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**Production, Characterization and Utilization of the Bacteriocin
Produced by *Enterococcus faecalis* B9510**

**A thesis presented in partial fulfilment of the requirements for the degree of
Doctor of Philosophy in Biotechnology
at Massey University, Palmerston North, New Zealand**

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ABSTRACT

Bacteriocins are antimicrobial proteins and peptides produced by bacteria, antagonistic to other bacterial species but not affecting the producer species. The bacteriocins from Lactic Acid Bacteria (LAB) are particularly well-studied and exploited as safe and natural food preservatives. The current research aimed to identify and characterize a bacteriocin-like substance produced by *Enterococcus faecalis* B9510, a local isolate from silage. The production of this antimicrobial during the growth phase, destruction of antimicrobial activity by proteolytic enzymes and self-immunity of the producer strain indicated that the antimicrobial is a bacteriocin. The bacteriocin was heat-labile as the antimicrobial activity was destroyed by heating at 60 °C for one hour. This bacteriocin was also found to lyse sensitive cells.

To further characterize the bacteriocin to the protein level, the producer strain was grown in a completely defined medium, devoid of any proteins and peptides, to facilitate downstream processing. Purification was done by passing the culture supernatant through 10 kDa and 30 kDa ultrafiltration membranes. The 30 kDa ultrafiltration retentate showed antimicrobial activity and was then subjected to SDS-PAGE. The in-gel bacteriocin activity was then determined by incorporating dead cells of a sensitive strain *Lactococcus lactis* ssp. *cremoris* 2144 in a parallel SDS-PAGE gel, which was renatured after the electrophoresis. After renaturation a zone of clearance was observed around the active band, at approximately 35 kDa. The active band was excised and analyzed by mass spectrometry. The results revealed that the amino acid sequence matched a known bacteriocin enterolysin A. This was confirmed when the enterolysin A gene was amplified from the producer strain using PCR followed by DNA sequencing.

The earlier studies on enterolysin A primarily focused on the structural gene, and primary structure of enterolysin A. No information is available on the function of neighbouring genes of the enterolysin A structural gene. An attempt was made in the current study to elucidate the function of genes found in close proximity to the structural gene, with the aim to find the immunity gene.

Experiments were also conducted to find the mode of action of enterolysin A. Earlier studies have reported that enterolysin A is an endopeptidase which degrades the cell walls of sensitive Gram-positive bacteria. However, the cleavage site within the cell wall moiety has not been reported. The current study has revealed that enterolysin cleaves a peptide bond between D-glutamic acid and L-alanine in the stem peptide and N-

terminus of L-lysine and C-terminus of D-aspartic acid within the interpeptide bridge of peptidoglycan units of sensitive bacterial strains. Furthermore, transmission electron microscopy of enterolysin A treated cells gave new insight into the morphology of damaged cells.

The antimicrobial spectrum of enterolysin A already reported was also extended to other species in the current study. The results revealed that in addition to its activity against the bacterial species already reported, enterolysin A is also active against *Lactobacillus helveticus*, *Lactobacillus casei* and *Lactobacillus delbrueckii* ssp. *bulgaricus*.

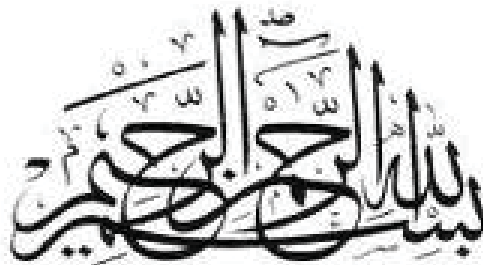
To conclude the project, enterolysin A was coated on polyethylene film. This film was found to effectively control the growth of *L. casei* and thus can be incorporated into antimicrobial packaging against spoilage microorganisms.

DEDICATED

TO

MY KIND AND LOVING MOTHER

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In the name of Allah, most benevolent, ever merciful

The PhD degree programme is a process of learning to be an independent researcher, a process which involves three to four years of dedication. However, this journey of learning is not possible without the help and support of other people. This section is an acknowledgement of the help and support of all those, without whom I would not be able to complete my PhD.

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FREQUENTLY USED ABBREVIATIONS

aa	Amino acids
ABC	ATP Binding Cassette
API	Analytical Profile Index
AS	Ammonium Sulphate precipitation
ATCC	American Type Culture Collection
ATP	Adenosine Triphosphate
AU	Arbitrary Units
AWDA	Agar Well Diffusion Assay
bp	Base pairs
CAT	Catalytic Domain
CDM	Complete Defined Medium
CEX	Cation Exchange Chromatography
Cm	Chloramphenicol
Cm-r	Chloramphenicol resistance cassette
Da	Daltons
DNA	Deoxyribonucleic Acid
FPLC	Fast Protein Liquid Chromatography
FRC	Fonterra Research Centre, Palmerston North, New Zealand
GF	Gel Filtration Chromatography
GRAS	Generally Regarded As Safe
h	hour
HIC	Hydrophobic Interaction Chromatography
IAC	Immuno-Affinity Chromatography
IEX	Ion-Exchange Chromatography
IPTG	Isopropyl- β -D-galactopyranoside
kDa	kilodaltons
LAB	Lactic Acid Bacteria
LDPE	Low Density Polyethylene
MALDI, TOF	Matrix Assisted Laser Desorption Ionisation, Time of Flight
min	minutes

MIC	Minimum Inhibitory Concentration
ML, MU	Microbiology Laboratory, Massey University, Palmerston North
NAG	N-acetyl Glucosamine
NAM	N-acetyl Muramic Acid
NCBI	National Centre for Biotechnology Information
NCTC	National Collection of Type Cultures
NMWCO	Nominal Molecular Weight Cut-Off
OD	Optical Density
ORF/ <i>orf</i>	Open Reading Frame
PCR	Polymerase Chain Reaction
pH	Negative decadal logarithm of the [H ⁺] ion concentration
pI	Iso-electric point
PTS	Mannose Phospho-Transferase System
rcf	Relative Centrifugal Force
RP-HPLC	Reverse Phase-High Performance Liquid Chromatography
SD	Standard Deviation
SDM	Simplified Defined Medium
SDS-PAGE	Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis
sec	Seconds
SRD	Substrate Recognition Domain
ssp.	Subspecies
TEM	Transmission Electron Microscopy
TEMED	Tetramethyl Ethylene Diamine
TLC	Thin Layer Chromatography
UF	Ultrafiltration
μ	Specific growth rate (h ⁻¹)

ABBREVIATIONS OF NUCLEIC ACIDS

A	Adenine
T	Thymine
G	Guanine
C	Cytosine
U	Uracil

ABBREVIATIONS OF AMINO ACIDS

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

Chapter 1

INTRODUCTION

Bacteriocins are ribosomally-synthesized peptides and proteins produced by one bacterial strain, which are antagonistic to other bacterial strains and species and the producer strain is protected from its own bacteriocin by a dedicated immunity mechanism (Cotter *et al.*, 2005). The production of such antimicrobial proteinaceous compounds was first described in *Escherichia coli*, a Gram-negative bacterium, and these compounds were termed as colicins (Gratia 1925). However, it was the report of antimicrobial activity in *Streptococcus lactis*, a Gram-positive Lactic Acid Bacterium (Rogers 1928; Whitehead, 1933) that created great interest among researchers. This antimicrobial substance was later named as nisin (Mattick and Hirsch 1944). Since Lactic Acid Bacteria (LAB) have been utilized as starter cultures for the production of a variety of dairy products for thousands of years and are Generally Regarded As Safe (GRAS), their bacteriocins can also be regarded as safe (Cleveland *et al.*, 2001). The discovery of nisin thus opened a new chapter in bacteriocin research and paved the way for studies related to the identification of novel bacteriocins from LAB, their characterization, and possible utilization as food preservatives.

The last twenty years can be regarded as “the golden era” for the discovery of novel bacteriocins produced by LAB, due to the financial support of bacteriocin-related projects from funding agencies such as European Union (Nes 2011). To date the number of bacteriocins purified to homogeneity and characterized at the amino acid level is more than one hundred and the majority of them were discovered in the last two decades. These bacteriocins have been reported to be produced by a diverse range of bacterial species but the most important genera include *Lactobacillus*, *Lactococcus*, *Streptococcus*, *Carnobacterium* and *Enterococcus*.

The bacteriocin-related studies are not only confined to identification and description of the primary structure of bacteriocins. The investigations include the description of bacteriocin genetics, mechanisms of extracellular transport and regulation of bacteriocin synthesis inside the cells. Furthermore, many studies have focused on elucidating the factors responsible for conferring self-immunity to the producing strains and the mechanisms of self-immunity. Detailed studies have also been conducted to understand the three dimensional structure and mode of action of bacteriocins. These

studies have greatly helped in understanding how the bacteriocins interact with the sensitive strains and how they can be used against undesirable bacteria such as pathogens and spoilage organisms.

Experiments have also been conducted on the practical application of these natural antimicrobials. The main focus in this regard has been their utilization as food preservatives. Many bacteriocins have been tested as preservatives in a wide range of food products which include fruits, vegetables, seafood, dairy and meat products (Galvez *et al.*, 2008; Khan *et al.*, 2010). However, to date nisin is the only bacteriocin which has been approved as a food preservative and has been commercially available since 1953 (Delves-Broughton 2005). In addition to their utilization as food preservatives, bacteriocins have also been tested for their medical and veterinary applications e.g. nisin has been included in mouthwashes for the treatment of plaque and gingivitis (Van Kraaij *et al.*, 1999) and is also used to control mastitis in animals (Sears *et al.*, 1992). Similarly BLIS K12, a commercial product containing a strain of *Streptococcus salivarius* that produces salivaricin A2 and B, is successfully used to control foul breath (Tagg 2004; Burton *et al.*, 2011).

Keeping in view the importance of bacteriocins and the continuing interest of researchers in this area of research, the current study was planned with the aim to discover and characterize a potentially novel bacteriocin. The bacteriocin-producing isolate *Enterococcus faecalis* B9510 was selected from among a number of LAB isolates due to its broad range of antimicrobial activity against many bacterial isolates tested which also included *Listeria monocytogenes*. The bacteriocin identified was found to be enterolysin A, a heat-labile lytic bacteriocin first described to be produced from *E. faecalis* LMG2333, a strain isolated from fish in Iceland (Nilsen *et al.*, 2003). It has also been reported to be produced by *E. faecalis* strains isolated from raw milk (Hickey *et al.*, 2003), bovine rumen (Nigutova *et al.*, 2007) and partridge (Almeida *et al.*, 2011). This is, therefore, the first report of production of enterolysin A by an enterococcal isolate from silage in New Zealand.

Enterolysin A is a well-studied bacteriocin; however, the current project includes several novel aspects. Earlier studies involved growing the producer strains in complex commercial media followed by a series of purification steps to purify the enterolysin A from the media contaminants. In the current study *E. faecalis* B9510 was grown in a chemically defined medium devoid of any contaminating proteins and peptides, thus greatly helping in reducing the purification steps which only involved ultrafiltration.

The amino acid sequence of enterolysin A and its structural gene has been described in literature. However, no information is available on the gene(s) responsible for conferring self-immunity to the producer strain. Therefore, experiments were conducted to find the immunity gene.

Investigations were also conducted to study the mode of action of enterolysin A. It is known that enterolysin A acts by degrading cell walls of sensitive strains resulting in lysis. This knowledge was carried further by visual examination of the enterolysin A treated cells of the sensitive strain by Transmission Electron Microscopy (TEM). The TEM pictures give new insight into the morphological appearance of damaged cells after treatment with enterolysin A. Furthermore, experiments were also conducted to find the cleavage site of enterolysin A within the peptidoglycan chain of sensitive bacterial cell walls.

Finally, a crude enterolysin A preparation was coated onto polyethylene on which it was found to successfully control the growth of a sensitive strain of *Lactobacillus casei*, when tested on a lawn of bacterial cells. Such coatings may be used as packaging films to control microbial growth in food products.

Chapter 2**LITERATURE REVIEW**

Bacteriocins have been reported to be produced by a large number of bacterial species, which include Gram-positive as well as Gram-negative bacteria. It has even been postulated that all bacterial species are capable of producing bacteriocins, however many have not yet been studied for bacteriocin production (Tagg 1992). Bacteriocins produced by LAB have dominated the literature because of their potential to be used as food preservatives. The current research project aimed to identify and characterize a bacteriocin produced by *Enterococcus faecalis* B9510, a lactic acid bacterium. The following review, therefore, primarily focuses on the bacteriocins produced by LAB.

2.1 Production, purification and characterization of bacteriocins

The journey for the discovery of a bacteriocin involves several steps. It starts from the screening for bacteriocin producing strains from a large number of isolates. These strains are then grown in the most appropriate medium to produce the maximum amount of bacteriocin, which is then purified to homogeneity through a series of steps leading to identification and characterization.

The origin of strains selected to be screened for bacteriocin activity may be environmental isolates or already purified and well-characterized strains. Lactic Acid Bacteria (LAB) secrete many substances that may have antimicrobial activity, including hydrogen peroxide, lactic acid, aldehydes and ketones (Naidu *et al.*, 1999). The antimicrobial activity due to these substances must be eliminated before detection of antimicrobial activity due to bacteriocins can be determined.

The methods used for the detection of bacteriocin activity are based on classical antibiotic detection methods, in which the antimicrobial activity of the suspected bacteriocin is tested against a bacteriocin sensitive indicator strain. Direct detection methods are usually used for initial screening and usually involve growing the colonies of potential producer strain on the agar surface seeded with cells of a sensitive strain. During incubation the potential producer cells and indicator strain grow simultaneously and any antimicrobial activity due to bacteriocin like inhibitory substances is indicated by the presence of zones of inhibition around the producer cells after incubation. Other commonly used antagonism assay methods include the spot-on-the-lawn assay (Fleming *et al.*, 1975), agar well diffusion assay (Tagg and McGiven 1971; Yanagida *et al.*, 2005)

and microtitre plate assay. The microtitre plate assay involves preparing a series of serial two-fold dilutions of the crude bacteriocin preparation in a microtitre plate which is then inoculated with a sensitive strain. This assay is used to quantify bacteriocin concentration in terms of Arbitrary Units (AU), which are defined as the reciprocal of highest dilution of bacteriocin preparation which can inhibit the growth of a sensitive strain more than or equal to 50% to that of a control treatment (Nissen-Meyer *et al.*, 1992).

The next step after selection of the bacteriocin producing strain, involves growing the strain in an appropriate medium under optimum conditions for maximum bacteriocin production. The bacteriocin is extracellularly secreted into the medium during growth of the producer strain. The bacteriocin is then purified and separated from the contents of the medium in a series of steps, followed by identification at the molecular level. Since the bacteriocins are heterogeneous in nature, therefore, the purification strategy varies for each bacteriocin, but usually involve an initial volume reduction step followed by final purification in series of chromatographic steps which exploit the charge and hydrophobic properties of bacteriocins, which are necessary for their activity against the sensitive strains. The purification protocols of selected bacteriocins are given in Table 2.1.

Table 2.1 Purification of Bacteriocins of Lactic Acid Bacteria

Organism	Bacteriocin	Purification Scheme		Reference(s)
		Volume reduction/ concentration	Final purification	
<i>Enterococcus faecalis</i> ssp. <i>liquefaciens</i> S-48	Enterocin AS-48	-	CEX, GF, RP-HPLC	(Galvez <i>et al.</i> , 1989)
<i>E. faecalis</i> BFE 1071	Enterocin 1071A	AS	CEX	(Balla <i>et al.</i> , 2000)
<i>E. faecalis</i> BFE 1071	Enterocin 1071B	AS	CEX	(Balla <i>et al.</i> , 2000)
<i>E. faecalis</i> SE-K4	Enterocin SE-K4	pH mediated cell adsorption-desorption	Solid-phase extraction, RP-HPLC	(Eguchi <i>et al.</i> , 2001)
<i>E. faecalis</i> LMG 2333	Enterolysin A	AS	UF, CEX, RP-HPLC	(Nilsen <i>et al.</i> , 2003)
<i>E. faecalis</i> RJ-11	Enterocin AS-48 RJ	AS	Dialysis, GF	(Yamamoto <i>et al.</i> , 2003)
<i>E. faecalis</i> MR99	Enterocin MR99	AS	HIC	(Sparo <i>et al.</i> , 2006)
<i>E. faecalis</i> WHE 96	Enterocin 96	Ultrafiltration	CEX, RP-HPLC	(Izquierdo <i>et al.</i> , 2009)
<i>Enterococcus faecium</i> CTC492	Enterocin A	AS	CEX, HIC, RP-HPLC	(Aymerich <i>et al.</i> , 1996)
<i>E. faecium</i> T136	Enterocin B	XAD-16	CEX, HIC, RP-HPLC	(Casaus <i>et al.</i> , 1997)

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<i>E. faecium</i> CRL35	Enterocin CRL35	AS	GF, CEX, RP-HPLC	(Fariás <i>et al.</i> , 1996)
<i>E. faecium</i> P13	Enterocin P	AS	GF, CEX, HIC, RP-HPLC	(Cintas <i>et al.</i> , 1997)
<i>E. faecium</i> L50	Enterocin L50 A	XAD-16	CEX, HIC, RP-HPLC	(Cintas <i>et al.</i> , 2000)
<i>E. faecium</i> L50	Enterocin L50 B	XAD-16	CEX, HIC, RP-HPLC	(Cintas <i>et al.</i> , 2000)
<i>E. faecium</i> L50	Enterocin Q	XAD-16	CEX, HIC, RP-HPLC	(Cintas <i>et al.</i> , 2000)
<i>E. faecium</i> F-58	Enterocin F-58		CEX, RPC, RP-HPLC	(Achemchem <i>et al.</i> , 2005)
<i>E. faecium</i> T8	Bacteriocin T8	AS		(De Kwaadsteniet <i>et al.</i> , 2006)
<i>E. faecium</i> E 50-52	Bacteriocin E50-52	AS	CEX, HIC	(Svetoch <i>et al.</i> , 2008)
<i>E. faecium</i> KU-B5	Enterocin X (α and β)	-	CEX, HIX, RP-HPLC	(Hu <i>et al.</i> , 2010)
<i>Enterococcus mundtii</i> ATO6	Mundticin	AS	HIC, CEX, GF, RP-HPLC	(Bennik <i>et al.</i> , 1998)
<i>E. mundtii</i> CUGF08	Mundticin L	pH mediated adsorption-desorption	-	(Feng <i>et al.</i> , 2009)
<i>Enterococcus hirae</i> DCH5	Hiracin JM79	AS	GF, CEX, HIC, RP-HPLC	(Sanchez <i>et al.</i> , 2007; Sanchez <i>et al.</i> , 2008)
<i>Enterococcus avium</i> XA83	Avicin A	AS	CEX, RP-HPLC	(Birri <i>et al.</i> , 2010)
<i>Enterococcus durans</i> QU 49	Durancin TW-49M	-	CEX, HIC, RP-HPLC	(Hu <i>et al.</i> , 2008)
<i>Enterococcus</i> spp.	Enterocin E-760	-	CEX, HIC	(Line <i>et al.</i> , 2008)
<i>Lactobacillus plantarum</i> C-11	Plantaricin A	AS	CEX, HIC, RP-HPLC	(Nissen-Meyer <i>et al.</i> , 1993)
<i>L. plantarum</i> LPCO10	Plantaricin S	AS	CEX, HIC, RP-HPLC	(Jimenezdiaz <i>et al.</i> , 1995)
<i>L. plantarum</i> TMW1.25	Plantaricin 1.25 α	AS	CEX, HIC, RP-HPLC	(Remiger <i>et al.</i> , 1999)
<i>L. plantarum</i> TMW1.25	Plantaricin 1.25 β	AS	CEX, HIC, RP-HPLC	(Remiger <i>et al.</i> , 1999)
<i>L. plantarum</i> C19	Plantaricin C19	pH mediated adsorption-desorption	RP-HPLC	(Atrih <i>et al.</i> , 2001)
<i>L. plantarum</i> NC8	Plantaricin C8 α and Plantaricin C8 β	AS	CEX, HIC, RP-HPLC	(Maldonado <i>et al.</i> , 2003)
<i>L. plantarum</i> 423	Plantaricin 423	AS	Dialysis, Chloroform-methanol extraction, CEX,	(Van Reenen <i>et al.</i> , 2003)
<i>L. plantarum</i> A-1	Plantaricin ASM-1	AS	CEX, HIC, RP-HPLC	(Hata <i>et al.</i> , 2010)
<i>Lactobacillus sake</i> L45	Lactocin S	AS	AEX, CEX, HIC, GF, RP-HPLC	(Mortvedt <i>et al.</i> , 1991)
<i>L. sake</i> LB706	Sakacin A	AS	CEX, HIC and RP-HPLC	(Holck <i>et al.</i> , 1992)

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<i>L. sake</i> LTH673	Sakacin P	AS	CEX, HIC, RP-HPLC	(Tichaczek <i>et al.</i> , 1992)
<i>L. sake</i> C2	Sakacin C2	Cold ethanol precipitation	GF	(Gao <i>et al.</i> , 2010)
<i>Lactobacillus acidophilus</i> JCM 1132	Acidocin J1132 α	AS	CEX, GF, RP-HPLC	(Tahara <i>et al.</i> , 1996)
<i>L. acidophilus</i> JCM 1132	Acidocin J1132 β	AS	CEX, GF, RP-HPLC	(Tahara <i>et al.</i> , 1996)
<i>L. acidophilus</i> DSM20079	Acidocin D20079	AS	Dialysis, CEX, HIC	(Deraz <i>et al.</i> , 2005)
<i>Lactobacillus curvatus</i> LTH 1174	Curvacin A	AS	CEX, HIC and RP-HPLC	(Tichaczek <i>et al.</i> , 1992)
<i>L. curvatus</i> CWBI-B28	Curvalicin 28a, 28b and 28c	AS	HIC, RP-HPLC	(Ghalfi <i>et al.</i> , 2010)
<i>Lactobacillus pentosus</i> 31-1	Pentocin 31-1	AS	Dialysis, SP-Sepharose fast flow (IEX), Dialysis,	(Liu <i>et al.</i> , 2008)
<i>Lactobacillus amylovorus</i> DCE 471	Amylovorin L471	AS	Chloroform-Methanol extraction, RP-HPLC	Callewaert <i>et al.</i> , 1999
<i>Lactobacillus helveticus</i> 481	Helveticin J	AS	GF	(Joerger and Klaenhammer 1986)
<i>Lactobacillus casei</i> CRL 705	Lactocin 705	pH mediated adsorption-desorption	RP-HPLC	(Palacios <i>et al.</i> , 1999)
<i>Lactobacillus divergens</i>	Divergicin M35	-	CEX, Solid-phase extraction (C-18 Sep-Pak), RP-HPLC	(Tahiri <i>et al.</i> , 2004)
<i>Lactobacillus johnsonii</i> VPI 11088	Lactacin F	AS	GF, UF, RP-HPLC	(Muriana and Klaenhammer 1991)
<i>Lactobacillus gasseri</i> SBT2055	Gassericin T	Dialysis	HIC	(Kawai <i>et al.</i> , 2000)
<i>Lactobacillus reuteri</i> LA6	Reutericin 6	Dialysis	HIC, RP-HPLC	(Kawai <i>et al.</i> , 2001)
<i>Lactobacillus brevis</i> SB27	Brevicin 27	AS	CEX, HIC, RP-HPLC	(Benoit <i>et al.</i> , 1997)
<i>Lactobacillus rhamnosus</i> 68	Rhamnosin A	Lyophilization, Ethanol precipitation	RP-HPLC	(Dimitrijevic <i>et al.</i> , 2009)
<i>Lactococcus lactis</i> ssp. <i>lactis</i> CNRZ 481	Lacticin 481	AS	GF, RP-HPLC	(Piard <i>et al.</i> , 1992)
<i>L. lactis</i> ssp. <i>lactis</i> 2081	Lactococcin G α	AS	CEX, HIC, RP-HPLC	(Nissen-Meyer <i>et al.</i> , 1992)
<i>L. lactis</i> ssp. <i>lactis</i> 2081	Lactococcin G β	AS	CEX, HIC, RP-HPLC	(Nissen-Meyer <i>et al.</i> , 1992)
<i>L. lactis</i> ssp. <i>lactis</i> IPLA 972	Lactococcin 972	Acetone precipitation	CEX	(Martinez <i>et al.</i> , 1996)
<i>L. lactis</i> ssp. <i>lactis</i>	Nisin A	-	IAC	(Suarez <i>et al.</i> , 1997)
<i>L. lactis</i> ssp. <i>lactis</i> 61-14	Nisin Q	XAD-16	CEX, RP-HPLC	(Zendo <i>et al.</i> , 2003)
<i>Lactococcus lactis</i> IFPL105	Lacticin 3147 A1	AS	CEX, HIC, RP-HPLC	(Martinez-Cuesta <i>et al.</i> , 2000)

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<i>L. lactis</i> IFPL105	Lacticin 3147 A2	AS	CEX, HIC, RP-HPLC	(Martinez-Cuesta <i>et al.</i> , 2000)
<i>L. lactis</i> MMT 24	Lactococcin MMT 24 (α and β)	AS	Solid phase chromatography (C 18), RP-HPLC	(Ghraiiri <i>et al.</i> , 2005)
<i>L. lactis</i> QU 14	Lacticin Z	XAD-16	CEX, RP-HPLC	(Iwatani <i>et al.</i> , 2007)
<i>Lactococcus lactis</i> ssp. <i>cremoris</i> LMG 2130	Lactococcin A	AS	CEX, RP-HPLC	(Holo <i>et al.</i> , 1991)
<i>L. lactis</i> ssp. <i>cremoris</i> IL 1403	Lactococcin B	Ethanol precipitation	Preparative isoelectric focusing, Ultrafiltration	(Venema <i>et al.</i> , 1997)
<i>Lactococcus</i> sp. QU 12	Lactocycelicin Q	-	CEX, HIC, RP-HPLC	(Sawa <i>et al.</i> , 2009)
<i>Lactococcus garvieae</i> DCC43	Garvicin ML	-	CEX, RP-HPLC (two cycles)	(Borrero <i>et al.</i> , 2011)
<i>Pediococcus acidilacti</i> PAC-1.0	Pediocin PA1	-	GF, CEX, Dialysis, RP-HPLC	(Henderson <i>et al.</i> , 1992)
<i>Pediococcus pentosaceus</i> SA131	Pediocin SA131	Ethanol precipitation	IEX, Ultrafiltration	(Lee <i>et al.</i> , 2010)
<i>Pediococcus damnosus</i> NCFB1832	Pediocin PD1	AS	Dialysis, Chloroform-methanol extraction, CEX	(Bauer <i>et al.</i> , 2005)
<i>Streptococcus thermophilus</i> SF113	Thermophilin 13 (A and B)	Trichloroacetic acid precipitation	HIC, RP-HPLC	(Marciset <i>et al.</i> , 1997)
<i>S. thermophilus</i> SBT1277	Thermophilin 1277	-	HIC, RP-HPLC	(Kabuki <i>et al.</i> , 2007; Kabuki <i>et al.</i> , 2009)
<i>S. salivarius</i> 20P3	Salivaricin A	XAD-2	CEX, GF, RP-HPLC	(Ross <i>et al.</i> , 1993)
<i>Streptococcus uberis</i> 42	Nisin U	pH mediated cell adsorption-desorption	RP-HPLC (3X)	(Wirawan <i>et al.</i> , 2006)
<i>Leuconostoc mesenteroides</i>	Leucocin B	pH mediated cell adsorption-desorption	RP-HPLC	(Papathanasopoulos <i>et al.</i> , 1998)
<i>L. mesenteroides</i>	Leucocin C	-	CEX, RP-HPLC	(Fimland <i>et al.</i> , 2002b)
<i>L. mesenteroides</i> ssp. <i>mesenteroides</i> Y105	Mesentericin Y105	-	AC, UF, RP-HPLC	(Hechard <i>et al.</i> , 1992)
<i>Leuconostoc gelidium</i> UAL 187	Leucocin A	AS	HIC, GF, RP-HPLC	(Hastings <i>et al.</i> , 1991)
<i>Leuconostoc pseudomesenteroides</i>	Leucocin Q	XAD-16	CEX, RP-HPLC	(Sawa <i>et al.</i> , 2010)
<i>L. pseudomesenteroides</i>	Leucocin N	XAD-16	CEX, RP-HPLC	(Sawa <i>et al.</i> , 2010)

Abbreviations: AS: Ammonium Sulphate precipitation; GF: Gel filtration; IEX, Ion-Exchange; CEX, Cation Exchange; AEX, Anion Exchange; IAC, Immuno-affinity Chromatography; AC: Affinity Chromatography, RP-HPLC, Reverse-phase high performance liquid chromatography; FPLC, Fast Protein Liquid Chromatography; HIC, Hydrophobic Interaction Chromatography; UF, Ultrafiltration; SDS-PAGE, Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis; MS, Mass Spectrometry.

A look at Table 2.1 shows that ammonium sulphate precipitation, based on the salting out principle, is most commonly used for initial volume reduction and concentration of bacteriocins. Other methods for initial volume reduction include the use of amberlite resins such as XAD-16, ultrafiltration and lyophilization. The initial volume reduction is necessary since bacteriocins are produced in small quantities in large volumes of culture media. However, this initial step is not very selective, as proteins and peptides of the growth medium are also concentrated along with the bacteriocins. Therefore, further purification steps are necessary to separate bacteriocins from these contaminants and to purify them to homogeneity. Most of the bacteriocins are low molecular weight, cationic, and contain hydrophobic amino acid residues, therefore, these properties are usually exploited to purify bacteriocins to homogeneity (Parada *et al.*, 2007). Commonly used purification schemes include cation exchange chromatography and hydrophobic interaction chromatography followed by Reverse-Phase High Pressure Liquid Chromatography (RP-HPLC) as a final step (Table 2.1). Gel filtration chromatography and other steps may also be included to further purify some of the bacteriocins, but RP-HPLC is usually the final step in the purification scheme which purifies the bacteriocin to homogeneity and separates it from any remaining contaminants (Saavedra and Sesma 2011). The purity is then confirmed by running the purified fraction in a SDS-PAGE gel, followed by in-gel activity testing. The presence of a single active band in the gel confirms that the bacteriocin has been purified to homogeneity, and the primary structure is then determined using techniques such as N-terminus sequencing and mass spectrometry.

The purification scheme described above is most commonly used and has been helpful in the purification and identification of many novel bacteriocins, however, there are several drawbacks. The scheme involves a number of steps, and some of them are quite time consuming and laborious. For example ammonium sulphate precipitation requires overnight stirring at 4 °C. Furthermore, there are losses at each step and, therefore, the greater the number of steps, the greater the loss in bacteriocin activity. Since the bacteriocins are produced in small quantities, the entire bacteriocin activity may be lost after final purification.

Alternative purification schemes have been suggested to either reduce the time or the number of purification steps thereby reducing the losses in activity. Novel methods have been developed which selectively separate bacteriocins from contaminants in minimal steps. One such novel procedure was developed by Yang *et al.* (1992), which

exploits the cationic nature of bacteriocins. It is based on the principle that cationic bacteriocins are selectively adsorbed to the cells of the producer strain at neutral pH (maximum adsorption of about 90% at pH 6.0), and are desorbed at pH of 2.0 (about 99% desorption). The procedure involves the production of bacteriocin in growth medium followed by heating the medium to about 70 °C to kill the cells. The pH of the medium is then adjusted to 6.0 which results in the adsorption of the bacteriocin to heat-killed cells. The cells are then removed from the production broth and then resuspended in a small volume of saline buffer at pH 2 at a temperature of 4 °C. This results in desorption of cationic bacteriocins from the cells into the buffer. The bacteriocin containing buffer is then dialyzed after the removal of cells, followed by lyophilization. The resulting bacteriocin is highly purified and may require only one more step such as RP-HPLC to purify bacteriocin to homogeneity. Yang *et al.* (1992) were able to recover more than 90% of the bacteriocin in the case of Pediocin AcH, nisin and leuconocin Lcm1. However, only 44% bacteriocin was recovered for sakacin A, indicating that this method although very efficient, but may not be suitable for all bacteriocins.

Another novel strategy was developed by Venema *et al.* (1997) for the purification of lactococcin B, which involves the addition of precooled ethanol at 4 °C to the cell-free supernatant resulting in precipitation of the bacteriocin. The precipitated bacteriocin can then be purified to homogeneity in one or two steps. This method has recently been used for the purification of sakacin C2 (Gao *et al.*, 2010) and pediocin SA131 (Lee *et al.*, 2010) in minimal steps, as shown in Table 2.1.

A different approach for purifying the bacteriocin in minimal steps was used by Pingitore *et al.* (2009). They developed a simplified chemically-defined medium for the production of salivaricin 1328 by *Lactobacillus salivarius* 1328. Since the defined medium was devoid of any contaminating peptides and proteins, a single ultrafiltration step was sufficient to purify and concentrate the bacteriocin from cell-free supernatant. This was evident by the presence of a single active band in a tricine SDS-PAGE gel.

To conclude it can be said that with the development of new and improved methods for the purification of bacteriocins or innovative approaches for producing bacteriocins in defined media, many novel bacteriocins may be identified and characterized at the molecular level thus adding to the database of existing bacteriocins.

2.2 Classification of bacteriocins

Although bacteriocins are very heterogeneous in nature, they do have certain characteristics in common which can be used for classification. Initially physicochemical characteristics such as heat resistance, sensitivity to trypsin and host range were used to divide LAB bacteriocins into eight groups (Kozak *et al.*, 1978; Geis *et al.*, 1983). However, this division did not gain any support from researchers. In 1993 Klaenhammer presented a classification scheme based on the primary structure of bacteriocins. This classification was readily accepted and all future classifications are mere modifications of this scheme. According to this scheme bacteriocins are divided into four distinct classes, class II being further divided into three sub-classes (Figure 2.1).

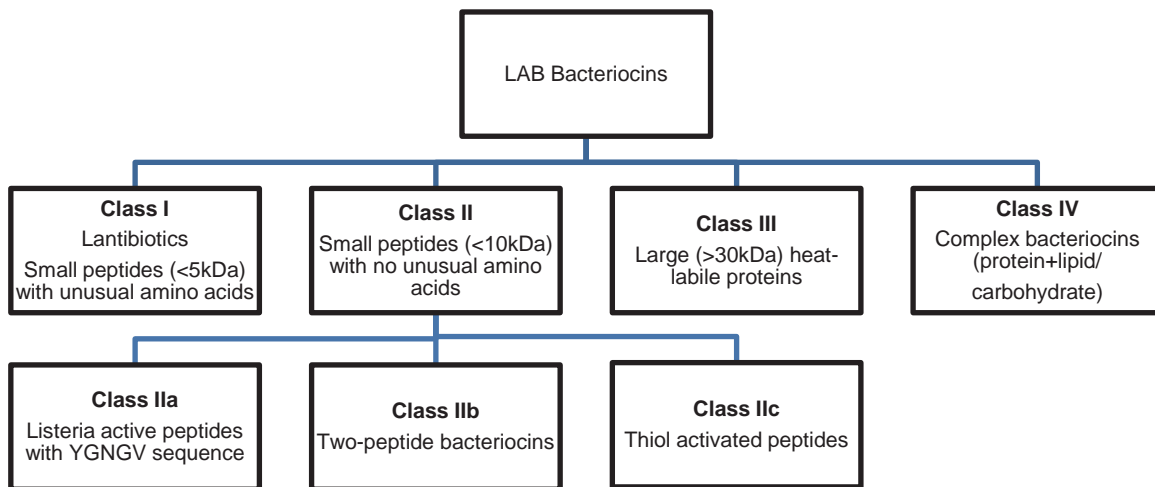


Figure 2.1 Classification of LAB bacteriocins according to Klaenhammer, 1993

In this classification scheme class I include the small peptides termed lantibiotics containing unusual amino acids such as lanthionine. These unusual or modified amino acids are formed by post-translational modifications of normal amino acids. Nisin is an example of this class. Class II bacteriocins are small (< 10 kDa), heat-stable peptides which do not contain modified amino acids. This class is further divided into three subgroups *viz.* Ila, I Ib and I Ic. The Ila subgroup covers the pediocin like bacteriocins having a conserved amino acid sequence (YGNGV) at the N-terminus, and they are very active against *Listeria*. The I Ib subgroup comprises bacteriocins that require two peptides for antimicrobial activity. The subgroup I Ic bacteriocins require reduced cysteine residues for their activity. The class III consists of large (>30 kDa) heat-labile bacteriocins and

Class IV comprises complex bacteriocins which require lipid or carbohydrate moieties in addition to proteins for their activity. A detailed examination of this scheme reveals that the peptides of molecular mass less than 10 kDa are included in class I and II and proteins of greater than 30 kDa molecular mass are included in class III and, therefore, any bacteriocin with a molecular mass between 10 - 30 kDa is out of scope of this scheme. All subsequent classification schemes recognize class I and II as the major classes of bacteriocins. The recognition of class III (heat-labile proteins) as bacteriocins is, however, disputed. Most of the classification schemes emphasize class I and II bacteriocins and their sub-classification, giving least importance to class III, while some have altogether refused to recognize these antimicrobial heat-labile proteins as bacteriocins (Cotter *et al.*, 2005b). However, in a classification scheme proposed by Heng *et al.* (2007) these biologically-active heat-labile proteins have been recognized as an important class of bacteriocins, and have been further sub-divided into two sub-classes based on their mode of action— class IIIa (cell wall degrading bacteriolysins) and IIIb (non-lytic proteins).

Class I was initially divided into two sub-classes; type A consisting of linear, screw-shaped peptides, up to 34 amino acid residues in length acting primarily by membrane disruption of sensitive cells, and type B consisting of globular and compact peptides, up to 19 amino acid residues in length which act by disruption of enzyme function involved in cell wall biosynthesis (Jung 1991). Mersacidin is an example of type B lantibiotic. However, many lantibiotics (Class 1) have intermediate properties. For example, nisin possesses both modes of action and many novel lantibiotics were discovered which could not be placed in either groups. Therefore, a new division of lantibiotics was proposed and they were divided into eight groups (Pag and Sahl 2002). However, this scheme is also incomplete and it has been suggested to divide lantibiotics into 11 subgroups based on similarities in unmodified peptide sequences (Cotter *et al.*, 2005a).

The class II bacteriocins are also very heterogeneous and, therefore, the sub-division of this class has been the subject of debate similar to that of class I. The Klaenhammer scheme divided the non-modified antimicrobial peptides into three groups as discussed above. Nes *et al.* (1996) while retaining the subgroups IIa and IIb, changed the subgroup IIc to include bacteriocins secreted by a general cellular translocase (sec) pathway. While differing from these schemes, van Belkum and Stiles (2000) proposed a division of the class II bacteriocins based on cysteine residues. Six groups (IIa to IIf) were

formed according to this criterion, with the last group consisting of atypical bacteriocins. However, this sub-division did not gain any acceptance in any future classification schemes.

Kemperman *et al.* (2003) while principally agreeing with the Klaenhammer classification, recommended the addition of class V. They proposed inclusion of head-to-tail ligated cyclic antibacterial peptides to this new class, based on the argument that head-to-tail ligation is a post-translational modification and, therefore, cannot be included in class II, and since they do not contain any modified amino acid residues thus do not belong to the class I bacteriocins either. Cotter *et al.* (2005b) disagreeing with this argument, placed the cyclic bacteriocins as a sub-division of class II. They divided the class II bacteriocins into four sub-classes. While sub-class IIa and IIb were similar to Klaenhammer's classification, class IIc consisted of cyclic bacteriocins and class IId forming a repository for all the linear non-modified peptides that do not fall into class IIa (*Listeria* active peptides) and class IIb (two-peptide bacteriocins).

In 2007 Heng *et al.* presented their classification scheme in which they eliminated Klaenhammer's class IV of complex bacteriocins and in agreement with Kemperman *et al.* (2003) raised the status of cyclic peptides to a separate class (class IV). With cyclic peptides now belonging to a separate class, class II was sub-divided into three sub-classes with class IIc now forming a repository of linear non-modified peptides. They also divided class I lantibiotics into three groups; Type A linear, Type B globular and Type C multi-component lantibiotics, thus adding only one more sub-class to the initial sub-division of lantibiotics. However, they further subdivided linear lantibiotics into two sub-types on the basis of size, charge and sequence of leader peptides *viz* subtype A1 (nisin like) and subtype AII (SA-FF22 like). All the heat labile bacteriocins of molecular mass greater than 10 kDa have been included in class III of bacteriocins, which has been further sub divided into two sub classes - the heat labile lytic bacteriocins and the non-lytic bacteriocins. This classification scheme, therefore, include all heat labile bacteriocins of molecular mass greater than 10 kDa in contrast to Klaenhammer's scheme which apparently excluded bacteriocin of molecular mass between 10 – 30 kDa. The classification scheme is shown in Figure 2.2, which tries to encompass not only LAB bacteriocins, but also bacteriocins of other Gram-positive bacteria. While the above mentioned general classification schemes were developed for all the LAB or Gram-positive bacteriocins, a classification scheme was proposed by Franz *et al.* (2007) to include the bacteriocins of the genus *Enterococcus* only. In this classification scheme the

enterococcal bacteriocins are divided into four classes, with class II further sub-divided into sub-classes (Figure 2.3).

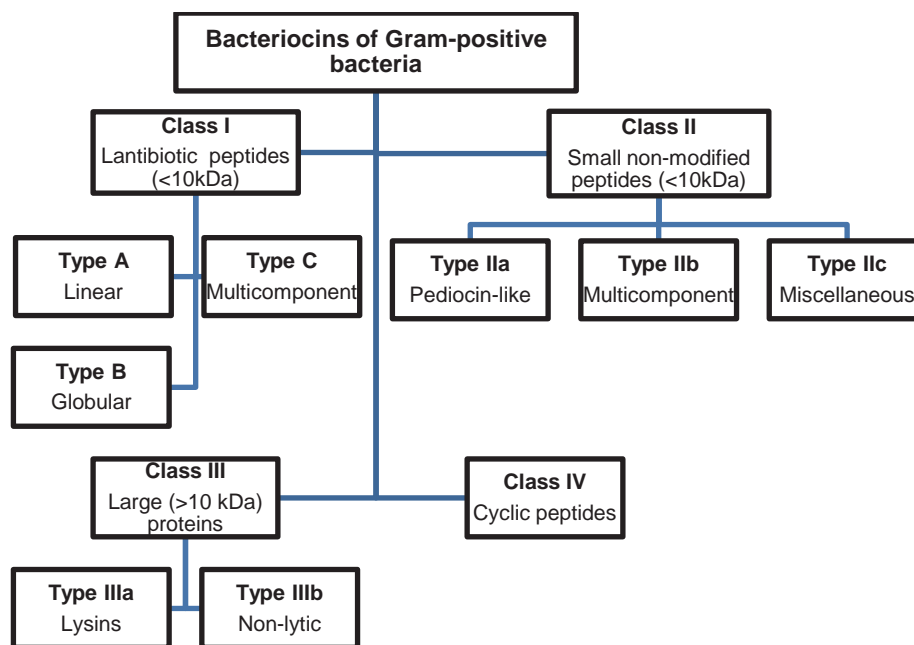


Figure 2.2 Classification scheme of Heng *et al.* (2007). Adapted from Heng *et al.* (2007)

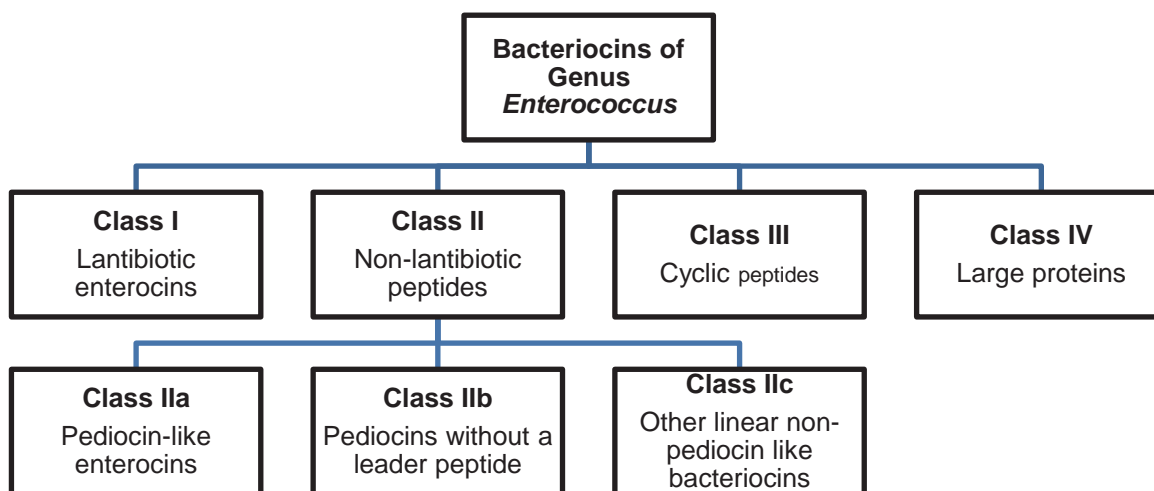


Figure 2.3 Classification scheme of Franz *et al.* (2007) for enterococcal bacteriocins

A comparison of this scheme with other classification schemes reveals that this classification is a simplified version of the scheme presented by Heng *et al.* (2007). The class I lantibiotic enterocin has only one member i.e., cytolysin which is a two peptide lantibiotic. Similarly there is only one large protein identified to date from the genus

Enterococcus named Enterolysin A, which is a cell wall-degrading bacteriocin. Most of the enterocins described to date are non-modified heat stable linear peptides and thus belong to class II, with majority of the members in class IIa, the pediocin-like enterocins, active against *Listeria*.

As discussed in the earlier lines that the classification scheme proposed by Klaenhammer (1933) described a class of complex bacteriocins, which included all the bacteriocins that require lipid or carbohydrate moiety for their activity. The presence of such bacteriocins has long been disputed since no convincing data was available and, therefore, this class was excluded in the later classification schemes. But recent findings have convincingly revealed the presence of complex bacteriocins. It has been reported that sublancin produced by *Bacillus subtilis* 168 (Oman *et al.*, 2011) and glycocin F secreted by *Lactobacillus plantarum* KW30 (Stepper *et al.*, 2011) are glycoproteins that require carbohydrate moiety for antimicrobial activity. Therefore, a modified version of the classification scheme of Heng *et al.*, 2007 is being proposed in this review which adds class V of complex bacteriocins to the scheme (Figure 2.4).

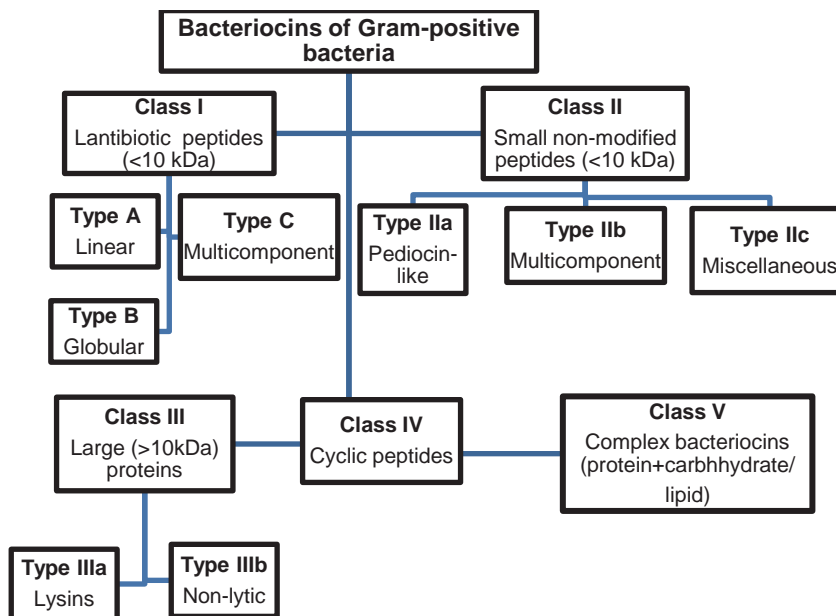


Figure 2.4 Modified version of classification scheme adapted from Heng *et al.* (2007)

It is expected that as the knowledge about bacteriocins gets more advanced, a consensus may be reached for a universal classification scheme.

2.3 The Lantibiotics

2.3.1 Primary structure and physico-chemical properties

The lantibiotics group is one of the most extensively studied group of bacteriocins consisting of peptides (<5 kDa) having unusual amino acids such as lanthionine and methyllanthionine in their primary structure, formed by extensive post-translational modifications (Jack and Sahl 1995). Indeed the term Lantibiotic is derived from L(anthionine) containing antibiotics (McAuliffe *et al.*, 2001). To date about 60 lantibiotics from Gram-positive bacteria have been characterized, and more or less 20 of these are produced by LAB (Bierbaum and Sahl 2009). Some important properties of these LAB lantibiotics are summarized in Table 2.2.

The primary translation product of lantibiotics is a prepeptide consisting of a leader peptide at the N-terminus. The length of the leader peptide varies from 23 to 59 amino acids. No amino acid modification takes place in the leader peptide region however, extensive modifications occur in the propeptide region while the leader peptide is still attached to the propeptide. Only three amino acids, serine, threonine and cysteine are involved in post-translational modifications. Occasionally lysine, alanine and isoleucine may also be post-translationally modified (Sahl and Bierbaum 1998).

Table 2.2 Physico-chemical properties of selected lantibiotics

Bacteriocin	Producer strain	Amino acid residues		Molecular mass (Da)	References
		Leader peptide	Mature bacteriocin		
Nisin A	<i>L. lactis</i>	23	34	3353	(Gross and Morell 1971; Pag and Sahl 2002)
Nisin Z	<i>L. lactis</i>	23	34	3330	(Mulders <i>et al.</i> , 1991)
Lacticin 481	<i>L. lactis</i>	24	27	2901	(Vanden Hooven <i>et al.</i> , 1996)
Streptococcin A-FF22	<i>Streptococcus pyogenes</i>	25	26	2795	(Jack <i>et al.</i> , 1994)
Salivaricin A	<i>S. salivarius</i>	29	22	2315	(Ross <i>et al.</i> , 1993)
Lactocin S	<i>L. sake</i>	31	37	3764	(Mortvedt <i>et al.</i> , 1991)
Lacticin 3147A1	<i>L. lactis ssp. lactis</i>	29	30	3307	(Martinez-Cuesta <i>et al.</i> , 2000; Martin <i>et al.</i> , 2004)
Lacticin 3147A2	<i>L. lactis ssp. lactis</i>	36	29	2847	(Martinez-Cuesta <i>et al.</i> , 2000; Martin <i>et al.</i> , 2004)
Cytolysin L _L	<i>E. faecalis</i>	24	44	4164	(Gilmore <i>et al.</i> , 1994; Booth <i>et al.</i> , 1996)
Cytolysin L _S	<i>E. faecalis</i>	36	27	2705	(Gilmore <i>et al.</i> , 1994; Booth <i>et al.</i> , 1996)

The post-translational modifications are brought about by specific enzymes. For lantibiotics such as nisin two enzymes are involved which are encoded by genes designated as LanB and LanC. The enzyme encoded by the LanB gene is responsible for the dehydration of serine and threonine to dehydroalanine (Dha) and dehydrobutyrine (Dhb) respectively. The LanC protein then catalyzes cyclization of cysteine residues onto Dha or Dhb resulting in the formation of lanthionine and methyllanthionine respectively (Chatterjee *et al.*, 2005).

Another group of lantibiotics which include lactacin 481, cytolysin and salivaricin A possess only one gene, LanM, for post-translational modifications. The gene encodes a 900-1000-amino acid enzyme which catalyzes both the dehydration and cyclization reactions (Chatterjee *et al.*, 2005). The structures of the selected lantibiotics of these two groups are shown in Figure 2.5.

A look at Figure 2.5 shows that post-translational modifications result in the formation of multiple thioether rings in the lantibiotic structure. These ringed structures and other post-translational modifications have been considered to confer stability to the molecule against thermal and proteolytic degradation and also have a role in the antimicrobial activity of the lantibiotics (Sahl and Bierbaum 1998; McAuliffe *et al.*, 2001).

The next step after post-translational modification is the export of the peptide outside the cell. The export of lantibiotics involves a dedicated ATP-Binding Cassette (ABC) transport system. The leader peptide is cleaved off by specific proteases either before or after export from the cell, resulting in the mature active peptide (McAuliffe *et al.*, 2001).

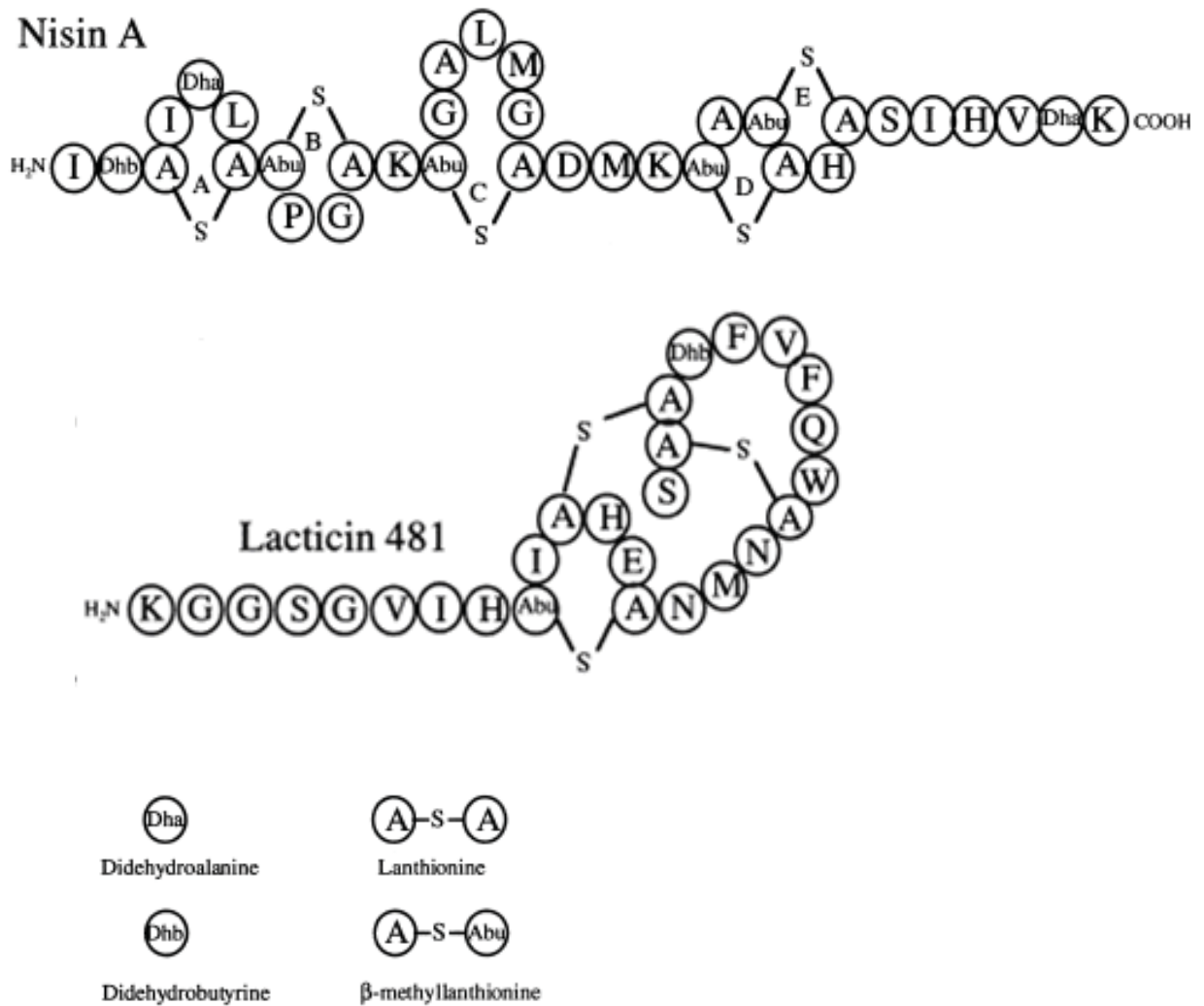


Figure 2.5 Structures of selected lantibiotics Nisin A and Lacticin 481. From McAullife *et al.* (2001)

2.3.2 Genetics

The production of a mature lantibiotic molecule involves synthesis of prelantibiotic peptide (with leader peptide attached), post-translational modification and finally transport and cleavage of the leader peptide to yield mature active bacteriocin. Immunity proteins are also synthesized which give self-immunity to the producing strain. The genes encoding all these processes are arranged in clusters located either on a transposon (e.g. nisin), on plasmids (e.g. lacticin 481) or occasionally on the chromosome (e.g. streptococci A-FF22) (McAullife *et al.*, 2001). The genetic determinants of some of the representative lantibiotics are graphically presented in Figure 2.6.

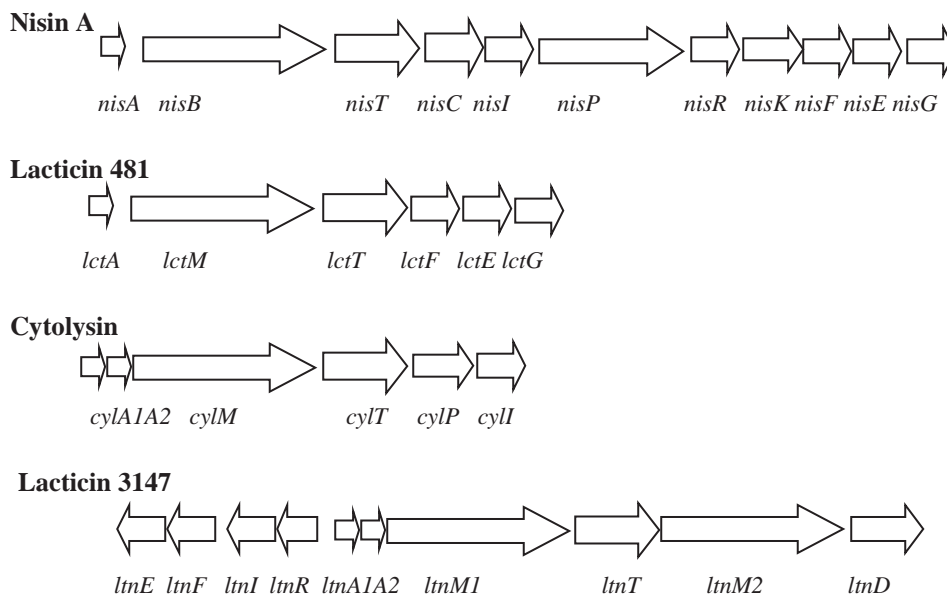


Figure 2.6 Graphical representation of gene clusters of selected lantibiotics. Adapted from McAuliffe *et al.* (2001)

The genes related to lantibiotic synthesis have been given the generic locus symbol *lan*, with a more specific symbol for individual lantibiotics (e.g. *nis* for nisin, *lct* for lacticin 481, *cyl* for cytolysin and *ltn* for lacticin 3147 (Chatterjee *et al.*, 2005) (Figure 2.6). The structural gene (*lanA*) produces prelantibiotics, which have an extension of 23-59 amino acid leader peptides at their N-terminus. These prelantibiotics have no antimicrobial activity and become active after the cleavage of the leader peptide. For two-peptide lantibiotics two genes (A1 and A2) produce the two prepeptides which are then separately post-translationally modified. Currently only two lantibiotics (cytolysin and lacticin 3147) have been identified which require synergistic effects of two peptides for their antimicrobial activity.

Based on the genes responsible for post-translational modifications and transport, the lantibiotics can be divided into two groups. Group I lantibiotics (e.g. nisin) possess two genes (*lanB* and *lanC*) for post-translational modifications. The cleavage of leader peptide is performed by a serine protease encoded by *lanP* gene, and the *lanT* gene encodes the ABC-transporter responsible for the export of the mature peptide. On the other hand group II peptides (e.g. lacticin 481) possess a single gene (*lanM*) for post-translational modifications and are simultaneously exported by an ABC-transporter

encoded by a *lanT(P)* gene with intrinsic proteolytic activity and, therefore, no separate protease is required (Chatterjee *et al.*, 2005).

In addition to genes responsible for modification and transportation, dedicated genes responsible for self-immunity designated as *lanI* are also present in the lantibiotic gene clusters. Additional genes (*lanFEG*) for conferring self-immunity are also present in gene clusters of some lantibiotics such as nisin and lactacin 481. These genes encode a second ABC-transport system, which confers extra immunity to the producing strain by expelling the lantibiotic into the surrounding medium and keeping the concentration at the membrane below a critical level. Many lantibiotics such as nisin, streptococcin A-FF22 and salivaricin A also possess genes for regulation of lantibiotic biosynthesis. These genes designated as *lanK* and *lanR* are involved in a self-inducing regulatory mechanism. In this mechanism, few lantibiotic molecules are synthesized initially and these are exported extracellularly. These molecules are sensed by the regulatory system of the producing cells which stimulates production of further bacteriocin molecules in sufficient quantities (Chatterjee *et al.*, 2005).

2.3.3 Mode of action

All the lantibiotics primarily act on the membranes of the sensitive cells causing their disruption through the formation of pores. This results in dissipation of the Proton Motive Force (PMF) which drives ATP synthesis and accumulation of ions. Thus the destruction of PMF deprives the cell of its energy leading to death.

The mode of action of nisin has been studied in detail. Experiments involving artificial model membranes have shown that a trans-membrane electric potential (negative inside) of 50-100 mV is required for pore formation. This membrane potential probably helps in attracting the positively-charged C-terminus of the nisin molecules towards the negatively-charged membrane. Upon interaction with the membrane the hydrophobic amino acid residues insert into the lipophilic core of the membrane resulting in the formation of pores. These experiments involving artificial membranes require micromolar (μM) quantities of nisin for pore formation. However, it has been observed that the efficacy of nisin is greatly increased against live bacterial cells and only Nanomolar (nM) concentration is required for pore formation. Nisin uses lipid II (a precursor involved in cell wall biosynthesis) as a docking molecule prior to forming pores in the membrane. Initially the N-terminal part of nisin binds to the carbohydrate moiety of lipid II in the target membrane followed by insertion of C-terminal part into the lipid phase of the

membrane. Finally the pore complex formed by nisin and lipid II consist of eight nisin and four lipid II molecules as shown in Figure 2.7 (Hasper *et al.*, 2004). The binding of nisin to lipid II also interferes with the cell wall biosynthesis which increases its efficacy against the sensitive cells. Furthermore, it also binds to negatively charged teichoic and lipoteichoic acids in the cell wall thus activating the autolytic cell wall enzymes resulting in lysis of the cells (Chatterjee *et al.*, 2005; Bierbaum and Sahl 2009).

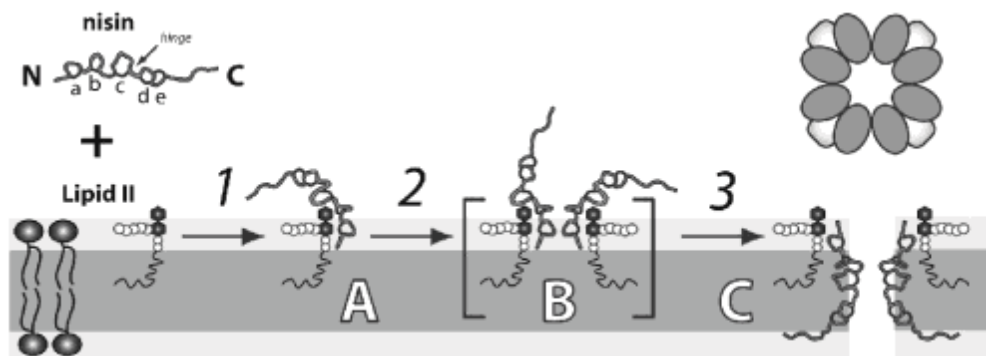


Figure 2.7 Model for the formation of nisin-lipid II pores in lipid bilayers. The polypeptide antibiotic nisin is presented here in a very simplified way, showing only the main structural properties such as the five thioether rings (a-e) and the position of the hinge region (top left). The formation of the nisin-lipid II pore (C) occurs in 3 steps, namely, via the formation of two intermediate structures (A and B). The topview of complex C (top right) shows the proposed arrangement of the molecules in a pore complex composed of 8 nisin (grey ellipse) and 4 lipid II (white triangle) molecules. From Hasper *et al.* (2004).

In addition to the vegetative cells, nisin has also been found active against spores of *Bacillus* and *Clostridium* spp. Initial findings suggested the reaction of thiol groups in the spore wall react with the Dha5 residue in nisin. However, recent experimental evidence shows that removal of the Dha5 residue from nisin does not affect its sporicidal activity. However, truncation of the C-terminus of the nisin molecule does interfere with the activity of nisin against spores which indicates that nisin possibly acts on the membranes of germinating spores (Rink *et al.*, 2007).

2.3.4 Self-immunity

Lantibiotic producing bacteria are protected from the destructive effects of their own bacteriocins by two distinct immunity systems. One encoded by *lanI* genes consists of specific immunity proteins whereas the second system encoded by *lanFEG* genes, consists of specialized ABC transporter proteins which confer enhanced immunity to the producer strain (Draper *et al.*, 2008). A 245-amino acid protein is encoded by the *nisI*

gene, found in the nisin gene cluster. It is a lipoprotein having an N-terminal cysteine residue which anchors in the membrane of the producer strain thus protecting it against nisin (Kuipers *et al.*, 1993). Similarly *ltnI* encodes a 116-amino acid hydrophobic protein which protects the lactacin 3147 producing strain. This protein has three transmembrane domains which indicate its localisation in the cytoplasmic membrane thus preventing the bacteriocin molecules attacking the membrane (McAuliffe *et al.*, 2000).

A second immunity system has also been found in many lantibiotics such as nisin and lactacin 481. This system consists of three proteins encoded by *lanFEG* genes. The *lanF* proteins contain an ATP binding site, whereas *lanE* and *lanG* are membrane-spanning proteins. This system is specifically involved in expelling the lantibiotic into the surrounding medium thus keeping its concentration near the membrane of the producer cells below a critical level thereby providing additional protection to the producer strain (Draper *et al.*, 2008).

2.4 Pediocin-like peptide bacteriocins

In contrast to the lantibiotics which contain post-translationally modified amino acids in their primary structure, many peptide bacteriocins have been described which do not undergo any post-translational modifications. A sub-group of these unmodified bacteriocins includes those peptide molecules which share a consensus motif YGNGV(X)C(X)₄C(X)V(X)₄A (where X is any amino acid) at their N-terminus. Since pediocin PA1 was one of the earliest bacteriocins reported to have this motif in its primary structure, these peptides are also known as the pediocin-like bacteriocins.

2.4.1 Primary structure and physico-chemical properties

In the early nineties bacteriocins such as leucocin A and pediocin PA-1 were described which were found very active against the food pathogen *L. monocytogenes* (Hastings *et al.*, 1991; Henderson *et al.*, 1992). Later many other peptide bacteriocins were discovered which had anti-listerial properties. Studies of the primary structures of these bacteriocins revealed a consensus motif YGNGV(X)C(X)₄C(X)V(X)₄A at their hydrophilic N-terminus with a more varying hydrophobic or amphiphilic C-terminus. These bacteriocins have, therefore, been grouped together and to-date more than 20 pediocin-like bacteriocins have been described (Drider *et al.*, 2006). The physico-chemical properties of some of the representative bacteriocins are listed in Table 2.3.

Table 2.3 Physico-chemical properties of pediocin-like bacteriocins

Bacteriocin	Producer strain	Amino acid residues		Molecular mass (Da)	pI	References
		Leader peptide	Mature Bacteriocin			
Pediocin PA-1	<i>P. acidilactici</i> PAC 1.0	18	44	4628	8.7	(Henderson <i>et al.</i> , 1992)
Leucocin A	<i>L. gelidium</i> UAL 187	24	37	3932	8.8	(Hastings <i>et al.</i> , 1991)
Enterocin SE-K4	<i>E. faecium</i> K-4	27	49	5358	9.6	(Eguchi <i>et al.</i> , 2001)
Enterocin P	<i>E. faecium</i> P13	26	44	4493	8.3	(Cintas <i>et al.</i> , 1997)
Enterocin A	<i>E. faecium</i> CTCA92	18	43	4829	8.9	(Aymerich <i>et al.</i> , 1996)
Bacteriocin T8	<i>E. faecium</i> T8	30	44	5094	9.2	(De Kwaadsteniet <i>et al.</i> , 2006)
Avicin A	<i>E. avium</i> 208	18	43	4289	9.2	(Birri <i>et al.</i> , 2010)
Sakacin P	<i>L. sake</i> 674	18	43	4434	8.8	(Tichaczek <i>et al.</i> , 1994)
Sakacin A	<i>L. sake</i> 706	18	41	4306	9.6	(Holck <i>et al.</i> , 1992)
Mundticin	<i>E. mundtii</i> ATO6	15	43	4288	9.7	(Bennik <i>et al.</i> , 1998)
Mesentericin Y105	<i>L. mesenteroides</i> Y105	24	37	3868	8.8	(Fleury <i>et al.</i> , 1996)

A look at the Table 2.3 reveals that pediocin-like peptides have high isoelectric points which make them positively charged at physiological pH. Their molecular masses are less than 10 kDa, and the number of amino acid residues in the mature peptides range from 37 to 49.

The peptides are synthesized as prebacteriocins on the ribosome, which contain a leader peptide (ranging from 15 to 30 amino acids) with a double glycine motif. The double glycine motif is the cleavage site of prebacteriocin and it also indicates that the bacteriocin is exported out of the cell by the ABC transport system (Van Belkum *et al.*, 1997). A few pediocin-like bacteriocins (e.g. bacteriocin 31 and enterocin P) do not have the double glycine motif in their leader peptides and they are secreted by the Sec-dependent general secretory pathway (Cintas *et al.*, 1997; De Kwaadsteniet *et al.*, 2006). The presence of the leader peptide at the N-terminus of pre-bacteriocin makes the peptide inactive thus protecting the producing cell at the cytosolic side. Furthermore, the leader peptide also acts as a recognition signal for the transport system. After cleavage of the

leader peptide, the mature bacteriocin is exported out of the cell into the environment where it is fully active against the sensitive bacterial strains (Drider *et al.*, 2006).

The structural studies have revealed that in addition to the conserved motif, all the pediocin-like bacteriocins have a disulfide bond formed by the two cysteine residues at their N-terminus. Some bacteriocins (e.g. pediocin PA-1, sakacin G and enterocin A) have an additional disulfide bond near their C-terminus. The presence of these disulfide bonds not only confers stability on the molecule but also enhances the antimicrobial activity of the bacteriocins (Fimland *et al.*, 2000).

2.4.2 Genetics

A majority of the pediocin-like peptides are encoded by genes which are located on plasmids. The exceptions are enterocin A (Aymerich *et al.*, 1996), sakacin P (Huhne *et al.*, 1996), divercin V41 (Metivier *et al.*, 1998) and carnobacteriocins B2 and BM1 (Quadri *et al.*, 1997) whose gene clusters are located on the chromosome. In addition to the structural gene which encodes the prebacteriocin, a minimum of three other genes are required for bacteriocin production. These genes encode proteins which are involved in providing self-immunity, transport of the bacteriocin and an accessory protein with unknown function. The gene clusters of many pediocin-like bacteriocins also possess genes responsible for the regulation of bacteriocin biosynthesis. Three genes are involved in the regulation of biosynthesis which encodes for an inducer peptide, transmembrane histidine kinase (the receptor of inducer peptide) and a response regulator (which stimulates bacteriocin transcription) (Drider *et al.*, 2006).

2.4.3 Mode of action

The pediocin-like bacteriocins have been found active particularly against *L. monocytogenes*. In addition they have also been found active against other Gram-positive bacterial species belonging to the genera *Enterococcus*, *Lactococcus* and *Clostridium*. Detailed studies of the mode of action of these listericidal peptides against the sensitive strains have indicated that the target of these peptides is the membrane of sensitive cells. They act on the membrane in two steps. The first step involves interaction of the peptide with a receptor in the membrane and the second step is the formation of pores or channels.

Specific proteins of the Mannose-Phosphotransferase System (M-PTS) act as receptors for the pediocin-like peptides. This system is responsible for import and translocation of sugars in the bacterial cells, and is composed of three major components. One of the components of this system designated as enzyme II (EII) is in turn further composed of four subunits, IIA, IIB, IIC and IID. The last two subunits (IIC and IID) are associated with the membrane where they form a complex through which the sugars enter the cell (Postma *et al.*, 1993). Since these two subunits are associated with the membrane, therefore, they have been predicted as the possible candidates for receptors of pediocin-like bacteriocins (Diep *et al.*, 2007). Heterologous expression of the *mptC* gene (responsible for encoding IIC subunit) in the resistant *L. lactis* strains make them sensitive to pediocin-like bacteriocins (Ramnath *et al.*, 2004). Detailed studies have revealed that a 40 amino acid extracellular loop within the IIC sub-unit of sensitive strains acts as a receptor for the bacteriocins (Kjos *et al.*, 2010).

After interaction with the receptor, the next step is the formation of pores in the membrane. This is achieved by insertion of the more hydrophobic or amphiphilic C-terminus region of the bacteriocin into the lipid membrane. After insertion, the more hydrophobic region faces the lipid bilayer whereas the less hydrophobic region faces the lumen of the pore. The formation of the pores results in efflux of small molecules particularly ions such as K^+ ions, which dissipates the membrane potential and deprives the cell of its energy resulting in death (Ennahar *et al.*, 2000).

2.4.4 Self-immunity

As with lantibiotics and other bacteriocins, immunity proteins are responsible for conferring self-immunity to the pediocin-like bacteriocins. Immunity genes have been identified for many of these bacteriocins and the amino-acid sequences have been deduced. A comparison of amino acid sequences of immunity proteins shows that similarities ranging from 5 to 85%. Based on these similarities these immunity proteins have been divided into three groups (Drider *et al.*, 2006). However, despite these sequence similarities, each immunity protein is very specific to its own bacteriocin. Cross-immunities have only rarely been observed in case of very closely-related bacteriocins such as immunity proteins for sakacin A and pediocin PA-1 that confer immunities against both of these bacteriocins (Fimland *et al.*, 2002a).

Heterologous expression of the immunity proteins in the sensitive strains confers immunity to these strains against the associated bacteriocin. However, extracellular

addition of the immunity proteins has not been found effective in protecting the sensitive strains, which indicates that the protective effect of these proteins is from the cytosolic side (Quadri *et al.*, 1995). The cognate immunity protein recognizes the mannose phosphotransferase-bacteriocin complex and binds to it, thus somehow inhibiting the formation of pores by its own bacteriocin (Diep *et al.*, 2007). The precise mode of action of these immunity proteins is, however, yet to be elucidated.

2.5 Non-pediocin like linear un-modified one-peptide bacteriocins

This is one of the most diverse groups of bacteriocins which include all the non-modified one peptide linear bacteriocins that do not contain a YGNGV motif in their primary structure. There is also diversity between the structures and mode of action. A brief discussion of this group follows.

2.5.1 Primary structure and physico-chemical properties

Many bacteriocins in this group are biosynthesized as pre-peptides which contain a leader peptide with a double glycine motif, which indicates that they are exported extracellularly by a dedicated ABC transport system. However, some members of this group do not contain a double glycine motif in their leader peptides and are secreted by the Sec-dependent mechanism or general secretory pathway of the cell. Divergicin A, propionicin T1 and lactococcin 972 are examples of such bacteriocins (Worobo *et al.*, 1995; Martinez *et al.*, 1999; Faye *et al.*, 2000). In contrast other bacteriocins, such as lacticin Q and enterocin EJ97, are secreted without any leader peptide, (Sanchez-Hidalgo *et al.*, 2003; Fujita *et al.*, 2007). The important properties of selected bacteriocins of these three types are given in Table 2.4.

Table 2.4 Physico-chemical properties of non-pediocin like un-modified bacteriocins

Bacteriocin	Producer strain	Amino acid residues		pI	Molecular mass (Da)	References
		Leader peptide	Mature Bacteriocin			
Bacteriocins secreted by ABC transport system						
Lactococcin A	<i>L. lactis</i> ssp. <i>cremoris</i> 9B4	21	54	8.4	5778	(Holo <i>et al.</i> , 1991)
Lactococcin B	<i>L. lactis</i> ssp. <i>cremoris</i> 9B4	21	47	8.9	5328	(Vanbelkum <i>et al.</i> , 1992)
Lactococcin M	<i>L. lactis</i> ssp. <i>cremoris</i> 9B4	21	48	10.3	4325	(Vanbelkum <i>et al.</i> , 1991)
Sec-dependent bacteriocins						
Divergicin A	<i>Carnobacterium divergens</i>	29	46	9.4	4224	(Worobo <i>et al.</i> , 1995)
Propionicin T1	<i>Propionibacterium theoni</i> 419	31	65	9.5	7130	(Faye <i>et al.</i> , 2000)
Lactococcin 972	<i>L. lactis</i> ssp. <i>lactis</i> IPLA972	25	66	10.6	7381	(Martinez <i>et al.</i> , 1999)
Leaderless bacteriocins						
Enterocin Q	<i>E. faecium</i> L50	No leader peptide	34	9.4	3980	(Cintas <i>et al.</i> , 2000)
Lacticin Q	<i>L. lactis</i> QU 5	No leader peptide	53	10.1	5926	(Fujita <i>et al.</i> , 2007)
Lacticin Z	<i>L. lactis</i> QU 14	No leader peptide	53	9.9	5968	(Iwatani <i>et al.</i> , 2007)
Enterocin EJ97	<i>E. faecalis</i> EJ97	No leader peptide	44	10.8	5322	(Galvez <i>et al.</i> , 1998; Sanchez-Hidalgo <i>et al.</i> , 2003)

2.5.2 Genetics

The Sec-dependent non-pediocin like one peptide bacteriocins possess only two genes associated with bacteriocin production, since no specific genes are required for the production of the ABC transport system and the bacteriocin uses the general secretory pathway of the cell for export. The genetic determinants of divergicin A secreted by *C. divergens* LV 13 are, therefore, located on a small plasmid pCD3.2. This plasmid contains two ORFs associated with divergicin production, a 225 bp ORF which encodes the pre-bacteriocin peptide. The second ORF encodes a putative 56 amino acid immunity protein (Worobo *et al.*, 1995). Similarly propionicin T1 and lactococcin 972 producing strains have only two genes for bacteriocin production - the structural gene and gene encoding for self-immunity (Worobo *et al.*, 1995; Martinez *et al.*, 1999).

In contrast to the Sec-dependent bacteriocins other bacteriocins of this group, which include the leaderless bacteriocins, are transported by a dedicated ABC transport system and thus require additional genes. The most interesting case in this group is that of the lactococcal plasmid p9B-4 harbored by *L. lactis* ssp. *cremoris* 9B-4 (Van-belkum *et al.*, 1992). This plasmid carries genes associated with the production of three one-peptide bacteriocins, lactococcin A, B and M. The genes for production of these three bacteriocins have also been reported to be present in *L. lactis* ssp. *lactis* biovar diacetylactis DPC938 and DPC3286 strains (Morgan *et al.*, 1995). The genes for these bacteriocins are present in separate operons on the same plasmid. Each operon has the structural gene for production of pre-bacteriocin peptide as well as the immunity gene for that bacteriocin. Despite being present on the same plasmid, the mature peptides of three bacteriocins do not share significant homology. However, the N-terminal leader peptides of the three bacteriocins are almost identical which indicates that they are processed and exported by the same pathway i.e. the dedicated ABC transport system, and genes for this transport system are also present in the operons of these bacteriocins (Stoddard *et al.*, 1992; Vanbelkum *et al.*, 1992).

2.5.3 Mode of action

The members of this group of bacteriocins vary greatly in their primary structure and, therefore, have diverse modes of action. Lactococcin A and lactococcin M bind to the mannose phospho-transferase system in the cell membrane resulting in pore formation and, therefore, act in a manner similar to those of pediocin-like bacteriocins (Diep *et al.*, 2007). In contrast lactacin Q does not bind to any receptor in the cell membrane. Instead the positively-charged peptide is attracted by the negatively-charged phospholipid membrane. Upon interaction with the membrane it forms an α helical structure and results in a flip flop of the lipid bilayer causing the formation of a huge toroidal pore (4.6 -6.6 nm in diameter). This is one of the largest pores reported to be formed by the peptide bacteriocins and is permeable to many macromolecules such as proteins (Yoneyama *et al.*, 2009).

In opposition to the pore-forming activity of these bacteriocins, a different mode of action has been observed for lactococcin 972. This bacteriocin has been found to be active only against the dividing cells where it inhibits septum formation. No antimicrobial activity of this bacteriocin has been reported for non-dividing cells of sensitive strains. Detailed analyses have shown that lactococcin 972 binds to lipid II, a precursor involved

in cell wall biosynthesis. In this respect it is similar to the lantibiotic mersacidin which also inhibits cell wall biosynthesis by binding to lipid II. However, mersacidin also affects the non-dividing cells whereas lactococcin 972 is only active against dividing cells which indicate that other components associated with cell division may also be additional targets of lactococcin 972 (Martinez *et al.*, 2008).

2.5.4 Self-immunity

Immunity genes have been identified in the operons of non-pediocin like linear bacteriocins and the protein structures of many of these have been deduced from the DNA sequences, however, the self-immunity mechanism of only lactococcin A has been studied in detail. This has been found to be similar to pediocin-like bacteriocins, i.e. the immunity protein does not bind directly to the bacteriocin rather it binds to the bacteriocin-receptor complex and thus prevents the bacteriocin from causing any further damage to the membrane of the producer cell (Diep *et al.*, 2007).

2.6 Two-peptide un-modified bacteriocins

In contrast to other peptide bacteriocins which require only one peptide for antimicrobial activity, several bacteriocins have been identified which require two-peptides for bacteriocin activity. Some of these two-peptide bacteriocins are post-translationally modified lantibiotics such as cytolysin and lacticin 3147. However, the majority of the two-peptide bacteriocins described to-date are non-modified peptides and are described as follows.

2.6.1 Primary structure and physico-chemical properties

Like other peptide bacteriocins, the two-peptide bacteriocins are 30-60 amino acid long cationic molecules having high isoelectric points, with amphiphilic or hydrophobic structures which are necessary for antimicrobial activity against the membranes of sensitive strains (Garneau *et al.*, 2002). The two-peptide bacteriocins, however, require the complement of two peptides for antimicrobial activity and can be divided into two groups, enhanced (E-type) and synergistic (S). In case of E-type bacteriocins, the individual peptides of the two-component system are active against the sensitive strains; however the combined effect of two-peptides results in significant enhancement of antimicrobial activity. Thermophilin 13 and enterocin L50 are examples of such bacteriocins. In contrast the individual peptides of S-type bacteriocins have no

antimicrobial activity. It is only the combination of two-peptides which displays the killing activity against sensitive strains. Lactococcin G and lactacin F represent the S-type bacteriocins (Heng *et al.*, 2007).

Since the discovery of lactococcin G, the first two-peptide bacteriocin to be described, more than 15 two-peptide bacteriocins from LAB have been described (Garneau *et al.*, 2002). The important properties of some of the selected two-peptide bacteriocins are presented in Table 2.5.

Enterocin L50 is the only two-peptide bacteriocin secreted without a leader peptide and, therefore, is similar to many one-peptide non-pediocin like bacteriocins. However, since its activity is dependent upon two peptides, it is included in this group. All the other two-peptide bacteriocins are synthesized with a leader peptide having a double glycine motif and ranging in length from 15 to about 30 amino acid residues, which is excised prior to export out of the cell by a dedicated ABC transport system.

2. Literature review

Table 2.5 Physico-chemical properties of two-peptide bacteriocins

Bacteriocin	Producer strain	Individual component	Amino acid residues		pI	Molecular mass (Da)	References
			Leader peptide	Mature Bacteriocin			
Lactococcin G	<i>L. lactis</i> LMG 2081	α	15	39	10.9	4346	(Nissen-Meyer <i>et al.</i> , 1992)
		β	25	35	10.4	4110	
Lactococcin Q	<i>L. lactis</i> QU4	α	15	39	10.3	4260	(Zendo <i>et al.</i> , 2006)
		β	26	35	9.8	4018	
Enterocin 1071	<i>E. faecalis</i> BFE 1071	A	18	39	9.5	4285	(Balla <i>et al.</i> , 2000)
		B	28	34	9.9	3899	
Enterocin L50	<i>E. faecium</i> L50	A	No leader peptide	44	10.0	5190	(Cintas <i>et al.</i> , 1998)
		B	No leader peptide	43	10.2	5178	
Plantaricin E/F	<i>L. plantarum</i> C11	E	22	33	11.6	3545	(Anderssen <i>et al.</i> , 1998)
		F	18	34	10.3	3703	
Plantaricin J/K	<i>L. plantarum</i> C11	J	28	25	10.9	2929	(Anderssen <i>et al.</i> , 1998)
		K	25	32	10.5	3503	
Thermophilin 13	<i>S. thermophilus</i>	A	23	62	8.0	5777	(Marciset <i>et al.</i> , 1997)
		B	21	43	8.1	3910	
Enterocin W	<i>E. faecalis</i> NKR-4-1	α	30	30	6.9	3257	(Sawa <i>et al.</i> , 2012)
		β	32	29	8.5	2729	

2.6.2 Genetics

The genes associated with the production of the two-peptide bacteriocins are organized in one or two operons. A minimum of five genes are related to bacteriocin production, including two structural genes encoding the pre-bacteriocins, an immunity gene, a transporter gene responsible for export of bacteriocin and a gene responsible for production of an accessory protein with unknown function. In addition to these genes some two-peptide bacteriocin producers also carry genes for regulation of bacteriocin synthesis, similar to those as described for lantibiotics and pediocin-like bacteriocin producers. *L. plantarum* C11, which produces two two-peptide bacteriocins, plantaricins E/F and J/K is one example of such a strain which carries genes for the regulation of bacteriocin biosynthesis (Nissen-Meyer *et al.*, 2011).

The two structural genes for all the two-peptide bacteriocins (genetically characterized to-date) are located next to each other on the same operon, and, therefore, the two pre-peptides are believed to be produced in a 1:1 ratio. However, there is one immunity gene encoding a single immunity protein for both the peptides (Oppegard *et al.*, 2007).

The genes responsible for transport of the two-peptide bacteriocins encode the proteins for a dedicated ABC transport system which is involved in cleavage of the leader peptide and export of bacteriocins out of cell.

2.6.3 Mode of action

Similar to other peptide bacteriocins the target of two-peptide bacteriocins is the cell membrane of Gram-positive bacteria. They induce the formation of pores resulting in leaking of ions and small molecules causing cell death.

Studies have indicated that the individual peptides of a two-peptide bacteriocin are unstructured in aqueous solutions. Upon exposure to a hydrophobic membrane or membrane mimicking environment, these peptides become organized and behave as a single antimicrobial entity (Hauge *et al.*, 1999). The primary structure of all two-peptide bacteriocins contains GxxxG motifs which play an important role in interaction of the individual peptides with each other and penetration of the membranes (Nissen-Meyer *et al.*, 2011). The individual peptides (α and β) of lactococcin G form a helical structure within the membrane of the sensitive strain with the two helices bonded by the GxxxG motifs. The positively charged C-terminus of the α -peptide helps in penetration of the membrane of sensitive cell due to the membrane potential (negative inside). Within the

membrane, the α and β peptides form a well-organized helix-helix structure with the C-termini of both peptides inside the cell. This results in the formation of pores with leakage of ions causing cell death (Moll *et al.*, 1996; Oppegard *et al.*, 2008). Similar results have been obtained for plantaricin E/F and J/K (Fimland *et al.*, 2008; Rogne *et al.*, 2009). Since lactococcin Q and enterocin 1071 share sequence similarity with lactococcin G, they may have the same mode of action (Nissen-Meyer *et al.*, 2010). Differences, however, have been observed with respect to the type of ions released after the formation of pores. The pores formed by plantaricin E/F and J/K seem to be permeable to monovalent ions but not to divalent ions such as Mg^{+} . However, plantaricin E/F conducts cations more efficiently, whereas pores formed by plantaricin J/K seem to render membranes permeable to anions (Moll *et al.*, 1999). Similarly lactococcin G permeabilizes membranes for cations, such as Na^{+} , K^{+} , Li^{+} , Cs^{+} , Rb^{+} , and choline, but not for H^{+} (Moll *et al.*, 1996; Moll *et al.*, 1998).

2.6.4 Self-immunity

As stated above, only one immunity gene is present in the bacteriocin operons of two-peptide bacteriocins. Thus the immunity proteins of cognate two-peptide bacteriocins recognize the two peptides as a single entity while protecting the producer cell. It has been proposed that these immunity proteins bind to the bacteriocin-receptor complex in a similar manner as indicated for pediocin-like bacteriocins. However, no such receptor has been identified (Nissen-Meyer *et al.*, 2010).

2.7 The circular bacteriocins

2.7.1 Primary structure and physico-chemical properties

The circular bacteriocins are distinct from linear bacteriocins as their N-terminus is ligated to C-terminus via an amide bond giving them a cyclic structure. They are ribosomally synthesized as linear peptides, which then undergo post-translational cyclization after cleavage of the leader peptide. The exact mechanism and the enzymes involved in the ligation of the N-terminus with the C-terminus have not been identified. However, it has been postulated that the leader peptides play an important role in cyclization (Craik *et al.*, 2003; Conlan *et al.*, 2010). Due to the cyclic nature, they are resistant to many proteolytic enzymes, and are also more stable at varying pH and temperature (Maqueda *et al.*, 2008). In contrast to linear peptides, only nine circular

bacteriocins from Gram-positive bacteria have been described, of which six are produced by LAB (Table 2.6).

Table 2.6 Physico-chemical properties of circular bacteriocins

Bacteriocin	Producer strain	Amino acid residues		Molecular mass (Da)	pI	References
		Leader peptide	Mature Bacteriocin			
Enterocin AS-48	<i>E. faecalis</i> S-48	35	70	7150	10.1	(Galvez <i>et al.</i> , 1989)
Uberolysin	<i>S. uberis</i>	6	70	7048	9.6	(Wirawan <i>et al.</i> , 2007)
Lactocyclin Q	<i>Lactococcus</i> sp. strain QU12	2	61	6060	9.7	(Sawa <i>et al.</i> , 2009)
Garvicin ML	<i>L. garvieae</i> DCC43	3	60	6007	10.1	(Borrero <i>et al.</i> , 2011)
Gassericin A	<i>L. gasseri</i> LA39	33	58	5664	6.8	(Kawai <i>et al.</i> , 1998)
Leucocyclin Q	<i>L. mesenteroides</i> TK41401	2	61	6115	9.5	(Masuda <i>et al.</i> , 2011)

Enterocin AS-48 is the most extensively studied of the circular bacteriocins, and was the first bacteriocin of this group to be purified to homogeneity and identified (Galvez *et al.*, 1986; Galvez *et al.*, 1989). It, therefore, stands as the prototype of circular bacteriocins. The mature AS-48 molecule is a 70 amino acid globular protein consisting of five α helices, and a high proportion of hydrophobic amino acids (49%) present at the centre of the globular structure. This hydrophobic core plays an important role in the antimicrobial activity, by inserting into the lipid bilayer of cell membrane (Sanchez-Barrena *et al.*, 2003). Secondary structure prediction of other circular bacteriocins have shown a similar α -helical structure, and a common saposin-like motif which helps in interaction with the lipid membranes thus making these circular peptides membrane active (Martin-Visscher *et al.*, 2009).

The linear form of the AS-48 molecule has been shown to be 300-fold less active than the circular form, and has also been found very sensitive to temperature changes. Similar results have also been observed for gassericin A, which indicate the importance of the circular form in conferring stability and greater potency against the sensitive strains (Montalban-Lopez *et al.*, 2008).

2.7.2 Genetics

The genes involved in the production of most of circular bacteriocins have been identified. While only the structural genes for garvicin ML, lactocyclicin Q and leucocyclicin Q have been identified (Sawa *et al.*, 2009; Borrero *et al.*, 2011; Masuda *et al.*, 2011), the genetic loci for enterocin AS-48, uberolysin and gassericin A have been well-studied (Figure 2.8).

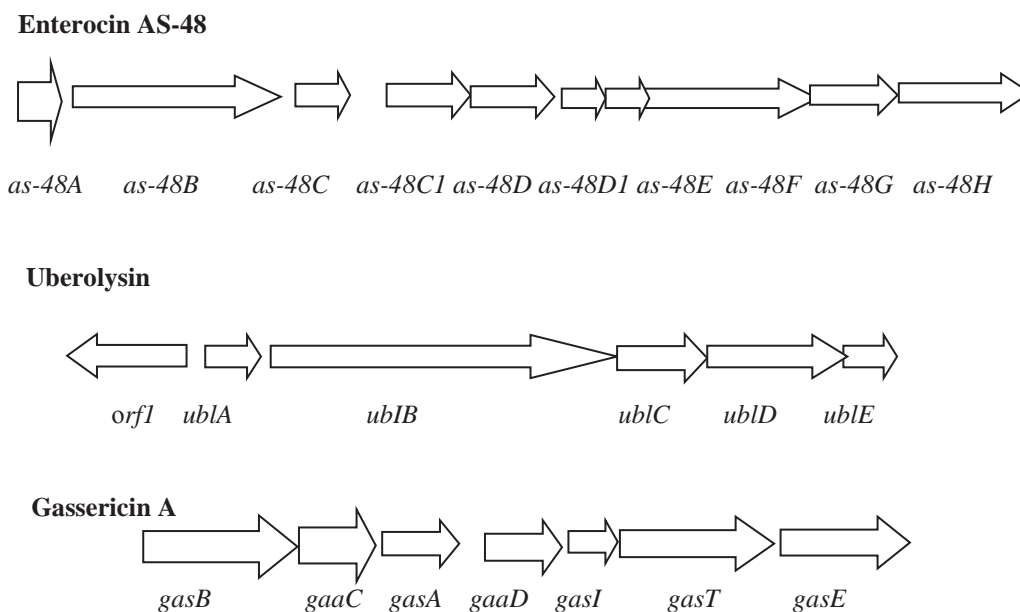


Figure 2.8 Graphical representation of gene clusters of selected circular bacteriocins. Adapted from Van Belkum *et al.* (2011)

The genes encoding enterocin AS-48 production, transport, regulation and self-immunity are located on a conjugative plasmid pMB2 and are organized in two operons. Six genes (*as48ABCC₁DD₁*) in one operon are necessary for production, transport and self-immunity, while four genes (*as48EFGH*) on a second operon encode an additional ABC transporter protein. This supplementary transport system provides additional immunity against the exogenously applied AS-48 (Martinezbuena *et al.*, 1990; Diaz *et al.*, 2003).

An *E. faecium* RJ 16 strain isolated from goat cheese has also been reported to carry the complete gene cluster involved in enterocin AS-48 production and immunity. However, in contrast to *E. faecalis* S-48 strain, the gene cluster is located on the chromosome which indicates the possibility of horizontal gene transfer among the species. Furthermore, there is a difference of one base pair in the structural gene (*as-48A*), where thymine (T) is present at position number 164 in *E. faecium* RJ16, instead of

adenine (A) in *E. faecalis* S-48. This also changes the amino acid composition of enterocin AS-48 RJ (a variant of AS-48) where a valine is present at position number 20 instead of glutamine (Yamamoto *et al.*, 2003).

The genetic determinants of gassericin A are also located on a 33.3 kb conjugative plasmid (pLgLA39). Seven genes (*gaaBCADITE*) located on this plasmid are related to gassericin A phenotype. *GaaA* and *GaaI* are structural and immunity genes respectively, *gaaT* and *gaaE* are possibly involved in export of gassericin A outside cell while *gaaC* and *gaaD* are membrane associated, however, their exact function remains unknown (Ito *et al.*, 2009). These gassericin A related genes are also present on a plasmid (pLrLA6) in a closely related species *L. reuteri* LA6 which strongly indicates natural transfer of plasmids between the species (Kawai *et al.*, 2001).

Unlike AS-48 and gassericin A, the genes related to uberolysin production are present on the chromosome. Six genes (*orf1* and *ublA, B, C, D* and *E*) have been predicted to be related to uberolysin production. The *orf1* located in opposite orientation and upstream of structural gene (*ublA*) is the response regulator while *ublE* is responsible for conferring self-immunity to the producer strain. The *ublB* gene has been predicted to be involved in circularization of the linear form of the bacteriocin and *ublC* and *ublD* are possibly involved in the transport of uberolysin (Wirawan *et al.*, 2007).

A comparison of gene clusters of circular bacteriocins reveals that while there is only one structural gene and immunity gene responsible for the production of prebacteriocin and immunity protein, several genes are involved in transportation of the bacteriocins. These genes encode the proteins which constitute the ABC transport system, which is responsible for export of the circular bacteriocins outside the cell. Furthermore, the presence of gene clusters of the same bacteriocin in closely related species indicates frequent transfer of circular bacteriocin genes across the species.

2.7.3 Mode of action

Similar to most other peptide bacteriocins, the cell membrane is the primary target of circular bacteriocins. The enterocin AS-48 molecule has a cluster of basic amino acids on the surface of the molecule which gives a positive charge. This positive charge helps in the attraction of the peptide to the negatively charged membrane. On approaching the membrane the central hydrophobic core is exposed which helps in the insertion of the molecule into the lipid membrane of the sensitive bacterial cell. In contrast to nisin and similar other peptides, AS-48 does not require any surface receptors and interacts directly

with the membrane as indicated by its effect on artificial membranes, liposomes and bilayers. Upon interaction with the membrane, AS-48 forms pores (about 0.7 nm in diameter) resulting in leakage of low molecular weight ions (such as potassium) and amino acids. This disrupts the membrane potential causing cell death. In contrast to most bacteriocins of Gram-positive bacteria, AS-48 has also been found active against some Gram-negative bacteria such as *E. coli* and *Salmonella typhimurium* although higher concentrations are required than for Gram-positive bacteria. This may be due to the fact that AS-48 does not require any surface receptors for antimicrobial activity and thus higher concentrations can permeate the outer membrane as well as the inner membrane (Galvez *et al.*, 1991; Sanchez-Barrena *et al.*, 2003). Some other cyclic bacteriocins from LAB such as lactocyclicin Q and leucocyclicin Q also have weak antimicrobial activity against Gram-negative bacteria, indicating they may have a mode of action similar to enterocin AS-48.

2.7.4 Self-immunity

Genes encoding dedicated self-immunity proteins have been identified for most circular bacteriocins. The genes are usually located in close proximity to the structural genes. The proteins, encoded by these genes, are small peptides which have high pI and show homology with the membrane associated proteins. This homology indicates that the immunity is conferred by the interaction of these proteins with the cell membrane (Van Belkum *et al.*, 2011). However, the detailed studies of these immunity proteins have not been conducted and, therefore, the mechanism of immunity for circular bacteriocins is poorly understood.

2.8 Heat-labile lytic bacteriocins

2.8.1 Primary structure and physico-chemical properties

The majority of bacteriocins studied to date are heat-stable peptides, as discussed above. But in addition to these peptides, a few large proteinaceous (>10 kDa), heat-labile bacteriocins have also been described. A sub-group of these proteinaceous bacteriocins are the bacteriolysins which are endopeptidases that lyse the cell walls of the sensitive strains in an enzymatic manner. A handful of lytic bacteriocins from Gram-positive bacteria have been identified. The properties of some of the important ones are listed in Table 2.7.

Table 2.7 Physico-chemical properties of heat-labile lytic bacteriocins

Bacteriocin	Producer strain	Amino acid residues		Molecular mass (Da)	pI	References
		Leader peptide	Mature Bacteriocin			
Enterolysin A	<i>E. faecalis</i> LMG2333	27	316	34501	9.2	(Nilsen <i>et al.</i> , 2003)
Zoocin A	<i>Streptococcus zooepidemicus</i> 4881	23	262	27877	7.8	(Simmonds <i>et al.</i> , 1995)
Millericin B	<i>Streptococcus milleri</i> NMSCC 061	18	259	28822	8.7	(Beukes <i>et al.</i> , 2000)
Lysostaphin	<i>Staphylococcus simulans</i> bivoar <i>staphylolyticus</i>	36	246	25000	9.5	(Schindler and Schuhardt 1964; Bastos <i>et al.</i> , 2010)

A look at Table 2.7 shows that the leader peptides of lytic bacteriocins range from 18-amino acid to 36-amino acid in length. Although there is little sequence homology between these leader peptides they all, however, have the characteristics of typical bacterial signal sequence which indicate that these bacteriocins are secreted by the General Secretory Pathway (GSP) or Sec-dependent mechanism. A typical bacterial signal sequence has three domains, a positively charged N terminal domain consisting of 1 – 5 amino acids, a central hydrophobic region consisting of 7 - 15 amino acids and a more polar C-terminus domain which includes the cleavage site (Von Heijne 1990). The function of the signal peptide is to direct the bacteriocin towards the Sec translocase complex which is located in the cell membrane. The Sec translocase complex consists of a motor domain protein SecA, the protein conducting channel formed by three proteins SecY, SecE and SecG and the accessory proteins SecD and SecF. The pre-bacteriocin after synthesis in the ribosome is directed towards the SecA motor protein due to the signal peptide. SecA protein helps in translocation of the pre-bacteriocin in the SecYEG excretory channel. This is an energy dependent step which is provided by ATP hydrolysis. The SecYEG channel is closed in its natural state, which opens once the secretory protein is in the channel. This results in the release of bacteriocin out of the cell with the concomitant cleavage of signal peptide by signal peptidase, a membrane bound peptidase (Driessen *et al.*, 1998). Once the bacteriocin is secreted it folds to form active mature bacteriolysin.

The primary structure of mature bacteriolysins consists of two domains, an N-terminus catalytic domain (CAT) which belongs to the M37/M23 endopeptidase family, and a C-terminus substrate recognition domain (SRD). These two domains are connected by a Threonine-Proline (TP) rich linker sequence (Cooper and Salmond 1993; Simmonds *et al.*, 1997; Nilsen *et al.*, 2003; Maliničová *et al.*, 2010). The N-terminus domains of lysostaphin, zoocin A, millericin B and enterolysin A show sequence homology with each other (Nilsen *et al.*, 2003).

The C-terminus of zoocin A has slight homology with bacteriophage lysins (Lai *et al.*, 2002). However, the C-terminus domain of enterolysin A has significant homology with bacteriophage lysins of *Lactobacillus casei* (Nilsen *et al.*, 2003). The lysostaphin A retains its enzymatic activity even after removal of its C terminal (Baba and Schneewind 1996), however, for zoocin A and enterolysin A, both CAT and SRD domains are necessary for lytic action, and truncated proteins missing either of the domains have no antimicrobial lytic activity (Lai *et al.*, 2002; Nigutová *et al.*, 2008).

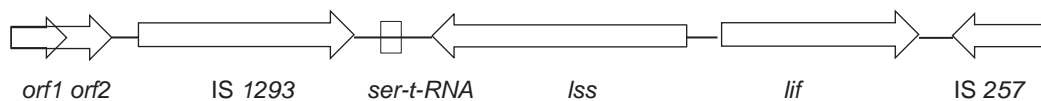
2.8.2 Genetics

The genetic determinants for the lytic bacteriocins lie either on the chromosome or plasmids. The genetic factors of lysostaphin, one of the most thoroughly studied of the lytic bacteriocins, are located on a large pACK1 plasmid (Heath *et al.*, 1987; Gargis *et al.*, 2010a). The plasmid harbors the lysostaphin structural (*lss*) and lysostaphin immunity factor (*lif*) genes organized in opposite directions. The *lss* gene is 1479 bp in length and encodes a 493-amino acid preprolysostaphin protein. The protein comprises a 36-amino acid signal peptide at the N-terminus. The signal peptide is cleaved and a 457-amino acid prolysostaphin is secreted out of the cell. A 195-amino acid region of this propeptide at the N-terminus is arranged into 15 tandem repeats of a 13-amino acid sequence, which is cleaved by a cysteine protease to yield the fully active 246-amino acid mature lysostaphin. Immediately upstream of *lss*, lies the *lif* gene in opposite direction to the *lss* gene. The *lif* gene encodes a 415 amino acid immunity protein which is homologous to FemABX type proteins. The immunity protein inserts unusual amino acids in the cell wall interpeptide bridge of the producer strain thus rendering it resistant to the action of lysostaphin (Thumm and Gotz 1997).

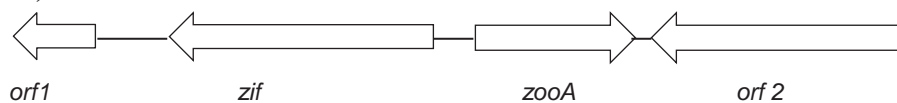
Lysostaphin shares strong sequence homology with other lytic bacteriocins zoocin A, millericin B and enterolysin A. This sequence homology is in the N-terminus region of these enzymes which is the catalytic domain. Similarly the immunity factors of

lysostaphin (*lif*), zoocin A (*zif*) and millericin B (*milF*) also show strong sequence similarity with each other (Beatson *et al.*, 1998; Beukes and Hastings 2001). While the immunity factors of lysostaphin, zoocin A and millericin B have been identified, no gene responsible for self-immunity has been identified for enterolysin A. However, three open reading frames located downstream and upstream of enterolysin A structural gene have been identified (Genbank No. HE585879 Appendix 3). Since the immunity genes for lysostaphin, zoocin A and millericin B are located in close proximity to the structural gene (as shown in Figure 2.9), therefore, it can be hypothesized that the enterolysin A immunity gene may also lying in close proximity to the structural gene, and one of the open reading frames identified upstream and downstream of enterolysin A producing gene may be the immunity gene.

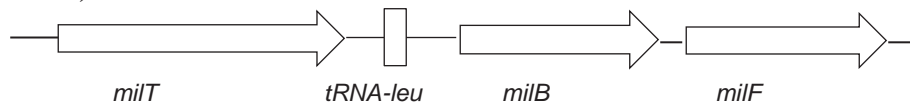
A) Lysostaphin



B) Zoocin A



C) Millericin B



D) Enterolysin A

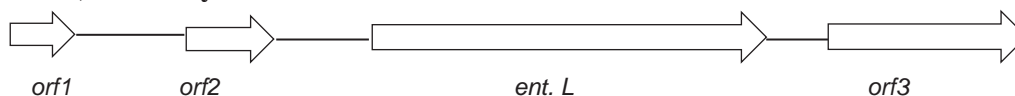


Figure 2.9 Graphical arrangement of gene cluster of bacteriolysins. A) Lysostaphin gene cluster showing the lysostaphin structural gene (*lss*) and lysostaphin immunity factor (*lif*) (Thumm and Gotz 1997). B) Zoocin A gene cluster showing zoocin A structural gene (*zooA*) and zoocin A immunity factor (*zif*) (Beatson *et al.*, 1998). C) Millericin B gene cluster showing the millericin B structural gene (*milB*), millericin B transporter gene (*milT*) and millericin B immunity factor (*milF*) Beukes and Hastings 2001. D) Enterolysin A gene cluster showing enterolysin A structural gene (*entL*), and three open reading frames (*orf1*, *orf2* and *orf3*) (Genbank No. HE585879).

It has also been observed that *lss* and *lif* genes and *zooA* and *zif* genes are flanked by insertion sequences (identified as IS1293 and IS257 for lysostaphin and *orf1* and *orf2* for zoocin A in Figure 2.9). Furthermore the sequence homologies and the similarity in position and orientation of the *zooA* with *lss* gene and *lif* with *zif* gene indicate a strong possibility of horizontal gene transfer between the lysostaphin and zoocin A producing strains (Beatson *et al.*, 1998). Similarly the C-terminus region of enterolysin A has strong sequence similarities with bacteriophage lysins of *L. casei* (Nilsen *et al.*, 2003), which may be due to the gene transfer from the bacteriophages to the producer strain of enterolysin A.

2.8.3 Mode of action

The target of the lytic bacteriocins is the cell wall of Gram-positive bacteria. The cell wall of the Gram-positive bacteria is much thicker (25 nm) compared to that of the Gram-negative bacteria (2 – 3 nm). In Gram-negative bacteria the cell wall is surrounded by an additional outer layer of lipopolysaccharides and lipoproteins, which is absent in Gram-positive bacteria. In the absence of this outer layer the cell wall is the sole protective layer in Gram-positive bacteria which helps to maintain the cell shape and size and protect against osmotic lysis.

The cell wall of the Gram-positive bacteria is made up of alternating units of N-acetyl glucosamine (NAG) and N-acetyl muramic acid (NAM) linked by β -1 \rightarrow 4 bonds. In each NAM residue the D-lactoyl group is replaced by a side peptide chain of four amino acids which is called the stem peptide. Two adjacent stem peptides are connected by cross-linked peptide chains also known as an interpeptide bridge. While the composition of stem peptides is more or less the same across the species, the cross-linked peptides vary in their composition and are species-specific. The stem peptides and cross-linked chains are the target for lytic bacteriocins (Figure 2.10). After the cleavage of a specific peptide bond, depending on the nature of bacteriolysin, the insoluble peptidoglycan layer is solubilized, the cell wall cannot maintain its structure and is destroyed. After the destruction of the cell wall the cell membrane can no longer withstand the cell's internal turgor of up to 25 atmospheres thus resulting in lysis of the cell (Maliničová *et al.*, 2010).

Depending on the nature of the bacteriocin, the enzymatic cleavage of the peptide bond can occur either at the stem peptide or cross-linked peptide chain or both. Lysostaphin has been found to target only the pentaglycine cross-linking peptides of all

known staphylococcal species (Schleifer and Kandler 1972; Grundling and Schneewind 2006). Similarly zoocin A, produced by *S. zooepidemicus* 4881, also targets only the cross-linking peptides. However, in contrast to lysostaphin, it targets the sensitive streptococcal strains which are closely related to the producer strain. These include *Streptococcus oralis*, *S. pyogenes*, *Streptococcus equi* and *Streptococcus gordonii*. The cross-linking peptide chains of all these species consist of two to three L-alanine residues, and experiments have shown that zoocin A acts as a D-alanyl - L-alanine endopeptidase which hydrolyzes the bond between the terminal D-alanine of the stem peptide and the first L-alanine of the cross-linking bridge (Simmonds *et al.*, 1996; Gargis *et al.*, 2009b).

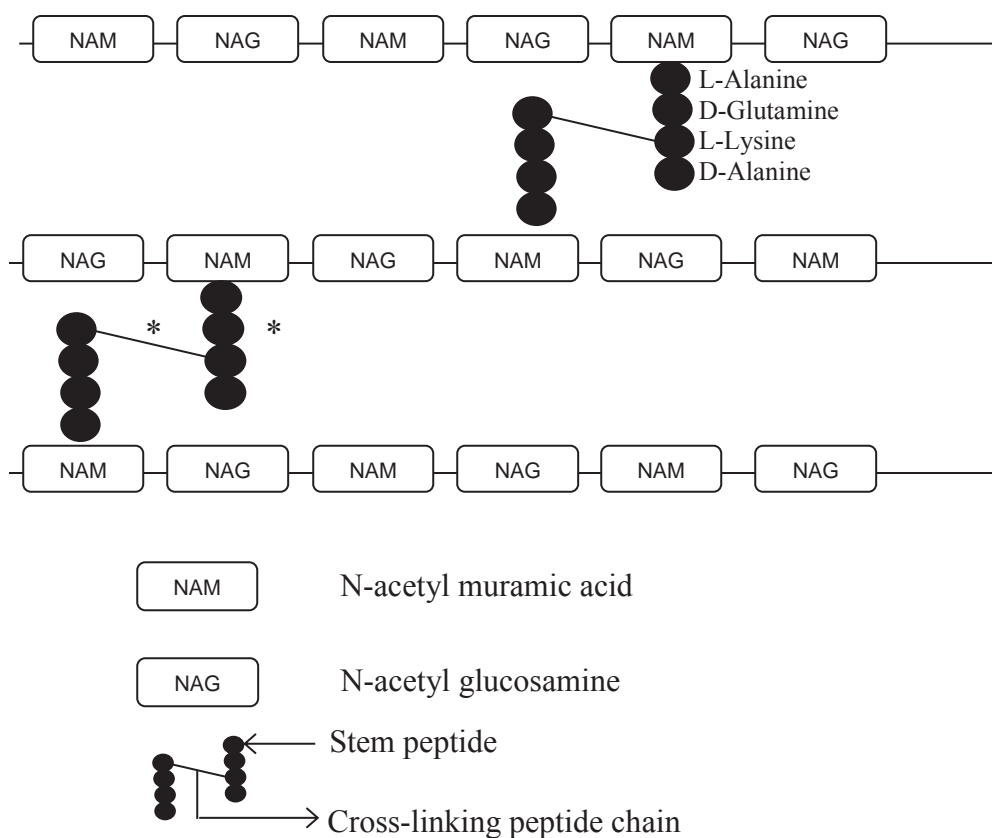


Figure 2.10 Schematic representation of peptidoglycan network structure in a Gram-positive bacterial cell wall showing the specific target sites (shown with *) of lytic bacteriocins. Adapted from Maliničová *et al.* (2010).

In contrast to lysostaphin and zoocin A, millericin B cleaves the peptide bonds both in the stem peptide as well as in the cross-linking bridge of the cell wall. Millericin B is distinct from these bacteriocins in that it is active against a broad range of bacterial

species which not only include non-producing strains of *S. milleri* but also *L. monocytogenes*, *Micrococcus luteus*, *Streptococcus agalactiae* and *L. lactis*. Millericin B most probably hydrolyzes the bond at the glutamic acid of the stem peptide and the N-terminus of alanine within cross-linking peptides of sensitive strains (Beukes *et al.*, 2000).

Like millericin B, enterolysin A is also a broad-spectrum lytic bacteriocin. It is active against a range of bacterial species which include *E. faecalis*, *E. faecium*, *L. sake*, *L. lactis* ssp. *cremoris*, *L. lactis* ssp. *lactis*, *Listeria innocua* and *Bacillus subtilis*. Since the composition of interpeptide bridges is different in these sensitive species and there is no common denominator, therefore, it has been predicted that the site of action of enterolysin A is within the stem peptide whose composition is the same among all sensitive strains (Nilsen *et al.*, 2003).

2.8.4 Self-immunity

All the cell wall-degrading enzymes which have been classified as bacteriocins provide self-immunity to the producer strain. Without this important characteristic these enzymes cannot be categorized as bacteriocins. The factors responsible for conferring this self-immunity have been identified for several of these lytic bacteriocins. Both lysostaphin and zoocin A producing strains also produce FemABX-like immunity proteins which insert unusual amino acids in the cross-linking bridge of the cell wall. All the FEM (Factors Essential for Methicillin resistance) factors play an important role in cell wall biosynthesis, and fem A, B and X proteins are peptidyl transferases which add specific amino acids during the synthesis of a cross-linking bridge of Gram-positive cell walls. In case of staphylococci, the FemA, B and X proteins add glycine residues form the pentaglycine interpeptide bridge of staphylococcal cell walls. The Lysostaphin Immunity Factor (*lif*) encodes FemABX-like proteins which incorporates serine residues instead of glycine at positions 3 and 5 of the pentaglycine interpeptide bridge of the producing strain (*S. simulans* biovar *staphylolyticus*) (Dehart *et al.*, 1995; Gargis *et al.*, 2010b). Similarly zoocin A immunity factor (*zif*), incorporates an additional L-alanine in the interpeptide bridge of the producing strain (*S. equi* ssp. *zooepidemicus* 4881) (Gargis *et al.*, 2009a). These modifications in the interpeptide cross-bridges of cell walls affect the binding as well as the catalytic action of lysostaphin and zoocin A, thus conferring resistance to the producer strains against their own lytic bacteriocins (Gargis *et al.*, 2010b).

A gene encoding for a 302-amino acid protein has been identified in the millericin B operon. This protein has strong sequence similarity (68%) with FemA and B of *S. aureus* and *lif*. This protein has been predicted to add lysine in place of a threonine in the cross-linking bridge of *S. milleri* NMSCC 061 cell wall, thus giving immunity to the host strain (Beukes and Hastings 2001).

While immunity factors have been identified for lysostaphin, zoocin A and millericin B, no immunity genes have been described for enterolysin A producing strains. The present study aims to identify the immunity genes for enterolysin A.

2.9 Heat-labile non-lytic bacteriocins

2.9.1 Primary structure and physico-chemical properties

In contrast to lytic bacteriocins, there is another group of high molecular mass (>10 kDa) bacteriocins which kill their target in a non-lytic fashion. To date three such bacteriocins from LAB have been described, and their important physicochemical properties are listed in Table 2.8.

Table 2.8 Physico-chemical properties of heat-labile non-lytic bacteriocins

Bacteriocin	Producer strain	Amino acid residues		Molecular mass (Da)	pI	References
		Leader peptide	Mature Bacteriocin			
Helveticin J	<i>L. helveticus</i> 481		334	37511	6.0	(Joerger and Klaenhammer 1986; 1990)
Dysgalacticin	<i>Streptococcus dysgalactiae</i> ssp. <i>equisimilis</i> W2580	28	192	21504	4.7	(Heng <i>et al.</i> , 2006)
Streptococcin A-M57	<i>S. pyogenes</i> M-57	27	152	16900	5.2	(Heng <i>et al.</i> , 2004)

Helveticin J was the first non-lytic heat-labile bacteriocin to be described. It has been found as an aggregate of over 300 kDa, which when dissociated yields the bacteriocin with a molecular mass of 37.5 kDa (Joerger and Klaenhammer 1990). It has a narrow spectrum of activity and is active only against the closely related species and strains which include non-producing strains of *L. helveticus*, *L. bulgaricus* and *L. lactis* (Joerger and Klaenhammer 1986). Similar to helveticin J dysgalacticin, produced by *S.*

dysgalactiae ssp. *equisimilis*, this bacteriocin also has a narrow spectrum of activity principally showing antimicrobial activity against the human pathogen *S. pyogenes*. It is synthesized initially as a 220-amino acid protein, which is then cleaved after the 28th amino acid from the N-terminus end to yield a signal peptide which helps in secretion of the mature dysgalactin out of the cell by the sec-dependent mechanism. The mature dysgalactin molecule has an unstructured N-terminus region and a helical C-terminus region which also contains two disulphide bonds. These disulphide bonds have been found essential for the antimicrobial activity and their reduction results in loss of the function of the bacteriocin (Heng *et al.*, 2006).

The secondary structure of dysgalactin is very similar to streptococcin A-M57, a bacteriocin produced by *S. pyogenes*, which also consist of a flexible N-terminus, and a helical C-terminus region with a putative disulphide bond. However, the primary structure of streptococcin has no homology with dysgalactin (Heng *et al.*, 2006). The spectrum of activity of streptococcin A-M57 is also very broad and unusual, as it is not active against other *S. pyogenes* strains, but does inhibit other Gram-positive bacteria such as *Micrococcus luteus*, *L. lactis* sssp. *lactis* (including biovar diacetylactis), *L. lactis* ssp. *cremoris*, *Bacillus megaterium*, *S. simulans*, *Listeria grayii*, and *E. hirae* (Heng *et al.*, 2004).

2.9.2 Genetics

Helveticin J is a chromosomally encoded bacteriocin, as determined by cloning and sequencing of chromosomal DNA containing the helveticin J (*hlv*) gene. An Open Reading Frame (ORF) of 1002 bp was identified encoding a protein of molecular mass 37,500 Da. This was further confirmed when this ORF was cloned and expressed into *L. acidophilus* 249 which showed similar inhibitory spectrum and properties to that of helveticin J. Although the producing strain is self-immune, no genes encoding any immunity factor have been identified (Joerger and Klaenhammer 1990).

In contrast to helveticin J, the genetic determinants of dysgalactin lie on a 3043 bp plasmid (pW2580). The 660 bp structural gene of dysgalactin (*dysA*) has been found in the opposite direction with respect to other ORFs on this plasmid. This gene encodes the 220 amino acid predysgalactin which is then cleaved to form a 192 amino acid mature dysgalactin as it is exported out of the cell. The dysgalactin structural gene is flanked by hexanucleotide promoter motifs on the upstream side and a potential transcriptional terminator on the downstream side. The curing of the pW2580 plasmid renders the

dysgalactacin producing strain susceptible to its own bacteriocin which indicates that gene for self-immunity is also located on the same plasmid. (Heng *et al.*, 2006). This gene has been identified as *dysI* which encodes a 57-amino acid protein.

Similar to dysgalactacin, the gene for the production of streptococcin A-M57 is also located on a plasmid (pDN571), and likewise the gene for dysgalactacin is also in the opposite direction with respect to other genes on the plasmid. This gene encodes the 179 aa prebacteriocin, which is cleaved to yield the active 152 aa streptococcin A-M57. The prebacteriocin has significant similarity (71%) to a hypothetical protein of *E. faecalis* V583, while the C-terminus has homology to a hypothetical protein (YpkK) from *Corynebacterium jeikeium* (Heng *et al.*, 2004).

2.9.3 Mode of action

Dysgalactacin is the only non-lytic high molecular mass bacteriocin whose mode of action has been studied in detail. The dysgalactacin is a narrow spectrum bacteriocin mainly active against *S. pyogenes*. Dysgalactacin inhibits glucose fermentation in the sensitive cells of *S. pyogenes* as revealed by no decrease in external pH, as compared to a control (without dysgalactacin) where the pH drops by 1 unit within 10 min. This indicates that dysgalactacin arrests cell growth by interfering either with glucose uptake or with glucose metabolism inside the cell. Further experiments have shown that dysgalactacin binds to the phosphoenolpyruvate-dependent glucose- and mannose-phosphotransferase system (PTS) within the cell membrane of *S. pyogenes* as evidenced by the low-rate of glucose uptake (1.57±1.13 nmol/mg protein/min) in the starved cells pretreated with dysgalactacin, while untreated cells showed a high-rate of glucose uptake (32.8±2.20 nmol/mg protein/min). The binding of dysgalactacin also dissipates membrane potential resulting in the loss of K⁺ ions. To maintain the membrane potential, the cell uses its store of ATP which depletes the cell of its energy resulting in death (Swe *et al.*, 2009).

Although helveticin J and streptococcin A-M57 have been shown to kill the cells of sensitive bacterial strains in a non-lytic fashion, their detailed mode of action and target sites have not been studied in detail.

2.9.4 Self-immunity

The strains producing the non-lytic heat-labile bacteriocins are immune to the effect of their own bacteriocins. The immunity mechanism of dysgalactacin has been studied in

detail. The immunity gene for dysgalactin (*dysI*) is located on the the plasmid pW2580, which also carries the structural gene. The gene encodes a 57-amino acid membrane associated protein. Transformation of the dysgalactin sensitive strain *S. pyogenes* FF-22 with the *dysI* gene rendered it resistant to dysgalactin. It was further observed that after exposure to dysgalactin, glucose uptake and fermentation occurred normally in the resistant transformant in contrast to the wild-type sensitive strain where glucose fermentation was inhibited. This indicates that the *dysI* encoded protein prevent the interaction of dysgalactin with the glucose/mannose-phosphotransferase system (PTS) without interfering with the glucose uptake by the cell (Swe *et al.*, 2010).

2.10 The Complex Bacteriocins

The presence of the complex bacteriocins, which require carbohydrate or lipid moiety in addition to protein component, has long been debatable. Earlier reports of such bacteriocins included plantaricin S (Jimenez-Diaz *et al.*, 1993) and leuconocin S (Lewus *et al.*, 1992). These bacteriocins were supposed to be complex bacteriocins since they were found sensitive to glycolytic or lipolytic enzymes. However, it was later found that plantaricin S secreted by *Lactobacillus plantarum* LPCO10 was in fact a two peptide bacteriocin and no convincing data about the complex nature of leuconocin S has been presented and, therefore, the complex bacteriocins were eliminated in the recent classification schemes.

However, recent findings demand the revival of complex bacteriocins as an important class. It has recently been confirmed that sublancin (initially thought to be a lantibiotic) and glycocin F are glycoproteins that require carbohydrate moiety for their activity. Both bacteriocins are post-translationally modified to form active cysteine S-linked glycopeptides with the help of glycosyltransferase enzymes.

Sublancin 168 produced by *B. subtilis* 168 was initially presumed to be a lantibiotic (Paik *et al.*, 1998) but later it was confirmed that sublancin is in fact a glycopeptide (Oman *et al.*, 2011) which has a predicted mass of 3717.7207 Da, whereas the mass determined by FT-ICR-MS was 3875.7454 Da. This additional mass is due to hexose sugar covalently attached to the cysteine residue of the peptide moiety of 37 amino acid residues via sulphur atom (S-linked glycopeptide). In addition there are two disulphide bonds in the molecule (Stepper *et al.*, 2011), Sublancin 168 has been found particularly active against non-producing strains of *B. subtilis*, *Bacillus megaterium*, *Staphylococcus aureus* and *Staphylococcus epidermidis*.

Glycocin F produced by *L. plantarum* KW30 is a heat stable bacteriocin whose activity is limited only to lactobacilli. It is particularly active against the non-producing strain *L. plantarum* ATCC 8014. The structural gene for glycocin F lies on the chromosome which encodes a 64-amino acid preglycocin F including a 21-amino acid double glycine leader peptide. The predicted molecular mass of mature glycocin F is 4796.9197 Da while the measured molecular mass is 5199.0488 Da. The difference between the two masses is due to the S-glycosylation of the peptide moiety with the hexose similar to sublancin (Stepper *et al.*, 2011).

The role of carbohydrate moiety in the antimicrobial activity and the mode of action of the complex bacteriocins is yet to be elucidated. It is expected that interesting findings will be revealed as more detailed studies on the mode of action of glycobacteriocins are carried out.

2.11 Bacteriocins as food preservatives

For centuries several physical or chemical treatments have been employed to increase the shelf-life of foods. But now there is an increasing concern about the foods which have undergone extensive physical processing or contain chemical preservatives, and now there is increasing demand for foods that are minimally processed with minimum or no chemical preservatives. However, pathogenic and spoilage microorganisms that are already present in the food may survive and grow due to minimal processing and in the absence of preservatives. The bacteriocins therefore, offer an alternative to chemical preservatives, as they are safe, natural antimicrobial products. Although bacteriocins are produced by many Gram-positive and Gram-negative species, those produced by LAB are of particular interest to the food industry, since these bacteria have generally been regarded as safe.

Among the lactic acid bacteria, a high diversity of bacteriocins is produced and several have been patented. The most successful of them is Nisin, a lantibiotic produced by various strains of *L. lactis*.

The antimicrobial activity of *L. lactis* was first reported by Rogers, 1928, and later the bacteriocin was named as nisin by Mattick and Hirsch (1944). In 1957 Aplin and Barret (Ltd) developed a commercial preparation containing 2.5% nisin. The product was marketed with the trade name of Nisaplin®. Today nisin is approved for use in food in over 50 countries including EEC, USA, Australia and New Zealand (Delves-Broughton *et*

al., 1996). Nisaplin has been used as preservative in various food products at various levels as given in Table 2.9.

Table 2.9 Typical addition levels of nisin and Nisaplin® in food applications. From Delves-Broughton (2005)

Food application	Typical target organisms	Level of nisin (mg/kg or mg/L)	Level of Nisaplin® (mg/L)
Processed cheese	<i>Clostridium spp.</i> , <i>Bacillus spp.</i>	5–15	200–600
Pasteurised milk and milk products	<i>Clostridium spp.</i> , <i>Bacillus spp.</i>	0.25–10.0	10–400
Pasteurised chilled soups	<i>B. cereus</i> , <i>C. pasteurianum</i>	2.5–6.25	100–200
Crumpets	<i>B. cereus</i>	4–6.25	150–250
Canned foods (high acid)	<i>C. botulinum</i> and <i>C. thermosaccharolyticum</i>	2.5–5.0	100–200
Ricotta cheese	<i>L. monocytogenes</i>	2.5–5.0	100–200
Continental type cooked sausage	Lactic acid bacteria, <i>Brochothrix thermosphacta</i> , <i>L. monocytogenes</i>	5–25	200–1000
Dipping sauces	Lactic acid bacteria	1.25–6.25	50–250
Salad dressings	Lactic acid bacteria	1.25–5	50–200
Beer: pitching yeast wash	Lactic acid bacteria, e.g. <i>Lactobacillus</i> , <i>Pediococcus</i>	25.0–37.5	1000–1500

Another bacteriocin which is commercially available is Pediocin PA-1, a crude fermentation product with the trade mark of Alta®, which is mostly used in meat products to extend shelf-life and inhibit pathogens (O'Connor *et al.*, 2006). The pediocin PA-1 is produced by *Pediococcus acidilactici* (Gonzalez and Kunka, 1987), and has anti-listerial properties. When used in combination with other treatments Alta has been found to inhibit the growth of *L. monocytogenes* and extend the shelf-life in frankfurters (Chen, *et al.*, 2004a), chicken (Rozum and Maurer, 1997), cheese (Glass *et al.*, 1995) and crab-meat (Degnan *et al.*, 1994).

Currently, there are investigations to exploit the food preservative properties of many other bacteriocins. These include the enterocins that have many properties which make them suitable candidates for use as safe, natural food preservatives. Most of the enterocins have anti-listerial properties, and have a wide spectrum of activity as compared with other bacteriocins such as nisin and pediocin.

Enterocin AS-48 is one of the most thoroughly studied enterocins, and has been studied as a food preservative. It is a broad spectrum bacteriocin active against most Gram-positive bacteria such as *S. aureus*, *B. cereus* and some Gram-negative pathogens, such as *Salmonella* and *E. coli* (Lucas *et al.*, 2006; Mendoza *et al.*, 1999). It has been found to inhibit the growth of *L. monocytogenes* and *S. aureus* in sausages (Ananou, *et al.*, 2005a, 2005b), *Bacillus cereus* in commercially available ready-to-eat vegetable soups and purees and rice-based foods (Grande *et al.*, 2007; Grande *et al.*, 2006a), *L. monocytogenes*, *B. cereus* and *S. aureus* in lettuce juice (Grande *et al.*, 2005b), rope-forming *B. licheniformis* in apple cider (Grande *et al.*, 2006b), *Alicyclobacillus* spp. in fruit juices (Grande *et al.*, 2005a) and *S. aureus* in dairy products such as skimmed milk and fresh cheese (Munoz *et al.*, 2007).

In addition to AS-48, several other enterocins have been tested as preservatives in food products. Enterocins A and B act synergistically and also have independent antimicrobial activity. When applied as anti-Listerial additives in dry fermented sausages, they significantly diminished *Listeria* counts by 1.13 log ($P < 0.001$). However, when the producer strain *E. faecium* CTC492 was added as a starter culture, no significant reduction in *Listeria* counts ($P > 0.1$) was found compared with the standard starter culture (Aymerich *et al.*, 2000). Enterocin 416K1 is produced by *E. casseliflavus* IM 416K1, and has been shown to significantly reduce *L. monocytogenes* counts in sausages when the bacteriocin is produced *in situ* by the live culture of the producer strain (Sabia *et al.*, 2003). Enterocin CRL35, produced by *E. faecium* CRL35, when added to goat cheese was found to decrease the pathogen population by nine log cycles by the end of ripening period with no change in the product quality (Farias *et al.*, 1999). Enterocin EJ97, produced by *E. faecalis* EJ97, when tested against strains of spore forming *Bacillus macroides*/*B. maroccanus* in vegetable puree was found to effectively decrease the populations of these bacteria. When the anti-microbial effect was tested at different pH values, the inhibitory effect was highest at neutral pH, but at lower or higher values, the spore forming strains did not grow noticeably and the inhibitory effect of enterocin EJ97 was also limited. The enterocin EJ97 activity was enhanced by the presence of sodium nitrite, sodium benzoate, sodium lactate and sodium tripolyphosphate in the puree (Garcia *et al.*, 2004).

Another approach in the application of bacteriocins as food preservatives is their coating on the surface of bioactive antimicrobial films. This approach has become popular for about a decade and is considered more appropriate than the direct addition of

bacteriocins to the food products for the reasons that bacteriocin is released gradually on the food surface and the negative effect of various food components on the bacteriocin activity is nullified. The effect of food components greatly increases the quantity of bacteriocin required to achieve the desirable shelf life. The bacteriocins that have been found to be effective as antimicrobial films include nisin to control *M. luteus* (Mauriello *et al.*, 2005), bacteriocin 32Y, produced by *L. curvatus*, to control *L. monocytogenes* (Ercolini *et al.*, 2004), pediocin PA-1 to control *L. innocua* (Quintero-Salazar *et al.*, 2005), enterocins A and B to control *L. monocytogenes* (Marcos *et al.*, 2007), enterocin 416K1 to control *L. monocytogenes* (Iseppi *et al.*, 2008).

It can be concluded from the above discussion that as more and more bacteriocins are being purified, characterized and tested as effective anti-microbials against a range of food pathogens, some of the promising ones may find their commercial utilization as safe and natural food preservatives, in a similar manner as nisin (Nisaplin) and pediocin (Alta 2341).

2.12 Objectives and contributions of the current study

This study focused on the production and characterization of the bacteriocin produced by a local isolate of *E. faecalis* termed as B9510. The main objectives of the study were:

- (i) Production of the bacteriocin in a defined medium and its purification in minimal steps
- (ii) Biochemical and genetic characterization of the bacteriocin
- (iii) Identification of the gene responsible for self-immunity to the producer strain.
- (iv) Studying the mode of action of the bacteriocin
- (v) Testing the efficacy of the bacteriocin as a food preservative

***Chapter 3* MATERIALS AND METHODS**

3.1 Bacterial strains and working cultures

The bacterial isolate used in the study for bacteriocin production was *E. faecalis* B9510, which was obtained from the Fonterra Research Centre in Palmerston North, New Zealand. The strain was stored and maintained at -70 °C in M17 broth as well as on M17 agar plates at 4 °C.

The other bacterial species used as indicator strains in this study are listed in Table 3.1 along with the source, growth conditions and media used for inoculation. Among the strains listed in Table 3.1 *L. lactis* ssp. *cremoris* 2144 was selected for routine antimicrobial activity assays due to its high sensitivity to the bacteriocin produced by *E. faecalis* B9510.

The working cultures of the bacterial species were prepared by streaking frozen bacterial culture onto appropriate media. Inoculated plates were incubated overnight and single colonies were taken from these plates and again inoculated and incubated to get the working cell cultures. These plates were stored at 4 °C during experimental trials, and were periodically refreshed during the entire research period.

In addition to streaking on M17 agar plates, the broth was also transferred to sterile vials (1.5 ml/vial). The culture vials were then stored at -70 °C. These culture vials were stored as a backup to the main culture vial, so that these could be used whenever required during the entire research period.

Table 3.1 Bacterial strains/isolates used in the current study

Bacterial strain	Source	Incubation temperature	Growth medium used
<i>Enterococcus faecalis</i> B9510	FRC*	37 °C	M17
<i>E. faecalis</i> No. 40	ML, MU	37 °C	M17
<i>Lactococcus lactis</i> ssp. <i>cremoris</i> 2144	FRC	30 °C	M17
<i>L. lactis</i> ssp. <i>lactis</i> F-1	FRC	30 °C	M17
<i>L. lactis</i> ssp. <i>lactis</i> RM-1	FRC	30 °C	M17
<i>L. lactis</i> ssp. <i>lactis</i> RM-5	FRC	30 °C	M17
<i>L. lactis</i> ssp. <i>lactis</i> K-30	FRC	30 °C	M17
<i>L. lactis</i> ssp. <i>lactis</i> MG1363 (plasmid free for cloning experiments)	FRC	30 °C	M17
<i>Pediococcus pentosaceus</i> 43200	ML, MU**	30 °C	MRS
<i>P. pentosaceus</i> 43201	ML, MU	30 °C	MRS
<i>Streptococcus thermophilus</i> ST 49	ML, MU	37 °C	M17
<i>S. thermophilus</i> V38	ML, MU	37 °C	M17
<i>Lactobacillus helveticus</i> 1109	ML, MU	37 °C	M17
<i>L. helveticus</i> 1113	ML, MU	37 °C	M17
<i>Lactobacillus casei</i> ML-1	ML, MU	37 °C	MRS
<i>Lactobacillus plantarum</i> ML-2	ML, MU	30 °C	M17
<i>Lactobacillus acidophilus</i> ML-3	ML, MU	37 °C	M17
<i>Lactobacillus delbrueckii</i> ssp. <i>bulgaricus</i> ATCC 11842	ATCC	37 °C	MRS
<i>Listeria monocytogenes</i> NCTC 10884	NCTC	30 °C	TS Agar/broth
<i>Bacillus cereus</i> ML-4	ML, MU	37 °C	TS Agar/broth
<i>Staphylococcus aureus</i> NCTC 4163	NCTC	37 °C	TS Agar/broth
<i>Proteus vulgaris</i> ML-5	ML, MU	37 °C	TS Agar/broth
<i>Staphylococcus epidermidis</i> ML-6	ML, MU	37 °C	TS Agar/broth
<i>Escherichia coli</i> 0157:H7	ML, MU	30 °C	TS Agar/broth
<i>E. coli</i> 0111	ML, MU	30 °C	TS Agar/broth
<i>E. coli</i> JM109 (plasmid free for cloning experiments)	Promega Corp., WI, USA	37 °C	LB broth
<i>Salmonella enteridis</i> ML-7	ML, MU	30 °C	TS Agar/broth

*Fonterra Research Centre, Palmerston North, New Zealand

**Microbiology Lab Collection, Massey University, Palmerston North, New Zealand

3.2 Growth media

The commercially available complex media used for the growth of LAB were M17 and MRS as indicated in Table 3.1. For the growth of non-LAB bacterial strains, tryptic soy agar/broth (TS Agar/Broth) was the most commonly used medium. In addition LB

(Luria-Bertani) medium was also used for the growth of *E. coli* strains especially during gene cloning experiments. All media were manufactured by Difco (BD Diagnostics Systems, Sparks, MD, USA).

3.3 Measurement of Optical Density (OD)

To monitor the growth of *E. faecalis* B9510, the optical density (OD) was measured from hourly samples at 620 nm using UV/Visible spectrophotometer (Ultrospec 2000 80-2106-00; Pharmacia Biotech, Cambridge, UK). Sterile culture media were used as blanks to subtract background value. Samples having an OD₆₂₀ of more than 0.7 were diluted with the sterile culture medium and the corrected OD value was obtained by multiplying by the dilution factor.

3.4 Assay for bacteriocin activity

The antimicrobial activity of the bacteriocin produced by *E. faecalis* B9510 was determined by two methods. The first method used was an Agar Well Diffusion Assay (AWDA) as described by (Yanagida *et al.*, 2005), with some modifications. The method involved inoculation of a 12 h culture of the appropriate indicator strain (Table 3.1) at a final concentration of 10⁴ cfu/ml to 50 ml of molten soft M17, MRS or TS agar (at 55 °C). The agar was then poured into a Petri dish and allowed to solidify. After solidification, 4 mm wells were drilled in the agar at regular intervals and each well was filled with 50 µl of the sample. The Petri dishes were then placed at 4 °C for two hours to allow diffusion of the samples in the agar. After incubation at 30 °C or 37 °C for 15 h, the plates were then observed for zones of inhibition around the wells.

The bacteriocin activity was quantified by a microtitre plate assay method adapted from Nissen-Meyer *et al.*, (1992). Serial two-fold dilutions of the sample (cell-free culture supernatant or concentrated bacteriocin) were prepared in sterile distilled water in a 96-well microtitre plate along with a control well with no bacteriocin. Each well was then inoculated with 2x10⁴ cfu/ml of the log phase culture of indicator strain in appropriate medium (Table 3.1) to give a final concentration of 1x10⁴ cfu/ml with a final volume of 100 µl in each well. The microtitre plate was then incubated at 30 or 37 °C for 12-15 hours. The inhibitory effect of the bacteriocin was then observed by measuring the OD₆₂₀ in a Spectro-star Nano (BMG Labtech, Ortenberg, Germany) microplate reader. The reciprocal of the highest dilution that inhibited at least 50% of the growth of indicator

strain as compared to that of control was taken as the critical dilution and was expressed as Arbitrary Units /ml (AU/ml).

3.5 Identification of the bacteriocin producing isolate

3.5.1 API kit identification

The bacteriocin-producing isolate used in this study was originally isolated from silage and obtained from the Fonterra Research Centre, Palmerston North, New Zealand. This isolate had been tentatively identified as *Enterococcus* spp. B9510. The isolate was initially characterized and identified to species level using an API rapid ID 32 Strep kit (bioMerieux SA, Marcy l'Etoile, France). The kit uses 32 miniaturized enzymatic tests in small cupules for the identification of streptococci and enterococci and related bacterial species. The kit was inoculated with a pure culture of the enterococcal isolate in accordance with the instructions of the manufacturer and incubation was done at 36 ± 2 °C for 4 hours.

The results obtained were converted into a numerical profile and the isolate was identified to the species level using the APIWeb™ identification software.

3.5.2 16S RNA gene amplification and sequencing

Further confirmation of the API based biochemical identification was done by amplification and sequencing of the 16S ribosomal RNA (16S rRNA) gene. The whole 1540 bp 16S RNA gene was amplified in two separate PCR reactions, so that the entire gene sequence could be determined. The primers were designed based on the 16S RNA sequence of the representative strains of *E. faecalis* and are listed in Appendix 1.

The DNA was extracted by resuspending a single colony in 50 µl of sterile (DNase free) water followed by boiling for 20 minutes. The PCR was done in a 50 µl volume containing PCR Master Mix (2X) from Fermentas (Vilnius, Lithuania) which was diluted to 1X, 2 µl of DNA template (from *E. faecalis* B9510) and 0.5 µM of forward and reverse primers. A control reaction mixture was also prepared which contained all the reagents except the template DNA. The reaction mixture was then vortexed for 15 seconds, and the reaction was carried out in a thermocycler (TECHNE TC-4000, Cambridge, UK), with these conditions: a 5 min denaturation at 94 °C, followed by 30 cycles of denaturation at 94 °C for 45 sec, annealing at 55 °C for 45 sec and polymerization at 72 °C for 1 min; this was followed by 5 min at 72 °C and a final cool down to 4 °C.

The product of the PCR reaction was run on a pre-cast 2% agarose gel (E-gel® Ex agarose gel from Invitrogen, Carlsbad, CA, USA) along with a DNA ladder (E-gel® 1kb plus DNA ladder). The gel was run for 10 minutes and then visualized under UV light, and the picture was saved for documentation.

For DNA sequencing, the PCR product was purified using DNA clean and concentrator™- 5 kit by Zymo Research (Irvine, CA, USA). Sequencing was performed with an ABI3730 DNA Analyzer using BigDye® Terminator v3.1 cycle sequencing kit.

3.6 Haemolytic activity and antibiotic resistance

In order to determine any potential pathogenic activity, *E. faecalis* B9510 was tested for haemolytic activity, by the method described by (Cariolato *et al.*, 2008). Fresh active culture of *E. faecalis* B9510 was streaked on Columbia Agar plates supplemented with 5% sheep blood. After incubation at 37 °C for 24 hours under aerobic conditions, plates were examined for haemolysis. A zone of clearing around the colonies was interpreted as β-haemolysis, a partial discoloration or greening as α haemolysis and no clearing as γ haemolysis. *S. aureus* NCTC 4163 was used as a positive control in this experiment.

To indicate any potential for antibiotic resistance in *E. faecalis* B9510, the Mastring STM (Mast Group, Liverpool, UK) test was used. This consisted of six antibiotic discs (10 µg ampicillin, 25 µg chloramphenicol, 1 unit of penicillin G, 10 µg streptomycin and 200 µg of sulphatriad).

In addition to the Mastring test, the *E. faecalis* B9510 strain was also tested for vancomycin susceptibility. Wells were drilled in the M17 agar (inoculated with a final concentration of approximately 1×10^4 cfu/ml from a 12 hours culture of *E. faecalis* B9510) and different concentrations (10 µg/ml, 30 µg/ml, 50 µg/ml and 100 µg/ml) of vancomycin were added to the wells and incubated at 37 °C for 10 hours, then observed for any zones of clearing indicating antibiotic sensitivity.

3.7 Effect of enzymes, heat and pH on bacteriocin activity

The cell-free supernatant from M17 broth was treated with these enzymes: trypsin, α-chymotrypsin, proteinase K, papain, β-glucosidase, and catalase. All the enzymes were supplied by Sigma-Aldrich Corporation (St Louis, MO, USA). Each enzyme was dissolved in 10 mmol/L sodium phosphate buffer (pH 7), and the solutions were added to

the cell-free supernatant of M17 broth to a final concentration of 1 mg/ml with a control preparation containing cell-free supernatant, buffer and no enzyme. All preparations were then incubated at 37 °C for 2 hours, and the residual activity was then determined.

To determine the effect of heat on bacteriocin activity, the cell-free supernatant from M17 broth having antimicrobial activity was heated at 50 °C, 55 °C, or 60 °C, for 15, 30, and 60 min in a Dri-block 08-3 (Techne, Cambridge, UK), then removed and immediately placed in ice. The residual activity was then determined for each treatment, including a control (no heat treatment), by AWDA as well as microtitre plate assay.

To evaluate the effect of pH on bacteriocin activity, the supernatant pH levels were adjusted between 2.0 and 10.0 using either 1M HCl or 1M NaOH, as required. The residual activity of all the treated samples was then determined by AWDA and microtitre plate assay.

3.8 Production and purification of bacteriocin

3.8.1 Chemically defined medium

In order to identify the bacteriocin, the bacteriocin producing isolate was grown in a chemically-defined medium with the aim to minimize purification steps. A Complete Defined Medium (CDM) adapted from the formulation given by Murray *et al.* (1993), as given in Table 1, was used as the starting point for the defined medium trials. The medium consisted of 39 components. All chemicals used for the preparation of defined medium were of analytical grade and obtained from either Sigma-Aldrich (St Louis, MO, USA) or BDH Chemicals Ltd. (Poole, UK). For all the experiments, aqueous stock solutions of the individual components were prepared in appropriate concentrations and were sterilized by autoclaving, with the exception of heat-sensitive components. The heat-sensitive amino acids (asparagine, glutamine and tryptophan), all the B vitamins and FeSO₄ were filter sterilized (pore size 0.22 µm membrane; Millipore Corp., Billerica, MA, USA). All the stock solutions were then stored at 4 °C, except FeSO₄ which was freshly prepared before each experiment as it is prone to oxidation during storage.

In addition to defined medium, modified GM17 with 15 g/L glucose was used as a reference for comparison with the defined medium.

Table 3.2 Composition of Completely Defined Medium (CDM) and Simplified Defined Medium (SDM)

Constituent	Concentration	
	CDM	SDM
Amino acids (g/L)		
L-Alanine	1	-
L-Arginine	1	1
L-Asparagine	1	-
L-Aspartic acid	1	-
L-Cysteine	1	1
L-Glutamic acid	1	1
L-Glutamine	1	-
Glycine	1	1
L-Histidine	1	1
L-Isoleucine	1	1
L-Leucine	1	1
L-Lysine	1	1
L-Methionine	1	1
L-Phenylalanine	1	1
L-Proline	1	-
L-Serine	1	1
L-Threonine	1	1
L-Tryptophan	1	1
L-Tyrosine	1	-
L-Valine	1	1
B vitamins (mg/L)		
Biotin	20	-
Cyanocobalamin	2	-
Ca-pantothenic acid	20	20
Folic acid	2	2
Nicotinic acid	2	2
Para-amino Benzoic acid	2	-
Pyridoxal	20	20
Riboflavin	2	2
Thiamine	10	-
Nucleic Acid Bases (mg/L)		
Uracil	20	20
Adenine.SO ₄	20	20
Guanine	20	-
Minerals (g/L)		
Dipotassium hydrogen phosphate	7	7
Potassium dihydrogen phosphate	2	2
Ammonium sulphate	1	-
Magnesium sulphate	0.1	0.1
Sodium citrate	0.5	-
Ferrous sulphate. 7H ₂ O	0.008	0.008
Carbon source (g/L)		
Glucose	15	15

3.8.2 Preparation of simplified defined medium

To determine the nutritional requirements of *E. faecalis* B9510, a series of media formulations were prepared by modifying CDM using the single omission/Leave One Out

(LOO) technique (Zhang *et al.*, 2009), with the original formulation (CDM) used as the control. To further confirm the effect of some amino acids and nucleic acid bases, some media formulations were prepared by multiple omission technique (Hebert *et al.*, 2004). The inoculum for these experiments was prepared by propagating *E. faecalis* B9510 in M17 broth at 37 °C for 15 hours. To eliminate carry-over nutrients, the cells were harvested by centrifugation (5856 ×g; 15 min), washed twice in sterile saline solution (8.5 g/L NaCl), and finally resuspended in sterilized distilled water to the original volume. This cell suspension was used to inoculate the different treatments of defined medium, with each treatment lacking in one component, except the control.

All growth studies were carried out in 25 ml sterilized screw cap bottles, each bottle containing 15 ml of modified CDM having an initial pH of 7.10 ± 0.1 . Each treatment was inoculated with the above mentioned inoculum at the rate of 5%, and incubation was then done at 37 °C for 7 hours (early stationary phase). These growth experiments were repeated three times.

Growth was monitored by measuring the optical density at 620 nm (OD_{620}) at regular intervals. The optical density values were then used to measure the growth rate (h^{-1}), as described by (Monod 1949). The following equation was used to measure the growth rate.

$$\mu = \frac{\ln OD_2 - \ln OD_1}{t_2 - t_1}$$

where μ is the growth rate per hour, \ln is the symbol for natural logarithm, OD_1 and OD_2 are the corrected optical density values (as described in section 3.3) measured during the exponential growth phase and t_1 and t_2 are the corresponding time points.

A component was considered essential if its absence resulted in 25% or less growth as compared with the control (CDM). Any component whose absence resulted in 26 – 80% of the growth rate of CDM was considered stimulatory, while the components that when absent resulted in more than 80% growth compared with the control were considered non-essential (Terrade and de Orduna, 2009).

3.8.3 Bioreactor experiments

After determination of the nutritional requirements and components required for good growth of *E. faecalis* B9510, a Simplified Defined Medium (SDM) was developed which included the essential and stimulatory components of the CDM (Table 3.2). This

SDM was then used for the growth and bacteriocin production experiments in a bioreactor and comparison with a complex medium (modified M17 containing 15 g/L glucose).

The growth and bacteriocin production of *E. faecalis* B9510 was carried out either in SDM or modified M17 broth using a 1 L MultiGen F-1000 bioreactor (New Brunswick Scientific Co. Inc., Edison, NJ, USA), with temperature, pH and agitation control. The temperature was monitored using a thermometer and was kept constant at 37 °C. For pH control, 5M sodium hydroxide was used to neutralize any decrease in pH during fermentation. The pH was measured and recorded by a pH electrode (Mettler Toledo, Greifensee, Switzerland) and pH controller (Model 5997-20, Horizon Ecology Company, Chicago, IL, USA), and sodium hydroxide was introduced via a Cole-Parmer (Chicago, IL, USA) pump and solid state Masterflex pump controller (Cole Parmer Instrument Co., Vernon Hills, IL, USA). The pH was not allowed to drop below 6.5. The batch was harvested after 24 hours of fermentation and processed for bacteriocin purification and characterization.

During the growth, samples were periodically taken after each hour to monitor the growth rate and bacteriocin production. Total viable counts were also determined from the hourly samples by spreading appropriate dilutions on M17 agar plates.

3.8.4 Purification of bacteriocin

The *E. faecalis* B9510 strain was allowed to grow in the SDM for 6-7 hours, the time required for maximum bacteriocin production as determined from initial trials. The cells were then removed by centrifugation (11,800 ×g, 25 min) in a Sigma 6-16 (St Louis, MO, USA) centrifuge. The cell-free supernatant was then filtered through a 0.22 µm filter (low-protein binding HVLP filter, Millipore Corp., Billerica, MA, USA) to remove any remaining cells. This cell-free supernatant was referred to as crude bacteriocin.

The crude bacteriocin was subjected to ultrafiltration using low-protein binding regenerated cellulose membrane, in Amicon centrifugal units (Millipore Corp, Billerica, MA, USA), having a Nominal Molecular Weight cut-off Limit (NMWCO) of 10 kDa. The retentate from this step was further subjected to ultrafiltration using a 30 kDa NMWCO ultrafiltration unit. The retentate from the 30 kDa unit was then subjected to SDS-PAGE.

3.9 Detection of bacteriocin in SDS-polyacrylamide gel

SDS-PAGE was carried out according to the method described by Walker (1996) with modifications as described below.

3.9.1 Preparation of reagents

- (a) Acrylamide stock solution

Acrylamide	30.0 g
Bis-acrylamide	0.8 g
Distilled water	upto 100 ml

- (b) Resolving gel buffer

1.5M Tris-HCl (pH 8.8)

- (c) Stacking gel buffer

0.5M Tris-HCl (pH 6.8)

- (d) Ammonium persulphate

10% solution (freshly prepared just before gel preparation)

- (e) N,N,N',N'-tetramethylethylenediamine (TEMED)

- (f) Sample buffer (2X)

0.5M Tris-HCl, pH 6.8	2.5 ml
10% SDS (w/v)	4.0 ml
Glycerol	2.0 ml
Bromophenol blue	2.0 mg

The Milli Q water was added to the buffer to a final volume of 10 ml.

- (g) Running Buffer (Tank buffer) 1X

Tris	3.0 g
Glycine	14.4 g
SDS	1.0 g

Milli Q water was added to a total volume of 1L. The pH of the buffer was 8.3.

- (h) Staining solution

Coomassie brilliant blue R250	0.1 g
Methanol	50.0 ml
Acetic acid	10.0 ml

Milli Q water was added to a final volume of 100ml.

- (i) Destaining solution

10% methanol and 7% glacial acetic acid in Milli Q water

3.9.2 Preparation of resolving gel

Before preparation of the resolving gel, the spacer plate and the short glass plate were assembled together on the gel casting stand (Bio-Rad Laboratories, Hercules, CA, USA) with a spacing of 0.75 mm. The resolving gel was then prepared as follows with a final acrylamide concentration of 13% in the gel.

Resolving gel buffer	1.25 ml
Acrylamide stock solution	2.15 ml
Milli Q water	1.60 ml
10% APS solution	25.00 μ l
TEMED	2.50 μ l

The above reagents were mixed together in a flask by swirling, with APS and TEMED added just before casting the gel. The mix was then carefully poured between the glass plates of the gel cassette with care being taken to avoid incorporation of any air bubbles in the gel. A small volume of water was then distributed over the surface of gel, and the gel was allowed to polymerize (approximately 30 min).

3.9.3 Preparation of stacking gel

The stacking gel was prepared as follows, with a final acrylamide concentration of 4% in the gel.

Stacking gel buffer	1.25 ml
Acrylamide stock solution	0.65 ml
Milli Q water	3.05 ml
10% APS solution	25.00 μ l
TEMED	5.00 μ l

The above reagents were mixed together in a flask by swirling, and approximately 20 μ l was used to wash the surface of polymerized resolving gel (after decantation of surface water). The stacking gel mixture was then carefully poured over the resolving gel trying to avoid any air bubbles. A 10-tooth comb was then inserted and the gel was allowed to polymerize for about an hour.

3.9.4 Preparation of the sample

The retentate from the 30 kDa ultrafiltration step was filtered through a 0.22 μ m filter (low-protein binding HVLP filter, Millipore Corp.). The retentate was then mixed

with sample buffer in a 1:1 ratio. The mix was thoroughly vortexed and no heating of the sample was done.

3.9.5 Preparation of molecular weight standards

Low range molecular weight standards (Bio-Rad Laboratories, Hercules, CA, USA) were used for estimation of molecular weight of the bacteriocin. The standards were prepared by diluting them in reducing sample buffer (prepared from stock sample buffer) in the ratio of 1:20. The composition of the stock sample buffer is as follows:

0.5M Tris-HCl (pH 6.8)	1.2 ml
Glycerol	1.0 ml
10% (w/v) SDS	2.0 ml
0.1% (w/v) Bromophenol blue	0.5 ml
Distilled water	4.8 ml

The stock sample buffer was divided into 475 μ l aliquots. For preparation of reducing sample buffer 25 μ l of β -merceptethanol was added to an aliquot of sample buffer just before use. The standard was mixed with this reducing sample buffer in 1:20 ratio, and the mixture was heated at 95 °C for five minutes and then cooled to room temperature.

3.9.6 Running the samples on the gel

After the polymerization of resolving and stacking gels, the comb was removed from the stacking gel and the gel cassette was assembled on the electrode assembly of the Bio-Rad Mini-PROTEAN® 3 electrophoresis cell (Bio-Rad Laboratories, Hercules, CA, USA). The assembly was then lowered into the Mini Tank of the cell. The inner chamber of the tank was filled with the running buffer. The outer chamber of the tank was then also filled with same running buffer.

Samples were then loaded in the wells (10-15 μ l), with the molecular weight standards being loaded in one of the wells. The gel was then run at 100 V constant voltage for about 1.5 – 2.0 hours until the dye front reached the bottom of the gel. The gel cassette was then removed from the assembly and stained as described below.

3.9.7 Staining and destaining of gel

After completion of the run the gel was carefully removed from the cassette, and soaked in coomassie blue staining solution for about 2 hours with gentle shaking. The staining solution was then replaced with destaining solution which was periodically replaced with fresh solution. Destaining was continued until the protein bands could easily be distinguished from the blue background.

3.9.8 Preparation of zymogram SDS-PAGE gel

To detect the lytic activity of the bacteriocin, a parallel zymogram SDS-PAGE gel was prepared and run in a similar manner as described above. The only difference was that 0.2% (w/v) autoclaved cells of sensitive strain *L. lactis* ssp. *cremoris* 2144 were incorporated in the resolving gel as described by Potvin *et al.* (1988). Furthermore, after completion of the electrophoresis run, the gel was not stained with Coomassie blue but was renatured according to the method described by Beukes *et al.* (2000). Briefly, the gel was initially soaked in distilled water followed by treatment with renaturation buffer (25 mM Tris-HCl [pH 7.5] containing 0.1% Triton X-100 and 10 mM MgCl₂) for 16 hours. Clear lytic bands were observed around the bacteriocin band against the opaque background of dead cells. The gel was compared with the Coomassie stained gel to locate the active band and determine its molecular mass.

3.10 Mass spectrometry

The mass spectrometry analysis was done by the Centre for Protein Research, University of Otago, Dunedin, New Zealand using MALDI TOF/TOF mass spectrometry.

For mass spectrometry the active band in the SDS-PAGE gel was excised and subjected to in-gel digestion with trypsin (Shevchenko *et al.*, 1996). Eluted peptides were then dried using a centrifugal concentrator. Peptides were resuspended in 30% [v/v] ACN (acetonitrile) and 0.1% [v/v] TFA (trifluoroacetic acid) in water. One μ l of peptide solution was premixed with 2 μ l of matrix (10 mg/ml alpha cyano-4-hydroxycinnamic acid (CHCA) dissolved in 65% [v/v] aqueous acetonitrile containing 0.1% [v/v] TFA and 10 mM ammonium dihydrogen phosphate). The sample/matrix mixtures (0.8 μ l) were spotted onto a MALDI sample plate (Opti-TOF 384 well plate, Applied Biosystems, MA, USA) and air dried.

Samples were analysed on a 4800 MALDI tandem Time-of-Flight Analyzer (MALDI TOF/TOF, Applied Biosystems, MA, USA). All MS spectra were acquired in a

positive-ion mode with 800-1000 laser pulses per sample spot. The 15 - 20 strongest precursor ions of each sample spot were selected for MS/MS collision-induced dissociation (CID) analysis. CID spectra were acquired with 2000-4000 laser pulses per selected precursor using the 2 kV mode and air as the collision gas at a pressure of 1*E-6 torr. The data acquired by the mass spectrometer were processed by the PROTEINPILOTTM 4.0 software (Applied Biosystems, MA, USA).

For protein identification, the MS/MS data were compared against the NCBI nr *E. faecalis* amino acid database (downloaded in May 2010) using the MASCOT v 2.3 search engine (<http://www.matrixscience.com>). The search was set up for full tryptic peptides with a maximum of three missed cleavage sites. Carboxyamidomethyl cysteine, oxidized methionine and pyroglutamate (E, Q) were included as variable modifications. The precursor mass tolerance threshold was 75 ppm and the maximum fragment mass error was 0.4 Da.

The protein(s) identified from the database were given a score, which was the sum of the ions score for each unique peptide sequence from the MS/MS data. The ions score in turn was $-10 \cdot \log(P)$, where P is the probability that the observed match is a random event. Individual ions scores of greater than 60 indicated identity or extensive homology ($P < 0.05$).

3.11 Amplification of bacteriocin gene by PCR

To confirm the result of mass spectrometry a PCR experiment was set up and the following primers were designed based on NCBI database and published sequence (Nilsen *et al.*, 2003).

Enl. A (Forward): 5'- CGATTTCTGTTGTAGAACC - 3'

Enl. A (Reverse): 5'- GTACATCTCCATATACTTTCC - 3'

The primers were obtained from Invitrogen (Carlsbad, CA, USA), and were expected to amplify a 1770 bp region. The PCR was done in a 50 µl volume containing 25 µl PCR Master Mix (2X) from Fermentas (Vilnius, Lithuania), 2 µl of DNA template (from *E. faecalis* B9510) and 0.5 µM of forward and reverse primers. A control reaction mixture was also prepared which contained all the reagents except the template DNA. The reaction mixture was then vortexed for 15 seconds, and the reaction was carried out in a thermocycler (TECHNE TC-4000, Cambridge, USA), with these conditions: a 5 min

denaturation at 94 °C, followed by 30 cycles of denaturation at 94 °C for 45 sec, annealing at 55 °C for 45 sec and polymerization at 72 °C for 1 min; this was followed by 5 min at 72 °C and a final cool down to 4 °C.

The product of the PCR reaction was run on a pre-cast 2% agarose gel (E-gel® Ex agarose gel from Invitrogen (Carlsbad, CA, USA) along with a DNA ladder (E-gel® 1kb plus DNA ladder). The gel was run for 10 minutes and then visualized under UV light, and the picture was saved for documentation.

For DNA sequencing, internal primers were designed to divide the 1770 bp region into three short sequences as DNA sequencing method used cannot read the entire 1770 bp region in a single reaction. The primers and the product lengths are given in Table 3.3.

Table 3.3 Primers used for amplification of short sequences of 1770 bp enterolysin A gene for DNA sequencing

Primer name	Sequence	Product size (bp)	Region amplified (bp)
Enl. A1 F	5'- CGATTTCTGTTGTAGGAACC -3'	600 bp	1-600
Enl. A1 R	5'- ACCTACAGGATCCCAACCAGCA -3'		
Enl. A2 F	5'- GCAAGCGATCCAACGGTTCTCC -3'	737 bp	598-1335
Enl. A2 R	5'- TGCTGGTTGGGATCCTGTAGGT -3'		
Enl. A3 F	5'- GGAGAACCGTTGGATCGCTTGC -3'	477 bp	1294-1770
Enl. A3 R	5'- GTACATCTCCATATACTTTTCC -3'		

The PCR products of the above reactions were purified using DNA clean and concentrator TM- 5 kit by Zymo Research Corporation (Irvine, California, USA). Sequencing was performed with an ABI3730 DNA Analyzer using BigDye® Terminator v3.1 cycle sequencing kit. The DNA sequencing facility was provided by the Allan Wilson Centre Genome Service at Massey University, Palmerston North, New Zealand.

3.12 Cloning of putative immunity genes

3.12.1 PCR amplification of putative immunity genes

Since the immunity genes for bacteriocins lie in close proximity to the structural gene, therefore, the neighbouring genes of enterolysin A structural gene (both upstream and downstream) were suspected as putative immunity genes. The sequences of the

3. Materials and methods

neighbouring genes of enterolysin A were obtained from the NCBI database (www.ncbi.nlm.nih.gov) using the enterolysin A structural gene as a query. Among the results of the query a sequence of 5156 bp from *E. faecalis* BGPT1-10P showed three open reading frames lying upstream and downstream of the enterolysin A structural gene (Genbank No. HE585879). The open reading frames and the whole sequence are shown in Appendix 3. The schematic arrangement of these open reading frames is shown in Figure 3.1, and the primers designed based on these open reading frames are listed in Table 3.4.

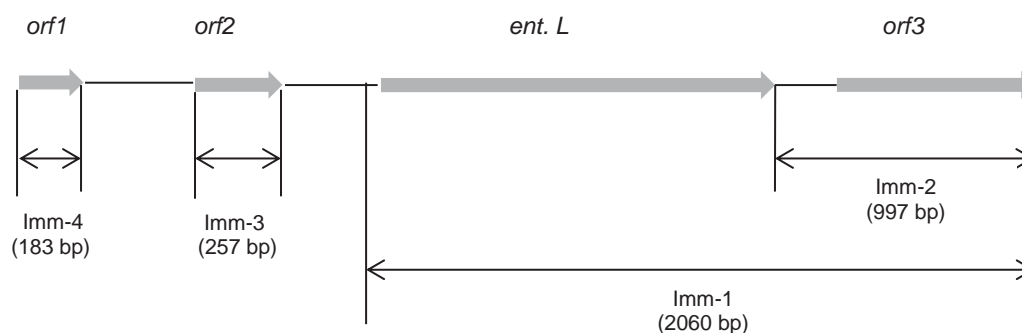


Figure 3.1 Schematic arrangement of Enterolysin A structural gene (*ent. L*) and the neighbouring ORFs. Primers were designed to amplify the regions named as Imm-1, Imm-2, Imm-3 and Imm-4.

Table 3.4 Primers used for the amplification of putative immunity genes

Primer name	Sequence	Restriction site	Product size (bp)
Imm-1 F	5'- GAGCGAATTCTCTTAATTAGGAGGGGTTTCT-3'	Eco RI	2060
Imm-1 R	5'- TGCGAAGCTTTTAAATCTCTTAATTGTTTTA-3'	Hind III	
Imm-2 F	5'-GAGCGAATTCATGACAGTAGGATTATGCATT-3'	Eco RI	997
Imm-2 R	5'-TGCGAAGCTTTTAAATCTCTTAATTGTTTTA-3'	Hind III	
Imm-3 F	5'-GAGCGAATTCATGCTTTTAAAGGTCTTTTGC-3'	Eco RI	257
Imm-3 R	5'-TGCGAAGCTTTTATTTTTTTTATTTTACTAACA-3'	Hind III	
Imm-4 F	5'-GAGCGAATTCATGGAAATTACTTTTTCTAA-3'	Eco RI	183
Imm-4 R	5'-TGCGAAGCTTTCAATCGTTTTCAAATTCTCT-3'	Hind III	

The primers were purchased from Invitrogen (Carlsbad, CA, USA), and the above mentioned four regions were amplified in separate PCR experiments. The PCR was done

in a 100 µl volume containing 50 µl PCR Master Mix (2X) from Fermentas (Vilnius, Lithuania), 2 µl of DNA template (from *E. faecalis* B9510) and 0.5 µM of forward and reverse primers. A control reaction mixture was also prepared which contained all the reagents except the template DNA. The reaction mixture was then vortexed for 15 seconds, and the PCR reactions for amplification of the four genes were carried out in a thermocycler (TECHNE TC-4000, Cambridge, UK), with these conditions: a 5 min denaturation at 94 °C, followed by 30 cycles of denaturation at 94 °C for 45 sec, annealing at 48 °C for 45 sec and polymerization at 72 °C for 1 min; this was followed by 5 min at 72 °C and a final cool down to 4 °C.

The products of the PCR reactions were run on a 1.5% agarose gel along with a DNA ladder prepared by digestion of lambda DNA with *Pst*I restriction enzyme. The gel was run for about 4 hours and then visualized under UV light, and the picture was saved for documentation.

The PCR products for all the above regions were purified using DNA Clean and Concentrator™-5 Kit (Zymo Research Corporation, Irvine, California, USA). These four putative immunity regions were then used for insertion into a pFX-3 plasmid after digestion with restriction enzymes.

3.12.2 Extraction of plasmid DNA

A pFX3 expression vector (Xu *et al.*, 1991) was selected for cloning the putative immunity genes. The shuttle vector pFX3 was selected because it enables direct screening of recombinant plasmids in *E. coli* by inactivation of the *LacZ* function. Furthermore, it has been found to be stably maintained in the Gram-positive bacterium *L. lactis* ssp. *lactis* and the T3 and T7 promoters flanking the multiple cloning region facilitate direct transcriptional studies (Xu *et al.*, 1991). The *E. coli* JM109 strain carrying the vector was grown in LB medium supplemented with 25 µg/ml chloramphenicol. Extraction of the vector was then done according to the alkaline lysis method described by (Birnboim and Doly 1979). The method involves resuspending the overnight culture in a protoplasting solution (2 mg/ml lysozyme, 50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl (pH 8.0) followed by treatment with alkaline SDS solution (0.2 N NaOH + 1% SDS). Finally the extracted DNA was treated with high salt solution of 3M sodium acetate (pH 4.8) followed by treatment with ice-cold 70% ethanol solution to precipitate the plasmid DNA, which was resuspended in TE buffer.

3.12.3 DNA digestion with restriction endonucleases

The sequence specific digestion of vector DNA and putative immunity genes was carried out independently using *EcoRI* and *HindIII* restriction enzymes (Roche, Germany) in a single reaction. Each enzyme was added at a concentration of 10-20 U to 1 µg of DNA along with 1x Sure Cut buffer B in the reaction mix. Incubation was then done at 37 °C for 1-4 hours followed by denaturation of the enzymes by heating the reaction mixture at 65 °C for 15 minutes. The vector DNA as well as the putative immunity genes (digested in separate reactions) were then purified from the reaction mix using a DNA Clean and ConcentratorTM-5 Kit (Zymo Research Corporation, Irvine, California, USA).

3.12.4 Ligation of DNA

After treatment with restriction enzymes and purification, the vector DNA and putative immunity genes (amplified by PCR reaction) were ligated using T4 DNA ligase (Roche, Germany) in separate reaction mixtures. For each ligation mix the insert to vector ratio was 1:5. The T4 DNA ligase was added at a concentration of 2U along with 1X DNA ligase buffer (3 µl in 30 µl volume). The ligation mixtures were then incubated at 10 °C for 12 hours and then purified by using DNA Clean and ConcentratorTM-5 Kit (Zymo Research Corporation, Irvine, California, USA). The purified products were used to transform *E. coli* JM109 strain by electroporation.

3.12.5 Preparation of electrocompetent cells of *E. coli* JM109

The method of Sheng *et al.* (1995) was followed to prepare electrocompetent *E. coli* cells. An overnight culture of *E. coli* JM109 was prepared by inoculating a single colony in LB broth followed by incubation at 37 °C with vigorous shaking. A 4 ml aliquot of the overnight culture was then added to 400 ml of fresh LB medium in a 1 L flask and incubation was done in a shaking incubator. The cells were allowed to grow until an OD₆₀₀ of 0.3 – 0.6 was reached. The cells were then harvested by centrifugation at 4 °C and were resuspended in chilled 10% glycerol. The cells were washed twice with 10% glycerol and then finally resuspended in 2 ml of wash solution. This suspension was then divided into 80 µl aliquots and stored at -80 °C.

3.12.6 Transformation of *E. coli* JM109 cells

An 80 µl aliquot of *E. coli* JM109 cells was mixed with 2 µl of recombinant DNA vector prepared by ligation (pFX3+insert) and transferred to an ice-cold cuvette. The mixture was kept on ice for two minutes and then electroporation was done in a Micro-pulser apparatus (Bio-Rad Laboratories, Hercules, CA, USA) set for bacteria. After the pulse, 800 µl of SOC medium at room temperature was immediately added to the cuvette and the cell suspension was transferred to a sterile 1.5 ml eppendorf tube which was then placed on ice, which was then incubated with shaking at 37 °C for 1 hour. Appropriate dilutions of the cell suspension were then prepared and 100 µl of each dilution was then spread on LB agar plates containing 30 µg/ml chloramphenicol and 40 µg/ml X-gal and 1mM isopropylthio-β-D-galactoside (IPTG) for blue/white screening.

All the four putative immunity genes were electroporated independently in a separate batch of *E. coli* JM109 cells, along with a control treatment of unligated pFX3 vector being transformed into *E. coli* JM109.

3.12.7 Selection of colonies with insert gene

The inoculated electroporated cells, were incubated at 37 °C for 15 hours. The plates were then stored at 4 °C for several hours to allow the blue colour to develop in the colonies containing non-ligated vector. Only the white colonies were selected for further inoculation into LB broth, as white colour was the positive indication of insertion of the foreign gene in the PFX3 vector. The broth was incubated at 37 °C for 15 hours. The recombinant plasmid was then extracted as described above, and confirmed for the presence of immunity gene by doing a PCR experiment. This plasmid preparation was then used for transformation of *L. lactis* ssp. *lactis* MG1363 strain.

3.12.8 Preparation of competent cells of *L. lactis* ssp. *lactis* MG1363

The method of Kaneko *et al.* (2000) was used to prepare electrocompetent cells. Overnight culture of *L. lactis* ssp. *lactis* MG 1363 was used to inoculate 50 ml fresh M17 medium supplemented with 1% glycine to obtain an OD₆₀₀ of about 0.05. The cells were grown at 30 °C for 4 hours until an OD of 0.3 - 0.6 was reached and then harvested by centrifugation at 2700 ×g. The cells after washing three times with sterile distilled water were resuspended in 0.5 ml of 30% PEG1500. This suspension was then divided into 60 µl aliquots and stored at -80 °C.

3.12.9 Transformation of *L. lactis* ssp. *lactis* MG1363

A 60 μ l aliquot of the lactococcal cell suspension was mixed with 1-2 μ l (less than 0.1 μ g) recombinant DNA plasmid (obtained from *E. coli* JM 109 cells) and transferred to an ice-cold electroporation cuvette (from Bio-Rad Laboratories, Hercules, CA, USA) with 0.2 cm inter-electrode distance. Electroporation was then done in a Micro-pulser electroporation apparatus (Bio-Rad Laboratories, Hercules, CA, USA) set for bacteria. The cuvette was immediately placed in ice after electroporation and 500 μ l M17 expression broth (GM17+0.5M sucrose) was added to the cuvette and incubation was done at 30 °C for two hours. Appropriate dilutions of the cell suspension were then prepared and 100 μ l of each dilution was then plated on M17 agar containing 5 μ g/ml chloramphenicol.

A control treatment was also prepared in a similar manner except that a non-recombined plasmid vector was added to the cells instead of the recombinant plasmid.

Selected colonies from the M17 chloramphenicol agar were independently grown in M17 broth with chloramphenicol, and tested for the presence of the putative immunity gene by PCR. The culture which was positive for the presence of the immunity gene was then tested against the bacteriocin produced by *E. faecalis* B9510 by agar well diffusion assay to confirm the development of immunity against the bacteriocin.

3.13 Mode of action studies

3.13.1 Lytic activity and Transmission Electron Microscopy (TEM)

3.13.1.1 Determination of lytic activity

An overnight culture of *L. lactis* ssp. *cremoris* 2144 was used to inoculate fresh M17 broth. Incubation was then done for about 4 hours to an OD₆₂₀ of 0.5-0.6. The log phase cells were then harvested by centrifugation and resuspended in 10 mM phosphate buffer (pH 7). The suspension was then divided into aliquots and crude enterolysin A (retentate of 30 kDa ultrafiltration step) was added to each aliquot at a final concentration of approximately 5000 AU/ml. A suspension of cells without any added enterolysin A was used as control. The samples were then incubated at 30 °C and OD₆₂₀ was measured at regular intervals. In addition to measurement of optical density, samples were also obtained from the aliquots for TEM. For this purpose the sample obtained was centrifuged to collect the cell pellet after removal of buffer. The cell pellets thus obtained after different intervals of treatment with enterolysin were then processed as follows for

visualization under TEM. Cell pellet was also obtained from the control sample for comparison.

3.13.1.2 Preparation of samples for TEM

The cell pellets collected at different intervals of enterolysin A treatment were sent to Manawatu Microscopy and Imaging Centre (MMIC), Massey University, Palmerston North, New Zealand for sample preparation and viewing under transmission electron microscope. The samples for TEM were prepared as follows.

The cell pellets collected at different intervals of enterolysin A treatment were resuspended in primary fixative (3% glutaraldehyde + 2% formaldehyde in 0.1 M phosphate buffer pH 7), and fixed for two hours. The suspension was then centrifuged for five minutes and the supernatant was discarded. A few drops of 20% Bovine Serum Albumin (BSA) were then added to the pellet and mixed well. A drop of 25% glutaraldehyde was then added to the top of the pellet to coagulate BSA. The pellet was then removed from the eppendorf tube and sliced into thin pieces. The thin pieces were then again treated with primary fixative as described above to ensure complete coagulation of BSA. The sliced pieces of pellet were then washed with 0.1M phosphate buffer (pH 7.2) three times, and postfixed for 1 hour with 1% osmium tetroxide. The sliced pellet was then dehydrated in graded acetone series ranging from 25% to 100% acetone. Finally the pellet was embedded in resin and sectioned.

3.13.1.3 Preparation and viewing of ultrathin sections

Ultrathin sections were cut with a diamond knife using Leica Ultracut-R microtome (Leica, Vienna, Austria), and were mounted on copper grids. The samples were then stained for 20 minutes with 2% uranyl acetate (in 70% methanol). The grids were then washed with milli Q water and dried on a filter paper. Samples were then viewed at various magnifications with a Philips CM10 transmission electron microscope (Royal Philips Electronics, the Netherlands), and selected pictures of the untreated control and enterolysin A-treated samples were saved for record.

3.13.2 Determination of cleavage site of enterolysin A within the cell wall

For determination of cleavage site of enterolysin A within the peptidoglycan moiety of cell walls of selected sensitive strains method of Ghuysen *et al.* (1966) was followed with some modifications as detailed below.

3.13.2.1 Preparation of purified cell walls of sensitive strains

Cell walls from *L. lactis* ssp. *cremoris* 2144, *P. pentosaceus* and *L. bulgaricus* ATCC 11842 were prepared from overnight cultures grown in M17 and MRS broth respectively. The media were centrifuged at 8000 $\times g$ to collect the cells which were washed twice with 20 mM phosphate buffer (pH 7.0). The cell wall associated proteins from these cells were then extracted as described by Sahl *et al.*, (1985) with some modifications. Approximately 1 g of the cells were suspended in 10 ml of 10 mM phosphate buffer (pH 7) and were lysed by ultrasonication using a Misonix® (Farmingdale, NY, USA) 3000 sonicator. The cell suspension was kept on ice throughout sonication period to prevent heating of the sample. Sonication was done at 20 kHz with a power of 40 W for 20 minutes for each sample. After sonication the cell suspension was heated at 95 °C for 15 minutes which was then centrifuged at 2000 $\times g$ for 15 minutes to remove unbroken cells. The clear supernatant was then subjected to a second centrifugation at 20000 $\times g$ for 30 minutes. This step yielded a cell wall containing pellet, that was suspended in 5 ml of 50 mM phosphate buffer (pH 7), and 1 mg each of DNase (Sigma, St. Louis, MO, USA) and RNAase (Sigma, St Louis, MO, USA) were added. The incubation was then done at 37 °C for four hours. After four hours the buffer was removed by centrifugation at 20000 $\times g$ and the cell wall was resuspended in 5 ml of 50 mM phosphate buffer (pH 7.8), 1 mg trypsin (Sigma, St Louis, MO, USA) was then added and incubation was done at 37 °C for 16 hours. The trypsin containing buffer was then removed by centrifugation at 20000 $\times g$ for 30 minutes. The cell walls were then washed twice with distilled water and freeze dried.

3.13.2.2 Digestion of cell walls with enterolysin A

Purified *L. lactis* ssp. *cremoris* 2144, *Pediococcus pentosaceus* 43201 and *L. bulgaricus* ATCC 11842 cell walls (0.3 mg) were resuspended in 10 mM phosphate buffer (pH 7.0) to an OD₆₂₀ of about 0.5. Crude enterolysin A preparation (retentate of 30 kDa ultrafiltration step) was then added at a final concentration of about 160 AU/ml and incubation was done at 37 °C for 12 hours. Undigested cell walls were then harvested at 16,000 $\times g$ for 10 minutes, and the supernatant was lyophilized.

3.13.2.3 Derivatization of liberated N-terminal amino acids

Lyophilized samples of the enterolysin A digested supernatant were resuspended in 100 μ l of 1% potassium tetraborate (K₂B₇O₄) and 10 μ l of fluorodinitrobenzene (Sigma

Aldrich (St Louis, MO, USA) reagent was added (the reagent was prepared by dissolving 130 μ l of fluorodinitrobenzene to 10 ml of 100% ethanol). The mixture was then incubated at 60 °C for 30 minutes. After acidification with concentrated HCl (50 μ l), the N-dinitrophenyl (DNP) derivatives of free amino acids were extracted three times with ether (100 μ l). All of the free amino acids are not liberated at this stage, therefore, the residual ether in the aqueous phase was evaporated at 60 °C which was then hydrolysed for 6 hours at 95 °C. The remaining N-terminal amino acids were then extracted three times with ether. The ether was then evaporated at 37 °C with gentle agitation. The samples were then dried under vacuum, and redissolved in 0.05 M NH₃. The derivatized lysine still remaining in the aqueous phase was then extracted with water saturated n-butanol which was evaporated under vacuum, and the samples were redissolved in 0.05 M NH₃.

In addition to the samples, analytic reagent grade alanine, aspartic acid, glutamic acid and lysine (the amino acids present in the cell walls of LAB) were also derivatized for N-terminal to be run along with the samples (as reference standards) on thin layer chromatography plates.

3.13.2.4 Derivatization of liberated C terminal amino acids

The lyophilized samples (digested with enterolysin A) were resuspended in 40 μ l hydrazine and incubated for 16 hours at 60 °C. The excess reagent was dried under vacuum and the residue was resuspended in 100 μ l of water. Benzaldehyde (20 μ l) was then added to the samples and the tubes were thoroughly mixed for 10 seconds after every five minutes for a period of one hour. An aliquot (90 μ l) of the supernatant was then removed from the tubes, 20 μ l of benzaldehyde was added to it and thoroughly mixed for 1 hour as described above. An 80 μ l aliquot of supernatant was then removed and the derivatized amino acids were then extracted with 100 μ l ether twice. The ether was evaporated under vacuum, and the samples were redissolved in 0.05M NH₃.

In addition to the samples, analytic reagent grade alanine, aspartic acid, glutamic acid and lysine (the amino acids present in the cell walls of LAB) were also derivatized for C-terminal to be run along with the samples (as reference standards) on thin layer chromatography plates.

3.13.2.5 Thin layer chromatography of derivatized samples

The N-terminal and C-terminal derivatized amino acids were detected by Thin Layer Chromatography (TLC). The samples were spotted on 20×20 cm aluminium sheets coated with silica gel (Merck, Darmstadt, Germany) using a micropipette, and the standard amino acids of the cell walls of LAB (derivatized in a similar manner) were also spotted. The plates were then developed with isopropanol (containing 0.1% trifluoroacetic acid) at room temperature.

The TLC sheets were then dried in air and the liberated N- and C-terminal amino acids from the enterolysin A-digested cell walls were determined by comparison with the standard amino acids run alongside the samples. The sample spots were visualized by viewing under UV light (short wavelength).

3.14 Coating of enterolysin A on polyethylene film

Crude enterolysin A (ultrafiltered retentate of 30 kDa ultrafiltration step) was coated on Low Density Polyethylene Film (LDPE) as described by Franklin *et al.* (2004), with some modifications as detailed below.

3.14.1 Preparation of coating mixture

The antimicrobial coating was prepared by adding 3.5 g of methyl cellulose (Sigma-Aldrich, St Louis, MO, USA) to 100 ml of crude enterolysin A. the mixture was then homogenized for about two minutes at 16000 rpm in a Sorvall Omni Mixer 17105 (Ivan Sorvall Inc., Conn., USA). Ethanol (20 ml) and 3 ml of Polyethylene Glycol 400 (Sigma-Aldrich, St Louis, MO, USA) were also added to the mix and homogenization was again done for further two minutes. A control coating mix was also prepared in a similar manner except that Milli Q water was added to the coating mix instead of crude enterolysin A.

3.14.2 Coating on polyethylene film

A manual thin layer chromatography (TLC) plate coater (Camag, Muttenz, Switzerland) was used for coating Low Density Polyethylene Film (LDPE) with antimicrobial coating mixture. The LDPE film was cut and securely fixed on glass plates (20x20 cm) using a cellophane tape. The plate was then placed in the TLC plate coater and the antimicrobial mixture was then casted on the plate at a thickness of 1 mm. The

control mix was also casted in a similar manner. The plates were then allowed to dry at 37 °C for about 2 hours. After 2 hours the LDPE film was detached from the glass plate. The film was assayed for its antimicrobial activity as described below.

3.14.3 Antimicrobial activity assay of Enterolysin A film

A 2×2 cm (approximately) piece of enterolysin A film was placed on the surface of M17 soft agar (0.8% agar) previously inoculated with 10⁴ cfu/ml of *Lactobacillus casei* ML-1 indicator strain (as described for agar well diffusion assay). The treated face of the film was in contact with agar. The control film was also tested similarly. The plates were then incubated at 30 °C for 15 hours and observed for presence or absence of zones of inhibition around the films in a lawn of bacterial cells.

Chapter 4 **EXPERIMENTAL RESULTS**

4.1 Identification of the enterococcal isolate to the species level

The current project was aimed at identification of potential novel bacteriocin(s) from selected bacterial isolates belonging to *Enterococcus* spp., *Lactococcus* spp. and *Lactobacillus* spp. Enterococcal isolate B9510 was selected from many isolates screened for bacteriocin like activity, for further research and detailed investigations due to its large zones of inhibition in the lawns of sensitive bacterial species and antimicrobial activity against a broad range of Gram-positive bacteria which included lactococci, lactobacilli and pediococci (Graham Davey, Fonterra Research Centre, Palmerston North, New Zealand).

The Enterococcal isolate B9510 was originally obtained from silage and was preliminarily identified as belonging to *Enterococcus* spp. Therefore, the first step in the investigation was the identification of the isolate to the species level. This was achieved initially by the use of the API rapid ID 32 Strep kit. This kit has been designed for the rapid identification of streptococci and enterococci by using 32 miniaturized enzymatic biochemical tests. The results of the biochemical tests are given in Appendix 2. These results were manually entered in the APIweb™ identification software and a numerical profile was generated which was 70731515331. This numerical profile was used by the software to identify the species. The species identified by the software was *E. faecalis* with 99.9% confidence given by the API software.

The result of the biochemical identification was further confirmed by amplification of the 16S rRNA gene. To amplify the gene, primers were designed based on the representative strains of *E. faecalis* (*E. faecalis* V583 and *E. faecalis* OG1RF). A 1572 bp region was amplified and was used as a query for a nucleotide BLAST (Altschul *et al.*, 1990) search at the NCBI website (<http://www.ncbi.nlm.nih.gov/>). The results (Table 4.1) show >99% sequence similarity with several *E. faecalis* strains. Since there is universal agreement that >98.5 sequence homology identifies the isolate/strain to the rank of species (Janda and Abbott 2007), therefore, it can be reliably concluded that B9510 is a strain of *Enterococcus faecalis*.

Table 4.1 Nucleotide BLAST of 16S rRNA gene sequence of *E. faecalis* B9510

Accession Number	Strain	Query coverage	E value	Maximum identification
CP002621.1	<i>E. faecalis</i> OG1RF, complete genome	99%	0.0	100%
CP002491.1	<i>E. faecalis</i> 62, complete genome	99%	0.0	99%
AB530697.1	<i>E. faecalis</i> gene for 16S rRNA, partial sequence, strain: LPS18	98%	0.0	100%
AE016830.1	<i>E. faecalis</i> V583, complete genome	99%	0.0	99%
AY692453.1	<i>E. faecalis</i> strain SL5 16S ribosomal RNA gene, complete sequence	99%	0.0	99%

4.2 Haemolytic activity and antibiotic resistance

E. faecalis B9510 was tested for haemolytic activity using sheep blood agar plates, and was found to be non-haemolytic, in contrast to a positive control (*S. aureus*) which clearly showed zones of clearance around the colonies thus showing β -haemolysis.

The results of sensitivity against selected antibiotics are given in Table 4.2 which shows that *E. faecalis* B9510 is sensitive to ampicillin, vancomycin, tetracycline and chloramphenicol.

Table 4.2 Antibiotic resistance/sensitivity of *E. faecalis* B9510 against selected antibiotics

Antibiotic (μg)	Zone of inhibition (mm)*
Cell wall synthesis inhibitors	
Ampicillin (10)	21.75 \pm 0.5
Vancomycin (10)	13.00 \pm 0.9
Penicillin G (1 unit)	Nil
Protein synthesis inhibitors	
Tetracycline (25)	29.30 \pm 1.5
Chloramphenicol (25)	22.80 \pm 0.6
Streptomycin (25)	Nil

*Values are means \pm SD (standard deviation) of three observations

4.3 Effect of enzymes, heat and pH on bacteriocin activity

Lactococcus lactis ssp *cremoris* 2144 was chosen as the indicator strains to test the effect of enzymes and heat on bacteriocin activity and effect of pH on bacteriocin stability. Enzyme sensitivity assays demonstrated that the antimicrobial activity exhibited by *E. faecalis* B9510 was completely destroyed by treatment with trypsin, α -chymotrypsin and proteinase K, while no change in activity was observed after treatment with catalase or β -galactosidase. These results clearly indicate the proteinaceous nature of the antimicrobial substance produced by *E. faecalis* B9510 and give a strong indication that it is a bacteriocin.

The pH stability studies on the bacteriocin revealed that the antimicrobial activity was lost at pH values of 3 or below, which was partially regained at pH 4, while maximum stability was observed between pH 5 to 9.

Table 4.3 clearly highlights the heat sensitivity of the bacteriocin which loses its antimicrobial activity when heated at 60 °C for one hour.

Table 4.3 Effect of heat treatment on the bacteriocin activity (against *L. lactis* ssp. *cremoris*) in the cell-free supernatant of M17 broth

Heat treatment		Residual activity (AU/ml)
Control (no heat treatment)		640
55 °C	15 min	320
	30 min	320
	60 min	80
60 °C	15 min	40
	30 min	40
	60 min	Nil
65 °C	15 min	Nil
	30 min	Nil
	60 min	Nil

4.4 Bacteriocin production in defined medium

The effect of proteolytic enzymes and heat on the antimicrobial substance produced by *E. faecalis* indicated that the antimicrobial is a heat-labile bacteriocin. The next step after these preliminary tests was to characterize and identify the bacteriocin to the amino acid level. For this purpose the bacteriocin producing isolate *E. faecalis* B9510 was grown in a chemically defined medium devoid of any peptides and proteins with a view to minimize the number of purification steps. A Simplified Defined Medium (SDM) was developed from a Complete Defined Medium (CDM).

4.4.1 Effect of omitting amino acids on the growth of *E. faecalis* B9510

According to the Leave One Out (LOO) technique each amino acid was sequentially omitted in the series of formulations prepared, along with a control (CDM). The results (Table 4.4) indicate that eight amino acids viz. arginine, glycine, histidine, isoleucine, leucine, methionine, tryptophan and valine were essential for the growth of *E. faecalis* B9510, whereas cysteine, phenylalanine, serine and threonine were found stimulatory for growth. Their absence in the medium did not stop the growth completely, but caused an appreciable decrease in the growth. Similarly the absence of lysine in the medium slowed the growth rate resulting in a long lag phase (5 hours), and, therefore, was considered stimulatory for good growth.

Table 4.4 Effect of single omission of amino acids on the growth of *E. faecalis* B9510 and bacteriocin production in the defined medium

Omitted amino acid	**Growth rate (h ⁻¹)	*Relative growth rate	**OD _{max} (620 nm) after 6 hours
None (CDM)	1.25 ± 0.02	100	1.70 ± 0.14
L-Alanine	1.25 ± 0.01	100	1.70 ± 0.14
L-Arginine	n.g.***	n.g.	0.18 ± 0.01
L-Asparagine	1.20 ± 0.01	96.29	1.60 ± 0.15
L-Aspartic acid	1.19 ± 0.02	96.29	1.60 ± 0.23
L-Cysteine	0.93 ± 0.02	74.04	1.34 ± 0.23
L-Glutamine	1.10 ± 0.01	88.89	1.55 ± 0.18
L-Glutamic acid	1.06 ± 0.01	85.20	1.50 ± 0.20
Glycine	n.g.	n.g.	0.17 ± 0.01
L-Histidine	n.g.	n.g.	0.19 ± 0.02
L-Isoleucine	n.g.	n.g.	0.16 ± 0.02
L-Leucine	n.g.	n.g.	0.17 ± 0.01
L-Lysine	1.00 ± 0.01	81.49	1.44 ± 0.17
L-Methionine	n.g.	n.g.	0.17 ± 0.01
L-Phenylalanine	0.98 ± 0.02	79.63	1.40 ± 0.17
L-Proline	1.20 ± 0.01	96.29	1.60 ± 0.11
L-Serine	0.34 ± 0.01	27.78	0.50 ± 0.09
L-Threonine	0.74 ± 0.01	59.25	1.20 ± 0.18
L-Tryptophan	n.g.	n.g.	0.20 ± 0.01
L-Tyrosine	1.18 ± 0.02	96.29	1.60 ± 0.20
L-Valine	n.g.	n.g.	0.17 ± 0.02

*Calculated as percentage of growth in CDM

**Values are Means ± SD of three observations

***n.g.= no growth

Another important observation was the effect of glutamine or glutamic acid in single omission experiments. Both of them seemed to be non-essential, their absence resulting in a good growth (Table 4.4). However, no growth was observed when they were removed together. The add-back experiments indicated that either of them can be added for the growth of *E. faecalis*, however, addition of glutamic acid seemed to be

better in terms of supporting growth and, therefore, was retained as an important component in the SDM.

4.4.2 Effect of omitting B vitamins, nucleic acid bases and glucose on growth of *E. faecalis* B9510

Single omission of vitamins from the CDM showed that Ca-pantothenic acid, nicotinic acid and pyridoxal were absolutely essential for growth, and their absence resulted in no growth (Table 4.5). Folic acid and riboflavin were found to be stimulatory and their presence was required for appreciable growth. All the other B vitamins were found to have no effect on the growth of *E. faecalis* B9510, and, therefore, were omitted in the SDM.

The nucleic acid bases were not found essential for growth (Table 4.5); however, uracil was found to have a stimulatory effect. Adenine and guanine seemed to be non-essential in the single omission experiments; however, the absence of both of them resulted in a long lag phase of 4 – 5 hours and slow growth. The addition of either of them to the defined medium resulted in shortening the lag phase, but the presence of adenine seemed to have more positive effect on the growth and, therefore, was retained as a component of the defined medium.

Glucose was used as carbon source in the defined medium, and its absence resulted in no growth (Table 4.5) and, therefore, was retained as an important component in the SDM.

Table 4.5 Effect of single omission of B vitamins, nucleic acid bases and glucose on the growth of *E. faecalis* B9510 in the defined medium

Omitted vitamin	**Growth rate (h ⁻¹)	*Relative growth rate	**OD _{max} (620 nm) after 6 hours
None (CDM)	1.25 ± 0.03	100	1.70 ± 0.14
B vitamins			
Biotin	1.18 ± 0.02	96.29	1.60 ± 0.10
Cyanocobalamin	1.24 ± 0.02	100	1.65 ± 0.16
Calcium pantothenic acid	n.g.***	n.g.	0.16 ± 0.05
Folic acid	0.83 ± 0.01	66.67	1.30 ± 0.08
Nicotinic acid	n.g.	n.g.	0.12 ± 0.02
Para-amino benzoic acid	1.18 ± 0.02	96.29	1.58 ± 0.14
Pyridoxal	n.g.	n.g.	0.20 ± 0.05
Riboflavin	0.74 ± 0.01	59.26	1.20 ± 0.11
Thiamine	1.25 ± 0.02	100	1.72 ± 0.23
Nucleic acid bases			
Adenine	1.11 ± 0.01	88.89	1.54 ± 0.12
Guanine	1.25 ± 0.01	100	1.70 ± 0.18
Uracil	1.00 ± 0.01	79.63	1.40 ± 0.13
Carbon source			
Glucose	n.g.	n.g.	0.16±0.02

*Calculated as percentage of growth in CDM

**Values are Means ± SD of three observations

***n.g.= no growth

4.4.3 Influence of minerals on the growth of *E. faecalis* B9510

In the final set of experiments, the effect of minerals on the growth of *E. faecalis* was observed (Table 4.6). Ammonium sulphate, and tri-sodium citrate were of low importance for the growth of *E. faecalis* and were, therefore, omitted from the medium. Magnesium sulphate, however, was the only mineral which was essential for growth, whereas potassium dihydrogen phosphate and ferrous sulphate were found stimulatory for growth (Table 4.6). The phosphate salts, i.e. potassium dihydrogen phosphate and dipotassium monohydrogen phosphate also act as buffers in the defined medium, and, therefore, were retained as important components in the defined medium.

Table 4.6 Effect of omitting minerals on the growth of *E. faecalis* B9510 in the defined medium

Omitted salt	**Growth rate (h ⁻¹)	*Relative growth rate	**OD _{max} (620 nm) after 6 hours
None (CDM)	1.25 ± 0.02	100	1.70 ± 0.14
Ammonium sulphate	1.22 ± 0.01	98.15	1.65 ± 0.16
Ferrous sulphate	1.00 ± 0.01	79.63	1.40 ± 0.08
Magnesium sulphate	n.g. ^{***}	n.g.	0.24 ± 0.04
Potassium dihydrogen phosphate	0.70 ± 0.01	55.56	0.94 ± 0.07
Di-potassium monohydrogen phosphate	1.04 ± 0.02	83.33	1.47 ± 0.21
Tri-sodium citrate	1.20 ± 0.03	96.29	1.62 ± 0.18

*Calculated as percentage of growth in CDM

**Values are Means ± SD of three observations

***n.g. = no growth

4.4.4 Comparison of the Simplified Defined Medium (SDM) with a complex medium (M17)

The information obtained from the single omission experiments was used to develop a simplified defined medium (SDM), containing only the essential and stimulatory components. However, such a medium resulted in a very long lag phase of about five hours compared with the complete defined medium. Further addition of one amino acid (lysine) and one of the purines (adenine) resulted in shortening the lag phase to one hour producing a medium enabling a growth rate similar to that of CDM. Therefore, based on these observations a Simplified Defined Medium (SDM) was formulated, as shown in Table 3.2. The efficiency of this medium was then compared with modified glucose M17, a complex medium commonly used for the growth of lactic streptococci and enterococci.

A comparison of growth of *E. faecalis* B9510 in SDM and M17 broth (Figure 4.1 and Figure 4.2) showed a lag phase in the first hour of growth in defined medium, which was not observed in M17 broth. However, after two hours in defined medium, the growth in defined medium was comparable with that in M17 broth. A similar bacteriocin production was observed in SDM as well as in M17 broth (Figure 4.1 and 4.2), probably due to the semi-quantitative nature of the critical dilution assay which was used for the quantification of bacteriocin.

4. Experimental Results

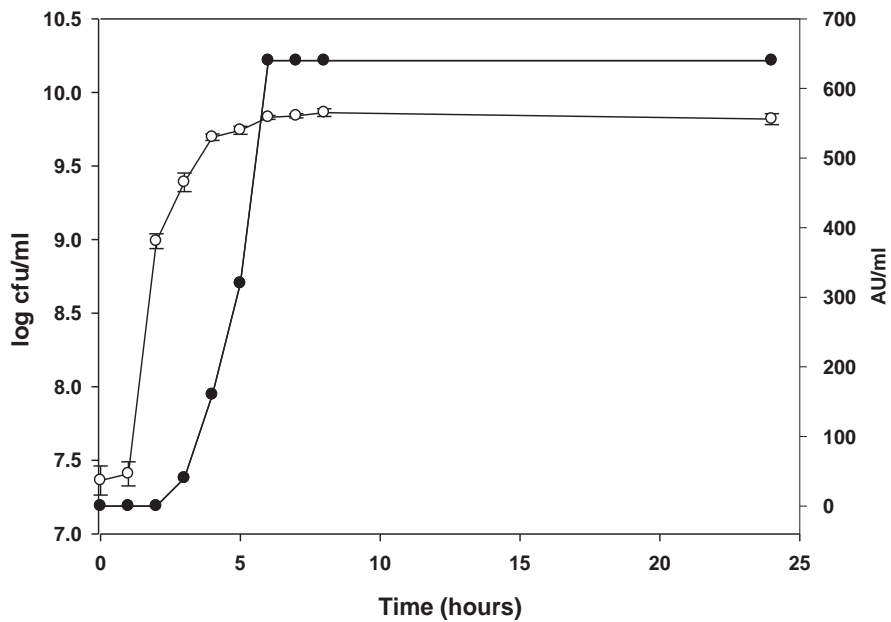


Figure 4.1 Growth of *E. faecalis* B9510 (○) and bacteriocin production in Arbitrary Units/ml (AU/ml) (●) in Simplified Defined Medium (SDM). Values are Means \pm SD of three observations. Due to the semi-quantitative nature of bacteriocin detection method, no difference was observed between the values of three observations and, therefore, no standard deviation for bacteriocin production values is shown.

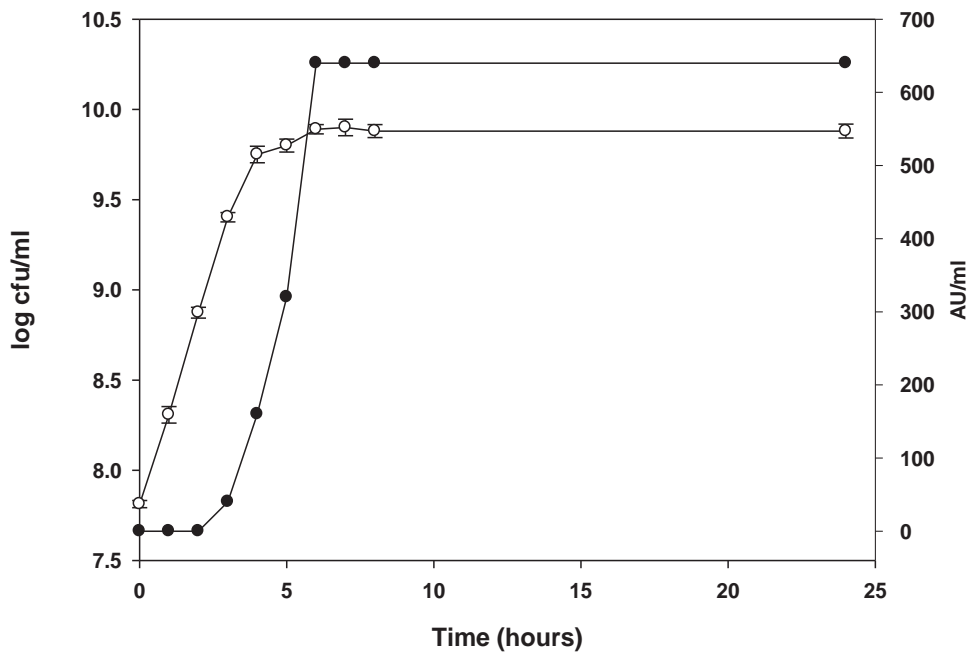


Figure 4.2 Growth of *E. faecalis* B9510 (○) and bacteriocin production in Arbitrary Units/ml (AU/ml) (●) in M17 broth. Values are Means \pm SD of three observations. Due to the semi-quantitative nature of bacteriocin detection method, no difference was observed between the values of three observations and, therefore, no standard deviation for bacteriocin production values is shown.

4.5 Purification and characterization of bacteriocin

After removal of cells from the defined medium, the cell-free supernatant was subjected to ultrafiltration through a 10 kDa ultrafiltration membrane. The active retentate was then further subjected to a second ultrafiltration step of 30 kDa NMWCO. The antimicrobial activity was again found in the retentate (20480 AU/ml) when tested by agar well diffusion assay using *L. lactis* ssp. *cremoris* 2144 as the indicator strain. However, some of the activity (320 AU/ml) was also found in the permeate, which indicated that the molecular mass of the bacteriocin was near to 30 kDa. Therefore, no further ultrafiltration was done, and the retentate of 30 kDa step was subjected to SDS-PAGE for characterization and identification of bacteriocin.

4.5.1 SDS Polyacrylamide Gel Electrophoresis and Zymogram

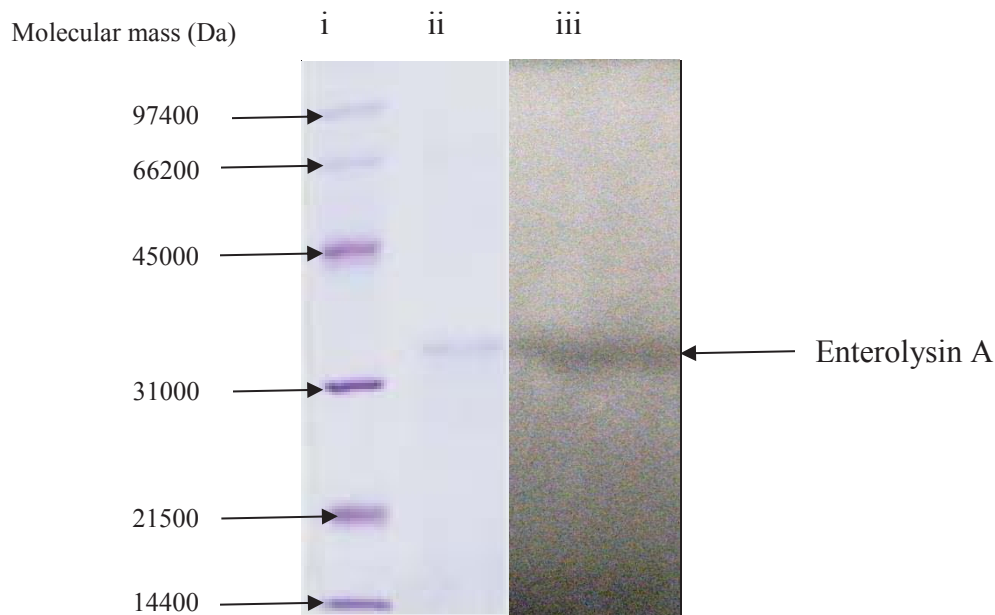


Figure 4.3 SDS-PAGE of purified bacteriocin. Lanes: i, low-range molecular weight standards (Bio-Rad Laboratories, Hercules, CA, USA); ii, purified bacteriocin after ultrafiltration (30 kDa NMWCO). iii Gel slice of a 13% polyacrylamide zymogram containing 0.2% dead cells of *L. lactis* ssp. *cremoris* 2144 following renaturation of the bacteriocin in 0.1% Triton X-100 and 10 mM MgCl₂ showing the lysis of cells (zone of clearance) around 35 kDa.

The results of staining and zymogram gel (Figure 4.3) indicated an active band around 37 kDa in the SDS-PAGE gel. For molecular mass determination and amino acid sequencing, the active band was excised from the gel, and subjected to mass spectrometry analysis.

4.5.2 Mass spectrometry and amino acid sequence

The active band excised from the non-denaturing gel was analysed by mass spectrometry. The results showed that eight unique peptides (from the mass spectrometry data) matched the sequence of a heat labile class III bacteriocin enterolysin A (Nilsen *et al.*, 2003). The accession number of enterolysin A in NCBI *Inr* database is 11078549. The sequence coverage of the experimental sample was 58% with a score of 831. The sequence of enterolysin A is shown in Figure 4.4, with the underlined letters showing the unique sequences from mass spectrometry data that matched with enterolysin A.

```

1   ASNEWSWPLG KPYAGRYEEG QQFGNTAFNR GGTYFHDGFD FGSAIYNGS
51  VYAVHDGKIL YAGWDPVGGG SLGAFIVLQA GNTNVIYQEF SRNVGDIKVS
101 TGQTVKKGQL IGKFTSSHLH LGMTKKEWRS AHSSWNKDDG TWFNPIPILO
151 GGSTPTPPNP GPKNFTTNVR YGLRVLGGSW LPEVTNFNNT NDGFAGYPNR
201 QHDMLYIKVD KGQMKYRVHT AQSGWLPWVS KGDKSDTVNG AAGMPGQ AID
251 GVQLNYITPK GEKLSQAYYR SQTTKRSGWL KVSADNGSIP GLDSYAGIFG
301 EPLDRLQIGI SQSNPF

```

Figure 4.4 Amino-acid sequence of mature enterolysin A (without leader sequence) obtained from NCBI database (Accession No. 11078549). The underlined sequences reveal those that matched with that of experimental sample from SDS-PAGE active band. The sequence coverage was 58% with a score of 831 [This score was sum of the individual ions score of eight unique peptide sequences from MS/MS spectra where a ions score of >60 indicated identity or extensive homology ($P < 0.05$)].

4.6 Amplification and sequencing of enterolysin A gene

To confirm the result of mass spectrometry, the enterolysin A gene was amplified by PCR. The PCR product was purified and run on a 2% agarose gel (Figure 4.5). The amplification of the enterolysin A gene is evident from the figure which was further confirmed when the purified PCR product was subjected to DNA sequencing. The sequence obtained is shown in Figure 4.6.

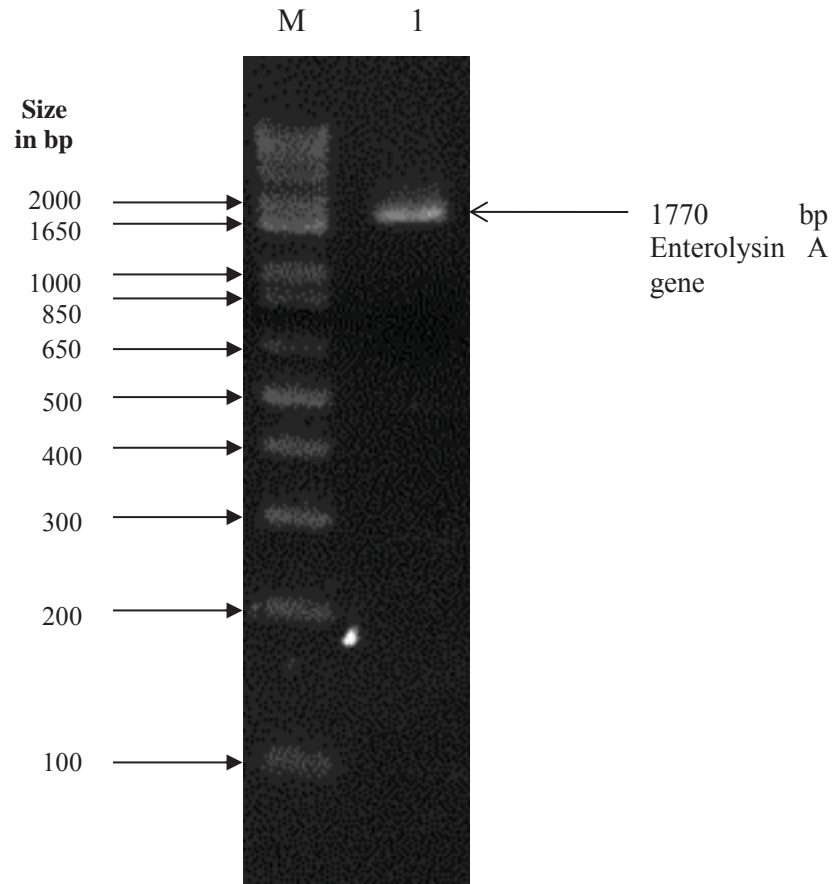


Figure 4.5 Agarose-gel (2%) electrophoresis of PCR amplified enterolysin A gene from the genome of *E. faecalis* B9510. Lane M= 1Kb+ DNA ladder (Invitrogen, Carlsbad, CA, USA); Lane 1= 1770 bp band containing the enterolysin A gene

The 1770 base pairs completely matched the Enterolysin A gene as described by Nilsen *et al.* (2003) with the exception of a single base pair at position 1085, where instead of cytosine there is thymine (Figure 4.6). However, this does not alter the amino acid sequence of Enterolysin A protein as determined by NCBI BLASTX software.

4. Experimental Results

```

1    CGATTTCTGT  TGTAGGAACC  ATTATTTTTG  AATAGGACTT  TTATGTTAGT  AAAATAAAAA
61   AATAAATCAC  AATTAAGGTT  CTGGTTGTTA  TTAATCTATC  TCATGAAGCA  TTAGATGAAT
121  TAGTTCTAGA  AGTACCTGTT  GTECTAGTTA  AAAATACTGT  TAAATCAAAT  TTTTGTGTTA
181  AAAGAATCAT  TAAGTTGGTG  CCTAACTATA  AAATCAAAT  GACTAAAATC  CAATAACATT
241  GGGGATACT  CTGTAAATCG  TGTGTCGTAG  TACGTTAGTC  TTGTAATAAA  TAAATATTTA
      RBS                Start
301  TTAGGAGGGG  TTTCTATGAA  AAATATTTTA  CTTTCTATTC  TAGGGGTATT  ATCTATCGTT
361  GTTTCTTTGG  CGTTTTCTTC  TTATTCTGTC  AACGCAGCTT  CTAATGAGTG  GTCGTGGCCA
421  CTGGGCAAAAC  CATATGCGGG  AAGATATGAA  GAAGGACAAC  AATTCGGGAA  CACTGCATTT
481  AACCGAGGAG  GTACTTATTT  CCATGATGGG  TTTGACTTTG  GTTCTGCTAT  TTATGGAAAT
541  GGCAGTGTGT  ATGCTGTGCA  TGATGGTAAA  ATTTTATATG  CTGGTTGGGA  TCCTGTAGGT
601  GGAGGCTCAT  TAGGTGCATT  TATTGTACTA  CAAGCGGGAA  ACACAAATGT  GATTTATCAA
661  GAATTTAGCC  GAAATGTTGG  AGATATTTAA  GTTAGCACTG  GACAAACTGT  TAAAAAAGGA
721  CAGCTGATAG  GAAAGTTTAC  TTCTAGTCAT  TTACATTTAG  GAATGACAAA  AAAAGAATGG
781  CGTTCTGCTC  ATTCTTCTTG  GAATAAAGAT  GATGGCACTT  GGTTTAACCC  AATTCCTATA
841  CTTCAAGGAG  GATCTACGCC  TACGCCTCCA  AATCCAGGAC  CAAAAAATTT  CACAACAAAT
901  GTTCGTTACG  GATTGCGGGT  CCTCGGAGGT  TCATGGTTAC  CAGAAGTAAC  CAACTTTAAC
961  AATACCAATG  ATGGTTTCGC  AGGTTACCC  AATCGTCAAC  ATGATATGCT  TTATATAAAG
1021 GTAGATAAAG  GGCAAATGAA  ATATCGTGTT  CACACGGCTC  AAAGTGGATG  GTTGCCTTGG
1081 GTAAGTAAAG  GGGATAAGAG  CGATACAGTA  AATGGAGCGG  CAGGTATGCC  TGGACAAGCA
1141 ATTGATGGTG  TTCAGCTAAA  CTATATAACT  CCTAAGGGAG  AAAAAATTATC  ACAGGCTTAC
1201 TATCGTTCAC  AAAC TACGAA  ACGATCAGGC  TGGTTAAAAG  TAAGTGCAGA  TAATGGTTCT
1261 ATTCTTGGAC  TAGACAGTTA  TGCAGGAATC  TTTGGAGAAC  CGTTGGATCG  CTTGCAATA
      Stop
1321 GGTATTTTAC  AGTCAAATCC  ATTTTAAAT  TAGAAAATGA  CAGTAGGATT  ATGCATTTAA
1381 TAAATTTTAA  AGAGGTGAAC  GAGTATAAAT  AGAAATACAG  TTAATACTACT  AAAAAAATCG
1441 TTTATTTTAC  CTTTACTTGG  CTTATTCTTT  ATAGTTGGTA  CAATGCTTTT  GCTTCTGAAG
1501 ATTACAACCT  ACTTGGAGTG  AAAAATTATG  ATCAGTATGC  CTTAGGAGCG  CTTAGTGGGT
1561 GTGAGGGGGC  TTCACTATTA  CAAGGCTTAC  AGTATAAAGG  AAAAAATCCT  GATTGGGACT
1621 TAATCAAAT  TTTAAAAACA  ATTCTATCT  CTCCTAATGG  AAATCCTTAT  GATGGATTTG
1681 TAGGTAGTCC  TTTTGTGAA  AACTCATGGA  CTTACTCTGC  TATCTATCCT  GTGCCTTTAG
1741 TAAATGGGG  AAAAGTATAT  GGAGATGTAC

```

Figure 4.6 Nucleotide sequence of the 1770 bp nucleotide fragment from *E. faecalis* B9510 with the enterolysin A structural gene highlighted. The thymine at position 1085 is underlined to show that this nucleotide is different from the sequence described by Nilsen *et al.* (2003). The putative Ribosomal Binding Site (RBS), Start and Stop codons are underlined.

4.7 Sequence homology of enterolysin A

The primary structure of enterolysin A consists of two domains, an N-terminus catalytic domain (CAT) which belongs to the M23/M37 endopeptidase family, and a C-terminus substrate recognition domain (SRB). These two domains are connected by a Threonine-Proline (TP) rich linker sequence (Cooper and Salmond 1993; Simmonds *et al.*, 1997; Nilsen *et al.*, 2003; Maliničová *et al.*, 2010). The N-terminus domain of enterolysin A shares sequence homology with other bacteriocins having M23/M37 domain in their N-terminus as shown in Figure 4.7.

A look at Figure 4.7 shows that enterolysin A is 34% identical with zoocin A produced by *S. zooepidemicus* 4881 (Simmonds *et al.*, 1997) and lysostaphin M produced by *S. aureus* (Ramadurai and Jayaswal 1997), 29% identical with lysostaphin produced

by *S. simulans* biovar *staphylolyticus* (Recsei *et al.*, 1987) and ALE-1 produced by *Staphylococcus capitis* EPK1 (Sugai *et al.*, 1997), and 44% identical to millericin B produced by *S. milleri* NMSCC061 (Beukes *et al.*, 2000).

The C-terminus of enterolysin A shares significant sequence homology with the lysins of *L. casei* and its bacteriophages (Figure 4.9 and 4.10) which indicate possible horizontal transfer of genes from the bacteriophages into the bacterial genomes (Hickey *et al.*, 2003).

Analysis of Figure 4.9 shows that the C-terminus of enterolysin A shares 66% identity with lysin produced by bacteriophage PL1 (Kashige *et al.*, 2000) and 67% identity with N-acetyl muramoyl-L-alanine amidase produced by *L. casei* (Maze *et al.*, 2010) and its temperate bacteriophage A2 (García *et al.*, 1997).

On the basis of the sequence alignments phylogenetic tree of enterolysin A with other lytic bacteriocins (Figure 4.10) and lysins of bacteriophages and *L. casei* (Figure 4.8) were created using the NCBI BLAST program by the fast minimum evolution method (Desper and Gascuel 2004). The phylogenetic tree is an alternate way of showing the sequence similarity between related proteins in a graphical manner.

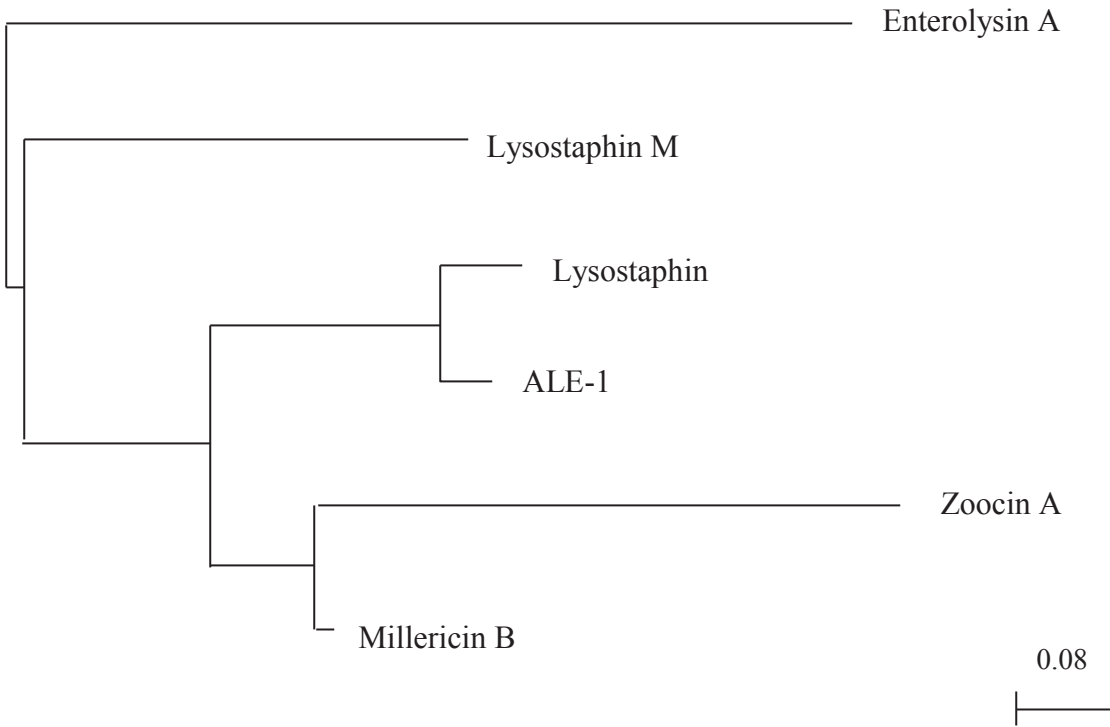


Figure 4.8 Phylogenetic relationship between enterolysin A and other cell wall degrading lysins

4. Experimental Results

Enl A	1	MKNILLSILGVLSTIVVSLAFSSYSVNAASNEWSWPLGKPYAGRYEEG-----QOFGNTAFNRGGTYFHDGFDFG	69
Cas	1	M-----SYT-----INKEFALG-ANEGSSQV-----ANRLYIILHDVGAESGARANAAYFKNNIAAE	51
PL-1	1	M-----SYT-----INKEFALG-ANEGSSQV-----ANRLYIILHDVGAESGARANAAYFKNNIAAE	51
A-2	1	M-----SYT-----INKEFALG-ANEGSSQV-----ANRLYIILHDVGAESGARANAAYFKNNISAE	51
Enl A	70	SA-----IYGNCS-VYAVHDGKILYAGWDPVGGGSLGAFIVLQAGNTN---VIYQEFNRVGDIKVSTGQTVKKGQLIG	139
Cas	52	IAYTAFVV-GDGGQVYQVGEPPGYVQWAGTVANANSPVQIELGHTS---DPETFKKDYAVYIELARDMAARYGIPTSLDA	127
PL-1	52	IAYTAFVV-GDGGQVYQVGEPPGYVQWAGTVANANSPVQIELGHTS---DPETFKKDYAVYIELARDMAARYGIPTSLDA	127
A-2	52	IAYTAFVV-GDGGQVYQVGEPPGYVQWAGTVANANSPVQIELGHTS---DPETFKKDYAVYIELARDMAARYGIPTSLDA	127
Enl A	140	KFTSSHLHLGMTKKEWRSASHSSWNKDDGT-----W----FNPIPLQGGSTPTPPNPGPKNF-----TTN	195
Cas	128	GGAGT---PGIKSHLWVTQHI-WGDHTDP---YGLARWGITKEKLAADLANGTTTVDASKSALAAQSARPOAIVTRNVN	200
PL-1	128	GGAGT---PGIKSHLWVTQHI-WGDHTDP---YGLARWGITKEKLAADLANGTTTVNPSPSAPAAESLRPOATISGNVN	200
A-2	128	GGAGT---PGIKSHLWVTQHI-WGDHTDP---YGLARWGITKEKLAADLANGTTTVDASTSAPATQSTRPOATVSGNVN	200
Enl A	196	VRYGLRVLGGSWLPEVNTNNTNDGFAGYPNRQHDMLYIKVDKQMKYRVHTAQSGWLPWVSKGDKSDTVNGAAGMPGQA	275
Cas	201	VAYGLHLLGTRWLDEVTNFGSDNGFAGMPNYQHDLLEYIKVDHGSVKYRVHTVQSGWLPWVAKGDRNDTVNGCAGNAGEV	280
PL-1	201	ATYALHLLGGSWLDEVTNFGSDNGFAGMPNHQHDLLYIKVDHGSVKYRVHTVQSGWLPWVTKGDRNDTVNGCAGNAGEV	280
A-2	201	ATYGLHLLGGSWLDEVTNFGSDNGFAGMPNHQHDLLYIKVDHGSVKYRVHTVQSGWLDVWVTKGDRNDTVNGCAGTAGEA	280
Enl A	276	IDGVQLNYITPKGKLSQAYYRSQTTKRSGLWLVKVSADNG-SIFGL-DSYAGIFGEPLDRLQIGISQSNPF	343
Cas	281	IDGVQIIFLTPAGESYKQAYYRSQTTQRAGWLGVCDDGTSLPQYTDYAGLFGPEPLDRLQIGISSINPF	350
PL-1	281	IDGVQIIFLTPAGESYKQAYYRSQTTQRAGWLGVCDDGTSLPQYTDYAGLFGPEPLDRLQIGISSVSPF	350
A-2	281	IDGVQIIFLTPAGEFYQQAYYRSQTTQRAGWLGVCDDGTSLPQYTGTYAGLFGPEPLDRLQIGISSINPF	350

Figure 4.9 Alignment of Enterolysin A with lysins from *Lactobacillus casei* BL23 (cas) and bacteriophages PL-1 and A-2. Adapted from Nilsen *et al.* (2003)

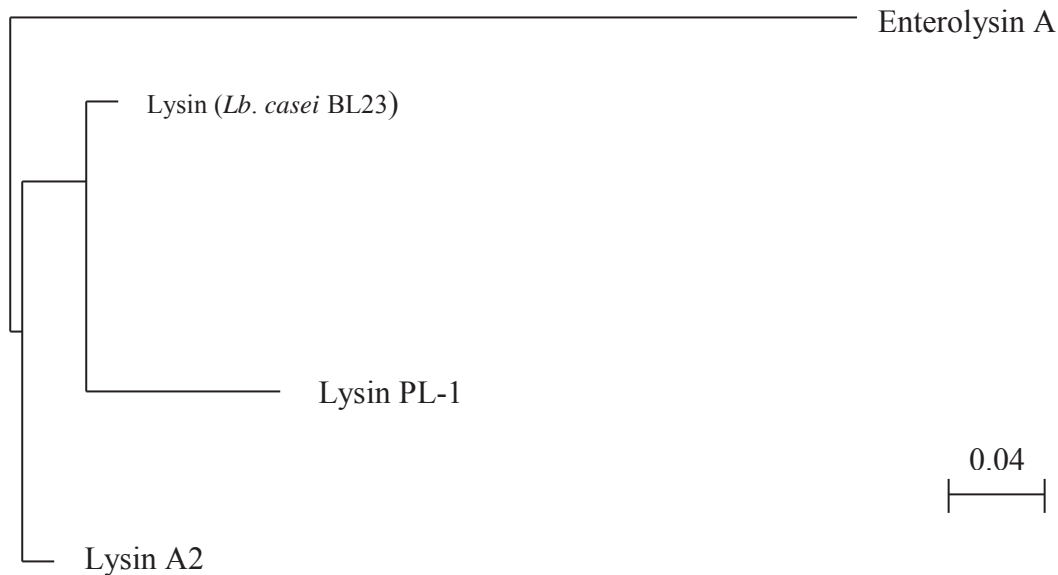


Figure 4.10 Phylogenetic relationship between Enterolysin A and lysins of *Lactobacillus casei* BL23 and bacteriophages PL-1 and A2

4.8 Antimicrobial spectrum of enterolysin A

The enterolysin A was tested for its antimicrobial activity against several pathogenic and non-pathogenic strains of selected bacterial species (Table 4.7). The selected strains included some Gram-negative species (*E. coli*, *Salmonella enteridis* and *Y. enterocolitica*) as well. However, no activity was found against these Gram-negative bacteria. The antimicrobial activity of enterolysin A has already been reported for many LAB and non-LAB species (Nilsen *et al.*, 2003; Nigutova *et al.*, 2007). The current study extended this knowledge further by testing the antimicrobial activity of enterolysin A against many species which have not been reported previously, and it was observed that enterolysin A is also active against *Lactobacillus helveticus* 1109, *Lactobacillus bulgaricus* ATCC 11842 and *Lactobacillus casei* ML-1. Enterolysin A was also found to be active against some pathogenic strains which included *Listeria monocytogenes* NCTC 10884 and *Staphylococcus aureus* NCTC 4163. However, the antimicrobial activity was much lower against these pathogens as compared to the LAB strains and was only detectable in the agar well diffusion assay.

Table 4.7 Inhibitory spectrum of enterolysin A against selected pathogenic and non-pathogenic bacteria

Bacterial strain	Sensitivity to enterolysin A (AU/ml)
<i>E. faecalis</i> B9510	-
<i>E. faecalis</i> No. 40	(+)*
<i>Lactococcus lactis</i> ssp. <i>cremoris</i> 2144	20480
<i>L. lactis</i> ssp. <i>lactis</i> F-1	2560
<i>L. lactis</i> ssp. <i>lactis</i> RM-1	2560
<i>L. lactis</i> ssp. <i>lactis</i> RM-5	2560
<i>L. lactis</i> ssp. <i>lactis</i> K-30	5120
<i>L. lactis</i> ssp. <i>lactis</i> MG1363	2560
<i>Pediococcus pentosaceus</i> 43200	5120
<i>P. pentosaceus</i> 43201	5120
<i>Streptococcus thermophilus</i> ST 49	-
<i>S. thermophilus</i> V38	-
<i>Lactobacillus helveticus</i> 1109	5120
<i>L. helveticus</i> 1113	5120
<i>Lactobacillus delbrueckii</i> ssp. <i>bulgaricus</i> ATCC 11842	5120
<i>L. casei</i> ML-1	10240
<i>L. plantarum</i> , ML-2	-
<i>L. acidophilus</i> , ML-3	-
<i>Listeria monocytogenes</i> NCTC 10884	(+)*
<i>B. cereus</i> ML-4	-
<i>P. vulgaris</i> ML-5	-
<i>Staphylococcus aureus</i> NCTC 4163	(+)*
<i>Staphylococcus epidermidis</i> ML-6	-
<i>E. coli</i> 0157:H7	-
<i>E. coli</i> 0111	-
<i>Salmonella enteridis</i> ML-7	-

*No activity detected in microtitre plate assay. However, zones of inhibition (5 – 6 mm diameter) were detected in agar-well diffusion assay

4.9 Mode of action of enterolysin A

Enterolysin A is a cell wall degrading bacteriocin which lyses the cell walls of sensitive bacterial strains, thus resulting in the killing of cells. The mode of action studies, therefore, focused on the lytic action of enterolysin A followed by Transmission Electron

Microscopy (TEM) of treated cells. Finally experiments were conducted to find the cleavage site of enterolysin A within the peptidoglycan moiety of cell walls of sensitive strains.

4.9.1 Lytic activity of enterolysin A

To test the lytic activity of enterolysin A, a suspension of *L. lactis ssp. cremoris* 2144 cells at an initial concentration of about 10^6 cfu/ml, was treated with crude enterolysin A at a final concentration of 5000 AU/ml. A rapid decrease in turbidity of the suspension was observed with concomitant decrease of total viable counts (Figure 4.11). The figure shows that the counts of viable bacteria reached below detectable limits (< 10 cfu/ml) within two hours of enterolysin A treatment as compared to the untreated control in which the bacterial counts remained almost the same.

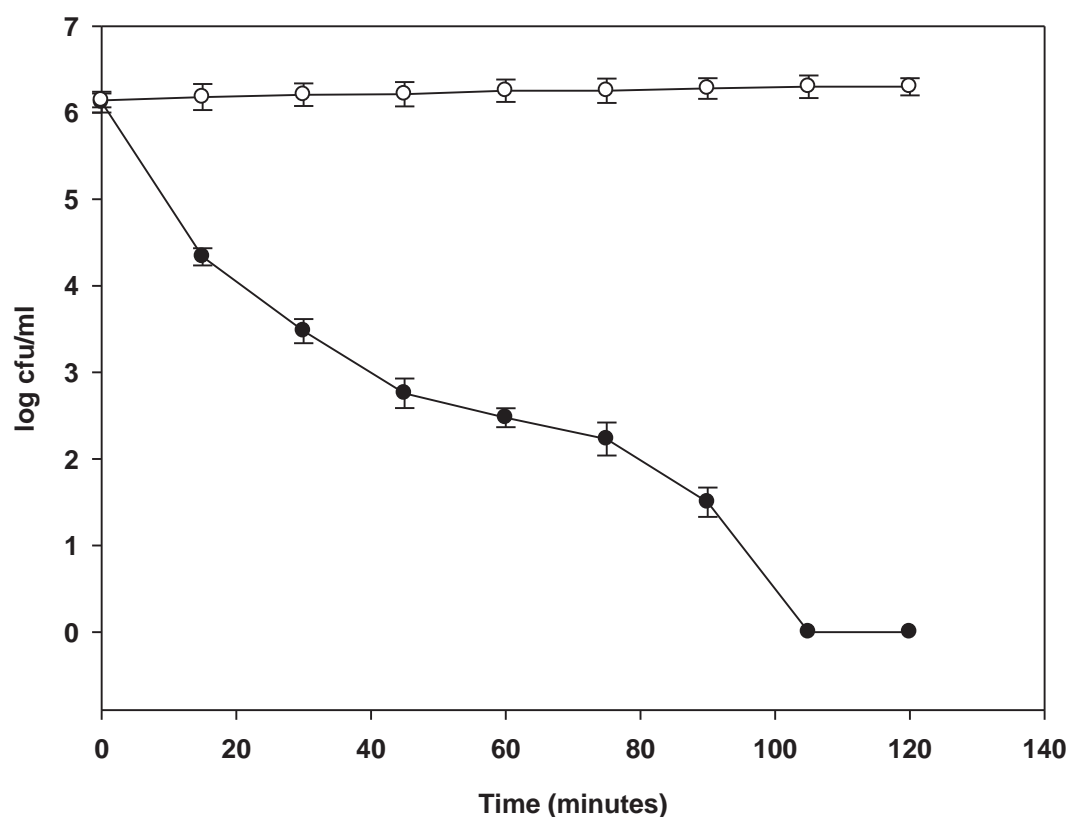


Figure 4.11 Killing of *L. lactis ssp. cremoris* 2144 cells (●) suspended in 10mM phosphate buffer by crude enterolysin A (5000 AU/ml) as compared to untreated control (○). Values are Means \pm SD

4.9.2 Transmission Electron Microscopy (TEM) of enterolysin A treated cells

During measurement of lytic activity samples were collected at intervals for visualization of damaged cells under TEM. Selected pictures of the untreated control cells and cells treated with enterolysin A show a clear difference with regard to the integrity of cell walls (Figure 4.12A). The untreated cells clearly show intact cell walls with visible dark staining cytoplasm (A and B). Figure 4.12C shows the initial stages of cell wall damage with the dark cytoplasmic material still visible, while Figure 4.12D shows the advanced stage of damage with the complete loss of cytoplasmic material into the surroundings with only few dark stained cells visible. Observation of the individual damaged cells at higher magnifications clearly shows the rupture of cell wall and its separation from the cytoplasmic membrane (E), oozing out of cytoplasmic material in the surroundings (G) and remains of a ghost cell with cytoplasmic material completely lost (H) in the final stages of damage.

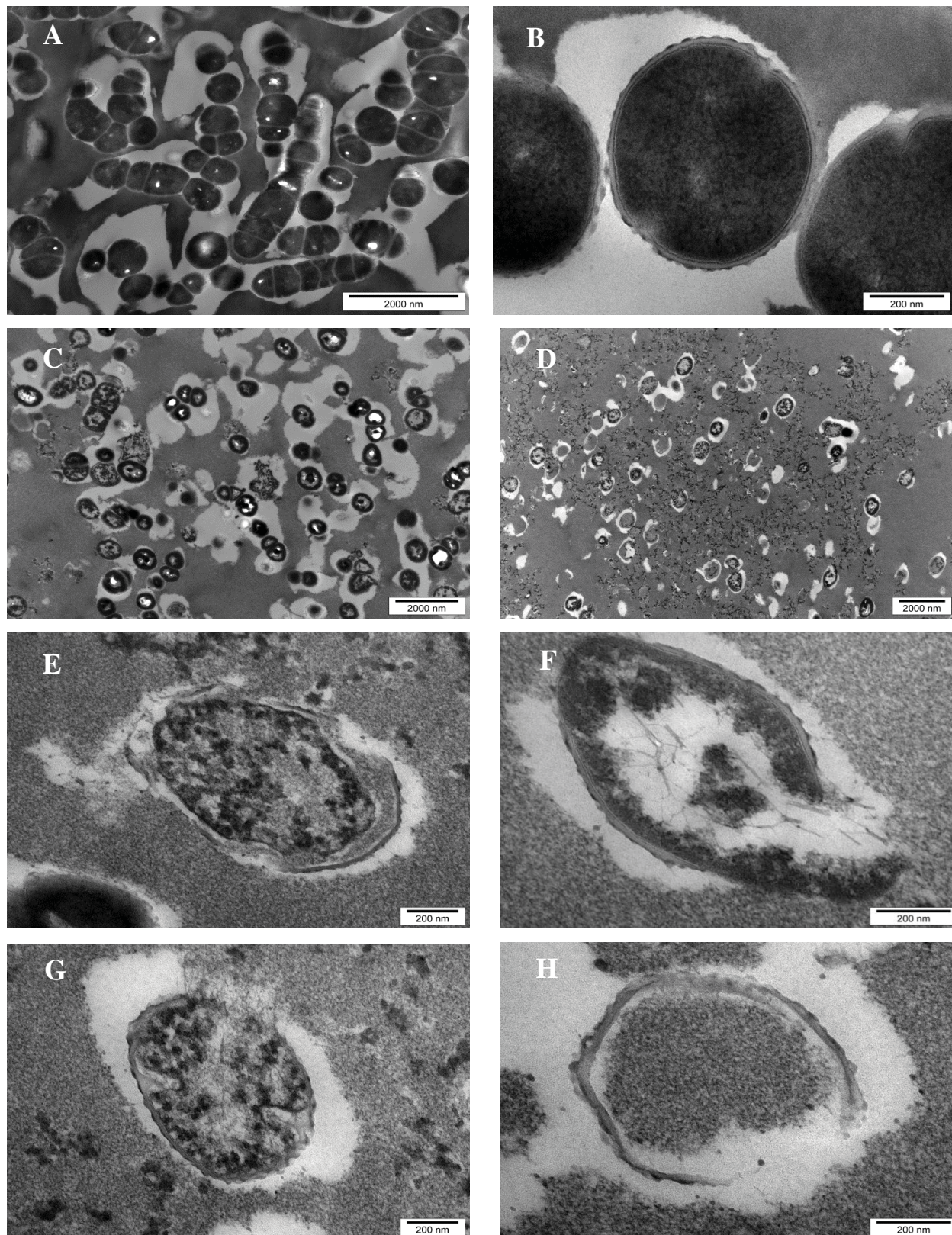


Figure 4.12 Transmission electron microscopy of enterolysin A treated *L. lactis* ssp. *cremoris* cells. (A) *L. lactis* ssp. *cremoris* 2144 cells without enterolysin A treatment; (B) An intact *L. lactis* ssp. *cremoris* 2144 cell without enterolysin A treatment (C) and (D) *L. lactis* ssp. *cremoris* 2144 cells treated with enterolysin A illustrating the range of cellular disruption observed after 15 and 45 minutes of enterolysin A addition respectively; (E) to (H) *L. lactis* ssp. *cremoris* 2144 cells treated with enterolysin A showing rupture of cell wall and extrusion of the cytoplasmic membrane and cell contents from the ruptured cells

4.9.3 Determination of the cleavage site of enterolysin A in the cell walls of sensitive strains

Enterolysin A has been known to kill the sensitive cells by causing lysis of the cell walls as has been reported in the literature and discussed above. It has also been reported that enterolysin A is an endopeptidase belonging the M23/M37 family and cleaves the bond within the peptide moiety of peptidoglycan subunit of bacterial cell walls. However, the exact site of cleavage within the peptide moiety has not been reported before. Therefore, an attempt was made in the current study to extend the knowledge further by finding the exact cleavage site of enterolysin A within the peptide moiety.

The method originally developed by Ghuysen *et al.* (1966) and followed by Beukes *et al.* (2000) for determination of cleavage site of millericin B was followed. Purified cell walls of three sensitive strains *L. lactis* ssp. *cremoris* 2144, *P. pentosaceus* 43201 and *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 were prepared and digested with crude enterolysin A (retentate of 30 kDa ultrafiltration step). They were then derivatized for N- and C- termini and the liberated amino acids were determined by running the derivatized samples on TLC silica sheets along with standard amino acids of the peptidoglycan units derivatized in a similar manner.

The results of the N-terminus derivatized samples showed the presence of lysine and glutamic acid in the samples of purified cell walls. The results of the C- terminal derivatized samples showed the presence of aspartic acid and alanine in the purified cell walls when compared with the pure amino acids (Figure 4.13). This indicated that after digestion with enterolysin A, the N- terminal of lysine and alanine are liberated and available for derivatization and similarly the C-terminal of aspartic acid and alanine are free.

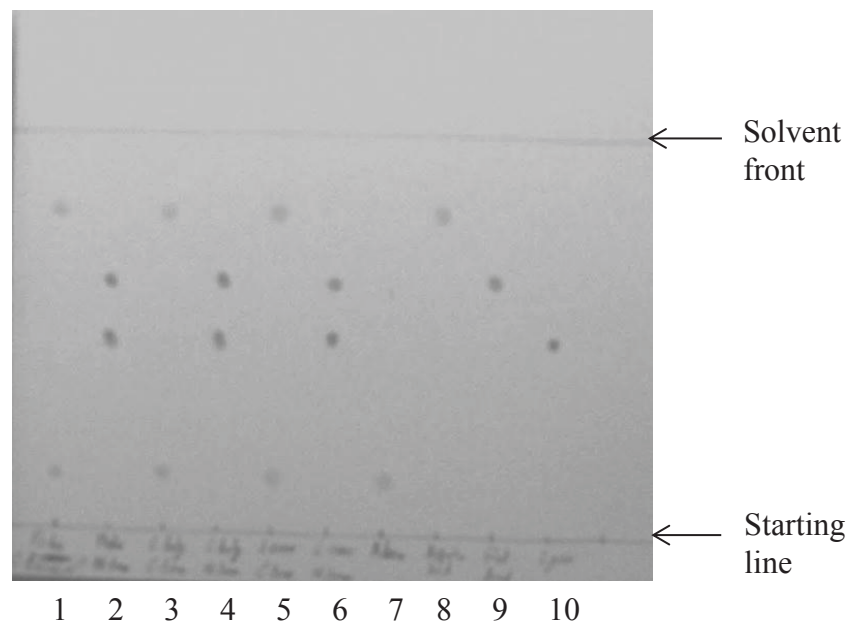


Figure 4.13 Chromatogram of thin layer chromatography of liberated N and C terminal amino acids from purified cell walls of sensitive strains after treatment with enterolysin A, run on silica gel using isopropanol (with 0.1% TFA) as solvent. The TLC plate was viewed under UV light (short wavelength) for visualization of spots. 1, 3 and 5 C-terminal derivatized samples of *L. lactis* ssp. *cremoris*, *P. pentosaceus* 43201 and *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 respectively, 2, 4 and 6 N-terminal derivatized samples of *L. lactis* ssp. *cremoris*, *P. pentosaceus* 43201 and *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 respectively. 7 to 10 are pure amino acids run as standards for comparison (7 - Alanine, 8 - Aspartic acid, 9 - Glutamic acid and 10 - Lysine).

Viewing these results in the light of composition and structure of peptidoglycan units of *L. lactis* ssp. *cremoris*, *P. pentosaceus* and *L. delbrueckii* ssp. *bulgaricus* (Schleifer and Kandler 1972) indicates that enterolysin A cleaves the bond within the peptide moiety at two locations. The first location is between L-alanine and D-glutamic acid of the stem peptide and the other location is between L-lysine of the stem peptide and D-aspartic acid of interpeptide bridge (shown by A and B respectively in Figure 4.14).

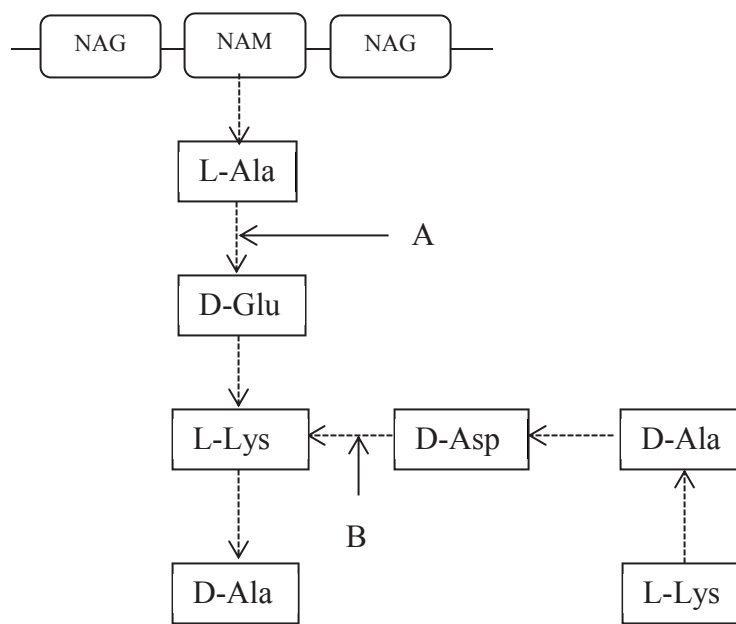


Figure 4.14 Structure of the peptide moiety of peptidoglycan unit of cell walls of *L. lactis* ssp. *cremoris*, *P. pentosaceus* and *L. delbrueckii* ssp. *bulgaricus*. Enterolysin A cleavage sites have been marked as A and B in the stem peptide and interpeptide bridge respectively (NAG = N-acetyl glucosamine, NAM = N-acetyl muramic acid). Adapted from Schleifer and Kandler (1972)

4.10 Determination of enterolysin A immunity gene

Self-immunity of the producer strain is an important characteristic of bacteriocin production by Gram-positive bacteria, and immunity genes for many bacteriocins have been identified. The genes responsible for conferring self-immunity have also been described for many lytic bacteriocins, however, no immunity genes have been described for enterolysin A. Therefore, an attempt was made in the current study to identify the gene responsible for conferring self-immunity to the host strain.

The self-immunity genes for lysostaphin (Dehart *et al.*, 1995), zoocin A (Gargis *et al.*, 2009a) and millericin B (Beukes and Hastings 2001) and most of the bacteriocins described to date lie in close proximity to the structural gene, therefore, it was assumed that gene responsible for conferring self-immunity to the enterolysin A producing strain must also be lying in close proximity to the structural gene.

The sequences of the neighbouring genes of enterolysin A were obtained from the NCBI database (www.ncbi.nlm.nih.gov) using the enterolysin A structural gene as a query. Among the results of the query a sequence of 5156 bp from *E. faecalis* BGPT1-10P showed three open reading frames lying upstream and downstream of the enterolysin A structural gene (Genbank No. HE585879 Appendix 3). The Genbank sequence showed

4. Experimental Results

that the gene fragments of 182 bp and 257 bp were lying directly upstream of the enterolysin A structural gene while a 530 bp gene was lying directly downstream of the enterolysin A gene. The details of these sequences are shown in Appendix 3. The sequences of these open reading frames were used to design primers and amplify them from the *E. faecalis* B9510 genome, as given in the Materials and Methods chapter. The PCR experiments successfully amplified the desired regions (Figure 4.15), which indicated that these genes are indeed present in the *E. faecalis* B9510 genome.

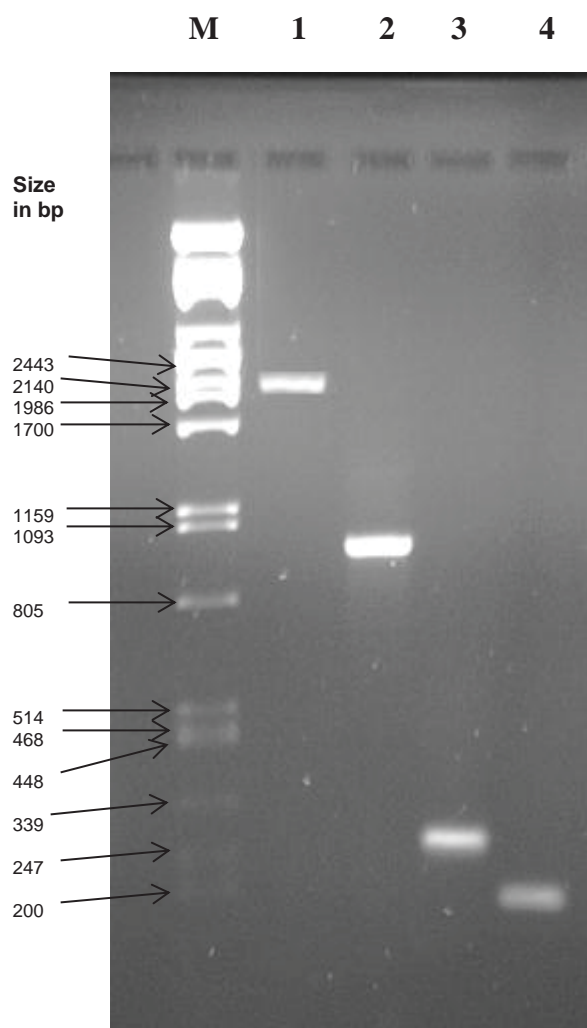


Figure 4.15 Agarose-gel (1.5%) electrophoresis of PCR amplified regions of enterolysin A and neighbouring genes. Lane M= DNA ladder obtained by digestion of lambda DNA with Pst I restriction enzyme. Lane 1 = Imm-1 (2060 bp), Lane 2 = Imm-2 (997 bp), Lane 3= Imm-3 (257 bp), Lane 4= Imm-4 (183 bp)

4. Experimental Results

The four amplified regions (named as Imm-1, Imm-2, Imm-3 and Imm-4) were then purified and ligated independently to pFX3 vector in separate reactions. Thus four different types of vectors were obtained which were then used to transform four batches of *E. coli* JM109 strain. Successful transformants of *E. coli* JM109 containing the desired amplified regions (as confirmed by blue-white screening and PCR experiments) were then selected for plasmid extraction and subsequent transformation of *L. lactis* ssp. *lactis* MG1363 strain. The whole strategy is graphically represented in Figure 4.16.

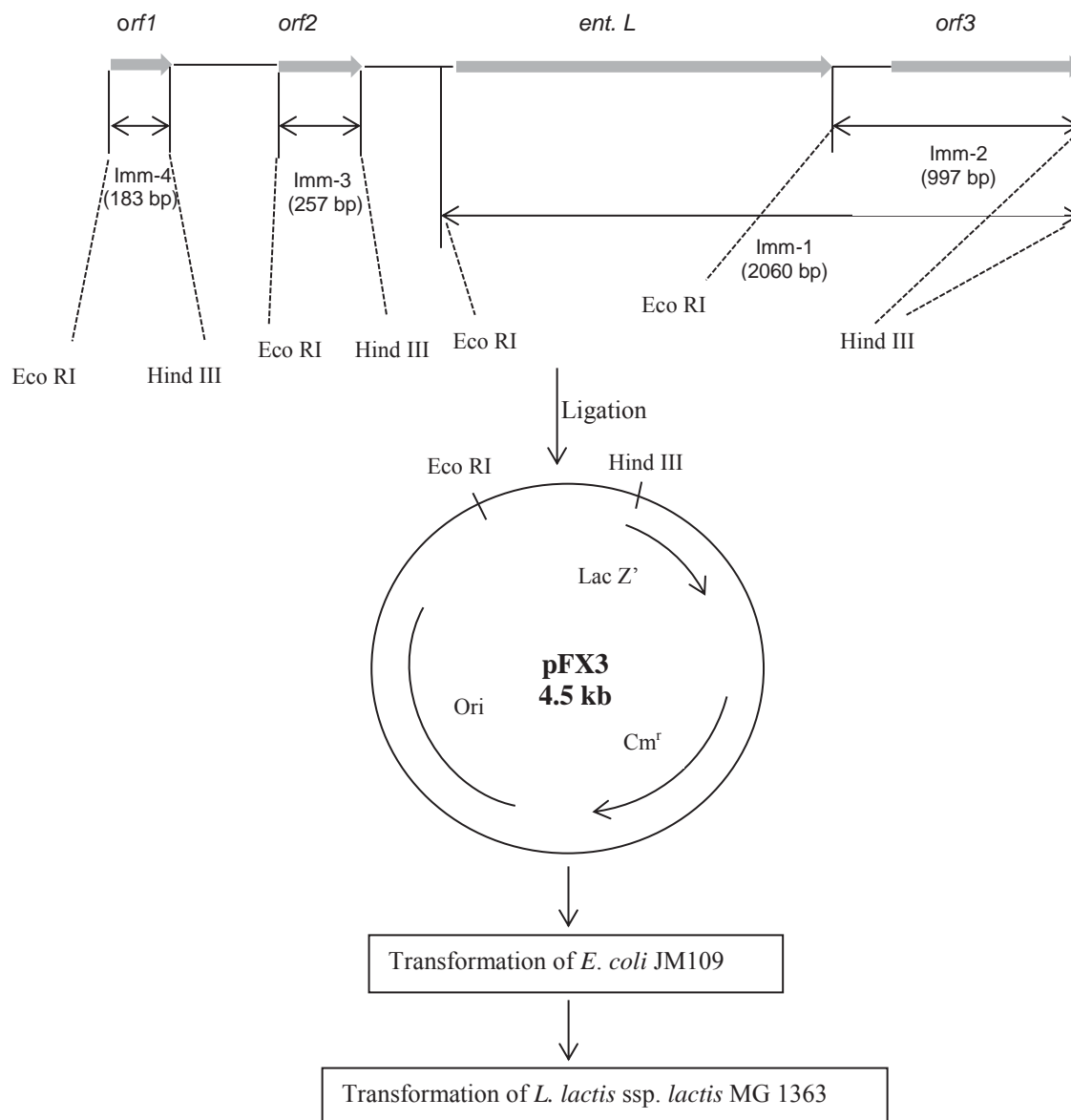


Figure 4.16 Cloning strategy for putative immunity regions. A pFX3 vector (Xu *et al.*, 1991) containing chloramphenicol resistance gene was linearized by digestion with Eco RI and Hind III restriction enzymes. The PCR amplified putative immunity regions (Imm-1, Imm-2, Imm-3 and Imm-4) were also digested with Eco RI and Hind III restriction enzymes, followed by ligation with pFX3 vector in separate reactions. Thus four pFX3 recombinants were obtained, which were then independently electroporated into *E. coli* JM109 competent cells followed by transformation of *L. lactis* ssp. *lactis* MG 1363. Cm^r = Chloramphenicol resistance gene. Ori = Origin of replication, Lac Z' = Lac. Z operon

The first primer pair amplified a 2060 bp region (*EntL+Orf1*) which included the enterolysin A structural gene as well as the region lying directly downstream to it which included the 530 bp gene. This amplified region was ligated to pFX3 vector (Xu *et al.*, 1991) and the construct thus obtained was used to transform the enterolysin A sensitive strain *L. lactis* ssp. *lactis* MG1363. However, no transformants were obtained when the transformed cells were grown on chloramphenicol (5 µg/ml) M17 agar plates. In the second attempt only the 997 bp region (which included the 530 bp gene) lying directly downstream of enterolysin A structural gene (named Imm-2) was amplified and ligated to the pFX3 vector. Electroporation of this construct in *L. lactis* ssp. *lactis* MG1363 resulted in successful transformants, as confirmed by PCR experiments. The transformants were tested for any acquired resistance against enterolysin A by agar well diffusion assay. However, the transformants were still sensitive to enterolysin. Based on these results it can be hypothesized that cloning the Imm-1 gene (which includes enterolysin A producing gene) into a sensitive strain results in the production of enterolysin A by the sensitive strain which causes autolysis of the cells and thus no transformants are obtained. If the gene located downstream of the structural gene would have been related to self-immunity then successful transformants containing the Imm-1 should have been obtained. This was further confirmed when only the gene located downstream of enterolysin A producing gene was transformed into sensitive strain and successful transformants were obtained but still found sensitive to enterolysin A. Therefore, it can be predicted that the gene lying directly downstream of enterolysin A structural gene may not be responsible for providing self-immunity to the producer strain.

Similar results were obtained when the two genes lying upstream of enterolysin A structural gene were PCR amplified and cloned into pFX3 vector. None of the gene inserts gave resistance to the *L. lactis* ssp. *lactis* 1363 and the transformants were still found sensitive to enterolysin A. This indicates that these three neighbouring genes are most probably not responsible for conferring self-immunity to enterolysin A producing strain.

4.11 Coating of enterolysin A on polyethylene film

An antimicrobial film with a coating of enterolysin A on LDPE was prepared along with a control film using methylcellulose as a carrier. Both the films were tested for antimicrobial activity against *L. casei* ML-1 as an indicator strain. The results of the assay are shown in Figure 4.17. The figure clearly shows the presence of a zone of inhibition around the enterolysin A film as compared to that of control film where no such zone of inhibition was observed.

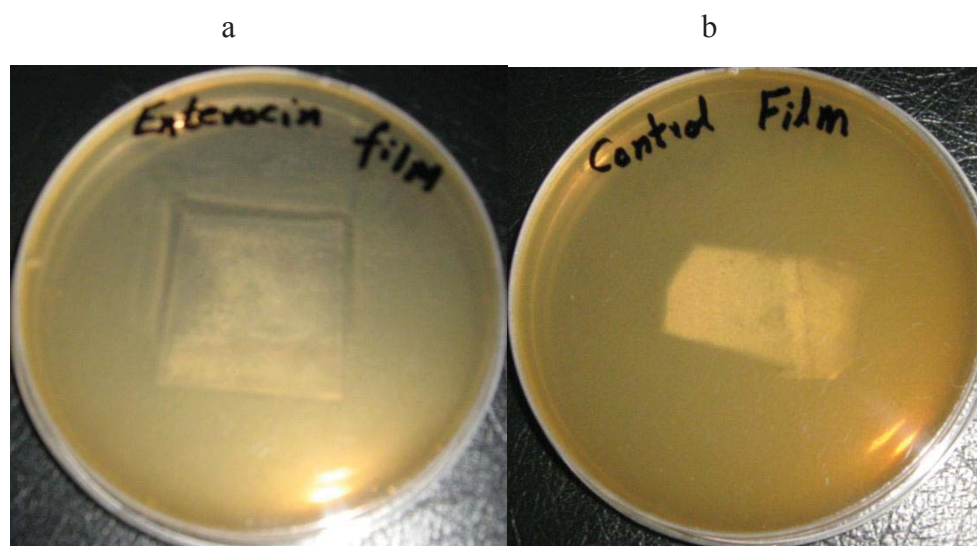


Figure 4.17 Antimicrobial activity of Enterolysin A film against the indicator strain *L. casei* ML-1. (a) Enterolysin A coated film (b) Control film without any enterolysin A.

Chapter 5 DISCUSSION OF RESULTS

5.1 Haemolytic activity and antibiotic resistance

Enterococcus is an important genus of the Lactic Acid Bacteria (LAB), whose members have been frequently found in the intestine of humans and animals, and some form an important component of the artisanal cultures used for preparation of traditional fermented dairy products (Giraffa 2003). However, many enterococcal strains acquire various pathogenic traits such as haemolytic activity in a clinical environment and have been reported to be involved in nosocomial infections (Kayser 2003). Many of these develop resistance against antibiotics. These also include bacteriocin producers such as *E. faecalis* FA2-2 which produces cytolysin (Booth *et al.*, 1996) and *E. faecalis* DPC5280 which produces cytolysin as well as enterolysin A (Hickey *et al.*, 2003). These strains particularly those resistant against vancomycin are of particular concern since vancomycin is often the last resort for treatment of enterococcal infections. Therefore, only the strains which are non-haemolytic and sensitive to commonly used antibiotics especially vancomycin can be regarded as safe, especially when they are intended to be incorporated directly into a food product (De Vuyst *et al.*, 2003). Therefore, *E. faecalis* B9510 was also tested for presence of any haemolytic activity or antibiotic resistance to determine its safety especially for any possible future experiments involving the direct incorporation of this strain in a food product for *in situ* bacteriocin production. The strain was found non-haemolytic and also sensitive to commonly used antibiotics such as tetracycline, ampicillin, chloramphenicol and vancomycin and considered suitable for bacteriocin production and characterization studies.

5.2 Preliminary characterization of the bacteriocin-like substance

The LAB produce a number of substances which have antimicrobial properties. These include acids, ethanol, hydrogen peroxide, aldehydes and bacteriocins (De Vuyst and Leroy 2007). Therefore, it was considered necessary to do preliminary characterization of the antimicrobial substance produced by *E. faecalis* B9510, before detailed studies on its production and identification can be carried out. The most important property of bacteriocins is their proteinaceous nature which distinguishes them from other antimicrobials. To confirm the proteinaceous nature the supernatant of growth

medium showing antimicrobial activity was treated with proteolytic and non-proteolytic enzymes. The loss of antimicrobial activity after treatment with proteolytic enzymes confirmed the proteinaceous nature and gave a strong indication that *E. faecalis* B9510 is producing a bacteriocin. Furthermore, the expression of antimicrobial activity under neutral pH conditions and in the presence of catalase eliminated the possibility of any antimicrobial effect due to the presence of any acid or hydrogen peroxide. Similar results have been reported by various researchers during the preliminary characterization of bacteriocins and the loss in activity was observed only after treatment with proteolytic enzymes (Park *et al.*, 2003; Todorov and Dicks 2006; Ben Belgacem *et al.*, 2008).

After the confirmation that the antimicrobial activity was due to a bacteriocin the next step was to test the heat and pH sensitivity of bacteriocin. Heat sensitivity of bacteriocin is an important criterion and can help to determine whether the bacteriocin belongs to the class of heat-labile large proteins or is a heat-stable peptide (Heng *et al.*, 2007). In the current study it was observed that the bacteriocin was heat-labile being inactivated when heated at 60 °C for 60 minutes. This was a positive indication that *E. faecalis* B9510 is most probably producing a large antimicrobial protein (>10 kDa) belonging to class III of bacteriocins (Heng *et al.*, 2007).

5.3 Bacteriocin production in defined medium

After preliminary characterization the next step is production of bacteriocin in large batches followed by purification and identification at the amino acid level. For this purpose the bacteriocin producing strains are usually grown in complex commercial media rich in peptides and proteins due to the fastidious nature of LAB. Since the bacteriocins themselves are proteinaceous in nature they are very difficult to purify from these complex media, especially when the bacteriocin to be identified is very closely related to the peptides and proteins of the growth medium. Furthermore, the bacteriocins are produced and secreted in very small quantities in the production broth and, therefore, need to be concentrated with the simultaneous removal of closely related contaminants from the medium as well as from other proteins or proteinaceous substances produced by the bacterium during growth. This increases the complexity of the purification protocol which normally consists of one or two initial steps to reduce the working volume, followed by a series of several chromatography steps such as ion exchange, gel filtration or RP-HPLC (Reverse Phase-High Performance Liquid Chromatography) to achieve significant purification. There are losses at each step, and sometimes the entire

bacteriocin activity may be lost during purification. For these reasons, many strains of bacteria have been described to produce bacteriocins or bacteriocin like substances, but only a few have been purified to homogeneity, identified and characterized (Parada *et al.*, 2007).

An alternative approach to minimize the number of purification steps is to grow the producing strain in a completely defined medium devoid of any peptides and proteins. To fulfill the nutritional requirements crystalline amino acids and pure B vitamins can be incorporated in the defined medium in appropriate quantities along with mineral salts. Such chemically defined media have usually been developed for determination of the nutritional requirements of various bacterial strains (Moretro *et al.*, 1998; Letort and Juillard 2001; Hebert *et al.*, 2004; Torino *et al.*, 2005; Saguir and de Nadra 2007; Terrade *et al.*, 2009; Zhang *et al.*, 2009), and only few studies describe the production of bacteriocins in defined media, which include production of nisin (De Vuyst 1995), sakacin P (Moretro *et al.*, 2000), pregallidermin (Medaglia and Panke 2010) and salivaricin 1328 (Pingitore *et al.*, 2009). The current study explored the bacteriocin production by *E. faecalis* B9510 in a completely defined medium and is the first report of the production of a bacteriocin by an *Enterococcus* species in a chemically defined medium.

The original Complete Defined Medium (CDM) consisted of 39 components, which included amino acids, B vitamins, nucleic acid bases and minerals (Murray *et al.*, 1993). The effect of these components on the growth rate of bacteriocin producing strain (*E. faecalis* B9510) was studied by single omission/Leave One Out (LOO) technique (Zhang *et al.*, 2009).

The experiments to determine the essential amino acids revealed that eight amino acids viz. arginine, glycine, histidine, isoleucine, leucine, methionine, tryptophan and valine were necessary for the growth of *E. faecalis*, whereas, cysteine, lysine, phenylalanine, serine and threonine were found to have a stimulatory effect. Similar findings were reported in an earlier study in which it was observed that histidine, isoleucine, methionine and tryptophan were essential for the growth of all *E. faecalis* strains tested, whereas absence of arginine, glutamic acid, glycine, leucine or valine resulted in no or poor growth for some strains. Similarly serine and threonine were also found to enhance the growth of some of the *E. faecalis* strains (Murray *et al.*, 1993). A recent study for the development of a simplified defined medium for the lactococci, enterococci and streptococci, however, states the necessity of only six amino acids for

good growth, and these are arginine, histidine, isoleucine, leucine, methionine and valine (Zhang *et al.*, 2009). In another study growth and nisin production by a strain of *L. lactis* ssp. *lactis* NIZO 22186 was tested in a minimal synthetic medium (De Vuyst 1995). The omission of single amino acids from the medium indicated that histidine, isoleucine, arginine, threonine, leucine, valine, methionine and glutamic acid were essential for growth. The results are also similar to the current study with the exception of glycine, which has been found essential for the growth of *E. faecalis* B9510.

Another interesting fact observed in the current study was that glutamine and glutamic acid seemed to be non-essential for growth during single omission experiments. However, no growth was observed when a simplified medium was formulated omitting both amino acids, indicating that one of them needs to be present in the defined medium for supporting growth. Similar results have been reported while studying the nutritional requirements of *Lactobacillus delbrueckii* ssp. *lactis* in a defined medium (Hebert *et al.*, 2004).

The B vitamins are also essential for the growth of LAB and, therefore, must be provided in the medium. However, the specific requirement of B vitamins can vary among various species and strains. In case of *E. faecalis* B9510, the omission experiments indicated that pyridoxal, nicotinic acid and Ca-pantothenic acid were essential for the growth, whereas folic acid and riboflavin were found stimulatory for the growth of *E. faecalis* B9510. These vitamins have also been found essential when studying the nutritional requirements of other LAB species. *L. delbrueckii* ssp. *lactis* CRL 581 required nicotinic acid, pyridoxal and pantothenic acid for growth, while *L. delbrueckii* ssp. *lactis* CRL 654 required only niacin and pantothenic acid for growth, while riboflavin and cyanocobalamin were found stimulatory for both strains (Hebert *et al.*, 2004). *L. helveticus* ATCC 15807 failed to grow in a chemically defined medium devoid of nicotinic acid, Ca-pantothenic acid, pyridoxal and riboflavin (Torino *et al.*, 2005). Similarly growth studies of wine LAB in a chemically defined medium showed that Ca-pantothenic acid and nicotinic acid were essential for all the strains tested while riboflavin was found essential for only two strains (Terrade *et al.*, 2009). Experiments with several *S. thermophilus* strains (ST1, ST7, ST8, ST11, ST18, ST21) indicated that riboflavin was essential for growth for all strains, while Ca-pantothenic acid and nicotinic acid were found to be required for growth by four strains only (Letort and Juillard 2001).

None of the nucleic acid bases was found essential for growth, but uracil was found to be stimulatory for growth. Further experiments during formulation of a simplified

defined medium revealed that absence of both adenine and guanine resulted in a long lag phase and lower growth rate when compared with a complete defined medium. This was corrected by addition of adenine which helped in shortening the lag phase and getting results similar to that of CDM. A similar trend was observed when two *L. delbrueckii* ssp. *lactis* strains failed to grow in the absence of both the purines, and the addition of adenine and guanine resulted in a growth rate similar to that of CDM (Hebert *et al.*, 2004). In the present study, however, the addition of only one purine base, i.e. adenine was found sufficient to give a growth rate similar to that of CDM.

In addition to the organic nutrients, mineral salts also play a significant role in the growth of bacteria, and are normally a part of many commercial media such as M17 and MRS (De Man *et al.*, 1960; Terzaghi and Sandine 1975). The single omission of the minerals from the CDM showed that only magnesium sulphate was essential for growth, while ferrous sulphate and potassium dihydrogen phosphate were stimulatory. The necessity of magnesium and phosphate has also been reported by other researchers working on various LAB species (Saguir and de Nadra 2007; Terrade *et al.*, 2009; Zhang *et al.*, 2009).

The above discussion shows the auxotrophy of amino acids, B vitamins, nucleic acid bases and minerals by various LAB species. This is expected due to the fastidious nature of LAB, and their inability to synthesize many of these nutrients by themselves (Terrade *et al.*, 2009). However, exact requirements of various components are genus, species and strain specific. Some microorganisms such as *S. thermophilus* can be grown in a simplified or minimal medium consisting of only 20 components (Letort and Juillard 2001), while some extremely fastidious lactobacilli require 34 components (Moretro *et al.*, 1998). In the present study a Simplified Defined Medium (CDM) was developed which consisted of 26 components (Table 1). The growth rate in this medium was comparable to that of CDM, and it was selected for bacteriocin production and comparison studies.

The growth and bacteriocin production in SDM was compared with a modified M17 medium (containing 15 g/L glucose). A visible lag phase was observed during the first hour of growth in the defined medium, while no such lag was detected in the M17 medium. However, afterwards the growth trend and bacteriocin production in defined was almost similar to that of M17. The comparable performance of a defined medium to that of a complex medium is also an achievement because simplified defined media usually give a lower growth rate and bacteriocin production (De Vuyst 1995). In contrast a

different trend was observed when the growth and bacteriocin production by *L. salivarius* CRL 1328 strain was observed in a defined medium and a complex medium (LAPTg). The specific growth rate was higher in the complex medium but the bacteriocin production in both the media was similar (Pingitore *et al.*, 2009).

To conclude this work has revealed new information on the nutritional requirements of an enterococcal strain and its suitability for the bacteriocin production in a Simplified Defined Medium (SDM). The production of bacteriocins in chemically defined media offers the possibility of reducing the number of steps involved in purification, thereby helping to minimize the losses during downstream processing and avoid the risk of complete loss of bacteriocin activity during purification. This strategy can be helpful in the identification and characterization of novel bacteriocins from various LAB strains.

5.4 Purification, characterization and identification of the bacteriocin

As mentioned in the preceding section *E. faecalis* B9510 was grown in the defined medium with the aim to minimize the number of purification steps. Therefore, ultrafiltration was used as the only step for purification of cell-free supernatant from defined medium. The retentate of 30 kDa showing the maximum activity was used for further characterization and identification of bacteriocin. When run on an SDS polyacrylamide gel a single major band was observed in the gel (Figure 4.3) and was easily identified as bacteriocin band when a single zone of clearance was observed around the 35 kDa band in a parallel zymogram gel containing the dead cells of *L. lactis* ssp. *cremoris* 2144, indicating that the bacteriocin has been purified to homogeneity. This clearly indicates that defined medium can be used as a strategy to minimize the number of purification steps. Similar results were obtained when *L. salivarius* CRL 1328 was grown in a chemically defined medium and the ultrafiltered supernatant was subjected to tricine SDS-PAGE. A major band at 4.5 kDa was shown to have antimicrobial activity due to salivarin CRL1328 (Pingitore *et al.*, 2009).

To determine the molecular mass and also the amino acid sequence, the active band from the SDS gel was excised and analysed by mass spectrometry. Mass spectrometry in recent years has developed as a technique for the identification of already existing and novel bacteriocins based on accurate mass measurements. In the present study mass spectrometry was successfully used to identify the unknown bacteriocin produced by *E. faecalis* B9510. The bacteriocin was identified as enterolysin A a cell wall degrading lytic bacteriocin which has been reported to be produced by many enterococcal

strains which include *E. faecalis* DPC5280, a raw milk isolate (Hickey *et al.*, 2003) and *E. faecalis* II/1, a ruminal isolate (Nigutova *et al.*, 2007). In addition the gene has been reported to be present in *E. faecalis* P13CS2 isolated from partridge (Almeida *et al.*, 2011), a number of isolates from faeces and blood (Hassan *et al.*, 2012) as well as from ruminal isolates (Nigutova *et al.*, 2007). In addition to *E. faecalis* some related species have also been reported to carry the enterolysin A gene which include *Enterococcus malodoratus* NCDO846 and *Streptococcus bovis* isolates (Nigutova *et al.*, 2007). The current study is, therefore, the first report of production of enterolysin A by an isolate from silage in New Zealand.

5.5 Antimicrobial spectrum of enterolysin A

A comparison of antimicrobial activity of enterolysin A with other lytic bacteriocins show that in contrast to the majority of bacteriolysins it is a broad spectrum bacteriocin active against many species of Gram-positive bacteria particularly against LAB. Lystostaphin and its closely associated lysins ALE-1 and lysostaphin M are only active against *S. aureus* strains (Ramadurai and Jayaswal 1997; Sugai *et al.*, 1997; Bastos *et al.*, 2010). Similarly zoocin A is also a narrow spectrum lysin active only against selected members of the genus *Streptococcus*. However, millericin B is a broad spectrum bacteriolysin found to be active against non-producer strains of *S. milleri*, *Micrococcus luteus*, *L. monocytogenes*, *Streptococcus agalactiae* and *L. lactis* (Beukes *et al.*, 2000). Thus millericin B is more similar to enterolysin A as compared to other bacteriolysins. This is also evident from the 44% sequence identity of the two bacteriocins which is much higher as compared to sequence identity of enterolysin A with other bacteriolysins.

5.6 Mode of action of Enterolysin A

The majority of bacteriocins produced by LAB are peptides and proteins which act primarily on the cell membrane of the sensitive bacterial strains. In contrast the primary target of the lytic bacteriocins is the cell wall of the Gram-positive bacteria. The cell wall of the Gram-positive bacteria is much thicker (15 - 30 nm) compared to that of the Gram-negative bacteria (2 – 3 nm). In Gram-negative bacteria the cell wall is surrounded by an additional outer layer of lipopolysaccharides and lipoproteins, which is absent in Gram-positive bacteria. In the absence of this outer layer the cell wall is the sole protective layer in Gram-positive bacteria which helps to maintain the cell shape and size and protect against osmotic lysis (Vollmer *et al.*, 2008).

The cell wall of the Gram-positive bacteria is made up of alternating units of N-acetyl glucosamine (NAG) and N-acetyl muramic acid (NAM) linked by β -1 \rightarrow 4 bonds. In each NAM residue the D-lactoyl group is replaced by a side peptide chain of four amino acids which is called stem peptide. Two adjacent stem peptides are connected by cross-linked peptide chains also known as an interpeptide bridge. While the composition of stem peptides is more or less the same across the species, the cross-linked peptides vary in their composition and are species specific. The stem peptides and cross linked chains are the target for lytic bacteriocins. After the cleavage of a specific peptide bond, depending on the nature of bacteriolysin, the insoluble peptidoglycan layer is solubilized; the cell wall cannot maintain its structure and is destroyed. After the destruction of cell wall the cell membrane can no longer withstand the cell's internal turgor of up to 25 atmospheres resulting in lysis of the cell (Maliničová *et al.*, 2010).

The lytic activity of enterolysin A was tested in the current study when crude enterolysin A was added to a cell suspension of sensitive strain *L. lactis* ssp. *cremoris* 2144. A rapid decrease in optical density with the concomitant decrease of total viable counts was observed. Similar results have been reported when enterolysin A was added to the purified cell walls of *L. lactis* IL1403 and *E. faecium* CTC492 (Nilsen *et al.*, 2003) or to a suspension of live cells of *L. lactis* HP (Hickey *et al.*, 2003). These results clearly indicate that enterolysin A is a cell wall degrading bacteriocin and kills the cells of sensitive strains by solubilisation of peptidoglycan units of cell walls.

When the enterolysin A treated cells of *L. lactis* ssp. *cremoris* 2144 were visualized by TEM a lysis of cell wall and release of cytoplasmic material was observed as compared to control cells which were intact (Figure 4.12 A to H). Similar observations were recorded when zoocin A treated cells of *Streptococcus pyogenes* FF22 were examined by TEM and a clear separation of cell membranes from cell walls was observed. The late stages of zoocin A treated cells showed complete loss of cytoplasmic structure resulting in ghost cells (Simmonds *et al.*, 1996). When cells of *Staphylococcus aureus* NCTC 4163 were treated with SMAP29 (an antimicrobial peptide isolated from sheep blood), damaged cells without cell walls and leaking of cytoplasmic material was obvious (Anderson *et al.*, 2004)

While the lytic activity of enterolysin A has been reported in earlier studies the exact site of cleavage within the peptidoglycan units of sensitive strains has not been described. An attempt was made in the current study to find out the site of cleavage of enterolysin A. Purified cell walls of three sensitive strains *L. delbrueckii* ssp. *bulgaricus* ATCC 11842,

L. lactis ssp. *cremoris* 2144 and *L. casei* ML-1 were digested with enterolysin A and derivatized for the N and C terminal of liberated amino acids. The results indicate that enterolysin A cleaves the interpeptide bonds within the peptidoglycan units at two locations (Figure 4.14). The first location is within the stem peptide between L-alanine and D-glutamic acid while the second location is between L-lysine of stem peptide and D-aspartic acid of interpeptide bridge. A comparison of these results with the list of sensitive species (Table 4.7) and the cell wall composition of these species (Schleifer and Kandler 1972) reveals that the first cleavage site within the stem peptide is present in all sensitive strains which include *L. monocytogenes* NCTC 10884, *S. aureus* NCTC 4163 and *E. faecalis* No. 40. However, the second cleavage site between L-lysine of stem peptide and D-aspartic acid is only available in *L. helveticus*, *L. casei*, *L. delbrueckii* ssp. *bulgaricus*, *P. pentosaceus*, *L. lactis* ssp. *lactis*, *L. lactis* ssp. *cremoris* and *L. helveticus* (Kandler 1970). The presence of a cleavage site within the interpeptide bridge gives a possible explanation of higher antimicrobial activity of enterolysin A against these species as compared to *L. monocytogenes*, *E. faecalis* and *S. aureus*. The interpeptide bridge within the peptidoglycan units gives considerable strength to the cell walls of Gram-positive bacteria. In fact it is the cleavage of a bond within the interpeptide bridge which brings about solubilisation of water-insoluble peptidoglycan units rather than the cleavage of a bond within the stem peptide thus ultimately causing the rapid decrease in optical density of cell suspensions (Strominger and Ghuyesen 1967). This gives one of the possible reasons of failure of detection of antimicrobial activity of enterolysin A against *L. monocytogenes*, *E. faecalis*, *B. subtilis*, *B. cereus*, *S. aureus*, and *Propionibacterium jensenii* in the microtitre plate assay which relies on optical density measurements. The antimicrobial activity for these organisms is only detectable in agar overlay assays (Nilsen *et al.*, 2003). These bacterial species do not have D-aspartic acid in their interpeptide bridge and, therefore, no solubilisation of cell walls occurs (Schleifer and Kandler 1972; Pucciarelli *et al.*, 2007).

The cleavage of interpeptide bonds at two locations gives a possible explanation of the broad spectrum of activity of enterolysin A similar to that of millericin B which is also a broad spectrum lysin and cleaves the peptide bonds at two locations. The cleavage site in the stem peptide is the same for enterolysin A and millericin B, however, the interpeptide cleavage site is different for the two bacteriocins. Enterolysin cleaves the bond between L-lysine of stem peptide and D-aspartic acid of interpeptide bridge. This type of interpeptide bridge is present in the majority of members of the family

Lactobacillaceae (Kandler 1970) and enterolysin A is, therefore, particularly active against members of this family. Millericin B cleaves the bond between L-threonine and L-alanine in the interpeptide bridge and is, therefore, very active against the species having this type of interpeptide bridge such as non-producer strains of *S. milleri*.

In contrast to enterolysin A and millericin B the other lytic bacteriocins i.e. lysostaphin and zoocin A have narrow spectrum of activity as they only cleave the bond within interpeptide bridge of cell walls. Lysostaphin has been found to target only the pentaglycine cross-linking peptides of all known staphylococcal species (Schleifer and Kandler 1972; Grundling and Schneewind 2006). Similarly zoocin A targets the sensitive streptococcal strains which are closely related to the producer strain. These include *Streptococcus oralis*, *S. pyogenes*, *Streptococcus equi* and *Streptococcus gordonii*. The interpeptide bridge of all these species consist of two to three L-alanine residues, and experiments have shown that zoocin A acts as D-alanyl - L-alanine endopeptidase which hydrolyzes the bond between the terminal D-alanine of the stem peptide and the first L-alanine of the cross-linking bridge (Simmonds *et al.*, 1996; Gargis *et al.*, 2009b). Therefore, it can be concluded that enterolysin A is more closely related to millericin B as compared to other bacteriolysins in terms of spectrum of activity.

5.7 Immunity gene of Enterolysin A

All the cell wall degrading enzymes which have been classified as bacteriocins provide self-immunity to the producer strain. The factors responsible for conferring this self-immunity have been identified for several of these lytic bacteriocins. However, no immunity gene has been described for enterolysin A, therefore, an attempt was made in the current study to identify the gene responsible for conferring self-immunity to the producing strain of enterolysin A.

The bacteriocin related immunity genes are found in close proximity to structural genes often organized in operons. Similarly the immunity genes for conferring immunity to producing strains of zoocin A (Beatson *et al.*, 1998), lysostaphin (Thumm and Gotz 1997) and millericin B (Beukes and Hastings 2001) have been found directly downstream of the structural gene for these bacteriolysins. Furthermore, the sequence homology (as determined from NCBI database) of *orf3* (lying directly downstream of enterolysin A structural gene) indicated that this open reading frame encodes a protein that is predicted ($7.71e^{-23}$) to be a peptidase_C39_2 (peptidase_C39 like family) protein, and that C39 peptidase domains are often found at the N-terminus of dedicated proteolytic ABC

transporter involved in the export of peptide bacteriocins. The C_39 peptidase domains cleave double glycine leader sequences, and enterolysin A contains four GG motifs (FNRGGTYF; DPVGGGSL; ILQGGSTP and RVLGGSWL). Since the proteolytic degradation of bacteriocins by proteases in the cell envelopes of producer strains is an acceptable mechanism of self-immunity. Therefore, it was hypothesized that *orf3* may be responsible for conferring self-immunity gene to the enterolysin A producing strain. Based on this assumption *orf3* was cloned into an enterolysin A sensitive strain (*L. lactis* ssp. *lactis* MG1363). However, no acquired self-immunity was observed. Similar results were obtained when the genes (*orf1* and *orf2*) lying upstream of enterolysin A structural gene were cloned into an enterolysin A sensitive strain. Therefore, it can be concluded that unlike most other bacteriocins which include the bacteriolysins, the immunity gene of enterolysin A is not located in close proximity to the structural gene. It may be possible that more than one gene is responsible for conferring self-immunity or the immunity may operate by a different mechanism which may be the absence of cleavage site in the interpeptide bridge of *E. faecalis* species (as indicated in the mode of action studies) or may be due to the absence of specific enterolysin A binding receptor in the producer strain (Piknová *et al.*, 2011).

5.8 Enterolysin A as potential food preservative

Bacteriocins produced by LAB are considered safe natural antimicrobials for utilization as food preservatives (Cleveland *et al.*, 2001). The current project was, therefore, aimed at the identification of a novel bacteriocin with a potential to be used as a food preservative against pathogenic and spoilage bacteria. The bacteriocin identified was, however, enterolysin A a heat-labile bacteriocin known to kill the sensitive bacterial strains by lysis of cells.

Heat-labile bacteriocins cannot be added to foods during processing since they are prone to inactivation during any heating step. Furthermore, any bacteriocin which completely lyses the cells cannot be used against pathogenic bacteria since the lysis can release the toxins from within the cells into the food product thus making it unsafe for consumption (Heng, N.C.K., personal communication).

Therefore, the only option of utilization of enterolysin A as a food preservative is to use it against spoilage bacteria for prevention of post-processing contamination. For this purpose the bacteriocin can either be added directly to the food product after processing or can be coated on the packaging material. The direct incorporation of bacteriocin in the

food product has certain disadvantages. It may diffuse rapidly in the food product or may be inactivated completely or partially by the food constituents. The other alternative is the innovative concept of active packaging which involves coating of antimicrobial substance on the packaging films. When the food product is packaged with such film, the antimicrobial substance is slowly released onto the surface of food product thus providing protection for extended periods (Quintavalla and Vicini 2002).

Keeping in view the benefits of antimicrobial packaging, enterolysin A was coated onto the surface of LDPE film and was found to effectively inhibit the growth of *L. casei* ML-1 on the agar surface (Figure 4.17). Similar results have been reported by (Mauriello *et al.*, 2004) when an antilisterial bacteriocin 32Y produced by *L. curvatus* was tested against *L. monocytogenes* V7. The bacteriocin 32Y was found to effectively control the growth of *L. monocytogenes* on the agar surface as indicated by the zone of clearance around the treated film similar to that obtained in the current study against *L. casei* ML-1.

L. casei has often been involved in spoilage of food products such as pickled cucumbers (Perez-Diaz *et al.*, 2007). Therefore, utilization of enterolysin A containing packaging films is a good alternative to control the growth of *L. casei* (or other spoilage microorganisms against which enterolysin A is active) in food products.

Chapter 6 CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

This study aimed at the identification and characterization of the bacteriocin produced by *E. faecalis* B9510, a local isolate. Following are the main conclusions which can be drawn from the results obtained in this study:

1. *E. faecalis* B9510 produces enterolysin A, a cell wall-degrading bacteriocin.
2. The use of a chemically defined medium for bacteriocin production greatly helped in the downstream purification and only an ultrafiltration step was sufficient to purify the bacteriocin to homogeneity as indicated by a single active band in a SDS-PAGE experiment.
3. Enterolysin A has a broad spectrum of activity. In addition to the strains and species already reported to be sensitive against enterolysin A, the current study also showed that enterolysin A is also active against *L. delbrueckii* ssp. *bulgaricus* ATCC 11842, *L. helveticus* 1113 and *L. casei* ML-1.
4. Enterolysin A is a cell wall degrading endopeptidase bacteriocin which cleaves the interpeptide bonds within the cell walls of sensitive species at two points. The first location is between L-alanine and D-glutamic acid of the stem peptide and the other location is between L-lysine of the stem peptide and D-aspartic acid of interpeptide bridge of peptidoglycan units.
5. The gene located directly downstream of the enterolysin A structural gene is not responsible for providing self-immunity to the host strain.
6. Enterolysin A coated on polyethylene films can successfully control the growth of sensitive strains as indicated by the zones of inhibition on the lawn of sensitive indicator strain *L. casei* ML-1.

6.2 Future Recommendations

Enterolysin A is a well-studied bacteriocin which has been further characterised by the present study. However, there are some areas which need to be studied as indicated below:

1. An attempt was made in the current study to find out the gene responsible for conferring self-immunity to *E. faecalis* B9510 against enterolysin A. However, the self-immunity gene could not be elucidated. Therefore, further investigations are needed to determine the self-protection mechanism of *E. faecalis* B9510.
2. Enterolysin A coated polyethylene film was shown to control the growth of sensitive strain by agar well diffusion assay. Further studies can be designed to check the efficacy of enterolysin A coated antimicrobial films against sensitive spoilage strains in a real food environment.

PUBLICATIONS AND CONFERENCE PRESENTATIONS

1. Khan H., Flint, S.H. and Yu, P-L. 2013. Development of a chemically defined medium for the production of enterolysin A from *Enterococcus faecalis* B9510. *Journal of Applied Microbiology*. doi: **10.1111/jam.12115**.
2. Khan H., Flint, S.H. and Yu, P-L. 2013. Determination of mode of action of enterolysin A produced by *Enterococcus faecalis* B9510. (Submitted to *Journal of Applied Microbiology*).
3. Khan H., Flint, S.H. and Yu, P-L. 2011. Identification and characterization of bacteriocin produced by *Enterococcus faecalis* B9510. Presented at the 2011 New Zealand Microbiological Society (NZMS) Annual Conference held at Palmerston North, New Zealand, November, 2011.
4. Khan H., Flint, S.H. and Yu, P-L. 2010. Enterocins in food preservation. *International Journal of Food Microbiology*. **141**: 1-10.
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APPENDICES

APPENDIX 1

Results of API Rapid ID32 Strep kit identification

Cupule label	Test	Active ingredient	Reactions/Enzymes	Result
1.0	ADH	L-arginine	Arginine DiHydrolase	+
1.1	β GLU	resorufin- β D-glucopyranoside	β GLUcosidase	+
1.2	β GAR	resorufin- β D-galactopyranoside	β GALactosidase	+
1.3	β GUR	resorufin- β D-glucuronide	β GIUcURonidase	-
1.4	α GAL	α GAL 4-nitrophenyl- β D-galactopyranoside	α GALactosidase	-
1.5	PAL	4-nitrophenyl- β D-galactopyranoside-2-CHA	ALkaline Phosphatase	-
1.6	RIB	D-ribose	RIBose (Acidification)	+
1.7	MAN	D-mannitol	MANnitol (Acidification)	+
1.8	SOR	D-sorbitol	SORbitol (Acidification)	+
1.9	LAC	D-lactose (bovine origin)	LACtose (Acidification)	+
1.A	TRE	D-trehalose	TREhalose (Acidification)	+
1.B	RAF	D-raffinose	RAFfinose (Acidification)	-
1.C	SAC	D-saccharose (sucrose)	SACcharose (Acidification)	+
1.D	LAR	L-arabinose	L-ARAbinose (Acidification)	+
1.E	DARL	D-arabitol	D-ARAbitoL (Acidification)	-
1.F	CDEX	α cyclodextrin	CycloDEXtrin (Acidification)	+
0.0	VP	sodium pyruvate	Acetoin production (Voges Proskauer)	+
0.1	APPA	L-alanyl-L-phenylalanyl-L-proline β -naphthylamide	Alanyl-Phenylalanyl-Proline Arylamidase	-
0.2	β GAL	2-naphthyl- β D-galactopyranoside	β GALactosidase	-
0.3	PyrA	pyroglutamic acid- β -naphthylamide	Pyroglutamic acid Arylamidase	+
0.4	β NAG	6-bromo-2-naphthyl-N-acetyl β D-glucosaminide	N-Acetyl β -Glucosaminidase	-
0.5	GTA	L-glycyl-L-tryptophan β -naphthylamide	Glycyl-Tryptophan Arylamidase	+
0.6	HIP	sodium hippurate	Hydrolysis of HIPpurate	+
0.7	GLYG	glycogen	GLYcoGen (Acidification)	-
0.8	PUL	pullulan	PULlulane (Acidification)	-
0.9	MAL	D-maltose	MALtose (Acidification)	+

0.A	MEL	D-melezitose	MeLeZitose (Acidification)	+
0.B	MLZ	D-melezitose	MeLeZitose (Acidification)	+
0.C	MBDG	methyl- β D-glucopyranoside	Methyl- β D Glucopyranoside (Acidification)	+
0.D	TAG	D-tagatose	TAGatose (Acidification)	+
0.E	β MAN	4-nitrophenyl- β D-mannopyranoside	β MANnosidase	-
0.F	URE	urea	UREase	-

Identification: *Enterococcus faecalis*

%ID = 99.9%

APPENDIX 2

Identification of enterococcal isolate at species level

1.1 Polymerase Chain Reaction (PCR)

PCR was carried out to amplify 16S rRNA gene for identification of B9510 at species level.

1.2 Primers for amplification of 16S RNA gene:

(i) For amplification of first 772 bp:

Forward primer: 5'-AGAGTTTGATCCTGGCTCAG-3'

Reverse primer: 5'-CCTCAGCGTCAGTTACAG-3'

(ii) For amplification of last 786 bp:

Forward primer: 5'-GACGCTGAGGCTCGAAAGCG-3'

Reverse primer: 5'-GGTGATCCAGCCGCACCT-3'

Complete 16S RNA gene sequence of *E. faecalis* B9510 (Experimentally determined by PCR followed by sequencing):

AGAGTTTGATCCTGGCTCAGGACGAACGCTGGCGGCGTGCCTAATACATGCA
AGTCGAACGTTTCTTTTCTCCCGAGTGCTTGCCTCAATTGGAAAGAGGAGT
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GTGGATC

APPENDIX 3

Mass spectrometry analysis of active band from SDS-PAGE gel

Mascot Search Results (Protein View)

Match to: **gi|11078549** Score: **831**

Enterolysin A [Enterococcus faecalis LMG2333]

Found in search of 11183_B1_EnLA_conc.txt

Nominal mass (M_r): **37300**; Calculated pI value: **9.30**

NCBI BLAST search of gi|11078549 against nr

Unformatted sequence string for pasting into other applications

Taxonomy: Enterococcus faecalis LMG2333

Links to retrieve other entries containing this sequence from NCBI Entrez:

gi|257087105 from Enterococcus faecalis D6

gi|257419583 from Enterococcus faecalis T11

gi|256987309 from Enterococcus faecalis JH1

gi|256995135 from Enterococcus faecalis D6

gi|257161411 from Enterococcus faecalis T11

Variable modifications: Carbamidomethyl (C), Deamidated (NQ), Gln->pyro-Glu (N-term Q), Glu->pyro-Glu (N-term E), Oxidation (M)

Cleavage by Trypsin: cuts C-term side of KR unless next residue is P

Sequence Coverage: **58%**

Matched peptides underlined

1 MKNILLIILG VLSIVVSLAF SSSVNAASN ESWPLGKPY AGRYEEGQOF
 51 GNTAFNRGGT YFHDGDFGS AIYNGSVYA VHDGKILLYAG WDPVGGGSLG
 101 AFIVLQAGNT NVIYQEFRN VGDIKVSTGQ TVKKGQLIGK FTSSHLHLGM
 151 TKKEWRSAHS SWNKDDGTFW NPIPILQGG TPTPNPGPK NFTTNVRYGL
 201 RVLGGSWLPE VTNFNNTNDG FAGYPNRQHD MLYIK VDKG QMKYRVHTAQ
 251 SGWLPWVSKG DKSDITVNGAA GMPGQAIDGV QLNYIITPKGE KLSQAYYRSQ
 301 TTKRSGWLKY SADNGSIPGL DSYAGIFGEP LDRLQIGISQ SNPF

Ions score is $-10 \cdot \log(P)$, where P is the probability that the observed match is a random event. Individual ions scores > 60 indicate identity or extensive homology ($p < 0.05$). Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.

Start - End	Observed	Mr (expt)	Mr (calc)	ppm	Miss	Sequence	(Ions score)
9 - 22	1660.7354	1659.7281	1659.7277	0	0	R.YEEGQQFGNTAFNR.G	112
23 - 50	2938.2891	2937.2818	2937.2995	-6	0	R.GGTYFHDGDFGSAIYNGSVYAVHDGK.I	66
23 - 50	2938.3030	2937.2957	2937.2995	-1	0	R.GGTYFHDGDFGSAIYNGSVYAVHDGK.I	98
106 - 117	1358.6809	1357.6736	1357.6813	-6	0	K.FTSSHLHLGMTK.K	66
106 - 117	1374.6727	1373.6654	1373.6762	-8	0	K.FTSSHLHLGMTK.K Oxidation (M)	42
130 - 155	2703.3362	2702.3289	2702.3341	-2	0	K.DDGTWFNPIPILQGGSTPTPPNPGPK.N	61
167 - 192	2839.3477	2838.3404	2838.3362	2	0	R.VLGGSWLPEVTNFNNTNDGFAGYPNR.Q	101
210 - 223	1595.8245	1594.8172	1594.8256	-5	0	R.VHTAQSGWLPVWSK.G	104
227 - 252	2633.2722	2632.2649	2632.2803	-6	0	K.SDITVNGAAGMPGQAIDGVQLNYIITPK.G Oxidation (M)	58
274 - 297	2450.1877	2449.1804	2449.1761	2	0	K.VSADNGSIPGLDSYAGIFGEPLDR.L	146
298 - 308	1203.6147	1202.6074	1202.6295	-18	0	R.IQIGISQSNPF.-	85

Appendices

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 VERSION AAG29099.1 GI:11078549
 DBSOURCE accession AF249740.1
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 ORGANISM *Enterococcus faecalis*
 Bacteria; Firmicutes; Bacilli; Lactobacillales; Enterococcaceae;
 Enterococcus.
 REFERENCE 1 (residues 1 to 343)
 AUTHORS Nilsen,T., Nes,I.F. and Holo,H.
 TITLE Enterolysin A, a cell wall-degrading bacteriocin from *Enterococcus faecalis* LMG 2333
 JOURNAL Appl. Environ. Microbiol. 69 (5), 2975-2984 (2003)
 PUBMED 12732574
 REFERENCE 2 (residues 1 to 343)
 AUTHORS Nilsen,T., Nes,I.F. and Holo,H.
 TITLE Direct Submission
 JOURNAL Submitted (26-MAR-2000) Laboratory of Microbial Gene Technology, Agricultural University of Norway, P.O. Box 5051, Aas 1430, Norway
 COMMENT Method: conceptual translation supplied by author.
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APPENDIX 4

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DEFINITION *Enterococcus faecalis* scpE gene, ORF1, ORF2, entL gene and ORF3, strain BGPT1-10P.

ACCESSION HE585879
VERSION HE585879.1 GI:345500236
KEYWORDS
SOURCE *Enterococcus faecalis*
ORGANISM *Enterococcus faecalis*
Bacteria; Firmicutes; Lactobacillales; Enterococcaceae;
Enterococcus.

REFERENCE 1
AUTHORS Veljovic,K., Topisirovic,L., Terzic-Vidojevic,A. and Kojic,M.
TITLE Cloning and expression of enterolysin A gene from *Enterococcus faecalis* BGPT1-10P
JOURNAL Unpublished

REFERENCE 2 (bases 1 to 5156)
AUTHORS Kojic,M.
TITLE Direct Submission
JOURNAL Submitted (31-AUG-2011) to the INSDC. Kojic M., Lmgim, Imgge, Vojvode Stepe 444a, Belgrade, 11010, SERBIA AND MONTENEGRO

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ORIGIN

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4141 TACAGTTACT ATACTAAAAA AATCGTTTAT TCTACCTTTA CTTGGCTTAT TCTTTATAGT
4201 TGGTACAATG CTTTTGCTTC TGAAGATTAC AACTTACTTG GAGTGAAAAA TTATGATCAG
4261 TATGCCTTAG GAGCGCCTAG TGGGTGTGAG GGGGCTTCAC TATTACAAGG CTTACAGTAT
4321 AAAGGAAAAA TTCCTGATTG GGACTTAATC AAATTTTTTAA AAACAATTCC TATCTCTCCT
4381 AATGGAAATC CTTATGATGG ATTTGTACGT AGTCCTTTTG TTGAAAATC ATGGACTTAC
4441 TCTGCTATCT ATCCTGTGCC TTTAGTAAAA TGGGGAAAAG TATATGGAGA TGTACAAGAT
4501 ATGTCAGGTC ATTCTTTAGA TGAATTACTA GAGGAAGTGA AAAATAATAA TCCAATTGTT
4561 GCTTGGGTGA CGATAAAATT TCAACCGGCA CGATGGGGAA TATCGGATTT TGGTAAAGCG
4621 GTAAATAATA ATCATGCAGT GACATTAGAT GGCTACGATT TAAAAAATGG AACGGTTCAC
4681 GTATCTGATC CGATTTCCGG TAAATATTGG ATCAATAAAA ATACCTTTGA AAGTATATAT
4741 AATGAGAGGA ATCTTGCAGT TGTAATTCGT TAAGCCCTAT TTTTCAGTAC TATATGGGAA
4801 TAAATGAAAG CACCTTTAAA ATTTAAAGTCA TACGAGAGTG TACAGAAAAG TTTTCCTTTC
4861 TGTACTACTCT GTTGTTGTAT TTTCTTATAT GAGTATATTT AGTGTTTATA AAACATAACA
4921 AAAAAACAAA GGAGGAAAGT AAAACAAAAA AATCTCTCCA GTTTTAAATA TAGTGAAATT
4981 AGTTTAAATGG TTAAAACAAT TGAAGGCAAG TTATTAATAC TTATAGGCTC TGCTTTAAAC
5041 TATTATATTA GATGTCATTA AAACAATTAA GAGATTTAAA AAGACAAAAA CAAAAGAACA
5101 AACAGCAAAA ATATTTTTTT ATTTTATAGGT ATTATTTCTA AATTATATATA AAGCTT

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Note: The highlighted area shows the region where the *orf1*, *orf2*, *Ent. L* and *orf3* are located