THE INFLUENCE OF THE LEADER SEQUENCE ON ANTIMICROBIAL ACTIVITY OF LEUCOCIN A, AN ANTILISTERIAL BACTERIOCIN PRODUCED BY *LEUCONOSTOC GELIDUM* UAL187-22

by

JIREN REDDY

Submitted in fulfilment of the academic requirements of the degree of Master of Science in the Discipline of Genetics, School of Biochemistry, Genetics, Microbiology and Plant Pathology, University of Kwazulu-Natal, Pietermaritzburg

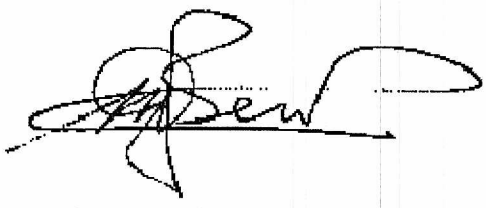
February 2008

PREFACE

All experimental work described in this thesis for a Masters in Science was carried out at the School of Biochemistry, Genetics, Microbiology, and Plant Pathology; University of Kwa-Zulu Natal; Pietermaritzburg, under the supervision of Dr. Mervyn Beukes.

The experimental strategies and results 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 duly acknowledged in the text.

Jiren Reddy (Mr)



Dr. Mervyn Beukes, PhD (supervisor)

DECLARATION

I, JIREN REDDY declare that

1. The research reported in this thesis, except where otherwise indicated, is my original research.

2. This thesis has not been submitted for any degree or examination at any other university.

3. This thesis does not contain other persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.

4. This thesis does not contain other persons' writing, unless specifically acknowledged as being sourced from other researchers. Where other written sources have been quoted, then:
   a. Their words have been re-written but the general information attributed to them has been referenced
   b. Where their exact words have been used, then their writing has been placed in italics and inside quotation marks, and referenced.

5. This thesis does not contain text, graphics or tables copied and pasted from the Internet, unless specifically acknowledged, and the source being detailed in the thesis and in the References sections.

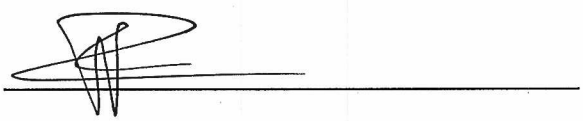
Signed: 

TABLE OF CONTENTS

ABSTRACT

Bacteriocin leader pepides are currently receiving much attention due to their possible functions. It is predicted that these leaders prevent cytoplasmic toxicity within the producer organism by rendering the bacteriocin inactive. Leucocin A, a class IIa bacteriocin produced by *Leuconostoc gelidum* UAL187-22 is synthesized with a 24 amino acid leader pepide which is cleaved during extracellular translocation. The antimicrobial activity of the leucocin A precursor, pre-leucocin A, was determined to gain insight into whether, the presence of a leader peptide has an impact on anti-listerial activity. The leucocin A and pre-leucocin A genes were generated by PCR of *L. gelidum* UAL187-22 plasmid DNA. Recombinant plasmids, pLcaA and pPreLcaA were isolated by cloning the amplified genes into the *Escherichia coli* pMAL.c2 vector, and by screening transformant colonies using blue white selection methods. The *malE-LcaA* and *malE-preLcaA* fusion genes were expressed, and resulting maltose binding fusion proteins, were purified using amylose affinity chromatography. Fractions collected, contained partially pure forms of MBP-LcaA (46.433 kDa) and MBP-preLcaA (49.088 kDa) fusion proteins. Following Factor Xa digestion, the MBP affinity tag was removed; and recombinant peptides, leucocin A and pre-leucocin A were further purified by reverese phase high performance liquid chromatography. It was determined that leucocin A was eluted with a retention time of 24.893, while pre-leucocin A was eluted with a retention time of 31.447. Fractions of pure leucocin A and pre-leucocin A were thereafter assayed for activity using a deferred antagonism assay, with *Listeria monocytogenes* being the indicator strain. Pre-leucocin A tested positive for antimicrobial activity. However, when compared to leucocin A it was found that the leucocin A precursor inhibits *Listeria* to a lesser degree than leucocin A. The relative bactericidal activities of leucocin A and pre-leucocin A was calculated at 6.0×10^5 AU and 4.0×10^5 AU. Taking this into consideration, it was estimated that the leucocin A precursor is ~66.667 % active as mature leucocin A. Hence the presence of a leader peptide does not have an influence on leucocin A antimicrobial activity.

LIST OF FIGURES

LIST OF TABLES

LIST OF SYMBOLS AND ABBREVIATIONS

ABC-transporter: ATP binding cassette transporter

amp: ampicillin

ATP: adenosine triphosphate

ATZ: anilinothiazolinone

AU: activity units

BCA: bicinchoninic acid

bp: base pairs

BSA: Bovine Serum Albumin

DIG-ddUTP: Digoxigenin dideoxyuridine-triphosphate

Dha: dehydroalanine

Dhb: dehydrobutyrine

DPC: dodecyl phosphocholine

EDTA: Ethylenediamine tetraacetic acid

HPK: histidine protein kinase

HPLC: high performance liquid chromatography

IF: induction factor

IPTG: Isopropyl-β-D-thiogalactopyranoside

KAc: Potassium acetate

kDa: kilodaltons

LAB: lactic acid bacteria

Lan: lanthionine

LB: Luria Bertani

Lca: leucocin

malE: maltose E gene

MBP: maltose binding protein

MBP-LcaA: maltose binding protein – leucocin A fusion protein

MBP-preLcaA: maltose binding protein – pre-leucocin A fusion protein

MeLan: β –methyllanthionine

Mes: mesentericin

MRS: De Man Rogosa Sharpe

OD_{600}: optical density at 600 nm

PCR: polymerase chain reaction

PITC: phenylisothiocyanate

pLcaA: recombinant pMAL.c2 containing the leucocin A gene within its polylinker

pPreLcaA: recombinant pMAL.c2 containing the pre-leucocin A gene within its polylinker

PMF: proton motive force

PTC: phenylthiocarbamyl

PTH: phenylthiohydantoin

PVDF: Polyvinylidene fluoride

RMS: Root mean square

RP-HPLC: reverse phase high performance liquid chromatography

RR: response regulator

SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis

Sec: signal sequence

Spp.: species

SSC: Salinesodium citrate

subsp.: sub-species

TAE: Tris acetate buffer

TFA: trifluoroacetic acid

TFE: trifluoroethanol

Tm: melting temperature

TSA Tryptone soy broth

TSB Tryptone soy agar

UV: ultra violet

v/v: volume per volume

w/v: weight per volume

X-gal: X-galactosidase

ACKNOWLEDGMENTS

I would like to express my gratitude to the following persons and organizations for their contributions to this thesis:

Dr. M. Beukes for his assistance during the year.

To members of the Microbial Genetics Research Group: Abhita Jugdave, Prisca Mbele, Rizwana Mia, Aletia Naidoo, Clint Schleidrecht, and Melisha Sukku. Thank you all, for being such a great team of researchers to work with, and for words of encouragement and support during the trying times.

To members of the academic and support staff: Dr Alain Boulange, Mrs Colleen Mycock, Megan Brunkhorst, Denzil Lakay, Robin Hillerbrand, Charmaine Ahrens, Colleen Le Roux, Mrs Agnes Zondi, and Mr Goodman Zondi. Thank you all, for being so approachable in times of need. It has been a pleasure working beside you.

My fellow postgraduate students and most of my friends: Akshanee, Sheryllyn, Viksha, Yashini, Abitha, Melisha, Darshan, Prisca, Parushka, and Vishen. Thank you for all the good and crazy memories, for being there in times of need, and most of all, for all the laughter. You guys ROCK!

To my mom, Sharm, for her endless support, encouragement and love.

To my aunt, Wendy, and brother, Saiyush. Thank you for the support.

To Ms. Prisca Mbele for supplying the partilly purified leucocin A sample, which was used as a control in various tests.

To Mr. R. Rajagopaul for his valued guidance.

To the National Research Foundation for financial assistance.

CHAPTER ONE

LITERATURE REVIEW

1.1 Introduction to Antimicrobial Peptides or Bacteriocins

The control of detrimental microorganisms in our environment has received scientific recognition since the era of Louis Pasteur and Robert Koch and even more so, now in the 21st century. The discovery of penicillin by Alexander Fleming in 1929, allowed for the use of therapeutic antibiotics by the medical and veterinary communities to combat specific disease-causing organisms. However, therapeutic antibiotics are prohibited for use in foods, which did not benefit food safety and preservation (Chen & Hoover, 2003). Over the past decade, lactic acid bacteria (LAB) have become economically important, as they have been shown to be capable of inhibiting the growth of a wide variety of food spoilage organisms (Jack *et al.*, 1995). Bacteriocins are proteinaceous inhibitory agents produced by some LAB (Diep & Nes, 2002). The utilization of these antagonistic additives with preservative or antimicrobial properties has since become a trademark approach in the food industry (Soomro *et al.*, 2002).

Bacteriocins, as defined by Tagg *et al.* in 1976, are a heterogeneous group of antimicrobial proteins or peptides produced by a number of different bacteria, whose activity is generally directed towards species that are closely related or share the same ecological niche (Stiles, 1993). Owing to their significance, these ribosomally synthesized proteins/peptides have been characterized both on a genetic and biochemical basis. This has led to the classification of these bacteriocins into various classes. Of all the classes, class I (lantibiotics) and class II (small heat-stable non-lanthionine containing peptides) bacteriocins have been the most documented (Ennahar *et al.*, 2000). Bacteriocins belonging to class II are further divided into subclasses with class IIa bacteriocins forming the majority (Diep & Nes, 2002). Class IIa bacteriocins are classified on the basis of their strong amino acid sequence similarity, and their activity directed towards *Listeria monocytogenes* (antilisterial activity) (Quadri *et al.*, 1994).

A major topic in bacteriocin research is the mode of action which class IIa peptides adopt. It has been postulated that these peptides act by causing a dissipation in the proton motive force of the target organisms which leads to their lysis (Hechard and Sahl, 2002). Various studies have shown that the mode of action of class IIa bacteriocins is directly related to their protein structure. The N-terminal conserved consensus sequence is believed to play a

role in a recognition step during the mode of action, while the C-terminal amphiphilic α-helix is vital for membrane perturbation (Miller *et al.*, 1998[a]).

The biosynthesis of class IIa bacteriocins is associated with one to three operon-like structures located on plasmids (Chen & Hoover, 2003). These operons consist of genes encoding bacteriocin production and immunity proteins, which confer protection to the producer strain. Also present are genes, which encode for a putative ABC-transporter responsible for the processing and extracellular translocation of the bacteriocin (Jack *et al.*, 1995; Yarmus *et al.*, 2000). Class IIa bacteriocins are synthesized as pre-peptides consisting of an N-terminal extension (leader peptide), and the active peptide or pro-peptide. These sequences are separated by two glycine residues which are cleaved by the ABC-transporter during processing (Aucher *et al.*, 2005). The specific function of the leader peptide and its effect on activity has not yet been fully elucidated. Studies have shown that the leader peptide is vital for the maturation and secretion of the bacteriocin during synthesis (Jack *et al.*, 1995). Another suggestion is that the N-terminal extension could be involved in the loss of activity of the bacteriocin during processing (Quadri *et al.*, 1997; Aucher *et al.*, 2005). This suggestion seems plausible as inactivation of the pro-peptide acts by protecting the host producer from the action of its own bacteriocin. Recent findings have shown that certain class IIa pre-peptides are active. However, the activity of the prep-peptide is lower in intensity than the activity of the mature bacteriocin (Quadri *et al.*, 1997).

In the present study, the primary objectives were to determine if the leader peptide has an influence on the antimicrobial activity of leucocin A; and to compare the antimicrobial activity of leucocin A with its precursor. Using techniques such as molecular cloning, recombinant pMAL.c2 expression vectors, containing the leucocin A and pre-leucocin A genes were constructed. Both leucocin A and pre-leucocin A, were produced and purified as MBP fusion constructs using affinity chromatography. The MBP affinity tag was removed by Factor Xa cleavage and the resulting recombinant peptides (leucocin A and pre-leucocin A), were tested for activity directed against *Listeria monocytogenes*. In this way the inhibition of *L. monocytogenes* by both leucocin A and its precursor was tested.

1.2 Classification of Bacteriocins Produced by Lactic Acid Bacteria

Several classification criteria have been used to group the different bacteriocins produced by LAB. Some systems take into consideration the presence of disulfide and monosulfide bonds as the basis for their classification, while others are based on the molecular mass, thermostability, enzymatic sensitivity, mode of action, and presence of post-translationally modified amino acids (Oscariz & Pisabarro, 2001). The Klaenhammer, (1993) classification divides LAB bacteriocins into two major classes i.e. class I and class II based on the modification that takes place after the synthesis of the bacteriocin (van Reenen *et al.*, 2003). Class I bacteriocins are post translationally modified and are also referred to as lantibiotics as they consist of unusual amino acid residues such as lanthionine (McAuliffe, Ross & Hill, 2001). Bacteriocins belonging to class II are further divided into sub-classes based on their molecular mass, sequence similarity and thermostability apart from others. The classification of LAB bacteriocins presently used is outlined in figure 1.1 (Jack *et al.*, 1995).

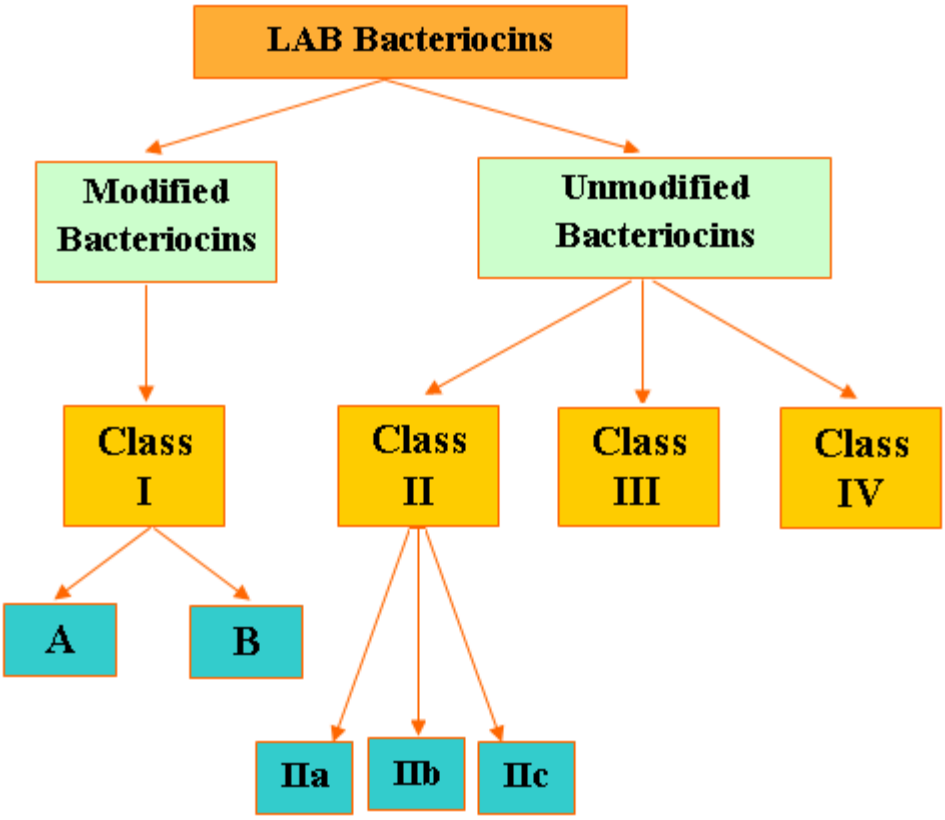


Figure 1.1. The Klaenhammer method used to classify bacteriocins. Lactic acid bacteriocins can be first classified into modified and unmodified bacteriocins according to the post-translational modification that occurs after synthesis. Further sub-divisions into various classes are based on various biochemical and genetic properties (Jack *et al.*, 1995).

1.2.1 Modified bacteriocins: class I

Bacteriocins belonging to the class I group are termed Lantibiotics. This is due to the presence of a high proportion of unusual amino acids, synthesized by post-translational side-chain modifications of ribosomally produced precursor peptides (Bauer & Dicks, 2005). The most prominent modifications include the thioether amino acids, lanthionine (Lan) and β -methyllanthionine (MeLan), and a number of dehydrated amino acids, such as the α, β -unsaturated amino acids, dehydroalanine (Dha) and dehydrobutyrine (Dhb) (McAuliffe, Ross & Hill, 2001). This group is further subdivided into type A and type B lantibiotics according to chemical structures and antimicrobial activities (Chem & Hoover, 2003).

The best-characterized Type A lantibiotic is nisin, produced by *Lactococcus lactis* subsp *lactis*, which has been approved for use as a preservative in foods in over 45 countries (Hastings *et al.*, 1991). Nisin is a pentacyclic peptide consisting of 34 amino acid residues of which 13 have been post-translationally modified (Bauer & Dicks, 2005). Two naturally occurring nisin variants, nisin A and nisin Z, are produced by *lactococci* (Breukink & de Kruij, 1999). Nisin A differs from nisin Z in a single amino acid residue at position 27, being histidine in nisin A, and asparagine in nisin Z. Nisin is active against a wide range of Gram-positive bacteria and is regarded as a pore forming bacteriocin. It is also active against gram-negative bacteria such as *Escherichia coli* and *Salmonella* spp., if the outer membranes of these targets are damaged (Bauer & Dicks, 2005).

Type B lantibiotics are smaller, globular peptides of approximately 20 amino acids and have a negative or no net charge (Hoffman *et al.*, 2001). Their antimicrobial activity is related to the inhibition of specific enzymes within the target cell (Ennahar *et al.*, 1999). They are further divided into the cinnamycin and mersacidine subtypes, and exert their activity by binding to specific membrane lipids. The cinnamycin-like peptides inhibit phospolipases by forming a complex with phosphatidylethanolamide, which interferes with several membrane transport systems (Bauer & Dicks, 2005).

1.2.2 Unmodified bacteriocins: class II

Bacteriocins belonging to this group have been classified mainly on the basis that they do not undergo post-translational modification. Moll *et al.* (1999) states that class II bacteriocins are small, heat stable, non-lanthionine-containing peptides, varying between 30-60 amino acid residues in length (<10-kDa). Similar to nisin, class II bacteriocins have also proved valuable for use in food preservation. These bacteriocins have a narrow inhibition spectrum, limited to species or strains related to the producer. Accordingly, class II bacteriocins are mainly active against low G-C Gram-positive bacteria such as other lactic acid bacteria, *Listeria*, *Enterococcu*s and *Clostridium* (Hechard & Sahl, 2002). It has been hypothesized that the activity of these positively charged peptides are characterized by their interaction with the negatively charged cell wall of the target strain, resulting in an electrostatic interaction leading to pore formation and lysis (Papthanasopoulos *et al.*, 1998). An abundant amount of research has been carried out on class II bacteriocins, which have led to the subgrouping of this class into sub-classes IIa, IIb, and IIc (van Belkum & Stiles, 2000).

A. Class IIa bacteriocins

The class IIa is the largest and most extensively studied subgroup of class II bacteriocins that are especially strong inhibitors of the food borne pathogen, *Listeria monocytogenes* (Ennahar *et al.*, 2000). Among the bacteriocins identified, more than 20 share 34 to 80.5% sequence identity in their primary structure (Hechard & Sahl, 2002). Leucocin A and mesenterocin Y105 are the exception as they vary only in the amino acids at positions 22 and 26. Furthermore, independently investigated class IIa bacteriocins have been shown to be identical to others. Among these, are include carnobacteriocin BM1 and piscicocin B1b that have identical amino acid sequences (Jack *et al.*, 1995). Class IIa bacteriocins are characterized primarily by a conserved N-terminal consensus sequence. This has been designated as the KYYGNGV consensus sequence (Diep & Nes, 2002). Another common feature of class IIa bacteriocins is the presence of particular groups of amino acid residues. Class IIa bacteriocins have a high content of amino acid residues with ionizable side chains especially basic and non-polar amino acids (Jack *et al.*, 1995; Hechard & Sahl, 2002).

B. Class IIb bacteriocins

Bacteriocins belonging to this sub-class are termed two-peptide bacteriocins and range from 25 to 62 amino acids in size (Diep & Nes, 2002). Many of these bacteriocins contain a high degree of sequence identity in the N-terminal amino acid residues, whereas the C-terminal amino acids are relatively diverse. This characteristic is indicative of class IIa bacteriocins (van Belkum & Stiles, 2000). However, the main feature, which allows class IIb bacteriocins being distinguished from the other sub-classes, is their non-antilisterial activity.

The sub-class IIb includes bacteriocins whose activity depends on the complementary action of the two peptides (Hechard & Sahl, 2002). Thus, maximum activity requires the action of two different peptides. Individual peptides of these bacteriocins often show a defined amphipathic α-helical structure on a helical wheel, which suggests these bacteriocins are pore forming (Diep & Nes, 2002).

Plantaracin S is an example of a class IIb bacteriocin produced by *Lactococcus plantarum* LPCO10, which is isolated from a green-olive fermentation. This bacteriocin was initially believed to belong to class IV; however, it was soon identified that this bacteriocin is composed of two peptides, which allowed plantaracin S to be classified as a class IIb bacteriocins (Stephens *et al.,* 1997).

C. Class IIc bacteriocins

This subclass includes all the bacteriocins that do not fall into groups IIa and IIb. They are termed thiolbiotics or cystibiotics and contain a disulfide bridge that spans the N- and C-sections of the molecule (van Belkum & Stiles, 2000). They are thiol-activated bacteriocins owing to the fact that activation of the bacteriocin is brought upon by the reduction of cysteine residue present in bacteriocin amino acid chain, usually at position 24 (Oscariz & Pisabarro, 2001). Class IIc lack the YGNGVXC motif of class IIa and include bacteriocins, carnobacteriocin A and enterocin B (van Belkum & Stiles, 2000).

1.2.3 Class III bacteriocins

This group houses bacteriocins with a molecular mass larger than 30 kDa, which are not well characterized (Oscariz & Pisabarro, 2001). Furthermore, these bacteriocins are heat labile and are of less interest in food preservation (van Belkum & Stiles, 2000). Class III bacteriocins are mainly produced by bacteria of the genus *Lactobacillus*. Members of this group are heveticin J that is produced by *L. helveticus* 481, and lacticin B, produced by *L. acidophilis* (Rodriguez *et al.*, 2003). However, of the few class III bacteriocins that have been identified, it was noticed that their mode of action and physical properties are different from that of class II and I. This has raised the question of whether they should be termed as bacteriocins (Diep & Nes, 2002).

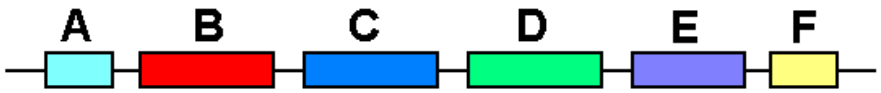
1.2.4 Class IV bacteriocins

Class IV bacteriocins consist of either glycoproteins (lactocin 27), or lipoprotein (lacstrepcins) that require non-protein moieties for their activity (Nissen-Meyer *et al.*, 1992; Oscariz & Pisabarro, 2001). However, bacteriocins in this class have not been characterized adequately at the biochemical level to the extent that the definition of this class requires additional descriptive information (Chen & Hoover, 2003).

1.3 Genetic Organization and Biosynthesis of Class IIa Bacteriocins

Bacteriocins are ribosomally synthesized as pre-bacteriocins that contain a N-terminal extension that is cleaved to form the active peptide. For class IIa bacteriocins, the N-terminal extension is cleaved by a serine protease that forms part of the N-terminal domain of the ABC transporter protein (Jack *et al.*, 1995; van Belkum & Stiles 2000). However, in order to fully understand the biosynthesis of class IIa bacteriocins, the genetic elements responsible for bacteriocin production require investigation.

The genes encoding bacteriocin production and immunity are usually organized in operon clusters. These operon clusters can be located on chromosomes, transposons, or on plasmids as in the case of class IIa bacteriocins (Chen & Hoover, 2000). Information on the genetic requirements and organization for the expression of class IIa bacteriocins, is still scant. However, comparative analysis on this organization revealed that the structural gene for class IIa bacteriocins and its surrounding regions, are confined to one to three operon-like structures, which is outlined in figure 1.2. These operons may be divergently transcribed and are involved in the bacteriocin production and extracellular translocation, the immunity of the producers, and in several cases, the regulation of bacteriocin synthesis (Ennahar *et al.*, 2000).



A - Promotor
B - Gene for bacteriocin production
C - Immunity gene which protects the producer organism against the bacteriocin
D - ATP-binding cassette also known as an ABC- transporter which is involved in the extracellular transport of the bacteriocin.
E - Three component signal transduction auto-regulatory cassette involved in regulation of the operon
F - Terminator

Figure 1.2. A simplified version for the gene organization encoding class IIa bacteriocins. The various open reading frames and their functions are also listed (adapted from Ennahar *et al.*, 2000).

Class IIa bacteriocins are produced by LAB, such as *Lactobacillus* spp., *Pediococcus* spp., *Leuconostoc* spp., and *Carnobacterium* spp. (Hastings *et al.*, 1991). Comparative overviews of the genetic organization of studied DNA fragments encoding class IIa bacteriocins revealed considerable amounts of genetic diversity among bacteriocin biosynthesis.From figure 1.3, it can be seen that in some cases, two genes have been identified to be necessary for bacteriocin production as in the case of curvacin A and enterocin P. However, the minimum requirements for class IIa bacteriocin production consist of the structural gene and a cognate immunity gene. This is apparent for leucocin A, mesentericin Y105, Pediocin AcH, and divercin V41 in figure 1.3 (van Belkum and Stiles, 2000).

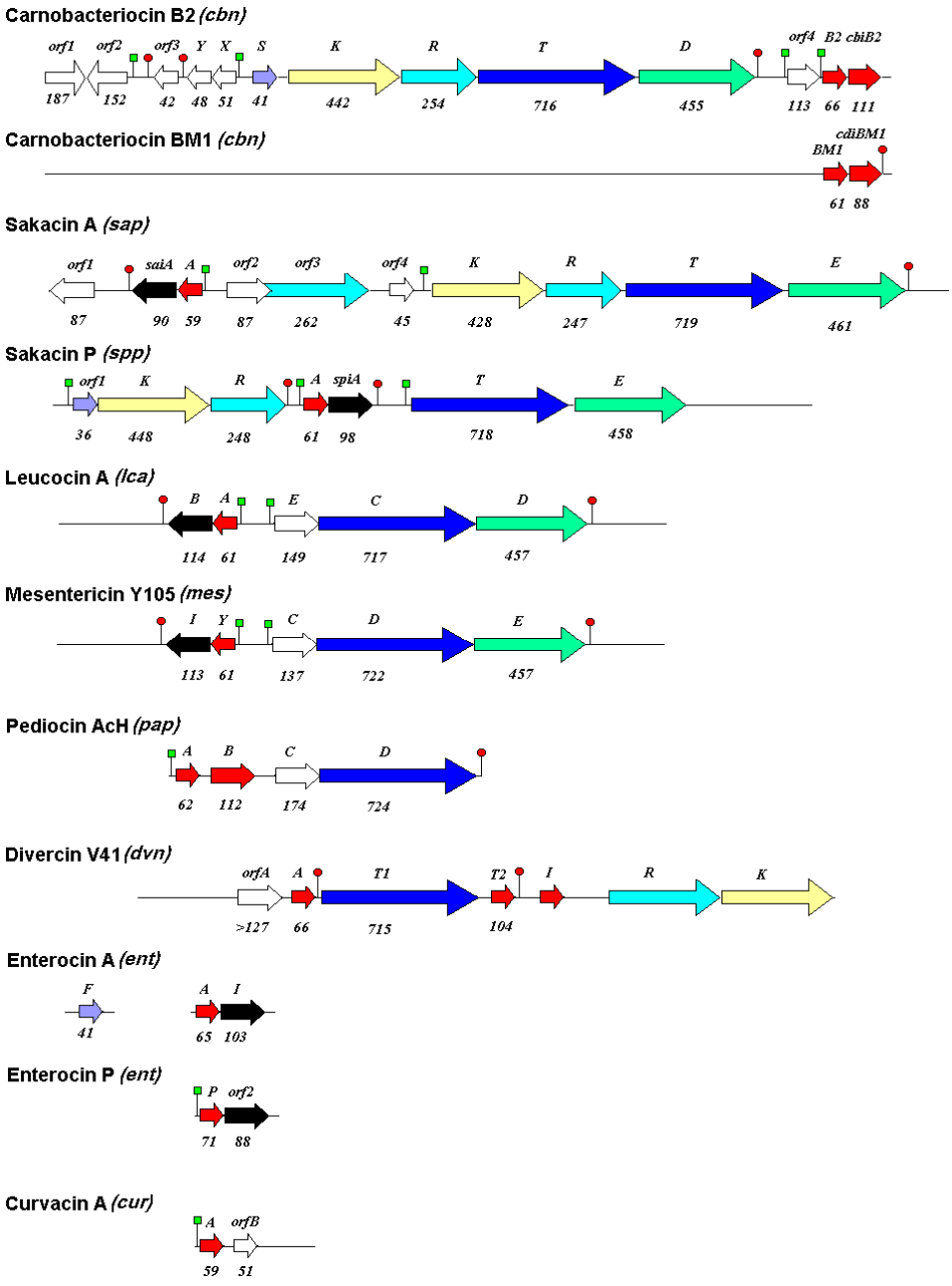


Figure 1.3. Arrangement of gene clusters associated with the production and immunity of several class IIa bacteriocins. Open reading frames are represented by arrows with the corresponding colors: bacteriocin pre-peptide (red); immunity genes (black); ABC-transporter (dark blue); regulatory genes (light blue, yellow, purple); product with unidentified function (white). The number of amino acid residues encoded by each ORF is indicated below each arrow. The size of the sequenced fragment is also indicated. Promoters are represented by green boxes, terminators (red lollipops), and overlapping sequences (overlapping arrows). With respect to carnobacteriocin B2 and BM1, the same ORFs exist (Ennahar *et al.*, 2000).

1.3.1 The bacteriocin structural gene

The bacteriocin structural gene encodes bacteriocins as pre-peptides (red arrowhead, figure 1.3), which contains an N-terminal extension that is cleaved to form the active peptides (van Belkum & Stiles, 2000; Ennahar *et al.*, 2000). One important feature of the majority of these leaders is the presence of two conserved glycine at positions –2 and –1 relative to the processing site, though this is not distinctive of the class IIa (Ennahar *et al.*, 2000). It is hypothesized that during the biosynthesis of class IIa bacteriocins the leader sequence may serve as a recognition signal for a *sec*- (signal sequence) independent ATP-binding cassette transporter (ABC-transporter), which cleaves at the Gly-Gly-1Xaa proteolytic processing site on the N-terminal extension. This is referred to as bacteriocin maturation and results in the formation of the active peptide (pro-peptide) (figure 1.4) (Allison *et al.*, 1995).

1.3.2 ATP - binding cassette transporter

The ATP - binding cassette transporter (ABC-transporter) is important for the activation of class IIa bacteriocins as well as their extracellular translocation following synthesis (dark blue arrowhead, figure 1.3). The ABC-transporter and accessory proteins are two membrane bound proteins that form a transport system within the membrane of the producer (Hastings *et al.*, 1991). Structural analysis of this complex revealed that its functions are similar to that of the ABC-transport system HlyB/HlyD of *Escherichia coli* responsible for the secretion of hemolysin A (O' Keeffe *et al.*, 1999).

Putative class IIa bacteriocin ABC-transporters are proteins ranging from 715 to 725 amino acids and display a high degree of homology at their N- and C-terminal regions. The C-terminus of this transporter contains a highly conserved ATP-binding domain (unique to ABC-transporters). The N-terminal region is a hydrophobic integral proteolytic membrane domain and can be distinguished by two conserved motifs, called the cysteine and the histidine motifs (C/H motifs). These motifs are responsible for recognition and cleavage of the GG-motif containing peptides (Dirix *et al.*, 2004). The proteolytic domain of the ABC-transporter described above binds to the pre-peptides leader sequence, which then triggers the ATP hydrolysis and subsequent conformational changes in the transporter. This results in the leader's removal by cleavage of the GG-motif by the C/H motifs and translocation of the

mature bacteriocin across the cytoplasmic membrane (Michiels *et al.*, 2001). Accessory proteins facilitate the membrane translocation and/or help in the processing of the leader peptide, and have been shown to be required for successful externalization of class IIa bacteriocins. These proteins consist of about 460 amino acids and contain large hydrophobic N- and C- termini (Ennahar *et al.*, 1999).

1.3.3 Producer immunity

Class IIa bacteriocins are co-expressed with cognate immunity proteins (black arrowhead, figure 1.3), which are hypothesized to be associated with the cytoplasmic membrane of the bacteriocin producer (Yarmus *et al.*, 2000). These proteins confer protection to the producer from the antimicrobial activity of its own bacteriocin (Kim *et al.*, 2005). However, in addition to protecting the producer strain, these immunity proteins appear to also provide partial protection against other class IIa bacteriocins. This was displayed in a study conducted by Eijsink *et al.* (1998) where strains producing class IIa bacteriocins displayed various degrees of resistance toward noncognate class IIa bacteriocins. This study concluded that certain genera of class IIa producing LAB generally possess one or more immunity genes. Furthermore, they stated that these genes may show various degrees of homology and may be expressed to various extents. These results could provide an explanation for the variation in bacteriocin sensitivity that is observed for closely related LAB strains.

Immunity proteins vary in length (51 to 154 amino acids), have a high pI, and often contain hydrophobic domains (van Belkum & Stiles, 2000). Furthermore, immunity proteins are cationic and are largely α-helical. The mode of action of the immunity protein was identified by a study conducted by Fimland *et al.* (2002[a]). It was identified that the C-terminal domain of the immunity protein acts by preventing an interaction between the bacteriocin and the producer's receptor, thereby inhibiting pore formation (Johnsen *et al.*, 2004). Observations obtained by Quadri *et al.* (1997), suggest that these proteins are free intracellular molecules and prevent the action of the bacteriocin at the membrane site indirectly via a membrane-bound receptor. However, this remains speculative and more research is required (Ennahar *et al.*, 2000).

1.3.4 Regulatory genes and bacteriocin biosynthesis

For transcriptionally regulated bacteriocins, additional open reading frames are present in the vicinity of the structural gene. These open reading frames encode a putative three-component signal-transduction auto-regulatory cassette, which consists of an induction factor and a two-component regulatory system (Ennahar *et al.*, 2000). These are indicated by light blue, yellow and purple arrowheads on figure 1.3. The two-component regulatory system comprises a membrane-bound histidine protein kinase that binds DNA resulting in the activation of the other regulatory genes (Michiels *et al.*, 2001). The induction factor is synthesized as a pre-peptide with a double-glycine-type leader sequence, which is cleaved during transport. This secreted induction factor is subsequently sensed by the dedicated two-component regulatory system, resulting in the induction of transcription of the operons involved in bacteriocin production (Michiels *et al.*, 2001). Figure 1.4 provides a schematic representation of the biosynthesis of class IIa bacteriocins and how specific genes regulate protein expression.

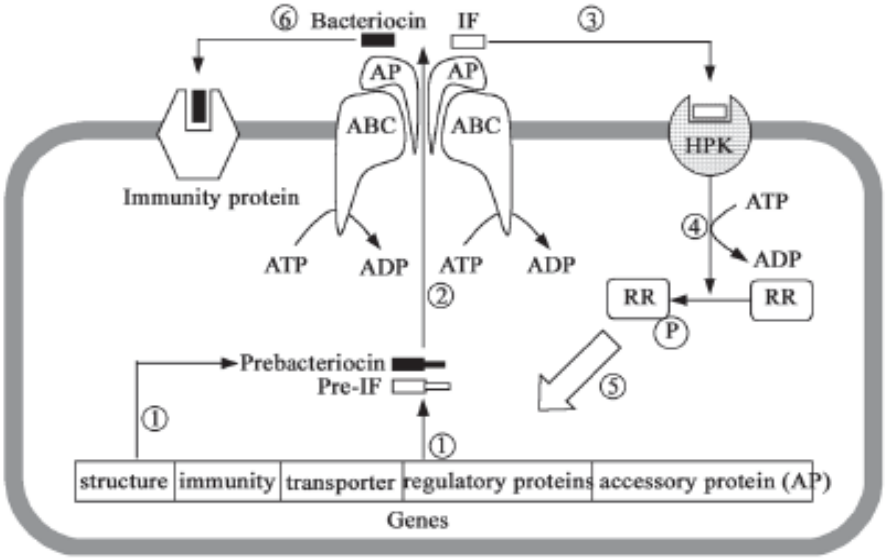


Figure 1.4. Schematic diagram of the biosynthesis of class IIa bacteriocins: (1) Formation of pre-peptide and pre- induction factor (IF); (2) The pre-peptide and pre-IF are processed and translocated by the ABC-transporter, resulting in the release of mature bacteriocin and IF; (3) Histidine protein kinase (HPK) senses the presence of IF and autophosphorylates; (4) The phosphoryl group (P) is subsequently transferred to the response regulator (RR); (5) RR activates transcription of the regulated genes; and (6) Producer immunity (Chen and Hoover, 2003).

1.4 Bacteriocins Produced by *Leuconostoc* Species

Leuconostocs are heterofermentative lactic acid bacteria, which play a major role in the fermentation of foodstuffs (Hemme & Scheunemann, 2005). They are traditionally used with other lactic acid bacteria to preserve food by natural or induced fermentations (Choi *et al.*, 1999). This genus comprises Gram-positive cocci that are claimed to be taxonomically and ecologically related to group N-streptococci (Stiles, 1993). Bergys manual of systematic bacteriology (1984) identified four species belonging to this genus: *L. mesenteroids spp.; L. paramesenteriodes spp.; L. lactis spp.; and L. oenos spp.*. However, since then, five new species have been identified, two of which are *L. gelidum* and *L. carnosum*, which are important in the lactic microflora of chilled-stored, vacuum packaged meats (Stiles, 1993).

Leuconostoc species display antimicrobial activity against other LAB and *Listeria* spp. (Hastings *et al.*, 1991). It was originally reported that the antimicrobial activity of *Leuconostoc* spp. against pathogenic and spoilage microorganisms was attributed to organic acids produced by these LAB. However, in 1984, Orberg and Sandine reported the production of bacteriocins by three strains of *Leuconostoc* (Stiles, 1993). This led to extensive studies being conducted on the bacteriocin production by *Leuconostoc* spp. Table 1.1 shows the various bacteriocins produced by the different strains of *Leuconostoc*.

Table 1.1. Bacteriocins produced by different *Leuconostoc* strains (Stiles, 1993).

Lactic acid bacteria	**Bacteriocin**
Leuconostoc mesenteroides	mesentericin Y105
Leuconostoc gelidum	leucocin A
Leuconostoc carnosum	carnosin 44A
Leuconostoc paramesenteroides	leuconocin S

Some of the *Leuconostoc* strains may sometimes even produce more than one type of bacteriocin. *Leuconostoc mesenteroides* subsp. *mesenteroides* FR52 is a good example of this scenario as this strain produces mesentericin 52A (which is identical to mesentericin Y105) mesentericin 52B, and leucocin C (Corbier *et al.*, 2001; Fimland *et al.*, 2002[b]). Furthermore, mesentericin 52B has been detected in culture extracts of *L. mesenteroides* Y105. Papthanasopoulos *et al*. (1998) reported that *L. mesenteroides* TA33a produces leucocin A-; B- and C-TA33a. This study found that the partial sequence of leucocin C-TA33a shares 80 to 78% homology with leucocin A UAL187-22, leucocin B-TA11a, and mesentericin Y105 (table 1.2). In addition, it was also found that leucocin C-TA33a appears to be similar to other *Leuconostoc* bacteriocins at the N-terminus, but more similar to other known class II bacteriocins at the C-terminus (Papthanasopoulos *et al.*, 1998). This high homology indicates the close relation within the different strains of the same species.

Table 1.2. Multiple alignment of the amino acid sequence of leucocin C-TA33a and other class IIa bacteriocins. Dashes represent gaps introduced to optimize the alignment, and consensus amino acids are highlighted (Papthanasopoulos *et al.*, 1998).

Bacteriocin	**Sequence**
Leucocin C-TA33a	--KNYGNG-VHCTKKGCSVDWGYAAT---NIANNSVMNGLTG---
Leucocin B-TA11a	--KYYGNG-VHCTKSGCSVNWGEAFS---AGVHRLANGGNGFW--
Leucocin A-UAL187-22	--KYYGNG-VHCTKSGCSVNWGEAFS---AGVHRLANGGNGFW--
Mesentericin Y105	--KYYGNGVVHCTKSGCSVNWGEAAS---AGIHRLANGGNGFW--

1.4.1 *Leuconostoc gelidum*

Leucocin A is a class IIa bacteriocin produced by *Leuconostoc gelidum* UAL187-22, which is isolated from vacuum packaged meat (Hastings *et al.*, 1991). This bacteriocin inhibits a wide spectrum of LAB, meat spoilage bacteria, and *Listeria monocytogenes*, a food-borne pathogen widely distributed in the environment (van Belkum & Stiles, 1995; Gaeng *et al.*, 2000).

Molecular characterization of the genes involved in bacteriocin production revealed that the genetic determinants are located on two of the three plasmids namely pLG 7.6 and pLG 9.2 (van Belkum & Stiles, 1995). Studies involving molecular cloning revealed that the genetic determinants for leucocin A production are arranged in an operon like structure arranged as two open reading frames (ORFs) (figure 1.5a) (Stiles, 1993). The red arrow on figure 1.5a represents the ORF encoding 61 amino acids and was identified as the leucocin structural gene. Van Belkum and Stiles (1995) determined that the second ORF (*lcaB*) encodes the immunity protein, which is responsible for conferring immunity to the producer organism. Molecular cloning by van Belkum and Stiles (1995) further confirmed the presence of additional open reading frames termed *lcaC*, *lcaD*, and *lcaE*. Homology comparisons of *lcaC* with other proteins showed distinctive similarities with secretory proteins that differ from those involved in the general signal sequence-dependent export pathway. *LcaC* contains a highly conserved ATP-binding domain in the C-terminus and several hydrophobic domains towards the N-terminus. Furthermore, a high degree of homology of *lcaC* with other ABC transporters including *mesD* and *pedD* was also observed. This data indicated that *lcaC* belongs to the family of ABC-transporters and is responsible for the extracellular translocation and activation of leucocin A (Fregeau-Gallagher *et al.*, 1997). *LcaD* was shown to be associated with bacteriocin production while the role of *lcaE* remains unclear (van Belkum & Stiles, 1995).

1.4.2 *Leuconostoc mesenteroides*

Leuconostoc mesenteroides subsp. *mesenteroides* is used industrially to produce dextrans and is the most common species isolated from fermented feed and food products. In addition, its recognized antagonistic or synergistic properties in mixed microflora populations have led to studies on its importance in some starter cultures (Moschetti *et al.*, 2000).

Mesentericin Y105 is a 37 amino acid class IIa bacteriocin produced by *Leuconostoc mesenteroides* Y105 (Morisset *et al.*, 2004). The structural gene encoding mesentericin Y105 designated *mesY* is located on a 35-kb plasmid (pHY30). All genes responsible for mesentericin Y105 production are organized in an operon like fashion constituting five ORFs (figure 1.5b). Each cluster, however, formed a separate operon that was surprisingly transcribed in a different and opposite direction from the other (Fremaux *et al*., 1995). The immunity gene (*mesI*) is situated directly downstream from *mesY.* A unique feature of these gene clusters was the presence of a stem loop structure, downstream from *mesI*. This structure is believed to be the terminator flanking *mesI* gene (Morisset *et al.*, 2004). Three additional ORFs have also been located and have been termed *mesC*, *mesD* and *mesE*. *MesD* resembled atypical ATP-dependent transporter, while *mesE* encodes for the accessory protein that acts as a membrane anchor and assists the transporter protein. The function of *mesC* however, remains unknown (Fremaux *et al.,* 1995).

A large amount of studies have been conducted on the genes encoding bacteriocins, mesentericin Y105 and leucocin A (Morisset *et al.*, 2004). These revealed the presence of sequence homology between *mesI* and *lcaB*. Additional homology was found between *mesD* and the gene encoding an ATP-dependent transporter for leucocin A (*lcaC)* (van Belkum & Stiles, 1995). This raised questions about the similarities existing between mesentericin Y105 and leucocin-A UAL187 both on their antimicrobial activities and structure.

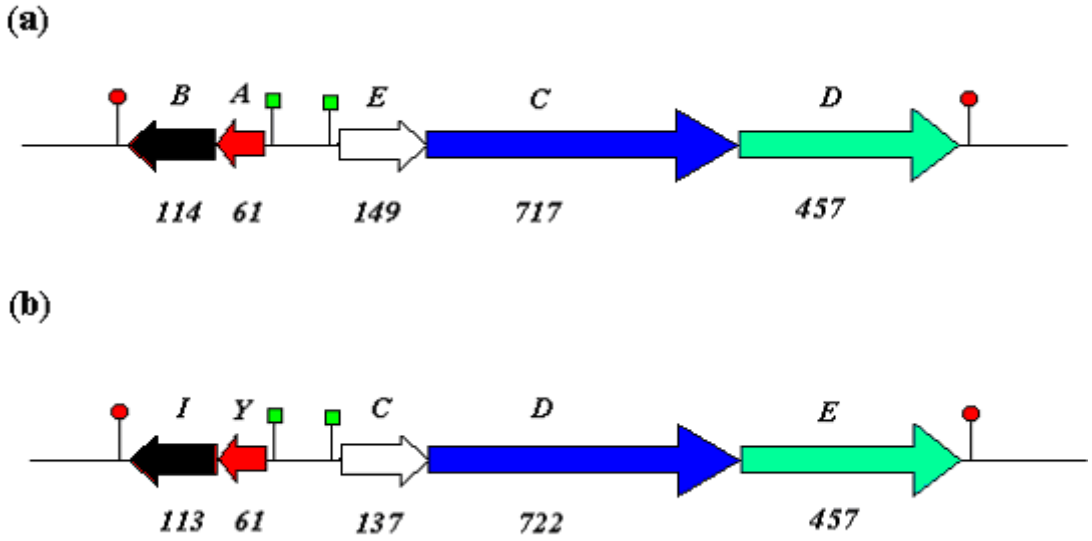


Figure 1.5. Organization of the gene clusters involved in the production and immunity of (a) leucocin A and (b) mesentericin Y105. Open reading frames are represented by arrows with the corresponding colors: bacteriocin pre-peptide (red), immunity genes (black), ABC-transporter (dark blue), product with unidentified function (white). The number of amino acid residues encoded by each ORF is indicated below each arrow. Promoters are represented by green boxes and terminators by red lollipops. With respect to carnobacteriocin B2 and BM1, the same ORFs exist (Ennahar *et al.*, 2000).

1.5 Structure of Class IIa Bacteriocins

1.5.1 Pre-bacteriocin leader sequences

The first pre-peptide to be isolated from the producing cell was pre-Pep5, a lantibiotic produced by *Staphylococcus epidermidis* 5 (Weil *et al.*, 1990). Since then, additional pre-peptides have been isolated, particularly from class IIa bacteriocins. The most abundant double-glycine (GG)-type extensions, characterized by a glycine doublet at the C-terminal end, were found in class II, as well as in some class I LAB pre-bacteriocins (Aucher *et al.*, 2005). The leader peptides of class IIa bacteriocins have between 14 to 30 amino acid residues and are characterized by the LSXKEL/MXXI/VXGG consensus sequence at positions -12 to -1 (van Belkum & Stiles, 2000). The primary structure consists primarily of hydrophobic and hydrophilic residues while secondary structure predictions of the double-glycine-type leader peptides indicate an α-helical structure (Aucher *et al.*, 2005). A further characteristic is the high degrees of homology between leaders of similar bacteriocins from the same species. This suggests that within the same species, the leader peptide amino acid sequences may be particularly highly conserved (table 1.3) (Jack *et al.*, 1995).

Studies involving the leader peptides of class II bacteriocins are scant and as a result the exact function(s) of these leaders have not yet been determined (Ennahar *et al.*, 2000). However according to Aucher *et al.* (2005), the possible functions include stabilizing the pre-peptide during translation, keeping the molecule biologically inactive thereby preventing producer cell lysis, maintaining the specific conformation of the pro-peptides (active bacteriocin) during processing, and assisting with the translocation of the pre-peptides by specific transport systems.

Table 1.3. Pre-bacteriocin leader sequences class IIa bacteriocins. Amino acids in red represent the glycine doublet involved in the processing of the bacteriocin (Aucher *et al.*, 2005).

Bacteriocin	Leader peptide sequence
Pediocin AcH/pediocin PA-1	MKKIEKLTEKEMANII GG
Sakacin674/sakacin P	MEKFIELSLKEVTAIT GG
Leucocin A	MMNMKPTESYEQLDNSALEQVV GG
Sakacin A/ curvacin A	MNNVKELSMTELQTIT GG
Lactacin F	KKQFNYLSHKDLAVVV GG
Carnobacteriocin A/ piscicolin 61	MNNVKELSIKEMQQVT GG
Carnobacteriocin B2	MNSVKELNVKEMKQLH GG
Lactococcin A	MKNQLNFNIVSDEELSEAN GG
Lactococcin B	MKNQLNFNIVSDEELAEVN GG
Lactococcin M	MKNQLNFEILSDEELQGIN GG

1.5.2 Pro-bacteriocin domain and the structure of active bacteriocins

The Class IIa bacteriocins share an overall amino acid sequence identity of between 40 and over 70% at the N-terminal domain of the active peptide. Table 1.4 represents sequence alignment of the N-terminal regions, which revealed the presence of a YGNGVXaaC consensus motif (Ramnath *et al.,* 2000; Gutierrez *et al.*, 2005). However, with the emergence of new bacteriocins such as sakacin A, it is seen that they contain an altered N-terminal sequence and thereby, share an incomplete consensus motif with the rest of class IIa bacteriocins. A similar case is represented by acidocin A, in which the YGNGVXaaC motif is split by a threonine residue, resulting in a YGTNGVXaaC N-terminal motif. For these reasons, the motif YGNG is considered as the "real" consensus motif (Ennahar *et al.*, 2000).

Table 1.4. Sequence alignment of class IIa bacteriocins, on the basis of the N-terminal YGNGV consensus motif, and of bacteriocin 31 and acidocin A. The red areas highlight residues conserved in at least 10 of the sequences shown. Blue areas highlight C-terminal residues conserved in at least two sequences (Ennahar *et al.,* 2000).

Bacteriocin	Amino acid sequence
Leucocin A	KYYGNGVHCTKSGCSVNWGEAFSAGVHRLANGGNGFW
MesentericinY105	KYYGNGVHCTKSGCSVNWGEAASAGIHRLANGGNGFW
Mundticin	KYYGNGVSCNKKGCSVDWGKAIGIIGNNSAANLATGGAAGWSK
Piscicolin 126	KYYGNGVSCNKNGCTVDWSKAIGIIGNNAAANLTTGGAAGWNKG
Bavaricin A	KYYGNGVHCGKHSCTVDWGTAIGNIGNNAAANXATGXNAGG
Sakacin P	KYYGNGVHCGKHSCTVDWGTAIGNIGNNAAANWATGGNAGWNK
Pediocin PA-1	KYYGNGVTCGKHSCSVDWGKATTCIINNGAMAWATGGHQGNHKC
Bavaricin MN	TKYYGNGVYCNSKKCWVDWGQAAGGIGQTVVXGWLGGAIPGK
Divercin V41	TKYYGNGVYCNSKKCWVDWGQASGCIGQTVVGGWLGGAIPGKC
Enterocin A	TTHSGKYYGNGVYCTKNKCTVDWAKATTCIAGSIGGFLGGAIPGKC
Enterocin P	ATRSYGNGVYCNNSKCWVNWGEAKENIAGIVISGGWASGLAGMGH
Carnobacteriocin BM1	AISYGNGVYCNKEKCWVNKAENKQAITGIVIGGWASSLAGMGH
Sakacin A	ARSYGNGVYCNNKKCWVNRGEATQSIIGGMISGWASGLAGM
CarnobacteriocinB2	VNYYGNGVSCSKTKCSVNWGQAGQERYTAGINSFVSGVASGAGSIGRRP
Bacteriocin 31	ATYYGNGLYCNKQKCWVDWNKASREIGKIIVNGWVQHGPWAPR
Acidocin A	KTYYGTNGVHCTKRSLWGKVRLKNVIPG……

The C-terminal half moiety of class IIa bacteriocins is characterized by large structural diversity as seen in table 1.4. It is hypothesized by Guyonnet *et al.* (2000), that this diversity aids in host specificity. However, as new members of this class emerge, the degree of homology at the C-terminus increases, suggesting that this sub-class may be defined based on C-terminal sequence similarity (Ennahar *et al.*, 2000). Examples of this scenario are bavaricin MN, divercin V41, and enterocin A (table 1.4), which all share high sequence similarity at their C-terminus.

Class IIa bacteriocins have also been analyzed on the basis of their secondary and tertiary structures. An important characteristic of class IIa bacteriocins is the presence of four cysteine residues that form two disulfide bridges, one in the N-terminal half and another in the C-terminal half of the molecule (van Belkum & Stiles, 2000). For this reason, class IIa bacteriocins are termed cystibiotics (Chen & Hoover, 2000). The N-terminal cysteine residues are present in conserved positions (table 1.4), and consequently, the disulfide bridge which forms a six-membered ring over these two residues is well conserved in all class IIa bacteriocins (Ennahar *et al.*, 2000). The N-terminus of class IIa bacteriocins is believed to contain β-sheets maintained in a β-hairpin conformation that is stabilized by the N-terminal disulfide bridge. This conformation allows for the amphiphilic characteristic in the N-terminal region. The C-terminal domain is characterized by an amphiphilic α-helix which is vital for antimicrobial activity (Diep & Nes, 2002).

1.5.3 Leucocin A

Leucocin A, an anti-listerial bacteriocin produced by *Leuconostoc gelidum* UAL187-22 was the focus of this study. The *lcaA* gene encodes a 61 amino acid peptide identified as LeuA with a 24-residue N-terminal extension that is essential for secretion by an ABC-transporter (van Belkum *et al.*, 1998) (figure 1.6). Leucocin A is a cationic peptide of 3933.03 Da, while the pre-peptide has a molecular weight of 6587.71 Da (Fregeau-Gallagher *et al.*, 1997).

MMNMKPTESYEQLDNSALEQVV G G KYYGNGVHCTKSGCSVNWGEAFSAGV HRLANGGNGFW

Figure 1.6. Amino acid sequence of pre-leucocin A, a 61 amino acid bacteriocin produced by *Leuconostoc gelidum* UAL187-22. Amino acid residues in blue represent the 24-residue N-terminal extension while the mature form of leucocin A is depicted in orange. The glycine doublet, which is essential for the cleavage of the leader sequence from the mature peptide, is highlighted in red.

Very few studies have reported the three dimensional structure of leucocin A and the relevance of structure to the peptides mode of action. Leucocin A exists as a random coil in water but adopts a defined conformation consisting of an α-helix and 3 β-sheets in a hydrophobic environment (Henkel *et al.*, 1992). Fregeau-Gallagher *et al.* (1997) determined the structure of leucocin A in trifluoroethanol and dodecylphosphocholine micelles using NMR spectroscopy. It was reported that residues 17-31 assumes an amphiphilic α-helix conformation while a three-strand antiparallel β-sheet domain is present for residues 2-16, anchored by the disulfide bridge. The overall three dimensional structure is shown in figure 1.7.

Using the results obtained, Fregeau-Gallagher *et al.* (1997) then determined the importance of each structure on the activity of leucocin A. It was found that the amphipathic α-helix is likely to be very important for biological activity, possibly by interaction with a lipophilic region on the receptor protein. Leucocin A contains specific amino acids, which are in close proximity on the hydrophobic side of the helix and are thought to be a receptor binding site. It was concluded that the C-terminal α-helix determines target cell specificity through receptor binding, but the correct special arrangement of key amino acid side chains are essential. The N-terminal β-sheet is also essential for activity possibly by interacting with the target membrane. Leucocin A also contains one disulfide bridge, which is required for a defined protein structure. However, its exact role is unknown.

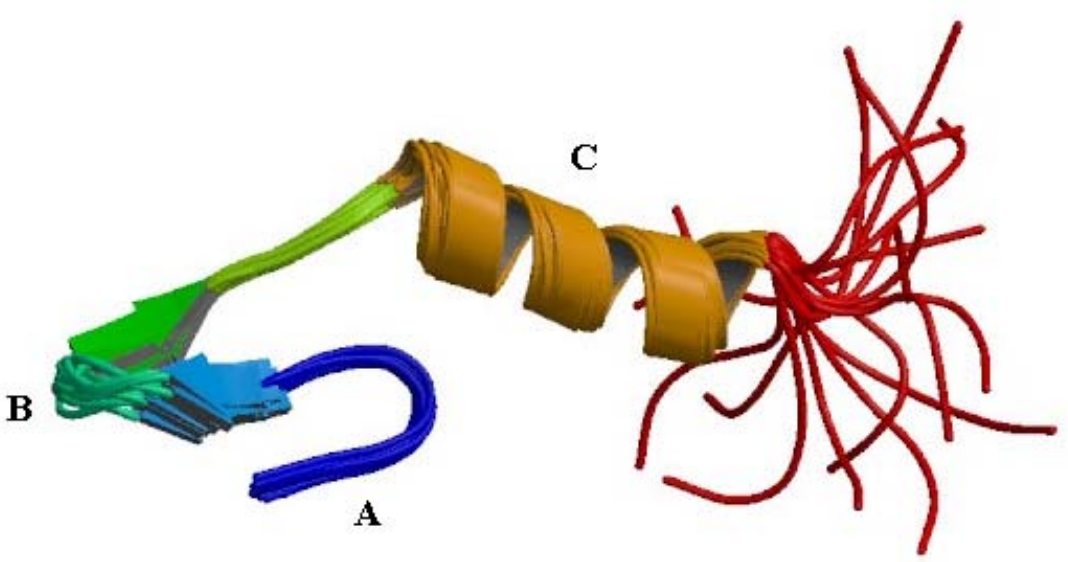


Figure 1.7. Tertiary structure of leucocin A depicting an amphiphilic α-helix region (C) and a three-strand antiparallel β-sheet domain anchored by the disulfide bridge (B). The N-terminal extension is alco represented (A) (Wang *et al.*, PDB ID: 1CW6).

1.6 Mode of Action of Class IIa Bacteriocins

The mode of action of class IIa bacteriocins has been extensively studied. The presence of amphiphilic segments, their water solubility and membrane binding activity, suggest that these bactericidal peptides act primarily by permabilizing the membranes of susceptible microorganisms, through the formation of poration complexes (Castano *et al.*, 2005). These complexes cause ionic imbalances and leakage of inorganic phosphate from the target strain (Hechard & Sahl, 2002). This action is refereed to as a disruption in the proton motive force (PMF) of the sensitive bacteria (Miller *et al.*, 1998[b]; Hechard & Sahl, 2002). The lethal activity of class IIa bacteriocins is seen by a depletion of intracellular adenosine triphosphate (ATP), and a blockage in the active transport of amino acids (Bruno & Montville, 1993). However, although postulated, the mechanism of pore formation has yet to be proven (van Belkum & Stiles, 2000).

As already mentioned, class IIa bacteriocins are cationic and contain a high degree of structural similarity at their hydrophobic N-termini. Furthermore, the N-terminus is believed to contain β-sheets maintained in a β-hairpin conformation that is stabilized by the N-terminal disulfide bridge. This conformation allows for the amphiphilic characteristic at the N-terminal region. It is suggested that the initial step during activity is the binding of the N-terminal consensus motif to a receptor protein with the membrane of the target cell. Thus, the N-terminus is often referred to as a hypothetical receptor-binding site (Oscariz & Pisabarro, 2001). An interaction with the membrane surface of the target cell is then mediated by an electrostatic interaction between the positively charged and polar residues (N-terminus) of class IIa bacteriocins with the anionic phospholipid head groups in the membrane (Figure 1.8a). As a subsequent step, hydrophobic interactions occur between the hydrophobic/amphiphilic domains within the C-terminal half of the bacteriocin and the lipid acyl chains. The amphiphilic α-helix located at the C-terminus is believed to be the transmembrane segment during pore formation in a sensitive cell membrane (Moll *et al.*, 1999). Bennik *et al.* (1998) states that these adopted α-helical conformations in the secondary structure of most class IIa bacteriocins are vital in membrane perturbation during the mode of action of class IIa bacteriocins (Figure 1.8a,b).

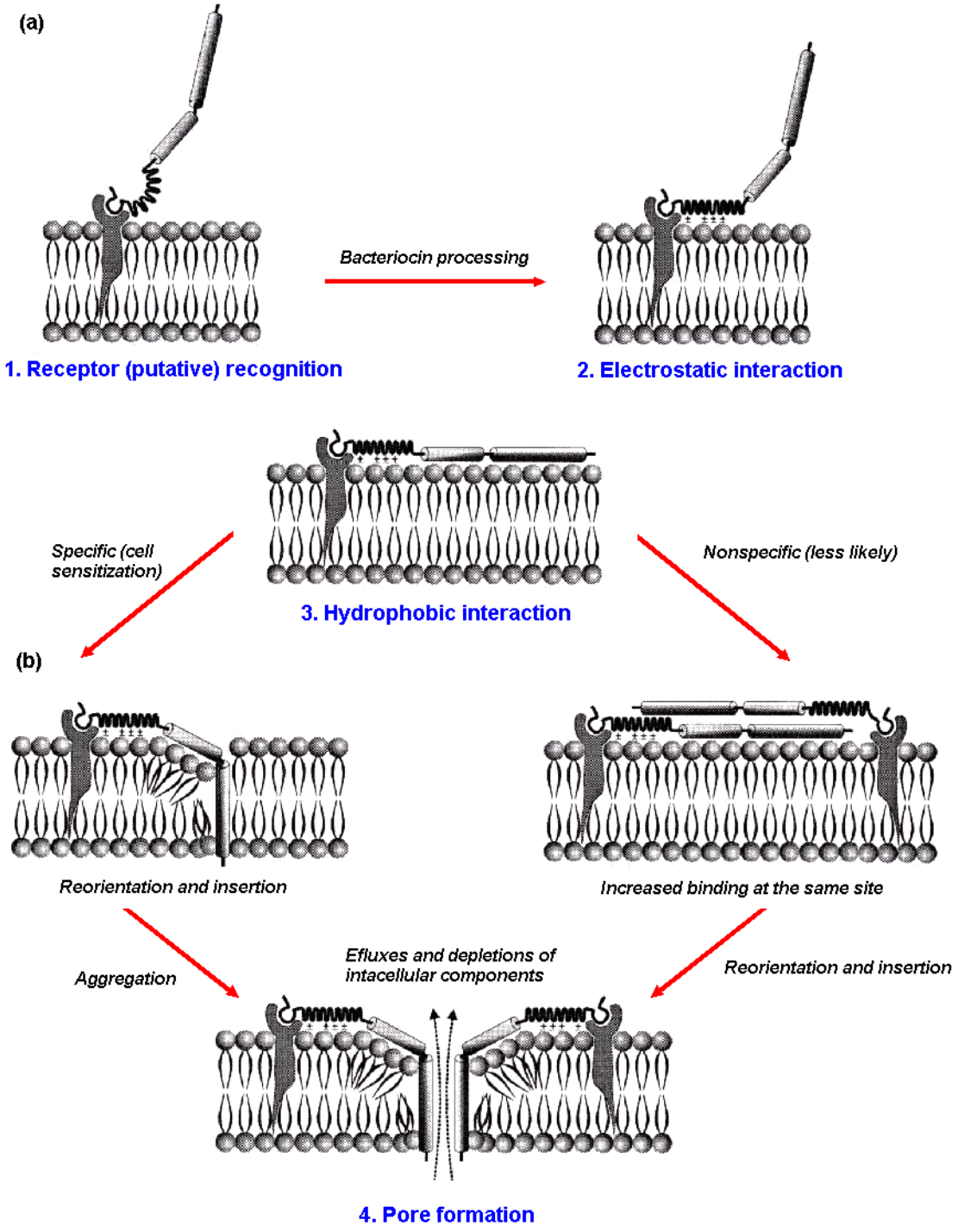


Figure 1.8. Schematic representation of the common mechanistic action of class IIa bacteriocins. (a) Possible interactions of each domain with the membrane surface; (b) Bacteriocin insertion and formation of hydrophilic pores. The hydrophobic face of the peptide is shaded dark and hydrophilic face is shaded light (Ennahar *et al.*, 2000).

1.7 Structure-function relationship of Class IIa Bacteriocins

The structure-function analysis of class IIa bacteriocins is useful for elucidating how potent membrane-permeabilizing peptides function at a molecular level (Fimland *et al.*, 2006). Their amino acid sequence and predicted secondary structure has been the basis of much research with the aim of identifying regions playing a crucial role in cell recognition and/or bactericidal action of these peptides. The mode of action of class IIa bacteriocins indicates that pore formation occurs through, not one, but a series of recognition steps which would involve different domains and structural features in the bacteriocin (Fleury *et al.*, 1996; Quadri *et al.*, 1997; Miller *et al.*, 1998[a]). Specific amino acid residues within the primary structure of the antimicrobial peptide have a major impact on its overall conformation. These amino acids dictate hydrophilic/hydrophobic profiles as well as α/β structures, which are important for activity. However, the significance of each domain and it role in antimicrobial activity is under debate (Hechard & Sahl, 2002).

1.7.1 The leader peptide

It is postulated that class IIa leader peptides are involved in the loss of activity of the precursor. Observations suggest that in many cases, producer cells are protected from the action of precursors mainly because these molecules display little, or no activity (Sprules *et al.*, 2004). Leader peptides are polar and increase the solubility of pre-bacteriocins in water. This may cause them to partition more into the aqueous phase than into the membrane, thereby preventing activity within the producer cell (Shnell *et al.*, 1988). Furthermore, both leader peptides and mature regions are moderately amphiphilic, and their non-polar regions may interact in water and shield non-polar membrane interactions. This prevents interaction of the mature domains with the lipid membrane of the producer cell (Quadri *et al.*, 1997). As plausible as the above statements may seem, studies have indicated that many class IIa precursors do exhibit some activity, however less than that of the mature peptide (Biet *et al.*, 1998).

The bacteriocin precursor of carnobacteriocin B2 displays an α-helical structure containing 18 amino acids in a lipophilic environment (Quadri *et al.*, 1995; Sprules *et al.*, 2004). Quadri *et al.* (1997), tested both the pre-peptide and mature peptide of carnobacteriocin B2 and results obtained indicate that both the pre and mature peptide of carnobacteriocin B2 display antimicrobial activity. However activity of the pre-peptide was much less than that of the mature carnobacteriocin B2. Similar conclusions were obtained by Biet *et al.* (1998) in which the mesentericin Y105 precursor, was demonstrated to be less active than the mature bacteriocin. The pediocin AcH was also found to be biologically active by Ray *et al.* (1999). The pediocin AcH precursor appeared to be ~80 % as active as the mature form. These results disprove the theory that the leader peptide keeps the mature bacteriocin biologically inactive but rather suggest that these leaders suppress antimicrobial activity within the producer cell (Aucher *et al.*, 2005). Based on the above findings, it becomes necessary to consider alternative ways for how producer cells overcome the potentially lethal action of the pre-peptide. The existing hypothesis, states that the immunity protein neutralizes the pre-peptide in the cytoplasm, thereby preventing activity. However, the mechanism by which this occurs is currently unknown (Ray *et al.*, 1999). Ray *et al.* (1999) also investigated the importance of disulfide bonds for pediocin AcH activity. Removal of these bonds completely suppressed activity of both pre- and mature forms of pediocin AcH. It was therefore suggested that the reducing state of the cytoplasm prevents disulfide bond formation and that this characteristic allows the precursor to be biologically active. Therefore disulfide bond formation aids in protecting the producer cell from its own bacteriocin.

A further possible function of the N-terminal extension is maintaining the conformation of the mature peptides, thereby allowing cleavage of the leader peptide during processing (Jack *et al.*, 1995). Aucher *et al.* (2005), analyzed the influence of amino acid substitutions in the leader peptide on maturation and secretion of mesentericin Y105. To analyze the roles of specific residues, 29 mutations were introduced in the leader peptide encoding region of the *mesY* gene. This study showed that mutations in the leader peptide resulted in lower production of mesentericin Y105. It was concluded that hydrophobic amino acids at positions -7 and -12, which are involved in the α-helical conformation, are of major importance for positioning the peptide at the active site of the ABC-transporter protease.

1.7.2 Role of the N-terminus

The presence of the YGNGV consensus motif led to speculation about its importance in activity (van Belkum & Stiles, 2000). The current view however, is that this motif could be involved in a recognition step of the mechanism of action (Fimland *et al.*, 1996). This is based on the secondary structure as this motif adopts a β-turn structure, which enables it to be exposed to and recognized by a putative membrane receptor (Bhugaloo-Vial *et al.*, 1996). No clear evidence for the above observation has been reported. However, various studies involving the N-terminal motif suggest that it is vital for antimicrobial activity. Modifications and/or deletions in the YGNGV motif have serious consequences on the anti-*Listerial* activity of these peptides. For example, when the N-terminal region of pediocin PA-1/AcH was deleted, this peptide did not show any activity against *Listeria* when tested (Miller *et al.*, 1998[a]). In a similar study, Quadri *et al.* (1997), induced amino acid substitutions at the N terminus of carnobacteriocin B2, which drastically altered its antimicrobial activity. However, alterations within the N-terminal domain of bacteriocin 31 and acidocin A, did not bring about altered activity to *Listeria* (Kanatani *et al.*, 1995). These class IIa bacteriocins contain modified N-terminal motifs.

The N-terminus of class IIa bacteriocins also contain positively charged amino acids and adopts β-sheet conformation, which plays a significant role in target membrane interaction. However, this is not a consensus among researchers. When the N-terminal β-sheet of carnobacteriocin B2 was compared to that of leucocin A in trifluoroacetic acid (TFA), it was found that this conformation differs dramatically, although these two bacteriocins share > 66 % sequence homology (Wang *et al.*, 1999). This suggests that this domain is not critical for antimicrobial activity.

A more promising view on the β-sheet conformation states that it plays a significant role in membrane primary interaction (Kazazik, Nissen-Meyer & Fimland, 2002). The electrostatic interaction of the N-terminal domain of pediocin PA-1 has been widely researched. This region consists of positively charged amino acid residues Lys-1, Lys-11 and His-12 and hydrophobic amino acid residues Val-7, Cys-9 and Cys-14, Val-16 and Trp-18. The positively charged patch mediates bacteriocin binding to target membranes, while the hydrophobic patch may be involved in the membrane insertion process (Chen *et al.*, 1997[a]). Site directed mutations for positively charged amino acid residues have led to less efficient binding of the

bacteriocin binding to the membrane surface. This was displayed by Chen *et al.* (1997[a]), where tryptophan fluorescence was used to monitor the binding of pediocin PA-1 without specific positively charged residues in the N-terminal β -hairpin loop. Results showed that these amino acids are vital for the peptides primary interaction with the target membrane. Miller *et al.* (1998[a]) further characterized the N-terminus of pediocin PA-1 by inducing mutations at Lys-1, His-42, and Lys-43 in pediocin AcH. When the chimeric peptide was tested against *Listeria*, it showed a decreased level of activity. A similar study by Morisset *et al.* (2004) involving mesentericin Y105, also showed reduced activity. Here, site directed mutations were used to introduce negative charges at the N-terminal moiety, which resulted in no interaction between the mutant peptide and lipid micelles. Therefore, the above observations support the view that the N-terminal β-sheet conformation may be involved in a membrane-surface recognition and binding step.

1.7.3 Role of the central amphiphilic α-helix

Oblique α – helical structures spanning residues 15, 16-27, and 28 of class IIa bacteriocins are believed to contribute to the destabilization of the phospholipid bilayers of target strains (Bennik *et al.*, 1998). This destabilization facilitates the insertion of the bacteriocin in the cytoplasmic membrane. However, a study conducted by Bughaloo-Vial *et al.* (1996), involving the class IIa bacteriocins piscicoins V1a and V1b, showed that the central hydrophilic or slightly amphiphilic α-helix anchors the bacteriocin to the surface of lipid bilayers, without inserting the membrane.

1.7.4 Role of the C-terminal hydrophobic/amphiphilic α-helix

Class IIa bacteriocins, when resolved in trifluoroethanol (TFE) and dodecyl phosphocholine (DPC) micelles, hydrophobic environments similar to the biological bilayer membrane, show a predominant propensity of α-helicity at their C-terminal half (Diep and Nes, 2002). This conformation is often referred to as the 'barrel-starve' poration complex, which allows the C-terminal domain to insert into the membrane of target cells (Ojcius & Young, 1991; Dennison *et al.*, 2005). The hydrophobicity of this domain, allows a stronger and specific interaction with the target membrane (Chen *et al.*, 1997[b]). Furthermore, due to the high sequence

diversity present within this domain, it is assumed that the C-terminus aids in target cell specificity (Diep & Nes, 2002).

The importance for hydrophobicity at the C-terminal domain has been tested by a number of studies. Johnsen *et al.* (2000), substituted a methionine residue present at the C-terminus of pediocin PA-1 with various hydrophilic negatively charged residues. When tested, mutant pediocin PA-1 displayed a marked decrease in potency, which is consistent with the proposal that the region interacts with the hydrophobic part of target cell membranes. Similarly, replacement of methionine with a hydrophilic but uncharged threonine residue, also reduced the activity. Miller *et al*. (1998[b]) also investigated the importance of the C-terminus of pediocin PA-1 by performing I26T, M31T, A34D, and G37E/R amino acid substitutions. These residues are thought to mediate membrane binding. The mutant proteins were tested against several indicator strains and substitutions resulted in either total loss in antimicrobial activity or partial loss. Furthermore, A34D mutants displayed differences in antimicrobial spectra, which state that this mutation may have a species-specific effect on activity.

1.7.5 Role of the disulfide Bonds

The cysteine content of class IIa bacteriocins allows for the presence of either one or two disulfide bridges, which allow for stability within the peptide structure (Kaur *et al.*, 2004). It has also been determined that the activity spectra of a bacteriocin increases as the number of disulfide bridges increases (van Belkum & Stiles, 2000). Eijsink *et al.* (1998) have shown that the two disulfide bonds of bacteriocins pediocin PA-1 and enterocin A are more efficient antimicrobials against *Listeria* than sakacin P and curvacin A, which possess a single disulfide bond. Furthermore, pediocin PA-1 has a broader spectrum of activity than leucocin A, which has one disulfide bond (Jack *et al.*, 1995).

Pediocin PA-1 contains two disulfide bridges (C9 – C14 and C22 – C44) within its tertiary structure (Oscariz and Pisabarro, 1998). The C9 – C14 disulfide bond may be required to establish the conformation of the putative β hairpin within the N-terminal region. The C22 – C44 disulfide bond brings positively charged Lys and His residues closer together to form a patch with positively charged side chains. This allows for a tighter junction between the bacteriocin and the target membrane (Chen *et al.*, 1997[b]). Serine substitutions conducted by Miller *et al.* (1998[b]) for Cys9 and Cys14 verified that these residues are necessary for activity

of pediocin PA-1. When Cys22 and Cys24 were substituted for serine residues, mutant proteins displayed a dramatic loss in activity revealing that these cysteine residues are important for pediocin activity. Leucocin A, sakacin A, and carnobacteriocin B2, are bacteriocins that contain one disulfide bond (Fregeau-Gallagher *et al.*, 1997). Reducing treatments for these residues resulted in a partial loss in activity, which indicates that a single disulfide bond is not crucial for activity.

Kaur *et al.* (2004), further determined that the presence of a C-terminal disulfide bond contributed significantly to the over stability of bacteriocin. It was found that pediocin PA-1 that has a second disulfide bond at it C-terminus, maintained its overall structure at elevated temperatures, whereas bacteriocins such as leucocin A experienced partial disruption of the C-terminal α-helix. These results suggest that there is a correlation between the presence of disulfide bonds and the potency of the class IIa bacteriocin. Furthermore, an extra disulfide bond in the C-terminal region, as in the case of pediocin PA-1, allows for a higher and stable level of activity (Chen *et al.*, 1997[b]; Kaur *et al.*, 2004).

1.8 Overview of Major Techniques used in this Study

1.8.1 Inhibition assays

The inhibitory activity of *Leuconostoc gelidum* UAL187-22 was tested using three types of inhibition assays. Inhibition assays as suggested by Kemperman *et al.* (2003) can be used to phenotypically assess the production of an inhibitory agent/s. Lactic acid bacteria are capable of producing inhibitory compounds such as hydrogen peroxide. However, in this study, these assays were used as preliminary tests to determine if the *Leuconostoc* strain contains the genes responsible for the production of active leucocin A. Leucocin A, a class IIa bacteriocin, is also referred to as an anti-listerial bacteriocin due to its activity against *L. monocytogenes* (Ramnath *et al.*, 2000). Therefore, *L. monocytogenes* was the indicator strain used in the inhibition assays. The aim of these assays was to determine if the operon for bacteriocin production is present for the amplification of the leucocin A and pre-leucocin A gene sequences.

1.8.2 The pMAL Protein and Purification System

The pMAL protein and purification system provides a convenient method of expressing and purifying recombinant proteins or peptides. This system developed and customized by New England Biolabs, consists of two major components i.e. the pMAL expression vector and affinity purification using the maltose binding tag.

A. pMAL-c2 expression vector

The pMAL-c2 expression vector is commonly used for the expression of recombinant proteins tagged with the maltose binding protein (Guan *et al.*, 1987). These vectors express the *malE* gene (encodes the maltose binding protein) fused to the *lacZα* gene. However, restriction sites present between these genes allow for the insertion of a coding sequence of interest. Upon induction, the cloned gene is expressed fused with the maltose binding protein (MBP) also referred to as a MBP fusion protein. Insertion of a gene inactivates the β-galactosidase α-fragment activity of the *malE-lacZα* fusion which provides an easy and rapid method for screening of recombinants when the construction is transformed into an α-complementing host such as *E.coli* JM103. The presence of a strong P_{tac} promoter provides

high-level expression of the MBP fusion proteins when induced with Isopropyl-β-D-thiogalactopyranoside (IPTG) (Lauritzen *et al.*, 1991). Located directly between the *malE* gene and the 5' end of the polylinker insertion site, is a specific protease cleavage site. This allows cleavage (usually with Factor Xa) of the MBP tag from the protein of interest after purification (Waugh, 2005).

B. Maltose binding protein tag

The production of purified and well-characterized recombinant proteins has become a major task within the biotechnology sector. Several different strategies exist which allow for the purification of recombinant proteins; however, in recent years, affinity handles or tags fused to the recombinant protein/peptide have been a popular choice (Terpe, 2003). The maltose binding protein is natural periplasmic *E.coli* protein which provides a convenient method for the production and purification of recombinant proteins by the generation of affinity handles (Bach *et al.*, 2001). When expressed in the cytoplasm, the MBP-tag enhances the solubility and stability of proteins that are fused to it (Kapust & Waugh, 1999). Furthermore an advantage of cytoplasmic expression of MBP fusions is that the expression level is much higher when compared to that of MBP fusions that are exported to the periplasm (Riggs, 2000). In a large majority of cases, fusion protein expressed from a pMAL-c2 plasmid constitutes 20-40 % of the total cellular protein.

C. Amylose affinity chromatography

Affinity chromatography is a type of liquid chromatography that makes use of biological-like interactions for the separation and specific analysis of sample components (Hage, 1999). Amylose affinity chromatography relies on the specific interaction between the maltose binding protein and the complementary ligand amylose, immobilized on a polymeric support (Cattoli & Sarti, 2002). Binding is due to the presence of asparagine residues between the MBP and the protein of interest, which increases the chances that a particular fusion will bind tightly to the amylose resin (Terpe, 2002). This characteristic allows the MBP-fusion proteins to bind to the amylose resin while all other non-specific proteins flow through the column. The bound protein can then be eluted with maltose which has a higher affinity for amylose and therefore, by competition, the fusion protein is released and isolated. A schematic representation of Amylose affinity purification is shown in figure 1.9.

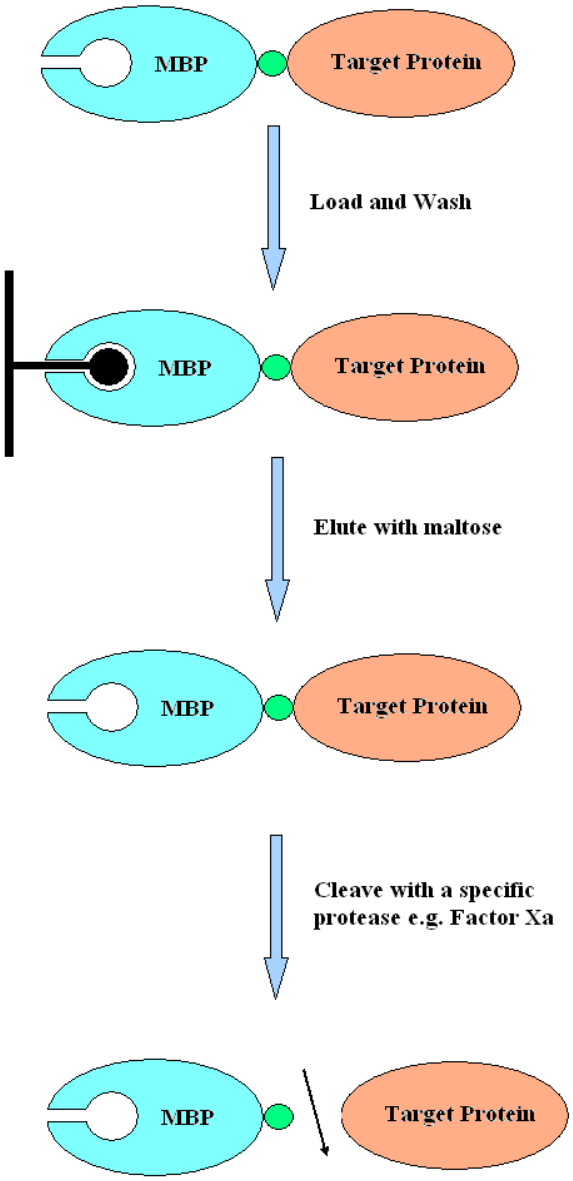


Figure 1.9. Schematic representation of the principles behind amylose affinity purification of MBP tagged proteins. The maltose binding protein has an affinity for amylose, and binds to the resin while other non-specific proteins flow through the column. Further non-specific proteins are removed by washing the amylose resin, and bound fusion protein is thereafter, eluted with maltose (adapted from New England Biolabs).

1.8.3 N-terminal Protein Sequencing

Peptide sequencing was performed using the Procise™/Procise cLC system (Applied Biosystems). During sequencing, N-terminal amino acids are sequentially derivitised and analyzed via the HPLC system. The chemical process of amino acid derivatization (figure 1.11), was developed by P. Edman in 1949 and for this reason, protein sequencing is often referred to as Edman degradation (Edman & Begg, 1967). Automated Edman degradation chemistry consists of three steps:

1. Phenylisothiocyanate (PITC) is coupled with the α-amino group of the protein/peptide under basic conditions to form a phenylthiocarbamyl derivative (PTC-protein).
2. Trifluoroacetic acid then cleaves off the first the coupled amino acid to generate an anilinothiazolinone (ATZ) amino acid.
3. The ATZ derivative is converted to the more stable phenylthiohydantoin (PTH) derivative.

The Procise sequencer is able to sequence proteins/peptides either within solution or bound to a solid phase such as polyvinylidene diflouride (PVDF). A specially designed reaction chamber, referred to as a Blott cartridge is used for membrane-bound samples. This cartridge is a vertical cross-flow reactor that facilitates optimization of the cartridge chemistry. During sequencing, the sample is retained in the temperature-controlled Blott cartridge and at the end of each degradation cycle, the terminal amino acid is removed as an ATZ derivative. The ATZ derivative is automatically transferred from the reaction cartridge to a separately heated conversion flask where it is converted to the PTH derivative. The PTH derivative is then transferred from the conversion flask to the injection valve for the subsequent separation and quantitation on the Procise HPLC System. Different PTH derivatives have unique relative affinities for the HPLC column and therefore exit the column at different times. This allows for the determination of the protein/peptide sequence by comparing the retention times of each PTH derivative with those of the standard amino acids (Kellner, Lottspeich, & Meyer, 1994). Figure 1.10 represesnts a typical arrangement of the components of a protein sequencer.

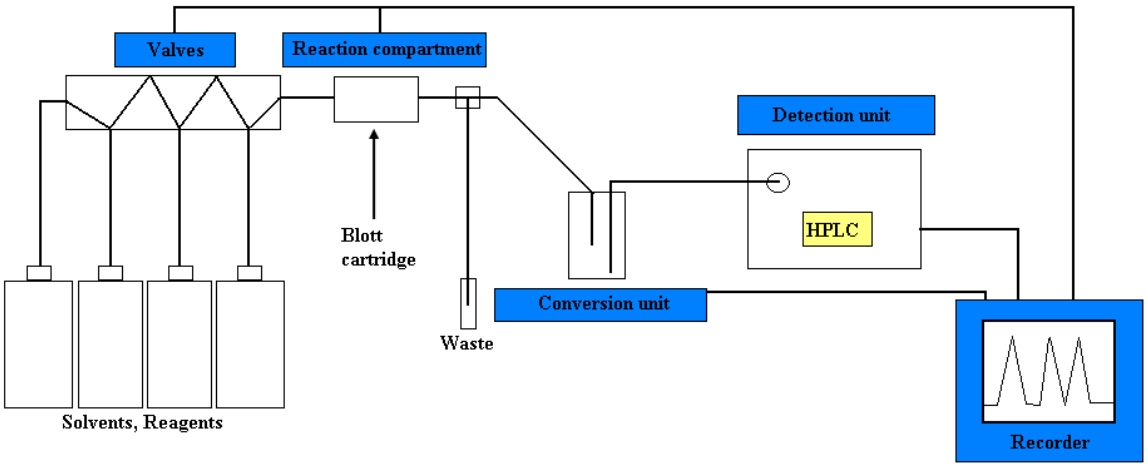


Figure 1.10. Typical arrangement of a protein sequencer. Edman degradation occurs within the reaction chamber and conversion unit. Derivitised PTC amino acids are then transferred to the HPLC detection unit (Kellner, Lottspeich, & Meyer, 1994).

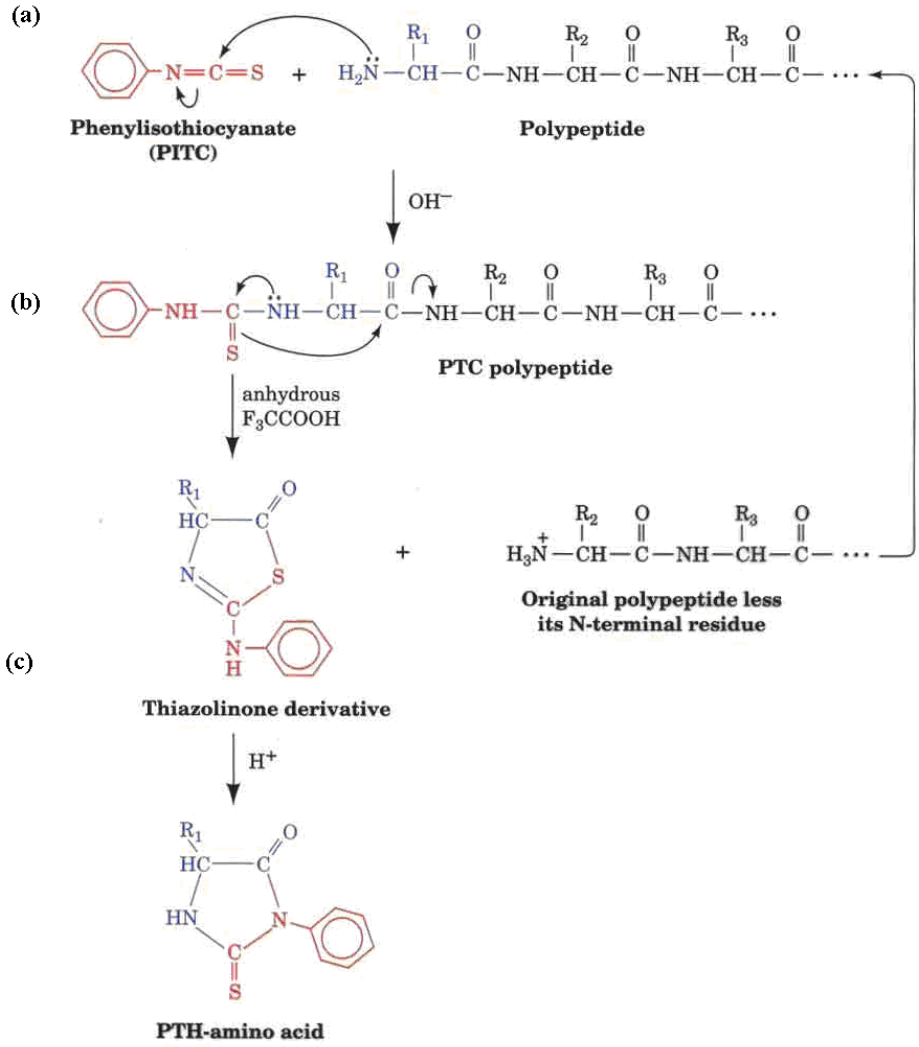


Figure 1.11. Chemical reactions that constitute Edman degradation. (a) A coupling reaction in which phenylisothiocyanate (PITC) reacts with the α-amino group of the protein/peptide to form a phenylthiocarbamyl (PTC) derivative; (b) The PTC derivative is cleaved off by anhydrous acid, which results in the formation of an anilinothiazolinone (ATZ) amino acid; (c) The final step involves the conversion of the ATZ derivative to a detectable phenylthiohydantoin (PTH) derivative (Kellner, Lottspeich, & Meyer, 1994).

1.8.4 High performance liquid chromatography

Reversed phase high performance liquid chromatography (RP-HPLC) has found both analytical and preparative applications in the area of biochemical separation and purification. The principle method of separation depends on the hydrophobic binding interaction between the solute molecule in the mobile phase and the immobilised hydrophobic ligand, i.e. the stationary phase (Dorsey & Cooper, 1994). Typical stationary phases are non-polar hydrocarbons, waxy liquids or bonded hydrocarbons (such as C_{18}, C_8, C_4, etc.), and the solvents (mobile phase) are polar aqueous-organic mixtures such as methanol-water or acetonitrile-water. Polar molecules have a shorter retention time (time of elution) as they interact more strongly with the mobile phase than with the stationary phase. On the other hand, interaction of non-polar molecules with the stationary phase is much stronger, and they elute from the column more slowly than polar molecules (Tanford, 1961). Therefore, separations in reversed phase chromatography depend on the reversible adsorption/desorption of solute molecules with varying degrees of hydrophobicity to a hydrophobic stationary phase. Therefore, reversed phase chromatography is an adsorptive process which takes advantage of the hydrophobic binding interaction of the biological sample (e.g. protein) to the stationary phase (matrix within the column). Subsequently, the mobile phase composition is modified to favour desorption of the solute from the stationary phase back into the mobile. Biomolecules adsorb strongly to the surface of the matrix under aqueous conditions and desorb within a small change in organic concentration. Each biomolecule has a unique adsorption affinity; therefore a complex biological sample consists of a diverse range of adsorption affinities. Therefore, biological samples are usually eluted under gradient conditions of the mobile phase, which automatically changes the polarity of the mobile phase during the course of the analysis and greatly affects the retention of the compounds. The system used in this study makes use of a gradient of acetonitrile in 0.1% TFA as the mobile phase. As each biomolecule is eluted, their retention times are detected at a specific wavelength, resulting in the formation of a chromatogram (Corran, 1989).

The RP-HPLC instrument (figure 1.12) consists of two mobile phase reservoir, a dual pump system that provides a constant high pressure which continuously supplies the mobile phases to the column under gradient conditions, a column containing the matrix, and a detector and a recorder. The sample is applied to the system at the injector component (Kellner, Lottspeich, & Meyer, 1994).

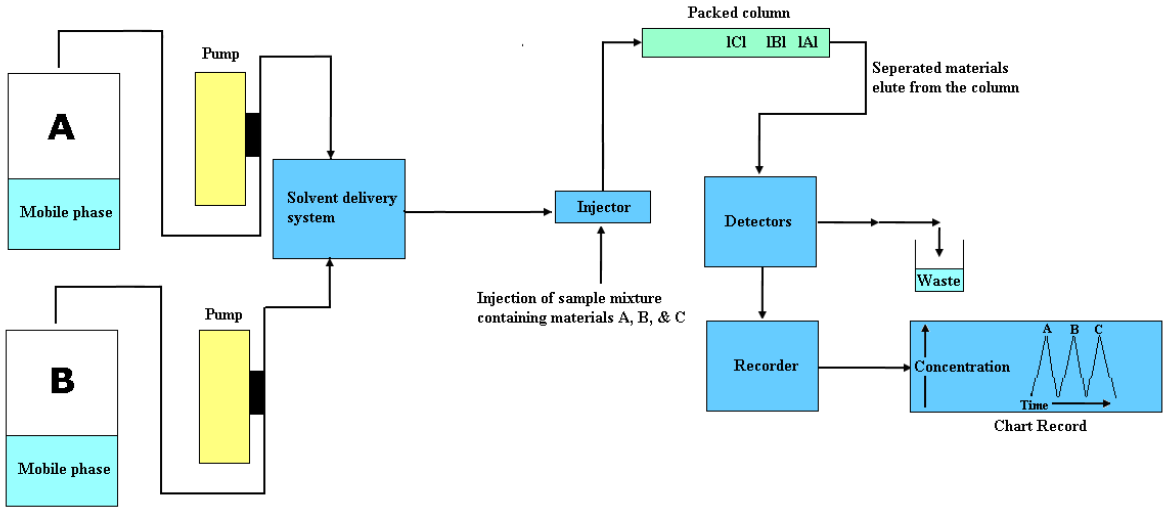


Figure 1.12. Components of a reverse phase high performance liquid chromatography system. Components consist of a solvent reservoir which supplies the mobile phase, a column containing the stationary phase, and a HPLC detector and recorder (Kellner, Lottspeich, & Meyer, 1994).

CHAPTER TWO

MATERIALS AND METHODS

2.1 Bacterial Strains, Plasmids and Growth Conditions

Bacterial strains and plasmids used in this study are listed in table 2.1. All strains were obtained from the stock culture collection maintained in laboratory B23, Dicsipline of Genetics, University of Kwa-Zulu Natal, Pietermaritzburg. Plasmid DNA harbouring the *LcnABCDE* operon responsible for leucocin A production and secretion, was isolated from *Leuconostoc gelidum* UAL187-22. Expression vector pMAL-c2 was isolated from *Escherichia coli* K12 TB1. Plasmid pJF5.5 (constructed previously in laboratory B23 Department of Genetics, University of Kwa-Zulu Natal) was used as control DNA in various techniques, and was isolated from *E. coli* JM103. *L. gelidum* UAL187-22 was cultured in De Man Rogosa Sharpe (MRS) (Merck, South Africa) broth at 25 ^{o}C. *E. coli* K12 TB1 and *E. coli* JM103 which was used as a host for the various recombinant plasmids were grown in Luria Bertani (LB) (Merck, South Africa) broth (Sambrook *et al.*, 1989). All cultures *E. coli* were grown in a shaking incubator maintained at 37 ^{o}C by a fan. The selective pressure of the LB media was maintained by adding ampicillin (Roche Biochemicals, USA) to a final concentration of 100 µg/ml. The indicator strain *Listeria monocytogenes* B73 which is susceptible to leucocin A activity, was grown in tryptone soy broth (TSB) (Merck, South Africa) at 30 ^{o}C. Tryptone soy agar (TSA) (Merck, South Africa) prepared to half strength (sloppy agar), was used as overlays in the inhibition assays. Stock cultures were prepared in 30 % (v/v) glycerol and stored at -70 ^{o}C. Working cultures were prepared on LB or MRS agar slants and plates and maintained at 4 ^{o}C. All agar plates were subcultured every two weeks at 37 ^{o}C.

Table 2.1. Bacterial strains and plasmids used in this study.

Strain	Description	Reference or source
Escherichia coli K12 TB1	F^- *ara* Δ*(lac-proAB) [Φ80dlac* Δ*(lacZ)M15] *rpsL*(Str^R) *thi hsdR*	New England Biolabs, UK
Escherichia coli JM103	*thr rpsL endA sbc-15 hsdR14* Δ (lac pro AB) F' *traD36 pro* AB, *lacIZ*ΔM15	Sambrook *et al.*, 1989
Leuconostoc gelidum UAL187-22	*LcaA*+ *Imm*+ containing native plasmids pLG 9.2 and pLG 7.6	Hastings *et al.*, 1991
Listeria monocytogenes B73	leucocin A sensitive strain	Ramnath *et al.*, 2000

Plasmid	Description	Reference or source
pLG 9.2	*LcaA*+ *Imm*+	Hastings *et al.*, 1991
pLG 7.6	*LcaA*+ *Imm*+	Hastings *et al.*, 1991
pMAL-c2 6.6	*malE, lacZ α, Amp*$^+$	New England Biolabs, UK
pJF 5.5	*LcaB*$^+$, *Imm*$^+$, *Amp*$^+$	Laboratory B23, Discipline of Genetics, UKZN
pLcaA[a]	*malE-LcaA, lacZ α, Amp*$^+$	This study
pPreLcaA[a]	*malE-preLcaA, lacZ α, Amp*$^+$	This study

[a] recombinant pMAL-c2 derivatives

2.2 Inhibition Assays

2.2.1 Colony overlay assay

This assay was performed in order to determine if the *Leuconostoc* strain under investigation has the ability to inhibit *L. monocytogenes*. An overnight culture of *L. gelidum* UAL187-22 was prepared in 10 ml MRS broth by incubating at 25 ^{o}C overnight. An inoculum of the overnight culture was streaked onto an MRS agar plate which was incubated overnight at 25 ^{o}C. Following incubation, the plate was analyzed for the presence of individual bacterial colonies. A 1 ml aliquot of an overnight culture of *L. monocytogenes* was sub-cultured into 10ml tryptone soy broth. This was allowed to grow at 30 ^{o}C for +/- 7 hrs. Thereafter 250 µl of this culture was added to 6 ml of TSA sloppy agar and overlayed onto the MRS plate containing the *L. gelidum* UAL187-22 colonies. Plates were allowed to dry, inverted, and incubated at 25 ^{o}C overnight. Zones of inhibition produced by each colony were then inspected.

2.2.2 Deferred antagonism assay

A deferred antagonism assay was used to test for the production of an inhibitory agent/s within the supernatant of *L. gelidum* UAL187-22. MRS broth (250 ml) was inoculated with a single colony of *L. gelidum* UAL187-22 and incubated overnight at 25 ^{o}C. Following incubation, the culture was centrifuged at 6000 x g for 10 min in a Beckman centrifuge using a JA-20 rotor. Supernatant was transferred to a fresh tube and stored on ice. Tryptone soy broth (10 ml) was sub-cultured with 1ml of an overnight culture of *L. monocytogenes* and allowed to grow at 30 ^{o}C for +/- 7 hrs. Thereafter 250 µl of this culture was added to 6 ml of TSA sloppy agar and overlayed onto an MRS plate. Plates were allowed to dry and thereafter 10µl of supernatant from *L. gelidum* UAL187-22 was spotted onto the overlayed plates. A control sample consisting of partially purified leucocin A was also spotted. This control was used in all inhibition assays. Plates were allowed to dry, inverted, and incubated at 25 ^{o}C overnight. Following incubation zones of inhibition were inspected. A further deferred antagonism assay was carried out using concentrated *L. gelidum* UAL187-22 supernatant. A single colony of *L. gelidum* UAL187-22 was inoculated into 250 ml MRS broth and incubated at 25 ^{o}C overnight. The supernatant was extracted from the overnight culture by

centrifugation (10 min; 6000 x g at 4 ^{o}C) and snap freezed in liquid nitrogen. The frozen sample was then freeze dried (water was removed) using an Edwards Modulyo freeze dryer for a period of 24 hrs. Freeze dried samples were resuspended in 1 ml 0.1% trifluoroacetic acid (TFA) which was thereafter tested for the presence of an inhibitory agent by a deferred antagonism assay. Zones of inhibition were inspected the following day.

The activity of the MBP fusion proteins, Factor Xa cleavage mixtures, and purified recombinant peptides i.e. leucocin A and pre-leucocin A were tested for activity using the deferred antagonism assay procedure described above.

2.2.3 Half moon assay

The same procedure as that used for the deferred antagonism assay was followed. Plates (MRS) overlayed with *L. monocytogenes* were prepared as already described. Concentrated supernatant (10 µl) obtained from an overnight culture of *L. gelidum* UAL 187-22 was spotted onto the overlayed agar plate. This was allowed to dry and thereafter 2 µl of Proteinase K (prepared to 100 mg/ml) (Roche Biochemicals, USA), was spotted adjacent to each test spot. The plates were allowed to dry, inverted and incubated overnight at 25 ^{o}C. Half moon zones of inhibition were inspected which would indicate the action of a proteinaceous inhibitor.

2.3 Plasmid DNA Isolation

2.3.1 *Leuconostoc gelidum* UAL 187-22 plasmid isolation

Leuconostoc gelidum UAL187-22 was screened for the presence of plasmid DNA using an alkaline lysis method described by Holt *el al.* (2001). A 50 ml overnight culture of *L. gelidum* UAL187-22 was centrifuged at 7000 x g for 10 min at 4 ^{o}C in a Beckman centrifuge using a JA-20 rotor. The pelleted cells were resuspended in 1 ml GTE buffer [25 mM Tris-HCl, pH 8.0; 50 mM glucose and 10 mM EDTA]. A lysozyme solution (50 mg/ml) was added to the mixture (400 µl) and incubated at room temperature for 60 min. Thereafter, 2 ml lysis solution containing 1% sodium dodecyl sulfate and 0.2 N NaOH was added, mixed by inverting three times and placed on ice for 5 min. Ice-cold potassium acetate buffer [1500 µl, 29.5 ml glacial acetic acid, KOH pellets to pH 4.8; H_2O to 100 ml], was then added. The mixture was vortexed for 10 sec and incubated on ice for 5 min. Following incubation the sample was centrifuged for 5 min at 14000 x g and 600 µl of the supernatant was transferred to a fresh microfuge tube. An equal volume of phenol/chloroform (50:50) was added to the supernatant, mixed by inverting and centrifuged at 5000 x g for 10 min. The aqueous layer (top layer) was extracted and transferred to a new microfuge tube. An equal volume of chloroform/isoamyl alcohol (24:1) was added, mixed by inverting, and centrifuged at 5000 x g for 10 min. The top layer was extracted and transferred to a new microfuge tube. DNA was precipitated by adding two-volumes ice-cold 100% ethanol and centrifuging at 14000 x g for 5 min. The resulting pellet was washed twice with 500 µl 70% ethanol and centrifuged at 15000 x g for 5 min. Supernatant was extracted and discarded and the pellet was air-dried for 15 min. The DNA pellet was resuspended in 50µl 1X TE buffer [10 mM Tris-Cl, pH 7.5; 10 mM EDTA]. RNAse A (2 µl) (Roche Biochemicals, USA) was added to each DNA sample and incubated on ice for 10 min. Plasmid DNA was analyzed on a 0.6 % (w/v) agarose gel and viewed using a UV transilluminator.

2.3.2 Expression vector pMAL-c2 isolation

Expression vector pMAL-c2 was isolated from *E. coli* K12 TB1 using the Nucleobond® AX 100 kit (Machery-Nagel, Germany). Overnight cultures of *E. coli* K12 TB1 bearing the pMAL-c2 vector was prepared by inoculating a single bacterial colony into 50 ml LB broth supplemented with ampicillin (100 µg/ml). This was grown at 37 ^{o}C, overnight in a shaking waterbath. Bacterial cells were harvested by centrifugation at 7000 x g for 10 min at 4 ^{o}C in a Beckman centrifuge using a JA-20 rotor. The supernatant was discarded and the cells (pellet) was resuspended in 4 ml of buffer S1 [50 mM Tris-HCl; 10 mM EDTA; 100 µg/ml RNAas A, pH 8.0]. To each tube 4 ml of buffer S2 [200 mM NaOH; 1% SDS] was added and the solution was mixed immediately by inverting the tube 6 to 8 times and thereafter, incubated at room temperature for 5 min. Pre-cooled buffer S3 [4 ml, 2.8 M KAc, pH 5.1, 4^{o}C] was added to each tube, mixed by inverting 6 to 8 times until a homogenous suspension was formed, and incubated on ice for 5 min. The mixture was filtered through a Nucleobond® filter to remove cellular material and the filtrate was saved on ice. A Nucleobond® AX 100 column was equilibrated with 2.5 ml buffer N2 [100 mM Tris; 15 % ethanol; 900 mM KCl; 0.15 % Triton X-100]. The column was allowed to empty by gravity flow. The filtrate from the cell lysate was loaded onto the pre-equilibrated column and the flow through collected. Thereafter the column was washed twice with 5 ml buffer N3 [100 mM Tris; 15 % ethanol; 1.15 M KCl pH 6.3). Plasmid DNA was eluted with 5 ml buffer N5 [100 mM Tris; 15 % ethanol; 1 M KCl, pH 8.5). The plasmid DNA was then precipitated with 3.6 ml isopropanol (room temperature), and centrifuged at 15000 x g for 30 min at 4 ^{o}C. Supernatants were discarded and the pelleted DNA was washed with 2 ml 70 % ethanol (4 ^{o}C). Samples were centrifuged at 15000 x g for 10 min at room temperature. The supernatant was carefully removed with a micropipette and the plasmid DNA (pellet) was air-dried at room temperature for 15 min. Plasmid DNA was resuspended in 50 µl 1X TE buffer.The presence of DNA was confirmed by electrophoresis on a 0.8 % (w/v) agarose gel and viewed using a UV transilluminator.

2.3.3 Plasmid DNA pJF5.5 isolation

Plasmid pJF5.5, which was used as control DNA is various procedures during this study was also isolated from *E. coli* JM103 using Nucleobond® AX 100 kit as already described.

2.3.4 Recombinant Plasmid Isolation

All recombinant plasmids were isolated using the mini prep alkaline lysis method described by Birnboim & Doly, 1979. LB medium (50 ml) supplemented with ampicillin (100 µg/ml) was inoculated with a single bacterial colony of *E.coli* JM103 containing recombinant plasmid. This was grown overnight with good aeration at 37 ^{o}C with vigorous shaking. Cells were harvested by centrifugation (5 min; 7000 x g at 4 ^{o}C) in a Beckman centrifuge using a JA-20 rotor. Supernatant was discarded and the pellet was resuspended in 1 ml GTE buffer. The suspension was incubated at room temperature for 5 min and thereafter 10 µl of RNAse A was added. Freshly prepared lysis solution (1.2 ml) containing 1 % sodium dodecyl sulfate and 0.2 N NaOH was added, mixed gently by finger tapping and placed on ice for 5 min. Thereafter, 1.5 ml of ice-cold potassium acetate buffer [29.5 ml glacial acetic acid, KOH pellets to pH 4.8; H_2O to 100 ml] was added. The solution was mixed thoroughly and placed on ice for 5 min. Centrifugation to 13000 x g at 4 ^{o}C for 5 min separated cellular debris from plasmid DNA. The supernatant was transferred to a fresh 50 ml centrifuge tube and 3 ml of isopropanol was added. Plasmid DNA was precipitated by incubating at -20 ^{o}C for 30 min. Following incubation, plasmid DNA was pelleted by centrifugation at 13000 x g at 4 ^{o}C for 5 min. The supernatant was discarded and the pellet was washed twice with 2.5 ml 70 % ethanol (4 ^{o}C). Following the wash step, plasmid DNA was air dried to remove excess ethanol and thereafter resuspended in 50 µl 1X TE buffer. Plasmid DNA was analyzed on a 0.8 % (w/v) agarose gel and viewed using a UV transilluminator.

2.3.5 Quantification of plasmid DNA

Plasmid DNA was quantified using a Beckman DU 640 Spectrophotometer. Isolated DNA (5 µl) was added to 495 µl of 1X TE buffer in a quartz cuvette. Absorbance readings of isolated DNA was then taken at wavelengths of 260 nm and 280 nm. The purity of the DNA samples was calculated as the ratio between the readings at 260 nm and 280 nm. Pure isolates of DNA have a 260 nm/280 nm value between 1.8 and 2.0 (Sambrook *et al.*, 1989). The DNA concentration was calculated as follows: dilution factor x A_{260} x constant = X, where X is the concentration of DNA in µg/ml and constant is 50 µg/ml.

2.3.6 Analysis of plasmid DNA by agarose gel electrophoresis

Isolated DNA was confirmed using horizontal agarose gel electrophoresis. Electrophoresis was performed in 1X Tris acetate buffer [0.04 M Tris-acetate; 0.001 M EDTA, pH 8.0]. Agarose gels were prepared using electrophoresis grade agarose D-5 (Pronadisa, South Africa) and contained 5 µg/ml ethidium bromide (Promega, USA). DNA samples were prepared by adding 10 µl DNA to 2 µl sample buffer [0.25% (w/v) Ficoll; 0.2% (w/v) bromophenol blue in 1 X TAE buffer). Electrophoresis was performed at 8 V/cm for 90 min. DNA bands present on the gels were viewed under UV light with a transilluminator and documented using a Versadoc Imager.

2.4 Construction of MBP fusion expression plasmids

The experimental procedures used to clone the leucocin A and pre-leucocin A genes into pMAL-c2 and to express the recombinant proteins are shown schematically in figure 2.1. All DNA manipulations and cloning procedures were carried out according to Sambrook *et al.* (1989).

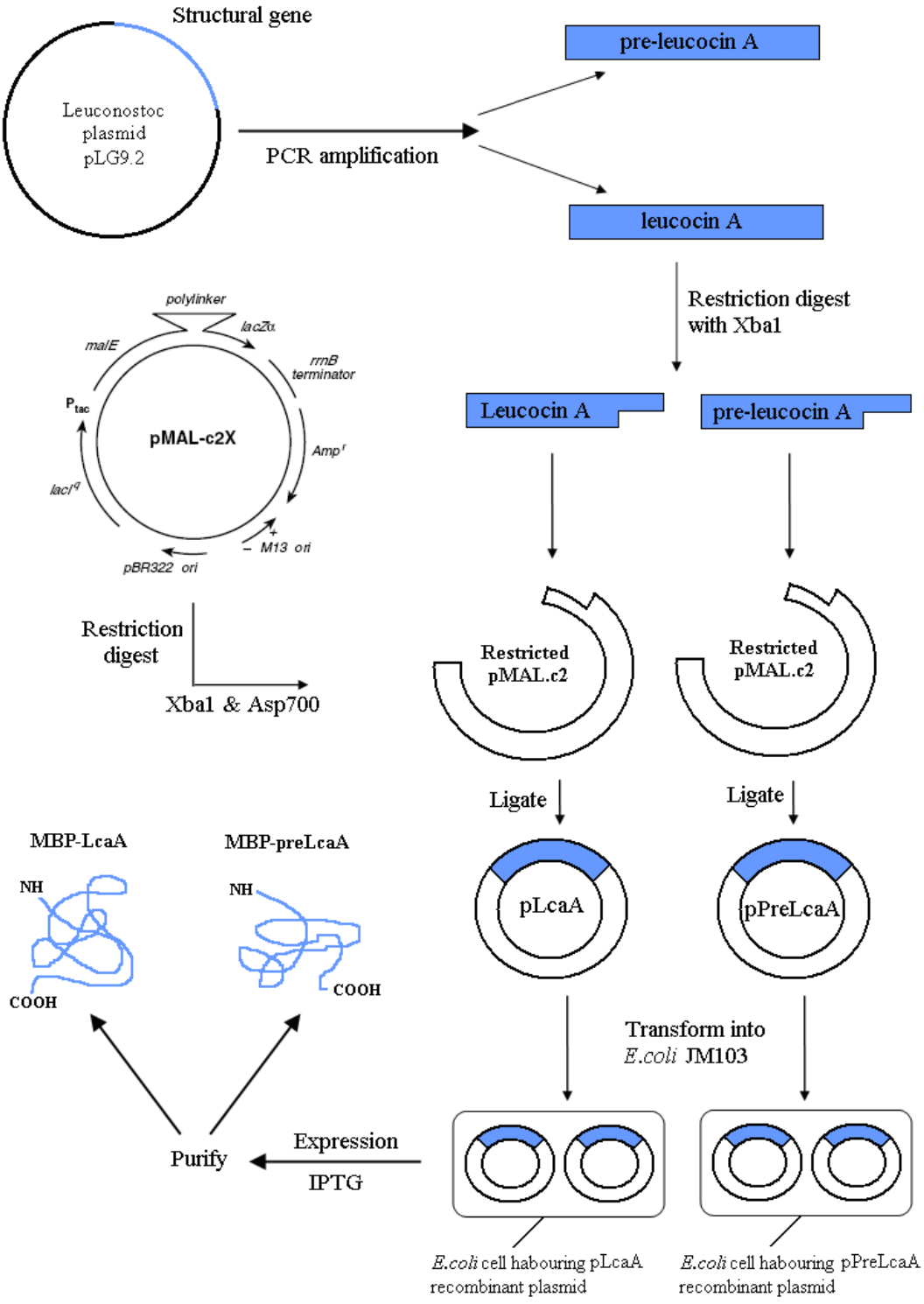


Figure 2.1. Schematic representation of the experimental procedures used to isolate MBP fusion proteins. The construction of recombinant plasmids consisted of amplification of the leucocin A and pre-leucocin A genes, DNA restriction and ligation into the pMAL-c2 vector. Recombinant fusion proteins, MBP-LcaA and MBP-preLcaA were expressed and purified.

2.4.1 Amplification of the target genes by Polymerase Chain Reaction (PCR)

Two DNA fragments i.e. the leucocin A and pre-leucocin A gene (leucocin A including the leader sequence) were amplified from plasmid DNA isolated from *L. gelidum* UAL187-22 by PCR. Primers were designed by obtaining *L. gelidum* UAL187-22 sequence data from the NCBI database, accession number M64371 (figure 2.2). Forward and reverse primers designed from this sequence are shown in table 2.2. The 5' ends of the reverse primers contain an *Xba1* restriction enzyme site (ATTCTAGA). All primers contain a GC clamp at their 3' ends. Furthermore, primers were subjected to a final check using an Oligo Calculator program to ensure that they were designed accordingly (Dana-Farber Cancer Institute, USA). These included calculating the GC content and melting temperature (Tm), and ensuring that secondary structure and primer dimer formation were prevented.

Table 2.2. Forward and reverse primers designed to amplify the leucocin A and pre-leucocin A genes. The *Xba*1 restriction site is represented in bold text.

Target	Primer	Sequence
Leucocin A	Leu-f	5'AAGTATTATGGTAACGGAGTTCATTGC 3'
	Leu-r	5'**ATTCTAGA**CCAGAAACCATTTCCACCATTTGCTAAACG 3'
Pre-Leucocin A	Pre-f	5'ATGATGAACATGAAACCTACGGAAAGC 3'
	Pre-r	5'**ATTCTAGA**TTACCAGAAACCATTTCCACCATTTGC 3'

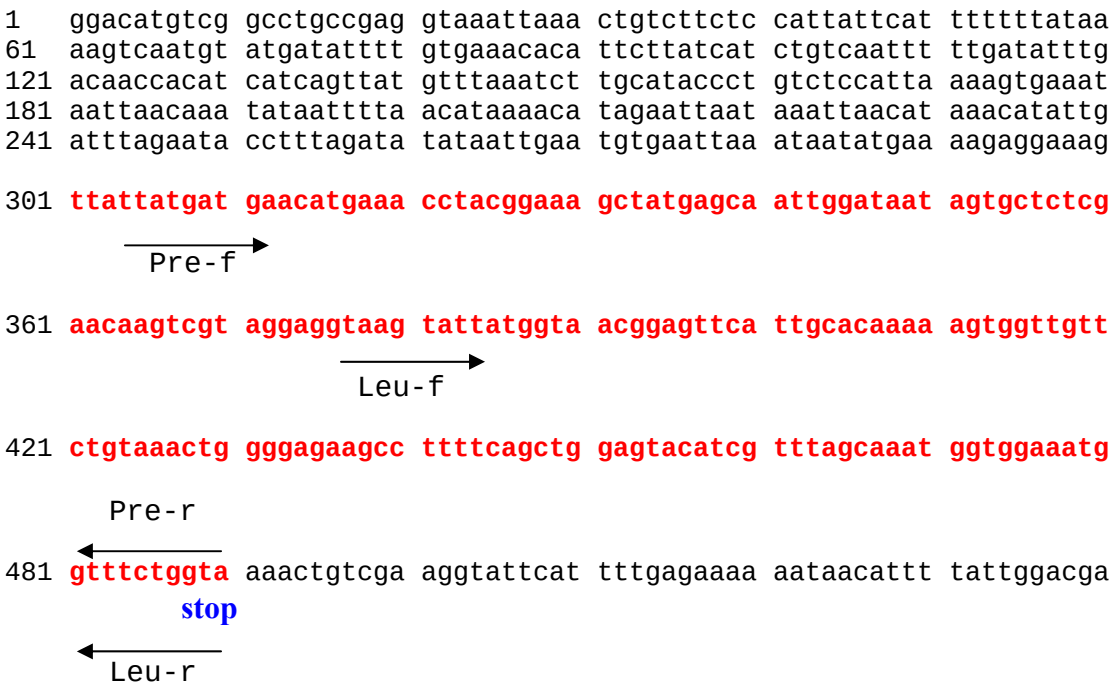


Figure 2.2. *Leuconostoc gelidum* UAL187-22 sequence data depicting regions of the *LcaABCD* involved in the production of leucocin A and the immunity protein (accession number M64371). The sequence was used to design primers Leu-r/Leu-f and Pre-r/Pre-f for the amplification of the leucocin A and pre-leucocin A genes. Text in red represents the leucocin A gene together with the leader sequence. Vertical arrows represent the binding sites of both forward and reverse primers for leucocin A and pre-leucocin A genes. The stop codon for these genes is also indicated.

PCR amplification was performed in an automated GeneAmp® PCR System 2700 thermal cycler (Applied Biosystems, USA). The contents of each reaction mixture (100 µl) used for PCR is outlined in table 2.3.

Table 2.3. Reaction components used for the PCR amplification of the leucocin A and pre-leucocin A genes.

Reagent	**Leucocin A**		**pre-Leucocin A**	
	Vol (µl)	**Final conc.**	**Vol (µl)**	**Final conc.**
10 X PCR buffer with $MgCl_2$	10	1 X	10	1 X
10mM dNTP mix (Roche Biochemicals, USA)	2	0.2 M	2	0.2 M
Primer Leu-f	1	0.3 M	0	0
Primer Leu-r	1	0.3 M	0	0
Primer Pre-f	0	0	1	0.3 M
Primer Pre-r	0	0	1	0.3 M
Template DNA (9.00 µg/ml)	2	0.18 µg/ml	2	0.18 µg/ml
Faststart Taq polymerase (50 U/ml) (Roche Biochemicals, USA)	2	1 U/ml	2	1U/ml
Sterile dH_2O	82		82	
Total reaction	100		100	

Various controls were included in the PCR reaction. These included a positive control using plasmid pJF5.5 as template DNA. Two negative controls were used, one without template DNA and the other using pMAL-c2 DNA. Faststart Taq DNA polymerase (Roche Biochemicals, USA) was added last to each reaction mixture. After 5 min denaturation at 94 ^{o}C, 30 cycles of the following program was run: denaturation for 30 sec at 94 ^{o}C, primer annealing for 30 sec at 50 ^{o}C, and polymerization for 30 sec at 72 ^{o}C. This was followed by a final extension step at 72 ^{o}C for 7 min.

Products obtained after PCR were analyzed by electrophoresis (8 V/cm, 90 min) on a 2.5 % (w/v) agarose gel. An O'RangeRuler 100 bp DNA ladder (Fermentas, South Africa) was included on the gel to confirm the size of each PCR product. Amplified DNA was quantified at 260 nm and 280 nm in order to determine the DNA concentration and purity.

2.4.2 Purification of PCR products

Following PCR, the amplified leucocin A and pre-leucocin A genes were purified to remove excess salts and to ensure that subsequent reactions such as restriction digests would not be inhibited. Purification was performed using the Nucleospin® DNA purification kit according to the manufacture's instructions. NT buffer (200 µl) was added to 100 µl of the PCR reaction. A Nucleospin column (supplied in the kit) was placed into a 2 ml collecting tube and the sample added to the column. The column was centrifuged at 11000 x g for 1 min in Biofuge Heraeus bench top centrifuge, the flow through discarded, and the column, placed back into the collecting tube. The column was washed with 600 µl of buffer NT3 by centrifugation at 11000 x g for 1 min and the flow through discarded. To remove excess buffer NT3, the silica membrane was re-centrifuged for 2 min at 11000 x g. The column was then placed into a sterile 1.5 ml microcentrifuge tube, 50 µl prewarmed (50 ^{o}C) buffer NE added, and incubated at room temperature for 1 min to increase the yield of DNA. DNA was eluted by centrifugation at 11000 x g for 1 min. The eluted DNA was quantified at 260 nm and 280 nm in order to determine the DNA concentration and purity.

2.4.3 Converting 5' overhangs to a blunt end terminus

Purified PCR products were subjected to exonuclease activity of Klenow DNA polymerase (Roche Biochemicals, USA). This reaction ensured that each termini of the PCR product was blunt ended. The contents of each reaction are shown in table 2.4.

Table 2.4. Components used for the Klenow reaction for leucocin A and pre-leucocin A.

Reagent	Klenow reaction 1		Klenow reaction 2	
	Vol (µl)	Final conc.	Vol (µl)	Final conc.
Klenow polymerase (100 U/ml) (Roche Biochemicals, USA)	1	5 U/ml	1	5 U/ml
10mM dNTP mix (Roche Biochemicals, USA)	0.2	0.2 M	0.2	0.2 M
Buffer H[a] (10 X conc.)	2	1 X	2	1 X
Leucocin A (2.98 µg/ml)	16.8	2.50 µg/ml		
Pre-leucocin A (5.12 µg/ml)			16.8	4.30 µg/ml
Total reaction	20		20	

[a] Buffer H (20 mM Tris-Cl pH 7.5 at 37 ^{o}C; 10 mM $MgCl_2$; 100 mM NaCl; 1 mM Dithioerythritol

Each reaction was performed in a sterile microcentrifuge tube and incubated at 30 ^{o}C for 15 min in a water bath. The reaction was stopped by heating in a water bath at 75 ^{o}C for 10 min.

2.4.4 Molecular cloning and transformation

A. Restriction digestion of the pMAL-c2 expression vector

Procedures for DNA manipulations and cloning were performed as described by Sambrook *et al.* (1989). Expression vector pMAL-c2 was restricted using enzymes *Xba*1 (creates "sticky" ends) and *Asp*700 (creates blunt ends) (Roche Biochemicals, USA). Reactions were carried out according to manufacturer's instructions and in a sterile microcentrifuge tube. Two single digest reactions of pMAL-c2 using enzymes *Xba*1 and *Asp*700 served as digest controls. Components of each restriction reaction are shown in table 2.5.

Table 2.5. Reaction components used for the restriction of expression vector pMAL-c2. Single digest reactions which served as controls are also listed.

Reagent	**Double (*Xba*1/*Asp*700)**	**Single (*Xba*1)**	**Single (*Asp*700)**	
	Vol (µl)	**Vol (µl)**	**Vol (µl)**	**Final conc.**
pMAL-c2 (12.62 µg/ml)	10	10	10	6.31 µg/ml
*Xba*1 (1000 U/ml)	1	1	0	50 U/µl
*Asp*700 (500 U/ml)	1	0	1	25 U/µl
Buffer B[a] (10 X conc.)	2	0	2	1 X
Buffer H[b] (10 X conc.)	0	2	0	1 X
Sterile dH_2O	6	7	7	
Total	20	20	20	

[a] Buffer B (10 mM Tris-Cl pH 7.5 at 37 ^oC; 10 mM $MgCl_2$; 0.1 mg/ml BSA) (Roche Biochemicals, USA)

[b] Buffer H (20 mM Tris-Cl pH 7.5 at 37 ^oC; 10 mM $MgCl_2$; 100 mM NaCl; 1 mM Dithioerythritol) (Roche Biochemicals, USA)

All restriction digests were incubated at 37 ^oC, overnight in a waterbath. Reactions were stopped and enzymes inactivated by heating at 65 ^oC for 10 min in a waterbath. Restricted DNA fragments were resolved on a 1.2 % agarose gel (8 V/cm, 90 min) to verify restrictions were successful. Samples were stored at -20 ^oC until future use.

B. Restriction digestion of PCR products

The amplified leucocin A and pre-leucocin A genes were restricted with an enzyme compatible to that used to restrict the pMAL-c2 vector. Restricting the amplified inserts with *Xba*1 ensures complementary sequences when ligating the vector and insert. Reactions were prepared in sterile microcentrifuge tubes and the composition of each reaction is outlined in table 2.6.

Table 2.6. Reaction components used for the restriction of amplified pre-leucocin A and leucocin A gene sequences.

Reagent	leucocin A digest		pre-leucocin A digest	
	Vol (µl)	Final conc.	Vol (µl)	Final conc.
Pre-leucocin A[a] (4.30 µg/ml)	10	2.15 µg/ml		
Leucocin A[a] (2.98 µg/ml)			10	1.49 µg/ml
Buffer H[b] (10X conc.)	2	1 X	2	1 X
*Xba*1 (1000 U/µl) (Roche Biochemicals, USA)	1	50 U/µl	1	50 U/µl
Sterile dH_2O	7		7	
Total reaction	20		20	

[a] Purified DNA from each Klenow reaction was used

[b] Buffer H (20 mM Tris-Cl pH 7.5 at 37 °C; 10 mM $MgCl_2$; 100 mM NaCl; 1 mM Dithioerythritol) (Roche Biochemicals, USA)

All restriction digests were incubated at 37 °C, overnight in a waterbath. Reactions were stopped and enzymes inactivated by heating at 65 °C for 10 min in a waterbath. Restricted DNA fragments were resolved by electrophoresis on a 1.2 % agarose gel (8 V/cm, 90 min). Samples were stored at -20 °C until future use.

C. Preparation of *Escherichia coli* JM103 Competent Cells

Escherichia coli JM103 cells were made competent using standard procedures (Sambrook *et al.*, 1989). An overnight culture of *E.coli* JM103 was prepared by inoculating a single colony into 50 ml LB broth. Following incubation at 37 °C with vigorous shaking, 2 ml of this starter culture was inoculated into 200 ml LB broth. This was incubated in a 37 °C waterbath with vigorous shaking. The culture was grown to an optical density (OD_{590}) of 0.375 and dispensed into 4 x 50 ml pre-chilled sterile centrifuge tubes (Beckman Coulter, South Africa). Cells were incubated on ice for 5 min and thereafter harvested by centrifugation in a Beckman centrifuge using a JA-20 rotor (5 min; 5000 x g at 4 °C). The supernatant was discarded, and each pellet was resuspended in 10 ml ice-cold $CaCl_2$ solution [60 mM $CaCl_2$; 10 mM PIPES, pH 7.0]. Cells were centrifuged at 5000 x g for 10 min at 4 °C. Supernatants were again

discarded, pellets resuspended in 10 ml ice-cold $CaCl_2$ solution and incubated on ice for a further 30 min. Cells were pelleted at 5000 x g for 10 min at 4 ^oC, supernatants discarded and pellets resuspended in 2 ml ice cold $CaCl_2$ solution containing 15 % (v/v) glycerol. Cell solutions were dispensed into pre-chilled sterile microcentrifuge tubes in 250 µl aliquots. Each aliquot was immediately stored at -70 ^oC until future use.

D. Ligation and Transformation of the cloned vector

The *Xba*1 restricted inserts were ligated into the *Xba*1/*Asp*700 site of pMAL-c2. All ligations were carried out in sterile microcentrifuge tubes using T4-DNA ligase (Roche Biochemicals, USA) according to manufacturers' recommendations. Vector, insert and sterile distilled water were added to a microcentrifuge tube and heated to 65 ^oC for 5 min. Thereafter T4-DNA ligase and ligation buffer were added. A reaction using restricted pMAL-c2 vector without insert DNA, was used as a control ligation. Specific volumes of each component in the reaction are shown in table 2.7.

Table 2.7. Reaction components used for the ligation of leucocin A and pre-leucocin A into the *Xba*1/*Asp*700 site of the pMAL-c2.

Reagent	**leucocin A**		**pre-leucocin A**	
	Vol (µl)	**Final conc.**	**Vol (µl)**	**Final conc.**
Restricted pMAL-c2 (6.31 µg/ml)	1	0.32 µg/ml	1	0.32 µg/ml
Restricted pre-leucocin A (2.15 µg/ml)	10	1.08 µg/ml		
Restricted leucocin A (1.49 µg/ml)			10	0.75 µg/ml
Sterile dH_2O	5		5	
T4-DNA Ligase (1000 U/ml) (Roche Biochemicals, USA)	2	100 U/µl	2	100 U/µl
Ligase Buffer (10 X conc.) (Roche Biochemicals, USA)	2	1 X	2	1 X
Total reaction	20		20	

The reactions were incubated overnight at 16 ^{o}C in a circulating waterbath. Following incubation, ligations were heated at 65 ^{o}C for 10 min in a waterbath to inactivate the ligase enzyme and cooled on ice. Ligation reactions were stored at -20 ^{o}C until further required.

For transformation, *E.coli* JM103 competent cells were thawed on ice and mixed by gentle finger tapping. Once thawed, 50 µl of competent cells were added to a pre-chilled microcentrifuge tube. Ligation mixture (15 µl), was added to the competent cells. The microcentrifuge tube was gently mixed and the mixture incubated on ice for 30 min. Following incubation the mixture was heat shocked at 42 ^{o}C for 2 min. Thereafter, 1 ml pre-warmed LB broth (37 ^{o}C) was added to the mixture and incubated at 37 ^{o}C for 1 hr. The positive transformation control consisted of uncut pMAL-c2 vector (10 µl of a 12.62 µg/ml stock) and 50 µl of competent cells. Negative controls consisted of restricted pMAL-c2 vector (10 µl of a 6.31 µg/ml pMAL-c2 restriction stock), and 50 µl of competent cells. Competent cells (50 µl) were also added to 10 µl of the ligation control. This mixture was also used for transformation. Aliquots of 100 µl of the test transformation mix and 50 µl of the controls were plated on LB agar plates supplemented with ampicillin (100 µg/ml), X-galactosidase (X-gal) (80 µg/ml) and IPTG (0.3 mM) (Roche Biochemicals, USA). Controls for *E.coli* JM103 competent cells constituted 100 µl competent cells and 1 ml LB broth. These were plated in 50 µl aliquots on LB agar plates containing no ampicillin and LB agar plates with ampicillin (100 µg/ml). Plates were incubated at 37 ^{o}C overnight and thereafter, viewed for the presence of blue and white colonies.

2.5 Screening for Recombinants

Following overnight incubation, LB agar plates containing transformation mixtures were observed for the presence of recombinants using the blue/white selection method (Sambrook *et al.*, 1989). Blue colonies represented non-recombinants i.e. cells containing pMAL-c2 vector. White colonies on X-gal, IPTG, amp plates were indicative of β-galactosidase negative phenotypes also known as recombinants. Recombinant colonies were selected using sterile toothpicks and transferred to fresh LB agar plates containing ampicillin (100 µg/ml) and incubated overnight at 37 ^{o}C. Recombinant DNA isolated from these colonies (as described in section 2.3.3) was used in various screening methods, i.e. southern blot hybridization and colony PCR. Vector pMAL-c2 containing the leucocin A gene within the polycloning site was designated as pLcaA, while pMAL-c2 vector containing the pre-leucocin A gene was named pPreLcaA.

2.5.1 Southern blot hybridization

The southern blot hybridization procedure was carried out using the JIR-1 probe (5' – AAATATTATGGTAATGGTGT – 3'), which was designed such that it is homologous to the 5' end of the leucocin A sequence. True recombinants could be selected by binding the probe to this gene sequence. Plasmid DNA to be hybridized were resolved on a 0.8 % agarose gel (8 V/cm, maximum current). Also included in the gel were control DIG-ddUTP labeled DNA (Roche Biochemicals, USA), pJF5.5 plasmid DNA (positive controls) and pMAL-c2 plasmid DNA (negative control). Following electrophoresis, DNA was transferred onto MS1 Nylon Transfer Membrane (GE Osmonics, USA).

A. Oligonucleotide tailing with DIG-dUTP dATP

The DIG Oligonucleotide 3'-end labeling kit (Roche Biochemicals, USA) was used to label the JIR-1 oligonucleotide probes. The JIR-1 probe used was designed in such way that it binds to the 5' end of each form of the bacteriocin structural gene i.e. leucocin A and pre-leucocin A. The end of the gene codes for the N-terminal region of the bacteriocin which is represented by the conserved KYYGNGV consensus sequence. The tailing reaction was prepared by adding the following solutions to a sterile microcentrifuge tube:

i. 4 µl of 5x reaction buffer [1 M Potassium cacodylate; 0.125 M Tris-Cl; 125mg/ml Bovine serum albumin (BSA), pH 6.6]
ii. 4 µl $CoCl_2$ (25 mM);
iii. 1 µl DIG-dUTP solution;
iv. 1 µl (50 U) terminal transferase;
v. 0.2 µl JIR-1 probe (100 pmoles) and 9.8 µl sterile distilled water.

The reaction mixture was mixed, incubated at 37 ^{o}C for 30 min, and placed on ice. Hybridization solution [100 µl, 6 x SSC, 0.5 % SDS and 5 x Denhardt's solution] was added to the tailing reaction and stored at –20 ^{o}C for use in hybridization experiments. Denhardt's solution was prepared to a 100X concentration and contained 2 % Ficoll 400; 2 % polyvinylpyrrolidone; 2 % Bovine serum albumin, Fraction V (Roche Biochemicals, USA), and stored at -20 ^{o}C.

B. Transfer of DNA onto nitrocellulose membrane

The VacuGene XL Protocol No. 1 (Pharmacia, USA), was used to transfer DNA onto a nitrocellulose membrane of 110 x 70 mm in size. The nitrocellulose membrane was incubated prior to transfer, in sterile distilled water for 1 min and 20 x SSC for 10 min respectively. The agarose gel containing plasmid DNA was subjected to depurination for 20 min by immersing the gel in 50 ml solution I [0.2 N HCl]. A denaturation step then involved immersing the agarose gel in 50 ml solution II [0.5 M NaOH; 1.5 M NaCl] for 20 min. Following denaturation the gel was subjected to neutralization for a further 20 min by immersion in 50 ml solution III [1 M Tris, pH 7.5; 1.5 M NaCl]. DNA was transferred onto nitrocellulose membrane with transfer solution, 20X SSC [3 M NaCl; 0.3 M Na Citrate.$2H_20$] for 60 min.

Following transfer the wells were clearly marked, and the gel removed. The agarose gel was viewed using a UV transilluminator in order to determine whether all DNA was successfully transferred. The nitrocellulose membrane was washed in 2 x SSC for 5 min to remove any debris. The filter was then air dried for 30 min on Whatman 3 mm filter paper (Whatman, UK).

C. Pre-hybridization and hybridization of the probe to the DNA

The blotted membrane was placed in a Hybaid HB-OV-BL hybridization tube (Labnet, USA) containing 10 ml of hybridization solution; and pre-hybridized overnight at 42ºC in a Hybaid™ Micro-4 hybridization oven. Following incubation, the hybridization solution was decanted and replaced with 10 ml hybridization solution containing 100 µl labeled JIR-1 probe. The membrane was hybridized with the probe at 42 ºC overnight. The membrane was washed twice in 2 x SSC, 0.1 % SDS for 15 min each and twice in 0.1 x SSC, 0.1 % SDS, for a further 15 min each. All washes were carried out with gentle agitation. The membrane was allowed to air dry on Whatman 3mm filter paper.

D. DIG nucleic acid detection

The detection procedure was carried out using the Roche DIG Detection kit. The hybridized membrane was washed in buffer 1 [0.1 M Tris-Cl, pH 7.5; 0.15 M NaCl] for 1 min. The membrane was blocked using freshly prepared Buffer 2 [1 % skim milk powder in 100 ml Buffer 1] at room temperature for 30 min with continuous shaking. After blocking, the membrane was washed for 60 min in Buffer 1. Anti-digoxigenin-AP conjugate was diluted in buffer 2 to give a final concentration of 150 mU/ml and the membrane was incubated for 30 min in 20 ml of this solution. The membrane was thereafter washed twice for 15 min in buffer 1 to remove any unbound antibody conjugate. The membrane was equilibrated for 2 min in 20 ml of Buffer 3 [0.1 M Tris-Cl, pH 9.5; 0.1 M NaCl; 50 mM $MgCl_2$]. Freshly prepared colour substrate solution [200 µl NBT/BCIP solution in 10 ml Buffer 3], was prepared and the membrane incubated in colour substrate solution in the dark. A colour precipitate was allowed to develop and when the desired bands were detected, the membrane was washed in 1X TE buffer to stop the reaction.

2.6 Sequence analysis of Recombinant Clones

Automated DNA sequencing of recombinant plasmids was performed by Inqaba Biotech. The submitted plasmid DNA was sequenced using the BigDye version 3.1 dye terminator cycle sequencing kit (Applied Biosystems, USA). PCR reactions were prepared in a 96-well plate followed by PCR purification using ethanol/EDTA precipitation. The purified PCR products were thereafter sequenced using the Genetic analysis system SCE2410 containing 24 capillaries from SpectruMedix LLC. Sequenced data was analyzed using the BaseSpectrum V2.1.1 (SpectruMedix, USA).

2.7 Production and Purification of MBP fusion peptides

Each fusion protein to be expressed was referred to as MBP-leucocin A (MBP-LcaA) and MBP-preleucocin A (MBP-preLcaA) according to the particular peptide fused to the affinity tag. Protein expression and purification for MBP fusion proteins was performed as described by the pMAL™ Protein Fusion and Purification System (New England Biolabs, UK). A schematic representation of the steps used to express and purify the MBP fusion proteins is displayed in figure 2.3.

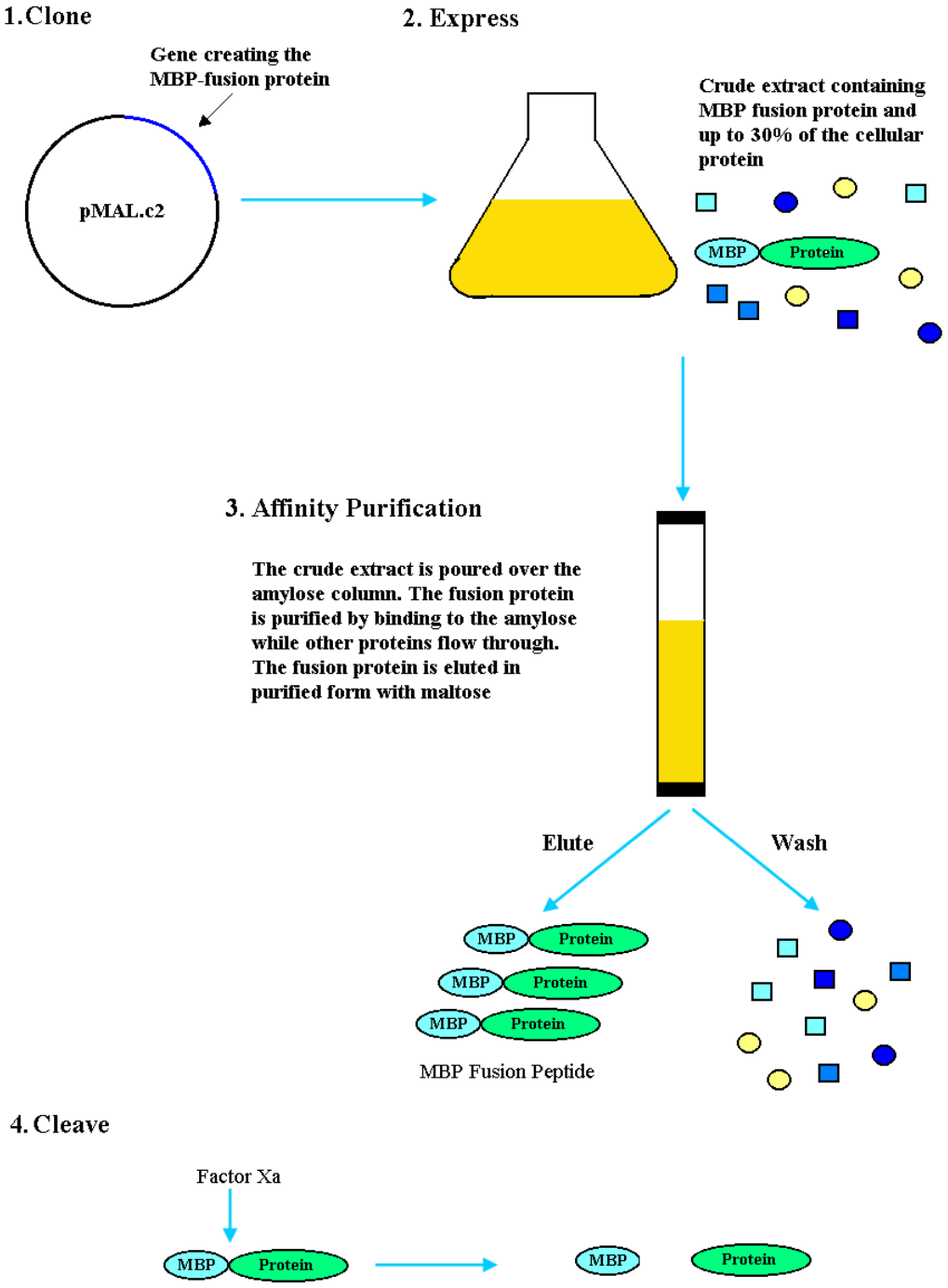


Figure 2.3. Schematic representation of the affinity purification procedure used to isolate MBP fusion proteins. Expressed MBP fusion proteins were subjected to amylose affinity chromatography. The maltose binding protein has an affinity for amylose and binds to the resin while non-specific proteins flow through the column. The MBP fusion protein was isolated by eluting with maltose. Purified MBP fusion proteins were cleaved with Factor Xa to remove the MBP affinity tag (Adapted from New England Biolabs, 1991).

2.7.1 Expression of the MBP fusion peptides by induction with IPTG

Initially protein expression and purification was conducted on a small scale using 200 ml LB and then up scaled to 1 L. Overnight, cultures of recombinant plasmids pLcaA and pPreLcaA, were prepared in 50 ml LB broth supplemented with ampicillin (100 µg/ml). Cultures were grown overnight at 37 ^{o}C with vigorous shaking and good aeration. LB broth (2 x 1 Litre) containing ampicillin (100 µg/ml) was subcultured with 10 ml of each of the overnight cultures. Cells were grown with good aeration to approximately 2 x 10^8 cells/ml (A_{600} of ~0.5). When an optical density of 0.5 was reached, an uninduced sample (1 ml) was removed from each culture and micro-centrifuged for 2 min at 5000 x g. The supernatant was discarded and pellets were resuspended in 50 µl 2X SDS-PAGE sample buffer [0.125 M Tris-Cl; 4% SDS; 20 % (v/v) glycerol; 0.2 M dithiothreitol; 0.02 % bromophenol blue, pH 6.8]. This was considered the uninduced sample. To the remaining culture, IPTG (0.1 mM) to a final concentration of 0.3 mM was added, and further incubated at 37 ^{o}C for 2 hrs. A 0.5 ml sample was thereafter removed from each culture and micro-centrifuged at 5000 x g for 2 min. The supernatant was discarded and the pellet was resuspended in 100 µl 2X SDS-PAGE sample buffer. This was considered the induced sample. Cells were harvested from the remaining cultures by centrifugation at 4000 x g for 20 min in a Beckman centrifuge using a JA-20 rotor. Supernatants were discarded and pellets resuspended in 50 ml chilled column buffer [200 mM Tris.Cl pH 7.4; 200 mM NaCl; 1 mM EDTA; 3 mM DTT] and stored at -20 ^{o}C overnight. Induced and uninduced samples were boiled in a water bath for 5 min and protein expression was analyzed by 10 % SDS-PAGE (Laemmli buffer system, 1970). The gel was electrophoresed at 18 mA for 2 hrs, Coomassie stained overnight, destained, and viewed. A detailed protocol for SDS-PAGE is provided in section 2.15.

Stored inducted samples were thawed in ice-cold water and cells were sonicated using a VirSonic 60 sonicator (Virtis, USA) to release their cytoplasmic content. Sonication was performed at 0.10 watts (RMS) in 1 min intervals. The release of protein was monitored at 595 nm by removing 10 µl of the sonicate and adding it to 1.5 ml Bradford reagent [50 mg Serva Blue G, 88 % phosphoric acid, 99.5 % ethanol, dH_2O. Sonication was continued for 5 min until the maximum amount of cytoplasmic content was released and a 1 ml sample was extracted for analysis. The sonicated samples were centrifuged at 9000 x g for 30 min at 4 °C. The crude extracts (supernatants) containing fusion proteins, were saved on ice. Pelleted insoluble matter was resuspended in 50 ml column buffer. Aliquots of 5 µl were removed

from the sonicated sample, crude extract and insoluble matter and added to 5 µl 2X SDS sample buffer in a sterile microcentrifuge tube. These were boiled for 5 min and the protein profiles were compared by 10 % SDS-PAGE. The gel was electrophoresed at 18 mA for 2 hrs, Coomassie stained overnight, destained, and viewed

2.7.2 Fusion protein purification by affinity chromatography

Fusion proteins were purified using amylose affinity chromatography. Amylose resin (New England Biolabs, UK) with a bed volume of 14 ml was prepared in a 2.5 x 10 cm ion exchange columns (BioRad, South Africa). The columns were equilibrated with 8 column volumes column buffer. Crude extracts were loaded onto each column and the flow through was collected at a flow rate of 1 ml/min. The flow through was retained and stored at -20 °C for further analysis. The resin was washed (3 x) with 60 ml column buffer to remove non-specific proteins. A 1 ml aliquot of each wash was collected and stored at -20 °C for further analysis. Following the wash step fusion protein was eluted with column buffer containing maltose [200 mM Tris.Cl pH 7.4; 200 mM NaCl; 1 mM EDTA; 10 mM maltose]. Ten 3 ml fractions were collected and assayed using the BCA protein assay to determine protein concentration.

Aliquots (5 µl) of the crude extracts, flow through and washes were each added to 5 µl of 2X SDS sample buffer, boiled for 5 min and analyzed on a 10 % SDS-PAGE gel. The first nine fractions eluted were similarly analyzed but using 20 µl of sample added to 10 µl of 2X SDS sample buffer. All SDS gels were electrophoresed at 18 mA for 2 hrs, Coomassie stained overnight, destained, and viewed. The purification process for MBP-LeuA and MBP-preLeuA was monitored by taking absorbance readings of the crude extract, flow through, washes and elutions at 280 nm. These values were then used to construct a chromatogram by plotting the sample type against the absorbance at 280 nm.

The amylose column was regenerated by washing with 3 column volumes sterile distilled water followed by a further wash using 3 column volumes 0.1 % SDS. Thereafter, sterile distilled water (1 column volume) was passed through the column. The final step consisted of a wash using 3 column volumes column buffer.

2.7.3 Quantification of MBP Fusion Proteins

Fractions collected by amylose chromatography were quantified for total protein content using the Pierce Micro BCA™ (bicinchoninic acid) Protein Assay (Pierce, USA). The BCA assay involved the preparation of a standard curve using Bovine Serum Albumin from which the concentration of each eluent was calculated.

A. Preparation of Diluted Albumin (BSA) Standards

Table 2.8 outlines the procedure used to prepare a fresh set of BSA protein standards. The contents of one Albumin Standard ampule (1 ml containing 2.0 mg/ml Albumin Standard) was diluted into several clean test tubes, using the same diluent as the protein to be assayed, in this case column buffer.

Table 2.8. Preparation of diluted Albumin (BSA) standards.

Test tube	Volume of diluent (ml)	Volume of BSA (ml)	Final BSA concentration (µg/ml)
A	4.5	0.5 of stock	200
B	8.0	2.0 of A	40
C	4.0	4.0 of B	20
D	4.0	4.0 of C	10
E	4.0	4.0 of D	5.0
F	4.0	4.0 of E	2.5
G	4.8	3.2 of F	1.0
H	4.0	4.0 of G	0.5
I	8.0	0	0 (blank)

B. Sample preparation

The working solution used to analyze each fraction was prepared using the MA [sodium carbonate and sodium tartrate in 0.2 N NaOH], MB [4 % bicinchoninic acid in water], and MC [4% cupric sulphate pentahydrate in water] reagents all of which were supplied in the Pierce Micro BCA™ Protein Assay kit. The working solution was made up to 5 ml using 2.5 ml MA, 2.4 ml MB, and 0.1 ml MC. Thereafter, 250 µl of each elution was added to 250 µl of

the prepared working solution in a sterile microfuge tube. Bovine serum albumin standards (250 µl) were similarly added to 250 µl of working solution in a sterile microfuge tube. All microfuge tubes (eluent samples and BSA standards) were incubated at 60°C for 60 min. Following incubation, absorbance readings were taken at 562 nm of each BSA standard sample and each eluent sample. A standard curve was constructed by plotting the absorbance of each BSA standard at 562 nm against the known concentration of BSA in µg/ml. A regression line was obtained from the standard curve and using the equation $y = mx + c$, the protein concentration of each fraction was calculated. The y value in this equation represented the absorbance taken at 562 nm for each fraction, while m is the regression value provided with the regression curve. The y-intercept of the regression curve was substituted for c. Fractions containing the highest MBP fusion concentration were verified for MBP-LcaA and MBP-preLcaA by results obtained by SDS-PAGE analysis. Once fractions were chosen, each MBP fusion protein was cleaved with Factor Xa.

2.8 Western blot of the Purification Components

A western blot was performed using anti-MBP monoclonal antibodies to detect the MBP fusion protein. The Mini Trans-Blot Electrophoretic Transfer Cell (BioRad, South Africa) was used to transfer proteins onto a nitrocellulose membrane. The apparatus and setup used for the transfer is shown in figure 2.4. Two western blots were performed for purification of each MBP fusion protein.

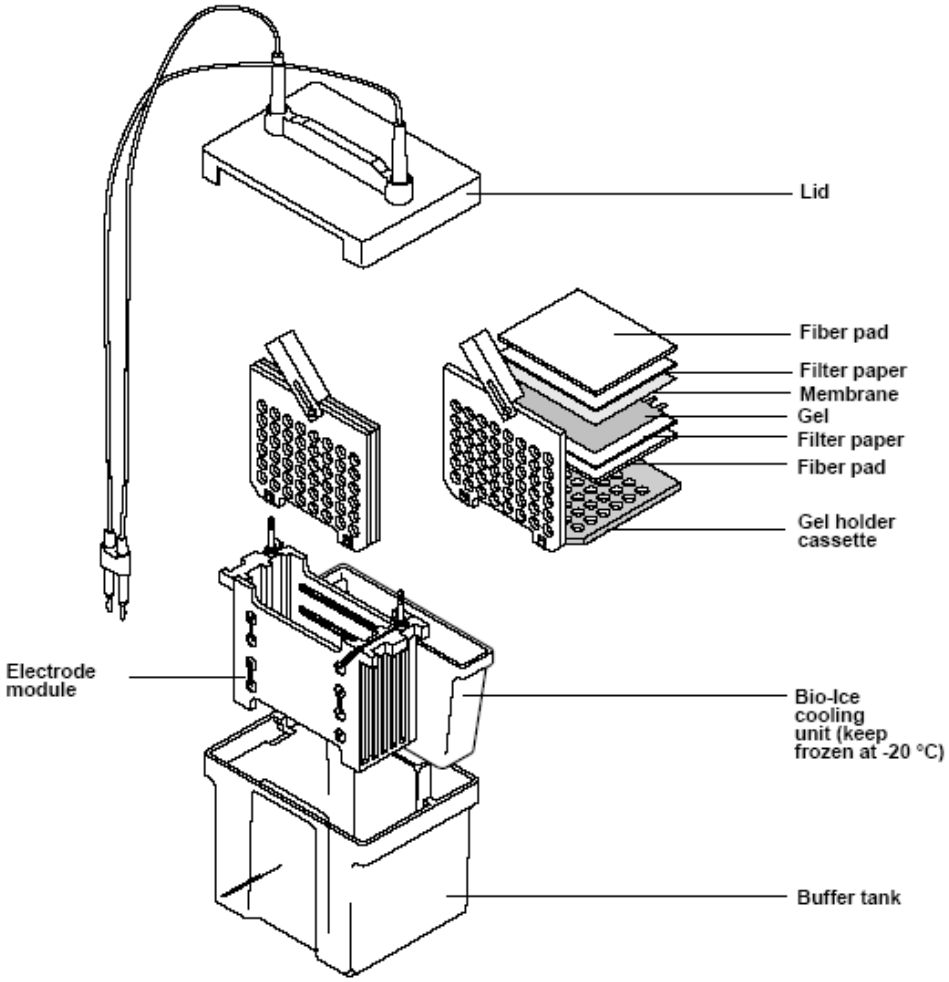


Figure 2.4. A description and assembly of The Mini Trans-Blot Electrophoretic Transfer Cell used for the transfer of proteins onto nitrocellulose membrane (Biorad Laboratories).

2.8.1 Transfer of fusion proteins onto nitrocellulose membrane

Samples chosen to be blotted included the uninduced and induced cytoplasmic crude extracts, sonicated samples, crude extracts, flow-through, wash and MBP fusion protein. A BioRad protein standard and maltose binding protein (positive control) were also included. SDS-PAGE was carried out in duplicates, with one gel being used as a reference gel, while the other was used in the transfer procedure. Following electrophoresis, the reference gel was removed and the Coomassie staining procedure was carried out. The gel to be blotted was soaked in ice-cold Towbin buffer [25 mM Tris; 192 mM glycine; 20 % methanol (v/v), pH 8.3] (Towbin *et al.*, 1979). The gel cassette holder and the nitrocellulose membrane (6 x 9 cm), were similarly soaked. All components were soaked for 30 min in Towbin buffer and thereafter, the sandwich (gel holder cassette) consisting of two filter pads (BioRad, South Africa), the SDS gel, nitrocellulose membrane and Whatman 3 mm filter paper, was assembled as indicated in figure 2.4. During assembly the formation of air bubbles was prevented by rolling the membrane onto the SDS gel using a test tube. The sandwich was inserted into the Mini-Trans Cell which was thereafter filled with ice-cold Towbin buffer. The transfer was conducted for 90 min at 90 V. The efficiency of the transfer was assessed by carefully removing the membrane and gel from the gel holder cassette and briefly staining the membrane in Ponceau S [0.2 % Ponceau S (w/v) (BioRad, South Africa) in 1 % acetic acid (v/v)]. Upon staining with Ponceau S, the protein standard was marked in pencil. The Ponceau S stain was removed by immersing the membrane in sterile distilled water containing a few drops of NaOH [1 M]. The membrane was then allowed to air dry on Whatman 3 mm filter paper for approximately 2 hrs. The SDS gel was Coomassie stained overnight, destained and viewed for the presence of protein bands (if present).

2.8.2 Detection of the MBP fusion proteins

For the detection of the MBP fusion proteins, anti-MBP antibodies were used. The nitrocellulose membranes containing blotted protein, were blocked for 1 hr with 5 % (w/v) non-fat milk powder in phosphate buffered saline (PBS) [100 mM phosphate; 1.5 M NaCl, pH 7.2]. The membrane was washed (3 x 5 min) in 1X PBS containing 0.1 % (v/v) Tween 20 (Merck, South Africa). Anti-MBP monoclonal antibodies (10 µl of a 1 mg/ml stock solution) (New England Biolabs, UK) was added to 10 ml PBS containing 0.5 % (w/v) BSA (Roche

Biochemicals, USA). The membrane was incubated in the antibody solution at room temperature for 1 hr with gentle agitation. Following incubation, the membrane was washed (3 x 5 min) in 1X PBS containing 0.1 % (v/v) Tween 20. BM Blue peroxidase (POD) precipitating chromogenic substrate (5 ml) (Roche Biochemicals, USA) was added to the membrane in a foil covered container, until the protein bands were visible. Detection was stopped by removing the membrane from the substrate solution and washing in sterile, distilled water. The membrane was then air dried between Whatman 3 mm filter paper. Banding patterns formed by western blotting were then compared to each reference SDS gel.

2.9 Bactericidal activity of MBP fusion protein

The purified MBP-LcaA and MBP-preLcaA were tested for activity against *L. monocytogenes* using the deferred inhibition method described in section 2.2.1. Aliquots of 2 to 20 µl of each fusion protein were spotted onto an MRS plate overlaid with TSA containing *L. monocytogenes*. Plates were allowed to dry and incubated at 25 °C, overnight. Plates were then analyzed for active MBP fusion constructs by viewing for zones of inhibition.

2.10 Cleavage of MBP-Leu A and MBP-preLeu A using Factor Xa

A pilot experiment using a portion of each MBP fusion protein was performed to determine the optimal time required for cleavage. In separate sterile microcentrifuge tubes 20 µl of the fusion peptide was added to 1 µl of Factor Xa (1 mg/ml) (New England Biolabs, UK) and incubated at 25 °C in a waterbath. At 2, 4, 8 and 24 hrs, 5 µl of the reaction was removed, and added to 5 µl 2X SDS-sample buffer and saved on ice. A control reaction with 5 µl of uncut MBP fusion protein without Factor Xa (uncut control) was also incubated at 25 °C for 24 hrs and thereafter added to 5 µl 2X SDS-sample buffer. All samples were boiled for 5 min and analyzed by 10 % SDS-PAGE. The cleavage experiment was then scaled up using 1 ml of each fusion peptide i.e. MBP-LeuA (2.7536 µg/ml) and MBP-preLeuA (2.8048 µg/ml) and 50 µl Factor Xa (1 mg/ml) in a sterile microcentrifuge tube. These reactions were incubated overnight in a 25 °C waterbath. An uncut control reaction consisting of MBP fusion protein (20 µl) without Factor Xa was also included. Following incubation, 20 µl of each reaction (including controls) was added to 10 µl of 2X SDS sample buffer, boiled for 5 min, and analyzed by SDS-PAGE at 90 V for 30 min.

2.11 Bactericidal activity of cleaved MBP fusion protein

Cleavage mixtures containing maltose binding protein, Factor Xa, and either leucocin A or pre-leucocin A, were tested for activity against *L. monocytogenes* using the deferred inhibition method described in section 2.2.1. Aliquots of 20 µl of each cleavage reaction were spotted onto an MRS plate overlaid with TSA containing *L. monocytogenes*. Plates were allowed to dry and incubated overnight, at 25 °C. The plate was analyzed for active leucocin A and pre-leucocin A by viewing for zones of inhibition.

2.12 N-terminal protein sequencing of leucocin A and pre-leucocin A

Protein bands produced after Factor Xa cleavage were verified for the presence of recombinant peptides, leucocin A and pre-leucocin A, by N-terminal amino acid sequencing. SDS-PAGE was carried out on duplicate gels for each Factor Xa reaction. The cleaved components of the Factor Xa reaction were transferred onto a polyvinylidene fluoride (PVDF) membrane by electroblotting and each band of interest excised and sequenced using a Procise cLC Protein Sequencer (Applied Biosystems, USA). A 60 µl aliquot from each Factor Xa reaction, i.e. pre-leucocin A and leucocin A, was added to 20 µl of 2X SDS sample buffer, and boiled for 5 min. These samples were electrophoresed together on duplicate 15 % SDS PAGE gels at 18 mA constant current for 90 min. A BioRad protein standard was also included on each of the SDS gels. One gel was stained with Coomassie Blue and used as a reference gel, while the other was transferred onto PVDF membrane.

2.12.1 Transfer of cleaved fusion protein onto PVDF membrane

The same procedure used for western blotting (shown in figure 2.4), was used for transfer of cleaved proteins onto polyvinylidene difluoride membrane. However, there were variations in the buffers and solvents used in the transfer step. The gel holder cassette together with the SDS gel to be blotted, were soaked in ice-cold CAPS buffer [10 mM CAPS, pH 11; 10% methanol] for 30 min. BioTrace™ PVDF membrane (6 x 9 cm) (PALL Life Sciences, USA) was soaked in 100 % methanol for 15 min and thereafter, transferred to CAPS buffer for an additional 15 min, to remove excess methanol. The sandwich was assembled and bubbles were removed as described in 2.8.1. The sandwich was inserted into the Mini-Trans Cell filled with ice-cold CAPS buffer and transfer was conducted at 90 V for 2 hrs. Following the transfer procedure the PVDF membrane together with the SDS gel were treated as described in 2.8.1.

2.12.2 N-terminal protein sequencing

Automated N-terminal protein sequencing of leucocin A and pre-leucocin A, was conducted on a 476A sequencer equipped with a 120A HPLC system (Procise cLC Protein Sequencer, Perkin-Elmer Applied Biosystems, USA). All reagents and solvents used for sequencing were obtained from Perkin-Elmer Applied Biosystems, USA. The sequencer program was modified so that 13 amino acid residues were sequenced from the N-terminal region of leucocin A and pre-leucocin A. The bands of interest were excised from the PVDF blot, placed into the reaction chamber, and the sequencing run was initialized. The first cycle of the sequencing run consisted of a standard mixture of 19 PTH-amino acids which was injected into the column for separation. The chromatogram produced for the 19 PTH-amino acids provided standard retention times for comparison with each Edman degradation cycle chromatogram of the peptides that were sequenced. For specific chemistries behind Edman degradation and principles of N-terminal sequencing, refer to chapter one, section 18.2.

The HPLC chromatograms for each derivitised amino acid of leucocin A and pre-leucocin A, were collected using a computer data analysis system. These chromatograms were overlayed one on top of the other in the order in which they were derivitised, in order to determine the amino acid present at a particular residue number. The standard retention times produced by the 19 PTH-amino acids were used to determine the N-terminal sequence of leucocin A and pre-leucocin A.

2.12.3 N-terminal sequence analysis for leucocin A and pre-leucocin A

Once the sequences were obtained, a BLAST search was conducted using EXPASY. By this method, the sequence obtained from N-terminal sequencing was verified against the leucocin A and pre-leucocin A sequences found in the EXPASY database.

2.13 Isolation of peptides by high performance liquid chromatography

High performance liquid chromatography was used to separate each recombinant peptide from the MBP affinity tag and Factor Xa. Each peptide was purified from the Factor Xa reaction by reverse phase HPLC on a C-18 column (Perkin-Elmer, USA). The column was 25 cm x 4.6 mm, had a particle size of 5 μm, and a pore size of 12.5 nm. Before analysis, a blank run was performed with acetonitrile containing 0.1 % TFA, to ensure that the column was free of contaminants. Aliquotes of 200 μl of the cleavage reaction was injected and the elution of each protein/peptide (flow rate of 1 ml per minute) was monitored at an absorbance of 220 nm. Profiles generated for each cleavage sample were kept aside, and thereafter, controls were analyzed. Control runs consisted of maltose binding protein (1.0023 μg/ml) and Factor Xa (0.1 mg/ml). These samples were individually injected into the HPLC system. Profiles generated for controls were overlapped with profiles from the corresponding cleavage sample. Overlapping peaks were identified and cancelled out. In this way, the peak representing the recombinant peptide (leucocin A or pre-leucocin A), was identified and samples were eluted in acetonitrile containing 0.1 % TFA. Maltose binding protein and Factor Xa were similarly collected. All samples collected were tested for activity by deferred inhibition of *Listeria*. The program used for the purification of leucocin A and pre-leucocin A is outlined below in table 2.9.

Table 2.9. The method used for the purification of leucocin A and pre-leucocin A from cleavage mixtures using reverse phase HPLC on a C-18 column.

Step	Time (min)	Flow rate (ml/min)	% A (Acetonitrile, 0.1 %TFA)	Curvature
E[a]	2.0	1.0	0	
1	20.0	1.0	0	1.0
2	20.0	1.0	50	1.0
3	5.0	1.0	90	1.0
4	5.0	1.0	0	1.0

[a] Equilibration step

2.14 Bactericidal activity of leucocin A and pre-leucocin A

Fractions collected by reverse phase HPLC containing maltose binding protein, Factor Xa, leucocin A , and pre-leucocin A were tested for activity against *L. monocytogenes* using the deferred inhibition method described in section 2.2.1. Aliquots of 20 µl of each fraction were spotted onto an MRS plate overlaid with *L. monocytogenes*. Plates were allowed to dry and incubated at 25 °C, overnight. The plate was analyzed for active leucocin A and pre-leucocin A by viewing for zones of inhibition.

Relative activities of leucocin A and pre-leucocin A, were determined by a double dilution assay. A serial double dilution (10^{-2} to 10^{-10}), was prepared for each peptide (20 µl) using 0.1 % TFA. Aliquots of 10 µl of each dilution were spotted onto an MRS plate overlaid with *L. monocytogenes*. The agar plate was allowed to dry and incubated at 25 °C, overnight. Following incubation, the plate was analysed for zones of inhibition produced by each dilution. Since the zone of inhibition becomes fainter, as the dilution factor increases, the relative activites in activity units per a milliliter (AU/ml), was based on the highest dilution able to inhibit *L. monocytogenes*. The method used to calculate the AU of leucocin A and pre-leucocin A is shown with the aid of a sample calculation below.

Sample calculation to determine activity units:

Highest dilution factor able to inhibit indicator strain = 1/6 → reciprocal = 6

Activity per milliliter = 6 x (1000 µl/ 10 µl), where 10 µl is the volume of sample spotted
= 600 AU/ml

Therefore, activity units within the stock solution = 600 x 1000 µl, for a 1 ml stock
= 6.0×10^{-5} AU
= total relative activity

2.15 Analysis of protein samples

2.15.1 Sodium dodecyl sulfate polyacrylamide gel electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis or SDS-PAGE was used to analyze all protein manipulations. The Hoefer SE 600 gel apparatus was used to assemble all polyacrylamide gels (BioRad, South Africa). The vertical slab gel unit was assembled and placed into the dual casting stand according to manufacturer's instructions. The resolving/running gel was prepared according to table 2.10 and the solution pipetted to a level about 3cm from the top of the gel caster. Once the resolving gel polymerized, a stacking gel (4 %) was prepared, as in Table 2.10, and pipetted above the resolving gel. Thereafter, a comb was immediately inserting into the stacking gel, which was then allowed to polymerase for 60 min. Samples containing the protein sample to be analyzed and 2X treatment buffer (0.125 M Tris-Cl, 4 % SDS, 20 % v/v Glycerol, 0.2 M DTT, 0.02 % bromophenol blue, pH 6.8) were placed in a microcentrifuge tube which was heated for 5 min in boiling water. Samples were allowed to cool, centrifuged for 2 min at 5000 x g, and placed on ice until ready for use. The comb was slowly removed from the gel unit and the caster containing the gel was removed from the casting unit. The gel was attached to the vertical slab gel electrophoresis unit with the lower chamber filled with ice cold tank buffer [0.025 M Tris-Cl, 0.192 M glycine, 0.1 % SDS, pH 8.3]. Each well was similarly filled with tank buffer. Approximately 10-40µl of each sample was loaded into each well. Electrophoresis was performed at 18 mA until the dye front reached the bottom of the gel. The gels were removed from the electrophoresis apparatus and viewed using standard Coomassie staining techniques (Laemmli, 1970).

Table 2.10. Composition of SDS-PAGE gel for separation of protein samples.

Solutions	Running Gel (15 %)	Running Gel (10 %)	Stacking Gel (4 %)
Acrylogel (BDH Electran, UK)	4.72 ml	3.15 ml	650 µl
Distilled water	3.82 ml	5.40 ml	3.00 ml
0.5M Tris pH 6.8	0	0	1.25 ml
3M Tris pH 8.8	1.25 ml	1.25 ml	0
Glycerol 50 % (v/v)	200 µl	200 µl	0
SDS 20 % (w/v)	50 µl	50 µl	25µl
TEMED (Promega, USA)	15 µl	15 µl	5µl
Ammonium persulfate 10 % (w/v)	36 µl	36 µl	25 µl

2.15.3 Staining and destaining of polyacrylamide gels

In order to view the relevant protein bands SDS-PAGE and Tris-tricine gels were stained overnight with gentle agitation, using Coomassie Blue R250 staining solution (0.025 % Coomassie brilliant blue R250 (Sigma-Aldrich, South Africa), 40 % methanol, 7 % acetic acid). Protein bands were viewed by destaining the polyacrylamide gel for 30 min in destaining solution I [40 % methanol; 7 % acetic acid]. Destaining solution I was removed and replaced with destaining solution II [7 % acetic acid; 5 % methanol], and the destaining process was continued until proteins bands were clearly visible.

CHAPTER THREE

RESULTS AND DISCUSSION

3.1 Inhibition Assays

A colony overlay assay was used to determine if *L. gelidum* UAL187-22 is a natural producer of an agent which is able to inhibit the growth of *L. monocytogenes*. From the results obtained in figure 3.1(a), it can be concluded that *L. gelidum* UAL187-22 is able to obstruct the growth of *Listeria*. Distinct zones of inhibition surrounding each *Leuconostoc* colony are present. These are indicated with the use of an arrow on figure 3.1(a). A deferred-antagonism assay was used to qualitatively test for the presence of an inhibitory agent within the supernatant of an overnight culture of *L. gelidum* UAL187-22. Zones of inhibition produced for the deferred antagonism assay do suggest that *L. gelidum* UAL187-22 was able to produce an agent that is able to inhibit the growth of *L. monocytogenes* as seen in figure 3.1(b). A similar result to that obtained in figure 3.1 was obtained by Van Belkum & Stiles (1995), in which zones of inhibition were produced by leucocin A against *L. monocytogenes*. Zones of inhibition where intense; however, not as intense as the control leucocin A sample (sample C). This is expected since the control sample consisted of partially purified leucocin A. Upon concentration of the *Leuconostoc* supernatant, zones produced were of similar intensity to the control leucocin A sample (figure 3.1c). From these results, it can be assumed that the supernatant of *L. gelidum* UAL187-22 does contain an inhibitory agent of which *L. monocytogenes* is susceptible.

Leuconostoc gelidum UAL187-22 is a lactic acid bacterium, which produces a wide variety of inhibitory agents other than bacteriocins. These include lactic acid and peroxides (Chen & Hoover 2003). Therefore, a half moon assay was conducted in order to test if the inhibition of *Listeria* was due to the action of a protein/peptide. Proteinase K is used to digest the bacteriocin if present which results in the formation of half moon zone of inhibition. From figure 3.1(d), the results obtained indicate that the inhibitory agent present is proteinaceous as half moon zones of inhibition were produced for the *L. gelidum* UAL187-22 supernatant (concentrated). Distinctive half moon zones of inhibition were produced by sample 2 and the control. These zones are indicated by arrows on figure 3.1(d). The results obtained for all inhibition assays suggest that leucocin A is produced and therefore the *lcaABCD* operon is present.

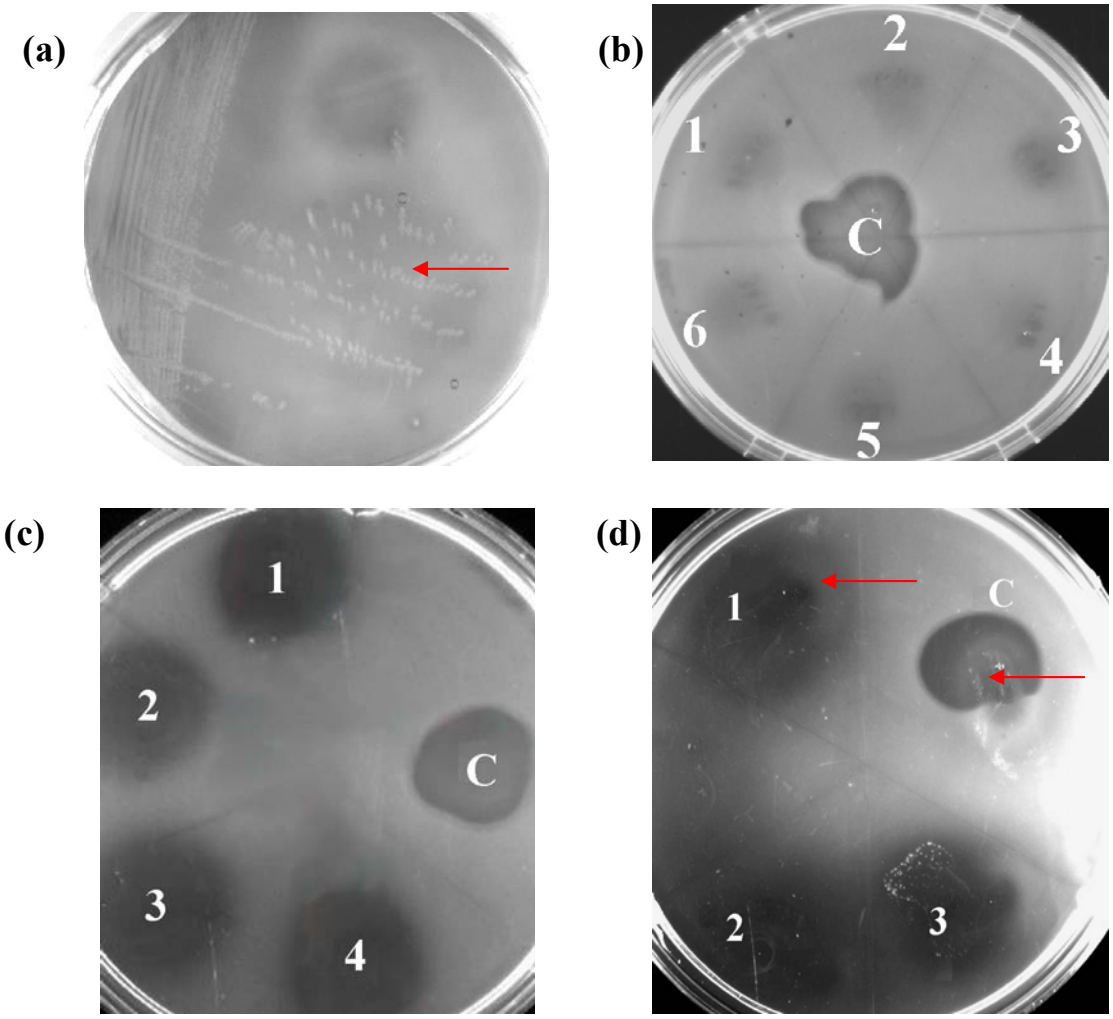


Figure 3.1. (a) Colony overlay assay used to determine if individual colonies of *Leuconostoc gelidum* UAL187-22 are able to inhibit the growth of *Listeria monocytogenes*. Individual colonies are present with distinct zones of inhibition surrounding each colony; (b) Deferred inhibition assay using the supernatant extracted from a saturated culture of *L. gelidum* UAL187-22 where zones of inhibition were produced by the sample tested; (c) *L. gelidum* UAL187-22 supernatant tested after freeze drying. The intensity of the zones has increased following concentration; (d) Half moon assay using concentrated supernatant obtained from *L. gelidum* UAL187-22. Zones produced are indicative of half moons (indicated by the arrows) due to digestion of bacteriocin by Proteinase K. All test spots are numbered while the control leucocin A sample is labelled C

3.2 Isolation of DNA

Over the years, a number of different methods have been developed to purify plasmid DNA. The Nucleobond® AX100 Plasmid DNA Purification Kit is based on the alkaline lysis technique developed by Birnboim and Doly (1979). This kit uses a silica-based anion-exchange resin, which provides efficient binding of nucleic acids and has been shown to elute DNA of a pure form (Macherey-Nagel, 2005). The Nucleobond® kit was used to isolate pMAL-c2 plasmid DNA from *E.coli* K12 TB1. *L. gelidum* UAL187-22 is a gram-positive bacterium and therefore requires an additional lysis step when isolating plasmid DNA, due to the presence of a peptidoglycan layer (Presscott, 1990). A method devised by Holt *et al.* (2001), was used to isolate plasmid DNA from this strain as this method uses Lysozyme to lyse the cell. A phenol/chloroform extraction step ensured that isolated DNA was of a pure form.

The pMAL-c2 expression vector is approximately 6721 bp and contains a polycloning site located downstream from the *malE* gene of *E.coli*, resulting in easy production of maltose binding protein (MBP) fusion proteins. *L. gelidum* UAL187-22 contains three plasmids of approximately 5.0, 7.6, and 9.2 MDa in molecular mass (Stiles, 1994). Both plasmids pLG 9.2 and pLG 7.6 contain the leucocin A operon, which is responsible for leucocin A production and secretion (Hastings *et al.*, 1991). The study required the use of the pre-leucocin A and leucocin A gene sequences. Therefore, by isolating a plasmid containing the genes encoding these proteins, one could easily amplify them using PCR. Plasmid pJF5.5 isolated from *E.coli* JM103 was used as control DNA in various procedures conducted in this study. This vector was constructed at the Microbial Genetics Department of the University of Kwa-Zulu Natal and contains the leucocin A gene sequence cloned into the polycloning site. For this reason, pJF5.5 was used as a positive control in PCR and southern blot hybridization.

When pMAL-c2 and pJF5.5 plasmid DNA were analyzed by electrophoresis, three bands were observed, signifying the three main conformations of a single uncut plasmid, which are supercoiled, nicked circular and linear (Snyder & Champness, 2003) (figure 3.2a & b). Upon restriction with *EcoR1*, the pMAL-c2 plasmid was linearized and produced a fragment of ~6700 bp (figure 3.2a, lane 3). This is a good indication that the expression vector was isolated, since this is the approximate size of pMAL-c2. Isolated pJF5.5 plasmid DNA is

shown in figure 3.2(b). Plasmid DNA isolated from *L. gelidum* UAL187-22, is represented by figure 3.2(c), lanes 1, 2, and 3.

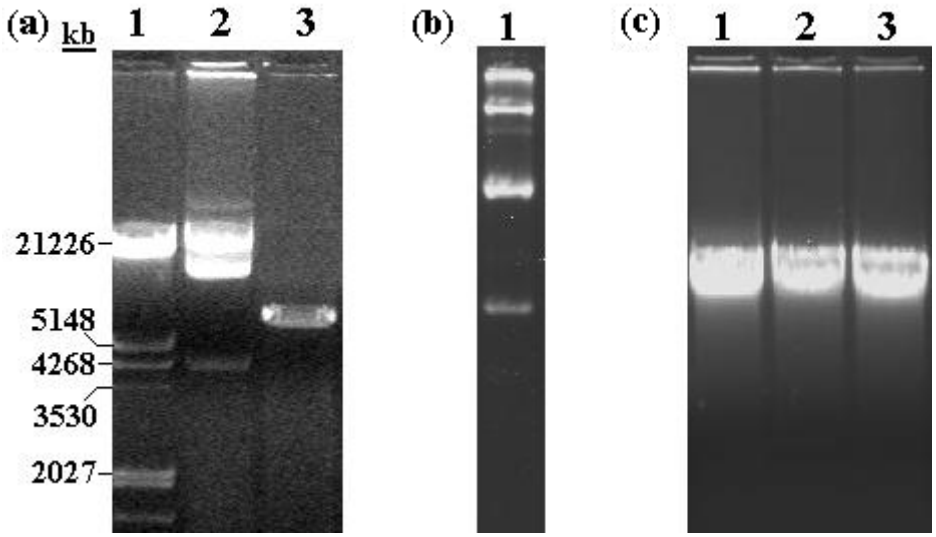


Figure 3.2. An agarose gel (0.8 % (w/v)) indicating the presence of the isolated plasmids used in this study. (a) Expression vector pMAL-c2 isolated from *E.coli* K12 TB1. Lane 1: Molecular weight marker III (Roche Biochemicals, USA), lane 2: pMAL-c2 plasmid DNA, lane 3: restricted pMAL-c2 showing a single band of about 6700 bp; (b) pJF5.5 plasmid DNA isolated from *E. coli* JM103; (c) Plasmid DNA isolated from *L. gelidum* UAL187-22.

3.2.1 Spectrophotometric Quantification of Isolated Plasmid DNA

Spectrophotometric analysis confirmed that the isolated DNA is pure. This is due to OD values of about 1.8 (table 3.1). The isolation procedures produced DNA concentrations of 9.00 µg/ml for *Leuconostoc* plasmid DNA and 12.62 µg/ml for pMAL-c2 expression vector. The concentration of pJF 5.5 DNA was estimated to be 7.815 µg/ml.

Table 3.1. Absorbance readings for isolated pMAL.c2 and for *Leuconostoc* plasmid DNA, resuspended in TE buffer.

Plasmid DNA	**A_{260}**	**A_{280}**	**OD (A_{260}/A_{280})**	**DNA Concentration (µg/ml)**
Leuconostoc DNA	1.799	0.977	1.84	9.00
pMAL-c2	2.523	0.229	1.85	12.62
pJF 5.5	1.563	0.859	1.82	7.815

3.3 Amplification of the Target Genes by Polymerase Chain Reaction

Polymerase chain reaction provides an efficient method to selectively amplify regions of DNA out of much longer DNAs (Snyder & Champness, 2003). By using this method, the leucocin A and pre-leucocin A genes were isolated in a large quantity for further analysis. Very few studies involved the PCR amplification of the *lcaA* gene; therefore, primers were designed for this purpose. *Leuconostoc gelidum* UAL187-22 sequence data was obtained from the NCBI database (accession number M64371). The *lcaA* coding regions were identified and used to design primers; which were used for the amplification of the leucocin A and pre-leucocin A gene sequences. Leucocin A is synthesized by the *lcaABCD* operon which contains a 72 bp sequence which encodes the N-terminal extension of leucocin A (24 residues) (Hastings *et al.*, 1991). This sequence is followed by a 111 bp sequence which codes for the mature form of leucocin A (Ennahar *et al.*, 2000).

The Leu forward and reverse primers, designated as Leu-f and Leu-r, were designed such that they are homologous to the 5' and 3' end of the gene sequence encoding mature leucocin A. These primers were used for the amplification of leucocin A without its leader sequence. For the amplification of pre-leucocin A, primers Pre-f and Pre-r were designed. The 5' end of primer Pre-f, binds to the gene sequence encoding the leader peptide, thereby allowing amplification of leucocin A with its leader sequence. Pre-r binds to the 3' end of the leucocin A sequence. The binding of PCR primers to their associated gene sequences is shown schematically in figure 3.3. Primer design also involved the addition of a *Xba*1 restriction site at the 5' end of each reverse primer. This allowed for the incorporation of this restriction site at the 5' ends of the leucocin A and pre-leucocin A genes after PCR, so as to facilitate restriction upon cloning.

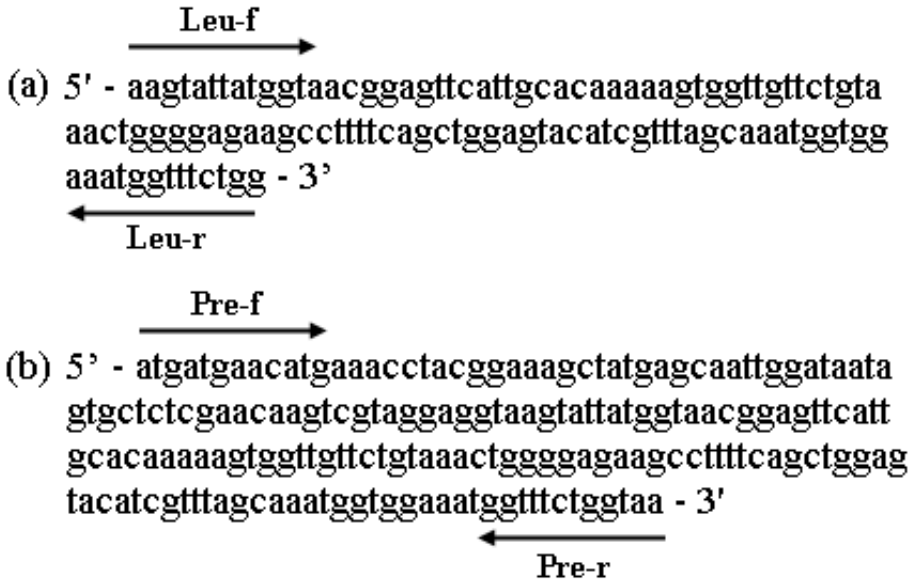


Figure 3.3. Schematic representation of the amplification of the leucocin A and pre-leucocin A gene sequences. (a) Leucocin A sequence and the Leu reverse and forward primers used to amplify this segment of DNA; (b) Forward and reverse primers used to amplify the pre-leucocin A gene.

Upon analysis of the gene sequences, it was found that leucocin A is approximately 111 bp in length, while pre-leucocin A is 186 bp. When PCR products were electrophoresed on a 2.5 % agarose gel the products obtained for leucocin A and pre-leucocin A were within the expected range, which is indicated by arrows in lanes 2 and 3 on figure 3.4. Band size was also verified using the BIO-RAD Quantity One software (BioRad, South Africa) where it was found that the leucocin A and pre-leucocin A bands were 109 bp and 180 bp respectively. Thus, Quantity One gave a rough estimate that PCR was successful in amplifying the respective gene sequences. Lane 4 represents the amplification of plasmid pJF5.5, which was used as a positive control using primers Leu-f and Leu-r. This plasmid contains the leucocin A gene cloned into its multiple cloning site. Amplification using the above primers gave a product of similar size to that obtained for leucocin A in lane 3, which suggests that primers were able to bind and amplify DNA. A negative control was included in lane 5, which used pMAL-c2 as a template and primers, Pre-r and Pre-f, for its amplification. This did not produce any product, as there were no genes present on the vector to which primers could bind. The negative control using no template DNA also did not produce a band (lane 6). Since positive and negative controls produced the expected results it can be concluded that the PCR occurred at the optimal stringency, and was successful in amplifying the leucocin A and pre-leucocin A gene sequences.

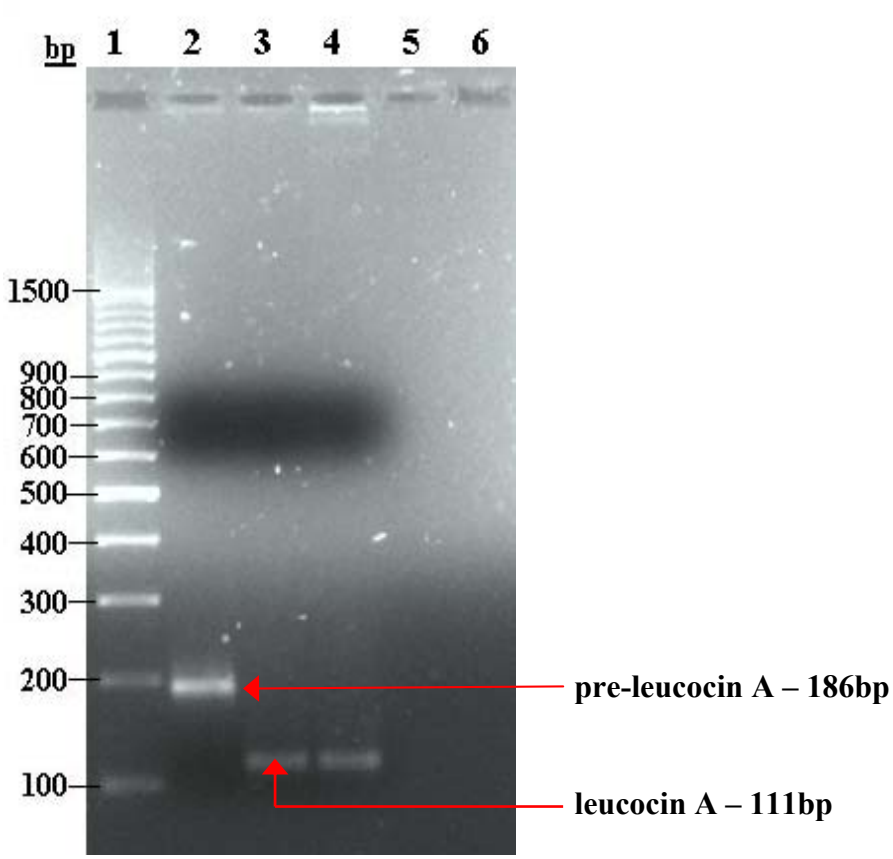


Figure 3.4. An agarose gel (2.5 % (w/v)) of the amplification products, leucocin A and pre-leucocin A. Lane 1: O'RangeRuler 100 bp DNA ladder (Fermentas, South Africa), lane 2: leucocin A gene, lane 3: pre-leucocin A gene, lane 4: pJF5.5 positive control, lane 5: pMAL-c2 negative control. Arrows indicate expected products for each PCR reaction. The expected size of each gene is also depicted.

The amplified DNA obtained by PCR was used in subsequent molecular reactions such as restriction digests in order to achieve recombinant clones. Polymerase chain reaction uses high salt concentrations that usually inhibit restriction enzymes (Sambrook *et al.*, 1989). Therefore, PCR products were cleaned using the Nucleospin® DNA Purification Kit prior to the cloning procedures. Following purification, amplified DNA was analyzed at absorbencies of 260 and 280 nm in order to determine the concentration of DNA obtained after PCR and purification. Leucocin A was calculated to be 29.8 μg/ml, while pre-leucocin A was present at a concentration of 43.0 μg/ml. Following this procedure, the cleaned amplified DNA sequences were subjected to a Klenow reaction in order to fill in either 3'/5' overhangs. These ends are usually produced following PCR and are termed 5'/3' overhangs (Promega, 1996). With the use of Klenow DNA polymerase, the leucocin A and pre-leucocin A sequences were blunt ended. This facilitated unidirectional cloning as the 3' end of each insert required a blunt end, as seen in figure 2.1 of chapter 2.

3.4 Molecular cloning and transformation

The general steps, which were employed for cloning the pre-leucocin A and leucocin A gene into the pMAL.c2 vector are listed below:

1. Prepare the vector and DNA to be cloned by digestion with restriction enzymes to generate complementary ends.
2. Ligate the foreign DNA into the vector with the enzyme DNA ligase.
3. Introduce the recombinant vector into bacterial cells, (or yeast cells for YACs), by transformation.
4. Select cells containing foreign DNA by screening for selectable markers (usually drug resistance) (Brown *et al.*, 2001).

3.4.1 Restriction of Vector and Insert DNA

Plasmid pMAL-c2 (figure 3.5a) was the expression vector of choice for the construction of the *malE-lcaA* and *malE-prelcaA* translational fusion genes. Expression of these genes produces MBP fusion proteins, where the protein of interest can be cleaved from MBP with serine protease, Factor Xa (Terpe, 2003). The signal sequence of the *mal*E gene on this vector is deleted which allows the fusion protein produced to remain in the cytoplasm (Waugh, 2005). The polycloning site of pMAL-c2, shown in figure 3.5(b) contains unique restriction sites that allow insertion of foreign DNA by molecular cloning. The leucocin A and pre-leucocin A genes were cloned into the *Xmn*1/*Xba*1 site of pMAL-c2. However, restriction enzyme *Asp*700 was used in place of *Xmn*1, as these are enzymes that have the same recognition sequence and are termed isoschizomers (Sambrook *et al.*, 1989). Further characteristics of the pMAL-c2 vector system are explained in 1.8 of chapter 1.

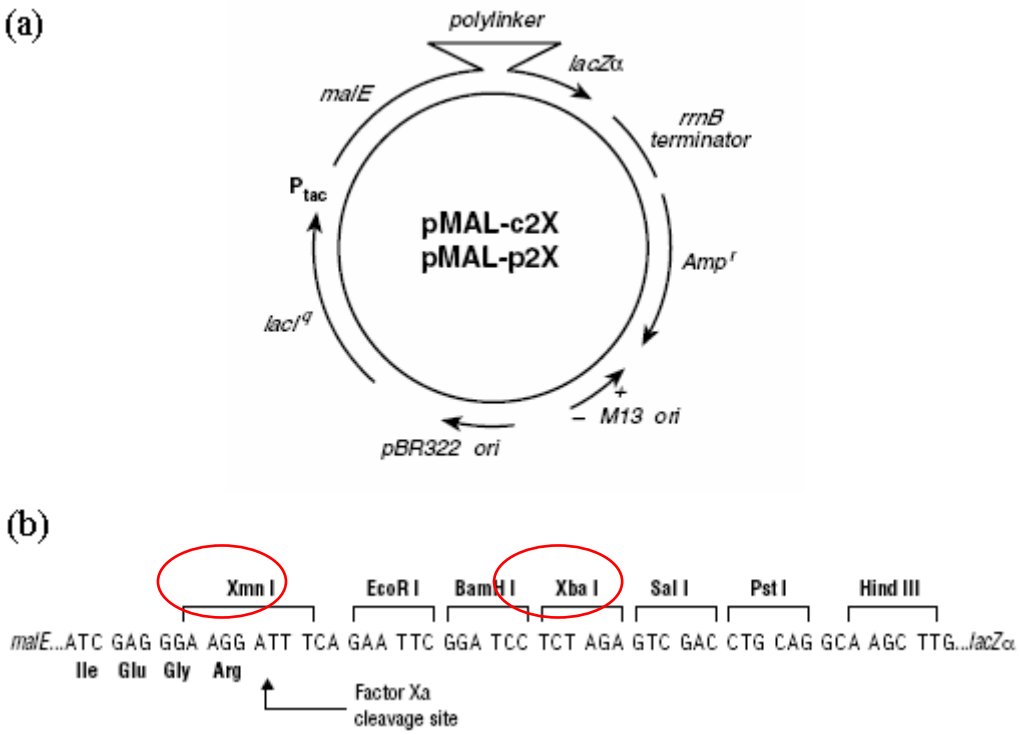


Figure 3.5. (a) The pMAL-c2 expression vector indicating the location of the *malE* gene, polylinker, and the *lacZ* gene; (b) Restriction sites found with the polylinker of pMAL-c2. Restriction sites *Xmn*1/*Asp*700 (isoschizomers) and *Xba*1 were used. The Factor Xa cleavage site used for the separation of MBP from the target protein is also indicated (New England Biolabs).

The polycloning site of the pMAL-c2 vector was subjected to a double digest using *Asp*700 and *Xba*1 restriction enzymes. *Asp*700 creates blunt ends upon restriction while *Xba*1 creates sticky ends (Promega, 1996). Re-circularization of the vector is prevented by creating one sticky end and one blunt end within the polylinker region which increased the possibility for ligation of the inserts (leucocin A or pre-leucocin A) into the pMAL-c2 vector (Brown, 2001). Figure 3.6 is a 0.8 % (w/v) agarose gel on which each pMAL-c2 restriction digest was analyzed. The double digest is represented in lane 3. Single digests with *Asp*700 and *Xba*1 served as control reactions and are shown in lanes 4 and 5 respectively. These controls were used to verify if each restriction enzyme used was able to restrict DNA. Uncut pMAL-c2 vector was included in lane 2, where the three conformations are present. Single bands of about 6721 bp in lanes 3, 4, and 5 indicate that restrictions using *Asp*700 and *Xba*1 were successful as this is the approximate size of pMAL-c2. The leucocin A and pre-leucocin A gene sequences were restricted with *Xba*1 and contained 3' blunt end via a Klenow reaction. The use of these enzymes allowed for unidirectional cloning of the inserts into the pMAL-c2 vector (Sambrook *et al.*, 1989). Furthermore, by using compatible enzymes as that used for the vector, ligation of the leucocin A and pre-leucocin A inserts were achieved. Restrictions of the inserts are shown in lanes 6 and 7 of figure 3.6. No noticeable differences can be seen following the restriction of leucocin A and pre-leucocin A, however these were included in order to make sure that DNA was not lost during procedures prior to cloning.

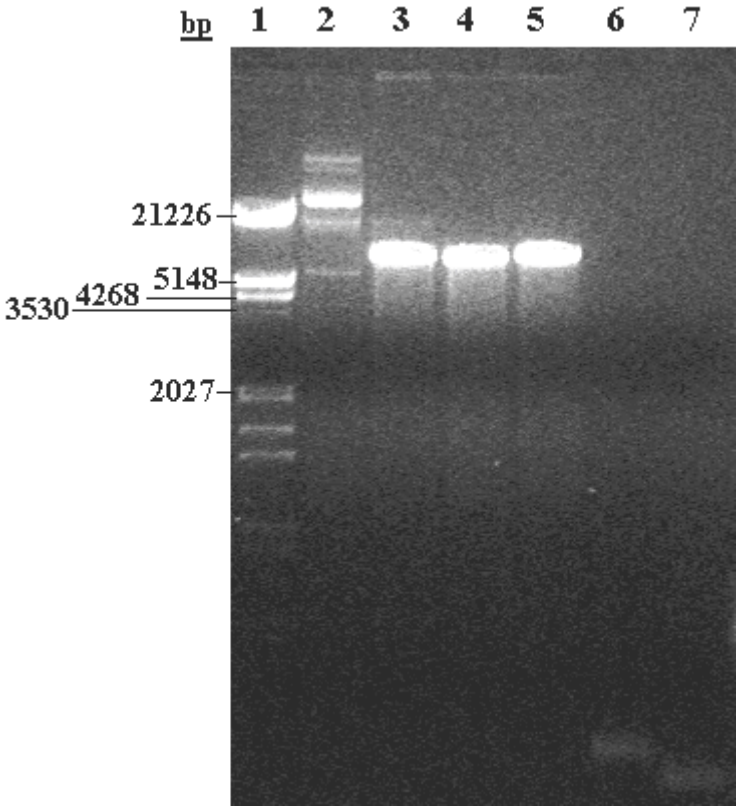


Figure 3.6. An agarose gel (0.8 % (w/v)) of the various restrictions digests prior to cloning. Lane 1: Molecular weight marker III (Roche Biochemicals, USA), lane 2: uncut pMAL-c2 vector, lane 3: pMAL-c2 double digest using *Asp*700 and *Xba*1 restriction enzymes, lane 4: pMAL-c2 single digest with *Asp*700, lane 5: pMAL-c2 single digest with *Xba*1. Lane 6: Restriction of pre-leucocin A with *Xba*1, lane 7: Restriction of leucocin A with *Xba*1. All vector restrictions produced single bands of about 6721 bp, which is the size of pMAL-c2.

3.4.2 Transformation into the Host strain

Following ligation of leucocin A and pre-leucocin A into the pMAL-c2 vectors, the resulting plasmids were transformed into freshly prepared *E.coli* JM103 competent cells. Cells were made competent using the $CaCl_2$ method. The amount of competent cells used was considerably reduced from 100 µl to 50 µl in order to increase the chances of a vector being taken up by a cell. Aliquots of the transformation mix containing the ligation reaction were plated onto LB agar plates supplemented with ampicillin (100 µg/ml), IPTG (0.1 mM), and X-gal (80 µg/ml). Addition of ampicillin ensures growth of cells containing the pMAL-c2 vector due to the ampicillin selectable marker located on this plasmid (Brown, 2001). Thus, *E.coli* cells containing either parental forms of pMAL-c2 vector or the recombinant form, will grow. Recombinants were selected using the blue/white selection method or α-complementation. This method relies on the activation of the *lacZ* gene, which is located within the multiple cloning site of the pMAL-c2 vector. The *lacZ* gene codes for a subunit of the β-galactosidase enzyme (α-fragment) which metabolizes X-gal to form a blue precipitate (Brown, 2001). Recombinant pMAL-c2 are $lacZ^-$ as they contain a gene cloned within the polycloning site resulting in inactivation of this gene (Watson, 1987). Thus, recombinant colonies will be white as their cells do not express β-galactosidase subunit and therefore, cannot metabolize X-gal. Cells bearing parental forms of pMAL-c2 are $lacZ^+$ and are able to express β-galactosidase, which results in the production of blue colonies. Transcription of the *lacZ*, leucocin A, or pre-leucocin A genes is brought about by the presence of IPTG (Brown, 2001).

Figure 3.7 represents the plates containing cells transformed with recombinant vectors. Transformation of pMAL-c2 vector containing the leucocin A gene is shown in figure 3.7(a) while figure 3.7(b) represents the plate used for transformation of pMAL-c2 containing the pre-leucocin A. Growth was abundant on both plates, in the form of numerous single blue and white colonies. Blue colonies represent non-recombinants or parentals i.e. pMAL-c2 vector. White colonies (indicated by arrows on figure 3.7a and b), were indicative of recombinant clones.

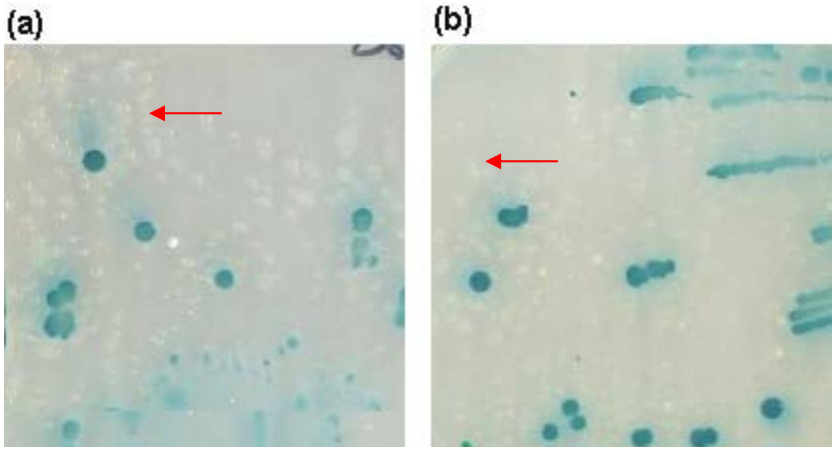


Figure 3.7. Blue and white colonies produced from the transformation of recombinant vectors into *E.coli* JM103 competent cells. (a) Transformation of pMAL-c2 vector containing the leucocin A gene; (b) Transformation of pMAL-c2 vector containing the pre-leucocin A gene. Blue and white colonies produced by α-complementation were seen on LB agar plates supplemented with ampicillin, X-gal, and IPTG.

The transformation experiment consisted of various controls in order to confirm that each component of the reaction was effective. These controls are shown in figure 3.8. *E.coli* JM103 competent cells were tested for viability and transformation capabilities. The viability of competent cells was tested by growing on unsupplemented LB agar plates and LB agar plates supplemented with ampicillin. Competent cells do not contain a plasmid and therefore no antibiotic resistant genes are present. Figure 3.8(a) shows that no growth was observed for competent cells grown on LB agar containing ampicillin while white colonies were seen on unsupplemented LB plates (figure 3.8b). This indicates that *E.coli* JM 103 cells were viable. By transforming *E.coli* JM103 competent cells with either unrestricted or restricted pMAL-c2 vector, one is able to test the transformation capabilities of these cells. These also serve as controls for transformation, as growth is an indication that plasmid DNA is being taken up by the competent cells. Transformation of *E.coli* JM103 with restricted pMAL-c2 vector did not produce any colonies, which are seen in figure 3.8(c). Growth on the other hand was observed for *E.coli* JM103 transformed with unrestricted pMAL-c2 plasmid (figure 3.8d). These results prove that the competent cells used were viable and transformation of DNA can occur. The efficiency of T4 DNA ligase was tested by restricting pMAL-c2 vector, re-ligating with the ligase enzyme, and transforming into *E.coli* JM103. The presence of blue colonies (figure 3.8e), indicates that T4 DNA ligase was successful in ligating the plasmid. All controls suggest that components/procedures of the cloning procedure i.e. competent cells and T4 DNA ligase, and transformation, were producing the expected results.

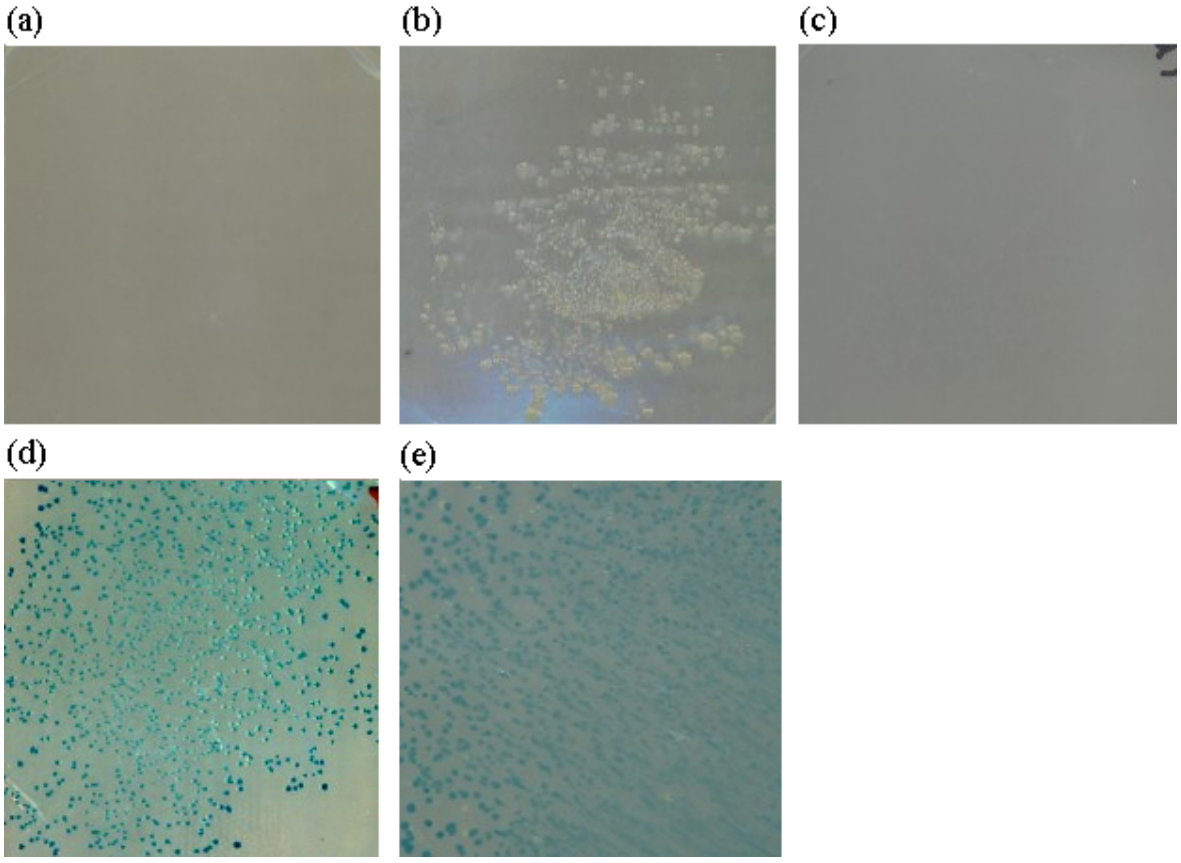


Figure 3.8. Controls used in the ligation reaction, competency of *E.coli* JM103, and transformation of *E.coli* JM103; (a) *E.coli* JM103 competent cells grown on LB agar plates supplemented with ampicillin; (b) Competent cells grown on unsupplemented LB agar plates; (c) Transformation negative control using restricted pMAL-c2 vector; (d) Transformation positive control using unrestricted pMAL-c2; (e) Transformation using the ligation control reaction.

White colonies produced were tested for the presence of a plasmid containing the ampicillin resistant selectable marker by growing on fresh LB agar plate containing ampicillin. Colonies were selected from plates in figure 3.7 using sterile toothpicks, transferred onto fresh LB agar plates supplemented with ampicillin and incubated at 37 ^{o}C overnight. Each colony transferred produced a distinct white colony following incubation (data not shown).

DNA was isolated from each of the selected colonies and then screened for the presence of the bacteriocin insert. The new recombinant plasmids were named accordingly with pLcaA representing the pMAL-c2 vector containing the leucocin A gene sequence. The vector containing the pre-leucocin A gene sequence in the multiple cloning site, was designated as pPreLcaA. Screening procedures included southern blot hybridization and colony PCR. These two procedures were used to distinguish between true positives and false positives.

3.4.3. Isolation of Recombinant DNA

Recombinant DNA was isolated from each colony using the mini prep isolation method. Isolated DNA was analyzed by electrophoresis on a 0.8 % (w/v) agarose gel (figure 3.9). Lane 3 contains the pPLcaA.1 plasmid while plasmid pLcaA.1 is seen in lane 4. Plasmid pMAL-c2 was electrophoresed in lane 2 in order to compare differences between recombinant DNA and uncut pMAL-c2. Upon analysis of figure 3.9, a considerable number of differences are seen between recombinant DNA and pMAL-c2. These differences, however do no suggest that the plasmid contains the respective genes. Therefore a specific screening method such as southern blot hybridization is required to ensure that each recombinant vector contains the insert of choice.

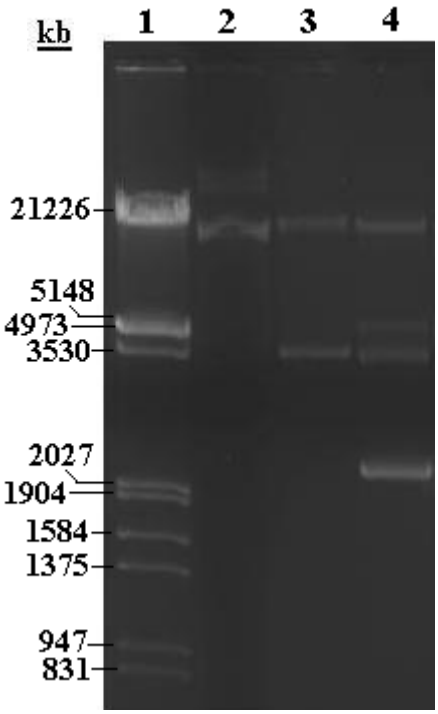


Figure 3.9. An agarose gel (0.8 % (w/v)) depicting recombinant plasmids pLcaA and pPreLcaA. Lane 1: Molecular weight marker III (Roche Biochemicals, USA), lane 2: pMAL-c2 vector, lane 3: recombinant plasmid pPreLcaA containing the leucocin A insert, lane 4: recombinant plasmid pLcaA containing the pre-leucocin A insert. The various fragments in lanes 3 and 4 represent the different conformations that the plasmid can exist in.

3.5 Verification of Recombinant Clones

Two methods were used to verify if the selected clones contained either the leucocin A or pre-leucocin A genes. Firstly, isolated recombinant DNA was subjected to Southern Blot Hybridation using a probe specific to class IIa bacteriocin gene sequences. Recombinant plasmids were also screened using colony PCR. Primers used for the amplification of leucocin A and pre-leucocin A from *Leuconostoc* DNA was used again for colony PCR.

3.5.1 Southern Blot Hybridization

Southern blot hybridization, done by probing with the sequence probe JIR-1 was used to confirm the presence of the bacteriocin genes. With this method, plasmids carrying the leucocin A or pre-leucocin A gene sequences (true positives) can be distinguished from those that are not. The JIR-1 probe was designed to be homologous to the bacteriocin gene sequence encoding the conserved N-terminal protein consensus sequence, KYYGNGV of class IIa bacteriocins (Sprules *et al.*, 2005). Therefore, if the inserts were successfully cloned into pMAL-c2 vector, the probe will hybridize to the DNA and produce a signal upon detection indicating the presence of a true recombinant (Sambrook *et al.*, 1989). Hastings *et al.* (1991), used southern blot hybridization as a preferred screening method for the detection of the *lcaA* gene following insertion into the polycloning site of pUC118. Probing was carried out using the JHA-3 primer (homologous to an internal region of the leucocin A sequence) which produced positive results for the detection of the *lcaA* sequence.

Five white colonies were selected for each clone and the isolated DNA was tested for the presence of the bacteriocin insert. The results obtained for southern blot hybridization are shown in figure 3.10, where positive signals were produced for all DNA tested. Lanes 1 to 5 contain DNA isolated from clones pLcaA, while lanes 6 to 10 contain pPreLcaA DNA. Bands produced within these lanes imply that the JIR-1 probe has hybridized to a DNA sequence, which indicates that the leucocin A and pre-leucocin A genes are present within the vector sequence. Further analysis of figure 3.10, shows that in certain lanes sometimes 1,2 or even 3 bands were produced. A possible explaination of this is that the plasmid could have been isolated in either either conformation rather than all three. Plasmid pJF5.5 served as a positive control as this plasmid contains the leucocin A gene sequence cloned into its polycloning site.

Hybridization of the probe to pJF5.5 DNA did take place, which is seen in lane 11. DIG-labeled DNA, which is present in lane 12, served as a positive control for the detection procedure. A signal within this lane suggests that the detection procedure used for southern blot did occur at the optimal conditions. A negative control, pMAL-c2, was included in lane 13. Here, no signal was produced, as the probe did not have a complementary sequence to bind to (pMAL-c2 without the bacteriocin gene cloned into the polylinker region). Thus, from the above results it can be concluded that all white colonies tested are true positives, and contain either the leucocin A or pre-leucocin A gene sequence within the polycloning site.

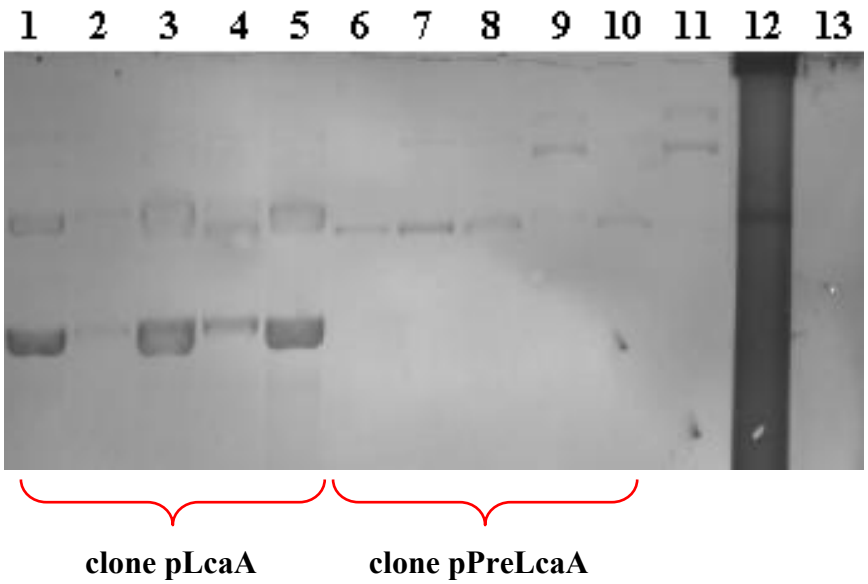


Figure 3.10. Southern blot hybridization of recombinant plasmids pPreLcaA and pLcaA in order to test for the presence of the leucocin A and pre-leucocin A gene sequences. Bands were detected for all recombinant DNA tested which suggests the presence of the insert genes. Lanes 1 to 5: DNA isolated from 5 pLcaA colonies, lanes 6 to 10: DNA isolated from 5 pPreLcaA colonies, lane 11: plasmid pJF5.5 (positive control), lane 12: DIG-labelled DNA (positive control for the detection procedure), lane 13: pMAL-c2 vector (negative control).

3.6 DNA Sequencing and Analysis of the Sequence Data

DNA sequencing did not produce the expected results. Sequencing results for clones pLcaA and pPreLcaA obtained from Inqaba Biotech failed to show the presence of an insert. An alignment of the sequences (figures 3.11 and 3.12) for clones pLcaA and pPreLcaA were with the pre-leucocin A DNA sequence gave no positive results. The sequences were then subjected to a blast search, which stated that they were homologous to regions of the pMAL-c2 sequence. Furthermore, sequences were of a poor quality and consisted of many unidentifiable nucleotides. As a result of this, sequencing results where not taken into consideration as screening methods such as southern blot hybridization proved that the respective genes were present. Expression studies (discussed in section 3.7) further revealed the presence of a protein band of expected size, upon induction.

GAAACGCCAGGCCAACTTGCCTGCAGTCGACTCTAGCCTTCCCTCGATCCCGAGTTGTTGT
TATTGTTATTGTTGTTGTTGTTCGAGCTCGAATTAGTCTGCGCGTCTTTCAGGGCTTCATCG
ACAGTCTGACGACCGCTGGCGGCGTTGATCACCGCAGTACGCACGGCATACCAGAAAAA
GCGGACAA

Figure 3.11. Nucleotide sequence obtained from automated DNA sequencing of clone pLcaA.

CKRSMSSGCMRMCTATTTCGAGCTCGAACAACAACAACAATAACAATAACAACAACCTC
GGGATCGAGGGAAGGAAGRGTCGACCTGCAGGCAAGCTTGGCACTGGCCGTCGTTTTAC
AACGTCGTGACTGGGAAAACCCTGGCGTTACCCAACTTAATCGCCTTGCAGCACATCCCC
CTTTCGCCAGCTGGCGTAATAGCGAAGAGGCCCGCACCGATCGCCCTTCCCAACAGTTGC
GCAGCCTGAATGGCGAATGGCAGCTTGGCTGTTTTGGCGGATGAGATAAGATTTTCAGCC
TGATACAGATTAAATCAGAACGCAGAAGCGGTCTGATAAAACAGAATTTGCCTGGCGGC
AGTAGCGCGGTGGTCCCACCTGACCCCATGCCGAACTCAGAAGTGAAACGCCGTAGCGC
CGATGGTAGTGTGGGGTCTCCCCATGCGAGAGTAGGGAACTGCCAGGCATCAAATAAAA
CGAAAGGCTCAGTCGAAAGACTGGGCCTTTCGTTTTATCTGTTGTTTGTCGGTGAACGCTC
TCCTGAGTAGGACAAATCCGCCGGGAGCGGATTTGAACGTTGCGAAGCAACGGCCCGGA
GGGTGGCGGGCAGGACGCCCGCCATAAACTGCCAGGCATCAAATTAAGCAGAAGGCCAT
CCTGACGGGATGGCCTTTTTTGCGTTTTCTACAAACTCTTTTTGTTTAWTTTTTCTAAAATA
CATTCAAAATATGTATCCCGCTTCATGAGACAATAAMCCCTGATAAATGCTTTCAATAAT
ATTGGAAAAGGGARGAGKWTGAGTATTYCAMCATTTCCGGKGTCGSCCTTWWTTCCCYT
TTTTTKSSGGCWTTTTTGCYTTYCCTTGTTTTTTGCYCMCCCMRGAAAMSSYTGGGTGAAA
AKTAAAAARAARGSYTRAARAAYKAGTTTGGGGKGGCARGAAGTKGGKTTTAMCYT

Figure 3.12. Nucleotide sequence obtained from automated DNA sequencing of clone pPreLcaA.

3.7 Production and Purification of MBP fusion proteins

The general steps used for the expression and purification of MBP fusion proteins according to the pMAL protein and purification manual (New England Biolabs) are listed below:

1. Express each fusion protein within the cytoplasm of *E. coli* JM 103.
2. Subject host cells to a lysis step, thereby releasing the fusion proteins into the supernatant.
3. Purify the MBP fusion proteins by amylose affinity purification.

Recombinant plasmids pLcaA and pPreLcaA, were constructed such that the leucocin A and pre-leucocin A genes were fused to the 3' end of the *malE* gene of pMAL-c2. Upon expression, the maltose binding protein tag was fused to the N-terminus of each recombinant peptide resulting in the production of MBP-LcaA and MBP-preLcaA. Furthermore, the MBP tag was separated from the leucocin A and pre-leucocin A peptides by a cleavage site with Factor Xa which allowed for the separation of the tag from each peptide. The two types of fusion proteins constructed and analyzed, are shown in figure 3.13.

The main aim of this study was to analyse the activity of the pre-peptide of leucocin A compared to that of the mature form. The activity of each peptide i.e. leucocin A and pre-leucocin A against *L. monocytogenes*, was tested at three levels. This consisted of testing each MBP fusion peptide, each Factor Xa cleavage mixture, and finally, testing the activity of the pure forms of leucocin A and pre-leucocin A.

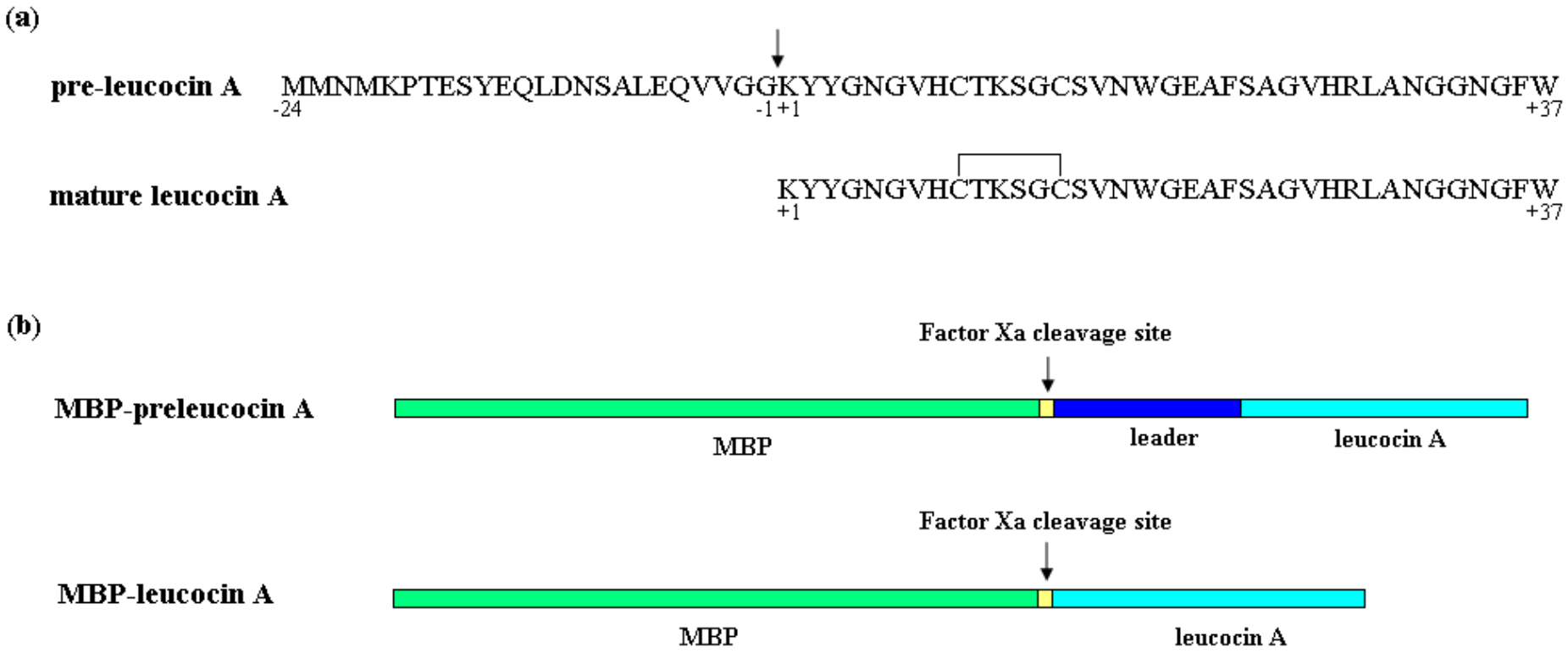


Figure 3.13. (a) Amino acid sequences of pre-leucocin A and mature leucocin A formed after processing of the 24 amino acid leader peptide and oxidation of the two cysteines; (b) Properties of MBP-LcaA and MBP-preLcaA fusion proteins. MBP-preLcaA contains the 61 amino acid pre-leucocin A while MBP-LcaA consists of leucocin A fused to the MBP affinity tag. The Factor Xa cleavage, which separates the MBP from the recombinant peptide, is also shown (Jack *et al.*, 1995).

3.7.1 Expression of the *malE-lcaA* and *malE-prelcaA* Fusion Constructs

Expression of the *lac* operon of recombinant plasmids pLcaA and pPreLcaA, was induced with Isopropyl-β-D-thiogalactopyranoside or IPTG. Induction is defined by the binding of RNA polymerase to the *lac* operon, thereby allowing transcription of the cloned inserts (Sambrook *et al.*, 1989). The aim of this experiment was to achieve a high level of expression for each *malE* fusion construct. High levels of MBP-LcaA and MBP-preLcaA expression within the cytoplasm will ensure high yields of each MBP fusion protein after amylose affinity chromatography.

Expression of fusion proteins, MBP-LcaA and MBP-preLcaA was induced at the mid log phase (OD_{600} ~ 0.5) during the growth of *E.coli* JM103 bearing clones pLcaA and pPreLcaA. Absorbance readings were taken prior to induction and a growth curve was constructed by plotting the absorbance at 600 nm against the time taken to reach an optical density of 2×10^8 cells/ml (figures 3.14 and 3.15). Clone pLcaA was induced at an OD_{600} of 0.4970, while clone pPreLcaA was grown to an OD_{600} of 0.5003 before IPTG was added.

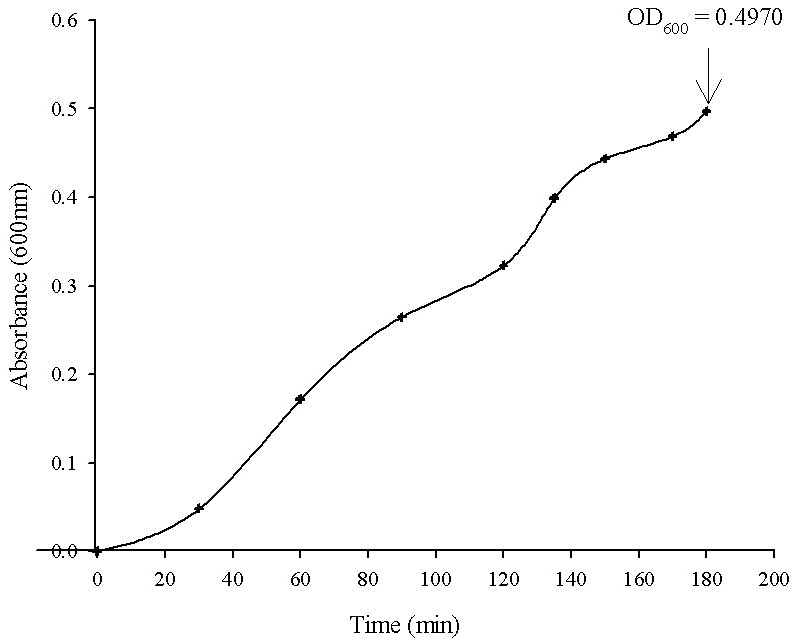


Figure 3.14. Induction of clone pLcaA with IPTG for the expression of the MBP-LcaA fusion constructs. Absorbance readings were taken at 600 nm until the culture reached an optical density of 0.4970 and thereafter, IPTG was added to a final concentration of 0.3 mM.

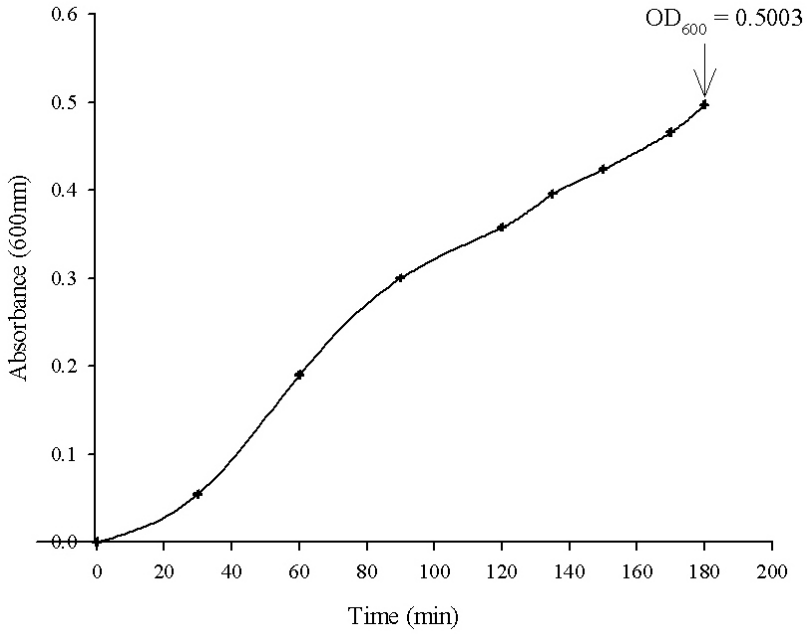


Figure 3.15. Expression of the MBP-preLcaA fusion construct by induction of clone pPreLcaA with IPTG. The culture was grown to an optical density of 0.5003 and thereafter, IPTG was added to a final concentration of 0.3 mM.

Upon SDS-PAGE analysis, induction of recombinant clones pLcaA and pPreLcaA revealed the expression of each fusion protein (figure 3.16). The crude induced and un-induced cytoplasmic extracts showed a distinct difference which is indicated by arrows on figure 3.17, lanes 2 and 4. An induced sample is distinguished from an un-induced sample, merely by the presence of a dark band, which is not seen in the un-induced sample. This band represents the recombinant proteins as the addition of IPTG allows for its expression (Promega, 1996). A band between the 37 and 50 kDa range can be seen for both induced leucocin A (lane 2) and pre-leucocin A (lane 4) samples. These bands are not present in the un-induced samples and are of high intensity indicating a high level of expression. The maltose binding protein is approximately 42.5 kDa in mass (Bach *et al.*, 2001). The calculated mass for leucocin A and pre-leucocin A is 3.933 kDa and 6.588 kDa respectively according to data obtained from UniProtKB/Swiss-Prot, entry number P34034. Therefore, the estimated size of the MBP-LcaA is 46.433 kDa and MBP-preLcaA is 49.088 kDa. From figure 3.16, it is seen that induced bands lie within the 37 and 50 kDa range, which is a good indication that expression of the fusion proteins did occur. Furthermore, since there is a 2.655 kDa difference between the two fusion proteins, one would not expect to see any differences, especially by 10 % SDS-PAGE analysis.

Cells containing the MBP fusion proteins were harvested by centrifugation and resuspended in column buffer which was the suggested lysis buffer used for the purification of each MBP fusion protein. The column buffer, was chilled to prevent disruption of the cells and contained a pH of 7.4, which was optimal for the binding of the MBP fusion proteins to the amylose resin (Lee *et al.*, 2005). DTT served as an additive to the column buffer as this increased the binding of MBP fusion proteins to the resin thereby allowing a high yield during purification (Hennig & Schafer, 1998).

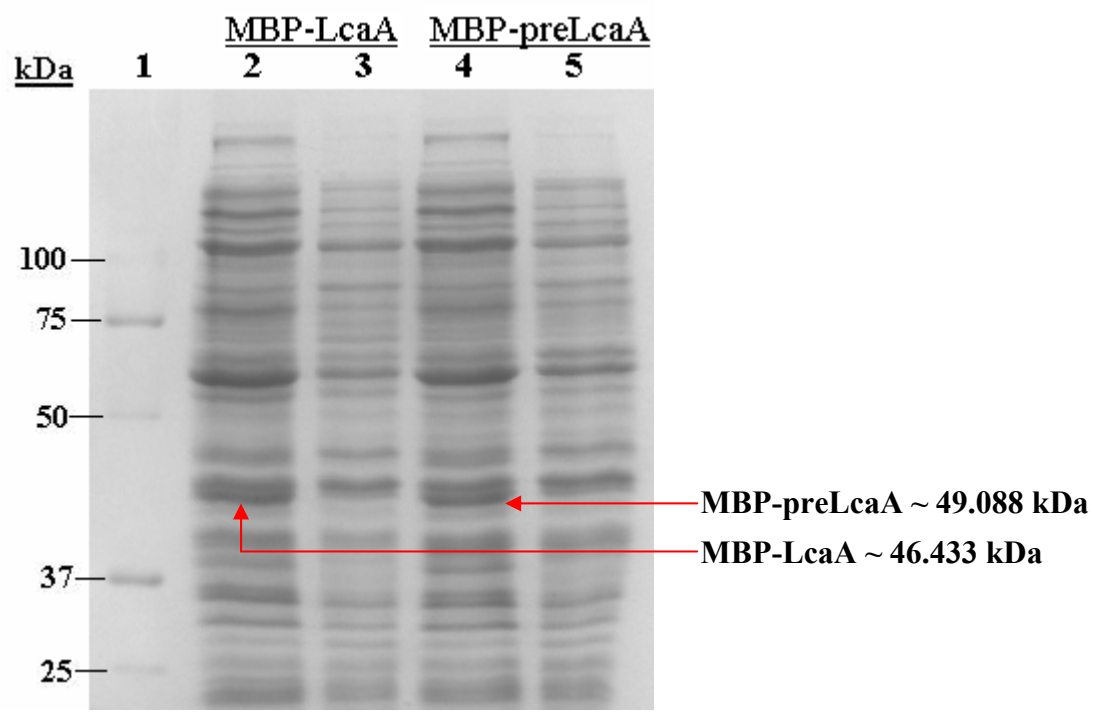


Figure 3.16. Coomassie stained 10 % SDS-PAGE gel indicating the induced and un-induced cytoplasmic extract of clones pLcaA and pPreLcaA. Lane 1: BIORAD Precision Plus Molecular Weight Marker (BioRad, South Africa), lane 2: crude induced extract of clone pLcaA, lane 3: pLcaA.1 crude un-induced extract, lane 4: crude induced extract of clone pPreLcaA, lane 5: pPreLcaA crude un-induced extract. Arrows indicate the positions and expected molecular weights of MBP-LcaA and MBP-preLcaA within each induced samples.

Within the pMAL-c2 system, fusion proteins are expressed within the cytoplasm of the host cell (*E. coli* JM103). Therefore, in order to bind and purify the MBP-LcaA and MBP-preLcaA proteins, *E. coli* cells require lyses in order to release that cytoplasmic content into the supernatant. Following ample expression time, cells bearing the fusion proteins were subjected to overnight lyses by freezing at -20 ^{o}C. Further cell lysis was carried out by sonication. This procedure uses short sound pulses which force cells to lyse thereby releasing their cytoplasmic content into the supernatant, where MBP fusion proteins can be purified. Sonication was performed in 1-minute intervals at 0.10 watts (RMS). After each 1 min burst, an aliquot was removed from the sample, added to Bradford reagent, and the absorbance at 595 nm taken. As the protein content within the supernatant increased, the absorbance at 595 nm increased. This procedure was used to monitor release of protein such that once the maximum amount of protein was released into the supernatant, sonication could be stopped. Absorbance readings obtained for each clone are listed in table 3.3. The protein content within the supernatant increased for the first seven bursts after which it began to decrease. This was the case for both clones pLcaA and pPreLcaA.

Table 3.2. Absorbance readings taken at 595 nm during the sonication of expressed clones pLcaA and pPreLcaA.

Number of Bursts (1 min each)	**Absorbance at 595 nm**	
	Clone pLcaA	**Clone pPreLcaA**
1	0.5388	0.6154
2	0.9244	1.0853
3	1.3320	1.2782
4	1.3607	1.4514
5	1.5271	1.5549
6	1.5559	1.6397
7	1.6030	1.6988
8	1.5662	1.6549

In order to visualize the recombinant protein content after sonication, samples were removed from the pLcaA and pPreLcaA sonicated culture, and compared to the unsonicated crude induced cytoplasmic extract by 10 % SDS-PAGE. Upon centrifugation, the sonicated sample was divided into the crude extract (supernatant) containing the MBP-fusion proteins, and the insoluble matter (pellet). These were also analyzed together with the sonicated sample. The 10 % SDS-PAGE containing sonicated samples, crude extracts, and insoluble matter for each fusion protein is shown in figure 3.17. Marked difference in the fusion protein content can be seen between the unsonicated and sonicated samples for each clone. These differences are apparent in lanes 2 and 3 (unsonicated and sonicated samples for clone pLcaA), and lanes 6 and 7 (unsonicated and sonicated samples for clone pPreLcaA). The MBP fusion protein yield has increased as a result of sonication and this is especially seen by the presence of a much larger and darker band (marked by arrows on figure 3.17). Therefore, sonication was successful in releasing fusion protein into the supernatant so that they could be purified. Fusion proteins, MBP-LcaA and MBP-preLcaA within the crude extracts, were purified using affinity chromatography. Crude extracts for clone pLcaA and pPreLcaA are seen in lane 4 and lane 8. These samples have higher fusion protein content than the insoluble matter, which is seen in lanes 5 and 9 for the respective clones. Therefore, a high proportion or fusion protein is present which can now be bound onto the amylose column and separated from all unwanted cellular protein.

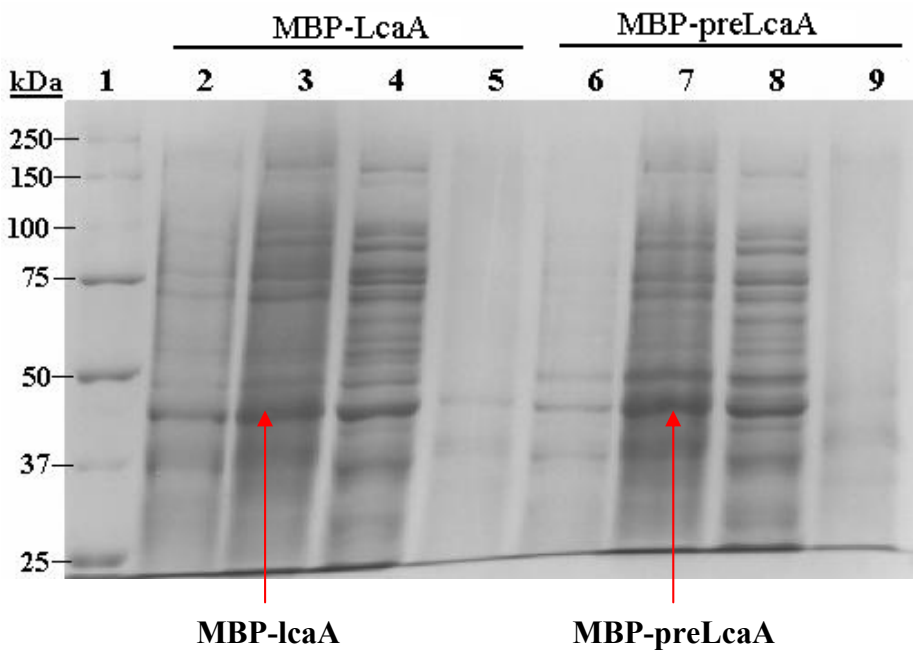


Figure 3.17. Expressed clones pLcaA and pPreLcaA following sonication and centrifugation. Samples were sonicated in 1 min intervals at 0.1 watts (RMS). Following sonication, samples were centrifuged and the supernatant extracted (crude extract). The resulting pellet constituted the insoluble matter. Lane 1: BIORAD Precision Plus Molecular Weight Marker (BioRad, South Africa), lane 2: crude induced extract of clone pLcaA, lane 3: clone pLcaA after sonication, lane 4: crude extract containing MBP-LcaA, lane 5: insoluble matter of clone pLcaA, lane 6: crude induced extract of clone pPreLcaA, lane 7: clone pLcaA after sonication, lane 8: crude extract containing MBP-preLcaA, lane 9: insoluble matter of clone pPreLcaA. Marked differences are seen between the MBP fusion yield before and after sonication (indicated by arrows).

3.7.2 Purification of MBP Fusion Proteins by Affinity Chromatography

The expressed fusion proteins were purified in one-step by affinity chromatography on amylose matrices. The maltose binding protein, connected via a short linker to the N-terminus of MBP-LcaA and MBP-preLcaA, binds to the amylose resin while other non-specific proteins flow through (Hage, 1999). The MBP fusion proteins are then eluted by competitive displacement with maltose. Miller *et al.* (1998[a]) used the MBP tag to successfully purify chimeric forms of pediocin. This method provided high yields of each fusion protein following purification. Furthermore, the MBP tag did not affect the biological activity of pediocin since the activity of the bacteriocin was tested while fused to the tag. In a similar way Quadri *et al.* (1995), expressed the immunity protein and carnobacteriocin B2 as a MBP fusion construct; which were purified with affinity chromatography.Therefore, based on past research, the MBP tag fusion protein system provides an efficient and reliable method for the purification of MBP-LeuA and MBP-preLeuA.

During the affinity purification of MBP-LcaA and MBP-preLcaA, aliquots of the crude extract, flow through, and wash samples were collected and analyzed by 10 % SDS-PAGE that is shown in figure 3.18. The fusion proteins present in these samples are present in lanes 2, 3, and 4 for MBP-LcaA and lanes 6, 7, and 8 for MBP-preLcaA. The induced band in lanes 2 and 6 for each fusion protein are also present in the flow through (lanes 3 and 7). This indicates that the binding capacity of the amylose resin was poor and not able to bind some of the protein. A plausible explanation for this is that the concentration of protein within the crude extract was too high. Absorbance readings at 280 nm of the crude extracts gave an absorbance value of 3.6251 for MBP-LcaA and 3.2294 for MBP-preLcaA. In general, the protein concentration of the crude extract should be about 2.5 mg/ml to allow efficient binding of the fusion protein. A proportion MBP fusion protein was also washed out during the wash step as a faint band is present with lanes 4 and 8 for MBP-LcaA and MBP-preLcaA. However, it is a minute amount when considering the intensity of the fusion protein band with the crude samples.

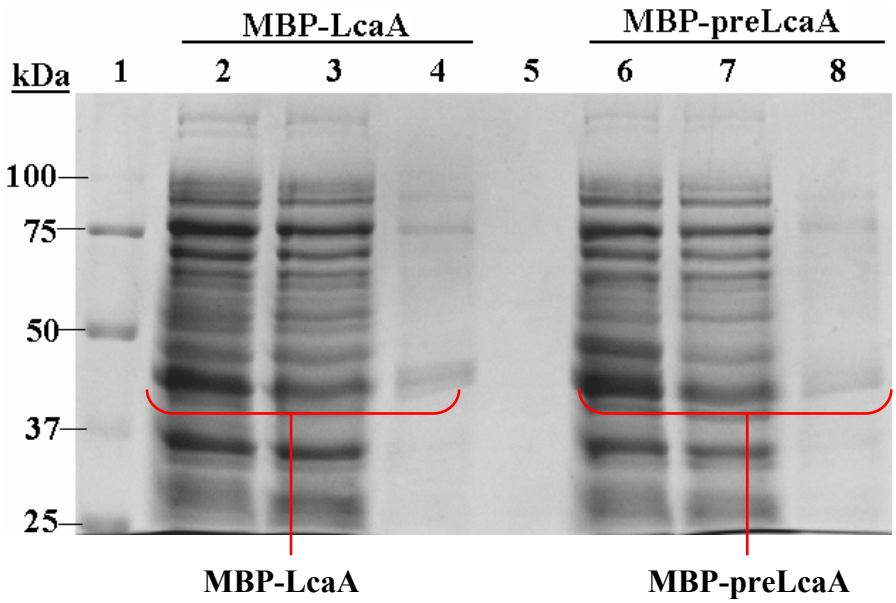


Figure 3.18. SDS-PAGE analysis of the amylose affinity chromatography used for the purification of MBP-LcaA and MBP-preLcaA. Lane 1: BIORAD Precision Plus Molecular Weight Marker (BioRad, South Africa), lane 2: crude extract of clone pLcaA containing the MBP-LcaA fusion protein, lane 3: flow through, lane 4: wash, lane 5: empty, lane 6: crude extract of clone pPreLcaA containing the MBP-preLcaA fusion protein, lane 7: flow through, lane 8: wash. Bands representing fusion proteins are highlighted in the respective lanes. Dark bands in lanes 2 and 6 indicate that the concentration of MBP-LcaA and MBP-preLeuA is high. These bands are also present in the flow through and wash samples indicating poor binding of each fusion protein to the amylose resin.

Following binding of MBP-LcaA and MBP-preLcaA onto the amylose matrix, the fusion proteins were eluted under mild conditions using maltose. Maltose has an affinity to bind to amylose and thereby displaces the bound MBP-LcaA and MBP-preLcaA by competitive binding (Hage, 1999). In general practice, MBP fusion proteins are usually eluted within the first five fractions (Lauritzen *et al.*, 1991). For the elution of MBP-LcaA and MBP-preLcaA nine 3 ml fractions were collected for each fusion protein. These fractions recovered during purification were analyzed by 10 % SDS-PAGE in order to determine if the procedure was successful in purifying both fusion proteins. The expected results for SDS-PAGE analysis is a single band representing each fusion protein. The results obtained for fractions collected for MBP-LcaA and MBP-preLcaA are shown in figures 3.19(a) and 3.20(a) respectively. The first three fractions of MBP-LcaA and four fractions of MBP-preLcaA collected, resulted in the elution of many non-specific proteins for both purification procedures. This can be due to low stringency washes of the column. Furthermore, the majority of the fusion proteins were eluted within these fractions and this is seen by the intense bands produced in lane 3 and 4 on figures 3.19(a) and 3.20(a). These fractions required further purification before subsequent analysis and this can be achieved by passing fractions 3 and 4 of both fusion proteins through the amylose column again, washing the column, and eluting new fractions. Pure MBP-LeuA was eluted from the fourth fraction collected and this seen by the presence of single bands (~ 46.433 kDa) in lanes 5 to 10 on figure 3.20(a). MBP-preLcaA was eluted from fraction 5 to 9. Single bands of ~ 49.088 kDa are seen in lanes 6 to 10 on figure 3.20(a). The intensity of single bands for MBP-LcaA and MBP-preLcaA is high, indicating that fusion proteins are concentrated within these fractions. Therefore, subsequent concentration steps by freeze drying were not required.

The overall purification procedure for each fusion protein was monitored at 280 nm. This involved taking absorbance readings of aliquots of each crude extract, flow through, washes, and the fractions collected. Chromatograms for the affinity separation of MBP-LcaA and MBP-preLcaA were then constructed using these values. These are represented in figures 3.19(b) for the purification of MBP-LcaA, and figure 3.20(b) for MBP-preLcaA. From these chromatograms, it is seen that the protein concentration of each fusion sample is decreasing at the flow through and wash steps. This is due to non-specific protein passing through the amylose column. The A_{280} values then begin to peak at particular fractions, which indicates those fractions that contain the highest concentration of each MBP fusion protein. However, analysis of the chromatograms together with the SDS-PAGE containing the fractions

collected, indicate that peaks at fractions 1 and 2 for MBP-LcaA, and fractions 2, 3, and 4 for MBP-preLcaA, are due to non-specific proteins. This is due to the multiple bands seen in figure 3.19(a) (lanes 3 and 4), and figure 3.20(a) (lanes 3, 4, and 5). Peaks for fraction 6 and 7 for MBP-LcaA and MBP-preLcaA and single bands for these fractions (figures 3.20a and 3.20a) represent those fractions that contain the highest concentration of the respective fusion proteins. From the results seen in figures 3.19 and 3.20, it can be concluded that affinity chromatography was successful in isolating pure forms of each MBP fusion protein.

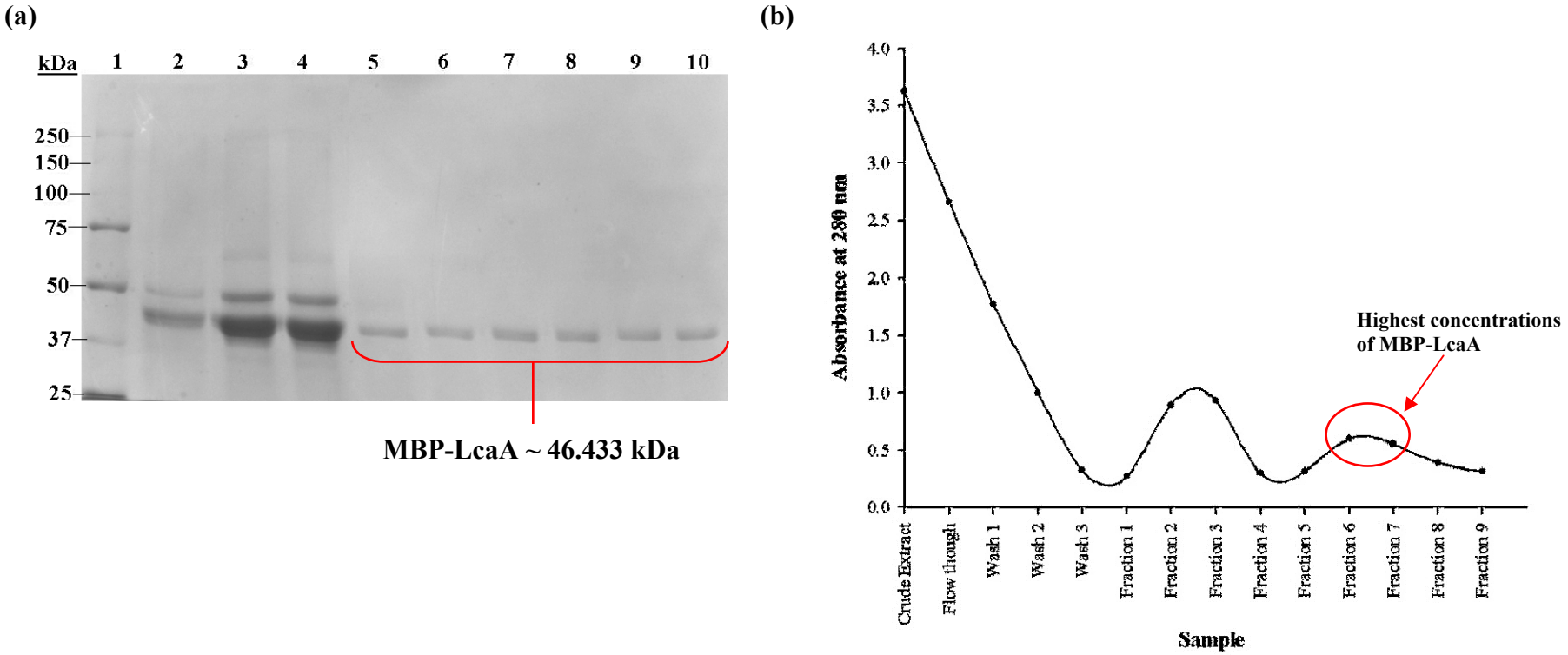


Figure 3.19. (a) SDS-PAGE analysis of MBP-LcaA fractions collected by amylose affinity chromatography. Lane 1: BIORAD Precision Plus Molecular Weight Marker (BioRad, South Africa) , lane 2 to lane 10: fraction 1 to 9 of MBP-LcaA. Fractions containing pure MBP-LeuA were obtained from fraction 4 onwards; (b) A chromatogram constructed for the affinity purification of MBP-LcaA. The absorbance at 280 nm of each component of the purification i.e. crude extract, flow through, washes, and fractions 1 to 9 were determined. Fractions 6 and 7 which are indicated by an arrow contain the concentration of MBP-LcaA.

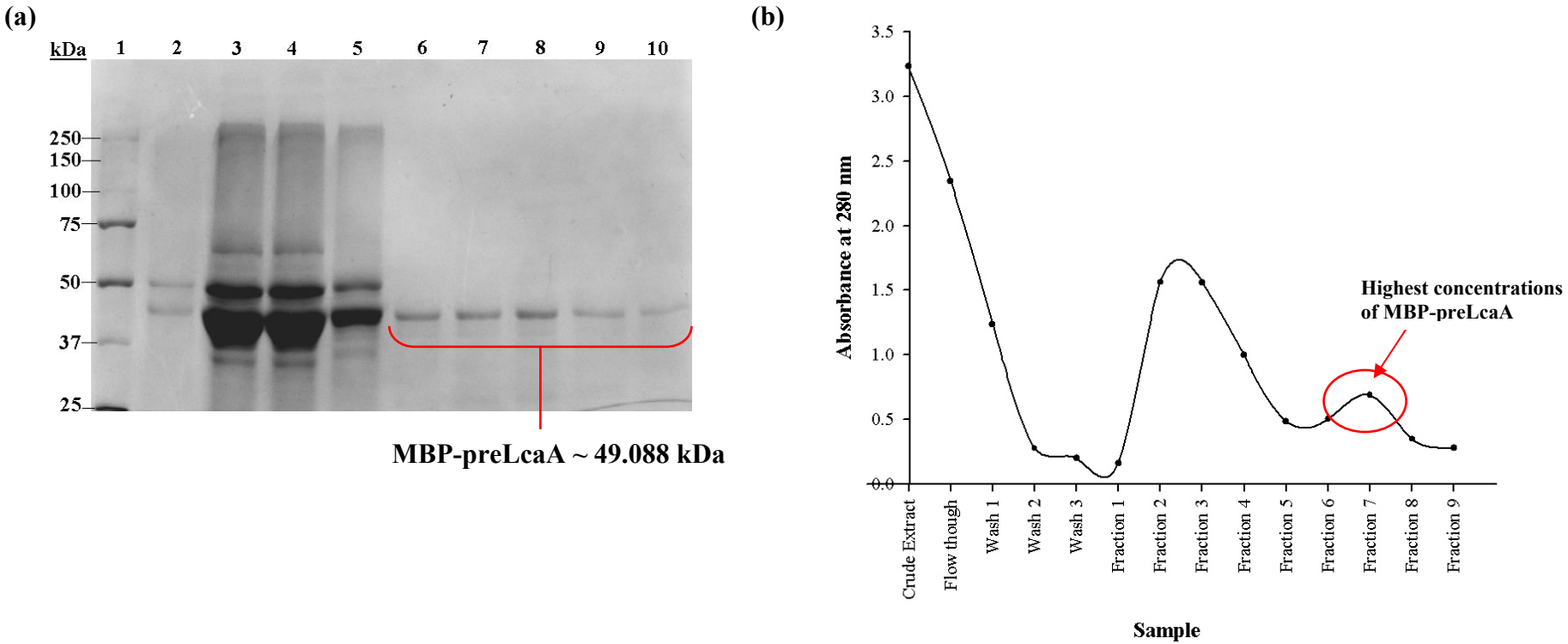


Figure 3.20. (a) SDS-PAGE analysis of MBP-preLcaA fractions collected by amylose affinity chromatography. Lane 1: BIORAD Precision Plus Molecular Weight Marker (BioRad, South Africa), lane 2 to lane 10: fraction 1–9 of MBP-preLcaA. Pure fractions of MBP-preLcaA were obtained from fraction 5 onwards; (b) A chromatogram constructed for the affinity purification of MBP-preLcaA. The absorbance at 280 nm of each component of the purification i.e. crude extract, flow through, washes, and fractions 1 to 9 were determined. Fractions 6 and 7 which are indicated by an arrow contain the concentration of MBP-preLcaA.

3.7.3 Quantification of purified protein by BCA protein assay

Fusion proteins collected by amylose affinity purification were assayed using the BCA protein assay. With this method, the protein concentration of MBP-LcaA and MBP-preLcaA within each fraction, was determined. The BCA assay detects Cu^{1+}, which is formed when Cu^{2+} is reduced by proteins in an alkaline environment. Two molecules of BCA chelates to one cuprous ion (Cu^{1+}) resulting in the formation of a purple-coloured reaction. This water soluble complex exhibits a strong absorbance at 562 nm that in linear with increasing protein concentration (Pierce Biotechnology). Figure 3.21 represents the BSA standard curve used to calculate the protein concentration in each fraction. This curve was obtained by reacting standard concentrations of BSA with bicinchoninic acid and thereafter, reading the absorbance at 562 nm. A non-linear relationship exists between the absorbance and concentration of BCA, seen in figure 3.21. Certain protein assay kits allow more linearity over a particular protein concentration range. For instance, if a concentration of 0 – 50 μg/ml a linear curve would have been obtained. As a result of a non-linear relationship, a regression curve was included in the plot. With the use of the straight line equation, $y = mx + c$ the protein concentration of each fraction was calculated. Aliquots of each fraction were reacted with bicinchoninic acid and the absorbance at 562 nm was determined. A graph representing the absorbance at 562 nm of MBP-LcaA and MBP-preLcaA, is shown in figure 3.22, which shows that the protein concentration within each fraction increases as the fraction number increases.

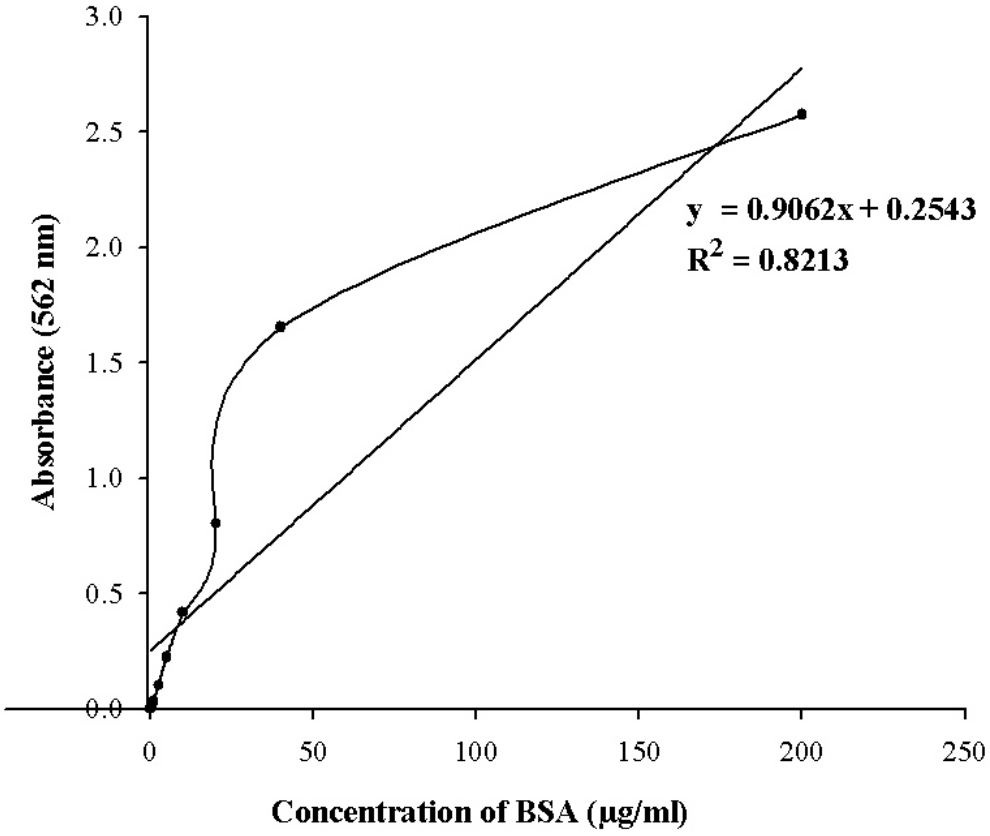


Figure 3.21. A Bovine Serum Albumin standard curve obtained by reacting standard concentrations of BSA with bicinchoninic acid and determining the absorbance at 562 nm of each reaction. A regression curve was determined and the straight line equation, y = 0.9062x + 0.2543 was used to calculate the protein concentration of MBP-LcaA and MBP-preLcaA in each fraction. The y value in the equation represented the absorbance of each fraction at 562 nm while the x value represented the protein concentration.

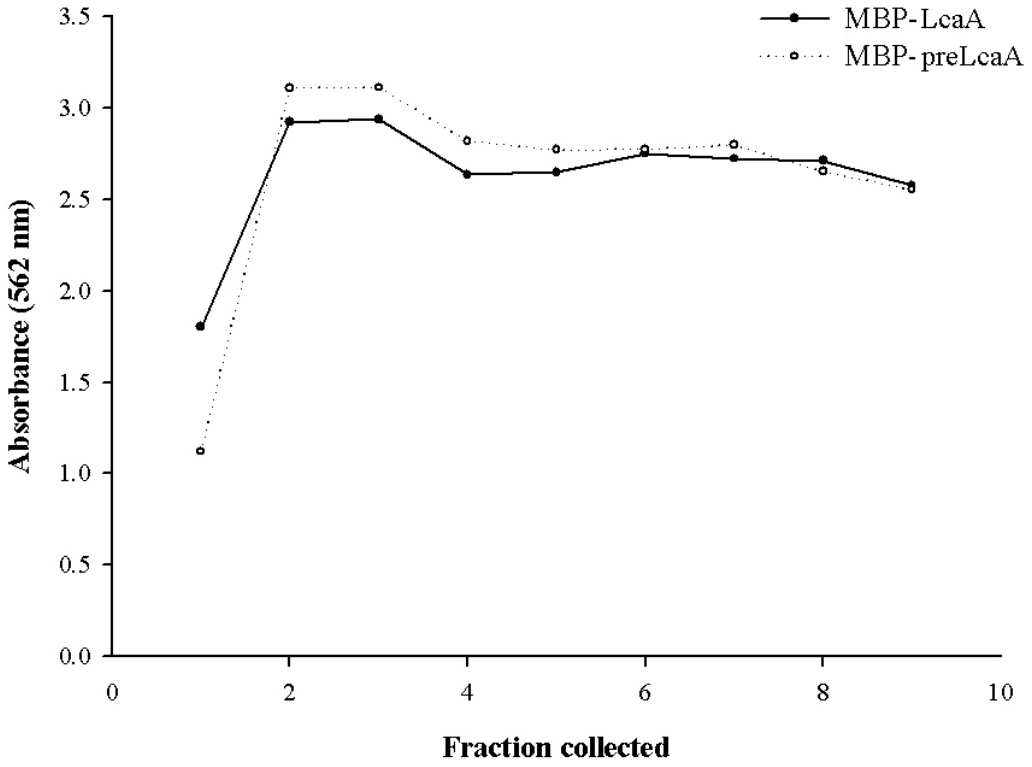


Figure 3.22. Absorbance readings taken at 562 nm after reacting an aliquot of each fraction of MBP fusion protein with BCA. The purple-colored reaction product exhibits a strong absorbance at 562 nm. Absorbance values obtained were substituted in the straight line equation in figure 3.21 and solved for x (protein concentration of MBP-LcaA and MBP-preLcaA).

The absorbance values of each fraction at 562 nm represented the y value in the straight line equation. The y value, was then substituted in the equation, y = 0.9062x + 0.2543 and x was determined which represented the concentration in μg/ml of MBP-fusion protein within each fraction. The calculated concentration of each fraction is shown in table 3.4 below.

Table 3.3. The concentration of MBP-LcaA and MBP-preLcaA within each of the nine fractions collected by amylose affinity chromatography.

Fraction	**Concentration (μg/ml)**	
	MBP-LcaA	**MBP-preLcaA**
1	1.7092	0.9566
2	2.9431	3.1508
3	2.9612	3.1523
4	2.6273	2.8315
5	2.6420	2.7759
6	2.7536	2.7804
7	2.7248	2.8048
8	2.7097	2.6450
9	2.5627	2.5362

Protein concentrations of purified MBP-LcaA in fractions 4 to 9 ranged from 2.5627 μg/ml to 2.7536 μg/ml with fraction 6 containing the highest concentration. Fraction 7 contained the higest concentration of MBP-preLcaA. Therefore, fraction 6 of MBP-LcaA and fraction 7 of MBP-preLcaA were used for subsequent analysis.

3.7.4 Western blot of the Purification Components

Anti – MBP monoclonal antibodies (murine anti-maltose binding protein antibody) were used to test for the presence of the MBP affinity tag by western blotting. These antibodies have an affinity to bind maltose binding protein and are conjugated with peroxidase. Due to the latter, the need for secondary antibodies were eliminated, and the time required for detection was reduced. Isolated maltose binding protein was used in the western blot procedure as a positive control and samples tested included the crude induced and uninduced, sonicated extract, crude extract, flow through, washes and purified MBP fusion protein.

The transfer of proteins onto nitrocellulose membrane was verified by temporary staining with Ponceau S. Upon addition of this stain, bands were seen in the appropriate lanes, which suggested that the transfer step was successful in transferring the majority of the proteins onto the membrane. This stain also allowed the detection of the protein standard marker. Upon staining, protein bands within the standard marker were marked in pencil as these are not detectable during western blotting.

Banding patterns obtained from the detection procedure are depicted in figures 3.23 and 3.24. The maltose binding protein was unnoticeable in figures 3.23(a) (lane 9) and 3.24(a) (lane 9). This could be due to low concentrations of protein within the purified fraction. Furthermore, the MBP affinity tag (42.5 kDa) was detected at an unexpected molecular weight, which is seen in lane 1 of figure 3.23(b) and 3.24(b). By comparing the reference gel (figure 3.23a), with the corresponding blot (figure 3.23b), it is apparent that the MBP affinity tag is present. However, upon closer inspection of figures 3.23(a) and 3.24(a) bands are present within the crude uninduced extract. Catabolic repression was not inforced the expression experiment and due to the nature of the lac promoter, low levels of constitutive expression did occur. Hence, the presence of a putative band in lanes 2 of figures 3.23(a) and 3.24(a). Bands in figure 3.23(b) were detected for the MBP-LcaA crude induced (lane 7), sonicated extract (lane 6), and the crude extract (lane 5) at the expected molecular weight. An intense band is seen in lane 2 of figure 3.23b, which represents MBP-LcaA. This suggests that a MBP tagged protein is present. Conversely, the detection procedure for MBP-preLcaA purification samples (figure 3.24b), did not produce the expected results. Intense multiple bands due to the over detection of protein by the anti-MBP antibodies are seen in lanes 4 to 7. This could be the result of insuffient blocking and increased binding of the antibody to the membrane. Therefore, due to

poor experimental procedure no conclusions were obtained for the western blot of MBP-preLcaA although a single band A was detected in lane 2 of figure 3.24(b)

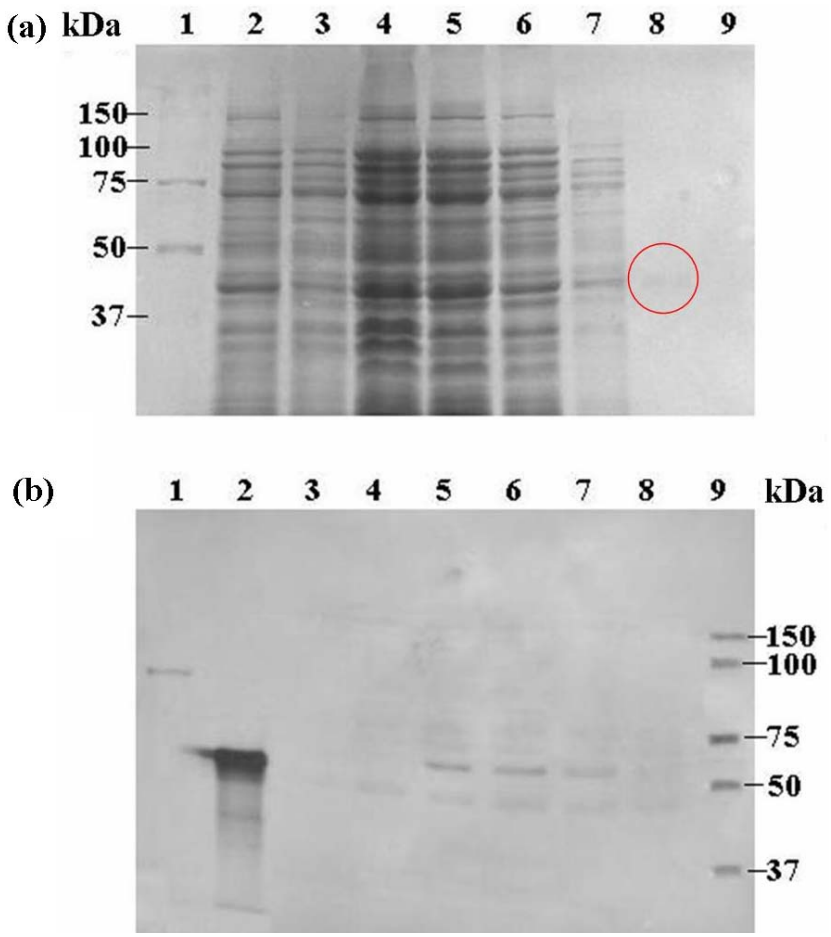


Figure 3.23. Western blot for the purification procedure of MBP-LcaA. The detection of the MBP affinity tag was carried out using anti-MBP antibodies. (a) Reference gel used to analyse the bands produced by detection procedure. Lane 1: BIORAD Precision Plus Molecular Weight Marker (BioRad, South Africa), lane 2: crude uninduced extract of clone pLcaA.1, lane 3: crude induced extract, lane 4: sonicated extract, lane 5: crude extract, lane 6: flow through, lane 7: wash sample, lane 8: MBP-LcaA fusion protein, lane 9: MBP affinity tag; (b) Nitrocellulose membrane containing detected bands. Lane 1: MBP affinity tag, lane 2: MBP-LcaA fusion protein, lane 3: wash sample, lane 4: flow through, lane 5: crude extract, lane 6: sonicated extract, lane 7: crude induced extract, lane 8: crude uninduced extract of clone pLcaA.1, lane 9: BIORAD Precision Plus Molecular Weight Marker (BioRad, South Africa). Bands were detected at the expected molecular weight indicating the presence of the MBP affinity tag. MBP-LcaA is highlighted in lane 8 of figure 3.23(a).

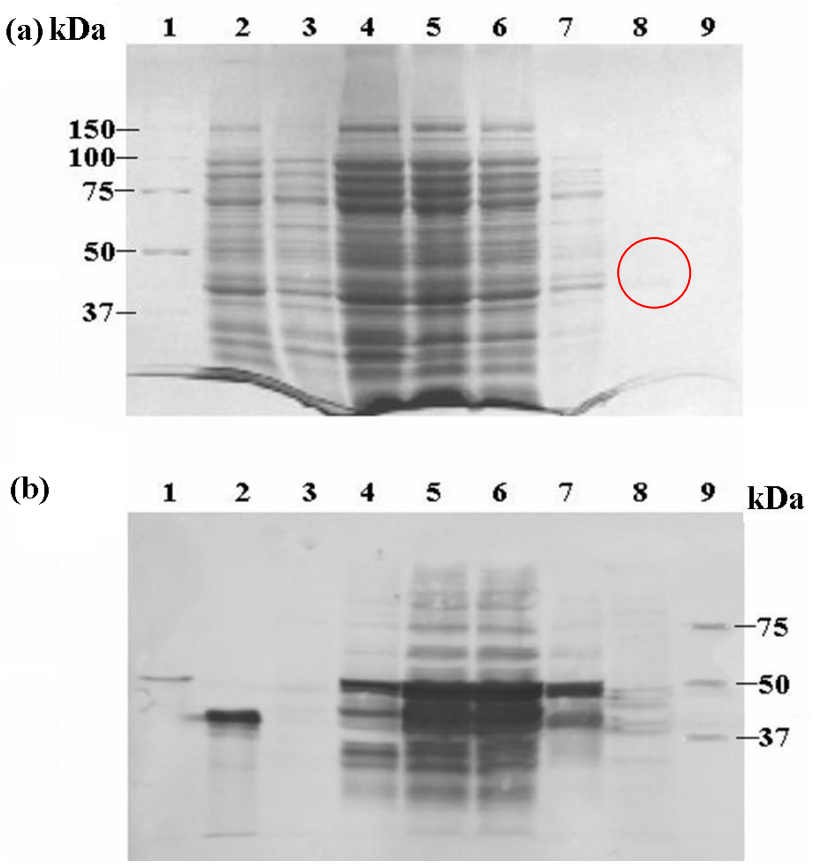


Figure 3.24. Western blot used to detect MBP-preLcaA in various samples during the purification procedure. The detection of the MBP affinity tag was carried out using anti-MBP antibodies. (a) Reference gel used to analyse the bands produced by detection procedure. Lane 1: BIORAD Precision Plus Molecular Weight Marker (BioRad, South Africa), lane 2: crude uninduced extract of clone pLcaA.1, lane 3: crude induced extract, lane 4: sonicated extract, lane 5: crude extract, lane 6: flow through, lane 7: wash sample, lane 8: MBP-preLcaA fusion protein, lane 9: MBP affinity tag; (b) Nitrocellulose membrane containing detected bands. Lane 1: MBP affinity tag, lane 2: MBP-preLcaA fusion protein, lane 3: wash sample, lane 4: flow through, lane 5: crude extract, lane 6: sonicated extract, lane 7: crude induced extract, lane 8: crude uninduced extract of clone pPLcaA.1, lane 9: BIORAD Precision Plus Molecular Weight Marker (BioRad, South Africa). Over detection on protein by the anti-MBP antibody occurred and thus a conclusion was not made for the western blot of MBP-preLeuA. MBP-preLcaA is highlighted in lane 8 of figure 3.23(a).

3.7.5 Inhibition assay using MBP fusion proteins

A deferred antagonism assay was carried out in order to determine if MBP-LcaA and MBP-preLcaA have activity directed against the growth of *L. monocytogenes*. This was carried out as certain studies have shown that bacteriocins expressed with an MBP tag do retain activity. Miller *et al.* (1998[a]) and Ray, Schamber & Miller, (1999) determined that MBP-pediocin AcH exhibits activity against *Listeria innocua Lin 11*. Aliquots of up to 20 µl of MBP-LcaA (2.7536 µg/ml) and MBP-preLcaA (2.8048 µg/ml), were spotted onto an agar lawn supplemented with the indicator strain (*L. monocytogenes*). From the results obtained in figure 3.25(a) and (b), it is clear that each fusion protein is not active as no zones of inhibition were produced. Partially purified leucocin A was included in the assay as a positive control and this produced an intense zone of inhibition, suggesting that the experimental procedure was carried out correctly.

The mode of action of class IIa bacteriocins relies on proper conformational folding of particular domains. These include the C-terminal amphiphilic α-helix and the N-terminal conserved domain, as research has shown that these regions play a vital role in the mode of action (Fimland *et al.*, 1996; Chen *et al.*, 1997[b]). The MBP tag was introduced at the N-terminus of leucocin A and pre-leucocin A. Positively charged amino acids within this domain are said to be involved in a recognition step during class IIa mode of action (Fimland *et al.*, 1996). Therefore, the introduction of the maltose binding protein may have hindered the role of the N-terminal domain. Furthermore, MBP is estimated to be 42.5 kDa, while leucocin A and pre-leucocin A correspond to molecular weights of 3.933 kDa and 6.588 kDa (Hastings *et al.*, 1991; Bach *et al.*, 2001). The affinity tag is much larger than the antimicrobial peptides. As a result, proper conformational folding could have been prevented due to sterical hindrance introduced by the MBP tag. The findings, which suggest that MBP-pediocin AcH is active, could be attributed to that fact that pediocin AcH is much larger that leucocin A and/or that leucocin A relies more on its N-terminus for antimicrobial activity.

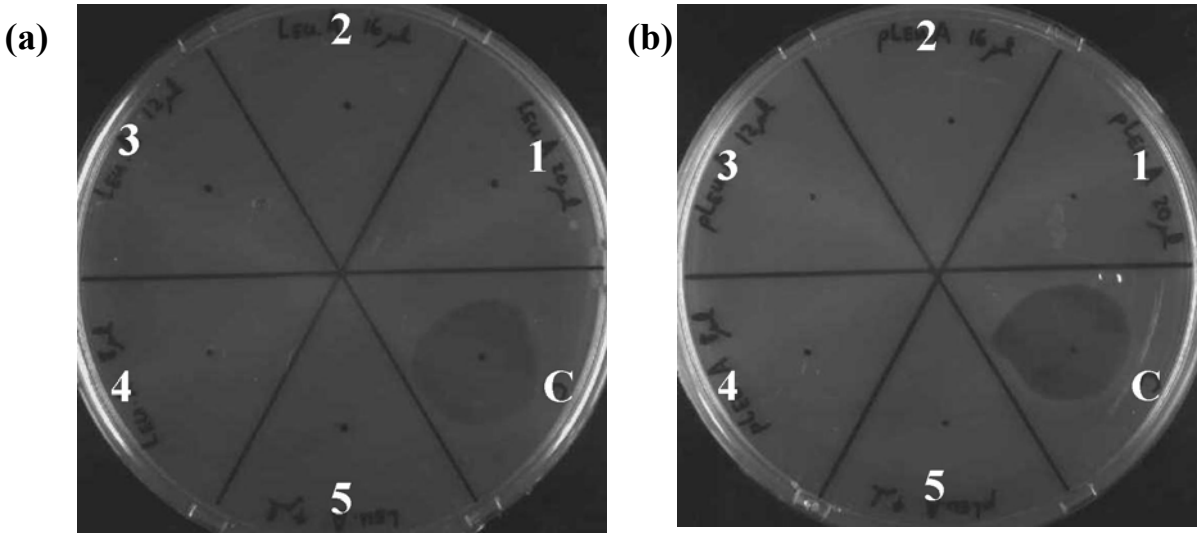


Figure 3.25. Deferred inhibition of *Listeria monocytogenes* by MBP-LcaA and MBP-preLcaA fusion proteins. (a) Deferred inhibition assay using MBP-LcaA; (b) Deferred inhibition assay using MBP-preLcaA. The positive control which produced a zone of inhibition is labelled as C on each plate. Aliquots of 2 to 20 µl of each fusion protein were spotted onto the agar lawn containing the indicator strain *L. monocytogenes*. No zones of .inhibition were seen after incubation for both fusion proteins.

3.8 Factor Xa cleavage of MBP Fusion Proteins

The removal of the MBP tag from recombinant peptides, leucocin A and pre-leucocin A was necessary, since each fusion construct did not exhibit antimicrobial activity. It was suggested that the presence of the MBP affinity tag obstructed proper folding of leucocin A and pre-leucocin A thereby preventing antimicrobial activity. The maltose binding tag was removed from fusion proteins, MBP-LcaA and MBP-preLcaA, by cleavage with Factor Xa. Factor Xa is a serine protease isolated from bovine plasma and cleaves after the arginine residue in its preferred cleavage site, Ile-(Glu or Asp)-Gly-Arg (Maina *et al.*, 1988; Gardella *et al.*, 1990). According to the pMAL protein and purification manual (New England Biolabs), MBP fusion proteins contain the Ile-Glu-Gly-Arg recognition site at their fusion joints. Therefore, Factor Xa cleavage after the arginine residue in this recognition site of MBP-LcaA and MBP-preLcaA, results in the removal of the MBP affinity tag.

A test digestion of each fusion protein with Factor Xa was performed in order to determine the optimal time required for total cleavage of the MBP tag from leucocin A and pre-leucocin A. It was found that an incubation time of 24 hrs is adequate for complete removal of the affinity tag from the recombinant proteins. Once this was established the reaction was scaled up. Factor Xa cleavage of the MBP fusion proteins was analyzed by 15 % (w/v) SDS-PAGE, which is seen in figure 3.26. As already mentioned, MBP-LcaA is approximately 46.433 kDa, while MBP-preLcaA corresponds to a molecular weight of 49.088 kDa. Slight differences in size can be seen between each fusion protein in lanes 2 and 4 (uncut controls) of figure 3.26.

Under optimal conditions, MBP fusion protein cleavage by Factor Xa produces two products, which consist of the MBP tag and the recombinant protein/peptide (Jenny *et al.*, 2003). From figure 3.26, it can be seen that Factor Xa cleavage of MBP-LcaA and MBP-preLcaA yielded two of the above mentioned products, which are labelled on figure 3.26. The maltose binding protein in lanes 3 and 5 corresponds to a molecular weight of ~42.5 kDa, while leucocin A (3.933 kDa) and pre-leucocin A (6.588 kDa) are migrating along the 25 kDa level. These peptides are cationic in nature and aggregate during pore formation. Reports have suggested that certain bacteriocins are able to aggregate in an SDS environment. A study by Osmanagaoglu *et al.* (1998), reported differences in the molecular weight of pediocin F up to 16.6 kDa. Similar results were seen by Bhunia *et al.* (1987), and increases in the molecular weight of pediocin PA-1 were attributed to the ability of these peptides to aggregate in a SDS

environement. This explains the high molecular weight seen for leucocin A and pre-leucocin A.

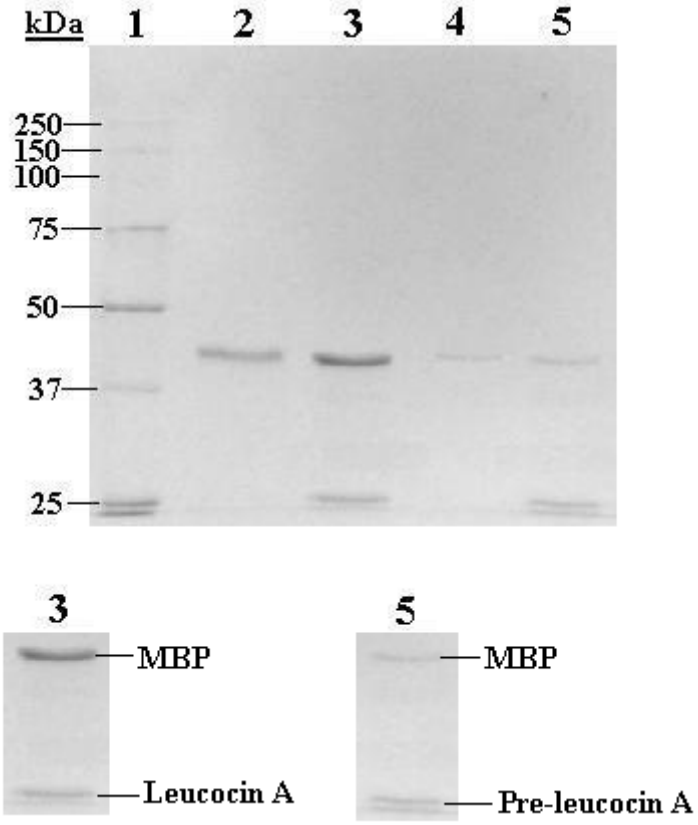


Figure 3.26. 15 % (w/v) SDS-PAGE analysis of MBP-LcaA and MBP-preLeuA following Factor Xa cleavage. Lane 1: BIORAD Precision Plus Molecular Weight Marker (BioRad, South Africa), lane 2: MBP-LcaA uncut control, lane 3: cleavage of MBP-LcaA with Factor Xa, lane 4: MBP-preLcaA uncut control, lane 5: cleavage of MBP-preLcaA with Factor Xa. Size differences are clearly seen for MBP-LcaA and MBP-preLcaA uncut controls in lanes 4 and 6. Factor Xa cleavage generated two products, each of which are labelled. The high molecuar weight seen for leucocin A and pre-leucocin A is attributed to aggregation as already explained.

3.8.1 Inhibition assay using cleaved MBP fusion protein

Once the MBP tag was removed by Factor Xa cleavage, each recombinant peptide within the cleavage mixture was tested for antimicrobial activity. The cleavage mixture consisted of maltose binding protein, Factor Xa, and the peptide of interest (leucocin A or pre-leucocin A). Activity was tested by a deferred inhibition assay with *L. monocytogenes* as the indicator strain. From figure 3.27, it is apparent that each of the cleavage mixtures did not exhibit any activity against *Listeria*, as zones of inhibition were not seen for either of the samples tested. The positive control (labelled C), did inhibit the growth of *Listeria* as a distinct zone of inhibition is seen.

The results can be interpreted by examining the properties of the solution, which contained the cleavage products. During affinity chromatography, MBP-LcaA and MBP-preLcaA, were eluted in column buffer containing 10 mM maltose. Factor Xa cleavage was performed in the same buffer which consists primarily of salts. Control leucocin A was purified using high performance liquid chromatography and was eluted in a volatile solvent (acetonitrile containing 0.1 % trifluoroethanol (TFE)). The mode of action of bacteriocins depends on their solution structure as this plays a vital role in the three dimensional folding of these peptides. Fregeau Gallagaher *et al.* (1997) tested the effects of two media, namely doecylphosphocholine (DPC) micelles and 90 % TFE, on the three dimensional structure of leucocin A. Here, it was established that this peptide adopts a more defined structure with regards to the C-terminal α-helix in 90 % TFE. As already mentioned in chapter one, the C-terminal α-helix is vital for activity, specifically pore formation, and site directed mutagenesis in this region results in a decrease in antimicrobial activity. In a similar study by Kaur *et al.* (2004) it was concluded that peptides such as sakacin P, pediocin PA-1, carnobacteriocin B2, and leucocin A adopt more stable structures in TFE compared to water. Peptides in TFE were shown to maintain a conserved amphiphilic α-helix in the C-terminal region and a β-sheet or random coil in the N-terminal region. Thus, it can be concluded that volatile solvents such as TFE and TFA, are more suitable for the antimicrobial activity of class IIa bacteriocins.

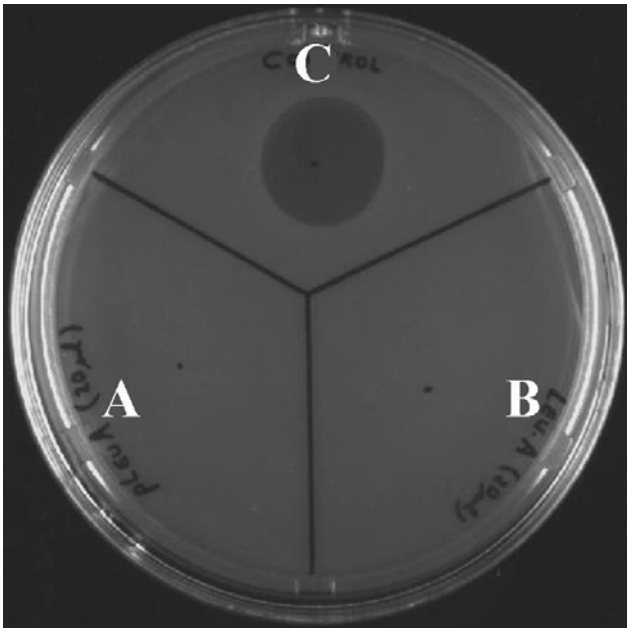


Figure 3.27. Deferred inhibition of *Listeria monocytogenes* using Factor Xa cleavage mixtures for MBP-LcaA and MBP-preLcaA fusion proteins. Both cleavage mixtures did not inhibit the growth of *L. monocytogenes*, as no zones of inhibition were produced. The positive control, however, did produce a zone of inhibition. Samples spotted are labelled as follows: Cleaved MBP-preLcaA (A); cleaved MBP-LcaA (B); Control (C).

3.9 N-terminal Protein Sequencing of Isolated Peptides

Cleaved MBP-LcaA and MBP-preLcaA were transferred onto PVDF membrane so that the suggested leucocin A and pre-leucocin A bands could be excised and sequenced. Since both MBP fusion proteins and Factor Xa cleavage mixture did not exhibit antimicrobial activity, N-terminal sequencing was used to verify if the correct peptides were isolated. Figure 3.28 represents the result obtained for the membrane transfer step. The reference gel containing each of Factor Xa cleavage sample, is shown in lanes 3 and 6 of figure 3.28(a). Lane 5 contains the uncut MBP-preLeuA control which is not visible. However, this does not affect the N-terminal sequencing procedure as only Factor Xa cleavage samples are required. Bands speculated to be leucocin A (lane 3) and pre-leucocin A (lane 6) migrating at a 25 kDa level. This was due to the ability of these peptides to form aggregates in an SDS environment (as explained section 3.8). Figure 3.29(b) represents the PVDF membrane containing the transferred protein samples. Lanes 1 and 4 contain the Factor Xa cleavage reactions for MBP-preLcaA and MBP-LcaA which have transferred successfully.

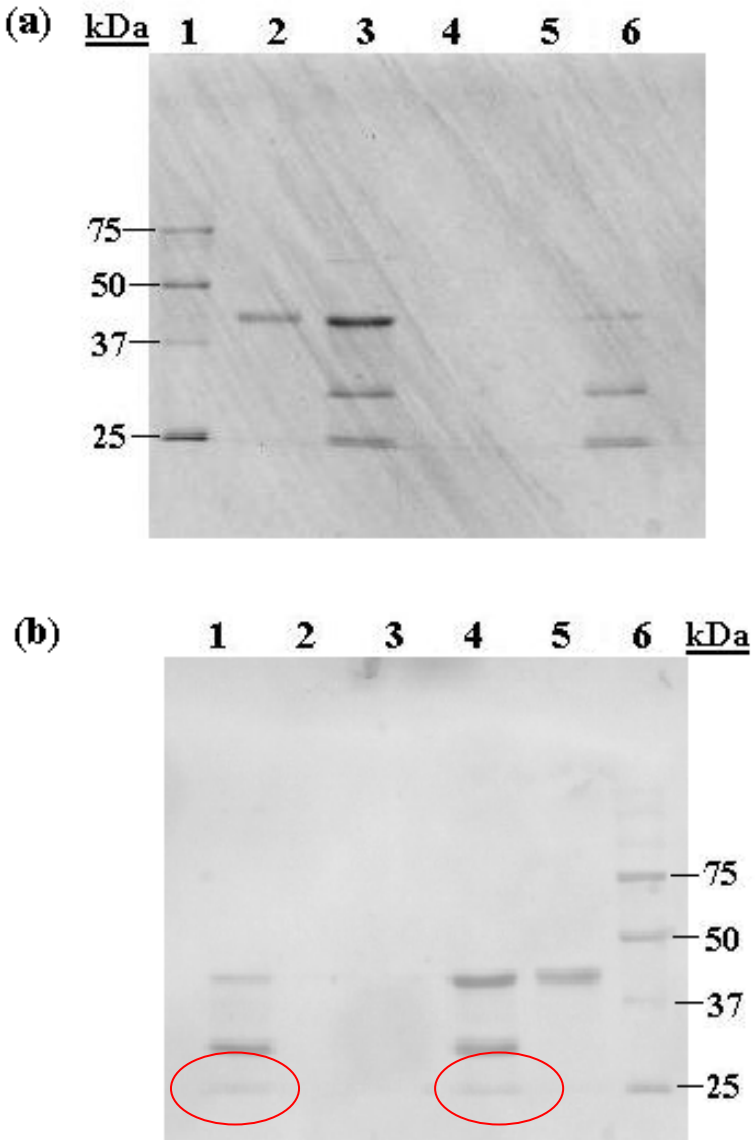


Figure 3.28. (a) SDS-PAGE analysis of the Factor Xa cleavage of MBP-LeuA and MBP-preLeuA. The gel was used as a reference for the transfer procedure of the cleaved protein onto PVDF membrane. Lane 1: BIORAD Precision Plus Molecular Weight Marker (BioRad, South Africa), lane 2: MBP-LcaA, lane 3: Factor Xa cleavage of MBP-LcaA, lane 4: Empty, lane 5: MBP-preLcaA, lane: Factor Xa cleavage of MBP-preLcaA; (b) PVDF membrane containing transferred proteins. Lane 1: Factor Xa cleavage of MBP-preLcaA, lane 2: MBP-preLcaA, lane 3: Empty, lane 4: Factor Xa cleavage of MBP-LcaA, lane 5: MBP-preLcaA, lane 6: BIORAD Precision Plus Molecular Weight Marker (BioRad, South Africa). By comparing figures 3.29(a) and 3.29(b), it is apparent that the procedure was successful in transferring each cleavage mixture onto the PVDF membrane. The high molecuar weight seen for leucocin A and pre-leucocin A is attributed to aggregation as already explained.

Circled protein bands in figure 3.28(b) which were assumed to be leucocin A (lane 4) and pre-leucocin A (lane 1), were excised and sequenced using the Procise cLC Protein Sequencer. The sequencing program was modified such that 13 N-terminal amino acids were sequenced. Analysis of the obtained chromatograms following each run, produced protein sequences depicted in table 3.4. A much shorter sequence was obtained for pre-leucocin A compared to that of leuconcin A. However, after comparing the sequences present in table 3.4 with the documented protein sequence of pre-leucocin A it was apparent the proteins tested were in fact leucocin A and pre-leucocin A.

Table 3.4. N-terminal sequences of proteins obtained after Factor Xa cleavage. The alignment of these sequences with pre-leucocin A is also shown

Peptide	**N-terminal sequence**
Leucocin A	KYYGNGVHCTK……
Pre-leucocin A	MMNMKP……

Peptide	**Amino acid alignment**
Pre-leucocin A	MMNMKPTESYEQLDNSALEQVVGGKYYGNGVHCTKSGCSVN……
	:::::: ::::::::::::
	MMNMKP KYYGNGVHCTK

3.10 Isolation of Peptides by High Performance Liquid Chromatography

Reverse phase high performance liquid chromatography was the purification method of choice used to separate each recombinant peptide from the MBP affinity tag and Factor Xa. Aside from being very sensitive, this method provided an additional benefit in that each peptide was eluted in a volatile solvent. As already mentioned, class IIa bacteriocins adopt a stable structure in volatile solvents such as acetonitrile containing 0.1 % TFE (Kaur *et al.*, 2004). Therefore, in order to compare the activity of leucocin A and pre-leucocin A, peptides were purified and eluted in acetonitrile containing 0.1 % TFA by RF-HPLC.

Factor Xa cleavage reactions for MBP-LcaA and MBP-preLcaA were applied to the injector compartment of the HPLC system in 200 µl aliquots and analysed as independent runs. The HPLC chromatograms obtained for these samples are depicted in figures 3.29 and 3.30. Each Factor Xa cleavage sample consists of three components i.e. the MBP affinity tag, Factor Xa, and the recombinant peptide. Therefore, HPLC analysis of a cleaved sample should produce three peaks. However, this was not the case for cleavage mixtures of MBP-LcaA and MBP-preLcaA due to the additional peaks are seen in figures 3.29 and 3.30. These could represent trace contaminants within the HPLC column or unwanted proteins obtained from amylose affinity chromatography. However, the absorbance of a number of these peaks is low, which indicates that these proteins are present in low concentrations. Peaks considered to be of importance (possibly leucocin A or pre-leucocin A), are numbered on each chromatogram present in figures 3.29 and 3.30. These peaks were eluted and stored for future use. Purified MBP as well as Factor Xa were similarly applied to the column. These were analyzed independently and served as control runs. Chromatograms obtained for MBP and Factor Xa are shown in figures 3.31 and 3.32 respectively. The peak with a retention time of 3,283 (figure 3.31), was suggested to represent the MBP affinity tag as this protein is present at a high concentration within solution. Upon analysis of figure 3.32, it was determined that the Factor Xa has a retention time of 11,623. Taking the above statements into account, peaks with similar retention times were identified on figures 3.29 and 3.30. It was therefore concluded that peaks with retention times of 3,283 and 10,593 on figure 3.29, were that of MBP and Factor Xa. Similarly, peaks with retention times of 3,333 and 11,263 on figure 3.31, were suggested to be MBP and Factor Xa.

The retention times for leucocin A and pre-leucocin A were indistinguishable due to the presence of multiple peaks in figures 3.29 and 3.30. For example in figure 3.29, peak 5 (24,893) was suggested to be leucocin A (high concentration). However, due to the presence of peak 2 (4,100) and peak 4 (16,267), this was not concluded. For this reason, all peaks labelled in figures 3.29 and 3.30, were assayed for antimicrobial activity.

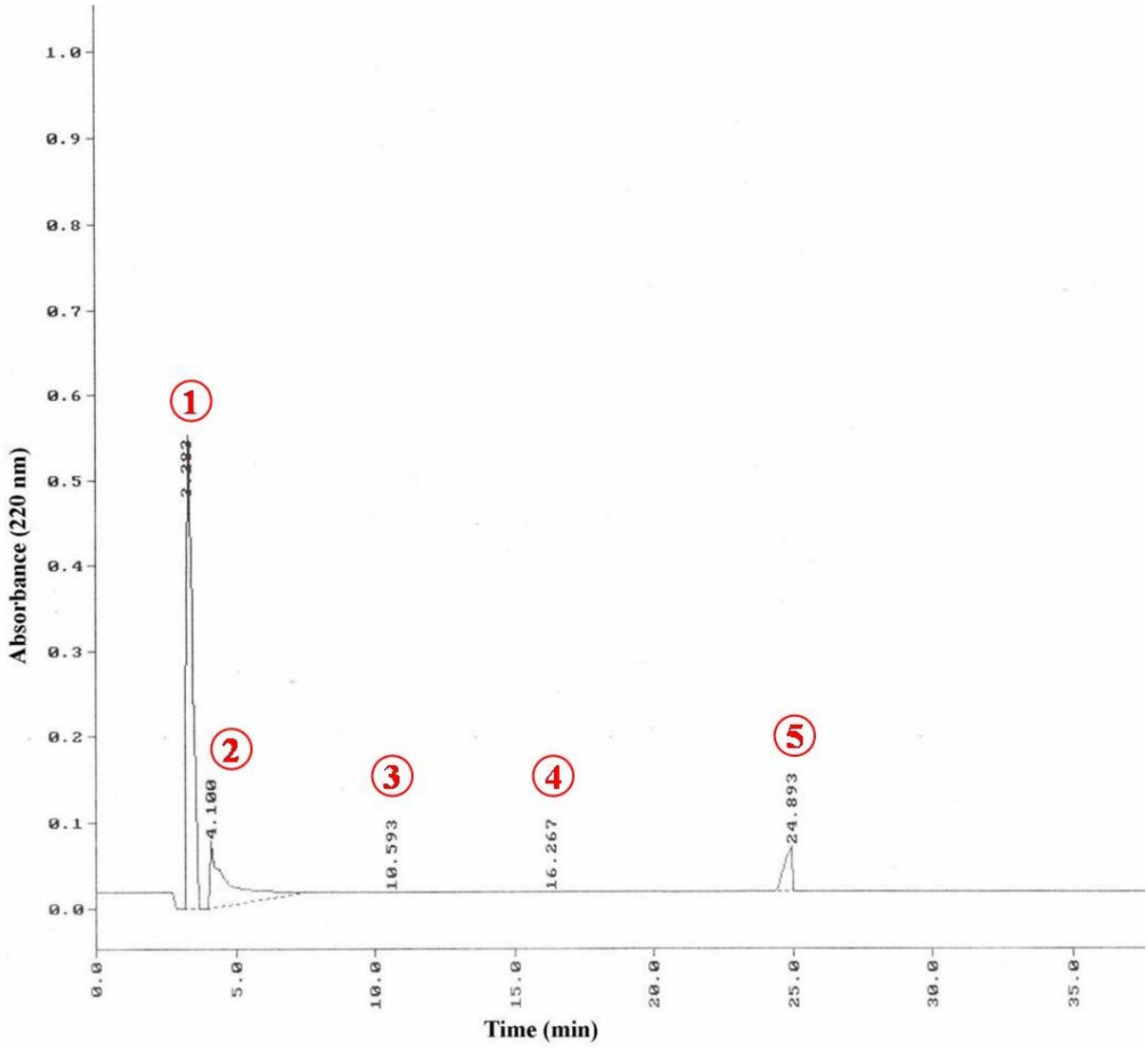


Figure 3.29. Chromatogram obtained from the HPLC analysis of MBP-LcaA cleaved with Factor Xa. Peaks, together with their retention times, are clearly shown. Numbered peaks were eluted in acetonitrile containing 0.1 % TFA.

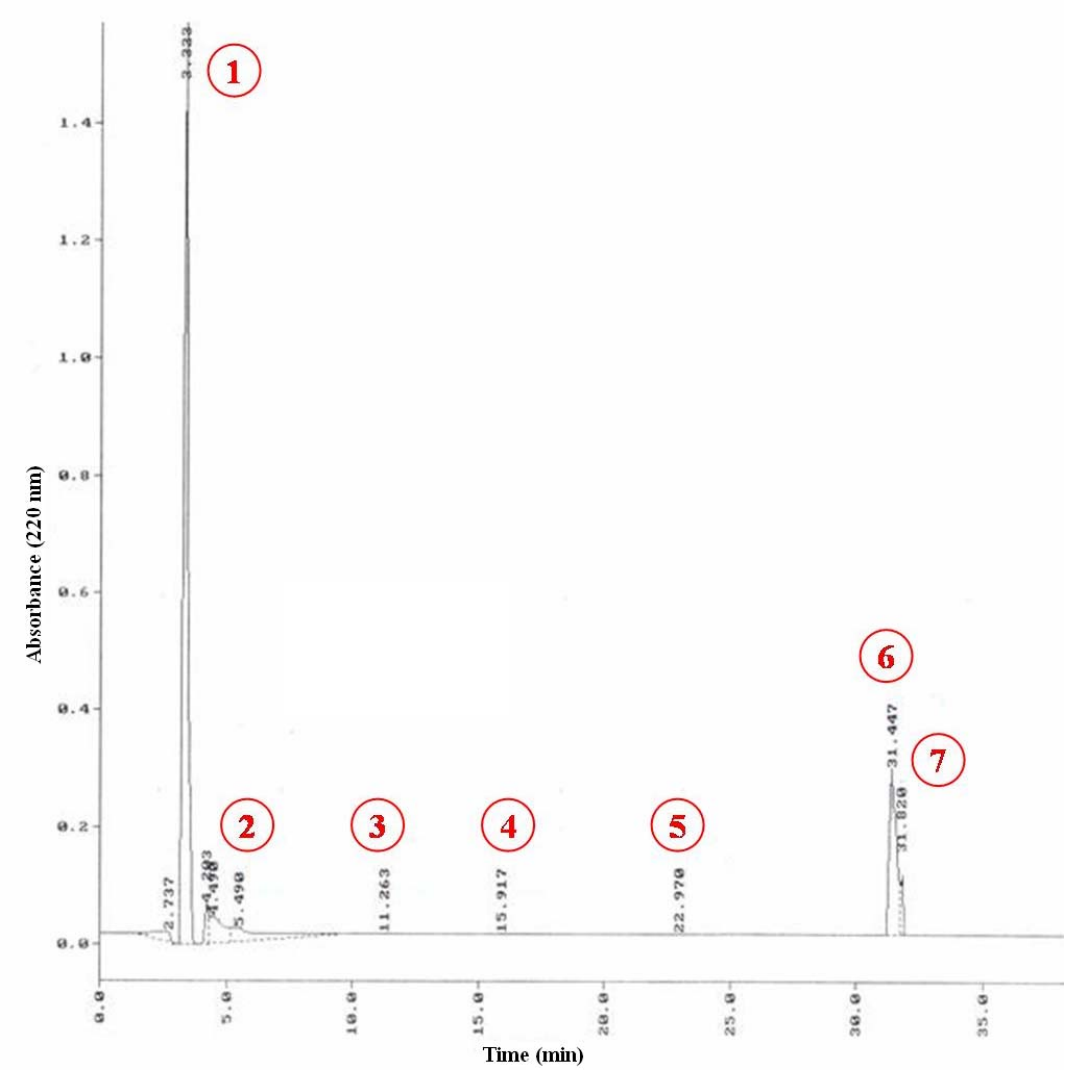


Figure 3.30. Chromatogram obtained from the HPLC analysis of MBP-preLcaA cleaved with Factor Xa. Peaks, together with their retention times, are clearly shown. Numbered peaks were eluted in acetonitrile containing 0.1 % TFA.

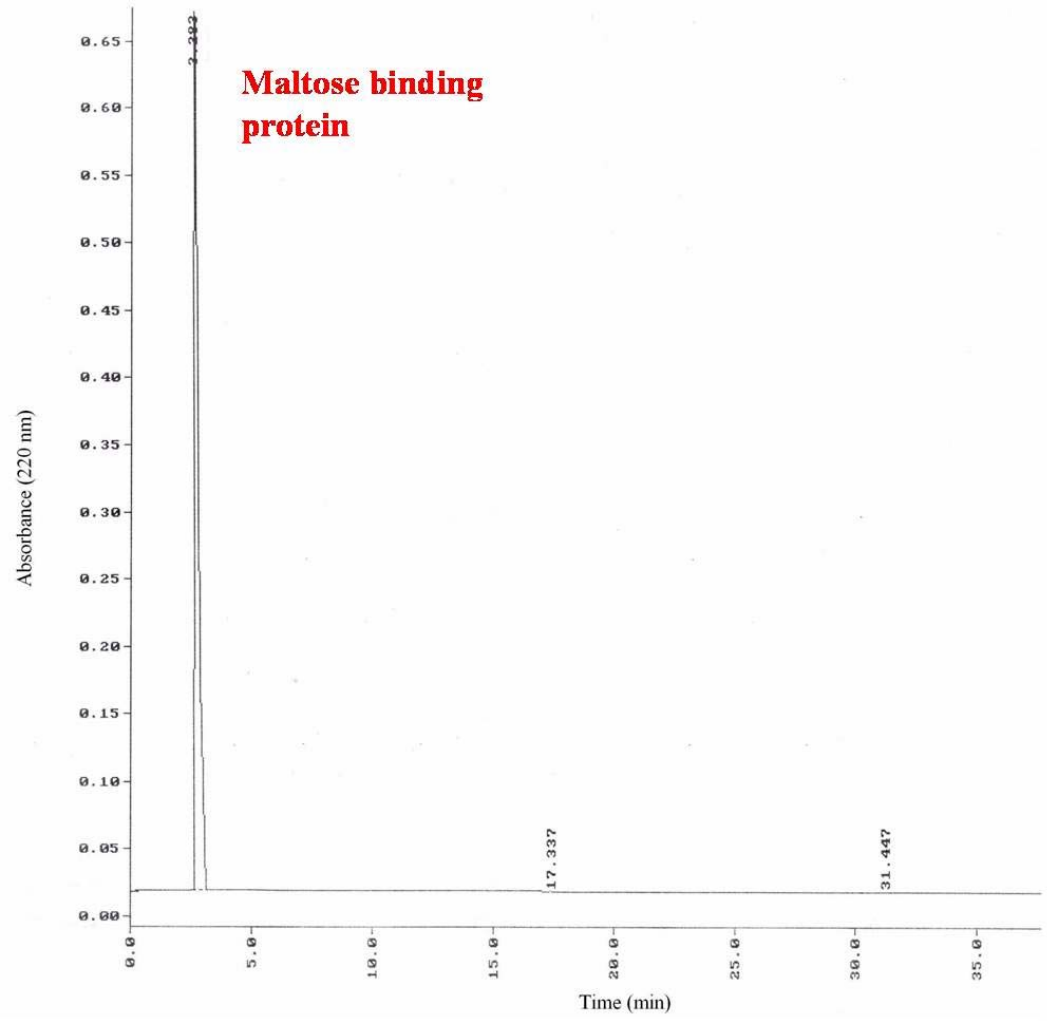


Figure 3.31. Chromatogram obtained from the HPLC analysis of maltose binding protein (1.0023 µg/ml) which served as a control run. Peaks, together with their retention times, are clearly shown. Furthermore, the peak suspected to be MBP is labelled.

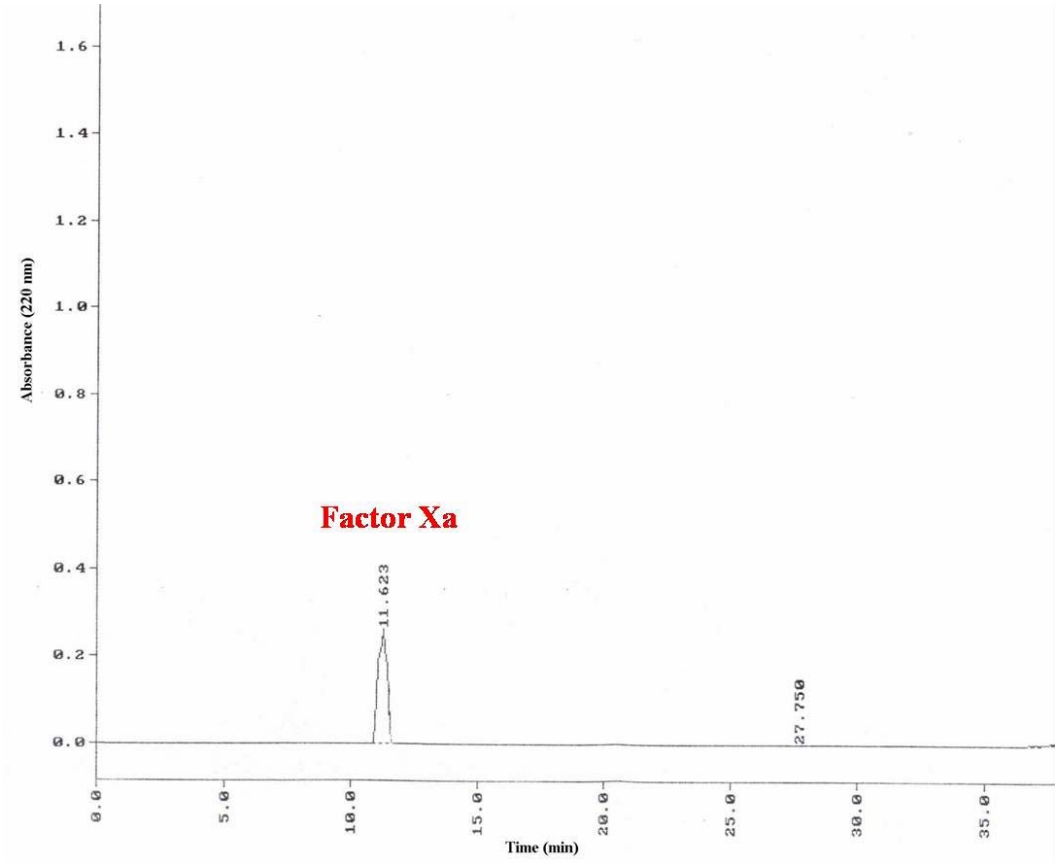


Figure 3.32. Chromatogram obtained from the HPLC analysis of Factor Xa (0.1 mg/ml), which served as a control run. Peaks, together with their retention times, are clearly shown. Furthermore, the peak suspected to be Factor Xa is labelled.

The antimicrobial properties of samples collected by RF-HLPC were tested for the deferred inhibition of *L. monocytogenes*. These assays, depicted in figure 3.33(a) and (b), were carried out in order to determine which eluent contains leucocin A and pre-leucocin A. The analysis of cleaved MBP-LcaA yielded 5 peaks (figure 3.29), of which peak 5 produced a zone of inhibition seen in figure 3.33(a). It can therefore be concluded that this peak represents the retention time of leucocin A (24,893). Seven peaks were collected for the analysis of cleaved MBP-preLeuA (figure 3.30). However, from figure 3.33(b), it is seen that peak 6 with a retention time of 31.447, was able to inhibit the growth of *L. monocytogenes*. This suggests that this peak represents pre-leucocin A. The control sample labelled C on figures 3.33(a) and (b) produced a distinct zone of inhibition, which suggests that the experimental procedure for the deferred inhibition assays were carried out correctly.

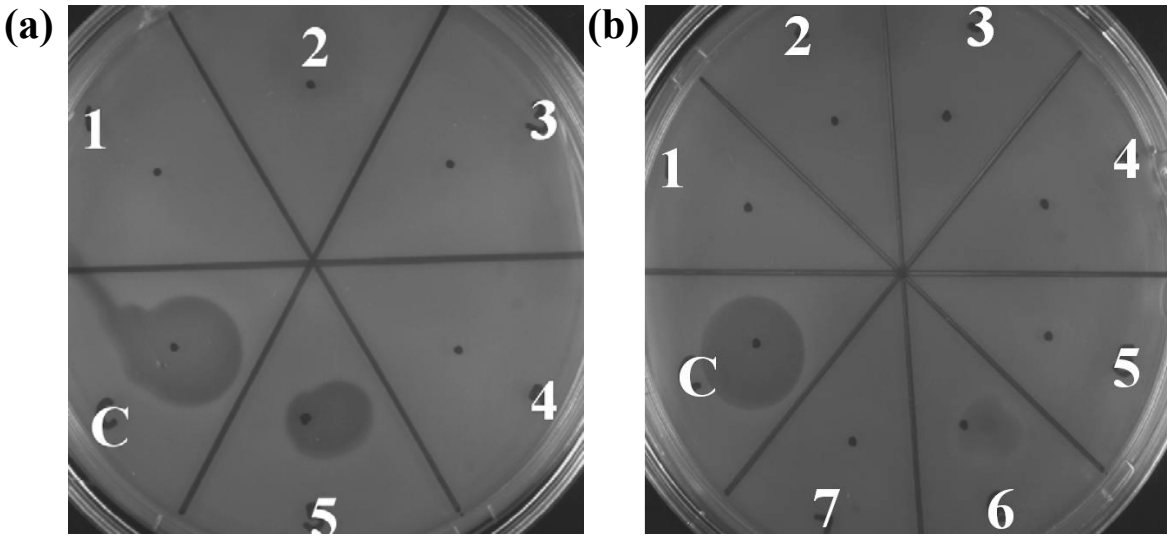


Figure 3.33. Deferred inhibition of *L. monocytogenes* by samples collected by RF-HPLC. (a) Samples eluted from the HPLC analysis of cleaved MBP-LcaA; (b) samples eluted from HPLC analysis MBP-preLcaA. Samples spotted are labelled according to their corresponding peak on figures 3.29 and 3.30. Samples which were able to inhibit the growth of *L. monocytogenes* (produced zones of inhibition) include the control (labelled C), sample 5 (leucocin A), and sample 6 (pre-leucocin A).

3.11 Bactericidal activity of leucocin A and pre-leucocin A

The leucocin A and pre-leucocin A antimicrobial peptides were tested and compared for activity against *L. monocytogenes*. Although differences in antimicrobial activity are seen in figure 3.33, peptides were tested on the same plate to avoid any experimental error. The deferred antagonism assay depicted in figure 3.34 revealed both peptides to be active. However, the zone of inhibition produced by pre-leucocin A is much lighter than the zone produced by leucocin A. The experimental procedure was carried out correctly as the control leucocin C sample (labelled C) was able to inhibit the growth of *L. monocytogenes*. The results indicate that the presence of a leader peptide does not inhibit antimicrobial activity.

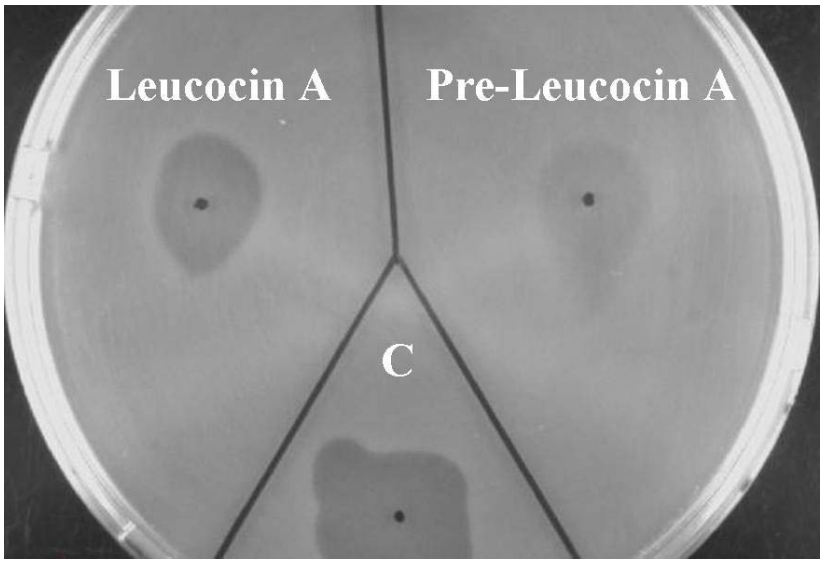


Figure 3.34. The deferred inhibition of *L. monocytogenes* by leucocin A and pre-leucocin A. Zones of inhibition were produced by both leucocin A and pre-leucocin A, indicating that the leader peptide does not hinder antimicrobial activity. The sample labelled C, represents the leucocin A positive control.

The relative activity of the leucocin A precursor was estimated by calculating the specific activities of the leucocin A and pre-leucocin A peptides. This method was employed by Ray *et al.* (1999), to quantify the relative antimicrobial activity of the pediocin AcH precursor. The specific activities or activity units (AU) of leucocin A and pre-leucocin A, was determined using a double dilution assay. A double dilution of each peptide solution was prepared in 0.1% TFA, from 10^{-2} to 10^{-10}. Aliquots of each dilution were spotting each dilution onto a TSB agar lawn supplemented with *L. monocytogenes*. The zones of inhibition produced by each sample become lighter as the dilution factor increases. The AU of each leucocin peptide was based on the dilution, which produced the smallest zone of inhibition. Figure 3.35 represents the result obtained for the double dilution assay, and dilutions, which produced the smallest zone of inhibition, are indicated by arrows. Leucocin A inhibited *L. monocytogenes* up to a 10^{-6} dilution, while *L. monocytogenes* was inhibited by pre-leucocin A up to a dilution of 10^{-4}. The activity units of leucocin A and pre-leucocin A (shown in table 3.5) were calculated using these dilution factors according to the formula provided in chapter 2, section 2.14 and are.

Table 3.5. Relative bactericidal activities of leucocin A and pre-leucocin A peptides.

Protein	Dilution factor	Activity units (AU)
Leucocin A	10^{-6}	6.0×10^{5}
Pre-leucocin A	10^{-4}	4.0×10^{5}

The activity units obtained for leucocin A and pre-leucocin A indicates that the leucocin A precursor is ~66.667 % as active as mature leucocin A. This level of activity is relatively high; although the present hypothesis suggests that class IIa precursors are inactive. However, it should be noted, that these are merely estimations on relative antimicrobial activities, as the concentration of leucocin A and pre-leucocin A does play a major role when comparing the activity of two or more proteins/peptides.

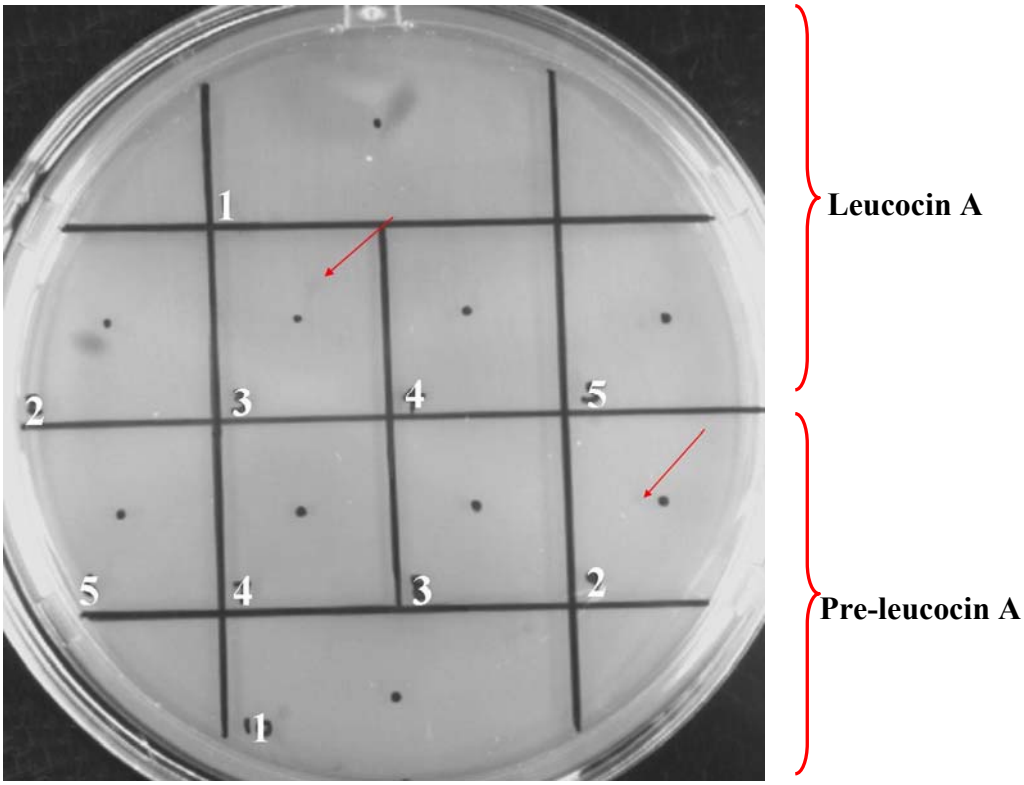


Figure 3.35. Double dilution inhibition assay to estimate the relative bactericidal activity of leucocin A and pre-leucocin A. The activity units (AU) of leucocin A and pre-leucocin A is based on the dilution which inhibited *L. monocytogenes* the least, which are indicated by arrows. Leucocin A inhibited *L. monocytogenes* up to a dilution of 10^{-6} (sample 3), while *L. monocytogenes* was inhibited by pre-leucocin A up to a dilution of 10^{-4}. Certain zones of inhibition were faint, and are not very visible on a photographed image.

CHAPTER FOUR

CONCLUSION

Class IIa bacteriocins consist of the largest group of LAB and have been shown to be potent antimicrobial agents. They have also been shown to pocess a complex secondary structure consisting of α-helices and β-sheets. The present hypothesis suggests that these compounds mode of action consists of series of steps based on their secondary structure. Over the past decade, a significant amount of research has stated that class IIa bacteriocin biosynthsis, structure, and mode of action are all inter-related (structure/function relationship). However, the presence of the leader peptide and its impact on antimicrobial activity remains unclear. Currently, a number of theories exist for the possible functions of these leaders. One such theory, suggests that the leader peptide renders the bacteriocin inactive, thereby preventing cytoplasmic toxicity in the producer cell. The focus of this study, was to determine if the precursor of leucocin A (pre-leucocin A) was active and if so comparing its antimicrobial activity with mature leucocin A.

Leucocin A and pre-leucocin A were expressed and purified as MBP fusion constructs. Further purification methods yielded each recombinant peptide in a partially pure form, which were then tested and compared for antimicrobial acivity. The main outcome of this study found both leucocin A and pre-leucocin A active against *Listeria monocytogenes*. The degree of antimicrobial activity, between these peptides was comparable in that pre-leucocin A inhibited *Listeria* to a lesser extent than the mature form. The relative activity of the isolated peptides indicated that the leucocin A precursor is ~66.667 % active as mature leucocin A. The above estimate suggests that the leucocin A precursor has significant biological activity in comparison with its mature form. It can therefore be concluded, that the presence of the 24 amino acid leader peptide does not have a detrimental effect on the antimicrobial activity of leucocin A.

The finding that pre-leucocin A is active raises the question of why its leader peptide is removed; and it becomes necessary to consider alternative ways for how producer cells are resistant from their own bacteriocin. The results from the study were based on phenotypic analysis, and possible future studies could involve molecular interaction analysis between the leucocin A precursor, and other components that confer producer immunity. Since the immunity protein is proposed to be involved in producer cell resistance, surface plasmon resonance can be used to determine if pre-leucocin A interacts directly with the immunity protein of *Leuconostoc gelidum* UAL187-22. Furthermore, similar interaction studies could

involve chimeric forms of the precursor and the immunity protein; in order to determine the specific region of the precursor to which the immunity protein binds.

CHAPTER FIVE

REFERENCES

Allison, G. E., Ahn, C., Stiles, M. E. and Klaenhammer, T. R. 1995. Utilization of the leucocin A export system in *Leuconostoc gelidum* for production of a *Lactobacillus* bacteriocin. FEMS Microbiology Letters, **131**: 87-93.

Aucher, W., Lacombe, C., Hequet, A., Frere, J. and Berjeaud, J. M. 2005. Influence of amino acid substitutions in the leader peptide on maturation and secretion of mesentericin Y105 by *Leuconostoc mesenteroides.* Journal of Bacteriology, **187**: 2218-2223.

Bach, H., Mazor, Y., Shaky, S., Berdichevsky, A. S. Y., Gutnick, L. and Benhar, I. 2001. *Escherichia coli*: maltose-binding protein as a molecular chaperone for recombinant intracellular cytoplasmic single-chain antibodies. Journal of Molecular Biology, **312**: 79-93.

Bauer, R. and Dicks, L.M.T. 2005. Mode of action of lipid II-targeting lantibiotics. International Journal of Food Microbiology, **101**: 201-216.

Bennike, M. H. J., Vanloo, B., Brasseur, R., Gorris, L. G. M. and Smid, E. J. 1998. A novel bacteriocin with a YGNGV motif from vegetable-associated *Enterococcus munditt*: full characterization and interaction with target organisms. Biochemical and Biophysical Research Communications, **1373**: 47-58.

Bhugaloo-Vial, P., Dousset, X., Metivier, A., Sorokine, O., Anglade, P., Boyaval, P. and Marion, D. 1996. Purification and amino acid sequences of piscicocins V1a and V1b, two class IIa bacteriocins secreted by *Carnobacterium piscicola* V1 that display significantly different levels of specific inhibitory activity. Applied Environmental Microbiology, **62**: 4410 - 4416.

Bhunia, A. K., Johnson, M. C., and Ray, B. 1987. Direct detection of an antimicrobial peptide of Pediococcus acidilactici in sodium dodecyl sulfate polyacrylamide gel electrophoresis. Journal of Industrial Microbiology, 2: 319-322

Biet, F., Berjeaud, J. M., Worobo, R. W., Cenatiempo, Y. and Fremaux, C. 1998. Heterologous expression of the bacteriocin mesentericin Y105 using the dedicated transport system and the general secretion pathway. Microbiology, **144**:2845-2854.

BioRad Laboratories, 1985. Mini trans-blot electrophoretic transfer cell instruction manual.

Birnboim, H. C. and Doly, J. 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA: a method for preparing plasmid DNA. Nucleic Acid Research, **7**: 1513-1523.

Breukink, E. and de Kruij, B. 1999. The lantibiotic nisin, a special case or not?. Biochemical and Biophysical Research Communications, **1462**: 223-234.

Brown, T. A. 2001. Gene cloning and DNA analysis, An introduction. 4th ed. Blackwell Science Ltd, Oxford.

Bruno, M. E. C. and Montville, T. J. 1993. Common mechanistic action of bacteriocins from lactic acid bacteria. Applied and Environmental Microbiology, **59**: 3003-3010.

Castano, S., Desbat, B., Delfour, A., Dumas, J. M., da Silva, A., and Dufourcq, J. 2005. Study of structure and orientation of mesentericin Y105, a bacteriocin from Gram-positive *Leuconostoc mesenteroides*, and its Trp-substituted analogues in phospholipid environments. Biochimica et Biophysica Acta, **1668**: 87-98.

Cattoli, F. and Sarti, G. C. 2002. Characterization of amylose affinity membranes through adsorption of pure recombinant MBP-intein-CBD: equilibrium and kinetics data. Desalination, **149**: 465-470.

Chen, H. and Hoover, D. G. 2003. Bacteriocins and their food applications. Comprehensive Reviews in Food Science and Food Saftey, **2**: 82-100.

Chen, Y., Ludescher, R., and Montville, T. J. 1997[a]. Electrostatic Interactions, but Not the YGNGV Consensus Motif, Govern the Binding of Pediocin PA-1 and Its Fragments to Phospholipid Vesicles. Applied and Environmental Microbiology, **63**: 4770-4777.

Chen, Y., Shapira, R., Eisenstein, M, and Montville, T. J. 1997[b]. Functional Characterization of Pediocin PA-1 Binding to Liposomes in the Absence of a Protein Receptor and Its Relationship to a Predicted Tertiary Structure. Applied and Environmental Microbiology, **63**: 524-531.

Choi, H. J., Lee, H. S., Her, S., Oh, H. and Yoon, S. 1999. Partial characterization and cloning of leucocin J, a bacteriocin produced by *Leuconostoc* spp. J2 isolated from the Korean fermented vegetable Kimchi. Journal of Applied Microbiology, **86**: 175-181.

Chung, C. T., Niemela, S. L. and Miller, R. H. 1989. One-step preparation of competent *Escherichia coli*: transformation and storage of bacterial cells in the same solution. Proceedings for National Academic Science USA, **86**: 2172-2175.

Corbier, C., Krier, F., Mulliert, G., Vitoux, B. and Revol-Juvelles, A. M. 2001. Biological activities and structural properties of the atypical bacteriocins Mesentericin 52B and Leucocin B-TA33a. Applied and Environmental Microbiology, **67**: 1418-1422.

Corran, P.H. 1989. HPLC of Macromolecules, A Practical Approach., 1st ed. IRL Press, Oxford.

Dana-Farber Cancer Institute. Oligo Calculator. 14 April 2006 <http://mbcf.dfci.harvard.edu/docs/oligocalc.html>

Dennison, S. R., Wallace, J., Harris, F. and Phoenix, D. A. 2005. Amphiphilic a-Helical Antimicrobial Peptides and Their Structure/Function Relationships. Protein and Peptide Letters, **12**: 31-39.

Diep, D. B. and Nes, I. F. 2002. Ribosomally synthesized antibacterial peptides in Gram-positive bacteria. Current Drug Targets, **3**: 107-122.

Dirix, G., Monsieurs, P., Dombrecht, B., Daniels, R., Marchal, K., Vanderleyden, J. and Michiels, J. 2004. Peptide signal molecules and bacteriocins in Gram-negative bacteria: a genome-wide in silico screening for peptides containing a double-glycine leader sequence and their cognate transporters. Peptides, **25**: 1425-1440.

Dorsey, J. G., Cooper, W. T. 1994. Retention mechanisms of bonded-phase liquid chromatography. Analytical Chemistry, **66**: 857-867.

Edman, P. and Begg, G. 1967. A protein sequenator. European Journal of Biochemistry, **1**: 80-91.

Eijsink, V. G., Skeie, M., Middelhoven, P. H., Brurberg, M. B. and Nes, I. F. 1998. Comparitive studies of class IIa bacteriocins of lactic acid bacteria. Applied and Environmental Microbiology, **64**: 3275-3281.

Ennahar, S., Sashihara, T., Sonomoto, K. and Ishizaki, A. 2000. Class IIa bacteriocins: biosynthesis, structure and activity. FEMS Microbiology Reviews, **24**: 84-106.

Ennahar, S., Sonomoto, K. and Ishizaki, A. 1999. Class IIa bacteriocins from lactic acid bacteria: antibacterial activity and food preservation. Journal of Bioscience and Bioengineering, **87**: 705-716.

Fimland, G., Blingsmo, O. R., Sletten, K., Jung, G. and Nes, I. F. 1996. New biologically active hybrid bacteriocins constructed by combining regions from various pediocin-like bacteriocins: the C-terminal region in important for determining specificity. Applied and Environmental Microbiology, **62**: 3313-3318.

Fimland, G., Eijsink, V. G. J. and Nissen-Meyer, J. 2002[a]. Comparative studies of immunity proteins of pediocin-like bacteriocins. Microbiology, **148**: 3661-3670.

Fimland G., Pirneskoski, J., Kaewsrichan, J., Jutila, A., Kristiansen, P. E., Kinnunen, P. K. J. and Nissen-Meyer, J. 2006. Mutational ananlysis and membrane-interactions of the β-sheet-like N-terminal domain of the pediocin-like antimicrobial peptide sakacin P. Biochemica et Biophysica Acta, **1764**: 1132-1140

Fimland, G., Sletten, K. and Nissen-Meyer, J. 2002[b]. The complete amino acid sequence of the pediocin-like antimicrobial peptide leucocin C. Biochemical and Biophysical Reseach Communications, **295**: 826-827.

Fleury, Y., Dayem, A. M., Montagne, J. J., Chaboisseau, E., Le Caer, J. P. Nicolas, P. and Delfour, A. 1996. Covalent structure, synthesis, and structure-function studies of mesentericin Y 10537, a defensive peptide from gram-positive bacteria *Leuconostoc mesenteroides*. Journal of Biological Chemistry, **271**: 14421-14429.

Fregeau-Gallagher, N. L., Sailer, M., Niemczura, W. P., Nakashima, T. T., Stile, M. E. and Vederas, J. C. 1997. Three-dimentional structure of leucocin A in trifluoroethanol and dodecylphosphocholine micelles: spatial location of residues critical for biological activity in type IIa bacteriocins from lactic acid bacteria. Biochemistry, **36**:15062-15072.

Fremaux, C., Hechard, Y., Cenatiempo, Y. 1995. Mesentericin Y105 Gene Clusters in *Leuconostoc mesenteroides* Y105. Microbiology. **141**:1637-1645.

Gaeng, S., Scherer, S., Host, N. and Loessner, M. J. 2000. Gene cloning and expression and secretion of *Listeria monocytogenes* bacteriophage-lytic enzymes in *Lactococcus lactis*. Applied and Environmental Microbiology, **66**: 2951-2958.

Gonzalez, C. F. and Kunka, B. S. 1983. Plasmid transferrin *Pediococcus* spp.: intergeneric and intrageneric transfer of pIP501. Applied and Environmental Microbiology, **46**: 81-89.

Gardella, T. J., Rubin, D., Abou-Samra, A., Keutmann, H. T., Potts, J. T. Jr., Kronenberg, H. M. and Nussbaum, S. R. 1990. Expression of human parathyroid hormone-(1-84) in *Escherichia coli* as a factor X-cleavable fusion protein. Journal of Biological Chemistry, **265**: 15854-15859.

Guan, C., Li, P., Riggs, P.D. and Inouye, H. 1987. Vectors that facilitate the expression and purification of foreign peptides in *Escherichia coli* by fusion to maltose-binding protein. Gene, **67**, 21-30.

Gutierrez, J., Criado, R. Citti, R., Martin M., Herranz, C., Nes, I. F., Cintas, L. M. and Hernandez, P. E. 2005. Cloning, production and functional expression of enterocin P, a sec-dependent bacteriocin produced by *Enterococcus faecium* P13, in *Escherichia coli*. International Journal of Food Microbiology, **103**: 239-250.

Guyonnet, D., Fremaux, C., Cenatiempo, Y. and Berjeaud, J. M. 2000. Method of rapid purification of class IIa bacteriocins and comparison of their activities. Applied and Environmental Microbiology, **66**: 1744-1748.

Hage, D. S. 1999. Affinity chromatography: a review of clinical applications. Clinical Chemistry, **45**: 593-615.

Hasting, J. W., Sailer, M., Johnson, K., Roy, K. L., Vederas, J. C., Stiles, M. E. 1991. Characterization of leucocin A-UAL 187 and cloning of the bacteriocin gene from *Leuconostoc gelidum*. Journal of Bacteriology, **173**: 7491-7500.

Hechard, Y. and Sahl, H.G. 2002. Mode of action of modified and unmodified bacteriocins from gram positive bacteria. Biochimie, **84**: 545-557.

Hemme, D. and Scheunemann, C. F. 2005. *Leuconostoc*, characteristics, use in dairy technology and prospects in functional foods. International Dairy Journal, **14**: 467-494.

Henning, L. and Schafer, E. 1998. Protein purification with C-terminal fusion of maltose binding protein. Protein Expression and Purification, **14**: 367-370.

Hoffmann, A., Pag, U., Wiedemann, I. and Sahl, H. G. 2001. Combination of antibiotic mechanisms in lantibiotics. IL Farmaco, **57**: 685-691.

Holt, S. M., Al-Sheikh, H. and Shin, K. J. 2001. Characterization of dextran-producing *Leuconostoc* strains. Letters in Applied Microbiology, **32**: 185-189.

Jack, R. W., Tagg, J. R. and Ray, B. 1995. Bacteriocins of gram-positive bacteria. Microbiology Reviews, **59**: 171-200.

Jenny, R. J., Mann, K. G. and Lundblad, R. L. 2003. A critical review of the methods for cleavage of fusion proteins with thrombin and factor Xa. Protein Expression and Purification. **31**, 1-11.

Johnson, L., Fimland, G., Eijsink, V., and Nissen-Meyer, J. 2000. Engineering Increased Stability in the Antimicrobial Peptide Pediocin PA-1. Applied and Environmental Microbiology, **66**: 4798-4802.

Johnsen, L., Fimland, D., Mantzilas, D. and Nissen-Meyer, J. 2004. Structure-function analysis of immunity proteins of pediocin-like bacteriocins: C-terminal parts of immunity proteins are involved in specific recognition of cognate bacteriocins. Applied and Environmental Microbiology, **70**: 2647-2652.

Kanatani, K., Oshimuri, M. and Sano, K. 1995. Isolation and characterization of acidocin A and cloning of the bacteriocin gene from *Lactobacillus acidophilus*. Applied and Environmental Microbiology, **61**: 1061-1067.

Kapust, R. B. and Waugh, D. S. 1999. *Escherichia coli* maltose-binding protein is uncommonly effective at promoting the solubility of polypeptides to which it is fused. Protein Science, **8**:1668-1674.

Kaur, K., Andrew, L. C., Wishart, D. S. and Vederas, J. C. 2004. Dynamic relationships among type IIa bacteriocins: temperature effects on antimicrobial activity and on structure of the C-terminal amphipathic α-helix as a receptor-binding region. Biochemistry, **43**: 9009-9020.

Kazazic, M., Nissen-Meyer, J. and Fimland, G. 2002. Mutational analysis of the role of charged residues in target-cell binding, potency and specificity of the pediocin-like bacteriocin sakasin P. Microbiology, **148**: 2019-2027.

Kemperman, R., Jonker, M., Nauta, A., Kuipers, O. P. and Kok, K. 2003. Functional Analysis of the Gene Cluster Involved in Production of the Bacteriocin Circularin A by *Clostridium beijerinckii* ATCC 25752. Applied and Environmental Microbiology, **69**: 5839-5848

Kim, I. K., Kim, M. K., Yim, H. S., Cha, S. S., Kang, S. O. 2005. Crystallization and preliminary X-ray crystallographic analysis of the pediocin immunity protein (PedB) from *Pediococcus pentosaceus* at 1.35 A ° resolution. Biochimica et Biophysica Acta, **1751**: 205-208.

Klaenhammer, T. R. 1993. Genetics of bacteriocins produced by lactic acid bacteria. FEMS Microbiology Reviews. **12**:39-86.

Laemmli, U. K. 1970. Cleavage of structural protein during the assembly of the head of bacteriophage T4. Nature, **227**: 680-685

Lauritzen, C., Tüchsen, E., Hansen, P. E. and Skovgaard, O. 1991. BPTI and N-terminal extended analogues generated by factor Xa cleavage and cathepsin C trimming of a fusion protein expressed in *Escherichia coli*. Protein Expression and Purification, **2**: 372-378.

Lee, J. H., Rhee, S. K. and Kim, C. H. 2004. Expression and activation of an esterase from *Pseudomonas aeruginosa* 1001 in *Escherichia coli*. Enzyme and Microbial Technology, **35**: 563-567.

Maina, C. V., Riggs, P. D., Grandea III, A. G., Slatko, B. E., Moran, I. S., Tagliamonti, J. A., Mcreynolds, L. A. and di-Guan, C. 1988. A *Escherichia coli* vector to express and purify foreign proteins by fusion to and separation from maltose binding protein. Gene, **74**: 365-373.

McAuliffe, O., Ross, R. P. and Hill, C. 2001. Lantibiotics: structure, biosynthesis and mode of action. FEMS Microbiology Reviews, **25**: 285-308.

Michiels, J., Dirix, G., Vanderleyden, J. and Xi, C. 2001. Processing and export of peptide pheromones and bacteriocins in Gram-negative bacteria. TRENDS in Microbiology, **9**: 164-168.

Miller, K. W., Schamber, R., Chen, Y. amd Ray, B. 1998[a]. Production of active chimeric pediocin AcH in *Escherichia coli* in the absence of processing and secretion genes from the *Pediococcus* pap operon. Applied and Environmental Microbiology, **64**:1997-2005.

Miller, K. W., Schamber, R., Osmanagaoglu, O. and Ray, B. 1998[b]. Isolation and characterization of pediocin AcH chimeric protein mutants with altered bactericidal activity. Applied and Environmental Microbiology, **64**: 14-20.

Moll, G. N., Konings, W. N. and Driessen, A. J. 1999. Bacteriocins: mechanism of membrane insertion and pore formation. Antonie Van Leeuwenhoek, **76**: 185-198.

Moschetti, G., Blaiotta, G., Villani, F. and Coppola, S. 2000. Specific detection of *leucocnostoc mesenteroides* subsp. *mesenteroides* with DNA primers identified by randomly amplified polymorphic DNA analysis. Applied and Environmental Microbiology, **66**: 422-424.

Morisset, D., Berjeaud, J. M., Marion, D., Lacombe, C. and Frere, J. 2004. Mutational analysis of mesentericin Y105, an antilisterial bacteriocin for determination of impact on bactericidal activity, in vitro secondary structure and membrane interaction. Applied and Environmental Microbiology, **70**: 4672-4680.

New England BioLabs, 1991. pMAL protein fusion and purification system instruction manual.

Nissen-Meyer, J., Holo, H., Havarstein, L. S., Sletten, K. and Nes, A. 1992. A novel *lactococcal* bacteriocin whose activity depends on the complementary action of two peptides. Journal of Bacteriology, **174**: 5686-5692.

O'Keeffe, T., Hill, C., and Ross, P. R. 1999. Characterization and Heterologous Expression of the Genes Encoding Enterocin A Production, Immunity, and Regulation in *Enterococcus faecium* DPC1146. Applied and Environmental Microbiology, **65**:1506-1515.

Ojcius, D. M. and Young, J. D. E. 1991. Cytolytic pore-formation proteins and peptides: is there a common structural motif? Trends in Biochemical Science. **16**, 225-229.

Orberg, P. K. and Sandine, W. E. 1984. Common occurrence of plasmid DNA and vancomycin resistance in *Leuconostoc* spp. Applied and Environmental Microbiology, **48**: 1129-1133.

Oscariz, J. C. and Pisabarro, A. G. 2001. Classification and mode of action of membrane-active bacteriocins produced by gram-positive bacteria. International Microbiology, **4**: 13-19.

Osmanagaoglu, O., Gunduz, U., Beyatli, Y. and Cokmus, C. 1998. Purification and Characterization of Pediocin F, A Bacteriocin Produced By Pediococcus acidilactici F. Journal of Biology, 22: 217-228.

Papathanasopoulos, M. A., Dykes, G. A., Revol-Junelles, A., Delfour, A., von Holy, A., Hastings, J. W. 1998. Sequence and structural relationships of leucocins A-, B- and C-TA33a from *Leuconostoc mesenteroides* TA33a. Microbiology, **144**:1343-1348.

Presscott, L.M., Harley, J.P. and Klein, D.A. 1990. Microbiology, 2nd ed., Wm. C. Brown Publishers. Oxford.

Promega, (1996) Protocols and applications guide, 3rd ed., Promega Corporation, USA.

Quadri, L. E. N., Sailor, M., Roy, K. L., Vederas, J. C. and Stiles, M. E. 1994. Chemical and genetic characterization of bacteriocins produced by *Carnobacterium piscicola* LV17B. Journal of Biological Chemistry, **269**: 12204-12211.

Quadri, L. E. N., Sailor, M., Terebiznik, M. R., Roy, K. L., Vederas, J. C. and Stiles, M. E. 1995. Characterization of the protein conferring immunity to the antimicrobial peptide carnobacteriocin B2 and expression of carnobacteriocins B2 and BM1. Journal of Bacteriology, **177**: 1144-1151.

Quadri, L. E. N., Yan, L. Z., Stiles, M. E. and Vederas, J. C. 1997. Journal of Biological Chemistry, **272**: 3384-3390.

Ramnath, M., Beukes, M., Tamura, M. and Hastings, J. W. 2000. Absence of a putative mannose-specific phosphotransferase system enzyme IIAB component in a leucocin A resistant strain of *Listeria monocytogenes*, as shown by two-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Applied and Environmental Microbiology, **66**: 3098-3101.

Ray, B., Schamber, R. and Miller, K. W. 1999. The pediocin AcH precursor is biologically active. Applied and Environmental Microbiology, 65: 2281-2286.

Riggs, P. 2000. Expression and purification of recombinant proteins by fusion to maltose-binding protein. Molecular Biotechnology, **15**: 51-63.

Rodriguez, J. M., Martinez, M. I., Horn, N. and Dodd, H. M. 2003. Heterologous production of bacteriocins by lactic acid bacteria. International Journal of Food Microbiology, **80**: 101-116.

Sambrook, J., Fritsch, E. M., and Miniatis, T. 1989. Molecular Cloning, A Laboratory Manual., 2nd ed. Cold Spring Harbour Laboratory, Laboratory Press, USA.

Snyder, L. and Champness, W. 2003. Molecular Genetics of Bacteria, 2nd ed., American Society for Microbiology, USA.

Soomro, A. H., Masud, T. and Anwaar, K. 2002. Role of lactic acid bacteria (LAB) in food preservation and human health- a review. Pakistan Journal of Nutrition, **1**: 20-24.

Sprules, T., Kawulka, K. E. and Vederas, J. C. 2004. NMR solution structure of ImB2, a protein conferring immunity to antimicrobial activity of the type IIa bacteriocin, carnobacteriocin B2. Biochemistry, **43**: 11740-11749.

Stephens, S. K., Floriano, B., Cathcart, D. P., Bayley, S. A., Witt, V. F., Jimenez-Diaz, R., Warner, P. J., Ruiz-Barbin, J. L. 1997. Molecular analysis of the locus responsible for the production of plantaricin S, a two-peptide bacteriocin produced by *Lactobacillus plantarum* LPC010. Applied and Environmental Microbiology, **64**:1871-1877.

Stiles, M. E. 1994. Bacteriocins produced by *Leuconostoc* species. Journal of Dairy Science, **77**: 2718-2724.

Tagg, J.R., Dajani, A.S. and Wannamaker, L.W. 1976. Bacteriocins of Gram-positive bacteria. Microbiology Reviews, **40**: 722-756.

Tanford, C. 1961. Physical chemistry of macromolecules, Wiley, London.

Terpe, K. 2003. Overview of tag protein fusions: from molecular and biochemical fundamentals to commercial systems. Applied Microbiology and Biotechnology, **60**: 523-533.

Van Belkum, M. J. and Stiles, M. E. 2000. Nonlantibiotic antimicrobial peptides from lactic acid bacteria, **17**: 323-335.

Van Belkum, M. J. and Stiles, M. F. 1995. Molecular characteristics of genes involved in the production of the bacteriocin leucocin A from *Leuconostoc gelidum*. Applied and Environmental Microbiology, **61**: 3573-3579.

Van Reenen, C. A., Chikindas , M. L., van Zyl, W. H., and Dicks, L. M. T. 2005. Characterization and heterologous expression of a class IIa bacteriocin, plantaricin 423 from *Lactobacillus plantarum* 423, in *Saccharomyces cerevisiae*. International Journal of Food Microbiology, **81**: 29-40.

PDB ID: 1CW6 **Wang, Y., Henz, M. E., Gallagher, N. L. F., Chai, S., Yan, L. Z., Gibbs, A. C., Stiles, M. E., Wishart, D. S., Vederas, J. C.** Refined Solution Structure of Leucocin A.

Wang, Y., Henz, M. E., Fregeau-Gallagher, N. L., Chai, S., Gibbs, A. C., Yan, L. Z., Stiles, M. E., Wishart, D. S. and Vederas, J. C. 1999. Solution structure of carnobacteriocin B2 and implications for structure-activity relations among type IIa bacteriocins from lactic acid bacteria. Biochemistry, **38**: 15438-15447.

Watson, J. D., Baker, T. A., Bell, S. P., Gann, A., Levine, M. and Losick, R. 2003. Molecular Biology of the Gene., 5th ed., Benjamin Cummings Publishers, USA.

Waugh, D. S. 2005. Making the most of affinity tags. TRENDS in Biotechnology, **23**: 316-320.

Weil, H. P., Beck-Sickinger, A. G., Metger, J., Stefanovic, S., Jung G., Josten, M. and Sahl, H. G. 1990. Biosynthesis of the lantibiotic Pep5: isolation and characterization of a pre-peptide containing dehydramino acids. European Journal of Biochemistry, **194**: 217-233.

Yarmus, M., Mett, A. and Shapira, R. 2000. Cloning and expression of the genes involved in the production of and immunity against the bacteriocin lacticin RM. Biochimica et Biophysica Acta, **1490**: 279-290.