An Investigation into the Global Cellular Responses of *Listeria monocytogenes* During the Transition from Exponential to Stationary Growth Phase

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No part of this thesis has been submitted in support of an application for any degree or other qualification of the University of Kent, or any other University or Institution of learning.

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Abstract

Contamination of foodstuffs by microbial pathogens is a major ongoing concern in the food industry. Consequently, an understanding of the biological mechanisms that permit microorganisms to survive in food environments designed to preclude their growth is highly desirable. The aim of this study was to use a state-of-the-art proteomic approach to identify those cellular processes that enable *Listeria monocytogenes* to exhibit enhanced resistance to stress whilst in stationary phase. The following investigation relates functional competence (survival of the organism in food environment) to concerted regulation of gene expression via systematic identification of key changes in expression of specific genes or related groups of genes during entry into stationary phase.

A defined medium was shown to support the growth of *Listeria monocytogenes* Scott A to stationary phase. Monitoring amino acid and glucose utilisation within the medium has differentiated, exponential growth, stationary phase and growth arrest. Flow cytometry has been used as a means of defining the point of onset of stationary phase by monitoring the growth phase specific effects of a nisin challenge. The point at which an identifiable change in growth rate could be attributed to the onset of stationary phase occurred at an OD_{600} of 0.75.

Using a proteomic platform with 2-D SDS-PAGE as the core technology, consistent 'coverage' and protein resolution of the *Listeria* proteome at pH 4 – 7 separating range has been achieved. With this system up to 1200 discrete proteins were routinely observed. A comparison of functional gene (protein) expression during exponential growth and transition of the organism into stationary phase at specific time points has been undertaken. Analysis of the resulting gel images has revealed global changes in gene expression during that transition Overall, 64% of all proteins visualised showed a change in expression indicating that *Listeria monocytogenes* cells undergo a radical physical adaptation in preparation for stationary phase and growth arrest. Ten proteins showing major quantitative change throughout growth have been identified using MALDI MS. The expression of each identified at fixed time points throughout growth and stationary phase has been demonstrated and shows good correlation with the expected patterns of expression.

Additional experimental evidence supporting the role for quorum sensing in the onset of stationary phase of *Listeria monocytogenes* batch cultures is presented. The combined growth characterisation, medium utilisation and flow data also indicated the presence of sub-populations of cells responding to changing medium conditions and population growth. This may be a general indication that the culture is demonstrating phase shift behaviour.

The identification and characterisation of further growth phase specific proteins should lead to a greater understanding of the physiology of the stress response and a possible strategy for more effective microbial control strategies. This investigation has highlighted several gene products important to the early onset of stationary phase and shown that sub-populations may be present that expand the range of environmental conditions in batch culture over which the organism may survive.

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In a vision, or in none
Is it therefore the less gone?
All that we see or seem
Is but a dream within a dream

Extracted from 'A Dream Within A Dream'
By Edgar Allen Poe (1827)

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SCHEMES

Abbreviations used

ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
APS	Ammonium persulphate
ATP	Adenosine triphosphate synthase
ATR	Acid tolerant response
a_w	Water activity
B. subtilis	*Bacillus subtilis*
BHI	Brain heart infusion
Bis	N,N- methylene-bis-acrylamide
BSA	Bovine serum albumin
CAMP	Christie-Atkins-Munch-Peterson
CCFH	The Codex committee on food hygiene
CDC	US Centres for disease control and prevention
CFU	Colony forming units
CHAPS	(3-[(3-Cholamidopropyl) dimethylammonio]-1-propane-sulphonate)
CH_3CN	Acetonitrile
Co	Company
2-D	Two dimensional (as in 2-D SDS-PAGE)
2-DE	Two dimensional electrophoresis
2-D SDS-PAGE	Two dimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis
DNA	Deoxyribonucleic acid
DPI	Dots per inch
DTT	Dithiothreitol
E. coli	*Escherichia coli*
EDTA	Ethylenediamine tetraacetic acid
EF	Elongation factor
ETF	Electron transfer flavoproteins
ESI	Electrospray ionisation
FAO	Food and agriculture organisation of the United States
GAD	Glutamate decarboxylase
GDP	Guanidine diphosphate
GTP	Guanidine triphosphate
GLP	Good laboratory practice
HACCP	Hazard Analysis and Critical Control Point programs
HCl	Hydrochloric Acid
HK	Hexokinase/glucose assay kit
Hly	Haemolysin

HPLC	High pressure liquid chromatography
HPr	Phospho carrier protein
Hrs	Hours
HSP	Heat shock protein
ICAT	Isotope-coded affinity tags
IID	Infectious intestinal disease
IEF	Isoelectric focusing
IPG	Immobilised pH gradient
kDa	Kilodaltons
LAB	Lactic acid bacteria
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
LLO	Listeriolysin O
L. monocytogenes	*Listeria monocytogenes*
LPS's	Lipopolysaccharides
LTA	Lipoteichoic acids
Ltd	Limited
MALDI	Matrix-assisted laser desorption/ionization
MALDI-MS	Matrix assisted laser desorption ionisation mass spectrometry
MALDI-TOF	Matrix assisted laser desorption ionisation mass spectrometry-time of flight
MAP	Modified atmosphere packing
Max	Maximum
MBq	Megabecquerels
Milli Q	Distilled water conforming to 18.4 MΩ cm purity
mRNA	Messenger ribonucleic acid
MR/VP	Methyl Red and Voges-Proskauer
Min	Minimum
Mn	Magnesium
MS	Mass spectrometry
OD	Optical density
OD_{600}	Wavelength at which a particular sample optical density was determined
OPA	O–phthaladehyde
OsO	Osmium oxide
PAGE	Polyacrylamide gel electrophoresis
pI	Isoelectric point
PI	Propidium iodide
PEP	Phosphenolpyruvate
PMF	Proton motive force
PTM	A chemically defined medium
PTS	Phosphotransferase system
PVDF	Polyvinylidene fluoride
RNA	Ribonucleic acid

RPM	Revolutions per minute
RT	Room temperature
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate- polyacrylamide gel electrophoresis
SEM	Scanning electron microscopy
SMC	Structural maintenance of chromosome
SSR	Starvation survival response
SOD	Superoxide dismutase
Soln.	Solution
sp.	Species
SYTO 13	Cell permeant green fluorescent nucleic acid stain, absorption 488 nm, emission 509 nm (different dye numbers refer to different emission ranges)
TBP	Tributyl phosphine
TCA	Trichloroacetic acid
t_d	Doubling time
TEM	Transmission electron microscopy
TEMED	N,N,N,N,- tetramethylethylenediamine
Temp.	Temperature
TGS	Premixed tris-Glycine-SDS electrophoresis buffer
Tiff	Tagged-Image File Format
Tris	Trimethylol Aminomethane
UKC	University of Kent at Canterbury
UK	United Kingdom
USA	United States of America
UV	Ultra violet light
V/Hr	Volts per hour applied during IEF
WHO	World health organisation

1.0 Introduction

1.1 Bacterial diversity

Fossilised bacteria have been found dating back to 3.5 - 3.6 billion years ago, and for approximately 2 billion years prokaryotic-type cells were the only form of life on Earth. Since that time, the predecessors of modern day bacteria have undergone considerable evolution and speciation [1]. In terms of species diversity it has been estimated that the oceans contain two million bacterial species while up to four million species may be present in a ton of soil [2]. Bacteria are found in virtually every environment on the planet, they inhabit environments occupied by free-living organisms and exist within other organisms. The success of bacteria is due to their ability to rapidly deal with the changing stresses encountered during their interactions with the biotic and abiotic environments.

Prokaryotes are the smallest and least complex cells and most prokaryotic cells are very small in comparison to eukaryotic cells. A typical bacterial cell is about 1 micrometer in diameter while most eukaryotic cells range from 10 to 100 micrometers in diameter. Eukaryotic cells have a much greater volume of cytoplasm and a much lower surface-volume ratio than prokaryotic cells. The only 'sense' that a bacterium has results from its immediate contact with its environment. Its surface components assess the environment and respond in a way that supports its own existence and survival in that environment [3]. The various surface components of a bacterial cell are therefore extremely important in its ecology since they mediate the contact of the cell with its environment. For instance, pathogenic bacteria have various structures that enhance their ability to cause illness. One important property is the ability to attach to the intended host. Pili, proteinaceous surface structures on the bacteria, are critical in this process. Many bacteria are capable of movement in their environment either by flagellar or gliding motility. Flagellar can also help in the detection of favourable or unfavourable conditions and move the bacteria in an appropriate direction [4]. In many instances it is only while under stress that many of the attributes of bacteria are observed. These stresses can include shortage of nutrients, over-population, competition, extremes of pressure, temperature, osmolarity, pH, presence of toxic compounds, and the transitory nature of the environmental niche itself. Microbial genome sequencing projects have produced a wealth of information on microbial genetics, biochemistry, and evolution with important medical, environmental, agricultural and industrial applications [5].

Bacteria are used industrially in the manufacture of foods, drugs, vaccines, insecticides, enzymes, hormones and other useful biological products. Bacterial fermentations

can be used to produce lactic acid, acetic acid, ethanol or acetone. In the foods industry, lactic acid bacteria such as *Lactobacillus* and *Streptococcus* are used in the manufacture of dairy products such as yoghurt, cheese, buttermilk, sour cream, and butter. Lactic acid fermentations are also used in the pickling process. Bacteria affect the world through their interactions with insects, plants and animals. Bacteria or bacterial products (including their genes) can be used to increase crop yield or plant resistance to disease, or to cure or prevent plant disease. Understanding how bacteria respond to their environment by exploiting a wide variety of biochemical mechanisms is one of the most important ongoing challenges in microbial science. A better understanding of these mechanisms may lead to more effective control measures against pathogenic bacteria.

1.2 Pathogenic bacteria

Pathogenic bacteria can be parasitic to plants or animals and they grow at the expense of their eukaryotic host causing damage, harm, or even death (of the host) in the process. Human diseases caused by bacterial pathogens include tuberculosis (*Mycobacterium tuberculosis*), whooping cough (*Bordetella pertussis*), diphtheria (*Cornybacterium diptheria*), tetanus (*Clostridium tetani*), gonorrhoea (*Nesseria gonorrhoeae*), syphilis (*Treponema pallidum*) and pneumonia (*Streptococcus pneumoniae*) to name a few. The location from which the causative agent of a disease is immediately transmitted to the host can be directly through the environment or indirectly through an intermediate agent. To maintain an infectious disease in a population the causative agent must be transmitted from one susceptible host or source to another susceptible host. Transmission can be by four main routes, airborne, contact, vehicle and vector borne. In developed countries, bacteria cause 90 percent of documented infections in hospitalised patients [6]. These cases probably reflect only a small percentage of the actual number of bacterial infections occurring in the general population, and usually represent the most severe cases. In developing countries, a variety of bacterial infections often exert a devastating effect on the health of the inhabitants. Malnutrition, parasitic infections, and poor sanitation are a few of the factors contributing to the increased susceptibility of these individuals to bacterial pathogens [6]. Generally, the bacteria that cause these diseases have special structural or biochemical properties that determine their virulence or pathogenicity. These include; the ability of bacteria to colonise and invade their host, the ability to resist or withstand the antibacterial defences of the host and the ability to produce various toxic substances that damage the host [4].

1.2.1 Food borne pathogenic bacteria

Food borne disease can be defined as 'a disease resulting from the consumption of food contaminated with microorganisms or their toxins'. For the purposes of reporting such diseases the term infectious intestinal disease (IID) is often used and describes gastrointestinal symptoms (diarrhoea, vomiting, abdominal pain), although within this definition it is accepted that only a proportion of cases are food borne. There are other routes of transmission, such as person-to-person spread and direct contact with animals or environments contaminated with animal faeces. While it is relatively easy to obtain robust data on IID it is not possible to determine the exact proportion of cases that are the result of food borne pathogens [7]. Notifications are made to local public health officials and collected centrally by the Public Health Laboratory Service in England and Wales, by the Common Services Agency in Scotland and by the Department of Health, Social Services and Public Safety in Northern Ireland. Food borne pathogens are estimated to cause 76 million illnesses, 325,000 hospitalisations, and 5,200 deaths in the United States each year. Known pathogens account for an estimated 14 million illnesses, 60,000 hospitalisations, and 1,800 deaths annually in the USA [8]. In England and Wales the main sources of data on human food borne disease and other infectious intestinal disease are, the statutory notification system, reports of intestinal infectious disease made to the Royal College of General Practitioners 'weekly returns service' and laboratory reporting of the major food borne pathogens and reports of outbreaks to the national surveillance centres. Figure 1.0 shows the incidence of reported cases of food poisoning for the years 1986 – 2000 in the UK.

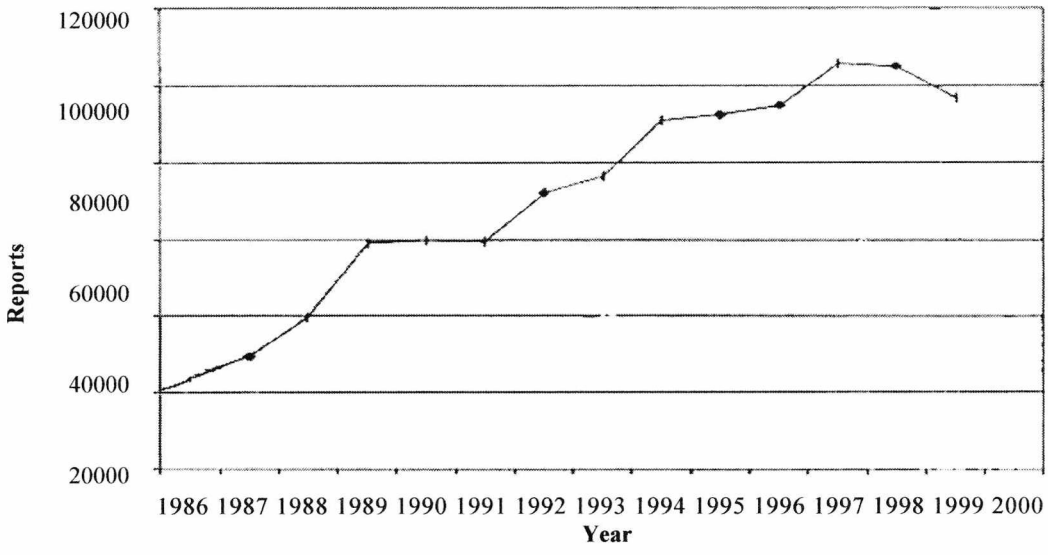


Figure 1.0 Statutory notifications of food poisoning 1986 – 2000 (UK data) [7]

The general trend in reported cases of food borne disease in Scotland has recently mirrored that seen in England and Wales, with notifications falling in 1999. In contrast in Northern Ireland, there has been a continuing upward trend. However the total UK figure decreased from 105,612 notifications in 1997 to 96,926 in 1999. The incidence has been falling since 1989. In 1994 and 1995, the mean weekly incidence was about 58 cases per 100,000, giving an annual estimate for England of just under 1.5 million cases [7]. The rise in cases of food borne disease associated with pathogenic bacteria may in part be due to better reporting methods. It is also possible that diets containing high levels of ready to eat and processed foods pose a greater risk of harbouring infection due to extended shelf life.

There are nearly 250 different types of bacteria, viruses and parasites that are known to cause food borne illness [9]. However the majority of cases of illness are caused by a relatively small number of common food borne bacterial pathogens [10]. Many bacteria are capable of causing food borne disease but only a few are commonly found in our everyday environment and these pathogens are capable of growth and survival in a wide range of environmental conditions. These 'commonly found' pathogenic bacteria and the environmental conditions they can tolerate are listed in Table 1.0 .

Table 1.0 Limiting conditions for pathogen growth [11]

Pathogen	Min.* a_w	Min. pH	Max. pH	Max. % salt	Min. temp.	Max. temp.	Oxygen requirement
Bacillus cereus	.92	4.3	9.3	18	4°C	55°C	Aerobe
Clostridium botulinum, type A, and proteolytic B and F	.935	4.6	9	10	10°C	48°C	Anaerobe**
Clostridium botulinum, type E, and non-proteolytic B and F	.97	5	9	5	3.3°C	45°C	Anaerobe**
Clostridium perfringens	.93	5	9	7	10°C	52°C	Anaerobe**
Escherichia coli (pathogenic)	.95	4	9	6.5	7.0°C	49.4°C	Facultative anaerobe***
Listeria monocytogenes	.92	4.4	9.4	10	-0.4°C	45°C	Facultative anaerobe***
Salmonella sp.	.94	3.7	9.5	8	5.2°C	46.2°C	Facultative anaerobe***
Shigella sp.	.96	4.8	9.3	5.2	6.1°C	47.1°C	Facultative anaerobe***
Staphylococcus aureus-growth	.83	4	10	25	7°C	50°C	Facultative anaerobe***
Staphylococcus aureus-toxin	.85	4	9.8	10	10°C	48°C	
Vibrio cholerae	.97	5	10	6	10°C	43°C	Facultative anaerobe***
Vibrio parahaemolyticus	.94	4.8	11	10	5°C	44°C	Facultative anaerobe***
Vibrio vulnificus	.96	5	10	5	8°C	43°C	Facultative anaerobe***
Yersinia enterocolitica	.945	4.2	10	7	-1.3°C	42°C	Facultative anaerobe***

* Water activity a_w * requires limited levels of oxygen ** requires the absence of oxygen *** grows both in the presence and absence oxygen

From the list above it can be seen that one of the most 'commonly found' pathogenic bacteria is *Listeria monocytogenes (L. monocytogenes)*. The organism was not originally associated with human disease although it had been reported in animals for some time. Its importance in public health has risen in recent times due to a number of outbreaks and a high mortality rate associated with it, usually close to 30%. *L. monocytogenes* has only been recognised as a

serious problem since about 1982 [12] when the first documented outbreak was recorded in Canada [13].

1.3 Characteristics of *L. monocytogenes*

Listeria is a genus of Gram-positive, non-sporing bacilli with a DNA G + C content of 36-38%. *L. monocytogenes* is aerobic and facultatively anaerobic, catalase positive, oxidase negative, indole negative and methyl red and Voges-Proskauer (MR/VP) positive and fermentative.

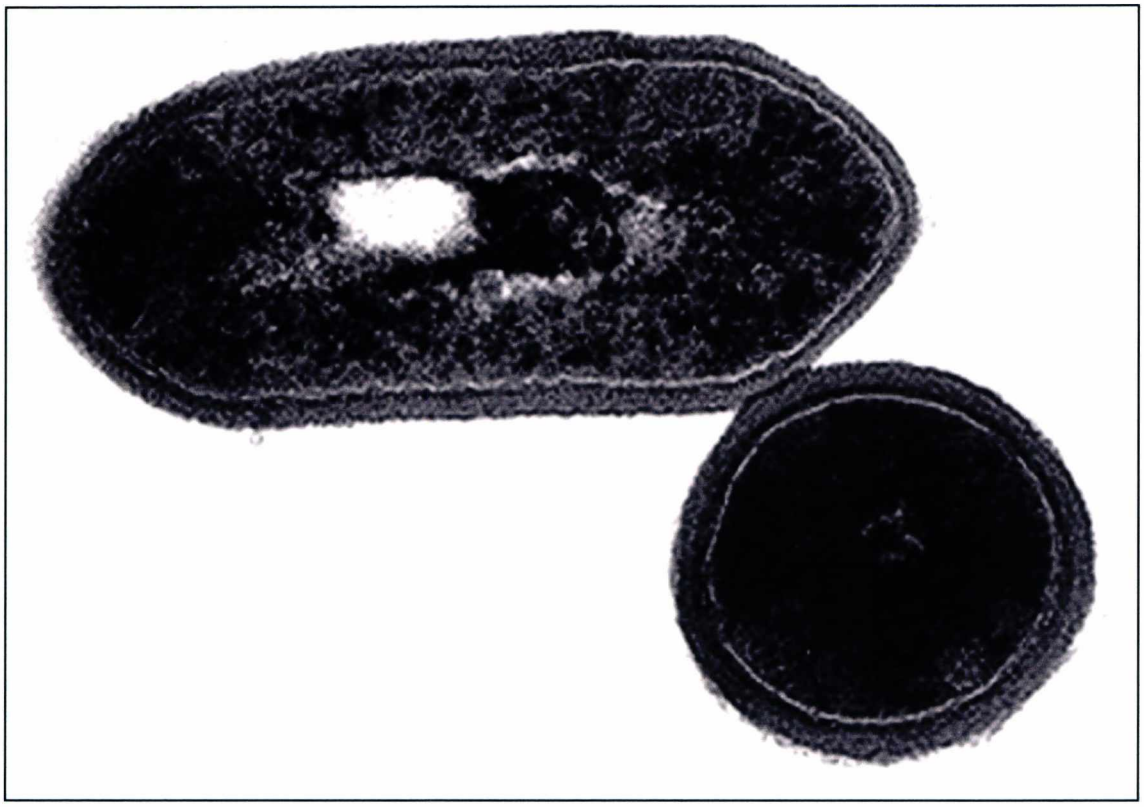


Figure 1.1 Scanning electron micrograph of a thin section through resin embedded *Listeria monocytogenes* showing lateral and longitudinal cell orientation. The cytoplasmic membrane and peptidoglycan coat are clearly defined (X19950) Source: BHI growth culture, harvest point OD_{600} 0.3. Cells fixed in 3% glutaraldehyde prior to embedding in resin and sectioning.

The cellular morphology of *L. monocytogenes* is variable and can be anything from coccoid to short rods. The cells are observed as either individual cells or may exist in chains, have peritrichous flagella, are characteristically motile but motility is temperature sensitive. At room temperature, one to six flagella may be present to give the cells motility. The tumbling motility characteristic of Listeria regularly used for diagnostic purposes is often not seen at 37°C. This is due to a loss of flagella at higher temperatures (above 30°C). *Bergey's Manual*

of Systematic Bacteriology lists eight species in the genus Listeria: *L. monocytogenes*, *L. innocua*, *L. seeligeri*, *L. welshimeri*, *L. ivanovii*, *L. grayi*, *L. murrayi* and *L. denitrificans* [14]. However the species *L. grayi* and *L. murrayi* are now considered to be subspecies of a single redefined species, *L. grayi* [15, 16]. All Listeria, except *L. grayi*, variously share 4 flagella antigens A, B, C, and D, of which flagella antigen B is common to all [14]. Of these six species, two are important pathogens, *L. monocytogenes* in humans and animals, and *L. ivanovii* in animals, especially sheep. The disease in both humans and animals is referred to as listeriosis, but this term covers a wide variety of clinical manifestations [14, 17].

Being aerobic and facultatively anaerobic, the organism can grow in varied atmospheric conditions although increased carbon dioxide will stimulate growth. The organism will grow at a range of temperatures from -0.4°C to 42°C with the optimum between 30 to 37°C [18-20]. The fact that the organism is common in the environment may explain why it is able to grow over a wide temperature range as well as in/on a variety of media. A wide range of pH values are tolerated from about 5.5 up to 9.5 as well as high NaCl (up to 12% w/v). Under certain circumstances, a pH as low as 3.5 can be tolerated and this is termed the acid tolerance response (ATR). Survival within a usually lethal pH range has been achieved by exposing the organism to its lowest normal tolerated pH (5.5) for one hour [21]. A study conducted by Davis *et al.* [22] described a lower pH tolerance, down to pH 3.0 after exposure to pH 5.0 for one hour.

The range of conditions at which growth is possible makes *L. monocytogenes* a potential food poisoning risk. Listeriosis, the disease associated with *L. monocytogenes,* has been shown to have devastating effects on pregnant women and their foetus, generating further interest in this bacterium. Pathogenic and non-pathogenic Listeria are ubiquitous in nature and can be isolated from soil, water and plant material, silage, sewage, sewage sludge and natural waters [17]. The organism has also been found in slaughterhouse waste, the milk of normal and mastic cows, human and animal faeces, cattle, sheep, goats and poultry [23]. Listeria is not common in wild animals but has been reported in, birds, amphibians, fish, crustaceans, insects and reptiles [24, 25].

1.4 Virulence factors and pathogenesis of *L. monocytogenes*

Listeriolysin, an enzyme capable of lysing red blood cells, is produced by *L. monocytogenes* and is associated with the ability of the organism to cause disease. The virulence of *L. monocytogenes* has been extensively studied, and a large number of virulence factors have been isolated and found to be significant in the pathogenesis of *L. monocytogenes*. Such

studies have been conducted using well-characterised strains of *L. monocytogenes* selected, or genetically modified, for the presence or absence of specific virulence genes. The majority of virulence studies have used genetically inbred mouse varieties as a surrogate animal model and have shown that genes involved with virulence of the organism are not expressed all of the time. In fact the majority of virulence genes identified are activated by the transcriptional regulator prfA. The expression of prfA and prfA dependent proteins is under the control of several environmental parameters such as temperature, pH, stress conditions and medium composition [26]. Additionally, the expression of some virulence genes has been shown to be partially regulated by environmental (medium) carbohydrate levels [27]. During host invasion *L. monocytogenes* succeeds in invading, surviving and replicating in a wide variety of phagocytes, including those regarded as non-professional phagocytes, and the virulence factors so far identified are mainly involved with its characteristic intracellular association and macrophages of varying origin are also affected in this way [28]. Many of the virulence genes (such as the haemolysin gene *hly*) are found in a cluster and are controlled by a single promoter [29]. The haemolysin gene protein product is not only a haemolysin, but a toxin as well. In the host the toxin aids the bacterium's survival in the phagosome and its transfer to the cytoplasm of the host cell. The toxin is known as the SH-activated haemolysin and is found in other bacterial species too, it has the same biological, biochemical and antigenic properties as its equivalent in other species, such as *Streptococcus pneumoniae* and *Clostridium perfringens* [29, 30]. This toxin/haemolysin (listeriolysin) is the most important factor in the pathogenic potential of *L. monocytogenes* as Listeria species lacking haemolysin, such as *L. innocua* are avirulent. Therefore pathogenic *L. monocytogenes* and non-pathogenic *Listeria innocua* are differentiated by their ability to produce listeriolysin [31]. Even mutant *L. monocytogenes* that are *hly* gene deficient have been demonstrated to be avirulent [32-35].

Others have shown that *hly* negative strains of *L. monocytogenes* are trapped within the phagosome of the macrophage and therefore fail to initiate phagosome/endosome fusion, although the frequency of naturally occurring strains that are deficient in one or more virulence markers is relatively rare among populations of food borne *L. monocytogenes* [36]. As stated previously food borne isolates generally have a complete array of virulence-associated genes. It is also assumed that except for atypical isolates such as listeriolysin-deficient mutants, all *L. monocytogenes* isolates are potentially pathogenic [37]. Listeria strains lacking listeriolysin O (LLO) are avirulent and in gastric infection studies have failed to colonise the liver or spleen in mice [35, 38, 39]. However, variation in the virulence of food borne isolates of *L. monocytogenes* has been observed. Ribotyping has identified a number of

disease-associated sub-types of *L. monocytogenes* and most disease is associated with a limited number of serotypes, these being 1/2a, 1/2b, 1/2c, 3, 4 and 4b [40-42].

No consistent pattern of increased virulence associated with any specific serotype or subtype has been highlighted in either *in vivo* or *in vitro* studies [35, 43]. It has however been reported that the frequency of 4b outbreaks was significantly greater in pregnancy cases whereas serovar 1/2b was most commonly associated with non-pregnancy cases [44]. The frequency with which these serotypes can be isolated from foods has not been paralleled by listeriosis distribution [45]. Therefore, the difference in the distribution of strains isolated from foods and humans does not necessarily reflect a difference in virulence, but it may be a reflection that the organism has undergone adaptations to different ecological niches [46]. During a small outbreak of listeriosis two serotypes (1/2a and 4b) of *L. monocytogenes* were isolated from contamination of hot dogs but, only the 4b strain resulted in disease even though it was present at lower concentrations than serovar 1/2b. This suggests that the 4b isolate was either more virulent, better able to survive transport through the stomach, or grew at a greater rate in the food. A large number of strains can be distinguished by different discriminatory techniques. However, it has also been shown that virulence can vary significantly between individual isolates of *L. monocytogenes* [30]. Such variability can influence the number of microorganisms required to produce infection, the potential for infections to become symptomatic and the severity of the subsequent illness.

The range of conditions that *L. monocytogenes* can adapt to make it a successful intracellular pathogen and it has a whole range of useful virulence factors. For example the *dlt* operon of Gram-positive bacteria comprises four genes (*dltA, dltB, dltC* and *dltD*) that catalyse the incorporation of D-alanine residues into the cell wall-associated lipoteichoic acids (LTA). The D-alaninylation of the LTA has been shown to contribute to the virulence of *L. monocytogenes* [47]. The production of the enzyme phospholipase C by virulent *L. monocytogenes* is important in relation to its ability to survive the early host neutrophil-mediated defence mechanism [48]. ActA and SvpA are surface associated *L. monocytogenes* proteins that are required for actin polymerisation, cell-to-cell spread of the organism in the host and promotion of bacterial escape from phagosomes of macrophages in the host [49, 50]. *L. monocytogenes* exploits the host cell machinery, enabling the pathogen to enter into cells and spread from cell to cell. Three bacterial surface proteins are crucial for these processes: internalin and InlB, which mediate entry into cells, and ActA induces actin polymerisation [51]. Among the surface-exposed proteins playing a role in this invasive process, internalin belongs to the family of LPXTG proteins. A recently discovered protein SrtA affects the

display of internalin at the bacterial surface, and plays a role in the pathogenicity of *L. monocytogenes* [52].

The elucidation of virulence factors has helped identify the steps of the infection process [53]. These steps include internalisation by eukaryotic cells, lysis of the resulting phagosome, replication as well as movement within the host cytoplasm; direct cell-to-cell spread, and subsequent lysis of a double-membrane vacuole when entering neighbouring cells [26, 54]. The listerial surface protein internalin A is involved in the internalisation of all types of cells. After invasion the bacterium is encapsulated in a membrane-bound compartment. In the phagocyte while encapsulated in the phagosome, a large number of *L. monocytogenes* are killed. Additionally, while in the macrophages superoxide dismutase (SOD) production by *L. monocytogenes* may aid survival of the bacterium [55]. Bacteria that survive or are in a non-activated phagocyte dissolve the phagosome by means of LLO [56]. *L. monocytogenes* then enters the cytoplasm, where conditions are suitable for growth. In the cytoplasm actin polymerase action can be initiated and the subsequent intracellular mobility allows the bacterium to invade adjacent host cells. The surface bound protein actin A then mediates the contact to the actin filament system of the host cell. This actin sheet grows and brings the bacteria to the surface of the host cell. It then forms long protrusions from the host cell that are phagocytosed by all kinds of cells, not only those that are generally known as being phagocytic [57]. Additional to the factors that have direct influence on the ability of *L. monocytogenes* to produce infection, the organism's virulence can also be influenced by characteristics that increase its chances of reaching the intestinal tract of the host. For example, *L. monocytogenes* has an adaptive acid resistance mechanism that once induced increases its survival at low pH allowing survival of a passage through the stomach [21, 22].

1.4.1 Occurrence of listeriosis

L. monocytogenes was first fully described in 1926 by Murray *et al.* who had isolated a small, Gram-positive rod bacterium that had been shown to be the causative agent of an epizootic outbreak in 1924 among rabbits and guinea pigs. In 1927 Pirie had described the same organism that had been isolated from gerbil in South Africa and it was he who subsequently suggested that the organism be named *Listeria monocytogenes* in 1940 [25].

The first case of human listeriosis was reported in 1929, although it was not associated with perinatal disease until 1936 [17]. *L. monocytogenes* infections can be life-threatening and fatality rates of 20 to 30% are common among hospitalised patients. A study of 84 cases of human listeriosis in Sydney in 1994 showed that listeriosis had a mortality rate

of 21% [58]. Among food borne pathogens, both the rate of hospitalisation (90%) and the case fatality rate (12%) in 1998 for the United States of America (USA) was highest with *L. monocytogenes* compared to other food borne pathogens (CDC, 1996; CDC, 1997; CDC, 1998). Listeriosis also had the highest hospitalisation rate (88%) and case fatality rate (20%) in 1997 [59].

In the majority of countries in mainland Europe, the United Kingdom and the United States there is a legal obligation for cases of listeriosis to be reported to the respective government health authorities. Although this was not always the case, in the United States listeriosis became a reportable disease in 1986 [8]. Most food-borne *listeriosis* outbreaks, and 50% of sporadic cases, are caused by strains of *Listeria monocytogenes* serotype 4b. Listeriosis is a relatively rare disease and typically has an incidence rate of 4 to 8 cases per 10^6 individuals in North America and Western Europe and most human cases occur in urban areas. Data from the U.S. Centres for Disease Control and Prevention (CDC) active food safety surveillance program, FoodNet, covering the years from 1996 to 1998 indicated that there were about 5 cases of listeriosis per 10^6 population during the past few years [59]. Estimates by the CDC suggest that it identifies approximately 50% of all listeriosis cases, as compared to a 3% identification rate for most other food borne pathogens [8]. Using 1996-97 surveillance data and extrapolating to the 1997 total U.S. population it has been estimated that there were 2493 cases including 499 deaths due to food borne listeriosis [8]. In England and Wales between 1983 and 2001 the number of case of listeriosis annually recorded has remained fairly static although an increase was recorded for the years 1987-89 (Table 1.1)

Table 1.1 *L. monocytogenes* human cases 1983 - 2001 in residents of England and Wales [60]

Year	Non-pregnancy associated	Pregnancy-associated	Not known	Total
1983	67	44	-	111
1984	77	35	-	112
1985	77	59	-	136
1986	87	42	-	129
1987	136	102	-	238
1988	164	114	-	278
1989	123	114	-	237
1990	93	26	-	119
1991	97	32	-	129
1992	83	25	-	108
1993	87	20	-	107
1994	90	26	-	116
1995	81	10	-	91
1996	101	18	-	119
1997	103	24	-	127
1998	88	21	-	109
1999	88	18	-	106
2000	87	13	-	100
2001	112	17	7	136

Note: a single pregnancy-associated case includes both mother and neonate. Source: Laboratory Reports to, PHLS Food Hygiene Laboratory

1.4.2 Symptoms and clinical signs of listeriosis

L. monocytogenes is ubiquitous in the environment and large numbers of people are exposed to the organism but do not necessarily become ill. *L. monocytogenes* causes illness by penetrating the lining of the gastrointestinal tract, and then establishing infections in normally sterile sites within the body. Once *L. monocytogenes* is ingested and penetrates the intestinal tissue, phagocytes take up the pathogen. However, it is capable of escaping from the phagosome, and subsequently grows within phagocytes. Phagocytes appear to be the means by which the bacterium can be transported to various parts of the body [61]. The likelihood that *L. monocytogenes* will invade the intestinal tissue depends upon a number of factors, including the number of organisms consumed, host susceptibility, and virulence of the specific isolate ingested [62]. Primarily listeriosis affects immunocompromised people such as those

being treated for cancer, those with organ transplants, and those with acquired immune deficiency syndrome (AIDS). Patients with AIDS are almost 300 times more likely to get listeriosis than people with normal immune systems. Additionally, other susceptible groups include persons taking glucocorticoid medication, the elderly and the very young (newborn). Pregnant women are 20 times more likely than other healthy adults to get listeriosis and up to a third of all reported listeriosis cases occur during pregnancy, despite this fact the incidence of listeriosis in males and females is approximately equal.

In general most cases reported are severe infections requiring some form of medical intervention. *L. monocytogenes* can cause a variety of clinical conditions including bacteraemia, bacterial meningitis, conjunctivitis, central nervous system infection, cutaneous infection, encephalitis, endocarditis, meningoencephalitis, miscarriage, neonatal disease, osteomyelitis, peritonitis, pleural infection, pneumonia, premature birth, and prodromal illness in pregnant women, septicaemia, and stillbirth. Incubation time for the organism is largely unknown although it has been reported to range from a few days up to three months [63]. These disease syndromes can vary considerably in mortality. The most common condition in the adult is meningoencephalitis and it is not usually possible to clinically distinguish it from other forms of purulent meningitis [64]. A variety of symptoms can be present which point to the involvement of the central nervous system, and such signs can be as obvious as comas and seizures or as subtle as a personality change. Low-grade fever is also seen along with motor disturbances. The only other significant disease caused by *L. monocytogenes* in the adult is a septicaemia. Symptoms are non-specific and can include common conditions such as general malaise, vomiting and nausea. It is generally not possible to distinguish a bacteraemia resulting from *L. monocytogenes* from that resulting from other bacterial pathogens without laboratory testing.

In pregnant women, listeriosis can be severe although not necessarily for the mother, however it may have serious effects on the foetus. Generally the mother may get a "flu-like" disease that is usually a self-limiting condition. Listeriosis in the neonate is possible and there are two forms of neonatal listeriosis, early and late onset disease. The early onset form of the disease occurs through infection in utero. The mother has the disease that is most often a "significant febrile illness" which usually results in a premature birth and the newborn will show signs of listeriosis just hours after the birth. These include hepatosplenomegaly, leucopoenia and respiratory distress. The late-onset meningitis of the newborn results from infection soon after birth or during parturition. No abnormalities are seen during birth but anywhere from three to 15 days after birth, the infant then develops symptoms such as fever and irritability [64]. *L. monocytogenes* can occur systemically, although this is rare.

1.5 Foods implicated in outbreaks of listeriosis

The most likely natural habitat of *L. monocytogenes* is soil and this probably accounts for the ease with which it can enter the food chain [63]. Although *L. monocytogenes* is an environmental organism, it is also a natural pathogen of a wide variety of animals. Animals can also serve as sources of the infection indicating that listeriosis is possibly a zoonosis, although transmission by this route appears to be of little significance [64]. Many animals can be infected with listeriosis (Section 1.3) and food animals have been shown to have the disease including chickens [65] and seafoods such as certain crabs [66]. Of great importance to veterinarians is the considerable increase amongst sheep of infection manifesting as abortion or encephalitis due largely to changing practices in silage manufacture [67]. Humans can also carry the organism asymptomatically, with 1% of the population thought to be carriers [68].

L. monocytogenes has many natural hosts and this gives the organism many opportunities to enter the food chain and infect humans. Food is the principal route of transmission [69]. Listeriosis cases are observed in conjunction with both common source outbreaks and individual sporadic cases. Foods of most concern include ready-to-eat products that (a) support growth of *L. monocytogenes*, (b) have a long refrigerated shelf life, and (c) are consumed without further listericidal treatments [13, 45, 70, 71]. This includes products that receive a listericidal treatment but are subject to post-processing re-contamination. This includes cross-contamination in both the retail and home setting. This most often occurs in milk or vegetables, although meat from both aquatic (seafood) and terrestrial (such as beef) sources can also harbour the bacterium [63]. Many categories of food have been associated with sporadic and epidemic listeriosis cases and outbreaks have been associated with consumption of the following food types; un-pasteurised milk products and milk [72-74]; butter [64], soft cheeses; soft, semi-soft and mature ripened cheeses [75]; cooked and processed meats (hot dogs, pork tongue, pate and salami), cooked and uncooked fish, mixed salads, salted mushrooms, pickled olives, raw vegetables and coleslaw. The organism can find its way into the food chain directly in infected animals or via the excretions of infected animals, which can be subsequently transferred to where vegetables are grown or stored. Uncooked vegetable foods have been implicated; an episode associated with consumption of coleslaw was linked with cabbage from a farm using sewage fertiliser [76].

Although the list of foods posing a risk is extensive, data collected over a 10-year period has shown that some foods may pose a higher risk to the individual than others. The highest risk foods are often found to be ready–to-eat, and are stored at refrigeration

temperatures for long periods of time, enabling Listeria to grow and reach infective doses. The safety of seafoods can vary significantly and is influenced by several factors, such as the origin of the fish, the microbial ecology of the product, processing and handling of the product and preparation prior to consumption (if any). Seafoods classed as high-risk have been identified as molluscs (including fresh and frozen mussels), clams, oysters, raw fish (eaten without cooking), lightly preserved (NaCl <6%, pH> 5.0) and mildly heat processed fish products. These can include salted, marinated, fermented and cold smoked fish. Other high-risk fish products include pre-cooked and hot smoked fish products and pre-cooked breaded fillets [77].

In the past decade a number of outbreaks of listeriosis have occurred throughout Europe and the USA and a variety of foods has been implicated. However in Scandinavian countries processed or home-prepared raw seafood is commonly consumed and it appears to pose an increased risk. In Sweden an outbreak of listeriosis occurred between 1994 and 1995 where all reported cases (nine) were attributed to the consumption of cold smoked rainbow trout or salmon. Two of the patients died, while six of the remaining patients had predisposing conditions [78].

More recently an outbreak of listeriosis occurred in Finland and was caused by the consumption of a vacuum-packed fish product. Twenty-three cases of listeriosis were identified throughout the country between June 1999 and February 2000, although overall the total number of cases was no higher than in previous years (34, 29, 53, 46, and 42 cases each years from 1995 to 1999). In ten of the reported case (8 sepsis, 1 meningitis, and 1 peritonitis) the causative agent was found to be *Listeria monocytogenes* serotype 1/2 and each was indistinguishable by pulsed field gel electrophoresis (PFGE). Half were male and half female, and were aged between 29 to 84. One was pregnant and the rest had predisposing underlying conditions, four of which were malignant. Four elderly patients (>70 years of age) died within one month of the positive identification of Listeria culture, two of them within one week. *L. monocytogenes* of the same PFGE type has also been identified in vacuum packed fish products and the association is being investigated.

During normal food testing conducted in Finland between 1996 and 1998, *L. monocytogenes* was identified in 8% to 25% of samples of Finnish vacuum-packed smoked and cold-salted fish products. However, the levels were usually low (<100 cfu/g) although high levels (1000-20 000 cfu/g) were sometimes detected. Hot-smoked products were rarely contaminated. Inspection of the hygiene of fishery establishments is one of the main topics in Finland's annual food control plan for the year 2000. In Finland the National Food

Administration have begun an intensified project to assess the occurrence of *L. monocytogenes* in vacuum packaged fish products at retail level, which includes the checking of storage temperatures [79]. Although seafood is high risk it is not the only food product to be linked with listeriosis. In a previous outbreak of listeriosis (15 cases) in Finland in 1998 and 1999 contaminated butter was identified as the cause [80].

A number of ready-to-eat foods have also been implicated in listeriosis outbreaks throughout Europe. In France in 1999 four cases of listeriosis were reported in two months, two further cases were identified in the following weeks. The cases had arisen between October and December 1999 in six different areas in France. Five of the cases were adults, four of whom had impaired immunity caused by serious illness, and one was an infant, born prematurely due to the infection. The baby and one of the adults with impaired immunity died. The vehicle for transmission was identified as rillettes, a ready-to-eat food prepared by cooking ham meat with grease [81]. A separate outbreak of listeriosis reported in France during the second half of December 1999 involved 26 cases including 7 deaths. The cases were widely distributed within France but on the basis of laboratory tests appeared to be from a single source, a pork tongue in jelly [82]. In 1992, the investigation of a large outbreak (279 cases) had also identified pork tongue in jelly as the main vehicle of transmission and showed that cross-contamination of other ready-to-eat meat products, especially those sold at the delicatessen counter, amplified transmission [83].

Ready-to-eat foods have also been implicated in outbreaks in the USA. From early August 1998 to January 1999, at least 50 cases caused by a rare strain of the bacterium *L. monocytogenes*, serotype 4b, were reported. Six adults died and 2 pregnant women had spontaneous abortions. The vehicle for transmission was identified as hot-dogs and possibly processed meats produced under many brand names by one manufacturer.

In the year 2000 listeriosis cases attributed to a food borne source have been reported in Algeria [84], Belgium [85], Canada [86], Denmark [87], France [88], Germany, Grand Canary [71], Hungary [89], Israel, Japan [90], Senegal [91], Spain [92], Switzerland [93], Ukraine [94], United Kingdom [60] and USA [95]. The case-fatality rate in recent outbreaks and sporadic cases is around 20%-30%. In agreement with the widespread occurrences of *L. monocytogenes*, the organism has been consistently isolated worldwide from a great variety of raw meats, poultry, seafood, dairy products and vegetables [96] and it is clear that food contaminated with *L. monocytogenes* is a significant source of illness and death worldwide.

1.5.1 Listeriosis and dose dependence

In general, the levels of *L. monocytogenes* in implicated foods have been elevated (>10^3 colony forming units/g {cfu/g}) however, there have been instances where the observed level of *L. monocytogenes* in the implicated food has been substantially lower. There is a great deal of uncertainty concerning these estimates since the actual level of the pathogen in a serving of food consumed by an individual may have varied considerably from that observed in other portions of the food during subsequent investigation. In reality the infectious dose of *L. monocytogenes* is currently unknown and cannot be tested directly in humans because of a potentially fatal outcome. In general, epidemiological studies do not accurately predict infectious dose because of the potential 3-4 week lag time between exposure and disease outcome. However listeriosis has been reported to affect pregnant non-human primates in the same manner as humans, causing stillbirth, abortion or neonatal death at dosages ranging from 1.2 x 10^3 to 3.5 x 10^6 cfu [97].

1.6 Diagnosis and identification of the organism

Where listeriosis is suspected, the source has to be found and the organism isolated and identified. Differentiation of the newly recognised subspecies of *L. ivanovii* is not needed in Listeria analysis [98]. *Brocothrix*, which is closely related phylogenetically to Listeria, is distinguished by its inability to grow at 35°C and its lack of motility. Listeria species isolates can be enriched on non-selective agar and purified on selective medium. Alternatively the organism can be speciated directly by a battery of conventional tests or rapid methods, which have a high specificity to detect *L. monocytogenes*, especially as the detection of the organism from clinical specimens is generally done with limited success. Enzyme-linked Immunosorbent Assay (ELISA) methods are common and these use a monoclonal antibody against a Listeria specific heat-stable protein [63]. Use of PCR in detection of the organism from foods can be difficult although a number of techniques are now in use [99, 100].

The Christie-Atkins-Munch-Peterson (CAMP) test is based on the formation of areas of haemolysis on blood agar at the boundary of contact between culture streaks of *S. aureus* and *R. equi*, this is due to the production of listeriolysin (a haemolytic protein) by pathogenic listerial species. *L. seeligeri* shows enhanced haemolysis at the *S. aureus* streak. *L. ivanovii* shows enhanced haemolysis at the *R. equi* streak. Of the non-haemolytic species, *L. innocua* may give the same rhamnose-xylose reactions as *L. monocytogenes* but is negative in the CAMP test. *L. innocua* is sometimes negative for utilisation of both rhamnose and xylose. *L. welshimeri*, which is rhamnose-negative, may be confused with a weakly haemolytic *L.*

seeligeri unless resolved by the CAMP test. Use of known control Listeria sp. on a separate sheep blood agar plate is usually recommended. Alternatively, isolate cultures can be rapidly speciated, by a chemiluminescent-labelled DNA probe kit or by equivalent *L. monocytogenes*-specific DNA probes or probe kits. Examination of Listeria plates by Henry illumination for suspect colonies of Listeria sp. can also be undertaken [101], and an API test is also available [102]. Table 1.2 shows some common traits that are used for the differentiation of Listeria sp.

Table 1.2 Differentiation of Listeria species [103]

Species	Haemolytic ([a]Beta)	Nitrate reduced	Acid produced from			Virulent (mouse)
			Mannitol	Rhamnose	Xylose	
L. monocytogenes	+	-	-	+	-	+
L. ivanovii	+	-	-	-	+	+
L. innocua	-	-	-	V [(b)]	-	-
L. welshimeri	-	-	-	V [(b)]	+	-
L. seeligeri	+	-	-	-	+	-
L. grayi [(c)]	-	V	+	V	-	-

[a] Sheep blood stab. [b] V, variable. [c] *L. grayi* now includes the former nitrate-reducing, rhamnose-variable species *L. murrayi*

Serology is rarely used as a single identification method and is usually only used as a final confirmation test. It can be unreliable because *L. monocytogenes* is antigenically similar to other pathogens such as *Staphylococcus aureus* and *Enterococcus faecalis*. Both of these organisms are pathogenic and it can be difficult to distinguish between these diseases based on serology. Both high numbers of false positives and false negatives have occurred. However, identification of serovars is widely undertaken and can be useful when epidemiological considerations are crucial. Table 1.3 shows the serological relationships of Listeria sp. (species). The majority of *L. monocytogenes* isolates obtained from patients and the environment are type 1 or 4, and more than 90% can be serotyped with commercially available sera [103]. All non-pathogenic species, except *L. welshimeri*, share one or more

somatic antigens with *L. monocytogenes*. Serotyping alone without thorough characterisation is not adequate for identification of *L. monocytogenes* [103].

Table 1.3. Serology of Listeria species [14]

Species	Serotypes
L. monocytogenes	1/2A, 1/2B, 1/2C, 3A, 3B, 3C, 4A, 4AB, 4B, 4C, 4D, 4E, "7"
L. ivanovii	5
L. innocua	4AB, 6A, 6B, Un [(a)]
L. welshimeri	6A, 6B
L. seeligeri	1/2B, 4C, 4D, 6B, Un

[a] Un, undefined.

1.7 Treatment of listeriosis

Effective control of the organism *in vivo* is not difficult as it is susceptible (to varying degrees) to a wide variety of antibiotics. *In vitro* it has been shown that *L. monocytogenes* is susceptible to ampicillin, penicillin G, erythromycin, trimethoprim, gentamycin, clindamycin, chloramphenicol and vancomycin [64]. Of these gentamycin, streptomycin and chloramphenicol in low concentrations (generally less than 5 micrograms per millilitre) are generally inhibitory but higher concentrations, up to a 50-fold increase are required to kill the bacteria. Ampicillin and penicillin G are the usual choice of antimicrobials and ampicillin is preferred. The treatment of listeriosis patients by drug intervention is usually for long periods, commonly around three weeks [64]. Treatment of immunocompromised patients and those with CNS infection tends to be longer than three weeks [64]. Listerial meningitis is best treated with penicillin G, which is usually prescribed for 10 to 14 days after defervescence. Both penicillin G and tobramycin should be given for endocarditis for up to 6 weeks and in the case of primary listeremia for 2 weeks beyond defervescence. Oculoglandular listeriosis and listerial dermatitis should respond to erythromycin estolate for 1 week after defervescence [104]. Like all bacteria, resistant strains of *L. monocytogenes* are emerging and studies into

the antibiotic resistance of Listeria sp. have shown that resistance to tetracycline is common [105]. More recently, observations made on *L. monocytogenes* isolated from a neonate have shown the organism to be resistant to many of the common antibiotics used to treat listeriosis [106]. Despite evidence of limited resistance to antimicrobials the patterns of susceptibility and resistance of *L. monocytogenes* have been fairly stable, unlike other bacteria, where the development of resistant strains are of greater concern [63].

1.8 Control measures in food

The expansion of the agro-food industry, the widespread use of systems of cold storage and changes in consumer demands have led to a large increase in the pool of Listeria that can cause food borne infection [107]. The range of foods (Section 1.4) implicated in listeriosis outbreaks is both extensive and varied. Many foods implicated are taste sensitive and cannot be subject to extensive processing to eliminate the organism. However, a wide range of control methods is available and have been adopted by the food industry to minimise the occurrence of Listeria in foods. Food industry initiated Hazard Analysis and Critical Control Point programs (HACCP programs) and increased sanitation efforts to eliminate contamination have been initiated. Because of the rising incidence of microbial food borne disease in the UK, particular attention is being given to the application of HACCP principles in all sectors of the food industry. The basic requirements of HACCP systems for the red meat and poultry industries are well known, but not yet uniformly applied in UK abattoirs.

HACCP involves seven principles (Table 1.4), The use of HACCP systems in the production of ready-to-eat meat and poultry products is considered essential for optimum pathogen control. It is not yet possible, however, to exclude *L. monocytogenes*, which occurs in various niches in the processing environment. Thus, low levels of Listeria are to be expected in a proportion of ready-to-eat products and are not regarded in the UK as hazardous to consumers [108].

Table 1.4 Seven principles of HACCP [109]

Principle	Action
Analyse hazards	Potential hazards associated with a food and measures to control those hazards are identified. The hazard could be biological, such as a microbe; chemical, such as a toxin; or physical, such as ground glass or metal fragments.
Identify critical control points	These are points in a food's production--from its raw state through processing and shipping to consumption by the consumer--at which the potential hazard can be controlled or eliminated. Examples are cooking, cooling, packaging, and metal detection.
Establish preventive measures with critical limits for each control point	For a cooked food, for example, this might include setting the minimum cooking temperature and time required to ensure the elimination of any harmful microbes.
Establish procedures to monitor the critical control points	Such procedures might include determining how and by whom cooking time and temperature should be monitored.
Establish corrective actions to be taken when monitoring shows that a critical limit has not been met	For example, reprocessing or disposing of food if the minimum cooking temperature is not met.
Establish procedures to verify that the system is working properly	For example, testing time-and-temperature recording devices to verify that a cooking unit is working properly.
Establish effective record keeping to document the HACCP system	This would include records of hazards and their control methods, the monitoring of safety requirements and action taken to correct potential problems. Each of these principles must be backed by sound scientific knowledge: for example, published microbiological studies on time and temperature factors for controlling food borne pathogens.

In a food medium (fermented smoked sausage recommended pH 5.5 or below [109]) a pH below 5.5 has an adverse effect on the growth of *L. monocytogenes*, however the effect of low pH is not always positive. Pre-exposure to acid can induce an acid tolerant response that makes the organism more resistant to low pH, ethanol and hydrogen peroxide if other barriers to growth are not present [110]. The listericidal effects of acid are attenuated in cured meats that contain salt and other preservatives. [111]. The nature of preserved meat

products means that a number of preservatives can be used in combination to limit the growth of *L. monocytogenes* and include, sodium chloride (NaCl), nitrite, trisodium phosphate, sorbic acid and chelators such as citrate and ethylenediamine tetra acetic acid (EDTA). [110-112].

Bacteriocins are a heterogeneous group of anti-bacterial proteins produced by bacteria (usually lactic acid bacteria {LAB}) that have antimicrobial effects on other bacteria usually closely related to the producer organism. The inhibitory activity of these substances is confined to Gram-positive bacteria. Bacteriocins can vary by activity, mode of action, molecular weight, genetic origin and biochemical properties [113]. Nisin has been shown to have inhibitory action against *L. monocytogenes*, however organisms that have adapted to acid conditions are more resistant to the effects of nisin and the development of nisin resistant strains and mutants [114] limit its effectiveness in some applications.

The use of thermal processes to control Listeria is dependent on many factors including the characteristics of particular strains and serovars. The susceptibility of *L. monocytogenes* to heat treatment can be affected by growth stage, temperature during growth and exposure to other stresses. In particular cells at stationary phase are more resistant to thermal inactivation [115]. As previously reported (nisin susceptibility) cells can demonstrate cross stress resistance due to stress responses induced by exposure to an unrelated stress [116]. Thermotolerance has also been shown to increase significantly when cells grown at 4°C were subjected to thermal shock. Although many thermal processes are successful at eliminating *L. monocytogenes,* high temperatures or prolonged heating can alter the sensory characteristics of some foods. In such cases combinations of mild heat with other measures must be considered [117].

In foods with subtle taste characteristics where the 'taste' of that product can be disrupted/modified by extensive bacterial control measures, a variety of methods can be adopted to exert control over the bacterial population within that product. The product can have a modified atmosphere packing (MAP). Atmospheres containing low oxygen and/or high carbon dioxide levels can suppress the growth of bacteria as well as extend the shelf life of the product. Numerous studies have been conducted on the effect of MAPs on the growth of *L. monocytogenes*, and the results achieved are inconsistent and may vary due to the fat content, acidity, storage temperature and the presence of other preservatives in foods under investigation. *L. monocytogenes* will grow in the absence of oxygen and has even been observed to multiply on vacuum-packed meat [118]. Apart from the variability in its effect another concern of using MAP is the fact that spoilage bacteria are effectively controlled while *L. monocytogenes* may still be present. Products with a long shelf life can appear sound while harbouring an infectious dose of Listeria [119].

Irradiation is an effective way of damaging and destroying food borne pathogens including *L. monocytogenes* [120] although *L. monocytogenes* has shown significant resistance to radiation particularly at low temperatures [121]. Other control methods under investigation include high pressure, high pressure in conjunction with pulsed electric fields and ultraviolet light. *L. monocytogenes* has been shown to be resistant to high-pressure during growth and more so at stationary phase [122-125]. Combinations of organic acids, low pH, and ethanol have been shown to be particularly effective bactericidal treatments; the most potent combination being pH 3.0, 50 mM formate, and 5 % (v/v) ethanol, which resulted in 5 log units of killing in just 4 minutes [126]. Artificial peptides have also been used experimentally to control food borne pathogens and cold adapted cells have been shown to be less susceptible to the effects of bacteriocins than those grown at higher temperatures.[127]

1.9 Control and prevention of *L. monocytogenes* in the environment

Due to its wide tolerance of a variety of conditions, *L. monocytogenes* can be hard to eliminate using standard procedures, such as refrigeration. As with all food-borne pathogens, the best way to eliminate the organism from foods or rather to prevent them from entering these foods in the first place is to observe good hygiene practice. The Codex Committee on Food Hygiene (CCFH) identified Listeria in ready-to-eat foods as a priority for risk assessment. In response, the World Health Organisation (WHO) and the Food and Agriculture Organisation of the United States (FAO) undertook risk assessments for *L. monocytogenes* in ready-to-eat foods. The preliminary report of a joint FAO/WHO consultation on microbiological risk assessment [128] was delivered to CCFH on 17 July 2000. As more information becomes available linking listeriosis with food consumption, food control agencies and private industry continue to developed programs to reduce the incidence of food borne listeriosis.

There have also been consumer education campaigns focusing on food safety. In the United States, a reduction in listeriosis from 7.9/million in 1989 to 4.4/million in 1993 was achieved [129]. In the United Kingdom rates of listeriosis simultaneously declined after the British government issued health warnings regarding *L. monocytogenes* [44, 130]. Similar declines have been reported as a result of public health initiatives in Europe and Australia. However, since that time, the incidence of listeriosis has remained relatively constant (CDC, 2000). The reported yearly incidence of human listeriosis in Europe ranges from 0.1 to 11.3 cases per 10^6 persons [131]. It is also important to control the disease in animals and vaccination has been used. One of the most recent types of vaccines being researched is a recombinant *Salmonella typhimurium* that carries either the superoxide dismutase gene or the p60 secreting construct of *L. monocytogenes* [132].

1.10 Gene regulation and the expression of gene products that allow adaptation to stress conditions

The expression of the bacterial genes involved in the different steps of the infectious process; invasion, intracellular multiplication and spreading, is temporally and spatially controlled. This ensures the presence of the respective gene products at the right moment and place. Some of the genes involved in virulence and stress responses are known. For example, PrfA is a well-characterised regulator of listerial virulence, and belongs to the Crp/Fnr family of transcriptional regulators. In addition to the PrfA regulon, the Clp regulated stress proteins have an impact on Listeria virulence. These two regulons interact with each other by an unknown mechanism [133].

Stress proteins play an important role in virulence, but little is known about the regulation of stress responses in pathogens. In *L. monocytogenes* the Clp ATPases, including ClpC, ClpP and ClpE, are required for stress survival and intracellular growth. Nair *et al.* have demonstrated that Listeria possesses a *CtsR* regulon controlling class III heat shock genes [134]. Additionally, environmental sensing by two-component signal transduction systems are likely to play a role in the growth and survival of *L. monocytogenes* both during transmission in food products and within a host organism [135, 136]. Two-component systems is the primary means by which bacteria sense and respond to environmental stimuli. These systems are comprised of a number of distinct elements, namely histidine kinases, response regulators and, in the case of phosphorylase, histidine phosphotransfer proteins [135, 137].

In the bacterium *E. coli* the Cpx envelope stress response is mediated by a typical two-component regulatory system consisting of the membrane-localised sensor histidine kinase (HK), CpxA and the cytoplasmic response regulator CpxR. CpxA responds to envelope stresses through auto-phosphorylation, probably at a conserved histidine residue, and subsequent phosphotransfer to CpxR [138]. As with other response regulators, this phosphorylation probably occurs at a conserved aspartate residue. Phosphorylation allows CpxR to function as a transcriptional activator of genes whose products are involved in protein folding and degradation in the bacterial envelope [139, 140]. Other examples of two-component regulatory systems are seen in Salmonella where the PhoQ/PhoP system responds to starvation and is essential for pathogenesis. In Bordetellae, BvgS and BvgA regulate all aspects of virulence.

A number of investigations have highlighted large groups of stress proteins that appear to be induced together by physical stress such as heat, salt, ethanol or acid as well as by glucose, oxygen, or phosphate starvation [141-147]. One of the strongest responses of a *Bacillus subtilis* cell (an organism homologous to *L. monocytogenes*) to a range of stress and

starvation conditions is the dramatic induction of a large number of general stress proteins. Sigma factors and alternate sigma factors will act on -10 and -35 promoter regions. The classic promoter region includes sequences -35 (consensus, TTGACA); -10 (consensus, TATAAT) and is recognised by RNA polymerase and the so called housekeeping sigma factor. Promoter strength can be varied by changes in the -35 and -10, as well as the spacing between these regions (17 base pairs is the ideal spacing). Over 1000 genes are regulated by the housekeeping sigma factor. Alternative sigma factors recognise different -10 and -35 regions. Examples in *E. coli* include sigma 32, for transcription of heat shock genes, and sigma 54 for transcription of genes regulated under nitrogen limitation, genes involved in flagella and chemotaxis, stationary phase. These alternate sigma factors control on average about 40 genes [137, 148]. The alternative sigma factor sigma B is responsible for the induction of the genes encoding these general stress proteins that occurs following heat, ethanol, salt or acid stress, or during energy depletion. Sigma B is absolutely required for the induction of this regulon, not only in *B. subtilis*, but also in other Gram-positive bacteria. More than 150 general stress proteins/genes belong to this sigma B regulon, which is believed to provide the non-growing cell with a non-specific, multiple and preventive stress resistance. Sigma B-dependent stress proteins are involved in non-specific protection against oxidative stress and also protect cells against heat, acid, alkaline or osmotic stress.

A cell in the transition from a growing to a non-growing state induced by energy depletion will be equipped with a comprehensive stress resistance machine to protect it against future stress. The protection against oxidative stress may be an essential part of this response. In addition, preloading of cells with sigma B-dependent stress proteins, induced by mild heat or salt stress, will protect cells against a severe, potentially lethal, future stress. Both the specific protection against an acute emerging stress, as well as the non-specific, prospective protection against future stress, are adaptive functions crucial for surviving stress and starvation in nature.

It is clear that the virulence of *L. monocytogenes* is a multi-factorial trait and the organism has evolved a wide range of responses to external stimuli that allow it to sense changing environments inside and outside the host. Additionally, the organism's ability to survive the varied conditions encountered during infection are the same responses that make elimination in food processing environments difficult. In attempts to control the organism during and after food processing, it has been noted that Listeria is more resistant to a number of stresses at stationary phase [137] and if pre-stressed the organism develops resistance to a number of stresses unrelated to the original stress. For example the starvation survival response (SSR) of *L. monocytogenes* EGD is induced under glucose or multiple-nutrient, but

not amino acid-limitation. The well-characterised regulators SigB and PrfA are both required for the full SSR and effect stress resistance during growth and starvation. Survivors from aged cultures showed a reduced cell size and increased cross-protection to several environmental stresses [135]. It has also been demonstrated that the alkaline stress response in *L. monocytogenes* provides resistance to thermal-processing conditions [149]. The glutamate decarboxylase (GAD) acid resistance system of *L. monocytogenes* plays a major role in its survival at low pH even in acidic foods when levels of free glutamate are low [150]. In addition F0F1-ATPase was found to greatly enhance survival at low pH in acid adapted cells[151]. The organism tolerates conditions of osmotic and chill stress by the accumulation of glycine betaine [152]. Clinical and food borne isolates of *L. monocytogenes* have been compared and demonstrate similar resistance responses to naturally occurring and intentionally added antimicrobial agents. All strains examined were able to use carnitine as an osmoprotectant, indicating the importance of this characteristic to the survival of *L. monocytogenes* in natural environments. Acid-sensitive strains were not found among the clinical isolates examined, highlighting the importance of acid stress resistance in the infection process [153]. It has also been suggested that *L. monocytogenes* possess a sigma (B)-dependent mechanism and a pH-dependent mechanism for acid resistance in the stationary phase. Sigma (B) contributions to viability during carbon starvation, and to acid resistance and oxidative stress resistance, supporting the hypothesis that sigma (B) plays a role in protecting *L. monocytogenes* against environmental challenge [137].

It has been well documented that stress can induce variations in the expression, synthesis and modification of existing and new cell components, especially proteins [154]. The approach to elucidation of the mechanisms of virulence and stress survival of bacterial pathogens is complex and has of necessity involved a wide range of differing techniques. Such an approach has been unable to take a global view of the changes in gene expression and gene products that occur during varied challenges to the organism. Protein expression is dynamic and can (using a given set of conditions) reflect the state of a biological system. In such biological systems the level of expression, state of modification, processing and macro-molecular association of proteins are controlled and modulated dependent on the state of the system. A greater understanding of such systems can be achieved by varying a number of defined environmental factors, thus inducing different protein expression profiles. Detection and quantitation of the proteins that make up such a system can highlight specific proteins that such changes promote [155].

1.11 The genomic revolution

At approximately the same time as proteomics was emerging as a new field of research, the successful elucidation of the complete genetic code of a replicating organism, *Haemophilus influenza* was achieved in 1995 [156]. This rapid progress in large-scale nucleic acid sequencing was mainly due to advances in the sequencing technologies themselves, including automated sequencing and improved PCR technology [157]. This single event spawned a considerable number of projects working on the elucidation of a wide variety of bacterial genomes and continued efforts on higher organism genome maps, in what has become known as the 'genomic revolution'. The initial approach involved the determination of a complete bacterial genome sequence by preparation of a physical map using cloned genome fragments in either a plasmid or phage library. The individual fragments were then sequenced and aligned to a physical map [158]. In 1997 the same method was used to successfully determine the genome sequence of *Escherichia coli* [159] and *Bacillus subtilis* [160]. The list of sequenced microorganisms continues to grow, and to date 69 bacterial genomes have been published (appendix I) and many are in progress [161]. The technique is not limited to microorganisms and recently the human genome project completed the draft human genomic sequence and to date has completed 85% of the entire genome [162].

In isolation however, DNA sequence information and mRNA profiling does have its limitations. DNA sequence information can only provide a static view of all the possible ways a cell might use its genes. In reality a cell is constantly reacting to its environment in a dynamic process. For example, the introduction of a pathogen may change how much gene product is made, when the genes are turned on, the type and extent of post-translational modifications that occur, and how these events affect other genes. Such effects can determine if the host successfully defends itself, or alternatively, if the pathogen successfully overcomes host defences thereby causing disease.

The study of this dynamic has the potential to reveal new targets for drug intervention in disease processes, or new targets for the control of pathogenesis of food borne organisms [163]. Research is now being directed towards gaining an understanding of how and when genome-encoded events (e.g., global induction of protein synthesis) occur. In many cases there is no correlation between abundance of mRNA and the functional protein product on which cell phenotypes depend [164]. In addition, the relationship that non-genome-encoded events such as posttranslational modifications of proteins and the interactions between proteins, nucleic acids, lipids and carbohydrates, have to particular physiological states is being investigated. Nor can gene over-expression or small gene occurrence be highlighted by

current molecular biological techniques [165, 166]. The degree and nature as to which gene products are post-transitionally translated, will also remain unresolved, without reference to the functional protein content of a given cell type, tissue or organism [164]. In order to highlight the ability of *L. monocytogenes* to adapt and survive conditions designed to preclude its growth and the role that stationary phase plays in the survival tactics of the organism a global technique is required. Proteomic analysis (proteomics) has the potential to fulfill the research criteria required to investigate global changes to gene and protein expression [167].

1.12 Introduction to proteomics

The word 'proteome' (all the PROTEins expressed by a genOME) was first used in late 1994 at the Sienna 2-D-Electrophoresis meeting and is now a widely accepted term despite not making a literary appearance until July 1995 [168]. An obvious extension to this term, ''proteomic analysis'' soon followed, which indicates the methodology of investigating and visualising the total protein compliment expressed by a given genome, cell, or tissue type. More recently proteomics as a field of research, has been defined as the 'use of quantitative protein-level measurements of gene expression to characterise biological processes (e.g., disease processes and drug effects) and decipher the mechanisms of gene expression and control' [164]. In the most common implementation of proteome analysis the core technology is two-dimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis (2-D SDS-PAGE). This technology is preferred due to its ability to separate proteins and successfully present them for analysis in a two-dimensional array [164]. Samples extracted from the cell or tissue analysed, are separated by high-resolution 2-D SDS-PAGE. The 1st dimension of a 2-D SDS-PAGE protocol is isoelectric focusing (IEF). Proteins are separated in a pH gradient by their isoelectric point (pI) this being the point on a pH gradient where a protein has a net zero charge. The 2nd dimension will separate proteins on the basis of molecular mass. Significant advances in the techniques and technology employed for 2-D SDS-PAGE have occurred since inception in 1970 [169] and subsequent development in 1975 [170-173] to make the protocols used recognisable as those currently used in research.

A problem long associated with the technology since its inception is vertical and horizontal streaking particularly when using concentrated samples [174]. Herbert *et al.* [175] proposed a key solution, modifying the standard reducing agent, changing from DTT to tributyl phosphine (TBP), thus giving less streaking and better spot resolution. This improvement is due to the fact that tributyl phosphine is uncharged and remains on the gel during the first dimension separation, isoelectric focusing. Problems associated with complete

sample protein solubilisation have also been solved by the use of a range of alternative reagents, such as stronger chaotropes like thiourea, and in some cases better non-ionic detergents such as sulfobetaines [176]. With *E. coli,* high copy number membrane proteins are found in the insoluble pellet after cell lysis. Heating samples in 0.3% SDS can be used to solubilise proteins for 2-DE analysis providing that urea is omitted from the lysis buffer in order to prevent carbamylation at high temperatures [177]. SDS can be removed from the proteins prior to IEF by adding high concentrations of detergent [178].

With the use of modified reagents, when the whole proteome is being visualised, 2-DE gels can become crowded with protein spots. With the use of narrow pH range IEF strips first dimension separations can now be more spread out. A complete set of overlapping narrow range IEF strips can be used to effectively visualise the whole proteome without over crowding and at the same time improving resolution [163].

1.12.1 Visualisation: Imaging technology

Having achieved maximum separation of a sample using 2-DE a limiting factor in proteomic analysis can be the detection of the protein on the gels. As proteomic technology evolves, protein staining sensitivity is constantly being improved, enabling researchers to better visualise the proteome of their system. The current challenge is to balance the limits of detection of protein visualisation with those of the mass spectrometric methods used to identify proteins [179]. Coomassie Blue is capable of visualising upwards of 500 spots on a gel down to the sub-picomole range. Although this remains a standard for protein staining, 2-DE gels can separate proteins out at attomole levels, which is well below the detection range of Coomassie Blue [180]. Sensitivity of detection can be increased beyond the sub-picomole range by the use of silver staining techniques, which have the potential to visualise in excess of 10,000 spots on a single gel [181]. However silver stain can be negatively selective for glycosylated proteins. This remains a problem for bacterial proteomic research in particular, as many stress responses may involve post transitional modification of existing proteins, including glycosylation [182]. Silver staining techniques also have to be modified if further analysis is undertaken, as standard protocols can interfere with mass spectrometric analysis results [179].

A number of investigators have opted to use new fluorescent stain methods, which can under some conditions reach attomole sensitivity [183]. It has been shown that the newest generation of fluorescent protein stains, compared with traditional staining methods, are more compatible to matrix-assisted laser desorption/ionisation (MALDI) and liquid chromatography-tandem mass spectrometry (LC-MS/MS) methods. However the equipment

required for the accurate visualisation and capture of fluorescent images is expensive and not universally available. The pre-fractioning of protein samples prior to 2-DE may be another way in which low level protein expression could be investigated. Corthals *et al.* [184] described a pre-fractionation approach using electro-kinetic membrane apparatus designed specifically to pre-fraction protein samples in a number of different ways. With this system it may also be possible to enrich some proteins present in physiological samples at attomolar concentrations.

An alternative approach would be the *in vivo* radiolabelling and detection of protein spots by autoradiography. This method has been regularly adopted for defining the proteomes of *E. coli* [185, 186] and *B. subtilis [187]*. More recently the stable isotope labelling and the isotope-coded affinity tags (ICAT) strategy has been shown to provide an ideal method for accurately quantitating low copy number proteins which play a key role in cells or tissues [188]. To characterise and identify unique spots on different gels computerised 2-D gel analysis is required. A number of software systems are currently available for the analysis and comparison of 2-D gels. These include systems by Photorex, BioRad, Melanie 1 and 11, and Quest 1 and 11, and ImageMaster [189].

1.12.2 Identification and characterisation of proteins

With the genomic revolution DNA amplification could be undertaken using PCR technology. The problem with proteomics is that no such analogous system exists for the amplification of trace amounts of proteins separated by 2-DE. The importance of the ability to visualise and select attomole protein spots from a 2-DE gel becomes obvious when the selection of unique stress response proteins is undertaken. The success of 2-DE technology for proteomic analysis is itself reliant on the ability of combining imaging techniques with rapid reliable and sensitive protein identification and characterisation. Protein identification itself can be performed in a number of classical ways, including Edman degradation [190]. As previously indicated 2-DE gels containing up to 10,000 distinct protein and peptide spots have been produced [181] however the main problem with this technique is that over 95% of the spots cannot be sequenced because they are beyond the limits of current high-sensitivity Edman sequencers. With the use of standard-format 2-D gels up to 2,000 spots can be separated all of which can be sequenced by Edman methods [191]. Gooley *et al.* modified an automated protein sequencer and the Edman chemistry to provide a more rapid sample throughput, despite this automation it remains a slow and time-consuming technology [192]. Proteins can also be identified by amino acid compositional analysis, N-terminal sequence tagging, immuno-detection and molecular weight and pI's [164]. However such approaches

cannot necessarily identify proteins that may differ due to post transitional modification, or when a single spot isolated from a gel contains more than one protein. Additionally, some protein spots may be present in too low a concentration, requiring enrichment prior to characterisation. A more recent approach involves the application of two soft ionisation mass spectrometric techniques, matrix assisted laser desorption ionisation mass spectrometry (MALDI-MS) and electrospray ionisation (ESI), both techniques being suited to accurate, rapid and sensitive detection of biological samples [193]. In the peptide mass finger printing approach proteins and peptides are digested either chemically or enzymatically to produce a unique degradation fingerprint that can then be analysed by mass spectrometry (MS) [194].

Proteins can be either digested within the gel matrix or after blotting to a Tropifluor™ polyvinylidene fluoride (PVDF) membrane [195]. Sequence specific proteases can be used to digest the protein and the masses of the peptides produced can be determined using MALDI-MS. These techniques in conjunction with rapidly expanding protein databases have extended the scope and range of protein characterisation. An extension of MALDI-MS peptide mass fingerprints is the use of tandem mass spectrometry to derive a peptide sequence 'tag' (usually comprising three or four amino acids) from the digested peptides. The tag can then be used in combination with information from peptide mass fingerprinting for protein identification [196, 197]. Recently Gatlin *et al.* report improvements in ESI sensitivity by reduction of injection pressure so that the sample spends more time being analysed, and the use of micro-columns to introduce the sample [198]. This has effectively reduced the spot sample concentration required to gain a useful result, down to femtomole level.

The majority of recently reported proteomic studies have used isoelectric focusing (IEF) immobilised pH gradient (IPG) gels for the first separation and sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) for the second dimension. Commercially produced IPG and SDS-PAGE gels in large (20 x 20 cm) and small format (8 x 8 cm) are reliable and readily available [199].

1.13 Proteomic applications in the global analysis of bacterial gene expression

The techniques of proteomics (high resolution two-dimensional electrophoresis and protein characterisation) are widely used in microbiological research to analyse global protein synthesis as an indicator of gene expression. The rapid progress in microbial proteomics has been achieved through the wide availability of whole genome sequences for a number of bacterial groups. Beyond providing a basic understanding of microbial gene expression, proteomics has also played a role in medical areas of microbiology. The characterisation of

the proteomes of bacterial pathogens growing in their natural hosts remains a future challenge [200]. In 1998 alone, large-scale proteome characterisation projects had already been undertaken for a number of different organisms and cell types. At present microbial proteome projects in progress include, *Saccharomyces cerevisiae, Salmonella enterica, Spiroplasma melliferum, Mycobacterium tuberculosis, Ochrobactrum anthropi, Haemophilus influenzae, Synechocystisn* sp., *Escherichia coli, Rhizobium leguminosarum* and *Dictyostelium discoideum* [201]. Many current proteomic projects were initiated prior to the emergence of proteomics as a separate field of research. However, all use the basic technique of 2-DE to investigate global changes in protein synthesis as a marker for altered states of gene expression.

2-DE is an ideal tool for the characterising and identification of protein mutations in eukaryotic as well as prokaryotic cells [200, 202]. The combination of site directed mutagenesis and 2-DE provides an ideal opening for the investigation of genetic interactions. This approach coupled with peptide mass fingerprinting and MALDI-MS has been responsible for highlighting the regulation of proteins during environmental perturbation in a number of bacteria [200, 203-208]. Apart from major stress-induced proteins, many other proteins with altered expression under stress conditions remain unidentified. Once these proteins have been identified by the techniques as described herein (and referred to in references) a greater understanding of the role of these stress proteins relative to metabolic changes during stress should be achieved.

1.13.1 Stress-specific protein purification from food borne bacterial isolates

The list of food-borne pathogens of concern is in constant flux. The adoption of new methods of food processing and microbiological control allows some organisms to gain significance, while others become less important. Existing and emerging food-borne pathogens are always a potential safety hazard to the food industry. In order to fully prevent (or gain acceptable control over) potential food poisoning organisms, it is essential that a complete understanding of the physiology of the response of organisms of concern, (to food related stresses), be achieved.

Food related stress can include, low pH (weak acid preservatives), heat, cold, salt (osmotic stress), oxidative stress, detergents, alcohol, antibiotics (in sub-lethal doses), and nutritional deprivations. *L. monocytogenes*, in common with other bacteria can survive and adapt to harsh conditions and hostile environments. To achieve this the bacterium has the ability to develop mechanisms able to resist the injury caused by stress. The stress can induce variations in the expression, synthesis, and modification of existing and new cell components,

especially proteins. An investigation of heat and cold shock induced proteins in *L. monocytogenes* and *L. innocua* indicated the existence of species-specific stress response proteins [209]. The stress responses to acidity have also been investigated using *L. monocytogenes* [22, 203, 210]. Quantitative analysis of various proteins showed no universal stress proteins, although a number of proteins were induced by different stresses [211].

O'Driscol *et al.* were able to isolate an acid tolerant mutant which demonstrated increased virulence [212]. For bacteria, which infect the host via the intestinal tract, an ability to adapt and survive low pH conditions gives organisms such as *L. monocytogenes* a selective advantage. *L. monocytogenes* in particular has been shown capable of survival at pH 3 for 90 minutes [210]. However the bacteria are only able to survive such conditions if pre- adapted at a higher pH. The specific and general stress responses of *L. monocytogenes* have been investigated using proteomics [203, 210, 213, 214]. The acid stress response important in virulence and food borne survival has been comprehensively investigated by a number of technologies. The ability of the organism to resist a wide range of environmental perturbation at stationary phase and the global changes in gene expression that occur during the transition from exponential growth to stationary phase are less well known.

Any proteomic profile generated must represent the state of the organism at the time of sampling and be reproducible when the organism is subjected to the same conditions. In particular, detailed maps must be created that identify universal house keeping proteins as being different to general stress and stress-specific proteins [211]. Investigators have found that in *E. coli* for example, up to 10% of the sample is not solubilised in normal solubilising agents used in standard 2-DE gels [215]. The separation and characterisation of bacterial proteins is further complicated by the diverse nature of proteins in any given system, and the level, timing, and post transitional modification of some proteins when expressed. In particular, stress related proteins could be expressed in femtomolar quantities that may defy all attempts to separate and visualise them in 2-DE gels. Hence, the need to use existing and new protocols to visualise and characterise very low concentration gel bound protein spots on 2-DE 'maps'. In comparisons of proteomes of different growth states of *L. monocytogenes* batch cultures the ability to reproduce consistent results becomes critical where stress-specific proteins are being sought.

1.14 *Strategy: aims and objectives of current project*

In their early history bacteria, developed protective shock responses, triggered when the organism was subject to some form of environmental stress. In the natural environment

bacterial growth can be limited by the availability of nutrients. Consequently the stress most frequently encountered is starvation. *L. monocytogenes* is widely dispersed throughout the natural environment, and that dispersal is dependent on the ability of the organism to survive for long periods in conditions not suited to growth. Therefore the ability of the organism to survive harsh environments is crucial to the transmission of the organism to the host by a food borne route [216]. These constitutive and induced systems of survival (developed throughout evolution) give the organism the capacity to survive conditions designed to preclude its growth during food processing [21]. When the organism is starved or encounters conditions unfavourable to growth, a large number of physiological changes are induced that lead to the transformation of the cells into metabolically less active, more resistant stationary state. This can be as a stress-adaptive response to nutrient depletion, "metabolic crowding", or, environmental perturbation [217]. The expression of up to 50% of all the gene products present during growth can be modified on transition to a stationary state. The roles that some induced changes play in increased resistance to environmental stress at stationary phase have been highlighted [218]. For example Sigma (B) appears central to the increased resistance of *L. monocytogenes* to heat, ethanol, acid, and oxidative stress resistance and during carbon starvation [137]. Additionally, it has been shown that the expression of the single *L. monocytogenes* superoxide dismutase (SOD) enzyme is constitutively produced in response to many environmental factors and may also be responsive to the cellular growth rate [219]. The transition to a stationary state is therefore a complex one that is central to the long-term survival of the organism in conditions unfavorable for growth.

In the present study the changes in gene expression and the expression of gene products of *L. monocytogenes* batch cultures throughout growth and stationary phase have been compared using a proteomic platform. Prior to the undertaking of the mapping of the bacterial proteomes the 2-DE protocol was standardised. Equally important, was the selection of a growth state, which formed the base standard against which other growth states were compared.

While a proteomic platform is a valuable technique that is able to monitor global changes in gene expression, a strategy for the elucidation of the changes occurring during transition to stationary phase should not rely on a single technique. Many of the methods adopted for the analysis of bacterial culture characteristics fail to detect change at single cell level and generally the data is viewed as a mean [220]. A proteomic map represents all the proteins expressed by all the cells in a culture at the point of sampling, it does not however provide information on culture heterogeneity. Traditional microbiology can be used in

conjunction with varied techniques such as flow cytometry to create a more holistic picture of the events leading to stationary phase. Flow cytometry allows measurements of single bacterial cells and other microorganisms. Flow cytometry has been used to simultaneously examine several physical and chemical cell characteristics, providing information on the heterogeneity of 1000's of bacterial cells in minutes [221].

The aim of this study was therefore to characterise and identify any critical growth phase specific proteins key to the induction of stationary phase in *L. monocytogenes* batch cultures. Temporally specific events important to the onset of stationary phase were sought. It was envisaged that the identification and characterisation of growth phase specific proteins would lead to a greater understanding of the physiology the general stress response (stationary phase) and yield possible targets for more effective microbial control strategies.

2.0 MATERIALS AND METHODS

2.1 Chemicals

All of the chemicals used were of analytical grade or better and were purchased from Sigma Aldrich Company (Co) Limited (Ltd), Dorset UK (unless specified separately in the text). The suppliers of any multi-component analysis kits are referenced as they occur in the following text.

2.2 Bacterial methods

2.2.1 Bacterial organism

Listeria monocytogenes Scott A (*L. monocytogenes)* was kindly donated by Unilever UK. The organism was maintained on BHI agar plates that were stored at 4°C until required. The stock culture plates were replaced each month from the original culture stock stored in 50% glycerol at –80°C (Section 2.4.2.1).

2.2.2 Brain heart infusion (BHI) broth

BHI was supplied by Oxoid (Hampshire UK) and is described as a highly nutritious infusion medium recommended for the cultivation of streptococci, pneumococci, meningococci, and other fastidious organisms. BHI broths were made in accordance with the manufacturers recommended procedures. The broth could be inoculated for immediate use or stored at 4°C for several weeks. BHI agar was supplied by Oxoid (Hampshire UK) and was used throughout for all agar plates. Agar plates could be used immediately or stored at 4°C for several weeks.

2.2.3 Defined medium

A chemically defined medium for the optimal culturing of *L. monocytogenes* was made generally in accordance with the materials and methods published by Phan-Thanh and Gormon (Appendix II) [222]. The medium will be referred to as PTM medium throughout. Medium amino acids and other components were of analytical grade and purchased from Fisher Scientific (Leicestershire, UK).

2.2.4 Long term storage of *Listeria monocytogenes* culture

Most strains of bacteria including *L. monocytogenes* can be stored for many years in a 50% glycerol solution at –80°C. To create a culture store of the original source material a sterile

loop was used to pick a single colony from the source agar plate. A few drops of 50% autoclaved glycerol were used to emulsify the culture. A small autoclaved holed plastic bead (C & H Fabrics, Canterbury, UK) was then mixed with the emulsified colony until the hole in the bead was filled with emulsified culture. The bead was placed in an autoclaved 1.2 mL cryogenic tube (Nalgene from Fisher Scientific UK). The process was repeated with five further colonies and the beads (loaded with culture) were placed in the same cryogenic tube. The tube complete with inoculated beads was then stored at -80°C. Care was taken not to mix individual colonies while emulsifying them with the carrier beads. The process was repeated to provide a large stock of original culture. A single bead carried sufficient bacteria to allow it to be used as an inoculum for future cultures. This allowed the parental strain to be preserved in a form available for easy recovery.

2.2.5 Conditions of bacterial growth

The growth of all *L. monocytogenes* starting cultures and batch cultures was completed using an Infors HT (Infors UK) temperature controlled rotary incubator set at 30°C with 150-rpm agitation. The growth of *L. monocytogenes* on agar plates was conducted using the same incubator, without agitation. The conditions of growth remained constant throughout. At the University of Kent (UKC) an Eppendorf Biophotometer (Eppendorf Cambridge UK) was used to monitor the optical density of bacterial cultures at OD_{600}. At Unilever Research, an Ultrspec 2000 (Amersham Pharmacia, Buckinghamshire) was used to monitor the optical density of bacterial cultures at OD_{600}.

2.2.5.1 Methods of bacterial growth

A single bead containing a single Listeria colony was retrieved from long-term (–80°C) storage (Section 2.2.4) and placed in a sterile loosely capped universal bottle containing a 10 mL BHI broth. The inoculated bottle was placed at an angle of 45° and grown to stationary phase (OD_{600} 1.4) using the standardised growth conditions (Section 2.2.5). To grow cultures on solid medium, a sterile loop was used to streak a small aliquot of a *L. monocytogenes* culture across a BHI agar plate. The streaked plate was incubated at 30°C for 48 hours. The plate could be stored at 4°C for up to one month. All 10 mL BHI starter cultures used to inoculate large volume batch cultures, were initially inoculated by picking a single colony from a BHI agar plate, that had itself been streaked from a bead inoculated starter culture. This practice ensured that any stock culture contamination could be eliminated. All *L. monocytogenes* batch cultures grown for analytical study were inoculated at 2% (v/v) from 10 mL BHI starter cultures.

2.2.6 Maintaining pure cultures of *L. monocytogenes*

At the University of Kent (UKC) a Zeiss standard 20 microscope and oil immersion lens (Carl Zeiss Ltd., Hertfordshire, UK) was used for the observation of bacterial cultures. To ensure that *L. monocytogenes* was a monoculture a number of standard microbiological confirmation tests were adopted as detailed in Table 2.0.

Table 2.0 Methods for the testing of *L. monocytogenes*

Test method	*L. monocytogenes* test result
Gram stain [223]	Gram positive
Catalase reactivity [224]	Catalase positive
Light microscopy	A small rod exhibiting typical tumbling motility [128] during exponential growth. Absence of contaminating organisms
Visual observations (I)	Consistent growth characteristics
Visual observations (II)	*L. monocytogenes* forms small off-white indented colonies on agar plates

The complete test series was carried out on every fourth culture. Every *L. monocytogenes* batch culture was examined by oil immersion light microscopy at X1000 magnification to confirm that no contaminating organisms were present. At Unilever a Leitz Aristoplan microscope and oil immersion lens (X1000 magnification) were used for the observation of bacterial cultures. Metamorph imaging software (v.4.01 Universal Imaging UK) and a Princeton CCD 1300 slow scan camera were used for the acquisition of microscope images of bacterial cultures at Unilever. The catalase reactivity test was performed on cultures sourced from agar plates and batch culture medium.

2.2.7 Determination of growth curves for *L. monocytogenes* in batch culture.

A Bioscreen C automated plate reading system at Unilever (Labsystems, Oy, Finland) was used to monitor the changing optical density of individual bacterial cultures in a 96 well plate. The growth temperature and amount of agitation applied to each plate was adjustable. The growth conditions were set at 30°C with continuous shaking throughout the monitoring period. The Bioscreen C equipment was utilised for the automated monitoring of the optical density of small volumes (300 μL) of *L. monocytogenes* culture over time at preset growth conditions. Optical density readings at a wavelength of 600 nm (OD_{600}) were automatically recorded every 45 minutes for up to 60 hours. The results were exported directly into Microsoft Xcell for further analysis and growth curve construction. The growth curves of

large volume bacterial cultures can also be determined by recording changes in culture OD_{600}. *L. monocytogenes* 1 L batch cultures were sampled at 1 hour timed intervals throughout growth and stationary phase for a period of 24 hours and thereafter at 12-hour intervals for a further 24 hours. At each sample point, a 1 mL aliquot was taken and the OD_{600} was measured and recorded. An Eppendorf Biophotometer (Cambridge UK) was used at UKC to measure the optical density of bacterial cultures at 600 nm.

2.2.8 The Miles-Misra population count [225]

This method was devised to accurately count the viable bacterial population. A *L. monocytogenes* batch culture was sampled at 1 hour timed intervals throughout growth and stationary phase for a period of 24 hours and thereafter at 12-hour intervals for a further 24 hours. At each sample point a 100 µl aliquot was taken and subjected to a 1 in 10 serial dilution in MilliQ water. Three 25 µl aliquots of each dilution (at each sample point) were placed on a BHI agar plate and allowed to dry. The plates were incubated at 30°C for 48 hours. After incubation the number of colonies that had grown from each sample set were counted. The results for each sample point were averaged and the dilution factors accounted for to give a cell count per mL. Only those samples that ranged between 50 and 300 colonies were counted. The results were used to construct growth curves based on viable population counts.

2.2.9 Flow cytometry

Flow cytometry was conducted at Unilever Research utilising an Epics Elite instrument and Expo 32 operating software (Beckman Coulter, Herts. UK). The analysis of results was completed using Expo 32 operating software and WinMDi software (v. 2.8 Joseph Trotter 1993 – 1999 http://facs.scripps.edu/software.html).

2.2.9.1 Stock bacterial culture

2 L PTM medium was inoculated at 2% (v/v) from a *L. monocytogenes* starter culture and placed in an incubator at 30°C with 150 rpm orbital shaking.

2.2.9.2 DNA stains

Two DNA stains were adopted for flow cytometry experiments. The stains SYTO 13 and propidium iodide were supplied by Molecular Probes Europe (Poort Gebouw, Netherlands).

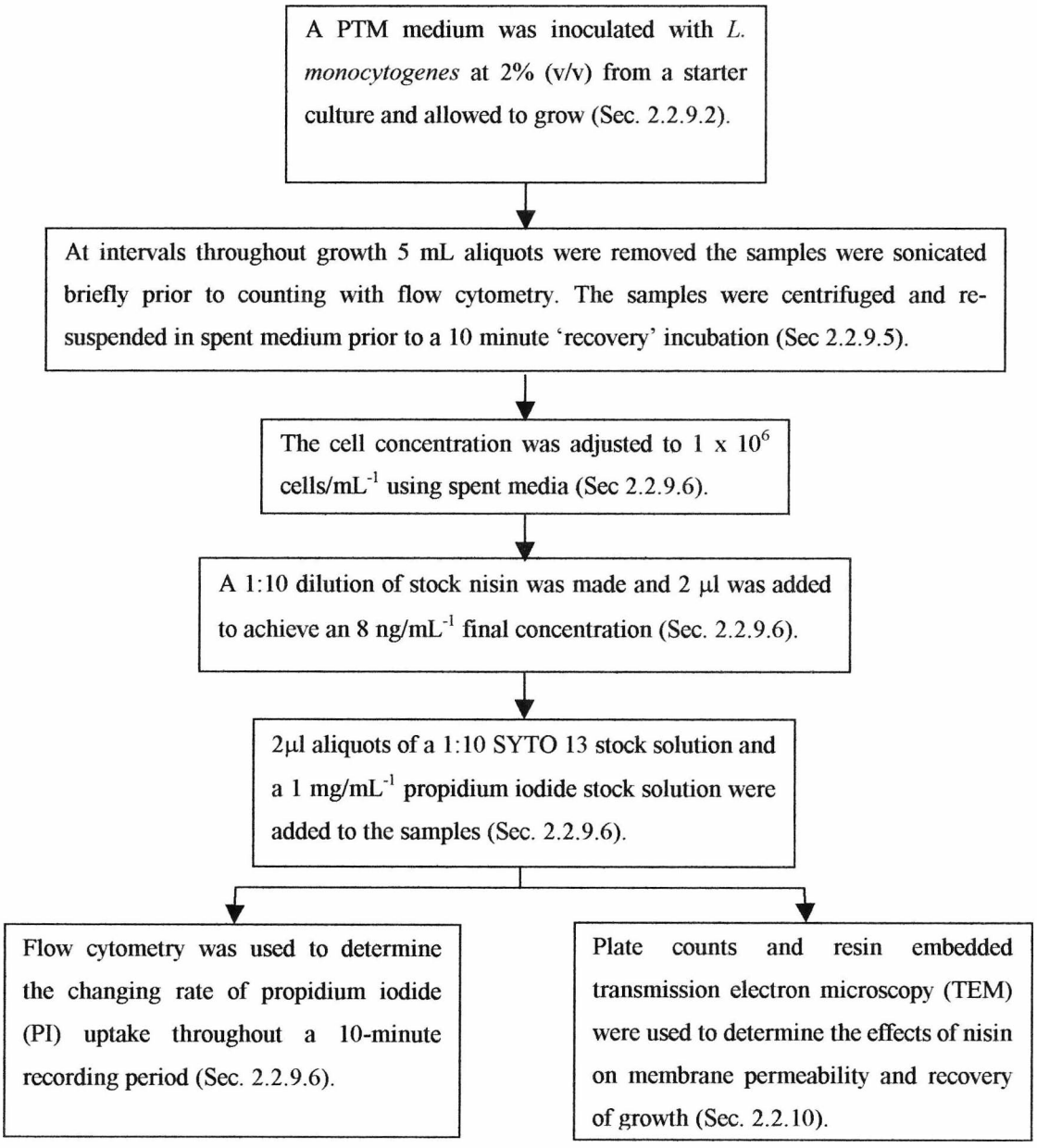
2.2.9.3 Stock solutions for flow cytometric analysis of the Listeria membrane

Nisin was solubilised in 0.2 M HCl at a concentration of 2 mg/mL^{-1} and the solution was used throughout. A stock solution of the dye SYTO 13 was prepared in MilliQ water from

the supplied concentrate at a 1 in 10 dilution. A stock solution of the dye propidium iodide was prepared in MilliQ water at a concentration of 2 mg/mL^{-1}.

2.2.9.4 Permeabilisation of the L. monocytogenes membrane by nisin

A scheme was developed to examine the changing state of the Listeria membrane during growth and its susceptibility to permeabilisation by a nisin challenge.



Scheme 2.0 The use of nisin to highlight the changing state of the Listeria membrane during growth using flow cytometry and TEM (see Sections 2.4.6, 2.4.6.1 and 2.4.6.2)

2.2.9.5 Flow cytometric determination of bacterial particle counts

Bacterial counts were determined by the addition of a solution of 660 nm yellow-green Polysciences (Warrington, USA) latex beads (1 x 10^6 $beads/mL^{-1}$) as an internal standard. The

bead solution was counted and determined at 7.74 x 10^6beads/mL^{-1} and the count cross-referenced with concentrations obtained from the rate of particle passage and the time of sample passage. To complete every sample count 25000 individual events were recorded. The counting of bacterial particles was based on the method published by del Giorgio *et al.* [226].

2.2.9.6 Flow cytometry control experiments

A number of control experiments were conducted to establish the basis for separation of *L. monocytogenes* cells in the same batch culture that were exhibiting different responses to a nisin challenge. An experiment was conducted to determine the volume of nisin that would effectively differentiate stationary phase cells from exponential phase cells by the rate at which the cell membrane was permeabilised. A number of aliquots of a population adjusted stationary phase culture (re-suspended in spent medium) were subjected to a 10 minute 'recovery' incubation. This allowed the cells a period of readjustment to medium conditions after centrifugation and re-suspension. After incubation, different volumes of the stock nisin solution and two dyes SYTO 13 and PI were added to the sample. The samples were viewed for 10-minutes using flow cytometry, any sample that did not show PI incorporation after 10 minutes was considered to contain a nisin concentration that did not permeabilise the membranes of *L. monocytogenes* cells at stationary phase. The experiment was repeated using exponential phase *L. monocytogenes* cells, which showed PI incorporation (and therefore membrane permeabilisation) after 10 minutes. The stock nisin solution was diluted and 2 µl of the diluted stock was added to each sample giving a final nisin concentration per sample of 8 ng/mL^{-1}. This nisin concentration was used in all subsequent nisin experiments.

2.2.9.7 Flow cytometry experimental methods

L. monocytogenes 1 L stock culture was sampled at 1-hour intervals throughout growth and at stationary phase (at intervals and optical density matching those used for the provision of spent medium diluent stock). The samples taken from the stock culture were sonicated in a water bath (Ultrawave U50/D, Fisher Scientific, UK) for ten seconds and were then counted using the flow cytometer. The sonication step ensured that any aggregating cells were separated and a more accurate count could be obtained. The sample was centrifuged and re-suspended in spent medium, the cell concentration was adjusted by dilution with spent medium so that the cell population of each sample was consistently 1 x 10^6 cells/mL^{-1}. This excluded concentration effects from the observed results, as the cell/nisin ratio remained constant. The sample was then incubated for 10 minutes with agitation at the standard growth conditions. After incubation 5 µl of the population adjusted cells, 2 µl diluted stock nisin solution, and 2 µl stock dye solutions were added to 490 µl of spent medium. The sample was

then passed through the flow cytometer. Individual events were recorded throughout a 10-minute period creating a kinetic record of the rate at which the cells became PI positive.

2.2.10 The effect of nisin on growth and membrane integrity of a *L. monocytogenes* batch culture

A *L. monocytogenes* batch culture was sampled at 1 hour intervals throughout growth and the samples subjected to a 10-minute incubation with nisin. The protocols adopted were as detailed in Section 2.2.9.4. The cell samples taken throughout growth were not subject to flow cytometric analysis but were fixed in glutaraldehyde and prepared for examination by TEM (Section 2.2.11). Aliquots of each sample were also subjected to Miles-Misra [225] population counts to determine the number of cells that would not survive exposure to the nisin concentration adopted for these experiments.

2.2.11 Transmission electron microscopy (TEM) sample preparation

The visualization of the external structures of bacterial cells such as flagella can be achieved using transmission electron microscopy (TEM) and negative staining procedures.

2.2.11.1 Negative staining of bacterial samples for TEM

Techniques for the preparation of negative stain specimens are direct. A sample of *L. monocytogenes* batch culture was taken and applied directly to the surface of a small copper grid that had a covering support film. The population of Listeria cells becomes adsorbed to the surface and attachment was sufficiently secure for the cells not to be removed by subsequent rinsing and staining procedures that did remove most of the medium salts. A methylamine tungstate stain was applied directly to the adhered sample at a concentration of 4%. The aim of the procedure was to embed the specimen in a uniform thin deposit of methylamine tungstate stain. Resolution of the bacterial external features was accomplished at the stain-specimen boundary where there was maximum contrast [227].

2.2.11.2 Resin embedding of bacterial samples for TEM

The membrane and internal structures of bacterial cells can be visualised by embedding the sample in a resin and viewing thin slices of the resin using TEM. Samples of Listeria were washed prior to fixing the cell pellet in a 3% glutaraldehyde 100 mM sodium cacodylate (pH 7.4) solution. After washing and post fixing in 2% osmium tetroxide the samples were washed again and stained with 1% uranyl acetate in maleate buffer pH 5.2. The samples were then washed and dehydrated in 70% ethanol prior to embedding in Spurr's resin. Once the resin has hardened, the samples were sectioned and viewed using TEM. The sample preparation

protocols and procedures for the TEM were as detailed in Principles and Techniques of Electron Microscopy [227]. Image acquisition by TEM was achieved with a Phillips 525 M based at the University of Kent. TEM images were also acquired at Unilever Research using a Joel 1200 Ex Mark II TEM.

2.3 Chemical Analysis

2.3.1 Monitoring medium pH during *L. monocytogenes* batch culture.

L. monocytogenes PTM medium culture was sampled at 1-hour intervals throughout growth and stationary phase for a period of 24 hours and thereafter at 12-hour intervals for a further 24 hours. Each sample was centrifuged and the cell pellet was discarded. The medium supernatant was filter sterilised by passage through a Sartorius Minisart blue filter unit (pore size 0.2 μm). The pH of each medium sample was recorded using a Jenway 350 portable pH meter (Jenway Ltd, Essex, UK)

2.3.2 The utilisation of glucose by a *L. monocytogenes* batch culture

Samples of medium were taken from a *L. monocytogenes* batch culture throughout the growth and stationary phase. The samples were taken at 1-hour intervals up to 24 hours then at 36, and 48 hours. The medium samples were prepared as detailed in Section 2.5.1 and glucose concentration determined for each sample. Determination of the medium glucose concentration was achieved with the use of a hexokinase/glucose (HK) assay kit supplied by Sigma Aldrich UK. The method was conducted in accordance with the manufacturer's supplied protocol and is based on the hexokinase/glucose–6–phosphate dehydrogenase enzymic assay [228].

2.3.3 The utilisation of amino acids by a *L. monocytogenes* batch culture

Medium samples were prepared as detailed in Section 2.5.1 and subjected to high-pressure liquid chromatographic (HPLC) separation. The equipment comprised, a Waters 474 scanning fluorescence detector, Waters 600 controller, Waters 626 pump, and Waters in-line degasser (Waters HPLC, Hertfordshire, UK). A Phenomenex sphereclone ODS (2) 150 mm x 4.6 mm bore 5μ (micron) column (Phenomenex, Cheshire. UK) and Jones Chromatography column heater (Mid Glamorgan, UK) were used. The column temperature was maintained at 25°C. Fluorescent measurements were made at an excitation wavelength of 337 nm and an emission wavelength of 452 nm. Data were collected online and processed by Millennium

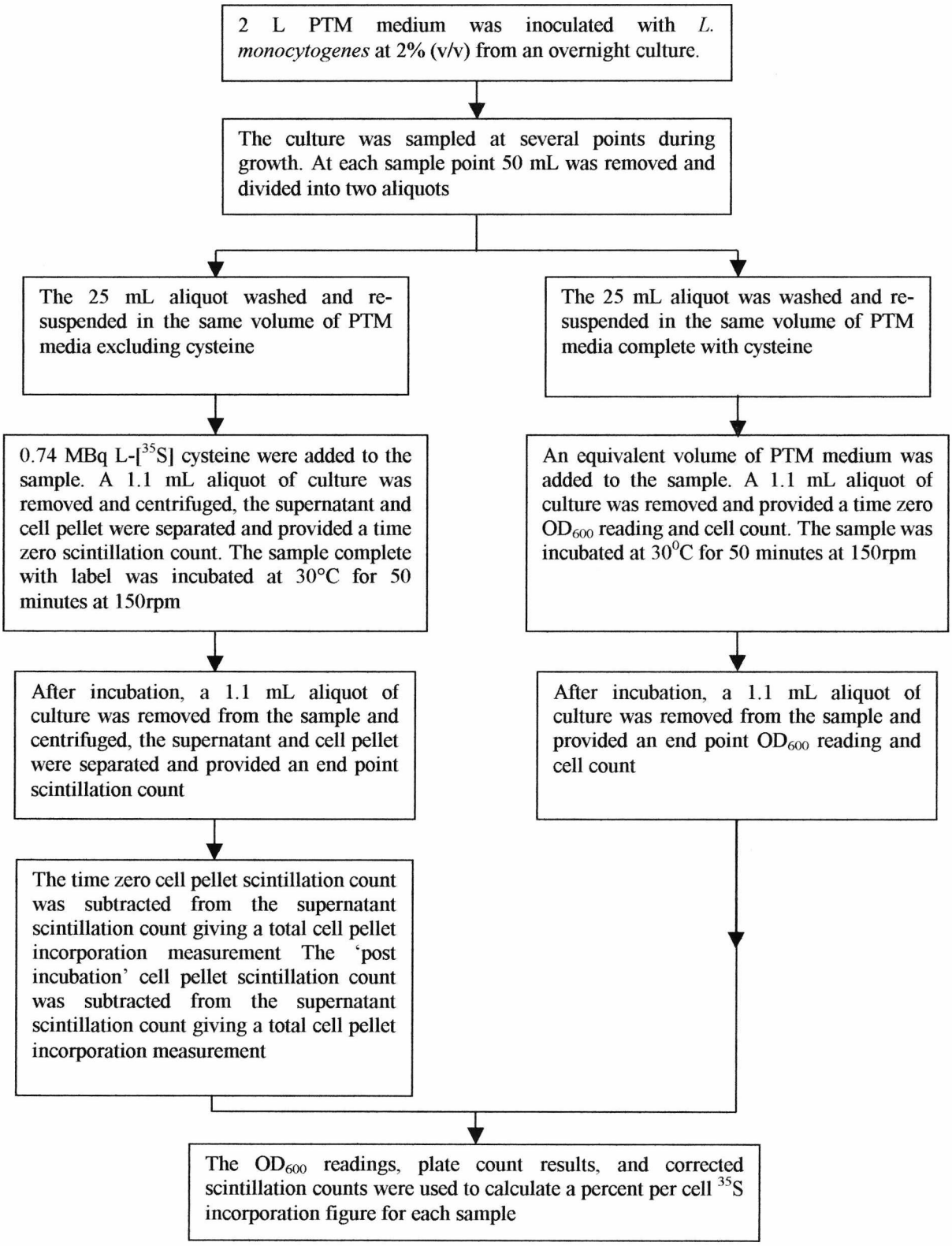
software (Waters UK). A known quantity of L–asparagine was added to each sample to achieve a concentration of 100 mg/L^{-1} immediately prior to the HPLC separation. All of the medium samples were compared to a time zero medium control that also contained L–asparagine at 100 mg/L^{-1}. Quantitation was achieved by measuring peak height and area relative to the introduced L-asparagine standard. The reagents and method used for the separation and quantitation of primary amino acids was based on the O–phthaladehyde (OPA) pre-column derivatisation of amino acids by gradient elution reverse phase HPLC and were as described by Fekkes *et al.* 1995 and 2000 [229, 230]. This method did not allow the differentiation and quantitation of the amino acid cysteine.

2.3.4 The incorporation of L-[^{35}S] cysteine by a *L. monocytogenes* batch culture during growth and stationary phase

L. monocytogenes 2 L PTM medium batch culture was inoculated at 2% (v/v) from an overnight BHI culture. Standard growth conditions were adopted for the growth of the culture. At 1 hour intervals a 1 mL aliquot was taken and the OD_{600} determined. When the culture OD_{600} reached 0.25 a 25 mL aliquot was taken and equally divided providing a sample and negative control. Both sample aliquots were centrifuged; one pellet was re-suspended in a complete PTM medium (the control culture) while the remaining pellet was re-suspended in a cysteine deficient PTM medium. A radioactive label, L-[^{35}S] cysteine was added to the cysteine deficient sample. The sample and control were used to determine the level of L-[^{35}S] cysteine incorporation after a 50 minutes incubation. After labelling, the Listeria samples were centrifuged and the supernatant and cell pellet separated. Each fraction was diluted in Optiphase 3 Hisafe scintillation fluid (APB UK) prior to the determination of uptake of ^{35}S by scintillation counting (Scintillation Counter, LS 6500 Value System, Beckman Coulter Buckinghamshire UK). Quenching was automatically calculated by monitoring an external standard adjacent to sample vials during counting giving a single label destructions per minute (DPM) count for each sample. A correlation between the spectral distribution of the external standard and the measured efficiency in the sample region is established by measuring a series of quenched standards. The external standard is positioned for 15 seconds adjacent to the vial before sample counting begins. The external standard spectrum is accumulated. During the first 15 seconds of the sample count, the sample contribution to the external standard spectrum is automatically subtracted to provide the external standard spectrum. Results will be presented as a percentage DPM relative to each sample and not relative to the total label available. The sample giving the highest DPM count was assigned 100% and remaining

samples were adjusted to that standard giving an incorporation percentage for each subsequent sample relative to the 100% sample. The experiment was conducted in accordance with the protocol detailed in Scheme 2.1. The protocol was developed to achieve a minimum incubation period over which reproducible labelling with L-[^{35}S] cysteine would occur. The protocol was based on the published methods of Phan-Thanh *et al.* (1999), and Esvan *et al.* (2000) [203, 231]. These methods were based on an L-[^{35}S] methionine/ L-[^{35}S] cysteine labelling mixture. The current method was adapted to use a single label. The protocol and analysis procedures for each sample were as detailed in Scheme 2.1. The radioactive label L–[^{35}S] cysteine was supplied by APB UK as hydrochloride in an aqueous solution.

A method was developed to quantify the incorporation of L–[^{35}S] cysteine by 1 hour separated aliquots of a *L. monocytogenes* batch culture during a 50 minute incubation.



Scheme 2.1 The incorporation of L-[^{35}S] cysteine by *L. monocytogenes*

2.4 Sample preparation for the analysis of the *L. monocytogenes* proteome

2.4.1 Listeria culture washing buffer

A pH 7 low salt buffer was used to wash Listeria cell samples (Table 2.1). A low salt concentration was used to limit the osmotic challenge to the cell during washing protocols. Ethylenediamine tetra acetic acid (EDTA) was included to inhibit the proteolytic action of metalloprotease enzymes. The washing buffer could be stored for several weeks at 4°C.

Table 2.1 *L. monocytogenes* washing buffer

Component	Concentration
Tris	100 mM
EDTA	100 mM
Protease inhibitor cocktail	1 Tablet per 5 mL
The buffer was adjusted to pH 7.0 with drop wise additions of 10 M HCl	

2.4.2 Centrifuge wash

A Beckman J2–21 centrifuge with JA-10 and JA-20 rotors (Beckman Coulter, Hertfordshire, UK) was used to centrifuge 50 mL to 250 mL culture volumes. Prior to further processing *L. monocytogenes* batch cultures were centrifuged and washed. The culture samples were centrifuged at 6000 rpm for 10 minutes at 4°C. The supernatant was discarded and the cells re-suspended in a washing buffer (Section 2.4.1). The cells were centrifuged as specified three times with fresh buffer prior to the re-suspension of the cell pellet in a lysis buffer (Section 2.4.2) of a sufficient volume to achieve a population of 1 $x10^9$ cells/ mL^{-1}. The samples could be held on ice for further processing or placed in a freezer at -80°C for prolonged storage.

2.4.3 Lysis buffer solutions

In normal biological systems protein recycling occurs as proteins are broken down and recycled by protease enzymes. These enzymes are either compartmentalised or controlled so that undamaged proteins remain intact. When a cell is lysed, the protease enzymes present are released and can act on all the proteins present. To control the effects of protease enzymes a protease inhibition cocktail in tablet form was used adding one tablet per 5 mL of sample (Boehringer Mannheim mini protease inhibitor cocktail). Two buffers were used for the re-suspension of washed Listeria cells prior to lysis. The first, lysis solution 1 was adapted from

the cell-washing buffer detailed in Table 2.1. When utilised as a lysis buffer dithiothreitol (DTT) was added at 1% (w/v). Protease inhibition cocktail Tablets (Boehringer Mannheim) were added as detailed above. An alternative buffer, lysis buffer solution 2 was a Tris base with EDTA, DTT, and a protease inhibitor cocktail tablet as detailed in Table 2.2. The molar concentrations of Tris and EDTA were low compared to lysis buffer solution 1.

Table 2.2 Components of cell lysis buffer solution 2

Component	**Concentration**
Tris	25 mM
EDTA	50 mM
DTT	1% (w/v)
Protease inhibitor cocktail	1 Tablet per 5 mL
Adjusted to pH 7.0 with drop wise additions of 10 M HCl	

2.4.4 The extraction of Listeria proteins by cell lysis

2.4.4.1 Extracted sample protein assay

The quantitation of protein extracted from *L. monocytogenes* batch culture was achieved using a modified Bradford protein assay [232], and a standard curve constructed using bovine serum albumin. Triple repeats of each sample were completed at each dilution and an average calculated. A calibration curve was constructed for each new protein assay (Appendix III). The determination of protein concentrations in isoelectric focusing (IEF) buffers were based on the published method of Ramagli, who stated that for consistent results readings should be taken directly from the calibration curve without reference to a line of best fit [232].

2.4.4.2 Sample lysis solutions

The term' lysis solution' refers to the buffer into which a cell pellet was suspended prior to the application of a specified cell lysis protocol. Two options were considered, a standard lysis solution based upon the washing buffer (Section 2.4.3) and lysis solution 2 (Table 2.2) based upon a method suggested by Phan-Thanh and Mahouin [143].

2.4.4.3 Cell lysis: freeze/thaw cycling

After completion of the wash protocol a *L. monocytogenes* cell pellet was re-suspended in sufficient volume of lysis buffer to achieve a concentration of 1 x 10^9 cells/mL^{-1}. The re-suspended pellet was then subjected to repeated freeze/thaw cycles by immersion in liquid

nitrogen prior to a thaw at room temperature. A protein assay was conducted on each sample on completion of each freeze/thaw cycle.

2.4.4.4 Cell lysis: French press

After completion of the wash protocol, a *L. monocytogenes* cell pellet was re-suspended in sufficient volume of lysis buffer to achieve a concentration of 1 x 10^9 cells/mL^{-1}. The cell suspension was frozen at -20°C. The frozen sample was placed in pre-cooled pressure cell (4°C) and subjected to 20,000 psi (pounds per square inch) at a high-pressure setting utilising Thermo-spectronic French pressure cell equipment (Rochester, USA). The samples were subjected to increased passages through the press and a protein assay conducted to determine the number of passages giving the highest protein yield.

2.4.4.5 Cell lysis: sonication

A cell pellet could be retrieved from low temperature storage -80°C or sourced from a same day extract. The cell pellet was re-suspended in a sufficient volume of lysis buffer to achieve a concentration of 1 x 10^9 cells/mL^{-1}. The cell suspension was then subjected to either a single timed sonication protocol of 10 minutes or a cyclic timed sonication protocol of 3 X 5 minutes using a LABSONIC® U sonication unit and small probe (B. Braun Biotech, Germany). The sonication power setting was high power with a frequency of 20 micrometers cycling at 0.6 sec/sec for the duration of the sonication step.

2.4.4.6 Estimating the efficiency of each lysis method

A simple method was adopted for an estimation of the efficiency of cell lysis. A sample was divided into two aliquots; one acted as a negative control while the other was subjected to one of the three lysis methods investigated. A known volume of the treated sample was placed on a microscope slide next to the same volume from the control sample, and both were heat fixed by passage through a Bunsen flame. The samples were then viewed using oil immersion light microscopy at x1000 magnification. The number of cells observed from three fields of view was averaged and a comparison of cell numbers was made between each sample and its control. The change in cell numbers was attributed to cell lysis.

2.4.5 Removal of contaminating DNA and RNA

Cell disruption released nucleic acids into the sample making it viscous and difficult to handle. Nucleic acids can also interfere with 2-D electrophoresis taking up protein stain and causing streaking in 2nd dimension gel images. The nucleic acids were digested by the addition an aliquot of a 2 μg/mL^{-1} DNAase/RNAase (bovine pancreatic deoxyribonuclease 1 and bovine pancreatic ribonuclease A, Sigma Aldrich, UK) stock solution giving a final

concentration within the sample of 10 ng/mL^{-1}. The samples were incubated at room temperature for 20 minutes after lysis and prior to the addition of IEF buffer constituents.

2.4.6 Acetone precipitation of proteins

After extraction of the Listeria proteins in a lysis buffer, further solubilisation could be achieved by the addition of IEF buffer constituents. Alternatively, proteins could be precipitated out of the lysis buffer and re-solubilised directly into an IEF protein solubilising buffer. On completion of the cell lysis and DNAase/RNAase incubation protocol, the samples were diluted with three times their own volume of ice-cold acetone containing 10% TCA (v/v) and 0.007% (v/v) β-mercaptoethanol. The sample solution was then incubated at -20°C for 2 hours. The sample was then centrifuged at 6000 rpm for 10 minutes and the supernatant discarded. The protein pellet was washed in ice-cold acetone and centrifuged again [233-235]. Once washed the protein pellet was placed in an IEF buffer and incubated at room temperature for 20 minutes prior to continuous vortex for 45 minutes. The vortex timing was adjusted where required to ensure complete solubilisation of the precipitated protein pellet in the IEF buffer.

2.5 Selection of a protein solubilisation buffer from two standards

Details of a urea-based iso-electric focusing (IEF) buffer are published by Amersham Biotech [236]. This buffer was adopted as a standard protein solubilising buffer but was modified according to differing protein solubilisation and gel resolving conditions. In 1997, Rabilloud *et al.* published details of an improved IEF buffer containing thiourea [237]. This buffer was adopted as second standard but was modified according to differing protein solubilisation and gel resolving conditions. A range of modified buffers (Table 2.3) were used to solubilise proteins extracted from *L. monocytogenes* into two lysis buffers (Section 2.4.3) using different cell lysis methods (Section 2.4.4.3-5). After lysis and DNAase/RNAase incubation (Section 2.4.5) additions to the lysed sample were made in accord with the protein solubilising buffers detailed in the Table 2.4. The protein solubilising buffers detailed included DTT as a reducing agent. It has been reported that replacing the thiol containing reducing agent with non-charged reducing agents such as tributyl phosphine (TBP) can enhance protein solubility during IEF and improve the transfer of proteins from the first dimension to the second dimension in a 2-D electrophoresis separation [175]. The protein solubilising buffer systems detailed in Table 2.3 were considered with DTT and TBP as a reducing agent.

Aliquots of the same *L. monocytogenes* protein extracts were solubilised in different protein solubilising buffers and subjected to separation by mini gel format 2-D electrophoresis (Section 2.9.1.6). Visual differences in the resultant 2-D gels were used to select the optimal solubilising conditions. The majority of the protein solubilising buffers were easily rejected due to poor resolution and separation on a 2-D gel. A limited number of the protein solubilising buffers detailed in Table 2.3 produced good resolution and separation of proteins on a 2-D gel. These solubilising buffers were selected for further analysis to determine which produced the greatest sample separation and resolution.

Table 2.3 Protein solubilisation buffers

Prefix	Constituents
A	9.5M Urea, 40mM Tris, 4% CHAPS (w/v) 1% DTT (w/v), 0.8% pharmalyte (v/v), Bromophenol blue (trace)
B	Buffer B differed from buffer A as follows: 9.0 M Urea, and exclude 40 mM Tris
C	Buffer C differed from buffer A as follows: 8M Urea, and exclude 40 mM Tris
D	Buffer D differed from buffer A as follows: 7M Urea, and exclude 40 mM Tris
E	Buffer E differed from buffer A as follows: 9M Urea 3M thiourea, and exclude 40 mM Tris
F	Buffer F differed from buffer A as follows: 8M Urea 3M thiourea, and exclude 40 mM Tris
G	Buffer G differed from buffer A as follows: 7M Urea 3M thiourea, and exclude 40 mM Tris
H	Buffer H differed from buffer A as follows: 9M Urea 2M thiourea, and exclude 40 mM Tris
I	Buffer I differed from buffer A as follows: 8M Urea 2M thiourea, and exclude 40 mM Tris
J	Buffer J differed from buffer A as follows: 7M Urea 2M thiourea, and exclude 40 mM Tris
K	Buffer K differed from buffer A as follows: 7M Urea 1M thiourea, and exclude 40 mM Tris
A1 – K1	REPEAT BUFFER EXPERIMENTS A - K REPLACING DTT WITH TBP

CHAPS: (3-[(3-Cholamidopropyl) dimethylammonio]-1-propane-sulphonate) TGS

2.5.1 Optimisation of protein solubilisation

The protein solubilising buffer 1 detailed in Table 2.4 was adapted from a protocol published in 1999 by Phan-Thanh *et al.* [203]. The constituents making up protein solubilisation buffers 1-4 detailed in Table 2.4 were added to samples that had been extracted in lysis buffer 2. The constituents making up protein solubilisation buffer 5 were added to samples that had been extracted in lysis buffer 1.

Aliquots of the same *L. monocytogenes* batch culture were re-suspended in a sufficient volume of the detailed lysis solution (Table 2.4) to achieve a cellular concentration of 1 x 10^9 cells/mL^{-1}. Aliquots of each sample were subject to cell lysis by sonication. After lysis and prior to further additions (as detailed in Table 2.4) a DNAse/RNAse incubation was carried

out. Additions were then made to the lysed sample to increase the protein solubilisation conditions, denaturing the proteins, and making the sample suitable for IEF separation. Once additions to the sample were completed, the volume was adjusted with MilliQ water to give a final volume double that of the initial sample volume. The samples were then assayed to determine their protein concentration. The different samples were subjected to separation by mini gel format 2-D electrophoresis.

Table 2.4 Protein Solubilisation Buffers Selected For Further Analysis

Prefix	Lysis Buffer	Constituents
1	1	9.5M Urea, 40mM Tris, 4% CHAPS (w/v) 1% DTT (w/v), 0.8% pharmalyte (v/v), Bromophenol blue (trace)
2	1	Buffer 2 differed from buffer 1 as follows: 8M Urea, and excluded 40 mM Tris
3	1	Buffer 3 differed from buffer 1 as follows: 8M Urea, 3M thiourea, and excluded 40 mM Tris
4	1	Buffer 4 differed from buffer 1 as follows: 7M Urea, 2M thiourea, and excluded 40 mM Tris
5	2	Buffer 5 differed from buffer 1 as follows: 7M Urea, 2M thiourea, and excluded 40 mM Tris

2.6 Electrophoresis

2.6.1 Visualisation of Proteins

A number of stains were used for the visualisation of proteins separated by 2-D electrophoresis, these were; Brilliant blue R-250 (Sigma Aldrich), Bio-safe colloidal coomassie (BIO-RAD, Hertfordshire, UK), Silver stain Plus silver stain kit (BIO-RAD), Fluorescent protein stains SYPRO® orange and SYPRO® ruby (Molecular Probes, Holland), and Plusone silver stain kit (Amersham Pharmacia). A silver stain based on the published method of Shevchenko [238] was also used; the silver nitrate was purchased from BIO-RAD, other chemicals used to complete the stain were of analytical grade and purchased from Fisher Scientific, Leicestershire, UK.

2.6.2 Electrophoresis buffer

A concentrated premixed Tris/glycine/SDS (TGS) electrophoresis buffer was purchased from BIO-RAD. This buffer was used as a standard buffer for all SDS-PAGE experiments.

2.6.3 1-D SDS PAGE sample buffer

Laemmli [239] premixed non-reducing sample buffer was purchased from BIO-RAD.

2.6.4 Equilibration buffer

After IEF and prior to the running of the second dimension acrylamide gel separation an equilibration buffer was used for the incubation of Immobiline immobilised pH gradient dry strip gels. The constituents were combined in a 0.05M Tris/HCl pH 8.8 buffer as detailed in Table 2.5. The equilibration buffer could be stored at room temperature for a few days or frozen at -80°C and stored for several months.

Table 2.5 Components of IEF equilibration buffer

Component	Concentration
Urea	6 M
Glycerol	30% (v/v)
SDS	2% (w/v)
Adjusted to pH 8.8 with drop wise additions of 10 M HCl	

2.6.5 Bromophenol blue solution

A bromophenol blue solution was used as an indicator to visualise the gel front during second dimension gel electrophoresis. To make the solution bromophenol blue was added to a 1 X TGS buffer to give a final concentration of 0.25% (w/v). The solution could be stored for several weeks at 4°C. A few grains of bromophenol blue could also be added directly to the extracted protein samples.

2.6.6 Agarose sealing solution

An ultra pure low melting point type VIII agarose solution was used to create an unbroken seal between the 1st and 2nd dimension gels when conducting 2D SDS-PAGE, allowing unhindered passage of proteins from the first to second dimension. To make the solution agarose was added to a 1 X TGS buffer to a final concentration of 0.5% (w/v). Agarose could be stored at room temperature for several months.

2.6.7 Protein separation by IEF

2.6.7.1 IEF with Immobiline immobilised pH gradient dry gel strips (IPG gels)

IEF is the separation of proteins by their iso-electric point. IPG gels could be purchased in a range sizes, 7 cm, 13 cm 18 cm, and 24 cm. Within these size categories, the gels were also available with different defined pH immobilised gradients (Amersham Biotech). Immobilised pH gradient buffer (IPG buffer) was available in a range that complimented the IPG gel pH separating range. The IPG buffer was added to samples prior to IEF and was designed to help maintain the pH gradient during IEF.

2.6.7.2 IEF protocols utilising the IPGphore (Amersham Biotech)

The IPGphore [240] is an integrated instrument that includes a Peltier element for temperature control and a programmable power supply. IPG gels and matching IPG buffer were utilised for IEF protein separation. *L. monocytogenes* whole cell protein extracts were extracted immediately prior to further analysis or retrieved from storage at -80°C. A protein assay of the sample was conducted and the buffer concentration was adjusted to achieve a protein concentration of 10 µg in 125 µl as recommended by the manufacturer. The sample was distributed evenly between the electrode contacts of an IPGphore ceramic sample boat and the pH 3 – 10 7 cm IPG gel placed gel side down within ceramic holder. The sample and strip was overlaid with silicone oil and covered with a plastic lid. The sample boat was then positioned between the contact plates on the IPGphore and a pre-programmed protocol (Table 2.6) instigated. The focusing was complete when the recommended Volt/hrs were achieved. When using an IPG gel in combination with the IPGphore IEF equipment the run time, applied voltage, and temperature can be adjusted to take account of gel protein loads and the physical length of the gel. The sample preparation procedure remained the same although when using 18 cm IPG gel the protein load was increased from 10 µg to 100 µg per gel strip. A subsequent increase in the run time and volt\hrs was also required (Table 2.6). The pH range over which protein mixtures were separated by IEF could be adjusted by the selection of a pH 4 – 7, 18 cm IPG gel. The sample preparation, loading procedures, and IEF running protocol for the analytical separation of proteins using pH 4 – 7 18 cm IPG gels did not differ from that detailed for pH 3 – 10 18 cm IPG gels (Table 2.6). After a protein assay the sample concentration was adjusted to achieve a protein concentration of 120 µg in 350 µl. The samples were loaded onto the IPG gel and a pre-programmed protocol (Table 2.6) was utilised for the IEF separation of the sample.

Table 2.6 IPGphore protocols for the analytical separation of protein mixtures derived from *L. monocytogenes*

Step	Voltage mode	7 cm Immobiline gel pH 3 – 10 separating range Total protein load10 μg		18 cm Immobiline gel pH 3 – 10 separating range Total protein load 100 μg		18 cm Immobiline gel pH 4 – 7 separating range Total protein load 100 μg	
		Volts	**Time**	**Volts**	**Time**	**Volt**	**Time**
1	Step'n'hold*	30	12	30	12	30	12
2	Step'n'hold	100	1	100	1	100	1
3	Step'n'hold	500	1	500	1	500	1
4	Gradient**	500 - 8000	0.5	500 - 8000	1	1	1
5	Step'n'hold	8000	1 - 2	8000	2 - 4	8000	2 - 5
Total V/hr		**8000**		**28000**		**28000**	

*The mode of voltage increase 'Step'n'hold' refers to a single applied voltage increase to the next specified voltage step without any graduation. **The mode of voltage increase 'gradient' refers to a gradual applied voltage increase throughout the specified time setting for that step.

A protein load of up to 3000 μg could be applied to an 18 cm IPG gel and separated using the protocol detailed in Table 2.7, the increased protein load required a subsequent increase in the running time to complete the IEF.

Table 2.7 IPGphore protocol for the separation of protein mixtures derived from *L. monocytogenes*

Step	Mode	Volt)	Time (h)	Total V/Hr
1	Step'n'hold	30	12	
2	Step'n'hold	100	1	
3	Step'n'hold	500	1	
4	Gradient	500 – 8000	1	
5	Step'n'hold	8000	8 - 10	**85000**

2.6.7.3 Equilibration of IPG gel strips after IEF

Equilibration is a two-step process that enhances the second dimension gel result and modifies the side chain of the amino acid cysteine. The equilibration steps for 7 cm IPG gels were completed using a plastic petri dish (Bibby Sterilin). The equilibration protocol for 18 cm IPG gels was completed using 10 mL graduated pipettes. A 10 mL volume of equilibration buffer (Section 2.6.4) containing 100 mg DTT was added to the appropriate vessel containing the IPG gel and was agitated gently for 15 minutes. The gel was rinsed with MilliQ water prior to

the second equilibration step, during which 260 mg iodoacetamide was added to a second 10 mL aliquot of equilibration buffer. As before the focused gel and equilibration buffer were agitated gently for 15 minutes before rinsing the gel with MilliQ water. At this point, the equilibrated IPG gel could be forwarded for 2nd dimension separation by SDS PAGE. On completion of the first dimension, the IPG strip can be stored at - 80°C for several months but equilibration should not be performed before storage.

2.6.8 Self casting of polyacrylamide gels

Independent of specific physical gel size or the gel running system adopted for sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), all gels were cast from a monomer stock solution of 30% acrylamide/bis 37.5:1 (BIO-RAD UK) adjusted to achieve a final acrylamide concentration of 12.5%. A 4% stacking layer was added to gels used for the one dimensional SDS-PAGE separation of proteins. Once cast, the gels could be stored for up to one week at 4°C in zip-lock bags containing a small volume of running buffer.

2.6.9 Small format vertical 2-D SDS PAGE

Two small format self-cast 12.5% polyacrylamide gels could be simultaneously run using the Novex Vertical Xcell Mini-Cell electrophoresis system (Invitrogen, Groningen, Holland). To use the Novex equipment for the 2-D separation of *L. monocytogenes* whole cell protein extracts, equilibrated 7cm IPG gels were joined to 12.5% acrylamide gels. The integrity of the join at the gel-gel interface was maintained by the addition of an agarose covering layer. A preset programme (Table 2.8) was used for the separation of proteins by 2-D electrophoresis. The separation was conducted at room temperature.

Table 2.8 Pre-set mini gel running protocol

Applied parameter	Applied units
Volts	125 Constant
Current start	30-40 mA
Current end	8-12 mA
Run time	80-100 minutes

2.6.10 Large format vertical SDS PAGE

Four large format self-cast 12.5% polyacrylamide gels could be simultaneously run using the BIO-RAD Protean II XL gel system (BIO-RAD). To use the Protean II XL gel system for the 2-D separation of *L. monocytogenes* whole cell protein extracts, equilibrated 18 cm IPG gels

were joined to 12.5% acrylamide gels. The integrity of the join at the gel-gel interface was maintained by the addition of an agarose covering layer. The core of the BIO-RAD Protean II XL system was connected to a refrigerated water bath (Techne TempHC TE-8A) allowing the gel temperature to be maintained at 10°C during electrophoresis. The electrophoresis was conducted in two stages, the first stage was conducted at a lower voltage setting than stage two (Table 2.9). This allowed a controlled passage of proteins from the first dimension IPG gel to the second dimension polyacrylamide gel before the voltage was increased to complete the protocol.

Table 2.9 Running conditions for BIO-RAD Protean II XI vertical electrophoresis system

Time	Voltage	Current	Power	Temperature
45 min	150 V	40 mA	300 W	10°C
360 min	275 V	40 mA	300 W	10°C

2.6.11 Horizontal 2-D SDS PAGE

All horizontal large gel format (18.5 x 20 x 0.05 cm) SDS-PAGE was performed using a Multiphore II electrophoresis system (Amersham Biotech). The two dimensions of 2-D electrophoresis, IEF, and SDS PAGE, could be conducted on the same equipment. A Techne TempHC TE-8A refrigerated bath was used to maintain the gel support core temperature at 10^0C during IEF and SDS-PAGE. The Multiphore II horizontal electrophoresis equipment was used in conjunction with 18 cm pH 4-7 IPG gels for the IEF separation of *L. monocytogenes* whole cell protein extracts. The materials and methods adopted for the separation of proteins by IEF using the Multiphore II were in accordance with the protocols supplied by the equipment supplier, Amersham Biotech. On completion of the IEF separation the equipment was prepared in accordance with the manufacturers instructions to allow the transfer of proteins from the IPG gel to the second dimension pre-cast Excel Gel (Amersham Biotech). This was a two-stage protocol applying different voltages at each stage (Table 2.10). The equilibrated IPG gels were placed gel side down on the surface of the Excel Gel, and the SDS-PAGE second dimension was started. When it was observed that the bromophenol blue tracking dye had moved 0.5 cm from the IPG gel strip, the run was interrupted to allow the IPG gel strip to be removed and cathode buffer strip repositioning. Electrophoresis continued until the dye front had reached the anodic buffer strip.

Table 2.10 Running conditions for Multiphore II horizontal electrophoresis system utilising pre-cast SDS Excel Gel

Time	Voltage	Current	Power	Temperature
45 min	100 V	20 mA	50 W	15°C
160 min	800 V	40 mA	50 W	15°C

2.7 Visualisation of proteins in polyacrylamide gels

2.7.1 Protein stains

The production of a 2-D gel provides a method of separating a complex protein mixture; once this has been achieved, a method must be selected that allows the proteins within the gel to be visualised. A number of standard protein stains were selected based on their sensitivity, ease of use, and compatibility with identification of protein fragments by mass spectrometric peptide mass fingerprinting. These are detailed in Table 2.11.

Table 2.11 Protein visualization stains

Type of Stain	Supplier	Protocol detail
Brilliant blue coomassie stain	Sigma-Aldrich	Detailed protocol given in Section 2.7.1.1
Colloidal coomassie	BIO-RAD	Used in accordance with suppliers instructions
Sypro® orange [241]	Molecular Probes	Used in accordance with suppliers instructions
Sypro® ruby [241]	BIO-RAD	Used in accordance with suppliers instructions
Silver stain plus [242]	BIO-RAD	Used in accordance with suppliers instructions
Plusone silver stain	APB	Used in accordance with suppliers instructions
Shevchenko silver stain method [238]	various suppliers	Detailed protocol given in Section 2.7.1.2

2.7.1.1 Brilliant blue coomassie stain

The staining solution contained 50% methanol (v/v), 7% glacial acetic (v/v) and 0.025% (w/v) brilliant blue stain. After staining, excess stain can be removed by a de-stain solution. The de-staining solution contained 40% methanol (v/v) and 5% glacial acetic acid (v/v). A small

piece of foam or tissue was placed in the solution to take up excess stain during de-stain procedure. To visualise proteins with this stain the protocol detailed in Table 2.12 was followed. The de-staining solution could be stored at room temperature for several months. On completion of the de-staining step, gels were thoroughly rinsed and could be stored at 4°C in distilled water for several months.

Table 2.12 Brilliant blue coomassie staining protocol

Step	Operation	Detail	Time
1	Stain	Place gel in stain solution of sufficient volume to cover gel and shake gently	1 – 2 h
2	Wash	Wash in MilliQ water of sufficient volume to cover the gel and shake gently	1 – 2 min
3	*De-stain	Drain excess water and add sufficient de-stain to cover gel and shake gently changing de-stain solution twice during operation	4 – 6 h
5	Wash	Wash in MilliQ water sufficient to cover gel	1 – 2 min

2.7.1.2 Shevchenko silver stain method

This silver stain method was developed to be compatible with mass spectrometric (MS) identification of proteins by peptide mass fingerprinting as it excludes the use of glutaraldehyde [238]. The fixation step required a minimum time of 30 minutes up to 12 hours without affecting the result. Altering the time the gel was exposed to the development solution could vary the degree of stain uptake. The purpose of this variation was to achieve the same degree of stain uptake for all gels independent of the sample type and concentration (see Section 2.7.3).

Table 2.13 Silver stain protocol

Step	Operation	Detail	Time (minutes)
1	Fixation	Fix 50% methanol (v/v) 7% glacial acetic acid (v/v)	≥30
2	Wash 1	50% methanol	10
3	Wash 2	MilliQ water	10
4	Sensitise	0.03% sodium thiosulphate solution	1
5	Wash	2 X in MilliQ water	1
6	Stain	Silver nitrate (1 g/L^{-1}) in MilliQ water	20
7	Wash	2 X in MilliQ water	1
8	Develop	Calcium carbonate 20 g/L formaldehyde 1 mL/L^{-1}	Variable
9	Stop	5% Glacial acetic acid	30
10	Store	4°C in 1% glacial acetic acid	2 Weeks

2.7.2 Molecular weight marker

BIO-RAD (UK) calibrated broad range molecular weight markers were used to determine the approximate molecular weight of bands or spots of interest visualised on 1-D and 2-D SDS-PAGE gel images. The molecular weight markers were also used as an indicator to standardise the intensity of the silver stain uptake between separately stained samples and gels (Section 2.7.3).

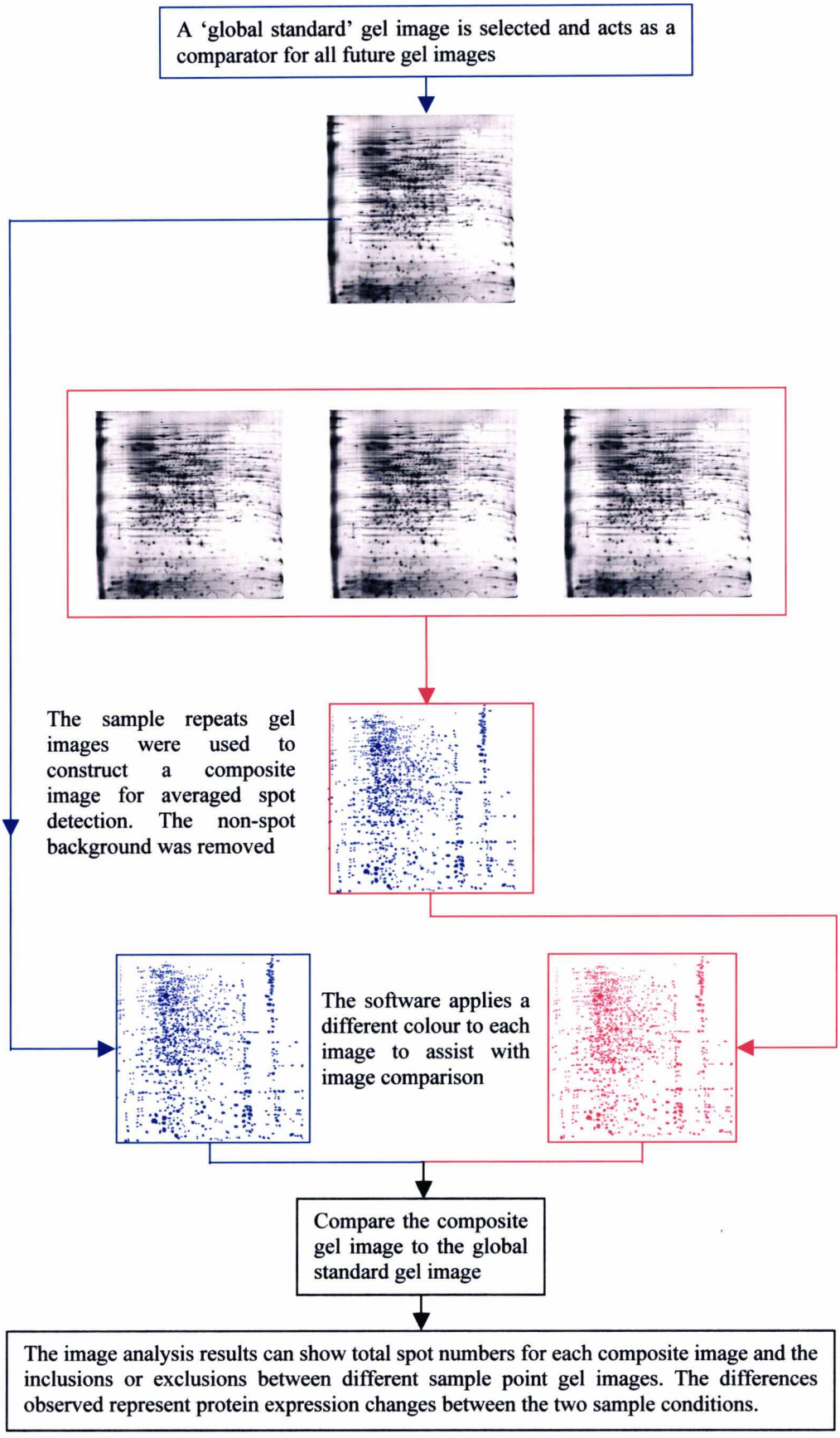
2.7.3 Consistent staining of separate gels with silver stain

The changes observed in the Listeria proteome based upon the comparative interpretation of a 2-D gel image must be independent of differences in stain intensity that arise between gels stained on different occasions. Calibrated broad range molecular weight markers (Section 2.7.2) were used as an indicator of silver stain uptake. Each gel was stained to the same degree by observing the point at which one of the broad range molecular weight standard proteins (BSA) began to over stain. A number of mini format 2-D SDS PAGE gels of the same sample were repeated. The gels contained the same protein load and each carried a fixed volume of BIO-RAD broad range molecular weight marker. The gels were silver stained increasing the stain development time for consecutive gels to a point where the majority of proteins were over stained. A gel was selected that represented the highest number of proteins stained with the minimum degree of over staining. On the selected gel image one of the broad range MW marker proteins (BSA) had just begun to over stain. The degree to which BSA had

stained was chosen as a visual indicator of fixed stain development point. All silver stained gels were matched to this visual standard. The volume of molecular weight standard loaded onto the indicator gel was consistent with that loaded onto all subsequent gels.

2.7.4 Image capture and analysis

Image capture requires high quality optical equipment with the ability to calibrate performance at the time of capture. The equipment used for this project was supplied by Amersham Biotech and based on an Umax 3000 scanner with lab scan software. The process of image capture involved scanning a gel image immediately after completion of the staining protocol. The scanner was calibrated prior to every use with a standard grey scale photographic Tablet (Kodak). All images were scanned at 300 dots per inch (dpi) and stored as tiff format image files. The scanning equipment and image capture software was operated without modification in accordance with the supplied instructions (APB UK). Image analysis was conducted using Image Master 2D software. The software was used to automatically detect separate protein spots using the same preset parameters that were maintained throughout for each image (sensitivity 8751, noise factor 9, operator size 81, background 9). Spots that did not represent proteins were removed. Software tools were also used to manually pick protein spots that had not been automatically detected. It has been observed that during spot detection the total number of spots detected on same sample repeats can be variable (+/- 5%). The software has the ability to combine the image information from multiple repeats of the same sample allowing the inconsistency of same sample image variability to be reduced. A method was adopted that combined the image information from three repeats of the same sample. The spots from each image were matched and those that did not appear in at least 2 of the three images were considered to be artefactual and were removed from the subsequent composite image. Image analysis was conducted in accordance with a scheme designed to eliminate erroneous spot information (Scheme 2.2).



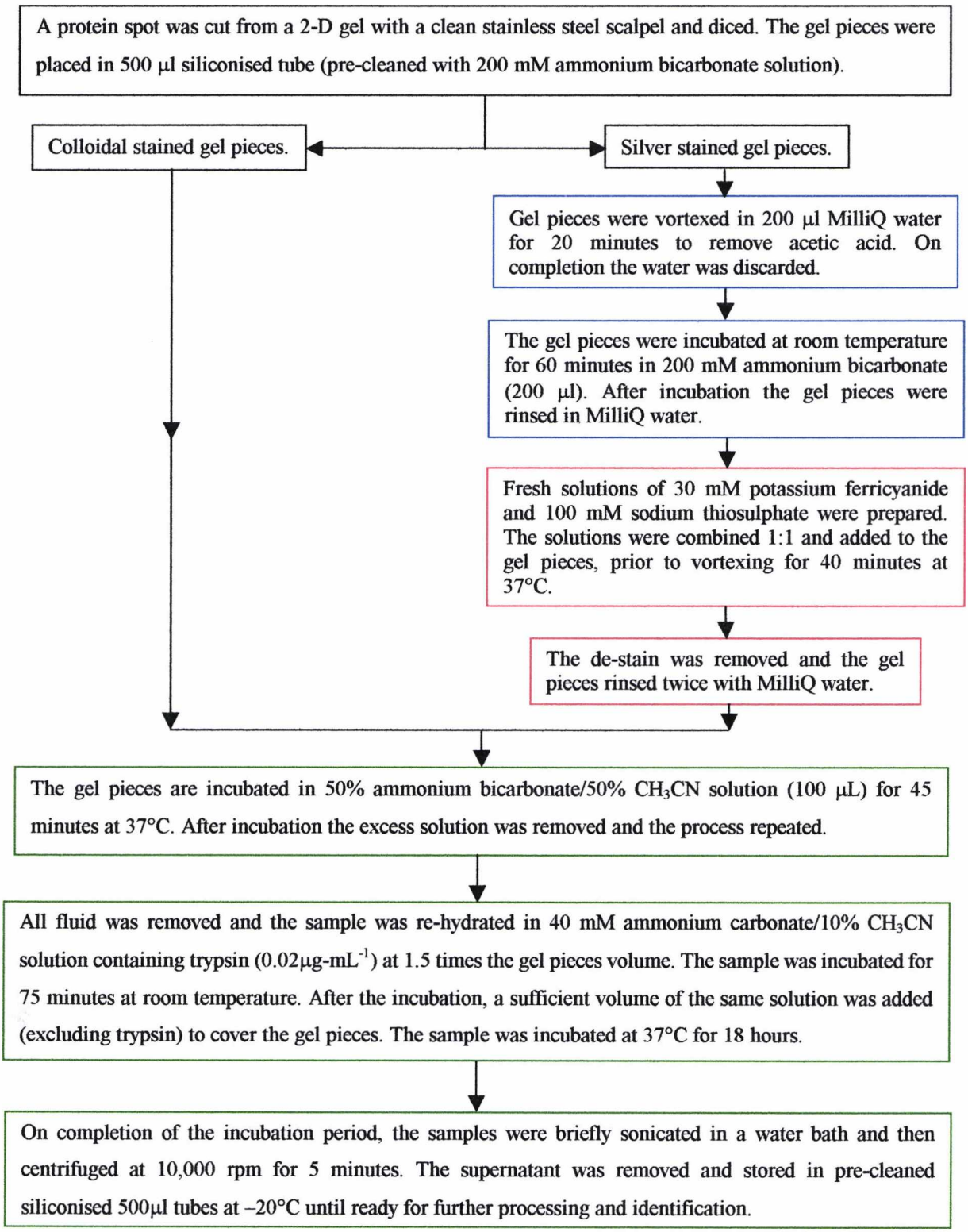
Scheme 2.2 A scheme for the construction and analysis of composite gel images

This analysis scheme (Scheme 2.2) was adopted for the comparison and analysis of every composite gel image produced using the proteomic platform detailed herein.

2.8 In-gel tryptic digest of protein spots

The image analysis of 2-D gels provides a means of identification of areas on the gels of different samples that show different patterns of protein expression. The areas of interest that contained spots showing a high level of change in expression could be excised from the gel and individual protein spots could be subjected to an in-gel tryptic digest (Scheme 2.3). The resultant peptide fragments can be used to identify the protein using peptide mass fingerprinting (Section 2.8.3). The protocol for the digestion of proteins with an excised gel fragment was conducted in siliconised 500 μL plastic tubes. Siliconised tubes were used to minimise the adhesion of peptide fragments to the walls of plastic tubes

A scheme was devised for the in-gel tryptic digest of proteins cut from silver stained and colloidal stained 2-D gels. Silver stained gel pieces required a washing and de-staining step prior to a common tryptic digest protocol.



Scheme 2.3 In-gel tryptic digest of excised protein spots

On completion of the tryptic digest the samples could be stored at –20°C for future analysis or subjected to a desalt protocol (Section 2.8.1) prior to preparation for identification by mass spectrometric analysis (Section 2.8.2).

2.8.1 Desalting in-gel tryptic digest samples using zip tip

In-gel tryptic digest samples were retrieved from –20°C store and allowed to equilibrate to room temperature. The samples were concentrated to a volume of 1 µL using a ThermoSavant SC210A speed vac concentrator (ThermoSavant, Holbrook, USA). After concentration the samples were subjected to a desalt procedure using Millipore C18 Zip tips in accordance with the manufacturers recommended protocol (Millipore, Watford, UK). All procedures were carried out in siliconised tubes to prevent surface attachment of the peptides to the tube walls.

2.8.2 Sample preparation for peptide mass fingerprint identification by MS

A matrix solution is required to prepare peptide samples for mass spectrometric analysis. An excess of α-cyano-4-hydroxy cinnamic acid was solubilised in 60% acetonitrile (v/v), 0.02% trifluoroacetic acid (v/v). A 0.6 µL aliquot of sample was combined with a 0.6 µL aliquot of saturated matrix solution on a mass spectrometer target disc. Once dried the target disc could be stored for several months prior to mass spectrometric analysis of the sample.

2.8.3 Identification of peptide fragments by laser desorption ionisation time of flight mass spectrometry

Previously prepared samples were subject to analysis using a laser desorption time of flight mass spectrometer at UKC. Alternatively, samples were subject to analysis at Unilever Research, Colworth UK. To identify proteins by peptide mass fingerprinting data, *pI* and *Mw*, experimentally measured, user-specified peptide masses were compared with the theoretical peptides calculated for all proteins in SWISS-PROT and TrEMBL databases using the site PeptIdent tools (http://www.expasy.org).

3.0 The growth characteristics of *L. monocytogenes* Scott A

3.1 Introduction

The evolutionary survival of prokaryotes has depended not only on their performance in favourable growth conditions, but also their ability to alter gene expression in response to external perturbation, to the point where long-term survival during conditions of growth arrest is possible. Most bacterial species are usually not more than 1 μm wide and 5 μm long whereas most eukaryotic cells may have a diameter at 10 – 50 μm [243]. The high surface area to volume ratio of microorganisms has led to extensive interaction with the environment and is the reason why many microorganisms have high metabolic rates. Metabolic flexibility in microorganisms is also far greater than that demonstrated by multi cellular higher organisms. In bacteria, this flexibility is a consequence of size: a micrococcal cell has space for only 100 000 molecules of protein. Enzymes not in use cannot be stored in reserve, therefore inducible enzymes can constitute up to 10% of the prokaryotic cellular protein compliment. The mechanisms for the regulation and control of protein production therefore play a greater role in microorganisms than other organisms [3].

Prokaryotic cells have evolved so that they may achieve rapid changes in gene expression in order to survive environmental change [244]. This evolutionary survival technique has resulted in major human pathogens possessing the ability to survive externally applied physical challenges related to food processing e.g. osmotic stress, pH, temperature. For many pathogenic bacteria, environmental dispersal is linked to the ability of the organism to survive unfavourable conditions outside the host for long periods. In the natural environment, changes in gene expression effecting bacterial growth rates are most likely to be caused by limited nutrient stress induction. The starvation response in particular can lead to a number of physiological changes that increase the ability of the pathogen to survive applied changes in pH, osmolarity and temperature [216]. As a result, the bacterium *L. monocytogenes* has developed such that it can survive food-processing conditions designed to preclude growth if already present in a growth-arrested state [245, 246]. For example it has been shown that the Listeria method of resistance to low pH during growth arrest is independent of the method employed to resist low pH during active growth where *de novo* synthesis of stress resistant proteins known as the acid tolerant response (ATR) occurs [141, 142]. Furthermore, it is unlikely that *de novo* synthesis of proteins would occur at a sufficient rate to confer protection against low pH in the growth arrested cell. Lee *et al.* have now shown that the mechanism of acid resistance during growth arrest is dependent on the presence of a growth arrest specific

sigma factor, Rpos [144]. The *RpoS* gene product is central to the onset of the stress related change in gene expression at growth arrest *[145, 146]*. In a number of bacteria, the *RpoS* (also called sigma factor) gene group has a role in virulence as well as stationary phase induction [247, 248] and some specific and general stress responses [249-252]. Most bacteria contain multiple sigma factors including both a primary sigma factor, controlling essential housekeeping functions, and alternative sigma factors, activated by specific signals or stress conditions [253]. A considerable body of work now exists that implicates the *RpoS* and Sigma gene family in stationary phase induction and stress responses in *L. monocytogenes* [137, 147, 254, 255].

It is clear that the environment in which a bacterial cell finds itself can have a determining effect on the pattern of gene expression throughout growth and at stationary phase. If an investigation into gene expression and the subsequent change of the cell protein complement is to be conducted, the conditions of growth must be defined and detailed. The growth conditions may apply selectivity to the pattern of gene expression observed during growth and stationary phase. An examination of the proteins expressed by the *L. monocytogenes* genome throughout growth, can increase the current knowledge base that has been used for the design of food preservation processes designed to be limiting to the growth of the organism. A further understanding of the 'systems' that a bacterium utilises for long term survival in limiting environmental conditions may allow current food processing to be adapted to the point where exit from a growth arrested state could be limited, or alternatively the growth arrested cell itself could be the target of anti microbial measures [256]. In this chapter, the parameters for the growth of the organism *L. monocytogenes* are determined. The factors influencing the growth kinetics that apply selectivity to gene expression and cellular protein complement are examined in more detail.

3.2 RESULTS

3.2.1 Maintenance of culture purity

A number of microbiological tests were adopted as standard practice (Section 2.2.6) to confirm that samples were free of other contaminating organisms and that the organism present was *L. monocytogenes*.

A **B**

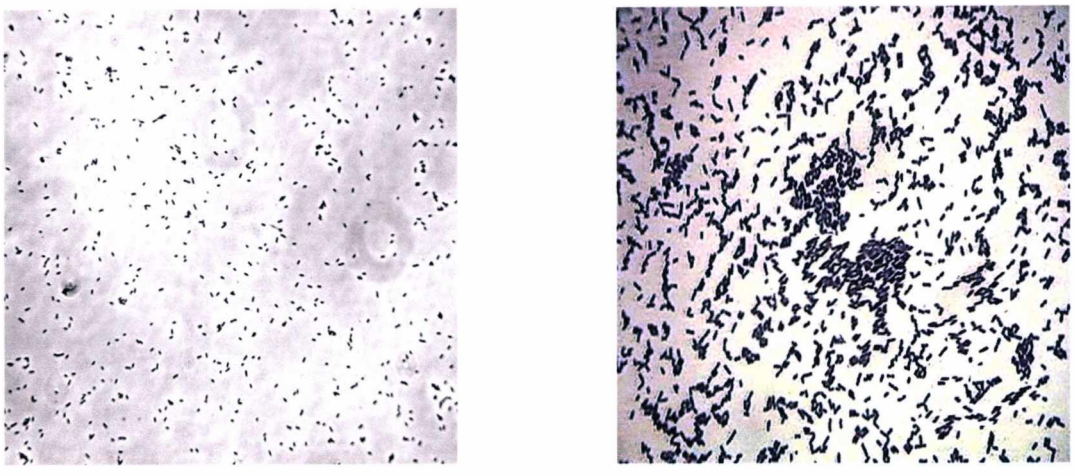


Figure 3.0 Wet slide and Gram stain images of *L. monocytogenes* at 0.25 OD_{600}. Image A: *L. monocytogenes* batch culture wet slide image of a culture sampled at 0.25 OD_{600} (magnification X1000). Image B: *L. monocytogenes* batch culture Gram stain image of a culture sampled at 0.25 $O.D._{600}$ (magnification X1000). The above images were captured at Unilever research using equipment previously detailed.

Figure 3.0 image A, clearly shows that the *L. monocytogenes* cells sampled from a PTM medium batch culture [222] during exponential growth were rod shaped and occurred singly and in short chains. The live image also clearly showed the tumbling motility that is typical of flagellated *L. monocytogenes* cells [128]. Meanwhile image B, a Gram stain image clearly shows that the *L. monocytogenes* cells sampled from a PTM medium batch culture during exponential growth were rod shaped and could be classed as Gram-positive giving a purple/blue stain result.

3.2.1.1 Catalase reactivity

The catalase reactivity test was used to confirm that the organism present in a batch culture (prior to protein extraction) was catalase positive. A standard protocol was adopted [257] which involved placing the organisms to be tested on a slide and then adding a few drops of 30% hydrogen peroxide. If the test organism possessed the enzyme catalase, small bubbles of oxygen was liberated where colonies were in contact with the hydrogen peroxide. Any samples showing negative results were discarded, as the organism *L. monocytogenes* Scott A

supplied by Unilever Research, was catalase positive. It should be noted that *L. monocytogenes* catalase activity can be strain specific and strains exist that give a catalase negative results [258].

3.2.2 *L. monocytogenes* growth in different media

During the exponential growth phase of a batch culture, the time taken for cells to double, the culture doubling time (t_d), should remain consistent and be reproducible when the growth conditions are standardised (Section 2.2.5) Environmental and/or chemical changes to these conditions could induce a change in the consistency and reproducibility of the observed culture t_d. Major change in the observed t_d was taken as an indicator that growth conditions, or the culture itself was compromised. Any samples that exhibited such inconsistency were discarded. Plotting the optical density of a batch culture against time can be used to graphically represent the growth of a bacterial culture. A typical curve will be sigmoid and can be divided into a number of discrete growth phases, typically lag phase, exponential phase, stationary phase and death phase. Where optical density is used to describe a bacterial growth curve, stationary phase describes the point after exponential growth where an increase in the recorded optical density readings stop and further readings are static. The growth of *L. monocytogenes* in BHI and PTM media have been compared and the results achieved are presented herein. The PTM medium was made in accordance with the published methods of Phan-Thanh and Gormon [222].

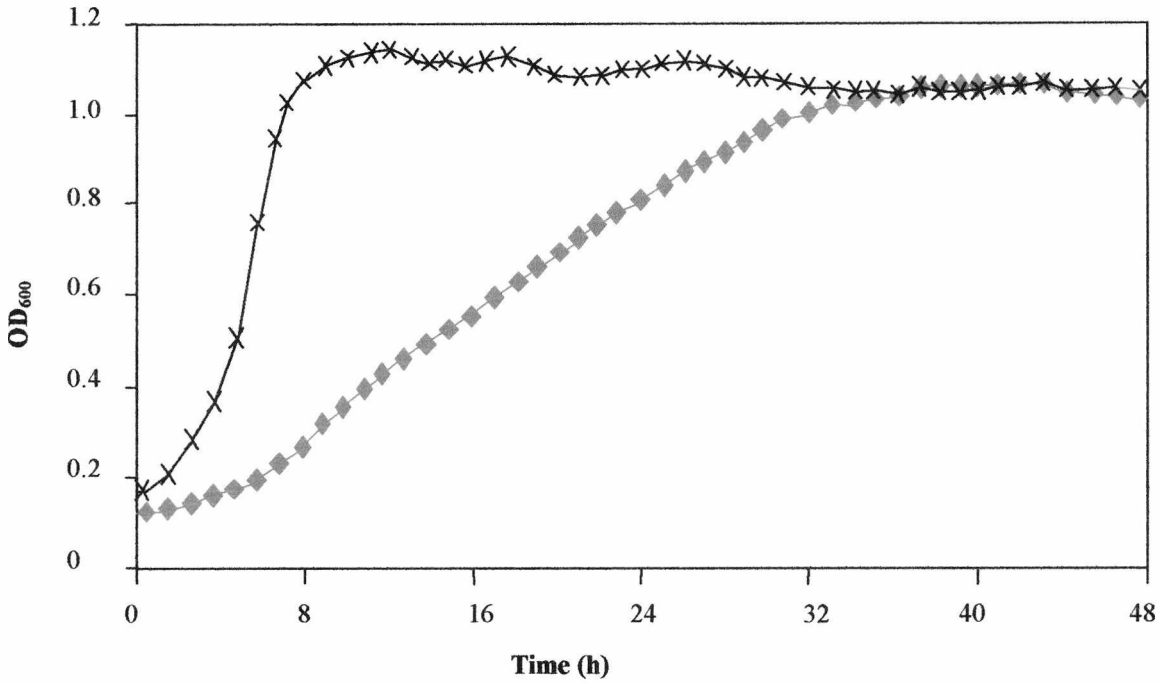


Figure 3.1 *L. monocytogenes* growth curves in 300 µL BHI and PTM medium batch culture at 30°C. Determined by optical density. × Complex undefined growth medium. ◆ PTM medium. The growth curves illustrated in Figure 3.1 were conducted using standardised inoculation and growth conditions (Section 2.4.5).

Figure 3.1 shows that *L. monocytogenes* will grow to stationary phase in BHI and PTM media. The point at which the BHI culture reached stationary phase was recorded at 1.15 OD_{600}. The culture had reached this point 10 hours after inoculation. The point at which the PTM medium culture reached stationary phase was recorded at 1.075 OD_{600}, the culture had reached this point 34 hours after inoculation. The results confirm that a 300 µL PTM medium was able to support the growth of *L. monocytogenes* to stationary phase, furthermore the optical density attained at stationary phase in a PTM medium closely matched that attained by the same organism in a nutritionally rich medium (BHI).

Figure 3.2 (over page), shows that a *L. monocytogenes* 1 L culture will grow to stationary phase in BHI and PTM media. The point at which the BHI culture reached stationary phase was recorded at 1.305 OD_{600}. The culture had reached this point 13.5 hours after inoculation. The point at which the PTM medium culture reached stationary phase was recorded at 1.20 OD_{600}, the culture had reached this point 23.5 hours after inoculation. The results confirm that in 1 L batch cultures PTM medium was able to support the growth of *L. monocytogenes* to stationary phase, furthermore the optical density attained at stationary phase in a 1 L PTM medium closely matched that attained by the same organism in a nutritionally rich medium (BHI). The culture volume is clearly shown to have an effect on the time and rate at which a *L. monocytogenes* culture attains stationary phase.

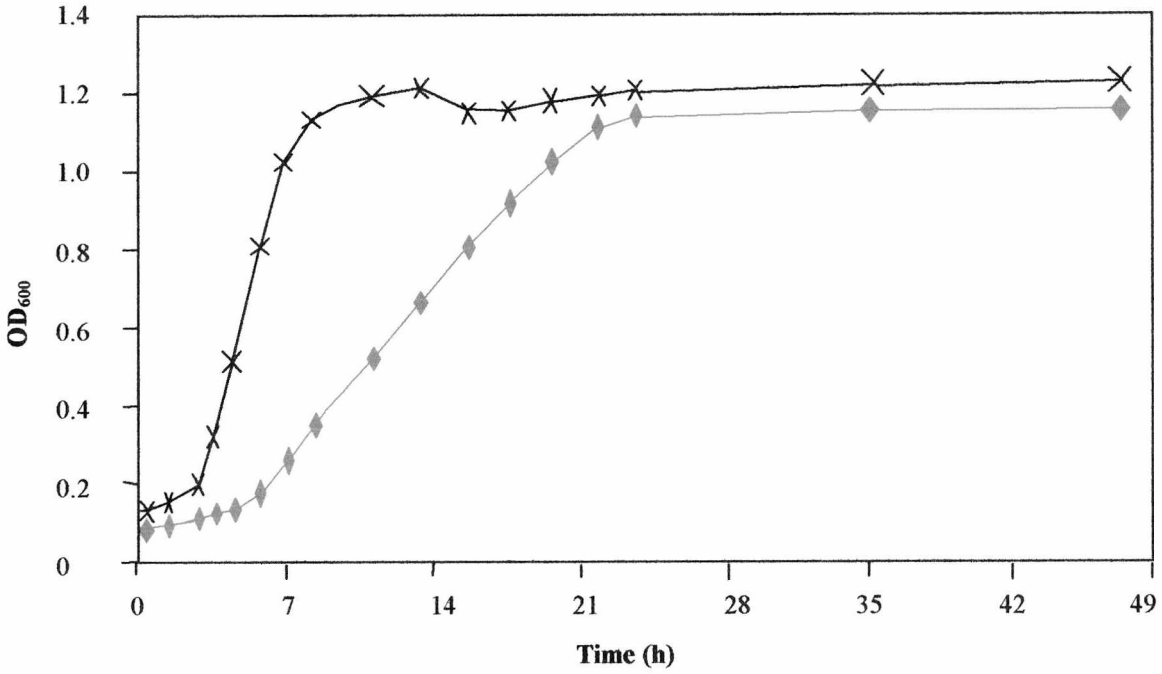


Figure 3.2 *L. monocytogenes* growth curves in 1 L BHI and PTM batch cultures. Determined by optical density. × Complex undefined growth medium (BHI). ◆ PTM medium.

3.2.2.1 L. monocytogenes growth in different media determined by cfu/mL^{-1}

In addition to the determination of growth curves by OD_{600} readings (Figures 3.1, 3.2 and 3.6), cell population counts were used to construct growth curves for *L. monocytogenes* large volume batch cultures in BHI and a PTM media. Cultures were sampled at 1-hour intervals and the number of cells in each sample determined (Section 2.2.8).

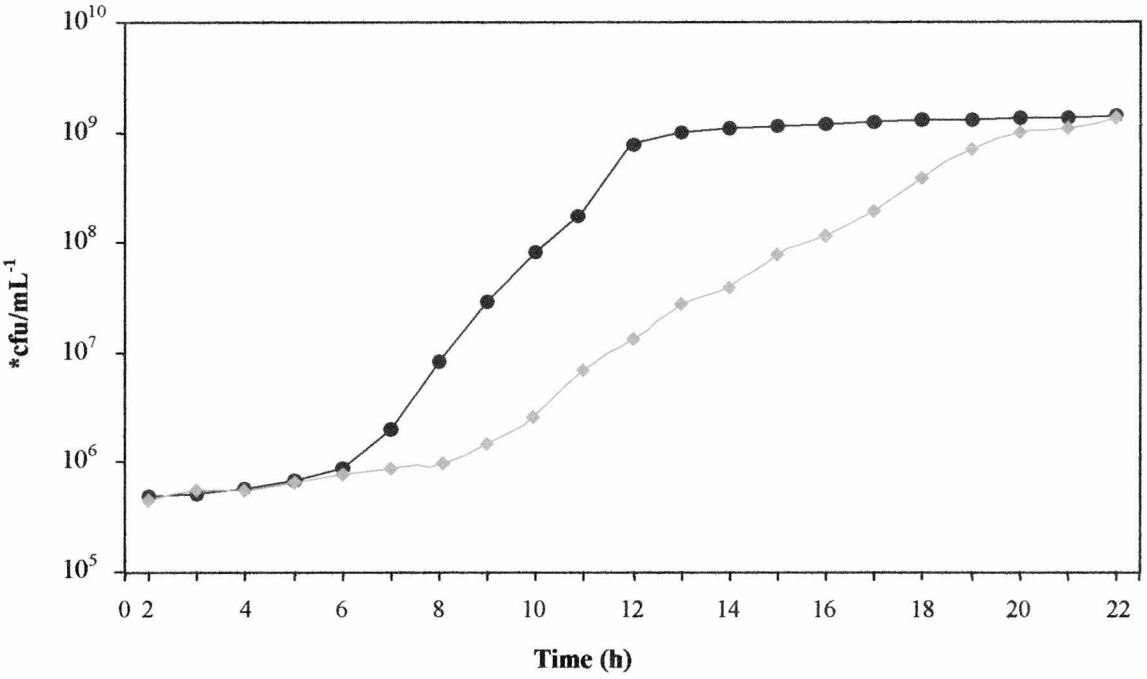


Figure 3.3 *L. Monocytogenes* growth curves in BHI and a PTM determined by cfu count. (•) BHI medium. (♦) PTM medium *cfu colony forming units

Figure 3.3 demonstrates that the growth to stationary phase of a *L. monocytogenes* 1 L batch culture was supported in BHI broth and a PTM medium. The BHI culture reached stationary phase 12 hours after inoculation at population concentration of 1 $x10^9$ cell/mL^{-1}. The PTM medium culture reached stationary phase 22 hours after inoculation at a population concentration similar to that recorded for the BHI culture. The population of cells recorded at stationary phase in the BHI culture was within 3% of the population recorded for the PTM medium culture. The result allowed the calculation of the rate at which the population doubled during exponential growth. The culture doubling time of a *L. monocytogenes* BHI batch culture was determined at 58 minutes, while in a PTM medium the doubling time was determined at 98 minutes. The culture t_d rates show that the rate of maximal cell division demonstrated by *L. monocytogenes* in BHI batch culture, was not matched by the rate of maximal cell division demonstrated by *L. monocytogenes* in a PTM medium batch culture. Where cell population was adopted as a method for monitoring the progression of growth in a bacterial culture, stationary phase described the point after exponential growth, where the number of cells counted no longer increased and the counts become static.

3.2.3 Images of *L. monocytogenes* at exponential phase and stationary phase

Images of Listeria cells at exponential phase and stationary phase in differing growth medium were acquired with the use of TEM using negative staining techniques (Section 2.2.11.1).

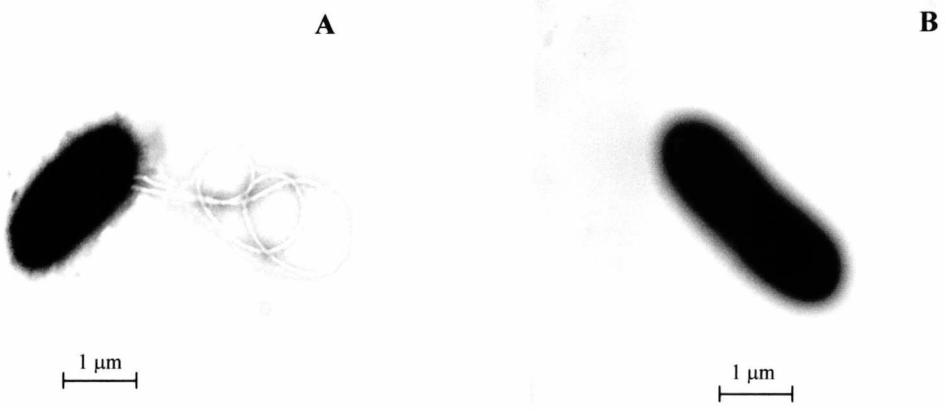


Figure 3.4 TEM of *L. monocytogenes* at exponential phase and stationary phase. Image A: Late exponential phase *L. monocytogenes* cell showing flagella typically associated with tumbling motility (magnification X40000). Image B: Early stationary phase *L. monocytogenes* cells in short chain formation no flagella present (magnification X40000). The images were acquired at Unilever Research using a Joel 1200 EX mark 2 electron microscope.

It was observed that during growth and in particular on transition to stationary phase the morphology of individual Listeria cells in batch culture changed. Figure 3.4 (A) shows *L. monocytogenes* cells sampled at an OD_{600} below 0.75 possessed flagella. It was also noted under light microscopy that the cells exhibited tumbling motility [128]. The *L. monocytogenes* cells pictured in Figure 3.4 (B) were sampled at an OD_{600} above 0.85 and did not possess flagella, were aggregating, and appeared to be smaller and more coccoid in appearance (Figure 3.3 B) than cells sampled earlier in the life of the culture. It was noted under light microscopy that cells sampled at an OD_{600} above 0.85 did not exhibit typical tumbling motility. A decrease in cellular size is a well-known characteristic demonstrated by bacterial cells in batch culture as the population enters stationary phase [259].

3.2.4 *L. monocytogenes* batch culture: The change of media pH throughout growth

L. monocytogenes 1 L PTM medium batch culture was inoculated and grown to stationary phase. The pH of the medium was recorded prior to inoculation and throughout growth to stationary phase (Section 2.3.1). The pH of the medium prior to inoculation was recorded at pH 6.9.

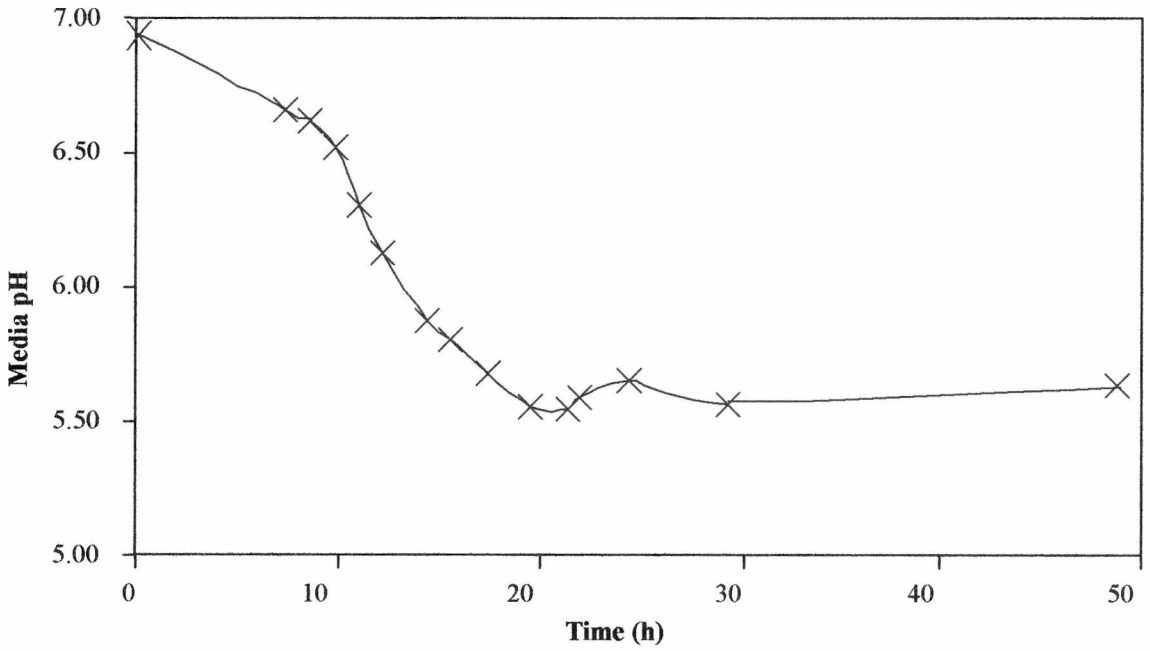


Figure 3.5 The change in medium pH recorded throughout the growth of a *L. monocytogenes* PTM medium batch culture.

It was noted that at stationary phase and thereafter the recorded changes in medium pH were small and more consistent with a static state than those recorded prior to stationary phase. Figure 3.5 clearly shows the change in medium pH of a *L. monocytogenes* PTM medium

batch culture recorded over time. At an OD_{600} of 0.25 at 8 hours after inoculation a medium sample was taken and the pH recorded at pH 6.65. Thereafter medium samples were taken at intervals to coincide with a 0.100 incremental increase in the culture optical density (OD_{600}). At the onset of stationary phase (OD_{600} 1.15) 20 hours after inoculation, the medium pH was recorded at pH 5.5, thereafter the medium pH was recorded for samples taken at 21, 22, 24, 30 and 48 hours.

3.2.4.1 The growth of L. monocytogenes in acidified medium

The growth of *L. monocytogenes* at different pH in BHI and PTM media small volume batch cultures was monitored by optical density using Bioscreen equipment based at Unilever Research. The cultures were grown using the standard conditions previously adopted for Bioscreen experiments (Section 2.2.7). Prior to inoculation, the pH of each medium was adjusted by the addition of 1 M HCl. The OD_{600} of each culture was recorded throughout growth and stationary phase and the results obtained were used to construct growth curves of the Listeria cultures at each medium pH. The experiment was repeated using acidified PTM medium at pH 4.5 and pH 4.1 (results not shown).

Figure 3.6 clearly shows that *L. monocytogenes* will grow to stationary phase in a BHI batch culture acidified to pH 5.5, the optical density at which stationary phase occurred (0.970) was lower than that recorded for a pH neutral BHI batch culture (1.200 OD_{600} Figure 3.1). The growth of *L. monocytogenes* to stationary phase in a BHI culture acidified to pH 5.0 is also shown. Additionally, Figure 3.6 shows that *L. monocytogenes* will grow to stationary phase in a PTM medium acidified to pH 5.5, as it will in a PTM medium acidified to pH 5.0. The time point at which each culture attained stationary phase was recorded. The growth curves presented in Figures 3.6 and 3.1 were used to calculate a growth rate for each culture and the results obtained were combined with those obtained from growth curves of *L. monocytogenes* in batch cultures acidified to pH 4.5 and pH 4.2. The growth rates were calculated from the linear portion of each curve and are based on the rate of change in OD over time. Table 3.0 clearly shows that the rate of growth and the time after inoculation at which each *L. monocytogenes* batch culture attained stationary phase was extended when the medium pH was below pH 7.1. The rate of growth of *L. monocytogenes* in batch culture below pH 5.0 was very slow compared to the rate achieved at higher pH.

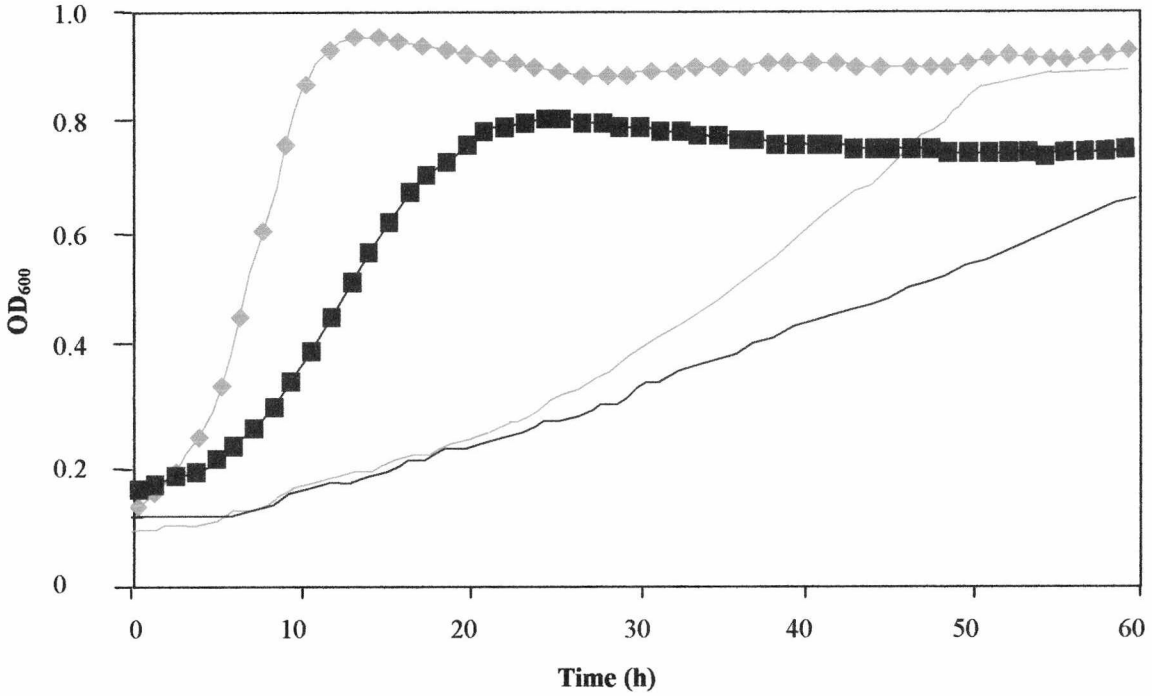


Figure 3.6 *L. monocytogenes* growth curves in acidified medium. Determined by optical density. ♦ Growth curve for *L. monocytogenes* in BHI at pH 5.5. ■ Growth curve for *L. monocytogenes* in BHI at pH 5.0. — Growth curve for *L. monocytogenes* in PTM medium at pH 5.5. — Growth curve for *L. monocytogenes* in PTM medium at pH 5.0

Table 3.0 The growth characteristics of *L. monocytogenes* batch cultures in media at neutral and acid pH

Growth medium	Medium pH prior to inoculation	Optical density at stationary phase (OD_{600})	Culture age at stationary phase (h)	Maximum exponential rate Δ OD/h	R^2
BHI	7.0	1.200	12	0.1533	.9917
PTM	6.9	1.168	21	0.0611	.9969
BHI	5.5	0.970	15	0.0939	.9901
PTM	5.5	0.910	48	0.0407	.9946
BHI	5.0	0.845	25	0.0112	.9813
PTM	5.0	0.720	60	0.0088	.9971
PTM	4.5	0.180	**60	0.0050	.9881
PTM	4.2	0.120	**60	0.0020	.9926

* The growth curves from which these results were taken are not shown here.** The data collection was halted at 60 hours at which point these cultures had not demonstrated stationary phase. Maximal exponential growth rate and R^2 were determined by application of linear regression calculation to linear portion of growth curves using Microsoft Excell.

3.2.5 *L. monocytogenes* batch culture: Chemical analysis of the growth medium

A number of protocols were used in order to follow the utilisation of medium amino acids and medium glucose throughout the growth and transition to stationary phase of *L. monocytogenes* PMT batch cultures.

3.2.5.1 Glucose utilisation

L. monocytogenes 1 L PTM medium culture was inoculated and grown to stationary phase. The culture was allowed to reach an OD_{600} of 0.240 at which point the culture medium was sampled every hour for 6 hours, and then every 1.5 hours for 4.5 hours, thereafter the culture was sampled once after 6 hours then again at 12 hours. These sample time points coincided approximately with 0.100 incremental increases in the observed culture optical density (OD_{600}). The culture was sampled and each sample was centrifuged to remove cells prior to glucose determination of spent medium.

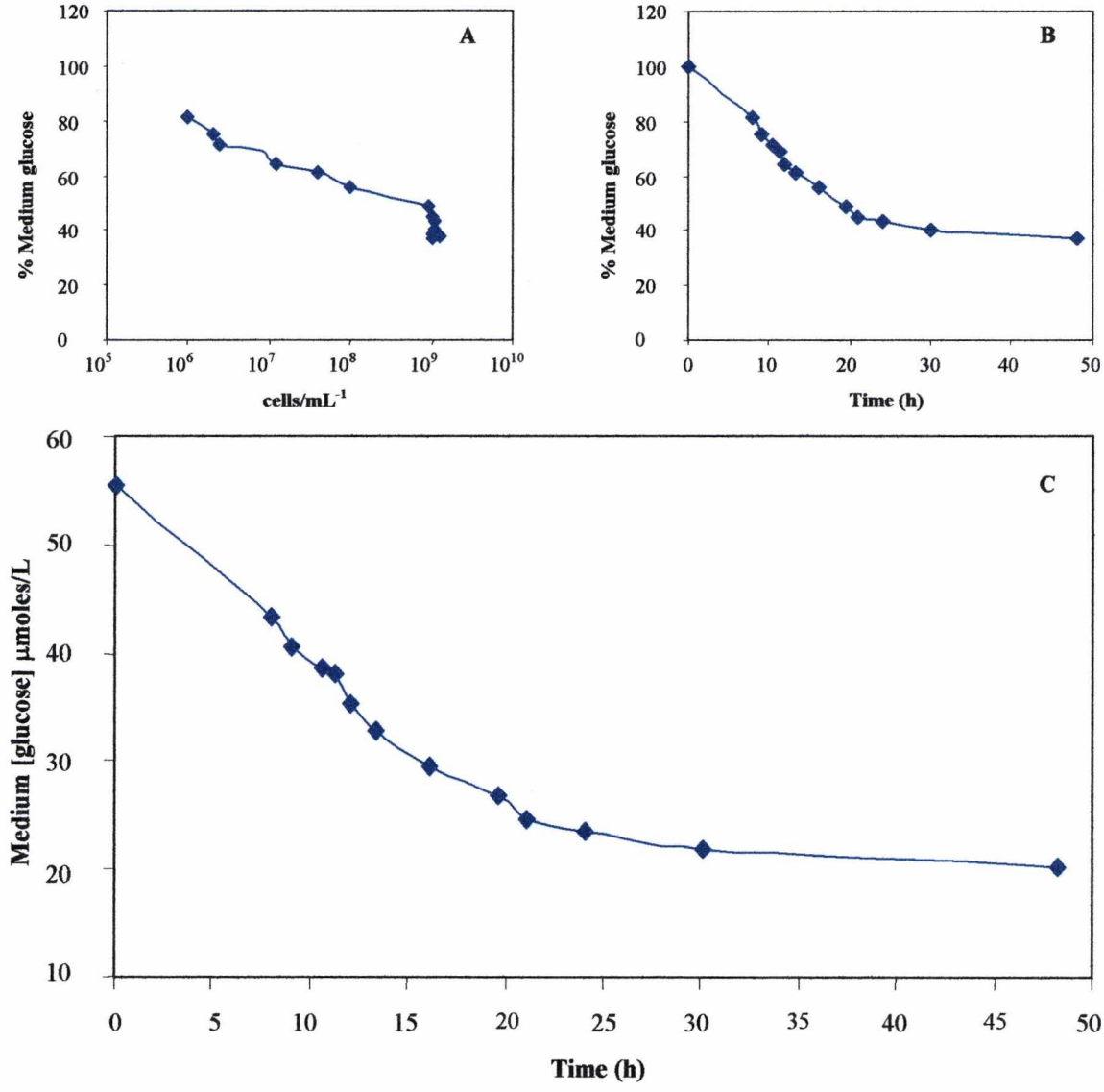


Figure 3.7 The utilisation of glucose by *L. monocytogenes* A) Glucose utilisation expressed as a % per cell. B) Glucose utilisation expressed as a % over time. C) Glucose utilisation expressed in µmoles/L over time

During the monitoring period, three phases of glucose utilisation were observed (Figure 3.7 B and C, and Table 3.1). The distinction between each growth phase is more clearly defined where the results are expressed as μmoles/L medium concentration over time (Figure 3.7 C). Specific growth phases were matched to the results achieved for the utilisation of amino acids (Section 3.2.5.2) and growth phase data detailed in Table 3.0 where growth phases were more apparent. Exponential growth continued for 11 hours from hour 8 (after inoculation) to hour 19.5. Stationary phase appeared to occur from hour 20 (after inoculation) to hour 30, thereafter a further decline in the utilisation off glucose was noted between hour 30 (after inoculation) and hour 48. The rate of utilisation of glucose was calculated from linear portions of the curve at exponential growth, stationary phase, and growth arrest (glucose calibration curve Appendix IV) as a time rate average per hour (by hand) and by calculation of the regression slope of each phase using Microsoft Excel (Figure 3.1 C). Determination of the medium glucose concentration was achieved with the use of an HK glucose assay kit (Section 2.3.2). The assay kit highlighted a concentration dependant colour change in the media samples that was detected using an Eppendorf Biophotometer.

Table 3.1 The rate of utilisation of glucose by a *L. monocytogenes* PTM medium batch culture.

Medium glucose utilisation					
Exponential growth		**Stationary phase**		**Growth arrest**	
Δ μmoles/h	R^2	Δ μmoles/h	R^2	Δ μmoles/h	R^2
1.79	0.9525	0.34	0.9903	0.078	0.9976

3.2.5.2 Amino acid utilisation

HPLC was used for the quantitative analysis of medium amino acids and the equipment used remained as detailed in section 2.3.3.

3.2.5.3 Sample preparation

L. monocytogenes 1 L PTM medium batch culture was inoculated and grown to stationary phase. The culture was allowed to reach an OD_{600} of 0.240 at which point the culture medium was sampled at incremental increases of OD_{600} at 0.100. The medium was filter sterilised by passage through a Sartorius Minisart Blue filter unit (pore size 0.2 μm).

3.2.5.4 Identification of amino acids by HPLC

The PTM medium [222] adopted for the batch culture growth of *L. monocytogenes*, contained ten amino acids. The concentration of each amino acid in the PTM medium prior to inoculation was 100 mg/L^{-1}. To identify the point at which an amino acid would be eluted

from a reverse phase column using HPLC, a single sample was run separately 10 times. On each consecutive run, a different medium amino acid was added to the sample. This established the order in which the medium amino acids would elute from the column and allowed non amino acid peaks to be identified and excluded from the results analysis. Using the established elution order amino acids could be assigned to the peaks produced by a medium sample. An HPLC trace produced from a medium blank (complete medium prior to inoculation) complete with an asparagine reference peak was used as a comparator against which all media samples could be compared. The change in peak area of the individual amino acids between different time point samples was determined.

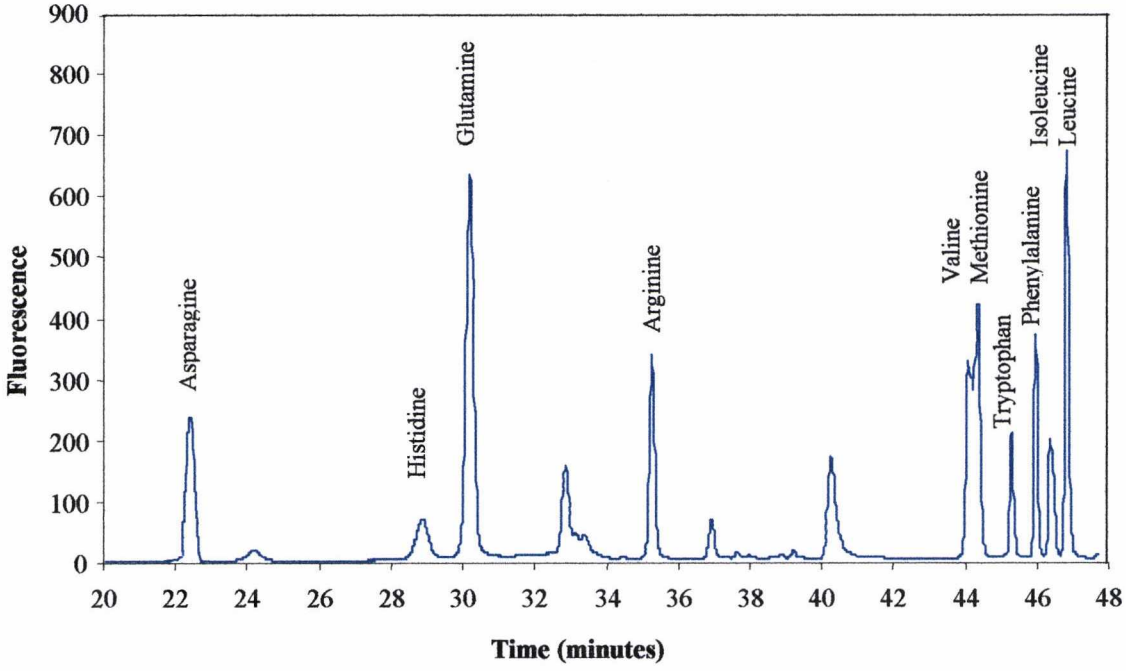


Figure 3.8 The identification and quantitation of amino acids by HPLC

Figure 3.8 is an example of a PTM medium HPLC trace, and it clearly shows that each peak representative of a medium amino acid was identified. The first peak eluted at approximately 22 minutes was identified as the amino acid asparagine. Peaks not assigned an identification (Figure 3.8) appeared in a medium blank that excluded all amino acids, therefore unidentified peaks were non-amino-acid-generated residues that could be excluded from results analysis.

3.2.5.5 Quantitation of PTM medium amino acids

The concentration of each amino acid prior to culture inoculation was known. Calculated peak areas were converted to a μM concentration, allowing the utilisation of amino acid against time to be graphically represented.

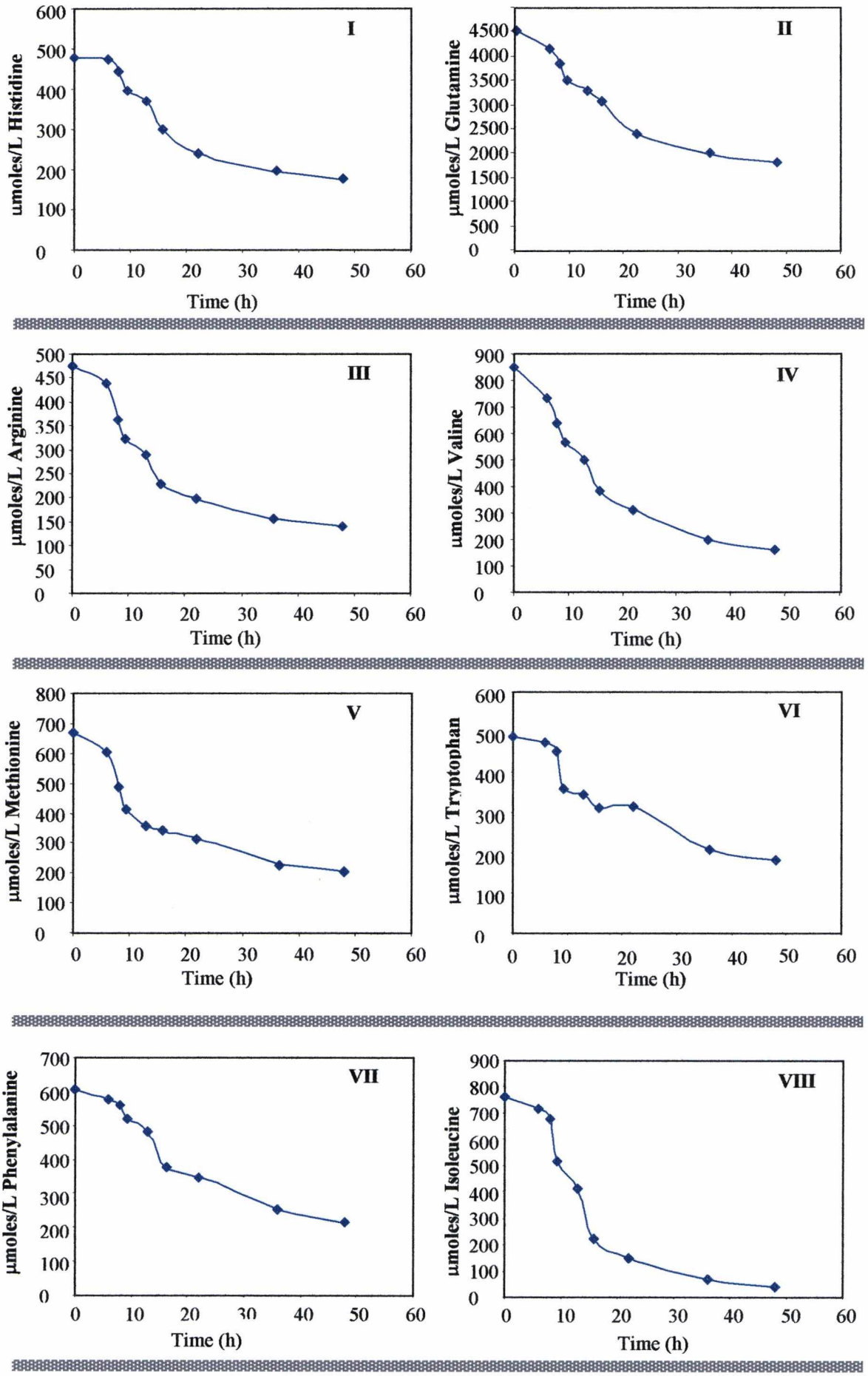


Figure 3.9 The utilisation of amino acids by a *L. monocytogenes* PTM medium batch culture

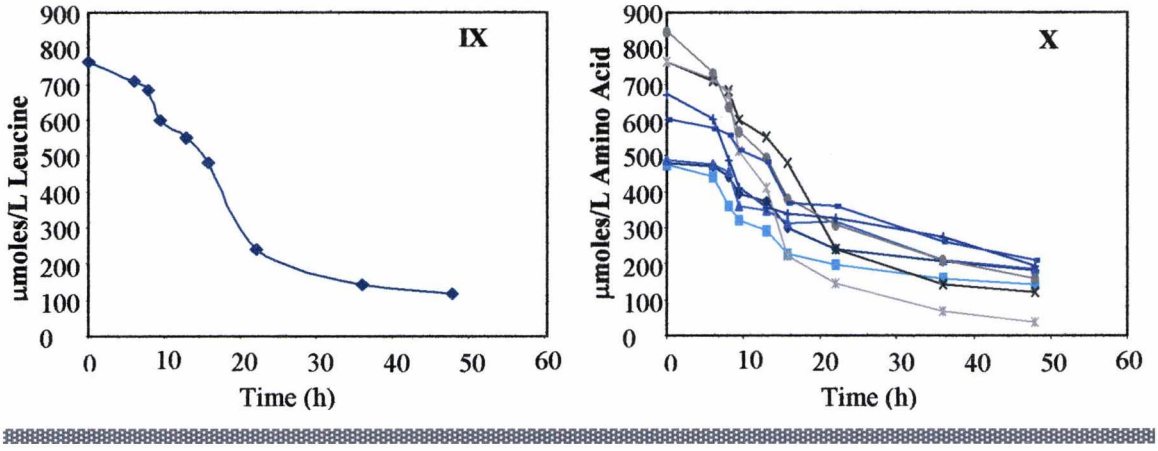


Figure 3.9 (Contd.) The utilisation of amino acids by a *L. monocytogenes* PTM medium batch culture

Figure 3.9 (graphs I – X) show the rate of utilisation of individual amino acids during the growth of a *L. monocytogenes* in a PTM medium batch culture. The utilisation of amino acids by the culture was monitored for 48 hours. Graph X is a composite image containing the utilisation curve for each amino acid (except glutamine which was present at a greater concentration than other amino acids and therefore outside the scale of Graph X). The composite image shows that the pattern of utilisation of each amino acid was similar. The results were used to calculate the rate of utilisation of each amino acid and the results achieved are presented in Table 3.2.

During the monitoring period, three phases of utilisation were observed. After inoculation, the rapid utilisation of amino acids continued for 20 hours. Stationary phase appeared to occur from hour 20 to hour 30, thereafter a further decline in the utilisation of each amino acid was noted between hour 30 and hour 48. The pattern of amino acid utilisation matched that observed for the utilisation of glucose. The rate of utilisation of each amino acid at each defined phase of growth was calculated from the linear portion of each curve at a point where a change in slope could be observed, using Microsoft Excel linear regression software facility.

Table 3.2 The rate of utilisation of amino acids by a *L. monocytogenes* PTM medium batch culture.

Amino acid		Amino acid utilisation					
		Exponential growth		Stationary phase		Growth arrest	
		Δ μmoles/h	R^2	Δ μmoles/h	R^2	Δ μmoles/h	R^2
I	**Histidine**	15.11	0.934	4.40	0.987	2.33	0.991
II	**Glutamine**	96.12	0.930	26.07	0.973	10.97	0.966
III	**Arginine**	19.55	0.933	3.80	0.985	1.66	0.996
IV	**Valine**	33.22	0.939	7.21	0.983	3.30	0.988
V	**Methionine**	25.48	0.947	3.97	0.958	2.83	0.990
VI	**Tryptophan**	14.9	0.889	4.60	0.929	2.11	0.965
VII	**Phenylalanine**	22.16	0.927	6.34	0.991	4.58	0.979
VIII	**Isoleucine**	50.83	0.959	7.53	0.942	2.66	0.999
IX	**Leucine**	23.43	0.919	4.28	0.919	1.88	0.988

3.2.6 L-[^{35}S] cysteine incorporation

A single 2 L PTM medium batch culture was inoculated from an overnight BHI culture and grown to stationary phase adopting standardised growth conditions. When the culture OD_{600} reached 0.25 an aliquot of 25 mL was taken and equally divided providing a sample and negative control. The sample and control were used to measure the uptake of a radioactive label, L-[^{35}S] cysteine. The experiment was conducted in accord with the previously detailed experimental protocol (Section 2.3.4, Scheme 2.1).

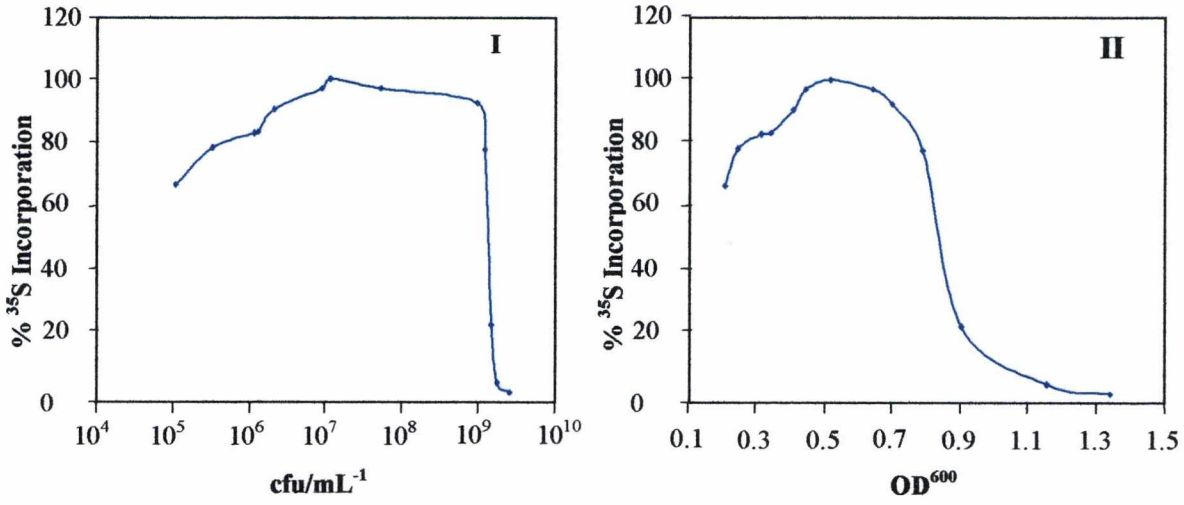


Figure 3.10 The incorporation of L-[^{35}S] cysteine by *L. monocytogenes* PTM medium batch culture

The results demonstrate three phases where the rates of incorporation of the radioactive label L-[^{35}S] cysteine changed. Figure 3.11, Graphs I and II clearly shows an increase in the % incorporation of the radioactive label L-[^{35}S] cysteine, as the bacterial cell numbers and optical density (OD_{600}) increase, to a point at 1 x 10^7 (OD_{600} 0.70) where the rate of label incorporation begins to fall. At a cell count of 5.5 x 10^8 (OD_{600} 0.850) the rate of incorporation of the radioactive label L-[^{35}S] cysteine dropped rapidly. Graphs show 100% label incorporation, this figure represents the point where incorporation of the label peaked rather than a point where all of the label present in the medium became incorporated.

3.3 Discussion

3.3.1 Maintenance of culture purity

Any microbiological investigation centred on a single specified organism must start with a high level of confidence that the organism being investigated remains the same throughout. All *L. monocytogenes* cultures were started using the same method and were seeded from an original culture source. The conditions of growth were standardised and remained the same throughout (Sections 2.2.5 and 2.2.5.1). The growth behaviour of bacterial cultures could be initially dependent on the physiological state of the bacteria prior to inoculation [222], therefore the method of production of starter cultures remained consistent throughout, although even where inoculum size is consistent variation in lag phase can still occur [260]. By maintaining the practice of inoculation from a second-generation overnight BHI starter culture, (Section 2.2.5) the bacteria were considered stress and injury free prior to inoculation in PTM medium cultures. The induction of cold shock and acclimatisation proteins has been noted in *L. monocytogenes* cultures during a down shift in temperature from 37°C to 4°C [213], additionally culture lag times have been reported to be noticeably shorter in cultures inoculated at 15°C than those inoculated at 20°C and 25°C [260]. All media prior to inoculation was pre-warmed to 30^0C to avoid any temperature induced stress responses at the time of inoculation. At the point of inoculation and beyond, consideration has to be given to the possibility of the presence of contaminating organisms in sample cultures.

A series of standard microbiological tests were adopted to test the cultural integrity of sample cells (Section 2.2.1.4). In conjunction with these test procedures a series of observations of the growth characteristics of *L. monocytogenes* were made including growth rate, culture t_d, OD, the cell count at stationary phase, and the age of the culture at stationary phase (Section 3.2.2, 3.2.2.1). During repeated sample preparation cycles, these parameters remained reproducible with limited variation (max 10% results not shown). Any cultures that demonstrated greater variation was discarded. The morphology and motility of the organism was periodically monitored throughout growth using oil immersion light microscopy and such observations formed part of the standard testing procedure that established the purity of each bacterial culture. Morphological changes were observed between Listeria cells sourced from differing growth medium. Cells sourced from a PTM medium at exponential phase and stationary phase appeared smaller than those sourced from BHI (results not shown). The standard test procedures were designed to provide rapid confirmation that the tested organism was *L. monocytogenes* and that the culture did not contain contaminating organisms. The

changes in morphology observed throughout growth (Section 3.2.3) may have masked the presence of contaminating organisms of similar morphology; therefore, samples examined by light microscopy were also streaked onto BHI agar plates and incubated for 24 hours. After incubation, the agar plates were examined for the presence of contaminating organisms. The *L. monocytogenes* colonies appeared off white and circular with an indented centre when grown on agar plates. In direct smears the cells may appear to be coccoid, so they could have been mistaken for streptococci. Longer cells can be suggestive of corynebacteria that are club shaped.

Taken in isolation, none of the tests adopted were totally conclusive for the identification of the organism as *L. monocytogenes,* neither can it be stated that by obtaining positives to the entire test sequence identification was confirmed. However given that the starting organism had been tested and confirmed as being *L. monocytogenes* these tests were accepted as suitable confirmation on that basis and equally importantly were adopted as a standard method of detecting contamination. The streak plate testing of cultures allowed the downstream identification of samples that had been sourced from contaminated cultures.

3.3.2 The selection of growth media

The growth of *L. monocytogenes* has been demonstrated between 4°C and 50°C and the low temperature at which growth is possible categorises the organism as being psychrotrophic [17]. The organism is tolerant of high salinities and has an ability to grow at low pH [261], although growth has been demonstrated at pH 4.5 and below, the organism is a neutrophile, and at pH 4.0, its growth can be inhibited. The lowest pH limit that Listeria can tolerate depends on the medium composition, the strain, and its physiological state [210]. The organism is adaptable and can survive a wide range of varying environmental perturbations, however unlike many bacteria, Listeria strains do not grow well in minimal medium [262]. It is however generally accepted that a nutritionally rich medium such as tryptic soy broth or bovine brain heart infusion (BHI) broth supports good growth of Listeria sp. It has been demonstrated that a nutritionally rich medium such as BHI supports the growth of *L. monocytogenes* to stationary phase [222]. The details of a PTM medium that supports the growth of *L. monocytogenes* to stationary phase have also been published [222]. Experiments were conducted to establish the growth characteristics of the organism when grown using the standard conditions adopted herein (Section 2.2.5 and 2.2.5.1). The results clearly demonstrated that at 30°C with continual agitation a BHI broth supported the growth of *L. monocytogenes* Scott A to stationary phase (Figure 3.1 and 3.2). In BHI batch culture the

maximal rate of cell division during exponential growth was defined by determination of the culture t_d, as being 58 minutes. The results presented here also clearly demonstrate that at 30°C with continual agitation PTM medium supported the growth of *L. monocytogenes* Scott A to stationary phase (Figure 3.1 and 3.2). The maximal rate of cell division during exponential growth, defined by determination of the culture t_d, was 98 minutes. During the growth of a Listeria culture the physically applied conditions of growth such as temperature, and agitation (motion applied to aerate the culture), were easily maintained and reproduced. A nutritionally rich medium such as BHI is not suited to experiments where a reproducible medium with a defined chemical composition is required. Not only is the precise composition of the protein component of the medium undefined it can also vary from batch to batch [222]. When adopting a chemically defined medium as standard, the chemical composition of the medium is known and easily reproduced. A chemically PTM medium also allows the monitoring of the rates of utilisation of individual medium components throughout growth. The PTM medium supported good growth of *L. monocytogenes*, although it did not match the growth rate demonstrated by BHI, the rate of growth demonstrated is considered good enough to adopt the medium as a standard for the culturing of *L. monocytogenes* in all further experiments.

3.3.3 Accurate determination of the growth characteristics of *L. monocytogenes*

A typical growth curve will be sigmoid and have a number of distinct phases of growth including lag phase, exponential phase and stationary phase, however cultures were not monitored for a sufficient length of time for a death phase to be observed. The optical density (OD_{600}) based growth curves of *L. monocytogenes* in batch culture using BHI broth and a PTM medium conformed to this standard, and a lag phase, exponential phase, and stationary phase, were clearly discernable (Figure 3.1 and 3.6). Low volume cultures were used for the automated production of multiple repeats of *L. monocytogenes* growth curves (Figure 3.1 and 3.6). The results produced allowed multiple comparisons of the rates of growth of *L. monocytogenes* in alternative growth medium. It should be noted however that the growth curve data from low volume batch cultures was not directly comparable to the growth curve data produced using large volume (500 mL to 2 L) cultures (Figure 3.2). Culture volume is not the only factor that can affect the accuracy of growth curves, optical density is a rapid convenient way to produce growth curve data but is limited and provides no information on cell numbers.

A more accurate method for the determination of bacterial growth curves is the viable count [225] and this was adopted as a standard method of determination of cell population. The assessment that each medium supported the growth to stationary phase of *L. monocytogenes* was based purely on the attainment of similar cell numbers and OD_{600} at stationary phase from the same starting point in each media. Even where cells are in the same physiological state at inoculation the act of inoculation itself will cause change to the physiological state of the cells. The degree of change in physiological state will depend on the new growth conditions, both work load (the amount of work required to adjust to a new environment and grow) and work rate (the speed at which such an adjustment occurs) can be independently affected [51]. The amount of work that a culture must do to achieve growth in different media may differ, and cells may require a different protein complement to achieve the same aims of adaptation to environment and preparation for cell division. The rate at which individual cells achieve this aim may also be linked to the need to make greater changes in protein complement when in different media. The increased lag phase noted after inoculation into a PTM medium may be indicative of the need to do more work to achieve exponential growth than the level of work required to achieve the same aim in BHI. The term work when applied to a batch culture, is not an easily measured quantity and is used as a convenient term to differentiate between the responses of *L. monocytogenes* inoculum in BHI broth and a PTM medium, where all other conditions remained constant.

3.3.4 Analysis of medium changes over time during the culturing of *L. monocytogenes.*

For the free-living organism, nutrient availability is an important environmental variable and it is apparent that functional differences exist between hungry (nutrient limited) and starving (nutrient depleted) bacterial cultures [263]. Thresholds exist where nutrients in excess become limiting and where that limitation then becomes starvation [264-266]. The hunger response is an adaptation to limiting nutrients and allows continued growth with the induction of nutrient scavenging proteins. Observations of the Gram-negative bacterium *E. coli* for example has revealed that the *osmY* gene, an example of an *RpoS* (σ^S) regulated gene undergoes increased expression during stationary phase. This *RpoS* dependent control begins to dominate at low growth rates and it is at this point that the hunger response ceases and the cell begins preparation for entry into stationary phase and eventual growth arrest [267]. In some bacteria, the main function of the *RpoS* (stationary phase specific) sigma subunit of RNA polymerase is control of the organism reaction to nutrient, carbon, phosphate, or nitrogen starvation. The

main function of the *RpoS* regulon is to instigate and control the changes that prepare the cell for prolonged starvation. This can be achieved by a change in the shape of the cell resulting in a decrease to the cell surface area [259] as seen in TEM images of *L. monocytogenes* (Section 3.2.3), or alternatively by an increase in osmo- and thermo-tolerance [268, 269]. The membrane permeability is also reduced as a cell enters stationary phase [270, 271]. A close examination of the various growth states can highlight specific phases of growth that can be directly related to the conditions in which the organism finds itself. The starvation response for instance, is different to the hunger response and it induces the production of proteins that allows the cell to survive prolonged starvation [267, 272]. Stationary phase itself is not a state of total growth arrest even in very old cultures (several weeks). It has been proposed that culture heterogeneity increases towards stationary phase [273] and in aged stationary phase cultures a small population of cells may still be replicating [272]. Stationary phase and the modification of cellular activity in response to limiting nutrients can be termed the starvation survival response (SSR). The SSR of *L. monocytogenes* has been induced under glucose or multiple-nutrient, but not amino acid limitation. The well-characterised regulators SigB and PrfA are both required for the full SSR and effect stress resistance during growth and starvation [135]. Expression of the PrfA-controlled virulence gene *hly* (encoding the pore-forming cytolysin listeriolysin) is under negative regulation by readily metabolised carbon sources in *L. monocytogenes* [274]. The determination of a growth state is therefore a complex issue and one not easily defined by simple observations of OD or cell population. A culture at stationary phase may contain cells adapted for long term growth arrest, cells adapted for short term growth arrest, cells at stationary phase and small sub-populations of cells which continue to replicate. As cultures in stationary phase age some of this heterogeneity is lost as sub populations of cells adapted for long-term growth arrest predominate while other short term growth arrested cells decrease in numbers.

It has been reported that a wide range of environmental stress factors can induce uncharacteristic early growth arrest during batch culture [263, 275]. A single medium component in a limiting concentration may induce the expression of stress proteins specific to that component. Different growth limiting factors have been shown to induce the expression of different subsets of stress proteins. Therefore, some of the changes (in protein expression) that occur in a cell population as it enters stationary phase or a growth-arrested state can be determined by the type of stress that instigated the growth arrested state. The heat-shock response involves the induction of a number of proteins generally referred to as heat-shock proteins (HSPs) in response to elevated temperature [276]. However the bacterial heat shock

response is not limited to temperature change and is a general stress response, as many HSPs are also induced by other environmental perturbations including starvation [277]. In bacteria, the major control of the expression of heat-shock genes is transcriptional. In low G + C Gram-positive bacteria, such as *Bacillus subtilis,* the heat-shock genes are transcribed by the vegetative housekeeping sigma factor (σ^{70}). In several bacterial species HSPs have been implicated in pathogenesis of the organism, moreover they have been shown to be essential for stationary phase [278]. Therefore stationary phase can be considered as a general stress response that allows survival of the organism when nutrient limitation or some other limiting factor precludes continued growth at an exponential rate.

3.3.4.1 Medium pH

In common with other lactic acid bacteria, *L. monocytogenes* produces lactic acid as the main by product of fermentation [279]. Many Listeria species grow on glucose aerobically, forming lactic acid and (or) acetic acid [280]. Lactic acid and acetic acid are not the only metabolites produced. Aerobic growth of *L. monocytogenes* in a semi-defined medium has resulted in the production of 10 acids as well as acetylmethylcarbinol [281]. During growth, the production of lactic acid can acidify the medium in which the organism is grown, and the extent to which the medium becomes acidic may be such that growth becomes unsustainable. Adjustments to the medium in which the organism is grown can be made by the addition of phosphates to increase the buffering capacity and thereby reduce the effects of medium acidification during growth [262]. The PTM medium adopted herein as a standard medium for the growth of *L. monocytogenes* in batch culture had a buffering capacity improved by the addition of phosphates [222]. The medium pH of *L. monocytogenes* PTM medium cultures was monitored throughout growth for 48 hours and low of pH 5.5 was recorded. In order to establish that growth was possible at pH 5.5 and that a medium pH of 5.5 was not limiting to growth, a number of PTM medium cultures were acidified, inoculated, and grown to stationary phase. The *L. monocytogenes* cells were not pre-shocked in any way prior to inoculation. The results achieved show that the lag phase for cultures at pH 5.5 was not extended beyond that observed for cultures at pH 6.9. This suggests that adjustments made to allow growth at low pH occurred at the same rate as adjustments made at neutral pH. Additionally cultures at low pH were able to demonstrate growth to stationary phase within the monitoring period. Duffy *et al.* 1994 [112] found that the growth rates of *L. monocytogenes* were linearly related to pH. The findings reported herein confirm that between pH 6.9 and pH 5.0 a linear relationship exists between growth rates and pH (Table 3.0), however below this point linearity was less pronounced and at pH 4.5 the linearity of response was no longer apparent and growth

appeared restricted. Robinson *et al.* 1998 [260] reported that the growth rate of *L. monocytogenes* in broth varied in a biphasic manner with respect to pH. It was also reported, as has been shown here, that lag phase was not affected by pH except close to the limit for growth. This suggests that the specific work required by the cell to adapt to acid conditions is slight except where conditions become limiting to growth and other factors such as cell damage may have a bearing on the extension of lag phase [260].

The results discussed and those results presented herein suggest therefore that at pH 5.0 and above the changes a cell needs to make to continue to grow are not major. It is also suggested that the rate of change of medium pH (Table 3.0) became static because stationary phase began, rather than a falling medium pH causing the inhibition of growth. This implies pH is not the agent responsible for the termination of exponential growth and the instigation of stationary phase. Further work was conducted to determine which (if any) medium component was limiting to growth at the induction of stationary phase.

3.3.4.2 Glucose utilisation

A PTM medium with glucose as the carbon source was used to observe the growth to stationary phase of a *L. monocytogenes* culture. Samples of media were taken prior to inoculation and throughout growth so that the utilisation of the carbon source could be monitored and quantified. The medium glucose levels determined throughout growth and at stationary phase showed three phases of utilisation. The general phases of glucose utilisation could be associated with the identification of exponential growth and stationary phase as defined by cell counts and OD_{600}. Between inoculation and the onset of stationary phase the OD_{600} and cell counts indicated that rapid growth and cell division were occurring. The rate of glucose utilisation during this phase of growth was high and consistent with exponential growth. The age of the culture at the onset of stationary phase was determined at 19.5 hours and at that time the medium glucose concentration was between 30 to35% of its starting concentration and not considered limiting to growth. The rate of utilisation of glucose at stationary phase was shown to be 27% of the rate of utilisation at exponential growth. The culture appeared to remain in stationary phase for 11 hours before another change in the rate of glucose utilisation was observed. Between stationary phase and the end of the monitoring period the rate of utilisation of glucose fell by 89%, indicating a state of growth arrest (Table 3.1). Between the same time point the OD_{600} and cell counts remained unchanged. The rate of utilisation of glucose at growth arrest was shown to be 3% of the rate observed during exponential growth. The glucose utilisation curve allowed the distinction of two phases or stages of stationary phase where cell counts and OD_{600} measurements did not differentiate specific phases of growth.

3.3.4.3 Amino acid utilisation

PTM medium using L–glutamine as the nitrogen source was used to observe the growth to stationary phase of a *L. monocytogenes* culture. The major catabolic fate of glutamine is hydrolysis to glutamate by glutaminase. Glutamate is possibly the most active amino acid and can be used in the biosynthesis of other amino acids including proline, arginine, histidine, and tryptophan. Intermediates of glutamate catabolism can also be used in the production of ornithine, glutathione, and glycoproteins [224]. Nine further amino acids were included in the medium at 100 mg/L^{-1} concentrations. Samples of media were taken prior to inoculation and throughout growth so that the utilisation of the individual medium amino acids could be followed and determined by HPLC.

The data presented detailing amino acid use shows (Figure 3.9) three phases of utilisation. Exponential growth and stationary phase are clearly separated by the point at which a distinct change in the rate of utilisation of each amino acid was observed. The results also clearly show that the separation of exponential growth and stationary phase (by the rate of amino acid utilisation) occurred at a different time points for different amino acids. The concentration of amino acid remaining at stationary phase for each medium constituent did not appear to be limiting at the point where stationary phase cells where determined to predominate. There are some reported differences in the amino acid requirements of different strains of *L. monocytogenes*, in 1977 Ralovich *et al.* [282] found that the most common demand was for leucine, iso-leucine, valine, and cysteine. In 1989, Siddiqi *et al.* confirmed that of six strains of *L. monocytogenes* tested, most strains required cysteine, valine, isoleucine, and leucine. Phenylalanine was a stimulatory growth factor for all six strains of Listeria, whilst tryptophan was essentially required by NCTC 7973, LM and C-286 and stimulatory for 4155 and C-294. Siddiqi also reported that none of the strains examined exhibited specific requirements for asparagine, glutamine, proline, histidine or tryptophan as essential/stimulatory growth factors. Later Premaratne *et al.* reported that the main amino acids required by *L. monocytogenes* Scott A were leucine, isoleucine, arginine, methionine, valine, and cysteine *[262]*.

The results presented here (Figure 3.9) show that the amino acids, arginine, methionine tryptophan, and phenylalanine show a distinct drop in their rate of utilisation at a culture age of 17 hours. The rate of utilisation of the amino acids histidine, glutamine, valine, leucine, and isoleucine did not change until the culture age reached 20 hours. The rate of utilisation of isoleucine and valine was higher than that exhibited by the remaining amino acids. The rate of utilisation of histidine and arginine was lower than that exhibited by the

other amino acids. The age of a culture at the onset of stationary phase as determined by glucose utilisation, amino acid utilisation, cell counts and medium pH generally coincide at an approximate culture age of 20 hours (+/- 15%). The two stages of stationary phase detected by observations of the amino acid utilisation graphs (Figure 3.9 and 3.1), were also apparent in the individual glucose utilisation data (Table 3.1). The rates of utilisation of some of the amino acids suggests that the accepted boundary for the division of exponential phase from stationary phase at 20 hours culture age, is more blurred than originally suggested by the medium pH, glucose utilisation, cell count, and OD_{600} results. The incorporation of a radio labelled amino acid could define more clearly the point at which exponential growth ceases and stationary phase began.

3.3.4.4 *The incorporation of L–[^{35}S] cysteine by L. monocytogenes during batch culture.*

A PTM medium was used to observe the growth to stationary phase of a *L. monocytogenes* culture. Aliquots of the culture were taken at timed intervals throughout growth and stationary phase (defined by OD_{600} 1.1). Each aliquot was re-suspended in a medium containing L–[^{35}S] cysteine after centrifugation. After a timed incubation, the incorporation of the label by each sample was determined by scintillation count, as described in Section 2.3.4. Cell counts were used to determine the percentage label uptake per cell at each sample point. The fixed incubation period for the uptake of the label served to highlight the difference in the rate of incorporation of the label as growth progressed to stationary phase. A rapid fall in the rate of label incorporation was noted at an OD_{600} of 0.85.

A number of methods have been used to define the point where a *L. monocytogenes* culture changes its pattern of growth from exponential growth and enters stationary phase. The definition of stationary phase in this instance being the point where OD_{600} readings and cell counts no longer increased. The point at which stationary phase was recognised in a 1 L *L. monocytogenes* PTM medium batch culture was not fixed and varied by the age of the culture and the OD_{600} recorded for each determining factor. Each culture was grown at the same conditions and in the same medium. Each method adressed a different aspect of growth as the determining factor for the selection of the point where stationary phase began.

It is clear that reliance on a single parameter (OD, glucose utilisation etc) as a determinate for the detection of stationary phase does not highlight the complexity or specific nature of the changes occuring as an organism changes from exponential growth to stationary phase. A number of parameters were adopted to define the point at which stationary phase predominated.

Table 3.3 Different methods of stationary phase determination produce variation in the observed OD_{600} of each point

Method of determination of stationary phase	**Culture age at stationary phase (h)**	**Culture OD_{600} at stationary phase**	**Cell count at stationary phase**
Optical Density (OD_{600})	23.5	1.200	4.5×10^9
Cell count	22.0	1.200	4.5×10^9
Medium pH	21.0	1.050	2.0×10^9
Glucose utilisation	19.0 - 20	0.900 – 1.000	5.0×10^8
Amino acid utilisation	17.0 - 20	0.850 – 1.000	5.0×10^8
^{35}S incorporation	16.5 - 17	0.800 – 0.850	2.0×10^8
Motility*	17.0 - 19	0.850 – 0.900	7.0×10^7

Table 3.3 gives motility* as a method of determining stationary phase, this was not the case. The cell count at this point also reflects the fact that not all cells have flagella therefore the count at the assumed stationary point is lower than that seen using other definitions of stationary phase. The point at which motility could no longer be detected coincided with the point at which amino acid utilisation and L–[^{35}S] cysteine incorporation were falling rapidly indicating the onset of stationary phase.

The results (Table 3.3) show that the process involves considerable change over an extended period of time. What is not clear from these results is the extent to which that change is reflected by all the cells in a batch culture. Synchronous growth is not occurring so the results presented represent an averaged view of the state of the culture at each sample point. What can be stated is that at stationary phase, cells that were not at exponential phasepredominate and at exponential phase cells that were not at stationary phase predominated. The transition from exponential growth to stationary phase appears complex and an event that spans several hours. The point at which cells in batch culture detect that conditions for continued growth are changing, appears to be a point several hours before stationary phase is attained. The results presented cannot determine whether this observation is due to a gradual increase in the total number of cells becoming stationary over time, or that the all of the cells are becoming stationary gradually over time.

The transition to stationary phase is a general stress response that can be induced by a single limiting factor or a combination of factors. The nature of the response can also be determined by the specific factor inducing the exit from exponential growth. For instance, the starvation survival response (SSR) of *L. monocytogenes* is induced under glucose- or multiple-nutrient-, but not amino-acid-limitation. In this instance glucose is not limiting,

results clearly show that glucose is available to the cultures at late exponential phase and stationary phase (Figure 3.7) and it has been reported that in a culture undergoing growth limitation, glucose consumption is uncoupled from cell proliferation [283]. The results presented here show glucose consumption continuing after stationary phase induction. The regulators SigB and PrfA are both required for the full SSR and effect stress resistance during growth and starvation. Herbert *et al.* found that 0.01-0.2% of a population remained viable even after 20 days and that the surviving cells showed a reduced cell size and increased cross-protection to several environmental stresses [135]. Expression of the PrfA-controlled virulence gene *hly* (encoding the pore-forming cytolysin listeriolysin) is under negative regulation by readily metabolised carbon sources in *L. monocytogenes* and the decrease in pH (Figure 3.5) associated with utilisation of sugars negatively regulates *hly-gus* expression, although sugars can affect *hly-gus* expression by another mechanism that is independent of pH [274].

4.0 Analysis of the heterogeneity of a *L. monocytogenes* culture using flow cytometry

4.1 Introduction

The majority of bacterial culture analysis methods do not operate at single cell level and the data collected is viewed as a mean. The distribution of a particular cellular property or properties within a population cannot be easily distinguished [217]. The results presented in chapter three demonstrated that a mean result could not be used for an in depth analysis of the growth characteristics of a bacterial culture. In particular optical density (OD), the most popular and least accurate determinate of bacterial growth, failed to discriminate between stationary phase cells and growth-arrested cells (Section 3.2.2). OD is a measurement of light scatter and is the product of cell number and cell size. The accuracy of the method is based on the assumption that cell size remains unchanged throughout growth. It is however well documented that bacterial cells change size during growth [256]. This means that a culture t_d based on OD can be longer than the actual rate [280]. As an alternative to OD, viable cell counts have been used herein to provide a more accurate method of determining population numbers, although viable counts fail to discriminate between stationary phase and growth arrested cells (Section 3.2.2.1). Although a cell count provided an accurate t_d and a more defined point at which stationary phase predominated (Section 3.2.2.1) it did not give any indication of the heterogeneity of the culture. In chapter three a number of methods were used to monitor the utilisation of nutrients, and these were able to discriminate between a stationary phase and growth arrested state (Section 3.3.4.2, 3.3.4.3, and 3.3.4.4). As with OD and viable counts, nutrient utilisation also recorded a culture mean. Results based on a culture mean can mask increasing/decreasing culture heterogeneity. This was apparent where no specific growth state predominated, in particular the point of transition between exponential growth and stationary phase.

It was not until the advent of flow cytometry in the late 1970s that measurements of single bacterial cells and other microorganisms were possible [217]. As early as 1977, observations of changes in the size and DNA complement of individual cells from bacterial cultures were being made using flow cytometry [280]. More recently flow cytometry has been used to simultaneously examine several physical and chemical cell characteristics, providing information on the heterogeneity of 1000s of bacterial cells in minutes [218]. Flow cytometry has also been used to investigate the effect of bacteriocins (anti-microbial peptides) on the bacterial cell [281] and has been used to study the effect of leucocin (a bacteriocin) B-TA11a

on *L. monocytogenes*. In this experiment mixed proportions of dead and live control populations were analysed by flow cytometry using two fluorescent dyes. The uptake of both dyes suggested that the cells remained viable but became leaky, possibly indicating bacteriocin-induced pore formation in the target membranes [282]. Working with the bacteriocin (lantibiotic) nisin, van Schaik *et al.* [201] has shown that acid adapted *L. monocytogenes* cells exhibited increased tolerance to the bacteriocin and to a lesser extent the bacteriocin lacticin 3147. Nisin forms transient pores in the membrane of *L. monocytogenes* dissipating the proton motive force and causing the efflux of cytoplasmic compounds [283]. Nisin is a Group A lantibiotic (antimicrobial peptide) that has antibacterial effects against a number of Gram-positive bacteria including *L. monocytogenes* [284]. Its action is in part dependent on the presence of precursor membrane constituents which are present during active growth but not at growth arrest [285, 286]. Nisin forms transient pores in the membrane of *L. monocytogenes* [287] and its action is attenuated by the presence of a bacterial cell membrane precursor peptidoglycan lipid II. Flow cytometry provides a sensitive means of monitoring cellular events that are occurring in bacteria [284]. The means of providing such sensitivity is the selection of indicator stains that highlight the aspects of cellular dynamics that are to be investigated.

Herein, it was proposed that flow cytometry be used to examine the heterogeneity of a *L. monocytogenes* culture on transition to stationary phase. To achieve this aim the lantibiotic nisin has been used to selectively create transient pores in the *L. monocytogenes* membrane while monitoring the uptake of a membrane impermeant dye. To investigate the effect that nisin had on the membrane of *L. monocytogenes* cells in culture during growth and at stationary phase two stains were adopted, SYTO 13 and propidium iodide (PI). SYTO 13 is an effective DNA stain that can be used as a determinate of total bacterial populations; the fluorescence signal that each stained bacterium emits can be measured. The stain will cross the bacterial membrane without hindrance and stains all the cells in a population [269, 288, 289]. PI is a cell impermeant DNA and RNA fluorescent dye that does not cross intact cell membranes of *L. monocytogenes* [290-292]. The basis for the monitoring of a *L. monocytogenes* culture throughout growth was that cells susceptible to the action of nisin were permeabilised allowing PI to enter. Cells not susceptible to nisin were not permeabilised and therefore do not take up the PI stain. A range of nisin concentrations were used to apply a challenge to a *L. monocytogenes* batch culture during growth and at stationary phase. The flow cytometer was used to look for different responses that such a challenge would produce.

4.2 RESULTS

4.2.1 Selection of an antimicrobial agent

In a series of preliminary experiments a range of antimicrobial peptides were tested to see if they would differentiate exponential from stationary *L. monocytogenes* cells. The difference in membrane permeability at different growth phases that each peptide brought about was examined by observing the rate of uptake of a membrane impermeant dye, propidium iodide (PI) using a flow cytometer. Rifampicin, chloramphenicol, ampicillin, and carbamicillin did not highlight any differences in the rate of stain uptake between exponential phase and stationary phase cultures of *L. monocytogenes* (results not shown). A further peptide, nisin was able to differentiate exponential from stationary cells.

4.2.2 The effect of nisin on *L. monocytogenes* at exponential and stationary phase

Nisin was added to *L. monocytogenes* culture samples at exponential and stationary phase. Nisin, a group A lantibiotic (antimicrobial peptide), has antibacterial effects against *L. monocytogenes* and demonstrated a different response to exponential and stationary phase cells when using the rate of PI uptake as an indicator of membrane permeabilisation. Data collection and results analysis was completed using Expo 32 and WinMDi (Ver. 2.8) software. Data from each sample was collected over a 15 minute period recording the change in fluorescence, as individual cells became PI positive. SYTO 13 was used to visualise PI negative cells, i.e. those cells within the population that had an intact membrane. Cells that became PI positive (and fluoresced red) could be separated from cells that were not PI positive. The point of separation between PI negative and PI positive cells (measured by fluorescence) could be fixed and applied to every sample. The number of cells from the total population that had become PI positive was monitored and has been expressed as a percentage.

Data collection for control experiments continued over 15 minutes, the degree of PI uptake was analysed at a single point within the final minute of the data collection period. Figure 4.0 clearly shows that nisin can differentiate between an exponential phase and stationary phase *L. monocytogenes* culture. Figure 4.0, Graph A shows the data recorded for a culture at 0.127 (OD_{600}), 59.9% of the cells are PI positive, The intact cells indicated in this image are thought to be cells at stationary phase from the initial inoculum that are yet to enter exponential growth phase, indicating non-synchronous growth. Figure 4.0, Graph B: shows the data recorded for a culture at 1.623 (OD_{600}), no cells during the recording period took up PI stain.

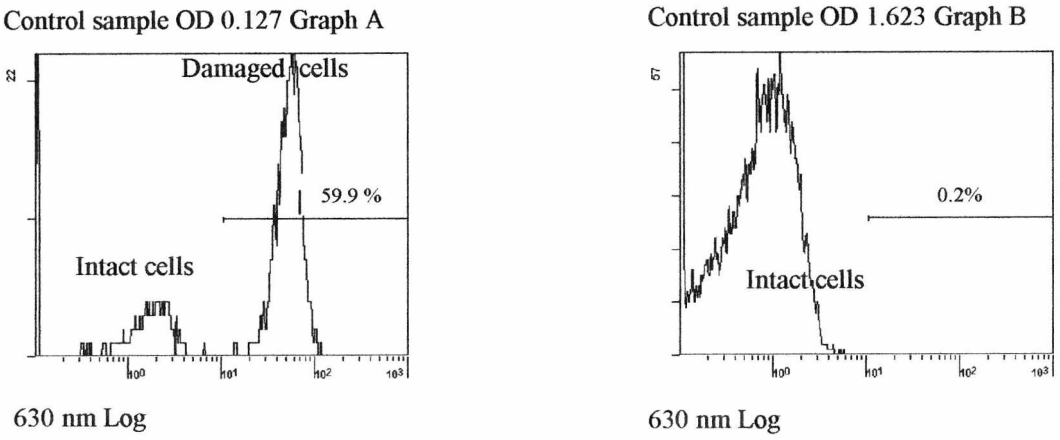


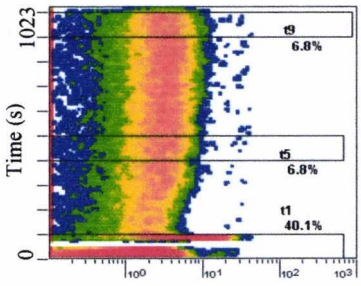
Figure 4.0: Differential uptake of the DNA stain PI by *L. monocytogenes* batch culture at exponential and stationary phase after exposure to an 8 ng/mL nisin incubation over 15 minutes.

4.2.1.1 Nisin permeabilisation of L. monocytogenes membrane in fresh and spent medium at different pH

Aliquots of *L. monocytogenes* grown in PTM medium batch culture to stationary phase (OD_{600} 1.309) were re-suspended in fresh PTM medium and spent PTM medium at different pH. Each sample was subjected to analysis by flow cytometry to determine the effect that changing medium conditions had on the membrane permeabilising action of nisin. The samples were adjusted so that each would contain the same number of cells. Spent medium diluent was taken from an earlier culture at various points throughout growth so an OD_{600} of the diluent could be selected to match that of the sample into which it was added. The data was filtered to remove any non-cell recorded events, i.e. background fluorescence and medium effects (medium precipitates). The data has been presented in the form of 'dot plots', each dot plot is a display of all the information collected over a ten minute period and represent: -

- Each dot on individual plots represents a single cell
- The colours represent cell density. Blue: low cell numbers. Red: high cell numbers
- Fluorescence was measured along the X-axis
- A shift in position from left to right along the X-axis represented a change in fluorescence and indicated PI uptake
- The boxed areas on each dot plot represent the percentage of the whole data that was used to create graphs of cells that had become PI positive at 1, 5 and 9 minute time intervals
- Sample time (in seconds) was recorded on the Y-axis

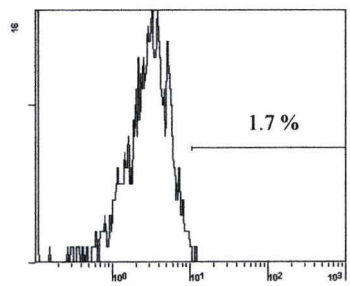
Plot I: OD 1.309



- Plot I Spent medium, pH 5.5. The pH and OD_{600} of the spent medium matched that of the sample into which it was added.
- Culture age at sample point, 19.3 hours
- Culture OD_{600} at sample point 1.309

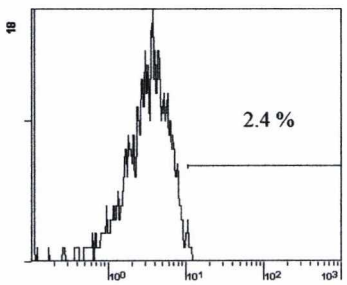
T1 = 1 min

A



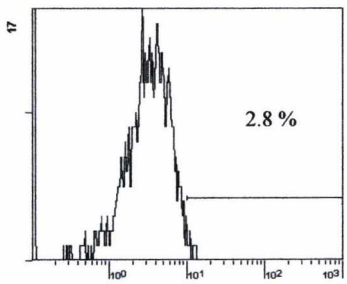
T5 = 5 min

B

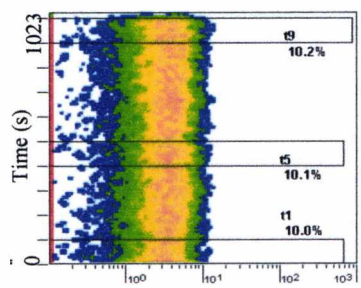


T9 = 9 min

C



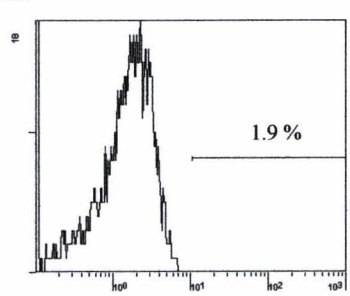
Plot II: OD 1.309



- Plot II Spent medium. The OD_{600} of the spent medium matched that of the sample prior to a medium change. The medium pH was adjusted to pH 6.9.
- Culture age at sample point, 19.3 hours
- Culture OD_{600} at sample point 1.309

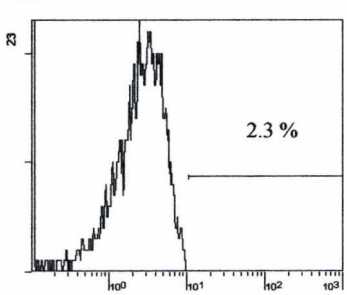
T1 = 1 min

A1



T5 = 5 min

B1



T9 = 9 min

C1

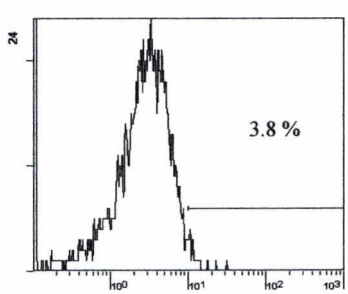
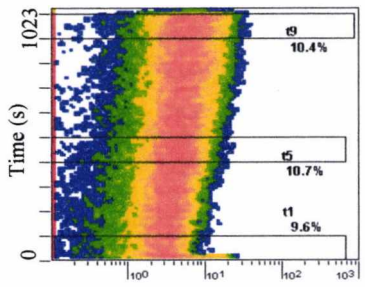


Figure 4.1: Uptake of the DNA stain PI by *L. monocytogenes* at stationary phase in spent medium at different pH

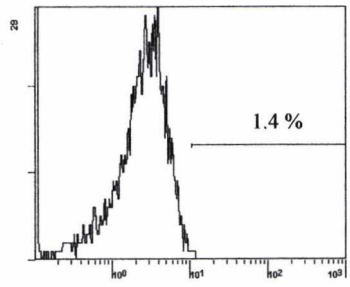
Plot I: OD 1II 1.309 (OD_{600})



- Culture OD_{600} at sample point 1.309
- Plot III fresh medium. The pH of the fresh medium matched that of the sample into which it was added.
- Culture age at sample point, 19.3 hours

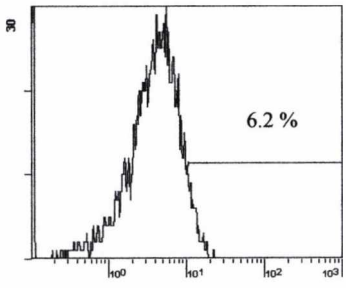
T1 = 1 min

A



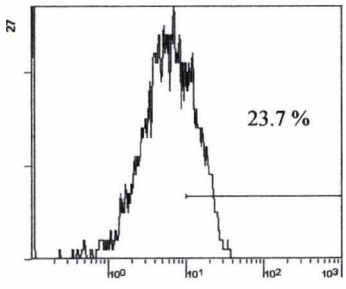
T5 = 5 min

B

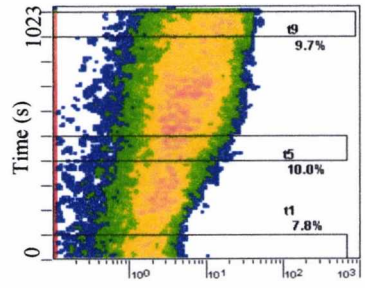


T9 = 9 min

C



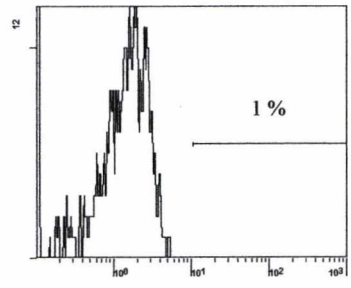
Plot IV: 1.309 (OD_{600})



- Culture OD_{600} at sample point 1.309
- Plot IV fresh medium. The pH of the medium did not match that of the sample into which it was added.
- Culture age at sample point, 19.3 hours

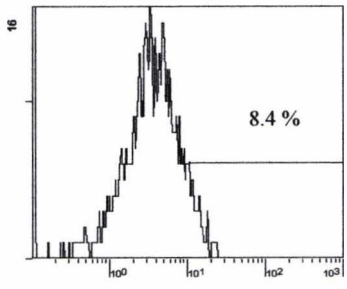
T1 = 1 min

A1



T5 = 5 min

B1



T9 = 9 min

C1

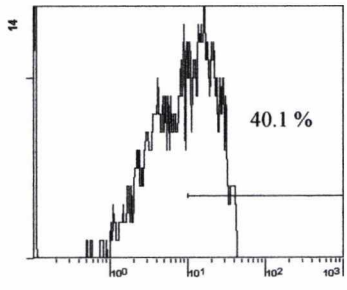


Figure 4.2: Uptake of the DNA stain PI by *L. monocytogenes* at stationary phase in fresh medium at different pH

Figures 4.0 showed that the action of nisin at 8 ng/mL^{-1} concentration acting on 1 x 10^6 cells/mL^{-1} was able to discriminate between exponential phase and stationary phase *L. monocytogenes* cells, by measuring PI uptake (Section 4.2.2). These concentrations were maintained for all flow experiments. Samples required counting (Section 2.2.9.5) and subsequent dilution to maintain the nisin molecules/cell ratio. The diluent used could be fresh medium or spent medium that matched the OD_{600} and pH of the original sample (prior to dilution). Control experiments were conducted on stationary phase cells to determine the effect that changing medium conditions would have on the action of nisin.

Figures 4.1 and 4.2 show the effect that changing medium pH and/or medium age (fresh or spent medium) had on the recorded rate of PI uptake. The results from Figures 4.1and 4.2 were collated and are presented in Figure 4.3.

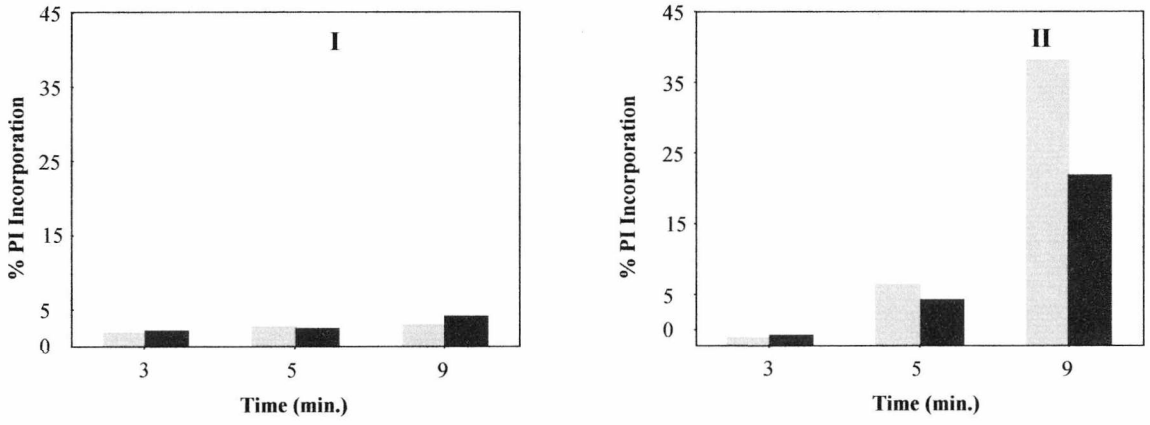


Figure 4.3 *L. monocytogenes* PI uptake in spent and fresh medium at different pH. Graph I ▫ spent medium at pH 5.5. ■ Spent medium at pH 6.9. Graph II ▫ fresh medium at pH 5.5, ■ fresh medium at pH 6.9.

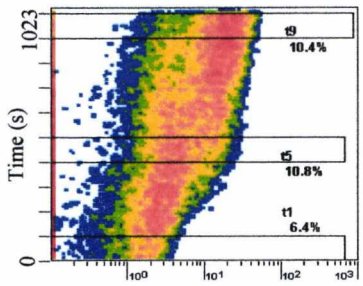
Figure 4.3 Graph I, shows that increasing the pH of the spent medium diluent from pH 5.5 (the pH of the sample medium prior to extraction) to pH 6.9, increased the number of cells becoming PI positive at 9 minutes by 1%. Figure 4.3 Graph II shows that using fresh medium as a diluent had a stimulatory effect on the action of nisin. Fresh medium at pH 5.5 (the pH of the sample medium prior to extraction) had a greater stimulatory effect on the rate of PI uptake than fresh medium at pH 6.9. The rate at which the cells became PI positive at each pH remained unchanged until a point at 6 minutes where the sample re-suspended in fresh medium at pH 6.9 became PI positive more rapidly than the sample re-suspended in fresh medium at pH 5.5. This shows that a change of medium pH in samples re-suspended in fresh medium has a greater effect on the rate of PI uptake over 10 minutes than the same change in spent medium. When Graph I and II (Figure 4.3) were compared it was clearly shown that

fresh medium stimulates the rate of PI uptake, and that a pH of 6.9 has a greater effect on the rate of PI uptake than a pH of 5.5. Re-suspension of samples in fresh medium (at a pH matching the extraction point) increased the rate of PI uptake to a greater degree at exponential growth than that observed at stationary phase (results not shown). Spent medium was used as a diluent so that the medium conditions at time of sampling were maintained even though cell volume was altered. The low rate of PI uptake shown in spent medium samples suggests the presence of a signal (in the medium) that acted to maintain the cell membranes relative insensitivity to nisin permeabilisation. When that signal was removed by the use of a fresh medium diluent, membrane susceptibility to nisin changed, allowing increased permeabilisation. This suggests individual cell perception of culture condition by external cell-to-cell signalling referred to as quorum sensing. [293]. The results already shown (figure 4.3) using spent and fresh medium at low (5.5) and high pH (6.9) confirmed that the observed increased susceptibility to nisin, was not specifically a function of changing medium pH.

4.2.3 The effect of nisin on *L. monocytogenes* batch culture throughout growth

L. monocytogenes PTM medium batch culture was sampled at 3.3, 6.2, 7.15, 7.55, 9, 9.45, 10.45, 12.45, and 13.5 hours after inoculation. The samples were counted and population adjusted by the addition of either spent or fresh medium. Each sample was stained with SYTO 13 and PI prior to a nisin addition. The rate of uptake of the dye PI was recorded by flow cytometry (Sections 2.2.9.3 - 7). The spent medium diluent matched the pH and OD_{600} of the original sample and the fresh medium diluent was pH adjusted to match the pH of the original sample (at each time point).

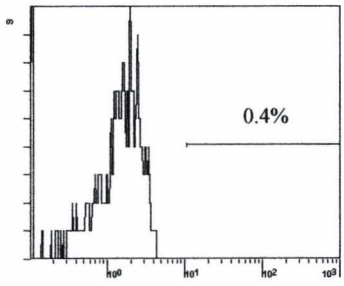
Plot I



- Culture OD_{600} at sample point 0.17
- Plot I spent medium. The pH and OD_{600} of the spent medium matched that of the sample prior to a medium change.
- Culture age at sample point, 9.3 hours

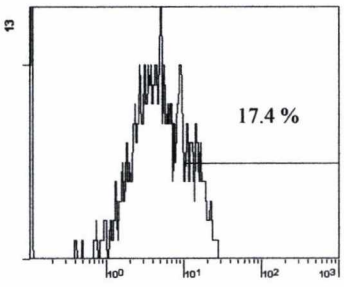
T1 = 1 min

A



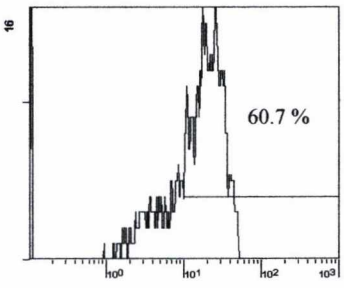
T5 = 5 min

B

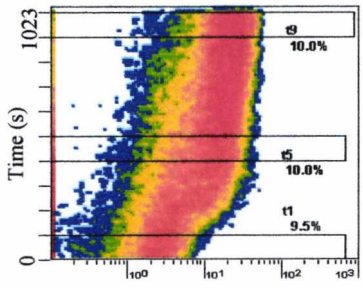


T9 = 9 min

C



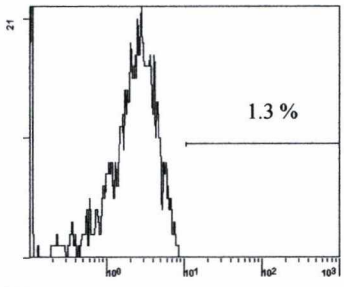
Plot II



- Culture OD_{600} at sample point 0.28
- Plot II spent medium. The pH and OD_{600} of the spent medium matched that of the sample prior to a medium change.
- Culture age at sample point, 11 hours

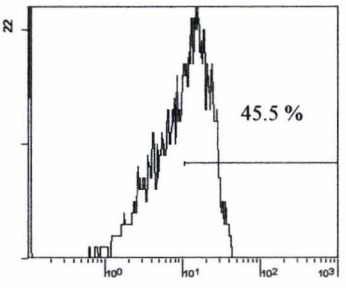
T1 = 1 min

A1



T5 = 5 min

B1



T9 = 9 min

C1

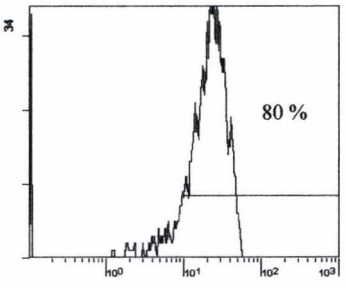
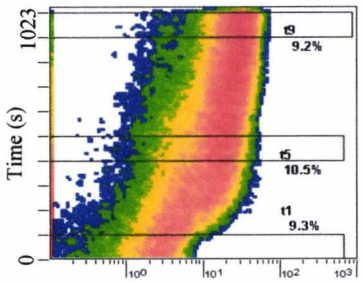


Figure 4.4 Uptake of the DNA stain PI by *L. monocytogenes* at OD_{600} 0.17 (plot I) and 0.28 (plot II) in spent medium (10 min.)

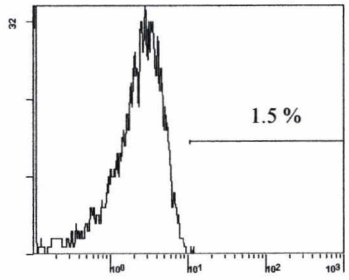
Plot III



- Culture OD_{600} at sample point 0.380
- Plot III spent medium. The pH and OD_{600} of the spent medium matched that of the sample prior to a medium change.
- Culture age at sample point, 12.30 hours

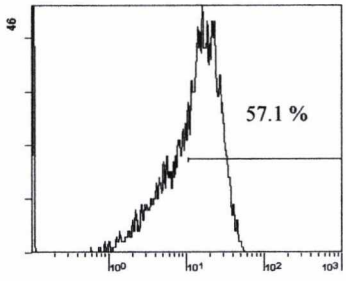
T1 = 1 min

A



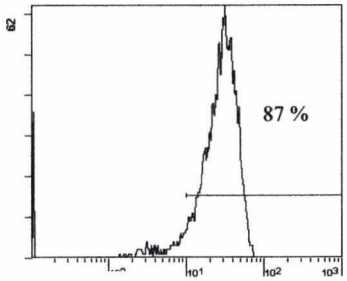
T1 = 5 min

B

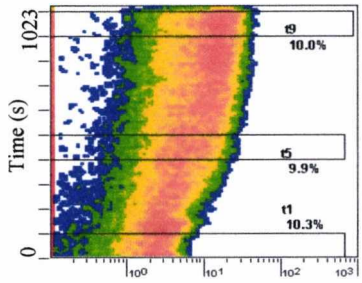


T1 = 9 min

C



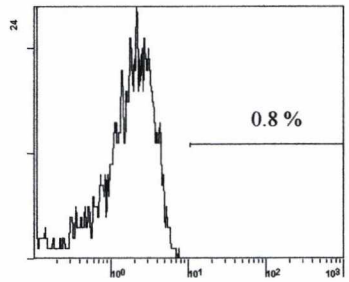
Plot IV



- Culture OD_{600} at sample point 0.560
- Plot IV spent medium. The pH and OD_{600} of the spent medium matched that of the sample prior to a medium change.
- Culture age at sample point, 14 hours

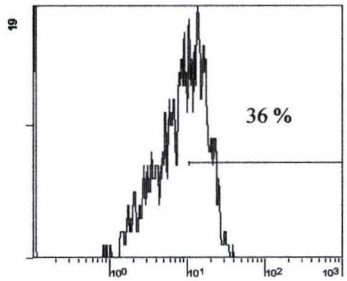
T1 = 1 min

A1



T1 = 5 min

B1



T1 = 9 min

C1

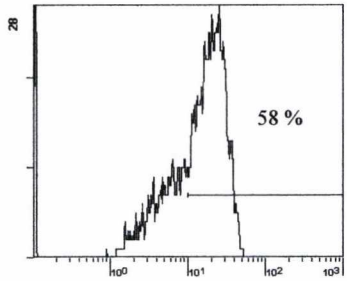
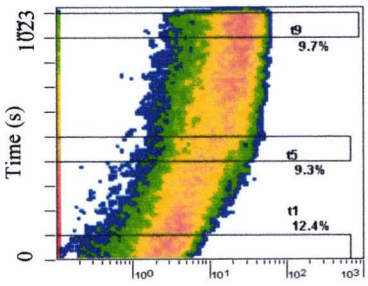


Figure 4.5 Uptake of the DNA stain PI by *L. monocytogenes* at OD_{600} 0.38 (plot III) and 0.560 (Plot IV) in spent medium (10 min.)

Plot V

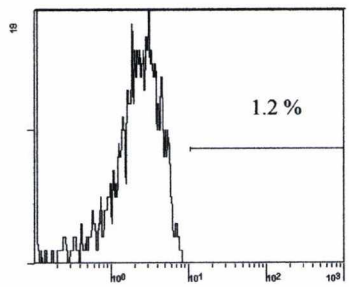


630 nm Log

- Culture OD_{600} at sample point 0.669
- Plot V spent medium. The pH and OD_{600} of the spent medium matched that of the sample prior to a medium change.
- Culture age at sample point, 15 hours

T1 = 1 min

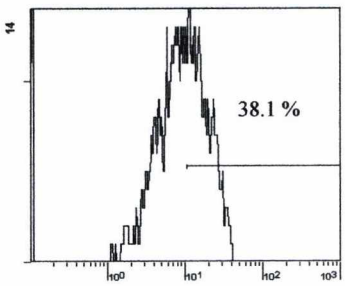
A



630 nm Log

T1 = 5 min

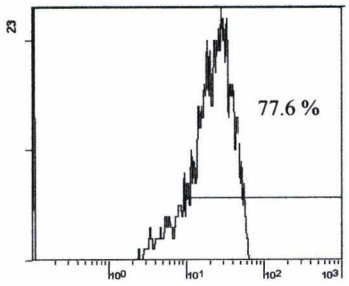
B



630 nm Log

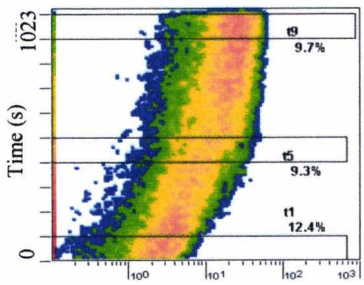
T1 = 9 min

C



630 nm Log

Plot VI

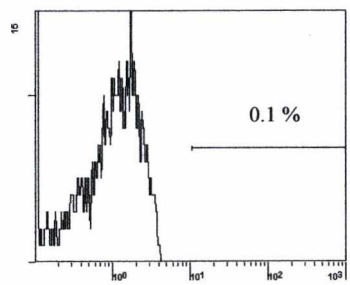


630 nm Log

- Culture OD_{600} at sample point 0.779
- Plot VI spent medium. The pH and OD_{600} of the spent medium matched that of the sample prior to a medium change.
- Culture age at sample point, 16 hours

T1 = 1 min

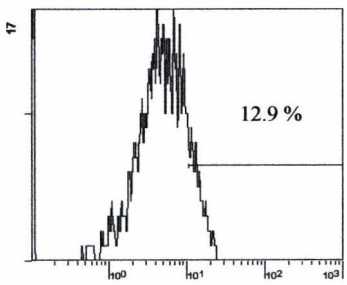
A1



630 nm Log

T1 = 5 min

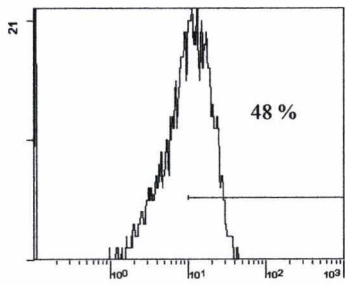
B1



630 nm Log

T1 = 9 min

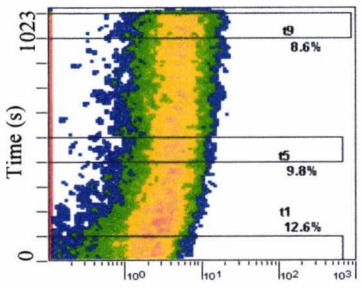
C1



630 nm Log

Figure 4.6 Uptake of the DNA stain PI by *L. monocytogenes* at OD_{600} 0.669 (Plot V) and 0.779 (Plot VI) in spent medium (10 min.)

Plot VII

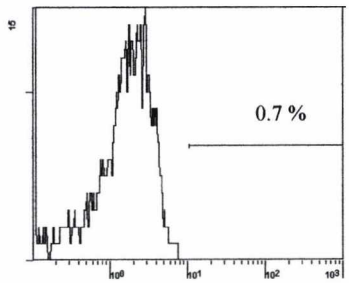


630 nm Log

- Culture OD_{600} at sample point 1.18
- Plot VII spent medium. The pH and OD_{600} of the spent medium matched that of the sample prior to a medium change.
- Culture age at sample point, 17.5 hours

T1 = 1 min

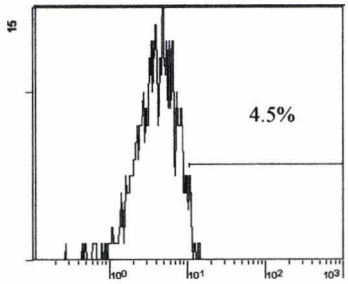
A



630 nm Log

T1 = 5 min

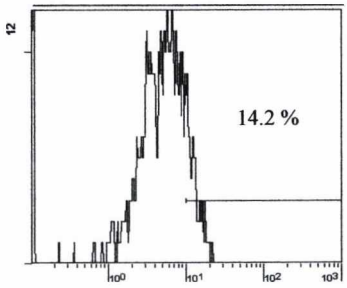
B



630 nm Log

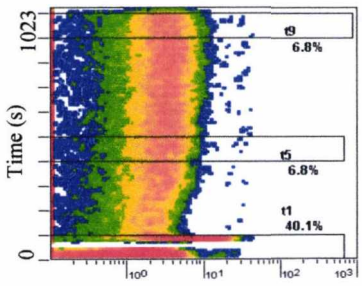
T1 = 9 min

C



630 nm Log

Plot VIII

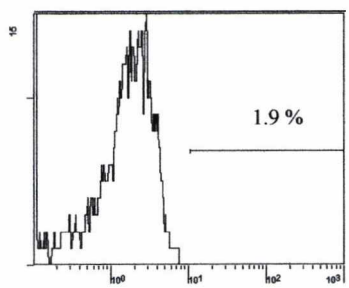


630 nm Log

- Culture OD_{600} at sample point 1.309
- Plot IV spent medium. The pH and OD_{600} of the spent medium matched that of the sample prior to a medium change.
- Culture age at sample point, 19 hours

T1 = 1 min

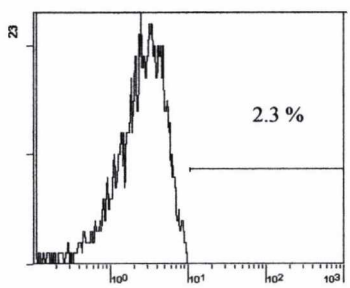
A1



630 nm Log

T1 = 5 min

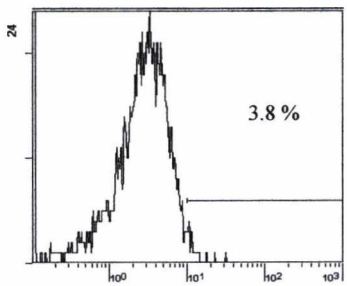
B1



630 nm Log

T1 = 9 min

C1



630 nm Log

Figure 4.7 Uptake of the DNA stain PI by *L. monocytogenes* at OD_{600} 1.180 (Plot VII) and 1.309 (Plot VIII) in spent medium (10 min.)

Figures 4.4 to 4.7 represent flow cytometry dot plot data collected over a 10 minute period after re-suspension in spent medium. Samples were taken at OD_{600} 0.17, 0.28, 0.38, 0.560, 0.669, 0.779, 1.180 and 1.309. The graphs A, B, C, and A1, B1 and C1 within each Figure detail the PI incorporation or total PI positive *L. monocytogenes* cells, at 1, 5 and 9 minutes after nisin addition. The experiment was repeated using pH adjusted fresh medium (dot plots not shown). The PI incorporation data for each set of experiments has been collated and is presented in Figure 4.8.

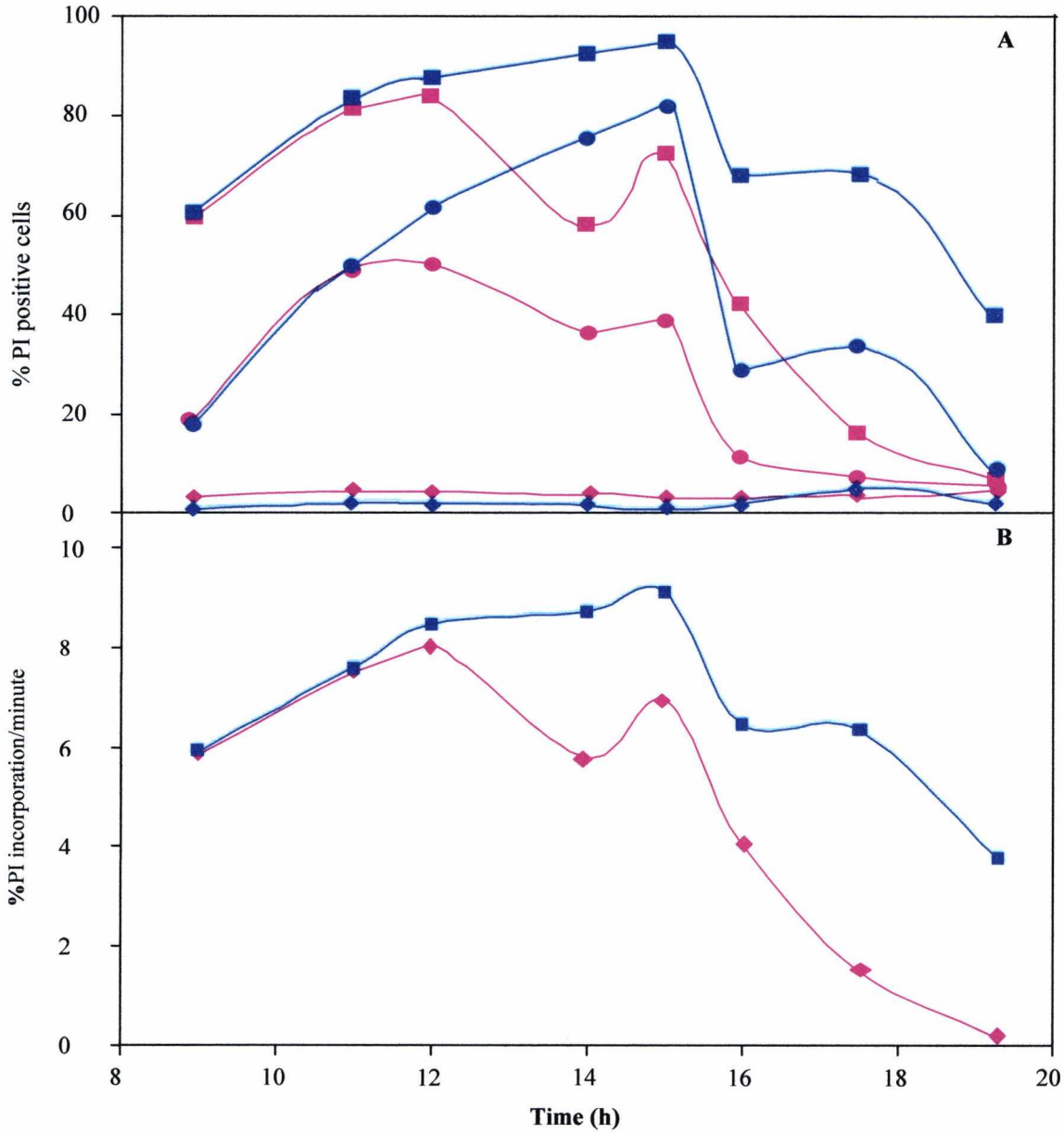


Figure 4.8 A) The incorporation of PI by *L. monocytogenes* cells re-suspended in spent medium or fresh medium throughout growth at 1, 5, and 9 minutes after nisin incubation. The plots 1 (◆◆), 5 (●●) and 9 (■■) respectively, represent the % PI incorporation throughout growth and stationary phase of a *L. monocytogenes* batch culture. (B) The % rate of PI incorporation/minute over the monitoring period (10 minutes) for *L. monocytogenes* cells re-suspended in spent medium or fresh medium samples.

The utilisation of medium constituents throughout growth and stationary phase has been previously shown (Tables 3.1 and 3.2) and the constituents of the spent medium used as a diluent were not expected to differ from previous determinations.

Figure 4.8 A, shows the PI incorporation attained by individual samples throughout growth after 1, 5 and 9 minutes nisin incubation. The results at 1 minute show that PI was not incorporated at that time point. Figure 4.8 A fresh medium plots, shows that at 5 and 9 minutes the PI incorporation increased, peaking at OD_{600} 0.38. Beyond 0.38 the PI incorporation decreased slowly to a point at OD_{600} 0.669. Beyond that point the PI incorporation fell more rapidly (excluding a point at OD_{600} 0.560 where a drop in PI incorporation was noted). Figure 4.8 A spent medium plots, shows the PI incorporation attained by individual samples throughout growth after 1, 5 and 9 minutes nisin incubation. The results show that at 1 minute, nisin was not incorporated at that time point. At 5 and 9 minutes PI incorporation continued to increase, peaking at OD_{600} 0.669, beyond which point the rate of PI incorporation dropped, remaining static until a point at stationary phase (OD_{600} 1.309) where a decrease was recorded. A clear divergence in the PI incorporation between fresh medium and spent medium after OD_{600} 0.380 was shown. The PI incorporation at OD_{600} 1.180 in fresh medium after 9 minutes (67%) was far greater than the PI incorporation recorded for the same sample re-suspended in spent medium (14%). Figure 4.8 B clearly shows a different rate of PI incorporation (due to nisin permeabilisation) when fresh medium was used as a diluent in place of spent medium.

The efficacy of nisin at stationary phase was less than that at exponential phase over a 10 minute period. A single sample at stationary phase (OD_{600} 1.18) was monitored for a further 10 minutes and PI uptake was determined at 11, 15 and 19 minutes, the results recorded are presented in Figure 4.9.

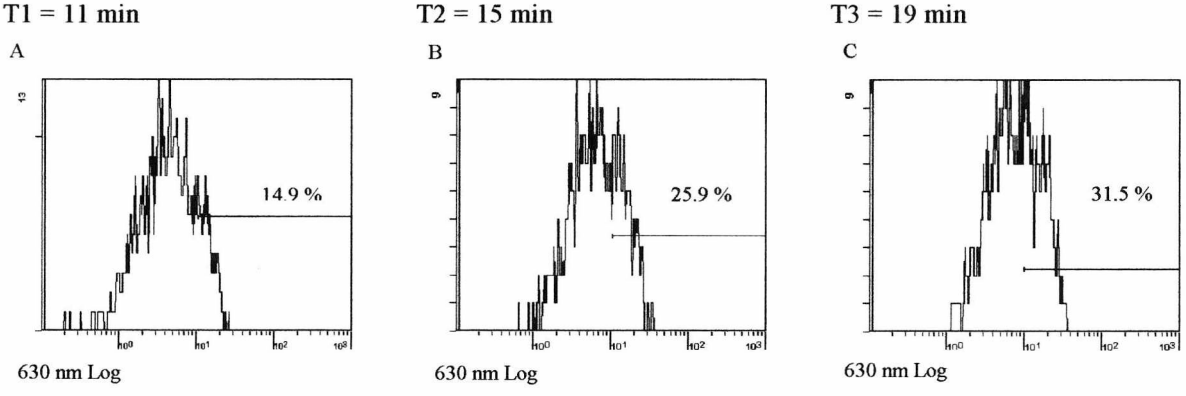


Figure 4.9 Uptake of the DNA stain PI by *L. monocytogenes* at OD_{600} 1.180 in spent medium (10 to 20 min.)

Figure 4.9 clearly shows that nisin remained active beyond the 10 minute initial recording period and continued to permeabilise the *L. monocytogenes* membrane during the recording period. The samples were also observed after incubation with nisin at 60 minutes, at that time point all the cells were PI positive (results not shown).

4.2.4 Viable counts of *L. monocytogenes* batch culture exposed to nisin

L. monocytogenes PTM medium culture was sampled throughout growth at OD_{600} and time intervals matching that of samples taken for flow cytometry experiments (Section 4.2.2). The samples were centrifuged and re-suspended in spent medium allowing each sample to be standardised to an OD_{600} equivalent to an approximate cell count of 1 x 10^6 cells/mL^{-1}. Aliquots were subjected to a 10 minute nisin incubation in accordance with Scheme 2.0, while a control sample was incubated without nisin.

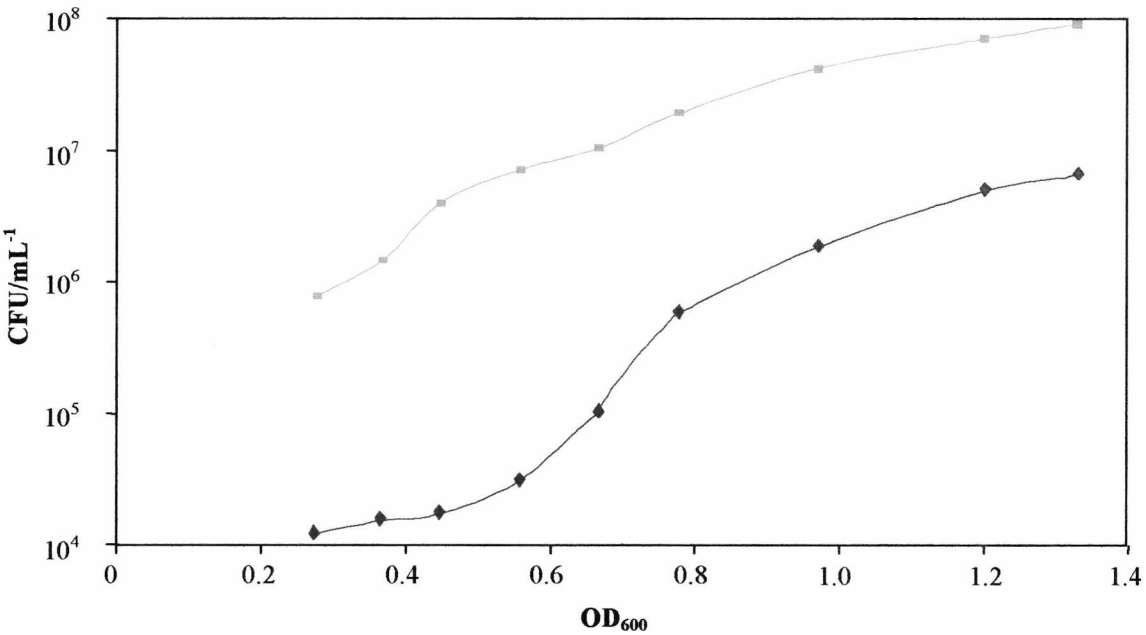


Figure 4.10 *L. monocytogenes* batch culture viable counts inclusive and exclusive of a 10 minute nisin incubation. ■ Viable count excluding nisin incubation ◆Viable count after nisin incubation.

After incubation the samples were counted (Figure 4.10). The plot ■ represents viable counts of *L. monocytogenes* that had not been subject to nisin incubation. The plot ◆ represents viable counts from samples of *L. monocytogenes* culture that had been subjected to a 10 minute nisin incubation. Each point represents the count obtained from a single culture aliquot that had been centrifuged, population adjusted, and incubated with (◆) or without nisin (■). The cell count totals presented in Figure 4.10 were adjusted to take account of dilution factor used during flow cytometry experiments. Figure 4.10 clearly shows that a nisin concentration

of 8 ng/mL^{-1} has an effect on the viability count of time separated aliquots of *L. monocytogenes* batch culture. The results also clearly show that as the culture approached stationary phase the number of viable cells increased. A maximum 2-log difference was observed at exponential growth between control and experimental samples, whilst at stationary phase a maximum 1-log difference was observed between control and nisin exposed samples.

4.2.5 The effect of nisin on aliquots of a *L. monocytogenes* culture at exponential phase and stationary phase using TEM

L. monocytogenes PTM medium batch culture was sampled and subjected to nisin incubation in accordance with Scheme 2.0. The samples were fixed in glutaraldehyde and prepared for imaging using TEM (Sections 2.11 – 2.11.2).

4.2.4.1 TEM images of L. monocytogenes inclusive and exclusive of a nisin incubation

The protocols adopted for the growth and nisin incubation of *L monocytogenes* culture were the same as those adopted for the flow cytometry experiments detailed in Scheme 2.0. TEM was used to examine the membrane of *L. monocytogenes*. Images of membranes were taken from cell samples extracted from sample culture before and after nisin exposure at exponential phase. The membrane images were created when a resin embedded sample of *L. monocytogenes* was thinly sliced. Within the sliced samples, the cells occurred at different orientations and those cells cut at acute angles did not present clear membrane images. Only cells that presented clear membrane images were examined in detail.

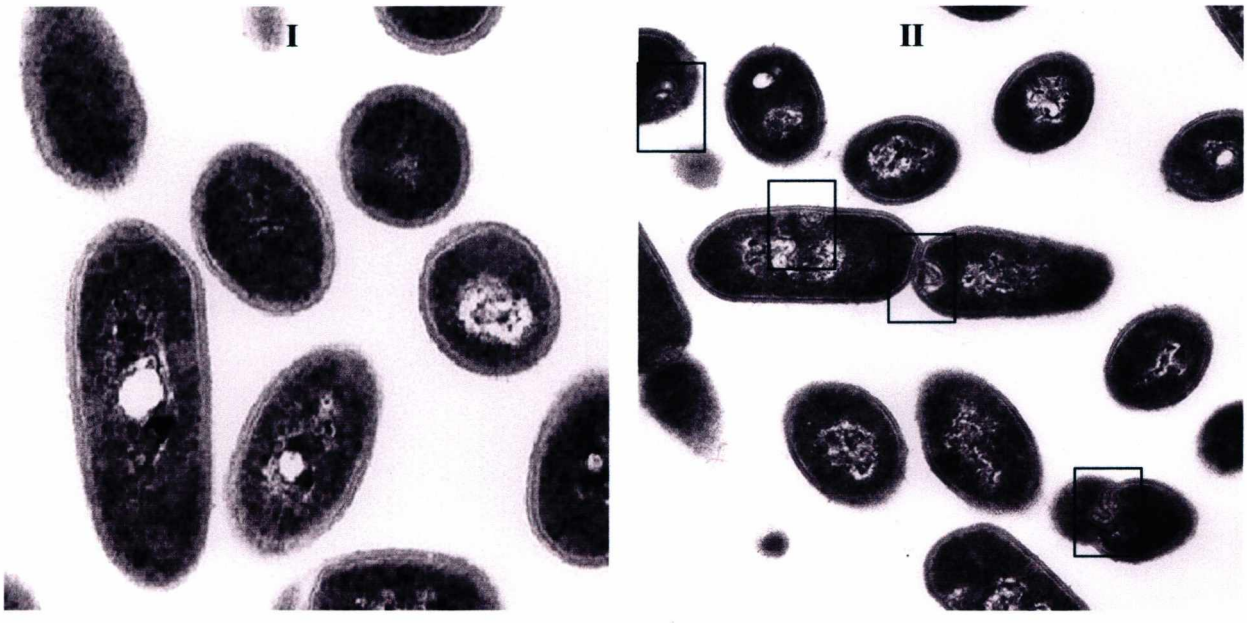


Figure 4.11 TEM images of resin embedded *L monocytogenes* cells at exponential phase (magnification X48000)

Figure 4.11 clearly shows that nisin has an effect on the integrity of *Listeria* membranes. In Image I, a culture that had not been subjected to nisin incubation, the cell membranes are intact. Image II shows cells sourced from a *L. monocytogenes* at OD_{600} 0.38 subjected to nisin incubation. The boxed areas of image II show where the cell membrane has been disrupted. A clear difference is shown between nisin incubated and nisin free *Listeria* samples. The observed difference between nisin incubated and non-nisin incubated cells was used to separate membrane intact cells from membrane disrupted cells for further samples.

4.2.5.2 Nisin permeabilised L. monocytogenes cells at exponential phase and stationary phase

L. monocytogenes PTM medium batch culture was sampled at 7.15 hours (OD_{600} 0.370) and 13.5 hours (OD_{600} 1.328) after inoculation. Each sample was subjected to 10 minute nisin incubation prior to concentrating and fixing for TEM analysis. The resultant images were used to assess the percentage of total cells imaged that showed nisin permeabilised membrane. The results from several images were collated and are presented in Figure 4.12

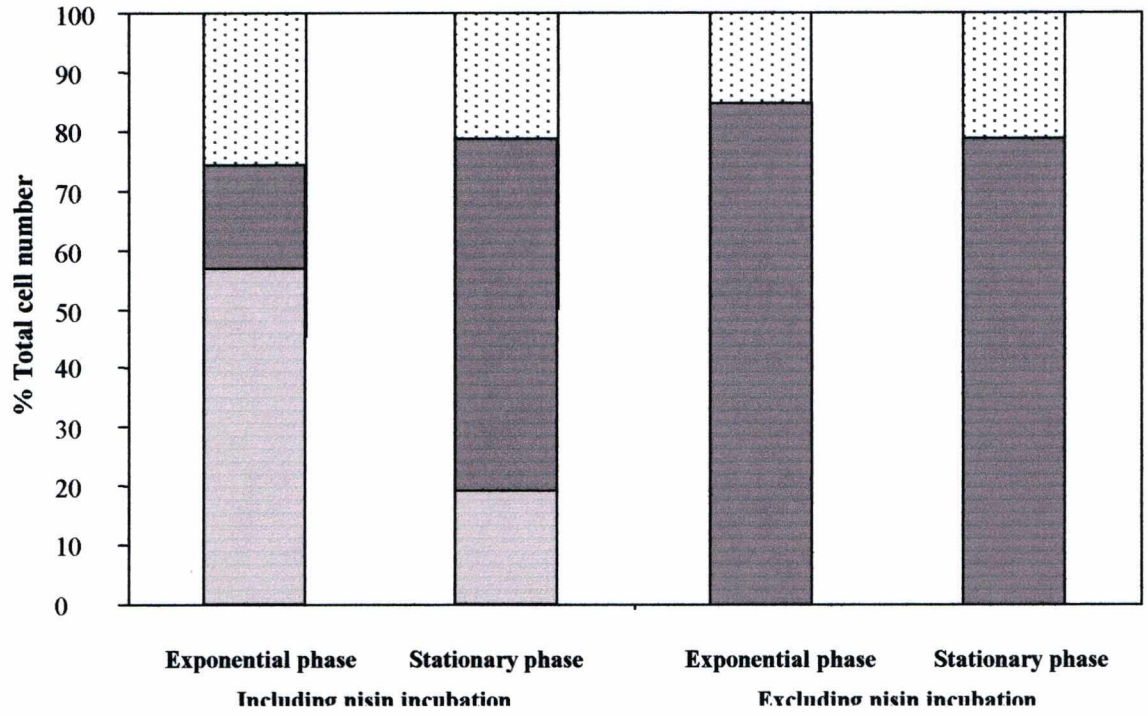


Figure 4.12 A comparison of nisin exposed to non-nisin exposed *Listeria* cells at exponential and stationary phase. Key: ■ %Total number of cells visualised that had clearly defined membrane structure ■ %Total number of cell visualised that had disrupted membrane. ⊡ %Total number of cells that did not have a clearly defined membrane

Figure 4.12 shows the proportions of imaged cells from *Listeria* cultures at exponential phase and stationary phase that showed evidence of cell membrane disruption after exposure to

nisin. The sample size was limited to approximately 50 cells or parts thereof at each sample point this being the number of cells that could be resolved in a single field of view at a magnification that allowed clear differentiation of membrane features. Any partial cells that did not show clear membrane definition were excluded, although they did contribute to the percentage total as cells that did not have a clearly defined membrane (Figure 4.12) The corresponding control images that were analysed have shown that no cells had membrane damage. Between 75% and 85% of all of the cells imaged had clearly defined membranes. Within the limited sample areas examined at exponential phase 80% of the clearly imaged cells showed membrane disruption. Within the limited sample areas examined at stationary phase 21% of the clearly imaged cells showed membrane disruption. The images used to provide the information presented in Figure 4.12 did not represent a sufficient sample size to allow a statistical analysis of result. The images did confirm that nisin permeabilised *L. monocytogenes* membranes, when present in cultures that had been exposed to 10 minute nisin incubation at a concentration of 8 ng/mL^{-1}, and a cellular concentration at 1×10^6 $cells/mL^{-1}$. The TEM image data confirmed the findings of earlier flow cytometry (Figure 4.8) and viable count experiments (Figure 4.10), all results showing the action of nisin to be growth phase dependent.

4.3 DISCUSSION

4.3.1 Analysis of the heterogeneity of a *L. monocytogenes* culture using flow cytometry

Results discussed in Chapter 3 used basic microbiological methods to define the growth states of a *L. monocytogenes* culture throughout growth from inoculation to stationary phase. However in a non-synchronously dividing culture, a specific change of physiological state from exponential growth to stationary phase cannot be determined by mass population observations, as has been demonstrated by the results achieved in Chapter 3. It is therefore necessary to make physiological measurements on individual cells. Flow cytometry is a technique that allows the analysis of cells rapidly and individually and permits the quantitative analysis of microbial heterogeneity. It offers many advantages over conventional measurements for both routine and more exploratory analyses of microbial properties. The measurement of microorganisms at the single cell level has therefore progressed greatly over the last few decades, the first papers on the use of flow cytometry to measure bacteria appeared in 1977 [280].

The use of flow cytometry in microbiology now allows rapid characterisation of cells from non-synchronously dividing cultures [294]. A number of methods based on flow cytometry have been devised to assess the effects of lethal agents and bacterial survival in starved cultures through the use of membrane potential, sensitive dyes, and nucleic acid markers [292, 295-297]. Flow cytometry provides a sensitive means of monitoring the dynamic cellular events that occur in bacteria exposed to antibacterial agents [298]. The results presented in Sections 4.2.1 and 4.2.2 were based on the premise that the cytoplasmic membrane of a cell is important in determining which molecules enter and leave the cytoplasm. A number of fluorescent dyes have been used to act as viability probes because they can detect changes in the physiology or metabolism of bacterial cells. A change in the ability of a cell membrane to control the flow of molecules in and out of the cell can compromise the cell and its ability to survive. A number of dyes rely on the fact that they are fluorescent only when bound to nucleic acids. Intact cells exclude such cell impermeant fluorescent dyes and fluorescence is not detected, however dead or damaged cells that can no longer exclude the dye become fluorescent. PI has frequently been used as an indicator of membrane integrity and has been used here to determine the point at which viability is compromised by an antimicrobial peptide, nisin. More specifically the influx of dye has been used to determine the point at which *L. monocytogenes* batch culture stops exponential

growth. The ability of the flow cytometer to examine single cells has also been used to highlight the presence/absence of sub-cultures within an exponentially growing batch culture that initiated an uncharacteristically early entry into stationary phase, alternatively highlighting subcultures that continue growth during stationary phase. The results presented in Chapter 2 confirm that the transition from exponential growth to stationary phase is a complex transition that appears to have a number of ill-defined phases between the two physiological states.

4.3.2 The *Listeria* membrane as a target for nisin binding

The lantibiotic nisin has been shown to have antimicrobial activity against a broad range of Gram-positive bacteria including *L. monocytogenes* [110, 299-301]. The presence of dehydrated residues and lanthionine rings (thioether bonds) in the structure of nisin impose restraints on the peptide [302, 303]. This and the relatively high activity (nM range) of nisin against Gram-positive bacteria indicated that the mode of action of nisin might be different to other peptide antibiotics in the large family of pore-forming peptides. Experiments conducted into the action of nisin on model membrane systems, composed of only phospholipids have shown that nisin behaves similar to the antimicrobial peptide magainin [304-306], although at an activity that is much lower than to its activity towards biological membranes. This difference can be linked to a missing factor which nisin needs for its high activity. Recent work has identified the factor as Lipid II, a membrane bound peptidoglycan precursor of bacterial cell membrane synthesis. The special high affinity interaction of nisin with Lipid II results in high activity and Lipid II plays an active role in the pore-formation process [285]. It has been further demonstrated *in vitro* and *in vivo* that lipid II serves as a docking molecule for nisin facilitating the formation of pores in the cytoplasmic membrane [286]. Nisin combines high affinity for Lipid II with its pore-forming ability making the peptide highly active (in the nanomolar range) [285]. Nisin has also been found to inhibit murein synthesis with concomitant accumulation of undecaprenyl-pyrophospho-MurHAc (pentapeptide) (lipid intermediate I). This inhibition is caused by the formation of a complex between the antibiotic and lipid I intermediate. Undecaprenyl-pyrophospho-MurNAc-GlcNAc (lipid intermediate II) also forms a complex with nisin. However, when murein synthesis is inhibited by nisin, this latter complex is not formed since lipid intermediate II is no longer synthesised [286, 307].

More recent data supports a model whereby lipid II-linked polypeptides are incorporated into the growing peptidoglycan via the transpeptidation and transglycosylation reactions of cell membrane synthesis, generating mature cell membrane-linked surface protein

[308]. During growth the replacement of membrane constituents is an ongoing process. As cells enlarge in preparation for the division process, new cell membrane synthesis must take place. During the process of membrane synthesis the peptidoglycan precursors lipid I and II would be abundant, however at stationary phase where cell division is not taking place the peptidoglycan lipid I and lipid II precursors would be less abundant. The disruption of the cell membrane would be less pronounced as has been shown here in Figures 4.10, 4.12 and 4.13. If it were the case that nisin only had an effect when lipid II were present no action would be detected in *L. monocytogenes* cultures at stationary phase where membrane synthesis is reduced. In contrast to the results reported here Budde and Jakobsen, have reported that the differences in nisin sensitivity between single cells in a *L. monocytogenes* population were insignificant for cells grown to the stationary phase in a liquid laboratory substrate [309].

The permeabilisation of *Listeria* membranes by nisin is concentration dependent and a number of investigations have determined the minimal inhibitory concentration (MIC) of nisin to be 2.5μg/mL [310, 311]. To determine the point at which nisin incubation would differentiate between exponential phase and stationary phase cells, within the same culture, a series of different nisin concentrations at 2.5 μg/mL and below were investigated. It is proposed that the changing morphology of the *Listeria* membrane would allow a change in the rate of membrane permeabilisation (by nisin) to be measured. The rate of membrane permeabilisation could be measured by monitoring a change in the rate of uptake of the cell impermeant dye PI. In this investigation, it was found that an 8 ng/mL concentration of nisin was able to discriminate between a stationary phase and exponential phase culture by observations of the rate of uptake of PI, as measured by flow cytometry (Figure 4.0). Based on these findings exponential phase and stationary phase cultures of *L. monocytogenes* were exposed to nisin at a concentration of 8 ng/mL and the flow cytometer was used to examine the rate of dye uptake (PI) over a 15 minute period. During the process of preparation of *L. monocytogenes* samples, the cells were counted by flow cytometry. The samples were then centrifuged and the supernatant discarded. The volume of the sample was adjusted (with spent medium matching the OD_{600} and pH of the original sample) to achieve a cellular concentration at 1 x 10^6 cells/mL. This maintained the cell/nisin ratio and removed the possibility of concentration effects, as it is known that the permeabilisation of target membranes by nisin is concentration dependent [312-315]. Each growth phase demonstrated a different response to nisin. At the end of the exposure period 59.9% of the exponential phase cells were PI positive (Figure 4.0 Graph A). At the end of the exposure period of the stationary phase culture 0.2% of the cells were PI positive (Figure 4.0 Graph B). Aliquots of each sample were then

combined and exposed to the same nisin concentration. At the end of the 15 minute exposure period 23.2% of the cells were PI positive. There was a clear differentiation between PI positive and PI negative cells (Figure 4.0 Graph C). The result clearly demonstrated that the action of nisin was affected by the growth phase at which it was applied. The results also clearly demonstrated that nisin could discriminate between stationary phase and exponential phase cells in a heterogeneous culture by monitoring the rate at which the cell impermeant dye PI was bound to cytoplasmic nucleic acids. This result did not confirm that lipid precursors were the specific target for nisin attachment at the membrane, only that cells actively replacing membrane are more susceptible to its action than cells that are not actively replacing membrane. It has been reported that the majority of the effect exerted by nisin on the membrane of *L. monocytogenes* is response time dependent [316], and that the maximum effect occurs within 7 minutes [317] to 10 minutes [318] of exposure. The period of nisin incubation adopted herein was changed from 15 minutes to 10 minutes.

A number of investigations conducted into the molecular mechanisms of nisin activity in the absence of lipid II [283, 304, 319] show that nisin can permeabilise membranes via two different mechanisms. At high concentration (μM range) pores can be formed without lipid II. Activity in the absence of lipid II has been shown to be primarily dependent on the presence of negatively charged membrane phospholipids, with the C terminus of nisin being important at the initial binding stage [304] and subsequent antimicrobial activity [283]. Pores formed under such conditions are anion selective and a membrane potential aids pore formation. Where lipid I and lipid II are available during membrane synthesis, nisin at nM concentration has been shown to be more effective at disrupting the barrier function of a *Listeria* membrane as demonstrated herein by Image II (Figure 4.11). In recent experiments using model membrane systems, nisin has been shown to perturb the hydrophobic region of both charged and neutral bilayers [320].

4.3.3 The effect of pH and fresh medium on nisin permeabilisation of the *Listeria* membrane

It has been shown that nisin kills cells by interfering with basic energy transduction occurring at the membrane [321, 322]. The action of nisin on the basal proton motive force (PMF) in *L. monocytogenes* has been demonstrated to be both time and concentration dependent. A nisin concentration at 2.5 μg/mL was shown to completely dissipate the PMF of several *Listeria* strains [323]. It has been further suggested that in addition to lipid I and lipid II precursors, nisin is thought to form pores in the target membrane by a mechanism that requires a

transmembrane electrical potential ($\Delta\Psi$) and its action involves local perturbation of the lipid bilayer structure. Moll *et al.* have shown that nisin does not exclusively form voltage-dependent pores: even in the absence of a $\Delta\Psi$, nisin was able to dissipate the transmembrane pH gradient. It has also been observed that the rate of dissipation of the transmembrane pH gradient increases with the magnitude of the ΔpH. However nisin will only form pores when the cell internal pH is more basic than the external (medium) pH. It was also reported that the external pH strongly affected the efficiency of $\Delta\Psi$induced pore formation, whereas ΔpH induced pore formation was insensitive to the external pH [324]. A number of investigations have confirmed that a falling external pH (pH 7.8 to pH 5.5) has been shown to increase nisin effectiveness against both *L. monocytogenes* and a number of other sensitive organisms [315, 325-327]. These are important observations when considering the use of nisin as a determinate of membrane physiology throughout growth.

It has been shown herein that during growth the pH of a *L. monocytogenes* PTM batch culture, drops from pH 6.9 to pH 5.5 over a 48 hour period (Section 3.2.4, Figure 3.5). In order to determine the effect that falling pH may have on the efficacy of nisin, a number of control experiments were conducted using pH adjusted medium. Using aliquots of a stationary phase culture samples were re-suspended in spent PTM medium at pH 5.5 and pH 6.9. Nisin additions were made to the samples and the uptake of PI monitored for 10 minutes. A change of pH did not increase the efficacy of nisin under these conditions (Scheme 2.0), no decrease in the rate of PI uptake was observed at pH 6.9 (Figure 4.3 Graph I). It was concluded that although the action of nisin could be synergistically increased by low pH the membrane physiology at stationary phase was such that it (the membrane) could nullify the increased efficacy of the peptide. This was not a permanent effect and it was observed that increased incubation times allowed nisin to produce an effect on stationary phase cells at low pH in spent medium. A culture aliquot at stationary phase (OD_{600} 1.180) was monitored using flow cytometry for 20 minutes after a nisin addition. At 10 minutes 14% of the cells were PI positive (Figure 4.7 Graph C: VIII), whilst at 20 minutes 31.5% of the cells had become PI positive (Figure 4.9 Graph C: X). In contrast a culture aliquot at exponential phase (OD_{600} 0.280) had 80% of the cells showing PI positive after 10 minutes (Figure. 4.4 Graph C:II). The results clearly demonstrated that the permeabilisation of *L. monocytogenes* membranes by nisin remained unaffected by changing pH. A further control was conducted to observe the effect that fresh medium at different pH would have on the rate of PI uptake during growth and at stationary phase. Samples of *L. monocytogenes* at stationary phase (OD_{600} 1,309) were re-suspended in fresh medium at pH 5.5 and pH 6.9. In each case fresh medium increased the

rate of PI uptake of the samples when compared to the rate observed at stationary phase (figure 4.3 Graph II).

Centrifuged cells were re-suspended in spent medium that matched the pH and OD_{600} of the original sample. This ensured that the medium conditions prevalent at the time the sample was taken were maintained. This was considered necessary, as lactic acid bacteria (LAB) are known to use specific molecules that act as signals within a population for the induction of genes. Although the induction of a gene will only occur when a certain threshold concentration of these molecules in the environment has been reached. The expression quorum sensing describes the use of these signal peptides as gene inducers. One of the best-characterised cases of quorum sensing in LAB is the auto regulation of nisin synthesis [328, 329]. Quorum sensing, the control of gene expression in response to cell density, is used by both Gram-positive and Gram-negative bacteria to regulate a variety of physiological functions [293]. Gene expression instigation in response to perception of cell density is an example of multi-cellular behaviour where a single cell is able to communicate and sense when a minimal population unit is achieved and certain phenotypes of the population can be efficiently expressed at the right time [330, 331] to ensure survival during extended periods of growth arrest. Quorum sensing provides a method of cell-to-cell communication and can be regarded as a form of multi-cellularity in these organisms. Gram-positive bacteria exclusively use peptides or post-translationally modified peptides as inducer molecules. The production and accumulation of these signal peptides in the environment (batch culture) makes the bacteria responsive to cell density. When adjusting the cellular concentration of samples taken throughout growth (to 1 x 10^6 cell/mL), spent medium was used as a diluent. The use of spent medium (matched to the original sample point OD and pH) was assumed to maintain the medium concentration of signal peptides (if present) to pre dilution levels. Quorum sensing is also a function of cell number and the ratio of signal molecule to cell was increased by dilution in spent medium. Although the cell numbers had been changed, the general accumulated concentration of signal peptides remained constant. A sudden dramatic change in the concentration of signal peptides/cell ratio could have an effect on the pattern of gene expression in a batch culture. To investigate the effect that a fresh medium pH change may have on the action of nisin fresh medium at pH 5.5 and pH 6.9 was used as a diluent. The medium at pH 5.5 (the pH of original sample point medium) produced a lower PI uptake rate than the use of fresh medium at pH 6.9, although the increase in the rate of PI uptake recorded was over and above the rate recorded for the same sample re-suspended in spent medium (Figure 4.3 Graphs I and II).

In the absence of a specifically defined inducer of stationary phase the change from exponential growth to stationary phase can be considered a general stress response that may in part be due to a quorum sensed population perception. When the perception of population signal was removed by a change of medium, the stationary phase cells responded by instigating active growth. In the presence of nisin this caused an increase in the rate of membrane permeabilisation, as observed in Figure 4.3 Graph II. This suggests that a change of pH and signal peptide concentration affect the rate of nisin induced PI uptake in re-suspended stationary phase cells. It can also be suggested that a low medium pH may play a role in the maintenance of stationary phase. Quorum sensing is a relatively new discovery and the extent to which signal peptides control gene expression is not well defined in *L. monocytogenes*. The change in medium nutrient concentration between spent and fresh medium could also be a contributory factor to the changing rate of PI uptake of nisin incubated stationary phase *L. monocytogenes* cells. An in depth explanation of the causes of changing PI uptake induced by a medium change is less of an issue here, than the fact that re-suspension of the cells in spent medium maintained the culture *status quo*. The control experiments conducted here allowed the monitoring of the effect of nisin on a *L. monocytogenes* membrane throughout growth while excluding pH effects, medium effects and concentration effects. Samples were examined throughout growth and the results presented herein show that as exponential phase growth continues, sensitivity to nisin permeabilisation increased to a maximum where 87% of the cells in the nisin incubated sample were PI positive. Beyond that point and towards stationary phase the number of cells that were PI positive after 10 minutes nisin incubation decreased to a minimum point at 1.309 OD_{600} where 3.8% of the cells in culture were PI positive.

4.3.4 The effect of nisin on aliquots of a *L. monocytogenes* culture at exponential phase and stationary phase imaged using TEM

The results presented in Section 4.2.4 show that the rate of PI uptake changed as growth progressed. It is proposed that the uptake of PI is directly linked to the permeabilisation of the *Listeria* membrane by nisin. To confirm that membrane permeabilisation had occurred in samples that had been nisin incubated, treated samples were fixed and prepared for TEM. The conditions adopted to produce *L. monocytogenes* cultures for examination of the bacterial membrane by TEM were the same as those adopted for flow cytometry experiments. Images of *L. monocytogenes* batch culture exposed to nisin at exponential phase and stationary phase show disrupted membranes (Figures 4.13 and 4.14). Images of *L. monocytogenes* batch

culture that were not exposed to nisin at exponential phase and stationary phase show intact membranes (Figure 4.15). However it should be noted that some debate as to the validity of assigning the appearance of membrane invaginations (in bacterial samples) to external actionas opposed to preparative artefacts, does exist. Membrane invaginations called mesosomes, have been detected in bacterial samples prepared for electron microscopy as early as 1953. Biochemists and bacteriologists debated their function for over a decade and such invaginations have been variously ascribed respiratory or photosynthetic functions [332]. Microscopists now generally construe them as artefacts of the sample preparation process , not genuine, or "real," structures in bacteria. However Silva *et al.* showed that the use of osmium oxide (OsO) was correlated temporally with progressive mesosome formation (in both number and size). They also considered other chemicals known to damage membranes. Those that damaged membranes (not just OsO) generated mesosomes; those that did not, generally did not (excluding other physical factors that might also damage membranes)[333, 334]. It has been concluded that mesosomes are "real," but they are produced only when the bacterial membrane is damaged in preparing cells for electron microscopy [335]. Here it is clear that mesosome structures appear in samples that had been the subject of a nisin incubation, while they do not appear in samples that had not been the subject of a nisin incubation. Nisin is known to cause damage (permeabilisation) to the membranes of *L. monocytogenes*. It is therefore suggested that the membrane invaginations observed here are the result of membrane damage brought about by nisin addition to the culture medium.

When exposed to nisin, more cells were permeabilised in 10 minutes at exponential phase than were permeabilised in 10 minutes at stationary phase. The results confirm that the membranes of a *L. monocytogenes* population (1 X 10^6 cells/mL) exposed to a 9 minute nisin incubation (at a concentration of 8 ng/mL) were permeabilised. Additionally the degree of permeabilisation observed was time and growth phase dependent (Sections 4.2.2 and 4.2.4).

4.3.5 Viable counts of *L. monocytogenes* batch culture exposed to nisin

Viable counts were conducted on *L. monocytogenes* batch culture samples that had been prepared in accordance with Scheme 2.0. The sample points matched those of samples that had been the subject of flow cytometry experiments. The samples were incubated with nisin for 9 minutes prior to a viable cell count. There was a 2-log reduction in cell numbers between nisin treated and nisin negative samples to a point at OD_{600} 0.695. Beyond that point the nisin treated sample results began to converge on the control sample result to a point where a 1-log difference was observed at OD_{600} 0.9 (and maintained to the end of data collection at OD_{600}

1.309). This result shows that nisin at 8 ng/mL can have a negative effect on the growth of an aliquot of *L. monocytogenes* culture at 1 X 10^6 cells/mL. The result also shows that at an OD_{600} between 0.695 – 0.850 the effect of nisin is less pronounced. Previous results on the growth characteristics of *L. monocytogenes* in a PTM medium (Section 3.2.2.1) have shown that at this point the culture is still actively growing.

It is proposed that the flow cytometry data (Section 4.2.2) and previously presented growth data results (Table 3.3) support the proposal that a distinct change in the physiology of the cell occurs before the onset of stationary phase during exponential growth, and that such a change is demonstrated by the change changing ability of nisin to permeabilise the organism membrane.

4.3.6 Selective membrane permeabilisation as a determinate of growth phase

The results presented herein show that the action of nisin on the membrane of *L. monocytogenes* in batch culture can be used to differentiate between cells at exponential phase and stationary phase (Section 4.2.4). The method as presented does not differentiate sub-cultures within a batch culture at transition to stationary phase. It is clear that in batch culture the age of the culture had an effect on the efficacy of nisin and that those changes were independent of medium pH, however where fresh medium was introduced nisin action did become pH dependent (Section 4.2.1.1). It is well established that nisin action is dependent on a number of physical factors including medium pH [336], a pH gradient (where the cells internal pH remains pH basic with respect to the external pH) [324], and trans-membrane electrical potential [337]. These factors should combine to increase the efficacy of nisin, as the medium pH drops and stationary phase approaches. Initially during early exponential growth this was observed (Figures 4.8 and 4.9) however as stationary phase approached and medium pH fell towards a low of pH 5.5 (Figure 3.5) the cells became more resistant to the action of nisin with less cells becoming PI positive (Figures 4.8 and 4.9). The internal pH of the cells was not determined but where cells were intact (not PI positive) and still growing it could be assumed that the cells were maintaining internal pH at near to neutrality and that a proton motive force and trans-membrane electrical potential were present as has been previously demonstrated by Bruno *et al.* [310]. The results support the proposal that the cell is becoming more resistant to the action of nisin due to some change of membrane physiology. Membrane precursor constituents lipids I and II, present during membrane growth, have been identified as necessary docking molecules that enhances the action of nisin particularly at nanogram concentrations [281, 285, 286, 308]. Where the rate of cell growth is slowing (therefore the

membrane concentration of lipid I and II is reduced) as a culture approaches stationary phase it would be expected (and was observed) that the action of nisin at ng concentrations, would be reduced. What is less clear is why a fall in the efficacy of nisin is observed early in the life of the culture when exponential growth is occurring (OD_{600} 0.380). If all the cells in a culture were demonstrating greater resistance to nisin the level of PI incorporation would be near to zero, this was not the case. Because flow cytometry can measure individual cells it is further proposed that some of the cells in batch culture at OD_{600} of 0.380 and above, show greater resistance to nisin than others in the same culture, and that as stationary phase approaches the proportion of cells showing greater resistance to nisin increased.

The introduction of fresh PTM into a *L. monocytogenes* batch culture, as it approached stationary phase, modified the observed action of nisin on the bacterial membrane. The efficacy of nisin was more pronounced during the early stages of exponential growth and the viable count results suggest that the *Listeria* membrane becomes less susceptible to permeabilisation well before the induction of stationary phase. It has been previously shown that medium pH and nutrient availability at this point are not limiting to growth (Sections 3.2.4.1, 3.2.5.1 and 3.2.5.2). Aliquots of the culture were individually exposed to nisin throughout growth and the exposure time limited the appearance of nisin resistant cells, which in any event usually require a single previous exposure to nisin or another factor (e.g. low pH) before specific resistance is observed [201, 317]. It has been shown that pH resistant strains of *Listeria* are more resistant to the action of nisin. The experimental method cannot determine whether or not at stationary phase, the cells increased resistance to nisin is a consequence of increased acid tolerance, although pH changes to spent medium suggested otherwise. Fresh medium at high pH induced a greater degree of membrane permeabilisation than fresh medium at low pH in batch culture, which suggests that low pH has a role in the maintenance of stationary phase. Perception of environment may also be a factor contributing to the efficacy of nisin and nutrient limitation may also induce increased nisin resistance.

The results reinforce the complexity of the bacterial response to specific stresses and general limiting conditions. The results also suggest that entry into stationary phase is a general stress response that confers protection against a number of factors. It is also clear that survival mechanisms with respect to a nisin challenge are induced earlier rather than later in the life of a culture (or are already present) when exponential growth is still apparent. The results achieved at 5 minutes and 9 minutes post nisin addition are similar suggesting that the observed changes are not due to specific adaptation to the challenge (Figures 4.8 and 4.9). At 5 minute nisin exposure, a *Listeria* culture could not respond rapidly enough to generate

resistant mutants when the culture t_d in PTM medium has been shown to be 98 minutes. This suggests that gene products already present are responsible for the observed resistance to nisin action early in the life of a culture. To investigate the hypothesis that bacteria carry some intrinsic protection against stress, in addition to induced stress specific responses and to examine the gene products (proteins) present at different phases of growth, a proteomic platform has been adopted and is discussed in Chapter 5.

5.0 Development of a proteomic platform for the analysis of the *Listeria* proteome

5.1 Introduction

Proteomic analysis has been defined as 'the use of quantitative protein-level measurements of gene expression to characterise biological processes and decipher the mechanisms of gene expression and control' [338]. Examining the proteome of an organism allows a closer study of the processes of a living cell [339] as changes in gene expression and post-translational changes to expressed proteins can be monitored directly by 2-D electrophoresis. The core technology of most proteomic projects since its inception in 1975 [170] has been two dimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis (2-D SDS-PAGE). The 2-D SDS-PAGE system separates complex biological protein extracts on the basis of charge in the first dimension (using IEF), followed by size in the second dimension (using SDS-PAGE) [340]. It would be desirable to move away from 2-D SDS-PAGE as a core technology due to the inherent difficulties of maintaining reproducibility and achieving complete proteome coverage, that is to say production of a gel image that represents every protein in a biological sample at the time of sampling [341]. In any organism the genome is a method of information storage and is a fixed feature. The proteome however is not fixed and is in a state of dynamic flux [163]. For example the proteome can change in response to an alteration in the external environment or may change during the development of the organism (growth phase). The proteins themselves may be varied by cellular/sub-cellular location, concentration, solubility, pI, molecular weight and degree/type of post-translational modification. There can also be temporal separation of certain proteins and variability in half-life. These variations are the factors that make mass protein separation a possibility but they also limit the scope of maximum coverage that can be achieved using current technologies.

Despite the inherent difficulties of 2-D SDS-PAGE, it remains the core technology of choice for proteomic investigations [342]. 2-D SDS-PAGE is still the only commonly accessible method available that is able to simultaneously separate and visualise a complex mixture of (up to) several thousand proteins from a single sample [343]. The key to the use of 2-D SDS-PAGE as a valid investigative tool within the sphere of proteomic platform is reproducibility. In theory it should be possible to produce a 2-D gel with a spot pattern representing every protein present in the organism at the time of sampling while maintaining the protein-protein ratio, molecular weight, and post-translational modifications [163].

However in order to achieve reproducibility some compromises must be accepted which inevitably reduce the ability of the technique to visualise every protein present in a complex biological mix. In particular, low abundance proteins can often have a pivotal regulatory role but may be difficult to detect or absent altogether due to the current detection limits of protein stain technology. Furthermore, highly basic proteins are difficult to separate using 2-D SDS-PAGE [344].

Although 2-D analysis can be applied to any protein mixture the route to successful analysis will differ depending on the source of that sample. Fractional separation of eukaryotic samples allows study of proteome maps of individual cellular structures [345, 346]. However this approach is not possible with prokaryotic cells, which do not have sub-cellular compartmentalisation. When a prokaryotic cell is lysed, all of the proteins soluble in the buffer system of choice should be present in the sample extract. Fractionation of such a sample beyond cytosolic and membrane fractions may introduce experimental weighting, whereby some protein-protein ratios are disturbed giving a false representation of the situation in existence in the original sample. Samples from the same organism subjected to different conditions can then be compared to identify differences in the protein complement of each condition.

Just as significant advances have been made in improving 2-D SDS-PAGE reproducibility, there has been simultaneous improvement in computerised image analysis [152]. Spot detection, background subtraction, spot matching (between different images) and database construction have all improved. The analysis of complex 2-D images is largely dependant on computer analysis and data processing. The up regulation, down regulation, appearance and disappearance of hundreds of spots per image may be detected. Such complex changes must be analysed quantitatively rather than qualitatively, for example during the creation of a composite image from three repeat images of a single sample a spot which appears in all the sample repeats can be given far greater significance than a spot that appears in only one sample repeat images.

The results presented in Chapters 2 and 4 highlight areas where traditional microbiological methods fall short in their ability to define the specific processes that lead to stationary phase and growth arrest in *L. monocytogenes* batch culture. As such, a proteomic platform has been developed and optimised to investigate the protein flux in a *L. monocytogenes* culture at the transition to stationary phase.

5.2 Results

5.2.1 Different methods of cell lysis

In order to extract proteins from *L. monocytogenes*, the cells must be lysed. Three methods of cell lysis were investigated to determine which would give the greatest protein yield; freeze/thaw cycling, use of a French press, and sonication (Section 2.4.4.3, 2.4.4.4 and 2.4.4.5). As cell lysis releases protease enzymes, DNA and RNA into the sample mixture, it was necessary to use a protease inhibitor cocktail (Section 2.4.3) to stop the unwanted proteolysis of extracted proteins by inherent protease enzymes. DNA and RNA in the samples were removed by conducting a 20 minute DNAse/RNAse incubation at room temperature on completion of cell lysis (Section 2.4.5). All samples were sourced from 500 mL BHI batch culture and the samples were centrifuge washed (Section 2.4.2) and re-suspended in 10 mL of lysis buffer 2 (Section 2.4.4.2). For each lysis method a lysed sample and non-lysed control were compared under oil immersion light microscopy (Section 2.4.4.6) providing an approximate measure of the efficiency of each method.

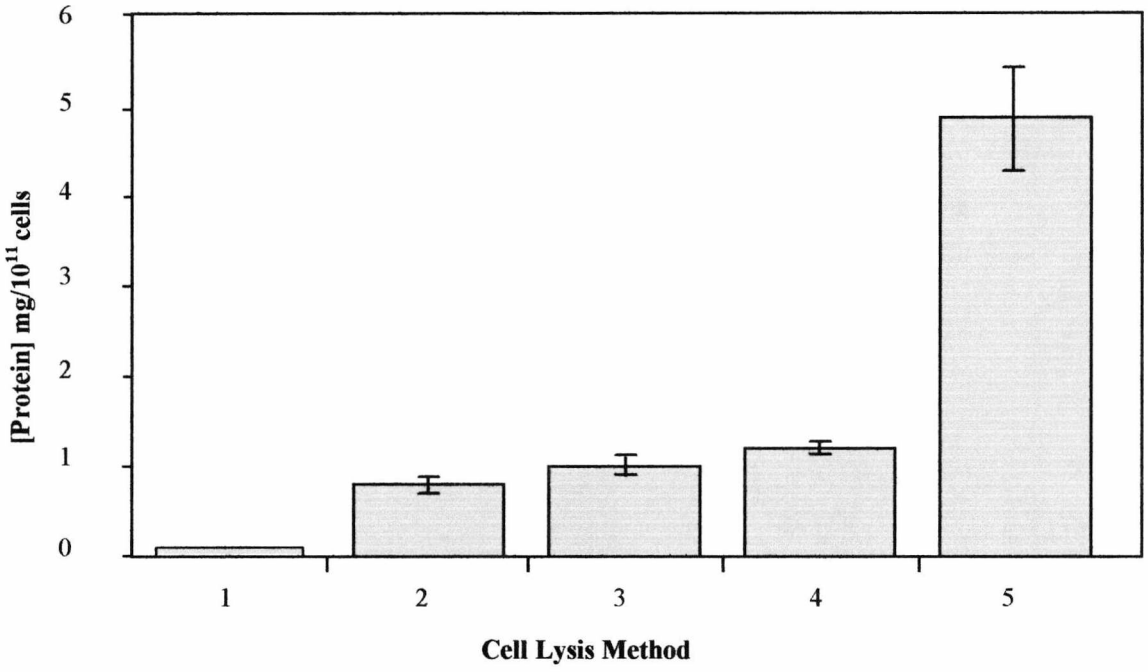


Figure 5.0 The protein yield of early exponential phase (OD_{600} 0.25) *L. monocytogenes* cells subjected to different cell lysis methodologies. 1. Five repeated freeze/thaw cycles (degree of error 11.5%) 2. Two passages French press. 3. Four passages French press. 4. Six passages French press 5. Single 10 minute sonication step.

Figure 5.0 represents the protein yield (determined by modified Bradford assay Section 2.4.4.1) from identical *Listeria* samples. The results clearly show that the protein yield achieved with sonication was greater than that achieved with other cell lysis methods. The use

of sonication achieved a four-fold increase in protein yield above that using 6 passages of the French press. A comparison of freeze/thaw treated cells to untreated cells viewed using oil immersion light microscopy showed that only 10% of the treated cells had been lysed after 5 cycles. Use of the French press gave a 10 fold increase in protein yield compared to freeze-thaw cycling. A comparison of French press lysed cells to untreated cells viewed using oil immersion light microscopy showed that more than half of the treated cells remained intact. A comparison of cells subjected to sonication against an untreated control (viewed using oil immersion light microscopy) showed that less than 15% of the treated cells remained intact.

5.2.1.1 Optimising cell lysis using a cyclic sonication protocol

During a single sustained sonication extraction the sample temperature increased despite being immersed in an ice bath during sonication. To reduce heat build up in the samples during sonication a cyclic protocol was devised allowing a rest period (on ice) between timed sonication steps. The samples were allowed 5 minutes to cool on ice between each 5 minute sonication step. Samples were extracted from a *L. monocytogenes* BHI batch culture at late exponential phase (OD_{600} 0.25) and stationary phase ($O.D_{600}$ 1.4). Samples were washed prior to re-suspension in lysis buffer of sufficient volume to maintain cell counts at 10^9 cells/mL^{-1}. Any increases in protein yield could therefore be attributed to the change in sonication procedures rather than changes in sample cell numbers.

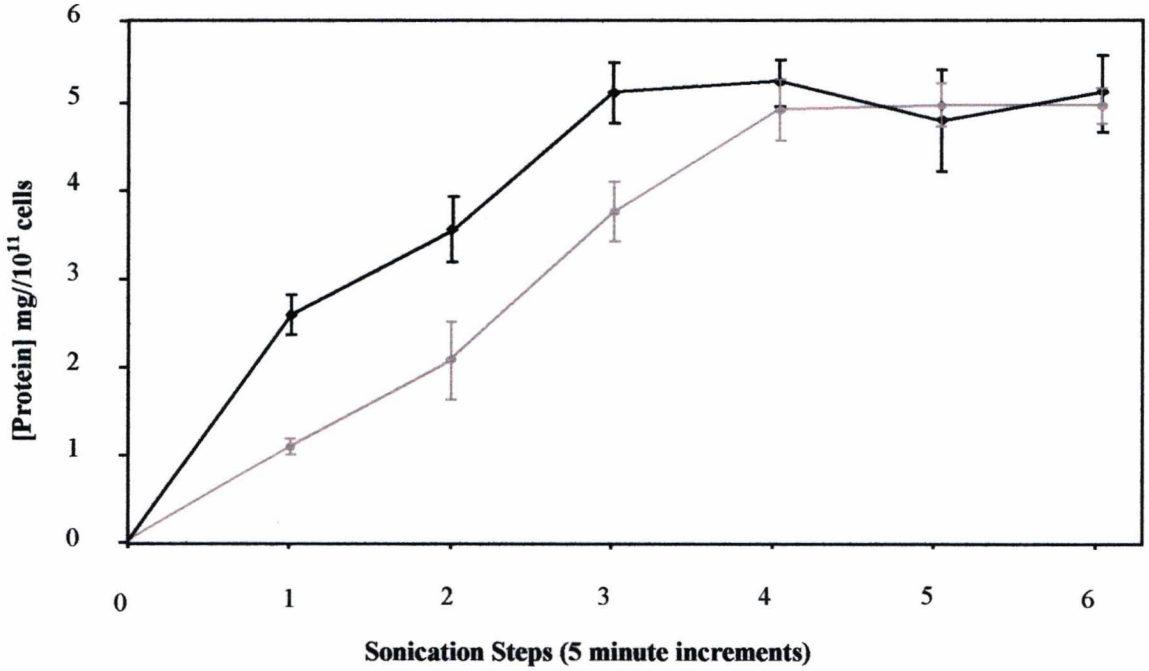


Figure 5.1 Protein yield of *L. monocytogenes* batch culture subjected to sonication at exponential phase and stationary phase. — Early exponential phase (0.25 OD_{600}). — Stationary phase (1.4 OD_{600})

Figure 5.1 shows that at stationary phase *L. monocytogenes* cells were more resistant to mechanical damage (sonication) than at exponential phase. The cyclic sonication protocol resulted in a small increase in the protein yield above that achieved using a single sonication step (Section 5.2.1, Figure 5.0). Figure 5.1 also shows that the resistance to damage by mechanical means observed at stationary phase could be overcome by a 4-step cyclic sonication protocol. This method achieved the greatest protein yields and more importantly, the yield was independent of the point in the growth phase at which samples were taken.

5.2.1.2 The effect of growth phase and cell volume on lysed cell protein yield

L. monocytogenes BHI batch culture (1 L) was sampled throughout growth and stationary phase (OD_{600} 1.575). At each time point the samples were divided such that one aliquot was volume adjusted so that the sample (independent of growth phase) contained the same cell number (1 X 10^9 cells/mL^{-1}), while the second aliquot volume and cell number remained unchanged. Each sample was subjected to a cyclic sonication protocol and the protein yield determined by the modified Bradford assay.

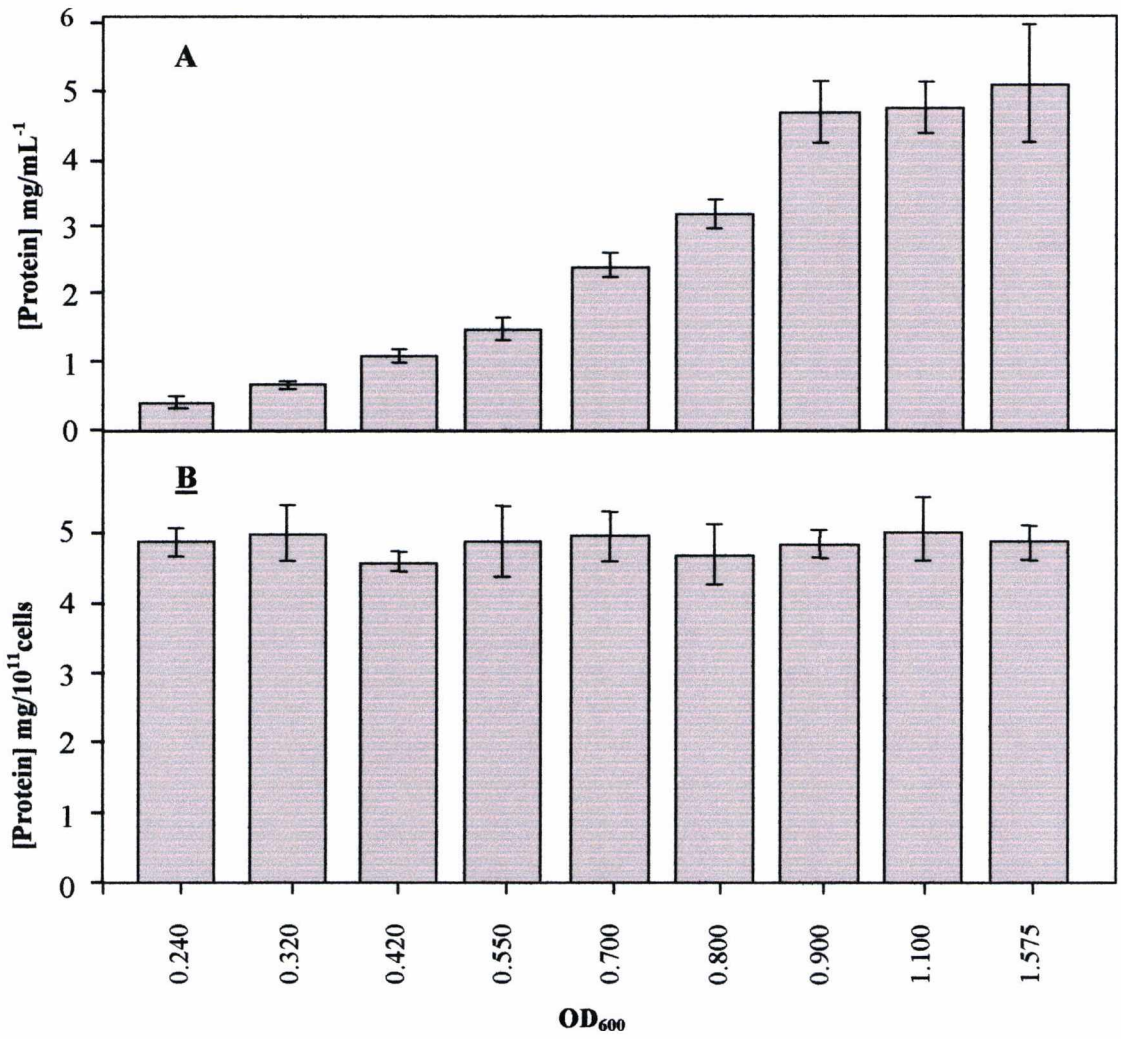


Figure 5.2 Protein yield using sonication at constant buffer volume (A) and constant cell number (B)

Three repeats of each sample set were undertaken. Figure 5.2 A, shows a clear increase in protein yield throughout growth from OD_{600} 0.24 to OD_{600} 0.9. However, throughout stationary phase (OD_{600} 1.1 to OD_{600} 1.575) the total protein yield did not change. Figure 5.2 B, shows that where sample cell numbers were maintained at 1 X 10^9 cells/mL^{-1} (independent of sample point) a cyclic sonication protocol consistently yielded the same protein concentration. The results presented in Figure 5.2 established that a cyclic sonication protocol would produce a consistent and high protein yield independent of the time point and growth phase at which the samples were taken. Furthermore, by maintaining a standard sample cellular concentration the protein yield was standardised.

5.2.2 A quantitative assessment of protein solubility

A wide range of buffer systems was investigated to assess the relative protein solubilising efficiency of each system and of 22 options investigated (results not shown) five were considered for further analysis (Table 5.0). *L. monocytogenes* BHI batch culture (500 mL) was grown to OD_{600} 0.25 and harvested. After washing the sample was re-suspended in lysis buffer 2 and subjected to lysis by sonication. After lysis the sample aliquots were adjusted such that each contained 4% CHAPS, 1% DTT, 0.8% pharmalyte and trace bromophenol blue. Further additions to each aliquot were made to optimise the sample for separation by IEF. (Table 5.0)

Table 5.0 Constituents added to a base sample buffer making for five protein solubilising buffers suitable for IEF

Prefix	Urea	Thiourea	Tris	MilliQ water
1	7 M	2 M	-	x 2 starting volume
2	8 M	3 M	-	"
3	8 M	-	-	"
4	9.5 M	-	40 mM	"
5	7 M	1 M	-	"

The relative solubilising efficiency of each buffer was evaluated by measuring the total protein concentration achieved using aliquots of the same cell extract. A difference in the protein concentration between individual samples was considered to be due to the different protein solubilising conditions that each buffer possessed. The effect of an acetone/TCA precipitation step on total protein concentration was also evaluated for each buffer. The results presented in Figure 5.3 were the product of an average of three repeats of each sample.

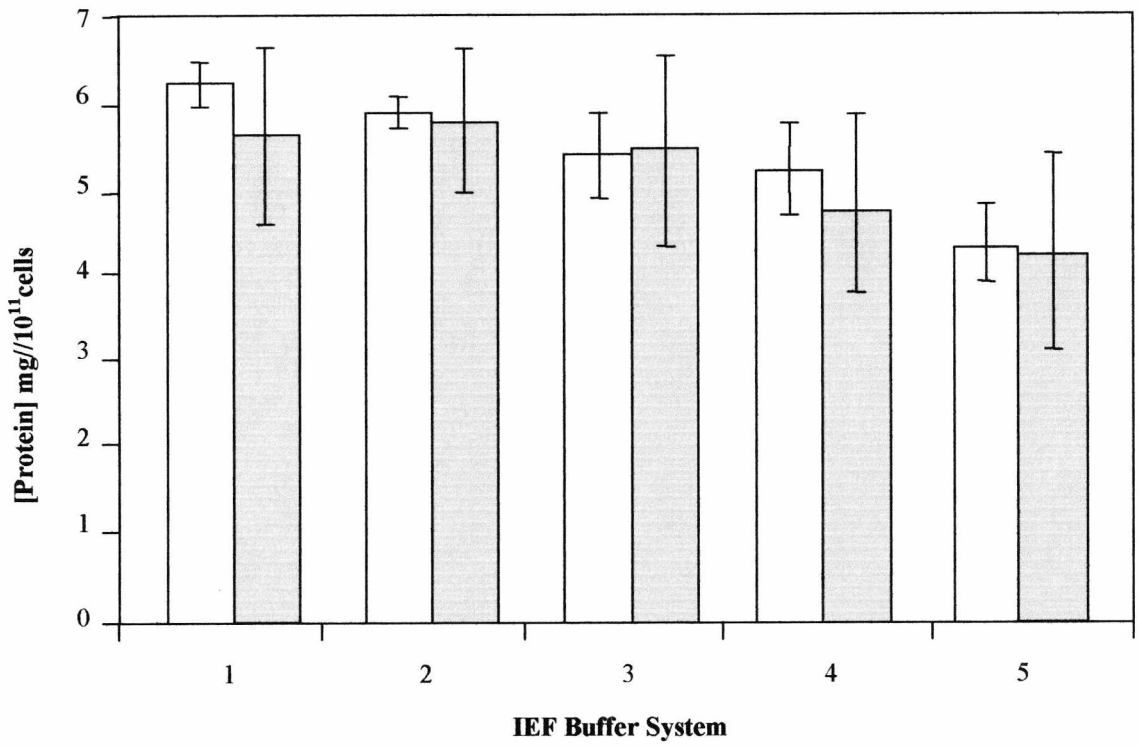


Figure 5.3. Solubilised protein concentration exclusive and inclusive of an acetone precipitation. □ Samples not subjected to an acetone precipitation. ▒ Samples subjected to an acetone/TCA precipitation.

Each buffer option was investigated using lysis buffer 1 and lysis buffer 2, samples lysed in buffer 2 had a higher protein content than samples lysed in buffer 1 (results not shown). The protein concentration of each sample differed dependant on the constituents of each solubilising buffer. Figure 5.3 shows the protein solubility of 10 aliquots of the same sample extracted from *L. monocytogenes (*after lysis in buffer 2) that were solubilised in IEF buffer systems 1 to 5 (Table 5.0). The protein concentration of samples that had been solubilised after an acetone/TCA precipitation varied by up to 2.5 mg/mL. The protein concentration of samples that were not subjected to an acetone/TCA precipitation varied by 1 mg/mL. The results showed that an acetone/TCA precipitation step introduced greater bias toward more easily solubilised proteins than solubilisation without a precipitation step. This was a quantitative assessment of IEF buffer solubilising capacity based on total protein and it could not determine which buffer system would produce the best resolved proteins for sample separation by 2-D electrophoresis. To make this buffer selection qualitatively the samples (excluding acetone/TCA precipitation) were separated by 2-D electrophoresis.

5.2.2.1 *The assessment of protein solubility using 2-D electrophoresis*

L. monocytogenes BHI batch culture (500 mL) was grown to OD_{600} 0.25 and harvested. After washing the sample was aliquoted into lysis buffer 2 and lysed by sonication. After extraction the sample was further aliquoted to provide three sample repeats for each sample

solubilising condition (Table 5.0). Each sample was subject to a 2-D SDS-PAGE separation using 7 cm, pH 3 – 10 IPG strips for the first dimension (Section 2.6.8) and Novex 7 x 7 x 0.05 cm self-cast gels for the second dimension (Section 2.6.10). The resultant gels were compared using ImageMaster software. The software defined spot count and operator defined spot count were recorded for three repeats of each buffer system and combined to give a total spot count.

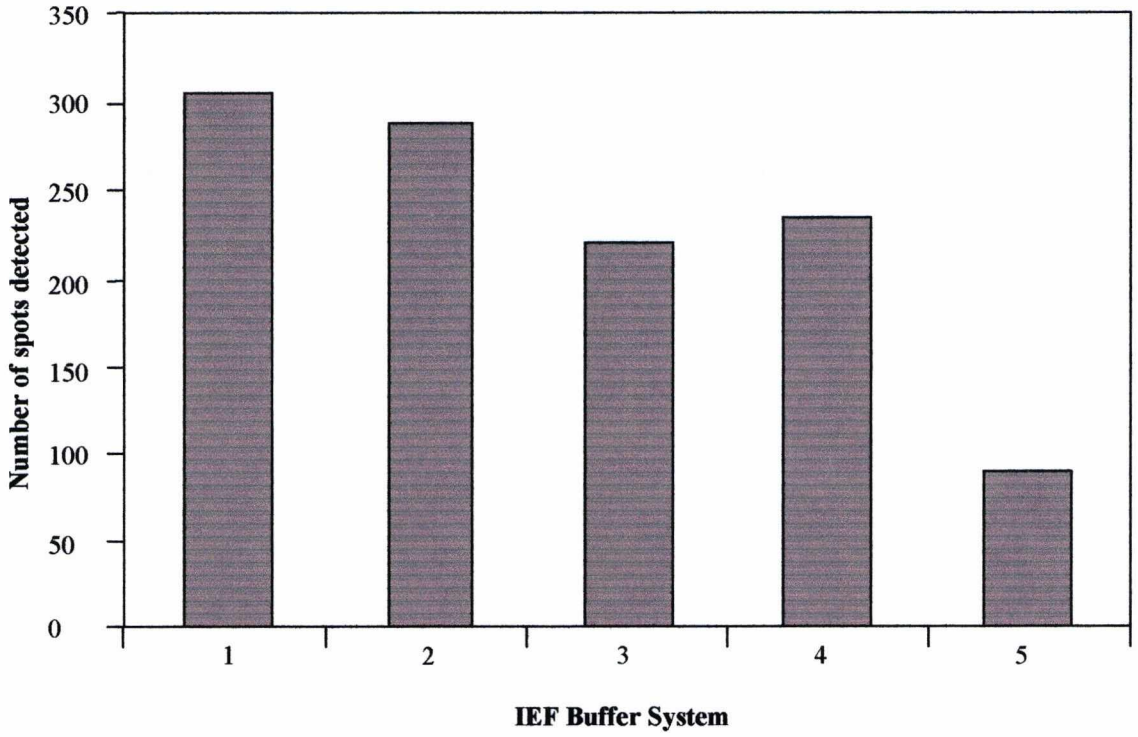


Figure 5.4 2-D SDS PAGE image analysis using 5 alternate IEF buffer systems. ■ Total number of spots detected in each IEF buffer system.

The number of spots detected was based on a software generated averaged image and as such were not subject to error bar inclusion. The averaged image excluded protein spots that could not be matched in at least two of the three images used for averaged gel construction. The averaged gel was a computer generated data source that did not alter the original gel images. Two sample gel images and analysis results of a *L. monocytogenes* whole cell extract separated using 2-D SDS-PAGE, are reported in Figure 5.5. The extracts were solubilised using IEF buffer 1 and buffer 5 (Table 5.0).

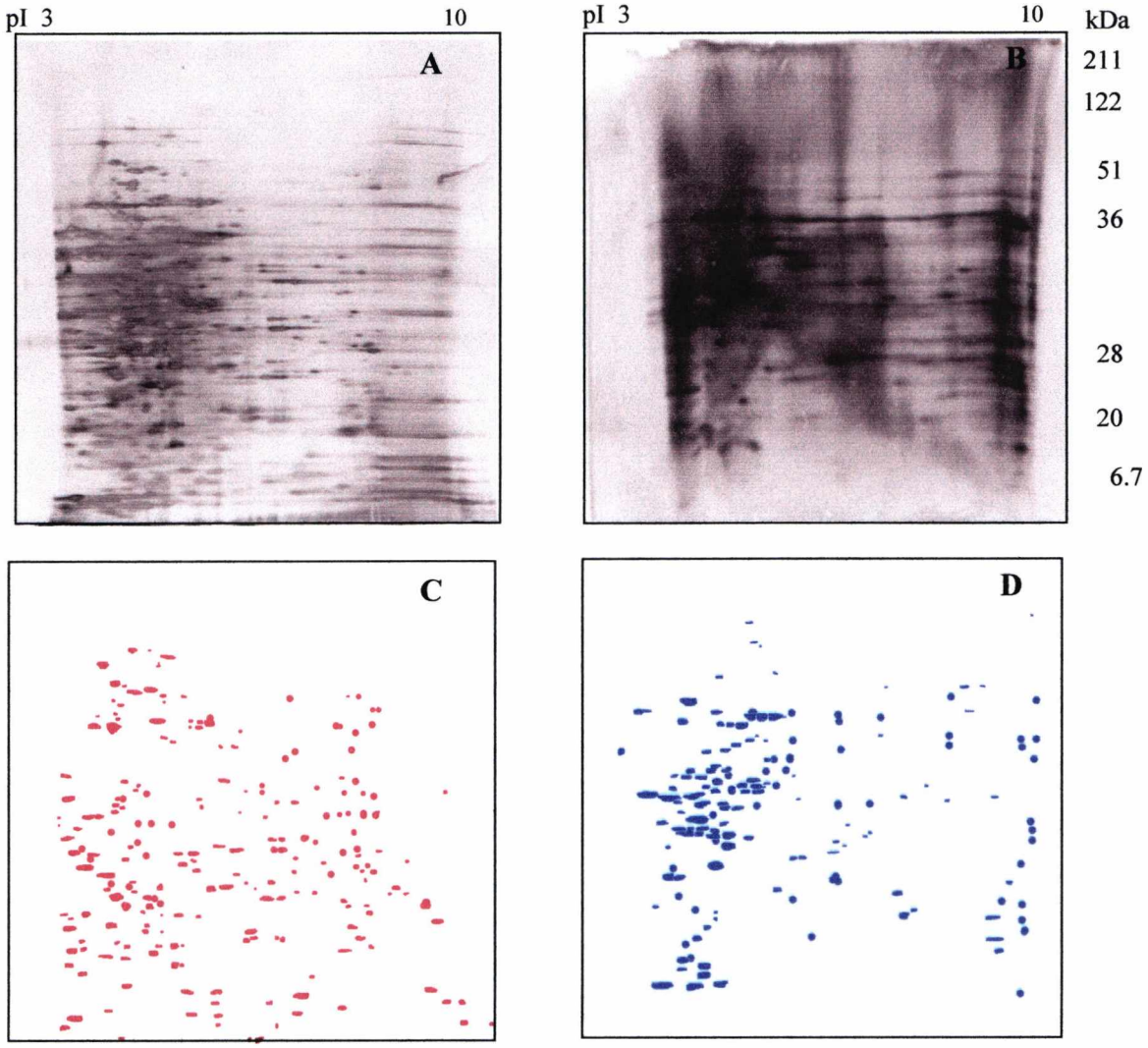
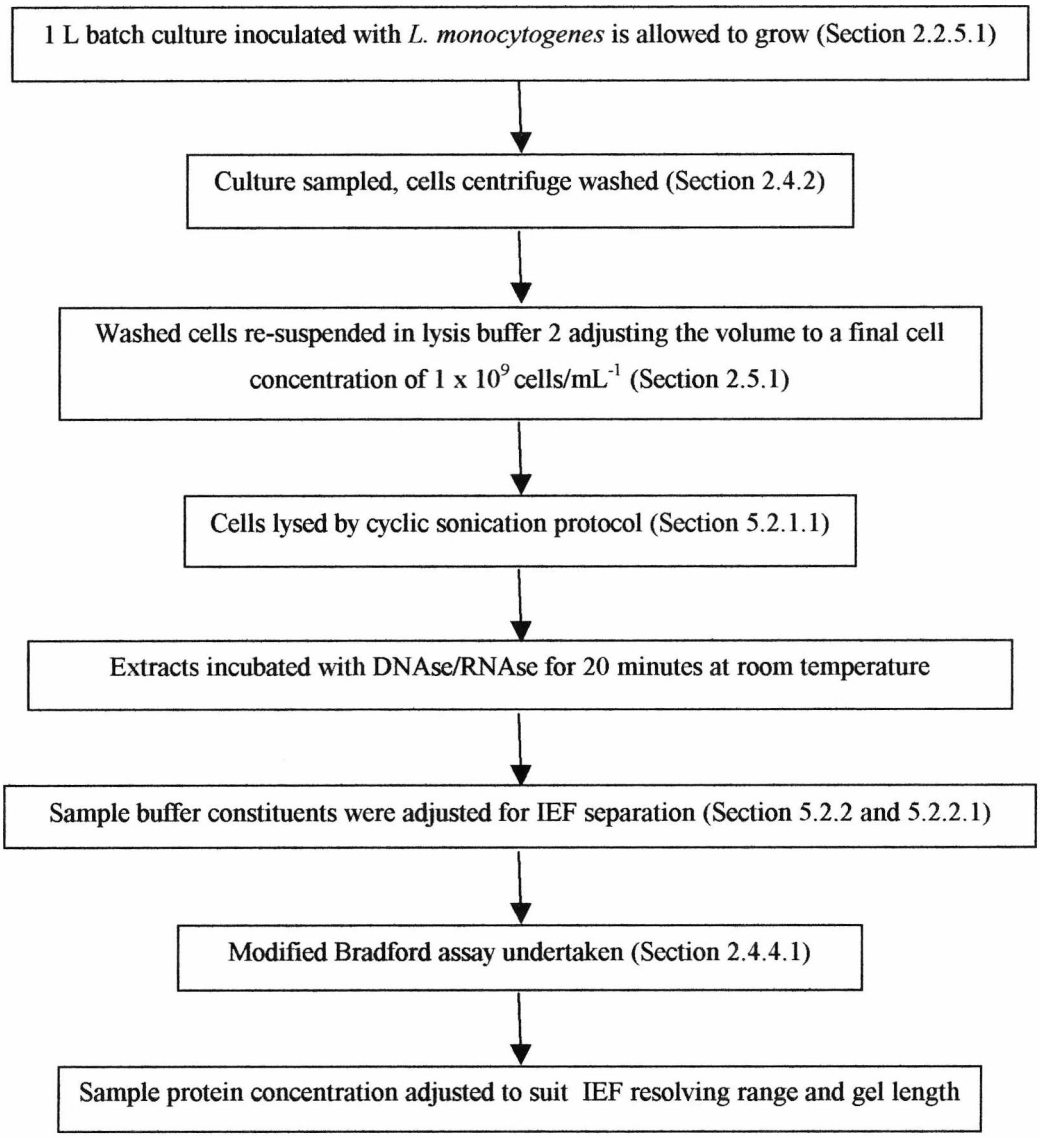


Figure 5.5 2-D SDS-PAGE of *L. monocytogenes* whole cell protein extract using different solubilising buffers. **A**, 2-D SDS-PAGE Shevchenko silver stained mini gel of *L. monocytogenes* whole cell extract using lysis buffer 2 and protein solubilising buffer 1. **B**, as for Figure A except proteins solubilised using buffer 5. **C**, ● 234 protein spots in Figure A detected using ImageMaster software. **D**, ● 150 protein spots in Figure B detected using ImageMaster software

Figure 5.5 clearly shows that a *L. monocytogenes* whole cell protein extract solubilised in protein solubilising buffer 1 and separated using 2-D electrophoresis produced a more defined and easily analysed gel image than a sample solubilised in IEF buffer 5, demonstrating that minor changes to solubilisation buffer constituents can have a large effect on the ability of that system to separate proteins effectively. This was also the case with images produced from IEF buffers 3 and 4. It should be noted that this was not detailed image analysis but a swift method for quantifying the ability of alternate buffer systems to separate the highest number of well defined spots on a gel using repeats of the same sample.

5.2.3 Optimised scheme for the preparation of *L. monocytogenes* whole cell extract

The results of the optimisation experiments lead to the development of a standardised scheme for the harvesting, extraction and solubilisation of *L. monocytogenes* whole cell extract in preparation for separation by 2-D SDS-PAGE.



Scheme 5.0 Optimised scheme for the harvest extraction and solubilisation of *L. monocytogenes* whole cell protein extract

5.2.4 Protein visualisation using small format 2-D SDS-PAGE

A number of protein stains were investigated on the basis of detection limitations and ease of use, these included brilliant blue coomassie [347, 348], colloidal coomassie, Sypro® orange, Sypro® ruby, Silver stain plus, Plusone silver stain and the Shevchenko silver stain method [235] (Section 2.7). A standard protein mix consisting of BSA (80 kDa), trypsin inhibitor (soybean 26 kDa), and lysozyme (16 kDa) was used to generate a range of concentrations (100, 50, 30, 20, 10, and 5 ng) and separated by 1-D SDS-PAGE. The dilution series was distributed in separate lanes, 100 ng lane 1, 50 ng lane 2, 30 ng lane 3, 20 ng lane 4, 10 ng lane 5 and 5 ng lane 6 (Figure 5.6 A, C and F). Lane 10 contained a BIORAD broad range MW marker. In addition a *L. monocytogenes* BHI batch culture (500 mL) was grown to 0.25 OD_{600} and harvested. The samples were prepared for 2-D SDS-PAGE in accordance with Scheme 5.0. The protein concentration was determined and aliquots of the same sample were subjected to 2-D SDS-PAGE separation using Novex 7 x 7 x 0.05 cm self-cast 12% gels. The individual samples were separated using 1-D and 2-D SDS-PAGE and the proteins were then visualised using the different staining methods described in Section 2.6.1.

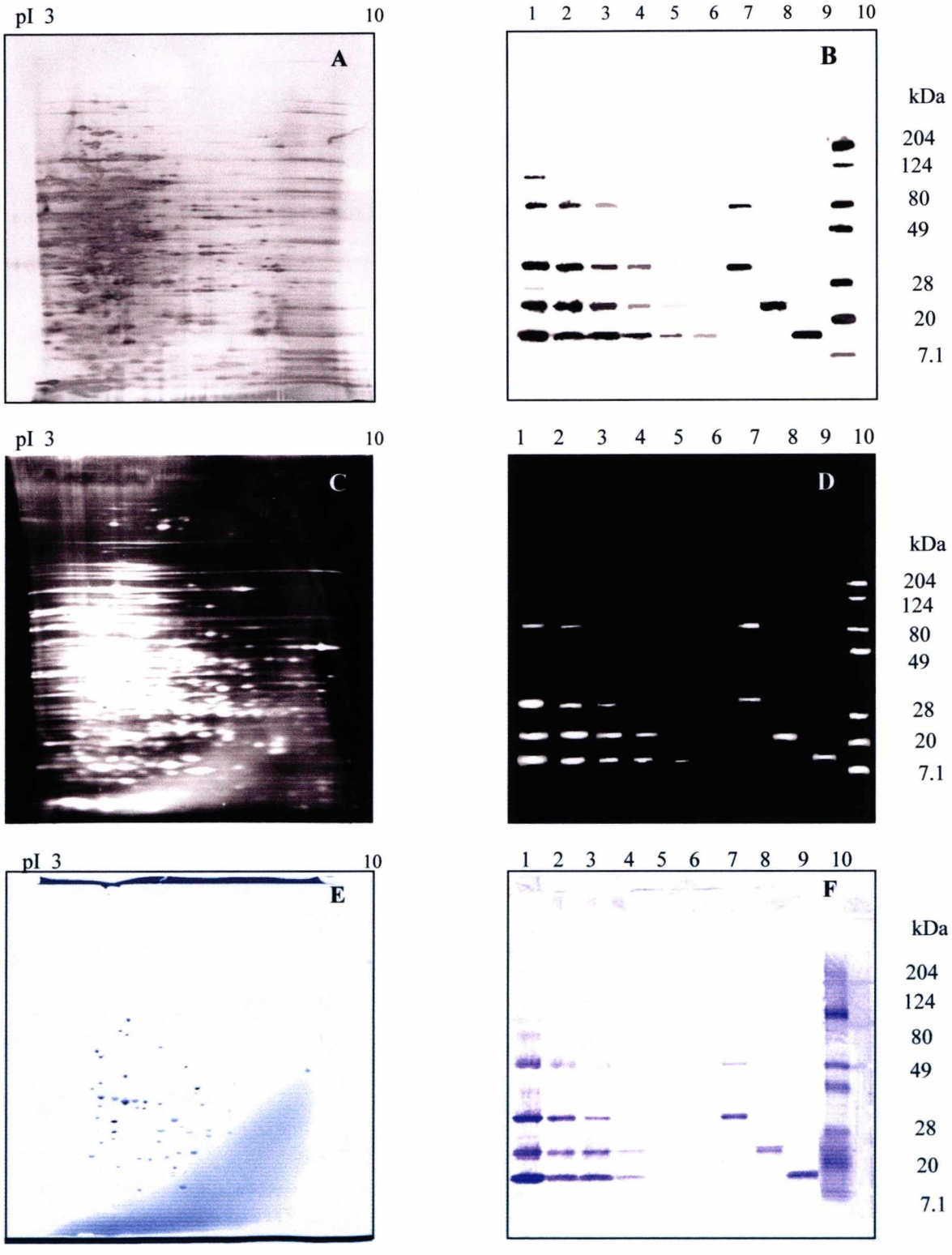


Figure 5.6 1-D and 2-D images used to define the detection limits of different protein stains. A) 2-D separation of a *L. monocytogenes* whole cell extract visualised using Shevchenko silver stain. C as for A, except proteins were visualised using SYPRO orange. E as in A, except proteins were visualised using colloidal coomassie. B) a 1-D separation of a standard protein mix dilution series covering a range from 100 ng to 5 ng concentration, proteins were visualised as in Figure A. D as in B, except proteins were visualised using SYPRO orange. Image F as in B, except proteins were visualised using colloidal coomassie.

The Figures A, B, E and F (Figure 5.6) were captured using a Umax scanner (Section 2.7.4). Figures C and D were imaged with UV light and captured using a BIORAD GelDoc 2000 image capture system and software (Section 2.7.4). The sensitivity of each stain is tabulated below.

Table 5.1 Sensitivity limits of different protein stain methods

Stain	Limit of sensitivity
Brilliant blue coomassie	50 – 100 ng
Colloidal coomassie	20 ng
Sypro® orange	10 ng
Sypro® ruby	5 – 10 ng
Silver stain plus	5 – 10 ng
Plusone silver stain	5 ng
Shevchenko silver stain	5 ng

Table 5.2 shows silver stain to be more sensitive than the other protein visualisation methods tested. It should be noted that the expected sensitivity of SYPRO ruby is 5 ng [349] or below, however this was not achieved and the poor result may in part be due to the limited imaging equipment available for this stain. Of the three silver stain methods used to visualise proteins on 1-D and 2-D gels, the Shevchenko method (Figure 5.6,A and B) was originally devised to be compatible with mass spectrometric identification of proteins that had been subjected to in gel tryptic digest (Section 2.8). The Shevchenko silver stain method was adopted as a standard and all further silver stain images used this method.

5.2.5 Transition to large format 2-D SDS-PAGE

Sections 5.2.2 and 5.3.3 describe the separation of a *L. monocytogenes* whole cell extract using small format (7 x 7 x 0.05 cm) 2-D SDS-PAGE (Figure 5.5 A and 5.6 A). Adopting larger gel formats for the 1st and 2nd dimension sample separations of *L. monocytogenes* whole cell extract increased the analytical area over which a sample could be separated. This was achieved with the use of 18 cm IPG strips in place of 7 cm IPG strips for first dimensional IEF separations. The maximum protein load that could be applied to a mini gel format 2-D separation visualised with silver stain was 10 µg. However, the maximum protein load applied that can be applied to 2-D large format separation for visualisation with silver stain is approximately 100 µg. The quantitation of proteins on SDS PAGE gels using silver stain is based on the total pixel intensity of individual spots on a greyscale image. An over-

stained protein spot will have a lower pixel intensity than one not over-stained, therefore the range over which proteins can be quantified using silver stain is narrow. Exceeding a 100 μg protein load increase the number of spots that over-stain and reduces the quantifiable data that can be extracted from such an image. A 100 μg protein load is a compromise between the desire to visualise the maximum number of proteins against the desire to extract the maximum amount of quantifiable data from each protein spot.

5.2.5.1 Vertical and horizontal 2-D SDS-PAGE

The transition to large format gel systems was achieved using the optimised sample preparation protocols described in Sections 5.2 – 5.2.3 and methods described in Sections 2.6.8 and 2.6.11.

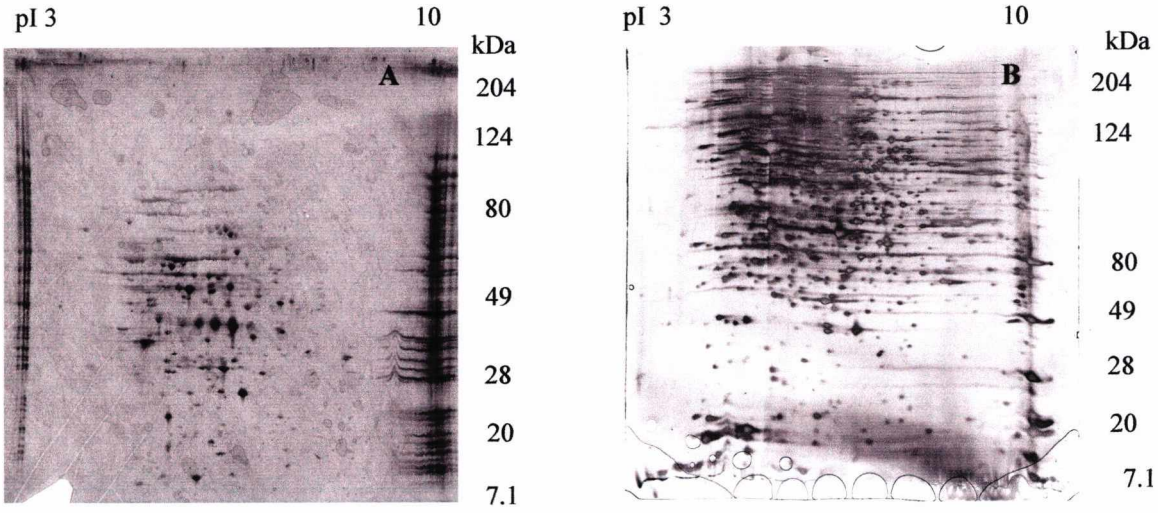


Figure 5.7 *L. monocytogenes* whole cell extract analysis by 2-D SDS-PAGE pH 3 - 10 resolving range. A) 12-14% gradient Excel gel 25 x 18 x 0.05 cm separating 2nd dimension, *L. monocytogenes* whole cell extract separated using Multiphore horizontal electrophoresis equipment. B) 12.5% self-cast large format gel, *L. monocytogenes* whole cell extract separated using Protean II vertical electrophoresis equipment.

The first dimension IEF separation was completed using IPGphor equipment and 18 cm pH 3 – 10 IPG strips (Section 2.6.6 and 2.6.8.1). Figure 5.7 compares two aliquots of the same *L. monocytogenes* whole cell protein extract separated by 2-D SDS-PAGE using the Multiphore II horizontal electrophoresis equipment (A) and Protean II vertical electrophoresis equipment (B). Table 5.3 compares the total number of protein spots detected using small and large format 2-D SDS-PAGE gel systems. Two SDS PAGE systems were investigated but the sample preparation and optimisation process had been more specifically directed to the use of the Protean II system. An accurate assessment of the Multiphore II equipment required further optimisation which was not undertaken.

Table 5.2 Number of protein spots visualised with silver stain using different 2-D SDS-PAGE equipment

Electrophoresis equipment	Total visualised spots with pH 3 – 10 resolving range
Novex mini gel system	234
Multiphore II horizontal system	368
Protean II vertical system	959

Table 5.3 shows that large gel formats significantly increased the number of spots that could be separated and visualised using 2-D electrophoresis. The totals were derived from virtual images constructed from three repeats of the same sample. Virtual images contained only protein spots that appeared in at least two of the three images used. The Protean II vertical system was adopted as standard for all further 2-D electrophoresis.

3.10 Selection of IEF resolving range

Large gel formats increased the area over which a *L. monocytogenes* whole cell protein extract could be separated which resulted in a ten fold increase in the amount of protein that could be loaded and resolved on 18 cm IPG strips. Consequently this resulted in an increase in the total number of proteins visualised by 2-D electrophoresis and hence the total number of low abundance proteins visualised. However, when an 18 cm pH 3 – 10 IPG gel was used in the first dimension, some overlapping protein spots were observed on the second dimension gel image within the pH 4 – 7 resolving range.

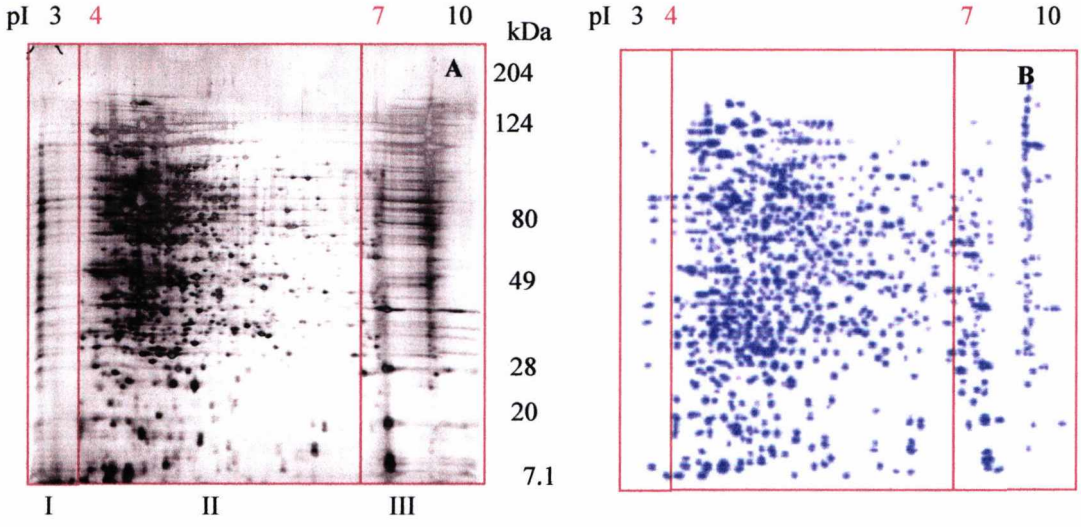


Figure 5.8 *L. monocytogenes* whole cell extract separated using 2-D SDS-PAGE with a pH 3 - 10 first dimension resolving range. Figure A, 2-D gel with pH 3 - 10 resolving range subdivided into distinct pH regions, I pH 3-4, II pH 4-7, III pH 7-10. Figure B, is an ImageMaster produced image of Figure A showing 959 software detected spots

Figure 5.8 A illustrates the division of a pH 3 – 10 resolving range into three sections (pH 3-4, pH 4-7, pH 8-10). An averaged image was used to examine each image section; the results obtained are illustrated in Figure 5.9. The averaged image was computer generated image and did not alter the original image information.

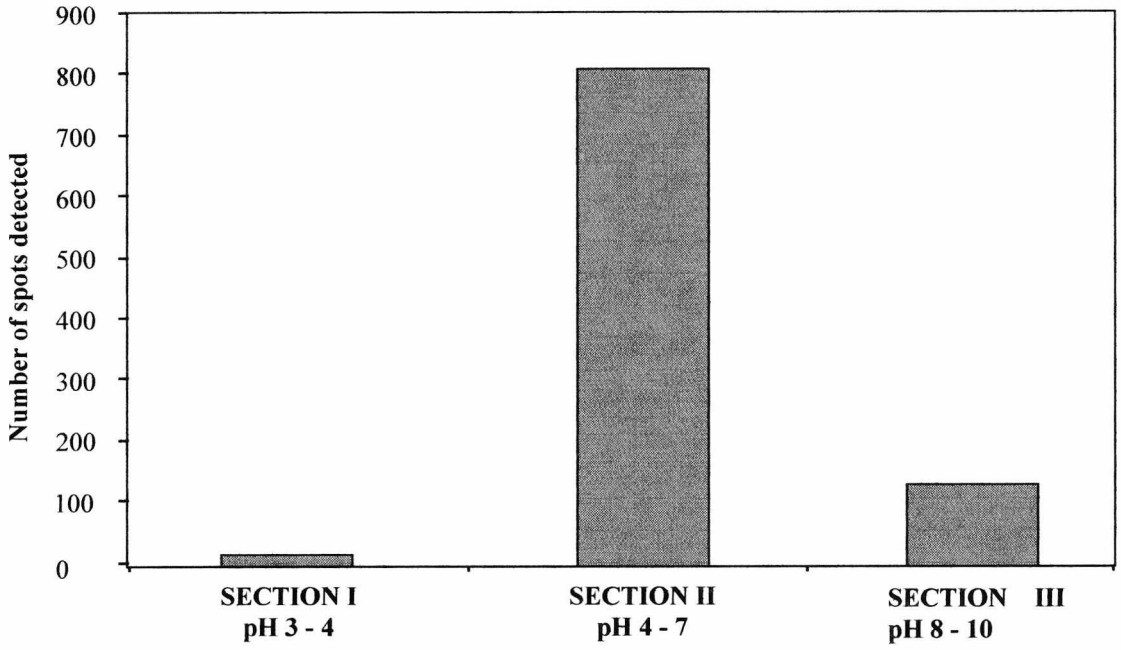


Figure 5.9 Total detected spots within defined pH regions on a *L. monocytogenes* whole cell extract separation using 2-D SDS-PAGE.

Sections (I) and (III) of image Figure 5.8 A, represent 40% of the total gel area over which 15% of the total spots detected were visualised. Section (II) Figure 5.8 A, represents 60% of the total gel area in which 85% of the total spots detected were visualised. The protein spots in areas (I) and (III) could not be automatically detected by image analysis software because separation between individual spots was poor. The separation of a *L. monocytogenes* whole cell extract across the defined gel area (II) was therefore expanded by the use of pH 4 – 7 IPG gels in place of the pH 3 – 10 IPG gels (Figure 5.10).

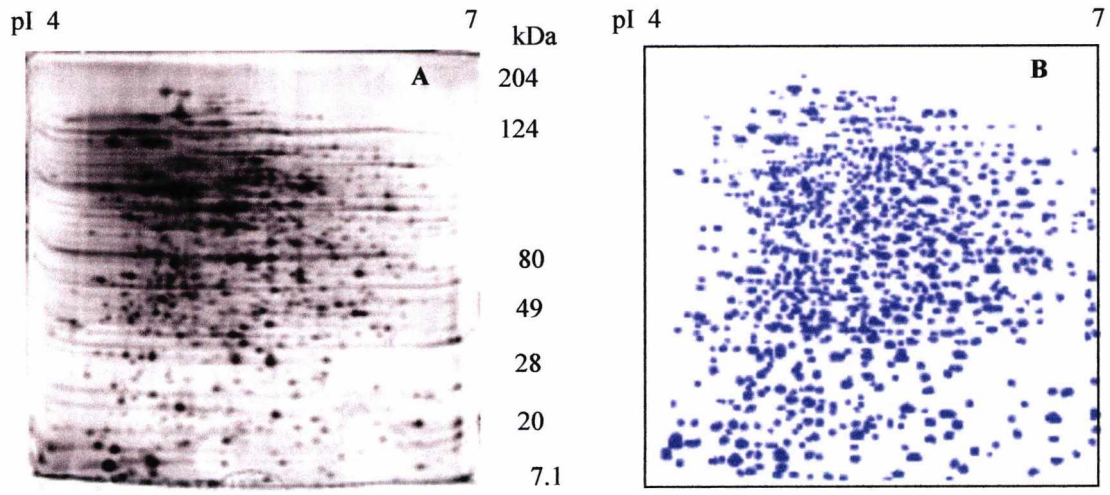


Figure 5.10 *L. monocytogenes* whole cell extract separated using 2-D SDS-PAGE and a pH 4 –7 first dimension resolving range. Figure A, gel visualised using silver stain. Figure B, ImageMaster produced image of Figure A showing 1025 software detected spots.

Within the pH 4 – 7 resolving range of a pH 3 – 10 separation of *L. monocytogenes* whole cell extract 816 protein spots were visualised, while the total number of spots visualised was 959 (Figure 5.8). The total number of spots visualised using large format 2-D SDS-PAGE with a pH 4 – 7 resolving range was 1025 (Figure 5.10). The Figures 5.8, 5.9 and 5.10 clearly show that increasing the area over which a *L. monocytogenes* whole cell extract was separated using 2-D SDS-PAGE increased the separation, resolution and detected spot total. The expansion of the gel area over which a pH 4-7 resolving range was separated, increased the total detected protein spots by 25%.

The standard protein load applied to an 18 cm pH 3 – 10 IPG gel for 2- D SDS-PAGE was 100 μg/350 μL [233]. However, a 100 μg/350 μL protein load did not give the optimum resolution when applied to an 18 m pH 4 – 7 IPG gel. A number of protein loads were investigated in order to determined that the optimum protein load for silver stain visualisation when using 18 cm pH 4 – 7 IEF separation. Discarding the pH 3-10 resolving range in favour of a pH 4-7 resolving range resulted in some of the unresolved protein to collect at the cathode and anode during IEF. This meant that not all of the protein being loaded onto pH 4-7 strips was resolved. Additional protein could be loaded increasing the ability to visualise low abundance protein. Care was taken not to increase the protein load to a point where quantitation was compromised by increasing the degree of over stain of high abundance proteins. The optimum protein load for these experiments was found to be 120 μg/350 μL.

5.2.7 Analysis of the *Listeria* proteome using samples grown in BHI and PTM media

Listeria is psychrotrophic in nature and can resist a wide range of environmental conditions [258, 350]. Although 'good' growth was achieved in PTM medium, an increased culture lag time was observed (Figure 3.1 and 3.2). Changing growth conditions has the potential to induce changes in gene expression [27, 271, 351, 352] and therefore the proteome of an organism. The adoption of PTM medium in preference to BHI broth as a standard for the growth of *Listeria* would only be of value if the changing growth conditions did not induce large-scale changes of gene expression. Samples of *Listeria* were sourced from different medium batch cultures (BHI and PMT) and harvested at the same OD_{600} and cell count, being 1 x 10^6 cells/mL^{-1} at exponential phase and 1 x 10^9 cells/mL^{-1} at stationary phase. These samples were then subjected to 2-D SDS-PAGE analysis and the resultant gel images compared in order to identify changes in gene expression (if any) that were induced by the change of medium.

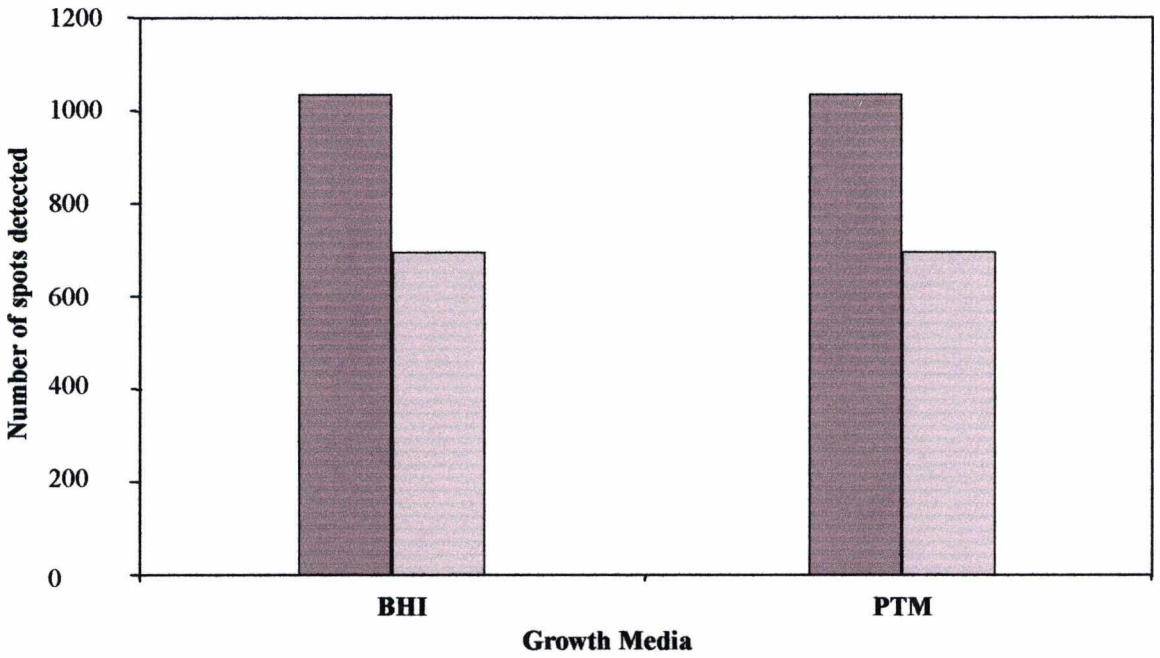


Figure 5.11 The total number of spots detected from *L. monocytogenes* samples grown in BHI and PTM medium separated by 2-D SDS-PAGE. ■ Number of spots detected at exponential phase. ■ Number of spots detected at stationary phase.

Figure 5.11 shows an averaged image spot count of a *L. monocytogenes* whole cell extract grown in BHI (sampled at exponential phase) or PTM medium (sampled at exponential phase). A total of 1025 protein spots were detected in each medium sample (Figure 5.11). The process was also repeated using samples extracted at stationary phase. A total of 675 protein

spots were detected in each medium sample. The results showed that a change of medium from BHI to a PTM did not induce an observable change in the total number of protein spots detected at exponential phase or stationary phase.

5.2.8 Artefactual stress response

Bacteria are capable of rapid changes in protein production in response to external perturbation [241]. It has previously been reported that during bacterial sample preparation a significant stress response can be initiated that would render different sample comparisons invalid. Rapid freezing at the point of harvest has been found to 'fix' the proteome so that a post harvest stress response is excluded [353]. In order to investigate the effect that a liquid nitrogen 'fixing' step had on the proteome of *L. monocytogenes,* whole cell extracts were analysed by 2-D electrophoresis. Samples harvested at exponential phase were divided such that one aliquot was immersed in liquid nitrogen and rapidly frozen prior to thawing, extraction and separation by 2-D SDS-PAGE, while the remaining aliquot was extracted and separated without immersion in liquid nitrogen. Averaged images of each sample extract were compared using the ImageMaster software.

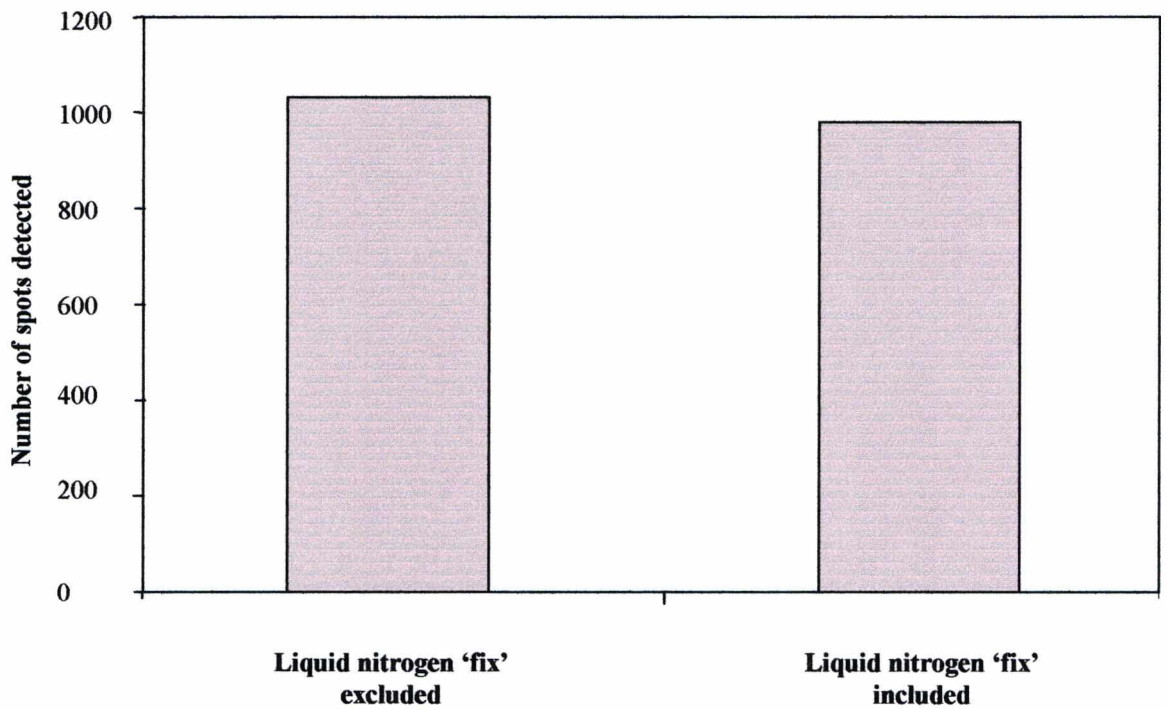


Figure 5.12 A comparison of *L. monocytogenes* whole cell extract separated by 2-D SDS-PAGE exclusive or inclusive of liquid nitrogen rapid freeze step

Figure 5.12 shows a difference in the total number of protein spots detected when a liquid nitrogen fix step was introduced during sample preparation prior to 2-D SDS-PAGE. Using

aliquots of the same sample, when a liquid nitrogen step was excluded 1025 protein spots were detected, while when a liquid nitrogen fix step was included 975 protein spots were detected. The ImageMaster software automatically compares the positions of like spots between gels assigning the same number to each protein spot that had a matched partner in the other gel. Spots that were not matched represented a change in the proteome that the different sample preparation techniques may have induced.

Using a single gel image, an area of gel where spot matching analysis had identified unmatched proteins has been expanded to highlight a protein that has not been matched (Figure 5.13).

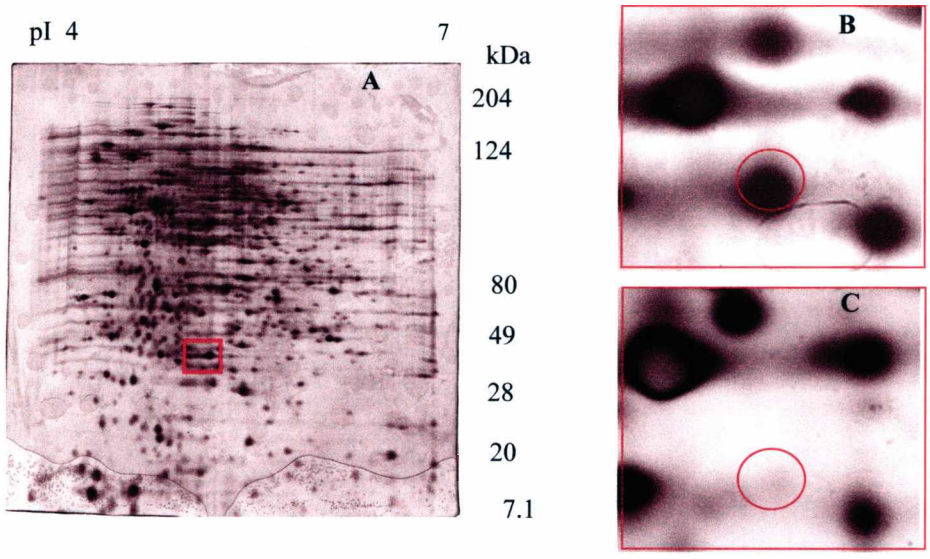


Figure 5.13 Changes to the *Listeria* proteome induced by different sample preparation techniques. Figure A, *L. monocytogenes* whole cell extract separated using 2-D SDS-PAGE pH 4-7 resolving range excluding a liquid nitrogen fixing step. Figure B, expanded area of image A. Figure C, expanded area from a *L. monocytogenes* whole cell extract separated using 2-D SDS-PAGE pH 4-7 resolving range including a liquid nitrogen fixing step (original image not shown).

Figure 5.13 B, shows a protein spot that appeared in the non-fixed sample image that was not matched to a protein in the liquid nitrogen fixed sample image (Figure 5.13 C). The protein was excised and subjected to in-gel tryptic digestion (Sections 2.8 and 2.8.1). The digested protein was then subjected to MALDI-TOF analysis to obtain a peptide mass fingerprint (Section 2.8.3). The resultant peptide fragments masses were entered into SWISS-PROT and TrEMBL databases (http://ca.expasy.org) using PeptIdent tools search engine (Section 2.8.3). The protein was identified as TrEMBL: O85743 RNA polymerase sigma factor B (Appendix V). The procedure was repeated for the same spot excised from a different gel and the identification was the same. The approximate kDa and pI of the protein could be calculated

from its position on the gel image and this was compared to its theoretical kDa and pI published in the TrEMBL database (Table 5.4).

Table 5.3 RNA polymerase sigma factor B theoretical kDa and pI compared to the experimentally observed kDa and pI

Protein characteristic	Experimental	Theoretical (SWISS PROT)
kDa	29 - 31	30.5
pI	5.2 - 5.5	5.59

A single protein spot was identified as a stress related protein and only appeared in samples not subjected to liquid nitrogen 'fixing'. The image analysis had highlighted 50 protein spots that were not matched between sample excluding or including a liquid nitrogen 'fixing step. These spots may have been part of a general stress response induced by sample preparation but were not identified. The experiment identified an artefactual stress specific protein induced after sample harvest during sample preparation; a liquid nitrogen 'fixing' step was able to stop the post harvest induction of this protein.

5.2.9 Visualisation of protein spots using colloidal coomassie protein stain

Colloidal coomassie protein stain (BIO-RAD, UK) is not suited to the detection and visualisation of low abundance proteins (below 20 ng, Table 5.2). When compared to silver stain, colloidal coomassie stain visualised less *L. monocytogenes* proteins on a 2-D gel using samples with the same protein concentration (Section 5.2.3 Figure 5.2). A simple way to increase the number of spots visualised by colloidal stain would be to increase the protein load [354]. However, increasing the protein load applied to 18 cm pH 4-7 IPG strips increases the time required for complete focusing from 24 to 60 hours. [355].

To investigate the use of colloidal coomassie stain as an alternative to silver stain *L. monocytogenes* whole cell extracts were separated by 2-D electrophoresis using a range of protein loads which were, 120 μg/350 μL, 525 μg/350 μL, 775 μg/350μL and 1050 μg/350 μL. The total number of spots visualised by colloidal coomassie stain at each protein concentration was compared with the number of protein spots visualised by silver stain.

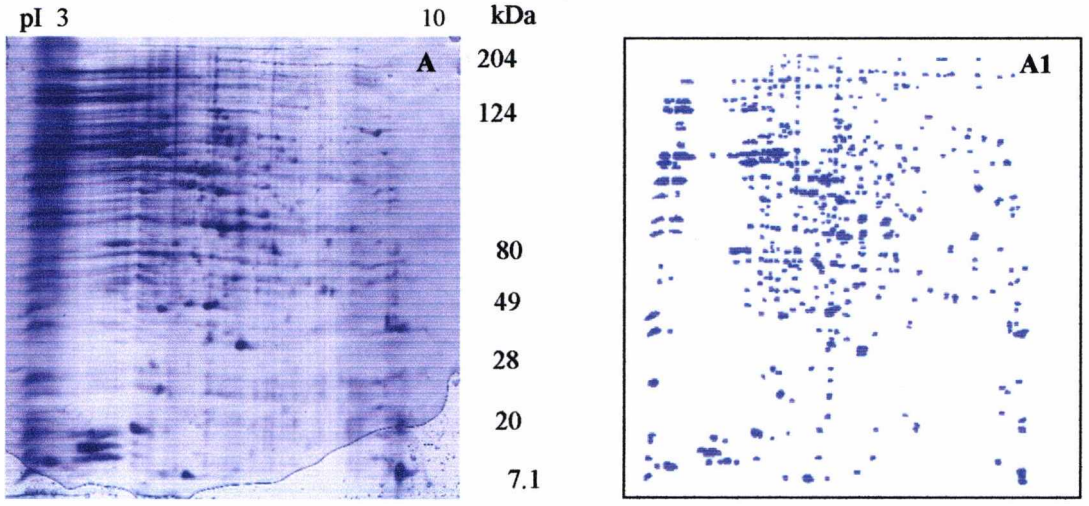


Figure 5.14 Large format 2-D SDS-PAGE *L. monocytogenes* whole cell extract. Figure A, a 1050 µg/350 µL protein load stained with colloidal coomassie. Figure B ImageMaster produced image of Figure A showing 520 software detected spots.

Figure 5.14 A shows a colloidal coomassie stained 2-D SDS-PAGE separation of a *L. monocytogenes* whole cell extract. Figure 5.14 B, shows the spot detection of this image. Aliquots of the same sample were also analysed using total protein concentrations of 775 µg/350 µL, 525 µg/350 µL and 120 µg/350 µL.

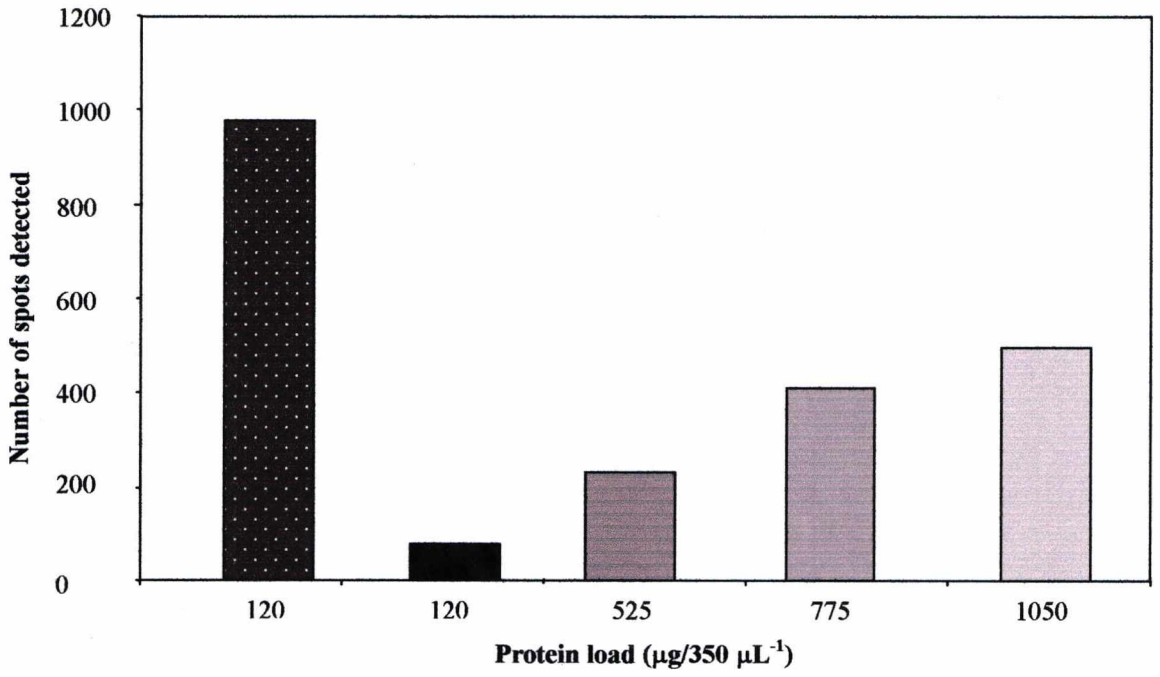
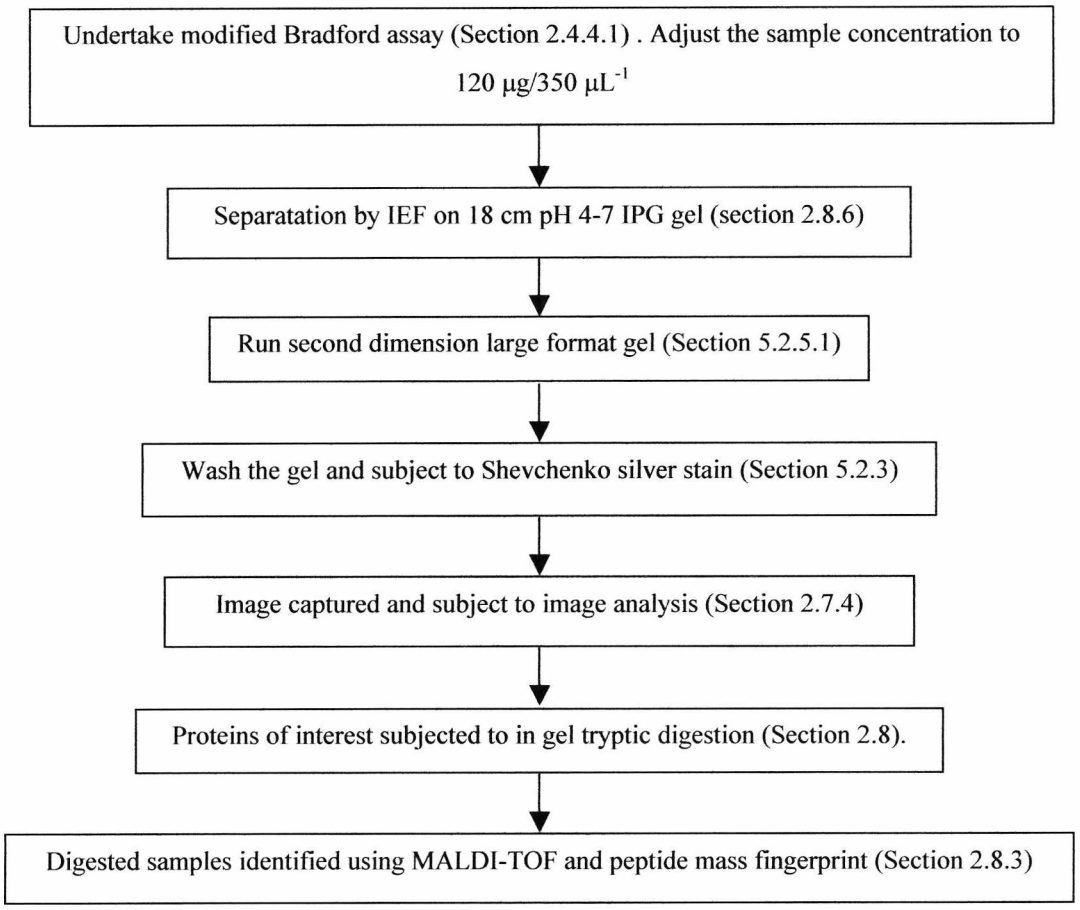


Figure 5.15 The use of variable protein concentrations to compare the sensitivity of silver stain and colloidal coomassie stain. **KEY** Number of spots detected from a *L. monocytogenes* exponential phase (OD_{600} 0.280) whole cell extract separated by 2-D SDS-PAGE visualised with silver stain. ■ ■, ■, and ■ samples visualised with colloidal coomassie stain.

The total number of *L. monocytogenes* proteins visualised by silver stain at 120 μg protein concentration (after fixing with liquid nitrogen) was 975. The total number of *L. monocytogenes* proteins visualised by colloidal coomassie at 120 μg protein concentration (after fixing with liquid nitrogen) was 115. Between 120 μg/350 μL and 775 μg/350μLthe number of spots detected increased linear to the protein load applied. However above that concentration (although stain intensity increased) the number of spots detected was not increasing at a linear rate and higher concentrations did not achieve comparably higher numbers of detected spots.

5.2.10 Optimised scheme for the separation of *L. monocytogenes* whole cell extract by large format 2-D SDS-PAGE

As a result of the gel optimisation experiments it was possible to develop a standardised scheme for the separation of *L. monocytogenes* whole cell extract by 2-D SDS-PAGE. This optimised scheme is presented here (Scheme 5.1).



Scheme 5.1 Optimised scheme for the separation of *L. monocytogenes* whole cell extract using 2-D SDS-PAGE

5.3 Discussion

5.3.1 Sample preparation

A proteomic platform can be used to compare changes in protein expression occurring in an organism over time. In order to observe as many proteins as possible an extraction method must be capable of providing reproducible results independent of the growth stage of the organism from which samples are taken. The method must also extract as many proteins as possible independent of any physiological changes (occuring during growth) that may make cells more resistant to disruption. The purpose of sample preparation is to ensure that the extraction method does not apply experimental selection to the relative abundances and concentration of proteins in a complex mixture. The aim therefore is to maintain the physiological state of each sample relative to the point at which it is taken.

A number of standard protocols were evaluated and modified to enable reproducible sample production without applying experimental selection to those samples. The preparation and extraction of proteins from bacterial samples for separation by 2-D SDS-PAGE using a proteomic platform includes cell lysis, protein solubilisation and the prevention of proteolytic degradation of extracted proteins. To extract protein from *L. monocytogenes* the cell membrane has to be disrupted. Gram-positive bacteria posses thick stable cell walls that are generally more difficult to lyse than the cell walls of Gram-negative bacteria [356]. Due to the variation in cell wall composition between prokaryotic species there is no single cell lysis method that is optimal for all bacterial cell walls. However, cell lysis can be achieved by homogenisers (French press), liquid nitrogen cooled mortar and pestle technique, ultrasonic disintegration (sonication), enzymic lysis (e.g. with lysozyme), detergents (e.g. NP-40, Triton X-100, CHAPS, SDS), osmotic shock, repeat freeze/thaw cycling or a combination of these methods [357]. Freeze/thaw cycling, French press and sonication methods were investigated to determine the most efficient method of cell lysis.

A number of standard lysis buffers can be used to assist the release of proteins from cells during lysis protocols [358], although for compatibility with IEF separation (the first dimension of 2-D SDS-PAGE) a lysis buffer with a low salt concentration is required [232]. The extraction of proteins from *L. monocytogenes* was conducted using a base buffer system containing a pH 7.0 tris/HCl base EDTA and DTT (Section 2.4.3), to which was added the constituents required to make a sample compatible with IEF (Section 5.2.2).

Cells (prokaryotic and eukaryotic) are known to contain protease enzymes and bacterial cells typically contain serine and metalloproteases [359]. EDTA can inhibit the

activity of metalloprotease enzymes released during cell lysis. In addition to EDTA a protease cocktail tablet (Boehringer Mannheim) that inhibited a broad range of protease enzymes was added to samples suspended in a base lysis buffer, prior to cell disruption protocols. DTT, also present in base lysis buffer acted as a reducing agent breaking disulphide bridges. Cell disruption also released nucleic acids into the sample mix and these made the sample viscous and can cause streaking on 2-D gels [233]. To improve the sample handling properties and reduce the risk of DNA/RNA streaking on 2-D SDS-PAGE gels, a 5 μL aliquot of DNAse/RNAse (from 2 mg/mL stock) was added to lysed samples, these were incubated at RT for 20 minutes prior to further processing. Lysis buffer 1 (Section 2.4.3) had a higher salt content than lysis buffer 2 and samples lysed in buffer 1 were less resolved making lysis buffer 2 the standard for all further extractions.

5.3.2 Evaluating cell lysis methods

Three methods, freeze-thaw cycling (2.4.4.3), French press (Section 2.4.4.4), and ultrasonic vibration or sonication (Section 2.4.4.5) were investigated to ascertain their effectiveness in achieving the complete lysis of samples of *L. monocytogenes* at variable and defined cell concentrations. Many cell types can be lysed by subjecting them to repeated rapid-freeze/thaw cycles [360, 361]. This method works by inducing ice crystal formation disrupting the cell membrane and causing cell lysis. Bacterial cells are generally less susceptible to cell lysis by freeze-thaw methods and the method will often be used in conjunction with enzymic lysis for the extraction of proteins and other cellular constituents such as DNA and RNA [362]. *Listeria* is psychrotrophic and capable of growth at low temperature (4°C) and is resistant to lysis at low temperature [363, 364]. The freeze/thaw cycling of *L. monocytogenes* cell samples was not an effective method of cell lysis (Figure 5.0).

The French press method of cell lysis is a high-pressure displacement pump that pumps a cell suspension through an adjustable orifice discharge valve applying mechanical lysis forces. This method of cell lysis works for most cultures except highly filamentous organisms [362]. Use of the French press to lyse *L. monocytogenes* cells gave a 10-fold increase in protein yield compared to that achieved using freeze-thaw cycling (Figure 5.0).

Ultrasonic disintegration (sonication) disrupts cells using short bursts of ultrasound. This technique is usually used for bacteria and has been used for disruption of *Listeria* cells in a number of investigations [365, 366]. Sonication was the most efficient cell lysis method and gave the greatest protein yield. The use of sonication achieved a four-fold increase in protein yield above that reported using 6 passages of the French press (Figure 5.0).

5.3.2.1 Optimisation of cell lysis by sonication

The evaluation of different cell lysis methods was undertaken on *L. monocytogenes* cells harvested at log phase (OD_{600} 0.280). It is known that during growth as stationary phase approaches *Listeria* cells become smaller, less active and more resistant to environmental perturbation [134, 367, 368]. All of the physiological processes of growth are likely to undergo varied alterations in the stationary phase [267], and such global changes in physiology lead to an organism that is generally resistant to a number of stresses. Therefore methods of membrane disruption leading to cell lysis must be independent of global changes in physiology during growth and at stationary phase. All of the cells present in a sample must be lysed to avoid favouring the extraction of proteins from easily lysed cells at the expense of lysis resistant cells [213].

The results presented in this Chapter have shown that *L. monocytogenes* whole cell extract protein yield was growth phase dependent. As exponential growth phase approached stationary phase, protein yield dropped (Figure 5.1). Results presented in Chapter 4 showed that *Listeria* cells at exponential phase were more susceptible to nisin challenge than cells at stationary phase (Section 4.2.2 and 4.2.4.2). This showed that increased resistance to mechanical and physiochemical challenge was growth phase dependent. To overcome the observed resistance to lysis at stationary phase, a single sonication step was abandoned in favour of a cyclic sonication protocol. The number of sonication cycles was increased to a point where growth phase was no longer selective with regard to total protein yield. This point was reached at four (5 minute sonication) cycles per sample (Section 2.4.4.5), the samples were 'rested' on ice for 5 minutes between cycles. The cyclic sonication protocol was adopted for the extraction of protein from all *L. monocytogenes* batch culture samples

5.3.2.2 The effect of growth phase and cell volume on lysed cell protein yield

Where a fixed volume (20 mL) sample of *L. monocytogenes* was taken throughout growth and stationary phase (Figure 5.2 A) the extracted protein yield over time increased. This demonstrated that cell numbers in combination with the point at which a sample was taken (growth phase), might have an effect on the reproducibility of an extracted protein sample yield. Increasing cell numbers in successive samples may therefore introduce experimental selection to the physiological abundances of proteins present at the time of sampling. This would compromise the ability of the sample preparation protocol to represent the relative abundance and concentration of proteins extracted a different growth stages. The aim therefore was to maintain the physiological protein levels at each sample point. The cellular concentration of each sample was adjusted so that it remained constant at 1 x 10^9 cells/mL^{-1},

the protein yield per sample (and therefore per cell) remained constant (Figure 5.2). A consistent protein yield from a known cell quantity confirmed that the physiological protein levels at the time of sampling were less likely to be biased by the extraction method. The observed results were not a function of the limit of the buffer protein carrying capacity. As a buffer approaches the limit of solubility the relationship between volume and concentration stops being linear. Higher cell concentrations achieved higher protein concentrations but these were beyond the linear range of the lysis buffer solubility, and a bias toward more soluble proteins could be introduced. The selected concentration was from the linear solubility range of the lysis buffer and did not bias proteins by solubility.

5.3.3 Evaluating protein solubility: a quantitative assessment

An important factor in successful 2-D electrophoresis is the solubilisation of proteins (after extraction) in a buffer compatible with IEF and SDS-PAGE. Since the inception of the method in 1975 [170] significant changes have been made, many relating to advances in IEF buffer constituents [231]. To avoid disrupting the physiological protein ratios in an extracted sample a solubilising buffer system for 2-D electrophoresis must achieve a number of aims.

1. Proteins must be denatured
2. Artefactual changes to the polypeptides must be prevented
3. Proteins/polypeptides must remain soluble throughout the extraction and separation process
4. Non-protein substances that interfere with separation protocols must be removed

To denature proteins the main forces that hold them together must be disrupted, i.e., disulphide bridges, ionic bonds, hydrogen bonds, and hydrophobic interactions. Disulphide bridges can be broken by the addition of a thiol compound to the solubilising medium, mercaptoethanol, DTT and tributylphosphine (TBP) have all been used in 2-D electrophoresis for this purpose [172]. Mercaptoethanol is not suited to IEF as it can ionise at basic pH destroying the gel embedded basic pH gradient resulting in poor separation of proteins at basic pH [369]. TBP is an effective denaturant but is volatile, toxic and requires stringent safety procedures in use. During the optimisation of protein solubilisation buffers (Section 5.2.2) TBP was considered as an alternative to DTT, but its use did not improve on the results achieved using DTT (results not shown). DTT was already present in the lysis solution (Section 2.4.3) and the concentration was increased for IEF by additions to the sample after

lysis (Table 2.4). DTT was therefore adopted as the preferred denaturing agent due to its ease of use and less stringent handling characteristics and effectiveness as a denaturing agent.

Non-covalent protein bond interactions can be disrupted by the addition of a chaotropic compound (such as urea) to the solubilising buffer. Urea is efficient at breaking hydrogen bonds but less efficient than other substituted ureas in breaking hydrophobic interactions [370]. The use of urea alone may create problems with the solubility of proteins at their isoelectric point and the only solution to this problem is to increase the chaotropicity of the solubilising solution by the use of both urea and thiourea as chaotropic compounds [172, 234]. Urea addition can also cause an increase in protein bond hydrophobic interaction and this was combated by the addition of an uncharged detergent. SDS is very effective at breaking hydrophobic interactions but is not compatible with 2-D electrophoresis unless removed prior to IEF. In the case of 2-D electrophoresis zwitterionic or non-ionic detergents such as Triton, Nonident P-40, Sulphobetaine, and CHAPS may be used. In this study Triton X100 and Nonident P-40 were considered but failed to produce 2-D gel images of the same quality as those produced when 4% CHAPS was used (results not shown). Sulphobetaine detergents are an expensive option and in previous studies have not improved upon results obtained with the use of 4% (w/v) CHAPS [371].

The majority of publications using 2-D electrophoresis show considerable variability of method, particularly with respect to protein solubilising conditions. These variations may well represent the variety of material and samples that have been under investigation. To determine the optimal buffer constituents for protein solubilisation a wide range of solubilising solutions was investigated (Table 2.3). The majority of options investigated produced very poor protein solubilisation and subsequent 2-D gel image results (results not shown). Five of the options initially investigated were subject to additional experimental investigation (Table 2.4). Aliquots of the same sample of *L. monocytogenes* extract were used to determine the protein solubilising capability of each buffer. After solubilisation each sample was subject to a modified Bradford assay to determine the protein concentration. The results showed that Buffer I (Figure 2.4) produced the maximum solubilised protein content when compared with the other buffer systems investigated. The observed difference in protein concentration between buffers was minimal (Figure 2.4). The result showed that changes in a protein solubilising solution affected the capacity of that buffer to solubilise all the proteins present in a given sample. However, the quantitative results produced from samples solubilised in each buffer were not sufficiently different to allow the selection of an optimised protein solubilising buffer.

5.3.3.1 The effect of acetone precipitation on sample protein concentration

The extracted cell lysate contains proteins of interest but will also contain other cellular constituents including DNA, RNA, membrane fragments, and protease enzymes. These constituents can alter the state of the extracted proteins and interfere with 2-D electrophoresis [357]. DNA in particular has a detrimental effect on the separation of proteins by IEF for a number of reasons. DNA complexes are dissociated and cause increased sample viscosity, this hinders the passage of proteins into the IPG gel and can slow migration within the gel [340]. DNA can also bind to proteins in cell extracts giving an artificial shift in position during IEF migration on an IPG gel, which causes streaking and poor resolution [372]. Particulates were removed by centrifugation but other constituents were difficult to remove and required some form of intervention. DNA and RNA were digested by the inclusion of a DNAse/RNAse incubation step immediately after sonication (Section 2.4.5). Protease inhibitor cocktail tablets and EDTA were also included in the lysis buffer to prevent the proteolytic degradation of extracted proteins (Section 2.4.4.2).

An alternative method for the removal of non-protein sample constituents is to include an acetone/TCA precipitation. This is an effective way of removing the majority of non-protein inclusions in a lysed cell sample although lipids and detergents would still remain in the sample [230-232]. Acetone/TCA precipitation was evaluated as a method for the removal of non-protein sample inclusions. After lysis samples the base sample buffer constituents were either adjusted to increase solubility and compatibility with IEF (Table 5.0), or subjected to an acetone/TCA precipitation. The total protein content of 10 aliquots of extracted protein solubilised in 1 of 5 buffer systems was compared (Figure 5.3). The results showed that an acetone precipitation step introduced considerable variability in protein concentration between aliquots of the same sample. Some loss of total protein volume was observed when the acetone precipitated protein concentration results were compared to non-acetone precipitated protein concentration results. Precipitated proteins pellets were difficult to solubilise and required considerable agitation over an extended period of time (up to 4 hours). This may have been the source of variation in protein concentration between sample repeats.

The 2-D separation and image analysis of samples subjected to acetone/TCA precipitation were also highly variable (results not shown). Therefore acetone/TCA precipitation was not adopted as a means of the removal of non-protein sample contaminants from *L. monocytogenes* whole cell extract prior to separation by 2-D electrophoresis.

5.3.4 Evaluating protein solubility: a qualitative assessment

The evaluation of protein solubility results discussed in Section 5.3.3 was comparative for total solubilised protein only. The results did not apply a qualitative assessment of the buffer systems resolving ability. Resolving ability is partially dependent on the ability of a given buffer system. Resolving ability can be defined as the ability of a buffer system to cleanly separate individual proteins with a low background from a whole cell extract by 2-D SDS-PAGE. The resolving ability of the protein solubilising buffers 1 to 5 (Table 5.0) was assessed by separating *L. monocytogenes* whole cell extracts using 2-D SDS-PAGE.

Image analysis was used as a measure of the ability of a specified buffer system to cleanly separate a complex protein mixture by comparing the total number of detected protein spots that each system produced. The spot detection results showed that protein solubilising buffers 1 and 2 produced better resolution and higher total detected spot numbers than buffers 3 to 5 (Figure 5.4). Protein solubilising buffer 2 had a higher molar concentration of urea and thiourea than that in buffer 1 and during IEF, samples solubilised in buffer 2, some crystallisation of urea out of solution, was noted. In conjunction with the protein solubilisation buffer quantitative results (Figure 5.3) it was clear that buffer 1 (Table 5.0) produced a higher protein yield (concentration) and total spot detection figure than alternate buffers, therefore this buffer was adopted as the standard for all future experiments.

5.3.5 Protein visualisation using small format 2-D SDS-PAGE

As with all other aspects of sample preparation for 2-D electrophoresis, the staining of proteins for visualisation and image analysis must be reproducible. There are a wide variety of protocols and manufactured stains available for the visualisation of proteins on acrylamide/bis gels such as colloidal coomassie, fluorescent stains and silver stains. Silver staining methods are about 10-100 times more sensitive than various coomassie blue staining techniques. Consequently, they are the method of choice when very low amounts of protein have to be detected on electrophoresis gels. A number of silver staining protocols have been published, using the silver nitrate staining technique of Merril *et al.* [373] and subsequent modifications by many others [235, 239, 374]. Fluorescent stains are also popular in proteomic research as they are at least as sensitive as silver stain and have the advantage of remaining quantitative over a wider linear concentration than silver stain [238, 349]. A number of standard protein stains were selected on the basis of sensitivity, reproducibility, ease of use and the protein concentration range over which quantitative analysis was possible.

The sensitivity of brilliant blue coomassie stain [347, 348] was low at 50-100 ng (Table 5.2) and was not suited to a proteomic investigation. Colloidal coomassie stain was more sensitive and detected protein at 20 ng concentration (Figure 5.6). The use of colloidal coomassie was investigated further with increased protein loads (Section 5.3.8). Fluorescent protein stains SYPRO orange and SYPRO ruby [238] detected proteins at between 5 ng and 10 ng concentrations and were suited to protein identification by peptide mass fingerprinting [176]. However, these stains required UV illumination for visualisation and specialised scanning equipment for quantitation that was not available. Additionally, SYPRO stained proteins of high abundance produced an area of flare that could mask less abundant proteins in close proximity within the same image (Figure 5.6, Image B). Three silver stain methods were investigated and shown to have similar sensitivity (5 ng Table 5.2). An important aspect of proteomic investigations is the ability to identify proteins of interest from a 2-D gel. In-gel tryptic digests and peptide mass fingerprinting (mass spectrometry) is commonly used to achieve this [235, 348, 375, 376]. The Shevchenko silver stain method [235] had been specifically developed for use with mass spectrometry and was adopted as a standard for all 2-D protein visualisation analyses.

5.3.5.1 Quantitative range and image reproducibility

Successful image analysis is based on the ability to produce gel images where the proteins from different gels that have the same concentration have been equally stained. If the stain protocol could be standardised for gels produced from different samples, then changes of protein expression occurring between samples could be quantified (Section 2.7.3). However, quantitation is dependent on the range of protein concentrations over which a stain remains quantifiable. This range for the SYPRO fluorescent stains spanned 1 ng to 1000 ng, although quantitation at this range is achieved only with a single protein using laser scanning devices that are not universally available [238, 349]. Colloidal coomassie also has a wide linear range over which proteins remained quantifiable but low sensitivity precluded its use. Of the stains investigated this leaves silver stain with a linear range of 5 to 100 ng. The selection of silver stain as a standard stain was a compromise between sensitivity and quantifiable range. Silver stain has the lowest quantifiable linear range but the highest sensitivity and does not require UV illumination or specialised equipment for image capture. The Shevchenko silver stain method (section 2.9.3) allowed easy manipulation of the stain development step and provided a limited means of standardising stain uptake between different gels. The amount of data that could be extracted from silver stained gel images relating to quantitation of individual protein spots was limited by the low linear range of this stain, and its selection as a standard stain was a compromise between low quantifying data and high sensitivity.

5.3.6 Transition to large format 2-D SDS-PAGE

Mini gel 2-D SDS-PAGE was used in all protocol development experiments, however to increase resolution large format gels were adopted. A large gel format allowed the protein load to be increased from 10 μg/125μL used for 7 cm IEF separations to 100 μg/350μL for 18 cm IEF separations. The increase in protein load and gel area (from 7 x 7 x 0.05 cm to 18 x 20 x 0.05 cm) increased the total detected spot count from 234 on a small gel to 959 on a large gel (Table 5.3). Many more proteins were visualised on larger gels and the increase of analytical area improved separation and resolution.

5.3.6.1 Vertical and horizontal 2-D SDS-PAGE

A comparison of two images produced from vertical and horizontal electrophoresis (Figure 5.7) equipment showed that the protean II SDS PAGE equipment (Section 2.6.11) was superior to the Multiphore horizontal SDS PAGE equipment (Section 2.6.12) in its ability to separate more proteins from aliquots of the same *L. monocytogenes* whole cell. This result probably reflected the increased optimisation preparations that had been undertaken when using the Protean II system. However, the Multiphore II system could not be used to run simultaneous gels, whereas the Protean II system allowed up to 4 gels to be run simultaneously, an important factor where consistent production is a goal. The Protean II SDS PAGE equipment was adopted as standard for large format 2-D electrophoresis in combination with the IPGphor IEF equipment.

5.3.7 Selection of IEF resolving range

It is a recognised limitation of proteomic analysis that basic proteins are generally poorly resolved [344, 377]. This limitation has in part been due to the limited availability of stable buffer systems for basic protein separations. Some proteomic investigations have separated basic proteins [203, 208]. However, despite advances in immobilised gradient technology [378] proteomic investigations still avoid the separation of basic proteins due to the inconsistent nature of the results produced [379, 380].

Using the optimised protocols for sample preparation and proteomic analysis of *L. monocytogenes* whole cell protein extract (Scheme 2.4), gel images could be compared from different growth states. Initial protein separations were conducted using a pH 3-10 IPG gel resolving range. This allowed the visualisation of 959 proteins. Image analysis showed that not all proteins were equally well resolved. Proteins separated between pH 3-4 and pH 7-10 were poorly resolved (Figure 5.8). Proteins in the basic range appeared less focused than proteins in the pH 4-7 resolving range. Within the pH 4-7 resolving range on a pH 3-10 gel,

815 protein spots were detected. When the same sample aliquot was separated on an 18 cm pH 4-7 resolving range IPG gel, 1025 protein spots were detected. Expanding the area over which the pH 4-7 proteins were separated increased, resolution, individual spot separation and total spot detection. The gel sections outside the pH 4-7 range on a pH 3-10 separation, represented 40% of the total gel area over which only 15% of the spots detected were present, whereas on the same gel the pH 4-7 separating range covered 60% of the total gel area over which 85% of the spots detected were separated. The proteins below pH 4 and above pH 7 were poorly resolved and it has been recently shown that the basic portion of wide pH (pH 3 – 10) range sample separations can contain a large number of spurious and incorrectly separated protein spots [381]. The 143 proteins that were separated outside the pH 4-7 resolving range were discarded in favour of better separation of the proteins within the pH 4-7 resolving range. IPG 'zoom gels' exist that separate proteins over a single pH range, and are available at basic pH, however the optimisation of samples has been directed toward improved resolution over a pH 4-7 range [379]. The pH 4-7 resolving range using IPG 18 cm gels was accepted as standard and used throughout. The *L. monocytogenes* genome has been shown to contain 2853 protein coding open reading frames. On this basis the pH 4 –7 18 cm 2-D gel image presented herein represent 36% of all the theoretically possible proteins represented by the number of open reading frames published.

5.3.8 Analysis of the *Listeria* proteome using samples grown in BHI and PTM media

When proteome maps of *L. monocytogenes* grown in different media (and harvested at the same growth point) were compared, no protein pattern differences were observed between samples. The result does not definitively confirm that no changes of protein expression were induced by a change of medium, it simply confirmed that within the parameters selected for the separation of a *L. monocytogenes* whole cell extract by 2-D SDS-PAGE no changes were apparent.

5.3.9 High protein loads visualised using colloidal coomassie

Colloidal coomassie stain is not as sensitive as silver stain and visualises very few protein spots when an 18 cm IPG gel is loaded with 100 μg/350mL protein. The number of spots detected at this concentration was low compared to silver stain (Figure 5.12). The simplest way to increase the number of spots detected on a 2-D gel would be to increase the protein load on the IPG gel [354]. Alternatively the thickness and width of the IPG gel could be increased allowing greater loading capacity [382]. This was not considered as an option here

as the commercially available IPG strips used were only supplied in 0.5 mm thickness and 3 mm width (Amersham Pharmacia, UK). It has also been demonstrated that protein load could be increased by increasing the sample volume loaded onto 18 cm IPG strips [382]. Herein, the sample volume (350 μL) was not increased beyond that recommended by the gel supplier (Amersham Pharmacia UK). The amount of protein loaded onto an 18 cm IPG gel was increased to loading 1050 μg/350 mL (3 mg/mL). At this protein load the IEF separation of the mixture was extended to 60 hours duration to ensure complete separation by isoelectric point. Colloidal coomassie protein stain at 1050 μg/350 μL did not visualise the same number of protein spots as a silver stained at an analytical protein loads (100 μg/350μL). Figure 5.15 clearly shows that the total number of protein spots detected by silver stain could not be reproduced using colloidal coomassie stain even when increased protein loads and an extended IEF focusing protocol was used. A high protein load with Colloidal coomassie was not adopted as an alternative to Shevchenko silver stain for the visualisation of proteins in 2-D gels.

5.3.10 Sample preparation initiated stress response

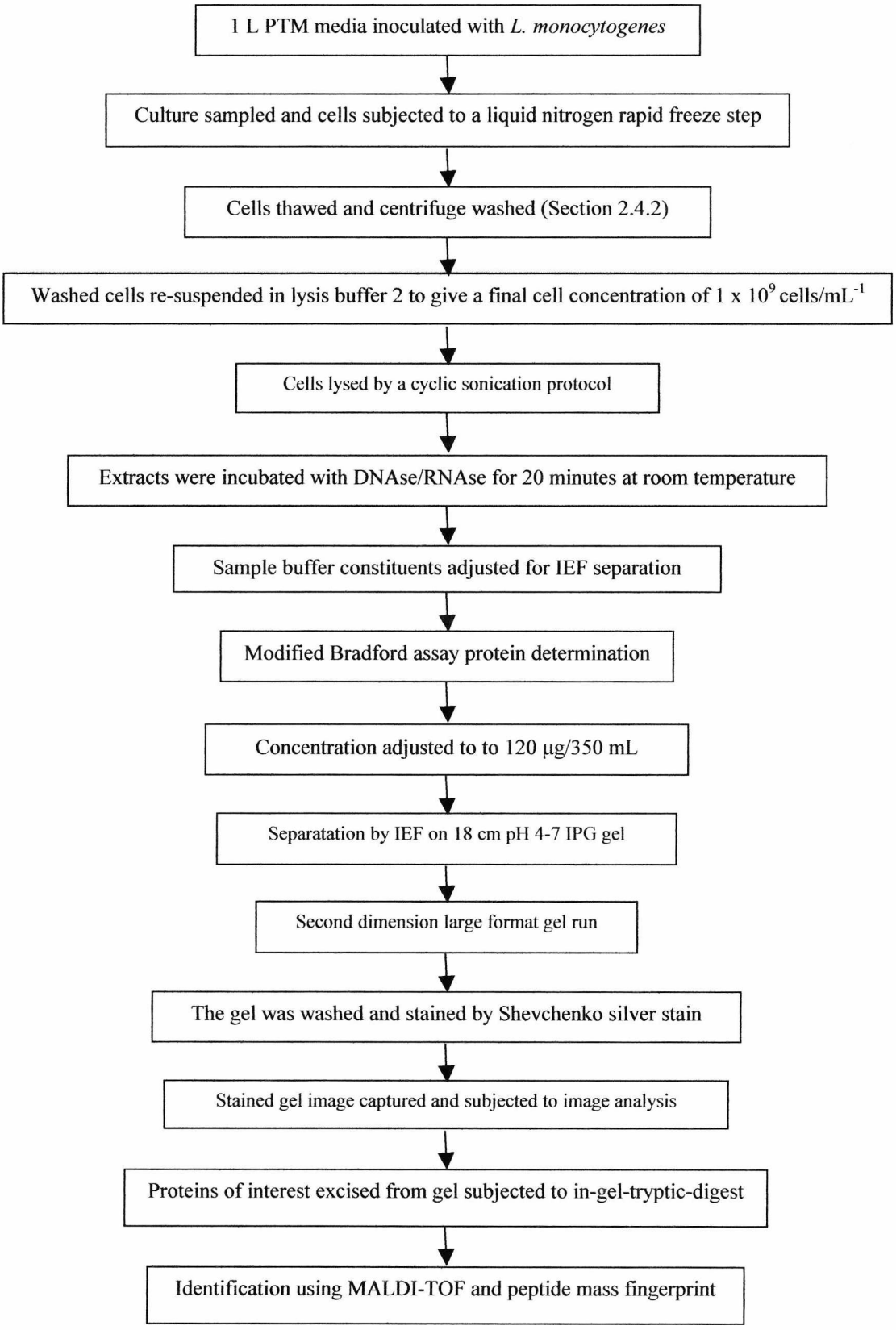
L. monocytogenes culture was sampled and prepared for separation by 2-D SDS-PAGE with the exclusion or inclusion of a liquid nitrogen fixing step (Section 3.9). Gels produced from samples that were not liquid nitrogen fixed had 50 more protein spots than gels that were liquid nitrogen fixed. The observed difference in total detected spot numbers between different aliquots of the same sample (Figure 3.3) was due to the inclusion or exclusion of a nitrogen fixing step, although variability between single gels produced from the same sample could also produce a difference of 50 protein spots or more. The conclusion drawn from these results was that the increase in spot numbers was due to the continuation of protein production beyond the required harvest point due to the induction of a stress response.

A protein spot excised from a non-fixed gel (a protein that did not appear in a fixed gel) was identified as RNA polymerase sigma factor (SigmaB). RNA polymerase sigma factor is an initiation factor that promotes attachment of the RNA polymerase to specific initiation sites and is then released (identified by similarity to *B. subtilis* homologue). The protein was originally identified in 1998 and its role has been linked to osmotolerance, acid resistance and virulence [132, 271]. Others have demonstrated that during cold shock, SigmaB contributes to adaptation in a growth-phase dependent manner and is necessary for efficient accumulation of betaine and carnitine as cryoprotectants [272]. The role of this protein has therefore been associated with a number of different stress related responses. Other sigma factors have been

identified in similar positions on 2-D gels that have been ascribed roles in the stress response [275, 276]. It is therefore most likely that stress related to sample preparation induced the expression of RNA polymerase sigma factor, whereas immersion of samples in liquid nitrogen fixed the proteome of the organism at the point of harvest, as the non-stressed sample did not contain the RNA polymerase sigma factor B. This demonstrates the value of fixing a sample at the point of harvest to halt any artefactual stress response appearing in subsequent 2-D gels. A liquid nitrogen fixing step was therefore included in all subsequent sample extraction protocols.

5.3.10 An intergrated proteomic platform for the production of proteome maps using *L. Monocytogenes* whole cell extract

As a result of the optimisation experiments undertaken here, it was possible to develop a standardised scheme for sample preparation and separation of *L. monocytogenes* whole cell extracts by 2-D SDS-PAGE. The optimised standardised Scheme is presented over page (Scheme 5.2)



Scheme 5.2 Optimised proteomic platform for sample preparation and analysis of the *Listeria* proteome by 2-D electrophoresis.

6.0 Analysis of the *Listeria* proteome throughout growth and stationary phase

6.1 Introduction

Up until now monitoring a *L. monocytogenes* PTM batch culture throughout exponential growth and stationary phase has shown that stationary phase occurs at or about OD_{600} 1.1 and at a culture age post inoculation of 21–22 hours. This is the point where cell numbers appear static (this maybe a state of dynamic equilibrium where the number of cells dying equals the number of cells dividing), although OD_{600} readings continued to rise to a point at 48 hours and OD_{600} 1.575 (culture age post inoculation) where data recording was halted (Chapter 3, Figures 3.2 and 3.3). These methods of growth curve determination provide a basis for the selection of reproducible sample extraction points between cultures but provide no information on the changing physiological and morphological state of the cells as they enter stationary phase. Chemical analysis of the culture PTM medium throughout growth and stationary phase has shown that the rate of amino acid and glucose utilisation falls towards stationary phase (Chapter 3, Figures 3.7, 3.9 and 3.10). Although these results indicated falling metabolic activity as growth advanced towards stationary phase the nature of changes taking place that lead to such a fall could not be shown.

In addition to OD and viable counts methods such as medium pH, cell motility, gross protein synthesis, medium chemical analysis and membrane permeability have been used to define a point during growth where stationary phase began. The results have show that identification of this point is difficult to define and each method provided a different result. Moreover, the results also varied between methods (Chapter 3 Table 3.3) when defining the length of the transition period from exponential to stationary phase. The transition to stationary phase is an important event as it represents the point where cells are preparing for long-term growth arrest. It can be viewed as a survival mechanism that prepares a cell for extended periods of starvation and leaves the cell more resistant to environmental challenge [273, 376]. During the transitional period global changes in gene expression are undertaken to bring about the physiological and morphological changes required for long term survival [267]. Moreover the point where a dynamic equilibrium (stationary phase) changes to a point of static equilibrium can be described as the point of transition from stationary phase to growth arrest.

6.0 Analysis of the Listeria proteome throughout growth and stationary phase

The majority of physiological and pathological processes including the transition from exponential growth to stationary phase are linked to a corresponding changes in quantitative gene expression. However studies undertaken at the DNA or mRNA level cannot provide data on relative protein expression as mRNA levels and protein levels within cells do not correlate[383]. A study of the changes that take place during the transition to stationary phase of *L. monocytogenes* PTM batch cultures must be capable of the simultaneous identification of global changes to protein expression. A proteomic platform utilising 2-D SDS-PAGE can be used to identify specific changes in gene expression in response to environmental change such as those encountered in batch culture at the onset of stationary phase [199]. A proteomic platfrom has been developed (Chapter Five) that allows proteome images of *L. monocytogenes* PTM batch cultures to be compared.

In this Chapter consecutive proteomes will be imaged and the comparative analyis of such images will be used to identify proteins important to the onset of stationary phase. The point in time that specific proteins were expressed was shown. Furthermore, once these proteins were identified semi-quantitative analysis provided a pattern of expression throughout growth and stationary phase. The identification and semi-quantitation of proteins important to the onset of stationary phase may highlight temporally importat proteins and identify the point where stationary phase begins.

6.2 Results

6.2.1 Analysis of the *Listeria* proteome at exponential and stationary phase

Stationary phase can be described as the point during growth where a culture shows no further increase in cell numbers (Section 3.2.2.1) but can be more accurately described as the point in the exponential phase when all cellular parameters cease increasing at equal rates (i.e. DNA, protein, and total cell mass, no longer increase together) to the point where no further increase in cell numbers is detected. In this investigation stationary phase has been defined as the OD_{600} where cell numbers become static (determined using viable count). *L. monocytogenes* PTM (500 mL) batch cultures reached this point at OD_{600} 1.1.

L. monocytogenes PTM batch cultures (500 mL) were inoculated and allowed to grow as described earlier. The culture was then sampled at an OD_{600} at 0.25 and 1.1. The resulting samples were then extracted and separated by 2-D electrophoresis using optimised protocols previously detailed in Scheme 5.2.

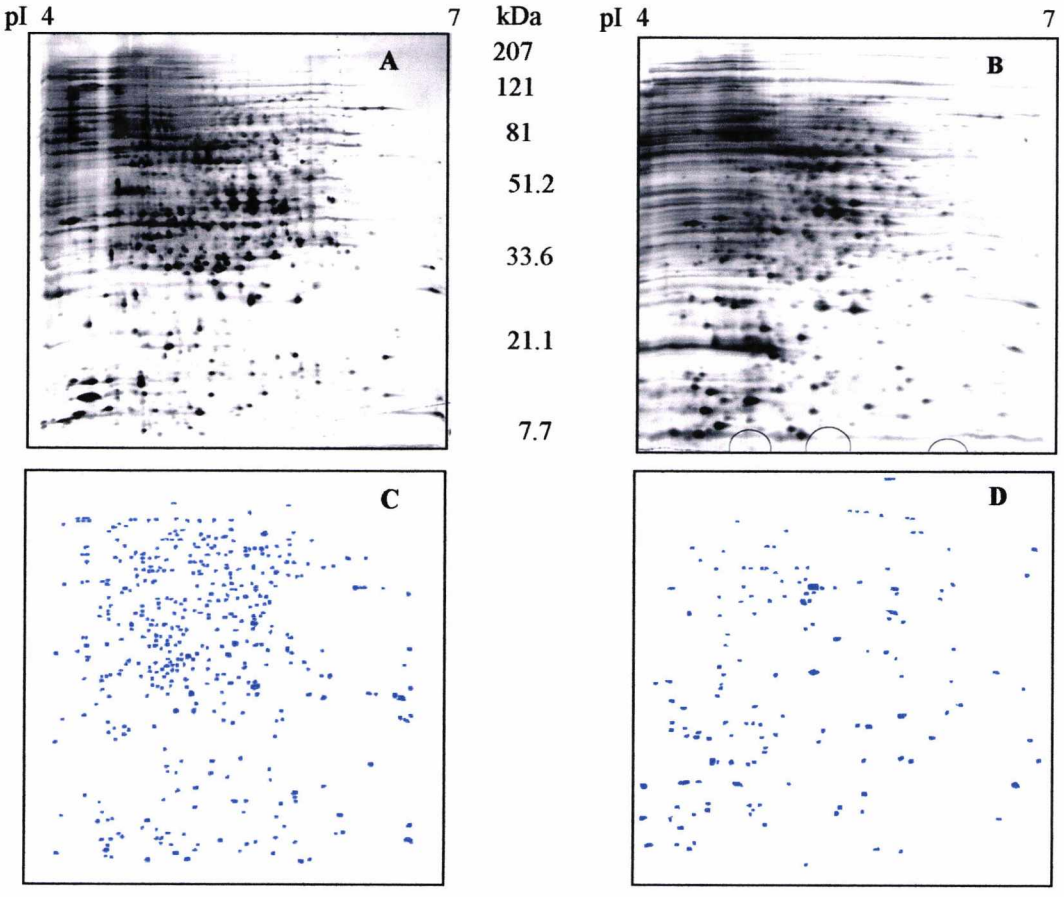


Figure 6.0 Differences in the *Listeria* proteome at exponential and stationary phase. Figure A, *L. monocytogenes* proteome at OD_{600} 0.250, 975 proteins. Figure B, *L. monocytogenes* proteome at OD_{600} 1.1, 701 detected proteins. Figure C, 414 spots detected using at exponential phase not detected at stationary phase. Figure D 140 spots detected at stationary phase not detected at exponential phase.

Figure 6.0 clearly shows that *L monocytogenes* PTM batch cultures entering stationary phase express fewer proteins than they were previously expressing at exponential phase (i.e. down regulation). Figures 6.0 also clearly shows a large change in the proteome of *L. monocytogenes* between active growth at exponential phase and stationary phase. The figures show that of the 975 proteins expressed at exponential phase, 414 (42%) were not expressed at stationary phase, whilst of the 701 proteins expressed at stationary phase, 140 (20%) were newly synthesised. Therefore 561 proteins (58%) are expressed at both exponential phase and stationary phase of *L. monocytogenes*. These results assume that all the spots visualised were proteins and not experimental artefacts or the breakdown products of existing proteins. Furthermore, these are only qualitative results and are based on observed numeric differences between samples at exponential and stationary phase. Nevertheless, a clear pattern of up and down regulation of specific proteins was observed as *L. monocytogenes* cultures enter stationary phase from exponential growth phase. Using the same images (Figure 6.0, A and B), ImageMaster software was used to identify proteins between exponential and stationary phase that showed at least a two-fold quantifiable increase or decrease in expression levels.

A proteomic map showing the position of matched spots undergoing a two-fold (or more) quantifiable change of expression is shown in Figure 6.1.

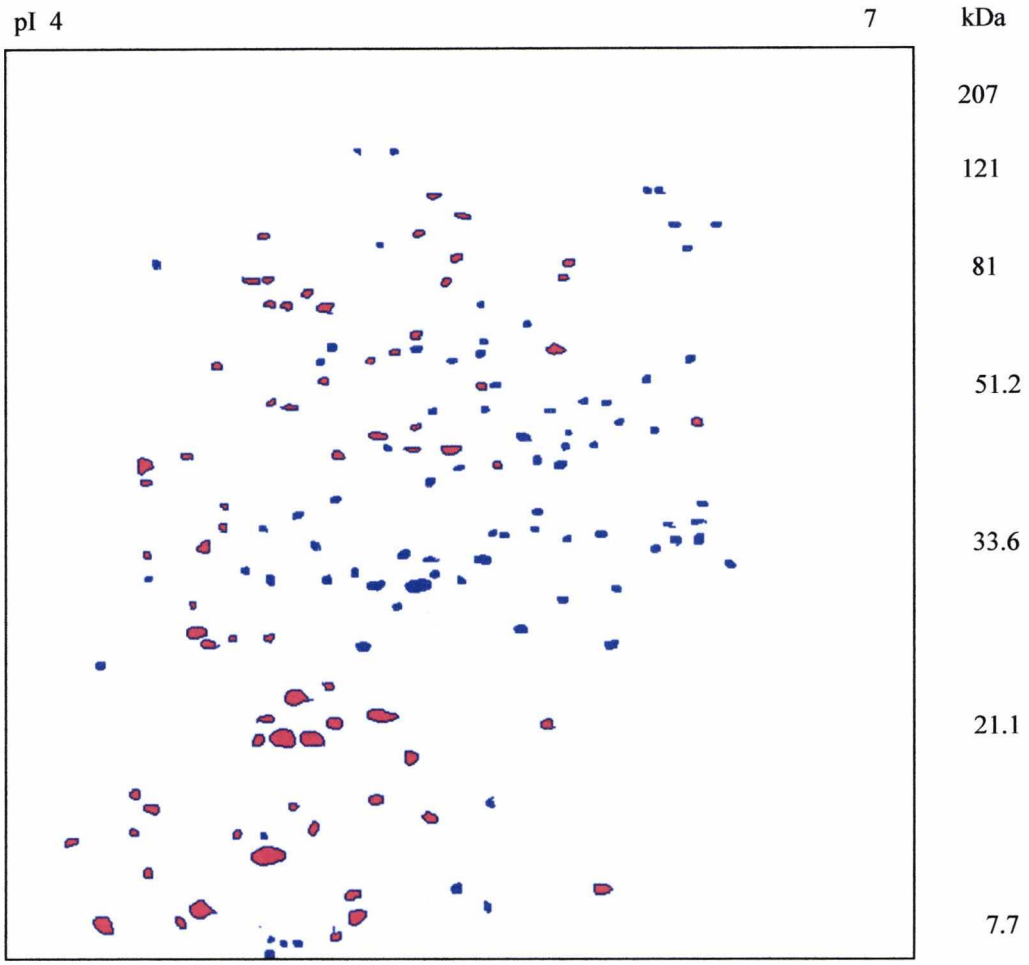


Figure 6.1 Difference map of *L. monocytogenes* derived from the analysis of exponential and stationary phase showing proteins that exhibited a two-fold change in expression levels. The image shows proteins that had a two-fold change in expression. Only proteins matched between each condition that had a two-fold or greater change of expression are shown. ● 66 matched proteins showed a two-fold increase in expression at stationary phase. ● 88 matched proteins showed a two-fold decrease in expression at stationary phase.

Figure 6.1 shows that of the 561 proteins conserved between exponential phase and stationary phase, 28% were either up or down regulated above the applied two-fold limit. The results from Figures 6.0 and 6.1 were collated and show that the total number of proteins expressed throughout growth (including *de novo* synthesis at stationary phase) totalled 1115. Therefore of all the proteins expressed at exponential and stationary phase only 36% did not show a two-fold or more increase or decrease in expression. This implies that 64% of all the detected proteins are undergoing a change in expression level between exponential phase and stationary phase. However, culture sampling at two fixed time points (exponential phase and stationary phase) does not provide any information of the temporal separation of the observed changes in protein expression. It is very unlikely that such a large change in expression would occur as a single event, and would more likely occur over an extended time scale throughout

growth and transition to stationary phase. Furthermore, it is possible that proteins observed in only one of the time points were actually present but at a level below the sensitivity of silver stain. In order to investigate the temporal separation of the changing expression of proteins throughout this time period samples were taken throughout growth and stationary phase and subjected to 2-D analysis.

6.2.2 Analysis of the *Listeria* proteome throughout growth and stationary phase

L. monocytogenes PTM batch cultures (500 mL) were inoculated and allowed to grow as previously described. The cultures were sampled throughout growth and stationary phase over a 48 hour period at OD_{600} of 0.25, 0.4, 0.55, 0.75, 0.9, 1.1 and 1.575. The samples were then subjected to 2-D electrophoresis analysis using the optimised protocols detailed in Scheme 5.2. Averaged images of each sample were made and compared (three images per averaged sample). The total number of visualised and detected protein spots at each sample point was determined and the results are shown in Figure 6.3.

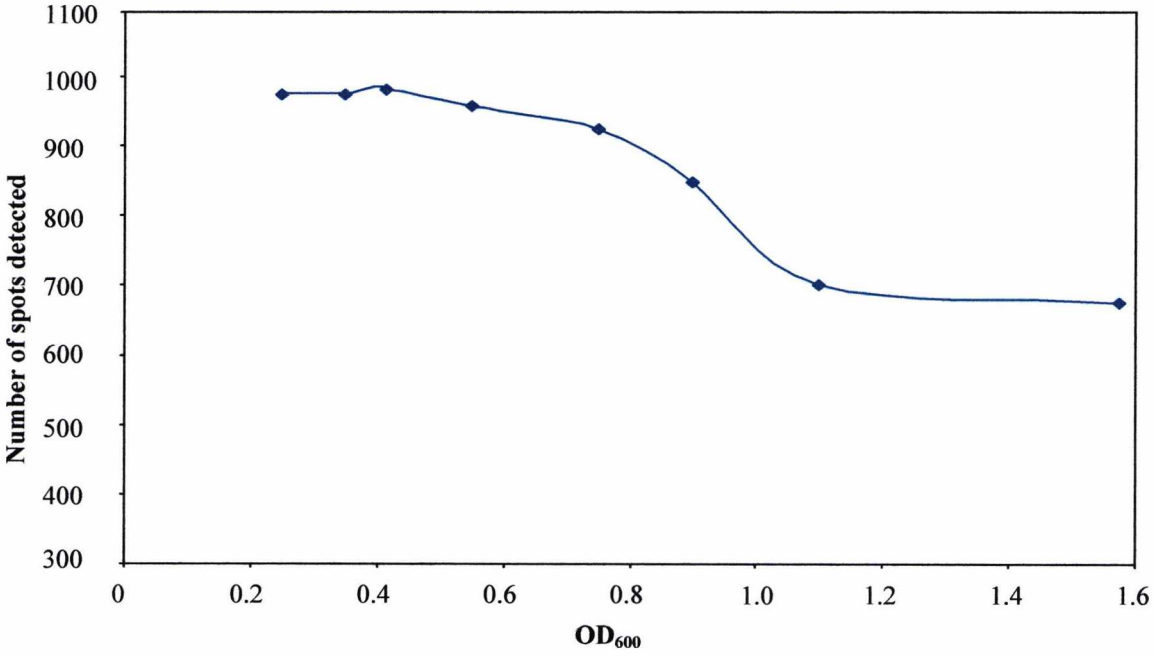


Figure 6.2 Total number of *L. monocytogenes* proteins visualised throughout growth and stationary phase by 2-D SDS-PAGE.

Figure 6.2 shows that the total number of proteins expressed by a *L. monocytogenes* PTM batch culture falls as growth progresses to stationary phase. Between inoculation and an OD_{600} of 0.75 the total number of detected proteins fell from 975 to 925, although an increase of 7 detected proteins was observed between OD_{600} 0.3 and 0.4. Between OD_{600} 1.1 and 1.575 the total number of detected proteins fell from 701 to 676. However, due to the inherent errors in

this type of proteomic analysis these differences are unlikely to be significant and we can say that the number of proteins was more or less constant over this period of growth (OD_{600} 0.25 to 0.75 and 1.1 to 1.575). However the majority of change occurred between OD_{600} 0.75 and OD_{600} 1.1 when the total number of protein spots detected fell from 926 to 701 (a difference of 225).

Image analysis of the changes observed in proteomic images based on total visualised spot counts does not reflect the extent to which proteins may be up or down regulated, or the extent to which proteins that are no longer expressed were replaced by newly synthesised proteins. In order to separate the loss of protein expression from *de novo* protein synthesis, averaged images at each sample point were compared to a single reference gel produced from a culture extract at OD_{600} 0.25. The analysis of these images in this way shows the number of down regulated proteins and newly synthesised proteins throughout growth and stationary phase. The collated results for each sample point are shown in Figure 6.3.

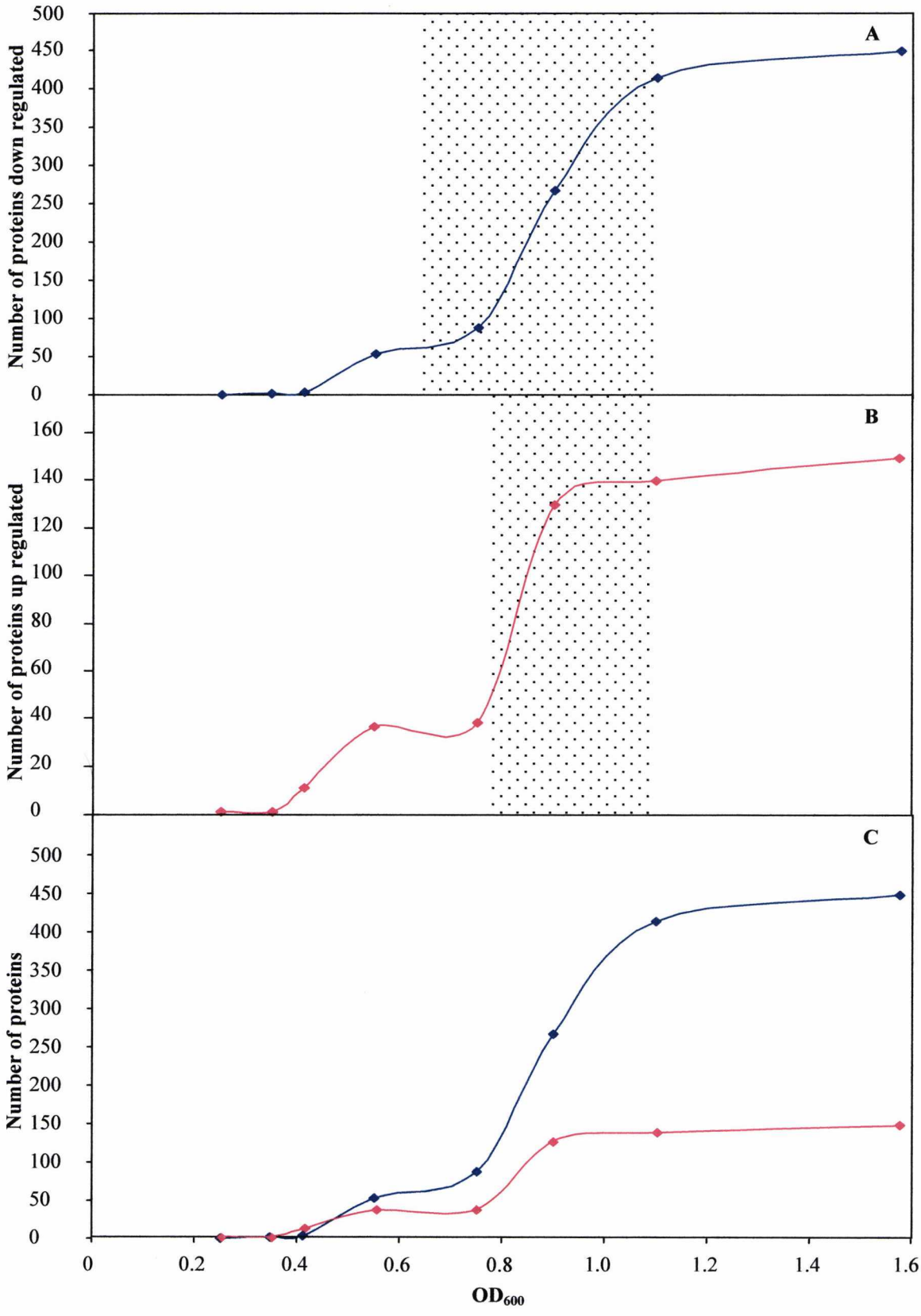


Figure 6.3 The number of up and down regulated proteins throughout growth and stationary phase relative to the total number of protein spots detected at OD_{600} 0.25. ◆ Number of protein spots detected at OD_{600} 0.250 not detected thereafter up to OD_{600} 1.575. ◆ Number of proteins not expressed at OD_{600} 0.25 that were expressed thereafter to OD_{600} 1.575. The shaded area ▒ represents the time span (OD_{600}) over which the majority of observed changes to the proteome were occurring. An up or down regulation of less than 50 spots was not considered significant due to the inherent errors in this type of proteomic analysis.

Figure 6.2 A, clearly showed that protein expression is fairly static throughout exponential phase up until OD_{600} 0.75 where a state of increasing flux develops. Proteins novel to stationary phase begin to appear and proteins novel to exponential phase are no longer expressed. This state of flux continues until stationary phase (determined at 1.1 OD_{600}) where a more static state returns and overall fewer proteins are expressed relative to the number expressed at exponential phase. The period of transition from exponential phase to stationary phase (shaded areas Figure 6.3, A and B) extends over a wide OD_{600} range and 6 hour time period. Furthermore, the observed numerical changes to the total number of proteins being expressed over this time were not linear (Figure 6.3). Between OD_{600} 0.25 and 0.75 (a period of 7.5 hours) a total of 40 proteins were induced while 90 were no longer expressed. Between OD_{600} 0.75 and 1.1 (a period of 7 hours) 100 proteins were induced while a further 324 were no longer expressed. Between OD_{600} 1.1 and 1.575 a period of 26 hours, a total of 10 proteins were induced while 35 were no longer expressed.

The results presented here show that the period of protein flux where the proteomic profile of *L. monocytogenes* undergoes the most change, extends over a majority of the growth curve. However, there are two points during growth when the proteome undergoes few changes, these being during early exponential growth when the culture was undergoing rapid cell division and protein production, and late stationary phase when the population count and protein expression levels were static. The image analysis results presented here accurately defined the time when the greatest changes in protein expression are occurring. An OD_{600} of 0.75 has been defined as the point during growth when the major changes in protein expression are induced.

6.2.3 The semi-quantitative analysis of *Listeria* proteins selected for identification by peptide mass fingerprinting

Image analysis has thus far been used to identify protein spots on 2-D gels whose level of expression was altered during growth and stationary phase. The time point at which changes of expression occurred was also identified. A number of proteins newly synthesised or no longer expressed at OD_{600} 0.75 and above were selected for further analysis on the basis that they exhibited a greater degree of change over time than other proteins exhibiting altered expression. The selected proteins were visualised at OD_{600} 0.25, 0.4, 0.55, 0.75, 0.9, 1.1 and 1.575. Images of the selected proteins are shown in Figures 6.4 and 6.5 and show that proteins 1, 3, 5 and 6 were newly synthesised during the transition to stationary phase of *L. monocytogenes* PTM batch culture. Proteins 2, 4, 7 and 8 were not expressed at stationary phase whereas proteins 9 and 10 were down regulated at stationary phase.

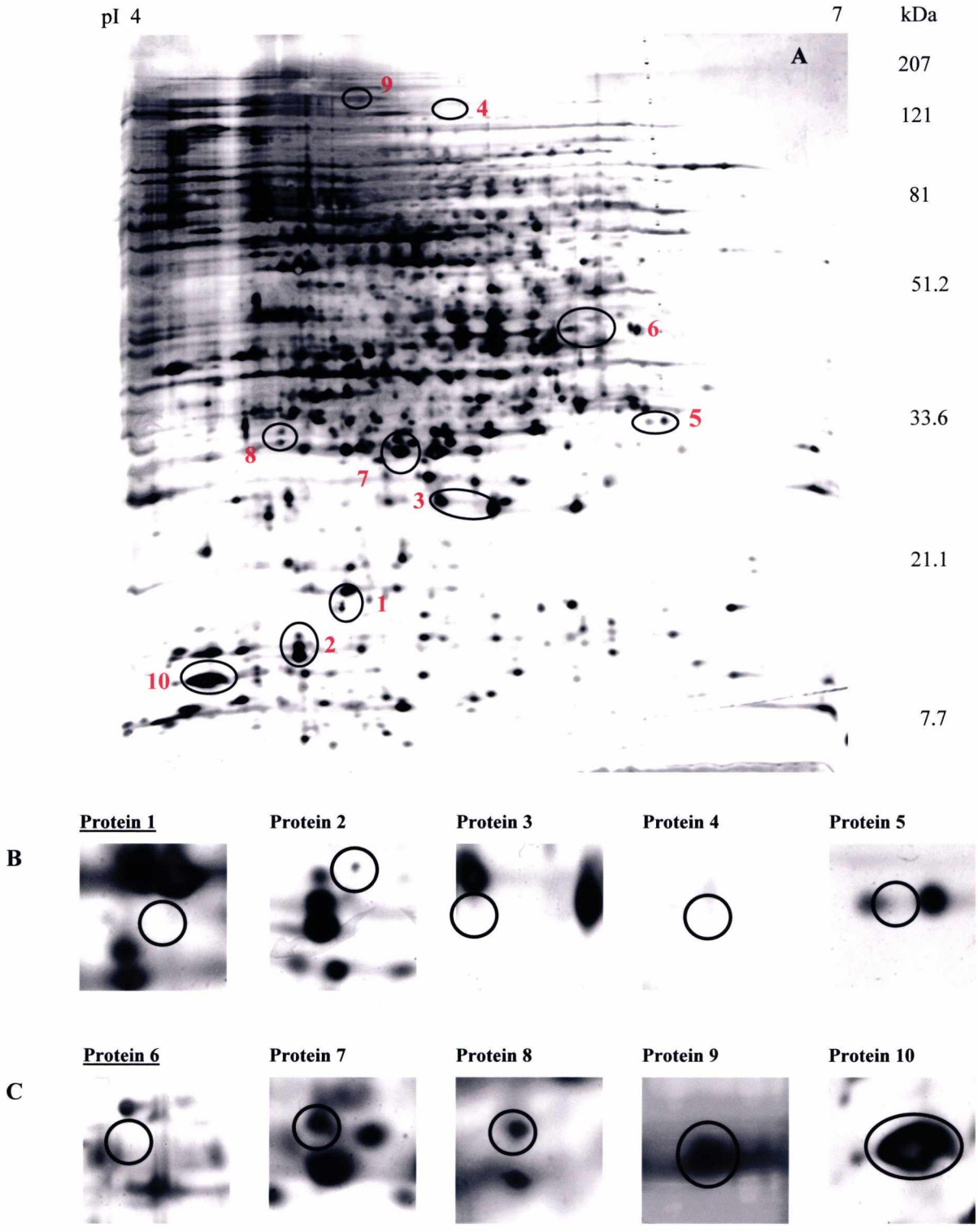


Figure 6.4 *L. monocytogenes* proteome at OD_{600} 0.25 showing areas of interest where the expression of specific proteins was up or down regulated. B) Expanded areas 1 – 5 of image A. C) Expanded areas 6 – 10 of image A

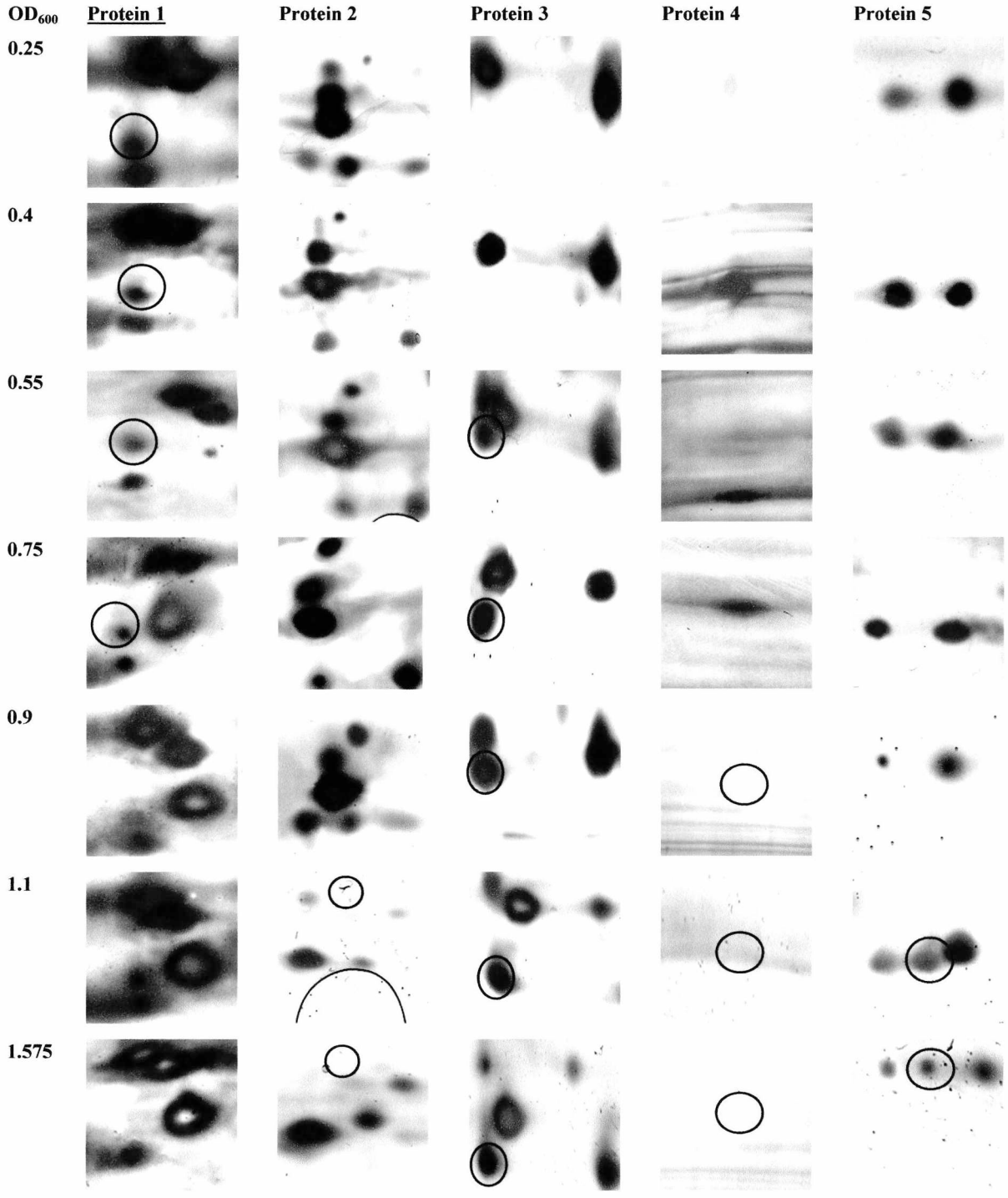


Figure 6.5 Proteins 1 – 5 from Figure 6.5 matched to protein spots at different sample points. Circled areas represent expanded image areas taken from different OD sample points (as detailed) Note: This is a reference gel image against which all samples were compared. The images below were taken from other gels; the image is presented to highlight the general area on other gels where matching proteins were found.

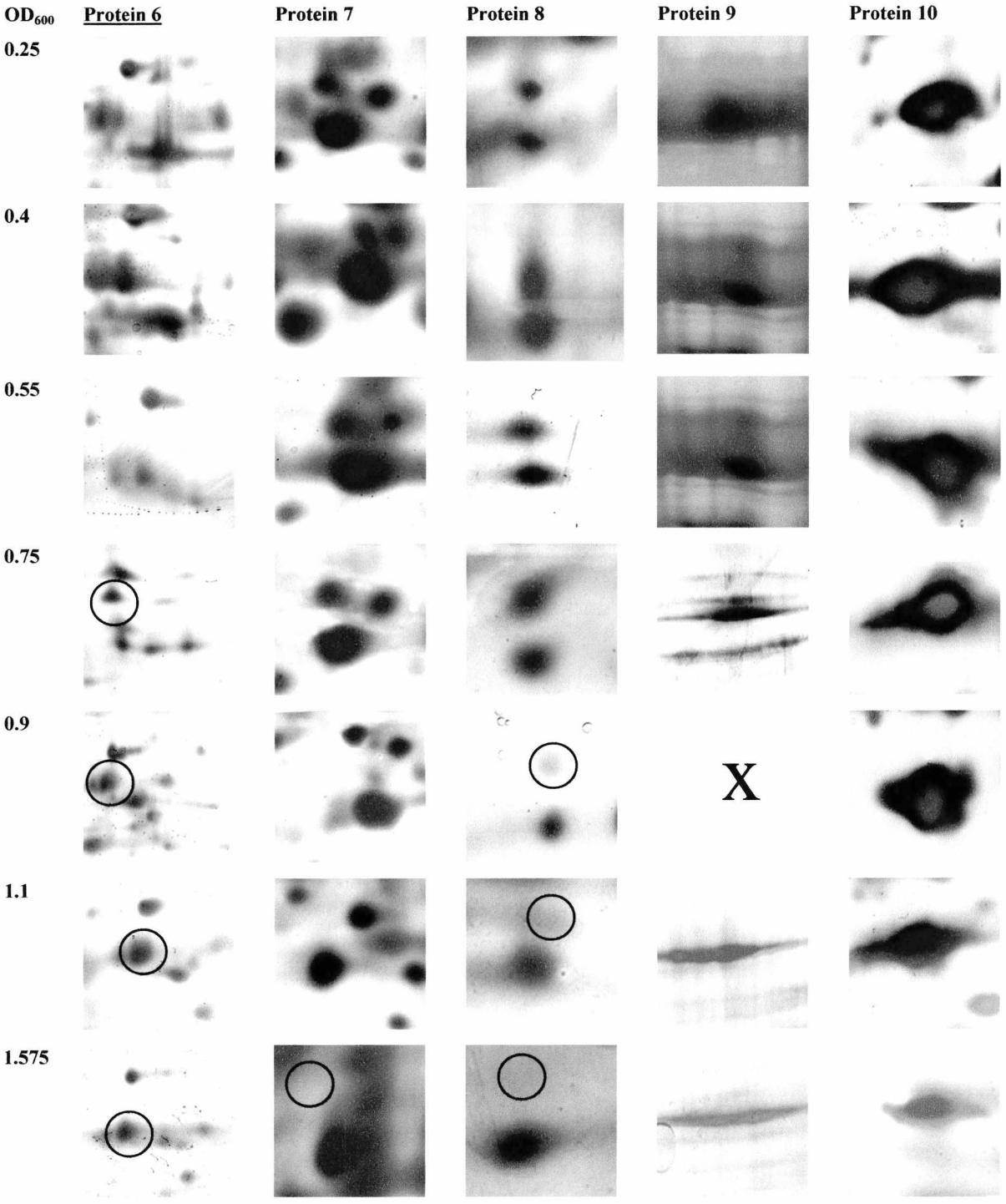


Figure 6.6 Proteins 6 – 10 from Figure 6.5 matched to protein spots at different sample points. Circled areas represent expanded image areas taken from different OD sample points (as detailed) Note: This is a reference gel image against which all samples were compared. The images above were taken from other gels; the image is presented to highlight the general area on other gels where matching proteins were found. (X) At this specific sample point the protein spot was not sufficiently resolved to extract reliable image or quantitation data

ImageMaster software was used to quantify the changing levels of expression of these selected proteins and the resulting semi-quantitation data is shown in Figures 6.7 and 6.8.

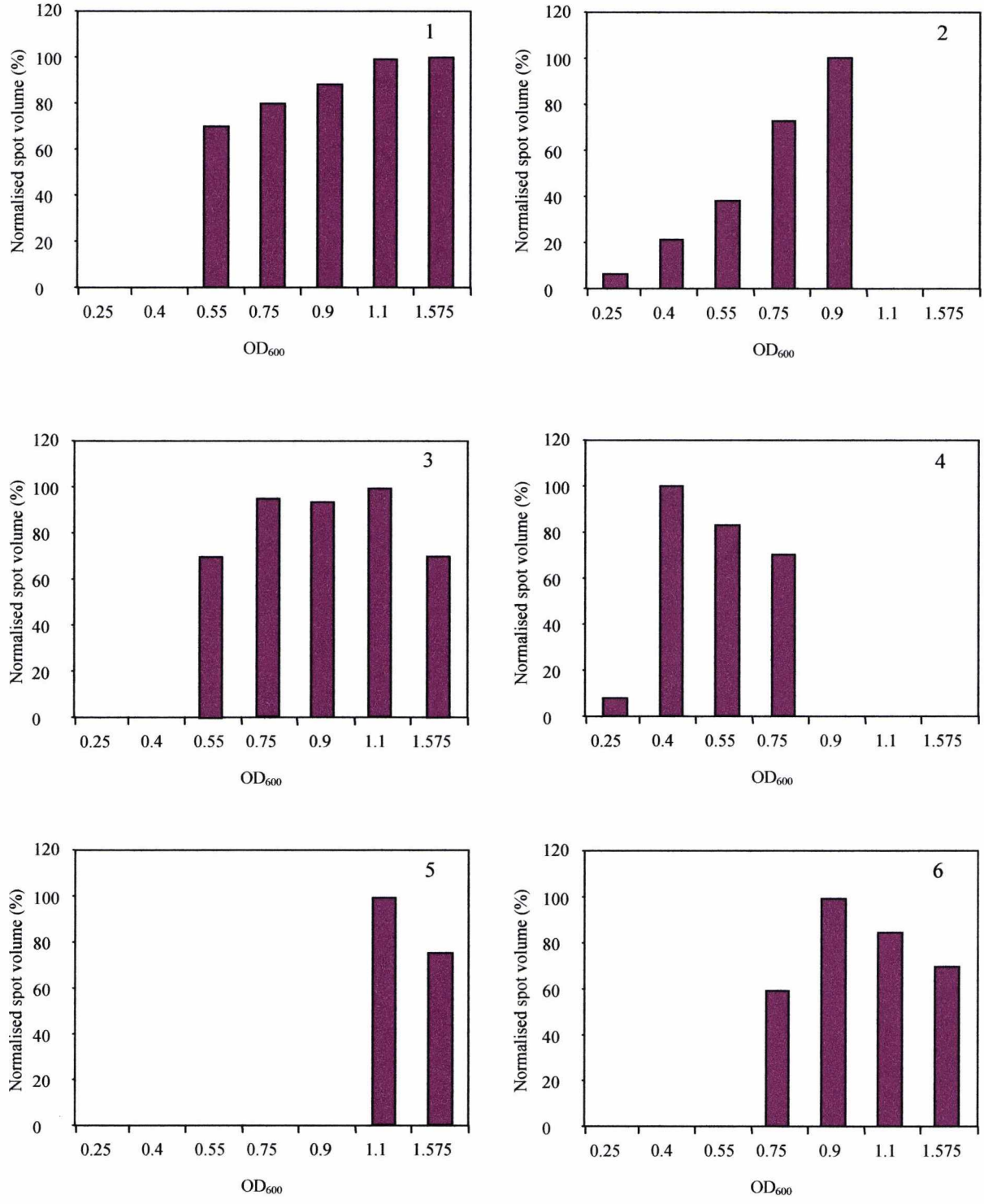


Figure 6.7 The semi-quantitative expression of proteins 1 to 6 throughout growth and stationary phase. Graphs show the semi quantitative information from *L. monocytogenes* sample extracts proteins detailed later in Table 6.0

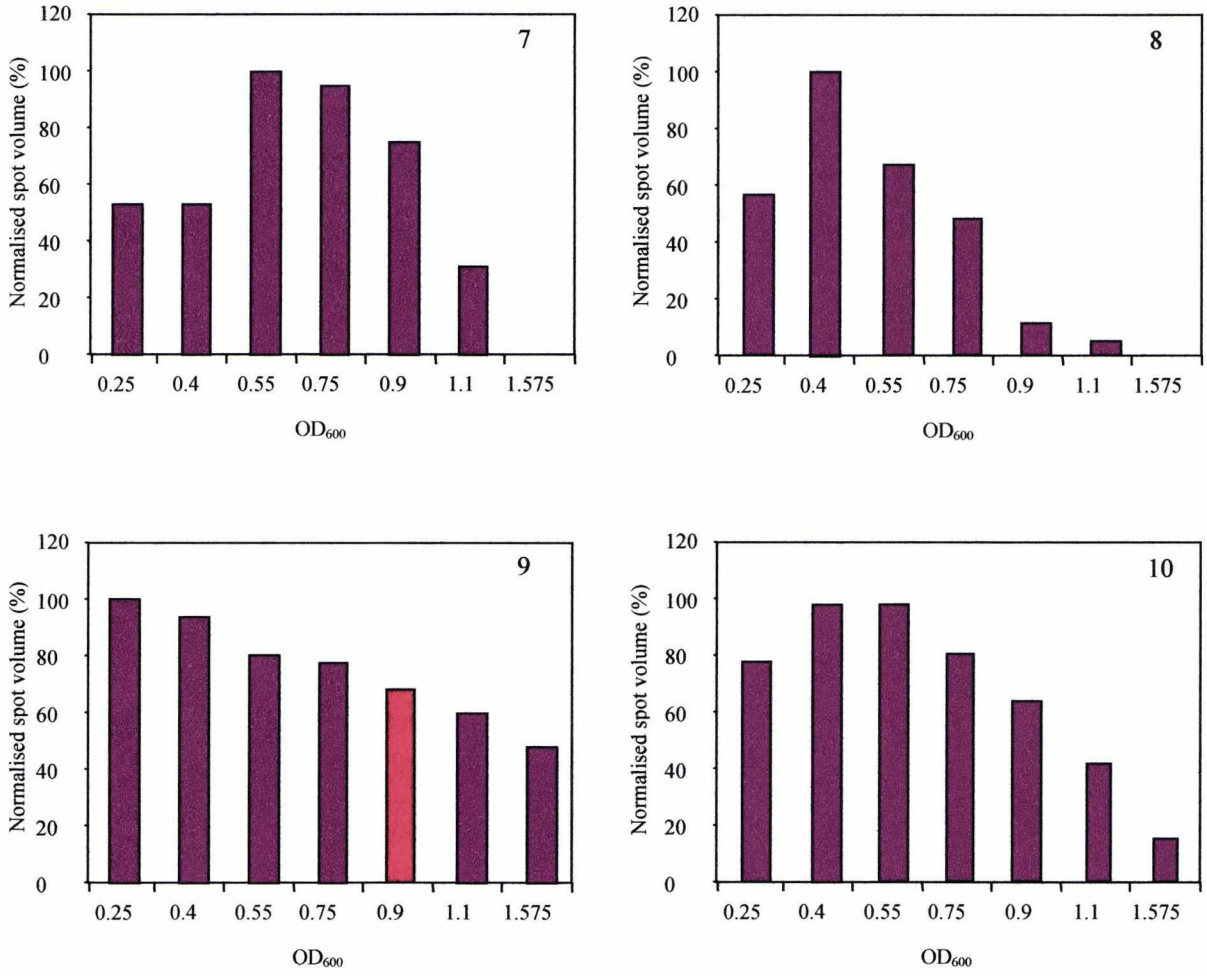


Figure 6.8 The semi-quantitative expression of proteins 7 to 10 throughout growth and stationary phase. Graphs show the semi quantitative information from *L. monocytogenes* sample extracts proteins detailed later in Table 6.0. Figure 9 point OD_{600} 0.9 ■, information for this sample point has been extrapolated from other points on this graph.

The images in Figures 6.5 and 6.6 and the semi-quantitation data presented in Figures 6.7 and 6.8 clearly show that the expression of selected proteins changed throughout growth and stationary phase. It should be noted that this data is only semi-quantitative as it is clear that different gels have been stained to different degrees. Furthermore, no attempt has been made to account for the large errors that are inherent in this type of analysis other than producing averaged gels. Nevertheless, for the ten proteins chosen for further analysis a clear pattern in expression levels (expressed as normalised % spot volume relative to OD_{600} 0.25) was observed. The selected proteins were excised from sample gels and were the subject of in-gel tryptic digestion and peptide mass fingerprinting identification. The resultant peptide masses were entered into SWISSPROT and TreMBLE databases and subsequent identifications and entry details are shown in Table 6.0.

6.0 Analysis of the Listeria proteome throughout growth and stationary phase

Table 6.0 Identification of proteins excised from proteomic maps of *L. monocytogenes* whole cell extracts

Protein number	SWISSPROT reference	Protein name	Synonym	By identification	Theoretical kDa	Theoretical pI	Experimental kDa	Experimental pI	Peptide matches
1	Q9S5A5	GRPE protein	HSP-70 cofactor	*L. monocytogenes*	21.9	4.6	18 - 20	4.7 - 4.9	3 + 3 + 3
2	Q8Y4C2	ATP synthase epsilon chain	ATP synthase F1 sector epsilon chain	*L. monocytogenes*	14.7	5.23	14 - 16	4.6 - 5.0	3 + 3
3	P28764	Superoxide dismutase	EC 1.15.1.1	*L. monocytogenes*	22	5.23	24 - 28	5.2 - 5.4	3 + 3 BM
4	P51834	Chromosome partition protein smc	None	*B. subtilis*	134	5.1	120 - 145	5.1 - 5.4	3+ 3
5	O52494	XyIS/AraC family transcriptional regulatory protein	Lmo 2164 protein	*L. monocytogenes*	33.8	5.73	32 - 34	5.8 - 6.1	3 + 3 BM
6	Q8YAT2	Transcriptional regulator LacI family	None	*L. monocytogenes*	39	5.8	40 - 44	5.6 – 5.9	3 + 3
7	P94550	Electron transfer flavoprotein β subunit	BETA-ETF	*B. subtilis*	28	5.0	27 - 30	5.1 – 5.3	3 + 3 + 3
8	Q8Y6M7	Elongation factor Ts	EF-Ts	*L. monocytogenes*	32.6	5.11	32 - 34	4.6 - 5.0	3 + 3 BM
9	P13267	DNA polymerase III polC-type	PolIII	*B. subtilis*	162	5.26	150 - 180	4.9 – 5.3	3 + 3
10	O31148	Phospho carrier protein HPr	Histidine-containing protein	*L. monocytogenes*	9.4	4.81	8 - 10	4.3- 4.7	3 + 3 BM

Note: Peptide matches, the plus sign + indicates that samples sourced from different gels produced the same identification. The letters BM indicate that previous to the publication of the *Listeria* proteome the same protein was identified by homology to *B. subtilis* genomic sequence. In some instances four matches were achieved by entering more peaks .

6.0 Analysis of the Listeria proteome throughout growth and stationary phase

The proteins that were identified by peptide mass fingerprint identification are listed in Table 6.0. Where a definitive identification specific to *L. monocytogenes* was not found *B. subtilis* was used as an organism homologous to *L. monocytogenes* (proteins 4 and 9). Each sample was excised from two gels at different time points to confirm that the observed changes in expression throughout growth were being ascribed to the same protein. The experimental pI and kDa values were entered as a search parameter giving a narrowed search field and a higher identification confidence.

6.3 Discussion

6.3.1 Differences in the *Listeria* proteome at exponential phase and stationary phase

Listeria can survive harsh conditions and hostile environments by utilising mechanisms that resist the injury processes associated with exposure to applied stress [210, 228]. Different stresses can induce variations in the pattern of protein expression [210] while some proteins exhibit induction across a range of stress conditions [134]. However, in common with many bacteria the survival of *Listeria* in the environment is not exclusively dependent on a single specific stress response. Bacteria will in the absence of favourable growth conditions adjust the expression of proteins or undertake the *de novo* synthesis of new proteins required to enhance survival during extended periods of growth arrest (stationary phase) [241, 260]. When bacteria are starved or encounter limiting nutrient conditions such as those that induce stationary phase during batch culture, a large number of physiological changes take place that lead to an organism that is generally resistant to a wide range of stresses [213]. Proteomic analysis is an ideal tool to use in order to identify important proteins central to the onset of stationary phase. Selective sampling allows the gathering of important information with regard to temporal protein expression as an organism begins to organise its 'internal affairs' in readiness for long-term growth arrest.

During initial image analysis it was noted that low abundance protein spots matched to create averaged gel images demonstrated a higher degree of stain variability (stain uptake) than other more defined spots. Whilst over stained proteins could not be quantified as they gave very low or negative quantitation results. However the majority of proteins that were over stained did not demonstrate changed expression throughout growth or stationary phase and were likely to be housekeeping proteins. Therefore the selection of proteins for further analysis was based on these limitations. Proteins that were newly expressed throughout growth or stationary phase and proteins that went from detectable to non-detectable levels were considered first. To ensure that the observed changes in protein expression were significant rather than a consequence of stain variability, proteins showing less than a two-fold up or down regulation (between comparative samples) were not selected for further analysis.

The results obtained by comparison of proteomic maps of *L. monocytogenes* whole cell extracts at exponential phase with those produced at stationary phase showed that approximately 400 proteins expressed during exponential growth ceased to be expressed during stationary phase or growth arrest. At the same time, 140 proteins not normally

expressed during exponential growth were expressed during stationary phase. Of the proteins visualised at exponential phase that were matched to proteins at stationary phase, 28% showed a two-fold (or greater) change in the quantity of protein expressed (Figure 6.1). In numeric terms within the confines of the experimental conditions applied here, this represents a 64% change in the expression of proteins between the exponential phase and stationary phase. Similar results have been observed for some stress responses of *L. monocytogenes* where the degree of change in protein expression observed has been above 50% [140].

As cells enter stationary phase they undergo radical physical adaptation to ensure that they can survive physical stresses while in a 'vegetative' (stationary) state. At the sub-cellular level the cytoplasm can become condensed while the volume of the periplasm can increase [384], and the composition of the cell membrane can be altered [213]. Phenotypically, bacteria in the stationary phase are more thermo-tolerant, for example *L. monocytogenes* cells are more resistant to heat destruction when in stationary phase [385]. Cells are also typically more resistant to oxidative stress [143], acid resistance [22], and can better survive osmotic stress and starvation [132, 243].

The results presented here have shown that increased resistance to an applied stress at stationary phase (specifically nisin permeabilisation Chapter 4) is achieved while at the same time the total number of expressed proteins (as visualised by 2-D electrophoresis) falls (Figure 6.2). In addition, at stationary phase protein synthesis as a whole is substantially reduced, as has been shown by the rapid fall in the rate of L-[^{35}S] cysteine incorporation as a culture approaches stationary phase (Section 3.2.6). It is believed that the onset of stationary phase is signalled by changes in metabolism that occur in response to nutrient limitation or any reduction in growth rate induced by a variety of environmental changes [213]. A number of investigators have identified an important regulator of gene function at stationary phase to be *rpoS (*sigma factor σ^S), which induces a specific set of gene products only expressed under stress conditions [143, 244]. In fact a number of investigators have identified sigma factor as an early inducer of multiple changes in protein expression in particular in response to applied environmental stress conditions [250-252], whiles others have identified stationary phase specific sigma factors (σ^s) [132, 204, 386]. In *E. coli,* the transcription factor sigma s, encoded by rpoS, controls the expression of a large number of genes involved in cellular responses to a diverse number of stresses, including starvation, osmotic stress, acid shock, cold shock, heat shock, oxidative DNA damage, and transition to stationary phase [387]. Transcription of the *rpoS* gene occurs throughout growth but can be specifically induced by entry into stationary phase where it mediates the selective transcription of stationary phase

specific genes [213]. Sigma factors are numerous and ubiquitous in bacteria including *Listeria* and share similar functions regarding stress responses and stationary phase induction [134].

6.3.2 Analysis of the *Listeria* proteome throughout growth and stationary phase

The comparison of proteomic maps of *Listeria* at exponential phase and stationary phase show that stationary phase represents a global response to changing conditions as growth progressed. During that transition a substantial change in the protein complement of a cell has been demonstrated. Rpos is a global regulator of stationary phase in a number of bacteria [388-390]. The response observed here is global and may indicate the involvement of a global regulator such as Rpos (as has been shown by others [132], in the orchestration of the many changes observed in *L. monocytogenes* proteome during transition from exponential to stationary phase.

In this Chapter the comparison of two fixed point proteome maps of *L. monocytogenes* sample extract did not provide information on the timing or duration of the transition to stationary phase. Therefore a number of proteomic maps were produced at consecutive points throughout the transition from exponential phase to stationary phase growth and these were compared in order to accurately define the duration and more specifically, the point at which stationary phase began. The results show that as growth progressed the total number of proteins visualised at each sample point, and therefore the overall protein complement of the cell, decreased as the cells descend towards stationary phase. During the 7.5 hours of growth from inoculation to OD_{600} 0.75 the overall number of detected proteins decreased by 50. Within the bounds of the experimental protocols this figure is unlikely to be statistically significant indicating that the number of proteins expressed during this time is more or less constant. Over a similar time period and up to OD_{600} 1.1 the overall number of detected proteins decreased from 975 to 701 a difference of 224. At that point the culture was allowed to grow for a further 25 hours attaining an OD_{600} of 1.575. A proteomic map of the culture at that point showed that the overall number of detected proteins had decreased by 25, once again within the bounds of the experimental protocol this is unlikely to be a statistically significant figure. This result suggests that the transition period from exponential phase to stationary phase begins earlier than that suggested by the growth curve data alone, which shows that the attainment of stationary phase occurred over a relatively short time period (three hours) between OD_{600} 0.9 and OD_{600} 1.1 (Chapter 3). However, conclusions based on protein complement alone ignore the fact that protein expression is undergoing change in both directions (up and down regulation). The total number of proteins visualised at each

sample point is the product of both increasing and decreasing protein expression. Image analysis was therefore used and revealed that a total of 449 proteins expressed at OD_{600} 0.25 were not expressed at growth arrest, whilst at the same time 150 proteins expressed at OD_{600} 1.575 (state of growth arrest) were not expressed at OD_{600} 0.25. This represents a mass change of 599 proteins over the 48 hours that the culture was sampled. The majority of changes occurred between OD_{600} 0.75 and 1.1 and confirmed earlier observations reporting the early onset and extended transition period between what is recognised as exponential growth and stationary phase (Figure 6.3).

Despite the non-quantitative nature of the total proteins detected result they do show a wide area of disparity between the timing and duration of the onset of stationary phase when compared to the earlier results discussed in Chapter 3. Thus far the data has not been used to provide information on the relative protein abundances or the functional importance of changing expression. In order to assign functionality to the observed changes in protein expression a number of protein spots were selected for further analysis by limited quantitative analysis and subsequent identification by in-gel tryptic digestion and peptide mass fingerprinting.

6.3.3 The semi-quantitative analysis and identification of *Listeria* proteins showing altered expression on 2-D gels

Proteins were selected for detailed analysis as they represented specific groups of proteins exhibiting either *de novo* protein synthesis, total loss of expression, or consistent expression throughout growth and stationary phase. Peptide mass fingerprints obtained from MALDI-time of flight (TOF) sample analysis were used to identify ten proteins excised from 2-D gels at different sample points. The position that each protein occupied on a 2-D gel analysis was known. The selected protein position in consecutive culture samples was matched and the level of expression at each point was compared (Figures 6.5, 6.6, 6.7 and 6.8). The results show the varied response that each protein exhibited during the transition to, and maintenance of, a stationary state (Figures 6.7 and 6.8). The proteins were initially identified using homology to *B. subtilis*, however the complete genome of *Listeria* species published in October 2001 [391] allowed further analysis of the identification of the mass spectrometry data and has allowed the identification of proteins to be species specific.

Of the proteins chosen for further study ten have been identified (Table 6.0) and are shown below (these are discussed in more detail hereafter),

- GRPE protein,
- Adenosine triphosphate synthase (ATP) epsilon chain
- Superoxide dismutase (SOD)
- Structural maintenance of chromosome (smc) chromosome partition protein
- XyIS/AraC family transcriptional regulator
- LacI transcriptional regulator
- Electron transfer protein β subunit
- Elongation factor Ts
- DNA polymerase II polC-polIII type
- Phosphocarrier protein HPr.

L. monocytogenes GRPE protein is a member of the 70-kDa family of molecular chaperones. This protein has been previously identified by transcriptional analysis of the DnaK heat shock operon of *L. monocytogenes* [392]. It has been shown to stimulate the ATPase activity of DnaK helping to release adenosine diphosphate (ADP) from DnaK allowing DnaK to recycle more efficiently. Semi-quantitative analysis of the protein spot at different sample points has shown that its expression did not reach detectable levels until OD_{600} 0.55 (at a culture age post inoculation of 12 hours). The protein reached stain saturation at 0.750 OD_{600} and beyond that point quantitation was unreliable. However, the protein was expressed at or above the level it attained at OD_{600} 0.75 until monitoring ceased at OD_{600} 1.575 (Figure 6.7 Graph 1). This suggests that its expression was maintained while stress conditions were prevalent. Its early induction and persistence throughout stationary phase indicates that the protein may be important for survival during stress conditions and for the long-term survival of the organism.

Adenosine monophosphate (AMP), RpoS and DnaK (the bacterial homologue of heat shock protein (HSP) 70, are known to affect the expression of certain starvation-inducible or stationary phase-inducible proteins [393]. Members of the 70-kDa family of molecular chaperones assist in a number of molecular interactions that are essential under both normal and stress conditions. These functions require ATP and co-chaperone molecules and are associated with a cyclic transition of intramolecular conformational changes. [394]. It has been previously observed in *E. coli* that the transcription factor sigma s is sensitive to

proteolysis by ClpPX in a reaction that is promoted by RssB and inhibited by the chaperone DnaK [387]. Here, it is possible that GRPE stimulates DnaK and forms part of a general stress response associated with the transition of a culture from exponential phase to stationary phase.

L. monocytogenes ATP synthase epsilon chain protein functions to produce ATP from ADP in the presence of a proton gradient across the membrane. Identification of this protein was by similarity to the published genome of *Listeria* spp. [391]. Semi-quantitative analysis of the protein spot at different sample points showed that it was expressed at OD_{600} 0.25 and that the level of expression increased as the population increased to a point at OD_{600} 1.1 where the level of expression fell below detectable limits (Figure 6.7 Graph 2). ATP is continually being broken down and re-synthesised, and it has been calculated that during the time that a cell doubles its ATP pool must turn over 10,000 times [254]. Related to this rapid turnover is the fact that if ATP is not immediately used for energy, growth, and biosynthesis, it is hydrolysed in reactions that do not yield energy. The protein was not expressed at stationary phase and this reflects the fact that cell division has stopped and the immediate energy requirements of the cell are changing in favour of the production of storable energy resources that can be accessed during long-term growth arrest.

L. monocytogenes superoxide dismutase [Mn] (SOD) protein belongs to the iron/manganese SOD family. This protein has been previously identified from genes encoding SOD [54], however identification here was by similarity to the published genome of *Listeria* species [391]. The protein function is to destroy oxygen radicals which are normally produced within cells and which are toxic to biological systems. Semi-quantitative analysis of the protein spot at different sample points has shown that it was not expressed at detectable levels until OD_{600} 0.55 (at a culture age post inoculation of 12 hours). The level of expression increased up to a point at OD_{600} 1.1 where the level of expression dropped slightly as the culture reached growth arrest at OD_{600} 1.575 (Figure 6.7 Graph 3).

Previous investigations of SOD in *L. monocytogenes* have found that levels increased as the cells progressed through the exponential phase of growth and into the stationary phase (as has been reported here) [216]. Furthermore, SOD activity decreased with decreasing growth temperatures and declined concurrently with decreased growth when higher concentrations of sodium chloride were added to the medium. Cells grown anaerobically possessed relatively high levels of SOD, although these levels were about 10 to 30% lower than those of aerobically grown bacteria [216]. Further work has shown *L. monocytogenes* Scott A (the strain of the organism used here) as having a high SOD activity compared to other *Listeria* strains [395]. The data here suggests that the single *L. monocytogenes* SOD

enzyme is expressed in response to increased population and may also be responsive to the cellular growth rate.

A *Listeria* equivalent could not be found during database searching for chromosome partitioning protein smc. However chromosome partition protein smc does occur in *B. subtilis*, which has been used as a homologue organism for *Listeria* proteins. This protein has been previously identified from an amino acid sequence [396], however identification here was by similarity to the published genome of the organism *Bacillus subtilis* (*B. subtilis*) [157]. The protein functions to stabilise chromosome structure and is essential for chromosome partitioning. Semi-quantitative analysis of the protein spot at different sample points has shown that it was not expressed at detectable levels from OD_{600} 0.25 to OD_{600} 0.75 (culture age post inoculation 22.5 hours). The level of expression increased rapidly at OD_{600} 0.4 before falling to a point at OD_{600} 0.9 where its expression fell below detectable levels (Figure 6.7 Graph 4).

SMC proteins are a ubiquitous protein family, present in almost all organisms thus far analysed except for a few bacteria [397]. Eukaryotic SMC proteins are implicated in a diverse range of chromosome dynamics including chromosome condensation, dosage compensation and recombinational repair [398]. SMC proteins and the structurally homologous MukB protein are unusual ATPases that form antiparallel dimers, with long coiled coil segments separating globular ends capable of binding DNA. The semi-quantitative results presented here suggest that the protein is involved in the cellular replication process, as it does not occur at detectable levels as stationary phase approaches.

L. monocytogenes transcriptional regulator protein belongs to the ARAC/XyIS family of transcriptional regulators. This protein was previously described in 1998 [399], however identification here was by similarity to the published genome of *Listeria* species [391]. The protein function is not known but roles as a stimulator of transcription during stress and or virulence activation have been suggested [400, 401]. Semi-quantitative analysis of the protein spot at different sample points showed that it was not expressed at detectable levels until OD_{600} 1.1 at a culture age post inoculation of 27 hours (Figure 6.7 Graph 5). This may indicate a role in stress related functions at stationary phase, however transcription and translation regulators occur in very low abundance and are difficult to detect. Therefore the level of expression and timing may be misleading regarding the association of possible functions of this protein.

The ArC/XylS family of prokaryotic positive transcriptional regulators includes more than 100 proteins and polypeptides derived from open reading frames translated from DNA

sequences. Members of this family are widely distributed and have been found in the gamma subgroup of the proteobacteria, low and high G + C-content gram-positive bacteria, and cyanobacteria [400]. Other transcriptional regulators identified in *L. monocytogenes* have had functions ascribed them, for example two pleiotropic regulatory elements acting at different levels, the transcription factor PrfA which controls virulence gene expression and the potential chaperone ClpC which is involved in tolerance to environmental stress, are required for *L. monocytogenes* survival within the host, although most virulence genes of *L. monocytogenes* are activated by the transcriptional regulator PrfA [402]. These transcriptional regulators have varied function but are associated with stress responses and here could be associated with stress responses at stationary phase.

L. monocytogenes transcriptional regulator protein belongs to the LacI family of transcriptional regulators. The identification of this protein was once again by similarity to the published genome of *Listeria* species [391]. This protein has no ascribed function in the SWISSPROT database. Semi-quantitative analysis of the protein spot at different sample points showed that it was not expressed at detectable levels until OD_{600} 0.75 at a culture age post inoculation of 21 hours and persisted to OD_{600} 1.575 (Figure 6.8 graph 6). This may once again indicate a role in stress related functions at stationary phase, however transcription and translation regulators occur in very low abundance and are difficult to detect. The level of expression and timing may be misleading regarding the association of possible functions for this protein.

No specific function for this protein was published in the SWISSPROT database however LacI transcriptional regulators do occur in *B. subtilis*. A CcpA protein has been identified as a key regulator of carbon metabolism in *B. subtilis* [403]. CcpA has also been shown to be a central regulator in low-G+C- content gram-positive bacteria. It confers carbon catabolite repression to numerous genes required for carbon utilisation and also operates as a transcriptional activator of genes involved in diverse phenomena, such as glycolysis and ammonium fixation [404]. Transcription of a new catabolic operon in *B. subtilis*, involved in the late stages of galacturonic acid utilization, has been studied. The operon is negatively regulated by the kdgR and ccpA gene products, which belong to the LacI family of transcription regulators [405]. The function of LacI regulators seems to be wide ranging and act as repressors or activators of gene functions related to the control of carbon flow in the cell. This may therefore suggest a role for this protein in *L. monocytogenes*

A *Listeria* equivalent could not be found during database searching for electron transfer flavoprotein beta subunit. However electron transfer flavoprotein beta subunit does

occur in *B. subtilis*, which has been used as a homologue organism for *Listeria* proteins. The protein belongs to the lectron transfer flavoproteins (ETF) beta–subunit/FIXA family. This protein serves as a specific electron acceptor for other dehydrogenases and the protein was originally identified in 1996 [406]. Semi-quantitative analysis of the protein spot at different sample points has shown that it was expressed at detectable levels from OD_{600} 0.25 and persisted to OD_{600} 1.1. The level of expression increased to a point at OD_{600} 0.55 where the level of expression fell continually to a point below detection at OD_{600} 1.575 (Figure 6.8 graph 7).

ETF proteins are alpha beta-heterodimers found in eukaryotic mitochondria and bacteria [407]. In the bacterium *B. subtilis* the transfer of electrons via ETF-ubiquinone oxidoreductase forms part of the main respiratory chain. The quantitation results show that the protein is expressed throughout growth to stationary phase where expression fell below detectable levels. The pattern of protein expression would be that expected of a component of central metabolism.

L. monocytogenes elongation factor (EF) protein belongs to the EF-TS family of protein elongation factors. The protein is involved in the elongation of proteins, during synthesis it associates with the EF-Tu.GDP complex and induces the exchange of guanidine diphosphate (GDP) to guanidine triphosphate (GTP). It remains bound to the aminoacyl-tRNA.EF-Tu.GTP complex up to the hydrolysis stage on the ribosome. The identification of this protein was again by similarity to the published genome of *Listeria* species [391]. Semi-quantitative analysis of the protein spot at different sample points has shown that it was expressed at detectable levels from OD_{600} 0.25 and persisted to OD_{600} 1.1. The level of expression increased to a point at OD_{600} 0.4 where the level of expression fell continually to a point below detection at OD_{600} 1.575 (Figure 6.8, Graph 8).

Elongation factors have been well characterised in *E. coli* and *Thermus thermophilus* [408, 409]. In *E. coli* it has been shown that the dissociation of GDP from EF-Tu in the presence of EF-Ts is not a rate-limiting process in protein synthesis [410, 411]. The pattern of expression shown would be that expected of a component of protein synthesis systems associated with elongation of proteins during synthesis. It has been shown here that the protein was down regulated towards stationary phase. This is generally in accordance with results achieved in Chapter Three using ^{35}S labeled cysteine where incorporation of the radioactive label was reduced towards stationary phase indicating a fall in protein synthesis (Chapter 3 Section 3.2.6).

A *Listeria* equivalent could not be found during database searching for polC-type DNA polymerase III. However polC-type DNA polymerase III does occur in *B. subtilis*, which has been used as a homologue organism for *Listeria* proteins. A complete sequence of the protein was first obtained in 1991 [412]. The identification of this protein was by similarity to the published genome of *B. subtilis* [225]. The protein is required for replicative DNA synthesis and also exhibits 3' to 5' exonuclease activity [413]. Semi-quantitative analysis of the protein spot at different sample points has shown that it was expressed at detectable levels throughout growth and stationary phase (Figure 6.9 Graph 9).

The inhibition of DNA polymerase III's has been used as a site of for novel antimicrobial compound activity against a broad range of Gram-positive bacteria, including methicillin-resistant staphylococci and vancomycin-resistant enterococci [414, 415]. The pattern of expression was not entirely as expected and the protein continued to be expressed at detectable levels throughout growth and stationary phase. Due to the involvement of this protein in replicative DNA synthesis it would be expected that the expression of this protein would fall at the onset of stationary phase and be minimal or below detectable levels at stationary phase. It has been shown here (by viable cell count) that at late

stationary phase (OD_{600} 1.1) cell numbers are static indicating that no cell division and hence no DNA replication is taking place (Section 3.2.6, Figure 3.3).

L. monocytogenes phosphocarrier protein (HPr) belongs to the HPR protein family. The protein is a component of the phosphenolpyruvate (PEP)-dependant sugar phosphotransferase system (PTS), which is a major carbohydrate active transport system. The phosphoryl group from PEP is transferred to the phosphoryl carrier protein HPR by enzyme I.phospho-HPR, which then transfers it to the permease (enzymes II/III). HPr is common to all PTS. The identification of this protein was again by similarity to the published genome of *Listeria* species [391]. Semi-quantitative analysis of the protein spot at different sample points has shown that it was expressed at detectable levels throughout growth and stationary phase. The level of expression increased to a point at OD_{600} 0.55 where the level of expression fell continually to the end of the monitoring period (Figure 6.8 Graph 10).

The phosphenolpyruvate (P-pyruvate)-dependent sugar PTS is a transport and signal-transduction system that is almost ubiquitous in bacteria but does not occur in eukaryotes. It catalyzes the uptake and phosphorylation of carbohydrates and is involved in signal transduction, e.g. catabolite repression, chemotaxis, and allosteric regulation of metabolic enzymes and transporters. [416]. The pattern of expression was not entirely as expected and the protein continued to be expressed at detectable levels throughout growth and stationary

phase. However, it has been shown here (Chapter 3, Figure 3.7) that glucose consumption and therefore uptake did continue at a greatly reduced rate throughout stationary phase.

The results here show that stationary phase appears to begin earlier than was expected and that the transition period is longer than expected or demonstrated by growth curve data alone (Chapter 3). Most of the proteins identified demonstrate patterns of expression that fit the growth data. Proteins related to stress do not appear at detectable levels until OD_{600} of 0.55 or above. Proteins related to metabolism were expressed throughout growth and show a decrease in expression as stationary phase approaches. The pattern of expression of proteins related to protein synthesis also fitted growth and radioactive labeling data. Two of the proteins, DNA polymerase III polC type and phospho carrier protein HPr were expressed throughout growth and stationary phase, this result did not match previously presented growth data.

It has been shown here using a *L. monocytogenes* whole cell extract that an optimised separation protocol (Scheme 5.2) has successfully separated semi-quantified and identified proteins with altered expression throughout growth and stationary phase. The results presented in this chapter provide information on the quantitation and temporal separation of protein expression events as a *L. monocytogenes* PTM batch culture grows from exponential phase to growth arrest. These and other results (from previous chapters) will be discussed further in Chapter 7.

6.3.4 Theoretical 2-D SDS-PAGE L. *monocytogenes* proteomic map

Since the publication of the *L. Monocytogenes* complete genomic sequence in Oct 2001 it has been used to construct a theoretical proteome of every protein ORF and assign each theoretical protein a position on an artificial image using kDa and pI. The proteomic images produced herein have been compared with the theoretical map and show similarity (Appendix VII). It should be noted that the theoretical map does not take account of any post-translational modification or position shifts (of proteins) due to sample preparation or artefactual modifications, neither can the theoretical image show temporal expression or different expression levels between housekeeping and stress induced proteins. Therefore an overlay match between theoretical and actual images was not expected or seen. As shown herein (Table 6.0) the theoretical and experimentally defined pI and kDa of 10 identified proteins was different.

7.0 General discussion and conclusions

7.1 Introduction

The small size of prokaryotes limits their ability to control their environment dependent on the perturbation, for example pH*i* can be controlled but cell temperature cannot [234]. Cells do not have sufficient internal capacity to produce the level of response required to actively alter the external environment in which they find themselves. Instead, prokaryotes must rely on rapid and efficient control of gene expression to respond to environmental challenges. A change of environment from conditions favourable for growth to conditions unfavourable for growth can induce global changes in gene expression that will ultimately lead to the induction of a stationary state. Bacterial cells at stationary phase are generally smaller, less motile, more resistant to mechanical and physiochemical damage [360, 379] and have adapted their 'internal affairs' to survive long-term nutrient deprivation [240, 266]. Therefore, stationary phase can be viewed as a survival tactic where environmental conditions do not favour growth. The reactions to unfavourable conditions can involve genetic control, bioenergetics and in particular (at stationary phase) the effective maintenance of homeostasis. Homeostasis can include cellular responses that keep key aspects of metabolism, composition and structure relatively unperturbed even when the surrounding environment is greatly perturbed [235]. For many human pathogens, the capacity to survive physical challenges during food processing is a critical step in their transmission to the host by the food-borne route [205].

Changes in gene expression that lead to a resistant stationary state are the obvious targets for food preservation and safety. Specifically growth rate mediated gene control and the expression of genes that prepare the cell for survival against a range of stresses are valid targets for investigation. The overall aim of the experiments described within this thesis was to highlight temporally important events that lead to a more resistant *L. monocytogenes* bacterium at stationary phase. A central regulator of stationary phase gene expression has been identified [240, 241, 410]. However the temporally important events that occur during preparation for stationary phase have not yet been adequately investigated. A proteomic platform has been developed and has been used in conjunction with some established and modern microbial protocols to provide a holistic approach to elucidation of events important to the induction and maintenance of a stationary state in *L. monocytogenes* batch cultures.

7.2 Growth characteristics of *L. monocytogenes*

Growth curves have been constructed for PTM and BHI batch cultures covering a 48 hour growth period using OD and viable counts (Section 3.2.2). These results were as expected and did not differ significantly from growth curves defined by others [211]. Bacterial cultures do not grow synchronously and a growth curve is the product mean of all the growth states prevalent at the time each sample/reading is taken. Therefore the ability of such data to accurately define specific growth states is limited, although clear delineation between (what was assumed to be) lag, exponential and stationary phase was observed for both media (Section 3.2.2). Stationary phase here was defined as the point where cell population counts became static. This point occurred at an OD_{600} of 1.1 and at a culture age 22 hours post inoculation (Section 3.2.2). Beyond OD 1.1 readings continued to rise but cell counts remained static. OD is based on sample turbidity and at static cell counts turbidity can increase due to cell debris, medium changes (changing medium pH can cause precipitation of some PTM medium components), and cell waste products. Although an experimentally convenient stationary point was selected it was unclear from the results whether numerical stasis was the point where the number of cells dividing matched the number of cells dying, or a state of complete stasis where the cells were no longer dividing or dying. The data is one-dimensional and does not provide information on the heterogeneity of the culture or the degree to which subpopulations (if present) may be contributing to the data mean. These methods gave reproducible growth curve data and allowed sample points between different cultures to be synchronised.

7.3 The effect of medium pH on *L. monocytogenes* in batch culture.

It has been shown that the exposure of *L. monocytogenes* to low pH during growth can induce an acid tolerant response (ATR), however increased acid resistance can also developed as a consequence of entry into the stationary phase. This response appears to be independent of the pH-dependent ATR seen during exponential growth [22]. It is known that low pH can induce stationary phase but it has been shown here that *L. monocytogenes* is capable of growth in medium at low pH, this suggests an inherent ability to survive low pH without adopting stationary phase as a means of survival. However falling pH may be one of a number of contributory factors that induce stationary phase of *L. monocytogenes* PTM batch cultures. There is no doubt that the transition is complex and that to examine the mechanics of stationary phase and the transition to it other methods of experimental analysis were required.

7.4 Different methods for the temporal separation of exponential and stationary phase

Like *E. coli*, *L. monocytogenes* loses viability and rRNA rapidly once exponential growth has ceased in batch culture [411]. Identification of the point at which exponential growth ceases can provide a fixed point in culture from which a closer pre- and post-event examination of the organism can be conducted. In order to define more clearly the growth state of *L. monocytogenes* batch cultures, a defined medium (PTM medium) was utilised. Glucose and amino acid utilisation was monitored throughout growth and stationary phase, as was the uptake of L-[^{35}S] cysteine. Glucose utilisation results showed the point at which exponential growth became stationary growth to be between OD_{600} 0.9–1.0 (Section 3.2.5.1). Amino acid utilisation results showed the point at which exponential growth became stationary phase to be between OD_{600} 0.85 and 1.1(Section 3.2.5.4). L-[^{35}S] cysteine utilisation results showed the point at which exponential growth became stationary phase to be between OD_{600} 0.8–0.85. L-[^{35}S] cysteine was also used as an indicator of protein synthesis (Section 3.2.6). It was also shown using amino acid utilisation data (and to a lesser extent glucose utilisation), that stationary phase could be subdivided into two distinct phases. OD_{600} measurements and viable counts did not highlight this distinction over the time frame examined (Section 3.2.5.5).

Each method used to define the point of transition to stationary phase showed the onset of stationary phase to be occurring at successively earlier points along the growth curve of *L. monocytogenes* PTM batch cultures. This showed that stationary phase as an event occurred over a 7 hour period between OD_{600} 0.85 and 1.1 when the majority of changes required for homeostatic control of the organism metabolism during stationary phase have already been made. Although chemical analysis of the medium showed that at stationary phase some limited utilisation of medium resources was ongoing indicating a dynamic equilibrium and/or replicating sub-cultures. However beyond a culture age of 24 hours up to a point at 48 hours when monitoring ceased (OD_{600} 1.2 to OD_{600} 1.575) a second static equilibrium stationary phase (growth arrest) was apparent. This period was thought to represent a more complete shutdown of cellular activity when the utilisation of medium resources was very low.

On transition to stationary phase it has been shown that *L. monocytogenes* has a starvation survival response (SSR) that is induced under glucose- or multiple-nutrient-, but not amino acid limitation. The initiation, but not the maintenance, of the SSR involves both protein and cell wall biosynthesis [135]. It is proposed that these events occur during stationary phase OD_{600} 0.85 – OD_{600} 0.11 which is actually a state of considerable change, and that only after this point OD_{600} 1.1 can the culture be in stasis at a state of growth arrest. It is

clear that the point of stationary phase determined by OD and viable counts is not the point when a stationary state has been fully attained but a period of intense change that prepares the organism for a stationary state.

7.5 Membrane permeability to nisin as an indicator of stationary phase in *L. monocytogenes* PTM batch culture using flow cytometry

The results discussed so far are the product of a culture mean. Results based on a culture mean (Chapter 3) mask the increasing/decreasing heterogeneity of bacterial cultures particularly at the point of transition to stationary phase. Flow cytometry can provide information on the heterogeneity of 1000s of bacterial cells in minutes [210]. Flow cytometry has also been used to investigate the effect of bacteriocins (anti-microbial peptides) on the bacterial cell [278]. It has been used here to show the behaviour of *L. monocytogenes* cells in response to a sub lethal exposure to nisin throughout growth and stationary phase.

Nisin will form transient pores in susceptible organisms dissipating the proton motive force and causing the loss of small molecules from the cytoplasm [281]. Its action has been shown in part to be dependant on the presence or absence of Lipid II and III precursor molecules in bacterial membranes [282]. Flow cytometry was used to show the point during growth where physiological changes to the *L. monocytogenes* membrane (as apart of the transition to stationary phase), were sufficiently large to affect the permeabilising action of a time controlled sub lethal dose of nisin on the membrane of the organism. The results have shown that the action of nisin was growth phase dependant and could be used as an analytical tool to discriminate between exponential and stationary phase *L. monocytogenes* cells. The results have also shown that the transition period from exponential to stationary phase (6 hours) was greater than that suggested by OD and viable counts (3-4 hours) (Section 3.2.2). The flow cytometry results were more in line with the analysis of medium results showing the early onset of stationary phase and the extended period of transition (up to 7 hours) between exponential and stationary phase (Section 3.3.4.2, 3.3.4.3, and 3.3.4.4). The protocol adopted did not show the presence of subcultures at stationary phase. It is suggested later that sub cultures may be present but in numbers below the detection limits of this protocol.

Additionally it was shown that when cells in spent medium at transition to stationary phase were re-suspended in fresh medium susceptibility to nisin increased. The response to nisin was shown to be independent of medium pH (Section 4.2.1.1). It is suggested that spent medium diluent from stationary cultures contained quorum-sensing peptides important for population perception and membrane function. The re-suspension of transition phase and

stationary phase cells (OD_{600} 0.55 – 1.1) in fresh medium removed that signal peptide and a different response to nisin was shown. It is further suggested that the different responses to nisin was as a consequence of a reversible change in membrane function instigated in response to increasing population. This may also indicate that a persistent signal is required for a quorum response. A different response to nisin was observed in fresh and spent media throughout growth and stationary phase. At an OD_{600} of 0.779 resistances to nisin became more pronounced in spent and fresh media samples. Although at no point was the fresh medium response as great as that seen in spent medium samples. There is limited evidence here from which to draw conclusions, however, it can postulated that cells at that point (OD_{600} 0.779) and beyond were more committed to a stationary phase than cells sampled prior to that point. Stationary phase and its initiation is a global commitment and this result may indicate that beyond a certain point irrespective of environmental conditions that commitment must be followed.

Quorum sensing is a common phenomenon in both Gram-positive and Gram-negative bacteria [412]. It has been shown that Gram-positive bacteria have two component quorum sensing systems that are involved in population-perception controlled expression of a number of gene products [327, 413]. More specifically, it has been shown that *B. subtilis* (an organism homologous to *L. monocytogenes*) antimicrobial peptide production is under the control of quorum sensing systems controls. In pathogenic bacteria many of the genes associated with virulence are also associated with resistance to stress and survival in the host. [26, 27, 249] It has also been shown in some pathogenic bacteria that the control of virulence is in part under the direction of quorum sensing [414, 415]. The control of virulence of *L. monocytogenes* has not been linked with quorum sensing but evidence here suggesting that the organism regulates membrane function in response to increasing population could suggest a role for quorum sensing in virulence. Should such a system exist, interference of that system could provide a novel approach to the control of infection [414].

The genome sequences of *B. subtilis* and *L. monocytogenes* were compared using NCBI web page and BLASTP (v. 2.1.2) sequence data. (http://www.ncbi.nlm.nih.gov). There were 4112 proteins listed for *B. subtilis* and 2787 showed homology to *L. monocytogenes.* Signal transduction systems occurred 117 times and of these 54 were two component response regulator systems. A random selection of these two component systems showed sequence homology ranging from 55% to 89%. The fact that such homology can be demonstrated for a high number of two component response regulator systems shows that *L. monocytogenes* has two component response regulator systems. In *B. subtilis* such systems are associated with quorum sensing. More specifically a histidine kinase sensory system identified by genome

sequence in *B. subtilis* (SWISSPROT ref. P96685) also exists in *L. monocytogenes* and 51% homology is shown by a comparison of the sequence of each protein.

The free-living marine bacterium *Vibrio harveyi* possesses two autoinducer-response systems that function in parallel to control the density dependant expression of the luciferase structural operon *luxCDABE*. This quorum sensing control system has features that have been found in both Gram-positive and Gram-negative bacteria. [290]. Highly conserved *luxS* homologues have been identified in Gram-positive and Gram-negative bacterial species including *B. subtilis* [416]. A homologue has also been reported in *L. monocytogenes* and has been ascribed the same function (by similarity), being involved in the synthesis of autoinducer 2 (AI-2) which is secreted by bacteria and is used to communicate both the cell density and the metabolic potential of the environment [384]. It is therefore clear that sensory systems involved in quorum sensing have been identified in *B. subtilis* and that those systems (by similarity) also occur in *L. monocytogenes*.

7.6 Analysis of the *Listeria* proteome throughout growth and stationary phase

A proteomic platform was developed (Chapter 5) that allowed proteome images of *L. monocytogenes* PTM batch cultures to be compared. *L. monocytogenes* PTM batch cultures were sampled throughout growth and stationary phase over a 48 hour period at OD_{600}'s of 0.25, 0.4, 0.55, 0.75, 0.9, 1.1 and 1.575. Averaged proteome images were compared and differences between samples were shown. Quantitative results have shown that the transition to stationary phase is a global event occurring throughout a 7-9 hour monitoring period and the transition involved more than 50% of the proteins imaged. The number of proteins down regulated was greater than the number of proteins upregulated at stationary phase, relative to the total number expressed at exponential phase (Section 6.2.2). The results also show that at OD_{600} 1.1 and beyond (22-48 hours post-inoculation) the total number of expressed proteins did not change significantly. This was in accordance with previous results that indicated that stationary phase could be subdivided into two distinct phases, early stationary phase and growth arrest.

The genus *Listeria* contains six species including *L. monocytogenes* [16], the genus belongs to the low G+C content Gram-positive bacteria and is closely related to the *Bacillus* and *Streptococcus* genera. The lineage of these organisms is as follows, bacteria, *Firmicutes*, *Bacilli*, *Bacillales*, *Listeriaceae*, *Listeria*, and *L. monocytogenes*. Prior to the publication of the complete *Listeria* genome, identification of the proteins of *L. monocytogenes* was conducted by homology to *B. subtilis*. Ten proteins have been identified by peptide mass

fingerprinting and comparison to the genome sequence of both *L. monocytogenes* and *B. subtilis*. A temporal pattern of expression was produced from proteomic images for each protein. Conclusions regarding the functional significance of these quantitation results are at best highly speculative when viewed in isolation, however results presented throughout can provide limited confirmation of discussion points.

Four proteins, GRPE, SOD, XyIS/AraC transcriptional regulator and transcriptional regulator LacI were upregulated towards stationary phase. GRPE and SOD were first observed at OD_{600} 0.55 indicating that stress perception and the possible induction of stationary phase was far earlier than had been indicated by OD and viable counts alone (Section 3.22). Transcriptional regulators are expressed at low levels and quantitation information can be variable. However, the pattern of expression observed here suggested a role for these proteins in the onset of stationary phase. The easily expression of SOD and GRPE coincided with the observed change in membrane permeability to nisin (Section 4.2.2) suggesting both results show that a stationary phase response has been initiated. Two proteins, ATP synthase and chromosome partitioning protein were down regulated towards stationary phase. This result supports the early induction of a stationary phase response. Chromosome partitioning protein is involved in cell division and its down regulation at OD_{600} 0.9 coincided with a decrease in the rate of growth, amino acid utilisation, and glucose utilisation and viable counts of *L. monocytogenes* in PTM batch culture. Two proteins, electron transfer protein flavoprotein β subunit and elongation factor Ts were down regulated between OD_{600} 1.1 and 1.575. Electron transfer proteins are components of central metabolism and its presence at OD_{600} 1.1 shows that nutrient utilisation is ongoing at stationary phase. This coincided with amino acid and glucose utilisation results that show low levels of utilisation at OD_{600} 1.1 (Section 3.2.5) Elongation factor is involved in protein synthesis and its presence at stationary phase suggests that some protein synthesis is occurring at that point. This coincided with L-[^{35}S] cysteine incorporation results that showed low levels of incorporation (and therefore protein synthesis) at OD_{600} 1.1(Section 3.2.6).

Two proteins, DNA polymerase and phospho carrier protein were expressed throughout growth and stationary phase and growth arrest. DNA polymerase in involved in the replication of DNA and its presence at growth arrest suggests that some DNA replication (and therefore cell division) is ongoing. Phospho carrier protein is involved in the metabolism of sugars and its presence at growth arrest suggests that some cells are metabolically active. Proteins involved in DNA replication are persistent and can have an extended half-life.

In isolation this result is inconclusive, however when viewed in conjunction with other it is proposed that this indicates the presence of a replicating subculture at growth arrest.

Amino acid and glucose utilisation fell continually from the onset of stationary phase (OD_{600} 0.75) and beyond that point utilisation was low but at detectable levels. The same pattern was demonstrated with L-[^{35}S] cysteine incorporation. The pattern of expression of the proteins electron transfer flavoprotein, elongation factor Ts, DNA polymerase and phospho carrier protein, shown here also suggests the presence of a small subculture of replicating cells was present at stationary phase and growth arrest. A subculture undergoing replication at this stage of growth suggests that a stationary phase phenotype is capable of growth despite or perhaps in response the presence of a large and persistent quorum sensing signal. The results support the suggestion that a subculture of replicating cells exists at stationary phase and that a sub-population persists into growth arrest.

SigB a central regulator of stationary phase was not identified here as a clear precedence exists for its involvement in stationary phase induction [133, 137, 249, 250]. The results presented here have shown how early that involvement may be as preparations for stationary phase are being made during mid to late exponential phase. What is not clear at this stage is whether those preparations are on a culture wide basis or limited to sub populations. However the fact that proteomics is based on a culture mean may suggest that specific changes to the proteome observed here must be occurring to a large number of cells for the changes in protein expression to be detected with silver stain. This suggested that culture survival is more important than culture growth and may indicate quorum sensing has a role to play earlier in the age of a culture where numbers are relatively low compared to stationary phase induction where numbers become limiting to growth

Numerous investigations have shown that *L. monocytogenes* can respond rapidly to environmental stress and that pre stressing the organism at a sub lethal level can increase the survival of the organism against a subsequent lethal stress [110, 115, 137, 417, 418]. Here the length of time over which transition to stationary phase occurs (6 – 7 hours) suggests a complex response. The apparent readiness of the organism to become stationary even when conditions for growth are still favourable may also indicate that stationary phase is a preferred state that ensures survival of the organism outside the host. This may be evidence of a graduated survival technique where some cells hypersensitive to nutrient limitation or pH respond swiftly becoming 'partially' stationary but ready to respond rapidly to improved conditions. This ensures culture survival over short tern environmental perturbation. If poor conditions persist these cells are likely to die rapidly. Other cells with long-term survival

tactics enter stationary phase more slowly but more completely and are able to survive long term environmental perturbation. The suggestion that a number of subcultures respond differently to the same environmental conditions may indicate that phase variation is occurring. Phase variation is an adaptive process whereby bacteria can undergo frequent reversible phenotypic changes as the result of genetic alterations in specific loci of their genomes [419]. This process is crucial for the survival of pathogens in a hostile and ever changing host environments. The best documented loci are those involved in the biosynthesis of surface-exposed antigen structures, such as outer lipopolysaccharides (LPSs) and lipoproteins, pili, flagella and other secreted proteins that play a role in the interactions between bacteria and their host. This can be by modulation of their tissue tropism or their ability to use locally available nutrients. Variation of these structures may allow the bacteria to respond to host defence mechanisms and assist in the colonisation of varied ecological niches [420]. The complete genome of a virulent strain of *Streptococcus pneumoniae* confirms that the organism uses a variety of genetic mechanisms to adapt to its environment. It also contains a number of potential phase variable genes that contain tandem DNA repeats as well as multiple DNA inversion systems [421]. The molecular and genetic mechanisms of phase variation are as yet unresolved. It is however clear that mechanisms allowing local modification of the structure and sequence of DNA exist that allows high levels of reversible genetic diversity [420].

Work continues with a number of gram-positive bacteria that has defined the role of quorum sensing in stationary phase, virulence, and gene competence [414, 422-424]. Quorum sensing in *L. monocytogenes* may well have similar roles and further work is required to provide confirmation of the findings here and the role that quorum sensing plays in stationary phase cell competence. Proteomic analysis has been successfully used here to identify numerous protein targets that undergo change during the transition from exponential phase to stationary phase, and that change has temporal significance. A systematic identification of all these targets and the temporal significance of each change of expression are required for a more complete understanding of the transition to stationary phase. As has been demonstrated here, proteomic analysis can be easily integrated with other methods of research (flow cytometry, chemical analysis) to provide supported results. A complete picture of important protein expression events during growth and transition to stationary phase could help define specific targets for more effective control of the organism in food and the host

Here a *L. monocytogenes* whole cell extract was separated over a pH 4-7 resolving range on a single gel giving a 364 cm^2 analytical area. Proteomics as a field of research

continues to develop, IPG gels are now available at 24 cm. To compare current technology to newly available technology a single sample has also been separated over a 24 cm pH 4 – 7 resolving range using recently acquired Ettan DALT II electrophoresis equipment (Amersham Pharmacia Biotech Ltd). This provided a 480 cm^2 analytical area over which a *L. monocytogenes* whole cell extract was separated (Appendix VI).

Recently available 24 cm narrow pH range IPG gels and large vertical electrophoresis systems (Ettan DALT II) can now separate the same sample over a pH range of 3.5-9 (separate gels) providing a possible analytical area of 2880 cm^2. The increased analytical area allows more protein to be separated at each pH increment and greatly increases the ability to visualise low abundance proteins. The equipment base for large-scale automated proteomic analysis has developed extensively since this project was undertaken. The Ettan DALT II large vertical electrophoresis system can run up to twelve gels simultaneously with separations completed in 4-6 hours. This has greatly increased the inter gel reproducibility of the system.

7.7 Conclusions

It has been clearly demonstrated in conjunction with traditional microbial methods that a proteomic platform can provide detailed information on the protein complement of a *L. monocytogenes* culture throughout growth and stationary phase. Evidence of quorum sensing has also been presented. The results have also shown that stationary phase and its induction occurred earlier than has been previously suggested by OD and viable counts. The transition from exponential phase to stationary phase is a graduated response that may be reversible prior to OD_{600} 0.75, beyond that point the commitment to global change in preparation for stationary phase appears irreversible. Stationary phase and the transition period is therefore a global response that affects over 50% of the expressed proteins visualised here, similar change has been observed with *L. monocytogenes* on application of acid stress conditions [199]. There is evidence of temporal separation of protein expression events; proteins unique to stationary phase are expressed before proteins unique to exponential phase are down regulated. This may explain why cultures at the beginning of transition to stationary phase can still respond to changing environmental conditions while cultures that are more advanced (in time) cannot. Once proteins associated with metabolic processes and cell division are down regulated stationary phase will continue to completion irrespective of changing environmental conditions.

It has also been demonstrated that subcultures of replicating cells may be present even at conditions of growth arrest. This has previously been demonstrated in aged cultures of *Salmonella typhimurium* [268]. Within the culture at stationary phase while the majority of cells are static a small subculture may exist that continues to replicate in opposition to presence of (an assumed) quorum signal. Proteins have been identified and semi quantified and the results of that analysis support the conclusions made herein. This thesis provides information on the complex nature of the bacterial stationary phase. The protocols described herein are reproducible and provide a basis for the complete identification of all proteins that *L. monocytogenes* PTM batch cultures express as it enters and maintains stationary phase and growth arrest.

L. monocytogenes continues to be a foodborne pathogen of concern. Despite extensive control measures the incidence of listeriosis has not fallen over the past few years (Section 1.2.1). This investigation has highlighted several gene products important to the early onset of stationary phase and shown that subcultures may be present that expand the range of environmental conditions in batch culture over which the organism may survive. A concerted effort to identify and characterise all temporally important growth phase specific proteins should lead to a greater understanding of the physiology of the stress response. Such an undertaking may also lead to effective microbial control strategies that are independent of or growth phase specific.

The techniques of 2-D SDS-PAGE and mass spectrometry are central to a proteomic platform and recent advances have produced fully integrated systems that offer automation from sample preparation through to protein identification. Mass spectrometry itself has advanced to the point where complex protein mixtures can be analysed and individual proteins automatically identified. Many recent advances are associated with software development and better integration of associated systems. However, just as important was the publication of the complete genome of *L. monocytogenes* published in October 2001. Work continues on elucidation of many other organism genome sequences. This has provided an important database against which proteomic identification of proteins can be compared.

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Appendix I

Published complete microbial genomes (listed alphabetically)

Genome	Strain	Domain	Size (Mb)	Publication
Aeropyrum pernix	K1	A	1.67	Kawarabayasi *et al.*, *DNA Research* 6: 83-101 (1999)
Agrobacterium tumefaciens	C58	B	5.3	Wood *et. al.*, *Science* 294:2317-2323 (2001) / Goodner *et. al.*, *Science 294:2323-2328 (2001)*
Aquifex aeolicus	VF5	B	1.50	Deckert *et al.*, *Nature*392:353 (1998)
Archaeoglobus fulgidus	DSM4304	A	2.18	Klenk *et al.*,*Nature* 390:364-370 (1997)
Bacillus halodurans	C-125	B	4.2	Takami *et.al.*, *Nuc. Acid Res.*28: 4317-4331 (2000)
Bacillus subtilis	168	B	4.20	Kunst *et.al.*, *Nature*390: 249-256 (1997)
Borrelia burgdorferi	B31	B	1.44	Fraser *et al.*, *Nature*, 390: 580-586 (1997) / Casjens *et al.*, *Mol Microbiol*, 35: 490-516 (2000)
Buchnera sp.	APS	B	0.64	Shigenobu *et al.*, *Nature* 407: 81-86 (2000)
Caulobacter crescentus		B	4.01	Nierman *et al.*, *Proc. Natl. Acad. Sci. USA*, 98: 4136-4141 (2001)
Campylobacter jejuni	NCTC 11168	B	1.64	Parkhill *et al.*, *Nature* 403: 665-668 (2000)
Chlamydia pneumoniae	CWL029	B	1.23	Kalman *et al.*, *Nat Genet* 21: 385-389 (1999)
Chlamydia pneumoniae	AR39	B	1.23	Read *et al.*, *Nuc. Acids Res.* 28: 1397-1406 (2000)
Chlamydia pneumoniae	J138	B	1.22	Shirai *et. al.*, *Nuc. Acid Res.* 28:2311-2314 (2000)
Chlamydia trachomatis	serovar D (D/UW-3/Cx)	B	1.05	Stephens *et al.*, *Science* 282: 754-759 (1998)
Chlamydia muridarum	Nigg	B	1.07	Read *et al.*, *Nuc. Acids Res.* 28: 1397-1406 (2000)
Chlorobium tepidum	TLS	B	2.10	Eisen *et al.*, *Proc. Natl. Acad. Sci. USA* 99: 9509-9514 (2002)
Clostridium acetobutylicum	ATCC 824	B	4.1	Nolling *et al.*, *J. Bacteriol.* 183: 4823-4838 (2001)
Deinococcus radiodurans	R1	B	3.28	White *et al.*, *Science* 286: 1571-1577 (1999)
Escherichia coli	K-12 Strain MG1655	B	4.60	Blattner *et. al.*, *Science* 277:1453-1474 (1997)
Escherichia coli	O157:H7 strain EDL933	B	5.5	Perna *et. al.*, *Nature* 409:529-533 (2001)
Escherichia coli	O157:H7 (RIMD 0509952)	B	5.6	Hayashi *et. al.*, *DNA Research* 8:11-22 (2001)
Haemophilus influenzae Rd	KW20	B	1.83	Fleischmann *et al.*, *Science* 269:496-512 (1995)

Halobacterium sp.	NRC-1	A	2.57	Ng *et. al., Proc Natl Acad Sci USA* 97:12176-12181 (2000)
Helicobacter pylori	26695	B	1.66	Tomb *et. al., Nature*388:539-547 (1997)
Helicobacter pylori	J99	B	1.64	Alm *et.al., Nature*, 397:176-180 (1999)
Methanobacterium thermoautotrophicum	delta H	A	1.75	Smith *et.al., J. Bacteriology*, 179:7135-7155 (1997)
Lactococcus lactis	IL1403	B	2.36	Bolotin *et.al., Genome Res*, 11:731-753 (2001)
Listeria innocua	Clip11262, rhamnose-negative	B	3.01	Glaser *et. al., Science* 294:849-852 (2001)
Listeria monocytogenes	EGD-e	B	2.94	Glaser *et. al., Science* 294:849-852 (2001)
Methanococcus jannaschii	DSM 2661	A	1.66	Bult *et. al., Science* 273:1058-1073 (1996)
Mesorhizobium loti	MAFF303099	B	7.59	Kaneko *et.al., DNA Res*, 7:331-338 (2000)
Mycobacterium leprae		B	3.26	Cole *et al., Nature*409:1007-1011 (2001)
Mycobacterium tuberculosis	H37Rv (lab strain)	B	4.40	Cole *et al., Nature*393:537 (1998)
Mycoplasma genitalium	G-37	B	0.58	Fraser *et. al., Science* 270:397-403 (1995)
Mycoplasma pneumoniae	M129	B	0.81	Himmelreich et. al., *Nuc. Acid Res.* 24:4420-4449 (1996)
Mycoplasma pulmonis	UAB CTIP	B	0.96	Chambaud et. al., *Nuc. Acid Res.* 29:2145-2153 (2001)
Neisseria meningitidis	MC58 (ATCC BAA-335)	B	2.27	Tettelin *et al., Science* 287: 1809-1815 (2000)
Pasteurella multocida	Pm70	B	2.4	May *et. al., Proc. Natl. Acad. Sci. USA* 98: 3460-3465 (2001)
Pseudomonas aeruginosa	PAO1	B	6.30	Stover *et al., Nature* 406: 959-964 (2000)
Pyrococcus horikoshii	OT3	A	1.80	Kawarabayasi *et al., DNA Research* 5: 55-76 (1998)
Rickettsia conorii	Malish 7	B	1.27	Ogata et. al., *Science* 293:2093-2098 (2001)
Rickettsia prowazekii	Madrid E	B	1.10	Andersson *et al., Nature* 396: 133-140 (1998)
Saccharomyces cerevisiae chromosome publications	S288C	E	13	Goffeau et. al., *Science* 274: 563-567 (1996) / Goffeau et. al., *Nature* 387: (Suppl.) 5-105 (1997)
Salmonella typhi	CT18	B	4.8	Parkhill et. al., *Nature* 413: 848-852(2001)
Salmonella typhimurium	LT2	B	4.8	McClelland et. al., *Nature* 413: 852-856 (2001)
Sinorhizobium meliloti	1021	B	6.7	Galibert et. al., *Science* 293: 668-572 (2001)
Staphylococcus aureus	N315	B	2.81	Kuroda *et. al., Lancet* 357:1225-1240 (2001)

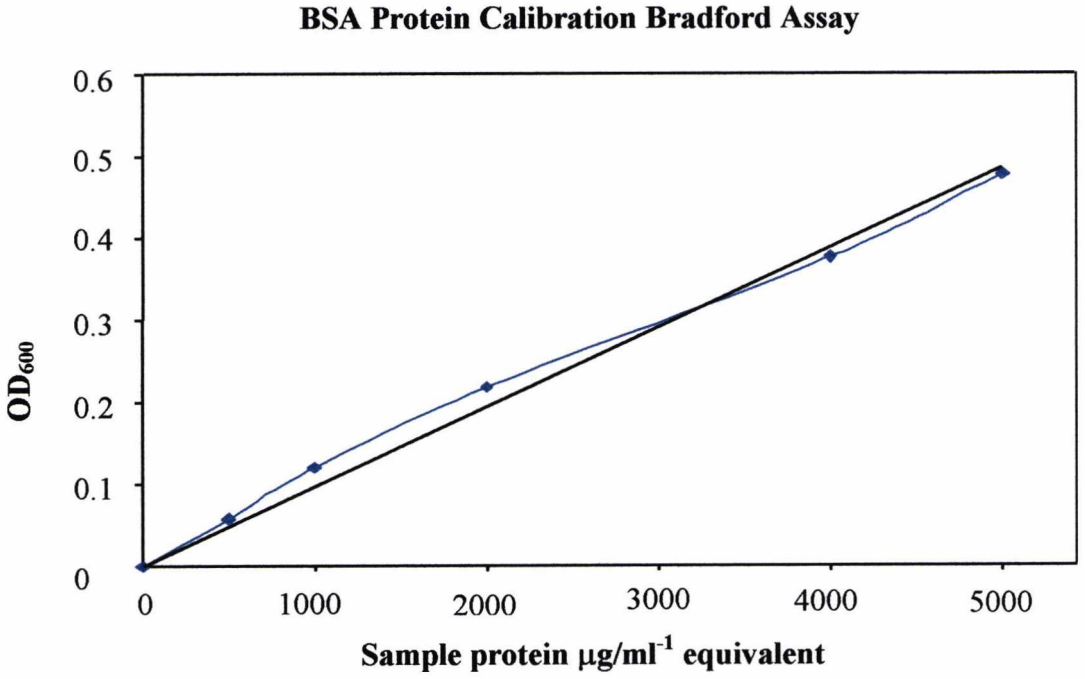
Streptococcus pneumoniae	TIGR4 (ATCC BAA-334)	B	2.20	Tettelin *et al.*, *Science* 293: 498-506 (2001)
Streptococcus pyogenes	M1	B	1.85	Ferretti *et. al.*, *Proc. Natl. Acad. Sci USA* 98: 4658-63 (2001)
Streptococcus pyogenes M3	MGAS315	B	1.90	Beres *et. al.*, *Proc. Natl. Acad. Sci USA* 99: 10078-83 (2002)
Streptococcus pyogenes M18	MGAS8232	B	1.89	Smoot *et. al.*, *Proc. Natl. Acad. Sci USA* 99: 4668-73 (2002)
Streptomyces avermitilis	ATCC31267	B	8.7	Omura *et. al.*, *Proc. Natl. Acad. Sci USA* 98: 12215-12220 (2001)
Streptomyces coelicolor	A3(2)	B	8.7	Bentley *et. al.*, *Nature* 417: 141-147 (2002)
Sulfolobus solfataricus	P2	A	2.99	She *et. al.*, *Proc. Natl. Acad Sci. USA* 98: 7835-7840 (2001)
Synechocystis sp.	PCC 6803	B	3.57	Kaneko *et. al.*, *DNA Res.* 3: 109-136 (1996)
Thermoanaerobacter tengcongensis	MB4	B	2.69	Bao *et. al.*, *Genome Research* 12: 689-700 (2002)
Thermoplasma acidophilum		A	1.56	Ruepp *et. al.*, *Nature* 407: 508-513 (2000)
Thermoplasma volcanium	GSS1	A	1.58	Kawashima *et. al.*, *Proc. Natl. Acad. Sci. USA* 97: 14257-14262 (2000)
Thermotoga maritima	MSB8	B	1.80	Nelson *et al.*, *Nature* 399: 323-329 (1999)
Treponema pallidum	Nichols	B	1.14	Fraser *et al.*, *Science* 281: 375-388 (1998)
Ureaplasma urealyticum	serovar 3	B	0.75	Glass *et al.*, *Nature* 407: 757-762 (2000)
Vibrio cholerae	serotype O1, Biotype El Tor, strain N16961	B	4.0	Heidelberg *et al.*, *Nature* 406: 477-483 (2000)
Xanthomonas axonopodis pv. citri	306	B	5.17	da Silva *et al.*, *Nature* 417: 459-463 (2002)
Xanthomonas campestris pv. campestris	ATCC33913	B	5.07	da Silva *et al.*, *Nature* 417: 459-463 (2002)
Xylella fastidiosa	9a5c	B	2.68	Simpson *et al.*, *Nature* 406: 151-157 (2000)
Yersinia pestis	CO-92 Biovar Orientalis	B	4.65	Parkhill *et al.*, *Nature* 413: 523-527 (2001)

Appendix II

Defined media composition

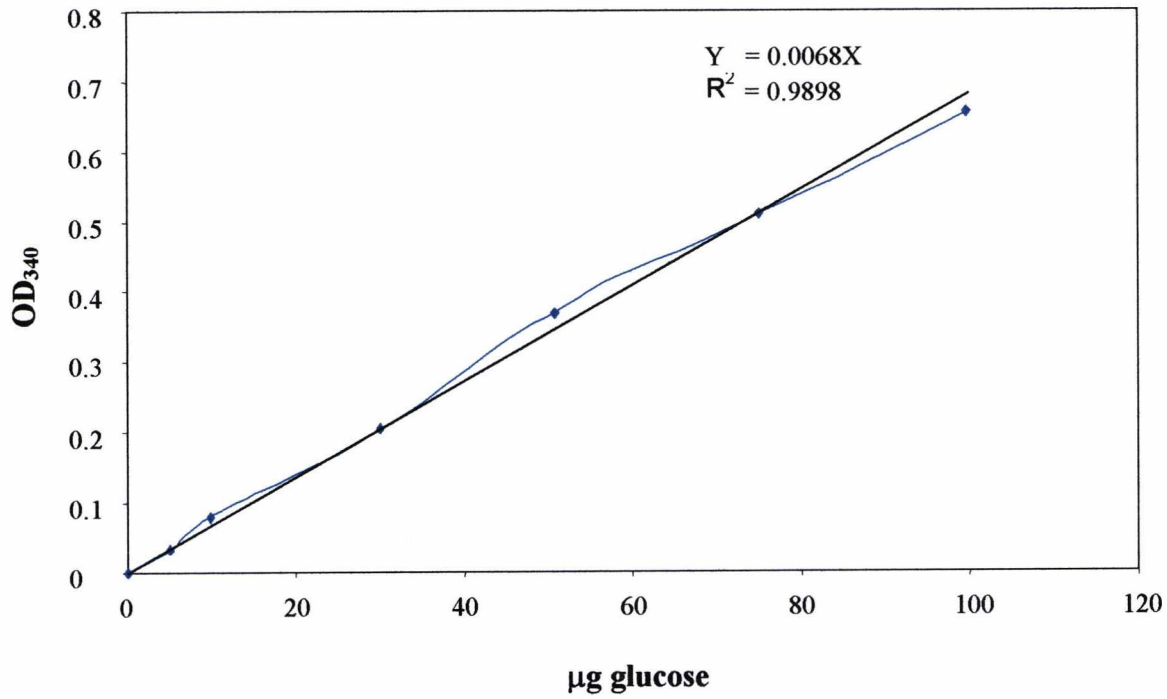
Component	Molecular weight	Conc. (g/L^{-1})	Molarity (mM)
Inorganic salts			
Sodium phosphate, dibasic ($Na_2HPO_4 \cdot 7H_2O$)	268	30.96	115.52
Potassium phosphate, mono. (KH_2PO_4)	136	6.560	48.23
Magnesium sulphate ($MgSO_4.7H_2O$)	246	0.410	1.66
Iron (lll) citrate (Fe $C_6H_5O_7$)	244	0.088	0.360
Other compounds			
D Glucose	180	10.00	55.55
Adenine (6- aminopurine)	184	2.500	13.58
Amino acids			
L – Arginine .HCl	210	0.100	0.476
L – Cysteine.HCl	157	0.100	0.636
L – Glutamine	146	0.600	4.110
L –Histidine.HCl.H_2O	209	0.100	0.478
DL – Isoleucine	131	0.100	0.763
L – Leucine	131	0.100	0.763
DL – Methionine	149	0.100	0.671
L – Phenylalanine	165	0.100	0.606
L – Tryptophan	204	0.100	0.490
DL – Valine	117	0.100	0.085
Vitamins			
Biotin	244	0.500	2.049
Riboflavin	376	5.000	13.297
Thiamine HCl	337	0.001	0.000296
p-Aminobenzoic Acid sodium salt ($C_7H_7NO_2Na$)	159	0.001	0.000628
DL – 6,8 - Thiotic acid	206	0.000005	0.00000242
Nicotinamide	122	0.001	0.000819
D- Calcium pantothenate	476	0.001	0.00021
Pyridoxal.HCl	203	0.001	0.000492

Appendix III


BSA Protein Calibration Bradford Assay
0.6
0.5
0.4
0.3
0.2
0.1
0
OD600
0
1000
2000
3000
4000
5000
Sample protein µg/ml-1 equivalent

Appendix IV

Glucose calibration curve



Appendix V

NiceProt View of TrEMBL: O85743

General information about the entry

Entry name	O85743
Primary accession number	O85743
Secondary accession numbers	None
Entered in TrEMBL in	Release 08, November 1998
Sequence was last modified in	Release 08, November 1998
Annotations were last modified in	Release 18, October 2001

Name and origin of the protein

Protein name	RNA polymerase sigma factor
Synonyms	None
Gene name	SIGB
From	Listeria monocytogenes [TaxID: 1639]
Taxonomy	Bacteria; Firmicutes; Bacillus/Clostridium group; Bacillales; Listeriaceae; Listeria.

References

[1] SEQUENCE FROM NUCLEIC ACID.
STRAIN=LO4035;
MEDLINE=98389670; PubMed=9721294;
Becker L.A., Cetin M.S., Hutkins R.W., Benson A.K.;"Identification of the gene encoding the alternative sigma factor sigmaB from Listeria monocytogenes and its role in osmotolerance.";J. Bacteriol. 180:4547-4554(1998).

[2] SEQUENCE OF 8-266 FROM NUCLEIC ACID.
STRAIN=2289, AND 689426;
MEDLINE=98324975; PubMed=9658010;
Wiedmann M., Arvik T.J., Hurley R.J., Boor K.J.;"General stress transcription factor sigmaB and its role in acid tolerance and virulence of Listeria monocytogenes.";J. Bacteriol. 180:3650-3656(1998).

Comments

- ***Function***: the sigma factor is an initiation factor that promotes attachment of the RNA polymerase to specific initiation sites and then is released *(by similarity)*.
- ***Similarity***: belongs to the sigma-70 factor family.

Cross-references

EMBL	AF074855; AAC34827.1; -. AF032446; AAC38792.1; -. AF032444; AAC38789.1; -.
InterPro	IPR000943; Sigma_70.
Pfam	PF00140; sigma70; 1.
PROSITE	PS00715; SIGMA70_1; 1.
Implicit links to	ProDom; ProtoMap; PRESAGE; ModBase; SWISS-2DPAGE.

Sequence information

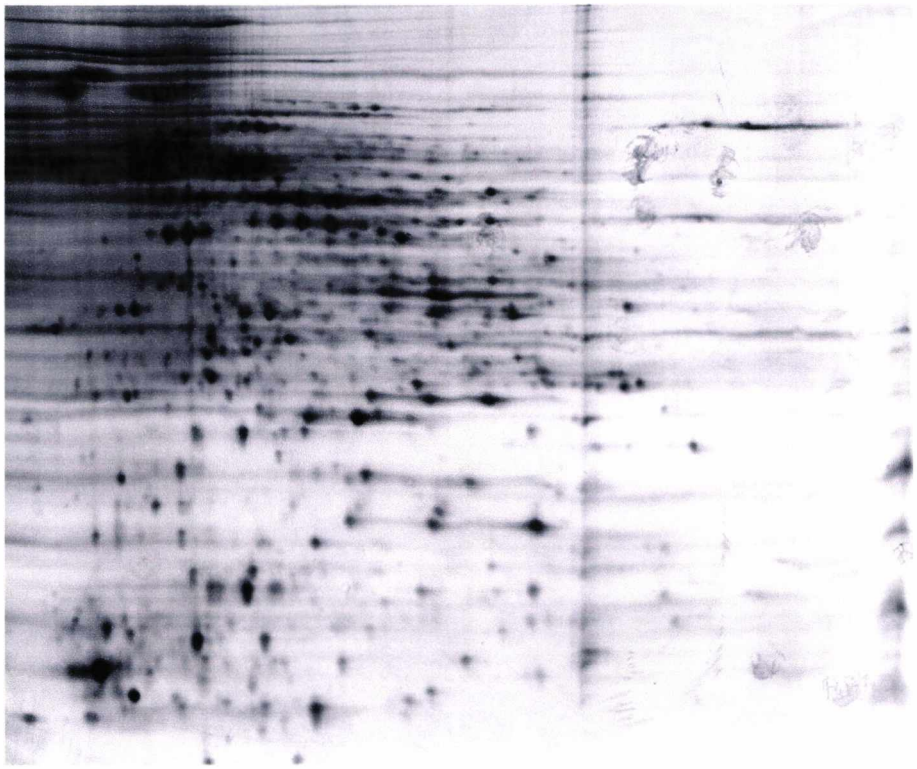
Length: **266 AA**. Molecular weight: **30510 Da**. CRC64: **473489A7DEE7223F** [This is a checksum on the sequence]

```
        10         20         30         40         50         60
         |          |          |          |          |          |
MKSRWRRMPK VSQPDKEAKE KVYIWIAAYQ ENGDQDAQYN LVVHYKNLVE SIARKYSQGK

        70         80         90        100        110        120
         |          |          |          |          |          |
SFHEDLVQVG NIGLLGAIRR YDATFGKSFE AFAVPTIVGE IKRFLRDKTW SVHVPRRIKE

       130        140        150        160        170        180
         |          |          |          |          |          |
LGPKIKNAVE ELTRELQSSP QISDIADFIG VTEEEVLEAM EMGKSYQALS VDHSIEADSD

       190        200        210        220        230        240
         |          |          |          |          |          |
GSTITLLDVV GGTDDGFERV NQRMLLEKVL PVLDEREQKI LQFTFIENRS QKETGELLDI

       250        260
         |          |
SQMHVSRIQR QAIKKLREAL QNEEVE
```

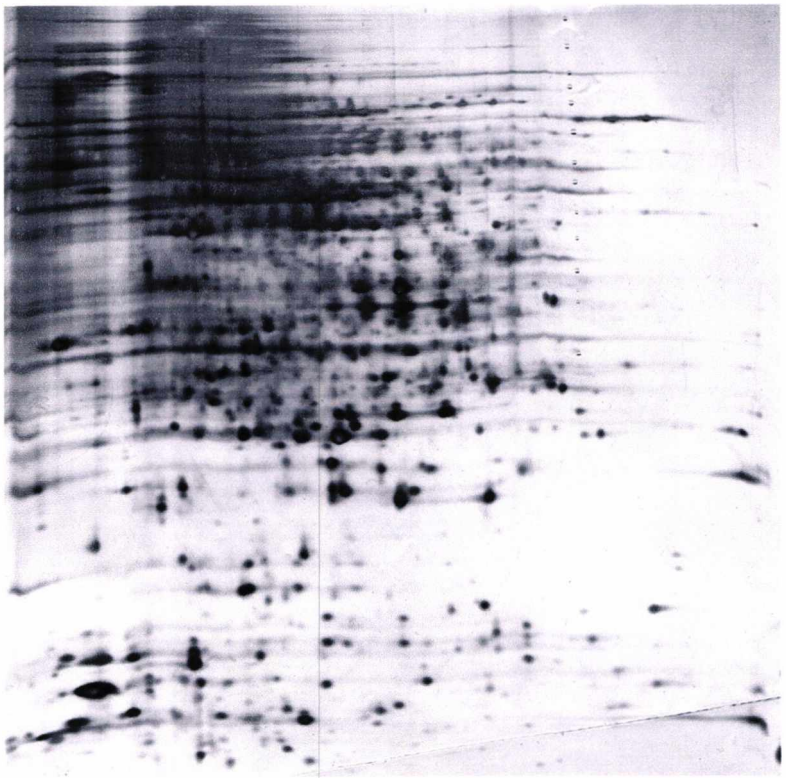
SOURCE http://ca.expasy.org/cgi-bin/niceprot.pl/printable?ac=O85743

Appendix VI

Silver stain image of 24 cm 2-D SDS PAGE separation of *L.monocytogenes* whole cell extract

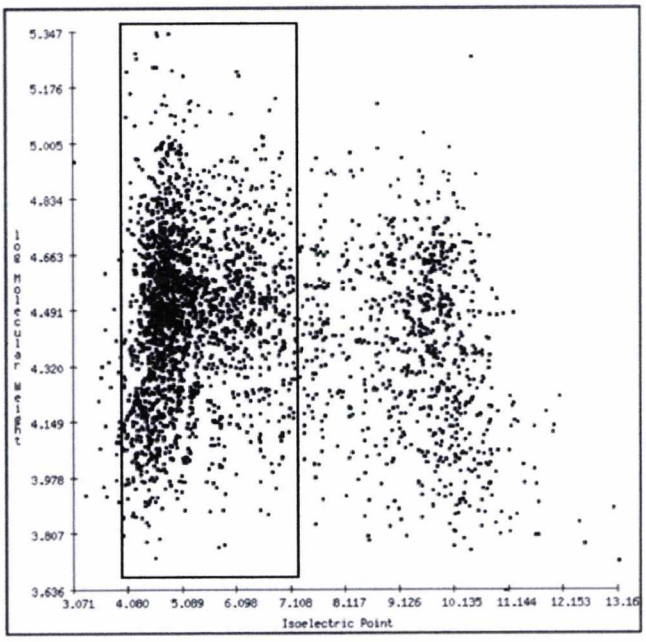


Silver stain image of 18 cm 2-D SDS PAGE separation of *L.monocytogenes* whole cell extract

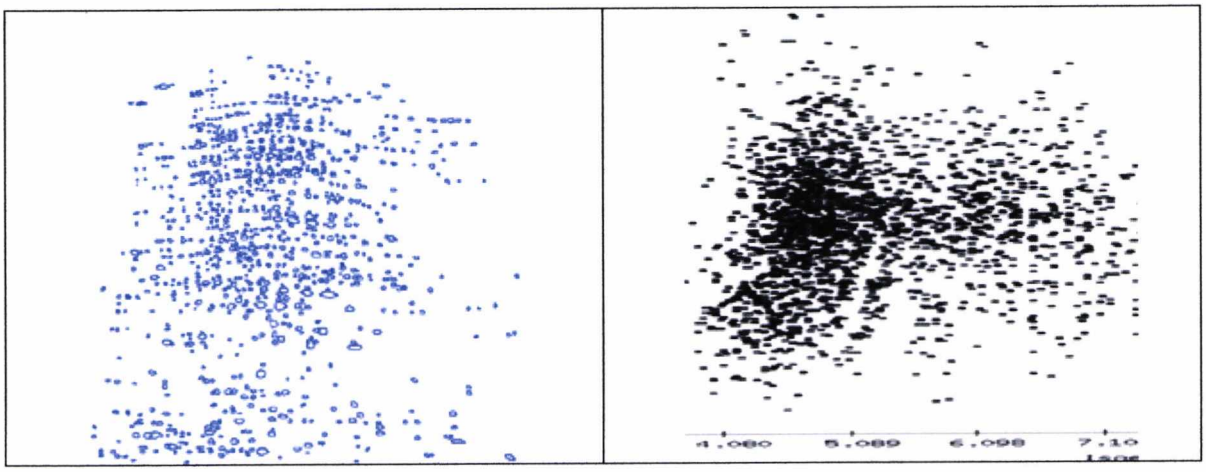


Appendix VII

Theoretical pseudo 2-D gel based on the published complete genome sequence of *L. monocytogenes.*



Boxed area has been expanded to compare an actual gel image with a theoretical gel image



Source http://www.tigr.org/tigt-scripts/CMR2/pseudo2Dgel.spl?db_data_id