CLONING, EXPRESSION AND PURIFICATION OF THE IMMUNITY FACTOR ASSOCIATED WITH LEUCOCIN A PRODUCTION

By

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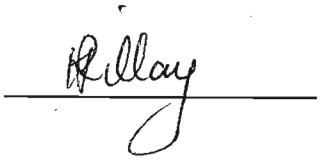
PREFACE

The experimental work described in this thesis for M.Sc was carried out in the School of Molecular and Cellular Biosciences, University of Kwa-Zulu Natal, Pietermaritzburg, under the supervision of Dr Mervyn Beukes.

These studies represent the original work by the author and have not otherwise been submitted in any form for any degree or diploma to any University. Where use has been made of the work of others, it is dually acknowledged in the text.



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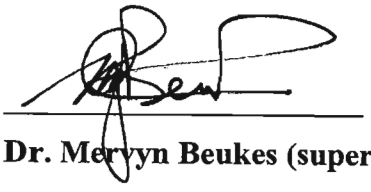

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ABSTRACT

Leucocin A is a bacteriocin produced by *Leucoconostoc gelidium* UAL 187-22. Bacteriocin producer strains possess an immunity protein, which enables the strain to protect itself against its own bacteriocin. The immunity gene from *Leucoconostoc gelidium* was isolated via PCR from a recombinant clone pJF5.5. This fragment was cloned by amplifying the immunity gene from pJF5.5 and ligating it into pMALc2. The resulting recombinant plasmid pKP1 was then transformed into *Escherichia coli* strain JM103. The clone putative, was confirmed by DNA sequencing and southern blot hybridization using the primers EAL-2 and EAL-3. It was shown to contain an insert of 3.6 kb. Expression analysis showed the construct as an in frame malE fusion protein expressed within *E. coli*. The fusion construct was isolated by affinity chromatography. Leucocin A was purified to 90% purity, from the supernatant of *Leucocnostoc gelidium* UAL 187-22 by ion-exchange chromatography and HPLC. It was found to elute from a C_{18} reverse phase column at 55% actetonitrile, 0.1% TFA. Binding interaction and the stability of the immunity gene fusion protein were compared using a Biacore 2000. The supernatant and cytoplasmic extract isolated from *Leucocnostoc gelidium* UAL 187-22 were tested for interaction with the fusion construct. Surface Plasmon resonance studies indicated that there was no binding partner present in the supernatant which would influence the immunity process. However, a stable interaction was found between the immunity protein and an orphan ligand within the cytoplasm.

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LIST OF ABBREVIATIONS

A_{260}	absorbance at 260nm
A_{280}	absorbance at 280nm
EDTA	ethylenediamine tetraacetic acid
HPLC	high phase liquid chromatography
HBS	HEPES buffered saline
IPTG	isopropyl-β-D-thiogalactopyranoside
LAB	lactic acid bacteria
LB	Luria Bertani
MRS	De Man Rogosa Sharpe Agar
OD_{260}	optical density at 260nm
OD_{280}	optical density at 280nm
OD_{590}	optical density at 590nm
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PTS	phosphophotransferase system
RT	room temperature
SDS	sodium dodecyl sulfate
SPR	surface plasmon resonance
TFA	tri flouro acetic acid
TSA	tryptone soy agar
TSB	tryptone soy broth

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CHAPTER ONE

LITERATURE REVIEW

1.1 Introduction to bacteriocins

Bacteriocins have been defined as antimicrobial peptides or proteins with bacteriocidal activity against closely related bacterial species (Hoffmann *et al*, 2004; Klaenhammer, 1993), however the producer strain is immune.

As early as 1877, Pasteur and Joubert, described the antagonistic interaction between competing bacteria (Oscariz and Pisabaro, 2001) when they noticed that some *Escherichia coli* strains interfered with the growth of *Bacillus antracis* present in infected animals (Jack *et al.*, 1995). Andre' Gratia, who observed that the growth of certain *E. coli* strains was inhibited by the presence of an antibacterial compound discovered the first detected bacteriocin in 1925 (Oscariz and Pisabaro, 2001). Bacteriocins are low molecular weight antimicrobial peptides often produced by lactic acid bacteria and are found to be associated with food spoilage (Felix *et al.*, 1994).

LAB are found to be inhibitory to other bacteria because of pH, organic acids, hydrogen peroxide, nutrient depletion, decrease in redox potential, other chemicals and bacteriocin production which are found to be produced during their growth (Bhugaloo-Vial *et al.*, 1996; Stiles, 1994). The conversion of sugars into organic acids by LAB is a well-known fermentation process to improve flavor and texture of certain food products. LAB have also been found to inhibit the growth of undesirable bacteria (Bhugaloo-Vial *et al.*, 1996).

LAB is known to be widely used as starter cultures for dairy, meat and vegetable fermentations. The growth of pathogenic and spoilage bacteria is also inhibited by LAB, this allows the shelf life of different meat products to be extended and also for the hygiene quality to be improved (Cintas *et al.*, 1995). *Leuconostoc* species, lactobacillus and pediococcus strains are LAB commonly associated with foods. *Leuconostoc* species are used as starter bacteria in

certain dairy fermentations and lactobacillus and pediococcus strains are used for the manufacture of fermented sausages that contribute to flavor and color development (Cintas *et* al., 1995; Stiles, 1994).

LAB play an essential role in food fermentation processes and their bacteriocins are not known to cause harm to humans or other eukaryotes and are therefore regarded as safe (Carolissen-Mackay *et al.*, 1997; Eijsink *et al.*, 2002). LAB, many of which have been isolated over the past years, have produced a wide variety of antibacterial peptides. Many LAB produce a high diversity of many types of bacteriocins (Jack *et al.*, 1995). *Leuconostoc gelidium* is one of the strains that are known to exhibit a wide spectrum of inhibition towards lactic acid bacteria, meat spoilage and food related human pathogens including the well-known *Listeria monocytogenes* (Hastings *et al.*, 1991).

In the studies of certain bacteriocins carried out they are non-toxic and non-antigenic to animals (Felix *et al.*, 1994). Bacteriocins are ribosomally synthesized antimicrobial peptides and may inhibit target cells by permeabilizing the cell membrane (Papathanosopoulos *et al.*, 1997). Bacterial produced antimicrobial peptides have been grouped into different classes on the basis of the producer organism, molecular size, chemical structure and mode of action (Jack *et al.*, 1995; Klaenhammer, 1993). Different classifications have resulted in different names (microcin, colicin, bacteriocin, lantibiotic, thiolbiotic, cytibiotic), which can be misleading as the same compound may be found under a different name in a different classification.

1.2 Bacteriocin diversity

The four major types of compounds that are produced by lactic acid bacteria have been identified: Class I membrane active and heat stable peptide lantibiotics. Class Ia lanthionine and β-methyllanthionine (Gajic *et al.*, 2003) and also consists of screw shaped, amphipathic, small cationic peptides that produce high voltage-dependent pores by interaction with target cell membranes, e.g. nisin. Nisin is the most widely used natural food preservative especially in dairy products and was discovered in 1928 being produced by some *Lactococous lactis* strains (Sahl and Bierbaum, 1998). Class Ib consists of anionic or neutral peptides that have a globular shape and includes antibiotics like mersacidin (Oscariz and Pisabarro, 2001).

Class II: consists of unmodified heat-stable peptides with a molecular mass less than 10kDa. These peptides are hydrophobic with a high isoelectric point (Van Belkum and Stiles, 1995). The gene from which the peptide is derived, code for prepeptides that contain an N-terminal extension (leader peptide). This leader peptide shows some sequence homology especially at the proteolytic processing site where two glycine molecules are at this position. The leader peptides are predicted to be amphiphillic; a hydrophobic stretch of signal peptides of export proteins is missing. Flanking regions upstream and downstream of the structural gene contain additional open reading frames for which a function of bacteriocin production could be postulated on the basis of homology. These genes code for immunity peptides, regulatory proteins, as well as the transporter protein (Boman and Marsh, 1994). Class II bacteriocins are further divided into three subgroups: Class IIa, pediocin like bacteriocins, class IIb, two peptide bacteriocins and class IIc, unmodified bacteriocins (Gajic *et al.*, 2003; Oscariz and Pisabarro, 2001).

Class IIa bacteriocins have the consensus sequence YGNGY at their N-terminal sequence which is involved in *Listeria* strain recognition (Bhugaloo-Vial *et al.*, 1996; Gibbs *et al.*, 2004) (e.g.) pediocin PA-I/AcH, leucocin A, sakacin P, and

curvacin). Class IIb bacteriocins consists of pore-forming complexes that require two peptides for their activity e.g. lactacin; class IIc bacteriocins are peptides containing thiolbiotics and cystibiotics, lactococcin B being a member of this group (Oscariz and Pisabarro, 2001; Van Belkum and Stiles, 1995).

Table 1.1: Bacteriocins produced by Class II LAB

Bacteriocin	Producer	References
Class IIa bacteriocins		
Pediocin PA-1/AcH	*Pediococcus acidilactici* PAC1.0/ H	Fremaux *et al.*, 1993; MacKenzie *et al.*, 1997
Leucocin A-UAL 187	*Leuconostoc gelidium* UAL 187	Barefoot and Kleanhammer, 1984; Harris and Angal, 1989; Riley and Wertz, 2002
Mesentericin Y105	*Leuconostoc mesenteroides* Y105	Enhnahar *et al.*, 2000; Liedberg *et al.*, 1995
Sakacin A	*Lactobacillus sake* Lb 706	Fimland *et al.*, 2002; Harris *et al.*, 1989
Sakacin P	*Lactobacillus sake* LTH 674	Fremaux *et al.*, 1993; Harris *et al.*, 1989
Carnobacteriocin A	*Carnobacterium piscicola* LV 17A	Fimland *et al.*, 2002; Harris *et al.*, 1989
Carnobacteriocin BM1	*Carnobacterium piscicola* LV 17B	Fremaux *et al.*, 1993; Harris *et al.*, 1989
Class IIb bacteriocins		
Lacticin F	*Lactobacillus acidophilus* 11088	Fimland *et al.*, 2002; Harris *et al.*, 1989
Mersacidin	*Bacillus species*	Cintas *et al.*, 1995; Harris *et al.*, 1989
Lacticin 481	*Lactococcus lactis* 481	Gonzalez *et al.*, 2001
Lactococcin G	*Lactococcus lactis*	Altschul *et al.*, 1997

Class III consists of peptidic antibiotics that are heat labile with a molecular mass of larger than 30kDa. Members of this group are helveticin (produced by *L. acidophilus*) (Oscariz and Pisabarro, 2001). The bacteriocins of this group are synthesized as precursor peptides with an N-terminal leader sequence.

Class IV bacteriocins consists of either glycoproteins or lipoproteins that require protein moieties for their activity. They are peptides with very few disulfide bridges. Members of this group are pediocin SJ-1, leucocin S (Hastings *et al.*, 1991; Stiles, 1994; Van Belkum and Stiles, 1995) and lactocin 27. None of these operons controlling biosynthesis or immunity have been characterized for those bacteriocins already identified (Klaenhammer, 1993).

1.3 Bacteriocins produced by *Leuconostoc* species

In 1997, Papathanasoupoulos and co workers found that *L. mesenteroides* TA33a produced three different bacteriocins, each having a different inhibitory activity. These bacteriocins were named Leucocin A, B and C-TA33a respectively. Leucocin A TA33a was found to be identical to leucocin A-UAL 187 (Papathanosopoulos *et al.*, 1997). Leucocin B-TA33a has a high sequence homology to mesentericin 52B but they differ greatly in their modes of action and antibacterial spectra (Corbier *et al.*, 2001). Both mesentericin52B and leucocin B TA33a don't contain the YGNGV consensus and also don't have a disulfide bridge which is shared by other bacteriocins (Corbier *et al.*, 2001).

Leucocin A-UAL 187 the bacteriocin by *L. gelidium* UAL 187 consists of two open reading frames and putative upstream promoter. The structural gene was found in the first open reading frame downstream of the promoter (Hastings *et al.*, 1991). The first open reading frame encodes 61 amino acids and the second open reading frame can encode the immunity protein for leucocin A but this has not yet been confirmed (Stiles, 1994). Leucocin A contains 24 amino acids and has a bacteriocin cleavage site. Leucocin B-Ta11a, produced by *L. carnosum* Ta11a, is active against *L. monocytogenes* and other LAB. After sequencing of a clone of *L. Carnosum*, it was found that two open reading frames are present. ORF 1 consisted of 61 amino acid, 37 of which was determined to be leucocin

B-Tal1a (Felix *et al.*, 1994; Hastings *et al.*, 1991) and a 24 amino acid N-terminal extension. The ORF 2 with 113 amino acid is identical to amino acid of leu A UAL 187.

Several *Leucocstoc* spp produce the bacteriocin leucocin which is known to inhibit the growth of the potential food borne pathogen *L. monocytogenes*, (Ramnath *et al.*, 2000). LAB cause inhibitory activity against other bacteria due to competitive growth in specific food environments. LAB are inhibitory to other bacteria because of pH, organic acids, hydrogen peroxide and other chemicals produced during their growth (Stiles, 1994). Two known types of leuconostoc spp are *L. carnosum* and *L. gelidium*. They are also heterofermentative and can be distinguished on the basis that they each have different carbohydrate fermentation patterns (Stiles, 1994). Leucocin A-UAL 187, a bacteriocin produced by *L. geldium* UAL 187 was previously purified by Hastings *et al.*, 1991. Leucocin A-UAL 187 is known to produced early in the growth cycle of the producer strain and has a molecular mass of ~ 3930Da (Harris *et al.*, 1989; Hastings *et al.*, 1991) Recently Fimland *et al.*, (2002) purified and completely sequenced the antimicrobial peptide leucocin C which is produced by a strain of *L. mesenteroides*, using a rapid two-step procedure and Edman degradation respectively. Previously the amino acid sequence of leucocin C was reported to be 36 sequenced residues (Papathanosopoulos *et al.*, 1998), however this was incomplete as its molecular mass was not consistent with the theoretical mass as calculated from it's sequence. The correct sequence of leucocin C is now found to contain 43 residues with a molecular mass of 4595 as determined using mass spectroscopy, which is consistent with the theoretical mass 4596 (Fimland *et al.*, 2002 (b)).

The treatment of leucocin with protease, trypsin, chymotrypsin, pepsin or papain causes complete loss of activity whereas treatment with lysozyme, lipase and phospholipase has no effect on the activity of leucocin (Harris *et al.*, 1989; Hastings *et al.*, 1991).

1.4 Bacteriocin resistance

There is an increasing interest in the use of bacteriocins from lactic acid bacteria in foods. Certain bacteriocins appear to inhibit potential food-borne pathogens including *Clostridium botulinum, Enterococcus faecalis, Listeria monocytogenes* and *Staphylococcus aureus* (Barefoot and Klaenhammer, 1984). Knowledge of the mechanism of action, immunity and resistance and the production of bacteriocins are of utmost importance in obtaining a natural defense against illness than to use chemically synthesized drugs.

The major problem now being faced is that there is a major development of bacterial resistance. This directly affects the use of chemotherapeutic agents and bacteriocins. Food safety has become a growing concern due to the numerous food-poisoning outbreaks and also the concern about preservation of processed foods. New approaches for inhibiting food-borne pathogens and for prolonging the shelf life of food products have increased.

Nisin, the most widely used natural food preservative is known to act mainly by the formation of pores in the bacterial cytoplasm membrane resulting in bacterial killing (Brotz and Sahl, 2000; Moll *et al.*, 1996). A major problem has however been reported on an enzyme called nisinase. This enzyme has been isolated from *Lactococcus lactis* species (Robichon *et al.*, 1997).

Research on gram positive bacteria is important with respect is important to the prevention and treatment of bacterial infection, since there is a rapid rise and spread of multi-resistant bacterial pathogens which have forced alternative methods of combating infection (Riley and Wertz, 2002). Bacteriocins of gram-positive bacteria differ from that of gram-negative bacteriocins in that the production of bacteriocin is not the lethal event for gram-negative bacteria and the transport mechanisms which gram-positive bacteria encode to release bacteriocin toxin (Riley and Wertz, 2002). Bacteriocins of gram-positive bacteria are of a low molecular weight and appear to be translated as pre-peptides that are subsequently modified into the mature, biologically active form.

The advantage associated with the use of bacteriocins in food is that they are considered to be normal constituents of the human diet, in that meat and dairy systems are rich in bacteriocinogenic lactic acid bacteria, therefore they should not alter the digestive tract ecology or cause any harm. Specific auxiliary functions required of bacteriocin-producing cells include mechanisms for extra cellular translocation of the bacteriocin and for conferring immunity to the bactericidal activity of the molecule.

Resistance has been found to be spontaneous or can be induced by exposure to bacteriocins. In recent studies carried out, it was suggested that the high level of resistance to class II *L. monocytogenes* is associated with one mechanism, irrespective of wild-type strain, class II bacteriocin or environmental conditions (Gravesen *et al.*, 2002). However, studies carried out by Ramnath *et al*, (2000) and Gravesen *et al.*, (2002) shows that the mechanism of class II bacteriocin resistance involves the absence of the EIIAB subunit of a mannose-specific phosphophoenolpyruvate-dependent phosphotransferase system (Vadyvaloo *et al.*, 2004) in *L. monocytogenes* strains. High-level resistance to class II bacteriocins in *L. monocytogenes* involves the removal of the mannose-specific phoshoenolpyruvate dependant phosphotransferase system (PTS). PTS is encoded by a *mpt*ACD operon (Ramnath *et al.*, 2004), is transcribed by the sigma factor δ^{54} which is encoded by the rpoN together with the activator ManR (Gravesen, 2004; Vadyvaloo *et al.*, 2002). Genetic inactivation of the *mpt*ACD operon was found by Hechard *et al.*, (2001), to result in resistance to mesemtericin Y105 in *L. monocytogenes* and *Enterococcus faecalis*. In another mechanism of resistance the subunit IIAB of mannose permease of the PTS, which has resistance to leucocin A, was found to be missing in a spontaneous mutant of *L. monocytogenes* (Ramnath *et al.*, 2000). Studies carried out by Gravesen *et al.*, (2002), made similar observations for a number of spontaneous mutants of *L. monocytogenes* which showed resistance to class II bacteriocins. Reports of cross-resistance between class II bacteriocins with similar or identical resistance mechanisms have also been noted (Dykes and Hastings, 1998; Ramnath *et al.*, 2000). Mutants of *L. monocytogenes* which are resistant to bacteriocins could be rendered less sensitive to defensins due to non-specific cross protection between cationic peptides (Gravesen *et al.*, 2004).

1.5 Mode of action of bacteriocins

The mode of action of bacteriocins is to produce proteins that interrupt the cell membrane to allow valuable nutrients out and in essence destroy the bacteria. Class Ia lantibiotics and their mode of action is based upon formation of voltage dependent, short-lived pores in the cytoplasmic membrane (Boman and Marsh, 1994). Nisin acts in a voltage dependent manner on energized cells, membrane vesicles and liposomes (Harris *et al.*, 1989). The interaction between the hydrophobic part of nisin and the bacterial target membrane generates specific ionic channels whose formation is aided by the presence of high transmembrane potential (Oscariz and Pisabarro, 2001). The peptides rapidly induce leakage of ions and small metabolites from bacterial cells and collapse the electrochemical proton gradient (Boman and Marsh, 1994). Nisin generated membrane pores efflux K^+ and Mg^+ ions passively as well as amino acids and ATP (Oscariz and Pisabarro, 2001). Peptide molecules form pores by moving through the membrane in response to the electrical potential while remaining surface-bound (Sahl and Bierbaum, 1998).

Cessation of biosynthetic processes occurs due to the loss of energy and precursors, which eventually leads to cell death (Boman and Marsh, 1994). Killing of cells sets in immediately after the addition of the peptides. Inhibitors of cell wall synthesis usually induce cell lysis (Sahl and Bierbaum, 1998). Pore formation decreases in the presence of divalent cations (Mg^{2+} and Ca^{2+}) because they neutralize the negative charges of the phospholipids, reducing the fluidity of the membrane (Oscariz and Pisabarro, 2001).

The proton motif force is a requirement for Type A lantibiotics in order for pore formation to occur (Figure 1.1). Activity is promoted by electrical potential or chemical potential gradient, provided it is sufficient magnitude (Boman and Marsh, 1994). Lysine is the cationic amino acid involved in the electrostatic interaction (Oscariz and Pisabarro, 2001). Pore formation or pore stability could be influenced by factors such as: (i), phospholipid composition of the membrane, (ii) interaction of the peptides with integral membrane components and (iii) the presence of surface layers. They could also exert secondary effects

and (iii) the presence of surface layers. They could also exert secondary effects that contribute to the bactericidal activity. This effect was observed with *Staphylococcus* species, which is sensitive to Pep 5 and nisin. This is due to the fact that lantibiotics induce autolysis of cells by activating the cell wall, hydrolyzing enzymes of the target cell (Boman and Marsh, 1994).

The antibacterial effect of Type B lantibiotics is rather weak when in comparison with Type A lantibiotics. They are known to bind to the head group of phospholipids and therefore inhibit phospholipase A_2 in a non-competitive way. Mersacidin is active against staphylococci and is also effective against Vancomycin and evidence has been found to prove that it also blocks peptidoglycan biosynthesis (Boman and Marsh, 1994).

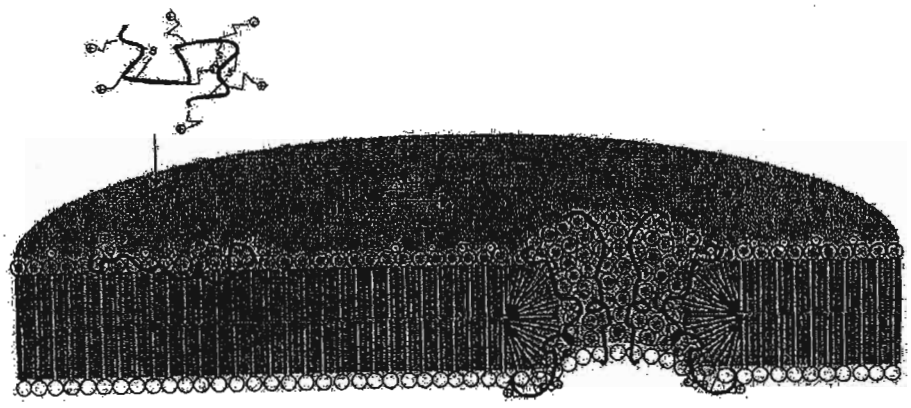


Figure 1.1: A model to show the pore formation by cationic type-A lantibiotics: The peptides adopt an amphiphillic conformation once they are bound to the membranes which then enables insertion into the outer leaflet of the membrane. Once the membranes have sufficient energy the peptides that remain surface-bound can carry the phospholipids through the membrane. When there are many peptides at the same site, transient forms start to form (Sahl and Bierbaum, 1998).

The mode of action of class II bacteriocins contains many hydrophobic amino acids, which suggest that the plasma membrane could be the target for antimicrobial activity of such peptides. The peptide dissipates the membrane potential of sensitive bacterial cells; insensitive strains and the immune producer strain are not affected at comparable bacteriocin concentrations (Boman and Marsh, 1994). Class IIa bacteriocins show a great amount of anti-listerial activity due to the presence of the sequence YGNGY in their N-terminal region (Oscariz and Pisabarro, 2001). Their mode of action includes electrostatic binding of the antibiotic to the target membrane that is mediated by a receptor molecule (Oscariz and Pisabarro, 2001). They are thought to act primarily by permeabilizing the target membrane by forming pores (Vadyvaloo *et al.*, 2002). The pores cause leakage of ions and inorganic phosphates that

dissipate the proton motive force. A receptor type molecule and electrostatic functional binding of cationic peptides to the anionic groups of phospholipids membranes are involved in the mediation of class IIa bacteriocin activity. Lipid composition of the target cell membrane interaction plays a vital role in modulating the membrane interaction of the bacteriocin. The interaction of bacteriocins with phospholipids is reported to largely influence membrane permeability. Different levels of resistance are associated with different resistance mechanisms (Vadyvaloo *et al.*, 2002) (Figure 1.2).

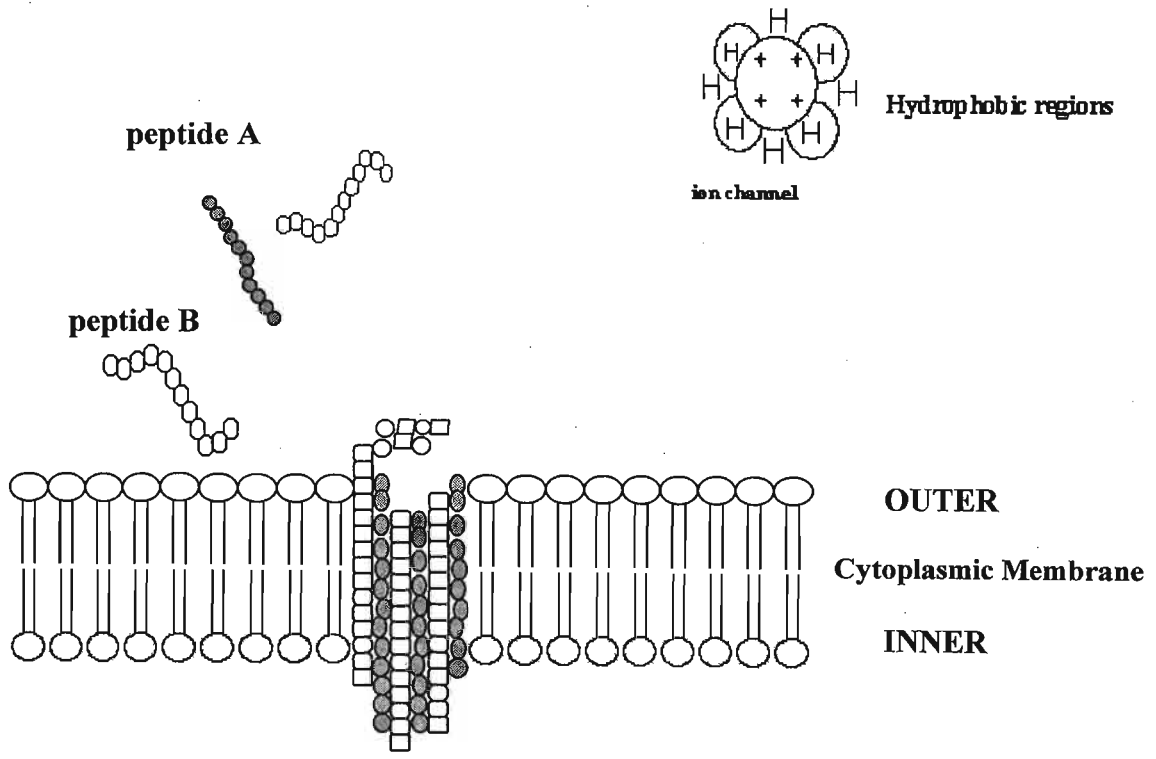


Figure 1.2: Barrel-stave poration complex formed by class II peptide bacteriocins. These complexes are able to form between one or many species of amphiphilic peptides that oligomerize to form pores in the membranes and ion-channels (Klaenhammer, 1993).

1.6 Genetics of lantibiotics from LAB

Bacteriocin sensitivity of the LAB strain has been shown to depend on the immunity gene, the presence of extra genes and the membrane composition or receptors that may be present (Fimland *et al.*, 2002 (a)). Many of the bacteriocins of gram-positive bacteria seem to be encoded by plasmid borne genes. Some, like nisin, have shown to be transposon associated. The bacteriocin-associated genes of gram-positive bacteria appear to be characteristically arranged in multigene operon like structures, the first gene typically encoding the structural protein. Additional gene products may be required for transcription, regulation, posttranslational modification (e.g. lantibiotics), processing translocation to the exterior of the cell and producer strain self-protection (Eisjink *et al.*, 2002).

Lantibiotics are derived from ribosomally synthesized prepeptides with an N-terminal extension (Sahl and Bierbaum, 1998; Sahl *et al.*, 1995; Twomey *et al.*, 2002). These are encoded by structural genes LanA, which have been deduced from the amino acid sequence. LanA prepeptides, which carry the N-terminal extension, are called the leader peptide (Sahl *et al.*, 1995; Twomey *et al.*, 2002). Leader peptides are known, not to contain cysteine residues, but they may contain seriene and theroine, which are the substrate required for most of the modifications (Twomey *et al.*, 2002). The short-lived precursor peptide, is converted into the mature peptide by proteolytic cleavage or via posttranslational modification by processing and export from the producing cell, in which unusual amino acid changes are introduced (Sahl and Bierbaum, 1998; Sahl *et al.*, 1995; Twomey *et al.*, 2002). The process of proteolytic cleavage can occur before, during or after export (Sahl and Bierbaum, 1998). The propeptide exists when the C-terminal segment of the pre-peptide, in which Ser, Thr, and Cys residues are modified into unusual amino acids (Sahl and Bierbaum, 1998).

The lantibiotic bacteriocins of gram-positive bacteria have two different methods by which protection is mediated: (i) the immunity peptide defined as

LanI that are associated with the membrane and, (ii) the ATP-binding cassette transporters LanEFG (Felix *et al.*, 1994; Hoffmann *et al.*, 2004). In some instances e.g. nisin both LanI and LanEFG mechanisms may exist within the same lantibiotic gene cluster (Twomey *et al.*, 2002). Regulation is usually achieved through a two-component regulatory system (LanR, LanK) (Sahl and Bierbaum, 1998). Regulatory proteins, proteases, transporters and immunity proteins can be found in the gene clusters necessary for production of some unmodified bacteriocins (Klaenhammer, 1993). Immunity for Pep5 and nisin has been described.

Open reading frames found in the flanking DNA regions were assumed to be involved in the biosynthesis of lantibiotics when the first structural genes for lantibiotics were detected (Sahl *et al.*, 1995). Apart from the LanA and LanEFG peptides, additional genes encoding for other functions were found. The additional functions included: (i) proteins with similar sequence to those of the two-component regulatory proteins, (ii) proteins with similar sequence to translocators of the ATP-binding cassette transporter protein (LanT proteins) and, (iii) proteins with similar sequence to immunity proteins (LanT proteins) (Sahl *et al.*, 1995). The leader peptide may have the ability to keep the lantibiotic inactive in order to protect the producer strain. The properties of the leader peptide enable it to play an essential role in biosynthesis, in that it could contain a specific recognition sequence which would direct the precursor toward biosynthetic enzymes or that it may interact with the propeptide region which enables the prepeptide conformation to be stabilized (Sahl *et al.*, 1995). Unususal amino acids such as lanthionine, β-methyllanthionine and dehydrated amino acids are introduced by post-translational modification of the precursor peptides which is thereafter followed by the removal of the leader peptide and translocation across the membrane (McAuliffe *et al.*, 2001). It was revealed by circular dichroism (CD) spectra however, that immunity proteins do not interact extensively with membranes as in the case of bacteriocins. This was noted when studies carried out on enterocin A and leucocin A revealed that there was no further increase in the structuring when the immunity proteins were exposed to micelles or liposomes of a specific nature (Johnsen *et al.*, 2004).

Additional accessory proteins, which are involved in the biosynthesis of lantibiotics, have also been found. Proteases (*lanP* genes) are involved in the final step of biosynthesis where the leader sequence is cleaved off by a leader peptidase. This occurs at a processing site (Sahl *et al.*, 1995). Transporters (*lanT* genes) have been found in all lantibiotic gene clusters so far described and aid in transportation (Sahl *et al.*, 1995). The others are regulatory proteins (*lanR*, *lank* genes), which are involved, in a number of physiologically important processes. The regulatory proteins, which are under the control of the two-component response regulatory protein, are also involved in biosynthesis of lantibiotics (Sahl *et al.*, 1995).

1.6.1 The immunity protein and its gene

Bacterial strains producing antibiotic peptides need to protect themselves against the action of their own antimicrobials and therefore they have genetically linked immunity mechanisms linked to the production of the bacteriocin. Each bacteriocin has its own immunity protein, which is expressed concomitantly with the bacteriocin. The immunity gene is generally located next to, and down stream from the bacteriocin gene. The two genes constitute an operon, which may or may not contain other genes involved in bacteriocin production (Fimland *et al.*, 2002 (a)). Primary structures of at least 17 immunity proteins of pediocin-like bacteriocins have been deduced from the DNA sequences (Fimland *et al.*, 2002 (a); Cintas *et al.*, 1997). Immunity proteins have been found to consist of 88 to 115 amino acid residues and show 5 to 85% sequence similarity (Eijsink *et al.*, 1998). Two basic mechanisms have been proposed for immunity to bacteriocins. The first example suggests that a direct interaction between the colicin and immunity protein occurs and this results in inactivation of the pore forming domains of colicins (Lagos *et al.*, 1999). A second example for immunity is that the immunity protein exerts its specific effect in the cytoplasmic membrane through the interaction with the translocation apparatus (Lagos *et al.*, 1999). Class II bacteriocins immunity mechanisms information is scarce and is thought the immunity protein binds the same membrane receptor as the bacteriocin. It can be debated as to the direct interaction with the C-terminal part of the bacteriocins or direct interaction

(blocking of receptors) of immunity proteins of pediocin-like bacteriocins affect on bacteriocin activity. Cross immunity was observed almost exclusively in situations where either the bacteriocin or the immunity protein belonged to the same sequence-based subgroup. In a few cases, the functionality of the immunity proteins was strain dependent, e.g. the leucocin A immunity gene provided immunity to enterocin A (Fimland *et al.*, 2002 (a)). Johnsen *et al.*, (2004) suggested that the immunity protein does not interact extensively with membranes but may be loosely associated as peripheral membrane proteins with the membrane. This enables the immunity protein to interact with their cognate bacteriocin. Functional analysis of two immunity proteins which confer immunity to mesentericin Y105 and carnobacteriocin B2 has shown that these immunity proteins are located intracellularly and that their intracellular pool is divided into a minor membrane –associated fraction and a major cytoplasmic fraction (Quadri *et al.*, 1995). Studies have also shown that the intracellular immunity protein is unlikely to act by directly preventing binding of the bacteriocin to the membrane (Quadri *et al.*, 1995). Based on the observations made, suggestions have been made that the immunity protein acts either by affecting the process of bacteriocin aggregation and pore formation or by causing a disturbance in the interaction between the bacteriocin and a (putative) membrane located bacteriocin receptor (Nissen-Meyer *et al.*, 1993; Quadri *et al.*, 1995; Johnsen *et al.*, 2004).

1.6.2 Molecular genetics of lantibiotics

Most type-A and type-B lantibiotics have been sequenced and the genes that are necessary for modification i.e. *lanB* / *lanC*, or *lanM* and *land*, for proteolytic processing (*lanP*), transport (*lanT*), producer self-protection, and regulation are in close proximity to the structural genes, forming biosynthetic clusters (Figure 1.3). Peptides (Lanl) are associated with the membrane that code for self-protection or immunity. Regulation is usually achieved through two-component regulatory systems (LanR, LanK). Biosynthetic gene clusters consist of several transcription units, there is normally a separation between the structural gene and that, which is associated with biosynthetic proteins, which is caused by a weak terminator structure that allows moderate read through. This ensures the

high level of transcription of the peptide mRNA in comparison to the mRNA encoding for the biosynthetic enzymes (Sahl and Bierbaum, 1998).

Figure 1.3: The genetic organization displayed by nisin operons of *Lactococcus lactis* (Sahl and Bierbaum, 1998).

Separate LanB and LanC enzymes process all nisin-like antibiotics, whereas solely LanM in other lantibiotic subclasses performs this function. The nisin biosynthetic gene cluster is located on three classes of 70kb transposons that also utilize sucrose (Sahl and Bierbaum, 1998). Production starts at early to mid logarithmic growth phase and increases to a maximal production level in the early stationary phase.

The first three genes in the enterocin cluster, *entA, entl* and *entF* were identified as the structural gene for enterocin A, the putative immunity gene as the gene encoding the enterocin induction factor (Figure 1.4). To confirm the role of the *entI* in immunity, the *entAI* region was introduced into previously sensitive strains on the plasmid pENT01. This fragment resulted in neither complete nor partial immunity in transformants and as *entA* has been confirmed as the structural gene. More than one immunity protein is needed for full immunity (O'Keefe *et al.*, 1999).

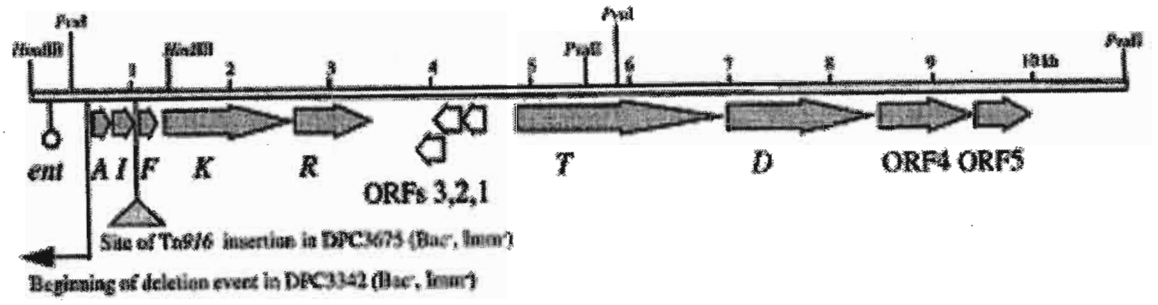


Figure 1.4 :Organisation of the gene cluster of enterocin A (O'Keefe *et al.*, 1999).

Most lactic acid bacteria are known to produce more than one bacteriocin. DPC1146 is capable of producing at least both enterocin A and B. Open reading frame (ORF) 2 located in the intergenic region of *entR* and *entT* is another candidate for a structural gene. The possible roles of ORF1, -2 and –3 are encoded on the opposite strand to all other genes in the enterocin gene cluster suggests that this intergenic region may be a result of a recombination event and, as such, may play no role in enterocin A synthesis (O'Keefe *et al.*, 1999).

Two genes, *entT* and *entD*, are necessary for secretion. They have homology to the bacteriocin ABC transporters and accessory factors resulted in production of active enterocin A when introduced on a cassette with the bacteriocin structural and immunity genes under control of the promoter. The structural genes and immunity genes alone resulted in no bacteriocin production. Open reading frame 4 and 5 are co-transcribed with *entT* and *ent*D indicates possible role in enterocin synthesis. Open reading frame 4 encodes a serine protease that degrades signal peptides in the cell membrane to maintain proper secretion of mature peptides. A role for ORF4 in removing membrane-bound leader peptides from secreted enterocin and induction factors might be envisaged in DPC1146 (O'Keefe *et al.*, 1999).

The *entT* codes for the induction factor / pre-peptide of a short amino acid leader sequence of the double glycine type (Enhnahar *et al.*, 2000). The induction peptide and the two component signal transduction system encoded

by *entK* (encodes histidine protein kinase) and *entR* (encodes response regulator protein) combines to provide the cell with a means to monitor and respond to cell density. Quorum sensing does not only control biosynthesis; it is also responsible for onset of a state of competence, as well as regulation of virulence response (O'Keefe *et al.*, 1999).

1.7 The Lacticin F operon

Molecular analysis characterized a small operon that encodes for three open reading frames, designated *lafA, lafX* and ORFZ (Figure 1.5). The peptide-encoded *lafA*, the lactacin F structural gene, was compared with various peptide bacteriocins from other LAB producer strains and similarities were identified in the amino and carboxy termini of the pro peptides. Site-directed mutagenesis of the *lafA* precursor a two-glycine residue in positions –1 and –2 defined an essential motif for processing of mature lactacin F. The involvement was investigated by sub cloning various fragments from the lactacin F region into the shuttle vector PGKV210. In addition to *lafA*, the expression of *lafX* is essential to lactacin F activity (Fremaux *et al.*, 1993). The Lactacin F operon resembles the genetic organization of lactococcin M. Although no function has been assigned to ORFZ by genetic analysis, both peptide Z and lactococcin M (Barefoot and Klaenhammer, 1984) immunity protein are predicted to be integral membrane proteins with four putative transmembrane segments. Lactacin F activity, defined by bacterial action on *Lactobacillus delbrueckii*, is dependent on the expression of two genes, *laf*A and *laf*X (Fremaux *et al.*, 1993).

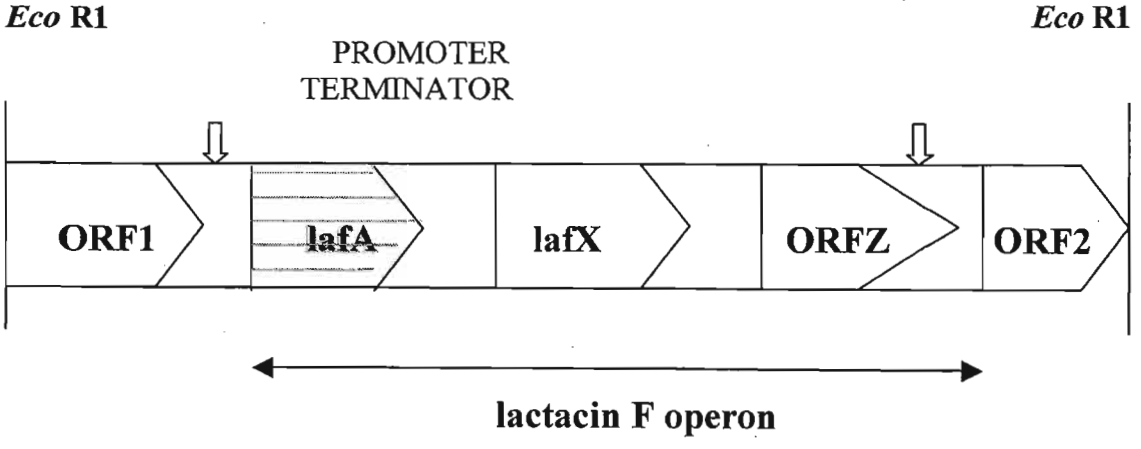


Figure 1.5: The genetic organization displayed by the lacticin F operon of *Lactobacillus acidophilus* (after Allison *et al.*, 1994).

1.8 Gene regulation

The genetics of the bacteriocin operon provides the basis to the production of these bacteriocins. Two types of gene regulation processes exist for the production of bacteriocin. The first is associated with the lantibiotics is a two component regulatory system termed signal transduction e.g. nisin auto regulates its own production. Class II bacteriocins are regulated by the second type, a three-component system called quorum-sensing phenomenon. This technique is a bacterial survival method. The bacteria sense the nutrients are low and the competition high. Production of the bacteriocins are then induced. The induction of bacteriocin depends on a pre-peptide as for the nisin bacteriocin. If the mechanism of production is further understood the application could be numerous for the food preservative industry.

Regulatory genes required for lantibiotic biosynthesis, secretion and immunity are generally organized in a cluster. Production starts in the early to mid logarithmic growth phase and increases to a maximal production level in the early stationary phase. The nisin biosynthetic gene clusters include genes encoding a sensor protein (*nisK*) and a response regulator (*nisR*) that have shown to be essential for lantibiotic production. NisR and nisK are the only components required for the signal transduction pathway (Eijsink *et al.*, 2002). Nisin acts as a signal molecule inducing its own synthesis. The nisA is the promoter gene for the production of nisin. It has been established that both antimicrobial and signaling activity depends on the expression of this gene.

1.8.1 Two component regulatory systems

A large number of bacterial responses involve the interaction of two regulatory proteins, one of which controls the other. A histidine protein kinase is the first component that receives an extra cellular signal directly or indirectly, possibly through its amino acid domain, which often induces transmembrane sequences. The signal is then transduced via the carboxy-terminal of the kinase to the second component, a response regulator (Figure 1.6). This second component is generally a transcription activator. The common mechanism underlying this

signal transduction process involves a phosphotransfer reaction between the two proteins. The protein kinase is first autophosphorylated in an ATP-dependent reaction at a conserved histidine residue and in a second step the phosphoryl group is transferred to an aspartate residue in the amino-terminal domain of the response regulator (De Ruyter *et al.*, 1996). Following the transfer of the phosphoryl group to the conserved aspartate residue in the response regulator, this affects the binding of target DNA sequences (Bijlsma and Groisman, 2003). Hyper variable residues located around the phosphorylatble aspartate cause the mediation of specificity between a sensor and regulator pair (Bijlsma and Groisman, 2003).

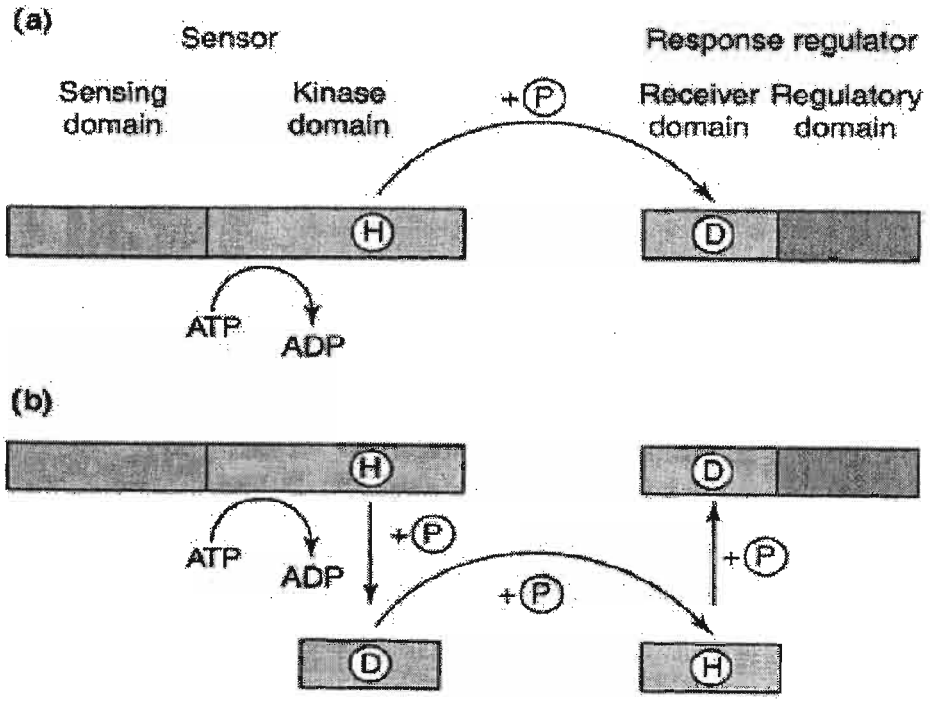


Figure 1.6 : A two-component system consisting of a sensor protein. (a) The sensor protein autophosphorylates onto a conserved histidine residue in response to a signal sensing. (b) The His-Asp-His-Asp three-step phosphorelay in which a phosphoryl group is transferred from the conserved histidine from the sensor domain to additional aspartate and histidine residues in the auxiliary domain before being transferred to the receiver domain of the response regulator (Bijlsma and Groisman., 2003).

Due to the integration of multiple signals in the location of the sensor protein in the membrane, two-component systems are ideally suited to its function. Sensor proteins have residues of phosporylatable histidine around them, resulting in the division of the sensor proteins into two major families. Most two-component systems involve a single histidine to aspartate phosphotransfer between a histidine kinase and a response regulator. There is however some which consist of a three-step phosphorelay (Figure 1.6b) (Bijlsma and Groisman, 2003).

1.8.2 Quorum sensing phenomenon

Quorum sensing is a density dependent regulation mechanism that enables a bacterial population to act in a coordinated manner. In gram-positive bacteria, quorum sensing regulates various processes, such as competence development, virulence and the production of bacteriocins. Some genetic elements found in connection with bacteriocin production in LAB display high homology to two-component signal transduction systems. In addition to genes encoding the histidine protein kinase and response regulator and a gene encoding a peptide pheromone, also termed inducing peptide or inducing factor, is found on the same transcriptional unit, thus comprising what is now termed a three-component regulatory system (Risoen *et al.*, 2000). Such three-component regulatory systems permit quorum sensing-based regulation of bacteriocin production. It is generally assumed that binding of the inducing peptide to its cognate histidine protein kinase receptor results in activation of the response regulator, which then contributes to transcriptional activation of all operons necessary for bacteriocin production, including the regulatory operon itself (Figure 1.7).

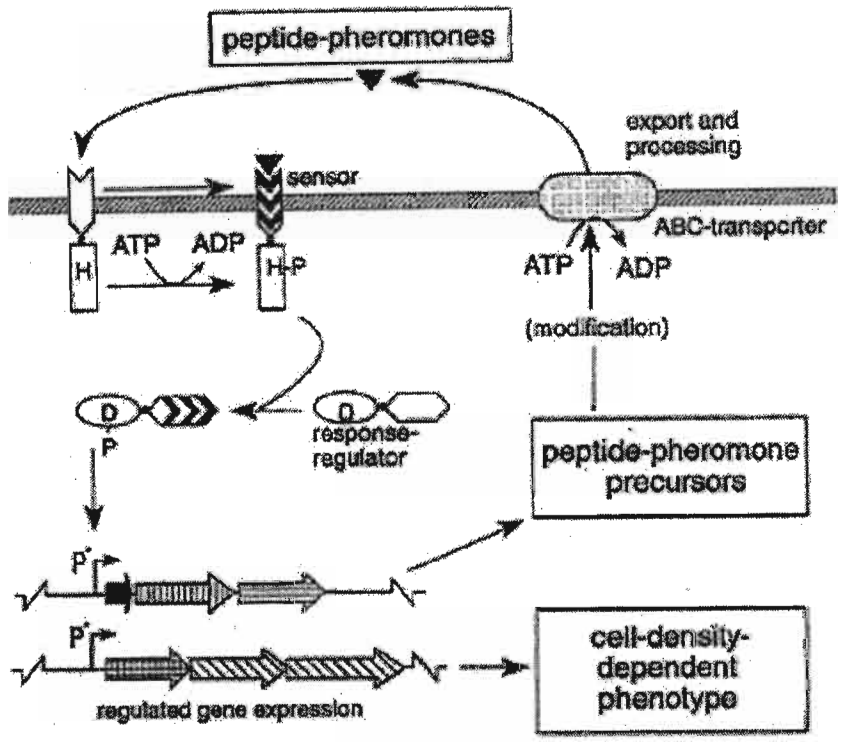


Figure 1.7: Schematic presentation of the model for molecular modes involved in the quorum-sensing modules that are mediated by peptide pheromones and two-component regulatory systems in Gram-positive bacteria (Kleerebezem *et al.*, 1997).

Cell density dependent gene expression appears to be widely spread in bacteria. It is well established in gram-negative bacteria. Cell density-dependent regulatory modes in these systems appear to follow a common theme, in which the signal molecule is a posttranslationally processed peptide that is secreted by a dedicated ATP-binding cassette exporter. This secreted peptide pheromone functions as the input signal for a specific sensor component of two-component signal-transduction system. Moreover, genetic linkage of the common elements involved results in autoregulation of peptide-pheromone production (Pestova *et al.*, 1996).

1.9 Surface Plasmon Resonance (SPR)

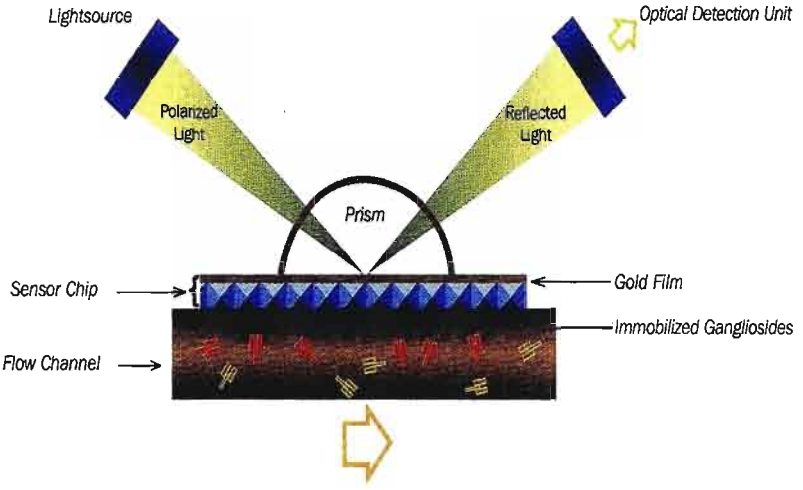
The biomolecular interaction analysis system (BIAcore) was launched in 1990 (Liedberg *et al.*, 1995; McDonnell, 2001) which is used to monitor binding reactions between low affinity proteins in real-time without the use of labeled molecules (Liedberg *et al.*, 1995; McDonnell, 2001; Zhang and Oglesbee, 2003). The BIAcore system is a microfluidic system that has the ability to minimize mass transport limitation for kinetic analysis (Quinn *et al.*, 2000). The basis for BIAcore is the use of surface plasmon resonance (SPR).

Surface plasmon resonance analyses molecular recognition events of binding under conditions that mimic the natural cell surface of these interactions (MacKenzie *et al.*, 1997). Once again there is no requirement for labeling. SPR is an optical technique and occurs at the interface of a metal and a dielectricum (Liedberg *et al.*, 1995). It can be excited by light and results as a charged density wave. Longitudinal oscillations of free charges on the surface of a metal film are surface plasmons. These surface plasmons are created when the metal film is excited under specific conditions of total internal reflection of plane-polarized light (Quinn *et al.*, 2000; Nedelkov and Nelson, 2003). SPR technology gives kinetic data, which is often difficult to obtain by other means (Liedberg *et* al., 1995; MacKenzie *et al.*, 1997). It is a very useful technique for immunosensing and biospecific interactions (Liedberg *et al.*, 1995). This technique works by a ligand which is the interaction partner being conjugated onto the surface of a sensor chip and the analyte, which is the binding partner flows over the surface (Zhang and Oglesbee, 2003). The ligand is covalently immobilized onto the sensor chip via the amino-terminal group of the ligandary protein. The analyte is in solution and their interaction results in a change in the refractive index (McDonnell, 2001), called the resonance angle and is measured in resonance units (RU) (Zhang and Oglesbee, 2003). The ligand is immobilized onto the sensor chip. A sample or the analyte is passed over the chip, where association between the ligand and analyte occurs. This binding capacity proceeds until the ligand is saturated with the analyte. The peak establishes the highest response where sufficient analyte has bound to the ligand, this establishes how much bioactivity there is. After this point dissociation occurs where the binding stability can actually be established. This process

continues until no analyte is in excess and only ligand is present. The refractive index change is directly related to a change in the protein concentration (Quinn *et al.*, 2000; Krotkiewska *et al.*, 2002). Fixed-angle variable wavelength modes or multiple-angle fixed wavelength modes both enable the biomolecular interaction to be monitored by detecting a change in refractive index within the probed layer (Quinn *et al.*, 2000).

When a binding event occurs, the result is a change in protein mass. This alters the SPR, which is an electromagnetic effect that decreases the intensity of light, which is reflected off of the surface of the sensor chip at the resonance angle. The new sensor chips include a carboxymethylated surface which has either a: (i) dextran layer on top of the metal, (ii) a dextran free-ligand, (iii) a chelated nickel surface for binding His-tagged ligands or (iv) a hydrophobic surface to analyze membrane systems (McDonnell, 2001) (Figure 1.8).

(A)



(B)

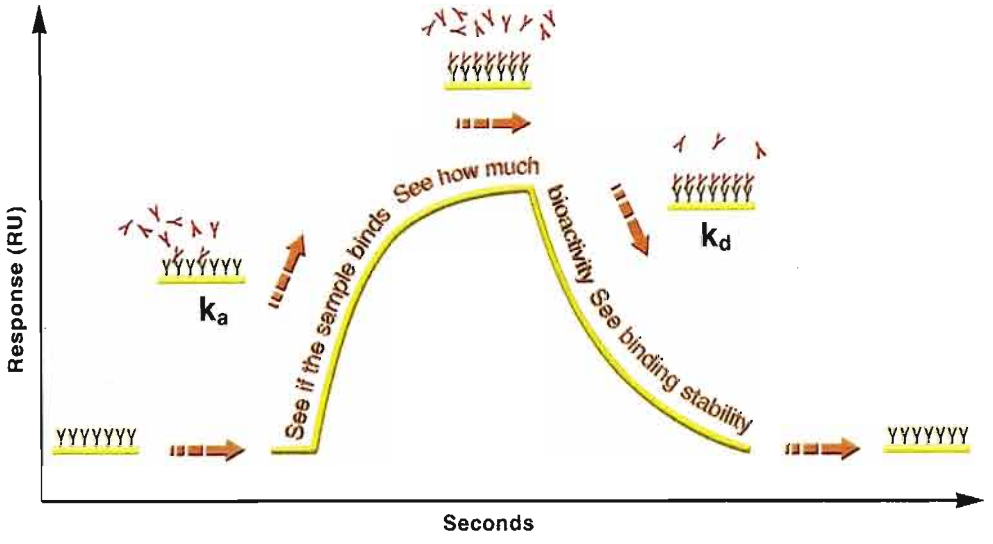


Figure 1.8: (A) Illustration of detector with sensor chip and IFC (Alaedini and Latov, 2002), (B) A sensogram depicting association and dissociation reactions that occur.

The strengths of using SPR are that it is easy to use and interaction analysis can be performed over a wide range of chemical and environmental conditions (temperature, ionic strength, pH, etc) (McDonnell, 2001). It also allows the analysis of receptor-legend interactions with a range of different molecular weights and binding rates (McDonnell, 2001). A benefit of SPR is that it allows the comparison of the binding properties of different interactants, including mutated recombinant proteins as well as the screening of unknown interactions in crude samples (Amano *et al.*, 1999). The weakness however, lies in the difficulty of measuring high interactions, which are related to very slow dissociation rates, and drift from the instrument may occur (McDonnell, 2001).

1.9.1 Applications of surface plasmon resonance

This advanced technique has provided experimental results on biomolecular interactions which would otherwise have been difficult or impossible to obtain (Gonzalez *et al.*, 2001) have used the SPR technology, which have provided good results for protein binding to glycolipid receptors, using a CM5 sensor chip. It has also been to obtain kinetic and affinity parameters which characterize the interactions of various neuropeptides with surfaces containing glycopeptides (Gonzalez *et al.*, 2001; Salamon *et al.*, 1998). SPR was also used to analyze the binding interaction of cholera toxin to gangliosides which were directly self-assembled onto alkylthiol surfaces (MacKenzie *et al.*, 1997). The kinetic and affinity constants which were obtained to characterize receptor specificities has shown that it can be specifically applied to the study of clostridial neurotoxins e.g., tetanus toxin (MacKenzie *et al.*, 1997). Other applications of SPR involve the structural and functional characterization of lipid and lipoprotein membranes (Salamon *et al.*, 1998) or small peptides to glycolipids. With the use of the biacore instrument, interactions between antigen and antibody, a ligand and its receptor and and enzyme and its substrate can be measured. This enables the study of the binding of molecules such as nucleic acids, lipid micelles, peptides and other drugs (Krotkiewska *et al.*, 2002). Uchida et al., (2004) conducted a specific study in which the interaction between bacterial cells and a human intestinal surface was determined. A variety of interaction studies based on the use of SPR exist. Further studies have yet to be reported based on SPR.

1.10 Applications of bacteriocins

Due to the rapid rise and spread of multi-resistant bacterial pathogens, alternative methods of combating infection have been considered (Riley and Wertz, 2002). Recently bacteriocins that are produced by lactic acid bacteria have been used in food processing and preservation due to their ability to inhibit growth of undesirable organisms like *L. monocytogenes* and *Clostridium* spp., it is for this reason that they have attracted a great amount of attention. Immunocompromised, children, pregnant women and senior citizens are at high risk of they ingest products that are contaminated with listeria. An estimate of 1100 and 2500 people in the United States develop listeriosis each year, and it is known that 20% of *Listeria* infections are fatal (Vadyvaloo *et al.*, 2002). The safe use of genetically modified LAB requires the development of cloning systems that are composed solely of DNA from food-grade organisms (Takala and Saris, 2002).

The use of bacteriocins is a potential means of preserving fermented foods (Vadyvaloo *et al.*, 2002). The traditional method of preservation was through naturally occurring fermentations but for new large-scale productions the exploitation of defined starter strains is used, that ensure consistency of the quality of the final product (Ross *et al.*, 2002). Nisin is the most widely used bacterioicn, and is used in 48 different countries and has been granted the status of GRAS (generally recognized as safe) in the United States (Mendoza *et al.*, 1999).

It is currently the only bacteriocin that is widely used for food preservation (Jack *et al.*, 1995). It has been used for decades while there are still up and rising bacteriocins that have very good potential for food applications (Ross *et al.*, 2002). It does however have several deficiencies: it is unstable at neutral to alkaline pH's, this is seen by a decline in its antimicrobial activity, it has a low solubility and its activity spectrum is limited only to gram-positive bacteria (Mendoza *et al.*, 1999). Many of the present and traditional methods of food preservation may not inhibit the growth of *L. monocytogenes* and therefore improved safety and shelf life of food products are required (Harris *et al.*, 1989).

Nisin was first introduced in the UK about 30 years ago as a commercial food preservative in processed cheese products and is now being used in other food applications as well as beverages. The latest uses of nisin are a preservative in high moisture, hot baked flour products and pasteurized liquid egg. Nisin has been viewed as a control of spoilage lactic acid bacteria in beer, wine, alcohol production, and low pH foods like salad dressings (Klaenhammer, 1993).

Nisin has been known since 1928 to be produced by *Lactococcus lactis* isolates. Genetic technology has proved that nisin is formed by posttranslational processing of a prepeptide molecule. Various regulatory hurdles and ethical issues need to be crossed in order for bacteriocins to be used commercially, once this path is clear, toxic nitrates that are being used as food preservatives can be reduced (Jack *et al.*, 1995). Foods that have an adequate shelf life are in high demand by the consumer and therefore its obvious that a need for expansion and legal use of bacteriocins is needed (Vadyvaloo *et al.*, 2002).

CHAPTER TWO

MATERIALS AND METHODS

2.1 Bacterial strains and growth conditions

Bacterial strains and plasmids used in this study are listed in Table 2.1. *Escherichia coli* JM103 was grown in Luria Bertani (LB) medium (Sambrook *et al.*, 1989) and on LB plates incubated at 37°C. Plasmid containing strains of *E. coli* JM103 was grown in LB with ampicillin (Amp) at a final concentration of 100μg/ml. *Listeria monocytogenes*, a food isolate, obtained from the laboratory collection at the University of Kwa-Zulu Natal (Pietermaritzburg), was grown on tryptone soy agar [TSA] (Biolab) or broth at 30°C for all experimental procedures. *Leuconostoc gelidium* UAL-187-22, the leucocin A producer strain, and *Leuconostoc gelidium* UAL-187-13, a leucocin negative strain was grown in MRS broth and on De Man Rogosa Sharpe Agar (MRS) (Biolab) plates at 25°C.

Table 2.1: Bacterial strains and plasmids used in this study

Strain or Plasmid	Description	Source or Reference
Eshcerichia coli		
JM103	*thr rpsL endA sbc-15 hsdR4* Δ (lac pro AB) F' *traD36 pro AB, lacZΔM15*	Sambrook *et al.*, 1989
Leuconostoc gelidium		
UAL 187-22	$LcnA^+ Imm^+$ containing native plasmids pLG9.2 and pLG 7.6	Hastings and Stiles, 1991
UAL 187-13	$LcnA^- Imm^-$ containing native plasmids pLG 9.2	Hastings and Stiles, 1991
Listeria monocytogenes	LcnA sensitive	Ramnath *et al.*, 2000
Plasmids		
pJF5.5	Amp^r, $LcnA^+$ Imm^+	Felix *et al.*, 1994
pMALc2 6.6	malE, lacZ α, Amp^r	New England BioLabs
pKP 7.0	Amp^r, malE-Imm, pMALc2 derivative	This study

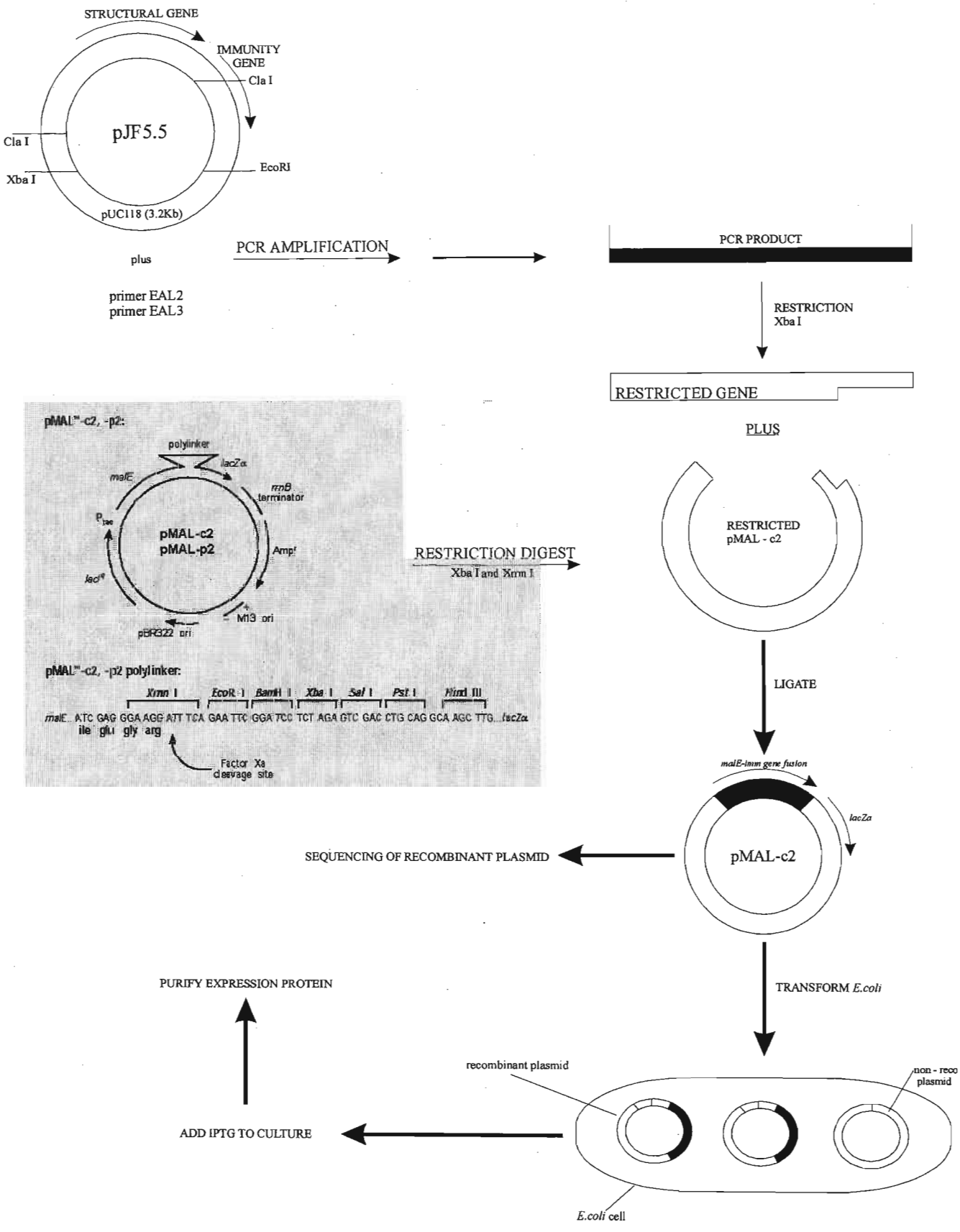


Figure 2.1: Schematic representation of the cloning and isolation procedure of the immunity gene construct and fusion protein.

2.2 DNA manipulations

2.2.1 Mini-Prep plasmid DNA isolation

Mini-prep DNA isolations were performed using the alkaline lysis method (Sambrook *et* al., 1989). LB medium (10ml) containing amp (100μg/ml) was inoculated with a single bacterial colony and grown overnight at 37°C with vigorous shaking. A glycerol stock of the culture was made using 30% glycerol. The remaining cells were centrifuged at 7520x g for 5min at 4°C in a JA 20.1 rotor in a Beckman centrifuge. The supernatant was discarded and the remaining cells were resuspended in 200μl GTE solution (50mM glucose, 25mM Tris-CL pH 8.0, 10mM Ethylene diamine trifluro acetate [EDTA]) at room temperature for 5min in a microfuge tube. Two μl of RNase A and 400μl of freshly prepared NaOH/SDS (0.2M NaOH, 0.1% SDS) was added to the resuspended cells and placed on ice for 5min. Three hundred μl of potassium acetate solution (29.5ml glacial acetic acid, KOH pellets to pH 4.8, H_2O to 100ml) was added, placed on ice for 5min in order for a precipitate of cell debris and chromosomal DNA to form. The mixture was centrifuged at maximum speed for 5min at room temperature (RT). Eight hundred μl of the supernatant was transferred to a fresh tube, to which 600μl of isopropanol was added and left at -20°C for 30min. The cells were centrifuged for 5min at 4°C to pellet plasmid DNA and the supernatant discarded. The remaining pellet washed in 0.5ml ice cold 70% ethanol and the pellet dried in a speed vac for about 2min. The pellet was resuspended in 50μl TE (100mM Tris-Cl, pH 7.5; 100mM EDTA) and stored at -20°C until further required.

2.2.2 Nucleobond AX 100 DNA isolation

Plasmids pJF 5.5 and pMAL-c2 were isolated using the Nucleobond AX 100 kit (Machery-Nagel, Duren, Germany). Overnight cultures of the two plasmids were grown in 30ml LB medium containing ampicillin (100μg/ml) at 37°C in a shaking water bath. Bacterial cells were harvested by centrifugation at 6000x g, for 15min at 4°C in a JA 20 rotor in a Beckman centrifuge. The supernatant was discarded and to the pellet, 4ml each of buffer S1 (50mM Tris-Cl, 10mM EDTA, 100Mg/ml RNase A, pH 8.0), buffer S2 (200mM NaOH, 1% SDS), incubated at room

temperature for 5 min and buffer S3 (2.8M KAc, pH 5.1), incubated in ice for 5 min, were added, mixed and stored on ice in order to lyse the cells. The NucleoBond AX 100 column was equilibrated using 2.5 ml of buffer N2 (100mM Tris, 15% ethanol, 900mM KCl, 0.15% Triton X-100, pH 6.3). The lysed cells resulted in a white precipitate, which was filtered, using NucleoBond filters and the clear lysate was loaded onto the equilibrated column. The column was thereafter washed using 10ml buffer N3 (100mM Tris, 15% ethanol, 1.15M KCl, pH 8.5). The DNA was eluted using buffer N5 (100mM Tris, 15% ethanol, 1M KCl, pH 8.5) and precipitated with 3.5 ml isopropanol. The DNA was pelleted by centrifugation at 15 000 x g for 30 min at 4°C. The supernatant was discarded, the pellet washed in 2ml of 70% ethanol and centrifuged at 15 000 x g for 10 min at room temperature. The pellet was allowed to dry at RT and redissolved in 50µl of TE buffer. The DNA was analyzed using agarose gel electrophoresis and viewed on a UV transilluminator.

2.2.3 Quantitation of DNA

Plasmid DNA was quantified using spectrophotometery. Purified DNA was diluted 1: 100 in 1 x TE buffer. The absorbance readings were taken at wavelengths of 260nm and 280nm. The reading at 260nm allows for calculation of the concentration of nucleic acid present within the sample. The ratio between the 260nm and 280nm (OD_{260}/OD_{280}) readings provides an estimate of the purity of the nucleic acid. Pure preparations of DNA and RNA have a ratio value of higher than 1.8 and 2.0 respectively (Sambrook *et al.*, 1989)

2.2.4 Analysis of Purified DNA by Agarose gel Electrophoresis

Agarose gels of 0.8% (w/v) were prepared using electrophoresis grade agarose (Hispanagar, Spain), which was dissolved in 1 x TAE buffer. The agarose gel contained 0.5µg/ml of ethidium bromide. Purified plasmid DNA samples containing 2µl DNA loading buffer for every 10µl sample of DNA was electrophoresed on the agarose gels at 80V for 90min. DNA bands were visualized using a UV transilluminator light.

2.3 Restriction endonuclease digestion of pJF 5.5 DNA

The isolated pJF 5.5 DNA was restricted with restriction endonuclease *EcoR*1 in a reaction tube containing the following to a final volume of 20µl: (i) 10µl DNA (1.57µg), (ii) 2µl 10x restriction buffer, (iii) 7µl sdH_2O, (iv) 1µl *Eco*R1. The contents were mixed by pulse spinning and incubated in a water bath at 37°C for 2hrs. The reaction was stopped by heating at 65°C for 10min. The digested product was analyzed on a 0.8% agarose gel and viewed by use of a UV transilluminator.

2.4 Polymerase chain reaction (PCR) amplification of the immunity gene

PCR amplification was performed with an automated Perkin Elmer 9600 thermal cycler (Perkin-Elmer, Applied Biosystem), using pJF 5.5 as a template. The PCR reaction mixtures were constituted as outlined in Table 2.2. The DNA template and *Taq* polymerase were added last to the reaction mixture. The sequences of the forward and reverse primers were as follows:

Forward:

EAL-2 5' ATT CTA GAC AAA GTC ATT TAT CTT TCA AAG ATA 3'

Reverse:

EAL-3 5' ATG AGA AAA AAT AAC ATT **TAT TGG ACG** 3'

XbaI site

The PCR amplification profile used was as follows: (i) initial denaturation at 95°C (5min), (ii) 30 cycles of denaturation at 94°C (30sec), annealing at 55°C (30sec), elongation at 72°C (30sec), (iii) final elongation at 72°C (7min) and (iv) cooling to 4°C, long term storage was at -20°C.

Table 2.2: Reaction components used for the PCR amplification of DNA target sequence from pJF 5.5 and pMALc2.

Reagent	pJF 5.5		pMALc2	
	Vol (µl)	Amount	Vol (µl)	Amount
10x PCR Buffer with $MgCl_2$	10	1x	10	1x
10mM dNTPs mix	2	0.2mM	2	0.2mM
Primer EAL-2	1	300nM	1	300nM
Primer EAL-3	1	300nM	1	300nM
DNA template	2.5	0.983µg	2.5	0.72µg
Taq (Roche)(1U/µl)	1	1U	1	1U
Sterile dH_2O	82.5		82.5	
Total	100		100	

The amplified PCR product was analyzed on a 2% agarose gel and viewed with a UV transilluminator. An O' Range ruler, 200bp marker (Fermentis) was included on the gel to confirm the approximate size of the amplicon.

2.5 Purification, Blunt ending and Restriction of PCR product

2.5.1 Purification of PCR product from agarose Gel

Following electrophoresis, the band of interest was excised with a sterile scalpel blade under UV light and collected in sterile microfuge tubes. The excised gel sample was grounded with a sterile pasteur pestle in an eppindorf tube.

PCR fragments were recovered from the gel slices using the GenElute™ agarose Spin column kit (Sigma) according to manufactures instruction. Briefly a spin column was pre-washed by the addition of 100µl of 1 x TE buffer and centrifuged at 14 000 x g for 10 sec. The grounded agarose gel piece containing the fragment of interest was placed into the spin column and centrifuged at 14 000 x g for 10min. The DNA and soluble molecules passed through the filter and were

collected in a microcentrifuge tube. The DNA obtained was quantified using spectrophotometery as previously described.

2.5.2 Blunt ending of PCR product

The following reaction mixture was prepared in a sterile microfuge tube: (i) 2µl of reaction buffer H, (ii) 1µl Klenow enzyme, (iii) 1µl of dNTP (2mM) and (iv) 16µl of PCR product (8.12ng/µl). The reaction was incubated at 30°C for 15min in a water bath, stopped by heating at 75°C for 10min on a dry heat block, and thereafter stored at -20°C, until further use.

2.5.3 Restriction digestion of pMALc2 plasmid DNA

Plasmid pMALc2 was restricted using the enzymes *Asp*700 (creates "blunt" ends) and *Xba*I (creates "sticky" ends) with reaction buffer B (10x conc. 10mM Tris-Cl, pH 7.5 at 37°C, 10mM $MgCl_2$, 0.1mg.ml^{-1} BSA). A single digest of pMAL-c2 with *Asp*700 was carried out and a double digest using both *Asp*700 and *Xba*I. The single digests consisted of 5µl of pMALc2 (0.785µg), 2µl of 10 x buffer B, 12µl of distilled water and 1µl of each enzyme i.e. *Asp*700 and *Xba*I (10 units), resulting in a 20µl reaction. The double digest consisted of 15µl of pMALc2 (2.355µg), 2µl of 10 x buffer B, 1µl of distilled water and 1µl each of enzymes *Asp*700 and *Xba*I. The reactions were set up and incubated in a water bath at 37°C for 2hrs. Reactions were stopped by heating at 65°C for 10min using a dry heater block. The restricted pMALc2 was analyzed on a 1% agarose gel and viewed with a UV transilluminator. Uncut pMALc2 DNA was used as a control and DNA molecular weight marker III (Roche) was included on the gel to estimate the approximate sizes of the DNA fragments obtained.

2.5.4 Restriction digest of immunity gene (PCR product)

The PCR product was digested with *Xba*I to make the ends compatible with that of the vector for ligation. Reaction mixtures were constituted in a sterile eppindorf tube as follows: (i) Klenow reaction (17µl) (Paragraph 2.5.2), (ii) reaction buffer H (10x conc. 20Mm Tris-Cl, pH 7.5 at 37°C, 10mM $MgCl_2$, 100mM NaCl, 1mM

Dithioerythritol (2µl) and (iii) restriction enzyme *Xba*I (1µl). The reaction was mixed by pulse spinning and incubated in a water bath at 37°C for 2hrs. The reaction was stopped at 65°C for 10min. Digests were stored at -20°C until further use.

2.6 Cloning of PCR Product into Expression Vector pMALc2

2.6.1 Ligation

The vector pMALc2 was used for cloning the PCR product. A restriction digest of the vector and the insert was prepared as described previously. Ligation reactions (1:5 molar ratio) were performed in sterile microfuge tubes and constituted the following: (i) Insert 10µl (Klenow-XbaI digest) (1.57µg), (ii) Vector 2.5µl (pMALc2 restricted with *Asp*700 and *Xba*I) (0.288µg), (iii) Ligation buffer (2µl), (iv) T4 DNA ligase (2µl) and (v) sdH_2O (3.5µl). The reactions were incubated overnight (15hrs) at 16°C in a water bath and the reaction was stopped by heating at 65 °C for 5min. Ligation reactions were stored at -20°C until further use (Sambrook *et al.*, 1989).

2.6.2 Preparation of competent cells using $CaCl_2$ treatment

A single colony from a LB plate containing *E. coli* JM103 was inoculated into 50ml LB medium and grown overnight at 37°C in a water bath with vigorous shaking. Two milliliters of the overnight culture was inoculated into 200ml LB medium and grown at 37°C in a water bath with vigorous shaking to an optical density (OD_{590}) of 0.375. The culture was dispensed into 4 x 50ml prechilled sterile tubes and left on ice for 5min to cool. Cells were centrifuged at 5000x g at 4°C for 5min, the supernatant discarded, and each pellet was resuspended in 10ml of cold $CaCl_2$ (60mM $CaCl_2$, 10mM PIPES, pH 7) solution. Cells were centrifuged at 756 x g at 4°C for 5min, the supernatant discarded and each pellet was resuspended in 10ml ice cold $CaCl_2$ and kept on ice for 30min. After 30min the cells were pelleted at 5000x g at 4°C for 5min, after discarding the supernatant the pellet was resuspended each pellet in 2ml of ice-cold $CaCl_2$ solution containing

15% glycerol. Cells were then dispensed into prechilled sterile microfuge tubes in 250µl aliquots and immediately frozen in a dry ice bath and stored at -70°C until required for further use (Sambrook *et al.*, 1989).

2.6.3 Transformation

Competent cells were thawed on ice and mixed by finger tapping. Competent cells (100µl) were added to sterile microfuge tubes, to which 10µl of ligation mixture was added. The mixture was incubated on ice for 30 min, followed by heat shock treatment at 42°C for 2min. Prewarmed LB (1ml) was added to the mixture and incubated at 37°C for 1hr. Aliquots of 200µl of the transformation reaction were plated on agar plates containing ampicillin (100µg/ml), X-galactosidase (80µg/ml) and Isopropyl-β-D-thiogalactopyranoside (IPTG) (0.1mM) and incubated at 37°C for 16hrs. The positive control used was uncut pMAL-c2 and the negative control was distilled water. Each of the controls (10µl) was added to the competent cells instead of the ligation mixture. After incubation at 37°C overnight, plates were observed for the presence of blue and white colonies (Sambrook *et al.*, 1989). The white colonies on the X-gal plates were indicative of β-galactosidase negative phenotypes. Using sterile toothpicks, white colonies were picked from transformation plates and transferred fresh LB plates containing 100µg/ml ampicillin and incubated overnight at 37°C.

Overnight cultures of the recombinant clones were grown in LB broth containing Amp 100µg/ml and DNA was prepared using the mini-prep procedure to verify the recombinant clones. Plasmid pJF 5.5 was used as a control. Once the presence of the cloned DNA was identified, a large-scale isolation was performed using the Nucleobond AX100 kit in order to obtain pure DNA for sequencing. The DNA was analyzed on a UV transilluminator and thereafter quantified using the spectrophotometer at OD_{260nm} and OD_{280nm}.

2.7 Screening of Recombinant clones

2.7.1 PCR Screening

Putative recombinant DNA was subjected to PCR as previously described. Amplicons were analyzed on 2% agarose gel and viewed with a UV transilluminator.

2.7.2 SDS-PAGE Screening

Expressions of fusion products from putative clones were induced with IPTG. Briefly, recombinant clones and *E. coli* harboring pMALc2 were grown to an optical density OD_{600} of ~0.5 at 37°C. A 1ml sample of each culture was removed, centrifuged for 2 min, the supernatant discarded and the cells resuspended in 50µl of SDS-PAGE treatment buffer. These were the uninduced samples. To the rest of the cultures 0.3mM IPTG was added and incubated at 37°C for 2hrs, shaking. A 500µl sample was removed from the induced cultures, microcentrifuged for 2 min, the supernatant discarded and the cells resuspended in 100µl of SDS-PAGE treatment buffer. These were considered to be the induced samples. Induced and uninduced samples were boiled for 5min, cooled to room temperature and analyzed on a 10% SDS-PAGE gel (Laemmli Buffer system), using BIORAD unstained Precision Plus Protein Standard molecular weight marker as a standard. The gel was stained with Coomassie Blue R250 for an hour and destained with destaining solution (40% methanol, 7% acetic acid, distilled water).

2.7.3 Southern Blot / Hybridization screening

2.7.3.1 Transfer of DNA

The VacuGene XL Protocol No. 1 was used to transfer DNA onto nitrocellulose membrane. The nitrocellulose membrane was pre-treated prior to transfer. A 1.2% agarose gel containing DNA from putative clones was run. The gel was subjected to depurination, denaturation and neutralization for 20min each on a

Vacu Gene XL apparatus. DNA from the gel was transferred onto the nitrocellulose membrane with transfer solution (20 x SSC) for a period of 60min. After the wells were clearly marked for easy referencing later, the membrane was washed, air dried and baked at 80°C for 2hrs to fix the DNA and stored until further required.

2.7.3.2 Oligonucleotide tailing with DIG-dUTP

Oligonucleotide, EAL-3 was labeled with dig-dUTP using a Roche 3' end tailing kit. The tailing reaction constituted the following solutions, and prepared in a sterile microfuge tube: (i) 4µl of 5x reaction buffer (1M Potassium cacodylate, 0.125M Tris-Cl, 125mg BSA pH 6.6); (ii) 4µl $CoCl_2$; (iii) 1µl DIG-dUTP solution; (iv) 1µl (50U) terminal transferase and (v) 3.3µl probe EAL-3 (100pmoles). The solutions were mixed and incubated at 37°C for 30min, thereafter placed on ice. Hybridization solution (100µl) was added to the tailing reaction and stored at-20°C for use in hybridization experiments (Sambrook *et al.*, 1989).

2.7.3.3 Hybridization

The baked membrane was placed in a sealable container to which 10ml of prehybridization solution was added. It was prehybridized for one and a half hours at 37°C. A probe was prepared as described in section 2.7.3.2, and added to the hybridization solution. The hybridization solution was poured out and the labeled hybridization solution added to the container containing the membrane. Hybridization occurred overnight at 37°C. Following hybridization the membrane was removed and washed in 2 x SSC, 0.1% SDS and 0.1 x SSC, 0.1% SDS (2 x each for 15min). The filter was allowed to air dry and subsequent detection followed.

2.7.3.4 Dig detection

The membrane was washed in buffer 1 (0.1M Tris-Cl, pH 7.5; 0.15M NaCl) for 1min. Thereafter it was blocked using freshly prepared buffer 2 (1% skim milk

powder in 100ml buffer 1) at room temperature with continuous shaking. After blocking, the membrane was washed briefly in buffer 1. Anti-digoxigenin-AP-conjugate (150 mU/ml) was diluted into buffer 2 and the membrane was incubated in 20ml of this solution for 30min. The membrane was thereafter washed in buffer 1, to remove any unbound antibody conjugate. An equilibration step in buffer 3 (0.1M Tris-Cl, pH 9.5; 0.1M NaCl; 50mM $MgCl_2$), 20ml was performed. Freshly prepared colour substrate solution (200µl NBT/BCIP in 10ml buffer 3), 10ml was prepared, added to equilibrated membrane and incubated in the dark until a colour precipitate developed . The reaction was stopped by washing the membrane in 1 X TE, pH 8.0 (Sambrook *et al.*, 1989).

2.8 DNA sequencing

Automated DNA sequencing utilizes fluorescent tracers instead of radioisotopes to detect the DNA, thereby eliminating or significantly reducing the use of radioactive materials in some research laboratories.

2.8.1 Big Dye Terminator v3.0 Ready Reaction Cycle Sequencing Kit

The PCR cycle sequencing reaction was performed using a Gene Amp PCR System 2700. The following reaction components used for PCR were added to a sterile PCR tube as follows: (i) DNA of pKP2, (2.35µl) 400ng; (ii) 5 x Big Dye terminator v3.1 buffer (3µl); (iii) 5mM primer EAL-3, (0.64µl) 3.2pmoles; (iv) Big Dye Terminator v3.1 cycle sequencing ready reaction mix-100 (2µl) and sterile distilled water (12.01µl) to give a total volume of 20µl reaction. The PCR profile used was as follows: 35 cycles of 96°C for 10sec, 55°C for 30sec, 60°C for 4 sec. The reaction tube was immediately removed and the extension product purified.

2.8.2 Purification of Extension Products

To each tube 50µl of 100% ethanol was added, 2µl of 125mM EDTA and 2µl of 3M NaAc (sodium acetate, pH4.6-5.2). The entire 20µl of the DNA sequencing reaction was added to the tube and vortexed gently for a few seconds. Tubes were placed at 4°C for 20 min to precipitate sequencing reaction extension products. Samples were

placed in a centrifuged for 20 min at maximum speed. The supernatant was very carefully removed, and the pellet washed with 120µl of 100% ethanol and briefly vortexed. Samples were pelleted by centrifugation for 15min at maximum speed and all traces of supernatant removed. The pellet was washed using 100% ethanol, and was dried in a laminar flow hood for 20 min.

2.8.3 Sequence reaction analysis

The resulting sequencing reaction was loaded onto a 96 well optical reaction plate and placed into a Genetic Analyzer 3100 (Applied Biosystems). The reaction was run for 4hrs. Data was analyzed using BioEdit.

2.9 Expression and Purification of Recombinant fusion Protein

2.9.1 Expression

This experiment was initially conducted on a small scale as a pilot experiment (see screening of recombinants) and thereafter upscaled (pMAL Protein Fusion and Purification System, New England Biolabs). A 1000 ml LB broth containing glucose and ampicillin (100µg/ml) were inoculated with 10ml of an overnight culture of cells containing the plasmid harboring the recombinant construct. The cells were grown to an optical density (OD_{600}) ~ 0.5 at 37°C in a water bath while shaking. IPTG was added to the culture at a final concentration of 0.3mM, the culture was incubated in a water bath for 2hrs at 37°C, shaking. The cells were harvested by centrifugation at 4000x g for 20min at 4°C. The supernatant was discarded and the pellet resuspended in 50ml column buffer (20mM Tris-Cl, 200mM NaCl, 1mM EDTA). Samples were stored at -20°C until further required.

The samples were thawed in cold water and sonicated using VirSonic 60 (Polychem Supplies CC, Laboratory consumables) in short pulses of 15sec to release the cytoplasmic content. The release of protein was monitored using Bradford reagent. Sonication was continued until the maximum amount of protein was released for ~ 2min. The sonicated samples were centrifuged at 9000x g for

30min at 4°C and the supernatant, called the crude extract, was diluted with column buffer (1:5).

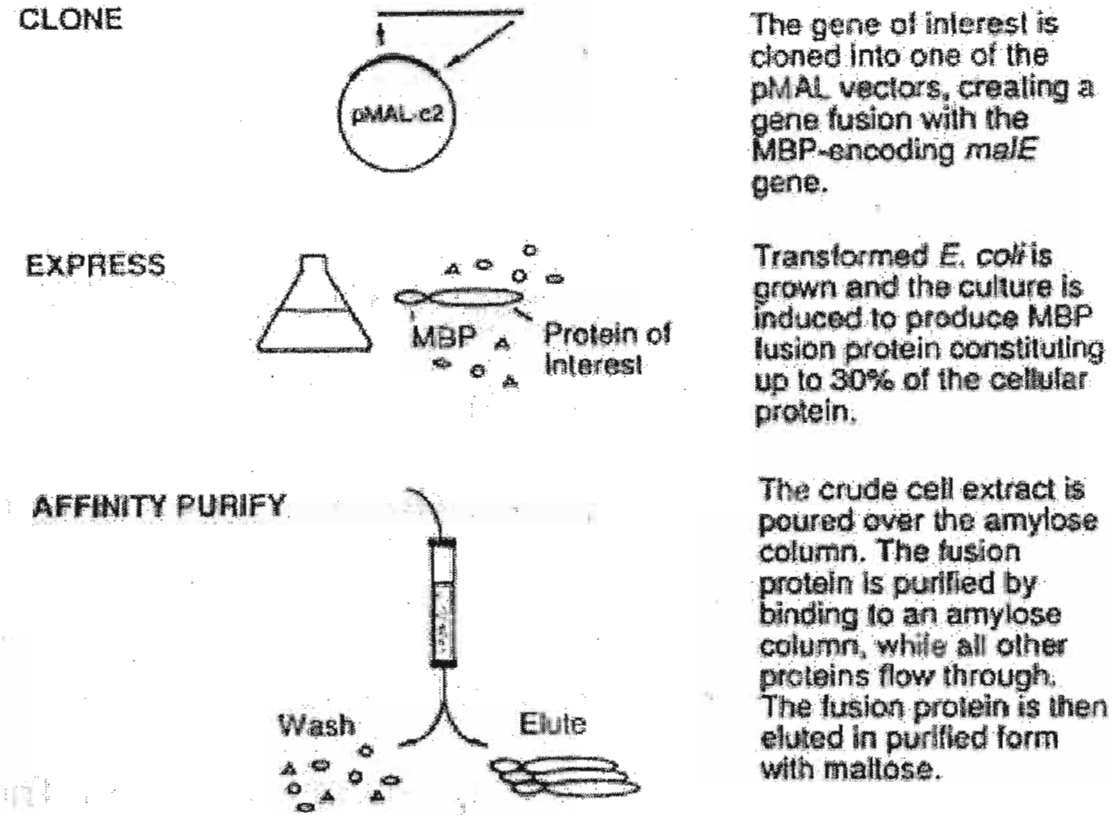


Figure 2.2 A schematic representation of the isolation of the fusion protein via affinity purification.

2.9.2 Purification of the fusion protein using Affinity Chromatography

The protocol used for this experimental procedure was according to the pMAL Protein Fusion and Purification System, New England, BioLabs.

Amylose resin in a 2.5 x 10cm column with a 14ml bed volume was prepared. The column was equilibrated using column buffer (20mM Tris-HCl, 200mM NaCl, 1mM EDTA) and the diluted crude extract was loaded onto the column (1ml of the flow through was collected and stored). The column was washed with column buffer, 5x column volume (1ml of the wash was collected and stored) and the fusion protein eluted using column buffer containing 10mM maltose. Fractions (3ml) were collected and using Bradford reagent (50 mg Serva Blue G, 88% phosphoric acid, 99.5% ethanol, dH_2O) monitored the eluted protein. Fractions containing the eluted protein were pooled and freeze dried. The column was regenerated by washing with column buffer for further use. The 1ml samples, which were collected, and the samples showing the eluted proteins were analyzed on a 10% SDS-PAGE gel.

2.10 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

A vertical slab gel unit (Hoefer, SE 600) was assembled in a dual casting stand was assembled. A resolving gel of 10%, Table2.3, was made and the solution was pipetted to a level about 4cm from the top and allowed to polymerize. A stacking gel of 4% was freshly made just before use as in Table 2.3 and placed above the running gel. A comb was inserted into the viscous stacking gel. The stacking gel was allowed to polymerize for 60min. Samples containing equal parts protein sample and 2x treatment buffer (0.125M Tris-Cl, 4% SDS, 20% v/v Glycerol, 0.2M DTT, 0.02% Bromophenol blue, pH 6.8) were placed in a microcentrifuge tube in boiling water for 5min, and placed on ice until ready for use. The comb was slowly removed from the gel unit and the wells were well rinsed with tank buffer (0.025M Tris, 0.192M Glycine, 0.1%SDS, pH 8.3). Each well and the lower chamber were filled with 1000ml of tank buffer. Approximately 10-20µl of

each sample was loaded into each well. Electrophoresis was performed at 10mA until the dye front reached the bottom. The gels were immediately stained using Coomassie Blue R250 staining solution (0.025% Coomassie brilliant blue R250, 40% methanol, 7% acetic acid) for a period of 2-12 hours after which they were destained in methanol-acetic acid water [5:1:4 (v/v/v)] solution for 30min. The first destaining solution was then replaced by destaining solution II (7% acetic acid, 5% methanol) until the background was entirely clear (Laemmli, 1970).

Table 2.3: Composition of SDS-PAGE gel for separation of protein samples.

Solutions	**Running Gel (10%)**	**Stacking Gel (4%)**
Acrylogel	3.15ml	650µl
Distilled water	5.40ml	3.0ml
0.5M Tris pH 6.8	0	1.25ml
3M Tris pH 8.8	1.25ml	0
Glycerol 50%(v/v)	200µl	0
SDS 20% (w/v)	50µl	25µl
TEMED	15µl	5µl
10 % (w/v)Ammonium persulfate	36µl	25µl

2.11 Purification of Leucocin A

2.11.1 Culture conditions for Leucocin A production

The bacteriocin was purified from the supernatant of *Leuconostoc gelidium* UAL 187-22. *L. gelidium* UAL 187-22 was grown in 600ml MRS broth to an optical density OD_{600} of 0.5 after which it was centrifuged at 10 000x g for 15min and the supernatant containing crude bacteriocin was collected.

2.11.2 SP-Sepharose Fast Flow Column

The crude bacteriocin preparation was applied to a 5ml SP-Sepharose Fast Flow (Amersham Pharmacia Biotech.) cation exchange column, equilibrated with 20mM sodium phosphate buffer pH 5.8. The column was subsequently washed with sodium phosphate buffer. The bacteriocin from *L. gelidium* UAL 187-22 was eluted with 1M NaCl. Eluted fractions were tested for bacteriocin using the spot on-lawn assay. The samples containing the leucocin A peptide (based on activity) were pooled and freeze dried. Freeze dried samples were stored at 4°C until further use.

2.11.3 High Pressure Liquid Chromatography (HPLC)

Freeze dried crude peptide samples were reconstituted with 0.1% Triflouroacetic acid (TFA) to a concentration of 40mg/ml. The sample was purified by reversed phase HPLC on a C-18 column (25cm x 4.6mm, 5µm particle size, 125 A; pore size, 12.5nm, Brownlee column) (Uteng *et al.*, 2002). A sample of 150µl from a stock of 40mg/ml leucocin A was injected per analysis. The profile used is outlined in Table 2.3. Leucocin A was eluted with acetonitrile, 0.1% TFA gradient. The elution (A_{220}) was monitored and the fractions tested for activity.

Table 2.4: Elution conditions of leucocin A on a C_{18} reverse phase column using actetonitrile

Step#	Time (min)	Flow rate (ml/min)	%A (Acetonitrile, 0.1%TFA)	Curvature
0	2.0	1.0	0	
1	5.0	1.0	40	1.0
2	35.0	1.0	60	1.0
3	5.0	1.0	90	1.0
4	2.0	1.0	0	1.0

2.12 Activity Assays

2.12.1 Dilution Assay of Leucocin A

Aliquots of 0.1% TFA (20µl) were placed on a sheet of parafilm. Leucocin A 40mg/ml (20µl) was added, mixed and double diluted with 0.1% TFA. In order to observe the activity of the dilution series, a TSA plate with TSA soft agar overlay containing *L. monocytogenes* was prepared. Undiluted leucocin A (40mg/ml) was used as a control. An aliquot of 10µl was plated on the TSA plate and incubated overnight at 37°C. The activity of each dilution was observed by checking for zones of inhibition.

2.12.2 Purified Leucocin A

Agar plates containing *L. monocytogenes* were used to assay for leucocin A activity. An aliquot of 10µl (40mg/ml) of leucocin A isolated from the SP Sepharose Fast flow column and 10µl of leucocin A purified using HPLC was placed on a TSA plate containing *L. monocytogenes* in TSA soft agar overlay and incubated at 37°C overnight, and checked for zones of lysis.

2.12.3 Interaction Assay

Freeze dried crude peptide samples were reconstituted with 0.1% TFA. A mixture containing 20µl of immunity fusion protein (40mg/ml) and 20µl of leucocin A (40mg/ml) was made. Another mixture containing 20µl of the cytoplasmic extract

from *L. gelidium* UAL 187-22 and 20µl of leucocin A (40mg/ml) was also made. An aliquot of 10µl of each mixture was spotted onto the TSA plate seeded with *L. monocytogenes*. Aliquots of 10µl of the immunity fusion peptide (40mg/ml), leucocin A (40mg/ml) and cytoplasmic extract mixtures were spotted onto the plate and incubated overnight at 37°C as in Table 2.5. The plates were observed for zones of inhibition.

2.13 Biomolecular Interaction Assay

2.13.1 Surface Plasmon Resonance (Biacore)

Interactions between the peptide leucocin A, the supernatant and cytoplasmic fractions of *L. gelidium* UAL 187-22 and 187-23 were analyzed with a Biacore model 2000 system, from Biacore (Biacore, Uppsala, Sweden). The running buffer used was HBS (10mM HEPES, pH 7.4, 150mM NaCl, 3.4mM Na_2EDTA), which was supplemented with 0.05% (v/v) surfactant P20 (Biacore, Uppsala, Sweden). The running buffer was degassed under a vacuum. Biacore system is equipped with the sensory chip CM5, a small metal chip with a carboxymethyldextran surface, to allow ligand immobilization via an amine group (Amano *et al.*, 1999). Four flow channels were used on the sensor chip. Flow channel 1, contained no antibodies and was used as a negative control. Flow channel 2-4, contained the immobilized rabbit anti-mouse anti-maltose antibody together with the mouse anti-MBP antibodies. Flow channel 3, was tested by passing a sample of the supernatant fraction over the surface. Flow channel 4, was tested by passing the cytoplasmic fraction over the surface of the chip. Unreacted moieties were blocked on the surface using ethanolamine. The CM5 sensor chip was washed several times with 10mM glycine, pH 2.2 to remove any unbound antibodies. All the measurements were carried out in HBS. Analyses were performed at 25°C. Peptides were dissolved in HBS to ~6mg/µl. The surface of the sensor chip was regenerated upon injection of a 50mM HCl solution after each run.

CHAPTER THREE

RESULTS AND DISCUSSION

3.1 Isolation of DNA

Plasmids are used more often than phage DNA molecules, as plasmid DNA often contains a single restriction site for a particular enzyme and therefore the ratio of inserted DNA to plasmid DNA is fairly high and the supercoiling of plasmids allows for easy isolation and manipulation. High quality intact plasmid DNA free of contaminating agents is essential for the process of obtaining a cloned immunity gene fragment. Complete digestion of DNA depends upon the absence of contaminating substances. Plasmid DNA of pJF5.5 and pMALc2 was isolated using the Nucleobond AX 100 kit and their concentrations were found to be 0.157μg/μl, and 0.115μg/μl respectively (Figure 3.1). These isolated plasmids were used in the cloning of the immunity gene.

A B

Figure 3.1: Isolated plasmids (A) pMALc2 and (B) pJF5.5.

3.2 PCR and DNA Quantitation

Following restriction digestion of pJF5.5 using *Eco*R1 for verification based on size (6.5 kb) the plasmid was used as a template for amplification of the immunity gene. Primers EAL-2 and EAL-3 were used in the amplification reaction. These primers were deduced from the sequence of the previously cloned leucocin A immunity factor (Hastings *et al.*, 1991). The amplicon obtained (Figure 3.2) fell within the 300bp range as expected, since the immunity gene has a calculated size of 365 bp. The band size was more accurately determined using the BIO-RAD Quantity One software and

gave a weight of 340 bp. This PCR product was purified using the GenElute™ agarose spin column. The concentration of the DNA obtained was found to be 8.12ng/µl. This isolated DNA was then used as the insert, which was ligated into the digested pMALc2 vector, and transformed, in order to obtain putative clones.

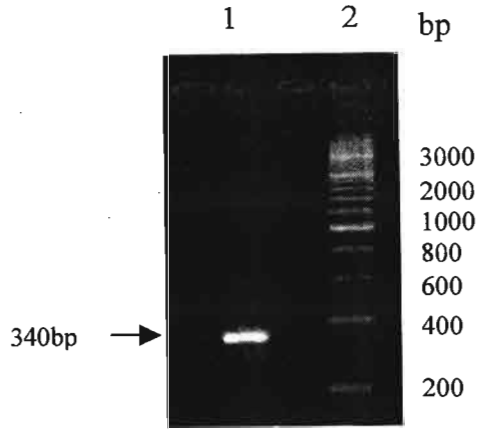


Figure 3.2: Agarose gel (2%) indicating the PCR product obtained containing the amplified immunity gene. Lane 1: PCR product using primers EAL-2 and EAL-3; Lane 2: O' Range Ruler 200 base pair, DNA ladder, used as the molecular weight standard.

3.3 Restriction Digestion

The plasmid pMALc2 was restricted with *Asp*700, an isoschizomer for *Xmn*I of which the recognition sequence is found in the polycloning site of pMALc2. Restriction with *Asp*700 creates a blunt end in pMALc2. The pMALc2 was double digested with *Asp*700 and *Xba*I, which created a blunt end and a sticky end (Figure 3.3). This allowed for uni-directional insertion of the insert into the pMALc2 vector.

The restricted pMALc2, using both *Asp*700 and *Xba*I were used for the ligation of the insert, obtained from the PCR. The insertion of the immunity gene occurs between the malE gene and the lac zα gene fragment, at the polylinker site on the cloning vector pMALc2, resulting in a complete plasmid containing the insert to be cloned. This construct codes for a malE/immunity fusion protein.

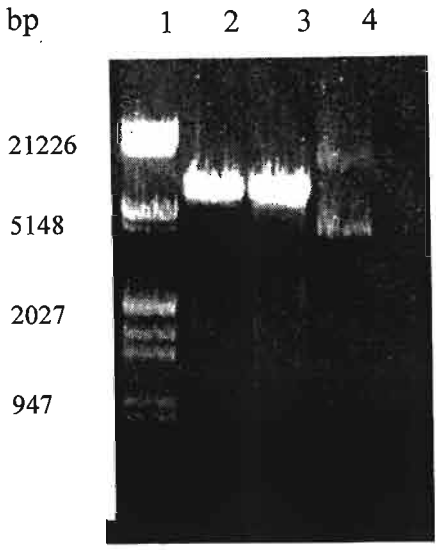


Figure 3.3: Agarose gel (1%) of restriction digests reactions run at 90V. Lane 1: DNA MWM III (Roche); Lane 2: pMALc2 restricted with *Asp*700; Lane 3: pMALc2 restricted with *Asp*700 and *Xba*I; Lane 4: unrestricted pMALc2.

The ligated plasmids were transformed into freshly prepared competent *E. coli* JM103 cells. After incubation at 37°C overnight, transformants were observed as blue and white colonies (Figure 3.4). The blue colonies were representative of non-recombinants, or plasmids that religated. White colonies represented recombinants, which was pMALc2 ligated with the immunity gene insert. The new recombinant plasmid was termed pKP. True positive clones can be distinguished from false positive clones, as the latter does not contain the immunity gene. Again, the verification test used previously was to detect for the immunity gene, using probes EAL 2 and EAL 3.

Figure 3.4: White and blue colonies of *E. coli* (JM103), grown on Luria bertani agar containing ampicillin, tetracycline and X-galactosidase at 37°C. White colonies represent bacterial cells carrying recombinant plasmid. Blue colonies represent parental cells.

3.4 Analysis of recombinant clones

A mini-prep isolation of the clones revealed the presence of DNA all of similar size (Figure 3.5). The plasmid pMALc2 was used as a control. In lanes 2-5, 4 of the clones obtained from the transformation were isolated. Plasmid DNA from lanes 2-5 (putative clones) migrated slower through the gel, as compared to pMALc2, indicating the presence of an insert (Figure 3.5). These were assumed to be putative clones. Confirmation of the clones was established after southern blot hybridization using the oligonucleotide EAL 3 as a probe.

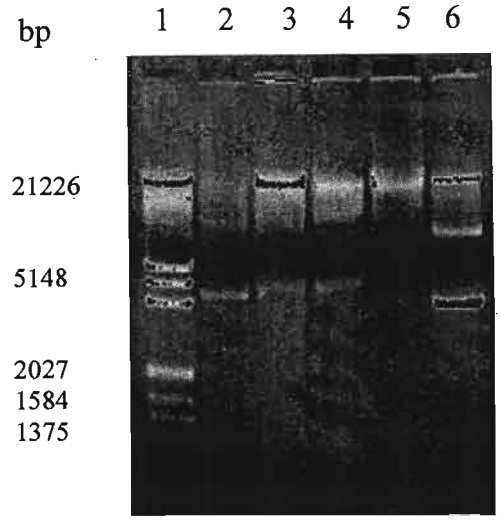


Figure 3.5: Agarose gel (0.8%) of recombinant plasmid clones obtained. Lane 1: DNA MWM III (Roche); Lane 2: Clone pKP1; Lane 3: clone pKP2; Lane 4: clone pKP3; Lane 5: clone pKP4; Lane 6: pMALc2 (115µg/ml).

In order to obtain DNA, for sequencing, the DNA from the clones were re-isolated using the Nucleobond AX 100 kit. The isolated clones pKP1, pKP2 and pKP3, (Figure 3.6), revealed that all of the clones contained plasmid DNA of the same sizes. The concentrations of these clones were quantified using spectroscopy and found to be: pKP1 0.17µg/µl; pKP2 0.12µg/µl; pKP3 0.13µg/µl. The recombinant plasmids were sent for DNA sequencing to determine the sequence of the insert.

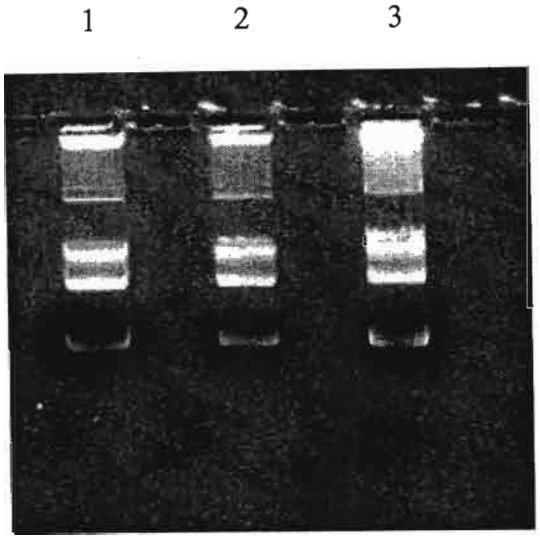


Figure 3.6: Agarose gel (0.8%) showing the isolation of recombinant clones obtained. Lane1: clone pKP1; Lane 2: clone pKP2; Lane 3: clone pKP3.

3.5 Southern Blotting

Southern and colony blot hybridization done by probing with the primer EAL 3 were used to confirm the presence of the immunity gene (Figure 3.4). The results obtained for the southern blot revealed that the clone pKP2 was a true positive clone. Lane 1 contained a Dig-labeled MWM, and served as a positive control for the detection procedure (Figure 3.7). The control, pMALc2 was in lane 2, resulted in no bands being detected. This occurred since pMALc2 contained no immunity gene insert. In lane 3, containing the clone pKP1, bands were visualized. This indicates the presence of the immunity gene insert. A colour precipitate develops due to the hybridization of the dig-labeled probe to its complementary sequence on the plasmid. This result confirmed the sequence results obtained for the clone pKP1 after cloning and isolation of the immunity gene. The plasmid pJF5.5 served as a positive control as it was used to amplify the insert to be cloned.

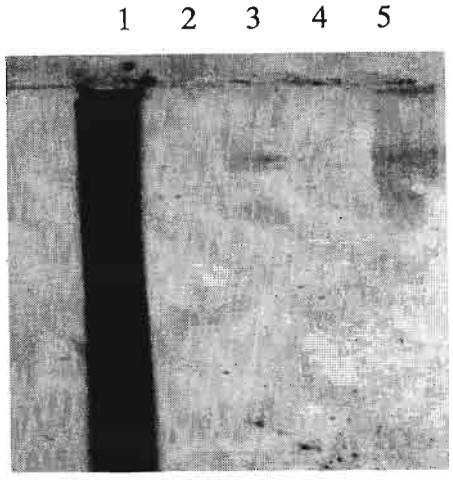


Figure 3.7: Southern blot hybridization of clone pKP1. Lane 1: Dig labeled control DNA (Roche); Lane 2: pMALc2 (115μg/ml); Lane 3: clone pKP1; Lane 4: blank; Lane 5: pJF5.5 (157μg/ml). EAL-3 [100pmoles], which binds the 3' end of the immunity gene, was used as a probe.

3.6 DNA Sequencing

DNA sequencing revealed the presence of the insert (Figure 3.8 and 3.9). The fragment was cloned adjacent to the malE gene within the polycloning site of the vector pMALc2. Conversion of the DNA sequence to amino acid sequence also showed the fragment was cloned in frame and upon expression would result in a fusion construct with malE protein (Figure 3.10). The theoretical mass and pI was calculated at 12 946.88 Da and 7.76 respectively.

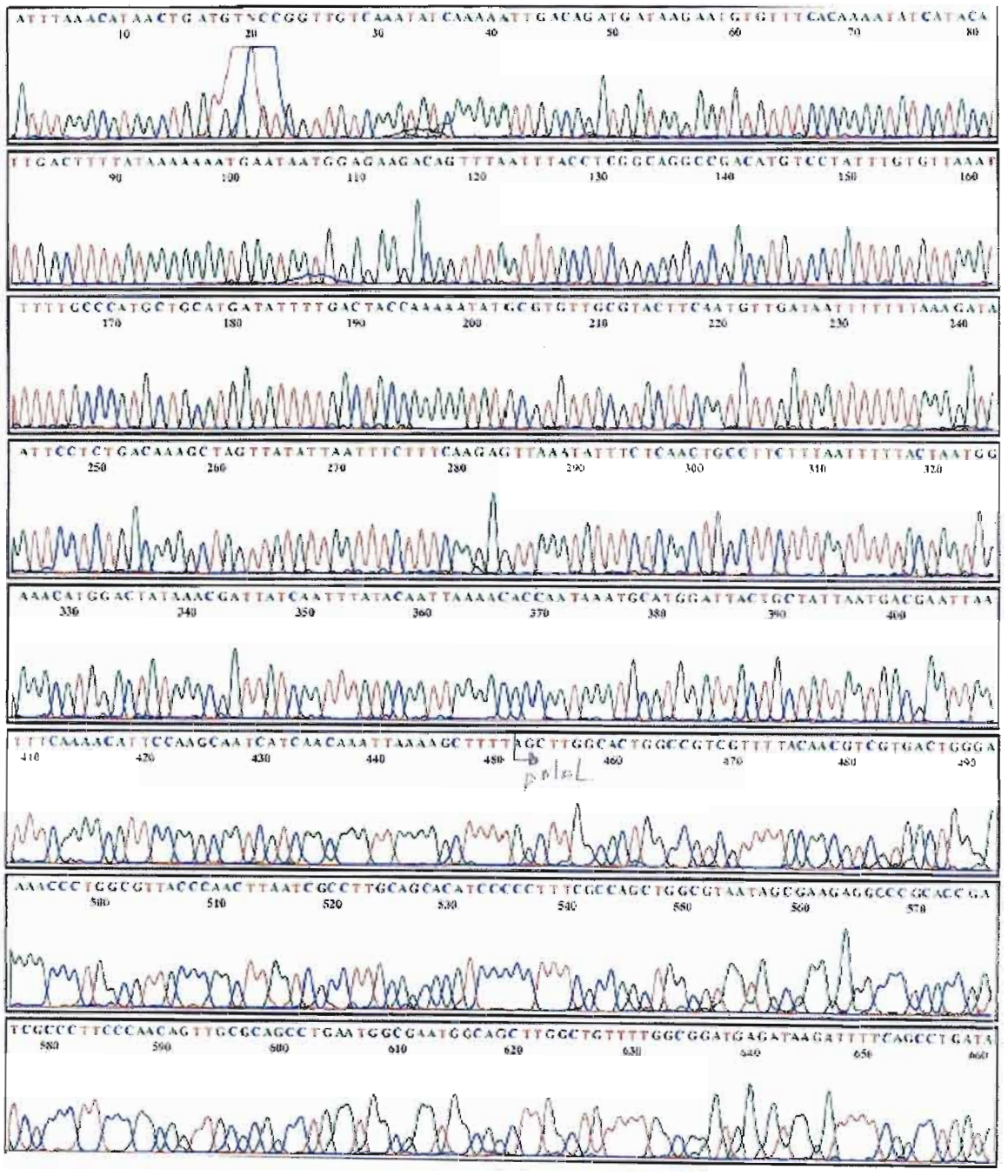


Figure 3.8 DNA sequencing results of the recombinant clone containing the inserted immunity gene.

1 TTAAACATAA CTGTATGTCC CCTGGTTGTC AAATATCAAA

41 AATTGACAG**A** **TGATAAGAAT** **GTGTTTCACA** **AAATATCATA**

81 **CATTGACTTT** **TATAAAAAAA** **TGAATAATGG** **AGAAGACAGT**

121 **TTAATTTACC** **TCGGCAGGCC** **GACATGTCCT** **ATTTGTGTTA**

161 **AATTTTTGCC** **CATGCTGCAT** **GATATTTTGA** **CTACCAAAAA**

201 **TATGCGTGTT** **GCGTACTTCA** **ATGTTGATAA** **TTTTTTTAAA**

241 **GATNATTCCT** **CTGACAAAGC** **TAGTTATATT** **AATTTCTTTC**

281 **AAGAGTTAAA** **TATTTCTCAA** **CTGCCTTCTT** **TAATTTTTAC**

321 **TAATGGAAAC** **ATGGACTATA** **AACGATTATC** **AATTTATNCA**

361 **ATTAAAACAC** **CAATAAATGC** **ATGGATTACT** **GCTATTAATG**

401 **ACGAATTAAT** **TTCAAAACAT** **TCCAAGCCAT** **CATCAACAAA**

441 **TTAAAAACTT** **TT**AGCTTGGC ACTGGCCGTC GTTTTTACAA

481 CGTCGTGACT GGGAAAAACC CNGGCGTTAN NCCCNCNTTN

521 ATCCNCCTGG NGGNNCANTC CCCCCTTTCG CCAGCTGGCN

561 CNATANNGNA TAAAGGCCCN CCCCCGAATC GCCCTTTNCC

601 AACNNTTGCG CCNGCCTG

Figure 3.9: DNA sequence of the recombinant plasmid pKP1. Bold font indicates the sequence of the immunity gene as determined by the Sanger dideoxy method.

```
ttaaacataactgtatgtcccctggttgtcaaatatcaaaaattgacagatgataagaat
L  N  I  T  V  C  P  L  V  V  K  Y  Q  K  L  T  D  D  K  N
gtgtttcacaaaatatcatacattgacttttataaaaaaaatgaataatggagaagacagt
V  F  H  K  I  S  Y  I  D  F  Y  K  K  M  N  N  G  E  D  S
ttaatttacctcggcaggccgacatgtcctatttgtgttaaatttttgcccatgctgcat
L  I  Y  L  G  R  P  T  C  P  I  C  V  K  F  L  P  M  L  H
gatattttgactaccaaaaatatgcgtgttgcgtacttcaatgttgataatttttttaaa
D  I  L  T  T  K  N  M  R  V  A  Y  F  N  V  D  N  F  F  K
gatnattcctctgacaaagctagttatattaatttctttcaagagttaaatatttctcaa
D  X  S  S  D  K  A  S  Y  I  N  F  F  Q  E  L  N  I  S  Q
ctgccttctttaatttttactaatggaaacatggactataaacgattatcaatttatnca
L  P  S  L  I  F  T  N  G  N  M  D  Y  K  R  L  S  I  Y  X
attaaaaacaccaataaatgcatggattactgctattaatgacgaattaatttcaaaacat
I  K  T  P  I  N  A  W  I  T  A  I  N  D  E  L  I  S  K  H
tccaagccatcatcaacaaattaaaaacttttagcttggcactggccgtcgtttttacaa
S  K  P  S  S  T  N  -  K  L  L  A  W  H  W  P  S  F  L  Q
cgtcgtgactgggaaaaacccnggcgtta
R  R  D  W  E  K  P  X  R
```

Figure 3.10: Translated amino acid sequence of the immunity gene derived from clone pKP1.

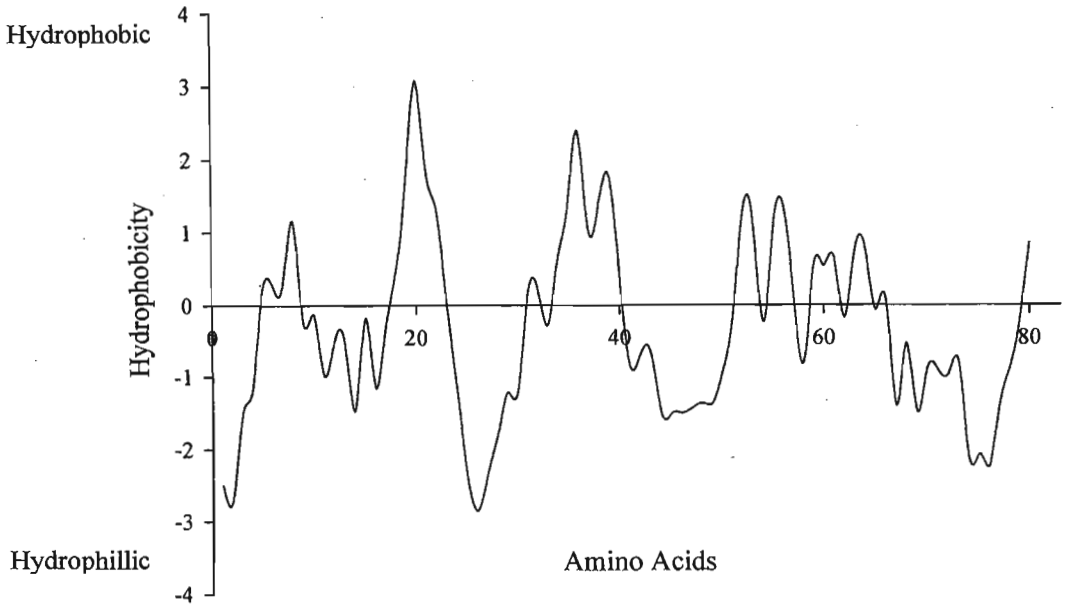


Figure 3.11 Hydrophobicity profile of leucocin A immunity protein.

The hydropathy profile of the immunity protein is shown in Figure 3.11, in which their hydrophobicity profiles are well conserved. The profile suggests that the immunity protein is unlikely to be an integral membrane protein, with no significant transmembrane regions (motifs). This is most likely due to the fact that the area of hydrophobicity within the protein is too short. To confirm these results an algorithm was run, showing that the immunity protein would most likely be extracellularly located. The pepI immunity protein of pep5 lantibiotic produced by *Staphylococcus epidermis* was shown to be extracellularly located (Hoffman *et al.*, 2004). The C-terminus of the pepI is very hydrophilic and is an important component to the protein for conferring the immunity phenotype. Pep5 is a pore forming type A lantibiotic. The difference between the leucocin A immunity protein and the pepI is their mechanism of action. Analysis with InterProScan algorithm shows that Leucocin A immunity protein has no significant domains.

3.7 Expression of fusion construct

IPTG was used as an inducer, in the expression of the fusion construct. The SDS-PAGE analysis of the recombinant clone pKP1 and pMALc2 showed the expression of the fusion protein (Figure 3.12).

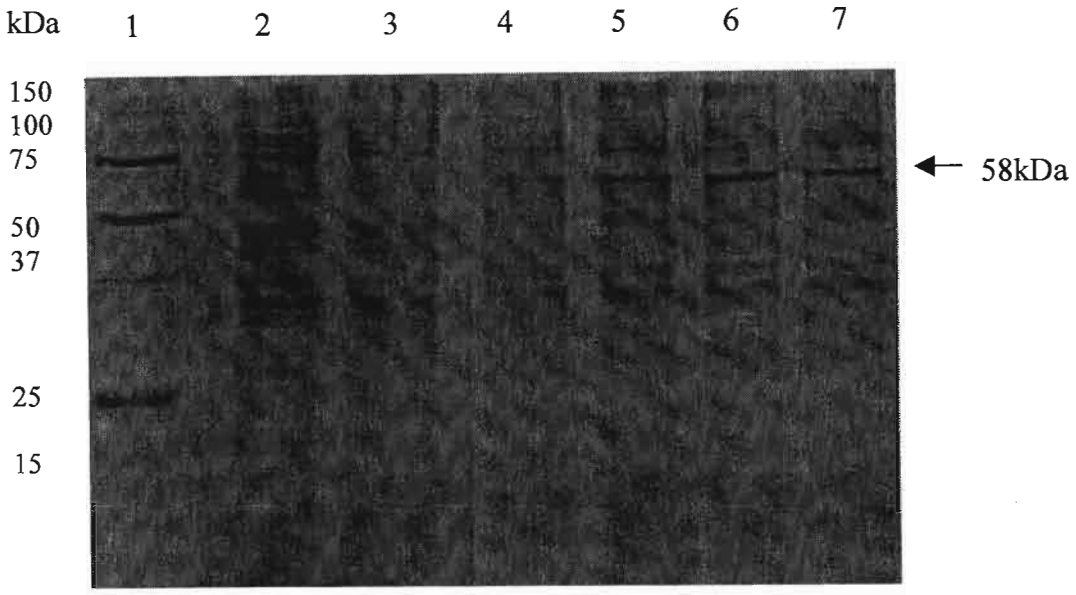


Figure 3.12: SDS-PAGE analysis, showing the expression of the fusion constructs. Lane 1: BIORAD MWM; Lane 2: uninduced pMALc2; Lane 3: induced pMALc2; Lane 4: clone pKP1 induced; Lane 5: clone pKP2 induced; Lane 6: clone pKP3 induced; Lane 7: clone pKP4 induced. Samples were induced using 0.3mM IPTG.

In lane 1, a BIORAD molecular weight marker was used as the standard in order to determine the sizes of the proteins. An uninduced sample of pMALc2 was in lanes 2. The induced sample of pMALc2 was in lane 3. The induced sample of pMALc2 contains a protein band at position 58kDa. A 43kDa band was visible, which correspond to the malE protein, in both the induced and non induced parental strains. This is due to the fact that the lac promoter is not as tightly controlled as the tac/tet promoter and were constitutively expressed at a lower level. Lanes 4-7 contained the induced samples of the recombinant clones pKP1-pKP4, all of which contained an expressed fusion protein at position 58kDa. Molecular weight sizes were calculated using the BioRad Quantity One software package with BioRad prestain MWM as internal standards.

3.8 Affinity Chromatography of MBP-Immunity Fusion Protein

Expressed proteins were purified with an Amylose affinity resin. These were analyzed on a 10% SDS-PAGE gel (Figure 3.13). Proteins from induced samples were extracted with a sonicator at 2min intervals for 60sec. Proteins were found to be completely released from the cytoplasm after ± 20min. This was monitored by adding Bradford reagent to sonicated samples. Analysis on SDS-PAGE shows the release of the proteins.

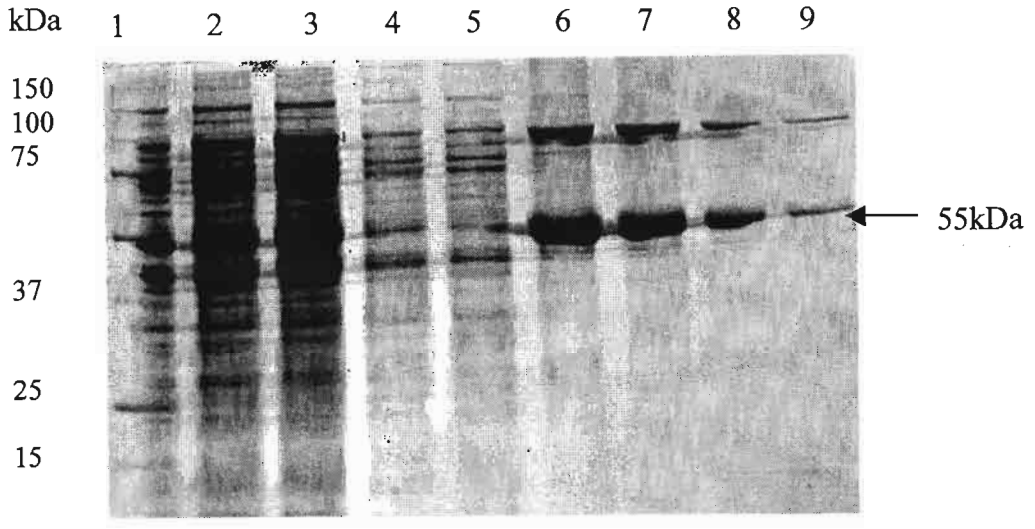


Figure 3.13: Tricine SDS-PAGE analysis using affinity chromatography to identify the MBP-fusion protein. Lane 1: BIORAD MWM; Lane 2-3: supernatant after centrifugation; Lane 4: flow through; Lane 5: first wash; Lane 6: fraction 4; Lane 7: fraction 5; Lane 8: fraction 6; Lane 9, fraction 7.

Lane 1 containing the BIORAD MWM was used as the indicator, in order to determine the sizes of the proteins present on the SDS-PAGE gel. Lane 2 and 3, containing the supernatant, clearly contained multiple proteins, including the MBP-fusion protein. The sample containing the flow through was run in lane 4 resulting in a decrease of the amount of proteins present. A reason for this is that some of the unwanted proteins are removed as they pass through the column and malE containing proteins bind to the amylose resin. After passing the pKP fraction containing the MBP-fusion protein through the column, it was washed and a sample of this wash was collected and ran in lane 5. This clearly shows a decrease in the amount of proteins

present as compared to the initial supernatant fraction. Lanes 6, 7, 8 and 9 contained the eluted MBP-fusion protein. The presence of only the MBP-immunity fusion protein indicates the effectiveness of the purification. However, the presence of a second band may be due to aggregation of the fusion construct. The sizes of the 2 common bands in lanes 6,7, 8 and 9 are approximately 55kDa and 115kDa as determined by the BIORAD Quantity one software. The MBP is known to have a theoretical mass of ± 43 kDa (pMAL Protein Fusion Purification System) and the fusion construct a theoretical mass of ± 55.9kDa. It is assumed that due to aggregation of the MBP-fusion protein, a dimer at ± 115 kDa was present.

3.9 Purification of leucocin A

Leucocin A was purified from the supernatant of *L. gelidium* UAL 187-22. The purification was a two-step procedure involving ion exchange chromatography and reverse phase HPLC.

3.9.1 Ion exchange chromatography

Ion-exchange chromatography is a technique in which molecules are separated on the basis of their charge. With the stationary phase being the actual ion-exchange resin, while the mobile phase is an aqueous solution. Ion-exchange chromatography is usually performed in columns and the charged molecules are retarded in their movement through the column depending on the sign and magnitude of their charge. An ion-exchange resin that is negatively charged and binds cations is called a cation exchanger (Stenesh and Wiley, 1975).

In our studies, the SP Sepharose Fast Flow cation-exchange resin (Amersham Pharmacia Biotech) was used for the purification of the bacteriocin leucocin A from the producer strain *L. gelidium* UAL 187-22. The bacteriocins, which bind to the resin, were detected using the Bradford reagent upon elution with 1M NaCl. Binding of the bacteriocin to the resin depends on the concentration of the sodium phosphate ions used for washing of the column before elution of the bacteriocin, as this can affect the overall net positive charge of the resin (Uteng *et al.*, 2002). The results obtained showed activity for leucocin A, in a few of the eluted fractions. The

fractions showing activities were pooled freeze dried and re-tested for activity as described in materials and methods.

3.9.2 Reverse phase HPLC

Freeze dried fractions from the Ion exchange chromatography step were resuspended in 0.1% TFA at a concentration of 40mg/ml. This was applied to a C_{18} reverse phase column at 150µl per injection. The HPLC chromatogram (Figure 3.14) shows the presence of two peaks having retention times of 3.4min and 28.0min respectively. This corresponds to 24% actetonitrile, 0.1% TFA and 55% acetonitrile, 0.1% TFA respectively. Peak 1 and 2 were tested for the presence of bacteriocin by testing each peak for inhibitory activity. Activity was detected in peak 2 with no activity shown to be present in peak1 (Figure 3.14). Quantifying peak 2, showed it to have 128AU/ml. After reverse phase HPLC leucocin A was considered to be 90% pure. This material was used in molecular interaction studies.

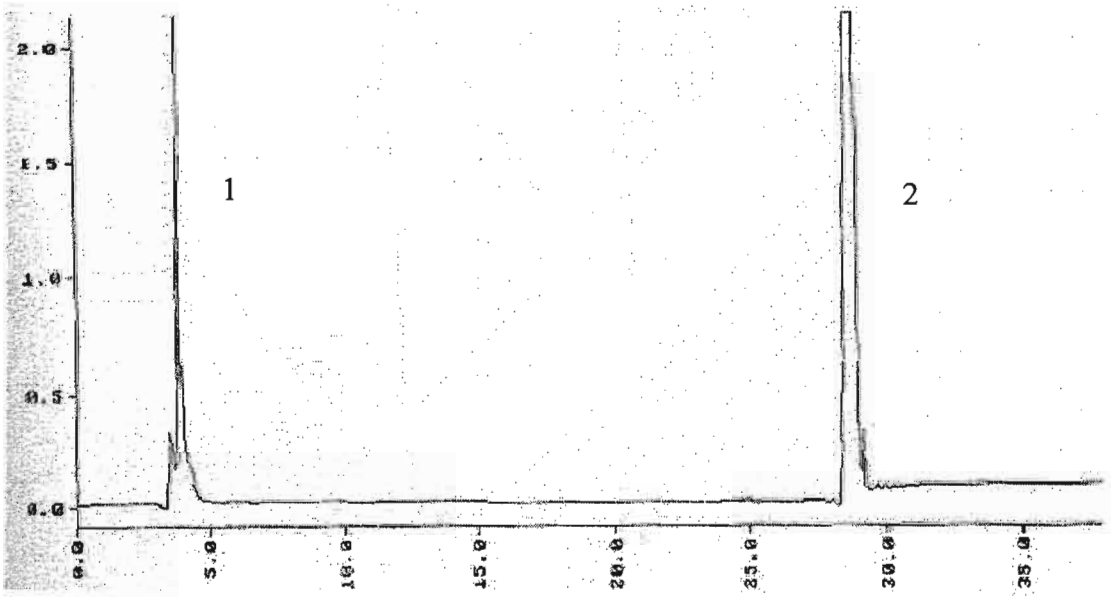


Figure 3.14: A chromatogram of a 150µl (40mg/ml) injection of a crude sample of the antimicrobial peptide, leucocin A, in 0.1% TFA on a reverse phase HPLC C_{18} column.

3.10 Activity Assay

Arrow 1 indicates the position at which leucocin A, the control was spotted resulting in the inhibition zone. This proves that the bacteriocin produced by *L. gelidium* UAL 187-22 causes inhibition of *L. monocytogenes*. Arrow 2 indicates the position at which the pure eluted protein from the HPLC was spotted and the eluted protein is the experimentally produced leucocin A. This result obtained confirms tests previously carried out by Hastings *et al.*, 1991, in which the isolated bacteriocin Leucocin A causes the inhibition of *L. monocytogenes.*

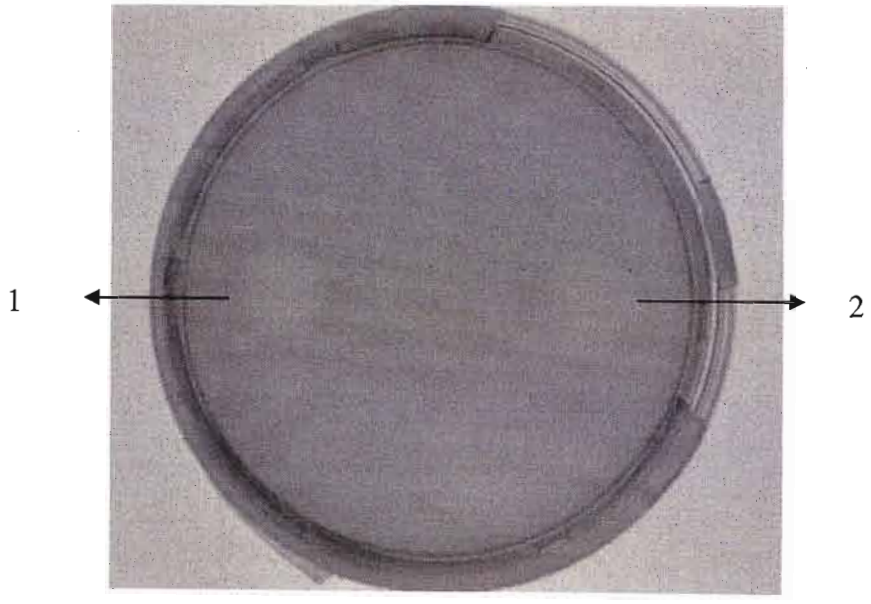


Figure 3.15: A Tryptone Soy agar plate overlaid with *Listeria monocytogenes* indicating the proteinaecous nature of the bacteriocin Leucocin A (40mg/ml) produced by *Leuconostoc gelidium* UAL 187-22, giving rise to the visible inhibition zones.

3.11 Biomolecular Interactive Assay

3.11.1 Plate assay

Interaction between the immunity fusion protein and several extracts from *L. gelidium* was tested (Table 3.1). From this it is noted that the immunity protein doesn't show any interaction between the supernatant and the cytoplasm.

Table 3.1: Interaction assays to analyze activity against *L. monocytogenes*.

Components	Inhibition
Leucocin A (40mg/ml)	+
Immunity fusion protein (40mg/ml)	-
Cytoplasmic extract from *L. gelidium* UAL 187-22	+
Leucocin A + Immunity fusion protein	+
Leucocin A + Cytoplasmic extract of *L. gelidium* UAL 187-22	+
Leucocin A + Cytoplasmic extract + immunity fusion protein	+
Supernatant from *L. gelidium* UAL 187-22	+

+ Zone of inhibition present

- Zone of inhibition absent

Purified leucocin A showed activity against *L. monocytogenes*. The immunity fusion peptide showed no activity, which was as expected. Inhibition of *L. monocytogenes* occurred from the supernatant and cytoplasmic fractions. It is interesting to note that the assay containing the fusion protein mixed with the cytoplasmic fraction caused inhibition of *L. monocytogenes*. This would suggest that the cytoplasmic fraction contains some leucocin A peptide. However, this would be unprocessed leucocin A that still contained its leader sequence. The leader sequence is shown to be important for transport out of the cell. It was also postulated that unprocessed leucocin A is inactive to protect the producer strain and are only activated once the leader sequence has been cleaved off. Our results clearly show that the unprocessed Leucocin A within the cytoplasmic fraction still contained some low level activity, hence the inhibition zone from the interaction assay between the fusion construct and cytoplasmic fraction. From this it can be deduced that the immunity fusion protein does not inhibit the activity of leucocin A and there is no interaction between them.

3.11.2 Biacore assay

Figure 3.16 represents the sensogram for the immobilization of the ligand used in the biacore assays. The individual section (1 – 4) of the sensogram shows the different steps of the immobilization process. During phase 1, rabbit anti-mouse antibodies were immobilized (via amine coupling) giving a relative response of 500RU. Mouse-anti MBP was allowed to interact with the immobilized rabbit –antimouse antibodies coupled to the dextran matrix. The relatively large increase in response value indicates the interaction between the ligand and mouse-anti MBP antibody. The amount interacted is proportional to the relative response (phase 2, Figure 3.16). To remove unbound antibody the surface was washed (phase 3) with HBS, this was followed by several regeneration steps (phase 4) to prepare the surface for the biacore assays to follow.

Since the activity of the peptide is believed to cause pores in the cytoplasmic membrane, we wanted to verify whether, protection against leucocin A is due to direct interaction between the immunity protein and leucocin A. However, from the Biacore experiment it was seen that there was no direct interaction between the immunity protein and leucocin A (Figure 3.17). This confirms the results obtained in table 2.5. The Plato after injection of sample indicated no interaction and the increase in relative response is due to a change in buffer flow over the sensor chip surface. Therefore, we would speculate that self-protection of *L. gelidium* via. the immunity protein is not based on leucocin A being chaperoned away from its target site.

We proceeded to check for the interaction of the immunity protein with other possible binding partners in the supernatant and cytoplasm of *L. gelidium* UAL 187-22.

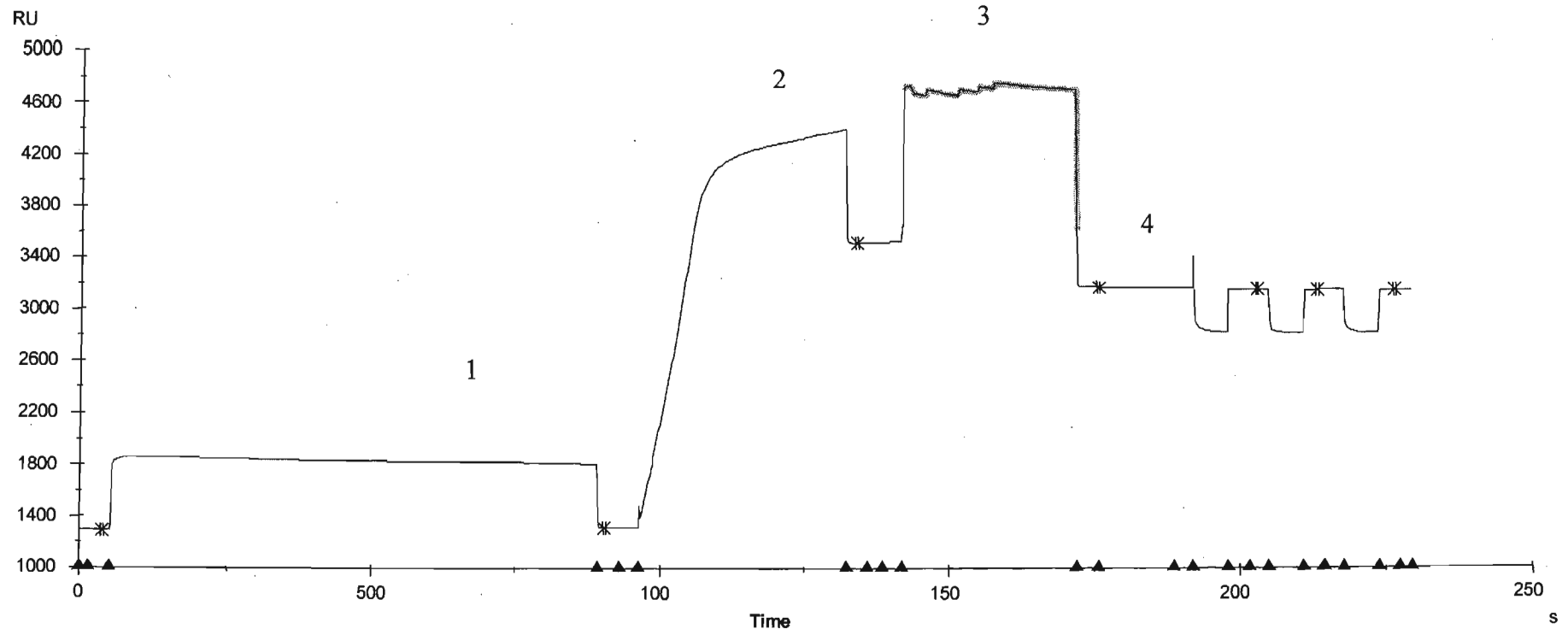


Figure 3.16: Sensogram showing the immobilization of rabbit anti-mouse antibodies and mouse anti MBP antibodies onto a CM5 sensor chip.

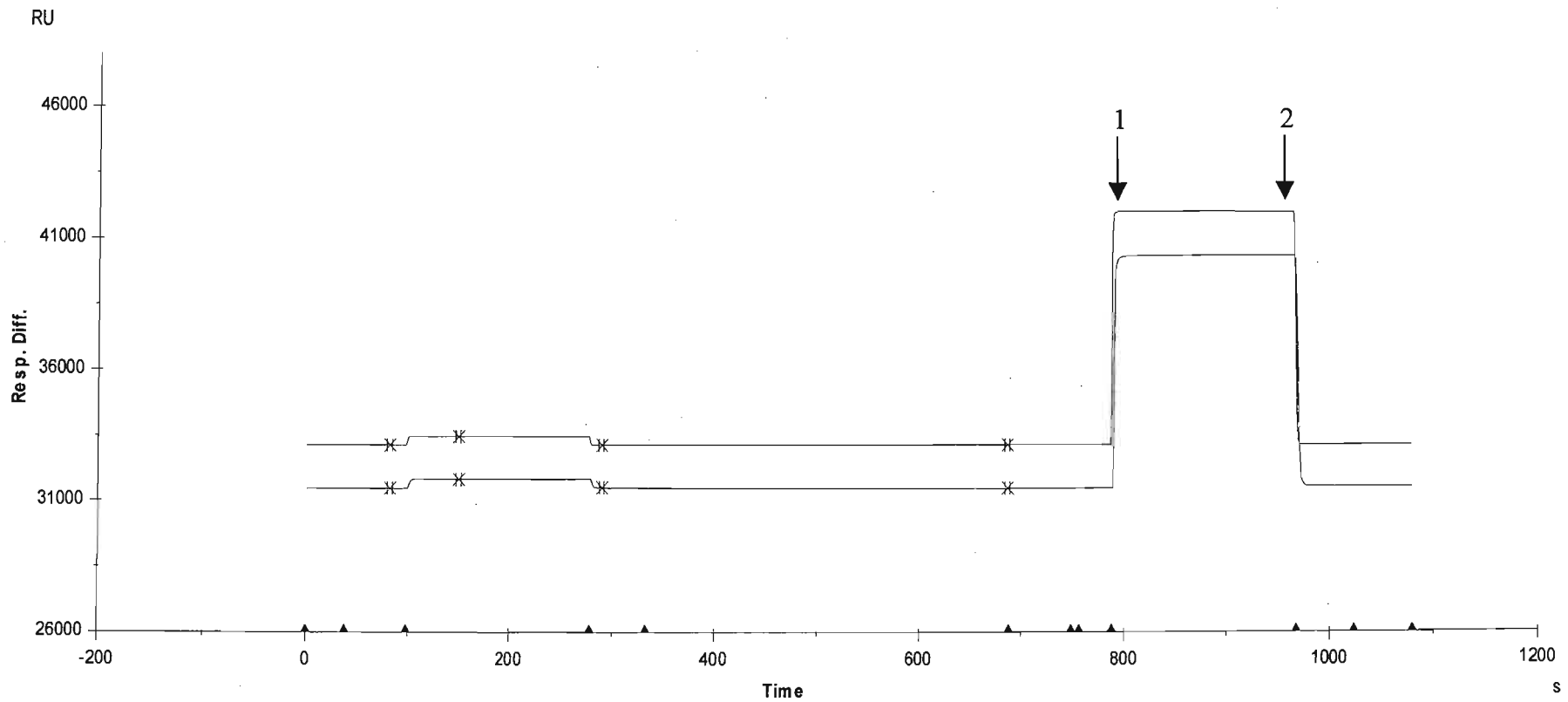


Figure 3.17: Sensogram showing the interaction experiment between the immunity protein and leucocin A. The arrows (↓) 1 and 2 indicate the start and end of the injection of sample.

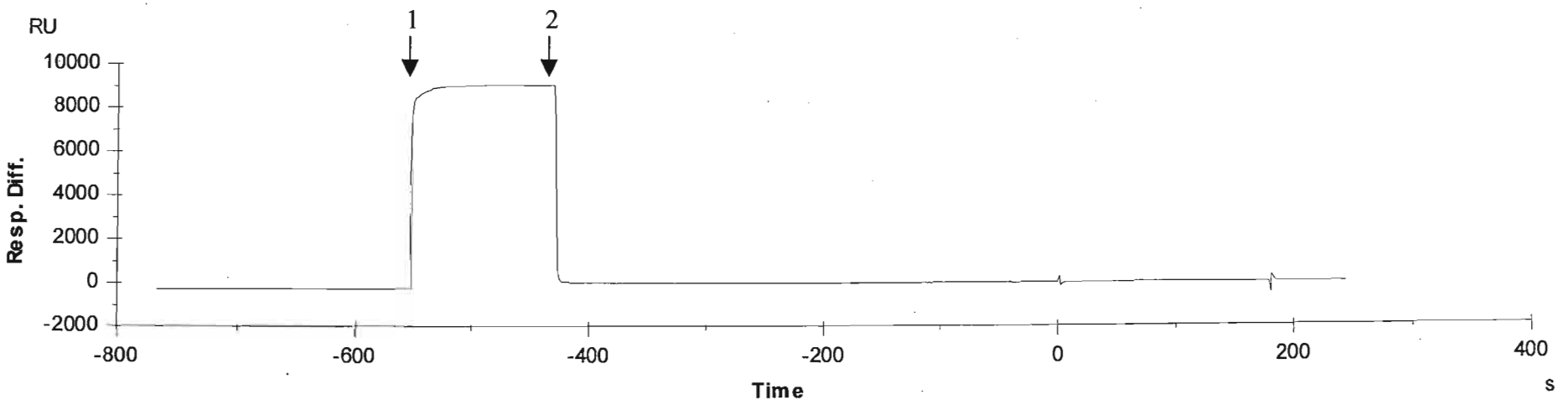


Figure 3.18: Sensogram showing the interaction of the supernatant extract of *Leuconostoc gelidium* UAL 187-22 with the immunity protein. The arrows (↓) 1 and 2 indicate the start and end of the injection of sample.

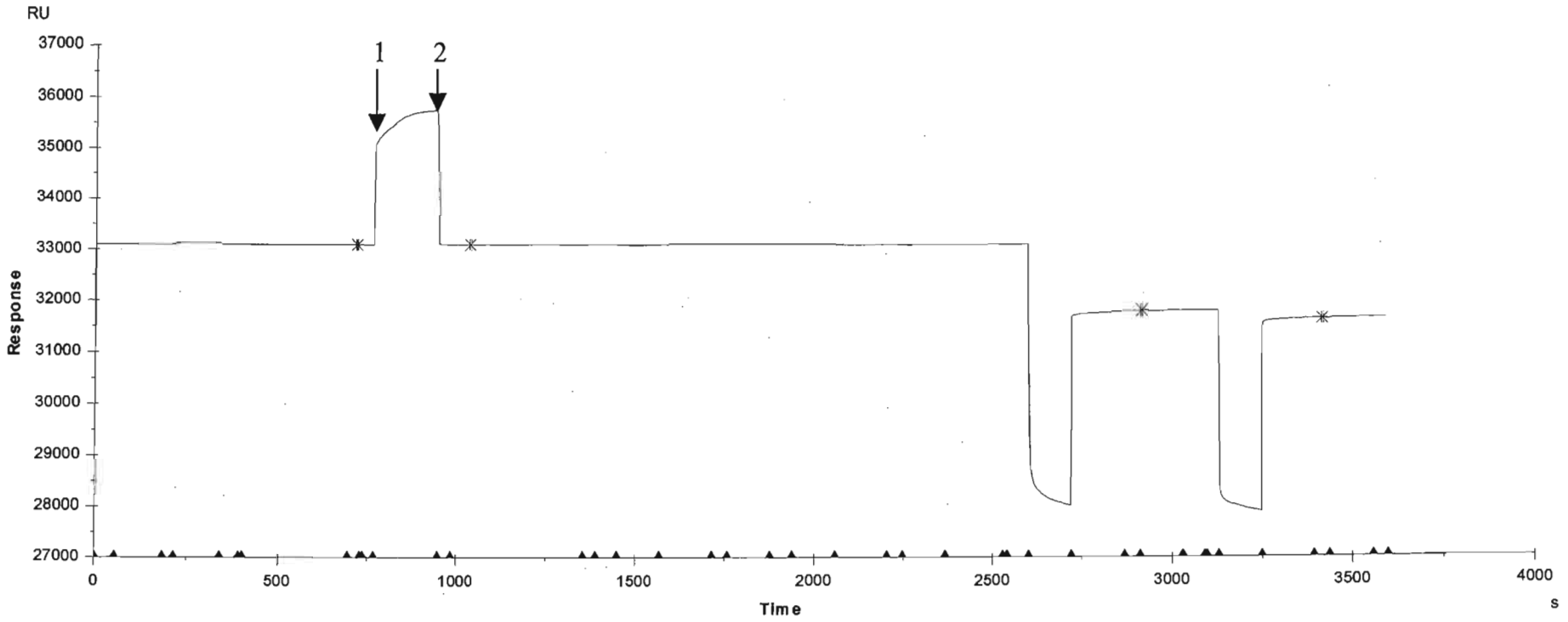


Figure 3.19: Sensogram showing interaction of the cytoplasmic extract of *Leuconostoc gelidium* UAL 187-22 and leucocin A

The sensogram in Figure 3.18 shows the interactive assay when a supernatant fraction was passed over the immunity protein surface. After injection of sample the sensogram plato, and there were no increase in relative response at this point. Thus we observed no direct interaction between the immunity protein and any possible binding partners in the supernatant. We might thus speculate that there is no secondary binding partner in the supernatant, which might influence the immunity process.

Following injection of the supernatant fraction the surface was regenerated. A fraction of cytoplasm extract from *L. gelidium* UAL 187-22 was passed over the immunity protein surface. Figure 3.19 shows the result obtained from this experiment. After injection (arrow 1 of figure 3.19) there was an increase in response value from 35 000RU to 35 750RU, giving a relative response of 750RU. Also visible from the sensogram is the lack of a dissociation phase between the immunity protein and the binding partner in the cytoplasm.

The results from this experiment clearly indicate the presence of a binding partner in the cytoplasm to that of the immunity protein. Moreover, the interaction between the immunity protein and an orphan ligand in the cytoplasm seen to be a very stable interaction. The obvious second step to this experiment would be to characterize its function and role as a binding partner to the immunity protein. This will allow us to formulate a more concrete hypothesis on the role of this ligand within the mechanism of producer self-protection.

CHAPTER FOUR

CONCLUSION

In the past few years there has been significant progress made in our understanding of class II bacteriocins. Apart from cloning and sequencing of the operons, we now begin to understand the mode of action of various bacteriocins and in this study, the way the leucocin A immunity protein works.

The role of the immunity protein for leucocin A has partly been revealed in this study. The main outcome of this study found that there is no direct interaction with the bacteriocin leucocin A. Interaction studies carried out using surface plasmon resonance showed a stable interaction found between the cytoplasmic extract of *L. gelidium* UAL 187-22 but not with the supernatant fraction of *L. gelidium* UAL 187-22. Possible reasoning for this could be that a binding partner for the immunity protein is located in the cytoplasm and not in the supernatant.

In order to identify the binding partner of the leucocin A immunity protein, various other experimental procedures would have to be carried out. One can use surface Plasmon resonance to isolate the binding partner by ligand fishing. Once this binding partner has been isolated, mass spectroscopy or N-terminal sequencing can be used to characterize this ligand. This will provide a better understanding to the process of immunity of the bacteriocin, and allow us to identify if the C-terminus or N-terminus is important in conferring the immunity phenotype.

Another possible method in determining its localization would be to raise antibodies against the immunity protein and determine where it is located experimentally. Although the leucocin A receptor has not yet been identified, it is likely to be the site of action of the immunity protein.

CHAPTER FIVE

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