RESISTANCE OF LISTERIA MONOCYTOGENES TO THE BACTERIOCIN NISIN

by

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SUMMARY

Resistance of *Listeria monocytogenes* to the bacteriocin nisin was investigated. *Listeria monocytogenes* NCTC 5105 and F6861 were evaluated for sensitivity to nisin. The results confirmed those previously published indicating marked differences in the sensitivity of the two strains. Mutants with increased resistance to nisin could be isolated from the less sensitive F6861 strain at a frequency of $10^{-6}$ to $10^{-7}$. The MIC of the mutant strain in broth at pH 5.5, after incubation at 30°C for 47 days, was $> 1000$ IU/ml as compared with 300 IU/ml for its parent strain. The nisin resistance phenotype of the F6861 mutant strain was completely stable after undergoing ten passages of growth in nisin-free media. The growth characteristics of *L. monocytogenes* F6861 wild type and its nisin-resistant mutant were similar, but the doubling time of the mutant was slightly longer, particularly under sub-optimal conditions such as acid pH (5.5) and low temperature (4°C). There was no obvious difference in the basic morphology of each strain.

Adsorption of nisin to the different *L. monocytogenes* strains was studied by measuring the residual nisin in solution. This was done routinely by means of a nisin-specific ELISA and compared with results obtained from a nisin bioassay. At a range of nisin concentrations, the amount adsorbed was found to reflect the sensitivity of the strain to nisin, with the more sensitive cells showing greater adsorption. These results eliminated the possibility of a nisinase enzyme reported in some resistant bacteria, but indicated that the mechanism of resistance is based on exclusion and inhibition of nisin binding to the cells. Immunogold labelling and transmission electron microscopy did not visibly show the incorporation of nisin into the cell membrane, probably because, if bound, the nisin epitopes were unable to form an antigen-antibody complex.

The initial rate of $K^+$ efflux induced by different nisin concentrations was measured in all three *L. monocytogenes* strains. In each strain, the rate of $K^+$ efflux increased with increasing nisin concentration until it reached a maximum rate. As the nisin resistance of each strain increased, $K^+$ efflux increased at a slower rate and had a
lower maximum rate. The saturation kinetics of $K^+$ efflux indicates that the mechanism of resistance involves a reduction in the accessibility or presence of suitable attachment sites, preventing the incorporation of nisin into the membrane. This resistance acquisition could result from adaption of either the cytoplasmic membrane or cell wall or both.

Nisin inactivation of protoplast cells showed that the cell wall of *L. monocytogenes* F6861 plays an active role in the acquired nisin resistance of its mutant. Without its cell wall, the increased nisin resistance of the mutant was lost, with a phenotypic reversion back to the wild type. In contrast, the resistance of the wild type remained unchanged. Further evidence for cell wall involvement was indicated by the fact that cell surface hydrophobicity was shown to correlate with nisin sensitivity, the wild type strain being more hydrophobic than its mutant. The possible role of surface-layer (S-layer) proteins in the acquisition of nisin resistance by *L. monocytogenes* was eliminated due to the fact that several procedures (freeze-etching, atomic force microscopy, and S-layer extraction/SDS-PAGE) indicated that S-layers did not exist on any of the *L. monocytogenes* strains studied, when compared with a *Lactobacillus* positive control. In addition, chloramphenicol did not adversely affect the frequency of isolation of nisin-resistant mutants, indicating that *de novo* protein synthesis is not involved.

Subsequently, the involvement of other cell surface components, namely teichoic and lipoteichoic acids, was investigated. Removal of these components and subsequent inactivation of the cells with nisin, reduced the resistance of both strains when compared with their respective whole cells. The resistance of the mutant strain lacking these components, however, was still significantly greater than whole cells of its wild type indicating the involvement of an extra factor in acquired nisin resistance. Resistance of the wild type lacking these components was significantly lowered when compared with its respective whole cells, and the fact that the resistance of wild type protoplast cells remained the same as whole cells suggests the possible involvement of lipoteichoic acids in intrinsic nisin resistance, conditional on
their partial retention in protoplast formation.

The role of the cytoplasmic membrane in the acquired nisin resistance of *L. monocytogenes* F6861 was also investigated. From the results obtained, qualitative and quantitative fatty acid analysis of *L. monocytogenes* F6861 wild type and its nisin-resistant mutant showed no obvious difference in content between strains. Any slight differences in quantity observed could not be verified from only one analysis. Modification of the lipid composition of *L. monocytogenes* was conducted by exogenous application of fatty acids (C14:0 or anteiso-C15:0) during growth, in order to determine the resistance response to nisin. Results from nisin inactivation and MIC determinations of these modified strains indicated that fatty acid C14:0 generally increased nisin resistance of both *L. monocytogenes* F6861 wild type and its nisin-resistant mutant, although MIC determinations of the wild type were similar to the control. Fatty acid anteiso-C15:0 decreased the nisin resistance of the wild type during both experiments, but increased the nisin resistance of the mutant during nisin inactivation only.

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DEDICATED TO MY PARENTS, MAIR AND LESLIE DAVIES
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CHAPTER 1  *LISTERIA MONOCYTOGENES*: A FOODBORNE PATHOGEN
1 LISTERIA MONOCYTOGENES: A FOODBORNE PATHOGEN

1.1 INTRODUCTION

Listeriosis is a bacterial infection from which both humans and animals are susceptible. In more unfortunate cases, it can result in death. Listeriosis is caused by only some species of the genus Listeria, in particular Listeria monocytogenes. Documented cases of listeriosis were extremely uncommon more than 50 years ago. Until 1945, fewer than forty cases of human listeric infection were recorded in the medical literature (Kaplan, 1945).

The first major recorded outbreak of human listeriosis occurred in East Germany between 1949 and 1957, and resulted in a dramatic increase in the number of stillborn infants. However, the source of infection was unknown (Ryser and Marth, 1991). This prompted an awareness of listeriosis, but it was not until the 1980s that "Listeria hysteria" dominated the headlines as a result of several major outbreaks of foodborne listeriosis. The first outbreak occurred in 1981 in Canada with 41 documented cases of which 17 were fatal. Infection involved the consumption of contaminated coleslaw from which L. monocytogenes was later isolated (Schlech et al., 1983). In this outbreak, cabbage had become contaminated with the organism from infected sheep manure, which was used as fertilizer in the soil in which the cabbages were grown. This provided the first conclusive evidence that humans can contract listeriosis by consuming contaminated food. It also demonstrated that foods other than dairy products, which had already been suggested as a probable vehicle of infection, can become contaminated with L. monocytogenes (Ryser and Marth, 1991).

Subsequently in 1985, consumption of contaminated Jalisco brand Mexican-style cheese manufactured in California was directly linked to more than 142 cases of listeriosis, including 48 deaths (Linnan et al., 1988). In Switzerland (1983-1987), another epidemic, which affected 122 people resulting in 31 deaths, was linked to the consumption of contaminated Vacherin Mont d’Or soft ripened cheese (Bille, 1990).
Other cases involved: a possible milkborne outbreak in Massachusetts, USA (Fleming et al., 1985); pâté in the UK (McLauchlin et al., 1991; Gilbert et al., 1993) and pork tongue in France (Halligan, 1993).

With greater awareness of this organism as a foodborne pathogen in the 1980s, *L. monocytogenes* was subsequently discovered in a variety of cheeses, ice cream and other dairy products. Thus, numerous product recalls led to staggering financial losses for the industry, including several lawsuits. In addition to dairy products, *L. monocytogenes* was isolated from raw and ready-to-eat meat, poultry, seafood and vegetables (Ryser and Marth, 1991).

Even though the incidence of listeriosis appears to be on the increase worldwide, it is uncertain whether this reflects a true increase in numbers or is due to better diagnosis and/or increased awareness of the disease. However, there is no doubt that the susceptible population is increasing, as are the numbers and types of foods in which *L. monocytogenes* is able to survive and grow (Farber and Peterkin, 1991). It was estimated in 1986 that 1600 cases of listeriosis occur annually in the USA with an annual incidence of ca. 6.7 cases/10^6 population. Western European data collected since 1987 indicate an incidence rate of ca. 3.6-8.0 listeriosis cases/10^6 population in all countries except France with the latter rate being two to four times higher. Thus, excluding France, the annual incidences of listeriosis in most European countries are generally similar to rates of illness observed in the USA and probably Canada (Ryser and Marth, 1991).

Laboratory reports of listeriosis in England, Wales and Northern Ireland (1985-90) indicated a near doubling in incidence between 1985 and mid-1989 followed by a sharp decline. This dramatic increase in the number of listeriosis cases was attributed to the consumption of contaminated pâté, as the subsequent decline coincided with government health warnings on pâté consumption and the suspension of a certain manufacturer (McLauchlin et al., 1991). In one study, an improvement in the microbiological quality of some foods, with respect to *Listeria* contamination, was
observed during the late 1980s (Anon., 1991). This was possibly due to changes in
UK legislation and implementation of improved hygiene standards by the food
industry.

1.2 THE ORGANISM LISTERIA MONOCYTOGENES

Previously, the genus *Listeria* contained eight different species namely, *L.
monocytogenes*, *L. ivanovii*, *L. innocua*, *L. welshimeri*, *L. seeligeri*, *L. denitrificans*,
*L. murrayi*, and *L. grayi* (Seeliger and Jones, 1986). Subsequent evidence suggested
that *L. denitrificans* was not a member of the genus *Listeria* and Rocourt et al. (1987)
proposed that the organism be transferred to a new genus *Jonesia* as *Jonesia
denitrificans*. This has now been formally approved (Anon., 1994). Human infection
has only been caused by *L. monocytogenes*, *L. ivanovii* and *L. seeligeri*, but incidences
involving the latter two organisms are extremely rare with only a few documented
cases (Busch, 1971; Rocourt and Seeliger, 1985; Rocourt et al., 1986). Thus, *L.
monocytogenes* is currently responsible for virtually all cases of human listeriosis.

The first accurate description of the causative agent of listeriosis, *L. monocytogenes*,
has been attributed to Murray et al. (1926), who originally named it *Bacterium
monocytogenes*, due to a typical monocytosis observed in diseased animals.
Morphologically, *L. monocytogenes* is a Gram-positive, non-spore-forming, non-acid
fast, pleomorphic rod-shaped bacterium with rounded ends (Seeliger and Jones,
1986). It does not form capsules but is motile via peritrichous flagella and exhibits
a characteristic tumbling motion. The microscopic appearance of the cells is
dependent on their age. Fresh isolates appear as short diptheroid-like rods measuring
1-2 µm x 0.5 µm and are in the smooth, pathogenic form. The organism sometimes
exhibits palisade formation, along with some V and Y forms. Older, avirulent rough
strains, which develop after a 3-5 day incubation, produce much longer rods,
typically measuring 6-20 µm. In contrast, very young cells of *Listeria* may appear to
be coci or diplococci (Ryser and Marth, 1991).
Listeria monocytogenes is a facultatively anaerobic, mesophilic organism. Its optimal growth temperature is 30°-37°C, although growth does occur between 1° and 45°C. The fact that L. monocytogenes is a psychrotrophic organism, and has the ability to grow at low temperatures, albeit with a slower growth rate, has particular relevance for the food industry. Once the organism has been isolated, it grows readily on common bacteriological media such as tryptose agar. Typical colonies, after a 24-hour incubation at 30°-37°C, are round, translucent, slightly raised with a finely textured surface, watery in consistency, bluish-grey under normal illumination, and have an entire margin. The colonies vary in diameter between 0.3 and 1.5 mm, but after further incubation (5-10 days), may reach 3-5 mm in diameter. The older, rough form is characterized by a coarsely textured surface, undulating border, and striations radiating toward the periphery of the colony (Ryser and Marth, 1991).

Biochemically, L. monocytogenes possesses the following characteristics: catalase-positive, oxidase-negative, urease-negative, indole-negative, methyl red-positive and Voges-Proskauer (VP)-positive (Lovett, 1990). Its fermentation of glucose produces acid but no gas, and the fact that it does not hydrolyze gelatin, casein, or milk is of particular importance to food microbiologists. The CAMP test is particularly useful for differentiating species of Listeria and is based on their pattern of β-haemolysis (if any) on sheep blood agar in the presence of β-haemolytic Staphylococcus aureus and Rhodococcus equi (Rocourt et al., 1983).

For detection and isolation of L. monocytogenes from food and other clinical and environmental samples, direct plating, cold enrichment, warm enrichment and several rapid methods (e.g., DNA hybridization and ELISA techniques) can be used in various combinations (Ryser and Marth, 1991).

Four basic serological types of L. monocytogenes based on somatic (O) and flagella (H) antigens have been described (Paterson, 1939; 1940). Serotypes 1, 3 and 4 were differentiated on the basis of O antigens, whereas identification of serotype 2 was based on a unique H antigen. These four serotypes have subsequently been divided
into a total of 13 subgroups. As an identification technique for use in listeriosis outbreaks and epidemiological studies, serotyping alone is of limited value. This is because cases of human listeriosis are caused mainly by only three serotypes: 4b, 1/2a and 1/2b. More useful techniques are phage typing, DNA restriction analysis and electrophoretic enzyme-typing (Ryser and Marth, 1991).

*Listeria monocytogenes* is classified as an enteroinvasive pathogen, although the mechanism of pathogenicity by which the organism causes affliction is by no means clear cut. It has been suggested that the primary site of entry for *L. monocytogenes* is the intestinal tract (Gaillard *et al.*, 1987), with replication of the organism occurring within the host tissue. The reason why carriers of *Listeria* (approximately 5% of the population) do not develop listeriosis, has been attributed to the fact that numerous subclinical infections that are thought to occur may lead to a state of immunity in large segments of the population (Seeliger, 1988).

*Listeria monocytogenes* possesses a variety of virulence factors that damage the host’s tissue, thus allowing the organism to invade the bloodstream and produce illness. These include one or more haemolysins, a monocyte-promoting agent, various cell wall/cell membrane constituents, toxic oxygen species, and several undefined toxins. Once in the bloodstream, the pathogen gains access to target organs (i.e., placenta and central nervous system, including the brain), causing abortion or symptoms in the host that are characteristic of meningioencephalitis (Ryser and Marth, 1991).

### 1.3 HUMAN LISTERIOSIS

Compared with other notifiable illnesses, human listeriosis is a rare disease. It is of minimal concern to healthy individuals, and even though between 1 and 9% of the population carries *L. monocytogenes* in its faeces, most of these individuals remain asymptomatic (Ryser and Marth, 1991). However, consequences of a listeric infection can be devastating. The overall mortality rate amongst cases of listeriosis is 25-50%.
Certain segments of the population, including the elderly, neonates, pregnant women and the foetus, and immunosuppressed or immunocompromised adults, are more susceptible to life-threatening illness. Currently, listeriosis most typically takes the form of meningitis or septicaemia. In pregnant women, a flu-like illness generally occurs, which leads to infection of the foetus resulting in miscarriage, stillbirth or birth of a severely ill or permanently damaged infant. At present, 25% of listeriosis cases in England and Wales are pregnancy associated. Only a small proportion (ca. 15% in England and Wales) of listeriosis cases occur amongst people with no known risk factors. Two surveys made in the USA indicate that most cases occur in the very young and the very old (Albritton, 1984). It is thought that infected infants have an immature defense system and lack both a specific antibody response and a cell-mediated immune response to *L. monocytogenes* (Issekutz *et al.*, 1984).

The incubation period between infection and the onset of clinical symptoms of listeriosis can vary from a few hours to 90 days, although it is not understood why. The infective dose for listeriosis is not clear, although food recovered from a small number of patients who developed listeriosis has been generally relatively heavily (>10³/g) contaminated (Farber and Peterkin, 1991).

Listeriosis is often confused with other illnesses as the clinical characteristics of listeric infection can vary widely. However, human listeriosis is generally characterized by pus-forming, miliary granulomas (or more specifically listeriomas) and focal necroses. Based on the most prominent clinical symptoms, Seeliger and Finger (1976) identified nine manifestations of listeric infection: listeriosis during pregnancy; listeriosis of the newborn (granulomatosis infantisepctica); meningitis, meningioencephalitis and encephalitis; cutaneous form; septicemia with pharyngitis and mononucleosis; oculoglandular form; cervicoglandular form; granulomatosis septica and typhoid-pneumonic form; and other forms (e.g., focal infections can result in arthritis, osteomyelitis, spinal or brain abscesses, peritonitis and cholecystitis). However, cases of human listeriosis cannot always be classified into one of these nine categories since combinations of two or more manifestations may occur.
simultaneously or in succession (Ryser and Marth, 1991).

With regards to treatment of listeric infection, early antibiotic therapy is usually required to prevent permanent disabilities and possible death, although some individuals may recover spontaneously. Clinical results concerning susceptibility of *L. monocytogenes* to various antibiotics are somewhat conflicting. Most clinical isolates of *L. monocytogenes* are sensitive *in vitro* to penicillin, ampicillin, tetracycline, erythromycin, chloramphenicol and cephalothin; however, many of these antibiotics have adverse side effects when administered *in vivo* (Ryser and Marth, 1991). Generally, the literature indicates that listeric infections are best treated with ampicillin (Marget and Seeliger, 1988; Sethi *et al*., 1989). However, several ampicillin (Rapp *et al*., 1984; Pollock *et al*., 1986) and also penicillin-resistant (Trautmann *et al*., 1985) strains of *L. monocytogenes* have been reported. To overcome the problem of antibiotic resistance, a double hurdle effect has been used successfully with the synergistic use of two antibiotics, ampicillin and gentamicin (Larsson *et al*., 1985; Espaze and Reynaud, 1988).

1.4 SOURCES OF *LISTERIA MONOCYTOGENES*

*Listeria monocytogenes* is ubiquitously distributed in the environment, so it can be transferred to foods from a wide variety of sources. For example, it has been isolated from water, soil, vegetation, faecal material, silage and sewage. The organism is of particular threat to the food industry as it can survive longer under adverse environmental conditions than most other non-spore-forming bacteria of importance in foodborne disease (Ryser and Marth, 1991). However, evidence for tracing the transmission of human listeriosis suggests that post-process contamination within the food manufacturing unit is of particular importance.

Numerous animal species, including nearly all domestic animals, are susceptible to listeric infection, although a large proportion of healthy animals are asymptomatic
carriers of *L. monocytogenes*, and shed the organism in their milk and faeces. Listeriosis in animals can occur either sporadically or as epidemics and often leads to fatal forms of encephalitis. However, milder infections in pregnant animals generally give rise to a damaged, dead, or aborted foetus. Early comparative studies revealed no differences in biological and biochemical properties of *L. monocytogenes* strains isolated from human and animal sources. Thus, listeriosis traditionally has been recognized as a zoonosis, an infectious disease of animals that is transmissible to humans (Ryser and Marth, 1991). A higher faecal carriage rate of *L. monocytogenes* has been reported in particular groups of people connected with animals, for example, slaughterhouse employees. Nevertheless, it has been concluded that human excretors do not appear to be a source of foodborne infection.

Contamination of food by *Listeria* can occur at any stage in the food chain, from the farm, through processing and distribution, to the consumer’s kitchen. As mentioned earlier, *L. monocytogenes* has been isolated from a wide variety of foods such as: soft cheese, milk, vegetables, seafoods, raw and pre-cooked chicken and meats, fermented sausage and pâté. Alarmingly, very high numbers of *L. monocytogenes* exceeding 1000/g have been found in food distributed in the UK, particularly in soft cheese (Farber and Peterkin, 1991) and pâté (McLauchlin *et al.*, 1991). However, these are exceptions, as numbers of *L. monocytogenes* isolated from foods in the UK are generally very low. *Listeria monocytogenes* serotype 1/2 is most commonly isolated from foods, although pâté has frequently been found to contain serotype 4, the serotype most commonly isolated from humans.

The presence of other *Listeria* spp. in foods, although they are not normally responsible for human listeriosis, may be indicative of poor hygiene practices, especially where foods have supposedly undergone a listericidal process.
1.5 *LISTERIA MONOCYTOGENES* AND FOOD SAFETY

Food processing is a fundamental prerequisite for the production of a microbiologically safe product with a suitable shelf life. The basic food preservation treatments involve heating, chilling, freezing, drying, fermenting or modified-atmosphere packaging (MAP). In addition to these, chemical and natural preservatives are also used, as will be discussed later.

The fact that *L. monocytogenes* is a psychrotrophic organism, with the ability to initiate growth at low, refrigeration temperature (ca. 4°C), poses a serious threat to the food industry, especially with dairy products. However, the growth rate of the pathogen is much slower at low temperature, taking several weeks to attain stationary phase. Although *L. monocytogenes* is usually unable to grow below 0°C, it can survive extended frozen storage, which has particular significance for the icecream manufacturer. It has also been reported that the virulence of the organism is increased when it is grown at low rather than high temperatures (Ryser and Marth, 1991).

Thermal processing is the most widely used method to preserve food. Due to the facts that *L. monocytogenes* is not a particularly heat-resistant organism and is not a spore-former, it can be destroyed by pasteurization treatments. With milk, it is generally agreed that pasteurization at 71.7°C for 15 seconds will destroy normal levels of *L. monocytogenes* (WHO Working Group, 1988). In ready meals or similar products, the Department of Health advised a heat treatment of at least 70°C for 2 min at or equivalent to ensure the destruction of *Listeria*. Generally, D-values (i.e., time necessary to inactivate 90% of the population) in foods range between 5-8 min at 60°C and 0.1-0.3 min at 70°C, depending on strain and substrate. However, thermal inactivation can sometimes be detrimental to food quality and aesthetic appearance, thus other processes such as irradiation or hydrostatic pressure are used in some circumstances to reduce thermal processing (Ryser and Marth, 1991).
The minimum pH at which *L. monocytogenes* can grow is greatly influenced by both incubation temperature and acidulant. The lowest value at which growth has been recorded in laboratory media is pH 4.3, with HCl as acidulant and incubation temperature of 30°C (Farber *et al.*, 1989). In foods, the lowest pH value to limit growth is likely to be considerably higher, especially at refrigeration temperatures and where acetic acid is used as acidulant. Generally, data indicate that *L. monocytogenes* is unlikely to initiate growth in food products which have a pH $\leq 5.2$. However, the survival of *L. monocytogenes* in foods at pH $\leq 5.2$ and low temperature is quite common. It has been shown, both in laboratory media (Ahamad and Marth, 1989) and foods (Conner *et al.*, 1986), that near-refrigeration temperatures provide some protection to *L. monocytogenes* against the stressful effects of low pH when compared with ambient temperatures, as at low temperatures the average D-values were considerably greater. Such behaviour raises concerns about the safety of refrigerated low-acid foods that may have been subjected to post-processing contamination (Ryser and Marth, 1991).

Antibacterial action of acidulants, for example, weak organic acids such as acetic, citric, lactic, and malic acid, is related to pH and the degree of dissociation, with the undissociated form being most bactericidal. Studies have shown that acetic acid is the most inhibitory, followed by lactic, citric and malic (Sorrells *et al.*, 1989; Ahamad and Marth, 1989).

*Listeria monocytogenes* is relatively tolerant of high sodium chloride/low water activities ($a_W$). Growth has been shown at 10% sodium chloride, which is a higher level than that found in most foods. The organism grows optimally at a $a_W$ of ca. 0.97 (Petran and Zottola, 1989), but has been shown to be able to grow at a $a_W$ level of 0.92, under otherwise optimal conditions. Tolerance of low water activity levels is influenced by temperature, pH and type of solute. For example, a slightly lower $a_W$ minimum, 0.90, has been observed with glycerol rather than sodium chloride or sucrose at 30°C (Nolan *et al.*, 1992).
Preservatives often used by the food industry to inhibit the growth of *L. monocytogenes*, as well as other food poisoning and spoilage organisms include sodium chloride, sodium nitrite, sodium benzoate, various antioxidants, and sorbic, propionic and lactic acid. In more recent years, the consumer demand to abolish the use of artificial preservatives, resulting in preservative-free food with reduced shelf-life capacity, has lead to a renewed interest in the use of "natural" preservatives, for example, certain antibiotics.

Going back to the farm level, the use of antibiotics to control bacterial pathogens in domestic livestock has proven successful, although somewhat controversial. Widespread fears that routine use of medically important antibiotics might increase the antibiotic resistance of bacterial pathogens has recently become evident. The unlimited use of the antibiotic ciprofloxacin in Spain has caused serious problems. Ciprofloxacin is a relatively new antibiotic and had great potential medically as it was effective against a wide range of organisms and only had to be administered as a single dose. Its subsequent widespread use in domestic animals has now led to multi-resistant strains of bacteria that are unaffected by the antibiotic, thus inflicting serious consequences medically.

However, one group of non-medically important antibiotics, namely bacteriocins, have a great potential as natural food preservatives. Nisin, is by far the most important polypeptide bacteriocin, as it is the only one currently permitted in food for human consumption. It has proven to be extremely useful in preventing outgrowth of *Clostridium* spp. (including *Clostridium botulinum*) in certain foods, particularly fermented dairy products (Ryser and Marth, 1991). In 1981, Hurst stated "It is a mistake to lump together everything that is called an antibiotic. Nisin is evidently very different from the antibiotics used in medical and veterinary practices and it has not been used for the purposes of growth promotion. Progress in the area of food preservation will be greatly hampered as long as the popular confusion remains over all substances called antibiotics being kept out of foods. It might be better to call nisin a biological food preservative and class it together with lactic or acetic acid
and hydrogen peroxide, the other antibacterial products of the lactic streptococci" (Hurst, 1981).

The association between *L. monocytogenes* and foodborne illness in the 1980s, prompted renewed interest in the apparent antilisterial activity of nisin and other bacteriocins (Mohamed *et al.*, 1984; Benkerroum and Sandine, 1988; Carminati *et al.*, 1989; Harris *et al.*, 1989; Spelhaug and Harlander, 1989). Considerable research has been conducted on the antilisterial properties of nisin in foods, and a number of applications have been proposed. This is discussed in Chapter 2.
CHAPTER 2 NISIN: A NATURAL FOOD PRESERVATIVE
2 NISIN: A NATURAL FOOD PRESERVATIVE

2.1 INTRODUCTION

Nisin is a bacteriocin-like inhibitory substance (BLIS) or bactericidal protein, produced by certain strains of the lactic acid bacterium, *Lactococcus lactis* subsp. *lactis*, during fermentation. Nisin and other related bacteriocins have been designated as a BLIS as they are not completely equivalent to the prototype bacteriocins, the colicins, produced by *Escherichia coli* (de Vos *et al.*, 1991). However, for the purpose of this work the term bacteriocin will be used.

Nisin was first discovered more than sixty years ago when problems arose during cheese-making. Batches of milk starter culture used in the process had become contaminated with a nisin producing strain of *Lactococcus lactis* (then called *Streptococcus lactis*), and as a result of nisin’s inhibitory properties, the development of the cheese was detrimentally affected (Delves-Broughton, 1990). Nisin was named accordingly from group N (streptococci) Inhibitory Substance (Mattick and Hirsch, 1947). Subsequently, it was shown to have antimicrobial activity against a wide range of Gram-positive bacteria, in particular sporeformers, but not Gram-negative bacteria, yeasts, fungi or viruses.

Initial research on nisin focused on its potential therapeutic qualities for clinical and veterinary uses. However, it was found to be unsuitable for such purposes due mainly to its relatively narrow antibacterial spectrum; its low solubility in body liquids; its breakdown by digestive proteases, and its instability at physiological pH (pH 7.0-7.5) (Hurst, 1983). The potential use of nisin as a food preservative was first suggested by Hirsch in 1951, who demonstrated that clostridial gas formation in cheese could be prevented with the use of nisin-producing starter cultures (Hirsch *et al*., 1951). McClintock *et al.* (1952) later suggested that nisin was most effective in preventing clostridial spoilage in pasteurized processed cheese. Subsequently, numerous other applications of nisin were identified, and in 1969, nisin was approved
for use as an antimicrobial in food by a Joint Food and Agriculture Organization/World Health Organization Committee (Delves-Broughton, 1990).

The suitability of nisin as a food preservative arises from the facts that it is non-toxic (nisin has been ingested naturally in raw milk over past centuries without ill effect); the producer strains of *Lactococcus lactis* are regarded as safe (termed food-grade); it is not used clinically; there is no apparent cross-resistance in bacteria that might affect antibiotic therapeutics; it is digested immediately and it is heat stable at low pH (Hurst, 1981; Delves-Broughton, 1990; Harris *et al.*, 1992a). Nisin is sold as a commercial preparation under the tradename of Nisaplin by Aplin and Barrett Ltd (Beaminster, Dorset, UK), and is currently permitted as a food additive (labelled E234) in at least 47 countries (Delves-Broughton, 1990). The activity or potency of a nisin preparation is expressed in terms of International Units (IU), where 1 g of pure nisin is usually equivalent to 40 x 10^6 IU and 1 g of Nisaplin is equivalent to 1 x 10^6 IU (Ray, 1992).

The principal commercial applications of nisin are in canned foods and processed cheeses to inhibit the outgrowth of spores (Delves-Broughton, 1990). Recently, nisin has attracted renewed interest as a "natural" agent to control vegetative organisms in foods, particularly the Gram-positive pathogen *Listeria monocytogenes*. Sensitivity of *L. monocytogenes* to nisin has been demonstrated (Mohamed *et al.*, 1984; Benkerroum and Sandine, 1988; Carminati *et al.*, 1989; Harris *et al.*, 1989; Spelhaug and Harlander, 1989). However, it has also been shown that nisin-resistant strains are likely to arise (Harris *et al.*, 1991).

### 2.2 APPLICATIONS OF NISIN

Nisin has become established as a most effective preservative in pasteurized processed cheese and pasteurized processed cheese spreads (Delves-Broughton, 1990). This is because heat processing (85-105°C) of the raw cheese during melting, is not adequate
in eliminating the presence of clostridial spores originally present, and without the addition of nisin, the composition of the pasteurized processed cheese would favour the outgrowth of the spores. Spore formers associated with processed cheese include *Clostridium butyricum*, *C. tyrobutyricum*, *C. sporogenes* and *C. botulinum* (Delves-Broughton, 1990). Spore outgrowth of the first three *Clostridium* species may result in spoilage due to the production of gas, off odours and liquefaction of the cheese, whereas *C. botulinum* more seriously produces a potentially fatal toxin. The level of nisin required to inhibit the outgrowth of spores in processed cheese and other products is dependent on a number of factors: the level of clostridial spores present (Scott and Taylor, 1981); the compositional factors of the food such as sodium chloride, disodium phosphate, pH and moisture content (Tanaka *et al.*, 1986; Somers and Taylor, 1987); the shelf life required; and the temperature of storage. Generally, levels of nisin used to control non-botulinal spoilage in processed cheese vary from 250 to 500 IU/g. For antibotulinum protection, levels required are 500 IU/g or higher (Delves-Broughton, 1990). The use of nisin *in situ* has also been proposed for cheesemaking, as the addition of exogenous nisin is not allowed in many naturally produced cheese types. A nisin-producing starter culture for Gouda cheese production has been developed to inhibit clostridial blowing, using the food-grade genetic transfer technique of conjugation (Hugenholtz and de Veer, 1991).

Benkerroum and Sandine (1988) studied the inhibitory effect of nisin against *L. monocytogenes* ATCC 7644 in cottage cheese. When *L. monocytogenes* (ca. \(10^5\) cells) was inoculated into both sterile and non-sterile cottage cheese treated with nisin (2500 IU/g), a 5 log reduction of cells was observed within 1 day. No *Listeria* were detected during storage of the cottage cheese at 4°C for 30 days. Although the results were encouraging, it must be noted that strain ATCC 7644 is relatively nisin sensitive when compared with more resistant strains such as F6861 (isolated from the Jalisco cheese listeriosis outbreak) and Scott A.

Other pasteurized dairy products, such as chilled desserts, cannot be subjected to full sterilization without damaging their organoleptic qualities, and are thus sometimes
preserved with nisin to extend their shelf life. For example, results with chocolate dairy dessert demonstrated a 20 day increase in shelf life with 150 IU/g of nisin added at 7°C (Anon., 1985). Similarly, canned evaporated milk has an extended shelf life with nisin (Gregory et al., 1964).

Nisin addition to milk is permitted in some Middle Eastern countries due to shelf life problems associated with the climate, long distance transport and inadequate refrigeration (Delves-Broughton, 1990). The use of nisin at levels of 30-50 IU/ml has been demonstrated to more than double the shelf life of the product. However, it is not permitted in the UK and other countries with temperate climates. A study by Jung et al. (1992) determined the influence of fat and emulsifiers on the efficacy of nisin in inhibiting *L. monocytogenes* in fluid milk. Nisin (10 IU/ml), reduced *L. monocytogenes* Scott A by roughly 5 logs (cfu/ml) in skim milk (0% fat), and by about 0.5 logs (cfu/ml) in milk with a fat content of 12.5% within 2 hours. Nisin activity had decreased 33% when added to skim milk and 88% when added to milk containing 12.5% fat. The addition of Tween 80 counteracted the reduction in nisin activity in high fat milk and increased the viability loss of *L. monocytogenes*. Addition of nisin to high-heat-treated-flavoured milk (Heinemann et al., 1964) and yogurt (Gupta and Prasad, 1988) has also been shown to extend shelf life.

Nisin is used in canned foods principally for the control of thermophilic spoilage. It is mandatory in the UK that cans must receive a heat treatment sufficient to ensure the destruction of *C. botulinum* spores. Survival of heat-resistant spores of thermophiles *Bacillus stearothermophilus* and *C. thermosaccharolyticum* during this process are responsible for spoilage, particularly under warm conditions. Addition levels of nisin used in canned foods are generally between 100 and 200 IU/g and product examples include canned vegetables, soups and cereal puddings (Delves-Broughton, 1990).

In processed meat products, nisin has been considered as an alternative preservative system to that of nitrite, due to the concern of toxicological safety. Nitrite is a
precursor of nitrosamine, which is potentially carcinogenic (Rayman et al., 1981). Partial and total replacement of nitrite with nisin to prevent outgrowth and toxin production by C. botulinum spores has been investigated (Rayman et al., 1983; Taylor and Somers, 1985; Taylor et al., 1985). Generally, the results indicated that only high, uneconomic levels of nisin would achieve good control of C. botulinum. Proposed reasons for the inadequacy of a nisin preservative system in meats include: poor solubility in meat systems; binding of nisin onto meat particles and surfaces; uneven distribution, and possible interference with nisin's mode of action from phospholipids (Bell and De Lacy, 1987). However, it has been shown that nisin has some bactericidal activity against L. monocytogenes on meat (ca. 1-1.6 log reductions), although the levels of nisin used (4 x 10^4 IU/ml) were also quite high (Chung et al., 1989; El-Khateib et al., 1993). More recently, Fang and Lin (1994) demonstrated that a modified-atmosphere packaging (MAP)/nisin combination system (100% CO_2, 80% CO_2 + 20% air/10^3, 10^4 IU/ml nisin) was effective in inhibiting L. monocytogenes on cooked pork. MAP alone is not an effective inhibitor (Hintlian and Hotchkiss, 1987; Hotchkiss, 1988). This effect was more pronounced at 4°C than at 20°C, as at 20°C the activity of nisin decreased rapidly with time.

Taylor et al. (1990) studied the antibotulin properties of nisin in fresh fish packaged in an atmosphere of carbon dioxide. Nisin treatment of cod, herring, and smoked mackerel fillets inoculated with C. botulinum spores delayed toxin production by 5 days over the control at 10°C, but only by half a day at 26°C. However, nisin treatment did not interfere with the growth of nonpathogenic spoilage bacteria. In smoked mackerel at 10°C and all three types of control samples at 26°C, toxin was formed before the samples were judged to be unacceptable or spoiled.

Nisin has a potential role in the production of alcoholic beverages. It has been demonstrated that nisin is effective in controlling spoilage lactic acid bacteria in beer (Ogden and Tubb, 1985; Ogden 1986; Ogden and Waites, 1986; Ogden et al., 1988) and wine (Radler, 1990a; 1990b). The fact that yeasts are completely unaffected by nisin allows its addition during the fermentation. Thus, identified applications of nisin
in the brewing and wine industry include: its addition to fermenters to prevent or control contamination; increasing the shelf life of unpasteurized beers or reduction of pasteurization regimes; and washing pitching yeast to eliminate contaminating bacteria as an alternative method to acid washing as this affects yeast viability. Previously, nisin could not be used during wine fermentations that depend on a desirable malolactic acid fermentation. However, this problem was overcome by developing nisin-resistant strains of *Leuconostoc oenos* that can grow and maintain malolactic fermentation in the presence of nisin (Daeschel *et al.*, 1991). According to Henning *et al.* (1986a), the addition of nisin during the production of fruit brandies reduces the growth of competitive lactic acid bacteria, and directly favours growth of the fermenting yeast to increase alcohol content by 10%. Additional uses of nisin to control spoilage of lactic acid bacteria have been identified in low-pH foods. Examples include, salad dressings (Delves-Broughton, personal communication, 1995), and a novel paired starter culture system for sauerkraut, consisting of a nisin-resistant *Leuconostoc mesenteroides* strain and a nisin-producing *Lactococcus lactis* strain (Harris *et al.*, 1992b).

Delves-Broughton (1990) suggested that the use of nisin to prevent Gram-positive bacteria contaminating fermentations carried out by nisin-insensitive microorganisms such as Gram-negative bacteria, yeasts or fungi could be applicable in other industrial fermentations. Examples include, the production of single cell protein, organic acids, polysaccharides, amino acids and vitamins. Its use as a processing aid to control contaminating bacteria in industrial processes other than fermentations has also been considered.

More recent applications of nisin include its use as a preservative in pasteurized liquid egg (Delves-Broughton *et al.*, 1992) and high-moisture, hot-baked flour products (e.g., crumpets) (Delves-Broughton, personal communication, 1995). With pasteurized liquid whole egg, nisin (5 mg/l) resulted in a significant increase in refrigerated shelf life from between 6-11 days to 17-20 days. In the first of two trials, nisin also protected the liquid egg from growth of *Bacillus cereus*. *Bacillus cereus*
was not present in the egg in the second trial. Effective residual levels of nisin were detected in the liquid egg post-pasteurization.

It is known that nisin can adsorb to both hydrophilic and hydrophobic silicon surfaces and still retain much of its original activity (Daeschel et al., 1992). In a recent novel approach to the problem of microbial contamination of food-contact surfaces, the adsorption of nisin on to silica surfaces suppressed the colonization of \textit{L. monocytogenes} Scott A (Bower et al., 1995). However, when these studies were repeated with a nisin-resistant strain of \textit{L. monocytogenes} Scott A, no inhibition of growth on surfaces with adsorbed nisin occurred.

According to Delves-Broughton (personal communication, 1995), future developments of nisin are likely to include: synergistic action with chelators for the inhibition of Gram-negative bacteria (e.g., Stevens et al. (1991) reported sensitivity of \textit{Salmonella} and other enteric pathogens to nisin following EDTA (ethylenediamine tetra-acetic acid) treatment); synergistic action with other bacteriocins (Hanlin et al., 1993); and as an adjunct in novel food processing technology such as high-pressure sterilization (e.g., Kalchayanand et al. (1994) showed that nisin, in combination with either ultrahigh hydrostatic pressure (UHP) or electroporation (EP) had greater antibacterial effectiveness against \textit{L. monocytogenes} Scott A than UHP or EP alone).

Concern that the use of nisin might hide the use of poor quality materials or poor manufacturing practice is unfounded as nisin has a narrow antimicrobial activity, and because efficacy is dependant upon a low microbial load (Hurst, 1981; Delves-Broughton, 1990).

\subsection{2.3 STRUCTURE AND BIOSYNTHESIS OF NISIN}

Gross and Morell (1971) have elucidated the complete structure of the nisin molecule (Fig. 2.1). Nisin was at that time a novel oligopeptide, but subsequently, a number
Fig. 2.1   Structure of nisin (Dha, dehydroalanine; Dhb, dehydrobutyryne; Ala-S-Ala, lanthionine; Aba, α-aminobutyric acid; Aba-S-Ala, β-methyl-lanthionine) (Based on Gross and Morell, 1971)

of similar bacteriocins have been identified and characterized (Klaenhammer, 1988). Although nisin is, as yet, the only commercially accepted bacteriocin for food preservation, most of the lactic acid bacteria that produce these other bacteriocins can be used commercially in starter cultures (Ryser and Marth, 1991).

Nisin is one of a group of bacteriocins collectively known as lantibiotics (Jung, 1991). The other major classes of bacteriocins include, small heat-stable peptides, large heat-labile proteins, and complex proteins that require carbohydrate or lipid moieties for bacteriocin activity (Klaenhammer, 1993). Lantibiotics are produced by Gram-positive bacteria of different genera, for example, Staphylococcus, Lactococcus, Streptococcus, Bacillus and Streptomyces. Like nisin, the other lantibiotics are effective against a range of Gram-positive bacteria. On the basis of their different ring structure, charge and biological activity, the lantibiotics are classified into two subgroups: Type A, lantibiotics of the nisin type; and Type B, lantibiotics of the duramycin type. Other established Type A lantibiotics include: Pep5 (Sahl and Brandis, 1981), epidermin (Allgaier et al., 1986) and gallidermin (Kellner et al.,
1988), all of which are produced by *Staphylococcus epidermidis*; subtilin (Jansen and Hirschmann, 1944), produced by *Bacillus subtilis*; mersacidin, produced by *Bacillus* and actagardine, produced by *Actinoplanes* (Jung, 1991). Recently a few new lantibiotics have been identified: staphylococcin Au-26, produced by *Staphylococcus aureus* (Scott et al., 1992); lactocin S, produced by *Lactobacillus sake* (Mörtvedt et al., 1991); lacticin 481, produced by *Lactococcus lactis* (Piard et al., 1992); carnocin U149, produced by *Carnobacterium piscicola* (Stoffels et al., 1992); streptococcin A-FF22, produced by *Streptococcus pyogenes* (Jack and Tagg, 1991), and salivaricin A, produced by *Streptococcus salivarius* (Ross et al., 1993). The previously identified lantibiotic staphylococcin 1580, has been re-identified as epidermin (Sahl, 1994).

Lantibiotics are relatively small peptides, with nisin consisting of 34 amino acids (3354 Daltons). They are named as such because they all contain the same unusual amino acids, one of which is lanthionine. Of these unusual amino acids, nisin has 2 dehydroalanine (Dha), 1 dehydrobutyryne (Dhb), 1 lanthionine and 4 β-methyl-lanthionine residues (Fig. 2.1). Dha and Dhb arise from dehydration of serine and threonine respectively, while condensation of Dha or Dhb with cysteine generates thio-ether bonds and the amino acids lanthionine and β-methyl-lanthionine, respectively (Fig. 2.2). Subtilin is a natural analog of nisin. They both contain the same number of dehydro residues and lanthionine rings with conserved locations of the Dhas and rings. However, there are 12 amino acid differences, and nisin has 34 residues whereas subtilin has 32 (Hansen, 1994).

Lanthionine is known to introduce a high level of hydrophobicity (Kaletta et al., 1989), and a high proportion of basic amino acids gives nisin a net positive charge (Harris et al., 1992a). According to Liu and Hansen (1990), nisin can form dimers or even oligomers, which possibly arise through a reaction between dehydroamino acids and amino groups of two or more nisin molecules. In aqueous solution, nisin is most soluble at pH 2. At a high pH, the presence of nucleophiles make Dha and Dhb susceptible to modification, which may explain the decreased solubility and instability of nisin under basic conditions. Using proton nuclear magnetic resonance
Fig. 2.2  Mechanism for the synthesis of unusual amino acids (dehydroalanine, dehydrobutyryne, lanthionine and β-methyllanthionine) found in nisin (Based on Ingram, 1970)
(1H NMR) analysis and computer simulation, it has been shown that nisin exists in a rigid three-dimensional structure due to the constraints imposed by the 5 thio-ether rings (Chan et al., 1989a; Slijper et al., 1989; van de Ven et al., 1991).

Nisin preparations were resolved into 5 polypeptides (nisins A to E) by countercurrent distribution between solvents (Berridge et al., 1952), but nisins B to E are thought to be degradation products of nisin A. It is not, as yet, clear which components of the nisin molecule are essential for activity (Harris et al., 1992a). Apart from chemically derived modifications, nisin A variations can arise through changes in DNA sequence. Nisin-like molecules with different activity spectra are produced by different strains of Lactococcus lactis subsp. lactis (Hirsch and Grinsted, 1951; Geis et al., 1983). This phenomenon is accounted for by minor differences in amino acid sequence. Nisin Z, which is produced by Lactococcus lactis subsp. lactis strain NIZO 22186, is identical to nisin A except for a substitution of Asn for His at amino acid residue 27 (Mulders et al., 1991; de Vos et al., 1993). This amino acid change is a result of a single nucleic acid substitution. A single amino acid sequence substitution (Leu^ for Ile^) is also observed for the two Staphylococcus epidermidis lantibiotics, epidermin (Schnell et al., 1988) and gallidermin (Schnell et al., 1989).

Nisin is initially synthesized ribosomally as a precursor peptide which is then enzymatically cleaved (to give pronisin) and post-translationally modified to generate the mature lantibiotic. This was first suggested by Hurst (1966), who showed immediate inhibition of nisin synthesis by chloramphenicol, a protein synthesis inhibitor at the ribosomal level. The presence of amino acids in nisin not encoded by DNA implied that post-translational modification of the pronisin occurred. This hypothesis was confirmed by Ingram (1969; 1970) who used radiolabelled amino acids to show that during nisin synthesis, either serine or threonine combine with cysteine to give lanthionine and β-methyl-lanthionine, respectively. The author also proposed that the dehydration of serine and threonine residues lead to the dehydro forms, some of which could condense with neighbouring cysteine residues generating thio-ether cross-linkages. The prepronisin structural gene has subsequently been
cloned and sequenced and has been designated *spa*N and *nis*A by different workers (Buchman *et al*., 1988; Kaletta and Entian, 1989; Dodd *et al*., 1990). It was found that serine, threonine, and cysteine located the precise positions required to produce a mature nisin molecule as suggested by Ingram (1969; 1970). The primary transcript of the nisin structural gene, prepronisin, consists of an N-terminal leader peptide of 23 amino acids, followed by a C-terminal propeptide of 34 amino acids from which the lantibiotic is matured.

Horn *et al.* (1991) showed that nisin biosynthesis genes are encoded by a novel conjugative transposon. (A transposon is a discrete DNA sequence capable of moving, independent of DNA homology, from one location to another). Sucrose fermentation, nisin immunity, conjugal transfer factors, N5-(carboxyethyl) ornithine synthase and bacteriophage resistance determinants have all been linked with nisin production (Hirsch and Grinsted, 1951; Gasson, 1984; Gonzalez and Kunka, 1985; Steele and McKay, 1986; Murphy *et al*., 1988; Donkersloot and Thompson, 1990). Sequence analysis of a transconjugant (conjugation of *Lactococcus lactis* subsp. *lactis* MG1614 and nisin producing NCFB894) revealed a 70-kilobase-pair transposon, designated Tn5301 (Horn *et al*., 1991). Similarly, in *Lactococcus lactis* NIZO R5 the production of nisin is encoded by a 70-kilobase-pair conjugative transposon, designated Tn5276 (Rauch and de Vos, 1992).

The location of the genes encoding nisin production was unclear. Tsai and Sandine (1987) detected an additional plasmid of 17.5 MDa in a nisin-producing transconjugant of *Leuconostoc dextranicum*, and Kaletta and Entian (1989) reported isolation of the nisin structural gene from plasmid DNA prepared from *Lactococcus lactis* subsp. *lactis* 6F3. In contrast, considerable evidence has been presented and generally accepted that the genes encoding nisin production are located on the chromosome of nisin-producing strains of *Lactococcus lactis* subsp. *lactis* (Dodd *et al*., 1990; Horn *et al*., 1991; Steen *et al*., 1991; Gireesh *et al*., 1992; Rauch and de Vos, 1992).
The genes involved in nisin biosynthesis are organized into an operon-like structure. A 10-kilobase-pair DNA fragment of Tn5276 was cloned from *Lactococcus lactis* NIZO R5, and was found to contain the gene cluster *nisABTCIPR* (Kuipers et al., 1993; van der Meer et al., 1993). The genes *nisABTCIPR* have also been found in strain *Lactococcus lactis* 6F3, plus the additional genes *nisKFEG* (Engelke et al., 1992; Engelke et al., 1994; Siegers and Entian, 1995). It has been suggested that *nisB* and *nisC* encode the enzymes needed for modification of the lantibiotic precursor peptides (Steen et al., 1991; Engelke et al., 1992; Kuipers et al., 1993). It is thought that NisT is involved in transport of (precursor) nisin molecules across the cytoplasmic membrane, since it shares significant homology with ATP-dependent translocator proteins (Engelke et al., 1992; Kuipers et al., 1993). The *nisI* gene encodes a putative lipoprotein, which was shown to be involved in immunity to nisin (Kuipers et al., 1993; Engelke et al., 1994). The *nisP* gene was shown to encode a subtilisin-like serine protease, involved in cleavage of the leader peptide sequence from the final precursor peptide (van der Meer et al., 1993; Engelke et al., 1994). The *nisR* gene encodes a positive regulator protein needed for activation of expression of the *nis* genes (van der Meer et al., 1993; Engelke et al., 1994) and *nisK* encodes a histidine kinase (Engelke et al., 1994). The deduced amino acid sequences of *nisR* and *nisK* exhibit marked similarities to SpaR and SpaK, which were identified as the response regulator and the corresponding histidine kinase of subtilin biosynthesis (Klein et al., 1993). The recent identification of genes *nisFEG* (Siegers and Entian, 1995) has led to the suggestion that they may also be involved in immunity to nisin. The encoded proteins, NisF, NisE and NisG, possibly form an ATP-binding cassette (ABC) transporter in a manner similar to that reported for the subtilin gene cluster (Klein and Entian, 1994). Engelke et al. (1994) have also shown that nisin biosynthesis is regulated by the expression of its structural and biosynthetic genes. Prenisin expression starts in the exponential growth phase and precedes that of the NisB protein by about 30 min. Both proteins are expressed to a maximum in the stationary growth phase.

The role of the nisin leader peptide sequence in the biosynthetic process of nisin
maturation has also been studied by creating site-directed mutations at various positions in the leader peptide sequence (van der Meer et al., 1994). It was confirmed that cleavage of the leader peptide is the last step in nisin maturation and is necessary to generate a biologically active peptide. It was also suggested that conserved residues in the leader peptide are involved in the maturation process and may interact with lantibiotic-specific modifying enzymes.

As the number of known natural lantibiotics has increased and their useful characteristics have been explored, it has become desirable to synthesize structural analogs of nisin and other lantibiotics that do not occur naturally (Hansen, 1994). The fact that lantibiotics are gene-encoded peptides, allows structural variants to be generated by mutagenesis. According to Hansen (1994), the construction and expression of mutant genes cannot be performed easily unless one is working with an organism that is both well characterized and amenable to a wide variety of genetic manipulations. These requirements are not easily met with \textit{Lactococcus lactis}, the natural producer of nisin. In contrast, \textit{B. subtilis}, which produces subtilin, is second only to \textit{Escherichia coli} in the amount of genetic information and genetic methodologies that are available for it. The likelihood that nisin and subtilin evolved from a common ancestor suggests that they are formed by the same biosynthetic mechanism and fundamentally have the same antibacterial mechanism of action. Thus, it is likely that information gained about subtilin will be highly relevant to gaining an understanding of nisin. Consequently, a host-vector pair has been engineered that permits the construction of mutants of the structural gene for subtilin. The vector is designed in such a way that the mutant gene can be substituted for the natural subtilin gene in the chromosome of \textit{B. subtilis}, which in turn directs the transcription, translation, post-translational modifications, and secretion of the mature form of the structural analog (Liu and Hansen, 1991; 1992).

However, this has actually been achieved with a lactococcal expression system for engineered nisins (Dodd et al., 1992). A nisin-producing \textit{Lactococcus lactis} strain that was deficient in the \textit{nisA} gene product but expressed those determinants necessary for
prenisin maturation, secretion, and immunity was generated. Complementation of the lesion in the \textit{nisA} gene by plasmid-encoded \textit{nisA} genes containing site-specific mutations resulted in the exclusive production of altered nisins containing specific amino acid substitutions. A number of alterations were made to the primary sequence of prenisin, and the effect of these changes on the biological activity of the mature molecule were described.

A novel approach by Rintala \textit{et al.} (1993) has shown that production of nisin is possible in a host other than \textit{Lactococcus lactis}, such as \textit{B. subtilis}. Conceivably, this could lead to an increased nisin yield.

2.4 MODE OF ACTION OF NISIN

The primary target of nisin in sensitive Gram-positive cells is the cytoplasmic membrane (Ruhr and Sahl, 1985). It was originally thought that nisin acted as a cationic surface-active detergent due to strong adsorption of nisin to cells causing leakage of ultraviolet-absorbing material and subsequent lysis (Ramseier, 1960). However, it is now thought that membrane disruption is due to incorporation of nisin into the membrane with subsequent ion channel or pore formation (Henning \textit{et al.}, 1986b; Sahl \textit{et al.}, 1987; Kordel \textit{et al.}, 1989; Gao \textit{et al.}, 1991; Garcerá \textit{et al.}, 1993). Adsorption does not appear to involve specific protein receptors (as is the case for colicins), but interaction between nisin and the membrane is perhaps facilitated by negatively-charged cell wall components (Hill, 1995).

Sahl \textit{et al.} (1987) observed that nisin produced transient multistate pores in black lipid bilayers and that pore formation required the existence of a sufficient \textit{trans}-negative membrane potential (negative inside). The pores had predicted diameters in the range of 0.2-1.0 nm, which allowed the efflux of hydrophilic solutes with molecular masses up to 500 Daltons. Sahl (1991) has proposed that due to the amphiphilic nature of nisin, charges are exposed on one side of the molecule which makes it rather unlikely
that a nisin monomer could adopt a transmembrane orientation; moreover, a monomer should not be able to form a channel of the size determined in planar membrane experiments (Benz et al., 1991). It is not known whether nisin inserts into the membrane as a monomer and then self assembles into an oligomer to form the pore, or whether this aggregation event precedes membrane binding or insertion. It has been suggested that firstly nisin monomers bind to the membrane with subsequent oligomerization at the membrane surface (Sahl, 1991). Upon energization, the channels are formed by the peptide oligomers, in which the single peptides are arranged as the staves in a barrel (Benz et al., 1991). This would be conducted in such a way that the hydrophobic face of the aggregate is exposed to the surrounding lipids while the hydrophilic face forms the inner lumen of the pore (Sahl, 1991). However, the observation that nisin aggregated at pH values above 7-7.5 was not capable of inducing pores (Liu and Hansen, 1990) suggests that insertion has to precede aggregation for activity (Garcera et al., 1993).

Sahl (1991) advises that one should also take into consideration that in vivo, nisin may interact with intergral constituents of the membrane such as proteins or membrane-bound cell wall precursors. Such interaction could have considerable influence on the kinetics of pore formation, on pore stability and pore lifetime and may explain the differences in susceptibility of individual bacterial species and genera which are observed for the different lantibiotics.

Efflux of K\(^+\), ATP and amino acids through the membrane pores was shown to result in destruction of the membrane potential (Ruhr and Sahl, 1985; Kordel and Sahl, 1986). Subsequent investigation with *L. monocytogenes* demonstrated that nisin dissipates the membrane potential and pH gradient in energized cells, leading to collapse of the proton-motive force (PMF) (Bruno et al., 1992; Winkowski et al., 1994). Depletion of the PMF has been suggested as the common mechanistic action of bacteriocins from lactic acid bacteria (Bruno and Montville, 1993).

However, Hansen (1993) has suggested that the dehydro residues and thioether rings
of nisin play a role in its mechanism of action, because they are so highly conserved. This is supported by the fact that the dehydro residues are required for antimicrobial activity (Lui and Hansen, 1990; Hansen et al., 1991). Hansen (1993) suggests that the dehydro residues act as Michael acceptors to react with nucleophiles such as sulfhydryl groups in the target cell, as originally proposed by Gross and Morell (1967). It has been shown that nisin inactivates sulfhydryl groups in the membranes of germinated bacterial spores, so that they will no longer react with iodoacetate (Morris et al., 1984). It has also been shown that unmodified sulfhydryl groups are necessary to permit spore outgrowth (Morris and Hansen, 1981; Buchman and Hansen, 1987). Hansen (1993) has further suggested that the mechanism of action of nisin could involve all these aspects discussed (reaction of dehydro residues with sulfhydryl groups followed by membrane leakage and collapse of gradients), or alternatively, the nisin molecule may be able to function by two distinct mechanisms depending on whether its activity is against spores or vegetative cells. This later hypothesis has been suggested for subtilin, due to the observation that an intact Dha$_5$ is required for subtilin to inhibit spore outgrowth, but is not required for it to lyse vegetative cells (Hansen, 1994).

Gram-negative cells are normally protected from nisin by the presence of an outer membrane in their cell walls. However, when the outer membrane is weakened, Gram-negative cells also show sensitivity to nisin indicating that their cytoplasmic membranes are susceptible. Kordel and Sahl (1986) showed that E. coli exhibited nisin sensitivity when the outer membrane was bypassed by osmotic shock or by formation of cytoplasmic membrane vesicles. Alternatively, Stevens et al. (1991) showed the inhibitory activity of nisin in combination with a chelating agent, disodium EDTA, against several Salmonella species and other Gram-negative bacteria. (Chelating agents bind magnesium ions which serve to stabilize the lipopolysaccharide layer of the outer membrane). Also, mutants of Salmonella typhimurium, which had reduced membrane lipopolysaccharide, were sensitive to nisin with a positive correlation between sensitivity and degree of aberration in the lipopolysaccharide (Stevens et al., 1992).
In artificial membrane vesicles (liposomes), nisin dissipates both the membrane potential and pH gradient and, to a smaller extent, inhibits oxygen consumption by cytochrome c oxidase-containing proteoliposomes (Gao et al., 1991). It has been suggested that membranes (both cellular and artificial) have to be in an energized state to allow nisin action (Sahl et al., 1987; Gao et al., 1991; Sahl, 1991). In contrast, Garcera et al. (1993) reported that an energized membrane (artificial) is not essential for nisin action, but the total PMF enhances its effect. Incorporation of nisin into the membrane is affected by the phospholipid composition of the liposome and may account for differences in nisin sensitivity between bacterial species or strains (Gao et al., 1991; Garcera et al., 1993).

It has been suggested that peptidoglycan synthesis is also partially inhibited by nisin (Linnett and Strominger, 1973; Henning et al., 1986b). Reisinger et al. (1980) showed formation of a complex between nisin and the lipid intermediates of murein biosynthesis in vitro. However, it is unlikely that this is the primary site of action as high levels of nisin are necessary to inhibit murein biosynthesis. Possibility of a nisin-murein complex involved in initial nisin-cell interaction and/or in transport to the membrane has not been eliminated.

It has also been shown that nisin and Pep5 induce cellular autolysis in Staphylococcus simulans (Bierbaum and Sahl, 1991). Electron micrographs demonstrated that the cell wall is degraded. Two autolytic enzymes were purified and are characterized by high isoelectric points, as are Pep5 and nisin. Experiments employing the autolysins, different types of cell walls, lipoteichoic acids and cationic peptides showed that the cationic peptides and autolysins bind to the polyanionic constituents of the cell wall (because of their high isoelectric points), and influence the rate of cell wall hydrolysis. At low ionic strength the enzymes are bound to the teichoic and teichuronic acids of the cell wall, which act as noncompetitive inhibitors of enzyme activity. Pep5 and nisin replace the enzymes from the anionic polymers and thereby induce autolysis.
The action of nisin against spores has been shown to be both sporostatic and sporicidal (Hirsch and Grinsted, 1954; Campbell and Sniff, 1959). Nisin affects the post-germination stages of spore development (Ramseier, 1960). It inhibits pre-emergent swelling and thus outgrowth and formation of vegetative cells (Hitchins et al., 1963). As mentioned earlier, it has been suggested that the active site in spores is membrane sulphydryl groups present in newly germinated spores, reactivity occurring with the nisin dehydro groups (Morris et al., 1984). Further support to this hypothesis is given by the facts that the dehydro residues in the nisin molecule are reactive with mercaptans and complete loss of nisin activity occurs with loss of intact Dha5 (Chan et al., 1989b; Liu and Hansen, 1990).

2.5 NISIN RESISTANCE

Nisin-producing strains of Lactococcus lactis subsp. lactis have a natural immunity to nisin, which appears to be co-ordinately controlled with nisin production. Nisin added to the producing organism before synthesis, inhibits and lyses the cells, whereas addition of nisin during its synthesis causes only a transient delay in growth (Hurst and Kruse, 1972). The actual role of nisin and other bacteriocins in nature is unclear. They may provide a competitive advantage over non-producing neighbours, but this remains to be convincingly demonstrated in the natural world (Hill, 1995). However, resistance to nisin also occurs in non-producing Gram-positive bacteria. Naturally nisin-resistant, non-producing, Gram-positives have been isolated from food products such as bacon, cured and fermented meat, and fermented vegetables (Harris et al., 1992a).

As mentioned, sensitivity of L. monocytogenes to nisin has been demonstrated (Mohamed et al., 1984; Benkerroum and Sandine, 1988; Carminati et al., 1989; Harris et al., 1989; Spelhaug and Harlander, 1989), but sensitivity can vary between strains (Mohamed et al., 1984). It has also been shown that acquired nisin-resistant strains can arise in the presence of sublethal nisin concentrations (Harris et al., 1991).
Mutant strains of *L. monocytogenes* resistant to 50 μg/ml (1850 IU/ml) nisin were detected at frequencies of 10^-6 to 10^-8 (at pH 6.5). The development of acquired nisin resistance has also been observed for *Streptococcus agalactiae, Staphylococcus aureus, Clostridium butyricum,* and *Pediococcus pentosaceus* (Harris et al., 1992a).

The ability of *L. monocytogenes* to develop resistance to nisin is clearly an obstacle to the development of new food applications. The mechanism of this resistance has not, as yet, been determined for *L. monocytogenes,* and may differ from strain to strain. However, bacterial strains that become resistant to nisin do not develop cross-resistance to antibiotics used in chemotherapy (Hossack et al., 1984).

Determinants of nisin resistance have been associated with plasmid DNA in the non-nisin-producers *Lactococcus lactis* subsp. *lactis* biovar *diacetylactis* (pNP40, McKay and Baldwin, 1984) and *Lactococcus lactis* subsp. *lactis* (pTR1040, Klaenhammer and Sanozky, 1985; pSF01, von Wright et al., 1990). However, nisin resistance in *L. monocytogenes* strains could not be attributed to the presence of plasmid DNA (Harris et al., 1991). Subsequent cloning (Froseth et al., 1988; Simon and Chopin, 1988) and sequencing (Froseth and McKay, 1991) of the resistance gene from *Lactococcus lactis* subsp. *lactis* biovar *diacetylactis* encoded a protein of 319 amino acids, the function of which is undetermined. The protein contained a hydrophobic region at the N terminus that was predicted to be membrane associated. A DNA probe prepared from the resistance gene did not hybridize to genomic digests of nisin-producing *Lactococcus lactis* subsp. *lactis* ATCC 11454, suggesting that the resistance and immunity factors are not the same (Froseth and McKay, 1991). As mentioned earlier, the immunity factors have been identified as being part of the putative nisin operon, and involve the genes *nisI* (Kuipers et al., 1993; Engelke et al., 1994) and *nisFEG* (Siegers and Entian, 1995).

A nisin-degrading enzyme, nisinase, has been described for a range of bacterial species (Kooy, 1952; Galesloot, 1956; Carlson and Bauer, 1957; Alifax and Chevalier, 1962; Jarvis, 1967). Nisinases from *Streptococcus salivarius* subsp.
thermophilus (Alifax and Chevalier, 1962) and Bacillus cereus (Jarvis and Farr, 1971) were partially purified but have not been fully characterized. The nisinase isolated from B. cereus was identified as a dehydroalanine reductase (Jarvis and Farr, 1971). However, Jarvis (1967) suggested that a general mechanism of resistance other than the production of a nisin-inactivating enzyme was prevalent within the Bacillus genus, and that it was possible that the enzyme was produced by the mature vegetative organisms for some function other than the inactivation of nisin. No evidence of a nisin-inactivating enzyme has been found in nisin-producing Lactococcus lactis (Hurst, 1981) and in L. monocytogenes this has not, as yet, been investigated.

A variety of biochemical mechanisms for antibiotic resistance have been described (Russell and Chopra, 1990). These include: alteration (antibiotic inactivation); insensitivity of the target site in the cell to the inhibitor whilst maintaining normal physiological function; decreased antibiotic accumulation (impaired uptake or enhanced efflux); bypass of antibiotic sensitive step by duplication of the target site, the second version being insusceptible to drug action; overproduction of target so that higher antibiotic concentrations are needed to inhibit bacterial growth; and absence of an enzyme/metabolic pathway. However, it should be noted that resistance to a particular antibiotic can result from the combined expression of several of the listed mechanisms.

Alternative mechanisms of nisin resistance to that of nisinase production could involve adaption of the cytoplasmic membrane and/or cell wall. Like nisin, the polymyxin antibiotics and some of the colicin bacteriocins destroy membrane integrity. The polymyxins are a group of cyclic, polycationic, peptides with a fatty acid chain attached to the peptide through an amide linkage. The bactericidal activity of these compounds results from their interaction with the bacterial cytoplasmic membrane causing gross disorganization of its structure. Membranes containing the phospholipid phosphatidylethanolamine are particularly sensitive to polymyxins, which explains why Gram-negative bacteria are more susceptible to polymyxins than Gram-positive organisms, because membranes of the latter generally do not contain phosphatidyl-
ethanolamine. In order to reach their target site in Gram-negative bacteria, polymyxins need to cross the cell wall outer membrane. This is achieved primarily by self-promoted uptake of polymyxins across the outer membrane, whereby divalent cations are displaced leading to membrane destabilization and further insertion of antibiotic molecules into the outer membrane bilayer. Plasmid-determined resistance to polymyxin has not been described, but chromosomal mutants of *Pseudomonas aeruginosa* resistant to polymyxin (and the divalent ion chelator EDTA) have been isolated. Resistance is associated with an increase in the content of an outer membrane protein (HI) which decreases the requirement for divalent cations in the outer membrane. Since the initial binding of polymyxin to sensitive cells depends upon displacement of divalent cations from the outer membrane, it is suggested that the protein HI replaces cations at sites in the outer membrane that would otherwise be susceptible to the antibiotic (Russell and Chopra, 1990).

Colicins are bacteriocins that are synthesized by *E. coli* and that kill *E. coli* cells and sometimes closely related species such as *Shigella* and *Salmonella* (Braun et al., 1994). They are elongated, single polypeptide chains, each chain consisting of three regions. The hydrophobic amino termini are responsible for translocation of colicins across the cell membrane, while the lysine-rich carboxy termini mediate lethal activity. Between these two domains lies an area associated with colicin binding to the outer membrane receptors of sensitive cells (Barefoot et al., 1992). Three putative stages involved in colicin A (a membrane-active colicin) entry and insertion into the cytoplasmic membrane may be visualized as follows: firstly, colicin A adsorbs to a receptor. Secondly, colicin adsorption results in a conformational change in the polypeptide chain, and α-helices associated with the carboxy terminus form a pore or channel in the membrane. The amino terminus then translocates across the cell membrane. Thirdly, after entering the periplasmic space, the polypeptide realigns so that the carboxy terminus may penetrate the inner membrane and exert additional lethal activity. Other pore-forming colicins include colicins DF13, E1, K, Ia and Ib. Like nisin, they form voltage-dependent channels, cause leakage of ions, and destroy the energy potential of the cell. Colicin A immunity protein (Cai) has been well
characterized. It is a minor component of the cell membrane and is arranged in a zigzag fashion across it. Pore-forming colicins are not toxic to the producer cells until they have been secreted, thus, the immunity proteins are necessary only to protect the cells from exogenous colicin. Colicin resistance, shown by the inability of some cells, generally mutants, to adsorb colicins, occurs. The mechanisms by which a sensitive cell becomes resistant vary. Changes in the binding site, elimination of the binding site, or production of a substance that competes with the colicin for the site are possible colicin resistance mechanisms. However, the fact that colicins often adsorb to receptors that are vital to cell survival minimizes the possibility of a resistant mutant being viable in nature. In addition, there is the mechanism of colicin tolerance, which refers to the ability of some cells, usually mutants, to bind a colicin without being killed by it (Barefoot et al., 1992).

As resistance or tolerance mechanisms to the bacteriocins of lactic acid bacteria have not been studied, Barefoot et al. (1992) suggested that it seems reasonable to assume that they are similar to mechanisms for insensitivity to colicins. However, Gram-positive cells do not possess an outer membrane in their cell walls, thus it is only conceivable that a similar effect may occur in the cytoplasmic membrane. The target of biocide action is frequently the cytoplasmic membrane, but at this stage, there is no evidence for changes at the target site as a basis either of plasmid or chromosomally-determined acquired resistance. It has been suggested that changes at this level to confer resistance may not be compatible with cell survival (Russell and Chopra, 1990). Alternatively, cell wall-associated proteins, polyanionic cell wall polymers (teichoic, teichuronic and lipoteichoic acids) and peptidoglycan all have a potential role in nisin resistance.

Harris et al. (1991) has suggested that further research into the mechanism of nisin resistance may provide insight into the mode of action of nisin and allow development of strategies to minimize the occurrence of nisin-resistant populations. Structural analogs of nisin could be designed to overcome nisin resistance. In addition, nisin-resistance determinants have application as selective markers for food-grade cloning
vectors (von Wright et al., 1990; Froseth and McKay, 1991) and their usefulness in starter cultures (nisin-resistant bacteria and nisin or nisin-producing strains) has been demonstrated (Daeschel et al., 1991; Harris et al., 1992b;).

Thus, the aim of this project was to gain an insight into the resistance mechanism of *L. monocytogenes* to the bacteriocin nisin by comparison of sensitive and resistant strains.
CHAPTER 3 GENERAL MATERIALS AND METHODS
3 GENERAL MATERIALS AND METHODS

Bacteria, media and culture conditions

*Listeria monocytogenes* NCTC 5105 serotype 3a and F6861 serotype 4b (an isolate from Jalisco cheese incriminated in an outbreak of listeriosis in 1985) were obtained from Dr B. Lund, IFR Norwich. They were grown in tryptose phosphate broth (TPB) (Oxoid) and on tryptone soya agar (TSA)(Oxoid) at 30°C. The media were prepared according to the instructions of the manufacturer and where necessary, the pH was adjusted to the required level (± 0.01) with HCl prior to sterilization. Cultures were maintained on TSA slopes stored at 4°C.

*Micrococcus luteus* NCIB 8166 was maintained on slopes of nisin bioassay medium (NBM) at 4°C, prepared as described by Fowler *et al.* (1975). NBM contained (g/l): bacteriological peptone (Oxoid), 10; Lab-lemco (Oxoid), 3; sodium chloride (Fisons), 3; yeast extract (Difco), 1.5; natural, soft, raw cane sugar (Muscovado, Sainsbury’s), 1; and Agar No. 1 (Oxoid), 10. The pH of the medium was adjusted to 7.5 ± 0.1 prior to sterilization.

*Lactobacillus buchneri* ATCC 4005 was grown on de Man, Rogosa and Sharpe agar (MRS)(Oxoid) at 37°C, prepared according to the specifications of the manufacturer. Plate cultures were stored at 4°C.

Gram stain

Cells were prepared for staining by smearing in water and heat fixing on a glass slide. Crystal violet was applied for 30 s and replaced with Lugol’s iodine for 30 s to 1 min. This was rinsed with absolute alcohol until no more colour appeared to flow from the preparation. After washing with water, safranin was applied for 2 min. This was again rinsed with water, blotted and dried with gentle heat.
Enumeration of bacteria by colony counting

Decimal, serial dilutions were made in maximum recovery diluent (MRD). MRD contained: bacteriological peptone (Oxoid), 0.1%; sodium chloride (Fisons), 0.85% (w/v).

Bacteria were enumerated either as spread plates (50 or 100-μl) or as spiral plates in which 50 μl of the appropriate dilutions were applied to TSA plates using a Spiral Plate Maker (Don Whitley Scientific Ltd, Shipley).

Colonies were counted after incubation of the plates at 30°C for 48 h.

Preparation of nisin stock solution

Pure nisin (50 x 10^6 International Units (IU)/g) was obtained from Aplin and Barrett Ltd (Beaminster, Dorset, UK). Stock solutions (1 mg/ml) were prepared by dissolving 1 mg of nisin in 0.02 M HCl (100 μl) and making up to 1 ml with distilled water. Solutions were sterilized by membrane filtration (0.2 μm, Minisart NML, Sartorius) and appropriate dilutions made with sterile water.
CHAPTER 4  DETERMINATION OF THE SENSITIVITY OF
LISTERIA MONOCYTOGENES TO NISIN AND
ISOLATION OF NISIN-RESISTANT MUTANTS
4 DETERMINATION OF THE SENSITIVITY OF \textit{LISTERIA MONOCYTOGENES} TO NISIN AND ISOLATION OF NISIN-RESISTANT MUTANTS

4.1 INTRODUCTION

Sensitivity of \textit{L. monocytogenes} to nisin has been demonstrated extensively (Mohamed \textit{et al.}, 1984; Benkerroum and Sandine, 1988; Carminati \textit{et al.}, 1989; Harris \textit{et al.}, 1989; Spelhaug and Harlander, 1989; Ferreira and Lund, 1991). Essentially, the degree of sensitivity is variable between strains. Ferreira and Lund (1991) compared nisin sensitivity in 27 strains of \textit{L. monocytogenes} by determining minimum inhibitory concentration (MIC) values on solid media. At pH 5.5 and 37°C the most sensitive strain was NCTC 5105 with a MIC of 10-50 IU/ml, but for the majority of strains the MIC was in the range 200-400 IU/ml.

Mohamed \textit{et al.} (1984) reported that when a population of \textit{L. monocytogenes} (10^5 cfu/ml) was treated with nisin (16 IU/ml) at pH 7.4, it decreased initially to below the detectable limit but on further incubation regrew to a stationary phase population (ca. 10^8 cfu/ml). No growth occurred within the same time period (72 h) at a nisin concentration of 32 IU/ml. Studies by Somers and Taylor (Doyle, 1988) showed that the Scott A strain of \textit{L. monocytogenes} could eventually grow at a nisin concentration of 2000 IU/ml (pH not stated) making it very nisin-resistant. In this case too, growth occurred after initial reduction of the population and a few days lag period. This greater nisin resistance was also seen to a lesser extent with other strains of \textit{L. monocytogenes} which grew at a nisin concentration of 500 IU/ml.

The development of a subpopulation of \textit{L. monocytogenes} with increased resistance to nisin could seriously limit the potential of nisin as a practical means of controlling this organism in food. With this problem in mind, the objectives of this initial study were to:
(1) Confirm the sensitivity of *L. monocytogenes* NCTC 5105 and relative resistance of *L. monocytogenes* F6861 to nisin by determination of MIC values using solid and liquid media at selected pH values.

(2) Isolate nisin-resistant mutants displaying higher MIC values than the respective parent strain. (During the course of this project the parallel work of Harris *et al.* (1991) was published. This is discussed in section 4.4).

(3) Determine the stability of the nisin resistance phenotype.

(4) Compare the morphology and growth kinetics of wild-type strains of *L. monocytogenes* and nisin-resistant mutants.
4.2 MATERIALS AND METHODS

Minimum inhibitory concentration (MIC) of nisin

MIC values were determined in duplicate on TSA (pH 5.5) and individually in TPB (pH 5.5, 6.5 and 7.3).

For TSA, plates of increasing nisin concentration (0-600 IU/ml) were prepared, on the day of use, by adding 1 ml of the appropriate nisin solution (Chapter 3) to 24 ml of molten agar to obtain each nisin concentration required. The Miles and Misra procedure was used to determine the growth of L. monocytogenes, in accordance with the protocol of Ferreira and Lund (1991). Overnight broth cultures were diluted in MRD to give ca. $10^6$ cfu/ml and triplicate 10-μl drops were inoculated on to the surface of each agar plate. Incubation was conducted at 30°C and 37°C for 24 h.

For TPB, broths of increasing nisin concentration (0-2500 IU/ml) were prepared, on the day of use, by adding 100 μl of the appropriate nisin solution to 9.8 ml of TPB before inoculating with 100 μl of diluted overnight broth culture to give the required nisin concentration and an initial inoculum level of ca. $10^6$ cfu/ml, respectively. This inoculum concentration caused no visible turbidity in the medium. Tubes were incubated at 30°C and growth was monitored by the appearance of turbidity. In the event of turbidity, the possibility of contamination was checked by streak plating each culture on TSA.

The MIC was defined as the lowest level of nisin that inhibited growth.

Effect of nisin on the survival of L. monocytogenes NCTC 5105 in TPB

A 24-h culture of L. monocytogenes NCTC 5105 in TPB, was diluted in MRD and inoculated into six, 250-ml volumes of TPB (pH 5.5, 30°C) to give an estimated
initial population of $10^5$ cfu/ml. As growth proceeded, nisin solution was added to
five of the cultures at different stages to give a final concentration of 50 IU/ml. The
remaining culture was left nisin-free. Samples were taken and enumerated on TSA
using the spiral plater (Chapter 3).

Isolation of nisin-resistant mutants

Nisin-resistant mutants of *L. monocytogenes* were isolated essentially as described by
Harris *et al.* (1991). Overnight cultures (TPB, pH 5.5 and 7.3) containing ca. $10^9$
cfu/ml, were diluted in MRD where necessary, and plated (spread and spiral) on to
TSA (pH 5.5) containing different concentrations of nisin (0-600 IU/ml)(page 45). Mutants were isolated at the lowest pH (5.5) as *L. monocytogenes* has greater nisin
sensitivity the more acidic the pH (Mohamed *et al.*, 1984; Harris *et al.*, 1991), and
so less nisin was needed in the medium. Plates were incubated at $30^\circ$C for 7 d.
Resistance frequencies were determined by comparison of colony counts on nisin-free
TSA and nisin-supplemented TSA (600 IU/ml).

Resistant mutants isolated were confirmed as *L. monocytogenes* using the Accuprobe
*L. monocytogenes* Culture Identification Test (Gen-Probe Inc., San Diego). The
principles of this procedure are described in Appendix I.

Nisin stability

The stability of nisin in TSA (pH 5.5) was monitored by incubating uninoculated agar
plates containing nisin (600 IU/ml) under the conditions used for the isolation of
resistant mutants ($30^\circ$C, 7 d). Nisin-resistance frequencies were then determined for
*L. monocytogenes* F6861 using these plates and the results compared with those from
fresh plates.
Stability of nisin resistance in *L. monocytogenes* F6861 mutant

Stability of nisin resistance in the *L. monocytogenes* F6861 mutant (isolated as described on page 46) was tested using: (1) a mutant that had been repeatedly subcultured on nisin-supplemented TSA (600 IU/ml, pH 5.5) and (2) a freshly isolated mutant that had only one exposure to nisin. Both organisms were grown through ten, 24-h passages in nisin-free TPB ((1) pH 7.3; (2) pH 7.3 and 5.5), each initial population size being ca. 10^7 cfu/ml. Nisin resistance was redetermined after each passage by enumeration on nisin-supplemented TSA (600 IU/ml, pH 5.5).

**Growth of *L. monocytogenes***

Growth of *L. monocytogenes* NCTC 5105, F6861 wild type (WT) and F6861 nisin-resistant mutant (M) in TPB (pH 5.5 and 7.3) were compared at 30° and 4°C (± 2°C). Broths (10-ml) were inoculated at a level of ca. 10^4 cfu/ml and samples taken periodically for enumeration on TSA (pH 7.3) using spread and spiral plating techniques.

**Morphology of *L. monocytogenes***

Gram stain preparations (Chapter 3) of each strain of *L. monocytogenes* were compared microscopically under oil immersion.
4.3 RESULTS

4.3.1 MIC of nisin for \textit{L. monocytogenes}

Determination of nisin MIC values for \textit{L. monocytogenes} on TSA (pH 5.5) at 30º and 37ºC confirmed the sensitivity of \textit{L. monocytogenes} NCTC 5105 and the relative resistance of \textit{L. monocytogenes} F6861 to nisin reported by Ferreira and Lund (1991). NCTC 5105 had a MIC of 10-40 IU/ml compared with 200-400 IU/ml for F6861.

Nisin MIC values for \textit{L. monocytogenes} in TPB (pH 5.5, 6.5 and 7.3) at 30ºC increased with time, thus two MIC values (initial (24 h) and final (d)) are reported in Table 4.1. No further change of the MIC values occurred after the stated time period. The rates of increase between MIC values are shown graphically in Fig. 4.1. Final MIC values for TPB (pH 5.5) corresponded with those for TSA (pH 5.5) and were, in fact, more definable due to there being a clearer cut-off point. As the pH of TPB decreased, sensitivity to nisin increased.

Table 4.1 MIC of nisin for \textit{L. monocytogenes} NCTC 5105 and F6861 in TPB at 30ºC

<table>
<thead>
<tr>
<th>pH</th>
<th>Initial</th>
<th>Final</th>
<th>Initial</th>
<th>Final</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.5</td>
<td>10 (24 h)</td>
<td>20 (3 d)</td>
<td>40 (24 h)</td>
<td>300 (35 d)</td>
</tr>
<tr>
<td>6.5</td>
<td>20 (24 h)</td>
<td>40 (10 d)</td>
<td>200 (24 h)</td>
<td>&gt; 2500 (&gt; 26 d)</td>
</tr>
<tr>
<td>7.3</td>
<td>30 (24 h)</td>
<td>&gt;250 (30 d)</td>
<td>400 (24 h)</td>
<td>&gt; 2500 (20 d)</td>
</tr>
</tbody>
</table>
Fig. 4.1 MIC of nisin for *L. monocytogenes* in TPB
4.3.2 Effect of nisin on the survival of *L. monocytogenes* and isolation of nisin-resistant mutants

Strain NCTC 5105

The effect of nisin (50 IU/ml) on the survival of *L. monocytogenes* NCTC 5105 in TPB (pH 5.5) at 30°C, during successive phases of its growth, was determined in duplicate (Fig. 4.2). At each stage of growth, addition of nisin reduced the population to below the detectable limit (< 20 cfu/ml) by the next sampling time 1 h later. This occurred even after 22 h growth at a population level of $1.4 \times 10^8$ cfu/ml. Thus it appeared that nisin-resistant mutants of *L. monocytogenes* NCTC 5105 were not obtainable.

![Fig. 4.2 Effect of nisin on the survival of *L. monocytogenes* NCTC 5105 at different phases of growth in TPB](image-url)
In a repeat experiment extra samples were taken after 48 h and 7 d incubation with nisin (Table 4.2). On these occasions viable cells were detected and each of the isolates was confirmed to be *L. monocytogenes* using the gene probe.

**Table 4.2** Effect of nisin on the survival of *L. monocytogenes* NCTC 5105 at different phases of growth in TPB, sampling 48 h and 7 d later

<table>
<thead>
<tr>
<th>Time of nisin addition (h)</th>
<th>Counts at nisin addition (cfu/ml)</th>
<th>Counts after nisin addition (cfu/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1 h</td>
</tr>
<tr>
<td>0</td>
<td>1.6 x 10^5</td>
<td>&lt; 20</td>
</tr>
<tr>
<td>2</td>
<td>1.9 x 10^5</td>
<td>&lt; 20</td>
</tr>
<tr>
<td>5</td>
<td>4.0 x 10^5</td>
<td>&lt; 20</td>
</tr>
<tr>
<td>8</td>
<td>1.6 x 10^6</td>
<td>&lt; 20</td>
</tr>
<tr>
<td>22</td>
<td>1.4 x 10^8</td>
<td>&lt; 20</td>
</tr>
<tr>
<td>No nisin added at 24 h</td>
<td>1.1 x 10^8</td>
<td>1.0 x 10^8</td>
</tr>
</tbody>
</table>

Further tests were conducted to determine whether these isolates displayed greater nisin resistance. Each was inoculated directly into TPB (pH 5.5) supplemented with nisin (50 IU/ml). After incubation at 30°C for 5 d none of the cultures showed visible growth. Low, invisible levels of growth were checked by taking plate counts after 3 days incubation and optical density (OD) readings (600 nm) after 3, 4 and 5 d incubation (Table 4.3).
Table 4.3  Growth of suspect nisin-resistant mutants of *L. monocytogenes* NCTC 5105 in nisin-containing medium

<table>
<thead>
<tr>
<th>L. monocytogenes</th>
<th>OD values (600 nm) of cultures after incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3 d (cfu/ml)</td>
</tr>
<tr>
<td>5105</td>
<td>0.011 &lt; 20</td>
</tr>
<tr>
<td>5105 Isolate 1</td>
<td>0.008 &lt; 20</td>
</tr>
<tr>
<td>5105 Isolate 2</td>
<td>0.028 40</td>
</tr>
<tr>
<td>5105 Isolate 3</td>
<td>0.006 &lt; 20</td>
</tr>
<tr>
<td>5105 control *</td>
<td>0.337 4.5 x 10^7</td>
</tr>
<tr>
<td>F6861</td>
<td>0.074 1.0 x 10^6</td>
</tr>
<tr>
<td>F6861 control *</td>
<td>0.339 1.9 x 10^8</td>
</tr>
</tbody>
</table>

* no nisin added

A low count was obtained from one isolate only (40 cfu/ml), but on repeating the above procedure with the new colonies no growth was observed. As an additional step, all isolates were subcultured on TSA and in TPB before inoculation into nisin-supplemented TPB, but still no growth was observed.

Further still, using the method of Harris et al. (1991), where serial dilutions of a population are plated on to solid medium containing increasing concentrations of nisin, nisin-resistant mutants of *L. monocytogenes* NCTC 5105 could not be isolated.

**Strain F6861**

However, continuing with the method of Harris et al. (1991), mutants with increased resistance to nisin (MIC > 600 IU/ml on TSA at pH 5.5) could be isolated from the less sensitive F6861 strain grown in TPB at pH 5.5 and pH 7.3 (Fig. 4.3).
Fig. 4.3 Isolation of nisin-resistant mutants from *L. monocytogenes* F6861

Fig. 4.4 Confirmation of nisin resistance in *L. monocytogenes* F6861 mutants
A biphasic survival curve was observed for both pH values, with the frequency of resistant mutants from $10^{-6}$ (pH 7.3) to $10^{-7}$ (pH 5.5).

To determine whether survivors were nisin-resistant mutants, or perhaps had escaped the effect of nisin, two single colonies were selected from the plate of highest nisin concentration (600 IU/ml), subcultured in TPB (pH 5.5) and re-evaluated for nisin susceptibility (Fig. 4.4). Both clones were more resistant to nisin than the original parent strain, and each was completely uninhibited at nisin levels of 600 IU/ml. Control experiments demonstrated the stability of nisin under the experimental conditions.

Subsequently it was found that the MIC of nisin to the mutant (M) in TPB (pH 5.5) was $> 1000$ IU/ml after a period of 47 d incubation at 30°C, compared with that of its wild type (WT) which had a MIC of 300 IU/ml (Fig. 4.5).

Fig. 4.5 MIC of nisin for L. monocytogenes F6861 nisin-resistant mutant in TPB at pH 5.5
4.3.3 Stability of nisin resistance in the *L. monocytogenes* F6861 mutant

Nisin resistance in the *L. monocytogenes* F6861 mutant, which had been repeatedly subcultured in nisin-containing media, was completely stable after undergoing ten passages of growth (ca. 65 generations) in nisin-free TPB (pH 7.3) (Fig. 4.6). Mutants isolated from only one exposure to nisin were also completely stable through ten passages in nisin-free TPB at pH 7.3, although at pH 5.5, after six passages, the viable number of nisin-resistant cells began to decrease (Fig. 4.7). After ten passages at pH 5.5 (ca. 65 generations) there was an overall reduction of 2.4 log cycles in the number of nisin-resistant mutants.

4.3.4 Growth and morphology of different strains of *L. monocytogenes*

Growth curves of *L. monocytogenes* NCTC 5105, F6861 wild type (WT) and its F6861 nisin-resistant mutant (M) in TPB (pH 7.3 and 5.5) at 30° and 4°C are presented in Figs. 4.8 and 4.9, respectively. The doubling times \( t_d \) (calculated from the relationship \( t_d = \log 2 \frac{(t-t_0)}{(\log N - \log N_0)} \)) and final cell concentrations (\( \log_{10} \text{cfu/ml} \)) are shown in Table 4.4.

At 30°C, *L. monocytogenes* F6861 WT and F6861 M behaved similarly. Both gave the same final cell concentration at pH 7.3 and 5.5, although the doubling time of the mutant was slightly longer than that of the wild type, particularly at pH 5.5. There were inadequate data to determine whether these differences were significant.

Strain NCTC 5105 had a distinctly longer doubling time than the other two strains at both pH values and a lower final cell concentration at pH 5.5. This slower growth rate of 5105 was also noted when the rates of colony formation on solid media were compared.
Fig. 4.6 Stability of nisin resistance in *L. monocytogenes* F6861 mutant in TPB (pH 7.3) after multiple exposures to nisin

Fig. 4.7 Stability of nisin resistance in *L. monocytogenes* F6861 mutant in TPB (pH 7.3 and 5.5) after one exposure to nisin
Fig. 4.8 Growth of *L. monocytogenes* at 30°C in TPB at a) pH 7.3 and b) pH 5.5
Fig. 4.9 Growth of *L. monocytogenes* at 4°C in TPB at a)pH 7.3 and b)pH 5.5
Table 4.4  Growth parameters of *L. monocytogenes* NCTC 5105, F6861 WT and F6861 M in TPB (pH 7.3 and 5.5) at 30° and 4°C

<table>
<thead>
<tr>
<th>L. monocytogenes strain and pH of growth</th>
<th>Doubling times (t_d) (h)</th>
<th>Final cell concentration (log_{10} cfu/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>30°C</td>
<td>4°C</td>
</tr>
<tr>
<td>NCTC 5105</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH 7.3</td>
<td>1</td>
<td>83.93</td>
</tr>
<tr>
<td>pH 5.5</td>
<td>1.23</td>
<td>NG</td>
</tr>
<tr>
<td>F6861 WT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH 7.3</td>
<td>0.73</td>
<td>38.63</td>
</tr>
<tr>
<td>pH 5.5</td>
<td>0.77</td>
<td>NG</td>
</tr>
<tr>
<td>F6861 M</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH 7.3</td>
<td>0.78</td>
<td>42.37</td>
</tr>
<tr>
<td>pH 5.5</td>
<td>0.95</td>
<td>NG</td>
</tr>
</tbody>
</table>

NG: no growth

At 4°C, pH 7.3, F6861 M had a slightly longer doubling time than its wild type but both gave the same final cell concentration. Strain 5105, however, had a much longer doubling time and lower final cell concentration at this temperature. It was noted that each cell population formed a sediment at the bottom of the culture vessel and that F6861 M formed a more dispersed pellet of cells than its wild type.

At 4°C, pH 5.5, no growth was apparent and the initial cell concentration of each strain remained constant over a period of 50 d.

Microscopic examination of each strain (Gram stained using oil immersion) showed no obvious difference between strains (Plates 4.1, 4.2 and 4.3).
Plate 4.1 Gram stain of *L. monocytogenes* NCTC 5105 under oil immersion (magnification x 2000)
Plate 4.2  Gram stain of *L. monocytogenes* F6861 wild type under oil immersion (magnification x 2000)
Plate 4.3  Gram stain of *L. monocytogenes* F6861 nisin-resistant mutant under oil immersion (magnification x 2000)
4.4 DISCUSSION

The variability of nisin sensitivity between *L. monocytogenes* strains initially reported by Mohamed *et al.* (1984) and Benkerroum and Sandine (1988) was observed. Determination of nisin MIC values for *L. monocytogenes* on TSA confirmed the sensitivity of *L. monocytogenes* NCTC 5105 and the relative resistance of *L. monocytogenes* F6861 to nisin reported by Ferreira and Lund (1991).

The difference in nisin sensitivity between the two strains was also apparent in TPB (Fig. 4.1), although after 24 h growth nisin had a greater inhibitory effect in TPB than on TSA. This was especially evident with strain F6861. This phenomenon might be accounted for by the difference in properties of each medium, with cells being more accessible to nisin in a liquid than a solid matrix.

Mohamed *et al.* (1984) noted that the effectiveness of nisin was greater the more acidic the pH. This trend was also apparent here with *L. monocytogenes* having increased sensitivity to nisin as the pH decreased from 7.3 to 6.5 to 5.5. Mohamed *et al.* (1984) partially attributed the effect to increased stability of nisin at acid pH as compared with alkaline or neutral pH where nisin is relatively insoluble and unstable (Hurst, 1983). However, the authors referred to earlier work (Mohamed *et al.* 1981) where they had reported that nisin in salt concentrations of 6, 7 and 8%, at pH 7.4, was as stable as in acidic medium (pH 5.5), but with less activity, which indicates that the activity of nisin is distinct from its stability. Harris *et al.* (1991) detected no difference in nisin stability at pH 6.5 compared with pH 5.5 using solid media, thus indicating the greater effectiveness of nisin at pH 5.5 to pH 6.5 is a genuine phenomenon. Results from this present study confirm the stability of nisin in solid media at pH 5.5 for at least 7 d (page 54) although this was not determined at the higher pH values.

At nisin concentrations below the MIC value, the time interval to visible growth of both strains depended on the nisin concentration. The higher the nisin concentration
the longer before turbidity was apparent. These findings correspond with previous reports of the growth of *L. monocytogenes* in broth with nisin after a lag of a few days (Mohamed *et al.*, 1984; Doyle, 1988).

At pH 5.5, it is unlikely that growth of F6861 in TPB containing < 300 IU/ml nisin was due to the presence of nisin-resistant mutants. This is because the frequency of resistance acquisition in TPB at pH 5.5 is *ca.* $10^{-7}$ (Fig. 4.3) and so with an inoculum size of *ca.* $10^6$ cfu/ml, the chance of mutant cells being present was small. Also, nisin-resistant mutants of strain F6861 can grow in TPB (pH 5.5) at nisin concentrations of > 1000 IU/ml (Fig. 4.5). It is more probable that the cells were sub-lethally injured after contact with low concentrations of nisin with recovery giving an extended lag phase and/or slower growth rate. If some cells had escaped the effect of nisin due to its depletion by adsorption to other cells, such long lag phases would be unlikely since a single healthy cell could produce turbidity within 24 h at a doubling time of 0.77 h (page 59).

At pH 7.3 and high nisin concentrations, it is possible that nisin-resistant mutants of strain F6861 became established upon extended incubation whilst the wild type cells of the population, with a naturally lower nisin resistance, were eliminated. This is because the nisin resistance frequency of F6861 in TPB at pH 7.3 is *ca.* $10^{-6}$ (Fig. 4.3) and with an inoculum size of *ca.* $10^6$ cfu/ml, the chance of mutant cells being present is better than at pH 5.5. An alternative explanation might be the instability of high levels of nisin at pH 7.3 over a long incubation period. Mohamed *et al.* (1984) showed that low levels of nisin (8 IU/ml) in broth at pH 7.4 became unstable during an incubation period of 3 d. In this case, growth would be due to the F6861 wild type. These two alternatives could have been distinguished if the culture grown at 2500 IU/ml nisin had been enumerated on nisin plates to see whether it had high nisin resistance at low pH and was, therefore, a nisin resistant mutant of the wild type. This was not done however.

The same phenomenon was observed at pH 6.5, with growth occurring at the same
high nisin concentration (2500 IU/ml). Harris et al. (1991) reported nisin to be stable for at least 4 d at this pH (but considerably longer incubation periods were used here). This suggests that nisin-resistant mutants were probably responsible for the observed growth.

Consequently, it was difficult to define exact nisin MIC values for *L. monocytogenes* F6861 wild type in TPB at pH 6.5 and 7.3. A lower initial population level (< $10^5$ cfu/ml) would have lowered the probability of mutants, but this was not known at the time of the experiment.

Growth of strain NCTC 5105 was inhibited by very low concentrations of nisin at pH 5.5 and 6.5 (10-40 IU/ml) but at pH 7.3, after 30 d incubation, its MIC was > 250 IU/ml. Subsequently, it was not possible to isolate mutants from NCTC 5105 at pH 5.5 but it is not known whether they can develop at pH 7.3.

Suspect nisin-resistant mutants of NCTC 5105 isolated after exposure to nisin (50 IU/ml) in TPB were later shown, on further exposure to nisin, to be nisin sensitive. This raised the question of how wild type cells of NCTC 5105 had survived the effect of nisin initially. The possibility of contamination was eliminated as each of the isolates was positively identified as *L. monocytogenes* using the gene probe. Nisin stability in TSA at pH 5.5 for 7 d has been confirmed, so it is likely to be stable in TPB under the same conditions. As suggested previously for strain F6861, it is unlikely that some cells escaped the effect of nisin due to depletion by adsorption to other cells. If this had been the case, stationary phase would have been reached by 24 h ($t_d$ of 1.23 h) giving ca. $10^8$ cfu/ml as opposed to the low numbers isolated ($10^2$-$10^3$ cfu/ml).

It is possible that with a high concentration of cells (ca. $10^8$ cfu/ml) and a low concentration of nisin (50 IU/ml), even though the majority of cells were killed, some were only sub-lethally injured. Survival would result from adsorption of lower quantities of nisin after depletion by other cells. Recovery from sub-lethal injury
would reduce the growth rate and explain the low number of cells isolated. On re-exposure of these cells to nisin death was inevitable because they did not possess inherent nisin resistance. Thus it was shown that not all strains of *L. monocytogenes* are capable of producing nisin-resistant mutants.

The next objective was to look at the effect of nisin on strain F6861, at different phases of growth, in an attempt to isolate F6861 nisin-resistant mutants. However, at this stage, parallel work by Harris *et al.* (1991) was published. The authors had succeeded to isolate mutants resistant to 50 µg/ml (1850 IU/ml) nisin at pH 6.5 from *L. monocytogenes* ATCC 19115, Scott A and UAL500 at frequencies of 10⁻⁶ to 10⁻⁸ using a direct plating method. Sensitivity to nisin was enhanced by reduction of the agar pH from 6.5 to 5.5 with hydrochloric or lactic acid. At a nisin concentration of 10 µg/ml (370 IU/ml), the resistance frequency of Scott A dropped from 10⁻⁵ at pH 6.5 to 10⁻⁸ at pH 5.5. Thus, it was decided to use this method as nisin-resistant mutants are actually isolated in the presence of nisin.

Using this method, mutants with increased resistance to nisin (MIC > 600 IU/ml on TSA at pH 5.5) could only be isolated from the less sensitive F6861 strain (Fig. 4.3). The detection frequency of these nisin-resistant mutants was dependent on the pH of the overnight broth culture in which they were grown and ranged from *ca.* 10⁻⁶ at pH 7.3 to *ca.* 10⁻⁷ at pH 5.5. This might be explained by the additional stress factor of acid pH. The lower mutant frequency of Scott A (10⁻⁸) (Harris *et al.*, 1991) compared with F6861 (10⁻⁶) on solid media at pH 5.5 might be a reflection of the different strains used. However, it might also reflect a slight difference in the methods used. It was noted that for colony formation of nisin-resistant mutants the incubation time was critical to the total colony count. Colony forming units can more than double in number from 2 to 7 d incubation due to the slower growth of some colonies. Harris *et al.* (1991) incubated their mutants for up to a period of 4 d only, but might have obtained higher total colony counts on further incubation thus increasing the resistance frequency.
Recent work by Ming and Daeschel (1993) has also examined nisin MIC values and resistance frequencies of *L. monocytogenes* Scott A and Jalisco. It is not known whether this Jalisco strain is the same as F6861 used in the present study as several strains were isolated at the time of the listeriosis outbreak, both from patients and the cheese itself (George *et al*., 1988). Ming and Daeschel (1993) found that both the Scott A and Jalisco strains had the same nisin MIC of 200 IU/ml, determined in broth and recorded after 24 h at 37°C. The nisin resistance frequencies, calculated at a nisin concentration of 400 IU/ml, were, however, dependent on strain type with 1.7 x 10^-6 for Scott A and 9.2 x 10^-6 for Jalisco. It is difficult to make any direct comparison with the present study or that of Harris *et al.* (1991), because the authors presumably conducted this work at pH 7.4 (they used brain heart infusion broth and agar) although it was not specified. Also, the nisin resistance frequencies were determined after an incubation period of 24 h only. However, these results indicate that their *L. monocytogenes* Jalisco strain has a higher resistance frequency than Scott A, as does F6861.

Nisin resistance in the *L. monocytogenes* F6861 mutant was completely stable after undergoing ten passages (ca. 65 generations) in nisin-free broth at pH 7.3 (Figs. 4.6 and 4.7). This was independent of whether the mutants were isolated from only one exposure to nisin or had been repeatedly subcultured on nisin-containing media. When grown in nisin-free broth at pH 5.5, stability of nisin resistance in the F6861 mutant after one initial exposure to nisin decreased gradually only after six passages with an overall reduction of 2.4 log cycles after ten passages (Fig. 4.7). This reduction in stability at pH 5.5 might be accounted for by the additional stress factor of acid pH. However, Ming and Daeschel (1993) found that stability of nisin resistance in mutants of *L. monocytogenes* Jalisco and Scott A, after one exposure to nisin, was lost after three passages in nisin-free broth, with the level of nisin resistance having reverted back to the original MIC. Meanwhile, a nisin-resistant mutant isolated from *L. monocytogenes* Scott A after stepwise exposure to increasing concentrations of nisin, had stable resistance after three passages (ca. 30-35 generations) through nisin-free media. The difference in nisin stability, after one exposure to nisin, between their
strains and strain F6861 might indicate a difference between each type of mutation.

On comparing the growth characteristics of each *L. monocytogenes* strain (Figs. 4.8 and 4.9) it was evident that NCTC 5105 was the least robust having longer doubling times and generally lower final cell concentrations. The weakness of this strain is reflected by its high sensitivity to nisin. *Listeria monocytogenes* F6861 wild type and its F6861 mutant behaved similarly in the fact that they reached the same final stationary phase cell concentrations, but the doubling time of the mutant was slightly longer, particularly under conditions of stress such as acid pH (5.5) and low temperature (4°C). The fact that at 4°C (pH 7.3) both strains formed a sediment at the bottom of the culture vessel with the F6861 mutant forming a more dispersed pellet of cells than its wild type might suggest a difference in their density and surface properties and hence cell composition.

Ming and Daeschel (1993) stated that the specific growth rate (k) of their Scott A resistant mutant was only slightly lower than that of the parent at 37°C, whereas at the sub-optimal temperature of 20°C, the mutant’s k was significantly decreased (40.9% that of the parent). The authors suggested that at low temperatures, the membrane of the resistant mutant may be less flexible than that of the parent strain (due to its higher phase transition temperature) and so the transport of nutrients may be affected, leading to the slower growth rate. However, in the present study, at an even lower temperature of 4°C, the specific growth rate of the F6861 mutant was only slightly lower than that of its parent strain, at 92.3%. Ming and Daeschel (1993) calculated their specific growth rates from optical density readings at 600 nm and as the turbidity of bacteria can only be detected at a level of 10^7 cfu/ml or higher, it is again difficult to make a comparison with the present study. An alternative possible explanation for the slightly slower growth rate observed for the F6861 mutant as compared with its wild type might be that the adaptation of the mutant for nisin resistance could have put an extra burden on it biochemically. This was seen by the slightly longer doubling time of the mutant under the optimum conditions of pH 7.3 and 30°C. Under added conditions of stress, such as low temperature and pH, the
slower doubling time of the mutant was more pronounced.

From the work described in this chapter, it can be concluded that nisin-resistant mutants of *L. monocytogenes* are likely to arise when nisin is used as an antimicrobial in food. This occurs at low frequency and would, therefore, be most favoured given conditions such as high levels of contamination; high storage temperatures allowing rapid growth; long shelf lives at low temperatures and/or low levels of nisin. Good hygienic practices would minimise some of these factors. However, understanding the mechanism of nisin resistance might suggest alternative ways of overcoming this problem, perhaps through biochemical adaptation of the nisin molecule or its use in synergistic combinations with other antimicrobials.
4.5 SUMMARY

Listeria monocytogenes NCTC 5105 and F6861 were evaluated for sensitivity to nisin. The results confirmed those previously published indicating marked differences in the sensitivity of the two strains. NCTC 5105 had a MIC of 10-40 IU/ml compared with 200-400 IU/ml for F6861 on solid media at pH 5.5 and 30°C.

Mutants with increased resistance to nisin could be isolated only from the less sensitive F6861 strain at a frequency of $10^{-6}$ to $10^{-7}$. The MIC of the mutant strain in broth at pH 5.5 and 30°C was > 1000 IU/ml as compared with 300 IU/ml for its parent strain.

The nisin resistance phenotype of the L. monocytogenes F6861 mutant strain was completely stable after undergoing ten passages of growth (ca. 65 generations) in nisin-free media at pH 7.3, although at pH 5.5 there was an overall reduction of 2.4 log cycles in the number of nisin-resistant mutants after ten passages.

The growth characteristics of both L. monocytogenes F6861 wild type and mutant strains were similar in the fact that they reached the same final stationary phase cell concentrations, but the doubling time of the mutant was slightly longer, particularly under conditions of stress such as acid pH (5.5) and low temperature (4°C). Strain NCTC 5105 had longer doubling times and generally lower final cell concentrations. There was no obvious difference in the basic morphology of each strain.
CHAPTER 5  ADSORPTION OF NISIN BY LISTERIA MONOCYTOGENES
5 ADSORPTION OF NISIN BY LISTERIA MONOCYTOGENES

5.1 INTRODUCTION

It has become widely established that the primary target of nisin in sensitive cells is the cytoplasmic membrane. Nisin is incorporated into the membrane to form ion channels or pores (Henning et al., 1986b; Sahl et al., 1987; Kordel et al., 1989, Gao et al., 1991) through which efflux of K⁺, ATP and amino acids results in destruction of the membrane potential (Ruhr and Sahl, 1985; Kordel and Sahl, 1986) leading to collapse of the proton-motive force (Bruno et al., 1992; Okereke and Montville, 1992). Sahl (1991) and Benz et al. (1991) have proposed a model of the mode of action of Type A lantibiotics. They suggested that in the first step the peptides bind to the membranes leading to increasing concentrations of the peptides at the membrane as compared with the concentration in solution. It was further suggested that oligomerization and formation of peptide pre-aggregates occurs at the membrane before they are incorporated to form transient multistate pores.

As it has been shown, resistance of L. monocytogenes to nisin varies between strains (Chapter 4). The resistance mechanism(s) could involve: (1) the enzymatic degradation of nisin; (2) a change in cell wall permeability or (3) modification or non-availability of the target site. These may operate independently or in combination.

By measuring the amount of nisin that adsorbs to cells, as opposed to that remaining in solution, it could be determined whether the variation in nisin sensitivity of the different L. monocytogenes strains was related to their affinity for nisin. The effect of different nisin concentrations on adsorption could also be determined. In this vein the first insight into the resistance mechanism(s) of L. monocytogenes might be achieved.

Location of nisin in the cell membrane, if present, could be visualized using immunogold labelling and transmission electron microscopy (TEM). By comparison
of nisin-sensitive and nisin-resistant strains of *L. monocytogenes*, it may be possible to determine whether nisin reaches the target site in each type of cell (even if binding does not occur in resistant cells), or whether a mechanism of exclusion or degradation is in operation. In the event of exclusion or degradation of nisin, immunogold labelling alone could not distinguish the two possibilities.

Thus, the objective of this study regarding nisin adsorption by *L. monocytogenes* was to:

(1) Determine the adsorption of nisin to the different strains of *L. monocytogenes* by means of an enzyme-linked immunosorbent assay (ELISA) and bioassay.

(2) Use immunogold labelling and TEM to locate nisin at its site of action in sensitive cells and to see whether it was absent or present (and if so to what extent) in resistant cells.
5.2 MATERIALS AND METHODS

5.2.1 Production of polyclonal sheep anti-nisin antibodies

Affinity purified anti-nisin immunoglobulin (IgG) was prepared for use in both the nisin adsorption assay, determined by means of a nisin ELISA, and gold immunocytochemical labelling.

Antiserum production

Antiserum was raised in a Suffolk sheep with nisin conjugated to egg albumin using the glutaraldehyde condensation technique (Reichin et al., 1968). Basically, this involved mixing 1 mg of nisin (Chapter 3) dissolved in 50 µl of 0.02 M HCl with 2 mg of egg albumin (Sigma) dissolved in 1 ml of distilled water. Conjugation was achieved by adding 100 µl of a 1 in 10 (v/v) dilution of a 25 % aqueous solution of gluteraldehyde (Sigma). This constituted the aqueous phase. For the initial priming injection, 200 µl of BCG (Guildhay Antisera, Guildford, UK) was also added, but this was omitted for subsequent boost injections.

The conjugate was emulsified with non-ulcerative Freund's adjuvant (Guildhay Antisera, Guildford, UK) at a ratio adjuvant:immunogen, 2:1 using two 5 ml syringes and a double hub connector. This procedure involved pushing the aqueous phase into the oil phase, very slowly approximately 50 times, after ensuring that all air bubbles were removed. To test for complete emulsion a drop of the preparation was placed in water and if dispersion of the droplet occurred then further mixing was required. When the droplet remained intact, emulsification had completed meaning that the preparation should remain stable when injected intramuscularly.

Serum used for the assay was obtained 7 d after the first boost at 9 months and subsequently from boost injections administered after the serum antibody titre had
declined. Protocol for determination of the serum antibody titre is presented in Appendix II. Serum samples collected were preserved with 0.1% (w/v) sodium azide (Sigma) and stored indefinitely at 4°C. IgG was prepared from whole serum by the method of Hurn and Chantler (1980) and immunospecific antibody was isolated by affinity chromatography, described as follows.

**Preparation of immunoglobulin fractions from whole serum**

This was conducted by precipitation with rivanol and ammonium sulfate according to the method of Hurn and Chantler (1980):

The antiserum was adjusted to pH 8.5 by careful addition of 0.1 N NaOH. For each 10 ml of antiserum 35 ml of rivanol solution (0.4%)(Sigma) was added dropwise from a separating funnel. The serum was stirred gently on a magnetic stirrer throughout. The supernatant (containing the immunoglobulins) was decanted into universal bottles and centrifuged in a bench centrifuge to remove the remaining sediment. The supernatant was then decanted into a conical flask and activated charcoal added (1-1.5 g/100-ml)(Sigma) to decolorize the solution. This was gently agitated for ca. 10 min. The charcoal was removed from the protein solution by filtration through a double layer of moistened filter paper (Whatman No. 42) in a Büchner funnel. The filtrate was transferred to a beaker and an equal volume of saturated ammonium sulfate solution (542 g/l) was added dropwise from a separating funnel, with gentle stirring on a magnetic stirrer throughout. When all the ammonium sulfate solution had been added, the beaker was placed at 4°C for at least 6 h to allow the immunoglobulin precipitate to flocculate. Centrifugation was conducted at 4000 g for 20 min, in a refrigerated centrifuge, and the supernatant discarded. The precipitate was dissolved in a volume of saline approximately equivalent to half the volume of the original antiserum. The immunoglobulin solution was placed in visking tubing (The Scientific Instrument Centre Ltd, London) and dialyzed (at 4°C) extensively against several changes of saline to remove sulfate ions. The preparation
was then stored at 4°C prior to affinity chromatography.

**Affinity chromatography**

Nisin (200 mg) (Chapter 3) was dissolved in 5 ml, 0.1 M acetate buffer (pH 4.5) and mixed with 1 g activated glass (gift, P. Kwasowski) at room temperature for 4 h. Solid glycine (Sigma) was added to yield a final concentration of 0.1 M, and mixing continued at room temperature for 4 h. The matrix was left at 4°C overnight before packing into a column. The column was washed through with an excess of distilled water followed by 0.1 M glycine-HCl (pH 2.2) and distilled water. The column was equilibrated with phosphate-buffered saline (PBS) (0.15 M, pH 7.4).

The IgG fraction was applied to the column, which was washed through with PBS until all non-specific protein had been removed (OD<sub>280</sub> < 0.05). Nisin-specific antibody was eluted in 1.5-ml fractions with 0.1 M glycine-HCl (pH 2.8). Fractions were neutralized with 2 M Tris (pH 7.0). The concentration of anti-nisin IgG was calculated using the equation:

\[
(IgG) \text{ mg/ml} = \frac{A_{280} \times \text{dilution factor}}{1.34}
\]

Fractions containing specific antibody were pooled and dialysed at 4°C against several changes of PBS. Samples (containing 1 mg of nisin-specific IgG) were freeze-dried for storage at -70°C and resuspended in 1 ml of PBS when required.

**5.2.2 Nisin adsorption assay**

Overnight cultures of cells, grown to a concentration of ca. 10<sup>9</sup> cfu/ml in TPB (pH 7.3) were adjusted with TPB to the same optical densities (OD) at 600 nm. The
cell concentration was determined using spread plates prepared from decimal serial dilutions in MRD (Chapter 3). Cells were harvested from suspension by centrifugation (1500 g for 15 min); washed twice in 5 ml of phosphate-buffered saline (0.15 M, pH 7.4) containing 0.1% (w/v) gelatin (Sigma)(PBSG), and resuspended to the original volume in PBSG. Plate counts were determined again at this stage to check for cell loss during this procedure.

The cell suspension (8 ml, ca. $10^9$ cfu/ml) was mixed with nisin solution (0.8 ml)(see section 5.3.1) to give the required final concentration (10-1000 IU nisin/ml). Cell-free controls consisting of buffer plus nisin were set up simultaneously. After incubation at 30°C for 15 min, the cells were removed by centrifugation. The nisin concentration in the supernatants was determined using a nisin enzyme-linked immunosorbent assay (ELISA) as described by Falahee et al. (1990) or by the nisin bioassay (Fowler et al., 1975), as specified. Relevant dilutions of the supernatants, controls and standards were made in PBSG that contained 0.05% (v/v) Tween 20 (Sigma)(PBSGT).

[Note: PBSG was used in the protocol as opposed to PBS in order to keep the conditions as similar to the ELISA as possible. PBSGT was not used other than for dilution of the test samples due to the fact that Tween 20 has detergent-like properties and adversely affects nisin adsorption to cells. Both buffers were made up with sterile water but had to be filter sterilised for the bioassay procedure in order to avoid contamination].

5.2.3 The nisin ELISA

A nisin ELISA using polyclonal antiserum was used, as developed by Falahee et al. (1990). The method has a limit of detection of $1.9 \times 10^{-2}$ IU/ml. Affinity-purified antinisin IgG was obtained as described (section 5.2.1) and conjugated to horseradish peroxidase (Sigma type VI-A) as indicated subsequently. ELISA microtitration plates (Nunc Immunoplate I; Gibco, Paisley, UK) were washed (Titertek S8/S12 plate
Conjugation of anti-nisin IgG to horseradish peroxidase

The specific anti-nisin IgG fraction was prepared for conjugation by dialysis at 4°C against two changes each of 1 mM sodium acetate (adjusted to pH 5 with glacial acetic acid), PBS and 0.1 M carbonate/bicarbonate buffer at pH 9.5.

Anti-nisin IgG was conjugated to horseradish peroxidase as described by Beyzavi et al. (1987). Basically, 5 mg of horseradish peroxidase was dissolved in 1 ml of distilled water before addition of 0.2 ml of 0.1 M sodium periodate (Sigma). The solution was mixed at room temperature for 30 min and then dialysed against 1 mM sodium acetate buffer (pH 4.4) overnight at 4°C. A volume containing 10 mg of affinity-purified antibody in 0.1 M carbonate/bicarbonate buffer (pH 9.5) was mixed with the enzyme for 2 h at room temperature. Sodium borohydride (5 mg)(Sigma) was added and the solution allowed to stand for 2 h at 4°C. The solution was then dialysed against two changes of PBS (2 l). The conjugate was then freeze-dried for storage at -70°C and rehydrated in 200 μl of PBS when required.

ELISA protocol

Microtitration plates were prewashed by soaking in coating buffer (0.1 M carbonate-bicarbonate, pH 9.6) for 15 min. Wells were coated with affinity-purified anti-nisin IgG (5 μg/ml) in coating buffer (0.2 ml/well). After a 2 h incubation at 37°C the plates were washed three times with PBS (0.15 M, pH 7.4) containing 0.1% (w/v) gelatin and 0.05% (v/v) Tween 20 (PBSGT). Subsequently they were blocked for 1 h with casein buffer (g/l: NaCl, 9; Tris-aminomethane (Sigma), 1.2; casein (Sigma), 5)(pH 7.6) at 37°C and washed three times with PBSGT. Samples, diluted in PBSGT...
(0.2 ml/well), were added and incubated at 4°C for 16 h. After washing three times with PBSGT, 0.2 ml of the horseradish peroxidase conjugate (diluted 1:20000 (v/v) in PBSGT) was added to each well and the plates incubated at 37°C for 2 h. The plates were washed as before and 0.15 ml substrate solution (0.4 mg/ml o-phenylene diamine (Sigma) in 0.024 M citrate-0.05 M phosphate buffer (pH 5.0) containing 0.04% (v/v) hydrogen peroxide (Sigma)) added. After 30 min at 37°C the reaction was stopped by the addition of 0.05 ml of 2.5 M H₂SO₄. The absorbances were read at 492 nm.

The protocol to determine optimal antibody-coating level and optimal conjugate dilution is presented in Appendix III.

**ELISA conditions for the nisin adsorption assay**

For each separate determination, the supernatants, control and standard curve (in the range 0-1.0 IU/ml) were assayed in duplicate on the same ELISA plate and the mean values used. The nisin content was determined for those supernatant dilutions with absorbances corresponding to the linear portion of the standard curve and the mean value of at least three dilutions was taken. The correlation coefficient of each standard curve was ≥ 0.995.

**Statistical analysis**

The results of the nisin adsorption experiments by means of the ELISA were analysed statistically using the Kolmogorov-Smirnov Goodness of Fit Test to show that the data were normally distributed and Student’s t-test to show a difference between the means at each nisin concentration.
5.2.4 The nisin bioassay

The bioassay method of Fowler et al. (1975) was used as follows:

The bioassay medium and test organism (*Micrococcus luteus*) used were described in Chapter 3. The inoculum of test organisms was prepared by emulsifying the growth from a slope culture with 10 ml of quarter-strength Ringer solution (BDH). The suspension of cells was adjusted to give a transmission of 50% (equivalent to an optical extinction of 0.3) in a 10 mm cuvette at 650 nm. To the melted and tempered assay medium was added 2% (v/v) of a 1 + 1 dilution of Tween 20 which had been held for 20-30 min at 50 ± 2°C. The medium was mixed thoroughly and 2% (v/v) of the inoculum was added to the medium. After mixing well, the medium was left for a few minutes in the tempering bath, to permit dispersion of the foam, and the medium was then poured into a sterile, previously labelled assay plate to give a depth of 3-4 mm agar. After the agar had set, the plate was inverted and stored at 4-7°C for 1 h to facilitate the cutting of wells. Wells were punched in the agar using a cork borer or other suitable punch of 7-9 mm diameter, allowing at least 30 mm between adjacent wells and between peripheral wells and the edge of the assay plate. The discs of agar were removed from the plate using a sterile mounted needle. Equal volumes (0.1-ml) of standard and test solutions were delivered into the wells (as indicated subsequently) and the plate incubated at 30 ± 1°C for 18-20 h.

Bioassay conditions for the nisin adsorption assay

Nisin standards were prepared at 5, 10 and 20 IU/ml, the correlation coefficient of the standard curve being 0.994. A latin square was used for random distribution of the samples and the mean value of 6 replicates taken for each sample. For each replicate, the diameter of zones of inhibition was measured twice at right angles and the mean value used.
5.2.5 Localization of nisin in *L. monocytogenes* by gold immunocytochemical labelling

Treatment of cells with nisin, fixation and resin embedment

*Listeria monocytogenes* 24-h cultures were prepared in TPB at pH 5.5 (9.9 ml) in duplicate. Nisin solution (100 µl)(Chapter 3) was added to only one of each pair of cultures at the required concentration. The remaining cultures were used as controls. After a 1-h incubation at 30°C, 1-ml samples of each culture were taken, placed in eppendorf tubes and centrifuged at 2500 g for 10 min in order to obtain cell pellets. The supernatants were discarded and the cells fixed for 2 h in a 3% (v/v) solution of glutaraldehyde (Sigma, EM grade) in 0.1 M sodium cacodylate buffer (BDH). Dehydration of the cells was then conducted in a graded series of ethanol, which consisted of 15-min steps in 30%, 50% and 70% (v/v) ethanol followed by two, 15-min steps in 100% ethanol and two, 30-min steps in a mixture of 1:1 (v/v) LR white (hard)(Agar Scientific) to 100% ethanol. Finally, the samples were given two further changes in pure LR white over a 24-h period at 4°C to ensure complete penetration of the resin. The low temperature was necessary to avoid premature polymerization of the resin. The cells were then transferred to Beem capsules (gift, Micro Structural Studies Unit (MSSU), Surrey University) and fresh LR white added again before curing at 60°C for 24 h.

Ultrathin sections of the embedded bacteria were cut (60-70 nm) using a microtome and mounted on uncoated copper electron microscopy grids (gift, MSSU).

Immunogold labelling

Careful handling of the grids was imperative and conducted with the use of finely tipped forceps which were rinsed thoroughly after use to avoid cross contamination. All buffers and washing solutions were filter sterilized (0.2 µm, Minisart NML,
Sartorius). The post-embedding immunogold labelling technique of Slot and Geuze (1984) was used as recommended by Beesley (1989).

The mounted grids were floated sequentially, section side downwards, on droplets (100-μl for buffers and 25-μl for antibody and gold probe) of the following reagents placed on wax sheets:

Phosphate buffered (0.01 M, pH 7.2) saline (0.15 M) containing 1% (w/v) bovine serum albumin (Sigma)(PBS BSA) for 5 min; phosphate buffered saline (PBS) containing 1% (w/v) gelatin (Sigma) for 10 min; PBS containing 0.02 M glycine (Sigma) for 3 min; suitable dilution of antibody (purified sheep anti-nisin IgG was used, prepared as described in section 5.2.1) in PBS BSA for 1 h; five rinses in PBS BSA for 1 min each; suitable dilution of gold probe (donkey anti-sheep IgG conjugated with gold at a particle size of 10 nm was used)(Biocell, Cardiff, UK) in PBS BSA for 1 h; 1 rinse in PBS for 1 min; fixed with 1% (v/v) glutaraldehyde (Sigma, EM grade) in PBS at room temperature for 3 min; five rinses in milli-Q water for 1 min each.

Additional controls were set up during the procedure by omitting the addition of antibody (PBS BSA only).

The sections were stained with uranyl acetate and lead citrate and later examined using a Philips 400T transmission electron microscope.
5.3 RESULTS

5.3.1 Assay of nisin adsorption by *L. monocytogenes* determined by means of a nisin ELISA and bioassay

a) The nisin ELISA

i) Determination of a high percentage recovery of nisin from control samples using the nisin ELISA

After completion of the first nisin ELISA adsorption assay, when a low concentration of nisin was used (20 IU/ml), the percentage recovery of nisin from the control samples (no cells) was lower than expected (ca. 33%) which gave cause for concern. Three possible explanations were proposed: (1) low levels of nisin were unstable at pH 7.4 (Mohamed *et al.* 1984); (2) nisin had adsorbed to the glassware or (3) nisin had been retained in the filter sterilization apparatus.

The first possibility was considered unlikely due to the short incubation time (15 min) of nisin. Mohamed *et al.* (1984) have shown that nisin instability at pH 7.4 does not occur that quickly as when residual nisin concentrations were measured over an incubation period of 24 h at 37°C, the detectable level of nisin decreased from *ca.* 7.5 IU/ml to 5 IU/ml.

To examine the second possibility, the ELISA was conducted with controls only, which had been prepared under different conditions. Glassware was coated with either of the blocking agents casein (0.5% in buffer) or gelatin (0.5% in PBS) prior to use or left untreated. The untreated glassware was rinsed with PBSGT after incubation with nisin in order for the detergent-like properties of Tween 20 to remove any residual nisin remaining adsorbed to the glass. The percentage recovery of nisin from the controls after the various glassware treatments is shown in Table 5.1.
Table 5.1 Recovery (%) of nisin from controls after various glassware treatments

<table>
<thead>
<tr>
<th>Treatment of glassware</th>
<th>Recovery (%) of nisin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein blocked</td>
<td>33.05</td>
</tr>
<tr>
<td>Gelatin blocked</td>
<td>33.10</td>
</tr>
<tr>
<td>Unblocked</td>
<td>37.05</td>
</tr>
<tr>
<td>PBSGT rinsed</td>
<td>0</td>
</tr>
</tbody>
</table>

There was no significant difference between each treatment with similar low levels of nisin being recovered. Furthermore, after rinsing the unblocked glassware with PBSGT no nisin was recovered. It was now evident that unrecovered nisin had not become adsorbed to the glassware.

The third possible explanation was tested by omitting the filter sterilization step and alternatively making the nisin solution with sterile water. This time a much higher recovery of the control was obtained (77.75%) suggesting that nisin had previously been lost through adsorption to the filter even though it was supposedly low protein-binding. Thus all further adsorption experiments for ELISA assay were conducted without the filter sterilization of nisin but with sterile water being used.

However, when much higher concentrations of nisin were filter sterilized the percentage recovery was good (76.87%) indicating that any loss of nisin is far more significant at low concentrations. It was realised at this stage that to minimize any loss of nisin, filter sterilization should be conducted with nisin stock solution only (50 x 10^3 IU/ml) and not from subsequent dilutions. For bioassay purposes, filter sterilization (of nisin stock solution) was used to avoid the possibility of contamination.
ii) ELISA assay results

The affinity of the different *L. monocytogenes* strains for nisin was determined by measuring residual nisin levels in solution after exposure to cells. Results of triplicate experiments with *L. monocytogenes* NCTC 5105 and F6861 at four different nisin concentrations are presented in Fig. 5.1. At each concentration, the sensitive 5105 strain adsorbed more nisin than the more resistant F6861 strain. This was most marked at the lower nisin concentrations of 10 and 20 IU/ml where adsorption by F6861 was significantly lower than adsorption by 5105 (*P* < 0.02) in all experiments. At higher nisin concentrations, only in two out of three experiments at 100 IU/ml and at 200 IU/ml did the more resistant strain show significantly lower adsorption (*P* < 0.05).

![Graph showing adsorption of nisin by *L. monocytogenes* NCTC 5105 and F6861](image)

**Fig. 5.1** Adsorption of nisin by *L. monocytogenes* NCTC 5105 and F6861 (Standard error of mean indicated)
The same pattern was seen when nisin adsorption to F6861 was compared with that of its more resistant mutant which consistently adsorbed less nisin ($P < 0.05$). With nisin solutions containing 1000 IU/ml, F6861 adsorbed on average 33% of the nisin in solution while the resistant mutant adsorbed only 19%.

b) The nisin bioassay

Nisin levels were routinely determined using the nisin ELISA but a similar effect was seen when nisin was measured using the bioassay procedure. At a nisin concentration of 20 IU/ml, the sensitive 5105 strain adsorbed 23% of the nisin in solution while F6861 adsorbed only 6% (determined as a % of the control, as in the ELISA).

5.3.2 Localization of nisin in *L. monocytogenes* by gold immunocytochemical labelling

The procedure was initially conducted at a nisin concentration of 20 IU/ml for *L. monocytogenes* NCTC 5105 and F6861, and repeated again at a higher nisin concentration of 200 IU/ml for both strains plus the F6861 nisin-resistant mutant.

The optimum nisin-specific antibody concentration was found to be a 1:1000 dilution of the antibody stock solution (1 mg/ml) and a suitable dilution of the gold probe was a 1:20 of the commercial stock solution. These were based on optimal labelling and minimal background interference.

From Plates 5.1 and 5.2, which are typical representatives, it can be seen that TEM showed no significant accumulation of gold particles at the cytoplasmic membrane of cells; this was irrespective of strain or nisin concentration. Controls where nisin-specific antibody was eliminated had a marked decrease in gold particles, but controls where nisin was eliminated were no different to the samples that contained nisin.
Plate 5.1 Immunogold labelling of *L. monocytogenes* NCTC 5105 after nisin (20 IU/ml) addition (Bar 100 nm)
Plate 5.2 Immunogold labelling of *L. monocytogenes* F6861 after nisin (20 IU/ml) addition (Bar 100 nm)
5.4 DISCUSSION

Results from the nisin adsorption assay showed that the amount adsorbed reflected the sensitivity of the strain to nisin, with the more sensitive cells showing greater adsorption (Fig. 5.1). The fact that this was observed over a range of nisin concentrations supports the view that multiple adsorption is possible at suitable membrane sites (Benz et al., 1991; Sahl, 1991). Parallel work by Ming and Daeschel (1995) similarly showed that less nisin adsorbed to mutant cells of *L. monocytogenes* Scott A than the parent strain regardless of the nisin concentration used (10, 50 or 100 IU/ml).

The results do not indicate the presence of a nisinase enzyme reported in some resistant bacteria (Kooy, 1952; Galesloot, 1956; Carlson and Bauer, 1957; Alifax and Chevalier, 1962; Jarvis, 1967). If this were present, lower levels of residual nisin would be expected in supernatants exposed to more resistant species rather than the converse. Since similar results were obtained with the bioassay, the possibility that a nisinase was destroying biological activity of the molecule while retaining epitopes recognised in the ELISA is excluded. Daeschel and Ming (1993) also found that nisin was not inactivated after exposure to culture supernatants from mutant or parent cells cultivated in BHI medium. Nisinases from *Streptococcus salivarius* subsp. *thermophilus* (Alifax and Chevalier, 1962) and *Bacillus cereus* (Jarvis and Farr, 1971) were partially purified but have not been fully characterized and thus the position of a target site in the nisin molecule has not been determined.

With the elimination of nisinase production, the fact that nisin-sensitive cells show greater adsorption of nisin than more resistant cells could be accounted for by the presence or accessibility of a higher proportion of nisin attachment sites. This is supported by the fact that greater adsorption of nisin by sensitive cells was more significant at lower rather than higher nisin concentrations. It is suggested that as increasing concentrations of nisin are added to cells, the nisin attachment sites become occupied until a point is reached when all the available sites are filled and no more
nisin is adsorbed. Low concentrations of nisin are unlikely to be so readily adsorbed in resistant cells as compared to sensitive cells due to the limited presence or availability of attachment sites. At high nisin concentrations the fewer attachment sites of resistant cells are more likely to be found and filled by nisin causing eventual lysis. For example, when nisin was added to strain F6861 at a concentration of 200 IU/ml or below (< its MIC), the amount of nisin adsorbed did not exceed 15% whereas at 1000 IU/ml (> its MIC) a much higher adsorption of 33% was observed. Thus at high nisin concentrations, the difference in amount of nisin adsorption between sensitive and resistant cells is less significant. However, sensitive cells should always adsorb more nisin than resistant cells whatever the nisin concentration added due to the greater number of attachment sites.

The observed adsorption behaviour suggests that in *L. monocytogenes* nisin resistance is based on exclusion and inhibition of nisin binding to the cells. Resistance is acquired by adaption of either the cytoplasmic membrane or cell wall to reduce the presence or accessibility of attachment sites preventing the incorporation of nisin into the membrane to produce pores, cell lysis and death.

Immunogold labelling did not show significant amounts of nisin present in the cytoplasmic membrane of cells (Plates 5.1 and 5.2), and control cells that did not contain nisin were no different from those with nisin. According to Beesley (1989), this could be attributed to several factors: personal (sic), no antigen, no antibody, no gold, and the wrong buffer. A faulty buffer system normally occurs if the pH is very low causing the antigen-antibody complex to dissociate, but as standard buffer recipes were followed in the method this seems unlikely. A faulty gold probe can be eliminated due to the high commercial preparation standards and the fact that gold particles were readily detected in the procedure. The presence of nisin-specific antibodies (polyclonal) was confirmed by use of the nisin ELISA and a significant advantage of using polyclonal as opposed to monoclonal antibodies is that they contain several populations of different antibodies directed to various portions of the antigen molecule. However, no immunolabelling results if the antibody cannot get to
the antigen. This seems highly plausible if nisin, on incorporation into the cytoplasmic membrane, is either inaccessible or has altered its configuration to such an extent that the nisin-specific epitopes would be unrecognizable to the nisin-specific antibodies. A similar argument could be used for no antigen, whereby nisin had undergone drastic physical alteration during the preparation procedure. For example, fixation causes cross-linking of amino acids which could alter the normal antigenicity. On the other hand, if weak fixation is employed to protect the antigen, it may leach out of the sample during processing.

It was decided not to pursue with the immunogold labelling due to the time consuming nature of the work. It has since become known that other workers have also not yet managed to detect nisin in the cytoplasmic membrane of cells by this method (Richard, personal communication, 1994). Also recently, Stringer et al. (1995) similarly failed to detect nisin bound to sensitive vegetative cells by anti-nisin antibody-mediated staining, and also suggested that insertion of nisin into the membrane may prevent antibody binding.
5.5 SUMMARY

The adsorption of nisin to *L. monocytogenes* NCTC 5105, F6861 and its nisin-resistant mutant was studied routinely by means of a nisin-specific ELISA and compared with results obtained from a nisin bioassay. At a range of nisin concentrations the amount adsorbed was found to reflect the sensitivity of the strain to nisin, with the more sensitive cells showing greater adsorption. This was most significantly marked at lower nisin concentrations. The results eliminated the possibility of a nisinase enzyme reported in some resistant bacteria. These results indicate that the mechanism of resistance involves a reduction in the accessibility or presence of suitable adsorption sites.

Immunogold labelling and TEM did not show significant amounts of nisin accumulated at the cytoplasmic membrane of *L. monocytogenes*, regardless of strain type. It is likely that the nisin epitopes were unable to form an antigen-antibody complex either because of their attachment to the cytoplasmic membrane or because of damage during the preparation procedure.
CHAPTER 6  THE KINETICS OF NISIN-INDUCED K⁺ EFFLUX FROM
LISTERIA MONOCYTOGENES AND NISIN RESISTANCE
6 THE KINETICS OF NISIN-INDUCED K⁺ EFFLUX FROM
LISTERIA MONOCYTOGENES AND NISIN RESISTANCE

6.1 INTRODUCTION

As stated, in nisin sensitive cells, nisin is incorporated into the cytoplasmic membrane
to form ion channels or pores (Henning et al., 1986b; Sahl et al., 1987; Kordel et
al., 1989, Gao et al., 1991) through which efflux of K⁺, ATP and amino acids
results in destruction of the membrane potential (Ruhr and Sahl, 1985; Kordel and
Sahl, 1986) leading to collapse of the proton-motive force (Bruno et al., 1992;
Okereke and Montville, 1992).

According to Russell and Chopra (1990), the first indication of membrane injury is
K⁺ leakage, followed by inorganic phosphates, amino acids and u.v.-absorbing
materials. Leakage is best considered as a measure of the generalized loss of function
of the cytoplasmic membrane as a permeability barrier. The rate and extent of leakage
may depend on the concentration of the inhibitor and the time and temperature of
exposure. Leakage may be related to bacteriostasis but not necessarily to cell death.

The aim of this present study was to investigate the effect of nisin at different
concentrations on cell leakage (efflux of K⁺ and u.v.-absorbing materials) from L.
monocytogenes, and to examine any relationship between the amount or rate of efflux
and nisin resistance.
6.2 MATERIALS AND METHODS

Determination of nisin-induced cellular efflux

Overnight broth cultures (300-ml, TPB, pH 7.3) of *L. monocytogenes* were centrifuged (1500 g for 15 min) and the cell pellets resuspended in phosphate buffer (5 mmol/l, pH 6.6) to give the same cell concentration (ca. 10⁹ cfu/ml), as measured by OD (600 nm) and confirmed by plate counts (TSA, pH 7.3).

Each suspension was divided into equal volumes and incubated in a waterbath at 30°C. A fixed volume of cell suspension was removed prior to addition of nisin and filter sterilized (0.2 µm, Minisart NML, Sartorius) to represent the level of efflux at time zero. Sterile water and nisin stock solution (50 x 10³ IU/ml) (Chapter 3) were added to the remaining suspension in the required proportions to obtain the desired final nisin concentration. Samples were taken at selected time intervals and filter sterilized prior to K⁺ and u.v. determination. Nisin-free controls were processed in the same way. The concentration of K⁺ ions in each filtrate was measured by atomic absorption spectroscopy at a wavelength of 383 nm (Perkin Elmer 306). The correlation coefficient of each standard curve was ≥ 0.996. The u.v. absorbance of the filtrates was measured at 260 nm in a u.v. spectrophotometer.

Determination of total cellular K⁺

Cell suspensions were prepared and centrifuged as described above. Concentrated HNO₃ (1 ml) was added to each pellet and the suspensions heated at 100°C for 30 min to dissolve the biomass. The pH was adjusted to 6 with 4 M NaOH. Samples were made up to a final volume of 10 ml with distilled water and filter sterilized before K⁺ determination.
6.3 RESULTS

6.3.1 Nisin-induced cellular efflux from \textit{L. monocytogenes}

Preliminary experiments studied the effect of nisin on the efflux of $K^+$ and u.v.-absorbing materials from \textit{L. monocytogenes} NCTC 5105 and F6861 over a 2 h time period (Figs. 6.1 and 6.2). $K^+$ efflux was most significant during the first minute after nisin addition, excepting strain 5105 at a nisin concentration of 20 IU/ml where $K^+$ efflux was more gradual. With both strains, the initial rate and final amount of $K^+$ efflux increased with increasing nisin concentration. A similar pattern was seen with the efflux of u.v.-absorbing materials, although the effect was not as pronounced as with $K^+$ efflux. The nisin-sensitive 5105 strain lost both more $K^+$ and u.v.-absorbing materials at a lower nisin concentration (40 IU/ml) than the more resistant F6861 strain at a higher nisin concentration (80 IU/ml).

Subsequently, the initial rate of $K^+$ efflux (mg/l/min) induced by different levels of nisin was measured in all three strains of \textit{L. monocytogenes} (Fig. 6.3). In each case, the rate of $K^+$ efflux increased with increasing nisin concentration until it reached a maximum level. The increase in rate of $K^+$ efflux with increasing nisin concentration and the maximum rate observed both increased with the cells' sensitivity to nisin.

The different maximum rates for $K^+$ efflux did not reflect differences in total cellular $K^+$ levels between strains. These were similar in the 5105 and the mutant F6861 strains (0.14 and 0.13 $\mu$g/10$^6$ cfu, respectively) which showed the biggest difference in $K^+$ efflux, while F6861 had the lowest $K^+$ content (0.10 $\mu$g/10$^6$ cfu) but an intermediate rate of $K^+$ efflux. The maximum $K^+$ efflux rates recorded corresponded to differing proportions of the total cellular potassium (5105, 78%; F6861, 63%; F6861 mutant, 42%).
Fig. 6.1 Effect of nisin on K⁺ efflux from *L. monocytogenes*
Fig. 6.2 Effect of nisin on the efflux of u.v.-absorbing materials from \textit{L. monocytogenes}
Fig. 6.3  Effect of nisin on the rate of K⁺ efflux from L. monocytogenes cells (Ranges of duplicate experiments indicated)
6.4 DISCUSSION

Preliminary experiments looking at the effect of nisin damage on *L. monocytogenes* cells showed that K⁺ efflux was a much better indicator of cell leakage than that of u.v.-absorbing materials (Figs. 6.1 and 6.2). This was also observed by Bhunia *et al.* (1991) on treatment of *Lactobacillus plantarum* with pediocin AcH.

With each strain of *L. monocytogenes*, the initial rate of K⁺ efflux increased with increasing nisin concentration until it reached a maximum rate, indicating saturation kinetics (Fig. 6.3). The rate of increase and the maximum level of efflux rate both increased with the cells’ sensitivity to nisin, and the different maximum rates for K⁺ efflux did not reflect differences in total cellular K⁺ levels between strains. These results indicate that as more nisin is added to the cells suitable nisin attachment sites become occupied until a point is reached when all the available sites are filled and no further increase in K⁺ efflux occurs. In very resistant cells, such as the F6861 nisin-resistant mutant, a greater amount of nisin is required to find and fill these fewer sites. It has been shown in Chapter 5 that very sensitive cells such as strain 5105 show significantly greater adsorption of nisin at low concentrations than more resistant cells, supposedly due to the extra adsorption sites.

Winkowski *et al.* (1994) have similarly shown that nisin-induced ATP efflux from *L. monocytogenes* Scott A depends on nisin concentration and follows saturation kinetics. In addition, Abee *et al.* (1994) have recently studied nisin Z-induced K⁺ efflux from *L. monocytogenes* Scott A with a K⁺-valinomycin-selective electrode. They found that nisin Z caused immediate and rapid efflux of K⁺ such that the cells lost all K⁺ in *ca.* 20 s. However, at low nisin Z concentrations, the internal K⁺ level did not reach zero indicating that the cells either lost only part of their K⁺ or that only a limited number of cells were hit and subsequently lost all their K⁺. On confirming the latter hypothesis and normalizing the results to 100% of the cells hit, the authors observed that the rate of K⁺ efflux ([K⁺]/min) increased with increasing nisin Z concentrations and at a high nisin Z/cell ratio, approached saturation level. They
stated that clear saturation could not be observed, probably because of the limit in accuracy in the K⁺ electrode response.

It is likely that a similar effect occurred on addition of nisin to strain F6861 and its nisin-resistant mutant. Thus, if at low nisin concentrations only a proportion of the cells had been hit by nisin, this would account for the increase in the rate of K⁺ efflux as nisin concentration increases. However, the differences in rates of K⁺ efflux at low nisin concentrations and the different maximum rates at high nisin concentrations observed between the strains, does still indicate the fundamental difference in sensitivity between strains.

The saturation kinetics of K⁺ efflux adds further evidence to the hypothesis (Chapter 5) that in L. monocytogenes nisin resistance is based on exclusion and inhibition of nisin binding to the cells. Resistance is acquired by adaption of either the cytoplasmic membrane or cell wall to reduce the presence or accessibility of attachment sites preventing the incorporation of nisin into the membrane.
6.5 SUMMARY

The initial rate of $K^+$ efflux (mg/l/min) induced by different levels of nisin was measured in *L. monocytogenes* NCTC 5105, F6861 and its F6861 nisin-resistant mutant. In each strain, the rate of $K^+$ efflux increased with increasing nisin concentration until it reached a maximum rate. As the nisin resistance of each strain increased, $K^+$ efflux increased at a slower rate and had a lower maximum rate indicating that the mechanism of nisin resistance involves a reduction in the accessibility or presence of suitable adsorption sites.
CHAPTER 7  THE CELL WALL OF LISTERIA MONOCYTIOGENES
AND ITS INVOLVEMENT IN NISIN RESISTANCE
7 THE CELL WALL OF *LISTERIA MONOCYTOGENES* AND ITS INVOLVEMENT IN NISIN RESISTANCE

7.1 INTRODUCTION

So far, it has been determined that the mechanism of nisin resistance in *L. monocytogenes* does not involve the presence of a nisinase enzyme, but instead is based on exclusion and inhibition of nisin binding to the cells. This chapter concentrates on the possible role of the cell wall in resistance acquisition, where some adaptation in its structure could result in a reduction of the presence or accessibility of nisin attachment sites. Thus, the question of the composition of the cell wall of *L. monocytogenes* arises, and which component(s) might play an active role in nisin resistance. Fiedler (1988) has discussed the biochemistry of the cell surface of *Listeria* strains and presented a probable model of the cell envelope of a *Listeria* cell in a cross-sectional view (Fig. 7.1).

![Fig. 7.1 General organization of the cell wall of strains of *Listeria* showing the possible arrangements of polymers (From Fiedler, 1988)](image-url)
The cell wall of a *L. monocytogenes* cell shows criteria typical for Gram-positive bacteria. In electron-micrographs of thin sections through cells it appears as a rather thick homogeneous layer which surrounds the cytoplasmic membrane and establishes the outer region of the cell, the cell surface. The cell wall maintains the shape of the cell and protects the mechanically fragile cytoplasmic membrane from rupture due to the high internal osmotic pressure generated by the cytoplasm. An outer membrane characteristic of Gram-negative eubacteria was not detected and there was no evidence for the presence of capsules and structures such as fimbriae (Fiedler, 1988).

The cell wall consists of a multilayered peptidoglycan, murein. Murein is a heteropolymer composed of glycan chains which are crosslinked by short peptides. The glycan moiety consists of alternating molecules of *N*-acetylglucosamine and *N*-acetyl muramic acid, linked by 1,4-β-glycosidic bonds. There are also many structurally-specific teichoic acid chains attached through specific linking regions to certain hydroxyl groups at the exposed C-6 of muramic acid residues of the glycan strands. The teichoic acid chains are partially embedded within the peptidoglycan layer and partially exposed at the outermost surface of the cell, and can become released from the walls by chemically cleaving the attachment site. The attachment sites consist of phosphodiester bonds which can be broken by the addition of phosphodiesterase. Cell wall teichoic acids are usually composed of the polyols glycerol and ribitol (or more rarely mannitol), neutral sugars, *N*-acetylamino sugars and phosphate. The polyol residues are also joined through phosphodiester links. Similarly, lipoteichoic acids, which are rooted in the cytoplasmic membrane, may partially reach the cell surface. Lipoteichoic acids are amphipathic molecules; that is, they have both hydrophobic and hydrophilic regions. The hydrophilic moiety consists of a 1-3-phosphodiester-linked polymer of glycerophosphate residues. The hydrophobic moiety is covalently linked to the terminal phosphomonoester of the hydrophilic chain and consists of glycolipids. This lipid region anchors the polymer chain to the outside of the cytoplasmic membrane, while the hydrophilic chain penetrates the cell wall.
Fiedler suggested that the surface of a *Listeria* cell seems to be made up mainly of teichoic acid chains, but the question of whether proteins are associated with the surface of the cell still needs to be explored. There is some evidence that a few are present, which may be autolytic enzymes, transiently excreted proteins or, possibly structural proteins. However, protein covalently linked to the peptidoglycan is lacking (Fiedler, 1988).

By removing the cell wall of *L. monocytogenes* to form protoplast cells, it would be possible to determine whether the cell wall has a role in nisin resistance. This could be achieved by comparison of nisin resistance in protoplast cells and whole cells. Protoplast formation can be accomplished by the action of lysozyme, which attacks the 1,4-β-glycosidic bonds of the peptidoglycan. When the rigid "basal" layer of the cell wall is digested by lysozyme the cell ordinarily lyses, but in a hypertonic medium (e.g., 20% sucrose or 0.5 M KCl) such treatment releases the membrane with its contents as an osmotically-sensitive sphere. With Gram-positive organisms such as *L. monocytogenes*, the protoplast appears to be free of cell wall constituents, by both microscopic and chemical criteria. Gram-negative organisms similarly yield osmotically-sensitive spheres, but these retain an outer wall layer and are called spheroplasts, to distinguish them from the presumably wall-free protoplasts. Dissolution of the cell wall does not affect metabolism.

If the cell wall is shown to play an active role in nisin resistance, it would subsequently be determined which component(s) of the cell wall are involved. For instance, a difference in cell surface hydrophobicity between strains, especially F6861 and its nisin-resistant mutant, could indicate a structural difference in their outer cell surfaces, with the adaptation of the mutant relating to increased nisin resistance.

Outer cell surfaces are often composed of regular crystalline arrays of proteinaceous subunits termed surface layers (S-layers), and are in fact one of the most commonly observed prokaryotic cell envelope structures (reviewed by Sleytr *et al.*, 1993a; 1993b). They are ubiquitous amongst Gram-positive and Gram-negative bacteria and,
if present, account for the major protein species produced by the cells. However, there have been no reports of S-layers in *Listeria* species. Although relatively few firm data are available, there is strong evidence that S-layers can provide organisms with a selection advantage. The various functions attributed to S-layers include protective coats, molecular sieves, ion traps and structures involved in cell surface interactions.

S-layers cover the cell surface completely and are endowed with the ability to maintain a complete covering during cell growth by an entropy-driven assembly process. Commonly, the subunits are linked together and also to the underlying cell envelope layers by non-covalent forces. Most S-layer proteins are acidic with a large proportion of hydrophobic amino acids and contain little or no sulfur-containing amino acids. Most S-layer lattices show oblique, square or hexagonal symmetry and the morphological units generally have centre-to-centre spacings of approximately 3-30 nm. S-layers are 5-15 nm thick and possess pores of identical size and morphology in the 2-6 nm range. The porosity of the protein network can reach 70%.

One of the most suitable electron microscopical procedures for identifying S-layers on cell surfaces is freeze-etching (Willison and Rowe, 1980; Beveridge and Graham, 1991). In this method, a copy, which is suitable for examination in the transmission electron microscope (TEM), is made from the fractured surface of a frozen aqueous specimen. The term "freeze-etching" is widely used to encompass both freeze-fracturing and freeze-etching, regardless of the extent to which etching has occurred. "Etching" refers to the vacuum sublimation of a small part of the ice present in the sample in order to reveal additional detail in the specimen. A carbon replica of the freeze-etched surface is prepared before or after metal shadowing and the specimen is itself removed. Examination of cell surface profiles by freeze-etching relies on the fact that a fracture plane will frequently occur along the ice-cell interface, as this is the weakest point.

Alternatively, S-layer proteins can be isolated from cell wall fragments or sometimes
whole cells and identified by SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis). The molecular weight of S-layer protomers, as determined by SDS-PAGE, can range from 40,000 to 200,000.

According to Sleytr et al. (1993b), very little information is available about how the synthesis, transport and assembly of S-layers is controlled at the cellular level. Assuming a generation time of 20 min, at least 500 subunits per second have to be incorporated into the S-layer lattice of a cell in order to maintain a closed surface crystal. The authors also reported that there is a strong indication that altered environmental and physiological conditions can stimulate cells to change their S-layers leading to selection advantage in a competitive environment.

Thus, if S-layers are shown to be associated with the outer surface of each L. monocytogenes strain tested, or are strain dependent, it is conceivable that they have a role in the acquisition of nisin resistance. Irrespective of S-layer involvement, the fact that cell wall teichoic acids and lipoteichoic acids project to the outer cell surface and might be involved in nisin resistance, albeit independently or in conjunction with S-layers, should also be investigated.

Thus, to determine whether the cell wall of L. monocytogenes is involved in nisin resistance and if so, which components play an active role, the following experiments were undertaken:

(1) Comparison of nisin resistance in whole cells and protoplast cells of L. monocytogenes F6861 and its nisin-resistant mutant to determine whether the cell wall is involved in nisin resistance.

(2) Determination of cell surface hydrophobicity to see if there is a structural difference in the outer components of the cell wall between strains, which could be related to nisin resistance.
(3) Determination of the involvement of de novo protein synthesis in nisin resistance. This may be achieved by the addition of the antibiotic chloramphenicol to wild type cells of *L. monocytogenes* F6861, to see whether it affects the frequency of isolation of nisin-resistant mutants. Chloramphenicol is a broad-spectrum bacteriostatic agent that inhibits protein synthesis. It binds to 70S (bacterial) ribosomes and prevents peptide bond formation by inhibiting the peptidyl transferase reaction (Russell and Chopra, 1990).

(4) To resolve whether S-layers are a common feature of the cell wall of *L. monocytogenes* with respect to the strains under investigation, strain dependent or absent. If S-layers are detected in one or more strains, their role in nisin resistance could possibly be elucidated.

(5) Determination of the involvement of cell wall teichoic acids and/or lipoteichoic acids in nisin resistance. This may be achieved by addition of phosphodiesterase to chemically disrupt the phosphodiester bonds linking each network, leading to their release from the cell wall. Comparison of nisin resistance in whole cells and cells lacking teichoic acid/lipoteichoic acid from *L. monocytogenes* F6861 and its nisin-resistant mutant would determine whether these cell wall components played any role in nisin resistance.
7.2 MATERIALS AND METHODS

7.2.1 Determination of cell wall involvement in nisin resistance

Protoplast formation

The formation of protoplasts from *L. monocytogenes* cells was based on the method of Ghosh and Murray (1967) and conducted as follows:

*Listeria monocytogenes* F6861 and its nisin-resistant mutant were inoculated separately into TPB (pH 7.3, 100 ml) and incubated at 30°C for 20 h to give ca. $10^9$ cfu/ml. The respective cell concentrations were determined using spread plates (TSA). Cells were harvested from suspension by centrifugation at 7000 g for 20 min (at 4°C), washed with water and weighed. The cell pellets were then resuspended in lysozyme incubation buffer (0.015 M NaCl; 0.03 M Tris-HCl at pH 6.7 (Sigma) and 0.4 M sucrose (BDH)) at ca. 20 mg/ml wet weight before addition of lysozyme (Sigma) to give a final concentration of 600 µg lysozyme/ml. The lysozyme had previously been dissolved in water (6 mg/ml) and filter sterilized (0.2 µm, Minisart NML, Sartorius). The samples were shaken for 15 min at 37°C and an appropriate volume of MgCl$_2$ (1 M)(BDH) added to give a final concentration of 0.02 M MgCl$_2$. After a 45-min incubation at 37°C without shaking, the samples were centrifuged at 1500 g for 15 min and the pellets washed in protoplast buffer (0.03 M Tris-HCl at pH 6.7; 0.01 M MgCl$_2$ and 0.5 M sucrose). The pellets were resuspended to their original volume (100 ml) with protoplast buffer and stored briefly on ice before use.

Determination of the efficacy of protoplast formation

The total number of cfu/ml present in each protoplast preparation was determined using spread plates. Due to the osmotic fragility of protoplasts, all enumeration was conducted using protoplast buffer as a diluent and TSA that had been hydrated with
protoplast buffer. By preventing osmotic shock and subsequent lysis in this way, the cells are viable enough to produce colonies.

To determine the actual % of protoplasts present, a small volume of each protoplast suspension was centrifuged (1500 g for 15 min) and resuspended in an equal volume of water. The cells were incubated at 30°C for 30 min with constant shaking to ensure lysis. Control whole cells were treated in the same manner. Plate counts were conducted again as described above.

Inactivation of whole cells and protoplasts by nisin

Overnight cultures of *L. monocytogenes* were grown to ca. 10⁹ cfu/ml in TPB (10 ml) at 30°C. After centrifugation (1500 g for 15 min), they were washed and resuspended to their original volume in protoplast buffer (0.03 M Tris-HCl at pH 6.7; 0.01 M MgCl₂ and 0.5 M sucrose) and incubated in a water bath at 30°C. After a 15-min equilibration, samples (100-μl) were removed, diluted in MRD (decimal, serial) and cells enumerated on TSA using the spiral plater. Nisin solution (100 μl) (Chapter 3) was added to the remaining cultures at the required concentration and samples taken for cell enumeration up to a period of 3 h.

Protoplasts were prepared as described above and inactivated by nisin in exactly the same way as whole cells, excepting the use of buffered diluent and agar.

7.2.2 Cell surface hydrophobicity determination

Several methods exist to determine the surface hydrophobicities of bacterial cells namely, contact angle measurements (CAM); hydrophobic interaction chromatography (HIC); bacterial adherence to hydrocarbons (BATH); adhesion to polystyrene; salt aggregation test (SAT) and latex particle agglutination (LPA).
According to Dillon et al. (1986) and Mozes and Rouxhet (1987), there is a good correlation of the methods for very hydrophobic and very hydrophilic organisms but measurements vary according to the method used when applied to less pronounced examples of cell surface hydrophobicities. Thus, reliance on one method for such tests is inadequate.

Method 1  Bacterial adherence to hydrocarbons (BATH)

The partition coefficient of cells between a n-hexadecane and aqueous phase was measured as an index of their relative surface hydrophobicities, as initially described by Rosenberg et al. (1980) and later modified by Beck et al. (1988). BATH involves the movement of cells from the aqueous to n-hexadecane phase during a brief period of mixing and the greater the decrease in cell concentration from the aqueous phase, the greater the hydrophobicity of the organism. The method was conducted as follows:

Listeria monocytogenes strains were cultured in TPB (pH 7.3, 10 ml) to stationary phase at 30°C. The cells were harvested by centrifugation at 1500 g for 15 min, washed twice in phosphate-urea-magnesium (PUM) buffer (pH 7.1) containing (g/l) (BDH): K_2HPO_4.3H_2O, 22.2; KH_2PO_4, 7.26; urea, 1.8; anhydrous MgSO_4, 0.1, and resuspended in the same buffer to give ca. 5 x 10^8-10^9 cfu/ml. Samples were taken for plate counts using the spread plate technique and optical density (OD) determination at 320 nm. n-Hexadecane (50 µl)(Sigma) was added to 1-ml volumes of each cell suspension and the phases equilibrated in a water-bath at 30°C for 10 min. The two phases were vortex-mixed for 1 min and the mixture allowed to stand for 15 min at room temperature in order to obtain a maximal rise in the n-hexadecane phase. The samples were then centrifuged at 2500 g for 5 min at low temperature (4°C), which solidified the n-hexadecane phase in a thin pellicle. This was removed with tweezers. After mixing, another sample was removed for enumeration by plate counts and the remainder used for OD determination. Bacterial hydrophobicity was
expressed as the % change in OD (ΔOD%) of the aqueous phase before and after the hexadecane addition, and as the % change of the bacterial concentration before and after the assay (Δcfu/ml%).

Method 2 Hydrophobic interaction chromatography (HIC)

HIC is a chromatographic procedure based on hydrophobic interaction between nonpolar groups on a gel bed and nonpolar regions of a solute, e.g., of a protein. The proportion of cells retained by a hydrophobic gel gives an indication of their hydrophobicity, the greater the retension the greater the hydrophobicity. The extent of hydrophobic interaction varies according to the conditions used such that it increases with an increase in ionic strength or with a decrease in the pH of the medium. The method used was based on those described by Smyth et al. (1978) and Mozes and Rouxhet (1987) as follows:

Duplicate overnight cultures of all three strains of *L. monocytogenes* were grown at 30°C (TPB, 10 ml) to stationary phase (ca. 10^9 cfu/ml). Following centrifugation at 1500 g for 15 min, the cell pellets were resuspended in 4 M NaCl (1 ml) buffered with either 0.1 M sodium phosphate buffer (pH 7) or 0.1 M citrate buffer (pH 5) to give ca. 10^10 bacteria/ml for HIC.

Hydrophobic, electrically-neutral derivatives of Sepharose, namely phenyl-Sepharose CL-4B and octyl-Sepharose CL-4B (Sigma) were both used as gel beds. Columns comprised of glass Pasteur pipettes (internal diameter, 5 mm) plugged with a little glass wool and fitted with clamped Teflon tubing. The gel beds were packed to a fixed height (0.8 ml gel bed volume) by gravity feed and washed with buffered 4 M NaCl (10 ml) to remove any residual ethanol preservative and for equilibration purposes. The gel suspensions were left to stabilize at room temperature before continuing, with care being taken that they didn’t dry out.
The preprepared bacterial suspensions (100-µl, ca. 10^9 bacteria) were then applied on to the gel beds and allowed to drain. The columns were washed through with buffered 4 M NaCl (3 ml) to elute the non-adsorbed bacteria and the absorbance of each fraction (A₀) measured at 600 nm. Similarly, NaCl-free buffers (0.1 M sodium phosphate buffer (pH 7) and 0.1 M citrate buffer (pH 5)) were washed through their respective columns (3 x 3-ml) to desorb the bacteria and the absorbance of each fraction (A_i) determined. In addition, 100 µl of each original cell suspension was diluted in the appropriate 4 M NaCl-buffer (3 ml) and the absorbancies measured (A_t at 600 nm 0.2-0.3). From the results obtained, two parameters expressing percentage of retention (R) were determined using the following equations:

\[
R_F = \frac{A_t - A_0}{A_t} \times 100 \quad \text{and} \quad R_U = \frac{A_t - \Sigma A_i}{A_t} \times 100
\]

Where A_t is related to the total amount of cells used in the test; A_0 is related to the amount of cells that are not retained by the gel under conditions favouring hydrophobic interactions, i.e., high ionic strength (4 M NaCl) and low water activity; \( \Sigma A_i \) is related to the amount of cells that are not retained by the gel under conditions that do not favour hydrophobic interactions, i.e., low ionic strength; \( R_F \) is the proportion of cells retained by the gel at high ionic strength (≥ 4M) which is favourable to observation of hydrophobic interactions - the water activity is low, the surface charges are screened at a very short distance and, consequently, electrostatic interactions are minimized; \( R_U \) is the proportion of cells that remain retained in conditions of lower ionic strength, the latter being imposed by the pH-controlling reagent.

Statistical analysis

The results of the BATH and HIC tests were analysed statistically using the Student's \( t \)-test to determine whether the difference in hydrophobicity between each \( L. \ monocytogenes \) strain was significant.
7.2.3 Determination of the involvement of *de novo* protein synthesis in nisin resistance

The effect of chloramphenicol on the frequency of isolation of nisin-resistant mutants from *L. monocytogenes* F6861 was studied.

Initially, the MIC of chloramphenicol for *L. monocytogenes* F6861 was determined. A stock solution of chloramphenicol (Sigma) was prepared at a concentration of 10 mg/ml and filter sterilized (0.2 µm, Minisart NML, Sartorius). Broths (TPB, pH 5.5, 10-ml) of increasing chloramphenicol concentration (0-100 µg/ml) were set up accordingly by adding chloramphenicol solution and water in the required proportions (100 µl total volume), before inoculation with 100 µl of diluted overnight broth culture to give an initial inoculum level of ca. $10^6$ organisms/ml. This inoculum concentration caused no visible turbidity in the medium. Tubes were incubated at 30°C for 7 d and growth was monitored by the appearance of turbidity. The MIC was defined as the lowest level of chloramphenicol that inhibited growth and the concentration determined was used subsequently.

*Listeria monocytogenes* F6861 was cultivated in TPB (pH 7.3, 100 ml) to stationary phase to give ca. $10^9$ cfu/ml. After centrifugation at 7000 g for 20 min, the cells were resuspended in TPB (pH 5.5, 100 ml) and dispensed in 9.8-ml volumes. Both chloramphenicol and nisin solution (Chapter 3) were added together or separately (200 µl total volume) to the individual cell cultures to give final concentrations of 10 µg/ml and 500 IU/ml, respectively. When adding both solutions together, the addition of chloramphenicol was conducted before the addition of nisin. Mutants were enumerated on TSA plates containing nisin (600 IU/ml, pH 5.5) as described in Chapter 4, and the total number of cfu/ml enumerated on nisin-free TSA plates (pH 5.5). Enumerations were conducted initially after immediate addition of chloramphenicol and/or nisin and subsequently after incubation at 30°C for 90 min, using decimal, serial dilutions in MRD and the spread plate technique. Total counts (nisin-free TSA plates) were taken after 48 h incubation and mutant counts (nisin-
supplemented plates) after 7 d incubation at 30°C.

7.2.4 S-layer determination

Various methods were used to detect S-layers as follows:

**Freeze-etching**

All three *L. monocytogenes* strains were examined for the presence of S-layer proteins by freeze-etching and transmission electron microscopy (TEM). *Lactobacillus buchneri* ATCC 4005 was used as an S-layer positive control to ensure that S-layers could be detected given the conditions used. For the procedure, cells were taken directly from their respective agar plates (Chapter 3) after a 48-h period of growth. A few colonies of each strain were suspended in a small volume of water, washed and centrifuged. The cell pellets were mounted on metal grids and freeze-dried with liquid nitrogen. The samples were then freeze-fractured and freeze-etched. Metalically-shadowed replicas (carbon/platinum) of the cells were removed and cleaned by soaking in bleach for approximately 2 h, before being mounted on copper grids in preparation for TEM (Philips 301G and 400T used).

**Atomic force microscopy (AFM)**

Samples of *L. monocytogenes* F6861 wild type and *Lactobacillus buchneri* ATCC 4005 (an S-layer positive control) from overnight broth cultures (Chapter 3) were used for AFM. Silicon wafer squares (1 cm x 1 cm), which had been pretreated with 0.25% (w/v) poly-L-lysine (Sigma), were used as substrata for the bacteria. The organisms were examined in air or in phosphate-buffered saline. A Nanoscope II SPM system (Digital Instruments, USA) was employed throughout.
Extraction of S-layer protein and SDS-PAGE

Guanidine hydrochloride was used in initial attempts to isolate S-layer protein from *L. monocytogenes* F6861 wild type and its nisin-resistant mutant (Messner and Sleytr, 1988) as follows:

Reagents:
- 50 mM Tris-HCl buffer, pH 7.2 (buffer 1)
- 50 mM Tris-HCl buffer, pH 7.2, containing 0.5% (w/v) Triton X-100 (buffer 2)
- 5 M guanidinium hydrochloride in distilled water (solution 3)
- 5 mM calcium chloride in distilled water containing 0.01% (w/v) sodium azide (solution 4)

(a) *Listeria monocytogenes* was grown in TPB (pH 7.3) to give 10 g (wet weight) of cells, washed twice with 50 ml of buffer 1 at 4°C, then resuspended in 50 ml of ice-cold buffer 1.

(b) The cells were disrupted by treatment in an ultrasonic disintegrator at maximum power output, with continuous cooling in an ice-bath, for a total of 3 min in 30-s periods with intervals of cooling.

(c) The broken cell suspension was centrifuged at 45,000 g for 20 min at 4°C. The upper, opaque part of the pellet, consisting of crude cell envelope, was carefully scraped off the lower pellet of unbroken cells. The unbroken cells were resuspended and treated again as in (a) and (b).

(d) The combined crude envelopes (peptidoglycan layer with attached S-layer, contaminated with soluble proteins and cytoplasmic membrane) were washed by resuspension in cold buffer 1 and centrifuged as in (c). This was repeated three times, and each time the upper layer of the pellet was retained, while any residual whole cells in the lower pellet layer were discarded.

(e) The washed envelope preparation was thoroughly resuspended and mixed for 15 min at room temperature in buffer 2 to solubilize any contaminating cytoplasmic membrane.
(f) The envelopes were recovered by centrifugation at 45,000 g for 20 min, then washed in cold buffer 1 as in (c), four times.

(g) S-layer subunits were extracted from the envelopes by homogenization of the pellet in 10 volumes of solution 3 with gentle stirring overnight at room temperature. The envelopes were recovered by centrifugation at 45,000 g for 30 min and re-extracted as above. The combined extracts were dialysed for up to 24 h against several changes of solution 4 at 4°C, to remove the chaotropic agent.

(h) The S-layer assembly products that had precipitated in the dialysis bag were collected by centrifugation at 6000 g for 15 min, and stored frozen at -20°C.

Subsequently, lithium chloride was used for both *L. monocytogenes* strains and *Lactobacillus buchneri* (Lortal *et al*., 1992) as follows:

*Listeria monocytogenes* was grown in TPB (pH 7.3) to stationary phase (ca. 10⁹ cfu/ml), the cells harvested and washed once with distilled water. For extraction of the S-layer protein, 10-15 mg of the moist pellet were suspended per ml of 5 M-LiCl and kept at 20°C for 15 min, followed by centrifugation (30,000 g, 15 min). For purification of the S-layer protein, the supernatant or crude extract was dialysed against distilled water overnight at 4°C. Protein was determined by the Lowry method as follows:

**Reagents:**

(a) Lowry protein assay reagent consisted of 9 parts stock solution A (2% (w/v) Na₂CO₃, 0.05% (w/v) Na-K-tartrate and 0.4% (w/v) NaOH) and 1 part stock solution B (0.1% (w/v) Cu₂SO₄·5H₂O). Chemicals were obtained from BDH.

(b) Folin-Ciocalteau reagent (Sigma) diluted 1:1 with water.

Lowry reagent (1 ml) was added to each sample (200 μl), mixed, and left for 15 min at room temperature. The diluted Folin-Ciocalteau reagent (100 μl) was added subsequently and the samples mixed. After standing for 30 min at room temperature,
the samples were mixed again and the absorbancies (750 nm) recorded. The concentration of protein in the samples was calculated from a calibration curve prepared from a dilution series of bovine serum albumin (Sigma).

In all experiments SDS-PAGE was performed using a 10% acrylamide separating gel (8 x 8 cm) and a 4% acrylamide stacking gel. Samples of isolated S-layer protein were suspended in a 3% (w/v) SDS solution in 0.05 M-Tris/HCl buffer, pH 7.2, and treated for 10 min in a boiling water-bath. Samples were centrifuged and 25 μl of the supernatant was mixed with 40 μl of the following solution: 0.1% SDS, 0.1% 2-mercaptoethanol, 30% (w/v) glycerol, 0.2% Orange G in 0.01 M-Tris/HCl buffer, pH 7.2. Gels were stained with Coomassie blue G250. Molecular mass standard proteins were obtained from Pharmacia.

7.2.5 Determination of the involvement of cell wall teichoic acids and/or lipoteichoic acids in nisin resistance

Phosphodiesterase treatment

*Listeria monocytogenes* F6861 and its nisin-resistant mutant were grown in TPB (pH 7.3, 10 ml) to stationary phase to give ca. 10⁹ cfu/ml. After centrifugation (1500 g for 15 min), the cell pellets were resuspended in 9.9 ml of protoplast buffer (0.03 M Tris-HCl at pH 6.7; 0.01 M MgCl₂ and 0.5 M sucrose) in preparation for phosphodiesterase treatment. Protoplast buffer was used instead of ordinary buffer (e.g., phosphate buffer) as a precautionary measure to avoid possible cell lysis due to osmotic shock. However, it is not known for certain whether phosphodiesterase would render the cell wall osmotically unstable and if so, to what extent. Treatment of the cell suspensions with phosphodiesterase in the form of crude extract of rattlesnake venom (*Crotalus atrox* type IV, Sigma) was conducted with great care. Phosphodiesterase (25 mg/ml, 100 μl) was added to give a final concentration of
0.25 mg/ml and the samples incubated at 37°C for 1 h. Subsequently, after centrifugation (1500 g for 15 min), the cell pellets were washed and resuspended in protoplast buffer (10 ml). The samples were placed in a water-bath at 30°C, and equilibrated for 15 min before continuing.

**Inactivation of whole cells and phosphodiesterase-treated cells by nisin**

This method was conducted exactly as that described for "Inactivation of whole cells and protoplast cells by nisin" in section 7.2.1, excepting the fact that protoplast cells were replaced by phosphodiesterase-treated cells (as described above).

**N.B.** Phosphodiesterase is an extremely hazardous chemical and residual enzyme activity should be destroyed by thorough boiling of solutions.
7.3 RESULTS

7.3.1 Involvement of the cell wall of *L. monocytogenes* in nisin resistance

The inactivation of *L. monocytogenes* F6861 wild type (WT) and its nisin-resistant mutant (M) by nisin was initially determined at a nisin concentration of 500 IU/ml (Fig. 7.2a). At this nisin concentration, the death rates of the two strains were similar, with both strains having decreased by ca. 2.5 log cycles after 3 h. At a lower nisin concentration of 200 IU/ml, there was a significant difference in the death rates of the two strains (Fig. 7.2b). After a 3-h incubation period with nisin (200 IU/ml), the cell concentration of strain F6861 WT decreased by ca. 3 log cycles whereas its nisin-resistant mutant decreased by ca. 1 log cycle only. Thus, in order to compare the inactivation of whole cells and protoplasts of both strains, a nisin concentration of 200 IU/ml was selected for use in the study.

Hitherto, protoplasts of both strains showed no significant difference between their death rates (Fig. 7.3). After a 3-h incubation with nisin the F6861 M protoplasts had decreased in concentration by ca. 3 log cycles compared with a 1 log cycle reduction of the F6861 M whole cells. In contrast, the reduction in concentration of the F6861 WT protoplasts was similar to the F6861 WT whole cells, with about a 3 log reduction after 3 h. However, the initial death rate of the F6861 WT protoplasts was not as rapid as the F6861 WT whole cells.

Several controls were conducted to ensure that the results obtained were not due to artifacts of the procedure as follows:

(1) Inactivation of whole cells was also conducted using osmotically-buffered diluent and agar and the resulting death curve obtained was no different from those conducted without the use of buffer.
Fig. 7.2 Effect of nisin at a) 500 IU/ml and b) 200 IU/ml on the inactivation of *L. monocytogenes* F6861 WT and F6861 M
Fig. 7.3 Effect of nisin on the inactivation of L. monocytogenes F6861 WT and F6861 M whole cells and protoplast cells (Ranges of duplicate experiments indicated)
(2) In the experimental protocol the incubation temperature of the protoplasts was increased from 4° to 30°C. To eliminate the possibility of lysis caused by the change in temperature and the subsequent 3-h incubation period, counts of nisin-free protoplasts were taken on completion of the experiment. There was no decrease in cell concentration.

(3) The nisin resistance of F6861 M whole cells that had regenerated from F6861 protoplasts was rechecked from cells taken from the inactivation experiments (plates at time 0) to rule out the possibility of reversion back to the wild type.

The efficacy of protoplast formation of those cells surviving lysozyme treatment as determined by subsequent lysis in water, was very high, with conversion of > 90% of each strain (Table 7.1). Control cells, which had no treatment with lysozyme and were thus intact, had exactly the same cell concentration before and after potential lysis with water.

Table 7.1  The efficacy of protoplast formation

<table>
<thead>
<tr>
<th>L. monocytogenes strain</th>
<th>Viable cell number (cfu/ml)</th>
<th>Protoplast formation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before lysozyme treatment</td>
<td>After lysozyme treatment</td>
</tr>
<tr>
<td>F6861 WT</td>
<td>$1.02 \times 10^9$</td>
<td>$4.19 \times 10^8$</td>
</tr>
<tr>
<td>F6861 M</td>
<td>$1.39 \times 10^9$</td>
<td>$6.47 \times 10^8$</td>
</tr>
</tbody>
</table>

* Standard deviation (n=2)
7.3.2 Cell surface hydrophobicity of \textit{L. monocytogenes}

Bacterial adherence to hydrocarbons (BATH)

Bacterial hydrophobicity was expressed as the \% change in OD (\(\Delta\) OD\%) of the aqueous phase before and after the \textit{n}-hexadecane addition, and as the \% change of the bacterial concentration before and after the assay (\(\Delta\) cfu/ml\%). Results of triplicate samples from all three strains of \textit{L. monocytogenes} are shown in Table 7.2a.

Table 7.2a Determination of cell surface hydrophobicity by measuring bacterial adhesion to \textit{n}-hexadecane (Expt 1)

<table>
<thead>
<tr>
<th>% Change of aqueous phase</th>
<th>\textit{L. monocytogenes} NCTC 5105</th>
<th>\textit{L. monocytogenes} F6861 WT</th>
<th>\textit{L. monocytogenes} F6861 M</th>
</tr>
</thead>
<tbody>
<tr>
<td>OD (320 nm)</td>
<td>8.4 (± 1.5*)</td>
<td>37.2 (± 12.8)</td>
<td>23.9 (± 1.4)</td>
</tr>
<tr>
<td>cfu/ml</td>
<td>4.3 (± 1.5)</td>
<td>35.4 (± 23.5)</td>
<td>32.1 (± 8.9)</td>
</tr>
</tbody>
</table>

* Standard deviation (\(n=3\))

Results from both the OD and cell concentration determinations show a much lower \% change for \textit{L. monocytogenes} NCTC 5105 as compared with the other two strains indicating that it is more hydrophilic. Statistically, the difference in \% change between 5105 and F6861 mutant (M) was very significant (OD, \(P < 0.001\); cfu/ml, \(P < 0.01\)), but the difference in \% change between 5105 and F6861 wild type (WT) was only significant for one determination (OD, \(P < 0.02\)).

Results from both determinations also show a higher \% change for \textit{L. monocytogenes} F6861 WT than for its nisin-resistant mutant indicating that strain F6861 WT is more
hydrophobic. However, these results were not significant, presumably because the standard deviations for the WT and M strains were concerningly high. Thus it was decided to repeat the hydrophobicity determinations for these strains only. This time a slightly lower cell concentration (ca. $10^8$ cfu/ml) was used (Table 7.2b).

**Table 7.2b** Determination of cell surface hydrophobicity by measuring bacterial adherence to $n$-hexadecane (Expt 2)

<table>
<thead>
<tr>
<th>% Change of aqueous phase</th>
<th><em>L. monocytogenes</em> F6861 WT</th>
<th><em>L. monocytogenes</em> F6861 M</th>
</tr>
</thead>
<tbody>
<tr>
<td>OD (320 nm)</td>
<td>54.3 ($\pm$ 3.8*)</td>
<td>40.8 ($\pm$ 1.9)</td>
</tr>
<tr>
<td>cfu/ml</td>
<td>59.9 ($\pm$ 8.0)</td>
<td>43.4 ($\pm$ 3.6)</td>
</tr>
</tbody>
</table>

* Standard deviation (n=3)

The results showed a significantly higher % change in both OD ($P < 0.01$) and cell concentration ($P < 0.05$) for *L. monocytogenes* F6861 WT as compared with its nisin-resistant mutant indicating that strain F6861 WT is more hydrophobic. This time the standard deviations were suitably lower.

**Hydrophobic interaction chromatography (HIC)**

Cell hydrophobicity was expressed as percentage retention using two parameters $R_F$ and $R_U$, with the higher the $R_F$ or $R_U$ value the more hydrophobic the cells (page 114) (Table 7.3). Statistical analysis of the results by the Student's $t$-test determined whether there was a significant difference between the mean hydrophobicity values of each *L. monocytogenes* strain (Table 7.4).
Table 7.3  Determination of cell surface hydrophobicity by hydrophobic interaction chromatography (HIC), using a) phenyl Sepharose and b) octyl Sepharose

a) Phenyl Sepharose

<table>
<thead>
<tr>
<th></th>
<th>L. monocytogenes NCTC 5105</th>
<th>L. monocytogenes F6861 WT</th>
<th>L. monocytogenes F6861 M</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>pH 5.0</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$R_F$ (%)</td>
<td>99.3 (± 0.9*)</td>
<td>96.0 (± 0.8)</td>
<td>87.7 (± 3.5)</td>
</tr>
<tr>
<td>$R_U$ (%)</td>
<td>75.4 (± 9.0)</td>
<td>58.4 (± 7.0)</td>
<td>55.9 (± 7.0)</td>
</tr>
<tr>
<td><strong>pH 7.0</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$R_F$ (%)</td>
<td>97.3 (± 1.0)</td>
<td>92.6 (± 4.0)</td>
<td>85.9 (± 4.5)</td>
</tr>
<tr>
<td>$R_U$ (%)</td>
<td>38.5 (± 9.1)</td>
<td>44.4 (± 9.8)</td>
<td>49.4 (± 7.8)</td>
</tr>
</tbody>
</table>

* Standard deviation (n=5)

b) Octyl Sepharose

<table>
<thead>
<tr>
<th></th>
<th>L. monocytogenes NCTC 5105</th>
<th>L. monocytogenes F6861 WT</th>
<th>L. monocytogenes F6861 M</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>pH 5.0</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$R_F$ (%)</td>
<td>96.3 (± 2.4*)</td>
<td>91.2 (± 7.2)</td>
<td>79.5 (± 8.4)</td>
</tr>
<tr>
<td>$R_U$ (%)</td>
<td>80.7 (± 14.1)</td>
<td>76.2 (± 13.4)</td>
<td>80.9 (± 10.9)</td>
</tr>
<tr>
<td><strong>pH 7.0</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$R_F$ (%)</td>
<td>86.1 (± 1.7)</td>
<td>89.3 (± 3.3)</td>
<td>75.9 (± 1.7)</td>
</tr>
<tr>
<td>$R_U$ (%)</td>
<td>56.4 (± 11.2)</td>
<td>64.0 (± 11.2)</td>
<td>61.3 (± 26.1)</td>
</tr>
</tbody>
</table>

* Standard deviation (n=5)
Table 7.4 Statistical analysis of HIC by the Student’s t-test to determine whether the difference in hydrophobicity between *L. monocytogenes* strains is significant, using a) phenyl Sepharose and b) octyl Sepharose

a) Phenyl Sepharose

<table>
<thead>
<tr>
<th></th>
<th><em>L. monocytogenes</em> 5105 + F6861 WT</th>
<th><em>L. monocytogenes</em> 5105 + F6861 M</th>
<th><em>L. monocytogenes</em> F6861 WT + M</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>pH 5.0</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>R</em>&lt;sub&gt;F&lt;/sub&gt; (%)</td>
<td><em>P</em> &lt; 0.001</td>
<td><em>P</em> &lt; 0.001</td>
<td><em>P</em> &lt; 0.001</td>
</tr>
<tr>
<td><em>R</em>&lt;sub&gt;U&lt;/sub&gt; (%)</td>
<td><em>P</em> &lt; 0.02</td>
<td><em>P</em> &lt; 0.01</td>
<td>-</td>
</tr>
<tr>
<td><strong>pH 7.0</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>R</em>&lt;sub&gt;F&lt;/sub&gt; (%)</td>
<td><em>P</em> &lt; 0.05</td>
<td><em>P</em> &lt; 0.001</td>
<td><em>P</em> &lt; 0.05</td>
</tr>
<tr>
<td><em>R</em>&lt;sub&gt;U&lt;/sub&gt; (%)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

- Not significant

b) Octyl Sepharose

<table>
<thead>
<tr>
<th></th>
<th><em>L. monocytogenes</em> 5105 + F6861 WT</th>
<th><em>L. monocytogenes</em> 5105 + F6861 M</th>
<th><em>L. monocytogenes</em> F6861 WT + M</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>pH 5.0</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>R</em>&lt;sub&gt;F&lt;/sub&gt; (%)</td>
<td>-</td>
<td><em>P</em> &lt; 0.01</td>
<td><em>P</em> &lt; 0.05</td>
</tr>
<tr>
<td><em>R</em>&lt;sub&gt;U&lt;/sub&gt; (%)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>pH 7.0</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>R</em>&lt;sub&gt;F&lt;/sub&gt; (%)</td>
<td>-</td>
<td><em>P</em> &lt; 0.001</td>
<td><em>P</em> &lt; 0.001</td>
</tr>
<tr>
<td><em>R</em>&lt;sub&gt;U&lt;/sub&gt; (%)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

- Not significant
The results indicate that all three strains of *L. monocytogenes* are hydrophobic in nature, as reflected by the high $R_F$ values.

With phenyl Sepharose, *L. monocytogenes* NCTC 5105 was more hydrophobic than both the other strains as its $R_F$ value was significantly higher at both pH values, although its $R_U$ value was only significantly higher at pH 5. With octyl Sepharose, *L. monocytogenes* 5105 was more hydrophobic than strain F6861 M only, with a significantly higher $R_F$ value but not $R_U$ value.

With all four sets of experimental conditions, strain F6861 WT had a significantly higher $R_F$ value than its F6861 mutant indicating that F6861 WT is more hydrophobic. However, none of the $R_U$ values were significantly different.

As expected, $R_F$ values were always higher than $R_U$ values. Both $R_F$ and $R_U$ values decreased with increasing pH, although with $R_U$ values the effect was more pronounced. Phenyl Sepharose gave similar $R_F$ values but lower $R_U$ values than octyl Sepharose and generally the results were more consistent with phenyl Sepharose as indicated by the respective standard deviations.

### 7.3.3 Involvement of *de novo* protein synthesis in the nisin resistance of *L. monocytogenes*

The effect of chloramphenicol on the frequency of isolation of nisin-resistant mutants from *L. monocytogenes* F6861 was studied and the means of duplicate results are presented in Table 7.5. On comparison of the control culture and the culture to which chloramphenicol had been added, the results indicate that chloramphenicol did not adversely affect the frequency of isolation of nisin-resistant mutants. This suggests that *de novo* protein synthesis was not required for nisin resistance.

The addition of nisin expectedly reduced the total cell population, with a 2 log
reduction on immediate sampling and a 6 log reduction after 90 min. However, the mutant population was only reduced after 90 min incubation with nisin where it fell below the detectable limit. No change in the mutant population occurred on immediate sampling.

When chloramphenicol was added with nisin, it demonstrated a slight protective effect against nisin when total cell counts were enumerated. Again no change in the mutant population occurred on immediate sampling, but this time after 90 min, nisin-resistant mutants were detectable also demonstrating the protective effect of chloramphenicol against nisin.

Table 7.5 Involvement of de novo protein synthesis in nisin resistance

<table>
<thead>
<tr>
<th></th>
<th>Cell concentration after &lt;1 min (cfu/ml)</th>
<th>Cell concentration after 90 min (cfu/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total counts&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Mutant counts&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Control culture</td>
<td>7.5 x 10&lt;sup&gt;8&lt;/sup&gt;</td>
<td>6.4 x 10&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
<tr>
<td>Culture + nisin</td>
<td>4.4 x 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>1.9 x 10&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
<tr>
<td>Culture + chloramphenicol</td>
<td>7.5 x 10&lt;sup&gt;8&lt;/sup&gt;</td>
<td>2.3 x 10&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
<tr>
<td>Culture + chloramphenicol+ nisin</td>
<td>4.2 x 10&lt;sup&gt;7&lt;/sup&gt;</td>
<td>1.9 x 10&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>: Determined on TSA plates  
<sup>b</sup>: Determined on nisin-supplemented TSA plates
7.3.4 S-layer determination of *L. monocytogenes*

**Freeze-etching and atomic force microscopy**

Examination of strains by both freeze-etching (Plates 7.1, 7.2, 7.3, and 7.4) and atomic force microscopy (Plates 7.5 and 7.6) did not demonstrate the presence of S-layers as compared with the *Lactobacillus* control.

**S-layer extraction and SDS-PAGE**

S-layer extraction procedures isolated much lower protein concentrations from *L. monocytogenes* F6861 wild type (WT) and its nisin-resistant mutant (M) than the *Lactobacillus* control (Table 7.6). Lowest protein concentrations were generally obtained from *L. monocytogenes* F6861 WT.

**Table 7.6 S-layer extraction**

<table>
<thead>
<tr>
<th>S-layer preparation procedure</th>
<th>Protein content (mg/g wet weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>L. monocytogenes</em> F6861 WT</td>
</tr>
<tr>
<td>Guanidine-HCl <em>(n=4)</em></td>
<td>0.23 (0.02*)</td>
</tr>
<tr>
<td>Lithium chloride <em>(n=2)</em></td>
<td>0.19 (0.02)</td>
</tr>
</tbody>
</table>

* Standard deviation
Plate 7.1  Freeze-etching of *Lactobacillus buchneri* showing S-layer proteins

(Bar 100 nm)
Plate 7.2  Freeze-etching of *L. monocytogenes* NCTC 5105 showing no S-layer proteins (Bar 100 nm)
Plate 7.3  Freeze-etching of *L. monocytogenes* F6861 wild type showing no S-layer proteins (Bar 100 nm)
Plate 7.4  Freeze-etching of *L. monocytogenes* F6861 nisin-resistant mutant showing no S-layer proteins (Bar 100 nm)
Plate 7.5 Atomic force microscopy of *Lactobacillus buchneri* indicating the presence of S-layers
Plate 7.6 Atomic force microscopy of *L. monocytogenes* F6861 wild type indicating the absence of S-layers
On SDS-PAGE, both *L. monocytogenes* F6861 WT and its nisin-resistant mutant did not show a band corresponding to the *Lactobacillus* S-layer proteins (MW 55 kDa) (Plate 7.7). A faint band (MW ca. 80 kDa) was visible in both *Listeria* strains but with no marked difference in concentration between the two.

Plate 7.7  SDS-PAGE of S-layer preparations (1 Molecular weight markers; 2 *Lactobacillus buchneri*; 3 *L. monocytogenes* F6861 WT; 4 *L. monocytogenes* F6861 M; 5 Molecular weight markers)
7.3.5 Involvement of cell wall teichoic acids and/or lipoteichoic acids in the nisin resistance of *L. monocytogenes*

The inactivation of *L. monocytogenes* F6861 wild type (WT) and its nisin-resistant mutant (M) by nisin was conducted at a nisin concentration of 200 IU/ml, as previously determined in section 7.3.1 (Figs. 7.2b and 7.3). At this nisin concentration, there was a significant difference in the death rates of the two strains (ca. 2 log cycles). Thus, phosphodiesterase (PDase)-treated cells of both strains, which had their cell wall teichoic acid and lipoteichoic acid components removed, were also inactivated at a nisin concentration of 200 IU/ml to compare their resistance with whole cells (Fig. 7.4).

Both PDase-treated wild type and mutant strains of *L. monocytogenes* F6861 showed reduced resistance to nisin as compared with their respective whole cells. This effect was more pronounced in the wild type PDase-treated strain which on average had its cell concentration reduced by ca. 1.5 log cycles more than its respective whole cells. In contrast, the mutant PDase-treated strain had its cell concentration reduced on average by only ca. 0.75 log cycles more than its respective whole cells. This increased reduction in cell concentration by PDase-treated cells as compared with whole cells, when inactivated by nisin, was consistent in both strains over the 3 h sampling period. However, the mutant PDase-treated strain retained a greater resistance to nisin than wild type whole cells.
Fig. 7.4 Effect of nisin on the inactivation of *L. monocytogenes* F6861 WT and F6861 M whole cells and PDase-treated cells (Ranges of duplicate experiments indicated)
7.4 DISCUSSION

To investigate a possible involvement of the *L. monocytogenes* cell wall in nisin resistance, protoplast cells were produced from lysozyme-treated whole cells and the effect of nisin studied. Initially, the objective was to look at nisin-induced K⁺ efflux, as conducted in Chapter 6, with whole cells. However, this was not possible due to extensive K⁺ leakage of the protoplast cells before any addition of nisin. Schved *et al.* (1994a) successfully examined the effect of nisin and pediocin SJ-1 on *Escherichia coli* spheroplasts by lysis experiments which involved measuring the decrease in optical density. Similarly, the effect of pediocin SJ-1 on protoplasts prepared from pediocin-sensitive and pediocin-resistant cells of *Lactobacillus plantarum* was studied (Schved *et al*., 1994b). Due to the fact that the turbidity of bacteria can only be detected at a level of 10⁷ cfu/ml or higher, and our experiments required a much lower detection limit, enumeration of protoplast cells by total plate counts was tried using buffered diluent and agar. This proved successful with the regeneration of whole cells from protoplast cells enabling cfu to be counted, and so the inactivation of protoplasts by nisin was determined by this means.

The efficacy of protoplast formation regarding *L. monocytogenes* F6861 wild type (WT) and its nisin-resistant mutant (M), determined by their subsequent lysis in water, showed a high percentage conversion for both strains (92.8% and 94%, respectively). Schved *et al.* (1994a) obtained a 90% yield in spheroplasts as detected by phase-contrast microscopy. This was also achieved for protoplast production (Schved *et al*., 1994b), a similar percentage to protoplast production in the present study. Although protoplasts are technically free of cell wall constituents, it is probable that a mixed extent of sub-lethally injured cells were present, depending on their contact with lysozyme. That is, cells with their walls virtually intact; cells with partial cell walls and protoplasts with no cell wall at all. Thus, it was important to show that the majority of cells did not have the same osmotic stability as whole cells indicating cell wall injury and protoplast formation.
By comparison of protoplast cells with whole cells, when inactivated by nisin, it was possible to determine the significance of the cell wall in nisin resistance. After a 3-h incubation of whole cells with nisin (200 IU/ml), *L. monocytogenes* F6861 WT had decreased in population size by ca. 2 log cycles more than its nisin-resistant mutant (Fig. 7.3). Protoplasts of both strains, however, showed no significant difference between their death rates and after a 3-h incubation with nisin, each cell concentration resembled that of the F6861 WT whole cells.

Thus, when the cell wall was removed from the *L. monocytogenes* F6861 mutant, it appeared to lose its acquired resistance with a phenotypic reversion back to the wild type. In contrast, when the cell wall was removed from the F6861 WT, its resistance remained unchanged from its whole cell counterpart. However, the fact that the initial death rate of the F6861 WT protoplasts was not as rapid as the F6861 WT whole cells is difficult to explain. Due to certain overlap of the experimental error ranges, it is conceivable that this was not a true effect. On the other hand, if the observation was real and wild type protoplasts are indeed more resistant to nisin than whole cells over an initial period, then the following hypothetical explanation is plausible. For instance, if adsorption sites exist in the cell wall, which attract several nisin molecules, a concentrated number of nisin molecules would then be channelled to, and reach the same site on, the cytoplasmic membrane, which has been suggested to be necessary for their aggregation in pore formation (Benz *et al*., 1991; Sahl, 1991). Without the presence of a cell wall with receptors, nisin molecules would not be concentrated together in the same way but would instead be dispersed randomly around the cell membrane. Possibly, only at higher nisin concentrations would there be enough nisin molecules in the same vicinity to aggregate and form as many pores in the cytoplasmic membrane as wild type whole cells, or as in this case, after a longer incubation period where nisin had had more time to aggregate. Indeed, Schved *et al*. (1994a) showed that with their spheroplasts, the higher the concentration of nisin, the higher was the initial rate of lysis.

Whatever, the results imply that the cell wall has a significant role in the aquisition
of nisin resistance when comparing the increased resistance of the F6861 mutant to its original wild type strain. Schved et al. (1994b) also attributed the pediocin SJ-1 resistance of Lact. plantarum strains, to the barrier properties of the cell wall. In contrast, Zajdel et al. (1985) showed that the bacteriocin lactostrepcin (Las) 5, did not kill protoplasts prepared from either sensitive or resistant bacterial cells. The authors suggested that interaction with the cell wall is a condition necessary for Las 5 activity.

To investigate further the role of the cell wall in acquired nisin resistance and resistance between strains, cell surface hydrophobicities were determined for all three strains of L. monocytogenes to ascertain whether a difference exists in their outer cell components which might be related to nisin resistance.

Hydrophobicity determination is a complicated procedure and the methods used to assess hydrophobic properties of molecules or particles are numerous (Dillon et al., 1986; Mozes and Rouxhet, 1987). There is no general consensus concerning scales of hydrophobicity. The problem is mainly due to the fact that hydrophobicity is a general concept and can be measured only indirectly, through phenomena which reflect more or less clearly the nature of molecular interactions (Mozes and Rouxhet, 1987). To gain a more accurate picture of cell surface hydrophobicity, a combination of methods should be employed as stated in section 7.2.

As mentioned, determination of bacterial cell surface hydrophobicity was attempted for all three strains of L. monocytogenes. The relative hydrophobicity of strain NCTC 5105 varied according to the test used. Using BATH (Table 7.2a) it was hydrophilic, with the hydrophobicity of 5105 being significantly lower than both the other L. monocytogenes strains. Results using HIC (Table 7.3a) with phenyl Sepharose, indicated that strain NCTC 5105 was hydrophobic and predominantly more hydrophobic than both the other strains. With octyl sepharose, the hydrophobicity of strain 5105 was only significantly higher than strain F6861 M when determined by \( R_F \) values. Conflicting results regarding these two methods was also reported by
Dillon et al. (1986) for some strains of bacteria. The authors suggest that discrepancies between assays occur when applied to less pronounced examples of cell surface hydrophobicity. However, no conclusions regarding strain 5105 could be made without further experimentation.

More importantly, the results generally indicated that *L. monocytogenes* F6861 WT was significantly more hydrophobic than its nisin-resistant mutant using both BATH (Table 7.2b) and HIC (Table 7.3) tests. With the BATH test, the hydrophobicity of *L. monocytogenes* F6861 WT was 13.5% (OD) and 16.5% (cfu/ml) greater than F6861 M. With the HIC test, the results were more complicated. All four sets of $R_F$ values were significantly higher with F6861 WT as compared with F6861 M and even though two sets of $R_U$ values were also higher, they were not significant ($P > 0.05$). However, $R_F$ measurement is the conventional parameter of hydrophobicity as it reflects hydrophobic adsorption under conditions favouring hydrophobic interactions and the eluates measured contain non-retained bacteria. $R_U$ reflects the desorption of cells under conditions that do not favour hydrophobic interactions and is more suitable for the detection of very hydrophobic organisms.

The fact that hydrophobic interaction decreases with increasing pH was observed for both $R_F$ and $R_U$ values, with the effect being more pronounced with the latter. Phenyl Sepharose gave similar $R_F$ values but lower $R_U$ values than octyl Sepharose which can be explained by the fact that octyl Sepharose has a stronger hydrophobic nature at low ionic strength than phenyl Sepharose.

In a study by Mafii et al. (1991) a total of 22 strains of *L. monocytogenes* were compared for relative surface hydrophobicity with the salt aggregation test. All strains tested aggregated at high ammonium sulfate molarities indicating hydrophilicity. The hydrophilicity of *L. monocytogenes* Scott A was examined further with HIC and the results showed that adherence to octyl Sepharose only occurred when the pH was low. The surface free energy of Scott A also classified this strain as hydrophilic.
In the present study, it is not known why the HIC results indicated that all three strains of *L. monocytogenes* were hydrophobic. However, it should be noted that limitations exist for both tests used. For instance, with BATH, it has been shown by Mozes and Rouxhet (1987) that even though the test consists theoretically in assessing the partition ratio of cells in a two phase system, the cells excluded from the aqueous phase do not always disperse in the hydrocarbon phase. The cells excluded from the aqueous phase can be found at the two phase interface or may accumulate at the interface between hydrocarbon and the wall of the glass test tube. With HIC, if retention requires only some fraction of the cell surface to be hydrophobic, while other methods require a more general surface hydrophobicity, then HIC might give rise to discrepant results (Dillon *et al*., 1986).

With recommendation to use several methods for hydrophobicity determination (Dillon *et al*., 1986; Mozes and Rouxhet, 1987), other tests were attempted, namely contact angle measurement and bacterial adhesion to polystyrene. Contact angle measurement consists of depositing a drop of water on a film of cells and determining the angle between the film surface and the tangent to the drop at the solid-liquid-air meeting point. The method was attempted as described by Busscher *et al.* (1984) and Mozes and Rouxhet (1987). However, a difficulty inherent in determining the contact angle on a film of cells is the penetration of the water drop into the film, and the determinations have to be performed within 1-2 s after depositing the drop. This is normally overcome with the use of photographic equipment to capture the drop before penetration so that contact angle measurement can be conducted subsequently from photographs. Unfortunately, without the necessary equipment this was not possible. An attempt was made to measure the contact angles by eye, but this was found to be impossible given the time before the drop penetrated.

Bacterial adhesion to polystyrene is a test based on the assumption that there is a correlation between the hydrophobicity of the cells and their adhesion to polystyrene, as proposed by Rosenberg (1981). Trial of the test with *L. monocytogenes* indicated no obvious difference between strains although actual counts of the number of cells
per unit area were not undertaken (counts were not recommended). It appeared that this test was better suited for comparison of cells with extreme differences in hydrophobicity.

Thus, in conclusion from the results that were obtained, the general indication is that \textit{L. monocytogenes} F6861 WT is more hydrophobic than its nisin-resistant mutant. This indicates that the nisin-resistant mutant has adapted its cell surface in some way, and the factor responsible might possibly be involved in its nisin resistance mechanism. Nisin itself is predominantly hydrophobic in nature and so the decreased hydrophobicity of the more resistant cells may be related to their reduced avidity for nisin as observed in Chapter 5. Recently, Ming and Daeschel (1995) also reported that the cell surface hydrophobicity of strain Scott A was significantly greater than its nisin-resistant mutant, as determined by the BATH test.

The fact that the outer surface of many bacteria consists of an array of S-layer proteins, which have been shown in some Gram-positives to exhibit barrier functions against surface-active agents (Kalchayanand \textit{et al.}, 1992), led to their investigation regarding a possible role in the acquisition of nisin resistance by \textit{L. monocytogenes}. As S-layer proteins are predominantly hydrophobic in nature, it would seem that if involved in the nisin resistance mechanism of the F6861 mutant, then its hydrophobicity should be greater than its wild type lacking S-layers, which was not the case. Thompson \textit{et al.} (1982) examined the association of the surface array and the outer membrane of \textit{Deinococcus radiodurans} and found that the surface of the outer membrane was relatively more hydrophobic than the outer aspect of the surface array. Thus, it was considered possible that \textit{L. monocytogenes} F6861 WT was more hydrophobic than its nisin-resistant mutant because a surface lacking S-layers was more hydrophobic than a surface with S-layers. In contrast, the possibility that the wild type strain possessed S-layers and that the mutant was S-layer deficient was not eliminated, although in this scenario S-layers, instead of having a barrier function, would somehow enhance the uptake of nisin. Indeed, Garduño \textit{et al.} (1994) recently showed that the presence of an S-layer in \textit{Aeromonas salmonicida} predisposed this
bacterium to enhanced uptake of the hydrophobic antibiotics streptonigrin and chloramphenicol, with S-layer-positive cells being far more sensitive to the antibiotics than S-layer-negative cells. Alternatively, if both \textit{L. monocytogenes} strains possessed S-layers, then maybe the mutant had adapted them in some way to increase nisin resistance which was reflected by the difference in their hydrophobicities.

Thus with S-layers in mind, the question of whether \textit{de novo} protein synthesis is required in the acquisition of nisin resistance was determined. This was achieved by establishing the frequency of isolation of nisin-resistant mutants from the wild type F6861 strain after the addition of chloramphenicol (Table 7.5). The results indicated that chloramphenicol did not adversely affect the frequency of isolation of nisin-resistant mutants suggesting that \textit{de novo} protein synthesis was not required for nisin resistance. This implied that if S-layers were involved in the resistance mechanism, the presence of nisin would not activate their synthesis.

When nisin was added to the cultures without chloramphenicol, the total cell concentration expectedly decreased from a level of $7.5 \times 10^8$, with more than a 2 log reduction on immediate sampling and a 6 log reduction after 90 min. The frequency of mutant isolation with the control culture was \textit{ca.} $10^{-6}$, giving \textit{ca.} $10^2$ cfu/ml, but in this case mutants were isolated at a frequency of \textit{ca.} $10^{-4}$ on immediate sampling, again giving $10^2$ cfu/ml. This indicates that a certain number of cells in a given population have acquired nisin resistance and this number is more or less fixed. Nisin added to the cultures would only have eliminated the more sensitive cells, leaving the resistant mutants unaffected. However, after a longer incubation period with nisin (90 min), the mutant population did decrease to below the detectable limit. However, if the cultures were allowed to incubate for a much longer time with nisin, it is likely that very low numbers of nisin-resistant mutants would grow and become detectable again.

The fact that chloramphenicol had a protective effect against nisin, when added together, indicates that chloramphenicol, being a protein synthesis inhibitor, had
disrupted normal metabolism. Under these conditions the organism is marginally less sensitive to nisin due to its slower metabolism.

Examination of the *L. monocytogenes* strains by both freeze-etching (Plates 7.1, 7.2, 7.3 and 7.4) and atomic force microscopy (Plates 7.5 and 7.6) did not, after all, demonstrate the presence of S-layers as compared with the *Lactobacillus* control, thus discounting any role in the acquisition of nisin resistance. The inability to detect S-layer proteins on *L. monocytogenes* cells by freeze-etching was confirmed by Prof. U.B. Sleytr (personal communication, 1994). He stated that he "could not find any evidence for regular surface structures" but suggested that one should eliminate the presence of any major protein band on SDS-gels of whole cell extracts to be even more convinced that no S-layers existed.

The fact that S-layer extraction procedures isolated much lower protein concentrations from *L. monocytogenes* F6861 and its nisin-resistant mutant than the *Lactobacillus* control (Table 7.6) and that on SDS-PAGE, both *L. monocytogenes* strains did not show a major band corresponding to the *Lactobacillus* S-layer proteins (MW 55 kDa) (Plate 7.7), further indicates that *L. monocytogenes* does not possess S-layers.

With the elimination of S-layers, the involvement of other cell wall components in the nisin resistance of *L. monocytogenes* was investigated. As teichoic acids and lipoteichoic acids are partially exposed at the outermost surface of the cell it was conceivable that they had a role in nisin resistance. The bulk of the teichoic acids are embedded in the peptidoglycan while the lipoteichoic acids, which are amphipathic molecules, have their hydrophobic lipid region anchoring the hydrophilic chain to the outside of the cytoplasmic membrane, while the remainder of hydrophilic chain penetrates the cell wall. A difference in cell surface hydrophobicity between strains, as observed, might have indicated a difference in their teichoic acid/lipoteichoic acid components relating to nisin resistance.

Thus, phosphodiesterase (PDase)-treated cells of both strains, which had the effect
of removing the teichoic acid and lipoteichoic acid components, were inactivated by nisin at a concentration of 200 IU/ml to compare their resistance with whole cells (Fig. 7.4). The fact that both PDase-treated wild type and mutant strains of *L. monocytogenes* F6861 showed a consistently reduced resistance to nisin over the 3 h sampling period, as compared with their respective whole cells, indicated that teichoic acids and/or lipoteichoic acids were somehow involved in nisin resistance. As this effect was quite pronounced in the wild type PDase-treated strain, with a 1.5 log cycle reduction more than its respective whole cells, it would suggest that teichoic acids and/or lipoteichoic acids play an active role in intrinsic nisin resistance. And, even though the mutant PDase-treated strain showed a reduced resistance to nisin as compared with its respective whole cells (ca. 0.75 log cycle reduction), the resistance of the mutant PDase-treated strain was still greater than wild type whole cells, indicating that the involvement of teichoic acids and/or lipoteichoic acids are by no means exclusively responsible for acquired nisin resistance.

However, the fact that phosphodiester bonds also exist in the cytoplasmic membrane should not be ignored, as it is possible that they too were affected by the addition of phosphodiesterase. These phosphodiester bonds function as links between the hydrophilic head groups and bridging moieties attaching the hydrophobic fatty acid tails of the phospholipids. Breakage of these bonds would surely result in loss of membrane integrity and cell death, which was not observed before the addition of nisin suggesting that they were not affected to any great extent. Thus, it is likely that most of the phosphodiesterase was depleted at the cell wall before it targeted the cell membrane.

On comparison of the results from the protoplast and phosphodiesterase experiments it would appear that with the F6861 mutant, even though interference with the teichoic and lipoteichoic acids reduces its resistance to nisin, the complete cell wall must be removed for it to undertake a phenotypic reversion back to the wild type. This suggests the involvement of other cell wall components, possibly peptidoglycan, in acquired nisin resistance. Results from the wild type strain are more difficult to
assess as its resistance is significantly reduced on interference with its teichoic and lipoteichoic acids but not when the whole cell wall is removed. It is conceivable that on treatment of the cells with lysozyme, the teichoic acid chains being covalently attached to the peptidoglycan are removed, while fragments of the lipoteichoic acids remain as they are not attached to the peptidoglycan but instead anchored to the cell membrane by their hydrophobic lipid end. In this way, the lipoteichoic acids would continue to play their part in nisin resistance. In fact, lipoteichoic acids resemble lipopolysaccharides of Gram-negative eubacteria both in physicochemical characteristics and various biological properties (Fiedler, 1988). It is established that in Gram-negative bacteria, it is the lipopolysaccharide component of the outer membrane that confers resistance to the organism against hydrophobic molecules (Russell and Chopra, 1990). For instance, Stevens et al. (1992) have shown from work with Salmonella typhimurium lipopolysaccharide mutants, that the core oligosaccharide in lipopolysaccharide plays a role in nisin sensitivity. Phosphodiesterase treatment, on the other hand, would destroy the lipoteichoic acids thereby reducing the organism’s inherent resistance.
7.5 SUMMARY

The involvement of the cell wall in the acquisition of nisin resistance by *L. monocytogenes* was investigated using the F6861 wild type strain and its nisin-resistant mutant. Removal of the cell wall produced protoplast cells, which were subsequently inactivated by nisin and their resistance compared with whole cells. Results indicated that without its cell wall, the acquired nisin resistance of the mutant was lost, with a phenotypic reversion back to the wild type. In contrast, when the cell wall was removed from the wild type, its resistance remained unchanged from its whole cell counterpart. Thus, it would appear that the cell wall of *L. monocytogenes* F6861 does have an active role in the acquired nisin resistance of its mutant.

Cell surface hydrophobicity was shown to correlate with nisin sensitivity; the wild type strain being more hydrophobic than its mutant. Thus, in determining a difference in the outer surfaces of both strains, the possible role of S-layer proteins in the acquisition of nisin resistance by *L. monocytogenes* was investigated. Chloramphenicol did not adversely affect the frequency of isolation of nisin-resistant mutants, indicating that *de novo* protein synthesis was not involved. Examination of strains by freeze-etching and atomic force microscopy did not demonstrate the presence of S-layers in either strain as compared with a *Lactobacillus* positive control. S-layer extraction procedures isolated much lower protein concentrations from both *L. monocytogenes* strains than from the *Lactobacillus* and on SDS-PAGE no major bands corresponding to the S-layer proteins of the *Lactobacillus* were observed for either *L. monocytogenes* strain.

With elimination of S-layers, the involvement of other cell surface components, namely teichoic and lipoteichoic acids, were investigated. Removal of these components and subsequent inactivation of the cells with nisin, reduced the resistance of both strains when compared with their respective whole cells. The resistance of the mutant strain lacking these components, however, was still significantly greater than whole cells of its wild type indicating the involvement of an extra factor in acquired
nisin resistance. Resistance of the wild type lacking these components was significantly lowered when compared with its respective whole cells, and the fact that the resistance of wild type protoplast cells remained the same as whole cells suggests the possible involvement of lipoteichoic acids in intrinsic nisin resistance, conditional on their partial retention in protoplast formation.
CHAPTER 8 THE CYTOPLASMIC MEMBRANE OF LISTERIA MONOCYTTOGENES AND ITS INVOLVEMENT IN NISIN RESISTANCE
8 THE CYTOPLASMIC MEMBRANE OF *LISTERIA MONOCYTOGENES* AND ITS INVOLVEMENT IN NISIN RESISTANCE

8.1 INTRODUCTION

The previous chapter determined the role of the cell wall in the nisin resistance of *L. monocytogenes* and even though the evidence did indicate its involvement, this may not be the only factor. Thus, the role of the cytoplasmic membrane in the nisin resistance of *L. monocytogenes* was also investigated.

The cytoplasmic membrane acts as a selective permeability barrier between the cytoplasm and the cell environment and is also the site of many crucial cellular activities. Cytoplasmic membranes primarily consist of phospholipids and proteins, although the exact membrane composition depends on the cell of origin (Russell and Chopra, 1990). Membrane phospholipids are polar lipids which are also referred to as amphipathic, meaning that they incorporate both a hydrophobic tail and a hydrophilic head group within the molecule. The amphipathic phospholipid molecules are arranged in a bilayer with the hydrophilic polar groups directed outwards on both sides. The hydrophobic and hydrophilic regions in bacterial membranes are usually bridged by a glycerol moiety. The hydrophobic tail commonly consists of two long aliphatic chains (the commonest components of which are fatty acids) esterified to two hydroxy groups of glycerol, while the third, primary, hydroxy-group carries a hydrophilic phosphate ester grouping. However, phospholipids can contain phosphoric acid as a mono- or di-ester. Phosphatidylglycerol (1,2-diacyl-sn-glycero-3-phosphoglycerol) is probably the most widely occurring phospholipid in bacteria, being particularly abundant in the membranes of Gram-positive organisms (Harrison and Lunt, 1980).

Bacterial fatty acid components of the hydrophobic tails are generally 10-20 carbon atoms long and can be saturated or unsaturated, branched or straight. It is the saturated and branched chain fatty acids (iso and anteiso) that are widely found in
Gram-positive bacteria, with the fluidity of the membranes being controlled mainly by anteiso-C15:0.

Several antibacterial compounds disrupt the cytoplasmic membrane, one of which, of course, is nisin. The others include the polymyxin group of antibiotics, the ionophorous antibiotics and various types of antiseptics, disinfectants and preservatives. Bacterial resistance to membrane active antimicrobials is sometimes related to changes in the membrane components. For instance, the antibacterial activity of polymyxins is related to the nature of membrane phospholipids, and the absence of phosphatidylethanolamine in membranes from Gram-positive bacteria may account for their lack of sensitivity to these antibiotics (Russell and Chopra, 1990). Similarly, mutation of Bacillus firmus leading to duramycin (a Type B lantibiotic) resistance, results in substantial replacement of membrane lipid phosphatidylethanolamine by its plasmalogen form (Clejan et al., 1989). Also the role of lipid in butylated hydroxyanisole (BHA) (an antioxidant) resistance of L. monocytogenes has been suggested (Al-issa et al., 1984). In their study, the authors increased the lipid content of L. monocytogenes by successive subculturing in a glycerol medium. Fattened cells showed considerably greater resistance to BHA. Polar lipids and fatty acid composition of four cultures with different BHA sensitivity were analysed. They were basically similar but the resistant cultures had a lower proportion of unsaturated and anteiso to saturated and iso fatty acids.

With greater relevance to our study, Ming and Daeschel (1993) have investigated the role of the cytoplasmic membrane in the acquired nisin resistance of L. monocytogenes Scott A. In their study they conducted a gas chromatographic (GC) analysis of the fatty acids in L. monocytogenes Scott A and its nisin-resistant mutant. Results revealed significant differences in fatty acid composition such that the wild type contained a higher proportion of anteiso-C15:0, iso-C15:0 and iso-C17:0, whereas the mutant contained a higher proportion of C14:0. Thus comparatively, the resistant mutant contained a greater proportion of straight chain fatty acids while its wild type contained more of the bulkier branched fatty acids. The authors suggested
that these observations are consistent with the fact that the wild type had a lower phase transition temperature, as branched chain fatty acids disrupt acyl-chain packing in membranes thereby lowering the membrane lipid gel to liquid transition temperature.

According to Ming and Daeschel (1993), the difference in fatty acid composition was especially evident in the case of anteiso-C15:0, the branching fatty acid that plays an important part in controlling membrane fluidity. The authors suggested that 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 wild type, and that this may make it less susceptible to the action of nisin either directly or by altering protein configuration in the membrane. However, Ming and Daeschel (1993) commented that the change in fatty acid composition might simply be a general adaptation to an unfavorable environment as opposed to a specific response to nisin.

Juneja and Davidson (1993) showed that the sensitivity of *L. monocytogenes* Scott A to antimicrobial compounds was altered when bacterial membrane lipid composition was modified by growth in the presence of added fatty acids. This was not determined for nisin however, nor for the mutant strain of Scott A. GC analysis of cellular fatty acid composition showed that when the fatty acids C14:0, C16:0, C18:0, and C18:1 were applied exogenously, their relative percentages increased significantly indicating that they had been successfully incorporated into the cell. Resistance of the lipid-adapted cells was evaluated by MIC determination and inactivation over time. The results generally revealed an increased resistance with C14:0 and C18:0, and increased sensitivity with C18:1. Thus, the authors concluded that the susceptibility of *L. monocytogenes* to antimicrobial agents is related to the lipid composition of the cell membrane. Consequently, food preservation processes that alter fatty acid composition of *L. monocytogenes* could result in changes in antimicrobial susceptibility. Analogously, an acquired change in fatty acid composition of *L. monocytogenes* could result in increased resistance to nisin as suggested by Ming and

The fact that the difference in fatty acid composition between a wild type and nisin-resistant mutant strain of *L. monocytogenes* might simply be a general adaptation to an unfavorable environment, as opposed to a specific response to nisin, was subsequently investigated in this present study by using the protocol of Juneja and Davidson (1993).

Thus the objective of the study was to:

(1) Compare the natural fatty acid composition of *L. monocytogenes* F6861 and its nisin-resistant mutant by GC analysis to determine whether any differences exist between the strains.

(2) Modify the lipid composition of both strains by growing them in the presence of selected exogenous fatty acids, checked by GC analysis, and evaluate the nisin resistance of the lipid-adapted strains by MIC determinations and inactivation over time.
8.2 MATERIALS AND METHODS

8.2.1 Modification of lipid composition

On the basis of Ming and Daeschel's work (1993), two fatty acids were selected for incorporation into L. monocytogenes F6861 wild type and its nisin-resistant mutant. These were C14:0 (otherwise known as tetradecanoic acid or myristic acid), which would be expected to increase nisin resistance, and anteiso-C15:0 (12 methyl-tetradecanoic acid), which would be expected to decrease nisin resistance. C14:0 was obtained from Sigma and anteiso-C15:0 from Apin Chemicals Ltd.

The method was conducted as described by Juneja and Davidson (1993) with a few modifications as follows:

Bacterial cells were grown in TPB (pH 5.5, 15 ml) containing (50 \mu g/ml*) exogenous fatty acids (C14:0 or anteiso-C15:0) at 30°C for 24 h (ca. 10^8-10^9 cfu/ml) and harvested by centrifugation (1500 g for 15 min). The cell pellets were washed with MRD containing 0.1% (v/v) Tween 80 (Sigma) and centrifuged. The purpose of the Tween 80 was to remove any added fatty acid that was not incorporated into the bacterial cell membrane. The cell pellets were then washed with MRD (without Tween 80) and centrifuged; this washing and centrifugation was repeated three times.

*Note: The fatty acids were dissolved initially in ethanol and added to the broths to give their required concentrations so that the final ethanol concentration was 0.5% (v/v). At this ethanol concentration inhibition of growth of the cultures does not occur.

8.2.2 Fatty acid analysis

This procedure involved lipid extraction and conversion to methyl esters for subsequent GC analysis. Initial attempts for extraction and methylation of fatty acids
was conducted using the rapid method of Rozès et al. (1993), effectively for the use of Gram-positive and Gram-negative bacteria as follows:

*Listeria monocytogenes* cells were harvested from stationary culture (1 ml, TPB, pH 7.3) by centrifugation (5000 g for 10 min), then washed twice with sterile water. Methanolysis of cell materials was carried out with sodium methoxide (1 N)(Sigma) in anhydrous methanol. Sodium methoxide (0.5 ml) was added to the cell pellets in a glass tube and mixed for 1 min. Fatty acid methyl esters (FAMEs) were extracted by shaking with hexane (0.3 ml)(Sigma) for 20 s. The upper phase was removed after decanting and 1 µl was injected into the chromatograph.

However, after numerous attempts and the use of three different GCs this method proved to be unsuccessful as no peaks were detected.

Consequently, extraction and methylation of fatty acids was conducted by a method based on that of Bligh and Dyer (1959) as follows:

*Listeria monocytogenes* was grown to stationary phase (10 ml, TPB, pH 7.3) and the cells harvested by centrifugation (1500 g for 15 min). A minimum volume of distilled water was added to the cell pellet so that the final volume of suspension was 500 µl. After transferring to an acid washed extraction tube, methanol (1.25 ml) was added and the mixture briefly shaken before further addition of chloroform (625 µl). The mixture was again shaken and allowed to stand at room temperature for 2 h. After centrifugation (600 g for 15 min), the supernatant produced was transferred to a clean extraction tube. To the cell pellet was added a mixture of methanol/chloroform/water (2:1:0.8 v/v/v)(2.375 ml) and the suspension shaken before standing for a further 2 h. After another centrifugation step, the supernatant obtained was added to the supernatant obtained previously. To the pooled supernatants was added chloroform (2.5 ml) and water (2.5 ml), which after a brief centrifugation step, formed a two phase system. From this, the lower lipid-containing chloroform fraction was removed and transferred to an acid-washed glass tube. The sample was concentrated by gentle
heating under a nitrogen stream, which evaporated the chloroform. The extracted lipid could then be stored at -20°C overnight.

To the extracted lipid was added 2.5% (v/v) H$_2$SO$_4$ in anhydrous methanol (2 ml). The mixture was heated at 70°C for 2 h, before the addition of 10% (w/v) sodium chloride (3 ml) to stop the reaction. The FAMEs were extracted by mixing with 3 x 2-ml volumes of Petroleum Ether (60-80°C), removing only 90% of the total upper phase each time. The FAMEs were then concentrated by gentle heating under a nitrogen stream, and after evaporation to dryness, they were resuspended in a small fixed volume of Petroleum Ether ready for GC analysis.

GC analysis of the methyl esters was conducted using a Pye Unicam PU 4550 Gas Chromatograph with a flame ionization detector. Major fatty acids were identified by comparison of retention times with those of a pure bacterial fatty acid methyl esters standard (Matreya Inc., USA). The internal standard used was methyl n-decanoate (C10:0)(BDH). The chromatographic conditions were as follows: capillary column (10 m, BP 1-2, bonded phase, ID 0.33 mm); column temperature programmed from 120 to 240°C at 4°C/min; carrier gas hydrogen, 1 m/min; injection and detection temperature, 250°C; sample size, 1 μl.

8.2.3 Nisin resistance of lipid-adapted strains

Cultures of _L. monocytogenes_ F6861 wild type and its nisin-resistant mutant, which had been grown in the presence of the fatty acids C14:0 or anteiso-C15:0, were evaluated for resistance to nisin by MIC determination and inactivation over time. Using the prepared samples suspended in MRD (described in section 8.2.1), MIC determination was conducted in TPB (pH 5.5), as described previously in Chapter 4 (page 45) and inactivation of _L. monocytogenes_ was conducted at a nisin concentration of 200 IU/ml, as described previously in Chapter 7 (page 111).
8.3 RESULTS

8.3.1 Fatty acid analysis of *L. monocytogenes*

A commercial bacterial fatty acid methyl esters (FAMEs) standard mixture was spiked with a selected internal standard (FAME C10:0) and a gas chromatogram profile determined. The individual FAME peaks were identified from a data sheet (Fig. 8.1) as shown in Fig. 8.2a. To verify that the internal standard peak had been identified correctly, the FAME standard mixture was analysed again, but this time with double the original concentration of internal standard. This was reflected by a peak height double that of the original (Fig. 8.2b).

**Fig. 8.1 Commercial bacterial FAMEs technical data sheet (From Matreya Inc., USA)**
Fig. 8.2a  GC profile of a commercial bacterial FAMEs standard mixture spiked with a given concentration of internal standard (FAME C10:0)

Fig. 8.2b  GC profile of a commercial bacterial FAMEs standard mixture spiked with double the previous concentration of internal standard (FAME C10:0)
The relative retention times (RRT) of the individual FAMEs (calculated as: retention time of FAME/retention time of internal standard) for the duplicate FAME standard mixture profiles were similar, indicating the reproducibility of the technique. Results showing the RRT of C14:0 and anteiso-C15:0 FAMEs are presented in Table 8.1.

Table 8.1  Relative retention times of C14:0 and anteiso-C15:0 FAMEs

<table>
<thead>
<tr>
<th></th>
<th>Relative retention times (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C14:0 FAME</td>
</tr>
<tr>
<td>GC profile a (Fig. 8.2a)</td>
<td>4.34</td>
</tr>
<tr>
<td>GC profile b (Fig. 8.2b)</td>
<td>4.35</td>
</tr>
</tbody>
</table>

GC FAME profiles of *L. monocytogenes* F6861 wild type (WT) and its nisin-resistant mutant (M) were determined to reflect their respective fatty acid compositions (Figs. 8.3a and b). The RRT of the sample FAMEs were not sufficiently similar to those of the standard mixture to enable positive identification of all the peaks. Thus, presumptive identification was made by comparing the FAME profiles of the samples with the standard mixture on a qualitative basis. This took into account the order and relative positions of the peaks.

Qualitative comparison of the FAME profiles of the wild type and mutant strains indicated that their fatty acid compositions were similar. For quantitative analysis, peak areas were calculated and expressed as percentages of the total fatty acid content. FAMEs comprising of more than 1% of the total (except C14:0 and anteiso-C15:0 which were < 1%) are shown in Table 8.2.
Fig. 8.3 GC FAME profile of *L. monocytogenes* a) F6861 WT and b) F6861 M
Table 8.2 Quantitative analysis of the fatty acids present in *L. monocytogenes*, expressed as percentages of the total fatty acid content

<table>
<thead>
<tr>
<th>Peak number</th>
<th>Presumptive identification of fatty acid</th>
<th>L. monocytogenes F6861 WT</th>
<th>L. monocytogenes F6861 M</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>2-OH 10:0</td>
<td>3.06</td>
<td>2.89</td>
</tr>
<tr>
<td>3</td>
<td>12:0</td>
<td>1.22</td>
<td>0.95</td>
</tr>
<tr>
<td>6</td>
<td>3-OH 12:0</td>
<td>1.47</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>14:0</td>
<td>0.78</td>
<td>0.40</td>
</tr>
<tr>
<td>8</td>
<td>i-15:0</td>
<td>2.45</td>
<td>1.56</td>
</tr>
<tr>
<td>9</td>
<td>a-15:0</td>
<td>0.94</td>
<td>0.62</td>
</tr>
<tr>
<td>13</td>
<td>i-16:0</td>
<td>3.33</td>
<td>2.82</td>
</tr>
<tr>
<td>14</td>
<td>16:1&lt;sup&gt;9&lt;/sup&gt;</td>
<td>1.73</td>
<td>1.34</td>
</tr>
<tr>
<td>16</td>
<td>i-17:0</td>
<td>5.45</td>
<td>4.97</td>
</tr>
<tr>
<td>17</td>
<td>17:0&lt;sup&gt;Δ&lt;/sup&gt;</td>
<td>9.03</td>
<td>8.91</td>
</tr>
<tr>
<td>22</td>
<td>18:1&lt;sup&gt;9&lt;/sup&gt;/18:1&lt;sup&gt;11&lt;/sup&gt;</td>
<td>4.72</td>
<td>4.40</td>
</tr>
<tr>
<td>24</td>
<td>19:0&lt;sup&gt;Δ&lt;/sup&gt;</td>
<td>3.48</td>
<td>3.54</td>
</tr>
<tr>
<td>25</td>
<td>19:0</td>
<td>9.47</td>
<td>12.24</td>
</tr>
<tr>
<td>26</td>
<td>20:0</td>
<td>2.74</td>
<td>3.17</td>
</tr>
<tr>
<td>27</td>
<td>?</td>
<td>2.29</td>
<td>3.31</td>
</tr>
<tr>
<td>28</td>
<td>?</td>
<td>31.47</td>
<td>35.64</td>
</tr>
</tbody>
</table>

The relative proportions of the individual fatty acids of the wild type and mutant strains were similar but without further replication of the results it was not possible to determine whether any differences observed were significant. However, the proportion of fatty acids C14:0 and anteiso-C15:0, which were of particular interest, were both lower in the mutant as compared with the wild type.
The lipid composition of each strain was attempted to be modified by growing the cells in the presence of exogenous fatty acids (C14:0 or anteiso-C15:0). The FAME profiles of each strain with added C14:0 or anteiso-C15:0 are shown in Figs 8.4 and 8.5, respectively. As before, presumptive identification of the relevant peaks were made from the standard mixture and for quantitative analysis, peak areas were calculated and expressed as percentages of the total fatty acid content. The relative amounts of C14:0 and anteiso-C15:0 present in the samples were compared with those of the controls (Table 8.3).

Table 8.3 Quantitative analysis of the modified fatty acid composition of *L. monocytogenes* a) F6861 WT and b) F6861 M, expressed as percentages of the total fatty acid content

a) *L. monocytogenes* F6861 WT

<table>
<thead>
<tr>
<th>Fatty acid analyzed</th>
<th>Fatty acid incorporated in growth media</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>+ C14:0</td>
<td>+ anteiso-C15:0</td>
</tr>
<tr>
<td>C14:0</td>
<td>0.78</td>
<td>2.51</td>
<td>-</td>
</tr>
<tr>
<td>anteiso-C15:0</td>
<td>0.94</td>
<td>-</td>
<td>3.69</td>
</tr>
</tbody>
</table>

b) *L. monocytogenes* F6861 M

<table>
<thead>
<tr>
<th>Fatty acid analyzed</th>
<th>Fatty acid incorporated in growth media</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>+ C14:0</td>
<td>+ anteiso-C15:0</td>
</tr>
<tr>
<td>C14:0</td>
<td>0.40</td>
<td>0.91</td>
<td>-</td>
</tr>
<tr>
<td>anteiso-C15:0</td>
<td>0.62</td>
<td>-</td>
<td>3.31</td>
</tr>
</tbody>
</table>
Fig. 8.4 GC FAME profile of *L. monocytogenes* a) F6861 WT and b) F6861 M with added C14:0
Fig. 8.5 GC FAME profile of *L. monocytogenes* a) F6861 WT and b) F6861 M with added anteiso-C15:0
The results showed a slight increase in the amounts of C14:0 and anteiso-C15:0 present in each strain grown in the supplemented media, as compared with the controls. Thus, it would suggest that these fatty acids were incorporated into the cells. However, without replicate values it can not be determined whether these results are significant. It should also be noted that the amounts of other fatty acids, not incorporated in the growth media, had also increased as compared with the controls. Thus, these results are inconclusive.

8.3.2 Nisin resistance of lipid-adapted L. monocytogenes

Listeria monocytogenes F6861 wild type (WT) and its nisin-resistant mutant (M), which had been grown in the presence of exogenous fatty acids (C14:0 or anteiso-C15:0), were inactivated by nisin (200 IU/ml) and the number of viable cells enumerated over a 3-h period (Fig. 8.6). The cell concentration was expressed as percentage survivors due to the fact that when both strains were grown in the presence of anteiso-C15:0, the initial level of cells was nearly a log cycle lower (ca. $10^8$ cfu/ml) than those of the control and C14:0 (ca. $10^9$ cfu/ml). The results showed that with the addition of fatty acid C14:0, both strains had an increased resistance to nisin as compared with the controls. With the addition of fatty acid anteiso-C15:0, strain F6861 WT had a reduced resistance to nisin but its nisin-resistant mutant had an increased resistance to nisin. In fact, the resistance of F6861 M with added anteiso-C15:0 was greater than the resistance of F6861 M with added C14:0. To confirm this observation, the inactivation of F6861 M with added anteiso-C15:0 was repeated (Fig. 8.7). The second set of results (Expt 2) were practically identical to the first set (Expt 1), indicating that this was a true effect.

MIC values of the cultures that had grown in the presence of exogenous fatty acids (C14:0 or anteiso-C15:0) were recorded over an incubation period of 26 d and compared with untreated controls (Fig 8.8). The addition of C14:0 to strain F6861 WT, had no significant effect on the organisms' MIC values as compared with the
Fig. 8.6 Effect of altered fatty acid composition on the resistance of *L. monocytogenes* a) F6861 WT and b) F6861 M to nisin
Fig. 8.2  Effect of altered fatty acid composition (a-C15:0) on the resistance of \textit{L. monocytogenes} F6861 M to nisin
Fig. 8.8 Effect of altered fatty acid composition in L. monocytogenes
a) F6861 WT and b) F6861 M on the MIC of nisin
untreated control strain over a period of 16 d, at which time the MIC values were both 150 IU/ml. At 26 d, the MIC value of the control had risen to 250 IU/ml, whereas the MIC value of the C14:0-treated strain remained at 150 IU/ml. The addition of anteiso-C15:0 to strain F6861 WT, however, had the effect of decreasing the organisms' resistance to nisin over a 20 d period, giving an MIC of 100 IU/ml at 20 d.

The addition of C14:0 to strain F6861 M initially increased its resistance to nisin over a 16 d period, with an MIC of 600 IU/ml at 16 d as compared with the untreated control which had an MIC of 500 IU/ml. However, at 26 d they both had the same MIC value of 700 IU/ml. The addition of anteiso-C15:0 to strain F6861 M also decreased its resistance to nisin over a 20 d period with an MIC of 400 IU/ml at 13 d compared with that of the control which was 500 IU/ml.

However, irrespective of which fatty acid was added, the resistance of strain F6861 M at 24 h was greater than strain F6861 WT, even at 20 d.
8.4 DISCUSSION

Qualitative fatty acid analysis of *L. monocytogenes* F6861 wild type and its nisin-resistant mutant showed no obvious difference in content between strains (Fig 8.3). Quantitative analysis revealed similar proportions of the fatty acids in each strain, however, it was not possible to determine whether any slight differences observed were significant without repeating the analysis (Table 8.2). The relative amounts of both (what was thought to be) C14:0 and anteiso-C15:0 were slightly lower in the mutant as compared with the wildtype, as were all the other fatty acids of similar retention times. Thus, in consideration of the possibility of misidentification of these fatty acids, this observed difference still exists. A lower proportion of anteiso-C15:0 was also reported for the *L. monocytogenes* Scott A mutant as compared with its wild type. However, in contrast to the present results, a greater proportion of C14:0 was observed in the Scott A mutant as compared with its wild type (Ming and Daeschel, 1993). Thus to implicate roles for these fatty acids in nisin resistance, the present results possibly indicate the involvement of anteiso-C15:0 in the greater nisin sensitivity of *L. monocytogenes* F6861, but do not implicate the involvement of C14:0 in the acquired nisin resistance of the *L. monocytogenes* F6861 mutant.

Incorporation of exogenously-applied fatty acids C14:0 and anteiso-C15:0 into both *L. monocytogenes* strains could not be confirmed conclusively from the fatty acid analysis (Figs 8.4 and 8.5; Table 8.3). Even though an increase in the amounts of each fatty acid was observed on comparison with the controls, this increase was relatively small (< 3%) when compared to the work of Juneja and Davidson (1993) who observed increases of over 13%. However, both the nisin inactivation and MIC results indicated that the fatty acids C14:0 and anteiso-C15:0 had been incorporated into the cells, otherwise the results would have shown more similarity to the untreated controls (Figs. 8.6, 8.7 and 8.8).

Addition of fatty acid C14:0 to the *L. monocytogenes* F6861 mutant conferred even greater resistance to nisin during inactivation and MIC determination, as compared
with the controls. Thus, although added C14:0 does appear to increase nisin resistance, it was not apparent from the present results that the *L. monocytogenes* F6861 mutant had a naturally greater proportion of this fatty acid to its wild type as reported by Ming and Daeschel (1993) for *L. monocytogenes* Scott A and its nisin-resistant mutant. Addition of C14:0 to the *L. monocytogenes* F6861 wild type only increased its nisin resistance during inactivation. This suggests that if C14:0 is involved in increased nisin resistance, it may not play a significant role.

With the addition of fatty acid anteiso-C15:0, inactivation of strain F6861 WT showed a reduced resistance to nisin whereas its nisin-resistant mutant showed an increased resistance to nisin. And, the resistance of F6861 M with added anteiso-C15:0 was greater than the resistance of F6861 M with added C14:0. However, MIC determinations with added anteiso-C15:0 showed a decreased resistance by both strains. Ming and Daeschel (1993) partly attributed the reduced proportion of anteiso-C15:0 in the *L. monocytogenes* Scott A mutant to its increased resistance to nisin. Although this might also be true for the F6861 mutant, the inactivation results showing increased nisin resistance with added anteiso-C15:0, do not indicate this.

From the results of this present study, the function of the cell membrane in the acquired nisin resistance of *L. monocytogenes* F6861 is uncertain. It is possible that the presence of a higher proportion of fatty acid C14:0 confers increased resistance to the cell, as shown when applied exogenously, however, this was not observed from the fatty acid analysis of the natural compositions of each strain. The results did indicate a lower proportion of anteiso-C15:0 in the F6861 mutant, however, because exogenously applied anteiso-C15:0 then conferred greater nisin resistance in the mutant during inactivation, the role of anteiso-C15:0 is uncertain.
8.5 SUMMARY

The role of the cytoplasmic membrane in the acquired nisin resistance of *L. monocytogenes* F6861 was investigated. Qualitative fatty acid analysis of *L. monocytogenes* F6861 wild type and its nisin-resistant mutant showed no obvious difference in content between strains. Although quantitative analysis revealed similar proportions of the fatty acids in each strain, slight differences were observed. For example, the presence of slightly lower proportions of C14:0 and anteiso-C15:0 were indicated in the F6861 mutant as compared to its wild type. However, any significant differences could not be reported from only one analysis.

Modification of lipid composition by exogenous application of fatty acids C14:0 or anteiso-C15:0 during growth was not shown conclusively from subsequent fatty acid analysis. However, results from nisin inactivation and MIC determinations of these modified strains were sufficiently different from the controls indicating some incorporation. Fatty acid C14:0 generally increased nisin resistance of both *L. monocytogenes* F6861 wild type and its nisin-resistant mutant, although MIC determinations of the wild type were similar to the control. Fatty acid anteiso-C15:0 decreased the nisin resistance of the wild type during both experiments, but increased the nisin resistance of the mutant during nisin inactivation only.

Thus, it is possible that these fatty acids have a role in nisin resistance, however, it is suggested that other factors, such as the cell wall, are also involved as shown in Chapter 7.
CHAPTER 9  GENERAL DISCUSSION
The use of nisin, or nisin-producing starter cultures, as a means of natural food preservation to control the foodborne pathogen *L. monocytogenes* is potentially feasible. However, the capability of some strains of *L. monocytogenes* to acquire a considerably greater resistance to nisin than the respective parent strain, threatens the reliability of nisin as an effective antimicrobial agent (Harris *et al.*, 1991). Thus, acquiring an understanding of the mechanism(s) of nisin resistance by *L. monocytogenes* could lead to the development of strategies to overcome the problem.

It has been shown in this study and others, that the sensitivity of *L. monocytogenes* to nisin is variable between strains (Mohamed *et al.*, 1984; Ferreira and Lund, 1991), and that not all strains are capable of producing mutants with an increased resistance. However, one of the more virulent strains (F6861), actually isolated from an epidemic of fatal food poisoning connected with Jalisco brand Mexican-style cheese, has been shown in this study to produce nisin-resistant mutants. The MIC of the mutant strain in broth at pH 5.5, after incubation at 30°C for 47 days, was > 1000 IU/ml as compared with 300 IU/ml for its parent strain. The level of nisin used in cheese, and other products, is not normally as high as 1000 IU/g (Delves-Broughton, 1990), which indicates a possible danger of the growth of nisin-resistant mutants. However, it is likely that growth in food products would not be as prolific as that in broth, due to other inhibitory factors such as a lower $a_w$ and pH. One positive factor from this study and others (Harris *et al.*, 1991; Ming and Daeschel, 1993), is that nisin-resistant mutants are isolated at a relatively low frequency ($10^{-6}$ to $10^{-8}$), and so in practice, if high microbiological standards are maintained, the mutants should not pose a problem. However, in cases of high-level contamination, high storage temperatures allowing rapid growth, long shelf lives at low temperatures and/or low levels of nisin, nisin-resistant mutants of *L. monocytogenes* would be likely to arise.

During the course of this work it was reported elsewhere that the development of acquired nisin resistance by *L. monocytogenes* (Scott A) was a relatively unstable
feature (Ming and Daeschel, 1993). However, with the F6861 mutant strain used in the present study, the nisin resistance phenotype was completely stable after undergoing ten passages of growth (ca. 65 generations) in nisin-free media at pH 7.3. This suggests a further hazard in the use of nisin as a food antimicrobial to control *L. monocytogenes*, where contamination is a problem.

The fact that the doubling time of the F6861 mutant was slightly longer than its parent strain, particularly under conditions of stress such as acid and low temperature, suggests that maybe the mutant, in acquiring nisin resistance, has compromised on fundamental aspects of its biochemistry. For instance, similarly to certain types of colicin resistance, this could occur with adaption of non-specific nisin receptors which normally function for another purpose but are subsequently hindered. However, in contrast to colicin resistance, the absence of an outer membrane in *L. monocytogenes* would necessitate the receptors to be located elsewhere in the cell wall or the cell membrane. It should be noted, however, that experiments with artificial membranes show that there is no need for an integral membrane component that serves as a receptor for nisin (Gao et al., 1991).

Thus at the early stages of this study, it seemed feasible to hypothesize that the nisin resistance mechanism of *L. monocytogenes* involves either: modification or non-availability of the target site; a change in cell wall/membrane permeability; the enzymatic degradation of nisin; or possibly a combination of these factors. By comparing sensitive and resistant strains, results from *L. monocytogenes* nisin adsorption assays, which measured residual nisin in solution, showed an increased amount of nisin in the supernatants as the resistance of the cells increased. Thus the greater the sensitivity of the strain, the greater the nisin adsorption. This effect was shown over a range of nisin concentrations, but was most significantly marked at lower nisin concentrations. This experiment indicated that a resistance mechanism involving a nisinase enzyme, as reported in some resistant bacteria (Kooy, 1952; Galesloot, 1956; Carlson and Bauer, 1957; Alifax and Chevalier, 1962; Jarvis, 1967), is unlikely, as if this were present, lower levels of residual nisin would be expected.
in supernatants exposed to more resistant species rather than the converse. Subsequently, a resistance mechanism involving a nisinase enzyme was also eliminated in *L. monocytogenes* Scott A (Ming and Daeschel, 1995). Thus instead, it is thought that resistance is based on exclusion and inhibition of nisin binding to the cells. It was not possible to show this visibly by immunogold labelling and TEM probably because if cellularly incorporated, the nisin epitopes were unable to form an antigen-antibody complex. This has also been indicated by other workers (Richard, personal communication, 1994; Stringer *et al*., 1995).

Since the first indication of membrane injury is cell leakage, particularly K\(^+\) efflux, it was decided to examine any relationship between the amount or rate of efflux and nisin resistance. The initial rate of nisin-induced K\(^+\) efflux from *L. monocytogenes* increased with increased nisin concentration until it reached a maximum rate, indicating saturation kinetics. Subsequently, Winkowski *et al*., (1994) have similarly shown that nisin-induced ATP efflux from *L. monocytogenes* Scott A depends on nisin concentration and follows saturation kinetics. But, in the K\(^+\) efflux experiment, the fact that as the nisin resistance of each strain increased, K\(^+\) efflux increased at a slower rate and had a lower maximum rate, added further evidence to the hypothesis that in *L. monocytogenes* nisin resistance is based on exclusion and inhibition of nisin binding to the cells. Thus, by this stage, all experimental results indicated that resistance is acquired by adaption of either the cytoplasmic membrane or cell wall or both, to reduce the presence or accessibility of attachment sites preventing the incorporation of nisin into the membrane.

Subsequently, by removing the cell walls from *L. monocytogenes* F6861 wild type and its nisin-resistant mutant to produce protoplast cells, and comparing their resistance to each other and with respective whole cells when inactivated by nisin, it was evident that the cell wall has a role in acquired nisin resistance. Without its cell wall, the increased nisin resistance of the mutant was lost, with a phenotypic reversion back to the wild type. In contrast, the resistance of the wild type remained unchanged. In the same vein, Schved *et al*., (1994b) attributed the pediocin SJ-1
resistance of *Lactobacillus plantarum* strains to the barrier properties of the cell wall.

The fact that cell surface hydrophobicity determinations indicate that the wild type strain is more hydrophobic than its nisin-resistant mutant, suggests that there is a difference in outer surface structure possibly connected to nisin resistance. Interestingly, the hydrophobicity of *L. monocytogenes* Scott A wild type was also significantly greater than that of its mutant (Ming and Daeschel, 1995). However, in the present study, the possibility that surface-layer (S-layer) proteins have a role in the acquisition of nisin resistance in *L. monocytogenes* F6861, was eliminated. This was due to the fact that several procedures (freeze-etching, atomic force microscopy, and S-layer extraction/SDS-PAGE) indicated that S-layers did not exist on any of the *L. monocytogenes* strains studied, when compared with a *Lactobacillus* positive control. The general inability to detect S-layer proteins on *L. monocytogenes* cells by freeze-etching was confirmed by Prof. U.B. Sleytr (personal communication, 1994). In addition, chloramphenicol did not adversely affect the frequency of isolation of nisin-resistant mutants, indicating that *de novo* protein synthesis was not involved.

With elimination of S-layers, continuing work focused on other cell surface components of *L. monocytogenes*, namely teichoic and lipoteichoic acids (Fiedler, 1988). Removal of these components with phosphodiesterase treatment reduced the resistance of both strains compared with their respective whole cells, when inactivated by nisin. Although this indicates the involvement of teichoic acids and/or lipoteichoic acids in nisin resistance, the fact that the resistance of the mutant strain lacking these components was still significantly greater than whole cells of its wild type, also indicates the involvement of an extra factor in acquired nisin resistance. However, with the wild type strain, even though its resistance was significantly reduced on interference with its teichoic and lipoteichoic acids, no change in resistance was seen on removal of the whole cell wall. Thus, it is possible that the lipoteichoic acids are involved in its intrinsic nisin resistance, if they are partially retained in protoplast formation due to the fact that they are anchored to the cell membrane and not covalently attached to the peptidoglycan as are the teichoic acids (Fiedler, 1988).
During the course of this work, Ming and Daeschel (1993) proposed that as a resistance response to nisin, fundamental changes occurred in bacterial membrane structure and function. The authors reported that the *L. monocytogenes* Scott A nisin-resistant mutant had a higher percentage of straight chain fatty acids (C14:0), and a lower percentage of branched chain fatty acids (predominantly anteiso C15:0) than its wild type. However, it was not shown whether this difference was related to nisin resistance. Subsequently, the role of the cytoplasmic membrane in the acquired nisin resistance of *L. monocytogenes* F6861 was also investigated. From the results obtained, qualitative and quantitative fatty acid analysis of *L. monocytogenes* F6861 wild type and its nisin-resistant mutant showed no obvious difference in content between strains. Any slight differences in quantity observed (e.g., the presence of slightly lower proportions of C14:0 and anteiso-C15:0 were indicated in the F6861 mutant as compared with its wild type) could not be verified from only one analysis.

Modification of the lipid composition of *L. monocytogenes* was attempted by exogenous application of the fatty acids C14:0 or anteiso-C15:0 during growth, in order to determine the resistance response to nisin. Successful incorporation of the fatty acids was not shown conclusively from subsequent fatty acid analysis, although slight increases in content were observed. Nisin inactivation and MIC determinations of these modified strains indicated some incorporation. Fatty acid C14:0 generally increased nisin resistance of both *L. monocytogenes* F6861 wild type and its nisin-resistant mutant, although MIC determinations of the wild type were similar to the control. Fatty acid anteiso-C15:0 decreased the nisin resistance of the wild type during both experiments, but increased the nisin resistance of the mutant during nisin inactivation only. Thus, these results suggest that it is possible that fatty acid C14:0 has a role in increased nisin resistance, however, the fact that conflicting results were obtained with fatty acid anteiso-C15:0, whereby it increased the nisin resistance of the mutant during inactivation, poses some doubt as to whether it contributes to nisin sensitivity.
Ming and Daeschel (1995) also compared phospholipid composition in *L. monocytogenes* Scott A and its nisin-resistant mutant. They reported that the total phospholipid content of the mutant was significantly decreased compared with the parental strain. The types of phospholipids isolated from each strain were identical, but there was a decrease in the amount of three individual phospholipids (phosphatidylglycerol, diphosphatidylglycerol and bis-phosphatidylglyceryl phosphate). In addition, when exposed to nisin, the resistant mutant cells lost a smaller amount of phospholipid than their sensitive parent cells. Thus, the authors concluded that nisin resistance in *L. monocytogenes* Scott A may be correlated with the reduction in cellular phospholipid content, and that the phospholipids may serve as the initial contact sites for nisin. However, continued work in our laboratory has shown no significant difference (5 replications) in phospholipid content between *L. monocytogenes* F6861 wild type and its nisin-resistant mutant (Falahee and Adams, personal communication, 1995). Thus, it is possible that a different nisin resistance mutation exists in *L. monocytogenes* strains Scott A and F6861.

However, as mentioned earlier, the hydrophobicity of *L. monocytogenes* Scott A wild type was significantly greater than that of its mutant (Ming and Daeschel, 1995), which was similar to the findings with *L. monocytogenes* F6861. The fact that hydrophobicity is a cell surface phenomenon, rules out the involvement of the cell membrane in isolation from the cell wall in nisin resistance, unless of course, decreased hydrophobicity of the mutant is just a coincidental change and has nothing to do with nisin resistance. In agreement with findings in this present study, Ming and Daeschel (1995) have also suggested that the less hydrophobic cell surface of nisin-resistant cells may provide another factor that contributes to nisin resistance; and that some cell wall components such as proteins, lipoteichoic acids, and lipids were reported to be implicated in the hydrophobic reactions of Gram-positive bacteria. In addition, differences in the cell surface components of methicillin-resistant cells of *Staphylococcus aureus*, were attributed to the production of different anionic polymers on the surface (Ming and Daeschel, 1995).
Thus, in conclusion of the work presented in this study, it is suggested that the acquired nisin resistance of *L. monocytogenes* F6861 does not involve a nisinase enzyme, but instead is based on exclusion and inhibition of nisin binding to the cells. Resistance is acquired by adaption of the cell envelope to reduce the presence or accessibility of attachment sites preventing the incorporation of nisin into the membrane. Furthermore, it has been shown that the cell wall plays a fundamental role, with probable involvement of teichoic and/or lipoteichoic acids and other factors not investigated (possibly peptidoglycan). The possibility that surface-layer proteins are involved has been eliminated, due to the fact that they were not detected. However, there is no firm evidence to suggest that the cell membrane also plays a role in the acquired nisin resistance of *L. monocytogenes* F6861 as suggested by Ming and Daeschel (1993; 1995) for *L. monocytogenes* Scott A. But, the fact that lipoteichoic acids comprise part of the cell membrane and cell wall and are projected to the outer surface would suggest that the whole cell envelope is somehow involved in acquired nisin resistance.

It is suggested that continued work in this area should focus on other cell wall components not investigated, such as peptidoglycan. Addition of penicillin to *L. monocytogenes* cells to break the glycosidic bonds present in peptidoglycan, before a nisin inactivation experiment, could indicate a difference in resistance to whole cells, as did the addition of phosphodiesterase to teichoic acids and lipoteichoic acids. In addition, analysis of the cell wall polymers could indicate a difference in composition between strains. Further work on the cytoplasmic membrane fatty acid composition is also suggested. As mentioned, it has been reported that an integral membrane component that serves as a receptor for nisin is not required in artificial membranes (Gao *et al.*, 1991), but whether this is true *in vivo* has yet to be determined. In addition, the analysis of the plasmid-linked nisin resistance gene from *Lactococcus lactis* subsp. *lactis* biovar diacetylactis (a non-nisin producer) encoded a protein which was predicted to be membrane associated (Froseth and Mckay, 1991). Thus the possibility of a resistance mechanism in *L. monocytogenes* involving a membrane protein cannot be eliminated, although nisin resistance in *L. monocytogenes*
could not be explained by the presence of plasmid DNA (Harris et al., 1991). For instance, it has been shown that pediocin PA-1 (also known as SJ-1) functions in a voltage-independent manner but requires a specific protein in the target membrane (Chikindas et al., 1993).
BIBLIOGRAPHY
BIBLIOGRAPHY


Appendix I  Gen-Probe analysis

The Accuprobe *L. monocytogenes* Culture Identification Test is a rapid DNA probe test which utilizes the technique of nucleic acid hybridization for the identification of *L. monocytogenes* isolated from culture. Nucleic acid hybridization tests are based on the ability of complementary nucleic acid strands to specifically align and associate to form stable double-stranded complexes. The Accuprobe System uses a chemiluminescent-labelled, single-stranded DNA probe that is complementary to the ribosomal RNA of the target organism. After the ribosomal RNA is released from the organism, the labelled DNA probe combines with the target organisms’ ribosomal RNA to form a stable DNA:RNA hybrid. The selection reagent allows for the differentiation of non-hybridized and hybridized probe. The labelled DNA:RNA hybrids are measured in the Gen-Probe luminometer. A positive result is a luminometer reading equal to or greater than the cut-off. A value below this cut-off is a negative result (Table A.1).

**Table A.1**  Gen-Probe analysis results to confirm the identity of *L. monocytogenes*

<table>
<thead>
<tr>
<th>Organism tested</th>
<th>Relative light units (RLU)</th>
<th>Identification</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L. monocytogenes</em> control</td>
<td>15502</td>
<td>+ ve</td>
</tr>
<tr>
<td>Isolate 1</td>
<td>8600</td>
<td>+ ve</td>
</tr>
<tr>
<td>Isolate 2</td>
<td>10640</td>
<td>+ ve</td>
</tr>
<tr>
<td>Isolate 3</td>
<td>12392</td>
<td>+ ve</td>
</tr>
<tr>
<td><em>L. innocua</em> control</td>
<td>49</td>
<td>- ve</td>
</tr>
</tbody>
</table>

*RLU < 250 is a negative result; RLU > 1500 is a positive result.*
Appendix II  Determination of serum antibody titres

Microtitre plates were coated with nisin (1 μg/ml) in coating buffer (0.2 ml/well) and incubated overnight at 4°C. The plates were washed three times with PBSGT and the sera added (0.2 ml/well)(diluted 1:500 to 1: 512000 (v/v) in PBSGT). After a 2-h incubation at 37°C the plates were washed as before with PBSGT. Donkey-anti-sheep horseradish peroxidase conjugate (Guildhay Antisera, Guildford, UK)(diluted 1:10000 or 1:15000 (v/v) in PBSGT as specified) was added (0.2 ml/well) and the plates incubated at 37°C for 2 h before washing with PBSGT. The substrate solution was added (0.15 ml/well) and incubated at 37°C for 30 min. The reaction was stopped by the addition of 2.5 M H₂SO₄ (0.05 ml/well) and absorbances read at 490 nm. Each titre was calculated by plotting the absorbance versus the serum dilution, using a fixed value of absorbance (eg. $A_{490} = 1.0$) to give the titre.
Appendix III  Optimal antibody-coating level and conjugate dilution

Microtitre plates were coated (0.2 ml/well) with a series of antibody concentrations from 0 to 10 μg/ml (in coating buffer) with two rows of wells allocated for each concentration (Fig. A.1). After a 2 h incubation at 37°C the plates were washed three times with PBSGT, blocked for 1 h with casein buffer at 37°C and washed with PBSGT as before. For each antibody-coating concentration, PBSGT (blank) was added to the first row of wells and nisin (1 IU/ml in coating buffer) to the second (0.2 ml/well). Plates were incubated at 4°C overnight and washed three times with PBSGT. The conjugate was diluted in PBSGT (1:5000 to 1:40000) using a doubling dilution series and each conjugate dilution was applied to two columns of wells (0.2 ml/well). After incubation at 37°C for 2 h the plates were washed again with PBSGT and the substrate applied (0.15 ml/well) for 30 min at 37°C. The reaction was stopped by the addition of 2.5 M H₂SO₄ (0.05 ml/well) and absorbances read at 490 nm. The optimal antibody-coating level and conjugate dilution are chosen as the lowest concentrations to yield an optimum response with low background readings.

<table>
<thead>
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<th>Column number</th>
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<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nisin (IU/ml)</td>
<td>AB</td>
<td>CD</td>
<td>EF</td>
<td>GH</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Antibody (μg/ml)</td>
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<td>1</td>
<td>0</td>
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<td>0</td>
<td>1</td>
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<tr>
<td>1:40K</td>
<td>1:20K</td>
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Fig. A.1  Representation of the ELISA plate layout for optimal antibody-coating level and conjugate dilution
Short Communication

Resistance of *Listeria monocytogenes* to the bacteriocin nisin

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Aims and Scope

International Journal of Food Microbiology publishes full-length original research papers, short communications, review articles and book reviews covering all aspects of microbiological safety, quality and acceptability of foods. Contributions dealing with the following fields are invited: bacteriology, immunology, mycology, parasitology, virology and food fermentation. Emphasis will be placed on papers dealing with: microbiological quality assurance, intrinsic and extrinsic parameters of foods affecting microbial survival and growth, methods for microbiological and immunological examinations of foods, indices of the sanitary quality of foods, incidence and types of food microorganisms, food spoilage, microbiological aspects of food preservation, microbial interaction, predictive microbiology, food-borne diseases of microbial origin and the safety of novel food products. Achievements in rapid methods and automation in food microbiology are also included. It is a policy of this Journal to also publish Proceedings of suitable meetings, workshops, conferences etc. in the field of food microbiology.

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Short Communication

Resistance of *Listeria monocytogenes* to the bacteriocin nisin

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(Received 13 July 1993; accepted 7 November 1993)

*Listeria monocytogenes* NCTC 5105 and F6861 were evaluated for sensitivity to nisin. The results confirmed those previously published indicating marked differences in the sensitivity of the two strains. Mutants with increased resistance to nisin could be isolated from the less sensitive F6861 strain at a frequency of $10^{-6}$ to $10^{-7}$. Using a nisin specific enzyme-linked immunosorbent assay, the adsorption of nisin to the different strains and the resistant mutant were studied. At a range of nisin concentrations the amount adsorbed was found to reflect the sensitivity of the strain to nisin, with the more sensitive cells showing greater adsorption. In resistant cells, K⁺ efflux increased at a slower rate with increasing nisin concentration and had a lower maximum rate indicating that the mechanism of resistance involves a reduction in the accessibility or presence of suitable adsorption sites.

Keywords: Nisin; *Listeria monocytogenes*; Antibiotic resistance

Introduction

The bacteriocin, nisin, was approved for use as an antimicrobial in food by a Joint Food and Agriculture Organization/World Health Organization Committee in 1969 and is currently approved in 47 countries (Delves-Broughton, 1990). It is active against a range of Gram-positive bacteria but its principal commercial applications are in canned foods and processed cheeses to inhibit the outgrowth of spores which show particular sensitivity (Hurst, 1981; Delves-Broughton, 1990). Recently nisin has attracted renewed interest as a 'natural' agent to control vegetative organisms in foods, particularly the Gram-positive pathogen *Listeria monocytogenes*.

The primary target of nisin in sensitive vegetative cells is the cytoplasmic membrane. Nisin is incorporated into the membrane to form ion channels or pores (Henning et al., 1986; Sahl et al., 1987; Kordel et al., 1989) through which efflux of K⁺, ATP and amino acids results in destruction of the membrane potential (Ruhr...
and Hans, 1985; Kordel and Sahl, 1986). Sensitivity of *L. monocytogenes* to nisin has been demonstrated (Mohamed et al., 1984; Benkerroum and Sandine, 1988; Carminati et al., 1989; Harris et al., 1989; Spelhaug and Harlander, 1989) and it has been shown that in energised cells nisin dissipates the membrane potential and pH gradient leading to collapse of the proton-motive force (Bruno et al., 1992). However, it has also been shown that nisin-resistant strains are likely to arise (Harris et al., 1991).

The ability of *L. monocytogenes* to develop resistance to nisin is clearly an obstacle to the development of new food applications. The mechanism of this resistance has not, as yet, been determined for *L. monocytogenes*. Determinants of nisin resistance have been associated with plasmid DNA in *Lactococcus lactis* subsp. *lactis* biovar *diacetylactis* (McKay and Baldwin, 1984) and in *L. lactis* subsp. *lactis* (Wilkins et al., 1972; Klaenhammer and Sanozky, 1985). Subsequent sub-cloning and sequencing of the resistance gene from *L. lactis* subsp. *lactis* biovar *diacetylactis* encoded a protein of 319 amino acids, the function of which is undetermined (Froseth et al., 1988; Simon and Chopin, 1988; Froseth and MacKay, 1991). However, nisin resistance in *L. monocytogenes* strains could not be attributed to the presence of plasmid DNA (Harris et al., 1991).

A nisin degrading enzyme, nisinase, has been described for a range of bacterial species (Kooy, 1952; Galesloot, 1956; Carlson and Bauer, 1957; Alifax and Chevalier, 1962; Jarvis, 1967). Nisinases from *Streptococcus salivarius* subsp. *thermophilus* (Alifax and Chevalier, 1962) and *Bacillus cereus* (Jarvis and Farr, 1971) were partially purified but have not been fully characterised.

Hitherto, studies of this kind have relied upon a nisin bioassay which is subject to non-specific interference (Fowler et al., 1975). Here we report a study of the variability of nisin resistance in *L. monocytogenes* using an ELISA for nisin which shows greater sensitivity and specificity than the bioassay (Falahee et al., 1990).

**Materials and Methods**

**Media, culture conditions and reagents**

*L. monocytogenes* NCTC 5105 serotype 3a and F6861 serotype 4b (an isolate from Jalisco cheese incriminated in an outbreak of listeriosis in 1985) were obtained from Dr B. Lund, IFR Norwich. They were grown in tryptose phosphate broth (TPB)(Oxoid) and on tryptone soya agar (TSA)(Oxoid) at 30°C. Where necessary, the pH of media was adjusted to the required level (± 0.01) with HCl. Cultures were maintained on TSA slopes at 4°C. *Micrococcus flavus* NCIB 8166 was maintained on slopes of the nisin bioassay medium prepared as described by Fowler et al. (1975) at 4°C.

Pure nisin (50 × 10⁶ International Units (IU)/g) was obtained from Aplin and Barrett, Ltd. (Trowbridge, UK). Stock solutions (1 mg/ml) were prepared by dissolving 1 mg of nisin in 0.02 M HCl (100 μl) and making up to 1 ml with distilled water. Solutions were sterilised by membrane filtration (0.2 μm, Minisart NML, Sartorius, USA).
Determination of nisin resistance

Minimum inhibitory concentration (MIC) values were determined on TSA (pH 5.5) at 37°C in accordance with the protocol of Ferreira and Lund (1991). The slightly acidic pH also reflected the situation in most foods. Nisin-resistant mutants were selected as described by Harris et al. (1991). Resistant mutants selected were confirmed as L. monocytogenes using the ‘Accuprobe L. monocytogenes Culture Identification Test’ (Gen-Probe Inc., San Diego).

Nisin adsorption assay

Overnight cultures of cells, grown to a concentration of approximately 10^9 cfu/ml in TPB (pH 7.3) were adjusted with TPB (pH 7.3) to the same optical densities (OD) at 600 nm. The cell concentration was determined using spread plates (TSA) prepared from decimal serial dilutions in Maximum Recovery Diluent (Oxoid). Cells were harvested from suspension by centrifugation (4000 rpm, 20 min); washed twice in 5 ml of phosphate-buffered saline (pH 7.4) containing 0.1% gelatin (PBSG), and resuspended to the original volume in PBSG. Plate counts were determined again at this stage to determine cell loss in this procedure.

The cell suspension (8 ml, approx. 10^9 cfu/ml) was mixed with nisin solution (0.8 ml) to give the required final concentration (10–1000 IU nisin/ml). Cell-free controls consisting of buffer plus nisin were set up simultaneously. After incubation for 15 min at 30°C, the cells were removed by centrifugation. The nisin concentration in supernatants was determined using a nisin ELISA as described by Falahee et al. (1990) or by the nisin bioassay (Fowler et al., 1975), as specified.

ELISA conditions

For each separate determination, the supernatants, control and standard curve (in the range 0–1.0 IU/ml) were assayed in duplicate on the same ELISA plate and the mean values used. The nisin content was determined for those supernatant dilutions with absorbances corresponding to the linear portion of the standard curve and the mean value of at least three dilutions was taken. The correlation coefficient of each standard curve was \( \geq 0.995 \).

Bioassay conditions

Nisin standards were prepared at 5, 10 and 20 IU/ml, the correlation coefficient of the standard curve being 0.994. A latin square was used for random distribution of the samples and the mean value of six replicates taken for each sample. For each replicate, the diameter of zones of inhibition was measured twice at right angles and the mean value used.

Determination of the rate of K^+ efflux

Overnight broth cultures (300 ml, TPB, pH 7.3) were centrifuged at 5000 rpm for 15 min and resuspended in phosphate buffer (5 mmol/l, pH 6.6) to give the same cell concentration (approx. 10^9 cfu/ml) as measured by OD (600 nm) and confirmed by plate counts (TSA, pH 7.3).
Each suspension was divided into equal volumes and incubated in a waterbath at 30°C. A fixed volume of cell suspension was removed prior to addition of nisin and filter sterilized to represent the K⁺ concentration at time zero. Sterile water and nisin stock solution (50 × 10³ IU/ml) were added to the remaining suspension in the required proportions to obtain the desired final nisin concentration. Samples (5 ml) were taken after 1 min and filter sterilized prior to K⁺ determination. Nisin-free controls were processed in the same way. The concentration of K⁺ ions in each filtrate was measured by atomic adsorption spectroscopy at a wavelength of 383 nm (Perkin Elmer 306).

Determination of total cellular K⁺

Cell suspensions were prepared and centrifuged as described above. Concentrated HNO₃ (1 ml) was added to each pellet and the suspensions heated at 100°C for 30 min to dissolve the biomass. The pH was adjusted to 6 with 4 M NaOH. Samples were made up to a final volume of 10 ml with distilled water and filter sterilized before K⁺ determination.

Statistical analysis

The results of the nisin adsorption experiments were analysed statistically using the Kolmogorov-Smirnov Goodness of Fit Test to show that the data were normally distributed and Student's t-test to show a difference between the means at each nisin concentration.

Results and Discussion

Determination of nisin MIC values for L. monocytogenes confirmed the sensitivity of L. monocytogenes NCTC 5105 and the relative resistance of L. monocytogenes F6861 to nisin reported by Ferreira and Lund (1991). At pH 5.5, NCTC 5105 had an MIC of 10–40 IU/ml compared with 200–400 IU/ml for F6861. Mutants with increased resistance to nisin (MIC > 600 IU/ml) could be isolated only from the less sensitive F6861 strain by plating serial dilutions of a population on to solid medium containing increasing concentrations of nisin. The frequency of resistant mutants was 10⁻⁶ to 10⁻⁷, a similar rate to that observed by Harris et al. (1991).

The affinity of the different strains for nisin was determined by measuring residual nisin levels in solution after exposure to cells. Results of triplicate experiments at four different nisin concentrations are presented in Fig. 1. At each concentration, the sensitive 5105 strain adsorbed more nisin than the more resistant F6861 strain. This was most marked at the lower concentrations of 10 and 20 IU nisin/ml where adsorption by F6861 was significantly lower than adsorption by 5105 (P < 0.02) in all experiments. At higher nisin concentrations, only in two out of three experiments at 100 IU/ml and at 200 IU/ml did the more resistant strain show significantly lower adsorption (P < 0.05). Nisin levels were routinely determined using a nisin ELISA but similar results were obtained when nisin was measured using the bioassay procedure.
The fact that adsorption reflected the sensitivity of the strain to nisin over a range of nisin concentrations supports the view that multiple adsorption is possible at suitable membrane sites (Sahl, 1991; Benz et al., 1991).

The results do not indicate the presence of a nisinase enzyme reported in some resistant bacteria (Kooy, 1952; Galesloot, 1956; Carlson and Bauer, 1957; Alifax and Chevalier, 1962; Jarvis, 1967). If this were present, lower levels of residual nisin would be expected in supernatants exposed to more resistant species rather than the converse. Since similar results were obtained with the bioassay, the possibility that a nisinase was destroying biological activity of the molecule while retaining epitopes recognised in the ELISA is excluded.

The rate of $K^+$ efflux induced by different levels of nisin was measured in all three strains (Fig. 2). In each case, $K^+$ efflux increased with increasing nisin concentration until it reached a maximum level. The rate of increase and the maximum level of efflux both increased with the cells’ sensitivity to nisin. The different maximum rates for $K^+$ efflux did not reflect differences in total cellular $K^+$ levels between strains. These were similar in the 5105 and the mutant F6861 strains (0.14 and 0.13 $\mu$g/10^6 cfu, respectively) which showed the biggest difference in $K^+$ efflux, while F6861 had the lowest $K^+$ content (0.10 $\mu$g/10^6 cfu) but an intermediate rate of $K^+$ efflux. The maximum $K^+$ efflux rates recorded corresponded to differing proportions of the total cellular potassium (5105, 78%; F6861, 63%; F6861 mutant, 42%).

These results show that as more nisin is added to the cells suitable nisin attachment sites become occupied until a point is reached when all the available
sites are filled and no further increase in $K^+$ efflux occurs. In resistant cells, a greater amount of nisin is required to find and fill these fewer sites.

The observed adsorption behaviour and saturation kinetics of potassium efflux suggest that in *Listeria monocytogenes* nisin resistance is based on exclusion and inhibition of nisin binding to the cells. Resistance is acquired by adaption of either the cytoplasmic membrane or cell wall to reduce the presence or accessibility of attachment sites preventing the incorporation of nisin into the membrane to produce pores, cell lysis and death.

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The authors would like to thank Dr B.M. Lund for the *Listeria monocytogenes* strains and MAFF for provision of a studentship to EAD.

**References**


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Work presented in Chapter 7 of this thesis will be submitted as a paper to the Journal of Applied Bacteriology.
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Supplement to Journal of Applied Bacteriology, 1994, 77 (1)
A, B and C and are clustered with NTNH and BoNT. Structural mapping of genes upstream of BoNT and sequence analysis has lead to the identification of possible promoter regions controlling expression of the components of the toxin complex. Analysis of the regulation of the toxin gene with in vivo studies is not easy due to the extreme toxicity of the BoNT. An alternative means of examining factors affecting BoNT expression is being developed using reporter gene technology. PCR fragments of the region upstream of the toxin gene will be fused to a lacZ reporter gene and incorporated into a suitable shuttle vector in E. coli. Plasmids containing reporter gene fusions will be transferred to a suitable clostridial host and gene expression followed by measurement of β-galactosidase activity.

10. S-layer proteins and nisin resistance in Listeria monocytogenes

E. A. Davies, M. R. Adams, A. Brain1, A. H. L. Chamberlain, M. B. Falahee and P. A. Zhdan2, School of Biological Sciences, University of Surrey, Guildford, Surrey GU2 5XH1; Electron Microscope Unit, King's College, Manresa Road, London SW3 6LX and 2Department of Materials Science and Engineering, University of Surrey, Guildford, Surrey GU2 5XH.

The various functions attributed to the crystalline arrays of bacterial surface proteins, known as S-layers, include acting as a protective barrier to antimicrobial agents such as bacteriocins. We have investigated the possible role of S-layers in the acquisition of nisin resistance by Listeria monocytogenes. L. monocytogenes shows variable resistance to nisin and mutants showing high levels of resistance can be isolated at a rate of 10-4–10-7. Cell hydrophobicity correlates with nisin sensitivity: the more sensitive strain showing the most hydrophobic character. Chloramphenicol has no effect on the rate of isolation of resistant mutants indicating that de novo protein synthesis is not required. Examination of strains by freeze–etching and by atomic force microscopy failed to demonstrate the presence of S-layers in either the wild type strain or its more nisin–resistant mutant. An S-layer was clearly visible using both techniques in a lactobacillus control. S-layer extraction procedures isolated much lower protein concentrations from L. monocytogenes than from the lactobacillus. Lowest protein concentrations were generally obtained from the resistant mutant.

11. Rapid differentiation of Listeria monocytogenes into three major genotypes by PCR–RFLP of the listeriolysin O gene

L. M. Lawrence1,2, M. Linton3, J. T. M. McGuiggan2 and A. Gilmour12, Food Microbiology Department, 1The Queen's University of Belfast and 2Department of Agriculture for Northern Ireland, Agriculture and Food Science Centre, Newforge Lane, Belfast BT9 5PX.

Listeria monocytogenes can be readily identified using the polymerase chain reaction (PCR) and primers specific for the listeriolysin O gene. Digestion of the product of this reaction with the restriction endonuclease Taq 1 can then be used to detect restriction fragment length polymorphism (RFLP) within this gene. This technique was applied initially to 30 strains of L. monocytogenes isolated from various sources and two distinct PCR–RFLP profiles were identified. These two genotypes correlated to grouping of these isolates by multilocus enzyme electrophoresis (MEE) and was related to their antigenic structure as determined by serotyping. When the technique was applied to further strains of different serotypes of L. monocytogenes a third PCR–RFLP profile was identified. This third genotype was also related to results from MEE studies and antigenic structure, and was produced only by serotype 4a and 4c strains. This third PCR–RFLP profile was found to be closely related to that which encompassed serotypes 1/2b, 3b, 4b, 4d, 4e and 7. The PCR–RFLP method described can be used to confirm and characterise L. monocytogenes within 12 hours, and since one of the genotypes recognised by this technique is known to encompass L. monocytogenes 4b and 1/2b serotypes which are responsible for the majority of all food–borne outbreaks, the technique could be of value in epidemiological studies.

12. The effect of pH on the survival of Escherichia coli 0157/H7 and Yersinia enterocolitica in goats milk yoghurt

J. Rowe and A. Campbell, Seale–Hayne Faculty of Agriculture, Food and Land Use, University of Plymouth, Newton Abbot, Devon TQ12 6NQ.

The nature of goats milk production is such that small, on–farm enterprises are frequently involved in processing the raw product into yoghurt for retail sale. Yersinia enterocolitica has been isolated from raw goats milk and although E. coli 0157/H7 is not currently regarded as a problem from this source, it is conceivable that contamination could occur. Relative lack of control in small, on-farm enterprises and a demand for yoghurt with a relatively high pH could lead to contamination and development of these pathogens in yoghurt and present a hazard to health.

The survival of these pathogens in goats milk fermented to different pHs and held at refrigeration temperature was studied. From an initial inoculation of Log10 4.57 orgs/ml milk Yersinia enterocolitica was not detectable in yoghurt after 2 days at pH 3.4, after 4 days at pH 4.2 and 4.4 and after 8 days at pH 4.6. E. coli 0157/H7 was not detectable in yoghurt after 3 days at pH 3.4 and survived longer than 10 days at pH 4.0, 4.2 and 4.4 when inoculated at levels of Log10 5.88 orgs/ml milk. Thus the final pH of yoghurt can affect the safety of the product.
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Gram positive cocci, fusobacteria and coliforms remained relatively unaffected. These results demonstrate the potential for manipulation of the colonic flora by dietary addition which may have positive implications for host health.

Resistence of Listeria monocytogenes to the bacteriocin nisin

E. A. Davies and M. R. Adams, School of Biological Sciences, University of Surrey, Guildford, Surrey GU2 5NZ.

Listeria monocytogenes NCTC 5105 and F6861 were evaluated for sensitivity to nisin. The results confirmed those previously published indicating marked differences in the sensitivity of the two strains, pH being an influential factor on observed MIC values.

Mutants with increased resistance to nisin could be isolated from the less sensitive F6861 strain at a frequency of $10^{-6}$ to $10^{-7}$.

Using a nisin specific enzyme-linked immunosorbent assay, the adsorption of nisin to the different strains and the resistant mutant were studied. At a range of nisin concentrations the amount adsorbed was found to reflect the sensitivity of the strain to nisin with the more sensitive cells showing greater adsorption.

The initial rate of $K^+$ efflux in response to nisin was found to display saturation kinetics indicating specific nisin receptors on the cell surface. In resistant cells, $K^+$ efflux increased at a slower rate with increasing nisin concentration and had a lower maximum rate indicating that the mechanism of resistance involves a reduction in the availability of adsorption sites.

Effect of glucose on the bactericidal action of chloroquine on Escherichia coli

P. F. Olurinola1 and D. Wiseman2, 1National Institute for Pharmaceutical Research and Development, Abuja, Nigeria and 2Postgraduate Studies in Pharmaceutical Technology, The School of Pharmacy, Bradford University, Bradford BD7 1DP.

Glucose-starved cells of Escherichia coli (NCTC 1093), cultivated in a 3-(N-morpholino) – propane sulphonic acid (MOPS) buffered minimum salts medium, were treated with 2.0 to 16.0mM of chloroquine phosphate. Up to 8.0mM, there was little or no loss in viability for at least 2h, contrary to what obtains with normal growing cells. However, loss in viability became appreciable with the 16.0mM concentration. Addition of glucose to the growth medium led to a pronounced bactericidal action, which continued at an increased rate, even after subsequent removal of glucose from the growth medium. The presence of glucose (the carbon source) in the growth medium, at least for a short period, is apparently a necessity for the bactericidal action of chloroquine.

SESSION 4

Evaluation of the VIDAS Listeria system for the detection of Listeria in foods

A. R. Bennett, J. A. Bobbitt and R. P. Betts, Microbiology Department, Campden Food and Drink Research Association, Chipping Campden, Glos GL55 6LD.

The VIDAS Listeria assay was evaluated for its specificity, sensitivity and ability to detect Listeria spp. from a range of inoculated and uninoculated foods, enriched by four different methods: FDA method (Listeria Enrichment Broth Base with selective supplement); PALCAM (PALCAM Broth with selective supplement); USDA method (UVM1 and Fraser Broth); and a modified USDA method (UVM2 and Fraser Broth). The VIDAS system consisted of an automated enzyme–linked fluorescent assay (ELFA) that gave results from enriched food samples in 45 minutes. The assay was able to detect the 6 different Listeria spp. within the genus at a minimum level of $8 \times 10^4 - 1 \times 10^5$ cells per ml depending on species. The FDA procedure appeared the poorest enrichment method to use in conjunction with the VIDAS. The other three methods performed equally well. With uninoculated foods the USDA procedure gave no false negatives. A small number of false positives occurred when testing salads. PALCAM broth gave a number of false negative results. Success of the different enrichment procedures varied with food type and improved after 48 hours enrichment incubation compared with 24 hours. Different enrichment protocols are therefore required depending on the food type, the USDA procedure being the most universally acceptable enrichment method.

Evaluation of Rambach Agar and Salmosyst enrichment system for the detection of Salmonella in foods

A. R. Bennett and R. P. Betts, Microbiology Department, Campden Food and Drink Research Association, Chipping Campden, Glos GL55 6LD.

A new medium, Rambach Agar, has been developed to facilitate the differentiation of salmonellae. It has previously been shown to improve the detection of Salmonella in foods. It relies on the ability of Salmonella to form acid from propylene glycol and thus produce bright red colonies while other members of the family Enterobacteriaceae form colourless/biege colonies, or blue/green/violet colonies depending on the absence or presence of $\beta$-galactosidase activity respectively. The ability to metabolise propylene glycol is rare and so the number of false presumptive positives associated with other Salmonella–selective agars is reduced. False negatives can occur with $\beta$-galactosidase producing Salmonella. The current study further evaluated the performance of Rambach Agar compared to other selective agars in detecting a range of Salmonella strains inoculated into various foods. A rapid–two step enrichment system for sublethally damaged salmonellae, Salmosyst was incorporated for comparison with conventional pre–enrichment and selective enrichment procedures. Rambach Agar and Salmosyst