

Construction and characterisation of
aromatic amino acid dependent mutants
of *Listeria monocytogenes*.

Thesis submitted for the degree of
Doctor of Philosophy
at the University of Leicester

by

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May 1993

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Statement

The accompanying thesis is submitted for the degree of Doctor of philosophy entitled "Construction and characterisation of aromatic amino acid dependent mutants of *Listeria monocytogenes*" is based on work conducted by the author in the department of microbiology of the University of Leicester between October 1988 and September 1991.

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.

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Abstract

The severe forms of listeriosis, have a very high mortality rate. In farm animals, especially sheep, these losses can be of considerable economic importance. The increase in cases of listeriosis in both man and animals over the last decade has stimulated research to develop an effective vaccine to protect against *Listeria monocytogenes*. However, attempts at protection using killed or chemically attenuated live vaccines have been disappointing.

An alternative to these procedures is the development of strains with a defined mechanism of attenuation. Attempts were made to construct aromatic amino acid dependent mutant strains of *L. monocytogenes* and to investigate their efficacy as a vaccine.

Two strategies were used for the transposon mutagenesis of *L. monocytogenes*. Suicide vectors carrying transposon Tn917 and unable to replicate in *Listeria* were constructed. To facilitate the transformation of these vectors into *Listeria* species an efficient electrotransformation system was developed. However, this procedure was unsuccessful in generating Tn917 insertion mutants.

Insertional mutagenesis of *L. monocytogenes* EGD with Tn917 was achieved using a temperature sensitive plasmid. An aromatic amino acid requiring mutant deficient in chorismate mutase activity was isolated. The multiplication of this mutant was found to be unimpaired in both mouse tissues and cultured bone marrow derived macrophages. Organisms isolated from infected tissues were found to be prototrophic while still harbouring a Tn917 insertion. It was concluded that the original mutant carried a point mutation in the gene encoding chorismate mutase and that this had reverted on passage through the mouse.

A transposon induced aromatic amino acid dependent mutant of *L. monocytogenes* found to be deficient in prephenate dehydratase activity was obtained for investigation. The virulence and multiplication of this mutant were reduced in the mouse. Vaccination with this mutant was found to stimulate a protective immune response in mice. The results indicate that aromatic amino acid dependent mutants of *L. monocytogenes* protect against listeric infection and offer a new approach to the development of anti-listerial vaccines.

Acknowledgements

I would like to thank Dr Dorothy Jones for her supervision, help and advice throughout the course of this thesis. I would also like to thank Dr Ian Roberts and Dr Peter Andrew for their advice and interest in my work.

My gratitude must also go to all the members of the Department of Microbiology, past and present, who have assisted me in many ways. I would especially like to thank Jonathan Stephens and Dr Seamus O'Brien for their good humoured help, encouragement and commiseration.

In addition my thanks must go to Dr Dan Portnoy for the donation of bacterial strains and for generously performing intracellular growth assays.

For the provision of a maintenance grant I would like to thank the Medical Research Council.

In conclusion, I would like to thank my friends and family for their unfailing support and encouragement throughout the course of my work. Finally, my special thanks go to my husband Paul who has kept me going and without whom this thesis would not have been completed.

Abbreviations

<i>aro</i> ⁻	Aromatic amino acid dependent
BHI	Brain heart infusion
BMDM	Bone marrow derived macrophage
BMMM	Bone marrow macrophage media
bp	Base pair
BSA	Bovine serum albumin
cfu	Colony forming units
DME	Dulbecco's modification of Eagles medium
ED ₅₀	Fifty percent effective dose
EDTA	Diaminoethatetra-acetic acid
kb	Kilobase pair
kd	Kilo daltons
kV	Kilo Volts
MHC	Major histocompatibility complex
μF	Micro Farad
mic	Minimum inhibitory concentration
ms	Milli second
OD	Optical density
PBS	Phosphate buffered saline
PEG	Polyethylene glycol
rpm	Revolutions per minute
SDS	Sodium dodecyl sulphate
SMEM	Sucrose magnesium electroporation medium
TE	Tris-EDTA buffer
TS	Tryptose soya
X-gal	5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside
UV	Ultra violet
V	Volts

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Introduction

Members of the genus *Listeria* are nonmotile, Gram-positive rods that are widely distributed in the environment. Currently six species are recognised, *Listeria monocytogenes*, *Listeria innocua*, *Listeria ivanovii*, *Listeria welshimeri*, *Listeria grayi*, *Listeria seeligeri* and *Listeria murrayi*. (Seeliger and Jones, 1986, Rocourt *et al.*, 1987b). Of these only *L. monocytogenes* and *L. ivanovii* are frankly pathogenic, the former for man and the latter for animals mainly sheep. Symptoms are quite varied and are referred to as listeriosis, characterised by; encephalitis, septicaemia and abortion in both man and animals (Gray and Killinger 1966).

The marked increase in the number of cases of listeriosis since 1980 has rekindled interest in the listeriae. Significant advances have been made in the classification of the genus at both the inter and intrageneric level aided by the application of molecular biological techniques. Members of the genus are now more easily isolated and identified because of the development of new tools such as the Oxford and PALCAM selective differential media (Ryser and Marth 1991). Although our understanding of the epidemiology and virulence of the listeriae has been greatly advanced in recent years, further study is still required.

History

The organism now known as *Listeria monocytogenes* was isolated and first adequately described by Murray, Webb and Swan in 1926. Murray *et al.*, identified this organism as the causative agent of a septicaemic infection of laboratory rabbits and guinea pigs in Cambridge University in 1924. The organism was named "*Bacterium monocytogenes*" the indeterminate genus name *Bacterium* was chosen because Murray was uncertain of the exact taxonomic placement, while the specific epithet *monocytogenes* was chosen due to the marked mononucleosis observed in the blood of the infected animals. One year later in South Africa, Pirie described a bacterium isolated from the livers of wild gerbils (*Tatera labengula*) which he named *Listerella hepatolytica* in honour of the British microbiologist Lord Lister (Pirie 1927). This bacterium was later recognised by Pirie to be identical to that described by Murray *et al.*, and its name was changed to *Listerella monocytogenes* (Addendum, Pirie, 1927). In 1940 the name of the bacterium was changed to *Listeria monocytogenes* (Pirie

1940) because the genus name *Listerella* had previously been used for a mycetoan and a species of foraminifera. This name *Listeria monocytogenes* was adopted 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 (Judicial Commission [1954]).

The bacterium had almost certainly been detected prior to its first documented description. For example Hayer (1891), and Henle (1893) both refer to small Gram positive rods observed in sections of post-mortem tissue from patients who are thought to have died from listerial infections (Gray and Killinger 1966). Hülphers (1911) isolated an organism from rabbits which, although no extant strain remains, has been suggested to be of the genus *Listeria* (Murray 1953). An organism isolated from a case of human meningitis in 1918 and deposited at the Pasteur Institute Paris by Dumont and Cotoni in 1921 and later identified as *L. monocytogenes* (Cotoni 1942) is now regarded as the oldest authentic strain of *L. monocytogenes*

For some 25 years following its initial description cases of listeriosis were rarely reported and *L. monocytogenes* was regarded as being mainly an animal pathogen. The first confirmed isolation of *L. monocytogenes* in sheep was made by Gill in 1929 (Gray and Killinger, 1966). However sporadic human cases were reported (Burn, 1936, Kaplan, 1945). Nyfeldt (1929) made the first confirmed isolation of *L. monocytogenes* from a human infection. The organism was referred to as *L. monocytogenes* var. *hominis*, as it was thought to be a unique variant of the animal pathogen. This name was later dropped when it became more widely recognised that *L. monocytogenes* was also a human pathogen (Gray and Killinger, 1966). Retrospectively organisms isolated from cases of listerial infection by many workers, and given various names such as *Bacterium hepatis*, *Corynebacterium infantisepticum*, *Listerella ovis*, *Listerella cunicula* and *Corynebacterium parvulum* were proposed as being of the genus (Gill, 1931, Hülphers, 1911, Kankschewa, 1923, Potel, 1951).

Taxonomy

Intergeneric taxonomy.

For many years the genus *Listeria* was monospecific containing only *L. monocytogenes* and was classified in the family *Corynebacteriaceae*, in both the sixth and seventh editions of Bergey's Manual of Determinative Bacteriology (Breed *et al.*, 1948, 1957). In the eighth edition (Buchanan and Gibbons, 1974) this position was altered the genus *Listeria* being listed with *Erysipelothrix* and *Caryophanon* as "genera of uncertain affiliation" in the section containing the family *Lactobacillaceae*. Removal from the family *Corynebacteriaceae* was due to the findings of studies of cell wall composition (Cummins and Harris, 1956, Schleifer and Kandler, 1971), lipid analysis (Kosaric and Carroll, 1971, Shaw, 1974, Tadyon and Carroll, 1971), nucleic acids (Stuart and Welshimer, 1974), and numerical taxonomy (Davis and Newton, 1969, Davis *et al.*, 1969, Stuart and Pease, 1972, Stuart and Welshimer, 1974) of the genus. Subsequent evidence from further numerical taxonomic (Feresu and Jones, 1988, Fiedler *et al.*, 1984, Jones, 1975, Wilkinson and Jones, 1977), and chemical studies (Collins and Jones, 1981, Kamisango *et al.*, 1982), reinforced this division of *Listeria* from the coryneforms. 16S rRNA cataloguing studies of *Listeria* (Stackebrandt *et al.*, 1985) confirmed the location of *Listeria* as a distinct taxon in the low G+C Gram positive branch of the *Bacillus*, *Lactobacillus* *Clostridium* group most closely related to *Brochothrix* (Ludwig *et al.*, 1984). Analysis of 16S rRNA reverse transcriptase sequences of the genus has indicated that it is not closely related to *Lactobacillus* and should not be included in this family (Collins *et al.*, 1991). A new family *Listeriaceae*, to include *Listeria* and the genus *Brochothrix* has been suggested by Collins *et al.*, (1991).

Intragenetic taxonomy.

Of the species listed in the genus *Listeria* in the eighth edition of Bergey's Manual of Systematic Bacteriology (Seeliger and Jones, 1986), the classification of *L.denitrificans* has since been amended. Numerical taxonomic, biochemical, serological, morphological, and nucleic acid studies have shown that this species does

not belong to the genus *Listeria* (Collins *et al.*, 1983, Espaze *et al.*, 1986, Fiedler and Seger, 1983, Fiedler *et al.*, 1984, Jones, 1975, Jones *et al.*, 1986, Stuart and Pease, 1972, Stuart and Welshimer, 1973, 1974, Wilkinson and Jones, 1977). Ribosomal nucleic acid studies have clarified the position of *L.denitrificans* which has now been classified in a new genus *Jonesia* as *J. denitrificans* (Rocourt *et al.*, 1987a).

The isolation of nonpathogenic, nonhaemolytic, and markedly β -haemolytic organisms subsequently assigned to the species *L. monocytogenes* rendered the classification of the genus *Listeria* inadequate. DNA-DNA hybridisation studies allowed the redefinition of the genus to include five species; only one of which now includes the type strain *L. monocytogenes* (*sensu stricto*). The other four species of the genus include those nonpathogenic, nonhaemolytic strains given the name *L. innocua* (Seeliger and Schoofs 1977, Seeliger 1981), the markedly β -haemolytic *L. ivanovii* first described by Ivanov in 1962 (Ivanow 1975), and two other species *L. seeligeri*, and *L. welshimeri* (Rocourt and Grimont, 1983). These five genomic groups show a high degree of DNA relatedness ~87% (Rocourt *et al.*, 1982), and similarity of phenotypic, biochemical, and total protein characteristics (Jones *et al.*, 1986, Lamont *et al.*, 1986, Rocourt and Catimel, 1985). Multilocus enzyme electrophoresis studies have recently confirmed this classification and resulted in the detection of two subspecies in *L. ivanovii*, *L. ivanovii* subsp. *ivanovii* and *L. ivanovii* subsp. *londoniensis*, which could be distinguished biochemically on their ability to ferment ribose and *N*-acetyl- β -D-mannoside (Boerlin *et al.*, 1992).

The classification of *L. grayi* and *L. murrayi* was revised as numerical taxonomic and chemical studies indicate a close relationship between these two species and the rest of the genus. Stuart and Welshimer (1974), proposed that a new genus "*Murraya*" to contain "*Murraya grayi* subspecies *grayi*" and "*Murraya grayi* subspecies *murrayi*" should be created. However, DNA-DNA hybridisation studies indicated a more distant relationship to the genus and a very close relationship between the two species themselves (Rocourt *et al.*, 1987a). Their relationship with the genus *Listeria* was resolved by 16S rRNA cataloguing which showed *L. murrayi* to be closely related to *L. monocytogenes* (Rocourt *et al.* 1987b). However, the distinction between these two

species has been disputed by some workers who consider *L. murrayi* to be a strain of *L. grayi*. (Feresu and Jones, 1988, Stuart and Welshimer, 1974). This view has been supported by 16S rRNA reverse transcriptase sequence analysis of the type strains of *L. murrayi* and *L. grayi* which exhibit a 99.6% similarity (Collins *et al.*, 1991). Furthermore, recent DNA-DNA hybridisation, multilocus enzyme electrophoresis, and rRNA restriction fragment length polymorphism studies have indicated that these two species should be considered members of a single species named *L. grayi* on grounds of priority (Rocourt *et al.*, 1992).

These advances in the taxonomy of the genus have identified two distinct lines of descent among the six species, one via *L. monocytogenes*, *L. innocua*, *L. seeligeri*, *L. welshimeri*, *L. ivanovii* and its subspecies, the second via *L. grayi* (Collins *et al.*, 1991, Rocourt *et al.*, 1982) (Table 1.1).

Characteristics of *L.monocytogenes* .

Listeria monocytogenes, the type species of the genus, exhibits a morphology and motility common to all the species of the genus. Colonies of *L.monocytogenes* on nutrient agar incubated for 24-36 hrs at 37°C are 0.5-1.5 mm in diameter, round, translucent, low convex with a finely textured surface and entire margins. When grown on clear, solid medium, such as tryptose agar for 24-48h and viewed with a dissecting microscope using obliquely transmitted light (Henry, 1933), the colonies adopt a blue-green sheen, a characteristic often used for identification purposes. The cells are round ended, short, Gram-positive rods, 0.4-0.5 µm in diameter, 1-2 µm in length, occurring singly or in short chains and under microscopic examination are non-acid fast. No capsules or spores are seen.

The temperature range over which the *L. monocytogenes* will multiply is between 1°C and 45°C (Seeliger and Jones, 1986), with an optimum temperature of between 30-37°C. Strain variation may be evident at the higher temperatures, and the highest working temperature at which many strains will multiply is 41-42°C. Growth at low

Table 1.1. The six species of the genus *Listeria*, their subspecies, and the two lines of taxonomic descent.

	Species	Subspecies
	<i>L. monocytogenes</i>	
Line one	<i>L. ivanovii</i>	Subsp. <i>ivanovii</i> , Subsp. <i>londoniensis</i>
	<i>L. seeligeri</i>	
	<i>L. innocua</i>	
	<i>L. welshimeri</i>	
Line two	<i>L. grayi</i>	

temperatures may be greatly affected by the medium and pH in which it occurs. This ability to initiate growth at low temperatures allows the isolation of *L. monocytogenes* from mixed cultures by cold enrichment. This ability also poses a threat to the food industry as storage of products at low temperatures may still allow *L. monocytogenes* to multiply to numbers sufficient to cause disease. Flagella are expressed only when cultured at 20-25°C, and are predominately peritrichous. Tumbling motility is characteristic of the genus, and rotating movements may be followed by periods of rest. At temperatures over 30°C reversible damage of the flagella occurs and motility is lost (Seeliger, 1961).

Listeria monocytogenes is reported to grow at pH values of 5.6-9.6, in Bergey's Manual of Systematic Bacteriology (Seeliger and Jones, 1986), with maximum growth at neutral to slightly alkaline pH values. The minimum pH at which *L. monocytogenes* will grow has since been noted to be below pH 5.0 when near optimum incubation temperatures were applied and sufficient time allowed to overcome the extended lag phase (Farber *et al.*, 1989). Under these conditions growth was reported at a pH of 4.4-4.6 (Farber *et al.*, 1989).

Extended survival of listeriae at a wide range of salt concentrations at ambient temperatures has been observed, with persistence for at least 150 days in pure salt (Von Sielaff, 1968). In Bergey's Manual of Systematic Bacteriology (Seeliger and Jones, 1986) *L. monocytogenes* is reported to grow in nutrient broth supplemented with up to 10% NaCl (w/v), although strain variation is again observed, salt tolerant strains of *L. monocytogenes* having been seen to grow in brain heart infusion broth containing up to 12% salt at a pH of 5.0 (Ryser and Marth, 1991). Exposure to high salt concentrations leads to various morphological changes. Cells may become elongated or filamentous as cell division is inhibited without inhibition of cell growth (Ryser and Marth, 1991). Halotolerance may be increased when the organisms are present in salted foods, particularly in meat, and the survival of *L. monocytogenes* has been detected in infected beef immersed in a solution of 22% NaCl after 100 days storage at 15-20°C (Von Sielaff, 1968).

Biochemically *L.monocytogenes* is catalase positive, Voges-Proskauer test positive, aesculin hydrolysed, alkaline phosphatase-positive, urease-negative, and oxidase-negative (Rocourt and Catimel, 1985, Seeliger and Jones, 1986). The genus is facultatively anaerobic and produces acid without gas from glucose and certain other carbohydrates. The characteristic production of acid from L-rhamnose but not from D-mannitol, D-xylose or α -methyl-D-mannoside by *L.monocytogenes*, can be used to distinguish it from the other species of the genus (Table 1.2). Other characteristics that can aid differentiation between species of the listeriae include haemolysis, the reduction of nitrates to nitrites, and the CAMP reaction (Christie *et al.*, 1944). When streaked onto plates containing 5% (v/v) horse blood *L. monocytogenes* produces narrow zones of β -haemolysis, while *L. seeligeri* colonies produce very weak β -haemolysis which may only be noticeable under the colonies on their removal. *L. ivanovii* produces broad zones of haemolysis on blood agar which may be banded, consisting of an inner zone of clear β -type and an outer cloudy zone suggestive of phospholipase activity. The other three species of *Listeria* are not haemolytic, although weak contact haemolysis beneath some strains of *L. innocua* may be observed.

The CAMP test used to differentiate between species of *Listeria* is performed by streaking cultures of *Staphylococcus aureus* and *Rhodococcus equi* vertically on a plate of sheep blood agar. *Listeria* test cultures are then streaked at right angles to the other cultures, and after 48 hrs incubation at 35°C, β -haemolysis produced by *L. monocytogenes* and *L. seeligeri* is enhanced by the beta-toxin of *Staphylococcus aureus*. The haemolysis of *L. ivanovii* is not enhanced by the beta-toxin of *S. aureus*, but is enhanced by an exosubstance of *Rhodococcus equi*. The other species of *Listeria* do not produce any reaction in the CAMP test (Table 1.2).

The species *L. monocytogenes* can be divided serologically into 16 serotypes on the basis of their somatic (O) and flagella (H) antigens (Table 1.3). Serotypes 1, 3, and 4 were differentiated on the basis of their heat stable O antigens, whereas serotype 2 was identified on the basis of a unique heat labile H antigen. Subsequently Seeliger (1961)

Table 1.2. Abbreviated Biochemical Identification of *Listeria* species.

<i>Listeria</i> species	β -haemolysis	CAMP test		Production of acid from	
		<i>S. aureus</i> (a)	<i>R. equi</i> (b)	Rhamnose	Xylose
<i>L. monocytogenes</i>	+	+	-	+	-
<i>L. ivanovii</i>	+	-	+	-	+
<i>L. innocua</i>	-	-	-	v	-
<i>L. seeligeri</i>	v	+	-	-	v
<i>L. welshimeri</i>	-	-	-	-	+
<i>L. grayi</i>	-	ND	ND	-	-

(a) *S. aureus* NCTC 1803

(b) *R. equi* NCTC 1621

ND; not determined

v; variable

adapted from Ryser and Marth, 1991.

Table 1.3. Serotypes of *Listeria* spp.

<i>Listeria</i> spp.	Serotype	Somatic (O) antigenic structure	Flagellar (H) antigenic structure
<i>L. monocytogenes</i>	1/2a	I II (III)*	AB
	1/2b	I II (III)	ABC
	1/2c	I II (III)	BD
	3a	II (III) IV	AB
	3b	II (III) IV (XII) (XIII)	ABC
	3c	II (III) IV (XII) (XIII)	BD
	4a	(III) (V) VII IX	ABC
	4ab	(III) V VI VII IX X	ABC
	4b	(III) V VI	ABC
	4c	(III) V VI	ABC
	4d	(III) V VI VIII	ABC
	4e	(III) V VI (VIII) (IX)	ABC
	7	(III) XII XIII	ABC
<i>L. ivanovii</i>	5	(III) (V) VI (VIII) X	ABC
<i>L. innocua</i>	6a	(III) V (VI) (VII) (IX) XV	ABC
	6b	(III) (V) (VI) (VII) IX X XI	ABC
<i>L. grayi</i>		(III) XII XIV [XVI]	E
<i>L. grayi</i> subspp. <i>murrayi</i>		(III) XII XIV [XVII]	E

L. seeligeri cannot be differentiated serologically from *L. monocytogenes*.

* indicates not always present.

L. welshimeri cannot be differentiated serologically from *L. innocua* serotype 6b.

Adapted from Ryser and Marth (1991).

divided serotype 4 into serotypes 4a and 4b on the basis of additional O antigens and Donker-Voet extended this classification to include serotypes 4c, 4d, and 4e (Donker-Voet, 1965). Further investigation revealed slight differences in the H antigens of some cultures of serotypes 1, and 3, and this led to the creation of serotypes 1a and 3a (see Ryser and Marth, 1991). Table 1.3 indicates the serotypes of *Listeria* spp. currently recognised by their O and H antigens. *L. innocua*, *L. ivanovii*, *L. seeligeri*, and *L. welshimeri* often share somatic antigens with *L. monocytogenes*, but *L. grayi* is serologically distinct (Ralovich, 1984). 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 (McLauchlin 1987, Seeliger and Hohne, 1979).

Bacteriophages specific for *Listeria* spp. were initially discovered in 1945 (Schultz, 1945) and several groups have studied the possibility of phage-typing of *L. monocytogenes* (Audurier and Martin, 1989, Audurier *et al.*, 1984, McLauchlin *et al.*, 1986, Rocourt *et al.*, 1985). These studies were hampered by the lack of phages available, the overall percentage of *L. monocytogenes* strains typeable ranging from 52-78%. Recently a new set of phages derived from both environmental and clinical lysogenic strains has been described (Loessner *et al.*, 1990). These allow the typing of more than 90% of serotype 1/2 strains, and the majority of isolates of serotypes 4a, ab, c, d, and e, although strains of serotype 3 and 7 appear to be resistant. There is currently an international phage-typing system for *L. monocytogenes*, and an International centre for *Listeria* phage typing has been established at the Pasteur Institute (Jones and Seeliger, 1987).

Isoenzyme typing studies have identified subgroups within the serotypes, and forty five electrophoretic types were identified by Piffaretti *et al.* (1989), when strains of *L. monocytogenes* were analysed for 16 genetic loci encoding metabolic enzymes. These electrophoretic types could be further divided into two clusters; one containing *L. monocytogenes* strains of serotypes 1/2a and 1/2c, and the other serotypes 1/2b, 4a, and 4b (Piffaretti *et al.*, 1989). Similar results were reported by Bibb *et al.* (1990), although these workers identified 56 electrophoretic types, one cluster containing all *L. monocytogenes* strains of flagella H antigen type a, and another containing all *L.*

monocytogenes strains of flagella H antigen type b examined. In this study Bibb *et al.*, (1990) did not identify any epidemiological trend in electrophoretic type, but considered one cluster containing *L. monocytogenes* 4b isolates to represent a particularly virulent group. Recently multilocus enzyme electrophoresis studies by Boerlin *et al.*, (1991, 1992) using 18 enzyme loci confirmed these findings and showed that strains of *L. ivanovii* could be divided into two main genomic groups represented by the subspp. *L.ivanovii ivanovii* and *L.ivanovii londoniensis*, previously described.

Isolation.

Although *L. monocytogenes* is a non-fastidious organism that can grow on common bacteriological media, primary isolation from contaminated specimens often proves difficult (Gray and Killinger, 1966, Seeliger 1961). Cold enrichment, warm enrichment, and / or selective procedures can be used in conjunction with direct plating to isolate listeriae from clinical and environmental samples.

Cold enrichment isolation was first described by Gray *et al.* (1948) as a method for the isolation of *L. monocytogenes* from heavily contaminated samples, and is a successful though lengthy procedure. The method is based on the ability of listeriae to grow at 4°C samples are refrigerated in non-selective media for a prolonged period of up to six months. A number of enrichment procedures and selective media have been devised to reduce the time of analysis from months to less than one week. McBride and Girard (1960) developed a selective agar medium which, coupled with the oblique illumination of the colonies suggested by Henry (1933), contributed to successful isolations of *L. monocytogenes* from food. Various selective agents including potassium tellurite, nalidixic acid, and acriflavine have been proposed (Donnelly, 1988). Selective media incorporating these agents include the widely used Oxford and PALCAM media (Curtis *et al.*, 1989, Van Netten *et al.*, 1988). Conventional cultural methods have also been developed (Cassidy and Brackett, 1989, Donnelly, 1990), and alternative methods involving monoclonal antibodies and DNA probes, are reported (Datta *et al.*, 1987, Durham *et al.*, 1990, Farber *et al.*, 1988, Köhler *et al.*, 1990, Peterkin *et al.*, 1991). Detailed accounts of techniques for the isolation of *L.*

monocytogenes and *Listeria* species are given by Ralovich (1984), Prentice and Neaves (1988), and Ryser and Marth (1991).

Habitats.

Listeriae have been isolated from many diverse sources including at least 42 species of mammal in addition to man, 17 species of bird, insects, and from fish and shell fish (Armstrong, 1985, Fenlon, 1985, Fuchs and Surendran, 1989, Gray and Killinger, 1966). The geographical regions from which listeriae have been isolated are also diverse ranging from the Arctic to Africa (Ryser and Marth, 1991). After the initial description of the genus *Listeria* associated with a clinical infection of mammals, it was widely regarded as zoonotic, but in the mid 1960s the organism was isolated from other environmental sources such as silage, soil, waste water, effluents, rivers, and canals (Rocourt and Seeliger 1985, Watkins and Sleath, 1981, Welshimer, 1981). These isolates and certain phenotypic characteristics suggested that the listeriae were saprophytic rather than zoonotic in nature (Weiss and Seeliger, 1975). Although it has been suggested that their presence in these environments was due to faecal contamination from animals and birds (Donker-Voet, 1965, Fenlon, 1985) the listeriae are now generally regarded as saprophytes (Ryser and Marth, 1991, Welshimer and Donker-Voet, 1971).

The listeriae may be considered as an environmental contaminant in soil rich in organic material, whose primary means of transmission to man and animals appears to be contaminated foodstuffs. A link between *Listeria* infection and contaminated foodstuffs was first suggested by Pirie (1927), who succeeded in establishing infection in gerbils and mice by feeding them food soaked in emulsified culture, and histological studies of these animals revealed lesions in the small intestine indicating this area as the portal of entry. The transmission of *L. monocytogenes* to humans through food was suggested as early as 1915, when an outbreak of a "listeriosis-like" disease, linked to a food-borne route of infection, occurred in Australia (Atkinson, 1917). A link between outbreaks of listeriosis in cattle and the feeding of silage had been recognised for many years, before Gray (1960) supplied experimental evidence for this, isolating *L. monocytogenes* from poor quality silage with a pH of greater than

5.6. The findings of Gray were subsequently confirmed by several workers (Blenden *et al.*, 1966, Palsson, 1963) who detected *L. monocytogenes* in silage, in “numbers sufficient to cause disease”. Fenlon (1986) describe the increased incidence of listeriosis among animals when poor quality, high pH silage is used as fodder in late winter and spring.

Although in the past outbreaks of human listeriosis were thought to be due to the consumption of contaminated foodstuffs no definite source was established (Gray and Killinger, 1966, Seeliger, 1961). Several recent major outbreaks have been well documented and a major vehicle of infection has been reported to be contaminated food (see, Gellin and Broome, 1989, Lund, 1990). Sporadic outbreaks have occurred around the world. Foods implicated included; coleslaw (made from cabbage grown on soil fertilised with manure from an infected flock, Schlech *et al.*, 1983), milk (contaminated post-pasteurisation, Fleming *et al.*, 1985), soft cheese (prepared using unpasteurised milk, Linnan *et al.*, 1988), a Swiss cheese (ripened in contaminated cellars, Malinverni *et al.*, 1985), and in one case cook-chilled chicken (Gilbert *et al.*, 1989).

Listeria monocytogenes has been isolated from many foods for human consumption. Surveys of meats both cooked and uncooked, have indicated that the listeriae are able to survive on the surface of meat and in sarcoplasmic protein solutions from meat (Johnson *et al.*, 1986, Khan *et al.*, 1972, Nicholas, 1985). Poultry has been reported to be a major source of human listeriosis. Studies of fresh chicken carcasses in Britain indicated a contamination level of 33%-60% (Kwantes and Isaac, 1971, Pini and Gilbert, 1988). Listeriae have been isolated in low numbers from many different vegetables and processed vegetable products such as coleslaw salad (Heisick *et al.*, 1989, Schlech, 1984). Milk and dairy products have also been implicated as an important vehicle for listeriosis. A survey of raw milk in 1986 (Hayes *et al.*, 1986) indicated an isolation rate of 12% for *L.monocytogenes*, and growth has been reported in chocolate flavour milk, skimmed milk and cream (Rosenow and Marth, 1986). Reports that *L.monocytogenes* could survive pasteurisation in milk (Bearn and Girard, 1958) were refuted by more recent work in which exact pasteurisation

conditions were simulated (Twedt, 1986). The listeriae can survive the manufacture, ripening and storage of cottage, cheddar, and soft cheeses, and have been responsible for outbreaks of listeriosis linked with the consumption of these products (Linnan *et al.*, 1988, Ryser and Marth, 1986a, 1986b). The publicity generated by outbreaks of listeriosis has led to much greater awareness of food born disease and food hygiene in general.

Although transmission in food appears to be the most significant means for the dissemination of listeriosis, the extent of such transmission is not known, and other routes certainly exist. Infection can occur via respiratory, cutaneous and ocular routes, the death of a farmer from bronchopneumonia and meningitis following probable inhalation of contaminated faecal matter from sheep was reported by Ödegaard *et al.*, (1952). Furthermore, cases of pulmonary listeriosis in cattle have been traced to contaminated feed (Wohler and Baugh, 1983). Cutaneous listeriosis can be acquired by handling infected materials, and it has been reported that a veterinarian developed cutaneous listeric lesions after delivering an aborted foetus from an infected cow (Owen *et al.*, 1960). The ocular route of infection is well documented (Ralovich, 1984), and cases of conjunctivitis have been reported after accidental contamination of the eyes in the laboratory and after handling infected chickens (see Seeliger, 1961).

Route of infection.

Gastrointestinal symptoms are often associated with the onset of listeriosis, and evidence from food related outbreaks suggests that the intestine is an important natural route of infection. Conjunctival, nasal, and respiratory routes of infection have infrequently been noted (Anton, 1934, Lefford, *et al.*, 1978, Osebold and Inouye, 1954 a & b), but experimental evidence demonstrates that when high numbers of *L. monocytogenes* are administered orally to mice a systemic infection is developed (Audurier *et al.*, 1980, MacDonald and Carter, 1980). Unsubstantiated claims have been made that infection of the intestine is established via lesions in the mucosa (Armstrong, 1985). Contrary to this hypothesis is the observation that in an animal model of listerial enteritis intact epithelium can be invaded by the bacterium (Racz *et al.*, 1972). However, invasion via the Payer's patches rather than the intestinal

epithelium has also been reported (MacDonald and Carter, 1980). *L. monocytogenes* has been well documented in its ability to invade enterocyte-like Caco-2 cell lines by inducing phagocytosis (Gaillard *et al.*, 1987). The presence of a flaw in the epithelial mucosa does not therefore appear to be essential for invasion and such lesions may be a characteristic of listerial infection rather than the portal of entry (Pirie, 1927). Several predisposing factors may coincide with the ingestion of *L. monocytogenes* for an infection to ensue, and reports suggest that stool carriage is common in many asymptomatic individuals (Kamplmacher and van Noorle Jansen, 1972). Predisposing factors may include concurrent infection of the gastrointestinal (g.i.) tract by other organisms, altered composition of the normal gut flora, a reduction in the pH of the acids and secretions of the g.i. tract, and compromised immune status of the host (Armstrong, 1985, Gellin and Broome, 1989, Ho *et al.*, 1986, Zachar and Savage, 1979). Haemolytic strains of *L. monocytogenes* are able to translocate across the mucosal epithelium while non-haemolytic strains can not reflecting the importance of virulence factors to successful invasion (Roll and Czuprynski, 1990). Studies which have employed the intraperitoneal (i.p.), or intragastric (i.g.) routes of inoculation suggest that infection occurs more rapidly when the bacterium is introduced i.g. (Audurier *et al.*, 1980, Miller and Burns, 1970, Pine *et al.*, 1990). Although i.g. feeding experiments have not conclusively demonstrated that the carrier medium of the inoculum, for example contaminated milk, is of importance to the establishment of infection (Pine, *et al.*, 1990).

The infectious dose of *Listeria* is not known and most studies of listeric infection have employed a rodent model system in which the dose is administered intravenously (i.v.). Three stages are seen during the course of listerial infection after i.v. inoculation of mice (Mainou-Fowler *et al.*, 1988, Mitsuyama *et al.*, 1978). Within 6 hrs, 90% of the inoculum is cleared from the blood by liver Kupffer cells. Over the next 2-3 days a systemic infection develops, surviving organisms multiplying rapidly in the liver and spleen. Replication occurs even though polymorphonuclear neutrophils accumulate at the focus of infection followed closely by mononuclear phagocytes (Mandel and Cheers, 1980). If the infection proves to be sublethal immune T-cells overcome the infection forming granulomas rich in activated macrophages (Mainou-Fowler *et al.* ,

1988). Systemic *Listeria* infections may manifest themselves as many different symptoms in man and animals.

Epidemiology

The incidence of listeriosis worldwide is not known, and may be higher than current data suggests (Gellin and Broome, 1989). Clinical features of listeriosis are nonspecific especially when mild and may go undetected or unreported as statutory notification is required in only a few countries. Estimates of the incidence differ ranging from 1-12 per million of the population (Gellin and Broome, 1989, Jones, 1990) however a substantial increase in the number of cases has been noted over the last 20 years (Broome *et al.*, 1990, Campbell, 1990).

Clinical manifestations of human listeriosis.

The symptoms of human listeriosis vary widely but fall loosely into three groups, bacteraemia, meningitis, and pneumonia. Listeriosis, although rare among the immunocompetent, has a high mortality rate of up to 44% in adults and 50% in neonates (Farber and Peterkin, 1991).

Pregnant women are particularly susceptible to *Listeria* infection especially in the third trimester and this is often presented as a mild influenza-like or gastrointestinal illness. These symptoms represent a bacteraemia which is rarely life threatening to the woman but during which the fetus may become infected, resulting in septic abortion, still birth or delivery of an infected infant. Listeriosis in pregnant women does not always lead to fetal infection and if diagnosed and treated promptly the prospects for the neonate are much improved (Bucher *et al.*, 1989). Recurrent abortions and neonatal deaths have been claimed to be associated with the carriage of *L. monocytogenes* although experimental investigations have not been performed to support this (Gray, 1960, Saxbe, 1972).

Neonatal listeriosis may be early-onset (symptoms apparent at birth or shortly afterwards), or late-onset (symptoms apparent several days or weeks after birth). Early-onset neonatal listeriosis is a severe disease characterised by pneumonia, bacteraemia, and widely dissipated abscesses, which indicates intrauterine infection.

Neonates which appear healthy at birth may develop late-onset listeriosis due to peripartum contamination in the birth canal, exposure to fomites, or nosocomial transmission. Meningitis is the usual manifestation of late-onset neonatal listeriosis and the prognosis is much better in these cases (Gellin and Broome, 1989, McLauchlin, 1990).

Meningitis is the main clinical feature of listeriosis in apparently immunocompetent patients but less common infections, such as meningoencephalitis, abscesses of the brain stem and spinal cord, may also occur (Gellin and Broome, 1989, Gray and Killinger, 1966, Neiman and Lorber, 1980). These infections of the central nervous system present a complex spectrum of symptoms from personality changes to tremors, seizures, and coma (Neiman and Lorber, 1980). In these types of listeriosis the bacterium may be isolated from the cerebrospinal fluid and blood of the patient.

Listeric bacteraemia is very like other bacteraemias, the main indication being the presence of a fever. Sufferers also often display nonspecific symptoms such as nausea, vomiting, diarrhoea, muscle cramps, and fatigue. The presence of high numbers of the bacterium in the blood stream may lead to endocarditis usually in individuals who have existing cardiac damage (Farber and Peterkin, 1991, Gellin and Broome, 1989).

Infrequently cutaneous exposure to *L. monocytogenes* may result in localised skin lesions and abscesses, without any systemic illness. Cases of cutaneous infection have been recorded in those who come into contact with infected material including veterinarians, farmers, and slaughterhouse workers. Conjunctivitis due to listeric infection has also been reported in poultry workers and after accidental laboratory infection which may occasionally result in meningitis (Seeliger, 1961, Seeliger and Finger, 1976, Gray and Killinger, 1966, Gellin and Broome, 1989).

Immunocompromised individuals such as those undergoing immunosuppressive treatment, cancer patients, transplant recipients, the elderly and acquired immune deficiency syndrome sufferers are especially susceptible to listeriosis (Louria *et al.*,

1967, Mascola *et al.*, 1988, Neiman and Lorber, 1980, Stamm *et al.*, 1982). The spectrum of illness among these groups is diverse as focal infections occur due to dissemination of the organism during the bacteraemic phase of the disease. Reports of focal infections include septic arthritis, osteomyelitis, endophthalmitis, peritonitis, cholecystitis, liver granulomas, and pleuropulmonary infection (Farber and Peterkin, 1991).

Clinical manifestations of animal listeriosis.

Animal listeriosis does not vary greatly from human listeriosis, exhibiting the same three main symptomatic groups. Listeriosis in pregnant animals results in stillbirth and abortion, and usually occurs in sporadic outbreaks among cattle and sheep although outbreaks have been recorded among pigs (Prentice and Neaves, 1988). *L. monocytogenes* has been isolated from the faeces and milk of infected cattle (Løken *et al.*, 1982, Lovett *et al.*, 1987). Transmission in such sporadic outbreaks is thought to be due largely to faecal contamination (Prentice and Neaves, 1988). Bacteraemic infections in animals are most common in the first few weeks of life often due to postpartum contamination and symptoms include anorexia, pyrexia, and diarrhoea. Bacteraemia is most typical in new born lambs, while this manifestation is uncommon in calves and piglets (Gitter, 1985, Prentice and Neaves, 1988).

The most prevalent form of listeriosis among domestic animals is encephalitis, and the resulting brain lesions result in the typical "circling disease": facial paralysis, drooping ears, salivation, and walking in circles. *Listeria* encephalitis is often severe in sheep leading to death within 4-48 hrs of onset, and mortality may be up to 10%. In cattle encephalitis is less acute, survival for up to 14 days and spontaneous recovery being common. The bacteria are thought to move to the meninges via the cranial nerve, encephalitis having an incubation period of between 14 and 40 days (Low and Donachie, 1989). Entry of *L. monocytogenes* via the trigeminal nerve during tooth cutting has been postulated, because this would occur at the time when ovine listeriosis is most prevalent. Experimental infections via the tooth pulp cavity have not confirmed this theory, the incubation period of the encephalitis produced being only 6 days (Barlow and McGorum, 1985).

Other less common forms of animal listeriosis include infectious bovine keratitis, and bovine mastitis in which milk may be contaminated for 3 months after the symptoms have ceased (Gitter, 1985). Contaminated milk is a well documented source of human listeriosis (Fleming *et al.*, 1985, Linnan *et al.*, 1988, Lovett *et al.*, 1987).

Epidemiology of animal listeriosis.

Little data is available on the epidemiology of animal listeriosis in the UK most studies having been undertaken in Scandinavia, Eastern Europe, and the former Soviet Union (Bakulov, 1989, Grønstøl, 1979, Mencíková *et al.*, 1989). In Britain the incidence of listeriosis in sheep is best documented, and outbreaks occur most often in the North of England and Scotland (Gitter *et al.*, 1986, Wilesmith and Gitter, 1987). Reports over the last 20 years have noted a rise in the number of outbreaks of ovine listeriosis in Britain, Scandinavia, and Europe (Grønstøl, 1979, Ivanov and Masalski, 1979, Kamplmacher and van Noorle Jansen, 1977, Wilesmith and Gitter, 1987). The number of cases of ovine listeriosis reported in 1980 was 101 and this figure had risen to 480 by 1986, although as listeriosis is not a notifiable disease these figures do not represent the true incidence (Low and Donachie, 1989).

A change in the pattern of these outbreaks has also been observed the previous sporadic incidence of listeriosis has changed to a pattern of epidemic listeriosis (Gitter *et al.*, 1986, Wilesmith and Gitter, 1987). In these outbreaks abortions and encephalitis now appear together in the same flock which seldom occurred in the past (Gitter *et al.*, 1986, Low and Renton, 1985). The predominant *L. monocytogenes* serovar responsible for outbreaks of animal listeriosis is 1/2a as it is in humans, although serovar 4b is common in animal listeriosis abroad (Farber *et al.*, 1991, Ivanov 1985, Wilesmith and Gitter, 1986). *L. ivanovii* serovar 5 is not the main cause of listeriosis in Britain but does show preferential pathogenicity in sheep, being the major cause of ovine abortion in other countries (Ivanov, 1975).

There are many reports which associate the feeding of silage with listeriosis in sheep and cattle, and an increased incidence of listeriosis is reported during the seasons when silage feeding is most common (Fenlon, 1986, Gitter *et al.*, 1986, Grønstøl, 1980).

Evidence has been provided to link the spoilage of silage to outbreaks of listeriosis and the feeding of silage has increased with the number of outbreaks (Gitter *et al.*, 1986, Low and Renton, 1985, Wilesmith and Gitter, 1986). The use of the "big bale" method of silage preparation has also increased. Fermentation in this method takes place in large plastic bags rather than in the traditional silage clamp. The silage is more prone to spoilage in this system because damage of the bags allows aerobic conditions to become established, the pH to rise and contaminating *Listeria* to multiply (Gitter *et al.*, 1986, Low and Renton, 1985, Wilesmith and Gitter, 1986). Silage has been postulated to have an immunosuppressive effect on ewes (Grønstøl, 1980), although this has not been confirmed in other studies (Gitter *et al.*, 1986) and the exact epidemiological importance of silage in listeriosis is not known.

The carriage rate among domestic animals is considered to be between 1 and 5%, although rates of as high as 29% have been reported (Farber and Peterkin, 1991, Kamplmacher and van Noorle Jansen, 1977). More recently the use of modern isolation techniques has indicated that carriage rates may be higher Skovgaard and Morgan (1988) reported the isolation of *Listeria* species from 68% of bovine faeces examined from seven dairy herds.

Animal listeriosis is economically important because of the financial loss incurred due to the disease. The agricultural economy of Australia in the 1970s suffered the loss of approximately 1,000,000 sheep from listeric infection. In Britain, Low and Renton (1985) describe an outbreak of listeriosis in a housed flock in which 21 ewes and 88 lambs were lost. The financial cost to the farmer was calculated to be in excess of £5000 (as valued in 1985). Another important aspect of animal listeriosis is the possible contamination of raw foods and food production lines with *Listeria* from animal faeces and products. A reduction in the numbers of animal carriers would reduce this risk, and there are two possible approaches to achieve this. These approaches are; 1. the improvement of animal housing and feed, hygiene and quality, and 2. the effective bacteriological control of *Listeria* by active immunisation.

Immune response to *Listeria monocytogenes*.

Immunity to *L.monocytogenes* includes a humoral as well as a cell mediated response, although this is not as well defined. Studies suggest that while a strong antibody response is made to a spectrum of cell surface proteins and listeriolysin O these do not confer protection against infection (Barry *et al.*, 1992, Beattie *et al.*, 1990, Issekutz, *et al.*, 1984, Njoki-obi and Osebold 1962). Humans defective in their production of immunoglobulins do not have an increased risk of infection and no protection is given to animals inoculated with immune serum (Miki and Mackaness, 1964). Greater susceptibility to infection during pregnancy and in neonates may be due to the low levels of immunoglobulin (Ig) M present at this time. Immunoregulation at the placenta may lead to a local IgM deficiency and as the antibody response to *Listeria* is mainly IgM and does not appear to switch to IgG in the normal fashion perinatal infections can occur (Redine and Lu, 1987). Opsonisation is reported to be important in the host response to listerial infection (Bortolussi *et al.*, 1986). In neonates there is only low classical complement activity and also low levels of IgM which carries a complement binding site and is important in complement activation, and this may predispose members of this age group to infection (Bortolussi *et al.*, 1986, Issekutz, *et al.*, 1984).

The ability of *L.monocytogenes* to move from cell to cell without leaving the cytoplasm provides a biological explanation for the lack of protection offered by antibody, and the requirement of cell mediated immunity to listerial infection. T-cell mediated immunity is of primary importance in resistance to *L.monocytogenes* and recovery from infection is associated with the development of acquired cellular resistance (Hahn and Kaufmann 1981, Kaufmann 1984, Lane and Unane 1972, Mackaness, 1969). Evidence for this includes the finding that resistance to *L.monocytogenes* infection can be transferred adoptively by lymphoid cells and the association of listeriosis with immunodeficient states (Gellin and Broome, 1989, Mackaness, 1969). The involvement of T-cell subsets L3T4+ and Lyt2+ in immunity to *L. monocytogenes* has been demonstrated by Kaufmann (1984, *et al.*, 1979) and verified by the use of *in vitro* stimulated T-cell subsets (Bishop and Hinrichs, 1987). It has also been established that *L. monocytogenes* infection elicits the induction of CD4⁺ and CD8⁺ T cells. Lymphokines have been implicated in the T-cell mediated

activation of mononuclear phagocytes in cellular resistance to *L. monocytogenes*. CD4⁺ T cells act primarily through the secretion of lymphokines (Hahn, 1983, Hahn and Kaufmann 1981, Magee and Wing, 1988). The action of lymphokines γ interferon and interleukins 2, 3, and 4, regulated by prostaglandins, has been observed to be important in anti-listerial resistance (Denis and Gregg 1990, Jacobs and Murray, 1986, Kaufmann *et al.*, 1983, Magee and Wing, 1989, Petit *et al.*, 1985).

Pathogenesis.

The current body of information on the basis of listerial pathogenicity is far from complete, but stems from the fundamental work of Mackaness (1962). Mackaness demonstrated that *L. monocytogenes* multiplies within macrophages, thus evading the host immune response. 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. To survive and replicate in cells *L. monocytogenes* must possess an array of factors that permit invasion and assist in evasion of immune defences. Suggested virulence factors include the production of haemolysin, lipases, cell wall components, extracellular invasion proteins and the enzymes catalase and superoxide dismutase (see Chakraborty and Goebel, 1988, Goebel *et al.*, 1991, Portnoy *et al.*, 1992). The application of gene cloning techniques and transposon mutagenesis in recent years has allowed the identification and characterisation of these determinants of listerial pathogenicity at the molecular level.

Haemolysin.

All pathogenic strains of *L. monocytogenes* are haemolytic although there is strain variation in the degree of haemolytic activity. The level of haemolysin production is not directly proportional to the severity of infections in mice (Kathariou *et al.*, 1988). The haemolysin (listeriolysin O) produced by *L. monocytogenes* is a sulfhydryl activated protein of 59 kilodaltons (kd) which is of the family of cholesterol binding cytolysins characterised by streptolysin O (SLO) (Gaillard *et al.*, 1987 Njoki-Obi, *et al.*, 1963, Parisius *et al.*, 1986). Listeriolysin O is antigenically related to SLO. When cloned and sequenced the *lisA* gene which encodes listeriolysin O, was found to share a

strong homology at the protein level with both SLO and pneumolysin (Mengaud *et al.*, 1987, 1988, Vicente *et al.*, 1985). Transposon mutagenesis studies have demonstrated listeriolysin to be essential to the virulence of *L. monocytogenes*. Nonhaemolytic transposon mutants are avirulent (Cossart *et al.*, 1989, Gaillard *et al.*, 1987, Kathariou *et al.*, 1987). During intracellular growth a major part appears to be played by listeriolysin which binds to the membrane cholesterol of phagocytic vacuoles and disrupting them allows the escape of the organism into the cytoplasm where multiplication takes place (Gaillard *et al.*, 1987). The release of the organism from the phagocytic vacuoles does not take place when cells are infected with nonhaemolytic transposon mutants and little multiplication of these mutants can be observed intracellularly (Cossart *et al.*, 1989, Portnoy *et al.*, 1988). The disruption of the phagocytic vacuole allows the bacteria to evade the action of phagosomal enzymes, and makes available cell components and Fe^{3+} for bacterial iron acquisition. The growth of *L. monocytogenes* is enhanced under iron rich conditions (Cowart, 1987, Cowart and Foster, 1985, Portnoy *et al.*, 1988), and the haemolysin in this event acts indirectly as a growth factor increasing lysis of erythrocytes as a source of iron (Cowart and Foster, 1985).

Listeriolysin O has also been shown to inhibit macrophage mediated antigen presentation and it has been suggested that this ability in haemolysin producing strains of *Listeria* contributes to their virulence (Cluff and Ziegler, 1987). Peptides derived from listeriolysin O have been reported to be major targets for anti-listerial CD8^+ T cells (Bouwer *et al.* 1992). Furthermore, a nonapeptide of listeriolysin O from amino acid 91-99 has been described which induces significant protection against *L. monocytogenes* infection in H-2^d mice (Harty and Bevan, 1992). This protection was mediated by CD8^+ T cells specific for this nonapeptide bound to the H-2K^d molecule (Harty and Bevan, 1992).

Growth of *L. monocytogenes* at 4°C has been suggested to enhance production of haemolysin. Increased virulence has also been reported under these conditions (see Gray and Killinger, 1966, Wood and Woodbine, 1979), with serious implications for

the refrigerated storage of food. Further investigations have verified that the virulence of *L. monocytogenes* is increased at 4°C for intravenously but not orally infected mice (Czuprynski, *et al.*, 1989a), and that this effect is dose dependent (Stephens *et al.*, 1991). Neither study supported claims of increased haemolysin production under low temperature conditions. A recent study of the expression of a number of *L. monocytogenes* virulence genes, including *lisA* which encodes listeriolysin O, reported them to be thermoregulated by the transcriptional activator *prfA* (Leimeister-Wächter *et al.*, 1992). The expression of *lisA* as well several other virulence genes were found to be coordinately repressed at lowered temperatures of growth (Leimeister-Wächter *et al.*, 1992). Increased virulence of *L. monocytogenes* after low temperature growth may be associated with the synthesis of stress proteins, at least five of which have been demonstrated to be coinduced with haemolysin under stress conditions such as temperature-shock, and in the intracellular environment (Sokolovic *et al.*, 1990).

Recently workers in Denmark have studied the sequences of the *lisA* gene from 175 isolates of *L. monocytogenes* and found that they could be put into two main groups (Rasmussen *et al.*, 1991). These two groups appear to correspond to the two electrophoretic groups noted by Bibb *et al.* (1990) and it is suggested that there may be a link between the sequence of *lisA* and the flagella antigens produced (Rasmussen *et al.*, 1991). *L. ivanovii* and *L. seeligeri* both produce sulfhydryl listeriolysins, the genes for which have recently been cloned and sequenced (Haas *et al.*, 1992). These haemolysins are reported to show homology to listeriolysin O (Leimeister-Wächter and Chakraborty, 1989).

The amino acid sequences deduced for these haemolysins showed seeligolysin O (LSO) to be a 59 kd protein, and ivanolysin (ILO) to be a 58.5 kd protein, which when compared to listeriolysin O showed a high degree of homology; 82 % and 80% respectively (Haas *et al.*, 1992). However analysis of the LSO and ILO amino acid sequences also revealed significant differences from that of listeriolysin O, which may explain the variation in their haemolytic activities. LSO was reported to have a non-conservative amino acid substitution of alanine to phenylalanine at position 489, the

sixth position in the cysteine motif. This motif is highly conserved in all thiol-activated cytolysins including listeriolysin O (Boulnois *et al.*, 1990). This substitution while retaining the hydrophobicity of this region adds the large aromatic ring of phenylalanine which may alter the folding of the protein and explain the reduced haemolytic activity of the protein compared with that of listeriolysin O. In all the thiol-activated cytolysins sequenced so far the conserved cysteine motif contains only one cysteine residue which has been found to be important, but not essential for their haemolytic activity (Boulnois *et al.*, 1990, Michel *et al.*, 1990). However analysis of the amino acid sequence of ILO revealed an extra cysteine residue located 26 residues from the conserved cysteine motif (Haas *et al.*, 1992). This addition may allow the protein to form intramolecular disulphide bonds upon oxidation, possibly accounting for its increased haemolytic activity compared with Listeriolysin O (Haas *et al.*, 1992). *L. ivanovii* also produces other proteins also thought to be virulence factors including a second cytolysin, phospholipase C and a 24 kd sphingomyelinase which co-purifies with the *L. ivanovii* haemolysin (Vazquez-Boland *et al.*, 1989).

Lipases.

The exact importance of these proteins in listerial pathogenesis is not yet clear. The production of a phospholipase C by *L. monocytogenes* was first noted by Leighton *et al.*, (1975) under anaerobic conditions and subsequently by Barclay *et al.*, (1989). In tissue culture studies of haemolysin negative transposon mutants a small number were observed to be capable of escape from the phagocytic vacuole of many different cell lines (Camilli *et al.*, 1989, Kathariou *et al.*, 1990, Mounier *et al.*, 1990). It has been suggested that this phenomenon was due to the presence of a phospholipase C, (Camilli *et al.*, 1991, Kathariou *et al.*, 1990, Mounier *et al.*, 1990) and on investigation of the area upstream of the *lisA* gene an open reading frame encoding a 36 kd phosphatidylinositol specific (PI-Plc) phospholipase was identified (Camilli *et al.*, 1991, Leimeister-Wächter *et al.*, 1991, Mengaud *et al.*, 1991a). The nucleotide sequence of *plcA*, the gene encoding phospholipase C, predicts a protein with approximately 30% amino acid identity to the PI-Plc phospholipase in *Bacillus thuringiensis* and *Bacillus cereus*. Of the members of the genus *Listeria* the *plcA* is unique to *L. monocytogenes* and *L. ivanovii* (Camilli *et al.*, 1991, Leimeister-Wächter

et al., 1991, Mengaud *et al.*, 1991a). Mutation of the gene *plcA* and loss of phospholipase C activity has been demonstrated to result in a corresponding reduction of virulence implicating this protein as a possible virulence factor, although the polar effect of these mutations on the regulatory gene *prfA* may possibly account for this loss (Camilli *et al.*, 1991, Mengaud *et al.*, 1991a).

Two distinct extracellular lecithinases of 29 and 32 kd have also been purified from *L. monocytogenes* supernatant (Geoffroy *et al.*, 1991). These enzymes have a wide specificity for substrates such as phosphatidylethanolamine, and phosphatidylserine, but not phosphatidylinositol, a characteristic which distinguishes them from phospholipase C. The sequence of the gene *plcB* which encodes the 29 kd lecithinase shows similarity to the zinc dependent phosphatidylcholine-phospholipases of *Clostridium perfringens* (alpha-toxin) and *B. cereus*, and is contained in the recently designated lecithinase operon (Vazquez-Boland *et al.*, 1992). Little similarity between the lecithinases and phospholipase C of *L. monocytogenes* and those of *L. ivanovii* has been reported however *L. ivanovii* when cultured on egg yolk agar exhibits lecithinase activity and it has been suggested that this 29 kd protein may be responsible for this activity (Geoffroy *et al.*, 1991).

The first gene of the lecithinase operon immediately downstream from listeriolysin has been demonstrated to encode a metalloprotease which is antigenically related to those of many *Bacillus* species and typified by thermolysin (Domann *et al.*, 1991, Mengaud, *et al.*, 1991b). This metalloprotease gene *mpl* is present only in pathogenic strains of *L. monocytogenes* and encodes an amino acid motif common to zinc-dependent metalloproteases (Domann *et al.*, 1991, Mengaud, *et al.*, 1991b). Proteases of *Pseudomonas aeruginosa* and *Legionella pneumophila* preserve this motif and have been implicated as virulence factors in these pathogens (Domann *et al.*, 1991, Bever and Iglewski, 1988). The contribution of this metalloprotease to the pathogenesis of *L. monocytogenes* is not yet clear. However, mutants with transposon insertions in *mpl* are of reduced virulence (Raveneau *et al.*, 1992), expressing only the 32 kd form of lecithinase suggesting that metalloprotease may proteolytically process lecithinase (Vazquez-Boland *et al.*, 1992). Under laboratory conditions the metalloprotease is

synthesised predominantly in its inactive pro-form, although it is thought that production of active protease may be dominant within the host cell (Domann *et al.* , 1991).

Cell wall components.

The cell surface of *L. monocytogenes* contains many putative virulence factors including monocytoxis producing agents, immunosuppressive activity, delayed type hypersensitivity (DTH) factor, and several invasion proteins. The characteristic monocytoxis observed during animal listeriosis is induced by the monocytoxis producing agent (MPA) which in *L. monocytogenes* is a lipid associated with the plasma membrane (Shum and Galsworthy 1982). The MPA appears to have a molecular weight of 1.0 kd (Galsworthy, *et al.*, 1977, Shum and Galsworthy 1982, Stanley, 1949). MPA does not stimulate monocytoxis in humans, although it appears to damage macrophages. In rodents and *Cavia* it appears to stimulate the production of new monocytes (Galsworthy, 1987) however, the importance in *L. monocytogenes* infection of the MPA is not clearly understood.

Another cell surface component of *L. monocytogenes* is the so called immunosuppressive agent (ISA). This agent was initially recognised due to the observation that spleen cells from listeric animals were defective in their ability to synthesise antibody (Kim *et al.*, 1976). The ISA has a molecular weight of 150 kd, made up of amino acids, carbohydrates, phosphorous, and glycerol. Acting as a B-cell mitogen the ISA stimulates the production of suppressor T-cells *in vivo* (Galsworthy, 1987).

Cell wall proteins have also been cited to induce a DTH reaction and to be mitotic for lymphocytes in immunised animals (Galsworthy and Fensler 1984). Pathogenic and nonpathogenic strains of *Listeria* induce such a DTH, expressing antigens recognised by T cells (Hof and Chatzipanagiotou, 1987). Recently the gene for a listerial antigen (*ImaA*) capable of eliciting a specific DTH response in *Listeria* immune mice has been identified and cloned (Göhmann *et al.*, 1990). The gene product of *imaA* was found to be a protein of 170 amino acids with a molecular weight of 21 kd, which is predicted

to lie within the bacterial plasma membrane and cell wall. Only the pathogenic strains *L. monocytogenes* and *L. ivanovii* were found to possess *imaA*, although as other workers have noted that all species induce DTH on immunisation many other factors are probably involved. Subsequent nucleotide sequence analysis has demonstrated that *ImaA* is part of an operon and is preceded by the *imaB* gene, which encodes a 14 kd polypeptide. However, the role of this operon is undetermined (Portnoy *et al.*, 1992).

The expression of putative cell surface virulence factors may be linked to the expression of other factors such as lecithinase. The product of the second gene in the lecithinase operon *actA*, predicts a 639 amino acid surface protein with a signal sequence and a membrane anchor, necessary for *L. monocytogenes* to assemble actin. (Vazquez-Boland *et al.*, 1992). *actA* mutants do not express lecithinase, do not form plaques in monolayers of mouse fibroblasts, and do not nucleate the polymerisation of actin (see Portnoy *et al.*, 1992). Whether *actA* encodes an actin nucleator or a protein with another function is not yet known, but it does appear to have a role in the pathogenicity of *L. monocytogenes*.

Invasion of epithelial cells is a key virulence mechanism of *L. monocytogenes*. Recently a gene *inlA* encoding a surface protein internalin has been identified and found to mediate such entry (Gaillard *et al.*, 1991). The sequence of *inlA* predicts an 80 kd repeat protein reminiscent of surface antigens from other Gram-positive cocci, for example the *Streptococcus pyogenes* M protein. When introduced into *L. innocua* internalin confers the ability to invade epithelial cells upon this noninvasive species. Internalin was the first sequenced *L. monocytogenes* membrane protein and the first example in a Gram-positive rod of a protein with the carboxyl terminal motif LPTXGD thought to play a role in membrane attachment in Gram-positive cocci (Fischetti *et al.*, 1990). *inlA* was also found to be part of an operon including *inlB*, which encodes a leucine rich protein the function of which is not yet known (Gaillard *et al.*, 1991). The presence of these factors suggests that the components of the cell wall of *L. monocytogenes* enhance its pathogenicity.

Extracellular products.

Extracellular proteins other than those already mentioned appear to be necessary for cellular invasion by *Listeria*. Although haemolysin supports the survival of *L.monocytogenes* intracellularly it is not essential for entry into professional and nonprofessional phagocytes (Kuhn *et al.*, Goebel, 1988). Invasion of nonprofessional phagocytes by endocytosis appears to be in some way induced by *L.monocytogenes* itself because it cannot be inhibited by cytochalasin B (Kuhn *et al.*, Goebel, 1988). Furthermore, a group of avirulent transposon insertion mutants constructed by Kuhn and Goebel (1989) were distinguished by their rough colony appearance and designated rough (R) mutants. These R-mutants were fully haemolytic, with a growth rate comparable to their parent strain, but exhibited impaired septum development forming chains of cells. Invasion of macrophages by the R-mutants was as normal but they were not taken up by nonphagocytic cells even when separated into single cells by sonication. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the extracellular proteins of R-mutants revealed that their production of a major 60 kd protein (p60) was much reduced (Kuhn *et al.*, 1988). On application of partially purified wild type p60, chains of R-mutant cells disaggregated and invasiveness was restored, implying that p60 is a virulence factor (Kuhn and Goebel, 1989). The protein p60 is very positive in charge (pI 10.5), and is produced by all virulent strains of *L. monocytogenes* (Goebel *et al.*, 1991). The gene expressing p60 (*iap*) has been cloned and sequenced and a 400 base pair internal fragment of the gene specific to strains of *L. monocytogenes* has been suggested as a possible DNA probe for the detection of the species (Köhler *et al.*, 1990).

A subset of R-mutants were detected in SDS PAGE studies which produced at least normal levels of p60 but were nevertheless non-invasive. However these mutants adhered to mammalian cells more efficiently than p60 deficient R-mutants suggesting that more than one bacterial factor is involved in cell invasion. The presence of surface carbohydrates able to interact with eucaryotic cell surface receptors may be a factor in the adherence of virulent strains of *L.monocytogenes* to the cell surface. Cowart *et al* (1990) described the attachment of *L.monocytogenes* to a human hepatocarcinoma cell line (HepG2) by the interaction of α -D-galactose and the eucaryotic galactose receptor.

The virulent EGD strain of *L.monocytogenes* was found to have a surface α -D-galactose residue while two avirulent strains did not and were unable to invade the nonprofessionally phagocytic HepG2 cell line (Coward *et al.*, 1990).

Defences against activated phagocytes.

To survive intracellularly *L. monocytogenes* must overcome the antibacterial mechanisms of the host cell, and a most important component of these mechanisms is the respiratory burst. Within seconds of phagocytosis the respiratory burst is triggered releasing superoxide anions (O_2^-), and hydrogen peroxide (H_2O_2), at the phagosomal membrane in an attempt to eliminate the invading bacteria. The enzymes superoxide dismutase (SOD) and catalase can neutralise these reactive molecules and may therefore act as virulence factors, as has been demonstrated in *Mycobacterium tuberculosis* (Beaman and Beaman 1984). Studies on the catalase and SOD activities of strains of *L.monocytogenes* have failed to establish a correlation between their activity and virulence, although a trend towards high activities in virulent strains has been observed (Welch *et al.*, 1979, Welch, 1987). Transposon mutagenesis has also been employed in the investigation of the role of catalase in *L.monocytogenes* pathogenesis, and catalase negative insertion mutants were found to have unimpaired virulence in a mouse model system (Leblond-Francillard *et al.*, 1989). The SOD levels of mutants defective in catalase activity have been reported to be higher than normal which may compensate for the reduced catalase activity to aid their survival intracellularly. A catalase encoding gene from *L.seeligeri* has recently been cloned and expressed in *Escherichia coli*, and on nucleotide sequencing was found to include a region next to its promoter highly homologous to the ferric uptake regulon of *E. coli* (Haas, *et al.*, 1991). This finding is consistent with the observation that iron concentration has a regulatory effect on catalase activity in *L. monocytogenes* (Haas, *et al.*, 1991). The genes encoding the SOD of *L.monocytogenes* and *L. ivanovii* have also been cloned (Brehen *et al.*, 1992, Haas and Goebel, 1991) and in common with the catalases of *L.monocytogenes* and *L.seeligeri* their activity appears to be regulated by the concentration of available iron (Brehen *et al.*, 1992, Haas, *et al.*, 1991, Haas and Goebel, 1991, Welch *et al.*, 1979). These reports suggest that together these enzymes contribute to the virulence of the listeriae in the iron rich phagosomal

environment but are not critical to it.

Intracellular movement

L. monocytogenes has been recognised to spread from cell to cell and the morphological stages of this movement observed under the electron microscope (Mounier, *et al.*, 1990, Tilney *et al.*, 1990, Tilney and Portnoy, 1989). Almost immediately after *L.monocytogenes* has been phagocytosed, the phagosomal membrane is disrupted by the action of listeriolysin O and other factors such as phospholipase C. The listeriae then escape into the cytoplasm where they can be seen as free cells, which rapidly multiply. Within a few hours the bacteria induce the polymerisation of actin and become coated with short actin filaments and other actin binding proteins (Mounier, *et al.*, 1990, Tilney *et al.*, 1990). The coating actin structure is then rearranged to form a filamentous actin tail which advances the movement of the bacteria through the cytoplasm to the cell surface. Propelled by the actin tail, protrusions of the cell membrane form around the listeriae and are pushed into the neighbouring cells (Dabiri, *et al.*, 1990, Mounier, *et al.*, 1990, Tilney *et al.*, 1990). The listeriae containing protrusion becomes coated with cell membrane from the neighbouring cell forming a double membrane which is then recognised and internalised into the next cell (Mounier, *et al.*, 1990, Tilney *et al.*, 1990). In the cytoplasm of the new cell the double membrane is quickly dissolved and the cycle repeated.

To study the factors involved in this cell to cell spread and their regulation libraries of transposon mutants have been constructed and screened in plaque assay systems (Kuhn *et al.*, 1990, Sun *et al.*, 1990). Mutants defective in intracellular growth and cell to cell spread were identified in these studies including mutants which were unable to move intracellularly but could still polymerise actin (Kuhn *et al.*, 1990). These mutants were not able to rearrange the actin coat to generate movement, remaining trapped in the cytoplasm, and displayed a much reduced level of virulence in mice (Kuhn *et al.*, 1990). In further studies ten classes of mutants were isolated by Sun *et al.*, (1990), several of which were impaired in haemolysin and/or phospholipase activity, as well as actin association. Many mutations were found to lie within the

haemolysin structural gene *lisA* or within the open reading frame (ORF U) adjacent to *lisA*. This evidence suggests that these factors act in concert for intracellular growth and spread possibly with a surface or secretory actin nucleator, as yet unknown (Kuhn *et al.*, 1990, Sun *et al.*, 1990). Mutants of the intracellular parasite *Shigella flexneri* unable to associate actin lack a cell surface protein of 120 kd (Pal *et al.*, 1989). Further studies will determine whether such a protein is present in *L.monocytogenes*.

Immunoprophylaxis of listeriosis.

Research studies into active immunisation against listeriosis in animals have employed many different types of both live and killed vaccines. Experiments in the 1940s and 1950s using bacterins as sheep vaccines, were the first attempts to immunise against listeriosis. These vaccines did not provide any protection even when administered in large doses by Graham *et al.* (1943), although Eveleth *et al.* (1953) did note some protection by bacterin administration. These observations prompted the development of potential inactivated vaccines.

Killed vaccines against *Listeria* have yielded conflicting results in protection studies. Exposure to either avirulent or nonviable virulent *Listeria* strains has been demonstrated by many authors to fail in the induction of a protective immune response (Berche *et al.*, 1987a, Kearns and Hinrichs, 1977, Wirsing von Koenig and Finger, 1982), unless associated with an adjuvant (Van Der Meer, 1977, Van Dijk *et al.*, 1980). In contrast with these results extremely large doses of the avirulent species *L. innocua* were found to induce protective immunity to listeriosis in mice (Wirsing von Koenig *et al.*, 1983). Constraints placed on the use of live vaccines in Hungary prompted the development of a formol vaccine absorbed to aluminium hydroxide gel (Szemerédi and Nagy, 1962). This vaccine reduced the losses of sheep due to listeriosis from 3.0 to 0.36% over a ten year period when two vaccinations were administered (Szemerédi and Padányi, 1989). Although in some studies losses were reduced significantly by the use of inactivated vaccines, it was suggested that the disease could not be eradicated by the use of formol vaccines. Furthermore, other researchers having performed experiments with killed vaccines have obtained no satisfactory results (Armstrong and Sword, 1964, Coppel and Youmans, 1969).

Protection against listeriosis requires the stimulation of the cell mediated immune system because macrophages activated by sensitised T-cells eliminate *L. monocytogenes* from the body. It has been well documented that replicating antigens are more potent in stimulating this system than nonreplicating antigens (Baldrige *et al.*, 1988, Hahn and Kaufmann, 1981). Antigen preparations derived from *Listeria* fail to induce acquired resistance as intracellular growth has been reported to be a prerequisite for the induction of cell mediated protection against listeriosis (Baldrige *et al.*, 1988, Brunt *et al.*, 1990). In the light of these observations it is not surprising that the use of live vaccines consisting of avirulent or moderately virulent strains has yielded more positive results than killed vaccines. Olson *et al.*, (1950) and Ivanov (1981) have both reported some success in the use of attenuated vaccine strains, as have many other authors (Kearns and Hinrichs, 1977, Linde *et al.*, 1991, Osebold and Sawyer, 1955, Potel and Schulze-Lammers, 1985, Selivanov *et al.*, 1974).

The most widely used live vaccine against ovine listeriosis was developed by Ivanov *et al.*, (1977), using attenuated strains of *L. monocytogenes* 1/2a and 4b together with the adjuvant saponin. These strains attenuated through successive passages in media supplemented with an unnamed bacteriostatic agent and were reported to be avirulent for sheep, guinea pigs and albino mice. In recent years this vaccine has been used extensively in Bulgaria and has been licensed for use in Norway since 1983 under the name of "Listervac". In both of these countries the vaccine has been reported to be effective at a dose of 10^{18} organisms per sheep, although its use is recommended only in flocks well before the mating season (Gudding *et al.*, 1989, Ivanov, 1985). Vaccination is subcutaneous and immunity develops 10 to 12 days after a single injection and lasts from 6 to 10 months (Gudding *et al.*, 1989). The short lived nature of the immunity developed, the incomplete nature of the protection, and the need to vaccinate at precisely the right time to give maximum protection, has limited the use of this vaccine which is not licensed for use in Great Britain.

Attenuation of strains by passage with bacteriocides may alter their antigenic structure, so that they may not induce an immune response and memory specific to the wild type

organism. The use of strains of naturally reduced virulence as vaccine strains avoids this problem but incurs the possibility of infection on inoculation. The development of new vaccine strains of *Listeria* is therefore of value. Haemolysin negative strains of *L. monocytogenes* which are avirulent have been suggested as possible vaccine strains (Michel *et al.*, 1990). These strains although highly attenuated (5 orders of magnitude increase in 50% effective dose, ED₅₀) are defective in listeriolysin O which has been demonstrated to be a major stimulant of the immune response and protection against *L. monocytogenes* (Barry *et al.*, 1992, Berche *et al.*, 1987a, Berche *et al.*, 1987b). The lack of such a major antigen means that these strains are seen as less than ideal vaccine strains. One strategy for the development of vaccine strains which has been successfully employed for several species of Gram negative bacteria and especially for *Salmonella* is the construction of stable auxotrophic mutants (Dougan *et al.*, 1987, Hoiseth and Stocker, 1981, Mukkur *et al.*, 1991, O' Callaghan *et al.*, 1988). Many such mutant strains have been engineered, characterised and their immunogenicity assessed in protection studies (Dougan *et al.*, 1987, Hoiseth and Stocker, 1981, Mukkur *et al.*, 1991, O' Callaghan *et al.*, 1988).

The use of Gram negative aromatic amino acid dependent strains as live vaccines.

In the late 1940s Bacon and others made the first investigations into the virulence of auxotrophic mutants of *Salmonella typhi*. This group tested large numbers of auxotrophic mutants in a mouse model system and of these only those with a requirement for the purine p-amino-benzoic acid (pAB) or aspartic acid were seen to have consistently reduced virulence (Bacon *et al.*, 1951). pAB requiring mutants are attenuated because they cannot synthesise folic acid which is not a mammalian metabolite and cannot be gained from host tissues. In mammals folic acid is acquired as a vitamin in the diet which is reflected by the low concentrations of purines and their ribosides in mammalian tissues where purines are synthesised as mononucleotides (Bacon *et al.*, 1951, Stocker, 1988).

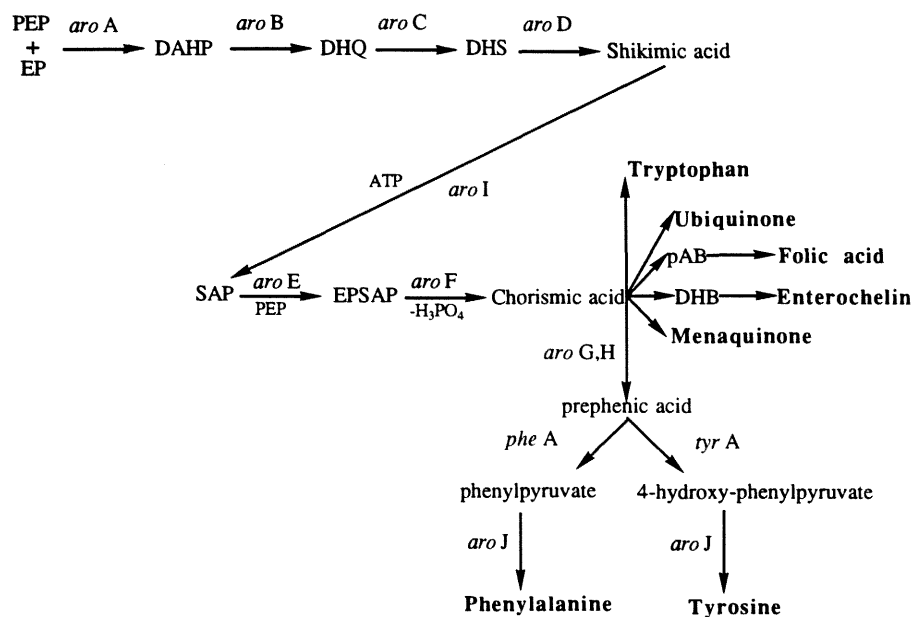
Some time later Yancey *et al.* (1979) reported the isolation of an avirulent mutant of *S. typhimurium* which required 2,3-dihydroxybenzoate (DHB). DHB is a precursor of

the bacterial iron acquisition compound enterochelin, and like pAB is not available in mammalian tissues. Bacteria synthesise both pAB and DHB from a single intermediate chorismic acid, the final product of the aromatic biosynthetic pathway (Figure 1.1). A block in any step of this pathway renders the bacterium auxotrophic for compounds not found in vertebrate tissue, and thus avirulent (Hoiseth and Stocker, 1981, Stocker *et al.*, 1988).

Prompted by this work Hoiseth and Stocker (1981) constructed auxotrophic *S. typhimurium* strains carrying nonreverting transposon induced lesions in the *aroA* gene. These mutants were found to be avirulent having a ED_{50} of $> 10^6$ bacteria in mice compared to < 20 of the wild type bacteria (Hoiseth and Stocker, 1981). When administered to mice *aroA*⁻ *S. typhimurium* were demonstrated to be an effective live vaccine against salmonellosis (Hoiseth and Stocker, 1981, Mukkur *et al.*, 1987, O'Callaghan, 1988). Administered by the oral route these vaccine strains proved to be as effective in stimulating protection as when administered parenterally, a single dose conferring excellent protection (Hoiseth and Stocker, 1981). Stocker (1988) went on to construct *S. typhimurium* strains harbouring two nonreverting lesions in the *aroA* gene and the *purA* gene involved in purine biosynthesis. This double mutation was necessary to ensure against reversion to prototrophy *in vivo*, and allowed trials as a human vaccine to be undertaken. When fed to human volunteers the *S. typhimurium aroA purA* mutant was found to be avirulent and caused no serious clinical reactions. A good cell mediated response was reported although only a poor humoral antibody response was induced (Levine *et al.*, 1987).

The behaviour of *S. typhimurium aro*⁻ and *pur*⁻ mutants in murine model systems has been characterised (Nnalue and Stocker, 1987, O'Callaghan *et al.*, 1988). When administered intravenously, *S. typhimurium aro*⁻ and *pur*⁻ mutants set up low level persistent infections in the liver and spleen which may last for up to 70 days. An initial phase of multiplication of strains carrying certain lesions such as *aroA* has been noted and is thought to represent the utilisation of any residual intermediates which are quickly exhausted.

Figure 1.1. General pathway for aromatic amino acid synthesis



Abbreviations: PEP: phosphoenolpyruvate, EP: erythrose 4-phosphate, DAHP: 3-deoxy-D-arabio-heptulosonic acid 7-phosphate, DHQ: 5-dehydroquinic acid, DHS: 5-dehydroshikimic acid, SAP: shikimic acid 5-phosphate, EPSAP: 3-enol-pyruylshikimic acid, pAB: para-amino benzoic acid, DHB: dihydroxy benzoic acid. Adapted from Hoch and Nester (1973).

In subsequent studies *purA*⁻, *purE*⁻, and *galE*⁻ mutants have been found to be less effective in the induction of protective immunity than *aroA* mutants and mutants carrying these lesions with an *aroA* lesion are not as effective as *aroA* alone (Nnalue and Stocker, 1987, O'Callaghan *et al.*, 1988). Purine and galactose mutants are highly attenuated and do not display any initial multiplication which may account for their reduced immunogenicity as persistence alone is not sufficient to establish immunity (Collins, 1974, Hormaeche, 1981, O'Callaghan *et al.*, 1988).

The majority of Gram negative auxotrophic vaccine strains have been constructed by transposon mutagenesis in which the transposon, a self transmissible sequence of DNA, enters the chromosome inactivating any gene it inserts into.

Transposon mutagenesis of *L. monocytogenes*.

Transposon mutagenesis has been used successfully to identify certain listerial virulence factors. Encouraged by the successful use of transposon mutagenesis of *Enterococcus faecalis*, and *Streptococcus pyogenes*, Gaillard *et al.* (1986) first described the use of transposon mutagenesis in *L.monocytogenes*. Gaillard *et al* (1986) employed the large (25.3 kb) *E. faecalis* conjugative transposon Tn1545, and this tetracycline resistance encoding (*tetM*) element has subsequently been employed in several studies to construct listeriolysin O and catalase negative mutant strains (Cossart 1988, LeBlond *et al.*, 1989). Another *tetM* encoding *E. faecalis* conjugative transposon very similar to Tn1545, although smaller in size (16.4 kb) is Tn916. This transposon has also been employed successfully for the insertional mutagenesis of *L.monocytogenes* (Kathariou *et al.*, 1987, Kuhn *et al.*, 1990, Sun *et al.*, 1990). Both Tn916 and Tn1545 are self transmissible, encoding conjugation systems independent of the normal plasmid specific mechanisms (Murphy, 1989). Transfer of these transposons by conjugation between *Listeria* species and *E. faecalis* has been reported, and can be easily achieved by the use of basic mating techniques (Gaillard *et al.*, 1986, Kathariou *et al.*, 1989). 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 Tn916) 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).

To avoid the problems associated with the use of Tn1545 and Tn916, the nonconjugative *E. faecalis* transposon Tn917 has also been employed successfully in mutagenesis of the listerial chromosomal genes (Camilli *et al.*, 1990, Cossart *et al.*, 1989). Tn917 was first isolated by Tomich *et al.*, (1980) as a plasmid associated erythromycin resistance determinant, unusual in that both its transposition and its erythromycin resistance encoding *erm* gene can be induced by erythromycin. This element is transposed in a Tn3-like process which is not site specific and is therefore random in nature, and the insertion of Tn917 has been demonstrated to be random in species of *Bacillus* (Youngman *et al.*, 1983). Tn917 has also been demonstrated to generate extremely stable insertional mutations, its frequency of excision being relatively low (Youngman *et al.*, 1983). The small size (5 kb) of Tn917 when compared to that of Tn1545 (25.3 kb), and Tn916 (16.4 kb) simplifies the cloning and mapping of the DNA flanking its insertion.

Derivatives of Tn917 carrying additional drug resistance genes, and translational fusions to reporter genes, such as *lacZ* and *cat-86* have all been constructed to enhance the usefulness of this element for the analysis of chromosomal genes (Youngman, 1987). The fusion generating derivatives of Tn917 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, 1984, Youngman *et al.*, 1985).

One approach used to recover transpositions of Tn917 to chromosomal sites in *B. subtilis* is the construction of suicide vectors carrying Tn917 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 Tn917.

The current method of choice for the recovery of Tn917 transpositions is based on the use of temperature sensitive plasmid vectors, which carry the pE194 replicon derived from *Staphylococcus aureus* (Youngman *et al.*, 1985, Youngman, 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 again predominantly bacteria which have acquired a chromosomal insertion of Tn917 (Youngman *et al.*, 1984b). The temperature sensitive vectors pTV32ts and pLTV3 which carry Tn917-*lac* fusions have been employed for the construction of insertion mutants of *L. monocytogenes* (Camilli *et al.*, 1990, Sun *et al.*, 1990).

Listeriosis in animals is economically important because of the financial losses incurred. Epidemiologically animal listeriosis is also important as dissemination of *Listeria* to the human population may occur by consumption of contaminated animal products. The risk of contamination could be lessened if the numbers of animal carriers were reduced, and active immunisation against *Listeria* is one way in which this can be achieved. The success of live and killed vaccine strains of *Listeria* has so far been limited, as major virulence factors and replication appear to be essential for the induction of a protective immunity.

The use of attenuated aromatic amino acid dependent mutant strains as live vaccines has been successful in protecting against the intracellular parasite *S. typhimurium*. The aim of this thesis is to document the endeavours made to construct and isolate *aro* transposon insertion mutants of *L. monocytogenes* by the use of strategies well documented for the transposon mutagenesis of *Bacillus*, the development of an optimised electroporation system for *Listeria* species and the investigation of the potential of aromatic amino acid dependent mutants as live vaccine strains of *L. monocytogenes*.

Materials and Methods

2.1. Bacterial Strains and Plasmids

The bacteria and plasmids used are listed in Table 2.1.

Growth conditions and Media

Unless otherwise stated, all strains of *Listeria* species were cultured in Tryptose Soya broth (TSB) or Tryptose Soya supplemented with 1.5% agar (TSA), at 30°C.

Escherichia coli was cultured at 37°C, in Luria broth subsequently referred to as L broth (per litre: 10g Tryptose, 5g Yeast extract, 5g NaCl) with the addition of 1.5% agar (BBL) as required. The minimal medium of Friedman and Roessler (1961) was used for the selection of auxotrophic insertion mutants (section 2.10). The modified basal medium of Wilkinson and Jones (1977) (per litre: 10g peptone, 10g Lab-Lemco, 5g yeast extract, 20g glucose, 1 ml polyoxyethylene sorbitan mono-oleate [Tween 80], 2g K₂HPO₄, 34g CH₃COON.3H₂O, 2g triammonium citrate, 200 mg MgSO₄.7H₂O, 50 mg MnSO₄.4H₂O) was used for the selection of mutants unable to utilise rhamnose. DM3 protoplast regeneration medium (Chang and Cohen, 1979) (0.5 M sodium succinate [pH 7.0], 0.5% Casamino Acids, 0.5% yeast extract, 0.35% K₂HPO₄, 0.5% glucose, 0.02 M MgCl₂, 0.01% bovine serum albumin, 0.8% agar) was also used where stated. Where necessary the antibiotics (Sigma), erythromycin, chloramphenicol, and lincomycin, were added to the media at a concentration of 25 µg/ml unless otherwise stated.

2.2. Procedures for the transformation of Bacterial Cells.

Production of competent cells of *E. coli*.

An inoculum (100 µl) of an overnight culture (*ca.* 18 hrs) of *E. coli* was added to L-broth (10 ml) and grown to mid-log phase (OD₆₀₀ 0.5). The cells were harvested, washed in 10 mM NaCl, and resuspended in 4 ml of ice-cold CaCl₂ (100 mM). Cells were placed on ice for 30 mins before harvesting by gentle centrifugation (1800 x g) at 4°C for 5 mins. The resulting cell pellet was resuspended in 1 ml ice-cold CaCl₂ (100 mM) and transformed immediately.

Table 2.1. Strains and plasmids used.

Strain	Code	Characteristics	Source /reference
<i>Listeria monocytogenes</i>	NCTC 7973		
<i>Listeria monocytogenes</i>	NCTC 7973	pLTV3-Tn917	This study
<i>Listeria monocytogenes</i>	EGD		M. W.
<i>Listeria monocytogenes</i>	EGD	pTV32ts-Tn917	M. W.
<i>Listeria monocytogenes</i>	Lm.1070138		D. P.
<i>Listeria monocytogenes</i>	Lm.918.6	pLTV3-Tn917	D. P.
<i>Listeria monocytogenes</i>	Lm.6b.	pTV32ts-Tn917	This study
<i>Listeria ivanovii</i>	ACTC 19119		
<i>Listeria innocua</i>	NCTC 11288		
<i>Listeria seeligeri</i>	CIP 100100		
<i>Escherichia coli</i>	LE392		Barck <i>et al.</i> , 1976

Plasmid	Characteristics	Reference
pACYC184	Cloning vector	Chang and Cohen, 1978
pBR328	Cloning vector	Bolivar <i>et al.</i> , 1977
pTV52	Carries Tn917::cat	Youngman <i>et al.</i> , 1984b
pTV32	Carries Tn917::lac	Youngman <i>et al.</i> , 1984b
pTV32ts	Temperature sensitive carries Tn917::lac	Youngman <i>et al.</i> , 1987
pLTV3	Temperature sensitive carries Tn917::lac	Camilli <i>et al.</i> , 1990
pGK12	Cloning vector	Kok <i>et al.</i> , 1984

M. W. indicates Dr M. Wuenscher. Institute für Genetik und Mikrobiologie Würzburg, FRG.
D. P. indicates Dr D. Portnoy. Dept. Microbiology, University of Pennsylvania, Philadelphia, USA.
NCTC indicates National Type Culture Collection., London, UK.
ACTC indicates American Type Culture Collection, Rockville, USA.
CIP indicates collection Institut Pasteur, Paris, France.

Transformation of *E. coli* with plasmid DNA.

Competent cells of *E. coli* (100 µl) were mixed with up to 25 µl of the appropriate plasmid DNA dissolved in sterile water or TE buffer and placed on ice for 1 hr. The cells were then heat shocked at 42°C for 3 mins, L-broth (500 µl) was added and the whole incubated for a further 1 hr at 37°C before plating onto L-agar containing selective levels of the appropriate antibiotic. Plates were incubated at 37°C overnight.

Preparation of *L. monocytogenes* protoplasts.

Protoplasts of *L. monocytogenes* were prepared by a modification of previously described procedures (Vicente *et al.*, 1987; Camilli *et al.*, 1990). A 10% (v/v) inoculum of a mid log phase (OD₆₀₀ 0.5 - 0.6) culture was added to 100 ml of Brain Heart Infusion broth (BHI) (Oxoid) in a 500 ml flask and incubated for 2 hrs at 30°C (OD₆₀₀ 0.6). The cells were harvested by centrifugation at 10,000 x g for 10 min and washed once with distilled water and once with 0.155 M NaCl. The cells were then suspended in lysis buffer (0.015 M NaCl, 0.03 M Tris buffer pH 6.7, 0.4 M sucrose, and 0.3 mg/ml lysozyme), to a concentration of 30-40 mg/ml dry weight. After incubation with gentle shaking, for 15 mins at 37°C, MgCl₂ was added to a final concentration of 0.03 M. Incubation was then continued at 37°C for 3 hrs. Protoplast formation was monitored by phase contrast microscopy and osmotic susceptibility - 1 ml of the "protoplast suspension" was centrifuged, resuspended in water and the OD₆₀₀ compared with that of the untreated solution. When no further reduction in optical density was observed the protoplasts were centrifuged at 7,600 x g at 25°C for 15 mins, washed once in 5 ml of 2 x Difco antibiotic medium no.3, 0.5 M sucrose, 0.02 M MgCl₂ [pH 6.5], adjusted with NaOH (SMMP), and resuspended in SMMP (1/10 th vol. of the starting culture).

Transformation of *L. monocytogenes* protoplasts with plasmid pLTV3 DNA.

For transformation 200 µl of the protoplast suspension in SMMP was mixed with 1 µg of DNA by gentle swirling, 600 µl of 40% PEG (Sigma; Mw 2,500) in 2 x SMM (SMMP without Difco medium: 0.5 M sucrose, 0.02 M MgCl₂ [pH 6.5]), was added. The solution was again gently mixed by swirling and left at room temperature for 3 min, before 2 ml of SMMP added. The protoplasts were pelleted at 7,600 x g for 15 min at 25°C, resuspended in 1 ml of SMMP containing a subinhibitory but plasmid replication inducing concentration (for plasmid pLTV3 erythromycin at a concentration of 0.04 µg/ml), and incubated at 30°C for 1 hr. Transformed protoplasts (100 µl) were then plated on to DM3 regeneration plates containing a selective level (25 µg / ml) of erythromycin, and incubated at 30°C for 3-4 days. To estimate regeneration efficiency, and transformation control equal volumes of untreated protoplast suspension were also plated onto DM3 medium.

2.3. Electroporation

Preparation of competent cells for the electroporation of *Listeria* species.

Two methods were used :

Preparation of cells for electroporation of *Listeria* species with plasmid DNA of less than 20 kb.

A 10% (v/v) inoculum of an overnight culture of *L. monocytogenes* in brain heart infusion broth (BHI) was added to 500 ml of BHI and incubated with shaking at 37°C to an OD₆₀₀ of 0.6-0.8. The bacteria were harvested by centrifugation at 5000 x g for 10 min at 4°C, washed twice in 1/10 th the original volume with 3.5 x sucrose magnesium electroporation medium (SMEM: 272 mM sucrose, 1 mM MgCl₂, pH 7.2) and resuspended in 1/100 th the original volume with 3.5 x SMEM. Portions of the cell suspension were stored at -70°C until required.

Electroporation of *Listeria* species with plasmid DNA of less than 20kb.

The prepared cells were thawed slowly in ice, 40 μ l ($\approx 4 \times 10^{10}$ bacteria) were mixed gently with 1 μ l of DNA dissolved in sterile nanopure distilled water and then left on ice for 1 min. The mixture was transferred to a chilled electroporation cuvette (0.2 cm²) and placed between the chilled electrodes of a Gene-Pulser™ electroporation apparatus with pulse controller (Bio-Rad, Richmond, California). The cells were electroporated at a field strength of 8.5 kV/ cm, 200 Ohms resistance with a time constant of approximately 5.0 ms. The cuvette was immediately placed on ice for 1 min before 1 ml of BHI broth was added and the culture incubated at 37°C for 2 hrs with gentle shaking. The cells were then plated onto Blood Agar Base no.2 (Difco) containing selective levels of antibiotic, and the plates incubated at 30°C for 48 hrs.

To determine the optimal conditions for electroporation the procedure was conducted at various field strengths for different time constants. The field strength is defined as the voltage gradient between the electrodes; the time constant is a function of the field strength and the resistance applied (Bio-Rad Manual, 1988). A range of time constants at each field strength studied was achieved by the connection of different resistors in parallel with the sample.

Preparation of cells for electroporation of *Listeria* species with plasmid DNA of greater than 20 kb.

A 20% (v/v) inoculum of an overnight culture of *L. monocytogenes* in brain heart infusion 0.5 M sucrose (BHI/s) was added to 500 ml of BHI/s and incubated at 37°C with shaking (~150 rpm) until an OD₆₀₀ of 0.2 was attained. Penicillin G was then added to a final concentration of 10 μ g/ml, and incubation was continued for a further 2 hrs (OD₆₀₀ 0.35-0.4). The cells were then harvested by centrifugation at 7,000 x g for 10 min at 4°C and washed twice in an equal volume of 1 mM HEPES (pH 7.0), 0.5M sucrose. The cell pellet was drained carefully and resuspended in 1/400 th vol. of HEPES-sucrose. The cells were placed on ice and used immediately for

electroporation.

Electroporation of *Listeria monocytogenes* with plasmid DNA greater than 20 kb.

100 µl of the prepared cell suspension ($\approx 1 \times 10^{10}$ bacteria) were gently mixed with 25 µl of DNA dissolved in sterile nanopure distilled water and left on ice for 1 min. The mixture was then transferred to a chilled electroporation cuvette (0.2 cm²) and placed between the chilled electrodes of a Gene-Pulser™ electroporation apparatus with pulse controller (Bio-Rad, Richmond, California). The cells were electroporated at a field strength of 10 kV/ cm with a time constant of approximately 4.0 ms. The cuvette was placed immediately on ice for 1 min before 1 ml of BHI sucrose was added and the culture incubated at 37°C for 4 hrs. The cells were then plated onto BHI - 1.5% agar, containing selective levels of antibiotic, and the plates incubated at 30°C for 48 hrs.

2.4. Procedures for DNA extraction.

Large scale extraction of chromosomal DNA.

The following solutions were used:-

Sucrose-Tris buffer,
0.05 M Tris-HCl pH 7.5
0.25 M sucrose

Phenol : Chloroform mixture,
Phenol water 1 kg phenol equilibrated in 200 ml NaCl-EDTA (0.1 M NaCl, 0.01 M EDTA [pH 8.0]) mixed 1:1 with chloroform and 0.1% (w/v) 8-hydroxyquinoline.

Tris EDTA buffer (TE) : 10mM Tris Hcl, 1mM EDTA, pH 7.5.

The method was adapted from that described by Saito and Muria (1963). A 1% inoculum of an overnight bacterial culture was added to 500 ml of TSB supplemented with 0.2% glucose, and incubated at 35°C with shaking for 24 hrs. The purity of the culture was then checked by Gram staining before the cells were harvested by centrifugation at 10,000 x g for 10mins at 4°C. The pellet was then washed once in distilled water and once in 1 M NaCl before being resuspended in 10 ml sucrose Tris buffer per 2-3g of dry cell weight. Lysozyme was then added to a final concentration of 1 mg/ml and the cells then incubated at 37°C for 1 hr. TE was then added 1: 5 (v/v) with self digested (37°C for 1 hr) proteinase K to a final concentration of 1 mg/ml. Incubation was continued for 1-2 hrs until lysis was complete.

Prewarmed SDS was then added to the lysed cell suspension 1:10 (v/v). This mixture was gently mixed with 1/3 (v/v) of freshly prepared sodium perchlorate (66.5% w/v in 1 x TE), before the addition of an equal volume of phenol : chloroform mixture (1:1). The mixture was shaken until a stable emulsion was formed and centrifuged at 4,000 x g for 30 min at 4°C. The aqueous phase was then removed and washed twice with chloroform : isoamyl alcohol (24:1 v/v), before centrifugation at 4,000 x g for 15-20 min at 4°C. The aqueous phase was again removed and 2 vols. of ice cold ethanol added to precipitate the nucleic acids which were spooled gently onto a glass rod, briefly air dried and dissolved in 10 ml 0.1 x TE.

The dissolved nucleic acids were treated with heat treated (100°C 1 min) RNAase (final concentration of 60 mg/ml), by incubation at 37°C for 1 hr, before the addition of self digested Proteinase K (final concentration of 0.1 mg/ml) and a further 2 hrs incubation. An equal volume of chloroform : isoamyl alcohol was added, and the mixture shaken for 5 min to obtain an emulsion, which was then centrifuged at 4,000 x g for 15 min at room temperature. The upper aqueous layer was removed and washed with phenol : chloroform until no white precipitate was formed at the interface. DNA was precipitated by the addition of 1/10 th vol. of 3 M sodium acetate in EDTA , with an equal volume of ice cold isopropanol. The DNA was collected by spooling

onto a glass rod, briefly air dried, dissolved in 0.1 x TE and reprecipitated with ethanol before being dissolved in a small volume of 0.1 x TE and stored at 4°C.

Small scale preparation of DNA.

The method was based on that of Flamm *et al.*, (1984): 10 ml of an overnight bacterial culture in brain heart infusion broth (BHI) was centrifuged at 11,700 x g for 10 min at 4°C and washed with 5 ml of 0.1 x SSC (1 x SSC: 0.15 M NaCl, 0.015 M trisodium citrate [pH 7.0]). The pellet was resuspended in 1 ml of 0.01 M sodium phosphate buffer in 20% sucrose (pH 7.0) with 2.5 mg/ml lysozyme and incubated at 37°C for 45 min. 9 ml of 1%SDS in TE was then added with 1 mg/ml proteinase K and the incubation continued for 30 mins. An equal volume of phenol : chloroform mixture was then added and shaken to form an emulsion before centrifugation at 4,000 x g for 15 min. The upper aqueous layer was removed and washed with phenol : chloroform until no white precipitate was formed at the interface. Ice cold ethanol and sodium acetate were then added to precipitate the DNA as described for large scale preparation and the nucleic acids were then collected by spooling and dissolved in a small volume of TE for storage at 4°C.

Large scale extraction of plasmid DNA.

The following solutions were used:-

Solution I,

50 mM glucose

25 mM Tris-HCl pH 8.0

10 mM EDTA

4 mg/ml lysozyme (Sigma)

Solution II,

0.2 M NaOH

1% sodium dodecyl sulphate (SDS)

Solution III (100 ml)

5 M acetate (11.5 ml glacial acetic acid)

3 M potassium ions (60 ml 5 M potassium acetate)

Tris EDTA buffer (TE) : 10mM Tris Hcl, 1mM EDTA, pH 7.5.

Stationary phase bacterial cultures (400 ml) were used for the large scale preparation of plasmid DNA as described by Birnboim and Doly, (1979). Cells were collected by centrifugation at 4,000 x g for 10 min at 4°C and resuspended in 10 ml of solution I, left on ice for 30 mins, before the addition of 20 ml of freshly prepared solution II, and after mixing well incubation continued on ice for 10 mins. 15 ml of ice cold solution III was then added, the mixture placed on ice for a further 10 mins, and cell debris removed by centrifugation at 4°C for 20 mins at 3,500 x g. The resulting supernatant was removed and mixed well with 0.6 vols. of isopropyl alcohol then left to stand for at least 15 min. Nucleic acids were collected by centrifugation at 4,000 x g for 30 mins at room temperature, and the pellet dried *in vacuo*, and resuspended in sterile distilled water to a final volume of 17 ml. Caesium chloride and ethidium bromide were added, to a final concentration of 1 mg/ml and 50 mg/ml respectively and the preparation placed in sealed polyallomar tubes.

Plasmid DNA was separated from other nucleic acids by centrifugation at 40,000 rpm in a Sorvall OTD 60 centrifuge for 20 hours at 20°C using a Sorvall TV850 rotor. DNA was visualised in the tubes under UV light and the lower band of plasmid DNA extracted. 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 ethanol precipitated by standing at -20°C for a minimum of 30 mins with the addition 1/10 th vol. of 3 M sodium acetate, and 2 vols. of ethanol. DNA was collected by centrifugation at 4,500 x g for 30 mins at room temperature.

For the large scale preparation of plasmid DNA from *Listeria* species the procedure was modified: all steps stated to take place on ice were performed at 37°C.

Small scale extraction of plasmid DNA.

Solutions were used as for large scale extraction:-

1.5 ml of a stationary bacterial culture were centrifuged in a bench top minifuge at 5,000 x g for 1 min and the supernatant removed. The pellet was resuspended in 100 µl of solution I and left on ice for 30 mins. Freshly prepared solution II (200 µl) was added to the preparation, which was gently mixed, left on ice for 5 min, and 150 µl of ice cold solution III were then added. After incubation on ice for at least 10 mins, the mixture was centrifuged in a minifuge at 13,000 x g for 5 min, and the supernatant removed. Protein was removed from the preparation by phenol extraction - an equal volume of phenol : chloroform mixture was added and the preparation vortexed before being centrifuged at 13,000 x g for 2 minutes. The upper aqueous phase was removed, mixed with 2 vols. of room temperature ethanol, allowed to stand for 2 mins at room temperature and centrifuged at 13,000 x g for 5 min. After careful removal of the ethanol, the DNA pellet was dried *in vacuo* and dissolved in 50 µl of 0.1 x TE containing 20 µg/ml free pancreatic RNase. DNA was stored at -20°C.

For the small scale preparation of plasmid DNA from *Listeria* species the procedure was modified. All steps stated to take place on ice were performed at 37°C.

2.5. Techniques for routine DNA manipulation.

Restriction endonucleases and DNA modifying enzymes were purchased from Pharmacia Biochemicals Inc or Life Technologies Ltd. (BRL-GIBCO). Restriction endonuclease cleavage of DNA was performed according to the manufacture's recommendations, usually in 20 µl reactions with one unit of enzyme at 37°C. T4 DNA ligase was used at 14°C overnight.

DNA fragments were separated by agarose gel electrophoresis using 0.7% Seakem agarose in Tris-acetate EDTA buffer (TAE: 40 mM Tris-acetate, 1 mM EDTA) with 0.5 mg/ml ethidium bromide and visualised using a longwave UV transilluminator. DNA fragments were separated on a 0.7% agarose gel. Electrophoresis was performed in TAE containing 0.5 mg/ml ethidium bromide at 140 V for 2 hours or at 10 V overnight. DNA samples were mixed with the appropriate volume of 6 x gel-loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol, 15% w/v Ficoll) prior to loading. The DNA size markers used were : 1 kb ladder (BRL-GIBCO) or bacteriophage λ DNA either uncut or digested with *Hind* III. λ DNA markers were incubated at 65°C in gel-loading buffer for 10 mins before use.

DNA fragments were recovered from agarose gels for subcloning and preparation of DNA probes by electroelution. Agarose containing the fragment required was excised from the gel and placed in dialysis tubing containing 300 μ l Tris EDTA buffer (TE). The sample was subjected to 100 V for 30 mins in TAE buffer and the polarity was then reversed for a further 30 s to free any DNA attached to the tubing. TE from around the gel slice was carefully removed, and the DNA recovered from the TE by ethanol precipitation as detailed in section 2.4.

2.6. Construction of suicide vectors pJA1 and pJA2.

Plasmid pJA1 was generated by ligating the 10.4 kb *Pst*I-*Eco*R1 restriction fragment of pTV32 (figure 2.6.1.) predicted to contain Tn917::*lac*, with the 3.4 kb *Pst*I-*Eco*R1 of pBR328 (figure 2.6.2.). After the digestion of pTV32 with *Pst*I and *Eco*R1 12 μ l of the reaction (~ 2.5 μ g of pTV32 DNA) was ligated with 6 μ l (~ 1 μ g of pBR328 DNA) of reaction mixture from the *Pst*I, *Eco*R1 digestion of pBR328. The orientation of the pTV32 fragment was verified by the digestion of 2 μ l of the ligation mixture with the restriction endonucleases *Kpn*I, *Bam*H1, *Hind*III, and *Pst*I-*Eco*R1.

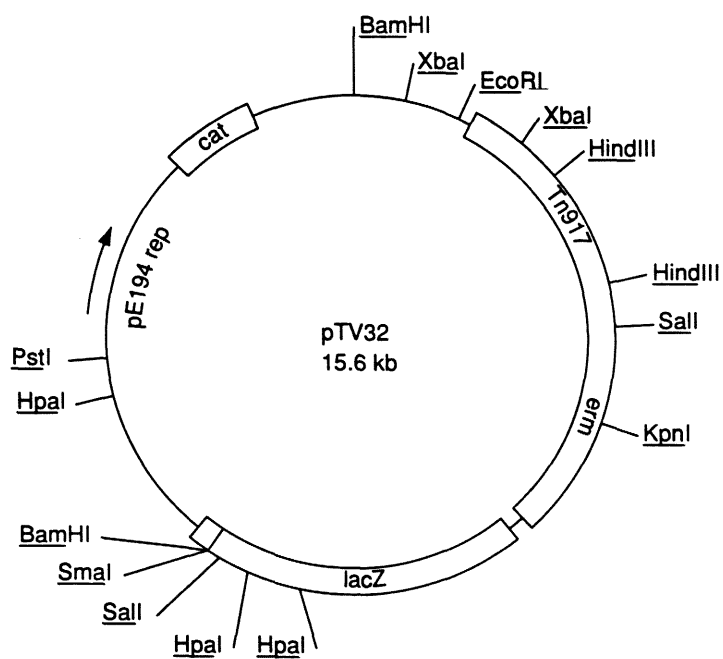


Figure 2.6.1. Restriction enzyme map of plasmid pTV32. Youngman *et al.*, (1984b).

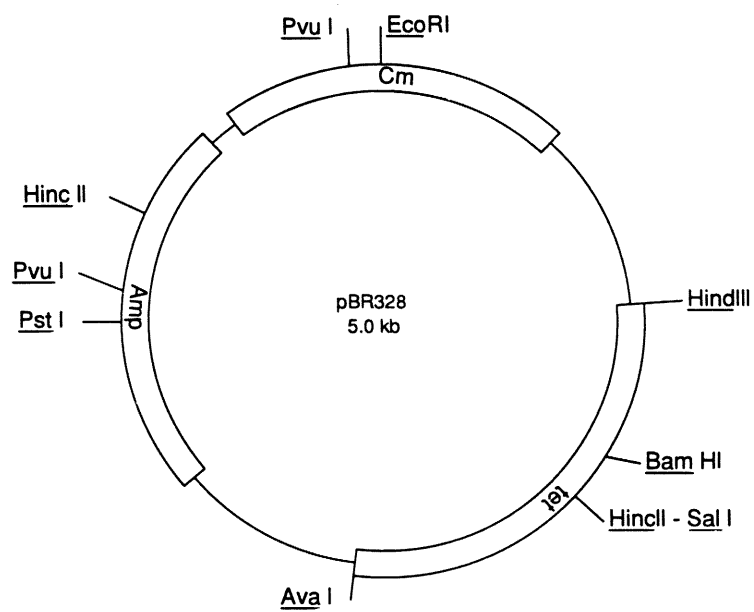


Figure 2.6.2. Restriction enzyme map of plasmid pBR328. Bolivar *et al.*, (1977).

The remaining (28µl) ligation mixture was used to transform competent *E. coli*. Recombinant bacteria carrying pJA1 were selected on L agar containing erythromycin and tetracycline. Mini-plasmid DNA preparations of twelve recombinant colonies were performed and the plasmid DNA from these digested with *Kpn*1, *Bam*H1, *Hind*III, and *Pst*1-*Eco*R1 to confirm that they carried pJA1.

To generate pJA2, the 10 kb *Eco*R1 restriction fragment of pTV52 (figure 2.6.3.) predicted to contain Tn917::cat, was ligated with the pACYC184 DNA (figure 2.6.4.) linearised with *Eco*R1. After the digestion of pTV52 with *Eco*R1 15 µl of the reaction mixture (~ 2.0 µg of pTV52 DNA), were ligated with 6 µl of reaction mixture (~ 1 µg of pACYC184 DNA) from the *Eco*R1 digestion of pACYC184.

The orientation of the pTV52 fragment was verified by the digestion of 2µl of the ligation mixture with the restriction endonucleases *Kpn*1, *Bam*H1, *Hind*III, and *Sal*1 and analysis by agarose gel electrophoresis. The remaining (28µl) ligation mixture were used to transform competent *E. coli*. Recombinant bacteria carrying pJA2 was selected on L agar containing erythromycin and tetracycline. Mini plasmid DNA preparations of twelve recombinant colonies were performed and the plasmid DNA from these was digested with *Kpn*1, *Bam*H1, *Hind*III, and *Sal* to confirm that they carried pJA2.

2.7. Transfer of DNA to nylon filters by Southern blotting.

DNA was transferred to filters as described by Southern (1975). DNA samples were separated by agarose gel electrophoresis as described in section 2.4, and the gel photographed next to a linear scale. The DNA was depurinated by soaking the gel in 0.25 M HCl for 7 mins. After rinsing briefly in distilled water the gel was placed in denaturing solution (0.5 M NaOH, 1.5 M NaCl) for 30 mins with occasional shaking. The gel was again rinsed in distilled water and placed in neutralising solution (0.5 M

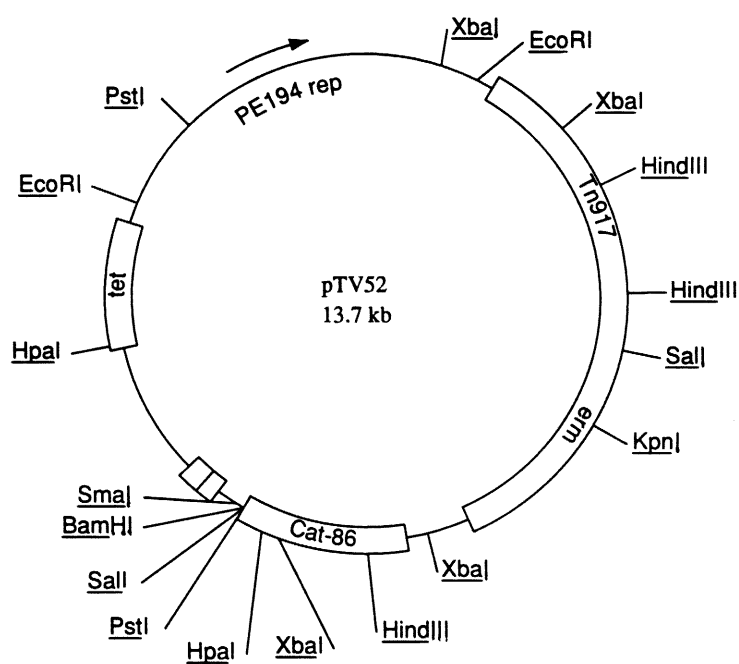


Figure 2.6.3. Restriction enzyme map of plasmid pTV52. Youngman *et al.*, (1984b).

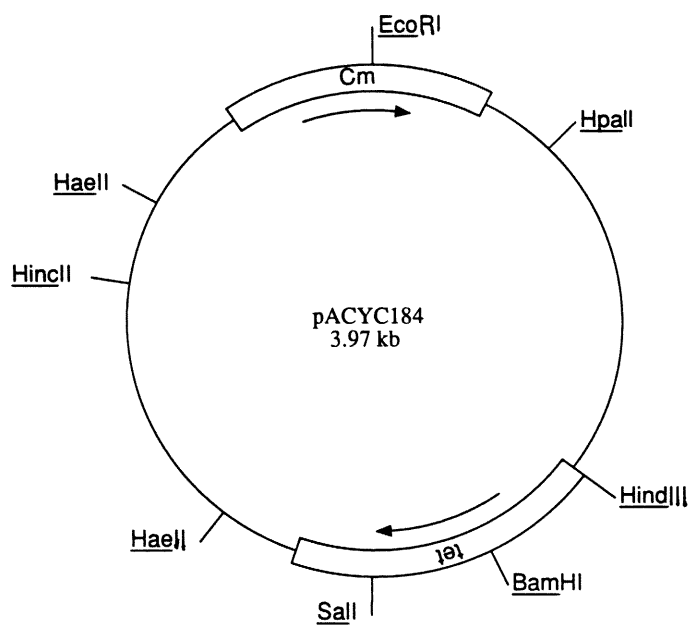


Figure 2.6.4. Restriction enzyme map of plasmid pACYC184. Chang and Cohen, (1976).

Tris-HCl pH 7.5, 3 M NaCl) for 30 mins with occasional shaking, and after rinsing as before was placed on six sheets of Whatman paper (3 mm) presoaked in 10 x SSC (1 x SSC: 0.15 M NaCl, 0.015 M trisodium citrate [pH 7.0]) making sure no air bubbles were trapped. A presoaked (5 x SSC) sheet of nylon membrane (Hybond-N, Amersham International PLC) was placed on the gel and covered with a presoaked (10 x SSC) sheet of Whatman paper, again avoiding air bubbles between layers. Four dry sheets of Whatman paper were placed on top and covered with a stack of paper towels cut to size. The whole assembly was weighed down with a 200-500 g weight, the lower sheets were regularly soaked in 20 x SSC and the paper towels regularly changed. The assembly was left overnight to allow DNA to transfer. The filter was then removed, briefly rinsed in 5 x SSC and thoroughly air dried on dry Whatman paper. The filter was then wrapped in Saran wrap and exposed to UV light from a long wave transilluminator for 5 mins to fix the DNA for hybridisation.

2.8. DNA hybridisation procedures.

Production of a radiolabelled probe.

Plasmid DNA was cleaved with the appropriate restriction endonucleases and the fragments separated by agarose gel electrophoresis on a 1% low melting point agarose gel (BRL). The required DNA fragment was excised from the gel and treated as described in section 2.4. The recovered DNA was again subjected to electrophoresis and the required DNA fragment excised from the gel and added to sterile distilled water (1.5 ml water /g agarose). The sample was placed in a boiling water bath for 7 mins then stored at -20°C. Prior to use the sample was boiled for an additional 5 mins and incubated at 37°C for 10 mins.

DNA was radiolabelled using random hexanucleotide primers as described by Feinberg and Vogelstein (1983). Nucleotides and hexanucleotides were obtained from Pharmacia and [α - 32 P] dCTP from Amersham International PLC. To confirm that the DNA probe had been successfully labelled and to remove any extraneous unbound 32 P, G50 Sephadex column chromatography was used. Sephadex slurry was prepared by slowly adding G50 Sephadex resin to water (10 g: 160 ml) with stirring and then

left to swell overnight. The swollen resin was washed several times with distilled water to remove any soluble dextran present and then equilibrated in Tris EDTA buffer (pH 7.6), before being autoclaved at 10 lb/square inch for 15 mins and stored at room temperature.

A Pasteur pipette was plugged with a small amount of sterile glass wool to form a column, and was filled with sephadex slurry (avoiding the inclusion of air bubbles) to within 1 cm of the top. The column was washed with several volumes of Tris EDTA NaCl buffer (TEN: 1 x TE, 100 mM NaCl [pH 8.0]). The Radiolabelled DNA probe preparation was made up to 100 μ l with sterile TEN buffer and applied to the top of the column. As soon as the DNA probe had entered the sephadex 100 μ l of 1 x TEN buffer were applied. Fractions (100 μ l) were collected at the bottom of the column and 1 x TEN buffer applied as necessary at the top. The fractions were monitored for radioactivity with a Geiger counter until two peaks of emission were observed and the fractions were then transferred to a scintillation counter (Packard Mininaxi Tricarb 4000) and the emissions of ^{32}P radioactivity counted for 1 min. The most radioactive fractions from the leading peak of emission was pooled to a volume not exceeding 500 μ l and used as the DNA probe, the second peak of emission represented unbound ^{32}P and was safely discarded.

Hybridisation of DNA immobilised on filters with a radiolabelled probe.

Hybridisation used the following solutions :

Prehybridisation solution

3 x SSC, (1 x SSC: 0.15 M NaCl, 0.015 M trisodium citrate [pH 7.0]).

5 x Denhardts (1 x Denhardts: 1% each Ficoll, BSA, polyvinolpyrrolidone).

0.1% SDS,

6% PEG 6000,

Hybridisation solution

6 x SSC, (1 x SSC: 0.15 M NaCl, 0.015 M trisodium citrate [pH 7.0]).

5 x Denhardtts (1 x Denhardtts: 1% each Ficoll, BSA, polyvinolpyrrolidone).

0.1% SDS,

6% PEG 6000,

Both solutions were stored at -20°C.

Prior to use 200 µg/ml salmon sperm DNA (sheared by forcing through a narrow gauge syringe needle and denatured by boiling for 5 min), were added.

Southern blot filters were shaken at 65°C in 25 ml of prehybridisation solution for 2 hrs. This solution was then replaced by 25 ml of hybridisation solution containing radiolabelled probe DNA which had been boiled for 5 mins before addition. To allow hybridisation the filter was shaken under the same conditions overnight.

After hybridisation the filter was washed four times by shaking for 15 mins in 250 ml of 1 x SSC at 65°C. The filter was then completely air dried for 1 hr, wrapped in Saran wrap and placed in an autoradiography cassette carrying intensifying screens. Kodak X-Omat AR film was exposed to the filters at -70°C. Films were developed in an Agfa-Geveart automatic film processing machine.

Removal of bound probe from a filter.

The probe was stripped from some filters following autoradiography then rehybridised with another probe. The probe was removed according to the manufacturers instructions: the filter was shaken in 0.4 M NaOH at 45°C for 30 mins and then transferred to 100 ml prewarmed buffer (0.1 x SSC, 0.1% SDS, 0.2 M Tris-HCl [pH 7.5]) and shaken at 45°C for a further 30 mins. Removal of the probe was confirmed by the reduction of deflection of a Geiger counter to background levels.

2.9. Insertional mutagenesis of bacteria.

Transposon mutants of *L. monocytogenes* strain EGD were generated in liquid culture by modification of the method of Youngman et al., (1987). A single bacterial colony from plate containing erythromycin, chloramphenicol, and lincomycin was used to inoculate a 10 ml of Tryptose Soya (TS) broth containing erythromycin, chloramphenicol, and lincomycin. The culture was incubated at 30°C until an OD₆₀₀ of at least 0.5 and no greater than 0.7 was achieved. The culture was then diluted 1:100 into prewarmed (48°C) TS broth containing erythromycin, and lincomycin, but no chloramphenicol. This culture was incubated at 48°C with shaking for 12-16 hrs. A 1: 50 dilution of this culture was made in TS broth as before and the resulting culture incubated at 48°C to an OD₆₀₀ of 2.0-3.0. The bacteria were collected by centrifugation at 4,000 x g for 10 mins. Cells were resuspended in 1/25 th the original culture volume of TS broth containing 10% glycerol and dispensed into 500 µl vols., which were stored at -70°C.

L.monocytogenes. strain EGD did not grow at temperatures over 41°C, a number of overnight outgrowth stages at 41°C were incorporated (section 3.4) before the last 1: 50 dilution and final incubation to an OD₆₀₀ of 2.0-3.0 which also took place at 41°C.

2.10. Selection and quantitation of insertion mutants.

A sample of harvested bacteria was serially diluted in sterile distilled water and dilutions plated in triplicate onto agar plates containing erythromycin, lincomycin, and for quantification onto tryptose soya agar (TSA) plates containing erythromycin, lincomycin, and chloramphenicol. Plates were incubated at 30°C for 24 hrs and the colonies present counted. To allow the frequency of transposition to be determined as detailed by Youngman *et al.*, (1989), dilutions of the harvested bacteria were plated out

in triplicate onto agar plates containing erythromycin, lincomycin, and the number of colony forming units (cfu) present at permissive (30°C), and nonpermissive (41°C) temperatures for plasmid replication determined. The transposition frequencies were calculated by dividing the number of cfus at the nonpermissive temperature (41 °C) by those at the permissive temperature (30°C). Frequencies were performed in triplicate and the mean found.

To detect any Tn917 insertions into transcriptionally active regions of the *L. monocytogenes* EGD chromosome, dilutions of the insertion mutants were plated onto TS agar plates containing 40mg of X-gal (5-bromo-4-chloro-3-indoyl-β-D-galactoside), and incubated for 48 hours at 30°C.

2.11. Determination of minimal inhibitory concentrations.

The loss of plasmid antibiotic resistance markers from insertion mutants was determined by comparison of their minimum inhibitory concentration of erythromycin and chloramphenicol. Single insertion mutant colonies were streaked onto agar containing erythromycin, and lincomycin, and the plates were incubated for 24 hrs at 30°C. Cells from each culture were added to 1 ml of TSB to form a suspension which was adjusted to an OD₆₀₀ of 0.5. In triplicate 100 µl of each suspension was added to 5 ml serial dilutions of antibiotic, and the dilutions were incubated at 30°C for 24 hrs. The minimum inhibitory concentration was taken as the lowest concentration which inhibited growth and was expressed as mean minimum inhibitory concentration of ten insertion mutants.

2.12. Selection of insertion mutants unable to utilise rhamnose.

To detect those mutants unable to produce acid from rhamnose, (the ability to ferment rhamnose is an important criterion in the identification of *Listeria monocytogenes*), insertion mutants were patched onto the modified basal medium of Wilkinson and Jones (1977) supplemented with 2% (w/v) rhamnose and 0.004% (w/v) chlorophenol red. Mutants were serially diluted in sterile distilled water and dilutions plated for single colonies onto tryptose soya agar (TSA) plates containing erythromycin, lincomycin, and incubated for 24 hrs at 30°C. Single colonies were picked from the agar using a sterile tooth-pick and patched consecutively onto modified basal medium and onto TSA plates containing erythromycin, lincomycin, plates were incubated at 30°C for 24 hrs. Mutants unable to produce acid from rhamnose, were those which did not produce a change in the colour of the media from purple to yellow.

2.13. Comparison of minimal media for selection of auxotrophic insertion mutants.

The following minimal media were compared :

Friedman and Roessler (1961):

Salts A :	/100 ml	Salts B :	
NaCl	250 mg	K ₂ HPO ₄	600 mg
MgSO ₄ .7H ₂ O	80 mg		
FeSO ₄ .7H ₂ O	4 mg		
Na-citrate	40 mg		
Glucose	600 mg		
Amino acid supplement :			
DL-Alanine	80 mg	L-Tyrosine	0.01%
DL-Aspartic acid	80 mg	DL-Valine	20 mg
L-Arginine	80 mg	DL-Tryptophan	20 mg
L-Lysine-HCl	20 mg	L-Cytosine-HCl	10 mg
L-Histidine-HCl	10 mg	DL-Serine	20 mg

DL-Isoleucine	20 mg	Glycine	10 mg
L-Leucine	10 mg	L-Glutamine	60 mg
DL-Methionine	20 mg	L-Asparagine	50 mg
L-Phenylalanine	10 mg	β -Alanine	80 mg
L-Proline	10 mg		
L-Threonine	10 mg		

Vitamins :

Riboflavin	5 mg
Thiamine-HCl	5 mg
Biotin	5 mg
α -Lipoic acid	5 mg

Welshimer (1963):

Basal:	/100 ml
KH_2PO_4	328 mg
Na_2HPO_4	820 mg
MgSO_4	20 mg
Glucose	1 mg

Amino acids:	10 mg
L-Cysteine-HCl	10 mg
L-Leucine	20 mg
DL-Isoleucine	20 mg
DL-Valine	60 mg
L-Glutamine	20 mg
DL-Methionine	20 mg
L-Histidine-HCl	20 mg
L-Arginine-HCl	20 mg
DL-Tryptophan	20 mg

Vitamin supplement:

Riboflavin	100 μg
Biotin	10 μg
Thiamine-HCl	100 μg
DL-Thioctic acid	0.1 μg

Ralovich *et al* (1977):

Basal:	Concn./ 100 ml
KH_2PO_4	450 mg
K_2HPO_4	1.05 g
trisodium citrate. $2\text{H}_2\text{O}$	50 mg
$(\text{NH}_4)_2\text{SO}_4$	100 mg
dextrose (anhydrous)	1.0 g
sodium thioglycollate	110 mg
$\text{FeSO}_4 \cdot 2\text{H}_2\text{O}$ (dried)	0.7 mg
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	20 mg
$\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$ (hydrated)	1.3 mg

Amino acids:

DL-Isoleucine	20 mg
L-Leucine	10 mg
DL-Valine	20 mg
L-Cysteine-HCl	40 mg

Vitamins:

Riboflavin	0.2 mg
D-Biotin	0.2 mg
Thiamine-HCl	1.0 mg

Siddiqi and Khan (1989):

Basal:	/100ml
Glucose	400 mg
$(\text{NH}_4)_2\text{SO}_4$	500 mg
NaCl	500 mg
KH_2PO_4	150 mg
Na_2HPO_4	250 mg
KCL	50 mg
FeCl_3	10 mg

Amino acids:

Cysteine	50 mg
Isoleucine	50 mg
Lysine	50 mg
Leucine	50 mg
Methionine	50 mg
Phenylalanine	50 mg

Threonine	50 mg
Tryptophan	50 mg
Valine	50 mg

Vitamins:

Riboflavin	0.5 mg
Folic acid	0.5 mg
D-Biotin	0.5 mg
Thiamine-HCl	0.5 mg

Nitrogen bases:

Adenine	0.5 mg
Cytosine	0.5 mg
Guanine	0.5 mg
Thymine	0.5 mg

Minimal media were made up as recommended with the addition of 1.5 % purified agar. Plates were streaked with 100 µl of a fresh overnight culture of *L. monocytogenes* (NCTC7973), and a TS agar plate inoculated as a control, inoculated plates were incubated at 30°C for one week and were examined for growth every 24 hrs. Media supporting good growth was then inoculated in the same way with *L. innocua* (NCTC 11288), *L. ivanovii* (ATCC 19119), and *L. seeligeri* (CIP 100100), to check for any species variation.

2.14. Selection of auxotrophic insertion mutants.

The minimal medium used for the selection of auxotrophic insertion mutants was that of Friedman and Roessler (1961). Samples of harvested insertion mutants were serially diluted in sterile distilled water and dilutions were plated for single colonies onto tryptose soya agar (TSA) plates containing erythromycin, lincomycin, and incubated for 24 hrs at 30°C. Single colonies were then picked from the agar using a sterile tooth-pick and patched consecutively onto minimal media plates without the amino acids of interest and onto TSA plates containing erythromycin, and lincomycin. The plates were incubated for 24 hrs at 30°C and were then compared. Any mutant

which did not grow on the minimal media but grew on the TSA plate containing erythromycin and lincomycin was repicked and the process repeated. If the phenotype was maintained the mutant was selected for further investigation. The specific amino acid requirements of the auxotrophs were determined by addition of amino acids and biosynthetic intermediates to the media at a concentration of 20 µg/ml.

2.15. Confirmation of insertion site by the measurement of enzyme activities.

Preparation of enzyme extracts.

Extracts were prepared by a modification of the method of Nester and Jensen (1966) from cells grown in minimal media, plus 0.5% glucose, and the required supplements (Tyrosine or phenylalanine 20 µg/ml). The cells were harvested by centrifugation at 4,000 x g for 10 mins, and washed once with the buffer (ice cold) to be used in the enzyme assay. Cell lysis was performed by periodic ultrasonic treatment on ice with a Labsonic 2000 at low power for 10 mins. Cell debris was sedimented by centrifugation at 25,000 x g for 30 min and the supernatant dialysed against 500 vols of Tris buffer (10 mM Tris-HCl, 1 mM K₂HPO₄ [pH 7.5]) overnight at 4°C. The protein concentration of the enzyme preparation was determined using a Bio-Rad protein assay kit according to the manufacturers instructions, before its storage at -20°C.

Measurement of chorismate mutase activity.

To confirm the identity of the enzyme defective in the aromatic amino acid auxotrophic mutants isolated, the specific activity of chorismate mutase was measured as detailed by Lorence and Nester (1967). The reaction mixture contained: 0.1 ml chorismic acid (10 µM/ml), 0.1 ml enzyme preparation, 0.3 ml Tris-maleate buffer (0.05 M Tris-HCl, 300 µM maleate, [pH 6.4]). Chorismic acid solutions were freshly prepared in Tris-maleate buffer for each assay.

The mixture was incubated at 37°C for 30 min and the reaction terminated by the addition of 0.15 ml 20% trichloroacetic acid. The prephenate formed was converted to phenylpyruvate by incubation for 10 min at 0°C, and a series of prephenate standards in Tris-maleate buffer were treated similarly. The optical density of the solution, prephenate standards and phenylpyruvate standards in Tris-maleate buffer were measured at 310 nm after the addition of 1.5 ml arsenate-borate solution (2 M arsenate, 1 M borate [pH 6.5]), and incubation at room temperature for 15 min. Specific activity of chorismate mutase was calculated using the definition of 1 unit of chorismate mutase as 1 nM prephenic acid formed per minute/ mg of protein. The molar absorption coefficient of phenylpyruvate in arsenate-borate solution at 300 nm was taken to be 9292, and the limit of detection was 0.001 units.

Measurement of prephenate dehydratase activity.

To confirm the identity of the enzyme defective in the aromatic amino acid auxotrophic mutants isolated, the specific activity of prephenate dehydratase was measured as detailed by Cotton and Gibson (1965). The reaction mixture contained: 0.1 ml barium prephenate (6 µM/ml), 0.1 ml enzyme preparation, 0.2 ml buffer (10 mM Tris-HCl, 0.1 mM EDTA, 1 mM MgCl₂, 6 mM mercaptoethanol [pH 8.1]), and 0.1 ml 25 µM K₂HPO₄. The reaction mixture was incubated at 37°C for 30 min and the reaction terminated by the addition of 0.15 ml 20% trichloroacetic acid. The phenylpyruvate formed was measured by the optical density of the solution at 320 nm after the addition of 1.5 ml of 0.8 N NaOH and incubation at room temperature for 15 min. Specific activity of prephenate dehydratase was calculated using the definition of 1 unit of prephenate dehydratase as 1 nM prephenate formed per minute/ mg of protein. The molar absorption coefficient of phenylpyruvate in NaOH solution at 320 nm was taken to be 17500, and the limit of detection was 0.001 units.

2.16. Materials and methods for *In vivo* investigations of Lm.918.6 and Lm.6b. *aro*⁻ virulence in mice.

Animals.

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

Growth and preparation of bacteria.

The bacteria were grown in tryptose soya broth (TSB) in stationary culture at 30°C to late log phase (OD_{600} 0.7-0.8), harvested by centrifugation at 4,000 x *g* for 10 min and resuspended in TSB containing 10% glycerol (v/v). Portions of this suspension were then stored at -20°C for several months with no significant loss of viability. When required the suspension was thawed, and bacteria were harvested by centrifugation before resuspension in sterile distilled water to the required concentration.

Infection of mice.

Mice were inoculated in groups of five by the intravenous (i.v.) route. For each i.v. injection the appropriate dose of viable bacteria contained in a total vol. of 100 μ l of distilled water was injected into the tail vein of each mouse. In all cases the number of bacteria inoculated was confirmed by plating serial dilutions on TS agar.

Determination of 50% effective dose.

Virulence was estimated by determination of the 50% effective dose (ED_{50}) i.e. the dose which results in 50% of the group injected becoming moribund. Doses of bacteria over the range 1.0×10^3 - 1.0×10^8 colony forming units (cfu) were administered to groups of five mice. Infection was allowed to proceed for 6 days by which time any recovery from infection was apparent. The number of mice per group that had reached the end point of the assay (i.e. became moribund) between day 0 and day 6 was recorded, and used to calculate the ED_{50} by the log-probit method (Finney, 1962).

Enumeration of bacteria in host tissue.

Following infection, groups of five mice were killed by cervical dislocation after 1,3,5,7,9 and where the animals survived, 15 days. To follow bacterial numbers over the first 24 hrs after infection, groups of five mice were killed by cervical dislocation every 4 hrs. In all cases the spleen and liver were removed, weighed and homogenised separately in 10 ml of sterile distilled water in a Seward Stomacher-Lab blender (Seward Medical) for 1 min. The homogenates were then serially diluted in sterile distilled water and counts performed in duplicate as described by Stephens *et al.*, (1991) on tryptose soya agar containing erythromycin and lincomycin when necessary. The plates were incubated for 12-24 hrs and results were expressed as mean counts of viable listeriae / g of tissue. Organisms isolated from tissues were also routinely checked for aromatic auxotrophy on the minimal medium of Friedman and Roessler, (1961).

Splenomegaly.

The spleens from groups of 5 mice were weighed individually before homogenisation. Splenomegaly was expressed as a splenic index:

$$\text{splenic index} = \frac{\text{mean spleen weight (g)} \times 200}{30\text{g}}$$

The constant, 30, is the mean weight of the mice in the study. The factor 200 was chosen to give a splenic index of 1 for a healthy 30g MF1 mouse.

Vaccination and challenge of mice.

Mice were vaccinated (i.v.) with a dose of 1.0×10^4 cfu of Lm. 918.6 in water. When given, the booster vaccination was an identical dose of Lm. 918.6 given 14 days after the initial vaccination. Twenty eight days after the initial dose of Lm. 918.6 the ED₅₀ of Lm. 1070138 in the mice was determined as described earlier.

2.17. Statistical analysis.

95% confidence limits were calculated by the log-probit method (Finney, 1962). Data to be compared were analysed for statistical significance by the Mann-Whitney U-test (Jones, 1973), and the Kolmogorov-Smirnov test (Jones, 1973), was used to compare the clearance of bacteria.

2.18. Serum antibody response.

Harvesting of serum.

Blood was withdrawn from the tail vein of mice approximately every 3-5 days, and was allowed to clot for 3 hrs at room temperature. The clot was freed from the walls of the container and left to stand overnight at 4°C to retract. The serum was taken from around the clot and centrifuged at 1,300 x g for 2 mins to remove any remaining red blood cells, before storage at -20°C until use.

Preparation of whole cell antigens.

Antigen was prepared as described by Miettinen *et al.*, (1990); bacteria were grown in 1 L of TS broth at 20°C for 18 hrs and harvested by centrifugation at 5,000 x g for 10 mins. The cell pellet was then washed twice with PBS (pH 7.2), and resuspended in 5 ml of PBS. Cell lysis was performed on ice by periodic ultrasonic treatment with a Labsonic 2000 at low power for 10 mins. Remaining whole bacteria were removed by centrifugation at 5,000 x g for 30 mins. The supernatant was dialysed against PBS for 18 hrs at 4°C, and the resulting antigen stored at -20°C.

Determination of circulating antibody.

Antibody determination was performed using an enzyme linked immunosorbant assay system (ELISA) according to Jalonen *et al.*, 1989. The ELISA was performed using Nunc-Immuno Maxisorp plates (Nunc Gibco Europe, Uxbridge, UK.). Plates were sensitised with diluted antigen (30 µg/ml, protein) in 0.25 M sodium carbonate buffer, (1 M Na₂CO₃, 1 M NaHCO₃, 0.14 M NaCl [pH 9.6]), at 4°C overnight. To remove unbound antigen the plates were washed three times with PBS (pH 7.2), containing

0.01% Tween 20, and blocked with 1% caseine, 3% foetal calf serum, in sodium carbonate buffer, for 2 hrs at 37°C. After washing three times as before, the plate was incubated for 2 hrs with serial dilutions of a 1 : 50 dilution of serum in dilution buffer (PBS, 0.01% Tween 20, 1% bovine serum albumin [BSA]). To remove unbound antigen plates were washed three times as before and incubated for 1 hr at 37°C with horseradish peroxidase-conjugated goat immunoglobulins to mouse immunoglobulins, diluted 1 : 2000 in sodium carbonate buffer 1% caseine, 3% foetal calf serum. Unbound conjugate was removed by washing three times as before and bound conjugate was reacted with o-phenylenediamine at a concentration of 0.34 mg/ml in freshly prepared buffer (0.1 M citrate- Na_2HPO_4 [pH 5.5]), activated with 0.002% v/v H_2O_2 . After 30 min incubation at 37°C the reaction was terminated by the addition of 30% H_2SO_4 and the colour produced, measured at 490 nm in an MR600 microplate reader (Dynatech).

2.19. Intracellular growth of auxotrophic *Listeria* strains in bone marrow derived macrophages.

This procedure was kindly performed by Dr. D. Portnoy; Dept. of Microbiology, University of Pennsylvania, as described in Portnoy *et al.*, (1988). Briefly:

Tissue culture cells and growth medium.

Bone marrow macrophage cells were extracted from the femur of killed disease free female CD-1, ICR mice (Charles River Laboratories, Wilmington MA). Cells (1.0×10^7 / ml) were then grown in 50 ml of Bone Marrow Macrophage Media (BMMM : 50 ml Dulbecco's modification of Eagles medium [DME], 20 ml foetal calf serum, 30 ml L-cell supernatant, 1 ml 0.1 M pyruvate, 100 x Eagle basal medium, 100 x PBS), for three days. 20 ml of BMMM was then added to the cells and incubation was continued for a further three days. Cells were then washed in 2 x PBS. Macrophages (2.0×10^6) in BMMM were then used to seed sterile coverslips which were incubated overnight under CO_2 , and examined for growth of a monolayer before use the next day.

Preparation bacterial cells.

Bacteria were grown overnight at 30°C in 20 ml of BHI to an OD₆₀₀ of 0.9, and harvested by centrifugation at 4,000 x g for 10 min. The cell pellet was washed twice in 2 x PBS and diluted in PBS.

Intracellular growth assay.

Macrophage seeded cover slips were infected with 10⁶ bacteria and cultured in prewarmed (37°C) medium (PBS, DME, 10% foetal calf serum, glutamine), and incubated at 37°C in a CO₂ incubator. At the desired time points 10 µg/ml gentamycin was added and after 1 hr the coverslips were removed. The number of bacteria per coverslip was determined in triplicate by washing the slip in PBS and vortexing for 15 s in 5 ml of distilled water to lyse the infected cells. Dilutions of the cell lysate were then plated onto brain heart infusion (BHI) agar and incubated overnight at 37°C, the number of bacteria per coverslip was calculated and represented the mean of three coverslips.

Results

3.0. Construction of suicide vectors.

To facilitate the construction of *aro*⁻ strains of *L. monocytogenes* by transposon mutagenesis, the vectors pJA1, and pJA2 were constructed. These vectors were designed to deliver the transposon Tn917 *lacZ*, and *car86* gene fusions into *L. monocytogenes* to permit the transposition of these elements into the chromosome. Plasmid pJA1 was generated by ligating the 10.4 kb *Pst*I-*Eco*R1 restriction fragment of pTV32, with the 3.4 kb *Pst*I-*Eco*R1 restriction fragment of pBR328. Plasmid pJA2 was generated by the ligation of the 10 kb *Eco*R1 restriction fragment of pTV52, to pACYC184 DNA linearised with *Eco*R1.

Construction of plasmid pJA1.

Plasmid pTV32 DNA (5 µg) was digested with the endonucleases *Pst*I and *Eco*R1 and 1 µl of the reaction mixture analysed by agarose gel electrophoresis to confirm that the restriction had reached completion. A 10.4 kb restriction fragment predicted to contain Tn917::*lac* was distinguishable (figure 3.0.1. track 1).

Plasmid pBR328 DNA (5 µg) was digested with the endonucleases *Pst*I and *Eco*R1, 1 µl of this digest was removed, and analysed by agarose gel electrophoresis indicating that restriction was complete and that a 1.6 kb fragment had been removed leaving a 3.4 kb fragment (figure 3.0.2. track 1).

After ligation the orientation of the pTV32 fragment was determined by the digestion of 2 µl of the ligation mixture with the restriction endonucleases *Kpn*I, *Bam*H1, *Hind*III, and *Pst*I-*Eco*R1 (figure 3.0.3 tracks 5 - 8). The results of these digests allowed the restriction map of pJA1 to be constructed (figure 3.0.4). The remaining (28µl) ligation mixture was used to transform competent *E. coli*, and recombinant bacteria carrying pJA1 were selected on L agar containing erythromycin and tetracycline. Small scale plasmid DNA preparations of twelve recombinant colonies

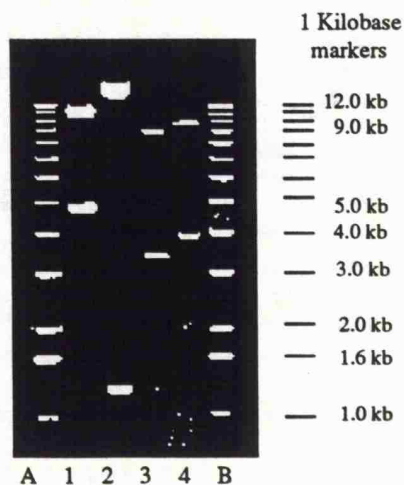


Figure 3.0.1. Agarose gel electrophoresis of : track 1: pTV32 cleaved with restriction endonucleases *Pst* I and *EcoR*I. Track 2: pTV32 cleaved with restriction endonuclease *Hind* III. Track 3: pTV52 cleaved with restriction endonuclease *Hind* III, track 4: pTV52 cleaved with restriction endonuclease *EcoR*I. Tracks A and B: 1 kilobase marker DNA.

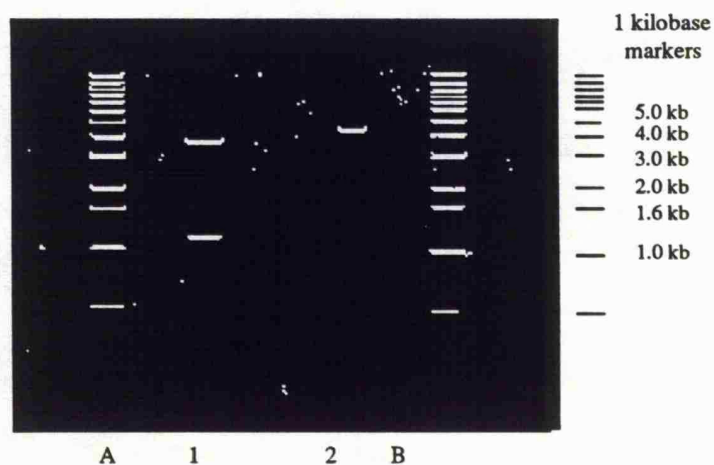


Figure 3.0.2. Agarose gel electrophoresis of : track 1: pBR328 cleaved with restriction endonucleases *Pst* I and *EcoR*I. Track 2: pACYC184 cleaved with restriction endonuclease *EcoR*I. Tracks A and B: 1 kilobase marker DNA.

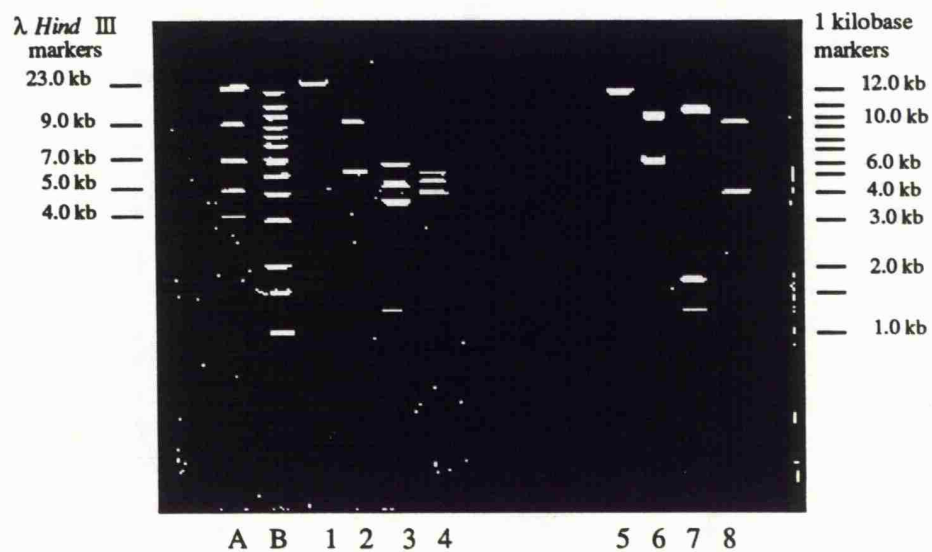


Figure 3.0.3. Agarose gel electrophoresis of : tracks 1 - 4: pJA2 cleaved with restriction endonucleases *Kpn* 1, *Bam* H1, *Hind* III, and *Sal* 1. Tracks 5 - 8: pJA1 cleaved with restriction endonucleases *Kpn* 1, *Bam* H1, *Hind* III, and *Eco*R 1 - *Pst* 1. Track A: marker λ phage DNA restricted with endonuclease *Hind* III, track B: 1 kilobase marker DNA.

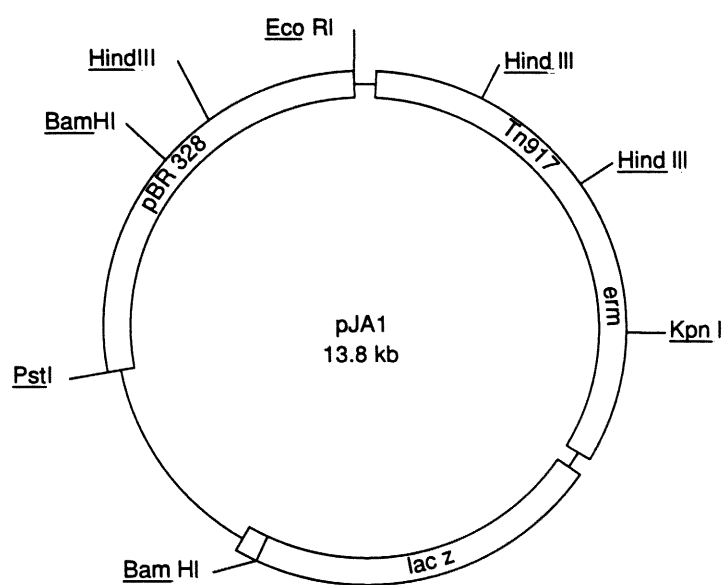


Figure 3.0.4. Restriction map of plasmid pJA1.

were performed and the plasmid DNA from these digested with *Kpn*1, *Bam*H1, *Hind*III, and *Pst*1-*Eco*R1 to confirm that they carried pJA1. The results indicated that eight of the twelve recombinant colonies carried pJA1 DNA of the correct size and restriction pattern and one of these was used to make large scale preparations of pJA1.

Construction of plasmid pJA2.

Plasmid pTV52 DNA (5 µg) was digested with endonuclease *Eco*R1, and 1 µl was similarly analysed by agarose gel electrophoresis. A 10 kb restriction fragment predicted to contain Tn917:: *cat* was visible (figure 3.0.1. track 4).

Plasmid pACYC184 DNA (5 µg) was digested with the endonuclease *Eco*R1, 1 µl of this digest was removed, and analysed by agarose gel electrophoresis. The results indicated that digestion had been complete and that the plasmid had been linearised giving a single 4.0 kb fragment (figure 3.0.2. track 2).

To generate pJA2, the 10 kb *Eco*R1 restriction fragment of pTV52, was ligated with the pACYC184 DNA linearised with *Eco*R1. The orientation of the pTV52 fragment was determined by the digestion of 2 µl of the ligation mixture with the restriction endonucleases *Kpn*1, *Bam*H1, *Hind*III, and *Sal*1 and analysis by agarose gel electrophoresis (figure 3.0.3. tracks 1 - 4). The results of these digests allowed the restriction map of pJA2 to be constructed (figure 3.0.5). The remaining (28 µl) ligation mixture was used to transform competent *E. coli*, and recombinant bacteria carrying pJA2 were selected on L agar containing erythromycin and tetracycline. Small scale plasmid DNA preparations of twelve recombinant colonies were performed and the plasmid DNA from these digested with *Kpn*1, *Bam*H1, *Hind*III, and *Sal*1 to confirm that they carried pJA2. The results indicated that all of the twelve recombinant colonies carried pJA2 DNA of the correct size and restriction pattern and one of these was used to make large scale preparations of pJA2.

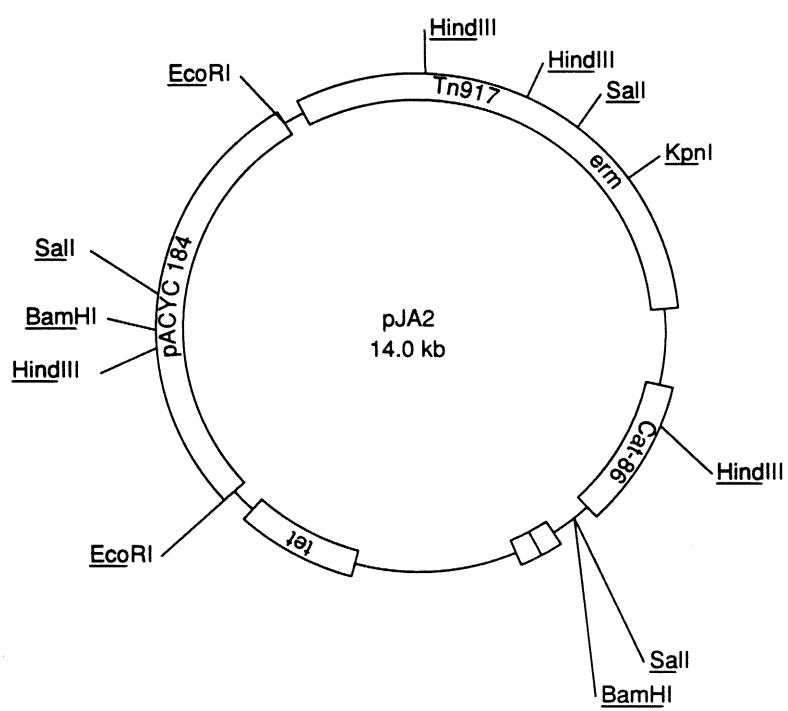


Figure 3.0.5. Restriction map of plasmid pJA2.

3.1. Electroporation of *Listeria* species.

To facilitate the entry of pJA1, and pJA2 into *L. monocytogenes*, an efficient transformation system was required. Conjugation and protoplast transformation have been reported with low levels of frequency. Electrotransformation offered a more efficient system for the introduction of DNA into listeriae and an optimised procedure was developed for this purpose. The results of the experiments with *L. monocytogenes* (NCTC 7973) and the plasmid pGK12, a 4.3 kb plasmid known to replicate in a wide range of host bacteria, are listed in table 3.1.1. This plasmid was chosen because it had previously been successfully electroporated into *L. monocytogenes* (Luchansky *et al* 1988). As can be seen (table 3.1.1.) both the field strength and time constant influence the degree of transformation but for each field strength there was an optimal time constant. The application of a field strength above 8.5 kV/cm exceeded the conductivity of the electroporation medium and resulted in arcing. With a low time constant, approximately 2 ms, at field strengths of 3.1 or 6.2 kV/cm no transformants were recovered. With field strengths of 3.1 and 6.2 kV/cm transformation was maximal at 10 ms. At 8.5 kV/cm the transformation rate increased with time constant and was maximal at 5 ms. The survival of electroporated bacteria was reduced when the time constant exceeded 10 ms for field strengths of 3.1 and 6.5 kV/cm, and 5 ms for a field strength of 8.5 kV/cm (table 3.1.1.).

On the basis of these results the optimum conditions for the recovery of transformants of *Listeria monocytogenes* (NCTC 7973) with plasmid pGK12 were judged to be a field strength of 8.5 kV/cm, 200 Ohms resistance, 25 μ F capacitor, with a time constant of ~ 5 ms. Under these conditions, a transformation frequency of approximately $4 \times 10^6/\mu\text{g}$ pGK12 DNA was achieved.

The results of the transformation of *Listeria innocua* (NCTC 11288), *Listeria ivanovii* (ATCC 19119), and *Listeria seeligeri* (CIP 100100) with plasmid pGK12 DNA using the protocol devised to optimise transformation of *L. monocytogenes* are given in table 3.1.2. *L. innocua* and *L. ivanovii* transformed at approximately the same rate of

Table 3.1.1. Electroporation of *Listeria monocytogenes* NCTC 7973 with pGK12, pJA1 and pJA2 DNA.

Plasmid	Field strength (kV/cm)	Time constant (ms)	Actual time constant* (ms)	Percentage survival	Transformant No. (/µg DNA)
pGK12	3.1	5.0	4.4	57.3	0
" "	3.1	10.0	11.3	51.4	3.2×10^2
" "	3.1	15.0	14.6	42.6	2.9×10^2
" "	3.1	20.0	16.8	9.0	2.5×10^2
" "	6.2	5.0	4.4	33.6	3.3×10^4
" "	6.2	10.0	11.7	35.5	7.3×10^4
" "	6.2	15.0	13.8	0.4	3.5×10^3
" "	6.2	20.0	Arc†	-	-
" "	8.5	2.0	2.3	89.8	2.4×10^4
" "	8.5	4.0	4.3	59.9	1.3×10^5
" "	8.5	5.0	4.4	61.9	3.9×10^6
" "	8.5	8.0	6.8	24.8	1.1×10^4
" "	8.5	10.0	Arc	-	-
" "	8.5	15.0	Arc	-	-
" "	8.5	20.0	Arc	-	-
pJA1	8.5	5.0	4.4	61.9	0
pJA2	8.5	5.0	4.4	61.9	0
pLTV3	8.5	5.0	4.4	61.9	0

* Actual time constants are given due to small variations in conductivity of samples and cuvettes.

† Arc indicates pulse did not pass through sample.

Table 3.1.2 Electroporation of *Listeria innocua*, *L. ivanovii*, and *L. seeligeri* with pGK12 DNA.

Species	% Survival	No. of transformants
		(/μg DNA)
<i>L. innocua</i>	71.3	2.5 x 10 ⁶
<i>L. ivanovii</i>	59.7	1.2 x 10 ⁶
<i>L. seeligeri</i>	69.6	5.0 x 10 ³

Electroporation conditions used: 8.5 kV/cm field strength, 200 Ohms resistance, 25 μF capacitor 5 ms time constant.

efficiency as *L.monocytogenes* ($\sim 10^6$ transformants / μg pGK12 DNA). *L. seeligeri* transformed at a lower rate of 5×10^3 / μg pGK12 DNA.

These conditions were also used to attempt to introduce pJA1, pJA2 into *L. monocytogenes* NCTC 7973 (Table 3.1.1). Since neither of these plasmids would be expected to replicate in listeriae, any erythromycin resistant colonies should represent Tn917 insertion mutants. No such resistant colonies were generated under the conditions that allowed the optimal transformation of pGK12 (Table 3.1.1). The same conditions were also used in an attempt to introduce pLTV3, a temperature sensitive vector carrying Tn917:*lac*, developed to optimise Tn917 transposition in Gram-positive bacteria and known to replicate in *L. monocytogenes* (Camilli *et al.*, 1990; Sun *et al.*, 1990). However this electrotransformation did not result in the isolation of erythromycin resistant *L. monocytogenes* transformants carrying pLTV3.

3.2. Electroporation of penicillin treated *L. monocytogenes* strain NCTC7973 with pLTV3 DNA.

As electroporation under the conditions that allowed the optimal transformation of pGK12, did not yield any pJA1, pJA2 or pLTV3 transformant colonies, the application of electroporation to penicillin treated cells was used initially with pLTV3 DNA in an attempt to facilitate the entry of these plasmids. 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 4×10^6 / μg DNA). Using this method a transformation efficiency of 2.3×10^2 transformants / μg of pLTV3 DNA was achieved.

3.3. Protoplast transformation of *L. monocytogenes* strain NCTC7973 with pLTV3 DNA.

Due to the failure of electrotransformation under the conditions that allowed the optimal transformation of pGK12 and the low transformation efficiency achieved by penicillin treatment, other protocols for the introduction of plasmid DNA into *L. monocytogenes* were attempted. When protoplast transformation was used to facilitate the entry of plasmid pLTV3 into *L. monocytogenes* NCTC 7973, a low level of transformation, 1.2×10^2 transformants / μg of pLTV3 DNA was achieved with a regeneration efficiency of 80%.

3.4. Insertional mutagenesis of *L.monocytogenes* strain EGD using pTV32ts.

Transposon insertion mutants were generated by modification of the method of Youngman *et al.*, (1987), using *L.monocytogenes* EGD carrying temperature sensitive pTV32ts. (Youngman *et al.*, 1984, kindly supplied by M. Wuenscher). The plasmid pTV32ts will not replicate at temperatures above 42°C and contains a copy of Tn917::lac identifiable erythromycin resistance as well as a plasmid encoded chloramphenicol resistance gene (Youngman *et al.*, 1987). After growth at a temperature non-permissive for pTV32 replication, any erythromycin resistant survivors that had lost chloramphenicol resistance were assumed to be predominately bacteria which had acquired a chromosomal insertion of Tn917. Since strain EGD did not grow at temperatures over 41°C, a number of passages at this temperature were incorporated into the method to increase the numbers of bacteria that had lost pTV32. This modification raised the frequency of transposition, determined as described by Youngman (1987) to a maximum of 5.1×10^{-5} / recipient, after 6 passages (Table 3.4.).

Table 3.4. Effect of repeated cultivation at 41°C on the transposition frequency of Tn917 from the temperature sensitive vectors pTV32 and pLTV3 in *L. monocytogenes* strain EGD and NCTC7973 respectively.

Number of times cultivated	Frequency of transposition /recipient*	
	Vector pTV32	Vector pLTV3
1	9.8×10^{-7}	2.6×10^{-6}
2	1.5×10^{-6}	4.0×10^{-5}
3	2.5×10^{-6}	6.9×10^{-5}
4	4.5×10^{-5}	7.3×10^{-4}
5	4.8×10^{-5}	7.3×10^{-4}
6	5.1×10^{-5}	7.3×10^{-4}
7	5.1×10^{-5}	ND**

* determined as described by Youngman (1987)

**ND indicates not done.

On plating dilutions of the insertion mutants onto agar plates containing x-gal (5-bromo-4-chloro-3-indoyl- β -D-galactoside (section 2.10.) to detect any insertions into transcriptionally active regions of the chromosome it was found that all the colonies were a similar intensity of blue after 48 hours at 30°C.

Repeated growth at 41°C resulted in the isolation of insertion mutants that displayed a reduced chloramphenicol resistance. The determined minimum inhibitory concentrations (mic) of chloramphenicol for these mutants of 5 µg/ml was compared with the mic of the EGD parent strain carrying pTV32: mic 30 µg/ml. This persistent low level of chloramphenicol resistance was unexpected, and suggested that pTV32 had not been lost completely from the bacteria. Complete loss would have resulted in the same level of chloramphenicol resistance as that displayed by *L. monocytogenes* EGD without pTV32 (mic 1.0 µg/ml). Southern hybridisation analysis was performed to check whether pTV32 was still present at a low copy number in these bacteria and if transposition had occurred.

Chromosomal DNA from 12 insertion mutants was digested with restriction enzyme *Eco*R1, which has no cleavage sites in Tn917 (Youngman *et al.*, 1987). The digested DNA was then probed with a transposon-specific 1.5 kb *Hind* III restriction fragment DNA probe (figure 3.4.1.). The probe hybridised to single bands of different molecular weight this confirmed that Tn917 had inserted randomly into the chromosome as a single copy (figure 3.4.2.). The 2 bands visible in tracks 1 and 11 of figure 3.4.2., suggests that 2 copies of Tn917 have inserted into the chromosome of these mutants.

The *Eco*R1 digested chromosomal DNA was also probed with a pTV32-plasmid specific 5.0 kb *Pst*I - *Bam*H1 restriction fragment DNA probe (figure 3.4.1.). The probe hybridised to single bands of different molecular weight, suggesting that the plasmid was still present (figure 3.4.3.). To determine whether the plasmid was present chromosomally or extrachromosomally a third hybridisation was performed. The 2 bands visible in track 2 of figure 3.4.3., suggests that 2 copies of pTV32 have inserted into the chromosome of this mutant.

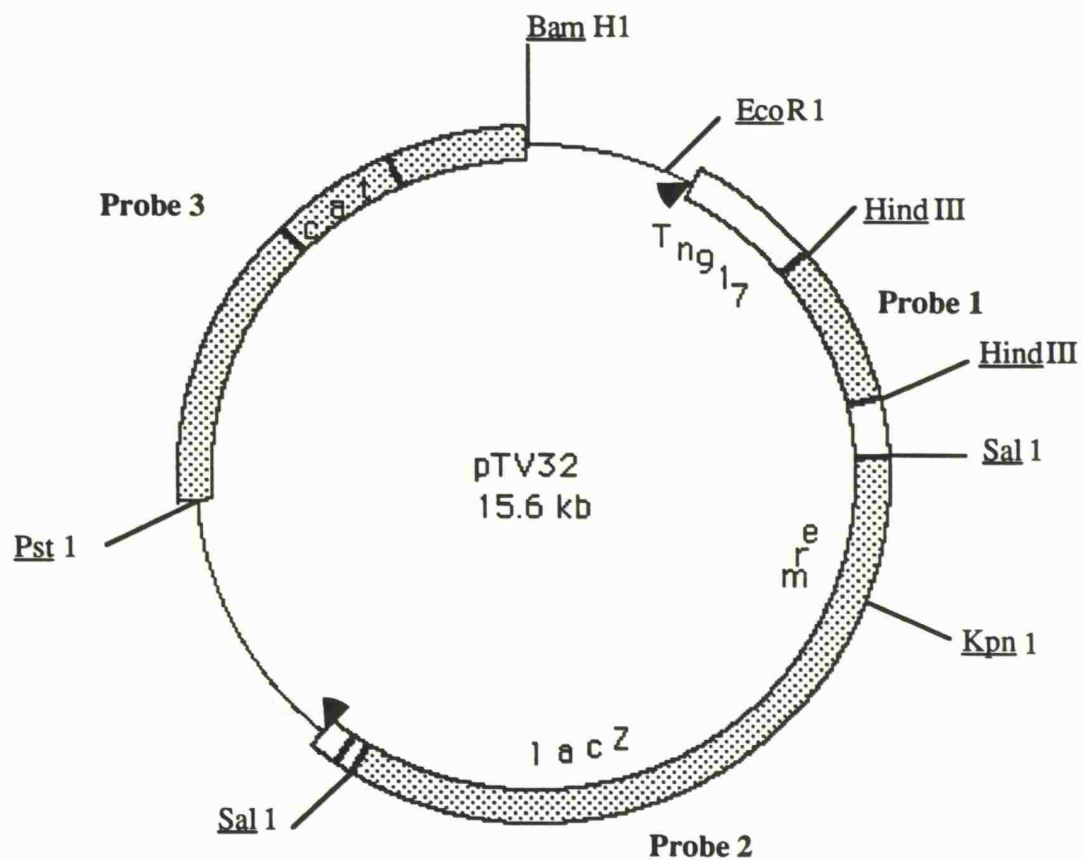


Figure 3.4.1. Diagram to represent the position of DNA probes from pTV32, used in the Southern hybridisation analysis of transposon insertion mutants. Arrow heads indicate extent of *Tn917::lacZ* fusion, Probe 1 represents a 1.5 kb *Hind* III restriction fragment specific to the transposon *Tn917*. Probe 2 represents a 4.5 kb *Sal* I restriction fragment spanning the *Kpn* I restriction site. Probe 3 represents a 5.0 kb *Pst* I-*Bam* H1 restriction fragment specific to the plasmid pTV32.

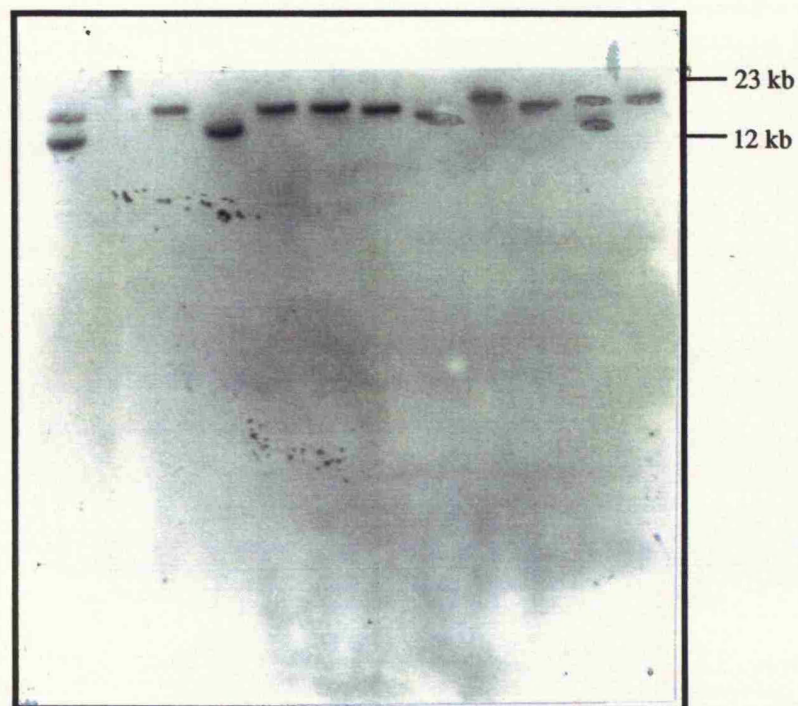


Figure 3.4.2.

Chromosomal DNA from 12 *L. monocytogenes* strain EGD, insertion mutants from a pTV32 derived transposon Tn917 insertion library digested with restriction enzyme *EcoR*I, probed with a 1.5 kb transposon Tn917 specific DNA probe (Figure 3.4.1.). Track 6: contained DNA from Lm.6b. The probe hybridised to single bands of different molecular weight, the 2 bands visible in tracks 1 and 11 suggest that 2 copies of Tn917 have inserted into the chromosome of these mutants.

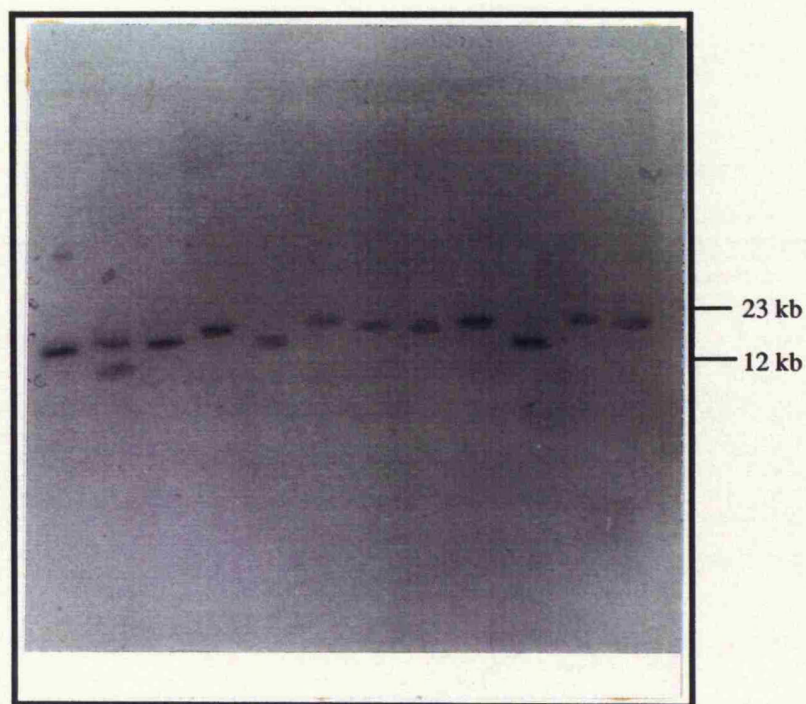


Figure 3.4.3.

Chromosomal DNA from 12 *L. monocytogenes* strain EGD, insertion mutants from a pTV32 derived transposon Tn917 insertion library digested with restriction enzyme *Eco*R1, probed with a 5.0 kb plasmid pTV32 specific DNA probe (Figure 3.4.1.). Track 6: contained DNA from Lm.6b. The probe hybridised to single bands of different molecular weight, the 2 bands visible in track 2 suggest that 2 copies of pTV32 have inserted into the chromosome of this mutant.

Chromosomal DNA was digested with restriction enzyme *Kpn*I which has no cleavage sites in the plasmid but which cuts once in Tn917 itself (Youngman *et al.*, 1987). The DNA was then probed with a Tn917-specific 4.5 kb *Sal*I restriction fragment DNA probe spanning the *Kpn*I cleavage site (figure 3.4.1). The probe hybridised to 2 fragments of the digested chromosomal DNA (figure 3.4.4.). This confirmed that both the plasmid and Tn917 had inserted into the chromosome as the probe would have hybridised to a single fragment if Tn917 was present in an extrachromosomal plasmid. This insertion of Tn917 with the plasmid appears to be random as the hybridised fragments are of different sizes it can also be seen in track 2 of figure 3.4.4., that 2 copies of both the plasmid and Tn917 have inserted into the chromosome of this mutant.

3.5. Insertional mutagenesis of *L.monocytogenes* strain NCTC7973 using pLTV3.

The vector pLTV3 in *L. monocytogenes* NCTC7973 transposed at a frequency of 7.3×10^{-4} /recipient, approximately 14 times higher than that of pTV32 (5.1×10^{-5} /recipient) when the Tn917 insertional mutagenesis protocol of Youngman *et al.* (1987) was used. This method was modified by the addition of repeated growth steps at 41°C as was successful for pTV32 mediated mutagenesis. Four overnight replication steps at 41°C were required to achieve maximum transposition of pLTV3, two fewer than were required for pTV32 (Table 3.4).

3.6. Comparison of minimal media for selection of auxotrophic transposon insertion mutants.

To find the most suitable minimal medium for the selection of auxotrophic mutants of *L. monocytogenes* NCTC7973, four previously published minimal media were assessed. Minimal media plates were examined by eye for growth every 24 hrs. Growth was scored by comparison to that on the tryptose soya agar (TSA) plates

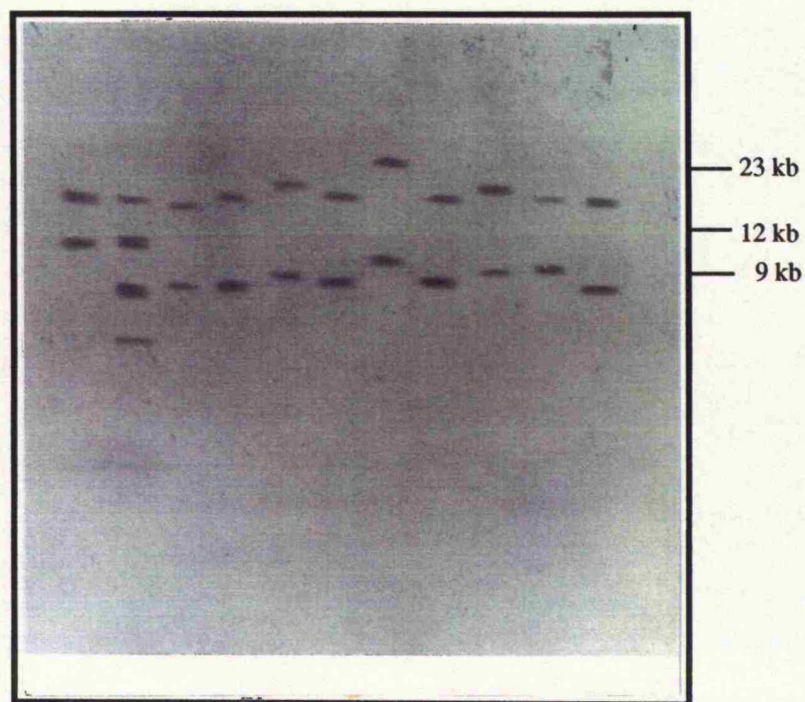


Figure 3.4.4.

Chromosomal DNA from 12 insertion mutants digested with restriction enzyme *Kpn*1, and probed with a 4.5 kb Tn917 specific DNA probe spanning the *Kpn*1 cleavage site (Figure 3.4.1.). Track 6: contained DNA from Lm.6b. The probe hybridised to 2 fragments of the digested chromosomal DNA which were a different size in the case of each mutant, however in track 2, four bands can be seen.

(Table 3.6.1). The minimal media of Friedman and Roessler (1961) and Welshimer (1963) both supported good growth of *L. monocytogenes* NTCT7973 but better growth was achieved on the former (Table 3.6.1). Significantly less growth of *L. monocytogenes* NTCT7973 was evident on the minimal medium of Ralovich *et al.* (1977), and no growth was observed on that of Siddiqi and Khan (1989) (Table 3.6.1).

Representatives of other *Listeria* species *L. innocua* (NCTC 11288), *L. ivanovii* (ATCC 19119), and *L. seeligeri* (CIP 100100), exhibited good growth also (Table 3.6.2). The minimal medium of Friedman and Roessler (1961) was selected for further use as it sustained good growth of all species after 48 hrs incubation, whereas the medium of Welshimer (1963) did not support growth of all the species comparable to that on the control TSA plate, until seven days after inoculation (Table 3.6.2).

3.7. Characterisation of insertion mutants.

To identify auxotrophic insertion mutants of *L. monocytogenes* in excess of 20,000 insertion mutants from 2 separate transposon libraries were streaked onto the minimal medium of Friedman and Roessler (1961). Screening for adenosine and glycine as well as aromatic amino acid dependent insertion mutants was undertaken to confirm the random nature of Tn917 in the *L. monocytogenes* libraries prepared. Two auxotrophic mutants were isolated from pTV32ts derived Tn917 insertion mutant libraries, Lm.6b, which required aromatic amino acids, and Lm.12c., which required glycine.

To screen for other mutations caused by Tn917 insertion, approximately 1000 insertion mutants were also streaked onto the modified basal medium (Wilkinson and Jones, 1977) to detect those unable to produce acid from rhamnose. Of the insertion mutants screened 1% were found to be unable to produce acid from rhamnose.

Table 3.6.1. Comparison of the growth of *L. monocytogenes* strain NCTC7973 on minimal media after incubation at 30°C.

Media	Day after inoculation						
	1	2	3	4	5	6	7
Tryptose soya agar	+++	+++	+++	+++	+++	+++	+++
Freidman and Roessler (1961)	+/-	+++	+++	+++	+++	+++	+++
Welshimer (1962)	-	+/-	+	++	+++	+++	+++
Ralovich <i>et al.</i> , (1977)	-	-	-	-	+/-	++	++
Siddiqi and Khan (1989)	-	-	-	-	-	-	-

+/- indicates growth just visible

+

 indicates scant growth

++ indicates moderate growth

+++ indicates growth equivalent to that of control on tryptose soya agar

Table 3.6.2. Comparison of the growth of *Listeria* species on the minimal media of Freidman and Roessler (1961) and Welshimer (1962) after incubation at 30°C.

	Day post inoculation						
	1	2	3	4	5	6	7
Tryptose soya agar	+++	+++	+++	+++	+++	+++	+++
<i>L. innocua</i> (NCTC 11288)	++	+++	+++	+++	+++	+++	+++
<i>L. ivanovii</i> (ATCC 19119)	+++	+++	+++	+++	+++	+++	+++
<i>L. seeligeri</i> (CIP 100100)	+++	+++	+++	+++	+++	+++	+++
Freidman and Roeseler (1961)							
<i>L. innocua</i> (NCTC 11288)	-	++	+++	+++	+++	+++	+++
<i>L. ivanovii</i> (ATCC 19119)	+/-	+++	+++	+++	+++	+++	+++
<i>L. seeligeri</i> (CIP 100100)	+/-	+++	+++	+++	+++	+++	+++
Welshimer (1962)							
<i>L. innocua</i> (NCTC 11288)	-	-	+/-	++	+++	+++	+++
<i>L. ivanovii</i> (ATCC 19119)	-	-	+/-	+	++	+++	+++
<i>L. seeligeri</i> (CIP 100100)	-	-	-	+	++	++	+++

- indicates no growth
 +/- indicates growth just visible
 + indicates scant growth
 ++ indicates moderate growth
 +++ indicates growth equivalent to that of control on tryptose soya agar

3.8. Confirmation of transposon Tn917 chromosomal insertion.

Southern hybridisation analysis was performed to check whether Tn917 was present in the chromosome of auxotrophic insertion mutant Lm.6b. Chromosomal DNA from Lm.6b was digested with restriction enzyme *Eco*R1, which has no cleavage sites in Tn917 (Youngman *et al.*, 1987). The digested DNA was then probed with a transposon-specific 1.5 kb *Hind* III restriction fragment DNA probe (figure 3.4.1.). The probe hybridised to a single band, which confirmed that Tn917 had inserted into the chromosome as a single copy (figure 3.4.2. track 6).

To check whether the plasmid pTV32 had inserted into the chromosome of Lm.6b, with the transposon, *Eco*R1 digested chromosomal DNA was also probed with a pTV32-plasmid specific 5.0 kb *Pst*I - *Bam*HI restriction fragment DNA probe (figure 3.4.1.). The probe hybridised to a single band, suggesting that the plasmid was present chromosomally and that Tn917 had inserted randomly into the chromosome together with the plasmid (figure 3.4.3. track 6). To confirm this a third hybridisation was performed.

Chromosomal DNA was digested with restriction enzyme *Kpn*I which has no cleavage sites in the plasmid but which cuts once in Tn917 itself (Youngman *et al.*, 1987). The DNA was then probed with a Tn917-specific 4.5 kb *Sal*I restriction fragment DNA probe spanning the *Kpn*I cleavage site (figure 3.4.1). The probe hybridised to 2 fragments of the digested chromosomal DNA (figure 3.4.4. track 6). This confirmed that both the plasmid and Tn917 had inserted into the chromosome of Lm.6b as the probe would have hybridised to a single fragment if Tn917 was present in an extrachromosomal plasmid.

An aromatic amino acid dependent insertion mutant Lm.918.6 from a pLTV3 derived transposon insertion library (Camilli *et al.*; 1989) was also acquired from Dr. D Portnoy. Lm.918.6 had been described as an insertion mutant that requires aromatic amino acids for growth (Camilli *et al.*; 1989). Southern hybridisation analysis was

performed to check whether Tn917 was present in the chromosome of Lm.918.6. Chromosomal DNA from Lm.918.6 was digested with restriction enzyme *EcoR*I, which has no cleavage sites in Tn917 (Youngman *et al* 1987). The digested DNA was then probed with a transposon-specific 1.5 kb *Hind* III restriction fragment DNA probe (figure 3.4.1.). The probe hybridised to a single band, which confirmed that Tn917 had inserted into the chromosome as a single copy (figure 3.8.1. track 2). No further Southern hybridisation analysis of Lm.918.6 was carried out as complete removal of the plasmid pLTV3 from Lm.918.6 had previously been confirmed by Dr. D Portnoy in hybridisations with a pLTV3 specific probe (Camilli *et al.*; 1989).

3.9. Confirmation of insertion site by the measurement of enzyme activities.

To localise more precisely the biochemical defects in Lm.6b, aromatic amino acids and intermediates of the aromatic amino acid pathway were assayed for their ability to support growth of Lm.6b in minimal media. The results showed that tyrosine, phenylalanine, phenylpyruvic acid and prephenic acid could support growth whereas their precursor chorismic acid did not. These observations suggested that insertional activation had occurred either in the structural or regulatory sequences of the *aroG/H* gene that encodes the enzyme chorismate mutase. This enzyme catalyses the conversion of chorismic acid to prephenic acid (figure 3.9.). It was expected that the growth of Lm.6b would not be supported by tryptophan but in its presence a limited amount of growth was seen.

To determine the biochemical defects in Lm.918.6 aromatic amino acids and intermediates of the aromatic amino acid pathway were assayed for their ability to support its growth in minimal media. The results indicated that while phenylalanine and its precursor phenylpyruvic acid could support the growth of Lm.918.6, neither tyrosine nor prephenic acid (a precursor for both tyrosine and phenylalanine) did so. These observations suggested that insertional activation had occurred either in the structural or regulatory sequences of the *pheA* gene that encodes the enzyme prephenate dehydratase. This enzyme catalyses the conversion of prephenic acid to phenylpyruvic acid (figure 3.9.).

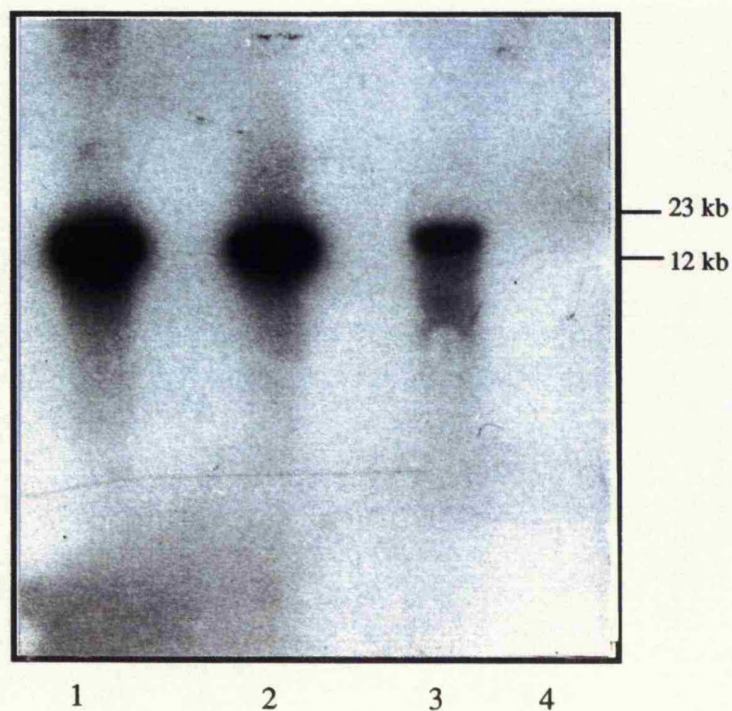
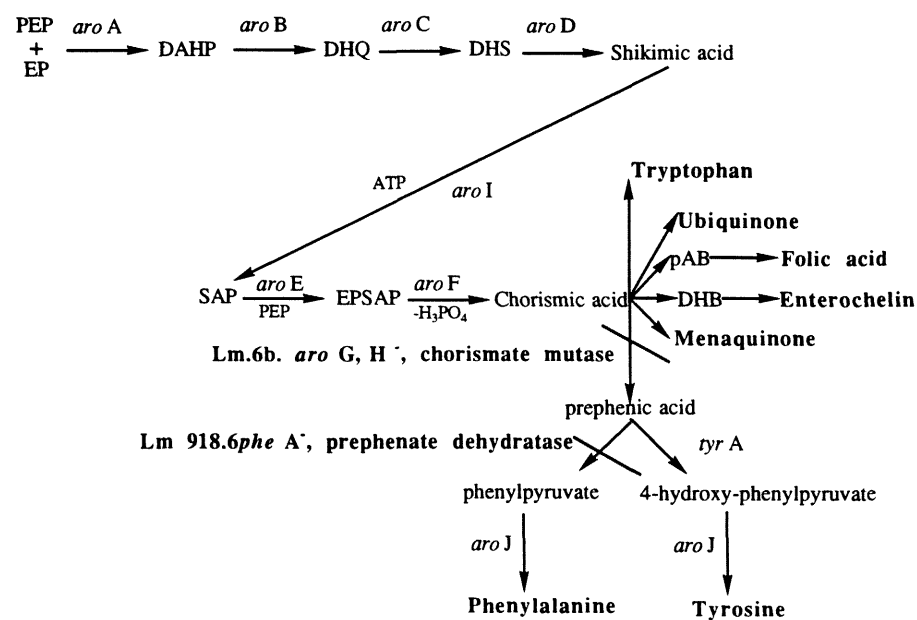


Figure 3.8.1. Tracks 1 and 2: chromosomal DNA from Lm.918.6 digested with restriction enzyme *Eco*R1, probed with a 1.5 kb *Hind*III transposon Tn917 specific DNA probe (Figure 3.4.1). The probe hybridised to a single band in each case indicating a single copy of Tn917 to be present. Track 3: plasmid pTV32ts DNA carrying Tn917 digested with restriction enzyme *Eco*R1, probed with the same 1.5 kb *Hind*III transposon Tn917 specific DNA probe. Track 4: chromosomal DNA from Lm.1070138 digested with restriction enzyme *Eco*R1, probed with the same 1.5 kb *Hind*III transposon Tn917 specific DNA probe, no band present indicating that no non-specific hybridisation occurred.

Figure 3.9. General pathway for aromatic amino acid synthesis showing enzymes not deficient in mutants Lm 918.6 *aro*⁻ and Lm 6b *aro*⁻.



Abbreviations: PEP: phosphoenolpyruvate, EP: erythrose 4-phosphate, DAHP: 3-deoxy-D-arabio-heptulosonic acid 7-phosphate, DHQ: 5-dehydroquinic acid, DHS: 5-dehydro-shikimic acid, SAP: shikimic acid 5-phosphate, EPSAP: 3-enol-pyruylshikimic acid, pAB: para-amino benzoic acid, DHB: dihydroxy benzoic acid. Adapted from Hoch and Nester (1973).

Enzyme analysis of auxotrophic mutants; Lm.6b, and Lm.918.6.

To confirm these possible insertion sites, the chorismate mutase and prephenate dehydratase activities of the aromatic amino acid dependent auxotrophic strains (Lm. 6b, Lm.918.6), and those of the respective parent strains (EGD, Lm.1070138) were determined (Table 3.9.). The results suggested that insertional inactivation had occurred either in the structural or regulatory sequences of the *aro* G/H gene (figure 3.9.) encoding the enzyme chorismate mutase in the case of Lm.6b, and in the structural or regulatory sequences of the *phe* A gene encoding the enzyme prephenate dehydratase in the case of Lm.918.6 The activity of chorismate mutase was reduced to below the detection level of the assay (< 0.01 units / mg of protein) in Lm. 6b and the activity of prephenate dehydratase was similarly reduced (0.02 units / mg of protein) in Lm 918.6 compared to those of the wild type parents 16.67 units / mg of protein, and 7.00 units / mg of protein respectively.

3.10 Results of *In vivo* investigations of Lm.918.6 and Lm.6b virulence in mice.

Determination of 50% effective dose (ED_{50}).

The virulence of the auxotrophic mutants Lm.6b, and Lm.918.6, was estimated by determining the ED_{50} i.e. the number of organisms which when administered resulted in 50% of the mice becoming moribund. The numbers of mice which became moribund after administration of different doses of the mutants Lm.6b, Lm.918.6 and their parent wild type strains EGD and Lm.1070138, were recorded (Tables 3.10.1. a. b. c. & d.). The ED_{50} values of Lm.6b, and Lm.918.6 were then determined, and compared to that determined for their wild type parent strains (Table 3.10.2). The strain Lm.918.6 had a significantly ($p < 0.05$) higher ED_{50} , 2.6×10^6 colony forming units (cfu), than its wild type parent strain Lm.107013 of 5.5×10^4 cfu. In contrast the ED_{50} of strain Lm.6b 1.4×10^4 cfu was not significantly greater ($p < 0.05$) than that of its wild type parent strain *L. monocytogenes* EGD 1.8×10^4 cfu. This result could be explained by the presence of wild type *L. monocytogenes* in the inoculum of Lm.6b

Table 3.9. Specific enzyme activities of aromatic amino acid dependent Tn917 insertion mutants of *L. monocytogenese*.

Strain	Specific activity units / mg of protein	
	Chorismate mutase	Prephenate dehydratase
EGD wt	16.67	8.00
Lm. 1070138 wt	11.46	7.00
Lm. 6b. <i>aro</i> ⁻	< 0.01	6.80
Lm. 918.6 <i>aro</i> ⁻	10.73	0.02

wt indicates wild type parent strain.

Strain EGD is the parent of Lm. 6b.

Strain Lm.1070138 is the parent of Lm. 916.6.

Table 3.10.1.a. Number of MF1 mice per group of five, which became moribund six days after the administration of wild type Lm. 1070138.

Dose cfu.	Number of mice moribund
5.0×10^3	0.0
7.3×10^3	0.0
1.2×10^4	1.0
1.0×10^4	2.0
3.5×10^4	2.7
7.0×10^4	4.0
1.0×10^5	5.0
1.0×10^6	5.0

Numbers represent the mean of three experiments.

Table 3.10.1.b. Number of MF1 mice per group of five, which became moribund six days after the administration of Lm. 918.6.

Dose cfu	Number of mice moribund
7.1×10^4	0.0
1.4×10^5	0.0
2.9×10^5	1.0
5.8×10^5	1.7
6.0×10^5	2.0
7.1×10^5	2.0
3.1×10^6	2.7
7.1×10^6	3.0
2.8×10^7	5.0
5.0×10^7	5.0

Numbers represent the mean of three experiments.

Table 3.10.1.c. Number of MF1 mice per group of five, which became moribund six days after the administration of wild type EGD.

Dose cfu.	Number of mice moribund
1.0 x 10 ³	0.0
7.1 x 10 ³	0.0
1.0 x 10 ⁴	1.0
1.6 x 10 ⁴	3.0
5.5 x 10 ⁴	4.0
6.7 x 10 ⁴	4.7
2.3 x 10 ⁵	5.0
1.1 x 10 ⁶	5.0

Numbers represent the mean of three experiments.

Table 3.10.1.d. Number of MF1 mice per group of five, which became moribund by six days after the administration of Lm.6.b.

Dose cfu.	Number of mice moribund
1.0 x 10 ³	0.0
8.0 x 10 ³	0.0
1.0 x 10 ³	1.0
1.4 x 10 ⁴	3.0
1.8 x 10 ⁴	4.0
5.0 x 10 ⁴	4.3
7.0 x 10 ⁴	5.0
2.4 x 10 ⁵	5.0
1.1 x 10 ⁶	5.0

Numbers represent the mean of three experiments.

Table 3.10.2 Comparison of ED50 values of wildtype and aromatic amino acid dependent strains of *L. monocytogenes*.

Strain	ED50 ^a	SE ^b	95% CI ^c
i. Lm. 1070138	5.5 x 10 ⁴	5.0 x 10 ²	1.3 x 10 ⁴ - 2.4 x 10 ⁵
ii. Lm. 918.6	2.6 x 10 ⁶ *	3.7 x 10 ³	7.0 x 10 ⁵ - 9.7 x 10 ⁶
iii. EGD	1.8 x 10 ⁴	3.5 x 10 ²	9.5 x 10 ³ - 7.3 x 10 ⁴
iv. Lm.6b.	1.4 x 10 ⁴ **	5.7 x 10 ²	6.6 x 10 ³ - 8.7 x 10 ⁴

a. data are the 50% effective dose which is the mean dose required to render 50% of the mice moribund.

b. standard error.

c. confidence limits.

* indicates difference in median ED50s of strains is significant (p < 0.05).

** indicates difference in median ED50s of strain is not significant (p < 0.05)

This possibility was ruled out by plating the inoculum onto minimal medium and tryptose soya agar (TSA). 1.0×10^8 cfu / 100 μ l of inocula were present when plated onto TSA, while no growth occurred on minimal medium. Growth on both the TSA plates and on minimal medium plates would be expected if the inoculum contained a mixed population of auxotrophs and prototrophs, so this was discounted.

Growth of auxotrophs Lm.6b, and Lm.918.6, in host tissue.

The ability of Lm.918.6 and its wild type parent strain Lm.1070138, to colonise and persist in the livers and spleens of MF1 mice after i.v. inoculation was determined (figures 3.10.2 a & b). The colonisation of these organs was investigated as they are the site for Listerial multiplication in natural infection (Armstrong and Sword, 1964). Compared with the wild type strain, Lm.1070138, significantly ($p < 0.05$) lower numbers of Lm.918.6 were recovered from these organs. After a dose of 1.0×10^4 colony forming units (cfu) of Lm.1070138, peak counts of 6.0×10^9 cfu/g of spleen tissue, and 7.0×10^7 cfu/g of liver tissue were obtained after 3 days of infection. When given at the same dose, Lm.918.6 multiplied more slowly reaching peak counts of 2.0×10^6 cfu/g and 9.0×10^5 cfu/g of spleen and liver tissue respectively after 5 days (figure 3.10.2 a & b). Many large abscesses were observed on livers and spleens of mice infected with the parent strain, while few abscesses were observed in mice infected with Lm.918.6 and these were much smaller in size. The results demonstrate that the ability of Lm.918.6 to colonise and multiply in the liver and spleen of mice is impaired.

To investigate the early course of infection, mice were infected with a higher dose (1.0×10^5 cfu) of each strain and growth in the tissues was monitored for 24 hrs (figure 3.10.2 c & d). The counts in the spleen 4 hrs after infection were significantly ($p < 0.05$) higher for Lm 1070138 (4.9×10^4 cfu/g of tissue), than for Lm.918.6 (8.7×10^3 cfu/g of tissue). Over the next 20 hrs Lm.1070138 multiplied in both the liver and spleen to reach counts of 6.7×10^6 cfu/g of tissue, and 7.8×10^7 cfu/g of tissue

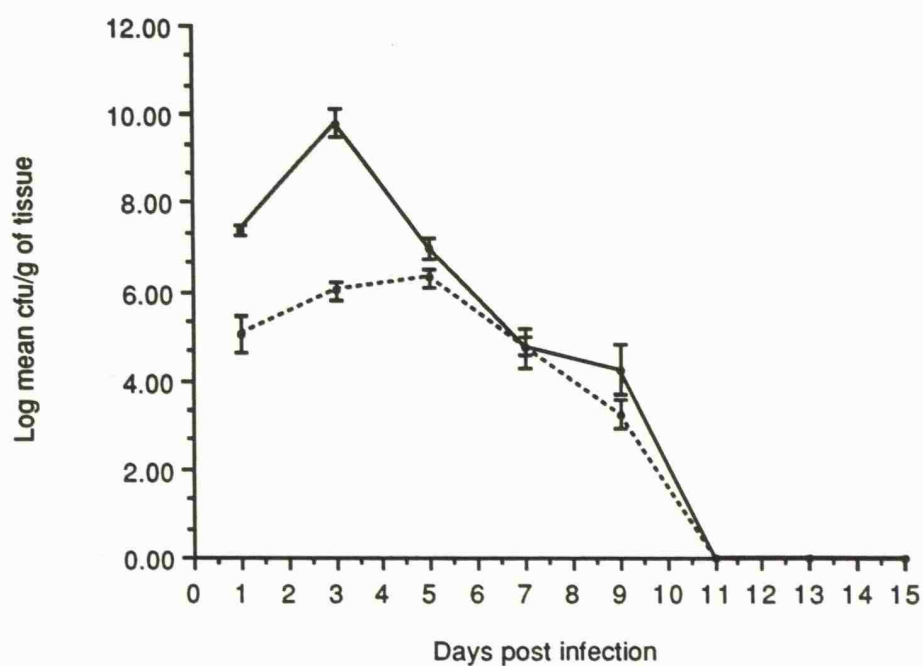


Figure 3.10.2.a. Numbers of viable Lm.1070138 — and Lm.918.6 - - - -, in spleens of MFI mice over 15 days after i.v. infection with 1.0×10^4 cfu, each point represents the geometric mean of five mice $\pm$ standard error of the mean.

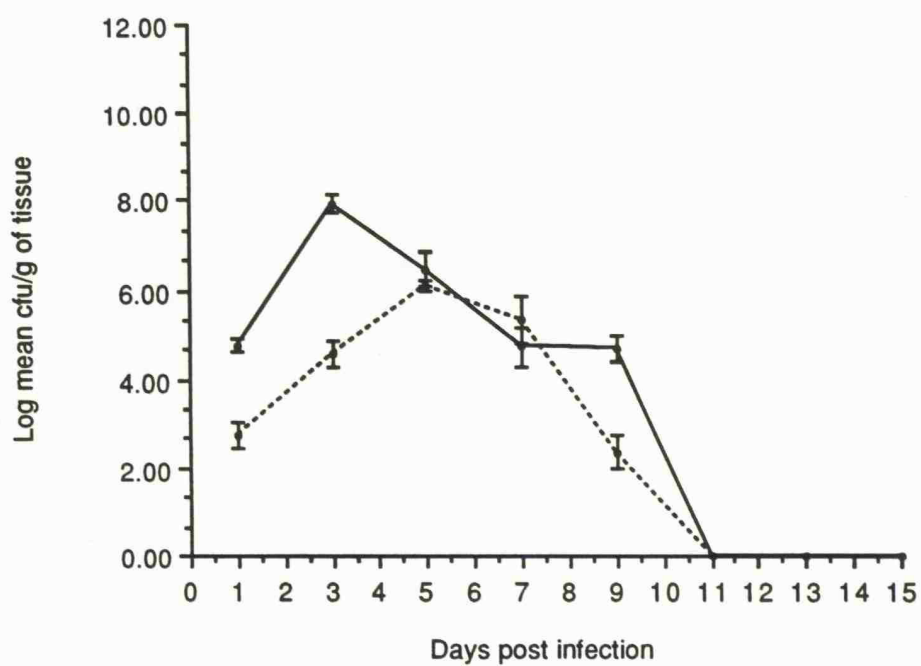


Figure 3.10.2.b. Numbers of viable Lm.1070138 —.—, and Lm.918.6 - - - -, in livers of MF1 mice over 15 days after i.v. infection with 1.0×10^4 cfu, each point represents the geometric mean of five mice $\pm$ standard error of the mean.

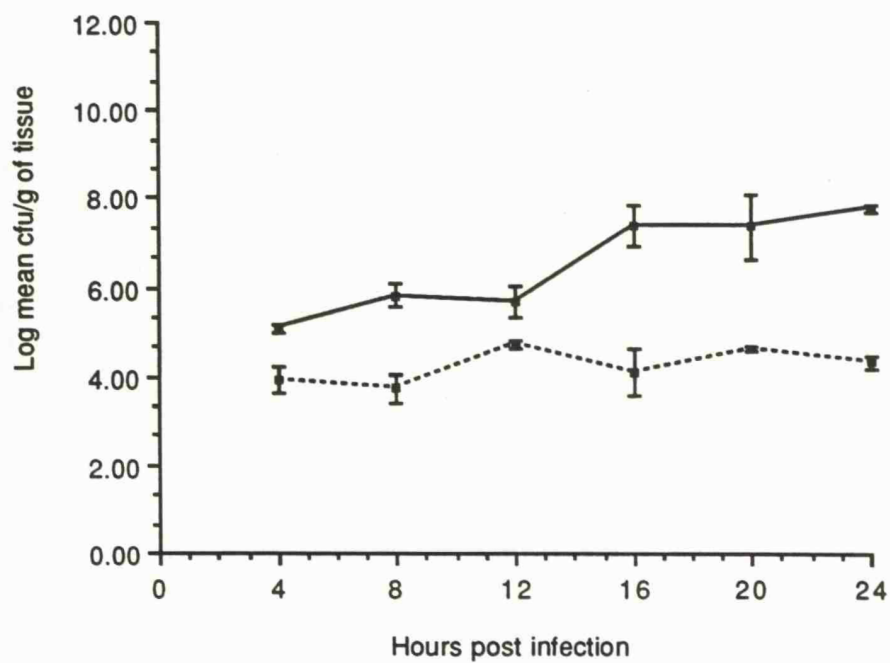


Figure 3.10.2.c. Numbers of viable Lm.1070138 —, and Lm.918.6 - - - -, in spleens of MF1 mice over 24 hours after i.v. infection with 1.0×10^5 cfu, each point represents the geometric mean of five mice $\pm$ standard error of the mean.

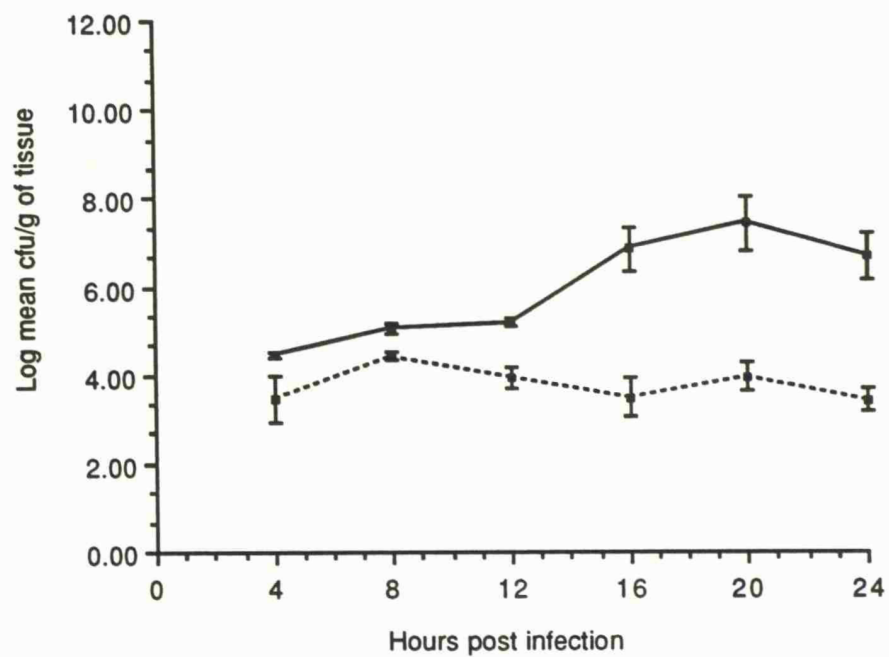


Figure 3.10.2.d. Numbers of viable Lm.1070138 —, and Lm.918.6 - - -, in livers of MF1 mice over 24 hours after i.v. infection with 1.0×10^5 cfu., each point represents the geometric mean of five mice $\pm$ standard error of the mean.

respectively. This pattern was not followed by Lm.918.6, which multiplied more slowly to reach counts of to 2.1×10^4 cfu/g of spleen tissue, while in the liver reduced counts (2.5×10^3 cfu/g of tissue) were obtained after 24 hrs (figure 3.10.2. c & d).

The determined ED_{50} of Lm.6b was not significantly different from that of its parent strain EGD. Consequently its ability to colonise and persist in host tissue was not determined quantitatively. However, following inoculation with Lm.6b an extended period of time (2 days) before the onset of visible sickness was observed in infected mice, and during this time little evidence of infection was seen in the tissues. The appearance of granulomas in the liver and spleen was slower in Lm.6b infected tissues than in the wild type infected tissues during the first two days of infection, but after this period the infection appeared to advance rapidly, reaching lethality on day 3 as did the wild type infection. Although not quantified by enumeration of the bacteria in the tissues, these observations suggested that excision of Tn917 may have occurred, and this was supported by the prototrophic phenotype of the of organisms isolated from tissues after infection with Lm.6b. However the erythromycin resistant phenotype of Lm.6b remained unchanged which did not support this argument. To confirm whether excision had taken place southern hybridisation analysis of the organisms was carried out (figure 3.10.2.e.).

Chromosomal DNA was prepared from organisms isolated from the tissues of mice infected with Lm.6b and subcultured once after isolation with erythromycin selection. The chromosomal DNA was digested with restriction enzyme *EcoR*I, which has no cleavage sites in Tn917. The digested DNA was then probed with a 1.5 kb *Hind* III restriction fragment transposon-specific DNA probe (figure 3.4.1.). The probe hybridised to single bands of different sizes suggesting that Tn917 was still present in the chromosome. (figure 3.10.2.e.).

Persistence and splenomegaly.

To investigate whether Lm.918.6 stayed in the tissues long enough to induce an immune response, mice were infected with a sublethal dose (1.0×10^4 cfu) of Lm.1070138 and Lm.918.3 and the persistence of the organisms in the tissues studied

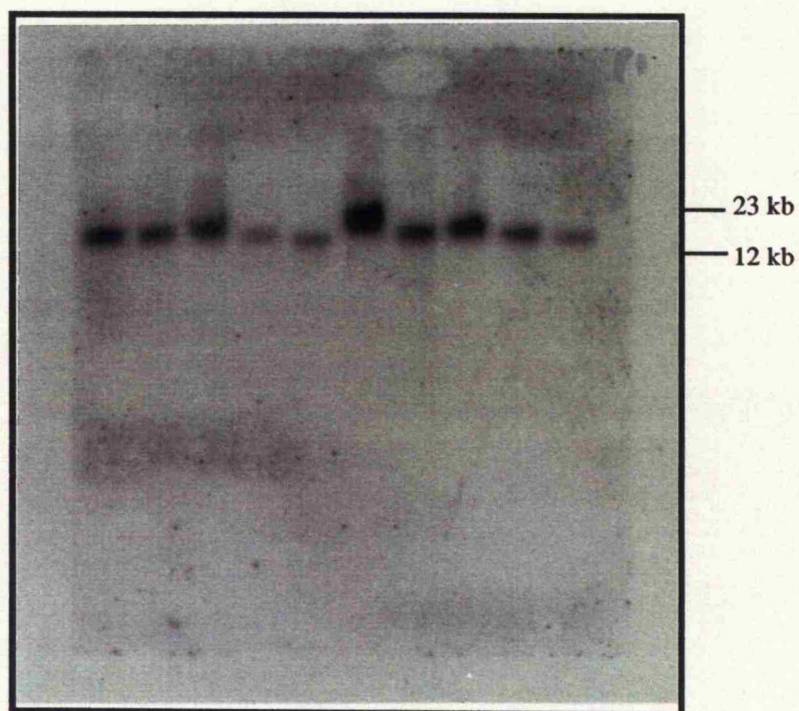


Figure 3.10.2.e.

Chromosomal DNA from 12, Lm. 6b. insertion mutants recovered from liver and spleen tissue of infected mice, digested with restriction enzyme *Eco*R1, probed with a 1.5 kb *Hind*III transposon Tn917 specific DNA probe (Figure 3.4.1.). The probe hybridised to single bands of different molecular weight in each case.

over 15 days. No significant difference ($p < 0.05$) was seen in the clearance of the two organisms between days 3 - 11 of infection. After this time Lm.918.6 appears to be cleared 1-2 days earlier than the wild type, but both were cleared to below detectable levels from the liver and spleen 15 days after infection.

Maximum splenomegaly occurred four days after the maximum number of organisms were detected in the organs; day seven post-infection for wild type strain Lm.1070138, day nine for Lm.918.6 (figure 3.10.3). The maximum splenomegaly induced by Lm.918.6 was not significantly ($p < 0.05$) different from that induced by Lm.1070138. In mice infected with Lm.1070138, splenomegaly reduced slowly and spleens had not returned to normal size fifteen days after infection, In contrast, the splenomegaly induced by Lm.918.6 was not sustained, the spleens returned to normal size 11 days after infection.

Protection of mice after immunisation with Lm.918.6

A vaccinating dose of 1.0×10^4 cfu of Lm.918.6 was administered to mice. Twenty eight days later the mice were challenged with wild type strain Lm.1070138 and the numbers of vaccinated mice which became moribund were recorded (Table 3.10.4.a.). Protection against infection with Lm.1070138 was noted, the ED_{50} of the wild type Lm.1070138 was significantly ($p < 0.05$) greater in mice vaccinated with one dose of Lm.918.6 (1.7×10^6 cfu) than in non-vaccinated mice (5.5×10^4 cfu) (Table 3.10.4.b). To determine whether this protection could be increased, in a subsequent experiment a second identical vaccinating dose of Lm.918.6 (1.0×10^4 cfu) was administered 15 days after the first. Twenty eight days after the administration of a second vaccinating dose of Lm.918.6 the mice were challenged with Lm.1070138, and the numbers of vaccinated mice which became moribund were recorded (figure 3.10.4.c). The ED_{50} of the Lm.1070138 was increased significantly ($p < 0.05$) by this second dose of Lm.918.6 to 6.00×10^6 cfu, compared with that achieved by one vaccinating dose (1.7×10^6 cfu) (Table 3.10.4.b.).

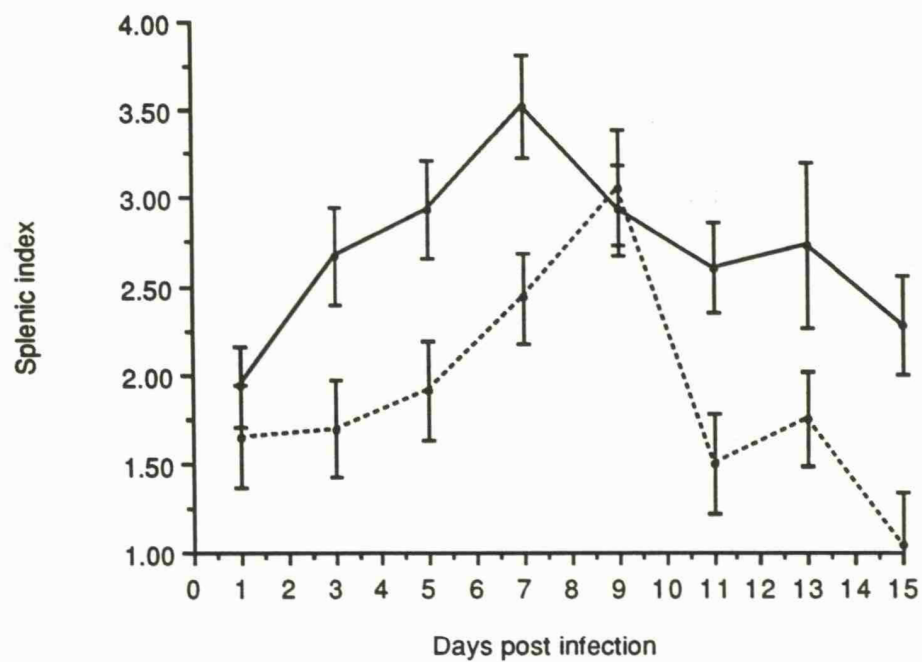


Figure 3.10.3. Splenomegaly induced over 15 days by infection with 1.0×10^4 cfu of Lm.1070138 —, and Lm.918.6 - - - -, each point represents the arithmetic mean of five mice $\pm$ standard error of the mean.

Table 3.10.4.a. Number of MF1 mice vaccinated with Lm.918.6.,which became moribund by six days after the administration of wild type Lm. 1070138.

Dose cfu.	Number of mice moribund
1.0 x 10 ⁵	0.0
5.0 x 10 ⁵	0.0
1.0 x 10 ⁶	1.0
1.3 x 10 ⁶	2.0
1.6 x 10 ⁶	3.0
3.3 x 10 ⁶	4.0
4.2 x 10 ⁶	4.0
5.0 x 10 ⁶	5.0
7.0 x 10 ⁶	5.0
1.0 x 10 ⁷	5.0

Numbers represent the mean of three experiments.

Table 3.10.4.b. Effect of vaccination of mice with Lm 918.6 on ED50 of wildtype *L. monocytogenes* 1070138.

Vaccination ^a	ED50 of wild ^b type	SEM	95% CI. ^c
i. none	5.5 x 10 ⁴	5.0 x 10 ²	1.3 x 10 ⁴ - 2.4 x 10 ⁵
ii. 1 dose Lm. 918.6 <i>aro</i> ⁻	1.7 x 10 ⁶ *	5.4 x 10 ³	1.7 x 10 ⁵ - 8.8 x 10 ⁶
iii. 2 doses Lm. 918.6 <i>aro</i> ⁻	6.0 x 10 ⁶ **	6.1 x 10 ³	1.4 x 10 ⁶ - 2.6 x 10 ⁷

a. Each vaccinating dose contained 1.0x 10⁴ cfu of Lm 918.6 given i.v.

b. Data are the 50% effective dose of Lm.1070138 which is the mean dose required to render 50% of the mice moribund.

c. Confidence limits.

* Difference between i & ii was significant p<0.05.

** Difference between i i & iii was significant p<0.05.

Table 3.10.4.c. Number of MF1 mice per group of five, vaccinated twice with Lm.918.6., which became moribund six days after the administration of wild type Lm. 1070138.

Dose cfu.	Number of mice moribund
1.0×10^5	0.0
5.0×10^5	0.0
1.0×10^6	0.0
1.9×10^6	1.0
2.0×10^6	2.0
5.0×10^6	3.0
8.0×10^6	4.0
1.4×10^7	5.0
5.0×10^7	5.0

Numbers represent the mean of three experiments.

The ability of Lm.1070138 to colonise and persist in the livers and spleens of MF1 mice after two vaccinating doses of 1.0×10^4 cfu of Lm.918.6 was determined and compared to that in non-vaccinated mice (figure 3.10.4.d & e.). On challenge with the Lm.1070138, no multiplication of the bacteria was observed in the livers and spleens of vaccinated mice, the bacteria were quickly cleared from the liver and spleen of vaccinated mice, numbers falling to below detectable levels by day 3 post infection. As expected multiplication was seen in the tissues of non-vaccinated mice, and peak counts of the bacteria were reached in the tissues of non-vaccinated mice on day 3 post infection.

3.11. Serum antibody response.

Although currently not considered to be significant in protection against listeric infection, the ability of Lm.918.6 to stimulate a serum antibody response may enhance its protective ability. Mice infected with Lm.918.6 developed a total IgG / IgM immunoglobulin antibody response, and this response reached a peak, approximately eight times that of nonimmune serum, on day thirteen post infection (figure 3.11.). The levels of immunoglobulin fell rapidly from this peak to a level approximately three times that of nonimmune serum on the fifteenth day post infection. This raised antibody level was then maintained until the end of the experiment on day 28 post infection (figure 3.11.).

3.12. Intracellular growth of auxotrophic *Listeria* strains.

Experiments to investigate the intracellular growth of Lm.918.6 and Lm.6b were performed by Dr. D. Portnoy in the Department of Microbiology, University of Pennsylvania. When the growth of Lm.918.6 and Lm.6b in mouse bone marrow-derived macrophages was observed and compared with that of their parent strains Lm.1070138 and EGD respectively, no significant difference in growth rate could be seen. Over 8 hrs, Lm.6b multiplied to a mean count of 1.8×10^5 cfu/ monolayer with

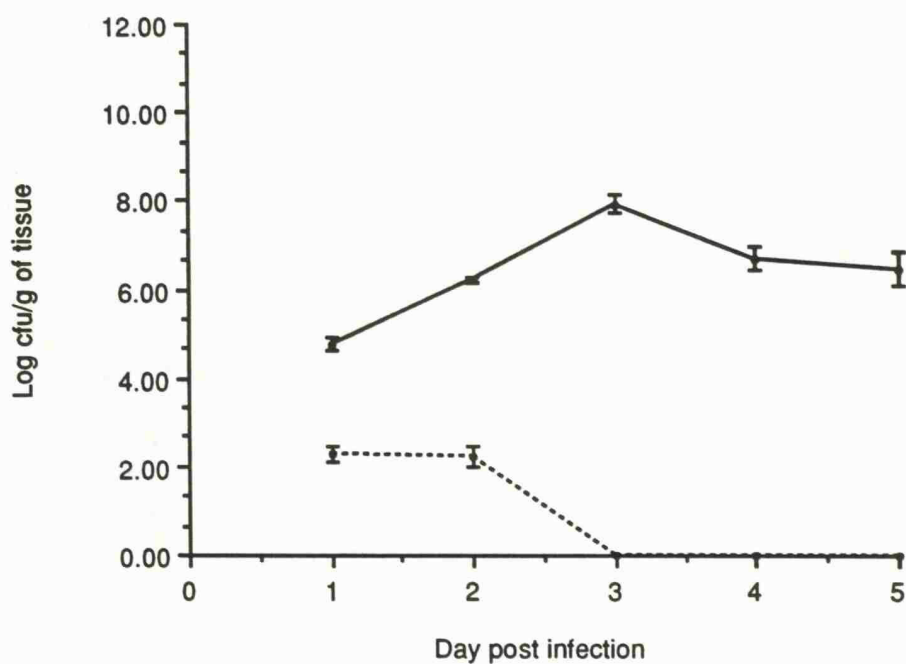


Figure 3.10.4.d. Numbers of viable *L. monocytogenes* Lm.1070138 in livers of immune --- and nonimmune — MFI mice over 5 days infection with 1.0×10^4 cfu of Lm.1070138. Each point represents the geometric mean of five mice $\pm$ standard error of the mean.

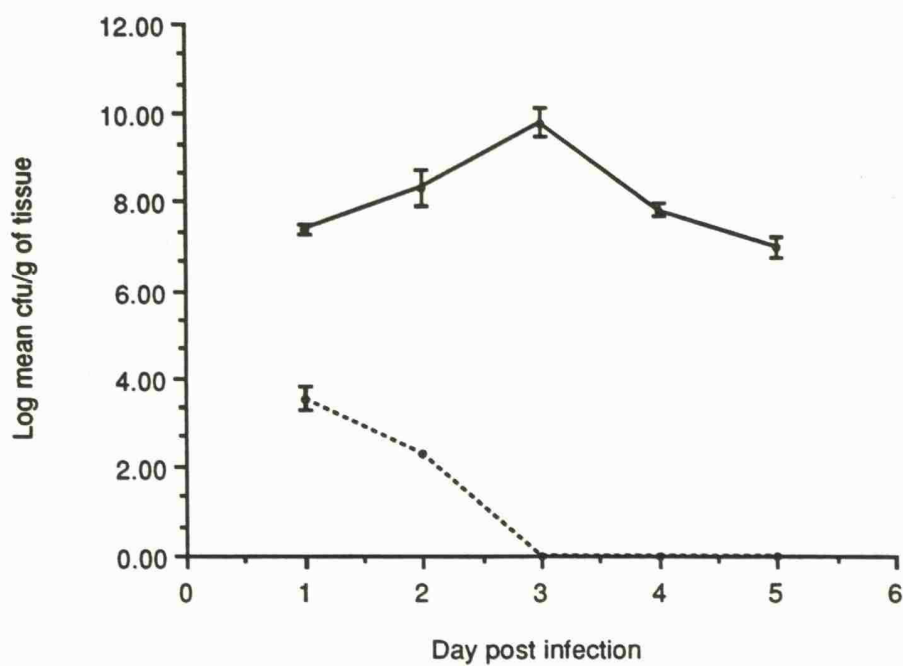


Figure 3.10.4.e. Numbers of viable *L. monocytogenes* Lm.1070138 in spleens of immune - - - - and nonimmune ——— MFI mice over 5 days infection with 1.0×10^4 cfu of Lm.1070138. Each point represents the geometric mean of five mice $\pm$ standard error of the mean.

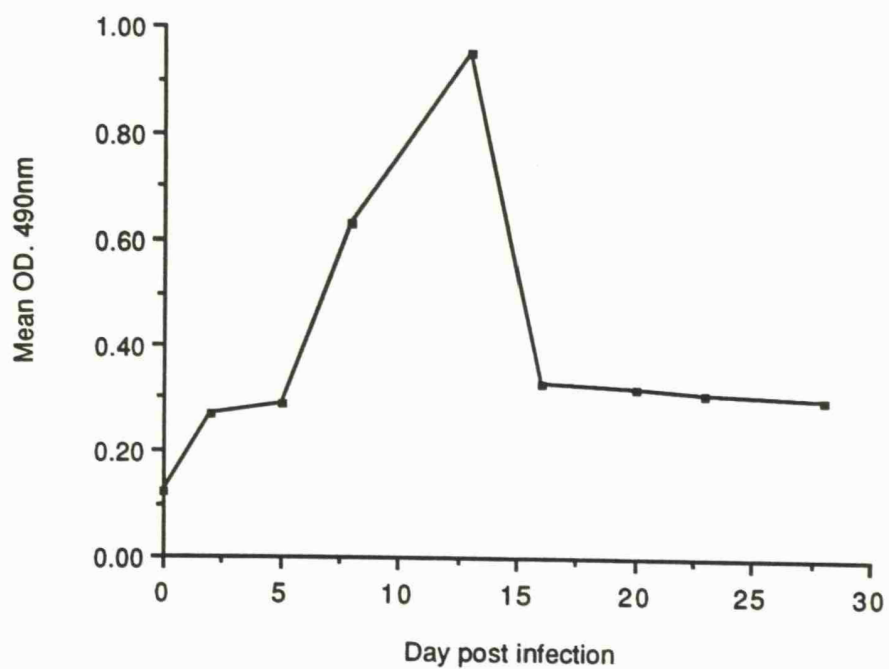


Figure 3.11. Levels of total immunoglobulin in serum of MF1 mice after vaccination with 1.0×10^4 cfu Lm.918.6 measured by ELISA, each point represents the arithmetic mean of five mice.

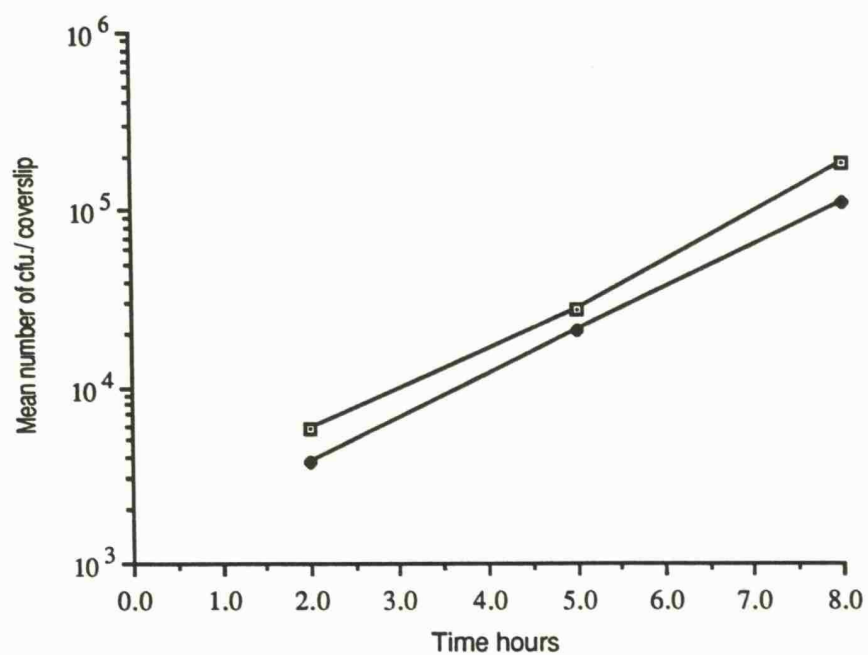


Figure 3.12. The growth of *L. monocytogenes* EGD —.—, and Lm.6b ----, in bone marrow derived macrophages over 8 hrs, after infection with 1.0×10^6 cfu per coverslip. Each point represents the arithmetic mean of three coverslips.

Discussion

The aim of work presented in this thesis was to construct aromatic amino acid dependent (*aro*⁻) transposon insertion mutants of *L. monocytogenes* and to assess their potential as live vaccines.

To have potential as a live vaccine a strain should be non-reverting and genetically defined. Naturally occurring *aro*⁻ mutant strains are unsuitable because of their undefined nature and the likelihood of spontaneous reversion to a virulent prototrophic phenotype. Transposon mutagenesis was selected for the construction of genetically defined *aro*⁻ strains of *L. monocytogenes*. Alternative techniques such as site-directed or indirect transposon mutagenesis could not be used because the *L. monocytogenes* *aro* genes had not been cloned. Successful mutagenesis to generate an *aro*⁻ mutant would facilitate the subsequent cloning of these genes for further studies.

Insertion elements such as the bacteriophages Mu and lamda (λ) can be used for insertional mutagenesis of many Gram-negative bacterial species (see Pato, 1989, Thompson and Landy, 1989). However, these elements are not appropriate for the construction of *aro*⁻ mutants of *L. monocytogenes* and other Gram-positive species. Transposon mutagenesis was chosen for the insertional mutagenesis of *L. monocytogenes* because transposons have been well characterised in Gram-positive bacteria and have several advantages over bacteriophages. Transposon insertion mutants are typically nonreverting and can be selected easily by transposon encoded resistance markers, whereas mutants carrying bacteriophage insertions can be selected only by prophage immunity. In general, transposons are smaller in size than bacteriophage. This characteristic facilitates the cloning and mapping of DNA. Furthermore, transposons provide restriction sites and mobile primer binding sites useful for such cloning and mapping. Transposons can be used to analyse translational and transcriptional mechanisms, protein conformation, gene expression, and to introduce genes into new hosts (for review see Berg *et al.*, 1989).

The nonconjugative *Enterococcus faecalis* transposon Tn917 was chosen for the transposon mutagenesis of *L. monocytogenes* because it has been shown to generate very stable insertion mutations in Gram-positive bacteria and has been employed successfully in the mutagenesis of the *L. monocytogenes* chromosomal genes (Camilli *et al.*, 1990, Cossart *et al.*, 1989, Murphy 1989, Youngman *et al.*, 1983, Youngman *et al.*, 1984b). Isolated from the *E. faecalis* plasmid pAD2 (Clewell *et al.*, 1982), Tn917 encodes MLS (erythromycin, lincomycin and spiramycin) drug resistance, mediated by an rRNA methylase, the product of the *ermAm* gene (Lai *et al.*, 1973, figure 4.1).

A member of the Tn3 family of insertion elements, Tn917 transposes replicatively as described by the Shapiro model (Shapiro, 1979, figure 4.2). In this model, the formation of a donor-recipient cointegrate, joined by a single copy of the transposon, is initiated by a transposon encoded transposase (figure 4.1). Replication of the transposon is followed by recombination with the target sequence in the recipient DNA. Separation of the cointegrate is then 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 use of this transposon has several advantages over the conjugative transposons Tn1545 and Tn916 previously used for insertional mutagenesis of *L. monocytogenes* (Cossart 1988, LeBlond *et al.*, 1989, Kathariou *et al.*, 1987, Khun *et al.*, 1990, Sun *et al.*, 1990). The small size (5.4 kb) of Tn917, compared with that of Tn1545 (25.3 kb) and Tn916 (16.4 kb), simplifies the cloning and mapping of the DNA flanking its insertion. Furthermore, Tn917, unlike other Tn3 type transposons, displays insertion specificity for chromosomal rather than plasmid DNA (Sherratt, 1989). Slight differences in transposition specificity are thought to be due to factors which are sensitive to local conformation of DNA (Weaver and Clewell, 1987).

The transposition of Tn917 also shows very little site specificity in bacteria other than *Bacillus subtilis* (Murphy, 1989). The element exhibits preference for A+T-rich sequences and those with similarity to the transposon ends (figure 4.1) i.e. short,

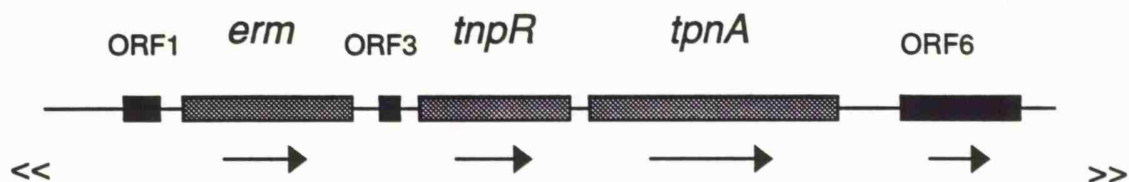


Figure 4.1. Genetic organisation of Tn917, ORF1: openreading frame 1 is the *erm* leader peptide, *erm*: erythromycin resistance gene, ORF3: is a short openreading frame of unknown function, *tnpR*; is the gene encoding resolvase, *tpnA*: is the gene encoding transposase, ORF6: is an openreading frame also involved in transposase production. <<, >> represent terminal repeat sequences. Adapted from Murphy 1989.

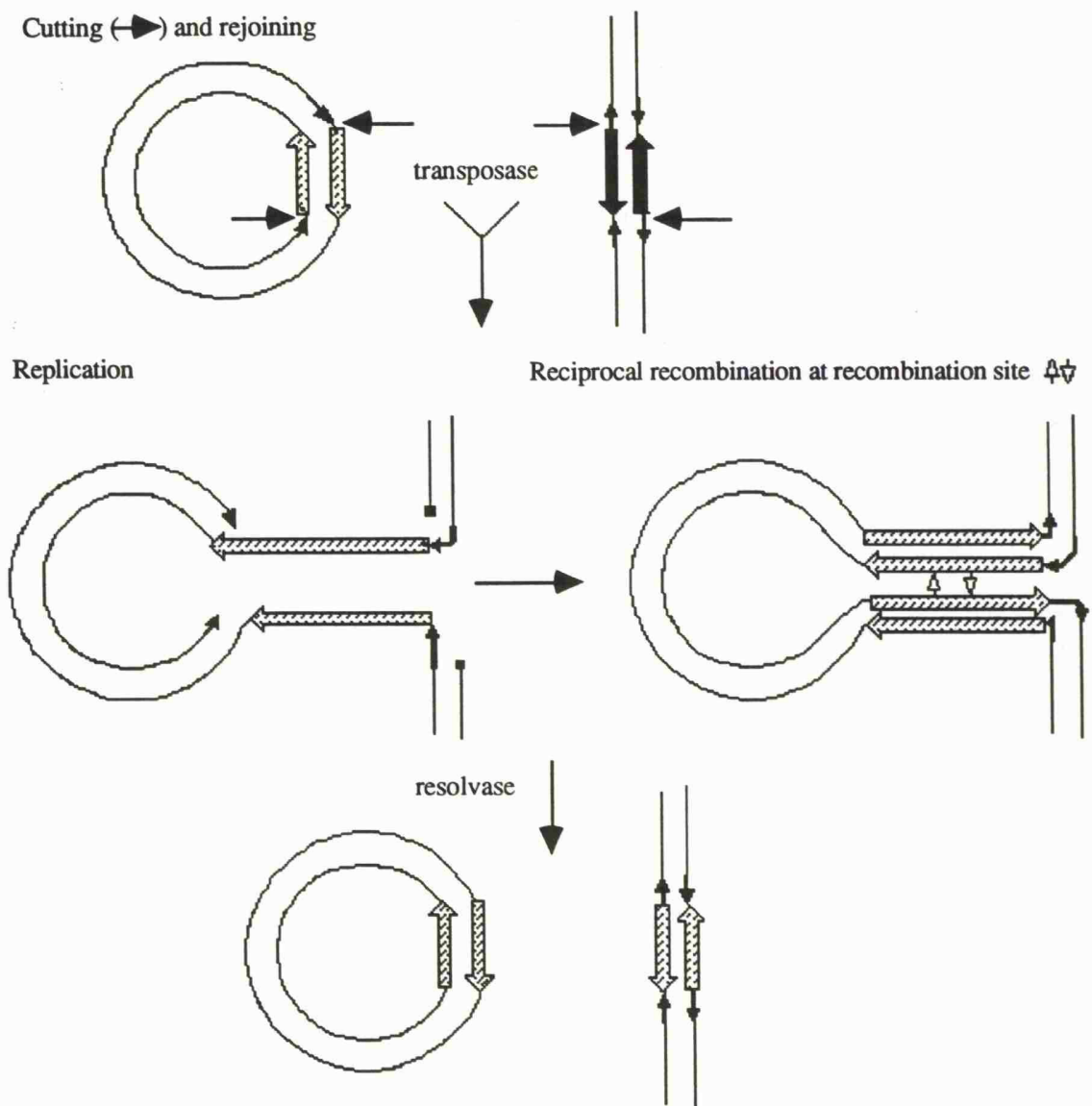


Figure 4.2. Postulated mechanism of transposition of the Tn3 family of transposons from plasmid to chromosomal DNA. The DNA double helix of each is simplified to parallel lines. The Tn3 single strands are shown as cross hatched arrows and the sequences which become duplicated in the chromosomal DNA as solid arrows. Arrowheads indicate 3' ends and solid squares indicate 5' ends. Adapted from Fincham (1983).

terminal inverted repeats of 38 base pairs (Perkins and Youngman, 1984, Tu and Cohen, 1980). Because of this lack of site specificity the transposition of Tn917 is more random than that of the conjugative transposons Tn916, and Tn1545. These Tn5-like elements, are highly site specific in that they require sequence homology between both ends of the element and sequences surrounding the integration sites (Caillaud and Courvalin, 1987, Clewell, *et al.*, 1988, Scott *et al.*, 1988). The preferential insertion of Tn917 into A+T-rich regions makes this element very suitable for insertional mutagenesis of *L. monocytogenes* which has a low mol. % G+C ratio of approximately 38% (Seeliger and Jones, 1986, Stuart and Welshimer, 1974).

Several strategies, based on suicide plasmids, have been used to obtain Tn917 insertions in chromosomal sites in strains of the genus *Bacillus* (Youngman *et al.*, 1983, Youngman, 1987, Youngman *et al.*, 1989). One such strategy is the construction of a suicide vector carrying Tn917 together with replicons from the ColE1-derived vectors pBR322 and pBR328, or the p15A-derived vector pACYC184. These two replicons are unable to replicate in Gram-positive bacteria (Bolivar *et al.*, 1977, Chang and Cohen, 1978) thus, on transformation into such bacteria they fail to replicate but deliver the transposon they carry into the host cell before they are lost. Any erythromycin resistant organisms recovered after this event are predominantly those that have acquired a chromosomal insertion of Tn917. This strategy was improved subsequently by the construction of Tn917 derivatives carrying additional drug resistance genes and/or reporter genes inserted near the end of the transposon (Perkins and Youngman, 1984, Youngman *et al.*, 1984a, Youngman *et al.*, 1985). Incorporation of additional drug resistance genes increases the potential host range of the modified transposon. Further, the presence of reporter genes allows the expression of the disrupted gene to be monitored as was first demonstrated by Casabadan and Cohen (1979). Such gene fusions have been used to analyse gene regulation and to detect genes that respond to environmental signals such as heat shock, osmolarity, anaerobiosis, and phosphate starvation (Neidhardt *et al.*, 1987). Gene fusions can be used also to generate hybrid proteins to aid the study of genes encoding cell surface and exported proteins and to identify their cellular localisation (Manoil and Beckwith, 1986, Silhavy and Beckwith, 1985). On insertion of the transposon into a gene in the

correct orientation, the reporter genes of the transposon are expressed under the regulation of the promoter for the insertionally inactivated gene. Consequently, mutants harbouring insertions in transcription units can be identified by markers such as β -galactosidase activity (blue colonies on X-gal - see section 2.10.), or chloramphenicol resistance.

Based on this strategy, the suicide vectors pJA1 and pJA2 were constructed to apply the same approach for the transposon mutagenesis of *L. monocytogenes*. Plasmid pTV32 was selected for the construction of pJA1, engineered to utilise the pBR322 origin of replication in *E. coli*. Plasmid pTV32 is a 15.6 kb vector which generates transcriptional fusions to *lacZ* (Youngman *et al.*, 1985). 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 275 base pairs (bp) from the terminal inverted repeat at the *erm*-proximal end of Tn917 (figure 2.6.1). Plasmid pTV52, selected for the construction of pJA2, designed to utilise the pACYC184 origin of replication, is a 13.7 kb vector which carries a tetracycline *tet* resistance gene as a selectable marker independent of the Tn917 derivative it carries (Youngman *et al.*, 1985). This derivative of Tn917 generates fusions to a *Bacillus pumilus* derived *cat86* reporter gene inserted 275 base pairs (bp) from the terminal inverted repeat at the *erm*-proximal end of Tn917 (figure 2.6.3).

The successful insertion of either of the Tn917 derivatives from pTV32 or pTV52 generates type I or transcriptional fusions, because the reporter genes (*lacZ* and *cat86*) carry their own Shine-Delgarno sequence but lack a promoter. Expression of the reporter gene in transcriptional fusions takes place if insertion of the transposon occurs in the correct orientation. Type II or translational fusions formed by transposons such as *TnphoA*, which encodes alkaline phosphatase, require insertion in the correct reading frame and with the appropriate orientation for expression (Casabadan and Chou, 1984). The reporter proteins made by type I fusions are of constant length and amino acid sequence because the fusion occurs upstream of the reporter translation initiation site. Hybrid proteins formed in type II fusions have an amino terminus of

variable length encoded by the gene into which the transposon has inserted and a constant carboxyl-terminal region encoded by the transposon reporter gene (Casabadan and Chou, 1984).

The restriction patterns resulting from endonuclease digestion of pJA1, and pJA2, constructed in this study (figures 3.0.4, & 3.0.5.), indicated that they had been successfully constructed and carried transposon Tn917 together with either the pBR328 or pACYC184 origins of replication respectively. Concurrent with the construction of the vectors pJA1 and pJA2, the potential of electrotransformation was investigated as an efficient route of entry for these vectors into *L. monocytogenes*.

The optimum electroporation conditions for the electrotransformation of *L. monocytogenes* were determined using plasmid pGK12, a 4.3 kb plasmid known to replicate in a wide range of host bacteria. In addition, this vector had been used previously with moderate success by other workers in electrotransformation studies with *L. monocytogenes* (Luchansky *et al.*, 1988). As can be seen from the results of the electroporation experiments (table 3.1.1.), the main limiting factor was the intensity of field strength. Increasing the field strength increased the number of transformants recovered, but it could be raised only until the conductivity limit of the electroporation medium was reached. When the conductivity limit of the electroporation medium was exceeded arcing occurred because the current could not pass through the sample. It is possible that the frequency of transformation may have been improved by the use of a medium with a higher ionic strength and greater conductivity limit. Improved conductivity of the medium could have been achieved by the omission of osmotic agents such as the sucrose in the electroporation medium (SMEM). However, sucrose is an important agent for the survival of the permeabilised cells. The ionic strength of the electroporation medium not only determines the current passing through the sample but also the rate of heating. Higher conductivity is likely to reduce cell survival, due to increased heating of the sample by the greater current passing through it.

The results of the same experiments (table 3.1.1) also indicate that transformation efficiency was increased by the application of the current for longer time constants. However, this was true only up to a certain point after which efficiency declined due to cell damage and killing because of the longer exposure to the charge applied. The optimum time constant and field strength for electrotransformation were those that limited the percentage of the bacterial population killed but still allowed maximum polarisation of the cell wall for DNA entry.

The transformation frequency of 4.0×10^6 transformants / μg of pGK12 DNA achieved for *L. monocytogenes* NCTC 7973 is a considerable improvement on the rates of $6 - 8 \times 10^3$ transformants / μg of DNA previously reported for conjugation and protoplast transformation of other *L. monocytogenes* strains (Flamm *et al.*, 1984, Lucas and Levine, 1989, Trieu-Court *et al.*, 1987, Vicente *et al.*, 1987, Wuenscher *et al.*, 1991). This frequency is also a considerable improvement on rates reported for *L. monocytogenes* strain B-67 (5.8×10^3 transformants / μg pGK12 DNA), when an electroporation protocol designed for *Lactobacillus acidophilus* was employed (Badi *et al.*, 1989, Luchansky *et al.*, 1988). Apart from the different methods used, these variations in reported transformation efficiency may be due, at least in part, to the strains used in the studies. Variation in transformation competence has been noted previously between species and strains of *Listeria* (Wuenscher *et al.*, 1991).

The results obtained when the same electroporation procedure was used to transfer plasmid pGK12 into strains of other *Listeria* species are somewhat puzzling (table 3.1.2.). Not surprisingly, in view of the close phenotypic similarity between members of the genus *Listeria* (Seeliger and Jones, 1986), the type strain of *L. innocua* (NCTC 11288) and *L. ivanovii* (ATCC 19119) were transformed at about the same efficiency ($\sim 10^6$ transformants/ μg pGK12 DNA) as *L. monocytogenes* under these conditions.

Furthermore, the transformation efficiency achieved for *L. innocua* (2.50×10^6 transformants/ μg pGK12 DNA) was considerably greater than that reported for *L. innocua* strain B-66 (2.6×10^2 / μg pGK12 DNA) using the protocol determined for

Lactobacillus acidophilus (Luchansky *et al.*, 1988). However, strain CIP 100100, the type strain of *L. seeligeri*, was transformed at a lower frequency ($5 \times 10^3 / \mu\text{g pGK12 DNA}$), compared with the other species. One explanation for this may be the variation in transformation competence reported between strains of different species of *Listeria* by Wuenscher *et al.*, (1991), and also between *Bacillus* species (Belliveau and Trevors, 1989).

This variation could be due to a number of factors for example *L. seeligeri* may be more sensitive to the presence of divalent cations during electroporation than the other species of *Listeria* investigated. The presence of as little as 1 mM of divalent cations such as Ca^{+2} , Mn^{+2} , or Mg^{+2} in an electroporation medium has been reported to reduce transformation efficiency in strains of a variety of bacterial genera (Shigekawa and Dower, 1988). The sucrose-magnesium electroporation medium used in this study contains MgCl_2 as a stabilising agent, because Mg^{+2} has been shown to reduce cell kill during electroporation possibly by stabilising the cell wall (Miller, 1988). However, during electroporation Mg^{+2} may enter the cells and it is known that some bacteria are inhibited by such cations (Miller *et al.*, 1988). Little information is available on the effect of Mg^{+2} on strains of the genus *Listeria*. If it were to be shown that the bacteria were inhibited by the low levels of free Mg^{+2} in SMEM, it could be replaced with an alternative stabilising agent such as phosphate or HEPES. No attempt was made to optimise the electroporation conditions, including the electroporation medium, for species other than *L. monocytogenes* as the frequencies of transformation obtained with this protocol are sufficiently high to allow the introduction of genetic material into other *Listeria* species. However, slight alterations to the basic protocol voltage and resistance settings might have increased the transformation efficiency for these species.

Variation in electrotransformation efficiency may be due also to a number of other factors which modify the ability of different strains to establish and maintain plasmid function. These include barriers to DNA entry, nonspecific nucleases, restriction endonucleases and factors affecting DNA conformation. In *L. seeligeri* DNA

supercoiling may affect plasmid function, but little is known about plasmid DNA conformation in *Listeria* species. Restriction barriers to the entry of DNA prepared in *E. coli* may be present in *L. seeligeri*. Such barriers have been reported previously in other bacteria (Shigekawa and Dower, 1988). The electrotransformation frequency of *Campylobacter jejuni* is decreased by at least four orders of magnitude when the transforming DNA is prepared from *E. coli* HB101 (Miller *et al.*, 1988). Although it is unlikely that *L. seeligeri* differs from the other strains of *Listeria* in this respect, the problem could be overcome by replicating the DNA of interest in *L. monocytogenes* before extraction for electroporation into *L. seeligeri*.

Electrotransformation efficiency variation has also been noted between strains and species of the genus *Bacillus* (Belliveau and Trevor, 1989). These workers implicated cell wall composition. However, this explanation is unlikely in the case of *L. seeligeri*, because the cell wall composition of all the members of the genus *Listeria* is very similar: directly cross-linked meso-diaminopimelic acid-containing murein, and ribitolphosphate type teichoic acid (Fiedler *et al.*, 1984). Some structural variation in teichoic acid composition, due to glycosidic substitution of ribitolphosphate units, has been associated with serovar (Fiedler *et al.*, 1984). No difference in the teichoic acid composition of serovars 1/2b and 1/2a, of which *L. seeligeri* (CIP100100) and *L. monocytogenes* (NCTC 7973) are members, was noted (Fiedler *et al.*, 1984). Nevertheless, detailed structural studies of the cell wall chemistry of *Listeria* strains (Fiedler *et al.*, 1984), revealed that the cell wall of *L. seeligeri* (CIP100100) contains less rhamnose, glucose, and glucosamine, than those of the other *Listeria* species. Furthermore, in recent analyses of surface proteins of *Listeria* species differences in SDS-PAGE and immunoblotting profiles of serotypes of *L. innocua*, *L. welshimeri*, *L. seeligeri* and *L. ivanovii* compared with *L. monocytogenes* have been reported (Tabouret *et al.*, 1992). It is possible that these differences in surface protein, rhamnose, glucose, and glucosamine composition, may affect the polarisation of the *L. seeligeri* cell wall, reducing DNA entry.

The failure to recover any erythromycin resistant transformants when plasmids pJA1 and pJA2 (which carry Tn917) were used to transform *L. monocytogenes* NCTC7973

(table 3.1.1) suggests that either transformation had not been successful or transposition of Tn917 had not taken place. Transposition into the chromosome of *L. monocytogenes* NCTC 7973 may not have occurred for several reasons. One explanation could be transposition immunity. This phenomenon inhibits the transposition of the Tn3 family of transposons into a replicon containing certain Tn3 sequences (Arthur *et al.*, 1984, Wallace *et al.*, 1981). The presence within a replicon of even a single 38 bp transposon terminus has been reported to be sufficient to induce immunity to further transposition (Wallace *et al.*, 1981). The presence of transposons in *Listeria* species has not been studied, but it is possible that Tn3 like transposons may be present in the chromosome of *L. monocytogenes* NCTC 7973. Tn3 family transposons have been detected in Gram-positive species phylogenetically related to *Listeria* such as *Staphylococcus aureus*, *Enterococcus faecalis*, *Bacillus thuringiensis*, and *Clostridium perfringens* (see Sherratt, 1989). *Listeria monocytogenes* NCTC 7973 may have acquired sequences from a Tn3 family transposon by contact with bacteria carrying such transposons. There is strong evidence for such movement between species, for example a drug resistance encoding plasmid detected in a clinical isolate of *L. monocytogenes* has been found to have originated in enterococci or streptococci (Poyart-Salmeron *et al.*, 1990). Furthermore, conjugative transfer of plasmid DNA from *E. faecalis* to *L. monocytogenes* in the digestive tracts of gnotobiotic mice has been reported (Doucet-Populaire *et al.*, 1991, Pérez-Díaz, *et al.*, 1982).

Although Tn917 transposition in strains of *L. monocytogenes* has been reported previously (Camilli *et al.*, 1990, Cossart *et al.*, 1989), competent host factors which may be required for Tn917 transposition may not have been provided by *L. monocytogenes* NCTC7973. Replication functions supplied by the host bacteria can be needed for the replication step and for the final covalent ligation in transposition. Accessory proteins such as integration host factor (IHF), a histone-like protein which induces a strong connection on binding to inverted repeats at the transposon termini, are reported to have some function in transposase gene expression (Sherratt, 1989). Also, mutations in the *polA* gene encoding DNA polymerase 1, have been reported to reduce transposition (Syvanen *et al.*, 1982). It is not clear, however, whether this is

because of the direct involvement of DNA polymerase I in transposition or as a consequence of the *polA* mutation on DNA. The conformation of recipient DNA has also been reported to affect transposition, and mutations that affect DNA supercoiling have been reported to reduce transposition frequencies of Tn3 related elements (Heffron, 1983). Although DNA supercoiling in strains of *L. monocytogenes* is likely to be similar, this could possibly be one reason for the apparent lack of Tn917 transposition in *L. monocytogenes* NCTC7973. It may be that electroporation alters *L. monocytogenes* in a way that inhibits transposition and that this method of transposon delivery may not be suitable for insertional mutagenesis of this species.

The most likely explanation for the failure to introduce pJA1 and pJA2 into *L. monocytogenes* by electroporation is their large size (13.8 kb and 14.0 kb respectively) compared with that of pGK12 (4.3 kb), the plasmid used to determine the optimum conditions for electrotransformation. Gram-positive bacteria appear to be particularly sensitive to the size of the transforming plasmid DNA. For example, plasmids between 7.6 and 26.5 kb have been reported to have a reduced transformation efficiency in species of *Clostridium* (Leonard and Seding, 1990). In general, a greater degree of cell wall permeabilisation appears to be required for the transformation of Gram-positive bacteria with plasmid DNA in excess of 10 kb (Trevors, *et al.*, 1992). However, the degree of permeabilisation required is partially dependent on the strain and method used (Trevors, *et al.*, 1992).

Using the protocol optimised for pGK12, attempts to determine whether *L. monocytogenes* could be electrotransformed with another suitable large vector, were made. For this purpose the 22.1 kb plasmid pLTV3 was chosen because it was known to replicate in *L. monocytogenes* (Camilli *et al.*, 1990). This plasmid, developed to optimise Tn917 transposition in Gram-positive bacteria, is a pE194ts-derived vector which carries Tn917::lac (Camilli *et al.*, 1990). However, no transformants were recovered (table 3.1.1.). This result supports the theory that the failure of pJA1 and pJA2 to enter *L. monocytogenes* NCTC 7973 is due to their large size.

In attempts to overcome this problem, penicillin treatment was incorporated into the electroporation protocol to increase the permeabilisation of the cell wall and thus allow

the entry of large vectors. The use of penicillin pretreatment of cells for electroporation has been reported to be successful for *L. monocytogenes* by Park and Stewart (1990). These workers achieved efficient electrotransformation of *L. monocytogenes* at a frequency of 4×10^6 transformants / μg plasmid DNA. Cell wall peptide crosslinking between glycan chains is inhibited by Penicillin G treatment. This destabilises the cell wall and increases the degree of permeabilisation during electroporation required for the entry of large vectors into the cell. Unfortunately, during this process the cells become very osmotically fragile because cell wall renewal is retarded, making the recovery of electrotransformants difficult. For this reason sucrose was included to osmotically stabilise the recovery medium, and the incubation period after electroporation was extended from the 2 hrs used for the untreated electrotransformants, to 4 hrs. These steps allow regeneration of a functional cell wall, as well as expression of antibiotic resistance, before plating onto agar.

Penicillin treatment before electroporation allowed the successful transformation of *L. monocytogenes* with pLTV3 at a frequency of 2.3×10^2 transformants / μg pLTV3 DNA (section 3.2). This suggests that the electrotransformation of pJA1 and pJA2 might have been achieved by the use of this method. However, the frequency of transformation achieved with pLTV3 (2.3×10^2 / μg pLTV3 DNA), was not high enough to allow it to be used for the insertional mutagenesis of *L. monocytogenes*. Further, it was reasoned that because of the difficulty in recovering penicillin treated electrotransformed bacteria the use of other strategies for the insertional mutagenesis of *L. monocytogenes* could be more successful.

A second strategy for the insertional mutagenesis of *L. monocytogenes* based on the temperature sensitive plasmid pTV32ts was successful. Plasmid pTV32ts a highly temperature sensitive version of pTV32 (figure 2.6.1), the vector used for the construction of pJA1 (Youngman, 1987), and was obtained in *L. monocytogenes* strain EGD. Plasmid pTV32ts has been used for insertional mutagenesis of *Bacillus* species (Youngman *et al.*, 1983, Youngman *et al.*, 1984b, Youngman *et al.*, 1989) and replicates in a range of Gram-positive bacteria. It carries a pE194ts derived replicon,

which is temperature sensitive because of a point mutation in the *repF* gene (Villafane *et al.*, 1987). Vectors derived from this replicon exhibit progressively decreasing copy-numbers with temperatures above 32°C, and completely fail to replicate at temperatures above 45°C (Gryczan *et al.*, 1982).

To recover Tn917 insertion mutants, bacteria carrying pTV32ts are at first cultured at a low temperature (32-35°C) without selection for the plasmid encoded chloramphenicol resistance but with selective levels of erythromycin and lincomycin. These antibiotics induce the transposition of Tn917 because expression of the transposase occurs via transcription from the *erm* gene into the *tpnA* gene (figure 4.1). To remove the plasmid from the Tn917 carrying population the bacteria are then cultured at a high temperature (45-48°C) nonpermissive for the plasmid with selection for the Tn917 encoded erythromycin and lincomycin resistance. Survivors of this period of growth at high temperature were predominantly bacteria that had acquired a chromosomal Tn917 insertion. Transposon insertion mutants maintained erythromycin resistance but lost plasmid encoded chloramphenicol resistance. When insertion occurred within a gene 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 existing chromosomal promoter when the Tn917:: *lacZ* fusion inserts in the correct orientation to allow expression.

The reported temperature range over which the *L. monocytogenes* will multiply is between 1°C and 45°C, although strain variation is evident at the higher temperatures, (Seeliger and Jones, 1986). Unfortunately *L. monocytogenes* strain EGD carrying pTV32ts was found not to grow at temperatures above 41°C. This proved to be a serious problem because a temperature of 41°C was not sufficiently high to remove pTV32ts from the bacterial population when the method developed for the recovery of Tn917 chromosomal insertion mutants of *Bacillus subtilis* was used (section 2.10, Youngman, 1989). Incorporated into this method for *Bacillus* species are a series of dilution steps and incubations at 48°C which enrich for Tn917 insertions. These steps

are required as three to four cell-generations are needed before plasmid copy number is depleted to one per cell. Furthermore, these steps enrich the population for insertion mutants by diluting out any members of the background population with inherited methylated ribosomes for erythromycin resistance. Because the replication of pTV32ts is reduced rather than arrested at 41°C extra dilutions and replication steps at 41°C were introduced to remove the plasmid from the population and to allow maximum transposition frequency to be achieved. As a result of the introduction of additional replication steps the transposition frequency was successfully raised to a maximum of 5.1×10^{-3} recipients (table 3.4), although this is considerably less than the frequency reported for Tn917 transposition from pTV32ts in *B. subtilis* (Youngman *et al.*, 1989).

The blue appearance of all the Tn917 insertion mutants from an "insertion library" of *L. monocytogenes* EGD, when plated onto media containing X-gal, (section 3.4), was not expected. Such high numbers could not represent transposon insertions in active regions of the *L. monocytogenes* EGD genome. This observation suggested that in this strain, a low level of endogenous β -galactosidase activity was in some way augmented by the *lacZ* gene on pTV32ts. Other workers have also reported this phenomenon (Camilli *et al.*, 1990, M. Wuenscher personal communication). This restricts the use of *lacZ* transcriptional fusions to look for transcriptional units in this strain of *L. monocytogenes*. It is possible that this problem could be overcome by using other strains of *L. monocytogenes*. For example, transposon insertions forming *lacZ* fusions in *L. monocytogenes* strain 10403S have been detected by expression of above background levels of β -galactosidase activity (Camilli *et al.*, 1990). These insertion mutants were found to produce greater than 1,000-fold more β -galactosidase activity than wild-type bacteria in liquid culture (Camilli *et al.*, 1990).

The results of Southern hybridisation analyses used to evaluate the randomness of Tn917 insertion in pTV32ts libraries indicated that insertion into the chromosome of *L. monocytogenes* EGD was random (figure 3.4.2.). The Tn917 specific probe bound to chromosomal fragments of different sizes (figure 3.4.2.). This random insertion of Tn917 is consistent with its Tn3-like transposition, which favours A+T-rich sequences

and those with similarity to the 38 bp terminal inverted repeats of the transposon (Tu and Cohen, 1980). However, Tn917 insertions into the chromosome of *B. subtilis* and *B. megaterium* have been found not to be random (Bohall and Vary, 1986, Vandeyser and Zahler, 1986). In Tn917 mutagenesis of the *B. subtilis* chromosome, 99% of all inserts were clustered in several "hotspots", and approximately 90% of all auxotrophic insertion mutants isolated were glutamine requiring, and harboured insertions in or near the *gltA* and *gltB* loci (Perkins and Youngman, 1984, Youngman *et al.*, 1983, Youngman *et al.*, 1984b).

Southern hybridisation analysis of chromosomal DNA with a plasmid specific probe (figure 3.4.1.), to confirm that the plasmid pTV32ts had been lost from the insertion mutants, revealed that plasmid DNA was still present (figure 3.4.3.). This result was not expected because the bacteria did not show the plasmid encoded chloramphenicol resistance. An explanation for these observations is that the plasmid had integrated into the chromosome with the transposon. To investigate whether such an abnormal transposition event had taken place, Southern hybridisation analysis was carried out on *Kpn*I digested DNA with a *Sal*I restriction fragment DNA probe which spanned the *Kpn*I restriction site cutting the transposon once (figure 3.4.1.). The results of this hybridisation (figure 3.4.4.) showed that the plasmid was present chromosomally because the probe bound to two bands, if the plasmid DNA had been extrachromosomal only one band of the same size as linearised pTV32ts (15.6 kb) would have been seen. The presence of a single copy of the plasmid encoded chloramphenicol resistance gene present in the chromosome of a majority of the mutants led to low level chloramphenicol resistance evident only after 2-3 days of incubation. 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 having been used to inactivate the listeriolysin *lisA* gene (Wuenschel *et al.*, 1991). However insertion of the plasmid into the chromosome with the transposon negated the advantage of the small size of Tn917. Cloning, and mapping of DNA flanking transposon insertions as well as mutation analysis, are simplified if the insertion element is small.

Insertion of plasmid DNA into the host chromosome with the transposon may be due to impaired resolution of the plasmid-chromosome cointegrate formed during transposition *tnpR* (figure 4.3). The cointegrate is resolved by the transposon encoded resolvase protein (figure 4.1), and cointegrates are detected only when mutant elements defective in resolvase are used (Heffron, 1983). One explanation for a lack of cointegrate resolution of the cointegrate is that the copy of Tn917 in pTV32ts harboured by the initial strain was defective in the production of resolvase. There have been a number of reports of mutations in the resolvase *res* genes of Tn3 family transposons (Hatfull and Grindley, 1986, Newman and Grindley, 1984). The mutant resolvases tend to fall into two groups: mutations in the C-terminal of the resolvase protein which eliminate or impair binding to *tnpR*, and those in the N-terminal which impair binding to *tnpR* and the recombination required for resolution. 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). However, Southern hybridisations to *EcoR*I digested chromosomal DNA with the transposon specific probe (figure 3.4.1.), would not have revealed whether resolution had not occurred.

The formation of unresolved cointegrates has been noted with Tn917 in certain strains of *E. faecalis* in which stable unresolved cointegrates were formed (Tomich & Clewell 1980). These strains were thought to lack certain host factors required for the replication stage of transposition (Tomich & Clewell 1980). Replication functions supplied by the host bacteria may also be needed for the final covalent ligation in transposition, and accessory proteins such as integration host factor (IHF), are reported to have some function in transposase gene expression in *E. coli* (Sherratt, 1989). Because the replication of *L.monocytogenes* strain EGD is restricted at 41°C, the temperature used for the insertional mutagenesis, such factors needed for the replication stage of transposition, may not have expressed.

Another possible explanation for the insertion of the donor plasmid into the host chromosome with the transposon is that recombination with another transposon may have occurred. Such an event could occur if the transposon carrying plasmid remains in the cell after successful insertion of the transposon into the host chromosome.

Cutting (→) and rejoining

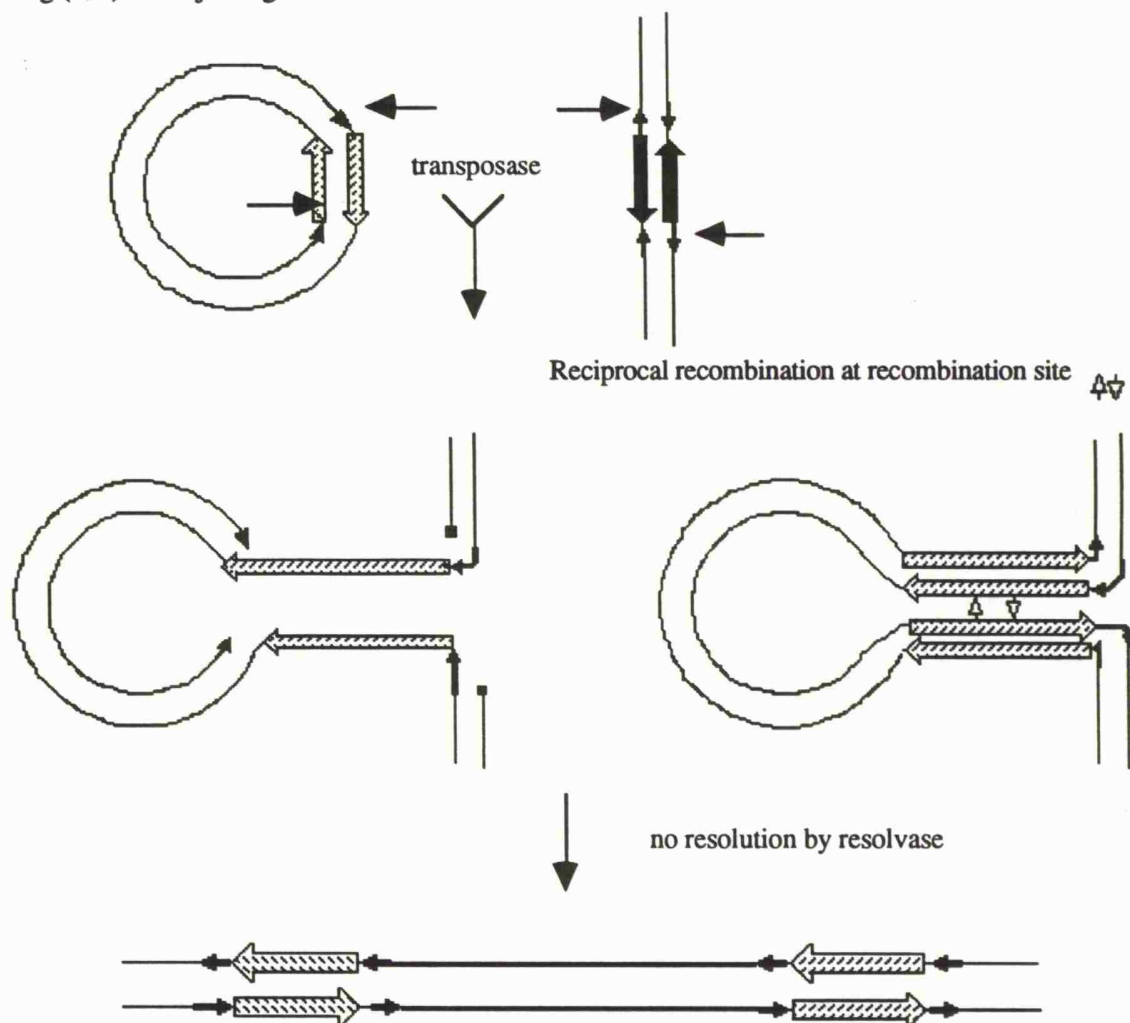


Figure 4.3. Postulated mechanism of plasmid insertion into chromosomal DNA, mediated by the transposition of Tn917 if resolution does not take place. The Tn917 single strands are shown as cross hatched arrows and the sequences which become duplicated in the chromosomal DNA as solid arrows. Arrowheads indicate 3' ends and solid squares indicate 5' ends.

Recombination between the two copies of the transposon would result in the insertion of the plasmid into one copy of the transposon in the chromosome (figure 4.4). Secondary recombination events would not normally be expected, because the donor suicide vector carrying a copy of the transposon should be lost from the cell. However the reduced temperature employed for the mutagenesis (41°C) may not have removed the donor from the cell directly after transposition allowing secondary recombination to occur.

Several workers have observed insertion of the donor replicon into recipient DNA during Tn3-like transposition due to one-ended transposition (Arthur *et al.*, 1984, Heritage and Bennet, 1985, Mötsch and Schmitt, 1984, Schmitt *et al.*, 1981). Normal replicative transposition requires two transposon termini in inverted orientation (figure 4.2). Nevertheless, aberrant replicative transposition can take place in which an initial recombinant connection between one donor transposon terminus and one end of the cleaved target DNA is made. Such a connection is then followed prematurely by rolling circular replication before a second connection at the other transposon termini has been made (figure 4.5). Subsequently, a second cleavage is made in a section of the newly replicated donor molecule and is ligated to the other end of the target DNA (figure 4.5). The frequencies of such transposition events are lower than those of normal transposition, a factor of 1,000 lower in the case of transposon Tn3 (Arthur *et al.*, 1984, Mötsch *et al.*, 1985). As a member of the Tn3-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 as a consequence of Tn917 insertion. Although such events are rare the application of an increased number of replication steps at the partially nonpermissive temperature, may have resulted in the selection of bacteria carrying chromosomal insertions of this type. However, as all the insertion mutants analysed appeared to have undergone abnormal transposition, it is unlikely that an event as rare as one ended transposition could be responsible.

The apparently random insertion of Tn917 (with the plasmid) into the chromosome as indicated by Southern hybridisation (figure 3.4.2) was not consistent however with the observations made when insertion libraries were screened for mutants defective in

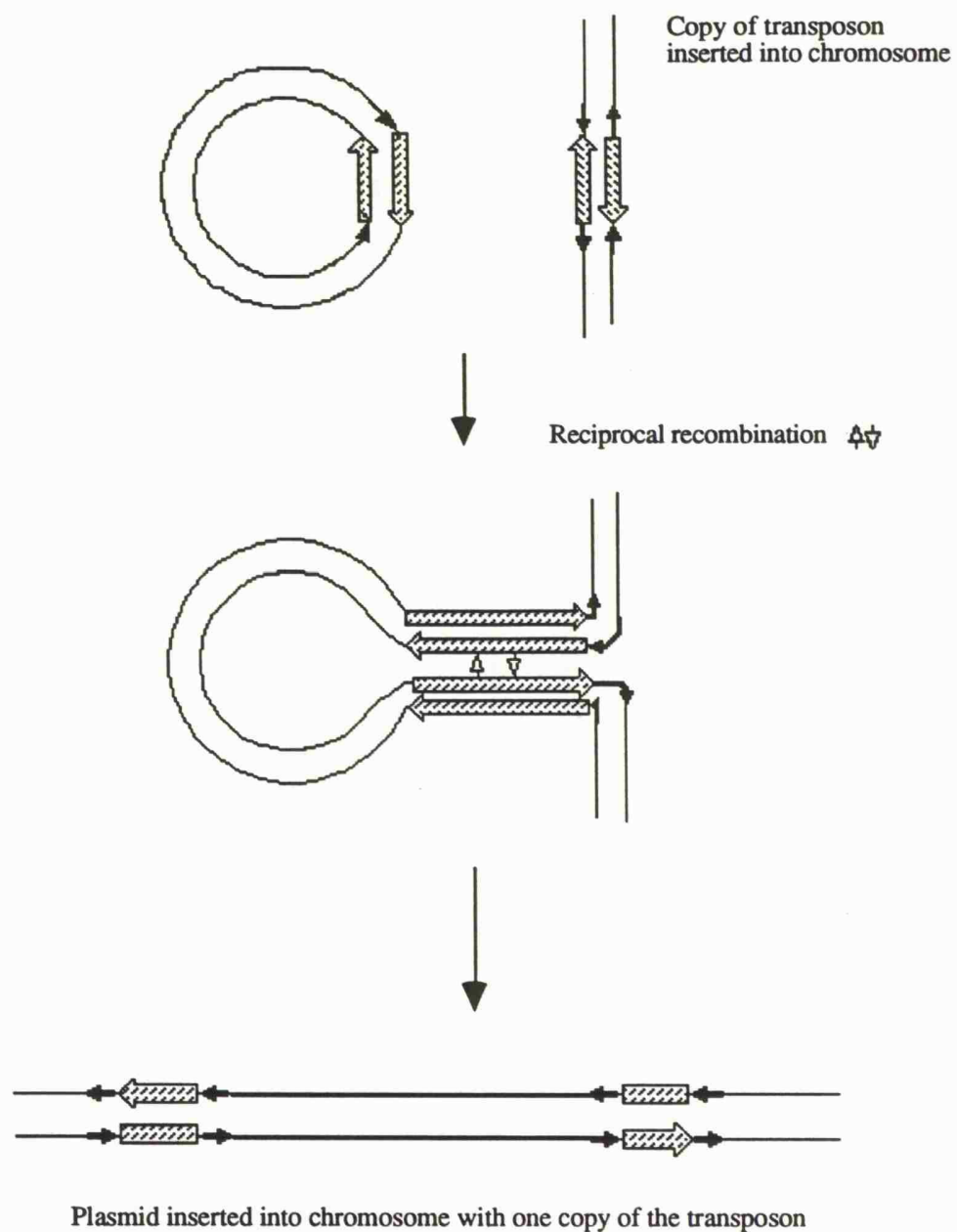


Figure 4.4. Postulated mechanism of plasmid insertion into chromosomal DNA, mediated by the reciprocal recombination between copies of Tn917, after Tn917 chromosomal insertion. The Tn917 single strands are shown as cross hatched arrows.

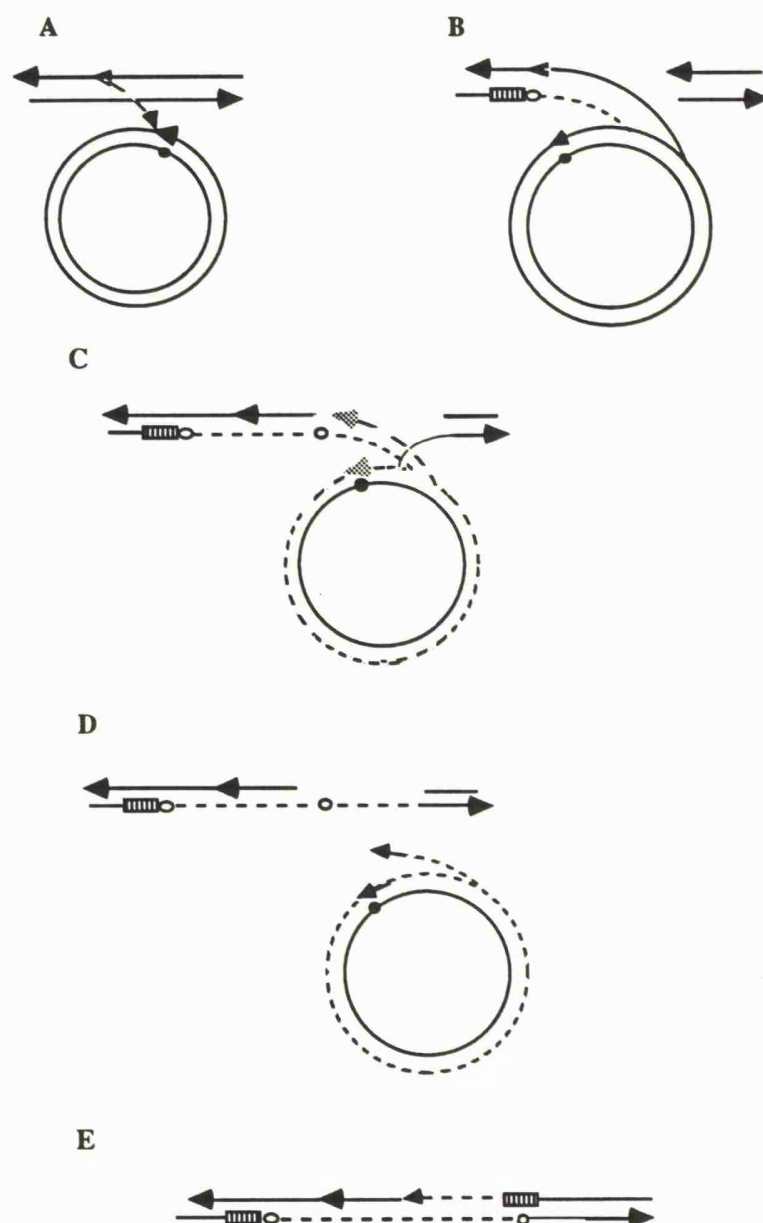


Figure 4.5. Schematic representation of one-ended transposition.

A. Initiation, as indicated by arrows, a nick at the IR in the donor DNA and 5bp staggered nicks in the recipient are produced. The IR on the cut strand of the donor DNA is then ligated to the recipient DNA, as indicated by the dotted line. B. Elongation, a replication fork is formed 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 the recombinant molecule is filled to produce a duplicated segment of donor DNA. Key: Donor replcon indicated 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, ■■■ indicates the direct repeats of recipient DNA. Adapted from Mötsch *et al* (1985).

rhamnose utilisation (section 3.7.). Utilisation of this carbohydrate is an important differential characteristic in the identification of *Listeria* species (Rocourt and Catimel, 1985, Seeliger and Jones, 1986). It was chosen as a suitable marker of Tn917 insertion, because defective mutants could be isolated easily on an appropriate solid medium such as the modified basal medium of Wilkinson and Jones (1977). This medium contains rhamnose as the sole carbon source and chlorophenol red as an indicator (section 2.12). This indicator changes colour from red to yellow under colonies that produce acid from rhamnose thus allowing the identification of non-rhamnose utilising mutants. The detection of a high percentage of mutants unable to utilise rhamnose (section 3.7), suggests that a hotspot for Tn917 insertion might exist in the *L. monocytogenes* EGD genes encoding the proteins responsible for this function. Tn917 insertion in this region of the chromosome may be favoured if it is particularly A+T-rich or has homology with the 38 bp terminal inverted repeats of the transposon. However, the DNA sequences of these genes in *L. monocytogenes* is not known. Such hotspots have been reported previously, in some regions of the *B. subtilis* chromosome (Youngman *et al.*, 1983). For example, approximately 90% of all auxotrophic insertion mutants from pTV1 derived Tn917 insertion mutant libraries of *B. subtilis* required glutamine, harbouring insertions in or near the *gltA* and *gltB* loci (Youngman *et al.*, 1983, Youngman, 1989). Preferential insertion has also noted by Camilli *et al.*, (1990) 59% of the auxotrophs isolated by these authors from a pLTV3 derived Tn917 insertion libraries of *L. monocytogenes* required adenosine, suggesting the presence of an insertion hotspot in this region.

To determine a suitable medium for the screening of insertion libraries for aromatic amino acid-dependent mutants and those of other auxotrophic phenotypes, several minimal media were compared (section 2.13). The results (table 3.6.1) indicated that the minimal medium of Friedman and Roessler (1961) was the most suitable for further use as it sustained good growth of all species after 48 hrs incubation. Although the medium of Welshimer (1963) offered good growth of *L. monocytogenes* NCTC7973, it did not allow as rapid growth of all the species as that of Friedman and Roessler (1961), (Table 3.6.2). The minimal media of Ralovich *et al.*, (1977), and Siddiqi and Khan (1989), were not found to be suitable for the growth of *L.*

monocytogenes NCTC7973 (Table 3.6.1). These differences in the growth of *Listeria* species on minimal media may be due to strain and species variation in nutritional requirements which have been noted in many other studies (Ralovich *et al.*, 1977, Siddiqi and Khan, 1982, Siddiqi and Khan, 1989). In these reports the vitamin, nitrogen base, and amino acid requirements of different strains have been reported to vary considerably between strains. The source and quality of the components of the minimal media may also affect the results of such comparisons. Furthermore, it has been noted that the growth of *L. monocytogenes* is sensitive to iron concentration and the form of the iron available in these media may affect their ability to support growth. *Listeria monocytogenes* binds Fe^{+2} and ferric citrate but does not take up ferric ferroxamine, ferric EDTA, or FeCl_3 (Adams *et al.*, 1990). In the minimal media of Siddiqi and Khan (1989), iron is supplied in the form of FeCl_3 (section 2.13), and this may be one reason for its inability to support the growth of *Listeria* species.

Screening for adenosine and glycine as well as aromatic amino acid dependent insertion mutants was undertaken to confirm the random nature of Tn917 in the *L. monocytogenes* libraries prepared. Screening resulted in the isolation of these phenotypes at an unexpectedly low frequency of 0.0001% (section 3.7). The frequency of isolation of these phenotypes was compared with the frequencies reported by other workers from Tn917 insertion libraries (Camilli *et al.*, 1990, Youngman *et al.*, 1983, Youngman *et al.*, 1984b). The frequency of isolation was much lower than that of 0.006% reported by Camilli *et al.*, (1990) for the isolation of adenosine, glycine and aromatic amino acid dependent Tn917 insertion mutants of *L. monocytogenes*. Further, in contrast to the observation of these workers that 59% of the *L. monocytogenes* auxotrophs isolated from constructed Tn917 insertion libraries required adenosine, no adenosine dependent insertion mutants were isolated (section 3.7). The frequency of auxotrophic mutant isolation is also comparatively lower than the 5% isolation of mutants of several auxotrophic phenotypes reported from pTV1 derived insertion mutant libraries of *B. subtilis* (Youngman *et al.*, 1983, Youngman *et al.*, 1984b).

The low frequency of auxotrophic mutant isolation and the relatively high frequency of isolation of rhamnose utilisation mutants, suggests the preferential insertion of Tn917 into certain "hotspots" in the *L. monocytogenes* chromosome. Another possible explanation is that the application of an increased number of replication steps at the partially nonpermissive temperature of 41°C, may have resulted in the selection of a small number of sibling insertion mutants of the same phenotype rather than a random population. To reduce the need for prolonged replication at a nonpermissive temperature and so reduce the risk of sibling selection, the plasmid pLTV3 could be used. Plasmid pLTV3 has been reported to be more temperature sensitive than pTV32ts because it carries an engineered mutation in the *repF* gene of the pE194 replicon. This vector also promotes transposition at a higher frequency compared with other pE194 derived Tn917 carrying vectors and the conjugative transposons Tn1545, and Tn916 (Camilli *et al.*, 1990, Gaillard *et al.*, 1986, Kathariou *et al.*, 1987).

The increased transposition frequency of 7.3×10^{-4} recipients, observed in preliminary Tn917 insertion recovery experiments using pLTV3 in *L. monocytogenes* NCTC7973, incorporating a number of dilutions and periods of high temperature replication (table 3.4) correlated well with those of Camilli *et al.*, (1990). These workers reported an increased Tn917 transposition frequency of 8.2×10^{-4} recipients from pLTV3 in *L. monocytogenes* and a frequency of 6.1×10^{-3} recipients from pTV32ts in *B. subtilis*.

Further studies were carried out to characterise the aromatic amino acid requiring auxotrophic mutant Lm.6b isolated from my own pTV32 derived Tn917 insertion mutant libraries (section 3.7). The results of these studies to characterise the biochemical defect in Lm.6b suggested that insertional inactivation had occurred in the structural or regulatory sequences of the genes encoding the enzyme chorismate mutase (figure 3.9). This enzyme was implicated because tyrosine, phenylalanine, phenylpyruvate and prephenate, but not their precursors chorismate and shikimate were found to support the growth of Lm.6b in minimal medium (section 3.9). The reduced growth of Lm.6b supported by the aromatic amino acid biosynthesis intermediates phenylpyruvate and prephenate when compared with that supported by

tyrosine and phenylalanine (section 3.7), may be because of inefficient cellular uptake or because these intermediates are readily oxidised in solid media. Slow growth of aromatic amino acid dependent mutant strains of *E. coli* supported by intermediates such as shikimate and chorismate has been reported previously (Brown and Doy, 1976, Pittard, 1987). These workers noted that the efficiency with which aromatic amino acid biosynthesis intermediates gain entry to bacterial cells varied considerably. Because of these considerations enzyme assays were undertaken to confirm the biochemical defect present in Lm.6b. Variations in the cellular uptake of intermediates are not a problem in these assays because enzyme preparations rather than whole cells are used. Furthermore, because of their short duration, enzyme assays reduce the likelihood of oxidation of the intermediates.

The results of enzyme assay (table 3.9) confirmed that Lm.6b is defective in chorismate mutase activity, the enzyme that catalyses the conversion of chorismate to prephenate (figure 3.9). The genes which encode chorismate mutase are designated *aroG/H* in *B.subtilis* (Lorence and Nester, 1966, Piggot and Hoch, 1985), and the chorismate mutase catalysed conversion of chorismate to prephenate occurs at the branch point of the aromatic amino acid biosynthetic pathway where chorismate is directed into the pathways of tyrosine, phenylalanine, and tryptophan synthesis (figure 3.9).

An aromatic amino acid dependent transposon insertion mutant of *L. monocytogenes* strain Lm.1070138, Lm.918.6 was obtained from D. Portnoy, University of Pennsylvania, to study in parallel with Lm.6b. The strain Lm.918.6 is an aromatic amino acid dependent mutant of the wild-type strain Lm.1070138 (serotype 1/2a), and had been described previously as a Tn917 insertion mutant from a pLTV3 derived insertion mutant library (Camilli *et al.*, 1990). These workers reported that Lm.918.6 required aromatic amino acids for growth (Camilli *et al.*, 1990). The results of Southern hybridisation analysis (figure 3.8.1.) confirmed that Lm 918.6 contained a single Tn917 insertion.

Experiments to characterise the biochemical defect in Lm.918.6 (section 3.9), indicated that the growth could be supported by phenylalanine and its immediate precursor phenylpyruvic acid, but not by tyrosine nor prephenate (a precursor of both tyrosine and phenylpyruvic acid). This finding suggested that prephenate dehydratase activity had been lost and that insertional inactivation had occurred in the structural or regulatory sequences of the gene encoding this enzyme. Prephenate dehydratase catalyses the conversion of prephenic acid to phenylpyruvic acid - the penultimate stage in the biosynthesis of phenylalanine (figure 3.9), and is encoded by a gene designated *pheA* in *B.subtilis* (Piggot and Hoch, 1985). The hypothesis that Lm.916.8 is defective in prephenate dehydratase was confirmed when the activity of this enzyme was determined (table 3.9).

Both chorismate mutase and prephenate dehydratase catalyse reactions late in the aromatic amino acid biosynthetic pathway (figure 3.9). In *E. coli*, *S. typhimurium*, and *Enterobacter aerogenes* chorismate mutase and prephenate dehydratase activities have been found to be a product of a bifunctional chorismate mutase-prephenate dehydratase enzyme complex (Baldwin *et al.*, 1981, Pittard, 1987). In these species of Gram-negative bacteria, the loss of activity of one of these enzymes normally affects the activity of the other enzyme in the complex. However, in *B. subtilis* no association of chorismate mutase and prephenate dehydratase is evident (Gibson and Pittard, 1968). The detection of normal activities of prephenate dehydratase and chorismate mutase in Lm.6b and Lm.918.6 respectively, although chorismate mutase and prephenate dehydratase activities had been lost (table 3.9), indicates a similar situation in *L. monocytogenes*. This finding is not surprising considering the relative phylogenetic positions of the genera *Bacillus* and *Listeria* and may indicate a possible evolutionary relationship between Gram-positive genes involved in the biosynthesis of aromatic amino acids. These genes may be located close together, possibly forming a cluster with other genes in the biosynthetic pathway. Such aromatic amino acid biosynthesis gene organisation has been reported in *B.subtilis* (Nester, *et al.*, 1963). No linkage map of the *L. monocytogenes* chromosome is currently available.

The results of experiments to determine the ED₅₀ of Lm.6b in mice were disappointing (section 3.10.). The unaltered virulence of Lm.6b in mice when compared with that of its wild-type parent strain Lm.1070138 (table 3.10.2), suggested that either the bacteria had reverted to a prototrophic phenotype or the biochemical defect in this strain was not attenuating. One possible explanation for these results is that, under the selective pressures present *in vivo*, spontaneous excision of Tn917 had occurred. The prototrophic phenotype of the organisms isolated from the tissues after infection also indicated that excision had taken place. Spontaneous excision of Tn917 was not considered to be very likely since such events are noted to occur at very low frequencies (less than 10⁻¹⁰ / bacteria) for Tn3-induced mutations (Berg *et al.*, 1989). Tn917 was chosen for the insertional mutagenesis of *L. monocytogenes* because chromosomal insertions of this element have been found to be very stable (Murphy, 1989, Youngman *et al.*, 1983, Youngman *et al.*, 1989). The results of Southern hybridisation of a Tn917 specific probe with chromosomal DNA from organisms isolated from the tissues after infection with Lm 6b (figure 3.10.2.e.), indicated that excision had not taken place. Therefore other explanations were sought for the observed prototrophy of the bacteria isolated from the tissues of mice immunised with Lm.6b

One possible explanation was the occurrence of a point mutation in the *aro* genes of Lm.6b as well as a Tn917 insertion in an unknown region of the chromosome. Such an event could have been identified by transduction or transformation analysis of Lm.6b to confirm linkage between the transposon and the mutant phenotype. Selection for erythromycin resistance associated with Tn917 in a genetic cross should have resulted in 100% inheritance of the *aro*⁻ phenotype if this had been caused by its insertion rather than by a second independent mutation. It is likely that reversion of a point mutation would occur *in vivo* under the selective pressures of the intracellular environment. Studies on purine requiring *purA*⁻ strains of *S. typhimurium* in animal tissues revealed a greater than 10⁸ fold selection for a prototrophic *purA*⁺ phenotype (McFarland and Stocker, 1987, Mahan *et al.*, 1993). Following infection of mice with Lm 6b, the extended period of time before the onset of sickness observed (section

3.10), is consistent with the hypothesis that a point mutation was the cause of the aromatic amino acid dependence of Lm.6b. Also consistent with this hypothesis was the slower appearance of granulomas in Lm.6b infected tissues during the first two days of infection (section 3.10). These findings may indicate reduced multiplication of Lm.6b due to its initial auxotrophic phenotype. The subsequent rapid progress of the infection which reached lethality on the same day as the wild-type infection (section 3.10), represented the multiplication of the reverted organisms. Reversion of the mutation may occur because of selective pressure *in vivo* and the course of infection by the reverted bacteria may then proceed more rapidly than usual, because the host immune system may be already compromised by the initial *aro*⁻ inoculum.

The hypothesis that an aromatic amino acid dependent auxotroph of *L. monocytogenes* may exhibit reduced virulence was confirmed by analysis of the virulence of Lm.918.6 in mice (tables 3.10.1, 3.10.2). The determined reduction in the ED₅₀ of Lm.918.6 was significant, although less than that reported for *aroA*⁻ mutants of *Salmonella typhimurium* (Hoiseth and Stocker, 1981, O'Callaghan, *et al.*, 1988). Such *aroA*⁻ insertion mutants have been reported to have LD₅₀ values a factor of 10⁶ greater than their wild-type parent strain when delivered parenterally; i.p. (Hoiseth, and Stocker, 1981, O'Callaghan, *et al.*, 1988).

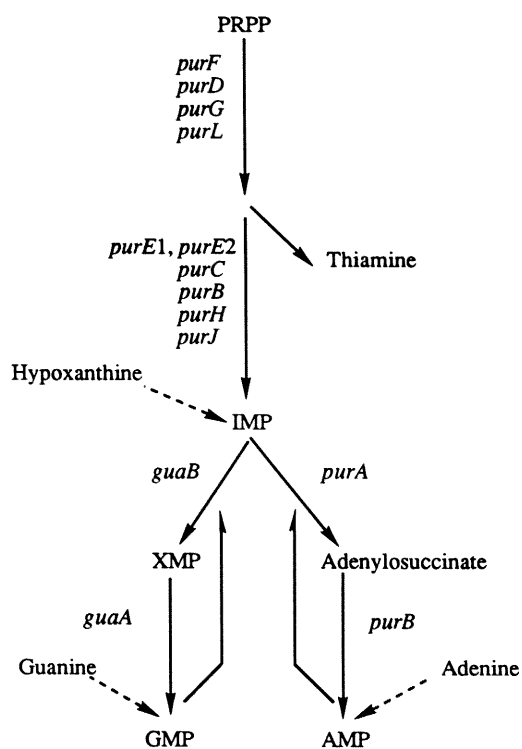
Care must be taken in comparison of the virulence of strains of *Listeria* and *Salmonella* because of the fundamental differences in the pathogenesis of the two species. Furthermore, comparisons are difficult to make because of the different route of infection (i.v. rather than i.p.), and the susceptibility to infection of the mouse strains used. However, differences in the degree of attenuation may be due to the respective positions in the aromatic biosynthetic pathway of the enzymes encoded by the inactivated genes. The product of the *Salmonella aroA* gene, 3-enol-pyruvoyl shikimate-5-phosphate synthase acts earlier in the aromatic amino acid biosynthetic pathway than the *pheA* gene product, prephenate dehydratase, deficient in Lm.918.6. No significant differences in the degree of attenuation of different *aro*⁻ mutants in *Salmonella* has been noted. *AroA*, *aroC*, and *aroD*, mutants have approximately the

same LD₅₀ in mice (Dogan *et al.*, 1988, Miller *et al.*, 1989, O'Callaghan *et al.*, 1988). In contrast with these findings, the location of mutations in the *pur* genes for purine biosynthesis have been reported to affect the degree of attenuation (O'Callaghan *et al.*, 1988, McFarland and Stocker, 1987). Strains of *Salmonella* carrying a *purA* mutation were found to show a greater degree of attenuation than the *Salmonella* carrying a mutation in the *purE* gene (figure 4.6), (McFarland and Stocker, 1987, O'Callaghan *et al.*, 1988). These differences can be explained partially by the branched nature of the purine biosynthetic pathway and the number of salvage pathways present (figure 4.6). The *pheA* gene inactivated in Lm.918.6 occurs in the branched region of the aromatic amino acid biosynthetic pathway (figure 3.9). Salvage pathways from the tryptophan and tyrosine synthesis pathways may operate, allowing partial restoration of the ability to synthesis phenylalanine thus reducing attenuation of Lm.918.6. *Listeria monocytogenes* may have salvage pathways not present in *Salmonella* which allow some degree of restoration, however there is, to my knowledge, no published information on the aromatic biosynthetic pathways of *Listeria* species which would allow this to be predicted.

The observed reduction in the multiplication of Lm.918.6 *in vivo* (figures 3.10.2.), is consistent with the findings of other workers that the growth of aromatic amino acid dependent intracellular bacteria is severely limited *in vivo* (Bacon *et al.*, 1951, Dogan *et al.*, 1988, Hoiseth and Stocker 1981, O'Callaghan *et al.*, 1988). This may be due, to an inability to synthesis p-amino-benzoic acid (pABA) and dihydroxy benzoate (DHB), as suggested for *aro*⁻ mutants of *Salmonella* (Hoiseth and Stocker 1981).

These workers reported experiments in which a large inoculum (2.0×10^8 bacteria i.p.) of non-virulent *aro*⁻ *S. typhimurium* caused fatal infections in inbred (BALB/c) mice provided with drinking water containing pABA and DHB. Because of the high levels of pABA and DHB taken in by the mice from the drinking water these compounds would become available to the bacteria in the tissues. The same inoculum caused no apparent ill effects in mice which received pABA, DHB or benzoate alone, suggesting that the requirement for pABA and for DHB individually is not enough to render an *aro*⁻ *S. typhimurium* strain non-virulent for mice on a normal diet. The

Figure 4.6. Diagrammatic representation of the main reactions of the purine biosynthesis pathway and of salvage pathways for the uptake of purines, or utilization of adenine as a source of guanine.



Abbreviations: PRPP: phosphoribosyl amine, IMP: inosine monophosphate, XMP: xanthosine monophosphate, GMP: guanosine monophosphate, AMP: adenosine monophosphate.
 — indicates purine biosynthesis pathway
 - - - indicates salvage pathways for the uptake of purines
 — indicate utilization of adenine as a source of guanine.
 Adapted from Stocker, (1988).

inability of *aro*⁻ *S. typhimurium* to synthesise other products of chorismate such as ubiquinone and menaquinone, was suggested to be irrelevant to the attenuation of *aro*⁻ *S. typhimurium* (Hoiseth and Stocker 1981).

The inability to synthesise p-amino-benzoic acid (pABA), and dihydroxy benzoate (DHB), would not at first appear to be likely for Lm.918.6. If the aromatic amino acid biosynthetic pathway in *L. monocytogenes* is similar to that of *Bacillus*, disruption of the *pheA* gene of Lm.918.6 would not impair the synthesis of pABA and DHB (figure 3.9). However, in *B. subtilis* the activity of 3-deoxy-D-arabino-heptulosonic acid 7-phosphate (DAHP) synthase, the first enzyme in the biosynthetic pathway encoded by the *aroA* gene (figure 3.9), is inhibited by high levels prephenate (Jensen and Nester 1966). A mutation in the *pheA* gene will normally lead to a build up of prephenate which may reduce DAHP synthase activity by feedback inhibition. Such feed-back inhibition would have an inhibitory effect on the synthesis of pABA and DHB, similar to that exerted by insertional inactivation of *aroA* (Lorence and Nester, 1966, Pittard and Gibson, 1970). Furthermore, feed-back inhibition of chorismate mutase activity due to the accumulation of prephenate, the substrate of prephenate dehydratase, has also been reported (Jensen, and Nester, 1966). This again reduces the synthesis of pABA and DHB (Jensen, and Nester, 1966). In this way feed-back of accumulated metabolites in Lm.918.6 may inhibit enzymes earlier in the aromatic pathway which impair the synthesis of pABA and DHB retarding growth (figure 3.9).

Although, the reduced growth of Lm.918.6 *in vivo* may therefore be due, at least in part, to an inability to synthesise pABA and DHB, the importance of these compounds in *L. monocytogenes* may be different from that in *Salmonella*. The aromatic amino acid biosynthesis intermediate pABA is a precursor for the synthesis of folic acid, a vitamin commonly required by bacteria for carbon metabolism, methyl group transfer, and nucleic acid synthesis. Folic acid is important for the growth of *L. monocytogenes* and the addition of folic acid to defined media has been found to be stimulatory for some strains (Siddiqi and Khan, 1982). The requirement of pABA and folic acid for the growth of *L. monocytogenes* is confirmed by the susceptibility of *L. monocytogenes* to sulphonamides, structural analogs of pABA which inhibit the

production of folic acid (Scheild, 1983).

The role of the intermediate DHB in the growth and virulence of *L. monocytogenes* to my knowledge has not been investigated. However, it is likely to be different from its role in *Salmonella* pathogenicity. DHB is a precursor of the *Salmonella* iron capturing compound 2,3-dihydroxy-N-benzoylserine or enterochelin. This phenolic siderophore binds iron in an assimilable form, by six phenolic hydroxyl groups. Although not proven the loss of ability to acquire iron for metabolic functions is thought to contribute to the reduced virulence of *aro*⁻ mutants of *Salmonella* (Stocker *et al.*, 1988). Iron has been shown to be important for the growth of *L. monocytogenes* during experimental infection (Cowart and Foster, 1985, Cowart, 1987, Sword 1966). It is therefore tempting to suggest that a diminished ability to sequester iron may contribute to the reduced virulence of Lm.918.6. However, recent studies on iron acquisition systems of *L. monocytogenes* indicate that iron is not acquired by the action of siderophores like enterochelin (Adams *et al.*, 1990). These workers reported that *L. monocytogenes* obtains both Fe²⁺ and Fe³⁺ *in vivo* by the reductive mobilisation of iron (Cowart *et al.*, 1988). The extracellular reductant involved in mobilisation was demonstrated to be a low molecular weight protein (Cowart *et al.*, 1988). Free iron is then acquired by the action of two independent cell surface receptors (Adams *et al.*, 1990). After binding to these receptors the uptake of Fe³⁺ (as Fe³⁺-citrate) is reported to be citrate inducible, while the uptake of Fe²⁺ is reported to be via facilitated diffusion (Adams *et al.*, 1990). The requirement of *aro*⁻ mutants of *L. monocytogenes* for DHB derived siderophores is therefore unlikely to affect virulence.

The ability of Lm.918.6 to grow and persist in mice, after i.v. infection, was compared with that of wild-type Lm.1070138 (figure 3.10.2. a & b). The liver and spleen were chosen for investigation because *L. monocytogenes* is known to multiply rapidly in these organs (Mainou-Fowler *et al.*, 1988, Mitsuyama *et al.*, 1978). The results demonstrated that although Lm.918.6 infected these tissues its multiplication was impaired. Limited multiplication in these tissues was probably due to low levels of

residual aromatic amino acids in the bacteria, or available in the tissues.

A degree of restricted replication would be advantageous for the initiation of acquired cellular resistance to *L. monocytogenes*. Avirulent strains of *L. monocytogenes* that are unable to replicate within a host have been found to be poorly immunogenic (Baldridge *et al.*, 1988, Berche *et al.*, 1987a, Brunt *et al.*, 1990). The protective cellular immune response to listerial infection is mediated by both CD4⁺ and CD8⁺ T cells (Kaufmann and Hahn, 1982, Magee and Wing, 1988). CD4⁺ T cells act primarily through the secretion of lymphokines (Czuprynski *et al.*, 1989b, Magee and Wing, 1988), and can be stimulated by live and killed *Listeria monocytogenes*. CD8⁺ cells are important for clearance of bacteria by cytolysis and the secretion of lymphokines (Baldridge *et al.*, 1990, Bishop and Hinrichs, 1987). This T cell subset has been proved to be induced only by *L.monocytogenes* when there is growth in the cytoplasm (Brunt *et al.*, 1990).

Reduced virulence of Lm.918.6 was also evident when an inoculum equivalent to the ED₅₀ of the wild-type, was administered to mice (figure 3.10.2. c & d). The results of this experiment (figures 3.10.2. c & d), indicated that Lm.918.6 invaded the spleen and liver in a similar pattern to the wild-type and that significant multiplication did not occur over 24 hrs. This result contrasted with those of earlier experiments in which a lower dose was administered (figures 3.10.2. a & b) and multiplication of Lm.918.6 occurred in the tissues over the first 24 hrs after infection (figures 3.10.2. c & d). One explanation for this difference in the rate of multiplication is that the available intracellular aromatic amino acids are exhausted more quickly when more bacteria are present.

The observation that reduced numbers of Lm.918.6 bacteria, compared with wild-type Lm.1070138 bacteria, were isolated from the liver and spleen, after 4 hrs of infection (figure 3.10.2. c & d) is similar to that reported of *aroA*⁻ mutants of *Salmonella*. When the numbers of *aro*⁻ *Salmonella* were compared with those of a wild-type *Salmonella* in the livers and spleens of mice, shortly after infection, fewer

aro⁻ bacteria were isolated (Killar and Eisenstein, 1985). This difference may be explained in part by the reduced multiplication of the *aro*⁻ bacteria in the tissues. However it may be that fewer bacteria reach these tissues because Lm.918.6 has a reduced ability to avoid clearance by phagocytes.

One explanation for this may be the diminished ability to synthesise proteins involved in the avoidance of host defence mechanisms, for example, the production of catalase, superoxide dismutase and other protective enzymes. A relationship between bacterial growth phase and intracellular killing of *L. monocytogenes* by oxidative agents has been suggested (Bortolussi *et al.*, 1987). Sensitivity to antibacterial cell products, for example, hydrogen peroxide, superoxide, and hydroxyl radicals, was found by these workers to be greater in lag or stationary growth phase bacteria. A possible explanation for this was the reduced production of catalase, and superoxide dismutase, by these bacteria (Bortolussi *et al.*, 1987). Many proteins important for the virulence of *L. monocytogenes* will not be synthesised by Lm.918.6 reducing its ability to avoid clearance by host defence mechanisms for example, haemolysin, phospholipase, "p60" protein, actin nucleators, and internalin.

Attempts to determine differences between the intracellular growth of Lm.6b and Lm.918.6 and their wild-type parent strains in bone marrow derived macrophages (BMDM), performed by D. Portnoy, indicated that the growth of both mutants was unimpaired (section 3.12). Good correlation between growth of nonhaemolytic mutants within these cells in culture and growth *in vivo* has been reported (Camilli *et al.*, 1990, Portnoy *et al.*, 1988). However, the growth of both *aro*⁻ mutants in BMDM did not reflect the differences in the virulence observed *in vivo* (section 3.12, figure 3.12). The apparently unrestricted growth of Lm.918.6 could have been due to the concentration of aromatic amino acids in the culture medium which was found to be greater than that found in normal serum (table 4.1). The high levels of aromatic amino acids in the medium may increase the intracellular concentrations sufficiently to allow growth of Lm.918.6. Purine dependent mutants of *L. monocytogenes* have also been noted to grow in BMDM (D. Portnoy personal communication), possibly due to

Table 4.1. Comparison of aromatic amino acid concentration in cell culture medium and normal serum.

Aromatic amino acid	Concentration in cell culture medium* (mg/100ml)	Concentration in normal serum** (mg/100 ml)
tyrosine	7.2	1.4
tryptophan	1.6	1.1
phenylalanine	6.6	0.8

* cell culture medium comprises Dulbecco's modified Eagles medium with 10% fetal calf serum.

** normal serum values represent mean range values as given by Sherwin, (1980).

the compounds available in the cell culture media.

Bone marrow derived macrophages are used in many investigations because of their ability to bind and ingest considerably more bacteria than other primary cell lines (Portnoy *et al.*, 1988). However, another explanation for the discrepancy between the growth of bacteria in this cell culture system apart from the availability of aromatic amino acids, may be the difference between BMDM and the liver and spleen derived macrophages in which *L. monocytogenes* usually multiplies *in vivo* (Armstrong and Sword, 1964, Portnoy *et al.*, 1988). The growth of *L. monocytogenes* in cell lines has been reported previously, not to correspond with strain virulence as determined by mouse LD₅₀. An *in vitro* model for listerial infection of enterocytes which employed a human colon carcinoma (Caco-2) cell line has been used to assess the virulence of strains of *L. monocytogenes* (Gaillard *et al.*, 1987). However, Pine *et al.*, (1991) reported that the results of these assays differed considerably from the determined virulence of several strains in the mouse. The results of these experiments indicate that this cell culture assay, although useful as an initial screen for some mutants with reduced virulence, does not accurately reflect the *in vivo* pathogenicity of aromatic amino dependent mutants. Modification of the culture medium used in the BMDM cell culture could overcome these problems. Alternatively, the chick embryo chorioallantoic membrane model system (Notermans *et al.*, 1991), could be employed. In this technique nutrients are supplied by the egg itself and culture media is not used, avoiding artificially high concentrations of aromatic amino acids. These authors reported good correlation between the pathogenicity of *Listeria* species assessed by this method with mouse bioassay results (Notermans *et al.*, 1991).

The ability to persist in host tissue is an important property in potential vaccine strains (Baldrige, 1988, Collins, 1974, Hahn and Kaufmann, 1981, Kaufmann, 1983, Mackaness, 1962). The persistence of Lm.918.6 (figures 3.10.2 a & b) suggested that this strain could potentially stimulate a protective immune response to *L. monocytogenes*. Such protection against *L. monocytogenes* is based on a population of immunologically committed CD4⁺ and CD8⁺ T-cells, produced in the spleen, between days two and four of infection (Coppel and Youmans, 1969, Kaufmann and

Hahn, 1982, Magee and Wing, 1988, North 1975). Early curtailment of a *Listeria* infection with antibiotics has been reported to greatly reduce the number of antigen-specific cells generated and to result in a rapid decay in the duration of the immune response (North *et al.*, 1981). Furthermore, Kaufmann *et al.*, (1983) reported that while both persistent and nonpersistent *Listeria* strains are recognised by protective T-cell clones only persistent strains induced cell mediated immunity. The persistence of Lm.918.6 in the spleens of infected mice up to nine days after infection indicated that it could be able to initiate the production of protective T-cells.

One indication that an immune response has been initiated is the detection of splenomegaly. Splenomegaly (enlargement of the spleen) occurs when high concentrations of blood monocytes capable of processing and presenting antigen arrive in the spleen and is a common feature of *L. monocytogenes* infection (Armstrong and Sword, 1964, North, 1973). Data from experiments to look for the stimulation of splenomegaly by Lm.918.6 indicated that maximal splenomegaly occurred when the mice began to clear the bacteria from their spleens (figures 3.10.2 a & b). This observation is consistent with reports that splenomegaly occurs in association with a period of nonspecific macrophage activation (Baldrige *et al.*, 1988, North, 1973, O'Callaghan 1988). Such activation can be indicative of a longer lasting cellular immune response (Baldrige *et al.*, 1988, North, 1973, O'Callaghan 1988). The results indicated that the degree of splenomegaly induced by Lm.918.6, although significant, was not as great as that induced by the wild-type bacteria (figure 3.10.3). This finding reflects the reduced multiplication of Lm.918.6 *in vivo* because multiplication is a prerequisite for the initiation of protection against listerial infection (Berche *et al.*, 1987a). However, the induction of splenomegaly by Lm.918.6 suggests successful initiation of the cellular immune response essential for immunity to *L. monocytogenes*.

The humoral immune response is thought not to contribute greatly to protection against listerial infection. It has been reported that *L. monocytogenes* does not elicit a protective humoral response (Miki and Mackaness, 1964). The results of experiments to investigate the humoral immune response to Lm.918.6 (section 3.11), indicated that

the humoral immune system was stimulated, an immunoglobulin response to immunisation being apparent (figure 3.11). These observations are in agreement with previously reported pattern of the primary antibody response to listerial infection (Miettinen *et al.*, 1990). These authors noted that increased antibody levels after infection represent raised levels of IgM. It may be that the elevated antibody levels indicated by the results similarly represent raised levels of this class of immunoglobulin. Although the humoral immune response is thought not to contribute greatly to protection against listerial infection, the serum antibody levels observed (figure 3.11), reflected the course of the infection (figures 3.10.2 a & b). The coincidence of the highest anti-listerial serum antibody titres with the disappearance of the bacteria from the tissues (figures 3.10.2 a & b), may indicate some role for humoral immune response in anti-listerial protection. Other workers have also observed this association which suggests that antibodies, in addition to cell mediated mechanisms, are implicated in resistance to listerial infections (Aalund *et al.*, 1966, Miettinen *et al.*, 1990). This supposition is supported by the susceptibility of neonates to infection attributed to their low IgM levels and reduced complement activity, (Issekutz, *et al.*, 1984). Further, in contrast to the current body of knowledge murine anti-listerial IgG given i.p. has been found to protect mice against *L. monocytogenes* infection (Rudnicka and Chimiela, 1992). The ability of Lm.918.6 to stimulate a serum antibody response may therefore enhance its protective ability.

Further studies could identify the classes of immunoglobulin stimulated by immunisation with Lm.918.6, by the use of specific immunoglobulin conjugates in an ELISA system. Following the immunoglobulin response after the administration of a booster dose would reveal whether the shift from IgM to IgG had been made and a specific memory to Lm.918.6 had been induced. Investigations to ascertain whether any secretory IgA response is induced by immunisation with Lm.918.6 would be of interest, because such a response may be important in protection against initial invasion of the gut epithelium by ingested *L. monocytogenes*.

The results of challenge experiments indicate that significant protection against wild-type infection was afforded after immunisation with Lm.918.6 (table 3.10.4.b). This protection is based on an accelerated recall of the previously induced cell mediated immunity. Experiments indicated that wild-type organisms were cleared from the tissues much quicker in immune mice (figure 3.10.4), and growth of the organism was controlled before clinical illness was apparent. Protection against approximately 100 ED₅₀s of wild-type *L. monocytogenes* generated by immunisation with Lm.918.6, is comparable with that generated by immunisation with a metabolic drift, streptomycin and rifampicin resistant mutant in a mouse model (Linde *et al.*, 1991). These workers reported protection from 100 ED₅₀s of wild-type bacteria, fourteen days after an immunising dose (10⁴ cfu) of the mutant *L. monocytogenes* strain, administered i.p. The degree of protection stimulated by the commercially available "Listervac" (a live vaccine consisting of serotypes 1/2a and 4b), also determined by these authors after i.p. immunisation, was less than that stimulated by Lm.918.6 (Linde *et al.*, 1991).

The most common *L. monocytogenes* serovar, responsible for animal listeriosis in this country is 1/2a (Farber *et al.*, 1991) and Lm.918.6 was found to be of this serovar when kindly serotyped by Dr. J. McLauchlin (Central Public Health Laboratory, Colindale Avenue, London). In other countries *L. monocytogenes* serovar 4b is significant together with 1/2a and *L. ivanovii* serovar 5 (Farber *et al.*, 1991, Ivanov, 1975, Ivanov and Masalski, 1977, Ivanov, 1985, Wilesmith and Gitter, 1986). It is therefore important that protection against these strains is offered by any potential vaccine. In this study no attempt was made to investigate whether any protection was induced against *L. ivanovii* or other serovars of *L. monocytogenes*, by inoculation with Lm.918.6. However, sublethal infection with virulent *L. monocytogenes* has been noted to afford cross-protection to several different serotypes of *L. monocytogenes* (Notermans and Chakraborty, 1992). These workers noted cross-protection only if the bacterial load of the spleens exceeded 5.0 x 10⁵ cfu at days 3 to 4 post infection. After the administration of 1.0 x 10⁴ cfu of Lm.918.6, the bacterial load in the spleen reached approximately 5.0 x 10⁵ cfu (figure 3.10.2.a.). Although

maximum numbers of Lm.918.6 in the spleens of mice were not reached until day 5 post infection, vaccination with Lm.918.6 may afford cross protection against other serotypes of *L. monocytogenes*. The degree of cross protection offered by Lm.918.6 inoculation should be investigated in future studies.

It may be possible to transfer the insertional mutation in LM.918.6 to *L. monocytogenes* serovar 4b and *L. ivanovii* serovar 5 by phage transduction. This route of transfer of genetic markers between bacterial strains via bacteriophage (grown on one and used to infect another) is widely used in Gram-negative bacteria (see Neidhardt, 1987). The use of bacteriophage P22 as a vehicle for transducing a stable mutation in the *aroD* gene between strains of *S. typhimurium* has been reported (Miller *et al.*, 1989). Although, temperate phages have not to my knowledge been identified for *L. monocytogenes* the use of lytic phage for the typing of *L. monocytogenes* is well documented (Audurier and Martin, 1989, Mclauchlin *et al.*, 1986, Rocourt and Catimel, 1989).

The intravenous route by which mice in this study were challenged resulted in an easily observed systemic infection (section 3.10.2.). However, this route does not simulate the course of infection via the gastrointestinal (g.i.) tract. Entry by this route is regarded as most likely in natural listerial infection (Farber and Peterkin, 1991, Miller and Burns, 1970, Schlech, 1984.). The induction of local immunity in the g.i. tract may be important for protection. It has been suggested that there is a delay in the induction of protection in the intestinal region, when immunisation is given i.v. rather than orally. Immunity is only induced in this region some time after extensive splenic infection, due to the different circulatory pathways of spleen and gut associated cells (MacDonald and Carter, 1980). On arrival in the gut, listeriae are phagocytosed by macrophages associated with M cells (Payer's patches) known to process soluble intestinal antigens. The oral route of inoculation could therefore avoid any delay in the initiation of a protective local immune response in this region (MacDonald and Carter, 1980).

Further work should include the oral challenge of Lm.918.6 i.v. immunised mice to determine whether satisfactory protection is induced by this route of administration. Experiments should also be carried out to establish whether greater protection is afforded by oral immunisation with Lm.918.6. Oral administration of a vaccine is more practical especially for livestock. Aromatic amino acid dependent mutants of *S. typhimurium* and *S. dublin* have proved to be successful as live oral vaccines in animals and man (Chatfield *et al.*, 1992, Dougan *et al.*, 1988, Hone *et al.*, 1991, Levine *et al.*, 1987). Furthermore, oral administration of a recombinant *L. monocytogenes* vaccine expressing *E. coli* β -galactosidase has been reported to elicit cell-mediated responses (Schafer *et al.*, 1992). The recombinant *L. monocytogenes* vaccine strain DP-L967, used by these workers is derived from the same wild-type strain as Lm.918.6 and carries an insertion of a derivative of transposon Tn917-*lac* that constitutively expresses β -galactosidase. It is likely therefore that Lm.918.6 would also elicit cell-mediated responses when given orally. In future studies the use of this route of administration could be investigated for the induction of protection with Lm.918.6.

When considering the suitability a vaccine for the use in any population, but especially animals, the administration of a single dose to induce maximum protection is preferable to the need for repeated booster immunisations. The results of experiments in which a second immunising dose of Lm.918.6 was administered (table 3.10.4.b.), indicated that a small (although significant) improvement in the level of immunity could be induced by a booster dose. This suggests that the administration of a second dose to improve the protection induced may not be necessary until some time after the first inoculation. Further trials would clarify this. The protection induced by immunisation with live *Listeria* strains has been reported as long lasting in mice (North 1975, Notermans and Chakraborty, 1992). However, in cattle immunised with live vaccine strains of *L. monocytogenes* the need for revaccination every year has been noted (Gudding *et al.*, 1985). These authors reported that after vaccination with these strains immunity was maintained for about 10 months (Gudding *et al.*, 1985). The period over which the persistence of immunity induced by Lm.918.6 was studied, was of necessity short. A more informative picture of persistence could be achieved if these

studies were extended over months or years.

Further studies could also include engineering *L. monocytogenes* strains earlier in the aromatic amino acid biosynthetic pathway. As previously mentioned, extrapolation from published information on *aro*⁻ mutants of *Salmonella* suggests that mutations earlier in the pathway could be more attenuating than that carried by Lm.918.6. Strains of *Salmonella* with double lesions in the *aroA*, and *aroD*, *aroC*, or *purA*, genes have been constructed and characterised, exhibiting greater attenuation than *aroA*⁻ strains (Hoiseth and Stocker, 1981, Dougan *et al.*, 1988, O'Callaghan *et al.*, 1988, Jones *et al.*, 1991). Furthermore, such multiple mutations reduce the probability of reversion by transposon excision in *aro*⁻ mutants. The results of Southern hybridisation analysis of bacteria recovered from the tissues after Lm.918.6 infection (figure 3.10.2.e.), indicated that Tn917 excision from the chromosome had not occurred *in vivo*. However, before auxotrophic mutants of *L. monocytogenes* could be tested as live vaccine strains in the field, such double mutations should be constructed and may yield a greater reduction in virulence.

A possible strategy for the identification of *aro* genes would be to construct an *L. monocytogenes* chromosomal DNA library in *aro*⁻ strains of *E. coli*. Recombinants carrying cloned *L. monocytogenes* *aro* genes could be identified by complementation and the restoration of a prototrophic phenotype. Another strategy would be the cloning of DNA flanking the Tn917 insertion in Lm.918.6 This may allow the identification of other listerial *aro* or *phe* genes, because as mentioned previously, some bacterial *aro* and *phe* genes have been shown to be closely linked, forming gene clusters (Nester *et al.* 1963, Pittard, 1987, Pittard and Wallace 1966). Mapping studies in *Salmonella* have indicated that the *pheA* gene is close to the *aroF* and *aroC* genes (Bachman, 1990), although in *B. subtilis* this gene maps at some distance from the *aro* gene cluster (Piggot and Hoch, 1985).

Preliminary work to clone the DNA flanking has been undertaken utilising the Tn917 insertion in Lm.918.6. The Tn917 fusion inserted in Lm.918.6 is from vector pLTV3, which was constructed to allow the direct cloning of DNA flanking transposon insertions (Camilli *et al.*, 1990). This vector contains a cluster of polylinker cloning sites, which facilitate the cloning of chromosomal DNA on the promoter-proximal side of transposon-mediated *lacZ* fusions. Insertions and flanking DNA can be cloned directly by cleaving genomic DNA with a restriction endonuclease which has a unique restriction site in the polylinker of Tn917-LTV3. The cleaved chromosomal DNA can then be ligated and transformed into *E. coli* using the ColE1 origin of replication present in Tn917-LTV3 and the Tn5 derived *neo* gene, as a selectable marker encoding kanamycin resistance (Camilli *et al.*, 1990). Subsequently, DNA flanking the insertion could be isolated and in turn used as a probe to clone a functional copy of the *pheA* gene or related *aro* genes. The construction of double or triple *aro*⁻ mutants would then be possible by site directed mutagenesis of other *aro* genes, although, to reduce the risk of recombination, such mutations are preferable in loci well separated on the bacterial chromosome.

The construction of *aro*⁻ mutant strains by site directed mutagenesis would have the advantage that transposon encoded antibiotic resistance markers would be avoided. Antibiotic resistance is considered to be an undesirable property in live vaccine strains. Acquired antibiotic resistance to chloramphenicol, erythromycin, streptomycin, and tetracycline has been reported in *L. monocytogenes*, and it has been reported that conjugative transposons and plasmids were involved in this development (Doucet-Populaire *et al.*, 1991, Poyart-Salmeron *et al.*, 1990). The non-conjugative nature of Tn917 and its affinity for insertion in chromosomal rather than plasmid DNA, mean that its transfer to other organisms is less likely than that of other insertion elements. The transposon Tn1545 for example has been reported to transfer in a two way process between *E. faecalis* and *L. monocytogenes* in the digestive tracts of gnotobiotic mice (Doucet-Populaire *et al.*, 1991). Tn917 encoded erythromycin resistance in Lm.918.6 *aro*⁻ would not interfere with the preferred antibiotic treatment of listeriosis (high doses of ampicillin or penicillin). However, future construction of

aro⁻ mutants of *L. monocytogenes* by site directed mutagenesis, could avoid the possibility of Tn917 transfer to other organisms if it became associated with a transferable plasmid.

Nonreverting *aro*⁻ mutants of *Listeria* may also serve as vaccine carriers of genes for protective antigens of other pathogens. This application of *aro*⁻ mutant strains has been reported for *Salmonella* (Brown *et al.*, 1987, Flynn *et al.*, 1990, Molina and Parker, 1990, Tite *et al.*, 1990). The genetic determinants of extracellular antigens such as the *E. coli* K88 fimbriae and enterotoxin B subunit have been expressed in a *aroA*⁻ mutants of *S. typhimurium* (Dogan *et al.*, 1987, Maskell *et al.*, 1986). Cloned secreted antigens have also been found to elicit both humoral and cellular responses when delivered to mice in *aroA*⁻ mutants of *S. typhimurium*, and similar responses have been induced to viral, bacterial, and parasitic proteins (Brown *et al.*, 1987, Flynn *et al.*, 1990, Molina and Parker, 1990, Tite *et al.*, 1990). Recombinant vaccine strains of mycobacterium BCG, have also been described that induce an immune response to exogenous antigens (Aldovini, and Young, 1991, Stover *et al.*, 1991). Furthermore, as mentioned earlier, a recombinant strain of *L. monocytogenes*, expressing *E. coli* β -galactosidase was used as a live vaccine vector in BALB/c mice and was observed to induce cellular and humoral immune responses when administered by both the oral and parenteral route (Schafer *et al.*, 1992).

Recombinant *L. monocytogenes* strains are good candidate vaccine vectors because the organism elicits a strong protective cellular immune response by both CD4⁺ and CD8⁺ T cells (Kaufmann and Hahn, 1982, Magee and Wing, 1988). Unlike several other facultative intracellular bacteria, for example *Salmonella* and BCG, *L. monocytogenes* escapes from the phagosome into the cell cytoplasm stimulating the class 1-restricted pathway of antigen processing for cytotoxic T cells. Furthermore, because *L. monocytogenes* spends time in the phagosome before entering the cytosol, it should also be able to stimulate an alternative vacuolar class 1 antigen processing pathway involving class 1 major histocompatibility complex (MHC) molecules reported recently (Pfeifer *et al.*, 1993). Class 1-restricted cytotoxic T lymphocytes are

crucial to the elimination of many viral and bacterial infections (Kauffman, 1988). The antigens of these organisms are processed intracellularly by proteolysis in the cytoplasm. The resulting peptides are transported into the endoplasmic reticulum where they associate MHC class 1 molecules before transportation to the cell membrane. Aromatic amino acid dependent strains of *L. monocytogenes* can multiply in the cytoplasm. Proteins they express are therefore likely to be processed and presented as peptides on MHC class 1 molecules at the T cell membrane. An aromatic amino acid dependent mutant of *L. monocytogenes* is therefore an attractive recombinant live vaccine candidate.

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Development of an optimized system for electroporation of *Listeria* species

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Received 1 December 1989 and accepted 4 December 1989

ALEXANDER, J.E., ANDREW, P.W., JONES, D. & ROBERTS, I.S. 1990. Development of an optimized system for electroporation of *Listeria* species. *Letters in Applied Microbiology* 10, 179–181.

Electroporation was used to facilitate transformation of *Listeria* species with plasmid DNA. Optimal conditions for transformation of *L. monocytogenes* were a field strength of 8.5 kV/cm, 200 Ohms resistance, 25 µF capacitor with a time constant of 5 ms. With these conditions, 3.9×10^6 transformants/µg DNA were obtained. Under the same conditions, *L. innocua* and *L. ivanovii* exhibited a frequency of transformation similar to that of *L. monocytogenes* but a somewhat lower level was obtained with *L. seeligeri*.

The increase in listeriosis in man and animals in the last decade (McLauchlin 1987) has stimulated interest in the basis of pathogenicity of the causative organisms, *Listeria monocytogenes* and *L. ivanovii*. The study of potential virulence factors is hampered by the lack of defined genetic systems for the listeriae. Conjugation and protoplast transformation have been reported (Flam *et al.* 1984; Vincente *et al.* 1987) but with low levels of frequency. Electroporation offers a more efficient system for the introduction of genetic material into listeriae. Electroporation involves the application of high intensity electric fields of short duration to permeabilize the bacterial cell membrane reversibly, thus facilitating the entry of DNA. Sufficient intensity of current must be applied to produce the optimum number of permeabilized areas for DNA entry and the intensity of current is a function of the field strength and time constant used (Sowers & Leiber 1986; Dower *et al.* 1988). Electrotransformation has been reported for lactobacilli and *L. monocytogenes* and *L. innocua* (Luchansky *et al.* 1988; Badii *et al.* 1989) but the frequency of transformation was very low. The work report-

ed here describes the development of an optimized system for the electrotransformation of *L. monocytogenes* and the application of this system to other *Listeria* species.

Materials and Methods

BACTERIAL STRAINS AND PLASMIDS

Listeria monocytogenes (NCTC 7973), *Listeria ivanovii* (ATCC 19119), *Listeria innocua* (NCTC 11288), *Listeria seeligeri* (CIP 100100), and pGK12, a 4.3 kb plasmid encoding for erythromycin and chloramphenicol resistance (Kok *et al.* 1984) were used.

PREPARATION OF BACTERIA CELLS

Overnight cultures in Brain Heart Infusion Broth (BHI; Oxoid) were inoculated into fresh BHI and incubated with shaking at 37°C until an O.D.₆₀₀ of 0.6–0.8 was attained. The bacteria were harvested by centrifugation at 5000 g for 10 min at 4°C, washed twice in 1/10th the original volume with 3.5 × sucrose magnesium electroporation medium (SMEM: 272 mM sucrose, 1 mM MgCl₂, pH 7.2) and then resuspended in 1/100th the original volume with 3.5 × SMEM.

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Portions of the cell suspension were stored at -70°C until required.

ELECTROPORATION

A Gene-PulserTM electroporation apparatus and pulse controller (Bio-Rad, Richmond, California) was used in all studies. The prepared cells were thawed on ice and an initial colony-forming unit (cfu) count made. Forty microlitres of cell suspension ($ca\ 4 \times 10^{10}$ bacteria) were mixed gently with $0.4\ \mu\text{g}$ of pGK12 DNA in a volume of $0.1\ \mu\text{l}$ and left on ice for 1 min. The mixture was then transferred to a chilled $0.2\ \text{cm}^2$ electroporation cuvette and electroporated between chilled electrodes. The cuvette was then placed immediately on ice and left for 1 min before 1 ml of BHI broth was added and a final cfu count made. The culture was then incubated at 37°C , with shaking, for 3 h to allow for the expression of the plasmid-encoded drug resistance genes. Serial dilutions in distilled water were plated onto Blood Agar Base No. 2 (Difco) to determine the number of viable bacteria and onto the same medium supplemented with erythromycin (Sigma) and chloramphenicol (Sigma) at $5\ \mu\text{g}/\text{ml}$ to select for transformants. The plates were incubated for 48 h at 30°C before enumeration of the colonies.

To determine the optimal conditions for electroporation the procedure was conducted at

various field strengths for different time constants. The field strength is defined as the voltage gradient between the electrodes; the time constant is a function of the field strength and the resistance applied (Bio Rad Manual 1988). A range of time constants at each field strength studied was achieved by the connection of different resistors ($100\text{--}0\ \text{Ohms}$) in parallel with the sample.

Results and Discussion

The results of the experiments with *L. monocytogenes* (NCTC 7973) and the plasmid pGK12 are listed in Table 1. As can be seen both the field strength and time constant influence the degree of transformation but for each field strength there was an optimal time constant.

The application of field strength above $8.5\ \text{kV}/\text{cm}$ exceeded the conductivity limit of the electroporation medium and resulted in arcing. Up to $8.5\ \text{kV}/\text{cm}$ an increase in field strength favoured successful transformation at each time constant. The efficiency of transformation also increased with the time constant at each of the field strengths used up to a certain time constant after which efficiency declined, possibly due to the increased killing of the bacteria. With a low time constant, approximately 2 ms, at field strengths of 3.1 or $6.2\ \text{kV}/\text{cm}$ no transformants were recovered.

Table 1. Electroporation of *Listeria monocytogenes* NCTC 7973 with pGK12 DNA

Field strength (kV/cm)	Time constant (ms)	Actual time constant* (ms)	Percentage survival	No. of transformants (/ μg DNA)
3.1	5.0	4.4	57.3	0
3.1	10.0	11.3	51.4	3.0×10^2
3.1	15.0	14.6	42.6	2.9×10^2
3.1	20.0	16.8	9	2.5×10^2
6.2	5.0	4.4	33.6	3.3×10^4
6.2	10.0	11.7	35.5	7.3×10^4
6.2	15.0	13.8	0.4	3.5×10^3
6.2	20.0	Arc†	—	—
8.5	2.0	2.3	89.8	2.4×10^4
8.5	4.0	4.3	59.9	1.3×10^5
8.5	5.0	4.4	61.9	3.9×10^6
8.5	8.0	6.8	24.8	1.1×10^4
8.5	10.0	Arc	—	—
8.5	15.0	Arc	—	—
8.5	20.0	Arc	—	—

* Actual time constants are given due to small variations in conductivity of samples and cuvettes.

† Arc indicates pulse did not pass through sample.

With 3.1 and 6.2 kV/cm transformation was maximal at 10 ms. At a field strength of 8.5 kV/cm transformation rate increased with time constant and was maximal at 5 ms. These results were probably due to incomplete permeabilization of the bacterial cell membrane at the lower time constant.

The percentage of bacteria killed during electroporation is an important consideration. At any field strength an increase in the time constant results in increased killing (Table 1). This limits the use of longer time constants, just as the conductivity limit of the electroporation buffer limits the use of increased field strengths. Therefore the optimum time constant and field strengths are those that limit the percentage of the bacterial population killed to a minimum, while still allowing maximum polarization of the membrane to allow DNA entry.

On the basis of our results the optimum conditions for the recovery of transformants of *L. monocytogenes* (NCTC 7973) with plasmid pGK12 are a field strength of 8.5 kV/cm 200 Ohms resistance, 25 μ F capacitor with a time constant of 5 ms. The transformation frequency of approximately $4 \times 10^6/\mu$ g pGK12 DNA represents a considerable improvement on the rates achieved by Luchansky *et al.* (1988). These workers, using a protocol developed for *Lactobacillus acidophilus*, reported a transformation frequency of $5.8 \times 10^3/\mu$ g pGK12 DNA for *L. monocytogenes* strain B-67 and $2.8 \times 10^2/\mu$ g DNA for *L. innocua* strain B-66.

The results of the transformation of *L. innocua* (NCTC 11288), *L. ivanovii* (ATCC 19119) and *L. seeligeri* (CIP 100100) with plasmid pGK12 DNA using the protocol devised to optimize transformation in *L. monocytogenes* are given in Table 2. *Listeria innocua* and *L. ivanovii* transform at approximately the same rate of efficiency as *L. monocytogenes* (10^6 transformants/ μ g pGK12 DNA). The lower rate exhibited by *L. seeligeri* ($5 \times 10^3/\mu$ g pGK12

DNA) may reflect differences in the nature of the cell wall or genome of this species.

No attempt was made to optimize the electroporation conditions for species other than *L. monocytogenes* and slight alterations to basic protocol could increase transformation frequencies. The frequencies of transformation obtained using our protocol for *L. monocytogenes* are sufficiently high to allow the introduction and expression of genetic material into other *Listeria* species so that the genetic basis of virulence in members of the genus *Listeria* can be studied.

We thank J. Kok, Department of Genetics, University of Groningen, Kerklaan 30, 9751 NN Haren, The Netherlands, for the gift of plasmid pGK12. One of us (J.E.A.) thanks the Medical Research Council for a Research Studentship.

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Table 2. Electroporation of *Listeria innocua*, *L. ivanovii* and *L. seeligeri* with pGK12 DNA

Species	% Survival	No. of transformants (μ g DNA)
<i>L. innocua</i>	71.3	2.50×10^6
<i>L. ivanovii</i>	59.7	1.25×10^6
<i>L. seeligeri</i>	69.6	5.00×10^3

Characterization of an Aromatic Amino Acid-Dependent *Listeria monocytogenes* Mutant: Attenuation, Persistence, and Ability To Induce Protective Immunity in Mice

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Received 6 November 1992/Accepted 8 February 1993

A transposon insertion mutant of *Listeria monocytogenes* was shown to be deficient in prephenate dehydratase, an enzyme acting late in the pathway for biosynthesis of phenylalanine. This mutant had reduced virulence in mice. The mutant and parent strains persisted to the same extent in the tissues of infected mice and elicited similar degrees of splenomegaly. Mice vaccinated with the mutant were protected significantly from subsequent challenge with virulent *L. monocytogenes*.

In farm animals, especially sheep, losses due to deaths from listeriosis can be considerable (21). To protect against infection, killed and live attenuated strains have been used as vaccines in animals, but with little or no success (1, 3, 8, 13). The failure of previous live attenuated strains as vaccines is probably due to the method of attenuation used (8, 13).

An alternative to the procedures previously used for making attenuated strains is the generation of strains with a defined mechanism of attenuation. This approach is the basis of the suggestion that hemolysin-deficient strains of *Listeria monocytogenes* could be suitable as attenuated strains for vaccines (15). However, use of these strains would exclude protection from the effects of hemolysin, an important virulence factor (18). Therefore, we decided to assess the potential of aromatic amino acid-dependent, hemolytic strains of *L. monocytogenes* for use as live vaccines. Aromatic amino acid-dependent (*aro*) strains of *Salmonella typhimurium* and *Salmonella typhi* are very successful examples of the use of defined mutants as vaccines (12, 17, 19) against salmonellosis. We assessed an aromatic amino acid-dependent transposon insertion mutant of *L. monocytogenes* as a candidate vaccine in a mouse model of listeriosis.

L. monocytogenes Lm.918.6 is an aromatic amino acid-dependent mutant of the wild-type strain Lm.1070138 (2). Both strains were obtained from D. Portnoy, University of Pennsylvania, and maintained as described before (20). When necessary, minimal medium (6), supplemented with the appropriate amino acid at 20 µg/ml, was used. Cells for inoculation into mice were grown at 30°C to late log phase in tryptose soya broth (Difco), supplemented when appropriate with erythromycin (25 µg/ml). The cells were then resuspended in tryptose soya broth containing 10% (vol/vol) glycerol and stored at –20°C for several months without significant loss of viability. When required, the bacteria were thawed rapidly, harvested by centrifugation, and resuspended in sterile distilled water. The specific activity of prephenate dehydratase was assayed by the method of Nester and Jensen (16). Female MF1 outbred mice, ca. 30 g in weight (Harlan Olac Ltd., Shaw's farm, Bicester, United Kingdom), were used throughout. Virulence was estimated

by determining the 50% effective dose (ED₅₀). Doses of bacteria between 10³ and 10⁸ CFU in 100 µl of distilled water were administered intravenously to groups of five mice. The number of mice per group that reached the end point of the assay (i.e., became moribund) between days 0 and 6 was recorded and used to calculate the ED₅₀ by the log-probit method (the percentage of mice surviving to end point versus the log of the CFU inoculated) (5).

In experiments to ascertain the numbers of bacteria in the livers and spleens, infected mice were killed by cervical dislocation at the desired times after infection. The spleens and livers were removed, weighed, and homogenized separately in 10 ml of sterile distilled water in a Stomacher-Lab blender (Seward Medical), and viable counts on tryptose agar were obtained (20). Results were expressed as mean counts of viable listeriae per gram of tissue. To determine whether vaccination with Lm.918.6 conferred resistance to subsequent infection, mice were first vaccinated (intravenously) with 10⁴ CFU of Lm.918.6 in water. The booster vaccination was an identical dose of Lm.918.6 given 14 days after the initial vaccination. At 28 days after the initial vaccination with Lm.918.6, the ED₅₀ of Lm.1070138 for the mice was determined as described above. Data were analyzed by the Mann-Whitney U test (10) or the Kolmogorov-Smirnov test (10).

Lm.918.6 had been described previously as a Tn917 insertion mutant that required aromatic amino acids for growth (2). To ensure that Lm.918.6 contained a single Tn917 insertion, a Southern blot was performed to *Eco*RI-digested chromosomal DNA with a radiolabelled Tn917-specific probe as described in reference 2. Uncut plasmid pTV32 was included as a positive control, as it carries a single copy of Tn917 (22). The probe hybridized to a single *Eco*RI fragment (Fig. 1). Since there are no recognition sites for *Eco*RI within Tn917 (2), Lm.918.6 appears to contain a single Tn917 insertion. To characterize more precisely the biochemical defect, aromatic amino acids and intermediates in the aromatic amino acid pathway were assayed for their ability to support the growth of Lm.918.6 in minimal medium. While phenylalanine and its immediate precursor, phenylpyruvic acid, supported growth, neither tyrosine nor prephenate (a precursor of both tyrosine and phenylpyruvic acid) did. To explain this, the activity of prephenate dehydratase, the enzyme that catalyzes the conversion of pre-

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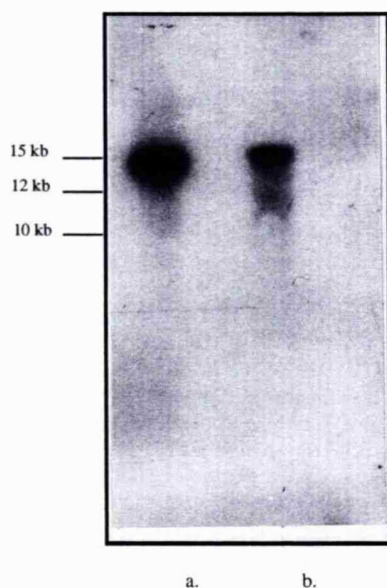


FIG. 1. Southern blot of *Eco*RI-digested Lm.918.6 chromosomal DNA (a) and uncut plasmid pTV32 DNA (b), probed with a 1.5-kb transposon Tn917-specific DNA probe (2). Lambda DNA digested with either *Xho*I or *Hind*III was used as molecular weight markers.

phenic acid to phenylpyruvic acid, in both Lm.1070138 and Lm.918.6 was determined. In Lm.918.6, the activity was 0.02 U/mg of protein, whereas the wild-type level was 7.00 U/mg of protein. This suggested that the single transposon insertion in Lm.918.6 was affecting expression of the *pheA* gene, which encodes prephenate dehydratase. As measured by ED_{50} , strain Lm.918.6 was significantly ($P < 0.05$) attenuated compared with the wild-type strain, Lm.1070138, in the mouse model of listeriosis (Table 1). Lm.918.6 multiplied to significantly ($P < 0.05$) lower numbers in both the liver and the spleen than Lm.1070138 (Fig. 2A and B). After a dose of 10^4 viable cells of Lm.1070138, peak counts of 6×10^9 CFU/g of spleen tissue and 7×10^7 CFU/g of liver tissue were obtained after 3 days. At the same dose, Lm.918.6 grew more slowly, reaching peak counts of 2×10^6 and 9×10^5 CFU/g of spleen and liver tissue, respectively, at day 5 (Fig. 2A and B). No significant difference ($P > 0.05$) in the clearance of the two strains between days 1 and 9 was noted, and by day 11, both strains had been cleared (Fig. 2A and B). Maximum splenomegaly, as measured by the mean spleen weight of the five animals at each time point, occurred 4 days after maximum numbers of listeriae were detected, i.e., on day 7 postinfection with the wild-type strain Lm.1070138 and on day 9 postinfection with Lm.918.6 (Fig. 2C). The extent of splenomegaly induced by Lm.918.6 was not significantly

TABLE 1. Comparison of ED_{50} s of Lm.1070138 and Lm.918.6

Strain	ED_{50} (CFU)		
	Mean ^a	SEM	95% CL ^b
Lm.1070138	5.51×10^4	4.97×10^2	1.28×10^4 – 2.37×10^5
Lm.918.6	2.61×10^6 *	3.67×10^3	7.00×10^5 – 9.71×10^6

^a Mean dose required to render 50% of the mice moribund. *, significantly different ($P < 0.05$) from value for Lm.1070138.

^b CL, confidence limits.

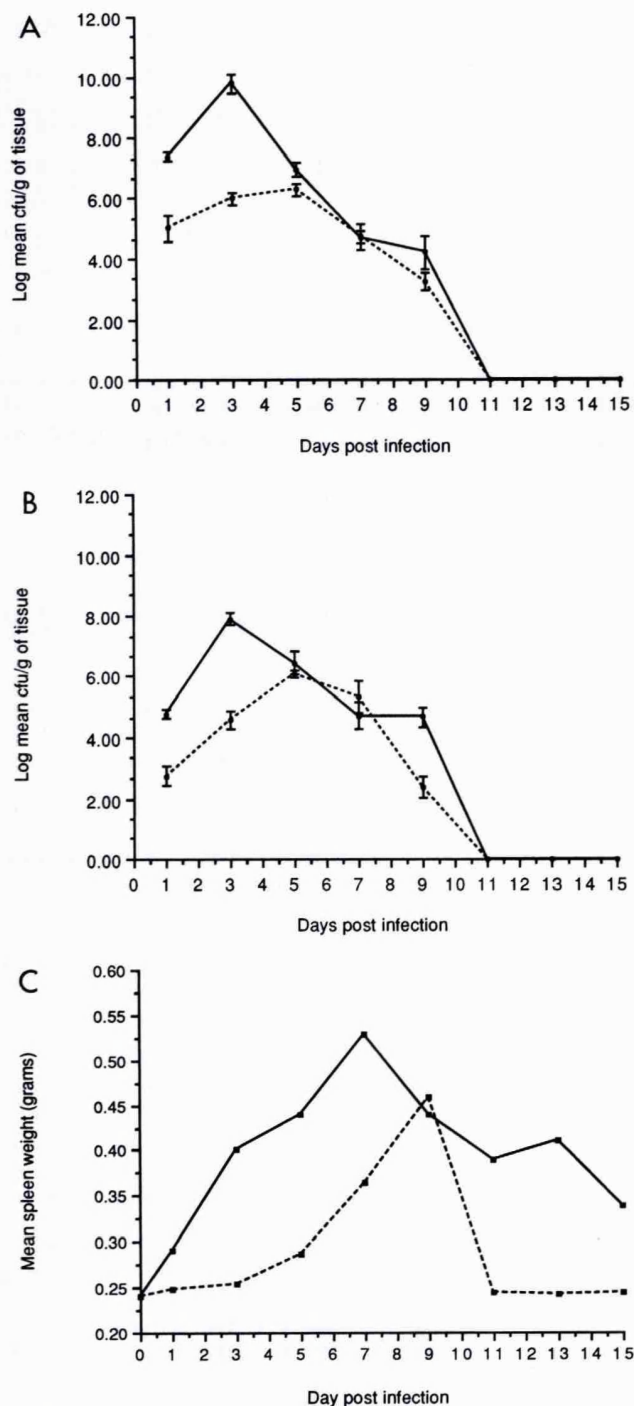


FIG. 2. (A and B) Concentrations of viable Lm.1070138 (—) and Lm.918.6 (---) in spleens (A) and livers (B) of MF1 mice over 15 days after intravenous infection with 10^4 CFU. Each point (A and B) represents the geometric mean for five mice. (C) Splenomegaly (expressed as mean spleen weight, in grams) induced over 15 days by infection with 10^4 CFU of Lm.1070138 (—) and Lm.918.6 (---). Each point represents the arithmetic mean for five mice.

TABLE 2. Effect of vaccination with Lm.918.6 on the ED₅₀ of wild-type Lm.1070138 in mice

Lm.918.6 vaccination ^a	Lm.1070138 ED ₅₀ (CFU)		
	Mean ^b	SEM	95% CL ^c
None	5.51×10^4	4.79×10^2	1.28×10^4 – 2.37×10^5
1 dose	$1.73 \times 10^{6*}$	5.35×10^3	1.73×10^5 – 8.82×10^6
2 doses	$6.00 \times 10^{6**}$	6.09×10^3	1.37×10^6 – 2.62×10^7

^a Each vaccinating dose contained 10^4 CFU of Lm.918.6, given intravenously.

^b Mean dose required to render 50% of the mice moribund. *, Significantly different ($P < 0.05$) from value for unvaccinated mice; **, significantly different ($P < 0.05$) from value for mice receiving one dose.

^c CL, confidence limits.

different ($P > 0.05$) from that induced by Lm.1070138 (Fig. 2C), but the duration was significantly reduced, with spleens returning to normal weight 11 days postinfection (Fig. 2C). In contrast, with Lm.1070138, splenomegaly was maintained for at least 15 days postinfection (Fig. 2C). In all these experiments, the phenotype and genotype of Lm.918.6 recovered from the infected animals were confirmed by growth on minimal medium and by Southern blotting with Tn917-specific DNA probes. In all cases, Lm.918.6 remained PheA⁻ with a single transposon insertion (data not shown).

To determine whether vaccination with Lm.918.6 conferred protection against subsequent infection, mice were first vaccinated with Lm.918.6 and then challenged 28 days later with Lm.1070138. The ED₅₀ of Lm.1070138 was significantly ($P < 0.05$) greater in mice vaccinated with one dose of Lm.918.6 than in nonvaccinated mice (Table 2). In addition, a significant ($P < 0.05$) booster effect was seen when mice were given a second dose of Lm.918.6 14 days after the initial vaccination (Table 2). Therefore, strain Lm.918.6 with a transposon insertion which affects *pheA* expression is attenuated in the mouse model for listeriosis. In the same system, it confers significant protection against infection by virulent *L. monocytogenes*, with a pronounced booster effect upon second vaccination. This is the first evidence that an auxotrophic mutant of *L. monocytogenes* can be attenuated in a manner similar to that described for *aro* mutants of *S. typhimurium* (17). Why a marked reduction in *pheA* gene expression should result in attenuation is not yet clear. It has been hypothesized that in *aroA* mutants of *S. typhimurium* attenuation may be due to an inability to synthesize *p*-aminobenzoate and dihydroxybenzoate (7). Since Lm.918.6 should be capable of making both *p*-aminobenzoate and dihydroxybenzoate, attenuation in this case may reflect the poor availability of phenylalanine in mammalian cells. In addition, feedback inhibition of earlier aromatic pathway enzymes by the accumulation of prephenate, the substrate of prephenate dehydratase (9), may also be occurring. Reduced virulence of *pheA* mutants of gram-negative or gram-positive bacteria has not, to our knowledge, been reported previously. Studies of *aro* mutants of *Salmonella* spp. (4, 11) suggest that a strain of *L. monocytogenes* with multiple lesions in the pathway of aromatic amino acid synthesis might prove to be more attenuated than Lm.918.6. Future studies will attempt to introduce such additional mutations into Lm.918.6. This should serve to increase the attenuation and also reduce the risk of possible reversions in vivo. It is entirely feasible that such strains could be useful as vaccines in farm animals.

In addition to their potential as vaccine strains, nonreverting aromatic amino acid-dependent mutants of *L. monocytogenes* may be exploited as carriers of cloned gram-positive

virulence factors and protective antigens of other pathogens, as has been reported for similar strains of *Salmonella* (4, 14).

We thank D. Portnoy, University of Pennsylvania, for strains Lm.918.6 and Lm.1070138.

One of us, J.E.A., thanks the Medical Research Council of Great Britain for the award of a postgraduate studentship.

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