MOLECULAR ANALYSIS OF A *LISTERIA MONOCYTOGENES* STRAIN THAT IS RESISTANT TO LEUCOCIN A

by

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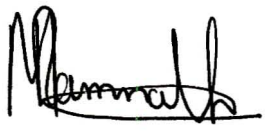
PREFACE

The experimental work described in this thesis for M.Sc was carried out in the School of Molecular and Cellular Biosciences, University of Natal, Pietermaritzburg, under the supervision of Professor John W. Hastings.

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



Manilduth Ramnath (Mr)

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ABSTRACT

Leucocin A is a class IIa bacteriocin produced by *Leuconostoc mesenteroides* TA33a that was previously shown to inhibit *Listeria monocytogenes*. A spontaneous resistant mutant of *L. monocytogenes* was isolated, and found to be resistant to leucocin A at levels in excess of 2 mg/ml. The resistant mutant had an eight-fold increased binding capacity for leucocin A in comparison to the parental strain. The mutant showed no significant cross resistance to nisaplin or ESF1-7GR. The resistant phenotype had a similar growth rate in monoculture, to the sensitive phenotype. DNA and protein analysis of the resistant and susceptible strains were carried out using silver stained amplified fragment length polymorphism (ssAFLP) and one and two-dimensional (2D) SDS polyacrylamide gel electrophoresis (PAGE). Two-dimensional SDS PAGE revealed two differences. The first was a 35 kDa protein which was present in the sensitive but absent from the resistant phenotype and, secondly there was a higher level of expression of a 18 kDa protein in the resistant phenotype compared with the sensitive phenotype. The 35 kDa protein was found to have a 83% homology to the mannose-specific phosphotransferase system IIAB of *Streptococcus salivarius.*

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LIST OF ABBREVIATIONS

aa amino acid

ADP adenosine diphosphate

AFLP Amplified Fragment Length Polymorphism

ATP adenosine triphosphate

AU/ml arbitrary units per milliliter

A_{600} absorbance at 600 nm

CAPS 3-cycohexylamino-1-propanesulfonic acid

CF carboxyfluorescein

DTT dithiothreitol

EI enzyme I

EII enzyme II

HPr histidine protein

IEF isoelectric focusing

LAB lactic acid bacteria

MIC minimum inhibitory concentration

OD_{600} optical density at 600 nm

1-D one-dimensional

PAGE polyacrylamide gel electrophoresis

PCR polymerase chain reaction

PEP:PTS phosphoenolpyruvate dependent phosphotransferase system

PFGE pulsed field gel electrophoresis

Pi inorganic phosphate

pI isoelectric point

PMF proton motive force

PTS phosphotransferase system

PVDF Polyvinylidine difluoride

rep-PCR repetitive DNA sequence-based PCR

SDS sodium dodecyl sulfate

2-D two-dimensional

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CHAPTER ONE

LITERATURE REVIEW

1.1 An introduction to bacteriocins

Low molecular-weight antimicrobial peptides, often produced by lactic acid bacteria (LAB), are collectively referred to as bacteriocins (Jack *et al.*, 1995; Klaenhammer, 1993). These antibacterial peptides are ribosomally synthesized as precursor polypeptides. In their active form they exert an antibacterial effect against a narrow spectrum of closely related bacteria (Jack *et al.*, 1995).

Bacteriocins of LAB are different from the large domain-structured protein toxins produced by *Escherichia coli* and related gram-negative bacteria (example colicins), that exert receptor-mediated lethality by either forming ion channels or displaying nuclease activities (Gouaux, 1997; Lazdanski, 1995; Lazdanski *et al.*, 1998). Bacteriocins are also different from peptide antibiotics which are made through enzymatic condensation of free amino acids (Montville and Kaiser, 1993). The bacteriocins of Gram-positive bacteria are mostly peptides (Jack *et al.*, 1995) that share a great deal of similarity with eukaryotic defense peptides. In general, a bacteriocin acts against LAB species that are closely related to the producing strain. The producing strain generally protects itself by an immunity protein that is specific to its own bacteriocin (Quadri *et al.*, 1995).

Bacteriocins from LAB have been classified according to the scheme of Klaenhammer (1993): Class I (lantibiotic peptides) contain unusual amino acids such as lanthionine, β-methyl lanthionine and dehydrated residues, which are introduced by post-translational modifications. Examples of class I bacteriocins are: nisin, lactocin S, carnocin, cinnanycin, daramycin, and mersacidin. Class II bacteriocins are small, stable peptides, which can be further subdivided as follows: IIa) are

active against *Listeria spp.* and contain the consensus motif (YGNGV) at the N-terminal end (e.g. pediocin PA-I/AcH, sakacin P, leucocin A, and curvacin); IIb) require two peptides for optimal activity (e.g. lactacin F, lactococcin M, and lactococcin G); IIc) contains peptides which are thiol-activated, hence requiring reduced cysteine residues for activity (e.g. lactococcin B). Class III and IV were originally defined as large heat-labile proteins and complex proteins that require a lipid or carbohydrate moiety for activity respectively (Klaenhammer, 1993).

1.2 Application of LAB bacteriocins as food preservatives

Artificial chemical additives such as sulfur dioxide, benzoic acid, sorbic acid, and nitrate (Lloyd and Drake, 1975), are currently added to food to suppress microorganisms. Increasing consumer awareness of potential health risks associated with these substances has led researchers to examine the possibility of using bacteriocins produced by LAB as biopreservatives (Abee *et al.*, 1995). The use of bacteriocins for the control of microbial populations, may provide the consumer with natural and minimally processed foods, with an assurance of microbial food safety.

The bacteriocin nisin, was approved for use by The Joint Food and Agriculture Organization/ World Health Organization Committee in 1969 as a biopreservative in food and is currently approved in forty-seven countries (Delves-Broughton, 1990). The use of pediocin PA-1 is covered by several European and U.S. patents (Bourdreaux and Matrozza, 1992; Gonzalez, 1988; van den Bergh *et al.*, 1989). Both pediocin PA-1 and nisin have applications in the preservation of dairy products, meat and fish products (Abee *et al.*, 1995). Studies of model food systems demonstrated

that pediocin-like class II bacteriocins, associated with meat and meat fermentations from *Pediococcus*, *Leuconostoc*, *Carnobacterium* and *Lactobacillus spp.*, were likely to have much greater potential as meat preservatives (Baccus-Taylor *et al.*, 1993; Jack *et al.*, 1996; Leisner *et al.*, 1996; Montville and Winkowski, 1997; Stiles and Hastings, 1991; Yousef *et al.*, 1991).

1.3 Bacteriocin resistance

The euphoria produced by the discovery of antibiotics led to confident predictions that bacterial diseases would soon be conquered and safely forgotten. Instead of witnessing the disappearance of bacterial diseases, there is now a resurgence of them (Salyers and Amabile-Cuevas, 1997). The development of bacterial resistance is a major world-wide problem that is complicating the use of chemotherapeutic agents (Percival, 1997).

The increasing use of bacteriocins as biopreservatives may lead to the generation of resistant strains. Such an event would severely compromise the use of bacteriocins in food preservation. Recently, there has been an increasing number of studies focusing on the resistance of the food borne pathogen *Listeria monocytogenes* to nisin.

An initial report on nisin resistance indicated that an enzyme was responsible for the inactivation of nisin. Nisinase was found to be produced by *Lactobacillus plantarum* (Kooy, 1952), *L. lactis* subsp. *lactis* and *cremoris*, *Enterococcus faecalis*, *Betacoccus* (*Leuconostoc spp.*) (Galesloot, 1956), *Staphylococcus aureus* (Carlson and Bauer, 1957), *Streptococcus salivarius* subsp.

thermophilus (Alifax and Chevaliar, 1962) and several *Bacillus* spp. (Jarvis and Farr, 1971). The enzyme was isolated from several *Bacillus* spp. and was shown to be a dehydropeptide reductase, because it specifically reduced the C-terminal dehydroalanyllysine of nisin to alanyllysine (Hurst, 1981).

The first report on the adaptive mutants of *L. monocytogenes* merely determined the frequency of resistant phenotypes to 50 µg/ml nisin (Harris *et al.*, 1991). Subsequently knowledge that nisin kills bacterial cells by the permeabilization of the plasma membrane has led researchers in the field of resistance to nisin to focus their attention on the cell envelop of the cell. Common trends on the properties of the resistant phenotypes can be observed. One trend included a decrease in the cell surface hydrophobicity and a corresponding reduction in the quantity of nisin adsorbed to the cell (Davies and Adams, 1994; Davies *et al.*, 1996; Ming and Daeschel, 1995; Maisner-Partin and Richard, 1996). The composition of the cell membrane was found to be modified in some studies (Crandall and Montville, 1998; Ming and Daeschel, 1993; 1995; Mazzotta and Montville, 1997). However, no change in the total phospholipid content was found by Davies *et al.* (1996). The phospholipid content of the cell membrane was not determined by these researchers.

There were also a number of contrasting properties found in the resistant strains. The study by Crandall and Montville (1998) found that the resistant and the parental strains had similar growth rates. However, other researchers have found a lower growth rate for resistant strains (Mazzotta and Montville, 1997; Ming and Daeschel, 1993). Crandall and Montville (1998) also found no difference in cell wall thickness or morphology using transmission electron microscopy. This was

in contradiction to work by Maisner-Partin and Richard (1996) who found localized thickening of the cell wall of resistant strains. Verheul *et al.* (1997) found that there were no changes in sensitivity of the cell wall to lysozyme, although other researchers found an increase in cell wall compounds (Crandall and Montville, 1998; Maisner-Partin and Richard, 1996). These contrasting properties could be explained by the different types of mutants used for these experiments because they were all adaptive mutants. In most of the studies, different strains of *L. monocytogenes* were used, which may have different mechanisms to survive the stress of exposure to bacteriocins.

Resistance to other classes of bacteriocins have been reported. The mechanism of resistance to class IIa bacteriocins remains poorly understood, but is reportedly a stable phenomenon (Rekhif *et al.*, 1994). Rekhif *et al.* (1994) established cross protection between different bacteriocins mesentaricin 52, curvacin 13, and plantaricin C19 belonging to the class IIa subgroup, while none where found for nisin belonging to class I. In these studies, resistance to the class IIa bacteriocins (mesentaricin 52, curvacin 13, and plantaricin C19) was neither due to inactivation of the bacteriocin nor a modification of their adsorption on the target cells (Rekhif *et al.*, 1994). Resistant strains were also found to have a lower growth rate (and thus relative fitness), and were found to be lost during co-culture with the sensitive strain (Dykes and Hastings, 1998). Transposon inactivation of the *rpoN* (σ^{54}) gene from *L. monocytogenes* was linked to resistance of mesentericin Y105 (Robichon *et al.*, 1997). These are the only reports on resistance to group II bacteriocins, and there is still little known about the molecular basis of resistance in naturally isolated strains.

The study of resistance to bacteriocins will not only be useful knowledge, as bacteriocins are increasingly used for biopreservation, but it could also aid in the understanding of the mode of action of bacteriocins.

Resistance to bacteriocins may result from the inhibition of complex formation between bacteriocin and target molecules on or in the target cells. To further understand the resistance to class I and class II bacteriocins, greater understanding of the mechanism of action, factors influencing bacteriocin activity, as well as structure-function relationships with regard to the respective classes of bacteriocins are imperative.

1.4 Primary mechanism of action of bacteriocins

Nisin has been widely used for a number of decades as a food preservative (Hurst, 1981). However, relatively little is known about the mechanism by which it is able to kills susceptible bacterial cells (Jack *et al.*, 1995). Nisin was found to cause the rapid efflux of amino acids and potassium ions from the cytoplasm of gram-positive cells (Rühr and Sahl, 1985). This resulted in a dissipation of the membrane potential. It has been shown that pediocin AcH and pediocin JD causes efflux of amino acids and potassium ions respectively (Bhunia *et al.*, 1991; Christensen and Hutkins, 1992). On the basis of amino acid sequence homology and structure-function relationships, other class II bacteriocins are likely to dissipate proton motive force (PMF) (Bruno and Montville, 1993). Hence the dissipation of PMF was identified as a common mechanism for the lethal activity of LAB bacteriocins.

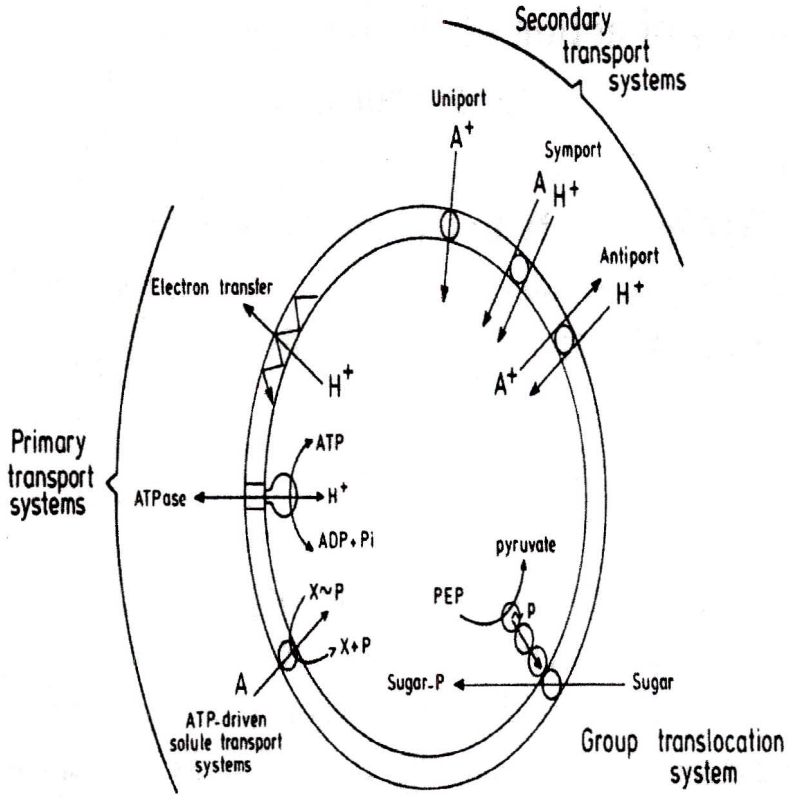


Figure 1: Schematic presentation of the mechanisms of carrier-mediated solute transport in bacteria. Solutes and protons are indicated by A and H^+, respectively (Abee *et al.*, 1991).

Both class I and class II bacteriocins cause the release of ultraviolet absorbant material from sensitive cells. A closer look at the functions of the cell membrane with regard to energy transduction should be considered.

Energy transducing systems are transport systems for solutes (including ions) across the cytoplasmic membrane (Figure 1) (Abee *et al.*, 1991). The systems can be grouped in three classes, according to the mode of energy coupling: i) Primary transport systems are responsible

for the conversion of chemical, light or redox energy into electrochemical energy of protons or other solutes (Figure 1). The electron transfer systems, the membrane bound ATPase and ATP driven transport systems belong to this class. Several of these systems were found to function as proton translocating systems which pump protons from the cytoplasm to the external medium. As a result of this activity, an electrochemical gradient of protons is formed which exerts an inwardly directed force on the protons. This force was the PMF. The PMF is composed of membrane potential ($\Delta\Psi$) and a chemical gradient of protons or pH gradient across the membrane (ΔpH) (Abee *et al.*, 1991). ii) Secondary transport systems convert one form of electrochemical energy to another form (Figure 1). The proton motive force-driven transport systems belong to this class. Secondary transport systems are the most common transport systems in bacteria. The uptake of most amino acids and other biosynthetic precursors is mediated by secondary transport systems (Abee *et al.*, 1991); iii) Group translocation systems couple the translocation of a solute to a chemical modification. As a result of this transport activity, the product released inside is chemically different from the solute outside. In bacteria, the only known group is the phosphoenolpyruvate-dependent phosphotranspherase system (PEP-PTS) which is responsible for sugar transport (Abee *et al.*, 1991).

LAB bacteriocins were found to be responsible for the dissipation of either or both $\Delta\Psi$ and ΔpH. Nisin dissipated both $\Delta\Psi$ and ΔpH completely (Bruno and Montville, 1993; Rühr and Sahl, 1985). The class IIa bacteriocins pediocin PA-1 (Bruno and Montville, 1993) and bavaricin (Kaiser and Montville, 1996), dissipated ΔpH completely and a substantial amount of $\Delta\Psi$. Another class IIa bacteriocin, mesentericin Y105, partially dissipated $\Delta\Psi$ in *L. monocytogenes* (Maftah *et al.*, 1993).

Lactococcin G, a class IIb bacteriocin, dissipated the $\Delta\Psi$ only, and not the ΔpH of sensitive lactococcal cells (Moll *et al.*, 1998). A unique feature of lactococcin G is that the two peptides required for its activity (a complex of α and β) forms a transmembrane pore that conducts monovalent cations but not protons. Nisin, pediocin PA-1 and bavaricin MN form pores that conduct both protons and the other charged ions.

PMF is the driving force for many vital energy-demanding processes in the cytoplasmic membrane, notably the synthesis of ATP (Harold, 1986). Its dissipation by LAB bacteriocins depletes intracellular ATP (Abee *et al.*, 1991; Chen and Montville, 1995; Waite *et al.*, 1998; Winkowski *et al.*, 1994). For nisin it was suggested that the cause of ATP dissipation was primarily the result of ATP hydrolysis in order to regenerate a proton gradient (Winkowski *et al.*, 1994) rather than the loss of Pi shifting the equilibrium of ATP hydrolysis toward ADP. The latter mechanism was proposed for the action of nisin Z (Abee *et al.*, 1994). For pediocin PA-1/AcH, the former was also determined to be responsible for mode of depletion of ATP.

LAB bacteriocins may also induce the leakage of various small intercellular substances from sensitive cells. Class II bacteriocins pediocin PA-1, lactococcin A and mesentericin cause the efflux of pre-accumulated amino acids from sensitive cells (van Belkum *et al.*, 1991; Chikindas *et al.*, 1993; Maftah *et al.*, 1993). The efflux of amino acids caused by pediocin PA-1 and lactococcin A may be a result of a combination of reflux via PMF transport systems and diffusion through bacteriocin pores. In addition to the efflux of amino acids, class II bacteriocins also induce the efflux of potassium (Chen and Montville, 1995; Moll *et al.*, 1998), and inorganic phosphate (Chen

and Montville, 1995). However, pediocin PA-1, did not to induce the efflux of ATP (Chikindas *et al.*, 1993; Waite *et al.*, 1998).

The addition of nisin to sensitive cells was also found to result in the efflux of amino acids, potassium, inorganic phosphate, and partial efflux of ATP (Abee *et al.*, 1994; Rühr and Sahl, 1985; Waite *et al.*, 1998). From the efflux of ATP, which has no active transport system in sensitive cells, and of glutamate which is transported by a PMF-driven system, it can be inferred that small substances diffuse through nisin pores (Rühr and Sahl, 1985; Sahl, 1991).

Dissipation of PMF was identified as a common mechanism for the lethal activity of LAB (Bruno and Montville, 1993; Rühr and Sahl, 1985). This would have a direct effect on the secondary transport systems in bacteria (Figure 1). All PMF-driven transport systems belong to this class. This has led to the inhibition of the active transport of amino acids into sensitive bacteria (van Belkum *et al.*, 1991; Chikindas *et al.*, 1993; Moll *et al.*, 1998; Sahl, 1991).

Cells treated with the lantibiotics nisin and pep5, led to the cessation of all macromolecular biosynthesis within the cell, including protein, DNA and RNA biosynthesis (Sahl, 1991). Likely explanations for this phenomenon were that treated cells lacked the energy to conduct any biosynthesis due to the dissipation of the primary transport system (Figure 1). Alternatively, the cells now devoid of a PMF, were not able to take up precursors from the medium via the secondary transport systems (Figure 1). Finally, the integrity of the cytoplasmic membrane was disturbed to such an extent that accumulated cytoplasmic compounds effluxed from the cells (Rühr and Sahl,

1985; Sahl, 1991). The effect of class II bacteriocins on macromolecular biosynthesis has not been investigated.

1.5 Secondary mechanisms of activity of bacteriocins

The primary mechanism by which LAB bacteriocins exhibit their mode of action is through the depolarization of the cytoplasmic membrane to form ion permeable pores (Bruno and Montville, 1993; Jack *et al.*, 1995; Sahl, 1991). The study of the treatment of cells over a longer period of time (one to two h) showed that the lantibiotics nisin and pep5 induced autolysis in certain strains of staphylococci (Bierbaum and Sahl, 1985; 1991). The activation of autolysis occurred most markedly in the region of the septa adjoining two daughter cells, as revealed by electron microscopic investigations (Bierbaum and Sahl, 1985; 1991). Cell wall turnover is under the regulation of autolysins (lytic enzymes) bonded to poly-anionic cell wall constituents such as teichoic, lipoteitoic and teichuronic acids. Further studies have shown that cationic peptides replace the enzyme from their anionic cell wall constituents (intrinsic inhibitors), probably by an ion exchange-like process, which results in an apparent activation of cell wall hydrolysis and an induction of autolysis (Bierbaum and Sahl, 1987; 1991).

In vitro, the activity of autolysins can also be increased by other highly cationic peptides such as poly-lysine or by the addition of cations, including Na^+ and Mg^{2+}, indicating the non-specific release mechanisms (Bierbaum and Sahl, 1985; 1987; 1988). Nisin and pep5-induced pores allow the release of only small solutes and are not large enough to allow efflux of high moleculer weight

components. This may lead to enhanced osmo-induced influx of water through the pores. Concomitantly, the activation of cell wall lytic enzymes would result in areas of degradation in the cell wall. The combination of increased osmotic pressure with a weakened cell wall could lead to the lysis observed after treatment of cells with some lantibiotics (Bierbaum and Sahl, 1991).

Treatment with pediocin PA-1 resulted in the cell lysis of *L. monocytogenes* and *Pediococcus pentosaceus*, which was observed as a reduction in optical density (Pucci *et al.*, 1988). However, Bhunia and co-workers (1991) showed that for *Lactobacillus plantarum* NCDO955, exposure to pediocin AcH prevented further increase in optical density. Alternatively, treatment of *Leuconostoc mesenteriodes* with pediocin AcH also caused a decrease in optical density, suggesting that cell lysis had occurred. This was further confirmed by transmission electron microscopy (Bhunia *et al.*, 1991). Several sensitive strains of *L. monocytogenes* were treated with pediocin AcH. There was a loss optical density in a portion of the strains, indicating lysis. The remainder of the strains showed no decrease in optical density (Molitor and Sahl, 1991). Such variation clearly demonstrates the conflicting results that have been reported concerning the ability of non-lanthionine bacteriocins to induce lysis in sensitive cells. The primary killing action of pediocin AcH and possibly other nonlantibiotics, is the dissipation of the plasma membranes, but cell death may activate autolytic systems and bring about lysis in some strains (Bhunia *et al.*, 1991).

Bacteriocins also effect other biochemical processes within the cell. Nisin inhibits murein synthesis by the formation of a complex with lipid intermediate I (Reisinger, 1980). Both nisin and pep5 inhibit the uptake of oxygen. This is due to the direct effect of these bacteriocins on the

cytochrome C oxidase (Sahl, 1991). Another inhibitory effect of nisin and subtilin is the inhibition of spore outgrowth (Chan *et al.*, 1996; Lian *et al.*, 1991).

Mesentaricin Y 105 was reported to inhibit ATP synthase and adenine nucleotide translocase. The action of mesentericin Y 105 on ADP transport could be another inhibiting factor of ATP synthesis (Maftah *et al.*, 1993). Group translocation systems were also affected by bacteriocins (Figure 1). The PEP-PTS glucose transport was inhibited in *L. monocytogenes* by nisin, pediocin JD and leuconocin S (Christensen and Hutkins, 1994; Waite *et al.*, 1998; Waite and Hutkins, 1998).

Loss of PMF was probably due to inhibition of low affinity PMF-mediated glucose transport systems (Figure 1). Efflux of intracellular ATP and Pi suggested that the high affinity glucose transport system was inhibited by the loss of intracellular metabolites, such as PEP and ATP, rather than the effects of PMF dissipation (Waite *et al.*, 1998). Pediocin JD and leuconocin S were considered to have an additional mechanism of inhibition of the PEP-PTS other than the loss of intracellular metabolites (Waite and Hutkins, 1998).

1.6 Models for bacteriocin-membrane interactions

Several reports indicate that bacteriocins form poration complexes in target membranes (Abee, 1995; Jack *et al.*, 1995; Klaenhammer, 1993; Montville *et al.*, 1995). The hypothesis that nisin and class II bacteriocins permeabilize target cell membranes through a multi-step process of binding, insertion, and pore formation, has provided a conceptual framework for studies on the molecular

mechanism of bacteriocin action (Driessen *et al.*, 1995; van den Hooven *et al.*, 1996(a); Ojcius and Young, 1991; Sahl, 1991). Precisely how pore complexes are formed is a major focus of ongoing research. Models for pore formation are largely based on studies of nisin interaction with membranes. These include synthetic phospholipid vesicles or planar lipid bilayers (Breukink *et al.*, 1998; Driessen *et al.*, 1995; Garcerà *et al.*, 1993; Giffard *et al.*, 1996; 1997; van Kraaij *et al.*, 1998), phospholipid monolayers (Demel *et al.*, 1996), detergent micelles (van den Hooven *et al.*, 1996b), or lipid vesicles derived from sensitive microorganisms (Winkowski *et al.*, 1996).

The alternative mechanisms for pore formation have been described , namely, the "barrel-stave" model (Ojcius and Young, 1991; Sahl, 1991) and the "wedge" model (Driessen *et al.*, 1995; van de Hooven *et al.*, 1996a) (Figure 2 A-C). Nisin has both water solubility and membrane-binding ability, which are necessary for both models. Electrostatic anchoring of the C-terminus to the membrane surface was found to be essential for membrane permeabilizing activity (Breukink *et al.*, 1997; 1998; van Kraaij *et al.*, 1998). The N-terminus portion of the nisin molecule was reported to penetrate the lipid phase (Breukink *et al.*, 1997; Demel *et al.*, 1996). Recent studies have illustrated an overall parallel average orientation of nisin in the membrane with respect to the membrane surface, with the N-terminus inserted more deeply than the C-terminus (Breukink *et al.*, 1998). The models differ in their consideration of the insertion of the nisin molecule into the membrane.

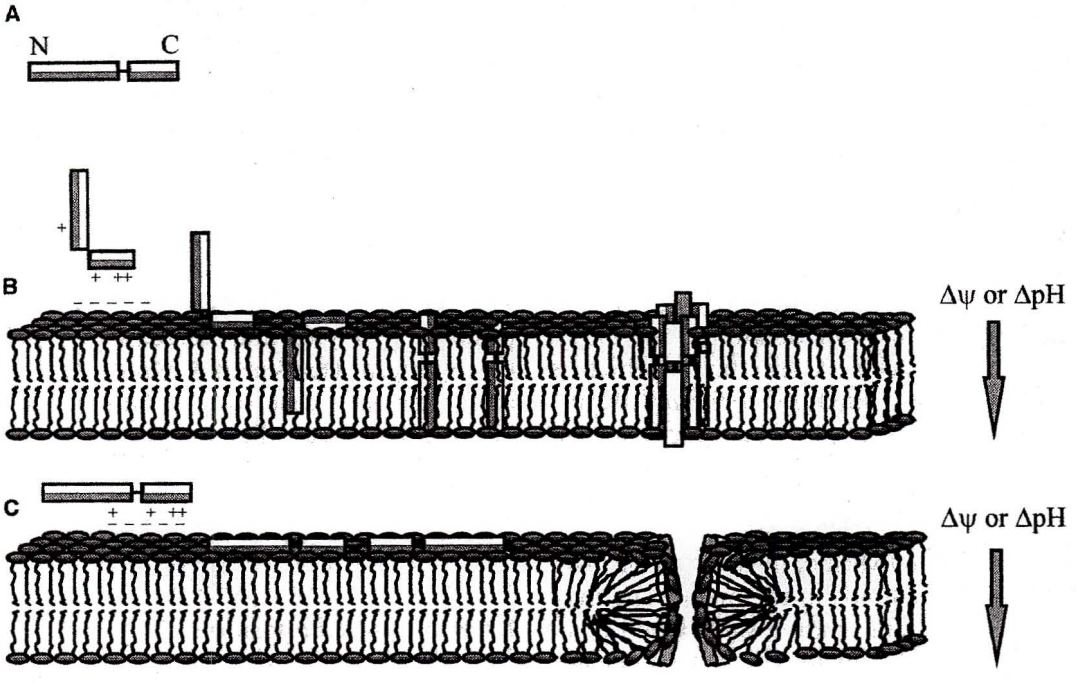


Figure 2 A-C: Models for nisin pore formation. **A** Schematic representation of the peptide showing the – and C-terminal domains connected by a flexible hinge region. The hydrophilic face is *shaded dark* and hydrophobic face is *white*. **B** The barrel-stave model, from Ojcius and Young (1991) and Sahl (1991). **C** The wedge model, from Driessen *et al.* (1995) and van den Hooven *et al.* (1996b). In both models, the initial binding involves electrostatic interactions to a certain degree, and possible ways of binding (not necessarily inherent to one or the other model) are illustrated. Proton motive force components ($\Delta\Psi$ or ΔpH) enhance (barrel-stave model) or mediate (wedge model) insertion and pore formation. In both models, the hydrophilic face of the nisin molecule faces the lumen of the pore (Montville and Chen, 1998).

For the barrel-stave model, nisin forms oligomers at the membrane surface after binding (Sahl, 1991). Once the membrane has been energized, this would force the peptide into a conducting state, presumably by adopting a transmembrane orientation in such a way that the hydrophobic face

of the aggregate is exposed to the surrounding lipids, while the hydrophilic face forms the inner lumen of the pore (Sahl,1991) (Figure 2B). It was hypothesized that the high dia pole moment of the peptides which responds to the application of the voltage, and that repulsion of parallely orientated dia poles opens the pore (Sahl, 1991). However, a contradiction exists in the initial insertion of the nisin molecule. Ojcius and Young (1991) proposed that pore-forming peptides bound as a monomer, which inserted into the lipid bilayers and then aggregated laterally like the staves of a barrel, to form pores. A large number of pore-forming proteins act according to the barrel-stave model (Ojcius and Young, 1991).

Studies by van den Hooven *et al.* (1996a,b) revealed important details contributing to an improved wedge model proposed by Driessen *et al.* (1995) (Figure 2C). They determined the conformation of the nisin molecule in the presence of membrane-mimicking micelles. Due to the amphiphilic nature of nisin, it was found that nisin not only interacts with the phospholipid head groups via ionic forces, but also inserted with its hydrophobic side into the outer leaflet of a bilayer (Sahl and Bierbaum, 1998). It was speculated that several peptides could form a pore when the $\Delta\Psi$ above a certain threshold pulls the nisin molecule with surrounding anionic lipids, while remaining surface-bound and carrying the lipids across the plasma membrane (van den Hooven *et al.*, 1996a). The orientation of the nisin molecule relative to the head groups was not changed. Hence in this model, the peptide does not come into contact with the hydrophobic core of the membrane. The resulting pore has the hydrophilic side of nisin and the attached lipid head-groups facing the center of the water-filled pore. The hydrophobic surface of nisin and the fatty acid chains of the lipids point into the lipid bilayer (van den Hooven *et al.*, 1996a). Due to this arrangement, both the polar

sides of the cationic nisin and the anionic lipids face the lumen of the pore, which explained the observed non-selective efflux of ions and other small solutes (Kordel and Sahl, 1986; Rühr and Sahl, 1985; Sahl, 1991). The peptide magainin (a frog defensin) was also considered to permeabilize the cellular membrane by the wedge model (Ludkte *et al.*, 1996).

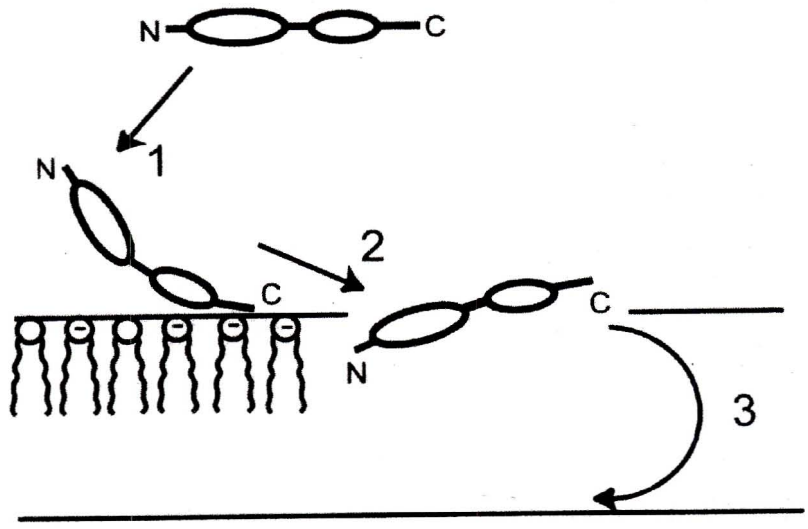


Figure 3: Model of the first steps of the interaction of nisin with a lipid bilayer. 1: Binding of nisin to the membrane. Electrostatic interactions between the positive residues of the peptide and acidic lipids play an important role. 2: Insertion of nisin into the membrane. The N-terminal part of nisin penetrates into the outer leaflet of the membrane. 3: Translocation of the C-terminal part of nisin across the membrane (van Kraaij *et al.*, 1998).

It was shown that the C-terminus of the nisin molecule is essential for membrane permeabilizing activity in artificial membrane systems (Breukink *et al.*, 1997; van Kraaij *et al.*, 1998). Findings to the contrary have been reported using artificial systems supplemented with lipid II (Brötz *et al.*,

1998; Breukink *et al.*, 1999) (see section on putative docking molecules). Studies have shown that a strong electrostatic interaction between the negatively charged phospholipids and the positive charges on the C-terminus of nisin were harmful because they trapped the C-terminus at the interface allowing N-terminal insertion to occur but without giving rise to membrane leakage (van Kraaij *et al.*, 1998). van Kraaij *et al.* (1998) demonstrated that pore formation of nisin involved translocation of the C-terminal region of the molecule across the membrane. In contrast to the mechanism of action of other pore forming peptides, after binding of nisin to the outer leaflet of the membrane bilayer and insertion of the N-terminus into the layer, the peptides might flip into a membrane-spanning orientation with the C-terminus with at least part of the peptide in the lumen of the vesicle (van Kraaij *et al.*, 1998) (Figure 3). It was shown that nisin adopted an overall parallel orientation at the membrane surface (Breukink *et al.*, 1998). It is therefore assumed that the membrane-spanning orientation of the peptides is only transient, such that the peptides might rapidly flip back to the parallel orientation in the outer leaflet. Alternatively, similar to other α-helical peptides, nisin molecules might translocate entirely across the membrane by formation of short-lived, transmembrane pores and take an orientation parallel to the plane of the membrane in both leaflets of the bilayer (van Kraaij *et al.*, 1998).

The complexity of the nisin-membrane interaction can be observed with the differing models attempting to explain the mechanism of pore formation. The discrepancies that exist between the models and the seemingly contradictory evidence supporting the models indicate that bacteriocin and membrane interaction is not fully understood. The extent to which the above mentioned models describe pore formation by other LAB bacteriocins, particularly class IIa bacteriocins is not

known. Pore formation by bacteriocins is further complicated by a number of *in vivo* effects, which will also have to be considered.

1.7 Factors influencing bacteriocin activity

1.7.1 The cell wall

Upon attack of a gram-positive bacterium, the bacteriocin would have to first pass the cell wall before it reaches the membrane of the cell. The cell wall of gram-positive bacteria forms a web that is composed of a peptidoglycan layer, which confers rigidity and shape to the cell. The basic structure consists of repeating units of sugar forming glycan chains (Bugg and Walsh, 1992). The mechanism(s) of bacteriocin passage through the cell wall has not yet been critically studied (Jack *et al.*, 1995).

Resistance of gram-negative bacteria and yeast to nisin could be due to the relative impermeability of their outer membranes. In order to interfere with the barrier properties of resistant gram-positive and gram-negative bacteria, sublethal stresses were applied to them (Kalchayanand *et al.*, 1997; Ray, 1993; Ray and Daeschel, 1992; Stevens *et al.*, 1991). When the cell wall was removed from a nisin resistant strains, both sensitive and resistant strain showed identical susceptibility to nisin, indicating that differences in the cell wall were responsible for nisin resistance in this strain (Davies *et al.*, 1996). Both gram-negative and resistant gram-positive bacteria were made sensitive to pediocin AcH and nisin following exposure to sublethal stress (Kordel and Sahl, 1986; Kalchayanand *et al.*, 1997; Ray, 1993; Ray and Daeschel, 1992; Stevens *et al.*, 1991). Intact yeast

cells are resistant to nisin, but removal of the cell wall facilitated access of nisin to the membrane and it is able to rupture the cells (Dielbandhoesing *et al.*, 1998). Furthermore, it was found that specific glucanase-extractable cell wall proteins are crucial in conferring resistance to the lantibiotic nisin (Dielbandhoesing *et al.*, 1998).

Several recent studies showed that the acquisition of resistance to nisin was attributed to cell wall changes. Nisin-resistance in *L. monocytogenes* was also reported to be accompanied by altered sensitivity to cell wall hydrolyzing enzymes (Crandall and Montville, 1998; Maisner-Partin and Richard, 1996). This indicates that changes in cell wall composition and/or structure may reduce the access of the cell wall hydrolyzing enzyme to its target. However, work carried out by Verheul *et al.* (1997), showed that resistance to nisin by *L. monocytogenes* was not due to modifications of the cell wall because both the resistant and mutant strain had comparable sensitivity to lysozyme. A nisin resistant strain of *Listeria innocua* also demonstrated resistance to cell wall-acting antibiotics (Maisner-Partin and Richard, 1996). Electron microscopy studies of this resistant strain revealed local thickening of the cell wall (Maisner-Partin and Richard, 1996). In contrast, Crandall and Montville (1998) showed that their resistant strain had no morphological differences and that both the wild-type and the resistant strain contained cell walls of comparable thickness.

Bacteriocins were found to adsorb to the cell surface of gram-positive bacteria in a pH dependent manner, irrespective of being bacteriocin producers, nonproducers, sensitive or resistant strains (Yang *et al.*, 1992). This supports the suggestion that initial adsorption occurs through ionic attraction between the bacteriocin molecules and the cell surface. It was suggested that the class

II bacteriocin mesentaricin Y 105^{37} may also bind to the anionic cell surface polymers (Fleury *et al.*, 1996). Bierbaum and Sahl (1985;1987) demonstrated that cationic peptides adsorb to the teichoic acids and lipoteichoic acids of the cell wall of bacteria and thereby activate the autolytic enzymes.

Further evidence of the cell wall's role in nisin resistance comes from the determination of cell surface hydrophobicity. With nisin being predominantly hydrophobic, the decreased hydrophobicity of the more resistant cells may be related to their reduced avidity for nisin (Davies *et al.*, 1996). Nisin resistant strains also have a less hydrophobic cell surface (Davies *et al.*, 1996; Maisner-Partin and Richards, 1996; Ming and Daeschel, 1995).

Because the chemical structure of the cell wall of gram-positive bacteria is complex, details of the nature of the changes that occur upon acquisition of bacteriocin resistance is not yet known. More research on the length of the peptidoglycan chains, the extent and manner of cross-linking of the chains which might result in greater rigidity of the wall and in turn inhibit the passage of the bacteriocin molecules, will aid in understanding the role played by the cell wall in bacteriocin action.

1.7.2 Role of lipid composition

The primary target for the activity of bacteriocins appears to be the bacterial cytoplasmic membrane (Bruno and Montville, 1993; Jack *et al.*, 1995; Sahl, 1991). Cell membranes consist

mainly of phospholipids. These molecules have a hydrophobic tail composed of two fatty acid chains and a hydrophilic phosphate group which can be attached to a small hydrophilic compound such as ethanolamine, choline or serine. The major phospholipids in the cell membrane of gram-positive bacteria are cardiolipin and phosphatidyl glycerol, each is negatively charged, and in some organisms phosphatidyl ethanolamine, which like phosphatidyl choline is zwitterionic. The relative amounts in which these phospholipids occur vary from one species to another.

The first step in the mechanism of action of nisin is considered to be the binding of the peptide to the cytoplasmic membrane of the target bacteria. Because nisin is positively charged, it was suggested that electrostatic interactions between positive charges of the peptide and negatively charged phospholipids play an important role in the binding step (Driessen *et al.*, 1995; Gao *et al.*, 1991; Garcerá *et al.*, 1993). Divalent and trivalent cations (Mg^{2+}, Ca^{2+}, or Gd^{3+}) decrease the action of nisin on *L. monocytogenes*, presumably by shielding electrostatic interactions between the peptide and the negatively charged head groups of the phospholipid molecules, and/or neutralizing the negative charges of the head groups (Abee *et al.*, 1994). Binding studies of nisin using fluorescently-labeled phospholipids showed that nisin interacted tightly with membranes containing the negatively charged lipid phosphotidylglycerol, and has little affinity for zwitterionic lipids predominantly found in gram-negative bacteria, yeast cells and human cells (Demel *et al.*, 1996). In lipid vesicles with varying ratios of anionic and zwitterionic phospholipids, nisin induced the release of potassium and CF with higher anionic lipid contents (Breukink *et al.*, 1997). It also appeared that anionic phospholipids, in particular cardiolipin, interacted strongly with nisin and encouraged nisin insertion (Giffard *et al.*, 1996; 1997; Martin *et al.*, 1996).

The affinity of pediocin PA-1 for the lipid vesicles increases with a higher content of negatively charged phospholipids (Chen *et al.*, 1998). There was little change in binding parameters for zwitterionic lipid vesicles (Chen *et al.*, 1998). Such evidence coupled with the finding that the electrostatic interactions, and not the YGNGV consensus motif, govern pediocin binding to the target membrane (Chen *et al.*, 1997) suggests that class IIa bacteriocins may also bind to membranes in an electrostatic manner.

However, the role of the negatively charged phospholipids is unclear, because several reports indicated that *in vitro* permeability studies on the activity of nisin was most efficient on lipid vesicles composed of zwitterionic lipids, while anionic lipids were strongly inhibitory (Driessen *et al.*, 1995; Gracerá *et al.*, 1993; van den Hooven, 1996a). It was suggested that nisin acts as an anion-carrier on zwitterionic vesicles, such that it translocates back to the outer surface where the anion is released (Driessen *et al.*, 1995). Because negatively charged lipids were shown to inhibit this process, a possible anion-carrier activity was expected to be low *in vivo*. Due to conflicting results of the *in vitro* and *in vivo* experiments, phospholipid dependence of nisin activity remains one of the key questions of membrane activity of nisin.

Reduction in the anionic phosphotidylglycerol and cardiolipin acted as mechanisms of resistance to the lantibiotic nisin (Crandall and Montville, 1998; Ming and Daeschel, 1995). Modifications to the phospholipid head groups in nisin resistant variants of *L. monocytogenes* was also demonstrated, with a decrease in diphosphatidylglycerol content in the resistant variants (Verheul *et al.*, 1997). A study by Demel *et al.* (1996) showed that nisin penetrated more deeply into the

lipid monolayers of diphosphatidylglycerol than into those of other lipids, including phosphatidyl glycerol. The interaction with diphosphatidylglycerol was apparently much stronger, which may be linked to the high charge density and its specific charge distribution (Demel *et al.*, 1996; Giffard *et al.*, 1996).

Further modifications to the cell membrane were implicated in nisin resistance, namely, the nisin-induced changes to the composition of the fatty acid chain (Crandall and Montville, 1998; Mazzotta and Montville, 1997; Ming and Daeschel, 1993). They all found a decrease in cell membrane fluidity due to the lower ratios of C15/C17. Evidence that a more rigid membrane affects nisin activity was also reported for nisin Z (Abee *et al.*, 1994). Cellular membranes with decreased fluidity may resist insertion of nisin. Alternatively, the protein distribution in the membrane may also be altered due to changes in membrane fluidity (Ming and Daeschel, 1993).

1.7.3 Putative docking molecules

Pore formation by the class I lantibiotic was thought not to require a protein receptor, because the bacteriocin dissipated the PMF and caused CF efflux from lipid vesicles which lacked membrane proteins (Breukink *et al.*, 1997; Gao *et al.*, 1991; Garcerá *et al.*, 1993; Winkowski *et al.*, 1996). From the number of observations, it has become clear that the membrane activity of nisin, as observed in the model membrane experiments, does not totally explain its *in vivo* action. *In vitro*, nisin is 1000-fold less active on membranes solely composed of phospholipids compared with the nM concentrations of nisin required for *in vivo* activity. For many modified nisin molecules, their

activity on model membrane systems was almost equal to the wild-type nisin, while their antimicrobial activity against bacterial strains was significantly reduced. The analysis of the phospholipid composition of bacterial strains showed that they all contain relatively high amounts of negatively charged phospholipids. The differences in level of these lipids can not explain the large variation in nisin sensitivity of the strains (Crandall and Montville, 1998; Ming and Daeschel, 1995; Verheul *et al.*, 1997). From these results, it is evident that the interaction with bacterial cells is more specific and such specificity cannot be explained by a mechanism of action for which the presence of anionic lipids is the only prerequisite.

A possible explanation for these results could be a recent observation concerning the involvement of peptidoglycan precursor molecules in the mechanism of action of nisin (Brötz *et al.*, 1998; Breukink *et al.*, 1999). The presence of the membrane-bound precursor, lipid II [undecaprenyl-pyrophosphoryl-MurNAc- (pentapeptide)- GlcNAc] in liposomes substantially increased the susceptibility of the liposomes to nisin, as indicated by the fact that liposomes are susceptible to nisin without previous energization. It was postulated that lipid II, which is present at the outer surface of the gram-positive bacteria, serves as a docking molecule for nisin, facilitating specific binding to the bacterial membrane. Interaction with nisin may involve the lipid moiety, disaccharide unit or the peptide side chain, which may be mediated by the N-terminal portion of nisin. The C-terminal portion of nisin was not involved in the permeabilization of the lipid II containing liposomes.

The amount and accessibility of lipid II in the membrane of bacteria could be an additional

determinant for susceptibility of bacteria to nisin. Lipid II was also found to be a specific docking molecule for nisin and epidermin, but not for Pep5 and epilancin K7. This has been correlated to the low *in vivo* activity of pep5 against *Micrococcus luteus*, which has high number of undecaprenol and undecaprenol-coupled molecules (Brötz *et al.*, 1998).

Nisin resistance might be altered by the degree of the biosynthesis and/or accessibility of lipid II molecules. This could in turn contribute to the observed changes in the bacterial cell wall of nisin-resistant strains (Crandall and Montville, 1998; Maisner-Partin and Richard, 1996). It was found that by inhibition of transglycoslyation and the subsequent concomitant accumulation of lipid II in the membrane by pretreatment with mersacidin, further binding sites for nisin and epidermin may be made available (Brötz *et al.*, 1998).

Studies on the necessity of a protein receptor for class II bacteriocins have shown that not all bacteriocins within this class require them. Initial work suggested that a protein receptor mediates pediocin PA-1 pore formation in pediococcal cells (Chikindas *et al.*, 1993). However, it has been demonstrated that a protein receptor was not essential for the activity of pediocin PA-I/AcH in other sensitive organisms (Chen *et al.*, 1997). Pediocin PA-1 caused CF efflux from complex lipid vesicles derived from *L. monocytogenes* cells, as well as from pure phospholipid vesicles (Chen *et al.*, 1997). Another class IIa bacteriocin, barvaricin MN, induced CF leakage from lipid vesicles derived from *L. monocytogenes*. This confirmed that pore formation could take place in the absence of a protein receptor (Kaiser and Montville, 1996).

A peptide fragment derived from pediocin PA-1 (residues 20 to 34) specifically inhibited the bactericidal activity of pediocin PA-1. The 15 mer fragment did not inhibit other class IIa bacteriocins to the same extent as it inhibited pediocin PA-1, and enterocin A. The interesting observation that pre-incubation of cells with the N-terminal fragment of nisin 1-12, was found to inhibit the antibiotic action of nisin (Chan *et al.*, 1996). This was one of the first indications that nisin may interact specifically with a particular component of the cytoplasmic membrane. Further work has demonstrated that nisin specifically interacted with lipid II (Brötz *et al.*, 1998). This preliminary result could indicate that pediocin may also interact in a specific manner with an entity on the target membrane, as was found for nisin. However, a protein receptor that was indispensable for activity (Brötz *et al.*, 1998) was implicated in the membrane permeability of lactococcin A because the protease-treated membrane vesicles isolated from sensitive cells lost sensitivity to the bacteriocin (van Belkum *et al.*, 1991). There is further evidence from the high degree of lactococcin A specificity against closely related *Lactococcus* strains that lactococcin A acts via a protein receptor (van Belkum *et al.*, 1991). Such a degree of specificity may be due to interactions of the bacteriocin with a specific protein receptor found only in lactococcal strains. The class IIb bacteriocin, lactococcin G, may be mediated by unidentified cellular factors, because it is only active against whole cells while neither membrane nor lipid vesicles are susceptible (Moll *et al.*, 1996).

The isolation and characterization of putative receptors and/or docking molecules would not only help determine their role in the binding of bacteriocins to target membranes, but also shed further light on the mode of action of bacteriocins, their specificity for particular bacterial strains which

differ in bacteriocin sensitivity, and its role in bacteriocin resistance.

1.7.4 Transmembrane potential and pH gradient

With the use of liposomes and vesicles which have been artificially energized to varying degrees, nisin and other type A lantibiotics require a threshold potential for activity (Abee *et al.*, 1991; Bruno and Montville, 1993; Gao *et al.*, 1990; Garcerá *et al.*, 1993; Rühr and Sahl, 1985; Sahl, 1985; 1991; Sahl *et al.*, 1998). In addition to the threshold potential, the orientation of the potential was also proven to be important for some peptides. Pep5 and nisin only function with a *trans*-negative membrane potential while others such as subtilin and SA-FF22 form pores irrespective of the orientation of the potential applied (Abee *et al.*, 1991; Benz *et al.*, 1991; Gao *et al.*, 1990; Garcerá *et al.*, 1993; Jack *et al*, 1994; Kordel *et al.*, 1988; Schuler *et al.*, 1989). However, liposomes supplemented with lipid II did not require prior energization for activity of nisin (Brötz *et al.*, 1998; Breukink *et al.*, 1999).

Depending on the experimental system used, nisin formed pores in an energy independent fashion where both $\Delta\Psi$ (inside negative) and ΔpH (inside alkaline) enhanced nisin activity, or in a voltage-gated fashion, where the energized state of the membrane was required. Nisin also permeabilized lipid vesicles in the absence of a $\Delta\Psi$, although the presence of a $\Delta\Psi$ increased poration (Breukink *et al.*, 1997; Garcerá *et al.*, 1993; Winkowski *et al.*, 1996). In sensitive lactococcal cells, nisin dissipated ΔpH in the absence of a $\Delta\Psi$. However, the rate of ΔpH dissipation increased with the magnitude of the ΔpH (Moll *et al.*, 1997). Brötz *et al.* (1998) demonstrated that liposomes which

had lipid II incorporated into them were susceptible to nisin without previous energization.

The class II bacteriocins, pediocin PA-1 and lactococcin A, permeabilized the target membranes *in vivo*, in the presence, as well as the absence of PMF (van Belkum *et al.*, 1991; Chikindas *et al.*, 1993). Pediocin PA-1 functions in a $\Delta\Psi$-enhanced fashion when acting on lipid vesicles (Chen *et al.*, 1997). *In vivo* and *in vitro* findings were consistent for bavaricin MN, where the presence of $\Delta\Psi$ stimulated PMF dissipation in cells and increased CF efflux from lipid vesicles (Kaiser and Montville, 1996).

1.7.5 Influence of external pH

The binding affinity of nisin to lipid vesicles was not affected, when the pH was decreased from 8.0 to 5.0, but the acidic pH raised the critical number of nisin molecules required to initiate CF efflux (Winkowski *et al.*, 1996). As a result of the pH decrease, CF efflux decreased from lipid vesicles (Winkowski *et al.*, 1996). The nisin-induced $\Delta\Psi$ dissipation was markedly reduced at pH 6.0 compared with pH 7.0. External pH had little influence on ΔpH dissipation in sensitive lactococcal cells (Moll *et al.*, 1996).

In contrast, the CF efflux caused by pediocin PA-1 and the binding of the peptide was higher at acidic than at alkaline pH values (Chen *et al.*, 1997). Pediocin PA-1 had a higher binding affinity than nisin, which demonstrated that pediocin PA-1 bound to the lipid vesicles more strongly than nisin at pH 6.0 (Chen *et al.*, 1997). A possible explanation for this phenomenon was that pediocin

PA-1 had a higher net positive charge than nisin at the lower pH. The arrangement of positively charged amino acids which form clusters of positively charged side chains may anchor the pediocin molecule more tightly to the negatively charged head groups of the bilayer, and thus decrease the dissociation rate of bound pediocin (Chen *et al.*, 1997). Bavaricin MN-induced CF efflux was maximal at pH 6.0 and decreased as pH deviated from the optimum (Kaiser and Montville, 1996).

No common trend was apparent for the influence of external pH on bacteriocin activity, but this is an important consideration in the biopreservation of food because pH of foods vary considerably.

1.8 In perspective

A considerable amount of information exists about the mechanism of action of the class I bacteriocin nisin. Much still remains to be discovered about class II bacteriocins. Although common aspects of bacteriocin action have been identified, clear differences exist with respect to the selectivity of the pore and the involvement of cell surface factors in pore formation. Models for the exact mechanisms of pore formation exist for nisin, but it still remains to be elucidated how well these models describe the mode of action of class II bacteriocins. Such models, in conjunction with continued structure-function relationship studies, would allow for the production of synthetic bacteriocins with enhanced activity. An important factor governing pore formation is the role of putative docking or receptor molecules at the cell surface. It would be imperative to determine the function of such molecules and their roles in pore formation.

An alternative approach to obtaining information about factors that determine the sensitivity of species to a bacteriocin is to isolate bacteriocin-resistant variants of a strain and to compare the properties of these bacteriocin-resistant strains with those of the parental strain. This strategy has been used for nisin. For the genus *Listeria*, nisin resistance has been correlated with both changes in membrane composition (Crandall and Montville, 1998; Mazotta and Montville, 1997; Ming and Daeschel, 1993; 1995; Verheul *et al.*, 1997), and cell wall changes (Crandall and Montville, 1998; Davies *et al.*, 1996; Maisner-Partin and Richards, 1996), but without study of the molecular basis of resistance.

Mechanisms of resistance to the class II bacteriocins are poorly understood, even though it is a stable phenomenon (Rekhif *et al.*, 1994). The only molecular work was done by Robichon *et al.* (1997), who found that transposon -mediated inactivation of σ^{54} in *L. monocytogenes* rendered it resistant to mesentaricin Y 105. In an attempt to discover phenomena with associated resistance at both the DNA and proteomic level, Amplified Fragment Length Polymorphism (AFLP) and two-dimensional (2-D) gel electrophoresis were employed, respectively.

Studies to date have demonstrated that AFLP is highly reproducible (Dijkshoorn *et al.*, 1996; Janssen *et al.*, 1996; Koelman *et al.*, 1998, Savelkoul *et al.*,1999; Vos *et al.*, 1995) and it has a good potential to differentiate clonally derived strains (Dijkshoorn *et al.*, 1996; Gibson *et al.*, 1998; Janssen *et al.*, 1996; Janssen *et al.*, 1997; Koelman *et al.*, 1998). The differentiating power of AFLP appears to be greater than that of PCR-based ribotyping (Koelman *et al.*, 1998). However, repetitive DNA sequence-based PCR (rep-PCR) revealed more strain variation within clusters

(Rademaker *et al.*, 2000) than AFLP and pulsed field gel electrophoresis (PFGE) (Desai *et al.*, 1998).

In contrast to the genome, the proteome is an entity that can be altered depending on the cell type, developmental change, or physiological conditions. For many genes, more than one protein product exists due to alternative splicing, post-translational modification, or cellular processing. Two-dimensional gel electrophoresis provides a powerful technique for monitoring these processes. Two-dimensional PAGE allows for the resolution of a complex protein mixture into more discrete components than 1-D PAGE, because it separates on the basis of protein charge in addition to molecular weight (O' Farrell, 1975).

The utility of this technique lies in its enormous resolving power of the 5 000 to 10 000 proteins present in a cell. Approximately 1 000 to 2 000 can be resolved in a single electrophoretic run. Not only does this provide a method for detecting small changes in the levels or properties of proteins in response to changes in cellular conditions (Huang *et al.*, 1997; Lasko *et al.*, 1997; Phan-Thanh and Gorman, 1995; 1997), it also allows for the isolation of proteins in quantities sufficient for structural analysis by a number of techniques, including amino acid analysis, amino-terminal sequencing, peptide mass fingerprinting, and tandem mass spectrometry.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Bacterial strains and growth conditions

Listeria monocytogenes B73, a food isolate from the laboratory collection at the University of Natal (Pietermaritzburg), South Africa, was grown on brain heart infusion (BHI) agar or broth at 30 °C for all experiments. The *L. monocytogenes* B73-MR1 resistant strain was isolated during the screening of crude bacteriocins (leucocin A, B, and C) from the producer organism *Leuconostoc mesenteroides* TA33a and was maintained in the same manner as the parental strain.

2.2 Bacteriocin preparation

Leucocin A was synthesized in the laboratory of Dr. Aimoto, Osaka, Japan as described previously (Dykes *et al.*, 1998). Lyophilized purified leucocin A was resuspended in 0.1 % TFA (trifluoroacetic acid) as described previously (Hastings *et al.*, 1996). Synthesized ESF1-7GR was prepared as for leucocin A (Dykes *et al.*, 1998). Nisaplin obtained from Aplin and Barrett Ltd. (Beaminsteri, UK) was prepared by dissolving in 0.2 N HCl. All bacteriocin stock solutions were stored at -20°C until used.

2.3 Minium inhibitory concentration (MIC)

Liquid MIC determination was done using a standard method of Hancock with minor modifications (Hancock- www.interchg.ubc.ca/bobh/peptides.htrr.). Bacterial strains were cultured to an OD_{600} of approximately 0.7 representative of mid-log growth phase. To ensure that an equal number of

cells was added each time, X µl of cells was made up to one ml with fresh broth each time, where X was calculated as $10/OD_{600}$. From the 1 ml of diluted bacterial cells, a further dilution was carried out by removing 100 µl of the cellular suspension and adding it to 10 ml of fresh broth. A 100 µl portion of this second cell suspension was added to each well of a microtitre plate (nunc plate, cat no. # 442587) Uninoculated media served as a sterility control. A 100 µl amount of bacteriocin suspension was added to the first and second well, the second well was mixed and 100 µl was then transferred to the third well. This process was continued until well number 30. Well number 31 served as a positive control, because it contained no inhibitory bacteriocin. Micotitre plates were incubated at 30°C and results were checked for indicator growth after 16 h and again at 24 h. MIC was recorded as the lowest concentration of bacteriocin at which no growth occurred. Bacteriocin activity was also determined using the spot-on-lawn method (Hastings *et al.*, 1996). Ten microlitres of each twofold dilution was spotted onto an indicator lawn of *L. monocytogenes* B73 or *L. monocytogenes* B73-MR1 and incubated for 16-18 h at 30^0C. The indicator lawn was prepared by adding 0.1% of an overnight culture to 10 ml of overlay of BHI soft agar (BHI broth plus 0.7% agar). The titre was defined as the reciprocal of the highest dilution showing a visible zone of inhibition, and was expressed in arbitrary units per milliliter (AU/ml). Experiments were repeated five times for both the liquid MIC and the spot on lawn assay and mean results were presented.

2.4 Cross resistance

The spot-on-lawn assay (Hastings *et al.*, 1996) (see, section 2.3) was used to determine the relative

resistance of strains to both Nisaplin (trade name for nisin) (Aplin and Barret, Trowbridge, Wilts, England) and ESF1-7GR, an analog of an α-helical portion of the antimicrobial peptide magainin PGLa which was isolated from a frog (Dykes *et al.*,1998), using *L. monocytogenes* B73 and *L. monocytogenes* B73-MR1 as indicator strains. The titre was determined as described above.

2.5 Stability of phenotypic resistance character

The resistant mutant strain *L. monocytogenes* B73-MR1 was subcultured, by the addition of 100 µl of the overnight culture into fresh media, 20 times in BHI broth without bacteriocin. An indicator lawn was prepared after each subculture and the level of resistance was determined using the spot-on-lawn assay as described previously. As a control, an overnight culture of *L. monocytogenes* B73-MR1 was always prepared and used as an indicator lawn.

2.6 Relative fitness

Growth curves were determined by growing cultures of *L. monocytogenes* strains B73 and B73-MR1 individually in BHI broth for 16 h at 30°C. Relative growth rates were determined by measuring optical density at 590 nm at each time interval using uninoculated BHI broth as a blank. This experiment was repeated three times and mean results and standard deviations were calculated.

2.7 Binding assays

Mid-log phase *L. monocytogenes* strains B73 and B73-MR1 were diluted to an A_{600} of 0.7. Cell suspensions were centrifuged (10 000 x *g*, 15 min, 4°C). Cell pellets were washed twice in phosphate buffer (5 mM, pH 6.8). Washed pellets were resuspended in 100 µl phosphate buffer (5 mM, pH 6.8). Phosphate buffer (5 mM, pH 6.8) without bacterial cells served as a control. A 5 µl sample portion of leucocin A stock (2 mg/ml) was added to each of the samples and the control. Samples were vortexed for 5 s and immediately placed at 30°C for 15 min. After incubation, cells were immediately pelleted (10 000 x *g*, 15 min, 4°C). The supernatant was removed and placed on ice. The activity of each sample was determined using the spot-on-lawn assay (Hastings *et al.*, 1996). *L. monocytogenes* B73 was used as an indicator lawn as described previously. This experiment was repeated five times.

2.8 Genomic analysis

2.8.1 Isolation of genomic DNA

Intact genomic DNA was isolated using the NucleoSpin C + T (Macherey-Nagel) kit according to the manufacturer's instructions with the following modifications: i)a 4 ml overnight culture suspension was used; and ii) eluted DNA was treated overnight at 37°C with RNAse I (Boehringer Mannheim). DNA was quantified electrophoretically using Lambda standards (Boehringer Mannheim) on 0.8% agarose gels.

2.8.2 AFLP reactions

Ligation and preselective PCR was done with an AFLP Ligation and Preselective amplification kit (Perkin-Elmer, Foster City, Calif.) according to the manufacturer's instructions with minor modifications. Tubes containing the *Mse*1 and *Eco*R1 adaptors were heated at 95^{0}C for five min and allowed to cool to room temperature over a ten minute period. Restriction and ligation reactions were prepared by the addition of 500 ηg of genomic DNA; 10x T4 ligase buffer (1µl) which included ATP (Boehringer Mannheim); 0.5 M nuclease-free NaCl (1 µl) (Boehringer Mannheim); 1 mg bovine serum albumin (0.5 µl) per ml (New England Biolabs, Beverly, Mass); 1µl each of the *Mse*1 and *Eco*R1 adaptors (Perkin-Elmer) and 1µl enzyme master mix containing 0.1 µl 10x T4 ligase buffer; 0.1 µl nuclease-free NaCl; 0.05 µl 1 mg bovine serum albumin per ml (New England Biolabs, Beverly, Mass); 2,5 U T4 DNA ligase (Boehringer Mannheim); and 1.5 U *Tru*1 (Boehringer Mannheim) which is an isoschizomer of *Mse*1 and 10 U of *Eco*R1 (Boehringer Mannheim). The restriction-ligation reactions were incubated at 37^{0}C for four h in the Perkin-Elmer 2400 thermal cycler. The reaction mixture was diluted with 0.1 M TE buffer (20 mM Tris-HCl, 0.1 mM EDTA; pH 8.0) to a final volume of 200 µl. PCR amplification was done on a mixture consisting of 4µl of the diluted restriction-ligation mixture, 1µl of *Eco*R1 and *Mse*1 preselective amplification primer pairs (Perkin-Elmer), and 15µl of AFLP core mixture (Perkin-Elmer). Both *Eco*R1 and *Mse*1 preselective primers had a single selective nucleotide on the 3' end (A and C respectively). Thermal cycler parameters for amplification using the Perkin-Elmer 2400 was as follows: an initial hold step consisting of 72^{0}C for 2 min; 20 cycles consisting of 94^{0}C for 20 s, 56^{0}C for 30 s, and 72^{0}C for 2 min; and a final hold step of 60^{0}C for 30 min. All ramp times

were set at 90% for the PCR amplifications. The PCR products were stored at -20^{0}C.

2.8.3 Separation of PCR products

2.8.3.1 Preparation of sequencing gel

AFLP products were run on a 6% denaturing polyacrylamide sequencing gel containing ultra-pure urea (ICN Biochemicals Inc, Aurora, Ohio). To cross-link the gel chemically to the short plate, it was treated with Bind Silane (Promega, Madison, Wisc.). This step was essential to prevent tearing of the gel during the silver staining procedure. A fresh 1 ml solution consisting of 950 µl of 99.6% ethanol (Merck, Darmstadt, Germany); 45 µl Milli-Q water; 5 µl glacial acetic acid [Associated Chemical Enterprise (ACE)] and 5 µl Bind Silane (Promega) was applied to the glass plate. After 4-5 min, the plate was wiped three times with 95% ethanol, to remove excess binding solution. To prevent the gel from binding to the long plate, it was treated with silanizing fluid [5% (v/v) dimethyldiclorosilane (Merck) in chloroform (ACE)]. A paper towel saturated with silanizing fluid was wiped across the plate until the plate was completely covered. After 10 min, excess silanizing fluid was removed by wiping the glass plate three times with 70% ethanol. Excess silanizing fluid could lead to the inhibition of silver staining. The two glass plates were separated by a 0.4 mm spacer and wells were formed using a sharks tooth comb.

2.8.3.2 Sample preparation and electrophoresis

Equal volumes of loading buffer (95% formamide, 20 mM EDTA (ACE), 0.05% bromophenol blue, and 0.05% xylene cyanol) and amplified product (5 µl) were loaded into each lane. Prior to loading of the gel, samples were heated at 90^{0}C for 3 min and snap cooled by placing them immediately on ice, to prevent DNA secondary structures from reannealing. Electrophoresis was done on a S2 sequencing gel apparatus (Gibco, BRL. Life Technologies, Gaithersburg, Md). Pre-electrophoresis was done at 55 W constant power for 30 min. Separation of AFLP products was done at 55W with 1x Tris -borate-EDTA (TBE) as the running buffer in the upper chamber, and 1x TBE supplemented with 0.5 M sodium acetate (Merck) in the lower compartment, to prevent gel "smiling" or "frowning" (Aarets *et al.*, 1998). The current was applied until the slower migrating tracking dye was two thirds down the length of the gel.

2.8.3.3 Detection of PCR products

After electrophoresis, AFLP fingerprints were detected by a modified silver staining method described by Bassam *et al.* (1991). Immediately after electrophoresis, the glass plate sandwich was split with the gel still attached to the short plate. It was fixed in 12% glacial acetic acid (ACE) for approximately 20-25 min or until the tracking dyes disappeared. The acetic acid was removed by rinsing the gel 3 times for 2 min with Milli-Q water. If acetic acid was not completely removed it could interfere with the downstream staining steps. The gel was immersed in a solution containing silver nitrate (1 g/L) (BDH, Poole, England) and 14.8% formaldehyde (2.5 ml/L)

(BDH), for 30 min. The gel was rapidly rinsed once in Milli-Q water. If the gel was rinsed for more than 10 s, the impregnation step would have to be repeated because the silver that was bonded to the DNA would be washed off. This would lead to decreased intensity of the bands. However, if insufficient rinsing occurred, it would cause an increase in the background staining. This could hamper the observation of the bands. The gel was then developed with a solution containing sodium carbonate (30 g/L) (Merck), formaldehyde (2.5 ml/L) (BDH) and 0.1 M sodium thiosulphate (125 µl/L) (BDH). These were added prior to use. This solution was stored at 4°C prior to use. Once the banding pattern was visible, the developing reaction was stopped with precooled 12% glacial acetic acid (ACE). The gel was rinsed twice in Milli-Q water for 2 min to remove the acetic acid and air-dried overnight. The stained gel was examined for polymorphic bands.

2.9 Proteomic analysis

2.9.1 Isolation of total cellular proteins for SDS PAGE

L. monocytogenes strains B73 and B73-MR1 were cultured in BHI broth for 16 h at 30°C. Bacterial cells were harvested (8 000 x *g*, 15 min, 4°C), washed once in 0.01 M phosphate buffered saline pH 7.0 and twice in Tris-HCl (32 mM, pH 7.0) (Boehringer Mannheim). The cell pellet was finally resuspended in 1 x Tris-EDTA (TE) (pH 7.0) containing the mini Complete™ tablet (Boehringer Mannheim), which is a mixture of several protease inhibitors with broad inhibitory specificity. Cells were sonicated on ice for 3 x 4 minute bursts at power setting 15 (Versonic, The Virtis Company, INC-Gardiner, N.Y). Insoluble cellular debris was removed by centrifugation

(6 000 x *g*, 45 min, 4°C). The supernatant was removed and dried under vacuum at 55°C. The samples were resuspended in a small volume of loading buffer [0.125 M Tris-Cl, 4% (w/v) SDS, 20% (v/v) Glycerol, 0.2 M dithiothreitol (DTT), 0.02% (w/v) bromophenol blue, pH 6.8)] and electrophoresed on 12.5% (44:0.8, acrylamide:bis-acrylamide) gel, containing a 4.5% stacking gel (30:0.8, acrylamide:bis-acrylamide), using the Hoefer SE600 series with the 18 x 16 cm gels by the method of Laemmli (1970). The stacking gel was run at 35 mA constant current and the separating gel at 75 mA constant current. Gels were stained with Coomassie Blue.

2.9.2 Isolation of total cellular proteins for 2D gel electrophoresis

Total cellular protein of *L. monocytogenes* strains B73 and B73-MR1 was isolated as described for the isolation of total cellular proteins for SDS PAGE, up to and including sonication. After sonication, the mixture was treated with 5.5 µl of a 10 mg/ml stock of DNAse I (Boehringer Mannheim) and 5.5 µl of RNAse I (Boehringer Mannheim) and incubated at 37°C for 30 min. Nucleic acids are also charged molecules that can interact with proteins as well as alter apparent pI ranges. They also stain with silver-staining procedures making protein interpretation more difficult. To solubilize the proteins, a solution containing 9.5 M ultra pure urea (ICN Biochemicals), 100 mM DTT (Boehringer Mannheim), 4% (v/v) Triton X-100 (BDH), 0.4% (v/v) pH 3.5 - 9.5 Ampholine® preblended (Pharmacia Biotech, Uppsala, Sweden) and 1.6% (v/v), pH 5 - 7 Ampholine® (Pharmacia Biotech) were added to give the final concentrations indicated. Ampholine (Pharmacia) was used exclusively for the initial 2D gel electrophoresis 2% (v/v) pH 3.5 - 9.5. In addition, the solution was incubated at 30°C for two h. Insoluble material was removed

by centrifugation (30 000 x *g*, 45 min, 25°C).

2.9.3 Alternative protein solubilization protocol

In the alternative protein solubilization protocol, the procedure for the isolation of total cellular protein for 2D SDS PAGE was followed up to the treatment of the cell homogenate with nucleases. In an attempt to increase solubilization of total cellular protein, the homogenate was treated with SDS and DTT to a final concentration of 1% and 100 mM, respectively. The samples were boiled for 5 min, after which the following reagents were added to 9.5 M ultra pure urea (ICN Biochemicals), 100 mM DTT (Boehringer Mannheim), 4% (v/v) Triton X-100 (BDH), 0.4% (v/v) pH 3.5 - 9.5 Ampholine® preblended (Pharmacia Biotech) and 1.6% (v/v) pH 5 - 7 Ampholine® (Pharmacia Biotech) at the above final concentrations. The solution was incubated at 30°C for 2 h. Samples were centrifuged (30 000 x *g*, 45 min, 25°C) and the supernatant containing the solubilized proteins was aspirated.

2.9.4 Isolation of water soluble proteins

Strain growth conditions, washing of cells, sonication, DNAseI and RNAseI treatment were carried out as described for the isolation of total cellular proteins for 2D SDS PAGE (see,section 2.9.2). Cellular homogenate was centrifuged (40 000 x *g*, 90 min, 4°C) to remove cellular debris and insoluble proteins. The cleared supernatant was removed and dried under vacuum at 55°C. This was resuspended in 2D sample buffer [9.5 M ultra pure urea, 100 mM DTT (Boehringer

Mannheim), 4% (v/v) Triton X-100 (BDH), 0.4% (v/v) pH 3.5 - 9.5] Amphopline® (Pharmacia Biotech) and 1.6% (v/v) pH 5-7 Ampholine® (Pharmacia Biotech) .

2.9.5 Determination of protein concentration for 2D gel electrophoresis

The protein concentration in samples was determined using the modified Bradford assay (Ramagli and Rodriguez, 1985; Dunbar, 1987). Protein standards of bovine serum albumin (Boehringer Mannheim) containing 10-50 µg were prepared in duplicate with 10 µl 2D sample buffer. To the standards, 10 µl of 0.1N HCl was added to each tube and the volume was brought up to 100 µl with 80 µl Milli-Q water. A 5 µl aliquot of the protein sample, solubilized in 2D sample buffer, was made up to 10 µl with the addition of 2D sample buffer and thereafter 10 µl 0.1N HCl (ACE) and 80 µl Milli-Q water were added. To the standards and the samples, 3.5 ml Coomassie dye reagent was added and thoroughly mixed. The absorbance was read at 595 nm. A blank containing all reagents except protein was used to set the zero. Samples of 12 µg and 350 µg of proteins were applied to first dimensional gels for silver staining and coomassie staining, respectively.

2.9.6 Isoelectricfocusing (IEF) and SDS PAGE

2-D electrophoresis was done according to a previously described procedure (O'Farrell, 1975) with the following modifications: i) the first dimensional separation was done in 16 cm x 1.5 mm (internal diameter) using the Hoefer tube gel adaptor of the SE6001-2D (Hoefer Scientific Instruments, San Francisco, Calif.). The glass tubes were first soaked overnight in chromic acid

and extensively rinsed in Milli-Q water. The tubes were placed in a KOH/ethanol solution (20 g KOH per 100 ml 95% ethanol) for two h. Hot, Milli-Q water was used to rinse the tubes. The glass tubes were dried at 100^0C.

The length of the gel affected reproducibility and care had to be taken to keep the measurements constant. The base of the glass tubing was sealed using parafilm. To produce 25 IEF gels the following components were combined: 3.45 g ultra pure urea (ICN Biochemicals); 798 µl IEF acrylamide (2.838 g acrylamide and 0.162 g bis-acrylamide dissolved in 7.1 ml Milli-Q water); 1.2 ml of a 10% (v/v) Triton X-100 solution; 1.2 ml Milli-Q water; 240 µl pH 5-7 Ampholine® (Pharmacia) and 60 µl pH 3.5 - 9.5 Ampholine® preblended (Pharmacia). For gels with a broad pH range between 3.5 and 9.5, 300 µl pH 3.5 - 9.5 Ampholine® preblended (Pharmacia) was only used for the preparation of 25 IEF gels. Once all of the components had been dissolved, the IEF gel solution was degassed. Immediately after the addition of 6 µl 10% APS and 4.2 µl TEMED, the IEF gel solution was loaded into the glass tubes using a 1 ml syringe with a steel needle spanning the length of the glass tube. The gel solution was added to the 11 cm mark and great care was taken to prevent bubbles within the gel and to ensure that the gels were all accurately at the 11cm mark. The gels were overlayered with 8 M urea. Gels were allowed to polymerize for at least two h.

After polymerization of the gels the 8 M urea was carefully removed by rinsing the surface of the gel with Milli-Q water. All liquid was removed from the gel surface and a 1 cm layer of sample overlay [2.75 g ultra pure urea (ICN Biochemicals); 2.5 ml Milli-Q water; 100 µl pH 5-7 Ampholine® and 25 µl pH 3.5 - 9.5 Ampholine® preblended] was added. The parafilm was

removed from the base of the gels and the gels were placed into the Hoefer tube gel adaptor of the SE6001-2D (Hoefer Scientific Instruments, San Francisco, Calif.). Gels were filled to the brim using the cathode solution containing 20 mM NaOH. The bottom tank contained the anode solution of 10 mM H_3PO_4. The IEF gels were pre-electrophoresed according to the following schedule (a) 200V for 15 min (b) 400V for 30 min and (c) 600V for 30 min. Samples were loaded with a 50µl Hamilton syringe and gels were run at 600V for 13 h and 1000V for one hour thereafter.

The gels were extruded from the tubes using air pressure from a syringe fitted with a 200µl Pipetteman tip and equilibrated in 4 ml SDS sample buffer (4.6 ml Milli-Q water; 1 ml 0.5 M Tris-HCL, pH 6.8; 0.8 ml Glycerol (BDH); 1.6 ml 10% SDS and 0.0618 g DTT (Boehringer Mannheim) for five min or immediately stored at -70°C until running.

The second dimensional separation (SDS PAGE) was done with 16.5% gels (44:0.8, acrylamide:bis-acrylamide) containing a stacking gel (30:0.8, acrylamide:bis-acrylamide) and was electrophoresed using the Hoefer SE600 series with the 24 x 16 cm gels to increase resolution. The IEF gel was placed on the surface of the slab gel ensuring that there were no bubbles between the IEF and the vertical gels. Melted agarose overlay (1 ml) [1.5 g Tris (Boehringer Mannheim), 7.2 g Glycine (BDH), 0.5 g SDS, 2.5 g agarose DI LE (Whitehead Scientific) and all components were made up to 500 ml with Milli-Q water] and was carefully layered over the tube gel to keep it in place. The stacking gel was run at 35 mA constant current and the separating gel was run at 75 mA constant current. Second dimensional gels were run at 10°C.

For all 2D of the gels presented, samples were isolated in duplicate and three gels of each sample were run before the pattern on a gel was considered to be reliable.

2.9.7 Gel staining

Gels were silver stained (Blum *et al.*, 1987) or they were stained with Coomassie Blue depending on the amount of protein loaded. All of the steps of silver staining were done at room temperature on an orbital shaker. After electrophoresis, the gel was soaked overnight in fixing solution [50% (v/v) methanol (Merck), 12% (v/v) acetic acid (ACE) and 0.2% (v/v) formaldehyde (ACE)]. To neutralize the gels for subsequent treatment with the acid labile sodium thiosulfate ($Na_2S_2O_3$) (BDH) gels were washed 3 times for 20 min in wash solution [50% (v/v) ethanol]. Gels were treated with pre-treatment solution [0.02% (m/v) $Na_2S_2O_3.5H_2O$] for 1 minute, and soaked in impregnation solution [0.2% (m/v) $AgNO_3$ and 0.03% (v/v) formaldehyde] for a further 20 min. After rinsing with Milli-Q water (3 x 20 s) to remove excess silver nitrate from the gel surface, the gel was immersed in development solution [6% (m/v) Na_2CO_3, 0.0004 % (m/v) $Na_2S_2O_3.5H_2O$ and 0.02% (v/v) formaldehyde] until spots were visible. The gels were rinsed in Milli-Q water (2 x 20 s), and treated with stop solution [50% (v/v) methanol and 12% (v/v) acetic acid]. Once staining was completed the gels were dried on the Hoefer gel drier (Drygel Sr, Slab gel dryer, Model SE 1160) and visually inspected for differences.

2.9.8 Electroblotting onto PVDF membrane

The second dimensional gel was run for an extra 2 h after the dye front reached the bottom of the gel in order to ensure better separation of the spot of interest. The region of the gel containing the 35 kDa spot was excised. Immediately after running, the excised gels were soaked in

electrotransfer buffer [10 mM CAPS (Sigma), 10% (v/v) methanol (Merck) and 0.1 mM thioglycollic acid, pH 11.0] for 5 min to reduce the amounts of Tris and glycine present in the gel. In the meantime, PVDF membranes (Boehringer Mannheim) were pre-treated in 100% (v/v) methanol for 15 s, Milli-Q water for 5 min and stored in electrotransfer buffer. The gel was sandwiched between a sheet of PVDF followed by a backup PVDF membrane and several sheets of 3 mm paper and arranged between the electroblotting cassette of the Mini Trans-Blot cell (Bio-Rad, Richmond, Calif.). Following electroblotting for 2 h at 200 mA, the PVDF membrane was washed in Milli-Q water (3 x 5 min), immersed in stain solution [0.025% (w/v) Coomassie Blue R-250 in 40% (v/v) methanol] for 7 min and in destaining solution [50% (v/v) methanol] for 10 min. Membranes were air dried and stored in a desiccator until the spot of interest was sequenced.

2.9.9 Protein sequencing

The approximately 35 kDa spot, was cut off the PVDF blot and the first 20 amino acids of the protein sequence were determined using automated Edman degradation on a 491 Procise automated sequencer (Perkin-Elmer Applied Biosystems). The identity of the protein was determined using the default settings on the Blast advanced database (Altschul *et al.*, 1997).

CHAPTER THREE

RESULTS AND DISCUSSION

3.1 Level of resistance

L. monocytogenes B73-MR1 was found to be resistant to levels of leucocin A in excess of 2 mg/ml, with *L. moncyotgenes* B73 having an MIC of 1.86×10^{-6} µg/ml using the liquid MIC method. This corresponds to a level of resistance in excess of 1×10^{11} AU/ml determined by the spot-on-lawn assay. These results indicate that *L. monocytogenes* B73-MR1 has a 1×10^{11} fold increased resistance to leucocin A, which has been confirmed by both liquid MIC and the spot-on-lawn assay. No variation in the duplicate experiments were observed for both the liquid and the spot-on-lawn assay. The level of resistance found for this class IIa bacteriocin was significantly higher than those found previously for class IIa and class I bacteriocins (Crandall and Montville, 1998; Dykes and Hastings, 1998; Ming and Daeschel, 1993; Rekhif *et al.*, 1994; Verheul *et al.*, 1997).

Noerlisa and Ray (1994) suggested that acquired resistance of bacterial cells to a bacteriocin produced by growing them in an environment containing that bacteriocin was a temporary trait, and was soon lost by growing them in an environment free of that bacteriocin. The high level of resistance in excess of 1×10^{11} AU/ml was still present after 20 subcultures in unsupplemented media. This indicates that the resistant phenotype of *L. monocytogenes* B73-MR1 was not merely an adaptation response due to the stresses imparted by the bacteriocin, but may be due to a genetic mutation. The stability of the resistant phenotype was similar to previous reports (Rekhif *et al.*, 1994) in which the resistant phenotype was stable for 10 sub-cultures in the absence of bacteriocin, but different from other resistant phenotypes that had lost its resistant phenotype after 10 transfers

in unsupplemented media (Dykes and Hastings, 1998). The bacteriocin resistant mutants isolated by Dykes and Hastings (1998) were possibly adaptive responses of the bacteria in response to the bacteriocin, because the bacteria was unable to maintain a resistant phenotype in the absence of the bacteriocins without the selective pressure of a bacteriocin. Nisin adaptive mutants or "trained mutants" obtained by sub-culturing bacterial cells on increasing concentrations of nisin led to a progressive increase of the MIC (Hanlin *et al.*, 1993; Hurst, 1981; Ray, 1992). Further sub-culturing on nisin-free media led to the loss of the resistant phenotype. However, the resistance phenotype in this study was stable, leading to the postulation of a genetic basis.

3.2 Cross resistance

Table 1: Minimum Inhibitory Concentrations[a] of *Listeria monocytogenes* B73 (wild type) and *Listeria monocytogenes* B73-MR1 (leucocin A resistant) toward antimicrobial peptides ESF1-7GR and nisin.

Antimicrobial compound	***Listeria monocytogenes*** **B73 AU/ml**	***Listeria monocytogenes*** **B73-MR1 AU/ml**
Nisaplin	6 400	3 200
ESF1-7GR	400	400

[a]Minimum Inhibitory Concentration determined using the spot-on-lawn assay (Hastings *et al.*, 1996).

The *L. monocytogenes* B73-MR1 did not show any significant cross-resistance to nisin, or the antimicrobial peptide ESF1-7GR (Table 1). It appears that leucocin A, as shown for mesentericin

Y 105, dissipates the proton motive force (PMF) of *L. monocytogenes*. Nisin acted against *L. monocytogenes* by depleting the PMF (Bruno and Montville, 1993; Maftah *et al.*, 1993). Even though the two bacteriocins have similar primary effects on *L. monocytogenes*, this did not result in cross resistance between them (Table 1). Spontaneous mutants of *L. monocytogenes* resistant to three class IIa bacteriocins were also found to be sensitive to nisin at the same levels as the parental strains (Rekhif *et al.*, 1994).

L. monocytogenes mutants resistant to mesentericin 52, curvacin 13 and plantaricin C19 were cross resistant to each other (Rekhif *et al.*, 1994). Dykes and Hastings (1998), generated resistant mutant strains to the class IIa bacteriocins leucocin A and sakacin A in *L. monocytogenes*. All were cross resistant to the various class IIa bacteriocins tested. Studies by Rekhif and co-workers (1994) and Dykes and Hastings (1998) showed that resistant mutants generated to a single class IIa bacteriocin resulted in cross resistance to other bacteriocins within this class. Further work regarding the susceptibility of the resistant strain to other class IIa bacteriocins would be essential to determine whether resistance to a single class IIa bacteriocin results in resistance to class IIa bacteriocins in general. This could indicate a common mode of action of class IIa bacteriocins.

Cross resistance was reported over a number of classes of bacteriocins (Crandall and Montville, 1998). It was found that nisin resistance in *L. monocytogenes* also led to resistance to the class IIa bacteriocin pediocin PA-1 and the class IV leuconocin S. However, the results of this study indicate that resistance to leucocin A did not lead to resistance to the nisin (Table 1). These preliminary results indicate that the mechanism of resistance in the resistant *L. monocytogenes* strain B73 may be specific for the mode of action of leucocin A.

3.3 Relative fitness

The resistant strain had a similar growth rate (relative fitness) compared with the sensitive strain (Figure 4). In a previous study (Dykes and Hastings, 1998), it was shown that the resistant strain had a lower relative fitness in comparison to the wild type strain. This suggests that there may be different mechanisms of resistance to the class IIa bacteriocins. Dykes and Hastings (1998) reported that the resistant strain of *L. monocytogenes* showed a lower relative fitness and the resistant trait was not stable, indicating a possible adaptive response. It may be possible that the unstable spontaneous mutants could be utilizing energy requiring pathways to mediate the resistance phenotype. In *L. monocytogenes* strain B73-MR1, resistance was clearly being mediated by a different mechanism.

Work on nisin, has shown that stable, resistant mutants of *L. monocytogenes* have similar growth rates (Crandall and Montville, 1998). Modifications to the fluidity of the cell membrane was reported for nisin resistant strains (Crandall and Montville, 1998; Mazzotta and Montville, 1997;

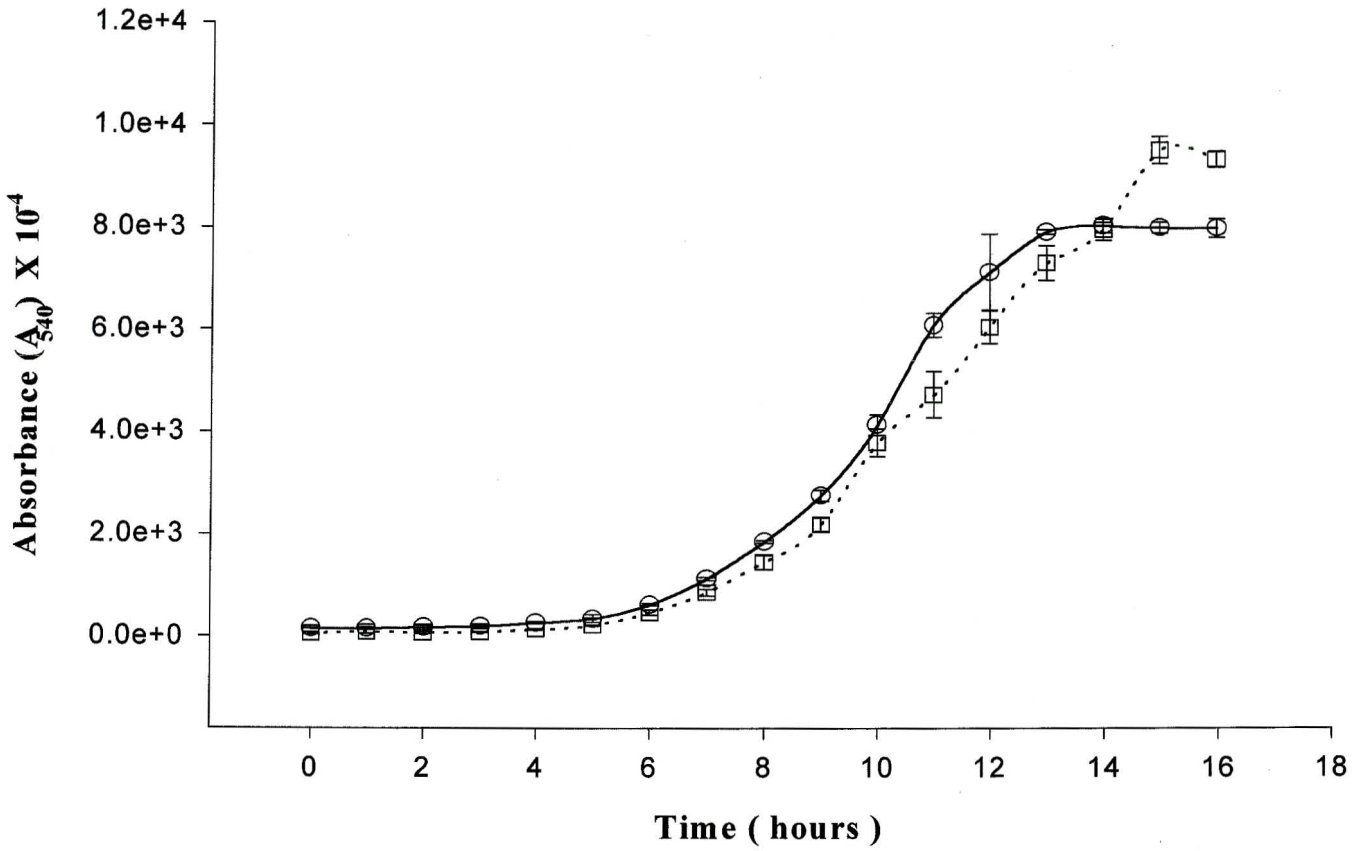


Figure 4: Growth curves of leucocin A sensitive strain of *Listeria monocytogenes* B73 (○) and resistant strain *Listeria monocytogenes* B73-MR1 (□) in brain heart infusion (BHI) broth without bacteriocin at 30°C. The mean of three trials are presented with standard deviations.

Ming and Daeschel, 1993; 1995). Evidence that a more rigid membrane affects nisin's activity was also reported for nisin Z (Abee *et al.*, 1994). A membrane at its optimal fluidity which allows for normal growth was found to be susceptible to the action of nisin, while a more rigid membrane that affects nisin activity does not permit normal growth (Mazzotta and Montville, 1997). Mazzotta and Montville (1997) demonstrated that nisin resistant strains with the modified membrane fatty acids had a slower growth rate than the wild type strain at all temperatures. The effect of membrane fluidity on class IIa bacteriocins was not determined.

Although the relative fitness of *L. monocytogenes* B73-MR1 was not compromised, it would be important to investigate the cell wall composition, phospholipid content and the composition of the cytoplasmic membrane fatty acids, to establish the effect of modifications to the above mentioned parameters on leucocin A resistance.

3.4 Binding assay

Studies on nisin established a correlation between nisin resistance in *L. monocytogenes* and decreased adsorption of the bacteriocin to the resistant strains (Davies and Adams, 1994; Ming and Daeschel, 1995). However, strains that were resistant to class IIa bacteriocins, appeared to bind the same level of bacteriocin as the parental strain (Rekhif *et al.*, 1994). Results shown in Table 2 indicated that *L. monocytogenes* B73-MR1 had a binding capacity for leucocin A that was eight times greater than *L. monocytogenes* B73. No variation in the duplicate experiments were observed.

Table 2: Residual amount of leucocin A activity detected after pre-incubation of leucocin A with *Listeria monocytogenes* strains B73 and B73-MR1.

Strain	Leucocin A (AU/ml)
No bacterial cells	819 200*
L. monocytogenes B73	51 200
L. monocytogenes B73-MR1	6 400

* Mean of three trials

Mesentericin Y 105 possibly interacts initially with non-specific sites, anionic cell surface polymers like teichoic acid and lipoteichoic acid (Fleury *et al.*, 1996). A similar mechanism was also proposed for pediocin AcH (Bhunia *et al.*, 1991). The decrease in the residual leucocin A activity detected for *L. monocytogenes* B73-MR1 could be due to modifications of the anionic cell components of the cell wall (Table 2). This indicates that further investigation into the cell wall composition may be necessary to elucidate the role of the cell wall components as non-specific docking sites for leucocin A.

3.5 Genomic analysis

High quality intact genomic DNA free of contaminating agents is essential for the AFLP process. Previous studies indicated that digestion of DNA with restriction endonucleases and amplification by PCR was often inhibited by the presence of polysaccharides. This broad class of substances was found to interfere with many types of DNA modifying enzymes (Lodhi *et al.*, 1994). Complete digestion of DNA was found to depend upon the absence of contaminating compounds and the

concentration of DNA (Figure 5).

Although only the preselective AFLP was carried out which contained only a single selective nucleotide on the primers, it should have been capable of detecting polymorphisms. Previous studies showed that for small bacterial and fungal genomes, a single PCR amplification with one and two selective nucleotides on both primers respectively, was sufficient in detecting polymorphisms (Janseen *et al.*, 1996; Koeleman *et al.*, 1998; Mueller *et al.*, 1996). No polymorphic bands were distinguishable between the resistant and wild type strains using the silver-stained AFLP process (Figure 6). The identical AFLP fingerprints indicates that the mutation which is responsible for the resistance phenotype may not be due to insertions or deletions in the portion of the genome scanned by the AFLP process

Janseen *et al.*, (1996) reported that AFLP was a highly reproducible technique when applied to bacterial genomes. In this study, the reproducibility was verified by triplicate extractions of genomic DNA from *L. monocytogenes* strains B73 and B73-MR1. The DNA samples were then subjected to restriction-ligation and preselective amplification. Banding patterns of lanes 2 to 4, and lanes 5 to 7 (Figure 6) indicated that AFLP was a highly reproducible process and that the results obtained were reliable.

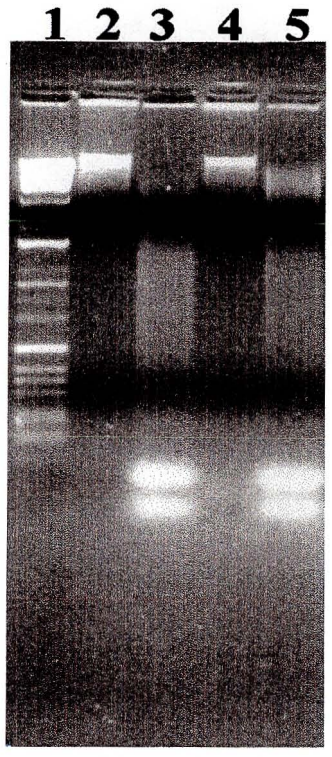


Figure 5: Agarose gel (0.8%) of restriction digests prior to AFLP reactions run at 70V. Lane 1:Molecular weight marker X (Boehringer Mannheim); Lane 2: 500 ng of undigested genomic DNA of *Listeria monocytogenes* B73; Lane 3: 500 ng of *Listeria monocytogenes* B73 genomic DNA after restriction ligation reaction (Perkin Elmer); Lane 4: 500 ng of undigested genomic DNA of *Listeria monocytogenes* B73-MR1; Lane 5: 500 ng of *Listeria monocytogenes* B73-MR1genomic DNA after restriction ligation reaction (Perkin Elmer). The two bright bands at the lower rnd of lane 3 and lane 5 are the excess *EcoR* I and *Mse* I adaptors.

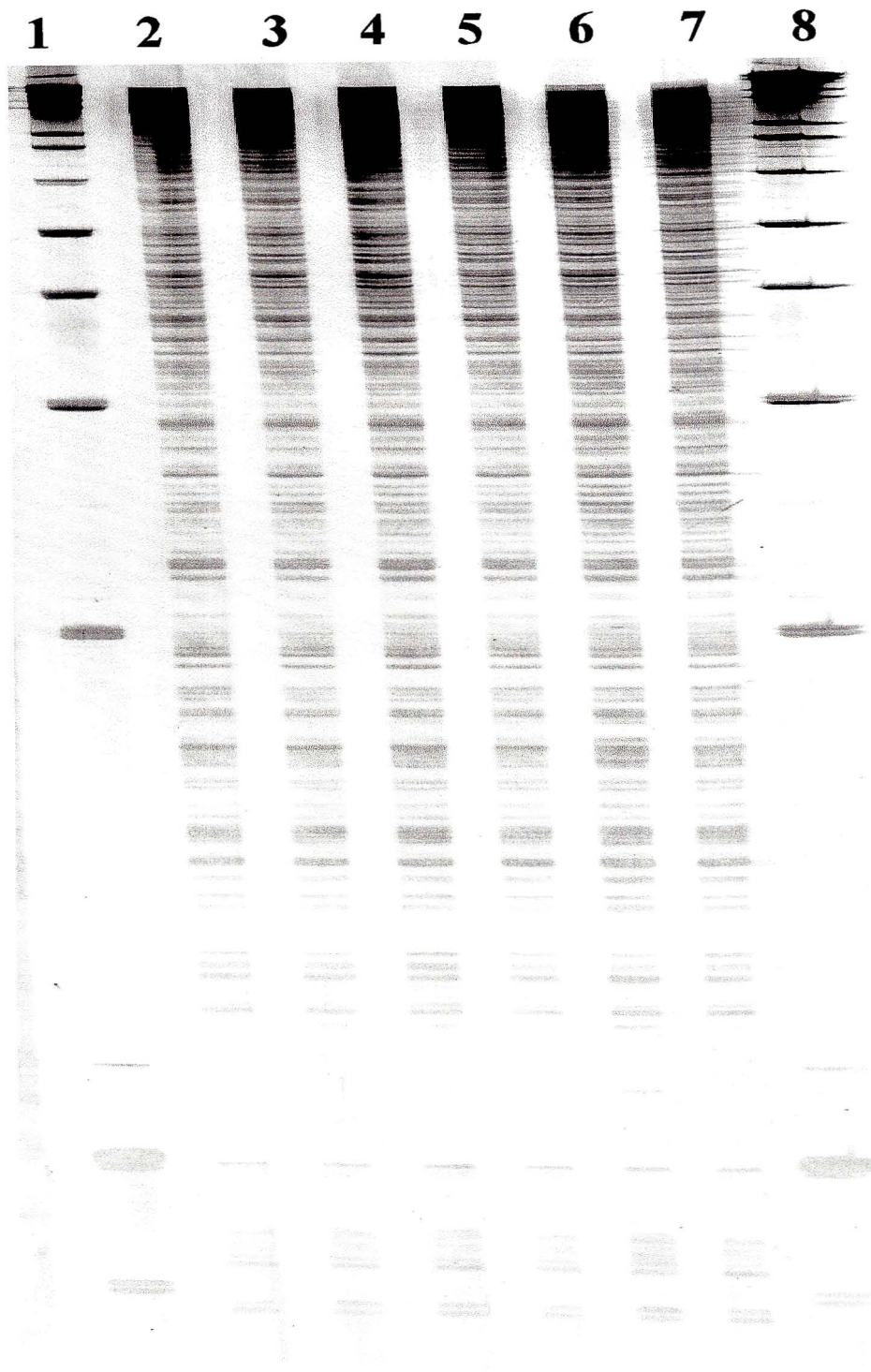


Figure6: Denturing polyacrylamide gel (6%) of preselective AFLP products electrophoresed at 55W. Lanes 1 and 8: 1kb ladder (Boehringer Mannheim); Lane 2 to lane 4: *Listeria monocytogenes* B73; Lanes 5 to 7: *Listeria monocytogenes* B73-MR1

3.6 Proteome analysis

No polymorphic bands were detected after SDS PAGE of total cellular proteins for *L. monocytogenes* strains B73 and B73-MR1. In an attempt to resolve the complex protein mixture of the total cellular proteins into their component polypeptides, a method of higher resolution was employed, namely, two-dimensional polyacrylamide gel electrophoresis (2D-PAGE).

In preliminary work using broad range ampholites (pH 3.5-9.5) exclusively for 2D gel electrophoresis, no differences in the protein pattern for *L. monocytogenes* strains B73 and B73-MR1 were produced (Figure 7). Utilization of a blend of ampholytes with a higher percentage of narrow range ampholytes (pH 5-7) (1.6% v/v) to broad range ampholytes (pH 3.5-9.5) (0.4% v/v) resulted in the separation of the central cluster of proteins obtained with the exclusive use of broad range ampholytes in Figure 7. Further use of different ampholyte ranges may result in the further separation of the clustering. Two differences were detected: (1) a 35 kDa protein that was present in the gels of protein from the sensitive strain but absent in the resistant strain, and (2) there was a difference in expression levels of a 18 kDa protein which showed a higher intensity signal in the resistant phenotype than in the sensitive phenotype (Figure 8).

The presence of the 35 kDa protein in *L. monocytogenes* B73 and the absence of this protein from *L. monocytogenes* B73-MR1 can clearly be seen in Figure 9.

The variation of the expression level of the 18 kDa protein in *L. monocytogenes* B73-MR1 (Figure

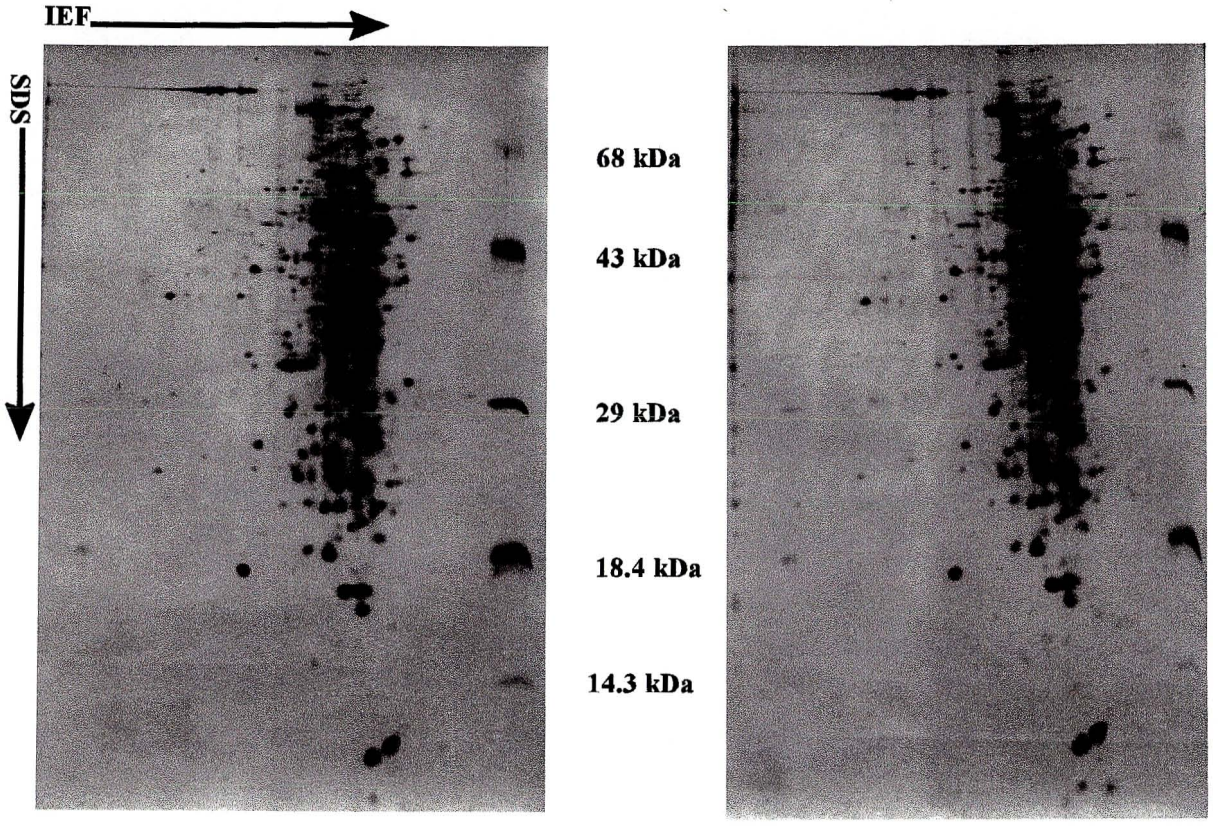


Figure 7: Silver-stained 2D SDS PAGE of total cellular proteins extracted from 16 hour cultures of: (i) *Listeria monocytogenes* B73 and (ii) *L. monocytogenes* B73-MR1 grown at 30°C, which were first separated by isoelectric focusing (IEF) [2% (v/v), pH 3.5 - 9.5 Ampholine (Pharmacia)] in the horizontal direction, followed by 16.5% (44:0.8, acrylamide:bis-acrylamide) SDS PAGE in the vertical direction. Directions of IEF and SDS PAGE are indicated by arrows.

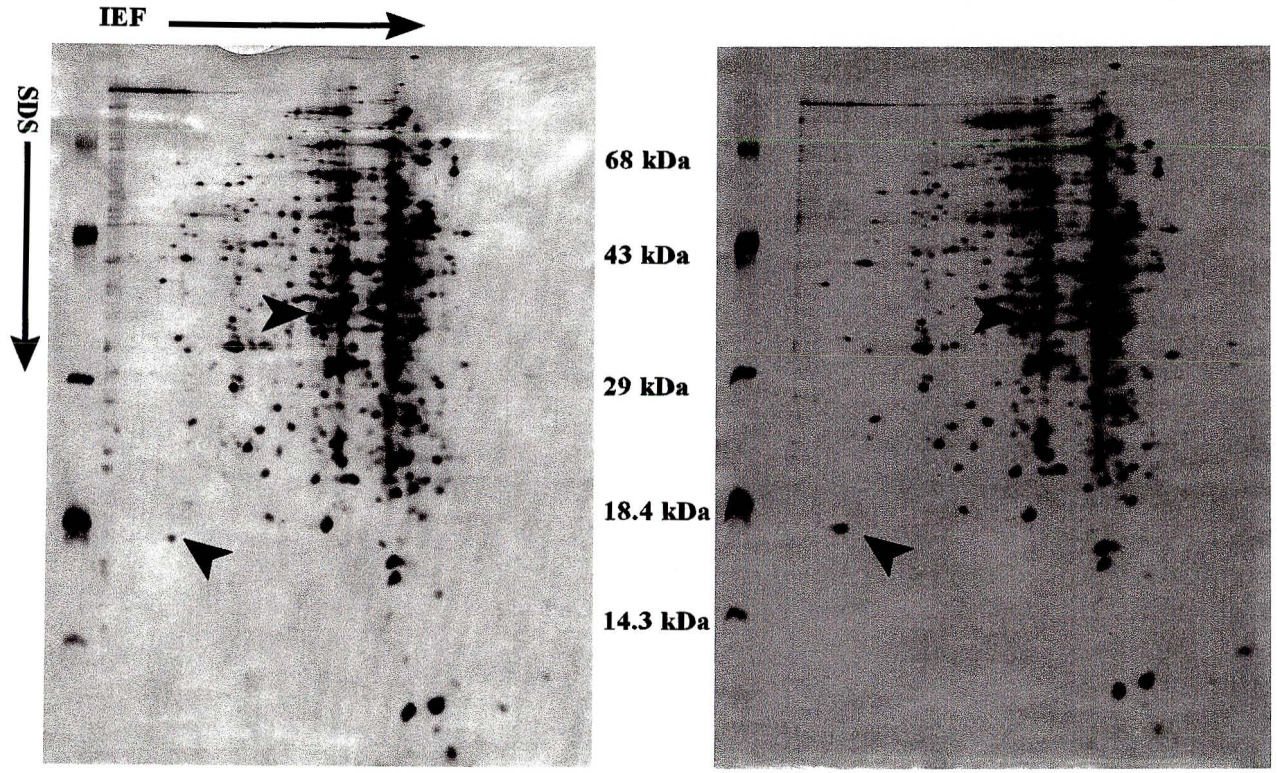
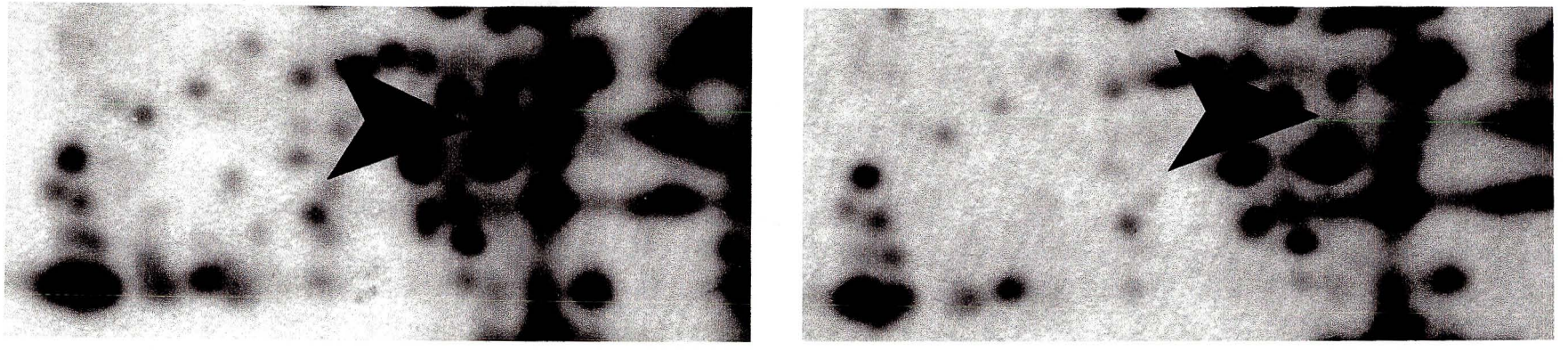


Figure 8: Silver-stained 2D SDS PAGE of total cellular proteins extracted from 16 hour cultures of: (i) *Listeria monocytogenes* B73 and (ii) *Listeria monocytogenes* B73-MR1 grown at 30^{0}C, which were first separated by isoelectric focusing (IEF) [1.6% (v/v) pH 5-7) Ampholine (Pharmacia) and 0.4% (v/v) pH 3.5-9.5 Ampholine (Pharmacia)] in the horizontal direction, followed by 16.5% (44:0.8, acrylamide:bis acrylamide) SDS PAGE in the vertical direction. Directions of IEF and SDS PAGE are indicated by arrows. Protein differences are indicated by arrow heads.



(i) (ii)

Figure 9: Magnification of the 29 kDa to 43 kDa region of a silver-stained 2D SDS PAGE gel of total cellular proteins, extracted from 16 hour cultures of: (i) *Listeria monocytogenes* B73 and (ii) *Listeria monocytogenes* B73-MR1, grown at 30^{0}C, which were firstly separated by isoelectric focusing (IEF) [1.6% (v/v) pH 5-7) Ampholine (Pharmacia) and 0.4% (v/v) pH 3.5-9.5 Ampholine (Pharmacia)] in the horizontal direction, followed by 16.5% (44:0.8, acrylamide:bis acrylamide) SDS PAGE in the vertical direction. Directions of IEF and SDS PAGE are indicated by arrows. The protein difference at 35 kDa is indicated by arrow heads.

8) was only apparent when the gels were silver-stained. However, coomassie-stained gels did not show any variation in intensity of the 18 kDa protein spot (Figure 10).

Instead, the 18 kDa protein was absent from *L. monocytogenes* B73 [Figure 10 (i)]. An extremely low intensity spot was visible for *L. monocytogenes* B73-MR1 [Figure 10 (ii)] when the gel was stained with Coomassie Blue. It was only when the stained gels were dried that the 18 kDa spot in the resistant strain was made more apparent. The absence of the spot in the coomassie-stained *L. monocytogenes* B73 gel could simply be due to the protein concentration being below the detection limit of the coomassie stain. Due to the low intensity of the 18 kDa spot, which is indicative of a low protein concentration, several spots would have to be collected in order to obtain a concentration which is required for automated Edman degradation amino acid sequencing of the protein.

The identification of this spot may provide additional insight into the complex changes in the regulatory cascade which gave rise to resistance to leucocin A.

3.6.1 Alternative Solubilization Technique

Sample pre-treatment with an SDS-based solubilization protocol in *Porphyromonas gingivalis*, in conjunction with the addition of a protease inhibitor, resulted in spots with improved resolution compared with the resolution obtained in the standard method

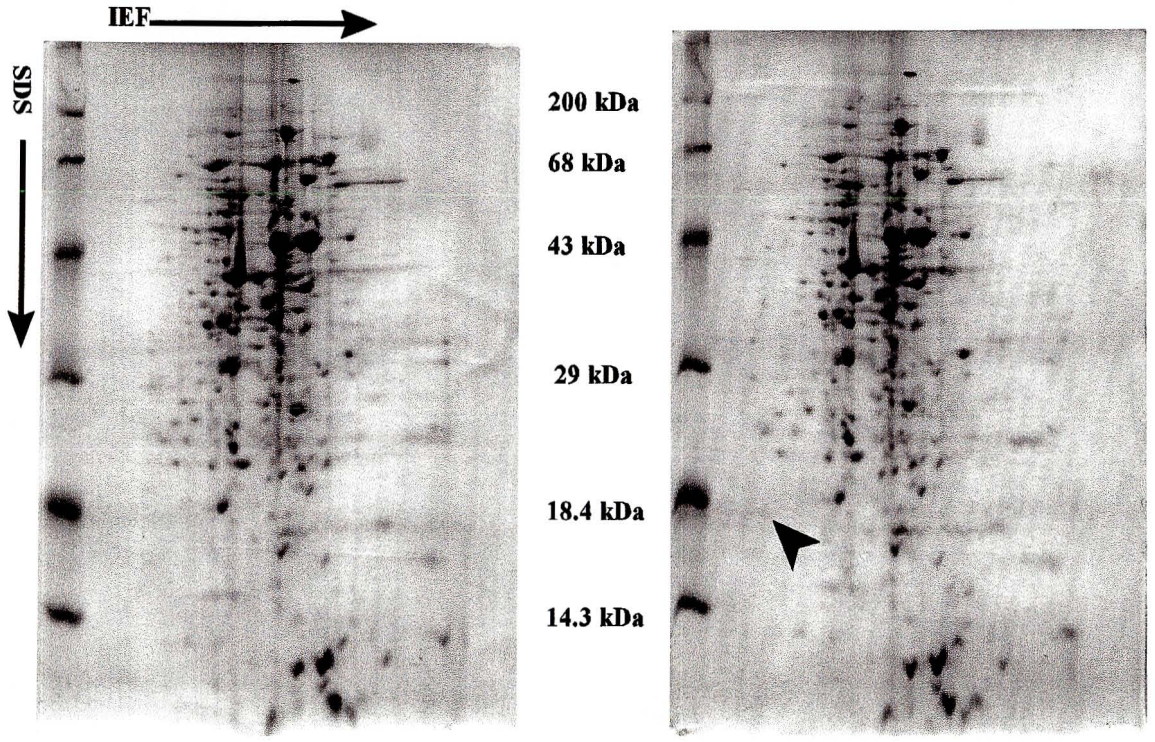


Figure 10: Coomassie-stained 2D SDS PAGE of total cellular proteins extracted from 16 hour cultures of: (i) *Listeria monocytogenes* B73 and (ii) *Listeria monocytogenes* B73-MR1 grown at 30°C, which were first separated by isoelectric focusing (IEF) [1.6% (v/v) pH 5-7) Ampholine (Pharmacia) and 0.4% (v/v) pH 3.5-9.5 Ampholine (Pharmacia)] in the horizontal direction, followed by 16.5% (44:0.8, acrylamide:bis acrylamide) SDS PAGE in the vertical direction. Directions of IEF and SDS PAGE are indicated by arrows. The 18 kDa protein is indicated by an arrowhead.

(Pridmore *et al.*, 1999). Non-specific staining was reduced and the spot definition was sharper. The inclusion of a SDS pre-treatment was also reported for the improved solubilization of membrane proteins (Ames and Kaido, 1976).

In an attempt to ensure the majority of proteins were solubilized the SDS pretreatment had to be assessed. Comparison of Figure 8 (i) with Figure 11 (i), and Figure 8 (ii) with Figure 11 (ii), indicated that identical patterns were obtained for samples pre-treated with SDS and the standard solubilization protocol. This shows that adequate disruption of protein complexes was achieved by the standard solubilization technique.

3.6.2 Water soluble proteins

Identical patterns were obtained for water soluble proteins from *L. monocytogenes* strains B73 and B73-MR1 (Figure 12). The two differences observed in the total cellular proteins were not observed in the water soluble proteins. This demonstrates that the differences observed (Figure 8), were not part of the water soluble component of proteins present in the cell.

Further compartmentalization of the proteins in the cell would not merely reduce the complexity of the 2D SDS PAGE gels due to small or number of proteins per gels. Isolation of various cellular protein fractions may indicate the location of the two differences observed between the resistant and wild-type strains, and possibly reveal more differences.

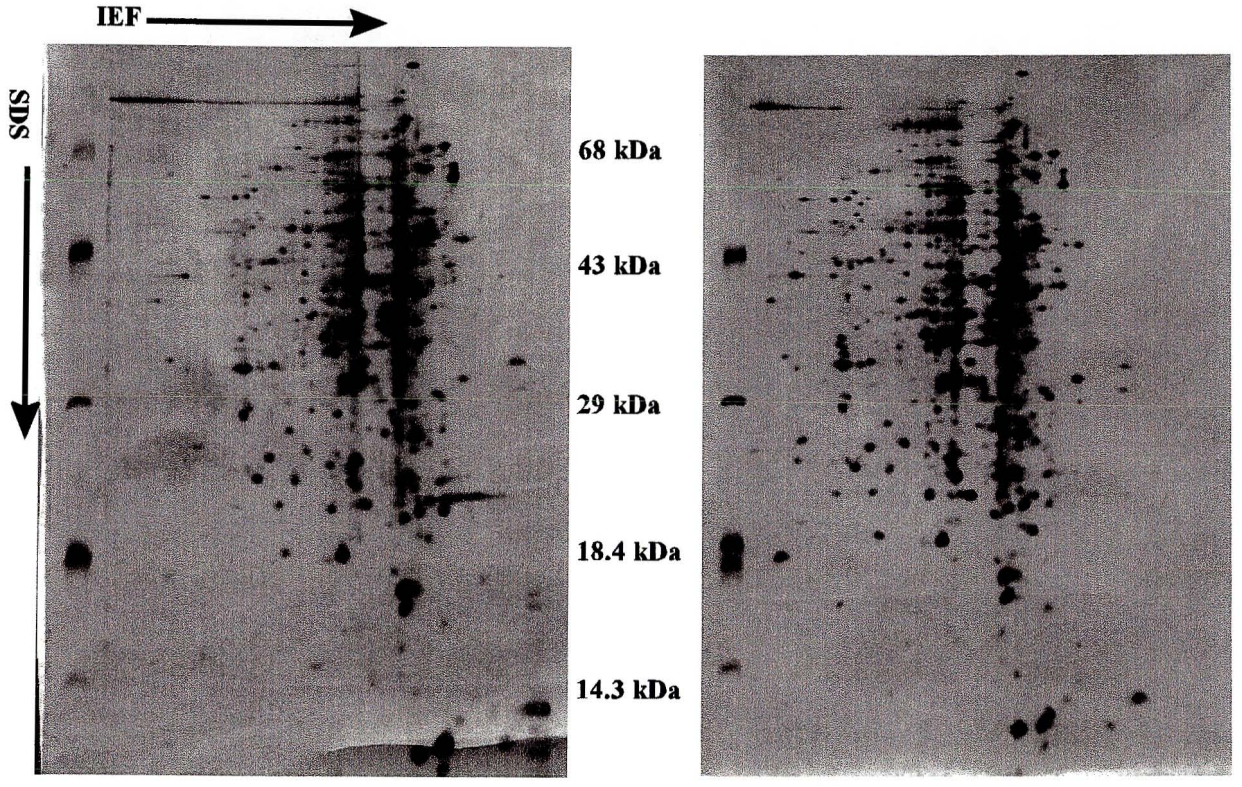


Figure 11: Silver-stained 2D SDS PAGE of SDS pre-treated total cellular proteins, extracted from 16 hour cultures of: (i) *Listeria monocytogenes* B73 and (ii) *Listeria monocytogenes* B73-MR1 grown at 30^{0}C, which were first separated by isoelectric focusing (IEF) [1.6% (v/v) pH 5-7) Ampholine (Pharmacia) and 0.4% (v/v) pH 3.5-9.5 Ampholine (Pharmacia)] in the horizontal direction, followed by 16.5% (44:0.8, acrylamide:bis acrylamide) SDS PAGE in the vertical direction. Directions of IEF and SDS PAGE are indicated by arrows.

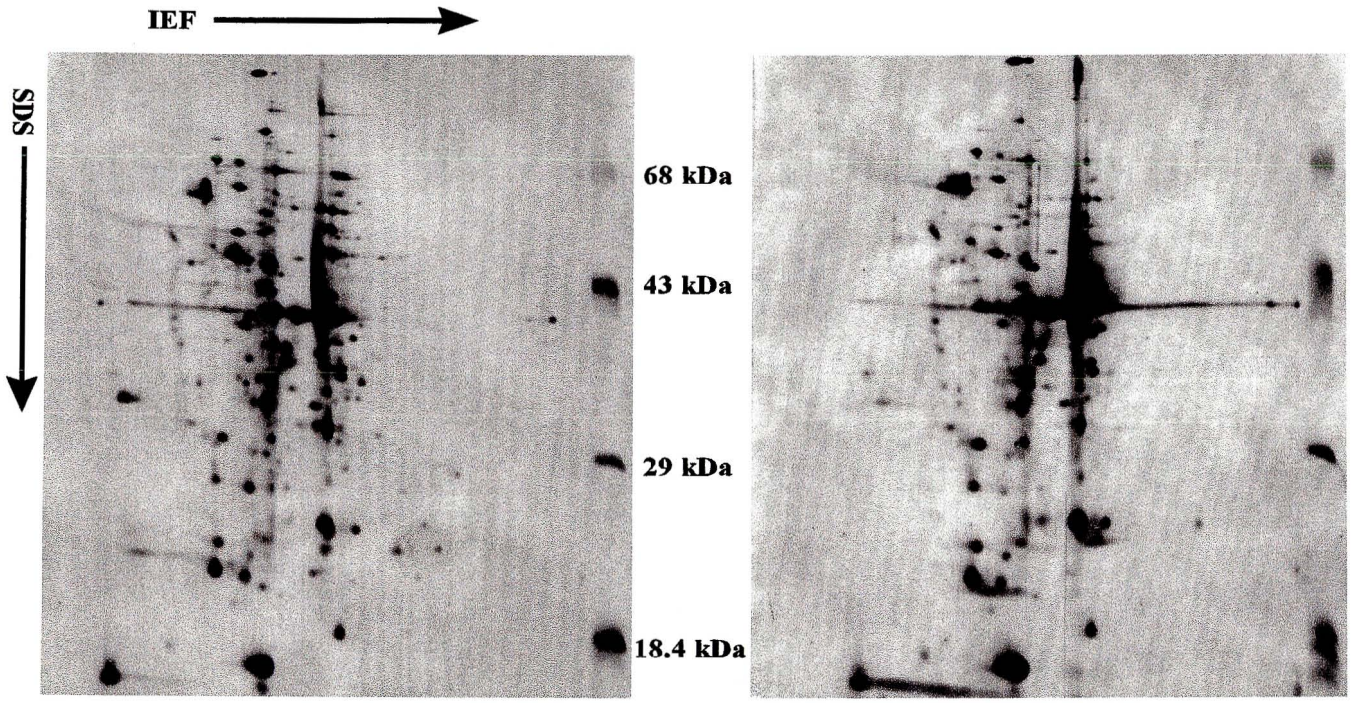


Figure 12: Silver-stained 2D SDS PAGE of water soluble proteins, extracted from 16 hour cultures of: (i) *Listeria monocytogenes* B73 and (ii) *Listeria monocytogenes* B73-MR1 grown at 30°C, which were first separated by isoelectric focusing (IEF) [1.6% (v/v) pH 5-7) Ampholine (Pharmacia) and 0.4% (v/v) pH 3.5-9.5 Ampholine (Pharmacia)] in the horizontal direction, followed by 16.5% (44:0.8, acrylamide:bis acrylamide) SDS PAGE in the vertical direction. Directions of IEF and SDS PAGE are indicated by arrows.

3.6.3 Electroblotting and sequence analysis of a spot of interest

The N-terminal 20 amino acids of the 35 kDa protein was determined using automated Edman degradation. The following sequence MVGIILAT/GHGWFAEGIKQWG was revealed. This sequence shares a 68% identity and 83% homology to the mannose-specific phosphotransferase system (PTS) II AB of *Streptococcus salivarius*.

The phosphotransferase system (PTS) mediates the uptake and concomitant phosphorylation of many carbohydrates in bacteria (Postma *et al.*, 1993; Saier and Reizer, 1994). Several phosphoryl transferases catalyze the relay of phosphate from phosphoenolpyruvate (PEP) to an incoming sugar. Some proteins of the PTS contain more than one phosphoryl-transfer-domain. Proteins containing these domains are functionally classified into two groups.

Enzyme I and histidine protein (HPr) compromise the soluble energy coupling PTS proteins and function to transfer phosphate from PEP to the sugar-specific phosphoryl carrier proteins. Enzyme II complexes are localized to the inner membrane (Postma *et al.*, 1993). These complexes consist of three (or four) proteins or domains, designated IIA, IIB, IIC (and sometimes IID) (Saier and Reizer, 1992; 1994; Vaneechoutte, 1996). The phosphate is transferred sequentially from PEP to Enzyme I, HPr, IIA, IIB, and finally to the incoming sugar, which is transported across the membrane via IIC (and sometimes IID) (Postma *et al.*, 1993). Each Enzyme II translocates specific sugar or subset of substrates (Postma *et al.*, 1993).

L. monocytogenes accumulates glucose by a high affinity PTS and a low affinity PMF (Christensen and Hutkins, 1994; Parker and Hutkins, 1997). Both the PTS and PMF-mediated uptake of glucose was affected by the class IIa bacteriocin pediocin JD (Christensen and Hutkins, 1994). PMF-mediated glucose transport and PEP-dependent PTS glucose transport inhibited by nisin, pediocin JD, and leuconocin S (class IV) (Waite *et al.*, 1998). The loss of the PMF by the dissipation of the cell membrane by the bacteriocins was probably the cause of inhibition of the low affinity, PMF-mediated glucose transport system.

Inhibition of the high affinity PEP-dependent PTS by nisin, pediocin JD, and leuconocin S may be due to loss of intracellular PEP or direct inhibition of PTS enzymes responsible for the transfer of the phosphate group from the PEP to the incoming sugar (Waite *et al.*, 1998). Studies on the effect of nisin on sensitive *L. monocytogenes* found that the PEP-dependent PTS activity increased in the presence of this bacteriocin and excess PEP. Similar results were also obtained for another class I bacteriocin produced by *Staphylococcus epidermidis* 5, pep 5 (Kordel and Sahl, 1986). This evidence suggests that inhibition of the high affinity glucose transport system by both class I bacteriocins was due to the loss of the intracellular metabolites, such as PEP and ATP rather than the effects of PMF dissipation (Waite *et al.*, 1998; Waite and Hutkins, 1998).

Initial work indicated that trypsin inactivated pediocin JD and had residual PTS-inhibitory activity. This indicates that PTS inhibition by pediocin may occur via interference of the bacteriocin or a trypsin-treated bacteriocin hydrolysis product with a PTS component rather than by leakage of PEP or an essential ion (Christensen and Hutkins, 1994). Later it was established that treatment of *L.*

monocytogenes with pediocin JD and leuconocin S resulted in partial inhibition of glucose phosphorylation by PEP:PTS in the presence of excess PEP. This indicated that the inhibition of PEP:PTS by leuconocin S and pediocin JD was due to an additional mechanism independent of the loss of intracellular PEP (Waite and Hutkins, 1998).

At the time when PTS was discovered, a single function was attributed to the PTS, namely sugar phosphorylation. It has recently been discovered that this system plays many roles in aspects of bacterial cellular physiology. The primary functions of the system include sugar reception, transport and phosphorylation. Secondary functions include various ramifications of metabolic and transcriptional regulation include: i) carbohydrate catabolic enzymes, sugar permeases, and cyclic AMP biosynthetic enzyme adenylate cyclase, regulated allosterically by the IIA^{glc} PTS protein in enteric bacteria; ii) a variety of non-PTS transport systems, a sugar-phosphate phosphatase which controls the process of inducer expulsion and possibly the PTS itself, regulated by HPr (ser-p)-dependent allostery in Gram-positive bacteria (Postma *et al.*, 1993); iii) transcriptional antiterminators regulated by direct phosphorylation in both enteric and Gram-positive bacteria (Saier *et al.*, 1994); iv) carbohydrate catabolic enzymes and permeases also regulated by direct phosphorylation in Gram-positive bacteria. Recent genetic evidence has indicated that other processes, including the net production of carbon and energy storage sources such as poly-β-hydroxybutyrate (Pries *et al.*, 1991), and the control of σ^{54}-dependent transcription of nitrogen metabolic genes in numerous Gram-negative bacteria (Merrick and Coppard, 1989; Reizer *et al.*, 1992) are also controlled by the PTS.

Bacillus subtilis the levanase operon is regulated by the LevR gene product. It behaves as a repressor in the uninduced state and as an activator after induction with fructose (Debarbouille *et al.*, 1991c). Two domains have been identified in LevR. One domain (residues 144 to 345) shows extensive similarity to NifA/NtrC regulators, which are activators of σ^{54}-dependent transcription. They are required for the formation of an open promoter complex by σ^{54} and the RNA-polymerase haloenzyme (Debarbouille *et al.*, 1991a). The levanase promoter of *B. subtilis* also contains two regions that are almost identical to the -12, -24 consensus regions present in σ^{54}-dependent promoters. Furthermore, mutants lacking σ^{54} (encoded by *sigL*) no longer expressed the *lev* genes (Debarbouille *et al.*, 1991a; 1991b). A second domain of LevR (residues 411 to 572) was homologous to the antiterminators, includes SacT and SacY, two regulatory proteins of the sucrose regulon of *B. subtilis* and BgIG from *Escherichia coli* (Debarbouille *et al.*, 1991a).

On the basis of sequence comparisons and structural organization of the different domains, EII's are grouped into six families: 1) the glucose-sucrose family; 2) the mannitol-fructose family; 3) the lactose-cellobiose family; 4) the mannose family; 5) the glucitol family; and, 6) the galactitol family (Pelletier *et al.*, 1998). The mannose family of EII's contains three highly similar PTSs (45% to 65% identical residues), namely, 1) the mannose PTS of *E. coli*, 2) the L-sorbase PTS of *Klebsiella pneumonae*, and, 3) the fructose PTS of *B. subtilis* (Postma *et al.*, 1993).

The 35 kDa protein of EIIAB was found to have an 83% homology to *S. salivarius*, which in turn has varying similarities ranging from 50% with the $EIIA^{man}$ domain of *Lactobacillus curvatus* to 73% with that of *E. coli*. The sequenced protein also contains the consensus sequence of the N-

terminal of IIA^{man} domains of the mannose EII family (M____I____HG) (Pelletier *et al.*, 1998), as well as a histidine residue at position nine that corresponds to the phosphorylation site found in all IIA domains of the mannose EII family (Lengler *et al.*, 1994). Such strong evidence would place the sequenced protein into the mannose family of PTS. Although a PEP:fructose PTS was discovered in *L. monocytogenes*, researchers were unable to isolate the EII of the fructose specific PTS (Mitchell *et al.*, 1993). The mannose PTS in *S. salivarius* has an unusually broad substrate specificity (Postma *et al.*, 1993). It transports mannose, glucose, and fructose. This could indicate that the fructose-PTS discovered by Mitchell and co-workers (1993), may be the same as the mannose-PTS. The sequenced protein could be the first isolated and partially sequenced EIIAB^{man} of *L. monocytogenes* to be characterized.

Although the composition of the cellular envelope has not yet been investigated within the context of this study, results of high levels of resistance to leucocin A, similar growth rates, and increased absorbance of bacteriocin by the resistant strain, could warrant the elucidation of the function of the cellular envelope and its role in bacteriocin resistance. In a study by Brochu and co-workers (1993), mutants of *S. salivarius*, lacking the EIIAB component of the mannose specific PTS had alterations in the cell envelop. Some of these modifications included altered membrane protein profiles, modifications of total lipid content and phosphorus contents, and altered lipid/protein ratio. One mutant also had variations in the phospholipid content. The mutants also exhibited altered sensitivity to antibiotics. A single mutant was found to have a higher resistance level to mutocins and demonstrated complete resistance to mutocins produced by some strains. The numerous and diverse modification of physiological functions suggests that the mutations may affect a gene

encoding a global regulatory component. The EIIAB component of the PTS in *S. salivarius*, or a protein regulating its synthesis, could regulate the expression of several genes (Bourassa and Vadeboncoeur, 1992; Gauthier *et al.*, 1990).

The fructose PTS (Mitchell *et al.*, 1993) and a glucose PTS (Christensen and Hutkins, 1994) has been previously characterized in *L. monocytogenes*. However, the regulation of both these systems, as well as the mannose PTS in this organism has yet to be determined. The fructose (*Lev*) PTS in *B. subtilis*, which also belongs to the mannose class of EII's (Postma *et al.*, 1993) is regulated by σ^{54} (Debarbouille *et al.*, 1991a,b). The levanase promotor in *B. subtilis* also contains two regions almost identical to the -12, -24 consensus regions present in σ^{54}-dependent promotors (Debarbouille *et al.*, 1991a,b). The σ^{54}-dependent regulons have been identified, which encode more than 50 proteins affecting diverse physiological functions, including nitrogen assimilation, nitrogen fixation, adaption of cellular respiration to anaerobiosis, use of unusual carbon sources, photosynthesis, developmental switches, and adjustments to symbiotic and virulent growth (Powell *et al.*, 1995).

Interestingly, the inactivation of the *rpoN* (σ^{54}) gene in *L. monocytogenes* led to resistance in the class IIa bacteriocin mesentericin Y105 (Robichon *et al.*, 1997), which has a 95% homology to leucocin A. Assessment of the activity of σ^{54} in *L. monocytogenes* B73-MR1 may shed light on the missing link between the activity of σ^{54} expression, resistance to class IIa bacteriocins and the regulation of the mannose PTS in *L. monocytogenes*.

CHAPTER FOUR

CONCLUSION

Due to the increased use of bacteriocins for biopreservation, there is a increase in interest in resistance to these antimicrobial compounds. Such studies of resistance to bacteriocins may not only access the viability of bacteriocins for future biopreservation, but it could aid in the understanding of the mode of action of bacteriocins.

The resistance-associated phenomena associated with the highly resistant stable genetic mutant of *L. monocytogenes* B73-MR1 to leucocin A, has only been partly unraveled in this study. The most significant aspect of the resistance of *L. monocytogenes* B73-MR1 is the discovery of the inactivation of the mannose PTS due to the lack of the EIIAB component of the mannose PTS. Although this system is primarily involved in the uptake and concomitant phosphorylation of a specific sugar source, the PTS of bacteria is also involved in metabolic and transcriptional regulation in the bacterial cell. In a previous study, *S. salivarius* mutants lacking the EIIAB component of the mannose specific PTS were found to harbor alterations in the cell envelop (Brochu *et al.*, 1993). Specific modifications to the cell envelop were not determined in this study but would have to be investigated in future studies to determine the role played by these modifications in bacteriocin resistance. This would shed more light on the regulatory functions of the mannose PTS in *L. monocytogenes*.

Evidence does exist that the class IIa bacteriocin pediocin JD directly interacts with the components of the glucose PTS (Waite and Hutkins,1998). It is possible that leucocin A also interacts specifically with the components of the mannose PTS or components which may be regulated by the mannose PTS. Because no gross modifications were detected at the genomic level by ssAFLP, it may be possible that the absence of the EIIAB component of the mannose PTS

could be due to the regulation of the mannose PTS operon. This warrants further investigations into the regulation of the mannose PTS in *L. monocytogenes* because the regulatory component may also be responsible for the regulation of cellular components required for bacteriocin activity. Once the identity of the 18 kDa protein has been determined, this may also shed more light on the regulatory function of the mannose PTS in *L. monocytogenes*.

To determine if the regulatory component of the mannose PTS or the mannose PTS itself is responsible for the resistance to leucocin A, gene knockout technique would have to be employed in order to knockout the EIIAB component of the mannose PTS. This would result in the inactivation of the mannose PTS. If the EIIAB inactivated strain also becomes resistant, a relationship could possibly be obtained between bacteriocin resistance and components of the mannose PTS or genes regulated by the mannose PTS. Gene chip technology could possibly assist in revealing the genes regulated by the mannose PTS. It may be possible that one of the genes under the regulation of the mannose PTS or the regulatory component of the PTS could encode a putative receptor molecule.

Identification of new points of lethal interaction between bacteriocin and *L. monocytogenes* may serve as a blueprint for the design of not only higher potency bacteriocins, but may also be useful for the design of a new class of antibiotics.

CHAPTER FIVE

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