually including the amino acid sequences for the antibody and running the peptide identification on the raw data independently by the MassAI (University of Southern Denmark, Denmark) and Peaks Studio (Bioinformatics Solutions Inc., Canada).

3 Results

3.1 MIC Assay of Cloned *L. lactis* and *S. aureus*

Expression of the garvicin KS immunity gene leads to a significant reduction in sensitivity towards GarKS in both *L. lactis* and *S. aureus*. The minimum inhibitory concentration (MIC) towards GarKS of *L. lactis* IL1403 expressing *gakI* was 17 μg/mL (see Table 3.1), a 130-fold increase compared to the negative control B100 at 0.13 μg/mL. Strains of *L. lactis* producing the FLAG-tagged GakI had a MIC equivalent to or better than for the native GakI at 25 μg/mL, corresponding to a 190-fold increase compared to B100.

Table 3.1: MIC assays of clones producing native GakI and FLAG-tagged variants towards garvicin KS, including negative controls B100 and HG/RN-GFP. Growth was measured following 24 hours of incubation. Values represent the average of three parallel assays.

Strain (name)	MIC (μg/mL GarKS)	Fold-increase with immunity
L. lactis IL1403		
B100 (empty plasmid)	0.13	
23 (native *gakI*)	17	130
FI6 (N-ter FLAG-tag)	25[1]	(>)190
FI8 (C-ter FLAG-tag)	25[1]	(>)190
S. aureus HG001		
HG-GFP	7.8	
HG23 (native *gakI*)	109	14
HG6 (N-ter FLAG-tag)	62.5	8
S. aureus RN4220		
RN-GFP	7.8	
RN23 (native *gakI*)	46.9	6
RN6 (N-ter FLAG-tag)	39.1	5

[1] MIC exceeded 25 μg/mL in two of three parallels.

S. aureus was less sensitive to GarKS than *L. lactis* IL1403 overall, and the effect of the immunity was less pronounced in both RN4220 and HG001. The native immunity gene made a 14-fold change in sensitivity to HG001 and 6-fold in RN4220, the presence of an N-terminal FLAG-tag reduced the effect of the immunity protein to 8-fold in HG001 and 5-fold in RN4220.

Because of the high sequence identity between the immunity proteins for GarKS and AurA70, the producer of the native GakI protein (23) was tested for cross-immunity against AurA70. The MIC is increased at least 97-fold towards AurA70 with GakI compared to the empty plasmid control.

Table 3.2: MIC assay of clone 23 and negative control B100 towards aureocin A70, measured after 24 hours of incubation. Both values have been averaged from four parallels.

Strain (name)	MIC (μg/mL pp[1])	Fold-change with immunity
B100 (empty plasmid)	<0.05[2]	
23 (native *gakI*	4.7	>97

[1] Concentration is given per peptide.
[2] No growth at the lowest concentration tested.

MIC values was all measured following 24 hours of incubation. To determine the maximum concentration of GarKS that is tolerated by the culture while maintaining normal growth characteristics, growth was measured over time at various concentrations (see Figure 3.1).

The empty plasmid negative control B100 reach stationary phase after 8 hours in GM17 at 30°C, while at 0.25 μg/mL of GarKS there is no appreciable growth over 16 hours of incubation in this strain. GakI producers show no change in growth characteristic at up to 0.25 μg/mL GarKS. However, at 1 μg/mL FI8 takes

an additional 2 hours to reach stationary phase. FI6 remains unaffected at up to 2 µg/mL, but both clone 23 and FI8 show severely delayed growth.

Based on the measured growth characteristic of FI6 and FI8 in the presence of GarKS, concentrations of 0.25 µg/mL and 1 µg/mL were used in the Co-IP experiments. Two concentrations were chosen to detect any concentration-dependent effects.

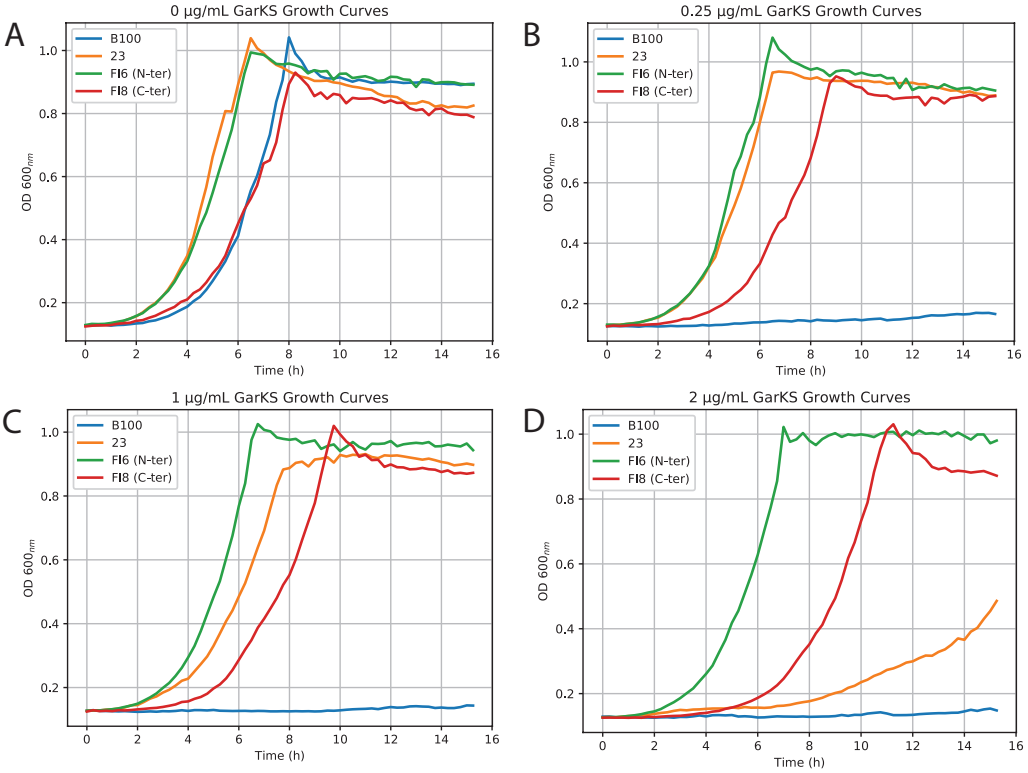

Figure 3.1: Effect of various GarKS concentrations on the growth characteristic of *L. lactis* clones and the empty plasmid control.

Growth characteristic for *S. aureus* HG001 expressing native *gakI* (HG23) was unchanged at 15 µg/mL of GarKS compared to no bacteriocin (see Figure **??** of Supplementary Material). N-terminal FLAG-tag clone HG6 displayed the same growth characteristic as HG23, reaching the end of the exponential phase at 4

hours of incubation. Increasing the concentration of GarKS to 30 µg/mL extended the exponential phase for HG23 to nearly 8 hours, while HG6 had no measureable growth in the same 8 hours. In the absence of IPTG to induce expression of *gakI*, no growth is observed at the two concentrations. A concentration of 15 µg/mL of GarKS was chosen for this strain in Co-IP experiments.

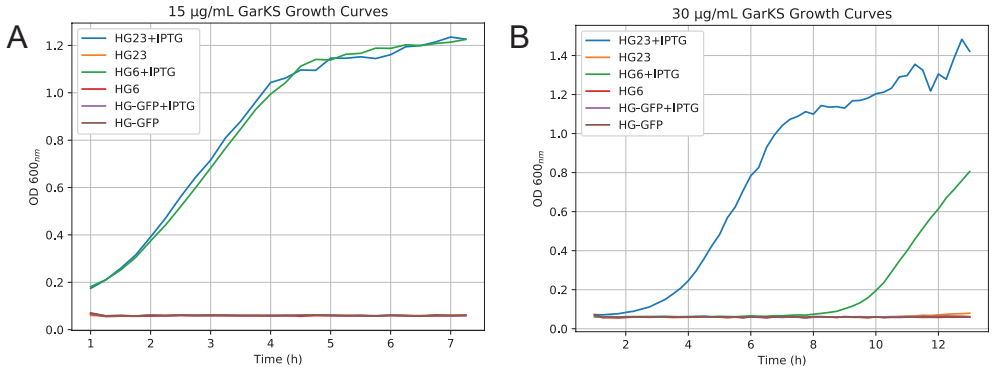

Figure 3.2: Growth curves of HG23 and HG6 at 15 and 30 µg/mL GarKS, A and B respectively. Microtiter plate was incubated for 1 hour at 37°C prior to readings. The jagged parts of the curve are due to colony formation in the microtiter wells that are partially resuspended by shaking prior to each reading.

3.2 Genomic Analysis of GarKS Mutants

Whole-genome sequencing data of *L. lactis* IL1403 with reduced sensitivity to garvicin KS was re-analyzed to see if there were mutations that had not previously been reported. In all but one mutant *ythA* was the only gene that differed significantly from the wild type. One mutant however had an intact *ythA*, the only other mutations found was a missense mutation in *cesR* (see Table 3.3).

Table 3.3: Mutations identified in GarKS mutants of IL1403

Mutant	Mutation	Consequence	Gene
1	34 nt duplication, pos 248	Asp94fs	*ythA*
2	G→A, pos 62	Gly21Glu	*ythA*
3	G→T, pos 50	Gly17Val	*ythA*
4	T→C, pos 163	Tyr55His	*ythA*
5	7 nt insertion, pos 404↓405	Lys138fs	*ythA*
	A →G, pos 47	Val16Ala	*ypfE* [1]
6	C→T, pos 329	Ala110Val	*llrD* [2]

[1] Multidrug Efflux MFS Transporter (NCBI: WP_003130061)
[2] *cesR*

3.3 Co-IP Optimization

Co-IP was initially carried out with lysate obtained from cultures of N-terminal FLAG-tag *L. lactis* (FI6) grown in the presence of 1 μg/mL (FI6 1), 0.25 μg/mL (FI6 0.25), 0 μg/mL (FI6 0) and the empty plasmid negative control B100. The anti-FLAG M2 antibody resin was incubated with lysate and washed under non-stringent conditions using TBS. The resulting immunoprecipitate had a high background of >20 proteins (see Figure 3.3). Multiple protein bands are of similar intensity, and even more intense than the immunity protein. A similar picture is also observed with FI8 samples (data not shown). However, in the FI6 1 sample two protein bands at just above 25 and 50 kDa marked II and I respectively, is observed. The two bands are not observed in any other sample. A more intense band for the immunity protein is seen in the sample obtained from 1 μg/mL GarKS in the culture. The approximate mass of the FLAG-tagged immunity protein is marked with a star (*) in the figures. The high background of unspecifically bound proteins would make further sample analysis difficult. To reduce unspecific binding to the antibody resin a detergent Triton X-100 was included in the washing buffer.

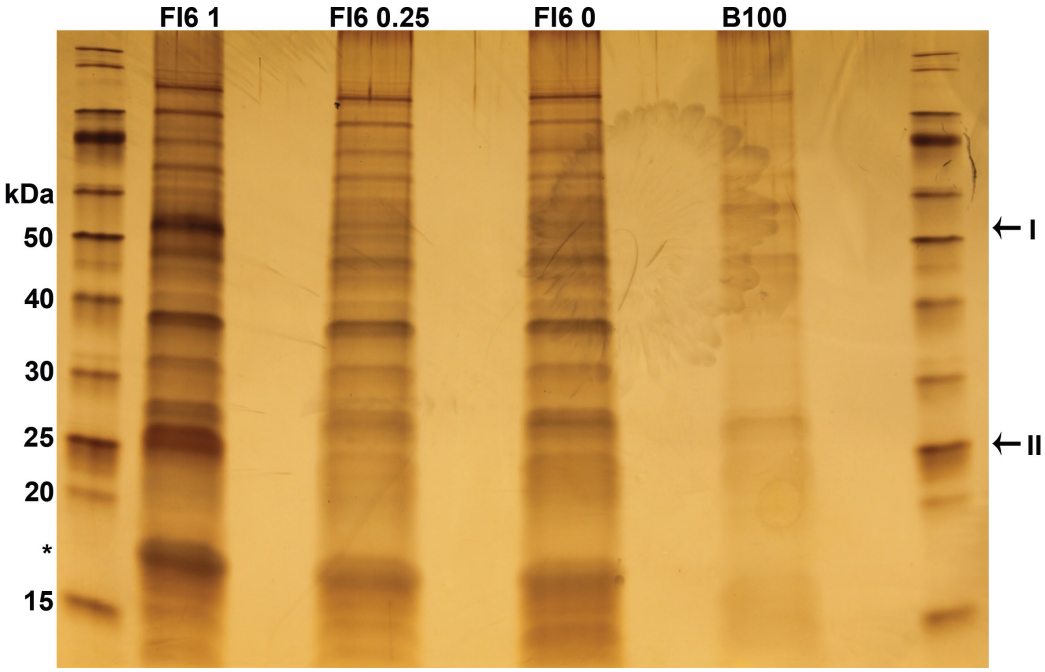

Figure 3.3: Silver staining of immunoprecipitate obtained from FI-6 samples (from 1, 0.25 and 0 μg/mL) and the empty plasmid negative control B100. Expected mass range of the bait protein is marked with a star (*). Two proteins unique to sample FI-6 1 is observed at I and II.

Addition of the detergent Triton X-100 to the washing buffer reduced background in a concentration-dependent manner (see Figure 3.4). All other co-precipitating proteins washed away equally, with one protein band at around 50 kDa (I) at 0% Triton X-100 disappearing at 0.1%. Two other bands at approximately 15 kDa (II) and 13 kDa (III) are visible only when a low concentration of $<$0.2% detergent is used (see Figure 3.4). The intensity of the immunity protein band is retained up to 0.8% Triton X-100, however other bands appear faint at this concentration. The detergent was not able to reduce background without interfering with FLAG-tag binding to the antibody. A different approach to reducing unspecific binding was attempted instead, using a high ionic strength wash buffer.

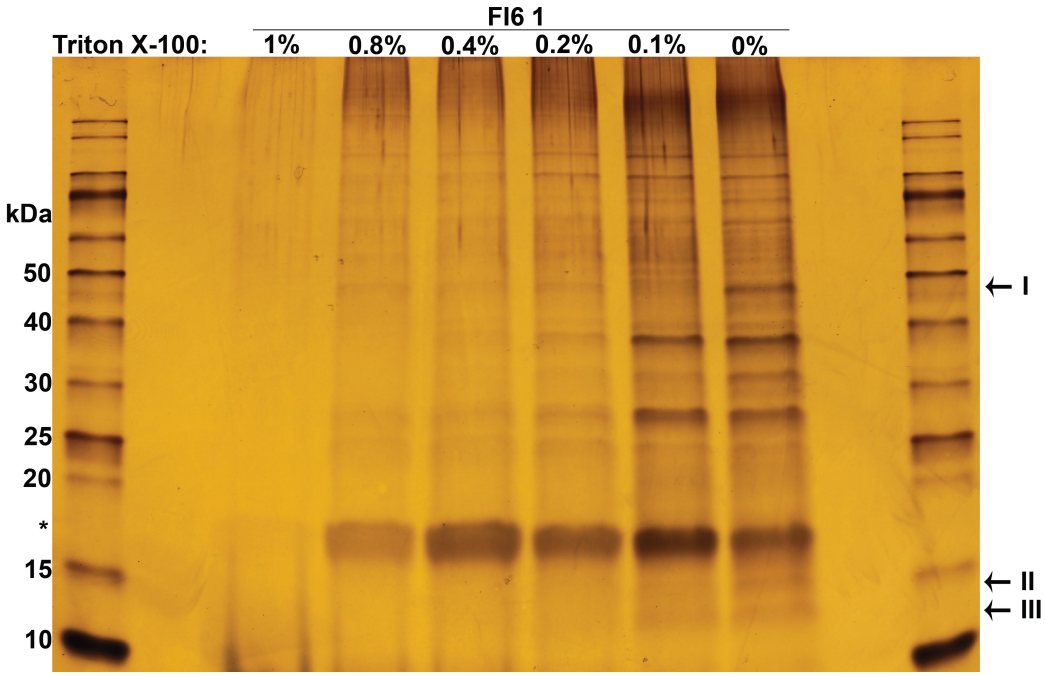

Figure 3.4: Immunoprecipitate of FI6 1 washed three times in TBS with varying concentrations of Triton X-100 (from left to right) 1%, 0.8%, 0.4 0.2%, 0.1%, 0%. Protein bands indicated with I, II and III disappear even at low concentrations of detergent. The well next to each ladder was left empty.

Multiple intense bands was observed when using a high ionic strength buffer (see Figure 3.5), here the samples has been incubated in 0.575 M NaCl and washed three times in TBS containing 1 M sodium chloride. The intensity of the immunity protein in FI8 show a correlation with bacteriocin concentration in the cultures. The negative controls with no FLAG-tag (23) and no immunity protein (B100) only had a faint band below 10 kDa, no other proteins are visible in the two samples. Next step was to include a detergent in addition to the high ionic strength to reduce background and try to simultaneously preserve hydrophobic interactions.

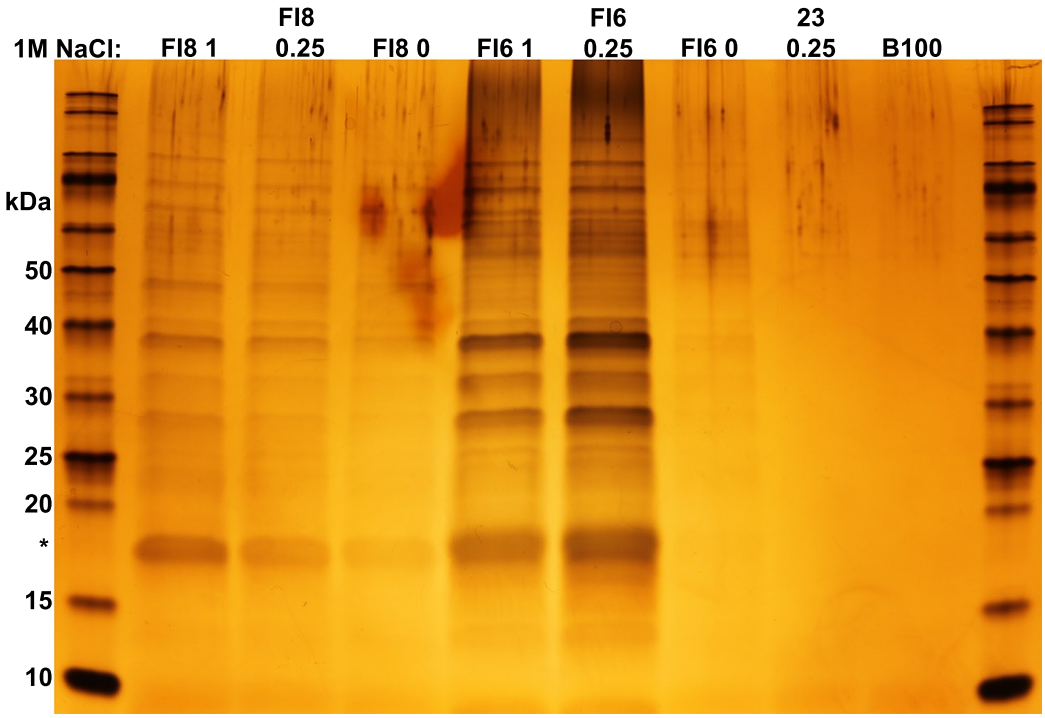

Figure 3.5: Immunoprecipitate of all *L. lactis* IL1403 samples following incubation with the antibody resin in 0.575 M NaCl and subsequently washed three times in TBS containing 1 M NaCl. Both negative controls B100 and 23 0.25 have no visible bands.

With the combination of a different detergent IGEPAL CA-630 (Nonidet P-40) at 1% and 0.575 M NaCl, the background was reduced significantly (see Figure 3.6). Immunity protein is indicated with a star (*) and appear intensely in all FI6 samples, especially in FI6 1. Again, the immunity protein appear more intense with increasing GarKS concentration. In the same sample a very faint band is observed at around 25 kDa (marked I).

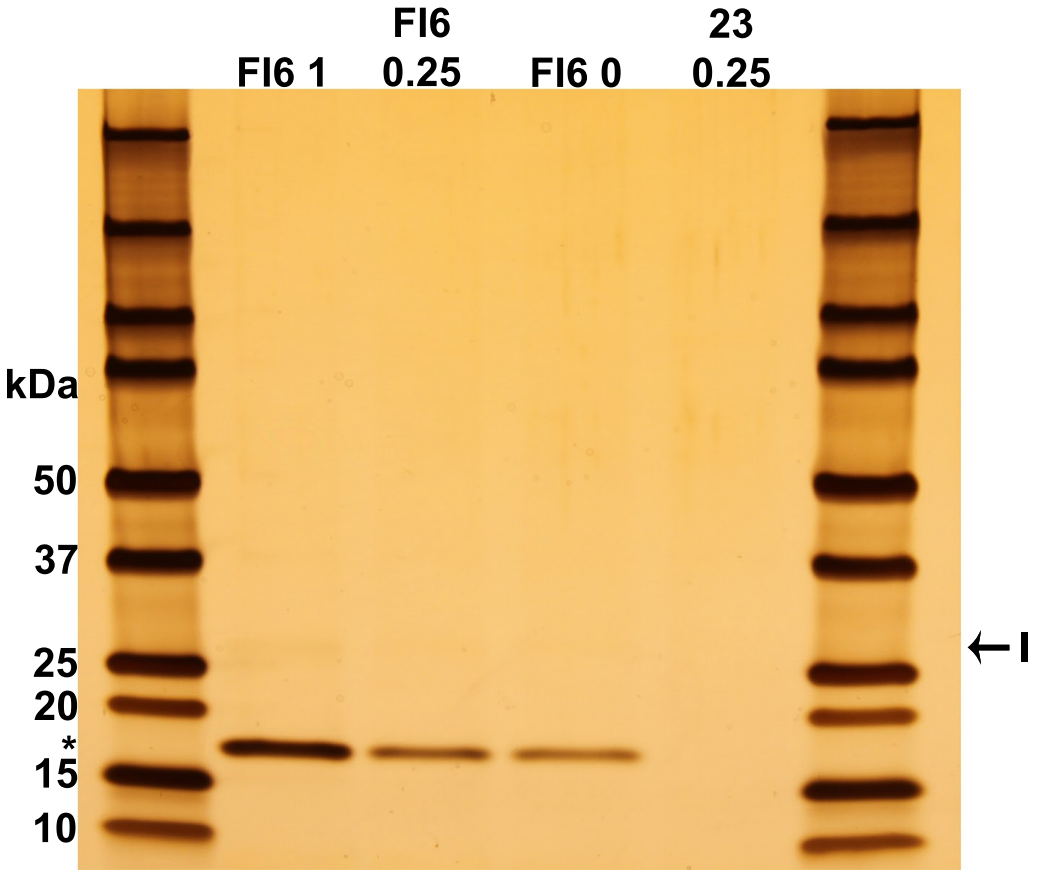

Figure 3.6: Immunoprecipitate obtained from the stringent protocol developed in this thesis. The combination of IGEPAL CA-630 and NaCl give a strong band for the immunity protein with no visible bandground proteins. A band was also faintly visible at just above 25 kDa in FI6 1 indicated at I.

This washing buffer significantly reduced background while retaining the immunity protein. Co-IP proceeded with this washing buffer to identity specific bands that co-purify with the immunity protein.

3.4 Co-Immunoprecipitation

After finally developing a protocol that could reduce background levels of proteins, Co-IP was replicated with an increased amount of resin. Proteins that co-precipitated with the immunity protein then became visible as two bands around 25 kDa and one at around 50 kDa (indicated with I, II and III respectively). One band at around 25 kDa indicated with I appear with increasing intensity from FI6 0 to FI6 0.25, to FI6 1. The other band at around 25 kDa (II) is present in FI6 1 and FI8 1 (see Figure 3.7). The third band at 50 kDa is only visible in FI6 1 (III). The two negative controls B100 and 23 0 have no visible protein.

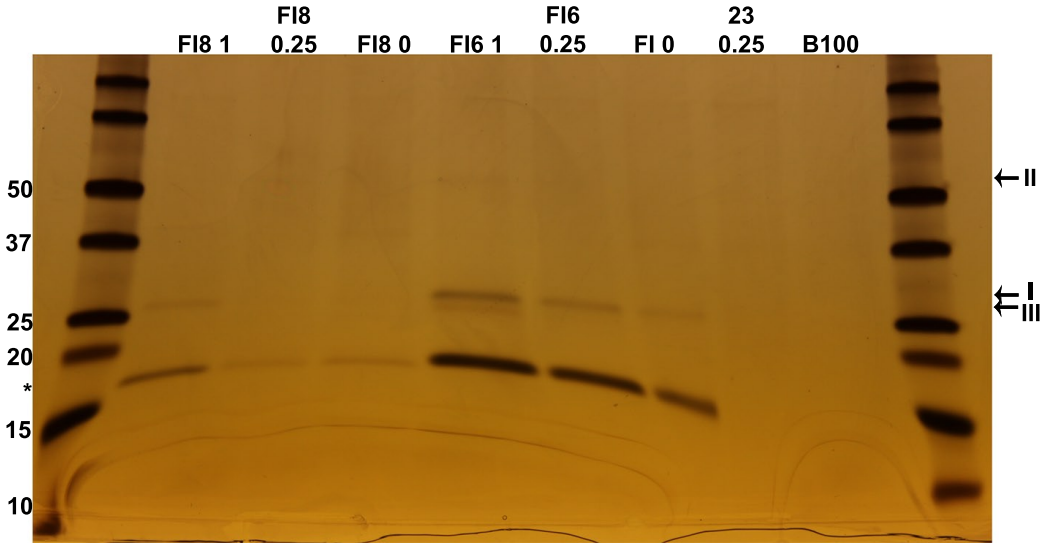

Figure 3.7: Immunoprecipitate of IL1403 samples had co-precipitating proteins at around 25 kDa and one at 50 kDa. GarKS immunity bait protein is marked with a star. The gel is distorted at the bottom due to buffer depletion during the run.

Co-precipitating proteins at around 25 kDa is also observed in *S. aureus* HG001, a band at around 27 kDa was observed in HG6 15 (I) while a band at around 25 kDa was visible in HG6 0 (II) (see Figure 3.8). Due to the increased effect of GakI in HG001 from the MIC assay, this strain was used in Co-IP over RN4220.

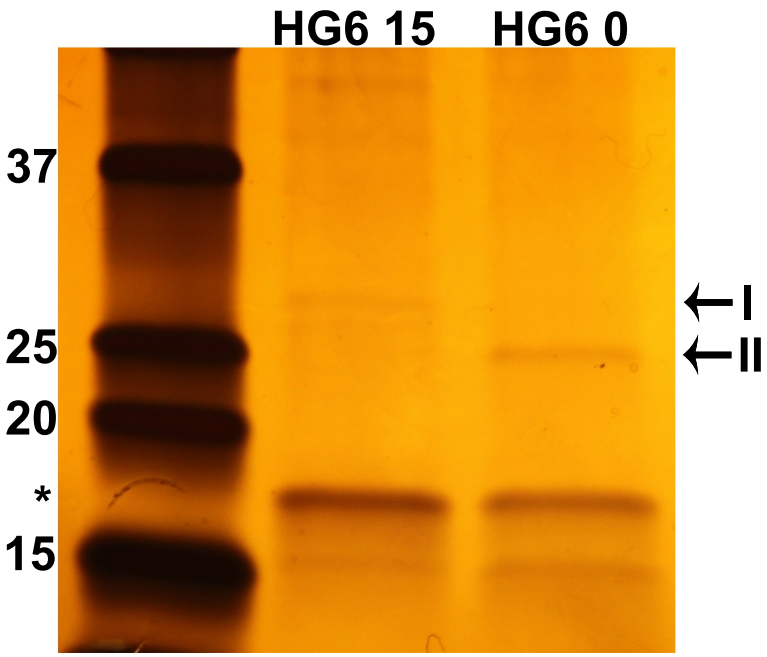

Figure 3.8: Samples obtained from *S. aureus* HG001 in the presence of GarKS at 15 and 0 μg/mL both show a unique band at approximately 27-29 kDa for HG6 15 and 25 kDa for HG6 0. A negative control with no visible protein was also included, but not shown.

To verify if the three co-precipitating proteins observed for FI6 1 in Figure 3.7 eluted with the immunity, as opposed to being residual background proteins. The lysate prior to (Lysate) and after incubation was analyzed on SDS-PAGE and silver staining, as well as the three washing fractions (W1-3) and final eluate (FI6 1). The lysate from FI6 1 (see Figure 3.9) has a large number of proteins, as well as after incubation with the antibody resin (W0). Only two bands persist in the first washing fraction (W1). Both the second (W2) and third (W3) washing fraction

has no visible bands on the gel. Following elution from the anti-FLAG M2 resin, three bands appear that are indicated I, II and III, at approximately 25, 27 and 52 kDa respectively (see Figure 3.9).

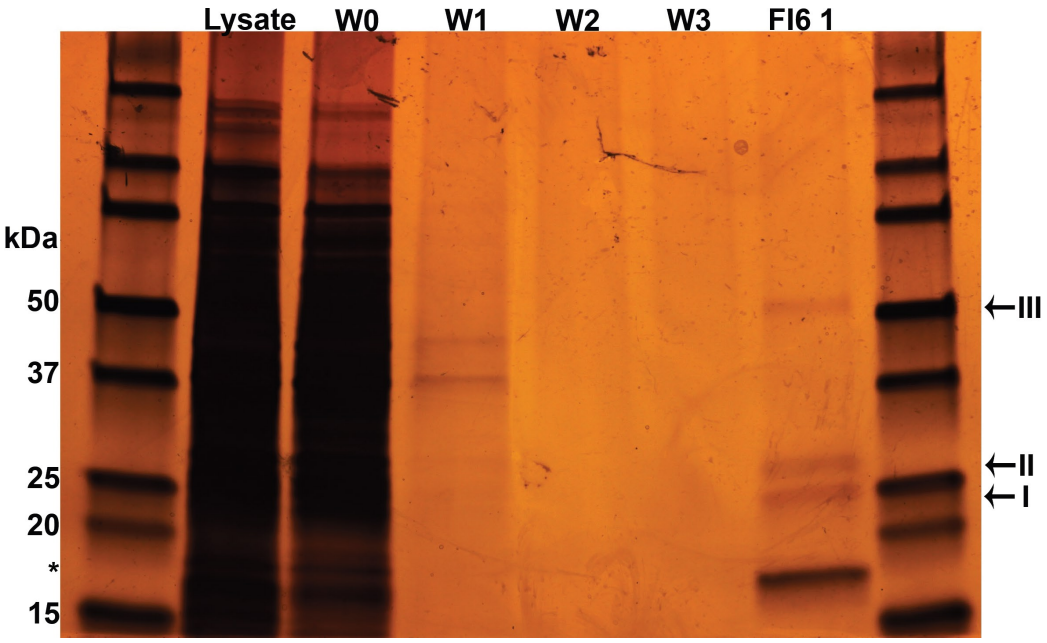

Figure 3.9: Washing efficiency of the stringent buffer, three proteins co-precipitate with FI6 1 following three washes (W1-W3) of the antibody resin.

3.5 MS Analysis of Immunoprecipitate

In order to identify the co-precipitating proteins of GakI, the immunoprecipitate from samples FI6 0, 0.25 and 1 and FI8 1 from Figure 3.7 was analyzed by mass spectrometry. As a negative control for the Co-IP the clone producing GakI without FLAG-tag 23 0.25 from Figure 3.7 was included. Following database searches against the IL1403 proteome, 737 unique proteins were detected at a very high confidence across the five samples. In many samples 30S and 50S ribosomal

proteins were of the highest calculated abundance, but were discarded from the results.

Among the most abundant in all but one sample was YthC, a member of the *yth*-operon in *L. lactis* together with *ythA*. The C-terminal FLAG-tag sample FI8 1 had YneH as the second most abundant protein, an SpxB homologue known to positively regulate OatA. A membrane-bound metalloprotease FtsH believed to be involved in the CesR response and known to degrade PspC in *E. coli* and *Y. enterocolitica* was detected at high concentration in 23 0.25 [99].

Table 3.4: Top 10 proteins identified in the immunoprecipitate of samples from Figure 3.7 after removal of ribosomal proteins. Estimated abundance is given in parentheses as the exponentially modified protein abundance index (emPAI) as calculated by MASCOT (relative abundance). Protein threshold of 99% and 0.1% peptide false discovery rate.

Sample (# identified proteins)				
23, 0.25 (602)	FI6, 0 (565)	FI6, 0.25 (560)	FI6, 1 (119)	FI8, 1 (261)
YthC (66)	YthC (27)	YthC (38)	PtnAB (22)	PfL (89)
PtnAB (30)	FabI (24)	FabI (31)	EzrA (20)	YneH[1](48)
YgaJ (20)	PtnAB (14)	PtnAB (25)	AsnB (20)	YthC (41)
PyrR (20)	YnbE (10)	Ldh1 (11)	HuP (17)	TuF (33)
Idh1 (19)	Ldh1 (10)	BmpA (10)	FabZ (16)	CtsR (29)
YnbE (18)	BmpA (9)	YnhD (9)	YraB (12)	Eno1 (16)
FabI (14)	PyrR (8)	YgaJ (8)	SepF (8)	AlS (15)
FtsH (13)	YgaJ (7)	PyrR (8)	YnbE (8)	DnaK (15)
AtpD (13)	RecA (7)	MaE (7)	YwfF (8)	MaE (15)
AdhE (12)	HuP (6)	CitF (7)	BmpA (8)	CarB (15)

[1] Positive regulator of OatA (SpxB)
Underlined proteins are putatively regulated by CesSR
Estimated abundance of YthC in FI6, 1 was 2.26.
YthA was detected in all samples but with no significant difference in abundance (1-2)

4 Discussion

4.1 Immunity Protein is Functional in *L. lactis*

Immunity from heterogeneously expressed *gakI* has not previously been reported, this thesis demonstrates the functionality of this immunity gene from *L. garvieae* in both *L. lactis* and *S. aureus*. Production of GakI in *L. lactis* IL1403 significantly reduce sensitivity to the cognate bacteriocin GarKS, showing that the GakI protein is functional in this species. It is not known how GakI confer immunity, but a protein-protein interaction is very likely to be involved due to the mechanism of immunity described for LcnA. Such an interaction could be disrupted by the presence of a FLAG-tag on the immunity protein, in which a reduced effect of the immunity would be expected. However, the presence of a FLAG-tag did not increase the sensitivity of the strain relative to the untagged strain 23. Surprisingly, both FLAG-tagged versions of GakI had improved immunity relative to the native protein. Improved immunity by the FLAG-tag persisted and was evident both from MIC assays and growth curves (see Figure 3.1). The explanation for this difference is unknown. If a protein-protein interaction is involved in the mechanism of immunity, this interaction is maintained by the presence of the FLAG-tag - a prerequisite for proceeding with Co-IP experiments.

A difference in immunity between the two FLAG-tag clones is not apparent from the MIC values, but the C-terminal FLAG-tag clone FI8 had a 50% longer generation time at 1 μg/mL of GarKS than the N-terminal clone (see Figure 3.1). This slower growth of FI8 in the presence of GarKS relative to FI6 suggested a reduced functionality of C-terminally tagged variant relative to N-terminal variant. FI6 and was thus preferred in the Co-IP experiments and MS analysis.

It is however interesting that *L. lactis* IL1403 is more sensitive to GarKS than *L. garvieae* strains more closely related to the native producer (4 to 8-fold) [50]. This suggests GarKS has a higher affinity for the receptor in *L. lactis* IL1403 and other *L. lactis* than that of its own producer. This would indicate that GarKS and GakI do not compete for the same binding site on the receptor. It would be interesting to assess the GarKS sensitivity of the native producer with the gak locus deleted, as this would likely represent the most ideal case for the immunity protein. Knowledge gained from GarKS in *L. lactis* is also transferable to the important human pathogen *S. aureus*.

4.2 GarKS Stabilizes the Immunity Protein

A consistent observation was a more prominent band for the FLAG-tagged immunity protein with increasing concentration of garvicin KS - suggesting a stabilizing effect of GarKS on GakI. A possible explanation is that GarKS is subject to proteolytic degradation, and in the absence of GarKS, as is the immunity protein. The presence of the bacteriocin could promote the formation of an immunity complex as described for lactococcin A, which could make it inaccessible to the protease. A strict regulation of this sort would be expected especially if the receptor is functionally important to the cell, as the immunity complex is likely to render it non-functional. It has been demonstrated for the lactococcin A immunity that producing strains show reduced growth when glucose and/or mannose is the only carbon source [32]. Deficits of that sort are likely accentuated in the natural environment. A decrease in fitness is unlikely to be apparent in the laboratory where complex growth media is the norm. Little is known about how bacteria regulate the degradation of immunity proteins. However, to properly establish a

stabilizing role of the bacteriocin would require further work using more appropriate techniques.

4.3 GakI Provide Immunity in *S. aureus*

S. aureus was far less sensitive to GarKS than *L. lactis*, as the MIC measured for the GFP-producing strains is 60 times higher than for B100. This was consistent with the range of MIC values measured by Ovchinnikov et al. [6] for *S. aureus* strains. Despite the relatively low sensitivity of HG001 and RN4220 to GarKS, expression of the immunity protein from *L. garvieae* increased the MIC by 14 and 6-fold respectively. The reduced sensitivity to GarKS of GakI producing strains was especially evident from the growth characteristic, and the effect was reproducible. As expected, the native GakI is a better immunity protein than the FLAG-tag variant in both strains tested. The highest concentration of GarKS where HG23 and HG6 both showed normal growth characteristics was 15 μg/mL (see Figure 3.2), this is the concentration used for Co-IP. The functionality of heterologously expressed *gakI* in *S. aureus* provide strong evidence of a similar receptor and mechanism in both species.

4.4 Class IIe Bacteriocins Have a Similar Mechanism

Identifying potential receptor components of GarKS would not only increase our understanding of this bacteriocin, but likely all multi-component bacteriocins discovered so far. All Class IIe bacteriocins share significant sequence similarity, also in their immunity proteins. The peptides are of similar length and physic-

ochemical properties. A similar mode of action by AurA70 and GarKS is supported by cross-immunity, *L. lactis* IL1403 expressing GakI is not only immune to GarKS, but also AurA70 (see Table 3.2). GakI provides immunity to GarKS also in *S. aureus* HG001 and RN4220, although the MIC is only increased 4 and 8-fold respectively (see Table 3.1). GakI provides immunity to all multi-peptide bacteriocins (A. Telke, personal communication, May 10, 2019).

4.5 Co-IP Optimization

Immunoprecipitate obtained from non-stringent conditions had high background levels of protein, proteins presumed to bind non-specifically to the resin, antibody or immunity protein. Surprisingly, the non-specific interactions survive multiple washes, also when extended to 15 minutes each with agitation on a rotator. Background levels were far higher in samples containing the FLAG-tagged protein, when washing the resin with high salt concentration the negative controls had no visible protein (see Figure 3.5). Despite the clean negative controls, the FLAG-tagged samples had in excess of 20 protein bands. It seems that unspecific interactions are with the immunity protein or somehow depends on its binding to the antibody. Considering the effectiveness of detergents at reducing background, a likely explanation is that of hydrophobic aggregates.

The immunity protein is predicted to be transmembrane and have poor water solubility, the sequence of GakI show an excess of aliphatic and aromatic amino acids (56%). Hydrophobic molecules will minimize their surface area in contact with the aqueous environment by aggregating, known as the hydrophobic effect. This is expected to occur between integral membrane proteins and lipid components.

A brief database search in the proteome of *L. lactis* IL1403 show predicted multi-pass membrane proteins in the 20-100 kDa range (e.g. from YkjJ to YlcG). A detergent is necessary to solubilize hydrophobic aggregates, but will also interfere with any interaction partners of the immunity protein if hydrophobic contacts are involved.

Triton X-100 was the detergent used initially as recommended by the manufacturer, however when used at a concentration necessary for removing background it also interfered with the FLAG-tag binding to the antibody. Co-IP experiments would frequently result in a very weak band for the immunity protein. In addition, various bands appeared intermittently between parallel experiments. In excess of 50 silver stained gels with 4-8 samples each was necessary to determine experimental variance from bands representing co-precipitating proteins.

A similar detergent IGEPAL CA-630 (Nonidet P-40) used at a concentration of 1% retained the immunity protein on the antibody while removing background figure **??**. However, no other proteins were detected when only IGEPAL CA-630 was used at that concentration. I reasoned that lowering the detergent concentration would increase all background equally as was the case with Triton X-100, simultaneously increasing the salt concentration was attempted instead. Increasing the ionic strength of a solution increases the strength of hydrophobic interactions [100](too high ionic strength will cause aggregation and precipitation; salting out). With the combination of salt and detergent, two co-precipitating proteins were visible. However, the two bands were very faint and could be residual background. To test whether the bands were residual background, the co-immunoprecipitation was reproduced using twice the amount of anti-FLAG M2 resin and with collection of the washing fractions. From the resulting gel a third band became visible at around 26 kDa and neither of the three bands are from residual background.

The three proteins persisted over three washing cycles with no bands at this size visible in the washing fractions. In fact, no background is visible in the second and third fraction, the three proteins appeared in solution following competitive elution with 3X FLAG-peptide. It is evident that the three proteins bind strongly to the immunity protein and are likely interaction partners *in vivo*, potentially as a single protein complex of three subunits.

4.6 Co-Precipitating Proteins Could Not be Identified

In an attempt to identify the specific proteins visible on the gel from the immunoprecipitate, all the proteins identified were filtered based on the following criteria: (i) only identified in samples with bacteriocin and a FLAG-tag, (ii) the estimated abundance is in increasing order with more bacteriocin in the culture ([FI6, 1] >[FI, 0.25]), (iii) 24-29 kDa or 50-53 kDa, and (iv) predicted to be transmembrane (subcellular location, Uniprot). No proteins matched the criteria. A second round of filtering without the criteria of increasing abundance was then performed, also with no results.

The most obvious explanation for the two bands visible in the 25 and 50 kDa region are unconjugated antibodies from the anti-FLAG M2 resin. The database used in peptide identification did not include the antibody, and would thus not be able to identify the bands. To check if the antibody was present in the samples, a custom database was constructed that included the amino acid sequence for the heavy and light chain of the anti-FLAG M2 Fab domain [101]. The MassAI software could not detect the antibody in FI6 1, but identified 60 peptides from the light chain in 23 0.25. PEAK Studio identified the light chain in both samples,

but estimated the abundance in 23 0.25 to be approximately 20-fold higher than in FI-6 1 (see Figure 6.3 of Supplementary Material). The heavy chain was not detected in the samples. In addition, negative controls did not show any bands when eluted with 3X FLAG-peptide over numerous parallels (data not shown). Elution from the anti-FLAG M2 resin with denaturing SDS-PAGE sample buffer did give strong bands for the antibody at approximately 24 kDa and 54 kDa (data not shown). However, a second band at the 25 kDa region from the antibody was not observed (e.g. band marked II in Figure 3.9).

Sample FI6 1 was unlike the two other FI6 samples, having only 119 identified proteins compared to 565 for FI6 0 and 560 for FI6 0.25. In addition, FI6 0 and FI6 0.25 share 6 of 10 proteins together among the top ten (FabI, PtnAB, Ldh1, BmpA, PyrR and YgaJ) while FI6 1 only share three proteins in common with either of the two other samples (PtnAB, YnbE and BmpA). YthC was detected in all other samples except FI6 1. It seems probable that the data for FI6 1 is suspect.

It was expected that co-precipitating proteins appearing on the silver stained gel would be orders of magnitude more concentrated than the background, however no specific proteins were predicted to be of significantly higher abundance than any other. In addition, 560 proteins was detected at a 100% probability in the immunoprecipitate of FI6 0.25. The bait protein N-terminal FLAG-tag GakI was ranked 401 in abundance of a total of 560 in this sample, despite the prominent band of this protein on silver stained SDS-PAGE gel (data not shown).

A likely explanation is that GakI and any co-precipitating proteins have properties that make them resistant to mass spectrometric analysis by the conventional sample preparation methods used in this thesis. Low representation of membrane proteins is a common problem in MS-assisted proteomics [102, 103]. Several

factors contribute to this difficulty, (i) lower abundance compared to cytoplasmic proteins, (ii) poor solubility in sample preparation solvents, and (iii) fewer tryptic cleavage sites [102]. Analysis of the tryptic peptides mapped to GakI revealed only three peptides: DNEIITR, TLFVNIQEK and TLFVNIQEKNDVLK that span from residue 13-19, 69-77 and 69-82 respectively. The cleavage sites for tryptic peptides correspond almost exactly to two cytoplasmic coil regions of GakI as predicted by MEMSAT-SVM [104]. The compact hydrophobic four-helix bundle structure of GakI likely resist tryptic cleavage and have poor solubility in solvents used in sample preparation. Membrane proteins appear underrepresented in the data overall and could explain why co-precipitating proteins could not be identified, as co-precipitating proteins are believed to be membrane proteins and possibly of similar physicochemical properties as GakI. Just based on the intensities on the gel picture, co-precipitating proteins are more dilute than the immunity protein by a factor of approximately 4 to 10 (1/4 for protein I to 1/10 for II in Figure 3.7. This would put the proteins below the confidence threshold assuming they have similar properties as GakI.

Estimates for protein abundance is given as the emPAI value, which is based on the ratio of observed peptides over observable peptides [105]. This calculation will further underestimate the abundance of membrane proteins because the theoretical number of cleavage sites (that determines the number of observable peptides) are much higher than are actually accessible. Papanastasiou et al. [106] acknowledge this issue and propose a modified calculation based on the number of ”trypsin-accessible surface peptides” as a replacement for total theoretical tryptic peptides.

In view of the aforementioned problems in analyzing membrane proteins by mass spectrometry, a different method and protocol would have to be used to identify the proteins of interest. (i) Increase the volume or number of washes to further

dilute background proteins. (ii) Excise the band of interest from the gel. (iii) Use proteases like chymotrypsin or peptidase I for more efficient digestion in hydrophobic regions [107]. (iv) Use a sample preparation protocol optimized for membrane proteins, e.g. include MS-compatible detergents to better solubilize the proteins. (v) Understand the pitfalls in LC-MS/MS based protein quantification.

Immunoprecipitate from Figure 3.7 was analyzed by mass spectrometry with the intention of identifying co-precipitating proteins indicated I, II, and III in Figure 3.7. However, in light of the problems discussed in the above interpretation of the data difficult. Due to the high sensitivity of the instrument, the data likely represent that of the cell lysate. The affinity purification performed in this thesis correspond to an approximate dilution of the original lysate by a factor of 1 to 8×10^7, this will dilute proteins below the detection limit of silver staining on SDS-PAGE. However, this is well within range of the mass spectrometer with a limit of detection in the atto- to femtomole range [108]. Some proteins will bind unspecifically to the antibody resin with a higher affinity than others, introducing further uncertainty in abundance estimates also for cytoplasmic proteins.

4.7 Proteomic Analysis of Cultures

One of the most abundant proteins in all but one culture was YthC, a member of the Psp-response in *L. lactis*. YthC also appear highly abundant in the culture not exposed to the bacteriocin (FI6 0). It is possible that the production of GakI by the cell is sufficient stressor to activate of the Psp-system. Production of membrane proteins in *L. lactis* has previously been shown to up-regulate the *ythA* and *ythC* genes and other genes believed to belong to the CesSR regulon by Marreddy et al.

[109] and Pinto et al. [92]. However, there is a 1.5 to 2.5-fold increase in YthC from the culture without GarKS to cultures with 0.25 μg/mL GarKS and FI8 1, suggesting that the bacteriocin could further activate Psp or CesSR. Up-regulation of the psp operon has been demonstrated in *L. lactis* exposed to Lcn972 [53]. The presence of FtsH and YneH among the top 10 abundant proteins in cultures exposed to GarKS could suggest an activation of the CesSR, as both contain a putative CesR-binding motif in their promoter region, as does YthC [53].

The PspC-protein YthA had the same abundance in 23 0.25 and FI6 0 despite a 2.5-fold difference in YthC. The two genes are believed to be on an operon and are similarly overexpressed on the level of transcription [53, 63, 92, 109]. A low abundance of the YthA protein in this case could suggest degradation/proteolysis of YthA as part of the Psp-response.

Many of the other proteins identified the samples are naturally of high abundance in *L. lactis* IL1403 according to the PaxDB Protein Abundance Database [110]. Among the 50 most abundant proteins are HuP, TuF, Eno1, YgaJ, PtnAB, PyrR and DnaK, ranked 1, 4, 11, 20, 21, 25 and 48 out of 1303 respectively. The naturally high abundance explains their presence in the samples. Despite being the most abundant protein in three samples, YthC is only ranked 248 of 1303. Further indication that YthC was overexpressed in the cultures. FtsH is ranked number 273, no abundance information was available for YneH.

4.8 YthA and CesR are Involved in a General Stress Response

Mutations in *ythA* was frequently observed in spontaneous mutants to GarKS, including mutations expected to render the YthA protein non-functional (see Table 3.3). This observation is rather puzzling, considering that YthA is believed to be part of a membrane repair system [85, 63, 111]. An attractive hypothesis consistent with observations is YthA as an anti-ces factor, whose function is to repress transcription of the CesSR regulon either directly or indirectly under normal conditions. The consequence of a non-functional YthA would then result in a constitutively activated CesSR response.

Genes that are regulated by CesSR include cell wall modifying enzymes like OatA that acetylates peptidoglycan and the psp-proteins which are associated with robust phenotypes [112]. YthA mutants only have a small decrease in sensitivity to GarKS at about 2 to 4-fold (P. Mikkelsen, personal communication, March 28, 2019), this is consistent with a general response against membrane active antimicrobials as seen with psp mutations in other organisms (see section below). An over-activation of the ces system has the same effect in Enterococci, where mutations in the *liaFSR* locus encoding a homologous stress response system are well-documented in mutants resistant to certain antibiotics [113, 114, 115]. Mutations in *liaF* is frequently observed in resistant mutants [116, 117, 118]. LiaF is a known negative regulator of the stress response system in *B. subtilis* [119].

The *cesR* mutant could also be consistent with this this hypothesis, as some mutations in *liaR* is known to increase resistance to daptomycin and vancomycin in *E. faecalis* and *E. faecium* [114, 118, 113]. A typical response regulator must be phosphorylated by the cognate HK sensor to activate transcription. However, a daptomycin resistant mutant of *E. faecalis* characterized by Davlieva et al. [114]

activated transcription by *liaR* even in the absence of phosphorylation. This LiaR Asp191Asn variant had an increased tendency to oligomerize spontaneously into a DNA-binding tetramer. Further, *liaR* mutants of *E. faecium* tend to have only a few types of mutations that recur [118]. A similar mechanism could be involved also for the *cesR* mutant in *L. lactis* IL1403, in which case selection for GarKS resistance would necessarily select for this mutation. Increased activation of CesSR as a general stress response mechanism against GarKS is further supported by the high abundance of YthC, FtsH and YneH from the mass spectrometry data (see Table 3.4).

4.9 The Role of Psp

The Psp-proteins of *L. lactis* is up-regulated following phage infection, exposure to the bacteriocin Lcn972 and probably GarKS, expression of membrane proteins, osmotic stress and starvation [63, 53, 109, 120]. Homologues of the Psp-proteins are similarly up-regulated in numerous species under conditions disruptive to the cell envelope. The putative role of the Psp-system is one of restoring cell envelope homeostasis, which appears to be at least partially mediated through the CesSR. YthA is unlikely to act as an anti-ces factor directly, as the *ces*-operon already encode a putative LiaF homologue likely to serve this function [70].

The putative *L. lactis* LiaF homologue is encoded by the first open reading frame of the *ces*-operon, named *yjbB* in *L. lactis* IL1403 and llmg1650 in *L. lactis* MG1363 and encode a protein with the same conserved domain as LiaF in *B. subtilis* DUF2154 (pfam: OF09922). LiaF is the first gene in the *liaFSR* operon, similarly the first gene in the *vraSR* operon of *S. aureus* encode a LiaF homologue

VraT (previously YvqF). Mutations in VraT has been associated with reduced sensitivity to vancomycin and dalbavancin [121]. However, homology of LiaF or VraT with Llmg1650/YjbB can only be inferred based on its physical location and conserved domain, as the sequence identity is relatively low at 15%.

Little is known about the Psp-proteins in lactococci, but homologs in other species has been investigated. Mutations in the cell envelope stress two-component system LiaRS and homologues of the Psp-proteins are associated with reduced sensitivity to daptomycin in Enterococci. The Psp-homologs in *E. faecalis* V583 are annotated as EF1753-1751 (ordered locus name), the first gene EF1753 encode a protein with the same DUF4097 domain as YthC and Llmg2164. Similar to YthA and Llmg2163, EF1752 is 107 aa and has a PspC domain. EF1751 share sequence homology with YvlD of *B. subtilis* (36.3% identity, 57.3% similarity), both belonging to a Mycobacterial 4 TMS phage holin of superfamily IV (pfam: PF04020).

Mutations in *liaF*, *liaR*, and EF1753 were among the most frequent in *E. faecalis* S613 adapted to increased daptomycin (DAP) resistance, among the most resistant (32-fold increased MIC) was an EF1753 V289fs mutant (C-terminal domain truncation) [115]. The EF1753 mutants also showed reduced biofilm formation. Miller [115] confirmed that EF1753 is on the same RNA strand as *pspC*. An EF1753 mutation was also found in 2 of 3 DAP resistant mutants generated by Palmer et al. [122] in a serial passage experiment with *E. faecalis* V583, but other mutations were also found. The genes of the psp-operon were one of the most upregulated in E. faecium 1,231,410 (VanA-type VRE) upon exposure to the antiseptic chlorhexidine [123]. In addition, a *pspC* mutation was identified in *E. faecium* DO with reduced DAP susceptibility. Curiously, the authors note a significantly thicker cell wall in the strain with a *pspC* mutation [124].

The putative YthC homologue in *E. faecium* was shown to interact with penicillin binding protein 5 (Pbp5) *in vivo* by Desbonnet et al. [125] using tandem affinity purification (a technique similar to co-immunoprecipitation). The protein the authors name P_5AP (Pbp5-associated protein) is 60% identical to EF1753 in *E. faecalis*, contain the same DUF4097 domain and is located just upstream of a PspC-protein (Genbank: AFC64111.1). Downstream of the PspC-protein is a YvlD/EF1751 homologue of the same holin family. Interestingly, holins oligomerize in the cytoplasmic membrane in a ”hole forming” fashion, holes formed by holins can function as export pathways for enzymes to access peptidoglycan [126]. The Pbp5 protein is considered the primary determinant of resistance to ampicillin and other β-lactams in *E. faecium*, Pbp5 has a low affinity for the aforementioned antibiotics while synthesizing PG [127]. Overexpression of P_5AP increased the MIC of ampicillin and ceftriaxone by 2-fold and 4-fold respectively. However, the interaction could involve the extended N-terminal which is not present in YthC of *L. lactis*. This association supports the view of the Psp-system as involved in the maintenance, repair or stabilization of the cell envelope.

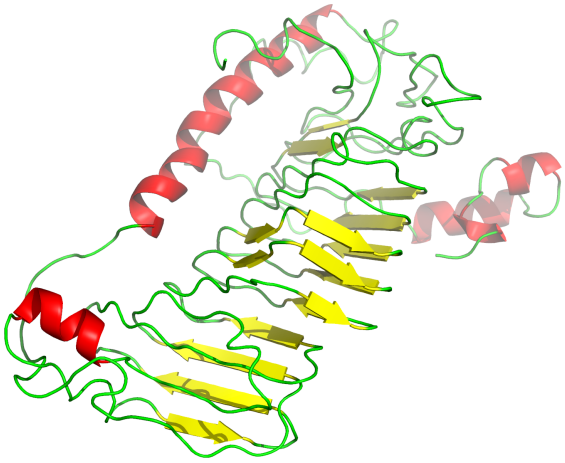

Figure 4.1: Structure of ythC generated by phyre2, the DUF4097 domain was modeled with a high confidence to other adhesins as β-sheets (yellow). Remaining sequence was modeled as α-helices (red) with a low confidence using parts of PFL-like glycyl radical enzymes, dna binding proteins and transcription factors as a template.

A *ab initio* structure prediction of YthC in *L. lactis* IL1403 using the phyre2 web server modeled a structure of primarily β-sheets (see Figure 4.1). Even though *ab initio* structural prediction is very inaccurate, the structure is roughly in agreement with other prediction tools. A protein of this structure is likely localized on the cell surface. The PspC-protein YthA is predicted to have a single transmembrane helix with the remaining 90 residues outside the cell, such that the two proteins might interact. A putative model for the Psp-system and its interaction with CesSR is presented in Figure 4.2.

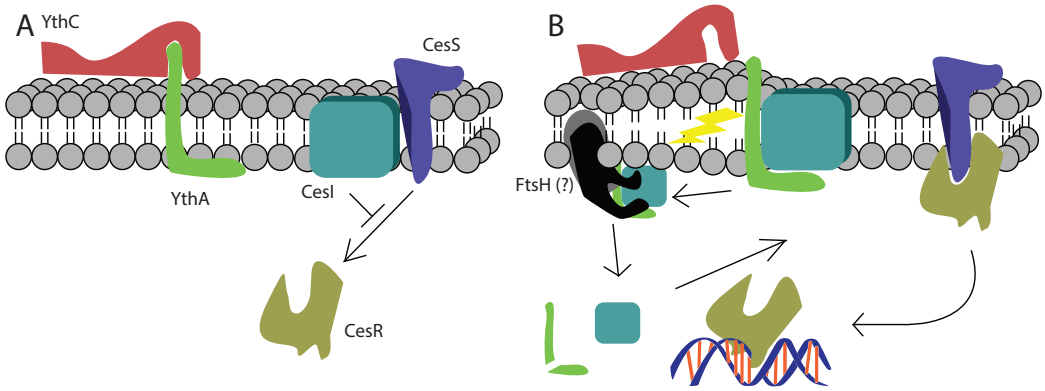

Figure 4.2: Putative working model of the psp-system and its interaction with CesSR in *L. lactis* IL1403. (left) Un-induced, YthC sequesters YthA by an extracytoplasmic interaction. CesI (llmg1650/YjbB) is proposed as the inhibitor of the ces response in this model. (right) An inducing condition represented by a yellow lightning symbol perturbs the membrane causing YthA to disassociate from YthC, unbound YthA associates with the inhibitor CesI. Signal is relayed from CesS to CesR that subsequently activates transcription of the regulon. YthA is subsequently degraded, possibly by FtsH, which releases CesI that might then be recycled.

In this model mutated forms of YthA and YthC can both cause activation of Ces, (i) if YthC is not able to sequester YthA, the inhibitor CesI will be bound to YthA. (ii) Mutations in YthA interfere with binding to YthC, such that YthA can interact with CesI. The transmembrane topology of YthA predicts residues 40 to 60 to be located in the membrane, with the remaining C-terminal region on the extracytoplasmic side. If CesI interaction involve the N-terminal region (residue 0-40) the majority of mutations will be on residues involved in YthC binding rather than CesI. Also interesting to note that the PspC-domain is located on residues 4-65, however the function of this domain is not known. In fact, PspC is annotated as a DNA-binding transcriptional activator in many databases and believed to dimerize and form a leucine-zipper motif [84]. Instead of interacting with CesI to activate Ces, YthA could dimerize when released from YthC and positively regulate the CesR regulon by binding DNA directly or together with CesR. Dimerization could instead occur only with the N-terminal PspC-domain part. Disassociation of YthA from YthC could expose it to a membrane-bound

protease like FtsH, following cleavage the cytoplasmic part of the protein form the activated transcriptional regulator. A membrane-bound metalloprotease FtsH has been shown to degrade PspC in *E. coli* and *Yersinia enterocolitica* specifically in an uncomplexed form [99]. The leucine-zipper motif is 60-80 aa, corresponding to the length of the predicted cytoplasmic region of YthA.

In bacteria ranging from clinically relevant human pathogens like *E. faecium*, *E. faecalis* and *Y. enterocolitica*, to the industrial food producer *L. lactis*, the psp-genes appear central in their defense mechanism to cell envelope disruption and other stressors. Even so, the Psp-proteins remain largely uncharacterized and with a mechanism of protection that is unknown.

4.10 GakI is a Four-Helix Bundle

GakI share 30% sequence identity with the immunity protein for AurA70, AurI [128]. The structure of AurI has only been characterized by the *in silico* prediction tools TMpred and PSIPRED. Assisted by the tools mentioned Coelho et al. [129] postulated a structure of four antiparallel helices likely inserted into the cytoplasmic membrane. The same results are also obtained for GakI (see Figure 4.3) [130, 131].

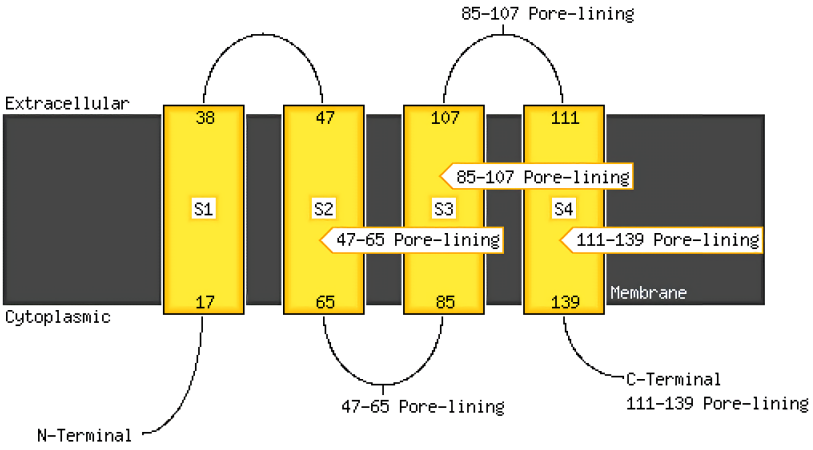

Figure 4.3: Transmembrane topology prediction of GakI by PSIPRED MEMSAT-SVM V4.0 [130].

In fact, all immunity proteins for multi-peptide bacteriocins are predicted to have four transmembrane helices. To further verify the predicted structure, the sequence of GakI and AurI was both submitted to the QUARK structure prediction server [132, 133]. QUARK is a computationally based algorithm for protein structure prediction as opposed to a template-based algorithm. Other algorithms rely on homologs in the Protein Data Bank. The structure generated by QUARK for both immunity proteins is a compact bundle of four antiparallel helices, in agreement with other predictions and similar to other immunity protein structures (see Figure 1.9). This further exemplifies the importance of the four-helix bundle structural motif in immunity proteins.

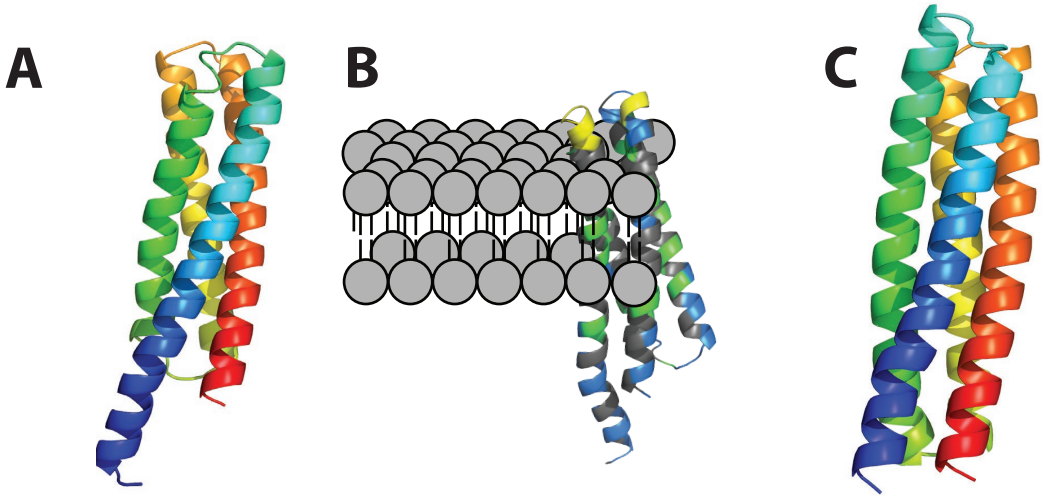

Figure 4.4: (A) Structure predicted for GakI. (B) Cartoon representation of GakI inserted into the cytoplasmic membrane as predicted by MEMSAT-SVM. Extracytoplasmic coil in yellow, hydrophilic residues in blue and hydrophobic residues in green. (C) Structure predicted for AurI. Structural models A & C were generated by QUARK [132, 133].

4.11 The Role of the Receptor

The premise of this thesis is based on the hypothesis that the immunity protein associates strongly with the receptor of the cognate bacteriocin when the bacteriocin is present. This is based on the model proposed for bacteriocins targeting the Man-PTS system, as proposed by Diep et al. [32] for lactococcin A. However, this model seems to require that the receptor itself is part of the pore that ultimately kill the cell. Such a mechanism is easy to imagine when the receptor is a transporter, as the transporter already possesses pore-like conformational states that can be ”locked” or induced by the bacteriocin to result in an ”open” leaky state. It is not as easy to imagine how the same mechanism or model would apply to e.g. lactococcin G that targets UppP [39]. Yet, LcnG also kill target cells by permeabilization of the CM [42]. Another example is LsbB that targets a membrane-bound protease, also with damage to the membrane [134].

Barraza et al. [135] has demonstrated both membrane permeabilization and immunity with the bacteriocin enterocin CRL35 (mundticin KS) in the absence of a receptor. This was achieved by fusing the bacteriocin to a protein that localizes to the membrane [135]. Enterocin CRL35 is a class IIa bacteriocin presumed to target Man-PTS, as is likely with all members of class IIa. In this alternate model the receptor merely act as an initial docking or anchoring point to bring the bacteriocin in close proximity to the membrane. The close proximity facilitate insertion of the bacteriocin into the membrane which then form pores that no longer depends on the receptor. Recently, Farizano et al. [136] demonstrated the down-regulation of Man-PTS in *L. monocytogenes* with an increase in sensitivity to enterocin CRL35 and pediocin PA-1. The authors attribute the increase in sensitivity to changes in lipid composition of the membrane. Hence, lipid-bacteriocin interactions are im-

portant for susceptibility Farizano et al. [136]. In this latter model, the immunity protein would interact directly with a distinct conformation adopted by the bacteriocin only within the membrane. Pediocin-like bacteriocins has been shown to be unstructured in aqueous solution, but become structured within a membrane-like environment [137].

The findings supporting each model are difficult to consolidate based on current knowledge, it is clear that the understanding of bacteriocins at the molecular level are still lacking. Demonstrating a direct interaction between between LcnA, LciA and Man-PTS by co-immunoprecipitation has been of great importance to our understanding of bacteriocin mechanisms, but equally important would be an understanding of why the technique works for this bacteriocin and immunity protein. Could LcnA and LciA be made to work in the absence of a receptor, as described for enterocin CRL35 by Barraza et al. [135]? If bacteriocin receptors are merely exploited for inserting the bacteriocin into the cytoplasmic membrane, including Man-PTS, Co-IP is unlikely to be viable in elucidating bacteriocin receptors. It is conceivable that the mechanism of immunity proposed by Diep et al. [32] is correct, but that it is unique for LcnA.

4.12 'To Fish or Not To Fish?'

Diep et al. [32] convincingly identified the receptor for lactococcin A by immunoprecipitation. However, no other bacteriocin receptors has been identified by this technique so far. A similar approach has been attempted previously for lactococcin G, but without success [39]. It is possible that the formation of an immunity complex sufficiently stable for Co-IP is unique to LcnA. Co-IP is a laborious technique that requires extensive optimization unique for every protein, no single protocol will work for every system. The GakI system could be additionally challenging due to the high hydrophobic character and transmembrane topology of this immunity protein. However, a stabilizing effect of the bacteriocin on the immunity protein could be indirect evidence of the formation of an immunity complex as hypothesized for LcnA. In this work three proteins co-precipitated with the garvicin KS immunity protein. Further work is needed to identify the three proteins and assess their potential role as a receptor for GarKS, this will establish the viability of Co-IP for this immunity protein.

Co-immunoprecipitation is by many researchers still considered the gold-standard technique in resolving suspected protein-protein interactions [138]. However, a few different approaches to Co-IP ought to be further developed for bacteriocin receptor identification. Cross-linking is the use of reagents that covalently link proteins and peptides that are in close proximity. Cross-linking can be performed both *in vivo* and *in vitro* to "lock in place" protein-protein interactions. A promising cross-linker for the application of bacteriocin receptor identification is formaldehyde, a small molecule that readily dissolves into the CM [139]. However, the FLAG-tag is not compatible with this cross-linker but His-tag and Strep-tag is compatible. Even if receptors for many bacteriocins merely act as docking

sites for insertion into the CM, cross-linking could potentially trap the bacteriocin during its initial interaction. A benefit of Co-IP following cross-linking is that it can be performed much more stringent without loss of the protein interaction. Another interesting approach would be Co-IP of the bacteriocin itself. In recent years synthetic peptides has replaced natural isolates for many unmodified bacteriocins. The custom synthesis of bacteriocins is a powerful tool for generating variants of bacteriocins with different properties and potency [6, 140]. A peptide tag like the FLAG-tag could be added to the synthetic bacteriocin with little added cost. Such an approach would skip the time-consuming cloning required when using the immunity protein.

Co-IP coupled with liquid chromatography tandem mass spectrometry is a powerful and important technique widely documented in the scientific literature for studying protein-protein interactions. Knowledge of the available tools in peptide identification also allows for MS analysis of cross-linked proteins. The issues reported in this thesis could be overcome by few modifications to conventional protocols and by including more parallels and negative controls. Shotgun proteomics of immunoprecipitate should in theory be a powerful technique, without the need of any optimized protocol. By simply treating a tagged immunity protein sample the same as a non-tagged negative control, even very weak interactions would lead to an increase in concentration of interacting proteins detectable by MS. However, the analysis should ensure a more equal coverage of proteins of hydrophobic and hydrophilic properties.

An expansion of conventional Co-IP that includes cross-linking and also using the bacteriocin as a bait in addition to the immunity protein, could make the technique more efficacious in receptor identification.

5 Future Work and Concluding Remarks

The Co-IP experiments with GakI ought to be reproduced and give the same three co-precipitating proteins as reported in this thesis. If identification of the three proteins yield a good candidate for the receptor, the obvious next step is to construct knock-out mutants for the gene. If the sensitivity to GarKS is reduced substantially, complementation of the gene should restore normal sensitivity. Together that would be strong evidence that the gene is responsible for GarKS sensitivity and involved in the mode of action of the bacteriocin. If Co-IP is not successful, generating spontaneous mutants with a much lower sensitivity to GarKS should be attempted.

What is missing for multi-peptide bacteriocins are resistant mutants. The so-called mutants to GarKS analyzed in this thesis has a 2-8-fold decrease in sensitivity, which is unlikely to involve the receptor [20]. Mutants generated for lactococcin G (LcnG), a two-component class IIb bacteriocin, showed a 700-2500-fold increase in resistance in a study that identified the likely receptor of this bacteriocin by sequencing [39]. Further attempts should be made at generating GarKS mutants with a more significant increase in resistance, subsequent sequencing would likely be sufficient to pinpoint the receptor.

The Psp-system in lactococci has not been characterized, mutations in *ythA* occur frequently in GarKS mutants while YthC appear to be of high abundance in cells exposed to GarKS. Co-IP of YthA would help resolve the significance of *ythA* mutations to GarKS resistance, and also provide experimental evidence for improving the model of Psp/CesSR proposed in this thesis. If a member of the ces-system co-precipitated with YthA, that would conclusively link the two systems.

Due to their relatively low size and complexity, there is a lot of potential in studying multiple aspects of bacteriocins by molecular dynamics simulations. Both the pore-forming ability of nisin, as well as class IIa bacteriocin-immunity interactions has been investigated with molecular dynamics [141, 142]. In addition, many lipid bilayer systems and other membrane-mimicking environments that are central to bacteriocins has been extensively modeled *in silico* [143]. Such simulations could give valuable insights into molecular aspects that are otherwise difficult to study. GarKS consist of three peptides believed to make a single antimicrobial unit, simulations could provide clues into the structure of this unit and how it interacts with the membrane. However, the structure of GarKS and GakI is required for accurate simulations, but the structures has not yet been solved.

The target receptor and mode of action of multi-peptide bacteriocins is still unknown, but believed to be same for all members of this group due to sequence similarities and cross-immunity demonstrated in this thesis. GarKS has a relatively broad antimicrobial spectrum, inhibiting growth of important human pathogens of the genera *Listeria*, *Bacillus*, *Enterococcus* and *Staphylococcus* [6]. A functional GakI in *S. aureus* demonstrated in this thesis suggest that the same receptor and mechanism is involved in this important human pathogen. Identifying the receptor of GarKS would be invaluable in devising commercial and clinical use cases for this bacteriocin - and likely all multi-peptide bacteriocins discovered to date.

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6 Supplementary Material

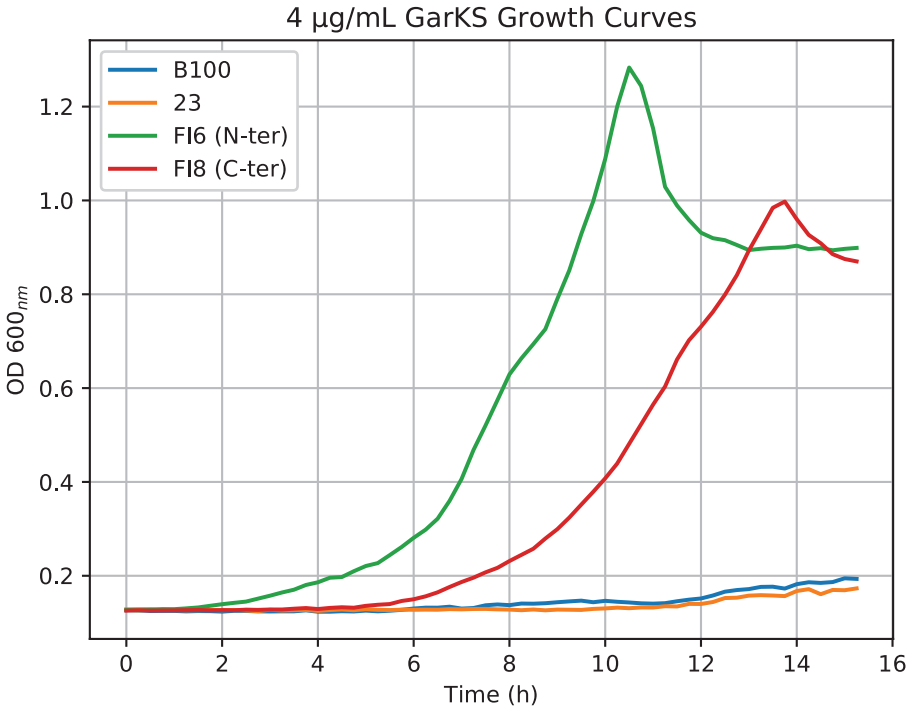


Figure 6.1: Growth characteristics of IL1403 strains at 4 µg/mL GarKS.

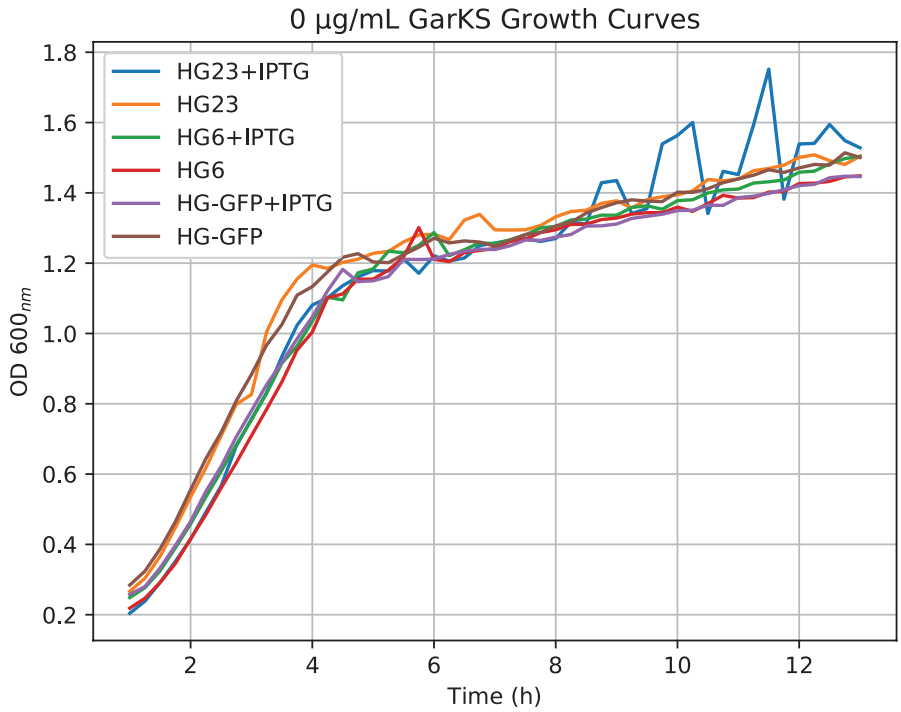
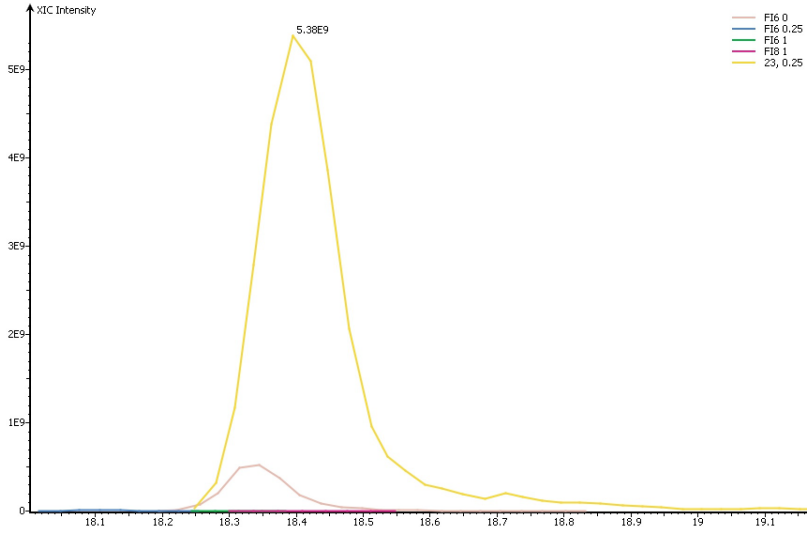


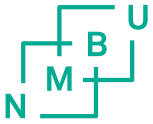
Figure 6.2: Normal growth characteristics of HG001 clones, with and without IPTG.

Sample	Area	Ratio
FI6 0	7.3E7	0.41
FI6 0.25	1.65E6	0.02
FI6 1	4.03E5	0.02
FI8 1	1.06E6	0.02
23, 0.25	8.09E8	4.54




```
  1 DVLMTQAPLT LPVSLGDQAS ISCRSSQAIV HANGNTYLEW YLQKPGQSPA LLIYKVANRF SGVPDRFSGS GSGTDFTLKI
 81 SRVEAEDLGV YYCFQGAHAP YTFGGGTKLE IKRADAAPTV SIFPPSSEQL TSGGASVVCF LNNFYPKDIN VKWKIDGSER
161 QNGVLNSWTD QDSKDSTYSM SSTLTLTKDE YERHNSYTCE ATHKTSTSPI VKSFNR
```

Figure 6.3: Extracted ion chromatogram of relative intensities of the peptide mapped to the light chain of the antibody (below). Ratios in the table in the upper left are relative to the average intensity of the five samples (log2 ratio). Sample 23 0.25 have a high ratio relative to the other samples. The x-axis is retention time.



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