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Eight foodborne pathogenic and spoilage Gram-positive bacteria were evaluated for their spontaneous resistance frequencies to the peptide antimicrobial nisin. In brain heart infusion (BHI) medium, nisin resistance frequencies were in the range of 10^{-6} to 10^{-8} when exposed to nisin at concentrations 2 to 4 times the minimal inhibitory concentrations.

A stable nisin resistant mutant of *Listeria monocytogenes* Scott A was obtained by increasing stepwise exposure to nisin and subsequently characterized. Phospholipid content, fatty acid composition, phase transition temperature (Tc), and specific growth rates of the resistant mutant and parent were determined. The Tc of the resistant mutant (44.4°C) in comparison of the parent (37.4°C) indicated that significant changes occurred in the lipid composition of the mutant. Gas chromatographic analysis of fatty acids of mutant and parent revealed significant differences ($P<0.05$) in the proportional ratios of fatty acids; 14:0, 15:0 iso, 15:0 anteiso and 17:0 iso.

cells and the concentration of nisin required for reaching a 50% maximum killing rate was five times that of sensitive cells. The bactericidal activity of nisin was more effective at 37°C than 4°C for both resistant and sensitive cells, but there was always higher survival of resistant cell than sensitive cells for both temperatures. Nisin degradation and plasmid involvement were demonstrated to not be the responsible resistance mechanism to nisin in *L. monocytogenes*. No cross-resistance was observed to therapeutically important antibiotics (rifampicin, chloramphenicol, vancomycin, erythromycin) and to metabolic inhibitors (CCCp, DCCD) by the nisin-resistant mutant.

A STUDY OF NISIN RESISTANCE IN LISTERIA MONOCYTOGENES SCOTT A

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A STUDY OF NISIN RESISTANCE IN LISTERIA MONOCYTOGENES SCOTT A

INTRODUCTION

Nisin is a polypeptide antibacterial agent produced by *Lactococcus lactis* subsp. *lactis*. It is effective in inhibiting Gram-positive bacteria, particularly spore formers. In the United States, nisin is generally recognized as safe for use in pasteurized cheese products to prevent both spore outgrowth and toxin production by *Clostridium botulinum*. In other countries, nisin has been used for a number of applications including extension of the shelf life of dairy products and prevention of spoilage of canned foods by thermophiles. Because of its low toxicity, use of nisin as a food preservative has attracted a number of researchers to look for potential applications. The study on nisin resistance mechanisms is of great interest due to the increased use of nisin as a food preservative. In this thesis, the nisin resistance in *Listeria monocytogenes* Scott A was studied. *Listeria monocytogenes* has been chosen because this bacterium is nisin sensitive and is of major concern to the food industry because of its ability to survive refrigeration temperatures and its high potencial for causing fatal foodborne illness, listeriosis.

Nisin resistance in *L. monocytogenes* as well as other bacteria has been reported by several laboratories (Jarvis, 1967; Lipinska, 1977 ; Daeschel, 1991;

Harris, 1991). Jarvis (1967) proposed a nisinase mechanism for nisin resistant strains of *Bacillus*. Daeschel et al. (1991) obtained strains of *Leuconostoc oenos* that exhibited 100-fold resistance to nisin for use in malolactic fermentation of wine. These strains were not resistant by means of a secreted nisinase. Harris (1991) did not detect plasmid DNA in either nisin resistant or sensitive strains of *L. monocytogenes* Scott A. Mechanisms for nisin resistance other than nisinase have not been reported. An understanding of nisin resistance in foodborne pathogens would increase our knowledge of the general resistance mechanism of bacteriocins as well as the mode of action of nisin which is still not clearly defined. A successful explorative study on nisin resistance in*L. monocytogenes* would also broaden our knowledge about this pathogen and provide useful information on enhancing the use of nisin for inhibition of *L. monocytogenes* and other foodborne pathogens.

The principal aim of these studies was to advance our understanding of nisin resistance in *L. monocytogenes*. This thesis began with a literature review which covered the properties and applications of nisin, the mode of action of nisin, and the resistance mechanisms of antibiotics and bacteriocins. The experimental section of the thesis comprised three chapters. The first of the three chapters summarizes a study on spontaneous nisin resistance frequency for eight common foodborne pathogens and the isolation and characterization of a nisin resistant mutant strain of *L. monocytogenes* Scott A (R-2000). The second chapter of the experimental section summarizes the

physiological and genetic characteristics of the resistant mutant *L. monocytogenes* Scott A R-2000 in comparison with its nisin sensitive parent. The final chapter summarizes the study on changes observed in the phospholipid composition of the mutant strain and how that property may be related to nisin resistance.

LITERATURE REVIEW

NISIN AND ITS APPLICATIONS

Structure and properties

Nisin is a member of a unique group of small ribosomally-synthesized peptide antibiotics which, because of their characteristic lanthionine bridges, are often referred to as "lantibiotics" (Schnell et al., 1988). Several other lanthionine-containing peptides have been discovered and characterized, including subtlin (Gross and Kiltz, 1973), Pep-5 (Sahl et al., 1985), and epidermin (Allgaier et al., 1986). Of this group, only nisin has achieved widespread recognition and application for its use as a food preservative.

Nisin's novel structure, as shown in Fig. 1 (Gross and Morell, 1971). has been studied for many years. It contains 34 amino acid residues which include two atypical forms; α,β-unsaturated amino acids dehydroalanine (DHA) and dehydrobutyrine (DHB) (Gross and Morell, 1967). These unusual amino acid residues which arise through posttranslational modifications of the ordinary amino acids serine and threonine (Hurst, 1981) contribute to the five internal thioether rings which are characteristic of lantibiotics.

There is a growing body of evidence that α,β-unsaturated amino acids such as DHA and DHB play an important role in the mechanism of nisin

antibiotic action by reacting with one or more nucleophiles in a sensitive cellular target (Gross and Morell, 1971; Morris, 1984; Liu, 1990). These molecules have been shown to be reactive and may crosslink with sulfur to form lanthionine or β-methyllanthionine rings. They may also react with available NH groups forming peptide bonds, and leading to the formation of nisin polymers.

The existence of nisin polymers was apparently responsible for the controversy surrounding nisin's true molecular mass. Originally, the molecular mass was reported to be 7000 daltons (Cheeseman and Berridge, 1959), but the researchers had been unable to detect either amino or carboxy end groups. Gross and Morell (1967) obtained a molecular mass of 3500 by using partial dinitrophenylation and countercurrent distribution. This result was confirmed by Jarvis et al. (1968). It is now accepted that nisin generally occurs in its most stable forms, as a dimer with a molecular mass of about 7000, or as a tetramer (Jarvis et al., 1968).

The solubility and stability of nisin have been found to depend on the pH of the solution. In dilute HCL at pH 2.5, the solubility of nisin is 12%, and it may be autoclaved without loss of activity (Tramer, 1964). The solubility decreases to 4% when the pH is raised to pH 5.0. At neutral and alkaline pH values, nisin is practically insoluble. The effect of processing temperature and pH on the stability of nisin in different foods was studied by Heinemann et al. (1965). Their results showed that in low-acid foods, at pH 6.1-6.9, heating for 3

minutes at 250° F destroyed 25 - 50% of the added nisin. A similar degree of destruction was reported for highly acidic foods (pH 3.3-4.5). Proteins and macromolecules from milk or broth were reported by Tramer (1964) to protect nisin from heat inactivation.

The inactivation of nisin at high pH was once believed to be simply a protein denaturation; however, it has been shown to occur as a consequence of pH-induced modifications (Liu and Hansen, 1990). The dehydro residues of nisin are potentially susceptible to modification by nucleophiles that are present at high pH, such as hydroxide ions, deprotonated amines, and deprotonated hydroxyl groups. Reactions with those nucleophiles could be intramolecular or intermolecular, the latter which could cause cross-linking. Since there are three dehydro residues per molecule, large multimolecular aggregates could form by intermolecular reactions (Gross and Morell, 1971).

Applications

The approved use of nisin varys from country to country. Britain was the first country to permit the use of nisin as a food preservative (1959). In 1969, the Joint Food and Agriculture Organization/World Health Organization (FAO/WHO) Experts Committee on food additives assigned international acceptance of nisin (WHO, 1969). WHO recommended that use of nisin as a food additive "be considered acceptable, the unconditional Average Daily Intake being 0-33,000 units/kg of body weight."

After 30 years of safe and efficacious use in many European and Third World countries, nisin was affirmed by the FDA (1988) as a GRAS (generally recognized as safe) substance for use as an antimicrobial agent to inhibit the outgrowth of *Clostridium botulinum* spores and toxin formation in certain pasteurized cheese spreads. This action by the FDA was based on the accumulated body of scientific data indicating that nisin is nontoxic, nonallergenic, and is safe and effective as an antimicrobial agent (Hurst, 1983; Delves-Broughton, 1990).

Since its initial use as a food preservative in Swiss cheese to prevent gas-induced defects attributable to the growth of clostridia (Hirsch, 1951), nisin has proven to be an effective preservative in pasteurized processed cheese and pasteurized processed cheese spreads. The clostridial spores, often present in raw cheese, can survive relatively high temperatures (85-105°C) which occur during the melting process. The outgrowth of these spores is then favored by the pH, moisture content, and ultimate anaerobiosis of the processed cheese. This may result in subsequent spoilage due to liquefaction or gas production and off-odors.

Trials have indicated that nisin at levels of 500-10,000 U/g (U= international units; 1 ug of pure nisin is 40 U/ml) in pasteurized processed cheese spreads can delay or prevent the growth and formation of toxin by inoculated *C. botulinum* strains (Somers and Taylor, 1987). The level of nisin required was dependent upon the number of clostridial spores present (Scott

and Taylor, 1981; Hirsch and Grinsted, 1954), as well as the storage time, and the temperatures likely to be experienced during the shelf life of the product. Levels of nisin used to control nonbotulinal spoilage varied from 250 to 500 U/g. For antibotulinal protection, levels required were 500 U/g or higher.

Nisin can also be used to extend the shelf life of dairy based desserts. Such products cannot be subjected to full sterilization without damaging either the appearance, flavor or texture of the product. Pasteurized products have a limited shelf life but the addition of nisin can give a significant increase (Heinimann et al., 1965).

In many European countries, the application of nisin to canned foods has been accepted. With nonacid foods, cans should normally receive a minimum heat treatment (F=3). F is the time in minutes at 121°C in the center of the can that ensures the destruction of *C. botulinum* type A, which is also the most nisin-resistant sporeformer. Even at F values above 3, heat resistant spores of thermophiles *Bacillus stearothermophilus* and *Clostridium thermosaccharolyticum* can survive and cause spoilage of canned foods, particularly under warm storage conditions (Eyles and Richardson, 1988). By using nisin, it is possible to control thermophilic spoilage of canned foods which are stored at warm storage condition or climates. A reduction in heat processing will also save energy, as well as improve the nutritional value, flavor, appearance, and texture of canned foods without the risk of increased spoilage by thermophiles.

Nisin has also been considered for use in cured meats to reduce the level of nitrite for reasons of toxicological safety (Scott and Taylor, 1981; Taylor and Somers, 1985; Calderon et al., 1985; Bell and De Lacy, 1987). Results have indicated that only high levels of nisin can effectively control *C. botulinum*, and that further work will be required before nisin can be considered for partial replacement of nitrite.

Research in the United Kingdom and Germany has demonstrated that nisin has potential for controlling spoilage lactic acid bacteria in beer (Ogden and Tubb, 1985; Ogden and Waites, 1986; Ogden et al. 1988) and wine (Radler, 1990). These studies indicated that although the spoilage lactic acid bacteria were sensitive to nisin, the yeasts were completely unaffected; which is an important factor permits nisin to be introduced during the fermentation. Other applications in the brewing industry include the use of nisin for washing the pitching yeast. This alternative to the present method of acid washing would have the advautages of decreasing the spoilage bacteria without affecting yeast viability. The use of nisin also has the potential for reducing pasteurization regimes, and increasing the shelf life of unpasteurized or bottle-conditioned beers. Recently, Daeschel et al. (1991) reported that nisin and nisin-resistant bacterial starter cultures of *Leuconostoc oenes* could conceivally be used to control malolactic fermentations in wines.

Studies have also shown that the spectrum of nisin activity could be extended to include Gram-negative bacteria. Blackburn et al. (1989) first

reported that nisin used in combination with a chelating agent (e.g. EDTA) exhibited a bactericidal effect against Gram-negative bacteria. Kelly et al. (1991) reported that nisin was bactericidal to *Salmonella* species and other Gram-negative bacteria. In their study, inhibition was observed for all of the *Salmonella* species tested when the bacteria were simultaneously exposed to nisin and EDTA. The inhibition was defined as greater or equal to a 1-log-cycle reduction of the initial population. Applications involving simultaneous treatment with nisin and an outer membrane modifying-chelating agent such as EDTA may be of value in controlling food-borne *Salmonella* species as well as other Gram-negative pathogens in foods (Stevens et al., 1992).

Nisin inhibition of Listeria monocytogenes

Listeria monocytogenes was recognized as a significant emerging human food-borne pathogen in the 1980s and continues to be a major concern of the food industry today (Farber and Peterkin, 1991; Ryser and Marth, 1991). This pathogen has been isolated from both the food-processing environment and finished food products. Because *L. monocytogenes* can grow slowly at refrigeration temperature, control of the organism is of particular concern in minimally processed refrigerated foods that are distributed with extended shelf life period (Farber and Peterkin, 1991; Leistner, 1978). For control of *L. monocytogenes* in such foods, it often becomes

necessary to incorporate additional microbial outgrowth barriers, including preservatives (Farber and Peterkin, 1991; Leistner, 1978; Ryser and Marth, 1990).

It has been reported that nisin can inhibit the growth of *L. monocytogenes* (Benkerroum and Sandine, 1987; Harris et al. ,1989; Doyle,1988; Monticello and O'Connor, 1990; Jung et al. 1992). The research results of these investigators has demonstrated that nisin can cause lysis of many strains of *L. monocytogenes,* but the degree of inhibition depends on the number of bacteria present. Nisin effectively inhibited the growth of *L. monocytogenes* at a concentration of 100 U/ml when the bacterial load was less than 10^6/ml. Since the levels of *L. monocytogenes* reported in even the worst contaminated foods is usually less than 10^6/ml, the potential for using nisin as an anti-Listerial agent in some foods appears favorable.

MODE OF ACTION OF NISIN

Target of nisin action

Numerous studies have been made to determine nisin's mode of action against susceptible bacterial cells and spores. Ramseier (1960) suggested that nisin behaves like a surface active cationic detergent, and that the point of action was the cytoplasmic membrane. The subsequent disruption resulted in leakage of essential cellular materials such as adenosine triphosphate from

the cell, or in some cases, total cell lysis. Linnett et al. (1973) reported that nisin could inhibit murein synthesis. Later, with cell-free systems, Reisinger et al. (1980) suggested that nisin could cause the accumulation of undecaprenyl-pyrophospho-MurNac(pentapeptide), which is an lipids intermediate of murein synthesis. This accumulation was caused by the formation of a complex between nisin and the lipid intermediate which resulted in a final inhibition of polymer synthesis.

Investigations by a group of German scientists provided a better understanding of the cellular target of nisin. They found that one of the earliest events after nisin addition was the rapid, nonspecific efflux of different substrates like amino acids and cations from sensitive cells and their membrane vesicles, followed by the immediate collapse of the membrane potential (Ruhr and Sahl, 1985). From these results, they concluded that the inhibition of murein synthesis should be regarded as a secondary effect of nisin action. Electron micrographs of nisin-sensitive bacteria provided physical evidence of damage to the cytoplasmic membrane when the bacteria were exposed to nisin (Andersson et al., 1988).

Molecular basis of nisin action

Even though the mode of action of nisin at the molecular level is not yet completely understood, there have been some interesting findings in recent years. Sahl and Kordel (1987, 1989) found that nisin disrupted

valinomycin-induced potassium diffusion potentials imposed on intact cells of *Staphylococcus cohnii* 22. The membrane depolarization occurred rapidly at high diffusion potentials, while at low potentials, nisin-induced depolarization was slower. Their results suggested that nisin required a membrane potential for activity. This assumption was later proven in their experiments with artificial lipid bilayers. Further, they observed that the potential must have a trans-negative orientation with respect to the addition of nisin and a sufficient magnitude (ca. -100mV).

As a peptide antibiotic, nisin shares striking structural similarities with staphylococcin-like peptide Pep-5 and subtilin (Sahl et al., 1985). They are all cationic peptides possessing intramolecular thioether bridges introduced by posttranslational modifications of precursor peptides (Gross, 1973; Sahl, 1985). The structural similarity of these three peptide antibiotics suggests a similar mode of action. Kordel and Sahl (1989) pointed out that nisin, Pep 5 and subtilin each depolarized bacterial and artificial membranes by formation of voltage-dependent multi-state pores. Single channel recordings resolved transient multi-state pores, which for nisin, had diameters in the range of 0.2-1 nm and a lifetime of several hundred milliseconds. Studies using non-energized liposomes indicated that the peptides failed to span the membrane in the absence of a membrane potential. The affinity between nisin and membrane was strongly dependent on the presence of negatively charged phospholipids, and was primarily based on electrostatic interactions (Kordel,

1989). Henning et al. (1986) observed that isolated membrane fractions from nisin-sensitive *Lactobacillus plantarum* and *Streptococcus agalactiae* could antagonize the antimicrobial effect of nisin. Their results indicated that some constituents of the membrane could interact with nisin, and that the interaction of nisin with phospholipids was the most important step of the antimicrobial effect of nisin.

Bierbaum and Sahl (1985) observed autolysis of *S. simulans* 22 induced by nisin and Pep-5; they noted a substantial amount of wall hydrolysis as the treated cells rapidly lysed. To elucidate the induction mechanism of cell wall degradation, they isolated and purified two autolysins from *S. simulans* 22 and demonstrated *in vitro* that addition of a small quantity of nisin or Pep-5 at low ionic strength led to the stimulation of lytic enzyme activity if teichoic or teichuronic acids were present in the cell wall. They observed that the effect of the peptides on autolysin activity depended on the length and charge of the peptide, the presence of anionic polymers in the cell wall, and the ionic strength of the assay condition. Their results indicated that at low ionic strength the enzymes are bound to the teichoic or teichuronic acids of the cell wall which act as noncompetitive inhibitors of enzyme activity. Nisin and Pep-5 replace the enzymes from the anionic polymers and thereby induce lysis. In the case of intact cells, the peptide-induced autolysis was enhanced after energization of the cells with glucose (Bierbaum and Sahl, 1985). However, treatment of cells with valinomycin,

which should have caused potassium leakage and thereby displayed a de-energization effect similar to that of nisin, did not stimulate autolysis. Therefore, they suggested that the membrane-disruptive effect, as well as the binding of basic peptide nisin to teichoic and teichuronic acids, was essential for the effective induction of autolysis.

The effect of nisin on spore formers

The inhibitory activity of nisin against spores is sporicidal rather than sporostatic. Hitchins et al. (1963) followed germination and outgrowth by measuring cell volume. Three stages of swelling were recognized: (1) germination swelling, followed by (2) pre-emergence swelling, an approximate doubling in volume which occurs before emergence from the spore envelope and (3) elongation, or the commencement of growth. Germination swelling (1) was not inhibited by nisin, whereas the pre-emergence swelling (2) was oxygen dependent and was inhibited by nisin (Hitchins et al., 1963; Lipinska, 1977).

Nisin was tested (Lewis et al. 1954; O'Brien et al. 1956) for its complementary action with heat against spore formers including *C. botulinum, Clostridium thermosaccharolyticum, Bacillus coagulans,* and *Bacillus stearothermophilus*. Nisin reduced the D value by 50-60%. Hawley (1962) reported that the spores of *C. botulinum* types A and B became injured when heated. This probably contributed to the greatly increased sensitivity of

the sublethally heated organisms to nisin.

Factors affecting the antimicrobial activity of nisin

In addition to the pH effect on solubility and stability of nisin, there are other factors which affect the antimicrobial activity of nisin. Many investigators have noticed that nisin activity was gradually lost in food. Inactivation was observed in processed Swiss cheese (McClintock et al., 1952), canned mushrooms (Denny et al., 1962), chocolate milk (Fowler and McCann, 1971), and cooked-ham (Rayman et al., 1981).

Nisin is vulnerable to enzymic inactivation by α-chymotrypsin, a component of pancreatin (Heineman et al., 1966) and nisinase; isolated from *L. plantarum* (Kooy, 1952), *Streptococcus thermophilus* (Alifax and Chevalier, 1962), and *Bacillus cereus* (Jarvis, 1967).

The effectiveness of nisin, and the subsequent concentration of nisin required to inhibit outgrowth, was found to be closely related to the initial spore load. Thus, increasing the spore load by 10-fold would require an increase in nisin concentration of about half a logarithmic cycle expressed in international units (Ramseier, 1960).

The components of foods also affected the activity of nisin. Jones (1974) observed that nisin was more effective in controlling *Staphylococcus aureus* in skim milk than in whole milk and attributed the effect to the milk fat content. Daeschel (1990) observed that nisin was consistently more active

on a per unit basis in low fat foods than in high fat foods. Jung et al. (1992) reported that nisin was far less effective in inhibiting *Listeria* in half-and-half than in skim milk.

RESISTANCE MECHANISMS OF ANTIBIOTICS

Genetics of antibiotic resistance

Basically, two theories have evolved to explain the genetics of antibiotic resistance. One school of thought professes that the development of a resistant cell population could be explained by the phenotypic adaptation of the cells to an inhibitory compound without much modification of their genotype. The opposing faction took the view that any large population of cells which was sensitive overall to an antibiotic was likely to contain a few genotypically- resistant cells, and the continued presence of the antibiotic would result in the emergence of a new population of resistant cells through a process of selection.

Evidence gathered over the years strongly supports the second of the two theories. Bacterial cells which have phenotypically adapted to antibiotics are always genotypically different from the sensitive cells, and do not constitute the majority of the wild-type population not previously exposed to the antibiotic (Franklin and Snow, 1989). When the selective pressure applied by an antibiotic is removed, a previously resistant microbial population may

revert to a sensitive state. Sometimes the resistant cells are at a selective disadvantage in an antibiotic-free environment, and are therefore eventually overgrown by the sensitive cells. In other cases of reversion, the genetic material that confers the resistance is lost from the cells, and the resistant cells are progressively diluted out of the population.

Plasmid-mediated antibiotic resistance has been extensively studied. Most clinically significant antibiotic resistance is determined by plasmids (Broda, 1979). A plasmid is an extrachromosomal segment of DNA. Some plasmids carry one or more genes for antibiotic resistance. They may be either self-transmissible through conjugation or non-self-transmissible. In the latter case, transmission may result from mobilization of the plasmid, or by transduction or transformation.

The size of plasmids which carry resistance determinants can vary greatly. Conjugative R plasmids generally have a molecular mass above 25 x 10^6. Nonconjugative plasmids are much smaller and usually range from 1 x 10^6 - 1 x 10^7. The number of copies of R-plasmid per cell is related to the molecular mass. R-plasmid may be lost spontaneously from cells upon storage or may be unstable at elevated temperatures. They may be artificially eliminated through selective inhibition of plasmid replication by curing agents (Bryan, 1981).

Although many antibiotic-resistance determinants are carried by plasmids, certain resistance genes appear to be restricted to a chromosomal

location in a number of bacteria, particularly Gram-positive organisms (El Salh et al., 1981). Genetic alterations in chromosomes result from spontaneous mutations. Mutations are said to be spontaneous when they occur unprovoked by experimental mutagenesis. Spontaneous mutations are relatively infrequent, e.g. one mutation per 10^5-10^7 cell division. However, when the vast numbers of cells involved in bacterial populations are considered, the probability of a mutation causing an increase in antibiotic resistance is quite high. A spontaneous mutation may occasionally cause a large increase in resistance, but more commonly, resistance develops as a result of numerous mutations, each one giving rise to a small increment in cell resistance. In this case, highly resistant cells emerge only after prolonged or repeated exposure of the cell population to the given antibiotic.

In general, resistance produced by mutation, unlike that associated with plasmids, is by a recessive mechanism. The result of the mutation in most cases is to produce a gene product with reduced or absent affinity for the antibiotic in question. Most often the product affected is the antibiotic target or a transport protein. Other possible effects of mutation might be to increase the amount of target or to reduce a cell's need for a particular metabolite. Mutations involve the addition, deletion or substitution of one or more nucleotides. They most likely occur during DNA replication or repair (Bryan, 1982).

Biochemical mechanisms of antibiotic resistance

A number of possible biochemical mechanisms for antibiotic resistance can be found in the literature:

1) Conversion of active antibiotic to an inactive derivative by enzymes produced by the resistant cells.

β-lactamase was found (Foster, 1983) to be capable of hydrolyzing antibiotics of β-lactams group such as penicillin and ampicillin. Chloramphenicol acetyltransferase was able to enzymatically inactivate chloramphenicol, and various aminoglycoside-modifying enzymes have been reported to be active against aminoglycoside (Britz and Wilkinson, 1978; Shannon et al., 1982).

2) Modification of the target site.

Cases have been reported of bacteria resistance to streptomycin, kanamycin, erythromycin, rifamycins and quinolones by a mechanism which involves modification of the target site (Foster, 1983; Bryan, 1981; Hotta, et al., 1981). This kind of resistance is related to altered proteins, rRNAs, RNA polymerase or DNA gyrase, and usually, results from chromosomal mutation.

3) Loss of cell permeability to an antibiotic.

The loss of permeability barrier may be due to any of several mechanisms: (a) Modification of an existing permeability barrier. Antibiotic resistance of Gram-negative bacteria depend more on this mechanism. (b)

Partial or complete loss of transport function. Some antibiotics gain intracellular access by a specific transport mechanism. Mutants with transport function loss may fail to take up the antibiotic and thus become resistant in that manner. (c) Specific antagonism of antibiotic transport. The most commonly encountered mode of resistance to tetracyclines in Gram-positive and Gram-negative bacteria (Levy, 1981).

4) Synthesis of an additional resistant enzyme.

Resistance to sulfonamides, trimethoprim and methicillin are reported due to the synthesis of additional resistant enzymes (Pattishall et al., 1977; Bryan, 1981). The resistance determinants are either plasmids or transposons (Shapiro et al., 1977; Foster, 1983), which encode more than one form of the target enzymes for the antibiotics, the enzymes have similar K_m for their substrates but highly resistant to the antibiotics.

5) Increased concentration of a metabolite that antagonized the inhibitor.

This type of resistance is exemplified by certain mutants resistant to sulphonamides (Sevderg et al., 1980). In these cells the concentration of *p*-aminobenzoic acid is said to be substantially higher than in sensitive cells, the inhibitor is competitively displaced from its enzyme binding site.

RESISTANCE MECHANISMS OF PEPTIDE ANTIBIOTICS OF BACTERIAL ORIGIN

Nisin

The development of acquired or spontaneous nisin resistance was first observed by Hirsch (1950). He reported that cells of *Streptococcus agalactiae* that survived exposure to nisin were able to grow in concentrations of nisin forty times higher. Carlson and Bauer (1957) were able to increase the resistance of *Staphylococcus aureus* from 2.5 to 2000 U/ml through successive stepwise transfers. Ramseier (1960) reported on the nisin resistance of *Clostridium butyricum*. Hirsch and Grinsted (1951) used spontaneous resistant mutants of *L. lactis* subsp. *cremoris* to differentiate bacteriocins of different Lactococci.

Observations were made which suggested the existence of a nisin inactivation substance in nisin resistance strains. Kooy (1952) described strains of *Lactobacillus plantarum* isolated from cheese and raw milk which could degrade the antimicrobial activity (presumably nisin) in *L. lactis* subsp. *lactis* culture filtrates. Galesloot (1956) also described similar observations with *L. lactis* subsp. *lactis* , *L. lactis* subsp. *cremoris* and *Enterococcus faecalis*. Alifax and Chevalier (1962) described nisinase activity in a strain of *Streptococcus salivarius* subsp. *thermophilus* and went on to partially purify the enzyme. They further observed that the optimum conditions for nisin-

degradation by their enzyme preparation included pH 7.0 (no activity at pH 3 or 4), with a six hour incubation at 37°C. Nisinase activity has also been reported for several *Bacillus* spp. (Javis, 1967). Nisinase preparations from *B. cereus* and *B. polymyxa* were found to inactivate nisin and subtilin, but not other polypeptide antibiotics. Further investigations (Jarvis, 1970; Jarvis and Farr, 1971) have provided details on substrate specificity, thermal stability and the mechanism of nisin inactivation. Jarvis et al. (1971) suggested that nisin inactivation by the nisinase preparation may not be a proteolytic degradation process but rather a reductase reaction on the dehydroalanine residue adjacent to the C-terminus. The inactivation of subtilin by the nisinase preparation was consistent with their hypothesis in that nisin and subtilin have a similar C-terminal sequence.

The appearance of spontaneous nisin resistance or the intentional exposure of strains to increasing amounts of nisin to obtain variants with high resistance has been frequently observed. Lipinska (1977) "trained" cheese starters to become nisin resistant but not to produce nisinase. Daeschel et al, (1991) obtained strains of *Leuconostoc oenos* 100-fold resistant to nisin for use in malolactic fermentation of wine. These strains were not resistant by means of a secreted nisinase. Harris et al. (1991) described the appearance of *L. monocytogenes* mutants resistant to 2000 U/ml nisin at frequencies between 10^{-6} to 10^{-8}. Mechanisms to explain nisin resistance other than "nisinase" have not been proposed.

It has been suggested (Klaenhammer et al., 1985; McKay et al., 1988, 1989) that nisin resistance was plasmid encoded among *Lactococcus lactis* , the genus in which nisin producers belong. Klaenhammer et al. (1985) reported that a plasmid (pTR1040) encoded nisin resistance in *L. lactis* ME2. McKay and Baldwin (1984; 1988; 1991) reported that a plasmid pNP40 from *L. lactis* subsp. *diacetylatis* DRC3 encoded nisin resistance. The nisin resistance determinant was located onto a 1.3-kb *EcoR* I-*Nde* I fragment. The fragment as well as its associated transcription and translation signal sequences were cloned into plasmid-free *L. lactis* subsp. *lactis* LM0230 and conferred an MIC of 160 U/ml of nisin. This level of nisin resistance was equivalent to that of the initial nisin-resistance strain (Froseth and Mckay, 1988, 1991). The inferred amino acid sequence would result in a protein with a molecular mass of 35,035 Da. This value was in agreement with the molecular mass of protein detected after in vitro transcription and translation of DNA encoding the nisin resistance gene, *nsr* . This protein contained a hydrophobic region at the N- terminus that was predicted to be membrane associated. Nevertheless, the mechanism by which the proteins encoded by these genes confer resistance is not yet understood.

Polymyxin

Polymyxin is a clinical peptide antibiotic which acts on cytoplasmic membrane and is the only peptide antibiotic in which resistance mechanisms

have been explained. The antibacterial action of polymyxin is caused primarily by its binding to the cytoplasmic membrane. The positively charged peptide ring is thought to bind electrostatically to the anionic phosphate head groups of the membrane phospholipid, displacing magnesium ions which normally contribute to membrane stability. At the same time the fatty acid side chain is inserted into the hydrophobic inner region of the membrane. This disrupts the normal organization of the membrane and alters its permeability.

The resistance mechanism of polymyxin has been extensively studied. *Pseudomonas aeruginosa* strains were used as a model system in these studies. Gilleland et al. (1976, 1977, 1982b) suggested that the resistance was due to an exclusion mechanism which prevented the antibiotic from penetrating the outer membrane to reach the sensitive sites on the cytoplasmic membrane. Freeze-etch analysis of polymyxin-resistance isolates of *P. aeruginosa* indicated that there was indeed an alteration in the ultrastructure of the outer membrane. Gilleland and Lyle (1979) documented that acquisition of polymyxin resistance in *P. aeruginosa* was associated with the loss of three outer membrane proteins having apparent molecular mass of 24,000, 36,500, and 47,000. There was also a reduction of approximately 25% in the lipopolysaccharide content. Following their findings, Conrad and Gilleland (1981; 1982a) compared the lipid composition of cells of *P. aeruginosa* polymyxin B resistant strains with the sensitive strains, and

observed consistent alterations in the cell envelope of four genetically unrelated *P. aeruginosa* polymyxin B resistant isolates. These alterations included a decrease in phospholipid content, with either a slight or insignificant change in the amount of readily extractable lipid fraction. The decreased phospholipid level was of particular interest since all of the resistant strains had a significant reduction in phosphatidylglycerol and phosphatidylethanolamine content with the concomitant appearance of a large amount of free fatty acids. Furthermore, Moore et al. (1984) suggested that the resistance to polymyxin in *P. aeruginosa* was a complicated phenomenon which resulted in a number of cell surface alterations. At least two mechanisms were proposed to account for the resistance. The first, as described by Gilleland and Conrad (1981), resulted from stepwise adaptation to increasing amounts of polymyxin, and was characterized by a variety of outer membrane alterations. The second mechanism involved mutational resistance, and was characterized by increased levels of the outer membrane protein H1, decreased Mg^{+2} content of the cell envelope, and increased resistance to polymyxin, EDTA and aminoglycosides (Nicas et al., 1980). Later, Conrad and Galanos (1989) proposed that the loss of hydroxy fatty acids from lipopolysaccharides disrupted the outer membrane hydrophobicity and was a contributing factor to polymyxin B adaptive resistance.

CHAPTER 2

NISIN RESISTANCE OF FOODBORNE BACTERIA AND THE SPECIFIC RESISTANCE RESPONSES OF *LISTERIA MONOCYTOGENES* SCOTT A*

INTRODUCTION

Nisin is an antibacterial peptide produced by *Lactococcus lactis* subsp. *lactis* (*L. Lactis*) that exhibits a broad spectrum of inhibitory activity against Gram-positive bacteria and spores. Since its first application as an effective food preservative in preventing butyric clostridial blowing in Swiss cheese (Hirst, 1951), nisin has been used in a large variety of fresh and processed foods in many countries and has been found to be an effective and safe food preservative. Recent investigations (Benkerroum, 1987; Carminati, 1989; Harris, 1989) have indicated that nisin or nisin-producing lactococci are inhibitory toward *Listeria monocytogenes*, a foodborne pathogen of increasing concern to the food industry. Nisin is also effective in preventing the outgrowth of *Clostridium botulinum* spores (Scott et al., 1981) and has found application as a GRAS ingredient in processed cheese to prevent clostridial growth (FDA, 1988). With the potential for increased use of nisin in food preservation and processing, foodborne pathogens will have a greater

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frequency of exposure to nisin. Analogous to drug-resistant bacteria that occur after exposure to clinical antibiotic agents, nisin sensitive bacteria may develop nisin resistance. Therefore, it is meaningful to investigate the occurrence of nisin resistance among some common foodborne pathogenic and spoilage bacteria. Information regarding spontaneous resistance frequencies of these undesirable bacteria may provide a better base of knowledge for the application of nisin in food processing and preservation. Furthermore, investigations into the mechanism of nisin resistance will broaden our knowledge about the mode of action of nisin.

The only type of bacterial nisin resistance that has been well characterized was reported by Jarvis and Farr in 1971. They isolated a protein from *Bacillus cereus* capable of inactivating nisin and similarly structured subtilin and characterized it as a dehydroalanine reductase. Other researchers have reported on strains *Streptococcus thermophilus, Leuconostoc oenos* and *Staphylococcus aureus* which are resistant to nisin, but the mechanism of nisin resistance has not been specifically identified (Hurst, 1981; Daeschel et al., 1991). Harris, et al. (1991) specifically addressed the frequency of nisin resistance in three strains of *L. monocytogenes* and were able to isolate mutants resistant to 50 ug/ml (2000 units) at frequencies of 10^{-6} to 10^{-8}.

In this study, we evaluated the spontaneous nisin resistance frequencies of eight common foodborne pathogenic and spoilage type bacteria

and characterized the phenotypic response of *L. monocytogenes* Scott A when purposefully made resistant to high levels of nisin.

MATERIALS AND METHODS

Bacterial strains

Test strains used in this study were from the author's laboratory and are listed in Table 1. Cultures were maintained in brain heart infusion (BHI) broth (Difco Laboratories, Detroit, MI), stored at 4 °C and transferred to fresh BHI broth 24 h prior to experiments.

Nisin

High potency grade nisin was obtained from Aplin and Barrett, Ltd. (Dorset, U. K.). Activity was indicated as 37 X 10^6 U/g. Stock solutions (10,000 U/ml) were prepared by solubilizing in distilled water acidified to pH 2 with 0.02 N hydrochloric acid. Aliquots were kept frozen at -18°C until use.

Minimum inhibitory concentrations

The minimum inhibitory concentrations (MIC) of nisin were determined for the eight test strains and for nisin resistant-mutants derived from the strains. Serial two-fold dilutions of nisin containing BHI broth were prepared according to Bailey and Scott (1974). Each tube was inoculated with

approximately 5 X10^5 (CFU/ml) contained in 4 ml final tube volume. Incubation was at 37°C for 24 h and the lowest concentration of nisin that resulted in complete inhibition of visible growth was read as the MIC. All dilution series were performed in duplicate.

Determination of spontaneous resistance frequencies

Nisin-containing agar medium was prepared by adding nisin stock solution to 50°C BHI agar. The final concentrations of nisin were two to eight times the MIC for the test strains. Bacterial strains were exposed to nisin by mixing 10^8 to10^9 log phase cells into tempered (50°C) BHI agar medium and pouring plates with 20 ml of cell and agar mixture. Solidified plates were incubated at 37°C for 24 h. Frequency of spontaneous resistance was determined by comparing the number of colonies arising at each concentration of nisin with the original inoculum size, which was determined by the same method using nisin-free BHI agar. The procedures were performed in duplicate, and the mean values recorded.

Selection of resistant mutants

Two types of nisin-resistant mutants were selected in this study: 1) mutants from a single exposure to nisin and 2) a highly resistant mutant of *Listeria monocytogenes* Scott A selected by stepwise exposure in BHI broth that contained increasing concentrations of nisin. For the first type, the

mutants were picked from nisin containing BHI agar plates and confirmed by streaking out the colony on plates that contained the same amount of nisin. With the second type, a pure colony isolate from nisin containing agar plate was transferred into BHI broth and sequentially transferred into BHI broth that contained increasing concentrations (400, 600, 800, 1000, 1200, 1500 and 2000 U/ml) of nisin.

Inactivation of nisin in culture supernatants and cell extracts

Loss of nisin activity in culture supernatants may indicate a specific or nonpecific mechanism by which cells are degrading or inactivating nisin exocellularly. The method described by Collins-Thompson, et al. (1985) was used for detecting what was termed nisinase activity. Residual nisin levels were measured in cell-free (filter-sterilized, 0.2um) supernatants from nisin resistant and nisin-sensitive isolates of *Listeria monocytogenes* Scott A. Experiment controls consisted of uninoculated medium with and without nisin.

Phase transition temperatures determined by differential scanning calorimetry (DSC)

A DuPont 910 differential scanning calorimeter was calibrated using manufacturer software and with distilled water for heat flow calibrations. Overnight cultures of *L. monocytogenes* Scott A mutant R-2000 and parent

strain were washed twice with distilled water by centrifugation at 5000 x g for 10 min. Cell pellets were sealed in aluminum pans for placement in the instrument. The heat adsorption was measured in the range of 10-100°C with a heating rate of 10 °C /min according to the manufacturers guidelines (Dupont instruments, 1988) and those of Wang et al. (1991).

Fatty acid composition of bacterial cells

The fatty acid profile of nisin resistant and sensitive *L. monocytogenes* were determined with gas chromatography analysis by contract with Five Star Laboratories (Branford, CT). The data were analyzed statistically with the two sample *t* test (Devore, 1986).

Growth rates

Specific growth rates (k) (Drew, 1981) of resistant and sensitive strains at 20, 25, 32, 37 and 42°C were measured by growing the strains in BHI broth and taking optical density (O D_{600}) readings hourly. Arrhenius plots of the growth rate (Ingraham et al., 1983) were constructed by plotting log (k) against 1/K.

RESULTS

Spontaneous nisin resistance frequency

Eight common foodborne spoilage and pathogenic strains were tested for their spontaneous resistance frequencies to nisin (Table 1). Five of the eight strains exhibited the same MIC value, however there was variation in their resistance frequencies at nisin concentrations twice the MIC. Furthermore, there was a significant difference in resistance frequency among strains within a given species. For example, *Staphylococcus aureus* 40 had a higher resistant frequency than*S. aureus* 618 and *L. monocytogenes* Jalisco demonstrated a higher resistance frequency than *L. monocytogenes* Scott A.

The stability of resistant mutants isolated from initial nisin exposures was determined by transferring colonies from nisin containing culture plates to nisin free BHI broth. It was observed (Table 2) that the resistant mutants from initial nisin exposure had developed a resistance 2-4 times the original MIC values and that after three passages in nisin free BHI broth (approx. 30-35 generations), the MICs of resistant mutants *S. aureus* 618 and *L. monocytogenes* Jalisco returned to the original MICs. Nisin resistance with *B. cereus* and *L. monocytogenes* Scott A remained at 2 times the original MIC value after passage through nisin free medium.

A nisin resistant isolate of *L. monocytogenes* Scott A was obtained after seven transfers through BHI broth that contained increasing amount of

nisin. The nisin resistance of the mutant (R-2000) that subsequently developed was ten times the original MIC and was very stable.

Nisin degradation in culture supernatants

No differences were observed in residual activity when nisin was exposed to cell-free culture supernatants from nisin sensitive and nisin resistant *L. monocytogenes* Scott A. Furthermore, there was no difference in residual nisin activity of the culture supernatants and uninoculated media in which the same amount of nisin was added.

Phase transition temperature determination

The phase transition temperature (T_c) of whole cells of *L. monocytogenes* Scott A and the derived nisin resistant mutant (R-2000) were measured by DSC (Fig. 2). The T_c value is the temperature at which the phase transistion of lipids from a solid gel matrix to a liquid starts to occur (Russel 1989). The T_c of the nisin resistant strain was 44.4°C while the sensitive parent was 37.4°C. The higher phase transition temperature of the resistant mutant compared to its parent indicated that significant changes likely occurred in cellular lipid composition of the mutant.

Fatty acids profile

The GC analysis of fatty acids of *L. monocytogenes* Scott A and mutant

R-2000 indicated that there were some significant differences in fatty acid composition (Fig. 3) which is consistent with the observed differences in phase transition temperature. The percentages of peak area of each fatty acid detected in the two strains were compared statistically by the two-sample *t* test. It was found that there was a significant difference in fatty acids: 14:0, 15:0 iso,15:0 anteiso and 17:0 iso ($P < 0.05$). Comparatively speaking, the resistant mutant contained a greater proportion of straight chain fatty acids while the sensitive strain contained more bulkier branched fatty acids (iso and anteiso).

Comparison of specific growth rate at different temperatures

The Arrhenius plot of culture growth rate (Fig. 4) served to illustrate the growth behavior of resistant and sensitive strains at temperatures that ranged from 42 to 20°C. The specific growth rate of resistant mutant is slightly lower than that of the sensitive parent strain at the higher temperatures but was much slower at lower temperatures. At 37°C, the specific growth rate of the resistant strain was 86.7% of that of parent strain while at 20°C, it was only 40.9% of the parent strain

DISCUSSION

The results of this study indicated that spontaneous nisin resistance among some common food-borne spoilage and pathogenic bacteria occurs at different

frequencies. Considering both the MICs and the resistance frequencies observed from our study, it appeared that the group of bacteria in this investigation are less sensitive to nisin in comparison with lactic acid bacteria (Collins-thompson, 1985; Harris, 1992; Radler, 1990). Although the background resistance (MIC values) of the bacteria in this study are generally higher than that described for lactic acid bacteria, the resistant mutants obtained through one exposure to nisin did not develop high resistance in terms of MIC (2-4 times original MIC). Furthermore, the resistance was lost when cultured without nisin. In contrast to mutants obtained after a single exposure to nisin, the resistance level of *L. monocytogenes* Scott A R-2000 remained stable after five passages through nisin-free medium. The latter, however, was isolated after multiple and increasing stepwise exposure to nisin.

It is often the case that antibiotic resistance in bacteria is plasmid determined with the potential consequence of a rapid distribution of the genotype among other strains. The genes for nisin resistance (more accurately called producer cell immunity) in nisin producing *Lactococcus lactis* strains have been described as plasmid encoded. McKay and Baldwin (1984) observed that a plasmid (pNp400) from *Lactococcus lactis* subsp. *Lactis* biovar *diacetylactis* DRC3 encoded nisin resistance. Klaenhammer and Sanozky (1985) also reported that a plasmid (pTR1040) encoded nisin resistance in *L. lactis* ME2. Plasmid or chromosomal genes for true nisin resistance as

opposed to nisin producer immunity have not been described for any bacteria. Harris et al (1990) did not detect plasmid DNA in any of three nisin-sensitive *L. monocytogenes* strains or nisin-resistant strains derived from them. Included among the three strains was *L. monocytogenes* Scott A. In our study, we were also unable to detect plasmid DNA (data not shown) in the parent strain or the resistant mutant of *L. monocytogenes* Scott A.

The significance of the above observations as pertains to the use of nisin as a food preservative or food processing aid is difficult to predict. As pointed out by Harris et al. (1991) when high levels of microbial contamination are present, the likelihood of spontaneous nisin resistant mutants arising is inevitable. We concur, however, that the transisent and relatively low degree of resistance with mutants derived from a single exposure to nisin, coupled with the lack of plasmid mediated resistance (only shown with *L. monocytogenes*) may serve to limit a more serious problem from developing. Certainly, multiple exposure to nisin will enhance the likelihood of stable resistance mutants which would be of more serious consequence. This would be quite relevant in a situation where nisin is used as the sole means of preservation. Nisin and other bacteriocins may provide a useful alternative to certain preservatives and preservation mechanisms. However, the observation of resistance to nisin by many bacteria should be a caution as how to effectively utilize this bacteriocin. Food product contamination load, potential food interactions with nisin and the residual of

nisin in products are parmeters that warrant careful consideration when developing applications for nisin.

The nisin resistant mutant of *L. monocytogenes* Scott A (R-2000) and its parent were used as a model system to study possible nisin resistance mechanisms. In this study we did not find any evidence that nisin resistance in strain R-2000 was related to a possible inactivation mechanism. Jarvis et al., (1971) described one such inactivation mechanism with *Bacillus cereus* as nisinase activity and characterized a specific reductase that inactivated nisin. This does not appear to be the resistance mechanism in *L. monocytogenes* Scott A R-2000 in that nisin activity was stable in culture supernatants. Based on the general consensus that nisin kills cells by disrupting membranes (Henning, 1986; Hurst,1981) and that some resistance mechanisms to antimicrobial agents are related to the changes in bacterial membrane components (Bryan et at., 1984; Conrad et al., 1989; Juneja et al., 1992; Krulwich et al., 1987), our attention was focused on finding changes that occurred in the resistant mutant in terms of membrane fatty acid composition and other related characteristics.

The cellular membrane of *Listeria monocytogenes* contains mainly branched-chain fatty acids, with anteiso compounds being preponderant (O'Leary et al., 1989; Tadayon et al., 1971). The fluidity of cell membranes composed of branched-chain fatty acids is controlled mainly by anteiso-C_{15} (Kaneda, 1991). In our study, we observed that the nisin resistant mutant

R-2000 had a higher phase transition temperature which corresponded to the higher percentage of straight chain fatty acids as well as the lower percentage of branched fatty acids compared with that of the parent strain. The above observations are consistent in that branched chain fatty acids disrupt acyl chain packing in membranes, thereby lowering the membrane lipid gel to liquid transition temperature (Russell, 1989). The difference in fatty acid composition was especially evident ($P=0.001$) in the case of anteiso-C_{15}, the branching fatty acid which plays an important part in controlling membrane fluidity (Kaneda, 1991). The lower amount of bulkier iso and anteiso fatty acids and higher proportions of straight chain fatty acids may make the cell membrane of the mutant more compact than that of the sensitive strain. We speculate that this may somehow make it less susceptible to the action of nisin. Alternatively, the protein distribution in the membrane may also be altered due to changes in membrane fluidity and this may affect membrane related enzymatic or receptor reactions which may make the mutant resistant to nisin. The lower growth rate of the nisin resistant mutant at lower temperatures also indicated membrane composition changes of the resistant strain. It is well established that transport across the membrane of bacterial cells is affected by the structure and composition of the membrane, and reflected by their phase transition temperature profiles (McElhaney, 1985). The specific growth rate of the resistant strain markedly decreased at low temperature. At low temperatures, the membrane of the resistant strain may

be less flexible than that of the parent strain due to its higher phase transition temperature; therefore, the transport of nutrients may be affected leading to the lower growth rate. In a study of protonophore resistance in *Bacillus subtilis* , the resistance was found to be related to the packing properties of fatty acids in the membrane (Krulwich et al., 1987). Protonophores are membrane disrupting agents which may induce some resistant responses similar to that observed with nisin in this study. From the standpoint that changes in the fatty acids composition of bacteria membranes have been observed when bacteria are grown at low temperatures or in high salt environments (Kaneda, 1991; Miller, 1985), a question arises is: can the change in fatty acid composition be simply a general adaption to an unfavorable environment or is it a specific response to nisin? Other researchers have described similar changes in fatty acid composition in salt (NaCl) resistant mutants of *L. monocytogenes* (Juneja et al. 1992). However, the nisin resistant mutant in our study did not exhibit any change in NaCl tolerance compared to the sensitive parent strain (both strain grew in BHI broth with 6, 7, 8% of NaCl; and did not grow in BHI broth with above 9.6% of NaCl).

It appears that nisin resistance is a multi-faceted and complicated phenomenon. The correlation between fatty acid composition and nisin resistance in *L. monocytogenes* Scott A may only represent one aspect of the entire effect. Currently, studies are in progress to more precisely describe mechanisms of resistance to nisin in *L. monocytogenes*.

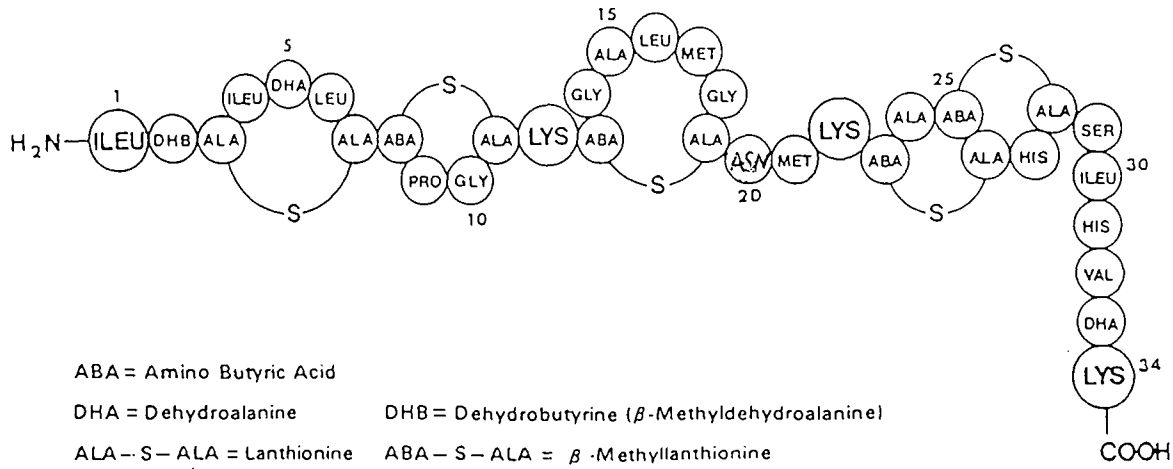

Fig. 1 The structure of nisin. From Gross and Morell (1971).

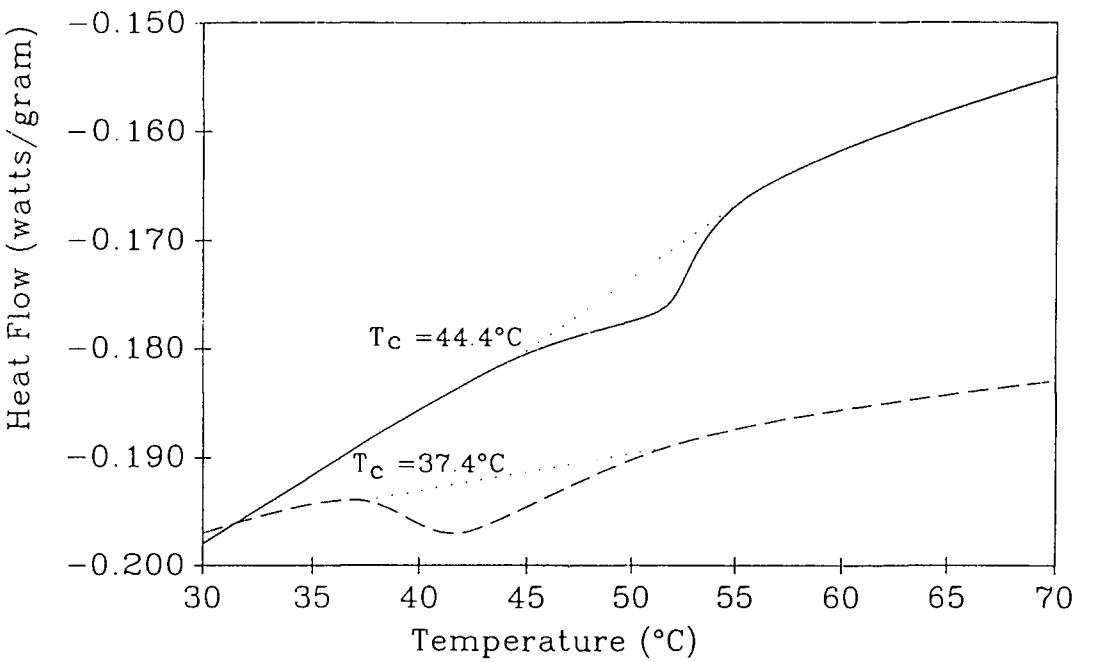

Fig. 2 The phase transition temperatures (Tc) of *L. monocytogenes* Scott A and R-2000 determined by differential scanning colorimetery. (_____) sensitive strain; (---------) resistant strain; (.....)reference baseline. Distilled water sealed in aluminium pans as reference material.

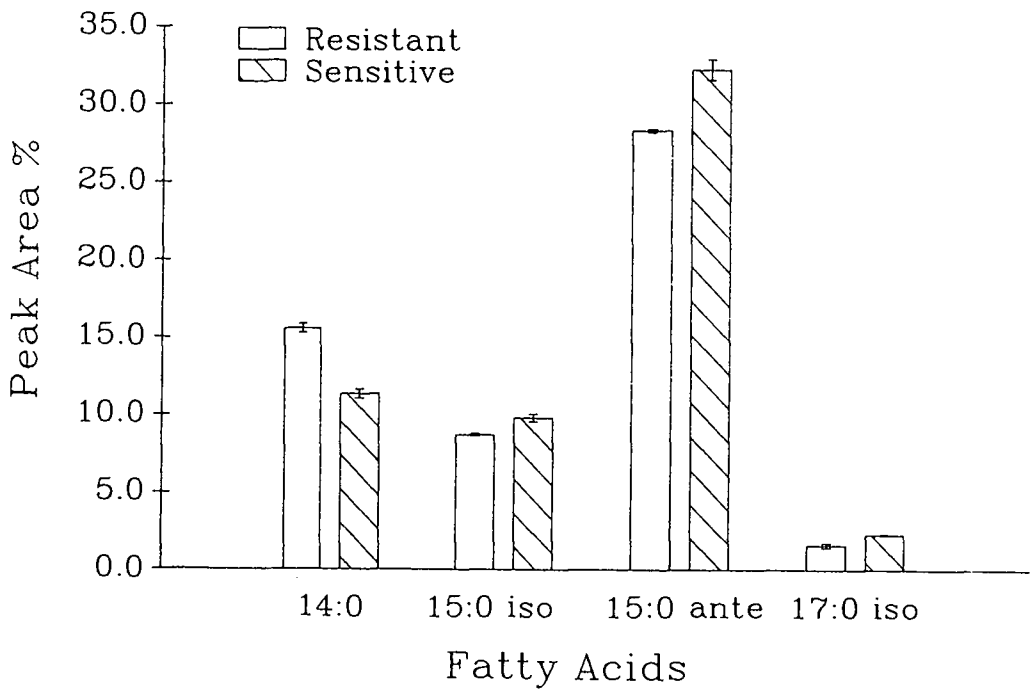

Fig. 3 Comparison of percentage of some straight chain and branch chain fatty acids of resistant and sensitive strains of *L. monocytogenes* Scott A. Results are means and SEM (standard error of mean) for four samples.

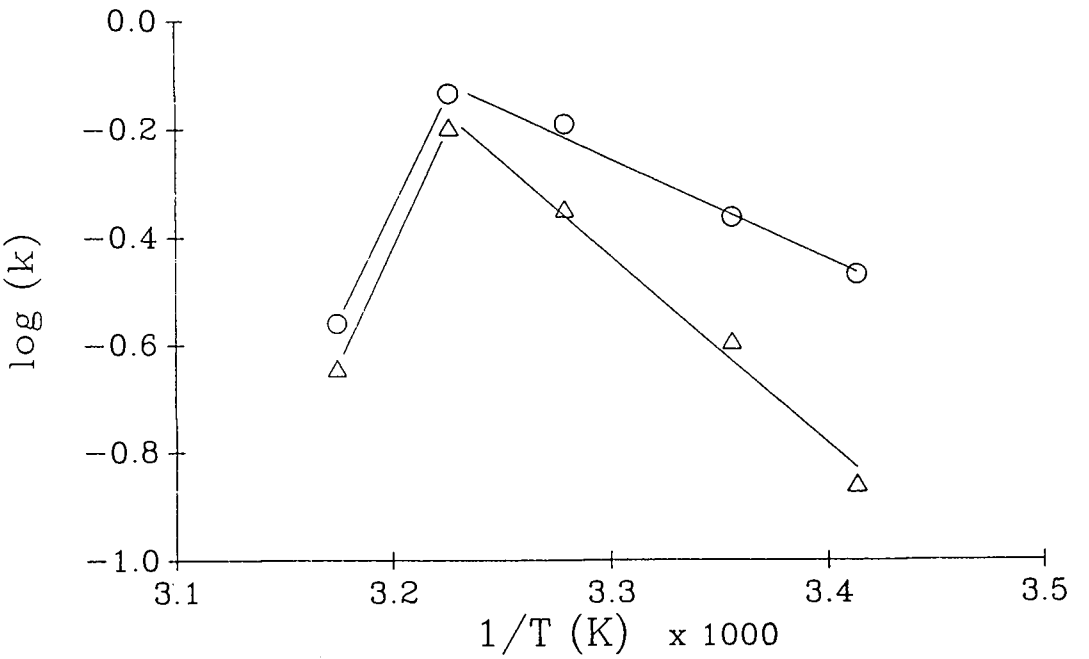

Fig. 4 Arrhenius plot of specific growth rate of resistant (△) and parental (○) strains of *L. monocytogenes* Scott A.

Table 1. Nisin resistance frequencies of some foodborne spoilage and pathgenic bacteria in BHI agar.

Strains	MIC (U/ml)	Resistance frequencies at 400 U/ml
S. aureus 618	200	$8.5 \times 10^{-8} \pm 5.6X10^{-9}$ *
S. aureus 40	200	$1.5 \times 10^{-6} \pm 4.2X10^{-7}$
L. monocytogene Scott A	200	$1.7 \times 10^{-6} \pm 4.5X10^{-7}$
L. monocytogene Jalisco	200	$9.2 \times 10^{-6} \pm 4.2X10^{-7}$
B. licheniformis	50	$5.6 \times 10^{-8} \pm 8.5X10^{-9}$
B. pumilus	50	$4.2 \times 10^{-8} \pm 2.8X10^{-10}$
B. subtilis	200	$2.1 \times 10^{-6} \pm 7.1X10^{-7}$
B. cereus	400	$4 X 10^{-6} \pm 5.6X10^{-7}$

* standard diviation

Table 2 . Minimum Inhibition Concentration (MIC) values of nisin resistant mutants and their resistance stability.

Strains	Minimum Inhibition Concentration (U/ml)		
	Initial	Mutants after a single exposure to nisin [a]	Mutants[a] after three passage on nisin-free medium
L. monocytogenes Scott A	200	400	200
L. monocytogenes Jalisco	200	400	200
B. cereus	400	1600	800
S. faecalis	600	800	600
S. aureus 40	200	800	200
L. monocytogenes Scott A R-2000[b]	2000	----	2000

a. Mutant were obtained from BHI agar plates containing nisin at the highest concentration which permitted mutant outgrowth into colonies.

b. Mutant were obtained from multiple and increased stepwise exposure to nisin.

CHAPTER 3

PHYSIOLOGICAL AND GENETIC CHARACTERISTICS OF A NISIN RESISTANT MUTANT OF*L. MONOCYTOGENES* SCOTT A

INTRODUCTION

Listeria monocytogenes was recognized as an imerging and serious significant human foodborne pathogen in the 1980s and continues to be a major concern to the food industry (Farber et al., 1991). Since this pathogen is sensitive to the bacteriocin nisin, using nisin as a barrier to control the pathogen has been reported for different foods and has shown great potential. The appearance of nisin resistant bacterial strains has been reported for several genera as well as for *L. monocytogenes* (Jarvis, 1972; Daeschel, 1990; Harris, 1991). In prior studies, we have investigated the spontaneous nisin resistance frequency and the specific nisin resistance responses of *L. monocytogenes* Scott A. In the present study, we examined the nisin killing rate for both resistant and sensitive *L. monocytogenes* Scott A, the temperature effect on nisin activity, the cell surface characteristics, the genetics characteristics of the resistant mutant, and the cross-resistance with other antibiotics for the nisin resistant mutant and its parental strain.

MATERIALS AND METHODS

Bacterial strains and growth media

Listeria monocytogenes Scott A was from the author's laboratory and was cultivated and enumerated with brain-heart infusion (BHI) broth and agar. The nisin resistant mutant R-2000 was derived from the nisin susceptible strain of *L. monocytogenes* Scott A by stepwisely induced the resistance in BHI broth containing incremental concentrations of nisin (described in previous chapter).

Nisin

High potency grade nisin was obtained from Alplin and Barrett, Ltd. (Dorset, U.K.). Activity was indicated as 37×10^6 U/g. Stock solutions (10,000 U/ml) were prepared by solubilizing in distilled water acidified to pH 2 with 0.02 N hydrochloric acid. Aliquots were kept frozen at -18°C until use.

Nisin killing rate

The loss of viability of resistant and sensitive bacterial cell populations after nisin treatment was determined in BHI broth and in phosphate buffer (50 mM, pH 6.5), as a function of time and amount of nisin added. Cells were added as a log phase culture to a final population of about 10^6-10^7 CFU/ml. Nisin was added in amounts to provide a final concentrations ranging from 5

to 500 U/ml. Samples were withdrawn at designated time intervals and viable cell counts were performed in duplicate. Average killing rates were calculated from the slopes of survivor curves for different nisin concentrations in a interval of 0 to 10 minunts.

Determination of cross-resistance between nisin and other antibiotics

The cross-resistance between nisin and other antibiotics were determinated by comparing the minimum inhibitory concentrations (MIC) of resistant and sensitive strains to several antibiotics and inhibitors. Serial 1 : 1 dilutions of antibiotic-containing BHI broth were prepared according to Bailey and Scott (1974). Each tube was inoculated with approximately 5×10^5 (CFU/ml) contained in a 4-ml final volume. Incubation was at 37°C for 24 h, and the lowest concentration of antibiotic resulting in complete inhibition of visible growth was read as the MIC. All dilution series were performed in duplicate and mean values were reported.

Determination of cell hydrophobicity

Hydrophobicity of the resistant and sensitive cells were determined by the BATH test (bacterial adherence to hydrocarbons) described by Rosenberg (1984). Log phase cultures (O D_{600} = 0.2-0.3) were centrifuged, washed twice with 50mM phosphate buffer (pH 7.0) and suspended in a suitable volume of the same buffer to adjust the optical density of the suspension in the range of

0.7 to 0.8. Hexadecane was added at 1/5 the volume of the cell suspension. The mixtures were then vortexed on a mixer for 1 min at maximum speed and kept at room temperature for 30 min for phase separation. Optical densities at 600 nm were recorded before the addition of hexadecane and after phase separation. The decrease in optical density after phase separation served as a relative index of cell surface hydrophobicity.

Bacterial cell protoplasts preparation and testing

Protoplasts from both resistant and sensitive cells *L. monocytogenes* Scott A were prepared as described by Lee-Wickner (1984) with some modifications. Log phase cells were washed twice with 0.85% saline and suspended in 1/5 original culture volume of protoplast buffer, which consisted of 0.02 M HEPES (N-2-hydroxyethylipiperazine-N'-2-ethanesulfonic acid) pH 7.0, 1 mM $MgCL_2$, 0.5% gelatin, and 0.5 M lactose as a stabilizer. Lysozyme was added to the cell suspension at 50 ug/ml. The cell suspension was incubated at 37°C for 6-8 hours to allow 99.999% of reduction in original plate count. Formation of protoplasts was judged by light microscopy, or enumeration of the number of osmotically fragile cells (the difference between the plate counts before and after protoplast formation treament). Nisin treatment of protoplasts was under the same conditions as for intact cells, except they were conducted in protoplast buffer, and the response was measured by recording the decrease in optical density at 600 nm.

Plasmid isolation

Plasmid DNA was isolated as described by Klaenhammer (1984). Electrophoresis was conducted on 0.7% agarose, horizontal slab gels in Tris acetate buffer (Maniatis et al., 1982) at pH 8.0 using a constant voltage of 75 V for 105 min. Lamda DNA/Hind III marker (BRL, Inc., Gaithersburg, Md.) was used as a standard for molecular mass determinations.

Restriction fragment length polymorphism analysis of genomic DNA of resistant and parental strains.

Restriction fragment length polymorphism (RFLP) was carried out with restriction endonuclease followed by fragment separation by pulsed-field gel electrophoresis. Chromosomal DNA embedded in agarose was prepared essentially by a previously described procedure (Smith et al., 1989). Each plug was cut into about five inserts, each insert was placed in a sterile microcentrifuge tube for restriction endonuclease digestion of embedded DNA as described by the enzyme manufacturer (Promega Corp., Madison, Wis.). A portion of 20 U of enzyme was added per insert. Following digestion for at least 16 h at the appropriate temperature with gentle shaking, 25 ul of 0.5 M EDTA (pH 8) was added to stop the reaction.

Samples were electrophoresised through 1% agarose gels in a solution consisting of 45 mM Tris, 45 mM boric acid (pH 8.3) and 1 mM sodium EDTA for 24 h at 200 V at 12°C in a Bio-Rad CHEF DR II electrophoresis cell (Vollrath

et al., 1987). Pulse times ramped from 1 to 19 s. Gels were stained with 0.5 mg of ethidium bromide per liter of water to detect DNA under UV illumination.

RESULTS AND DISCUSSION

Nisin killing rates of resistant and sensitive cells and protoplasts

Nisin killing effect on resistant and sensitive cells were compared in phosphate buffer and BHI broth. The survival plots (Fig.5. a, b, c, d) showed that nisin killed resistant cells less effectively than sensitive cells in both BHI broth and buffer. When cells were exposed to nisin in buffer (Fig. 5. a, b), the sensitive population decreased significantly (10^7 to 10^3 at 5 U/ml) in 30 min while the resistant population was only slightly reduced (0.5 log cycle at 5 U/ml). In BHI broth (Fig. 5. c, d), the sensitive population started decreasing when nisin concentration was at 50 U/ml, while for resistant cells, the population started showing significant reduction when the nisin concentration was 500 U/ml.

Considering the killing rate and nisin concentration used, it appeared that nisin killed cells more effectively in buffer than in BHI broth. In BHI broth, active cell multiplication and nisin killing occurred simultaneously; the observed survivor cells were the net result of growth and killing. Since the maximum nisin killing rate of resistant cells was only one third of that of

sensitive cells (Table 4) but their growth rates at 37°C were similar (Fig. 4), the difference in the growth/killing rate for resistant and sensitive cells would make resistance more significant in BHI broth than in buffer. The possibility that nisin may have bound to components of BHI broth, thus reducing the activity was desproved since nisin residual activity of BHI broth and phosphate buffer were essencially the same.

Different nisin lysis effects were observed for the protoplasts prepared from resistant and sensitive cells. The lysis rate of protoplasts from sensitive cells was higher than that of the resistant protoplasts when treated with 10 or 100 U/ml of nisin (Fig. 6). In the case of protoplasts, cell walls are removed so nisin may act directly on the cytoplasmic membrane. The relatively lower lysis rate of resistant protoplasts indicated that nisin resistance also exist in the protoplasts. This observation suggested that nisin resistance may not related to cell wall and more likely related to cell membrane.

The curves of killing rates *versus* nisin concentrations for cells and protoplasts (Fig.7, Fig.8) suggested that nisin killing rates were dependent on nisin/cell ratios instead of on nisin concentration alone. The killing of a definite fraction of a bacterial population in a chosen time interval requires the absorption of a given amount of antimicrobial agent by the cells. In the case of polymyxin B, 2×10^5 molecules/cell should be bactericidal toward *Salmonella* (Franklin and Snow, 1989). In our study, the 50% maximum killing rates were reached when the ratio at 0.63 U/10^6 cell (2.9×10^7

molecules/cell) for sensitive cells and 3.3 U/10^6 cells (1.5×10^8 molecules/cell) for resistant cells (Table 3). Considering the killing rates individually, it was found that the maximum nisin killing rate for sensitive cells was 3.2 times of that of resistant cells (Table 3).

The data on nisin killing rate indicated that resistant cells have a higher threshold for nisin killing action and a lower killing rate compared to that of sensitive cells. It is these two factors that made the nisin resistant population relatively more resistant to nisin.

Temperature effect on nisin activity

The effect of temperature on nisin's bactericidal and bacteriolytic activity was examined with both nisin resistant and sensitive cells. It was observed that nisin induced-cell lysis was temperature dependent for both resistant and sensitive cells (Fig. 9). At 4°C, both resistant and sensitive cells did not lyse when the nisin concentration was 10 U/ml and the sensitive cells slightly lysed at 100 U/ml. At 37°C, both populations severely lysed at nisin concentrations of 10 U/ml, however, the sensitive cells lysed to a greater extent than resistant cells. The significant temperature effect on cell lysis suggested that nisin-induced cell lysis may involve the action of autolytic enzymes. It has been documented (Bierbaum and Sahl, 1985, 1987) that many cationic peptides that exhibit antimicrobial activity also stimulate autolysis in Gram-positive bacteria. Nisin and Pep 5 have been reported to stimulate the

autolysis of *S. simulans* 22 in addition to its membrane-disturbing properties (Bierbaum and Sahl, 1985, 1987); the bacteriolytic effect of AS-48 (basic peptide produced by *Enterococcus faecalis* S-48) was also reported to be associated with autolysin activation (G'alvez et al., 1990). Since autolysis was the result of enzymatic reaction (autolysins) and could be retarded at low temperatures (Lortal et al., 1991), the poor lysis induced by nisin at a low temperature in the present study may serve to indicate the involvement of autolysins in cell lysis.

Fig. 10 portrays the viability of resistant and sensitive cells when incubated with nisin at 4°C and 37°C. It was observed that nisin was more effective at 37°C than at 4°C for killing both resistant and sensitive cells. It has been reported that nisin was more effective in killing *Salmonella* species (with EDTA) at 37°C than at 4°C (Stevens et al., 1992). At lower temperatures, nisin-induced cell lysis may be retarted, while at higher temperatures, this process may be more active and may facilitate the bactericidal action of nisin. Changes in the physical properties of cell membranes at lower temperatures which may make the bacteria more resistant to nisin, is yet another possible reason.

It was also observed that more resistant cells survived nisin exposure than sensitive cells at both 4°C and 37°C. At 4°C, even though sensitive cells did not exhibited much lysis, their viability was much less than that of resistant cells (Fig. 9, Fig. 10). These results suggested that nisin-killed cells

not necessarly resulted in cell lysis and that nisin resistance observed for *L. monocytogenes* Scott A may not be related to autolytic response. For nisin killing action against *L. monocytogenes* Scott A, we propose that membrane damage is the first, fatal event, while nisin stimulated cell lysis is functioning as a secondary, cooperative effect.

Hydrophobicity of resistant and sensitive cells

Bacterial hydrophobicity is a term used to describe the hydrophobic properties conferred on bacteria cells by their outermost cell surfaces. Since the major part of the amino acid content of nisin are non-polar in nature, hydrophobic interactions between nisin and cell surfaces may have some effects on nisin killing efficiency (Henning, 1986). The hydrophobicity of the resistant and sensitive cells were compared in this study. It was observed that sensitive cells had a relatively higher hydrophobicity than resistant cells (Fig. 12). The lower hydrophobicity observed for the resistant cells suggested that the affinity between nisin and resistant cells may be weaker then that between nisin and sensitive cells, which would make the resistant cells absorb less nisin compared to sensitive cells. Therefore, a less hydrophobic cell surface may provide another factor that contributes to nisin resistance.

Difference in bacterial cell surface hydrophobicity may be influenced by cell surface composition. Some cell wall components were reported to be implicated in the hydrophobic reactions of Gram-positive bacteria (Hancock,

1991), such as proteins, lipoteichoic acids and lipids. It has been reported that there were significant differences in the cell surface components of methicillin-resistant cells of *S. aureus* and were attributed to the production of different anionic polymers on the surface (James, 1972). The particular cell surface components of the resistant cells that may be responsible for the observed decrease in hydrophobicity was not further investigated in this study.

Cross-resistance between nisin and some antibiotics and inhibitors

Table 4 lists the MIC of resistant and sensitive cells to other antibiotics and inhibitors. With all the antibiotics and inhibitors tested, the nisin resistant strain showed only a slight increase in resistance to polymyxin B which is also a peptide membrane disrupting agent. With the other nine antibiotics and metabolic inhibitors, nisin resistant cells were slightly less resistant than the nisin sensitive parent. These results indicated that nisin resistance observed for *L. monocytogenes* Scott A did not contribute to resistance to therapeutically important antibiotics and some metabolic inhibitors. The slightly less resistance to other antibiotics may be due to the less metabolic activity of the resistant mutant which was reflected by their reduced growth rate (Fig. 4) as compared to the sensitive parent.

It has been reported in the literature that nisin resistant *S. aureus* (Carlson and Bauer, 1957) and lactic acid bacteria (Lipinska, 1977) did not

exhibit cross-resistance to medically important antibiotics. Our study with nisin resistant *L. monocytogenes* Scott A is consistent with these fundings.

Resistance to several antibiotics have been documented for *L. monocytogenes*. Resistance to tetracycline, chloramphenicol, erythromycin, and streptomycin were reported as transferable plasmid-mediated traits (MacGowan, 1990). We failed to detect any plasmids in both resistant and sensitive strains of *L. monocytogenes* Scott A which is consistent with the results of Harris et al. (1991). These results suggested that the resistance mechanism of *L. monocytogenes* to nisin is specific for nisin and is not related to resistance to clinically important antibiotics.

Restriction fragment length polymorphism analysis of genomic DNA of resistant and parent strains.

Restriction fragment length polymorphism (RFLP) has been used for several years as an epidemiological tool for the study of viral or bacterial infection outbreaks. RFLP analysis of chromosomal DNA can be carried out with restriction endonucleases which infrequently cleave sites in the DNA. The use of infrequently cleaving enzymes followed by fragment separation by pulsed-field gel electrophoresis has been demonstrated to be a sensitive and reproducible method for the typing of bacterial species or subspecies otherwise difficult to study. This approach has recently been used for differentiating *Listeria* spp. as well as other bacterial species (Le Bourgeois et al., 1989).

In initial experiments, we found that both resistant and sensitive strains of *L. monocytogenes* ScottA were plasmid-free, and the resistance was quite stable (Table 2). Based on these two facts, we assumed that the resistance was genetically determined by some alternations in chromosomal DNA, and we attempted to detect those alterations by RFLP.

Endonuclease *Sma* I and *Apa* I were reported (Howard et al., 1992) most useful for the separation of megabase-sized restriction fragments from strains representing different *Listeria* species. Generally, the number of sites recognized by a restriction endonuclease in a DNA molecule is dependent on the length of the recognition site and the respective base compositions of the DNA (McClelland et al., 1987). *Listeria monocytogenes* genomic DNA has a G+C content of 38% (Seeliger et al., 1986). Therefore, digestion with endonucleases recognizing G+C-rich sites would be expected to give rise to a small number of fragments. Enzymes *Apa* I and *Sma* I, which have recognition sites (GGGCCC) and (CCCGGG), were chosen for use in this study.

Apa I digestion of *L. monocytogenes* DNA generated 15 fragments, whereas 17 fragments were obtained after *Sma* I digestion (Fig. 13). The two restriction endonuclease digestion patterns of the nisin resistant strain *L. monocytogenes* R-2000 were identical to that of the parent (Fig.13). Possible explanations for the *Sma* I and *Apa* I digestion patterns may include : (1) Nisin resistance may not be related to chromosomal rearrangements

involving deletions, insertions and inversions which were comparatively large genetic changes and could be detected easily. (2) The resistance may be related to some minor genome changes which would not cause the appearance or disappearance of recognizably different cut sites of both *Sma* I and *Apa* I. (3) The nisin resistance was not genetically determined by alterations in chromosomal DNA.

It was reported that these two enzymes (*Sma* I and *Apa* I) could produce different digestion patterns among strains belonging to different serovars of *L. monocytogenes* (Carriere et al., 1991), but in a study using the same technique in identification of *Lactococcus,* it was also found (Tanskanen et al., 1990) that the *Sma* I digestion pattern of bacteriophage-resistant derivatives was identical to that of the parent strain. The genetic determinants of nisin resistance in *L. monocytogenes* Scott A R-2000 remains to be identified.

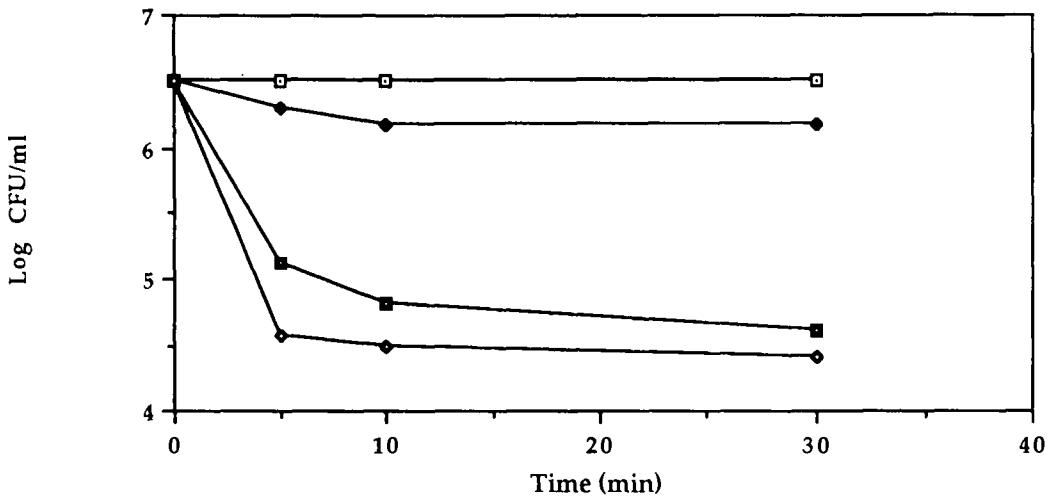

Fig. 5 a) Survival plots of *L. monocytogenes* Scott A R-2000 as a function of time at different concentrations of nisin in 50 mM phosphate buffer pH 6.5.

(—□—) 0 U/ml (—●—) 5 U/ml

(—■—) 50 U/ml (—◇—) 100 U/ml of nisin.

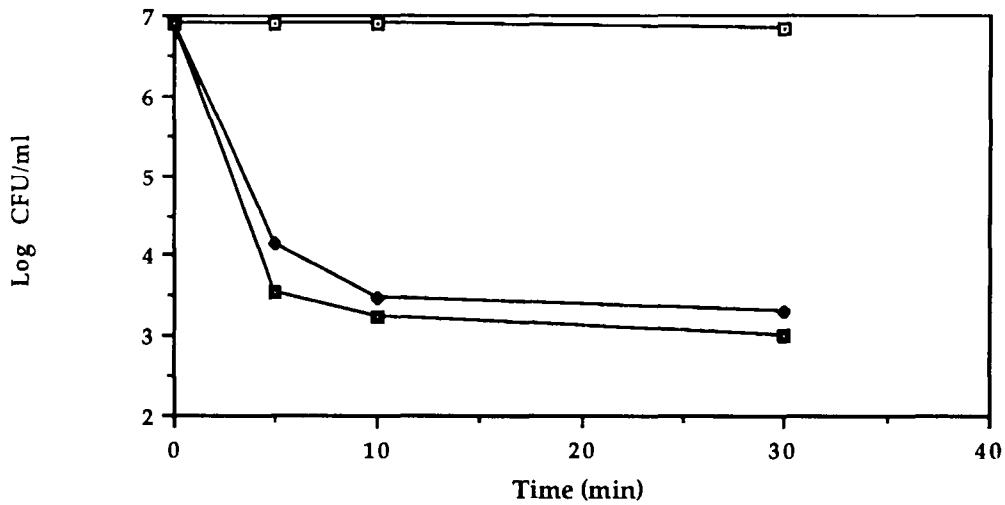

Fig. 5 b) Survival plot of *L. monocytogenes* Scott A (sensitive) as a function of time at different concentrations of nisin in 50 mM phosphate buffer pH 6.5,

(—□—) 0 U/ml (—◆—) 5 U/ml

(—■—) 50 U/ml of nisin.

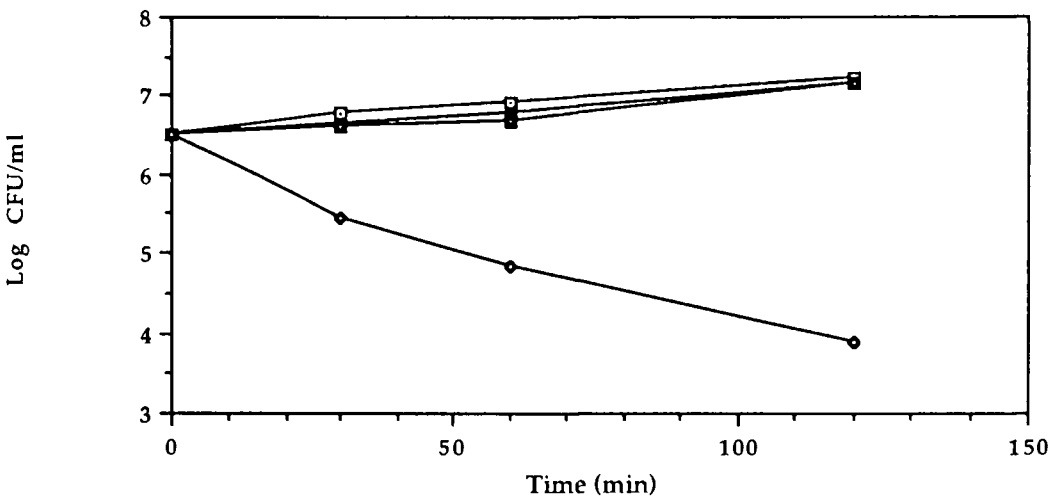

Fig. 5 c) Survival plot of *L. monocytogenes* Scott A R-2000 as a function of time at different concentrations of nisin in BHI broth.

(—□—) 0 U/ml (—■—) 10 U/ml

(—◆—) 100 U/ml (—◇—) 500 U/ml of nisin.

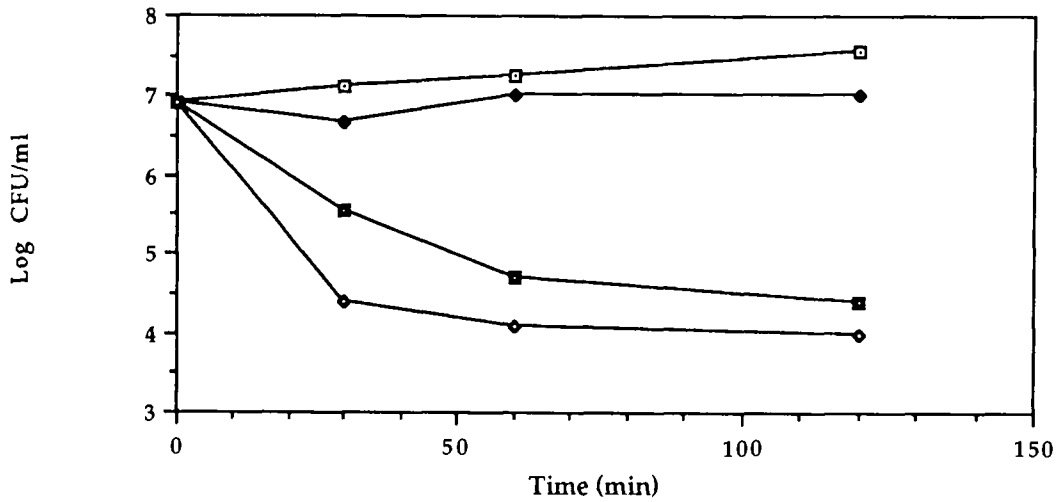

Fig. 5 d) Survival plot of *L. monocytogenes* Scott A (sensitive) as a function of time at different concentrations of nisin in BHI broth.

(—□—) 0 U/ml (—◆—) 10 U/ml

(—■—) 50 U/ml (—◇—) 100 U/ml of nisin.

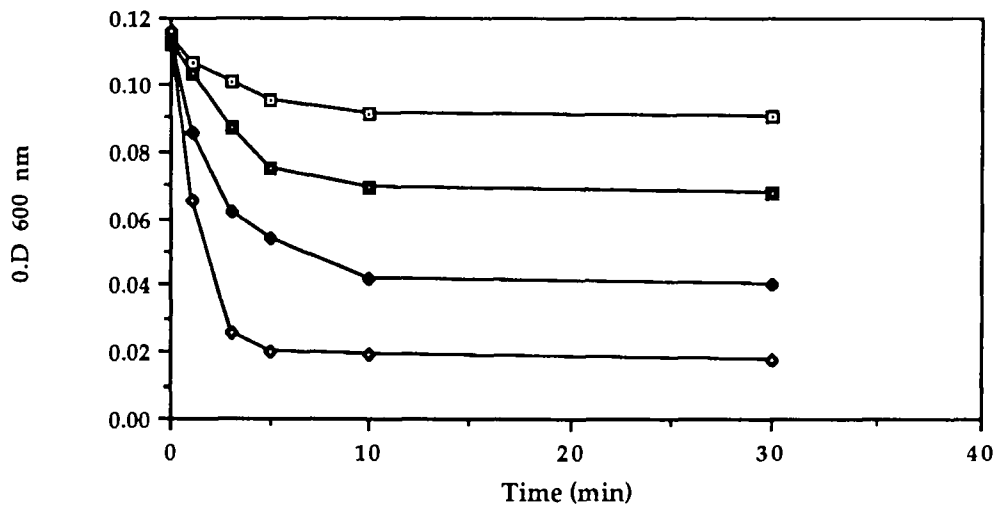

Fig. 6 Nisin lysis of protoplasts of *L. monocytogenes* Scott A resistant (R) and sensitive (S) strains in protoplast buffer containing nisin for one hour at 37°C. Lysis was indicated by the decrease in optical density ($0.D_{600}$).

(—□—) R-10 U/ml (—■—) R-100 U/ml

(—◆—) S-10 U/ml (—◇—) S-100 U/ml.

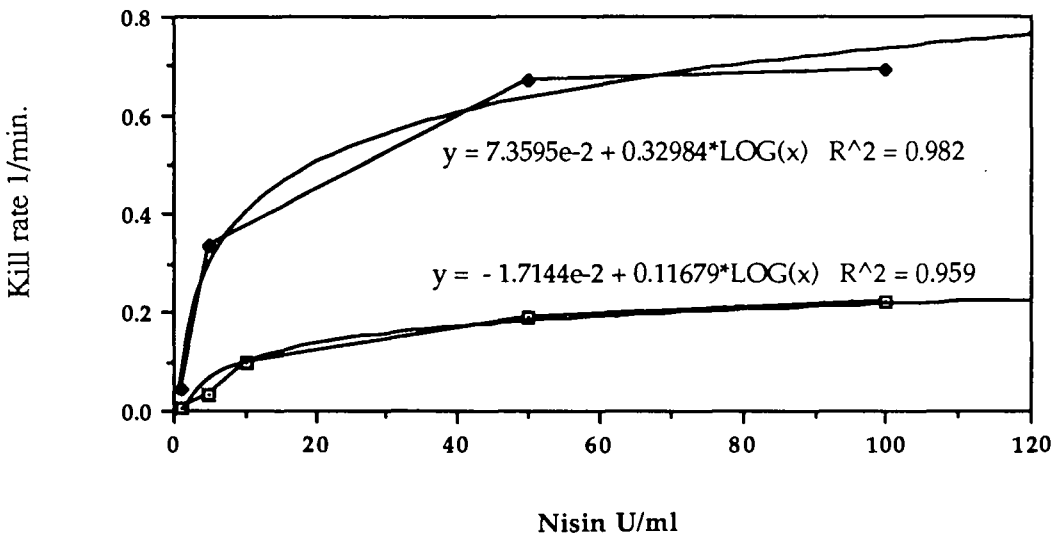

Fig. 7 Nisin killing rates of *L. monocytogenes* Scott A resistant (—□—) and sensitive (—◆—) cells as a function of nisin concentrations in 50 mM phosphate buffer, pH 6.5. Killing rates (K) were slopes of survival plots (Fig. 5 a and b).

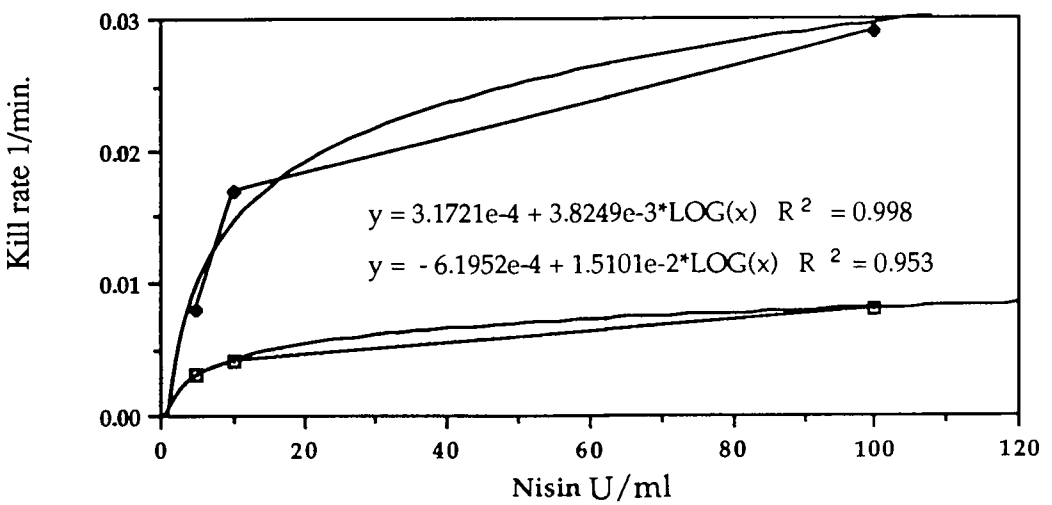

Fig. 8 Nisin lysis rates of protoplasts from *L. monocytogenes* Scott A resistant (—□—) and sensitive (—●—) cells as a function of nisin concentrations in protoplast buffer. Killing rates (K) were slopes of lysis curves of protoplasts from resistant and sensitive cells.

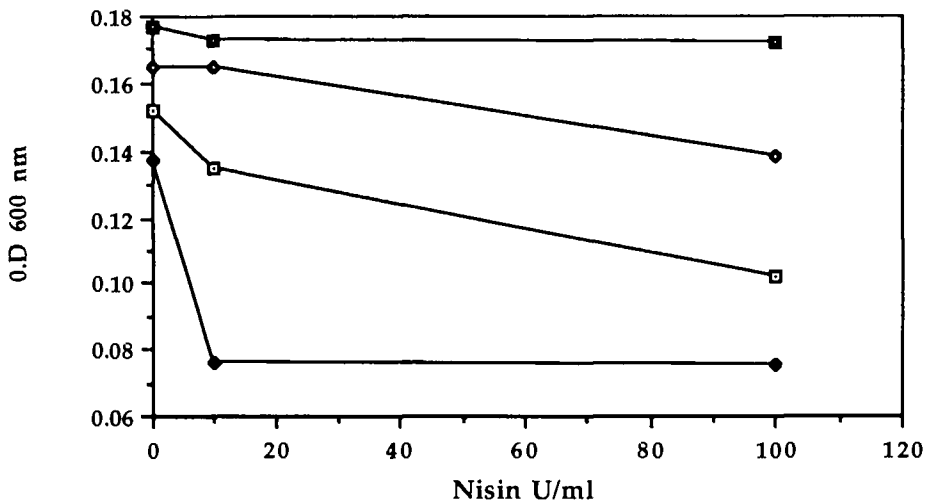

Fig. 9 Temperature effect on nisin induced lysis of *L. monocytogenes* Scott A resistant (R) and sensitive (S) cells. Log phase cells were washed once and suspended in 50mM phosphate buffer, pH 6.5. Nisin was added at different concentration, the samples were incubated at 4°C and 37°C respectively for one hour. Results are means of duplicates.

(—■—) R-4°C (—◇—) S-4°C

(—□—) R-37°C (—◆—) S-37°C

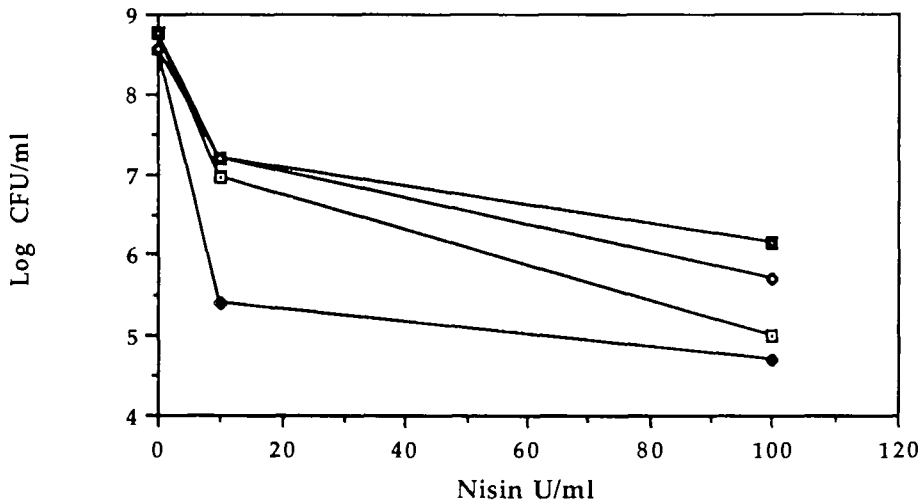

Fig. 10 Temperature effect on nisin bactericidal activity toward *L. monocytogenes* Scott A resistant (R) and sensitive (S) cells. Nisin treatment was the same as in Fig. 9. Results are the means of duplicates.

(—■—) R-4°C (—□—) S-4°C

(—◆—) R-37°C (—●—) S-37°C

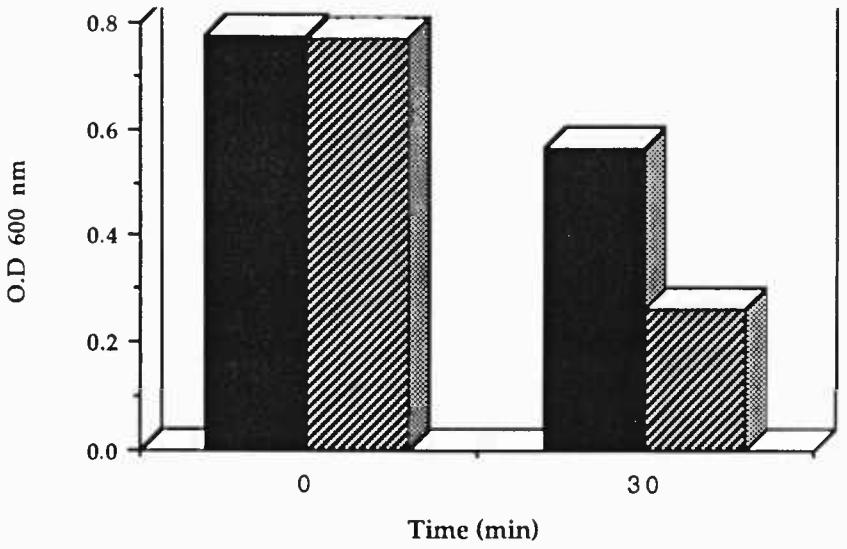

Fig. 11 The comparative hydrophobicity of *L. monocytogenes* Scott A resistant (■) and sensitive (▨) cells. The assay was conducted by theBATH procedure of Rosenberg (1984). The decrease in cell suspension optical density (O D $_{600}$) occured because more hydrophobic cells partitioned into the organic phase (hexadecane). Hence, this served as a relative index of cell hydrophobicity. Results are the means of duplicates.

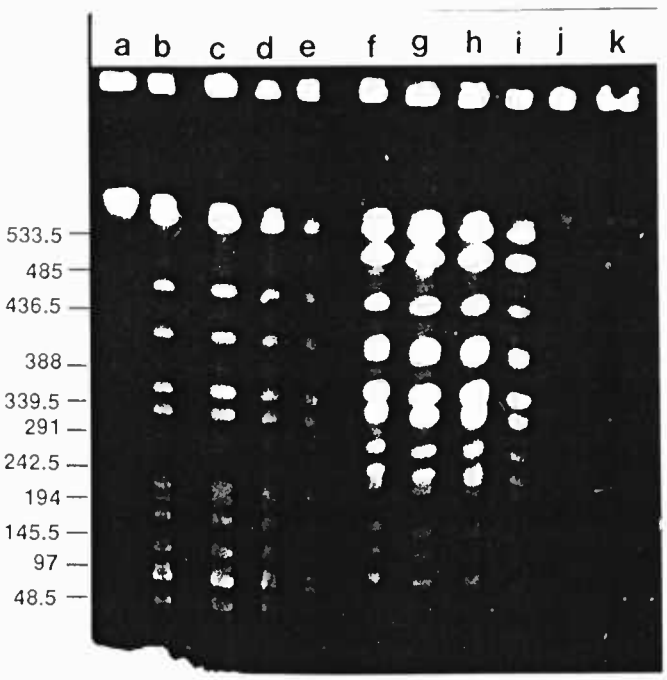

Fig. 12 Restriction fragment patterns of *Sma* I (b, c, d, e) and *Apa* I (f, g, h, i) digests of chromosomal DNA of *L. monocytogenes* Scott A resistant (R) and sensitive (S) cells.

Lane a: lambda concatemers

Lane b, d, f, h: R

Lane c, e, g, i : S

Lane j: undigested control of R

Lane k: undigested control of S

TABLE 3. Comparison of nisin killing rates of resistant and sensitive strains of *L. monocytogenes* Scott A.

Strains	Original CFU/ml	MKR*(1/min)	Nisin/cell ratio for 50% MKR
Resistant cells	3×10^6	0.219 (100 U/ml)	3.3 U/10^6
Sensitive	8×10^6	0.691 (50 U/ml)	0.63 U/10^6
Resistant/Sensitive	--	0.32	5.24

* MKR: Maximum killing rate ; data in parenthesis were nisin concentrations at which maximum killing rates were reached.

Table. 4 Minimum inhibition concentrations of *L. monocytogenes* Scott A and the resistant mutant R-2000 to antibiotics.

Antibiotics (ug/ml)	Scott A	R-2000
CCCP	16	8
DCCD	32	16
D-cycloserine	32	16
Cerulenin	16	8
Rifampicin	32	16
Chloramphenicol	16	8
Vancomycin	16	2
Streptomycin	32	8
Erythromycin	0.125	0.0625
Polymyxin B(U/ml)	64	128

CCCP : Carbonyl cyanide *m* -chlorophenylhydrazone.

DCCD : N, N'-Dicyclohexylcarbodiimide.

CHAPTER 4

CORRELATION OF CELLULAR PHOSPHOLIPID CONTENT WITH NISIN RESISTANCE OF *L. MONOCYTOGENES* SCOTT A

INTRODUCTION

Nisin is the most thoroughly characterized bacteriocin among the antimicrobial peptides produced by lactic acid bacteria. As an effective and safe food preservative, nisin received international acceptance in 1969 and has found applications in the United States as a GRAS (generally recognized as safe) ingredient in processed cheese to prevent clostridial growth (FDA, 1988). The inhibitory activities of nisin toward some Gram-positive foodborne pathogens including *L. monocytogenes* have been approved (Benkerroum, 1988; Scott, 1981; Somers, 1981). The recognition of a nisin-sensitive strain of *Streptococcus agalactiae* becoming resistant was first observed during early studies on nisin by Hirsch (1950). The development of acquired or spontaneous nisin resistance has been reported for *Staphylococcus aureus, Lactobacillus plantarum, Streptococcus thermophilus,* several species of *Bacillus* and more recently *L. monocytogenes* (Chevalier, 1962; Jarvis 1968; Lipinska 1977; Harris et al. 1991). Even though nisin resistance has been observed for a long time, the mechanisms of nisin resistance other than

nisinase have not been determined. In the previously described studies in this thesis, we reported on the nisin resistance frequencies for several foodborne pathogenic bacteria and one possible mechanism of nisin resistance in *L. monocytogenes* Scott A. The present study was a continued investigation on nisin resistance in *L. monocytogenes* and was undertaken in an attempt to elucidate the correlation of cellular phospholipid changes with nisin resistance.

MATERIALS AND METHODS

Bacterial strains and growth media

Listeria monocytogenes Scott A was from the author's laboratory and was cultivated and enumerated with brain-heart infusion (BHI) broth and agar. The nisin resistant mutant R-2000 was derived from *L. monocytogenes* Scott A by stepwise exposure to increasing concentrations of nisin in BHI broth (described previously in this thesis).

Nisin

High potency grade nisin was obtained from Alplin and Barrett, Ltd. (Dorset, U.K.). Activity was indicated as 37×10^6 U/g. Stock solutions (10,000 U/ml) were prepared by solubilizing in distilled water acidified to pH 2 with 0.02 N hydrochloric acid. Aliquots were kept frozen at -18°C until used.

Extraction and identification of phospholipids

Lipids were extracted directly from wet cell pellets of overnight cultures by the modified Bligh and Dyer procedure (Morris, 1972).

Individual phospholipid components were separated by two-dimensional thin-layer chromatography on silica gel 60 plates using chloroform-methanol-28% ammonia (65:35:5) in vertical direction. Plates were then air-dried and developed with chloroform-acetone-methanol-acetic acid-water (5: 2: 1: 1: 0.5) in the horizontal direction. Individual phospholipids were identified by spraying the plates with ammonium molybdate reagent and then comparing plate their mobilities with those of standards obtained from the Sigma Chemical Company. (St. Louis). Phospholipids were quantified by estimating lipid phosphorus by the method of Bartlett (1959), as modified by Marinetti (1962).

Release of phospholipids from cells

Log phase cells (O D $_{600}$ = 0.2-0.3) were washed once and suspended in 0.85% saline at 1/50 of the original volume of the culture. After being incubated with nisin at 37°C for 60 min, cells were removed by centrifugation at 8000 x g for 5 min. Lipids in the supernatant were extracted with the Bligh and Dyer procedure and then assayed for phospholipid phosphorus (1962).

Measurement of cell viability

Cell viability was measured for the same batch of cells that were measured for release of phospholipids. Samples were taken before cells had been removed from cell/nisin suspensions. Viability of cells was determined by plate count. Results reported represent means of duplicate determinations.

Electron microscopy

Nisin-induced physical damage to resistant and sensitive cells was visually evaluated using transmission electron microscopy. Early log phase cells (O D_{600} = 0.2-0.3) were washed twice with 50mM phosphate buffer (pH 6.5) and resuspended in the same buffer at the original volume. Nisin was added to a final concentration of 300 U/ml and cultures incubated at 37°C for 2 hours. Cells were removed from 3 ml of samples by centrifugation and resuspended in 0.5 ml fixative (McDowel and Trump, 1976). Preparation of samples for electron microscopy was carried out according to Wiegel and Dykstra (1984).

Nisin binding affinity

Early log phase cells were washed twice with 50 mM potassium phosphate buffer (pH 6.5) and suspended in the same buffer to the same volume. Nisin was added at different concentrations and incubated at 37°C for 2 hours. Portions (100 ul) of supernatant of the cell/nisin suspensions

were added to wells in MRS agar plates seeded with 0.1% (V/V) of indicator bacteria (*Pediococcus pentosaceus* FBB-61-2). Samples were allowed to diffuse for 24 h at 4°C prior to incubation at 37°C for outgrowth of the indicator. Estimates of residual nisin activity in samples were obtained by measuring inhibition zone widths (the distance from the edge of the inhibition zone to the edge of the well) with a dial micrometer. The residue nisin activity was reported as percentage of the control (nisin in buffer without cells).

RESULTS AND DISCUSSIONS

Changes of phospholipid content of nisin resistant cells.

It was observed (Table 5) that the development of nisin resistance was accompanied by a significant reduction in the content of total phospholipids (TPL) ($P < 0.001$). This decrease was also reflected in the content of individual phospholipids. Three phospholipids: phosphatidylglycerol (PG), diphosphatidylglycerol (DPG) and bis-phosphatidylglyceryl phosphate (BPG), were isolated from both resistant and sensitive cells by two-dimensional TLC (Fig. 17) which was consistent with the results of Kosaric (Kosaric, 1971). There was a significant ($P < 0.01$) decrease in content of each of the three phospholipids. Earlier studies on the mode of action of nisin have suggested that the antimicrobial effect of nisin is caused by its interaction with phospholipids components of the cytoplasmic membrane (Henning et al.,

1986), and that the phospholipid composition of artificial membranes will affect the activity of nisin (Abee et al., 1991). Nisin is an amphiphilic molecule with a net positive charge (Pi=10.5). Henning et al. (1986) proposed that hydrophobic interaction between nisin's amino acid residues and fatty acids of membrane phospholipids was responsible for interactions between nisin and membrane that decreased its activity. On the other hand, the electrostatic reaction between nisin molecules and negatively charged phospholipids has also been considered as the primary reaction of the bactericidal action of nisin (Sahl et al., 1991). In both cases, phospholipids of bacterial cell membrane were strongly implicated as the target of nisin. In our study, nisin resistance observed for *L. monocytogenes* Scott A correlated with the reduction of phospholipids, which perhaps implies that the resistant cells may be less damaged because of the reduced amount of phospholipids which function as the targets of nisin.

It has been proposed that a correlation exists between lipid composition and bacterial resistance to antibiotics (Vaczi, 1973). The reduction of phospholipid content was documented for polymyxin B (membrane disrupting peptide) resistance (Conrad et al., 1981; 1989) and ethambutol resistance in *Mycobacterium tuberculosis* (Sareen, 1990). The bacterial resistance to duramycin (related to nisin) was reported as originating from alterations of the phospholipid composition (Clejan et al., 1989). The nisin resistance observed for *L. monocytogenes* Scott A may share a similar

resistant mechanism with other peptide antibiotics promoted by the alteration of membrane phospholipids.

The release of phospholipids from cells

The experiment of phospholipids released by nisin from both resistant and sensitive cells provided additional evidence for the nature of nisin resistance. When treated with 300 U/ml of nisin in 0.85 % saline, sensitive cells lose about 45% of the cellular phospholipids while the resistant cells only lost 14% (Fig. 13). It was reported (Katsu et al., 1989) that amphophilic peptides gramicidin S and melittin stimulated the release of membrane phospholipids from bacterial cells. Sahl et al. (1991) has proposed that nisin forms pores on cell membranes which may be due to the formation of nisin-aggregate on the membrance. In this study, based on the data generated, we suggest that in the processes of pore formation, aggregated nisin molecules may pluck off cell membrane components which could be indicated by the release of phospholipids, analagous to the findings of Katsu et al. (1989). The lower amount of phospholipid released from resistant cells in this experiment indicated that nisin caused less damage to resistant cells, which made the population comparatively resistant to nisin. The reduction of phospholipids in the resistant cells may simply be the reason for the observed smaller amount of phospholipids being released by nisin.

Viability of resistant and sensitive cells after nisin treatment

To find out the correlation between cell viability and phospholipid release after nisin treatment, the viability of both resistant and sensitive cells were measured for the same batch of cells that were subjected to phospholipid release by nisin. It was observed (Fig. 14) that nisin kills sensitive cells more effectively than resistant cells. With 300 U/ml nisin treatment for one hour, 3.1% of resistant cells survived, while for the sensitive cells in the same treatment, only 0.5% of the population survived. The fact that nisin killed a smaller amount of resistant cells than sensitive cells of *L. monocytogenes* Scott A was correlated with the smaller amount of phospholipids released from resistant cells and may have also resulted from the reduction in their membrane phospholipids.

Cell membranes act as osmometers, permeability barriers and carry important functional units concerned with energy and macromolecular synthesis. In speaking about the bactericidal action of nisin, cell membrane damage has been suggested to be the major cause of cell death. It was demonstrated by single channel recording (Sahl et al., 1987) that nisin induced formation of transient multi-state pores and the pores had diameters in the range of 0.2-1 nm and a life time of a few to several hundred milliseconds. Although these pores were small, they were important to the viability of the cells. According to the pore-formation theory, we could assume that the threshold nisin concentration for cells to be killed depends on the number

and size of the pores formed on the target membrane. Nisin resistant cells have a reduced amount of phospholipids which may render them less susceptible because there are less target sites for nisin and thus less and perhaps smaller pores would formed on their membrane compared to that of sensitive cells. Although there is no direct evidence for this assumption yet, electron microscopy studies showed that resistent cells were less damaged than sensitive cells under the same nisin treatment (Fig. 17).

Nisin binding ability of resistant and sensitive cells

In our preliminary experiment, we found that no signifficant difference between nisin residual activity of pre-heated cell suspensions (80°C for 5 min.) and cell suspensions without heating for both resistant and sensitive cells. therefore, enzymatic digestion was eliminated as a major reason for reduced nisin activity since most of the proteins denatured during the heating. The decrease in nisin activity when cells incubated with nisin should be mainly resulted from cell binding of nisin. Fig. 15 illustrates the nisin binding ability of resistant and sensitive cells. We observed that more residual nisin activity was present in the supernatants of resistant cell suspension than in that of sensitive cells.This suggested that the resistant cells may be bind a lesser amount of nisin compared to sensitive cells. This observation may provide another explanation for nisin resistance. Nisin binding to a cell surface is the first event for its eventual bactericidal action

(Sahl et al., 1991). The data from nisin's activity on artificial membranes suggested (Abee et al., 1991) that there were no specific cell surface components that serve as the receptor for nisin binding, and that the activity of nisin was affected by phospholipid composition of the artificial membranes. This may indicate that it is the bulk membrane component (phospholipids) that serve as the initial nisin binding site. In this study, we found smaller amounts of nisin binding to resistant cells than to sensitive cells, which may be correlated with the reduction in the phospholipid content of resistant cell membranes. In the studies of a newly characterized bacteriocin AS-48, it was suggested that (G'alvez et al., 1989) the receptor for the bacteriocin was more general than specific, and cell membrane absorbed more bacteriocin than cell wall. Moreover, they found that phospholipid (DPG) could absorb and neutralize the bacteriocin in vitro which suggested phospholipid could be the binding site of the bacteriocin. AS-48 is a peptide produced by *Enterococcus faecalis* S-48 and shares a similar size (8000) and iso-electric point (10.5) (G'alvez et al., 1989) with nisin. Their results provided supportive evidence for the theory of phospholipids as the binding sites for nisin as well as other amphiphilic bacteriocins. The interaction with anionic phospholipids may be an essential step in biological activity of cationic peptides with a amphiphilic structure.

SUMMARY AND CONCLUSIONS

Nisin resistance responses of *L. monocytogenes* Scott A were studied. Characterization of the nisin resistant mutant was conducted by comparison with its parent. The mutant was then examined for its fatty acid profile, phospholipid composition, phase transition temperature, nisin binding affinity, nisin killing kinetics, cell hydrophobicity, growth rate as well as cross-resistance to other antibiotics and inhibitors.

From the data presented in this thesis, we present the following conclusions:

1. Nisin resistance observed for *L. monocytogenes* Scott A was related to cell membrane structure and property changes. These changes occurred in fatty acid and phospholipid composition and content. The significant decrease in phospholipid contents was proposed to be the major contribution to the nisin resistance.

2. The resistant cells may had less nisin binding affinity which may relate to the decrease in phospholipid content. The cell surfaces of the mutant were less hydrophobic than that of the sensitive parent, which may also be related to its weaker affinity for nisin binding and thus greater nisin resistance.

3. The resistant cells had a higher phase transition temperature and significantly decreased specific growth rate at lower temperatures, both of which may directly relate to the higher percentage of straight chain fatty acids

and lower percentage of branched fatty acids of the cell membrane.

4. The resistant cells had a higher threshold and lower killing rate for nisin killing action in comparison with the parent.

5. Nisin was more effective at 37°C than at 4°C for both resistant and sensitive cells of *L.monocytogenes* Scott A.

6. Nisin stimulated cell autolysis which may enhance nisin's bactericidal action. Membrane damage is the first, cidal event while nisin induced cell lysis is likely a secondary, cooperative effect.

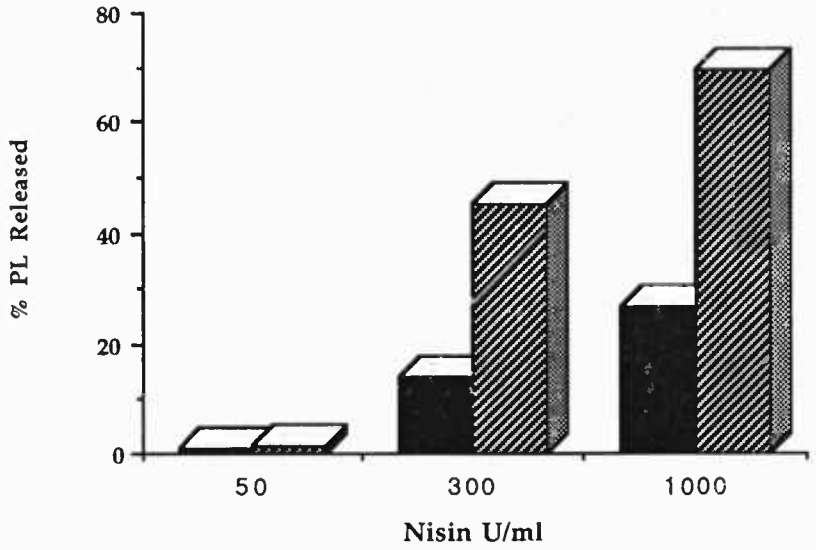

Fig. 13 Comparison of phospholipids released from *L. monocytogenes* Scott A resistant (▨) and sensitive (■) cells by nisin. Log phase cells were washed once and suspended in 0.85 % saline at about 10^{10} CFU/ml, different concentrations of nisin were added and incubated at 37°C for one hour. Phospholipids released from cells were analyzed after cells had been removed. The data is presented as percentages of equivalent amount of whole cell phospholipids.

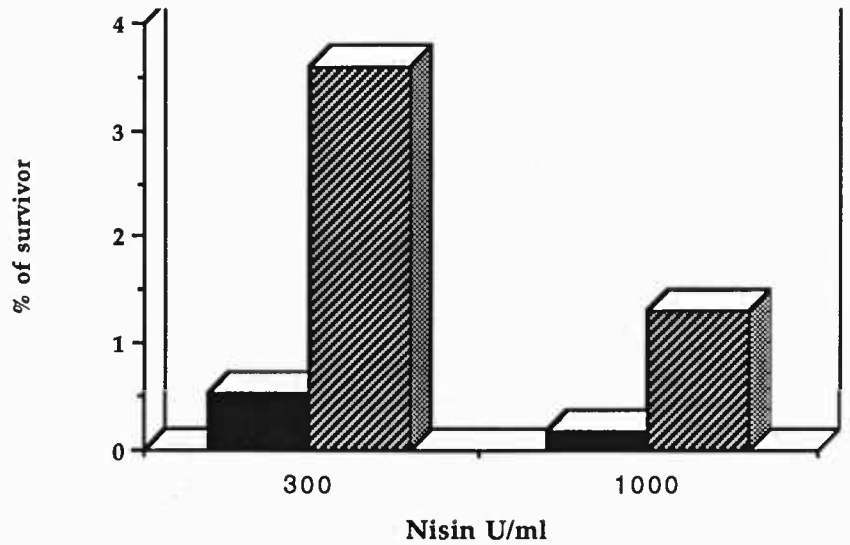

Fig. 14 The viability of *L. monocytogenes* Scott A resistant (▨) and sensitive (■) cells after nisin treatment (same treatment as in Fig. 13, samples were taken before cells been removed).

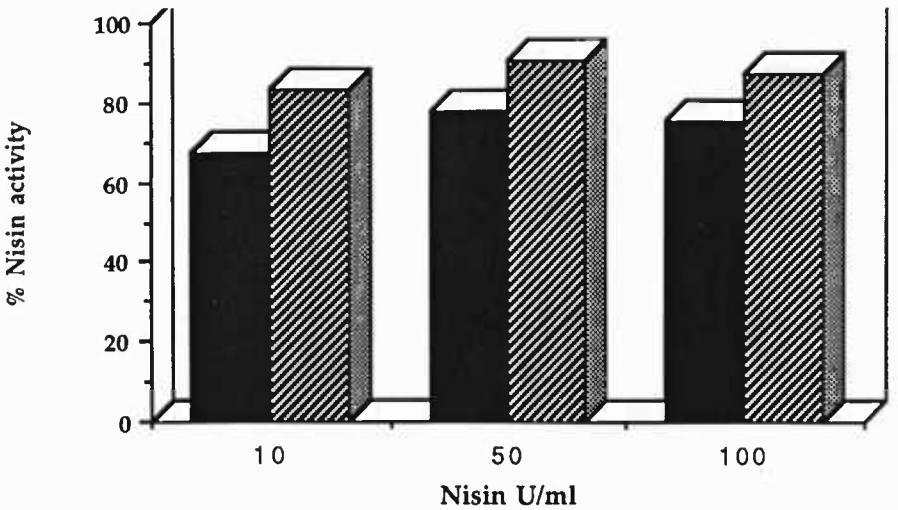

Fig. 15 The comparison of nisin binding ability of *L. monocytogenes* Scott A resistant (▨) and sensitive (■) cells. Log phase cells were washed once and suspended in 50mM phosphate buffer, pH 6.5, different concentrations of nisin were added and incubated at 37°C for one hour. Portions of 100 ul of supernatant were put into well plates seeded with indicator bacteria. Residue nisin activity was presented as percentage of control (nisin in same buffer without cells).

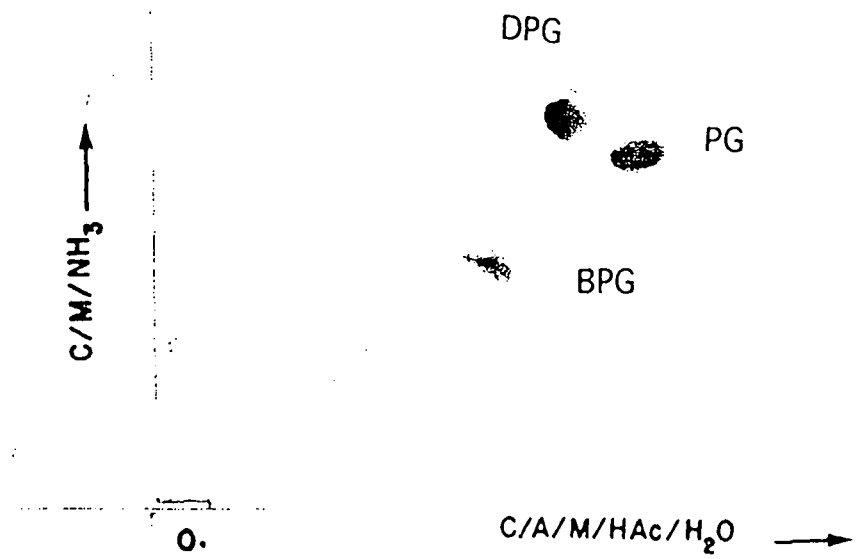

Fig. 16 Individual phospholipid components of *L. monocytogenes* Scott A were separated by two-dimensional thin-layer chromatography. The TLC was run on silica gel 60 plates using the solvent system chloroform-methanol-28% ammonia (65:35:5) in vertical direction, air-dried and then developed with chloroform-acetone-methanol-acetic acid-water (5: 2: 1: 1: 0.5) in the horizontal direction. The individual phospholipids were identified by comparing their mobilities on plates with those of standards obtained from Sigma chemical company (St. Louis).

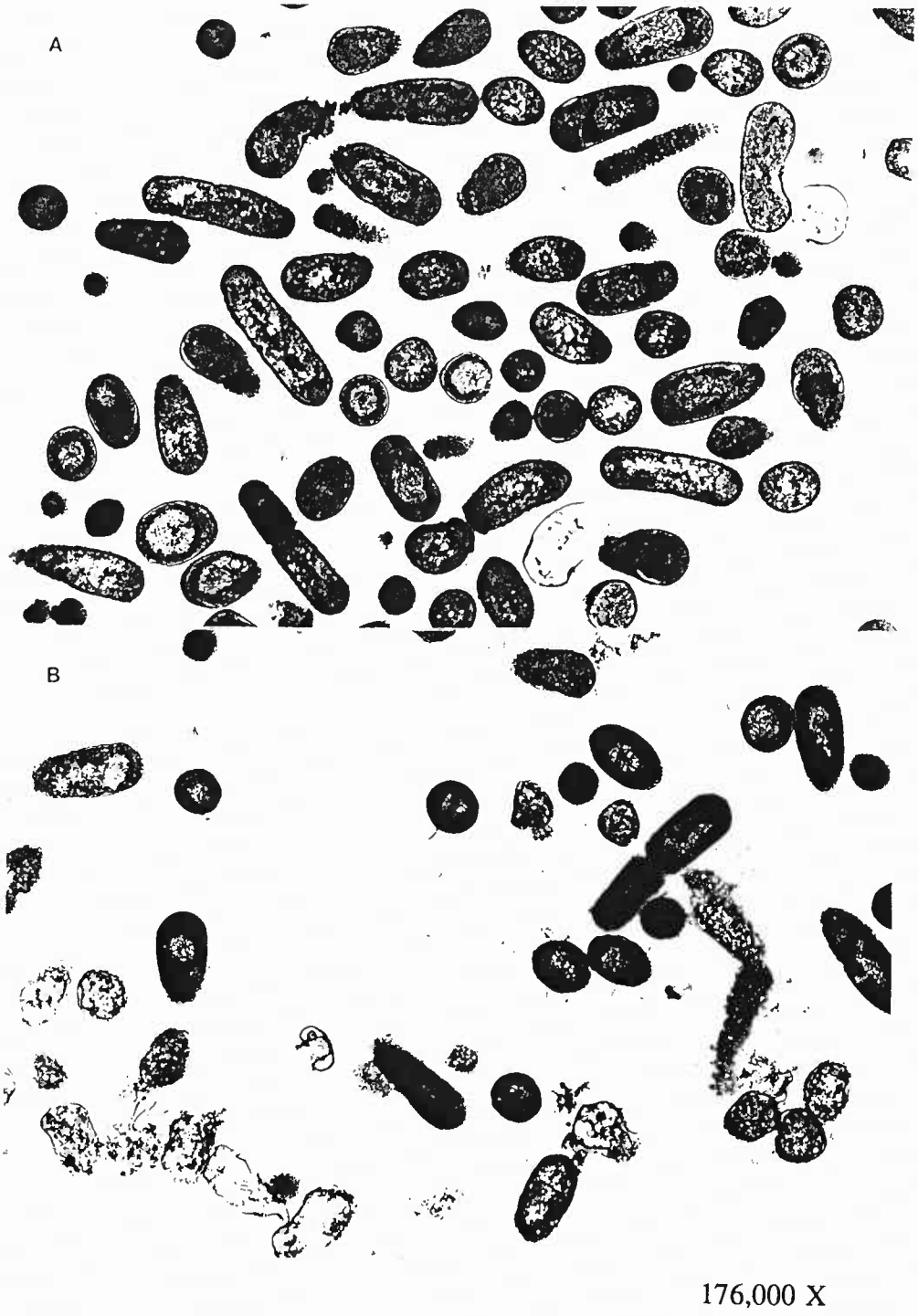

Fig. 17 Electron micrographs of *L. monocytogenes* Scott A resistant (A) and sensitive (B) cells exposed to 300 U/ml nisin for 2 hours.

Table 5. Comparsion of phospholipid (PL) contents of nisin resistant and sensitive strains of *L. monocytogenes* Scott A

			Individual PL*		
Strains	Total PL*	% of reduction	PG	DPG	BPG
Resistant	31.32±1.7	32	11.71±1.4	10.78±0.9	4.83±0.25
Sensitive	46.1±2.2	-	15.62± 2.0	18.43±1.3	5.65±0.4

* mg/g [dry weight] of cells. Values are means ± standard deviations of three different batches.
PG: Phosphatidylglycerol;
DPG: Diphosphatidylglycerol;
BPG: Bis-phospfatidylglyceryl phosphate.

BIBLIOGRAPHY

Abee T., F. H., Gao and W. N., Konings. 1991. The mechanism of action of the lantibiotic nisin in artifical membranes. *In* Nisin and Novel Lantibiotics. ESCOM Science Publishers B. V. The Netherlands. p. 373-385.

Alifax, R., and R. Chevalier. 1962. Study of the nisinase production by *Streptococcus thermophilus.* J. Dairy Res. 29: 233-240

Allgaier, H., G. Jung, R. G. Werner, U. Schneider, and H. Zahner. 1986. Epidermin: sequencing of a heterodetic tetracyclic 21-peptide amide antibiotic. Eur. J. Biochem. 160: 9-22

Andersson, R., M. A. Daesachel and H. M. Hassan. 1988. Antibacterial activity of plantaricin SIK-83, a bacteriocin produced by *Lcatobacillus plantarum.* Biochimie. 70: 381-390.

Bailey, W. R., and E. G. Scott. 1974. Determination of susceptibility of bacteria to antimicrobial agents. *In* Diagnostic Microbilogy. 4th ed. *edited by* W. R. Bailey and E. G. Scott. The C.V. Mosby Co., St. Louis, Mo. p. 313-317

Bartlett, G. R. 1959. Phosphorus assay in column chromatography. J. Biol. Chem. 234: 466-468.

Bell, R. G. and De Lacy, K. M. 1986. Factors influencing the determination of nisin in meat products. J. Food Technol. 21: 1-7.

Benkerroum, N., and W. E. Sandine. 1987. Inhibitory action of nisin against *Listeria monocytogenes.* J. Dairy Sci. 71: 3237-3245.

Bierbaum, C. and H. G., Sahl. 1985. Induction of autolysis of staphylococci by the basic peptide antibiotic Pep 5 and nisin and their influence on the activity of autolytic enzymes. Arch. Microbiol. 141: 249-254.

Bierbaum, C. and H. G., Sahl. 1987. Autolytic system of *Staphylococcus simulans* 22: Influence of cationic peptide on activity of N-acetylmuramoyl-L-amianine amidase. J. Bacteriology. 169: 5452-5458.

Blackburn, P., J. Polak, S. Gusik, and S. D. Rubino. 1989. Nisin compositions for the use as enhanced, broad range bacteriocins. International Patent Application No. WO89/12399. Applied Microbiology, Inc., New York.

Bligh, E. G. and W. J. Dyer. 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. 37: 911-917.

Brandis, H., and H. G. Sahl. 1984. Staphylococcin and other antibacterial substances produced by staphylococci, p. 173-186. *In* Staphylokokken und Staphylokokken-Erkrankungen. W. Meyer (ed), VEB Gustav Fischer Verlag, Jena, German Democratic Republic.

Britz, M.L., and R. G. Wilkinson. 1978. Chloramphenicol acetyltransferase of *Bacteriodes fragilis* . Antimicrob. Agents. Chemother. 14: 105-111.

Broda, P. 1979. Plasmids. W. H. Freeman & Co. London.

Bryan, L. E., and S. Kwan. 1981. Mechanisms of aminoglycoside resistance in clinical isolates of *Pseudomonas aeruinosa* of anaerobic bacteria and facultative bacteria grown anaerobically. J. Antimicrob. Chemother. 8 (Suppl D): 1-8

Bryan, L. E., T. Nicas, B. W. Holloway. 1982. Mutation of *Pseudomonas aeruinosa* specified reduced affinity for pencillin G. Antimicrob. Agents. Chemother. 21: 216-223.

Bryan L. E., Koji o' hara, and W. Sallene. 1984. Lipopolysacchaarid changes in impermeability-type aminoglycoside resistance in *Pseudomonas aeruinosa.* Antimicrob. Agents. Chemother. 26: 250-258.

Calderon, C., D. L. Collins-Thompson, and W. R. Usborne. 1985. Shelf life studies of vacuum packaged bacon treated with nisin. J. Food Protect. 48: 330-333.

Carlson, S., and H. M. Bauer. 1957. A study of problems associated with resistance to nisin. Arch. Hyg. Bakteriol. 141: 445-460.

Carminati, D., G. Giraffa, and M. G. Bossi. 1989. Bacteriocin-like inhibitors of *Streptococcus lactis* against *Listeria monocytogenes.* J. Food Prot. 52: 614-617.

Champlin, F. R., H. E. Gilleland, Jr., and R. S. Conrad. 1983. Conversion of phospholipids to free acids in response to acquisition of polymyxin resistance in *Pseudomonas aeruginosa.* Antimicrob. Agents Chemother. 24: 5-9.

Cheeseman, G. C. and N. J. Berridge. 1957. An improved method of preparing nisin. Biochem. J. 65: 603-608.

Cheeseman, G. C. and N. J. Berridge. 1959. Observations on the molecular weight and chemical composition of nisin A. Biochem. J. 71: 185-192.

Carriere, C. A. Allardet-Servent, Bourg, G. Audurier, A. and M. Ramuz. 1989. DNA polymorphism in strains of *Listeria monocytogenes*. J. of Clinical Microbiology. 29: 1351-1355.

Clejan, S. , A. Guffanti, M. A. Cohen, and T. A. Krulwich. 1989. Mutation of *Bacillus firmus* OF 4 to duramycin resistance results in substantial replacement of membrane lipid phosphatidylethanolamine by plasmalogen form. J. of Bacteriol. 17: 1744-1746.

Collins-Thompson, D. L., C. Calderon and W. R. Usborne. 1985. Nisin sensitivy of lactic acid bacteria isolated from cured and fermented meat products. J. of Food Prot. 48: 668-670.

Conrad, R. S. and H. E. Jr. Gilleland. 1981. Lipid alterations in cell envelopes of polymyxin-resistant mutant *Pseudomonas aeruginosa* isolates. J. Bacteriol. 148: 487-497.

Conrad, R. S. and C. Galano. 1989. Fatty acid alterations and polymyxin B binding by lipopolysaccharides from *Pseudomonas aeruginosa* adapted to polymyxin B resistance. Antimicrob. Agent Chemother. 33: 1724-1728.

Daeschel, M. A. 1990. Application of bacteriocins in food systems. *In* Biotechnology and Food Safety. Shain-Dow Kung (ed). Butterworths, London. p. 91-104

Daeschel, M. A. , D. S. Jung, and B. T. Watson. 1991. Controlling wine malolactic fermentation with nisin and nisin resistant *Leuconostoc oenos*. Appl. Environ. Microbiol. 57: 601-603.

Delves-Broughton, J. 1990. Nisin and its uses as a food preservative. Food Technology. 44: 100-112.

Denny, C. B., L. E. Sharpe. and C. W. Bohrer. 1961. Effects of tylosin and nisin on canned food spoilage bacteria. Appl. Microbiol. 9: 108-110.

Doyle, M. P. 1988. Effect of environmental and processing conditions on *Listeria monocytogenes*. Food Technology. 42:169-171

Drew, S. W. 1981. Liquid Culture. *In* Manual of Methods for General Bacteriology. P. Gerhardt (ed). American Socity for Microbiology. p.151-155.

Dupont Instruments. 1988. Differential scanning calorimeter 910 operators manual. Dupont Co. Wilmington, DE.

El Solh, N., J. Fouace, J. Pillet, and Y. A. Chabbert. 1981. Ann. Microbiol. 132B: 131-136.

Eyles, M. J. and K. C. Richardson. 1988. Thermophilic bacteria and food spoilage. CSIRO Food Res. Quart. 48: 9.

Farber, J. M. and P. J. Peterkin. 1991. *Listeria monocytogenes*, a food-borne pathogen. Microbiol. Rev. 55: 476-511.

Food and Drug Administration, 21 CFR part 184, Nisin preparation; Affirmation of GRAS status as a direct human food ingredient., Federal Register, 53 (66), 1988.

Foster T. J. 1983. Plasmid-determined resistance to antimicrobial drugs and toxic metal ions in bacteria. Microbiol Rev. 47: 361-409

Fowler, G. G. and B. McCann. 1971. The growing use of nisin in the dairy industry. Aust. J. Dairy Technol. 26: 44-46.

Franklin, T. J. and G. A. Snow. 1989. Biochemistry of Antimicrobial Action. 4th edition. Published by Chapman and Hall, New York. p.61-65.

Froseth, B. R., R. E. Herman, and L. L. McKay 1988. Cloning of nisin resistance determinant and replication origin on 7.6-kilobase EcoR fragment of pNP40 from *Streptococcus lactis* subsp. *diacetylactis* DRC3. Appl. Environ. Microbiol. 54: 2136-2139.

Froseth, B. R., and L. L. McKay. 1991. Molecular characterization of the nisin resistance region of *Lactococus lactis* subsp. *lactis* Biovar diacetylactis DR C3. Appli. Environ. Microbiol. 57:804-81.

Galesloot, T. E. 1956. Lactic acid bacteria which destroy the antibiotics (nisin). Neth. Milk and Dairy J. 10: 143-155.

Ga'lvez, A., M. Maqueda, M. Martinez-Bueno, and E. Valdivia. 1989 Bactericidal and bacteriolytic action of peptide antibiotic AS-48 against Gram-positive and Gram-negative bacteria and other organisms. Research in Microbiology 140: 57-68.

Ga'lvez, A E. Valdivia, M. Martinez-Bueno, and M. Maqueda. 1990. Induction of autolysis in *Enterococcus faecalis* S-47 by peptide AS-48. J. Applied Bacteriology, 69: 406-413.

Gilleland, H. E. JR. and R. D. E. Muffy. 1976. Ultrastructural study of polymyxin-resistant isolates of *Pseudomonas aeruginosa*. J. Bacteriol. 125: 267-281.

Gilleland, H. E. JR. 1977. Ultrastructural alterations of the outer membrane of *Pseudomonas aeruginosa* associated with resistance to polymycin B and EDTA. *In* Microbiology-1977. D. Schlessinger (ed). American Socieity for Microbiology, Washington, DC. p.145-150.

Gilleland, H. E. JR. and R. D. Lyle. 1979. Chemical alterations in cell envelopes of polymyxin-resistant mutant *Pseudomonas aeruginosa* isolates. J. Bacteriol. 138: 839-845.

Gilleland, H. E. JR, and R. S. Conrad. 1982a. Chemical alterations on cell envelopes of polymyxin-resistant mutant *Pseudomonas aeruginosa* grown in the absence or presence of polymyxin. Antimicrob. Agents Chemother. 22: 1012-1016.

Gilleland, H. E. JR, and L. Farley. 1982b. Adaptive resistance to polymyxin in *Pseudomonas aeruginosa* due to an outer membrane impermeability mechanism. Can. J. Microbiol. 28: 830-840.

Gross, E., and J. L. Morell. 1967. The presence of dehydroalanine in the antibiotic nisin and its relationship to activity. J. Am. Chem. Soc. 89:2791-2792.

Gross, E., and J. L. Morell. 1970. Nisin. The assignment of sulfide bridges of β-methyllanthionine to a novel bicyclic structure of identical ring size. J. Am. Chem. Soc. 92: 2919-2920.

Gross, E., and J. L. Morell. 1971. The structure of nisin. J. Am. Chem. Soc. 93: 4634-4635.

Gross, E., H. H. Kiltz. 1973. The number and nature of α,β–unsaturated amino acids in subtilin. Biochem. Biophy. Res. Commun. 50: 559-565.

Hancock, I. C. 1991. Microbial cell surface architecture. *In* Microbial Cell Surface Analysis, VCH Publishers, Inc. New York. p. 29-35.

Harris, L. J., M. A. Daeschel, M. E. Stiles, and T. R. Klaenhammer. 1989. Antimicrobial activity of lactic acid bacteria against *Listeria monocytogenes*. J. Food Prot. 52: 384-387.

Harris, L. J., H. P. Fleming, and T. R. Klaenhammer. 1991. Sensitivity and resistance of *Listeria monocytogenes* ATCC 19115, Scott A and UAL 500 to nisin. J. Food Prot. 54: 836-840.

Harris, L. J., H. P. Fleming, T. R. Klaenhammer. 1992. Novel paired starter culture system for sauerkraut, consisting of a nisin-resistant *Leuconostoc mesenteriodes* strain and a nisin-producing *Lactococcus lactis* strain. Appl. Environ. Micro. 58: 1484-1489.

Hawley, H. B. 1962. The uses of antibiotics in canning. *In* Antibiotics in Agriculture. M. Woodbine (ed). Butterworth, London. p. 272-288.

Heinemann, B., L. Voris, and C. R. Stumbo. 1965. Use of nisin in processing food products. Food Technol. 19: 592-596.

Heinemann, B. and R. Williams. 1966. Inactivation of nisin by pancreatin. J. Dairy Science 49: 312-314

Henning S., R. Metz and W. P. Hammes. 1986. Studies on the mode of action of nisin. Intern. J. Food Microbiol. 33: 121-134.

Hirsch, A. 1950. The assay of the antibiotic nisin. J. Gen. Microbiol. 4: 70-88.

Hirsch, A., E. Grinsted, H. R. Chapman, and A.T. R. Mattick. 1951. A note on the inhibition of an anaerobic sporeformer in swiss cheese by a nisin-producing *Streptococcus*. J. Dairy Res. 18: 198-204.

Hirsch, A., and Grinsted, E. 1954. Methods for the enumeration of anaerobic sporeformers from cheese with observations on the effect of nisin. J. Dairy Res. 21: 101-110.

Hitchins, A. D., Gould, G. W., and A. Hurst. 1963. The swelling of bacterial spores during germination and outgrowth. J. Gen. Microbiol. 30: 445-453.

Hotta, K., H. Yamaoto, Y. Okami, and H. Umezawa. 1981. Resistance mechanisms of kanamycin-, neomycin-and streptomycin-producing streptomycetes to aminoglycoside drugs. J. Antibiotic. 34: 1175-1182.

Howard, J. P., K. D. Harsono, and J. B. Luchansky. 1992. Differentiation of *Listeria monocytogenes, Listeria innocua, Listeria ivanovii,* and *Listeria seeligeri* by pulsed-field gel electrophoresis. Appl. Environ. Microbiol. 58: 709-712.

Hurst, A. 1981. Nisin. Adv. Appl. Microbiol. 27: 85-123.

Husrt, A. 1983. Nisin and other inhibitory substances from lactic acid bacteria. *In* "Antimicrobials in foods". A. L. Branen and P. M. Davidson (ed). Marcel Dekker, Inc., New York. P.327-351.

Ingraham, J. L., O. Maaloe, and F. C. Neihardt. 1983. Growth of the bacterial cell. Sinauer Assocs. Sunderland, MA.

James, A. M. 1991. Charge properties of microbial cell surfaces. *In* Microbial Cell Surface Analysis. VCH Publishers, Inc. New York. p. 245

Jarvis, B. 1967. Resistance to nisin and production of nisin-inactivating enzymes by several Bacillus species. J. Gen. Microbiol. 47: 33-48.

Jarvis, B., J. Jeffcoat, and G. C. Cheeseman. 1968. Molecular weight distributon of nisin. Biochim. Biophys. Acta. 168: 153-155.

Jarvis, B., and R. R. Mahoney. 1969. Inactivation of nisin by alpha-chymotrypsin. J. Dairy Sci. 52: 1448-1450.

Jarvis, B., and J. Farr. 1971. Partial purification, specificity and mechanism of action of the nisin-inactivating enzyme from *Bacillus cereus*. Biochim. Bioghys. Acta. 227: 232-240.

Jones, L. W. 1974. Effect of butterfat on inhibition of *Staphylococcus aureus* by nisin. Can. J. Microbiol. 20: 1257-1260.

Juneja, V. K. and P. M. Davidson. 1992. Influence of altered fatty acid composition on resistance of *Listeria monocytogenes* to antimicrobials. Instit. Food Tech. Annual meeting Abstracts. p.71.

Jung, D.S., F. W. Bodyfelt. and M. A. Daeschel. 1992. Influence of fat and emulsifiers on the efficacy of nisin in inhibiting *Listeria monocytogenes* in fluid milk. J. Dariy Sci. 75: 387-393.

Kaneda, T. 1991. Iso- and anteiso-fatty acids in bacteria: Biosynthesis, function and taxonomic significance. Microbiol Rev. 55: 288-302.

Katsu, T., M. Kuroko, T. Morikawa, K. Sanchika, Y. Fujita, H. Yamamura, and M. Uda. 1989. Mechanism of membrane damage induced by the amphipathic peptides gramicidin S and melittin. Biochim. Biophys. Acta. 983: 135-141.

Klaenhammer, T. R. 1984. A general method for plasmid isolation in lactobacilli. Curr. Microbiol. 10: 23-28.

Klaenhammer, T. R., and R. B. Sanozky. 1985. Conjugal transfer from *Streptococcus lactis* ME2 of plasmids encoding phage resistance, nisin resistance and lactose-fermenting ability: evidence for a high-frequency conjugative plasmid responsible for abortive infection of virulent bacteriophage. J. Gen. Microbiol.131: 1531-1541.

Kooy, J. S. 1951. Strains of *Lactobacillus plantarum* which destroy the antibiotic made by *Streptococcus lactis*. Neth. Milk Dairy J. 6: 223-330.

Kordel, M ., F. Schuller, and H. G. Sahl. 1989. Interaction of the pore forming-peptide antibiotics Pep 5, nisin, and subtilin with non-energized liposomes. FEBS Letters. 244: 99-102.

Kosaric, N., and K. K. Carroll. 1971. Phospholipids of *Listeria monocytogenes* . Biochem. Biophys. Acta. 239: 428-442.

Krulwich, T. A., S. Clejan, L. H. Falk, and A. A. Guffanti. 1987. Incorporation of specific exogenous fatty acids into membrane lipids modulates protonophore resistance in *Bacillus subtilis*. J. Bacteriology. 169: 4479-4485.

Le Bourgeois, P., M. Mata, and P. Ritzenthaler. 1989. Genome comparison of *Lactococcus* strains by pulsed-field gel electrophorsis. FEMS Microbiol. Lett. 59: 65-68.

Lee-Wickner L.-J. , and B. M. Chassy. 1984. Production and regeneration of *Lactobacillius casei* protoplasts. Appl. Environ. Microbiol. 48: 994-1000.

Leistner, L. 1978. Microbiology of ready-to-serve foods. Fleischwirtschaft. 58: 2008-2111.

Levy, S. B. 1981. The tetracyclines: microbial sensitivity and resistance, *In* New Trends in Antibiotics: Research and Therapy. G. G. Grassi and L. D. Sabath (ed). Elsevier/North-Holland, Amsterdam. P. 27-44.

Lewis, J. C. , H. D. Michener, C. R. Stumbo, and D. S. Titus. 1954. Antibiotics in food processing: Additives accelerating death of spores by moist heat. J. Agric. Food Chem. 2: 298-302.

Linnett, P. E. and J. L. Strominger. 1973. Additional antibiotic inhibitiors of peptidoglycans. Antimicrob. Agents Chemother. 4: 231-236.

Lipinska, E. 1977. Nisin and its applications. In Antibiotics and Antibiosis in Agriculture, M. Woodbine (ed). Butterworth. London, p. 103-130.

Liu, W. and J. N. Hansen, 1990. Some chemical and physical properties of nisin, a small-protein antibiotic produced by *Lactococcus lactis* . Appl. Environ. Microbiol. 56: 2551-2558.

Lortal S., M. Rousseau, P. Boyaval and J. V. Heijenoort. 1991. Cell wall and autolysis system of *Lactobacillus helveticus* ATCC 12046. J. General Microbiology. 137: 549-559.

MacGowan A. P., D. S. Reeves, and J. Mclaughlin. 1990. Antibiotic resistance of *Listeria monocytogenes* . Lancet. 336: 513-514.

Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, N. Y. p. 156.

Marinetti, G. V. 1962. Chromatographic separation, identification and analysis of phospholipids. J. Lipid Res. 3: 1-6.

McClatchy, J. K. 1980. Antituberculosis drugs: mechanisms of action, drug resistance, susceptobility testing, and assays of activity in biology fluid, p. 135-169. *In* V. Lorian (ed.), Antibiotica in labortory medicine. The William & Wilkins Co., Baltimore.

McClelland, M., R. Jones, Y. Patel, and M. Nelson. 1987. Restriction endonucleases for pulsed field mapping of bacterial genomes. Nucleic Acids Res. 15: 5985-6005.

McClintock, M., L. Serres, J. Marzolf, J. A. Hirsch, and G. Mocquot. 1952. Action inhibitrice des streptocoques producteurs de nisine sur le developpment des sporules anaerobies dans le fromage de gruyere fondu.. J. Dairy Res. 19: 187-193.

McDowel, E. M. and B. F. Trump. 1976. Histologic fixatives suitable for diagnostic light and electromicroscopy. Arch. Path. Lab. Med. 100: 405-414.

McElhaney, R. N. 1985. The effect of membrane lipids on permeability and transport in prokatyotes, *In* G. Structure and Properties of Cell Membeanes, Benga (ed). Boca Raton, Fla. p. 20-51,

McKay, L. L. and K. A. Baldwin. 1984. Conjugative 40-Megadalton plasmid in *Streptococcus latic* subsp. *diacetylactis* DRC3 is associated with resistance to nisin and bacteriophage. Appl. Environ. Microbiol. 47: 68-74.

Miller, K. J. 1985. Effect of temperature and sodium chloride concentration on phospholipid and fatty acid compositions of a halotolerant *Planococcus* sp. J. of Bacteriology. 162: 263-270.

Monticello, D. J. and D. O'Connor. 1990. Lysis of *Listeria monocytogenes* by nisin. *In* Food Listeriosis. Miller, A. J. et al. (eds) Elsevier,Amsterdam. p.81-83.

Moore, R. A , L. Chan, and R. E. W. Hancock. 1984. Evidence for two distinct mechanisms of resistance to polymyxin B in *Pseudomonas aeruginosa.* Antimicrob. Agents Chemother. 26: 539-545.

Morris K. 1972. Techniques of Lipidology. Elsevier. New York. p. 351-352.

Morris, S. L., R. C. Walsh, and J. N. Hansen. 1984. Identification and characterization of some bacterial membrane sulhydryl groups which are targets of bacteriostatic and antibiotic action. J. Biol. Chem. 259: 13590-13594.

Nicas,T. I., and R. E. W. Hancock. 1980. Outer membrane protein H1 of *Pseudomonas aeruinosa*: Involvmenent in adaptive and mutational resistance to ethylenediaminetetraacetate, polymyxin B, and gentamicin. J. Bacteriol. 143: 872-878.

O'Brien, R. T. , D. S. Titus, , K. A. Devlin, C. R. Stumbo, andJ. C. Lewis. 1956. Antibiotics in food preservation. II. Studies on the influence of subtilin and nisin on the thermal resistance of food spoilage bacteria. Food Technology. 10: 352-355.

Ogden, K., and R. S. Tubb. 1985. Inhibition of beer spoilage lactic acid bacteria by nisin. J. Inst. Brew. 91:390-392.

Ogden, K. 1986. Nisin: A bacteriocin with a potential use in brewing. J. Inst. Brew. 92:379-383.

Ogden, K., M. J. Waites and J. R. M Hammond. 1988. Nisin and Brewing. J. Inst. Brew. 94: 23-28.

O'Leary, W. M. and S. G. Wilkinson. 1989. Gram-positive Bacteria, *In* Microbial Lipids, Ratledge C. and S. G. Wilkinson (ed.), Vol 1 Academic press. p.179,

Pattishall, K.H., J. Acar, J. J. Burchall, F. W. Goldstein, and R. J. Harvey. 1977. Two distinct types of trimethoprim-resistant dihydrofolate reductase specified by R-plasmids of different compatibility groups. J. Biol. Chem. 252: 2319-2323.

Radler, F. 1990. Possible use of nisin in winemaking. 1. Action of nisin against lactic acid bacteria and wine yeasts in solid and liquid media. Am. J. Enol. Vitic. 41:1-6

Ramseier, H. R. 1960. The action of nisin on *Clostridiun butyricum*. Arch. Mikrobiol. 37: 57-94.

Rayman, M. K., B. Aris, and A. Hurst. 1981. Nisin: A possible alternation or adjunct to nitrite in the preservation of meats. Appl. Env. Microbiol. 41: 375-380.

Reisinger, P. H. Seidel, H. Tschesche and W. P. Hammes. 1980. The effect of nisin on murein synthesis. Arch. Microbiol. 127: 187-193.

Rosenberg, M. 1984. Bacterial adherence to hydrocarbons : A useful technique for studying cell surface hydrophobicity. FEMS Microbiology Letters. 22: 289-295.

Ruhr, E., and H.-G. Sahl. 1985. Mode of action of the peptide antibiotic nisin and influence on the membrane potential of whole cells and on cytoplasmic membrane vesicles. Antimicrobiol. Agents Chemother. 27: 841-845.

Russell, N. 1989. Functions of lipids: Structure Roles and Membrane Functions, *In* Microbial Lipids, Ratledge, C. and S. G. Wilkinson (eds) Vol 2, Academic Press. San Diego. p. 286-287.

Ryser, E. T. , and E. H. Marth. 1991. Listeria, Listeriosis, and Food Safty. Marcel Dekker, Inc., New York. p. 289-331.

Sahl, H. -G., M. Grossgarten, W. R. Widger, W. A. Cramer, And H. Brandis. 1985. Structure similarities of the Staphylococcin-like peptide Pep-5 to the peptide nisin. Antimicrobiol. Agent Chemother. 27: 836-840.

Sahl, H. -G., M. Kordel, and R. Benz. 1987. Voltage-dependent depolarization of bacterial membranes and artificial lipid bilayers by the peptide antibiotic nisin. Arch Microbiol. 149: 120-124.

Sahl, H. -G. 1991. Pore formation in bacterial membranes by cationic lantibiotics. *In* Nisin and Novel Lantibiotics. ESCOM Science Publishers B. V. The Netherlands. p. 347-358.

Sareen, M. and G. K. Khuller. 1990. Cell wall and membrane changes associated with ethambutol resistance in Mycobacterium tuberculosis H37 Ra. Antimicrob. Agents Chemother. 34: 1773-1776.

Schnell, N., K. D. Entian, U. Schneider, F. Gotz, H. Zahner, R. Kellner and G. Jung. 1988. Nature. 333: 276-278.

Scott, V. N., and S. L. Taylor. 1981. Effect of nisin on the outgrowth of *Clostridium botulinum*. J. Food Sci. 46: 117-120.

Seeliger, H. P. R. and D. Joues. 1986. Genus Listeria, *In* Bergey's Manual of Systematic Bacteriolog. P. H. A. Sneath, N. S. Mair, M. E. Sharpe, and J. G. Holt (ed), vol. 2. The William & Wilkins Co., Baltimore. p. 1235-1245.

Shannon, K., and I. Philips. 1982. Mechanisms of resistance to aminoglycosides in clinical isolates. J. Antimicrob. Chemother. 9: 91-102.

Shapiro, J. A., and P. Sporn. 1977. Tn *402* : A new transposable element determining trimethoprim resistance that insert in bacteriophage lambda. J. Bacteriol. 129: 1632-1635.

Smith, C. L. and C. R. Cantor. 1989. Pufication, specific fragmentation, and separation of large DNA molecules. Methods Enzymol. 155: 449-465.

Somers, E.B. and S. L. Taylor. 1987. Antibotulinal effectiveness of nisin in pasteurised processed cheese spreads. J. Food Protect. 50: 842-848.

Stevens, K. A. , B. W. Sheldon, N. A. Klapes, and T. R. Klaenhammer. 1992. Effect of treatment conditions on nisin inactivation of Gram-negative bacteria. J. Food Prot. 55: 763-766.

Svedberg, G., and O. Skold. 1980. Characterization of different plasmid-borne dihydropteroate synthases mediating bacterial resistance to sulpfonamides. J. Bacteriol. 142: 1-7.

Tadayon, R. A. and K. K. Carroll. 1971. Effect of growth conditions on fatty acid composition of *Listeria Monocytogenes* and comparison with the fatty acids of *Erysipelothrix* and *Corynebacterium* Lipids. 6: 820-825.

Tanskanen E. I., L. D. Tulloch, A. J. Hiller, and B. E. Davidson. 1990. Pulsed-field gel electrophoresis of *Sma* I digests of Lactococcal genomic DNA, a novel methol of strain identificaion. Appl. Environ. Microbiol. 56: 3105-3111.

Tramer, J., 1964. The inhibitory action of nisin on *B. stearothermopholus*, *In* Microbial Inhibitors in Foods, N. Molin. (ed) Almqvist and Wiksell, Stockholm, p. 25.

Vaczi, L. 1973. Correlation between the lipid composion and the resistance of bacteria, p. 39-52. *In* The Biology Role of Bacterial Lipids. Akademiai Kiado, Budapest.

Wang, D. Q. and E. Kolbe, 1991. Thermal properties of surimi analysis using DSC. J. Food Science. 56: 302-308.

WHO. 1969. Specification for the identity and purity of food addictives and their toxicologcial evolution: some antibiotics. 12th Report of the Joint FAO/WHO Expert Committee on Food Adictiveas. WHO Tech. Rept. Series No. 430. World Health Org., Geneva, Switzerland.

Wiegel & Dykstra M. 1984. Appl. Microbiol. Biotech. 20: 59-65.