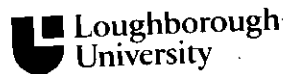


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**Attachment of *Listeria monocytogenes* to materials commonly found in a  
food-processing environment**


**by**

**Mark Richard Beresford**

**A Doctoral Thesis submitted in partial fulfillment for the award of  
Doctor of Philosophy of Loughborough University**

**September 20<sup>th</sup> 2002**

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## Abstract

*Listeria monocytogenes* is a facultative intracellular pathogen and the causative agent of listeriosis. Contaminated food is thought to be the major vector of listeriosis. Food products that have been implicated in cases of listeriosis include poultry, meat, seafood, milk and their derivative products.

Microbial contamination of food surfaces is an ongoing problem for the food industry and is a major risk to food quality and safety. The primary aim of this study is to investigate attachment of *L. monocytogenes* 10403S to different food processing surface materials. The mean number of cells recovered from short contact times and 2 hour contact times of coupons in bacterial culture significantly differ for each of the 18 materials examined with the exception of polypropylene.

*L. monocytogenes* transposon mutants were generated and a cellular staining assay was used for screening, selecting for those mutants attaching to glass coverslips in low frequency. One mutant was investigated further. An ORF disrupted by transposon mutagenesis in this mutant showed 48% identity to the *L. monocytogenes* strain EGD alpha-mannosidase and a conserved domain belonging to the glycosyl hydrolase family (alpha-mannosidases). An enzyme assay confirmed that expression of this enzyme was reduced in the mutant.

Alpha-mannosidase is part of the alginate pathway characterized in *Pseudomonas aeruginosa*. Alginate activity has been shown to be up-regulated during attachment of *P. aeruginosa* to materials (Davies *et al.*, 1993) and is implicated in bacterial attachment to surfaces and biofilm formation. We therefore suggest a hypothesis that interrupting an ORF coding for an alpha-mannosidase enzyme disrupts the alginate synthesis pathway responsible for extracellular polysaccharide (EPS) production and hence inhibits attachment to food processing surfaces. Further experiments such as site-directed mutagenesis are required to test this hypothesis.

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## List of Abbreviations

<b>Meso-DAP</b>	<b><i>meso</i>-diaminopimelic acid</b>
<b>MurNAc-GlcNAc</b>	<b>N-acetylmuramic acid-(<math>\beta</math>1-4)-N-acetylglucosamine</b>
<b>LPXTGX</b>	<b>Leucine-Proline-X-Threonine-Glycine-X</b>
<b>GPI</b>	<b>glucosyl-phosphatidylinositol</b>
<b>Gro-P</b>	<b>polyglycerol phosphate</b>
<b>Rit-P</b>	<b>polyribitol phosphate</b>
<b>Glc-P</b>	<b>polyglucosyl phosphate</b>
<b>LRR</b>	<b>leucine-rich repeat domain</b>
<b>PCR</b>	<b>polymerase chain reaction</b>
<b>SOD</b>	<b>superoxide dismutase</b>
<b>LLO</b>	<b>listeriolysin O</b>
<b>PI-PLC</b>	<b>phosphatidylinositol-specific phospholipase C</b>
<b>PC-PLC</b>	<b>phosphatidycholine-specific phospholipase C</b>
<b>EPS</b>	<b>exopolysaccharide / extracellular polysaccharide</b>
<b>HIC</b>	<b>hydrophobic interactive chromatography</b>
<b>BATH</b>	<b>bacterial adhesion to hydrocarbons</b>
<b>SAT</b>	<b>salting-out aggregation test</b>
<b>ESIC</b>	<b>electrostatic interaction chromatography</b>
<b>CIP</b>	<b>cleaning-in-place procedures</b>
<b>QUATAL</b>	<b>quaternary ammonium compound</b>
<b>CDC</b>	<b>Centre for Disease Control and Prevention</b>
<b>TEM</b>	<b>transmission electron microscopy</b>

<b>SEM</b>	<b>scanning electron microscopy</b>
<b>ESEM</b>	<b>environmental scanning electron microscopy</b>
<b>DIC</b>	<b>episcopic differential interference contrast microscopy</b>
<b>AFM</b>	<b>atomic force microscopy</b>
<b>HMC</b>	<b>Hoffman modulation contrast microscopy</b>
<b>SCLM</b>	<b>scanning confocal laser microscopy</b>
<b>TSA / TSB</b>	<b>tryptone soya agar/broth</b>
<b>IPTG</b>	<b>isopropyl <math>\beta</math>-D thiogalactopyranoside</b>
<b>EDTA</b>	<b>ethylenediaminetetraacetic acid</b>
<b>SDS</b>	<b>sodium dodecyl sulphate</b>
<b>MOPS</b>	<b>N-morpholinopropanesulfonic acid</b>
<b>CIP</b>	<b>calf alkaline phosphatase</b>
<b>RTG</b>	<b>Ready to go dCTP labelling kit</b>
<b>BSA</b>	<b>bovine serum albumin</b>
<b>TAE</b>	<b>Tris acetate EDTA buffer</b>
<b>CFU</b>	<b>Colony forming unit</b>
<b>SSC</b>	<b>standard saline citrate solution</b>
<b>HEPES</b>	<b>N-[2-Hydroxyethyl]piperazine-N-[2-ethanesulfonic acid]</b>
<b>PBS</b>	<b>Phosphate buffered saline</b>
<b>ORF</b>	<b>Open reading frame</b>
<b>MCS</b>	<b>Multiple cloning site</b>
<b>NCTC</b>	<b>National collection of type cultures</b>



## INTRODUCTION

### 1.1 The history and classification of *Listeria monocytogenes*

*L. monocytogenes* is a Gram-positive flagellated organism that occurs either singly or in chains. It is a non-encapsulated, non-spore forming, facultative anaerobe. The organism was first described in 1926 by Murray and co-workers (Murray *et al.*, 1926). It was isolated from an outbreak of septicaemia in rabbits and guinea pigs in an animal house at Cambridge University in the UK. The organism responsible for the infection was at first termed *Bacterium monocytogenes* (Murray *et al.*, 1926)). The occurrence of a similar organism isolated from the liver of infected gerbils was reported in 1927 (Pirie, 1927). He named the organism *Listerella hepatolytica* in honour of famous surgeon Lord Lister (Gray and Killinger, 1966). Fourteen years later he renamed the genera of the organism *Listeria* on discovering that the genera *Listerella* had been previously assigned to a different type of organism (mycetozoa). At this time, *L. monocytogenes* was the only recognised species.

Although Murray is credited with the isolation and description of *L. monocytogenes* in 1926, there are reports of earlier isolations of organisms matching the description of *L. monocytogenes*. The first of these was by Hülphers (1911) who termed the organism he isolated from a rabbit *Bacillus hepatis*. There were also isolations from humans by Atkinson (1917), Dick (1920) and Dumont and Cotoni (1921). The latter stored the organism they isolated from a human infection and it was conclusively identified as *L. monocytogenes* 19 years later by Paterson (1940). *L. monocytogenes* was originally

classified in both the sixth and seventh editions of Bergey's Manual of Determinative Bacteriology as belonging to the *Corynebacteriaceae* (Breed *et al.* 1948; 1957).

The eighth edition of Bergey's Manual of Determinative Bacteriology (Buchanan and Gibbons, 1974) places *Listeria* in the *Lactobacillaceae* family. In 1982 DNA / DNA hybridisation experiments by Rocourt proposed five distinct *Listeria* species (one of which is *L. monocytogenes*). Later the 16S rRNA studies of Stackebrandt placed *Listeria* as a taxon in a low G+C, *Bacillus-Lactobacillus-Clostridium* branch of Gram-positive bacteria closely related to *Brochothrix* (Collins *et al.*, 1991). In 1991, 16S rRNA reverse transcriptase sequence studies of members of the genus showed that they were sufficiently distinct to belong to their own family- the *Listeriaceae* that includes the genus *Brochothrix* (Collins *et al.*, 1991). This is in accordance with a number of detailed taxonomic investigations (Davis and Newton, 1969; Davis *et al.*, 1969; Stuart and Pease, 1972; Stuart and Welshimer, 1973), cell wall chemistry (Cummins and Harris, 1956; Schleifer and Kandler, 1972; Srivastava and Siddique, 1973), lipids (Shaw, 1974), and nucleic acid studies (Stuart and Welshimer, 1974). There are now six species of *Listeria* recognised: *L. monocytogenes*, *L. innocua*, *L. ivanovii*, *L. ivanovii* subsp. *londoniensis*, *L. grayi*, *L. seeligeri*, and *L. welshimeri* (Seeliger and Jones 1986; Rocourt *et al.* 1992). Only four of these species are known to cause infections in humans and animals. These species are *L. monocytogenes*, *L. innocua*, *L. ivanovii* and *L. seeligeri* (McLaughlin *et al.* 1987).

## 1.2 Habitats

Prior to 1960, *L. monocytogenes* was predominantly associated with animal rather than human infection (Seeliger 1961, Gray and Killinger 1966, Welshimer 1981). Since

then it has been widely isolated from a number of environmental sources including vegetation, soil, faeces, food, sewage, material surfaces, water, humans and animals (Kampelmacher and Van Noorle Jansen, 1969; Cox *et al.*, 1989; Ryser and Marth, 1991).

### **1.2.1 Isolation from environmental sources**

Reports by Weis and Seeliger, (1975); Welshimer, (1968); Welshimer and Donker-Voet, (1971); Ryser and Marth (1991) suggest that the main habitat of *L. monocytogenes* is soil and rotting vegetation where the organism survives as a saprophyte. Indeed, *L. monocytogenes* is easily isolated from mud and soil. Welshimer (1960) reported that *Listeriae* survive better in soil less subject to evaporation. This claim was based on the results of soil moisture loss experiments, in which the number of *Listeriae* isolated decreases with the amount of moisture lost by the soil. *L. monocytogenes* is present in large numbers in human and animal faeces and is therefore often present in sewage and water. Although Julianelle (1940) successfully infected mice with listeriosis from drinking water, there are to date no conclusive reports of human infection from water.

### **1.2.2 Isolation from humans and animals**

More than 50 species of mammals and birds are known to harbour *Listeriae*. *Listeriae* have even been isolated from crustaceans, fish, oysters, flies and ticks (Gray and Killinger, 1966; Kampelmacher and Van Noorle Jansen, 1969; Armstrong, 1985; Fenlon, 1985; Rocourt and Seeliger, 1985; Lamont *et al.*, 1988; Gellin and Broome,

1989). Although *Listeriae* are pathogenic, they have been isolated from the faeces of humans and animals not exhibiting symptoms of disease and are hypothesised to be commensal organisms living in as many as 9% of European people (Ralovich, 1984).

### 1.2.3 Isolation from Food

Contaminated food is thought to be the major vector of listeriosis. Food products that have been implicated in cases of listeriosis include poultry, meat, seafood, milk and their derivative products. Atkinson (1917), who had isolated and described a bacterium that was probably *L. monocytogenes* from human meningitis infections, was the first to suggest a relationship between infected food products and a listeriosis-like disease. Furthermore, when Murray *et al.* (1926), described *L. monocytogenes*, he succeeded in infecting rabbits by the oral route. Similarly, Pirie (1927) successfully infected mice and gerbils with *L. monocytogenes*-contaminated food. Julianelle succeeded in infecting mice with *L. monocytogenes* contaminated water 13 years later in 1940. It was not until the 1980's that listeriosis was widely linked to contaminated food. Schlech *et al.* (1983) isolated *L. monocytogenes* from coleslaw following an outbreak of listeriosis in Canada that killed 17 people and infected a further 24. The organism was successfully traced back to cabbage that had been fertilised by manure from sheep that had been infected with listeriae, indeed, two of the sheep had even died. Many more outbreaks involving different types of foods have been documented since the early eighties, firmly establishing the link between contamination of food products with *L. monocytogenes*, listeriosis. For example, in 1985, consumption of Mexican-style cheese was directly linked to over 142 cases of listeriosis, including 48 deaths (Linnan *et al.* 1988), and between 1983 and 1987, consumption of

contaminated Vacherin Mont d'Or soft-ripened cheese resulted in 31 deaths (Malinverni *et al.*, 1985). In the UK, it was concluded that paté was a contributory cause of the increase in the incidence of listeriosis between 1987 and 1989 (McLaughlin *et al.*, 1991). The widespread occurrence of the organism and incidence of infections from contaminated food products has created much concern for the food industry (Rijpens *et al.*, 1998). This has been exacerbated by the discovery that the organism has the ability to, not only survive on meat and in protein solutions, but also grow over a large range of temperatures, including refrigeration temperatures (Khan *et al.*, 1972; Johnston *et al.*, 1986). This is made possible by the bacterium's ability to modify its membrane composition in order to maintain membrane fluidity (Jones *et al.*, 1997).

Poultry are well known to harbour *L. monocytogenes* and to be responsible for a number of listeriosis cases, however, it is only since 1988 that the first of such cases was documented (Kerr *et al.*, 1988). It has long been suspected that contaminated egg can cause listeriosis. Desmarchelier *et al.* (1995) isolated *L. innocua* from over 1 in 8 eggs swabbed in a factory in Australia. Moreover, 69% of the factory produced egg products and 100% of egg products obtained from the farms contained *L. innocua* (though *L. monocytogenes* was isolated once only during the experiment). Despite this, no firmly linked cases of listeriosis have ever been attributed to contaminated egg.

*L. monocytogenes* has been detected in various seafoods including shrimps, lobster, crab and fish. Weagent *et al.* (1988) reported that the bacterium is present in over a quarter of frozen seafood products. At least one outbreak of listeriosis was caused by infected mussels (Brackett and Beuchat, 1990). However, the danger of infection

from contaminated seafood is probably reduced by the fact that most seafood is cooked prior to consumption.

Milk and milk products have been shown to enable growth of *L. monocytogenes* (Rosenow and Marth, 1987). Seeliger (1961), listed many cases of listeriosis in Germany between 1949 and 1957, directly linked to unpasteurised milk and various milk products including cottage cheese, and cream. However, it was suggested in 1985 that an outbreak in Massachusetts was linked to pasteurised milk (Fleming *et al.*, 1985). Whether listeriosis infection can arise from heated contaminated milk products is the subject of much debate. Bearns and Girard (1958) claimed that *L. monocytogenes* could resist heating and was even able to survive pasteurisation. This was challenged by Twedt (1986) who observed that *L. monocytogenes* would not survive pasteurisation when correctly carried out. Since Fleming *et al.* (1985) claimed that the milk in the Massachusetts outbreak had been correctly pasteurised, that no post-pasteurisation infection had occurred and furthermore that *L. monocytogenes* had not been isolated from the milk, it was concluded that the source of contamination could not be conclusively pinpointed. The World Health Organisation claims that *L. monocytogenes* does not survive even minimum pasteurisation requirements (Ryser and Marth, 1991).

### **1.3 Disease and Epidemiology**

*L. monocytogenes* is a facultative intracellular pathogen and the causative agent of listeriosis. Rocourt and Seeliger (1985) claimed that *L. monocytogenes* is responsible for nearly 100 % of all *Listeria* infections in man. The incidence of listeriosis worldwide is not known. Serovars 1/2a, 1/2b and 4b of *L. monocytogenes* are

responsible for the majority of cases of human listeriosis (Jones and Seeliger, 1991). As in many other food-borne diseases, a large number of mild cases not exhibiting severe enough symptoms to warrant treatment and diagnosis go unreported (Jones and Seeliger, 1991). It is estimated that the incidence of listeriosis varies somewhere from 1 to 15 cases per million of the population worldwide (Jones and Seeliger, 1991; Farber and Peterkin, 1991). A dramatic increase in reported cases has occurred in the last 25 years (Jones and Seeliger, 1991). The Public Health Laboratory Service (PHLS) have discovered that the bulk of listeriosis cases between 1983 and 1994 occur with onsets in late summer or early autumn but the reasons for this are not yet understood (McLaughlin and Newton, 1995). There are different epidemiology patterns exhibited by cases involving different outcomes of disease. The number of pregnancy-associated cases fell between 1990 and 1996 in comparison to 1983 and 1989. This may be attributed to improved education of pregnant mothers over the dangers of soft cheese and pâté. However, the number of cases associated with immunocompromised people rose in the 1990's (Communicable Disease Report, 1997).

### **1.3.1 Human Listeriosis**

Listeriosis is defined as the presence of the organism in the bloodstream or any otherwise sterile site of a human or animal. It can also cause septicaemia, encephalitis and meningitis. In less serious cases gastrointestinal complaints such as vomiting and diarrhoea are the outcome. Listeriosis is of particular danger to the elderly, the immunocompromised and pregnant women, and has a high mortality rate.

There are approximately 1600 cases of listeriosis per year in the U.S. of which one quarter are fatal. The clinical signs and symptoms of human listeriosis vary and can

lead to misdiagnosis. Human listeriosis is categorised in three groups: bacteraemia, pneumonia and meningitis. Listerial meningitis is almost clinically identical to other forms of bacterial meningitis. It has a mortality rate of 70% if untreated or if treatment is delayed (Seeliger and Finger, 1976). Sequelae of *Listerial* meningitis include brain abnormalities, oedema and cerebellar atrophy (Marrie *et al.*, 1984). *L. monocytogenes* is a frequent cause of meningitis in immunocompromised people. The illness usually begins with 'flu-like symptoms, followed by headaches, leg pain, fever, nausea, stiffening of the neck, chills, vomiting and aversion to bright light. The disease then progresses to convulsions, dehydration and coma followed by death (Ryser and Marth, 1991).

Listeriosis is a major cause of foetal damage and death. Infection of pregnant women can lead to spontaneous abortion of the foetus in the fourth month of pregnancy (Seeliger and Finger, 1976). This is thought to occur in approximately 1 in 20000 births. The symptoms are again those of 'flu, possibly including gastrointestinal illness and very rarely meningitis (Seeliger and Finger, 1976). Symptoms may be mild or not exhibited (Seeliger, 1961). If untreated, the chances of survival are poor and the mortality rate is close to 100% usually depending on spread to the central nervous system and meninges (Ryser and Marth, 1991).

### **1.3.2 Animal Listeriosis**

As in the case of human listeriosis, the worldwide incidence of animal listeriosis is unknown. The number of reported cases in Great Britain reached as many as 342 in 1984 (Wilesmith and Gitter, 1986). The onset of animal listeriosis differs to that of



human listeriosis in that livestock listeriosis onset usually occurs from late November to early May and peaks around February to March (Gray and Killinger, 1966). Similarly to human listeriosis, *L. monocytogenes* serovar 1/2a is associated with most animal cases (Wilesmith and Gitter, 1986). Animal listeriosis can have significant impact on the economy. Indeed, during the 1970's an outbreak of listeriosis in Australia caused the loss of 1 million sheep (Ryser and Marth, 1991). The scale of economic losses can be gauged from a smaller outbreak in Great Britain that caused the deaths of 21 ewes and 88 lambs at a cost to the farmer of £5000 (Low and Renton, 1985). Prevention of such outbreaks spreading to food products can be achieved by improvement of animal housing conditions, feed, sampling and bacterial control such as vaccination.

The disease can occur sporadically or in epidemics (Ryser and Marth, 1991). All domestic animals can become infected with listeriae, however sheep, cattle, goats, and chickens are the most frequently infected animals (Gray *et al.*, 1956; Gray, 1958; Sharma *et al.*, 1983). The first isolation of *L. monocytogenes* from a farm animal was by Gill (1931). Two years previously, he observed a disease in sheep that he dubbed a 'circling disease'. This symptomatic description is still used for listeric encephalitis and meningioencephalitis, the most common listeric infections in sheep (Seeliger, 1961). Symptoms may include raised temperature, aversion to drinking and subsequent neurological sequelae. This is the phase at which an animal may demonstrate the characteristic 'circling' behaviour. Death usually occurs 2 or 3 days later (Seeliger, 1961). Listeric abortion also occurs in animals (Seeliger, 1961). Importantly, *L. monocytogenes* can cause generalised mastitis in cows and the organism may be shed into milk by infected cows (Ryser and Marth, 1991).

#### 1.4 Growth and characteristics of *L. monocytogenes*

*Listeria* species grow on common laboratory media, though enrichment is recommended when isolating the organism from clinical specimens (Jones and Seeliger, 1991). Tryptone-based media are commonly used for culturing *Listeria* species. Tryptose agar (DIFCO), tryptose broth (DIFCO) and tryptose blood base agar (DIFCO) all support excellent growth. Using these media for primary isolation usually requires the presence of *Listeria* in large numbers (Jones and Seeliger, 1991). Other media have been developed for isolation and selection of *Listeria*. These include a modification of Stuart's Transport medium (Stuart, 1959) and Lovett's medium for enrichment of *Listeriae* in milk (Lovett *et al.*, 1987). The latter is itself a modification of the medium of Ralovich *et al.*, (1971). The media developed by Donnelly and Baigent (1986) and Curtis *et al.* (1989) are used extensively in the UK and USA for enrichment of food and isolation from clinical specimens from mixed flora.

The most frequently used methods of identifying *Listeria spp.* include examination of colony shape, cell morphology, staining reactions, growth at 37°C, and various biochemical tests (Jones and Seeliger, 1991). Colonial shape is similar for all species of *Listeriae*. They appear bluish-grey and exhibit a blue-green sheen under obliquely transmitted light (rough strains do not) (Jones and Seeliger, 1991). Colonies usually grow on nutrient agar between 24-48 hours at 37°C and are 0.5 to 1.5mm in diameter, circular, translucent, low convex with entire margins (Jones and Seeliger, 1991). Cellular morphology of *Listeria* species is not very distinctive. They are short Gram-positive, motile (at 20-25°C), rods of about 0.5µm in diameter and 1-2µm in length with rounded ends (Jones and Seeliger, 1991). They are non acid-fast, non-spore

forming, non-encapsulated facultative anaerobes. The rods can adopt a palisade formation, along with some V and Y forms. Rough strains can typically grow up to 20µm in length (Anton, 1934). Motile *L. monocytogenes* grown at 20-25°C exhibit a characteristic tumbling motility (Seeliger, 1961) and usually exhibit a small number of peritrichous flagella (Kressebuch *et al.*, 1988). *Listeria* species are most easily differentiated from each other by haemolysis, acid production from mannitol, rhamnose, D-xylose, α-methyl-D-mannoside and nitrate reduction (Jones and Seeliger, 1991). One of the most reliable methods of testing acid production from carbohydrates is the API 50 CH gallery (bioMérieux S.A., France), which comprises 49 different substrates.

Additional ways of identifying *Listeriae* include gas production from glucose (negative), Voges-Proskauer test, aesculin test, oxidase test (negative), urease test (negative), and alkaline phosphatase test (positive) (Jones and Seeliger 1986). Further non-biochemical tests include antigenic composition, specific bacteriophages (Schultz, 1945), multi-locus electrophoresis (Piffaretti *et al.*, 1989) and DNA techniques. Nowadays DNA techniques such as restriction endonuclease digests and the use of specific gene probes (McLaughlin *et al.*, 1988), polymerase chain reaction (PCR) and specific antibodies allow conclusive identification of bacterial species. Paterson (1940) identified four serological types of *L. monocytogenes* based on somatic (O) and flagella (H) antigens. Serotype 2 was based on the unique H antigen and serotypes 1,3 and 4 were based on differentiation O antigens. Further O antigens were identified and the serotype 4 divided into serotypes 4a and 4b. Serotypes 4c, 4d and 4e were subsequently added by Donker-Voet (1965). *L. monocytogenes* isolates comprise a

total of 13 different serotypes (Jones and Seeliger, 1991), most belonging to 1/2a, 1/2b and 4b (Seeliger and Hohne, 1979; McLaughlin, 1987).

All species of *Listeria* have a peptidoglycan cell wall containing *meso*-diaminopimelic acid (*meso*-DAP), glutamic acid, muramic acid, alanine, glucosamine, glucose and sometimes rhamnose (Keedler and Gray, 1960; Ullman and Cameron, 1969; Scheifer and Kandler, 1972; Kamisango *et al.*, 1982; Fiedler and Seger, 1983; Hether *et al.*, 1983; Fiedler *et al.*, 1984). The polar lipids of *L. monocytogenes* contain phosphatidylglycerol, diphospholipid, galactosyl-glucosyldiacylglycerol and a glycopospholipid (Kosaric and Carroll, 1971; Shaw, 1974). The fatty acid composition of these lipids depends on the growth temperature (Russell, 1990; Jones *et al.*, 1997). All *Listeriae* contain predominantly straight-chain, saturated, anteiso- and isomethyl-branched chain type fatty acids (Jones and Seeliger, 1991). When grown in a complex medium with shaking, cytochromes can be found but this is not reported when using a defined media (Trivett and Mayor, 1971). GC content of the DNA of *Listeria* varies from 36-42 mol% (Jones and Seeliger, 1991), with *L. monocytogenes* containing 37-39 mol% GC (Rocourt and Grimont, 1983).

### **1.5 The *L. monocytogenes* Cell Surface**

The cell walls of various serotypes of *L. monocytogenes* have been reported to contain abundant peptidoglycan, teichoic acid, lipoteichoic acid, and lipopeptidopolysaccharide (Hofman *et al.*, 1985).

There are three distinct cellular compartments of bacteria: the cytosol, a single cytoplasmic membrane, and the surrounding cell wall (Giesbrecht *et al.*, 1976). The main purpose of the cell wall is to provide an exoskeleton for protection against both mechanical and osmotic lysis (Salton *et al.*, 1952, Salton *et al.*, 1994). However, the cell wall of Gram-positive bacteria also serves as an attachment site for proteins that interact with the bacterial environment (Navarre and Schneewind, 1999).

The cell wall of Gram-positive bacteria is a peptidoglycan macromolecule with attached accessory molecules such as teichoic acids, teichuronic acids, polyphosphates, or carbohydrates (Hancock, 1997; Salton *et al.*, 1994). The glycan phase of the cell wall consist of the repeating disaccharide *N*-acetylmuramic acid-( $\beta$ 1-4)-*N*-acetylglucosamine (MurNAc-GlcNAc) (Ghuysen and Strominger, 1963a; Ghuysen and Strominger, 1963b). Peptides in the cell wall are cross-linked with other peptides attached to a glycan strand (Tipper and Strominger, 1968). It is widely assumed that surface proteins of Gram-positive bacteria might interact with eukaryotic proteins as a means of attaching at unique locations or evading the immune system (Navarre and Schneewind, 1999). Gram-positive bacteria synthesize several compounds on their peptidoglycan exoskeleton (Salton, 1994). These compounds can be divided up into teichoic acids, teichuronic acids, lipoteichoic acids, lipoglycans, fimbriae, adhesions, pili and flagella and are discussed in further detail below.

### 1.5.1 Cell Wall Sorting Signals

The Leu-Pro-X-Thr-Gly-X (LPXTG) motif is conserved within the sorting signals of all known wall-anchored surface proteins of Gram-positive bacteria and is located in the protein's C-terminus (Fischetti *et al.*, 1990). This is the only known mechanism allowing covalent linkage of surface proteins to the cell wall of Gram-positive bacteria (Navarre and Schneewind, 1999; Cabanes *et al.*, 2002). The first LPXTG motif discovered in *L. monocytogenes* was in internalin A (Gaillard *et al.*, 1991). The threonine (T) position in the motif displays some variation in that either alanine or serine can also be substituted here. Based on sequence homologies with the C-terminal attachment site for glucosyl-phosphatidylinositol (GPI) anchors in a number of eukaryotic membrane-anchored proteins, there have been suggestions that the LPXTG motif may have a similar function in Gram-positive bacteria (Panchioli and Fischetti, 1989; Kehoe, 1994).

### 1.5.2 Teichoic and Lipoteichoic Acids

Gram-positive bacteria synthesize anionic polymers that are covalently attached to the peptidoglycan or tethered to a lipid anchor moiety (Armstrong *et al.*, 1958; Baddiley, 1972). These polymers consist of polyglycerol phosphate (Gro-P), poly-ribitol phosphate (Rit-P), or poly-glucosyl phosphate (Glc-P) all of which may be glucosylated (Fischer, 1994; Pooley and Karamata, 1994).

Although the structures of cell wall teichoic acids are largely known, the physiological roles of these molecules are still not completely understood (Pooley *et al.*, 1991). Cell wall teichoic acids appear to be the binding sites for some enzymes that cleave the

bacterial peptidoglycan (Herbold and Glaser, 1975) and may allow Gram-positive bacteria to create an envelope structure chemically distinct from the envelope of other organisms.

Lipoteichoic acids are polyanionic polymers inserted in the outer leaflet of the cytoplasmic membrane via a lipid moiety (Fischer, 1994). The polymer extends through the cell wall peptidoglycan onto the surface of Gram-positive cells (Navarre and Schneewind, 1999). The cell wall teichoic acids and lipoteichoic acids possess different chemical structures in *Listeria* and most other Gram-positive organisms.

It is possible that lipoteichoic acids, serve as species-specific decorations of the peptidoglycan exoskeleton (Fischer, 1994). Although the exact function of lipoteichoic acids is unknown, lipoteichoic acids has been shown to be a potent structure of Gram-positive bacteria that can induce inflammation (Cleveland *et al.*, 1996).

### **1.5.3 Anchored Cell Surface Proteins**

Surface proteins are expressed to interact with a substrate located in the surrounding environment. These proteins can be involved in interaction with host tissues, binding to immune system components, protein processing and nutrient acquisition.

Anchored surface proteins must span the thick cell wall of Gram-positive bacteria in order to display their functional domains to the surrounding environment (Navarre and Schneewind, 1999). Although they have a wide range of functions, cell wall-anchored

proteins of Gram-positive bacteria have many features in common. The N-terminal domain of cell-wall anchored proteins is often followed by a set of repeat domains.

The first discovery of Gram-positive cell-wall-associated proteins were the streptococcal M proteins (Lancefield, 1928). Since then recombinant DNA technology has led to expansion in our knowledge of Gram-positive cell-wall-associated proteins (Kehoe, 1994).

The cell wall of Gram-positive pathogenic bacteria functions as a surface organelle that allows them to interact with their environment, in particular the infected host. For this to occur, surface proteins need to be properly targeted to the cell wall envelope. Two mechanisms control this, cell wall sorting and targeting (Navarre and Schneewind, 1999). Cell wall sorting is the covalent attachment of surface proteins to the peptidoglycan via a C-terminal sorting signal that contains a consensus LPXTG sequence (Navarre and Schneewind, 1999) as discussed above. Cell wall targeting involves the non-covalent attachment of proteins to the cell surface via specialized binding domains (Navarre and Schneewind, 1999). Several of these wall-binding domains appear to interact with secondary wall polymers that are associated with the peptidoglycan, for example teichoic acids and polysaccharides. Other proteins comprising the cell wall include the flagella, fimbriae, enzymes, internalins and other virulence factors.

Gram-positive flagella are very similar in structure to Gram-negative flagella (DePamphilis and Adler, 1971). However there have been far fewer studies of flagella in Gram-positive bacteria than Gram-negative bacteria (Kehoe, 1994). Wild-type *L. monocytogenes* has peritrichous flagella and their expression is temperature-dependent, being flagellated and motile at 20 to 25°C but non-motile and with few flagella above



35°C (Seeliger, 1961; Seeliger and Jones, 1986; Vantanyoopaisarn *et al.*, 2000). It has been suggested that flagella are involved in attachment of *Listeria* to surfaces (Vantanyoopaisarn *et al.*, 2000; Gorski *et al.*, 2003). This phenomenon will be investigated in this thesis. Some Gram-positive bacteria express fimbriae up to 4000nm long on their cell surface (Kehoe, 1994), however there is no evidence of such structures in *Listeria*.

There are also 69 genes encoding putative lipoproteins in the *L. monocytogenes* genome (Cabanes *et al.*, 2002). Lipoproteins are characterised in bacteria by the presence of a specific signal peptide (Cabanes *et al.*, 2002). They can be potent inflammatory molecules initiating innate and adaptive immune responses in mammals (Aliprantis *et al.*, 1999).

As explained in section 1.x, the ability of *L. monocytogenes* to enter eukaryotic cells relies on a family of surface and secreted proteins called the internalins. As yet, seven members of the internalin family have been discovered (InlA to InlC, InlC2, and InlD to InlF) (Dramsai *et al.*, 1997). It has been demonstrated that the terminal leucine-rich-repeat domain (LRR) and conserved inter-repeat region (IR) of InlA confer an invasive phenotype (Lecuit *et al.*, 1997). Similar findings have recently been made for InlB (Braun *et al.*, 1998).

The LRR and IR are both necessary and sufficient for the interaction of InlA and E-cadherin, and subsequent invasion of epithelial cell lines 475, (Mengaud *et al.*, 1996). No ligand has been identified for any of the other internalin family members (Navarre and Schneewind, 1999). The precise role of any of the internalins *in vivo* is not yet entirely clear (Cossart and Lecuit, 1998). This is discussed further in section 1.6.

*Listeria* p60 is required for cell growth and invasion of this organism (Hess *et al.*, 1996). The N- and C-terminal domains of p60 species are conserved in all *Listeria* species; however, the central sequences are variable (Bubert *et al.*, 1992). Cytotoxic T cells that recognize p60 epitopes are protective against *Listeria* infections in a mouse model system, suggesting that this surface protein presents an immunodominant antigen (Bouwer and Hinrichs, 1996; Navarre and Schneewind, 1999). *Listeria* p60 has murein hydrolase activity and is implicated in cell division (Cabanes *et al.*, 2002). SvpA (Lmo2185) has recently been shown to be linked to the cell-surface in a similar way to ActA (a surface protein involved in actin polymerisation) and to be required for bacterial escape from the phagosome (Borezee *et al.*, 2001). Other virulence factors are described in section 1.6.

The abundance and diversity of cell surface proteins in *L. monocytogenes* (133 in strain EGD, Cabanes *et al.*, 2002), reflects the nature of *Listeria* being able to survive and adapt in a large variety of environments by attachment to surfaces. Most of the characterised virulence genes of the organism are regulated by the PrfA regulon which is described in more detail in section 1.6.

Cell wall-anchored surface proteins of *Listeria* are important in attachment and pathogenesis and are discussed further in section 1.6. The types of function discussed are recognition of cell surfaces, host proteins and other surfaces. Other functions of cell-wall-anchored surface proteins include binding of immunoglobulins and binding of serum and extracellular matrix proteins.

Much is known about the Ig-binding surface proteins of Gram-positive bacteria. Since the discovery of staphylococcal protein A, several other Ig-binding proteins have been characterized and many of their genes have been cloned and sequenced (Navarre and

Schneewind, 1999). To my knowledge there are data published with regards to *L. monocytogenes* having Ig-binding surface proteins.

#### **1.5.4 Wall-anchored enzymes.**

Metabolic enzymes can also be surface attached, especially in cases where the function of the enzyme is to break down a large, nutrient polymer into smaller subunits for uptake into the cell e.g. the dextranases and fructosidases of the mutans streptococci and the casein peptidase of lactococci (Navarre and Schneewind, 1999). Conjugal transfer of DNA requires cells be able to adhere to one another through the specific interactions of surface proteins (Dunny and Leonard, 1997). *L. monocytogenes* has two genes to express sortases for anchoring LPXTG surface proteins to the cell wall (Bierne *et al.*, 2002). One is similar to *S. aureus srtA* and is involved in cell-wall anchorage, less is known about the other sortase gene (Bierne *et al.*, 2002). A similar gene (*srtB*) in *S. aureus* has been shown to be involved in iron-regulation possibly involved in pathogenesis (Mazmanian *et al.*, 2003). *L. monocytogenes* also has a cell-surface ferric reductase likely to be involved in iron-acquisition (Deneer *et al.*, 1995) and a cell-surface autolysin (Ami) is involved in adherence to eukaryotic cells (Milohanic *et al.*, 2001).

### 1.5.5 Bacterial S-Layers

S-layers are two-dimensional proteinaceous crystalline arrays formed by proteinaceous subunits that covering the outer surface of many types of unicellular organisms (Beveridge *et al.*, 1997). S-layers have been identified in several types of bacteria, including *Listeria* (Davies *et al.*, 1999).

They are formed by the aggregation of subunits exported from the cytoplasm via the general secretory pathway by an N-terminal leader peptide (Navarre and Schneewind, 1999). S-layer proteins are often the most abundant species in cells, comprising up to 15% of the total cellular protein (Sleytr *et al.*, 1993). Approximately  $5 \times 10^5$  subunits are required to cover an average-sized cell (Sleytr and Messner, 1988). To my knowledge there is no data linking S-layers with bacterial attachment.

### 1.6 Pathogenesis and virulence factors

Very few people actually become ill from *Listeria* infections, even though the organism is ubiquitous in the environment. This is probably due to the combination of only certain virulent strains being able to cause disease, the resistance of most of the population and increased food safety awareness with time.

DNA technology has led to the discovery of a number of genes implicated in the pathogenesis of *L. monocytogenes*. PCR related experiments have been used to investigate differences between invasive and non-invasive strains of *Listeria* (Franciosa *et al.*, 2001). The findings suggested that DNA sequence differences between these

strains and expression of different virulence factors are the cause for the variation in virulence.

A key to *L. monocytogenes* causing disease is its ability to induce phagocytosis. Once inside a cell, it can survive the oxidative burst and may replicate and transfer to other cells. One key factor involved in *Listeria* being able to become invasive, is the development of acid tolerance (Conte *et al.*, 2000). In the gut, M cells or enterocytes take up the listeriae, where they begin to multiply. They then move from the gut to the liver and other organs, where they are usually killed by neutrophils. For infection to occur, listeriae need to be present in high numbers, probably in the order of  $10^8$  cells. However, if host defences are compromised the cells can end up being transported further around the body and may end up in the placenta or cross the blood-brain barrier.

Portnoy *et al.* (1992) demonstrated that initial survival of *Listeria* within macrophages and other mammalian cells is essential for infection. This has stimulated much research interest in T-cell mediated immunity. The murine infection method provides a reproducible system for investigating *L. monocytogenes* virulence (Portnoy *et al.*, 1992). Following intra-peritoneal injection, virulent *L. monocytogenes* are quickly phagocytosed by macrophages. Many bacteria are then killed following ingestion but surviving bacteria begin to replicate and reach maximum numbers 2 to 3 days after infection. The animal recovers by cytotoxic and T-helper cell mediated immunity over a period of days (Sheehan *et al.*, 1994).

An overview of *L. monocytogenes* infection is described as follows. Once phagocytosed, the cells become membrane-bound in a vacuole which is subsequently

lysed by listeriolysin O (LLO), a pore-forming toxin, allowing the listeriae to escape. The production of LLO has shown to be essential for escape, though LLO mutants have been shown to survive in a vacuole for some time.

Once the listeriae have successfully escaped from the vacuole they begin to replicate and produce ActA, a multifunctional virulence factor. ActA is involved in actin-based motility and host cell tropism and invasion (Suarez *et al.*, 2001). As the bacteria begin actin polymerization, and the cell moves along the polarized actin in a 'treadmilling' fashion, to reach the cell membrane forming 'listeriopodal' bulges in the cell membrane (Kocks *et al.*, 1992). Nearby cells then enter these bulges and invade and disseminate further.

Invasion is initiated and propagated by a series of virulence factors. These are located on, and positively regulated by the PrfA regulon (Renzoni *et al.*, 1997). The regulon incorporates: *prfA* (encoding a positive regulatory factor), *plcA* (encoding phosphatidylinositol-specific phospholipase C), *hly* (encoding the Listeriolysin O haemolysin), *mpl* (encoding a metalloproteinase), *actA* (encoding a surface protein for actin polymerisation), and *plcB* (encoding a lecithinase). Downstream of *plcB* are three open reading frames of unknown function. Other genes that have been implicated in the virulence of *L. monocytogenes* include the internalin genes *inlA* and *inlB*, the *iap* gene, the *lmaA* gene and the superoxide dismutase (SOD) gene.

Figure 1.1 Virulence gene expression by PrfA in *L. monocytogenes*

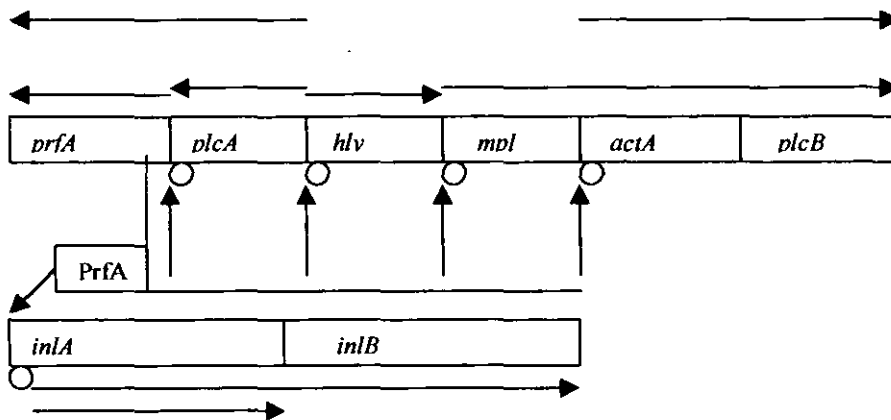


Figure 1.1 shows listerial gene expression as controlled by PrfA. Octagons represent putative PrfA binding sites. The putative negative-regulation of the *prfA* promoter by PrfA is shown by the blue lines (Sheehan *et al.*, 1995)

Perhaps the most important of these virulence factors are the internalins. Internalin A was the first of the virulence factors found to be involved in invasion (Gaillard *et al.*, 1991). The gene *inlA* encodes internalin A and is believed to be essential for the invasion of host epithelial cells (Dramsı *et al.*, 1995). This protein appears to induce phagocytosis by binding to E-cadherin on epithelial cells. Similarly, the gene *inlB* encodes a surface protein, internalin B that is thought to be essential for entry of cells into hepatocytes (Dramsı *et al.*, 1995; Braun *et al.*, 1998) as is the autolysin Ami which is involved in adherence to eukaryotic cell (Milohanic *et al.*, 2001). Aside from the internalins, there is a third protein (p104) involved in invasion. This protein is involved in adhesion to human intestinal cells (Pandiripally *et al.*, 1999). The product of the *iap* gene is a protein (p60) also thought to be essential for invasion (Bubert *et al.*, 1992). Bubert *et al.*, (1992) used purified p60 protein to restore the invasive

ability of mutants for entry into fibroblasts. More recently, Pilgrim *et al.*, 2003 have demonstrated a viable p60 deletion mutant, though the mutant is highly attenuated in a mouse model and fails to form actin filaments (Pilgrim *et al.*, 2003). Other proteins are implicated in virulence. The *lma* operon controls the transcription of *lmaA*, a gene encoding a strong hydrophobic protein capable of inducing delayed-type hypersensitivity reactions in *L. monocytogenes* immune mice (Gohmann *et al.*, 1990). However, its exact role in pathogenesis is currently unknown. Superoxide dismutase counteracts potentially harmful superoxide radicals, for example, those associated with the oxidative burst exerted by phagocytes on an engulfed bacterium (Fisher *et al.*, 2000). Phospholipase C has also been implicated in invasion. Phosphatidylinositol-specific phospholipase C (PI-PLC) participates with listeriolysin O to allow *Listeria* to escape from the vacuole, and phosphatidylcholine-specific phospholipase C (PC-PLC) is involved in damaging cell membranes during transfer to epithelial cells (Smith *et al.*, 1995). A zinc metalloprotease is involved in activation of PC-PLC by cleavage (Marquis *et al.*, 1997). Other virulence factors involved include Clp-C ATPase, which aids disruption of vacuolar membranes (Roquette *et al.*, 1998). It is also involved in ActA and internalin expression. Clp-E ATPase is also involved in pathogenesis (Nair *et al.*, 2000).



## **1.7 Bacterial attachment to surfaces and biofilm formation**

### **1.7.1 Introduction**

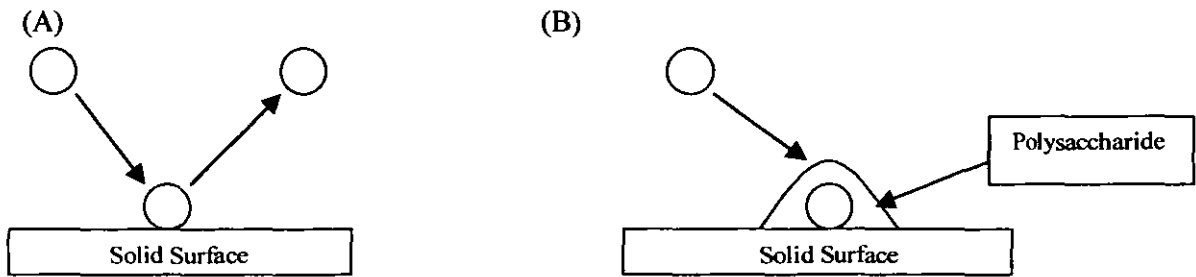
Bacterial attachment to surfaces in natural and artificial environments has important implications (Marshall *et al.*, 1971). The first report of bacteria sticking to surfaces was in 1943 (Zobell, 1943). The bacteria reported were marine bacteria stuck to submerged surfaces. Marine slime layers can easily be seen as slime layers on rocks at beaches but can be destructive, as is the case with biofilm formation on ships.

Bacteria may attach to a surface in a period of minutes and may then go on to form a matrix-enclosed population adherent to each other and / or to surfaces or interfaces (Costerton *et al.*, 1995). The term biofilm applies to microorganisms that have colonised a surface over a long period of time and is usually characterised by the production of sticky exopolysaccharide (EPS) material.

Attached populations of microorganisms are ubiquitous in the environment. A classic example of attached multispecies bacterial populations occurs on teeth. Bacteria progressively attach and add to previously attached bacteria forming a build-up of microorganisms capable of breaking down tooth enamel and causing dental caries. This phenomenon can also occur on medical prostheses and heart valves (endocarditis). Indeed, the ability of bacteria to attach and colonise surfaces is just as much a problem for hospitals as for the food industry.

Why do cells attach to surfaces? Forming an attached layer is a strategy of nutrient trapping and survival in harsh natural environments. This increased resistance has been attributed both to altered metabolism and also to the concentration gradients which exist across the layer and which protect cells from high concentration of disinfectants. Cells in a biofilm are significantly more resistant to phagocytosis (Meluleni *et al.*, 1995), disinfection and sanitizers than are planktonic cells (Mosteller and Bishop 1993, Momba *et al.*, 1998; Ronner and Wong, 1993; Ren and Frank, 1993; Yu and McFeters, 1994; Johansen *et al.*, 1997; Oh and Marshall, 1995; Stark *et al.*, 1999; Arizcun *et al.*, 1997). Once established, encased in a protective polysaccharide matrix they represent an efficient niche for enclosed cells to secure nutrients and grow. The cells are also protected from adverse environmental conditions and from biological and chemical antibacterial agents.

It is widely accepted that there are two types of attached (sessile) cells. One type is reversibly bound (see Figure 1.2A) and may subsequently become free-floating (or planktonic); the other is anchored to the surface EPS irreversibly (see Figure 1.2B). In comparison to well-characterised dental biofilms (Kolenbrander and Andersen, 1986), those found in the food industry are poorly characterised and were only hypothesised in the 1970's (Notermans and Kampelmacher, 1974). Quantitative analysis of attached bacteria in the food industry has only relatively short history.



**Figure 1.2 Reversible and Irreversible attachment**

A cell comes into contact with a solid surface and may detach and become planktonic or attach irreversibly over time and with polysaccharide extracellular production (EPS).)

### 1.7.2 The two-step and three-step hypotheses

Various mechanisms have been proposed for the adhesion of a cell to a surface and / or other cells. These include a two-step (Marshall *et al.*, 1971) and a three-step model (Busscher and Weerkamp, 1987).

Marshall's two-step hypothesis assumes that the first step is the reversible adsorption of the bacterium to a surface. Importantly he assumes that these reversibly bound cells may be removed by washing. The second step is time-dependent and is classified by the production of extracellular polysaccharides (EPS) by the organism.

The three-step hypothesis takes a more complex approach: adhesion is characterised according to forces of attachment (Busscher and Weerkamp, 1987). These forces are dependent on the distance between the cell and a surface. This hypothesis claims that long-range Van Der Waals' forces are initially at work, followed by long-range and

electrostatic forces, and finally additional forces and the production of EPS. Again the process is time dependent and the production of extra-polymeric substances is crucial for irreversible adhesion to a surface. The first step involves the cell being close enough to the surface for Van Der Waals' forces to attach it to the surface in a weak fashion. This is a reversible step. The second step involves electrostatic forces bringing the cell into contact with the surface and is also reversible, however these forces are stronger than the Van Der Waals' forces. The third step is when the cell is so close to the surface that long range and short-range forces are at work and the production of EPS makes binding irreversible. The first step occurs at distances between the cell and the surface of 50nm and more, the second at approximately 20nm and the third step at less than 20nm.

Extracellular material comprises acidic sticky polysaccharides that anchor adsorbed cells to a surface and to each other. Indeed, there is evidence to suggest that these polysaccharides can bridge the gap between cell and substrate in attached bacterial populations formed in aqueous solution Marshall (1986). Although production of polysaccharide material may be characteristic of biofilms formed in environmental or low-level culturing conditions, it is not generally produced in laboratory cultures containing enriched media (Costerton *et al.*, 1978). Deposition of these substances is time dependent (Davies *et al.*, 1993). *Pseudomonas aeruginosa* has been shown to preferentially adopt an attached phase, anchoring itself in a glycocalyx layer of exopolysaccharide alginate (Costerton *et al.*, 1987). 'Alginate' is common collective term for a copolymer of  $\alpha$ -1,4-linked D-mannuronic acid and L-guluronic acid (Davies *et al.*, 1993).

Whilst Mafu *et al.* (1990) claimed that *L. monocytogenes* could produce EPS on various types of food processing surface at adherence times as low as 1 hour, Sasahara and Zottola (1993) reported that *L. monocytogenes* only formed a confluent biofilm when aided by a primary colonising organism (for example *Pseudomonas aeruginosa*). In cultures, *L. monocytogenes* only appeared to form sparse monolayers of sessile cells. Wirtanen and Mattila-Sandholm (1992) used epifluorescence image analysis to view *L. monocytogenes* (amongst other organisms) in biofilms grown over a period of days at 25°C. They also concluded that *L. monocytogenes* only formed monolayers rather than a uniform biofilm matrix.

### **1.7.3 Factors influencing attachment**

For attachment to occur in a food-processing environment, the contact time between a microorganism and the surface it contaminates need only be short, although this depends on the surface, microorganism and the surrounding media (Mafu *et al.*, 1990). Examples of factors that influence attachment and colonisation include surface hydrophobicity and charge of the bacterial cell, smoothness and / or presence of crevices on the material surface and the ionic strength of the media.

Originally a hypothesis was that as EPS was required for attachment, and therefore decreasing nutrients would decrease bacterial attachment (Hood and Zottola, 1995). However, it soon became obvious that the opposite were true (Wrangstadh *et al.*, 1986; Hood and Zottola, 1995).

The exact contribution of the various forces of attraction has been the subject of much debate (Al-Makhlafi *et al.*, 1995; Cowan *et al.*, 1992; Absolom *et al.*, 1983; Busscher *et al.*, 1984; Busscher, 1989; Mafu *et al.*, 1991; Weerkamp *et al.*, 1988; Smoot and

Pierson, 1998). Van Der Waal's forces and electrostatic forces have already been discussed. Dickson and Koohmaraie (1989) found a linear correlation between the magnitude of the negative charge on a bacterial cell surface and strength of attachment to meat. Stoodley *et al.* (1997) found decreasing the pH of media compacts the structure of a biofilm by as much as 69 % of its original thickness. Smoot and Pierson (1998) found that over short contact times (30 minutes), less *L. monocytogenes* cells adhered to a surface in alkaline conditions and furthermore that cells adhered to bunanitril rubber and stainless steel in greater numbers with increasing temperature (10°C-45°C), with a peak at 30°C. Adhesion has also been postulated to be strain- (Norwood and Gilmour, 1999) or surface- (Blackman and Frank, 1996; Mosteller and Bishop, 1993; Spurlock and Zottola, 1991) specific.

#### **1.7.4 Hydrophobicity**

Hydrophobicity is not only a measure of how non-polar a molecule is, but also its persuasion to aggregate in aqueous solvent. A scale of hydrophobicity was calculated by Nozaki and Tanford (1971) from measurements of the free energy of transfer of amino acid side chains from a non-polar solvent to water, found tryptophan to be the least soluble. By the same scale the least hydrophobic was arginine. This is essentially for the same reason: the molecule is small, with a small accessible surface area and has several ionisable groups.

Several methods of measuring cell surface hydrophobicity have been proposed (Rosenberg, 1981; Rosenberg and Kjelleberg, 1986); Geertsema-Doornbusch *et al.*, 1993; Al-Makhlafi *et al.*, 1995). Rosenberg and Kjelleberg (1986) reviewed a number

of methods. These include hydrophobic interactive chromatography (HIC), bacterial adhesion to hydrocarbons (BATH), and the salting-out aggregation test (SAT). HIC involves the use of columns full of sepharose beads covalently bound to hydrophobic moieties. Cells are then eluted through the column. BATH involves mixing a cell suspension with a hydrocarbon. Hydrophobic cells then bind to the hydrocarbon and the decrease in absorbance the cell suspension can be measured. SAT involves suspending cells in a buffer solution to which ammonium sulphate is added until aggregation occurs. These three methods are the most commonly cited hydrophobicity-based tests for cell surfaces. Mafu *et al.*, (1991) examined the hydrophobicity of 22 strains of *L. monocytogenes*. The group used SAT, HIC and contact angle measurements. Under each test they found *L. monocytogenes* to be hydrophilic.

Attachment to surfaces may be influenced non-specifically by hydrophobicity. Hydrophobicity has been frequently implicated in adhesion of bacteria to surfaces and formation of biofilms (Dahlback *et al.*, 1981; Reynolds and Wong, 1983; Samuelsson and Kirchman, 1990; Rad *et al.*, 1998). Attachment studies evaluating the effects of hydrophobicity found that the great majority of microorganisms isolated from substrates submerged in aquatic media exhibit higher levels of adhesion to hydrophobic rather than hydrophilic surfaces (Pringle and Fletcher, 1983; Cunliffe *et al.*, 2000). Moreover, they suggest a quantitative relationship between the hydrophobicity of bacteria and their tendency to attach to living and material surfaces. Research has indicated that altering surface hydrophobicity reverses or reduces bacterial attachment (Rosenberg *et al.*, 1981; Van Loosdrecht *et al.*, 1987; Weerkamp and Busscher, 1988; Husmark and Ronner, 1990). Absolom *et al.* (1983) demonstrated that in liquids with

high surface tension, hydrophobic bacteria attach in higher numbers. They hypothesised that bacterial surface tension can partly determine the extent of phagocytosis, and furthermore the degree of non-specific opsonization (labelling for phagocytosis). The ambiguity surrounding hydrophobicity and its role in bacterial attachment to surfaces has led to the formation of hypotheses supporting attachment in which both hydrophobic and hydrophilic mechanisms are significant. Paul and Jeffery (1985) suggested that adhesion occurs by separate models depending on the relative hydrophobicity of the substrata. They postulate that proteins packaged into fimbriae or fibrils may impart cell surface hydrophobicity or be involved in adhesion (Paul and Jeffery, 1985). In organisms devoid of fimbriae or fibrils, the hydrophobic proteins may derive from a uniform layer across the cell surface such as the 'A protein' of *Aeromonas salmonicida* (Paul and Jeffery, 1985).

Attempts have been made to measure the distance over which long-range hydrophobic interactions occur, though results were not conclusive (Wood and Sharma, 1995). Samuelsson and Kirchman (1990) demonstrated bacterial growth rates to be higher on hydrophilic glass than on hydrophobic polyethylene. Marshall (1986) suggested that attachment is a multifactorial process and not just dependent on hydrophobicity, even though hydrophobic bacteria attach more strongly to surfaces than hydrophilic microorganisms. This evidence aside, it is quite obvious that hydrophobic interactions, however important in attachment, are part of a much wider multi-factorial process.



### 1.7.5 Surface Charge

It is widely known that bacterial cells have a net negative cell surface charge though this varies from strain to strain. It is also known that bacterial attachment is influenced by cell surface charge (Fletcher and Loeb, 1979). Cell surface charge has been measured by electrostatic interaction chromatography (ESIC) and electrophoretic mobility. Dickson and Koohmarie (1989) found a linear correlation between relative negative charge and initial adherence to beef muscle, though they found a much weaker correlation regarding attachment to fat. Gilbert *et al.*, (1991) demonstrated an inverse relationship between surface electronegativity and adherence to glass with *Escherichia coli*, but an opposite effect with *Staphylococcus epidermidis*. Weerkamp and Busscher (1988) demonstrated that attachment of strains of Streptococci to natural tooth surfaces was reduced significantly when the ionic strength of the surrounding experimental medium was lowered. They proposed that this was due to increased electrostatic repulsion. Dunne and Burd (1992) investigated adhesion of various strains of *Staphylococcus epidermidis* to microwell plates. They found adhesion was promoted by addition of divalent calcium (128 $\mu$ M) and magnesium (16 $\mu$ M) and reduced by addition of low concentrations of EDTA (0.25mM). They also found marginal decreases in adhesion at pH values lowered to 5.0 or 6.0 and slightly increased rates of adhesion at pH 8.0. Smoot and Pierson (1998) demonstrated that after short contact times, *L. monocytogenes* attached to Buna-N rubber and stainless steel in lower numbers under alkaline pH conditions. In addition they found that numbers of attached cells increased on both types of surface with increasing temperature (from 10°C to 45°C), though maximum numbers were achieved at 30°C.

Similarly, Herald and Zottola (1988) showed that *L. monocytogenes* could attach to stainless steel at a variety of temperatures (10°C to 35°C) and pH values (5.0 to 8.0).

#### **1.7.6 Surface conditioning**

The conditioning of surfaces with macromolecules has been hypothesised as affecting the attachment of bacteria to surfaces (Hood and Zottola, 1997; Fletcher and Marshall, 1982). Although attachment may be increased or decreased by conditioning a surface *in vitro*, little is known of the implications of natural conditioning of environmental surfaces. In general, surfaces that come into contact with protein-containing mixtures tend to become quickly covered by proteins (Bower *et al.*, 1995). Proteins present on the surface of the material may then influence subsequent interactions between the surface and microorganisms (Bower *et al.*, 1995).

#### **1.7.7 Other Microbial factors implicated in attachment**

There have been few conclusions drawn regarding the effect of bacterial proteins in attachment of bacteria, and no data implicating proteins of *L. monocytogenes* binding to surfaces. Flagella are tail like projections found protruding from many bacteria including *L. monocytogenes*. They are organelles of locomotion enabling the cell to move along concentration gradients (chemotaxis). There is a suggestion that flagella may aid contact of cells with a substratum by overcoming electrostatic repulsive forces (Fletcher and Loeb, 1979). This was not in accordance with the work of McSweeney and Walker (1986), who concluded that flagella played no role in bacterial adhesion. More recently, the findings of Herald and Zottola (1988) were that *L. monocytogenes*

isolated from Jalisco cheese could attach to stainless steel surfaces at various temperatures and pH levels and that this might be mediated by a combination of motility and polymers around the cells.

Another group of proteins that may be involved in adhesion to surfaces are the adhesins. Adhesins are macromolecules that bind to surface receptors on host cells. They have been well studied with respect to human pathogens e.g. the pili of *gonococci*. Pili are bacterial hair-like surface structures comprised of protein structures that bind to receptors on animal cells. A single organism may have many different types of pili, and may facilitate adhesion with different parts of the pili. Mack *et al.*, 1996 proposed an intercellular adhesin to be important in *Staphylococcus epidermidis* biofilm accumulation. This was done by constructing transposon knockout mutants of strain of *S. epidermidis* capable of attachment to surfaces.

### **1.8 Bacterial attachment in medicine and the food industry**

Much research has been conducted into attachment of *Pseudomonas* species (Nickel *et al.*, 1985; Ketyi, 1995; Klausen *et al.*, 2003) to surfaces. *Pseudomonas* are Gram-negative rods and can be found free-swimming or as attached populations. *Pseudomonas aeruginosa* is the epitome of a human opportunist pathogen. Although it is ubiquitous, it is primarily a nosocomial pathogen. Two problems caused by *Pseudomonas* infection are by the organism colonising prosthetic devices (Moussa *et al.*, 1996) or valves of the heart (Bicanic and Eykyn, 2002). *P. aeruginosa* infection during operations is a risk and equipment and prostheses should be effectively sanitised. Once attached, *Pseudomonas* have been shown to develop into thick

biofilms, dense at the attachment surface and diffuse at the outer edges, comprising over 70% extracellular material and space (Lawrence *et al.*, 1991). Once enclosed, entrapped cells are protected from chemical and biological attack (Costerton *et al.*, 1995).

Dental plaque has only been recognised as a multi-species biofilm by researchers in recent times. Oral infections in humans are still widespread and the average US citizen has 10-18 teeth that are decayed, missing or filled (Slavkin, 1997). Exactly how these plaque-dwelling microorganisms cause oral diseases is not completely clear (Overman, 2000). Back in 1917 *Amoebae*, *spirochetes*, *fusiforms* and *streptococci* were isolated from patients with periodontal diseases and, therefore, suggested as possible etiologies (Meyer, 1917). The characterisation of dental biofilms has been vastly aided by darkfield microscopy, transmission electron microscopy, scanning electron microscopy, DNA probes and immunoassay (Papapanou *et al.*, 1999). Currently, researchers agree that periodontal diseases are infections caused by specific pathogens such as oral streptococci, *Bacteroides forsythus*, *P. gingivalis* and *A. actinomycetemcomitans* as primary pathogens for most periodontal infections with moderate evidence linking another subset of microorganisms (*C. rectus*, *E. nodatum*, *F. nucleatum*, *P. intermedia/nigrescens*, *P. micros*, *S. intermedium*, and *T. denticola*) as possible pathogens (Papapanou *et al.*, 1999; Overman, 2000). The oral streptococci (e.g. *S. mutans*, *S. oralis*, *S. mitis*, *S. gordonii* and *S. sanguis*) have been the subject of much research and various surface proteins of these organisms have been implicated in adhesion, such as the coaggregation-mediating surface adhesions of *Streptococcus gordonii* (Clemans and Kolenbrander, 1995).

Microbial contamination of food surfaces is an ongoing problem for the food industry and is a major risk to food quality and safety. Under appropriate conditions microorganisms can not only survive, but grow on, attach to and reproduce on surfaces (Bos *et al.*, 1999): steel (Wirtanen and Mattila-Sandholm, 1993; Norwood and Gilmour, 1999; Kim and Frank, 1994; Hood and Zottola, 1997; Jeong and Frank, 1994), steel and iron (Spurlock and Zottola, 1991), steel and rubber (Ronner and Wong, 1993; Helke and Wong, 1994; Smoot and Pierson, 1998), steel, Teflon, nylon and polyester (Blackman and Frank, 1996), steel, glass, polypropylene and rubber (Mafu *et al.*, 1990). They may also alter their phenotypes during adhesion to a surface (Costerton and Lappin-Scott, 1995). The understanding of how these processes occur and can be prevented is therefore of concern to the food industry (Gourama and Bullerman, 1995). Bacteria have been shown to enter foods as a result of contact with a contaminated surface (Eginton *et al.*, 1995; Barnes *et al.*, 1996). This is exacerbated by the fact that attachment can occur in as little time as 15 minutes (Mafu *et al.*, 1990). Once attached to a surface, the microorganisms begin to trap nutrients and replicate or detach and become planktonic once again.

Residues that build up in food-processing areas such as in pipes, gaskets, drains and conveyers may act as ideal culturing conditions for detrimental microorganisms. In food-processing environments, surfaces such as these must therefore be hygienically designed with as few crevices as possible (Austin and Bergeron, 1995), and effectively cleaned and sanitized. It has been shown that even with cleaning-in-place procedures (CIP), bacteria can still remain and grow on contaminated surfaces (Austin and Bergeron, 1995). Once attached the organisms can survive for prolonged periods depending on sources of nutrients and environmental conditions (Wong, 1998).

Austin and Bergeron (1995) detected the presence of microorganisms in the crevices of PTFE gasket material in a milk-processing plant using scanning and transmission electron microscopy. They also discovered confluent growth on Buna-N gaskets in both pre- and post-pasteurisation gaskets. They did not find growth in steel endcaps, pipeline inserts or PTFE vacuum plugs and concluded that significant biofilm growth may arise on the side of gaskets in spite of standard cleaning procedures. Gibson *et al.* (1999) investigated the efficiency of food processing factory cleanliness procedures using test organisms *Pseudomonas aeruginosa* and *Staphylococcus aureus*. They concluded that the most effective methods were use of high-pressure sprays, and a mechanical floor scrubber. Use of alkaline, acidic or neutral detergent prior to spraying did not significantly increase removal of *P. aeruginosa* or *S. aureus* though they did reduce the viability of the organisms (Gibson *et al.*, 1999). Other important vectors of contamination include food workers, equipment, pests and air. Dunsmore *et al.* (1981) demonstrated that the major factors influencing food contamination and its severity are the number of individual microorganisms present on the surface of contact and the number of these microorganisms that move from the contact surface to the food product.

The presence and formation of attached microorganisms in drinking water conduits have been widely reported (Ridgway and Olson, 1981). Similarly, studies have implicated milking and transport of dairy products as major sources of psychrotrophic contaminants. It is also known that attached bacteria can form and build up in milk cooling and storage tanks, heat exchangers, stirrers and packaging products (Criado *et al.*, 1994). Biofilm formation or biofouling as it is often termed, may also occur on heat exchangers causing financial implications (Jass and Lappin-Scott, 1997).

Similarly, biofouling and corrosion of pipelines can lead to potential financial and utility losses. Attached microorganisms can cause pipeline corrosion by oxygen depletion on or near a metal surface producing anodic regions on the pipes. The rubber and PTFE-based components of industrial gaskets can also easily become contaminated with microorganisms (Mosteller and Bishop, 1993).

As we have already discussed earlier in the chapter, the ubiquitous nature of *Listeria* in the environment makes it a worry for the food industry. Many microorganisms are capable of forming dense single species biofilms. However with only one exception (Mafu *et al.*, 1990), *L. monocytogenes* has only ever been reported as forming attached monolayers on surfaces (Wirtanen and Mattila-Sandholm, 1993; Sasahara and Zottola, 1993). The highest incidence of *Listeria* is normally in moist environments such as conveyor belts and drains (Wong, 1998). There have been several attempts made to study biofilm formation by *L. monocytogenes* (Krysinski *et al.*, 1992; Zottola and Sasahara, 1994; Stone and Zottola, 1995; Blackman and Frank, 1996). The general consensus of results is that *L. monocytogenes* biofilm bacteria are more resistant to harsh environments. Krysinski *et al.*, (1992), evaluated the effects of cleaners and sanitizers on *L. monocytogenes* attached to stainless steel, polyester and polyester/polyurethane surfaces. Their discovery was that the resistance of *L. monocytogenes* differed between surfaces. Polyester / polyurethane provided the greatest resistance for the cells, followed by polyester and stainless steel respectively. They also found that cell removal was at its most efficient when the surfaces were cleaned prior to sanitising. Arizcun *et al.* (1997) investigated several decontamination procedures for removal of *L. monocytogenes* from glass surfaces. They found that the most efficient removal of *L. monocytogenes* biofilms was applied at 55°C, high

osmolarity (10.5% sodium chloride) together with acetic acid (pH 5.4, 76.7mM). Once removed from the biofilm cells would be susceptible to standard sanitising conditions applied in most food-processing plants. A surface with many pits and grooves may promote attachment of microbes. In this way, a material, which promotes biofilm growth, is inhibiting the effectiveness of an antimicrobial compound.

Roy *et al.* (1993) used the novel method of disinfecting stainless steel and polypropylene surfaces contaminated with *L. monocytogenes* using Listeriaphages. Using Listeriaphages H387, H387-A and 2671, they found that each of the Listeriaphages were just as efficient as a 20ppm quaternary ammonium compound (QUATAL) at cell concentrations of up to  $3.5 \times 10^8$  PFU/ml. This is an innovative approach to the decontamination process, and the specificity of Listeriaphages would probably not effect the food environment or products detrimentally.

### **1.9 *Listeria* and other food-borne pathogens**

*Listeria monocytogenes* is a food-borne pathogen attracting much attention from the media and scientific researchers. However, the number of *L. monocytogenes* cases per year is far fewer than the other pathogens described below. The Centre for Disease Control and Prevention (CDC) found that the incidence of listeriosis is 0.27 per 100,000 population, compared to a combined rate of 51.2 per 100,000 for all nine other food borne illnesses surveyed by the Communicable Diseases Report (CDC, 1998). The most common of these other organisms causing disease are discussed below. So why study *L. monocytogenes* if the number of cases of infection are comparatively few? Firstly, Listeriosis has a high mortality rate. The types of



individuals at risk from *Listeria* infection include the immunocompromised, the very young, the elderly and probably most notably pregnant women. The organism can cause septicaemia, meningitis, encephalitis, or intrauterine or cervical infections leading to spontaneous abortion in pregnant women due to its ability to cross the blood-brain barrier. This ability makes the *Listeria* infection an emotive subject and therefore poses great concern to the food industry. For this reason pregnant women are advised not to eat the types of food at great risk of harbouring the organism e.g. pate, cold meat and soft cheeses.

*L. monocytogenes*, has attracted considerable attention as a potential food-borne pathogen due to two major cheese-related outbreaks of listeriosis. As *L. monocytogenes* can not only survive, but grow at refrigeration temperatures, it is a serious threat to consumers and to the entire food industry. Although *L. monocytogenes* is responsible for fewer numbers of cases of food-poisoning than most of the other 'usual suspect' bacteria, it has one of the highest mortality rates making it of great concern to the food industry. Groups particularly at risk are the elderly, the immunocompromised and pregnant women. *Listeria* is not the only food-borne pathogen of significance. Indeed there are several organisms responsible for greater numbers of cases of disease each year

The most important food-borne pathogen by numbers of cases caused is *Salmonella*, which achieved notoriety in the UK during the late 1980's. *Salmonella* are a genus of gram negative, non spore-forming, usually motile, facultative anaerobic bacilli belonging to the family Enterobacteriaceae (Ekperigin and Nagaraja, 1998). There are

around 2000 different serotypes of *Salmonella* based on differences in somatic, flagellar, and capsular antigens. Infection with *Salmonella* may or may not lead to a sometimes fatal salmonellosis, a disease that can remain localized in the gastrointestinal tract as gastro-enteritis, or become generalized as a septicemia and affect several organ systems (Ekperigin and Nagaraja, 1998). Transmission to humans and animals is accelerated by survival of 'carrier' animals, which develop the disease but are not killed by it. Onset of *Salmonella* infection is much quicker and incubation time can be as little as 12 hours, and the infectious dose can be as few as 12 cells. Types of food of risk include raw meat, eggs and milk. The number of cases of salmonellosis is large 16.1 per 100,000 population per year in the US (CDC report, 2002). All persons are at potential risk especially the young, elderly and immunocompromised.

An organism responsible for a similar number of cases per year is *Campylobacter jejuni*. *C. jejuni* is a Gram-negative, motile, rod and is the most common food borne bacterial pathogen and leading cause of food borne disease in humans in the United States and other industrialized nations (Altekruse *et al.*, 1998). Although it is responsible for several million cases of food-poisoning in the US each year (13.37 per 100,000), it has only been recognized as a food-borne pathogen and a leading cause of gastroenteritis in the late eighties (CDC report, 2002). The majority of cases are trivial such as diarrhea, however more extreme cases of campylobacteriosis can lead to reactive arthritis and Guillain-Barre syndrome (Altekruse *et al.*, (1998). Incubation of the disease can be as long as a week and infection is possible even with a low infectious dose (as low as 400 bacteria). The organism is usually found in eggs, milk,

water and meat. Campylobacteriosis is most often associated with children and young adults.

*Shigella* are Gram-positive, nonmotile, nonsporeforming rod-shaped bacteria.

Shigellosis, the disease caused by *Shigella* infection is most commonly caught from feces infected water. Inset of infection can be as quick as half a day. The number of infections has risen in recent years to as high as 10.34 per 100,000 population (CDC report, 2002). The kinds of foods usually implicated in *shigella* infection are Salads (potato, tuna, shrimp, macaroni, and chicken), raw vegetables, milk and dairy products, and poultry (Shane *et al.*, 2003). Fatality from infection is not as high as *Listeria* and is usually associated with the young, elderly and immunocompromised.

Responsible for far fewer, but still significant numbers of cases of food-related infection are *Escherichia coli* O157:H7, *Yersinia enterocolitica*, *Staphylococcus aureus*, and *Clostridium botulinum*.

Over the last two decades *Escherichia coli* O157:H7 has become a pathogen of public health importance and has been much publicized in the English media. *E. coli* itself, is found in the normal flora of humans and other animals, but several invasive strains are capable of causing disease in both humans and animals (Riemann and Cliver, 1998). *E. coli* O157:H7 and other shiga-like toxin-producing strains have been transmitted via foods and caused disease ranging from bloody diarrhea, and in more severe cases, hemolytic uremic syndrome and thrombocytopenic purpura (Riemann and Cliver, 1998). Incubation is normally between 2 to 4 days and is found particularly in meat (ground beef) and milk. The organism is responsible for approximately 1.73 per

100,000 population (CDC report, 2002) and typically affects the very young and old, although anyone can be infected.

*Yersinia enterocolitica*, another psychrotrophic food-borne pathogen, was linked to several outbreaks of food poisoning associated with raw and pasteurized milk, pork, lamb, oysters and contaminated water (Ryser and Marth 1989). Food-borne infections involving *Y. enterocolitica* manifest as enterocolitis. Enterocolitis has similar symptoms to appendicitis and there have been cases of misdiagnosis (Ryser and Marth, 1989). There have even been cases of the disease being misdiagnosed as Crohn's disease. Onset of illness is approximately 24-48 hours after consumption of contaminated food-stuffs. The number of cases of yersiniosis (isolation of the organism from blood, faeces etc) is estimated at around 17000 per year in the US (Ryser and Marth, 1989), substantially greater than that of listeriosis. Similarly to listeriosis, the most at risk are the very young, elderly and immunocompromised.

*Staphylococcus aureus* is normally found on human skin, but growth on food allows production of enterotoxins. The organism itself is easily killed by heating, however the enterotoxins are heat stable and remain active. Gastroenteritis from staphylococcal enterotoxins is one of the most prevalent types of food poisoning, although exact numbers are difficult to estimate due to misdiagnosis (Holeckova *et al.*, 2002). The organism is most likely to cause disease in food prepared by hand and left at room temperature for long periods allowing ideal growth conditions. Foods such as ham, seafood, salads and sandwiches are of great risk. Onset of illness can begin as early as

4 hours (Holeckova *et al.*, 2002) and there are no particular types of people at greatest risk.

One of the most potent toxins known is produced by *Clostridium botulinum*. *C. botulinum* is an anaerobic, Gram-positive spore-forming rod that produces a potent neurotoxin. Food poisoning from *C. botulinum* is very severe and caused by the neurotoxin itself (St Louis, 1991). Fortunately, incidence of the disease is very low (approximately 10-30 cases per year in the US), however the mortality rate is extremely high. The toxin blocks neuromuscular junctions and causes flaccid paralysis. This eventually causes death by stopping the diaphragm from working. Onset of illness can be from as little as 4 hours to 8 days and organism is most associated with canned meat, vegetables and seafood (St Louis, 1991). Botulinum toxin poses a major biological weapons threat because of its extreme lethality. It is also potentially easy to make. Botulinum toxin is the single most poisonous substance known and has therefore provoked extensive research.

#### **1.10 Biofilm structure and microscopic study of attached organisms**

Microbial attachment is a very sensitive phenomenon, and the forces at play can easily be disturbed or unbalanced by experimental conditions such as tipping, pouring and rinsing. These conditions are important but often neglected in the construction of adhesion assays. Failure to take these factors into account could lead to inaccurate results, making it imperative to choose the correct system for the process under study.

The choice of method should best reflect the natural environment of the adhesion phenomena under study. Some of the most commonly used adhesion assays are : BATH (bacterial adhesion to hydrocarbons, see section 1.6.4), coupons, beads, flow systems and parallel plates with viewing chamber (Bos *et al.*, 1999) and microscopy methods.

Coupon systems include all assays where a microbial suspension is kept stationary with respect to an exposed substratum. Following a period of exposure for attachment, the coupons are usually rinsed in a variety of methods. One limitation of this process is that rinsing can expose attached organisms to a considerable shear force when passed through an air-liquid interface (Bos *et al.*, 1999). However, coupon methods allow a large number of experiments to be undertaken in a comparatively short space of time and are useful for monitoring the early stages of adhesion.

Bead methods are a variation on slide methods, though differ by having a large substratum with respect to suspension volume (Bos *et al.*, 1999). Beads are usually washed and agitated following incubation and adherent cells counted by either radio-labelling or spectrophotometry (Bos *et al.*, 1999).

Flow devices are one of the most commonly used systems and are designed to measure microbial attachment under controllable parameters such as temperature, pressure and nutrient concentration. The modified Robbins' device is frequently used as a chamber to contain coupons of substrata, allowing a flow-through of medium to run over them (Kharazmi *et al.*, 1999). Other types of flow-through devices include: stagnation point flow collectors (Dabros and Van de Ven, 1987), radial (Dickinson *et al.*, 1995), and parallel plate flow chambers (Christersson *et al.*, 1987, Sjollem *et al.*, 1989, Bos *et*

*al.*, 1999). These flow systems allow large numbers of materials to be tested at once but are limited by keeping medium conditions constant. They are also limited by distribution of forces of flow. In other words a material at the front of a modified Robbin's device will be subjected to different conditions of flow compared to a material situated at the rear of the device.

The parallel plate flow chamber system consists of nickel-coated brass section and a metal top plate. This chamber is situated on top of a microscope which is attached to a camera and computer (Bos *et al.*, 1999). The total number of bacteria can be enumerated as can deposition rates. A major advantage of parallel plate flow chambers is that deposition experiments can be carried out on metals when equipped with a microscope (Mueller *et al.*, 1992). The devices allow for application of conditioning films to alter hydrophobicity dynamics as well as providing the ability to study microbial detachment and spatial arrangement (Bos *et al.*, 1999). However the system is expensive as it requires a computer to collate meaningful results.

Many of the reports of microbial adhesion and methods of study and quantification are based on the use of computer-aided and microscopic techniques (Costerton *et al.*, 1995; Ridgway and Olson, 1981). Surman *et al.* (1996) evaluated microscope techniques for the study of biofilms developed from an aquatic environment. The methods investigated by the group were transmission electron microscopy (T.E.M), scanning electron microscopy (S.E.M.), environmental scanning electron microscopy (E.S.E.M), episcopic differential interference contrast microscopy (D.I.C.), Hoffman modulation contrast microscopy (H.M.C.), atomic force microscopy (A.F.M.), and scanning confocal laser microscopy (S.C.L.M.).

T.E.M. and S.E.M. are two of the most commonly used microscopic techniques. T.E.M. was found to give useful spatial information and S.E.M. was found to give good analysis of surface shape. However, the fixing process may damage the biofilm, as may electron beams. Both methods are also subject to background artefacts. E.S.E.M. is also prone to electron beam damage as it is a form of S.E.M. adapted to enable viewing of hydrated specimens. However it bypasses the detrimental staining process which can prematurely remove adherent cells. D.I.C. also shows surface topology and can show a 3D visualisation of the biofilm population. It requires little detrimental preparation and can be used to view thick populations in a detailed format. H.M.C. allows direct visualisation of hydrated specimens without staining or fixing as does A.F.M. A.F.M allows a 3D image and offers excellent topological information. S.C.L.M. can show viability of biofilms as well as interactions between cells and between the cells and the surface. It allows 3D imaging with little interference from artefacts and is non-destructive. These features allow it to be used for direct examination of complex spatial and chemical relationships that exist between bacteria, their extracellular products, and their environment (Costerton *et al.*, 1995).

Surman *et al.* (1996) reviewed the use of different types of microscopy for biofilm study. Scanning electron microscopy is one of the most common methods of investigation available. Some of the more recently available techniques such as A.F.M and E.S.E.M offer the obvious advantage of not requiring any detrimental fixing / preparatory steps and allow viewing of an intact hydrated specimen. Each of the techniques provides a useful insight into different aspects of bacterial attachment including population structure and morphology.



Each of the systems described has its own benefits and disadvantages, although the more resources available, the more parameters that may be investigated accurately. The coupon batch culture was method was selected for this project due to the large number of materials, strains and mutants to be investigated.

Many laboratory methods have been used for the dislodging step of slide-based assays. These include surface scraping of stainless steel (Jeong and Frank, 1994), sonication (Green and Pirrie, 1993), vortexing (Anwar *et al.*, 1992) and shaking with glass beads (Bloomfield *et al.*, 1994). According to Jeong and Frank (1994), scraping removes up to 97% of attached cells on stainless steel and vortexing removes 99% of attached cells from silicone rubber (Anwar *et al.*, 1992). The first direct comparison of various dislodging methods was carried out by Lindsay and Von Holy (1997). The findings were that there was no significant difference in the efficacy of *Pseudomonas fluorescens* and *Bacillus subtilis* biofilm removal from stainless steel and polyurethane by vortexing, sonication and shaking with beads. The enumeration was carried out by plate counts and scanning electron microscopy (SEM).

One part of this investigation was to investigate and compare several removal techniques for the efficiency of removal of *Listeria monocytogenes* from materials.

Many of the reports of microbial adhesion and methods of study and quantification are based on the use of computer-aided and microscopic techniques (Costerton *et al.*, 1995; Ridgway and Olson, 1981). Surman *et al.* 1996 evaluated a comparison of microscope techniques for the evaluation of biofilms developed from an aquatic environment. The methods investigated by the group were transmission electron microscopy (T.E.M), scanning electron microscopy (S.E.M.), environmental scanning

electron microscopy (E.S.E.M), episcopic differential interference contrast microscopy (D.I.C.), Hoffman modulation contrast microscopy (H.M.C.), atomic force microscopy (A.F.M.), and scanning co focal laser microscopy (S.C.L.M.).

T.E.M. and S.E.M. are two of the most commonly used microscopic techniques. T.E.M. was found to give useful spatial information and S.E.M. was found to give good analysis of surface shape. However, the fixing process may damage the biofilm, as may electron beams. Both methods are also subject to background artefacts. E.S.E.M. is also prone to electron beam damage as it is a form of S.E.M. adapted to enable viewing of hydrated specimens. However it bypasses the detrimental staining process, which can prematurely remove adherent cells. D.I.C. also shows surface topology and can show a 3D visualisation of the biofilm population. It requires little detrimental preparation and can be used to view thick populations in a detailed format. H.M.C. allows direct visualisation of hydrated specimens without staining or fixing, as does A.F.M. A.F.M allows a 3D image and offers excellent topological information. S.C.L.M. can show viability of biofilms as well as interactions between cells and between the cells and the surface. It allows 3D imaging with little interference from artefacts and is non-destructive. These features allow it to be used for direct examination of complex spatial and chemical relationships that exist between bacteria, their extracellular products, and their environment (Costerton *et al.*, 1995).

Surman *et al.* (1996) reviewed the use of different types of microscopy for biofilm study. Scanning electron microscopy is one of the most common methods of investigation available. Some of the more recently available techniques such as A.F.M and E.S.E.M offer the obvious advantage of not requiring any detrimental fixing /

preparatory steps and allow viewing of an intact hydrated specimen. Each of the techniques provides a useful insight into different aspects of bacterial attachment including population structure and morphology.

### **1.11 Transposon mutagenesis**

Transposon mutagenesis has become a powerful tool for investigating bacterial genes and gene function. Gaillard *et al.* (1986) were the very first group to use transposon mutagenesis to investigate *L. monocytogenes*. They successfully constructed Listeriolysin O-negative mutants and discovered that the loss of listeriolysin O resulted in a loss of virulence (Gaillard *et al.*, 1986). The nonconjugative transposon *Tn917*, has successfully been used in mutagenesis of the listerial chromosomal genes (Cossart *et al.*, 1989; Camilli *et al.*, 1990). This transposon was first isolated by Tomich *et al.* (1980) and was found in a plasmid (pAD2), encoding resistance to streptomycin, kanamycin and erythromycin. The erythromycin resistance gene was located on the transposable element *Tn917* (Tomich *et al.*, 1980). The transposon is transposed in a *Tn3*-like process, non-specific and random in nature (Youngman *et al.*, 1983). The transposon is also able to generate stable insertional mutants with very low excision rate (Youngman *et al.*, 1983; Camilli *et al.*, 1990). The relatively small size of *Tn917* allows simple cloning procedures for mapping flanking chromosomal DNA regions. Useful constructs of *Tn917* have been developed to include translational fusions such as *lacZ* and *cat-86*, and drug resistance genes (Youngman, 1987), allowing direct identification of transcriptional and regulatory functions.

Transposing bacteriophages, such as lambda and Mu, can be used for insertional mutagenesis of many Gram-negative bacterial species (Thompson and Landy, 1989). However bacteriophages of this kind are not appropriate for the construction of insertion mutants of *L. monocytogenes* and other Gram-positive species (Berg *et al.*, 1989). The findings of transposons carrying antibiotic resistance led to a revolution in the use of transposable elements for genetic engineering in bacterial species (Berg *et al.*, 1989). These elements carried resistance markers that were much easier to select than Mu and  $\lambda$ - prophage immunity, they could insert into many sites and transpose in many species, and their generally smaller size facilitated easier physical mapping and cloning of DNA (Berg *et al.*, 1989).

Transposons are not only used to identify genes by insertion mutation but are also utilised in characterising organisation of operons; analysing transcriptional and translational regulatory mechanisms, protein conformation, and cellular location; generating genome rearrangements such as deletions and replicon fusions; introducing specific genes into new hosts or into chromosomal sites; introducing origins of replication or of conjugative DNA transfer; providing mobile primer binding sites that facilitate DNA sequencing; to provide portable, regulatable promoters with which to manipulate patterns of gene expression; and to identify essential genes and sites (Berg *et al.*, 1989).

The transposon Tn917 is a member of the Tn3 family of transposons, and shares common features with this transposon family including generation of a 5-base-pair duplication on insertion and sequence homology within the terminal inverted repeats.

The size of Tn917 (5.4 kb), when compared with that of Tn916 (16.4 kb) and Tn1545 (25.3 kb), simplifies the cloning and mapping of the DNA flanking its insertion. For

that reason it is a suitable replacement for the conjugative transposon Tn916, and the related Tn1545, which had previously been the transposons of choice for insertional mutagenesis of *L. monocytogenes* (Kathariou *et al.*, 1987; Mengaud *et al.*, 1987; Kuhn *et al.*, 1988; Camilli *et al.*, 1989; Leblond-Francillard *et al.*, 1989; Sun *et al.*, 1990). Furthermore, the comparatively low transposition rates of Tn916 ( $10^{-6}$ ) and Tn1545 ( $10^{-8}$ ) makes it inconvenient to carry out large scale mutagenesis, in contrast to Tn917 which transposes at a frequency of  $10^{-5}$  (Youngman, 1989).

Members of the Tn3 family, like most other transposable elements, show some insertion site specificity. However, Tn917 is somewhat different without it shows very little site specificity in bacteria, other than *Bacillus subtilis* (Sherratt, 1989). Tn917 exhibits preference for A+T rich sequences and is attracted to sites showing some similarity to the transposon ends (Perkins and Youngman, 1984; Shaw and Clewell, 1985). Because of this lack of site specificity the randomness of insertion of Tn917 is higher than that of the conjugative transposons Tn916 and Tn1545 that require sequence homology between both ends of the elements and sequences surrounding the sites of integration (Clewell *et al.*, 1988). The low molecular % G+C ratio of *L. monocytogenes* (approximately 38%) (Stuart and Welshimer, 1974; Seeliger and Jones, 1986) makes Tn917 a convenient transposon for insertional mutagenesis in this organism since it prefers A+T-rich regions.

Several strategies have been used to deliver Tn917 in chromosomal sites of the *Bacillus* species. One strategy includes the use of suicide vectors carrying Tn917 together with replicons from the ColE1-derived vectors pBR322 and pBR328 or the

p15A-derived vector pACYC184 (Berg *et al.*, 1989). These vectors cannot replicate in Gram-positive bacteria, consequently on transformation into such bacteria they fail to replicate but deliver the transposon they carry into the host chromosome and are then lost. Any erythromycin resistant organisms recovered after this event are predominately those that have acquired a chromosomal insertion of Tn917 (Chang and Cohen, 1978).

The construction of Tn917 derivatives carrying additional drug resistance genes and, or, reporter genes, inserted near the end of the transposon has facilitated analysis of gene regulation (Youngman, 1989). Incorporation of additional drug resistance genes increases the potential host range of the modified transposon. Furthermore, the presence of reporter genes allows investigation of target gene expression (Berg *et al.*, 1989). Gene fusions have been instrumental in the discovery of genes that respond to particular environmental signals, such as DNA damage, changes in osmolarity, heat shock, nutrient starvation and anaerobiosis. The identification of genes for cell surface and exported proteins (potentially involved in pathogenicity) and the study of cellular localisation of proteins have been greatly aided by the ability to easily generate a variety of hybrid proteins (Berg *et al.*, 1989). 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 inactivated gene. Consequently, mutants harbouring insertions in transcription units can be identified by markers such as  $\beta$ -galactosidase activity.

Based on this strategy, the vector pLTV3 was constructed to apply the same approach for the transposon mutagenesis of *L. monocytogenes* (Camilli *et al.*, 1990). This

plasmid carries a modified form of Tn917, Tn917-LTV3, and utilises the pE194 origin of replication derived from *Staphylococcus aureus* (Iordanescu, 1976), which is naturally temperature-sensitive for replication. When DNA fragments containing Tn917 are inserted or cloned into pE194-derived plasmids, insertions of the transposon into *L. monocytogenes* chromosome can be obtained simply by raising the temperature of plasmid-containing bacteria while maintaining selection for Tn917-specified drug resistance (e.g. erythromycin resistance). The derivative, Tn917-LTV3, can generate transcriptional fusions to *lacZ*. Transcriptional fusions are generated because a promoterless *E. coli* sequence encoding *lacZ*, modified to utilise a Shine-Delgarno sequence from the *spoVG* gene of *B. subtilis* (Zuber and Losick, 1983), is inserted a close to the terminal inverted repeat at the *erm*-distal end of Tn917. This derivative contains, immediately downstream from the *lacZ* coding sequence, an *E. coli* cloning vector that includes a kanamycin *neo* resistance gene as a selectable marker in *E. coli*, a chloramphenicol *cat* resistance as a selectable marker in *B. subtilis* or *L. monocytogenes*, ColE1 replication functions, an M13 origin of replication, and a cluster of polylinker cloning sites (Camilli *et al.*, 1990). The polylinker sites facilitate the recovery in *E. coli* of chromosomal DNA adjacent to sites of insertion, particularly DNA on the promoter-proximal site of transposon-mediated *lacZ* fusions (Camilli *et al.*, 1990).

Finally, successful insertion of the Tn917-LTV3 derivative from pLTV3 generates “type I” fusions or transcriptional fusions, because the reporter gene (*lacZ*) lacks a promoter but contains its own translation initiation signals (Berg *et al.*, 1989). Expression of the reporter gene in transcriptional fusions takes place if insertion of the transposon occurs in the correct orientation (Berg *et al.*, 1989). “Type II” fusions or translational fusions monitor translation as well as transcription, because the reporter

gene lacks sequences needed to initiate both transcription and translation. Transposons that form “type II” fusions need to insert in both the correct orientation and the correct reading frame for expression (Casadaban and Chou, 1984). The reporter protein made by a “type I” fusion is constant in length and amino acid sequence because the fusion occurs upstream of the reporter translation initiation site. In contrast, hybrid proteins formed in “type II” fusions have an amino terminus of variable length encoded by the target gene and a carboxy-terminal region (reporter) that is constant for any given transposon (Casadaban and Chou, 1984).

One approach used to recover *Tn917* insertions in chromosomal sites in *B. subtilis* employed the use of suicide vectors carrying *Tn917* and replicons of the vector native to *E. coli*. These vectors could not replicate in Gram-positive bacteria. Upon transformation into *B. subtilis* organisms still containing the transposable element can be selected for by drug resistance (e.g. erythromycin).

Temperature sensitive plasmid vectors are the most common method of choice for *Tn917* recovery, especially those that carry the pE194 replicon from *Staphylococcus aureus* (Youngman *et al.*, 1985; 1989; Youngman, 1987). Replicons of pE194 origin show a reduced copy number with increasing temperature that terminates at 45°C (Gryczan *et al.*, 1982). Passaging of bacteria carrying pE194 at elevated temperature loses the vector and erythromycin resistant surviving bacteria predominantly display a *Tn917* chromosomal DNA insertion (Youngman *et al.*, 1984a). Camilli *et al.*, (1990) used the temperature sensitive vector pLTV3 (carrying a *Tn917-lac* fusion) for the construction of a *L. monocytogenes* mutant library.



The primary aim of this study is to investigate attachment of *L. monocytogenes* 10403S to different food processing surface materials. The application of transposon mutagenesis provides a method by which identification of genes responsible for *L. monocytogenes* attachment to materials may be discovered. For this purpose it was decided to use a transposon *Tn917* derivative (*Tn917*-LTV3) carried on plasmid pLTV3 (Camilli *et al.*, 1990). The transposon is carried by pE194T (Villafane *et al.*, 1987), a derivative of pE194, containing several drug resistance cassettes, a promoterless copy of the *E. coli lacZ* gene, *E. coli ColE1* and M13 origins of replication, together with a multiple cloning site.

## Chapter 2: Materials and Methods

### 2.1 Bacterial strains and plasmids

Tables 2.1 and 2.2 show the bacterial strains and plasmid vectors used.

#### 2.7.2 Growth conditions and media

*Listeria monocytogenes* strain 10403S was grown statically in Tryptone Soya Broth (TSB) or Tryptone Soya Agar (TSA) (OXOID, Unipath Ltd) medium at 30°C. *L. monocytogenes* DP-L910 was grown in the presence of tetracycline, erythromycin and lincomycin at concentrations of 12µg/ml, 1µg/ml and 25µg/ml respectively.

The transposon insertion mutants of *L. monocytogenes* (containing the plasmid pLTV3 see figure 2.1) were grown in the presence of erythromycin and lincomycin (Sigma-Aldrich Company Ltd. Dorset, England) at the concentrations stipulated above.

*Escherichia coli* DH5α and MC1061 were grown in Luria-Bertani (LB) medium (per litre: 10g Bacto-Tryptone (Sigma), 5g Bactone-Yeast Extract (Sigma), 5g Sodium chloride (Sigma); for Agar 15g Bacto-Agar per litre was added, Davis *et al.*, 1980) at 37°C.

*E. coli* transformants were recovered using SOC recovery medium (20g Bacto-Tryptone per litre, 5g Bacto-Yeast Extract per litre, 10mM Sodium chloride, 2.5mM Potassium chloride (Sigma), 10mM Magnesium chloride (Sigma), 10mM Magnesium sulphate (Sigma), 20mM Glucose (Sigma), Sambrook *et al.*, 1989). *E. coli*

transformants harbouring pGEM T-Easy vector (Promega UK, Romsey, Southampton, UK) were grown in the presence of ampicillin at a concentration of 50µg/ml. *E. coli* transformants harbouring pCK1 vector (Gasson and Anderson, 1985) were grown in the presence of kanamycin at a concentration of 20µg/ml. *E. coli* transformants harbouring pMK4 (Sullivan *et al.*, 1984) vector were grown in the presence of ampicillin at a concentration of 50µg/ml.

All media were prepared using distilled water and were autoclaved at 121°C at 15 psi for 15 minutes. Bacterial strains were maintained on agar plates at 4°C. Bacterial stocks were stored at -70°C in broth containing 10% (v/v) glycerol cryoprotectant.

#### **2.1.2 Growth conditions and media used for adhesion assay**

*L. monocytogenes* was grown on Tryptone Soya Agar (TSA). Tryptone Soya Broth (TSB) was prepared according to manufacturer's instructions. A culture of *L. monocytogenes* was grown overnight statically in TSB at 30°C. The overnight culture was then centrifuged at 1400g at 4°C for 15 minutes. The cell pellet was resuspended 1% (w/v) in fresh sterile 20% (v/v) TSB. For use, cell suspensions were prepared to an OD<sub>600nm</sub> of between 0.15 and 0.2, which corresponded to cell counts of between  $5.2 \times 10^8$  and  $8.6 \times 10^8$  cfu/ml.

**2.7.2 Other growth additives**

When necessary, 5-Bromo-4-chloro-3 indolyl-β-D-Galactosidase (X-gal) (Sigma) was added to growth media at a concentration of 40μg/ml. Isopropyl β-D thiogalactopyranoside (IPTG, Sigma) was used at a final concentration of 200mM.

**Table 2.1 Bacterial strains**

Bacterial strain	Genotype/Description	Reference
<i>Listeria monocytogenes</i> 10403S <sup>1</sup>		(1)
<i>Listeria monocytogenes</i> DP-L910 <sup>1</sup>	strain 10403S harbouring pLTV3	
<i>Listeria monocytogenes</i> , strain NCTC 7973	<i>fla2</i> mutant <sup>2</sup>	(2)
<i>Escherichia coli</i> DH5α	<i>supE44 ΔlacU169(φ80 lacZΔM15)</i>	(3)
	<i>hsdR17 recA1 end A1 gyrA96 thi-1 relA1</i>	
<i>Escherichia coli</i> MC 1061 <sup>3</sup>	<i>hsdR mcrB araD139 Δ(araABC-leu)</i>	(4)
	<i>7679 ΔlacX74 galU galK rpsL thi</i>	

<sup>1</sup>Obtained from Dr. D. Portnoy, Department of Microbiology, University of Pennsylvania, Philadelphia, USA.

<sup>2</sup>Obtained from Dr. C.E. Rees, Division of Food Sciences, School of Biological Sciences, University of Nottingham, Sutton Bonington Campus, Leicestershire, UK.

<sup>3</sup>Obtained from Dr. Gutierrez, Department of Oral Biology, University of Florida, Gainesville, Florida, USA.

(1) Bishop and Hinrichs, 1987. (2) Vatanyoopaisarn *et al.*, 2000. (3) Hanahan, 1983.(4) Casadaban *et al* 1983.

**Table 2.2 Plamids**

<b>Plasmid</b>	<b>Characteristics</b>	<b>Reference</b>
pBluescript Vector	<i>E. coli</i> vector, ampicillin resistance marker. Blue white selection.	(1)
pGEM T-Easy Vector	<i>E. coli</i> T-A cloning vector, ampicillin resistance marker. Blue white selection.	(2)
pCK1	<i>Listeria</i> / <i>E. coli</i> - <i>Bacillus</i> shuttle vector, kanamycin and chloramphenicol resistance.	(3)
pMK4	<i>Listeria</i> / <i>E. coli</i> - <i>Bacillus</i> shuttle vector, ampicillin resistance marker, blue / white selection.	(4)

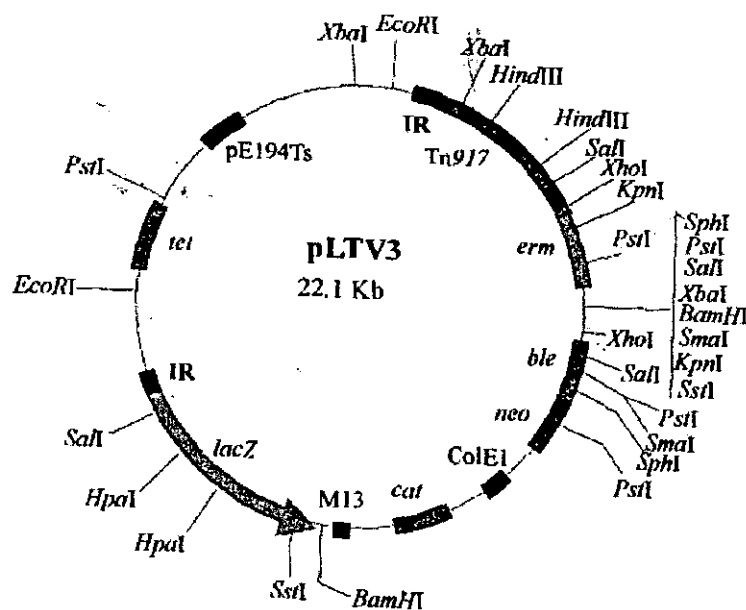
(1) Stratagene, Stratagene Europe, Amsterdam, Netherlands.

(2) Promega UK, Southampton, England.

(3) Gasson and Anderson, 1985.

(4) Sullivan *et al.*, 1984.

**Figure 2.1 Structure of plasmid pLTV3 (22.1 kilobases).**



The plasmid vector pLTV3 contains temperature sensitive replicon pE194Ts, the tetracycline resistance gene (*tet*), the chloramphenicol resistance gene (*cat*), the kanamycin resistance gene (*neo*), the bleomycin resistance gene (*ble*), the transposon Tn917 (containing the erythromycin resistance gene: *erm*), a promoterless copy of the *E. coli lacZ* gene, ColE1 replication functions, an M13 origin of replication, a multiple cloning site and Inverted Repeats (IR) (adapted from Camilli *et al.*, 1990).

## **2.2 DNA Extraction procedures.**

### **2.2.1 (a) Large scale extraction of *E. coli* plasmid DNA**

The plasmid Maxi-Prep Kit (QIAGEN Ltd Crawley, West Sussex, UK) was used for large scale plasmid DNA extraction. The method was carried out according to the manufacturer's instructions in the manual. Stationary phase bacterial cell cultures (500ml) were harvested by centrifugation at 6000g for 15 minutes at 4°C and the resulting pellet was completely resuspended in 10ml of Buffer P1 (50mM Tris-HCl (pH 8.0), 10mM EDTA, 100µg/ml RNase A). Then 10ml of a named "Buffer P2" (200mM NaOH, 1% (w/v) SDS) was added and mixed by careful inversion to produce a clear lysate. After 5 minutes incubation at room temperature, 10ml chilled Buffer "P3" (3M Potassium acetate (pH 5.0)) was added and the mixture cooled on ice for 20 minutes. Cell debris was discarded by centrifugation at 20000g for 30 minutes at 4°C in a Sorvall SS-34 rotor. The supernatant was removed immediately and re-centrifuged at 20000g for 15 minutes at 4°C to remove suspended material before elution through the QIAGEN column. The QIAGEN column was equilibrated by addition of 10ml Buffer "QBT" (750mM Sodium chloride, 50mM MOPS (pH 7.0), 15% (v/v) ethanol, 0.15% (v/v) Triton X-100). 30ml Buffer "QC" (1M Sodium chloride, 50mM MOPS (pH 7.0), 15% (v/v) ethanol) was used to wash the column twice. Plasmid DNA was eluted from the column by addition of 15ml Buffer "QF" (1.25M Sodium chloride, 50mM Tris-HCl (pH 8.5), 15% (v/v) ethanol. To pellet the plasmid DNA, 0.7 volumes of isopropanol were added to the eluate and mixed before immediate centrifugation at 15000g for 30 minutes at 4°C. The DNA pellet was

washed with 5ml 70% (v/v) ethanol and re-centrifuged. After the supernatant was removed, the pelleted DNA was air-dried for 10 minutes and resuspended in either 2ml of Tris EDTA (TE) buffer (10mM Tris-HCl, 1mM EDTA, pH 7.5) or 2ml nanopure water for sequencing.

### **2.2.1 (b) Small scale extraction of plasmid DNA**

Small scale extraction was carried out according to the method of Sambrook *et al* (1989), with slight modifications. The following is a list of solutions that were used:

#### **Solution I**

50mM glucose

25mM Tris-HCl pH 8.0

10mM EDTA

4 mg/ml lysozyme (Sigma)

#### **Solution II**

0.2M Sodium chloride

1% (w/v) Sodium dodecyl sulphate

#### **Solution III**

5M acetate (11.5ml glacial acetic acid)

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

Distilled water (28.5ml)



Stationary phase bacterial culture (1.5ml) was centrifuged in a Micro-centaur bench top microfuge at 5000g for 1 minute and the supernatant was discarded. The cell pellet was resuspended in 100µl of Solution I and incubated on ice for 30 minutes. Following this, 200µl of freshly prepared Solution II was added, carefully mixed and left on ice for no longer than 5 minutes. This was followed by addition of 150µl of ice-cold Solution III. The preparation was carefully mixed and the incubation on ice continued for at least 10 minutes. Following this incubation the preparation was centrifuged at 13000g for 5 minutes and the supernatant carefully removed. Protein was carefully removed from this preparation by addition of an equal volume of phenol : chloroform (1:1) and vortexed, before being centrifuged at 13000g for 2 minutes. The upper aqueous layer was removed, mixed with 1/10th of 3M Sodium acetate (pH 5.2) and 2 volumes of ice-cold absolute ethanol at room temperature. After a 2 minute incubation period the plasmid DNA was collected by centrifugation at 13000g for 5 minutes. The ethanol was carefully removed and the plasmid DNA pellet air-dried and dissolved in either 50µl of 0.1x TE buffer or 50µl nanopure water for sequencing. Plasmid DNA was stored at -20°C.

## **2.2.2 Extraction of listerial plasmid DNA**

### **2.2.2 (a) Large scale extraction of listerial plasmid DNA**

*Listeria monocytogenes* plasmid DNA was purified by ultra centrifugation in a caesium chloride/ethidium bromide gradient (Sambrook *et al* 1989).

This method requires use of Solutions I, II and III previously described in section 2.2.1

(b) 400ml of stationary phase bacterial cells were centrifuged at 4000g for 10 minutes at 4°C. The pellet was then resuspended in 10ml of Solution I and incubated at 37°C for 30 minutes. After this period 10ml of Solution II was added and the incubation continued for a further 10 minutes. Then 15ml of ice cold Solution III was added to the mixture and the mixture incubated at 37°C for 10 minutes. Cell debris was removed by centrifugation at 3500g at 4°C for 20 minutes. The supernatant was removed and mixed with 0.6 volumes of isopropyl alcohol. This was allowed to stand at room temperature for a minimum 15 minutes. DNA was collected by centrifugation at 4000g for 30 minutes at room temperature. Following this the pellet was air-dried and resuspended in sterile nanopure water to a volume of 17ml. Caesium chloride and ethidium bromide were added to the DNA at final concentrations of 1mg/ml and 10µg/ml respectively and placed in a polyallomer tube. Tubes were sealed, placed in a Sorvall OTD 60 centrifuge and the DNA was separated by centrifugation at 40000g for 20 hours at 20°C using a Sorvall TV850 rotor. The position of the plasmid DNA band was shown by visualisation under UV light. Ethidium bromide was removed by equilibration with saturated isopropanol, and caesium chloride was removed by exhaustive dialysis against distilled water at 4°C. Plasmid DNA was precipitated by adding 0.1 volumes of 3M Sodium acetate (pH 5.2) and 2 volumes of ice cold absolute ethanol. The plasmid DNA was then collected by centrifugation at 4500g for 30 minutes at room temperature and redissolved in either 500µl TE buffer or 500µl nanopure water for sequencing.

### **2.2.2 (b) Small scale extraction of listerial plasmid DNA**

This was carried out by the same method as in section 2.2.1 (b). All steps were as in the method described except for those carried out on ice which were performed at 37°C for efficient extraction.

### **2.2.3 Extraction of listerial chromosomal DNA**

Chromosomal DNA extraction from listeria was carried out by the method of Flamm *et al* 1984. For this method, 10 ml of overnight bacterial culture grown in Tryptone Soya Broth (TSB) was centrifuged at 11700g for 10 minutes at 4°C. The cell pellet was resuspended in 5ml of 0.1 x SSC (1 x SSC: 0.15M Sodium chloride, 0.015M trisodium citrate (pH 7.0)) and centrifuged at 11700g for 10 minutes at 4°C. The supernatant was then removed and 1ml of 20% (w/v) sucrose in 0.01M Sodium phosphate buffer (pH 7.0) containing 2.5mg/ml lysozyme (w/v) was added to the cell pellet and the preparation incubated at 37°C for 45 minutes. After this period 9ml of 1% (w/v) SDS in TE buffer, with proteinase K at a concentration of 1mg/ml, was added and the incubation continued for a further 30 minutes. Following this, an equal volume of phenol : chloroform (1:1) mixture was added and the preparation was mixed to form an emulsion before being centrifuged at 4000g for 20 minutes. After centrifugation the upper aqueous phase was removed and phenol : chloroform added until no white precipitate was shown at the interface of the two layers. DNA was precipitated from the aqueous phase by addition of 2 volumes of ice cold absolute ethanol and 0.1 volumes of 3M Sodium acetate (pH 5.2). The DNA was then centrifuged at 4000g for

10 minutes and the supernatant carefully removed. The pellet was air-dried for 10 minutes and then resuspended in either 1ml of 0.1x TE buffer or 1ml of nanopure water.

## **2.3 Procedures for transformation of bacterial cells**

### **2.3.1 Transformation of *E. coli* by electroporation**

#### **2.3.1 (a) Preparation of electro-competent *E. coli***

The method of electro-competent cell preparation used was based on the method of Dower *et al.*, (1988). One litre of LB-broth was inoculated with 10ml of an overnight culture of *E. coli* and the bacteria were grown with vigorous aeration to mid-log phase ( $OD_{600nm}$  of 0.5 to 1 unit). The culture was cooled on ice for 30 minutes and then centrifuged at 4000g for 15 minutes at 4°C. The cell pellet was then washed twice in 1L of ice cold sterile nanopure water times and centrifuged at 6500g for 20 minutes. The resulting pellet was the resuspended in 25ml ice cold 10% (v/v) glycerol, and centrifuged at 5000g for 20 minutes at 4°C. The final cell pellet was resuspended in 1ml of ice cold 10% (v/v) glycerol. This was then dispensed into 100µl aliquots and stored at -70°C until use.

### **2.3.1 (b) Electro-transformation with plasmid DNA**

Aliquots of electro-competent cells were thawed on ice and used as in the method of Sambrook *et al* (1989). Aliquots of 40µl cell suspension were mixed with 1ng of plasmid DNA and placed in a cold 0.2cm<sup>2</sup> electroporation cuvette (Bio-Rad). A Bio-Rad Gene Pulser and Pulse Controller were used in accordance with the manufacturers instructions to generate an electric pulse through the cuvette. The settings used were 25µF, 12.5kV/cm<sup>2</sup> and 200Ohms. The cold electroporation cuvette was placed in the safety chamber of the Gene pulser, inserted into the electrodes and given a single pulse. 1ml SOC (see section 2.1.1) medium was immediately added to the cell suspension, mixed and incubated for one hour at 37°C at 300 rpm (revolutions per minute). The suspension was then plated onto LB-agar containing the appropriate concentration of the appropriate selective antibiotic. Selective plates were incubated at 37°C overnight.

### **2.3.1 (c) Preparation of competent *E. coli* cells for transformation by heat shock**

This method was carried out according to the Hanahan method (Inoue *et al.*, 1990).

The bacteria were streaked out on LB-agar containing 10mM Magnesium chloride or Magnesium sulphate and allowed to grow overnight. A single colony was grown for 2 hours at 37°C in 5ml of TYM broth (2% (w/v) Bacto-Tryptone, 0.5% (w/v) Bacto Yeast Extract, 100mM Sodium chloride, 10mM Magnesium chloride or Magnesium sulphate) in a 50ml sterile flask at 300 rpm. The cells were then transferred to 100ml of fresh TYM broth and incubated at 300 rpm for a further 2-3 hours until reaching an

OD<sub>550nm</sub> of 0.8-0.9. The cells were then centrifuged at 4000g for 10 minutes in two polypropylene tubes. The supernatant was removed and the pellet resuspended in 40ml ice cold TFB1 (30mM Potassium acetate, 50mM Magnesium chloride, 100mM Potassium chloride, 10mM Calcium chloride, 15% (w/v) glycerol) per 100ml starting culture. This suspension was incubated on ice for 5-10 minutes. The cells were then centrifuged at 4000g for 8 minutes at 4°C. The supernatant was again discarded and the pellet resuspended in 4ml per 100ml starting culture of TFB2 (10mM Sodium-MOPS, 75mM Calcium chloride, 10mM Potassium chloride, 15% (w/v) glycerol). The cell suspension was then dispensed into 100µl aliquots in pre-cooled eppendorf tubes and stored at -70°C until use.

#### **2.3.1 (d) Transformation of *E. coli* by heat shock method**

Plasmid DNA (25ng) was added per aliquot of competent cells thawed on ice and left for 15 minutes. The cell and plasmid mixture was then heat shock at 37°C for 5 minutes in a water bath. Following this 0.9ml of pre-warmed LB was added to the mixture and the suspension incubated at 37°C for 1 hour at 300rpm. The suspension was then plated onto LB-agar containing the appropriate concentration of the appropriate selective antibiotic. Selective plates were incubated at 37°C overnight.

### **2.3.2 Transformation of *L. monocytogenes* by electroporation**

#### **2.3.2 (a) Preparation of *L. monocytogenes* cells for electroporation with plasmid DNA**

This method was carried out and adapted from the method of Park and Stewart (1990). A 20% (v/v) inoculum of an overnight culture of *L. monocytogenes* in brain heart infusion broth with 0.5M sucrose (BHI/s) was added to 500ml sterile BHI/s and incubated at 37°C at 150 rpm until reaching an OD<sub>600nm</sub> of 0.2. Penicillin G was added at a concentration of 10µg/ml, and the culture incubated until reaching an OD<sub>600nm</sub> of 0.4. The cells were harvested by centrifugation at 7000g for 10 minutes and two washing steps in an equal volume of 1mM HEPES (pH 7.0), in 0.5M sucrose. The supernatant was carefully removed and the pellet resuspended in 1/400 (w/v) in HEPES-sucrose. The cells were kept on ice until immediate use.

#### **2.3.2 (b) Electroporation of *L. monocytogenes* with plasmid DNA**

Plasmid DNA (1µg) in 25µl nanopure water was added per 100µl aliquot of competent listerial cells (*ca*  $1 \times 10^{10}$  bacteria) and were left on ice for 1 minute. The mixture was placed in a cold 0.2cm<sup>2</sup> electroporation cuvette and placed into the electrodes of a Gene-Pulser (Bio-Rad). The settings used for electroporation with *L. monocytogenes* were 25µF, 12.5kv/cm<sup>2</sup>, and 200Ohms. The cells were electroporated with a single pulse. The cuvette was immediately placed on ice for 1 minute before addition of 1ml BHI/s and incubation at 37°C for 4 hours. The cells were then plated onto BHI- 1.5%

(w/v) agar containing the appropriate concentration of the appropriate selective antibiotic. Selective plates were incubated at 30°C for 48 hours.

## **2.4 Routine Techniques for DNA manipulation**

### **2.4.1 Agarose gel electrophoresis**

Fragments of DNA were separated according to size by agarose gel electrophoresis (Sambrook *et al.*, 1989). Agarose (Lab M) concentrations used were 0.7% (w/v) for separation of large (>3kb) DNA fragments and 1% (w/v) for separation of smaller (<3kb) DNA fragments. Agarose was solubilised in TAE buffer (pH 7.7, 40mM Tris-acetate, 1mM EDTA) and electrophoresis was carried out in TAE buffer containing ethidium bromide at a concentration of 0.5µg/ml. DNA was mixed with 1/5th (v/v) of 6 x gel-loading buffer (0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol FF, 15% (w/v) Ficoll Type 400 (Pharmacia) in water) before being loaded into the wells of an agarose gel. The conditions of electrophoresis were 65-75 volts for one hour or 15-20 volts overnight. DNA bands were visualised by placing the agarose gel under UV light from a long-wave UV transilluminator. 1kb ladder (GIBCO-BRL, Invitrogen Ltd, Paisley, Scotland) and 50bp ladder (GIBCO-BRL) were used as DNA size markers.

### **2.4.2 DNA Restriction digests**

Restriction endonucleases were used for routine DNA manipulations. They were purchased from GIBCO-BRL, Promega UK and Roche Products Ltd, Welwyn Garden



City, Herts, UK, restriction endonuclease cleavage of DNA was carried out according to the manufacturer's instructions, typically in 20µl reactions with 10 units (1µl) of enzyme and 500ng DNA for 1-2 hours at 37°C.

#### **2.4.3 Ligation of DNA fragments**

T4 DNA ligase was purchased from GIBCO-BRL. And was used in synergy with the ligase buffer supplied by GIBCO-BRL. Ligations were carried out according to manufacturer's instructions with only the ratio of insert to vector changed for different experiments. Ligations were typically carried out at 14°C overnight. Ligase was removed by phenol : chloroform extraction (see section 2.4.4), and the fragment washed by ethanol precipitation (see section 2.4.4) before DNA was transformed.

#### **2.4.4 Phenol : chloroform extraction and ethanol precipitation**

This method was performed as described by Sambrook *et al.*, (1989). Equal volumes of phenol : chloroform (1:1) and phenol (pH 7.5), were added to the DNA and mixed to form an emulsion. The mixture was centrifuged at 13000g in a Microcentaur microfuge, the aqueous phase removed and the process repeated. DNA was then precipitated from the aqueous phase by adding 0.1 volumes of Sodium acetate (pH 5.2) and 2 volumes of ice cold absolute ethanol. The sample was cooled at -20°C for 30 minutes-1 hour and then centrifuged at 13000g. The supernatant was then removed and the DNA pellet was washed with 70% (v/v) ethanol to remove salt, air-dried and resuspended in either TE buffer or nanopure water.

#### **2.4.5 DNA concentration measurement**

DNA concentration was measured by ultraviolet absorbance spectrophotometry according to the method of Brown *et al.* (1990). The amount of DNA in a given solution is proportional to the amount of ultraviolet radiation absorbed by the same solution of DNA. Absorbance is typically measured at OD<sub>260nm</sub>, at which wavelength an absorbance (A<sub>260</sub>) of 1.0 corresponds to 50µg of double-stranded DNA per ml.

#### **2.4.6 Dephosphorylation of DNA termini**

Following complete digestion with a given restriction endonuclease, plasmid DNA (no more than 10pmoles of 5' termini) was treated with 0.1 units of calf intestinal alkaline phosphatase (CIP) (Promega) using the supplied reaction buffer according to manufacturer's instructions, to a final volume of 50µl. Protruding 5' termini were dephosphorylated by incubation at 37°C for 30 minutes. Following this incubation period, a subsequent 0.1 unit of CIP were added to the mixture and the incubation continued for a further 30 minutes at 37°C. This reaction was ceased by phenol : chloroform extraction and cleaned by ethanol precipitation (see section 2.4.4).

## **2.4.7 DNA extraction from agarose gels**

### **2.4.7.1 DNA extraction from agarose gels using Bandprep Kit**

DNA was extracted from agarose gels using a Bandprep kit (ABGene, Epsom, UK). DNA fragments were produced by restriction endonuclease digestion with an appropriate enzyme and separated by agarose gel electrophoresis (see section 2.4.1). The DNA fragment of interest was excised from the gel under a UV transilluminator (UVP, Cambridge, UK) with a scalpel and transferred to a 1.5ml eppendorf tube. The agarose containing the DNA band was dissolved at 65°C for 10 minutes in the Gel solubiliser solution (sodium iodide buffered in Tris-HCl (pH 7.0)), then 5µl Sephaglas BP was added to the solution per µg of DNA. The solution was mixed by gentle flicking of the tube periodically throughout the 5 minute incubation. The mixture was then centrifuged at 13000g for 15 seconds in a Microcentaur microfuge to collect the Sephaglas, and the pellet washed twice in the Wash Solution (buffered salt solution). The resulting Sephaglas pellet was air-dried before eluting in either 20µl TE buffer or 20µl nanopure water. A second elution was performed if deemed necessary. The DNA was then centrifuged at 13000g in a Microcentaur microfuge for 10 minutes to check all Sephaglas had been removed. The DNA was then stored at -20°C ready for use.

### **2.4.7.2 DNA extraction from agarose gels using QIAquick Gel Extraction kit**

The QIAquick Gel Extraction kit (QIAGEN) was used to extract fragments of DNA from agarose gels. DNA fragments were separated by agarose gel electrophoresis (see section 2.4.1). The DNA fragment of interest was excised from the gel under a UV transilluminator with a scalpel and transferred to a 1.5ml eppendorf tube. The agarose containing the DNA band was dissolved at 50°C in the presence of 3 volumes of Buffer QX1. One volume of isopropanol was then added to the mixture before application to a QIAquick elution. The mixture was then centrifuged at 13000g for 1 minute in a Microcentaur microfuge. The eluate was discarded and the column washed with 0.5ml Buffer QX1. Following centrifugation at 13000g for 1 minute, 0.75ml Buffer PE was added to the column and the centrifugation repeated for a further minute. DNA was eluted from the column by addition of either 50µl Tris-HCl (pH 8.5) or 50µl nanopure water. The column was then centrifuged at 13000g in a Microcentaur microfuge for 1 minute and the DNA was then stored at -20°C ready for use.

## **2.5 DNA hybridisation**

### **2.5.1 DNA transferral to nylon filter by the Southern blotting technique**

DNA transfer to nylon filters was carried out according to the method of Southern (1975). DNA fragments were separated by agarose gel electrophoresis (see section 2.4.1) and photographed beside a fluorescent ruler under UV light. The DNA was depurinated by soaking the agarose gel in 250ml of 0.25M hydrochloric acid for 7 to 10 minutes. After a brief rinse in distilled water, the gel was submerged in denaturing

solution (0.5M sodium hydroxide, 1.5M sodium chloride) for 30 minutes with occasional shaking. After rinsing again with distilled water, the gel was submerged in neutralising solution (0.5M Tris-HCl (pH 7.5), 3M sodium chloride) for 30 minutes with occasional shaking. The gel was then placed on three or four sheets of Whatman paper (3mm) pre-soaked in 20 x SSC solution (1 x SSC: 0.15M sodium chloride, 0.015M trisodium citrate (pH 7.0)). A sheet of nylon membrane (Hybond-N+, Amersham International PLC, Little Chalfont, Buckinghamshire, UK), was cut to the exact dimensions of the gel, pre-soaked in 3 x SSC and placed on top of the gel and covered with three or four sheets of pre-soaked (3 x SSC) Whatman paper ensuring removal of any air bubbles. Three further sheets of pre-soaked Whatman paper were placed on top and then covered with a stack of paper towels cut to the same size as the gel. A weight of approximately 500g was applied to the top of the stack of paper towels. The lower sheets were immersed in 20 x SSC and the top sheets changed if required. DNA transfer was allowed to take place over-night, the filter was removed and air-dried. DNA was fixed permanently to the filter by 5 minutes exposure to UV light under a long wave transilluminator. The filter containing fixed DNA could then be wrapped in Saran wrap (DuPont U.K. Ltd, Stevenage Herts) prior to hybridisation.

### **2.5.2 Preparation of radio-labelled probe**

DNA was radiolabelled using the 'Ready To Go kit' (Amersham-Pharmacia) according to manufacturer's instructions. 20µl nanopure water was added to the 'Ready to Go dCTP labelling kit tube' (RTG), this was then left at room temperature for 30 minutes to hydrate. Approximately 50ng of DNA in a volume of 20µl was denatured by boiling in a water bath for 5 minutes. The DNA was then snap cooled on ice for 5

minutes. The DNA was briefly centrifuged at 13000g for 10 seconds before being added to RTG tube. 2-3 $\mu$ l of [ $\alpha$ -<sup>32</sup>P]dCTP (10 $\mu$ Ci, specific activity 3000Ci/mmol) (Amersham plc) were added to the RTG tube. The mixture was placed in a water bath at 37°C overnight for labelling. Prior to use the labelled probe was boiled for 5 minutes in a heat block and snap cooled on ice for 2-3 minutes.

### **2.5.3 Prehybridisation and hybridisation of radiolabelled probe to target DNA on nylon filter**

Prehybridisation was performed by incubation of Southern blot filters under gentle agitation for 4-6 hours at 65°C in the presence of 25ml prehybridisation solution (6 x SSC, 5 X Denhardt's solution (50 x Denhardt's solution is 1% (w/v) Ficoll Type 400, 1% (w/v) BSA, 1% (w/v) polyvinylpyrrolidone), 0.1% (w/v) SDS and 200 $\mu$ g/ml denatured salmon sperm DNA. The radiolabelled DNA probe was boiled for 5 minutes, snap cooled on ice for 2-3 minutes and then added to the hybridisation solution. Hybridisation was performed at 65°C overnight with constant, gentle agitation. Following hybridisation the nylon filter was washed twice for 10 minutes at 65°C in a washing solution (2 x SSC, 1% (w/v) SDS (1:1)), and the wash solutions discarded. The filter was then further washed in 1 x SSC at 65°C for 10 minutes before the filter was removed, wrapped in Saran wrap and placed in a cassette where it was covered by Cronex X-ray film (DuPont). The filter was left in contact with the X-ray film for 8-72 hours at -70°C before the film could be developed using an automatic film processor (Agfa-Geveart, Brentford, Middlesex, UK).

#### **2.5.4 Removal of bound probe from a filter**

Radiolabelled probes could be stripped from filters that needed to be re-probed by the following method. Filters were rinsed in 5 x SSC for 2 minutes. A boiling solution of 0.1% (w/v) SDS was added to the filters containing approximately 5ml SDS per cm<sup>2</sup> of filter and the filter was incubated in the solution at room temperature for 15 minutes with gentle agitation. The process was repeated a further two times using fresh SDS. Filters could then be re-prehybridised, hybridised and probed as previously described (see section 2.5.3).

#### **2.6 The Polymerase Chain Reaction**

Polymerase Chain Reactions (PCR) were typically carried out in 100µl volumes, using approximately 100ng of template chromosomal DNA, primers at a concentration of 20 pmol and each deoxynucleotide at 200µM. PCR reaction buffer (1x) was 20mM Tris-HCl (pH 8.8), 10mM Potassium chloride, 0.1% (v/v) Triton®X-100, 1mM Magnesium sulphate, 6mM ammonium sulphate and 0.3 mg/ml Bovine serum albumin. *Pfu* DNA polymerase (Promega) was added at 2.5 units per 100µl reaction, and the reactions spun down for a few seconds at 13000g in a bench Microcentaur microfuge. Reactions were overlaid with 100µl mineral oil (Molecular biology grade, Sigma; oil was cleared of contaminating DNA by exposure to UV light for 15 minutes prior to addition of tube) and the reaction was performed in a OmniGene Thermal cycler (Hybaid, Ashford, Middlesex, UK). Cycle parameters carried out were as follows:

1 cycle	denaturing	95°C for 2 minutes
30 cycles	denaturing	95°C for 45 seconds
	annealing <sup>1</sup>	55°C for 1 minute
	elongation <sup>2</sup>	72°C for 5 minutes
1 cycle	elongation	72°C for 10 minutes

<sup>1</sup>annealing temperature selected was dependent on the melting temperature of the oligonucleotide primers used in the reaction.

<sup>2</sup>Elongation temperature selected was dependent on the length (kb) of the PCR product desired.

After the PCR reaction, the reaction products were removed from the Thermal cycler and stored at -20°C. PCR products were analysed by agarose gel electrophoresis (see section 2.4.1). When cloning into pGEM-T-Easy cloning vector, PCR products produced using *Pfu* DNA polymerase were first A-tailed with taq DNA polymerase (Promega) for 30 minutes at 72°C.

#### **2.6.1 PCR Primers used for amplification of ORF1 (see Section 3.10)**

PCR primers designed for the PCR experiments based on the sequence flanking the gene (ORF1 in Section 3.10) inactivated by Tn917-LTV3 in mutant B380. Sequences underlined in bold are the *Bam*HI and *Sma*I restriction sites in primers MBBam and MBSma respectively.



Name	Primer Sequence	Size (bp)
MBBam	5' AC <u>GGATCCT</u> GCCACGGGGATACTGATGTCAC 3'	31
MBSma	5' CCC <u>CCCGGGG</u> CGTTATGGTTGCCCCGTGAC 3'	30

## 2.7 DNA sequencing

### 2.7.1 Automated DNA sequencing

Automated DNA sequencing was performed using the ABI PRISM Dye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA) in synergy with an Applied Biosystems Model 373A DNA sequencing system. The kit comprises of dye-labelled dideoxynucleotides which, when incorporated, terminate extending chains to produce products that are labelled by dye. The kit also comprises deoxynucleotides with dITP in place of dGTP to minimise band compressions and AmpliTaq DNA polymerase. AmpliTaq DNA polymerase FS is a mutant *Taq* DNA polymerase absent in 5' to 3' nuclease activity and has greatly reduced discrimination for dideoxynucleotides. Reaction products are analysed colourimetrically on a 373A Sequencing Work Station. Sequencing reactions were carried out using approximately 400ng of double stranded plasmid DNA isolated from QIAquick QIAGEN columns (see section 2.4.7.2). Plasmid DNA was mixed in solution with 3.2pmole of primer, 8µl Terminator Ready Reaction Mix (containing 4 dye labelled dideoxynucleotides, dATP, dCTP, dITP, dTTP, Tris-HCl (pH 9.0), Magnesium chloride, thermo-stable pyrophosphate and AmpliTaq DNA polymerase) in a total volume of 20µl. Reactions

were done in duplicate, overlaid with 40µl of mineral oil and placed in a HYBAID OmniGene Thermal cycler under the following conditions:

#### Denaturation

Rapid thermal ramp to 96°C

Hold 96°C for 30 seconds

#### Annealing

Rapid thermal ramp to 50°C

Hold 50°C for 15 seconds

#### Extension

Rapid thermal ramp to 60°C

Hold 60°C for 4 minutes

Repeated for 25 cycles.

Following the reaction, the samples were cleaned by ethanol precipitation to remove excess of unincorporated terminators. The 20µl reactions were transferred to fresh 1.5ml eppendorf tubes containing 0.1 volumes 3M sodium acetate (pH 5.2) and 2.5 volumes 95% (v/v) ethanol. After mixing the mixture was cooled on ice for 10 minutes before centrifugation in a Microcentaur bench microfuge at 13000g for 20 minutes. The ethanol supernatant was removed and the pellet washed in 250µl 70% (v/v) ethanol. Following centrifugation at 13000g for 20 minutes the ethanol was discarded and the pellet air-dried and resuspended in 10µl water. Reaction products

were analysed on an ABI Model 373A DNA sequencer by Dr. Katherine Lilly at the Protein and Nucleic Acid Sequencing Laboratory, University of Leicester. Sequence data was analysed using the GeneTool program supplied by (BioTools Ltd, Edmonton, Canada), the programs BLASTN and BLASTX (National Centre of Biotechnology Information, Los Alamos, North Mexico) available on the internet (<http://www.bio.cam.ac.uk/seqsrch/blast.html>) and also Dr Pierre Dehoux (Institut Pasteur, Paris, France).

### 2.7.2 List of primers used for sequence analysis

The following is a list of primers designed for sequencing from pMB3 after sequence analysis using commercial primers T7 and SP6 (see section 3.10.2).

Name	Primer Sequence	Size (bp)
Primer1	5' TGGGGTCACGGAAGGTGCGATTC' 3'	23
Primer2	5' GGCATCGGCGTCCTTCCGGCTTTTTTTTAC 3'	29
Primer3	5' CGGGACCGGAAATGCCTTTCGGAAA 3'	25
Primer4	5' CTGTTTTTGATAGGTCATTTCGG 3'	24

## 2.8 Insertional Mutagenesis

### 2.8.1 Transposition of Tn917-LTV3

*L. monocytogenes* transposon mutants were generated in culture by the method of Camilli *et al.*, (1990). A single colony of *L. monocytogenes* DP-L910 (containing

pLTV3, see Figure 2.1) was taken from a plate containing selective levels of erythromycin, tetracycline and lincomycin, and inoculated into Brain Heart Infusion (BHI) (OXOID, Basingstoke, Hants, UK) containing the same levels of the same three antibiotics. The bacterial culture was grown to stationary phase overnight at 30°C. This overnight culture was diluted 1/800 into sterile BHI containing the same selective levels of only erythromycin and lincomycin. *L. monocytogenes* colonies containing the chromosomal insertions were selected by incubation at 41°C with shaking until stationary phase (equating to an OD<sub>260nm</sub> of 2.0). The transposon insertion library was then aliquoted in 5ml of culture containing 25% (v/v) glycerol as cryoprotectant and stored at -70°C. These aliquots are subsequently referred to as the transposon insertion library of *L. monocytogenes*.

### **2.8.2 Determination of transposition frequency**

Single *L. monocytogenes* DP-L910 colonies were picked from overnight LB-agar plates containing erythromycin, tetracycline and lincomycin grown at 30°C and were used to inoculate 10ml of LB broth containing the above antibiotics. This culture was grown to an OD<sub>600nm</sub> of approximately 0.4. The overnight culture was serially diluted in sterile distilled water and dilutions were plated in triplicate onto selective agar containing erythromycin and lincomycin. The plasmid, conferring resistance to tetracycline, does not replicate at temperatures above 37°C since it is a highly temperature-sensitive derivative of vector pE194Ts (Villafane *et al.*, 1987). Therefore the number of colony forming units (CFU) present at the permissive (30°C) and the non-permissive (41°C) temperatures for plasmid replication were calculated to attain

the transformation frequency. Any colonies surviving incubation at 41°C, (the non-permissive threshold for plasmid replication) which had the phenotypes Em<sup>r</sup>, Lm<sup>r</sup> (lincomycin resistance) and Tc<sup>S</sup> (tetracycline sensitive) were assumed to have acquired a chromosomal insertion of Tn917-LTV3 and had lost the plasmid portion of Tn917-LTV3.

Transposition frequency was determined by dividing the number of CFU present at the non-permissive temperature by those at the permissive temperature. This was performed in triplicate.

## 2.9 Alpha-Mannosidase Assay

An overnight culture of bacteria (250µl) was used to inoculate 10ml of fresh Tryptone Soya Broth and the bacteria were grown at 30°C to an OD<sub>600nm</sub> of 0.4. The bacteria were harvested at 6000g for 15 minutes at 4°C and the resulting pellet was washed in 0.5ml of sodium phosphate buffer (150mM, pH 7.5). The cell pellet was then resuspended in 300µl of sodium phosphate at the same pH and concentration. The cell suspension was lysed by sonication in a bath sonicator (Kerry Ultrasonics Ltd, Hitchin, UK) for 15 minutes. 0.4ml *para*-nitrophenyl-*alpha*-D-mannopyranoside (250µM) (Sigma) in Sodium phosphate (50mM, pH 5) (Sigma) was added to 10 tubes and warmed to 37°C in a water bath. Volumes of cell lysate were added to the substrate and the total volume standardised to 500µl with distilled water. The reaction was incubated at 37°C in a water bath for 5 minutes and then stopped by adding 0.6ml Sodium carbonate (1M, pH 10.4). The absorbance was determined at OD<sub>400nm</sub>.

## **2.10 Bradford Protein Assay**

A BioRad Bradford protein assay was carried out according to manufacturers instructions. The dye reagent was prepared by diluting 1 part Dye Reagent Concentrate with 4 parts distilled, deionised water. This was filtered to remove particles. Three to five dilutions of a protein standard representative of the protein solution to be used were prepared. The range of these dilutions for BSA was 1.2-10 µg/ml. 800 µl of each standard and sample were pipetted into a clean test tube. This was done in duplicate. 200µl of the reagent concentrate was added to each tube and vortexed. The solution was then left to incubate at room temperature for 15 minutes. The absorbance was then measured at OD<sub>595nm</sub>.

## **2.11 Adhesion of *L. monocytogenes* to food processing surfaces**

The adhesion assay was designed to compare the ability of *L. monocytogenes* to attach to materials commonly found in a food-processing environment. Coupons of each material used in this investigation were degreased and sterilised prior to use. The adhesion assay was designed on several adhesion assays used by other groups in the research of bacterial attachment to surfaces (Mafu *et al.*, 1990; Oh and Marshal, 1995; Lindsay and Von Holy, 1997; Norwood and Gilmour, 1999).

### **2.11.1 Preparation of coupons**

The materials tested are listed in table 2.3. All test materials were cut into flat coupons (0.8cm x 0.8cm) from sheets, the thickness of which is also shown in table 2.3.

Coupons were placed in a sterile universal tube (8 coupons per tube) containing 10ml (v/v) Teepol solution. These tubes were then sonicated for 15 minutes in a bath sonicator (Kerry Ultrasonics Ltd, Hitchin, UK). After sonication, the coupons were washed in 20ml sterile distilled water, transferred to a glass universal tube and autoclaved at 121°C for 15 minutes.

### **2.11.2 Giemsa's Solution Staining Assay**

Cultures of *L. monocytogenes* were prepared according to section 2.1.2. Glass coverslips (Chance Propper, West Middlesex, UK) were prepared for the adhesion assay according to the protocol in section 2.11.1. Each glass coverslip was transferred aseptically to a separate well of a 24 well 'nuncion' plate (Nunc GMBH, Weisbaden, Germany). An aliquot of culture (1ml) was then pipetted into each well of the nuncion plate. The glass coverslips were immersed in culture for a period of 2 hours at 30°C. The wells of the plates were then drained of culture and washed with a further 1ml of sterile 20% (v/v) TSB. This fresh media was drained and then 1ml of methanol was added to each well. After 10 minutes, 1ml of Giemsa's solution was added to each well and the incubation continued for a further 10 minutes. The wells were finally washed with distilled water. The extent of adhesion was determined by sight according to the intensity of the purple staining: an intense colour indicated prolific attachment. Transposon mutants were designated a letter according to which batch of the mutant library they were isolated from, and then numerically within each batch. For example, mutant B380 Was the 380<sup>th</sup> mutant isolated from batch B of the mutant library. The term batch refers to a separate container containing bacterial culture.

**Table 2.3 Materials**

Material	Sheet Thickness (mm)	Supplier
Stainless steel 304 (2B finish)	0.78	Ulbrich UK Ltd, Sheffield, UK
Stainless steel 304 (2B finish sand blasted)	0.80	“
Stainless steel 430 (bright annealed)	0.50	“
Stainless steel 316 (bright annealed)	0.30	“
Aluminium	0.54	Aalco UK Ltd, Nottingham, UK
Polycarbonate	1.05	Adtech Polymer Engineering Ltd, Stroud, UK
Polypropylene	1.20	“
Polyurethane	0.75	“
Polyvinylchloride	1.10	“
PETG	1.00	“
PTFE	1.60	“
Lexan	1.00	Cadillac Plastics Ltd, Swindon, UK
Nitryl Rubber	1.58	Martin’s Rubber Co. Ltd Southampton, UK
Silicone Rubber	1.10	“
Natural White Rubber	1.37	“
Natural White Rubber Type CNA-70	1.70	“
EPDM Rubber	1.88	“
Glass coverslips	TBA	“



### **2.11.3 Quantitative Adhesion assay - detachment of *L. monocytogenes* from coupons**

Coupons and cultures were prepared according to the protocols in section 2.11.1 and 2.1.2. respectively. Aliquots of cell suspension (10ml) were dispensed into universal tubes equilibrated to 30°C and the coupons immersed directly in the culture. For short contact experiments, coupons were completely immersed in the culture, and then immediately withdrawn using forceps. For all other experiments, the coupons were incubated statically at 30°C for 2 hours.

Following incubation, the coupons were removed from the universal tubes using sterile forceps to hold the coupon by two edges. Each coupon was touched lightly against the side of the tube to remove as much adherent liquid as possible, before transferral to a Petri-dish containing 10ml sterile phosphate-buffered saline solution (PBS). The dish was swirled twice gently and the coupon removed with sterile forceps. The coupon was then gently placed in 10ml sterile 20% (w/v) TSB in a sterile plastic universal tube for sonication in a bath sonicator for 1 minute. For enumeration of bacteria, 20µl of cell suspension was serially-diluted in PBS and plated on LA. The Miles and Misra (1938) plating technique was employed, with six 20µl aliquots of sample being deposited onto the surface of the agar using a micropipette. All samples were plated in replicates of four. Plates prepared in this way were incubated overnight at 30°C. Only dilutions which resulted in counts of between 30 and 150 colonies were enumerated.

#### **2.11.4 Estimation of mass of liquid adhering to coupons**

Sterile coupons were immersed in the planktonic culture and removed immediately. The coupons were then placed onto pre-weighed 90mm Whatman filter paper discs ('School grade', Fisher Scientific, Loughborough, UK), allowing any adherent medium to soak into the paper. The papers were then weighed to determine the mass of liquid transferred.

#### **2.11.5 Effects of handling coupons with forceps**

The protocol was carried out as stipulated in section 2.11.4 with some adjustments. One set of experimental replicates were removed from the planktonic culture gripping the coupon by its corners and another set of replicates were removed from the planktonic culture by gripping the coupon by its edges. The coupons were then placed onto pre-weighed 90mm Whatman filter paper discs, allowing any adherent medium to soak into the paper. The papers were then weighed to determine the mass of liquid transferred.

#### **2.11.6 Comparison of adherent cell removal techniques**

The protocol was performed as in section 2.11.3 with adjustments. Following 2 hours incubation in culture at 30°C the coupons were removed from the universal tubes using sterile forceps to hold the coupon by two edges. Each coupon was touched lightly against the side of the tube to remove as much as possible, before transferral to a Petri-dish containing 10ml sterile phosphate-buffered saline solution (PBS). The dish was

swirled twice gently and the coupon removed with sterile forceps. The coupon was then gently placed in 10ml sterile 20% (w/v) TSB in a sterile plastic universal tube. One set of experimental replicates was then sonicated in a bath sonicator for 1 minute as before, another set of replicates was vortexed for 1 minute on a bench vortex, and a third set of replicates was vortexed on a bench vortex for 1 minute in the presence of 5mm diameter glass beads (Fisher Scientific, Loughborough, Leicestershire, UK). Enumeration of bacteria was then performed as described in section 2.11.3.

## **2.12 Scanning Electron Microscopy**

### **2.12.1 Sample Fixation and Dehydration**

Electron microscopy was performed on coupons used for the adhesion assay (see section 2.11). Coupons were prepared as stipulated in section 2.11 and prepared for S.E.M. following a 2 hour contact time with *L. monocytogenes* culture. The sample was fixed in 4% (v/v) glutaraldehyde (in distilled water). The sample was allowed to fix at room temperature for 3 hours. The fixative was then washed with three fresh changes of distilled water leaving the sample in each change of water for 10 minutes. The fixed sample was then placed in 1% (v/v) osmium tetroxide in distilled water. It was then left for 90 minutes at room temperature. The fixative was then washed off with three changes of distilled water, leaving the sample in each change of water for 10 minutes. Following fixation, the sample was dehydrated through a series of acetone changes from 30% to 50% to 70% 90% to 100% acetone. The sample was left in each acetone change for a minimum of 15 minutes. The sample was then dehydrated in a further two changes of 100% acetone, for 30 minutes each time. Samples were then

transferred to containers, still immersed in 100% acetone for the critical point drying procedure.

### **2.12.2 Critical Point Drying Procedure**

Fixed and dehydrated samples were loaded into the chamber of the critical point dryer (CPD) whilst still immersed in 100% acetone in the sample holders. The cap of the chamber was firmly screwed down into place. All checks were carried out and the controls were set on the CPD. The chamber was filled with liquid carbon dioxide from the gas cylinder and the critical point drying process performed. When completed the chamber was allowed to come back to atmospheric pressure and the samples removed from the chamber ready for processing through the sputter coater.

### **2.12.3 Sputter Coating and viewing of samples under S.E.M.**

When the critical point drying process was complete the sample holders were removed from the chamber and the samples were mounted onto stubs with sticky tabs. The stubs were transferred to the stage of the Sputter Coating Unit (SCU) in the carrier. The implosion shield around the glass wall was replaced and the lid of the chamber closed making sure that the rubber 'O' ring sat on the glass wall evenly on all sides. The SCU was turned on as was the water flow and return supply. The argon gas supply control above the vacuum coating unit was turned on. The directions were followed according to the manufacturer's instructions and the coating procedure completed automatically. Once the samples were back at atmospheric pressure the

chamber was opened and the samples removed. Samples were then ready to be observed under a Scanning Electron Microscope.

### **2.13 The API test for identification of *Listeria***

The API system (Biomérieux) for *Listeria* was used to investigate the biochemical profile of the transposon insertion mutant and compare it to the wild type bacterium. The kit consists of 10 microtubes containing dehydrated substrates enabling the performance of enzymatic tests and sugar fermentations. The results of each reaction are revealed either by spontaneous colour production or by addition of subsequent reagents (DIM test). Following 24 hours incubation at 30°C, the reactions are read visually and compared to the identification table in the manual.

**Table 2.4 The API *Listeria* strip test**

<b>Tests</b>	<b>How to read results</b>	
	<b>Positive</b>	<b>Negative</b>
DIM	Orange	Pale orange, pink-beige, grey-beige
ESC	Black	Pale Yellow
$\alpha$ MAN	Yellow	Colourless
DARL	Yellow, Yellow-Orange	Orange-red, red
XYL	Yellow, Yellow-Orange	Orange-red, red
RHA	Yellow, Yellow-Orange	Orange-red, red
MDG	Yellow, Yellow-Orange	Orange-red, red
RIB	Yellow, Yellow-Orange	Orange-red, red
G1P	Yellow, Yellow-Orange	Orange-red, red
TAG	Yellow, Yellow-Orange	Orange-red, red

**2.14 Statistical analysis**

Data were analysed using one way analysis of variance (ANOVA) and the Tukey-Kramer multiple comparison test commercial software Instat (Instat Biostatistics: <http://www.graphpad.com/instat3/instat.htm>).

## **Chapter 3: Results**

### **3.1 Transformation of *E.coli* strains with pLTV3**

One aim of this project was to identify chromosomal genes involved in the attachment of *L. monocytogenes* 10403S to materials commonly found in a food-processing environment. To achieve this, transposon mutagenesis using a derivative of Tn917, Tn917-LTV3, carried on plasmid pLTV3 (Camilli *et al.*, 1990) was used for selection of mutant strains of *L. monocytogenes* that attached to materials in lower frequencies than the wild type bacterium following a two hour incubation period. Plasmid pLTV3 (500ng) was transformed into eletrocompetent *E. coli* HB101 and selection was performed on agar plates. Camilli *et al.* (1990) demonstrated that *erm*-containing fragments recovered from plasmids pTV20 or pTV21-derived integrants could not be maintained in a standard *E. coli* host. Although they were not able to exactly determine why this was the case, it was speculated that the *erm* gene product may have a toxic effect on *E. coli* strains due to its ability to methylate ribosomal RNA. *E. coli* Strain HB101 contains *rpsL20* mutation, which alters the ribosome in a way that confers streptomycin resistance.

### **3.2 Transposition of Tn917-LTV3 in *L. monocytogenes* 10403S**

Transposition of Tn917-LTV3 into *L. monocytogenes* 10403S was carried out according to the method of Camilli *et al.* (1990) using the temperature sensitive plasmid pLTV3. The plasmid does not replicate at temperatures above 37°C since it is a highly temperature-sensitive derivative of vector pE194Ts (Villafane *et al.*, 1987).

The plasmid also contains a copy of Tn917 conferring inducible Em<sup>r</sup> (erythromycin resistance), a promoterless copy of the *E. coli lacZ* gene positioned that insertions into chromosomal genes can generate transcriptional *lacZ* fusions, the kanamycin resistance gene (*neo*) selectable in *E. coli*, the chloramphenicol resistance gene (*cat*), the lincomycin (*ble*) and the tetracycline resistance gene (*tet*) selectable in *Listeria*, ColE1 replication functions, an M13 origin of replication, and a cluster of polylinker cloning sites which facilitate the recovery in *E. coli* of chromosomal DNA adjacent to sites of insertion (Camilli *et al.*, 1990). Any colonies surviving incubation at 41 °C, (the non-permissive threshold for plasmid replication) which had the phenotypes Em<sup>r</sup>, Lm<sup>r</sup> (lincomycin resistance) and Tc<sup>s</sup> (tetracycline sensitive) were assumed to have acquired a chromosomal insertion of Tn917-LTV3 and had lost the plasmid portion of Tn917-LTV3.

### 3.3 Adhesion assay design.

An early objective of the investigation was to devise an assay for determining the numbers of *L. monocytogenes* cells attaching to materials selected here as representative of those commonly found in the food-processing environment. It was decided that the assay was to be conducted by immersing coupons in listerial cultures (see section 2.11.3). This would allow large numbers of replicates to be generated in a short timespan. Following incubation, the coupons were removed from the universal tubes using sterile forceps. Each coupon was touched lightly against the side of the tube to remove as much liquid as possible before washing in PBS. Viable counts were made before and following cell removal by sonication. This procedure

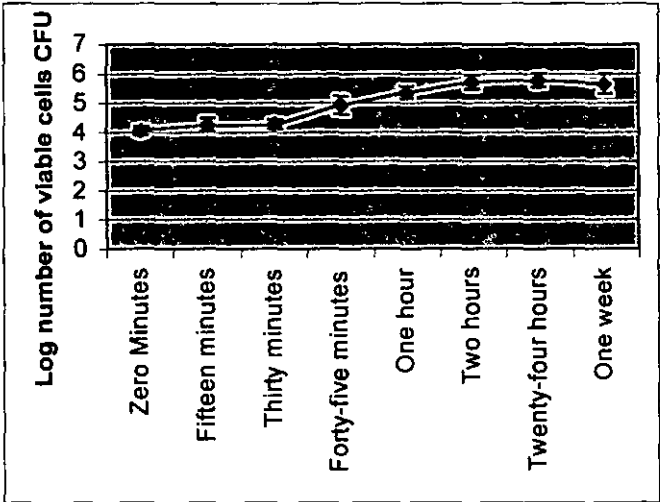


was used to investigate the attachment of wild type *L. monocytogenes* 10403S to 18 different materials used in the food industry.

Tukey-Kramer multiple comparisons tests were undertaken to evaluate the results obtained statistically. In comparing data, differences between results were assigned a 'P value', the probability of the difference having occurred by chance. A non-significant (NS) result was defined here as one in which  $P > 0.05$ . Levels of significance were graded in the tables and figures that follow thus: one star (\*  $P < 0.05$ ), two star (\*\*  $P < 0.01$ ), or three star (\*\*\*)  $P < 0.001$ , the latter being the most significant (1 in a thousand).

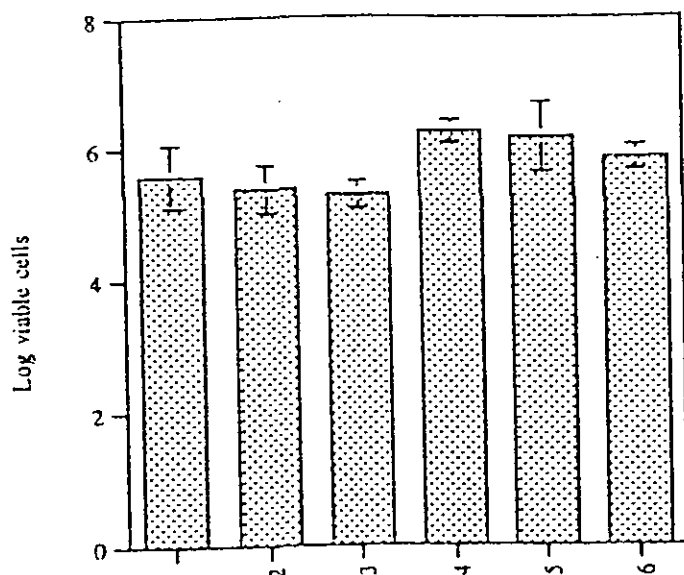
Several factors had to be considered during construction of the adhesion assay. One important consideration was the length of time that each material was to be immersed in bacterial culture. Preliminary assays were conducted by immersing coupons of stainless steel 304 into cultures of wild type 10403S for various lengths of time. These time periods varied from short contact (during which the coupon was simply immersed and immediately withdrawn from the culture) to times of 15 minutes, 30 minutes, 45 minutes, 1 hour, 2 hours, 4 hours, 12 hours, 24 hours and 1 week (see Figure 3.1). There was found to be no significant difference in the number of cells adhering to the coupons after 2 hours. There was a significant difference between the numbers of cells attaching to stainless steel 304 at 2 hours ( $P < 0.001$ ) and all shorter contact periods. Hence, a contact time of 2 hours was selected for the adhesion assay.

Figure 3.1 Attachment of *L. monocytogenes* to stainless steel 304 over time



Tests conducted to determine the best method of recovering cells from the coupons revealed that there was no significant difference in the number of cells removed between the techniques used (see Figure 3.2). These tests were carried out on *L. monocytogenes* 10403S and also strain Scott A as a control (Graham *et al.*, 1997). These were sonication, vortexing or vortexing with glass beads. This finding is in accordance with the results of Lindsay and Von Holy (1997). Sonication was therefore employed in this work due to speed and the ease of manipulations.

Figure 3.2 Comparison of methods of attached cell removal



- 1- Sonication of 10403s
- 2- Vortexing of 10403s
- 3- Vortexing (beads) of 10403s
- 4- Sonication of Scott A
- 5- Vortexing of Scott A
- 6- Vortexing (beads) of Scott A

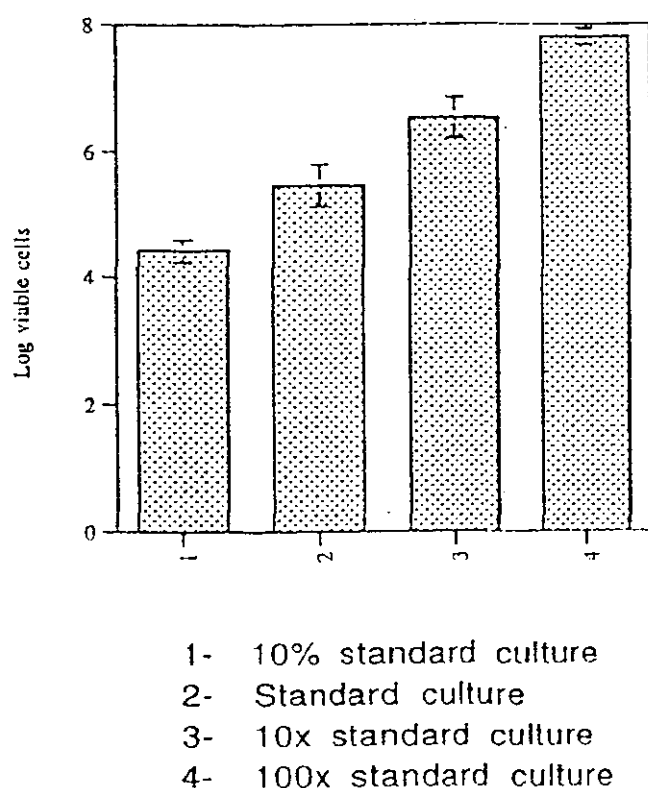
To limit liquid carry over, the washing of coupons with PBS and drainage on filter paper was compared because both methods are widely used in quantitative analysis of bacterial adherence to coupons (e.g. Mafu *et al.*, 1991; Lindsay and Von Holy, 1997). There was no significant difference ( $P>0.05$ ) in the carry over between these two methods of drainage. On average only 4.9 $\mu$ l of liquid were transferred by this method of handling the coupons (about three times the volume is transferred if the coupon is held by the corners). The coupon material did not significantly affect the volume transferred, thus, carried-over *Listeria* make up only a small fraction of the cells seen in the planktonic phase (in the worse case only about 690 cells from populations

numbering in the order of  $10^8$ ). As Table 3.1 shows, the number of cells shed following washing can represent a substantial fraction of the total population, in some cases as much as 54% of the total number of cells from the coupon.

An experiment was devised whereby coupons were carefully removed from culture following two hours and a fresh coupon introduced to the same culture. This was performed to test the hypothesis that the lack of difference in adhesion of cells between 2 hours and 24 hours could be attributed to a subpopulation of cells responsible for adhesion. The term subpopulation is defined here as a minority of cells being responsible to attach materials. The hypothesis was that once this subpopulation of cells had attached to the material all that would remain would be planktonic cells. Hence on addition of a subsequent coupon no attachment would occur. There was found to be no significant difference from the numbers of cells adhering to the first, second or third generation of coupons introduced to the culture (data not shown). This result suggests a subpopulation of cells present in a bacterial culture is not responsible for attachment.

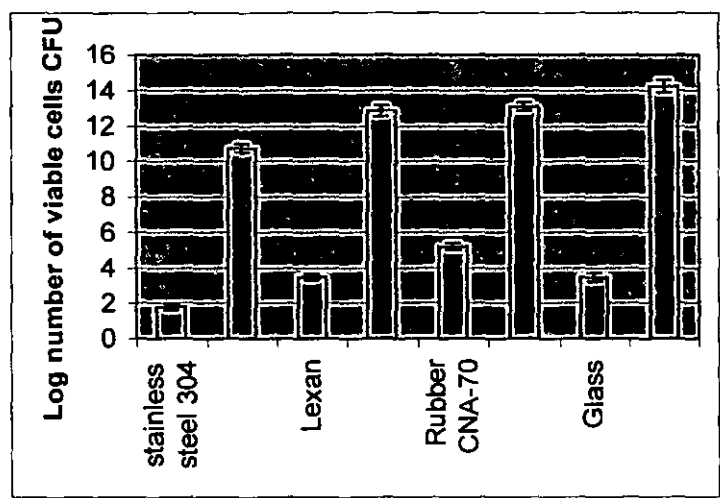
An experiment by which stainless steel coupons were immersed in diluted and concentrated volumes of bacterial culture showed that the number of cells attaching to materials increases linearly with planktonic cell concentration (see Figure 3.3). 'Standard' culture refers to the concentration of cells present at an  $OD_{600nm}$  of between 0.15 and 0.2 (see section 2.1.2).

**Figure 3.3 Increasing *L. monocytogenes* planktonic cell concentration alters the number of cells attaching to stainless steel 304**



Experimentation into the handling of coupons by forceps revealed that the manner in which the coupon is used to grip the coupon significantly influences the amount of cells carried over on an adherent droplet of water attached to the coupon. The method chosen here was to remove coupons by the edges so as to minimise carry over (Figure 3.4). Lifting the coupon by its edges minimises the meniscus of liquid carried between the angle of coupon edge and forcep. Lifting the coupon by its corner carries over a significant number of planktonic cells on adherent droplets of media.

**Figure 3.4 Numbers of cells carried over on adherent media as compared between two methods of forceps handling**



The First of each pair of results denotes removal of forceps by their edges.

The second of each pair of results denotes removal of forceps by their corners.

### 3.4 Attachment of *L. monocytogenes* to materials

The quantitative adhesion assay was designed to estimate the numbers of *L. monocytogenes* adhering to 18 commonly used food-processing materials. Adhesion was measured after a ‘short’ contact time and after two hours. In this context, a short contact time was achieved when coupons were introduced into cultures of *L. monocytogenes* and then immediately withdrawn.

The total number of *Listeria* associated with each material after a short contact time is shown in Table 3.1 (column 2). No significant difference was observed ( $P>0.05$ ). The numbers in this column are made up of cells strongly adherent, those that readily

detach into a planktonic population and those carried over in liquid adhering to the coupon. There was no significant difference between the number of *Listeria* weakly or strongly adhering to the different materials after a short contact time (Table 3.1, columns 3 and 4 respectively).

Table 3.2 shows the number of cells adhering to all materials following a contact time of two hours. There was no significant difference ( $P>0.05$ ) in the total number of *Listeria* adhering to each material, but for each material the number of adherent cells was greater after two hours contact time than after short contact time ( $P<0.05$ ). Also, the percentage of the cells that were weakly adherent fell over two hours. When the number of weakly adhering cells (those shed into the PBS) was subtracted from the 'total' number, the numbers adhering to polypropylene was found to be significantly lower ( $P<0.05$ ) than for the other materials. Indeed, the number of cells strongly adhering to this material after a two hour contact period did not differ significantly from those obtained following only short contact.

**Table 3.1 Number of *L. monocytogenes* cells associated with coupons after short contact time**      \*Data normalised to a 0.8 x 0.8cm coupon of zero thickness. †

Column 2 minus Column 3. Data are the mean of four replicates.

<b><u>Material</u></b>	<b><u>Total</u> <u>number of</u> <u>coupon-</u> <u>associated</u> <u>cells (mean</u> <u>log<sub>10</sub> ±</u> <u>S.D.)*</u></b>	<b><u>Number of cells</u> <u>shed from</u> <u>coupon following</u> <u>washing (mean</u> <u>log<sub>10</sub> ± S.D.)*</u></b>	<b><u>Number of</u> <u>strongly</u> <u>adhered cells</u> <u>(mean log<sub>10</sub> ±</u> <u>S.D.)*†</u></b>	<b><u>% of total</u> <u>coupon-</u> <u>associated</u> <u>listeria shed</u> <u>during</u> <u>washing</u></b>
Polypropylene	4.44 ± 0.22	3.97 ± 0.36	4.25 ± 0.16	35
Polycarbonate	4.70 ± 0.45	4.31 ± 0.58	4.46 ± 0.39	43
Polyurethane	4.34 ± 0.09	3.79 ± 0.14	4.21 ± 0.10	29
PETG	4.49 ± 0.29	4.10 ± 0.34	4.25 ± 0.27	42
PVC	4.37 ± 0.68	3.75 ± 0.82	4.24 ± 0.65	26
PTFE	4.30 ± 0.44	3.79 ± 0.54	4.13 ± 0.39	32
Lexan	4.29 ± 0.24	3.88 ± 0.26	4.08 ± 0.26	39
EPDM	4.35 ± 0.42	4.04 ± 0.47	4.00 ± 0.58	52
Silicone Rubber	4.67 ± 0.42	4.26 ± 0.48	4.44 ± 0.39	39
Nitryl Rubber	4.42 ± 0.29	3.82 ± 0.34	4.28 ± 0.27	26
Natural white Rubber	3.78 ± 0.44	3.09 ± 0.51	3.67 ± 0.43	22
Rubber CNA-70	4.51 ± 0.22	4.04 ± 0.34	4.32 ± 0.19	35
Stainless steel 304	4.02 ± 0.68	3.51 ± 0.95	3.80 ± 0.54	36
Stainless steel 304sb	5.04 ± 0.35	4.69 ± 0.10	4.78 ± 0.38	45
Stainless steel 316	4.01 ± 0.32	3.54 ± 0.05	3.76 ± 0.54	39
Stainless steel 430	4.48 ± 0.28	4.12 ± 0.33	4.22 ± 0.24	45
Aluminium	4.65 ± 0.33	4.34 ± 0.32	4.33 ± 0.39	51
Glass	4.47 ± 0.31	4.41 ± 0.15	4.34 ± 0.38	54



**Table 3.2    Number of *L. monocytogenes* cells associated with coupons after two hours contact time**

\*Data normalised to a 0.8 x 0.8cm coupon of zero thickness. † Column 2 minus Column 3. Data are the mean of four replicates.

<u>Material</u>	<u>Total</u> <u>number of</u> <u>coupon-</u> <u>associated</u> <u>cells (mean</u> <u>log<sub>10</sub> ±</u> <u>S.D.)*</u>	<u>Number of cells</u> <u>shed from</u> <u>coupon following</u> <u>washing (mean</u> <u>log<sub>10</sub> ± S.D.)*</u>	<u>Number of</u> <u>strongly</u> <u>adhered cells</u> <u>(mean log<sub>10</sub></u> <u>± S.D.)*†</u>	<u>% of total</u> <u>coupon-</u> <u>associated</u> <u>listeria shed</u> <u>during</u> <u>washing</u>
Polypropylene	4.97 ± 0.33	4.35 ± 0.42	4.58 ± 0.19	27
Polycarbonate	5.79 ± 0.27	5.18 ± 0.28	5.48 ± 0.26	25
Polyurethane	6.04 ± 0.27	5.07 ± 0.55	5.90 ± 0.21	13
PETG	5.56 ± 0.33	4.54 ± 0.49	5.45 ± 0.26	10
PVC	6.38 ± 0.40	4.94 ± 0.15	6.32 ± 0.40	6
PTFE	6.35 ± 0.15	5.48 ± 0.13	6.17 ± 0.23	16
Lexan	5.92 ± 0.32	5.15 ± 0.72	5.48 ± 0.42	33
EPDM	6.07 ± 0.34	4.91 ± 0.12	5.99 ± 0.35	8
Silicone Rubber	5.61 ± 0.40	4.65 ± 0.25	5.48 ± 0.41	12
Nitryl Rubber	6.44 ± 0.32	5.49 ± 0.27	6.31 ± 0.31	13
Natural        White Rubber	6.05 ± 0.10	5.49 ± 0.30	5.58 ± 0.18	31
Rubber CNA-70	5.70 ± 0.16	4.62 ± 0.15	5.61 ± 0.16	9
Stainless steel 304	5.98 ± 0.28	5.33 ± 0.28	5.66 ± 0.26	25
Stainless steel 304sb	6.13 ± 0.21	5.32 ± 0.17	5.87 ± 0.09	25
Stainless steel 316	6.16 ± 0.43	4.98 ± 0.35	6.09 ± 0.39	7
Stainless steel 430	6.37 ± 0.35	4.98 ± 0.26	6.33 ± 0.31	5
Aluminium	5.69 ± 0.326	4.64 ± 0.42	5.58 ± 0.22	11
Glass	5.58 ± 0.34	4.92 ± 0.33	5.45 ± 0.38	25

**Figure 3.5 Comparison of Short Vs. Two hours Contact Time**

**Number of strongly adhered cells (mean  $\log_{10} \pm S.D$ )**

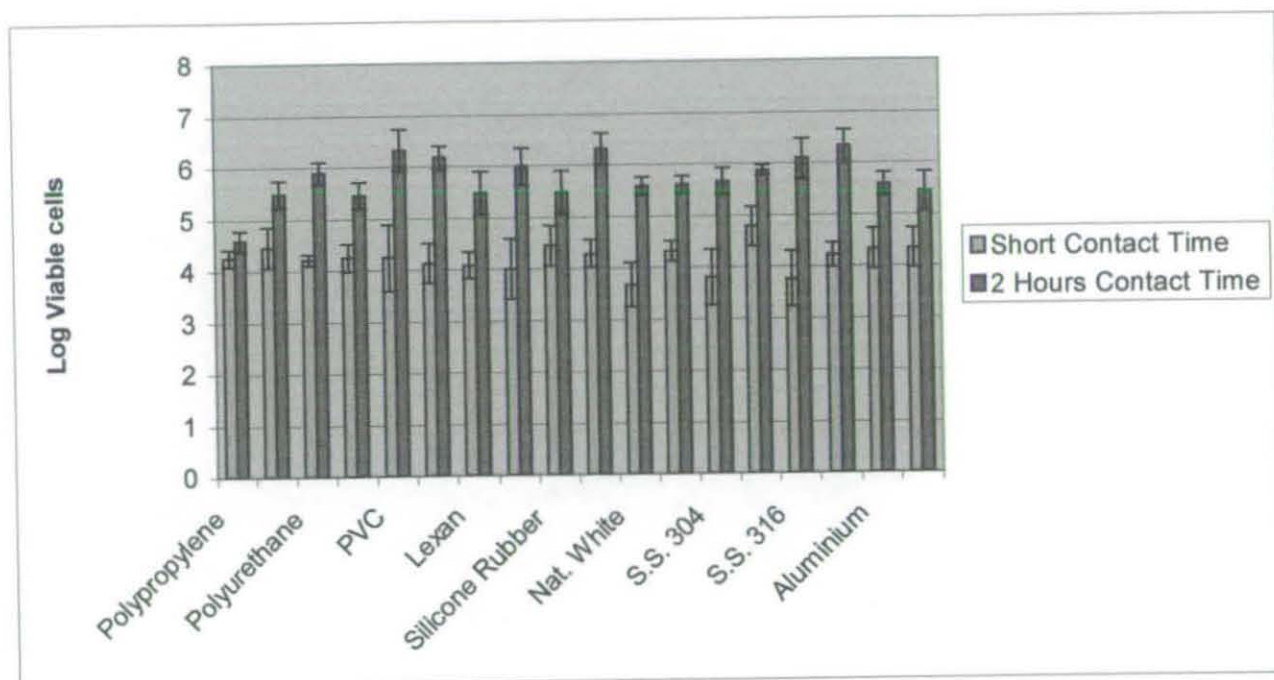


Figure 3.5 shows that, with the exception of polypropylene, there is a significant increase in the numbers of wild type bacteria attached to each material following two hours contact time compared to the numbers obtained for short contact.

### 3.5 Giemsa's Solution Staining Assay

A second assay was devised with the intention of screening a library of transposon insertion mutants (see section 2.11.2). This was a visual assay based on Giemsa's solution cellular staining assay.

The assay was designed to be rapid enough to screen a large number of mutants in a short space of time yet qualitatively reliable enough to select mutants with the

desirable phenotype. This was reduced ability to attach to glass. The assay was performed in a 24 well plate using glass coverslips as the test material. Mutant cultures of *L. monocytogenes* were inoculated into each well of the plate and allowed two hours of contact with the glass coverslip. Following this period, the coverslips were briefly washed before being fixed and stained. The colour of each coverslip was compared visually with that of the wild type control. Mutants exhibiting significantly less colouration than the wild type were retained, re-tested firstly using the Giemsa stain and then again by the quantitative adhesion assay.

This staining test was applied to preliminary experiments in which the number of cells adhering over a period of up to 2 hours was evaluated. The results are shown below:

Figure 3.6 Giemsa's solution staining of wild type *L. monocytogenes* 10403S following contact with glass coverslips for different time periods at 30°C.

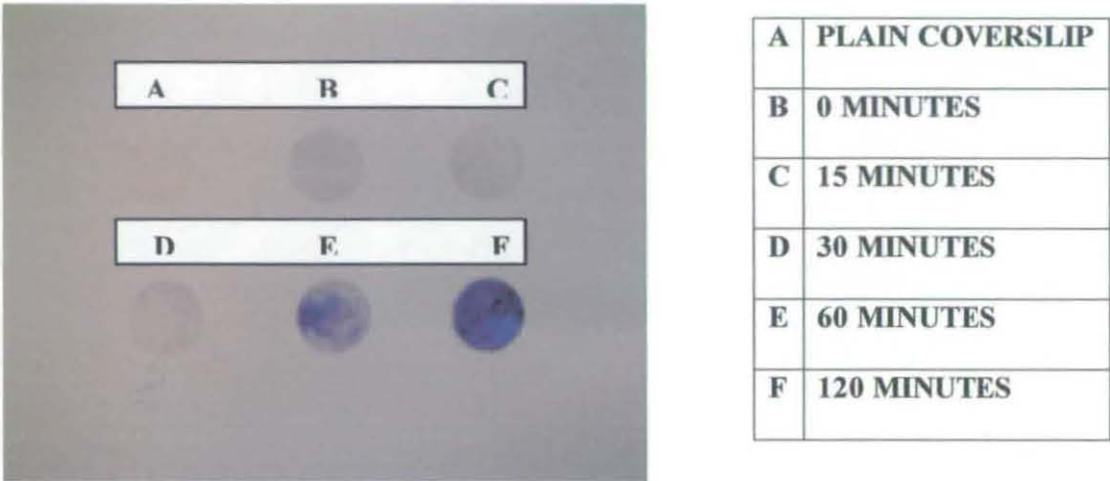


Figure 3.6 demonstrates the levels of staining on the glass coverslips at different contact times. After 60 minutes contact time the coverslip stains dark blue, and after 120 minutes the staining intensifies.

3.6 Adhesion properties of the transposon insertion mutants

Following investigation of the attachment of *L. monocytogenes* to the 18 selected materials, the adhesion assay was utilised to investigate the attachment of transposon insertion mutants to glass at different temperatures.

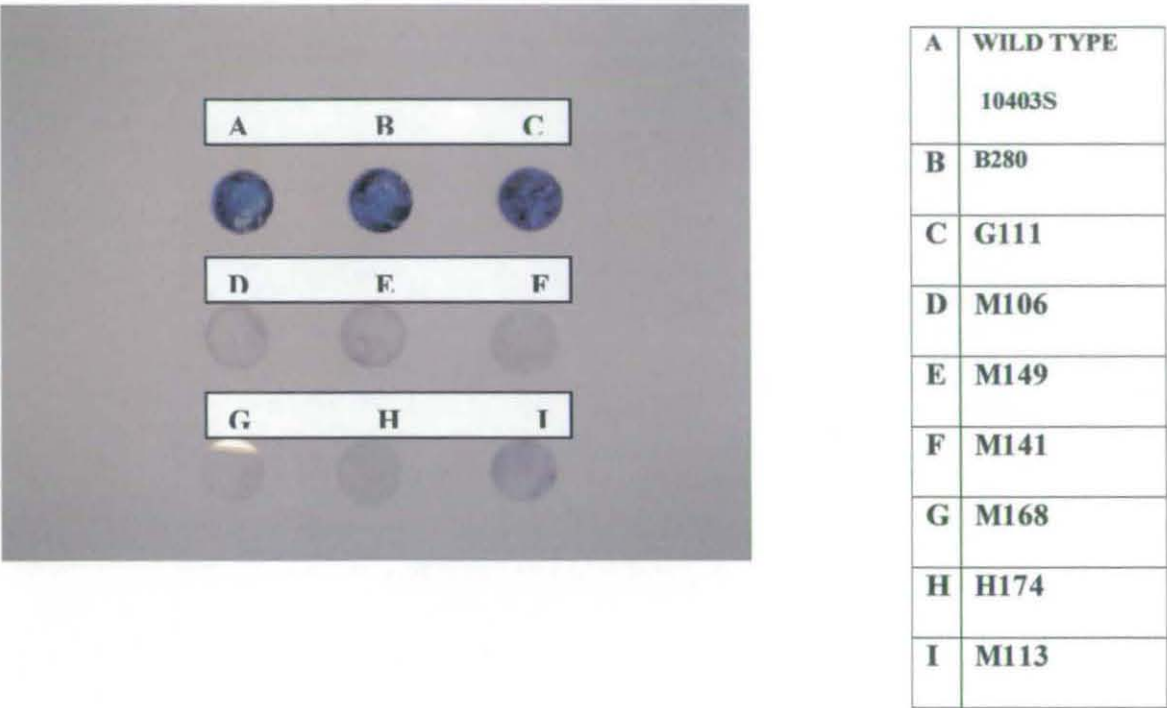
The cellular staining assay was used to screen 4000 of these mutants, selecting for those mutants attaching to glass coverslips in low frequency. Mutants were screened by the Giemsa's solution staining assay following a 2 hour exposure period to glass

coverslips in a 24-well plate (see section 2.11.2). The mutants were then classified by letter (A-I) according to which aliquot of the mutant library they were obtained from, and also numerically after subculturing onto a selective media agar plate. Of these mutants, 60 were isolated on the basis of relatively low attachment to glass compared to the wild type. In addition, one mutant (G111) adhered to glass coverslips at a higher frequency than the wild type. This mutant, along with a subset of 13 of the mutants exhibiting relatively poor attachment to glass, were retained and subjected to the quantitative adhesion assay.

Figure 3.7 shows the appearance of nine coverslips following the Giemsa's solution staining assay. Coverslip A was immersed in wild type culture for two hours. Each of the other eight coverslips were immersed in a different mutant selected from the library. Coverslips D through to I show barely any blue staining at all. Each of these coverslips has been immersed in culture from a mutant which would appear to be in some capacity defective in adhesion. Coverslip B (mutant B280) is shown in this figure as an example of an insertion mutant that does not differ significantly in frequency of attached cells to the wild type. Mutant G111 (Coverslip C) shows increased adhesion compared to the wild type.

The mutants were also analysed using the quantitative adhesion assay described in section 2.11.3. Table 3.3 shows the actual numbers of attached cells stainless steel 304. Each of the 14 mutants attached at a significantly lower frequency to stainless steel than the wild type with the exception of G111, which attached in a higher frequency, and B280 which did not significantly differ in frequency of adhesion to the wild type.

Figure 3.7 Giemsa's solution staining of *L. monocytogenes* wild type and mutants on glass coverslips at 30 °C



**Table 3.3 Number of strongly adhered cells of mutants to stainless steel coupons following a 2 hour contact period.**

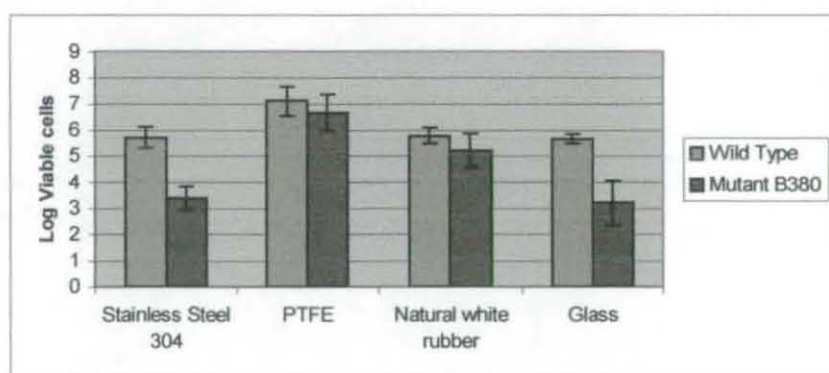
<u>Type of Listeria</u>	<u>Number of strongly adhered cells (mean log<sub>10</sub> ± S.D.)*†</u>
Wild Type	5.97 ± 0.10
Mutant B365	2.28 ± 2.28
Mutant B380	3.15 ± 2.10
Mutant B461	3.42 ± 2.36
Mutant B480	3.57 ± 2.40
Mutant D168	4.85 ± 0.38
Mutant G111	5.89 ± 0.08
Mutant G128	4.16 ± 1.88
Mutant G161	2.26 ± 2.60
Mutant H307	4.74 ± 0.57
Mutant M106	3.49 ± 2.37
Mutant M141	4.87 ± 0.25
Mutant M195	3.49 ± 2.33
Mutant M237	1.29 ± 2.58
Mutant M322	4.36 ± 0.71

Table 3.3 shows the numbers of strongly attached cells to stainless steel following a two hour contact period. The mutants were analysed using the quantitative adhesion



assay described in section 2.11.3 using a minimum of four replicates. The mean number of cells of each mutant attached to stainless steel 304 are significantly lower than that of the wild type, with the exception of G111 for which the mean number of cells is significantly greater. The results in table 3.3 show the mean number of cells attaching for each of the transposon-insertion mutants investigated. The standard deviations of several of the mutants are skewed by one of the four replicates. In most cases this relates to one of the four replicates being larger than the other three replicates by some margin, but still lower than the wild type. One mutant (B380) was selected for further investigation on the basis of a low mean number of cells attaching after 2 hours contact time. This mutant was the first mutant to be checked for correct insertion of the transposon–insertion by Southern hybridisation.

**Figure 3.8 Number of transposon mutant B380 versus wild type cells attached to each of three different food-processing materials at 30°C.**



The results in Figure 3.8 show that the ability of mutant B380 to attach to stainless steel 304 and glass at 30°C was significantly lower than that of the wild type and that



the transposon inserted into a region of the chromosome of *L. monocytogenes* involved in attachment to these materials. There was no significant difference in the numbers of cells adhering strongly to PTFE or natural white rubber.

### **3.7 Characterisation of the site of Tn917-LTV3 insertion**

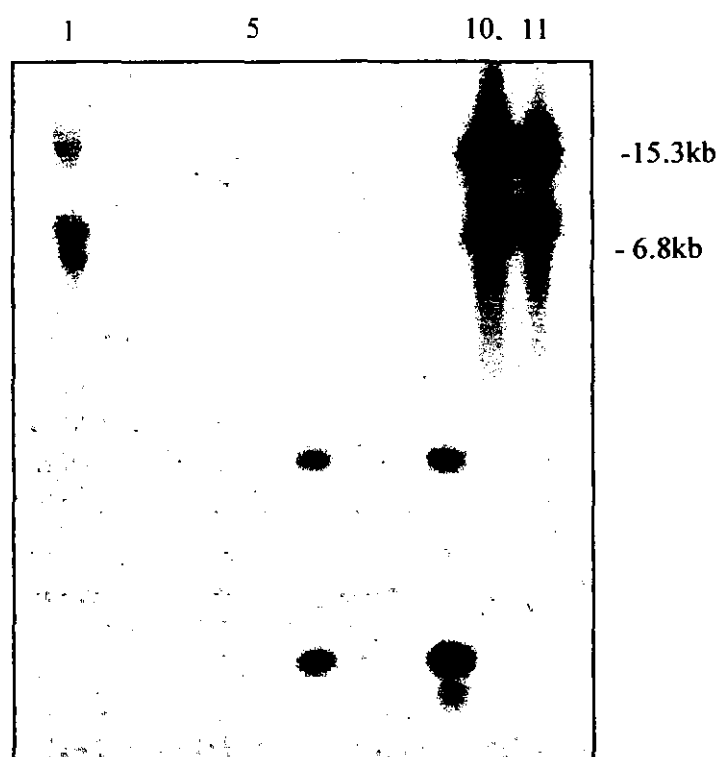
#### **3.7.1 Mutants defective in adherence to glass coverslips at 30°C**

Mutant B380 was selected at random as a candidate for further analysis after attaching to stainless steel and glass in comparatively reduced numbers in the quantitative adhesion assay. A Southern hybridisation reaction was carried out to characterise the transposon insertion mutant B380 further, and to check that Tn917-LTV3 had inserted singly into the chromosome of the mutants. Chromosomal DNA was digested with *Bam*HI, analysed on an agarose gel (0.6%), and blotted to a Hybond-N<sup>+</sup> nylon membrane. The probe used was pLTV3 digested with *Bam*HI, and labelled using the 'Ready to Go dCTP labelling kit tube' (Amersham PLC) (see Section 2.5). This was used as a probe against genomic DNA from mutant B380 also digested with *Bam*HI. Genomic DNA from wild type *L. monocytogenes* 10403S and pLTV3 DNA, also restricted by *Bam*HI, were used in the gel as a negative and positive control respectively (see Figure 3.7).

The conditions for the southern hybridization were carried out according to Section 2.5. The probe hybridised to three fragments of digested genomic DNA containing the insertion sequence. Firstly the probe hybridised to the internally digested 6.8kb fragment produced by digesting Tn917-LTV3 with restriction enzyme *Bam*HI. The

probe also hybridised to two strands of DNA that flank the internal 6.8kb fragment. The length of these fragments is not known in advance as this depends on the position of genomic *Bam*HI sites upstream and downstream of the insertion sequence. Figure 3.9 shows the lengths of DNA to which the probe hybridized. The estimated sizes of the DNA fragments which hybridised to the Tn917-LTV3 specific 6.8kb *Bam*HI-*Bam*HI probe were 6.5kb, 6.8kb and 14kb (Lane 1). These results suggest that a single copy of Tn917-LTV3 had inserted in the chromosome of this mutant. The *Bam*HI-*Bam*HI probe does not hybridise to the negative control (wild type *L. monocytogenes* 10403S) as it does not contain the insertion sequence (Lane 5). The probe does however hybridise to the *Bam*HI digested pLTV3control (Lanes 10 and 11). Lanes 6 and 9 contain 1 kb DNA ladder. The three bands shown in figure 3.9 for mutant B380 (one of which is approximately 6.8kb indicate a single insertion of Tn917 into the mutant. The probe also hybridises to several bands of the 1 kb ladder (lanes 6 and 9), this is quite common as ladders tend to be cut with similar sequences to those used in polylinkers of commercial plasmids. It is therefore likely that several of these ladder fragments contain similar sequences to those found in the multiple cloning site of pLTV3.

**Figure 3.9 Southern hybridisation of mutant B380 with labelled pLTV3 probe**



Lane 1: *Bam*HI digested mutant B380 DNA, Lane 5: Control DNA (*Listeria monocytogenes*) Lanes 6 & 9 1 kb ladder. Lanes 10 & 11: *Bam*HI digested pLTV control.

### **3.8 Cloning of chromosomal sequences flanking Tn917-LTV3 insertion sites**

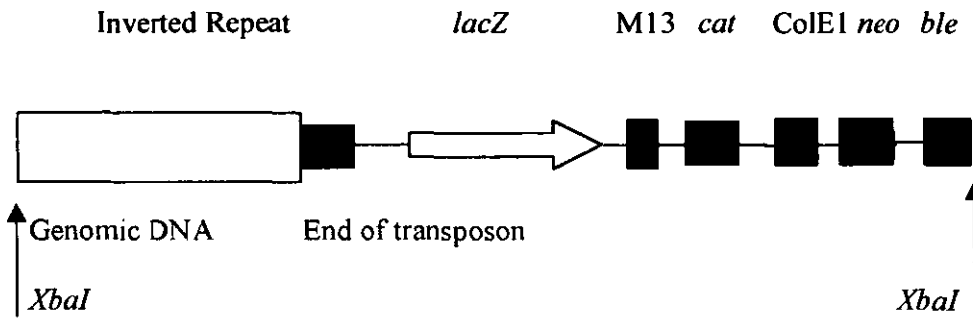
#### **3.8.1 Recovery and Cloning of listerial genomic DNA**

Following transposon mutagenesis, DNA adjacent to the transposon insertion may be rescued into *E. coli* by digesting total chromosomal DNA with an appropriate restriction enzyme, which cuts outside the selectable marker *neo* and the *ColE1* replication functions. This can then be re-circularised and transformed into an *E. coli* strain (Camilli *et al.*, 1990). Taking advantage of the fact that the chromosomal DNA flanking the sites of transposon insertions could be cloned directly into *E. coli* by

using the ColE1 replicon within Tn917-LTV3, DNA flanking the *erm*-proximal end of the insertion from mutant B380 was cloned.

For the cloning procedure, the method of Youngman (1990) was followed. For this, mutant genomic DNA was digested to completion with *Xba*I (enzyme known to cut outside of the selectable marker *neo* and the ColE1 replicon, producing fragments containing flanking chromosomal DNA), followed by ligation in a 150µl volume at a DNA concentration of 5 µg/ml. Such a diluted ligation will promote self-ligation. The inactivated ligation, was used to transform electro-competent *E. coli* HB101 and transformants were selected for kanamycin. Transformation efficiency in *E. coli* HB101 was 1.3 transformants/ µg genomic DNA.

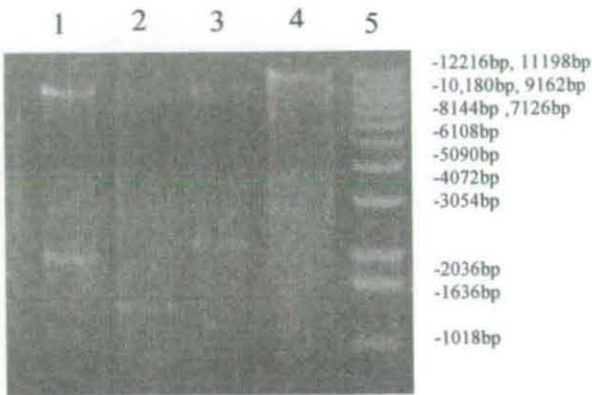
**Figure 3.10 Cloning listerial DNA sequences adjacent to a Tn917-LTV3 insertion with the recovery vector.**



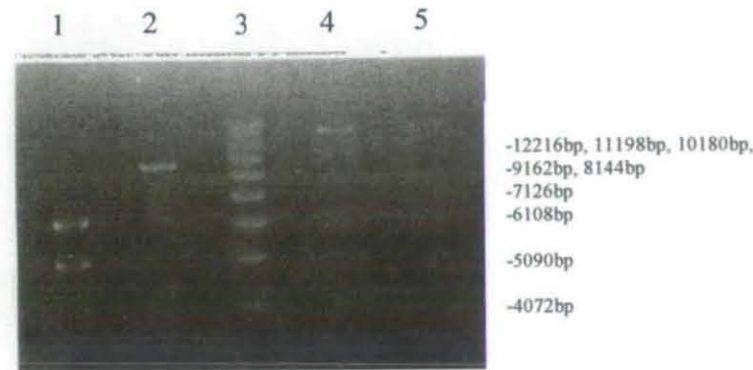
The *XbaI* digested fragment contains a portion of listerial genomic DNA, and inverted repeat of Tn917, the *lacZ* gene, M13, chloramphenicol resistance gene, ColE1, a neomycin resistance gene and the *ble* gene (kanamycin resistance gene).

To clone the DNA bordering the *erm*-distal end of the transposon insertion, chromosomal DNA from *L. monocytogenes* mutant B380 was digested with *XbaI*. *XbaI* cuts outside of the selectable marker *neo* and the ColE1 replicon (see Figure 3.10). The digests were then ligated at concentrations that promote self-ligation. Finally, ligation products are precipitated and used to transform competent *E. coli*, selecting for clones containing plasmids expressing kanamycin resistance. The recovery vector was termed pMB1.

Figure 3.11 Restriction enzyme digests of recovery vector pMB1.



A) Plasmid pMB1 digested with the restriction enzymes, *HpaI*, *PstI*, *SalI*, *XbaI* (lanes 1 through 4) respectively. Lane 5 contains 1 kb DNA size marker (GIBCO-BRL), fragment sizes are shown in the right hand column in kilobases (kb).



B) Lanes 1 and 2 show pMB1 digested with *BamHI* and *HpaI* respectively. It can be seen from lane 4 (digested with *XbaI*) that the approximate size of pMB1 is 11kb. Lanes 5 contains pMB1 DNA digested with *KpnI*. Lane 3 contains 1 kb DNA size marker (GIBCO-BRL).

Figure 3.11 shows various restriction enzyme digests pMB1. Complete sequence data for the pLTV3 vector is not available. The only map available for the vector is shown in figure 2.1. The total size of pMB1 is shown by the enzyme digest with *XbaI* (lane

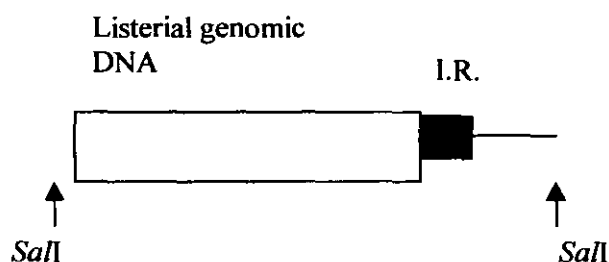
4) as this enzyme cuts the plasmid once only and linearises it. This is estimated to be approximately 11kb (see figure 3.10). The *SaII* digest (lane 3) should give a close indication of the size of the cloned genomic fragment as there are two *SaII* sites in the fragment of Tn917 present in pMB1. One of these sites is positioned in the *ble* cassette, the other is close to the inverted repeat (see figure 3.12). This digest should result in two DNA fragments. The first will contain *listerial* genomic DNA from the interrupted sequence plus a small amount of inverted repeat and *ble* cassette. The second will contain the *lacZ* gene through to the *ble* cassette. This fragment is known to be approximately 9.5-10kb. The results of lane 3 indicate a band of this size and a smaller band of approximately 1.8kb. This second band will be subsequently used for cloning into pBluescript. Lanes 1 and 2 show *HpaI* and *PstI* digests of pMB1 respectively.

### **3.8.1 - Subcloning of *Listeria* inserts for sequencing**

To facilitate the sequencing of the *L. monocytogenes* DNA insert in plasmid pMB1, it was necessary to subclone the fragments containing insert DNA into pBluescript (stratagene). The band selected from pMB1 was the approximately 1.8 kb *SaII-SaI* digested band (see Figure 3.12). pBluescript plasmid DNA was therefore also digested with *SaII* ready for ligation.

**Figure 3.12 *SalI* digested fragment of pMB1 to be cloned into pBluescript**

The *SalI*-digested fragment contains listerial genomic DNA, the inverted repeat of Tn917 and part of pLTV3.

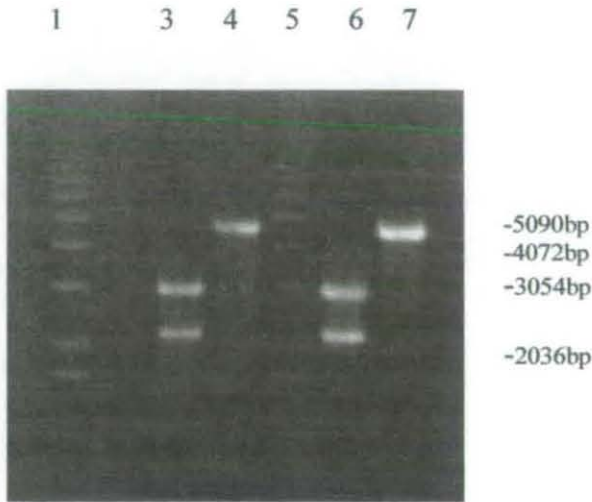


Plasmid pMB1 DNA (approximately 10 $\mu$ g) was digested with *SalI* to generate two fragments of approximately 1.8kb and a 9.2 kb and electrophoresed through a 1% (w/v) low-melting-point agarose gel. Gel slices containing the 1.8 kb *SalI* of pMB1, were excised, and DNA purified from the agarose as described in Section 2.4.7.2.

Ligations were performed as detailed in Section 2.4.3, using ratios of insert to vector of 1:1, 3:1 and 10:1. Ligation reactions were incubated at room temperature overnight, then ethanol precipitated, resuspended in nanopure water, and transformed by electroporation into *E. coli* DH5 $\alpha$  selecting for ampicillin resistance. Plasmid DNA was extracted from white transformants and analysed by restriction enzyme digestion in agarose gel electrophoresis prior to the sequencing. The DNA fragment was in this way successfully cloned in pBluescript (see figure 3.13).



**Figure 3.13** *SalI* digested DNA fragment from pMB1 cloned into pBluescript



Lane 1 and 5: 1 kb ladder. Lane 2: Nothing loaded. Lane 3: *SalI* cut pMB2. Lane 4: *XbaI* cut pMB2. Lane 6: *SalI* cut pMB2. Lane 7: *XbaI* cut pMB2

Plasmid pMB1 DNA was cloned into pBluescript and will be termed pMB2 from this point forward. pMB2 DNA was digested with the following restriction enzymes to show pMB1 and pBluescript separately: Lanes 3 (*SalI*) and 4 (*XbaI*) and 6 (*SalI*) and 7 (*XbaI*) are DNA from two colonies resulting from successful ligation into pBluescript. Lanes 1 and 5 contain 1 kb DNA size marker (GIBCO-BRL). In the case of each clone investigated the *SalI* digestion of pMB2 removes the cloned fragment from pBluescript, this suggests that the clones comprise the correct fragments. Digestion of pMB2 DNA with *XbaI* linearises the plasmid as there is now just one *XbaI* site present.

### 3.9 DNA sequencing of the plasmid pMB2

The sequencing method of choice was the “automated” sequencing using the ABI PRISM<sup>TM</sup> Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq<sup>TM</sup> DNA Polymerase, FS (Applied Biosystems) in conjunction with an Applied Biosystems Model 373A DNA sequencing system. This system relies on the incorporation of fluorescent dye-labelled dideoxynucleotides by the thermostable enzyme AmpliTaq DNA Polymerase, FS to terminate the extension of the primer. AmpliTaq DNA Polymerase, FS was developed specifically for fluorescent cycle sequencing with dye-labelled primers and terminators. This enzyme is a mutant form of *Taq* DNA polymerase which has no 5'----3' nuclease activity and has drastically reduced discrimination for dideoxynucleotides. This sequencing strategy has two advantages. Firstly the use of dye-labelled ddNTPs reduces signals due to false stops since the false stops won't be labelled. Secondly the results are analysed by laser densitometry and computer analysis, a process that allowed long reads of sequence to be obtained from a single run. Using this method, long sequencing reads of up to 700 bp or more were recovered. Sequence data was analysed following at least duplicate reactions and sequence chromatograms were consulted if necessary.

#### 3.9.1 DNA sequencing strategy of the listerial DNA at the *erm*-distal end of Tn917-LTV3

The first step in the sequencing strategy was to determine the sequence of the insert DNA in pMB2 (containing listerial DNA at the *erm*-distal end of Tn917-LTV3) subcloned in pBluescript, using the universal forward and reverse primers T3 and T7.

Sequence obtained from universal primers included part of the *erm*-distal inverted repeat of pLTV-3. The sequencing results showed the presence of the *erm*-distal inverted repeat of pLTV3 and therefore suggested that the correct fragment of pMB1 had been subcloned in pBluescript. Sequence data was then submitted for a nucleotide database search to Pierre Dehoux at the Institute Pasteur (Paris, France) to identify the *Listeria* sequence interrupted by Tn917-LTV-3.

**Figure 3.14 Sequence alignment of cloned mutant DNA against the sequenced *L. monocytogenes* genome sequence held at the Institut Pasteur.**

Key:

pMB2: refers to genomic DNA within the *SalI-SalI* fragment cloned into pBluescript.

Database: refers to the *L. monocytogenes* strain EGD sequence held at the Institut Pasteur.

Base pairs highlighted red indicate a mismatch between the submitted sequence and the database.

pMB2	ttttacttaatggatgcgcaaagctcgtttaatagaaagattattgcactactgcccg
Database	ttttacttaatggatgctcaaagttctttaatagaaagattattgcgctactgcccg
pMB2	gaagataaatcaagattagaaaaactgattactgaaaaacgccttattaccgggcatgg
Database	gaagataaatcgagattagaaaaacttattgctgaaaaacggcttattacggggccttgg
pMB2	tacacacaaacgggccaattagtcatttc-caagagtcgattggtagaaacttggtatat
Database	tatacacaaacggatcaattagttatttcacaagaatcgattgttagaaacttactttat
pMB2	ggtacgagaattgcacgcgaaatggacataccatggctgttggttatgtcccggatgca
Database	ggtacgagaattgcacgcgaaat-ggacatagcatggctgttggttatgtcccggatgca
pMB2	ttcgggcaaggcggaaacatgccgcaaattataaa
Database	ttcgggcaaggcggaaacatgccgcaaattataaa

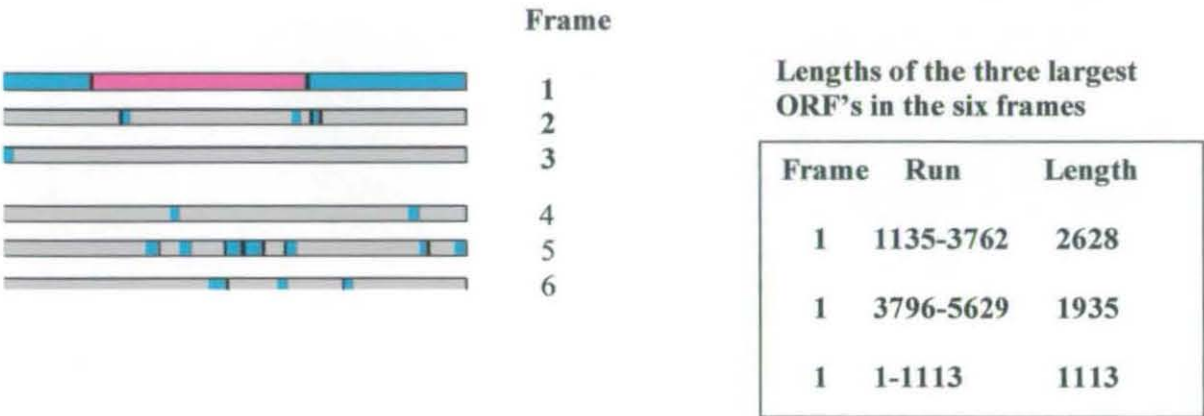
Figure 3.14 Shows the match between the interrupted genomic sequence and the sequence held in the *L. monocytogenes* database at the Institut Pasteur. There is significant homology (91% with no gaps) to a sequence in *L. monocytogenes* EGD thought to be part of a sugar hydrolase gene that is similar to the *ybgB* gene in *E. coli*.

To investigate this further the DNA sequence (plus sequences directly upstream and downstream of the interrupted sequence provided by P. Dehoux at the Institut Pasteur) was submitted to the 'ORF Finder' program at the NCBI website (<http://www.ncbi.nlm.nih.gov/gorf/gorf.html>) to analyse potential open reading frames (ORF's) in each of the six potential coding frames. The results are displayed in figure 3.15.

Potential open reading frames are indicated by green or purple shading. Regions shaded in grey are not part of an ORF. As can be clearly shown in figure 3.14, only coding in frame 1 displays ORF's large enough to be potential genes. The ORF shaded in purple is the ORF disrupted by the insertion of Tn917 (see figure 3.16). From this point onwards this shall be termed ORF1. Information of sequences upstream and downstream from ORF1 supplied by the Institut Pasteur allowed the design of PCR primers to allow amplification of ORF1. Once this ORF has been sequenced the DNA sequence can be converted in frame into an amino acid sequence using the DNA Strider program (Dr C Marck, Service de Biochimie de Genetique Moleculaire, France). This amino acid sequence can then be submitted to the BLASTX (Altschul *et al.*, 1990) amino acid database. This is discussed further in section 3.10.3.

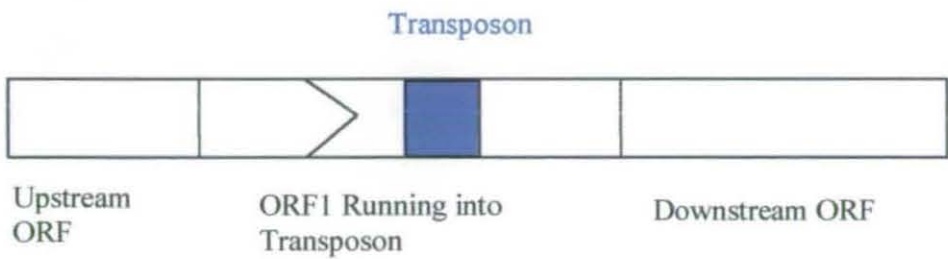
**Figure 3.15** Position of ORFs in the listerial DNA flanking the Tn917-LTV3 insertion in mutant B380 as hypothesised by sequence data aligned against that at the *L. monocytogenes* database at the Institut Pasteur.

Shows the presence and sizes of any potential ORF's in the sequence.



**Figure 3.16** Arrangement of ORF's

This diagram is an expansion of frame 1 in figure 3.15. It shows the position of the transposon insertion between the potential ORF's.



### **3.10 Isolation of the gene interrupted by Tn917-LTV3, in transposon insertion mutant B380**

In order to be able to clone the ORF1 (the ORF disrupted by Tn917-LTV3 insertion in mutant B380), the intact wild-type gene had to be isolated from the *L. monocytogenes* 10403S genome. The strategy chosen was PCR.

The aim of this experiment was to express the wild type gene in the deficient *L. monocytogenes* mutant B380. The PCR primers were designed upstream of the start codon and downstream of the stop codon of ORF1 (see section 2.6.1). Restriction endonuclease tags were added to the oligonucleotides in order to facilitate simple subcloning of the PCR product if necessary. PCR primers were designed with the aid of the 'Gene Tool' computer program and included a 5' *Bam*HI restriction site and a 3' *Sma*I restriction site. These primers were termed MBBam and MBSma, and contained 31 and 30 nucleotides respectively (see section 2.6.1). The approximate size of the PCR product generated using primers MBBam and MBSma (estimated from the Institut Pasteur database for strain EGD) would be 3530 base pairs which includes ORF1 together with flanking regions coding for ribosomal binding site and primers.

In the PCR reactions, the template DNA used was *L. monocytogenes* 10403S wild type genomic DNA at a range of 0.5 µg to 0.125 µg in 100 µl final volume reactions. The enzyme used in these reactions was *Pfu* DNA Polymerase (Promega) since it possesses a highly processive 5'-3' DNA polymerase and an 3'-5' exonuclease activity, also known as proof-reading activity, which increases approximately 12-fold the fidelity of DNA synthesis when compared to *Taq* DNA polymerase. The PCR

conditions were carried out as in Section 2.6. To visualise the products, 5 µl of each of the PCR reactions were analysed on 1% (w/v) agarose gels. Figure 3.17 shows the results of the PCR amplification products produced.

Lane 1 shows a fragment of approximately 3.5kb but also non-specific binding of the PCR primers to the template probably caused by the lowered annealing temperature (45°) used during this reaction. Lane 2 shows weak bands at the same annealing temperature but using a standard concentration of *Pfu* polymerase (units). Lane 3 shows a very strong band of the desired size (approximately 3.5 kb) was produced at increased concentration of *Pfu* polymerase (5 units) and increased annealing temperature (55°C) with little or no non-specific binding of PCR primers. Lane 4 shows 1 kb DNA size marker (GIBCO-BRL), fragment sizes are shown in the right hand column in kilobases (kb). No amplification was observed in the negative control reaction which consist of a PCR reaction with no template DNA added (lane 5).



**Figure 3.17 -Amplification products using MBBam and MBSma primers in PCR reactions.**



Various amplification products using MBBam and MBSma primers are shown below.

Lane 1: Amplification at 45°C. Lane 2: Amplification using 1 unit of *Pfu* polymerase at 55°C. Lane 3: Amplification using 5 units of *Pfu* polymerase at 55°C. Lane 4: 1 kb ladder. Lane 5: Amplification without DNA template.

### 3.10.1 Cloning into pGEM-T-Easy

PCR product prepared in the previous section, was electrophoresed through a 0.7% (w/v) low-melting-point agarose gel. The gel slice containing the 3.5 kb fragment was excised, and DNA purified from the agarose using the QIAGEN gel extraction kit method (see Section 2.4.7.2). Following extraction and clean-up, the fragment was then A-tailed at 72°C for 30 minutes in the presence of *Taq* DNA polymerase

(Promega). The purified A-tailed PCR fragment was then ethanol precipitated and cloned into the pGEM-T-Easy cloning vector using manufacturers instructions. pGEM-T-Easy derives from *EcoRV* cut pGEM-®-5Zf(+) and has a 3' terminal thymidine on each end to improve efficiency of ligation of PCR products produced from thermostable enzymes leaving A tails. This Vector also simplified subsequent cloning of our gene as there is an *EcoRI* site flanking the MCS thus avoiding the need for double digests into a shuttle vector. The resulting plasmid containing the cloned PCR product will be termed pMB3 from this point.

### **3.10.2 Direct sequencing of PCR product in pMB3**

Sequencing from within pMB3 was carried out initially using commercial T7 and SP6 primers. Following this subsequent primers were designed further into the sequence using the GeneTool computer program (BioTools Ltd). A list of these primers is shown in section 2.7.2.

Once fully sequenced the fragment cloned from *L. monocytogenes* strain 10403S (containing ORF1 and sequences upstream and downstream) was submitted to a BLAST sequence alignment (Altschul *et al.*, 1990) against the equivalent sequence for *L. monocytogenes* strain EGD. The results of the alignment are shown in the appendix. The DNA from strain 10403S (investigated in this thesis) displayed a 93% match (1% gap) to that of strain EGD (from the Institut Pasteur database). Once ORF1 was sequenced the DNA sequence was converted in frame into an amino acid sequence using the DNA Strider program. This amino acid sequence was then

submitted to the to the BLASTX (Altschul *et al.*, 1990) amino acid database. This is discussed further in section 3.10.3.

### **3.10.3 Sequence analysis of the listerial DNA sequence flanking the site of the insertion of Tn917-LTV3 in mutant B380**

Frame 1 of the ORF submitted to the BLASTX (Altschul *et al.*, 1990) amino acid database. The search found a significant match in the database (see figure 3.18).

Results of the BLASTX search show that the amino acid sequence subjected from ORF1 contains a putative conserved domain (Glycosyl hydrolase family 38).

Glycosyl hydrolase family 38 comprises enzymes of only one known function, the alpha- mannosidases (Coutinho P.M, Henrissat B. Carbohydrate-Active Enzymes server). This is discussed further in section 4.

The resulting translation product was then searched against the non-redundant GenBank CDS translations, PDB, SwissProt and PIR protein databases maintained at the National Centre for Biotechnology Information (NCBI) in Bethesda, Maryland, USA, for homologous sequences. Results of these comparisons are summarised in Table 3.4 for only the 10 top scores. Each of the top scores shown in Table 3.4 shows homology to and alpha mannosidase of the glycosyl hydrolase family 38. Based on this information, and the detection of the glycosyl hydrolase 38 conserved domain a series of biochemical experiments were constructed to characterise this ORF further (see section 3.12). From this point forward the ORF1 sequence will be termed a putative alpha mannosidase gene.

**Figure 3.18 Amino acid match to a putative sugar hydrolase in *Listeria monocytogenes* strain EGD**

**Key:**

Query: Amino acid sequence from DNA amplified and sequenced in *L.*

*monocytogenes* 10403S.

Subject: Amino acid sequence from BLASTX database (*L. monocytogenes* strain EGD : putative sugar hydrolase).

\* : Middle Line denotes matches.  
: Blue colouring indicates the detection of putative conserved domain  
Glycosyl hydrolase family 38.

Query: 1  
MAKTKVHVIPH<sup>\*</sup>HLHWDREWYFTSSRSTIYLVKHLKEVIETLEAKDDYHFYLMDAQSSSLIED 60\*  
M KTKVHVIPH HWDREWYFTSSRSTIYLVKHLKEVIETLEAK+DYHFYLMDAQSSSLIED  
Sbjct: 1  
MTKTKVHVIPHSHWDREWYFTSSRSTIYLVKHLKEVIETLEAKEDYHFYLMDAQSSSLIED 60

Query: 61  
YLHYCPEDKTRLEKLI<sup>\*</sup>AEKRLITGPWYTQTGQLVISKSRLVETCYMVRELHAKWGHTMAV 120  
YL YCPEDK+RLEKLI<sup>\*</sup>AEKRLITGPWYTQT QLVIS+ +V + + GH+MAV  
Sbjct: 61  
YLYRCPEDKSRLEKLI<sup>\*</sup>AEKRLITGPWYTQTDQLVISQESIVRNLLYGTRIAREMGHSMV 120

Query: 121  
SFCPRMPFRQGGNHATKFFKEFGNFKSFLFWPRSFRLSLZNQTRIFFWRRGINRD---RE 177  
+ P F QGGN + +KEFG FLFW R + QT F WR + +  
Sbjct: 121  
GYVPD-AFGQGGN-MPQIYKEFG-ISKFLFW-RGVADNRLKQTE-FIWRGDDGTEMLAEQ 175

Query: 178  
MPFGNQNSRLATTTERTFLKZSRTZNLFGZTNWRSRIKSFNAKCLFPKWLRPSASQKKFA 237  
+PFG A E ++ + + K+ FP L + +K  
Sbjct: 176  
IPFGYYYG--ANIPE----NEAELKTYLDEQIGALEEKASTPNVYFPNGLDQAPVRKNLP 229

Query: 238  
GIGCKFNELDSTREYQIASPETFFGDLEKDVTDLPEHCRRINRRKTZSPA<sup>\*</sup>ZIDFLYSSZF 297  
+ KFNELDSTREYQIASPETFF DLEKDVTDLP + K + F ++  
Sbjct: 230  
ELVAKFNELDSTREYQIASPETFFADLEKDVTDL<sup>\*</sup>PVIAGELTEGKHSRLHKSIFSTRADL 289

Query: 298  
KTSDESNZKLFKQCARAFSFHQLLTWESLPTQZTR-----RNLEADVZKCCTRZHWL 350  
K ++ + P + N D C +  
Sbjct: 290  
KQANNQIENFLSNVLEPVLSISYSLGNRYPHNELAEIWKLMFENAAHDSIGGCNSD<sup>\*</sup>TNR 349

Query: 351  
ZZZYDQPRRQTSLQIGFGFSQDQFTRFKHAPNQKRNRTKTTVPIYRFQPTPYEKSGVIKMT 410  
+ ++ R + + + D R +++ + TV F P PYEKSGVIKMT  
Sbjct: 350  
DVKH---RYKLASDLATNLLDLNMRLISEKIEQRQPFQFTV----FNPLPYEKSGVIKMT 402

Query: 411  
AYIPEDNFTVEDTQGTRLIHNS-RKNRPNRIRLNQHIDLNPSPVYLPEKVFLATMLVNV 469  
AYIPEDNFTVEDTQG L + K LNQHIDLNPSPVYLPEKVFLATMLVNV  
Sbjct: 403  
AYIPEDNFTVEDTQGNTLEYTILEKTDLTEYVLNQHIDLNPSPVYLPEKVFLATMLVNV 462

Query: 470  
NNVPALGYDTIYFNLEKETAEQEPTQSAATKIRKRLZ-NEVSSQZFTMIKKRAEPTPIK 528  
NN+PALGYDTIYFNLEKETAEQEPTQSAATKI + ++ T+ K+A T  
Sbjct: 463  
NNLPALGYDTIYFNLEKETAEQEPTQSAATKIENEFYEIQLAANNSLTIHDKKAGRTYTD 522

Query: 529  
ZFLW-ENGDDGDSYNYSPPRKDLVISSKEAVVERZSLAYQASTKHZLFPSSZMYGYNLEE 587  
+ ++ ENGDDGDSYNYSPPRKDLVISSKEAVVE + + + YNLEE  
Sbjct: 523  
QMIFVENGDDGDSYNYSPPRKDLVISSKEAVVESIESSISSVNQALTISFKLNVPYNLEE 582

Query: 588  
RANGEKNNRKZPIKNSIFFYVKNEELIRFZCKLLNQSIESSFMCYIRPEIASKFSNSZSI 647  
RANGEKNN + I ++ KNEELIRF+ ++ NQ + EIASKFS ++ +  
Sbjct: 583  
RANGEKNN--EMIIKTVISLRKNEELIRFDVQIANQVLSHRLCVTFATEIASKFSTADQL 640

**Table 3.4- Results of the BLASTX search of the protein databases for listerial DNA flanking both sides of the Tn917-LTV3 insertion in mutant B380.**

Sequences producing significant alignments:	Score (bits), E-Value, Identity (%)
NP_463931.1  Lmo0401 <i>L. monocytogenes</i> alpha mannosidase	502, e-140, 48%
NP_469769.1  ORF Lin0424 <i>L. innocua</i> alpha mannosidase	481, e-134,46%
NP_415260.1  ORF YbgG <i>E.coli</i> alpha mannosidase	125, 2e-27,23%
NP_466256.1 Lmo2724 <i>L. monocytogenes</i> alpha mannosidase	112, 2e-23, 41%
NP_463958.1 <i>L. monocytogenes</i> ORF Lmo0429 alpha mannosidase	89, 2e-16, 30%
NP_469793.1 Lin0449 <i>L. innocua</i> alpha mannosidase	89, 3e-16, 30%
NP_562331.1  <i>C.perfringens</i> ORF CPE1415 alpha mannosidase	87, 1e-15, 34%
NP_607667.1  <i>S. pyogenes</i> ORF SpyM18 alpha mannosidase	82, 4e-14, 29%
NP_665101.1  <i>S.pyogenes</i> Spy_M3 alpha mannosidase	82, 4e-14, 29%
NP_269660.1  <i>S. pyogenes</i> Spy_1604 alpha mannosidase	82, 4e-14, 29%

\*Acc. = Accession number

A Promoter sequence is a region of DNA to which RNA polymerase binds before initiating the transcription of DNA into RNA. The nucleotide at which transcription starts is designated +1 and nucleotides are numbered from this with negative numbers indicating upstream nucleotides and positive downstream nucleotides. Most bacterial promoters contain two consensus sequences that seem to be essential for the binding of the polymerase. The first, the Pribnow box, is at about -10 and has the consensus sequence 5'-TATAAT-3'. The second, the -35 sequence, is centred about -35 and has the consensus sequence 5'-TTGACA-3' (these are shown in Appendix II). The terminator program of the GCG Wisconsin Package (Genetics Computer Group, 1995), was used to investigate terminator sequences at the end of ORF1. The method used is described in detail by Brendel and Trifonov (1984). A putative *rho*-independent terminator sequence was detected shortly after the ORF1 stop codon (see Appendix II). The presence of promoter consensus sequences and a strong terminator sequence suggest that the phenotype change in mutant B380 caused by insertion of a transposon into ORF1 is not due to polar effects on other genes part of a larger ORF, although this cannot be ruled out completely.

### 3.11 Cloning of putative alpha mannosidase into pMK4 shuttle vector.

A genetic complementation experiment was devised to test the hypothesis that transposon insertion into this putative alpha mannosidase gene has resulted in a decreased ability of the mutant to attach to glass and stainless steel. In order to be able to complement the mutation in *L. monocytogenes* mutant B380, the PCR product (containing the putative alpha mannosidase gene), was to be cloned into a shuttle vector that will allow the expression of this gene in *L. monocytogenes*. The plasmid of choice was pMK4 (Sullivan *et al.*, 1984). This plasmid is a shuttle vector and can replicate in both *E. coli* and *L. monocytogenes*. This vector allows the use of the techniques for the isolation and manipulation of plasmids in *E. coli*. The resulting hybrid molecules can then be introduced into *L. monocytogenes* by transformation. This characteristic is important because direct transformation of ligation mixtures into *L. monocytogenes* is very inefficient. pMK4 contains the *lacZ* gene with several unique restriction sites. Insertion of DNA into any of these sites disrupts the *lacZ* peptide, causing a loss of  $\alpha$ -complementation and results in a Lac<sup>-</sup> phenotype in the appropriate *E. coli* host strain. Consequently transformants can be easily isolated and transformed into *L. monocytogenes*.

The insert DNA was digested from pMB3 using *EcoRI* and was ligated into pMK4 also digested with *EcoRI*. Ligations were performed as detailed in Section 2.4.3, using ratios of insert to vector of 4:1, 8:1 and 10:1. Ligation reactions were incubated at room temperature overnight, then ethanol precipitated, resuspended in nano-pure water, and transformed by electroporation into *E. coli* DH5 $\alpha$  selecting for ampicillin resistance. No colonies were recovered. Measures were taken to investigate the DNA preparation, purification,



restriction digestion and ligation steps of this experiment as well as the transformation and shuttle vector itself (pCK1, a similar shuttle vector was used as a control: Gasson and Anderson, 1985). The following controls were set up to test the cloning, ligation and transformation procedure. Another *EcoRI* digested insert from a piece of control DNA was successfully cloned into pMK4. This result confirms there were no problems with the *EcoRI* site in pMK4 itself.

- (1) Transformation of linearised and re-circularised pMK4, pGEM-T-Easy, pMB3 and pCK1 (digests with both *EcoRI* and other enzymes was attempted as a control).
- (2) Transformation of circular pMK4, pGEM-T-Easy, pMB3 and pCK1.
- (3) Transformation of *EcoRI* digested and re-ligated pMB3.
- (4) CIP-treating and non-CIP treating vector DNA prior to ligation.
- (5) Cloning of another *EcoRI* digested insert (1 kb fragment from plasmid pAV3 into pMK4 and pCK1).
- (6) Transformation of *EcoRI* digested pMB3 (not re-ligated).

Transformation of circular vectors yielded a high efficiency of colonies (approximately  $10^7$  colonies per  $\mu\text{g}$  of plasmid DNA). Transformation of re-circularised vectors (see 1 above) yielded approximately  $10^5$  colonies per  $\mu\text{g}$  of plasmid DNA. These two controls discount the stages of digestion, ligation and the transformation procedure itself as the failure to clone the fragment into pMK4. CIP treating pMK4 and pCK1 prior to ligation with the fragment also failed to yield colonies. As expected transformation of *EcoRI* digested pMB3 that had not been re-ligated did not yield any colonies.

Different preparations of pMK4 and pCK1 were isolated using both the QIAGEN midiprep kit and also the ABGene midiprep kit. Changing suppliers of the isolation kit and changing

the elution liquid to nuclease-free water did not yield colonies. It was hypothesised that DNA crosslinking may be occurring during bandprepping of the PCR product (Nejedly *et al.*, 2001). This may be causing damage to sites important for subsequent cloning. However, cloning of ethanol precipitated, but non-bandprepped DNA did not yield colonies either. No colonies were recovered from repeat ligations and transformations into pMK4 or vector pCK1.

### 3.11.1 Transformation of plasmid pMK4 into *L. monocytogenes*

Unfortunately, it was not possible to clone the DNA fragment into shuttle vector pMK4 (or the control pCK1). The reasons for this following a number of controls are not obvious and eventually time constraints did not allow this to be pursued any further. The final step of the complementation experiment would have been to transform plasmid pMK4 (containing the PCR product) into *L. monocytogenes*. The method chosen to transform pMK4 into *L. monocytogenes* mutant B380 was electroporation. Penicillin treatment of cells before electroporation to increase the permeabilization of the cells was developed by Park and Stewart (1990), who reported efficient electrotransformation of *L. monocytogenes* with plasmid DNA (transformation frequency of  $4 \times 10^6/\mu\text{g}$  DNA). Using this method, 1  $\mu\text{g}$  of pMK4 (as positive control) was electrotransformed into mutant B380 selecting for erythromycin and chloramphenicol. Chloramphenicol resistance is the selectable marker for pMK4, or pMK4 derived plasmids, in Gram-positives. This antibiotic resistance gene also exists in the genome of *L. monocytogenes* mutant B380, since it was carried by the Tn917-LTV3 derivative. Penicillin treated *L. monocytogenes* mutant B380 cells were electrotransformed on the day of the treatment or pre-stored at  $-70^\circ\text{C}$  for 18 hours prior to

the electroporation. This storage is thought to increase the efficiency of electroporation in *Listeria* cells. Plates were incubated at 37°C for 48 and 72 hours. Using this method control vector pMK4 was successfully cloned into *L. monocytogenes* mutant B380.

### **3.12 Biochemical tests on the wild type *L. monocytogenes* 10403S and transposon mutant B380**

#### **The API System for identification of *Listeria***

The API system (Biomérieux) for *Listeria* was used to investigate the biochemical profile of the transposon insertion mutant and compare it to the wild type bacterium. Table 3.5 shows mutant B380 exhibited the same API profile as the wild type with the exception of the  $\alpha$ -mannosidase test. Mutant B380 exhibited a distinct colourless solution indicating a negative result for  $\alpha$ -mannosidase production. The wild type exhibited a yellow colouration indicating a positive reaction for the  $\alpha$ -mannosidase test. This result suggests that  $\alpha$ -mannosidase production has been hindered in the transposon insertion mutant. In order to examine this further an  $\alpha$ -mannosidase assay was used to estimate activity of the enzyme. The API strips for the wild type and mutant are shown in Figure 3.19. The third microtube from the left shows the difference in colour between the wild type (yellow) and mutant B380 (colourless).

**Table 3.5 Results of API *Listeria* strip test for the wild type and mutant B380**

Tests	Reactions/Enzymes	Results in each strain	
		Wild Type	Mutant B380
DIM	Differentiation Test	Negative	Negative
ESC	Esculine (Hydrolysis)	Positive	Positive
$\alpha$ MAN	$\alpha$ -Mannosidase	Positive	Negative
DARL	D-Arabitol (Acidification)	Positive	Positive
XYL	D-Xylose (Acidification)	Negative	Negative
RHA	Rhamnose (Acidification)	Positive	Positive
MDG	$\alpha$ -Methyl-D-Glucoside (Acidification)	Positive	Positive
RIB	Ribose (Acidification)	Negative	Negative
G1P	Glucos-1-Phosphate (Acidification)	Negative	Negative
TAG	D-Tagatose (Acidification)	Negative	Negative

**Figure 3.19** API Strips for *L. monocytogenes* 10403S (wild type) and mutant B380

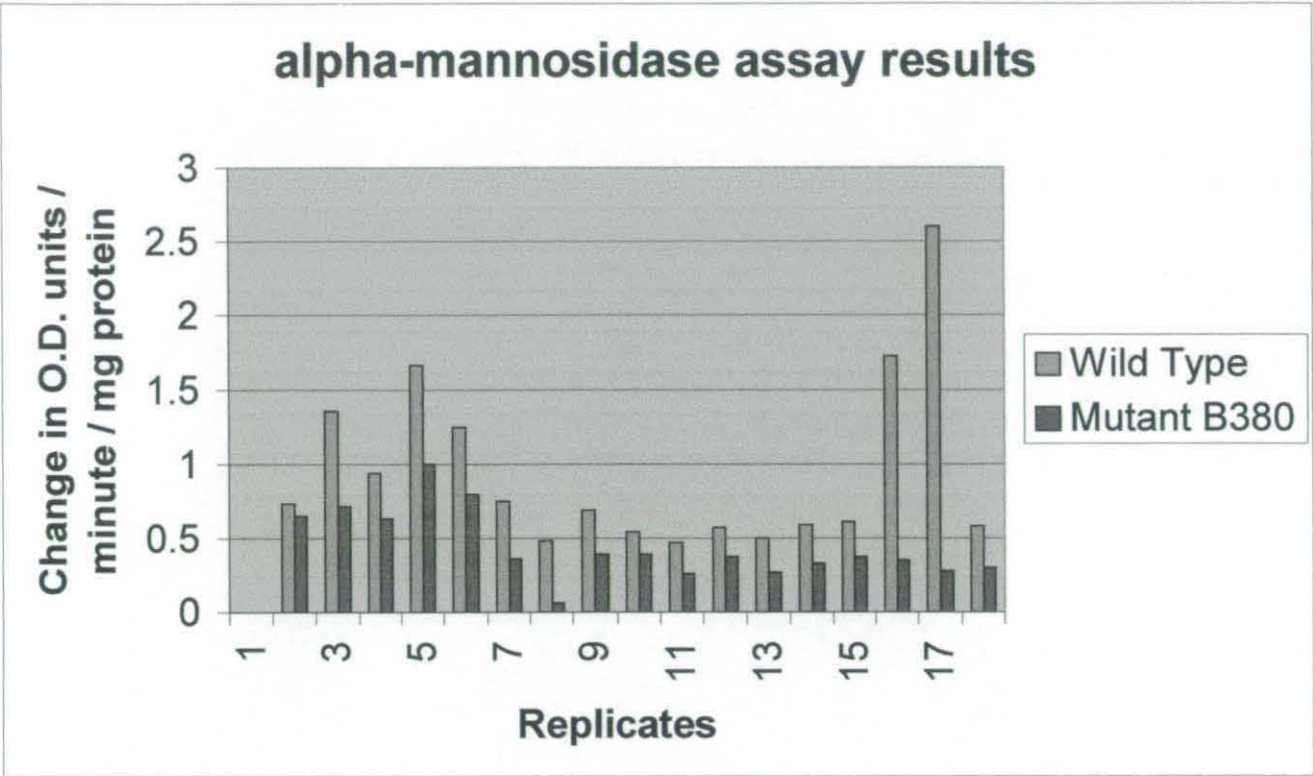


The top strip shows the API test results for mutant B380. The bottom strip shows the API test results for the wild type.

**3.13 Alpha-mannosidase assay**

The  $\alpha$ -mannosidase assay was carried out according to the method in section 2.9 using *para*-nitrophenyl- $\alpha$ -D-mannopyranoside in sodium phosphate as the substrate. Volumes of cell lysate were added to the substrate and the total volume equilibrated with distilled water. The reaction was incubated at 37°C in a water bath for 5 minutes and then stopped by adding Sodium carbonate. The absorbance was determined by OD<sub>400nm</sub>.

Table 3.6      Alpha-mannosidase assay results



The results in table 3.6 clearly show the change in O.D. unit per minute per milligram of protein is greater in each pair of replicates for the wild type bacterium than for the transposon insertion mutant. The experiment was carried out three times with a total of seventeen different replicates examined. The mean of the wild type was  $0.919 \pm 0.52$  O.D units / min / mg protein. The mean of mutant B380 was  $0.442 \pm 0.23$  O.D. units / min / mg protein. The results of a two-tailed paired T-test (Instat) showed that the mean of the wild type was significantly greater than that of the mutant. The two tailed P value was 0.0007, which is considered extremely significant. This result suggests that the mutant is expressing alpha-mannosidase at lower levels than the wild type.

### 3.14 Attachment of a flagellin mutant to food processing materials

The adhesion assay was used to determine the ability of a mutant of *L. monocytogenes* strain NCTC 7973 with a deletion/insertion in the *fla* gene to adhere to food processing materials at 30°C (Vatanyoopaisarn *et al.*, 2000). This mutant was obtained from Dr C.E. Rees at the University of Nottingham. A statistical comparison of adhesion of the flagellin mutant to each of the materials is shown in Table 3.7. The numbers of mutant cells adhering to metals, plastics, rubber and glass materials are compared to those of the wild type in figure 3.20. The flagellin mutant attaches to stainless steel 304 and 316L in significantly lower numbers than any other material.

The mutant adheres to stainless steel types 304 and 316L in significantly lower numbers than any other metal type, with respective means of 4.18 log cfu and 4.19 log cfu (see Figure 3.20). The numbers of mutant cells adhering to stainless steel types 304, and 316L are significantly lower ( $P < 0.001$ ) than for the rest of the metals, however they do not significantly differ from each other ( $P > 0.05$ ). The flagellin mutant also adheres to sandblasted stainless steel 304 at a significantly elevated frequency than to each of the other materials.

It is also evident that adherence of flagellin mutant cells is quite consistent between plastics, with means of attached cells ranging between 5.85 - 6.48 log cfu (see Figure 3.20) The exception is polycarbonate which has a mean number of attached cells of 5.46 log cfu.

Greater variability was obtained with the flagellin mutant when tested on different types of rubber. The highest mean number of attached cells is to commercial nitril rubber (6.59 log

cfu), and the lowest to EPDM rubber (4.74 log cfu). The numbers of flagellin mutant cells attaching to silicone and nitryl rubbers is comparable to the numbers of mutant cells attaching to the plastic materials (see figure 3.20). There is no significant difference between the number of cells adhering irreversibly to natural white rubber and EPDM ( $P>0.05$ ), or commercial nitryl and silicone ( $P>0.05$ ).

**Table 3.7 Tukey-Kramer multiple comparisons test results for flagellin mutant attachment to each material.**



Materials are grouped according to : Plastic, Rubber, Metal and Glass.

	PVC	Polypropylene	Polycarbonate	LEXAN	PETG	Polyurethane	SILICONE	Com. Nitril	Nat.W.Rub.	EPDM	CNA-70	S. Steel-304	Sand	S. Steel-430	S.Steel316L	Aluminium	GLASS
PTFE	NS	NS	**	NS	NS	NS	NS	NS	***	***	NS	***	NS	***	***	**	*
PVC	-	NS	***	NS	NS	NS	NS	NS	***	***	NS	***	NS	***	***	***	**
POLYPROPYLENE	-	-	**	NS	NS	NS	NS	NS	***	***	NS	***	NS	***	***	**	NS
POLYCARBONATE	-	-	-	NS	***	*	***	***	NS	NS	NS	***	NS	NS	***	NS	NS
LEXAN	-	-	-	-	NS	NS	NS	**	*	***	NS	***	NS	NS	***	NS	NS
PETG	-	-	-	-	-	NS	NS	NS	***	***	*	***	NS	***	***	***	**
POLYURETHANE	-	-	-	-	-	-	NS	NS	***	***	NS	***	NS	***	***	**	NS
SILICONE	-	-	-	-	-	-	-	NS	***	***	NS	***	NS	***	***	***	**
commercial nitril	-	-	-	-	-	-	-	-	***	***	***	***	NS	***	***	***	***
Natural White Rub.	-	-	-	-	-	-	-	-	-	NS	NS	**	***	NS	**	NS	NS
EPDM	-	-	-	-	-	-	-	-	-	-	***	NS	***	NS	NS	NS	**
CNA-70	-	-	-	-	-	-	-	-	-	-	-	***	NS	NS	***	NS	NS
S. Steel 304	-	-	-	-	-	-	-	-	-	-	-	-	***	**	NS	***	***
sandblasted S. Steel-304	-	-	-	-	-	-	-	-	-	-	-	-	-	***	***	*	NS
S. Steel-430	-	-	-	-	-	-	-	-	-	-	-	-	-	-	**	NS	NS
S.steel 316L	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	***	***
Aluminum	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	NS

NS Non-significant difference between data

*	<b>P&lt;0.05 : Significant difference between data</b>
**	<b>P&lt;0.01 :Very significant difference between data</b>
***	<b>P&lt;0.001 :Extremely significant difference between data</b>

Table 3.7 shows the levels of significant differences in attachment of the flagellin mutant to each of the 18 different materials. The mutant attached in lowest numbers to stainless steels 304 and 316 (see Figure 3.20).

**Figure 3.20** Comparison of the numbers of wild type and flagellin mutant cells attached to each of the 18 materials investigated

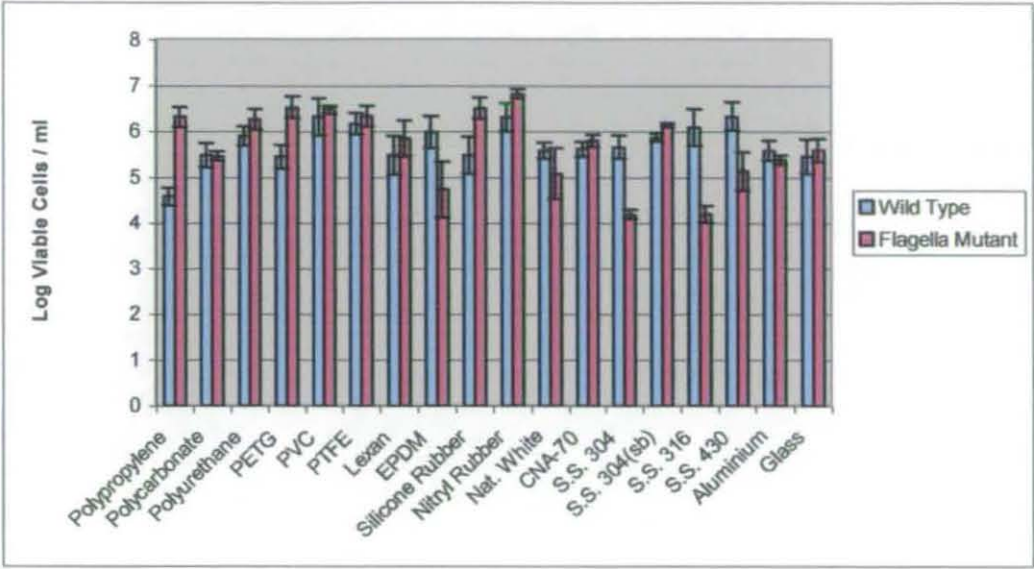


Figure 3.20 shows that the flagellin mutant attaches in significantly greater numbers than the wild type to polypropylene and in significantly fewer numbers than the wild type to stainless steels 304 and 316.

### 3.15 Investigation into the effects of temperature on adherence

The next series of results examined the effects of altering the temperature during the contact period. The assays were carried as described in section 2.11. Three different incubation temperatures were investigated, these were 4°C, 14°C, and 30°C. The first of these temperatures, four degrees was chosen as a direct comparison to refrigeration temperatures, and 14°C as an intermediary temperature. The four types of *L. monocytogenes* used were

the wild type (10403S), mutant M141 (a mutant that attaches to glass in low numbers), G111 (a mutant that attaches to glass in high numbers), and the flagellin mutant.

Figure 3.21 shows the number of wild type cells attached to glass at the three different temperatures. The differences in attached cells to glass at the three temperatures are statistically insignificant, with mean values of 5.97 log cfu at 4°C, 6.24 log cfu at 14°C, and 5.76 log cfu at 30°C.

**Figure 3.21** Number of cells attaching to glass coverslips at 4°C, 14°C and 30°C.

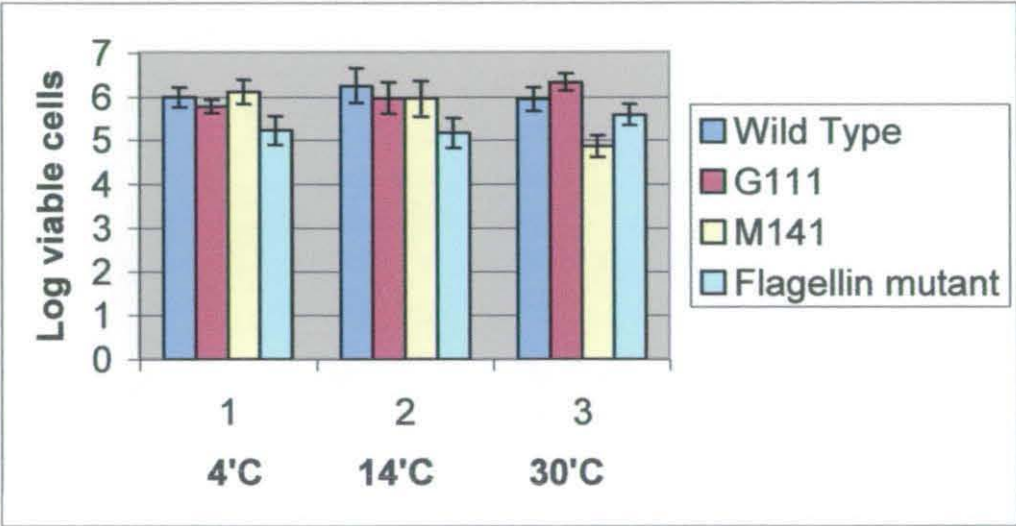


Figure 3.21 shows the number of mutant G111 cells attached to glass at the three different temperatures. The figure shows that mutant G111 (which had already demonstrated higher numbers of attached cells on glass at 30°C than the wild type), attaches to glass in a significantly higher frequency at 30°C (mean of 6.33 log cfu) than at 14°C (mean of 5.97 log

cfu) or 4°C (mean of 5.77 log cfu) ( $P<0.05$ ). It also shows that mutant M141 (which had already demonstrated lower numbers of attached cells on glass at 30°C than the wild type), attaches to glass in a significantly lower frequency at 30°C (mean of 4.87 log cfu) than at 14°C (mean of 5.95 log cfu) or 4°C (mean of 5.68 log cfu) ( $P<0.01$ ). The numbers of attached cells at each temperature of the flagellin mutant are statistically similar (mean values of 5.22 log cfu observed at 14°C, 5.59 log cfu at 4°C and 5.17 log cfu at 30°C respectively).

Comparing the wild type and the mutants to each other also uncovered some significant differences in attachment numbers. At 4°C the number of flagellin mutant cells attaching to glass (mean of 5.22 log cfu) was significantly lower than mutant G111 (mean of 5.77 log cfu) ( $P<0.05$ ), and the wild type 10403S (mean of 5.97 log cfu) ( $P<0.01$ ). At 14°C, the number of flagellin mutant cells attaching to glass (mean of 5.17 log cfu) was significantly lower than mutant G111 (mean of 5.97 log cfu) ( $P<0.05$ ), and the wild type 10403S (mean of 6.24 log cfu) ( $P<0.01$ ). Whilst at 30°C, the number of flagellin mutant cells attaching to glass (mean of 5.59 log cfu) was significantly lower than mutant G111 (mean of 6.33 log cfu) ( $P<0.05$ ), but not significantly different to the wild type (mean of 5.76 log cfu) ( $P>0.05$ ). There was also a significant difference in adherence of the flagellin mutant and mutant M141 to glass (M141 mean of 4.87 log cfu) ( $P<0.05$ ). There was a very significant difference between attachment to glass at 30°C between the mutants G111 and M141 ( $P<0.001$ ).

### 3.16 Scanning Electron Microscopy

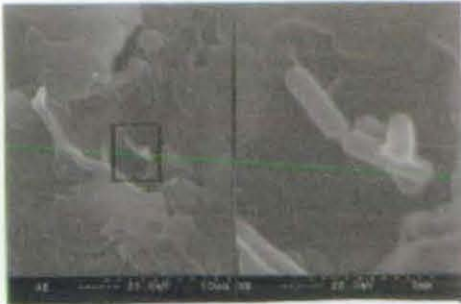
Samples of *L. monocytogenes* 10403S wild type bacterium were allowed two hours contact time with coupons of silicone rubber, PTFE and stainless steel 304. Following incubation the samples were fixed for Scanning Electron Microscopy (section 2.12). Figures 3.22 and 3.23 show *Listeria* cells attached to each of the three types of surface. *L. monocytogenes* 10403S does not appear to form complex biofilm structures following a two hour contact time, but instead forms a sparse monolayer of cells along the surface of the material. Cells can be clearly seen nestling in the pits of stainless steel 304 (Figure 3.23). There is no visual evidence of the production of anchorage material such as sticky exopolysaccharide.



Figure 3.22 SEM Microphotographs of *L. monocytogenes* attached to materials



Silicone Rubber



Silicone Rubber



Silicone Rubber



PTFE



PTFE



Silicone Rubber

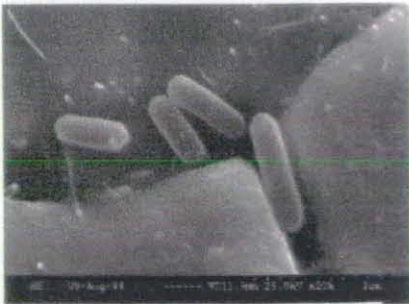


PTFE



PTFE

**Figure 3.23 SEM. Microphotographs of *L. monocytogenes* attached to materials**



**Stainless Steel 304**



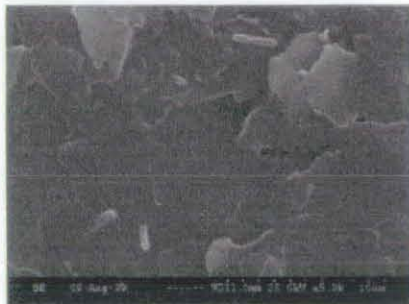
**Stainless Steel 304**



**Stainless Steel 304**



**Silicone Rubber**



**Silicone Rubber**



**Silicone Rubber**



**Silicone Rubber**



**Silicone Rubber**



## Chapter 4: Discussion

The adhesion assay was developed by comparing various methods of attached cell removal and by comparing different lengths of contact time of materials with bacterial culture. There was no significant difference between vortexing, vortexing with glass beads and sonication as methods of removal of cells from materials (see Figure 3.2). Several laboratory-based methods have been employed for removal and enumeration of attached bacterial samples. Many of these methods have been developed to test antimicrobials. The most commonly used methods are surface scraping (Jeong and Frank, 1994), sonication (Green and Pirrie, 1993), stomaching (Gagnon and Slawson (1999), vortexing (Anwar *et al.*, 1992), and shaking in the presence of beads (Bloomfield *et al.*, 1993). Jeong and Frank (1994) demonstrated that surface scraping removed up to 97% of all cells attached to stainless steel coupons. Scraping is the most popular removal method (Costerton *et al.*, 1978). Anwar *et al.*(1992), demonstrated that vortexing removed up to 99% of cells attached to silicone tubing. One of the best comparisons of removal methods was by Lindsay and Von Holy (1997) who compared attached cell removal by vortexing, sonication and shaking with beads. They found, as was found here, that there was no significant difference between the numbers of attached cells removed by each of the three methods. Scanning electron microscopy later revealed that shaking with beads removed extracellular polysaccharide most efficiently from the stainless steel and polyurethane surfaces used in the experiments (Lindsay and Von Holy, 1997).

An important consideration was the measurement of reversibly bound cells and those carried over as part of a droplet of media on the material. These cells account for up

to 54% (on glass) of all cells counted in the assay during short contact times (see Table 3.1), and up to 33% (on 'Lexan', a polymer) after 2 hours contact time (see Table 3.2). The magnitude of this population of cells shows that distinguishing them from irreversibly attached cells is very important in designing a reliable adhesion assay. Failure to distinguish these cells would have significantly altered the results just as the method of removal by forceps can further complicate the interpretation of results. The findings presented here were that holding the coupon by its corners significantly increased the volume of liquid droplet, and hence the number of planktonic cells carried over into the fresh media for counting (see Figure 3.4). Only Norwood and Gilmour (1999) have made an attempt to factor these other two types of transferred cells into the equation. Their research reported on the adhesion of over 100 strains of *L. monocytogenes* to stainless steel. In their study, they discounted from their total counts cells present in the carried-over liquid. However, in their account of carry-over they also may have counted some weakly attached cells. They assumed that all bacteria present instantaneously were carried over in adherent liquid. However, when the amount of adherent liquid was measured it was insufficient to account for the numbers of *Listeria* associated with the coupons. Thus it was concluded that some *Listeria* become instantaneously bound.

Although only semi-quantitative in terms of analysis of adhesive forces, tests in which samples or coupons of material are immersed in liquid culture and then withdrawn after a specified period of time, in order to estimate the number of adherent cells, are valid and widely used for comparative testing of adhesion. However, although apparently simple in concept, care has to be exercised in the undertaking and interpretation of these tests. For example, failure to account for planktonic cells

present in medium adherent to the coupon or very weakly adherent bacteria may lead to erroneous conclusions.

Tables 3.5 and 3.6 (section 3.1) show the mean number of cells recovered from short contact times and 2 hour contact times with each of the 18 materials. With the exception of polypropylene there is a significant different increase in the number of cells recovered. Similarly, with the exception of natural white rubber, a significant decrease in the percentage of coupon associated *Listeria* shed during washing. These results support the hypothesis that bacterial attachment is time dependent. This theory is confirmed by the results presented in Figure 3.1.

Marshall *et al.* (1971) proposed a two-step time-dependent model for adhesion of marine bacteria to a surface (glass). First there was an instantaneous reversible adhesion, with only weak interactions occurring between the bacteria and the surface, this was followed by irreversible adhesion mediated by the formation of extracellular material. The data presented here show that this model is generally true for *L. monocytogenes* binding to a wide variety of materials given the significant difference in numbers of cells attached to each surface (excepting polypropylene) between short contact and two hours contact time (see figure 3.5). This is underlined by the simple staining assay (see figure 3.6). The numerical data also support the notion of reversibly and irreversibly bound populations. However, the data apparently are at variance with the model of Marshall *et al.* in that reversibly and irreversibly bound populations are present instantaneously and reversibly bound cells continue to be found even after two hours contact time. However, in accordance with the theory of Marshall *et al.*, the proportion of irreversibly bound cells increases with time.

Another view of the comparison of these data and those of Marshall *et al.* is that while they both support the concept of different populations of bacteria with different adherence strengths, it may be erroneous to conclude that there are just two populations. Thus in the method used here where the washing was as gentle as possible, some bacteria may have been removed but there was a tendency to over estimate those irreversibly bound. Marshall used a more vigorous washing procedure and therefore may over estimate the reversibly bound fraction. Smoot and Pierson (1998) also examined short contact time adhesion and like Marshall *et al.*, employed a relatively harsh washing procedure with possibly the same outcome. An alternative conclusion is that there is a continuum of adhesive strengths within the populations of cells. This might occur if, following the initial cell-surface interactions, the strength of the bond formed between any cell and the surface increased with time in such a way that the force required for detachment was also time dependent. In this analysis no attempt has been made to quantify the adhesive forces that define each population. Terms such as 'reversible,' 'irreversible,' 'strong' and 'weak', are only operational terms within the limits of the assay used. For exact determination of the forces involved, test materials would need to be placed in well-characterised shear fields. Bos *et al.* (1999) go further, and claim that it is essential to eliminate any manipulations, such as drawing coupons through a liquid-air interface, that might subject the biofilms to unquantifiable shear forces.

My experiments revealed that a contact period of two hours recovered an equal amount of strongly attached cells over contact times of 2 hours to as long as one week (see Figure 3.1). This result is also in accordance with that of Mafu *et al.* (1990) who found that the attachment of *L. monocytogenes* cells to stainless steel, rubber,

polypropylene and glass, did not show an increasing trend with contact time (20 mins versus 1 hour). However, the presence of extracellular material was detected at contact times of around 1 hour. This result would suggest that according to the hypothesis of Marshall *et al.* (1971) irreversible attachment was starting to occur after approximately 1 hour contact time.

The lack of a significant increase in the numbers of attached cells after the a two hour contact time suggests that the listeria may have reached a maximum level of attachment to the material and will not continue attaching to form the advanced microcolonies or biofilm structures described by Costerton *et al.* (1978). This does not discount the theory that although the numbers may stay the same the biofilm may be maturing in other ways, e.g. the formation of extracellular polysaccharide (EPS) (Davies *et al.*, 1993). Electron microscopy experiments by Sasahara and Zottola (1993) also found *L. monocytogenes* to only attach to glass in sparse numbers. The scanning electron microscopy pictures presented here show a similar pattern, though there is always a possibility of damage and dislodging of attached cells during the harsh preparation conditions for electron microscopy. This result is discussed later in the thesis.

Alteration of the starting planktonic culture affected the number of cells adhering proportionally (see Figure 3.3). The number of cells attaching to materials increased linearly with increasing planktonic culture. This result suggests the possibility of a sub-population of cells within the total planktonic culture maybe responsible for attachment. However an experiment in which coupons were added sequentially to a planktonic culture and removed every two hours found no significant difference

between the first and last coupon added and removed. This experiment is difficult to standardise as it must be assumed that the planktonic population was increasing with time due to growth and therefore the final coupon added to the culture will be exposed to an increased population of cells compared to the first coupon added. Although the possibility that some increase in the numbers of planktonic cells occurred due to growth over the assay period cannot be ruled out, the results are nonetheless interesting. Future work should attempt to investigate or monitor the attachment and growth of these cells over time.

It has previously been shown that *L. monocytogenes* will adhere to surfaces found in a food processing environment (Mafu *et al.*, 1990; Helke and Wong, 1994; Smoot and Pierson, 1998). Disagreements between different groups of workers in the number of *L. monocytogenes* cells adhering to materials have been frequently reported. Smoot and Pierson (1998) showed that *L. monocytogenes* adheres to stainless steel in greater numbers than buna-nitril rubber. Whereas Blackman and Frank (1996) demonstrated that the organism adhered to polyester floor sealant in greater numbers than nylon, Teflon, and stainless steel. Some of the reported studies were quantitative whilst others were based on qualitative methods such as microscopic examination. Much of the previous work on the attachment of *L. monocytogenes* to surfaces has tended to focus on only a small number of materials, typically stainless steels and only one type of rubber (Buna-N).

Some of these studies have been quantitative whereas others have been primarily descriptive. This present study is the most comprehensive, quantitative analysis of the adherence of *L. monocytogenes* to materials that are encountered in the food

processing industry. Mafu *et al.* (1990) measured contact angles and surface energies for a restricted range of materials including some that were examined here. Given the range of surface properties of the coupons used in this study it was surprising that there were only minor insignificant differences in the degree of attachment that occurred either instantaneously or after two hours contact time. Mafu *et al.* (1990) had reported the ability of *L. monocytogenes* to adhere to materials of widely differing hydrophobicity but without quantification. These findings have been confirmed and extended. The adherence ability reinforces the view of *Listeria* as a micro-organism able to populate widespread niches.

At this stage there is no obvious explanation for the observation that numbers of *Listeria* strongly adhering to polypropylene do not increase with time. An explanation may come with a greater understanding of the mechanism of binding of *L. monocytogenes* to these various materials. The molecular basis of the capacity of *L. monocytogenes* to adhere to many materials is unknown but the findings of this work argue against a single mechanism.

It is likely that strain-to-strain differences in bacterial characteristics such as surface charge, surface hydrophobicity, and the ability of *L. monocytogenes* to produce other surface adhesins, including extracellular polysaccharides and flagella are responsible for differences in adhesion. However there is disagreement over the role of surface structures, since non-fimbriated and non-flagellated cells have been reported to attach in numbers similar to those of cells that possess these structures (Dickson & Koohmaraie, 1989). However, other reports indicate that motile bacteria attach to surfaces more rapidly than non-motile strains (Busscher & Weerkamp, 1987). It has

been demonstrated that at 37°C, flagellar development was so poor that listeriae appeared non-motile (Jones & Seeliger, 1986). The wild-type bacterium expresses peritrichous flagella according to temperature, being flagellated and motile at 20 to 25°C but non-motile and with very few flagella above 35°C. To investigate the potential effects of flagella expression in the attachment of *L. monocytogenes*, a flagellin mutant of *L. monocytogenes* obtained from Dr CE Rees at Nottingham University was used here.

The non-flagellated (*fla2*) flagellin mutant was constructed by insertion of the *luxAB* genes (Jacobs *et al.*, 1991) into the *flaA* gene by double homologous recombination using the temperature-sensitive shuttle vector pAUL-A (Chakraborty *et al.*, 1992). This mutant produces a 7-kDa truncate representing the N-terminal end of the flagellin protein. The use of a mutant which lacks the ability to produce flagella allowed direct investigation into the role of the flagellum in attachment and to separate this from the effect of temperature on the ability of *Listeria* to attach to a surface.

In the experiments reported here, at 30°C there was no difference between the numbers of flagellin mutant cells attached to glass and the wild type (see figures 3.20-3.21). At lower temperatures (4°C and 14°C) the flagellin mutant attached in significantly fewer numbers than the wild type (see figures 3.20-3.21). The reasons for this are as yet unclear. If at all, the lack of difference at 30°C between the mutant and the wild type suggest that flagella expression has the effect of reducing adhesion. Mutant G111 demonstrated elevated attachment to glass at 30°C, whereas mutant M141 attaches to glass in lowest frequency at 30°C, but in comparatively elevated frequency at 4°C and 14°C. Interestingly, the flagellin mutant did not attach to glass



in reduced numbers at 30°C though it did attach in reduced numbers at 4°C and 14°C (see figures 3.20-3.21).

The *L. monocytogenes* flagellin mutant was found to attach in lowest numbers to metals; stainless steel 304 and 316 in particular. Low numbers of adhered cells were found on glass but not as low as on metal materials. This result correlates with the findings (albeit with different assay conditions) of Chamberlain (1992), who also found that glass discouraged bacterial adhesion due to its low solid-solution interfacial energy characteristics, its high hydrophilicity, and also because the *L. monocytogenes* cell and the surface are similarly charged (Bower *et al*, 1995). For this reason hydrophilic materials such as glass are therefore frequently used for surfaces that will contact food. As glass (Chamberlain, 1992), and 22 strains of *L. monocytogenes* (Mafu *et al.*, 1991), are both described as hydrophilic, then theoretically they should repel and hinder attractive forces. Vantanyoopaisarn *et al.* (2000) found that at 22°C this same flagellin mutant of *Listeria monocytogenes* was found to attach to stainless steel at levels 10-fold lower than wild-type cells, even under conditions preventing active motility. At 37°C, when flagella are not expressed, attachment of both strains was identical and lower than the wild type at 22°C. Their conclusion was that flagella *per se* facilitate the early stage of attachment. If flagella are implicated in adhesion, then we would expect to see a similar trend of the wild type attaching in greater numbers than the flagellin mutant at 4°C and 14°C but not at 37°C when neither flagellin mutant nor wild type are expressing flagella. This is exactly what was found. The mutant attached in lower numbers than the wild type at both 4°C and 14°C but not at 30°C. A more useful control would have been to have used the wild type strain for the flagellin mutant (NCTC 7973) rather than strain 10403S as we may simply be

witnessing strain variation between results. Using an elevated temperature of 37°C for the assay would have allowed better comparison with the results of Vantanpooyaisarn *et al.* Peel *et al.* (1988) used microscopy to show that flagella expression in *L. monocytogenes* was temperature-dependent. They found flagella expressed in large numbers at 20°C whereas very few were expressed at 37°C. This result implies that flagella expression is not completely halted at 37°C. This raises questions of how gradual the decline in flagella expression is between 20°C and 37°C. This factor is obviously crucial in analysis of results. We can assume that the level of flagella expression was probably affected in the wild type at 30°C in our experiment, though it is difficult to estimate by what degree. A further investigation using the wild type for the flagellin mutant and a control monitoring actual flagella expression (for example silver-staining) would be necessary to give conclusive proof of an effect.

The high level of attachment of the *Listeria* (in particular the flagellin mutant) to sand blasted stainless steel 304 corroborates with the findings of Stone and Zottola (1985) who examined the microstructure of various stainless steel finishes by SEM. They found that these cracks, crevices, or other surface imperfections may have allowed the surface to harbour bacteria and reduce the effectiveness of PBS washing accounting for the elevated attachment to sandblasted stainless steel 304. They demonstrated that the number of cracks and irregularities varied greatly for the different finishes. Sandblasting changed the microscopic texture of the surface by creating crevices, scratches and other surface imperfections that could promote adherence of bacterial cells and nutrients with which to begin forming a biofilm layer. There is also an increased surface area for attachment. Microorganisms in these crevices are shielded from the standard cleaning procedures in food manufacturing environments and

represent a source of contamination. Figure 3.23 shows *L. monocytogenes* cells clearly residing in the grains of stainless steel and demonstrates how bacteria may become shielded. Even under conditions where biofilms are unlikely to form, if the texture of a surface makes it difficult to remove microorganisms, then the surface may play a role in contamination at a later time. For this reason, proper equipment design is essential to avoid cracks and 'dead' areas in which organic material could accumulate.

The aim of this project was to identify chromosomal genes involved in the attachment of *L. monocytogenes* 10403S to materials commonly found in a food-processing environment. Transposon mutagenesis using a derivative of Tn917, Tn917-LTV3, carried on plasmid pLTV3 (Camilli *et al.*, 1990) was used for selection of mutant strains of *L. monocytogenes* that attached to materials in lower frequencies than the wild type bacterium following a two hour incubation period. The results in table 3.3 show the mean number of cells attaching for each of the transposon-insertion mutants investigated. The standard deviations of several of the mutants are skewed by one of the four replicates. In most cases this relates to one of the four replicates being larger than the other three replicates by some margin, but still lower than the wild type. One mutant (B380) was selected for further investigation on the basis of a low mean number of cells attaching after 2 hours contact time. This mutant was the first mutant to be checked for correct insertion of the transposon-insertion by Southern hybridisation.

Mutagenesis generating attachment mutants led to cloning and identification of genes involved. The chosen derivative, Tn917-LTV3, has been demonstrated to transpose at a significantly elevated frequency, generating transcriptional *lacZ* fusions when

inserted into a chromosomal gene in the appropriate orientation. It also allows rapid cloning of flanking DNA into *E. coli* (Camilli *et al.*, 1990).

The nonconjugative *Enterococcus faecalis* transposon Tn917 was selected for transposon mutagenesis of *L. monocytogenes* due to its ability to generate very stable insertion mutations in Gram positive bacteria. It had previously been employed successfully in mutagenesis of the *L. monocytogenes* chromosomal genes (Cossart *et al.*, 1989; Youngman, 1989; Camilli *et al.*, 1990). Spontaneous excision of Tn917-LTV3 is known to occur at frequencies lower than  $10^{-10}$ /bacteria (Berg *et al.*, 1989). The transposon was isolated from the *E. faecalis* plasmid pAD2 and encodes MLS (i.e., resistance to erythromycin, lincomycin and spiramycin) drug resistance (Tomich *et al.*, 1980).

The electroporation method of DNA transformation was originally used with eukaryotic but has been subsequently used to transform *E. coli* (Dower *et al.*, 1988; Taketo, 1988, Luchansky *et al.*, 1988). Comparing to the other mechanisms for transferring plasmid DNA, electroporation is less time consuming and less tedious method available for recovering plasmid-containing transformant. Although the precise mechanism is unknown, presumably the high voltage produces transient pores at protein-lipid junctions in the cell membrane that allow for intracellular penetration by exogenously supplied DNA (Luchansky *et al.*, 1988).

Youngman *et al.* (1984a), found that the choice of *E. coli* strain used as a transformation recipient can be of critical importance when recovering chromosomal sequences adjacent to the *erm*-proximal end of a Tn917 insertion. They discovered that *erm*-containing fragments rescued from pTV20 or pTV21-derived integrants

could not be maintained in a “typical” *E. coli* host, such as MM294. Although the reason for this was not definitely determined, it was speculated that the *erm* gene product may be toxic to *E. coli* strains because of its ability to methylate ribosomal RNA. When strain HB101 was used this toxicity was not observed, however, perhaps due to the fact that this strain contains *rpsL20* mutation, which alters the ribosome in a way that confers streptomycin resistance. Thus, HB101 or similar strains are recommended whenever rescued fragments are expected to include the *erm* gene (Camilli *et al.*, 1990).

To recover Tn917-LTV3 insertion mutants, bacteria carrying pLTV3 were at first cultured at a low temperature (30°C) to stationary phase with selection for tetracycline resistance and for Tn917 encoded erythromycin and lincomycin resistance. To remove the plasmid from the Tn917-carrying population the bacteria were then cultured at the non-permissive temperature (41°C) with erythromycin and lincomycin resistance selection. These antibiotics induce the transposition of Tn917 because expression of the transposase via transcription from the *erm* gene into the *tnpA* gene. The high temperature of incubation does not allow the replication of pLTV3 since it contains pE194 origin of replication which is sensitive to temperatures higher than 37°C (Villafane *et al.*, 1987). Surviving bacteria should have acquired a chromosomal Tn917 insertion. Transposon insertion mutants maintained erythromycin and lincomycin resistance but lost plasmid encoded tetracycline resistance.

The randomness of 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 (Sherratt, 1989). Tn917 insertions into the

chromosome of *B. subtilis* and *B. megaterium* have also been found to be non-random (Vandeyser and Zahler, 1986). In Tn917 mutagenesis of *B. subtilis* chromosome, 99% of all insertions 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). Camilli *et al.* (1990), found that although the distribution of *L. monocytogenes* Tn917-LTV3 insertional auxotrophic mutations was not completely random, many different kinds were recovered. Insertions within the *hlyA* gene were more frequent than would be expected on a purely random basis, suggesting that this gene may be within one of the hotspot regions of the *L. monocytogenes* chromosome (Camilli *et al.*, 1990).

A key advantage of generating mutants by gene disruption is the fact that adjacent DNA can then be cloned easily into *E. coli*. In the case of disruptions mediated by Tn917, methods have been developed for integrating an *E. coli* replicon at the sites of existing transposon insertions, which then may be used to rescue adjacent sequences (Camilli *et al.*, 1990; Gutierrez *et al.*, 1996). An advantage of this approach is that vectors used to deliver Tn917 can easily be modified to introduce additional restriction sites. Therefore, from a single transposon insertion, an extensive set of integrants might be obtained that have different kinds of restriction sites in different configurations, which creates the possibility of using a large variety of restriction enzymes to generate clones extending in either direction from the site of insertion (Camilli *et al.*, 1990; Gutierrez *et al.*, 1996). In this investigation *L. monocytogenes* DNA adjacent to the transposon insertion, in mutants defective in attachment, was rescued into *E. coli* simply by digesting total chromosomal DNA with an appropriate restriction enzyme (*Xba*I), which cuts outside the selectable marker *neo*, the ColE1

replication functions and DNA sequences of the inactivated gene. This fragment was recircularised and transformed in an *E. coli* strain. A 1.8kb fragment containing listerial genomic DNA was then digested out and cloned into pBluescript for sequencing.

The automated sequencing system was selected for sequencing the listerial DNA flanking the site of Tn917-LTV3 insertion in mutant B380 (Section 2.7). Using this system reads of sequence as much as 700bp or more were recovered from a single experiment. The aim of sequencing the listerial DNA flanking both sites of the Tn917-LTV3 insertion in mutant B380 was to investigate DNA and amino acid homologies between the invaded sequence and sequences in the nucleic acids or protein databases.

Analysis of the sequence of the listerial DNA showed the transposon had inserted into an ORF. This was termed ORF1 (see figures 3.15-16). The nucleotide sequence of ORF1 was aligned against the sequenced *L. monocytogenes* EGD genome held at the Institute Pasteur. ORF1 matched a sequence (93%) in the sequenced genome that is annotated as a gene for a hypothetical sugar hydrolase similar to the *ybgG* gene of *E. coli* (see Appendix I). The position of the putative sugar hydrolase gene was downstream of a phosphotransferase (PTS) system and upstream of a transcriptional antiterminator. Using this information, and upstream and downstream data supplied by Pierre Dehoux (Institut Pasteur) it was possible to design primers to amplify ORF1 for further investigation.

Genetic complementation was the strategy selected to investigate the possibility of polar effects of genes adjacent to the gene interrupted by Tn917-LTV3. This approach would lead to restoration of wild type phenotype by complementing the

transposon mutant with a copy of the inactivated gene. This method is supposedly quicker and easier than creation of a site-directed complementation. However, the complementation is in the form of an autonomous plasmid and its limitations must therefore be considered. The most obvious limitation is the plasmid copy number. Therefore selection of a low copy number plasmid is essential to avoid complementing the mutant with a large number of copies of the invaded sequence.

To begin the complementation, the DNA fragment coding for the gene inactivated by Tn917-LTV3 was isolated. Then the fragment was cloned first into a cloning vector and secondly into a shuttle vector before introduction into the mutant host. Isolation of the gene sequence was performed by PCR (Section 3.10). As the ultimate aim was use in expression studies, the fidelity of DNA synthesis was of vital importance. For this reason the proof-reading *Pfu* DNA polymerase (Promega) was used. This polymerase has a high 5'-3' processivity and possesses a 3'-5' exonuclease activity also known as proof-reading activity. The inherent 3'-5' exonuclease proof-reading activity of *Pfu* results in approximately 12-fold increased fidelity of DNA synthesis compared to *Taq* DNA polymerase. When using *Taq* DNA polymerase [error rate of  $2 \times 10^{-4}$  errors/base (Cha and Thinly, 1993)] about 56% of a 200bp amplification product will contain at least a single error after one million fold amplification. In contrast when using *Pfu* DNA polymerase for amplification, only approximately 10% of the products will contain an error under the same conditions (Promega Corporation). A sequencing reaction was performed to confirm the fidelity of the PCR product. Sequencing data was compiled from at least two runs of reactions consulting chromatogram data where necessary.



A Promoter sequence is a region of DNA to which RNA polymerase binds before initiating the transcription of DNA into RNA. The nucleotide at which transcription starts is designated +1 and nucleotides are numbered from this with negative numbers indicating upstream nucleotides and positive downstream nucleotides. Most bacterial promoters contain two consensus sequences that seem to be essential for the binding of the polymerase. The first, the Pribnow box, is at about -10 and has the consensus sequence 5'-TATAAT-3'. The second, the -35 sequence, is centred about -35 and has the consensus sequence 5'-TTGACA-3'. The terminator program of the GCG Wisconsin Package (Genetics Computer Group, 1995), was used to investigate terminator sequences at the end of ORF1. A putative *rho*-independent terminator sequence was detected shortly after the ORF1 stop codon and is shown in Appendix II. The presence of promoter consensus sequences and a strong terminator sequence suggest that the phenotype change in mutant B380 caused by insertion of a transposon into ORF1 is not due to polar effects on other genes in a larger ORF, although this can not be ruled out completely. Investigation by RT-PCR would be the method employed to investigate this effect, however due to time constraints it was not possible to perform this experiment.

Using the BLASTX (Altschul *et al.*, 1990) amino acid database it was discovered that the amino acid translation of ORF1 in frame 1 showed 48% identity to the *L. monocytogenes* strain EGD putative alpha mannosidase, and showed similar homology to other glycosyl hydrolase family 38 alpha mannosidases in the database (see Figure 3.18 and Table 3.4). These findings will be discussed later in this section.

The shuttle vector chosen to clone the PCR product isolated, was pMK4 (Sullivan *et al.*, 1984). This plasmid is a bifunctional shuttle vector which replicates in both *Listeria* and *E. coli*, and has been successfully used in *Listeria* complementation studies (Cossart *et al.*, 1989; Michel *et al.*, 1990; Ripio *et al.*, 1997). Vectors of this type are very useful since they allow the use of the well-developed techniques for the isolation and manipulation of plasmids in *E. coli*. The resulting recombinant molecule can then be introduced into *Listeria* by transformation. This has been important because the generation of recombinant plasmids by direct transformation of ligation mixtures into Gram-positive bacteria is very inefficient (Trieu-Cout *et al.*, 1987). pMK4 contains the *lacZ* gene, which includes the respective operator and promoter, and encloses several unique restriction sites. cloning into any of these sites disrupts the *lacZ* gene, causing a loss of the  $\alpha$ -complementation and results in a Lac<sup>-</sup> phenotype in the appropriate *E. coli* host strain. The recombinant plasmid can then be readily isolated and transformed into *L. monocytogenes*. In the case of this investigation the *EcoRI* site lies outside of the *lacZ* gene and therefore blue white selection was not available.

The fragment to be cloned was removed from pMB3 by *EcoRI* restriction digest. This fragment was ligated into *EcoRI* digested pMK4. The ligation mixture was then transformed by electroporation into *E. coli* DH5 $\alpha$ . No colonies were obtained. The process was repeated using a selection of controls (see Section 3.11). These controls included a variation of ligation ratios (vector to insert) varying from 1:1 To 1:20, re-circularisation experiments, as well as using a substitute shuttle vector (pCK1), substitute insert DNA fragment and substitution of reagents and enzymes (see section

3.11). Due to time constraints there was not enough time to continue this experiment further.

As a control for the final section of the project pMK4 was transformed into *L. monocytogenes* using the electroporation of penicillin treated cells (Park and Stewart, 1990). This method takes advantage of the fact that cell-wall damage, caused either by degradative enzymes or by incorporating cell-wall active agents in the growth media, typically improves transformation efficiencies in Gram-positive bacteria. Park and Stewart (1990) observed that a pre-treatment with penicillin-G, caused a dramatic increase in transformation efficiency of *L. monocytogenes*, levels as low as 100ng/ml producing a 60-fold stimulation over the untreated control. The optimal conditions for electroporation of penicillin-G treated *L. monocytogenes* (optimal number of transformants  $4 \times 10^6/\mu\text{g}$  DNA) was achieved in the presence of 10 $\mu\text{g}$  penicillin-G/ml and electroporated at a field strength of 10kV/cm (pulse duration, 5ms) (Park and Stewart, 1990). Due to the lack of a Gene-Pulser which reached 10kV/cm, the voltage used to electroporate *L. monocytogenes* in this study was 2.5kV/cm (pulse duration approximately 5ms) (Section 2.3.2b). Using this method, control vector pMK4 (without insert) was successfully transformed into *L. monocytogenes* though due to the failure to clone the insert into the shuttle vector the complementation could not be completed.

The putative conserved domain detected in ORF1 is typical of the glycosyl hydrolase family 38 (Accession number: PF01074). The glycosyl hydrolase family 38 comprises enzymes with only one known activity; alpha-mannosidase (CAZy-Carbohydrate Active Enzymes website; Henrissat B, Coutinho P, Deleury E). The enzyme catalyzes the hydrolysis of terminal, non-reducing alpha-D-mannose residues

from alpha-D-mannosides, and can cleave all known types of alpha-mannosidic linkages.

Glycosyl hydrolases are part of the glycosidase family (Henrissat, 1991). These 'O-Glycosyl hydrolases' (EC 3.2.1.-) are a widespread group of enzymes responsible for the hydrolysis of the glycosidic bond between two or more carbohydrates or between a carbohydrate and a non-carbohydrate moiety (Henrissat, 1991). The enzymes are named and classified according to the IUB-MB Enzyme nomenclature (1984). This is based on substrate specificity, molecular mechanism and amino acid sequence (Henrissat, 1991).

Enzymatic hydrolysis of the glycosidic bond takes place via general acid catalysis that requires a proton donor and a nucleophile or base. This hydrolysis occurs via two major mechanisms giving rise to either an overall retention, or an inversion, of anomeric configuration (Henrissat, 1991).

An API kit was used to investigate the hypothesis that an alpha mannosidase enzyme was down-regulated by transposon insertion. The result (see table 3.5 and figure 3.19) was that mutant gave a negative result for the alpha mannosidase test whereas a positive test result was obtained with the wild type. This suggests that alpha mannosidase is either (a) not being expressed at all in the mutant, or (b) has been expressed at a lower level e.g. the organism may have two or more copies of the gene and only one gene has been knocked out. Both scenarios are possible as the API kit probably works with a threshold for enzyme activity, which might not be detected

when one gene is not being expressed. Analysis of the genome would tell if more than one alpha mannosidase is likely.

For this reason a second experiment was devised to confirm whether the disrupted gene product was an alpha mannosidase and to see what effects of the transposon insertion into this gene sequence have on its expression.

The test involved preparation of cellular extracts from the mutant and wild type and a Bradford protein assay to standardise total cellular protein for each replicate. Once cellular extracts were standardised the extracts were tested for hydrolysis of a specific substrate (p-nitrophenyl-alpha-D-mannopyranoside (pNP-alpha-D-Man). This substrate test has been used previously for testing alpha mannosidase activity (Nankai *et al.*, 2002). As the results in Section 3.13 show, The mean of mutant B380 was significantly lower than that of the wild type (a two tailed P value of P.0007). This result confirms the API test result that the mutant is expressing less of the mannosidase than the wild type. However, it would have been far more conclusive to have used a probe sonicator for cell lysis prior to the assay as a bath sonicator (albeit for a 15 minute burst) may not have been sufficient to lyse the cell suspension correctly. There was a significant difference in the change in OD units / minute / mg of protein between the wild type and mutant over 17 replicates carried in three independent batches. This would suggest that perhaps the cells were partially lysed by the prolonged period of sonication, though clearly not as effectively as if a sonicator probe had been used.

This significant reduction (though not elimination) of alpha mannosidase activity in the transposon mutant affirms the GenomeNet program (Bioinformatics Centre, Institute for Chemical Research, Kyoto University) model of mannose metabolism that *L. monocytogenes* has more than one copy of the alpha mannosidase gene. The fructose and mannose pathway model of *L. monocytogenes* held on the GenomeNet database is shown in Figure 4.1. The part of this pathway directly of interest to this study is the conversion of mannan and 1,4  $\beta$ -Mannan to D- mannose. This D- mannose is then a precursor for the alginate pathway shown in Figure 4.2 and also for the production of extracellular D- mannose, which is known to be a constituent of Extracellular Polysaccharide (EPS) (Wai *et al.*, 1998, Leriche *et al.*, 2000).

We will now put forward a model for implication of this enzyme in attachment and biofilm development of *L. monocytogenes* to food processing surfaces. This model evolves around the production of extracellular substances involved in adhesion.

Cell constituents have been implicated in the adhesion and cell accumulation process. These constituents include extracellular polysaccharides or exopolysaccharides (EPS) (Davies *et al.*, 1993, Ronner and Wong, 1992, Skillman *et al.*, 1999, Ortega-Morales *et al.*, 2001, Wingender *et al.*, 2001). Indeed EPS has even been implicated in protection of microorganisms against sanitizers (Ronner and Wong, 1992).

EPS is usually either homo- or heteropolysaccharides of neutral, more commonly positive, or most likely negative charge (Skillman *et al.*, 1999). The percentage of water in EPS is could be as much as 98-99% according to Christensen and Characklis, 1990. The rest of the matrix is comprised of extracellular polymers (polysaccharides

and glycoproteins). These extracellular polymers are as yet poorly understood and characterised.

As well as the hypothesis that a sticky EPS matrix secreted by adherent cells, acts as an anchor, there is also a hypothesis that EPS cancels repulsive charges that would otherwise make attachment electrochemically unfavourable (Skillman *et al.*, 1999). On the other hand, excess EPS expression may prevent cell adhesion to solid surfaces (Marshall *et al.*, 1985).

EPS production may influence whether or not cells are able to attach surfaces (Shea *et al.*, 1991). It has also been shown to vary in composition during stationary and exponential phases of growth (Christensen *et al.*, 1985). Exponential phase EPS was shown to promote attachment of *P. aeruginosa* to hydrophobic surfaces whereas late phase EPS had the opposite effect. Furthermore, Marshall *et al.*, (1986) showed EPS production by cells in suspension differs from that produced by cells in a polymer matrix.

It has been hypothesised that *Listeria monocytogenes* does not form classic densely-populated biofilms but more of a sparse monolayer of cells (Sasahara and Zottola, 1993). The microphotographs of our scanning electron microscopy work concurs with this theory (see Section 3.16, Figures 3.22-3.23). Single cells are shown attaching to each of the three materials studied (silicone rubber, stainless steel 304 and PTFE). At no point was a dense biofilm of cells observed in ours, or their, surfaces.

A similar scanning electron microscopy study by Ronner and Wong (1992) demonstrated *L. monocytogenes* attached to stainless steel and buna-nitryl rubber was

producing EPS matrix. They found EPS production to be elevated on stainless steel surfaces with compared to buna-nitryl surfaces.

The work of Mafu *et al.*, 1990 also demonstrated EPS production of attached *L. monocytogenes* even after contact times as short as one hour. Again, the scanning electron photomicrographs shown in this research paper greatly resemble those in section 3.16. Sparse *L. monocytogenes* cells are shown on each of the stainless steel, glass and polypropylene surfaces. They concluded that *L. monocytogenes* can attach to stainless steel, glass, polypropylene and rubber after short contact time at ambient and cold temperatures. They also concluded that although the exact mechanism of attachment by *L. monocytogenes* is not known it may be associated with the presence of EPS (Mafu *et al.*, 1990). Although many research groups have studied EPS production in *L. monocytogenes* attached to surfaces. To my knowledge, no have studied the production of EPS at the genetic level.

Alginate consists of a linear copolymer of D-mannuronic acid and L-guluronic acid (Davies *et al.*, 1993) and represent major components of the EPS of mucoid *P. aeruginosa* and have been implicated in development and stability of biofilms formed by the bacterium on living and abiotic surfaces (Davies *et al.*, 1999, Wingender 2001). Alginate activity has been shown to be upregulated during attachment of *P. aeruginosa* to Teflon (Davies *et al.*, 1993). Using reporter gene technology ( $\beta$ -galactosidase), Davies *et al.*, (1993) demonstrated a greater than threefold increase of *algC* reporter gene activity in biofilm cells grown on Teflon mesh in comparison to planktonic cells. From this, they concluded that activation of this gene and production of bacterial polysaccharide (EPS) coincided with attachment



to a surface. Alginate production was first reported in *P. aeruginosa* by Linker and Jones in 1966. The organism has since been used as a model of an organism that preferentially assumes an attached state, embedding itself in EPS composed of alginate (Costerton *et al.*, 1987). The alginate pathway of *P. aeruginosa* has been widely studied and the organism has been shown to become embedded in EPS consisting of alginate during attachment and colonisation (Costerton *et al.*, 1987). Occasionally, *P. aeruginosa* produces an alginate lyase enzyme, which cleaves the polymer into short oligosaccharides. This cancels the anchoring properties of the alginate and leads to increased detachment of the bacteria away from the surface. This underlines the importance of both alginate biosynthetic and degradative enzymes in cellular colonisation and spread (Boyd and Chakraborty 1995). To my knowledge the alginate pathway and its implications in biofilm formation has not been studied in other organisms although groups have studied EPS in other organisms. However the enzyme pathway responsible for alginate production is part of the pathway for fructose and mannose production in many bacteria including *L. monocytogenes*. (see Figure 4.1). The pathway shown in Figure 4.1 is the fructose and mannose pathway for *L. monocytogenes*. The pathway contains a more complex outline of the alginate pathway shown in Figure 4.2. The production of D-Mannose is partly dependent on the catalysis of 1,4- $\beta$  Mannan and Mannan to D-Mannose by three alpha mannosidase enzymes: mannan 1,2-(1,3)-alpha-mannosidase (1,4- $\beta$  Mannan catalysis, Eriksson, 1968), mannan 1,2-(1,3)-alpha-mannosidase (Mannan catalysis, Jones and Ballou 1969) and mannan exo-1,2-1,6-alpha-mannosidase (Mannan catalysis, Takegawa *et al.*, 1989) respectively. As the production of D-Mannose by these three enzymes is consequentially important in the synthesis of alginate, it would hence follow that removal of one of these enzymes would have a downstream effect of reducing

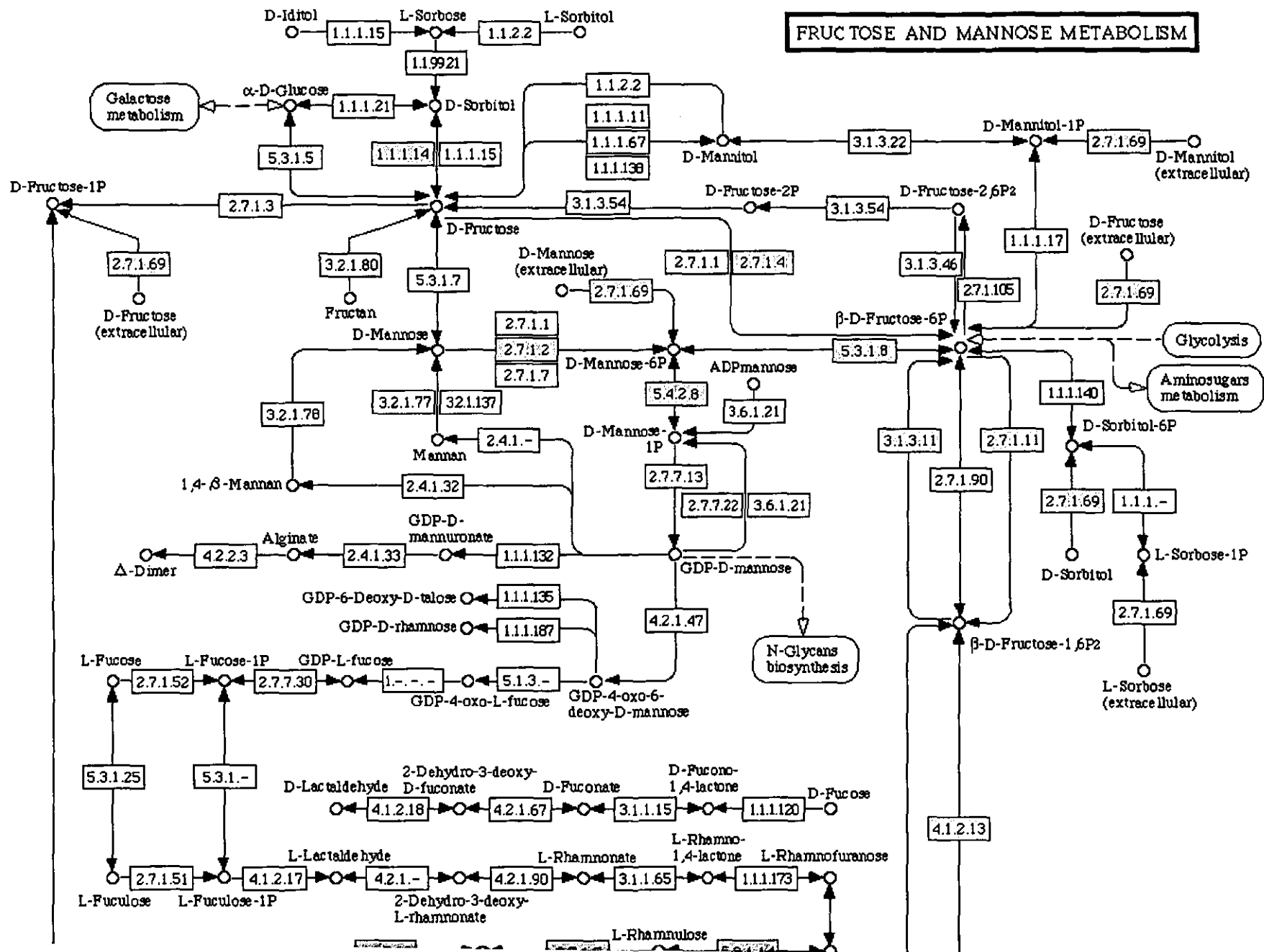
alginate synthesis and extracellular D-Mannose synthesis. This hypothesis is highly speculative as the model is circular. Further experiments perhaps involving site-direct mutagenesis to knock out other enzymes in this pathway would be necessary before any significant conclusions could be drawn.

While *L. monocytogenes* is known to form attached layers on various surfaces (Beresford *et al.*, 2000), nothing is known of the role of EPS in this attachment and colonisation of this bacterium. The results of this thesis suggest that transposon insertion into ORF1 has disrupted a region of the *L. monocytogenes* genome apparently responsible for expression of an alpha-mannosidase potentially important in the alginate pathway. The GenomeNet model for *L. monocytogenes* based on a collection of enzyme pathways from other organisms (Eriksson, 1968, Jones and Ballou 1969, Takegawa *et al.*, 1989) suggests *L. monocytogenes* has at least two of these enzymes although there is no published data to corroborate this or the existence of an alginate pathway in *listeria*. From this collection of data from other organisms we know that alpha mannosidase enzymes of the family glycosyl hydrolase 38 are responsible for hydrolysis of mannan and 1,4- $\beta$ - to D-mannose. This D-mannose (along with fructose-6-phosphate) is then converted to alginate via D-mannose-6-phosphate, D-mannose-1-phosphate, GDP-Mannose and GDP-Mannuronic acid intermediaries. We therefore suggest a hypothesis that decreased alpha mannosidase expression may lead to a downshift in D-mannose production and hence disruption of the alginate synthesis pathway responsible for EPS production and hence decreased attachment to food processing surfaces. As has been explained, this hypothesis is reasonable, but highly speculative, and further experiments would be required to investigate this effect further.

**Figure 4.1 Fructose and Mannose Pathway in *L. monocytogenes***

**Taken from GenomeNet service, Bioinformatics Center, Kyoto University.**

## FRUCTOSE AND MANNOSE METABOLISM





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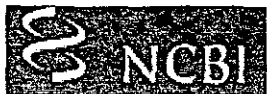
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# Blast 2 Sequences results

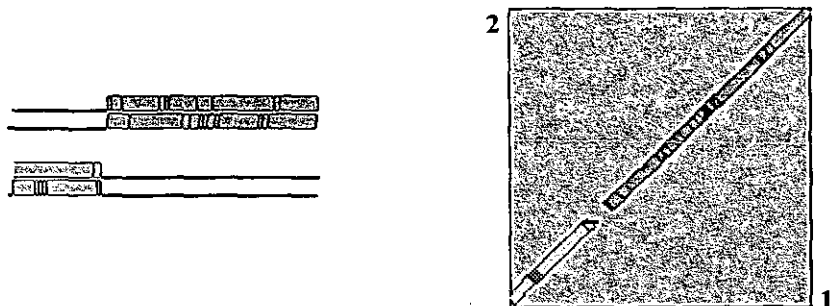
[PubMed](#)[Entrez](#)[BLAST](#)[OMIM](#)[Taxonomy](#)[Structure](#)

## BLAST 2 SEQUENCES RESULTS VERSION BLASTN 2.2.3 [Apr-24-2002]

Match:  Mismatch:  gap open:  gap extension:   
x\_dropoff:  expect:  wordsize:  ☒ Filter

Sequence 1 lcl|seq\_1 Length 3868 (1 .. 3868)

Sequence 2 lcl|seq\_2 Length 3916 (1 .. 3916)



NOTE: The statistics (bitscore and expect value) is calculated based on the size of nr database

NOTE: If protein translation is reversed, please repeat the search with reverse strand of the query sequence

Score = 3822 bits (1988), Expect = 0.0  
Identities = 2447/2625 (93%), Gaps = 27/2625 (1%)  
Strand = Plus / Plus

The figure shows a sequence alignment visualization. It consists of two horizontal bars representing the sequences. The top bar is labeled 'Query' and the bottom bar is labeled 'Sbjct'. The alignment is shown as a series of vertical lines connecting the two sequences.

Query: 1254 ggctactactacggagcgaacattcct-aaaatgaagccgaacttaaacctatttggat 1312

Sbjct: 1309 ggctactactacggagcgaacattcctgaaaatgaagccgaacttaaacctatttggat 1368

Query: 1313 gaacaaattggcgctctagaataaaaagcttcaacgccaatgtctatttcccaaattggc 1372

Sbjct: 1369 gaacaaattggcgctctagaagaaaaagcttcaacgccaatgtctatttcccaaattggc 1428

Query: 1373 ttagaccaagcgccagtcagaaaaaatttgccggaattggttgc-aaattcaatgaatta 1431

Sbjct: 1429 ttagaccaagcgccagtcagaaaaaatttgccggaattggttgcgaaattcaatgaatta 1488

Query: 1432 gatagcacgcgggaataccaaatcgcttcaccagaaacatttttcggggatttagaaaaa 1491

Sbjct: 1489 gatagcacgcgggaataccaaatcgcttcaccagaaacatttttcgcggaatttagaaaaa 1548

Query: 1492 gatgtgaccgatttaccagaacattgcccgcgaattaacagaaggaaaacatagtcgcct 1551

Sbjct: 1549 gatgtgaccgatttaccagt-cattgcccgcgaattaacagaaggaaaacatagtcgcct 1607

Query: 1552 gcataaatcgattttctctactcgagctgatttaaaacaagcgatgaatcaaattgaaaa 1611

Sbjct: 1608 gcataaatcgattttctctactcgagctgatttaaaacaagcgatgaatcaaattgaaaa 1665

Sbjct: 1608 gcataaatcgattttctctactcgagctgattttaaacaagcgaacaatcaaattgaaaa 1667

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Query: 1911 tatcaaaatgactgcgtacattccagaagacaatttcacagtagaagatacgcaaggaa- 1969  
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Sbjct: 1968 tatcaaaatgactgcgtacattccagaagacaatttcacagtagaagatacgcaaggaaa 2027

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Sbjct: 3216 tgcagtGaaaaaag--CCgaagacagcgatgcctt-Cattacgcgtttttcaatccgta 3272

Query: 3226 ttTgaaaaaagaaattccattccaagaagttttccaaggcaaggAACGCCactttggatg 3285  
| | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |  
Sbjct: 3273 ttTACAAAAAgaactcatcattccagaagttttCCAAGQCAAGGAACGCCac-tTggaTg 3331

```

= 1465 bits (762), Expect = 0.0
ities = 966/1053 (91%), Gaps = 7/1053 (0%)
nd = Plus / Plus

```

[illegible]



ry: 1020 attgcacgcgaaatggggacataccatggctgt 1052  
|||||  
ct: 1102 attgcacgcgaaat-gggacatagcatggctgt 1133

time: 0.12 user secs. 0.04 sys. secs 0.16 total secs.

dbda K H  
1.33 0.621 1.12

pped  
dbda K H  
1.33 0.621 1.12

rix: blastn matrix:1 -2  
Penalties: Existence: 5, Extension: 2  
ber of Hits to DB: 42  
ber of Sequences: 0  
ber of extensions: 42  
ber of successful extensions: 30  
ber of sequences better than 10.0: 1  
gth of query: 3868  
gth of database: 6,672,505,300  
ective HSP length: 26  
ective length of query: 3842  
ective length of database: 6,672,505,274  
ective search space: 25635765262708  
ective search space used: 25635765262708  
0  
30  
6 (11.5 bits)  
26 (50.0 bits)  
12 (23.8 bits)  
22 (43.0 bits)

**Appendix II Position of transcription consensus sequences  
in Cloned fragment of ORF1 and upstream / downstream  
sequences**

**Key:**

TAGAGA: -35 sequence  
TTTAAT: Pribnow box typically situated around -10  
ATG: start codon of ORF1  
AAA: stop codon of ORF1  
AACTACCTAAAAAGGAGCTG: Putative terminator sequence

TGCCACGGGGATACTGATGTCACTAATTCTCGGCGGACCGCTTGGTTGGTTAAACCAAACGTTACTTGATT  
GGGTAAC TGAAATGTGTAAAAACGATACAAACGTCATTATTCTAGCACTTATTTTAGGAGCAATGATTGGA  
TTTGACCTTGGTGGACCTGTAAACAAAGCTGCTTGGATGGCTGGGAACGCGCTATTTATGAGTGGAATTTA  
TCTACCTTCAATCATGATCAACGTCACAATTGCAATTCGCCCACTTGGCTACGGAATCGAAACACTTTTAC  
CGCAAAAAGAATTATTCTACTACATTCAAAGAATCTGGAAATGGCGCCGCTAATCATGGGACTAATTTGGG  
GTCACGGAAGGTGCGATTCCCAATTTTACACTACGTAGCCCTGGTAAATTAATTCGCTAAATGTGATTGCC  
TGCGCGATTGGTAGTGCCGTAAACGATGGGACTTGGCGCTTATGTGAAAATGCCGCGATTGGTGGAATGTA  
CGGTTTCTTCACTATTGGTAACGGCTGGGCTTACTTAGTTGGTGGCTTGGTTGGTGCATTTATTATTGGAA  
TTTGTGCGAACTTATTCGTCAATTTTACAGAAAGAAACAGCTGCTGCAGACGATGCTGTGGATGATATC  
GATATTAGTTTGTGAAATAGAGATTAAATAATAGTTGGAGGATTTAATGAAAATGGCTAAAACGAAAGT  
ACACGTAATTCGCGATTTGCACTGGGATAGAGAAATGGTATTTCACTTCAAGCCGTTCAACAATCTATTTAG  
TGAAGCATTTAAAGAAGTAATTGAAACATTGGAAGCGAAAGACGATTACCATTTTACTTAATGGATGCG  
CAAAGCTCGTTAATAGAAGATTATTTGCACTACTGCCCGGAAGATAAAACAAGATTAGAAAACTGATTGC  
TGAAAAACGGCTTATTACCGGGCCATGGTACACACAAACGGGCCAATTAGTCATTTCCAAGAGTCGATTGG  
TAGAAACTTGTTATATGGTACGAGAATTGCACGCGAAATGGGGACATACCATGGCTGTCAGTTTTTGTCCC  
CGGATGCCGTTCCGACAAGGCGGGAAACCATGCCACAAAATTTTTTAAAGAATTTGGGAATTTCAAAAGTTT  
CTTGTTTTGGCCCCGGAGTTTCCGATTATCCCTTTTAAACCAAACCCGAATTTTTTTTTTGGCGCCGGGGGA  
TTAATCGGGACCGGGAAATGCCTTTTCGGAACCAAATTTCCCGTTTGGCTACTACTACGGAGCGAACATTC  
CTAAATGAAGCCGAATTTAAACCTATTTGGATGAACAAATTTGGCGCTCTAGAATAAAAAGCTTCAACGC  
CAAATGTCTATTTCCCAAATGGCTTAGACCAAGCGCCAGTCAGAAAAAATTTGCCGGAATTGGTTGCAAT  
TCAATGAATTAGATAGCACGCGGGAATACCAAATCGCTTCACCAGAAACATTTTTCGGGGATTTAGAAAAA  
GATGTGACCGATTACCAGAACATTGCCGCGCAATTAACAGAAAGGAAACATAGTCGCCTGCATAAATCGA  
TTTTCTCTACTCGAGCTGATTTAAACAAAGCGATGAATCAAATTGAAAACTTTTTAAAGCAATGTGCTAGAG  
CCTTTTCTTTTCCATCAGCTACTCACTTGGGAATCGCTACCCACACAATGAACGCGAGAAATTTGGAAGCT  
GATGTTTGAAAATGCTGCACACGATAGCATTGGTGGCTGTAATAGTGATACGACCAACCGCGACGTCAAAC  
ATCGTTACAAATTTGGCTTCGGATTTAGCGACCAATTTACTCGATTTAAACATGCGCCTAATCAGCGAAAAA  
ATCGAACAAAGACAACCGTTCCAATTTACCGTTTTCAACCCACTCCATACGAAAAAAGTGGCGTTATCAA  
ATGACTGCGTACATTCCAGAAGACAATTTACAGTAGAAGATACGCAAGGAACACGCTTGATACACAATTC  
TAGAAAAAACAGACCTAACCGAATACGCTTAAATCAACATATCGATCTAAACCCAAGCAGACCCGTTTATT  
TACCAGAAAAGGTCTTTTAGCAACGATGCTAGTAAACGTAAATAACGTGCCAGCGCTAGGATATGACACC  
ATCTACTTTAATTTAGAAAAAGAAACAGCAGAGCAAGAACCGACACAATCCGCCGCGACAAAATTCGAAA  
ACGAATTTTATGAAATCCAGTTAGCAGCCAATAATTAACGTAACGATTCATGATAAAAAAGCGGGCAGAAC  
TACACCGATCAAATGATTTTTGTGGGAAAATGGCGATGATGGCGATTTCGTACAACCTATTCTCCGCCACGA  
AAAGACCTTGTAATTTTCATCGAAGGAAGCGGTAGTGGAAGATAGAGTCTAGCATATCAAGCGTCAACCAA  
GCATTAACTATTTCTTCAAGTTAAATGTACGGGTATAATTTAGAAGAACGTGCAATGGTGAAAAAATA  
ACCGGAAATGACCTATCAAAAAACGATTTTTCTTTTACGTAAAAAATGAAGAACTCATTGCTTTTGATGT  
AAGTTGCTTAATCAAAGTATTGAGTCATCGTTTATGTGTTACATTCGCCCCGAAATTGCTAGCAAATTC  
AAACAGCTGATCAATTATTCGCACCAATCCAGCGTCCGGGTTCTGCTTCCAGAAATGGATGTATGGGAAGC  
AGAAGAATGGCAAGAAGCGCCAATTTTCGATTGAAGCTATGCAAAGTTTTGTGAGCTTACACGATGAAGCTC  
ATGGGGTTGCTGTAATGACAGAGGGGTACGCGAATATGAAATCATTGGCGAAAATTACGATACGATCTCG  
CTAACCTGTTCCGCAATTTAGCCATATGGGTAAACCTGATCTACTATATCGTCCTGGTTCGCGCTTCCGG

TGAGTCAATCGTTGCGACACCAGACGCACAACCTGCTTGGCGAAATAAATGCCACATTTGCGCTAACTTTAT  
TCGAAAGCTCTTTTGACGAAGCGGATATCGCGAAAAAAGCAAAAGAATATCTATCGAACCCGCCAGTTTAC  
CAAATGTCTGATTTTCTAAACGGCCGCTTGATTTATGTTTACCGCGATGAAGAAAAAACGCTAGATGCCAC  
ATATAGCTTGGCACTTCCGACAATAGAGGAGCGGATTATTAGTGCAGTAAAAAAAAGCCGGAAGACGCCGA  
TGCCTTCCATTACACGCTTTTTCAATCCATATTTGCAAAAAGAAATTCATTCCAAGAAGTTTCCAAGGC  
AAGGAACGCCACTTTGGATGAAAGCGAATCAACGAGAACCAAACGAGATTAAAACATGCAAAAGTACAAAG  
CTATTTATTTGAAAAATAACGCTTAACCTAACCTAAAAAAGGAGCTGCTAATAATGGGATATTTTCGCTTATAA  
ACGTTTGGAAACACTATATGGCATGCTCCTTGATGCGGGTAGCCACTTATCAGCTCAGAAACTCAGCCAAG  
ATTTACATATTTCCGAACGTACGATACGAACAGATATTGCTAAACTAACTGAATTTCTCGAAAGCTACGGG  
GCAACGATAACGCTAACTCGAGGCGCGGGATACAAAATCGAAATCCTTGATTTCGTTGGTTTTTCAAGCTTT  
TCAGGCCGAAAAAATAAACCGAAAAATGCGGGTTATTTTGACCTTGATAATCCCGAAGAACGCGTGAAAT  
ACGAGATTTTCATGCTGCTATCAAGCGGCGACTATATTAAATTGGAAGACTTGGCAGATACGATTTTTCGCT  
AGCCGTGCAACCATTTCAAATGATATGAAGCAAGTACGAAAAGTTATTGCGGTGTATGATTTAACGCTCGT  
CTCAAAACCAGGTAGCGGCGTGAAAATTGTCGGCG



