IDENTIFICATION OF GENES NECESSARY FOR GROWTH OF *LISTERIA MONOCYTOGENES* AT LOW TEMPERATURES

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by

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To My Daughter Isabel With Love

STATEMENT

The accompanying thesis submitted for the degree of Ph.D. entitled "Identification of Genes Necessary for Growth of *Listeria monocytogenes* at Low Temperature" is based on work conducted by the author in the Department of Microbiology and Immunology of the University of Leicester mainly between October 1993 and September 1997.

All the work recorded in this thesis is original unless otherwise acknowledged in the text or by references.

None of the work has been submitted for another degree in this or any other University.

Signed:..... Date:.....

ABSTRACT

Listeria monocytogenes, the causative agent of listeriosis, has been recognised as a pathogen of man and animals for some 70 years. An important characteristic of the organism, unusual amongst pathogenic bacteria, is its ability to exhibit significant growth at refrigeration temperatures. This has serious consequences because refrigeration, commercial and domestic, is widely and increasingly used for food transportation and storage. Little is known about those factors that allow the bacterium to multiply and survive at low temperatures. In this study transposon mutagenesis has been used to identify genes encoding for proteins essential for the growth of *L. monocytogenes* at 4°C. A library of transposon mutants of *L. monocytogenes* was generated using Tn*917*-LTV3 and screened for the ability to grow at 30 and 4°C. One mutant which exhibit visibly reduced growth in liquid medium and five mutants which had visibly reduced growth on solid medium, when incubated at 4°C but unaltered growth at 30°C, were selected for further investigation. Southern blots analysis revealed that there is single insertion of the transposon in the chromosome and that the insertion was at different sites in each mutant. Southern blotting also showed that none of the mutants were in genes of the *cspL* family. These observation suggested that different genes have been inactivated in these mutants. Sequencing analysis of the gene inactivated in one of the "solid mutants", revealed that the inactivated gene encodes a protein with homology to *Bacillus subtilis* transmembrane proteins belonging to a multicomponent transport system involved in the uptake of the osmoprotectants such as glycine betaine. Such an osmoprotectant uptake transport system has not been characterised before, at the molecular level, in *Listeria monocytogenes*. Growth of this *L. monocytogenes* mutant in defined medium at high osmolarity, either at 30 or 4°C, showed to be affected when compared to the growth of the wild type confirming that the inactivated gene is not only involved in cryotolerance but also in osmotolerance in this organism.

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ABBREVIATIONS

BHI	Brain heart infusion
bp	Base pair
BSA	Bovine serum albumin
cfu	Colony forming units
CIP	Calf intestinal phosphatase
DNA	Deoxyribonucleic acid
EDTA	Diaminothantetra-acetic acid
HEPES	N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulphonic acid)
IPTG	Isopropyl-β-D-thiogalactosylpyranoside
kb	Kilobase
kd	Kilodalton
kV	Kilovolt
LB	Luria-Bertani medium
OD	Optical density
ORF	Open reading frame
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
rpm	Revolution per minute
SDS	Sodium dodecyl sulphate
TAE	Tris-acetate
TE	Tris-EDTA
TEMED	N, N, N, N'-Tetramethylethylethylenediamine
T+M	Trivett and Meyer medium
TRIS	Tris-(hydroxymethyl)-aminomethane
TSA	Tryptone soya agar
TSB	Tryptone soya broth
X-Gal	5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside
UV	Ultraviolet
V	Volts

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Chapter 3

Chapter 1

INTRODUCTION

The genus *Listeria* contains Gram-positive, non-sporeforming, motile, catalase positive, facultatively anaerobic, regularly rod-shaped bacteria. Six species are currently recognised: *Listeria monocytogenes, L. innocua, L. ivanovii* subsp. *ivanovii, L. ivanovii* subsp. *londoniensis, L. seeligeri, L. welshimeri* and *L. grayi* (Seeliger and Jones, 1986; Rocourt *et al.*, 1992). *Listeria monocytogenes* is pathogenic for humans and animals while *L . ivanovii* is pathogenic for animals, mainly sheep. In humans, bacterial meningitis is the most common form of listeriosis, while perinatal infections may result in abortion, stillbirth, and infant death. Abortion, encephalitis, septicaemia, and mastitis are the main clinical manifestations in cattle and sheep (Chakraborty and Goebel, 1988).

Listeriae, especially the species *L. monocytogenes*, have received a great deal of attention from microbiologists, clinicians, and veterinarians because of the problems associated with their classification, identification, isolation, epidemiology, and pathogenesis. While many of these problems have been solved, there is still much that is not understood, especially with regard to the epidemiology and pathogenesis of *L. monocytogenes* (Jones and Seeliger, 1991).

1.1 HISTORY

The first detailed description of the species now known as *L. monocytogenes* is that of Murray *et al.* (1926). These authors isolated a small, Gram-positive rod as the cause of a septicaemic syndrome among rabbits, and more rarely among guinea pigs, in an animal house at the University of Cambridge (UK). In recognition of the pronounced mononucleosis produced in the peripheral blood of experimental rabbits, they named the organism *Bacterium monocytogenes* (Murray *et al.*, 1926). Although Murray *et al.* (1926) are generally credited with the first accurate description of *L. monocytogenes*, early reports suggest that the organism was encountered in "listeric-like" infections as early as 1891 (Gray, 1960). In 1911, the Swedish worker Hülphers (1911) isolated an organism from necrotic foci of a rabbit liver. The description of this isolate, which he named *Bacillus hepatis*, corresponds the present-day description of *L. monocytogenes*. Probable isolations

of *L. monocytogenes* were also reported by Atkinson (1917) and Dick (1920) in 1919 from clinical cases of human meningitis. In 1918 Dumont and Cotoni (1921) isolated a diphtheroid organism from cerebrospinal fluid and deposited the culture at the Pasteur Institute of Paris. This organism was identified by Paterson (1940) as *L. monocytogenes*, therefore making this isolate the oldest known strain of *L. monocytogenes*.

One year after Murray *et al.*, (1926) provided the first detailed description of *L. monocytogenes*, Pirie (1927) in South Africa, independently reported the same organism as the cause of death among wild gerbils (*Tartera lobengulae*) and named it *Listerella hepatolytica* in honour of Lord Lister who pioneered antisepsis. After the recognition that the two names described the same bacteria, Pirie named the species *Listerella monocytogenes* (Addendum, Pirie, 1927). In 1939, it was discovered that the name *Listerella* had been applied to a mycetozoan. So, in 1940, Pirie proposed that the species name be changed to *Listeria* with *L. monocytogenes* as the type and only species. This name was used in the sixth edition of *Bergey's Manual of Determinative Bacteriology* (Breed *et al.*, 1948), and approved by the Judicial Commission on Bacteriological Nomenclature and Taxonomy in 1954 (Anonymous, 1954). Many other names have been used in the past, these include *Corynebacterium parvulum* (Schultz *et al.*, 1938), *Listerella ovis* (Gill, 1937), *Corynebacterium infantisepticum* (Potel, 1952), and *Listeria infantiseptica* (Potel, 1952). Some earlier authors employed such names as *Listerella bovina*, *L. gallinarium*, *L. cuniculi*, *L. suis*, and *L. gerbilli* (see Gray and Killinger, 1966).

1.2 CLASSIFICATION

Traditionally, bacterial classification has been based on similarities in phenotypic characteristics. This approach has been successful in defining, good, stable taxa for most bacteria, especially since the introduction of numerical taxonomy and chemotaxonomic techniques. It is frequently not sufficiently discriminatory, however, to distinguish between phenotypically similar groups of organisms nor informative enough to determine phylogenetic relatedness between bacterial groups. Most of these problems have begun to be solved by the application of molecular biological techniques that provide information at the genomic level. This is well illustrated by the changes that have taken place in both the intrageneric classification and intergeneric placement of the genus *Listeria* over the last decade (Jones, 1992).

1.2.1 Intergeneric Classification

The genus *Listeria*, was originally named by Pirie (1940) with *Listeria monocytogenes* as the type and only species, for the bacteria isolated, described and named "*Bacterium monocytogenes*" by Murray *et al.* (1926). As Gram-positive, non-sporeforming, catalase positive rods, the monospecific genus was placed in the family Corynebacteriaceae in the sixth edition of *Bergey's Manual of Determinative Bacteriology* (Breed *et al.*, 1948) and also in the seventh edition of the same publication (Breed *et al.*, 1957). Between 1961 and 1971 three additional species, *L. denitrificans, L. grayi* and *L. murrayi* were described (see Seeliger and Welshimer, 1974). On the basis of the results of a number of investigations, including numerical taxonomic (Davis and Newton 1969; Davis *et al.*, 1969; Stuart and Pease, 1972; Stuart and Welshimer, 1974), cell wall chemistry (Cummins and Harris, 1956; Scheleifer and Kandler, 1972; Srivastava and Siddique, 1973), lipid (Kosaric and Carroll, 1971; Tadayon and Carroll, 1971; Shaw, 1974), and nucleic acid (Stuart and Welshimer, 1974) studies, the genus *Listeria*, represented by the type species *L. monocytogenes*, was shown to be quite distinct from the genus *Corynebacterium*, the type genus of the family *Corynebacteriaceae*.

In the eighth edition of Bergey's Manual Determinative Bacteriology (Buchanan and Gibbons, 1974) the genus *Listeria*, containing four species *(L. monocytogenes, L. denitrificans, L. grayi,* and *L. murrayi*) was categorised as a "genus of uncertain affiliation" in the Section that contained the family *Lactobacillaceae*. Numerical taxonomic studies (Jones, 1975; Wilkinson and Jones, 1977; Feresu and Jones, 1988) and chemical studies (Collins and Jones, 1981; Kamisango *et al.*, 1982; Fieldler and Seger, 1983; Fiedler *et al.*, 1984; Ruhland and Fiedler, 1987; Feresu and Jones, 1988) all reinforced this division of *Listeria* from the corynebacteria and showed that phenotypically *Listeria* most closely resembled the genus *Brochothrix*.

In 1983 the 16S rRNA cataloguing studies of Stackebrandt *et al.*, placed *Listeria* (but not *L. denitrificans*) as a distinct taxon in the low G+C, *Bacillus-Lactobacillus-Clostridium,* branch of Gram positive bacteria and most closely related to *Brochothrix* (Ludwig *et al.*, 1984). Subsequent 16S rRNA reverse transcriptase sequence studies of members of the genus have indicated that *Listeria* is not closely related to the genus *Lactobacillus* and should not be included in the family *Lactobacillaceae* (Collins *et al.*, 1991) as suggested

earlier by Wilkinson and Jones (1977). Collins *et al.*, (1991) have suggested that a new family *Listeriaceae* be designated to include the genera *Listeria* and *Brochothrix*.

1.2.2 Intrageneric Classification

It is only recently, with the DNA-DNA hybridisation studies of Rocourt *et al.*, (1982a) that the classification of some species designated as *L. monocytogenes* has been resolved. Some strains of *L. monocytogenes* exhibited pronounced ß-haemolysis while others were non-haemolytic and also non-pathogenic for mice (Seeliger and Welshimer, 1974, Welshimer, 1981). In spite of Stuart and Welshimer (1973, 1974) demonstrating two DNA-DNA hybridisation groups within *L. monocytogenes* strains, they were not able to distinguish them by other criteria. The non-haemolytic and non-pathogenic strains were designated as *L. innocua*, which was a name originally given by Seeliger and Schoofs (1979) (Seeliger, 1981). DNA-DNA hybridisation studies, performed by Rocourt *et al.* (1982a), allowed the identification of five homology groups between strains designated *L. monocytogenes*. The first group contained the type strain of *L. monocytogenes*; a second contained strains originally determined *L. innocua*; and a third group contained strains, that exhibit a pronounced ß-haemolysis, which were first described by Ivanov in 1962 (Ivanov, 1975) and named by Seeliger and his colleagues as *L. ivanovii* (Seeliger *et al.*, 1984). The last two groups were named *L. seeliger* and *L. welshimer* (Rocourt and Grimont, 1983). On the basis of current data the genus *Listeria* contains six species grouped into two closely related but different lines of descendent; one line includes *L. monocytogenes, L. innocua, L. ivanovii* subsp. *ivanovii, L. ivanovii* subsp. *londoniensis, L. seeligeri* and *L. welshimeri;* the other line contains *L. grayi* (Seeliger and Jones, 1986; Rocourt *et al.*, 1992).

The intrageneric composition of the genus has been problematical. Three *Listeria* spp. - namely *L. denitrificans*, *L. grayi* and *L. murrayi* - were categorised as species of *incertae sedis* (Seeliger and Jones, 1986). Taxonomic studies indicated that *L. denitrificans* was more closely related to the genera *Corynebacterium* (Stuart and Welshimer, 1974), *Cellulomonas* (Chatelain and Second, 1976), or *Arthrobacter* (Jones, 1975). On the basis of DNA composition and DNA-DNA hybridisation, it was agreed that the species *L. denitrificans* showed a low degree of relatedness between other *Listeria* spp. and still could not be placed with confidence in any other taxonomic group (Jones and Seeliger, 1986). Rocourt *et al.*, (1987a) after analysing the 16S ribosomal RNA content, proposed that *L. denitrificans* be transferred to a new genus named *Jonesia* as *Jonesia denitrificans*.

Due to the conflict between the interpretation of numerical taxonomic and chemical studies on the one hand and those of DNA-DNA hybridisation studies on the other, *L. grayi* and *L. murrayi* were classified as *incertae sedis* (Seeliger and Jones, 1986). DNA-DNA hybridisation studies performed by Stuart and Welshimer (1974) showed that *L. grayi* and *L. murrayi* have lower DNA homology values than do the other *Listeria* spp. Consequently these authors suggested that *L. grayi* and *L. murrayi* should be reclassified as a new genus named "*Murraya*". Further numerical taxonomic and chemical (i.e., cell wall, cytochrome, fatty acid) studies, indicated that *L. grayi* and *L. murrayi* are closely related to *L. monocytogenes* (Seeliger and Jones, 1986). The 16S rRNA cataloguing studies showed a high degree of similarity (similarity coefficient (S_{AB}) value, 0.73) between *L. murrayi* and *L. monocytogenes* (Rocourt *et al.*, 1987b). Recently, *L. murrayi* has been no longer recognised; DNA-DNA hybridisation, multilocus enzyme electrophoresis, and rRNA restriction fragment length polymorphism studies have indicated that *L. grayi* and *L. murrayi* should be considered members of the same species, *L. grayi* (Rocourt *et al.*, 1992). Multilocus enzyme studies have also helped in the identification of two groups of subspecies status in *L. ivanovii*; *L. ivanovii* subsp *ivanovii* and *L. ivanovii* subsp *londoniensis* (Boerlin *et al.*, 1992).

1.3 HABITATS

Until about 1960, *L. monocytogenes* was associated almost exclusively with infections in animals and less frequently with humans (Gray and Killinger, 1966; Seeliger, 1961; Welshimer, 1981). During the last 30 years, listeriae, including the pathogenic species *L. monocytogenes* and *L. ivanovii* have been isolated from a variety of sources (Jones and Seeliger, 1991). These sources include soil, vegetation, faecal material, a variety of foods, sewage, and water (Ryser and Marth, 1991). Improperly fermented silage is another important niche for this organism and has been cited as the source of infection in numerous cases of listeriosis involving domestic farm animals. *Listerae* are now considered to be widely distributed in the environment (see Jones and Seeliger, 1991).

1.3.1 Soil and Vegetation

Although much of the epidemiology concerning *L. monocytogenes* remains obscure, reports suggest that the primary habitat of the organism is in soil and decaying vegetation where the bacterium leads a saprophytic existence (Ryser and Marth, 1991). The presence of *L. monocytogenes* in soil and vegetation is well documented (Weis and Seeliger, 1975; Welshimer, 1968; Welshimer and Donker-Voet, 1971).

Relatively large numbers of *L. monocytogenes* have been isolated from samples of mud, which suggest that a moist environment favours growth of the organism. Studies performed on different types of soil indicated that the survival of *L. monocytogenes* in soil depends on the type of soil and its moisture content (Welshimer, 1960). This worker observed that the number of *Listeriae* in fertile soil, protected from evaporation, remained at a relatively high level for a period of 295 days while in clay soil, under the same conditions, the organism was not isolated after 200 days. Where loss of moisture was not restrained, the number of *Listeriae* in both soils declined until no organisms survived after 67 days (Welshimer, 1960).

1.3.2 Animals and Humans

Listeria monocytogenes has been isolated, in addition to humans, from faeces of at least 42 species of wild and domestic mammals and 17 avian species including domestic and game fowl. *Listeriae* have also been isolated from other animals, some of which include seagulls, rooks, house sparrows, fish, crustaceans, oysters, ticks and flies (Larsen, 1964; Gray and Killinger, 1966; Kampelmacher and van Noorle Jansen, 1969; Armstrong, 1985; Fenlon, 1985; Rocourt and Seeliger, 1985; Lamont *et al.*, 1988; Gellin and Broome, 1989). The bacteria have also been detected in faeces of apparently healthy animals (Ralovich, 1984). Human faeces collected from asymptomatic individuals has also yielded *L. monocytogenes*. Ralovich (1984) summarised data, primarily of European origin, which indicated that between 1.8 and 9.0% of the normal healthy human population (differing by age, sex, and profession) may carry *L. monocytogenes* in the gut.

1.3.3 Sewage and Water

Considering the frequency with which *L. monocytogenes* appears in human and animal faeces, it is not surprising that is often encountered in sewage and water. No correlation was observed between populations of *Listeriae* and *Escherichia coli*, faecal streptococci, *Clostridium perfringens*, or *Salmonellae* (Watkins and Sleath, 1981).

To my knowledge no case of human listeriosis has been reported as consequence of consuming contaminated drinking water.

1.3.4 Food

Food has long been suspected as the route of infection for human listeriosis. Even before the isolation of the organism, Atkinson (1917) suggested that the listeriosis-like disease in 5 children in Australia, which occurred in 1915, was linked with eating of contaminated foodstuffs. In addition, early animal feeding studies also supported the idea that human listeriosis can be acquired through consumption of contaminated food. The *L. monocytogenes* description of Murray *et al.* (1926), included trials in which three of the six 32-day-old rabbits studied were infected via the oral route. Pirie (1927), also established a laboratory link between the onset of listeriosis in mice and gerbils with the consumption of feed artificially contaminated with *L. monocytogenes*. Julianelle (1940), reported that mice died of listeric infections after consuming drinking water contaminated with *L. monocytogenes*. These results led to the suggestion that ingestion of *L. monocytogenes* followed by penetration of the gastrointestinal tract by the bacterium is one means by which listeriosis can be acquired.

Even though consumption of raw milk was considered the cause of outbreaks of listeriosis, between 1949 and 1957, in Germany and the former Czechoslovakia (Seeliger, 1961; Gray and Killinger, 1966), it was only in the early 1980s that the connection between listeriosis and the consumption of foodstuffs was firmly established. The ability of *L. monocytogenes* to produce foodborne illness was proven when in 1981 in the Maritime Provinces of Canada, 17 of 41 (41.5%) people died of listeriosis after consuming coleslaw from which this organism was later isolated (Schlech *et al.*, 1983). This confirmed that humans can contract listeriosis by consuming contaminated food. Since then, several epidemics have been linked to the consumption of contaminated foodstuffs. In 1983, the consumption of

pasteurised milk was epidemiologically linked to 49 cases of listeriosis in Massachusetts, however, *L. monocytogenes* was never isolated from this milk (Fleming *et al.*, 1985). In June 1985, consumption of Jalisco brand Mexican-style cheese was directly linked to at least 142 cases of listeriosis, including 48 deaths, in Los Angeles (Linnan *et al.*, 1988). Between 1983 and 1987 another listeriosis outbreak in Switzerland, that resulted from consumption of contaminated Vacherin Mont d'Or soft-ripened cheese, was responsible for 31 deaths (Malinverni *et al.*, 1985). A list of listeriosis outbreaks associated with the consumption of contaminated foodstuffs is shown in Table 1.3.4.1.

These listeriosis outbreaks have generated world-wide concern over the presence of *L. monocytogenes,* not only in dairy products but also in many other foods including meat, seafood, poultry, eggs, and vegetables.

When listeric infections in domestic livestock began to be identified during the 1930s and the 1940s, some researchers began to speculate that consumption of meat products could play a role in the spread of human listeriosis (Ryser and Marth, 1991). In addition, surveys of cooked and uncooked meats, have indicated that the organism is able to survive on the meat surface and in sarcoplasmic protein solutions (meat drip) (Khan *et al.*, 1972; Johnson *et al.*, 1986).

Little is known about the epidemiology of *L. monocytogenes* in seafood. Weagant *et al.* (1988) reported that at least 26% of frozen seafood products contain *L. monocytogenes.* The bacterium was detected in raw shrimp and lobster, fin fish, surimi-based seafood, and cooked shrimp and crabmeat. Consumption of mussels have played a part in the listeriosis case in New Zealand during 1980 (Brackett and Beuchat, 1990). However, raw seafood may actually pose less hazard than ready-to-eat cooked seafood, since they are usually subjected to thermal treatment, sufficient to kill *L. monocytogenes,* immediately before being consumed. Surimi and seafood cooked and offered for sale at retail are often stored refrigerated for extended periods (2 weeks or more) (Brackett and Beuchat, 1990), increasing the potential for growth of *L. monocytogenes* and subsequent consumption. *Listeria monocytogenes* have also been shown to survive cold and warm smoking processes for fish (Fuchs and Nicholaides, 1994; Embarek *et al.*, 1995).

Table 1.3.4.1 - Food-borne outbreaks due to *L. monocytogenes* in North America and World-wide

Location (year)	No. of cases (no. of deaths)	Foods associated
Boston (1979)	20 (5)[a]	Salad vegetables[b]
New Zealand (1980)	29 (9)	Shellfish, raw fish
Maritime Provinces (1981) of Canada	41 (17)	Coleslaw
Massachusetts (1983)	49 (14)	Pasteurised milk[b]
California (1985)	142 (48)	Jalisco Cheese
Switzerland (1983-87)	122 (31)	Raw milk, cheese
Philadelphia (1986-87)	36 (16)	Ice cream, salami[b]
Connecticut (1989)	9 (1)	Shrimp[b]
United Kingdom (1987-89)	>300 (*)	Paté[b]
France (1992)	279 (*)	Pork tongue in jelly
France (1993)	39 (*)	Pork paté

[a] For two of these five deaths, an underlying disease, not listeriosis, was apparently the cause of death.

[b] Foods only epidemiologically linked.

* Exact number of related deaths unknown.

Adapted from Farber and Peterkin, (1991); McLauchlin *et al.* (1991); Goulet *et al.* (1995).

Only in November 1988, the transmission of *L. monocytogenes* by consumption of contaminated poultry was documented (Kerr *et al.*, 1988). Since then, poultry has been deemed as an important vehicle of foodborne listeriosis.

Acquiring listeriosis through consumption of contaminated eggs and egg products has been considered for nearly 40 years (Ryser and Marth, 1991); however, unlike poultry products, no such cases have been firmly documented. Recently a study by Desmarchelier *et al.* (1995), analysing possible listerial contamination in the Australian egg industry, revealed that *L. innocua* was recovered from 13.4% of samples collected from the factory (including environmental swabs, finished products and raw/semi-processed products). These workers also observed that 100% of the raw egg pulp obtained directly from the farms and 69% of the factory produced pulp was contaminated with *L. innocua*. Although *L. innocua* and *L. monocytogenes* are frequently isolated together, the latter was isolated only once (Desmarchelier *et al.*, 1995).

Given the evidence linking cases of animal listeriosis to consumption of contaminated plant material, it is reasonable to suspect that raw vegetables are an important vehicle for human listeriosis. Ho *et al.* (1986) reported an outbreak of human listeriosis amongst adult patients in eight Boston (USA) hospitals in 1979. Even though the victims had consumed different foods, the common feature among them was the serving of the foods with raw vegetables such celery, tomatoes, and lettuce (Ho *et al.*, 1986). The vegetables were not available for testing. In 1983, Schlech *et al.* (1983) published the first confirmed North American outbreak of foodborne listeriosis in which 41 Canadians became ill in 1981 after consuming coleslaw contaminated with *L. monocytogenes*. The origin of contamination was traced to the cabbage which was grown in fields fertilised by both composted and raw manure from a flock of sheep in which two have died of listeriosis (Schlech *et al.*, 1983). In the light of this Canadian outbreak it can be postulated that any outbreaks with raw vegetables or vegetables as the vehicle for human listeriosis, are likely to have been contaminated by animal faeces containing *L. monocytogenes*. Interestingly, it has been observed that raw carrot has an inhibitory effect on *L. monocytogenes* (Beuchat and Brackett, 1990). However, the anti-*Listeria* effect was eliminated when the carrots were cooked (Beuchat and Brackett, 1990).

Early reports suggested that *L. monocytogenes* is resistant to heat and are able to survive Pasteurisation (Bearns and Girard, 1958). However, until the mid 1980s human listeriosis

had only been linked to raw milk. Unpasteurised milk, sour milk, cream, and cottage cheese were suspected as possible vehicles of infection in several cases observed in Germany between 1949 and 1957 (Seeliger, 1961). In 1985, epidemiological studies strongly suggested that the Massachusetts outbreak was linked to the consumption of a specific brand of pasteurised whole milk and milk containing 2% fat (Fleming *et al.*, 1985). The microbiological studies were far less convincing since *L. monocytogenes* was never isolated from this milk. The milk was correctly pasteurised, plus no defect was identified that could have led to improper pasteurisation and no source of postpasteurisation contamination was ever found within the dairy factory (Fleming *et al.*, 1985). As a result the source of contamination was never conclusively revealed. Following the Massachusetts outbreak the work of Bearns and Girard (1958) has been questioned (Twedt, 1986; Donnelly *et al.*, 1987). Twedt (1986) observed that *L. monocytogenes* would not have survived proper pasteurisation and that even on intracellular location did not protect *L. monocytogenes* from thermal inactivation. Hence, the scientific community and the World Health Organisation claimed that *L. monocytogenes* will not survive minimum pasteurisation requirements (Ryser and Marth, 1991).

A diverse range of food types have been shown to be associated with both epidemic outbreaks and sporadic cases of human listeriosis. Milk products that have been shown to support the growth of *L. monocytogenes* include chocolate milk, cream and skimmed milk (Rosenow and Marth, 1987). Foods that are of special danger to susceptible individuals are poultry and associated products; meat and associated products, such as paté, and sausages; seafood; milk and associated products such as cheeses and milk drinks (Farber and Peterkin, 1991; Ryser and Marth, 1991).

The incidence of *L. monocytogenes* in cooked chicken, soft cheese and paté examined as part of planned surveys of foods on retail sale in England and Wales, in the late 1980s to mid 1990s (Table 1.3.4.2), have shown a dramatic fall in the number of reported cases towards the end of the surveys (Gilbert, 1995). These improvements in foods on retail sale are the result of the efforts made by the industry; (a) investigating the occurrence of Listeria in foods and within the factory environment, (b) implementing Hazard Analysis Critical Control Point (HACCP) procedures and (c) applying voluntary codes of practice (Gilbert, 1995). The high numbers of *L. monocytogenes* in paté in 1989 were due to only one manufacturer, the subsequent decline in numbers in 1990 were most likely due to the suspension of supplies from this manufacturer (McLauchlin *et al.*, 1991). Annual totals of

listeriosis in England and Wales ranged from 85 cases in 1995 to 278 cases in 1988. The rise in both cases associated with pregnancy and other cases between 1987 and 1989 was associated with eating paté from the above manufacturer (McLauchlin and Newton, 1995; Communicable Disease Report, 1997). The proportion of cases associated with pregnancy has fallen to between 8% and 26% of cases between 1990 and 1996, compared with 31% to 48% of cases between 1983 and 1989. This fall in cases associated with pregnancy is due to changes in diet in response to specific Government advice which warns pregnant woman (and also immunocompromised people) to avoid eating soft ripened cheeses and paté, and to reheat cooked chilled meals and ready to eat poultry until they are piping hot (Communicable Disease Report, 1997).

1.4 EPIDEMIOLOGY AND CLINICAL MANIFESTATIONS

1.4.1 Epidemiology of Human Listeriosis

Although human listeriosis may be caused by all 13 serovars of *L. monocytogenes*, serovars 1/2a, 1/2b and 4b cause most of the cases (Jones, 1990). Geographic differences in the distribution of serotypes exists, with serovar 4b predominating in most Europe and an even distribution of serovars 1/2a, 1/2b and 4b in Canada and United States (Farber and Peterkin, 1991).

The real incidence of human listeriosis world wide is not known. That it is higher than current data suggest is almost certain because the clinical features, especially in mild cases, are neither specific nor striking and sporadic cases frequently go undetected; laboratory investigation of the disease varies between and within different countries; statutory notification (usually only of the more overt forms) is required in only a few countries; and voluntary notification is patchy (Jones, 1990). Estimates of the incidence of the disease varies from 1 to 15 cases per million of the population (Gellin and Broome, 1989; Jones, 1990; Farber and Peterkin, 1991). Whether, this reflects the true incidence is not known, however, it is known that there has been a dramatic increase in cases of listeriosis in industrialised countries in the past twenty years (Jones, 1990). This increase may be 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).

Table 1.3.4.2 - Occurrence of *L. monocytogenes* in different food types examined as part of planned surveys of foods on retail sale in England and Wales.

Year	Number of samples examined	*L. monocytogenes*	
		Number	% of samples
Cooked Chicken			
1988/89	527	63	12.0
1989	102	27	26.5
1991	169	46	27.2
1991	983	91	9.3
1993	119	6	5.0
Soft Cheese			
1987	222	23	10.4
1988/89	1135	67	5.9
1989/90	131	0	0
1991/92	251	10	4.0
1995	1437	16	1.1
Paté			
1989	155	46	29.7
1989	1698	162	9.5
1989	65	13	20.0
1990	626	25	4.0
1990	96	3	3.1
1991/2	40	1	2.5
1994	3073	80	2.6

Adapted from Gilbert (1995).

Routine surveillance data collected by the Public Health Laboratory Service, in England, Wales and Northern Ireland, between 1983 and 1994 revealed a marked peak in the numbers of reported cases with onsets in the late summer or autumn (McLauchlin and Newton, 1995). The reason for this seasonal pattern is not known (McLauchlin and Newton, 1995). Between 1987 and 1989 there was a sharp increase in the annual totals of reported cases of human listeriosis in England, Wales and Northern Ireland which was associated with paté (McLauchlin *et al.*, 1991; McLauchlin and Newton, 1995; see Section 1.3.4). The numbers of cases fell in 1990 and has now returned to levels recorded in the early 1980s (Communicable Disease Report, 1997). The cases associated with pregnancy have fallen between 1990 and 1996, when compared with cases between 1983 and 1989, however the number of other cases, mainly immunocompromised adults, rose in the 1990s (Communicable Disease Report, 1997). The fall in cases associated with pregnancy may be due to changes in diet in response to the Government advice which warns pregnant women (and also immunocompromised people) to avoid eating potential risk foods such as soft ripened cheeses and paté (Communicable Disease Report, 1997). The rise in cases not associated with pregnancy included a rise in cases in the aged 75 years or over and may reflect demographic changes in the number of elderly people (Communicable Disease Report, 1997).

1.4.2 Clinical Manifestations in Humans

The clinical features of listeric infection vary widely and are often confused with other illnesses. The symptoms of human listeriosis can be arranged into three groups, bacteraemia, meningitis, and pneumonia.

Infection of pregnant women commonly results in a variety of influenza-like symptoms including fever, chills, headache, and backache. Gastrointestinal illness have been noted, but meningitis is rare (Seeliger and Finger, 1976; Schwarze *et al.*, 1989). However, infected pregnant women may be asymptomatic or exhibit only mild symptoms (Seeliger, 1961). These symptoms are an expression of listeric bacteraemia, and the bacterium can be isolated from maternal blood, umbilical cord blood, and placenta tissue (Seeliger and Finger, 1976). Listeriosis of the foetus have been proved after the fifth month of gestation but abortion only occurs before the fourth month of pregnancy (Seeliger and Finger, 1976). The time interval between maternal and foetal infections is poorly defined. In some cases, abortion or stillbirth occurred immediately after the mother experience the mild influenza-

like symptoms, whereas in others these events were separated by several weeks (Seeliger, 1960).

Neonatal listeriosis is among the most dangerous forms of listeriosis and is major cause of foetal damage and infant death, along with syphilis, erythroblastosis, and toxoplasmosis. As respiratory and gastrointestinal distress are commonly observed in new-born infants suffering from listeriosis, it has been postulated that the foetus becomes infected through aspiration of contaminated amniotic fluid (Seeliger and Finger, 1976). In untreated cases of perinatal listeriosis, prospects for survival are poor with the mortality rate approaching 100%; however, prompt antibiotic therapy has resulted in many survivors. Long-term prognosis depends on whether or not the infection has spread to the central nervous system and resulted in meningitis (Ryser and Marth, 1991).

The most dangerous category of listeric infections, which are usually a consequence of generalised bacteraemia, are meningitis, meningioencephalitis, and encephalitis. However, lymphatic spread from localised infections of the nose, throat, eye, and ear also have been reported (Seeliger and Finger, 1976).

Listeric meningitis, clinically cannot be differentiated from other forms of bacterial meningitis; hence, diagnosis rests solely on results from bacteriological tests. The course of the disease is often fatal (sudden onset of great severity) and has a mortality rate of approximately 70% in untreated patients or patients that are treated too late in the course of infection (Seeliger and Finger, 1976). Individuals who survive listeric meningitis may be afflicted with various brain abnormalities including hydrocephalus, cerebral oedema, and cerebellar atrophy (Marrie *et al.*, 1984).

Listeria monocytogenes is one of the most frequent causes of meningitis in immunocompromised individuals, such as those receiving immunosuppressive treatment, cancer patients, transplants recipients, the elderly and acquired immune deficiency syndrome sufferers (Louria *et al.*, 1967; Neiman and Lorber, 1980; Stamm *et al.*, 1982; Mascola *et al.*, 1988). The illness begins less suddenly in adults than in infants with the appearance of influenza-like symptoms, which are followed by development of headaches, leg pains, fever, chills, increasing rigidity of the neck, nausea, vomiting, and photophobia. Eventually, victims become somnolent with intermittent bouts of convulsions and dehydration and finally die in coma (Ryser and Marth, 1991).

1.4.3 Epidemiology of Animal Listeriosis

Listeriosis in domestic animals has been recognised around the world, however, the exact incidence of listeric infections remains unknown. In Great Britain the number of reported listeriosis cases in domestic animals increased substantially since 1975, increasing from 87 to 342 in 1984 (Wilesmith and Gitter, 1986). Seasonal variation in the number of cases of animal listeriosis has often been observed. In the Northern Hemisphere (England, Bulgaria, Hungary, United States, France and Germany), listeriosis in domestic livestock generally occurs from late November to early May with the greatest incidence during February and March (Gray and Killinger, 1966). Numbers of listeriosis cases increased when animals were fed silage during periods of extreme cold, whereas a fall in numbers of reported cases were observed when grass was available. Low *et al.* (1995) found a clear association between faecal excretion and silage feeding in a Scottish flock of sheep. After the introduction of the contaminated silage *L. monocytogenes* was isolated from up to 33% of the animals (Low *et al.*, 1995). Data from the Netherlands indicated that 40% of the cases of listeric abortion in cattle were directly associated with the consumption of contaminated silage and when the production of silage was changed the numbers of listeriosis cases in cattle were reduced markedly (Dijkstra, 1987).

As in humans, *L. monocytogenes* serovar 1/2a is associated with most cases of animal listeriosis, although serovar 4b has also been isolated (Ivanov, 1985; Wilesmith and Gitter, 1986; Low *et al.*, 1995). *Listeria ivanovii* serovar 5, is a common cause of ovine listeriosis abroad, however, in the United Kingdom it is of less concern (Ivanov, 1975).

Animal listeriosis can be of importance for the economy of a country. During the early 1970s, the agricultural economies of Australia and Norway were adversely affected by the loss of approximately 1 million and 2000-2500 sheep, respectively, from listeric infection (Ryser and Marth, 1991). As an example of the financial loss, in the United Kingdom an outbreak of listeriosis in a housed flock of sheep caused the loss of 21 ewes and 88 lambs costing the farmer an excess of £5000 (Low and Renton, 1985). Dijkstra (1987), also reported that 234-928 cases of listeric abortion occurred annually in cattle in the Netherlands between 1970 and 1985. During the same period, the annual percentage of abortions caused by *L. monocytogenes* in cattle ranged between 0.7 and 8.7% with an average of 3.2% (Dijkstra, 1987). Another concern, which relates with human foodstuffs, is the prevention of contamination of the production process by avoiding contact with infected

animal and faeces. This can be performed in several ways: improvement of animal housing and feed, and improved testing during manufacture, and also the effective bacteriological control of *Listeria*, by methods such as vaccination as was demonstrated in Norway during the 1980s (Gudding *et al.*, 1985; Gudding *et al.*, 1989). Vaccination, using two attenuated strains of *L. monocytogenes* resulted in a decrease of ovine listeriosis from 4 to 1.5% (Gudding *et al.*, 1985; Gudding *et al.*, 1989). A decrease (1.1% - 0.7%) in the amount of abortions was also observed in the vaccinated flocks (Gudding *et al.*, 1985; Gudding *et al.*, 1989).

1.4.4 Clinical Manifestations in Animals

Listeriosis in animals can occur either sporadically or as epidemics and often leads to fatal forms of encephalitis (Ryser and Marth, 1991). All domestic animals are susceptible to listeric infections. However, sheep (Ödegaard *et al.*, 1952; Gray *et al.*, 1956; Nilsson and Karlsson, 1959; Grønstøl, 1980; Wardrope and Macleod, 1983; Yousif *et al.*, 1984), cattle (Smith et al., 1955; Gray *et al.*, 1956; Nilsson and Karlsson, 1959; Oselbold *et al.*, 1960; Price, 1981; Rebhun and deLahunta, 1982; Wohler and Baugh, 1983), goats (Løken and Grønstøl, 1982; Løken *et al.*, 1982; Sharma *et al.*, 1983), and chickens (Gray, 1958; Nilsson and Karlsson, 1959; Nagi and Verma, 1967; Ramos *et al.*, 1988), succumb to infection more frequently.

"Listeric-like" infections were observed in sheep in 1925 (Seeliger, 1961). However, the first isolate of *L. monocytogenes* from domestic farm animals was reported by Gill (1931). In 1929, Gill observed an illness in sheep which he called "circling disease". This name is still in use to describe listeric encephalitis, encephalomyelitis, and meningioencephalitis, which are the most common manifestations of listeriosis in ruminants, including sheep (Seeliger, 1961). Clinical symptoms of ovine encephalitis may include elevated temperature and refusal to eat or drink. These initial symptoms are frequently followed by neurological disturbances. At this stage the animal moves in circles, depending on the side on which the head is bent. Death usually occurs within 2-3 days after onset of clinical symptoms (Seeliger, 1961).

Listeric infections in pregnant sheep often result in premature birth and infectious abortions (Gray *et al.*, 1956; Macleod and Watt, 1974; Carter *et al.*, 1976; Arda *et al.*, 1987). Listeriosis in young animals often assumes the form of a septicaemia which is characterised

by an elevated temperature, loss of appetite, and diarrhoea. Although death may eventually occur, the mortality rate is much lower for the septicemic than for the encephalitic form of listeriosis (Ryser and Marth, 1991).

Clinical manifestations of listeriosis in goats and sheep are essentially the same. Although meningoencephalitis predominates in goats, reports of listeric abortion are common (Seeliger, 1961).

In 1928, Matthews (1928) reported an outbreak of encephalitis of unknown origin in cattle which, was probably bovine listeriosis. During the early 1930s several cases of bovine encephalitis were recorded and probably resulted from infection with *L. monocytogenes* (Jones and Little, 1934).

Cattle can survive at least 4-14 days after the initial onset of symptoms, with a few reports of spontaneous recovery (Ralovich, 1984). Clinical symptoms normally appear after a incubation period of 1-3 days and may include self-imposed segregation from other animals and neurological disturbances that result in circular movement (Seeliger, 1961).

Infection of a pregnant cow with *L. monocytogenes* can give rise to an aborted foetus (Graham, 1939). Abortion in cattle as in sheep usually occurs during the second half of pregnancy (Osebold *et al.*, 1960; Seeliger, 1961; Spika *et al.*, 1973; Ralovich, 1984). *Listeria monocytogenes* is transmitted to the foetus via the intrauterine/intraplacental route, which generally culminates in death of the foetus, followed by abortion (Seeliger, 1961).

As in sheep (Grønstøl, 1979a; Grønstøl, 1979b; Grønstøl, 1980) and goats (Løken *et al.*, 1982), *L. monocytogenes* can also be shed in milk by dairy cattle (Ryser and Marth, 1991). In spite of not being particularly common, generalised listeric infections can give rise to mastitis, which is a condition of great concern to the diary industry. The role of *Listeria* in mastitic infections was not considered until 1944. In that year, Wramby (1944) isolated *L. monocytogenes* from milk and udders of mastitics cows in Sweden. When *L. monocytogenes* was isolated from a sample of raw milk during a human outbreak in East Germany (Seeliger, 1961), it was postulated that this organism can be a cause of atypical mastitis in dairy cows since the milk came from animals presumed to be healthy.

Meningioencephalitis in swine begins with a sudden refusal to eat and is typically followed by various neurological disorders including trembling, partial paralysis, incoordination, circling movements, and convulsions (Biester and Schwarte, 1940). Infections during pregnancy may be followed by miscarriages and abortions. If untreated, death often occurs within 48 hours (Seeliger, 1961; Gray and Killinger, 1966). Listeriosis in swine also can assume several other forms including septicaemia, localised internal abscesses, and pox-like lesions (Gray and Killinger, 1966).

1.5 ISOLATION

All *Listeria* species grow well on the common laboratory media, but primary isolation of the organism from infected or contaminated specimens is difficult (Gray and Killinger, 1966, Seeliger, 1961). Enrichment and/or selective procedures, are recommended and should always be used in parallel with any attempt at direct isolation of *Listeriae* on nonselective media (Jones and Seeliger, 1991).

Gray *et al.* (1948), observed that clinical specimens from suspected listeric infections failed to yield *L. monocytogenes* on primary plating but did so after long incubation at 4°C. This cold enrichment technique has been employed extensively with a number of selective and nonselective enrichment broths. Unfortunately, as it was referred before, the length of time required for cold enrichment is arbitrary and can take a few years (Jones and Seeliger, 1991). Cold enrichment technique developed by Gray *et al.* (1948), has been proven to be successful for the isolation of listeriae. However, as this method is based on the ability of these bacteria to grow at 4°C, samples are refrigerated in nonselective media for a period of up to six months. Therefore a number of other enrichment procedures and several selective media have been devised to reduce the time of analysis.

Tryptose-based media have been used extensively as nonselective media for the culture of listeriae. Tryptose agar (Difco), tryptose blood agar base (Difco) containing 5% washed sheep erythrocytes, and tryptose broth (Difco) all support excellent growth, as do other media of similar composition. Use of such media for primary isolation succeeds only when the suspected material contains relatively high numbers of *Listeriae* and few other bacteria (Jones and Seeliger, 1991).

Lovett *et al.* (1987), developed a medium for the enrichment of *Listeriae* in milk; it may also be used for food, faecal, and environmental samples. It is a modification of the medium of Ralovich *et al.* (1971). However, the enrichment medium of Donnelly and Baigent (1986) is the one chosen for the enrichment of food samples in both the UK and the USA. The reason for why this medium does not contain cycloheximide, which is toxic to humans (Jones and Seeliger, 1991).

Among the selective media devised for the isolation of *Listeriae*, the most successful employ a combination of two inhibitors, nalidixic acid, which inhibits Gram-negative bacteria, and an acridine dye at a concentration that inhibits many Gram-positive bacteria without suppressing the *Listeriae* (Jones and Seeliger, 1991). Detailed description of techniques for the isolation of *L. monocytogenes* and other *Listeria* species are given by Ralovich (1984), Prentice and Neaves (1988), and Ryser and Marth (1991).

1.7 IDENTIFICATION

Identification of isolates as *Listeria* species requires examination of colonial and cellular morphology, staining reactions, growth at 37°C, relationships to oxygen and catalase production. A number of conventional biochemical tests such as acid production from various sugars and hydrolysis of aesculin also helps in the identification of these species (Jones and Seeliger, 1991).

Listeria monocytogenes is usually referred as the type species of the genus and exhibits a morphology, and motility which is common to all its species. Colonies can be seen on nutrient agar after 24-48 h at 37°C and are 0.5 to 1.5 mm in diameter, round, translucent with a watery consistency, and low convex with finely textured surface and entire margins (Jones and Seeliger, 1991). These colonies appear bluish grey. New colonies exhibit a blue-green sheen when viewed by obliquely transmitted light, whereas old or rough cultures do not (Jones and Seeliger, 1991).

The morphology of *Listeriae* at the cellular level is not particularly distinctive. The Gram-positive rods are short, 0.4-0.5 μm in diameter and 1-2 μm in length, with rounded ends (Jones and Seeliger, 1991). They are not acid-fast, do not form spores, and capsules are not present. The rods can also exhibit typical palisade formation, along with some V and Y

forms. In contrast to the smooth form, long rods measuring 6-20 μm are typically observed in rough strains (Anton, 1934).

Listeria monocytogenes, when incubated in broth cultures at 20°C, exhibits a characteristic tumbling motility (Seeliger, 1961). This bacterium is motile by a few (usually 4-5) peritrichous flagella (Kressebuch *et al.*, 1988).

All species of the genus *Listeria* are facultatively anaerobic and produce acid but no gas from glucose (Seeliger and Jones, 1986). Some other biochemical tests can be used, these include a positive Voges-Proskauer (VP) test, aesculin hydrolysis, alkaline phosphatase positive, urease negative, and oxidase negative (Rocourt and Catimel, 1985; Seeliger and Jones 1986).

The most important characteristics differentiating the species are haemolysis; production of acid from mannitol, rhamnose, D-xylose, and α-methyl-D-mannoside; and nitrate reduction (Jones and Seeliger, 1991). Observation of haemolysis depends on the basal medium used and on the source of the blood (Pine *et al.*, 1987, Skalka *et al.*, 1982). A more trustworthy method, although expensive, for testing for acid production from carbohydrates is the API 50 CH gallery (bioMérieux S. A., France). However, information on all the 49 test substrates in this system is not required for routine identification (Jones and Seeliger, 1991).

The antigenic composition of *Listeriae* can be useful but is not essential for routine identification. Using the serological methods of Kauffmann and White, Paterson (1939, 1940) described four serological types of *L. monocytogenes* based on somatic (O) and flagellar (H) antigens. 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. Additional O antigens were identified and serotype 4 was split into serotypeas 4a and 4b. Donker-Voet (1965), added serotypes 4c, 4d, and 4e to the list of recognised *L. monocytogenes* serotypes (Seeliger, 1961). Small differences in H antigens were observed in some cultures of serotypes 1 and 3, and serotypes 1a and 3a were created (Seeliger, 1961). In total *L. monocytogenes* isolates can be classified into 13 serotypes (Jones and Seeliger, 1991). Most isolates from human and animal infections belong to a few common serotypes about 90% of isolates belong to serotypes 1/2a, 1/2b, and 4b (Seeliger and Hohne, 1979; McLauchlin, 1987).

Bacteriophages specific for *Listeria* spp. were initially discovered in 1945 (Schultz, 1945) and since then several groups have studied the possibility of phage typing of *L. monocytogenes* (Audurier *et al.,* 1984; Rocourt *et al.*, 1985; McLauchlin *et al.*, 1986; Audurier and Martin, 1989). These studies were not very successful by the lack of phages available with the overall percentage of *L. monocytogenes* strains typeable ranging from 52-78%. A new set of phages derived from both environmental and clinical lysogenic strain has been described (Loessner *et al.*, 1990). These allow the typing of more than 90% of serotype 1/2 strains, the majority of isolates of serotypes 4a, ab, c, d, and e, although strains of serotype 3 and 7 appear to be resistant.

Multilocus enzyme electrophoresis has shown a good potential for epidemiological studies with strains of *L. monocytogenes*. Piffaretti *et al.* (1989) analysed a number of strains of *L. monocytogenes* electrophoretically for allelic variation at 16 genetic loci encoding metabolic enzymes and identified forty-five distinct electrophoretic types (ETs). Recently multilocus enzyme electrophoresis studies by Boerlin *et al.* (1991, 1992) using 18 enzyme loci showed that strains of *L. ivanovii* could be divided into two main genomic groups represented by subsp. *L. ivanovii* sudsp *ivanovii* and *L. ivanovii* sudsp *londoniensis*, previously described.

In the last few years other methods for the identification of *L. monocytogenes* have been developed. These include the restriction endonuclease and the use of specific gene probes for DNA analysis (McLauchlin *et al.*, 1988); polymerase chain reaction (PCR) by using a species specific set of primers (Bansal *et al.*, 1995; Datta and Benjamin, 1995; Mingyuan *et al.*, 1995); specific immunological detection by using antibodies raised against p60-derived synthetic peptides (Bubert *et al.*, 1995) and cellular fatty acid analysis (Steele *et al.*, 1995).

1.7 GENERAL PROPERTIES

1.7.1 Chemical Composition

The chemical composition of the macromolecules of all species is alike. The peptidoglycan of the cell wall contains *meso*-diaminopimelic acid (*meso*-DAP), glutamic acid, alanine, muramic acid, and glucosamine; glucose, and rhamnose may or may not be present, and arabinose and galactose are never found (Keedler and Gray, 1960; Ullmann and Cameron,

1969; Schleifer and Kandler, 1972; Srivastava and Siddique, 1973; Kamisango *et al.*, 1982; Fiedler and Seger, 1983; Hether *et al.*, 1983; Fiedler *et al.*, 1984).

The polar lipid composition of *L. monocytogenes* consist of phosphatidylglycerol, diphosphatidylglycerol, galactosyl-glucosydiacylglycerol, and an uncharacterised glycophospholipid (Kosaric and Carroll, 1971; Shaw, 1974). All listeriae contain predominantly straight-chain, saturated, anteiso-, and isomethyl-branched chain types fatty acids(see Jones and Seeliger, 1991). The fatty acid composition of membrane polar lipids depends on growth temperature (Herbert, 1989; Russell, 1990; Gounot, 1991; Jones *et al.*, 1997; see Section 1.8).

When *Listeriae* are grown in a complex medium, with shaking, cytochromes $a_1$bdo can be found. Trivett and Meyer (1971) failed to detect any cytochromes when used a defined medium. The GC content of the DNA of the genus varies from 36-42 mol% (Jones and Seeliger, 1991); that of *L. monocytogenes* is 37-39 mol% (Rocourt and Grimont, 1983).

1.7.2 Metabolism and Nutritional Requirements

All *Listeriae* are facultatively anaerobic, however laboratory cultures exhibit better growth aerobically. Carbohydrates, usually glucose, are essential for growth of *Listeria*. Glucose can not be replaced as a carbon and energy source by gluconate, xylose, arabinose, or ribose (Miller and Silverman, 1959). Catabolism of glucose apparently proceeds by the Embden-Meyerhof (glycolysis) pathway both aerobically and anaerobically. Anaerobically, the end product is mainly lactic acid; aerobically, pyruvate, acetoin, lactic acid, and other end products are formed (Jones and Seeliger, 1991).

Trivett and Meyer (1971) observed that in a defined medium pyruvate, acetate, citrate, fumarate, nor malate supported growth of *L. monocytogenes* in the absence of glucose, nor did they allow increased growth in the presence of glucose. Pyruvate, malate, succinate, and α-ketoglutarate have, however, been reported to be oxidised at low rates by *L. monocytogenes* (Friedman and Alm, 1962, Kolb and Seidel, 1960).

Biotin, riboflavin, thiamine, thioctic acid, and several amino acids, including cysteine, glutamine, isoleucine, leucine, and valine, are required for growth in a defined medium (Jones and Seeliger, 1991). Iron is reported to have a growth-stimulating effect on *L.*

monocytogenes in stationary and aerated cultures (Sword, 1966, Trivett and Meyer, 1971); aeration improves growth only in the presence of adequate iron (Trivett and Meyer, 1971).

All *Listeriae* produce catalase, and cytochromes, but the production of both is dependent on the medium and conditions of cultivation. Strains grown on media containing low levels of meat and yeast extract may be catalase-negative, and aeration and adequate iron are necessary for the detection of cytochromes (Feresu and Jones, 1988).

1.8 GROWTH OF *Listeria monocytogenes* AT LOW TEMPERATURE

Listeria monocytogenes has become firmly established as a food-borne pathogen since, listeriosis outbreaks have been associated to the consumption of contaminated coleslaw (Schlech *et al.*, 1983), Pasteurised milk (Fleming *et al.*, 1985), soft cheese (Linnan *et al.*, 1988), and turkey frankfurters (Ryser and Marth, 1991). *Listeria monocytogenes* is of particular concern to the food industry and public health regulatory agencies because it can grow at refrigeration temperatures (4°C) and, being so widespread in the environment, can easily gain access into the food chain. Indeed, *L. monocytogenes* has been detected in virtually every food group (Brackett and Beuchat, 1990; see Section 1.3.4). The survival and growth of *L. monocytogenes* in a range of foodstuffs at low temperatures has been very well documented (Johnston *et al.*, 1986; Marth, 1986; Rosenow and Marth, 1987; Hudson and Mott, 1993).

The most widely accepted definitions for any microorganisms able to grow at low temperatures are those of Morita (1975). This author defined psychrophilic (cold-loving) bacteria as those microorganisms which have an optimal growth temperature of 15°C or lower, a maximal growth temperature of approximately 20°C and a minimum growth temperature of 0°C or lower. Microorganisms which grow at 0 to 5°C and at maximum temperatures exceeding 25°C are considered to be psychrotrophic (cold tolerant) or psychrotolerant. Compared with psychrotrophs, psychrophiles have a narrower growth range, with some having an optimum growth temperature as low as 10°C. Since *L. monocytogenes* has got an optimum growth temperature of between 30 and 37°C (Seeliger and Jones, 1986), but is able to grow at temperatures as low as -0.4°C (Walker *et al.*, 1990) this organism can be considered as psychrotrophic.

The psychrotrophic nature of *L. monocytogenes* has been characterised by a number of workers. Walker *et al.* (1990), observed that the minimum growth temperature for *L. monocytogenes* ranged from -0.1 to -0.4°C. This value is 1 to 1.5°C lower than the minimum (1°C) previously reported by Seeliger and Jones (1986). It is possible that *L. monocytogenes* can still grow at lower temperatures then -0.4°C, however, this could not be checked since the model growth media used froze (Walker *et al.*, 1990). These authors also observed that the type of growth medium used during chilled incubation affected the ability of the organism to grow. These results may reflect the differing nutritional and/or inhibitory properties of the media (Walker *et al.*, 1990). In addition, Walker *et al.* (1990), demonstrated that incubation of cultures at 4°C before inoculation caused a marked reduction in the lag time at very low growth temperature (<2°C) when compared with cultures which had been previously incubated at 30°C. These findings were also reported by Buchanan and Klawitter (1991). These results suggest that the microorganism may be adapted to growth at low temperatures before contamination of food occurs. In the large outbreak of listeriosis in Canada, during 1981, in which the consumption of contaminated coleslaw was implicated it was suggested that the source of contamination was the cabbage which had been subjected to prolonged cold storage (Schlech *et al.*, 1983). Cold storage may have allowed *L. monocytogenes* to proliferate by cold enrichment. These factors need to be taken into account when designing models for the prediction of bacterial growth and survival on foodstuffs.

Survival of *L. monocytogenes* at low pH can be affected by the temperature of incubation. George *et al.* (1988), observed that the minimum pH values at which growth was detected at 30, 20, 10, 7 and 4°C were, respectively, 4.39, 4.39, 4.62, 4.62 and 5.23. Similar results were found by Farber *et al.* (1989) and McClure *et al.* (1989). In contrast, Cole *et al.* (1990) observed that the minimum pH that permitted survival of *L. monocytogenes* was 4.66 at 30°C , 4.36 at 10°C and 4.19 at 5°C. The contradiction of results may be due to the use of different acidulants. Farber *et al.* (1989) had already suggested that the nature of the acidulant can greatly effect the growth of *L. monocytogenes* at low pH. Recently, Patchett *et al.* (1996) reported that growth conditions may affect *L. monocytogenes* susceptibility to environmental stress. These authors observed that growth temperature and growth rate had effects on the susceptibility of *L. monocytogenes* to acid stress conditions. In this report cells grown at 30°C were more resistant to acid stress at pH 2.5 than cells grown at the same growth rates at 10°C. Cells grown at low growth rate at 30°C gave greater resistance to acid stress than those grown at high growth rate (Patchett *et al.*, 1996). Knowledge of the

ability of *L. monocytogenes* to grow at low temperature under low pH is particularly important when a reduction in pH is used to control the growth of this pathogen in cold stored food.

The survival of *L. monocytogenes* at high salt concentration was also strongly temperature dependent. Cole *et al.* (1990), observed that at low temperatures (5°C, 10°C) *L. monocytogenes* survives high salt concentration for longer than at 30°C. McClure *et al.* (1989), also revealed that *L. monocytogenes* survived longer at 5°C than at 30°C in 5% (w/v) NaCl. These authors observed that *L. monocytogenes* was able of growing in 10% (w/v) NaCl at 30°C and 35°C, which agrees with the findings of Seeliger (1961). Cole *et al.* (1990) also observed that salt concentration of 4 - 6% (w/v) provided a slight protective effect against inactivation of *L. monocytogenes* at low pH values. Once again, these findings may have implications for cold stored foods, such as soft cheeses, which are commonly treated with brine leading to high salt concentrations.

Listeria monocytogenes accumulates glycine-betaine, proline, carnitine and peptides when grown under osmotic stress (see section on osmotic stress; Patchett *et al.*, 1992; 1994; Beumer *et al.*, 1994; Ko *et al.*, 1994; Amezaga *et al.*, 1995; Verheul *et al.*, 1995a; Smith, 1996; Gerhardt *et al.*, 1996). However, glycine-betaine and carnitine, albeit in a less effective way, were also shown to confer chill tolerance to the pathogen (Ko *et al.*, 1994; Smith, 1996). Ko *et al.* (1994), observed that the transport of glycine-betaine is stimulated by cold with maximal stimulation occurring at 7°C, at which temperature the rate was about 15-fold higher than that observed at 30°C. In addition, protein synthesis was not required for transport. In contrast to the glycine-betaine uptake which increases at low temperature (Ko *et al.*, 1994), the rate of carnitine uptake does not, and was less effective in enhancing chill tolerance (Smith, 1996). Interestingly, *L. monocytogenes* exhibited a preference for glycine-betaine over carnitine either at 7°C or 30°C. This preference, at low temperatures, may be due to the kinetics of transport, since the uptake rate for glycine-betaine is several-fold higher than that for carnitine (Ko *et al.*, 1994; Verheul *et al.*, 1995b). The mechanism by which glycine-betaine acts as a cryoprotectant is not known, however, Ko *et al.* (1994) suggested that as in osmotic stress, glycine-betaine may function as a stabilising agent in preventing aggregation and maintaining the solubility of cellular proteins or to alter the physical properties of the cell membrane. This mechanism may be related with changes in hydrophobic interactions among membrane proteins or between the membrane and proteins, since the hydrophobic interactions are weakened at low temperature (Ko *et al.*, 1994).

The effect of low temperature on the pathogenicity of *L. monocytogenes* is of particular interest with regard to the food safety, due to the extreme length of modern cold chains and the extended chilled storage life of foods provided by vacuum or carbon dioxide packaging (Buncic *et al.*, 1996). Early studies on virulence indicated that *L. monocytogenes* grown at refrigerated temperatures resulted in increased virulence (Gray and Killinger, 1966; Wood and Woodbine, 1977). The results presented in these reports were fragmentary and equivocal. To resolve these questions of the effect of growth temperature on the virulence of *L. monocytogenes*, mouse models of infection have been used in recent experiments (Czuprynski *et al.*, 1989; Stephens *et al.*, 1991). *Listeria monocytogenes* grown at 4°C was found to significantly increase their virulence for intravenously infected mice (Czuprynski *et al.*, 1989; Stephens *et al.*, 1991). However, virulence was apparent only at a dose of about or above 10^4 viable listerias (Stephens *et al.*, 1991). No increase in virulence was observed when cold incubated *L. monocytogenes* was administered orally to mice (Czuprynski *et al.*, 1989; Stephens *et al.*, 1991). These results suggest that in intragastric infection, external growth temperature may not have a significant effect on the virulence of *L. monocytogenes*. However, it must be taken in account that it is very difficult to infect mice orally and that, consequently, losses of the given dose may happen. Buncic and Avery (1996), reported that storage at 4°C for 4 weeks decreased the pathogenicity of non-growing *L. monocytogenes* cells for 14-day-old chick embryos, but effects were strain-, pH- and substrate-dependent. Interestingly, prolonged storage at 4°C decreased the production of listeriolysin O (Buncic and Avery, 1996; Buncic *et al.*, 1996). Czuprynski *et al.* (1989), also observed that listeriolysin O (LLO) production was much higher in cells grown at 37°C rather than 4°C. A reduction in the expression of LLO at lower growth temperatures (26°C when compared to 37°C) was also reported by Datta and Kothary (1993). Brackett and Beuchat (1990), and Myers and Martin (1994), have found no significant difference in the pathogenicity of *L. monocytogenes* strains grown at 4 - 5°C and at 37°C. These results suggest, that the amount of LLO released may not reflect the temperature-induced differences in virulence. Work performed at the molecular level have demonstrated that transcription of *L. monocytogenes* virulence genes is repressed at low temperature (20 to 25°C) (Leimeister-Wächter *et al.*, 1992; Dramsi *et al.*, 1993; Renzoni *et al.*, 1997). Renzoni *et al.* (1997), demonstrated that at low temperature PrfA, the pleiotropic transcriptional activator of virulence genes, was undetected. Thus, temperature modulates the levels of PrfA and hence virulence gene expression (Rezoni *et al.*, 1997).

Knowledge of how *L. monocytogenes* is able to sustain growth at low temperature is poorly understood. In general the mechanisms of thermal adaptation of *L. monocytogenes* and other microorganisms appear to involve changes in membrane lipids and fatty acids, variation in cellular enzymes and the induction of cold-shock proteins. The first two types of variation will be discussed bellow, the cold-shock proteins will be considered in great detail under the section on cold-shock response (Section 1.10.2).

Herbert (1989), suggested that bacterial membranes play a central role in the adaptation of microorganism to low temperatures. Membranes are important in the mediation of cellular activity by acting as a barrier to electrolyte diffusion, while remaining as solvents for a variety of membrane constituents (i.e. enzymes and proteins involved in transport systems). Early studies suggested that changes in fatty acid composition of membrane polar lipids may enable maintenance of membrane fluidity over a range of temperatures (Herbert, 1989; Russell 1990; Gounot, 1991). In a number of psychrophilic or psychrotrophic bacteria grown at low temperature, membrane fluidity is maintained by an increase in the proportion of unsaturated fatty-acyl residues (e.g. *Bacillus*, some *Vibrio*, *Pseudomonas* and *Brevibacterium* spp.) (Herbert, 1986; Russell, 1990). In psychrophilic *Vibrio* sp. there are no growth temperature-dependent changes in fatty-acid unsaturation, but there is a relative decrease in fatty-acyl chain length (Russell, 1989; Herbert, 1990). A variation in branched chain fatty acids was also observed in *Bacillus* (Kaneda, 1977; Russell, 1990). The effect of these raised amounts of unsaturated and shorter chain fatty acids is a reduction in the gel liquid-crystalline transition temperature (Russell, 1989).

Studies on the effect of temperature on the fatty-acid composition of *L. monocytogenes* revealed a progressive decrease in the levels of *anteiso*- 17:0 at temperatures below 30°C, accompanied by a small rise in *anteiso*- 15:0 (Puttmann *et al.*, 1993; Annous *et al.*, 1995). Recently, Jones *et al.* (1997), also found that when *L. monocytogenes* was grown in continuous culture at 10°C a lower proportion of *anteiso*- 17:0 and a higher proportion of *anteiso*- 15:0 was exhibited when compared to when grown at 30°C. These results suggests that *L. monocytogenes* may be able to maintain membrane function at low temperatures by a general reduction in the length of its fatty acids (Jones *et al.*, 1997) rather than by synthesising unsaturated fatty-acids.

How low temperature effects cellular enzymes is not very well documented. At 0°C and lower temperature, psychrophiles and psychrotrophs can, in general, exhibit all of the

biochemical activities evident at higher temperatures, but at considerably reduced rate (Margesin and Schinner, 1994). They show a slower metabolic rate than mesophiles (Jay, 1986). In cold-adapted microorganisms the effect of low temperature at the level of enzymes may be compensated. This compensation involves changes of the produced enzyme types as well as at the level of enzyme activity and changes in enzyme concentration (Margesin and Schinner, 1994). It has been shown that proteins of cold-adapted species are not prone to cold denaturation and enzymes have higher catalytic efficiencies than those from warm adapted species. Moreover, not only enzyme activity, but also the level of enzyme secretion was enhanced at low temperatures (Feller *et al.*, 1990; Gügi *et al.*, 1991; Margesin and Schinner, 1992). Margesin and Schinner (1994), suggested that formation of more enzyme at low temperatures may compensate for the slow rate of enzymatic activity and may ensure a high substrate utilisation in the cold environment.

1.9 PATHOGENESIS

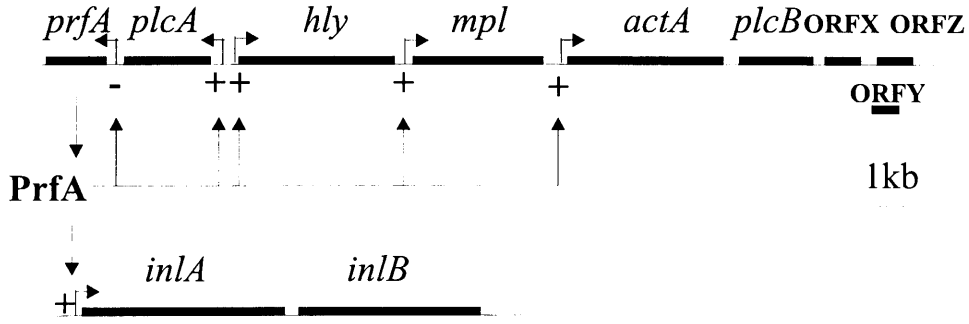
Knowledge of the pathogenesis of listeriosis remains imperfect. Early studies of Mackaness (1962) and subsequent work (see Portnoy *et al.*, 1992) demonstrated that initial survival within macrophages and other mammalian cells is essential for successful infection with *L. monocytogenes*. These findings have formed the basis for much research into T-cell mediated immunity, for which *L. monocytogenes* infection has become an accepted model system. The murine model of listeriosis provides a very reproducible system for the quantification of *L. monocytogenes* virulence (Portnoy et al., 1992; Kuhn and Goebel, 1995). After intraperitoneal injection into the mouse, virulent *L. monocytogenes* are rapidly phagocytosed (within minutes) by resident macrophages. Rapid killing of the ingested bacteria by the macrophage follows and the number of viable listeriae is greatly reduced during the first 6 hours. Surviving bacteria start replicating inside macrophages reaching a maximum, in the liver and spleen, 2-3 days after infection. This, is followed by the rapid inactivation of the bacteria mediated mainly by cytotoxic and helper T-cells over the next days, leading to the recovery of the infected animal (Mitsuyama *et al.*, 1978; Audurier *et al.*, 1980; for review see Sheehan *et al.*, 1994; Kuhn and Goebel, 1995).

With the tissue culture models of infection, the cell biology of *L. monocytogenes* intracellular infection has been characterised at the morphological level (Gaillard *et al.*, 1987; Tilney and Portnoy, 1989; Dabiri *et al.*, 1990; Mounier *et al.*, 1990, Tilney *et al.*,

1990, for reviews see Portnoy *et al.*, 1992; Sheehan *et al.*, 1994; Kuhn and Goebel, 1995) and is described as follows. Host cell infection begins with the internalisation of the bacteria either by phagocytosis, in the case of macrophages, or by induced phagocytosis, in the case of nonphagocytic cells. The bacteria are rapidly incorporated into a membrane-bound vacuole, which is subsequently lysed allowing the escape into the cytoplasm. There they start to replicate. Shortly thereafter, *L. monocytogenes* induces the nucleation of host actin filaments which form a cloud around the bacterial cell. The actin filaments are then rearranged into a polar tail which mediates bacterial movement through the cytoplasm to the cell periphery. It is assumed that the formation of the actin tail at one pole of the bacterial cell is the propulsive force which moves the bacteria through the cytoplasm. When moving bacteria contact the plasma membrane they induce the formation of pseudopod-like protrusions of the membrane. These pseudopods are apparently recognised by the neighbouring cell and phagocytosed, resulting in bacteria surrounded by a double membrane vacuole which is subsequently lysed to release the bacteria into the cytoplasm of the new host cell.

The application of gene cloning techniques and transposon mutagenesis has allowed the identification and characterisation of the bacterial factors required at each stage of the intracellular infectious process, at the molecular level. Most of the known virulence genes of *L. monocytogenes* are clustered on the chromosome in the so-called *prfA*-dependent virulence gene cluster. The cluster comprises six well-characterised genes, *prfA* (encodes a positive regulatory factor), *plcA* (encodes a phosphotidylinositol-specific phospholipase C), *hly* (encodes the haemolysin, listeriolysin O), *mpl* (encodes a metalloprotease), *actA* (encodes a surface protein necessary for actin assembly), and *plcB* (encodes a lecithinase) and three small open reading frames of unknown functions downstream of *plcB*, called ORF-X, -Y and -Z (Figure 1.9.1). These, and other genes which are also involved in the virulence of *L. monocytogenes,* will be discussed in the next sections.

Figure 1.9.1 - *Listeria monocytogenes* listeriolysin gene (*hly*), the two adjacent operons: the *plcA-prfA* operon and the lecithinase operon, and the internalin operon. *prfA* encodes a positive regulatory factor, *plcA* encodes a phosphatidylinositol-specific phospholipase C, *mpl* encodes a metalloprotease, *actA* encodes a surface protein necessary for actin assembly, *plcB* encodes a lecithinase, *inlA* encodes internalin a surface protein and *inlB* encodes a surface-bound protein with homology to internalin. ORFX, -Y and -Z are three open reading frames of unknown function. Arrows represent the co-ordinate regulation of virulence gene expression by PrfA. *Adapted* from Portnoy *et al.* (1992) and Sheehan *et al.* (1994).


prfA
plcA
hly
mpl
actA
plcB
ORFX
ORFZ
ORFY
-
++
+
+
PrfA
1kb
inlA
inlB
+

1.9.1 *hly* - Listeriolysin O

Listeriolysin O (LLO) is the best characterised determinant of *L. monocytogenes* pathogenesis. It is a member of a family of sulfhydryl-activated pore-forming cytolysins of which streptolysin O is the prototype (Smyth and Duncan, 1978). The role of LLO was studied by isolating nonhemolytic transposon mutants which were completely avirulent in a mouse model (Gaillard *et al.*, 1986; Kathariou *et al.*, 1987; Portnoy *et al.*, 1988; Cossart *et al.*, 1989). Virulence was restored in haemolytic revertants, which have lost the transposon insertion, or by the introduction of the cloned structural gene, *hly*, into a nonhaemolytic *L. monocytogenes* transposon mutant (Cossart *et al.*, 1989). Despite the clear correlation between haemolysis and virulence, the level of haemolysis production *in vitro* is not directly proportional to the virulence of the producing strains in the mouse (Kathariou *et al.*, 1988). Electron microscopy of infected macrophages and epithelial cells revealed that the nonhaemolytic *L. monocytogenes* mutants are unable to escape from the phagosome into the cytoplasm of the host cells (Gaillard *et al.*, 1987; Tilney and Portnoy, 1989). This strongly suggests that the role of LLO is to lyse the host vacuole membrane.

Further evidence for listeriolysin being essential for lysis of the phagosomal membrane and the intracellular growth was obtained by infection of macrophages with a *Bacillus subtilis* strain expressing listeriolysin (Bielecki *et al.*, 1990). The listeriolysin-producing *Bacillus* strain escaped from the phagosome into the cytoplasm and could even replicate in this compartment of the host cell, whereas the nonhaemolytic *B. subtilis* parental strain stayed in the phagosome. Interestingly, nonhaemolytic *L. monocytogenes* mutants were able to replicate in the human epithelial cell line Henle 407 (Portnoy *et al.*, 1988; Marquis *et al.*, 1995), suggesting that LLO is probably not essential for vacuolar lysis in certain cell types and that other gene products may be capable of mediating escape from a vacuole. This aspect will be discussed below.

1.9.2 *plcA* - Phosphotidylinositol-specific phospholipase C

Adjacent to *hly* and transcribed divergently is a gene which encodes a phosphatidylnositol-specific phospholipase C (PI-PLC) (Figure 1.9.1) (Sun *et al.*, 1990; Camilli *et al.*, 1991; Leimeister-Wachter *et al.*, 1991; Mengaud *et al.*, 1991). *plcA* was identified by both DNA sequencing and subsequent amino acid homology analysis and during a screen for *L. monocytogenes* mutants which formed small plaques in monolayers of mouse fibroblasts

(Sun *et al.*, 1990). Multiple assays gave evidence that secreted PI-PLC activity is encoded by the *plcA,* and that PI-PLC hydrolyses both PI and PI-glycan (Sun *et al.*, 1990). Early studies on the assessment of the role of PI-PLC in the intracellular life cycle of *L. monocytogenes*, suffered from the polar effect which the transposon insertion in *plcA* exerted on the downstream regulatory gene *prfA* with which *plcA* forms an operon (Mengaud *et al.*, 1991). The *prfA* gene encodes a transcriptional activator necessary for the transcription of all genes in the virulence gene cluster (see Section 1.9.6). In order to assess the precise role of *plcA* in the pathogenesis of *L. monocytogenes*, in-frame deletions within the *plcA* gene were performed (Camilli *et al.*, 1993). These, have shown that PI-PLC is required for the efficient escape of *L. monocytogenes* from the phagosome of mouse bone marrow-derived macrophages. However, the mutation in *plcA* had only a slight effect on virulence (Camilli *et al.*, 1993). It was postulated that PI-PLC acts in concert with LLO to mediate lysis of the vacuolar membrane.

To determine what effect *plcA* would have on intracellular survival, this gene was cloned and expressed in *Listeria innocua*, which lacks the *prfA*-dependent virulence gene cluster and is, therefore, unable to escape from the host cell vacuole (Schawn *et al.*, 1994). The PI-PLC-expressing *L. innocua* strain could not escape from the phagosome of J774 macrophages, but, exhibited limited intracellular growth inside the vacuoles which appear to be structurally intact (Schawn *et al.*, 1994). These results suggest that PI-PLC alone can not lyse the phagosomal membrane but seems to affect the vacuole in a way which alters its functions but not its integrity.

To study the potential co-operative interactions of *L. monocytogenes* PI-PLC and LLO in the lysis of the vacuolar membrane, a double internal in-frame deletion mutant was constructed (Marquis *et al.*, 1995). It was observed that the efficiency of bacterial escape from the primary vacuole, in Henle 407 cells which allowed intracellular growth of non-haemolytic bacteria, was reduced in this double mutant (Marquis *et al.*, 1995). These observations support a postulated accessory role for PI-PLC with LLO in lysing the primary phagosome of a macrophage.

1.9.3 *plcB* - Phosphotidylcholine-specific phospholipase C

Listeria monocytogenes also produces a second phospholipase C which hydrolyses phosphotidylcholine (lecithin), and is thus a phosphotidylcholine-specific phospholipase C

(PC-PLC) or lecithinase (Leighton *et al.*, 1975; Geoffroy *et al.*, 1991). Strains of *L. monocytogenes* which express high amounts of lecithinase activity produce a very dense zone of opacity surrounding the colony on egg yolk agar (Fuzi and Pillis, 1962). The purified protein exhibits a weak haemolytic activity but is not toxic in mice (Geoffroy *et al.*, 1991). The gene, *plcB*, encoding PC-PLC, is part of an operon which includes the genes *mpl*, *actA* and *plcB* (Figure 1.9.1) (Vazquez-Boland *et al.*, 1992). In contrast to PI-PLC, PC-PLC is secreted as an inactive pre-enzyme (proPC-PLC) whose activation is mediated *in vitro* by the metalloprotease of *L. monocytogenes* (see Section 1.9.4) (Raveneau *et al.*, 1992; Poyart *et al.*, 1993).

In order to analyse the function of PC-PLC in the intracellular life cycle of *L. monocytogenes*, *plcB* transposon mutants have been constructed (Vazquez-Boland *et al.*, 1992). These mutants were fully invasive, replicating and moving inside host cells as the wild type strain. However, *plcB* mutants produce only small plaques on fibroblast monolayers, suggesting that the cell-to-cell spread is impaired (Vazquez-Boland *et al.*, 1992). Electron microscopic analysis of the infected macrophages showed the bacteria trapped in vacuoles surrounded by a double membrane, indicating that lecithinase is involved in the lysis of the two-membrane vacuoles that surrounded the bacteria after cell-to-cell spread (Vazquez-Boland *et al.*, 1992).

1.9.4 *mpl* - Metalloprotease

The gene *mpl*, which is the first gene of the lecithinase operon (Vazquez-Boland *et al.*, 1992), is located immediately downstream of the *hly* gene (Figure 1.9.1) (Domann *et al.*, 1991; Mengaud *et al.*, 1991) and encodes a zinc-dependent metalloprotease. The role of this metalloprotease in pathogenecity and intracellular replication of *L. monocytogenes* is still not very well characterised. Transposon mutants in the *mpl* gene were found to have reduced virulence in mouse models but the uptake and the intracellular growth *in vitro* was not affected (Raveneau *et al.*, 1992). Raveneau and colleagues (1992) also observed that *mpl* transposon mutants also lost PC-PLC activity. These results suggested that the zinc metalloprotease of *L. monocytogenes* might play a role in the maturation of PC-PLC. The reduce virulence observed in mouse models was attributed to the lack of proteolytic processing of the PC-PLC proform (Poyart *et al.*, 1993; Sokolovic *et al.*, 1993). However, more recent studies showed mutants with in-frame deletions in *mpl*, also impaired in PC-

PLC maturation and *actA* degradation (see Section 1.9.5), are as virulent as the isogenic wild type strain when infected intraperitoneally into the mouse (Kuhn and Goebel, 1995).

1.9.5 *actA* - Actin assembly

Listeria monocytogenes is capable of directed movement within the cytoplasm of the infected host cells, propulsion is thought to be driven by actin polymerisation at the bacterial cell surface (Sanger *et al.*, 1992). *Listeria monocytogenes* mutants defective in intracellular motility were produced by transposon mutagenesis. These mutants had either lost the ability to initiate actin polymerisation (Sun *et al.*, 1990), or could still induce actin polymerisation but were unable to rearrange the actin filaments to actin tails (Kuhn *et al.*, 1990). *actA* the second gene of the lecithinase operon (Figure 1.9.1) (Vazquez-Boland *et al.*, 1992), which encodes the ActA protein, was shown to be essential for actin polymerisation. The ActA protein has a transport signal sequence (Kocks *et al.*, 1992) and represents a surface protein anchored to the bacterial membrane by a putative C-terminal membrane anchor (Domann *et al.*, 1992; Kocks *et al.*, 1992). The protein carries several repeats, in its central part, with homology to vinculin (Domann *et al.*, 1992). Mutations in the *actA* gene resulted in the loss of virulence in mice (Domann *et al.*, 1992), in the lack of intracellular actin polymerisation around the bacteria and in the inability of intracellular movement (Domann *et al.*, 1992; Kocks *et al.*, 1992). The distribution of the ActA protein on the bacterial surface is asymmetric and after bacterial cell division is concentrated at the original bacterial pole. Once ActA is present at the site of actin assembly it is suggested that ActA is involved in actin nucleation on the bacterial surface (Kocks *et al.*, 1993), however, the protein is not found within the actin tail (Neibuhr *et al.*, 1993).

The possible role of the ActA protein was demonstrated by expressing this protein in mammalian cells (Pistor *et al.*, 1994). This resulted in targeting of the protein to mitochondria, which subsequently recruited host-cell actin to these organelles, suggesting that ActA alone is sufficient to polymerise actin (Pistor *et al.*, 1994). Expressing ActA protein without its internal proline-rich repeats was also localised it in the mitochondrial membrane but did not induce polymerisation, suggesting that this domain is essential for actin nucleation (Pistor *et al.*, 1994). However, this type of approach can not address the role of ActA in the actin-driven bacterial propulsion, thus Lasa *et al.* (1995) generated several *L. monocytogenes* strains expressing different domains of ActA and analysed the ability of these domains to trigger actin assembly and bacterial movement in both infected

cells and cytoplasmic extracts. This experiments revealed that the amino-terminal part is critical for actin assembly and movement (Lasa *et al.*, 1995; 1997). Recently, Welch *et al.* (1997), purified an eight-polypeptide complex that possessed the properties of the host-cell actin polymerisation factor. The pure complex was sufficient to initiate ActA-dependent actin polymerisation at the surface of *L. monocytogenes*, and was required to mediate actin tail formation and motility (Welch *et al.*, 1997). The activity and subunit composition of this complex suggest that it forms a template that nucleates actin polymerisation.

1.9.6 *prfA* - Positive regulating factor A

A spontaneous nonhaemolytic mutant of *L. monocytogenes* was shown to have a deletion in a region downstream to *plcA* (Figure 1.9.1) (Gormley *et al.*, 1989; Leimeister-Wächter *et al.*, 1989). Cloning and sequencing of the region affected by the deletion led to the identification of the *prfA* (positive regulating factor A) gene (Leimeister-Wächter *et al.*, 1990; Mengaud *et al.*, 1991b), which encodes the cytoplasmic protein PrfA with homology to the Crp-Fnr family of transcriptional activators (Sheehan *et al.*, 1996). These mutants expressed low levels of *hly* mRNA, suggesting that *prfA* was a positive regulatory factor for *hly*. Complementation of this mutation with a recombinant plasmid expressing PrfA increased not only hly transcription (Leimeister-Wächter *et al.*, 1990) but also that of *plcA*, *mpl*, and *plcB* (Mengaud *et al.*, 1991). Site-specific mutations or transposon insertions in the *prfA* promoter or in the *prfA* coding region blocked the transcription of the entire gene cluster, i.e. *plcA*, *hly*, *mpl*, *actA*, and *plcB* (Mengaud *et al.*, 1991b; Chakraborty *et al.*, 1992; Domann *et al.*, 1992), demonstrating that the *prfA* gene encodes a transcriptional activator required for the expression of the *L. monocytogenes* virulence gene cluster. In addition, the role of PrfA was also indicated by the transcriptional activation of the *hly* gene by PrfA in *B. subtilis* (Freitag *et al.*, 1992). Most recently, *prfA* has been shown to be required for expression of the invasion-associated, *inlAB* locus (see Section 1.9.7) (Dramsi *et al.*, 1993).

The PrfA protein seems to recognise a 14-bp palindromic sequence found in the -35 region of the promoters for *hly*, *plcA*, and *mpl* (Mengaud *et al.*, 1989), suggesting that this palindrome may be the target site for PrfA-mediated activation. Indeed, a single amino acid substitution in the putative helix-turn-helix motif of PrfA reduced DNA binding and virulence-gene activation, and attenuated the virulence of *L. monocytogenes* in a mouse model of infection (Sheehan *et al.*, 1996). A hierarchy in the *prfA*-regulated promoters has been shown (Sheehan *et al.*, 1995). Activation by PrfA is more efficient at promoters which

posses a perfect 14-bp palindromic PrfA box than at promoters which have 1- or 2-bp substitutions in the PrfA box. *prfA* lies downstream from, and is cotranscribed with *plcA* (Figure 1.9.1). It has been shown that *prfA* can be transcribed from three different promoters, i.e., the *plcA* promoter and the P1 and P2 *prfA*-specific promoters located in the intragenic region between *prfA* and *plcA*, producing a 2.1-kb bicistronic *plcA-prfA* transcript and 0.9- and 0.8-kb monocistronic *prfA* transcripts, respectively. Since PrfA activates transcription from the *plcA* promoter it can positively regulate its own expression (Mengaud *et al.*, 1991).

Virulence determinants of many pathogenic bacteria are subjected to environmental modulation and similar strategies may be employed by *Listeria* to optimise gene expression within the host. Temperature influences the expression of the virulence genes in *L. monocytogenes*. Under heat-shock conditions the synthesis of listeriolysin (and possibly other PrfA-controlled proteins) is enhanced (Sokolovic and Goebel, 1989; Sokolovic *et al.*, 1990). At lower temperature (20°C), there is little or no transcription of PrfA-regulated genes although synthesis of the monocistronic *prfA* transcript(s) is still detectable (Leimeister-Wächter *et al.*, 1992). A shift to 37°C results in an increase of transcription of all PrfA-controlled virulence genes of *L. monocytogenes* (Leimeister-Wächter *et al.*, 1992).

A negative effector of virulence gene expression in *L. monocytogenes* was described by Park and Kroll (1993). These authors observed that the expression of *hly* and *plcA* was repressed by the plant-derived disaccharide, cellobiose. Cellobiose is derived from the degradation of cellulose and it is probably present in large amounts in the soil where *L. monocytogenes* can grow saprophytically. Cellobiose may thus act to repress virulence gene expression in the saprophytic environment (Sheehan *et al.*, 1994). Whereas temperature modulates the absolute amounts of the activator protein PrfA, cellobiose does not affect the amount of PrfA, on the contrary, PrfA is fully expressed in the presence of cellobiose (Rezoni *et al.*, 1997). These results strongly suggest that virulence gene activation depends on both the presence of PrfA and an additional regulatory pathway that either modifies PrfA or acts synergistically with PrfA (Rezoni *et al.*, 1997). These results are in accordance with Böckmann *et al.* (1996), which demonstrated that PrfA-mediated activation requires the presence of a coactivator which probably is a protein.

1.9.7 Internalin family

Listeria monocytogenes transposon mutants unable to invade culture epithelial cells resulted from a transposon insertion into a chromosomal region which represents an operon consisting of the *inlA* and *inlB* genes (Figure 1.9.1) (Gaillard *et al.*, 1991). *inlA* encodes, internalin (InlA), a surface protein (Gaillard *et al.*, 1991) and *inlB* encodes InlB also a surface-bound protein with homology to internalin (Dramsi *et al.*, 1995). Expression of *inlA* in *L. innocua*, a non-invasive *Listeria* species, confers on this species the ability to invade epithelial cells, suggesting that the *inlA* gene product is crucial for invasion into epithelial cells (Gaillard *et al.*, 1991; Dramsi *et al.*, 1995). To assess the role of *inlB* in invasiveness of *L. monocytogenes*, isogenic chromosomal deletion mutants in the *inlAB* locus were constructed (Dramsi *et al.*, 1995). It was found that *inlB* is required for entry of *L. monocytogenes* into hepatocytes, but not into intestinal epithelial cells and that internalin plays a role in entry into some hepatocyte cell lines. *In vivo* studies using the mouse infection model revealed that both internalin mutants were attenuated for virulence (Lingnau *et al.*, 1995).

The mechanism by which internalin mediates adhesion and invasion of *L. monocytogenes* into nonphagocytotic cells has only recently been revealed. By an affinity chromatography approach E-cadherin was identified as the cell-surface receptor for internalin (Mengaud *et al.*, 1996). By immunofluorescence and immunogold labelling internalin was shown to be present on the cell surface of *L. monocytogenes* in a polarised distribution similar to that of ActA (Lebrun *et al.*, 1996). Via genetic analysis these authors established that the C-terminal region of internalin is essential for cell-surface association and that its presence on the bacterial surface is necessary to promote entry. These results suggest that internalin on the bacterial surface mediates direct contact between the bacterium and the host cell.

Until recently nothing was known about how InlB is anchored to the *L. monocytogenes* surface. A region of 80-amino-acid containing repeats beginning with the sequence GW was shown to constitute the anchoring region (Braun *et al.*, 1997). Furthermore, addition of GW repeats to the C-terminal of InlB improved anchoring of the protein to the cell surface (Braun *et al.*, 1997). These results suggest that such "GW" repeats may constitute a novel motif for cell-surface anchoring in *Listeria* and other Gram-positive bacteria.

Internalin C a secreted protein with homology to InlA and InlB was identified by Engelbrecht *et al.* (1996). The gene, *inlC*, is strongly transcribed by *L. monocytogenes* in the cytoplasm of phagocytic J774 cells whereas *inlA* is poorly transcribed under these conditions (Engelbrecht *et al.*, 1996), suggesting that internalin C may play a role in a late stage of *L. monocytogenes* infection of cells. An *inlC* mutant exhibited reduced virulence in a mouse model, however, intracellular replication of this mutant in tissue culture cells was comparable with that of the wild type (Engelbrecht *et al.*, 1996). Other investigators identified and characterised the same protein with the same results, however, the gene was called *irp*A for internalin-related protein A (Domann *et al.*, 1997). By Southern hybridisation four more new members of the internalin family were identified (Dramsi *et al.*, 1997). These genes were named *inlC2*, *inlD*, *inlE* and *inlF*. The first three genes are contiguous on the chromosome of *L. monocytogenes* (Dramsi *et al.*, 1997). The *inlC2*, *inlD*, *inlE* and *inlF* null mutants were not affected for entry into various nonphagocytic cell (Dramsi *et al.*, 1997), suggesting that the role of these genes is other than invasion.

1.9.8 *iap* - Protein p60

Spontaneously occurring rough mutants of *L. monocytogenes* showed significantly reduced uptake by 3T6 fibroblast cells and reduced expression of a 60 kD extracellular protein, protein p60 (Kuhn and Goebel, 1989). These p60 mutants form long cell chains which possess double septa between the individual cells. Treatment with partially purified p60 protein deaggregates the cell chains to single bacteria which regain their ability to invade fibroblasts (Kuhn and Goebel, 1989; Bubert *et al.*, 1992). The reduced invasiveness of the p60 mutants is only observed with certain mammalian host cells (Bubert *et al.*, 1992). *Salmonella typhimurium*, expressing and secreting recombinant *L. monocytogenes* p60, seems to be more invasive for phagocytic cells but not for enterocytes (Hess *et al.*, 1995). Protein p60 is a major secreted protein of all *L. monocytogenes* isolates (Bubert *et al.*, 1992) but is also found on the cell surface of the bacterium (Khun and Goebel, 1995). The gene coding for this protein was termed *iap* for invasion associated protein (Köhler *et al.*, 1990). This gene could not be inactivated without a loss of cell viability, indicating that p60 is an essential housekeeping protein for *L. monocytogenes* (Wuenscher *et al.*, 1993). p60 possesses a murein hydrolase activity required for a late step in cell division (Wuenscher *et al.*, 1993). These authors also observed that p60 exhibited bacteriolytic activity. The N-terminal amino acid and multiple internal regions of p60 influence its stability in the cytosol

of the infected host cell (Sifts *et al.*, 1997). In contrast to the other virulence genes the *iap* gene is not under the control of transcriptional activator PrfA (Goebel *et al.*, 1993).

1.9.9 Other *L. monocytogenes* genes possibly involved in virulence

1.9.9.1 *lma* operon

The *lmaA* gene of *L. monocytogenes* encodes a protein capable of inducing delayed-type hypersensitivity reactions in *L. monocytogenes* immune mice (Gohmann *et al.*, 1990). The amino acid sequence of LmaA revealed a strong hydrophobic protein containing a putative transmembrane region at the N-terminal end suggesting that LmaA may represent an integral membrane protein (Gohmann *et al.*, 1990). Subsequent nucleotide sequence analysis has demonstrated that *lmaA* gene is part of an operon and is preceded by *lmaB* gene (Gohmann *et al.,* 1990). Recently, Schaferkordt and Chakraborty (1997) demonstrated that the *lma* operon comprises four genes, *lmaDCBA*. LmaA was found to be produced at 20 and 37°C, however it was secreted into culture supernatant only at 20°C. DNA hybridisation studies indicate that the *lma* operon is species specific being restricted to *L. monocytogenes* (Schaferkordt and Chakraborty, 1997). Since *lma* mutants are not yet available, the role of the different genes of the *lma* operon in virulence of *L. monocytogenes* is currently unknown.

1.9.9.2 Superoxide Dismutase and Catalase

Superoxide dismutase (SOD) and catalase act in concert to detoxify potentially harmful superoxide radicals. Superoxide radicals generated by the oxidative burst in a phagocytic cell are converted into hydrogen peroxide by the action of superoxide dismutase. Hydrogen peroxide is then cleaved by catalase into water and molecular oxygen (Haas and Brehm, 1993). Bacterial catalases and superoxide dismutase have long been suspected to be important virulence factors for intracellular bacteria since macrophages react upon ingestion of bacteria with greatly enhanced production of reactive oxygen intermediates. *Listeria monocytogenes* has a single SOD which utilises manganese as a metal cofactor (Vasconcelos and Deneer, 1994). Expression of superoxide dismutase was shown to vary with different environmental factors and to be dependent on different growth conditions (Vasconcelos and Deneer, 1994), but no correlation with virulence was found (Welch, 1987). The nucleotide sequence of the *L. monocytogenes* SOD gene, *lmsod*, showed close

similarity to manganese-containing SODs from other organisms (Brehm *et al.*, 1992). DNA/DNA-hybridisation experiments showed that the SOD gene is conserved within the genus *Listeria* (Brehm *et al.*, 1992). No difference in virulence or in the survival of the bacteria in the spleen and liver of the infected mice was observed between the *lmsod* mutant and the wild type strain (Kuhn and Goebel, 1995). The synthesis of SOD is not under the control of PrfA (Goebel *et al.*, 1993).

Catalase activity is a characteristic of almost all *L. monocytogenes* isolates albeit at different levels (Godfrey and Wilder, 1985; Dallmier and Martin, 1988; Swartz *et al.*, 1991; Bubert *et al.*, 1997). No correlation between virulence and catalase activity was found (Welch, 1987) and catalase-negative mutants obtained by transposon mutagenesis exhibited wild type virulence in a mouse model (Leblond-Francillard, *et al.*, 1989). Furthermore, isolation of catalase-negative *L. monocytogenes* strains from listeriosis patients (Swartz *et al.*, 1991; Bubert *et al.*, 1997) supports the notion that catalase does not seem to be necessary for the intracellular growth of *L. monocytogenes*. The catalase gene of *L. seeligeri* has been cloned and characterised (Haas *et al.*, 1991). The corresponding gene of *L. monocytogenes* has still not been isolated, however, the synthesis of catalase is not under the control of PrfA (Haas *et al.*, 1991).

1.10 MECHANISMS OF ADAPTATION TO ENVIRONMENTAL STRESS

Although *L. monocytogenes* may be subjected to several environmental stresses, only the two most relevant to this project are discussed in detail.

1.10.1 Adaptation of microorganisms to high osmolarity

One of the most widely ways used to preserve food products is by increasing the osmotic pressure. Desiccation or addition of high amounts of osmotically active substances, such as salts, lower the water activity in the food. Thus, understanding the processes by which microorganisms adapt to changes in the external osmolarity is of crucial importance in trying to design new ways for controlling growth of food-borne pathogens or spoilage bacteria in low and medium water activity foods (Gutierrez *et al.*, 1995).

The general mechanism of osmoadaptation can be summarised as follows. Under normal conditions the internal osmotic pressure in bacterial cells is higher than that of the surrounding environment. This results in pressure subjected outwards on the cell wall, named the turgor pressure (Csonka, 1989). A sudden increased in the osmolarity of the environment triggers a rapid efflux of water from the cell, resulting in a decrease in turgor. Changes in the external osmolarity must therefore be sensed by the microorganisms and this information must be converted into an adaptation process that aims at the restoration of turgor (Lucht and Bremer, 1994).

A universal response to the loss of turgor is accumulation of solutes in the cytoplasm, resulting in an increase in internal osmotic pressure. Non-ionic solutes are generally preferred since enzyme activity, DNA replication, protein synthesis and finally cell growth can be severely inhibited by the increase in the concentrations of ions. Thus, at high osmolarities bacteria have evolved to accumulate high cytoplasmic levels of a certain class of osmotically active solutes that can be accumulated to high intracellular concentrations without disturbing essential metabolic functions; the so called "compatible solutes" (Lucht and Bremer, 1994; Gutierrez *et al.*, 1995). These compatible solutes include polyols such as trehalose, amino acids such as proline, and methyl-amines such as glycine betaine (N, N, N-trimethylglycine). Glycine betaine is present in sugar beets and other foods of plant origin, whereas in certain food products increased amounts of amino acids and peptides are available as a consequence of proteolytic activity of bacteria present in this food (Verheul *et al.*, 1995a; 1995b).

The mechanisms of salt tolerance have been extensively studied in Gram-negative bacteria, and considerable information about the genetics and mechanisms of uptake of compatible solutes is available for *E. coli* and *S. typhimurium* (Csonka, 1989; Lucht and Bremer, 1994). Much less information is available for Gram-positive bacteria. Since the mechanisms of salt adaptation are so well documented for the enteric bacteria, these will be described, as a model system, in the following section, then the systems for Gram-positive *Bacillus subtilis* and *Listeria monocytogenes* will be discussed.

1.10.1.1 Osmoregulation in *Escherichia coli* and *Salmonella typhimurium*

The osmoadaptation process can be divided in two steps. First, high intracellular concentrations of K^+-glutamate are accumulated by increased K^+ uptake and concomitant *de novo* synthesis of counterions, such as glutamate. Second, the compatible solutes are accumulated by either synthesis or uptake from the environment. The accumulation of the compatible solutes then triggers an efflux of K^+ from the cytoplasm. Consequently, turgor is restored, and the cell can resume growth in high osmolarity environments (Csonka, 1989; Gutierrez *et al.*, 1995; Lucht and Bremer, 1994).

Turgor in *E. coli* and *S. typhimurium,* at the steady state, has been estimated in approximately 3-5 atm. When the osmolarity of the medium is increased, water is lost from the cells leading to a loss of turgor (Lucht and Bremer, 1994). The primary response to high-osmolarity stress is the controlled uptake of K^+ (Epstein, 1986; Dinnbier *et al.*, 1988). *Escherichia coli* has two active transport systems for K^+: Trk, which has a relatively low affinity for K^+ (K_m = 1.5 mM), and Kdp, which has a much higher affinity (K_m = 2 μM) (Dosh *et al.*, 1991). The former system is expressed constitutively, but its activity is increased in response to hyperosmotic shock. The expression of the Kdp structural genes responds to environmental signals and is also stimulated by hyperosmotic shock (Lucht and Bremer, 1994). To maintain the membrane potential, the increased intracellular K^+ concentration must be balanced by the accumulation of anions or the expulsion of cations. In *E. coli*, glutamate is the major counter-ion accumulated either by synthesis or transport from the environment. In high osmolarity environments the cytoplasmic levels of K^+-glutamate can reach 0.7 - 0.8 M in order to re-establish the osmotic pressure gradient across the cell. However these levels can have deleterious effects on cell physiology and can inhibit the functioning of key enzymes (Gutierrez *et al.*, 1995). Consequently, as a secondary response to high environmental osmolarity, the intracellular K^+ is replaced by compatible solutes also known as osmoprotectants.

In response to osmotic stress, trehalose has been found to be synthesised in the cytoplasm of a number of bacteria including *E. coli* (Csonka, 1989). Subsequently, a gradual release of potassium glutamate takes place until, at the steady state, there is a balance between potassium glutamate and trehalose (Dinnbier *et al.*, 1988). Mutations which result in an impairment in the accumulation of trehalose result in an increased sensitivity to osmotic stress (Giaever *et al.*, 1988). Expression of the genes for trehalose biosynthesis is stimulated

during the entry of the cells into stationary phase, and an alternative sigma factor, RpoS (σ^{38} or σ^{S}), the "stationary phase-sigma factor" is involved in the stationary phase and osmotic induction of these genes (Hengge-Aronis *et al.*, 1991). This central regulator is active in cells that have been subjected to a stress that slows the growth rate. The activity of σ^{S} is responsible for the induction of expression of genes and operons that potentiate survival of stress (Rees *et al.*, 1995).

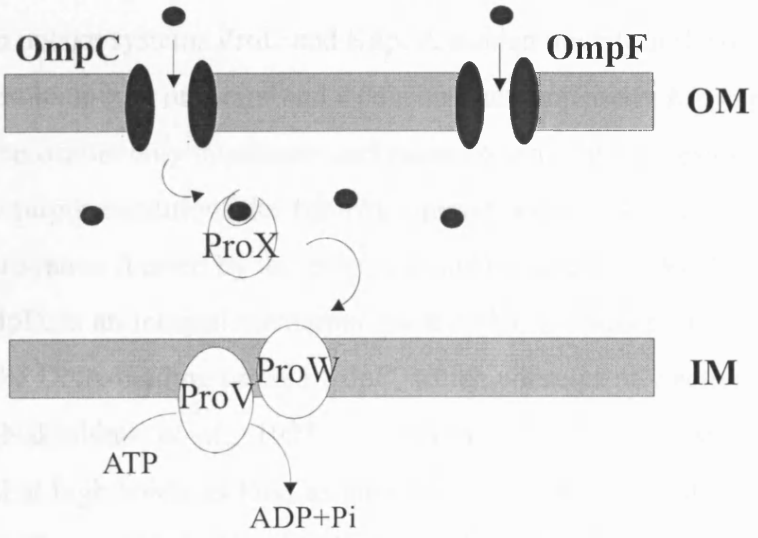
If glycine betaine or proline are available, the outcome of the adaptation process to high-osmolarity environments changes. After the onset of potassium and K^{+}-glutamate accumulation, the controlled accumulation of glycine betaine or proline from the environment takes place and the accumulated potassium may flow out of the cells as the compatible solutes accumulate (Sutherland *et al.*, 1986). *Escherichia coli* can not synthesise glycine betaine *de novo* but is able to accumulate it intracellularly when its precursor molecules, choline and glycine betaine aldehyde, are present in the growth medium (Lucht and Bremer, 1994). *Escherichia coli* possess an efficient transport system for scavenging these biosynthetic precursors from the environment, and their enzymatic conversion to glycine betaine allows the cells to grow in high-osmolarity environments (Lucht and Bremer, 1994).

Measures (1975) observed a large increase in the intracellular concentration of proline in the enteric bacteria *E. coli* and *S. typhimurium* when these were subjected to osmotic shock. The accumulation of this osmoprotectant cannot be accomplished by *de novo* synthesis, since the enzymes necessary for proline biosynthesis are subjected to feed-back inhibition by proline (Csonka, 1989). Both microorganisms have three independent proline transport systems: PutP, ProP and ProU. The PutP system is required for the transport of proline when this metabolite is used as a nutrient but it is not important for the transport of proline as an osmoprotectant (Csonka, 1989). The other two systems, ProP and ProU, are responsible for the intracellular accumulation of proline under hyperosmotic stress. These transport systems are also responsible for the uptake of other osmoprotectants (Cairney *et al.*, 1985a, 1985b). In "non-stressed" cells the ProP system is present but high osmolarity strongly stimulates its transcription. This system has a very low affinity (K_m = 0.3 mM) for proline. Nevertheless, for *S. typhimurium* it serves an important physiological function for osmoadaptation, since osmotically stimulated transport activity allows the cell to react rapidly to a sudden increase in medium osmolarity (Cairney *et al.*, 1985b).

The ProU system is present only after the imposition of osmotic stress. The level of expression of this system will reflect the availability of glycine betaine (and/or choline its precursor) in the environment, since the accumulation of this compatible solute represses expression of the *proU* operon (Sutherland *et al.*, 1986). ProU contributes little to total proline uptake but, this system from *E. coli* and *S. typhimurium* has a very high affinity for glycine betaine (K_m = 1.3 μM). ProU is a multicomponent, binding-protein-dependent transport system and in *E. coli* and *S. typhimurium* encodes three structural genes (*proV*, *proW* and *proX*) which are co-ordinately expressed under the control of one major osmoregulated promoter (Gowrishankar, 1989; Stirling *et al.*, 1989). The first gene of the operon, *proV*, encodes a 44.1 kDa hydrophilic polypeptide which is associated with the cytoplasmic membrane. The amino acid sequence of the ProV protein exhibited significant similarity to the energy-transducing components of other binding-protein-dependent transport systems (Gowrishankar, 1989). The following gene of the operon, *proW*, encodes a hydrophobic protein (37.6 kDa) that is integrated into the inner membrane (Gowrishankar, 1989). The third and last gene of the operon, *proX*, encodes the periplasmic glycine-betaine-binding protein of 33.8 kDa. The ProX protein of *E. coli* binds glycine-betaine with high affinity (K_m = 1 μm) (May *et al.*, 1986) and delivers it to a complex consisting of the ProV and ProW proteins. Together, these three proteins are responsible for the efficient translocation of glycine betaine across the cytoplasmic membrane (Figure 1.10.1.1.1). Like ProP, the ProU transport system also permits the uptake of osmoprotectants other than glycine betaine. Even though with low affinity, proline, taurine, ectoine and structural analogues of glycine betaine are also transported by the ProU system (Lucht and Bremer, 1994).

In *E. coli*, access of glycine betaine to the periplasmic glycine-binding protein (ProX) is provided by the integral outer membrane proteins OmpC and OmpF (Figure 1.10.1.1.1.1). These proteins constitute water-filled pores within the outer membrane through which low molecular weight compounds (≤600 Da) can diffuse (Lucht and Bremer, 1994).

Figure 1.10.1.1.1 - Model for the organisation of the binding protein-dependent glycine betaine transport system ProU in *E. coli*. The ProV protein is a ATP-binding subunit that couples ATP hydrolysis to the transport process. The ProW is a hydrophobic integral inner membrane (IM) protein. The substrate-binding protein of the transport system is in the periplasm (ProX). Permeation of glycine betaine (●) across the *E. coli* outer membrane (OM) occurs through the general diffusion pores OmpC and OmpF (Lucht and Bremer, 1994; Kempf and Bremer, 1995).


OmpC
OmpF
OM
ProX
IM
ProV
ProW
ATP
ADP+Pi

Escherichia coli and *S. typhimurium* also possess efflux systems for glycine betaine which are independent from the ProU and ProP uptake systems. This export system allows a rapid reduction of the intracellular concentration of the osmoprotectants in response to a reduction of the osmolarity in the environment and in order to preserve the normal osmotic pressure gradient across the cell membrane (Koo *et al.*, 1991; Lamark *et al.*, 1992). Lamark and colleagues (1992) observed that mutants that lack ProP and ProU are unable to accumulate glycine betaine derived from the oxidation of choline despite the fact that the oxidation takes place in the cytoplasm suggesting a very active efflux system against which the uptake process has to work.

1.10.1.1.1 Osmotic Regulation of *proU*

The molecular mechanism of osmotic induction of transcription has been best characterised for the two uptake systems ProU and Kdp. A sudden upshift in the osmolarity of the growth medium results in loss of turgor and a concomitant increase in *kdp* expression. Elevated *kdp* transcription occurs only transiently and ceases when turgor is restored by the uptake of K^+. Under low turgor conditions the *kdpABC* operon is expressed under the control of the two-component system formed by the proteins KdpD and KdpE (Walderhaug *et al.*, 1992). The sensor, KdpD, is an integral membrane protein that transduces the signal due to the loss of turgor to the DNA-binding protein KdpE, which consequently activates the transcription of *kdpABC* (Nakashima *et al.*, 1993). In contrast to the *kdp* system, *proU* expression is maintained at high levels as long as the osmotic stimulus persists. The presence of K^+ is a prerequisite for *proU* induction (Sutherland, 1986) and in an *in vitro* system, K^+-glutamate was identified as a signal stimulating *proU* expression. It is postulated that K^+-glutamate might stimulate *proU* transcription by facilitating a productive interaction between RNA polymerase and the *proU* promoter. K^+-glutamate is known to enhance the stability of protein-DNA interaction and to stimulate transcription in *in vitro* systems (Leirmo *et al.*, 1987).

Higgins and colleagues (1988; 1990) suggested an alternative model for osmoregulation of *proU* expression. These authors showed that high osmotic pressure results in an increased negative supercoiling of the DNA. They also showed that antibiotics that lower negative supercoiling also lower transcription of *proU*, suggesting that modulations of the DNA topology are involved in the osmotic regulation of transcription. In conditions of low osmolarity H-NS (an histone-like protein which binds DNA and it is believed to help

compaction in *E. coli*) inhibits *proU* transcription through binding to the *proV* site (Lucht *et al.*, 1994) and acting as a transcriptional road-block or by obstructing early steps of transcription initiation.

1.10.1.2 Osmoregulation in *Bacillus subtilis*

In its soil environment, *B. subtilis* often encounters osmotic challenges due to frequent variations in the availability of water. Exposure of *B. subtilis* to a hypersaline environment incites an integrated physiological adaptation reaction that is aimed at restoring the disturbed cellular water balance, maintaining optimal turgor, and protecting cell components from the detrimental effects of high ionic strength (Whatmore *et al.*, 1990; Whatmore and Reed, 1990).

Initially, large amounts of K^+ are accumulated after an osmotic upshock via turgor-sensitive transport systems (Whatmore *et al.*, 1990). Subsequently, the intracellular concentration of proline is strongly increased through *de novo* synthesis (Whatmore *et al.*, 1990; Whatmore and Reed, 1990). In *B. subtilis* proline is the predominant organic osmolyte synthesised in defined medium by cells exposed to a hypersaline environment (Whatmore *et al.*, 1990). However, several hours are required to reach a proline level that is sufficient for osmoprotection, leaving the cell at a growth disadvantage in harsh, high osmolarity environments (Boch *et al.*, 1994).

Bacillus subtilis can also use exogenously provided proline as an osmoprotectant. von Blohn and colleagues (1997) identified the transport system (OpuE), which mediates proline uptake for osmoprotective purposes, as a single integral membrane protein. This protein, OpuE, is a member of the sodium solute symporter family, comprising proteins from both prokaryotes and eukaryotes that obligatorily couple substrate uptake to Na^+ symport. Uptake of proline is under osmotic control and functions independently of the transport system for glycine betaine (von Blohn *et al.*, 1997). Two osmoregulated *opuE* promoters were identified; the activity of one of these promoters was dependent on sigma A and the second promoter was controlled by the general stress transcription factor sigma B (von Blohn *et al.*, 1997).

Bacillus subtilis response to osmotic upshock is markedly different in the presence of glycine betaine. Glycine betaine can be rapidly accumulated as a compatible solute with no

synthesis (Whatmore *et al.*, 1990) or uptake (von Blohn *et al.*, 1997) of proline. This potent osmoprotectant can be synthesised by *B. subtilis* from its precursor choline or taken up directly from the environment (Imhoff and Rodriguez-Valera, 1984; Whatmore *et al.*, 1990; Boch *et al.*, 1994). A strong increase in the growth rate, and proliferation under environmental conditions which usually are inhibitory for *B. subtilis,* can be attained when glycine betaine can be accumulated from the growth medium (Imhoff and Rodriguez-Valera, 1984; Whatmore *et al.*, 1990; Boch *et al.*, 1994). Glycine betaine is synthesised by plants and is brought in a varying supply into the habitat of *B. subtilis* through the degradation of plant tissues, thus necessitating effective mechanisms for the active acquisition of this important osmoprotectant (Kempf and Bremer, 1995).

Three osmotically controlled uptake systems of glycine betaine have been identified in *B. subtilis*: a single-component transporter, OpuD (Kappes *et al.*, 1996), and two multicomponent transport systems, OpuA (Kempf and Bremer, 1995) and OpuC (ProU) (Lin and Hansen, 1995), that are related to the binding protein-dependent transport system ProU from *E. coli.* These transport systems are members of a superfamily of prokaryotic and eukaryotic transporters known as ATP-binding cassette (ABC) transporters or traffic ATPases (Kempf and Bremer, 1995).

OpuD represents a new type of bacterial glycine betaine uptake system and is a member of a small family of transport proteins involved in the accumulation of trimethylammonium compounds. The *opuD* sequence predicts a hydrophobic protein of 512 amino acids residues that is likely to form an integral membrane protein with 12 transmembrane spans (Kappes *et al.*, 1996). This system does not exhibit any uptake activity for either carnitine or choline (Kappes *et al.*, 1996). Kinetics studies revealed high substrate affinity of OpuD for glycine betaine, with Km values of 13 μM and 9.5 μM in osmotically induced and uninduced conditions respectively (Kappes *et al.*, 1996).

DNA sequence analysis revealed that the components of the OpuA transport system are encoded by an operon (*opuA*) comprising three structural genes: *opuAA*, *opuAB*, and *opuAC*. The products of these genes exhibit features characteristic for binding protein-dependent transport systems and in particular show homology to the glycine betaine uptake system ProU from *E. coli* (Kempf and Bremer, 1995). The *opuAA* gene encodes a hydrophilic protein of 418 amino acids residues, and exhibits strong sequence identities to many prokaryotic and eukaryotic proteins involved in ATP hydrolysis. Approximately 58%

of the amino acid sequence is identical between OpuAA and ProV from *E. coli* and the *S. typhimurium* ProU system. Sequence conservation is particularly apparent in the N-terminal half of the OpuAA and ProV proteins. The *opuAB* reading frame codes for a hydrophobic protein of 282 amino acids that is homologous to the integral membrane protein ProW of the *E. coli* ProU transport system. ProW and OpuAB show 47% sequence identity over their entire length (Kempf and Bremer, 1995). *opuAC*, the last gene of the operon, encodes a 293 amino acids hydrophilic protein. The OpuAC protein is likely to be substrate-binding protein component for the OpuA transport system.

The substrate binding protein, ProX and OpuAC, show the least sequence conservation (33% identity in a 46-amino acid segment) among the components of the ProU and OpuA transport systems with the N-terminal and C-terminal ends of both proteins being entirely different (Kempf and Bremer, 1995). The OpuAC protein is likely to carry lipid modifications at the N terminus, anchoring it in the membrane (Kempf and Bremer, 1995). Characteristic of the binding protein-dependent transport systems of Gram-negative bacteria is the presence of a soluble, ligand-binding, periplasmic protein (ProX in *E. coli* and *S. typhimurium*) that serves to capture the substrate and deliver it to the membrane-bound components. Since Gram-positive bacteria have no perisplasm, Kempf and Bremer (1995) proposed that extacellular proteins anchored via lipid modifications in the cytoplasmic membrane can serve the physiological function of periplasmic proteins from Gram-negative bacteria. The OpuA system exhibits high substrate affinity for glycine betaine with K_m values of 2.4 μM in both conditions, either osmotically induced or not (Kappes *et al.*, 1996).

The third glycine betaine transport system in *B. bacillus* is the ProU system (Lin and Hansen, 1995) also known as OpuC (Kappes *et al.*, 1996). This system contains four open reading frames (ProV, ProW, ProX and ProZ also termed OpuCA, OpuCB, OpuCC and OpuCD respectively) with homology to the Gram-negative ProU proteins. The *proV* gene encodes an hydrophilic protein of 404 amino acids. The *B. subtilis* ProV protein, shows 35% identity over a 351-amino acid overlap with the *E. coli* ProV protein (Lin and Hansen, 1995). The *proW* reading frame encodes a protein of 217 amino acids. Approximately 29% of the amino acids in a 190-amino acid sequence are identical between the *E. coli* ProW and the deduced sequence of *B. subtilis* ProW (Lin and Hansen, 1995). The similarity of the putative *Bacillus* ProX protein (313 amino acids) to the *E. coli* ProX is considerably poorer, with only 17% identity obtainable (Lin and Hansen, 1995). The relatively low homology

between *E. coli* ProX and *B. subtilis* ProX may be due to the fact that *B. subtilis* ProX does not function in a periplasmic environment. A stretch of hydrophobic amino acids at the amino terminus of ProX suggested that this substrate-binding protein of *B. subtilis* is anchored in the cytoplasmic membrane (Lin and Hansen, 1995). However, a lipid modification of the ProX protein is suggested by the presence of a ProX signal peptide sequence that shows characteristics signatures (Leu^{-3}-Ser-Gly-Cys^{+1}) for the proteolytic processing and lipid modification site of bacterial lipoproteins (Kappes *et al.*, 1996). The last open reading frame of the *B. subtilis* ProU operon encodes the ProZ protein of 223 amino acids. The similarity between *E. coli* ProW and *B. subtilis* ProZ is 29.9% identity over a 291-amino acid sequence (Lin and Hansen, 1995). This fourth cistron is not present in the *proU* operon of either *E. coli* and *S. typhimurium*, and the function of the encoded protein is not known yet. ProZ may represent a new functionality that is not possessed by the Gram-negative *proU* locus (Lin and Hansen, 1995). The ProU transport system exhibits high affinity for glycine betaine with K_m values of 5.1 μM under uninduced osmotic conditions and 6 μM under induced osmotic conditions (Kappes *et al.*, 1996).

The OpuA transport system is certainly the predominant glycine betaine transporter in *B. subtilis*. The importance of the OpuA function for glycine betaine uptake is suggested by a mutant strain lacking the ProU (OpuC) and OpuD systems: this mutant grew with approximately the same efficiency as a wild-type strain producing all three uptake systems (Kappes *et al.*, 1996). *Bacillus subtilis* mutants which synthesised only one of the three glycine betaine uptake systems revealed that the contribution, to glycine betaine uptake, made by the OpuA system under low-osmolarity growth conditions exceeds by far that made by the ProU and OpuD transporters (Kappes *et al.*, 1996). The high basal level in OpuA activity is a reflection of the dual transcriptional regulation of the *opuA* operon. It is expressed both from an osmotically inducible promoter and from transcription initiation signals that mediate constitutive expression of *opuA* in log-phase cells (Kempf and Bremer, 1995).

In addition to transport systems for acquiring glycine betaine from the environment, *B. subtilis* can also accumulate this osmoprotective compound through synthesis (Boch *et al.*, 1994). This synthesis requires the presence of the precursor choline or glycine betaine aldehyde in the growth medium. Production of glycine betaine from choline is a two-step oxidation process with glycine betaine aldehyde as the intermediate compound. Uptake of choline is mediated by an efficient transport system that is osmotically regulated at the level

of transport activity and the expression of its structural gene(s). *Bacillus subtilis* possess two genes, *gbsA* and *gbsB*, whose products are essential to convert choline into glycine betaine (Boch *et al.*, 1994). In contrast to transport systems, however, the systems mediating the enzymatic conversion of choline into glycine betaine are only marginally stimulated by an increase in medium osmolarity (Boch *et al.*, 1994).

1.10.1.3 Osmoregulation in *Listeria monocytogenes*

The ability of *L. monocytogenes* to survive a wide range of environmental conditions including relatively high salt conditions (10%, w/v, NaCl, McClure *et al.*, 1991) and low temperatures allows the bacterium to resist traditional food preservation methods and to persist under conditions where the growth of other microorganisms is inhibited. *Listeria monocytogenes* accumulates glycine betaine when grown under osmotic stress and chill stress at refrigerator temperatures (Ko *et al.*, 1994). Exogenously added glycine betaine enhances the growth rate of stressed but not unstressed cells, i.e., it confers both osmotolerance and cryotolerance (Ko *et al.*, 1994). Both salt-stimulated and cold-stimulated accumulation of glycine betaine occur by transport from the medium rather than by biosynthesis (Ko *et al.*, 1994; Patchett *et al.*, 1994). At high salt concentration (4% w/v NaCl) cells transport glycine betaine 200-fold faster than in the absence of salt and at 7°C this transport is 15-fold faster than at 30°C. The kinetics of glycine betaine transport suggest that the two transport systems may be the same (Ko *et al.*, 1994).

Gerhardt *et al.* (1996), observed that the transport of glycine betaine into membrane vesicles of *L. monocytogenes* was dependent on both high concentrations of sodium ion and the presence of a hypertonic solute gradient. Transport was 65% lower in vesicles derived from cells that were grown under stress provided by KCl rather than NaCl, and approximately 94% lower in vesicles derived from cells that were not grown under osmotic stress (Gerhardt *et al.*, 1996). These workers also observed that this porter is specific for glycine betaine (Gerhardt *et al.*, 1996).

Another trimethyl amino acid that functions as an osmoprotectant in *L. monocytogenes* is carnitine (β-hydroxy-L-τ-N-trimethyl aminobutyrate). Carnitine is structurally related to glycine betaine and can be found in high concentration in food of animal origin (Beumer *et al.*, 1994). Beumer and colleagues (1994) observed that exogenously supplied carnitine significantly stimulated growth under osmotic stress conditions in minimal medium at both

37 and 10°C. Transport of L-[N-methyl-^{14}C]carnitine in *L. monocytogenes* was shown to be energy dependent (Verheul *et al.*, 1995b). Cell extracts analyses showed that L-carnitine was not further metabolised (Verheul *et al.*, 1995b), which supplies evidence for its function as osmoprotectant in *L. monocytogenes*. Uptake of L-carnitine proceeds in the absence of a proton motive force and is strongly inhibited by phosphate analogues, vandate and arsenate (Verheul *et al.*, 1995b). Thus, L-carnitine permease is most likely driven by ATP (Verheul *et al.*, 1995b). Kinetic analysis of L-carnitine transport revealed the presence of a high-affinity uptake system, with a K_m of 10 μM which, in *L. monocytogenes*, enables the bacterium to scavenge L-carnitine when it is available at traces levels in foods (Verheul *et al.*, 1995b). The transport capacity of the L-carnitine permease was very high in the absence of salt and this activity could not be stimulated upon imposition of an osmotic stress. This implies that carnitine accumulation in *L. monocytogenes* can occur even under conditions of low osmolarity (Verheul *et al.*, 1995b). L-carnitine was also shown to confer both chill and osmotic tolerance to the pathogen but was less effective than glycine betaine (Smith, 1996). The absolute amount of osmoprotectant accumulated by the cell was dependent on the temperature, the osmolarity of the medium and the phase of growth of the culture (Smith, 1996). Smith (1996), also demonstrated that *L. monocytogenes* accumulates high levels of osmolytes when grown on a variety of processed meats at reduced temperatures. However, the contribution of carnitine to the total intracellular osmolyte concentration was much greater in cells grown on meat than in those grown in liquid media. Verheul and colleagues (1995b) suggested that the L-carnitine transporter may facilitate the intracellular growth and survival of *L. monocytogenes* in mammalian cells, which is an essential component of the pathogenesis of this organism. Carnitine is present in the cytosol of mammalian cells at concentrations high above the K_m values of the L-carnitine transport system in *L. monocytogenes* of this compound (Verheul *et al.*, 1995b), thus the availability of this osmoprotectant might increase the capacity of the bacterium to grow in the host cell.

In contrast to most bacteria *L. monocytogenes* has a limited biosynthetic capacity. While the majority of bacteria are able to synthesise all 20 amino acids necessary for protein synthesis, *L. monocytogenes* demands five amino acids for growth (Verheul *et al.*, 1995a). Therefore, in order for *L. monocytogenes* to grow in food products, essential amino acids must be available and this is possible as a consequence of proteolytic activity of other bacteria present in the food (Verheul *et al.*, 1995a). Amezaga and colleagues (1995) found that during growth of *L. monocytogenes* in peptone (peptide)-containing media, the peptone plays two major roles: firstly as a supply of amino acids that either can be utilised directly

for protein synthesis or can be oxidised to provide ATP, and secondly as a sources of amino acids and peptides that serve, in addition to glycine betaine, as osmoprotectants. At low osmolarity the amino acids pools found in cells in the presence or absence of peptone are unchanged (Amezaga *et al.*, 1995). As *L. monocytogenes* does not grow with peptides as sole carbon source, the function of peptides during growth at low osmolarity is the supply of amino acids for protein biosynthesis (Amezaga *et al.*, 1995). At high osmolarity amino acids serve an additional function. There is an increase in amino acid pools in the cells, with particularly substantial pools of glutamate, asparate, proline, hydroxyproline and glycine. Interestingly, glycine- and proline- containing peptides have been shown to stimulate growth of *L. monocytogenes* at high osmotic strength (Amezaga *et al.*, 1995). When supplied exogenously at high concentrations (10 mM) proline has been shown to confer osmoprotection in *L. monocytogenes* (Beumer *et al.*, 1994). However, when supplied as peptides (prolyl-hydroxyproline), significant osmoprotection is provided at low concentration (1 mM) (Amezaga *et al.*, 1995). The peptide prolyl-hydroxyproline accumulates in cells to high levels in response to growth at high osmolarity, and the pools of the derived amino acids (proline and hydroxyproline) also show a dependence on the external osmotic pressure. Verheul *et al.* (1995a) characterised a proton motive force-dependent di- and tripeptide transport system in *L. monocytogenes* ScottA, which can supply the organism with amino acids essential for growth, thus facilitating the growth of this pathogen in foods. This transport system was shown to have a broad substrate spectrum, with high affinity for various proline-containing peptides, which suggests that it might play an important role in osmoregulation of *L. monocytogenes* during growth at high osmolarity (Verheul *et al.*, 1995a).

Little is known about the molecular mechanisms of osmotic induction in *L. monocytogenes.* However, Anderson *et al.* (1995), have cloned and sequenced two open reading frames from *L. monocytogenes,* which exhibited extensive similarity to the *E. coli* KdpD/E regulon and further work is still awaited. Knowledge about osmoregulation, including information about the regulation of the synthesis and activity of the transport systems offers exciting prospects for future research.

1.10.2 The Cold-Shock Response

Similar to the higher eukaryotes, microorganisms respond to sudden change of temperature by synthesising a new group of proteins. The literature is full of reports on the heat-shock proteins (HSPs) of microorganisms, however, the cold-shock proteins (CSPs) have only just started to be studied (Chattopadhyay, 1994).

Studies on the molecular and biochemical mechanisms that allow bacteria to survive cold-shock stress have been performed predominantly in mesophilic bacteria such as *E. coli* (Jones *et al.*, 1987; Goldstein *et al.*, 1990; Jones and Inouye, 1994) and *B. subtilis* (Willimsky *et al.,* 1992; Schindelin *et al.*, 1993; Shroder *et al.*, 1993). Much less information, about the mechanisms of cold-shock survival, is available for the psychrotrophic bacterium *L. monocytogenes* (Zheng and Kathariou, 1994; Francis *et al.*, 1995; Bayles *et al.*, 1996). Since there is most information available on the mechanisms of cold-shock survival for *E. coli*, this system will be outlined on the next section as a model system. Subsequently, the mechanisms for *B. subtilis* and *L. monocytogenes* will be described.

1.10.2.1 Cold-Shock Response in *Escherichia coli*

In summary, the cold-shock response in *E. coli* describes a specific pattern of gene expression in response to a downshift in temperature; this pattern includes the induction of several proteins called cold-shock proteins, continued synthesis of transcriptional and translational proteins despite the lag period, and specific repression of heat-shock proteins (Jones *et al.*, 1987; 1992a).

When the culture temperature of exponentially growing *E. coli* cells is shifted from 37 to 10°C, there is a growth lag period before reinitiation of cell growth (Jones *et al.*, 1987). During this growth arrest, the synthesis of the vast majority of proteins decreases. In contrast, the synthesis of 24 proteins continues and 14 of these are induced. These were named cold-shock proteins (Jones *et al.*, 1987; Jones *et al.*, 1992a; Jones and Inouye, 1994). The majority of these proteins are involved in the processes of transcription and translation (Jones *et al.*, 1987).

Proteins identified as cold-shock proteins because at 10°C they were made at rates 2-10 times greater than their rates at 37°C (Jones *et al.*, 1987) include: NusA, which is involved in termination and antitermination of transcription, initiation factor 2, which mediates the binding of charged $tRNA^{fmet}$ to the 30S subunit for initiation of translations, and polynucleotide phosphorylase, which may be involved in the degradation of mRNA (Jones and Inouye, 1994). Other cold-shock proteins include RecA, which has dual roles in recombination and in the induction of the SOS response (Jones and Inouye, 1994); H-NS, a nucleoid-associated DNA-binding protein (LaTeana *et al.*, 1991); Gyrase A, which is the α-subunit of the topoisomerase DNA gyrase A (Jones *et al.*, 1992b); RbfA, a 30S ribosomal binding factor (Jones and Inouye, 1996); and CsdA, a protein which is associated exclusively with ribosomes and has ability to unwind double-stranded RNA (Jones *et al.*, 1996).

The major cold-shock protein, CspA or CS7.4 (mol. wt 7.4kDa), has been identified as the sole member of the CSPs that is not detectable at 37°C, and its induction after cold-shock is most pronounced (about 200-fold) (Jones *et al.*, 1987; Goldstein *et al.*, 1990). CspA is a 70-amino-acid cytoplasmic protein encoded by the *cspA* gene (Goldstein *et al.*, 1990). Surprisingly, it was found that *E. coli* possesses nine CspA homologs of similar sizes, termed CspA to CspI, and that the corresponding genes are scattered on the *E. coli* chromosome (Goldstein *et al.*, 1990; Lee *et al.*, 1994; Nakashima *et al.*, 1996; Yamanaka and Ynouye, 1997). Of this protein family, only CspA, CspB and CspG are specifically induced by a temperature downshift (Lee *et al.*, 1994; Etchegaray *et al.*, 1996; Nakashima *et al.*, 1996). Induction of these proteins is reported to be regulated at the levels of transcription, mRNA stability, and translational efficiency (Tanabe *et al.*, 1992; Brandi *et al.*, 1996; Echegaray *et al.*, 1996; Goldenberg *et al.*, 1996; Fang *et al.*, 1997).

The physiological function(s) of the CspA family has not yet clearly emerged, however, sequence homology to other proteins of known function can give an insight into its role. CspA has remarkably high sequence similarity with the "cold-shock domain" (CSD) of eukaryotic DNA-binding proteins, called Y-box transcription factors (Wolffe *et al.*, 1992; Lee *et al.*, 1994). The Y-box factors bind preferentially to the Y-box, a *cis*-element found in the promoter region of mammalian major histocompatability complex class II genes, which has the consensus sequence ATTGG (Graumann and Marahiel, 1994). These results suggest that CspA may be a class of DNA binding proteins. Interestingly, CspA has been reported to enhance the transcription of two cold-shock genes, *hns* and *gyrA* (LaTeana *et al.*, 1991;

Jones *et al.*, 1992b). When the promoter of several cold-shock genes were sequenced a CCAAT motif was found to be conserved (Qoronfleh *et al.*, 1992). The same motif is found in the promoter of the gene which encodes for H-NS (*hns*) and is involved in the binding of CspA to this region. Similarly the ATTGG motif is also known to be involved in the binding of CspA to the promoter of *gyrA* and is found to be present in several cold-shock promoters including the promoter of *cspA* (Jones *et al.*, 1992b).

The three-dimensional structure of *E. coli* CspA, elucidated by X-ray crystallography (Schindelin *et al.*, 1994) and by nuclear magnetic resonance (NMR) spectroscopy (Newkirk *et al.*, 1994) also suggested that CspA probably has a general function interacting with DNA or possibly RNA. Indeed, it has been shown that CspA interacts with a 24-base single-stranded DNA (ssDNA) sequence corresponding to a region of its 5' leader mRNA sequence (Newkirk *et al.*, 1994) functioning as a cold-shock transcriptional activator by converting the close transcriptional machinery into an open complex during initiation of transcription (LaTeana *et al.*, 1991). CspA binds co-operatively to single-stranded RNA (ssRNA), however, more than 74 bases is required (Jiang *et al.*, 1997). Binding of CspA to RNA destabilises RNA secondary structures making them susceptible to ribonucleases. This confirms that CspA functions as an RNA chaperone to prevent the formation of secondary structures in RNA molecules at low temperature. Such a function may be crucial for efficient translation of mRNAs at low temperatures and may also have an effect on transcription (Jiang *et al.*, 1997).

Another cold-shock protein, CsdA, was found to increase translational efficiencies (Jones *et al.*, 1996). This protein was found exclusively localised in the ribosomal fraction and became a major ribosomal-associated protein at low temperature (Jones *et al.*, 1996). CsdA has a helix-destabilising activity and disruption of the gene resulted in a defect in growth and expression of certain genes, including heat-shock proteins, only at low temperature. Jones and colleagues (1996) proposed that at low temperature CsdA is essential for ribosomal function to increase translational efficiencies of mRNA by unwinding stable secondary structures formed at low temperature.

Reports by Jiang and colleagues (1993) revealed that transcriptional activation plays a major role for the expression of CspA after cold-shock. However, recent studies supports a mechanism in which post-transcriptional regulation mostly accounts for the induction of CspA. The mRNA of *cspA* has an extremely short half-life at 37°C, which is transiently but

drastically prolonged after a shift from 37 to 15°C (Brandi *et al.*, 1996; Goldenberg *et al.*, 1996; Fang *et al.*, 1997). Interestingly the stabilisation of *cspA* mRNA, which does not appear to be chemically modified, is reversed after adaptation of cells to the lower temperature. Furthermore, it was found that adaptation to cold-shock is blocked when the 143-base sequence of the 5' untranslated region 5' (UTR) of the *cspA* mRNA is overproduced (Jiang *et al.*, 1997). Indeed, the production of CspA, CspB and CsdA were no longer transient but rather prolonged (Jiang *et al*, 1996). In addition, when the CspA was overproduced together with the 5' UTR of its mRNA, the normal cold-shock response was resumed without a prolonged lag period (Jiang *et al.*, 1996), which would suggest that a specific mRNA-binding repressor has been titrated away (Graumann and Marahiel, 1996). Thus, CspA may be regulated at three levels: transcription, translation, and possibly protein stability (Graumann and Marahiel, 1996).

As referred to above, CspA has been suggested to be involved in transcriptional activation of other cold-shock genes such as *hns* (LaTeana *et al.*, 1991) and *gyrA* (Jones *et al.*, 1992b). Another possible regulator of cold-shock gene expression is guanosine 5' triphosphate-3'diphosphate (pppGpp) and guanosine 5' diphosphate-3' diphosphate (ppGpp) (collectively abbreviated (p)ppGpp). Jones *et al.* (1992a) observed that the decrease in the (p)ppGpp level, that occurs following a temperature downshift, positively affects the synthesis of not only transcriptional and translational proteins but also many cold-shock proteins. DNA supercoiling also appears to play a role, since cold-shock regulation of inducible promoters was reported to be lost in a gyrase-defective strain (Jones and Inouye, 1994).

Supplying inhibitors of translation (e.g. chloramphenicol, tetracycline, erythromycin, fusidic acid, spiramycin) resulted in the induction of the cold-shock response - the induction of cold-shock proteins, repression of heat-shock proteins, and continued synthesis of transcriptional and translational proteins (VanBogelen and Neidhardt, 1990). All these antibiotics are known to act on ribosomes. This information, combined with the observation that shifting cells to a lower temperature causes inhibition of protein synthesis, led to the proposal that the state of the ribosome is the physiological sensor for the induction of the response (VanBogelen and Neidhardt, 1990). The most likely explanation is that an abrupt downshift in temperature causes a physiological state whereby the translational capacity of the cell is insufficient relative to the supply of charged tRNA inducing a decrease in the (p)ppGpp level and of the cold-shock response (Jones *et al.*, 1992a). The function of the

cold-shock response is not yet revealed (Jones and Inouye, 1994), however in *E. coli* a possible function may be to overcome the partial block in translation that happens following a temperature downshift, thereby increasing the translational capacity of the cell (Jones and Inouye, 1994).

1.10.2.2 Cold-Shock Response in *Bacillus subtilis*

In the same way as *E. coli*, *B. subtilis* responds to a decrease in temperature with the induction of proteins that are classified as cold-induced proteins (CIPs) (Graumann *et al.*, 1996). After a shift from 37 to 15°C, the synthesis of a majority of proteins is repressed; in contrast, 37 proteins are synthesised at rates higher than preshift rates. One hour after cold-shock, the induction of CIPs decreases, and after 2 hours, general protein synthesis resumes at 37°C rate (Graumann and Marahiel, 1996). The identification of 16 of the 37 major CIPs in *B. subtilis* revealed functions at various levels of cellular physiology, such as chemotaxis (CheY), sugar uptake (Hpr), translation (ribosomal proteins S6 and L7/L12), protein folding (PPiB), and general metabolism (CysK, IlvC, Gap, and triosephosphate isomerase) (Graumann *et al.*, 1996). Three small acidic proteins that showed the highest relative induction after cold-shock are highly homologous and belong to the protein family of CspB (Graumann *et al.*, 1996). CspB is a cold-shock induced protein of *B. subtilis* which was identified by Willimsky *et al.* (1992). This protein exhibits 61% identity to the major cold-shock protein of *E. coli* (CspA) Willimsky *et al.* (1992).

Graumann and colleagues (1996) revealed that responses to cold-shock, heat-shock and general stress are connected since several cold-shock proteins are also induced by osmotic or heat stress. However, most cold-shock proteins are repressed after heat-shock and general stress (Graumann *et al.*, 1996), suggesting that the response to cold-shock has an contrary effect on heat-shock and general stress response.

After cold-shock in *B. subtilis* the greatest increase in synthesis was detected for CspB and the other two members of the family, CspC and CspD. This family possesses over 70% identity (Graumann *et al.*, 1996; Graumann *et al.*, 1997). A similar family of Csps also has been found in *Bacillus cereus* (Mayr *et al.*, 1996). *Bacillus subtilis* CspB shows 43% identity with the nucleic acid-binding domain of the Y-box factors (see Section 1.10.2.1), which is also known as cold-shock domain (CSD) (Graumann and Marahiel, 1994). Studies on the structure of CspB by nuclear magnetic resonance spectroscopy (NMR) and X-ray

(Schidelin *et al.*, 1993; Schnuchel *et al.*, 1993), revealed that this protein posses RNA-binding motifs. Gel retardation experiments showed that CspB binds to ssDNA that contains the ATTGG as well as the complementary CCAAT Y-box core sequence (Graumann and Marahiel, 1994; Schröder *et al.*, 1995). This results suggests that CspB can act as transcriptional activator of cold-shock genes by recognising putative ATTGG-box elements shown to be present in promoter regions of genes induced under cold-shock conditions (Graumann and Marahiel, 1994).

The function of *B. subtilis* CSPs still remains to be determined. However, early studies using a *B. subtilis* strain disrupted in the *cspB* gene revealed that the viability of cells at freezing temperatures was strongly affected. In addition, the effect of having no CspB could be slightly compensated for when cells were preincubated at 10°C before freezing (Willimsky *et al.*, 1992). These results indicate that CspB might protect *B. subtilis* cells from damage caused by ice crystal formation during freezing (Willimsky *et al.*, 1992). Graumann and colleagues (1996) observed that disruption of *cspB* not only caused a freezing-sensitive phenotype in *B. subtilis,* it also was shown to affect the level of induction of several CIPs and other proteins after cold-shock. A recent report showed that production of *B. subtilis* CspB in *E. coli* at 37°C, in which *E. coli* CspA and CspB are absent, resulted in either an increase or decrease in the levels of several proteins, accompanied by a marked reduction in cellular growth rate (Graumann and Marahiel, 1997). These results suggest an association of CSPs in the process of transcription and/or translation. Interestingly, deletion of *cspC* or *cspD* genes in *B. subtilis* did not change the phenotype; on the other hand, *csp* double mutants (*csp*B/C, *csp*B/D or *csp*C/D) exhibited severe reduction in cellular growth at 15°C as well as at 37°C (Graumann *et al.*, 1997). A reduction in survival during the stationary phase was observed only in *csp*B/C and *csp*B/D (Graumann *et al.*, 1997). These observations suggest that in the CSP family of *B. subtilis* a hierarchy of importance for cellular growth and stationary phase survival exists, with CspB being most important. Graumann and colleagues (1997) also observed that CspB, CspC and CspD bind to RNA in a co-operative and interactive manner, suggesting that Csps can function as RNA chaperones facilitating the initiation of translation under optimal and low temperatures.

The cold-shock response of *B. subtilis* is alike to, but different in detail from, the *E. coli* cold-shock response. The number of proteins synthesised after a drop in temperature is about three times higher than in *E. coli*, and the response is faster; 2 hours compared with 4 hours (Graumann *et al.*, 1996). This probably relates to *Bacillus* being a common soil

bacterium, which is relatively well adapted to life under conditions of constant fluctuations in temperature (Lottering and Streips, 1995), unlike the usual situation for *E. coli.*

1.10.2.3 Cold-Shock Response in *Listeria monocytogenes*

One of the salient characteristic of *L. monocytogenes* as a food-borne pathogen is its ability to grow at low temperatures (Seeliger, 1961; Walker *et al.*, 1990). Indeed, cold enrichment has been used extensively to isolate this pathogen (Hayes *et al.*, 1991). Since refrigeration is a commonly used method of extending food life, it is important to understand how *L. monocytogenes* is able to grow at low temperatures. In the present section the response to cold-shock stress by *L. monocytogenes* will be described.

Only recently the cold stress proteins induced in *L. monocytogenes* in response to temperature downshock has been studied. Phan-Thanh and Gormon (1995), observed that *L. monocytogenes* cellular proteins did not change appreciably within the normal range of temperature (10, 25, 37, and 42°C). However, when a mid-exponential phase *L. monocytogenes* culture grown at 25°C was downshift to 4°C, the protein patterns were quite distinctive (Phan-Thanh and Gormon, 1995). These authors observed that 32 protein were induced three-fold or more upon cold-shock with 12 of these exhibiting a fivefold or greater level of induction. In addition, cold-shock repressed approximately half the number of proteins synthesised at normal temperature and many decreased in level (Phan-Thanh and Gormon, 1995). These authors suggested that the proteins repressed, or that decreased in level, may play an important role in the metabolism of the microorganism. It was suggested that their absence may be responsible for the growth lag and the decreased final growth rate when the temperature of culture was dropped (Phan-Thanh and Gormon, 1995).

Bayles *et al.* (1996), in a similar study using a different strain of *L. monocytogenes*, identified 12 cold-shock proteins after cold-shocking from 37°C to 5°C. These authors also observed that when cultures were shocked they entered an extended lag phase and the growth rate achieved upon resumption of growth was much slower (Bayles *et al.*, 1996). The term cold-shock proteins, or Csps in this case, was used for proteins produced in response to a temperature downshock, while proteins exhibiting increased synthesis during growth at low temperature were termed cold acclimation proteins (Caps)(Bayles *et al.*, 1996). It was reported that 4 of the 12 Csps were also identified as Caps, exhibiting increased synthesis during balanced growth at 5°C when compared with 37°C (Bayles *et*

al., 1996). Some of the proteins identified by Phan-Thanh and Gormon (1995) as Csps correspond to proteins designated by Bayles and colleagues (1996) as Csps, however, many are different. Whether or not these proteins identified as Csps contain sequence homology to the Csps family of homologs of *E. coli* is not revealed yet.

Chasseignaux and Hebraud (1997), in a recent report, also observed that when transferring exponential growth-phase *L. monocytogenes* cells from different initial temperatures to 5°C, growth was always followed by a lag-phase. The duration of this lag-phase was proportional to the magnitude of the downshift. The SDS-PAGE protein patterns showed that the synthesis of numerous protein was strongly affected by the stress, with proteins synthesis being either stopped or greatly reduced depending on the magnitude of the cold-shock (Chasseignaux and Hebraud,1997). In this study at least 10 proteins were always induced in response to a drop of temperature. Among these Csps, the most significant induction was observed for a 18 kDa protein. The sequence of the 22 N-terminal amino acids did not match significantly any sequence from the protein databases. Studies to reveal the nature and function(s) of this Csp are in progress (Chasseignaux and Hebraud, 1997).

Discrepancies in the number of proteins identified, in the above studies, may be due not only to the use of different strains but also to different experimental conditions. In addition, the criteria for designation of a protein as a Csp, the protein separating systems used, and the means used to compare the intensities of spots corresponding to proteins in gels vary among investigators.

Six of the cold-shock proteins identified by Phan-Thanh and Gormon (1995) were found to be induced by heat-shock (25°C to 49°C) as well. One of these proteins had an induction of fiftyfold by heat-shock and more than eightfold by cold-shock. Those proteins concomitantly induced by heat- and cold-shock may be the products of certain inducible common regulons in a system of gene regulation with multiple signals as suggested by Van Boegelen and Neidhart for *E. coli* (1990).

A cold-sensitive mutant, produced by Bayles *et al.* (1996), did not exhibit obvious differences in the Csp profiles when compared to the parent strain under cold-shock stress. These results suggest that no major Csp was missing in the mutant. Zheng and Kathariou (1994) have also isolated transposon induced cold-sensitive mutants of *L. monocytogenes*. These mutants were unaltered in their haemolytic activity, motility and invasion of cultured

fibroblasts indicating that the loss of cold resistance may not interfere with other phenotypes (Zheng and Kathariou, 1994). Three genes of *L. monocytogenes*, named *ltrA*, *ltrB* and *ltrC*, which are essential for growth at 4°C, were cloned (Zheng and Kathariou, 1995). Whether these genes are induced at low temperature and whether they are involved in a cold-shock response similar to that described for *E. coli* (Jones *et al.*, 1987; Goldstein *et al.*, 1990; Jones and Inouye, 1994) and *B. subtilis* (Graumann *et al.*, 1997; Willimsky *et al.*, 1992) is still not known.

In order to investigate if the mechanisms of cold-shock survival found in *E. coli* were also found in *L. monocytogenes*, Francis and co-workers (1995), designed oligonucleotides primers based on the most conserved regions revealed by the alignment of the amino acid sequences from *E. coli* CspA, *B. subtilis* CspB and a number of other cold-shock proteins. Sequences of eukaryotic Y-box proteins were also used in the alignment (Francis *et al.*, 1995). When the oligonucleotides primers were used in a PCR reaction containing *L. monocytogenes* DNA, the resulting amplified DNA fragment was 79% and 65% identical at the DNA level to the major cold-shock gene sequences of *B. subtilis* (Willimsky *et al.*, 1992) and *E. coli* (Goldstein *et al.*, 1990) respectively (Francis *et al.*, 1995). Induction studies with the *L. monocytogenes* cold-shock gene, termed *cspL*, revealed that the regulation of this gene appears to be similar to *cspB* from *B. subtilis* rather than *E. coli*, in that it is transcriptionally active at 30°C but is more highly induced (approximately 8-fold) at 10°C (Francis *et al.*, 1995). In addition, low stringency hybridisation studies, using a *cspL* derived probe, revealed that *L. monocytogenes* contains at least 4 homologous genes sequences. These results suggest this bacterium contains a family of major cold-shock protein genes in similarity to *E. coli* (Goldstein *et al.*, 1990; Lee *et al.*, 1994; Jones *et al*, 1996; Nakashima *et al.*, 1996; Kunitoshi and Inouye, 1997) and *B. subtilis* (Graumann *et al.*, 1997).

Interestingly, homologs of CspA have been identified not only in *B. subtilis* (Willimsky *et al.*, 1992; Graumann *et al.*, 1997) but also in *Streptomyces* species (Av-Gay *et al.*, 1993), lactococcal strains (Kim and Dunn, 1997), and antarctic psychrotrophic bacteria (Ray *et al.*, 1994). Knowledge of the cold stress response is far more advanced in *E. coli* than the knowledge of this response in other bacteria. However, Csp and Cap responses have been identified in a limited number of diverse psychrophilic, psychrotrophic, and mesophilic bacteria, including a *Vibrio* sp. (Araki, 1991), *Aquaspirillum articum* (Roberts and Iniss, 1992), *Bacillus psychrophilus* (Whyte and Inniss, 1992), *Pseudomonas fragi* (Hebraud et

al., 1994), *Lactococcus lactis* (Panoff *et al.*, 1994), and *Vibrio vulnificus* (McGovern and Oliver, 1995). Once again the criteria for the designation of the proteins as Csps and Caps vary with the investigator.

1.11 TRANSPOSON MUTAGENESIS OF *L. MONOCYTOGENES*

Transposon mutagenesis has been very useful for the identification of listerial chromosomal genes. Encouraged by the successful use of transposon mutagenesis in *Enterococcus faecalis*, and *Streptococcus pyogenes*, Gaillard *et al.* (1986) were the first to use transposon mutagenesis in *L. monocytogenes*. These workers used the 26-kilobase (kb) conjugative transposon Tn*1545*, originally found in *Streptococcus pneumoniae* (Courvalin and Carlier, 1986), which encodes resistance to kanamycin, tetracycline and erythromycin, to construct listeriolysin O-negative phenotype mutants. The loss of listeriolysin O was associated with a complete loss of virulence in mice, and virulence was restored in the listeriolysin-producing revertant obtained by spontaneous elimination of the transposon (Gaillard *et al.*, 1986). The self-mobilising tetracycline resistance conjugative transposon Tn*916*, smaller in size with only 16.4 kb, has been used as well to produce specific mutants of *L. monocytogenes* (Kathariou *et al.*, 1987; Khun *et al.*, 1990; Sun *et al.*, 1990; Zheng and Kathariou, 1994; 1995). Transposons Tn*1545* and Tn*916* are self transmissible, encoding conjugation systems independent of the normal plasmid specific mechanisms (Murphy, 1989). Both transposons can be transferred through conjugation between *Listeria* species and *E. faecalis*, and can be accomplished by the use of basic mating techniques (Gaillard *et al.*, 1986; Kathariou *et al.*, 1987). Tn*1545* was delivered into *L. monocytogenes* at a frequency of approximately 10^{-8} (Gaillard *et al.*, 1986) and Tn*916* was introduced at a frequency of 10^{-6} (Kathariou *et al.*, 1987). These relatively low frequencies of transposition make it inconvenient to carry out large-scale mutagenesis, and the insertion of such conjugative transposons requires sequence homology between both ends of the elements and sequences surrounding the sites of integration (Clewell *et al.*, 1988; Hill *et al.*, 1985). This requirement predisposes insertion at certain sites in the chromosome referred to as hotspots, limiting the randomness of the insertion, and increasing the numbers of insertion mutants required for a complete transposon library. The frequency of excision (i.e. loss of Tn*916*) is often high, which has been exploited in the analysis of cloned Gram-positive genes in *E. coli* but is not advantageous for the construction of stable insertion mutants (Gawron-Burke and Clewell, 1984).

In order to overcome all the problems associated with the use of transposons Tn*1545* and Tn*916*, the nonconjugative *E. faecalis* transposon Tn*917* has also been employed successfully in mutagenesis of the listerial chromosomal genes (Camilli *et al.*, 1990; Cossart *et al.*, 1989). Tn*917* was first isolated by Tomich *et al.* (1980), and was identified in a plasmid, pAD2, encoding resistance to streptomycin, kanamycin, and erythromycin. The erythromycin resistance determinant was located on a 3.3-megadalton transposable element designated Tn*917* (Tomich *et al.*, 1980). Interestingly, the transposition functions of Tn*917* are also erythromycin inducible, and induction of transposition displays the same kinetics as induction of drug resistance (Tomich *et al.*, 1980; Clewell *et al.*, 1982). This element is transposed in a Tn*3*-like process which is not site specific and is therefore random in nature, and the insertion of Tn*917* has been demonstrated to be random in several species of *Bacillus* (Youngman *et al.*, 1983). This transposon has also been demonstrated to generate extremely stable insertional mutations, with the frequency of excision being relatively low (Youngman *et al.*, 1983; 1984a; Camilli *et al.*, 1990). The small size (5 kb) of Tn*917* when compared to that of Tn*1545* (25.3 kb), and Tn*916* (16.4 kb) simplifies the cloning and mapping of the DNA flanking its insertion.

Only recently a natural transposon of *L. monocytogenes* was described (Lebrun *et al.*, 1994a). This transposon was termed Tn*5422*, is of 6.4 kb and belongs to the Tn*3* family (Lebrun *et al.*, 1994a; 1994b). It contains two cadmium resistance genes together with the transposition genes (Lebrun *et al.*, 1994b). The genes involved in the transposition of Tn*5422* are very similar to those of Tn*917*, suggesting a common origin (Lebrun *et al.*, 1994b). Analysis of Tn*5422* sequences suggested that this transposon is functional, being capable of intramolecular replicative transposition-generating deletions (Lebrun *et al.*, 1994b). To my knowledge this transposon has not been used in the transposon mutagenesis of the *L. monocytogenes* chromosome.

To take full advantage of the fact that Tn*917* can function in *L. monocytogenes*, several derivatives carrying additional drug resistance genes, and translational fusions to reporter genes, such as *lacZ* and *cat*-86 have been constructed to enhance the usefulness of this element for the analysis of chromosomal genes (Youngman, 1987). The fusion generating derivatives of Tn*917* allow the isolation of gene fusions as a consequence of insertion and the direct identification of transcription units and regulatory functions, as well as conferring easily selectable markers (Perkins and Youngman, 1986; Youngman *et al.*, 1985).

One approach used to recover transpositions of Tn*917* to chromosomal sites in *B. subtilis* is the construction of suicide vectors carrying Tn*917* and replicons from the vectors native to *E. coli*, which are unable to replicate in Gram positive bacteria. On transformation into *Bacillus spp.* these replicons are not expressed, thus any erythromycin resistant organisms recovered are predominantly those which have acquired a chromosomal insertion of Tn*917*.

The most common method of choice for the recovery of Tn*917* transposition is based on the use of temperature sensitive plasmid vectors, which carry the pE194 replicon derived from *Staphylococus aureus* (Youngman *et al.*, 1985; Youngman *et al.*, 1987; Youngman *et al.*, 1989). Replicons originating from pE194 display a progressively reduced copy number with increasing temperature, and replication ceases completely at temperatures above 45°C (Gryczan *et al.*, 1982). When bacteria carrying these plasmids are passaged at elevated temperatures the vector is lost after a few rounds of replication and any erythromycin resistant survivors are predominately bacteria which have acquired a chromosomal insertion of Tn*917* (Youngman *et al.*, 1984b). The temperature sensitive vectors pTV32ts and pLTV3 which carry Tn*917*-lac fusions have been employed for the construction of insertion mutants of *L. monocytogenes* (Camilli *et al.*, 1990; Sun *et al.*, 1990).

The application of transposon mutagenesis provides a method for the identification of chromosomal genes responsible for the ability of *L. monocytogenes* to grow at low temperature. The aim of the current study is to use transposon mutagenesis in an attempt to identify genes encoding proteins necessary for the ability of *L. monocytogenes* to grow at low temperature.

In this study it was decided to use a derivative of transposon Tn*917*, Tn*917*-LTV3, carried on plasmid pLTV3 (Camilli *et al.*, 1990). In this system, the transposon is carried by the temperature-sensitive suicide vector pE194Ts (Villafane *et al.*, 1987) derived from pE194, which simplifies the recovery of chromosomal insertions. In addition, this modified transposon contains a promoterless copy of *E. coli lacZ* gene orientated such that insertions into chromosomal genes can produce transcriptional *lacZ* fusions. To facilitate the cloning of chromosomal DNA flanking the site of insertion *E. coli* ColE1 and M13 origins of replication, together with a polylinker of cloning sites, are also present in the modified Tn*917*.

Chapter 2
MATERIAL AND METHODS

2.1 Bacterial strains and plasmid

The bacteria and plasmids used are listed in Table 2.1.1 and 2.1.2.

2.1.1 Culture media and growth conditions

Listeria monocytogenes strain 10403S and *L. monocytogenes* strain DP-L910 (containing the plasmid pLTV3 see Figure 2.1.1.1) were grown in Tryptone Soya Broth (TSB) supplemented with 0.2% (w/v) glucose or on Tryptone Soya Agar (TSA) (OXOID, Unipath Ltd.) medium at 30°C. DP-L910 was grown in the presence of tetracycline, erythromycin and lincomycin (Sigma) at a concentration of 12.5μg/ml, 1μg/ml and 25μg/ml respectively. For the culture of the transposon insertion mutants of *L. monocytogenes*, erythromycin and lincomycin, at the same concentrations as above, were added to the media.

Escherichia coli JM101, LE392, DH5α and MC1061 were grown in Luria-Bertani (LB) medium (Davis *et al.*, 1980) (per litre: 10g of Bacto-Typtone, 5g of Bacto-Yeast Extract, 5g NaCl; for agar 15g of Bacto-Agar per litre was added) at 37°C. *Escherichia coli* HB101 (harbouring pLTV3) was grown in LB in the presence of kanamycin (Sigma) at 25 μg/ml.

SOC recovery medium (Sambrook *et al.*, 1989) (20g of Bacto-Tryptone per litre, 5g of Bacto-Yeast-Extract per litre, 10mM NaCl, 2.5mM KCl, 10mM $MgCl_2$, 10mM $MgSO_4$, 20mM Glucose) was used to recover transformants of *E. coli*.

Media were prepared using distilled water and sterilised by autoclaving at 121°C at 15 psi (pounds per square inch) for 15 minutes. For short-term storage, bacteria were maintained on agar plates at 4°C. For long-term storage, bacteria were stored at -70°C in broth containing 15% (v/v) glycerol as a cryoprotectant.

Table 2.1.1 - Bacteria Strains

Strain	Genotype and/or Description	Reference
Listeria monocytogenes		
Listeria monocytogenes 10403S*		(1)
Listeria monocytogenes DP-L910*	strain 10403S harbouring pLTV3	
Listeria monocytogenes 23074 (ATCC)		
Escherichia coli		
Escherichia coli DH5α	*supE*44 ΔlacU169 (ø80 *lacZ*ΔM15) *hsdR*17 *recA*1 *endA*1 *gyrA*96 *thi*-1 *relA*1	(2)
Escherichia coli HB101* (pLTV3)	[*hsdS20* (r_B^- m_B^-) *recA13 ara-14 proA2 lacY1 galK2 rpsL20*(Sm^r) *xyl-5 mtl-1 supE44* F^- λ^-]	(3)
Escherichia coli JM101	F' *traD36 lacI*^{q} *Δ(lacZ)M15 proA*$^+$*B*$^+$ */supE thi Δ(lac-proAB)*	(4)
Escherichia coli LE392	F-e14-(McrA-) *hsdR514*(r_k^- m_k^+) *supE44 supF58 lacY1 or Δ(laclZY)6 galK2 galT22 metB1 trpR55*	(5)
Escherichia coli MC1061Φ	[*hsdR mcrB araD139* Δ(*araABC -leu)7679 ΔlacX74 galU galK rpsL thi*]	(6)

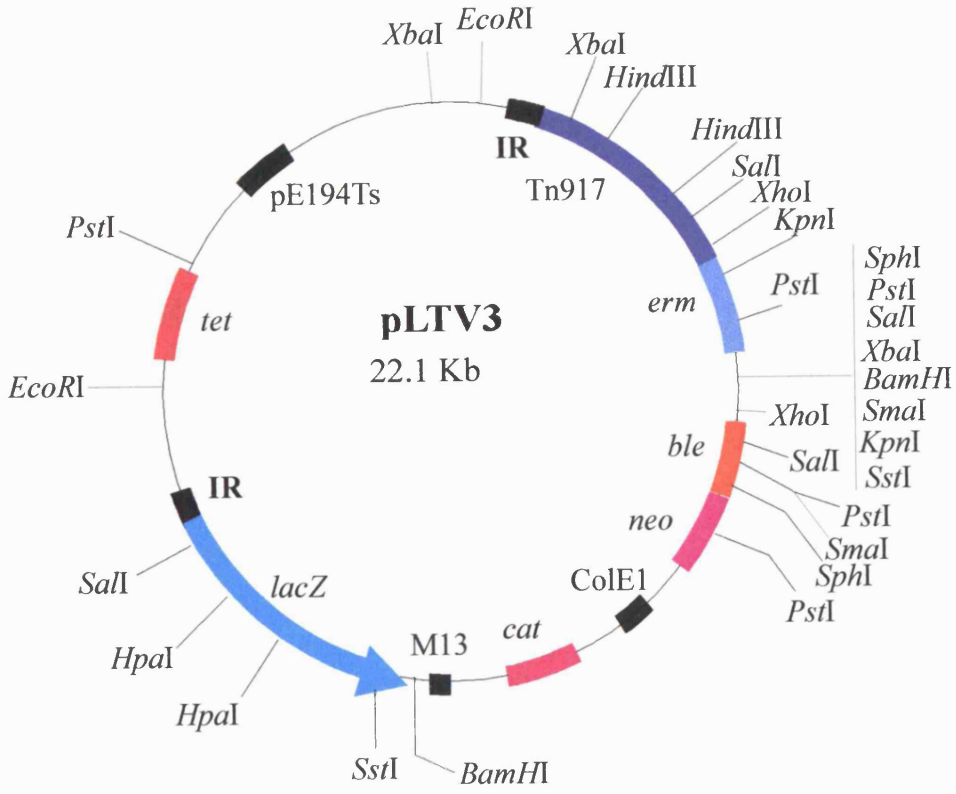
*Obtained from Dr Portnoy. Department of Microbiology, University of Pennsylvania, Philadelphia, USA. ΦObtained from Dr Gutierrez. Department of Oral Biology, University of Florida, Gainesville, Florida, USA.

(1) Bishop and Hindrichs, 1987; (2) Hanahan, 1983; (3) Boyer and Roulland-Dassioux, 1969; (4) Yanish-Perron *et al.*, 1985; (5) Sambrook *et al.*, 1989; (6) Casadaban *et al.*, 1983.

Table 2.1.2 - Plasmids

Plasmid	**Characteristics**	**Reference**
pACYC184	Low copy-number *E. coli* vector, chloramphenicol and tetracycline resistance markers	(1)
pBluescript II KS (+/-)	*E. coli* vector, ampicillin resistance marker, blue-white selection.	(2)
pMK4	*E. coli-Bacillus/Listeria* shuttle vector, ampicillin and chloramphenicol resistance marker, blue-white selection.	(3)

(1) Chang and Cohen, 1979; (2) Stratagene; (3) Sullivan *et al.*, 1984


pLTV3
22.1 Kb
XbaI
EcoRI
XbaI
HindIII
HindIII
SalI
XhoI
KpnI
SphI
PstI
SalI
XbaI
BamHI
SmaI
KpnI
SstI
PstI
SmaI
SphI
PstI
IR
Tn917
erm
ble
neo
ColE1
cat
M13
IR
lacZ
SalI
HpaI
HpaI
SstI
BamHI
tet
EcoRI
PstI
pE194Ts

2.1.2 Preparation of defined medium

When needed, *L. monocytogenes* 10403S and transposon insertion mutants were grown in defined medium. The medium chosen was that of Trivett and Meyer (1971). Nitrilotriacetic acid, vitamins and amino acids were purchased from Sigma, and all other chemicals were from Fisons (AR Grade). Each constituent of the medium was purchased specifically for this purpose, and removed from departmental circulation to ensure no contamination from other users.

The Trivett and Meyer (1971) medium consisted of the following parts which are shown for 1 litre of medium:

Salts

K_2HPO_4	8.5 g
NaH_2PO_4 $2H_2O$	1.7 g
NH_4Cl	500 mg
Nitrilotriacetic acid	480 mg
NaOH	240 mg
$FeCl_3$	29 mg
$MgSO_4$ $7H_2O$	410 mg

Amino Acids

L-cysteine-hydrochloride	100 mg
L-leucine	100 mg
DL-isoleucine	200 mg
DL-valine	200 mg
DL-methionine	200 mg
L-arginine-hydrochloride	200 mg
L-histidine-hydrochloride	200 mg

Vitamins

Riboflavin	1 mg
Thiamine-hydrochloride	1 mg
D-biotin	100 μg
Thioctic acid	10 μg

The preparation of the medium was as follows to stop precipitation and to provide for the sterilisation of the vitamins by filtration. (i) K_2HPO_4 and NH_4Cl were dissolved in 500ml of distilled water; (ii) NaOH and nitrilotriacetic acid were dissolved in 40ml of distilled water; (iii) $FeCl_3$ was dissolved in 40ml of distilled water; (iv) solution prepared in step ii was mixed with solution prepared in step iii; (v) $MgSO_4$ was dissolved in 30ml of dissolved water; (vi) the solutions from steps iv and v together with the amino acids and 390ml of distilled water were mixed with the solution prepared in step i to give a volume of 1000ml. This was sterilised by autoclaving at 121°C at 15 psi for 15 minutes. Once cool, 10ml of 100x concentrated vitamin solution was added. This 100x vitamin solution was made as follows. The 10µg of thioctic acid was dissolved in 200ml of 70% (v/v) ethanol. Two millilitres of this solution was mixed with 5mg of biotin, 50mg of riboflavine in 125ml of 95% (v/v) ethanol, subsequently the volume was made up to 500ml with distilled water. This solution was sterile filtered using Sartorius 0.2µm Midisart 2000 filtration units. The carbon source (glucose) was used at a final concentration of 0.2% (w/v). For this, 10ml of a 20% (w/v) solution (filter sterilised) was added to the medium.

2.1.3 Measurement of growth

Growth rates constants (μ_{max}) were calculated using the formula described by Herbert and Bell (1977):

$$\mu_{max} = 2.303 \times \frac{(\log OD_{600nm} T2) - (\log OD_{600nm} T1)}{T2 - T1}$$

T - represents the time point

2.1.4 Assay of ß-Galactosidase

The method used was based on the method of Miller (1972). 0.5ml of an overnight culture in TSB was used to inoculate 20ml of fresh Trivett and Meyer (T+M) (1971) broth and the bacteria was grown at 30°C to an OD_{600nm} of 0.4. After cooling the culture on ice for 20 minutes, the OD_{600nm} was registered and 0.5ml of the cell culture was mixed with 0.5ml of Z-buffer pH 7.0 (1 litre: 0.006M $Na_2HPO_4.7H_2O$, 0.04M $Na_2H_2PO_4.H2O$, 0.01M KCl, 0.001M $MgSO_4.7H_2O$, 0.05M β-mercaptoethanol), 2 drops of chloroform, 1 drop of 0.1% (w/v) SDS

and vortexed for 10 seconds. The mixture was then equilibrated at 28°C for 5 minutes before starting the reaction. This was started by adding 0.2ml of o-nitrophenyl-β-D-galactopyranoside (ONPG) (4mg/ml) in A medium pH 7.0 (1 litre: 60.2mM K_2HPO_4, 33.06mM KH_2PO_4, 7.5mM $(NH_4)_2SO_4$, 1.7mM $Na_3C_6H_5O_7.2H_2O$) to the mixture and the time of the reaction was recorded with a stop watch. The reaction was stoped by adding 0.5ml of a 1M Na_2CO_3 solution after sufficient yellow colour had developed. The cell debris was removed by centrifugation at 13,000rpm for 5 minutes in a MicroCentaur microfuge, the aqueous phase was carefully removed and the OD_{420nm} was recorded. The units of β-galactosidase was calculated by using the following formula:

$$\text{Units} = \frac{1000 \times OD_{420nm}}{t \times v \times OD_{600nm}}$$

Where t is the time of reaction in minutes and v the volume, in ml, of cell culture used in the assay.

2.2 Reagents

Where necessary, 5-Bromo-4-chloro-3 indolyl-β-D-Galactosidase (X-gal) (Sigma) was added to the medium at a concentration of 40μg/ml. When required O-nitrophenyl-β-D-galactoside (ONPG) (Sigma) was used at a final concentration of 800μg/ml, and isopropyl β-D thiogalactopyranoside (IPTG) was used at a final concentration of 200mM.

2.3 Procedures for DNA extraction

2.3.1 Extraction of *E. coli* plasmid DNA

2.3.1.1 Large scale extraction of plasmid DNA

For the large scale extraction of plasmid DNA from *E. coli* the Plasmid Maxi Kit, purchased from QIAGEN, was used. The instructions from the manufacturer were followed. For this method 500ml of stationary phase bacterial cultures were harvested at 6,000g for 15 minutes at 4°C and the resulting cell pellet was completely resuspended in 10ml of Buffer P1 (50mM Tris-HCl (pH 8.0), 10mM EDTA, 100μg/ml RNase A). Then 10ml of a named "Buffer P2" (200mM NaOH, 1% (w/v) SDS) was added and gently mixed by inversion to produce a clear lysate. After incubation for 5 minutes at room temperature, 10ml of chilled Buffer P3 (3.0M

potassium acetate (pH 5.0)) was added and the preparation incubated on ice for 20 minutes. Cell debris was removed by centrifugation at 20,000g for 30 minutes at 4°C in a Sorvall SS-34 rotor. Supernatant was immediately removed and re-centrifuged at 20,000g for 15 minutes at 4°C in order to avoid applying suspended material to the QIAGEN column. The solution was filtered through a QIAGEN column which had been equilibrated with 10ml of Buffer QBT (750mM NaCl, 50mM MOPS (pH 7.0), 15% (v/v) ethanol, 0.15% (v/v) Triton X-100). The QIAGEN column was then washed twice with 30ml of Buffer QC (1.0M NaCl, 50mM MOPS (pH 7.0), 15% (v/v) ethanol). The DNA was eluted from the column using 15ml of Buffer QF (1.25M NaCl, 50mM Tris-HCl (pH 8.5), 15% (v/v) ethanol). The eluate was mixed with 0.7 volumes of isopropanol at room-temperature and centrifuged immediately at 15,000g for 30 minutes at 4°C to pellet the DNA. The DNA pellet was washed briefly with 5ml of 70% (v/v) ethanol and recentrifuged. After removing the supernatant, the pelleted DNA was air-dried for 5 minutes and resuspended in 2ml of Tris EDTA (TE) buffer (10mM Tris HCl, 1mM EDTA, pH 7.5).

2.3.1.2 Small scale extraction of plasmid DNA

Plasmid DNA extraction was performed as described by Sambrook and colleagues (1989) with slight modifications. The following solutions were used:

Solution I
50mM glucose
25mM Tris-HCl pH 8.0
10mM EDTA
4mg/ml lysozyme (Sigma)

Solution II
0.2M NaOH
1% (w/v) sodium dodecyl sulphate (SDS)

Solution III (100ml)
5M acetate (11.5ml glacial acetic acid)
3M potassium ions (60ml 5M potassium acetate)
Distilled water (28.5ml)

In this procedure 1.5ml of stationary phase bacterial cells were centrifuged in a MicroCentaur bench top minifuge at 5,000g for 1 min and the supernatant was removed. The resulting pellet was resuspended in 100µl of solution I and incubated on ice for 30 minutes. After this incubation period 200µl of freshly prepared solution II was added, gently mixed by inversion, and left on ice for 5 minutes. This was followed by the addition of 150µl of ice cold solution III, gentle mixing, and incubation on ice for at least 10 minutes. The mixture was then centrifuged in the minifuge at 13,000g for 5 minutes and the supernate carefully removed. Protein was removed from the preparation by adding an equal volume of phenol:chloroform (1:1) and vortexed before being centrifuged at 13,000g for 2 minutes. The upper aqueous phase was removed, mixed with 1/10th of 3M sodium acetate (pH 5.2) and 2 vols. of absolute ethanol at room temperature. After an incubation period of 2 minutes at room temperature the plasmid DNA was collected by centrifugation at 13,000g for 5 minutes. The ethanol was careful removed and the resulting pellet was dried *in vacuo* and dissolved in 50µl of 0.1x TE buffer. DNA was stored at -20°C.

2.3.2 Extraction of listerial plasmid DNA

2.3.2.1 Large scale extraction of listerial plasmid DNA

Listeria monocytogenes plasmid DNA was purified by ultra centrifugation in caesium chloride\ethidium bromide gradients as described in Sambrook *et al.* (1989). Solutions I, II and III used in this method were the same as used on section 2.3.1.2. In this procedure, 400ml of stationary phase bacterial cells were collected by centrifugation at 4,000g for 10 minutes at 4°C. The resulting cell pellet was resuspended in 10ml solution I and incubated at 37°C for 30 minutes, before the addition of 10ml solution II, and further incubation for 10 minutes. Then 15ml of ice cold solution III was added and the mixture placed at 37°C for 10 minutes. The cell debris was removed by centrifugation at 3,500g at 4°C for 20 minutes. The resulting supernatant was removed, mixed well with 0.6 volumes of isopropyl alcohol and left to stand at room temperature for at least 15 minutes. Nucleic acids were collected by centrifugation at 4,000g for 30 minutes at room temperature. The resulting pellet was dried *in vacuo* and resuspended in sterile nanopure water to a final volume of 17ml. Caesium chloride and ethidium bromide were added to a final concentration of 1mg/ml and 10mg/ml respectively and the preparation was carefully placed in a polyallomer tube. Tubes were sealed, placed in a Sorvall OTD 60 centrifuge and plasmid DNA was separated by centrifugation at 40,000g for

20 hours at 20°C using a Sorvall TV850 rotor. The position of the plasmid DNA band was visualised by exposing the tube to UV light. This band was extracted by puncturing the tube with an hypodermic needle. Ethidium bromide was removed by equilibration with saturated isopropanol, and caesium chloride was removed by exhaustive dialysis against distilled water at 4°C. DNA was precipitated from the aqueous phase by adding 0.1 volumes of 3M sodium acetate (pH 5.2) and 2 volumes of absolute ethanol. DNA was collected by centrifugation at 4,500g for 30 minutes at room temperature and dissolved in 500µl of TE buffer.

2.3.2.2 Small scale extraction of listerial plasmid DNA

For the small scale preparation of plasmid DNA from *Listeria* species the procedure described on section 2.3.1.2. was modified in one detail, all steps stated in section 2.3.1.2 as taking place on ice were performed at 37°C for efficient extraction.

2.3.3 Extraction of listerial chromosomal DNA

Extraction of listerial chromosomal DNA was based on the method of Flamm *et al.*, (1984). In this method 10ml of bacterial cultures grown in Tryptone Soya Broth (TSB) at 37°C for overnight was centrifuged at 11,700g for 10 minutes at 4°C. Then the pellet was resuspended in 5ml of 0.1 x SSC (1 x SSC: 0.15M NaCl, 0.015M trisodium citrate (pH 7.0)) and centrifuged at 11,700g for 10 minutes at 4°C. The supernatant was removed and 1ml of 20% (w/v) sucrose in 0.01M sodium phosphate buffer (pH 7.0) containing 2.5mg/ml lysozyme (w/v) was added to the pellet and the preparation was incubated at 37°C for 45 minutes. Then 9ml of 1% (w/v) SDS in TE, together with proteinase K at a final concentration of 1mg/ml, were added and incubation continued for 30 minutes. An equal volume of phenol:chloroform (1:1) mixture was then added and the preparation was shaken to form an emulsion before centrifugation at 4,000g for 20 minutes. The upper aqueous layer was removed and washed with phenol:chloroform until no white precipitate was formed at the interface. DNA was precipitated from the aqueous phase on addition of 0.1 volumes of 3M sodium acetate (pH 5.2) and 2 volumes of ice cold absolute ethanol. The DNA was collected by spooling with a Pasteur pipette, briefly air dried, and dissolved in 1ml of 0.1 x TE.

2.4 Procedures for transformation of Bacterial Cells

2.4.1 Transformation of *E. coli*

2.4.1.1 Transformation of *E. coli* using electroporation

2.4.1.1.1 Preparation of electro-competent *E. coli*

The method used was based on the method of Dower *et al.* (1988). 10ml of an overnight culture in LB-broth was used to inoculate 1L of fresh LB-broth and the bacteria was grown with vigorous aeration to mid-log phase (OD_{600} of 0.5 to 1 unit). After cooling the culture on ice for 30 minutes, centrifugation in a cold rotor (4°C) at 4,000g for 15 minutes was used to harvest the cells. The cell pellet then was washed twice in 1L of ice-cold sterile nanopure water and centrifuged at 6,500g for 20 minutes at 4°C. The resulting cell pellet was resuspended in 25ml ice-cold 10% (v/v) glycerol, and centrifuged at 5,000g for 20 minutes at 4°C. The final pellet was resuspended in 1 to 2ml of ice-cold 10% (v/v) glycerol. The whole sample was disposed in aliquots of 90µl and stored at -70°C for up to 6 months.

2.4.1.1.2 Electro-transformation with plasmid DNA

Samples of electrocompetent cells were thawed on ice and immediately used as described in Sambrook *et al.* (1989). In this procedure 40µl of cell suspension was mixed with 1ng of plasmid DNA and placed in a cold 0.2cm electroporation cuvette (Bio-Rad). A Bio-Rad Gene Pulser and Pulse Controller apparatus was used to generate the electric pulse. The settings used were 25µF, 2.5kV/cm and 200Ohms. The cold electroporation cuvette was placed in the safety chamber of the apparatus, brought into contact with the electrodes, and given a single pulse. Immediately, 1ml of SOC medium (Section 2.1.1) was added to the transformed cell suspension, gently mixed, and incubated at 37°C for one hour before plating onto LB-agar containing selective levels of the appropriate antibiotic. Plates were incubated at 37°C overnight.

2.4.1.2 Transformation of *E. coli* using $CaCl_2$

2.4.1.2.1 Preparation of $CaCl_2$-competent *E. coli*

The preparation of $CaCl_2$-competent *E. coli* was as described by Sambrook and colleagues (1989). 100µl of an overnight culture of *E. coli* was used to inoculate 10ml of fresh LB-broth and grown to mid-log phase (OD_{600} of 0.5) at 37°C. The cells were harvested at 1,600g for 10 minutes at 4°C. The resulting cell pellet was resuspended in 4ml of ice cold 10mM NaCl and centrifuged at 1,600g for 5 minutes. Then the supernatant was removed and 4ml of ice cold $CaCl_2$ (100mM) was added to the pellet. The cell suspension was placed on ice for 30 minutes before harvesting by centrifugation at 4°C for 5 minutes. The final cell pellet was resuspended in 1ml of ice cold 100mM $CaCl_2$ and transformed immediately.

2.4.1.2.2 $CaCl_2$-Transformation with plasmid DNA

This transformation procedure was followed as described by Sambrook *et al.* (1989). $CaCl_2$-competent *E.coli* cells (100µl) were mixed with 25ng of the appropriate plasmid DNA dissolved in sterile water or TE buffer and incubated on ice for 1 hour. The cells were then transformed at 42°C for 3 minutes, 1ml LB-broth was added and the mixture was incubated at 37°C for 1 hour before plating onto LB-agar containing selective levels of the appropriate antibiotic. Plates were incubated at 37°C overnight.

2.4.2 Transformation of *L. monocytogenes* using electroporation

2.4.2.1 Preparation of cells for electroporation of *L. monocytogenes* with plasmid DNA

The method followed for the electroporation of *L. monocytogenes* was adapted from that described by Park and Stewart (1990). A 20% (v/v) inoculum of an overnight culture of *L. monocytogenes* grown in brain heart infusion broth with 0.5M sucrose (BHI/s) was added to 500ml of BHI/s and incubated at 37°C with shaking (±150rpm) until an OD_{600nm} of 0.2 was attained. Penicillin G was then added to a final concentration of 10µg/ml, and incubation was continued to an OD_{600nm} of 0.35-0.4. The cells were then harvested by centrifugation at 7000g for 10min at 4°C and washed twice in a equal volume of 1 mM HEPES (pH 7.0), 0.5M sucrose. The cell pellet was drained carefully and resuspended in 1/400th vol. of HEPES-sucrose. The cells were placed on ice and used immediately for electroporation.

2.4.2.2 Electroporation of *L. monocytogenes* with plasmid DNA

100µl of the prepared cell suspension (*ca* 1 x 10^{10} bacteria) were gently mixed with 1µg of plasmid DNA dissolved in 25µl sterile nanopure distilled water and left on ice for 1 min. The mixture was then transferred to a chilled electroporation cuvette ($0.2cm^2$) and placed between the chilled electrodes of a Gene-Pulser™ electroporation apparatus with pulse controller (Bio-Rad, Richmond, California). The settings used were 25µF, 2.5kV/cm and 200Ohms. The cells were electroporated with a single pulse with a time constant of approximately 4.2ms. The cuvette was placed immediately on ice for 1 min before 1ml of BHI/s was added and the culture incubated at 37°C for 4 hours. The cells were then plated onto BHI - 1.5% (w/v) agar, containing selective levels of antibiotic, and the plates incubated at 30°C for 48 hours.

2.5. Techniques for routine DNA manipulation

2.5.1. Agarose gel electrophoresis

DNA fragments were separated by agarose gel electrophoresis (Sambrook *et al.*, 1989). Agarose (Seakem) concentrations used were 0.7% (w/v) when separation of DNA fragments greater then 3kb was required and 1% (w/v) for fragments smaller than 3kb. Agarose was solubilised in TAE buffer pH 7.7 (40mM Tris-acetate, 1mM EDTA) and electrophoresis was performed in TAE buffer containing 0.5µg/ml of ethidium bromide. DNA samples were mixed with 1/5th of 6 x gel-loading buffer (0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol FF, 15% (w/v) Ficoll Type 400 (Pharmacia) in water) prior to loading into gel wells. The electrophoresis conditions were 70 volts for 1 hour or 20 volts overnight. The DNA bands were visualised by exposing the agarose gel to UV light from a long wave UV transilluminator. DNA size markers used were 1kb ladder (GIBCO/BRL), bacteriophage lambda (λ) DNA cut with *Hind*III (GIBCO/BRL) or cut with *Xho*I (GIBCO/BRL). λ DNA markers were incubated at 65°C in gel-loading buffer for 10 minutes before use.

2.5.2 DNA restriction digests

The restriction endonucleases used for routine DNA manipulations were purchased from GIBCO/BRL and Promega. Restriction endonuclease cleavage of DNA was performed according to the manufacturer's recommendations; usually in 20µl reactions with 1µl (10 units) of enzyme and 500ng of DNA at 37°C for 1 hour.

2.5.3 Measurement of DNA concentration

DNA concentrations can be accurately measured by ultraviolet absorbance spectrophotometry (Brown, 1990). The amount of ultraviolet radiation absorbed by a solution of DNA is directly proportional to the amount of DNA in the sample. Usually absorbance is measured at 260nm, at which wavelength an absorbance (A260) of 1.0 corresponds to 50µg of double-stranded DNA per ml.

2.5.4 DNA dephosphorylation

After complete digestion with the appropriate enzyme, plasmid DNA (no more than 10pmoles of 5' termini) was treated with 0.1 units of calf intestinal alkaline phosphatase (CIP) (Promega) using the reaction buffer supplied by the manufacturer, in a final volume of 50µl. For dephosphorylation of protruding 5' termini, DNA was incubated at 37°C for 30 minutes, then another 0.1 units of CIP was added and incubation continued for 30 minutes at 37°C. The reaction was stopped by phenol:chloroform extraction (Section 2.5.6), followed by ethanol precipitation (Section 2.5.6).

2.5.5 DNA ligation

T4 DNA ligase was purchased from GIBCO/BRL and was used with the ligase buffer supplied by the manufacturer. DNA ligation was performed according to the manufacturer's recommendations. Different ratios of vector to insert were used. Ligations were performed at room temperature overnight. Ligase was extracted by washing with phenol:chloroform (Section 2.5.6) and the DNA was ethanol precipitated (Section 2.5.6) before being used for transformation.

2.5.6 Phenol:chloroform extraction and ethanol precipitation

Phenol:chloroform extraction and ethanol precipitation were performed as described by Sambrook *et al.* (1989). An equal volume of phenol:chloroform (1:1), with phenol equilibrated to pH 7.5 with Tris-HCl, was added to the DNA and mixed to white emulsion. Then the mixture was centrifuged at 13,000rpm in a MicroCentaur bench top microfuge, the aqueous phase was removed and the whole process was repeated. To precipitate the DNA from the aqueous phase, 10% (v/v) 3M sodium acetate (pH 5.2) was added, followed by 2 volumes of absolute ethanol. The sample was placed at -20°C for 30 minutes, then the DNA was precipitated by centrifugation at 13,000rpm. The precipitated DNA was then washed with 70% (v/v) ethanol to remove residual salt, air dried and resuspended in an appropriate volume of sterile TE buffer or sterile nanopure water.

2.5.7 DNA extraction from agarose gels

2.5.7.1 DNA extraction from agarose gels using Bandprep kit

The Bandprep kit, purchased from Pharmacia, was used for extraction of DNA from agarose gels. Modifications to the manufacturer's instructions were performed as follows. The desired DNA fragments were produced by digestion with the appropriate enzyme and were separated by agarose gel electrophoresis as described above (Section 2.5.1). The band of interest was excised from the gel and placed in a 1.5ml eppendorf tube. The agarose slice was dissolved at 65°C using Gel solubiliser solution (sodium iodide buffered with Tris-HCl (pH7.0)), and Sephaglas BP was then added at 5µl/µg of DNA. In order to mix the different reagents well, the eppendorf tube was placed on a rotary wheel for 5 minutes. Then the mix was centrifuged at 13,000rpm for 15 seconds in a MicroCentaur microfuge to collect the Sephaglas, and the pellet was washed twice in Wash solution (a buffered salt solution). The resulting Sephaglas pellet was air dried before eluting the DNA twice using 20µl of TE buffer (pH 8.0). A final centrifugation at 13,000rpm in a MicroCentaur microfuge for 10 minutes was performed in order to remove any Sephaglas fines before storage at -20°C.

2.5.7.2. DNA extraction from agarose gels by electroelution

After agarose gel electrophoresis, the DNA band of interest was excised from the gel and placed in prepared dialysis tubing containing 300µl of TAE buffer. The sample was subjected to 100 volts for 30 minutes in TAE buffer and the polarity was then reversed for a further 30 seconds to free any DNA attached to the tubing. TAE buffer from around the gel slice was carefully removed, and the DNA was precipitated through ethanol precipitation as described before.

2.5.7.3 DNA extraction from agarose gels using QIAquick Gel Extraction kit

The QIAquick gel extraction kit (QIAGEN) was used to extract DNA from agarose gels. After agarose gel electrophoresis (Section 2.5.1) using low-melt agarose (GIBCO/BRL), the band of interest was excised from the gel and placed in a 1.5ml microfuge tube. Then 3 volumes of Buffer QX1 was added to the agarose slice and the mixture was incubated at 50°C for 10 minutes or until the gel slice was completely dissolved. Before applying the sample to the QIAquick column, 1 gel volume of isopropanol was added to the suspension. The column was then centrifuged at 13,000rpm for 1 minute in a bench top microfuge, the eluate was discarded and the column was washed with 0.5ml of Buffer QX1. After 1 minute centrifugation at 13,000rpm, 0.75ml of Buffer PE was added to the column and centrifugation repeated for another minute. 50µl of 10mM Tris-HCl (pH 8.5) was added to the column which was centrifuged at 13,000rpm for 1 minute to elute the DNA.

2.5.7.4 DNA extraction from agarose gels using lithium chloride

The method followed for the DNA extraction from agarose gels using lithium chloride was adapted from that of Sambrook *et al.* (1989). The desired DNA fragments were produced by digestion with the appropriate enzyme and were separated by agarose gel electrophoresis as described above (Section 2.5.1) using a low-melting point agarose. The DNA band of interest was excised from the gel, placed in a 1.5ml microfuge tube and weight. Then 1 volume of sterile TE buffer or sterile nanopure water was added to the agarose slice, the mixture was vortexed and incubated at 65°C for 10 minutes or until the slice was fully dissolved. An equal volume of phenol at room temperature was added to the mixture, mixed well and centrifuged at 13,000rpm for 3 minutes in a bench top microfuge. The aqueous phase was transferred to a new microfuge tube and the phenol stage was repeated. Then 0.1 volume of 4M LiCl was

added to the aqueous phase, briefly vortexed and incubated on ice for 5 minutes. After 5 minutes centrifugation at 13,000rpm, 2.5 volumes of ethanol was added to the aqueous phase, incubated at -70°C for 20 minutes and the DNA was precipitated by centrifugation at 13,000rpm. The precipitated DNA was then washed as described on Section 2.5.6.

2.6. DNA Hybridisation

2.6.1. Transfer of DNA to nylon filters by Southern blotting

The method used to transfer DNA from agarose gels to nylon filters was as described by Southern (1975). DNA samples were separated by agarose gel electrophoresis as described above, and the gel photographed next to a fluorescent ruler. The DNA in the gel was depurinated by soaking the gel in 250ml of 0.25M HCl for 7 minutes. After rising briefly in distilled water, the gel was placed in denaturing solution (0.5M NaOH, 1.5M NaCl) for 30 minutes with occasional shaking. After rising again with distilled water, the gel was placed in neutralising solution (0.5M Tris-HCl (pH 7.5), 3M NaCl) for 30 minutes with occasional shaking. The gel was then placed on three sheets of Whatman paper (3mm) pre-soaked in 20 x SSC (1 x SSC: 0.15M NaCl, 0.015M trisodium citrate (pH 7.0)). A pre-soaked (3 x SSC) sheet of nylon membrane (Hybond-N^+, Amersham International PLC) cut to the size of the gel was placed on top of the gel and covered with three pre-soaked (3 x SSC) sheets of Whatman paper making sure no air bubbles were trapped. Three sheets of pre-soaked Whatman paper were placed on top and covered with a stack of paper towels cut to the size of the gel. A 500g weight was placed on top of the assembly. The lower sheets were permanently immersed in 20 x SSC and the top paper towels were changed regularly. The DNA transfer was allowed to proceed overnight, the filter was then removed, air dried on dry Whatman paper and wrapped in Saran wrap. Finally the DNA was fixed to the filter by 5 minutes exposure to UV light from a long wave length transilluminator.

2.6.2 Transfer of DNA to nylon filters by colony lifts

The following procedure was adapted from the procedures used by Grunstein and Hogness (1975) and Benton and Davis (1977). This procedure was developed for low density colony, i.e. 50-200 colonies per 82mm plate. The nylon membranes used were the positively charged Colony/Plaque Screen™ circles of GeneScreen *Plus*® (NEN® Research Products). To transfer the DNA to the nylon filter, the dry membrane disc was carefully placed onto the agar plate

exhibiting the growth avoiding to trap any air under the membrane. The disc was allowed to sit on the agar plate for 2-3 minutes and its position was marked by using the position holes existent on the membrane. While the disc was in contact with the plate, sheets of plastic wrap were laid out with Whatman paper (3mm) on top of it. The membrane disc was carefully removed from the plate with plastic forceps and placed, with colony side up, onto the Whatman paper pre-soaked with denaturing solution (Section 2.6.1). After 5 minutes the disc was transferred onto Whatman paper pre-soaked with neutralising solution (Section 2.6.1) for more 5 more minutes. The membrane disc was then placed on dry Whatman paper and let dry for approximately 30 minutes. In order to fix the DNA to the nylon membrane, the disc was placed between two pieces of dry Whatman paper and placed in the oven at 80°C for 2 hours. The cell debris was then removed from the disc by scrubbing with polyamer wool soaked in 5 x SSC. The dry membrane disc could then be stored, in the dark, wrapped in Saran wrap until needed or immediately used for prehybridisation and hybridisation.

2.6.3 Preparation of labelled probe

2.6.3.1 Preparation of radiolabelled probe

DNA was radiolabelled as described by Feinberg and Vogelstein (1983). DNA (approximately 50ng) to be labelled was denatured by placing in a boiling water bath for 5 minutes before rapidly cooled on ice. To the denatured DNA was then added 3µl OLB buffer (100µM dATP, 100µM dTTP, 100µM dGTP, 3.58mM 2-mercaptoethanol, 1M HEPES (pH6.6) and 27OD/ml hexadeoxynucleotides), 1µl (4units/µl) of Klenow large fragment of DNA polymerase I (GIBCO/BRL), 1µl of [α-^{32}P]dCTP (10µCi, specific activity 400-3000Ci/mmole) (Amersham International) and sterile nanopure water to 25µl. The mixture was incubated at room temperature overnight. Prior to use, labelled probe was denatured by boiling for 5 minutes rapidly cooled on ice.

2.6.3.2 Preparation of non-radiolabelled probe

For the generation of a non-radiolabelled probe, the Fluorescein Gene Images labelling system (Amersham International) was used. In this system, monamers of random sequence are used to prime DNA synthesis on a denatured DNA template in a reaction catalysed by the Klenow fragment of *E. coli* DNA polymerase I. In the Fluorescein Gene Images random prime system, fluorescein-11-dUTP (FI-dUTP) partially replaces dTTP in the reaction so that a fluorescein-

labelled probe is generated. DNA (approximately 50ng) to be labelled was denatured by placing in a boiling water bath for 5 minutes, then chilled on ice for 5 minutes. The denatured DNA was then added to the nucleotide mix provided in the kit (stock solution of fluorescein-11-dUTP, dATP, dCTP, dGTP and dTTP in a reaction buffer containing Tris-HCl (pH 7.8), 2-mercaptoethanol and $MgCl_2$), primers, sterile nanopure water to a final volume of 50µl and 1µl (4units/µl) of Klenow large fragment. The reaction was gently mixed and incubated overnight at room temperature. To stop the reaction EDTA (pH8.0) to a final concentration of 20mM was added. Labelled probe could be stored at -20°C for at least six months. Prior to use, labelled probe was denatured by boiling for 5 minutes and then cooled on ice.

2.6.4 Hybridisation of probe to target DNA on filter

2.6.4.1 Prehybridisation and hybridisation of radiolabelled probe

Southern blot filters were prehybridised for 1 to 3 hours in 25ml of hybridisation solution at 65°C with gentle agitation. Hybridisation solution was 6 x SSC, 5 x Denhardt's solution (50 x Denhardt's is 1% (w/v) of Ficoll Type 400, 1% (w/v) BSA, 1% (w/v) polyvinolpyrollidine), 0.1% (w/v) SDS and 200µg/ml denatured salmon sperm DNA. The radiolabelled probe was boiled for 5 minutes, cooled on ice for 2 minutes, then added to the hybridisation solution. Hybridisation was continued at 65°C overnight with constant and gentle agitation. After hybridisation, the filter was washed three times in a wash solution (1 x SSC, 1% (w/v) SDS) at 65°C for 15 minutes. Then filters were wrapped in Saran wrap (DuPont) to prevent dryness and exposed to Cronex X-ray (DuPont) film in cassettes containing intensifying screens for 8 to 24 hours at -70°C. Films were developed in an Agfa-Geveart automatic film processor.

2.6.4.2 Prehybridisation and hybridisation of non-radiolabelled probe

For prehybridisation, Southern blot filters were incubated in 25ml of hybridisation solution (5 x SSC, 0.1% (w/v) SDS, 5% (w/v) dextran sulphate, 1/20 volume of liquid block supplied with the Fluorescein Gene Images system from Amersham) for at least 30 minutes at 65°C with agitation. The labelled probe was denatured by boiling for 5 minutes, rapidly cooled on ice, then added to the hybridisation solution. Incubation was continued overnight with gentle agitation at 65°C. After hybridisation filters were washed three times in 1 x SSC and 0.1% (w/v) SDS at 65°C for 15 minutes. Following the hybridisation washes, Southern blot filters

were incubated with gentle agitation, for 1 hour at room temperature in a 1 in 10 dilution of liquid blocking agent in diluent buffer (100mM Tris-HCl, 300mM NaCl [pH 7.5]). Filters were rinsed in diluent buffer before adding the 5000-fold diluted of anti-fluorescein-AP conjugated in 0.5% (w/v) BSA in diluent buffer and incubation proceeded at room temperature for 1 hour. The unbound conjugate was removed by washing 3 x 10 minutes in 0.3% (v/v) Tween-20 in diluent buffer at room temperature with agitation. For the signal generation, filters were placed on one half of an open bag (supplied with this kit) with DNA facing up and detection reagent (an aqueous solution of <1.5% (w/v) Disodium 2-chloro-5-(4-methoxyspirol[1,2-dioxetane-3,2'-(5'-chloro)-tricyclo[3,3,1,1]decan]-4yl) phenyl phosphate) was applied (30 - 40µl/cm^2) onto the filters. Then the plastic was folded over the top of the blots and the reagent was evenly spread by wiping the surface with a gloved hand. Cronex X-ray film (DuPont) was exposed to filters in cassettes carrying intensifying screens, overnight at room temperature or 1 hour at 37°C. Films were processed Agfa-Geveart automatic film processor.

For hybridisation of probe to target DNA on filters under conditions of low stringency, Southern blot filters were incubated at 42°C for 30 minutes in hybridisation solution (see Section 2.6.4.2). The labelled probe was denatured by boiling for 5 minutes, rapidly cooled on ice for 2 minutes, then added to the hybridisation solution. Hybridisation was continued at 42°C overnight. Filters were then washed three times in 5 x SSC, 0.1% (w/v) SDS at 42°C for 15 minutes. For signal generation and detection, filters were incubated in liquid block solution, in the anti-fluorescein-AP conjugate solution and washed as described on section 2.6.4.2). Filters were then exposed to X-ray film and film was developed as described above.

2.6.5 Removal of bound probe from a filter

The probe was stripped from some filters following autoradiography to allow rehybridisation with a different probe. When ready to commence re-probing, filters were rinsed in 5 x SSC for 1 - 2 minutes. Then a boiling solution of 0.1% (w/v) SDS was added to the filters using approximately 5ml of SDS solution per cm^2 of filter and incubated at room temperature for 15 minutes with gentle agitation. This operation was repeated twice more, using freshly boiling SDS each time. Filters were then prehybridised, hybridised and probe detected as described on section 2.6.3.2.

2.7 PCR Reactions

In general, PCR reactions were carried out in 100µl volumes, using 100ng of chromosomal template DNA, primers at a final concentration of 1µM and each deoxynucleotide at 200µM. Reaction buffer (x1) was 20mM Tris-HCl (pH8.3), 25mM KCl, 0.05% (v/v) Tween 80, with $MgCl_2$ added to give a Mg^{2+} concentration of between 1 and 4mM. *Taq* polymerase (Northumbria Bilogicals Ltd.) was added to 2.5 units per 100µl reaction, and the reactions briefly spun in a benchtop microfuge. Reactions were then overlaid with 100µl of mineral oil (molecular biology grade, Sigma; oil was exposed to UV light for 15 minutes to destroy any contaminating DNA) and placed in a HYBAID OmniGene thermal cycler. Thermal cycle parameters were as follows.

1 cycle	denaturing 96°C for 2 minutes
	annealing 60°C* for 2 minutes
	elongation 72°C for 3 minutes
25-35 cycles	denaturing 96°C for 30 seconds
	annealing 60°C for 30 seconds
	elongation 72°C for 1 minute
1 cycle	annealing 60°C for 1 minute
	elongation 72°C for 3 minutes

*annealing temperature was dependent on the melting temperature of the primers applied, usually up 10 degrees below the lowest melting temperature.

After the final elongation step, reactions were removed from the cycler for storage at -20°C and reaction products were analysed by electrophoresis through an agarose gel.

When using *Pwo* DNA polymerase (Boehringer Mannheim), reactions were carried out in 100µl volumes, using 100ng of chromosomal template DNA, primers at a final concentration of 300nM and each deoxynucleotide at 200µM. Reaction buffer (x1) was 10mM Tris-HCl (pH8.85), 25mM KCl, 5mM $(NH_4)_2SO_4$ and 2mM $MgSO_4$. *Pwo* DNA polymerase was added to 2.5 units per 100µl reaction, and the reactions briefly spun in a benchtop microfuge. Reactions were then overlaid with 100µl of mineral oil and placed in the thermal cycler. Thermal cycle parameters were as follows.

1 cycle	denaturing 96°C for 2 minutes
20 cycles	denaturing 96°C for 15 seconds
	annealing 60°C* for 30 seconds
	elongation 72°C for 45 seconds
1 cycle	elongation 72°C for 7 minutes

*annealing temperature was dependent on the melting temperature of the primers applied, usually up 10 degrees below the lowest melting temperature.

After the final elongation step, reactions were removed from the cycler for storage at -20°C. Reactions products were analysed by electrophoresis through a 0.7% (w/v) agarose gel.

2.8 DNA Sequencing

2.8.1 Manual DNA sequencing

2.8.1.1 Sequencing reactions using double stranded DNA

Manual DNA sequencing was achieved using the chain termination method developed by Sanger *et al.* (1977). DNA to be sequenced was cloned in pBluescript and plasmid DNA was purified using the QIAGEN columns (Section 2.3.1.1). The Sequenase Version 2.0 kit supplied by United States Biochemical Corporation was used to perform the sequencing reactions. Primers used in the sequencing were either the forward (T7) or reverse (T3) sequencing primers (purchased from GIBCO/BRL), or customised oligonucleotide primers synthesised by GIBCO/BRL.

Plasmid DNA to be sequenced had to be denatured before performing the sequencing reactions. 4 to 6μg of DNA, in a total volume of 16μl, was denatured by adding 0.1 volume of 2M NaOH, 0.1 volume of 2mM EDTA solution and incubating at 37°C for 30 minutes. Then DNA was precipitated by adding 0.1 volume of 3M ammonium acetate (pH 4.6), 2 volumes of absolute ethanol, and incubating at -70°C for 1 hour. DNA was pelleted by centrifugation at 13,000rpm in a microfuge for 20 minutes and the pellet was resuspended in 7μl of sterile

nanopure water. For the sequencing reactions, the manufacturer's instructions were carried out. DNA was radiolabelled by incorporating [α-^{35}S]dATP (4μCi, specific activity 600Ci/mmole) (Amersham International) in the extension reactions, and the radiolabelled fragments were separated by gel electrophoresis (Biggin *et al.*, 1983).

2.8.1.2 Gradient gel electrophoresis of reaction products

For gel electrophoresis a Sequi-Gen Nucleic Acid sequencing cell (Bio-Rad) was used. Before pouring the gel, sequencing plates and spacers were meticulously cleaned with ethanol, and the surface of one of the plates was siliconised with sigmacote (Sigma). Then the sequencing cell was assembled using 0.4mm spacers separating the plates. Sequencing gels (Biggin *et al.*, 1983) were prepared from the following solutions:

10 x TBE

109g Tris base
55g boric acid
40ml 0.5M EDTA pH 8.0
Final pH 8.3
Distilled water to 1L.

10% (w/v) Ammonium persulfate

1g ammonium persulfate (Bio-Rad Laboratories)
Distilled water to 10ml.

1 x TBE Acrylamide/urea solution

43g urea
20ml 30% (v/v) Acrylamide solution
(Accugel, National Diagnostics)
10ml 10 x TBE
Distilled water to 100ml.

To cast the gel, 900μl of the 10% (w/v) ammonium persulfate and 37.25μl of TEMED (N, N, N', N'-tetramethylethylenediamine; Bio-Rad Laboratories) were added to the 100ml of 1 x TBE Acrylamide/urea solution. The solution was then drawn up into a 25ml pipette and slowly poured between the sequencing plates avoiding the formation of air bubbles. Before

polymerisation the flat side of a shark's tooth comb was inserted to give the gel top a straight edge. The gel was allowed to polymerise for 3 to 18 hours before use.

Before running the gel, damp paper towels were used to wipe away any dried polyacrylamide/urea from the outside of the gel mould. Then the gel mould was attached to the electrophoresis apparatus, the top reservoir was filled with 0.5 x TBE and the bottom reservoir was filled with 1 x TBE. The gel was pre-run at a constant power of 47W until the gel temperature reached 50°C. Samples were denatured at 75°C for 2 minutes prior to loading onto the gel, and electrophoresis was performed at a constant 47W for 3 to 6 hours. Following electrophoresis, the gel plates were separated and the gel (together with its supporting plate) was fixed for 20 minutes in a shallow bath containing 4 to 5 volumes of fixing solution (10% (v/v) methanol, 10% (v/v) acetic acid). The gel was then briefly rinsed in distilled water before being transferred to Whatman 3mm paper. The gel was dried onto the paper under vacuum at 80°C for 30 minutes, then Cronex X-ray film (DuPont) was exposed to the gel for 12 to 24 hours. Film was processed in an Agfa-Geveart automatic film processor.

2.8.2 Automated DNA sequencing

Automated DNA sequencing used the ABI PRISM Dye Terminator Cycle Sequencing kit (Applied Biosystems) in conjugation with an Applied Biosystems Model 373A DNA sequencing system. This Cycle Sequencing kit contains dye-labelled dideoxynucleotides, whose incorporation terminates the extending chain to produce a dye labelled reaction product, deoxynucleotides with dITP in place of dGTP to minimise band compressions, and AmpliTaq DNA Polymerase, FS (Applied Biosystems). AmpliTaq DNA Polymerase, FS is a mutant form *Taq* DNA polymerase which has essentially no 5' to 3' nuclease activity and has drastically reduced discrimination for dideoxynucleotides. The reaction products are analysed colourimetrically on the 373A sequencing work station.

The sequencing reactions were performed using 400ng of double stranded plasmid DNA which was isolated by QIAGEN columns (see Section 2.3.1.1). Plasmid DNA was mixed with 3.2pmole of primer and 8µl of Terminator Ready Reaction Mix (Mix contains the 4 dye-labelled dideoxynucleotides, dATP, dCTP, dTTP, dITP, Tris-HCl (pH 9.0), $MgCl_2$, thermal stable pyrophosphate, and AmpliTaq DNA Polymerase) in a final volume of 20µl. Reactions were always done at least in duplicate. The reaction mixtures were then overlaid with 40µl of

mineral oil, placed in a HYBAID Omnigene thermal cycler and thermal cycling were performed as follows

Denaturation

Rapid thermal ramp to 96°C

Hold 96°C for 30 seconds

Annealing

Rapid thermal ramp to 50°C

Hold 50°C for 15 seconds

Extension

Rapid thermal ramp to 60°C

Hold 60°C for 4 minutes

Repeat for 25 cycles

After thermal cycling, the reactions were cleaned of any excess of unincorporated terminators by ethanol precipitation. The 20µl of the reactions mixture was transferred into a 1.5ml microcentrifuge tube which contained 2µl of 3M sodium acetate and 50µl of 95% (v/v) ethanol. After vigorous mixing, the sample was placed on ice for 10 minutes. Then the sample was centrifuged at 13,000rpm in a MicroCentaur benchtop microfuge for 20 minutes. The ethanol solution was then removed and the pellet was washed with 250µl of 70% (v/v) ethanol. Then the ethanol was carefully removed and the pellet was allowed to air-dry. The reaction products were analysed on an ABI Model 373A DNA sequencer by Dr. Katherine Lilly at the Protein and Nucleic Acid Sequencing Laboratory, University of Leicester. Sequence data was analysed using the SeqEd program supplied by Applied Biosystems Inc, the University of Wisconsin Genetics Computer Group (GCG Version 9.1) suite of programs (Genetics Computer Group, 1991), and the programs BLASTN and BLASTX (National Centre of Biotechnology Information, Los Alamos, N. Mex.) available via the Internet (http://www.bio.cam.ac.uk/seqsrch/blast.html).

2.9 Insertional Mutagenesis

2.9.1 Transposition of Tn*917*-LTV3

Transposon mutants of *L. monocytogenes* were generated in liquid culture by the method of Camilli *et al.* (1990). A single bacterial colony of *L. monocytogenes* DP-L910 (harbouring plasmid pLTV3, Figure 2.1.1.1) from a plate containing erythromycin, lincomycin and tetracycline was inoculated into 2ml of Brain Heart Infusion (BHI) (OXOID, Unipath Ltd) containing erythromycin, lincomycin and tetracycline. The culture was grown overnight at 30°C to stationary phase. The overnight culture was inoculated 1/800 into BHI containing erythromycin and lincomycin, and the bacteria containing chromosomal transposon insertions were selected by incubation at 41°C with shaking until stationary phase (OD_{600nm} of 2.0). Aliquots of 5ml culture in 25% (v/v) glycerol were directly frozen at -70°C, until later use. The frozen aliquots will be referred to as transposon insertion libraries of *L. monocytogenes*.

2.9.2 Determination of transposition frequency

Single colonies of *L. monocytogenes* DP-L910 (pLTV3) were picked after overnight growth at 30°C on LB agar containing erythromycin, lincomycin, and tetracycline and used to inoculate 10 ml of LB culture containing the above antibiotics. The culture was grown at 30°C to an OD_{600nm} of approximately 0.4. Serial dilutions of the overnight culture were performed in sterile distilled water, and dilutions plated in triplicate onto agar containing erythromycin, lincomycin, and the number of colony forming units (cfu) present at the permissive (30°C) and nonpermissive (41°C) temperatures for plasmid replication were determined. The transposition frequency was calculated by dividing the number of CFU at the nonpermissive temperature by those at 30°C. Transposition frequency determination was performed in triplicate.

2.9.3 Identification of insertional mutants defective in growth at low temperature

2.9.3.1 Identification of insertional mutants defective in growth at low temperature on solid medium

Samples of the *L. monocytogenes* transposon insertion library were thawed at room temperature, 100µl of culture was rapidly removed and serial dilutions were performed. 100µl of each dilution was plated on TSA plates containing erythromycin and lincomycin and incubated at 41°C for 48 hours. Transposon mutants were then replica plated on TSA containing the erythromycin and lincomycin together with X-gal, and incubated at 4°C, 10°C and 30°C for 30 days, 10 days and 48 hours respectively. From these, the mutants that showed visibly less growth at 10°C and/or 4°C but not at 30°C, when compared to the growth of the control, were selected.

2.9.3.2 Identification of insertional mutants defective in growth at low temperature in liquid medium

Single colonies recovered from the *L. monocytogenes* transposon insertion library were inoculated into individual wells of 96-well culture plates containing 200µl TSB with erythromycin and lincomycin. The plates were incubated at 30°C overnight. Subsequently 20µl of each culture was then transferred to a new microtitre plate and incubated at 4°C for up to 4 weeks. Mutants which grew normally at 30°C but showed defective growth at 4°C when compared to the growth of the control were selected. These mutants were later replica inoculated into TSB supplemented with erythromycin and lincomycin together with 0.2% (w/v) glucose, incubated at 30 and 4°C, and growth monitored at OD600nm. The original microtitre plates were kept frozen at -70°C, with 10% (v/v) glycerol as cryoprotectant, until later use.

2.10 Materials and methods for *in vivo* investigations of *L. monocytogenes* 10403S and *L. monocytogenes* G3 and J3 mutants virulence in mice

2.10.1 Animals

Female MF1 outbred mice, *ca* 30-35g in weight, were obtained from Harlan Olac Ltd (Shaw's Farm, Bicester, Oxon).

2.10.2 Growth and preparation of bacteria

The bacteria were grown in TSB at 37°C to late log phase, harvested by centrifugation at 4000g for 10 minutes and resuspended in the same volume, as the original culture, of sterile phosphate buffer (PBS).

2.10.2.1 Passage of bacteria

The number of bacteria in the suspension above was enumerated by microscopy by using a Thoma chamber. A concentration of $1x10^5$ cells per ml was obtained by diluting in PBS and 200µl were inoculated intravenously (i.v.) into mice. One mouse per bacterial strain was used. In all cases the number of bacteria inoculated was confirmed by preparing serial dilutions in PBS and plating on TSA.

2.10.2.1.1 Preparation of standard inoculum of passaged bacteria

Following injection, mice were culled by cervical dislocation after 2 days. The spleen was removed and homogenised separately in 10ml of sterile distilled water in a Seward Stomacher - Lab blender (Seward Medical) for 30 seconds.

In order to recover the passaged bacteria, the homogenates were serially diluted in sterile distilled water and plated on TSA. The plates were incubated for 24 hours and single colonies were used to inoculate 10ml of TSB. The bacteria were grown in stationary culture at 37°C for 18 hours. Bacterial growth was harvested by centrifugation and resuspended in TSB containing 10% (v/v) glycerol. Portions of this suspension could then be stored at -70°C for several days with no significant loss of viability. The concentration of the stocked standard inoculum was calculated by serial diluting in PBS and plating on TSA. When required the

suspension was thawed at room temperature, and bacteria were harvested by centrifugation at 3000rpm before resuspension in sterile PBS to the required concentration.

2.10.3 Infection of mice

Twenty five mice were inoculated by the i.v. route. For each i.v. injection a dose of approximately $5x10^5$ viable bacteria in a total of 100µl of PBS was injected into the tail vein of each mouse. In all cases the number of bacteria inoculated was confirmed by preparing serial dilutions and plating on TSA.

2.10.4 Enumeration of bacteria in infected host tissue

Following infection, predetermined groups of five mice were culled by cervical dislocation after 2, 4 and 6 days. The spleens were removed and homogenised separately in 10 ml of sterile distilled water in a stomacher for 30 seconds. The homogenates were then serially diluted in sterile distilled water and plated in duplicate on TSA. Plates were incubated for 24 hours before counting colonies. Results were expressed as a mean counts of viable listerias per ml of spleen.

2.11 Statistical analyses

Data were analysed by analysis of variance (ANOVA) and when post analysis was needed the multiple comparison Scheffé test (Scheffé, 1959) was used.

Chapter 3
RESULTS

3.1 Transformation of *E.coli* strains with pLTV3

In order to confirm that *L. monocytogenes* 10403S (pLTV3) was in fact carrying the plasmid pLTV3 and in order to obtain a sufficient amount of DNA for subsequent manipulations, plasmid DNA had to be extracted from this strain. Plasmid extractions from *L. monocytogenes* 10403S (pLTV3) only produced 0.125μg of DNA per ml of culture. This was not enough DNA for subsequent manipulations, so to improve it, it was decided to transform pLTV3 into *E. coli*. For this, 500 ng of pLTV3 DNA was electroporated into electrocompetent *E. coli* JM101 cells and selection was performed on agar containing kanamycin (the antibiotic resistance gene in pLTV3 which is selectable in Gram-negative bacteria). No transformants were recovered. This result could have been due to the low amount of plasmid DNA used or to the method used. Thus, it was decided to increase the amount of plasmid DNA used to transform, and to change the method of transformation. In a second attempt, 700 ng of pLTV3 were used to transform $CaCl_2$-competent cells and transformants were again selected with kanamycin. No transformants were recovered, although thousands of transformants were obtained from the control experiment with *E. coli* and pUC18.

Transformation of pLTV3 into electrocompetent or $CaCl_2$-competent *E. coli* JM101 cells never worked. It was speculated then, that the reason why pLTV3 failed to transform in *E. coli* was due to the size of the plasmid (22.1 kb) or due to a failure to express kanamycin resistance. Youngman (1990) found that *erm*-containing fragments rescued from plasmids pTV20 or pTV21-derived integrants could not be maintained in a "typical" *E. coli* host. Although he was not able to determine the reason for this, it was speculated that the *erm* gene product may be toxic to *E. coli* strains because of its ability to methylate ribosomal RNA. This toxicity was not observed in strain HB101 (Youngman, 1990), however, perhaps due to the fact that this strain contains an *rpsl20* mutation, which modifies the ribosome. Since pLTV3 carries the *erm* gene in association with Tn*917,* and *E. coli* strain JM101 do not carry the *rpsl* mutation, we can say that the reason pLTV3 failed to transform *E. coli* must probably due to the toxicity of the *erm* gene product. The reason why strain HB101 was not used in the transformation experiments was because it was thought to be a strain with a low efficiency of transformation when comparing to strains as JM101. In retrospect, HB101 should have been used but subsequent events made recovering of pLTV3 unnecessary.

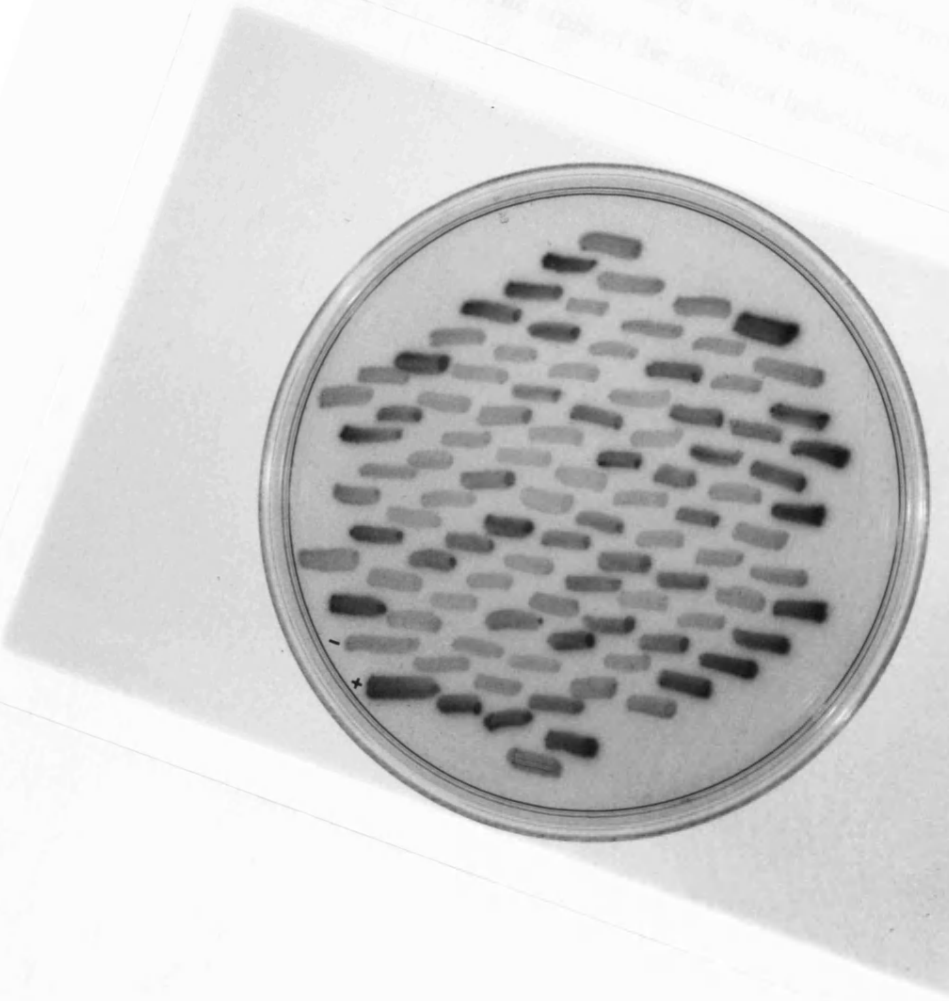
3.2 Transposition of Tn*917*-LTV3 in *L. monocytogenes* 10403S

To generate insertion mutants the method of Camilli *et al.* (1990) was followed since it had been shown to be a successful method. The strain used was the same as these workers which was *L. monocytogenes* 10403S carrying the temperature sensitive plasmid pLTV3 (Figure 2.1.1.1 in Section 2.1.1). The plasmid pLTV3 will not replicate at temperatures above 37°C since it is a highly temperature-sensitive derivative of vector pE194Ts (Villafane *et al.*, 1987). pLTV3 also contains a copy of Tn*917* conferring Em^r (erythromycin resistance) and Lm^r (lincomycin resistance), a promoterless copy of the *E. coli lacZ* gene orientated such that insertions into chromosomal genes can generate transcriptional *lacZ* fusions, the kanamycin resistance gene (*neo*) selectable in *E. coli*, the chloramphenicol resistance gene (*cat*), the bleomycin (*ble*) and the tetracycline resistance gene (*tet*) selectable in *Listeria*, ColE1 replication functions, an M13 origin of replication, and a cluster of polylinker cloning sites which facilitate the recovery in *E. coli* of chromosomal DNA adjacent to sites of insertion (Camilli *et al.*, 1990). After growth at 41°C, a temperature non-permissive for plasmid replication and in the presence of inducible Em concentration, any Em^r, Lm^r and Tc^s (tetracycline sensitive) survivors were assumed to be predominately bacteria which had acquired a chromosomal insertion of Tn*917*-LTV3 and had lost the plasmid.

The frequency of transposition was determined as described by Camilli *et al.* (1990) and this was 6.8×10^{-4}.

On plating the transposon insertion mutants onto agar plates containing x-gal (5-bromo-4-chloro-3-indolyl-ß-D-galactoside, see Section 2.2), to detect any insertions into transcriptionally active regions of the chromosome it was found that 30% of the colonies exhibited intense blue after 48 hours at 30°C (Figure 3.2.1), suggesting insertion downstream of an active promoter.

Figure 3.2.1 - Screening of transposon insertion mutants on plates containing x-gal. Around 30% of the mutants exhibited an intense blue (very dark grey in this Figure), indicating that the insertion of Tn*917*-LTV3 occurred in a transcriptional active region. (+) represents a colony expressing β-galactosidase; and, (-) represents a colony not expressing β-galactosidase.



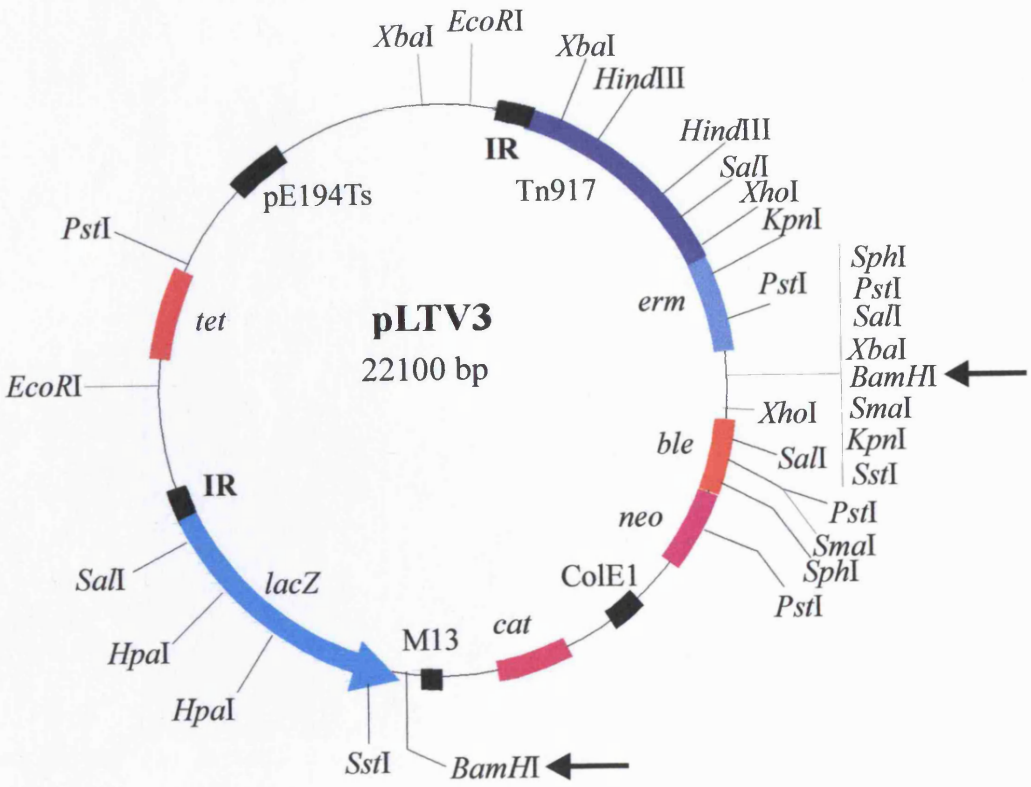
In order to confirm that Tn*917*-LTV3 had integrated randomly into the chromosome of *L. monocytogenes* 10403S, Southern Blots were performed to chromosomal DNA of 12 random transposon mutants. The chromosomal DNA of these insertion mutants was digested with *Bam*HI which cleaves twice in Tn*917*-LTV3. The digested DNA was then probed with pLTV3 DNA also digested with *Bam*HI (producing two fragments which were both used as a probe, see Figure 3.2.2) and labelled with [α-^{32}P]dCTP. Under conditions of high stringency [65°C in 5xSSC/0.1% (w/v) SDS overnight; washing three times in 1xSSC/0.1% (w/v) SDS at 65°C for 15 minutes], the probe hybridised to three different bands in each of these mutants (Figure 3.2.3, lanes 1 to 12). The sizes of the different hybridised bands are shown in Table 3.2.1. One band is of 6.8 kb, which corresponds to the internal *Bam*HI-*Bam*HI fragment specific of Tn*917*-LTV3 and is common to all of the mutants. The two other bands correspond to the reminder of the transposon plus flanking chromosomal DNA. These two "flanking" bands are of variable size dependent on the position of the insertion in the chromosome. Since the size of the "flanking" bands are identical in 3 (Figure 3.2.3; lanes 1, 9 and 10; see Table 3.21) of the 12 mutants tested we can say that a hot spot for insertion may exist or that these mutants represent siblings of the same mutation. The pattern of hybridisation with remaining mutants showed that a single but different site of insertion had occurred in each (Figure 3.2.3; Table 3.2.1). No hybridisation occurred with chromosomal DNA of wild type which was the negative control. These results suggest that although a hot spot for insertion exists, insertions outside of this site were sufficiently random to allow the use of this transposon to build a useful transposon insertion library.

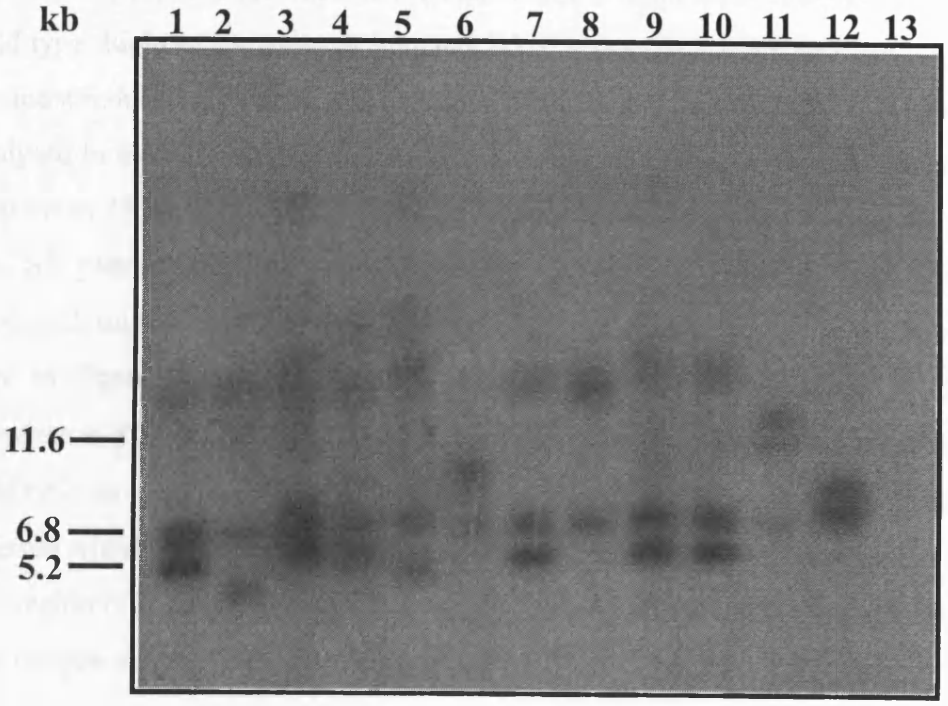
Table 3.2.1 - Estimated sizes of *L. monocytogenes* 10403S mutants DNA bands that hybridised to the probe derived from pLTV3.

Mutant	Sizes of the hybridised bands (kb)	Mutant	Sizes of the hybridised bands (kb)
1	17.3*	7	18.2*
	6.8		6.8
	5.2		5.2
2	17.3*	8	18.2*
	6.8		15.4*
	4.3		6.8
3	19.5*	9	17.3*
	6.8		6.8
	5.6		5.2
4	15.4*	10	17.3*
	6.8		6.8
	5.2		5.2
5	19.6*	11	12.9*
	6.8		11.6
	5.2		6.8
6	11.6	12	17.3*
	8.5		7.5
	6.8		6.8

* Sizes estimated by comparison to the distance to the 12.2 kb ladder band.

Figure 3.2.2 - Diagram to show the position of the *Bam*HI (arrow heads) restriction sites used to cleave pLTV3 when it was used as a probe in the Southern hybridisation analysis of the 12 transposon insertion mutants chosen at random from the insertion library. IR, represents the inverted repeats sequences (*Adapted* from Camilli *et al.*, 1990).


pLTV3
22100 bp
XbaI
EcoRI
XbaI
HindIII
HindIII
SalI
XhoI
KpnI
SphI
PstI
SalI
XbaI
BamHI
SmaI
KpnI
SstI
PstI
SmaI
SphI
PstI
IR
Tn917
erm
ble
neo
ColE1
cat
M13
IR
lacZ
tet
pE194Ts
PstI
EcoRI
SalI
HpaI
HpaI
SstI
BamHI


kb
1 2 3 4 5 6 7 8 9 10 11 12 13
11.6
6.8
5.2

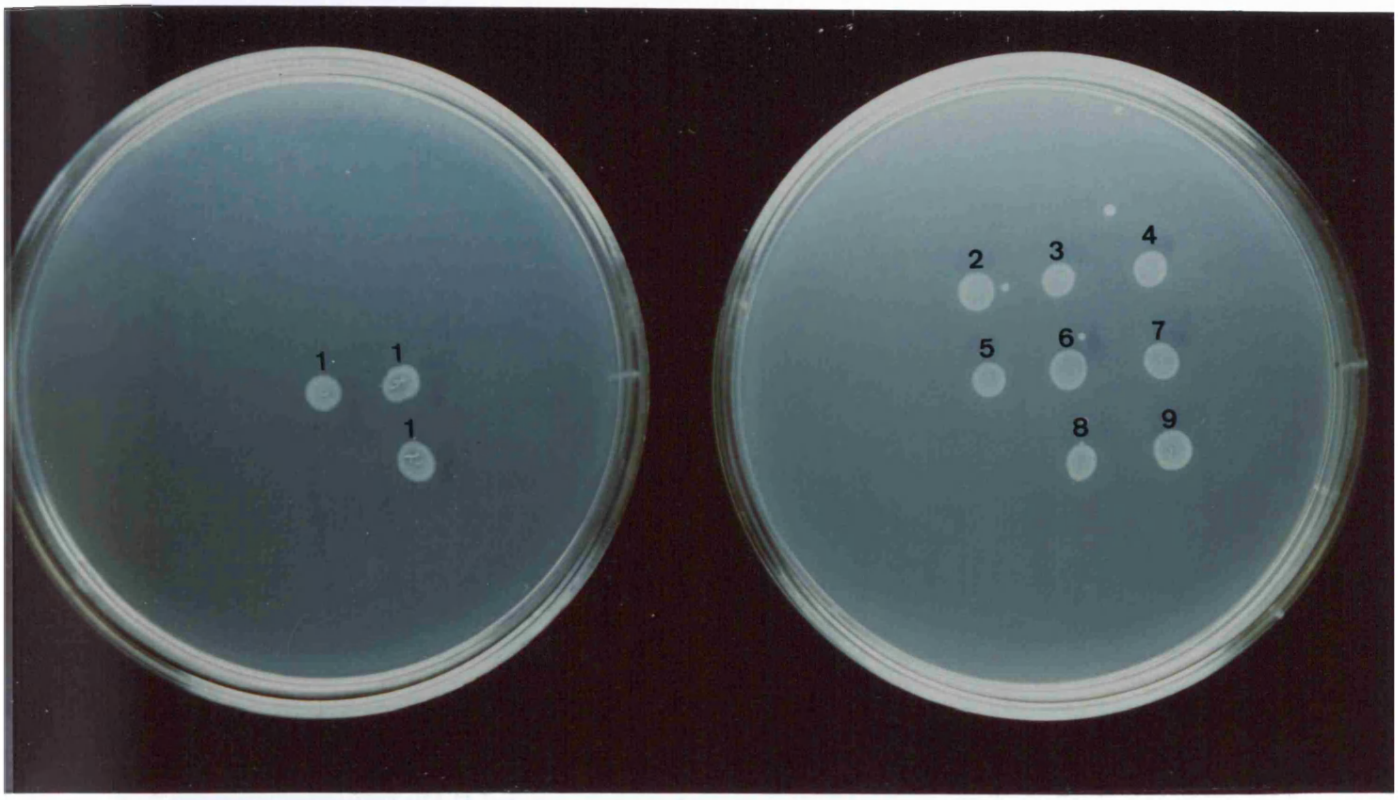
3.3 Characterisation of insertional mutants

3.3.1 Growth of mutants at low temperatures on solid medium

To identify transposon insertion mutants defective in growth at low temperatures a library of 7100 insertion mutants was screened on agar for growth at 4 and 10°C. Of these, 147 showed visibly less growth at 4°C, 48 reduced growth at 10°C and 13 showed reduced growth at 4 and 10°C when compared to the growth of the wild type as judged by eye. All of them had unaltered growth at 30°C when compared to the growth of the wild type. The 13 mutants with reduced growth at both 4 and 10°C, and 37 mutants with reduced growth at 4°C only, were grown at 4°C in TSB and growth monitored at OD_{600nm}. None of these mutants showed significantly reduced growth at low temperatures in broth when compared to the growth of the wild type. Eight of the mutants (mutants B3, C3, G3, J3, K3, L3, M3 and N3), that exhibited the most reduced growth at 4°C on TSA with a normal growth at 30°C (Figure 3.3.1.1) were analysed in more detail. Figure 3.3.1.1 was produced after growing the mutants and wild type 10403S in TSB at 30°C to an OD_{600nm} of approximately 0.6, then placing 1µl of each culture on TSA plates containing the appropriate antibiotics when needed. Two sets of plates were produced, one was incubated at 30°C overnight and the other at 4°C for 14 days. As can be seen in Figure 3.3.1.1 (A), mutants exhibited the same growth as the wild type at 30°C, however at 4°C (Figure 3.3.1.1, B) mutants showed defective growth when compared to the wild type growth. These results confirm that the ability of these mutants to grow at 4°C was affected when compared to the ability of the wild type suggesting that the transposon inserted in a region of the chromosome of *L. monocytogenes* which can be important for the growth at low temperature.

Figure 3.3.1.1 - Growth of wild type *L. monocytogenes* 10403S and mutants when incubated at 30°C (A) and 4°C (B) on solid medium (TSA).

Number 1 corresponds to wild type; and, numbers 2 through 9, corresponds to mutants B3, C3, G3, J3, K3, L3, M3 and N3. On the photographs mutants exhibiting defective growth at 4°C when compared with the wild type at the same temperature can be seen.


1
1
1
2
3
4
5
6
7
8
9


1
1
1
2
3
4
5
6
7
8
9

3.3.2 Growth of mutants at low temperature in liquid medium

In order to facilitate the screening for mutants in liquid cultures an alternative method was used. This, involved growth in TSB in microtitre plates and was used to screen 2726 mutants. Of these, five showed altered growth at 4°C, and unaltered growth at 30°C, when compared to the growth of the control. In the initial screening the OD_{630nm} of these mutants was 0.08, 0.07, 0.08, 0.06 and 0.08 (single reading only) when compared to a mean of 0.72 (mean of five readings) of the wild type after incubating 2 weeks at 4°C. When incubating at 30°C overnight, the OD_{630nm} of these mutants was 0.45, 0.91, 0.96, 0.68 and 0.83 (single reading only), when compared to a mean of 0.80 (mean of five readings) for the wild type. Although the first mutant exhibited a low OD_{630nm} at 30°C when compared to the wild type it was decided to proceed with this mutant since it exhibited a low OD_{630nm} at 4°C.

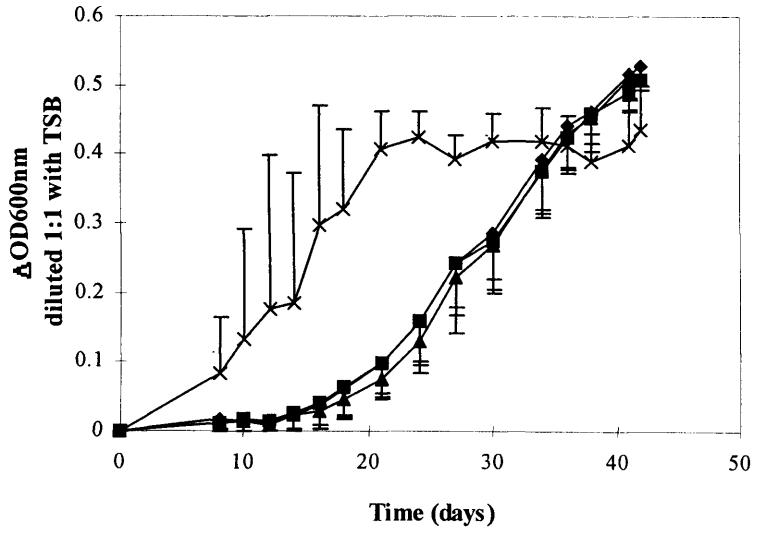
To further characterise these mutants the first three were grown in TSB and the OD_{600nm} measured, at regular intervals, to monitor growth. To facilitate subsequent analysis of the growth, growth rates were calculated and are shown in Table 3.3.2.1 and the estimated duration of the correspondent lag phases is shown in Table 3.3.2.2. At 4 and 10°C mutants displayed altered growth when compared to the growth of the wild type (Figure 3.3.2.1, A and B), exhibiting an extended lag phase prior to the onset of growth. At 4°C the mean duration of the wild type lag phase was of 9 days in contrast to that of the mutants which was of approximately 15 days, confirming that mutants take approximately twice of the time to start growth. At 10°C, mutants lag phase also lasted twice (25 hours) as that of the wild type (12 hours) further suggesting that mutants are defective in the ability to start growth at low temperatures when compared to the growth of the wild type. At 4°C the mean growth rate of the wild type was of 0.25/day as oppose to the lower mean growth rate of the mutants which ranged from 0.13 to 0.14/day. In order to demonstrate that this difference in growth between mutants and wild type was significant, data had were analysed for by analysis of variance (ANOVA). At 4°C the difference in mean growth rate between wild type and mutants was highly significant ($P<0.01$). At 10°C the mean growth rate of wild type was of 0.06/hour, while mutants were between 0.03 and 0.05/hour. ANOVA to these values revealed that the difference in mean of the growth rates was not significant ($P=0.49$). At 30°C, mutants and wild type exhibited a similar pattern of growth (Figure 3.3.2.2). At this temperature the mean duration of the mutants lag phase was 2 hours in consonance to the wild type lag phase duration which was of 1.6 hour. At this temperature the mean growth rate of wild type was 0.50/hour, with the mutants growth rates being between 0.51 and 0.55/hour. ANOVA of these

data revealed that the difference in the mean of growth was not significant (P=0.98). These results demonstrated that the mutants were defective for growth, in TSB, at 4°C, but not at 10 and 30°C, when compared to the growth of the wild type. Therefore, it can be speculated that the transposon in mutant 1, 2 and 3 inserted into a region of the *L. monocytogenes* chromosome that is significant for its growth at 4°C.

Figure 3.3.2.1 - Growth curves of *L. monocytogenes* 10403S (x) and transposon insertion mutants 1 (◆), 2 (■) and 3 (▲) in TSB incubated at 4°C (A) and 10°C (B).

Data in A are the mean ± standard deviation of 6 experiments. Data in B are the mean ± standard deviation of 3 experiments.

A


ΔOD600nm
diluted 1:1 with TSB
0.6
0.5
0.4
0.3
0.2
0.1
0
0
10
20
30
40
50
Time (days)

B

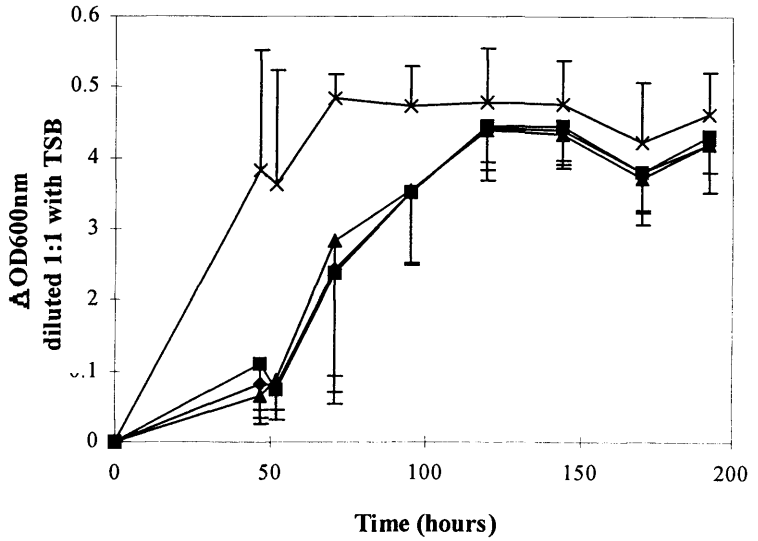

ΔOD600nm
diluted 1:1 with TSB
0.6
0.5
4
3
2
0
0
50
100
150
200
Time (hours)

Figure 3.3.2.2 - Growth curves of *L. monocytogenes* 10403S (x) and transposon insertion mutants 1(◆), 2 (■) and 3 (▲) in TSB incubated 30°C.

Data are the mean ± standard deviation of 5 experiments.

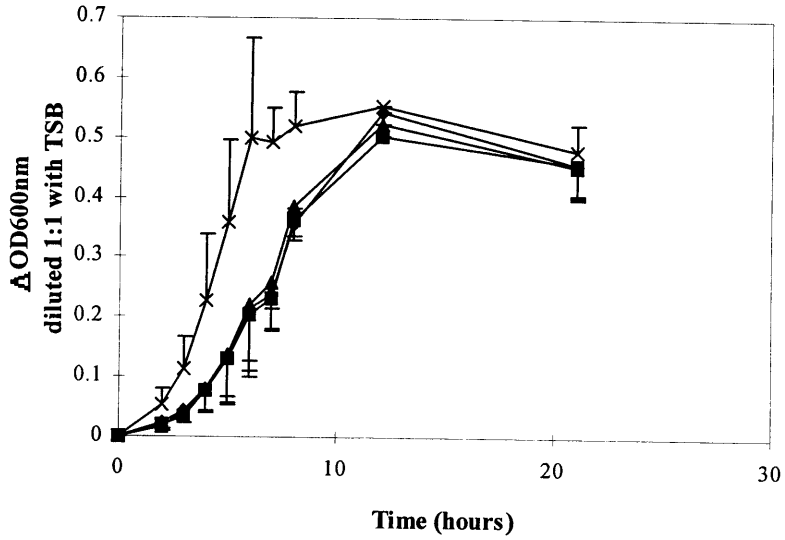

0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Δ OD600nm
diluted 1:1 with TSB
0
10
20
30
Time (hours)

Table 3.3.2.1 - Growth rates of mutants and wild type *L. monocytogenes* at 4, 10 and 30°C in TSB.

Number of Growth Curve	**Growth Rates (μmax)**			
4°C[a]	**Wild Type**	**Mutant 1**	**Mutant 2**	**Mutant 3**
1	0.26	0.12	0.11	0.13
2	0.35	0.13	0.15	0.15
3	0.32	0.13	0.14	0.15
4	0.25	0.10	0.10	0.13
5	0.24	0.15	0.13	0.15
6	0.21	0.14	0.13	0.12
Mean	0.25	0.13	0.13	0.14
Standard Deviation	±0.05	±0.02	±0.02	±0.01
10°C[b]	**Wild Type**	**Mutant 1**	**Mutant 2**	**Mutant 3**
1	0.06	0.03	0.03	0.03
2	0.04	0.06	0.03	0.09
3	0.07	0.04	0.04	0.04
Mean	0.06	0.04	0.03	0.05
Standard Deviation	±0.02	±0.02	±0.01	±0.03
30°C[b]	**Wild Type**	**Mutant 1**	**Mutant 2**	**Mutant 3**
1	0.50	0.37	0.42	0.45
2	0.43	0.62	0.57	0.52
3	0.49	0.64	0.84	0.62
4	0.56	0.46	0.46	0.47
5	0.52	0.45	0.47	0.47
Mean	0.50	0.51	0.55	0.51
Standard Deviation	±0.05	±0.12	±0.17	±0.07

[a] μmax/day

[b] μmax/hour

Table 3.3.2.2 - Duration of the lag phase correspondent to the growth curves of mutants and wild type *L. monocytogenes* at 4, 10 and 30°C in TSB.

Number of Growth Curve		**Duration of the lag phase**		
4°C[a]	**Wild Type**	**Mutant 1**	**Mutant 2**	**Mutant 3**
1	10	12	12	12
2	12	15	15	15
3	13	20	20	20
4	6	15	15	15
5	6	15	15	15
6	7	15	15	15
Mean	9	15.3	15.3	15.3
Standard Deviation	±3.1	±2.6	±2.6	±2.6
10°C[b]	**Wild Type**	**Mutant 1**	**Mutant 2**	**Mutant 3**
1	12	25	25	25
2	12	25	25	25
3	12	25	25	25
Mean	12	25	25	25
Standard Deviation	±0.0	±0.0	±0.0	±0.0
30°C[b]	**Wild Type**	**Mutant 1**	**Mutant 2**	**Mutant 3**
1	1	1	1	1
2	1	1	1	1
3	1	1	1	1
4	3	4	4	4
5	2	3	3	3
Mean	1.6	2	2	2
Standard Deviation	±0.9	±1.4	±1.4	±1.4

[a] values given in days

[b] values given in hours

3.4 Characterisation of the site of Tn*917*-LTV3 insertion

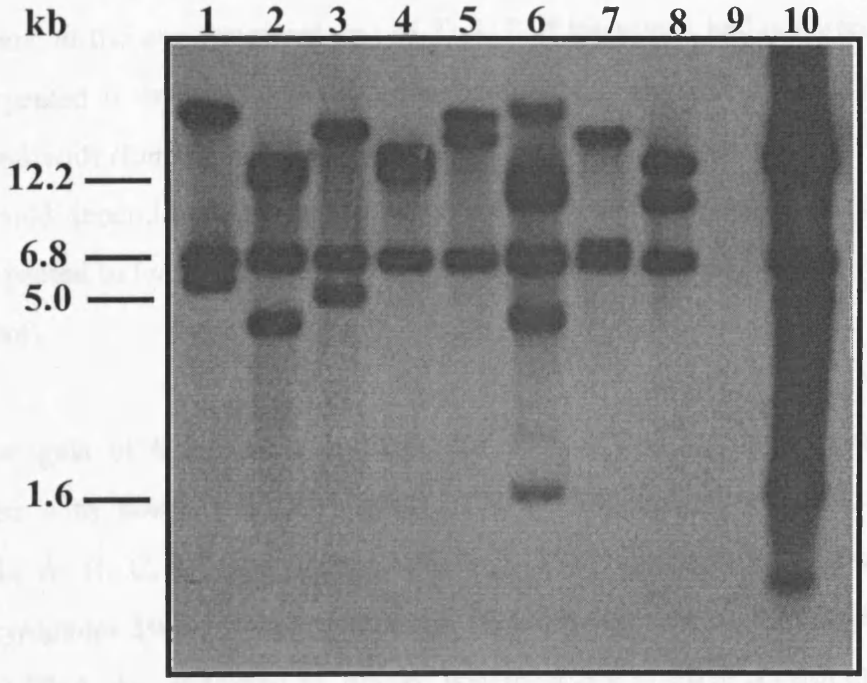
3.4.1 Mutants defective in growth at low temperature on solid medium

In order to characterise the chromosome of the insertional mutants at the site of the insertion and to confirm that Tn*917*-LTV3 had inserted singly into the chromosome of the mutants, Southern Blots experiments were performed. For the first Southern Blot the whole of pLTV3, digested with *Bam*HI (see Figure 3.2.2 in Section 3.2), was labelled by the Flourescein Gene Images system (see Section 2.6.3.2.) and used as a probe against genomic DNA from mutants B3, C3, G3, J3, K3, L3, M3 and N3 digested with *Bam*HI. Wild type DNA from *L. monocytogenes* 10403S and pLTV3 DNA, also restricted with *Bam*HI, was used in the gel as a negative and positive control respectively (Figure 3.4.1.1).

This probe, under high stringency conditions [60°C in 5xSSC/0.1% (w/v) SDS overnight, washing three times in 5xSSC/0.1% (w/v) SDS at 50°C for 15 minutes], was expected to hybridise to three bands in the chromosome of the mutants. One band should be the internal 6.8 kb *Bam*HI-*Bam*HI fragment, which is Tn*917*-LTV3 specific and should be in all of the mutants. The other two bands, represent the ends of the transposon and should include the remaining insertion sequences together with flanking chromosomal DNA. From Figure 3.4.1.1., it can be seen that the probe hybridised to the 6.8 kb band in all of the mutants (lanes 1 through 8) and that the two other flanking bands could be seen in mutants B3, C3, G3, J3, K3, M3 and N3 (lanes 1, 2, 3, 4, 5, 7 and 8). These results, indicates that a single copy of Tn*917*-LTV3 had inserted into the chromosome of these mutants. Mutant L3 (lane 6) exhibited 4 to 5 flanking bands, suggesting the existence of partial digests or that insertion did not occur in the expected way and multiple insertions had occurred in this mutant.

Figure 3.4.1.1 - Southern hybridisation of transposon mutants chromosomal DNA with a probe derived from pLTV3 showing localisation of Tn*917*-LTV3 in the chromosome of the mutants.

Chromosomal DNA was digested with *Bam*HI, analysed on 0.6% (w/v) agarose gels, and blotted to Hybond-N^+ nylon membranes. The probe used was the pLTV3 restricted by *Bam*HI (Figure 3.2.2.) resulting in two fragments, one of 6.8 kb and another one of 15.3 kb. These were labelled by the Fluorescein Gene Images system and used under conditions of high stringency [hybridisation conditions were 65°C in 5xSSC/0.1% (w/v) SDS overnight; washing was performed three times in 1xSSC/0.1% (w/v) SDS at 65°C for 15 minutes]. Lanes 1 through 8; chromosomal DNA from insertion mutants B3, C3, G3, J3, K3, L3, M3 and N3 restricted by *Bam*HI; lane 9, chromosomal DNA from wild type *L. monocytogenes* 10403S digested with *Bam*HI (is the negative control); lane 10, pLTV3 DNA digested with *Bam*HI (is the positive control).


kb
1
2
3
4
5
6
7
8
9
10
12.2
6.8
5.0
1.6

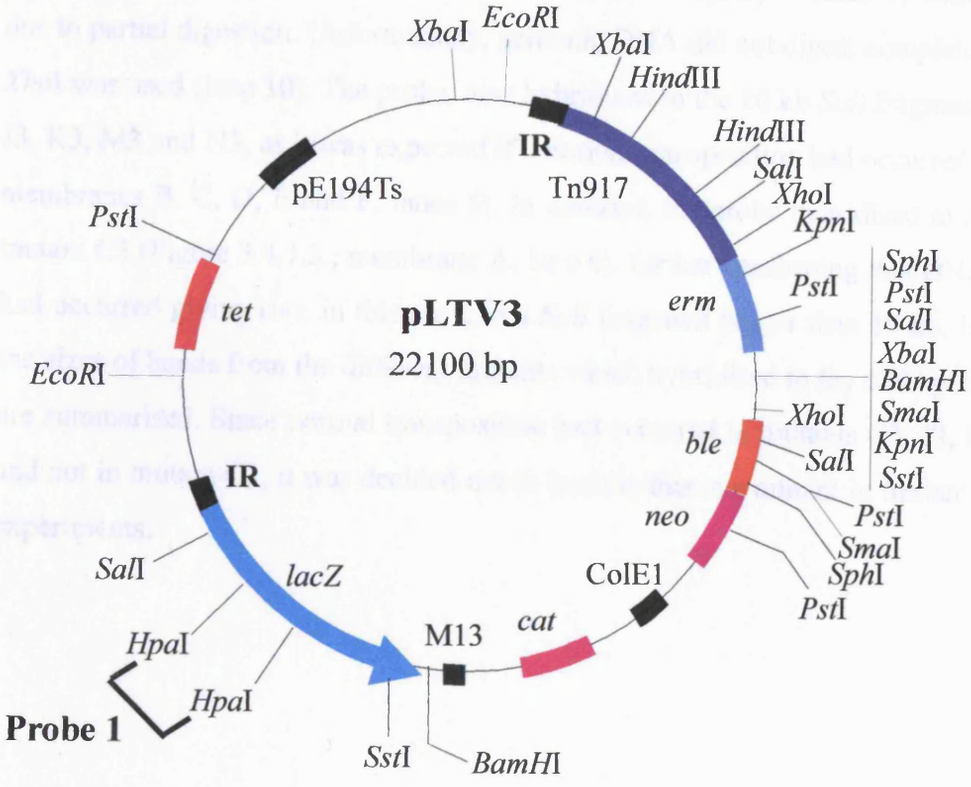
In order to check if the whole plasmid pLTV3 had also inserted into the chromosome of mutants, these were streaked on TSA plates containing erythromycin, lincomycin (antibiotics used to select for Tn*917*-LTV3 insertions) and tetracycline (antibiotic used to select for plasmid pLTV3). Mutant B3 and L3 showed growth on these plates after 48 hours of incubation at 30°C, confirming that pLTV3 had inserted into the chromosome of these mutants together with Tn*917*. Thus, it was decided to proceed with more Southern hybridisations, with the remaining mutants, in order to have a better characterisation of the chromosome at the site of the insertion.

This time, it was decided to use a probe which would hybridise to only one side of the flanking chromosomal DNA of mutants which had undergone a normal transposition. The probe chosen, was the 624 bp *Hpa*I-*Hpa*I (Probe 1, Figure 3.4.1.2) fragment specific for the *lacZ* gene, at the *erm*-proximal end of Tn*917*. If insertions had occurred normally, this probe was expected to hybridise to only one band of the genomic DNA from the mutants when restricted with *Bam*HI, *Eco*RI, *Hind*III, *Kpn*I, *Pst*I, *Sma*I, *Sst*I, *Xba*I or *Xho*I (to a band whose size would depend on the site where the insertion had occurred in the chromosome) and was also expected to hybridise to a *Sal*I fragment of 10 kb (a Tn*917*-LTV3 specific *Sal*I-*Sal*I DNA fragment).

Agarose gels of 0.6% were prepared for each of the mutants, containing genomic DNA digested with *Bam*HI, *Eco*RI, *Hind*III, *Kpn*I, *Pst*I, *Sal*I, *Sma*I, *Sst*I, *Xba*I or *Xho*I (Figure 3.4.1.3., A, B, C, D, E, F, lanes 1 through 10). Chromosomal DNA originated from *L. monocytogenes* 10403S was restricted by *Bam*HI and was used as negative control (lane 11). pLTV3 DNA also restricted by *Bam*HI was used as a positive control in lane 12. The extent of restriction digestion was checked by electrophoresis and DNA was transferred overnight to Hybond-N^+ membranes. The non-radioactive Fluorescein Gene Images system, was used for the labelling of the probe as for hybridisation and detection of the bands. High conditions of stringency were applied [65°C in 5xSSC/0.1% (w/v) SDS overnight, washing three times in 5xSSC/0.1% (w/v) SDS at 65°C for 15 minutes].

Figure 3.4.1.2 - Diagram to represent the position of a DNA probe from pLTV3, used in the Southern hybridisation analysis of the transposon insertion mutants.

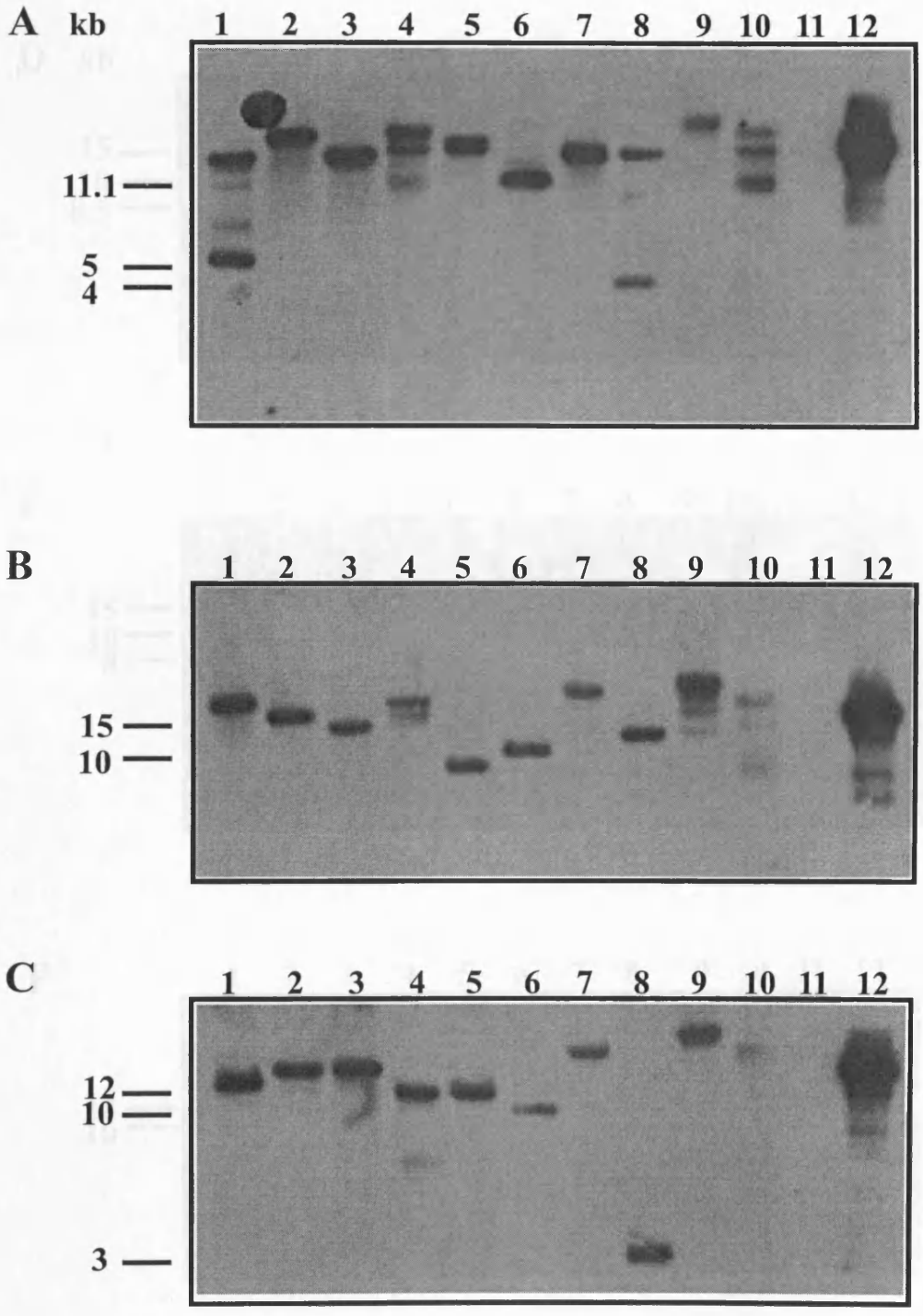
Probe 1, is a 624 bp *Hpa*I-*Hpa*I fragment specific for the *lacZ* gene at the *erm*-proximal side of transposon Tn*917*. IR, represents the inverted repeats (*Adapted* from Camilli *et al.*, 1990).


pLTV3
22100 bp
XbaI
EcoRI
XbaI
HindIII
HindIII
SalI
XhoI
KpnI
SphI
PstI
SalI
XbaI
BamHI
XhoI
SmaI
KpnI
SalI
SstI
PstI
SmaI
SphI
PstI
IR
Tn917
pE194Ts
PstI
tet
EcoRI
IR
SalI
lacZ
HpaI
HpaI
Probe 1
SstI
BamHI
M13
cat
ColE1
neo
ble
erm

As can be seen in Figure 3.4.1.3. (membrane A), the probe hybridised to 2 bands of the chromosomal DNA from mutant C3 restricted by *Bam*HI, *Kpn*I and *Sst*I (lanes 1, 4 and 8). The probe used was expected to hybridise to only one band of the chromosomal DNA digested with these enzymes since they do not have a restriction site within the *Hpa*I fragment. This results suggests that the insertion of Tn*917*-LTV3 into the chromosome of mutant C3 did not occur in the expected way, making use of the insertional sequences flanking the *lacZ* and Tn*917* genes. Thus, insertion seems to have disrupted Tn*917*-LTV3 within the *Hpa*I - *Hpa*I fragment region. Mutants G3, J3, K3, M3 and N3 (Figure 3.4.1.3., membranes B, C, D, E and F), exhibited only one band, when chromosomal DNA digested with *Bam*HI, *Eco*RI, *Hind*III, *Kpn*I, *Pst*I, *Sal*I, *Sma*I, *Sst*I and *Xba*I, hybridised to the probe. However, mutant G3 (membrane B), exhibited two bands when digested with *Kpn*I (lane 4) which most likely is due to partial digestion. Unfortunately, genomic DNA did not digest completely when enzyme *Xho*I was used (lane 10). The probe, also hybridised to the 10 kb *Sal*I fragment in mutants G3, J3, K3, M3 and N3, as it was expected if a normal transposition had occurred (Figure 3.4.1.3., membranes B, C, D, E and F, lanes 6). In contrast, the probe hybridised to a 11.1 kb *Sal*I in mutant C3 (Figure 3.4.1.3., membrane A, lane 6), further confirming that DNA rearrangement had occurred giving rise, in this case, to a *Sal*I fragment bigger than 10 kb. In Table 3.4.1.1., the sizes of bands from the different mutants which hybridised to the 624 bp *Hpa*I-*Hpa*I probe are summarised. Since normal transposition had occurred in mutants G3, J3, K3, M3 and N3, and not in mutant C3, it was decided not to include this last mutant in further characterisation experiments.

Figure 3.4.1.3 - Southern hybridisation of transposon mutants chromosomal DNA with a probe derived from pLTV3 showing localisation of Tn*917*-LTV3 in the chromosome of the mutants.

Chromosomal DNA was digested with different enzymes, analysed on 0.6% (w/v) agarose gels, and blotted to Hybond-N^+ nylon membranes. The probe used was the 624 bp *Hpa*I-*Hpa*I fragment specific from *lacZ* gene (Figure 3.4.1.2.), which was labelled by the Fluorescein Gene Images system and used under conditions of high stringency [hybridisation conditions were 65°C in 5xSSC/0.1% (w/v) SDS overnight; washing was performed three times in 1xSSC/0.1% (w/v) SDS at 65°C for 15 minutes]. Membranes A, B, C, D, E and F, corresponds to Southern Blots of mutants C3, G3, J3, K3, M3 and N3 respectively. In each of the membranes; lanes 1 through 10, mutant genomic DNA restricted by *Bam*HI, *Eco*RI, *Hind*III, *Kpn*I, *Pst*I, *Sal*I, *Sma*I, *Sst*I, *Xba*I and *Xho*I; lane 11, chromosomal DNA from wild type *L. monocytogenes* 10403S digested with *BamH*I (is the negative control); lane 10, pLTV3 DNA digested with *Bam*HI (is the positive control).


A
kb
1 2 3 4 5 6 7 8 9 10 11 12
11.1
5
4
B
1 2 3 4 5 6 7 8 9 10 11 12
15
10
C
1 2 3 4 5 6 7 8 9 10 11 12
12
10
3

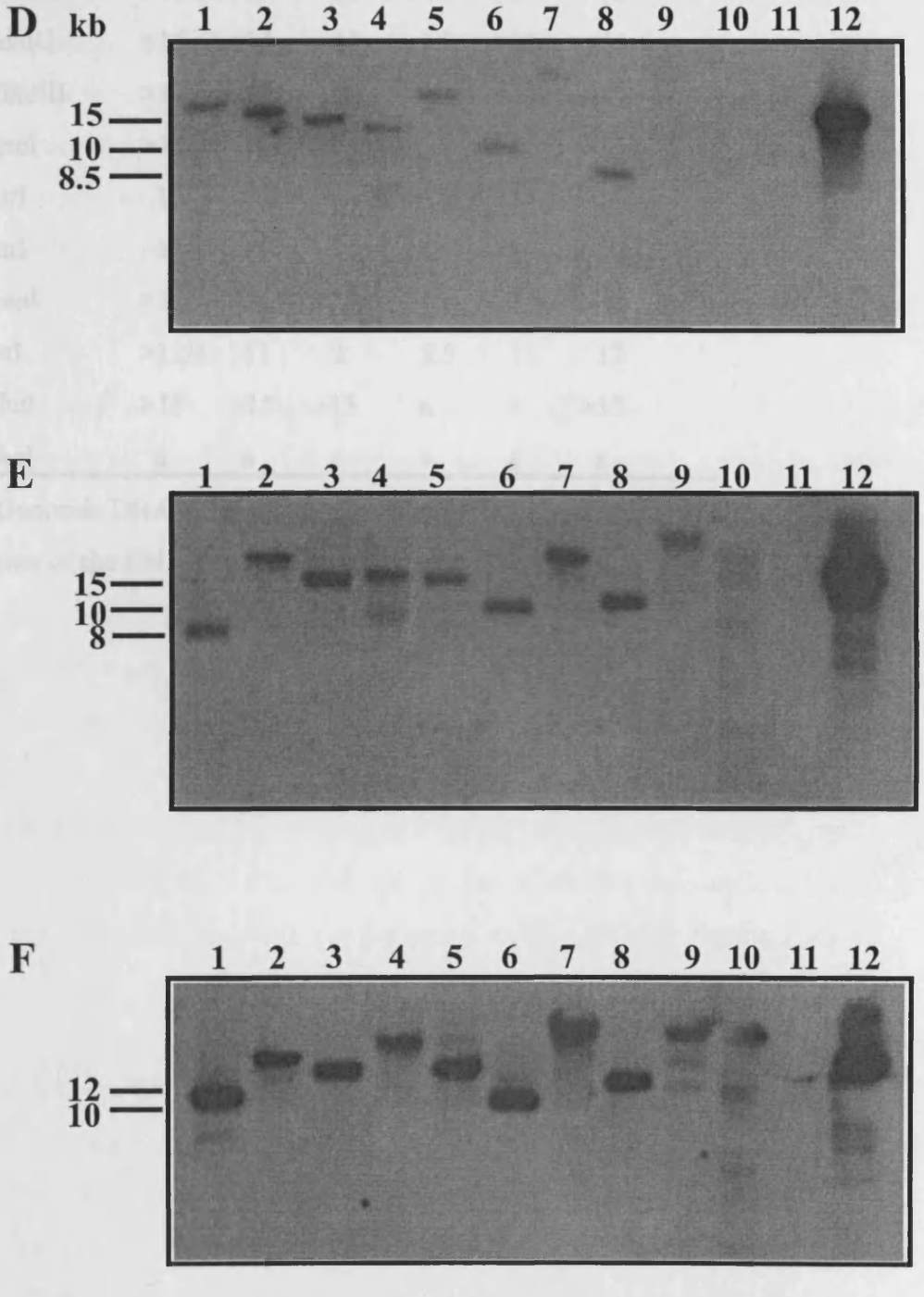

D
kb
1 2 3 4 5 6 7 8 9 10 11 12
15
10
8.5
E
1 2 3 4 5 6 7 8 9 10 11 12
15
10
8
F
1 2 3 4 5 6 7 8 9 10 11 12
12
10

Table 3.4.1.1 - Estimated sizes of the DNA fragments which hybridised to the Tn*917*-LTV3 specific 624 bp *Hpa*I-*Hpa*I band.

Enzyme	**Mutant**					
	C3	**G3**	**J3**	**K3**	**M3**	**N3**
*Bam*HI	>12/5	>15	12	>15	8	10
*Eco*RI	>15	15	15	15	>15	>15
*Hind*III	>12	12	15	>12	15	15
*Kpn*I	>15/15	>15	11	12	15	>15
*Pst*I	15	8.5	11	>15	15	15
*Sal*I	11.1	10	10	10	10	10
*Sma*I	>12	>15	>15	>15	>15	>15
*Sst*I	>12/4	11	3	8.5	11	12
*Xba*I	>15	>15	>15	a	a	>15
*Xho*I	a	a	a	a	a	a

[a] Genomic DNA did not digest to completion

Sizes of the DNA fragments are represented in kb.

3.4.1.1 Looking for homology to *cspL* (listerial cold shock protein gene)

In order to check if the gene disrupted by Tn*917*-LTV3 in the mutants was *cspL* (the gene encoding for the listerial cold shock protein) or any of the other 4 homologous genes belonging to the *csp* family (Francis *et al.*, 1995), Southern Blots were performed to genomic DNA of the mutants, using a probe derived from *cspL*. This probe contained 181 bp of *cspL* (nucleotides 380 - 561 in the sequence on the database with the accession number X91789) and had been shown that on probing chromosomal DNA from *L. monocytogenes* at high stringency one discrete band was produced but when hybridisation was repeated at low stringency, multiple bands were observed indicating that *L. monocytogenes* has a family of major cold shock protein genes, containing at least 4 homologous gene sequences (Francis *et al.*, 1995). This probe was kindly provided by Dr Cath Rees, University of Nottingham.

Genomic DNA from mutants G3, J3, K3, M3 and N3 was restricted by *Pst*I or *Xba*I (enzymes known not to digest the *cspL* gene in strain 23074) together with chromosomal DNA from *L. monocytogenes* 10403S, *L. monocytogenes* 23074 and pLTV3 plasmid DNA. *Listeria monocytogenes* 23074 is the strain in which *cspL* was first identified and was the positive control; pLTV3 was the negative control. Restriction enzyme digested DNA was electrophoresed in a 0.6% (w/v) agarose gel and transferred overnight into a Hybond-N^+ membrane. The DNA probe was labelled by the Fluorescein Gene Images system (see Section 2.6.3.2), and hybridisation was performed under low stringency conditions [42°C in 5xSSC/0.1% (w/v) SDS overnight; washing three times in 2xSSC/0.1% (w/v) SDS at 42°C for 15 minutes]. For the detection of the bands, the Fluorescein Gene Images system was also used and the manufacturer's instructions were followed (see Section 2.6.3.2).

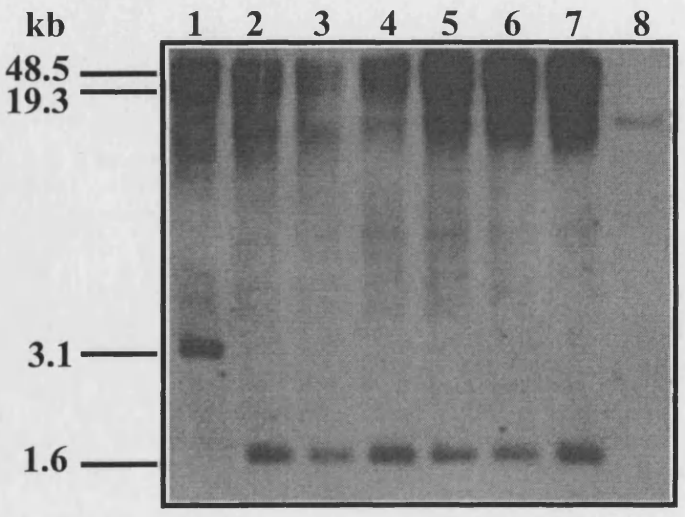
Figure 3.4.1.1.1 shows the pattern of hybridisation exhibited by *L. monocytogenes* wild type 23074 (lane 1), wild type 10403S (lane 2) and mutants (lanes 3 through 7) when restricted by *Pst*I. It can be seen, in this Figure, that the probe hybridised to *Pst*I fragments of high molecular weight and to a fragment of 1.6 kb in wild type 10403S and mutants genomic DNA. If insertion of Tn*917*-LTV3 into the chromosome of the mutants had occurred within one of the genes belonging to the *csp* family, the pattern of hybridisation exhibited by mutants would have been different from that exhibited by the corresponding wild type, since Tn*917*-LTV3 DNA would had introduced new *Pst*I sites into the genome. *Listeria monocytogenes* 23074 (Figure 3.4.1.1.1, lane 1) did not produce the same pattern of hybridisation as *L. monocytogenes* 10403S (lane 2). Both strains exhibited high molecular weight *Pst*I fragments

that hybridised to the probe and one smaller fragment, however, the size of the smallest fragment was 3.1 kb in *L. monocytogenes* 23074 and 1.6 kb in *L. monocytogenes* 10403S. These results suggest a polymorphism of the *csp* family in *L. monocytogenes* strains.

In order to further confirm the results obtained above, another Southern blot was performed to the chromosomal DNA of *L. monocytogenes* wild type 23074, wild type 10403S, and mutants digested with *Xba*I. Three fragments of sizes 48.5, 17 and 12.2 kb from wild type 10403S (Figure 3.4.1.1.2, lane 2) and mutants (lanes 3 through 7), hybridised to the probe. Once again the pattern of hybridisation exhibited by the wild type was the same as that exhibited by the mutants, confirming that the insertion of Tn*917*-LTV3 into the chromosome of the mutants did not occur within one of the genes belonging to the *csp* family. *Listeria monocytogenes* wild type 23074 (lane 1) also exhibited 3 bands that hybridised to the probe, however, the sizes were 48.5, 17 and 6.2 kb. In the same way as for the wild type genomic DNA digested with *Pst*I, the pattern of hybridisation of the *Xba*I digested wild type DNA differ in one of the fragments between both wild type strains further confirming the existence of a polymorphism within the *csp* family in these strains.

Figure 3.4.1.1.1 - Southern hybridisation of transposon mutants chromosomal DNA with a probe derived *cspL*, under low stringency conditions.

Chromosomal DNA was digested with *Pst*I, analysed on 0.6% (w/v) agarose gels, and blotted to Hybond-N^+ nylon membranes. The probe used was a 181 kb fragment from *cspL* (Francis *et al.*, 1995), which was labelled by the Fluorecein Gene Images system and used under conditions of low stringency [hybridisation conditions were 42°C in 5xSSC/0.1% (w/v) SDS overnight; washing was performed three times in 2xSSC/0.1% (w/v) SDS at 42°C for 15 minutes]. Lanes 1, *L. monocytogenes* 23074 restricted by *Pst*I as the positive control; lane 2, wild type *L. monocytogenes* 10403S digested with *Pst*I; lanes 3 through 7, chromosomal DNA from insertion mutants G3, J3, K3, M3, and N3 restricted by *Pst*I; lane 8, pLTV3 DNA digested with *Pst*I as the negative control.


kb
1
2
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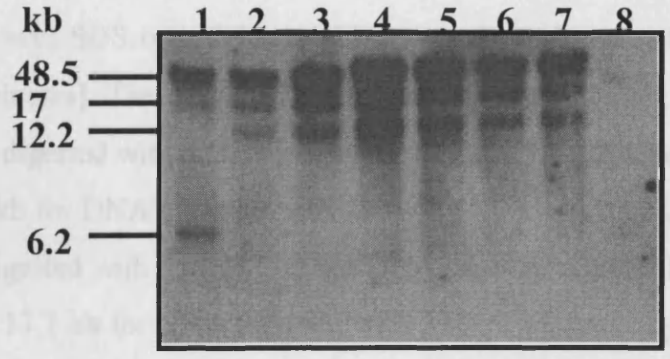
4
5
6
7
8
48.5
19.3
3.1
1.6

Figure 3.4.1.1.2 - Southern hybridisation of transposon mutants chromosomal DNA with a probe derived *cspL*, under low stringency conditions.

Chromosomal DNA was digested with *Xba*I, analysed on 0.6% (w/v) agarose gels, and blotted to Hybond-N^+ nylon membranes. The probe used was a 181 kb fragment from *cspL* (Francis *et al.*, 1995), which was labelled by the Fluorecein Gene Images system and used under conditions of low stringency [hybridisation conditions were 42°C in 5xSSC/0.1% (w/v) SDS overnight; washing was performed three times in 2xSSC/0.1% (w/v) SDS at 42°C for 15 minutes]. Lanes 1, *L. monocytogenes* 23074 restricted by *Xba*I as the positive control; lane 2, wild type *L. monocytogenes* 10403S digested with *Xba*I; lanes 3 through 7, chromosomal DNA from insertion mutants G3, J3, K3, M3, and N3 restricted by *Xba*I; lane 8, pLTV3 DNA digested with *Xba*I as the negative control.


kb
1 2 3 4 5 6 7 8
48.5
17
12.2
6.2

3.4.2 Mutants defective for growth at low temperature in liquid medium

To confirm that there is a single insertion of Tn*917*-LTV3 in the chromosome of the mutants and at the same time to construct a physical map of the chromosome at the site of the insertion, Southern blots were performed with the chromosomal DNA of the mutants using radiolabelled probes derived from Tn*917*-LTV3. For this, chromosomal DNA from mutant 1 digested with different enzymes (*Bam*HI, *Hind*III, *Pst*I, *Sal*I, *Xba*I or *Xho*I) and chromosomal DNA from mutant 2 and 3 only cleaved with *Hind*III was probed with DNA derived from Tn*917*-LTV3. The digested chromosomal DNA from mutant 2 and 3 was used in these blots to give an insight to whether these mutants had the insertion at the same site of the chromosome or in a different site from mutant 1. The first probe used was a 1.6 kb *Xba*I fragment which spans one end of Tn*917* (Figure 3.4.2.1, Probe 1). This, was expected to hybridise to the chromosome at only one of the ends of the inserted Tn*917*-LTV3. Consequently, the chromosome at this side could be characterised in detail. The probe was labelled with [α-32P]dCTP and used under conditions of high stringency [65°C in 5xSSC/0.1% (w/v) SDS overnight; washing was three times in 1xSSC/0.1% (w/v) SDS at 65°C for 15 minutes]. The probe hybridised to only one fragment of the chromosomal DNA from mutant 1 digested with different enzymes (Figure 3.4.2.2, lanes 1 to 8). The approximate sizes were 15 kb for DNA digested with *Bam*HI, 17.7 kb for DNA digested with *Hind*III, 7.9 kb for DNA digested with *Pst*I, 9.1 kb for DNA digested with *Sal*I, 1.6 kb for DNA digested with *Xba*I and 17.7 kb for DNA digested with *Xho*I. This probe hybridised to the same sized *Hind*III (17.7 kb) fragment in each of the three mutants (Figure 3.4.2.2, lanes 2, 7 and 8). Since the probe hybridised to only one fragment of the chromosomal DNA of mutant 1 digested with the different enzymes, it confirmed that there was a single insertion of the Tn*917*-LTV3 in the chromosome of the mutant 1. Considering that the hybridised *Hind*III fragment was the same in all three mutants, it suggests that the insertion was in the same site in these mutants.

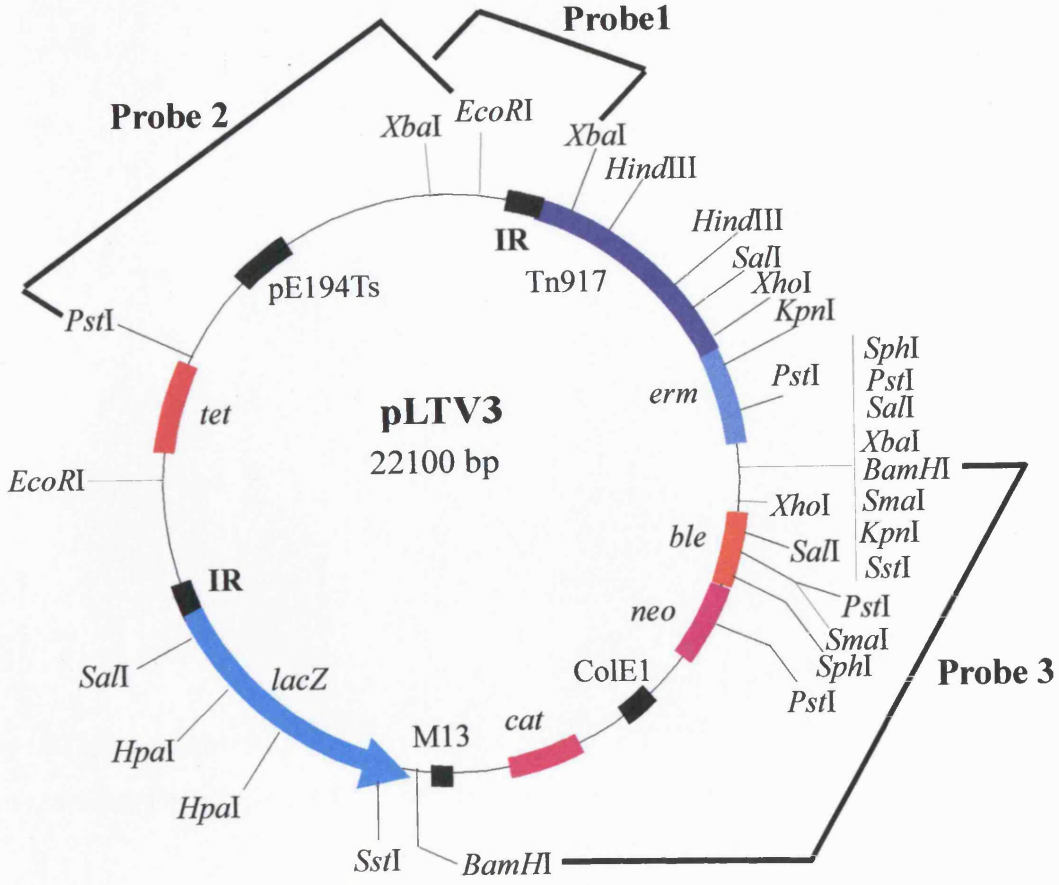
To check whether the plasmid pLTV3 had inserted into the chromosome of the mutants, together with the transposon, a 2.8 kb *Eco*RI-*Pst*I fragment external to the insertional sequences (Figure 3.4.2.1, Probe 2) was used as a probe in a Southern blot containing chromosomal DNA from mutant 1, 2 and 3 cleaved with the same enzymes as on the Southern Blot described in the previous paragraph (Figure 3.4.2.3). The probe was also labelled with [α-32P]dCTP and used under conditions of high stringency [65°C in 5xSSC/0.1% (w/v) SDS overnight; washing was three times in 1xSSC/0.1% (w/v) SDS at 65°C for 15 minutes]. This

probe hybridised to the chromosomal DNA of all three mutants (Figure 3.4.2.3, lanes 1 to 8). The approximate sizes of the mutant 1 chromosomal DNA fragments that hybridised to the probe were 15, 17.7, 7.9, 9.1, and 23.1 kb for digests with *Bam*HI, *Hind*III, *Pst*I, *Sal*I and *Xho*I respectively (Figure 3.4.2.3, lanes 1 through 4, and 6). For mutant 1 chromosomal DNA digested with *Xba*I, two fragment exhibited homology to the probe (Figure 3.4.2.3, lane 5) with 15 and 1.6 kb in size. Interestingly one of the *Xba*I hybridised fragments (1.6 kb) was of the same size as the first probe used in these Southern blots (Figure 3.4.2.2). The *Hind*III fragment from mutant 2 and 3 (Figure 3.4.2.3, lanes 7 and 8) that hybridised to the probe was of 17.7 kb, as for mutant 1. These results, suggest that vector also inserted into the chromosome of these mutants. Neither probe, 1 or 2, hybridised to chromosomal DNA of wild type *L. monocytogenes* 10403S (Figure 3.4.2.2 and 3.4.2.3, lane 9).

When mutants 1, 2 and 3 were streaked on plates containing erythromycin, lincomycin and tetracycline, no growth was detected after 24 hours of incubation at 30°C. However, when plates were left to incubate for longer then 48 hours some growth could be seen, confirming that the plasmid encoded tetracycline gene is present in these mutants.

Figure 3.4.2.1 - Diagram to represent the position of DNA probes from pLTV3, used in the Southern hybridisation analysis of the transposon insertion mutants.

Probe 1, represents a 1.6 kb *Xba*I restriction fragment spanning the insertional sequences of the transposon Tn*917*. Probe 2, represents a 2.8 kb *Eco*RI-*Pst*I restriction fragment from pLTV3 external to the insertional sequences. Probe 3, represents a 6.8 kb *Bam*HI-*Bam*HI restriction fragment from Tn*917*-LTV3 spanning the kanamycin and the chloramphenicol antibiotic resistance genes. IR, represents the inverted repeats (*Adapted* from Camilli *et al.*, 1991).


Probe1
Probe 2
Probe 3
XbaI
EcoRI
XbaI
HindIII
HindIII
SalI
XhoI
KpnI
SphI
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SalI
XbaI
BamHI
SmaI
KpnI
SstI
PstI
SmaI
SphI
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PstI
tet
pLTV3
22100 bp
EcoRI
IR
SalI
lacZ
HpaI
HpaI
SstI
BamHI
M13
cat
ColE1
neo
ble
erm
XhoI
SalI

Figure 3.4.2.2 - Southern hybridisation of transposon mutants chromosomal DNA with a probe derived from pLTV3 showing the position of Tn*917*-LTV3 in the chromosome of the mutants.

Chromosomal DNA was digested with different enzymes, analysed on 0.7% (w/v) agarose gels, and blotted to Hybond-N^+ nylon membranes. The probe used was the 1.6 kb *Xba*I fragment, spanning the insertional sequence (Figure 3.4.2.1, probe 1), which was labelled with [α-32P]dCTP and used under conditions of high stringency [hybridisation conditions were 65°C in 5xSSC/0.1% (w/v) SDS overnight; washing was performed three times in 1xSSC/0.1% (w/v) SDS at 65°C for 15 minutes]. Lanes 1 through 6; chromosomal DNA from insertion mutant 1 restricted by *Bam*HI, *Hind*III, *Pst*I, *Sal*I, *Xba*I and *Xho*I; lanes 7 and 8, chromosomal DNA from insertion mutant 2 and 3 digested with *Hind*III; lane 9, chromosomal DNA from wild type *L. monocytogenes* 10403S digested with *Hind*III (is the negative control); lane 10, pLTV3 DNA digested with *Bam*HI (is the positive control).

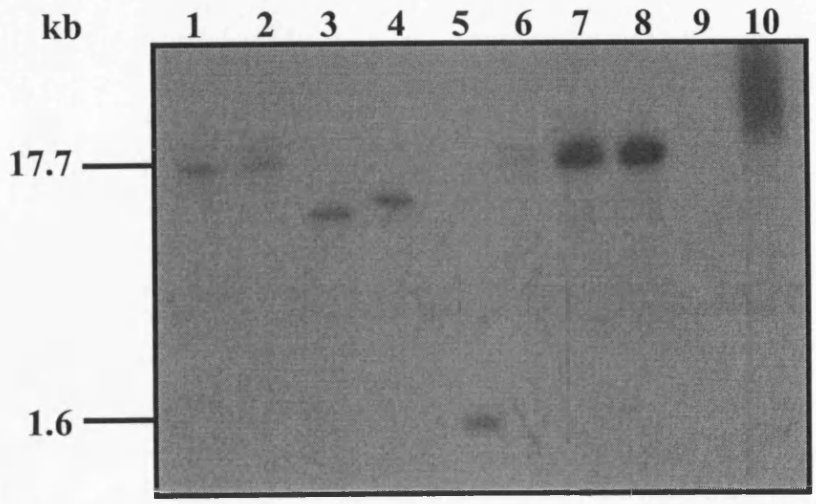
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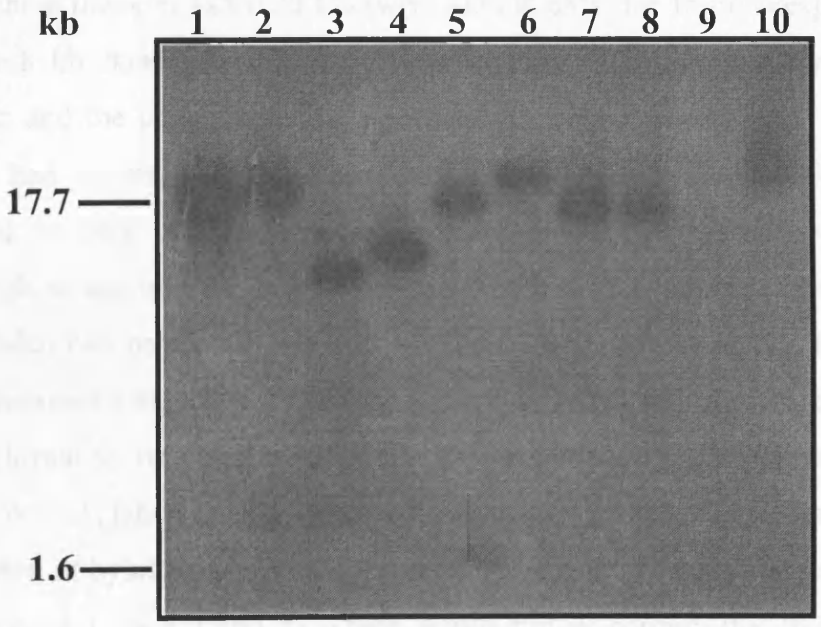
Figure 3.4.2.3 - Southern hybridisation of transposon mutants chromosomal DNA with a probe derived from pLTV3 showing the position of pLTV3 in the chromosome of the mutants.

Chromosomal DNA was digested with different enzymes, analysed on 0.7% (w/v) agarose gels, and blotted to Hybond-N^+ nylon membranes. The probe used was the 2.8 kb *Eco*RI-*Pst*I fragment external to the insertional sequences (Figure 3.4.2.1, probe 2), which was labelled with [α-32P]dCTP and used under conditions of high stringency [hybridisation conditions were 65°C in 5xSSC/0.1% (w/v) SDS overnight; washing was performed three times in 1xSSC/0.1% (w/v) SDS at 65°C for 15 minutes]. Lanes 1 through 6; chromosomal DNA from insertion mutant 1 restricted by *Bam*HI, *Hind*III, *Pst*I, *Sal*I, *Xba*I and *Xho*I; lanes 7 and 8, chromosomal DNA from insertion mutant 2 and 3 digested with *Hind*III; lane 9, chromosomal DNA from wild type *L. monocytogenes* 10403S digested with *Hind*III (as the negative control); lane 10, pLTV3 DNA digested with *Bam*HI (as the positive control). The fact that the probe hybridised to chromosomal DNA of all three mutants suggests that plasmid sequences had inserted into the chromosome.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3.4.2.1 Map of the chromosome at the site of insertion

Hybridisation patterns between the suicide vector and the chromosomal DNA of the insertion mutants suggested that these mutants resulted from some form of illegitimate recombination event, rather than transposition, in which the suicide vector also has become integrated into the chromosome. Therefore caution had to be taken in selecting the fragment containing the flanking chromosomal DNA in order to not clone mainly plasmid DNA. Thus, more Southern hybridisations were performed.

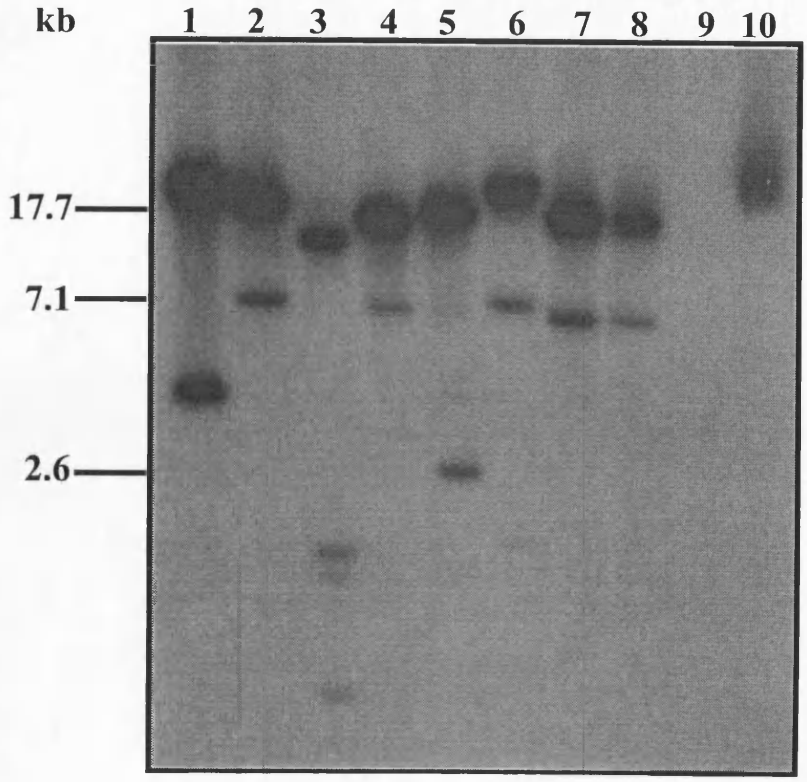
Chromosomal DNA from mutant 1 was digested with *Bam*HI, *Hind*III, *Pst*I, *Sal*I, *Xba*I and *Xho*I, and chromosomal DNA from mutants 2 and 3 was digested with *Hind*III. The DNA was then probed, under high stringency conditions [65°C in 5xSSC/0.1% (w/v) SDS overnight; washing three times in 1xSSC/0.1% (w/v) SDS at 65°C for 15 minutes], with Tn*917*-LTV3-specific 6.8 kb *Bam*HI-*Bam*HI restriction fragment DNA probe spanning the kanamycin, bleomycin and the chloramphenicol antibiotic resistance genes (Figure 3.4.2.1, Probe 3). If insertion had occurred taking advantage of the inverted repeats, this probe would had hybridised to only one band of the chromosomal DNA digested with *Bam*HI, which corresponds to the internal fragment of the Tn*917*-LTV3 insertion. However, the Southern Blot revealed two bands with sizes of 23.1 and 3.9 kb (Figure 3.4.2.1.1, lane 1), suggesting that the insertion occurred within this region creating these two flanking fragments. The probe also hybridised to two fragments of the mutant chromosomal DNA digested with *Hind*III (Figure 3.4.2.1.1, lane 2), with 17.7 and 7.1 kb in size, instead of only one flanking band. The same pattern of hybridisation was produced with mutants 2 and 3 when digested with *Hind*III (Figure 3.4.2.1.1, lane 7 and 8). These results further confirm that these three mutants are identical and that may be they are siblings of the same mutation. Chromosomal DNA digested with *Pst*I, *Sal*I, *Xba*I and *Xho*I produced 4, 2, 2 and 3 bands respectively that hybridised to the probe. The sizes of the *Pst*I fragments were 10, 1.4, 0.84 and <0.66 kb. If normal insertion had occurred only three bands would hybridise to the probe, two bands of less than 1 kb which corresponds to internal fragments and one flanking band bigger than 8 kb. The two internal bands were revealed by this probe together with two other flanking fragments. The bands expected for the *Sal*I digest were two internal bands, one of around 0.5 kb and another of 9 kb. The Southern blot produced two bands, one of 15 kb and another of 6.5 kb. Since these two fragments are bigger then the expected internal fragments it can be suggested that these are flanking fragments, however the smaller of the internal bands should had hybridised. The probe was expected to hybridise to one flanking *Xba*I fragment bigger than 9.6 kb, however,

the probe hybridised to two fragments, one of 15 kb and a smaller one of 2.6 kb. These are considered to be *Xba*I flanking fragments. The bands expected with the *Xho*I digest were an internal band of 1.7 kb and a flanking band bigger than 9.5 kb. The bands produced by the Southern blot were the internal 1.7 kb and two other bands of 23.1 and 6.5 kb. These last two bands are believed to be the insertional flanking bands. In general the pattern of hybridisation produced with these enzymes suggest that the flanking chromosomal DNA can be identified with this probe or probes chosen within the 6.8 kb *Bam*HI-*Bam*HI fragment.

The map of the chromosome of mutant 1 at the site of the insertion was constructed as described on Figure 3.4.2.1.2. A flanking *Bam*HI fragment of 3.9 kb was chosen for subsequent cloning since it was estimated that would contain approximately 3 kb of flanking chromosomal DNA.

Figure 3.4.2.1.1 - Southern hybridisation of transposon mutants chromosomal DNA with a probe derived from pLTV3 showing localisation of Tn*917*-LTV3 in the chromosome of the mutants.

Chromosomal DNA was digested with different enzymes, analysed on 0.7% (w/v) agarose gels, and blotted to Hybond-N^+ nylon membranes. The probe used was the 6.8 kb *Bam*HI-*Bam*HI fragment spanning the lincomycin, kanamycin and chloramphenicol resistance genes (Figure 3.4.2.1, probe 3), which was labelled with [α-^{32}P]dCTP and used under conditions of high stringency [hybridisation conditions were 65°C in 5xSSC/0.1% (w/v) SDS overnight; washing was performed three times in 1xSSC/0.1% (w/v) SDS at 65°C for 15 minutes]. Lanes 1 through 6; chromosomal DNA from insertion mutant 1 restricted by *Bam*HI, *Hind*III, *Pst*I, *Sal*I, *Xba*I and *Xho*I; lanes 7 and 8, chromosomal DNA from insertion mutant 2 and 3 digested with *Hind*III; lane 9, chromosomal DNA from wild type *L. monocytogenes* 10403S digested with *Hind*III (is the negative control); lane 10, pLTV3 DNA digested with *Bam*HI (is the positive control).


kb
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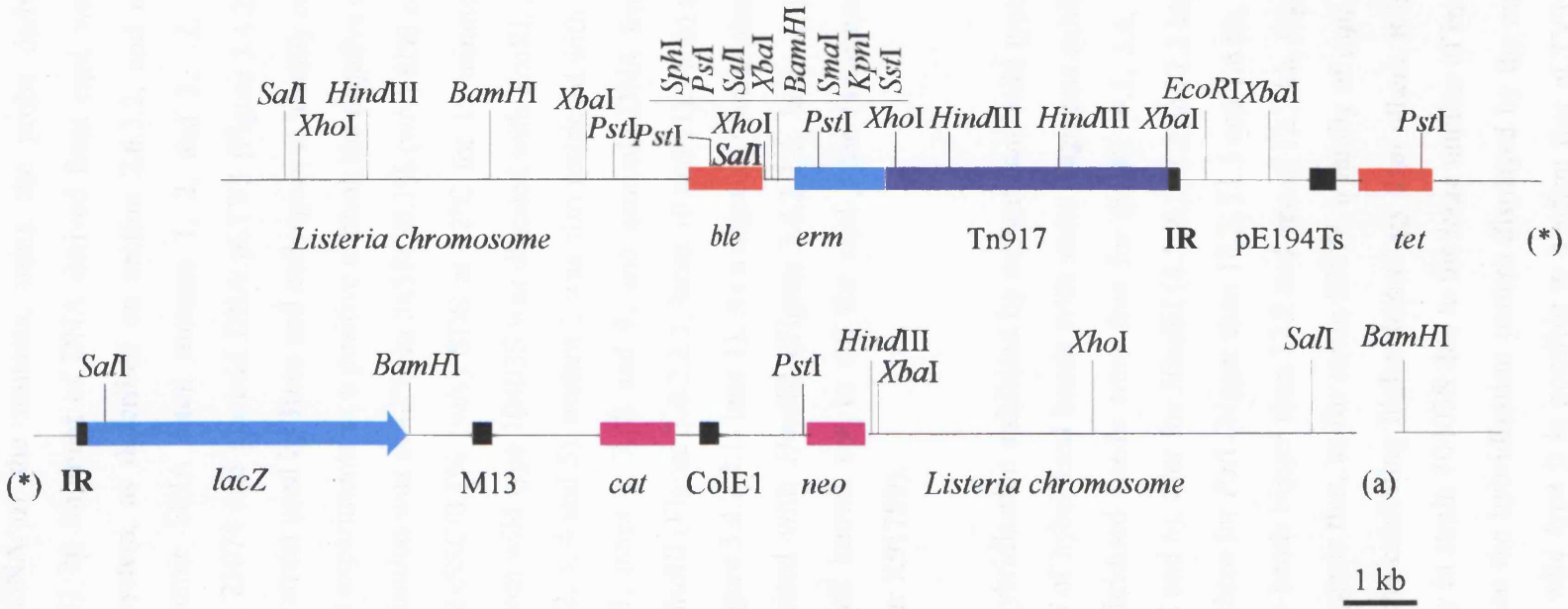
2
3
4
5
6
7
8
9
10
17.7
7.1
2.6

Figure 3.4.2.1.2 - Map of the chromosome of mutant 1 (and probably mutants 2 and 3) at the site of the insertion of pLTV3.

pLTV3 sequences are represented between the *ble* and *neo* gene which had to be separated due to the lack of space (*); line, represents the *Listeria* chromosome. (a) represents a gap of 10 kb in the *Listeria* chromosome.


SalI
HindIII
XhoI
BamHI
XbaI
PstI
PstI
SphI
PstI
SalI
XbaI
BamHI
SmaI
KpnI
SstI
XhoI
SalI
PstI
XhoI
HindIII
HindIII
XbaI
EcoRI
XbaI
PstI
Listeria chromosome
ble
erm
Tn917
IR
pE194Ts
tet
(*)
SalI
BamHI
PstI
HindIII
XbaI
XhoI
SalI
BamHI
(*)
IR
lacZ
M13
cat
ColE1
neo
Listeria chromosome
(a)
1 kb

3.4.2.2 Looking for homology to *cspL* (Listerial cold shock protein gene)

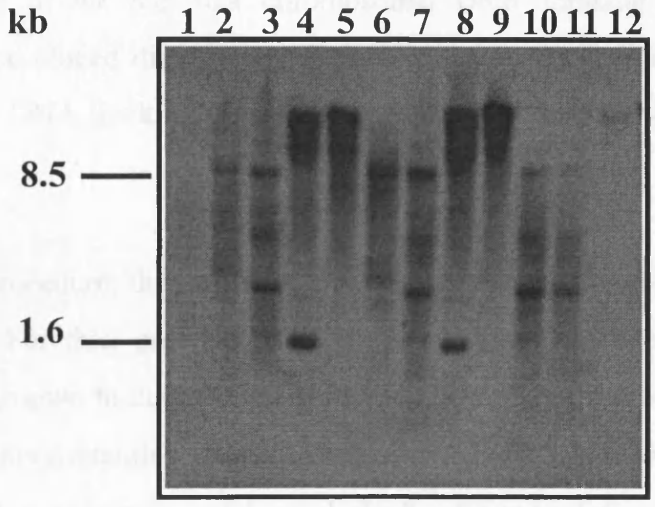
In order to check if the gene disrupted by pLTV3 in mutants 1, 2 and 3 was *cspL* or any of the 4 homologues of the *cspL* family (Francis *et al*, 1995), a Southern Blots was performed to the chromosomal DNA of the mutants, using the probe derived from *cspL* (see Section 3.4.1.1).The 181 bp fragment of DNA derived from *cspL* was labelled by the Fluorescein Gene Images system, as described on section 2.6.3.2, and used in hybridisations against digests of genomic DNA from mutants 1, 2, and 3, *L. monocytogenes* 10403S, *L. monocytogenes* 23074 and plasmid DNA pLTV3 (Figure 3.4.2.2.1). *Listeria monocytogenes* 23074, was the strain used by Rees and colleagues to identify *cspL* (Francis *et al.*, 1995) and, was used in this experiments as a positive control; the negative control was the plasmid DNA. For this, hybridisation was at 42°C in 5xSSC/0.1% (w/v) SDS overnight followed by washing three times in 2xSSC/0.1% (w/v) SDS at 42°C for 15 minutes. In this Southern, genomic DNA derived from wild type 10403S was digested with *Eco*RI, *Hind*III, *Pst*I and *Xba*I (Figure 3.4.2.2.1, lanes 2, 3, 4 and 5), mutant 1 was also restricted with *Eco*RI, *Hind*III, *Pst*I and *Xba*I (Figure 3.4.2.2.1, lanes 6, 7, 8 and 9) and genomic DNA from mutant 2 and 3 was only digested with *Hind*III (Figure 3.4.2.2.1, lanes 10 and 11). Wild type 23074 was only digested with *Hind*III (Figure 3.4.2.2.1, lane 1). As a negative control plasmid DNA (pLTV3) was used after being digested with *Hind*III (Figure 3.4.2.2.1, lanes 12). The enzymes used in this Southern were all known not to cut the *cspL* gene (sequence on the database under the accession number X91789).

The pattern of hybridisation exhibited by mutants and wild type 10403S was the same. This included a range of hybridised bands with some fragments exhibiting stronger hybridisation. The strongly hybridised bands were five for *Eco*RI (9.1, 5.6, 4.4, 3.3 and 3.0 kb, Figure 3.4.2.2.1, lanes 2 and 6), four for *Hind*III (8.5, 4.2, 2.2 and 1.3 kb, Figure 3.4.2.2.1, lanes 3, 7, 10 and 11), and three for *Pst*I (bigger than 12.2, 11.1 and 1.6 kb, Figure 3.4.2.2.1, lanes 4 and 8) and *Xba*I (two bands bigger than 12.2 and one of 12.2 kb, Figure 3.4.2.2.1, lanes 5 and 9). These results suggests that, as for strain 23074, a family of *cspL* homologues exists in strain 10403S, with some exhibiting higher homology than others. It is not possible to predict the size of this family in strain 10403S due to the large number of bands that exhibited homology to the probe. Since the hybridisation pattern displayed by the mutants was the same as that displayed by the wild type it is possible to confirm that pLTV3 did not disrupted the *cspL* gene or any of the homologues in mutants 1, 2 or 3. Unfortunately DNA from *L.*

monocytogenes 23074 got nucleased not displaying any hybridisation pattern (Figure 3.4.2.2.1, lane 1).

Figure 3.4.2.2.1 - Southern hybridisation of transposon mutants chromosomal DNA with a probe derived *cspL* (Francis *et al.,* 1995), under low stringency conditions.

Chromosomal DNA was digested with different enzymes, analysed on 0.6% (w/v) agarose gels, and blotted to Hybond-N^+ nylon membranes. The probe used was the 181 kb fragment from *cspL*, which was labelled by the Fluorescein Gene Images system and used under conditions of low stringency [hybridisation conditions were 42°C in 5xSSC/0.1% (w/v) SDS overnight; washing was performed three times in 2xSSC/0.1% (w/v) SDS at 42°C for 15 minutes]. Lanes 1 chromosomal DNA from wild type *L. monocytogenes* 23074 restricted by *Hind*III (is the positive control); lanes 2 through 5 chromosomal DNA from wild type *L. monocytogenes* 10403S digested with *EcoR*I, *Hind*III, *Pst*I and *Xba*I; lanes 6 through 9 chromosomal DNA from insertion mutant 1 restricted by *EcoR*I, *Hind*III, *Pst*I and *Xba*I; lanes 10 and 11, chromosomal DNA from insertion mutant 2 and 3 digested with *Hind*III; lane 12 pLTV3 DNA digested with *Hind*III (is the negative control).


kb
1 2 3 4 5 6 7 8 9 10 11 12
8.5
1.6

3.5 Cloning of chromosomal sequences flanking Tn*917*-LTV3 insertion sites

Following transposon mutagenesis, DNA adjacent to the transposon insertion may be rescued into *E. coli* simply by digesting total chromosomal DNA with an appropriate restriction enzyme, which cuts outside the selectable marker *neo* and the ColE1 replication functions; ligating at a dilute DNA concentration and transforming an *E. coli* strain.

3.5.1 Cloning of chromosomal sequences flanking Tn*917*-LTV3 insertion sites of mutants defective for growth on solid medium at low temperature

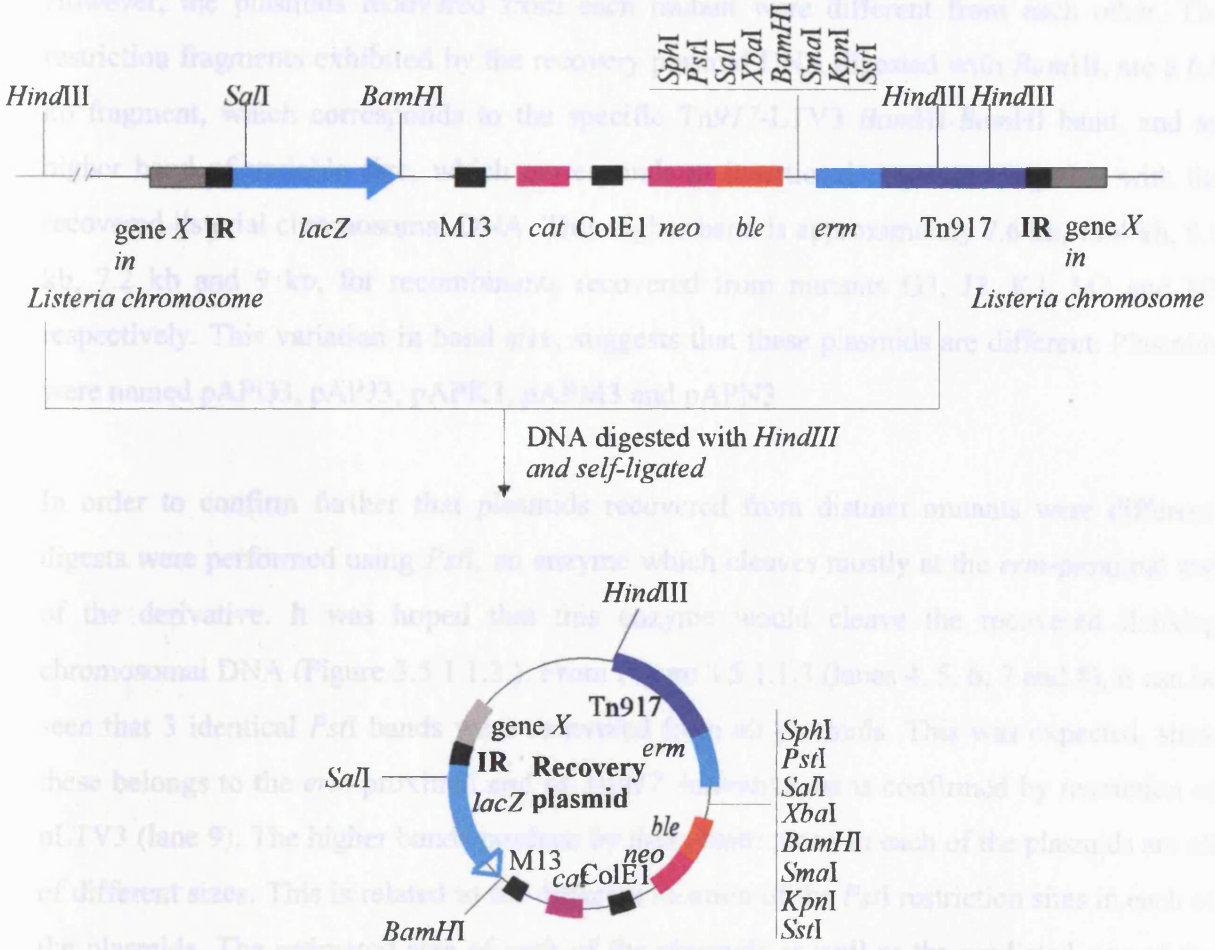
3.5.1.1 Cloning of chromosomal sequences flanking the *erm*-proximal end of Tn*917*-LTV3

Taking advantage of the fact that chromosomal DNA flanking the sites of transposon insertions could be cloned directly into *E. coli* by using the ColE1 replicon within Tn*917*-LTV3, we cloned DNA flanking the *erm*-proximal end of the insertion from mutants G3, J3, K3, M3 and N3.

For the cloning procedure, the method of Youngman (1990) was followed as is described in Figure 3.5.1.1.1. For this, genomic DNA from mutants was digested to completion with *Hind*III (enzyme known to cut outside of the selectable marker *neo* and the ColE1 replicon, producing fragments containing flanking chromosomal DNA), followed by ligation in a 150μl volume, at a DNA concentration of 5μg/ml. Such a diluted solution for ligation will promote self-ligation. After inactivation of the ligation reaction, DNA was used to transform electro-competent *E. coli* MC1061 and transformants were selected for kanamycin. The choice of the *E. coli* host is very important, since the Tn*917 erm* gene product may be toxic to typical *E. coli* strains due to its ability to methylate ribosomal RNA (Youngman, 1990). However, this can be avoided by using a strain, such as MC1061, that contains an *rpsl* mutation which alters the ribosome in a way that confers streptomycin resistance. Transformation efficiency in *E. coli* MC1061 were approximately $5x10^3$ transformants/μg genomic DNA

Figure 3.5.1.1.1 - Cloning listerial DNA sequences adjacent to a Tn*917*-LTV3 insertion with the recovery vector.

To clone the DNA bordering the *erm*-proximal end of the transposon insertion, chromosomal DNA from *L. monocytogenes* mutants was digested with restriction enzymes that cut outside of the selectable marker *neo* and the ColE1 replicon, such as *Hind*III. The digests are then ligated at concentrations that promote self-ligation to give the "recovery plasmid". Finally, ligation products were precipitated and used to transform *E. coli*, selecting for clones containing plasmids expressing kanamycin resistance. IR - represents inverted repeats.


SphI
PstI
SalI
XbaI
BamHI
SmaI
KpnI
SstI
HindIII
SalI
BamHI
HindIII HindIII
gene X IR
in
Listeria chromosome
lacZ
M13
cat
ColE1
neo
ble
erm
Tn917
IR
gene X
in
Listeria chromosome
DNA digested with HindIII
and self-ligated
HindIII
Tn917
gene X
IR
Recovery
plasmid
lacZ
erm
ble
neo
cat
ColE1
M13
SalI
BamHI
SphI
PstI
SalI
XbaI
BamHI
SmaI
KpnI
SstI

All kanamycin resistant transformants recovered from each mutant should contain the same plasmid, since only one fragment originated from the mutant genomic DNA should contain the ColE1 replicon together with the *neo* gene. To verify this, plasmid DNA was extracted from 3 transformants recovered from each mutant, digested with *Bam*HI and loaded alongside each other in a 0.7% agarose gel (Figure 3.5.1.1.2. (A) for mutants G3, J3 and K3; (B) for mutants M3 and N3). Figure 3.5.1.1.2. shows that, plasmid DNA originated from the 3 clones recovered from each of the mutants produced exactly the same bands when digested with *Bam*HI, consistent with them originating from the same self-ligated fragment.

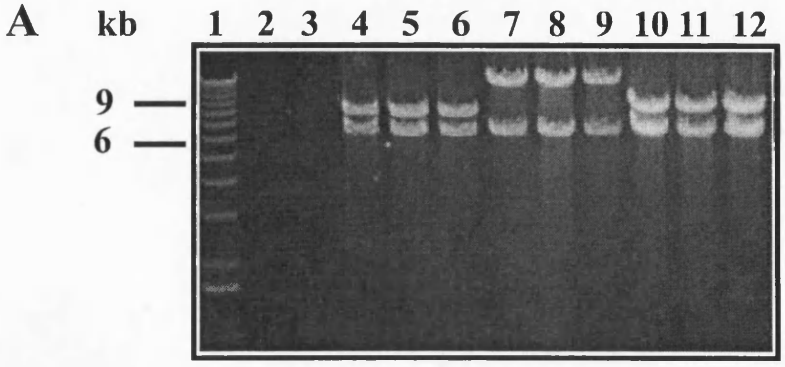
However, the plasmids recovered from each mutant were different from each other. The restriction fragments exhibited by the recovery plasmid DNA digested with *Bam*HI, are a 6.8 kb fragment, which corresponds to the specific Tn*917*-LTV3 *Bam*HI-*Bam*HI band, and an higher band of variable size, which corresponds to insertional sequences together with the recovered listerial chromosomal DNA. This higher band is approximately 7.6 kb, 11.4 kb, 8.6 kb, 7.2 kb and 9 kb, for recombinants recovered from mutants G3, J3, K3, M3 and N3 respectively. This variation in band size, suggests that these plasmids are different. Plasmids were named pAPG3, pAPJ3, pAPK3, pAPM3 and pAPN3.

In order to confirm further that plasmids recovered from distinct mutants were different, digests were performed using *Pst*I, an enzyme which cleaves mostly at the *erm*-proximal end of the derivative. It was hoped that this enzyme would cleave the recovered flanking chromosomal DNA (Figure 3.5.1.1.3.). From Figure 3.5.1.1.3 (lanes 4, 5, 6, 7 and 8), it can be seen that 3 identical *Pst*I bands were recovered from all plasmids. This was expected, since these belongs to the *erm*-proximal end of Tn*917* derivative, as is confirmed by restriction of pLTV3 (lane 9). The higher bands produce by these restrictions in each of the plasmids are all of different sizes. This is related to the different location of the *Pst*I restriction sites in each of the plasmids. The estimated size of each of the plasmids as well as the predicted size of the listerial chromosomal DNA recovered in each of them is detailed on Table 3.5.1.1.1. These results confirm that these plasmids are different from each other, however, it does not mean that it is a different gene that was inactivated in the listerial chromosome since the derivative could had inserted at different sites within the same gene.

Figure 3.5.1.1.2 - Restriction enzyme digests of plasmids recovered from transposon insertion mutants.

A) Plasmid DNA recovered from 3 transformants colonies from mutant G3 (lanes 4, 5 and 6), mutants J3 (lanes 7, 8 and 9) and mutant K3 (lanes 10, 11 and 12), digested with the restriction enzyme *Bam*HI. Lanes 1 and 13, 1 kb DNA size marker (GIBCO-BRL), fragment sizes are shown in the left hand column in kilobases (kb).

B) Plasmid DNA recovered from 2 transformants colonies from mutant M3 (lanes 2, 3 and 4) and mutant N3 (lanes 5, 6 and 7), digested with the restriction enzyme *Bam*HI. Lane 1, 1 kb DNA size marker (GIBCO-BRL), fragment sizes are shown in the left hand column in kilobases (kb).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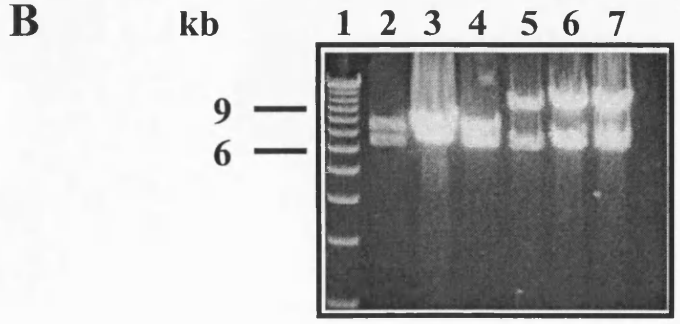
9
6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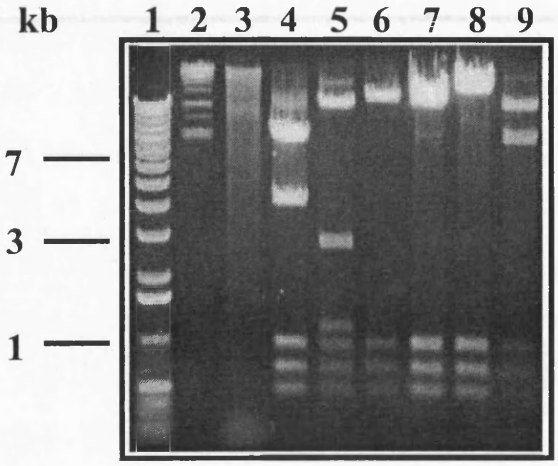

kb
1 2 3 4 5 6 7 8 9
7
3
1

Figure 3.5.1.1.3 - Restriction enzyme digests of plasmids recovered from transposon insertion mutants.

Lanes 4 through 8, *Pst*I digested pAPG3, pAPJ3, pAPK3, pAPM3 and pAPN3 respectively. Lane 3, *L. monocytogenes* 10403S digested by *Pst*I. Lane 9, pLTV3 restricted by *Pst*I. Lane 1, 1 kb DNA size marker (GIBCO-BRL). Lane 2, High Molecular Weight DNA markers (GIBCO-BRL).

Table 3.5.1.1.1 - Estimated sizes of the recovery plasmids and predicted sizes of the listerial chromosomal insert.

Plasmid	Total size of plasmid (kb)	Listerial DNA insert (kb)
pAPG3	14.4	1.7
pAPJ3	18.2	5.5
pAPK3	15.4	2.7
pAPM3	14.0	1.3
pAPN3	15.8	3.1

To generate a restriction map of the listerial chromosomal DNA recovered from each of the mutants, a range of restriction enzymes were used in single and double digests of pAPG3, pAPJ3, pAPK3, pAPM3 and pAPN3. The map deduced from these digests is detailed in Figure 3.5.1.1.4. In the insert of pAPG3, only a unique *Pst*I site was identified. Two *Kpn*I restriction sites together with 2 *Pst*I restriction sites, were identified in the listerial DNA in plasmid pAPJ3. Insert DNA of plasmid pAPK3, contains one restriction site for *Hpa*I, and one restriction site for *Xba*I. pAPM3 insert DNA, has a unique restriction site for *Hpa*I. Sites for *Bam*HI, *Hpa*I, *Kpn*I, *Pst*I, *Sal*I, *Sph*I and *Xho*I, were not found in the insert of pAPN3.

With the aim of confirming that listerial DNA was actually recovered in the collection of pAP plasmids, and at the same time to investigate if it was the same listerial gene that was inactivated in each of the mutants, Southern Blots analysis were performed. The probes chosen, for these experiments, were derived from each recovery plasmids and were used against wild type DNA digested with several enzymes, and *E. coli* K12 genomic DNA restricted by *Bam*HI (as a control). The probes used are described in Figure 3.5.1.1.4. These were: the approximately 2 kb *Sal*I-*Hind*III band from pAPG3, which contains the end of the *lacZ* gene together with the inverted repeats and insert DNA. The probe chosen from pAPJ3 was the approximate 5.5 kb *Sal*I-*Hind*III band containing insert DNA together with the *erm*-proximal end of the derivative. From pAPK3, the approximate 2 kb *Sal*I-*Xba*I was used, and contains the *erm*-proximal end of the derivative sequence together with 1.6 kb of insert DNA. From clones pAPM3 and pAPN3, the probes chosen were *Sal*I-*Hind*III which contains the inverted repeats sequence together with insert DNA. Under high stringency conditions [65°C in 5xSSC/0.1% (w/v) SDS overnight; washing was performed three times in 1xSSC/0.1% (w/v) SDS at 65°C for 15 minutes], all the probes hybridised to wild type DNA digested with *Bam*HI, *Eco*RI, *Hind*III, *Kpn*I, *Pst*I, *Sal*I, *Sma*I, *Sph*I, *Sst*I and *Xba*I (Figure 3.5.1.1.5.; membranes A to E, lanes 1 through 10). None of the probes used, hybridised to the *E. coli* DNA restricted by *Bam*HI (Figure 3.5.1.1.5.; membranes A to E, lanes 11). In previous Southern Blots when using pLTV3-derived probes (Sections 3.3), no hybridisation ever occurred to the *L. monocytogenes* 10403S genomic DNA. This time, probes selected from all of the transformants, hybridised to wild type DNA, confirming that *Listeria* DNA was recovered in each of the plasmids.

Figure 3.5.1.1.4 - Restriction map of the pAP recovery plasmids from *L. monocytogenes* transposon insertion mutants.

Boxed areas indicate pLTV3 DNA, while open areas represent the listerial DNA insert and filled areas show the inverted repeats. The full line below the insert region represents the probe used in Southern Blots analysis. The interrupted line below the insert sequences represents the DNA fragment which was subcloned into pBluescript for sequencing proposes.

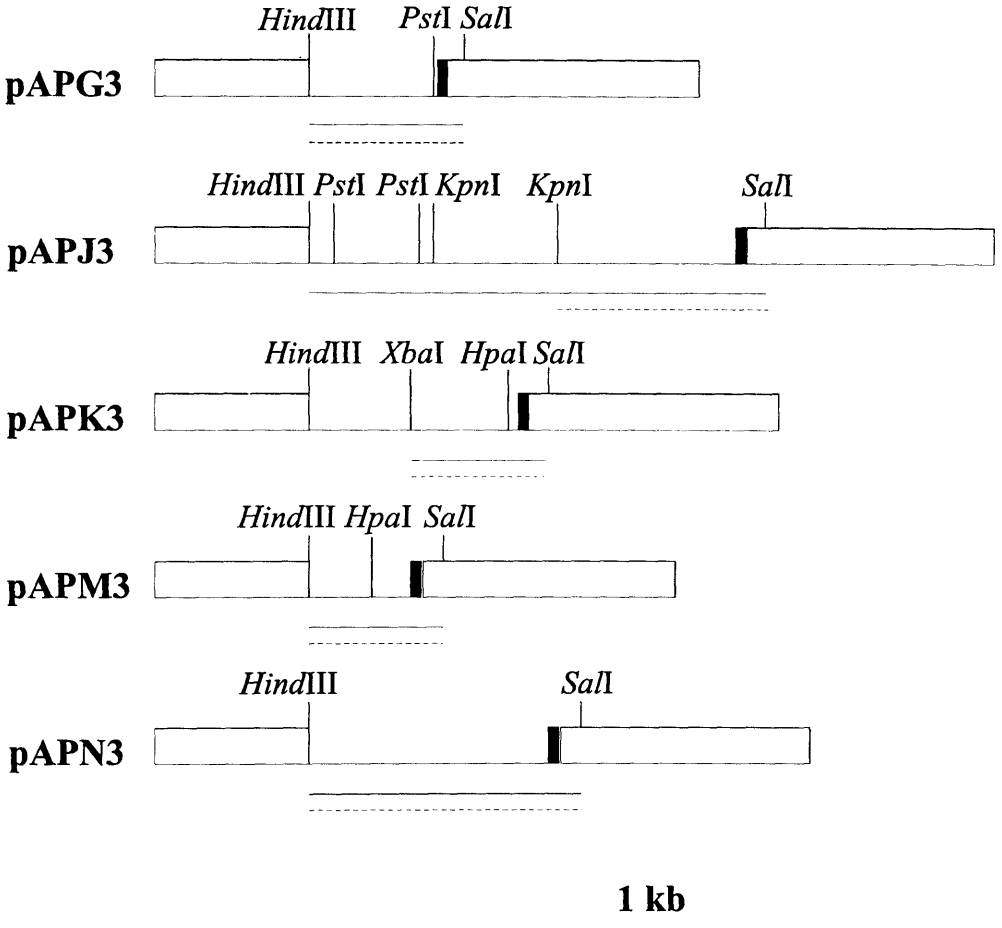
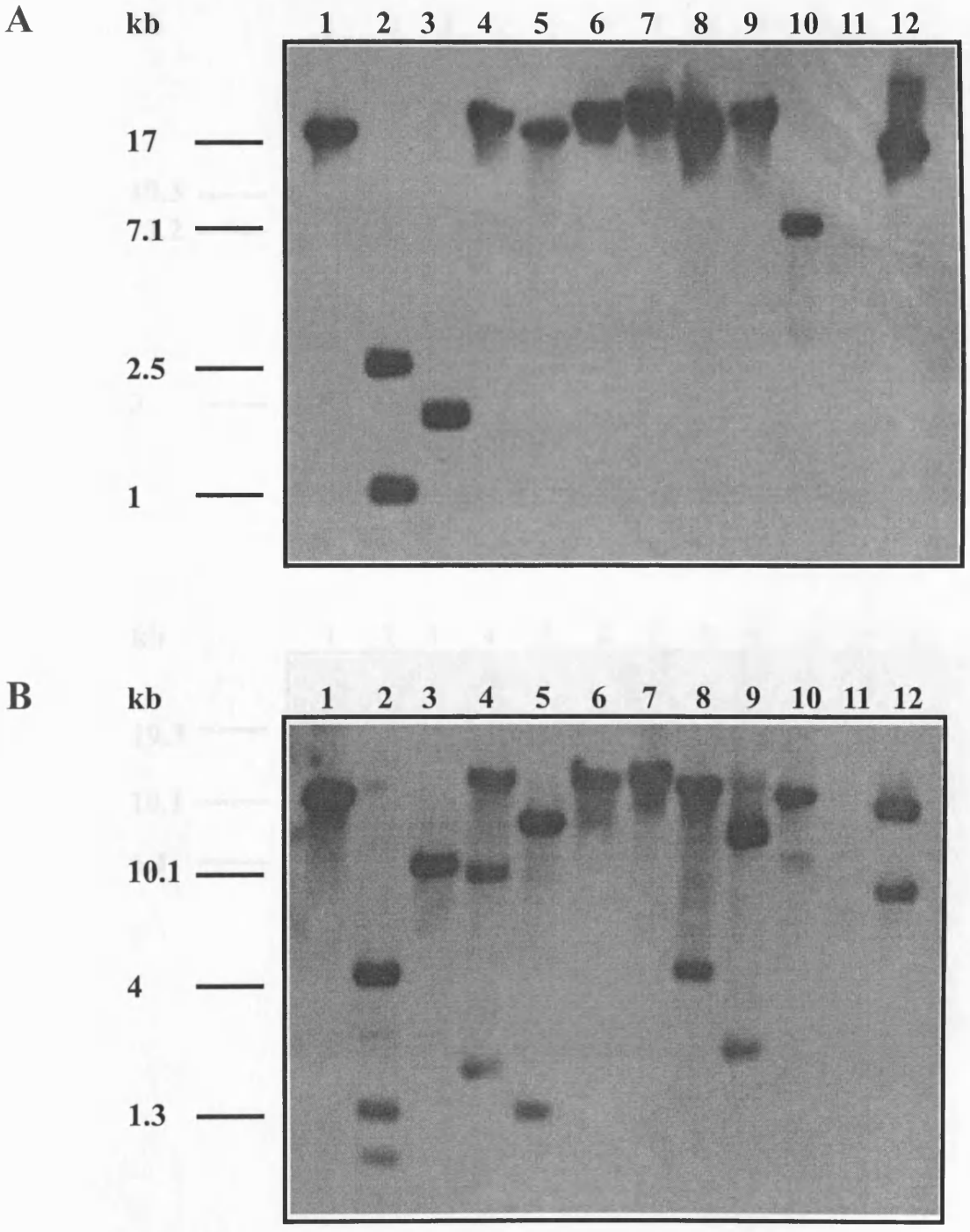

HindIII
PstI
SalI
pAPG3
HindIII
PstI
PstI
KpnI
KpnI
SalI
pAPJ3
HindIII
XbaI
HpaI
SalI
pAPK3
HindIII
HpaI
SalI
pAPM3
HindIII
SalI
pAPN3
1 kb

Figure 3.5.1.1.5 - Southern hybridisation of *L. monocytogenes* 10403S (wild type) genomic DNA with probes derived from insert DNA recovered by plasmids pAP.

Wild type genomic DNA was digested with different enzymes, analysed on a 0.6% (w/v) agarose gels, and blotted to Hybond-N^+ nylon membranes. The probes used were derived from the insert DNA in plasmids pAP and are represented on Figure 3.5.1.1.4., which were labelled by the Fluorescein Gene Images system (Section 2.6.3.2) and used under conditions of high stringency [hybridisation conditions were 65°C in 5xSSC/0.1% (w/v) SDS overnight; washing was performed three times in 1xSSC/0.1% (w/v) SDS at 65°C for 15 minutes]. Figures A, B, C, D and E, corresponds to Southern Blots using probes extracted from pAPG3, pAPJ3, pAPK3, pAPM3 and pAPN3 respectively. In each of the membranes; lanes 1 through 10, corresponds to wild type genomic DNA restricted by *Bam*HI, *Eco*RI, *Hind*III, *Kpn*I, *Pst*I, *Sal*I, *Sma*I, *Sph*I, *Sst*I and *Xba*I; lane 11, genomic DNA from *E. coli* K12 restricted by *Bam*HI; lane 12, pLTV3 restricted by *BamH*I.


A
kb
1 2 3 4 5 6 7 8 9 10 11 12
17
7.1
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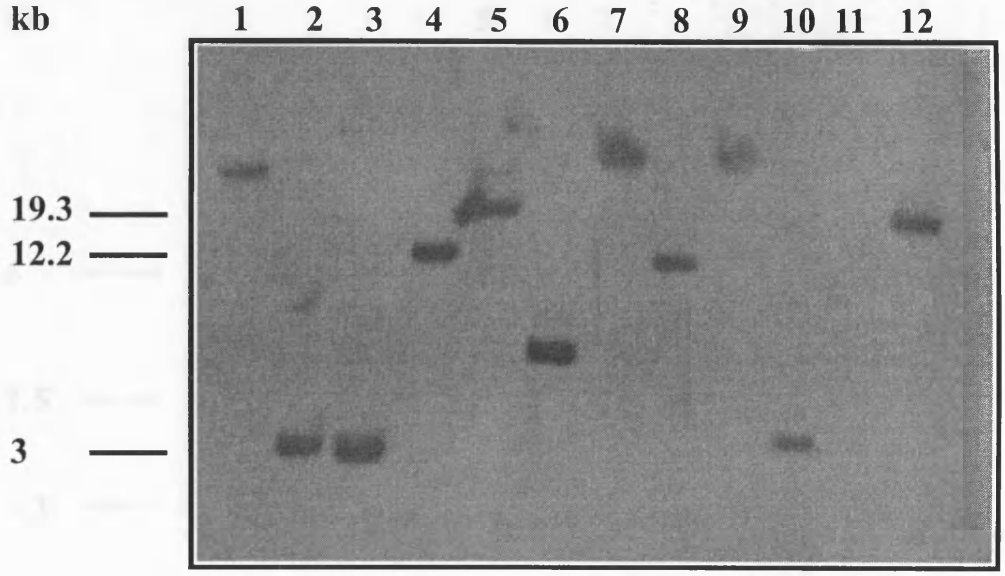
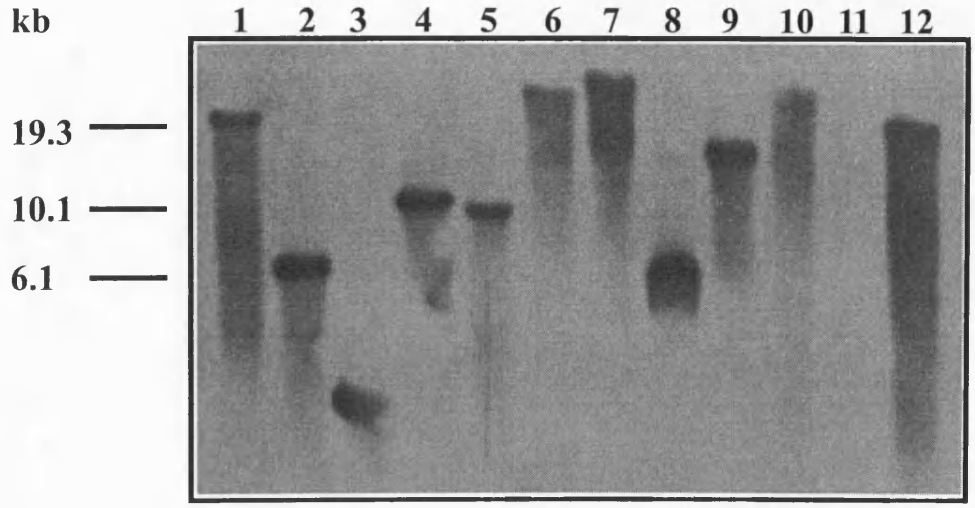
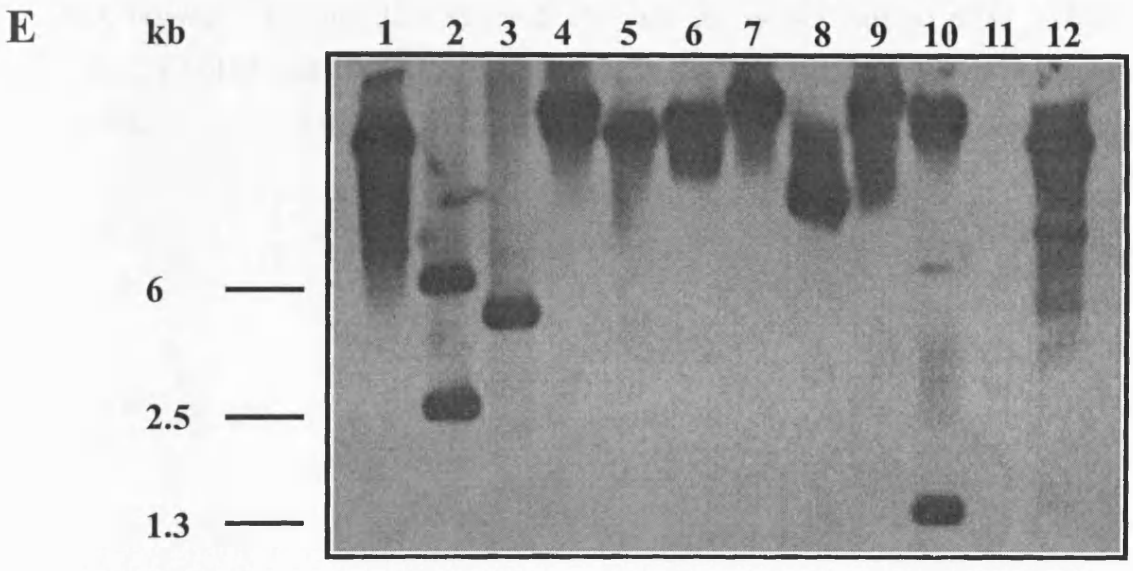
B
kb
1 2 3 4 5 6 7 8 9 10 11 12
10.1
4
1.3

C



D

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1 2 3 4 5 6 7 8 9 10 11 12
6
2.5
1.3

In Table 3.5.1.1.2, the sizes of the wild type DNA fragments that hybridised to the probes originated from the different recovery plasmids are shown. By analysing the sizes of the wild type hybridised bands, it was determined that the gene inactivated in each of the mutants was different. Because the probes, derived from the different recovery plasmids, hybridised to different size fragments of the wild type DNA restricted by *Sal*I (48.5 kb, bigger than 48.5 kb, 5 kb, between 19.3 and 22.6 kb, and, 19.3 kb, for probes derived from pAPG3, pAPJ3, pAPK3, pAPM3 and pAPN3 respectively) it can be concluded that different genes were inactivated in each of the mutants. The probes did not hybridised to the *E. coli* genomic DNA.

Table 3.5.1.1.2 - Estimated sizes of *L. monocytogenes* 10403S DNA bands that hybridised to the probes derived from plasmids pAP.

	Probes originated from plasmids shown below				
	pAPG3	pAPJ3	pAPK3	pAPM3	pAPN3
*Bam*HI	17	48.5	>48.5	19.3	17
*Eco*RI	2.5 1	4 1.3 0.9	3	6.1	6 2.5
*Hind*III	1.8	10.1	2.5	2.5	4.5
*Kpn*I	48.5	>48.5 9.1 1.8	12.2	10.1	>48.5
*Pst*I	17	>12.2,<15 1.2	19.3	9.1	>19.3,<22.6
*Sal*I	48.5	>48.5	5.5	>19.3,<22.6	19.3
*Sma*I	>48.5	>48.5	>48.5	>48.5	>48.5
*Sph*I	19.3	48.5 4.0	11.1	5.5	10.1
*Sst*I	48.5	>12.2,<15 2.2	>48.5	>12.2,<15	>48.5
*Xba*I	7.1	48.5	3	no data	48.5 1.3

3.5.1.1.1 - Subcloning of *Listeria* DNA inserts

To facilitate the sequencing of the *L. monocytogenes* DNA inserts from pAP plasmids, it was necessary to subclone the fragments containing insert DNA into pBluescript. The bands chosen are shown in Figure 3.5.1.1.4 and were the approximately 2 kb *Sal*I-*Hind*III of pAPG3; the 2.2 kb *Sal*I-*Kpn*I of pAPJ3; the 1.8 kb *Sal*I-*Xba*I of pAPK3; the 1.5 kb *Sal*I-*Hind*III of pAPM3 and the 3.1 kb *Sal*I-*Hind*III of pAPN3. All the bands included 361 bp of the *lacZ* gene, together with the inverted repeats sequence. During the analysis of the sequence of the insert, the existence of the sequences coding for the inverted repeats would allow us to confirm whether the right band was cloned.

pBluescript plasmid DNA was double digested with *Sal*I-*Hind*III, *Sal*I-*Kpn*I or *Sal*I-*Xba*I. The efficiency of digestion was checked by performing self-ligation with the digested DNA, then transforming into *E. coli* DH5α. The low number of transformants (approximately 400 transformants/μg plasmid DNA were obtained with the *Sal*I-*Xba*I self-ligation and no transformants were obtained with the *Sal*I-*Hind*III and *Sal*I-*Kpn*I ligation) indicated that the plasmid was sufficiently digested.

Plasmid DNA from pAP plasmids was digested, with the appropriate enzymes, using approximately 10μg of DNA and electrophoresed through a 1% (w/v) low-melting-point agarose gel. Gel slices containing the 2 kb *Sal*I-*Hind*III of pAPG3, the 1.8 kb *Sal*I-*Xba*I of pAPK3, the 1.5 kb *Sal*I-*Hind*III of pAPM3 and the 3.1 kb *Sal*I-*Hind*III of pAPN3 (for plasmids restriction map see Figure 3.5.1.1.4) fragments were excised, and DNA purified from the agarose using the LiCl extraction method (see Section 2.5.7.4).

A different strategy was used for the subcloning of the approximately 2 kb *Sal*I-*Kpn*I band of the plasmid pAPJ3. 10 μg of this DNA was double digested with *Sal*I-*Hind*III, the agarose slice containing the approximately 5.5 kb DNA band was excised and DNA extracted as before. This DNA was then cleaved with *Kpn*I producing the *Sal*I-*Kpn*I band of approximately 2.2 kb, the 1.6 kb *Kpn*I-*Kpn*I and an approximately 1.7 kb of *Kpn*I-*Hind*III fragment. This DNA was then used in the ligations with double digested *Sal*I-*Kpn*I pBluescript. Since pBluescript was digested with *Sal*I-*Kpn*I, only the 2.2 kb *Sal*I-*Kpn*I was expected to ligate; however, some ligations could occur with this band and the 1.6 kb *Kpn*I-*Kpn*I but this would be checked by restriction digestion and during the sequencing.

Ligations were performed as detailed in Section 2.5.5., using ratios of insert to vector of 4:1, 8:1 and 10:1. Ligation reactions were incubated at room temperature overnight, then ethanol precipitated, resuspended in nanopure water, and transformed by electroporation into *E. coli* DH5α selecting for ampicillin resistance. The transformation efficiency were $2.6x10^2$/μg, $6.6x10^2$/μg, 80/μg, $2.6x10^2$/μg, and $2.6x10^2$/μg of DNA for DNA originated from pAPG3, pAPJ3, pAPK3, pAPM3 and pAPN3 respectively. Although this was a very low efficiency of transformation it was decided to proceed since only one colony was needed. Plasmid DNA was extracted from white transformants and analysed by restriction enzyme digestion and agarose gel electrophoresis prior to the sequencing.

3.5.1.2 Cloning of chromosomal sequences flanking the *erm*-distal end of Tn*917*-LTV3

Because of time restraints, cloning of the chromosomal sequences flanking the *erm*-distal end of Tn*917*-LTV3 insertion was only performed for mutant J3. Genomic DNA from *L. monocytogenes* mutant J3 was partially digested with the restriction endonucleases *Bam*HI or *Sau*3AI to produce bands of high molecular weight. This was done in order to produce a band containing the ColE1 replication functions, the *neo* gene selectable in *E. coli* and some flanking chromosomal DNA. Thus, these bands had to be bigger then 12 kb for DNA digested with *Bam*HI, and bigger then 10 kb for digests with *Sau*3AI. The partial digestion reaction was inactivated and DNA was ligated in a final concentration of 5μg/ml, ligase extracted by phenol:chloroform washes and DNA used to transform *E. coli* MC1061. Selection was performed on plates with kanamycin and erythromycin.

The efficiency of transformation was approximately 10 transformants/μg which appears low, however, since these transformants were expressing resistance to kanamycin and erythromycin, it was decided to proceed. Plasmid DNA was purified from 6 *Bam*HI transformants and 6 *Sau*3AI transformants, digested with the restriction enzyme *Bam*HI (*Bam*HI sites flank the ColE1 and the *neo* gene in pLTV3, see Figure 3.2.2) and analysed by agarose gel electrophoresis. Figure 3.5.1.2.1 shows the results of this analysis (6 *Bam*HI transformants together with 1 *Sau*3AI transformant in the top gel and 5 *Sau*3AI transformants together with pLTV3 restricted by *Bam*HI in the bottom gel). Clone 7 (lanes 14 - 15, top gel) exhibited the 6.8 kb *Bam*HI-*Bam*HI band (pLTV3 derived) and a higher band of 10 kb. This digest revealed a plasmid of approximately 17 kb and hence since the derivative is of about 15 kb, at least 2 kb should be of listerial flanking DNA. However, the flanking DNA rescued in this plasmid could all be located at the *erm*-proximal end of Tn*917*-LTV3 rather than at the

erm-distal end. Clone 11 (lanes 8 - 9, bottom gel) did not exhibit the internal *Bam*HI pLTV3 derived fragment but displayed a *Bam*HI fragment bigger than 12 kb. All of the other transformants when restricted by *Bam*HI produced a single fragment of size smaller than 12 kb which suggested that they did not contain any flanking listerial DNA. However, in order to check if these clones contain any DNA flanking the *erm*-distal end of Tn*917*-LTV3 it was decided to perform a Southern Blot using a Tn*917*-LTV3 specific probe of the *erm*-distal end, against plasmid DNA extracted from the transformants (Figure 3.5.1.2.2). The probe chosen, was the *Hind*III-*Hind*III 1.5 kb band, a pLTV3 specific fragment. Under high stringency conditions [hybridisation conditions were 65°C in 5xSSC/0.1% (w/v) SDS overnight; washing was performed three times in 1xSSC/0.1% (w/v) SDS at 65°C for 15 minutes], the probe hybridised to DNA from clones 7 (lane 7), 10 (lane 10) and 11 (lane 11). However, these results, only confirmed that Tn*917* was recovered in these clones but did not reveal the existence of flanking chromosomal DNA. To check for the existence of listerial DNA, plasmid DNA from clones 7, 10 and 11 was extracted (Section 2.3.1.1) and sequenced with a primer which is homologous to the inverted repeats of the *erm*-distal end of Tn*917*.

Figure 3.5.1.2.1 - Restriction enzyme digests of plasmids recovered, after self-ligation of the *Bam*HI and *Sau*3AI partial digests of genomic DNA from mutant J3.

Top gel) Plasmid DNA recovered from 6 transformants colonies from *Bam*HI self-ligations, uncut plasmid DNA (lanes 2, 4, 6, 8, 10 and 12), plasmid DNA restricted by *Bam*HI (lanes 3, 5, 7, 9, 11 and 13). Plasmid DNA recovered from 1 transformant colony from *Sau*3AI self-ligations, uncut (lane 14), digested with the restriction enzyme *Bam*HI (lane 15). Lanes 1 and 16, 1 kb DNA size marker (GIBCO-BRL); fragment sizes are shown in the left hand column in kilobases (kb).

Bottom gel) Plasmid DNA recovered from 5 transformants colonies from *Sau*3AI self-ligations, uncut plasmid DNA (lanes 2, 4, 6, 8 and 10), plasmid DNA restricted by *Bam*HI (lanes 3, 5, 7, 9 and 11). Lane 12, represents pLTV3 undigested; and lane 13 represents pLTV3 restricted by *Bam*HI. Lanes 1 and 14, 1 kb DNA size marker (GIBCO-BRL); fragment sizes are shown in the left hand column in kilobases (kb).

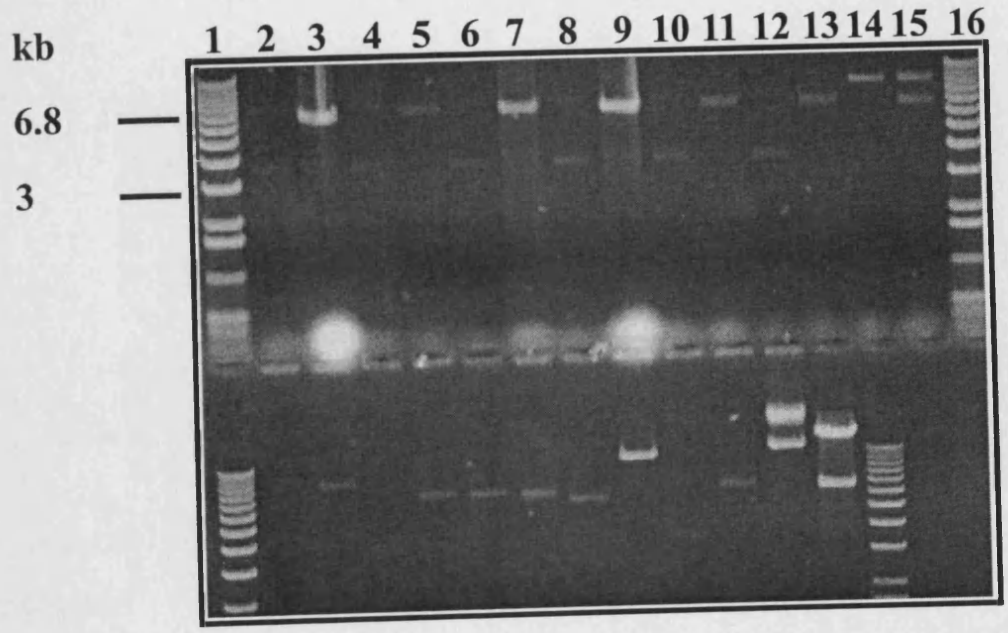
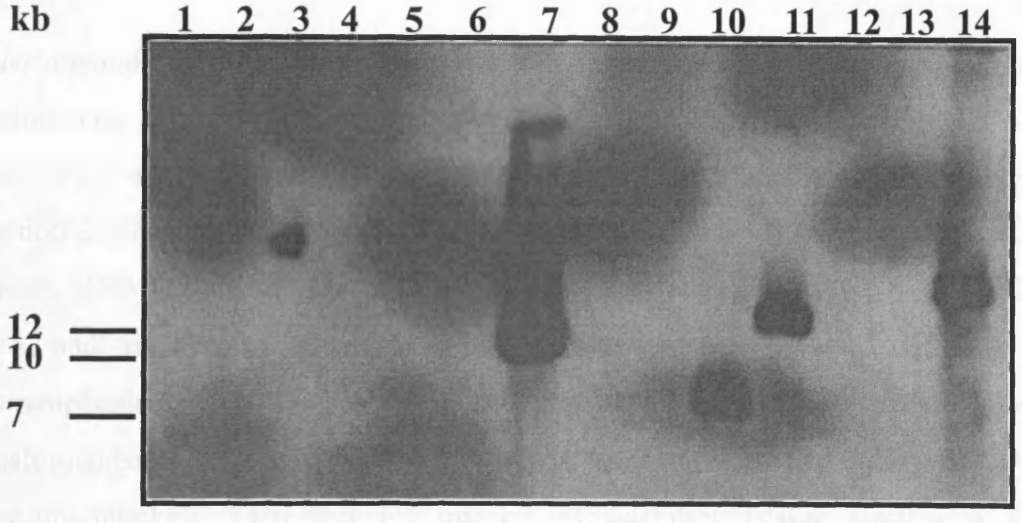

kb
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
6.8
3

Figure 3.5.1.2.2 - Southern hybridisation of plasmids recovered, from self-ligation of the *Bam*HI and *Sau*3AI partial digests, from mutant J3 genomic DNA with a probe derived from Tn*917 erm*-distal end.

Plasmid DNA recovered from 6 transformant colonies from *Bam*HI self-ligations and 6 transformant colonies from *Sau*3AI self-ligations was digested with *Bam*HI, analysed on a 0.7% (w/v) agarose gels, and blotted to Hybond-N^+ nylon membranes. The probe used was the 1.5 kb *Hind*III-*Hind*III specific to Tn*917*, which was labelled by the Fluorescein Gene Images system (Section 2.6.3.2) and used under conditions of high stringency [hybridisation conditions were 65°C in 5xSSC/0.1% (w/v) SDS overnight; washing was performed three times in 1xSSC/0.1% (w/v) SDS at 65°C for 15 minutes]. Lanes 1 through 6, corresponds to plasmid DNA of transformants recovered from *Bam*HI self-ligations; lanes 7 through 12, corresponds to plasmid DNA of transformants recovered from *Sau*3AI self-ligations; lane 13, genomic DNA from *L. monocytogenes* 10403S restricted by *Bam*HI; and lane 14, pLTV3 DNA restricted by *Bam*HI.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3.5.2 Cloning of chromosomal sequences flanking Tn*917*-LTV3 insertion sites of mutants defective for growth in liquid medium at low temperature

Since classical transposition had not occurred in these mutants the strategy for recovery of flanking listerial DNA had to be changed.

3.5.2.1 Construction of a partial library of *L. monocytogenes* mutant 1

Considering all of pLTV3 had inserted into the chromosome of these mutants, extra care had to be taken in order to clone listerial chromosomal DNA flanking the insertion. A detailed map of the chromosome at the insertion site had been prepared (Section 3.4.2, Figure 3.4.2.5) and the *Bam*HI band of 3.9 kb was selected for cloning, since it was estimated to contain approximately 3.5 kb of chromosomal DNA.

Listeria monocytogenes mutant 1 genomic DNA was digested to completion with *Bam*HI and electrophoresed through 0.6% preparative agarose gels. Gel slices containing DNA fragments in the 3 - 5 kb range were excised, and DNA purified from the agarose by electroelution (Section 2.5.7.2). Partial libraries were constructed using 3 - 5 kb size selected DNA. Size-selected DNA was ligated to *Bam*HI cut, dephosphorylated (Section 2.5.4) pACYC184 vector DNA, and transformed into *E. coli* DH5α. Colonies were selected on plates containing chloramphenicol. A low number of transformants was obtained; in the region of 1000 transformants/μg of vector DNA. Since the positive control worked well (uncut pACYC184), these low numbers of transformants may reflect instability of large inserts of A+T rich DNA in plasmid vectors, or poor ligation.

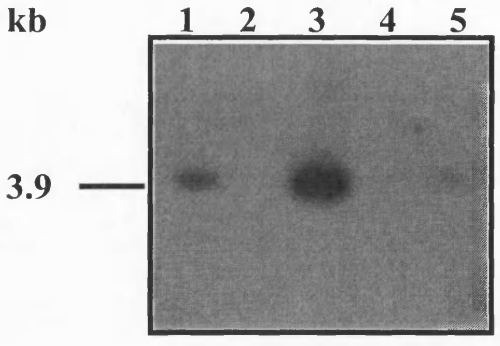
The method chosen to screen the partial library was colony hybridisation (Section 2.6) and the probe selected to screen the library was the 630 bp pLTV3-specific *Sma*I-*Sma*I fragment which partially spans the bleomycin resistance gene. To confirm that the fragment to be cloned was within the size-selected DNA used in the ligations and, at the same time to confirm that the probe would not hybridise to vector DNA, Southern Blots to mutant 1 chromosomal DNA were performed. The *Sma*I-*Sma*I probe was used, under high stringency conditions [hybridisation conditions were 65°C in 5xSSC/0.1% (w/v) SDS overnight; washing was performed three times in 1xSSC/0.1% (w/v) SDS at 65°C for 15 minutes], against size selected DNA prepared for the ligations, *L. monocytogenes* 10403S genomic DNA restricted by *Bam*HI, mutant 1 genomic DNA restricted by *Bam*HI, *E. coli* K12 genomic DNA restricted

by *Bam*HI, and plasmid pACYC184 DNA linearised by *Bam*HI. The probe hybridised to the 3.9 kb *Bam*HI band within the size selected DNA and within the mutant genomic DNA (Figure 3.5.2.1.1, lanes 1 and 3), confirming that the band to be cloned was within the size selected DNA. Unexpectedly the probe hybridised to the vector (Figure 3.5.2.1.1, lane 5). However, since this was a very weak signal when compared to the signal produced by the insert, it was decided to proceed with this probe. No hybridisation occurred to the wild type DNA and to the *E. coli* DNA.

Approximately 4850 clones from *L. monocytogenes* mutant 1 library were screened using the *Sma*I probe, and only one clone hybridised to the probe (Figure 3.5.2.1.2). This clone was named pAP34. In order to check for the size of the insert, plasmid DNA was extracted from pAP34 and digested by *Bam*HI. Digests were analysed by agarose gel electrophoresis, and no insert could be seen in the gel. In spite of this result, it was decided to do a Southern Blot with this gel onto a nylon membrane. The membrane was probed with the *Sma*I fragment as before, but no hybridisation signal could be detected (Figure 3.5.2.1.3; lane 4). The original pAP34 colony was replica plated on LA plates containing chloramphenicol alone, the selectable marker for transformants with pACYC184 containing insert and, chloramphenicol with tetracycline the selectable markers for the vector without insert. The aim of the plating was to check if the transformant was losing the insert. This clone grew defectively on the tetracycline plate, suggesting some loss of the insert. 10 colonies selected at random from the chloramphenicol plate, were again replica plated onto plates with chloramphenicol and chloramphenicol with tetracycline in order to select for a clone retaining the insert. Of these, 6 were unable to grow on tetracycline after overnight incubation at 37°C. Plasmid DNA was extracted from the 10 colonies and analysed by gel electrophoresis. No insert was seen in any of the plasmids. This results may reflect instability of inserts of A+T rich DNA in plasmid vectors, or the presence of DNA sequences encoding for inhibitors in the size selected DNA. As a result of this, it was decided to take a different approach to isolate the flanking chromosomal DNA.

Figure 3.5.2.1.1 - Southern hybridisation of the size-selected DNA prepared for the partial library of *L. monocytogenes* mutant 1.

Size-selected DNA from *L. monocytogenes* mutant 1 genomic DNA digested by *Bam*HI, *L. monocytogenes* 10403S genomic DNA digested with *Bam*HI, *L. monocytogenes* mutant 1 genomic DNA digested by *Bam*HI, *E. coli* K11 chromosomal DNA restricted by *Bam*HI, and pACYC184 linearised by *Bam*HI were analysed on a 0.7% (w/v) agarose gels, and blotted to Hybond-N^+ nylon membranes. The probe used was the 630 bp *Sma*I-*Sma*I specific of Tn*917*, which was labelled by the Fluorescein Gene Images system (Section 2.6.3.2) and used under conditions of high stringency [hybridisation conditions were 65°C in 5xSSC/0.1% (w/v) SDS overnight; washing was performed three times in 1xSSC/0.1% (w/v) SDS at 65°C for 15 minutes]. Lanes 1, size-selected DNA used in the partial library (3 - 5 kb of *Bam*HI restricted chromosomal DNA from mutant 1); lane 2, wild type genomic DNA restricted by *Bam*HI (as a negative control); lane 3, corresponds to chromosomal DNA from mutant 1 restricted by *Bam*HI (as a positive control); lane 4, *E. coli* K12 genomic DNA restricted by *Bam*HI (as a negative control); lanes 5, vector pACYC184 linearised by *Bam*HI.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3.9

Figure 3.5.2.1.2 - Colony hybridisation of the partial library of *L. monocytogenes* mutant defective for growth in liquid medium.

Recombinants were replica plated onto plates with chloramphenicol and chloramphenicol with tetracycline. *E. coli* DH5α containing pACYC184 and *L. monocytogenes* mutant 1, were included in the plate as negative and positive control respectively. Colonies were transferred onto nylon membranes, membranes were treated and DNA was fixed to the membrane. Membrane was hybridised, under high stringency conditions [hybridisation conditions were 65°C in 5xSSC/0.1% (w/v) SDS overnight; washing was performed three times in 1xSSC/0.1% (w/v) SDS at 65°C for 15 minutes], using the 630 bp *Sma*I-*Sma*I probe. Arrow head indicates a clone which exhibited a positive signal (pAP34). (+) indicates the positive control and (-) indicates the negative control.

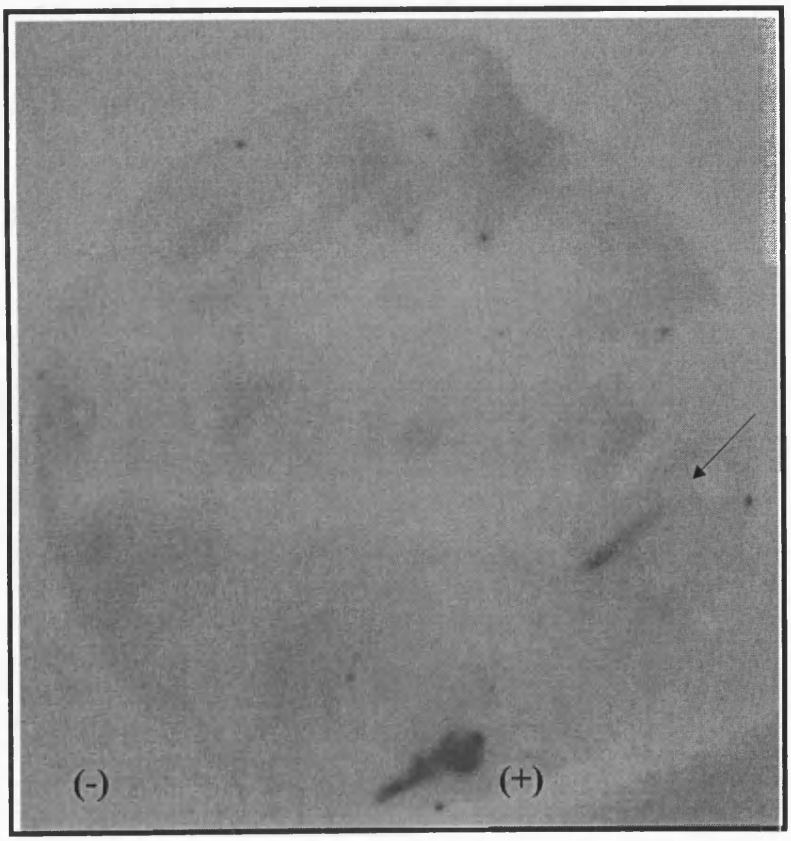
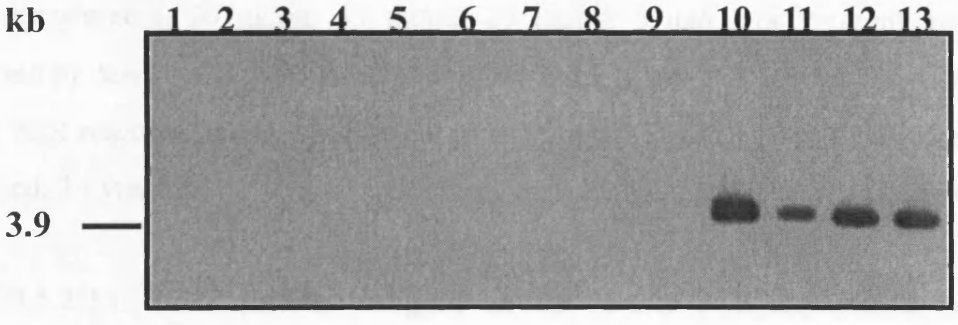


Figure 3.5.2.1.3 - Southern hybridisation of recombinants recovered from the partial library of *L. monocytogenes* mutant 1.

Plasmid DNA recovered from recombinants of the partial library digested by *Bam*HI, *L. monocytogenes* 10403S genomic DNA digested with *Bam*HI, *L. monocytogenes* mutant 1 genomic DNA digested by *Bam*HI, *E. coli* K12 chromosomal DNA restricted by *Bam*HI, and pACYC184 linearised by *Bam*HI were analysed on a 0.7% (w/v) agarose gels, and blotted to Hybond-N^+ nylon membranes. The probe used was the 630 bp *Sma*I-*Sma*I specific of Tn*917*, which was labelled by the Fluorescein Gene Images system (Section 2.6.3.2) and used under conditions of high stringency [hybridisation conditions were 65°C in 5xSSC/0.1% (w/v) SDS overnight; washing was performed three times in 1xSSC/0.1% (w/v) SDS at 65°C for 15 minutes]. Lanes 1, wild type genomic DNA restricted by *Bam*HI (as negative control); lane 2, vector pACYC184 linearised by *Bam*HI (as negative the control); lane 3, *E. coli* K12 genomic DNA restricted by *Bam*HI (as control); lane 4, plasmid DNA from pAP34 restricted by *Bam*HI; lanes 5 through 9, plasmid DNA recovered from colonies neighbouring pAP34 restricted by *Bam*HI; lane 10, corresponds to chromosomal DNA from mutant 1 restricted by *Bam*HI (as a positive control); lane 11 through 13, size-selected DNA used in the partial library (3 - 5 kb of *Bam*HI restricted chromosomal DNA from mutant 1, used as a positive control).


kb
1 2 3 4 5 6 7 8 9 10 11 12 13
3.9

3.5.2.2 Inverse PCR

The alternative strategy to isolate the flanking chromosomal DNA was inverse PCR. The requirement for primers complementary to both ends of the fragment to be amplified poses a problem for conventional PCR when the sequence of only one end of the fragment is known. Therefore inverse PCR was chosen. In brief, this procedure involves circularising the restriction fragment containing the region of interest, and use of primers oriented so that primer extension proceeds "outwards" from the region of known sequence to amplify the flanking DNA.

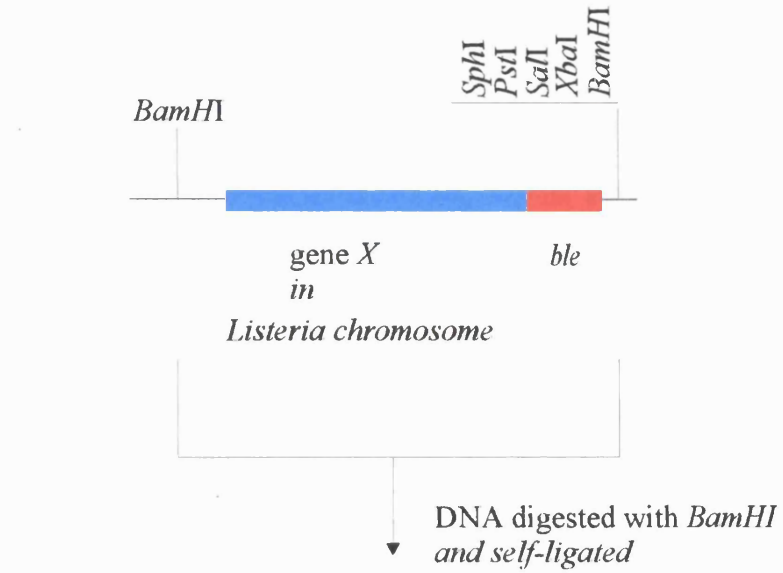
PCR primers, going in the opposite direction, were synthesised based on the bleomycin gene sequence (Figure 3.5.2.2.1). These primers were termed BINV1 and BINV2, and contained 21 and 22 nucleotides respectively (Table 3.5.2.2.1). The correct size of the DNA fragment generated using the primers BINV1 and BINV2 would be 3.9 kb. Template DNA was prepared by self-ligation of size-selected 3 - 5 kb *Bam*HI genomic DNA from mutant 1, at a final concentration 50 μg/ml, 33 μg/ml, 25 μg/ml, 5 μg/ml or 2 μg/ml. pLTV3 DNA, restricted by *Bam*HI and self-ligated at 5 μg/ml and 2 μg/ml, was used as the positive control. In the PCR reactions, a range of 1 to 30 ng of template DNA in 10 μl final volume reactions was used. To visualise the products, reactions were analysed on 0.6% (w/v) agarose gels.

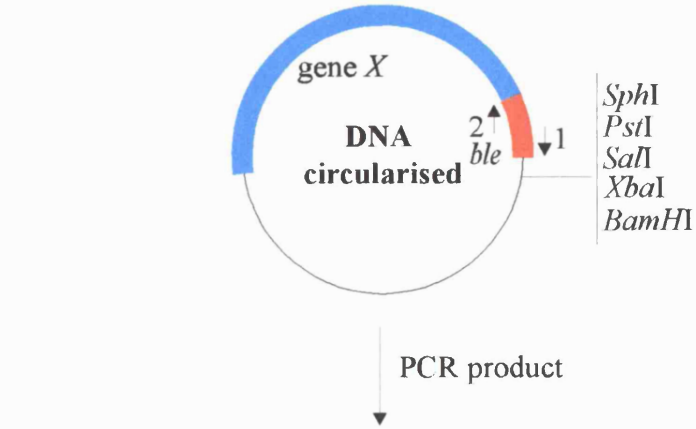
Table 3.5.2.2.1 - PCR primers designed for the inverse PCR experiments based on the Bleomycin sequence

Name	Primer sequence	Size (bp)
BINV1	5'AGGTTGGGCGTCGCTTGGTCG3'	21
BINV2	5'CTCATGCTGGAGTTCTTCGCCC3'	22

Figure 3.5.2.2.1 - Inverse PCR strategy used to isolate listerial DNA sequences adjacent to pLTV3 insertion in liquid mutant 1.

To isolate the DNA bordering the bleomycin gene of the pLTV3 insertion, chromosomal DNA from *L. monocytogenes* mutant 1 was digested with *Bam*HI. The digests were then ligated at concentrations to promote self-ligation to give a circularised fragment. PCR primers based on the bleomycin sequences were then used so that primer extension proceeds "outwards" from the region of known sequence to amplify the listerial flanking DNA. Arrowheads represents the primers BINV1 (1) and BINV2 (2).


SphI
PstI
SalI
XbaI
BamHI
BamHI
gene X
in
Listeria chromosome
ble
DNA digested with BamHI
and self-ligated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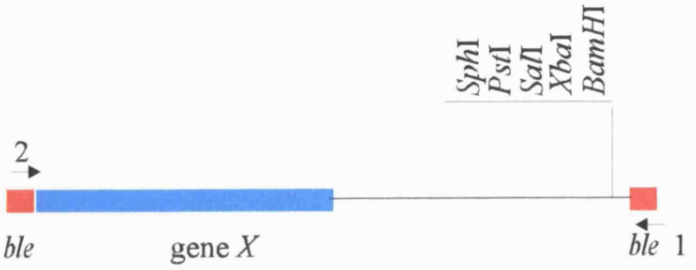
SphI
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SalI
XbaI
BamHI
2
ble
gene X
ble
1

A faint band of the desired size (3.9 kb) was produced (Figure 3.5.2.2.2, lanes 4, 6 7, 9 and 10). However, some non-specific amplification also occurred, producing a bright band of 1.2 kb. The positive control template (Figure 3.5.2.2.2, lanes 2 and 3) failed to produce amplification products of the correct size (6.8 kb). The failure of the positive control pointed to a technical problem. However, since 6.8 kb is quite a big band to be amplified using *Taq* this result may be is not surprising. It was decided to Southern Blot this gel and probe it, under high stringency conditions [65°C in 5xSSC/0.1% (w/v) SDS overnight; washing was performed three times in 1xSSC/0.1% (w/v) SDS at 65°C for 15 minutes], against the *Sma*I-*Sma*I probe. The probe hybridised to the 3.9 kb band (Figure 3.5.2.2.3, lanes 3, 5, 6, 8 and 9). However, there was some hybridisation to the 1.2 kb band on lane 5 and the positive control produced a smear of bands between 12 kb and approximately 500 bp (Figure 3.5.2.2.2, lanes 2 and 3).

In order to reduce or remove the non-target amplification products, and consequently to increase specificity, other approaches were tried. This included "Hot start", increasing the annealing temperature up to 10°C lower than the Tm (melting temperature) and using $MgCl_2$ in a range of final concentrations of 1 - 3 mM. This still led to the production of non-target amplification products and also to the disappearance of the desired 3.9 kb band. It was decided, therefore, to use other PCR enzymes since, a 3.9 kb might be a too big a fragment to be successfully amplified by *Taq*. It was decided to use Expand High Fidelity (BOEHRINGER Mannheim). However, this approach was unsuccessful, with the desired products not being generated with samples or the positive control.

Since all the modifications performed failed to optimise the PCR reactions, it was decided to go back and reproduce the PCR with the same conditions used on the first occasion. However, no products of the desired size could be generated. Fresh template DNA and primer stocks were prepared, but again, none of these modifications proved successful.

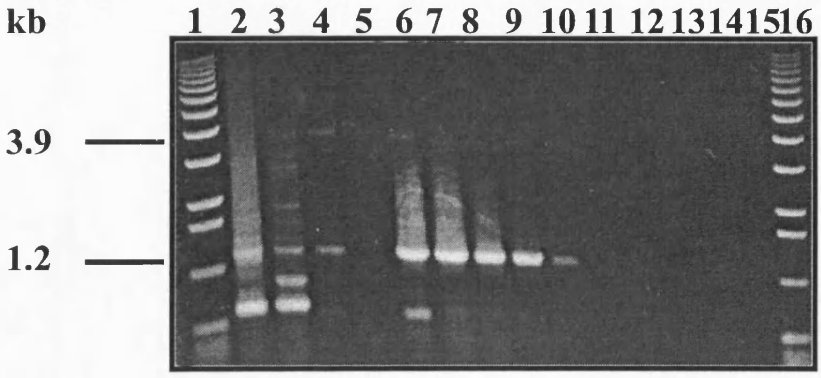
To circumvent the size problem, template prepared from the genomic DNA of mutant 1 was digested with *Xba*I, self-ligated and used in the inverse PCR reactions. The amplification product expected with this digest was of 2.6 kb but no amplification product of the desired size was produced.

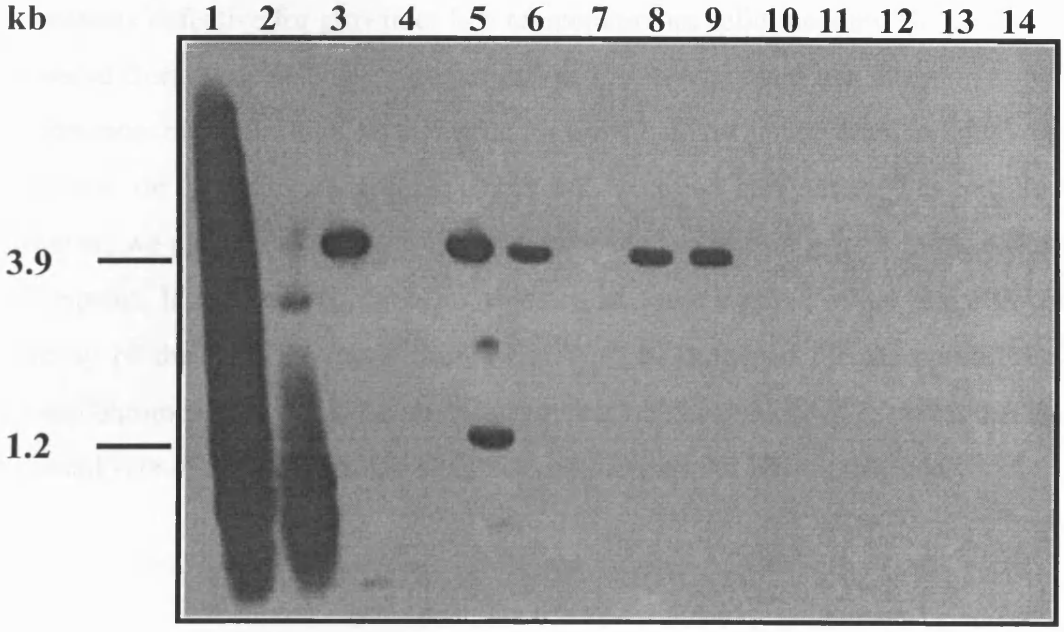
Figure 3.5.2.2.2 - Amplification products using the BINV1/2 primers in inverse PCR reactions.

*Bam*HI size-selected (3 - 5 kb) self-ligated *L. monocytogenes* mutant 1 genomic DNA, was the template for amplification reactions. Lanes 2 and 3, corresponds to amplifications products of 30 ng and 12 ng of the pLTV3 digested by *Bam*HI and self-ligated at a final concentration of 5 μg/ml (lane 2) and 2 μg/ml (lane 3) (these are the positive controls); lane 4 and 5, corresponds to amplifications products of 30 ng and 12 ng of the template DNA self-ligated at a final concentration of 5 μg/ml (lane 4) and 2 μg/ml (lane 5); lanes 6 through 8, corresponds to amplifications products of 10 ng, 2 ng and 1 ng of the template DNA self-ligated at a final concentration of 50 μg/ml; lanes 9 through 11; corresponds to amplifications products of 10 ng, 2 ng and 1 ng of the template DNA self-ligated at a final concentration of 33 μg/ml; lanes 12 through 14; corresponds to amplifications products of 10 ng, 2 ng and 1 ng of the template DNA self-ligated at a final concentration of 25 μg/ml; lanes 15, corresponds to the negative control (no template added to reactions); lanes 1 and 16, 1 kb ladder DNA size marker (GIBCO-BRL), with fragments sizes shown in kilobases (kb).

Figure 3.5.2.2.3 - Southern hybridisation of amplification products produced by inverse PCR of the partial library of *L. monocytogenes* mutant 1.

Gel from Figure 3.5.2.2.1 was blotted to Hybond-N^+ nylon membrane. The probe used was the 630 bp *Sma*I-*Sma*I specific of Tn*917*, which was labelled by the Fluorescein Gene Images system (Section 2.6.3.2) and used under conditions of high stringency [hybridisation conditions were 65°C in 5xSSC/0.1% (w/v) SDS overnight; washing was performed three times in 1xSSC/0.1% (w/v) SDS at 65°C for 15 minutes]. Lanes 1 and 2, corresponds to hybridisation to amplifications products of 30 ng and 12 ng of the pLTV3 digested by *Bam*HI and self-ligated at a final concentration of 5 μg/ml (lane 1) and 2 μg/ml (lane 2) (these are the positive controls); lane 3 and 4, corresponds to hybridisation to amplifications products of 30 ng and 12 ng of the template DNA self-ligated at a final concentration of 5 μg/ml (lane 3) and 2 μg/ml (lane 4); lanes 5 through 7, corresponds to hybridisation to amplifications products of 10 ng, 2 ng and 1 ng of the template DNA self-ligated at a final concentration of 50 μg/ml; lanes 8 through 10; corresponds to hybridisation to amplifications products of 10 ng, 2 ng and 1 ng of the template DNA self-ligated at a final concentration of 33 μg/ml; lanes 11 through 13; corresponds to hybridisation amplifications products of 10 ng, 2 ng and 1 ng of the template DNA self-ligated at a final concentration of 25 μg/ml; lanes 14, corresponds to the negative control (no template added to reactions) and no hybridisation occurred.


kb
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
3.9
1.2


kb
1 2 3 4 5 6 7 8 9 10 11 12 13 14
3.9
1.2

3.5.2.3 Classical self-ligation recovery

Since classical recombination had not occurred, it was thought that the standard recovery strategy could not be applied with this mutant. However, after analysing the map of the chromosome of mutant 1 (Figure 3.4.2.1.2), we considered that genomic DNA of mutant 1 digested with *Hind*III would produce a band of 17.7 kb containing the ColE1 replicon, the *neo* gene and approximately 200 bp of the flanking chromosomal DNA.

Genomic DNA from mutant 1 was digested to completion by *Hind*III, self-ligated at a final concentration of 5 μg/ml and used to transform electro-competent *E. coli* MC1061. Electroporation produced approximately 1500 transformants/μg of DNA, however, these were of smaller size, when compared to the *E. coli* transformants containing DNA recovered with the mutants defective for growth at low temperature on solid medium. No plasmid DNA was recovered from those colonies. Southern Blots results suggested that insertion of pLTV3, into the chromosome of the mutants defective for growth at low temperature in liquid medium, did not make use of the insertional sequences but, occurred between the *ble* and the *neo* gene. However, we can not still be sure that the complete kanamycin gene is in the chromosome of this mutant. If *neo* was disrupted or mutated in some way, it would make impossible the recovery of the flanking chromosomal DNA or, as suggested for the partial library, if the rescued chromosomal DNA encoded for proteins which are toxic in *E. coli* it could account to the small size of the colonies and the unsuccessful plasmid DNA extraction.

3.6 DNA sequencing of the pAPJ3 subclones

3.6.1 Choice of the sequencing method

Initially the Sequenase Version 2.0 kit supplied by United States Biochemical Corporation (Section 2.8.1) was used to sequence the subclones of plasmids pAP. Plasmid DNA was denatured using heat and alkali to produce single stranded template that could be used in the Sequenase reactions. Reaction products were analysed by electrophoresis through gradient buffer polyacrylamide gels and subsequent autoradiography (Section 2.8.1.2). Sequence data produced by this method were only of approximately 200 bp from a single experiment. With such a low efficiency, the sequencing of the listerial DNA inserts would be time consuming.

The sequencing method which gave the most satisfactory results was the "automated" sequencing using the ABI PRISM^{TM} Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq® DNA Polymerase, FS (Applied Biosystems) in conjunction with an Applied Biosystems Model 373A DNA sequencing system. This became the method of choice. This system relies on the incorporation of fluorescent dye-labelled dideoxynucleotides (ddNTPs) by the thermostable enzyme AmpliTaq DNA Polymerase, FS to terminate the extension of the primer. AmpliTaq DNA Polymerase, FS was developed specifically for fluorescent cycle sequencing with dye-labelled primers and terminators. This enzyme is a mutant form of *Taq* DNA polymerase which has essentially no 5'---- 3' nuclease activity and has drastically reduced discrimination for ddNTPs. This sequencing strategy has two main advantages; firstly, is that the use of dye-labelled ddNTPs reduces signals due to false stops since the false stops won't be labelled and, secondly, the reaction results are analysed by laser densitometry and computer analysis, a process that allows long reads of sequence to be obtained from a single experiment (700 bp or more).

3.6.2 DNA sequencing strategy

3.6.2.1 DNA sequencing strategy of the listerial DNA at the *erm*-proximal end of Tn*917*-LTV3 in pBluescript

The first step in the sequencing strategy was to determine the sequence of the pAP plasmids insert (containing listerial DNA at the *erm*-proximal end of Tn*917*-LTV3) subcloned in pBluescript, using the universal forward and reverse primers. Sequence obtained from universal primers included approximately 400 bp of the *lacZ* gene together with the inverted repeats of the Tn*917*-LTV3 derivative. The results further confirmed that the correct pAP fragment had been subcloned in pBluescript. Since there was not enough time in this project to fully sequence the inserts recovered from all of the mutants, it was decided to concentrate in only one of the subclones. The subclone of choice was the one containing chromosomal DNA of mutant J3 in pBluescript (pAPJ3 band containing the insert DNA).

On the basis of the sequence of the insert of this subclone obtained with the universal primer T7, oligonucleotide primers (18 - 24 bases in length) were synthesised to complete the sequence. Since we were interested in the gene inactivated by the transposon insertion, it was decided to fully sequence the end proximal to the *Sal*I site. A list of the primers used for this purpose is shown in Table 3.6.2.1.1. In Figure 3.6.2.1.1 the sequencing strategy for the listerial insert containing the *erm*-proximal end of Tn*917* insertion is detailed.

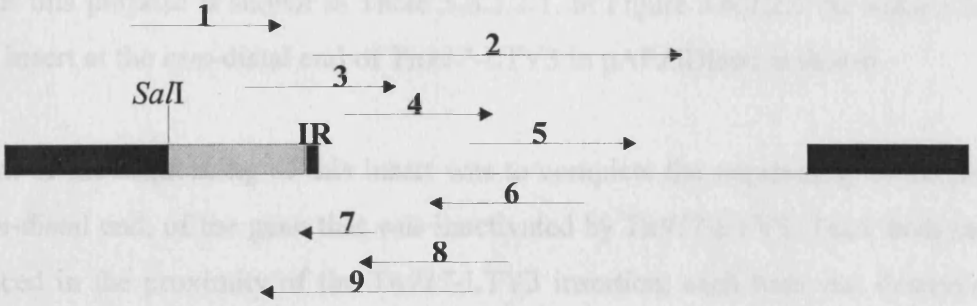
The initial aim of the sequencing of this insert DNA was to investigate if the cloned listerial DNA had any homologies to sequences in the nucleic acids or protein databases. For example, if any open reading frames (ORFs) present in the clone, which were inactivated by Tn*917*-LTV3, had homologies to genes that were known to be regulated by temperature. Also, the location of the derivative (Tn*917*-LTV3) in ORFs in the insert, could give a good indication of where a temperature regulated promoter might be located. Both strands were sequenced in the proximity of the Tn*917*-LTV3 insertion; each base was determined on the basis of at least duplicate reactions (with triplicate reactions performed in many cases).

Table 3.6.2.1.1 - List of primers used in sequencing the listerial DNA in the proximity of the *erm*-proximal end of Tn*917*-LTV3 (pAPJ3 DNA insert).

Primer Number	**Name**	**Sequence (5' - 3')**
(1)	T7 (Universal primer)	GTAATACGACTCACTATAGGGC
(2)	REM(REPEATERM)	GAGCGCCTACGAGGAATTTGT
(3)	Proximal Primer	GCAATAACCGTTACCTGTTTGTGCC
(4)	JAA(+)	GCCGGAATTGTTTGGATGATA
(5)	JAB(+)	CGTATGGTGGGAAGAATTTCT
(6)	JA(-)	AACGCGAAGGAGATGGCTATA
(7)	JB(-)	AATCATAGCGATACCGCTTGGAGT
(8)	JC(-)	ATCTGCATTAGCAACAGACGA
(9)	JD(-)	GCAGATGGCAAGTTGAAAGAACCA

Figure 3.6.2.1.1 - Sequencing strategy of listerial DNA in the proximity of the *erm*-proximal end of Tn*917*-LTV3 in pBluescript.

In the diagram, solid rectangles represent pBluescript DNA, the dotted rectangle represent pLTV3 DNA, while the pAPJ3 insert is represented by a line. Arrows indicate both the length of fragment sequenced from the oligonucleotide primer and the direction of primer extension, IR represents the inverted repeats. Numbers over the arrows indicate the oligonucleotide primer used as detailed in Table 3.6.2.1.1.


1
2
3
SalI
4
IR
5
6
7
8
9
1 kb

3.6.2.2 DNA sequencing strategy of the listerial DNA at the *erm*-distal end of Tn*917*-LTV3

Sequencing analysis of the three clones recovered from the *erm*-distal end of Tn*917*-LTV3 revealed that only clone 11 contained listerial DNA. The primer used was designed from Tn*917* *erm*-distal end (Table 3.6.2.2.1) and sequence was produced included the terminal inverted repeats of Tn*917* as well as listerial DNA. This primer failed to produce sequence from the two other clones (clone 7 and 10), suggesting that the region for primer homology did not exist in these clones. The clone containing insert DNA (clone 11) was termed pAPJ3Distal.

On the basis of the sequence obtained from the insert DNA in pAPJ3Distal, oligonucleotide primers (18 - 26 bases in length) were synthesised to complete the sequence. A list of primers used for this purpose is shown in Table 3.6.2.2.1. In Figure 3.6.2.2.1 the sequencing strategy for the insert at the *erm*-distal end of Tn*917*-LTV3 in pAPJ3Distal is shown.

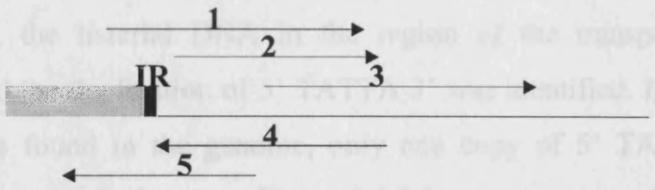
The aim of the sequencing of this insert was to complete the sequencing, in the proximity of the *erm*-distal end, of the gene that was inactivated by Tn*917*-LTV3. Thus, both strands were sequenced in the proximity of the Tn*917*-LTV3 insertion; each base was determined on the basis of at least duplicate reactions (with triplicate reactions performed in many cases).

Table 3.6.2.2.1 - Primers used in sequencing of listerial DNA in the proximity of the *erm*-distal end of Tn*917*-LTV3 in pAPJ3Distal.

Primer Number	**Name**	**Sequence 5' - 3'**
(1)	ERMDISTAL	GGCCTTGAAACATTGGTTTAGTGGGA
(2)	DISTAL11AA(+)	ACGTTGATGGAGCGCTTTTAG
(3)	DISTAL11AB(+)	TGGTGCTATTCCAACCGCAGT
(4)	DISTAL11B(-)	TGCTCCCTGACCTTCTTTCAT
(5)	DISTAL11C(-)	TGCTGCTACACCAATAGCGATAAC

Figure 3.6.2.2.1 - Sequencing strategy of listerial DNA in the proximity of the *erm*-distal end of Tn*917*-LTV3.

In the diagram, dotted rectangles represent pLTV3 DNA, while the listerial DNA insert in pAPJ3Distal is represented by a line. Arrows indicate both the length of fragment sequenced from the oligonucleotide primer and the direction of primer extension, IR represents the inverted repeats. Numbers over the arrows indicate the oligonucleotide primer used as detailed in Table 3.6.2.2.1.



1 kb

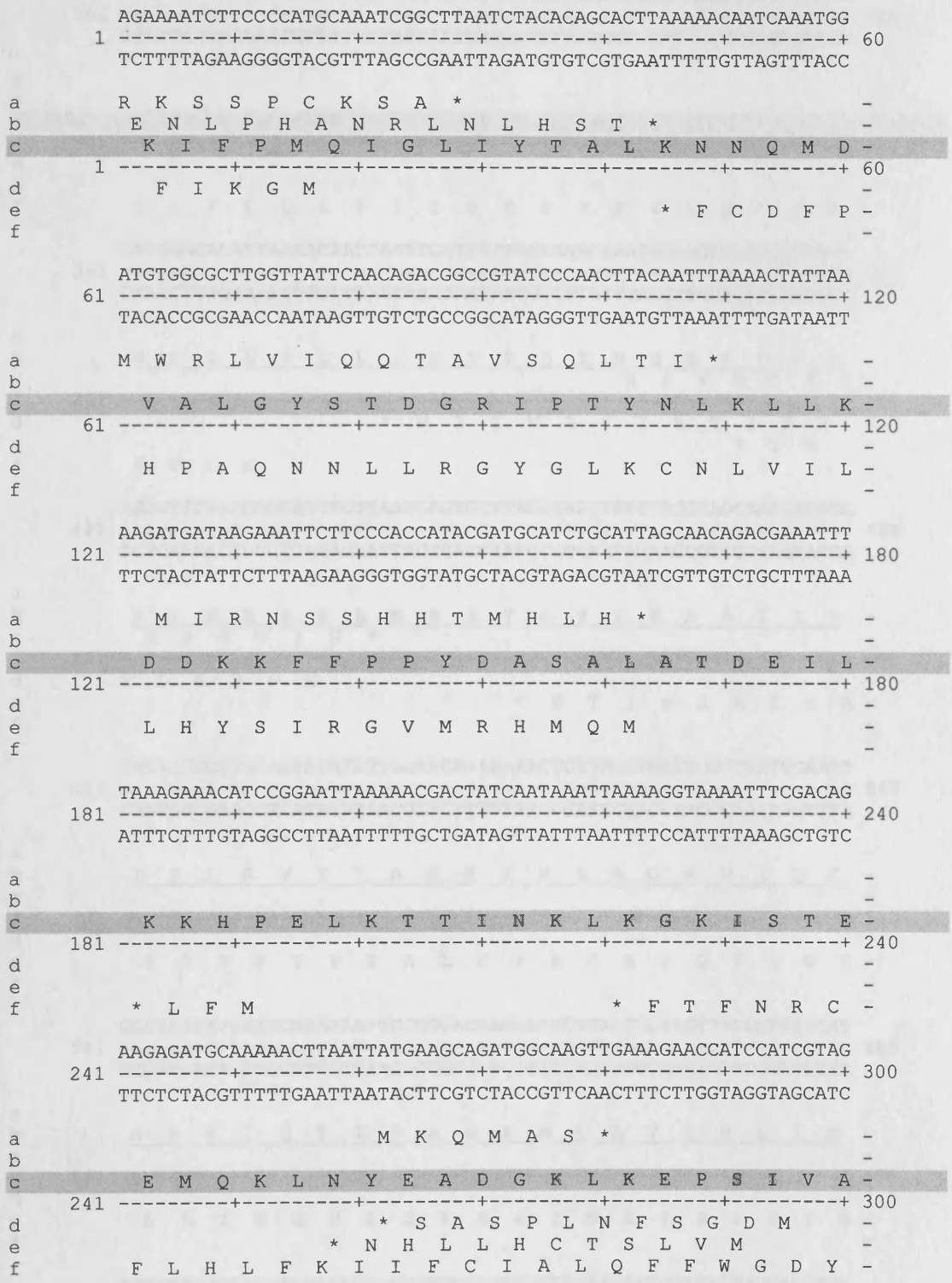
3.6.3 Sequence determination of the listerial DNA at the site of the insertion of Tn*917*-LTV3

The sequence of the listerial DNA, at the site of the insertion of Tn*917*-LTV3, was produced by joining the sequence of listerial DNA at the *erm*-proximal end of Tn*917*-LTV3 with the sequence of listerial DNA at the *erm*-distal end of Tn*917*-LTV3. This sequence is presented in Figure 3.6.3.1 and was produced using the 'map' program from the Genetics Computer Group (GCG) software package (Genetics Computer Group, 1995). The sequence is shown 5' to 3' in the direction of the listerial DNA flanking the *erm*-proximal end of Tn*917*-LTV3. The translation products from the six reading frames (three forward [a, b and c] and three reverse [d, e and f]) are shown under the sequence, with amino acids represented by the single letter code.

Tn*917* is known to generate a 5-base pair duplication upon insertion (Perkins and Youngman, 1984). Thus, the listerial DNA in the region of the transposon insertion was carefully examined and the duplication of 5' TATTA 3' was identified. In order to analyse the listerial DNA as it is found in the genome, only one copy of 5' TATTA 3' was kept. The final nucleotide sequence is shown on Figure 3.6.3.1.

Figure 3.6.3.1 - Sequence of the listerial DNA flanking the Tn*917*-LTV3 insertion in mutant J3.

Nucleic acid sequence of the joined listerial DNA flanking the *erm*-proximal and the *erm*-distal end of the Tn*917*-LTV3 insertion in mutant J3. The sequence is shown 5' to 3'. The translation products from the three forward reading frames (a, b and c) and from the three reverse reading frames (d, e and f) are shown under the nucleotide sequence, with amino acids represented by the single letter code. Open reading frames (ORF) are represented in Figure 3.6.4.1. ORF8 is shown with a single underline; ORF9 is shown in shading; the 5-base pair (5' TATTA 3'), which was duplicated by the insertion, is shown underlined with a doted line; the potential ribosomal binding site is shown in bold; and, the potential terminator sequence is shown underlined with a bold broken line. No evidence for a putative promoter was found. "*" denotes a translational stop.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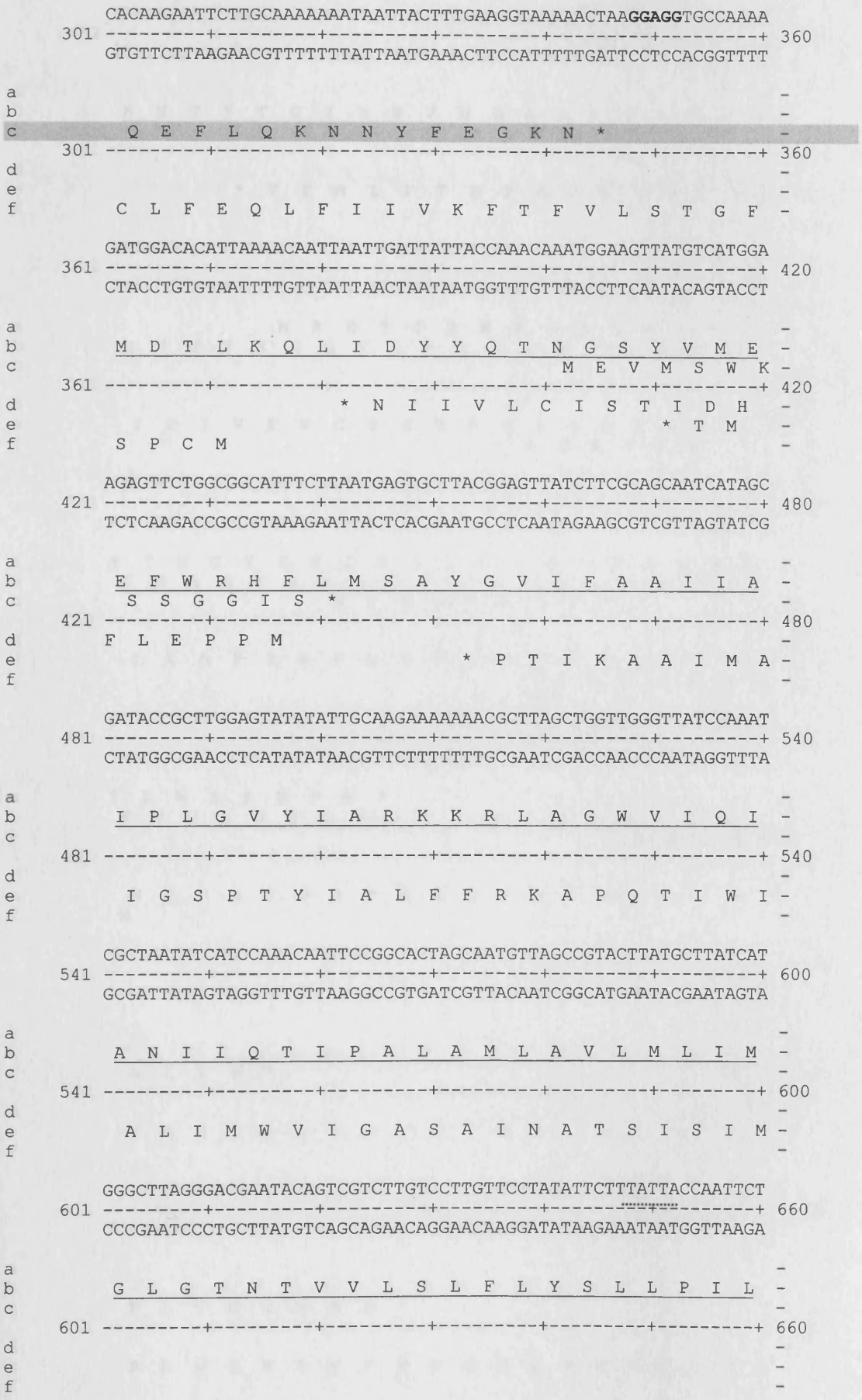
1 60
TCTTTTAGAAGGGGTACGTTTAGCCGAATTAGATGTGTCGTGAATTTTTGTTAGTTTACC
a R K S S P C K S A *
b E N L P H A N R L N L H S T *
c K I F P M Q I G L I Y T A L K N N Q M D
1 60
d F I K G M
e * F C D F P
f
ATGTGGCGCTTGGTTATTCAACAGACGGCCGTATCCCAACTTACAATTTAAAACTATTAA
61 120
TACACCGCGAACCAATAAGTTGTCTGCCGGCATAGGGTTGAATGTTAAATTTTGATAATT
a M W R L V I Q Q T A V S Q L T I *
b
c V A L G Y S T D G R I P T Y N L K L L K
61 120
d
e H P A Q N N L L R G Y G L K C N L V I L
f
AAGATGATAAGAAATTCTTCCCACCATACGATGCATCTGCATTAGCAACAGACGAAATTT
121 180
TTCTACTATTCTTTAAGAAGGGTGGTATGCTACGTAGACGTAATCGTTGTCTGCTTTAAA
a M I R N S S H H T M H L H *
b
c D D K K F F P P Y D A S A L A T D E I L
121 180
d
e L H Y S I R G V M R H M Q M
f
TAAAGAAACATCCGGAATTAAAAACGACTATCAATAAATTAAAAGGTAAAATTTCGACAG
181 240
ATTTCTTTGTAGGCCTTAATTTTTGCTGATAGTTATTTAATTTTCCATTTTAAAGCTGTC
a
b
c K K H P E L K T T I N K L K G K I S T E
181 240
d
e
f * L F M * F T F N R C
AAGAGATGCAAAAACTTAATTATGAAGCAGATGGCAAGTTGAAAGAACCATCCATCGTAG
241 300
TTCTCTACGTTTTTGAATTAATACTTCGTCTACCGTTCAACTTTCTTGGTAGGTAGCATC
a M K Q M A S *
b
c E M Q K L N Y E A D G K L K E P S I V A
241 300
d * S A S P L N F S G D M
e * N H L L H C T S L V M
f F L H L F K I I F C I A L Q F F W G D Y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301
360
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Q E F L Q K N N Y F E G K N *
C L F E Q L F I I V K F T F V L S T G F
GATGGACACATTAAAACAATTAATTGATTATTACCAAACAAATGGAAGTTATGTCATGGA
361
420
CTACCTGTGTAATTTTGTTAATTAACTAATAATGGTTTGTTTACCTTCAATACAGTACCT
M D T L K Q L I D Y Y Q T N G S Y V M E
M E V M S W K
* N I I V L C I S T I D H
* T M
S P C M
AGAGTTCTGGCGGCATTTCTTAATGAGTGCTTACGGAGTTATCTTCGCAGCAATCATAGC
421
480
TCTCAAGACCGCCGTAAAGAATTACTCACGAATGCCTCAATAGAAGCGTCGTTAGTATCG
E F W R H F L M S A Y G V I F A A I I A
S S G G I S *
F L E P P M
* P T I K A A I M A
GATACCGCTTGGAGTATATATTGCAAGAAAAAAACGCTTAGCTGGTTGGGTTATCCAAAT
481
540
CTATGGCGAACCTCATATATAACGTTCTTTTTTTGCGAATCGACCAACCCAATAGGTTTA
I P L G V Y I A R K K R L A G W V I Q I
I G S P T Y I A L F F R K A P Q T I W I
CGCTAATATCATCCAAACAATTCCGGCACTAGCAATGTTAGCCGTACTTATGCTTATCAT
541
600
GCGATTATAGTAGGTTTGTTAAGGCCGTGATCGTTACAATCGGCATGAATACGAATAGTA
A N I I Q T I P A L A M L A V L M L I M
A L I M W V I G A S A I N A T S I S I M
GGGCTTAGGGACGAATACAGTCGTCTTGTCCTTGTTCCTATATTCTTTATTACCAATTCT
601
660
CCCGAATCCCTGCTTATGTCAGCAGAACAGGAACAAGGATATAAGAAATAATGGTTAAGA
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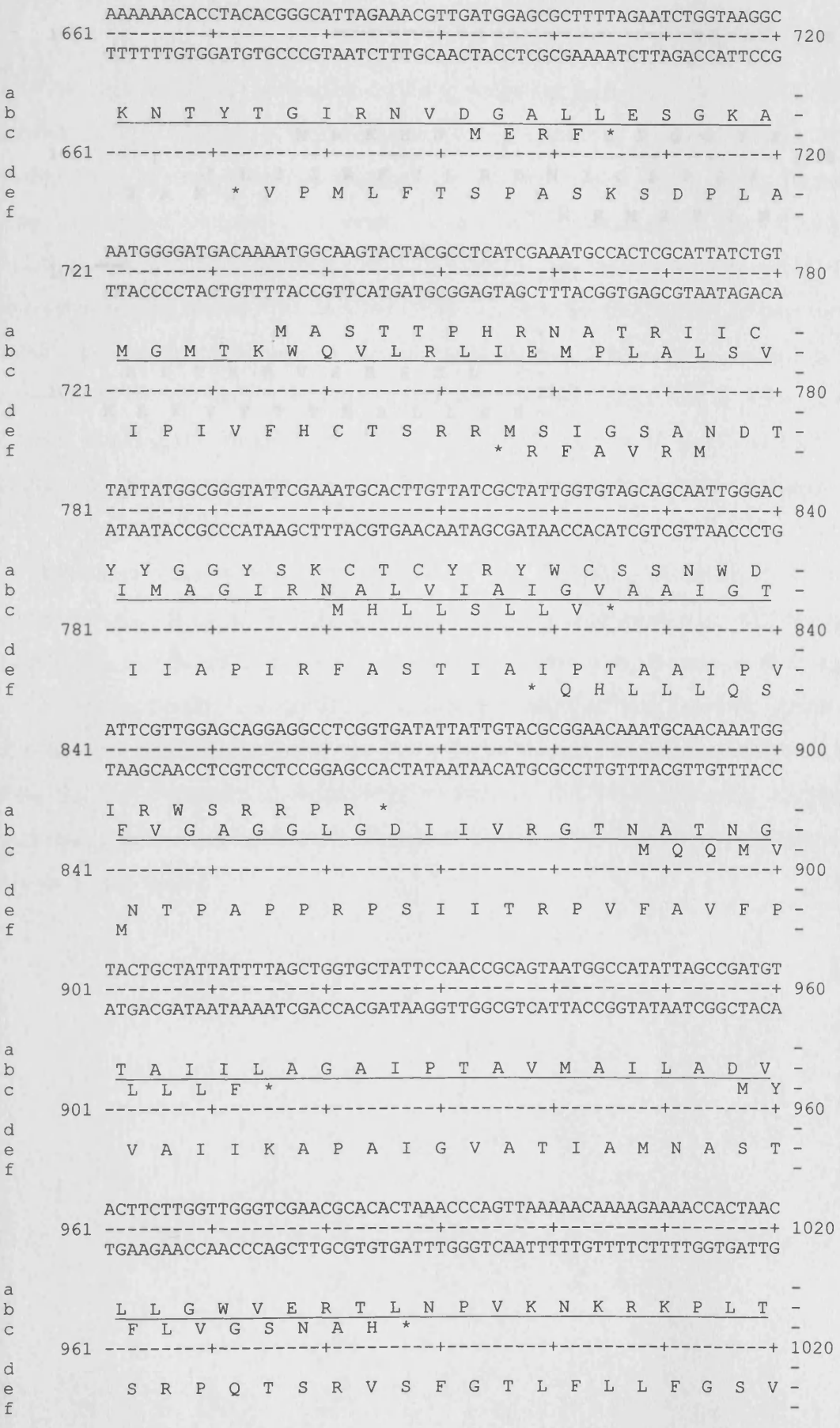

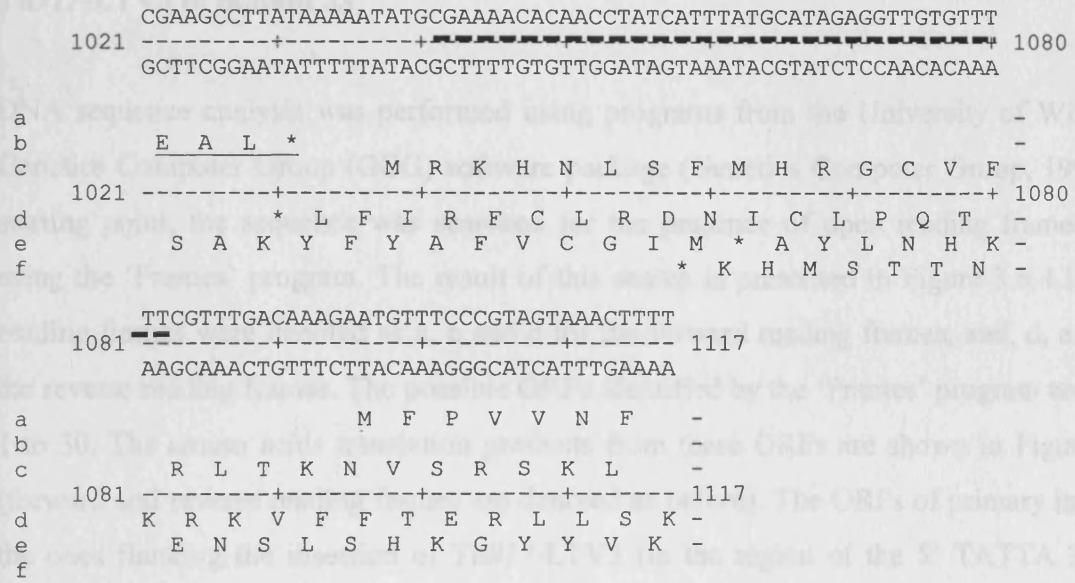
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1021
1080
GCTTCGGAATATTTTTATACGCTTTTGTGTTGGATAGTAAATACGTATCTCCAACACAAA
E A L *
M R K H N L S F M H R G C V F
1021
1080
* L F I R F C L R D N I C L P Q T
S A K Y F Y A F V C G I M * A Y L N H K
* K H M S T T N
TTCGTTTGACAAAGAATGTTTCCCGTAGTAAACTTTT
1081
1117
AAGCAAACTGTTTCTTACAAAGGGCATCATTTGAAAA
M F P V V N F
R L T K N V S R S K L
1081
1117
K R K V F F T E R L L S K
E N S L S H K G Y Y V K

3.6.4 Analysis of the sequence of listerial DNA flanking the site of the insertion of Tn*917*-LTV3 in mutant J3

DNA sequence analysis was performed using programs from the University of Wisconsin's Genetics Computer Group (GCG) software package (Genetics Computer Group, 1995). As a starting point, the sequence was searched for the presence of open reading frames (ORFs) using the 'Frames' program. The result of this search is presented in Figure 3.6.4.1. The six reading frames were denoted as a, b and c for the forward reading frames, and, d, e and f for the reverse reading frames. The possible ORFs identified by the 'Frames' program are labelled 1 to 30. The amino acids translation products from these ORFs are shown in Figure 3.6.3.1 (forward and reverse reading frames are denoted as before). The ORFs of primary interest are the ones flanking the insertion of Tn*917*-LTV3 (in the region of the 5' TATTA 3') which should correspond to the sequence of the listerial gene inactivated by the transposon.

The sequences were analysed with BLASTN and BLASTX (Altschul *et al.*, 1990), for homology comparisons at the DNA and amino acids levels, respectively, with gene sequences in databases. The BLASTX analysis is accomplish by translating the nucleic acid sequences in all six reading frames. The resulting translation product is then searched against the non-redundant GenBank CDS translations, PDB, SwissProt and PIR protein databases maintained at the National Centre for Biotechnology Information (NCBI) in Bethesda, Maryland, USA, for homologous sequences. Results of these comparisons are summarised in Table 3.6.4.1 for only the 12 top scores.

Figure 3.6.4.1 - ORFs in the listerial DNA flanking the Tn*917*-LTV3 insertion in mutant J3.
The listeria DNA, flanking the Tn*917*-LTV3 insertion in mutant J3, was searched for ORFs using the 'frames' program of the GCG software package. The three forward reading frames (a, b and c) and the three reverse reading frames (d, e and f) are shown, with ORFs labelled 1 to 30. The arrowhead represents where Tn*917*-LTV3 inserted.

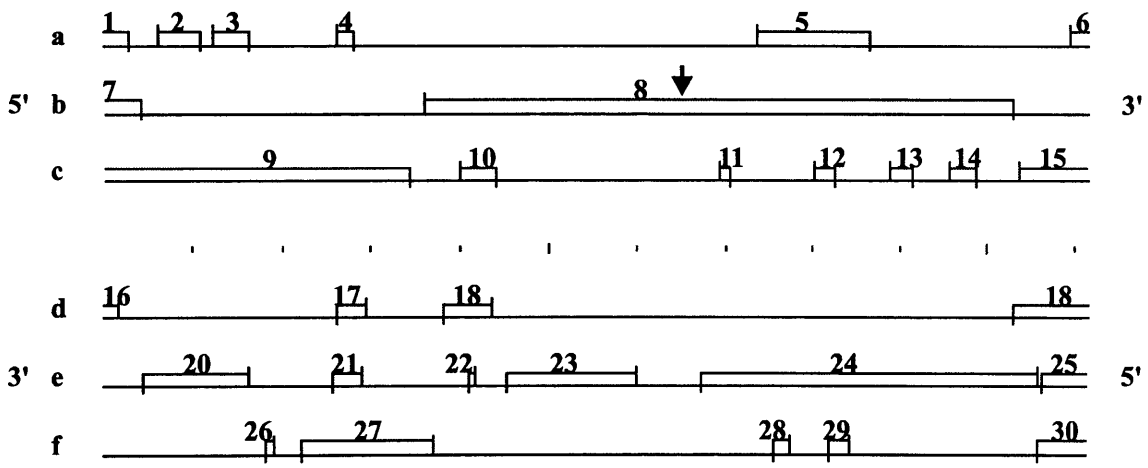

a
1
2
3
4
5
6
5'
b
7
8
3'
c
9
10
11
12
13
14
15
d
16
17
18
18
3'
e
20
21
22
23
24
25
5'
f
26
27
28
29
30

Table 3.6.4.1- Results of the BLASTX search of the protein databases for listeria DNA flanking both sides of the Tn*917*-LTV3 insertion in mutant J3.

ORF	Description	Organism/Acc*	% Identity
8	Transmembrane protein OpuCD	*B. subtilis*/AF009352	73
8	Transmembrane protein OpuBD	*B. subtilis*/AF008930	73
8	Glycine betaine transport ProZ	*B. subtilis*/U38418	71
8	Protein of product/function unknown	*B. subtilis*/M99263	73
8	Hypothetical protein (*spaE* 5' region)	*B. subtilis*	73
8	Transmembrane protein OpuCB	*B. subtilis*/AF009352	43
8	Transmembrane protein OpuBB	*B. subtilis*/AF008930	43
8	Glycine betaine transport ProW	*B. subtilis*/U38418	41
9	Osmoprotectant-binding-protein OpuCC	*B. subtilis*/AF009352	65
9	Osmoprotectant-binding-protein	*B. subtilis*/Z99121	65
9	Osmoprotectant-binding-protein OpuBC	*B. subtilis*/AF008930	61
9	Glycine betaine-binding-protein ProX	*B. subtilis*/U38418	60

*Acc. = Accession number

Special attention was given to the analysis of ORF8, because this ORF flanks the site of the transposon insertion and consequently should correspond to the inactivated gene. The regions of homology between ORF8 product and the different amino acids sequences found in the databases are detailed in Figure 3.6.4.2. The ORF8 translation product, a 224-residue protein, was found to have significant homology to the *Bacillus subtilis* transmembrane protein OpuCD (73% identity and 90% similarity in 216-amino-acids overlap of a total of 229-amino-acids [Kappes *et al.*, sequence submitted in 19.06.97]). The gene encoding for the OpuCD protein, *opuCD*, belongs to the *opuC* operon. This operon contains four ORFs constituting a multicomponent transport system for the uptake of the osmoprotectant glycine betaine (Kappes *et al.*, 1996). The components of this system are arranged in the following order and include: OpuCA, an ATPase (*opuCA*); OpuCB, a transmembrane protein (*opuCB*); OpuCC, an osmoprotectant binding protein precursor (*opuCC*); and OpuCD, a transmembrane protein (*opuCD*) (Kappes *et al.*, sequence submitted in 19.06.97).

Significant homology was also found between the translation product of ORF8 and the *B. subtilis* OpuBD transmembrane protein of 226-amino-acids (73% identity and 88% similarity in a 216-amino-acid overlap [Kappes *et al.*, sequence submitted in 17-06-97]). The OpuBD protein it is encoded by the fourth gene of the OpuB system (*opuBD*). The OpuB system, in similarity to the OpuC system, comprises four components in the following order: OpuBA, an ATPase (*opuBA*); OpuBB, a transmembrane protein (*opuBB*); OpuBC, a choline binding protein precursor (*opuBC*); and OpuBD, a transmembrane protein (*opuBD*). This is a multicomponent transport system for choline (Kappes *et al.*, sequence submitted in 17-06-97).

ORF8 product also exhibited 71% identity and 88% similarity in a 217-amino-acids overlap with *B. subtilis* ProZ protein. This protein is encoded by the *proZ* gene which is the fourth gene of the *proU* operon and consists of 223-amino-acids (Lin and Hasen, 1995). This operon contains four ORFs (ProV, ProW, ProX and ProZ) with homology to the Gram negative ProU proteins, consisting of three proteins (ProV, ProW and ProX) (Lin and Hansen, 1995). These proteins constitute a multicomponent transport system involved in the import of osmoprotectants such as proline and glycine betaine (Lin and Hansen, 1995). ProV and ProW are membrane associated proteins, with ProV sharing considerable sequence identity with ATP-binding proteins (Lin and Hansen, 1995). ProX encodes the glycine betaine-binding protein and ProZ it is believed to be a membrane-associated protein that participates in osmoregulation and complements the function provided by the VWX (Lin and Hansen, 1995).

The translation product of ORF8 yielded homology (73% identity and 88% similarity in an 197-amino-acid overlap) to a *B. subtilis* protein of unknown function encoded in the 5' region of the *spa* operon promoter. The *spa* operon, also known as the subtilin operon, comprises the *spaE*, *spaD*, *spaB*, *spaC* and *spaS* genes (Chung and Hansen, 1992). *spaS* is the structural gene for subtilin, which is an antimicrobial peptide belonging to the class of ribosomally synthesised peptide called lantibiotics (Chung and Hansen, 1992). *spaE* is the first gene of the subtilin operon and is thought to be involved in the production of active subtilin since the interruption of this gene destroys the ability of *B. subtilis* to produce subtilin (Chung and Hansen, 1992). SpaD it is involved in the translocation that follows the binding and insertion of the precursor peptide into the membrane (Chung *et al.*, 1992). The deduced SpaB protein sequence shows extensive homology to a variety of transport proteins and may be involved in subtilin export (Chung *et al.*, 1992). Another ORF situated at the 5' region of the *spaE* gene in *B. subtilis* displayed extensive homology to ORF8 product (73% identity and 89% similarity in an 193-amino-acid overlap), however, the function of the protein encoded by this ORF is still unknown (Chung and Hansen, 1992).

OpuCB and OpuBB also exhibited significant homology (43% identity and 65% similarity for OpuCB and, 43% identity and 64% similarity for OpuBB) to the translation product of ORF8. Both proteins are membrane proteins which belong to the ABC transporters OpuC and OpuB as referred before (Kappes *et al.*, sequence submitted in 19-06-97; Kappes *et al.*, sequence submitted in 17-06-97). The homology between ORF8 protein product/OpuCB and ORF8 protein product/OpuBB is over 198-amino-acid overlap.

Not surprisingly the ORF8 product holds 41% identity and 62% similarity to the *B. subtilis* membrane-associated protein ProW (on a overlap of 198-aminoacids [Lin and Hansen, 1995]). This protein belongs to the ProU operon and exhibits 29% homology to the other membrane-associated protein from the same operon (ProZ) (Lin and Hansen, 1995).

ORF8 has a potential GGAGG ribosome-binding site (shown with an interrupted underline in Figure 3.6.3.1) at an appropriate distance upstream from a putative transcription initiation codon. No evidence for a putative promoter, upstream from ORF8, was found. Each of the identified ORFs in the *B. subtilis proU* operon is preceded by a GGAGG ribosome binding site (Lin and Hansen, 1995).

The striking homology between the ORF8 protein product and the membrane proteins which belong to the different multicomponent transport systems involved in osmoprotection, suggest that the gene inactivated by Tn*917*-LTV3 in *L. monocytogenes* encodes for a membrane protein that may be a member of an osmoprotectant uptake transport system.

The general structure of transmembrane proteins comprise five hydrophobic helices spanning the membrane (Higgins, 1992). In these proteins the triplet Glu Ala Ala (EAA) is commonly found between the third and the fourth spanners of the minimum structure which is conserved in prokaryotes (Higgins, 1992). The hydropathy profile of the ORF8 product (Figure 3.6.4.3) computed by using the method of Kyte and Dolittle (1982) revealed six hydrophobic regions. The approximate locations of these transmembrane regions were assessed by using a Prediction of Protein Localisation Sites program (http://psort.nibb.ac.jp:8800/cgi-bi). These regions were identified between residues 32-48, 67-83, 182-198, 148-164, 129-145 and 84-100. The EAA motif was not found between the third and the fourth hydrophobic helices or in any other region. The differences between the hydropathy profile of the translation product of ORF8 and the profile of a typical transmembrane protein may be related to the unknown function of this new osmoprotectant system component.

Significant homology was also found with the translation product of ORF9, which is localised in a different frame, upstream from ORF8 but with the same orientation. The regions of homology between the ORF9 product and the different sequences found in the protein databases is detailed in Figure 3.6.4.4. In this we found homology to the 3' end of the *B. subtilis* OpuCC protein (65% identity and 84% similarity over 109-amino-acid overlap [Kappes *et al.*, sequence submitted in 19.06.97]). This protein is the osmoprotectant binding protein from the OpuC transport system (Kappes *et al.*, 1996). The same homology was found between the ORF9 protein product and the binding-protein for glycine betaine, carnitine and choline of a *B. subtilis* ABC transporter over 109 amino acids localised at the 3' end of this protein. The OpuBC protein, of the *B. subtilis* OpuB choline ABC transporter, also displayed 61% identity and 81% similarity on a 109-amino-acid overlap (Kappes *et al.*, sequence submitted on 17-06-97). Not surprisingly this partial ORF product also displays considerable homology to the glycine betaine-binding protein from the ProU operon of the same organism (ProX, 60% identity and 79% similarity over 104-amino-acid overlap [Lin and Hansen, 1995]).

In order to investigate for the location, in the cell, of the partially sequenced ORF9 product the Prediction of Protein Localisation Sites program (http://psort.nibb.ac.jp:8800/cgi-bi) was used. This program was unable to locate this fraction of protein in the cell, this result was not surprising since not all protein sequence was known.

The translation product of the partially sequenced ORF9 exhibited homology to the osmoprotectant-binding protein of various binding-protein-dependant uptake systems involved in osmoprotection. In all of these systems, OpuC, OpuB and ProU, the binding protein is encoded by the third of four genes (Lin and Hansen, 1995; Kappes *et al.*, sequences submitted in June 1997). The fact that ORF8, downstream from the ORF9, exhibits homology to the last gene of the operon suggests that these genes are members of a multicomponent uptake system involved in osmoprotection in *L. monocytogenes*. None of the other ORFs showed any significant homology with other proteins in the protein databases screened.

With the aim of investigating if some other sequences homology could be found, the whole insert sequence was analysed using BLASTN (Altschul *et al.*, 1990) for homology comparisons, at the DNA level, with gene sequences in databases. The nucleotide sequence was then searched against the non-redundant GenBank, EMBL, DDBJ and PDB sequences, nucleotide sequences databases maintained at the National Centre for Biotechnology Information (NCBI) in Bethesda, Maryland, USA, for homologous sequences. Searches based on DNA sequences are generally less sensitive than protein-based searches, so we must be careful in drawing conclusions from this type of search results.

The results from the search for homology comparisons at the DNA level did not revealed any new sequences of homology. The regions of DNA that coded for ORF8 exhibited homology, in the range of 64-66%, to regions of the coding sequences for the *B. subtilis* osmoprotectant transport system OpuC, choline transport system OpuB and glycine betaine transport system ProU. The region of DNA encoding for ORF9 displayed 65% identify, over 322 bp, to a region of the *B. subtilis* genome not coding for a specific protein. The same homology was displayed between ORF9 and a coding region of the *B. subtilis* osmoprotectant transport system OpuC. As can be perceived, this type of analyses revealed less sequences of homology than the protein databases searches.

Strong transcription terminators can be found at the 3' end of the last gene of an operon. This is the case with the *B. subtilis proU* operon where a GC-rich stemloop is followed by a stretch

of T residues (Chung *et al.*, 1992). In order to support further the suggestion that ORF8 and ORF9 are members of a similar operon, the DNA sequence at the 3' end of ORF8 was carefully examined using the 'stemloop' program from the Genetics Computer Group (GCG) software package (Genetics Computer Group, 1995). The listerial DNA sequence extending downward from position 1042 up to 1082 (Figure 3.6.3.1) constitutes a very strong stemloop which in homology with the ρ-independent terminator downstream from *proZ* also finish with a stretch of T residues (Figure 3.6.4.5). These results further support the conclusion that a *proU* type locus might exist in *L. monocytogenes*.

Figure 3.6.4.2 - Homology of the translation product of ORF8 protein product to transmembrane proteins involved in osmoprotection.

Significant homologies from the BLASTX search are shown. Transmembrane protein OpuCD, OpuBD, OpuCB and OpuBB of the *B. subtilis* osmoprotectant transport systems OpuC and OpuB share homology with the translation product of ORF8. "Unknown" represents the protein of function unknown (accession number M99263) which also displayed homology to ORF8 protein. Proteins ProZ and ProW from the *B. subtilis* glycine-betaine transport system ProU also displayed homology to ORF8 protein product. Protein sequences with homology are represented in bold.

```
          1                                                  50
  OpuBB   ~~~MHHIVQF LQTNGGELLY KTYEHITISL IAVILGVLVA VPLGVVL..T
   ProW   ~~~MHHIIQF LQTNGGELLY KTYEHITISL IAVILGVLVA VPLGVVLTFT
  OpuCB   ~~~MNQMMTF LQTNGGELLY KTGEHLYISL IAVVLGIIVA VPLGVAL..T
  OpuBD   MNVLEQLMTY YAQNGSYVMD EFGRHFLMSA YGVLFAAVVG VPAGI..LIA
   SpaE   ~~~~~~~~~~ ~~~~~~~~~~ ~~~~HFLMSV YGVLFAAVVG VPVGI..LIA
   ProZ   MNVLEQLMTY YAQNGSYVMD EFGRHFLMSV YGVLFAAVVG VPVGIFTLIA
Unknown   ~~~~~~~~~~ ~~~~~~~~~~ EFGRHFLMSV YGVLFAAVVG VPVGI..LIA
  OpuCD   MEVLQQLGTY YSQNGGYVLQ EFYRHFLMSV YGVLFAAIVG IPLGI..LIA
   ORF8   MDTLKQLIDY YQTNGSYVME EFWRHFLMSA YGVIFAAIIA IPLGVY..IA

          51                                                 100
  OpuBB   RMKKGAGTII GIVNIIQ..T LPSLAILAFF IPLLGVGKVP AIVALFFYSV
   ProW   RMKKGAGTII GIVNIIQ..T LPSPDSRHIF IPLLGVGKVP AIVALFFYSV
  OpuCB   RMKKGAGAVI GFVNIVQ..T LPSLAILAFF IPLLGVGKVP AIVALFFYSV
  OpuBD   HFRRLSAWVF AVTNVIQ..T IPALAMLAVL MLVMGLGANT VILSLFLYSL
   SpaE   HYRRLSAWVF AVTNVIQ..T IPALAMLAVL MLVMGLGANT VIISLFLYSL
   ProZ   HYRRLSAWVF AVTNVIQ..T IPALAMLAVL MLVMGLGANT VIISLFLYSL
Unknown   HYRRLSAWVF AVTNVIQFTT IPALAMLAVL MLVMGLGANT VIISLFLYSL
  OpuCD   RYRRLSGWVF AVTNVIQ..T IPALAMLAVL MLVMGLGANT VILSLFLYSL
   ORF8   RKKRLAGWVI QIANIIQ..T IPALAMLAVL MLIMGLGTNT VVLSLFLYSL

          101                                                150
  OpuBB   LPILRNTYTG I..RGVNKNL LESGKGIGM. .TPAEQVRLV ELPLAAPVIM
   ProW   LPILRNTYTG IFTRGVNKNL LESGKGIGM. .TPAEQVRLV DVPLAAPVIM
  OpuCB   LPILRNTYTG I..KGVNKNL LESGKGIGM. .TGWEQIRLV EIPLAIPIIM
  OpuBD   LPIIRNTY.. TGIISIEHAY LESGKAMGM. .TKFQVLRMV ELPLALSVIM
   SpaE   LPIIRNTY.. TGIVSIEHAY LESGKAMGM. .TKFQVLRMV ELPLALSVIM
   ProZ   LPIIRNTYFT TGIVSIEHAY LESGKAMGM. .TKFQVLRMV ELPLALSVIM
Unknown   LPIIRNTY.. TGIVSIEHAY LESGKAMGMT FTKFQVLRMV ELPLALSVIM
  OpuCD   LPIIRNTY.. TGIISIEHAY LESGKAMGM. .TKFQVLRMV ELPLALSVIM
   ORF8   LPILKNTY.. TGIRNVDGAL LESGKAMGM. .TKWQVLRLI EMPLALSVIM

          151                                                200
  OpuBB   AGIRTSTIYL IGWATLASFI GGGG..LGDY IFIGLNLYQP ...EYIIGGA
   ProW   AGIRTSTIYL IGWATLASFI GGGGFTLGDY IFIGLNLYQP ...EYIIGGA
  OpuCB   AGIRTSTIYL IGWATLASFI GGGG..LGDY IFIGLNLYQP ...EYIIGGA
  OpuBD   AGLRTALVIA IGITAIGTFV G..AGGLGDM IVRGSNATNG TAI..ILAGA
   SpaE   AGLRTALVIA IGITAIGTFV G..AGGLGDM IVRGSNATNG TAI..ILAGA
   ProZ   AGLRTALVIA IGITAIGTFV GFTAGGLGDM IVRGSNATNG TAI..ILAGA
Unknown   AGLRTALVIA IGITAIGTFV G..AGGLGDM IVRGSNATNG TAIFTILAGA
  OpuCD   AGLRTALVIA IGITAIGTFV G..AGGLGDI IVRGSNATNG TAI..ILAGA
   ORF8   AGIRNALVIA IGVAAIGTFV G..AGGLGDI IVRGTNATNG TAI..ILAGA

          201                                          241
  OpuBB   VPVTILAIVI DYVLAVAERK LTPAGMQRLK ELS~~~~~~~ ~
   ProW   VPVTILAIVI DYVLAVTERK LTPAGMQRLK EVS~~~~~~~ ~
  OpuCB   VPVTILAIII DYVLAVTERK VTPKGLQGMK EVS~~~~~~~ ~
  OpuBD   IPTAVMAVGA DLLMAWLERA LSPVKKKRTG AKHVQSAA~~ ~
   SpaE   TPTAVMAIGA DLIMAWIERF LNPVKQKSR~ ~~~~~~~~~~ ~
   ProZ   TPTAVMAIGA DLIMAWIERF LNPVKQKSRR KVISFTV~~~ ~
Unknown   TPTAVMAIGA DLIMAWIERF LNPVKQKSRR KVISV~~~~~ ~
  OpuCD   IPTALMAVIA DLVMGWLERA LSPIKKKKGN FIIADRKTTS I
   ORF8   IPTAVMAILA DVLLGWVERT LNPVKNKRKP LTEAL*~~~~ ~
```

Figure 3.6.4.3 - Hydropathy profile of *L. monocytogenes* ORF8 translation product.
The hydrophobicity of the protein is represented as a hydropathicity index, computed by using the method of Kyte and Doolittle (1982), which is plotted against the residue number.

ORF8 translation product:

MDTLKQLIDYYQTNGSYVMEEFWRHFLMSAYGVIFAAIIAIPLGVYVARK
KRLAGWVIQIANIIQTIPALAMLAVLMLIMGLGTNTVVLSLFLYSLLPILKN
TYTGIXNVDGALLESGKAMGMTKWQVLRLIEMPLALSVIMAGIRNALVIA
IGVAAIGTFVGAGGLGDIIVRGTNATNGTAIILAGAIPTAVMAILADVLLGW
VERTLNPVKNKRKPLTEAL

Individual values for the 20 amino acids of the hydropathicity scale of Kyte and Doolittle (1982) are:

Ala: 1.80	Arg: -4.50	Asn: -3.50	Asp: -3.50	Cys: 2.50	Gln: -3.50
Glu: -3.50	Gly: -0.40	His: -3.20	Ile: 4.50	Leu: 3.80	Lys: -3.90
Met: 1.90	Phe: 2.80	Pro: -1.60	Ser: -0.80	Thr: -0.70	Trp: -0.90
Tyr: -1.30	Val: 4.20	Asx: -3.50	Glx: -3.50	Xaa: -0.49	

Weights for window positions 1,..,9, using linear weight variation model:

1	2	3	4	5	6	7	8	9
1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
edge				center				edge

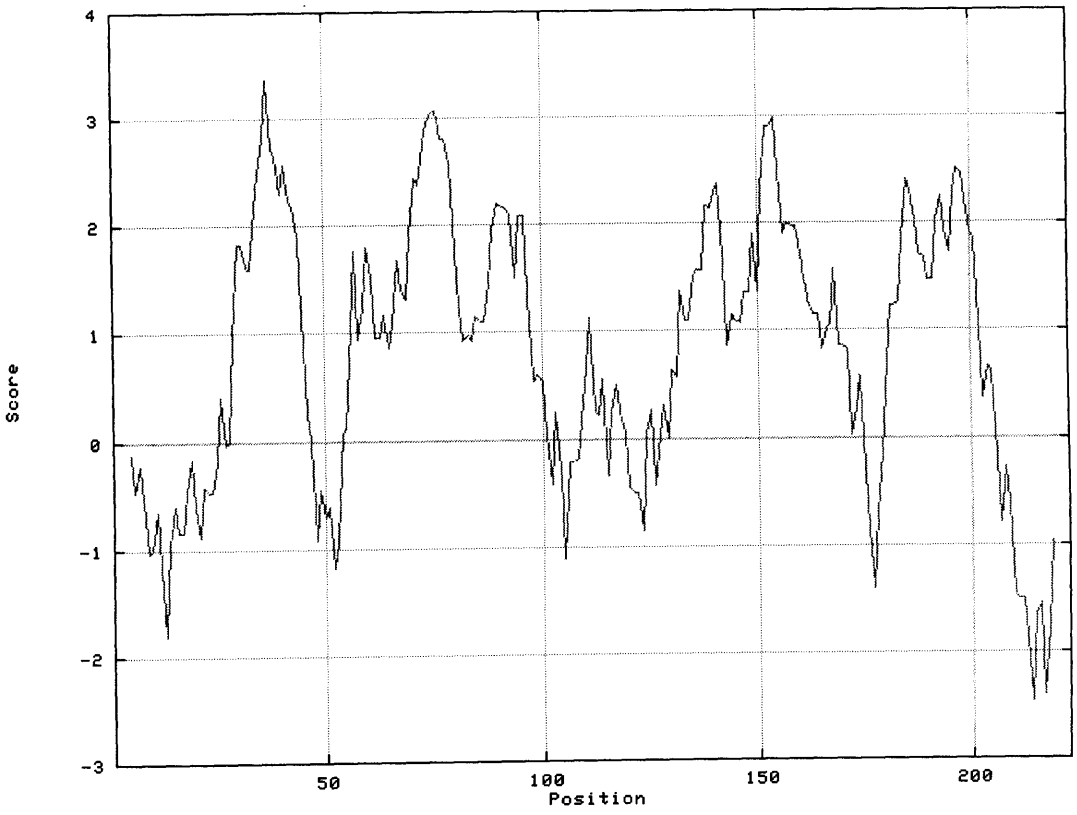

Score
4
3
2
1
0
-1
-2
-3
50
100
150
200
Position

Figure 3.6.4.4 - Homology of the ORF9 protein product to osmoprotectant binding proteins. Significant homologies from the BLASTX search are shown. Osmoprotectant binding protein precursor OpuCC, OpuBC and ProX from the *B. subtilis* transport systems OpuC, OpuB and ProU respectively, exhibited homology to the translation product of ORF9. Bacgen represents the protein sequence of the *B. subtilis* osmoprotectant binding protein with the accession number Z99121. Protein sequences with homology is represented in bold.

```
        1                                                   50
Bacgen  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
 OpuCC  MKMTKIKWLG AFALVFVMLL GGCSLPGLGG ASDDTIKIGA QSMTE..SEI
 OpuBC  MKRKYLKLMI GLALAATLTL SGCSLPGLSA AADQTIKIGA QSMSE..SEI
  ProX  MKRKYLKWMI GLTLAAMLPL SGCSLPGLSA ASDQTIKIGA QSMSEFTSEI
  ORF9  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~

        51                                                  100
Bacgen  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
 OpuCC  VANMIAHVIE HDTDLNTALV KNLGSNYVQH QAMLGGDIDI SATRYSGTDL
 OpuBC  IASMLGQLIE HHTDLKTTTI KNLGSNAVQQ QALMNGEIDI AATRYTGDAL
  ProX  IASMLGQLIE HHTDLKTTTI KNLGSNAVQQ QALMNREIDI AATRYTGDAL
  ORF9  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~

        101                                                 150
Bacgen  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
 OpuCC  TSTLGK..EA EKDPKKALNI VQNEFQKRFS YKWFDSYGFD NTYAFTVTKK
 OpuBC  TGTLRM..EP EKDPDKALAL TQREFKKRYD LKWYDSYGFD NTYAFTVSKE
  ProX  TGTLRMFTEP EKDPEKALAL TQREFKKRYD LKWYDSYGFD NTYAFTVSKK
  ORF9  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~

        151                                                 200
Bacgen  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
 OpuCC  FAEKEHINTV SDLKKNA..S QYKLGVDNAW LKRKGDGYKG FVSTYGFEFG
 OpuBC  LADQYHLETV SDVKKWA..P QLKLGVDNYW MKLKGNGYQD FTKTYGMTFG
  ProX  LADQYHLENV SDVKKWAFTP QLKLGVDKVL DEAQGERLSR FYENYGMTFS
  ORF9  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~

        201                                                 250
Bacgen  ~~YPMQIGLV YDAVKNGKMD AVLAYSTD.. GRIKAYDLKI LKDDKRFFPP
 OpuCC  TTYPMQIGLV YDAVKNGKMD AVLAYSTD.. GRIKAYDLKI LKDDKRFFPP
 OpuBC  GTYPMQIGLV YDAVKSGKMD IVLAYSTD.. GRIKSYGLKM LKDDKQFFPP
  ProX  GTYPMQIGLV YDAVKSGKMD IVLAYSTDFT GRIKSYGLKM LKDDKQFFPP
  ORF9  KIFPMQIGLI YTALKNNQMD VALGYSTD.. GRIPTYNLKL LKDDKKFFPP

        251                                                 300
Bacgen  YDCSPVIPEK VLKEHPELEG VINKLIGQID TETMQELNY. .EVDGKLKEP
 OpuCC  YDCSPVIPEK VLKEHPELEG VINKLIGQID TETMQELNY. .EVDGKLKEP
 OpuBC  YDCSPVVPEK VLKEHPELEG IIKKMLGKID TATMQELNY. .EVDGNLKEP
  ProX  YDCSPVVPEQ VLKEHPELEG TIQKMIGKID TATMQELNYF TEVDGNLKEP
  ORF9  YDASALATDE ILKKHPELKT TINKLKGKIS TEEMQKLNY. .EADGKLKEP

        301                 323
Bacgen  SVVAKEFLEK HHYFD~~~~~ ~~~
 OpuCC  SVVAKEFLEK HHYFD~~~~~ ~~~
 OpuBC  SVVAKEYLEK HRYFES~~~~ ~~~
  ProX  SVVAKAIFRK APLLRIVKGG RSQ
  ORF9  SIVAQEFLQK NNYFEGKN*~ ~~~
```

Figure 3.6.4.5 - Comparison of the potential terminator sequence located downstream from the ORF8 sequence to the ρ-independent terminator sequence located downstream from the *B. subtilis proZ* (Lin and Hanssen, 1995).

Underline represents the terminator inverted repeats.

B. subtilis AAAAAGGATTCTTTTCTGAGAGGGAAAAGAGTCCTTTTTT

L. monocytogenes GAAAACACAACCTATCATTTATGCATAGAGGTTGTGTTTTT

3.7 Phenotypic Characterisation of *L. monocytogenes* Insertional Mutants

3.7.1 Phenotypic characterisation of *L. monocytogenes* mutants defective for growth, at 4°C, on solid medium

3.7.1.1 Characterisation of *L. monocytogenes* mutants defective for growth at 4°C on solid medium, on TSA at 30 and 4°C

Mutants defective for growth on plates at 4°C were identified in the initial screening as described in Section 3.3.1. Subsequently, plates spot-inoculated with 1μl of mutants and wild type cultures, at the same OD_{600nm} (0.6), were incubated at 30 and 4°C as described in Figure 3.3.1.1 (see Section 3.3.1). In this experiment, at 4°C, the difference in growth between wild type and mutants started to be visible after 14 days of incubation. An identical experiment, set up a few months later, is shown in Figure 3.7.1.1.1. In this Figure, the growth of mutants and wild type at 30°C (A) and at 4°C (B) is displayed. The plates at 30°C were incubated for overnight, and plates at 4°C were incubated until the difference in growth between wild type and mutants could be seen, which happened after 9 weeks of incubation. At 30°C mutants and wild type still exhibited the same growth after overnight incubation, however, at 4°C the difference in growth between mutants (Figure3.7.1.1.1, B, numbers 2 - 7) and wild type (Figure3.7.1.1.1, B, number 1) was visible after more seven weeks of incubation than the original experiment (Figure 3.3.1.1). These results suggested that in order to investigate for the difference in growth between wild type and mutant strains at 4°C on solid medium, may be necessary to incubate the plates for longer than 14 days.

In order to confirm that differences in growth between mutants and wild type was exclusively due to the temperature of incubation and not to an antibiotic effect, mutants and wild type were grown together on plates of TSA with no antibiotic added. The results of this experiment are shown in Figure 3.7.1.1.2. As can be seen *L. monocytogenes* mutants and wild type exhibited the same growth after incubating overnight at 30°C (Figure 3.7.1.1.2, A). At 4°C after 9 weeks, mutants G3, J3, K3, M3 and N3 still exhibited less growth when compared to the growth of the wild type (Figure 3.7.1.1.2, B). Interestingly, mutant 7D (iron uptake defective mutant), which has been used in the last two experiments just as a "control", exhibited identical growth as the wild type, when incubating at 4°C (Figure 3.7.1.1.2, B, numbers 7 and 1). These results confirmed that mutants are defective for growth at 4°C on

solid medium, when compared to the growth of the wild type and that this difference is not an effect of growth on antibiotic.

3.7.1.2 Characterisation of *L. monocytogenes* mutants defective for growth at 4°C on solid medium, on TSA containing X-GAL

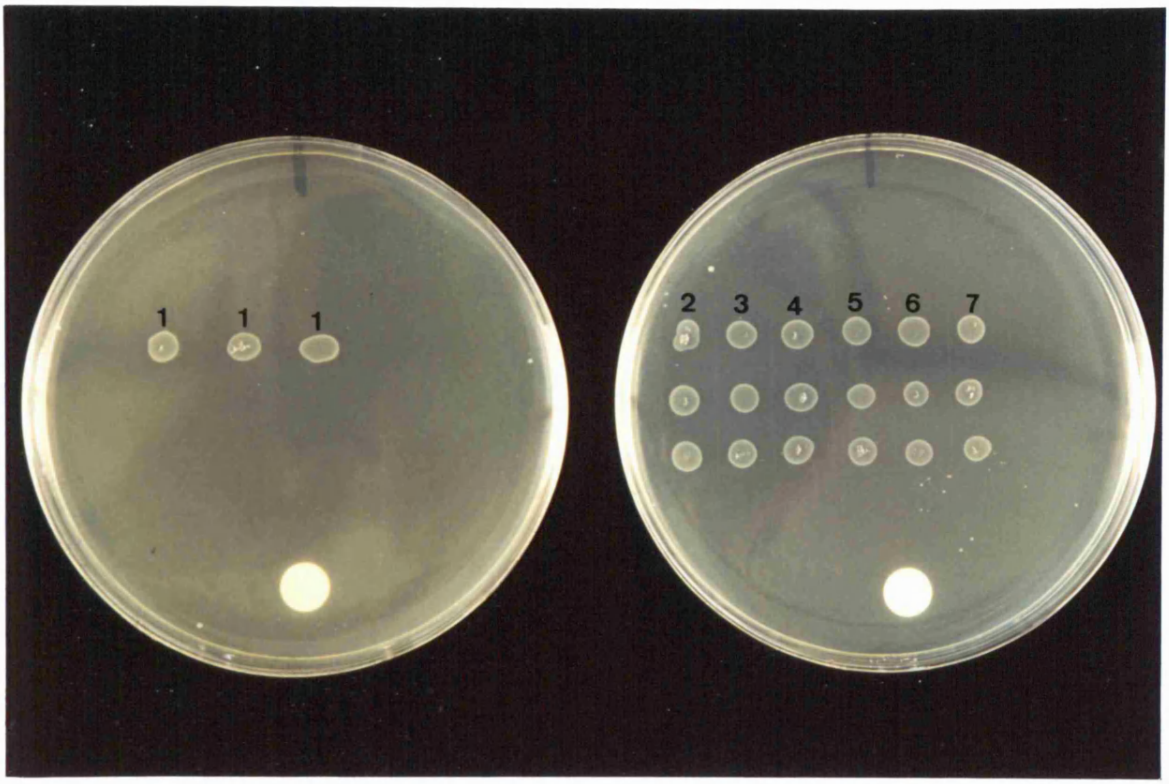
To investigate if the mutation occurred downstream of an active promoter, or a promoter regulated by temperature, mutants and wild type were streaked on TSA containing X-GAL, with the appropriate antibiotics when needed, and incubated at 30 and 4°C. None of the mutants expressed ß-galactosidase activity at either temperature. These results may suggest that the mutation did not occur downstream of an active promoter, that the insertion of the derivative Tn*917*-LTV3 was not in the same direction as the transcription of the gene inactivated or that the promoter of the gene inactivated was weak or too far away from the site where Tn*917*-LTV3 has inserted.

Figure 3.7.1.1.1 - Growth of *L. monocytogenes* wild type, 10403S, and transposon insertion mutants defective for growth medium at 4°C on TSA plates containing the appropriate antibiotics.

(A) Growth of wild type and mutants after overnight at 30°C. Number 1 corresponds to *L. monocytogenes* wild type; and, numbers 2 through 7, corresponds to *L. monocytogenes* transposon insertion mutants G3, J3, K3, M3, N3 and 7D. Each strain is represented by rows of 3 inoculation spots.

(B) Growth of wild type and mutants after 9 weeks at 4°C. Number 1 corresponds to *L. monocytogenes* wild type; and, numbers 2 through 7, corresponds to *L. monocytogenes* transposon insertion mutants G3, J3, K3, M3, N3 and 7D. Each strain is represented by rows of 3 inoculation spots.

A



B

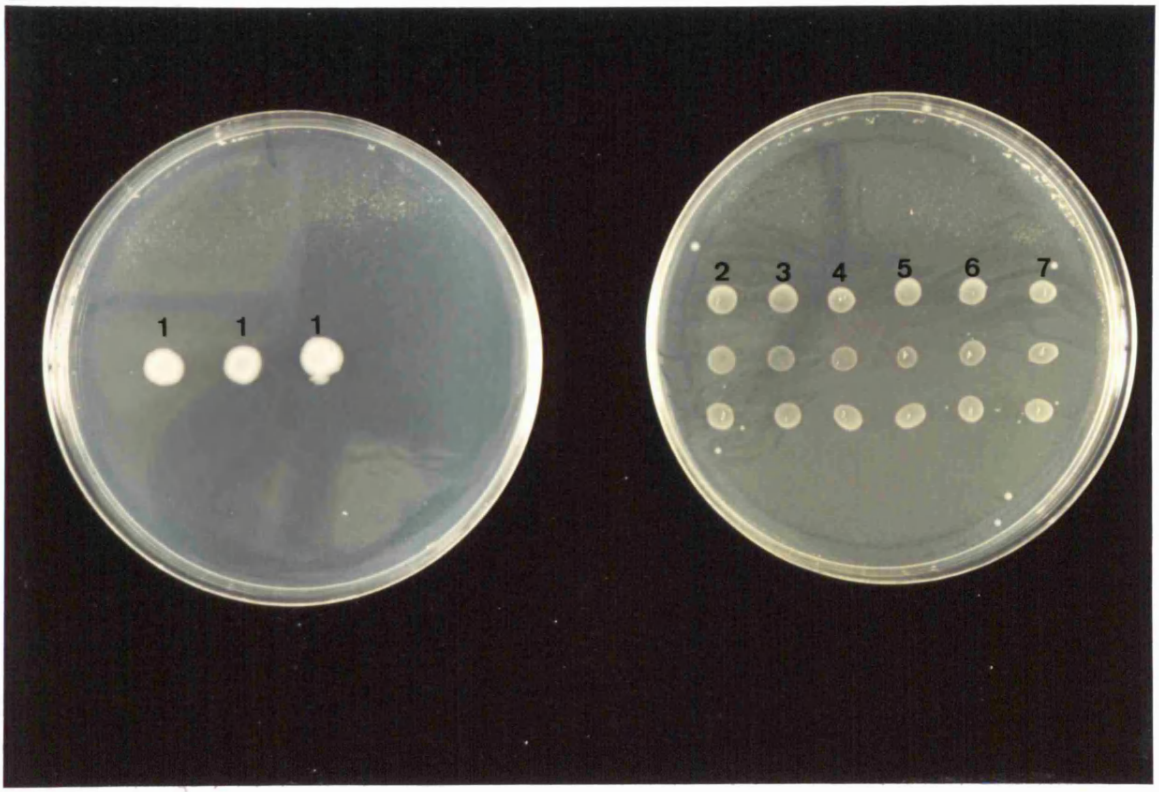
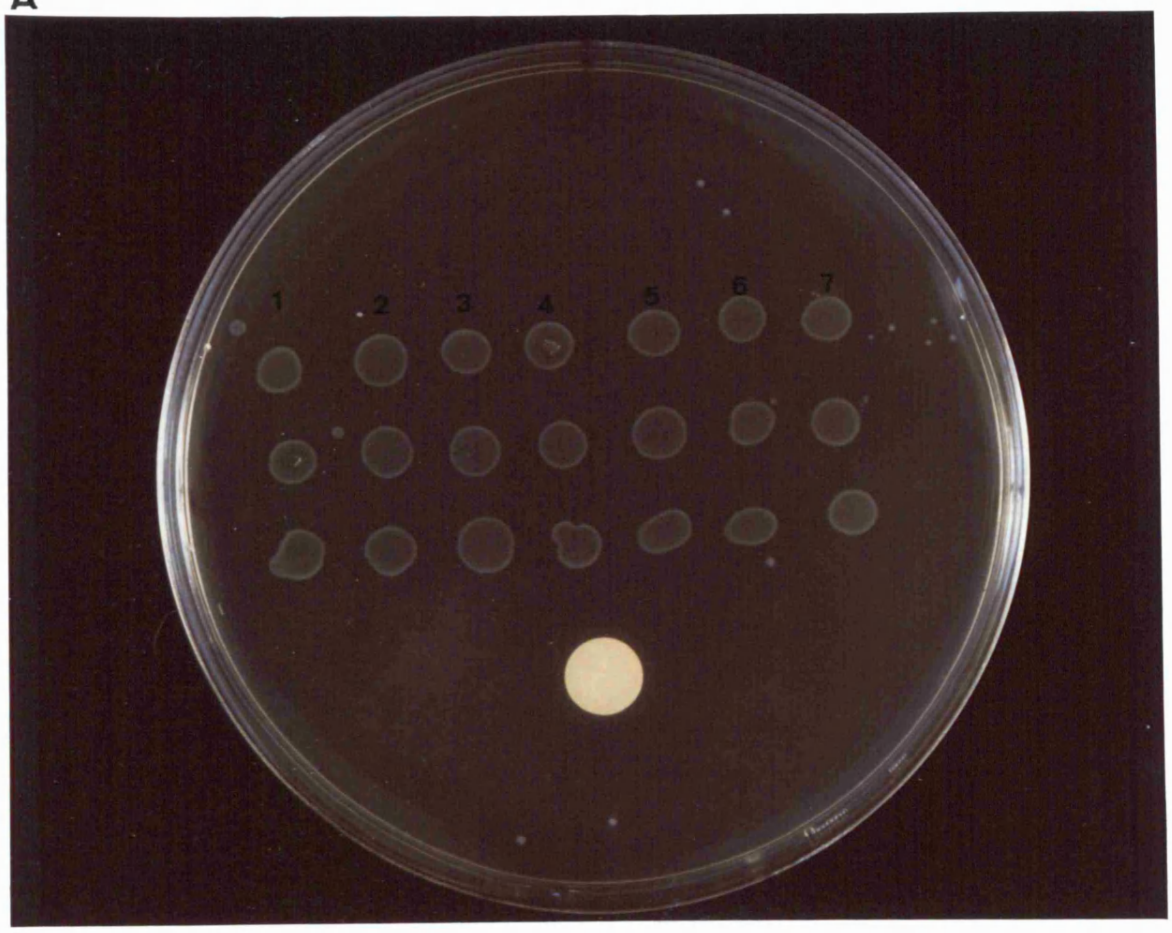


Figure 3.7.1.1.2 - Growth of *L. monocytogenes* wild type, 10403S, and mutants defective for growth on solid medium at 4°C on TSA plates without any antibiotics.

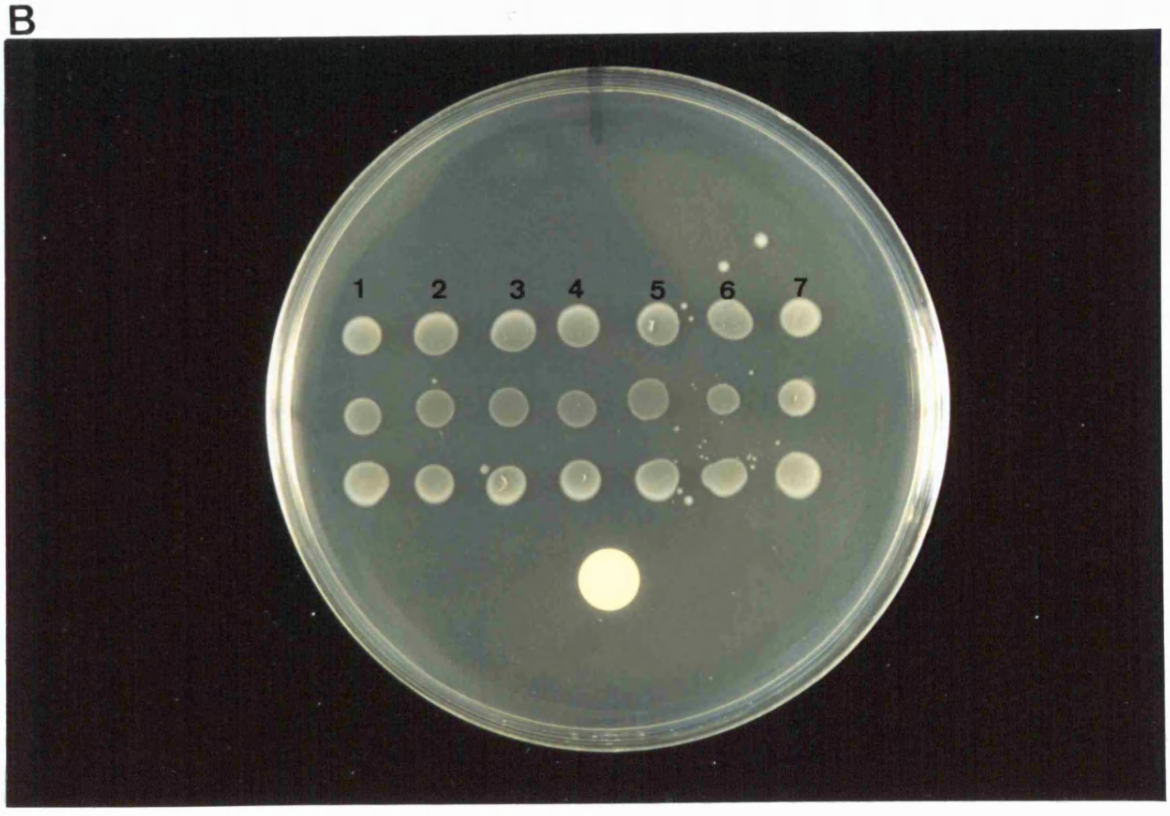
(A) Growth of *L. monocytogenes* wild type and mutants after overnight at 30°C. Number 1 corresponds to *L. monocytogenes* wild type; and, numbers 2 through 7, corresponds to transposon insertion mutants G3, J3, K3, M3, N3 and 7D. Each strain is represented by rows of 3 inoculation spots.

(B) Growth of *L. monocytogenes* wild type and mutants after 9 weeks at 4°C. Number 1 corresponds to *L. monocytogenes* wild type; and, numbers 2 through 7, corresponds to transposon insertion mutants G3, J3, K3, M3, N3 and 7D. Each strain is represented by rows of 3 inoculation spots.

A



B



3.7.1.3 Characterisation of *L. monocytogenes* mutants defective for growth at 4°C on solid medium, in TSB at 30 and 4°C

In order to analyse if the growth of the transposon insertion mutants, defective for growth at 4°C on solid medium, was also affected when grown in liquid medium, these mutants were grown in TSB at 30 and 4°C and OD_{600nm} measured, at regular intervals, to monitor growth. In Figure 3.7.1.3.1 the growth of the mutants and *L. monocytogenes* wild type 10403S, in TSB at 30 and 4°C is shown. As can be seen in Figure 3.7.1.3.1 (A), the pattern of growth exhibited by the *L. monocytogenes* mutants and wild type at 30°C was similar, with no major differences between them. At 4°C, Figure 3.7.1.3.1 (B), the growth of the transposon insertion mutants and wild type in TSB also displayed a similar pattern. Both, transposon insertion mutants and wild type, exhibited a lag phase of approximately 9 days with an exponential growth phase finishing on day 16 from the beginning of growth. One-way ANOVA was applied to the growth rates constants (μ_{max}) of growth curves to both temperatures.

In Table 3.7.1.3.1 the growth rate constants for growth curves at 30 and 4°C are given. As can be seen in this Table, growth rates at 30°C were approximately 40 times higher than those at 4°C. ANOVA indicated a statistically significant difference in the growth rates of the strains at 30 and 4°C respectively. This analysis, however, does not identify between which strains these differences occur. To do this, the multiple comparison Scheffé test (Scheffé, 1959), was used with a significance level of 0.05. This test revealed that at 30°C the significant difference occurs between the wild type strain and mutants J3, K3 and N3. These results indicate that the ability of mutants J3, K3 and N3 to grow at 30°C were affected when compared to the ability of the wild type strain. At 4°C, although ANOVA suggested a difference between strains ($P<0.05$), no two groups were significantly different after the Scheffé test. These results demonstrated that the ability of the mutants to grow in TSB at low temperature (4°C) was not affected when compared to the ability of the wild type.

Table 3.7.1.3.1 - Growth rate constants for *L. monocytogenes* wild type, 10403S, and transposon insertion mutants defective for growth on solid medium at 4°C, when grown in TSB at 30 and 4°C.

Number of experiment		**Mutant Strains**					
		Wild type	**G3**	**J3**	**K3**	**M3**	**N3**
30°C	1	477	454	431	438	474	442
	2	495	465	431	435	477	417
	3	514	438	449	419	465	458
4°C	1	11.5	9.2	9.2	9.2	9.2	11.5
	2	13.8	11.5	9.2	9.2	11.5	11.5
	3	11.5	9.2	9.2	9.2	11.5	9.2

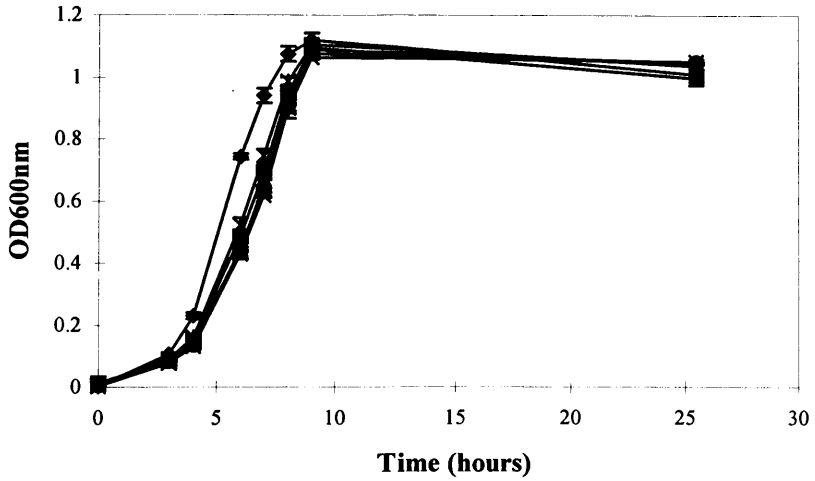
For simplification growth rate constant is multiplied by 1000 and units are per hour.

Figure 3.7.1.3.1 - Growth of the *L. monocytogenes* wild type, 10403S (◆), and *L. monocytogenes* transposon insertion mutants G3 (■), J3 (▲), K3 (x), M3 (*) and N3 (●), defective for growth at 4°C on solid medium, in TSB at 30°C (A) and 4°C (B).

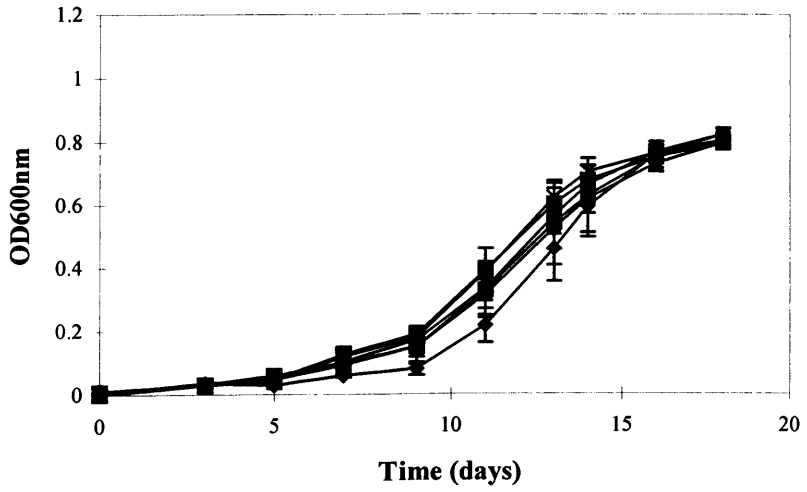
Data are the mean ± standard deviation of 3 experiments.

The pattern of growth exhibited by *L. monocytogenes* wild type, 10403S and transposon insertion mutants was similar at both temperatures.

A


1.2
1
0.8
0.6
0.4
0.2
0
OD600nm
0
5
10
15
20
25
30
Time (hours)

B


1.2
1
0.8
0.6
0.4
0.2
0
OD600nm
0
5
10
15
20
Time (days)

3.7.1.4 Comparison of the API profiles of the *L. monocytogenes* wild type, 10403S, and mutants defective for growth on solid medium at 4°C

To compare the phenotypes of the *L. monocytogenes* mutants, defective for growth on solid medium at 4°C, and wild type 10403S, API20STREP and API50CH (bioMérieux, France) galleries were used. These galleries were used following the manufacturer's instructions and incubated at 30 and 4°C for 24 hours and 4 weeks respectively. The results of the API20STREP and API50CH, when incubated at 30°C, are shown in Table 3.7.1.4.1 and Table 3.7.1.4.2 respectively.

As can be seen in Table 3.7.1.4.1 *L. monocytogenes* transposon insertion mutants J3, K3 and M3 produced the same biochemical profile as the wild type. Mutants G3 and N3, differ from the wild type in that they produced a positive β-galactosidase reaction, such reaction was not observed when growing on plates with X-Gal and incubated at 30°C. These results suggest that the biochemical test used in the gallery of the API20STREP, for the ß-galactosidase reaction, is a more sensitive test than that performed on plates. The difference in sensitivity may be related to the fact that the substrate for this reaction is different in both tests. In the API gallery the substrate was 2-naphtyl-ß-D-galactopyranoside and in the X-gal plates the substrate used was 5-Bromo-4-chloro-3 indolyl-ß-D-galactosidase which is the commonly used substrate.

The fermentation of the different carbohydrates by the *L. monocytogenes* wild type, 10403S, and transposon insertion mutants G3, J3, K3, M3 and N3 when incubating at 30°C for 24 hours, can be seen in Table 3.7.1.4.2. All mutants exhibited the same profile as the wild type strain.

In Table 3.7.1.4.3 and 3.7.1.4.4, the results of the API20STREP and API50CH respectively, when incubated at 4°C, can be seen.

Table 3.7.1.4.1 - Results of the API20STREP galleries reactions, when incubating at 30°C for 24 hours. The strains used were *L. monocytogenes* wild type, 10403S (WT), and transposon insertion mutants defective for growth at 4°C (G3, J3, K3, M3 and N3). Symbols: +, positive reaction; -, negative reaction.

Tests	Substrate	Reactions/Enzymes	Results for each strain					
			WT	G3	J3	K3	M3	N3
VP	Pyruvate	Acetoin production	+	+	+	+	+	+
HIP	Hippurate	Hydrolysis	+	+	+	+	+	+
ESC	Esculine	ß-glucosidase	+	+	+	+	+	+
PYRA	Pyrrolidonyl 2 naphtylamide	Pyrrolidonylarylamidase	-	-	-	-	-	-
αGAL	6-Bromo-2-naphtyl α-D-Galactopyranoside	α-galactosidase	-	-	-	-	-	-
βGUR	Napthol AS-BI β-D-glucuronate	β-glucuronidase	-	-	-	-	-	-
βGAL	2-naphtyl-β-D galactopyranoside	β-galactosidase	-	+	-	-	-	+
PAL	2-naphtyl phosphate	Alkaline phosphatase	+	+	+	+	+	+
LAP	L-leucine-2-naphtyl-amide	Leucine arylamidase	+	+	+	+	+	+
ADH	Arginine	Arginine dihydrolase	-	-	-	-	-	-
RIB	Ribose	Acidification	-	-	-	-	-	-
ARA	L-Arabinose	Acidification	-	-	-	-	-	-
MAN	Mannitol	Acidification	-	-	-	-	-	-
SOR	Sorbitol	Acidification	-	-	-	-	-	-
LAC	Lactose	Acidification	+	+	+	+	+	+
TRE	Trehalose	Acidification	+	+	+	+	+	+
INU	Inulin	Acidification	-	-	-	-	-	-
RAF	Raffinose	Acidification	-	-	-	-	-	-
AMD	Starch	Acidification	+	+	+	+	+	+
Glyg	Glycogen	Acidification	-	-	-	-	-	-

Table 3.7.1.4.2 - Results of the API50CH gallery reactions, when incubating at 30°C for 24 hours. The strains used were *L. monocytogenes* wild type, 10403S (WT), and transposon insertion mutants defective for growth at 4°C (G3, J3, K3, M3 and N3). Values: 0 is given to negative reactions and 5 to positive reactions of maximum intensity. Numbers 1, 2, 3, or 4 are given to the intermediate reactions (3, 4 and 5 being considered as positive).

Substrate	**Results for each strain**					
	WT	**G3**	**J3**	**K3**	**M3**	**N3**
Control	0	0	0	0	0	0
Glycerol	3	3	3	3	3	3
Erythritol	0	0	0	0	0	0
D-Arabinose	0	0	0	0	0	0
L-Arabinose	0	0	0	0	0	0
Ribose	0	0	0	0	0	0
D-Xylose	0	0	0	0	0	0
L-Xylose	0	0	0	0	0	0
Adonitol	0	0	0	0	0	0
β Methyl-D xyloside	0	0	0	0	0	0
Galactose	0	0	0	0	0	0
D-Glucose	5	5	5	5	5	5
D-Fructose	5	5	5	5	5	5
D-Mannose	5	5	5	5	5	5
L-Sorbose	1	1	1	1	1	1
Rhamnose	5	5	5	5	5	5
Dulcitol	0	0	0	0	0	0
Inositol	0	0	0	0	0	0
Mannitol	0	0	0	0	0	0
Sorbitol	0	0	0	0	0	0
α Methyl-D-mannoside	5	5	5	5	5	5
α Methyl-D-glucoside	5	5	5	5	5	5
N Acetyl glucosamine	5	5	5	5	5	5

Table continued on the next page

Substrate	Results for each strain					
	WT	G3	J3	K3	M3	N3
Continuation from page 191						
Amygdalin	5	5	5	5	5	5
Arbutin	5	5	5	5	5	5
Esculin	5	5	5	5	5	5
Salicin	5	5	5	5	5	5
Cellobiose	5	5	5	5	5	5
Maltose	5	5	5	5	5	5
Lactose	5	5	5	5	5	5
Melibiose	0	0	0	0	0	0
Sucrose	0	0	0	0	0	0
Trehalose	5	5	5	5	5	5
Inulin	0	0	0	0	0	0
Melezitose	0	0	0	0	0	0
D-Raffinose	0	0	0	0	0	0
Starch	1	1	1	1	1	1
Glycogen	0	0	0	0	0	0
Xylitol	3	3	3	3	3	3
β Gentiobiose	5	5	5	5	5	5
D-Turanose	0	0	0	0	0	0
D-Lyxose	0	0	0	0	0	0
D-Tagatose	0	0	0	0	0	0
D-Fucose	0	0	0	0	0	0
L-Fucose	0	0	0	0	0	0
D-Arabitol	5	5	5	5	5	5
L-Arabitol	0	0	0	0	0	0
Gluconate	0	0	0	0	0	0
2 ceto-gluconate	0	0	0	0	0	0
5 ceto-gluconate	0	0	0	0	0	0

Table 3.7.1.4.3 - Results of the API20STREP galleries reactions, when incubating at 4°C for 4 weeks. The strains used were *L. monocytogenes* wild type, 10403S (WT), and transposon insertion mutants defective for growth at 4°C (G3, J3, K3, M3 and N3). Symbols: +, positive reaction; -, negative reaction.

Tests	Substrate	Reactions/Enzymes	Results for each strain					
			WT	G3	J3	K3	M3	N3
VP	Pyruvate	Acetoine production	+	+	+	+	+	+
HIP	Hippurate	Hydrolysis	+	+	+	+	+	+
ESC	Esculine	ß-glucosidase	+	+	+	+	+	+
PYRA	Pyrrolidonyl 2 naphtylamide	Pyrrolidonylarylamidase	-	-	-	-	-	-
αGAL	6-Bromo-2-naphtyl α-D-Galactopyranoside	α-galactosidase	-	-	-	-	-	-
βGUR	Napthol AS-BI β-D-glucuronate	β-glucuronidase	-	-	-	-	-	-
βGAL	2-naphtyl-β-D galactopyranoside	β-galactosidase	-	+	-	-	-	+
PAL	2-naphtyl phosphate	Alkaline phosphatase	-	-	-	-	-	-
LAP	L-leucine-2-naphtyl-amide	Leucine arylamidase	+	+	+	+	+	+
ADH	Arginine	Arginine dihydrolase	-	-	-	-	-	-
RIB	Ribose	Acidification	-	-	-	-	-	-
ARA	L-Arabinose	Acidification	-	-	-	-	-	-
MAN	Mannitol	Acidification	-	-	-	-	-	-
SOR	Sorbitol	Acidification	-	-	-	-	-	-
LAC	Lactose	Acidification	-	-	-	-	-	-
TRE	Trehalose	Acidification	+	+	+	+	+	+
INU	Inulin	Acidification	-	-	-	-	-	-
RAF	Raffinose	Acidification	-	-	-	-	-	-
AMD	Starch	Acidification	+	+	+	+	+	+
Glyg	Glycogen	Acidification	-	-	-	-	-	-

Table 3.7.1.4.4 - Results of the API50CH gallery reactions, when incubating at 4°C for 4 weeks. The strains used were *L. monocytogenes* wild type, 10403S (WT), and transposon insertion mutants defective for growth at 4°C (G3, J3, K3, M3 and N3). Values: 0 is given to negative reactions and 5 to positive reactions of maximum intensity. Numbers 1, 2, 3, or 4 are given to the intermediate reactions (3, 4 and 5 being considered as positive).

Substrate	**Results for each strain**					
	WT	**G3**	**J3**	**K3**	**M3**	**N3**
Control	0	0	0	0	0	0
Glycerol	5	5	5	5	5	5
Erythritol	0	0	0	0	0	0
D-Arabinose	0	0	0	0	0	0
L-Arabinose	0	0	0	0	0	0
Ribose	0	0	0	0	0	0
D-Xylose	0	0	0	0	0	0
L-Xylose	0	0	0	0	0	0
Adonitol	0	0	0	0	0	0
β Methyl-xyloside	0	0	0	0	0	0
Galactose	0	0	0	0	0	0
D-Glucose	5	5	5	5	5	5
D-Fructose	5	5	5	5	5	5
D-Mannose	5	5	5	5	5	5
L-Sorbose	3	3	3	3	3	3
Rhamnose	5	5	5	5	5	5
Dulcitol	0	0	0	0	0	0
Inositol	0	0	0	0	0	0
Mannitol	0	0	0	0	0	0
Sorbitol	0	0	0	0	0	0
α Methyl-D-mannoside	5	5	5	5	5	5
α Methyl-D-glucoside	5	5	5	5	5	5
N Acetyl glucosamine	5	5	5	5	5	5

Table continued on the next page

Substrate	Results for each strain					
	WT	G3	J3	K3	M3	N3
Continuation from page 194						
Amygdalin	5	5	5	5	5	5
Arbutin	5	5	5	5	5	5
Esculin	5	5	5	5	5	5
Salicin	5	5	5	5	5	5
Cellobiose	5	5	5	5	5	5
Maltose	5	5	5	5	5	5
Lactose	3	3	3	3	3	3
Melibiose	0	0	0	0	0	0
Sucrose	0	0	0	0	0	0
Trehalose	5	5	5	5	5	5
Inulin	0	0	0	0	0	0
Melezitose	0	0	0	0	0	0
D-Raffinose	0	0	0	0	0	0
Starch	0	0	0	0	0	0
Glycogen	0	0	0	0	0	0
Xylitol	0	0	0	0	0	0
β Gentiobiose	5	5	5	5	5	5
D-Turanose	0	0	0	0	0	0
D-Lyxose	0	0	0	0	0	0
D-Tagatose	0	0	0	0	0	0
D-Fucose	0	0	0	0	0	0
L-Fucose	0	0	0	0	0	0
D-Arabitol	5	5	5	5	5	5
L-Arabitol	0	0	0	0	0	0
Gluconate	0	0	0	0	0	0
2 ceto-gluconate	0	0	0	0	0	0
5 ceto-gluconate	0	0	0	0	0	0

Enzymatic and fermentation of sugars reactions in the API20STREP at 4°C (Table 3.7.2.4.3) were the same for the wild type as for transposon insertion mutants J3, K3 and M3. Transposon insertion mutants G3 and N3 profiles differ from the wild type profile in that they produced a positive β-galactosidase reaction. These results were also observed when incubating at 30°C (Table 3.7.1.4.1). Interestingly, the alkaline phosphatase reaction and the fermentation of lactose was negative in all strains when incubating at 4°C (Table 3.7.1.4.3) in contrast to the positive results given when incubating at 30°C.

The fermentation profile of the different sugars by the transposon insertion mutants and the wild type strain, when incubating at 4°C (Table 3.7.1.4.4), was the same. These results suggest that the carbohydrate metabolism of *L. monocytogenes* was not affected by the mutations. With the API50CH galleries incubated at 4°C, the fermentation of lactose and xylitol did not occur in contrast to the positive results given when incubating at 30°C. Since the differences in the API galleries between 30 and 4°C were also observed for the wild type strain, it can be established that these differences reflect a natural difference of the *Listeria* metabolism at these temperatures.

3.7.1.5 Phenotypic characterisation of the *L. monocytogenes* transposon insertion mutant J3 in Trivett and Meyer medium containing different sodium chloride concentrations

Sequencing analysis of *L. monocytogenes* transposon insertion mutant J3 (Section 3.6) strongly suggested that the gene inactivated by Tn*917*-LTV3 is for a protein member of an osmoprotectant transport system necessary for the growth of micro-organisms in high salt conditions. In order to investigate how important this gene was for the ability of *Listeria* to grow in conditions of high salt and low temperature, several experiments were done as follows.

Growth of *L. monocytogenes* mutant J3 and wild type was followed by growing both strains in defined medium at different sodium chloride concentration (0, 2 and 4% (w/v)) and at two different temperatures (30 and 4°C). The defined medium used was that of Trivett and Meyer (T+M, 1971) (see Section 2.1.2). In Figure 3.7.1.5.1 the growth of *L. monocytogenes* wild type, 10403S, and mutant J3 at 30°C in T+M medium containing 0 (A), 2 (B) and 4% (w/v)

(C) NaCl is shown. Growth curves are the mean of 8 different experiments. In Table 3.7.1.5.1 the growth rates from these experiments are shown.

Table 3.7.1.5.1 - Growth rate constants from growth curves in Trivett and Meyer containing 0, 2 and 4% (w/v) NaCl and incubating at 30°C.

Strains	0% NaCl	2% NaCl	4% NaCl
Wild Type	50.7	43.8	41.5
	46.1	43.8	39.2
	43.8	41.5	36.8
	46.1	46.1	41.5
	46.1	27.6	34.5
	43.8	29.9	34.5
	48.4	34.5	39.2
	46.1	32.2	36.8
Mean value	46.4	37.4	38.0
Mutant J3	69.1	39.2	11.5
	73.7	43.8	13.8
	66.8	39.2	13.8
	64.5	41.5	13.8
	62.2	23.0	4.6
	59.9	16.1	9.2
	62.2	23.0	9.2
	66.8	18.4	6.9
Mean value	65.6	30.5	10.4

Growth rate constants were multiplied by 1000 for convenience and units are per hour.

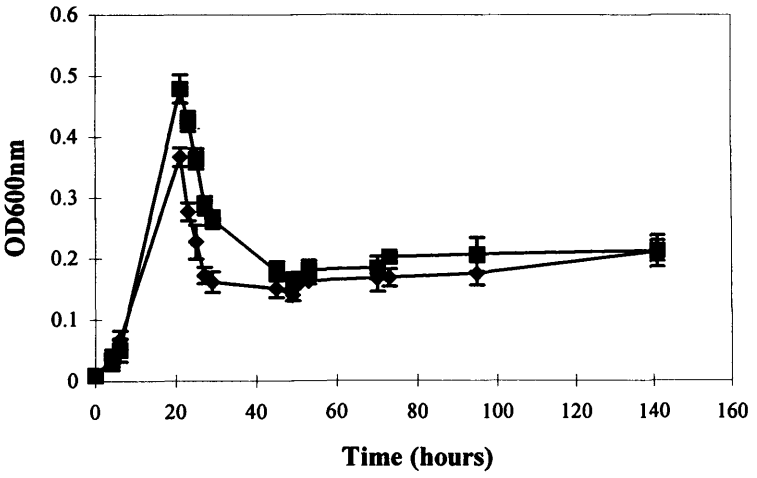
When growing in T+M medium containing 4% (w/v) NaCl (Figure 3.7.1.5.1, C), the growth rate of the wild type (38.0/hour; Table 3.7.1.5.1) is much higher than that exhibited by the mutant (10.4/hour; Table 3.7.1.5.1). These results suggest that in T+M medium with added NaCl and incubated at 30°C the ability of mutant J3 to grow is defective when compared to that of the wild type strain. ANOVA to the growth rate constants confirmed a highly significant ($P<0.01$) difference in the ability of both strains to grow under these conditions. These results are further proved when growing in T+M medium with no NaCl added and incubated at 30°C (Table 3.7.1.5.1; Figure 3.7.1.5.1, A). Under these conditions *L. monocytogenes* mutant J3 did not exhibited a defective ability to grow when compared to that of the wild type, in fact, exhibited a higher growth rate (65.5/hour) when compared to the wild type strain (46.4/hour) (Table 3.7.1.5.1; Figure 3.7.1.5.1, A). This results suggested that the growth of mutant J3 in T+M medium at 30°C was better when compared to that of the wild type. One-way ANOVA applied to the growth rate values revealed a highly significant difference ($P<0.01$) between the two strains confirming that the ability of the mutant to grow in T+M medium at 30°C was not affected. Another observation is that in T+M medium, with no salt added, at 30°C no stationary phase was observed with both strains, rather, they autolyse with a rapid decline OD600nm. Autolysis is not an uncommon phenomenon in strains of *L. monocytogenes* when entering the stationary phase of growth (Tyrrel, 1973; Pine *et al.*, 1989; Jones *et al.*, 1995).

In medium containing 2% (w/v) NaCl (Figure 3.7.1.5.1, B) the autolysis phenomenon did not occur in either of the strains. In these conditions the growth rate of wild type (37.4/hour) was approximately the same as that of the mutant J3 (30.5/hour) (Table 3.7.1.5.1). ANOVA to the growth rate values revealed a no significant difference between the ability of both strains to grow in T+M with 2% (w/v) NaCl at 30°C.

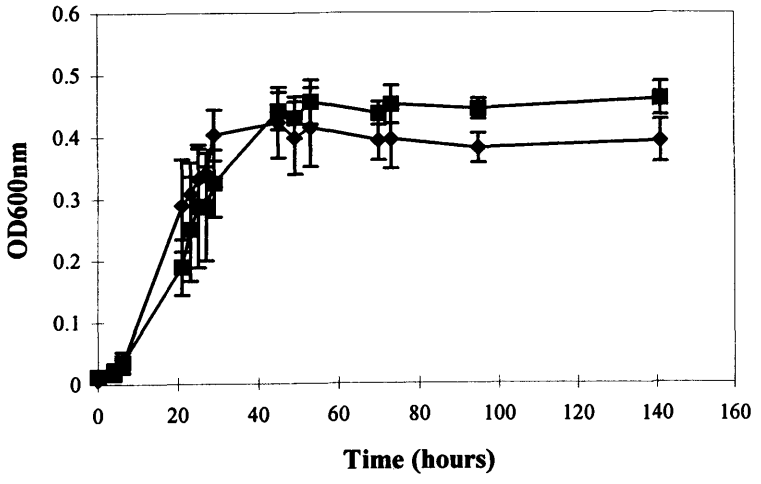
In Figure 3.7.1.5.2 the growth of *L. monocytogenes* wild type and mutant J3 at 4°C in T+ M medium containing no NaCl (A), with 2 (B), and 4% (w/v) NaCl (C) can be seen. In Table 3.7.1.5.2 the growth rate constants of these experiments are shown.

Figure 3.7.1.5.1 - Growth of *L. monocytogenes* wild type, 10403S (◆), and *L. monocytogenes* transposon insertion mutant J3 (■) at 30°C in Trivett and Meyer medium containing 0 (A), 2 (B) and 4% (w/v) (C) NaCl. Values are the mean ± standard deviation of 8 experiments.

A


OD600nm
0.6
0.5
0.4
0.3
0.2
0.1
0
0
20
40
60
80
100
120
140
160
Time (hours)

B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0.4
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0
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60
80
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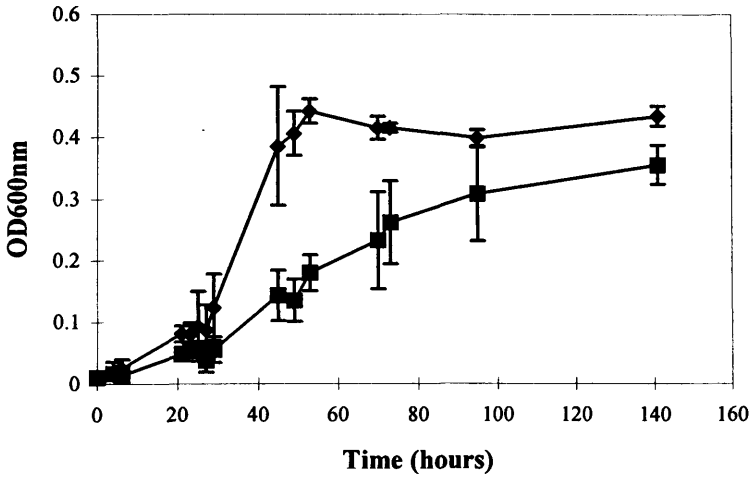
120
140
160
Time (hours)

C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0
20
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80
100
120
140
160
Time (hours)

Table 3.7.1.5.2 - Growth rate constants from growth curves in Trivett and Meyer containing 0, 2 and 4% (w/v) NaCl and incubating at 4°C.

Strains	0% NaCl	2% NaCl	4% NaCl
Wild Type	87.5	76.0	(a)
	82.9	57.6	
	82.9	71.4	
	62.2	62.2	
	105.9	85.2	
	82.9	27.6	
	110.5	87.5	
	101.3	18.4	
Mean value	89.5	60.7	
Mutant J3	78.3	20.7	(a)
	71.4	29.9	
	78.3	29.9	
	78.3	39.2	
	73.7	-	
	62.2	-	
	73.7	-	
	62.2	-	
Mean value	72.3	29.9	

- experiment not performed

(a) neither strains started growth within the first 64 days

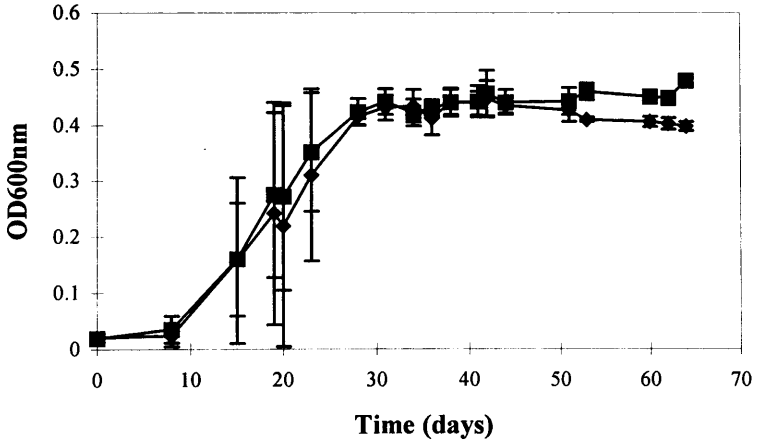
Growth rate constants were multiplied by 1000 for convenience and units are per day.

When incubated at 4°C in T+M medium with no NaCl added, the growth of wild type was better than that of mutant J3 with a growth rate of 89.5/day in contrast with J3 which exhibited a growth rate of 72.3/day (Table 3.7.1.5.2). ANOVA to the mean growth rate indicated that the difference between both strains is significant ($P<0.05$). These results confirm that these strains have a different ability to grow in T+M medium at 4°C, with mutant exhibiting a defective growth. In contrast to the cultures at 30°C with no NaCl added, the autolysis phenomenon was not observed at 4°C (Figure 3.7.1.5.2, A). These results suggested that under stress conditions (low temperature or presence of NaCl) the autolysis of *L. monocytogenes* does not occur.

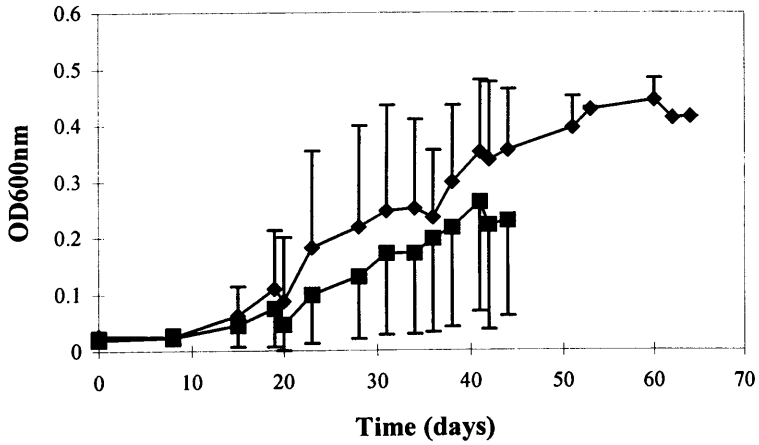
In medium containing 2% (w/v) NaCl (Figure 3.7.1.5.2, B) the ability of mutant J3 to grow was affected, with a growth rate of 29.9/day, when compared with that of the wild type strain which exhibited a growth rate of 60.7/day (Table 3.7.1.5.2).The statistical analysis (1-way ANOVA) to the growth rate values indicated a significant difference ($P<0.05$) in the ability of both strains to grow in T+M with 2% (w/v) NaCl. These results suggest that the growth of the mutant at 4°C is defective, when compared to that of the wild type, in T+M medium with 2% salt. When grown in medium containing 4% of NaCl and incubated at 4°C (Figure 3.7.1.5.2, C) neither strains started growth within the first 64 days.

Figure 3.7.1.5.2 - Growth of *L. monocytogenes* wild type, 10403S (◆), and *L. monocytogenes* transposon insertion mutant J3 (■) at 4°C in Trivett and Meyer medium containing 0 (A), 2 (B) and 4% (w/v) (C) NaCl. Values are the mean ± standard deviation of 8 experiments for 0 and 4% (w/v) NaCl containing medium. For 2%(w/v) NaCl containing medium, values are the mean ± standard deviation of 4 experiments.

A


OD600nm
0.6
0.5
0.4
0.3
0.2
0.1
0
0
10
20
30
40
50
60
70
Time (days)

B


OD600nm
0.6
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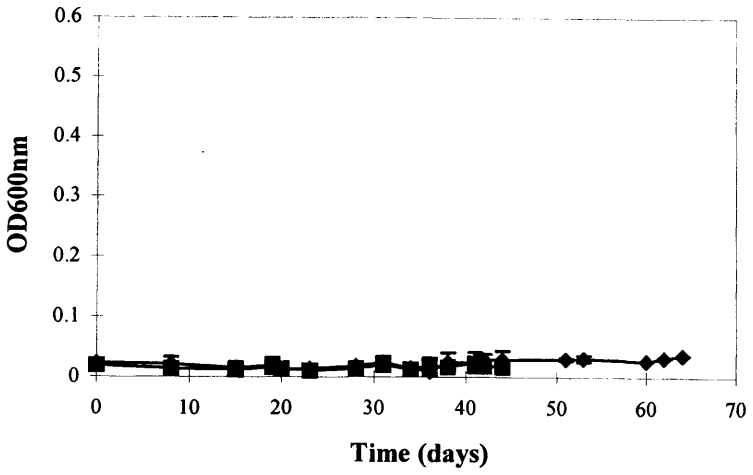
20
30
40
50
60
70
Time (days)

C



3.7.1.6 ß-Galactosidase expression of *L. monocytogenes* mutant J3 in Trivett and Meyer medium containing different sodium chloride concentrations

To investigate how salt and temperature influences the expression of the inactivated gene in the *L. monocytogenes* mutant J3, the ß-galactosidase expression was quantified for mutant J3 in T+M cultures, incubating at 30 and 4°C, without and with 2% (w/v) NaCl. These ß-galactosidase assays were performed in duplicate to two different cultures at an OD_{600nm} of approximately 0.4. Table 3.7.1.6.1 shows the ß-galactosidase activity values for the mutant J3. These values are very low and when compared to those described by Camilli *et al.* (1990) which reported values of thousands when Tn*917*-LTV3 had inserted, in the correct orientation, downstream to an active promoter suggest that no expression of ß-galactosidase is being measured. These results may indicate that at 4°C, NaCl is not affecting the expression of the inactivated gene in mutant J3 or that the expression of this gene is very low to be quantified by this assay.

Table 3.7.1.6.1 - ß-Galactosidase activity values found for the mutant J3 cultures in Trivett and Meyer medium when incubating at 30 and 4°C without NaCl and with 2% (w/v) NaCl.

Temperature	**Trivett and Meyer**	**Experiment**	
		1	**2**
30°C			
Mutant J3	0% NaCl	3.3	2.4
Mutant J3	2% NaCl	2.0	2.2
4°C			
Mutant J3	0% NaCl	8.2	7.2
Mutant J3	2% NaCl	0.4	0.1

OD600nm of the cultures was approximately 0.4, and values were originated from 2 different experiments each one performed in duplicate. Values ß-galactosidase are given in Miller units.

3.7.2 Phenotypic characterisation of *L. monocytogenes* mutants defective for growth, at 4°C, in liquid medium

3.7.2.1 Subculture of *L. monocytogenes* mutant 1 defective for growth at 4°C in liquid medium

The ability of *L. monocytogenes* transposon insertion mutant 1 to grow at 4°C, in liquid medium, was proved to be affected when compared to the growth of *L. monocytogenes* wild type, 10403S (see Section 3.3.2). In order to investigate if mutant 1 can adapt to grow at low temperature, cultures at 4°C were prepared from inocula incubating at 4°C for different periods of time.

In Figure 3.7.2.1.1 growth curves of *L. monocytogenes* wild type and mutant 1, in TSB at 4°C are shown. As can be seen in Figure 3.7.2.1.1, the wild type strain exhibited a lag phase of approximately 6 days in contrast to the lag phase of the transposon mutant 1 which was approximately 23 days. On day 10, 15, 20 and 27, time points 1, 2, 3 and 4 respectively (Figure 3.7.2.1.1), 0.5 ml of each culture was withdrawn and used to inoculate fresh pre-chilled 20ml vials of TSB, with the suitable antibiotics where appropriate, and incubated at 4°C. Different time periods were chosen to give an insight on when adaptation for growth at low temperature may occur.

In Figure 3.7.2.1.2 the growth curves of *L. monocytogenes* wild type, 10403S, and transposon insertion mutant 1 subcultured into fresh TSB, at the different time points and incubated at 4°C, are represented. The growth of *L. monocytogenes* wild type and transposon insertion mutant 1 after being subcultured at time point **1** (10 days into incubation at 4°C) is displayed in Figure 3.7.2.1.2 (A). In this Figure it can be seen that the wild type exhibited a lag phase of approximately 6 days before growth and that the transposon mutant 1 lag phase was at least of 33 days since growth had not begun by this time.

Figure 3.7.2.1.2 (B), represents the growth of both strains at 4°C after subculture into fresh TSB at time point **2** (15 days into incubation at 4°C). As can be seen in this figure, *L. monocytogenes* wild type displayed a lag phase of approximately 6 days before starting growth and mutant 1 still had not started growth on day 33 of incubation at 4°C.

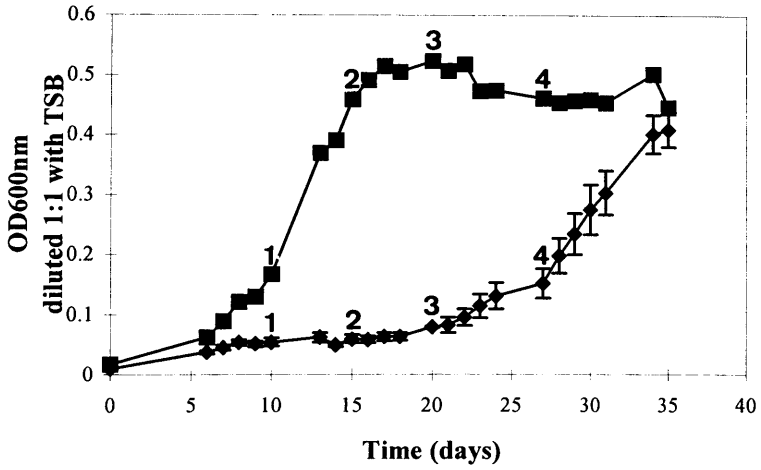
The growth of *L. monocytogenes* wild type and transposon mutant 1 after the subculture at point **3** (20 days into incubation at 4°C) is described in Figure 3.7.2.1.2 (C). The lag phase of the wild type strain was for 7 days before growth resumed, and the lag phase of the mutant lasted approximately 20 days. This time transposon mutant 1 started growth earlier than in the first 2 subcultures (A, B).

Figure 3.7.2.1.2 (D) shows the growth of *L. monocytogenes* wild type and mutant 1 after subculture at time point **4** (27 days into the incubation at 4°C). Once again wild type exhibited a lag phase of approximately 7 days in contrast to the transposon mutant 1 which exhibited a lag phase of 15 days.

In the four subcultures, the lag phase period of the *L. monocytogenes* wild type was of 6, 6, 7 and 7 days. These results are in agreement with the original growth curve used for the subculturing, which also exhibited a lag phase of approximately 6 days. In contrast, *L. monocytogenes* mutant 1 exhibited lag phase periods of 33, 33, 20 and 15 days. This reduction in the duration of the lag phase at 4°C, on the last two subcultures, suggested that the ability of mutant 1 to grow at low temperature improved. However, when compared to the duration of the lag phase in the original growth curve (23 days) no improvement occurred.

Growth of transposon insertion mutant 1 did not improve notably even after being previously incubated at 4°C for 27 days. It can be said therefore that the gene inactivated by Tn*917*-LTV3 it is involved in the ability of *L. monocytogenes* to grow at 4°C. These results also suggest that *L. monocytogenes* is unable to adapt to compensate for the loss of this gene.

Figure 3.7.2.1.1 - Growth curves of *L. monocytogenes* wild type, 10403S (■), and transposon insertion mutant 1 (◆), at 4°C in TSB. Labels 1, 2, 3 and 4, are the time points at which culture was withdrawn to inoculate fresh TSB and incubation at 4°C continued. Values for *L. monocytogenes* transposon insertion mutant 1 are the mean ± standard deviation of 3 experiments; values for *L. monocytogenes* wild type are from only one experiment.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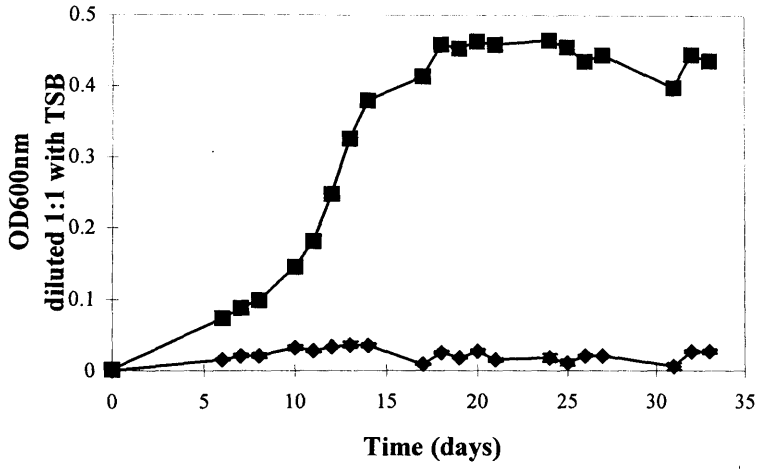
0.5
0.4
0.3
0.2
0.1
0
0
5
10
15
20
25
30
35
40
Time (days)
1
2
3
4
1
2
3
4

Figure 3.7.2.1.2 - Growth curves of *L. monocytogenes* wild type, 10403S (■), and transposon insertion mutant 1 (◆), subcultured at different time points and incubated at 4°C.

(A) Growth curves resulting from subculture at time point **1** (after 10 days of pre-incubation at 4°C) and incubated at 4°C; (B) Growth curves resulting from subculture at time point **2** (after 15 days of pre-incubation at 4°C) and incubated at 4°C; (C) Growth curves resulting from subculture at time point **3** (after 20 days of pre-incubation at 4°C) and incubated at 4°C; (D) Growth curves resulting from subculture at time point **4** (after 27 days of pre-incubation at 4°C) and incubated at 4°C. Values for *L. monocytogenes* transposon insertion mutant 1 are the mean ± standard deviation of 3 experiments; values for *L. monocytogenes* wild type are from only one experiment.

A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0.2
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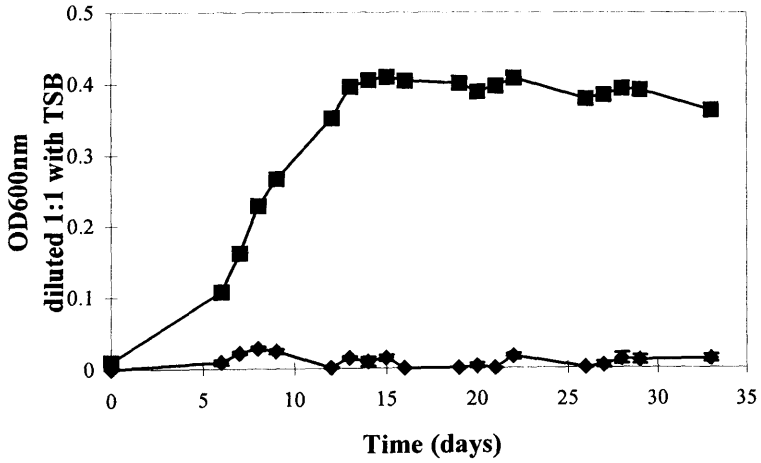
0
0
5
10
15
20
25
30
35
Time (days)

B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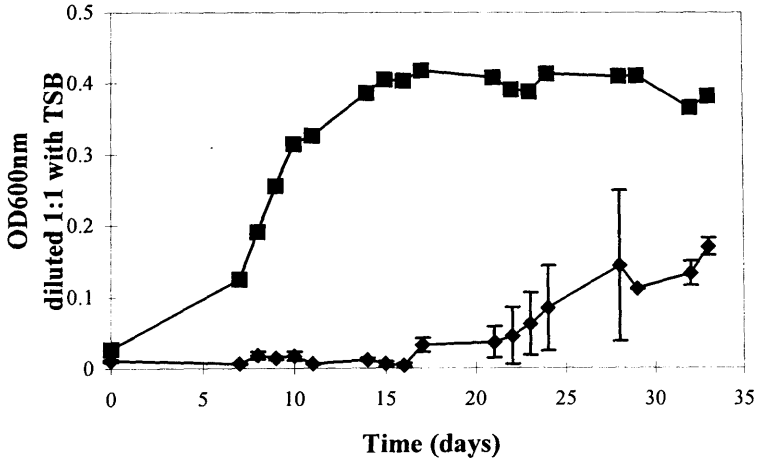
35
Time (days)

C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0
5
10
15
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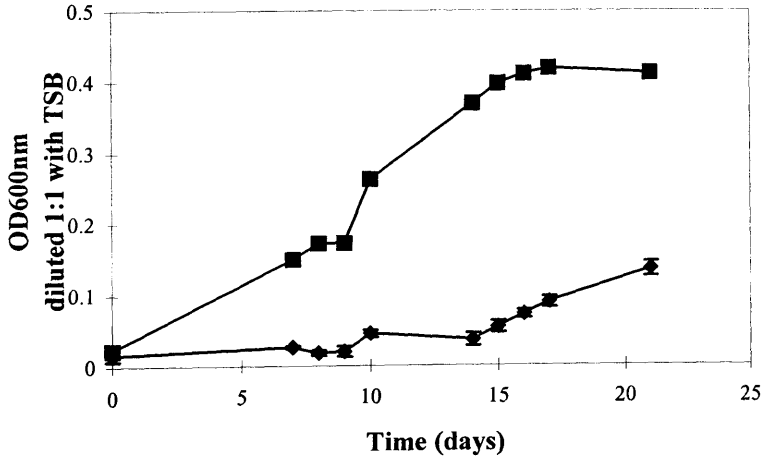
25
30
35
Time (days)

D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diluted 1:1 with TSB
0.5
0.4
0.3
0.2
0.1
0
0
5
10
15
20
25
Time (days)

3.8 Genetic complementation of the gene inactivated by Tn*917*-LTV3 in transposon insertion mutant J3

With the aim of restoring the wild type phenotype and at the same time to exclude the possibility of that a spontaneous mutation occurred in the chromosome of mutant J3 producing the cold sensitive phenotype, the wild type gene can be transformed into the mutant strain. To accomplish genetic complementation, an approach involving four steps was devised: (a) the DNA fragment coding for the gene inactivated by Tn*917*-LTV3, i.e. ORF8, was isolated by PCR; (b) this PCR product was then cloned in an *E. coli-Listeria* shuttle vector; (c) the sequence of the PCR product cloned into the shuttle vector was analysed; and (d) this plasmid was used to transform *L. monocytogenes* mutant J3.

3.8.1 Isolation of the gene inactivated, by Tn*917*-LTV3, in transposon insertion mutant J3

In order to be able to clone the gene inactivated by Tn*917*-LTV3 in mutant J3 (ORF8; see Section 3.6.2), the intact wild-type gene had to be isolated from the *L. monocytogenes* genome. The strategy chosen was PCR.

PCR primers were designed to encompass ORF8 and the associated ribosomal binding site. Also, examination of the *E. coli-Listeria* shuttle vector to be used, pMK4 (Sullivan *et al.*, 1984; Figure 3.8.2.1 in Section 3.8.2), together with analysis of the sequences adjacent to ORF8 allowed selection of the appropriate restriction sites. The primer with homology at the 5' end of ORF8, included an *EcoR*I restriction site already existent in a region upstream of the ribosomal binding site coding sequence. The PCR primer with homology to the 3' end, of the ORF8 flanking region, was synthesised to include a *BamH*I restriction site. These primers were termed Eco2 and Bam2, and contained 30 to 39 nucleotides respectively (Table 3.8.1.1). The correct size of the DNA fragment generated using primers Eco2 and Bam2 would be 813 bp.

Table 3.8.1.1 - PCR primers designed for the PCR experiments based on the sequence flanking the gene (ORF8 in Section 3.6.2) inactivated by Tn*917*-LTV3 in mutant J3. Sequences in bold are the *EcoR*I and *BamH*I restriction sites in primers Eco2 and Bam2 respectively.

Name	Primer Sequence	Size (bp)
Eco2	5' CCATCCATCGTAGCACAA**GAATTC**TTGCAA 3'	30
Bam2	5' GAAACATTC**GGATCC**AAACGAAAAACACAA CCTCTATGC 3'	39

In the PCR reactions, the template DNA used was *L. monocytogenes* wild type genomic DNA at a range of 0.5 μg to 0.125 μg in 100 μl final. The enzyme used in these reactions was *Pwo* DNA Polymerase (Boehringer Mannheim) since it is a highly processive 5'-3' DNA polymerase and possess an 3'-5' exonuclease activity, also known as proof-reading activity, which increases over 10-fold the fidelity of DNA synthesis when compared to *Taq* DNA polymerase. Since, the PCR product was to be cloned and used in expression studies, the fidelity of DNA synthesis was of vital importance. To visualise the products, 5 μl of each of the PCR reactions were analysed on 1% (w/v) agarose gels.

A very strong band of the desired size (813 bp) was produced (Figure 3.8.1.1; lanes 2, 3 and 4). No amplification was observed in the negative control reaction which consisted of a PCR reaction with no template DNA added (Figure 3.8.1.1; lane 5).

In order to confirm that the PCR product corresponded to ORF8 with adjacent flanking DNA, and not to some other region in the *Listeria* genome with sequence homology to the PCR primers used, it was decided to Southern Blot this gel and probe it, under high stringency conditions (Section 2.6.3.2), against the 5.5 kb *Sal*I-*Hind*III probe originated from pAPJ3 plasmid (Figure 3.5.1.1.4). This fragment contains the *erm*-proximal end of the Tn*917*-LTV3 derivative together with the 5' end of ORF8. The probe hybridised to the 813 bp band (Figure 3.8.1.2; lanes 1, 2 and 3) and to the Southern blot positive control (Figure 3.8.1.2; lane 5) but not to the PCR negative control (Figure 3.8.1.2; lane 4). These results suggest that the PCR product corresponds to the gene inactivated by Tn*917*-LTV3 in mutant J3. This PCR product was then cloned in the expression vector pMK4 as described in the next section.

Figure 3.8.1.1 - Amplification products using Eco2 and Bam2 primers in PCR reactions. *Listeria monocytogenes* wild type, 10403S, genomic DNA was the template for amplification reactions. Lanes 2, 3 and 4, reactions performed using 0.5, 0.25 and 0.125 μg of template DNA respectively; lane 5, negative control (no template added to reaction); lane 6, plasmid pAPJ3 double digested with *Sal*I-*Hind*III and used in this gel as a positive control for the subsequent Southern blot; lane 1 and 7, 1 kb ladder DNA size marker (GIBCO-BRL), with fragments sizes shown in kilobases (kb).

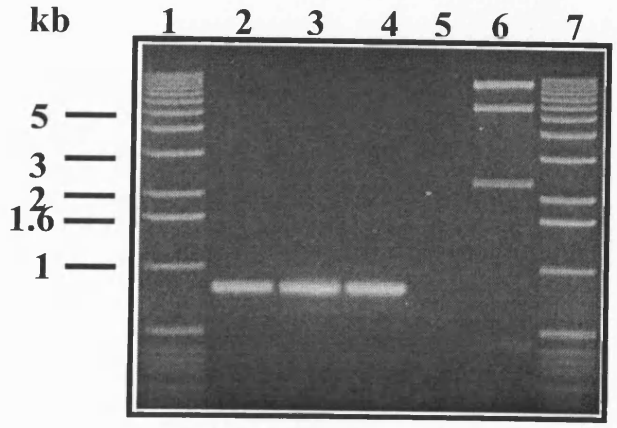
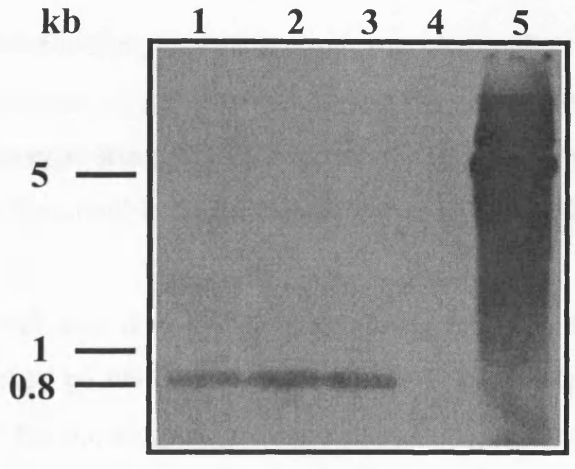

kb
1
2
3
4
5
6
7
5
3
2
1.6
1

Figure 3.8.1.2 - Southern hybridisation of amplification products produced by PCR of *L. monocytogenes* wild type, 10403S, using primers correspondent to the flanking region of the gene inactivated by Tn*917*-LTV3 in mutant J3.

Gel from Figure 3.8.1.1 was blotted to Hybond-N^+ nylon membrane. The probe used was the 5 kb *Sal*I-*Hind*III from pAPJ3 plasmid, which was labelled by the Fluorescein Gene Images system (Section 2.6.3.2) and used under conditions of high stringency [hybridisation conditions were 65°C in 5xSSC/0.1% (w/v) SDS overnight; washing was performed three times in 1xSSC/0.1% (w/v) SDS at 65°C for 15 mins]. Lanes 2, 3 and 4, corresponds to hybridisation to amplification products of 0.5 μg (lane 1), 0.25 μg (lane 2) and 0.125 μg (lane 3) of wild type genomic DNA used as template; lane 4, corresponds to the PCR negative control (no template added to reactions) and no hybridisation occurred; lane 5, corresponds to the DNA of plasmid pAPJ3 double digested with *Sal*I-*Hind*III (this is the positive control); with fragments sizes shown in kilobases (kb).


kb
1
2
3
4
5
5
1
0.8

3.8.2 Cloning, into pMK4, the PCR product coding for the gene inactivated by Tn*917*-LTV3 in transposon insertion mutant J3

In order to be able to complement the mutation in *L. monocytogenes* mutant J3, the PCR product, which corresponds to the gene inactivated by the transposition event, has to be cloned into a plasmid that will allow the expression of this gene in *L. monocytogenes*. The plasmid chosen was pMK4 (Sullivan *et al.*, 1984). This plasmid can replicate in both *E. coli* and *L. monocytogenes*. This characteristic is important because direct transformation of ligation mixtures into *L. monocytogenes* is very inefficient. pMK4 contains the *lacZ* gene with several unique restriction sites within it (Figure 3.8.2.1). Insertion of DNA into any of these sites disrupts the *lacZ* gene, causing a loss of α-complementation and results in a Lac^- phenotype in the appropriate *E. coli* host strain. Consequently, *E. coli* transformants can be readily isolated and DNA subsequently transformed into *L. monocytogenes*.

PCR product prepared in the previous section, was electrophoresed through a 0.7% (w/v) low-melting-point agarose gel. A gel slice containing the 813 bp fragment was excised, and DNA purified from the agarose using the LiCl extraction method (see Section 2.5.7.4). The purified PCR fragment was then double digested with *EcoR*I and *BamH*I.

pMK4 plasmid DNA was also double digested with *EcoR*I and *BamH*I. The efficiency of digestion was checked be performing self-ligation with the digested DNA, then transforming into *E. coli* DH5α. No transformants were obtained. This result demonstrates that the plasmid DNA was digested to completion.

Ligations were performed as detailed in Section 2.5.5, using ratios of insert to vector of 4:1, 8:1 and 10:1. Ligation reactions were incubated at room temperature overnight, then ethanol precipitated, resuspended in nanopure water, and transformed by electroporation into *E. coli* DH5α selecting for ampicillin resistance. The transformation efficiency was $2x10^3/\mu g$ of DNA. Plasmid DNA was extracted from 12 white transformants, double digested with *EcoR*I-*BamH*I and analysed by gel electrophoresis (Figure 3.8.2.2). The results of this analysis of the plasmid DNA of 6 of the transformants, together with pMK4 DNA double digested with *EcoR*I-*BamH*I (as a negative control), are shown in Figure 3.8.2.2 top gel. In this Figure, on the bottom gel, the digested plasmid DNA of the 6 remaining transformants together with the double digested plasmid pMK4 DNA are shown.

Since the ligations ends were *EcoR*I-*BamH*I, using these enzymes to digest would produce two bands, one corresponding to the plasmid itself (5.6 kb) and another corresponding to the insert (813 bp). All 12 transformants chosen for the analyses contained the insert DNA (Figure 3.8.2.2).

Plasmid DNA from 2 of the transformants, termed pMZ4 and pMZ5, was extracted (see Section 2.3.1.1) prior to the sequencing with primers that are homologous to sequences of the ORF8. The aim of sequencing was to confirm that the cloned ORF8 did not suffer any mutation during the PCR procedure. In section 3.8.3 the sequencing strategy of the cloned PCR product is described.

Figure 3.8.2.1 - Shuttle vector pMK4.

Shuttle vector used for the complementation of the gene inactivated by Tn*917*-LTV3 in *L. monocytogenes* mutant J3. Arrowheads in the plasmid map indicate the restriction sites used in the ligations with ORF8 PCR product. *Adapted* from Sullivan *et al.*, 1984.

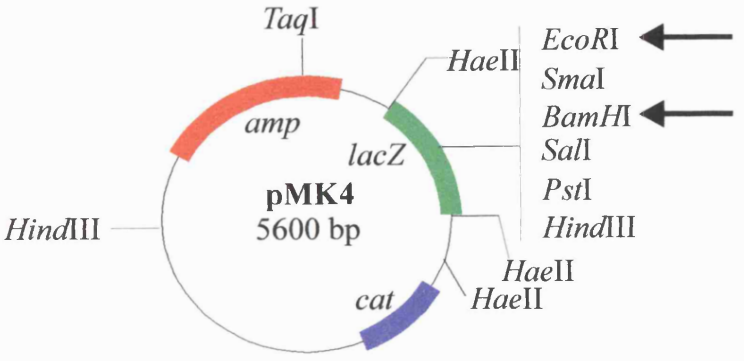
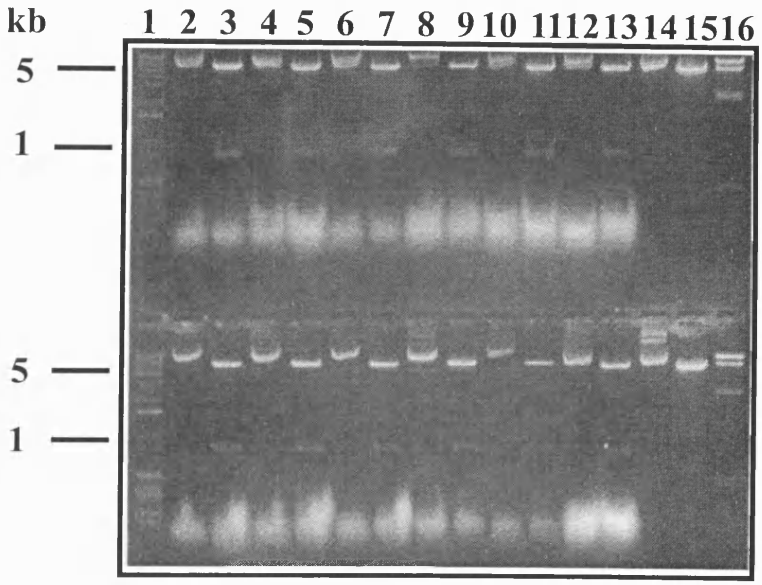

TaqI
HaeII
EcoRI
SmaI
BamHI
SalI
PstI
HindIII
HaeII
HaeII
amp
lacZ
pMK4
5600 bp
cat
HindIII

Figure 3.8.2.2 - Restriction enzyme digest of plasmids recovered after ligation of pMK4 with the digested *EcoR*I-*BamH*I PCR product originated from the amplification of the gene inactivated by Tn*917*-LTV3 in mutant J3.

Top gel) Plasmid DNA recovered from 6 transformants colonies from ligations of the plasmid pMK4 to the PCR product, uncut plasmid DNA (lanes 2, 4, 6, 8,10 and 12), plasmid DNA restricted by *EcoR*I-*BamH*I (lanes 3, 5, 7, 9,11 and 13). pMK4 plasmid DNA uncut (lane 14) and double digested by *EcoR*I-*BamH*I (lane 15). Lane 1, 1 kb DNA size marker (GIBCO-BRL); fragment sizes are shown in the left hand column in kilobases (kb).

Bottom gel) Plasmid DNA recovered from 6 transformants colonies from ligations of the plasmid pMK4 to the PCR product, uncut plasmid DNA (lanes 2, 4, 6, 8,10 and 12), plasmid DNA restricted by *EcoR*I-*BamH*I (lanes 3, 5, 7, 9, 11 and 13). pMK4 plasmid DNA uncut (lane 14) and double digested by *EcoR*I-*BamH*I (lane 15). Lane 1, 1 kb DNA size marker (GIBCO-BRL); fragment sizes are shown in the left hand column in kilobases (kb).


kb
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
5
1
5
1

3.8.3 Sequencing of the cloned PCR product in pMZ4

A list of the primers used, to determine the sequence of the PCR product cloned in the shuttle vector pMK4, includes some of the primers designed for the sequencing of ORF8 (see Section 3.6.2), and is shown in Table 3.8.3.1. In Figure 3.8.3.1 detail the sequencing strategy for the PCR product cloned in plasmids pMZ4 and pMZ5 is given.

Table 3.8.3.1 - List of primers used in sequencing the PCR product cloned in plasmids pMZ4 and pMZ5.

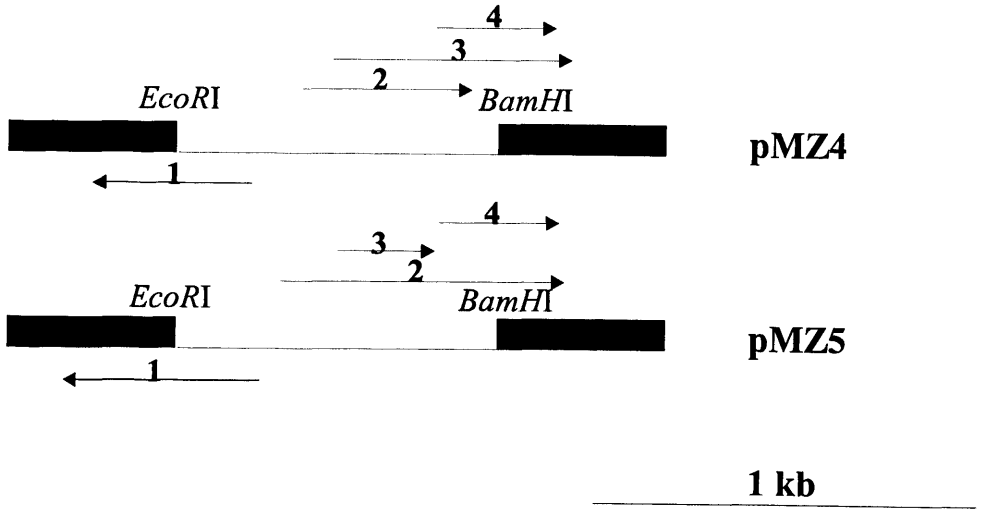
Primers		
Number	**Name**	**Sequence (5'-3')**
(1)	JAA(+)	GCCGGAATTGTTTGGATGATA
(2)	JB(-)	AATCATAGCGATACCGCTTGGAGT
(3)	DISTAL11AA(+)	ACGTTGATGGAGCGCTTTTAG
(4)	DISTAL11AB(+)	TGGTGCTATTCCAACCGCAGT

The aim of the sequencing of this insert DNA was to confirm that the cloned PCR product, in pMZ4 and pMZ5, contained an exact replica of the inactivated ORF in *L. monocytogenes* mutant J3. Due to the short time left in this project, it was not possible to complete the sequencing analysis on either strands of the insert. In Figure 3.8.3.2, a comparison of the partial sequenced insert PCR product, in plasmids pMZ4 and pMZ5, to the ORF inactivated by Tn*917*-LTV3 in mutant J3 is shown.

Figure 3.8.3.2 shows that the sequence of the PCR product in plasmid pMZ4, was not complete for 58 nucleotides at the central region (nucleotide 488 to 515) of the ORF. The sequencing of the PCR product in plasmid pMZ5 lacks 15 nucleotides in the same region (nucleotide 494 to 508) as in pMZ4, and 10 nucleotides (nucleotide 874 to 884) further in the sequence. The comparison also shows that partial sequence of pMZ4 and pMZ5 insert contains, 6 and 38 respectively, missidentified bases (e.g. bases where should be T a C was determined). These results may not mean that the cloned PCR product is mutated but is a reflection of the insufficient sequencing of both strands. Although insert DNA in both plasmids was not sequenced to completion and contains some unidentified nucleotides (e.g.

N), the homology with the remaining sequence indicates that the PCR product cloned corresponds to ORF8.

Figure 3.8.3.1 - Sequencing strategy of the PCR product cloned in plasmids pMZ4 and pMZ5. In the diagram, solid rectangles represent pMK4 DNA, while the PCR product insert is represented by a line. Arrows indicate both the length of the fragment sequenced from the oligonucleotide primer and the direction of primer extension. Numbers over the arrows indicate the oligonucleotide primer used as detailed in Table 3.8.3.1.


4
3
2
EcoRI
BamHI
pMZ4
1
4
3
2
EcoRI
BamHI
pMZ5
1
1 kb

```
          1                                                    50
   pMZ4   .......... .......... .......... .......... ..........
   pMZ5   .......... .......... .......... .......... ..........
General   AGAAAATCTT CCCCATGCAA ATCGGCTTAA TCTACACAGC ACTTAAAAAC

          51                                                   100
   pMZ4   .......... .......... .......... .......... ..........
   pMZ5   .......... .......... .......... .......... ..........
General   AATCAAATGG ATGTGGCGCT TGGTTATTCA ACAGACGGCC GTATCCCAAC

          101                                                  150
   pMZ4   .......... .......... .......... .......... ..........
   pMZ5   .......... .......... .......... .......... ..........
General   TTACAATTTA AAACTATTAA AAGATGATAA GAAATTCTTC CCACCATACG

          151                                                  200
   pMZ4   .......... .......... .......... .......... ..........
   pMZ5   .......... .......... .......... .......... ..........
General   ATGCATCTGC ATTAGCAACA GACGAAATTT TAAAGAAACA TCCGGAATTA

          201                                                  250
   pMZ4   .......... .......... .......... .......... ..........
   pMZ5   .......... .......... .......... .......... ..........
General   AAAACGACTA TCAATAAATT AAAAGGTAAA ATTTCGACAG AAGAGATGCA

          251                                                  300
   pMZ4   .......... .......... .......... .......... ..........
   pMZ5   .......... .......... .......... .......... ..........
General   AAAACTTAAT TATGAAGCAG ATGGCAAGTT GAAAGAACCA TCCATCGTAG

          301                                                  350
   pMZ4   .....GAATT NTTGCAAAAA AATAATTACT TTGAAGGTAA AAACTAAGGA
   pMZ5   .....GAATT CTTGCAAAAA AATAATTACT TTGAAGGTAA AAACTAAGGA
General   CACAAGAATT CTTGCAAAAA AATAATTACT TTGAAGGTAA AAACTAAGGA

          351                                                  400
   pMZ4   GGTGCCAAAA GATGGACACA TTAAAACAAT TAATTGATTA TTACCAAACA
   pMZ5   GGTGCCAAAA GATGGACACA TTAAAACAAT TAATTGATTA TTACCAAACA
General   GGTGCCAAAA GATGGACACA TTAAAACAAT TAATTGATTA TTACCAAACA

          401                                                  450
   pMZ4   AATGGAAGTT ATGTCATGGA AGAGTTCTGG CGGCATTT.C TTAATGAGTG
   pMZ5   AATGGAAGTT ATGTCATGGA AGAGTTCTGG CGGCATTTCC TTAATGAGTG
General   AATGGAAGTT ATGTCATGGA AGAGTTCTGG CGGCATTT.C TTAATGAGTG

          451                                                  500
   pMZ4   CTTACGGAGT TATCTTCGCA GCAATCAAAG CGATACC... ..........
   pMZ5   CTTACGGAGT TATCTTCGCA GCAATCAAAG CGATACCGTT TGG.......
General   CTTACGGAGT TATCTTCGCA GCAATCATAG CGATACCGCT TGGAGTATAT

          501                                                  550
   pMZ4   .......... .......... .......... .......... .....AATAT
   pMZ5   .........NC GCCCTANTAA AGAAAGGTGG ATCCGGGGGN TTCCCAATAN
General   ATTGCAAGAA AAAAACGCTT AGCTGGTTGG GTTATCCAAA TCGCTAATAT

          551                                                  600
   pMZ4   CAACCAAACA ATTNCGGCAC TAGCAATGTT AGCCGTACTT ATGCTTATCA
   pMZ5   CAACCAAACA ATTNNGGCAC TAGCAATGTT AGCCGTACTT ATGCTTATCA
General   CATCCAAACA ATTCCGGCAC TAGCAATGTT AGCCGTACTT ATGCTTATCA

          601                                                  650
   pMZ4   TGGGCTTAGG GACNAATACA GTCGTCTTGT CCTTGTTCCT ATATTCTTTA
   pMZ5   NGGGCTTNNG GACGAATACA GTCGTCTTGT CCTTGTTCCT ATATNCTTTA
General   TGGGCTTAGG GACGAATACA GTCGTCTTGT CCTTGTTCCT ATATTCTTTA

          651                                                  700
   pMZ4   TTACCAATTC TAAAAAACAC CTACACGGGC ATTAGAAACG TTGATGGAGC
   pMZ5   TTACCAATTC TAAAAAACAC CTACNCGGGC ATTAGAAACG TTGATGGAAC
General   TTACCAATTC TAAAAAACAC CTACACGGGC ATTAGAAACG TTGATGGAGC
```

Figure 3.8.3.2 - Alignment of the ORF inactivated by Tn*917*-LTV3 in *L. monocytogenes* mutant J3 to the partial sequences of the insert PCR product in plasmids pMZ4 and pMZ5. Primer Eco2 is shown with a single underline, primer Bam2 is shown with a double underline and the ribosomal binding site is shown in bold, on the acid nucleic sequence determined from *L. monocytogenes* mutant J3 (General); pMZ4 and pMZ5 represent the sequence of the insert in the respective plasmid. ORF8 is encoded from nucleotide 361 to nucleotide 1032. Positions where a base could not be identified with certainty are denoted "N" and positions where a base was not determined are represented by a dot.

```
          701                                                       750
   pMZ4   GCTTTTAGAA TCTGGTAAGG CAATGGGGAT GACAAAATGG CAAGT.CTAC
   pMZ5   GCTTTTANAA TCTGGTNAGG CAATGGGGAT GACAAAATGG CNAGTTCTAC
General   GCTTTTAGAA TCTGGTAAGG CAATGGGGAT GACAAAATGG CAAGTACTAC

          751                                                       800
   pMZ4   GCCTCATCGA AATG.CCACT CGCATTATCT GTTATTATGG CGGGTATTCG
   pMZ5   GCCTCATCGA AATGCCCACT CGCATTATCT GTTATTATGG CGGGTNTTCN
General   GCCTCATCGA AATG.CCACT CGCATTATCT GTTATTATGG CGGGTATTCG

          801                                                       850
   pMZ4   AAATGCACTT GTTATCGCTA TTGGTGTA.C AGCAATTGGG ACATTCGTTG
   pMZ5   ANATGCACTT GTTATCGCNA TTGGTGTA.N CNGCAATGGG ACATTCNTTG
General   AAATGCACTT GTTATCGCTA TTGGTGTAGC AGCAATTGGG ACATTCGTTG

          851                                                       900
   pMZ4   GAGCANGANG CCTCGGTGAT ATTATTGTAC GCGGAACAAA TGCAACAAAT
   pMZ5   GACNGGAANC TNTGTGATAT NTT....... ...GTACCNG AACNAATCAC
General   GAGCAGGAGG CCTCGGTGAT ATTATTGTAC GCGGAACAAA TGCAACAAAT

          901                                                       950
   pMZ4   GGTACTGCTA TTATTTTAGC TGGTGCTATT CCAACCGCAG TAATGGNCAT
   pMZ5   NAATGTATGC TATTTTTTAC TGGTGCNATC CANCCCNTAA T....NGCAT
General   GGTACTGCTA TTATTTTAGC TGGTGCTATT CCAACCGCAG TAATGGCCAT

          951                                                      1000
   pMZ4   ATTA.CCGAT GTNCTTCTTG GTTGGGTCGA ACGCACACTA AA.CCAGTTA
   pMZ5   ATAN.CCNAN GTT.TTCCTN GTTGGGTCCA ACG..CCCCT AAACCNGTTA
General   ATTAGCCGAT GTACTTCTTG GTTGGGTCGA ACGCACACTA AACCCAGTTA

          1001                                                     1050
   pMZ4   AAAACNAAAG AAAA.CACTA ACCGAAGCCT TATAAAAATA TGCGAAAACA
   pMZ5   NAA..AAANA CAAANCACNA ACCGANCC.. ..TTATAANA TATGCAAAAC
General   AAAACAAAAG AAAACCACTA ACCGAAGCCT TATAAAAATA TGCGAAAACA

          1051                                                     1100
   pMZ4   CAACCTATCA TTTATGCATA GAAGTTGTGT TTTTCGTTTG GATCCGTCGA
   pMZ5   ACNACTANCA CTTATGCATN GAAGTNGTGT TTTTCGTNTG GATCGTCCAC
General   CAACCTATCA TTTATGCATA GAGGTTGTGT TTTTCGTTTG ACAAAGAATG

          1101                                                     1150
   pMZ4   NCTGCAGCCA AGCTTGGCGT AATCATGGTC ATANCTGTTT CCTGTGTGAA
   pMZ5   CGCACCACNT GGGTNANCTG GCNAAGTGT. .......... .TTCTGTGTG
General   TTTCCCGTAG TAAACTTTT. .......... .......... ..........

          1151                                                     1200
   pMZ4   ATTGTTATCC GCTCACAATT CCACACAACA TACNACCNGN AACATAAAGT
   pMZ5   AATGTTACCC TC......CA NCCCCNCNCA TCCACCGGNA ATCANANTGT
General   .......... .......... .......... .......... ..........

          1201                                                     1250
   pMZ4   GTNAAGCCTG GGGTGCCTAA TGAATGANCT AACTCACATT AATTGCGTTG
   pMZ5   G......... .......... .......... .......... ..........
General   .......... .......... .......... .......... ..........

          1251                                                     1300
   pMZ4   CGCTCACTGC CCGCNTTCCA ATCGGGAAAA CTGTCGTGCC A.CTGCATTT
   pMZ5   .......... .......... .......... .......... ..........
General   .......... .......... .......... .......... ..........

          1301                                                     1350
   pMZ4   AAT.AANCCG GCNACGCCCG GGAAAAGCGG TTGCTATTGG GCGCTCTTCC
   pMZ5   .......... .......... .......... .......... ..........
General   .......... .......... .......... .......... ..........

          1351                                                     1400
   pMZ4   GCTTCCTCGC TCAATAATCN CTGNGCTCGG TCTTCGGGTG GGGGGAACNG
   pMZ5   .......... .......... .......... .......... ..........
General   .......... .......... .......... .......... ..........
```

```
         1401     1412
   pMZ4  TATCACTCAC TC
   pMZ5  .......... ..
General  .......... ..
```

3.8.4 Transformation of *L. monocytogenes* transposon insertion mutant J3 with plasmids pMZ4 and pMZ5

Because the sequencing of pMZ4 and pMZ5 was very encouraging, and because of shortage of time, it was decided to proceed before sequencing was complete. The method chosen to transform pMZ4 and pMZ5 into *L. monocytogenes* mutant J3 was electroporation. In an attempt to facilitate the entry of these plasmids into *L. monocytogenes*, cells were penicillin-treated prior to transformation. Penicillin treatment of cells before electroporation to increase the permeabilization of the cells was developed by Park and Stewart (1990), who reported efficient electrotransformation of *L. monocytogenes* with plasmid DNA (transformation frequency of $4x10^6$/μg DNA). Using this method, 1 μg of DNA from each of the recombinant plasmids and 1 μg of pMK4 (as positive control) was electrotransformed into mutant J3 selecting for erythromycin and chloramphenicol. Chloramphenicol resistance is the selectable marker for pMK4, or pMK4-derived plasmids, in *L. monocytogenes* (Michel *et al.*, 1990; Ripio *et al.*, 1997). This antibiotic resistance marker also exists in the genome of *L. monocytogenes* mutant J3, since it was carried by the Tn*917*-LTV3 derivative. The minimal inhibitory concentration of chloramphenicol for the growth of mutant J3 had to be estimated. This concentration, on plates, was estimated as 20μg/ml while *E. coli* DH5α carrying pMK4 could still grow at a concentration of 50μg/ml in plates incubating at 37°C for up to 72 hours. So, the selective chloramphenicol concentration, used for mutant J3 transformants, was 50μg/ml.

Penicillin-treated *L. monocytogenes* mutant J3 cells were electrotransformed on the day of the treatment or stored at -70°C for 18 hours prior to the electroporation. This storage is thought to increase the efficiency of electroporation in *Listeria* cells (Rees personal communication). Plates were incubated at 37°C for 48 and 72 hours.

With non-stored and stored cells no transformants were recovered when using either plasmids pMZ4 or pMZ5. No transformants were recovered with pMK4 DNA, the positive control. These results suggest that the transformation method was not the appropriate. Thus, the electroporation procedure needed to be optimised. However, due to the lack of time for this project, no other experiments were performed.

3.9 Results of *in vivo* investigations of *L. monocytogenes* 10403S and mutants G3 and J3

3.9.1 Comparison of the virulence of *L. monocytogenes* 10403S wild type, and mutants G3 and J3

The viable counts of *L. monocytogenes* strains recovered from the spleens of infected mice, at day 2, 4 and 6 after infection, are given in Table 3.9.1.1 and are expressed as $\log_{10}$ cfu per spleen.

The ability of *L. monocytogenes* mutants, and its wild type parent strain *L. monocytogenes* 10403S, to persist in the spleens of MF1 mice after i.v. inoculation was determined by calculating the mean of the $\log_{10}$ cfu per spleen from the five animals per group (Table 3.9.1.1 and Fig. 3.9.1.1). Colonisation of the spleen was investigated as this is one of the sites for listerial multiplication in natural infection (Armstrong and Sword, 1964).

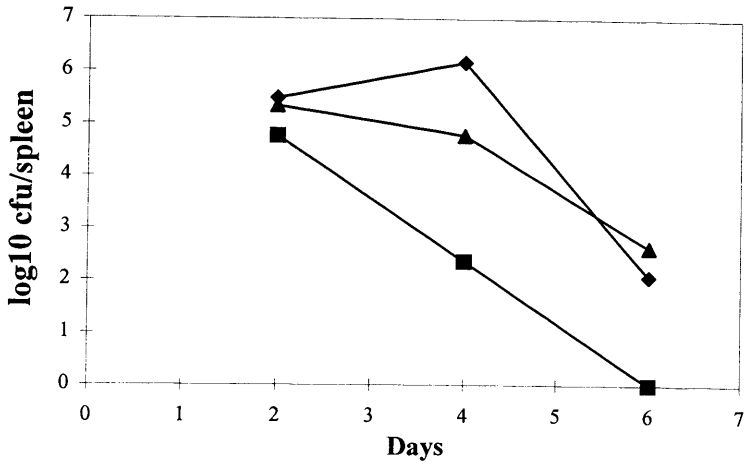
When compared to the wild type, lower numbers were recovered from the spleen of mutants G3 and J3 after 2 and 4 days of infection. After a dose of 4.47 $\log_{10}$ cfu *L. monocytogenes* 10403S, a mean peak count of 6.17 $\log_{10}$ cfu per ml of spleen homogenate was obtained after 4 days of infection. When given at a roughly same dose as wild type, 4.43 and 4.79 $\log_{10}$ cfu respectively, *L. monocytogenes* mutant G3 and mutant J3, appear to have a lower peak count and this was reached earlier after infection. These mean peak counts were 4.76 and 5.33 $\log_{10}$ cfu per spleen for mutant G3 and mutant J3 respectively (Table 3.9.1.1 and Fig. 3.9.1.1). In order to confirm that there is a significant difference between the numbers of viable listerias, recovered from the spleen of mice infected with the different strains, these results were analysed by ANOVA. A 1-way ANOVA revealed that the difference in the mean number of the viable listerias recovered on day 2 and 4 ($P=0.27$ and $P=0.13$) are not significant. On the sixth day of infection, no viable *Listeriae* were recovered from the spleens of all of the five mice infected with *L. monocytogenes* mutant G3. On the same day, only two mice harboured viable *L. monocytogenes* wild type and mutant J3 with one mouse dying overnight in each of the groups. One-way ANOVA revealed a P value of 0.19, which indicates a no-significant difference in the means of the strains on the sixth day of infection. These results demonstrate that the ability of *L. monocytogenes* mutants, G3 and J3 to colonise and multiply in the spleen of MF1 mice, is not affected by the mutation when compared to the ability of the wild type strain 10403S. These results are not due to excision of Tn*917*-LTV3 from the chromosome since mutants were expressing erythromycin and lincomycin after the recovery.

Table 3.9.1.1 - Counts of *L. monocytogenes* 10403S wild type, and mutants G3 and J3, per spleen on day 2, 4 and 6 of intravenous infection.

	Wild type	Mutant G3	Mutant J3
Dose size *	4.47	4.43	4.79
Animals number	**Day 2 of infection**		
1	5.46[+]	4.77	6.32
2	5.36	5.14	4.41
3	5.20	5.84	5.27
4	5.95	4.84	5.55
5	5.41	3.20	5.11
Mean value	5.48 (±0.28)[a]	4.76 (±0.97)	5.33 (±0.69)
	Day 4 of infection		
1	5.46	4.00	4.77
2	5.63	3.90	4.38
3	7.39	5.77	4.23
4	6.74	5.84	3.47
5	5.65	0	6.99
Mean value	6.17 (±0.85)	3.90 (±2.37)	4.77 (±1.32)
	Day 6 of infection		
1	3.77	0	5.27
2	4.55	0	5.30
3	0	0	0
4	0	0	0
5	✞	0	✞
Mean value	2.08 (±2.42)	0	2.64 (±3.05)

* Expressed as $\log_{10}$ cfu per dose; [+] Expressed as $\log_{10}$ cfu per spleen; [a] Represents the standard deviation; ✞ Mouse died.

Figure 3.9.1.1 - Numbers of viable *L. monocytogenes* 10403S (◆), and mutants G3 (■) and J3 (▲), in spleens of MF1 mice over 6 days after i.v. infection. Each point represents the mean of five mice.


log10 cfu/spleen
Days
0
1
2
3
4
5
6
7

Chapter 4

DISCUSSION

The aim of work presented in this thesis was to identify the chromosomal genes responsible for the ability of *L. monocytogenes* to grow at low temperature. To achieve this we used transposon mutagenesis.

Transposon mutagenesis using a derivative of Tn*917*, Tn*917*-LTV3, carried on plasmid pLTV3 (Camilli *et al.*, 1990) was selected for the construction of cold sensitive strains of *L. monocytogenes*. Successful mutagenesis to generate cold sensitive mutants would facilitate the subsequent cloning and identification of genes encoding proteins necessary for the ability of *L. monocytogenes* to grow at low temperature. The chosen transposon, Tn*917*-LTV3, was known to transpose at a significantly high frequency to generate transcriptional *lacZ* fusions when inserted into a chromosomal gene in the appropriate orientation and to allow the rapid cloning in *E. coli* of chromosomal DNA flanking transposon insertions (Camilli *et al.*, 1990).

The main reasons for choosing the nonconjugative *Enterococcus faecalis* transposon Tn*917* for the transposon mutagenesis of *L. monocytogenes* was because it had been shown to generate very stable insertion mutations in Gram positive bacteria and had been employed successfully in the mutagenesis of the *L. monocytogenes* chromosomal genes (Cossart *et al.*, 1989; Camilli *et al.*, 1990; Youngman, 1990). This transposon had been isolated from the *E. faecalis* plasmid pAD2 and encodes MLS (i.e., resistance to erythromycin, lincomycin and spiramycin) drug resistance (Tomich *et al.*, 1980).

Tn*917* is a member of the Tn*3* family of transposons. It shares many common features with these transposons including generation of a 5-base-pair duplication on insertion, sequence homology within the terminal inverted repeats, amino acid homology of the resolvases, and homology at a potential *res* site (Figure 4.1, Perkins and Youngman, 1984; Shaw and Clewell, 1985). Transposon Tn*917* transposes replicatively, as described by the Shapiro model (Figure 4.2, Sherratt, 1989). In this model, transposition of Tn*917* was shown to be a sequential two-step process involving, in the first transposition step, replication of the transposon and an initial fusion of the donor and recipient replicon to create co-integrate transposition intermediates. This is initiated by a transposon encoded transposase (Figure 4.1). The second site-specific recombination step involves the resolution of cointegrates to the normal transposition end products. The separation of the co-integrate is carried out by the transposon

encoded resolvase protein (Figure 4.1). This results in the insertion of a copy of the transposon into the chromosome and resolution of the plasmid extrachromosomally (Figure 4.2).

The conjugative transposon Tn*916*, and the related Tn*1545*, were the transposons originally used for insertional mutagenesis of *L. monocytogenes* (Kathariou *et al.*, 1987; Mengaud *et al.*, 1987; Kuhn *et al.*, 1988; Camilli *et al.*, 1989; Leblond-Francillard *et al.*, 1989; Sun *et al.*, 1990). However, the small size of Tn*917* (5.4 kb) when compared with that of Tn*916* (16.4 kb) and Tn*1545* (25.3 kb), simplifies the cloning and mapping of the DNA flanking its insertion. Furthermore, Tn*916* transposes at a frequency of 10^{-6} and Tn*1545* transposes at a frequency of approximately 10^{-8} which are very low and make it inconvenient to carry out large scale mutagenesis in contrast to Tn*917* which transposes at a frequency of 10^{-5} (Youngman, 1990).

The vector pLTV3 was constructed to include some features which can be useful for the transposon mutagenesis of *L. monocytogenes* (Camilli *et al.*, 1990). This plasmid carries a modified form of Tn*917*, Tn*917*-LTV3, and utilises a mutated pE194 origin of replication (pE194Ts) derived from *Staphylococcus aureus* (Iordanescu, 1976), which render it unable to replicate at temperatures above 37°C (Youngman, 1990). Insertions of the transposon into *L. monocytogenes* chromosome could be obtained simply by raising the temperature of plasmid-containing bacteria while maintaining a selection for Tn*917*-specified drug resistance (Em^r). Tn*917*-LTV3, enable the possibility of generating transcriptional fusions to *lacZ*. Transcriptional fusions are generated because a promoterless *E. coli* sequence encoding *lacZ*, modified to utilise a Shine-Delgarno sequence from the *spoVG* gene of *B. subtilis* (Zuber and Losick, 1983), is inserted a few (275) base pairs from the terminal inverted repeat at the *erm*-proximal end of Tn*917* (Figure 2.1.1.1). This derivative contains, downstream from the *lacZ* coding sequence, an *E. coli* cloning vector that includes a kanamycin *neo* resistance gene as a selectable marker in *E. coli*, a chloramphenicol *cat* resistance as a selectable marker in *B. subtilis* or *L. monocytogenes*, ColE1 replication functions, an M13 origin of replication, and a cluster of polylinker cloning sites (Camilli *et al.*, 1990).

Figure 4.1 - Genetic organisation of transposon Tn*917*.

ORF1, open reading frame 1 is the *erm* leader peptide; *erm*, erythromycin resistance gene; ORF3, is a short open reading frame of unknown function; *tnpR*, is the gene encoding resolvase; *tnpA*, is the gene encoding transposase; ORF6, is an open reading frame also involved in transposase production. Solid bars, represent terminal repeat sequences, left (LR) and right (RR). Arrows represent the direction of transcription. *Adapted* from Murphy (1989).

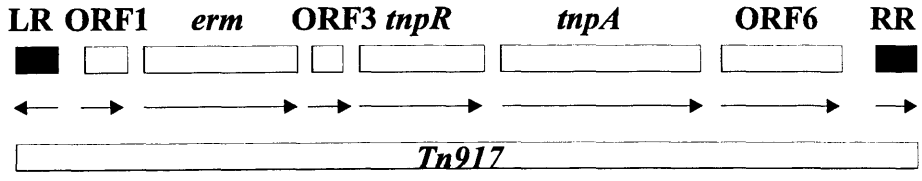
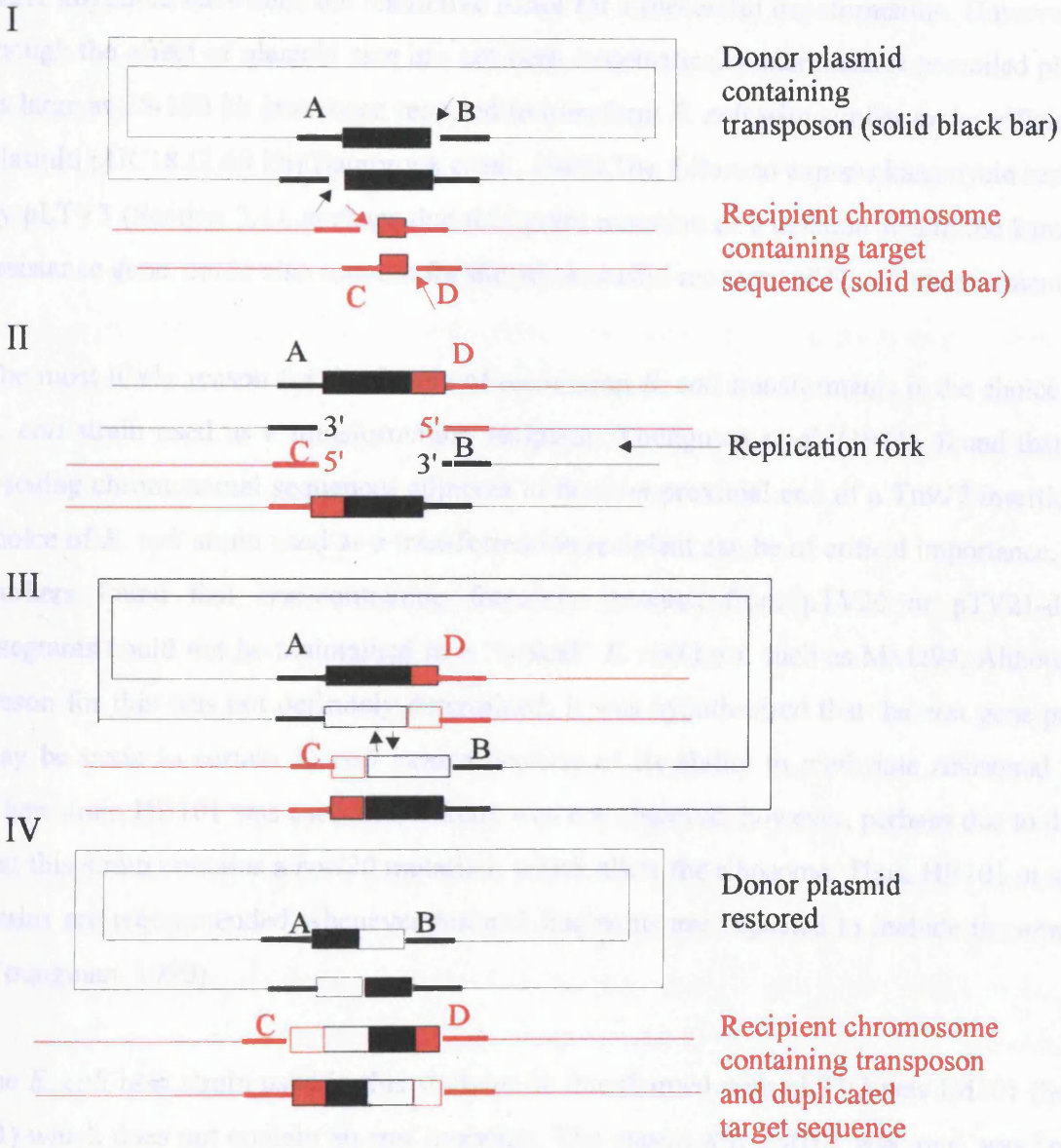

LR
ORF1
erm
ORF3
tnpR
tnpA
ORF6
RR
Tn917

Figure 4.2 - A model of transposition of the Tn*3* family of transposon proposed by Shapiro (1979).

The model is drawn for transposition from plasmid to chromosome. (I) Nicks are made at sites in the DNA molecule indicated by arrows. (II) Strand separation between nicks and joining of nonhomologous strands. Two replication forks result. (III) Replication of the transposon (clear bar) and initial fusion of the donor and recipient replicon to create a stable co-integrate intermediate. Site-specific recombination occurs between the internal resolution sites (arrows). (IV) Resolution of co-integrate. Plasmid and chromosome separate. Capital letters denote base sequences. *Adapted* from Shapiro (1979).


I
A
B
C
D
Donor plasmid containing transposon (solid black bar)
Recipient chromosome containing target sequence (solid red bar)
II
A
D
3'
5'
C
5'
3'
B
Replication fork
III
A
D
C
B
IV
A
B
C
D
Donor plasmid restored
Recipient chromosome containing transposon and duplicated target sequence

In order to obtain a sufficient amount of pLTV3 DNA for the manipulations required in this project, plasmid DNA was used to transform *E. coli*. However, transformation, either by electroporation or $CaCl_2$, was never successful (Section 3.1). The reason why pLTV3 failed to transform in *E. coli* did not seem to be related to the methods used since these has been extensively studied since the early 70s (Mandel and Higa, 1970; Cohen *et al.*, 1972; Dower *et al.*, 1988; Taketo, 1988) and it is now generally accepted that the efficiency of transformation has not been a limiting factor in molecular cloning since the late 70s. The size of the plasmid (22.1 kb) could have been the restrictive factor for a successful transformation. However, even though the effect of plasmid size has not been systematically examined, supercoiled plasmids as large as 25-130 kb have been reported to transform *E. coli* with similar molar efficiency as plasmid pUC18 (2.69 kb)(Sambrook *et al.*, 1989).The failure to express kanamycin resistance by pLTV3 (Section 3.1), perhaps due to a point mutation or a deletion within the kanamycin resistance gene, could also account for the unsuccessful recovery of *E. coli* transformants.

The most likely reason for the failure of recovering *E. coli* transformants is the choice of the *E. coli* strain used as a transformation recipient. Youngman *et al.* (1984), found that when rescuing chromosomal sequences adjacent to the *erm*-proximal end of a Tn*917* insertion, the choice of *E. coli* strain used as a transformation recipient can be of critical importance. These workers found that *erm*-containing fragments rescued from pTV20 or pTV21-derived integrants could not be maintained in a "typical" *E. coli* host, such as MM294. Although the reason for this was not definitely determined, it was hypothesised that the *erm* gene product may be toxic to certain *E. coli* strains because of its ability to methylate ribosomal RNA. When strain HB101 was used this toxicity was not observed, however, perhaps due to the fact that this strain contains a *rpsl20* mutation, which alters the ribosome. Thus, HB101 or similar strains are recommended whenever rescued fragments are expected to include the *erm* gene (Youngman, 1990).

The *E. coli* host strain used in this study to be transformed with pLTV3 was JM101 (Section 3.1) which does not contain an *rpsl* mutation. The reason why JM101 was used, was because of their high transformation efficiency. In retrospect this was not the correct approach. Subsequent events made transformation of *E. coli* with pLTV3 unnecessary and therefore this line was discounted. If, however, transformation of *E. coli* were required in future, use of strain HB101 is recommended.

To recover Tn*917*-LTV3 insertion mutants, bacteria carrying pLTV3 were at first cultured at a low temperature (30°C) to stationary phase with selection for tetracycline and for the Tn*917*-encoded erythromycin and lincomycin resistance. To promote transposition and to remove the plasmid from the Tn*917*-carrying population, the bacteria then were cultured at a high temperature (41°C) nonpermissive for the plasmid, with selection for Tn*917*-encoded erythromycin and lincomycin resistance. These antibiotics induce the transposition of Tn*917* because of expression of the transposase via transcription from the *erm* gene into the *tnpA* gene (Figure 4.1). The high temperature of incubation does not allow the replication of pLTV3 since it contains the pE194 origin of replication mutated in a way that renders it unable to grow at temperatures above 37°C (Villafane *et al.*, 1987). Survivors of this period of growth at high temperature were predominately bacteria that had acquired a chromosomal Tn*917* insertion. Transposon insertion mutants maintained erythromycin and lincomycin resistance but had lost plasmid encoded tetracycline resistance. When insertion occurred within a transcriptionally active region of the chromosome and in the correct orientation to form a transcriptional fusion, the insertion mutants were expected to appear blue on X-gal plates. This is because *lacZ* activity occurs under the regulation of an active chromosomal promoter.

All Tn*917* mutants from the transposon library of *L. monocytogenes* 10403S, when plated onto media containing X-gal, (Section 3.2, Figure 3.2.1) had a blue appearance but with some mutants being darker than others. According to Camilli *et al.* (1990) not all of these mutants contain the transposon insertion in an active region of the *L. monocytogenes* 10403S genome since this strain of *L. monocytogenes* has a low endogenous β-galactosidase activity, forming very light blue colonies on LB with X-gal. However, transposon mutants with insertions into transcriptionally active regions of the *L. monocytogenes* chromosome were easily detected above this background, having colonies that are dark blue (Camilli *et al.*, 1990). In this study the transposon mutants expressing above-background β-galactosidase were approximately 30% of the total when incubated at 37°C.

The conjugative transposons Tn*1545* and Tn*916* transposes at relatively low frequency into the chromosome of *L. monocytogenes*. Tn*1545* transposes at a frequency of approximately 10^{-8} (Gaillard *et al.*, 1986) and Tn*916* transposes into the chromosome of *L. monocytogenes* at a frequency of 10^{-6} (Kathariou *et al.*, 1987). Tn*917*-LTV3 was found to transpose, into the chromosome of *L. monocytogenes*, at a frequency of 6.8×10^{-4} (Section 3.2) which is similar to the transposition frequency of 8×10^{-4} achieved by Camilli *et al.* (1990). This derivative

exhibits a much higher frequency of transposition, than the conjugative transposons described above and previous versions of Tn*917*, which facilitates its use for insertional mutagenesis in *L. monocytogenes* by reducing the culture volumes necessary to produce transposon insertion libraries.

The results of the Southern hybridisation analysis, used to evaluate the randomness of Tn*917* insertion in pLTV3 libraries, indicated that a hotspot for insertion into the chromosome of *L. monocytogenes* 10403S may exist or that siblings of the same mutation were analysed (Figure 3.2.3). Thus, 3 out of 12 transposon insertion mutants tested had insertion in the same region. The pattern of hybridisation with the remaining mutants showed that a single but different site of insertion had occurred in each. Camilli *et al.* (1990), also found that although the distribution of *L. monocytogenes* Tn*917*-LTV3 insertional auxotrophic mutations was not completely random, many different kinds were recovered. Insertions within the *hlyA* gene were more frequent than would be expected on a purely random basis, suggesting that this gene may be within one of the hotspot region of the *L. monocytogenes* chromosome (Camilli *et al.*, 1990). The 25% identical insertions observed in this project probably are in the same hotspot region identified by Camilli *et al.* (1990). Although a hotspot region of insertion may exist, insertions outside this region are distributed quite randomly allowing the use of this transposon in the construction of a transposon library.

Out of the 7100 *L. monocytogenes* transposon mutants screened on solid medium for the inability to grow at 4°C when compared to the growth of the wild type, eight which were analysed in more detail exhibited the same growth as wild type at 30°C (Figure 3.3.1.1, A) and visibly defective growth at 4°C (Figure 3.3.1.1, B), when compared to the wild type. These findings suggested that the derivative Tn*917*-LTV3 had transposed into loci that produced cold-sensitive phenotypes in these mutants.

Out of the 2726 transposon mutants screened in liquid medium, 3 showed altered growth at 4 and 10°C when compared to the growth of the wild type (Figure 3.3.2.1, A and B), exhibiting an extended lag phase prior to the onset of growth. At 30°C, these mutants displayed a similar pattern of growth when compared to the growth of the wild type (Figure 3.3.2.2). One-way ANOVA applied to the growth rates of mutants and wild type growth curves incubating at 4, 10 and 30°C (Table 3.3.2.2) revealed a highly significant ($P<0.01$) difference in means of wild type and mutants growth rates at 4°C but not significant at 10 and 30°C. These results suggest that the transposon had inserted into a *L. monocytogenes* chromosomal region with molecular

functions required for growth at a low temperature (4°C) and that these functions are not necessary for growth at 10°C.

The results of Southern hybridisation analyses used to characterise the chromosome of the solid mutants (Section 3.4.1) at the site of the insertion and to confirm that Tn*917*-LTV3 had inserted singly into the chromosome of the mutants, revealed that in one (mutant L3) multiple insertions of the transposon had occurred (Figure 3.4.1.1). These results were not expected since, although multiple insertions have been reported for other transposons such as Tn*916* (Zheng and Kathariou, 1994), this was not reported for Tn*917*-LTV3 (Camilli *et al.*, 1990). To investigate if the whole plasmid pLTV3 had also inserted into the chromosome of the mutants, they were streaked onto plates containing tetracycline, the antibiotic used to select for pLTV3. Surprisingly mutants B3 and L3 exhibited growth after 2-3 days of incubation (Section 3.4.1). This result was not expected considering that in the initial screening the bacteria did not show the plasmid encoded tetracycline resistance. The presence of a single copy of the plasmid encoded tetracycline resistance gene present in the chromosome of these mutants may led to low level tetracycline resistance evident only after 2-3 days of incubation. From these observations we can conclude that the plasmid had integrated into the chromosome with the transposon. Although unexpected, the insertion of the plasmid into the chromosome would still result in disruption of the gene or genes into which insertion had occurred. Gene disruption by plasmid integration has been used in *L. monocytogenes* (Wuenscher *et al.*, 1991) and exploited for a wide range of genetic manipulations in *B. subtilis* (Youngman, 1990). However insertion of the plasmid into the chromosome deny the advantage of the small size of Tn*917*, mapping and cloning of the chromosomal DNA flanking the transposon insertions and mutation analysis becomes more difficult when the inserted element is large.

Insertion of plasmid DNA into the host chromosome together with the transposon was also observed in liquid mutants 1, 2 and 3. The plasmid specific probe used in Southern hybridisation analyses hybridised to the chromosomal DNA of all three mutants (Figure 3.4.2.1). Further confirmation that plasmid DNA was present in the chromosome of these mutants was obtained when they grew on agar containing tetracycline, the plasmid encoded antibiotic resistance gene, after 2-3 days of incubation (Section 3.4.2).

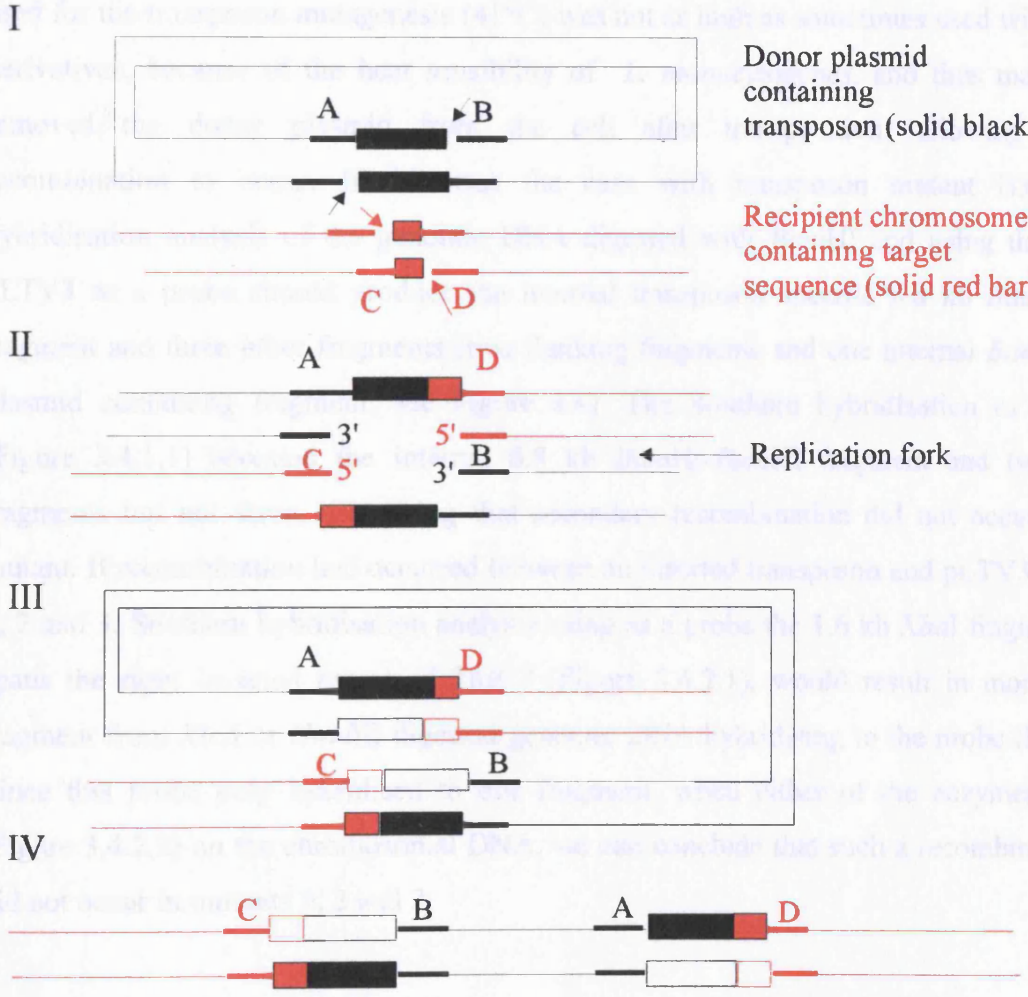
Insertion of plasmid DNA together with the transposon into the host chromosome may be due to impaired resolution of the plasmid-chromosome co-integrate formed during transposition (Figure 4.3). The co-integrate is resolved by the transposon-encoded resolvase protein, the

product of *tnpR* (Figure 4.1), and co-integrates are detected when mutant elements defective in resolvase are used (Sherratt, 1989). Experiments with mutants that form co-integrates, led to the conclusion that two transposon determinants are required for resolution of co-integrates to normal transposition products: *tnpR*, the gene encoding resolvase, and *res*, the recombination site (Sherratt, 1989). *res* (initially called the internal resolution site) was shown to be 5' of *tnpR* and *tnpA* in the Tn*3* subfamily of transposons. Footprinting experiments showed that resolvase binds to *res* and *in vitro* recombination experiments showed that recombination occurs at a unique site within *res*. This sequence also contains the promoters for the *tnpA* and *tnpR* genes (Sherratt, 1989). Therefore, binding of resolvase to *res* can repress transcription of *tnpA* and *tnpR* as well as lead to site-specific recombination. If the copy of Tn*917* harboured by the initial strain in mutants B3, C3, L3, 1, 2 and 3 was mutated at the *tnpR* gene and consequently defective in the production of resolvase, the co-integrate resolution would not happen and, plasmid DNA would stay in the chromosome with the transposon. Another explanation for the plasmid inserting into the host chromosome is through single recombination at a site of homology in the chromosome without transposition ever take place.

There have been a number of reports of mutations in the resolvase gene of Tn*3* family of transposons (Newman and Grindley, 1984; Hatfull and Grindley, 1986). The mutant resolvases fall into two phenotypic classes: those that bind *res* DNA but have lost recombinational activity, and those that neither recombine nor bind. Mutations in the C-terminal domain eliminate or impair binding to *res*, whereas those in the N-terminal domain can either eliminate binding to *res* or just block recombination. Two copies of the transposon, one at each end of the inserted plasmid, would be expected to be present if resolution had not occurred (Figure 4.3). This could be the case of mutant L3 since Southern hybridisation analyses when using pLTV3 digested with *Bam*HI would result in five hybridised fragments, one of 6.8 kb which corresponds to the internal transposon specific *Bam*HI-*Bam*HI fragment and four other flanking fragments, and this was observed in Figure 3.4.1.1.

Figure 4.3 - Formation of stable co-integrate of plasmid carrying Tn*917* in the host chromosome.

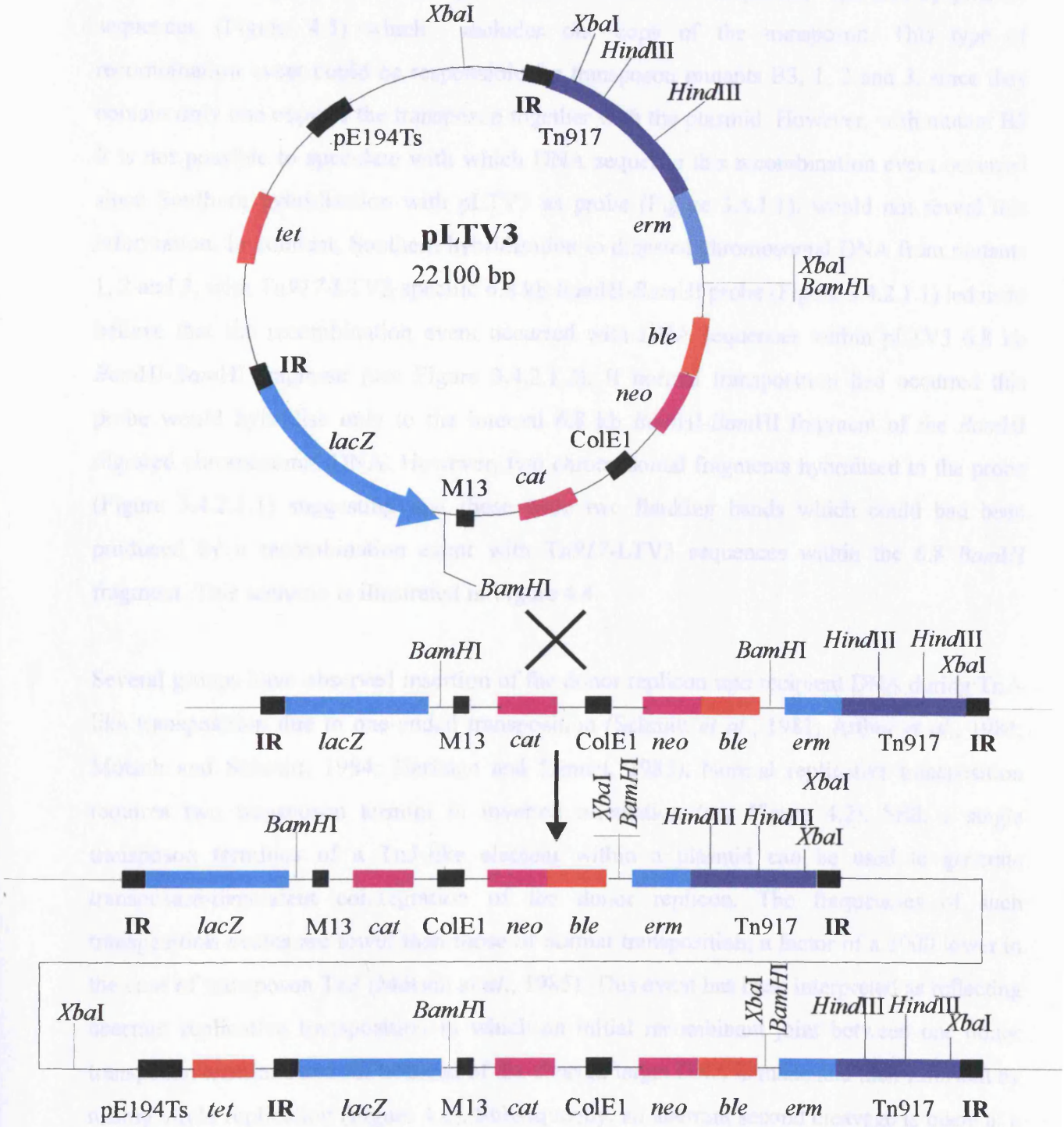
The model is drawn as in Figure 4.2. In step III the intermediate cointegrate is formed. However, the resolution of this co-integrate into normal transposition products does not occur resulting in the insertion of plasmid DNA together with the transposon into the host chromosome (IV). *Adapted* from Shapiro (1979).



Recipient chromosome containing the plasmid, 2 copies of transposon and duplicated target sequence (no resolution by resolvase)

Another way for the plasmid to insert into the host chromosome, together with the transposon, is through recombination with another transposon. This is possible if a copy of the plasmid carrying the transposon stays in the host cell after transposition of the transposon into the chromosome. The result of the recombination between the two copies of the transposon, would be two incomplete copies of the transposon separated by vector sequences (Figure 4.4). This type of recombination is not expected to be the explanation for the pattern of insertion reported here. This is because the donor vector carrying a copy of the transposon should be lost from the cell immediately after transposition had occurred. However, the temperature used for the transposon mutagenesis (41°C) was not as high as sometimes used with pE194Ts derivatives, because of the heat sensibility of *L. monocytogenes*, and thus may not have removed the donor plasmid from the cell after transposition, allowing secondary recombination to occur. If this was the case with transposon mutant B3, Southern hybridisation analysis of the genomic DNA digested with *Bam*HI and using the whole of pLTV3 as a probe should produce the internal transposon specific 6.8 kb *Bam*HI-*Bam*HI fragment and three other fragments (two flanking fragments and one internal *Bam*HI-*Bam*HI plasmid containing fragment; see Figure 4.4). The Southern hybridisation of mutant B3 (Figure 3.4.1.1) revealed the internal 6.8 kb *Bam*HI-*Bam*HI fragment and two flanking fragments but not three, suggesting that secondary recombination did not occurred in this mutant. If recombination had occurred between an inserted transposon and pLTV3 in mutants 1, 2 and 3, Southern hybridisation analysis using as a probe the 1.6 kb *Xba*I fragment, which spans the right inverted repeat of Tn*917* (Figure 3.4.2.1), would result in more than one fragment from *Xba*I or *Hind*III digested genomic DNA hybridising to the probe (Figure 4.4). Since this probe only hybridised to one fragment, when either of the enzymes was used (Figure 3.4.2.2) on the chromosomal DNA, we can conclude that such a recombination event did not occur in mutants 1, 2 and 3.

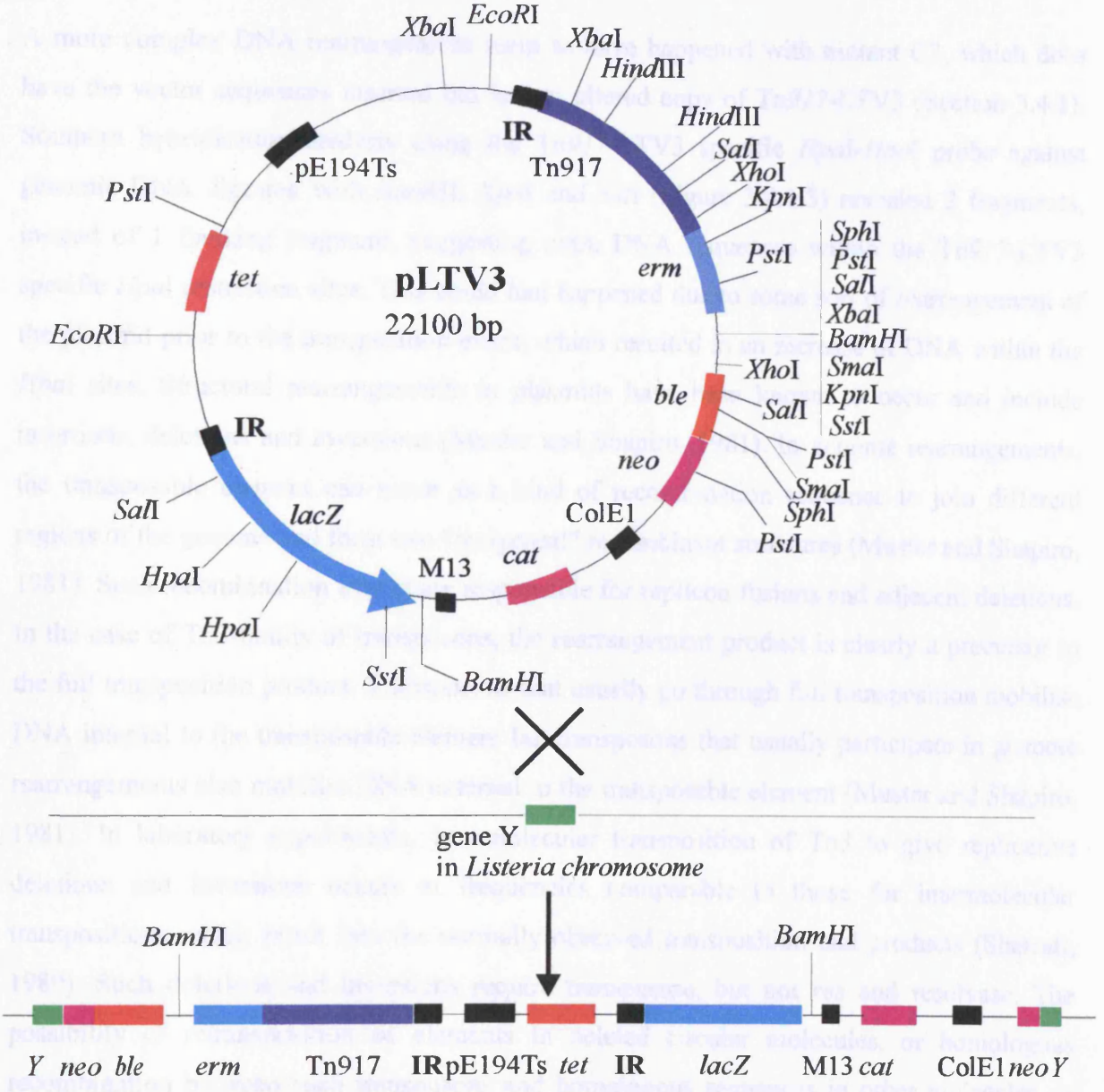
Figure 4.4 - Possible mechanism of plasmid insertion into chromosomal DNA, mediated by the reciprocal recombination between copies of Tn*917*-LTV3, after Tn*917*-LTV3 chromosomal insertion.


XbaI
XbaI
HindIII
HindIII
IR
Tn917
pE194Ts
tet
pLTV3
22100 bp
erm
XbaI
BamHI
ble
IR
neo
lacZ
ColE1
cat
M13
BamHI
BamHI
BamHI
HindIII
HindIII
XbaI
IR
lacZ
M13
cat
ColE1
neo
ble
erm
Tn917
IR
XbaI
BamHI
BamHI
HindIII
HindIII
XbaI
IR
lacZ
M13
cat
ColE1
neo
ble
erm
Tn917
IR
XbaI
BamHI
XbaI
BamHI
HindIII
HindIII
XbaI
pE194Ts
tet
IR
lacZ
M13
cat
ColE1
neo
ble
erm
Tn917
IR

Another possible explanation for the insertion of the donor plasmid and the transposon into the host chromosome is through recombination between a DNA sequence within the plasmid or the transposon and an homologuos DNA sequence in the chromosome. The result would be an interrupted copy of the homologous chromosomal DNA sequence, separated by plasmid sequences (Figure 4.5) which includes one copy of the transposon. This type of recombination event could be responsible for transposon mutants B3, 1, 2 and 3, since they contain only one copy of the transposon together with the plasmid. However, with mutant B3 it is not possible to speculate with which DNA sequence this recombination event occurred since Southern hybridisation with pLTV3 as probe (Figure 3.4.1.1), would not reveal this information. In contrast, Southern hybridisation to digested chromosomal DNA from mutants 1, 2 and 3, with Tn*917*-LTV3 specific 6.8 kb *Bam*HI-*Bam*HI probe (Figure 3.4.2.1.1) led us to believe that the recombination event occurred with DNA sequences within pLTV3 6.8 kb *Bam*HI-*Bam*HI fragment (see Figure 3.4.2.1.2). If normal transposition had occurred this probe would hybridise only to the internal 6.8 kb *Bam*HI-*Bam*HI fragment of the *Bam*HI digested chromosomal DNA. However, two chromosomal fragments hybridised to the probe (Figure 3.4.2.1.1) suggesting that these were two flanking bands which could had been produced by a recombination event with Tn*917*-LTV3 sequences within the 6.8 *Bam*HI fragment. This scenario is illustrated in Figure 4.4.

Several groups have observed insertion of the donor replicon into recipient DNA during Tn*3*-like transposition due to one-ended transposition (Schmitt *et al.*, 1981; Arthur *et al.*, 1984; Mötsch and Schmitt, 1984; Heritage and Bennet, 1985). Normal replicative transposition requires two transposon termini in inverted orientation (see Figure 4.2). Still, a single transposon terminus of a Tn*3*-like element within a plasmid can be used to generate transposase-dependent cointegration of the donor replicon. The frequencies of such transposition events are lower than those of normal transposition; a factor of a 1000 lower in the case of transposon Tn*3* (Mötsch *et al.*, 1985). This event has been interpreted as reflecting aberrant replicative transposition in which an initial recombinant joint between one donor transposon terminus and the one end of the cleaved target DNA is made and then followed by rolling circle replication (Figure 4.6). Subsequently, an aberrant second cleavage is made in a segment of the newly replicated donor molecule (often close to the transposon terminus) and this DNA is ligated to the other end of the recipient DNA molecule. Such insertions can be flanked by the normal 5-bp target duplication (Mötsch *et al.*, 1985). As Tn*917* is a member of the Tn*3*-like family of transposons, it is possible that one-ended transposition could be responsible for the insertion of the donor plasmid into the *L. monocytogenes* chromosome.

Figure 4.5 - A possible mechanism of plasmid insertion into chromosomal DNA, mediated by the reciprocal recombination between DNA sequences in the donor plasmid and homologous DNA sequences in the chromosome (gene y). For illustration in this Figure the cross over is within the *neo* gene.


XbaI
EcoRI
XbaI
HindIII
HindIII
SalI
XhoI
KpnI
SphI
PstI
SalI
XbaI
BamHI
SmaI
KpnI
SstI
PstI
SmaI
SphI
PstI
IR
Tn917
pE194Ts
PstI
tet
EcoRI
pLTV3
22100 bp
erm
ble
neo
ColE1
cat
IR
SalI
lacZ
HpaI
HpaI
M13
SstI
BamHI
gene Y
in Listeria chromosome
BamHI
BamHI
Y neo ble erm Tn917 IR pE194Ts tet IR lacZ M13 cat ColE1 neo Y

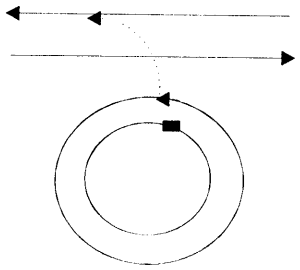
However, such events are very rare and since many insertion mutants analysed appeared to have undergone abnormal transposition, it is unlikely that an event as rare as one-ended transposition could be responsible.

A more complex DNA rearrangement seem to have happened with mutant C3, which does have the vector sequences inserted but has an altered copy of Tn*917*-LTV3 (Section 3.4.1). Southern hybridisation analysis using the Tn*917*-LTV3 specific *Hpa*I-*Hpa*I probe against genomic DNA digested with *Bam*HI, *Kpn*I and *Sst*I (Figure 3.4.1.3) revealed 2 fragments, instead of 1 flanking fragment, suggesting extra DNA sequences within the Tn*917*-LTV3 specific *Hpa*I restriction sites. This could had happened due to some sort of rearrangement of the plasmid prior to the transposition event, which resulted in an increase of DNA within the *Hpa*I sites. Structural rearrangements in plasmids have been known to occur and include insertions, deletions and inversions (Muster and Shapiro, 1981). In genome rearrangements, the transposable element can serve as a kind of recombination sequence to join different regions of the genome and form two "reciprocal" recombinant structures (Muster and Shapiro, 1981). Such recombination events are responsible for replicon fusions and adjacent deletions. In the case of Tn*3*-family of transposons, the rearrangement product is clearly a precursor to the full transposition product. Transposons that usually go through full transposition mobilise DNA internal to the transposable element but transposons that usually participate in genome rearrangements also mobilise DNA external to the transposable element (Muster and Shapiro, 1981). In laboratory experiments, intramolecular transposition of Tn*3* to give replicative deletions and inversions occurs at frequencies comparable to those for intermolecular transpositions which result into the normally observed transposition end products (Sherratt, 1989). Such deletions and inversions require transposase, but not *res* and resolvase. The possibility of retransposition of elements in deleted circular molecules, or homologous recombination between such transposons and homologous sequences in other molecules, is always present, and can generate more complex DNA rearrangements (Sherratt, 1989). In order to find out what type of rearrangement occurred in mutant C3, more detailed Southern hybridisations needed to be performed but this was not done because phenotipically identical mutants with normal transposition were isolated.

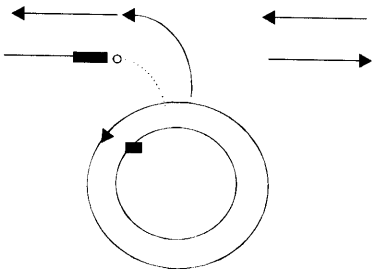
Figure 4.6 - Schematic representation of one-ended transposition.

(A) Initiation: as indicated by arrows, a nick at the IR in the donor DNA and 5 bp staggered nicks in the recipient are produced. The IR on the cut strand of the donor DNA in then ligated to the recipient DNA, as indicated by the dotted line. (B) Elongation: a replication fork is created that goes all the way round the donor replicon. (C) Termination: when the replication fork reaches the IR again it continues partially through it. The newly replicated IR is then cut precisely at the outer margin (arrow). The other strand is ligated to the recipient DNA (dotted line). (D) Resulting recombinant molecule with a gap, and donor molecule with short single stranded tail. (E) The gap in recombinant molecule is filled to produce a duplicated segment of donor DNA. Donor replicon is represented by large circle with the IR indicated ■ and ♦, tip of arrow indicates 5' outer margin of IR. Bold symbols and lines indicate original IR and DNA, dashed lines indicate newly synthesised DNA, ▬ represents the direct repeats of recipient DNA. *Adapted* from Mötsch *et al.* (1985).

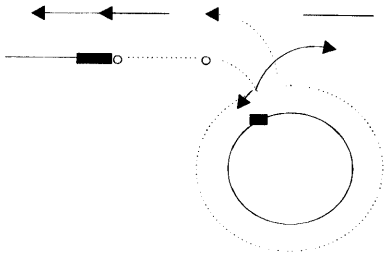
A



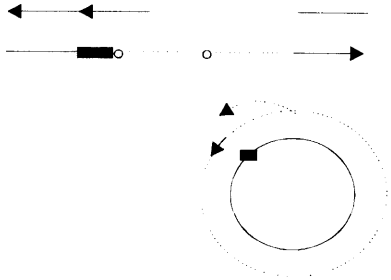
B



C



D



E

In summary, out of eight mutants exhibiting defective growth on plates at 4°C, three (mutants L3, B3 and C3) were shown to have resulted from non-classic transposition event. In mutant L3 and B3 the whole of the plasmid inserted into the chromosome together with the transposon. Southern blot analysis of the site of insertion in mutant L3 suggested that resolution of the plasmid-chromosome co-integrate formed during transposition had not occurred. In the case of mutant B3, Southern blot analysis of the chromosome at the site of insertion indicated that plasmid inserted into the chromosome through recombination between DNA sequences within the plasmid and an homologous DNA sequence in the chromosome. Mutant C3 does not contain the plasmid sequence inserted in the chromosome but it contains an altered copy of the transposon. Analysis of the chromosome at the site of insertion suggested that the transposon had suffered some rearrangement, prior to the transposition, which resulted in acquisition of extra DNA at the *erm*-proximal end of Tn*917*-LTV3. Furthermore, the three mutants isolated for their defective growth at 4°C in liquid medium were shown to be the same and to contain a copy of the plasmid in the chromosome. In this case, Southern analysis confirmed that plasmid had inserted into the chromosome through recombination between homologues sequences in the chromosome and the plasmid. All of these different ways through which "transposition" occurred indicates that when selecting for Tn*917*-LTV3 transposon mutants, the chromosome at the site of insertion should be carefully analysed in order to confirm what sort of transposition event took place.

Before proceeding onto the cloning of *L. monocytogenes* chromosomal DNA flanking the transposon insertion, with the aim of which was to identify the genes necessary for the organism to grow at low temperature, mutants were screened with a probe derived from the *Listeria* cold-shock gene (*cspL*). Since the mutants isolated were exhibiting a cold sensitive phenotype there was a possibility that the inactivated gene could be a cold-shock gene. The work of Francis and colleagues (1995) revealed that *L. monocytogenes* 23074 possesses a major cold-shock gene sequence (*cspL*) which is highly homologues to *cspB* from *B. subtilis* (Willimsky *et al.*, 1992) and *cspA* from *E. coli* (Goldstein *et al.*, 1990). In addition, these researchers observed that in low stringency hybridisation studies, using a *cspL* derived probe, *L. monocytogenes* 23074 revealed at least 4 homologous gene sequences (Francis *et al.*, 1995). In order to investigate if the gene disrupted by Tn*917*-LTV3 in the mutants defective for growth on solid medium at 4°C, was *cspL* or any of the 4 homologous gene sequences belonging to the family, a probe derived from *cspL* was used in low stringency Southern hybridisations against digested mutants (mutants G3, J3, K3, M3 and N3) genomic DNA. Since the pattern of hybridisation exhibited by all of the mutants was the same as the parent

wild type strain (*L. monocytogenes* 10403S) (Figure 3.4.1.1.1 and 3.4.1.1.2) we can conclude that the insertion of Tn*917*-LTV3 into the chromosome of the mutants did not occur within one of the *cspL* family of genes. The pattern of hybridisation exhibited by wild type 10403S was distinct to that of the wild type in which *cspL* was first identified, *L. monocytogenes* 23074 (Figure 3.4.1.1.1 and 3.4.1.1.2), in the size of the bands to which the probe hybridised. These results suggest that exist polymorphisms within the *csp* family of genes in the two *L. monocytogenes* strains.

In the case of mutants defective for growth in liquid medium at 4°C, and in order to investigate if the gene disrupted by pLTV3 was *cspL* or any of the homologues belonging to this family, Southern hybridisation analysis were performed at low stringency conditions (Figure 3.4.2.2.1). In this experiment the probe hybridised to a range of fragments, with some exhibiting stronger hybridisation. However, the pattern of hybridisation exhibited by all the mutants was the same as that exhibited by the parent strain confirming that the gene inactivated in the mutants is not *cspL* or any of the homologues gene sequences. As in the Southern analysis with the solid mutants (Figure 3.4.1.1.1 and Figure 3.4.1.1.2) in this Southern (Figure 3.4.2.2.1) the pattern of hybridisation of the two *L. monocytogenes* wild type strains (23074 and 10403S) differed, again suggesting a polymorphism of the *csp* family in these strains.

Although *L. monocytogenes* 10403S possesses gene sequences highly homologous to the *cspL* gene identified by Francis and co-workers (1995), these were not the gene sequence inactivated by the Tn*917*-LTV3, or pLTV3, insertion in the mutants identified in this work as exhibiting a cold-sensitive phenotype. These results suggests that not only does *L. monocytogenes* 10403S possess the *cspL* gene as well as genes homologous to the *cspL* family, but also other gene sequences that are involved in the mechanism of cold survival of this bacterium. Since *L. monocytogenes* is a psychrotrophic bacterium such additional loci may be involved in its survival at low temperature.

One of the advantages of having generated a mutation by gene disruption with a transposon derivative that contains an *E. coli* replicon, together with a large variety of restriction enzymes sites, is the fact that adjacent DNA, which can include the coding sequence of the gene and its *cis*-acting regulatory sequences, can then be cloned easily into *E. coli*. Therefore, in the present study *L. monocytogenes* DNA adjacent to the transposon insertion, in mutants defective for growth on solid medium at 4°C, was rescued into *E. coli* simply by digesting

total chromosomal DNA with an appropriate restriction enzyme (*Hind*III), which cuts outside the selectable marker *neo*, the ColE1 replication functions and DNA sequences of the inactivated gene, ligated at a dilute DNA concentration and transformed into an *E. coli* strain. Using this strategy, DNA flanking the *erm*-proximal end of Tn*917*-LTV3 insertions from mutants G3, J3, K3, M3 and N3 was cloned.

The recovered plasmids described in this work contained 1.3 to 5.5 kb (Table 3.5.1.1.1) insert derived from *L. monocytogenes* chromosomal DNAs flanking the *erm*-proximal end of Tn*917*-LTV3 insertion junctions and 12.7 kb of vector backbone derived from the recovery construct (Figure 3.5.1.1.1). Because of shortage of time, sequencing of *L. monocytogenes* DNA flanking the *erm*-distal end of Tn*917*-LTV3 insertion was only done for mutant J3. This DNA was recovered through partial digestion with *Sau*3AI (Section 3.5.1.2). Both types of recovery plasmids were transformed in *E. coli* MC1061 with selection for kanamycin in the case of transformants from the *erm*-proximal end of Tn*917*-LTV3, and selection for kanamycin together with erythromycin in the case of transformants from the *erm*-distal end of Tn*917*-LTV3. The use of this *E. coli* strain is very important, because Tn*917 erm* gene product may be toxic to typical *E. coli* strains due to its ability to methylate ribosomal RNA (Youngman, 1990). Gutierrez *et al.* (1996), from their work with *Streptococcus mutans,* found that *E. coli* MC1061 tolerated and expressed the Tn*917* Em^R determinant, and therefore could be used as a host to clone Tn*917* insertions from Streptococcal chromosomal libraries by selection of the *erm* gene. With the present work *E. coli* MC1061 was also shown to tolerate and express the erythromycin resistance gene. The efficiency of transformation, however, only was approximately 10 transformants/μg, which is low. This is most likely due to the fact that the transforming DNA was produced by partial digestion of the chromosome and not due to the inefficiency in transformation itself.

To investigate if the listerial chromosomal DNA recovered from the *erm*-proximal end of Tn*917*-LTV3 insertions in the different mutants, corresponded to DNA from different genes in the *Listeria* genome, the recovery plasmids were digested with *Pst*I (Figure 3.5.1.1.3). Although the results confirmed that these plasmids were different from each other, it still could not disclose if different genes had been inactivated in the *Listeria* chromosome since Tn*917*-LTV3 could have inserted at different positions within the same gene. To address this problem, Southern Blots analysis were performed using probes derived from each recovery plasmid against wild type DNA (Figure 3.5.1.1.5). Since the size of the hybridising fragment of the wild type DNA, restricted by an enzyme that produces a large fragment (*Sal*I), was

different for each of the probes different genes are likely to have been inactivated in each of the mutants. Such results are interesting because it suggests that an identical cold sensitive phenotype can be produced by inactivation of five different genes in the *Listeria* chromosome. In order to identify which genes were inactivated in each of the mutants, the flanking chromosomal DNA had to be sequenced.

To facilitate sequencing of the *L. monocytogenes* inserts from pAP plasmids (DNA sequences flanking the *erm*-proximal end of Tn*917*-LTV3 insertions), subcloning of the inserts into pBluescript was performed (Section 3.5.1.1.1). However, this step needed not have been done since *Listeria* DNA could had been sequenced directly from the pAP plasmids by using primers homologous to the inverted repeat at the *erm*-proximal end, then, when some chromosomal sequence was revealed, primers with homology to the new sequence could had been designed to proceed in the opposite direction. This way, some time could had been saved in this project. In the end the sequencing of the chromosomal DNA flanking the *erm*-distal end of Tn*917*-LTV3 insertion in mutant J3 (Section 3.5.1.2) was done directly from the recovery plasmid. This strategy proved to be time saving (Section 3.6.2.2).

The strategy to recover flanking chromosomal DNA, in *L. monocytogenes* mutants defective for growth in liquid medium at 4°C, was different. Because classical transposition had not occurred in these mutants, it was not possible to take advantage of the selectable marker *neo* and the ColE1 replication functions included in Tn*917*-LTV3 which facilitate the recovery of listerial DNA sequences adjacent to the insertion. The alternative approach was to clone the DNA fragment, containing the listerial flanking sequences, in a plasmid vector and only then transform into an *E. coli* strain (Section 3.5.2.1). In this way a partial library of 4850 clones of *L. monocytogenes* was created in *E. coli* and screened for the presence of the flanking chromosomal DNA. Only one of this clones exhibited a "positive signal" (Figure 3.5.2.1.2), however, when it was analysed in detail it proved not to contain insert. The fact that no clones were isolated may reflect instability of inserts of A+T rich DNA in *E. coli* vectors, or that the sequences included in the size selected DNA encoded for proteins which are toxic for *E. coli.* Youngman (1990) observed that it is not uncommon to find that *B. subtilis* chromosomal DNA sequences rescued into *E. coli* on a multicopy vector are toxic. This can lead to mutational alterations or rearrangements of rescued sequences, or prevent the recovery of clones altogether.

To circumvent the problems in isolating the flanking chromosomal DNA of the liquid mutants through cloning of the partial library, a different approach was taken. This was inverse PCR. With this procedure it was possible to amplify the desired listerial flanking DNA. However, some non-specific amplifications were also produced. Repeated attempts to generate a clean desired amplification fragment failed. The first result was never obtained on subsequent occasions. An inverse PCR system is a very laborious procedure and, when starting, it is important to test whether various steps in the procedure are working. However, this is not always possible. The most crucial and difficult step in inverse PCR is probably circularisation (Silver, 1991), and determining whether it has occurred is not easy in complex mixtures. One approach to monitoring circularisation is to dope the starting DNA with a surrogate radiolabelled restriction fragment comparable in size to that of the target. The mobility of this fragment in agarose gel electrophoresis can be monitored before and after ligation, and in the presence and absence of ethidium bromide (Silver, 1991). However, this test is not ideal as it provides only circumstantial evidence for circularisation of the actual target DNA. Thus, the positive result obtained on the first occasion suggested successful circularisation but it could not be obtained in the subsequent experiments. Silver (1991), suggested that in choosing primers and restriction enzymes for inverse PCR, it may be helpful to synthesise additional, "nested" primers. Note that in inverse PCR, "nested" primers are located "outside" of the primers used for initial amplification, when viewed from the perspective of the original, uncircularised DNA. The "nested" primers can be used to reamplify the products of the first PCR to enhance the sensitivity and specificity of the reaction (Silver, 1991). The nested primers would have been useful to reamplify the products of the first inverse PCR and consequently increase the amount of DNA of the desired product.

The automated sequencing system became the method of choice for sequencing the listerial DNA flanking the site of Tn*917*-LTV3 insertion in mutant J3 (Section 3.6.1). With this system, long "reads" of sequence were obtained (700 bp or more) from a single experiment, in contrast to the system used in the Sequenase kit which produced sequence data of approximately 200 bp from a single experiment. The aim of sequencing the listerial DNA flanking both sites of the Tn*917*-LTV3 insertion in mutant J3 was to give a putative identity to the gene inactivated, by comparison with sequences in the nucleic acid or protein databases. In addition, knowing the position of Tn*917*-LTV3 in the *Listeria* genome it is possible to have an insight into temperature regulated promoters. The final nucleotide sequence of the listerial DNA, at the site of insertion in mutant J3 (Section 3.6.3), was revealed by joining the sequence of listerial DNA at the *erm*-proximal end of Tn*917*-LTV3 (Section 3.6.2.1) with the

sequence of listerial DNA at the *erm*-distal end of Tn*917*-LTV3 (Section 3.6.2.2). Care was taken in order to account for the 5-bp direct duplication of the sequence adjacent to the insertion site which is known to be generated by all transposons of the Tn*3* family (Perkins and Youngman, 1984; Sherratt, 1989). The sequence 5'TATTA3' was identified as the duplication (Figure 3.6.3.1).

From the analysis of the sequence of the listerial DNA flanking the site of Tn*917*-LTV3 insertion in mutant J3, it is possible to observe that the transposon inserted into an ORF. This was termed ORF8 (Figure 3.6.4.1). Comparison at the amino acids level with sequences in the databases revealed that the ORF8 translation product exhibited significant homology (41-73% identity) to *B. subtilis* transmembrane proteins (Table 3.6.4.1) belonging to multicomponent transport systems involved in the uptake of osmoprotectants, such as glycine betaine. These transport systems are also known as ATP-binding cassette (ABC) uptake systems or traffic ATPases (Doige and Ames, 1993).

The glycine betaine transport systems to which the ORF8 product has homology are: OpuC (Kappes *et al.*, sequence submitted in 19.06.97), OpuB (Kappes *et al.*, sequence submitted in 17.06.97) and ProU (Lin and Hansen, 1995). Each of them is an operon comprising four ORFs. The first gene of these operons, encodes an ATPase; the second gene, encodes a transmembrane protein; the third gene, encodes an osmoprotectant binding protein precursor; and the fourth, a second transmembrane protein. ORF8 homology is to both transmembrane proteins, the second (41-43% identity) and the fourth (71-73% identity) in each of the operons above (Figure 3.6.4.2). The most homologous regions seems to be within the regions which encodes for hydrophobic helices spanning the membrane (residues 32-48, 67-83, 182-198, 148-164, 129-145 and 84-100) which are associated with its need to cross the membrane. Outside the sequences encoding for the membrane helices there are two other regions of homology (residues 67-83 and 114-123) which may be related to the function of the encoded protein, however, database searches did not provide any insight into the functionality. No noteworthy homology between ORF8 and transmembrane not proteins involved in osmoprotectant was revealed. Not much information on the functionality of the two ProU transmembrane proteins is available. In fact in the *B. subtilis proU* operon it is speculated that the first transmembrane protein, together with the ATPase, is likely to exist in the cell as homodimers (Kempf and Bremer, 1995), and that the function of the final transmembrane protein, although not known, is to enhance the functioning of the other proteins (Lin and Hassen, 1995). Both transmembrane proteins from the *proU* operon are known to display 29%

homology between them (Lin and Hansen, 1995), therefore ORF8 product has more homology to the two membrane proteins than they have to each other. Because of the striking homology between the ORF8 product and the transmembrane proteins which belong to the different multicomponent transport systems involved in osmoprotection, it is possible to speculate that the gene inactivated by Tn*917*-LTV3 in *L. monocytogenes* mutant J3 encodes for a membrane protein that is a member of an osmoprotectant uptake transport system. However, in order to be more specific, the sequence surrounding ORF8 was also analysed.

Upstream from ORF8 and with the same orientation, but in a different frame, another ORF (ORF9; Figure 3.6.4.1) is localised whose the translation product also exhibited significant homology (60-65% identity) to the third protein of the same transport systems to which ORF8 is similar. It should be noted that the determination of the 5' end of the nucleotide sequence of this ORF was not completed. Still, even the incomplete ORF9 product displayed homology to the third gene, encoding for the osmoprotectant-binding-protein, of the *opuC*, *opuB* and *proU* operons (Lin and Hansen, 1995; Kappes *et al.*, sequences submitted in June 1997). The most relevant characteristic of the osmoprotectant binding protein of the ProU system in *B. subtilis* is the fact that at the N-terminal region has a probable membrane helix corresponding to residues 8 to 28 (Lin and Hasen, 1995). However, this could not be investigated in the ORF9 sequence since not all of the amino acid sequence was known. The fact that ORF8, lying downstream from ORF9, exhibited homology to the last gene of *opuC*, *opuB* and *proU* suggests that these genes are members of a multicomponent uptake system that is involved in osmoprotection in *L. monocytogenes*. ORF9 being on a different frame from ORF8 is not unexpected since in the *B. subtilis* ProU system one of the components also is encoded on a different frame (Lin and Hansen, 1995) without affecting the organisation of the system. The existence of a putative ρ-independent transcription terminator (Section 3.6.4) downstream from ORF8 further supports the hypothesis that ORF9 and ORF8 constitutes an operon, or are part of an operon, with ORF8 encoding for the last component. This is in accordance to the *B. subtilis proU* operon which also contains this type of terminator downstream from the fourth gene. In addition, this terminator excludes the possibility of any transposon polar effect on genes downstream from ORF8 sequence due to interruption of transcription.

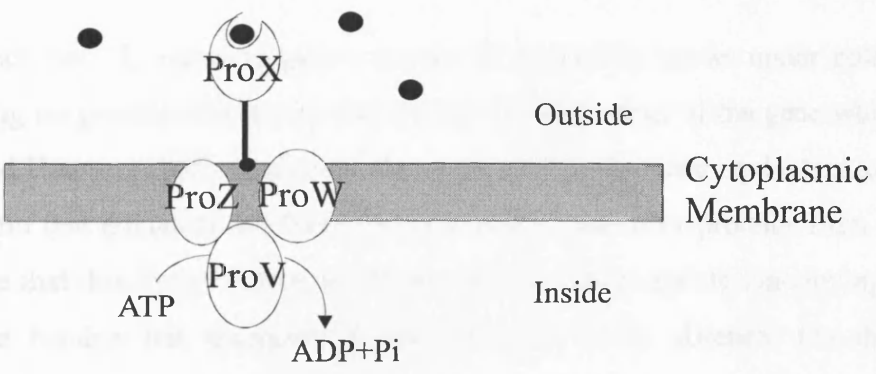
Multicomponent osmoprotectant transport systems, including those for osmoregulation, have been extensively studied in Gram-negative bacteria such as *E. coli* and *S. typhimurium* but only recently these studies have been extended to Gram-positive bacteria such as *B. subtilis*. Because high osmolarity and low-temperature conditions are those that favour *L.*

monocytogenes over its competitors, the knowledge of the processes of osmotic adaptation and low temperature adaptation are crucial to understanding its importance as a food-borne pathogen. An interesting aspect of the *opuC*, *opuB* and *proU* loci in *B. subtilis* is that they contain a fourth cistron, the last in the operon, that is lacking in the *proU* operon of either *E. coli* and *S. typhimurium* (Gowrishankar, 1989; Stirling *et al.*, 1989). Lin and Hansen (1995) speculated that this fourth cistron in *proU* encodes for a transmembrane protein that participates in osmoregulation in a new, although as yet undefined manner. Interestingly, *L. monocytogenes* ORFs identified in this work exhibited homology to the third and fourth gene of *B. subtilis* glycine betaine transport system, suggesting that in *Listeria* this type of transport systems also comprises four cistrons. This could be a characteristic of Gram-positive bacteria. In order to confirm the organisation of the operon in *Listeria* the nucleotide sequence upstream of ORF9 needs to be determined and analysed.

Another distinct characteristic between Gram-negative and Gram-positive ABC-type transporters is that the substrate-binding proteins in Gram-positive bacteria are extracellular lipoproteins that are tethered to the cytoplasmic membrane (Braun and Wu, 1994; Sutcliffe and Russell, 1995). The substrate-binding protein in Gram-negative bacteria is a soluble periplasmic protein that serves to capture the substrate and deliver it to the membrane-bound components (Kempf and Bremer, 1995). Since Gram-positive bacteria have no periplasm, it has been proposed that extracellular proteins possess lipid modifications at the amino-terminal residue of the mature substrate binding protein (the cysteine residue is modified through the covalent attachment of lipids) that would anchor it in the cytoplasmic membrane and prevent its loss into the surrounding medium (Braun and Wu, 1994; Sutcliffe and Russell, 1995; Kempf *et al.*, 1997). It would be interesting to investigate if such a lipid modification is present in the substrate-binding protein (ORF9) of the *Listeria* transport system. However, the sequence at the 5' end of the gene encoding for this protein was not determined during this work because the primary aim was to identify the ORF which has been inactivated by Tn*917*-LTV3 (ORF8). It would not be surprising that if ORF9 was one of the genes inactivated by the transposon in one of the other mutants obtained in this study.

A proposed model for the organisation of an osmoprotection transport protein system in *L. monocytogenes* is represented in Figure 4.7. This model is based on the homology between *L. monocytogenes* proteins to the *B. subtilis* proteins involved in the osmoprotectant transport system, ProU.

Figure 4.7 - Proposed model for the organisation of an osmoprotectant transport system in *L. monocytogenes*. By analogy to the *B. subtilis proU* operon, the *L. monocytogenes* proteins in this model were also called ProV, ProW, ProX and ProZ. The ProV protein is the ATP-binding protein that couple ATP hydrolysis to the transport process. ProW is an hydrophobic integral membrane protein, and in analogy to other binding-protein-dependent transporters, the ProV and ProW proteins are likely to exist in the cell as homodimers (Higgins, 1992). The substrate-binding protein, ProX, of the transport system is anchored to the membrane by a lipid modification. ProZ protein is also a transmembrane protein that may participate in osmoregulation in a new manner. (●) represents glycine betaine. *Adapted* from Kempf and Bremer (1995).


ProX
Outside
ProZ
ProW
Cytoplasmic
Membrane
ProV
ATP
Inside
ADP+Pi

As referred to before, *L. monocytogenes* is notably resistant to osmotic and cold stress. These two characteristics make it a serious threat to public health. Under osmotic stress *L. monocytogenes* accumulates glycine betaine, but, it also accumulates glycine betaine when grown under chill stress (Ko *et al.*, 1994). Ko and colleagues (1994) observed that the active transport of glycine betaine is stimulated by cold suggesting that glycine betaine confers both osmotolerance and cryotolerance. Carnitine was also observed to confer osmotic and cryotolerance to *L. monocytogenes* (Smith, 1996). However, the rate of carnitine uptake does not increase at low temperature (Smith, 1996) in contrast to glycine betaine (Ko *et al.*, 1994). In addition, these researchers observed that the kinetics of glycine betaine transport suggest that the two transport systems (i.e. the cold and the salt stimulated) are indistinguishable in terms of affinity for betaine and may be the same. Since sequencing analyses of the site of the transposon insertion in *L. monocytogenes* mutant J3 revealed a highly significant homology to *B. subtilis* proteins belonging to glycine betaine transport systems, this indicates that its cold sensitive phenotype is due to the inactivation of a glycine betaine transport system.

The fact that *L. monocytogenes* mutant J3 eventually grows under cold stress rather than showing no growth reflects the functioning of the product of the gene which was inactivated. Lin and Hansen (1995) speculated that in *B. subtilis* the gene equivalent to ORF8 encodes for a protein that enhances the functions provided by the other proteins from the operon. Thus it may be that this fourth cistron is not essential for the complete functioning of the transport of glycine betaine but transport is less efficient in its absence. On the other hand *L. monocytogenes*, as in *B. subtilis* (Kappes *et al.*, 1996), may possess more than one transport system for glycine betaine and the inactivation of one of these systems may be compensated by the activation of another and hence the difference in time to grow at 4°C that mutant J3 displayed, when compared to the wild type strain, reflects the time taken to induce this second system.

In order to further characterise the phenotype of the mutants defective for growth on solid medium at 4°C, plates were spot-inoculated with cultures of the mutants and wild type at the same OD600nm. In contrast to the initial screening (Section 3.3.1), these plates, at 4°C, had to be incubated for more than 2 weeks (9 weeks) in order for it to be possible to observe the difference in growth between wild type and mutant strains (Section 3.7.1.1). These results could suggest that the mutants became more able to grow on solid medium at 4°C when compared to the growth that they exhibited on the first occasion. These results are in agreement to what was speculated in the previous paragraph which referred to the existence of

an additional osmoprotectant transport system that could be compensating for the loss of the inactivated one. This second system may be is not as efficient as the ProU system or is only active for a short period of time after which it is not able to give the accessory help.

Certain antibiotics are known to induce a cold-shock response in *E. coli* (Van Bogelen and Neiddhart, 1990). Antibiotics such as chloramphenicol, tetracycline, erythromycin, fusidic acid and spiramycin are known to be inhibitors of translation (Van Bogelen and Neiddhart, 1990). Addition of these inhibitors to an *E. coli* culture resulted in the induction of the cold-shock response - the induction of cold-shock proteins, repression of the heat-shock proteins and continued synthesis of transcriptional proteins (Van Bogelen and Neidhart, 1990). To rule out the possibility that the differences in growth between mutants and wild type, when incubated at 4°C on plates, was not due to an antibiotic effect since mutants were grown on plates with erythromycin, an antibiotic recognised to inhibit translation by acting on ribosomes, mutants were grown together with the wild type on TSA plates with no antibiotic added (Section 3.7.1.1, Figure 3.7.1.1.2). The fact that mutants still exhibited defective growth at 4°C on TSA plates when compared to the growth of the wild type (Figure 3.7.1.1.2) demonstrates that the cold sensitive phenotype of these mutants is due to the mutation and not to an effect of antibiotic.

The presence of the reporter gene, *lacZ*, in the Tn*917*-LTV3 allows expression of the disrupted gene to be assessed. On insertion of Tn*917*-LTV3 into a gene in the correct orientation, the reporter gene can be expressed under the regulation of the promoter for the insertionally inactivated gene. Consequently, mutants harbouring insertion in transcription units can be identified by ß-galactosidase activity (Camilli *et al.*, 1990). When mutants defective for growth at 4°C were streaked on TSA containing X-Gal and incubated at 30 and 4°C (Section 3.7.1.2) none of the mutants expressed ß-galactosidase at either temperature. These results may indicate that the insertion of the transposon was not downstream to an active promoter, that the insertion may not have occurred in the same direction as the transcription of the gene inactivated or that the promoter was weak for its influence to be noticeable. In the case of mutant J3, in which the transposon has inserted in the same direction as the transcription of the inactivated gene (Section 3.6), it is possible to speculate that the promoter influencing the transcription of the disrupted gene may be too weak to express any visible levels of ß-galactosidase activity. It should be considered that may be not much ORF8 product is required for biological activity to be seen when compared to the ß-galactosidase activity required to produce any visible levels.

To investigate if the expression of the inactivated gene is regulated by salt, β-galactosidase activity from the inserted *lacZ* was measured in medium with different NaCl concentration (Section 3.7.1.6). *Listeria monocytogenes* mutant J3, at 30°C in T+M without and with 2% salt, expressed identical low levels of β-galactosidase suggesting that NaCl is not regulating the expression of the inactivated gene at this temperature. At 4°C the levels of β-galactosidase in medium without and with 2% salt, were also low suggesting that either temperature or salt is not regulating the expression of the inactivated gene. These results are not in complete agreement with the putative function of the inactivated gene which is expected to be active under high osmolarity conditions. However, it should be noticed that all of the values obtained were very low compared to those reported by Camilli *et al.* (1991) who observed activity levels of a thousand Miller units, when Tn*917*-LTV3 had inserted into an active gene in the correct orientation. These low levels of expression exhibited by the mutant suggest that no real activity is being measured in this assay and its in accordance to what was suggested in the previous paragraph that may be not much ORF8 product is required for biological activity to be seen. A more sensitive method for the quantification of such low expression should have been used. The alternative method known to be more sensitive than the Miller (1972) method is the fluorometric assay developed by Youngman (1990). This method uses MUG (4-methylumbelliferyl-β-D-galactopyranoside) as a substrate and is a substitute for the chromogenic substrate ONPG. In contrast to ONPG, cells need not be permeabilised for the MUG assay and being a more sensitive method smaller culture samples can be used. Although this is a more sensitive method, expression levels as high as those reported by Camilli *et al.* (1991) are not expected since the results above suggest that ORF8 may not be required in high amount.

In order to investigate if the growth of mutants, defective for growth at 4°C on solid medium, was also affected when grown in liquid medium, growth in TSB at 30 and 4°C was monitored (Section 3.7.1.3). At 30°C the growth of mutant J3, K3 and N3 was affected when compared to that of wild type, but at 4°C, the ability of the mutants to grow in TSB was not affected when compared to that of the wild type. This difference in growth at 30°C was not observed when growing on plates. As referred before, it was reasoned that in mutant J3, the difference in growth between mutants and wild type observed on solid medium at 4°C was probably due to the time taken to induce a second system for the transport of osmoprotectants such as glycine betaine. These differences in the results between liquid and solid medium may suggest that in TSB at 4°C this system is more rapidly induced or it is not required because of the

different environment of a liquid as compared to a solid medium. The reason why the mutants grow more slowly at 30°C in TSB than the wild type is not really explained.

In this study, galleries API20STREP and API50CH were used with the aim to investigate for differences between mutants and wild type phenotype, when incubated at 30 and 4°C (Section 3.7.1.4). At 30°C and 4°C the biochemical profile of mutants G3 and N3, in contrast to the wild type (Table 3.7.1.4.1 and Table 3.7.1.4.3), exhibit expression of ß-galactosidase. The reason why this reaction was not observed before on X-Gal plates, may be due to a more sensitive substrate used in the API20STREP gallery. These results suggested that in mutants G3 and N3 the insertion of Tn*917*-LTV3 occurred downstream to a promoter which is active at either 30 and 4°C. Considering the fermentation of different carbohydrates at 30°C and 4°C (Table 3.7.1.4.2 and Table 3.7.1.4.4), although the alkaline phosphatase reaction and the fermentation of lactose were negative at 4°C and not 30°C, the profile exhibited by the mutants was the same as that of the wild type demonstrating that the mutation in any of the mutants did not affect the carbohydrate metabolism of *L. monocytogenes*. Considering mutant J3 these results were expected since the analysis of sequencing (Section 3.6) suggested that the gene inactivated was not encoding for any enzyme involved in the carbohydrate metabolism of *L. monocytogenes*.

Sequencing analysis of mutant J3 (Section 3.6) strongly indicates that the gene inactivated by the transposon encodes for a protein member of an osmoprotectant transport system. To investigate how important this gene was for the *Listeria* ability to grow at low temperature and in high osmolarity, several experiments were done (Section 3.7.1.5). At 30°C the ability of mutants J3 to grow in media with NaCl becomes impaired as the salt concentration increases, when compared to the ability of the wild type (Figure 3.7.1.5.1). As was observed in Table 3.7.1.5.1, growth rate of wild type remained more or less constant when NaCl concentration was increased, whereas, mutant J3 exhibited decreased growth rate constants when NaCl was added. The fact that mutant J3 exhibited a defective ability to grow in medium with increasing salt concentration is not surprising since the inactivated gene belongs to a putative uptake transport system important for the survival of the organism in high osmolarity conditions. In contrast, the fact that the mutant exhibited a higher growth rate when compared to that of the wild type, when growing in T+M at 30°C with no NaCl added, may indicate that having to express ORF8, slows its growth since it is made but not used, whereas mutant J3 can use resources for other purposes. Other explanation is that it may be related to the fact that having ORF8 alters the membrane in a way that slows growth in the absence of

stress (e.g. reduces the uptake of nutrients). At 4°C, the growth rate of the wild type was higher than that of mutant J3 (Table 3.7.1.5.2) either in T+M without or with NaCl added. Statistical analysis revealed a significant difference, suggesting that the ability of the mutant to grow at 4°C was affected when compared to that of the wild type in both osmotic conditions. Considering that the gene inactivated in mutant J3 might be involved in the survival of the organism in high salt conditions accounts for the impairment of growth in increasing salt concentration. These results confirms that the inactivated gene is not only important for the growth of this organism in high osmotic conditions but also at low temperature. This results are in accordance to the findings of Ko *et al.* (1994) which observed that glycine betaine conferred osmotolerance as well as cryotolerance to *L. monocytogenes*. The fact that in T+M medium with no salt added, the mutant at 30°C exhibited a higher growth rate than that exhibited by the wild type but at 4°C the situation was reversed, confirms that the inactivated gene is not only important for the growth of this organism at high osmotic conditions but also at low temperature.

In order to investigate which osmoprotectant transport system was affected, by the mutation in mutant J3, several experiments could have been prepared. These, may include growth curves in which cells (wild type and mutant J3) are grown in T+M medium, at 30°C and at low temperature, in the presence of a single osmoprotectants known to be used by *Listeria*, such as glycine betaine, carnitine or proline. Accordingly to the osmoprotectant transport system mutated in mutant J3, will be unable to import its osmoprotectant from the medium when in conditions of low temperature. Thus, the wild type strain would be expected to maintain its growth since it is able to import the osmoprotectant, whereas the mutant would not. This uptake can easily be measured by using radiolabelled compounds. Identical type of experiments can be prepared on plates of T+M (1.5%) agar containing the osmoprotectants. It is relevant to investigate if the inactivated system also is important on solid medium and at the same time if will be possible to determine which osmoprotectant confers cryotolerance to cells growing on a solid surface, as would occur in solid food.

In T+M medium with no NaCl at 30°C both strains exhibited autolysis (Figure 3.7.1.5.1, A). The autolysis did not occur in the presence of added NaCl (Figure 3.7.1.5.1, B and C) or at 4°C (Figure 3.7.1.5.2). Autolysis is not uncommon in *Listeria* strains. Previously (Tyrrell, 1973), it was though that the lytic potential was common in the genus *Listeria* and that like other bacteriolytic system, was associated with cell wall degradation. Pine *et al.* (1989) more recently suggested that lysis may occur as a consequence of the substrate used, the activation

of a lytic bacteriophage, an autolytic enzyme being produced, or as a result of unbalanced growth, although these factors are not mutually exclusive. Wuenscher and co-workers (1993) studied the *L. monocytogenes* extracellular protein (p60) and concluded that it is a murein hydrolase with a role in cell division. Murein hydrolases which can degrade their own cell walls are also known as autolysins, accordingly p60 may be the listerial autolysin. As p60 is an autolysin together with the fact that the bacterial lysis is associated with the substrate used (Pine *et al.*, 1989), it can be said that T+M is an appropriate medium for the autolytic enzyme to be produced or activated. Wuenscher *et al.* (1993) also observed that p60 accumulates in the supernatant in the stationary phase rather than in the exponential phase. These results are in agreement with what was observed in Figure 3.7.1.5.1 (A) in which autolysis seem to occur at the beginning of the stationary phase. Interesting, this phenomenon is not observed when the substrate is modified, by adding NaCl (Figures 3.7.1.5.1 (B) e (C)), further indicating that the substrate may be an important factor effecting autolysis or that under stress conditions this protein is produced at a low level given that p60 is essential for viability. These last two hypothesis are further supported by the fact that in medium with no salt added but at low temperature the autolysis phenomenon was not observed. It would be interesting to investigate how environmental stresses control the expression of protein p60.

To rule out the possibility that the phenotype observed in mutant J3 was not the result of a spontaneous mutation somewhere in the chromosome, that could also be inactivating a gene involved in cryotolerance and/or osmotolerance, a genetic complementation approach was devised. Other ways of excluding the hypothesis that the phenotype observed with the mutant was due to a spontaneous mutation and not to the transposon insertion, is by reversion from the mutant phenotype into the wild type phenotype or creating an in-frame deletion of the wild type gene to check if the resulting phenotype is the same as that of transposon mutant. The reversion from the mutant into the wild type phenotype involves the loss of the transposon insertion by replica plating the mutant bacteria on plates with and without antibiotic selection. However, this procedure can be time consuming since Tn*917* is known to induce stable mutations (Berg *et al.*, 1989; Murphy, 1989; Youngman, 1990; Camilli *et al.*, 1990) with a reversion frequency as low as 10^{-10}/bacterium (Berg *et al.*, 1989). Creating an in-frame deletion can also be time consuming. One way to accomplish this is by introducing an antibiotic cassette within ORF8 and cloning it in an appropriate *Listeria* suicide vector, once transformed into the wild type strain promotes recombination between wild type and mutated ORF8 resulting in the transfer of the antibiotic/ORF8 on to the chromosome. This way selection for the antibiotic will reveal if recombination occurred. The subsequent step is to

introduce a copy of ORF8 with an internal deletion into the antibiotic mutated strain and replica plate for the loss of the antibiotic resistance. *Listeria* that have lost the antibiotic resistance should have resulted from recombination between ORF8 with the deletion and the antibiotic/ORF8. These recombinants will have on the chromosome a copy of ORF8 with an internal deletion. Since there is no selection thousands of recombinants may have to be screened. This procedure not only is time consuming as the recombination step has to be very well monitored in order to rule out the occurrence of any chromosomal rearrangements. Therefore the genetic complementation approach was chosen.

Following the decision to adopt genetic complementation, the intact wild-type gene which was inactivated by the transposon, ORF 8, had to be isolated. This was accomplished by PCR (Section 3.8.1, Figure 3.8.1.1). Because the PCR product was to be cloned and used in expression studies, the fidelity of DNA synthesis is of vital importance. Thus, the enzyme used in the DNA polymerisation was *Pwo* DNA polymerase (Boehringer Mannheim). This polymerase has a high 5'-3' processivity and possesses a 3'-5' exonuclease activity, also known as proof-reading activity. The inherent 3'-5' exonuclease proof-reading activity of Pwo results in an over 10-fold increased fidelity of DNA synthesis compared to *Taq* DNA polymerase. When using *Taq* DNA polymerase [error rate of $2x10^{-4}$ errors/base (Cha and Thinly, 1993)] about 56% of a 200 bp amplification product will contain at least a single error after one million fold amplification. In contrast when using *Pwo* DNA polymerase for amplification, only 10% of the products will contain an error under the same conditions (Boehringer Mannheim).

To verify that the PCR product corresponded to the ORF inactivated by Tn*917*-LTV3, and not to other DNA region of the *Listeria* genome, Southern hybridisation was performed. The positive hybridisation between the probe and PCR product (Figure 3.8.1.2) indicated that the amplified ORF was the one inactivated by the transposon in mutant J3. In order to confirm the fidelity of the PCR product, subsequent sequencing was performed. Although the sequencing analysis on both strands of the PCR fragment was not complete, the portions sequenced matched exactly with ORF8. Finishing of this sequence should be quick. In spite of the fact that is not possible to confirm that the PCR procedure did not mutate the ORF being amplified these results were encouraging and it was decided to proceed.

To circumvent the problems related with mutations created by the PCR procedure an alternative approach could have been taken. By using a probe derived from the ORF

inactivated with the transposon, a wild-type *Listeria* complete genomic library could have been screened and the complete intact wild-type gene would have been identified and isolated. However, this involves the availability of such a genomic library which at the time was not accessible.

The plasmid chosen to clone the PCR product for expression studies in *L. monocytogenes*, was pMK4 (Sullivan *et al.*, 1984). This plasmid is a shuttle vector which replicate in both *Listeria* and *E. coli*, and has been successfully used in *Listeria* complementation studies (Cossart *et al.*, 1989; Michel *et al.*, 1990; Ripio *et al.*, 1997). Vectors of this type are very useful since they allow the use of the well-developed techniques for the isolation and manipulation of plasmids in *E. coli*. The resulting recombinant molecule can then be introduced into *Listeria* by transformation. This has been important because the generation of recombinant plasmids by direct transformation of ligation mixtures into Gram-positive bacteria is very inefficient (Trieu-Cout *et al.*, 1987). pMK4 contains the *lacZ* gene, which includes the operator and promoter, and includes several unique restriction sites (Section 3.8.2, Figure 3.8.2.1). Insertion of DNA into any of these sites disrupts the *lacZ* gene, resulting in a Lac^- phenotype in the appropriate *E. coli* host strain. The recombinant plasmid can then be readily isolated and transformed into *L. monocytogenes*. Another characteristic of this plasmid, which is important for this study, is the fact that it contains the *lacZ* promoter and because ORF8 was identified as being the last gene of the operon and although the ribosomal binding site was included in the PCR amplification (Figure 3.8.3.2) there was no evidence for the existence of a promoter in the upstream sequence (Section 3.6.4) so this was not amplified. Thus, the aim was to use the *lacZ* promoter in the expression studies of ORF8 in *L. monocytogenes* mutant J3. In previous work, when using the pMK4 as expression vector, the promoter sequences associated with the gene to be cloned were also included (Cossart *et al.*, 1989; Michel *et al.*, 1990) This was not because the *lacZ* promoter does not function in *L. monocytogenes* but rather the natural promoter was close to the gene. In fact, the *lacZ* promoter is known to work in *L. monocytogenes,* however, may be not as efficiently as it would in *E. coli* (Cath Rees personal communication).

pMK4 can be transformed into *L. monocytogenes* through protoplast transformation (Cossart *et al.*, 1989) or electroporation (Michel *et al.*, 1990; Ripio *et al.*, 1997). The preparation of bacterial protoplasts involves the proper conditions and enzyme(s) for cell-wall removal, determination of the ideal molecular weight and concentration of the fusant for efficient fusion without significant loss of viability, and the precise formulation of a complex

regeneration medium to support subsequent synthesis of new cell-wall material. Moreover, these conditions do not guarantee consistently reproducible results from one experiment to the next (Trieu-Cuot *et al.*, 1987; Luchansky *et al.*, 1988). In contrast, electroporation has proved to be effective for transferring DNA into eukaryotic and prokaryotic systems (Luchansky *et al.*, 1988). Compared to the other mechanisms for transferring plasmid DNA, electroporation is less time consuming, less tedious, and less expensive method.

After successful electroporation of *E. coli* with the recombinant pMK4 (Section 3.8.2), transformants containing the insert, the intact ORF8, were selected (Figure 3.8.2.2) for subsequent transformation into *L. monocytogenes*. The method used was electroporation of penicillin-treated cells (Park and Stewart, 1990) which was also the adopted method of Ripio and co-workers (1997). This method takes advantage of the fact that cell-wall damage, caused either by degradative enzymes or by incorporating cell-wall active agents in the growth media, typically improves transformation efficiencies in Gram-positive bacteria. Park and Stewart (1990) observed that a pre-treatment with penicillin-G, caused a dramatic increase in transformation efficiency of *L. monocytogenes*, levels as low as 100ng/ml producing a 60-fold stimulation over the untreated control. The optimal conditions for electroporation of penicillin-G treated *L. monocytogenes* (maximum number of transformants $4x10^6$/μg DNA) was achieved in the presence of 10μg penicillin-G/ml and electroporated at a field strength of 10kV/cm (pulse duration, 5ms) (Park and Stewart, 1990). A Gene-Pulser which reached 10kV/cm was not available and consequently the voltage used to electroporate *L. monocytogenes* in this study was 2.5kV/cm (pulse duration approximately 5ms) (Section 2.4.2.1.2). Although this value was lower than the optimal value, it was felt that would be enough to obtain at least one colony which was the requirement. However, no transformants were recovered from the electroporation of *L. monocytogenes* mutant J3 either with recombinant pMK4 or pMK4 itself (Section 3.8.4). These results can suggest that the field strength used was not enough to produce a minimal permeabilisation of the bacterial cell membrane allowing the entry of the plasmid DNA. However, Michel *et al.* (1990) using a procedure which does not involve the cell-wall damage by penicillin-G and electroporating cells at a field strength of 2.3kV/cm was able to recover *L. monocytogenes* containing pMK4 at a frequency of 10^{-2} - 10^{-3}/μg DNA. The *Listeria* strain also is likely to affect the efficiency of transformation since, like in *E. coli*, there could be strains that are more easily transformable than others. Park and Stewart (1990) procedure was optimised for *L. monocytogenes* strain 23074, Michel *et al.* (1990) for strain LO28 and in this study the strain used was 10403S: may be *L. monocytogenes* 10403S is not a easily transformable strain. In

addition, in previous studies different plasmids of different sizes were used. These characteristics also may affect the efficiency of transformation.

Other factors also known to affect electroporation includes the plasmid DNA topology. Park and Stewart (1990) observed that relaxation of the DNA produces a reduction in transformation to 40% of the efficiency of the native supercoiled form and when linear plasmid DNA was used, no transformants were recovered. If pMK4-derived plasmid DNA became relaxed or nicked it would not be surprising that no transformants were recovered (Section 3.8.4). However, because transformation with both pMK4 and pMK4-derived plasmids failed, relaxation is unlikely to be the explanation. Due to the short time left in this project it was not possible to follow up these possibilities in an attempt to overcome the *Listeria* transformation failure.

Early studies on virulence of *L. monocytogenes* indicated that growth of this organism at low temperature increased its virulence (Gray and Killinger, 1966; Wood and Woodbine, 1979). Recently, mouse models of infection found that *L. monocytogenes* grown at 4°C has increased virulence for intravenously (i.v.) infected mice (Czuprynski *et al.*, 1989; Stephens *et al.*, 1991) but only at a dose of about or above 10^4 viable listerias (Stephens *et al.*, 1991). In order to investigate if virulence of *L. monocytogenes* mutants G3 and J3 was affected by the mutation, the ability of *L. monocytogenes* mutant G3 and J3 to grow and persist in mice after i.v. infection with a dose higher than 10^4 viable listerias, was compared with that of wild type *L. monocytogenes* 10403S (Table 3.9.1.1 and Figure 3.9.1.1). Although, sequencing analysis (Section 3.6) suggested that the inactivated gene in mutant J3 is probably involved in cryotolerance and osmotolerance it does not exclude the possibility of it also being related with the virulence of the organism. In addition, because cold has for a long time been connected with increased virulence in *L. monocytogenes,* a cold sensitive phenotype might suggest a reduced virulence. Virulence of the mutants was not affected, however, being the same as that of the wild type. These results demonstrate that the gene inactivated in mutant G3 and J3 does not contribute to the virulence of the organism when inoculated directly into the blood. Excision of Tn*917*-LTV3 from the mutants, within the intracellular environment, was excluded since mutants were expressing erythromycin and lincomycin resistance after the recovery from the animals.

The route of infection used in this project, intravenous, is not the natural infection route, in fact the gastrointestinal (g.i.) route of infection is the one that simulates the portal of entry in

naturally occurring outbreaks of listeriosis (Schlech, 1984; Farber and Peterkin, 1991). However, this infection route was adopted since it is the most common route used in virulence studies and excludes the need of *Listeriae* to translocate to the mesenteric lymph nodes, from where they then disseminate to the spleen and liver. Because of time limitations it was not possible to go on to investigate oral infection of the mice, however, if there had been more time available this type of infection would have been considered.

Overall the strategy used to identify genes encoding products (proteins) necessary for the growth of *L. monocytogenes* at low temperature has been proved to be successful. The Tn*917*-LTV3 transposon library constructed revealed that although a hotspot of insertion for this transposon may exist in the chromosome of *L. monocytogenes,* insertions outside this region were sufficiently random to allow the selection for mutants displaying a cold-sensitive phenotype. Screening for mutants defective for growth at 4°C and normal growth at 30°C revealed eight mutants when strains were grown on solid medium and three mutants when growth was in liquid medium. Southern blot analysis using probes derived from Tn*917*-LTV3 revealed that in some of the mutants classical transposition had not occurred. However, once detailed analysis at the site of insertion in five solid mutants that contained normal transposition was concluded, rescuing of flanking *Listeria* chromosomal DNA in the adequate *E. coli* strain was straight forward. Southern blot analysis of the chromosomal DNA surrounding the site of insertion in these five mutants confirmed that different genes had been inactivated, suggesting that *L. monocytogenes* contains several genes involved in growth at low temperature. Detailed sequencing analysis of one of the "solid" mutants, mutant J3, suggested that the gene inactivated by Tn*917*-LTV3, encodes for a membrane protein that is a member of an osmoprotectant uptake transport system not yet characterised, at the molecular level, in this organism. This suggests that the inactivated gene in mutant J3 not only is involved in cryotolerance but also in osmotolerance. Growth of *L. monocytogenes* mutant J3 in defined medium at high osmolarity, either at 30 or 4°C, was showed to be affected when compared to the growth of the wild type, confirming its importance in osmotolerance in this organism. Studies on the effects of the mutations on pathogenicity demonstrated that mutant J3 was not affected in its virulence when compared to that of the wild type parental strain. Although expression studies suggested only a low level of expression of ORF8, it would be interesting to develop a more sensitive method for the quantification of such a low gene expression. Unfortunately due to time constraints, analysis of the inactivated gene in each of the other cold sensitive mutants was not accomplished. However, to expand our knowledge of growth of *L. monocytogenes* at low temperature it would be worthwhile to identify the

function of these remaining mutated genes. In the first instance this would be done by sequencing the DNA flanking the site of Tn*917*-LTV3 insertion and looking for homology in the protein databases. It would also be interesting to confirm the *proU* operon structure in *L. monocytogenes*. This could be achieved by creating deletion mutations in each of the three ORFs upstream to ORF8. Subsequent analysis of the transcript size (Northern blots) would confirm if these genes function as an operon. Primer extension can be used to identify the operon promoter. Subsequently, expression studies based on reporter gene fusions with the promoter could be performed.

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