Characterisation of the Starvation-Survival Response in *Listeria monocytogenes*

by

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Summary

Listeria monocytogenes is a food-borne pathogen able to adapt and survive in a wide range of habitats in addition to being able to overcome host defences. The need to prevent *L. monocytogenes* entering the food chain and the role that stress plays during the course of an infection, means that the starvation-survival and stress resistance mechanism of this organism are thus of significant interest. The starvation-survival response of *L. monocytogenes* EGD in a chemically defined medium was induced under glucose- or multiple nutrient-, but not amino acid-limitation. This resulted in 90 to 99.9% loss of viability within 2 days, with viability maintained during prolonged starvation. Surviving cells were reduced in size and developed increased general stress resistance. *L. monocytogenes* EGD demonstrated density-dependent starvation-survival under multiple nutrient- but not under glucose-limitation. Protein synthesis was required for long-term survival only for the first 8 hours of starvation and survival became independent of cell wall biosynthesis during long-term starvation.

Strains bearing mutations in the gene regulators *sigB* (DES011) or *prfA* (DES012) showed a 10-fold reduction in starvation-survival compared to EGD after 20 days of glucose limitation. DES011 had reduced exponential phase acid stress resistance, but increased H_2O_2 resistance. Resistance to H_2O_2 in exponential phase and long-term starved DES012 cells was over 290-fold and 380-fold greater than in EGD (after 20 minutes and 50 minutes exposure respectively), whilst exponential- and post-exponential-phase acid resistance in the DES012 was at least 10-fold greater than in EGD. Both DES011 and DES012 also exhibited altered catalase expression. Four transposon insertion mutants (two pairs of siblings) defective in starvation-survival were isolated from a glucose-limitation screen. Both sets of mutations resulted in decreased starvation-survival and altered stress resistance properties. Characterisation of the transposon insertion sites in DES028 and DES029 revealed disruption of a putative ORF encoding for a homologue of YulB, a DeoR-family transcriptional regulator from *Bacillus subtilis*. In the isolates DES035 and DES045, the transposon insertion was found to disrupt a putative ORF encoding for a homologue of PhaQ, a protein associated with inclusion bodies of the storage polymer polyhydroxyalkanoic acid in *Bacillus megaterium*. The roles of these two loci in the starvation-survival response and in stress resistance are discussed.

Table of Contents

List of Figures

List of Tables

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Abbreviations

A_{600}	Absorbance at light wavelength of 600 nanometers
AMP	adenosine monophosphate
ATP	adenosine triphosphate
BSA	bovine serum albumin
cAMP	cyclic AMP
Ccp	catabolite control protein
CCR	carbon catabolite repression
CDM	chemically defined medium
CFU	colony forming units
cm	centimetres
CR	catabolite repression
CRP	catabolite repressor protein
°C	degrees Celsius
Da	daltons
DIG	digoxygenin
DNA	deoxyribonucleic acid
Em^R	erythromycin resistance
g	grams
GASP	growth advantage in stationary phase phenotype
kDa (kbp, kcal)	kiloDaltons (base pairs, calories)
l	litre
M	molar
m	metres
mg (l, m, M)	milligrams (litres, metres, molar)
µg (l, m, M)	micrograms (litres, metres, molar)
MIC	minimum inhibitory concentration
mRNA	messenger RNA
ORF	open reading frame
PBS	phosphate buffered saline

PCR	polymerase chain reaction
PHA	polyhydroxyalkanoate
PI	isoelectric point
ppGpp	guanosine tetraphosphate
PTS	phosphotransferase system
RNA	ribonucleic acid
S.D.	standard deviation
SDS	sodium dodecyl sulphate
sp.	species
TE	Tris-EDTA (buffer)
Tn	transposon
tRNA	transfer RNA
TSB	tryptone soy broth
Tris	tris(hydroxymethyl)aminomethane
u.v.	ultra violet
VBNC	viable but non-culturable
v/v	volume per volume
w/v	weight per volume
X-gal	5-bromo-4-chloro-3-indoyl-β-D-galactoside
σ	sigma factor

CHAPTER 1

INTRODUCTION

1.1 Starvation-survival

Starvation-survival of bacteria is generally defined as comprising the physiological adaptation to a medium containing insufficient nutrients for growth (i.e. increase in cell size) and division. In the natural life of bacteria, the uncertain nature of the environment and competition with other organisms will result in the feast/fast/famine lifestyle commonly recognised in microbial ecosystems (Koch, 1971; Poindexter, 1981), and this can be especially true of facultative intracellular pathogens. As these organisms alternate between different environments, both within and outside of the host, vast changes in nutrient availability, pH, osmolarity, temperature and redox potential can be experienced. The ability of *Listeria monocytogenes* to adapt and survive in a wide range of habitats and to overcome host defence mechanisms indicates a particular ability to deal with an array of stresses and nutrient limitation.

1.2 *Listeria monocytogenes* and stationary phase survival

The starvation-survival response of *L. monocytogenes* has a number of implications for survival within the environment, the subsequent colonisation of food products and during infection. *L. monocytogenes* is a motile Gram-positive, non-sporing, facultatively anaerobic, rod-shaped bacterium. Current taxonomy places *L. monocytogenes* amongst the low G+C content Gram-positive bacteria, which contains the genera *Bacillus*, *Staphylococcus*, *Streptococcus*, *Lactobacillus* and *Lactococcus* (Rocourt, 1988; Woese, 1987).

First identified as a human pathogen from the sporadic infection of farm workers and veterinarians through skin lesions after contact with diseased animals (Gray & Killinger, 1966), a variety of source for outbreaks of listeriosis have since been identified. Whilst *L. monocytogenes* is now well known as a food-borne pathogen, primarily associated with dairy products (Farber and Peterkin, 1991), other food types (meats, vegetables) have also been

implicated (MMWR, 1999). *L. monocytogenes* is frequently isolated from faecal samples, and seropositivity is common amongst the healthy population, with typically 5 to 10% of the population being carriers (Farber and Peterkin, 1991). In animals, the rate of carriage typically varies from 1 to 29%, but has been isolated from up to 52% of bovine faecal samples (Skovgaard and Morgen, 1988).

Since *L. monocytogenes* is generally opportunistic in nature, although healthy adults can contract listeriosis, particular groups tend to be most prone to infection. These include pregnant women, neonates and those carrying conditions that suppress T-cell mediated immunity, especially AIDS sufferers (Nieman & Lorber, 1980). Listerial infection can cause a variety of clinical syndromes, and whilst all conditions can vary from mild symptoms to life-threatening conditions, particularly high rates of mortality (10-50%) are seen (Farber and Peterkin, 1991). Infection of women during pregnancy can lead to the premature birth of a stillborn or infected baby, whilst transmission across the placenta can cause granulomatosis infantiseptica. The infant is born with disseminated abscesses or granulomas, and mortality can be as high as 100% (Armstrong, 1995). Sepsis of unknown origin in neonates is thought to be contracted during or after birth, and causes the development of symptoms (fever and chills) after about 3 days of age. Adult patients are in most cases immunosuppressed, and symptoms can be similar to that of Gram-negative sepsis. It is likely that this form of illness can be the precursor to infection of the meninges or brain. Infection of the central nervous system, and in particular the meninges is the most common syndrome observed, and where present, the associated mortality rates are particularly high (approximately 50%) (Armstrong, 1995). Focal infections can occur at a range of sites, though most commonly on the skin. Peritonitis, endocarditis and osteomyelitis are known, whilst other areas of infection include liver, lymph nodes, the eyes and around prosthetic joints (Armstrong, 1995).

In contrast to other pathogens, the development of multiresistant strains of *L. monocytogenes* has been relatively uncommon to date. *Listeria* is still generally considered as a genus that is uniformly susceptible to antibiotics. The emergence of the first multiresistant strain in 1988 (Poyart-Salmeron *et al.*, 1990), and the subsequent identification of resistant strains of *Listeria* spp. has cast significant doubt on this belief. Of major concern is the development of

resistance to trimethoprim, which in combination with sulfamethoxazole is used in the treatment of listeriosis, particularly where patients are allergic to penicillins (Charpentier *et al.*, 1995). The importance of the development of resistance cannot therefore be underestimated, as has been proven with other bacterial species, and adds further gravity to the threat posed by the emergence of *L. monocytogenes* as a food-borne pathogen.

A number of virulence factors have been identified within *L. monocytogenes*, the majority of which map to the same chromosomal region, whilst others, all associated with cell entry (*iap*, *inlAB*, *inlC*), have been found elsewhere on the chromosome (Dramsi *et al.*, 1993; Engelbrecht, *et al.*, 1996). Genes found in this virulence cluster include those encoding for the haemolysin Listeriolysin O (*hly*), an actin nucleator required for intracellular motility and cell to cell spread (*actA*), a phosphatidylinositol-specific phospholipase (*plcA*) and a lecithinase (*plcB*). The principal regulator of these genes is PrfA (positive regulatory factor of listeriolysin production), a member of the Crp/Fnr family of transcriptional activators (Kuhn & Goebel, 1995; Portnoy *et al.*, 1992; Sheehan *et al.*, 1994). PrfA-dependent regulation of the *L. monocytogenes* virulence genes is known to change in response to specific signals, particularly in response to environmental conditions, nutrient availability and during stationary phase. Since *L. monocytogenes* can survive in a variety of hostile environments and pathogenicity seems closely linked to the starvation and environmental stress responses, it is imperative to understand these processes, and to identify the key mediators involved.

1.3 Stationary phase and nutrient deprivation

The natural environment is highly competitive, with all organisms competing for the available nutrients. Bacteria are so well evolved to the role of nutrient scavengers, that any novel sources are quickly depleted, and so nutrient-limiting conditions are rapidly restored. Before an organism reaches the stage of starvation-survival however, there is a transitional period during which the cells within the culture begin adapting to new, nutrient-limiting conditions. Kolter *et al.* (1993) define this transition as the onset of stationary phase, where all cellular parameters (i.e. DNA replication, protein synthesis and total cell mass) cease increasing at equal rates. This process then continues until no further increase in cell numbers

can be detected. It is important however to separate the concepts of the cessation of growth in cells numbers from the metabolic activity of the cells. As will be discussed in greater detail later, significant evidence points to cells co-ordinately altering metabolic activity during the early stages of nutrient-limitation, retaining basal levels of metabolic activity during starvation and maintaining readiness for subsequent recovery from starvation.

Many nutrients are essential for the bacterial cell including carbon, nitrogen, phosphorus and iron. In the event of the levels of one or more of these nutrients becoming reduced, rather than immediately initiating a generalised response, bacteria trigger a variety of scavenging systems in a nutrient-specific manner. Firstly, an increase in the expression of activity of the normal enzymes responsible for uptake for the nutrient in question is seen. Should this prove insufficient, bacteria may either induce expression of a substitute uptake system with higher substrate affinity, or may express enzymes for the uptake of alternative sources of the nutrient involved. If these options fail to be sufficient, cellular growth is halted and the cells enter stationary phase.

1.3.1 Carbon limitation

Bacteria possess tremendous versatility in their ability to utilise a wide variety of different carbon sources. Bacteria will preferentially use the source that can be metabolised most readily, in most cases glucose, and whilst this is still available, expression of uptake systems for alternative sources is repressed by a process known as catabolite repression (Magasanik and Neidhardt, 1987).

The classic model for carbon catabolite repression (CCR) in bacteria is the mechanism that seen in the diauxic growth of *Escherichia coli* on glucose and lactose involving the catabolite repressor protein (CRP), cyclic AMP (cAMP) and the phosphoenolpyruvate-dependent phosphotransferase system (PTS) (Postma *et al.*, 1993). Bacteria simultaneously transport and phosphorylate many carbohydrates via the PTS, which is comprised of two general phosphotransfer proteins (Enzyme I [EI] and HPr), a substrate-specific permease (Enzyme II [EII]) which may be made up from several polypeptide subunits, and occasionally Enzyme III

(EIII), which can be found between HPr and EII. In the absence of glucose, the phosphorylated form of the glucose-specific EII subunit ($EIIA^{Glc}$) accumulates, as no phosphate sink for the system is present, and stimulates adenylate cyclase to synthesise cAMP from ATP. The cAMP produced binds CRP, the resulting complex activating transcription of the *lac* operon by binding a specific transcription-activating site. In the presence of glucose, non-phsophorylated $EIIA^{Glc}$ accumulates, which inhibits lactose permease by binding, thus preventing formation of the intracellular inducer allolactose, a process known as inducer exclusion.

In low GC content Gram-positive bacteria, carbon catabolite repression (CCR) appears to be principally dependent upon the HPr component of the PTS. In contrast to Gram-negative bacteria, the HPr of *Bacillus* species can be phosphorylated at two sites, the first (His-15) by EI, and the second (Ser-46) by an ATP-dependent HPr kinase (Stülke and Hillen, 2000). Phosphorylation at the first site forms part of the inducer exclusion regulatory pathway, whereas phosphorylation at the second is central to CcpA-mediated expression. CcpA, a member of the LacI/GalR family of regulators, is found in many Gram-positive bacteria, and mediates CCR of numerous catabolic genes in association with HPr by binding to a 14 bp region known as the catabolite responsive element (*cre*) (Stülke and Hillen, 1999). By binding to *cre* sequences located at varying positions relative to the promoter depending on the gene in question, CcpA can cause repression by the prevention of transcription initiation by causing a blockage in transcription, or by interference with the interaction between RNA polymerase and an activator (Stülke and Hillen, 1999). The *Bacillus subtilis* genome sequencing project discovered a gene encoding for an additional component of the CCR (Galinier *et al.*, 1997). Crh possesses 45% homology to HPr, but while Crh cannot be phosphorylated by EI, since the histidine residue at the first phosphorylation site is replaced with glutamine, phosphorylation by HPr kinase at the serine active site still occurs (Galinier *et al.*, 1997, 1998). In *B. subtilis*, expression of some catabolic enzymes become CCR-independent in a *ccpA* mutant. However, expression of several enzymes (β-xylosidase, inositol dehydrogenase and α-amylase), were only partially relieved from CCR or not at all in *hpr* mutants. In the case of β-xylosidase the phosphorylated Crh protein (P-Ser-Crh) was shown to stimulate the binding of CcpA to the *cre* sequence in the gene encoding for β-

xylosidase (Galinier *et al.*, 1998). Therefore some genes in the catabolite repression regulon are controlled independently of HPr. The full purpose of this alternative mode of catabolite repression is not yet clear.

1.3.2 Nitrogen limitation

In *E. coli* and *B. subtilis*, all cellular nitrogen for macromolecular synthesis is derived from the direct incorporation of ammonia, the amido group of glutamine (primarily for amino acid biosynthesis), or the amino group of glutamate (for purine, pyrimidine, histidine, tryptophan and asparagine biosynthesis) (Fisher 1994). Since many Gram-negative and Gram-positive bacteria preferentially use ammonia, the primary route for nitrogen assimilation in enteric bacteria is the production of glutamine from NH_4^+ (Figure 1.1). In *E. coli*, limitation of nitrogen in the form of ammonia is signalled by a drop in cellular glutamine. This results in the phosphorylation of NtrC, which in turn activates the transcription of Ntr-regulated genes via the alternative sigma factor RpoN (σ^N) (Magasanik and Neidhardt, 1987). Nitrogen limitation causes the induction of the expression of glutamine synthetase, together with numerous genes enabling the utilisation of alternative nitrogen sources for the synthesis of NH_4^+, glutamate and glutamine.

Nitrogen limitation via amino acid starvation induces the stringent response, which is signalled by the limitation of aminoacyl-tRNA available for protein synthesis (Cashel *et al.*, 1996). This response covers a broad spectrum of cellular processes (Table 1.1), and is governed by the *relA*-dependent accumulation of guanosine tetraphosphate (ppGpp). The stringent response can also be stimulated under carbon limitation, in part, by a *relA*-independent route by the activity of SpoT (Nyström, 1995).

1.3.3 Phosphorus limitation

Despite the fact that it is relatively abundant in nature, phosphorus is often growth-limiting since it mainly occurs in the form of insoluble salts. Owing to the importance of the supply of

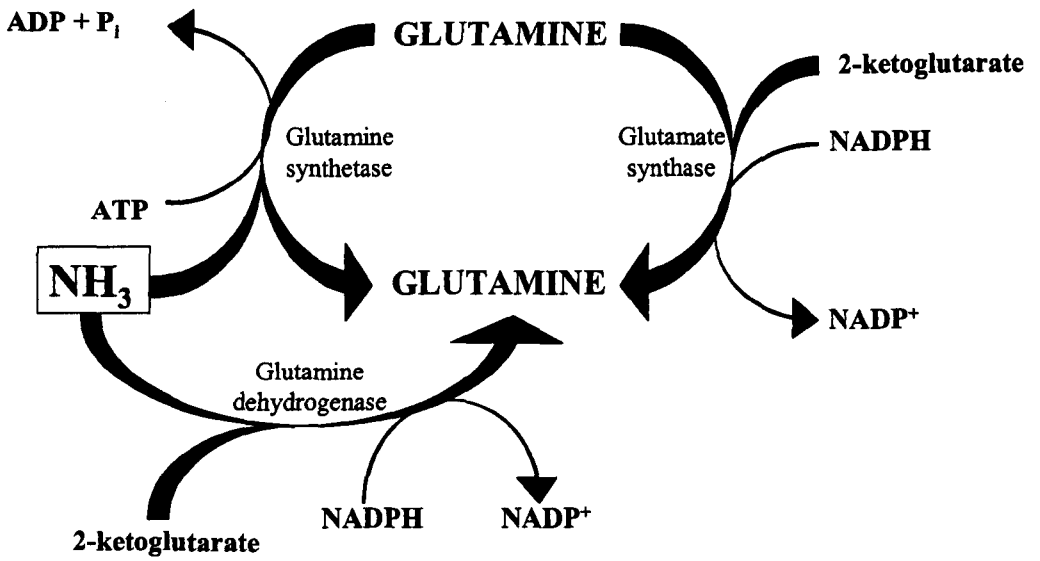


Figure 1.1

Ammonia assimilation in *E. coli*.

Activity	Effect (+/)
Stable RNA accumulation	-
Total carbohydrate synthesis	-
Membrane transport of glycosides	-
Glycolysis	-
Glucose respiration	-
Phospholipid synthesis	-
Lipid synthesis	-
Cell wall Lipopolysaccharide formation	-
Peptidoglycan formation	-
Nucleotide synthesis	-
Polyamine uptake	-
Size of dividing cells	Reduction
Amino acid operon transcription	-
Guanosine polyphosphate accumulation	+
Accumulation of adenosine polyphosphates	+

Table 1.1

Effects of aminoacyl-tRNA deprivation on various non-translational processes.

phosphate for cellular energy production and metabolic processes, a complex regulatory mechanism has evolved in *B. subtilis*, containing at least three two-component signal transduction systems (Hulett, 1996). PhoP-PhoR is the system primarily involved in response to phosphate deficiency controlling the Pho regulon, the phosphorelay system (SpoO) plays a role in sporulation initiation, and ResD-ResE in respiratory regulation. Genes that fall within the Pho regulon possess a Pho box within the promoter region, and latest estimates propose up to 130 or more genes being members (VanBogelen *et al.*, 1996). The potential for another tier of regulation by P-limitation exists in the discovery of a Pho box in the promoter region of *rpoH* in *E. coli*, a gene encoding the transcriptional activator σ^H (Smith and Payne, 1992).

The extent to which P-limitation affects bacterial gene expression is seen in the global study of *E. coli* protein synthesis (VanBogelen *et al.*, 1996). This revealed some 413 out of the 816 proteins monitored formed a PL (Phosphate-limited) stimulon and of these, 208 had induced expression, and 205 were repressed. Alternately, of 257 proteins that showed differential synthesis when phosphonate (commonly found in eukaryotic membranes) was used as a sole P source, 227 showed induced expression, and 30 were repressed. These results would mean that the expression of an estimated 10% of the *E. coli* genome responds to P-limitation, and thus many genes are likely to belong to overlapping stimulons in stress responses.

1.3.4 Iron limitation

In many bacteria, iron limitation initially induces the production of siderophores – scavenging molecules with high affinity for iron. Control of siderophore production falls under the control of the Ferric Uptake Regulator (Fur). Homologues of this regulator have now been identified in both Gram-negative and Gram-positive bacteria, and have since been identified in the regulation of genes outside of ferric uptake systems. These include the gene *sodA*, encoding a manganese-containing superoxide dismutase involved in protection from oxidative stress in *E. coli* (Privalle, and Fridovich, 1993), and virulence determinants in *E. coli*, *Vibrio cholerae*, *Serratia marcescens*, *Pseudomonas aeruginosa* and *Neisseria meningitidis* (Calderwood and Mekalanos, 1987; Karjalainen *et al.*, 1991; Goldberg *et al.*, 1991; Poole and Braun, 1988; Prince *et al.*, 1993; Thompson *et al.*, 1993). Regulation is

achieved as the Fur protein binds Fe(II) in iron replete conditions, the complex then repressing transcription of the siderophore synthetic genes. Under iron-limiting conditions, no Fur-Fe(II) complex is formed, and the siderophore genes are expressed.

In the Gram-positive bacteria *B. subtilis* and *Staphylococcus aureus*, three Fur homologues are found (Fur, Zur and PerR) (Bsat *et al.*, 1996; Gaballa and Helmann, 1998; Horsburgh *et al.*, 2001). Fur and Zur regulate iron and zinc homeostasis respectively, whilst PerR controls oxidative stress resistance, iron storage gene expression and also plays a role in the virulence of *S. aureus* (Horsburgh *et al.*, in press). An unrelated, but functionally similar group of metal ion-regulators are also found in some Gram-positive bacteria. DtxR in *Corynebacterium diptheriae*, regulates diptheria toxin and siderophore production, using operator boxes with some similarity to the Fur-box (Fourel *et al.*, 1989; Boyd, *et al.*, 1990; Schmitt, 1992), whilst a DtxR homologue in *B. subtilis* (MntR) regulates the transporters MntABCD and MntH in a manganese-dependent fashion (Que *et al.*, 2000).

The limitation of available iron by human transferrin and lactoferrin is a primary barrier to infection of the human host, and bacteria have developed a variety of mechanisms for obtaining iron under these circumstances. Some species such as *Neisseria* spp. and *S. aureus* produce receptors for direct use of these compounds (Modun *et al.*, 1994; Mickelsen *et al.*, 1981), whilst the production of iron-regulated haemolysins is a trait commonly seen in pathogens. *L. monocytogenes* does not produce siderophores (low-molecular weight iron chelating agents), and so must obtain iron via alternative mechanisms. The ability to bind of ferric citrate indicates the presence of a low-affinity mode of iron acquisition (Adams *et al.*, 1990). *L. monocytogenes* can also sequester iron via ferric reductase activity, which converts insoluble ferric iron to the more soluble ferrous iron, and requires the concomitant oxidation of NADH (Barchini and Cowart, 1996). Such is the extent of ferric reductase activity in *L. monocytogenes*, that iron can be obtained from not only iron salts, but also from siderophores and iron chelating compounds such as transferrin, lactoferrin, haemoglobin ferritin (Cowart and Foster, 1985; Deneer and Boychuk, 1993; Hartford *et al.*, 1993; Deneer *et al.*, 1995).

1.4 Cellular responses to starvation

The development of a starvation-survival phenotype is not an isolated mechanism and the transition to stationary phase can have a significant effect on maintenance of viability. As nutrients become limiting, bacteria may respond by attempting to maintain growth by either increasing expression of regular nutrient uptake systems, inducing alternate higher-affinity

scavenging mechanisms or using alternate substrates. Should these responses fail to be sufficient, net growth will cease and bacterial metabolism will become tailored to the maintenance of viability. In non-differentiating bacteria, the methods by which this is achieved involve highly ordered sets of regulatory networks, and can occasionally show distinct similarities to the spore-forming bacteria.

There are principally three phases to the starvation-survival response as outlined in *Vibrio sp.* S14 (Nyström *et al.*, 1990a). Within the first 30 minutes, the stringent response (described below) initiates rapid downregulation of tRNA, rRNA, protein and peptidoglycan synthesis (Nyström *et al.*, 1990a, Nyström and Kjelleberg, 1989). Also seen at this stage is an increase in intracellular proteolysis, expression of high affinity nutrient scavenging systems such as the broad substrate leucine uptake system (Mårdén *et al.*, 1987) and cellular exoprotease activity (Albertson *et al.*, 1990a). In the next phase, the stringent response is relieved, and 5 hours into starvation, a 10-fold reduction in total fatty acids is observed, along with a shift in their composition toward shorter, more unsaturated molecules. Over long-term starvation, the rate of protein synthesis falls further, though detectable levels are observed as long as the cells remain viable. Other changes include increased u.v. resistance, increased glucose uptake system affinity, and an increase in the mean mRNA half-life from 1.6 to 10.2 minutes (Nyström *et al.*, 1992; Albertson *et al.*, 1990b,c).

In studies on certain starved non-differentiating bacteria, significant discrepancies in the number of bacteria observed by microscopy and that obtained by viable plate counts have been recognised. The use of metabolic assays (Kogure *et al.*, 1979; Roszak and Colwell, 1987) and flow cytometry (Kaprelyants and Kell, 1992) has since suggested 'viable but non-culturable' (VBNC) forms of bacteria. The concept of a number of species entering the VBNC state via a specific reversible programmed or adaptive process is little supported by evidence of the resuscitation of such cells (Barer and Harwood, 1999), and it seems increasingly likely that temporary non-culturability actually stems from an accumulation of damage to the cell.

1.4.1 Cell envelope

Bacteria have limited scope to escape or alter the immediate environment, and hence a number of specific phenotypic changes are needed to enable survival until more favourable conditions prevail. One of the key responses to stress, including starvation, is the protection of the delicate cytoplasmic environment via alterations in the cell envelope. In Gram-negatives, carbon starvation causes an increase in the level of murein cross-linking (Schleifer *et al.*, 1976; Pisabarro *et al.*, 1985), with a concurrent increase in the thickness of the peptidoglycan layer as turnover falls to near zero (Leduc *et al.*, 1989). Major changes occur to both the inner and outer membrane of *E. coli* as fluidity and permeability are reduced. This is in contrast to *V. sp.* S14, where all unsaturated fatty acids are converted to the cyclopropyl form, phosphatidylglycerol is reduced, and cardiolipin levels rise (Wanner and Egli, 1990; Mukamlova *et al.*, 1995). In the outer membrane, lipopolysaccharide becomes more exposed on the cell surface, hydrophobicity increases and the lipoprotein becomes increasingly bound to peptidoglycan.

In Gram-positive bacteria, a variety of changes to the composition of the cell envelope are observed. Included is the saturation and epoxylation of unsaturated phospholipid fatty acids (Cronan, 1968; Guckert *et al.*, 1986; Hood *et al.*, 1986), changes in the fatty acid ratio and even the loss of phospholipids (Hood *et al.*, 1986; Lonsmann-Iversen, 1987). Within the envelope, increased incorporation of phosphorous-free teichoic acids in place of the phosphorylated version occurs (Ellwood and Tempest, 1972; Neidhardt *et al.*, 1990), which is possibly another mechanism for sequestering phosphorous during periods of starvation.

1.4.2 Morphology

Upon entry to stationary phase, many cells are actively involved in DNA replication, and so are initially committed to continued division. The completion of the current round of cell division is associated with a reduction in the average cell size, a phenomenon known as reductive division (Foster and Spector, 1995; Watson *et al.*, 1998a), and cells from rod shaped

bacterial species will also adopt a more coccoid form (Kjelleberg *et al.*, 1993; Kolter *et al.*, 1993). In *V. sp.* S14, the form of nutrient-limitation encountered can be signified by differences in the morphological characteristics of the cell. Phosphorous starvation results in swollen, elongated cells as PHB accumulates in the cytoplasm, whilst thin filamentous bacteria are created under nitrogen limitation and small, coccoid, nonmotile cells are found under either carbon- or multiple nutrient-limitation (Kjelleberg *et al.*, 1993).

In *E. coli*, the small, coccoid morphology characteristic of stationary-phase is seen during overexpression of the *bolA* gene (Aldea *et al.*, 1990). The precise role of the BolA protein is not fully understood, although involvement in the switching between cell elongation and septation mechanisms within the cell division cycle has been suggested (Aldea *et al.*, 1989, 1990). A helix-turn-helix motif implies that BolA may act by binding to DNA, and indeed two-dimensional electrophoresis revealed the induction of the penicillin-binding protein PBP6 (Aldea *et al.*, 1989). Constitutive expression of *bolA* occurs, implying a role for *bolA* in normal cell division, whilst increased expression of *bolA* was observed upon entry into stationary phase or in direct response to stress, and is dependent upon the alternative sigma factor RpoS (σ^S) (Aldea *et al.*, 1989; Santos *et al.*, 1999).

1.4.3 Storage Compounds

Bacteria can accumulate a number of storage compounds in response to stress, starvation in particular. In *E. coli*, if glucose is in excess, transition to stationary phase results in accumulation of glycogen, synthesis of which is mediated by the glycogen biosynthetic genes of the *glg*CAP operon, which is inducible by ppGpp (Preiss and Romeo, 1989) and by the σ^S-regulated *glgS* gene (Hengge-Aronis and Fischer, 1992). Possibly the most significant family of bacterial storage compounds are the polyhydroxyalkanoic acids (PHAs), the most common form of which is polyhydroxybutyric acid (PHB).

PHA storage has been found in both Gram-positive and Gram-negative bacteria, including species of *Vibrio, Pseudomonas, Bacillus* and *Staphylococcus* (Anderson and Dawes, 1990; Szewczk, 1992). PHA is typically accumulated under condition of excess glucose

accompanied by the limitation of another essential nutrient (e.g. nitrogen, phosphorus, oxygen, iron) (Anderson and Dawes, 1990). In *V. sp.* S14, phosphate starvation induces the significant accumulation of PHB (Malmcrona-Friberg *et al.*, 1986). In staphylococci, little PHB accumulation occurs - up to 0.15% (wt/wt), compared to 80% (wt/wt) in *Alcaligenes eutrophus*. This however enables the cell to utilise the polymer to delay the degradation of essential cell components during starvation (Szewczk, 1992).

Biosynthesis of PHAs can be achieved using many different substrates, and can vary between species, but is best understood in the synthesis of PHB from acetyl-CoA in the Gram-negative *A. eutrophus* (Anderson and Dawes, 1990). Synthesis occurs via the action of three enzymes – β-ketothiolase, acetoacetyl-CoA reductase, and PHB synthase (Figure 1.2). Condensation of two acetyl-CoA moieties by β-ketothiolase is followed by reduction of the acetoacetyl-CoA to D(-)-β-hydroxybutyryl-CoA. In the final step, PHB synthase links the D(-)-β-hydroxybutyryl moiety to an existing polymer molecule by an ester bond.

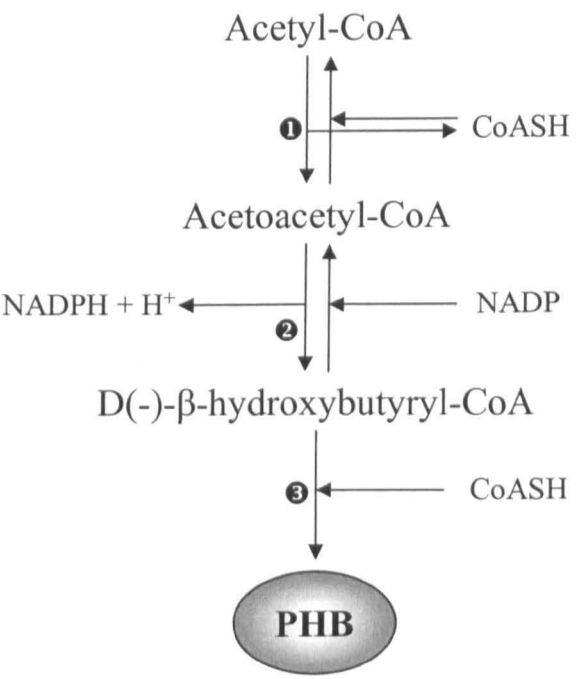


Figure 1.2

PHA biosynthetic pathway in *A. eutrophus*. Enzymes: 1. β-ketothiolase; 2. Acetoacetyl-CoA reductase; 3. PHB synthase.

The principal factor in the control of PHB synthesis is acetyl-CoA, which can act as a substrate for PHB synthesis or the tricarboxylic acid (TCA) cycle. In *Azotobacter beijerinckii*, PHB synthesis from glucose occurs under oxygen limitation. Citrate synthase and isocitrate dehydrogenase are inhibited by NADH, which accumulates under oxygen limitation. The rate of consumption of acetyl-CoA by the TCA cycle decreases, and acetyl-CoA enters the PHB biosynthetic pathway. In oxygen rich conditions, the release of CoA from acetyl-CoA upon entry to the TCA cycle inhibits the activity of β-ketothiolase, thus reducing PHB synthesis (Senior and Dawes, 1973)

1.4.4 Cellular DNA and RNA

Dependent upon the rate of growth approaching stationary phase, bacteria may contain the equivalent of four copies of the genome (Bremer and Dennis, 1987; Cooper, 1991). It was thought that already initiated DNA replications must be completed, and subsequently a number of cellular divisions will occur, since DNA and cell replication are out of sequence. The assumption is that further division will reduce the DNA content to a single DNA equivalent, though there is now a suggestion that some Gram-negative bacteria may be able to survive stationary phase with multiple DNA equivalents (Lebaron and Joux, 1994). In contrast, Gram-positive bacteria such as *Micrococcus luteus* show no variation in DNA content upon starvation for up to 150 days (Mukamolova *et al.*, 1995). In starved cells chromosomal DNA becomes condensed as levels of histone-like proteins (Dps, H-NS) are increased (Almirón *et al.*, 1992; Dersch *et al.*, 1993). This condensation is thought to play a part in both the repression of gene expression during starvation and the protection of the DNA from damage.

The net RNA content of the cells falls during starvation, whilst RNA synthesis continues, having been reduced to about 10% of that seen in exponential growth. The breakdown products act as an energy source via the release of ribose, whilst the release of phosphate helps to replenish the orthophosphate pool during phosphate starvation (Horinuchi, 1959; Medveczky and Rosenberg, 1971), and may also be rapidly incorporated into DNA. The observation that functional rRNA is retained at levels far higher than that required for

translation within the starved cells, and the rapid response of protein synthesis to nutrient upshift, indicate that non-differentiating bacteria attempt to remain primed for maximal growth well into starvation.

1.4.5 Proteins

Early on in carbon and nitrogen exhaustion, the bacteria must begin producing starvation-associated proteins and remove those previously used in exponential growth. As part of a generalised starvation response, this is aided by increased protein turnover from 1-3% to 5-7% per hour (Mandelstam, 1960; Pine, 1972; Goldberg and Dice, 1974), which helps provide the required amino acids for *de novo* protein synthesis, in addition to the removal of the now redundant proteins. During this stage, little change in overall protein concentration is witnessed (Wanner and Egli, 1990), and only when less vital cell components, storage compounds and RNA have been fully utilised does the actual breakdown of proteins become a primary source of energy (Strange *et al.*, 1961; Postgate and Hunter, 1962; Burleigh and Dawes, 1967; Scherer and Boylen, 1977; Nazley *et al.*, 1980; Boyaval *et al.*, 1985).

1.4.6 Starvation-associated stress resistance

The dramatic increase of resistance to a wide array of environmental stresses is a key development during the bacterial SSR (Kolter *et al.*, 1993; Siegele and Kolter, 1992). The most likely reason for this occurrence is that the cells are able to withstand exposure to additional stresses that may occur at times when nutrient limitation prevents a more active response to the stress in question. In particular, increased resistance to oxidative stress, low pH and heat have major significance for protocols for the elimination of *L. monocytogenes* from the food production environment.

Oxidative stress is caused by exposure to reactive oxygen intermediates such as superoxide anion ($O_2^{\bullet}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($HO^{\bullet}$), which can originate from exogenous or endogenous sources (Storz and Zheng, 2000). Damage to cells from oxidative stress can occur in proteins nucleic acids and cell membranes, and is increasingly suggested as a key factor in the ageing of bacterial cultures and ultimately cell death.

The adaptive responses to oxidative stress in Gram-negative bacteria are controlled by the *oxyR* and *soxRS* systems. In *E. coli*, the *oxyR* gene product induces the expression of at least 10 genes in response to H_2O_2 stress (Storz and Zheng, 2000). These include antioxidant enzymes (catalase, glutathione reductase and alkyl hydroperoxide reductase) and a DNA protection protein (Dps). Regulation is thought to be via a conformational change caused by oxidation of OxyR (Storz and Tartaglia, 1992). The *soxRS* system is a two-part O_2•-inducible regulatory mechanism, which induces at least 12 proteins including manganese-cofactored superoxide dismutase (MnSOD), glucose-6-phosphate dehydrogenase and O_2•-resistant forms of fumarase and aconitase (Nunoshiba, 1996). Regulation is dependent on the [2Fe-2S] centres present in SoxR. In the absence of the redox signal, the centres are removed, inactivating SoxR, whilst in the presence of the redox signal, SoxR becomes active as the [2Fe-2S] centres are reinserted. The active SoxR enhances transcription of SoxS, which in turn stimulates the *soxRS* stimulon (Demple, 1996). Understandably, given the increase in oxidative stress resistance in starved cells, overlap of the oxidative stress and starvation regulons is found. σ^S-dependent expression of both *oxyR* (*katG*, *gorA*, *dps*) and *soxRS* (*fumC*, *acnA*) regulated genes is seen (Storz and Zheng, 2000). In addition, *katE*, *xthA* and *sodC* which encode hydroperoxidase II, exonuclease III and copper-zinc superoxide dismutase respectively, all confer stationary-phase oxidative stress resistance in an σ^S-dependent fashion (Storz and Imlay, 1999).

Understanding of oxidative stress resistance in Gram-positive bacteria has been better defined in recent years. A number of H_2O_2-induced genes including *katA* (catalase), *ahpC* (alkyl hydroperoxide reductase) and *mrgA* (Dps), are co-ordinately regulated by PerR, a member of the Fur family transcriptional repressors (Bsat *et al.*, 1998). An additional regulator of several oxidative stress genes is the alternative sigma factor SigB (σ^B), which primarily induces expression in exponentially growing cells in response to stress (Reviewed in Chapter 1.7.2).

When the environment becomes sufficiently acidified, leakage of H^+ ions across the cell membrane can lead to acidification of the cytoplasm, resulting in damage to cell components and disruption of biochemical reactions. Bacteria are remarkably proficient at adapting to changes in pH. If previously exposed to nonlethal acidic conditions, they can survive over a

10^6-fold change in H^+ concentration (Foster, 2000), with the coordinate induction of up to 50 acid shock specific proteins (Foster, 1999). The first reaction of Gram-positive bacteria to acid stress is proton export, which is performed by the F_oF_1 ATPase in streptococci (Kobayashi *et al.*, 1982). In addition, some lactobacilli and streptococci are able to utilise the generation of ammonia by an arginine deaminase pathway to survive in an acidic environment (Marquis *et al.*, 1987). A further alternative, if glutamate is present, is the consumption of protons by the glutamate decarboxylation/antiporter system (Foster, 2000).

Both Gram-positive and Gram-negative bacteria exhibit a sophisticated acid tolerance system, the complexity of which requires several gene regulators. In *Salmonella typhimurium*, σ^S is a key regulator of both the stress-induced acid tolerance response (ATR) in exponential-phase cells, and the pH-independent carbon starvation-inducible ATR (Lee *et al.*, 1994), whilst the regulatory mechanism for which a third, pH-inducible ATR remains unknown (Lee *et al.*, 1994). The picture in Gram-positive acid stress resistance is also being elucidated. With the absence of σ^S in these bacteria, σ^B was identified as a stationary-phase acid tolerance regulator in *L. monocytogenes*, although whether σ^B also forms part of the pH-inducible exponential-phase ATR, as in the case of σ^S, has not been established (Wiedmann, *et al.*, 1998).

An array of heat shock proteins (hsps) can be induced by the cessation of growth caused by starvation. Two dimensional gel analysis of *E. coli* revealed 11 proteins common to both glucose starvation and heat shock (Jenkins *et al.*, 1988), including DnaK, GrpE, GroEL, GroES, ClpB, Lon, HtpH and the heat shock regulator RpoH (σ^H) (Nyström, 1995). Hsps perform a number of functions within the cell from the chaperonins GroEL, GroEs, DnaK, DnaJ and GrpE, to the Lon and Clp proteases, which bind and degrade misfolded and denatured proteins respectively (Foster and Spector, 1995). Regulation of these genes in response to starvation is dependent upon σ^H (Nyström 1994), which confers heat-shock promoter specificity on RNA polymerase. The mechanism at work during starvation has not been determined, but during heat-shock, preformed σ^H is released from a complex with DnaKJ and GrpE, whilst free DnaK/DnaJ binds denatured proteins, resulting in available σ^H for expression of target genes (Foster and Spector, 1995).

1.4.7 Starved culture dynamics

With readily available evidence from spore-forming bacteria, it had long been tempting to consider stationary phase cultures of non-sporing bacteria as being static in nature. This involved cells suspending the normal cell cycle until favourable conditions prevail, an idea that ultimately led to the concept of the VBNC state (Roszak and Colwell, 1987; Kaprelyants and Kell, 1992). While the VBNC phenomenon is still subject to much debate, evidence for significant activity within stationary-phase cultures has frequently been found. In *E. coli*, Lazar *et al.* (1998) identified a starvation-survival (*surA*) strain carrying a mutation in a gene involved in the assembly of cell wall synthesis apparatus. This resulted in a survival defect, where cells lysed whilst attempting to resume growth during stationary phase. Cell division within stationary-phase cultures has also been alluded to by the loss of viability observed after addition of cell wall biosynthesis inhibitors (ampicillin/penicillin G) to starved cultures of *Vibrio vulnificus* and *S. aureus* (Oliver *et al.*, 1991; Watson *et al.*, 1998).

The continuous adaptation of cells within long-term starved cultures has become more recognised over the last decade. In *E. coli*, the addition of cells from an overnight culture to those from a 10-day-old culture revealed that the 'aged' culture cells increased in number at the expense of the 'young' culture cells (Zambrano *et al.*, 1993; Kolter *et al.*, 1993). It was also possible to select mutant cells that possessed a growth advantage in stationary phase (GASP) phenotype, even after several exponential growth cycles (Zambrano *et al.*, 1993). The stability observed implied that the phenotype was the result of mutation(s), and not a reversible adaptive mechanism. Analysis of the mutation identified the reduction in the induction of several genes during starvation as the result of a mutation in the *rpoS* locus (Zambrano *et al.*, 1993). Since this initial discovery, the GASP phenotype has been observed a number of species, including *Pseudomonas putida*, and *Mycobacterium smegmatis* (Eberl *et al.*, 1996; Smeulders *et al.*,1999; Finkel *et al.*, 2000).

In phosphate-starved cultures of *P. putida*, a small colony variant was frequently isolated, gradually replacing the wild type colonies (Eberl *et al.*, 1996). In mixed culture experiments, both the mutant and wild-type cell numbers initially fell, before a rise in the numbers of the

mutant. Wild type cell numbers however, continued to fall (Eberl *et al.*, 1996). Surprisingly, the mutant strain was found to have no greater ability to survive phosphate limitation than wild type cells. Current models detail a continuous process of a succession of 'waves' of GASP mutants that arise to take over the current population (Finkel *et al.*, 2000). This has been exhibited for *E. coli* cells from up to 120 day-old cultures which were able to out-compete cells aged for up to 90 days (Finkel and Kolter, 1999). Significantly however, cells aged for 150 days could not take over fresh overnight LB cultures of the parental strain (Finkel and Kolter, 1999). This is thought to be due to the fact that the cells from older cultures have been selected for in conditions that differ significantly from those that prevail in overnight cultures. Therefore, the accumulation of selective mutations within cells from older cultures no longer confers a growth advantage for these cells in overnight culture conditions. The same study revealed that cells from parallel cultures differed in their competitiveness relative to each other, an indication of the independent evolution of GASP mutations (Finkel and Kolter, 1999).

1.4.8 Differential protein synthesis during starvation

Bacteria have evolved a geared system for reaction to a host of environmental stimuli, which is controlled by overlapping regulatory networks (e.g. cAMP, σ-factors, ppGpp, etc.). Central to this is the coordinated synthesis of novel proteins a fact highlighted by an increase in the loss of viability coupled with adverse affects in recovery after inhibition of protein synthesis in stationary phase cells (Reeve *et al.*, 1984; Nyström *et al.*, 1990a; Watson *et al.*, 1998). Comparisons from 2-D SDS polyacrylamide gel electrophoresis (2-D SDS-PAGE) have begun to reveal the extent of starvation-induced protein synthesis, and to identify the sets of proteins induced by specific stimuli. As witnessed in numerous bacteria, a core number of general stress proteins (gsps) are induced under a wide range of stresses, whilst subsets are induced only under specific forms of stress (Table 1.2).

Three phases of protein synthesis within the apdaptation process to multiple-nutrient starvation have been described for *Vibrio sp.* (Nyström *et al.*, 1990a,b; Kjelleberg, 1993). Within the first 30 minutes, the stringent control phase governed by the ppGpp synthases

	Stress-Induced Proteins				
	Carbon	Nitrogen	Phosphorous	Core GSP	Reference
E. coli	55	47	35	15-30	Matin, 1991
S. typhimurium	11	24	29	6	Spector *et al.*, 1986

Table 1.2 Starvation-induced proteins in *E. coli* and *S. typhimurium*

RelA and SpoT, causes a swift reduction in RNA and protein synthesis, whilst protein turnover is markedly increased (Kjelleberg *et al.*, 1993). Between 30 minutes and 3 hours of starvation, the stringent response is partially relieved, signalled by a drop in ppGpp levels, resulting in some recovery of protein synthesis (Nyström *et al.*, 1990a). As starvation occurs over the longer term, protein synthesis continues to decline to nominal levels. The suggestion from other *E. coli* and *S. aureus* however, is that sustained low-level protein synthesis is required for continued survival (Reeve *et al.*, 1984; Watson *et al.*, 1998).

1.5 RpoS (σ^S), a core Gram-negative starvation-survival/stress gene regulator

Sigma (σ) factors are subunits of RNA polymerase, which are primarily responsible for determining the promoter specificity for the core RNA polymerase enzyme and initiation of transcription (Loewen and Hengge-Aronis, 1994). As such, their promoter specificity provides a mechanism for regulation of stress-induced genes, and roles have been determined for σ^S, σ^N, σ^H and σ^B as regulators in starvation and stress responses. σ^S, encoded by the *rpoS* gene is a starvation-induced gene regulator controlling numerous regulons in a general stress response, homologues of which are found in a broad range of Gram-negative bacteria, including *S. typhimurium*, *Yersinia enterocolitica*, *Shigella flexneri* and *P. aeruginosa* (Loewen and Hengge-Aronis, 1994; Jorgensen *et al.*, 1999), but intriguingly none as yet in Gram-positive bacteria.

1.5.1 The σ^S regulon

The numerous genes that fall under the control of σ^S can be mostly classed relative to functions within stress resistance mechanisms and cellular physiology. Trehalose is synthesised by trehalose-6-phosphate synthase and trehalose-6-phosphate phosphatase, encoded for by the σ^S-dependent genes *otsA* and *otsB* respectively (Giaever *et al.*, 1988). The accumulation of trehalose by cells exposed to osmotic stress or starvation enables the cell to withstand both heat and osmotic stress, and both *rpoS* and *otsAB* mutants are sensitive to both heat and osmotic stress (Giaever *et al.*, 1988; Hengge-Aronis *et al.*, 1993). A significant number of σ^S-dependent genes are found to be components of the DNA damage protection systems. Included are the H_2O_2 stress resistance proteins HPI (KatG) and HPII catalase (KatE), and the Dps protein, which is thought to protect DNA by forming tight complexes with the bacterial DNA (Almirón *et al.*, 1992). The damage done by oxidative stress can also be repaired by proteins within the σ^S regulon, including AidB, which repairs DNA methylation, and Exonuclease III (XthA) (Volkert *et al.*, 1994; Sak *et al.*, 1989). Glycogen synthesis via the glgCAP operon is regulated by *glgS*, which encodes a 7.8 kDa protein and is positively regulated by σ^S and cAMP-CRP in stationary phase. Under conditions of nitrogen limitation and excess glucose however, cAMP-CRP dependent expression is relieved, and *glgS* falls under the sole control of σ^S (Loewen and Hengge-Aronis, 1994). Cellular morphology is influenced by σ^S via *bolA*, which itself regulates expression of the penicillin binding protein PBP6 (Aldea *et al.*, 1989), and *ficA*, whose function remains unknown (Tanaka *et al.*, 1993).

The close link between stress responses and virulence has been suggested by the role σ^S plays in the regulation of virulence genes in *Salmonella* species (Spector, 1998). This connection centres on the regulation of the *spv* plasmid virulence genes by both *rpoS* and *crp* through SpvR (Kowarz *et al.*, 1994). Induction of *spvR/spvABCD* occurs during carbon-, phosphorous- and nitrogen-starvation, as well as stationary-phase cells in LB (Spector, 1998).

1.5.2 Regulation of σ^S

The regulation of σ^S is highly complex, and differential expression of σ^S is seen to be dependent on the type and speed of starvation. Transcriptional control is seen during slow reduction of growth rate to stationary phase (Hengge-Aronis, 1996), whilst under sudden carbon-starvation cellular levels increase, despite reduced transcription due to turnover of σ^S being reduced between 7 and 16-fold (Zgurskaya, *et al.*, 1997). During osmotic stress, a reduction in σ^S turnover can also achieve an increase in cellular σ^S via regulation of the activity of the ClpX/ClpP protease by SprE (Pratt and Silhavy, 1996). Signals for a general stress response regulator would be expected to take more than one form, and this is the case for σ^S. Catabolite repression is known to control the expression of σ^S (Lange and Hengge-Aronis, 1994), and recent evidence has been found of a link to glucose metabolism via the PTS component $EIIA^{Glc}$ (Ueguchi *et al.*, 2001). The negative control of this regulator outside of stationary phase is thought to prevent the induction of a full-scale starvation response to mild carbon source limitation. σ^S is also subject to stringent control, as cellular levels are lowered in mutants with reduced cellular ppGpp (Xiao *et al.*, 1991), and the quorum sensing molecule homoserine lactone is thought to positively regulate *rpoS* via a signalling pathway (Huisman and Kolter, 1994).

1.5.3 Stationary phase gene regulation

Several regulators have been shown to interact with σ^S in the regulation of specific gene subsets during stationary phase, including integration host factor (IHF), the leucine responsive regulatory protein (Lrp), H-NS, and the aforementioned cAMP-CRP and ppGpp. IHF is a sequence-specific histone-like protein, which can positively or negatively regulate transcription initiation via its DNA bending properties (Friedman, 1988). The cellular content of IHF is increased during stationary phase, under the partial control of σ^S (Aviv *et al.*, 1994). As a continuation of the regulatory cascade in stationary phase, IHF induces the expression of *dps*, which itself increases the expression of a further 23 starvation-induced genes (Almirón *et al.*, 1992). H-NS, a second histone-like protein, represses a large number of starvation-specific genes during exponential phase (Barth *et al.*, 1995). H-NS differentially regulates

transcription initiation by σ^S and the highly similar housekeeping sigma factor σ^{70}, allowing only σ^S to override H-NS repression in starved cells, by an as yet unknown mechanism (Hengge-Aronis, 1999).

1.6 σ^B: a Gram-positive regulator of starvation-survival?

To date, no homologue for the Gram-negative σ^S protein has been found in Gram-positive bacteria. Given the number of parallel changes that occur in both groups in response to starvation, it would be logical to pursue the idea that another protein acts as a functional homologue in the absence of σ^S. Over recent years, the principal candidate for a major Gram-positive starvation-survival gene regulator has been σ^B.

1.6.1 The σ^B regulon

σ^B was the first of at least 17 alternative sigma factors to be discovered in *B. subtilis*, and was found to regulate a wide array of general stress genes (Haldenwang, 1995). σ^B was shown to induce expression of these stress proteins in exponentially growing cells in response to heat, ethanol, acid or salt stress (Varon *et al.*, 1996), and in cells starved for glucose, phosphate or oxygen (Hecker and Völker, 1998). In *B. subtilis*, σ^B-dependent genes comprise the class II subset of heat shock proteins. This class of proteins produced in response to multiple forms of stress, including starvation, contrast with class I (HrcA-dependent) dependent proteins, which are heat stress specific, and class III (HrcA/σ^B-independent) proteins, which are produced in response to various stresses excluding starvation (Hecker and Völker, 1998). Apart from stress-induced expression governed by σ^B, the profile of σ^B-dependent genes can be modulated by the presence of additional regulatory sequences, including promoters specific for σ^B and the housekeeping sigma factor σ^A, and recognition sequences for the stress response repressor CtsR (Hecker and Völker, 1998).

1.6.2 Functions of σ^B-regulated proteins

The number of genes that fall under the regulation of σ^B in *B. subtilis* continues to grow. Over 50 proteins have been identified with σ^B-dependent expression profiles from two-dimensional electrophoresis studies (Bernhardt *et al.*, 1997; Antelmann *et al.*, 1997b), though the regulon may contain many more not visible on the 2-D protein gels. The function of σ^B-dependent genes has been extensively reviewed by Hecker and Völker (1998), and can be classified into five main groups as follows. The first group incorporate sigB-dependent genes such as *clpP*, *clpC* and *clpX*, which encode for protease/ATPase subunits. These play a role in the renaturation or degradation of misfolded or denatured proteins that accumulate as a result of stress, and have been shown in *B. subtilis* to be crucial for general stress resistance, especially to salt and heat stress (Krüger *et al.*, 1994). Group two genes encode for proteins that provide cellular oxidative stress resistance. In addition to the aforementioned proteins ClpC and ClpP, this group includes KatE, a σ^B-dependent catalase, the DNA-binding protein Dps and two putative DNA repair proteins. A number of genes that possess putative roles in the adaptation of the cell to osmotic stress make up group three, whilst group four consists of genes which as yet have no defined role in stress resistance. Major scope for the extension of the role of σ^B is suggested by the fifth group of σ^B-dependent genes, which encode for at least ten genes with no homology to any in the protein databases available to date.

1.6.3 Regulation of σ^B activity

The regulation of σ^B in *B. subtilis* is coordinated by a complex signal transduction system, based upon the proteins produced from the *sigB* operon. The *sigB* operon is 5 kbp in length, and consists of eight genes (*rsbR-rsbS-rsbT-rsbU-rsbV-rsbW-sigB-rsbX*). Activity of σ^B is controlled by the anti-sigma factor RsbW, which by binding to σ^B during exponential growth, inactivates the regulator (Benson and Haldenwang, 1993). During stress or nutrient-limiting conditions, the non-phosphorylated form of the antagonist protein RsbV accumulates, and this form preferentially binds RsbW, releasing the active σ^B protein (Dufour and Haldenwang, 1994). Phosphorylation of RsbV can be controlled in response to two different classes of stress by two different sets of regulatory modules (RsbX-RsbS-RsbT and RsbU-

RsbV-RsbW) (Kang *et al.*, 1996). Both sets contain a serine phosphatase (RsbU/X), an antagonist protein (RsbS/V) and a switch protein/serine kinase (RsbT/W). In response to stress causing depletion of the cellular ATP pool (e.g. glucose/oxygen/phosphate starvation), phosphorylation of RsbV by RsbW is inhibited. In response to physical stress (e.g. ethanol, acid, heat or salt), the phosphatase activity of RsbU is stimulated, which dephosphorylates RsbV, thus inducing σ^B activity.

1.6.4 σ^B in other Gram-positive bacteria

The potential for σ^B as a Gram-positive gene regulator has been signified by the occurrence of homologues in a growing number of Gram-positive bacteria, including *Mycobacterium tuberculosis*, *S. aureus* and *L. monocytogenes* (De Maio *et al.*, 1996; Wu *et al.*, 1996; Becker *et al.*, 1998).

In *S. aureus*, σ^B governs the expression of at least 27 cytoplasmic proteins within the general stress response (Gertz *et al.*, 2000). The *sigB* operon structure matches that in *B. subtilis* excepting the fact that the operon lacks the RsbX-RsbS-RsbT module for regulation of σ^B activity in response to physical stresses (Kullik and Giachino, 1997). *sigB* null mutations have produced a number of effects of interest in *S. aureus*. Whilst *sigB* mutants were not impaired in starvation-survival or recovery, or for pathogenicity in a mouse abscess model (Chan *et al.*, 1998), the cells were more susceptible to heat and oxidative stress (Chan *et al.*, 1998; Kullik *et al.*, 1998). Interestingly, in a methicillin resistant *S. aureus* background, *sigB* mutant cells became reduced in resistance to methicillin (Wu *et al.*, 1996).

The discovery of a σ^B homologue in *L. monocytogenes* by Wiedmann *et al.* (1998), has enabled more detailed investigation of the stress responses of this bacterium than had previously been possible. The extent of the *sigB* operon determined to date (*rsbU-rsbV-rsbW-sigB-rsbX*) includes the regulator genes for regulation in response to ATP depletion (*rsbU-rsbV-rsbW*) and the first of the three physical stress regulator genes (*rsbX*) (Becker *et al.*, 1998). Regulation by these two modules was verified by the induction of σ^B activity under osmotic, acid, temperature and oxidative stress, and upon entry into stationary phase (Becker

et al., 1998, 2000). Of the conditions tested, activity of σ^B was greatest under osmotic stress, and the *sigB* mutant strain was deficient in the uptake of betaine and carnitine (Becker *et al.*, 2000). These compounds are imported into the cell for protection from osmotic stress and cold shock, and subsequent study identified a σ^B-dependent betaine transport system (BetL), mutants for which exhibited susceptibility to high salt concentrations (Sleator *et al.*, 1999). An extension of the role that σ^B plays in the stress response in *L. monocytogenes* was established by Wiedmann *et al.* (1998) who found that a *sigB* null mutation was linked to a reduction in acid stress resistance, but the strain was unchanged in virulence in a mouse model.

1.7 Recovery from Starvation

Recovery from starvation is an equally closely regulated process as the bacteria readjusts to nutrient replete conditions. In *S. aureus*, after addition of fresh nutrients, RNA synthesis recommenced immediately with protein synthesis following after 5 minutes (Clements and Foster, 1998). After approximately 30 minutes cells begin to enlarge, with DNA replication and cell division restarting after approximately 90 and 150 minutes respectively. Return to active growth witnesses a shift in the proteins required. This occurs in *S. aureus* within the first 30 minutes, and interestingly, the expression of two proteins could only be detected during the first 60 minutes of recovery, suggesting the existence of recovery-specific proteins. (Clements and Foster, 1998). In *V. sp.* S14, 18 immediate upshift proteins are synthesised from stable transcripts during the first 3 minutes of recovery, five of which are not present in exponentially growing cells (Marouga and Kjelleberg, 1996). Similarly, in *E. coli*, nine outgrowth specific proteins were observed by 2-D SDS-PAGE, with an additional five proteins found to have at least a five-fold increase in expression (Siegle and Guynn, 1996). It would be expected that proteins expressed during recovery in bacteria are regulated by regulators involved in nutrient shifts, and more specifically the general stress regulator σ^S in Gram-negative bacteria. However, while there may be some regulation by σ^S, many recovery proteins are still expressed in an *rpo*S mutant, and so some other form of regulation must occur. Whether cAMP or ppGpp are involved is not yet known.

The screening of *S. aureus* starvation-survival mutants by our laboratory actually identified a *ctaA* mutant defective in recovery from starvation (Watson *et al.*, 1998; Clements *et al.*,

1999). *ctaA* in *S. aureus* encodes for a homologue for haem A synthase, and the mutant strain lacked heme A-containing cytochrome terminal oxidases. The addition of catalase to recovering cultures was able to suppress the survival defect, implying the involvement of oxidative stress (Clements *et al.*, 1999).

1.8 *L. monocytogenes* and starvation

L. monocytogenes is widespread in nature, and has been isolated from soil, dust, food, animal feed (i.e. silage), sewage, plants and from almost all types of animals tested (Armstrong, 1995; Farber and Peterkin, 1991). Being able to exist in so many habitats, the capacity for survival of this bacterium under a variety of stress and nutrient-limiting conditions is evident. An early study of survival of *L. monocytogenes* in soil and pondwater was performed by Botzler *et al.* (1974). *L. monocytogenes* was inoculated in soil at approximately 1×10^5 CFU ml^{-1} (soil suspension) and viable cells were recovered for over 16 weeks. Similarly, viable cells could be isolated for over 8 weeks after inoculation at approximately 1×10^5 CFU ml^{-1} in pondwater samples. More recently, on a molecular level, the regulation of carbon source uptake by catabolite repression was found to occur similarly in *L. monocytogenes* as in other Gram-positive bacteria, with HPr playing a key role (Christensen *et al.*, 1998 and 1999).

L. monocytogenes is considered a good model of bacterial infection, and most studies performed regarding nutrient limitation investigate the effect of environmental conditions including nutrient limitation, on the expression of PrfA and the PrfA-dependent virulence genes. PrfA, the transcriptional activator of *L. monocytogenes* virulence genes exhibits regulated expression, yielding two maxima during batch culture. The first peak occurs during exponential growth, where a bicistronic *plcA-prfA* transcript is produced. As the bacteria enter stationary phase, a second peak occurs as the monocistronic *prfA* transcript becomes predominant. The switch of transcripts is induced specifically by nutrient limitation upon the shift of *L. monocytogenes* into Minimal Essential Medium (Bohne *et al.*, 1996). The most important evidence of the role of available carbon-sources in the regulation of *L. monocytogenes* virulence genes is that of the repression of PrfA-controlled regulon by a number of sugars, principally cellobiose, maltose, trehalose, glucose and fructose (Park &

Kroll, 1993; Milenbachs *et al.*, 1997). These sugars, which are readily metabolised by *L. monocytogenes*, are thought to induce this effect via catabolite repression. How this is achieved is still unclear, as *CcpA*, the principal catabolite repression mediator in low G+C content Gram-positive bacteria seems to play no part in the carbon source regulation of virulence genes (Behari and Youngman, 1998a). Interestingly, in the *L. monocytogenes* strain NCTC7973, cellobiose alone represses virulence gene expression (Parker and Kroll, 1993), indicating that catabolite repression comprises at least two pathways, only one of which functions in NCTC7973. Subsequently, Brehm *et al.* (1999) identified the *bvr* locus which mediated virulence gene regulation specifically in response to β-glucoside sugars. Physical levels of the PrfA protein remain unchanged during repression of the virulence genes, pointing to post-translational control of PrfA by an as yet unidentified PrfA-activating factor. This would be analogous to the Crp transcriptional regulator, where complexing with cAMP regulates gene expression (Sheehan *et al.*, 1996).

Not surprisingly, given that the potential human host uses the high-affinity iron binding compounds lactoferrin, transferrin and ferritin to restrict the level of available iron as a barrier to infection (Litwin and Calderwood, 1993), iron is also known to affect the regulation of *L. monocytogenes* virulence genes. An increase in PrfA specific DNA binding occurs upon the downshift of *L. monocytogenes* into Minimal Medium, whilst iron strongly inhibits PrfA activity (Böckmann *et al.*, 1996). The complexity of gene regulation based upon PrfA and the as yet unidentified cofactor Paf (PrfA activating factor), does not mirror the CRP model where cAMP is the sole cofactor, and the extended C-terminus of PrfA is proposed to play a role (Böckmann *et al.*, 1996).

Studies have failed to identify general stress proteins induced during phagocytosis of *L. monocytogenes* by macrophages (Hanawa *et al.*, 1995), suggesting that the stress response is not a major factor in listerial infection. The first evidence that they may play a role during *in vivo* survival was the discovery of a homologue of ClpC/MecB from *B. subtilis*, which is responsible for ATP-dependent proteolysis of toxic denatured proteins (Gottesman *et al.*, 1990). Mutations in this homologue produce bacteria highly susceptible to stress, especially iron limitation, elevated temperatures and high osmolarity. Interestingly this mutation also greatly impaired intracellular growth, both within host tissues and macrophages (Gottesman *et al.*, 1990).

The ability of *L. monocytogenes* to survive under acid, oxidative and heat stress is of key importance to the threat posed by *L. monocytogenes*, in the context of both food hygiene and the ability to cause infection. Acidification, particularly during food production and during the passage of the pathogen through the stomach – significant barriers to infection in food-borne illness – is a major factor in bacterial growth and survival. Acid tolerance therefore has particular relevance for the survival of *L. monocytogenes*. The previously described acid tolerance response is found in *L. monocytogenes*, in response to both weak (e.g. lactic acid) and strong (e.g. hydrochloric) acids (Davis *et al.*, 1996). Acid adapted *L. monocytogenes* cells possessed changed expression in 53 proteins, with the cells increased in tolerance to heat and osmotic stress (O'Driscoll *et al.*, 1997), and increased survival in dairy products (Gahan *et al.*, 1996). Prolonged exposure to pH 3.5 also produced mutants with increased acid tolerance within all stages of the life cycle (O'Driscoll *et al.*, 1996). Under oxidative and heat shock, 13 to 14 *L. monocytogenes* stress proteins were induced respectively, including homologues to GroEL and DnaK (Hanawa *et al.*, 1995). The induction of cross-resistance in *L. monocytogenes* in response to stress also occurs, with cells grown at 30°C more resistant to acid stress at pH 2.5, than those grown at 10°C (Patchett *et al.*, 1996), and increased thermotolerance of bacteria in beef samples induced by the presence of salt (Mackey *et al.*, 1990).

1.9 Project Objective

L. monocytogenes is a species that displays remarkable versatility in being able to survive in a variety of habitats, including soil, water, vegetation, dust, the intestinal tract and factory work surfaces (Farber and Peterkin, 1991). As a significant pathogen which causes a variety of infections with particularly serious consequences for pregnant women, neonates and immunosuppressed groups, it is likely that *L. monocytogenes* encounters nutrient-limited conditions in association with the various routes of transmission and in the course of an infection (Farber and Peterkin, 1991). Given the resilience of this bacterium to stress and starvation, understanding of the mechanism by which *L. monocytogenes* develops the starvation-survival response is of key importance.

The following work describes a) the characterisation of the starvation-survival response of *L. monocytogenes*, b) the analysis of two known gene regulators in this response and c) the identification and analysis of two novel starvation-survival loci.

CHAPTER 2

MATERIALS AND METHODS

2.1 Media

2.1.1 Chemically defined medium

The chemically defined medium (CDM) was modified from that previously described (Trivett and Meyer, 1971). Glucose-limiting CDM was composed as follows per litre: NaOH, 0.24 g; nitrilotriacetic acid, 0.48 g; $FeCl_3$, 0.048 g; $MgSO_4 \cdot 7H_2O$, 0.41 g; NH_4Cl, 1.0 g; K_2PO_4, 0.85 g; NaH_2PO_4, 0.15 g; 3-(*N*-morpholino)propanesulfonic acid (MOPS), 4.18 g; L-lysine.HCl, 0.1 g; L-leucine, 0.1 g; DL-isoleucine, 0.2 g; DL-valine, 0.2 g; DL-methionine, 0.2 g; L-arginine, 0.2 g; L-histidine, 0.2 g; glucose, 1.0 g; α-lipoic acid, 10 μg; biotin, 1 mg; thiamine, 5 mg; riboflavin, 1 mg. The liquid medium was prepared as described (Trivett and Meyer, 1971), excepting: (i) K_2HPO_4 and NH_4Cl were dissolved in only 480 ml distilled water (dH_2O); (ii) 20 ml of sterile 1M MOPS (pH 7.5) solution was added to the final medium; (iii) the amino acids dissolved in 390 ml of dH_2O replaced the water added finally. Glucose- and amino acid-limiting CDM was made by decreasing the glucose concentration to 0.1 % (w/v) from 0.2 % (w/v) and by reducing the total amino acid concentration from 0.12 % (w/v) to 0.0016 % (w/v). Glucose-free CDM was prepared with solution C (20 % [v/v] glucose) replaced with dH_2O. Oxoid Agar No.1 (1.5 % [w/v]) was used for CDM agar.

2.1.2 Brain Heart Infusion (BHI) Broth (Oxoid)

Brain Heart Infusion Broth $37\ g\ l^{-1}$

Oxoid Agar No.1 (1.5 % [w/v]) was used for BHI agar.

2.1.3 Tryptic Soy Broth (TSB) (Difco)

Tryptic Soy Broth	30 g l^{-1}

Oxoid Agar No.1 (1.5 % [w/v]) was used for TSB agar.

2.1.4 LB (Luria-Bertani) Medium

Tryptone (Oxoid)	10 g
Yeast Extract (Oxoid)	5 g
NaCl	10 g
dH_2O	to 1l

1.5 % (w/v) (Oxoid Agar No.1) was used for LB agar.
0.75 % (w/v) (Oxoid Agar No.1) was used for LB Sloppy agar (LBSA).

2.2 Antibiotics

Concentrations of antibiotics used for selection were as shown in Table 2.1 unless otherwise stated. Stock solutions of antibiotics used were prepared as in Table 2.1, filter sterilised, stored at -20°C and added to the media (cooled to below 60°C) as required.

Antibiotic	Stock Concentration (mg ml^{-1})	Concentration for Selection (μg ml^{-1})
Erythromycin	5 (50 % v/v Ethanol)	5
Tetracycline	5 (100 % v/v Ethanol)	5
Lincomycin	25 (50 % v/v Ethanol)	25
Kanamycin	50	40
Neomycin	50	5
Ampicillin	100	100

Table 2.1
Stock antibiotic solutions. Solutions made in dH_2O unless otherwise stated.

2.3 Buffers and Stock Solutions

2.3.1 DNA sample buffer (6 X)

Bromophenol Blue	25 mg
Xylene cyanol FF	25 mg
Ficoll (Mol. Wt. 400,000)	1.5 g
dH_2O	to 10 ml

2.3.2 Phosphate buffered saline

NaCl	8.0 g
Na_2HPO_4	1.4 g
KCl	0.2 g
KH_2PO_4	0.2 g
Distilled water	to 1l

2.3.3 Plasmid preparation solution I

20 % (w/v) glucose	5 ml
1 M Tris.HCl (pH 8.0)	2.5 ml
Na_2EDTA (0.5 M pH 8.0)	2 ml
Distilled water	to 100 ml

2.3.4 Plasmid preparation solution II

NaOH	0.2 M
Sodium dodecyl sulphate (SDS)	1 % (w/v)

2.3.5 Plasmid preparation solution III

Potassium acetate (5 M)	60 ml
Glacial acetic acid	11.5 ml
Distilled water	28.5 ml

2.3.6 SM Buffer

NaCl	5.8 g
$MgSO_4$	2.0 g
Tris.HCl (1M; pH 7.5)	50 ml
Distilled water	to 1l

2.3.7 SSC (20 X)

NaCl	175.3 g
Trisodium Citrate.$2H_2O$	88.2 g
Distilled water	to 1l

The pH was adjusted to 7.0 with 1M NaOH.

2.3.8 Southern prehybridisation solution

5x SSC

N-lauroylsarcosine	0.1 % (w/v)
SDS	0.02 % (w/v)
Blocking reagent (Boehringer Mannheim)	1 % (w/v)

2.3.9 TE Buffer

Tris	10mM
Na_2EDTA	1mM
Distilled water	to 1l

The pH was adjusted to 7.5 with 1M HCl.

2.3.10 TAE

Tris	4.84 g
Glacial Acetic Acid	1.14 ml
Na_2EDTA (0.5M; pH 8.0)	10 ml
Distilled water	to 1l

2.3.11 AB Buffer

NaCl	4g
K_2HPO_4	10.45g
KH_2PO_4	5.44g
Distilled water	to 1l

For ABT Buffer, add Triton X-100 (1 ml per litre).
For ABTN Buffer, mix ABT buffer with 0.4M Na_2CO_3 in equal volumes.

2.4 Chemicals and enzymes

Chemicals were obtained from Sigma, BDH or Fisons unless stated otherwise and were of analytical grade. Restriction enzymes, ligases and buffers used were obtained from Promega or Roche. Lysozyme and DNAse-free RNAse A were obtained from Sigma. Stock solutions

were prepared by dissolving in dH_2O to 50 mg ml^{-1} (Lysozyme) and 10 mg ml^{-1} (RNAse A) and stored at -20°C.

2.5 Bacterial strains, phages and plasmids

Bacterial strains, phages and plasmids used in this study are listed in Tables 2.2 and 2.3. *L. monocytogenes* strains were always grown fresh on BHI agar plates containing antibiotics as required for selection. The plates were then incubated at 37°C unless otherwise stated. Glycerol (30 % [v/v]) stocks were prepared for each strain for long-term storage at -20°C.

L. monocytogenes strain	Genotype/Markers	Source/Reference
EGD	Wild type isolate, serotype 1/2a	Wuenscher *et al.* (1993)
10403S	Laboratory strain, serotype 1	Portnoy *et al.* (1988)
LMA2B	10403S, *sigB* null mutant, km^R	Becker *et al.* (1998)
DES011	EGD, *sigB* null mutant, km^R	This study
DES012	EGD, *prfA* deletion mutant	C. Dickneite (Universität Würzburg, Germany)
DES028	Tn*917* insertion, ery^R	This study
DES029	Tn*917* insertion, ery^R	This study
DES035	Tn*917* insertion, ery^R	This study
DES045	Tn*917* insertion, ery^R	This study

Table 2.2
List of strains used

2.6 Evaluation of *L. monocytogenes* viability

Viable counts were determined by serial dilution in PBS, plating on TSB agar and incubation overnight at 37°C. The minimum detection level was 100 CFU ml^{-1}. Results were representative of at least two separate experiments which showed no greater than 10-fold variability between analogous time points.

L. monocyotgenes plasmid	Genotype/Markers	Source/Reference
pLTV3	tet^R, ery^R, cm^R, neo^R, ble^R Tn*917*-carrying temperature sensitive insertional vector	Camilli *et al.* (1990)
L. monocytogenes phage	Genotype/Markers	Source/Reference
ΦLMUP35	—	Hodgson (2000)

Table 2.3

List of plasmids and phages used. Plasmid DNA was stored in TE buffer at -20°C.

2.7 Starvation for glucose or amino acids in CDM

Starvation cultures were inoculated to an absorbance at 600 nm (A_{600}) of 0.01 from an overnight culture grown in glucose- or amino acid-limiting CDM and incubated for 18 hours at 37°C (50ml medium in 250ml conical flask, 250 rpm shaking). All cultures reached an A_{600} of 0.65 to 0.75, which corresponded to approximately 1×10^9 CFU ml^{-1}. The cultures were then incubated statically at 4, 25 or 37°C and viability determined over time.

2.8 Starvation for multiple nutrients in dH_2O or PBS

Multiple-nutrient-limited cells were prepared by harvesting stationary-phase glucose- and amino acid-limited CDM cultures by centrifugation (4,000 × *g* for 10 min at room temperature). The cells were washed twice by resuspension and centrifugation with an equal volume of PBS or dH_2O, before being resuspended in PBS or dH_2O respectively to the original culture volume. The cultures were then incubated statically at 25 or 37°C and viability determined over time.

2.9 Effect of cell density on survival

Cells from 50 ml glucose limited cultures incubated to 6 hours or 7 days glucose starvation were harvested by centrifugation (4000 rpm, 10 min, room temperature) and washed twice with sterile PBS by centrifugation and resuspension. The cells were then resuspended in sterile dH_2O or filter sterilised spent culture supernatant to the original concentration, or diluted 10-, 100-, 1000-fold and incubated at the same temperature. The spent culture supernatant was taken from glucose-limited CDM cultures of the same age, from which the cells were removed by centrifugation prior to filter sterilisation. Viability was then determined over time.

Cells incubated in sterile water after glucose limited growth were washed twice with sterile dH_2O by centrifugation and resuspension. The cells were then resuspended in sterile dH_2O to the original concentration, or diluted 10-, 100-, 1000-fold and incubated at the same temperature. Viability then was determined over time.

2.10 Effect of penicillin G on survival

Penicillin G was added to glucose-limited cultures at 6 hours post-exponential phase (25°C, 37°C), after 7 days incubation at 37°C or after 20 days incubation at 25°C, to a final concentration of 20 times the MIC (20 $\mu g\ ml^{-1}$). The cultures were then incubated without shaking at the same temperature and viability was determined over time.

2.11 Preparation of samples for electron microscopy

Samples were centrifuged (10,000 rpm) for 5 min at 4°C, the supernatant removed, the pellet snap frozen in liquid nitrogen and stored at -70°C until required. The sample was fixed in Karnovsky's fixative (2 % [w/v] paraformaldehyde, 2.5 % [v/v] glutaraldehyde in 0.1M phosphate buffer) for 3 hours at 4°C and then washed three times (30 minutes; 4°C) in 10 %

(w/v) sucrose (in 0.1M phosphate buffer). The samples were incubated in 2 % (w/v) aqueous osmium tetroxide for 1 hour at room temperature and washed in dH_2O. The samples were then incubated in 75 % (v/v) ethanol (15 minutes), 95 % (v/v) ethanol (15 minutes), then absolute ethanol (three changes; 15 minutes), before being dried over anhydrous copper sulphate (15 minutes). Following drying, samples were incubated in propylene oxide (two changes; 15 minutes) prior to incubation overnight (room temperature) in a 50/50 mixture of propylene oxide and araldite. The samples were incubated in 100 % (v/v) araldite followed by embedding in fresh 100 % (v/v) araldite resin (48 hours; 60°C). Ultrathin sections (70-90 nm thick) were cut on a Reichert Ultracut ultramicrotome and stained with 3 % (w/v) uranyl acetate (in 50 % ethanol) for 15 minutes, washed with dH_2O, then stained with Reynold's lead citrate for 2 minutes. The sections were studied with a Phillips CM-10 transmission electron microscope operated at 80kV. Mean cell dimensions were calculated from at least 60 individual cells viewed within a randomly selected area.

2.12 Stress resistance

A 50 ml culture of glucose-limiting CDM was inoculated to A_{600} 0.05 from an overnight culture of EGD in the same medium and incubated at 37°C (250 rpm). Mid-exponential phase (A_{600} 0.3) and 6-hour post-exponential phase cells were harvested by centrifugation (5,000 rpm, 3 min). The culture was then incubated without shaking for a further 7 days before cells were again harvested (5,000 rpm, 3 min). For challenge with heat stress (55°C), the harvested cells were diluted to approximately 5×10^6 CFU ml^{-1} in 37°C prewarmed PBS. A 1 ml sample was transferred to a 55°C prewarmed 1.5 ml microcentrifuge tube and samples removed after 0, 1, 2, 4, 8, 12 and 18 minutes incubation at 55°C. Viability was determined after serial dilution in PBS prewarmed to 37°C. Cells to be challenged with oxidative stress were diluted to approximately 5×10^6 CFU ml^{-1} in 37°C prewarmed PBS. H_2O_2 was added to a 10 ml sample of cells to a final concentration of 7.5mM and incubated at 37°C. Samples were removed after 0, 2.5, 5, 10, 15, 20, 30 and 50 minutes and serially diluted in 10 mg ml^{-1} catalase (Sigma) dissolved in PBS. Viability for each sample was then determined. Samples for challenge with acid stress were resuspended to approximately 5×10^6 CFU ml^{-1} in fresh

glucose-limited CDM at pH 3.5 (adjusted with HCl, filter sterilised). Samples were removed after 0, 15, 30, 45, 60, 75 and 90 minutes incubation at 37°C. The viability was then determined.

2.13 Chloramphenicol inhibition of protein synthesis

A 50 ml culture of glucose-limiting CDM was inoculated to A_{600} 0.05 from an overnight culture of EGD in the same medium and incubated at 37°C (250 rpm). Synchronous glucose limitation of cultures was achieved by harvesting mid-exponential (A_{600} 0.3) cells by centrifugation (5,000 rpm, 5 minutes, room temperature) and resuspension in the same volume of glucose-free CDM. The cultures were incubated at 37°C and chloramphenicol (100 μg ml^{-1}) was added after 0 h, 1 h, 2 h, 4 h, 8 h and 24 h. Control cultures to which no chloramphenicol was added were also monitored. Viability was determined by plating on TSB Agar.

2.14 Starvation-survival mutant screening

2.14.1 Tn*917* mutagenesis of *L. monocytogenes* EGD

Tn*917* insertion libraries of *L. monocytogenes* EGD were created using the vector pLTV3, which contains a temperature sensitive origin of replication (Youngman, 1990). A single colony of *L. monocytogenes* EGD carrying the vector pLTV3 was inoculated into 5 ml of BHI containing tetracycline and erythromycin and incubated overnight at 30°C. A 5 μl sample of the overnight culture was inoculated into 100 ml BHI containing tetracycline and erythromycin and incubated overnight at 30°C. Cells were harvested from a 3 ml sample of the overnight culture by centrifugation (5,000 rpm, 10 minutes, room temperature) resuspended in 1 ml BHI, inoculated into 100 ml of prewarmed BHI containing erythromycin and incubated at 40.5°C (200 rpm, 8 hours). The cell harvesting and subculture in 40.5°C prewarmed BHI containing erythromycin was repeated twice, before cells from the final 100

ml culture were harvested by centrifugation (5,000 rpm, 10 minutes, room temperature) and resuspended in 3 ml of BHI broth containing 10 % (v/v) glycerol. 100 µl aliquots were snap frozen in liquid nitrogen and stored at -20°C. Each library had >90 % transposon insertion and contained $>10^9$ individual clones.

2.14.2 Selection of starvation-survival mutants

The Tn*917* insertion library was diluted in PBS so that 100µl plated onto glucose-limited CDM agar plates resulted in 100 to 150 colonies after 24 hours incubation at 37°C. The colonies were then replica plated onto two glucose-limited CDM agar plates and incubated overnight at 37°C. One plate (the 'reference' plate) was then incubated at 4°C for 12 days, whilst the second ('test' plate) was incubated at 37°C for 12 days. The test plate was then replica plated onto fresh glucose-limited CDM agar plates and incubated overnight at 37°C. These plates were then compared to the reference plates and any colonies absent on the replicated plates were deemed to be putative survival mutants. Putative mutants were confirmed by a second plate screen.

2.15 Phage techniques

2.15.1 Preparation of phage lysates

Bacteriophage (10^7 and 10^8 plaque forming units [pfu]) was incubated with 10^8 CFU of exponential phase cells of the donor strain in LB broth for 40 minutes (room temperature). The mixture was overlaid onto LB agar plates containing 10 mM $MgSO_4$ and 10 mM $CaCl_2$, using LBSA containing 10 mM $MgSO_4$ and 10 mM $CaCl_2$. The plates were incubated overnight at room temperature. Lysates were harvested by soaking the plates with sterile TM buffer for 2 hours. The buffer was then filter sterilised.

2.15.2 Phage titre determination

Exponential phase cells of *L. monocytogenes* EGD (1×10^8 CFU) were incubated for 40 minutes (room temperature) with 100µl of 10-fold dilutions of the phage lysate. The cells were overlaid onto LB agar plates containing 10 mM $MgSO_4$ and 10 mM $CaCl_2$ using LBSA containing 10 mM $MgSO_4$ and 10 mM $CaCl_2$. The plates were incubated overnight at room temperature and the titre of the original phage lysate calculated.

2.15.3 Phage transduction into wild type EGD background

Exponential phase cells of *L. monocytogenes* EGD (1×10^8 CFU) were incubated with 1×10^8 PFU of phage lysate (40 minutes, room temperature). The mixture was overlaid onto BHI agar plates containing sodium citrate (10mM, pH 7.5) and kanamycin (40 µg ml^{-1}) using 3 ml BHI with 0.75 % (w/v) agar. For selection using erythromycin, the mixture was overlaid onto BHI agar plates containing sodium citrate (10mM, pH 7.5) and erythromycin (1.5 µg ml^{-1}), then incubated for 2 hours at 37°C. The plates were then overlaid with 3 ml BHI with 0.75 % (w/v) agar and 15 µg ml^{-1} erythromycin. Transductants were selected after the plates were incubated overnight at 37°C. To prevent selection of kanamycin or erythromycin resistant colonies arising through chromosomal point mutations, combinations of kanamycin and neomycin, or erythromycin and lincomycin were used for culture of the transductants.

2.16 DNA purification methods

2.16.1 Chromosomal DNA preparation

Chromosomal DNA from bacterial strains was purified using a QIAGEN 100/G kit using the following manual instructions. A single colony was incubated in 10 ml of TSB overnight at 37°C with shaking (250 rpm). The cells were pelleted (3000 rpm, 10 minutes) and resuspended in 3.5 ml of buffer B1 (QIAGEN) containing 200 µg ml^{-1} RNase A. 100 µl of QIAGEN proteinase (20 mg ml^{-1}) and 80 µl lysozyme (100 mg ml^{-1}) was added, and the

mixture incubated at 37°C for 30 minutes. After incubation, 1.2 ml of buffer B2 (QIAGEN) was added and vortexed briefly prior to incubation at 50°C for 30 minutes. The lysate was applied to a QIAGEN 100/G tip equilibrated with 4 ml of buffer QBT (QIAGEN). Once the flow had stopped, the tip was washed twice with 7.5 ml of buffer QC, before the DNA was eluted using 5 ml of 50°C prewarmed buffer QF. The chromosomal DNA was precipitated by mixing with 3.5 ml isopropanol and transferred to a fresh microcentrifuge tube. The DNA was washed with 300 µl of 70 % (v/v) ethanol, air-dried, then dissolved overnight in 0.2 ml TE buffer.

2.16.2 Ethanol precipitation of DNA

DNA was precipitated by the addition of 0.1 volume 3M sodium acetate (pH 5.2) and 2.5 volumes absolute ethanol and incubation at -20°C for at least 10 minutes. The DNA was recovered by centrifugation (10,000 rpm, 10 minutes), the pellet washed with 70 % (v/v) ethanol, air dried and resuspended in TE buffer.

2.17 *In vitro* DNA manipulation methods

2.17.1 Agarose gel electrophoresis

Fragments of DNA were separated by electrophoresis using either 0.7 % (w/v) or 1.0 % (w/v) submerged agarose gels containing 0.2 µg ml^{-1} ethidium bromide in TAE electrophoresis buffer. Samples to be run were mixed with 1/6 volume of 6 x DNA loading buffer. Gels were run at 3-4 volts per centimetre distance between the electrodes. 1 µg of phage λ DNA double digested with *Eco*RI/*Hind*III (Table 2.4) was run concurrently on the gels. The known λ DNA fragment sizes were plotted on a logarithmic scale against mobility in mm on a linear scale. Experimental DNA fragment sizes were determined from the best-fit curve.

2.17.2 Gel photography

Ethidium bromide-stained agarose gels were illuminated by u.v. light and photographed using Polaroid 667 (ASA 3000) film through a Kodak 203 red-orange filter.

λ size standards (*Eco*RI/*Hin*dIII) (kb)
21.23
5.15
4.97
4.27
3.53
2.02
1.90
1.58
1.38
0.95
0.83
0.56
0.13

Table 2.4

Restriction fragment sizes for agarose gel electrophoresis

2.17.3 DNA quantification (Sambrook *et al.*, 1989)

The absorbance of DNA solutions at 260 nm was measured spectrophotometrically. Absorbance of 1.0 corresponds to approximately 50 μg ml^{-1} of double stranded DNA and approximately 20 μg ml^{-1} for single stranded oligonucleotides of 31 bases in length.

2.17.4 DNA Ligation

To 250 µl of digested DNA in TE buffer, 30 µl of 10 x ligase buffer and 3 Weiss units (1µl) of T4 DNA ligase were added. The mixture was made up to 300 µl with dH_2O and incubated overnight at 15°C.

2.18 DNA Hybridisation Methods

2.18.1 Digoxigenin labelling of DNA probes

The Boehringer Mannheim DIG DNA Labelling and Detection Kit was used to produce Digoxigenin (DIG) labelled DNA probes for hybridisation by random primed DNA labelling. Approximately 1 µg of purified DNA template in 15 µl dH_2O was denatured in a boiling waterbath for 10 minutes, then chilled on ice. To the DNA, 2 µl each of 10 x hexanucleotide mixture and 10 x dNTP labelling mixture was added. Klenow enzyme (1 µl) was then added and the reaction incubated at 37°C overnight. After incubation, the reaction was stopped by the addition of 2 µl EDTA (200µM, pH 8.0).

Probe concentration was determined by comparison to labelled control DNA of known concentration. Serial dilutions of both the probe (1:1,000 to 1:1,000,000) and the control DNA (1:10 to 1:100,000) were spotted and then fixed onto nitrocellulose membrane by u.v. crosslinking (700 mJ, 15 seconds). The DIG-labelled DNA was detected as described below. Intensities of the probe and control spots were compared to estimate the probe concentration. The probe was made ready for used by diluting to between 5-25 ng ml^{-1} in prehybridisation solution.

2.18.2 Southern blotting

Agarose gel electrophoresis was carried out as described in Chapter 2.20.1. The gel was soaked in 250 mM HCl for 10 minutes to depurinate the DNA, rinsed with dH_2O and soaked

twice each in first denaturation buffer (0.5 M NaOH, 1.5 M NaCl) and then neutralisation buffer (1.0 M Tris.HCl pH 7.4, 1.5 M NaCl) for 15 minutes at a time. The DNA was transferred overnight by capillary action onto nitrocellulose membrane using 20x SSC (Figure 2.1).

2.18.3 Nylon membrane fixation

DNA was fixed by u.v. crosslinking (700 mJ, 15 seconds) to Amersham Hybond-N+ extra positively charged nylon membrane for all hybridisations.

2.18.4 Prehybridisation and hybridisation

Membranes to be probed with DIG-Labelled DNA were prehybridised for 1 hour at 68°C in 20 ml per 100 cm^2 of prehybridisation solution. The DIG-labelled probe was denatured in a boiling waterbath for 10 minutes, cooled on ice for 2 minutes and added to the membrane in place of the prehybridisation solution. The membrane was then incubated overnight at 68°C. After hybridisation, the probe solution was removed for future use and unbound probe DNA removed from the membrane by washing twice in 2x SSC containing 0.1 % (w/v) SDS for 5 minutes at room temperature and then twice in 0.5x SSC containing 0.1 % (w/v) SDS for 5 minutes at 68°C.

2.18.5 DIG-labelled DNA detection

The washed hybridised membranes were soaked in buffer 1 (100 mM Tris.HCl pH7.5 , 150 mM NaCl) for 1 minute and then blocked with gentle rocking in buffer 2 (1 % [w/v] blocking reagent [Boehringer Mannheim] in buffer 1) for 30 minutes. The membrane was transferred into buffer 2 containing Anti-DIG-alkaline phosphatase antibody (1:5000 dilution) and incubated with gentle rocking for 30 minutes. After incubation, the membrane was washed twice with gentle rocking (15 minutes each) in buffer 1. The membrane was subsequently transferred to 10 ml of buffer 3 (100 mM Tris.HCl pH 9.5, 100 mM NaCl, 50mM $MgCl_2$) containing 66µl nitrotetrazolium blue (NBT, 50 mg ml^{-1} in 70 % (v/v) dimethylformamide)

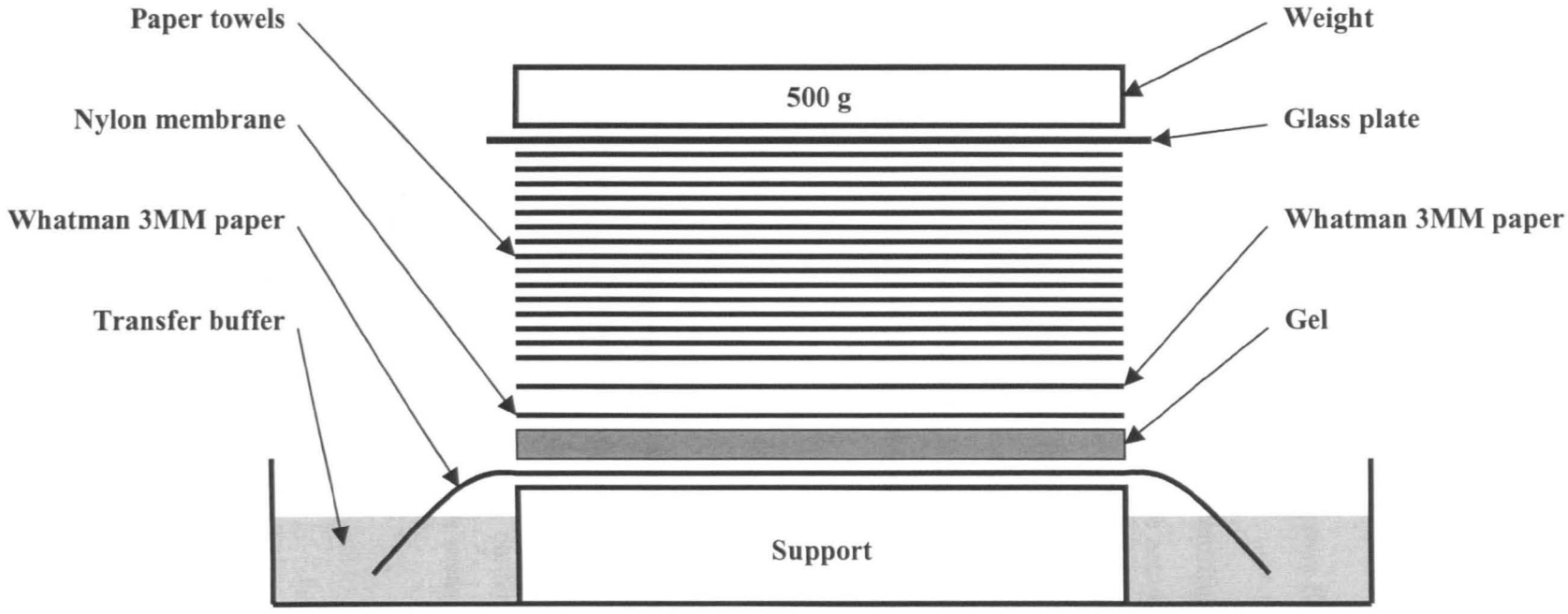


Figure 2.1
Capillary transfer of DNA from agarose gels.
The DNA elutes from the gel and is deposited onto the nylon membrane via the moving stream of buffer drawn from the reservoir into the stack of paper towels. The weight was applied to ensure a tight connection between the layers of material. Adapted from (Sambrook *et al.*, 1989).

and 33µl bromo-4-chloro-3-indolyl-phosphate (BCIP, 50 mg ml^{-1} in water) and incubated in the dark until the labelled DNA bands became visible (purple colouration). The reaction was stopped by washing the filter in a Tris.HCl pH 7.0, 0.2 M EDTA solution.

2.19 Catalase activity during growth of *L. monocytogenes* EGD

Cells from a 1 ml sample of culture were harvested by centrifugation (14,000 rpm, 2 min) and lysed in 2.5 mg ml^{-1} lysozyme (37°C, 30 min). Catalase activity was determined by decrease in H_2O_2 concentration (ϵ_{240} = 0.0435 mM^{-1} cm^{-1}) (Hildebrandt and Roots, 1975) at pH 7.0 (0.615M K_2HPO_4; 0.385M KH_2PO_4) and 25°C using a Shimadzu UV-2401 PC spectrophotometer. Protein concentration was determined colorimetrically (Bio-Rad protein assay reagent), using bovine serum albumin (Sigma) as a standard. One unit of catalase activity was defined as the degradation of 1 µmol H_2O_2 min^{-1} mg^{-1} protein.

2.20 Protein Concentration

Standards of 0, 2, 5, 10 and 20 µg bovine serum albumin (Sigma) were prepared in 800 µl of dH_2O and used to construct a calibration curve of protein concentration against A_{595}. To samples diluted appropriately in a volume of 800 µl and to the protein standards, 200 µl of BIORAD Protein Assay Reagent was added and the contents mixed by inversion. After incubation at room temperature for 2 minutes, A_{595} was measured and protein concentration calculated from the standard curve.

2.21 Genomic DNA sequencing PCR

DNA purified using a QIAGEN 100/G column and resuspended in 250 µl of TE buffer was incubated at 60°C for 30 minutes, mixed gently and quantified by measuring A_{260}. The PCR

reaction was set up in a thin walled PCR tube and performed in an Eppendorf 5330 Mastercycler, using a primer of 22 to 25 bases in length:

DNA	2.5 μg
Primer	13 pmol
BigDye™ Sequencing Premix (Applied Biosystems)	16 μl
dH_2O	to 40 μl

Reaction conditions:

(i)	95°C		5 minutes
(ii)	90 cycles of:	95°C	30 seconds
		55°C	20 seconds
		60°C	4 minutes
(iii)	Hold at 4°C		

The DNA was cleaned using a DyeEx column (Qiagen), by processing 20 μl of the sequencing reaction per column followed by 20 μl of TE buffer. The eluate was then vacuum dried in preparation for sequencing.

Primer sequence (*lacZ* proximal end of Tn*917*):

5′ CTC ACA ATA GAG AGA TGT CAC CGT C 3′

2.22 Automated sequencing and sequence analysis

Prepared samples for sequencing were loaded onto an Applied Biosystems 373 DNA sequencer according to manufacturers instructions. Sequence data obtained was edited using the Staden NIP program. Homology searches were performed using the protein database of the National Centre for Biotechnological Information (Bethesda, U.S.A.).

2.23 Assay for β-galactosidase activity

Samples (0.1 ml and 0.5 ml) were taken at various time-points from a culture inoculated to A_{600} 0.05 (incubated with shaking at 250 rpm, 37°C) and cells recovered by centrifugation (5,000 rpm, 10 minutes, room temperature). The cells were resuspended in 0.5 ml ABT buffer. 50 µl of 4-MUG (4-methylumbelliferyl β-D-galactoside) (10 mg ml^{-1} in dimethyl sulphoxide) was added and incubated at 25°C for 60 minutes. Next, 0.5 ml of 0.4M Na_2CO_3 was added, the sample mixed and 25 µl of each sample added to 225 µl of ABTN buffer. 1/10 and 1/100 dilutions of each sample in 225 µl of ABTN buffer were created by serial dilution.

A calibration curve of the fluorescent product 4-MU (4-methylumbelliferone) was created with final concentrations of 0.01, 0.05, 0.1, 0.25, 0.5, 1 and 2.5 µM in 225 µl ABTN buffer. Fluorescence (Excitation at 355_{nm}; Emission at 460_{nm}) was measured using a VICTOR^2 multilabel counter (Wallac) and for each sample the 4-MU concentration was related to β-galactosidase activity.

$$\beta\text{-galactosidase activity} = U/(D \times 60 \times OD)$$

Where:

U = pmols of 4-MU produced

$OD = OD_{600}$ at given time-point

60 = time of incubation at 25°C

D = dilution factor (for 0.5 ml of neat sample = 1050/25 ×2 = 1/84)

CHAPTER 3

CHARACTERISATION OF THE STARVATION-SURVIVAL RESPONSE OF *L. MONOCYTOGENES* EGD

3.1 Introduction

The adaptation of both Gram-positive and Gram-negative bacteria to nutrient limitation results in cells with altered morphology and which are capable of remaining viable for prolonged periods of time (Watson *et al.*, 1998a; Smeulders *et al.*, 1999; Kjelleberg *et al.*, 1993; Kolter *et al.*, 1993). The primary stimulus for the development of this response is seen to differ between bacteria. In *E. coli*, the development of the starvation-survival phenotype is induced regardless of the form that nutrient limitation takes (i.e. carbon- phosphorus - or nitrogen-limitation) (Matin *et al.*, 1989; Jenkins *et al.*, 1988). A similar case is seen in *S. typhimurium* although here, a greater degree of stress resistance is observed after carbon starvation in comparison to phosphorus- or nitrogen-starvation (Seymour *et al.*, 1996). In contrast, the development of starvation-survival potential in *V. sp.* and *S. aureus* occurs only under carbon- or multiple nutrient-starvation (Nyström *et al.*, 1992; Watson *et al.*, 1998a). The development of a high degree of resistance to numerous forms of stress in response to starvation is a well-known phenomenon and includes resistance to heat, high osmolarity, acid and oxidative stress (Kolter *et al.*, 1993; Hartke *et al.*, 1994). The great array of changes that occur within the cell during stasis requires a dramatic change in gene expression and protein synthesis (Morton and Oliver, 1994; Spector and Cubitt, 1992). These occur in a temporally controlled fashion over the first few hours of nutrient deprivation and low levels of protein synthesis are also required throughout long-term starvation (Nyström *et al.*, 1990a; Watson *et al.*, 1998a).

Most information on the starvation-survival of *L. monocytogenes* and related cellular characteristics focus on the incidence of the bacterium in foodstuffs – principally dairy and meat products (reviewed in Farber and Peterkin, 1991). Elsewhere, an early study on the survival of *L. monocytogenes* showed that cells could remain culturable for over 8 weeks in

pond water (Botzler *et al.* 1975). Also the viability of multiple nutrient-starved *L. monocytogenes* cells was found to fall by 1.3 log units over 24 hours when incubated at 30°C in phosphate buffer (Lou and Yousef, 1996). The heat resistance (D_{56}) of these cells rose significantly compared to exponential-phase cells (D_{56} = 13.6 and 1.0 minutes respectively), an effect also found in cells incubated in seawater for 7 days (Bremer *et al.*, 1998). A reduction in growth rate (from 0.389 h^{-1} to 0.05 h^{-1}) of *L. monocytogenes* cells in continuous culture at 30°C has been found to affect stress resistance, increasing D values at pH 2.5 (from 19.5 to 78.3 minutes respectively) (Patchett *et al.*, 1996).

Whilst no specific studies have yet been conducted on *L. monocytogenes*, the dynamic nature of starved cultures has been acknowledged for a number of bacterial species. Evidence of cryptic growth has been found in glucose-starved *S. aureus* cultures (Watson *et al.*, 1998a). In *E. coli* and *P. putida*, bacteria that accumulate advantageous mutations eventually take over the population, a phenomenon known as the growth advantage in stationary phase (GASP) phenotype (Zambrano *et al.*, 1993; Eberl *et al.*, 1996). Contrary to the development of GASP mutants, debate continues over whether bacteria enter a viable-but-non-culturable (VBNC) state (reviewed in Barer and Harwood, 1999), most notably in the pathogenic species from the genera *Vibrio*, *Salmonella*, *Shigella*, and *Legionella* (McKay, 1992). The possible existence of VBNC pathogenic bacteria has caused great concern over the use of traditional methods for the quality testing of water and foods. Besnard *et al.* (2000) have recently addressed the possibility of a VBNC state in *L. monocytogenes*, following previous studies in other bacteria (Xu *et al.*, 1982). Viable cells were deemed to be those that could become elongated after incubation in BHI with added DNA replication inhibitor (ciprofloxacin) and an estimation of viable cell numbers was compared to standard culture techniques. The study did not however, determine whether VBNC *L. monocytogenes* cells occur and the validity of extending this method to detecting the VBNC state has been questioned (Barer and Harwood, 1999).

The work described in this chapter details the development of a chemically defined medium and an examination of the physiological, morphological and stress-resistance characteristics of the starvation-survival-response of *L. monocytogenes*.

3.2 Results

3.2.1 Development of a chemically defined medium (CDM) for starvation-survival experiments in batch cultures

A chemically defined medium for the culture of *L. monocytogenes*, as described by Trivett and Meyer (1971), was modified for optimal growth yield (Chapter 2.1.1). To establish the level at which glucose and amino acid concentrations began to limit the final growth yield (and thus deemed to be the limiting factor), CDM was prepared with a range of concentrations of glucose or amino acids and growth monitored by A_{600}. Growth at limiting concentrations of glucose resulted in lower final A_{600} compared to that in the original CDM composition, which reached A_{600} 1.06 after 9 hours (Figure 3.1). The glucose concentration only became noticeably final growth yield limiting at 0.1 % w/v or below, indicating that glucose is in excess at 0.2 % w/v (Figure 3.1). Raising the glucose concentration above 0.2% (w/v) had no observable effect on the final growth yield, suggesting that alternative nutrients or other factors become limiting. It was notable that amino acid concentration only lowered final growth yield when reduced by at least 75-fold (Figure 3.2), suggesting that amino acids are in excess in the original CDM. For experiments using limiting glucose or amino acid concentrations, 0.1 % w/v and 0.0016 % w/v were used respectively, whilst all other nutrients were as in the original CDM composition (Chapter 2.1.1).

3.2.2 Kinetics of starvation-survival in glucose- and amino acid-limiting CDM at 4°C, 25°C and 37°C

Starvation-survival kinetics were studied under glucose- and amino acid-limitation at 4, 25 and 37°C. Cultures were inoculated to A_{600} 0.01 and grown at 37°C for 18 hours - approximately 6 hours post-exponential phase. The cultures were transferred to the long-term starvation temperature and incubated statically. All cultures attained A_{600} of 0.65 to 0.75, corresponding to approximately 1×10^9 CFU ml^{-1}. Culture viability was determined by plating serial dilutions onto TSB agar.

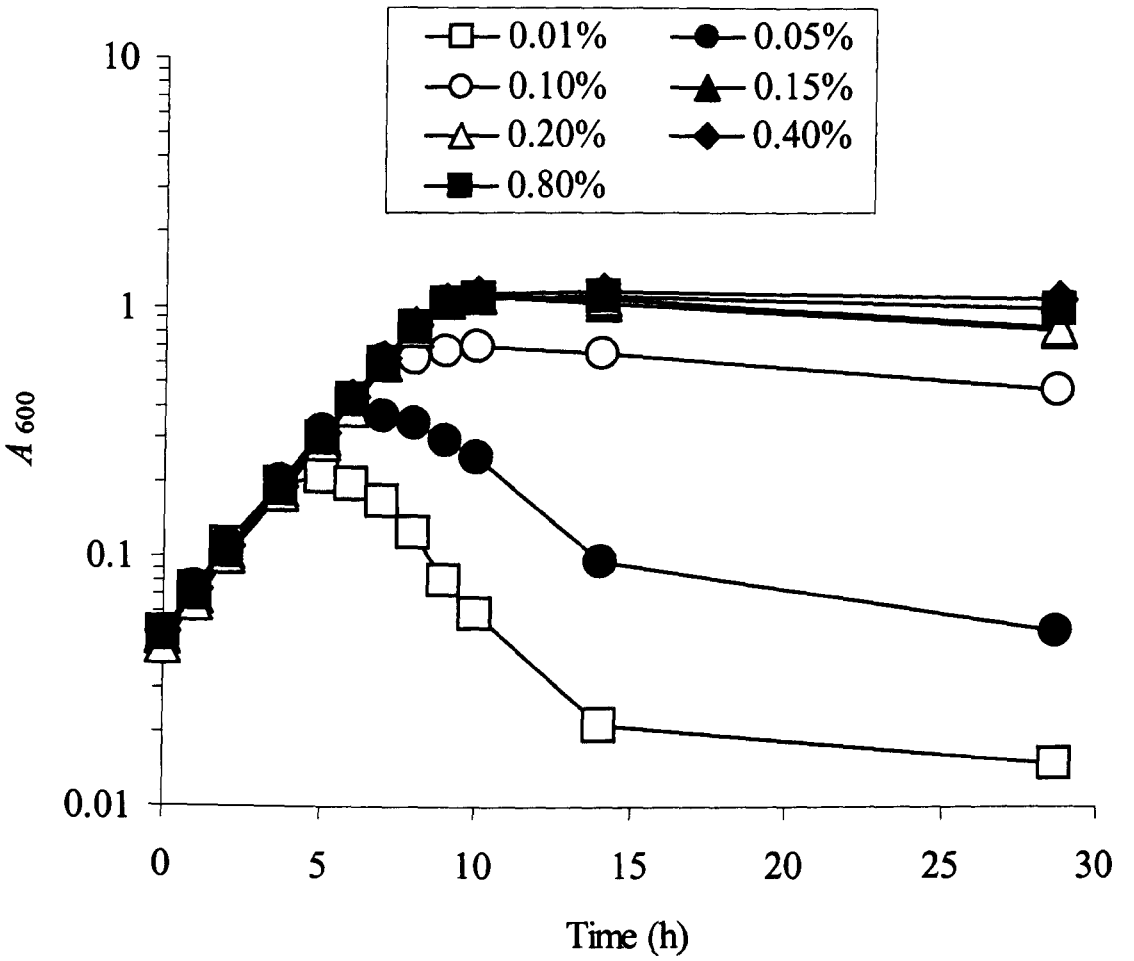


Figure 3.1
Growth kinetics of *L. monocytogenes* EGD at 37°C in CDM containing a range of glucose concentrations.

Cultures inoculated at A_{600} 0.05. % indicates concentration (w/v) of glucose in the medium.

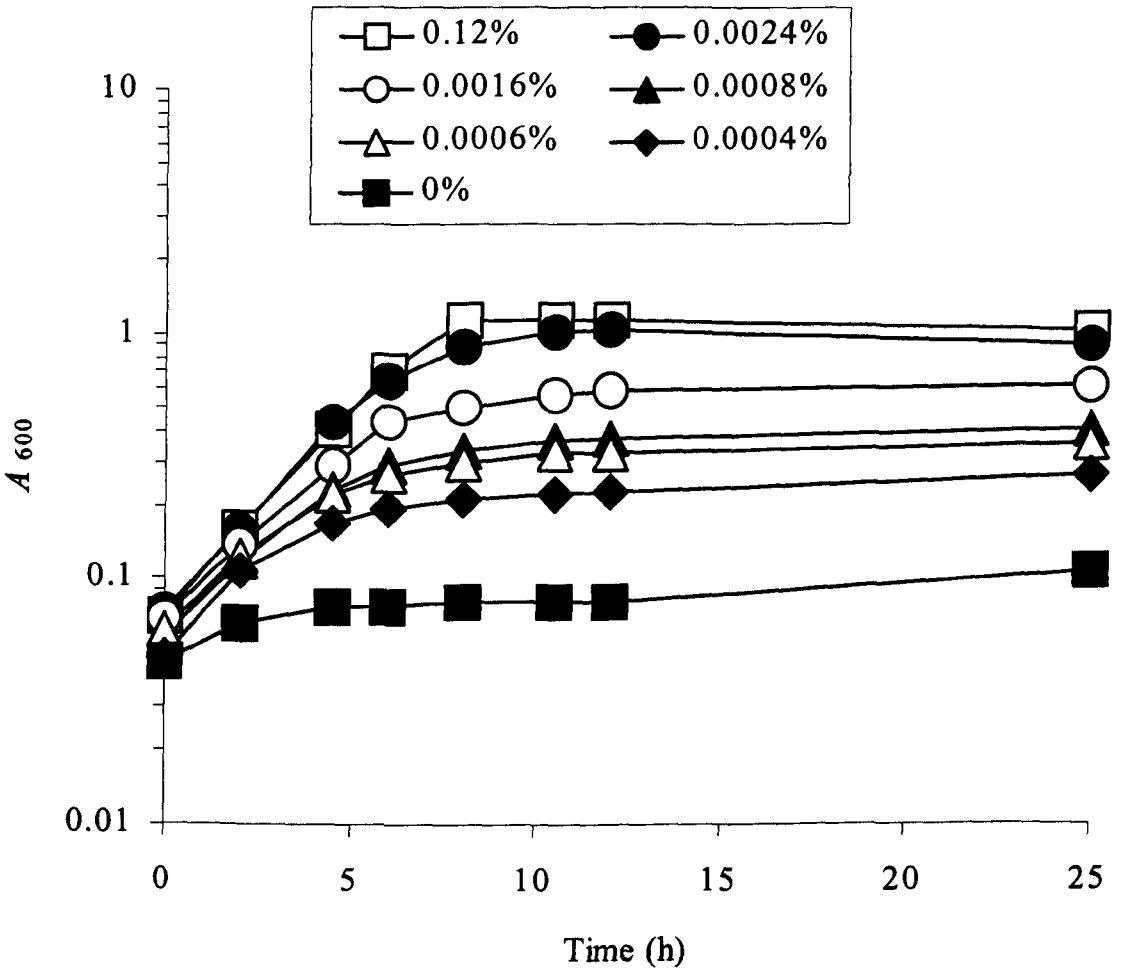


Figure 3.2
Growth kinetics at 37°C in CDM containing a range of amino acid concentrations.

Cultures inoculated at A_{600} 0.05. % indicates concentration (w/v) of amino acids in the medium.

In glucose-starved cultures incubated at 4, 25 and 37°C, viability fell to between 10 and 0.1 % after 20 days (Figure 3.3), with survival found to be **temperature variable**. Loss of viability was fastest at 37°C, although survival after 20 days was found to be greater at 25°C than at 4°C, despite the fact that the initial rate of loss of viability was greater at 25°C (Figure 3.3). The kinetics of starvation-survival was found to change with increasing temperature. At 4°C, the loss of viability occurred steadily throughout the 20 days of the experiment (Figure 3.3), whereas at 25°C and 37°C, a rapid loss in viability occurred in the first 1 to 3 days, before the rate of decline became reduced or stopped completely. Cells in batch cultures showed significantly greater survival under glucose limitation than when starved for amino acids (Figures 3.3 and 3.4). In amino acid starved cultures at 37°C, viability was undetectable after 3 days (Figure 3.4). Survival under amino acid limitation was also found to be **temperature variable**, with incubation at higher temperatures resulting in an increase in the rate of loss of viability (Figure 3.4). At 4°C, the loss of viability occurred steadily throughout the 20 days of the experiment, but at a greater rate than that observed in glucose-starved cultures (1 and 0.1% survival respectively; Figures 3.3 & 3.4). At 25°C, a rapid loss in viability occurred in the first 1 to 3 days, before the rate of decline slowed under both conditions (Figures 3.3 & 3.4).

3.2.3 Kinetics of survival of glucose- and amino acid-starved cells incubated in water and PBS at 25 and 37°C

Cells from 6-hour post-exponential glucose- and amino acid-starved cultures grown for 18 hours at 37°C were washed, resuspended in the same volume of sterile water or PBS and culture viability monitored over time. Cells from glucose-starved cultures retained approximately 0.7% of initial culture viability after 20 days when incubated in water at 25°C (Figure 3.5). A rapid fall in viability over the first 3 days was observed, followed by stable cell numbers for the remainder of the experiment. Resuspension in PBS at 25°C resulted in 10 % of viability being retained after 20 days for cells previously starved for glucose (Figure 3.6), which was reduced to 0.04 % after 20 days if incubation was at 37°C. At both temperatures however, similar kinetics were observed as the rate of decline in viability reduced after 1 to 3 days (Figure 3.6). Amino acid-starved cells incubated in water at 25°C

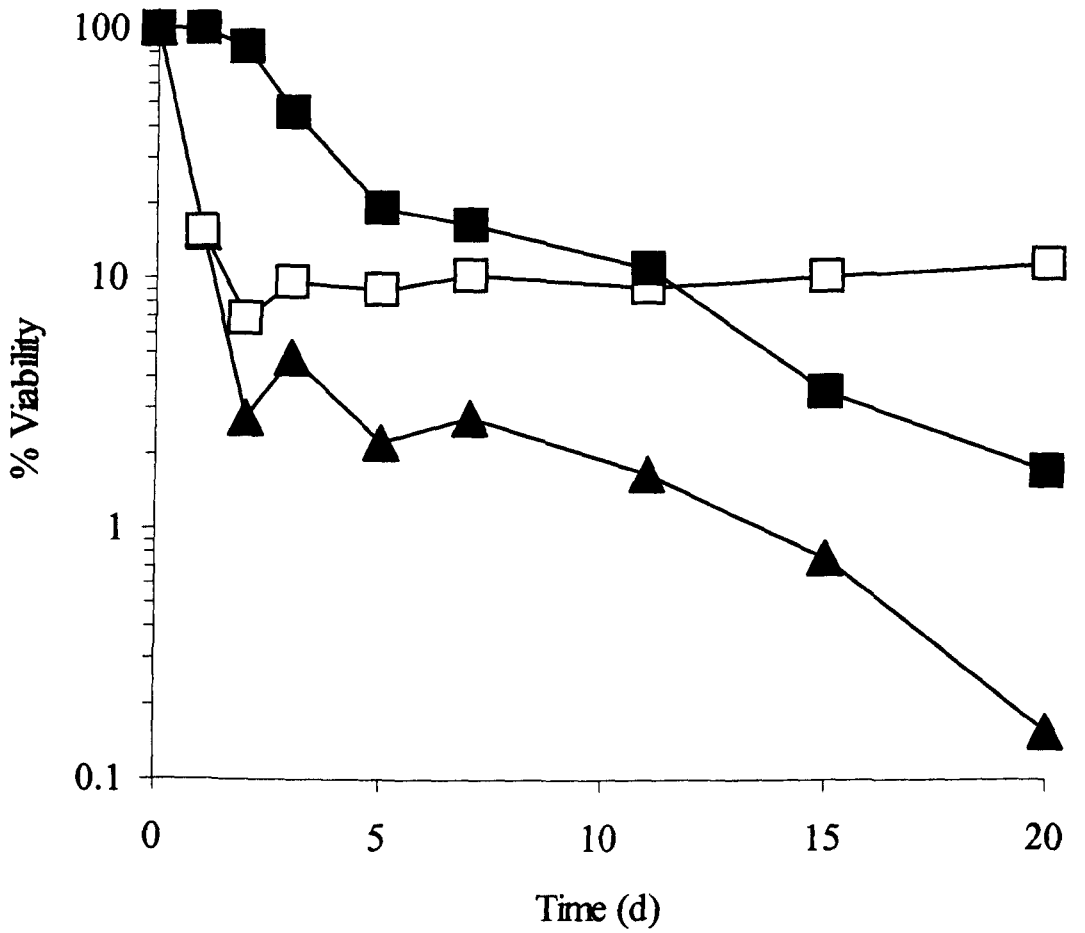


Figure 3.3
Starvation-survival kinetics in glucose-limiting CDM at a range of incubation temperatures.

Cells were incubated in glucose-limiting CDM at 37°C (▲), 25°C (□) and 4°C (■). 100% viability was 1×10^9 CFU ml^{-1}. Data are an average of at least two separate experiments for which the standard deviation was no greater than ±40% of the mean value at equivalent time points.

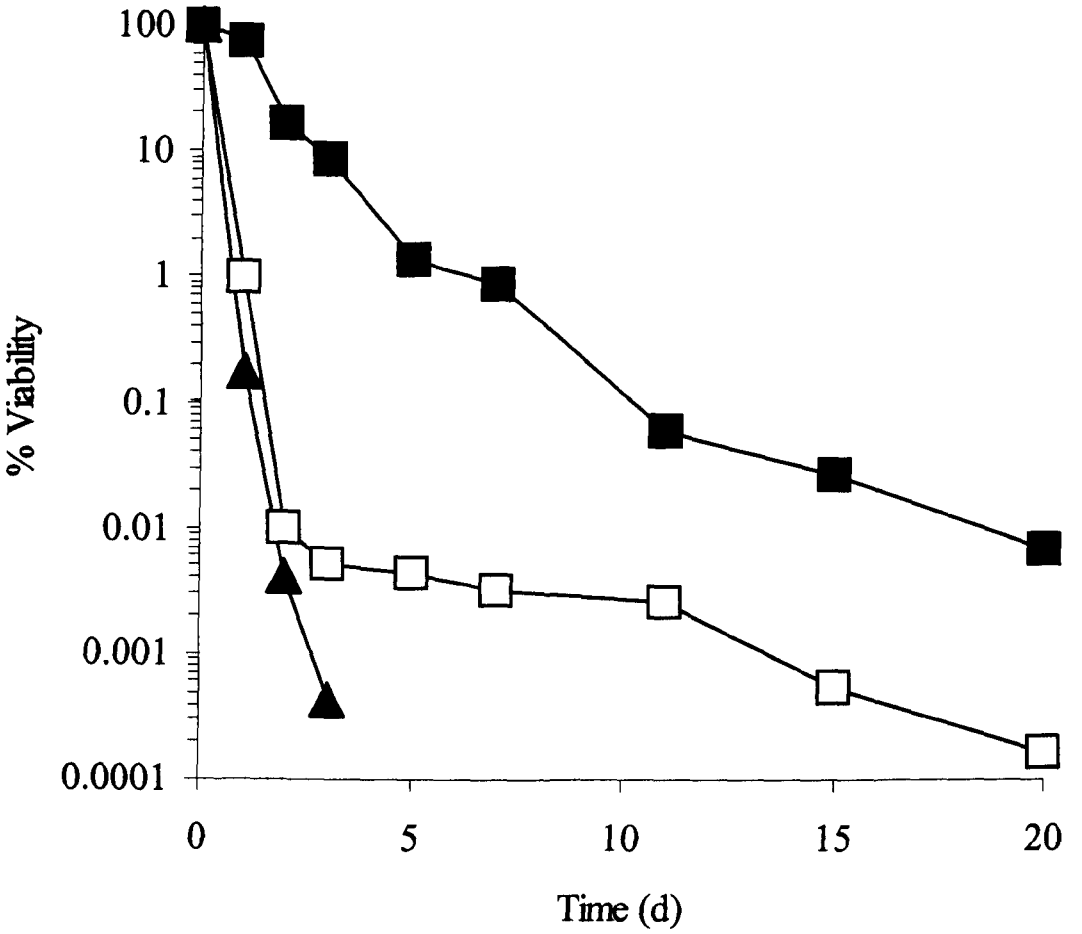


Figure 3.4
Starvation-survival kinetics in amino acid-limiting CDM at a range of incubation temperatures.

Cells were incubated in amino acid-limiting CDM at 37°C (▲), 25°C (□) and 4°C (■). 100% viability was 1×10^9 CFU ml^{-1}. Data are an average of at least two separate experiments for which the standard deviation was no greater than ±40% of the mean value at equivalent time points.

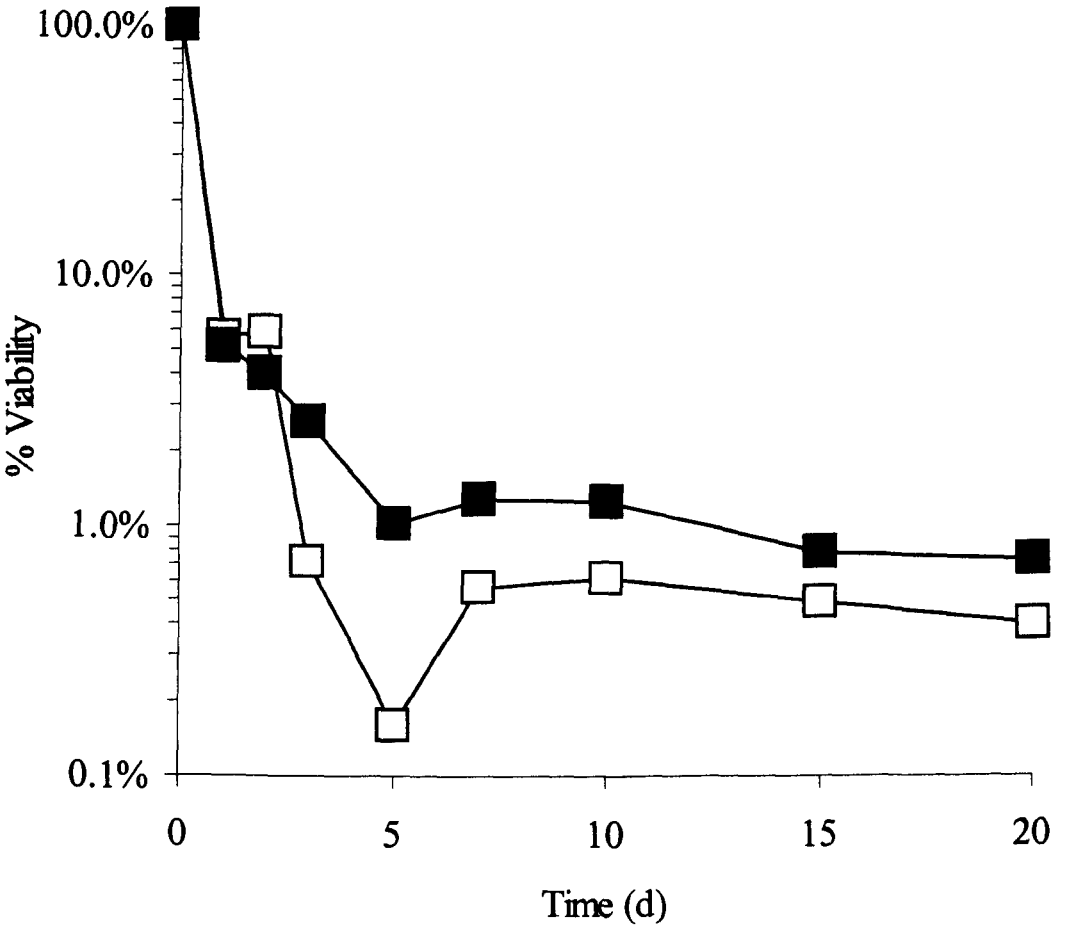


Figure 3.5
Kinetics of starvation-survival of glucose- and amino acid-starved cells in water.

Cells were incubated in glucose-limiting (■), or amino acid-limiting CDM (□) at 37°C, harvested, washed and resuspended in sterile distilled water at 25°C. 100% viability was 1×10^9 CFU ml^{-1}. Data are an average of at least two separate experiments for which the standard deviation was no greater than ±40% of the mean value at equivalent time points.

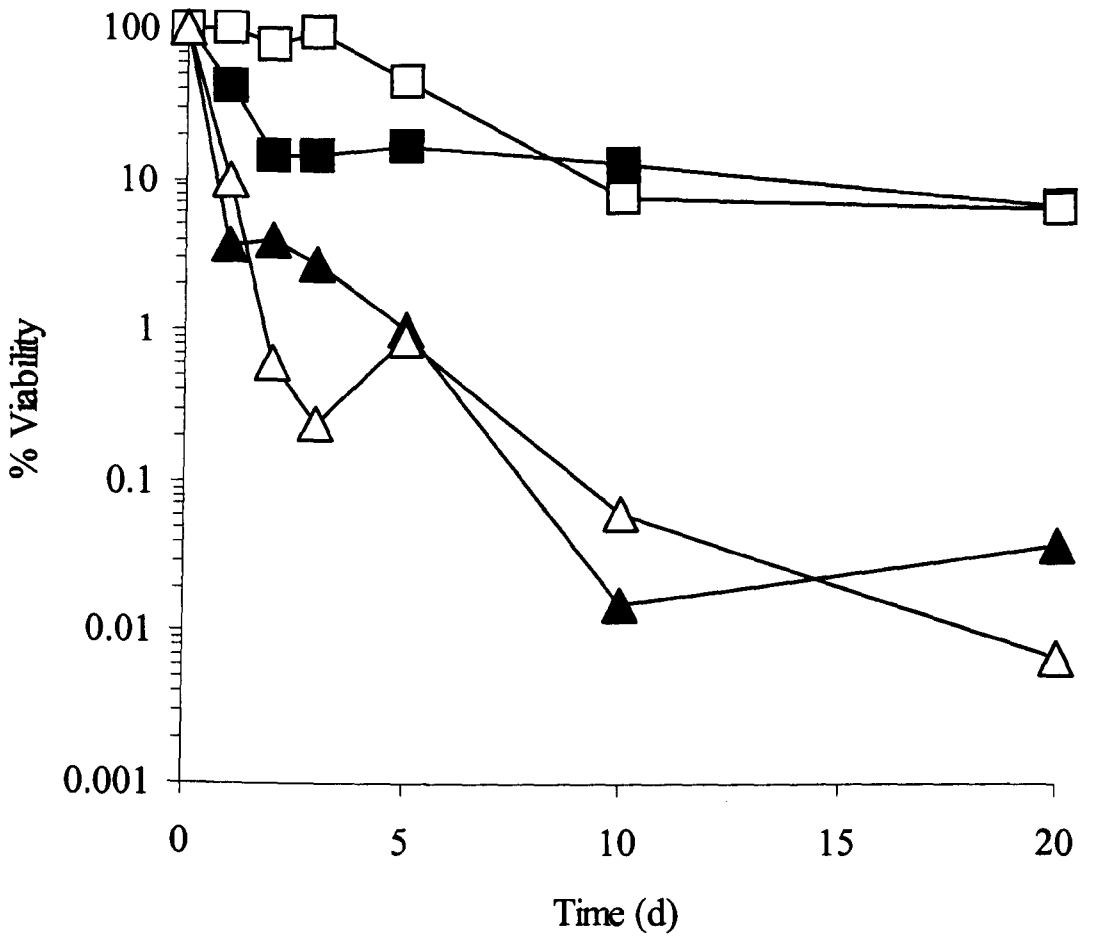


Figure 3.6
Kinetics of starvation-survival of glucose- and amino acid-starved cells in PBS at 25 or 37°C.

Cells were incubated in glucose-limiting (■,▲), or amino acid-limiting CDM (□,Δ) at 37°C, harvested, washed and resuspended in sterile distilled water at 25°C (■,□) or 37°C (▲,Δ). 100% viability was 1×10^9 CFU ml^{-1}. Data are an average of at least two separate experiments for which the standard deviation was no greater than ±40% of the mean value at equivalent time points.

exhibited a rapid fall in viability during the first 5 days, before cell numbers became stable at 0.4% of the initial culture viability after 20 days (Figure 3.5). When resuspended in PBS, 10 % of viability was retained after 20 days at 25°C and 0.006 % after 20 days at 37°C. As for glucose-starved cells incubated in PBS, the kinetics of an early rapid fall in viability was followed by a reduced rate of decline after 1 to 3 days (Figure 3.6).

The transfer of either glucose or amino acid-limited cells into water or PBS resulted in similar starvation-survival kinetics as seen for glucose-starved cultures (Chapter 3.2.2), with long-term survival of significant cell numbers. It would seem therefore, that the occurrence of glucose- or multiple nutrient-limitation is most important for eliciting a starvation-survival response.

3.2.4 The role of cell density in starvation-survival

To determine the role of initial cell density in the starvation-survival of *L. monocytogenes*, cells from 6-hour post-exponential-phase glucose-limited cultures (18 hours) were harvested by centrifugation and resuspended in water or the spent culture medium in which they had been grown at a range of initial cell densities. Upon resuspension in water at 37°C, survival was density dependent, whilst at 25°C survival was only density dependent below 1×10^7 CFU ml^{-1} (Figures 3.7 & 3.8). At 25°C, viability became undetectable within 3 days if resuspended at 6×10^5 CFU ml^{-1} or below, whilst at 2×10^7 CFU ml^{-1}, approximately 1 % of cells remained viable after 20 days. At 37°C, viability was only maintained over 20 days when the cells were resuspended at a density of 2×10^9 CFU ml^{-1} (Figure 3.8). Viability was lost after 2 days if cells were resuspended at 2×10^8 CFU ml^{-1} or within 24 hours when resuspended at 1×10^7 CFU ml^{-1} or below.

When cells were resuspended in filter-sterilised spent culture supernatant at initial densities of 5×10^5 CFU ml^{-1} to 1×10^9 CFU ml^{-1} and incubated at 25°C, the viability in all cultures reached approximately 2×10^7 CFU ml^{-1} within 3 days and was maintained at this level for the remaining 17 days (Figure 3.9). Upon incubation at 37°C in spent culture supernatant at initial densities of between 7×10^5 and 1×10^9 CFU ml^{-1}, viability in all cultures was retained

at approximately 5×10^5 to 5×10^6 CFU ml^{-1} after 20 days (Figure 3.10). It was noted that at both 25°C and 37°C, the viable counts of cultures with high initial cell densities converged with those inoculated at low initial cell densities, as viability in the former dropped rapidly over the first 2 to 3 days, whilst in the latter, regrowth to higher densities was observed in the same period (Figures 3.9 & 3.10).

3.2.5 The effect of cell density and resuspension medium on starvation-survival of long term glucose-starved cells

In order to establish whether cell density continued to play a role in the starvation-survival of long-term starved cells, cultures were treated as described (Chapter 2.9). Glucose-limited cultures incubated for 7 days at 37°C or 21 days at 25°C were washed, resuspended at a range of initial cell densities in either sterile water or the filter-sterilised spent culture supernatant in which they had been grown, and incubated at the same temperature.

The survival of cells taken from long-term (7 day) glucose-limited cultures and resuspended in water at 37°C was found to be density dependent when incubated in water, as all viability was lost within 3 to 7 days if resuspended at 2×10^5 CFU ml^{-1} or below, whereas at an initial density of 2×10^6 CFU ml^{-1}, approximately 1×10^3 cells remained viable after 20 days (Figure 3.11). Incubation of 7 day glucose-starved cells in spent culture supernatant at 37°C resulted in cell viability of approximately 5×10^6 CFU ml^{-1} after 20 days, irrespective of an initial cell density of between 4×10^3 to 5×10^6 CFU ml^{-1} (Figure 3.12).

Survival after 20 days of long-term (21 day) glucose-starved cells resuspended in water at 25°C occurred at all initial cell densities tested. The resuspension of cells at 10^7, 10^6, 10^5 and 10^4 CFU ml^{-1} resulted in a slow but steady loss in viability over 20 days (Figure 3.13).

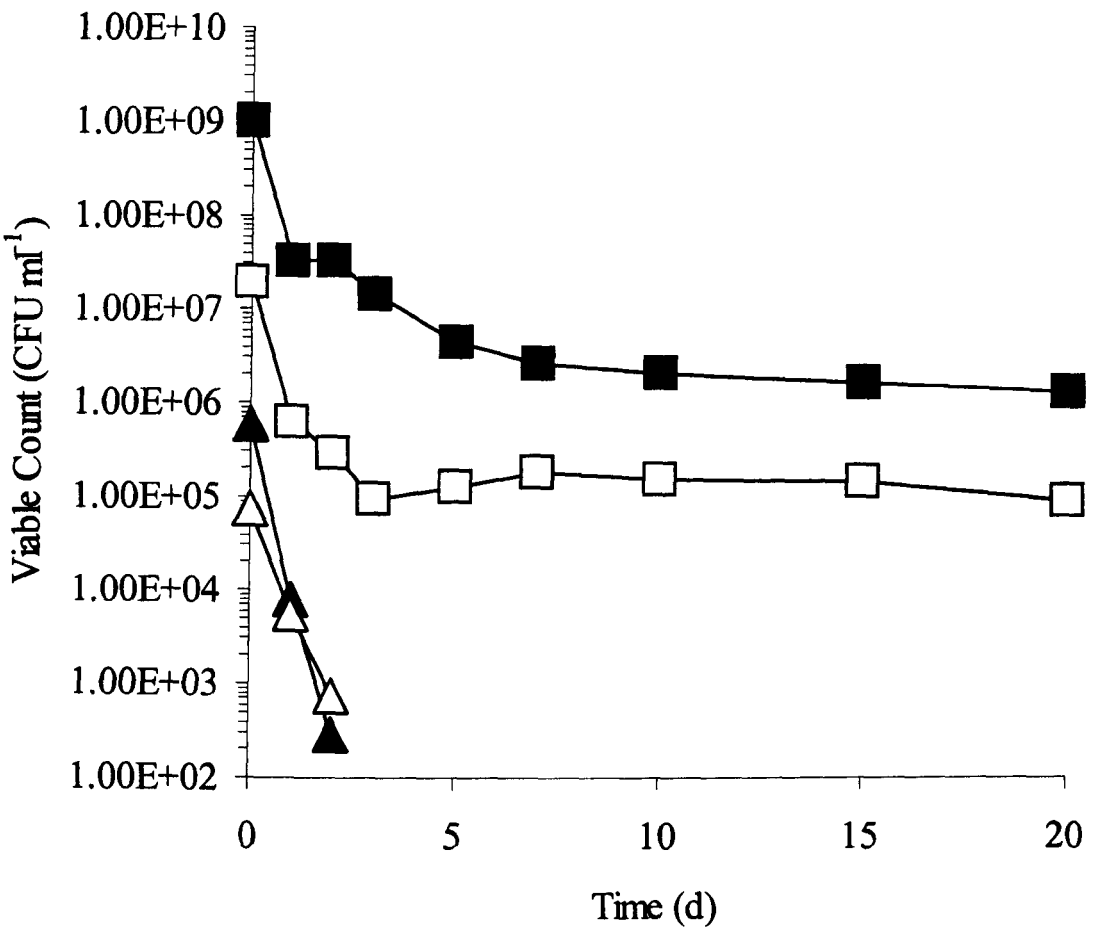


Figure 3.7
Starvation-survival kinetics of glucose-starved cells after resuspension in water at 25°C, at a range of cell densities.

6-hour post-exponential phase cells grown in glucose-limiting CDM were washed, resuspended and diluted in sterile distilled water. Cultures were then incubated at 25°C without shaking. Initial cell densities (CFU ml^{-1}) were approximately 1×10^9 (■), 2×10^7 (□), 6×10^5 (▲) and 8×10^4 (Δ). Data are an average of at least two separate experiments for which the standard deviation was no greater than ±40% of the mean value at equivalent time points.

Limit of detection (100 CFU ml^{-1}).

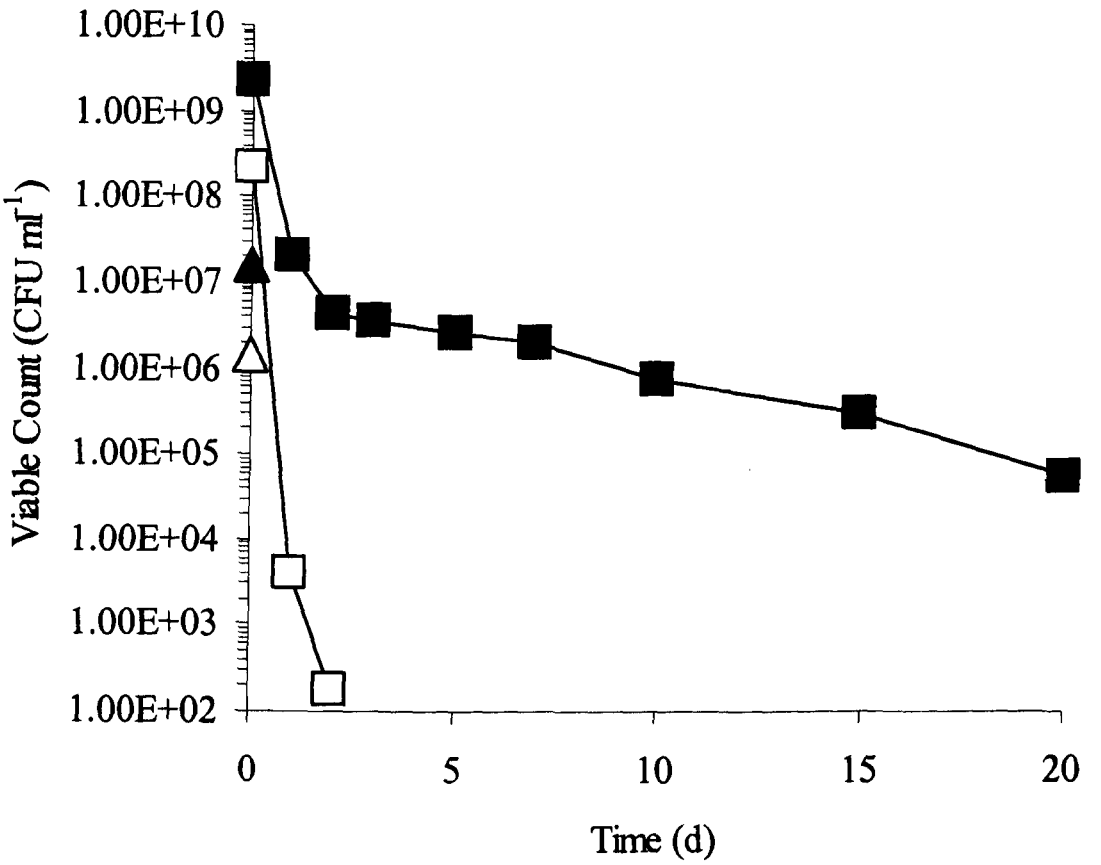


Figure 3.8
Starvation-survival kinetics of glucose-starved cells after resuspension in water at 37°C, at a range of cell densities.

6-hour post-exponential phase cells grown in glucose-limiting CDM were washed, resuspended and diluted in sterile distilled water. Cultures were then incubated at 37°C without shaking. Initial cell densities (CFU ml^{-1}) were approximately 2×10^9 (■), 2×10^8 (□), 1×10^7 (▲) and 2×10^6 (Δ). Data are an average of at least two separate experiments for which the standard deviation was no greater than ±40% of the mean value at equivalent time points.

Limit of detection (100 CFU ml^{-1}). Single time points indicate viability below limit of detection within 1 day.

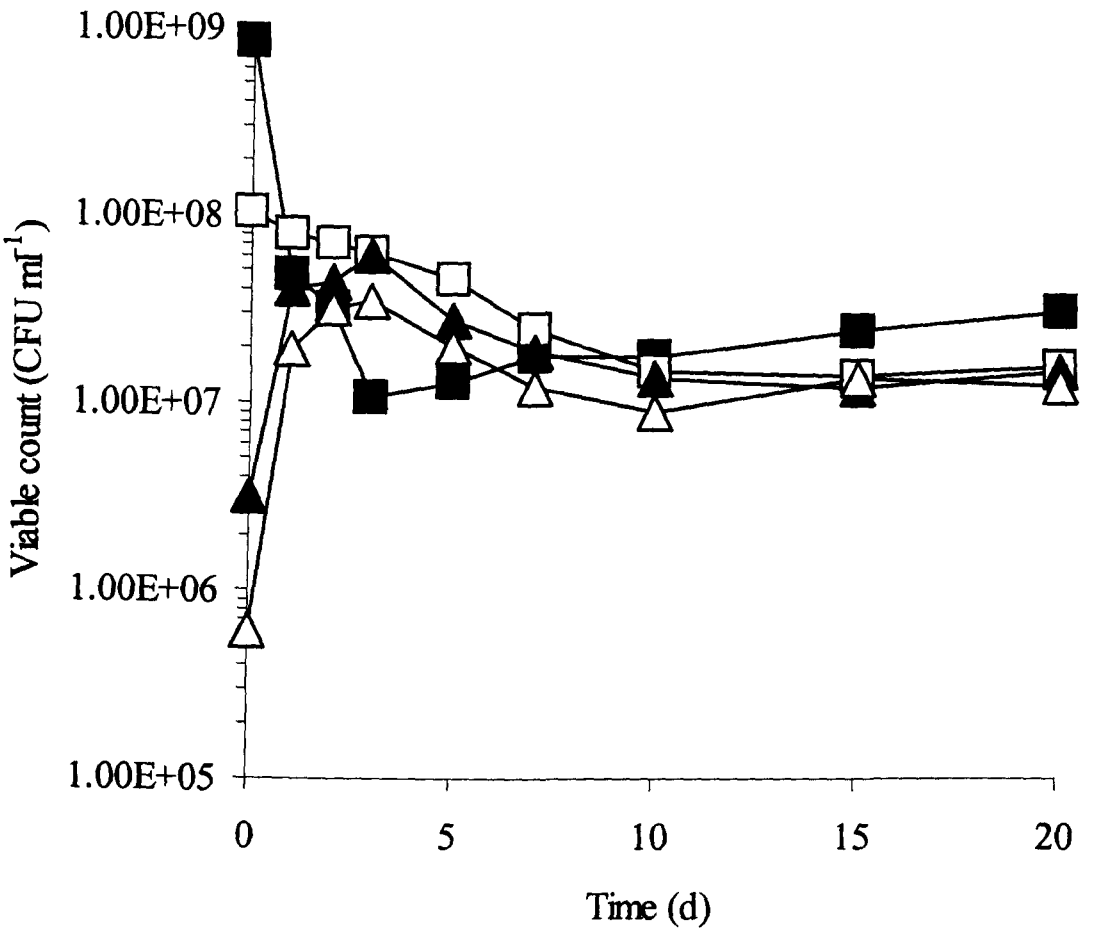


Figure 3.9
Starvation-survival kinetics of glucose-starved cells after resuspension in filter sterilised glucose-limited CDM conditioned culture supernatant at 25°C, at a range of cell densities.

6-hour post-exponential phase cells grown in glucose-limiting CDM were washed, resuspended and diluted in filter sterilised glucose-limiting CDM conditioned culture supernatant. Cultures were then incubated at 25°C without shaking. Initial cell densities (CFU ml^{-1}) were approximately 1×10^9 (■), 1×10^8 (□), 3×10^6 (▲) and 5×10^5 (Δ). Data are an average of at least two separate experiments for which the standard deviation was no greater than ±40% of the mean value at equivalent time points.

Limit of detection (100 CFU ml^{-1}).

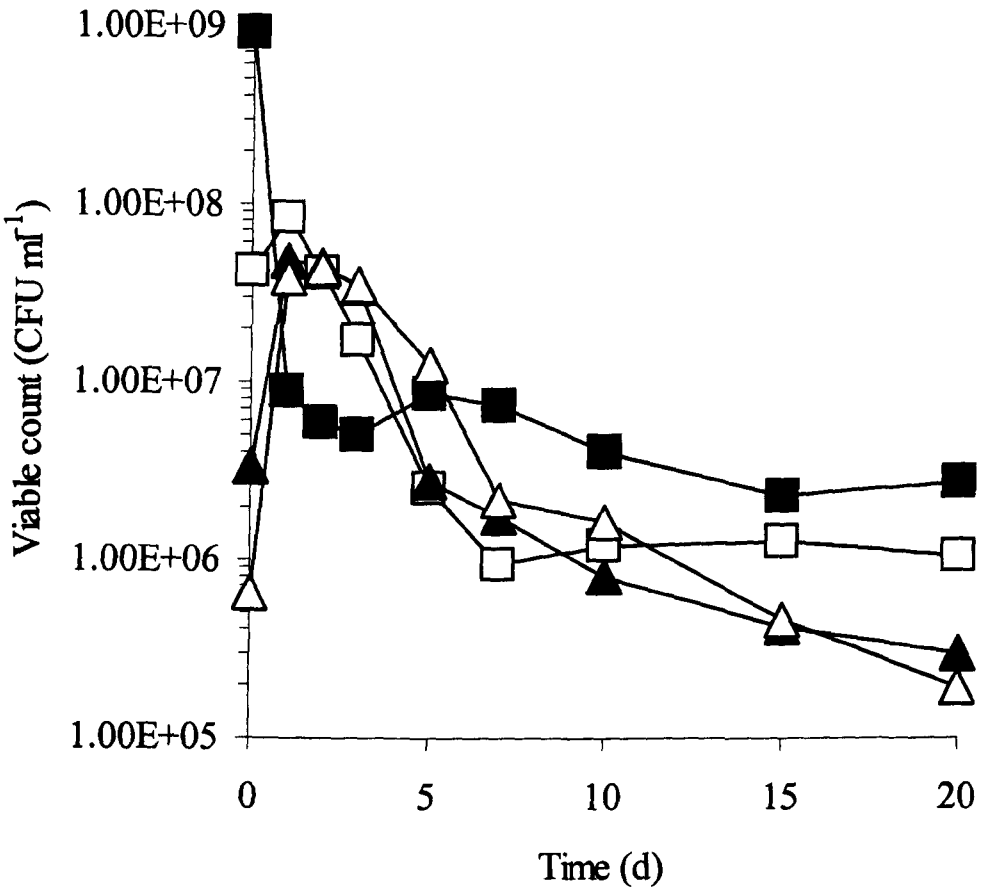


Figure 3.10
Starvation-survival kinetics of glucose-starved cells after resuspension in filter sterilised glucose-limiting CDM conditioned culture supernatant at 37°C, at a range of cell densities.

6-hour post-exponential phase cells grown in glucose-limiting CDM were washed, resuspended and diluted in filter sterilised glucose-limiting CDM conditioned culture supernatant. Cultures were then incubated at 37°C without shaking. Initial cell densities (CFU ml^{-1}) were approximately 1×10^9 (■), 4×10^7 (□), 3×10^6 (▲) and 7×10^5 (Δ). Data are an average of at least two separate experiments for which the standard deviation was no greater than ±40% of the mean value at equivalent time points.

Limit of detection (100 CFU ml^{-1}).

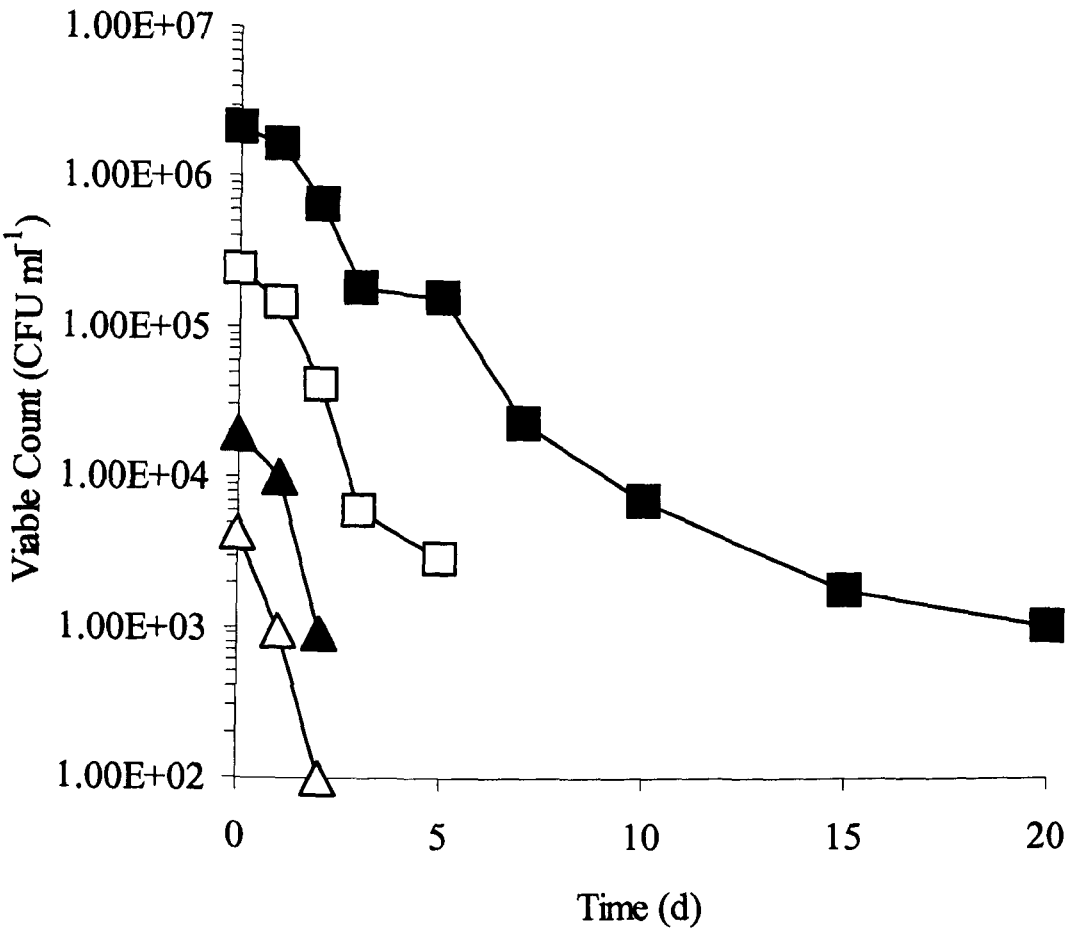


Figure 3.11
Starvation-survival kinetics of long-term (7 days, 37°C) glucose-starved cells after resuspension in water at 37°C, at a range of cell densities.

Cells from a 7 day-old, glucose-limited culture, incubated at 37°C, were washed, resuspended and diluted in sterile water. Cultures were then incubated at 37°C without shaking. Initial cell densities (CFU ml^{-1}) were approximately 2×10^6 (■), 2×10^5 (□), 2×10^4 (▲) and 4×10^3 (Δ). Data are an average of at least two separate experiments for which the standard deviation was no greater than ±40% of the mean value at equivalent time points.

Limit of detection (100 CFU ml^{-1}).

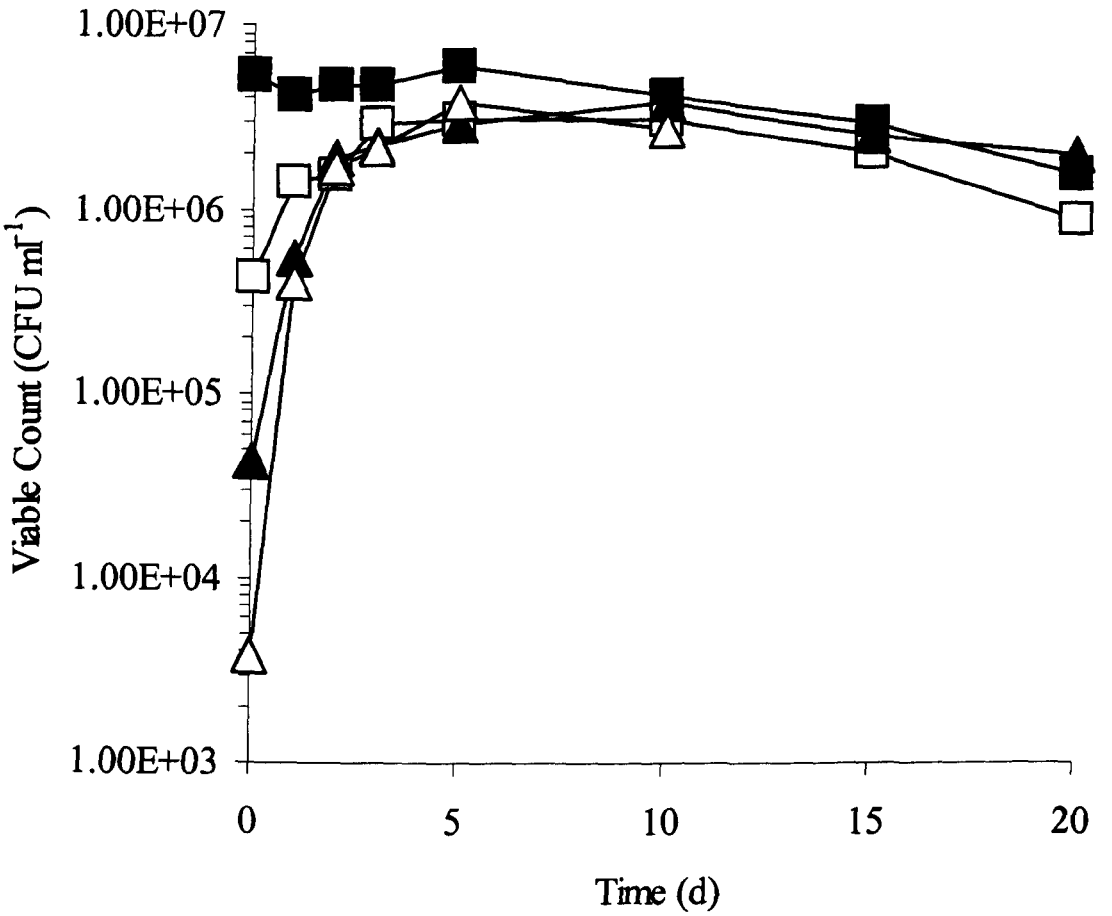


Figure 3.12
Starvation-survival kinetics of long-term (7 days, 37°C) glucose-starved cells after resuspension in filter sterilised glucose-limiting CDM conditioned culture supernatant at 37°C, at a range of cell densities.

Cells from a 7 day-old, glucose-limited culture, incubated at 37°C, were washed, resuspended and diluted in filter sterilised glucose-limiting CDM conditioned culture supernatant. Cultures were then incubated at 37°C without shaking. Initial cell densities (CFU ml^{-1}) were approximately 5×10^6 (■), 5×10^5 (□), 4×10^4 (▲) and 4×10^3 (Δ). Data are an average of at least two separate experiments for which the standard deviation was no greater than ±40% of the mean value at equivalent time points.

Limit of detection (100 CFU ml^{-1}).

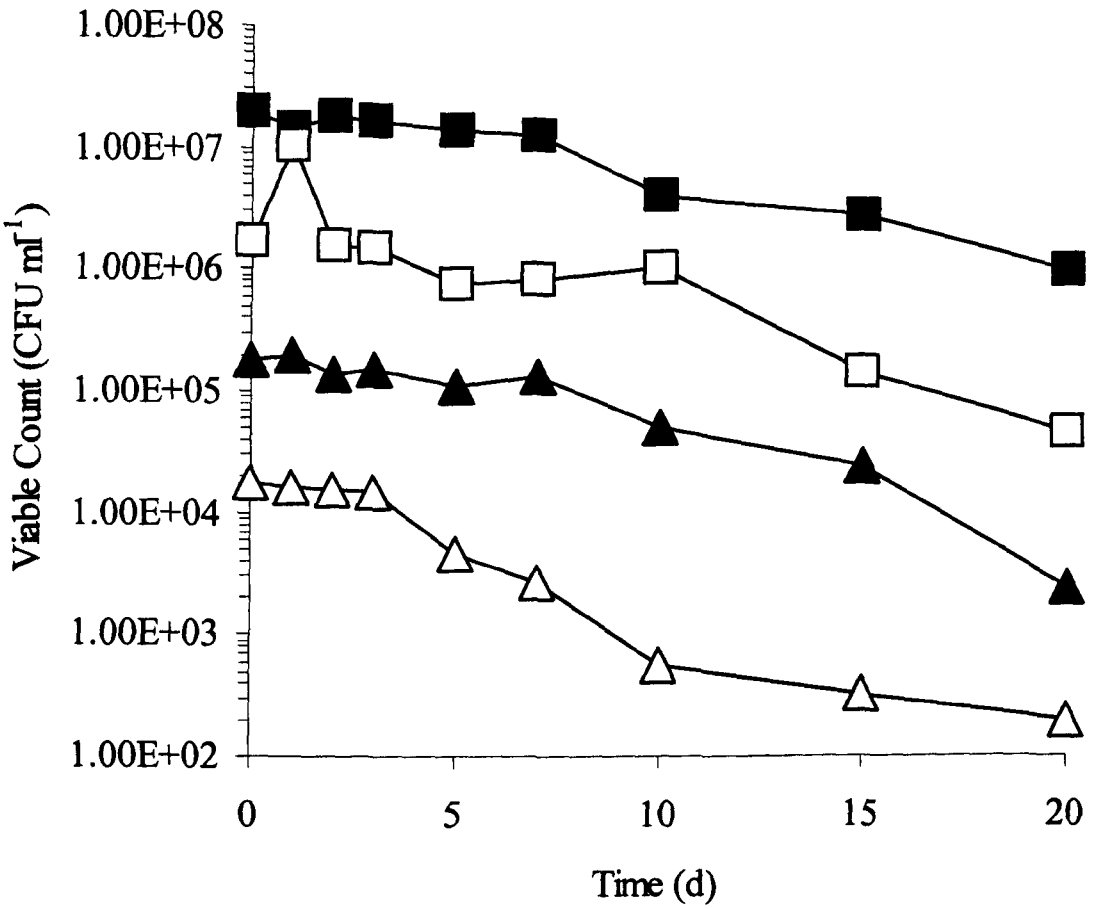


Figure 3.13
Starvation-survival kinetics of long-term (21 days, 25°C) glucose-starved cells after resuspension in water at 25°C, at a range of cell densities.

Cells from a 21 day-old, glucose-limited culture, incubated at 25°C, were washed, resuspended and diluted in sterile water. Cultures were then incubated at 25°C without shaking. Initial cell densities (CFU ml^{-1}) were approximately 2×10^7 (■), 2×10^6 (□), 2×10^5 (▲) and 2×10^4 (Δ). Data are an average of at least two separate experiments for which the standard deviation was no greater than ±40% of the mean value at equivalent time points. Limit of detection (100 CFU ml^{-1}).

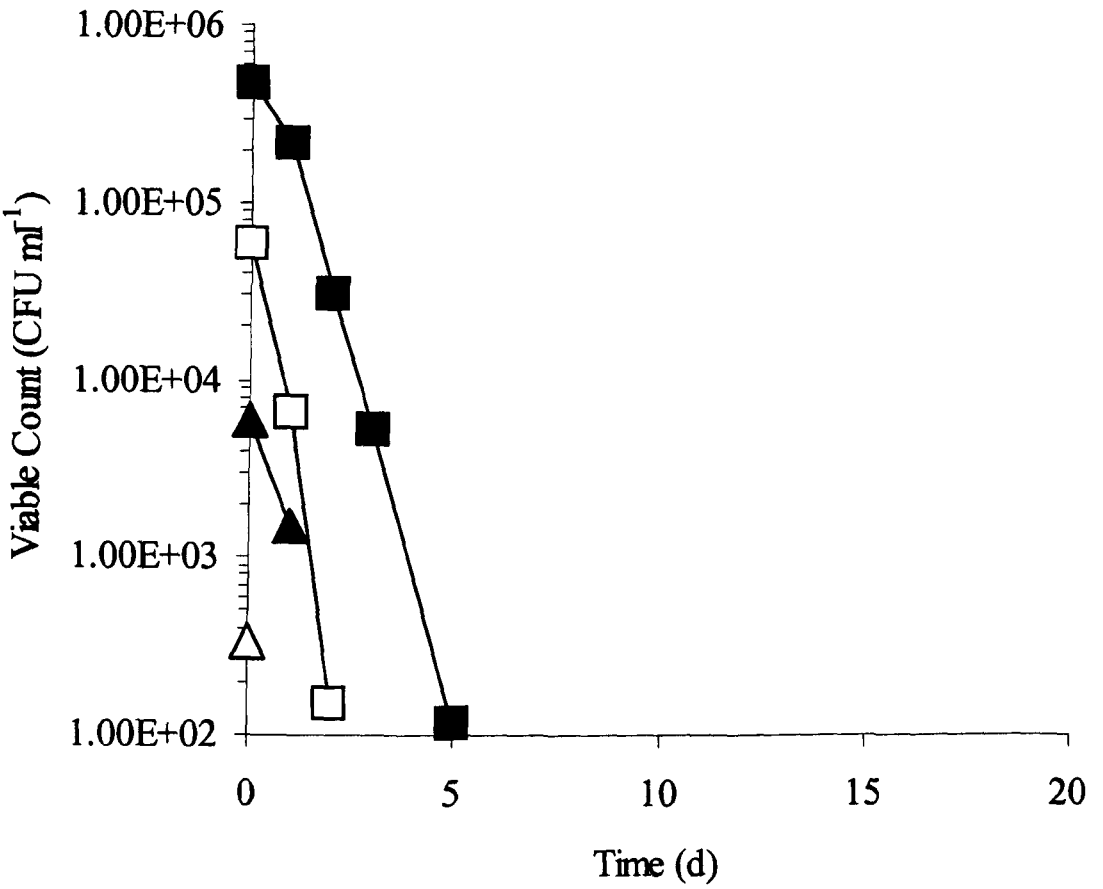


Figure 3.14
Starvation-survival kinetics of long-term multiple nutrient-starved cells (7 days, 37°C) after resuspension in water at 37°C, at a range of cell densities.

Cells from a 7 day-old, multiple nutrient-limited culture, incubated at 37°C, were washed, resuspended and diluted in sterile water. Cultures were then incubated at 37°C without shaking. Initial cell densities (CFU ml^{-1}) were approximately 5×10^5 (■), 5×10^4 (□), 6×10^3 (▲) and 3×10^2 (Δ). Data are an average of at least two separate experiments for which the standard deviation was no greater than ±40% of the mean value at equivalent time points. Limit of detection (100 CFU ml^{-1}). Single time points indicate viability below limit of detection within 1 day.

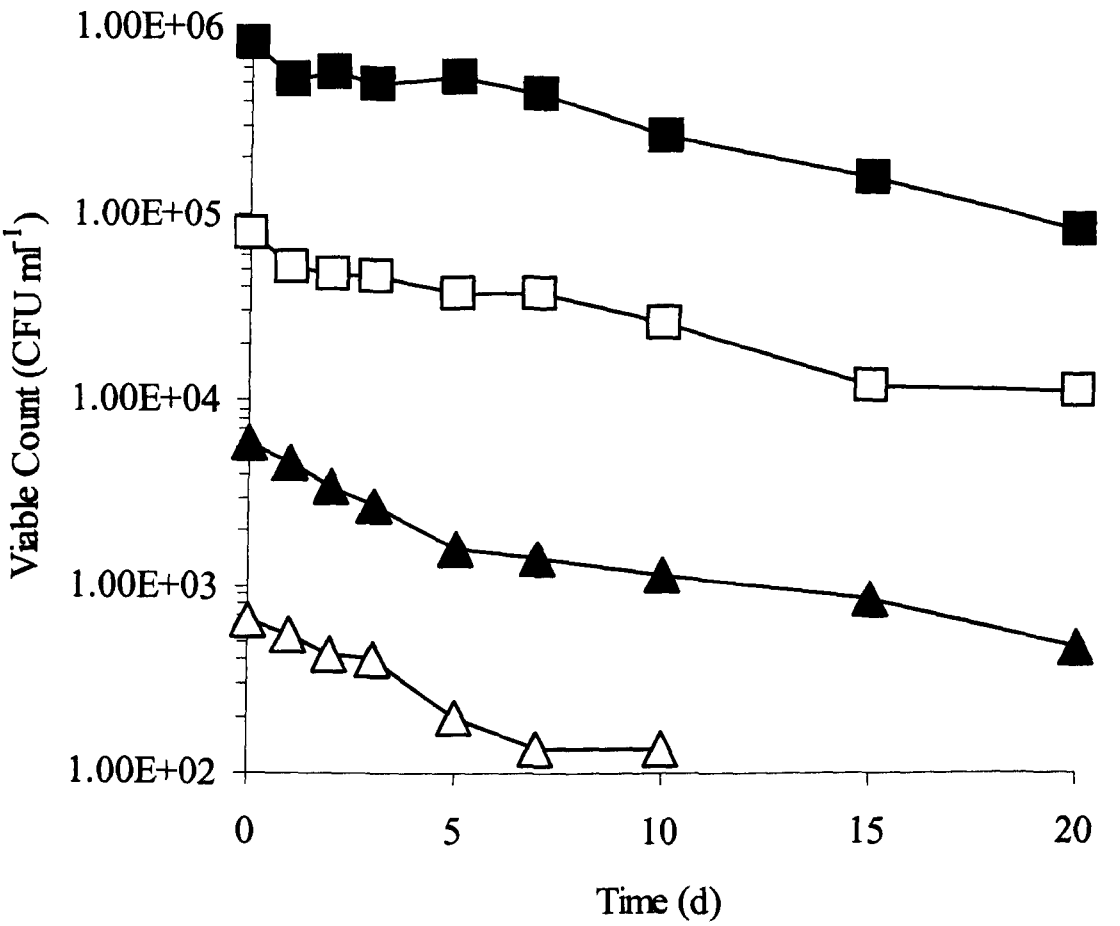


Figure 3.15
Starvation-survival kinetics of long-term multiple nutrient-starved cells (21 days, 25°C) after resuspension in water at 25°C, at a range of cell densities.

Cells from a 21 day-old, multiple nutrient-limited culture, incubated at 25°C, were washed, resuspended and diluted in sterile water. Cultures were then incubated at 25°C without shaking. Initial cell densities (CFU ml^{-1}) were approximately 8×10^5 (■), 7×10^4 (□), 5×10^3 (▲) and 6×10^2 (Δ). Data are an average of at least two separate experiments for which the standard deviation was no greater than ±40% of the mean value at equivalent time points.

Limit of detection (100 CFU ml^{-1}).

3.2.6 The effect of cell density and resuspension medium on starvation-survival of long term multiple nutrient-starved cells

In order to compare the characteristics of starvation-survival under multiple nutrient-starvation to that of glucose-starved cells, multiple nutrient limited cells were treated as in Chapter 2.9. If 7-day (37°C) multiple nutrient-starved cells are washed and transferred to water at 37°C, viability is lost within 7 days at all initial cell densities tested (5×10^5 to 3×10^2 CFU ml^{-1}) (Figure 3.14). In contrast, long-term multiple nutrient-starved cells (21 days at 25°C) remained viable in sterile water for at least 10 days at all initial cell densities tested (8×10^5 to 6×10^2 CFU ml^{-1}) when incubated at 25°C (Figure 3.15).

3.2.7 The role of cell wall biosynthesis in starvation-survival

The relative activity of penicillin G in causing cell death and lysis is known to be dependent on the rate of bacterial growth and division (Tuomanen *et al.*, 1986). As a result, actively dividing cells are sensitive to penicillin G, whereas stationary-phase cells that have ceased dividing are phenotypically tolerant. Growth inhibitory concentrations of penicillin G can therefore be used to determine whether starved cells are undergoing growth and division during starvation.

3.2.7.1 Effect of penicillin G on growth of *L. monocytogenes*

The minimum inhibitory concentration (MIC) of penicillin G for *L. monocytogenes* EGD in glucose-limited CDM was found to be approximately 1.0μg ml^{-1} (Figure 3.16), determined by the addition of penicillin G to EGD cultures to final concentrations of between 0.1 and 1.0 μg ml^{-1}. A small increase in A_{600} occurs even at growth inhibitory concentrations of penicillin G. An inhibitory concentration of 20μg ml^{-1} (20 × MIC) was subsequently chosen for the following experiments.

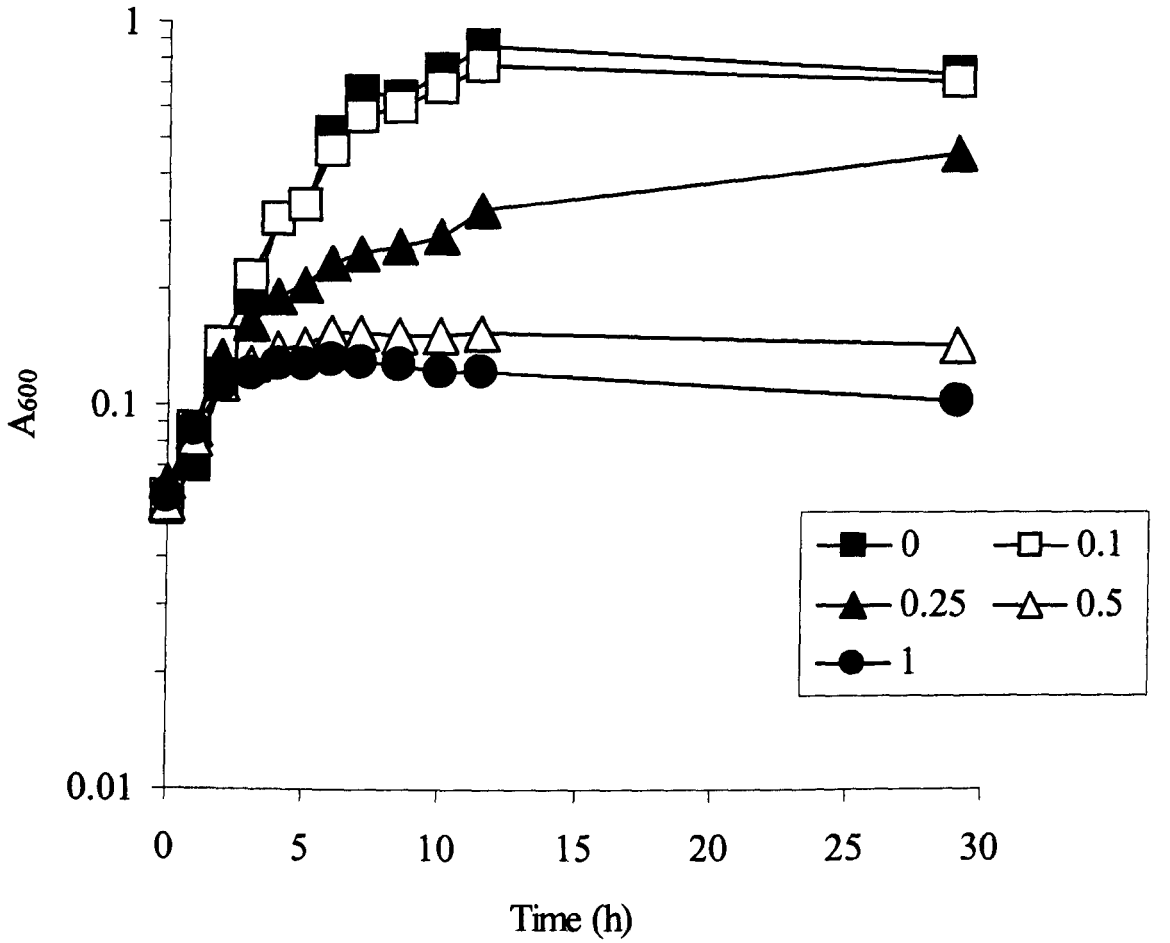


Figure 3.16
Growth kinetics in glucose-limiting CDM at 37°C, with or without addition of penicillin G.

Growth (A_{600}) was monitored after glucose limiting CDM was inoculated to A_{600} 0.05, and penicillin G added to final concentrations of 0 (■), 0.1(□), 0.25(▲), 0.5 (Δ) and 1.0 μg ml^{-1} (●). Data are representative of at least two separate experiments.

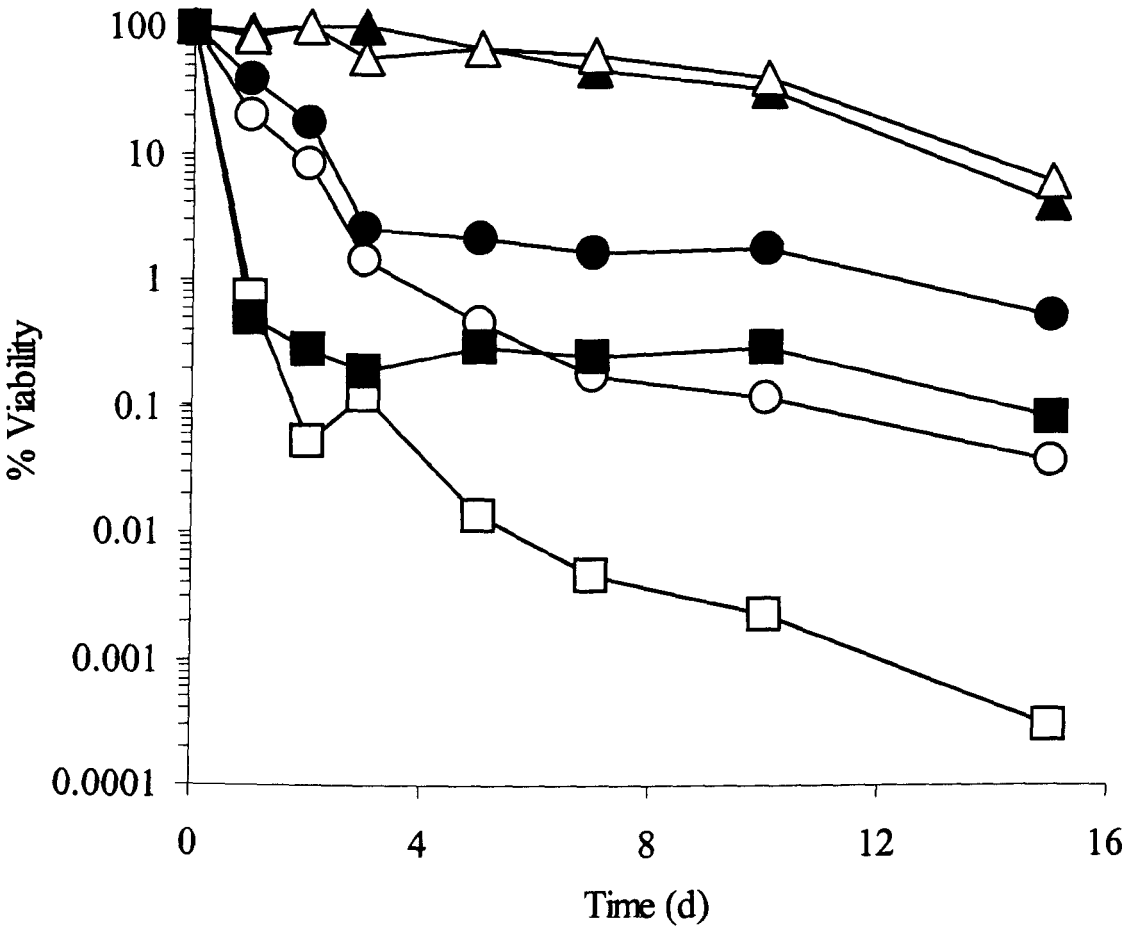


Figure 3.17
Starvation-survival kinetics of glucose-starved cells at 25°C and 37°C, and long-term glucose starved cells (7 days, 37°C) at 37°C with or without addition of penicillin G.

Penicillin G (20 μg ml^{-1}) was added to 6-hour post-exponential phase cultures and to 7 day-old glucose-limited cultures incubated at 37°C. The cultures were incubated without shaking (25°C, ○; 37°C, □; 7-day starved 37°C, Δ), and viability compared to their respective control cultures (●,■,▲). 100% viability was 1×10^9 CFU ml^{-1} (post-exponential) and 5×10^6 CFU ml^{-1} (7-day starved). Data are an average of at least two separate experiments for which the standard deviation was no greater than ±40% of the mean value at equivalent time points.

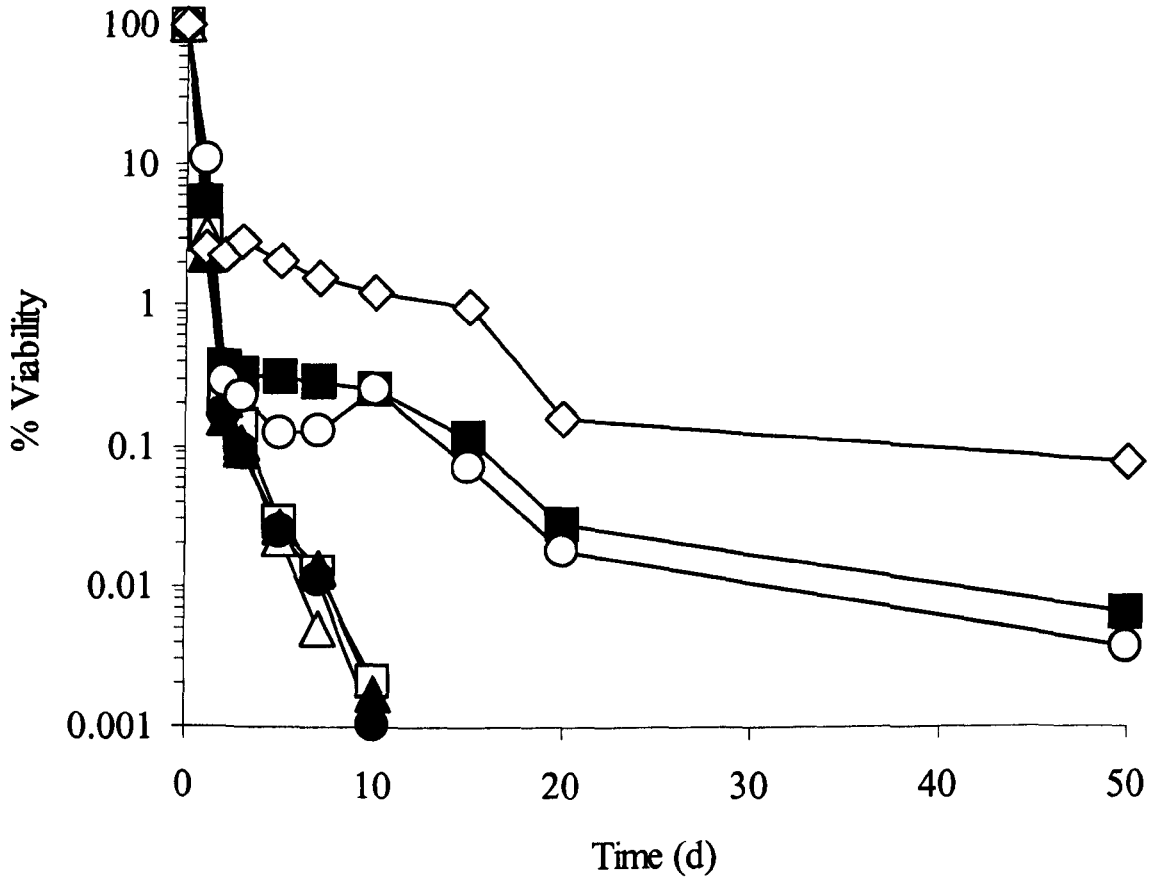


Figure 3.18
The effect of protein synthesis inhibition on the starvation-survival kinetics of glucose-starved cells at 37°C.

Chloramphenicol (100 μg ml^{-1}) was added at 0 (□), 1 (▲) 2 (Δ), 4 (●), 8 (○) and 24 hours (◇) after transfer of cells to glucose-free CDM. Viability was compared to control cultures (■). 100% viability was approximately 1×10^9 CFU ml^{-1} (0 to 8 h) and 1×10^8 CFU ml^{-1} (24 h). Data are an average of at least two separate experiments for which the standard deviation was no greater than ±40% of the mean value at equivalent time points.

Percentage viability is the percentage of cells surviving at a given time point relative to the initial culture viability (t = 0 hours).

3.2.7.2 The stability of penicillin G in long term starved cultures

The activity of 20 times the MIC penicillin G was monitored during incubation in starved cultures to ensure that sufficient penicillin G activity was retained throughout the course of the starvation experiments. Culture samples were taken at a number of time points, the cells removed by centrifugation and filter sterilisation, and 10 μl placed into a well cut into a freshly seeded lawn of *L. monocytogenes* EGD. Zones of clearing were measured after overnight growth at 37°C. Inhibition was not observed from a negative control of filter sterilised culture supernatant from a penicillin G free culture. Incubation at 37°C resulted in a 90 % loss of activity after 15 days. In relation to the starvation experiments (Chapter 3.2.8.3), penicillin G is still therefore growth inhibitory after 15 days.

3.2.7.3 The effect of growth inhibitory concentrations of penicillin G on starved cultures

In starved cultures to which penicillin G was added 6 hours into post-exponential-phase, incubation at 25 °C resulted in a gradual fall in viability,with only 2% of cells remaining viable after 3 days. During the same period, a rapid drop in viability (99.9 %) was observed. These initial changes were also observed in the corresponding untreated controls (Figure 3.17). Subsequent to this initial drop, the rate of decrease in cell viability for the untreated cultures ceased and viability was maintained at approximately 0.1 % (37°C) and 1.0 % (25°C) over the next 12 days. In contrast, after the initial fall in viability in penicillin G treated cultures, a further 40- to 400-fold decrease in viability occurred at 25°C and 37°C respectively over the ensuing 12 days (Figure 3.17).

The addition of penicillin G had no observable effect upon cells starved for glucose for 7 days at 37°C relative to the untreated controls (Figure 3.17). Both untreated and penicillin G treated cultures exhibited a slow gradual decrease in viability, with approximately 5 % of cells remaining viable after 15 days (Figure 3.17).

3.2.8 The role of protein synthesis in starvation-survival

To determine whether continued protein synthesis is necessary for long-term survival, the viability of glucose-limited cells was determined over time after treatment with 100 μg ml^{-1}

chloramphenicol – a protein synthesis inhibitor. To be able to determine how long into starvation protein synthesis is required to develop long-term starvation-survival potential, it was necessary to synchronise induction of the starvation-survival response. Mid-exponential-phase cells (A_{600} 0.3) were harvested and washed by centrifugation and resuspension. The cells were transferred into a glucose-free CDM, the culture incubated at 37°C and chloramphenicol added to 100 μg ml^{-1} to block protein synthesis at 0, 1, 2, 4, 8 and 24 hours after transfer.

After induction of starvation-survival by glucose limitation, a 99 % drop in viability occurred in the untreated control culture within 3 days. Viability then fell slowly, with approximately 0.01 % of cells remaining viable after 50 days (Figure 3.18). In comparison, the inhibition of protein synthesis up to 4 hours after the initiation of starvation resulted in all viability being lost after 10 days (Figure 3.18). When chloramphenicol was added 8 hours after transfer to glucose-free CDM, approximately 0.1 % of cells remained viable after 50 days (Figure 3.18). The kinetics of starvation-survival observed in this culture was comparable to in the untreated control. When chloramphenicol was added after 24 hours, an initial 50-fold fall in viability in the first 48 hours was followed by a slow rate of decline up to 20 days incubation, before viability stabilised at between 0.2 % and 0.3 % (Figure 3.18). The kinetics of survival paralleled that of the control culture, though it should be noted that by the time chloramphenicol was added, viability had already fallen by 10-fold. The results suggest that long-term starvation-survival ceases to be dependent upon continued protein synthesis beyond the first 8 hours of starvation.

3.2.9 The effect of starvation on cell morphology

A change in the morphology of non-sporing bacterial cells in response to nutrient limitation is a well-known occurrence (Watson *et al.*, 1998a; Kjelleberg *et al.*, 1993, Kolter *et al.*, 1993). Here, light and electron microscopy were used to study glucose- and amino acid-starved cells incubated at 25 and 37°C in comparison to exponential-phase cells.

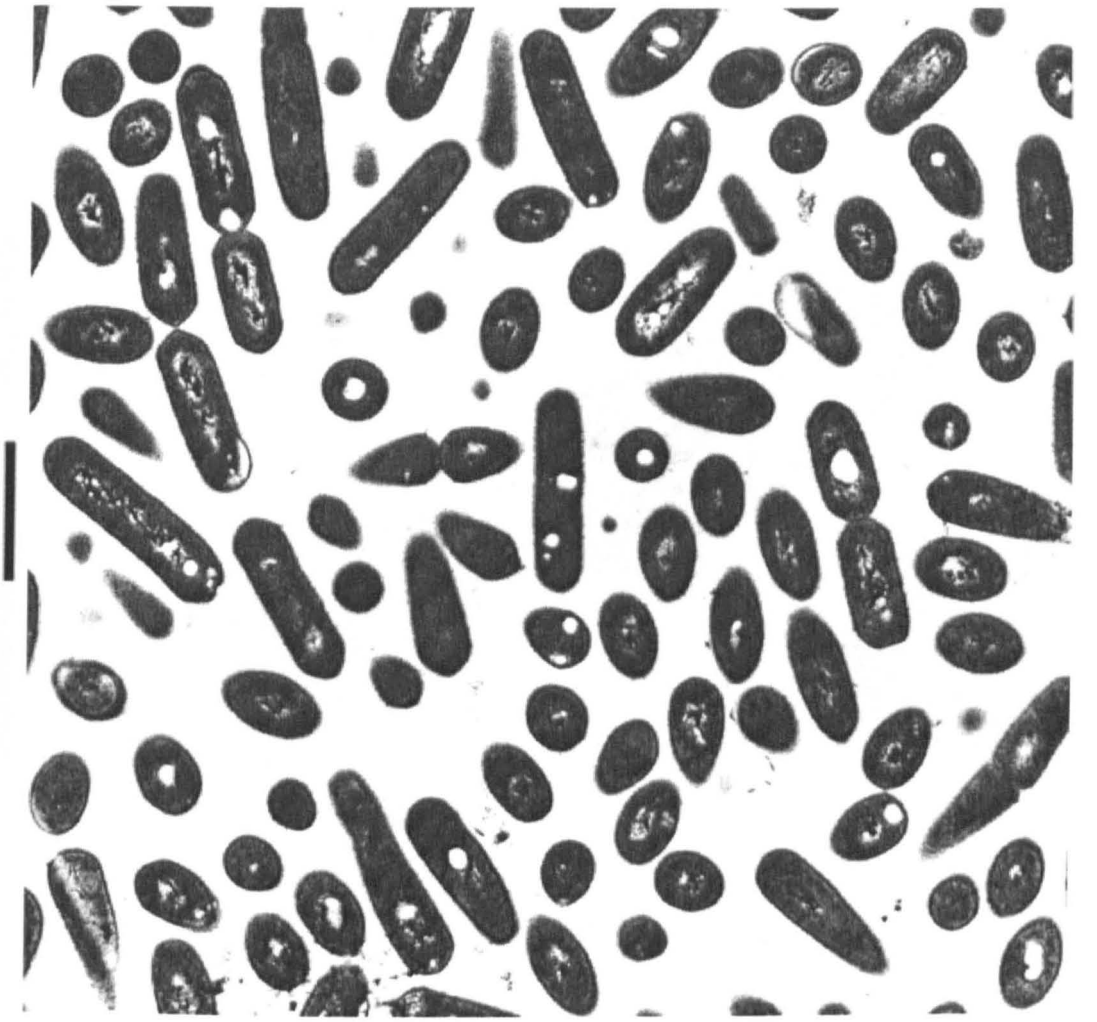


Figure 3.19
Transmission electron micrograph of mid-exponential phase cells at 37°C. Bar, 1 μm.

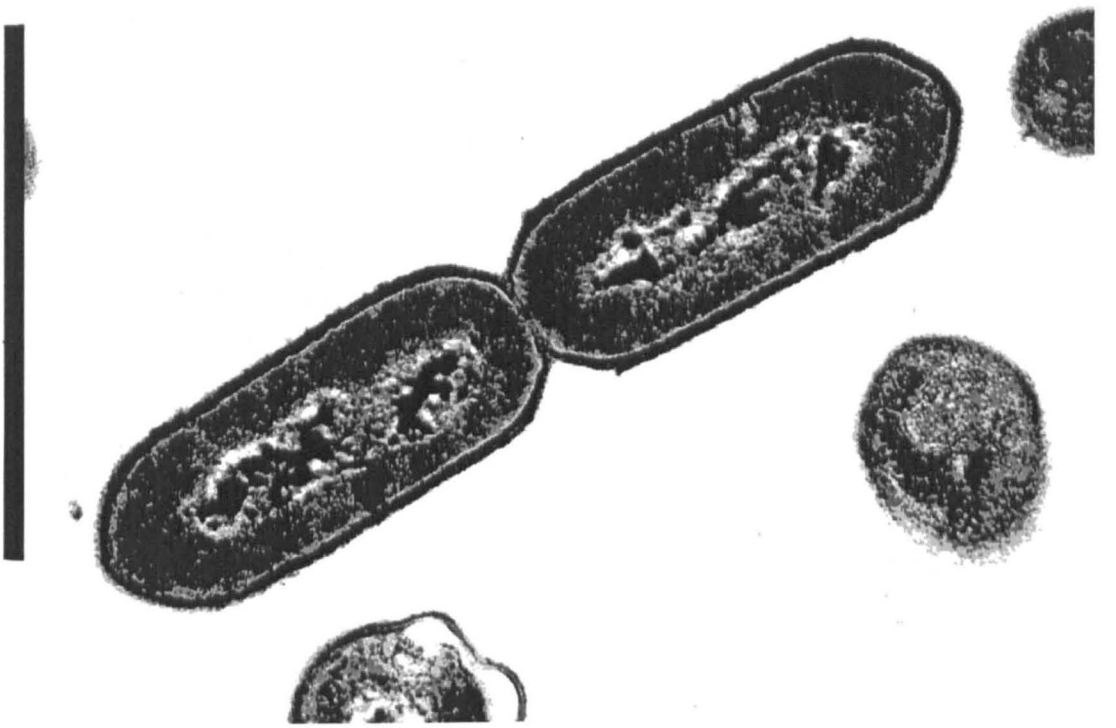


Figure 3.20
Transmission electron micrograph of mid-exponential phase cells at 37°C. Bar, 1 μm.

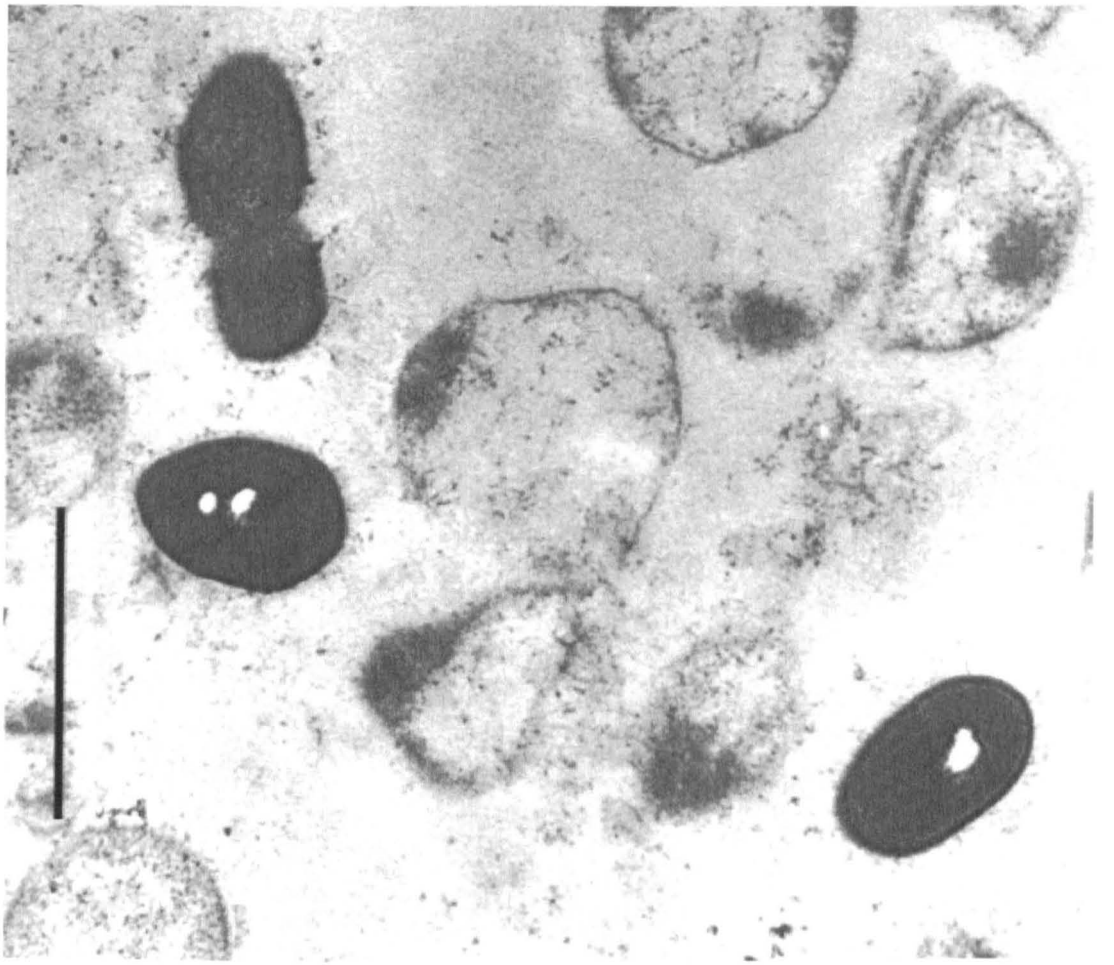


Figure 3.21
Transmission electron micrograph of cells starved for glucose for 7 days at 37°C. Bar, 1 μm.

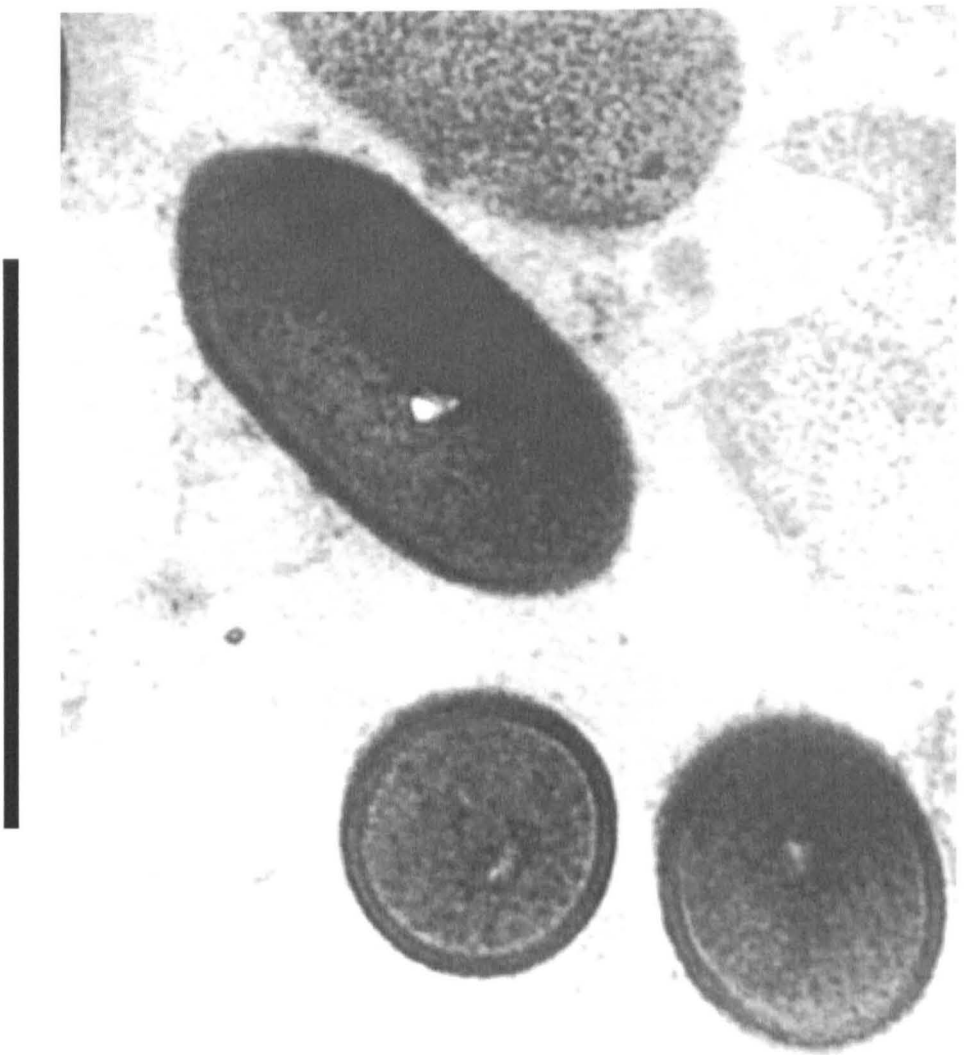


Figure 3.22
Transmission electron micrograph of cells starved for glucose for 20 days at 25°C. Bar, 1 μm.

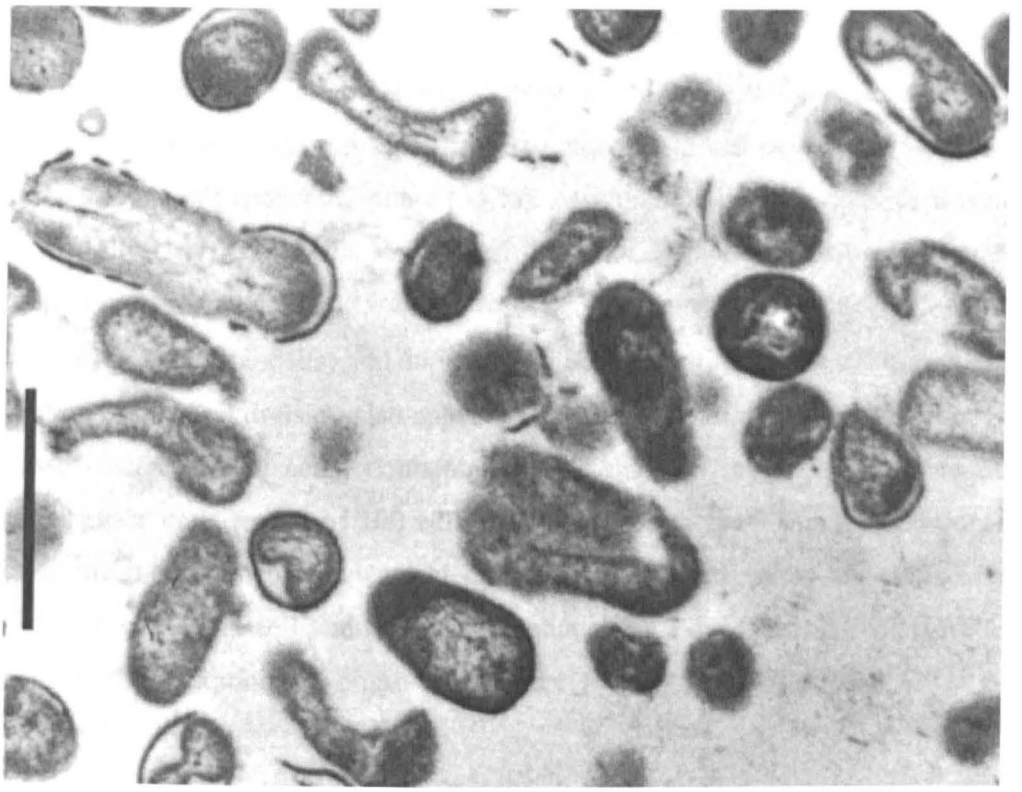


Figure 3.23
Transmission electron micrograph of cells starved for amino acids for 7 days at 25°C Bar, 1 μm.

Via electron microscopy it was determined that the average cell dimensions (± S.D.) for exponential-phase *L. monocytogenes* EGD cells were 1.29 μm ± 0.29μm in length by 0.41 μm ± 0.03μm in width, with 30 % of cells possessing partial division septa (Figures 3.19 & 3.20). In glucose limited cultures incubated for 7 days at 37°C, the mean cell length was reduced to 1.00μm ± 0.26μm (mean of 60 cells) compared to cells in exponential growth, whilst the mean cell width had increased slightly to 0.46μm ± 0.03μm (mean of 60 cells). The occurrence of partial division septa in 7-day glucose-starved cells (16.7 %) was seen to fall relative to cells in exponential-phase (30 %). Also notable in these cultures was the large number of 'ghost' cells present, indicating that cell lysis had occurred (Figure 3.21). Incubation of cells for 20 days at 25°C also resulted in a reduction in mean cell length to 1.09 μm ± 0.25μm (mean of 60 cells) and an increase in cell width to 0.45 ± 0.05μm (mean of 60 cells) (Figure 3.22). Cell division also appeared to be reduced, with only 7 % of cells starved for glucose (mean of 100 cells) containing partial division septa compared to 30 % in exponential phase cells (mean of 100 cells). In all stages of growth and starvation, a large variation in mean cell dimensions was noted. Despite this heterogeneity, the changes in mean values for cell length and cell width in cells incubated for 7 days at 37°C and for 20 days at 25°C compared to exponential-phase cells, were found to be significant ($P < 0.01$ %; student's *t* test).

Cultures incubated for 7 days at 25°C were chosen to analyse the morphology of amino acid limited cells, as significant viability (1×10^5 CFU ml^{-1}) was still detectable at this stage. Study of amino acid-limited cultures by electron microscopy revealed some likely viable cells, and electron-dense, fragmented cell wall material (Figure 3.23).

3.2.10 The effect of glucose-starvation on stress resistance

Studies in several bacteria have shown the development of resistance to a broad-spectrum of stresses (i.e. low pH, u.v.-radiation, heat and oxidative stress) during adaptation to starvation conditions (Watson *et al.*, 1998a; Kjelleberg *et al.*, 1993, Kolter *et al.*, 1993; Foster and Spector, 1995). To study the development of starvation-associated stress resistance, the stress resistance of cells taken from glucose limited cultures of different ages was tested during

exposure to pH 3.5, 7.5mM H_2O_2, or upon incubation at 55°C. All cultures were initially approximately 5×10^6 CFU ml^{-1}.

When exponential-phase cells were incubated in CDM adjusted to pH 3.5 at 37°C, a slight drop in viability over the first 15 minutes was followed by an increased rate of fall, resulting in viability being undetectable after 60 minutes (>10,000-fold reduction) (Figure 3.24). In comparison, 6-hour post-exponential phase cells were more resistant to acid stress, with only a 500-fold drop in viability over 90 minutes. Cells starved for glucose for 7 days were more resistant than both exponential- (2,250-fold more resistant after 60 minutes) and 6-hour post-exponential-phase cells (30-fold more resistant after 60 minutes), as approximately 38 % of cells remained viable after 90 minutes (Figure 3.24).

Cells from exponential phase, 6-hour post-exponential phase and 7-day glucose starved cultures were incubated in PBS at 55°C. Exposure of exponential phase cells to heat stress resulted in a 9,000-fold reduction in viability by 12 minutes, beyond which viability became undetectable (Figure 3.25). Post-exponential phase cells showed a 35,000-fold fall in resistance over 18 minutes. Long-term glucose starved cells were more resistant to heat stress than both exponential- and post-exponential-phase cells, with 2.8 % of initial viability retained after 18 minutes (Figure 3.25).

The incubation of exponential phase cells in PBS containing 7.5mM H_2O_2 resulted in viability becoming undetectable after 20 minutes (Figure 3.26). A transient increase in resistance to oxidative stress was found in post-exponential phase cells, with approximately 9% of cells remaining viable after 50 minutes. The H_2O_2 resistance of 7-day glucose-starved cells was greater than for exponential-phase cells, but they still exhibited a 650-fold reduction in viability over 50 minutes (Figure 3.26). Interestingly, culture supernatant appeared to confer a protective effect on cells exposed to oxidative stress. After 50 minutes, exponential phase cells incubated in culture supernatant with 7.5mM H_2O_2 retained 6.4 % viability, whilst post-exponential and 7-day glucose-starved cells were totally resistant (Figure 3.27).

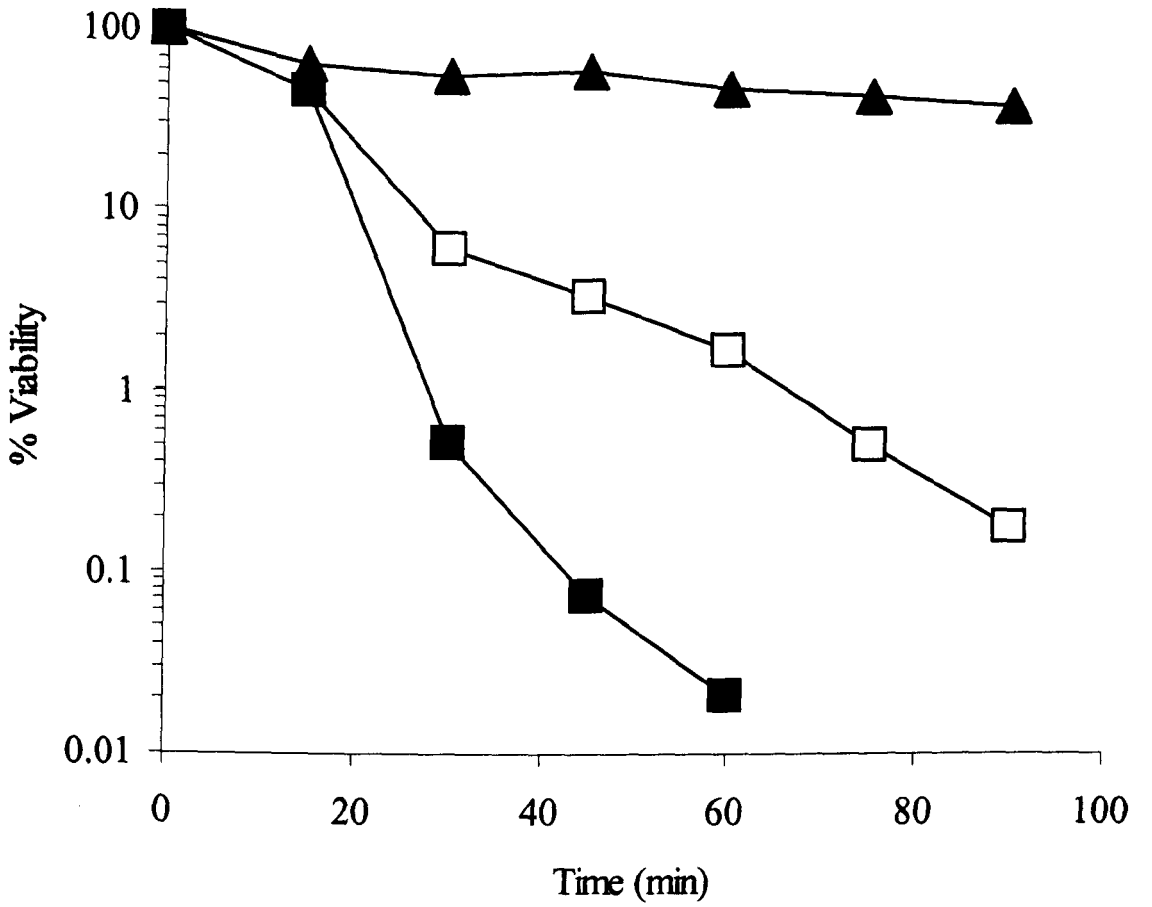


Figure 3.24
Effect of glucose starvation on resistance to acid stress (pH 3.5) in CDM at 37°C.

Exponential (■), post-exponential (□), 7-day glucose starved (▲). 100% viability was approximately 5×10^6 CFU ml^{-1}. Data are an average of at least two separate experiments for which the standard deviation was no greater than ±40% of the mean value at equivalent time points.

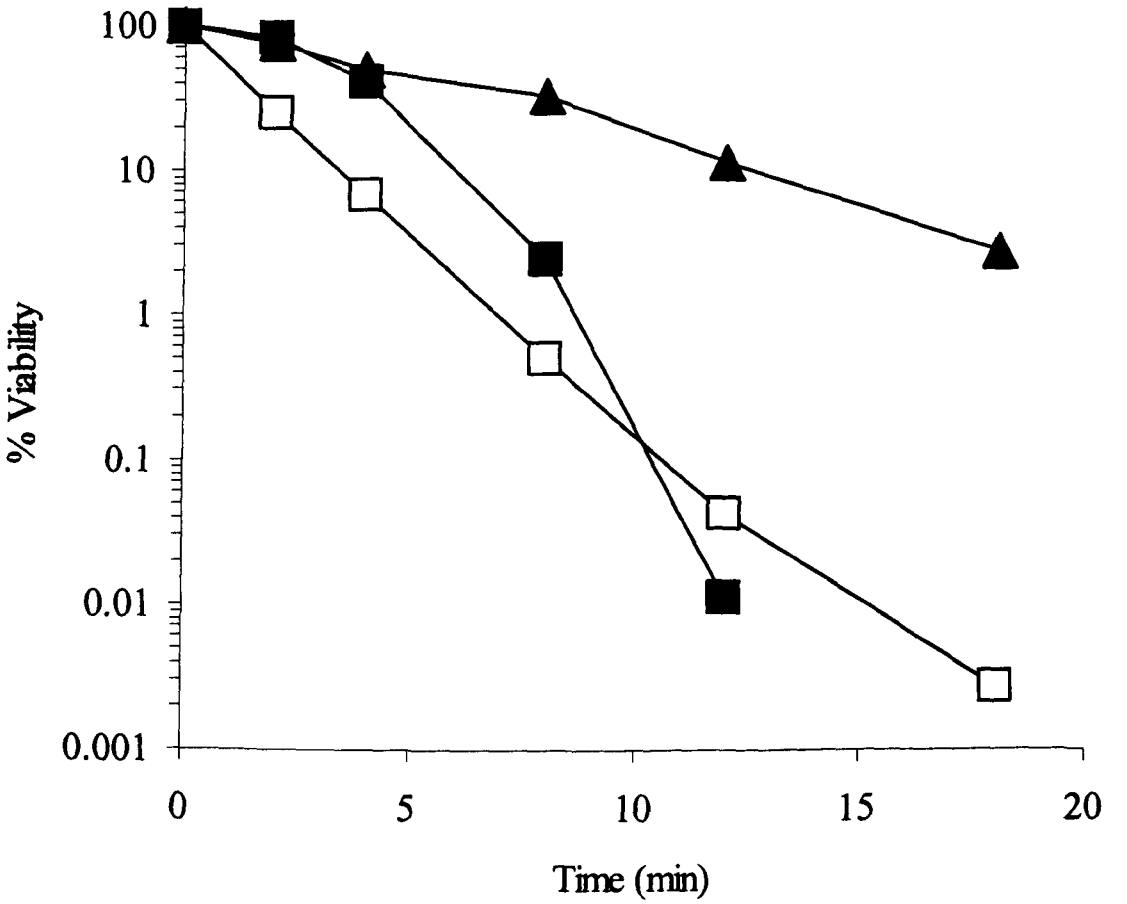


Figure 3.25
Heat resistance (55°C) of *L. monocytogenes* at different phases of growth in PBS.

Exponential (■), post-exponential (□), 7-day glucose starved (▲). 100% viability was approximately 5×10^6 CFU ml^{-1}. Data are an average of at least two separate experiments for which the standard deviation was no greater than ±40% of the mean value at equivalent time points.

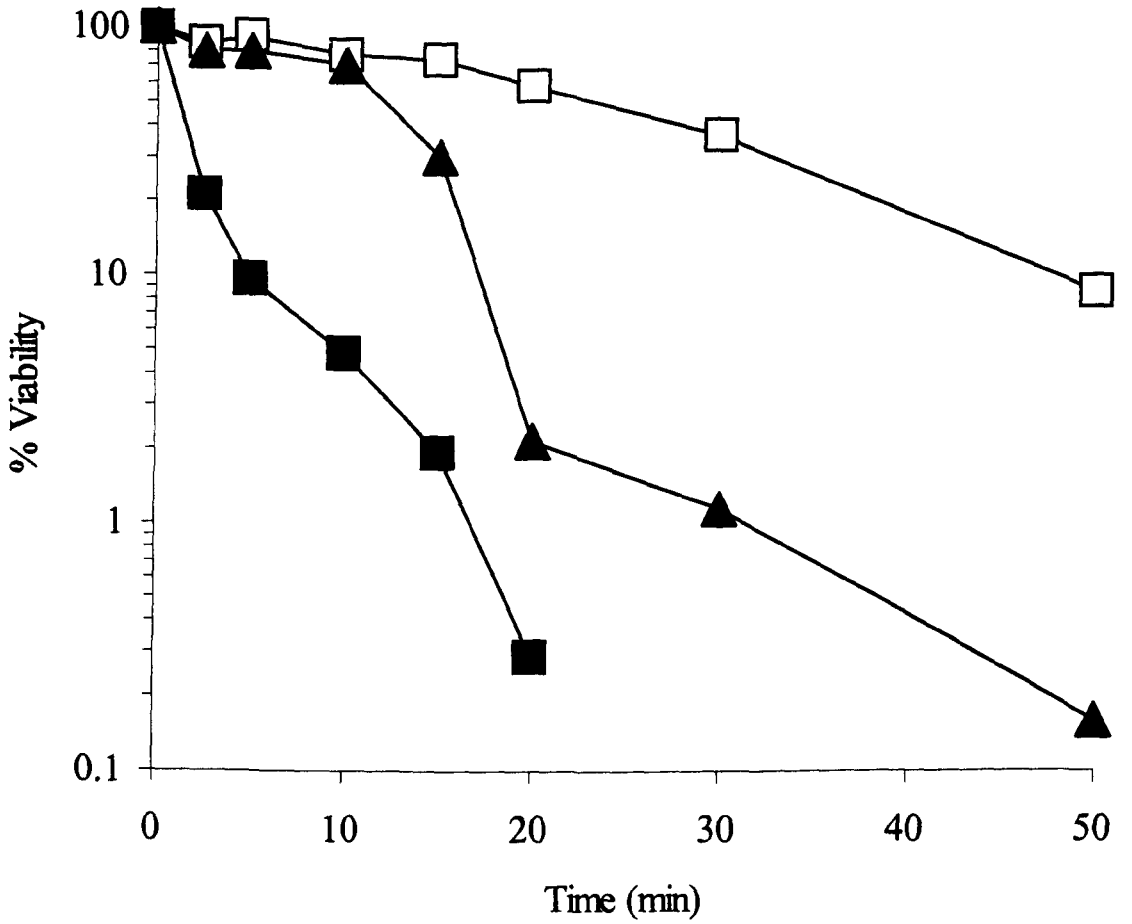


Figure 3.26
Effect of growth phase on resistance to H_2O_2 stress (7.5mM) in PBS at 37°C.

Exponential (■), post-exponential (□), 7-day glucose starved (▲) cells. 100% viability was approximately 5×10^6 CFU ml^{-1}. Data are an average of at least two separate experiments for which the standard deviation was no greater than ±40% of the mean value at equivalent time points.

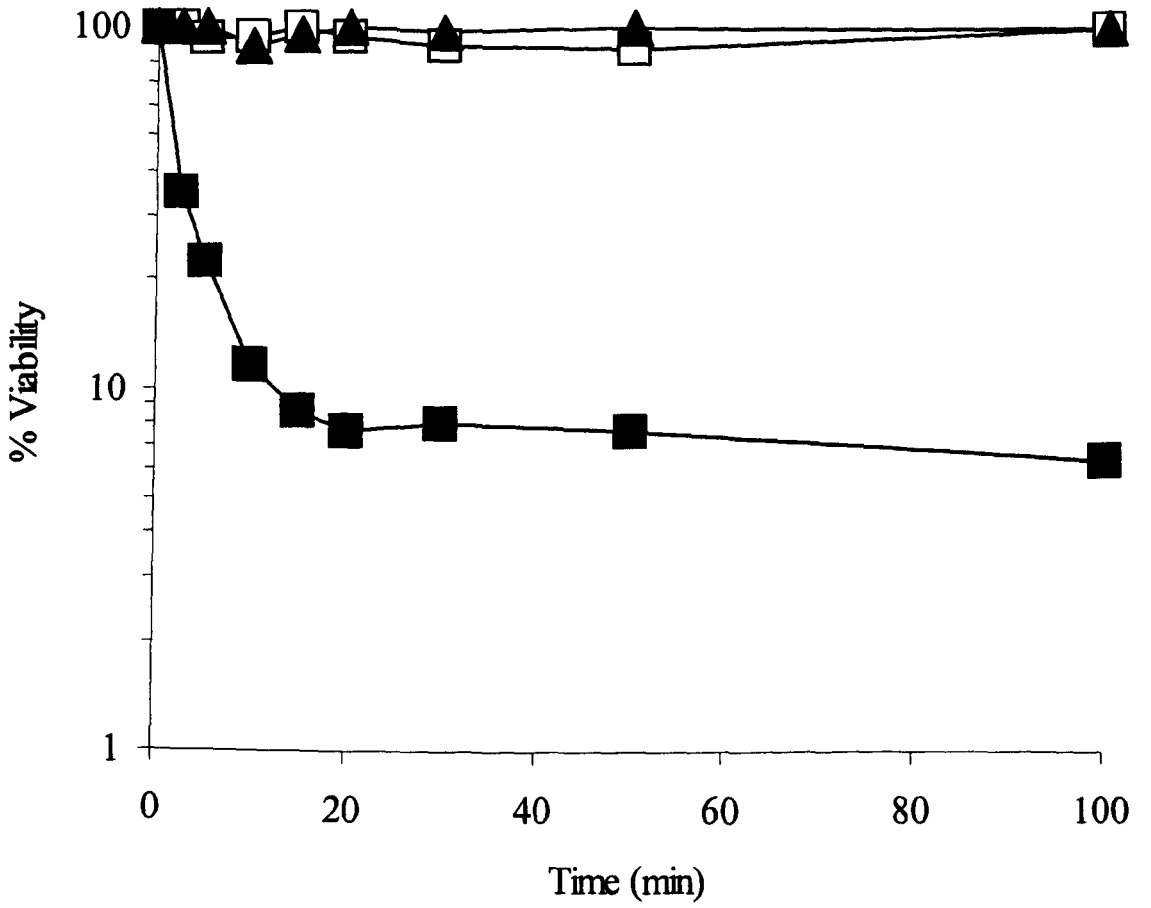


Figure 3.27
Effect of growth phase on resistance to H_2O_2 stress (7.5mM) in culture supernatant at 37°C.

Exponential (■), post-exponential (□), 7-day glucose starved (▲). 100% viability was approximately 5×10^6 CFU ml^{-1}. Data are an average of at least two separate experiments for which the standard deviation was no greater than ±40% of the mean value at equivalent time points.

3.3 Discussion

In the chemically defined medium described here, *L. monocytogenes* develops a starvation-survival response (SSR) in reaction to glucose or multiple-nutrient limitation, enabling maintenance of viability for prolonged periods. The specificity of the generation of SSR via carbon or multiple-nutrient limitation is analogous to the situation in *V. sp.* and *S. aureus* (Nyström *et al.*, 1992; Watson *et al.*, 1998a). This situation is indicative of depletion in the cellular ATP pool by glucose starvation being the primary signal for the development of the starvation-survival response. In *E. coli* and *S. typhimurium* however, a response is induced regardless of the type of limiting nutrient (Matin *et al.*, 1989; Seymour *et al.*, 1996). In Gram-negatives, *rpoS* expression can occur in a ppGpp-dependent fashion (Gentry *et al.*, 1993). ppGpp governs stringent control of gene expression in response to amino acid starvation (Cashel *et al.*, 1996), and the absence of σ^S in Gram-positive bacteria could signify a break in this regulatory chain, thus accounting for the lack of a response to amino acid starvation.

L. monocytogenes is able to maintain approximately 0.1 % to 1 % viability during long-term starvation, and exhibits survival kinetics similar to these have been seen with multiple nutrient limited *L. monocytogenes* Scott A cells over the first 48 hours when stored in phosphate buffer (Lou and Yousef, 1996). It is proposed that this phenomenon in *S. aureus*, *E. coli* and *S. typhimurium* (Watson *et al.*, 1998a; Siegele *et al.*, 1993; Spector and Cubitt, 1992) is the result of cryptic growth – the maintenance of a small population of cells via the release of utilisable nutrients from the death and lysis of the majority. The deleterious effect of penicillin G was relieved after glucose starvation for 7 days, indicating that cell division is greatly reduced or not occurring. In contrast, the addition of cell wall biosynthesis inhibitors to starved *S. aureus* and *V. vulnificus* cultures caused a gradual loss in viability (Watson *et al.*, 1998a; Oliver, 1993). The occurrence of low numbers of long-term starved cells with partial division septa (17 %) points toward continued cell division at a basal level. It would seem therefore, that growth and division in long-term starved *L. monocytogenes* populations might still occur, but at a significantly lower level than found elsewhere. Also, the possibility of the change being due to phenotypic tolerance to penicillin G in long-term starved *L. monocytogenes* cells cannot be discounted.

L. monocytogenes most likely undergoes reductive division during starvation accounting for the occurrence of the shortened cells observed under electron microscopy. A number of species undergo morphological changes during prolonged glucose starvation. In *E. coli*, the starvation-induced reduction in cell length is governed by the σ^S-regulated *bolA* gene (Aldea *et al.*, 1988; Lange and Hengge-Aronis, 1991a). A reduction in cellular dimensions is also witnessed in *S. aureus* and *M. luteus* (Watson *et al.*, 1998a; Mukamolova *et al.*, 1995), although the molecular basis in Gram-positive bacteria is still unknown. Interestingly, *L. monocytogenes* cells shaped similarly to those in glucose starvation have been observed in infected tissue samples (Armstrong, 1995), suggesting a potential link between listerial infection and nutrient limitation.

Starvation-survival in water is density dependent at 25°C and 37°C. Inoculation at 2×10^8 CFU ml^{-1} or below resulted in a total loss of viability within 3 days at 37°C, whilst at 25°C viability is lost at an initial cell density of 6×10^5 CFU ml^{-1} or below. A temperature dependent effect is also observed, as survival at 25°C is possible at 100-fold lower initial cell densities than at 37°C. The survival of cultures at higher cell densities after an initial 1- to 2-log fall would comply with the hypothesis of cryptic growth as liberation of nutrients from lysing cells enables the survival of a small proportion of the population (Roszak and Colwell, 1987). Starvation at different cell densities of post-exponential phase- and 7-day glucose starved-cells cells in filter-sterilised spent culture supernatant at 25°C and 37°C was density independent, resulting in viability of between 2×10^7 CFU ml^{-1} and 5×10^5 CFU ml^{-1} after 20 days. Notably, cultures inoculated at lower cell densities saw regrowth to these levels, implying that a significant level of nutrients, capable of sustaining a consistent number of bacteria, is still available within the culture fluid. Little difference was observed for survival in water between long-term glucose- and multiple-nutrient-starved cells, supporting evidence that both stimuli elicit similar responses. The survival of long-term glucose- and multiple nutrient-starved cells upon resuspension in water was also temperature dependent, with detectable viability after 20 days for initial cell densities as low as 1×10^4 CFU ml^{-1} at 25°C. Comparison of long-term glucose- and multiple nutrient-starved cells to post-exponential cells when resuspended in water, points to an increase in starvation-survival potential beyond the 6-hour post-exponential period. Viability was still detectable after 20 days at levels 1000-

fold lower than the minimum required for long-term survival in post-exponential phase cells. Thus long-term starvation results in the attainment of a cellular status with increased survival potential.

In *L. monocytogenes*, starvation-survival is dependent on gross protein synthesis, which correlates with the findings in *E. coli* and *S. aureus* (Reeve *et al.*, 1984; Watson *et al.*, 1998a) as the cells synthesise novel proteins in response to starvation conditions. The production of at least four classes of temporally expressed proteins is found in the Gram-negative bacteria, with continued synthesis required for long-term survival (Morton and Oliver, 1994; Spector, 1998). Interestingly, it appears that in *L. monocytogenes*, long-term survival is not dependent upon continued protein synthesis, as the addition of chloramphenicol after 24 hours starvation has no effect on viability. This raises the prospect that the cells may totally suspend their metabolic activity during starvation, rather than just down-regulation as observed in other non-sporing bacteria. Whether protein synthesis does still occur at a very low level requires further investigation, and the development of phenotypic tolerance in the starved cells cannot be ruled out.

An increase in cross-resistance to several stresses has been found to occur as cells become starved (Jenkins *et al.*, 1988; Seymour *et al.*, 1998; Watson *et al.*, 1998a), and several differentially expressed proteins have been found to either regulate or confer resistance to environmental stresses (Matin *et al.*, 1993; Seymour *et al.*, 1996). *L. monocytogenes* EGD also shows a starvation associated increase in resistance to environmental stress, along with increased tolerance to heat and acid stresses upon exposure to sublethal stress (Kroll and Patchett, 1992; Lou and Yousef, 1996; Patchett *et al.*, 1996). The use of low pH has long been a means of preserving foodstuffs and restricting the growth of pathogens, as resistance to acid stress, in particular adaptive acid tolerance, has been shown to be a key factor in the increased survival of *L. monocytogenes* in fermented foods. Gahan *et al.* (1996) noted over a 10,000 increase in survival when cells were acid adapted before incubation in natural yoghurt (pH 3.9). Oxidative damage is a major contributor to cell death and ageing in starved cultures (Dukan and Nyström, 1998; Nyström, 1999), and may also occur as a result of treatment with acid (Clements and Foster, 1999). Resistance to H_2O_2 is primarily mediated by an

extracellular catalase, which is produced throughout growth (Naclerio *et al.*, 1995; Watson *et al.*, 1998a). The effect of culture supernatant from aged cultures in increasing the apparent resistance of *L. monocytogenes* EGD cells to H_2O_2 is most likely via the protective action of a catalase secreted into the medium, an effect also seen in both *S. aureus* and *B. subtilis* (Naclerio *et al.*, 1995; Watson *et al.*, 1998a).

The increase in heat resistance during starvation supports previous findings on the effect of stress on thermotolerance (Lou and Yousef, 1996). It is known that exposure of *L. monocytogenes* to heat shock increases the expression of at least 14 proteins including GroEL and DnaK (Hanawa *et al.*, 1995). *B. subtilis* is known to possess at least 4 distinct classes of heat-induced proteins regulated by distinct mechanisms: Class I (HrcA repressor-dependent), Class II (σ^B-dependent), Class III (CtsR-dependent) and Class IV (whose mode of regulation is unknown) (Derré *et al.*, 1999). Studies to date provide evidence that *L. monocytogenes* possesses the elements of at least 3 of the classes found in *B. subtilis* (Derré *et al.*, 1999; O'Driscoll, *et al.*, 1996; Wiedmann *et al.*, 1998).

In summary, glucose- or multiple nutrient starvation of *L. monocytogenes* results in the development of a starvation-survival response. This phenomenon is characterised by long-term survival potential, a change in morphology and increased general stress resistance. Long-term survival is density dependent, and protein synthesis is essential for up to 8 hours into starvation. If one is to understand the molecular basis of this response, then it is important to identify components with a role in the process. In Chapter 4, the potential role of two known gene regulators in starvation-survival was investigated.

CHAPTER 4

THE ROLE OF THE GENE REGULATORS σ^B AND PrfA IN STARVATION-SURVIVAL AND STRESS RESISTANCE

4.1 Introduction

Differential gene expression is required for the starvation-survival response and the acquisition of stress resistance. Coordinate regulation of the expression of large numbers of genes allows full survival potential to be realised. In *L. monocytogenes*, continued protein synthesis is necessary for entering the starvation-survival state.

In many Gram-negative bacteria, RpoS (σ^S) is the principal regulator of starvation-associated stress resistance development. However, σ^S is absent from Gram-positive bacteria. In the absence of σ^S, the alternative sigma factor SigB (σ^B) found in several Gram-positive species shows some functional overlap with σ^S. Notably, the regulons for both σ^S and σ^B share a number of genes encoding for proteins involved in protection from both oxidative and osmotic stresses (Engelmann *et al.*, 1995; Antelmann *et al.*, 1997a; vonBlohn *et al.*, 1997; Völker *et al.*, 1998). σ^B is the general stress response regulator in *B. subtilis* (Völker *et al.*, 1999), controlling a regulon of over 50 genes (Antelmann *et al.*, 1997b; Bernhardt *et al.*, 1997). Activity and expression of σ^B is regulated in response to both stresses (i.e. ethanol, heat or salt) and extracellular signals such as glucose-, oxygen- and phosphate-starvation (reviewed in Hecker and Völker, 1998). In *S. aureus*, σ^B is principally expressed in stationary phase, with roles in acid and hydrogen peroxide resistance, and heat shock recovery (Chan *et al.*, 1998). It would appear however that the σ^B operon in *S. aureus* lacks the module controlling expression in response to physical stresses (Kullik *et al.*, 1997). This was partially reinforced by the work of Chan *et al.* (1998), who failed to identify any role in resistance to ethanol or osmotic shock. Also, σ^B in *S. aureus* was shown not to have a major role in starvation-survival (Chan *et al.*, 1998).

In *L. monocytogenes*, the *sigB* operon is found in the same *rsbU-rsbV-rsbW-sigB-rsbX* gene alignment as seen in *B. subtilis* (Wiedmann *et al.*, 1998). To date, the *sigB* operon regulatory components RsbR, RsbS and RsbT, which govern σ^B expression and activity in response to stationary-phase and stress signals in *B. subtilis* have not been located. Circumstantial evidence of their existence has been obtained through the detection of σ^B transcripts in response to increased temperature and osmolarity, and entry to stationary phase (Becker *et al.*, 1998). While σ^B has been found the regulate osmotolerance in *L. monocytogenes* (Sleator *et al.*, 1999), previous work has not attempted to define a role for σ^B in starvation-survival.

It is well known that the expression of virulence genes in bacteria changes in response to a variety of environmental signals. Evidence points to the control of a number of bacterial virulence genes by stress response regulators such as the σ^S/Crp-dependent *spv* expression in *Salmonella* (reviewed in Spector *et al.*, 1998). In *S. aureus*, σ^B has been shown to partially control Sar, a global regulator involved in virulence gene expression (Deora *et al.*, 1997) and the expression of several virulence determinants (Kullik *et al.*, 1998), whilst also regulating stress resistance (Chan *et al.*, 1998).

In *L. monocytogenes*, work has uncovered evidence that may signify a strong link between the regulation of both virulence gene and basic cellular physiology. Notably, the virulence gene regulator PrfA bears significant homology to the global regulators Crp and Fnr, which govern catabolite repression and the metabolic response to oxygen levels respectively (Lampidis *et al.*, 1994). The control of virulence gene expression by PrfA (in association with an unknown cofactor) in response to environmental signals (i.e. temperature, growth phase, iron concentration and pH) has also been well documented (Leimeister-Wächter *et al.*, 1992; Mengaud *et al.*, 1991; Böckmann *et al.*, 1996; Behari and Youngman, 1998b). A probable role for PrfA in regulating other cellular functions has been reinforced by the discovery that PrfA negatively regulates ClpC expression (Ripio *et al.*, 1998). ClpC, a member of Clp ATPase stress protein family, is known to have a dual role in stress resistance and virulence. *clpC* mutants are sensitive to low iron concentration, osmotic stress, exposure to H_2O_2 and have reduced virulence *in vivo* (Rouquette *et al.*, 1996).

Recently, the presence of β-glucosides such as cellobiose was found to repress virulence gene expression (Park and Kroll, 1993). This is presumably a signal that the bacteria are in a "soil" environment and so virulence gene expression is no longer required. The effect has been partially attributed to the *bvr* locus, which encodes a sensor that mediates repression in the presence of cellobiose and salicin (Brehm *et al.*, 1999). It has been proposed that a catabolite repression mechanism may govern the down-regulation of these genes by other fermentable carbohydrates. It is therefore logical to consider the possibility that virulence determinant regulators such as PrfA could either directly or indirectly affect the response to starvation and stress conditions.

This chapter describes the characterisation of the role of σ^B and PrfA in the starvation-survival response and associated stress resistance properties.

4.2 Results

4.2.1 Transduction of the *sigB* null mutation into *L. monocytogenes* EGD

In order to assess the role of σ^B in starvation-survival and associated stress resistance, it was necessary to transduce the null mutation from LMA2B (parental strain 10403S) into the wild type *L. monocytogenes* EGD, to create the strain designated DES011 (*sigB*).

Phage ΦLMUP35 was propagated on LMA2B and the resulting phage lysates used to infect an EGD recipient. Kanamycin resistant transductants were selected as previously described in Chapter 2.15.3. Transduction of the null mutation into the wild type EGD background was confirmed by selection of kanamycin resistant transductants. The insertion of the 1.5 kbp kanamycin resistance determinant was confirmed by the hybridisation of *Bgl*II and *Cla*II digested chromosomal DNA with a probe derived from the *sigB* gene (Figure 4.1A).

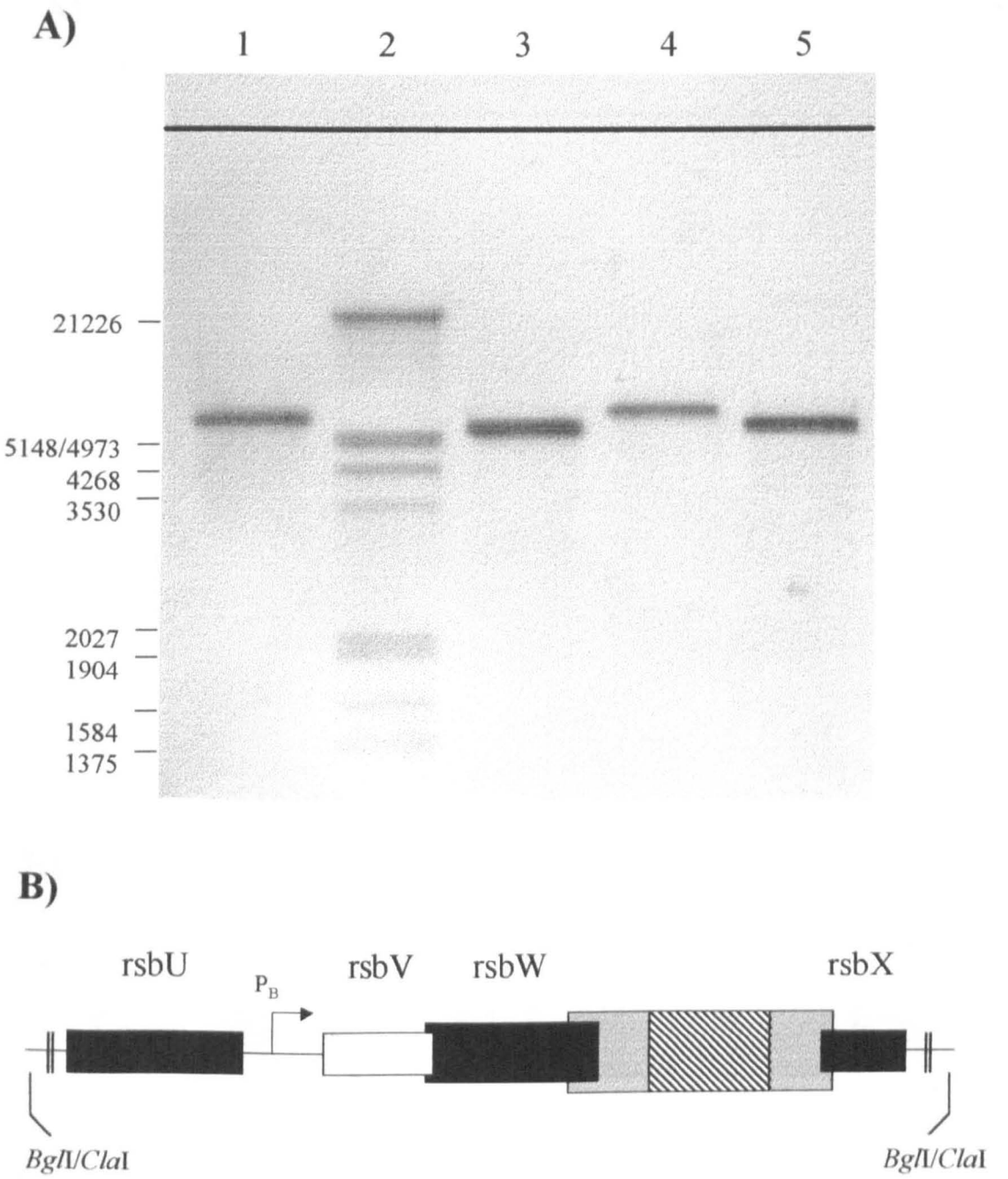


Figure 4.1

A) Southern blot of chromosomal DNA (*Bgl*II/*Cla*I digests) probed with 685 bp fragment of the *sigB* gene. Lanes: 1. DES011 *Cla*I; 2, λ DNA *Eco*RI/*Hind*III digest; 3, EGD *Cla*I; 4, DES011 *Bgl*II; 5, EGD *Bgl*II.
λ DNA *Eco*RI/*Hind*III digest fragment sizes (Top to bottom): 21226 bp, 5148/4973 bp, 4268 bp, 3530 bp, 2027 bp, 1904 bp, 1584 bp, 1375 bp.

B) Diagram of region of insertional inactivation of *sigB* gene in *L. monocytogenes* LMA2B. Not to scale. *sigB* (Grey) containing the 1.5 kbp kanamycin resistance determinant (Cross-hatch). σ^B-dependent promoter (P_B). Adapted from Becker *et al.* (1998).

4.2.2 Growth and starvation-survival of DES011 (*sigB*) cells in glucose-limiting CDM at 37°C

DES011 (*sigB*) was compared to that of the parental strain (EGD), for growth in glucose-limiting CDM at 37°C. DES011 (*sigB*) revealed a slight reduction in growth rate during exponential phase compared to EGD (0.75 and 0.83 A_{600} h^{-1} respectively), whereas the final yield achieved at stationary phase was unaffected by the *sigB* null mutation (Figure 4.2). The effect of a *sigB* null mutation on survival potential was investigated under glucose limiting conditions at 37°C. During glucose starvation, the viability of the DES011 (*sigB*) cultures fell to approximately 0.8% after 2 days (Figure 4.3). By 20 days the *sigB* mutant had undergone a 10-fold greater drop in viability compared to EGD. Reduced acid resistance, a likely consequence of the *sigB* null mutation was considered as a potential cause of any starvation-survival defect. To determine whether low pH was indeed a factor in glucose-limited CDM starved cultures, pH was monitored. During the experiment, the pH in both EGD and DES011 (*sigB*) cultures fell only slightly from pH 7.0 at the start of incubation to between 6.0 and 6.5 after 20 days.

4.2.3 The starvation-associated stress resistance of DES011 (*sigB*) cells

To further study the potential role of σ^B, the stress resistance of DES011 (*sigB*) was examined. The effect of glucose starvation upon the resistance of DES011 (*sigB*) to acid, heat and oxidative stress was determined as described in Chapter 2.12.

In wild type cells, resistance to pH becomes enhanced with increasing age of the culture, until cells become almost fully resistant after being starved for glucose for 7 days (Chapter 3.2.10). Exposure of exponential phase cells of DES011 (*sigB*) to pH 3.5 resulted in a greater loss of viability compared to exponential phase wild type cells (0.02% and 1% survival after 25 minutes respectively) (Figure 4.4). Post-exponential phase DES011 (*sigB*) cells were also over 10-fold more sensitive to acid stress than the corresponding EGD cells after 90 minutes. In contrast both DES011 (*sigB*) and EGD cells starved for glucose for 7 days were almost fully resistant to exposure to pH 3.5 (Figure 4.4).

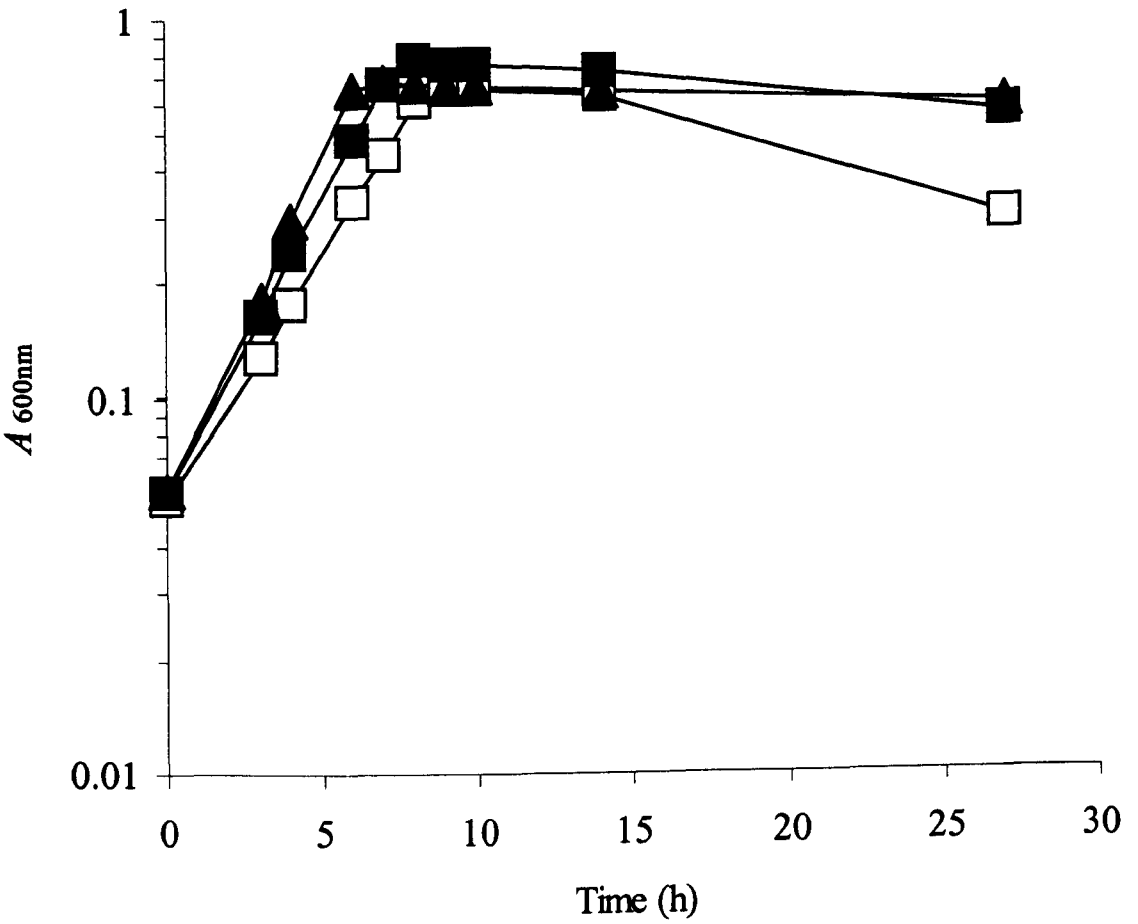


Figure 4.2

Comparison of growth of EGD (■), DES011 (*sigB*) (□) and DES012 (*prfA*) (▲) in glucose-limited CDM at 37°C. Cultures were inoculated at A_{600} 0.05.

Data are an average of at least two separate experiments for which the standard deviation was no greater than ±5% of the mean value at equivalent time points.

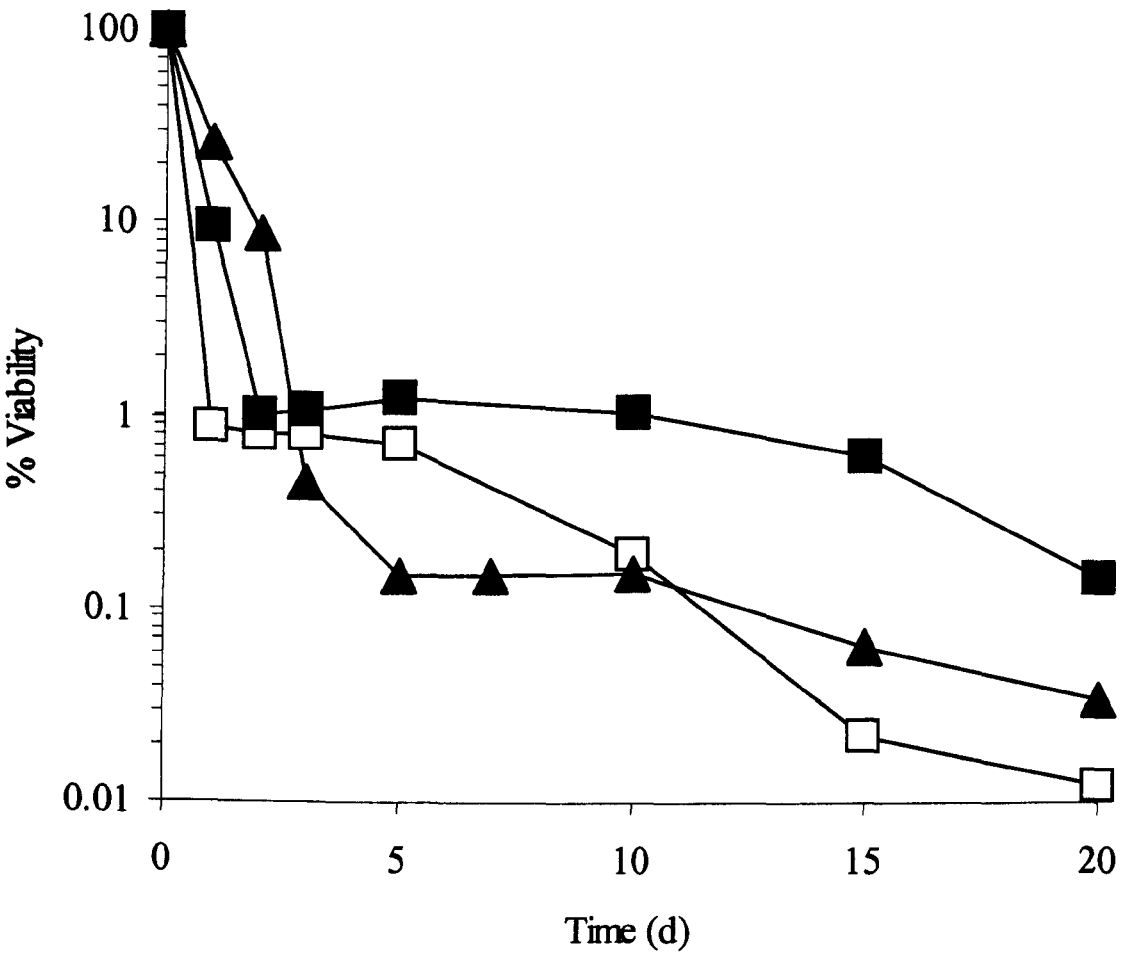


Figure 4.3
Comparison of the starvation-survival kinetics of EGD (■), DES011 (*sigB*) (□) and DES012 (*prfA*) (▲) in glucose-limited CDM at 37°C. 100% viability was 1×10^9 CFU ml^{-1}. Data are an average of at least two separate experiments for which the standard deviation was no greater than ±40% of the mean value at equivalent time points.

Investigation of the heat stress resistance of DES011 (*sigB*) found that the *sigB* null mutation had no significant effect on the resistance of exponential phase and post-exponential phase glucose-limited cultures to incubation in PBS at 55°C (Figure 4.5). However, long-term starved cells were slightly more sensitive to heat stress that starved EGD cells (Figure 4.5). When exposed to 7.5mM H_2O_2, exponential phase DES011 (*sigB*) cells exhibited greater resistance than EGD (50 and 1% survival after 15 minutes respectively), and viability of DES011 (*sigB*) cells was still detectable after 50 minutes (Figure 4.6). This increase in oxidative stress resistance is lost by post-exponential phase, as both post-exponential and 7-day starved cells showed wild type levels of resistance (Figure 4.6).

4.2.4 Cellular morphology of DES011 (*sigB*) under glucose-starvation conditions

Using electron microscopy (Chapter 2.11), the morphology of DES011 (*sigB*) cells taken from exponentially growing and 7 day-old glucose-starved cultures incubated at 37°C were studied. Exponential phase DES011 cells possessed average cell dimensions (± S.D.) of 1.62 μm ± 0.35 μm in length, by 0.59 μm ± 0.04 μm in width, with 28 % of cells possessing partial division septa (Figures 4.7 and 4.8). In glucose limited cultures incubated for 7 days at 37°C, the mean cell length was reduced from 1.29μm ± 0.35μm (mean of 60 cells), compared to cells in exponential growth, whilst the mean cell width had increased slightly to 0.60μm ± 0.04μm (mean of 60 cells). The occurrence of partial division septa in 7-day glucose-starved cells (15.0 %) was seen to fall relative to cells in exponential-phase (28 %) (Figures 4.7 and 4.8).

4.2.5 Growth and starvation-survival of DES012 (*prfA*) cells in glucose-limiting CDM at 37°C

A *prfA* null mutant strain (DES012) was compared to that of the parental strain (EGD), for growth in glucose-limiting CDM at 37°C. DES012 (*prfA*) exponential phase growth (μ=1.42h) and final yield comparable to that of the parental strain (Figure 4.2). Incubation of DES012 (*prfA*) in glucose-limited CDM at 37°C resulted in a 10-fold greater reduction in

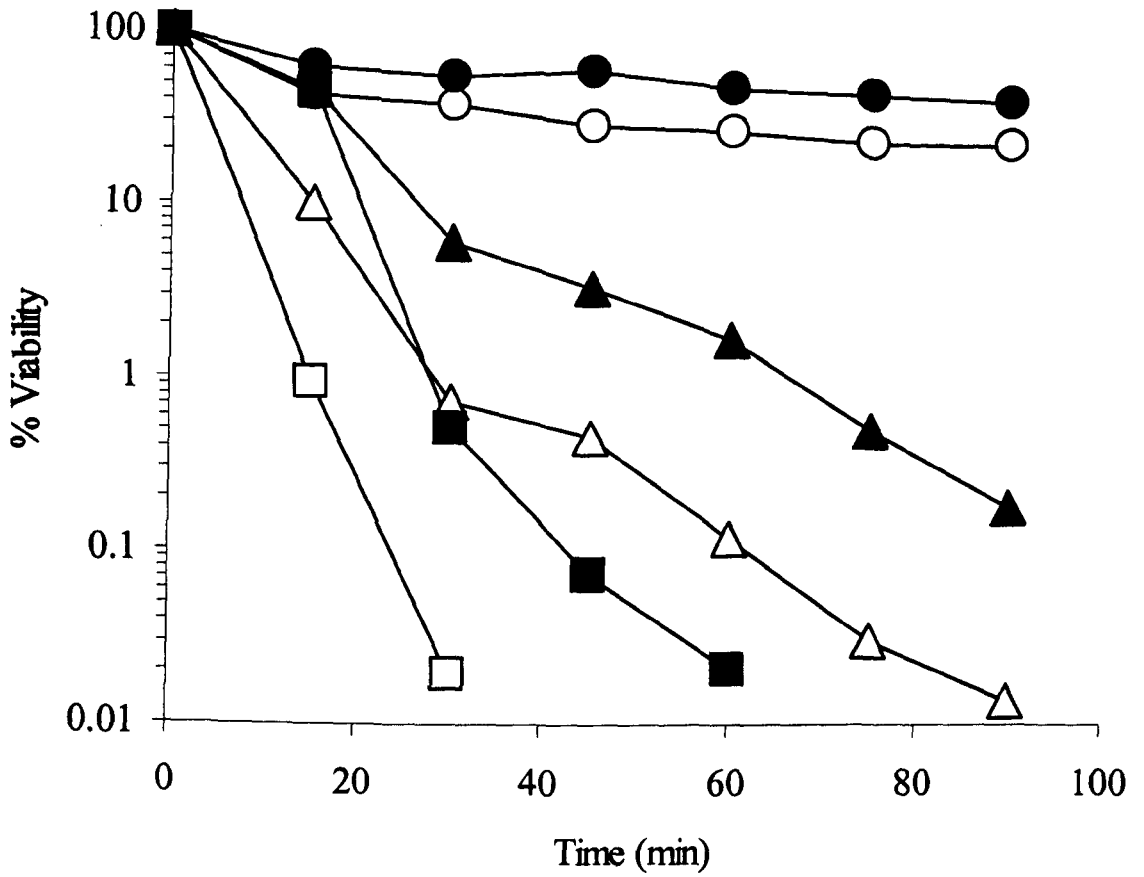


Figure 4.4
Comparison of the acid stress resistance (pH 3.5) of EGD (closed symbols) and DES011 (*sigB*) (open symbols) in PBS at 37°C.

Exponential (■,□), post-exponential (▲,Δ), 7-day glucose starved (●,○). 100% viability was approximately 5×10^6 CFU ml^{-1}. Data are an average of at least two separate experiments for which the standard deviation was no greater than ±40% of the mean value at equivalent time points.

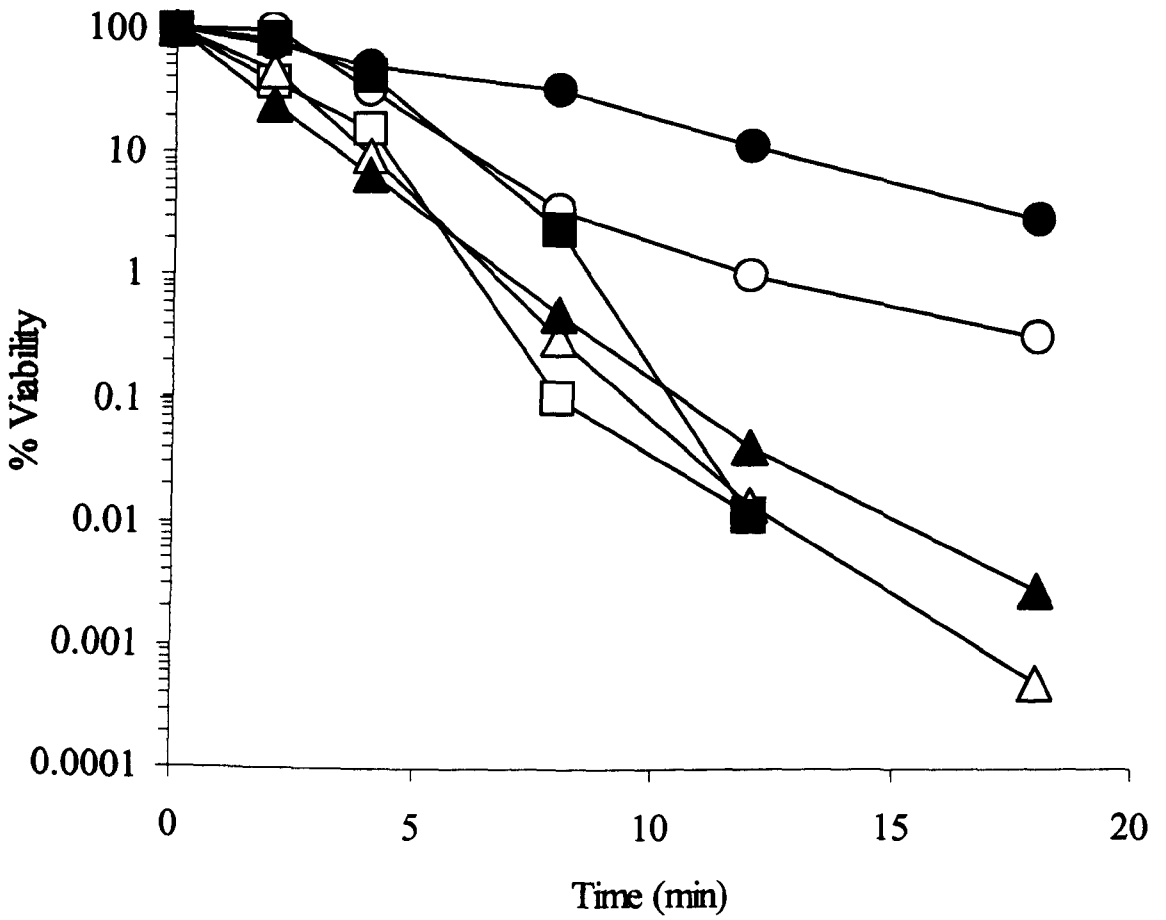


Figure 4.5
Comparison of the heat stress resistance (55°C) of EGD (closed symbols) and DES011 (*sigB*) (open symbols) in PBS.

Exponential (■,□), post-exponential (▲,Δ), 7-day glucose starved (●,○). 100% viability was approximately 5 × 10^6 CFU ml^{-1}. Data are an average of at least two separate experiments for which the standard deviation was no greater than ±40% of the mean value at equivalent time points.

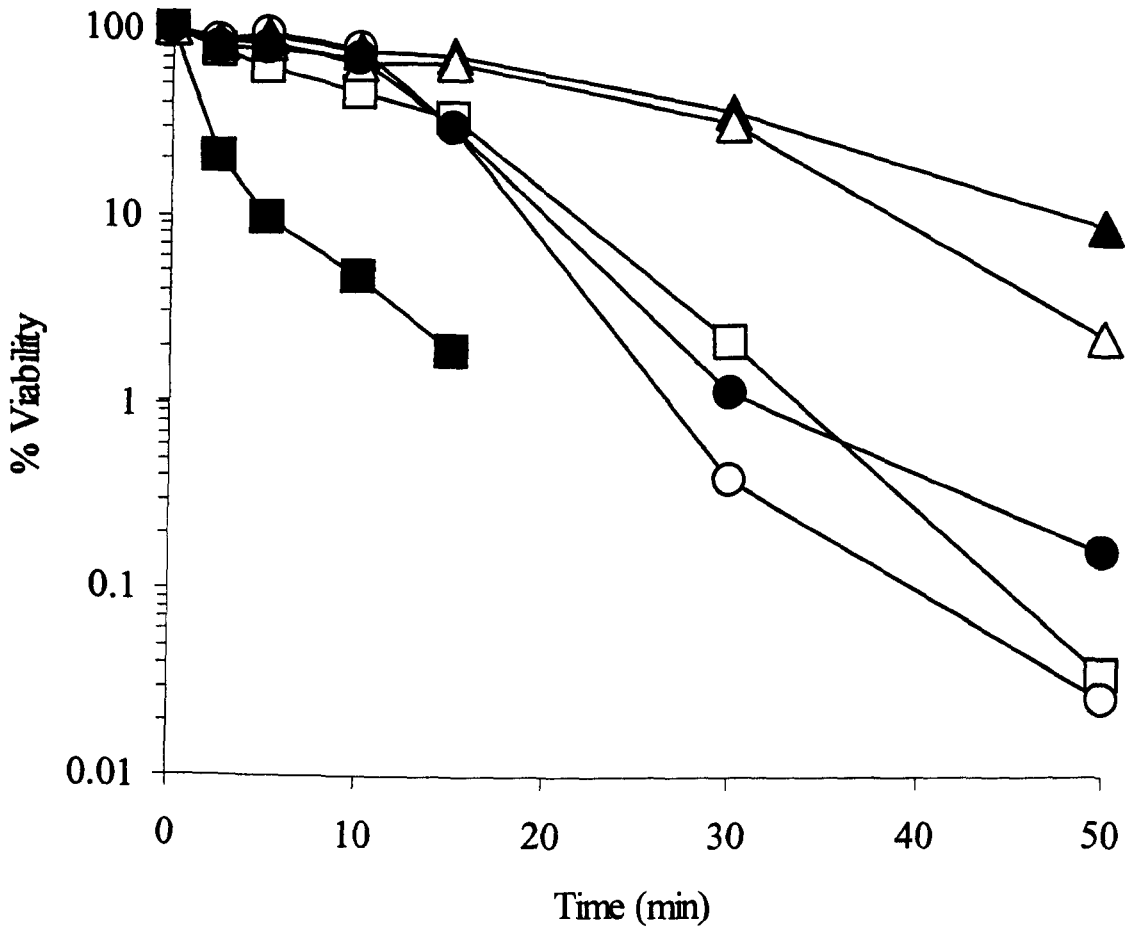


Figure 4.6
Comparison of the H_2O_2 resistance (7.5mM) of EGD (closed symbols) and DES011 (*sigB*) (open symbols) in PBS at 37°C.

Exponential (■,□), post-exponential (▲,Δ), 7-day glucose starved (●,○). 100% viability was approximately 5×10^6 CFU ml^{-1}. Data are an average of at least two separate experiments for which the standard deviation was no greater than ±40% of the mean value at equivalent time points.

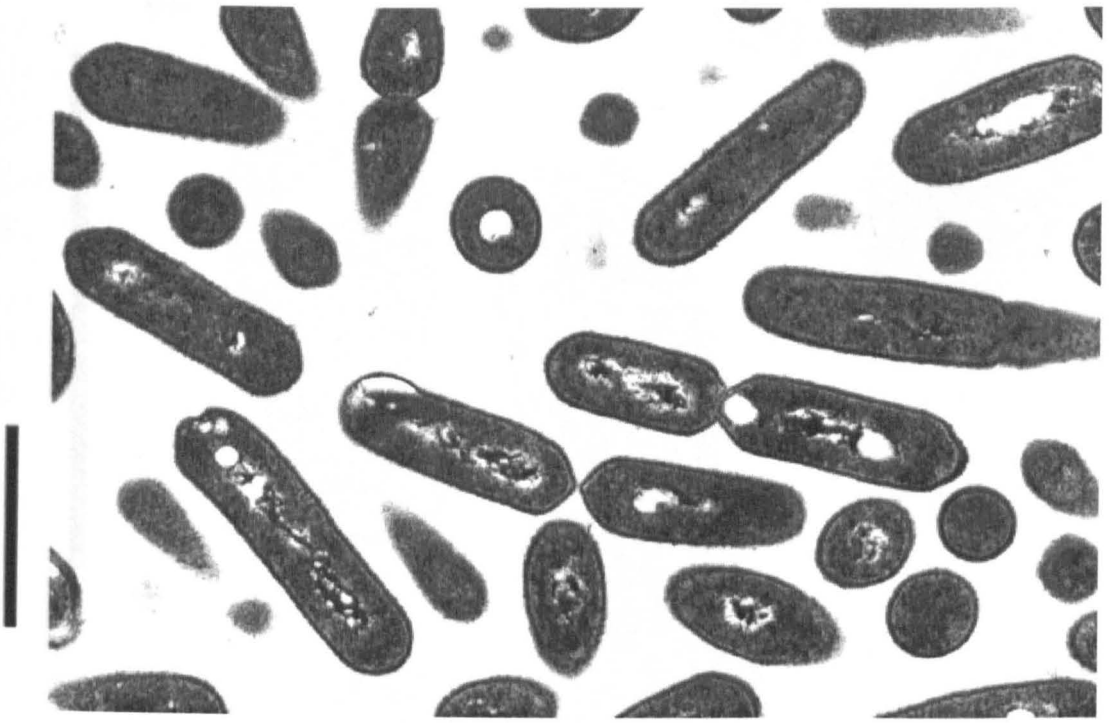


Figure 4.7
Transmission electron micrograph of exponential phase DES011 (*sigB*) cells grown at 37°C. Bar, 1 μm.

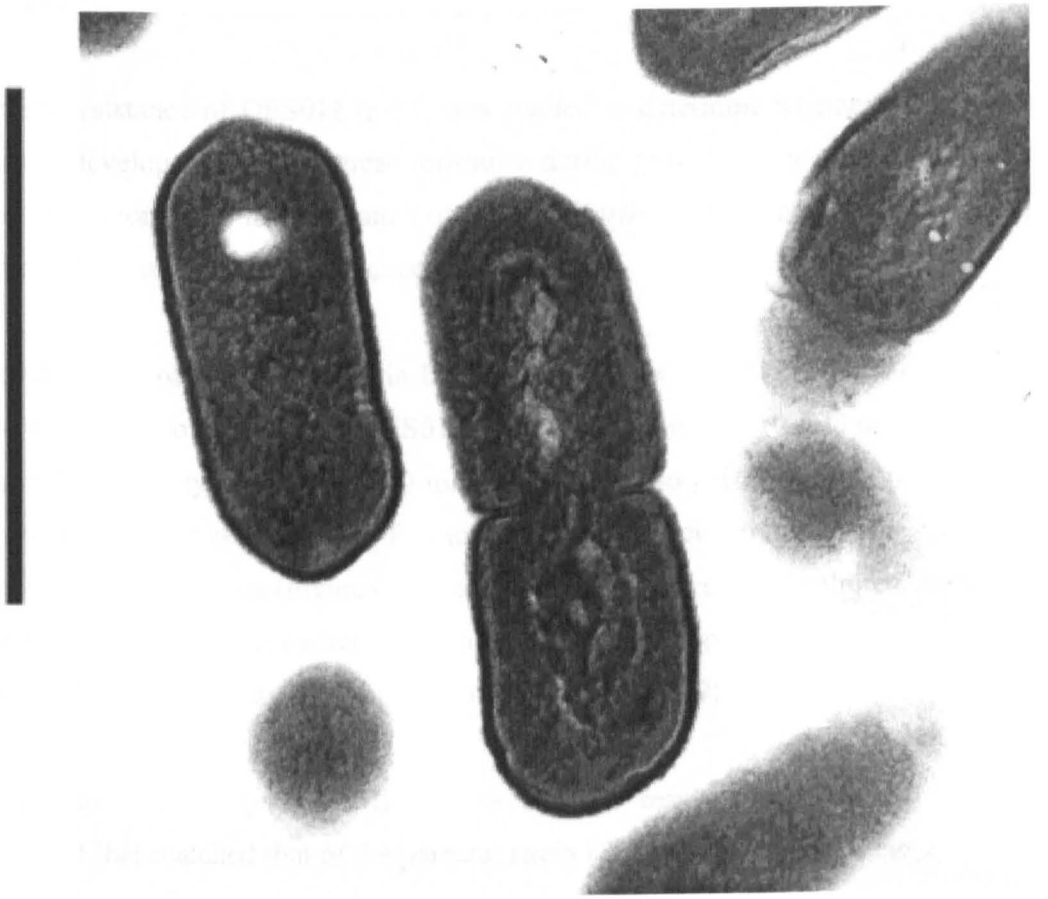


Figure 4.8
Transmission electron micrograph of DES011 (*sigB*) cells starved for glucose for 7 days at 37°C. Bar, 1 μm.

viability over the first 5 days (Figure 4.3). After 20 days 0.03% of cells remained viable after 20 days, compared to 0.14% in wild type cultures.

4.2.6 The stress resistance of DES012 (*prfA*) cells

The stress resistance of DES012 (*prfA*) was studied to determine whether PrfA played any role in the development of the stress resistance during growth and starvation. The effect of glucose starvation upon the resistance of DES012 (*prfA*) to acid, heat and oxidative stress was determined as described in Chapter 2.12.

The development of acid resistance in DES012 (*prfA*) was found to differ from that in EGD (Figure 4.9). In exponential phase DES012 (*prfA*) cells were over 10-fold more resistant than corresponding wild type cells after 60 minutes exposure to pH 3.5, whilst post-exponential phase DES012 (*prfA*) cells were 70-fold more resistant to acid stress than the corresponding EGD cells after 60 minutes (Figure 4.9). In long-term glucose starved cultures, the increase in acid resistance observed in earlier stages of growth is not apparent, as both DES012 (*prfA*) and EGD cells were almost fully resistant to pH 3.5 (Figure 4.9).

Incubation of DES012 (*prfA*) cells in PBS at 55°C resulted in a pattern of resistance development that matched that of the parental strain (Figure 4.10) and thus the *prfA* mutation had no obvious effect on starvation-associated heat resistance. DES012 (*prfA*) cells taken from exponential phase and 7-day starvation in glucose limited CDM exhibited high levels of resistance to oxidative stress. In exponential phase, DES012 (*prfA*) cells were 300-fold more H_2O_2 resistant than EGD after 20 minutes, and 8% of DES012 (*prfA*) cells remained viable after 50 minutes exposure to 7.5 mm H_2O_2 (Figure 4.11). Long-term, glucose-starved cells were fully resistant upon exposure to 7.5mM H_2O_2, whereas the viability of starved EGD cells exhibited a 3-log fall over 50 minutes (Figure 4.11). In contrast, DES012 (*prfA*) cells taken from post-exponential phase cultures were 45 times more sensitive to oxidative stress after 50 minutes exposure than the equivalent wild type cells.

4.2.7 Cellular morphology of DES012 (*prfA*) under glucose-starvation conditions

Using electron microscopy (Chapter 2.11), the morphology of DES012 (*prfA*) cells taken from exponentially growing and 7 day-old glucose-starved cultures incubated at 37°C were studied. Exponential phase DES012 (*prfA*) cells possessed average cell dimensions (± S.D.) of 1.83 μm ± 0.45 μm in length, by 0.66 μm ± 0.06 μm in width, with 32 % of cells possessing partial division septa (Figures 4.12 and 4.13). Mean cell length was reduced to 1.33μm ± 0.45μm (mean of 60 cells) in glucose limited cultures incubated for 7 days at 37°C, compared to cells in exponential growth, whilst the mean cell width had increased slightly to 0.68μm ± 0.04μm (mean of 60 cells). The occurrence of partial division septa in 7-day glucose-starved cells (18.3 %) was seen to fall relative to cells in exponential-phase (32 %). (Figures 4.12 and 4.13).

4.2.8 The role of *sigB* and *prfA* in catalase activity during growth of *L. monocytogenes* EGD

Since acid and oxidative stress resistance development was altered in strains DES011 (*sigB*) and DES012 (*prfA*), and given that a principal determinant in resistance to H_2O_2 is the production of catalase (Dallimer and Martin, 1988), profiles of the activity of this enzyme were determined. Catalase activity was monitored, as described in Chapter 2.20, during growth and starvation in glucose-limited CDM at 37°C, and compared to that of the parental EGD strain.

Catalase activity in EGD increased throughout growth in glucose-limited CDM, reaching a maximum of 100.0 Units approximately 6 hours post-exponential phase (T=14h), before activity fell by about 20% by 20 hours post-exponential phase (T=26h) (Figure 4.14). Residual levels of catalase activity (approximately 15.8 Units) were observed in long-term (7 day) glucose-starved cultures (data not shown). In DES011 (*sigB*), catalase activity gradually increased throughout growth, to a maximum approximately 6 hours post-exponential phase (T=14h) before a 20% fall by 20 hours post-exponential phase (T=26h) (Figure 4.14). The levels of activity observed however ranged between 45% and 65% of that found in EGD at

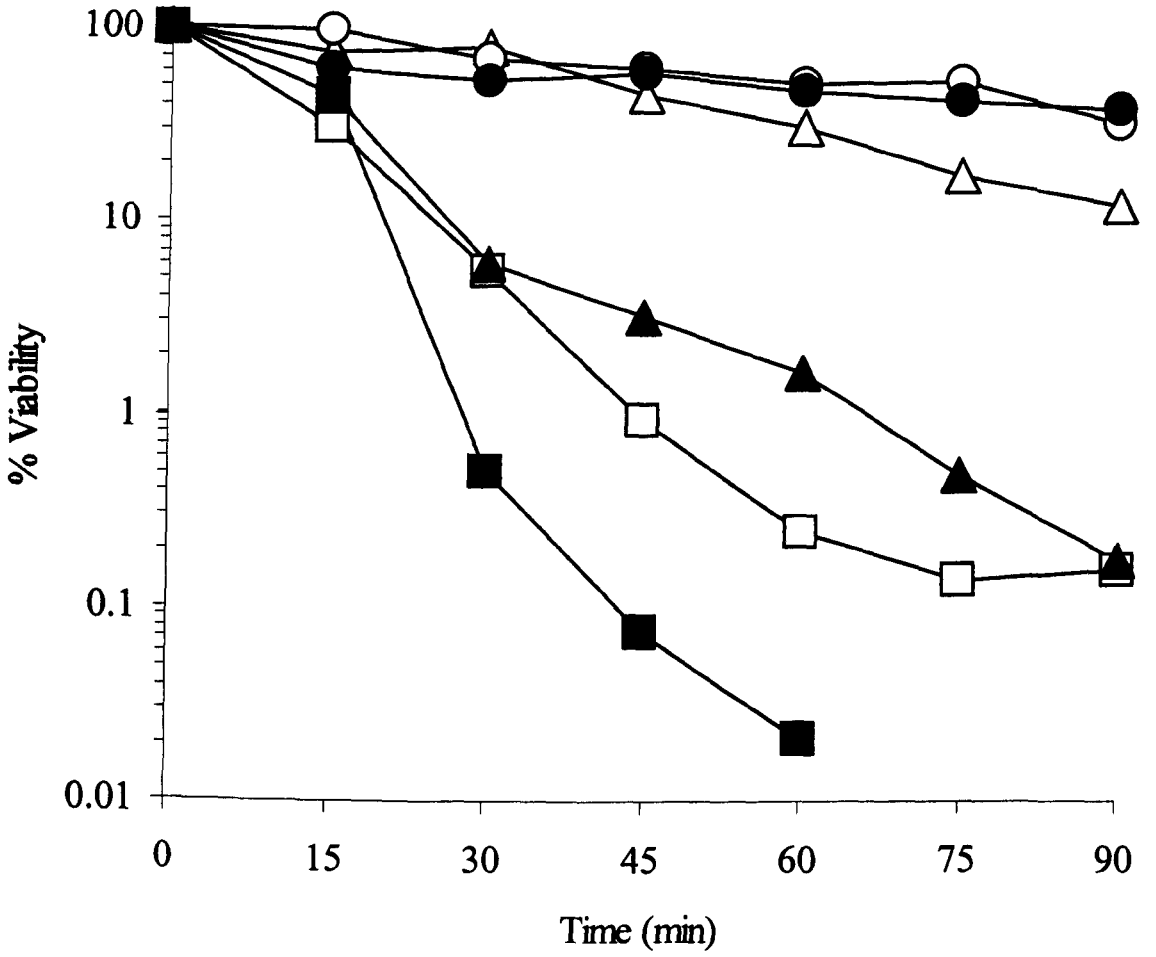


Figure 4.9
Comparison of the acid stress resistance (pH 3.5) of exponential phase, post-exponential phase and EGD (closed symbols) and DES012 (*prfA*) (open symbols) in PBS at 37°C.

Exponential (■,□), post-exponential (▲,Δ), 7-day glucose starved (●,○). 100% viability was approximately 5×10^6 CFU ml^{-1}. Data are an average of at least two separate experiments for which the standard deviation was no greater than ±40% of the mean value at equivalent time points.

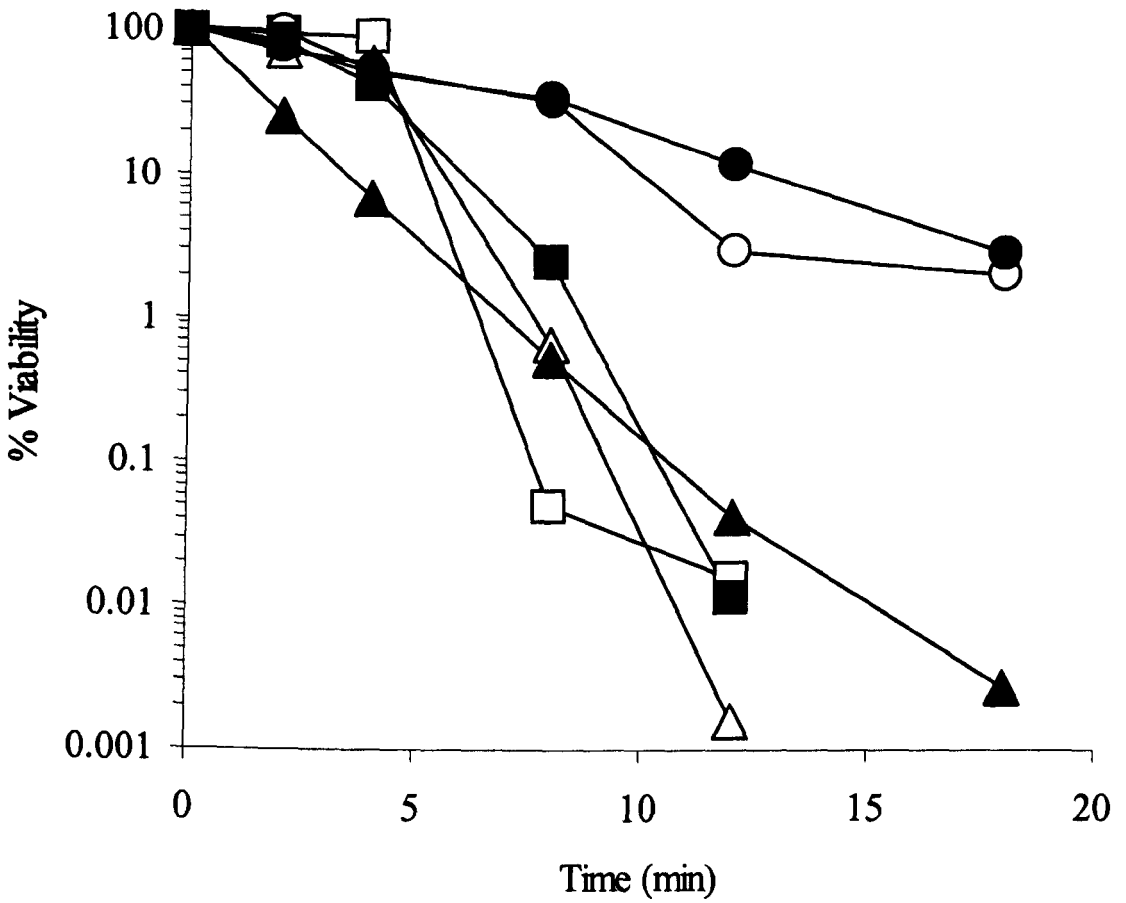


Figure 4.10
Comparison of the heat stress resistance (55°C) of EGD (closed symbols) and DES012 (*prfA*) (open symbols) in PBS.

Exponential (■,□), post-exponential (▲,Δ), 7-day glucose starved (●,○). 100% viability was approximately 5 × 10^6 CFU ml^{-1}. Data are an average of at least two separate experiments for which the standard deviation was no greater than ±40% of the mean value at equivalent time points.

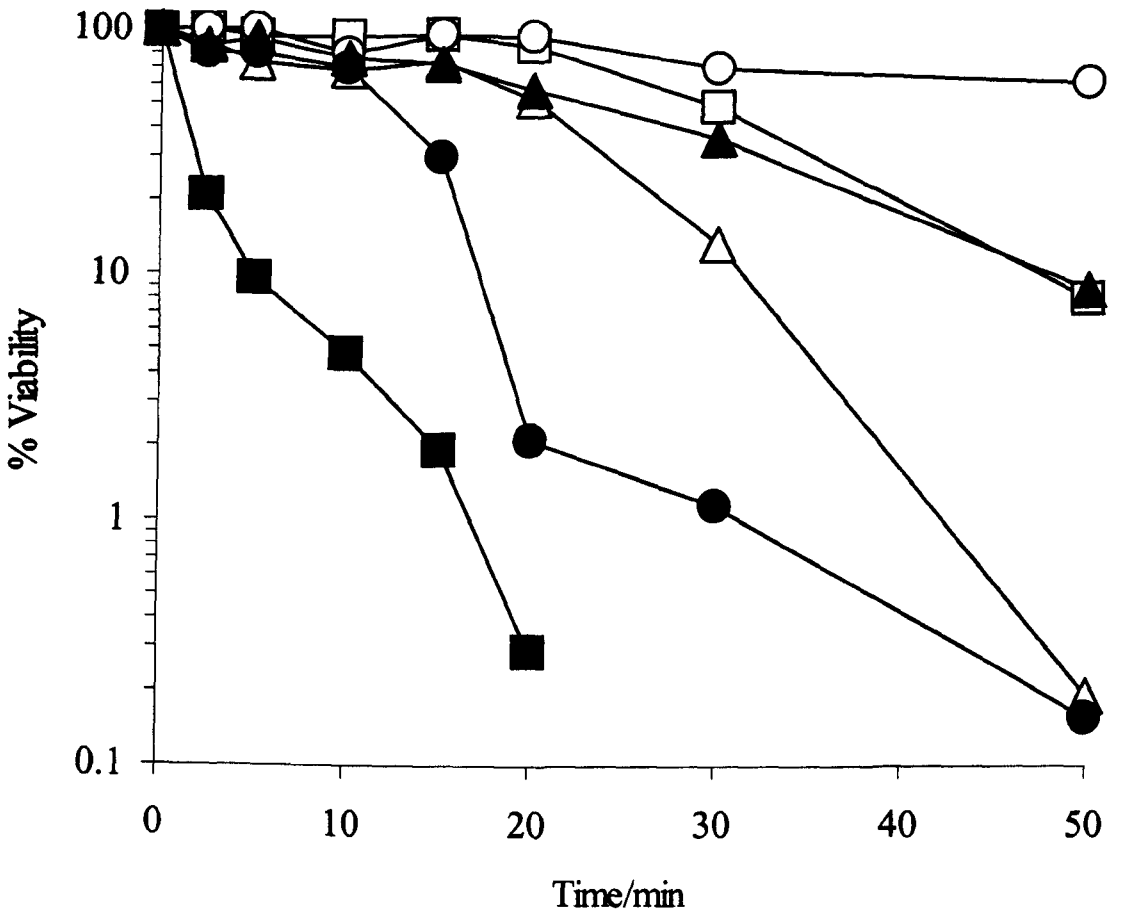


Figure 4.11
Comparison of the H_2O_2 stress resistance (7.5mM) of EGD (closed symbols) and DES012 (*prfA*) (open symbols) in PBS at 37°C.

Exponential (■,□), post-exponential (▲,Δ), 7-day glucose starved (●,○). 100% viability was approximately 5×10^6 CFU ml^{-1}. Data are an average of at least two separate experiments for which the standard deviation was no greater than ±40% of the mean value at equivalent time points.

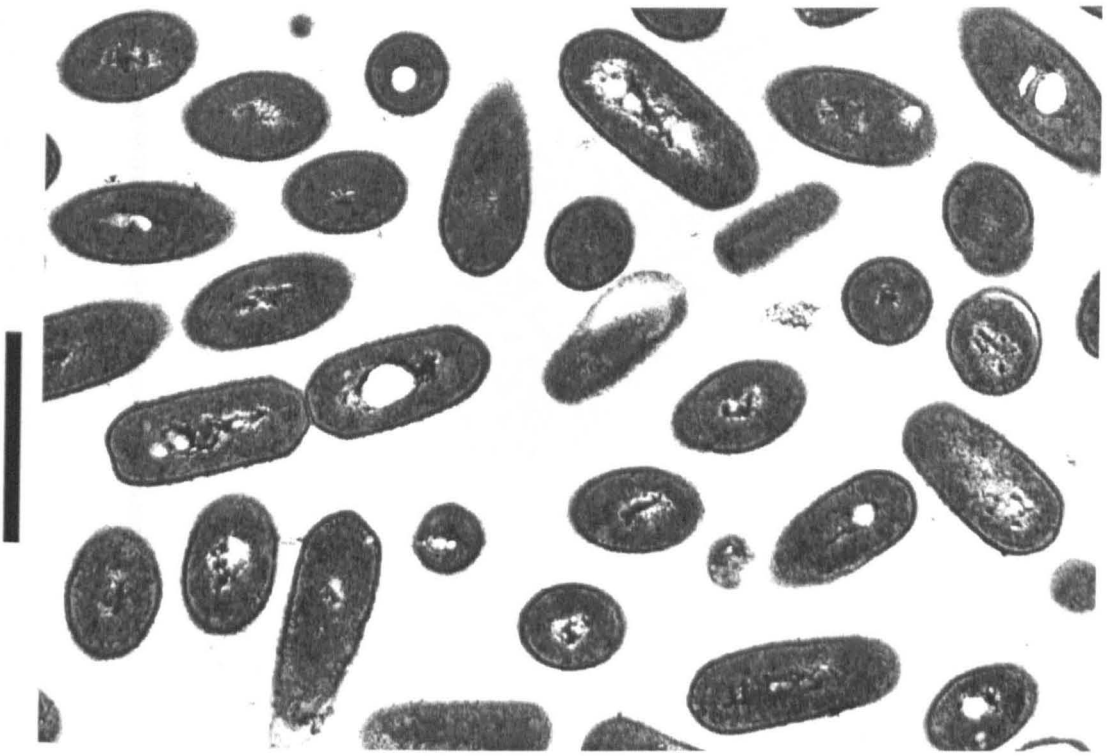


Figure 4.12
Transmission electron micrograph of exponential phase DES012 (*prfA*) cells grown at 37°C. Bar, 1 μm.

Figure 4.13
Transmission electron micrograph of DES012 (*prfA*) cells starved for glucose for 7 days at 37°C. Bar, 1 μm.

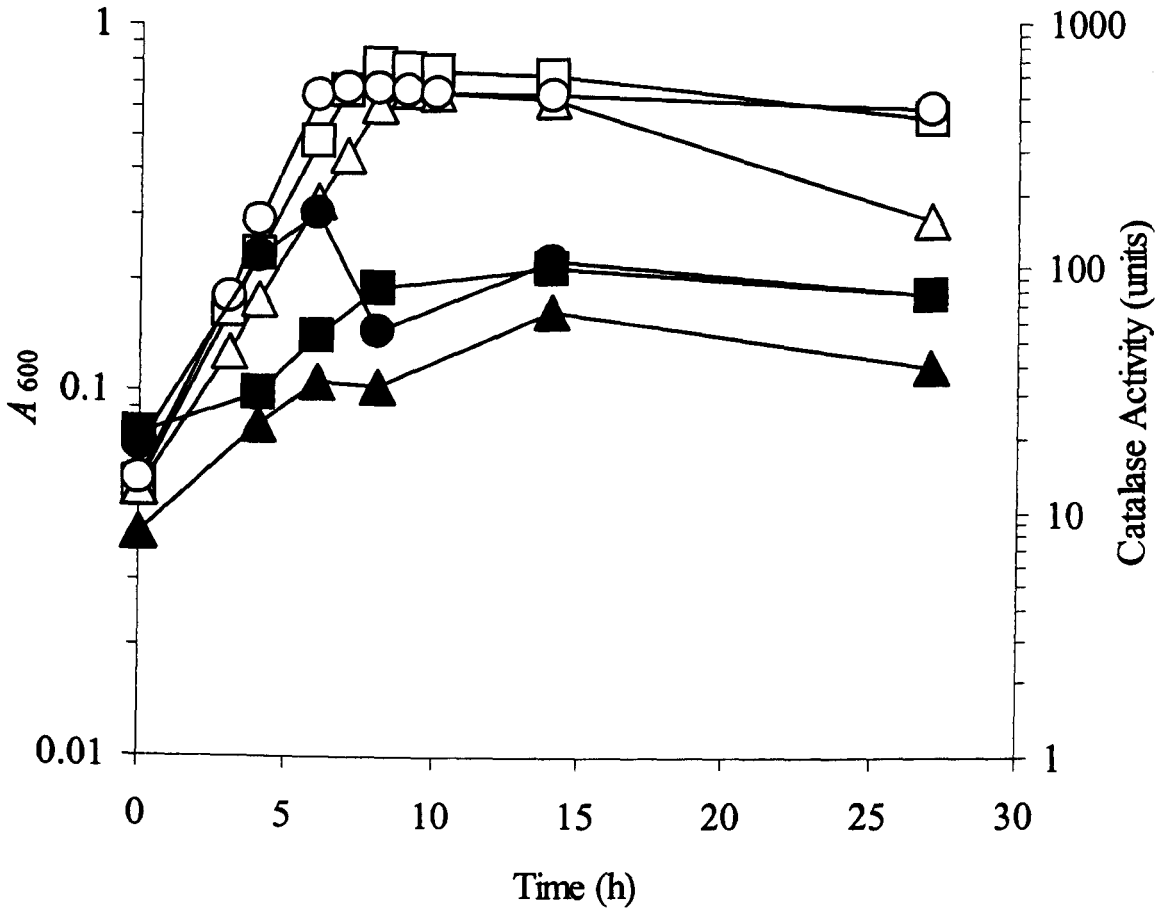


Figure 4.14
Catalase activity of EGD, DES011 (*sigB*) and DES012 (*prfA*) during growth in glucose-limited CDM at 37°C.

Catalase activity of *L. monocytogenes* EGD (wild type; ■), DES011 (*sigB*; ▲), and DES012 (*prfA*; ●). Growth (A_{600}) of EGD (□), DES011 (Δ), and DES012 (○) are also shown. One unit of catalase activity equals the degradation of 1 μmol H_2O_2 min^{-1} mg^{-1} protein.

equivalent time points. After 7 days glucose starvation, catalase activity in DES011 (*sigB*) cells (15.2 Units) was found to be comparable to that in wild type starved cells (data not shown). Catalase activity in DES012 (*prfA*) cells increased to four times wild type levels during mid- to late-exponential phase (Figure 4.14). After reaching a peak at the end of exponential phase (T=6h), activity fell to approximately 33% of peak levels and then remained relatively constant during the following 20 hours. The catalase activity of 7-day glucose-starved DES012 (*prfA*) cells (4.88 Units) was found to be 70% lower than that seen in EGD (data not shown).

4.3 Discussion

Null mutations in the genes encoding for the transcriptional regulators σ^B and PrfA resulted in reduced starvation-survival, and altered patterns of development of both stress resistance and catalase activity.

The *sigB* mutant strain (DES011) demonstrates approximately a 10-fold reduction in starvation-survival compared to wild type. This differs from both *S. aureus* (Chan *et al.*, 1998) and *B. subtilis* (Völker *et al.*, 1999), in which no significant role was observed. DES011 (*sigB*) cells exhibit a fall in viability of approximately 99% in the first two days, comparable to that in EGD cultures. Only after 20 days is a 10-fold lower viability than the parent apparent. This would suggest that the initial development of the starvation-survival response is not σ^B-dependent and that the loss of σ^B only becomes significant over the longer term. It has been postulated that accumulation of damage to cell components, principally as a result of the action of reactive oxygen species may be the key to the ageing and eventual death of cells in stationary cultures (Dukan and Nyström, 1998). Low pH is unlikely to be the principal cause of the reduced survival in this case, as the pH of the starved cultures only fell to between pH6.0-6.5. The possibility remains however, that the cumulative effect of the slightly reduced pH, over a prolonged period, could be sufficient to produce the phenotype seen. Included in the *B. subtilis* σ^B regulon are a number of genes involved in resistance to oxidative stress (Table 4.1). The lack of a protective contribution from these genes would

correlate with this hypothesis, and with the pattern of slow cell death observed in DES011 (*sigB*) cultures.

Strangely, exponential phase DES011 (*sigB*) cells possess increased resistance to hydrogen peroxide compared to corresponding EGD cells. The molecular basis for this is unclear, though it seems to occur only in washed cells, and be independent of catalase expression.

Gene	Function
katE	Catalase
trxA	Damaged protein repair (?)
clpC	Damage protein repair/degradation (?)
clpP	Damage protein repair/degradation (?)
clpX	Damage protein repair/degradation (?)
dps	DNA binding/protection
sms	DNA repair (?)
yakC	DNA repair (?)

Table 4.1
σ^B-dependent genes involved in oxidative stress resistance in *B. subtilis* (Hecker and Völker, 1998).

Determination of the development of oxidative stress resistance in the σ^B mutant failed to show any defect in post-exponential phase or long-term starvation. These results are different to that observed in *S. aureus* (Chan *et al.*, 1998), where increased sensitivity is observed in post-exponential phase and 6-day-starved *S. aureus* cells. σ^B therefore seems to take no part in short-term oxidative stress resistance during starvation of *L. monocytogenes* EGD, despite the fact that σ^B positively regulates numerous oxidative stress resistance genes in Gram-positive bacteria (Hecker and Völker, 1998). It is known that a number of these genes fall under the dual control of both σ^B and σ^A-dependent promoters, and although they contain a

σ^B-dependent promoter, no induction by glucose starvation occurs (Hecker and Völker, 1998). In *B. subtilis*, σ^B is known to regulate *katE*, encoding one of at least three cellular catalases. Expression of *katE* is induced by glucose starvation, though interestingly KatE does not determine H_2O_2 resistance in glucose-starved cells (Engelmann and Hecker, 1996). Instead, the σ^B-independent catalase KatA is required for the majority of the underlying oxidative stress resistance under most conditions (Engelmann and Hecker, 1996). KatA however also fails to account for the development of H_2O_2 stress resistance during glucose starvation.

Total catalase activity in DES011 (*sigB*) cells during growth and starvation is reduced by at least 35%. This appears to have no observable detrimental effect under the conditions studied, and therefore it seems that glucose-starvation induced oxidative stress resistance in *L. monocytogenes* EGD is also, at least partially, catalase-independent.

The lack of an effect of inactivation of *sigB* on heat resistance in exponential phase differs from other Gram-positive bacteria, where exponential phase *sigB* mutant cells are more sensitive than wild type (Chan *et al.*, 1998; Völker *et al.*, 1999). DES011 (*sigB*) cells starved for glucose for 7 days showed a marginal reduction in resistance to treatment at 55°C, implying that starvation-associated stress resistance is at least partially controlled by σ^B. A number of proteins that fall under the control of σ^B (ClpC, ClpP, ClpX) confer heat stress resistance to the cell, and have functions that overlap oxidative stress resistance (Hecker and Völker, 1998). Here, the effect of the *sigB* mutation would be mitigated, firstly by the multiple facets of general stress resistance and as previously mentioned in the case of *trxA*, *clpC* and *clpP*, by the fact that these genes are controlled by other transcriptional regulators (Hecker and Völker, 1998).

Exponential phase DES011 (*sigB*) cells were more sensitive to acid stress that the corresponding wild type cells, an effect also found in *B. subtilis* (Völker *et al.*, 1999). The increased post-exponential phase sensitivity observed was similarly found in a previous study on *L. monocytogenes* 10403S, where cells from overnight cultures were more sensitive than wild type to pH 2.5 (Wiedmann *et al.*, 1998). The development of stationary-phase acid

tolerance in Gram-negative bacteria is directed by σ^S (Lee *et al.*, 1994). Here we have established that the increased sensitivity to low pH was lost by the time the cells had been starved for glucose for 7 days. This would therefore imply that σ^B controls acid resistance during growth, but not the development of growth-phase dependent acid resistance. A two-component signal transduction system (*lisRK*) mediating acid resistance in *L. monocytogenes* was recently discovered (Cotter *et al.*, 1999). A deletion mutation in *lisK* resulted in a mutant that was acid tolerant in stationary phase, yet acid sensitive in early exponential phase. Further work on both σ^B, and the *lisRK* system may help move toward a full understanding of how these two regulatory mechanisms interact in the control of acid resistance in *L. monocytogenes*.

DES011 (*sigB*) cells were found to lack of any change in morphology relative to EGD during starvation. This implies that σ^B plays no part in the control of cellular morphology, an idea further reinforced by the absence of any morphology-related genes in those so far identified as part of the σ^B regulon in *B. subtilis* (Hecker and Völker, 1998). This is in contrast to σ^S, which in *E. coli* is known to positively regulate the morphogene *bolA* upon entry to stationary phase (Lange and Hengge-Aronis, 1991).

Inactivation of the *prfA* gene caused a significant alteration in the ability of the cell to adapt to glucose starvation and other stress conditions. Survival of the *prfA* mutant (DES012) cells was 10-fold reduced in glucose-limited CDM compared to that of wild type cells. Whilst there was no apparent effect of the *prfA* mutation on heat stress resistance, survival of DES012 (*prfA*) cells upon exposure to pH 3.5 was far greater than EGD during exponential- and post-exponential-phase. Loss of PrfA in DES012 (*prfA*) cells also appears to derepress oxidative stress resistance, principally during exponential phase and in long-term starved cells. This protective effect is still subject to additional regulation, as a transient drop in resistance is seen in post-exponential phase. The H_2O_2 resistance profile of DES012 (*prfA*) matches changes in catalase activity seen in exponential and post-exponential phase. At these times, KatA activity confers oxidative stress resistance in *B. subtilis*, so could the activity of a KatA homologue be controlled by PrfA in *L. monocytogenes*? During long-term starvation, DES012 (*prfA*) cells were almost fully resistant to H_2O_2 compared to a 3-log fall in wild type

cells, suggesting that the *prfA* mutation lifts repression of oxidative stress resistance during starvation. No change in the morphology of DES012 (*prfA*) cells was found relative to wild type during starvation, suggesting that PrfA is not involved in the regulation of cellular morphology.

The function of PrfA revealed here has not been previously reported for PrfA in *L. monocytogenes*. The question is why should a virulence determinant regulator also affect fundamental cellular physiological processes? The overlap between stress response and virulence is becoming more apparent with the discovery of the regulation of virulence determinants by stress response regulators in both Gram-positive and Gram-negative bacteria (Deora *et al.*, 1997; Spector *et al.*, 1998). The knowledge that PrfA, in association with an unknown cofactor, induces the expression of virulence genes in response to carbon starvation, pH and temperature implies that the starvation and stress responses may be tightly linked with virulence (Behari and Youngman, 1998b; Böckmann *et al.*, 1996; Leimeister-Wächter *et al.*, 1992). In *L. monocytogenes*, PrfA is well known to be homologous to the family of Crp/Fnr global regulators (Lampidis *et al.*, 1994), members of which control a number of key metabolic processes, including catabolite repression (CRP) and response to oxygen levels (FNR). Although related, whether or not PrfA plays a similar role to any of these regulators had not been investigated until now. Only recently has PrfA-dependent repression of the general stress protein ClpC been observed (Ripio *et al.*, 1998). The apparent importance of stress resistance in virulence was further highlighted by the fact that ClpC is also required for full virulence (Rouquette *et al.*, 1996). Since ClpC is regulated by PrfA in *L. monocytogenes*, and by σ^B in *B. subtilis*, regulation of starvation and stress resistance by PrfA via σ^B is possible. Certainly, relieving repression of ClpC in a PrfA mutant could account for the rises of pH and H_2O_2 resistance seen in DES012 (*prfA*). However, given that ClpC plays a role in heat shock, an increase in heat resistance may also have been expected. Also, regulation solely via σ^B may not be the whole story, since catalase expression differs distinctly between the *sigB* and *prfA* mutant strains.

The multiplicity of effects observed as a result of inactivation of *sigB* and *prfA* confirms that starvation-survival and stress resistance involve a number of sophisticated overlapping

regulatory mechanisms. In order to understand these processes, it is important to identify components with starvation-survival functions.

CHAPTER 5

THE ISOLATION, CHARACTERISATION AND MOLECULAR ANALYSIS OF *L. MONOCYTOGENES* STARVATION-SURVIVAL DEFECTIVE MUTANTS

5.1 Introduction

As with the study of any bacterial system, the isolation of mutants is essential if adequate progress in the understanding of the starvation-survival process is to be achieved. From mutants, we are able to obtain invaluable information on the genetic loci involved – from their initial discovery, through to DNA cloning and identification of the proteins encoded. This can ultimately lead to an understanding of the role and regulation of bacterial response mechanisms.

The pairing of the ability to generate a library containing clones with random chromosomal mutations to a suitable screening protocol can make a great number of mutants available for study. Currently, one of the most popular ways of generating a library of random mutants is by the use of transposable elements. Since the identification of the gene encoding σ^S (then named *katF*) in *E. coli* using Tn*10* mutagenesis by Loewen and Triggs (1984), a significant number of starvation mutants have been isolated using transposon mutagenesis. Watson *et al.* (1998b) were able to obtain ten *S. aureus* starvation-survival mutants from an amino acid starvation screen using the Tn*917*-based pLTV1. A diverse collection of genes was identified, including homologues to superoxide dismutase (*sodA*), haem A synthase (*ctaA*), a δ-subunit of RNA polymerase (*rpoE*), a component of the SOS response (*samB*) and a hypoxanthine-guanine phosphoribosyltransferase (*hprT*). More recently, Keer *et al.* (2000) were able to isolate 21 starvation-survival mutants in *M. smegmatis* using a library created with Tn*611*.

Transposon-mediated mutagenesis was initially demonstrated using conjugative transposons in *L. monocytogenes* (Kathariou *et al.*, 1987). The low frequencies of transmission and the requirement for sequence homology between the ends of the elements and that at the site of

insertion, restricted the use of these systems for large-scale mutagenesis. The problem was overcome by Cossart *et al.* (1989) who identified Tn*917* as a means of insertional mutagenesis. Importantly for the generation of a library of mutants, Tn*917* exhibited several favourable characteristics. Tn*917* inserts into the chromosome with a high degree of randomness, the products of which were extremely stable (typical reversion rates of less than 10^{-10}). The transposed strains can be selected using the incorporated Em^R determinant and the transposon can carry up to 8 kbp of additional DNA (Youngman, 1990).

Modified forms of Tn*917*, carried on a temperature-sensitive vector, were created to contain amongst other features a promoterless copy of the *lacZ* gene (pLTV1 and pLTV3) (Figure 5.1). If the transposon is inserted into the correct orientation relative to the interrupted chromosomal gene, a transcriptional fusion can be formed (Figure 5.2). This is of particular use since starvation-associated proteins are often expressed in a growth phase-dependent manner, and so expression during starvation of the *lacZ* fusions would be further evidence for the possible importance of a gene in survival. Confirmation of the fusion can be made by visual detection of the hydrolysis of X-Gal, producing an insoluble blue precipitate, whilst gene expression through growth can then be measured by assaying β-galactosidase activity via the hydrolysis of MUG (4-methylumbelliferyl-β-D-galactopyranoside) to β-D-galactopyranoside and the fluorescent 4-methylumbelliferone. In *L. monocytogenes* however, the potential for use of this method for the study of low-level gene expression is limited by the occurrence of natural β-galactosidase activity (Seeliger and Jones, 1986). Further features of the modified Tn*917* include erythromycin and chloramphenicol, resistance genes for selection in Gram-positive bacteria, neomycin and bleomycin resistance genes in *E. coli*, an *E. coli* origin of replication (ColE1) and a multiple cloning site.

pLTV3 is a plasmid 22.1 kb in size, containing a Tn*917* derivative sequence, flanked by plasmid sequences (Figure 5.1). pLTV3 was derived from pLTV1, which itself was created to enable the generation of insertion libraries from which flanking chromosomal DNA may be rescued (Camilli *et al.*, 1990). Insertion of *Tn917* into the chromosome of *L. monocytogenes* was determined to occur singly, and despite some hotspot areas of insertion was sufficiently

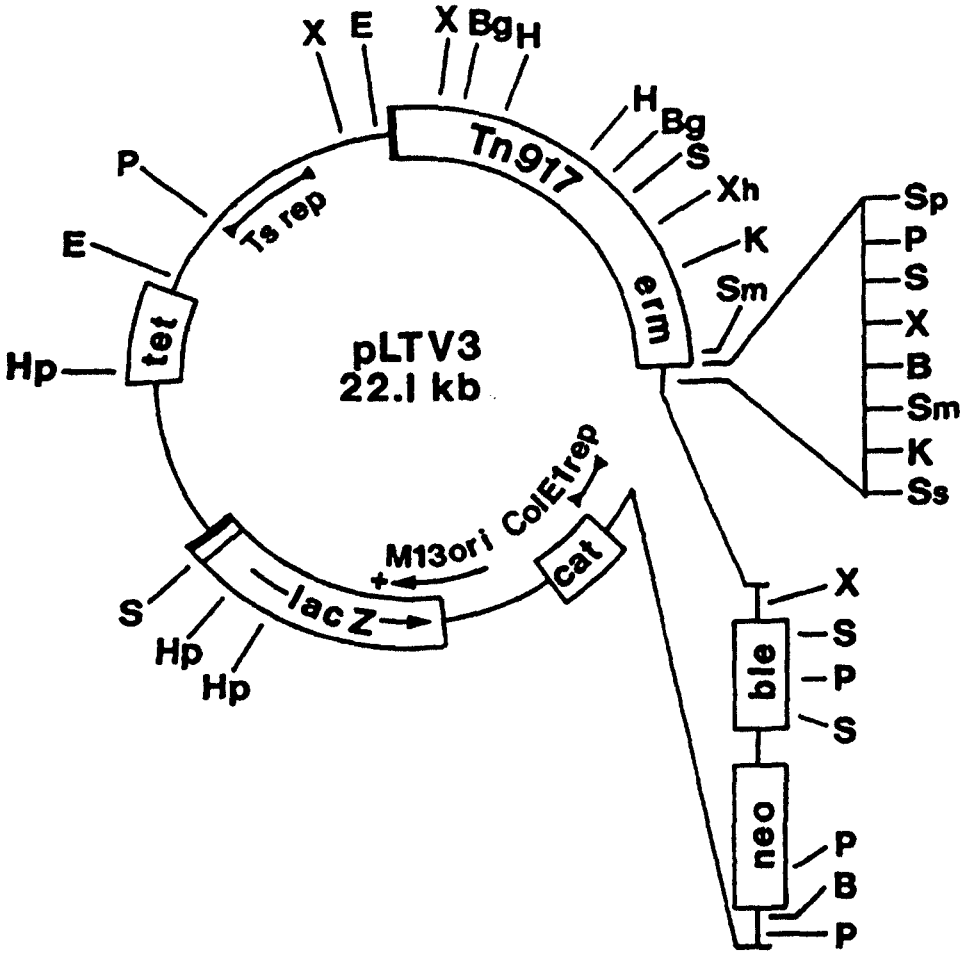


Figure 5.1
Physical map of pLTV3.
Vector harbouring a transposition-proficient derivative of Tn*917*. Thicker lines represent the ends of the transposon.
Features of this map: *erm*, Tn*917* erythromycin resistance determinant (ribosomal methyltransferase gene); *neo* and *ble*, neomycin phosphotransferase II and bleomycin resistance determinants of Tn*5* (Replaced pBR322 β-lactamase gene from pLTV1); ColE1rep, ColE1 origin of replication; M13ori, origin of replication for single stranded DNA synthesis; *cat*, chloramphenicol acetyl transferase gene derived from pC194; *lacZ*, promoterless *lacZ* gene from *E. coli*, with translation initiation signals derived from *B. subtilis*; *tet*, tetracycline resistance gene from pAMα1Δ1; Ts rep, $pE194_{ts}$ temperature sensitive replicon.
Restriction sites: B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; Hp, *Hpa*I; K, *Kpn*I; P, *Pst*I; S, *Sal*I; Sm, *Sma*I; Sp, *Sph*I; Ss, *Sst*I; X, *Xba*I; Xh, *Xho*I.

Reproduced from Youngman (1990).

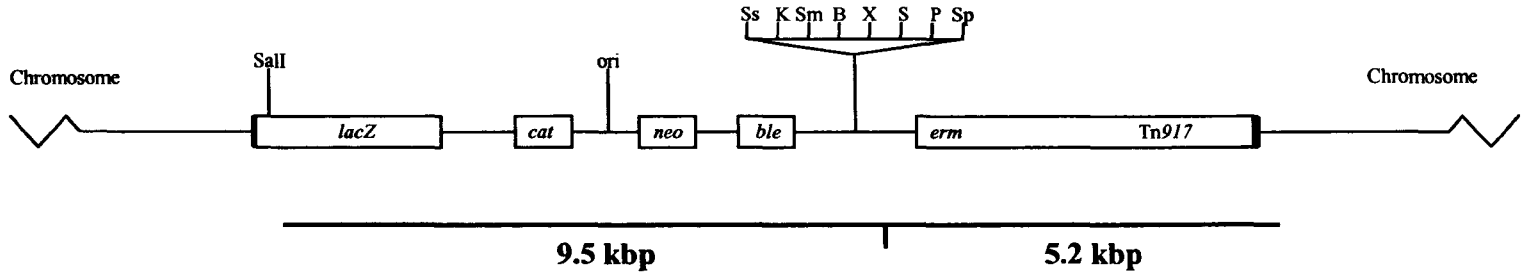


Figure 5.2
Map of Tn*917* inserted into chromosome.

Features of this map: *lacZ'*, a promoterless *lacZ* from *E.coli*, with translation initiation signals derived from *B. subtilis*; *neo* and *ble*, neomycin and bleomycin resistance determinants of Tn*5*; ori, ColE1 origin of replication; *cat*, chloramphenicol acetyl transferase gene derived from pC194; *erm*, a ribosomal RNA methyltransferase gene. Thick lines indicate the ends of the transposon. Restriction sites: B, *Bam*HI; K, *Kpn*I; P, *Pst*I; S, *Sal*I; Sm, *Sma*I; Sp, *Sph*I; Ss, *Sst*I; X, *Xba*I.

Adapted from Youngman (1990).

randomised for the purposes of generation of insertion libraries (Camilli *et al.*, 1990). The transposon is carried by a temperature sensitive derivative of the Gram-positive vector pE194Ts, replication of which is inhibited at temperatures above 37°C, and is flanked by an additional sequence encoding for a tetracycline resistance marker (Camilli *et al.*, 1990). Selection of Tn*917* insertion mutants can be achieved by culture at 40 to 42°C in the presence of erythromycin. Growth at the elevated temperature cures the bacteria of the plasmid, followed by selection of erythromycin resistant cells in which the transposon had been inserted into the chromosome.

The isolation of starvation-survival mutants described below was adapted from that employed by Watson *et al.* (1998b). Selection was based on the fact that cells were able to survive on an agar plate for longer periods at 4°C than when incubated at 37°C. The screen will identify starvation-survival as well as recovery mutants.

5.2 Results

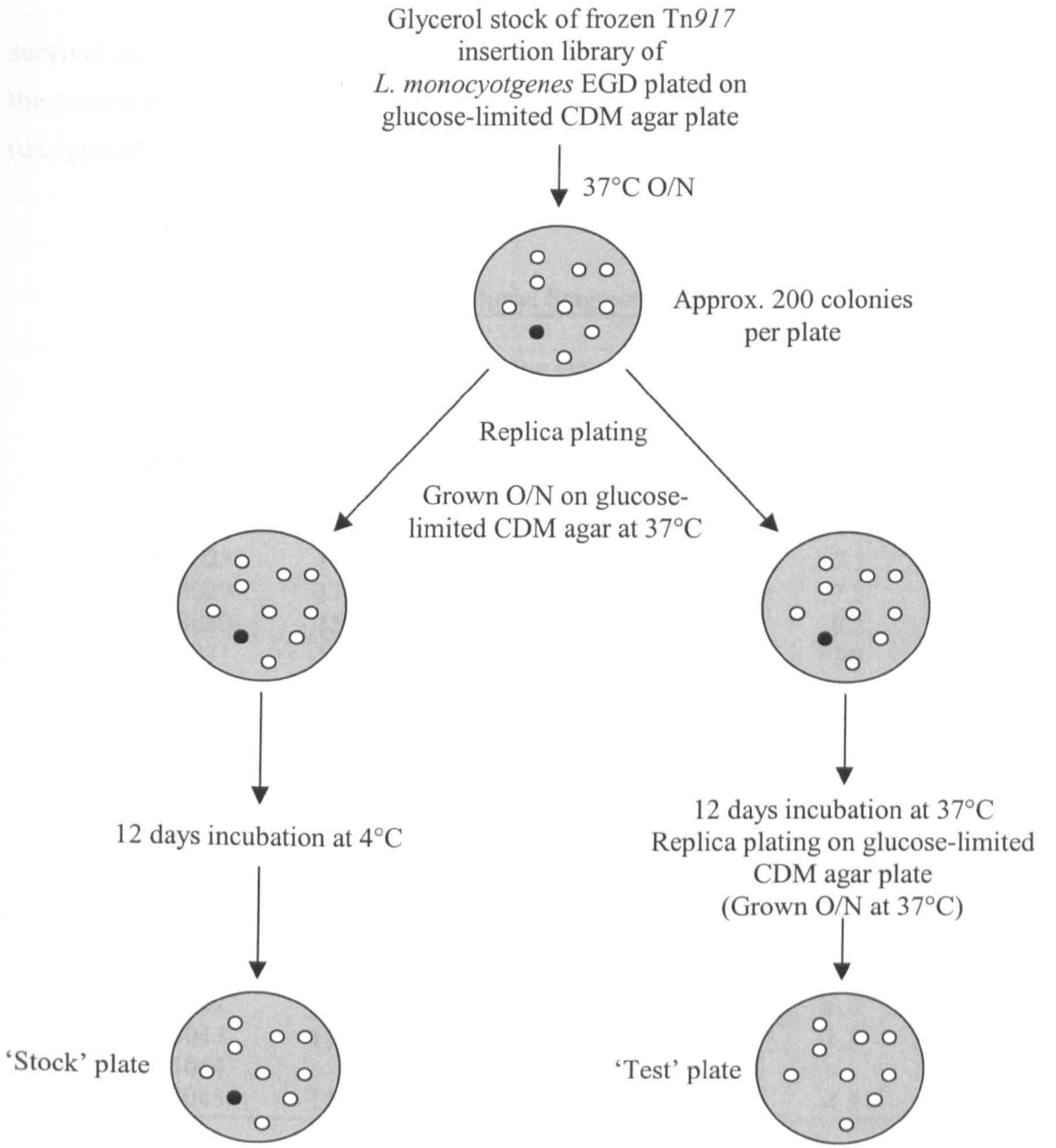
5.2.1 Tn*917* mutagenesis of *L. monocytogenes* EGD

A transposon library was created as described in Chapter 2.14.1. The library contained approximately 2.9×10^{10} CFU ml^{-1} with 94% of cells containing transposon insertions.

5.2.2 Starvation-survival mutant screening

The screen described in Chapter 2.14.2 bases the selection of putative starvation-survival mutants on the survival of individual clones as a colony during incubation for 12 days at 37°C on a glucose limiting CDM agar plate (Figure 5.3). The survival kinetics suggest that under glucose starvation, cells survive for longer periods at 4°C than at 37°C (Chapter 3.2.2). A 'reference' plate was therefore held at 4°C for comparison with the 'test' plate.

Approximately 9,000 colonies from the transposon library were screened and a total of 130 putative starvation-survival mutants were isolated. To validate that the isolates were indeed



Any colonies absent on the test plate (e.g. black colony) are deemed putative starvation-survival mutants. These are picked from the stock plate and screened again for confirmation of the phenotype.

Figure 5.3
Schematic representation of screening protocol for the isolation of starvation-survival mutants.

survival mutants, a second round of screening was performed on the putative mutants. From the secondary screen, only 22 of the 130 putative mutants obtained from the original screen (designated DES024 to DES045) failed to grow after replica plating.

	Digest Fragment Sizes			
	*Pst*I		*Sph*I	
Strain	Total DNA (kbp)	Flanking DNA (kbp)	Total DNA (kbp)	Flanking DNA (kbp)
DES024	17.3	9.3	15.3	7.3
DES025	—	—	—	—
DES026	—	—	—	—
DES027	—	—	—	—
DES028	17.3	9.3	15.3	7.3
DES029	17.3	9.3	15.3	7.3
DES030	15.0	7.0	17.2	9.2
DES031	14.6	6.6	15.3	7.3
DES032	11.4	3.4	15.3	7.3
DES033	12.2	4.2	11.0	3.0
DES034	11.1	3.1	13.0	4.9
DES035	13.6	5.7	12.3	4.3
DES036	—	—	—	—
DES037	—	—	—	—
DES038	14.1	6.1	14.0	6.0
DES039	14.1	6.1	14.0	6.0
DES040	11.0	3.0	11.0	3.0
DES041	12.4	4.4	11.0	3.0
DES042	14.3	6.3	14.0	6.0
DES043	11.0	3.0	11.3	3.3
DES044	—	—	—	—
DES045	13.6	5.6	12.3	4.3

Table 5.1
Genomic DNA hybridising fragment sizes. DNA probed with labelled *Xba*I Tn*917* fragment. The flanking DNA size was calculated from the total hybridising fragment minus the size of the transposon fragment (5kb).

5.2.3 Chromosomal mapping of Tn*917* insertions

To map the site of the transposon insertion, *Pst*I and *Sph*I digests of chromosomal DNA purified from the twenty-one starvation-survival mutant strains were analysed by Southern blot as described in chapter 2.19. The blot was probed using a labelled 5kb *Xba*I fragment from pLTV3 incorporating the *lacZ* distal end of Tn*917* (Figure 5.1). For *Pst*I and *Sph*I digests of each mutant, the probe would be expected to hybridise with a single Tn*917*-containing fragment. This would correspond to 5kb of complete Tn*917* sequence with additional flanking chromosomal DNA sequence (Figures 5.4-5.5) and would be indicative of a single insertion event.

Southern analysis of the digests revealed the occurrence of two transposon insertions into the chromosome in six of the survival mutants (DES025, DES026, DES027, DES036, DES037, DES044) (Table 5.1). Multiple insertions would restrict the ability to characterise the starvation-survival defect observed and as such, were not studied further. In the remaining strains, single bands of hybridisation confirm that only one transposon had inserted into the chromosome. The fact that the sizes of flanking chromosomal DNA vary between strains suggests that the transposon had inserted into several different sites within the chromosome (Table 5.1). Notably however, some strains show similar fragment patterns (DES024, DES028 and DES029; DES035 and DES045; DES038 and DES039). Since all the strains that were isolated originated from the same transposon library, it is possible that these are siblings derived from a single transposition event.

5.2.4 Phage transduction analysis

It was not known whether the starvation-survival phenotype observed in the mutants was due to the inactivation of the gene into which the transposon had inserted, or a naturally occurring mutation, and so it was thus necessary to eliminate the possibility that spontaneous mutation had led to the observed phenotype. Transduction of the transposon insertion for each isolate into the wild type parental (EGD) strain was used to confirm that the phenotype was 100% linked to the transposon insertion. The phage ΦLMUP35 was propagated on the 16 putative

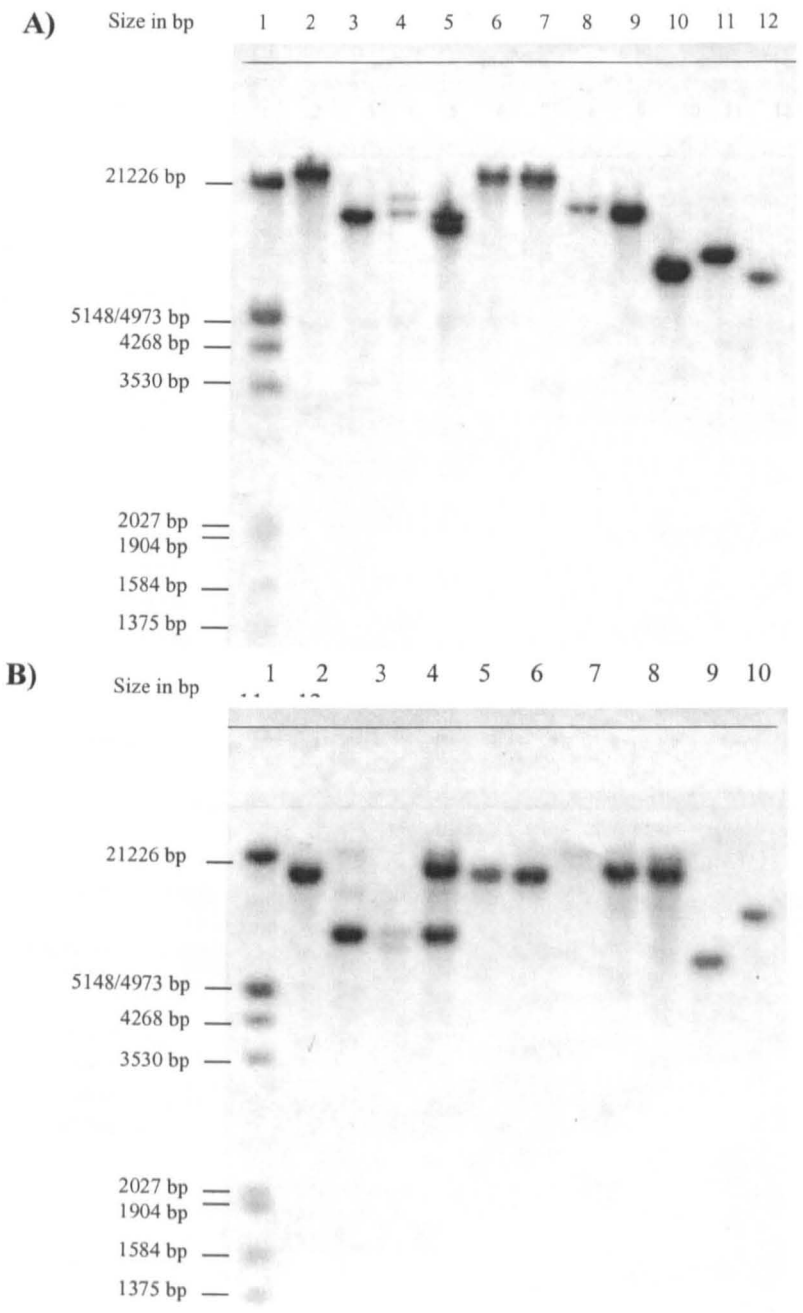


Figure 5.4
Southern blot of chromosomal DNA of putative starvation-survival mutant probed for Tn*917* insertions. Chromosomal DNA was restriction digested and separated by agarose gel electrophoresis, and probed with a fragment internal to Tn*917*. A) *Pst*I digested chromosomal DNA; B) *Sph*I digested chromosomal DNA.

Lanes: 1, λ DNA *Eco*RI/*Hin*dIII digest; 2, DES024; 3, DES025; 4, DES026; 5, DES027; 6, DES028; 7, DES029; 8, DES030; 9, DES031; 10, DES032; 11, DES033; 12, DES034.

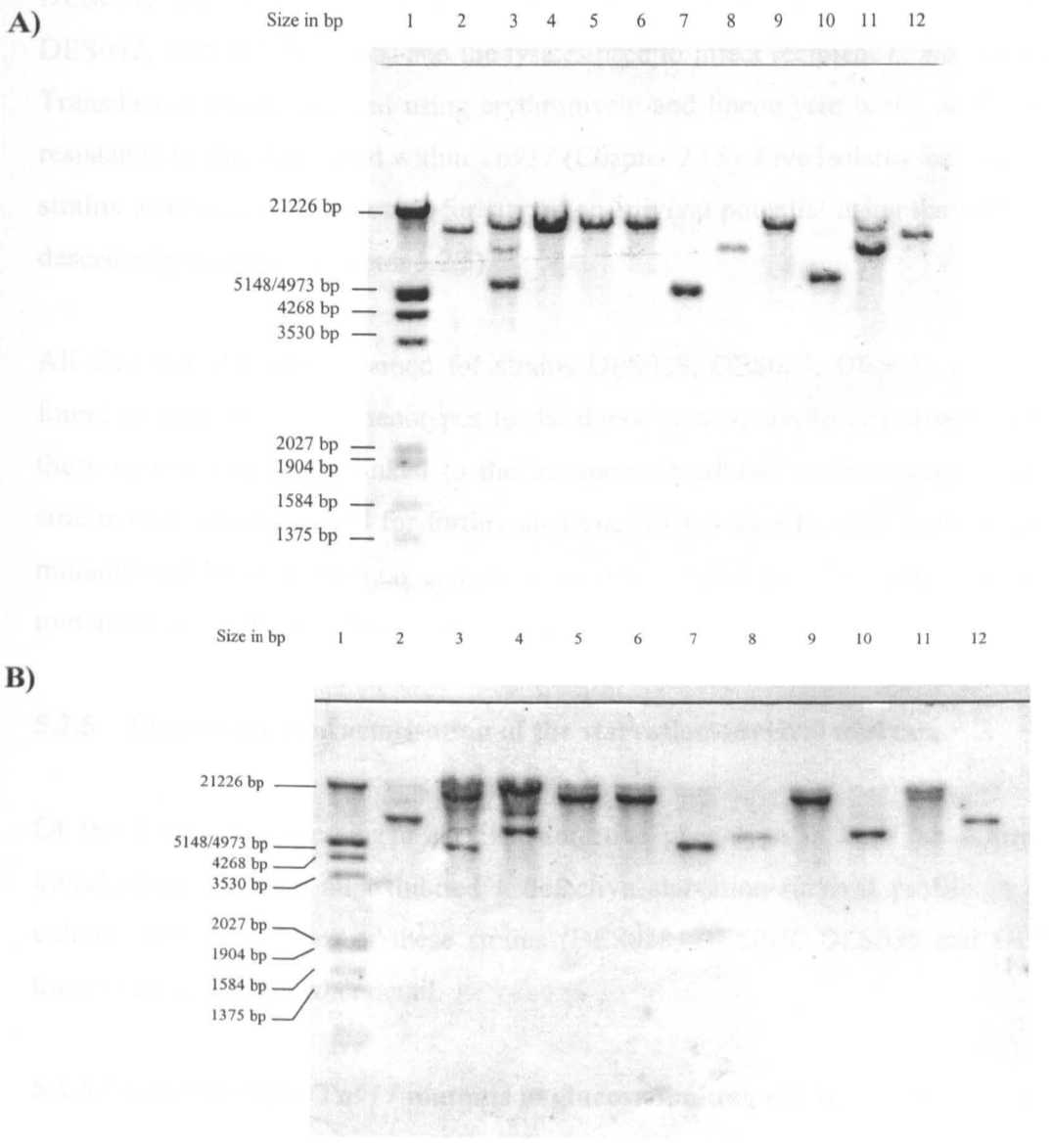


Figure 5.5
Southern blot of chromosomal DNA of putative starvation-survival mutant probed for Tn*917* insertions. Chromosomal DNA was restriction digested and separated by agarose gel electrophoresis, and probed with a fragment internal to Tn*917*. A) *Pst*I digested chromosomal DNA; B) *Sph*I digested chromosomal DNA.

Lanes: 1, λ DNA *Eco*RI/*Hin*dIII digest; 2, DES035; 3, DES036; 4, DES037; 5, DES038; 6, DES039; 7, DES040; 8, DES041; 9, DES042; 10, DES043; 11, DES044; 12, DES045.

mutants containing a single transposon insertion (DES024, DES028, DES029, DES030, DES031, DES032, DES033, DES034, DES035, DES038, DES039, DES040, DES041, DES042, DES043, DES045) and the lysates used to infect recipient *L. monocytogenes* EGD. Transductants were selected using erythromycin and lincomycin based on the erythromycin resistance marker contained within Tn*917* (Chapter 2.15). Five isolates for each of the mutant strains were taken and assessed for starvation-survival potential using the screening protocol described previously (Chapter 5.2.5).

All five transductants obtained for strains DES028, DES029, DES035 and DES045 were found to have identical phenotypes to the donor strains, confirming that the phenotype for these strains was 100% linked to the transposon-mediated erythromycin resistance. These strains were taken forward for further analysis. All the transductants from the other original mutants exhibited a parental starvation-survival phenotype, thus the phenotype of these mutants was not due to transposon insertion.

5.2.5 Phenotypic characterisation of the starvation-survival mutants

Of the 4 strains possessing a survival defective phenotype in both the screen and phage transduction analysis, all exhibited a defective starvation-survival profile in liquid batch culture. The phenotypes of these strains (DES028, DES029, DES035 and DES045) were therefore studied in further detail.

5.2.5.1 Growth of the Tn*917* mutants in glucose-limiting CDM at 37°C

The ability of strains DES028, DES029, DES035 and DES045 to grow in liquid batch culture in glucose-limiting CDM at 37°C was compared to that of the wild type parental strain. Inoculation at A_{600} 0.05 resulted in growth that was comparable to that of EGD, with all strains reaching a final yield of A_{600} 0.7 to A_{600} 0.8 after 10 hours (Figures 5.6 and 5.7).

5.2.5.2 Starvation-survival in glucose-limiting CDM at 37°C

From the starvation-survival screen, the isolates DES028 and DES029 were found to become unculturable after 12 days incubation on glucose-limiting CDM agar plates (Figure 5.8A). The kinetics of cell death over time for DES028 and DES029 in glucose-limiting CDM liquid culture was monitored and compared to that of the parental strain. Bacteria were recovered by plating on TSB agar. During glucose starvation, the number of viable cells drops by approximately 99% over the first 48 hours, a fall that parallels that of the wild type strain (Figure 5.9). Beyond this point, the fall in viability in cultures of DES028 and DES029 slowed, but continued to fall faster than that seen in EGD. As a result, viability in cultures of the mutant strains was approximately 10-fold lower than the wild type strain after 15 days. Over the following 5 days however, the viable count rises slightly resulting in wild type levels of viability after 20 days, possibly via the occurrence of suppressor mutations during starvation.

The mutant strains DES035 and DES045 became unculturable after 12 days incubation on glucose-limiting CDM agar plates (Figure 5.8B). Survival of DES035 and DES045 in liquid cultures of glucose-limiting CDM incubated at 37°C was compared to that of the wild type strain. When incubated at 37°C in glucose-limiting CDM, viability in cultures of DES035 and DES045 followed a wild type pattern of survival for the first 15 days of starvation (Figure 5.9). During the first 48 hours a 99% fall in viability was seen before the rate of cell death slowed considerably. After 15 days, approximately 0.1% of cells were still viable, a level similar to EGD. Over the ensuing five days however, viability fell to approximately 10% of that seen in the wild type cultures.

To determine whether the number of bacteria of the four Tn*917* mutant strains that could be recovered during starvation was affected by the type of recovery medium used, samples were also plated onto glucose-limiting CDM agar plates and incubated overnight at 37°C (Figure 5.10). The viable counts obtained by recovery on glucose-limiting CDM agar plates were comparable to those obtained on TSB agar (Figures 5.9 and 5.10). Likewise, all four

starvation defective mutant strains showed no significant difference in survival kinetics or the overall viable counts obtained by the two methods of recovery.

5.2.5.3 Starvation-associated stress resistance of the Tn*917* mutants

To further characterise the phenotype of the starvation mutants, the development of resistance to acid, heat and oxidative stress during glucose starvation was determined for the strains DES028, DES029 DES035 and DES045.

When exposed to pH 3.5, both the mutant strains DES028 showed levels of acid stress resistance throughout growth and starvation that were similar to those seen in the parental strain (Figures 5.11). Wild type levels of resistance were found upon exposure of DES028 cells to 55°C during exponential- and post-exponential-phase (Figures 5.12). In contrast, long-term starved cells were highly sensitive to heat stress, becoming unculturable after 5 minutes compared to EGD, where 10% of cells remain viable after 18 minutes. DES028 also exhibited sensitivity to oxidative stress during all phases of growth and starvation relative to wild type cells (Figures 5.13). Exponential phase cells were approximately 10 times more sensitive to H_2O_2 than the corresponding EGD cells (after 30 minutes), whilst viability of post-exponential phase DES028 cells was reduced 45-fold after 30 minutes. Significantly, the viability of long-term starved DES028 cells became undetectable after 30 minutes, whereas approximately 0.2% of EGD cells remained viable after 50 minutes exposure to 7.5 mM H_2O_2 (Figures 5.13). The starvation-survival mutant DES029 was found to possess patterns of heat, acid and oxidative stress identical to DES028. Molecular analysis confirmed that the two strains were siblings (see below).

During glucose starvation, DES035 cells were tested for their resistance to acid, heat and oxidative stress. The resistance of DES035 cells to acid stress (Figures 5.14) and heat stress (Figures 5.15) were found to be similar at all stages of growth and starvation to those observed in the corresponding EGD cells. DES035 cells were 10 times more sensitive to oxidative stress than the corresponding EGD cells in exponential phase (after 20 minutes).

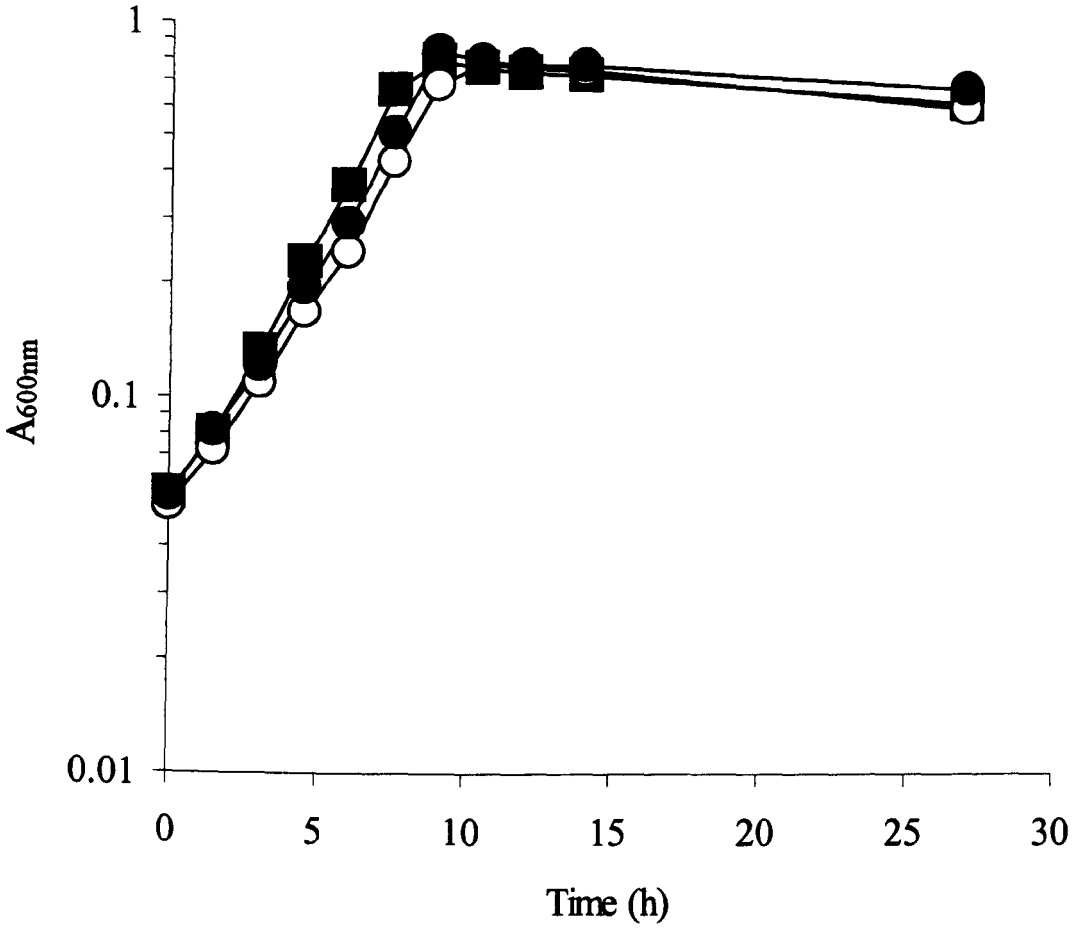


Figure 5.6

Comparison of the growth of EGD (■), DES028 (●) and DES029 (O) in glucose-limited CDM at 37°C. Data are an average of at least two separate experiments.

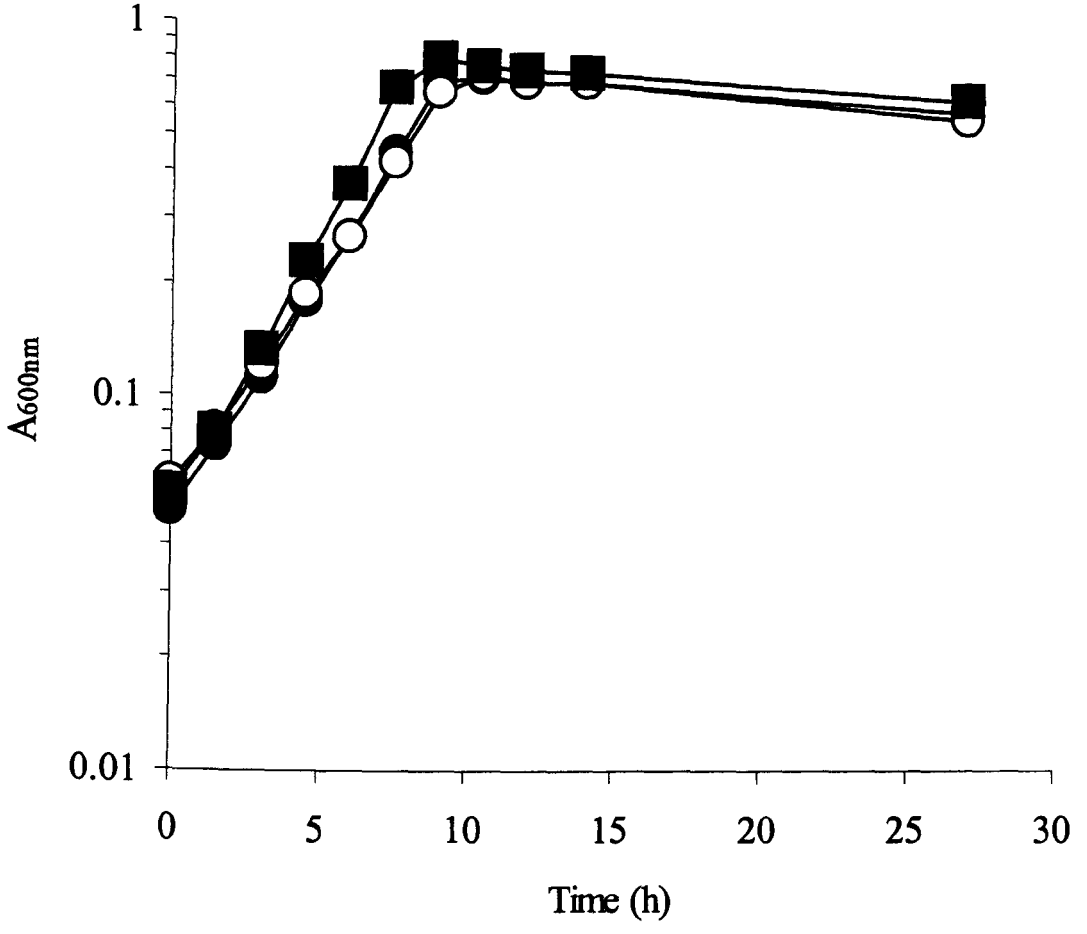


Figure 5.7

Comparison of the growth EGD (■), DES035 (●) and DES045 (O) in glucose-limited CDM at 37°C. Data are an average of at least two separate experiments.

A)

B)

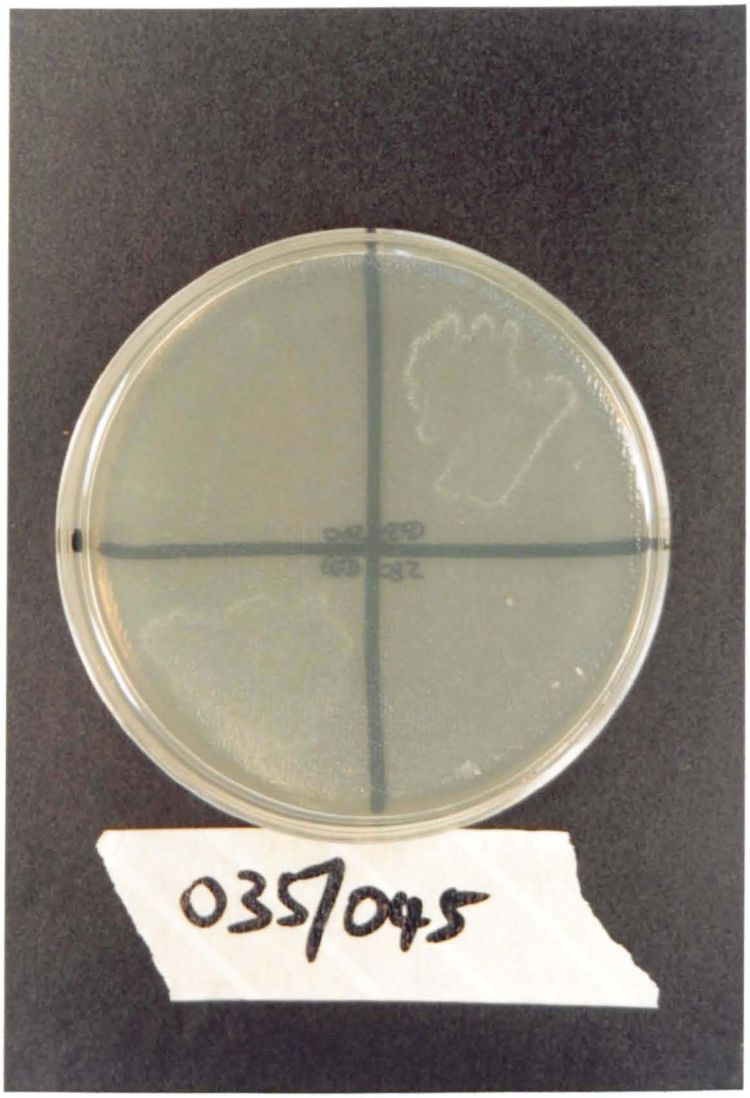


Figure 5.8

A) Previous page.
Recovery of *L. monocytogenes* EGD (Top left/Bottom right), DES028 (Bottom left) and DES029 (Top right) from glucose-limited plate screen. Screen conducted as previously described (Figure 5.3). After incubation at 37°C for 12 days, the test plate was replicated onto glucose-limited CDM agar and incubated overnight at 37°C.

B) Above.
Recovery of *L. monocytogenes* EGD (Top right/Bottom left), DES035 (Bottom right) and DES045 (Top left) from glucose-limited plate screen. Screen conducted as previously described (Figure 5.3). After incubation at 37°C for 12 days, the test plate was replicated onto glucose-limited CDM agar and incubated overnight at 37°C.

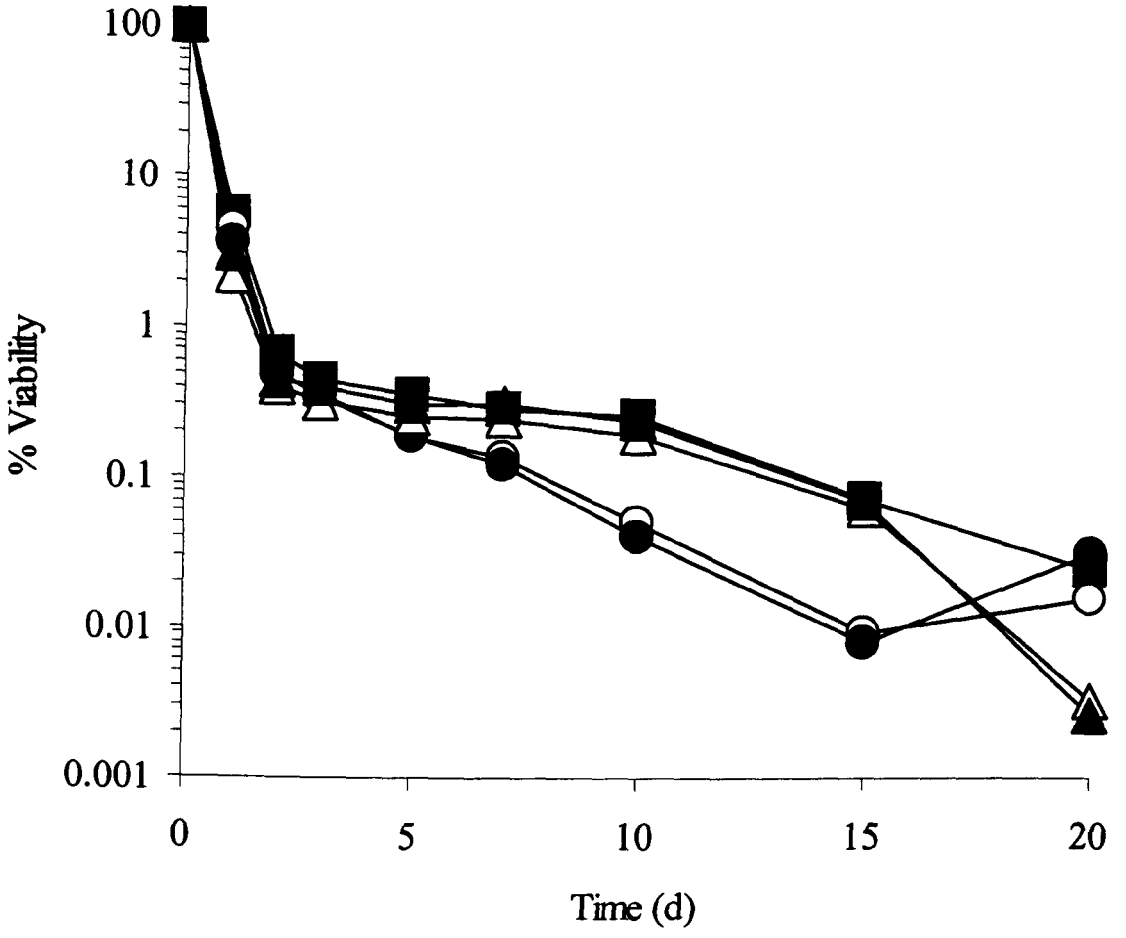


Figure 5.9

Comparison of the starvation-survival kinetics of EGD (■), DES028 (○), DES029 (●), DES035 (Δ) and DES045 (▲) in glucose-limited CDM at 37°C. Bacteria were recovered on TSB Agar plates. 100% viability was approximately 1×10^9 CFU ml^{-1}. Data are an average of at least two separate experiments for which the standard deviation was no greater than ±40% of the mean value at equivalent time points.

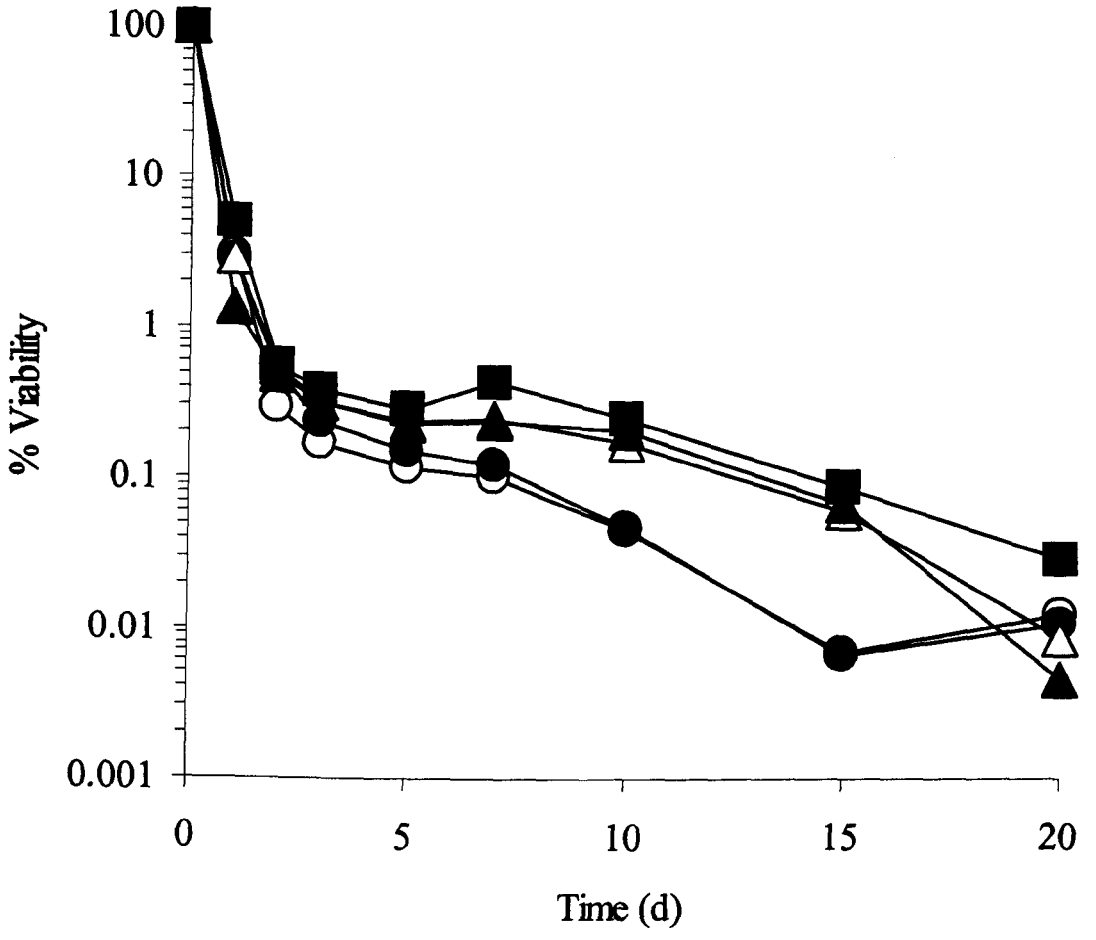


Figure 5.10

Comparison of the starvation-survival kinetics of EGD (■), DES028 (O), DES029 (●) DES035 (Δ) and DES045 (▲) in glucose-limited CDM at 37°C. Recovery of bacteria on glucose-limited CDM Agar plates. 100% viability was 1×10^9 CFU ml^{-1}. Data are an average of at least two separate experiments for which the standard deviation was no greater than ±40% of the mean value at equivalent time points.

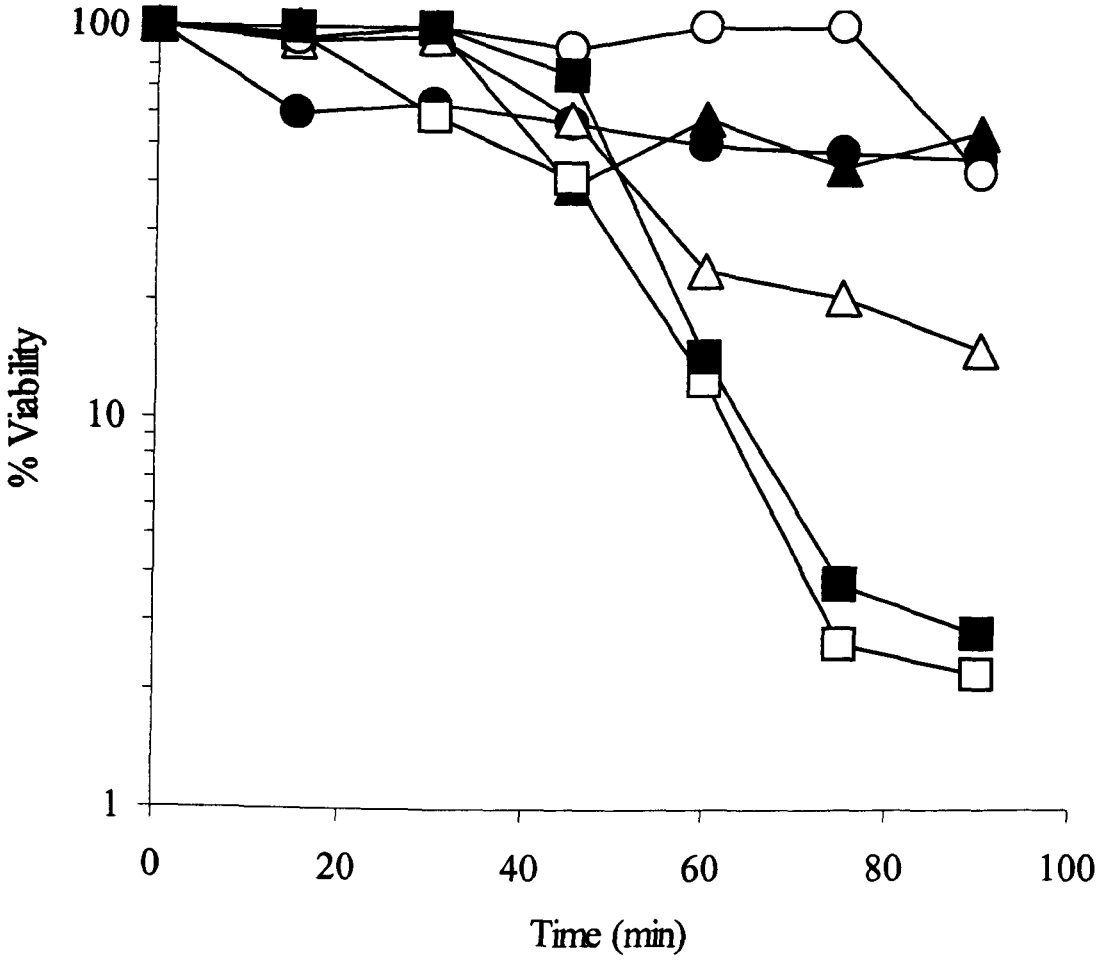


Figure 5.11

Comparison of the acid stress resistance (pH 3.5) of EGD (closed symbols) and DES028 (open symbols) in PBS at 37°C.

Exponential (■,□), post-exponential (▲,Δ), 7-day glucose starved (●,○). 100% viability was approximately 5×10^6 CFU ml^{-1}. Data are an average of at least two separate experiments for which the standard deviation was no greater than ±40% of the mean value at equivalent time points.

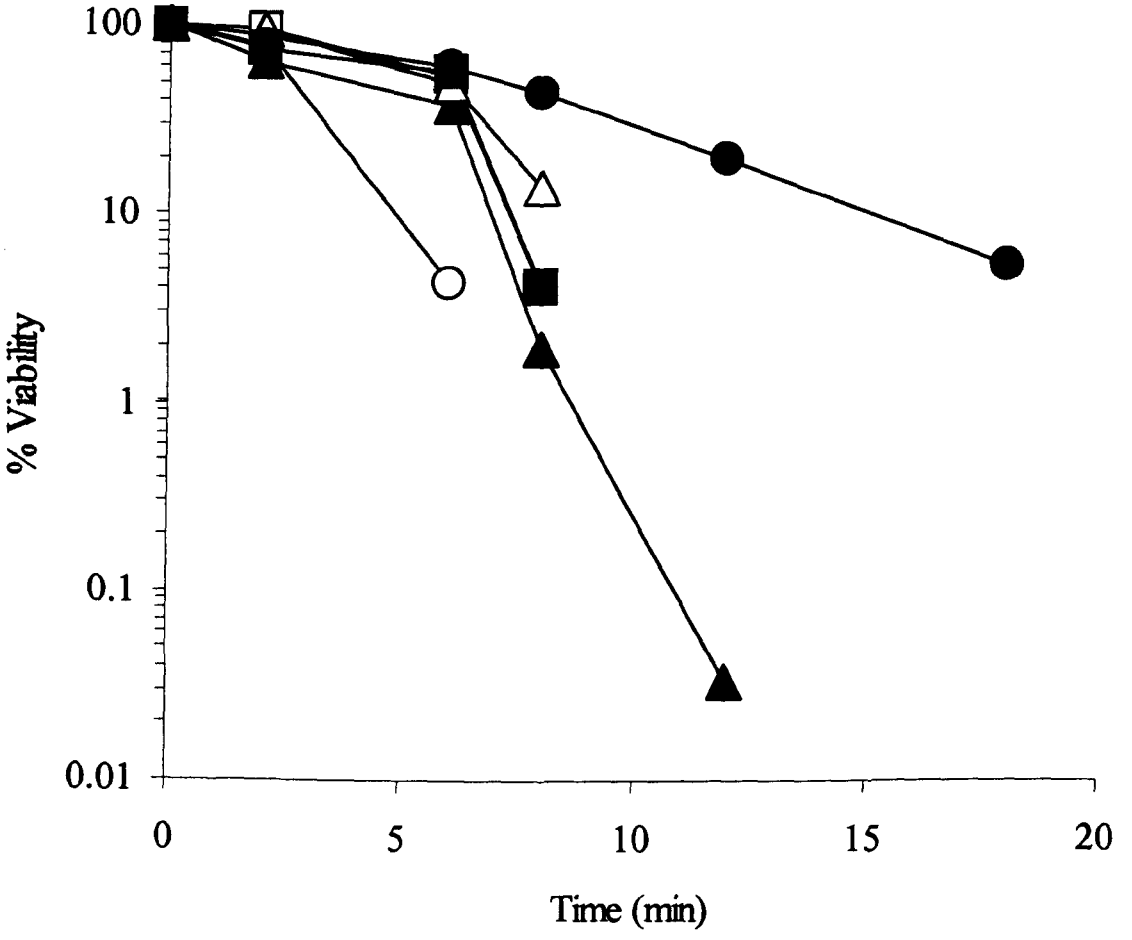


Figure 5.12
Comparison of the heat stress resistance (55°C) of EGD (closed symbols) and DES028 (open symbols) in PBS.

Exponential (■,□), post-exponential (▲,Δ), 7-day glucose starved (●,O). 100% viability was approximately 5 × 10^6 CFU ml^{-1}. Data are an average of at least two separate experiments for which the standard deviation was no greater than ±40% of the mean value at equivalent time points.

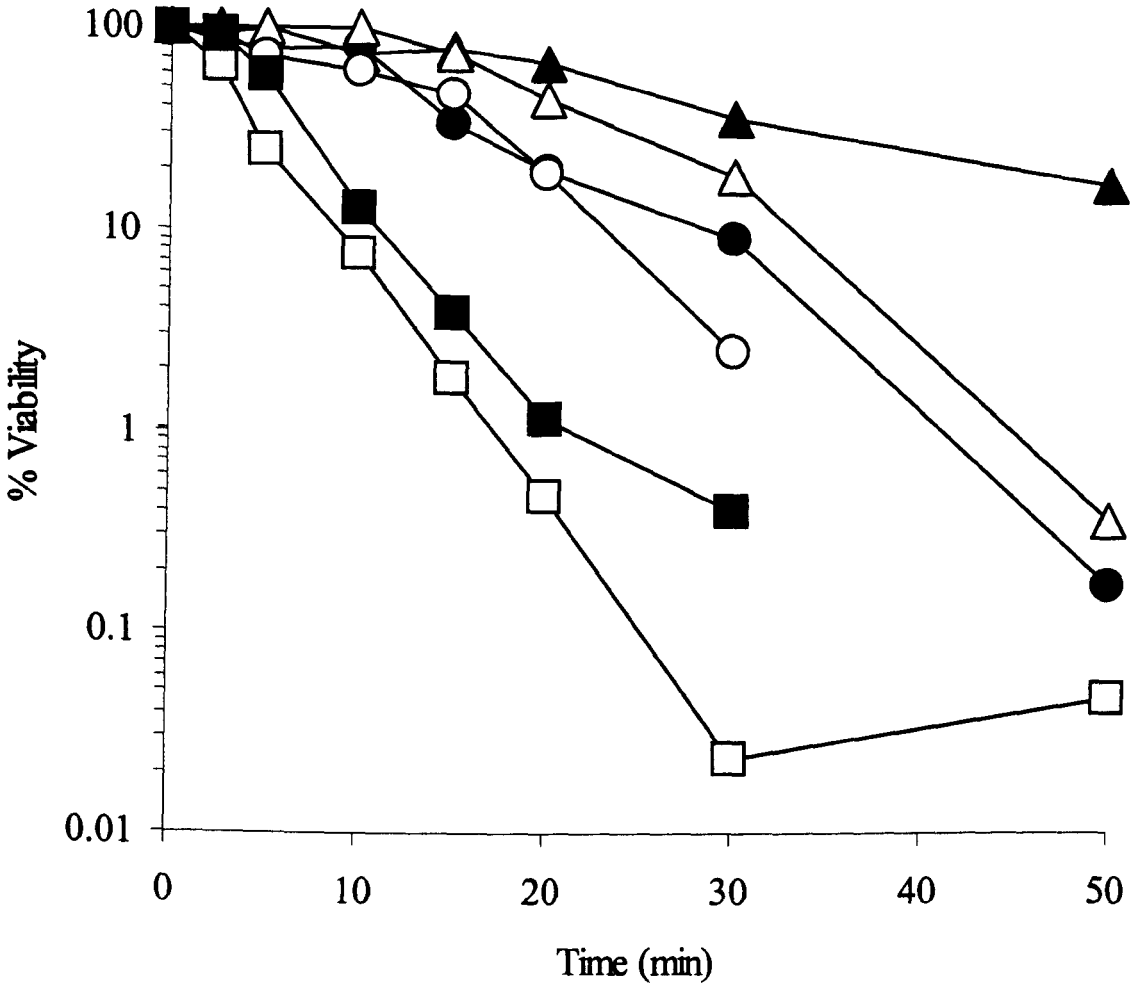


Figure 5.13
Comparison of the H_2O_2 resistance (7.5mM) of EGD (closed symbols) and DES028 (open symbols) in PBS at 37°C.

Exponential (■,□), post-exponential (▲,Δ), 7-day glucose starved (●,○). 100% viability was approximately 5×10^6 CFU ml^{-1}. Data are an average of at least two separate experiments for which the standard deviation was no greater than ±40% of the mean value at equivalent time points.

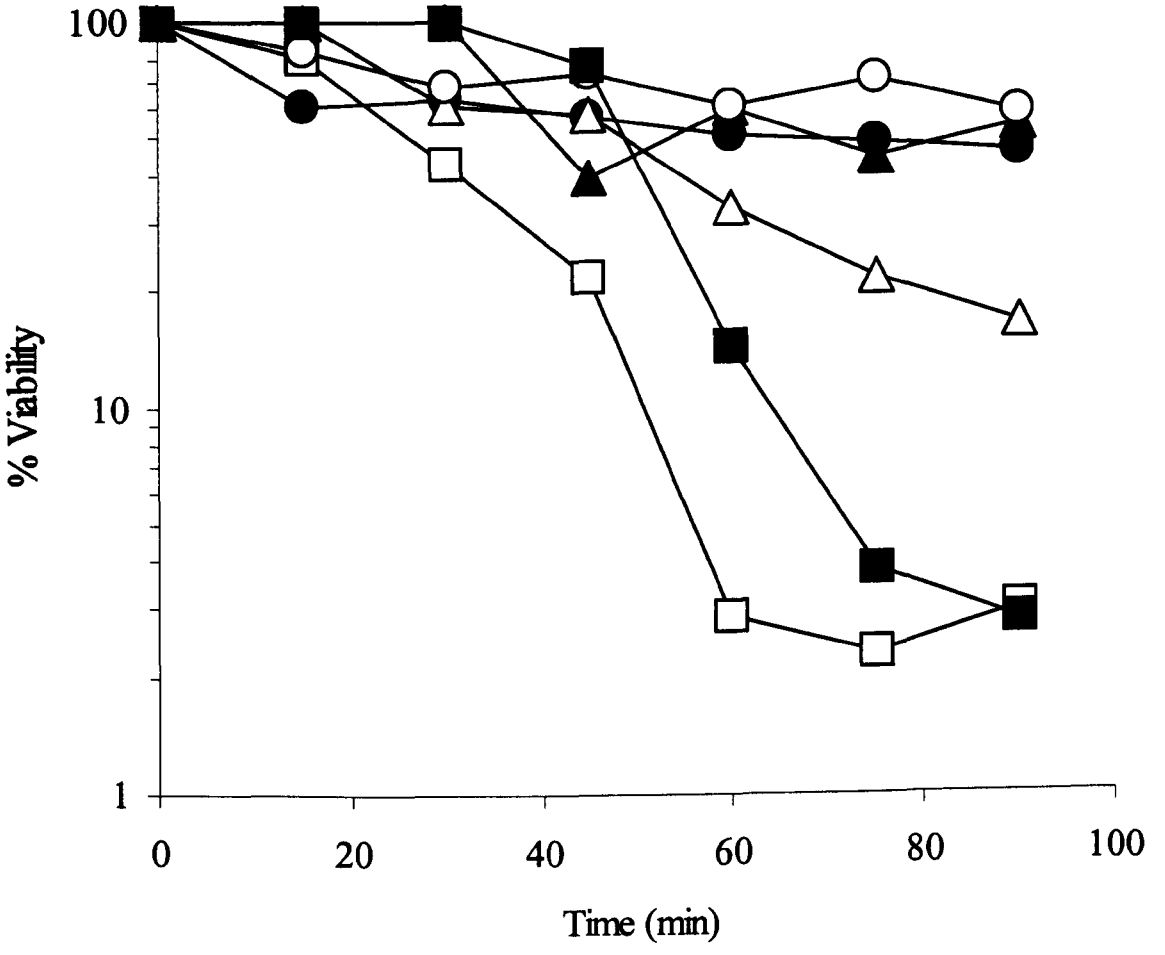


Figure 5.14
Comparison of the acid stress resistance (pH 3.5) of EGD (closed symbols) and DES035 (open symbols) in PBS at 37°C.

Exponential (■,□), post-exponential (▲,Δ), 7-day glucose starved (●,○). 100% viability was approximately 5×10^6 CFU ml^{-1}. Data are an average of at least two separate experiments for which the standard deviation was no greater than ±40% of the mean value at equivalent time points.

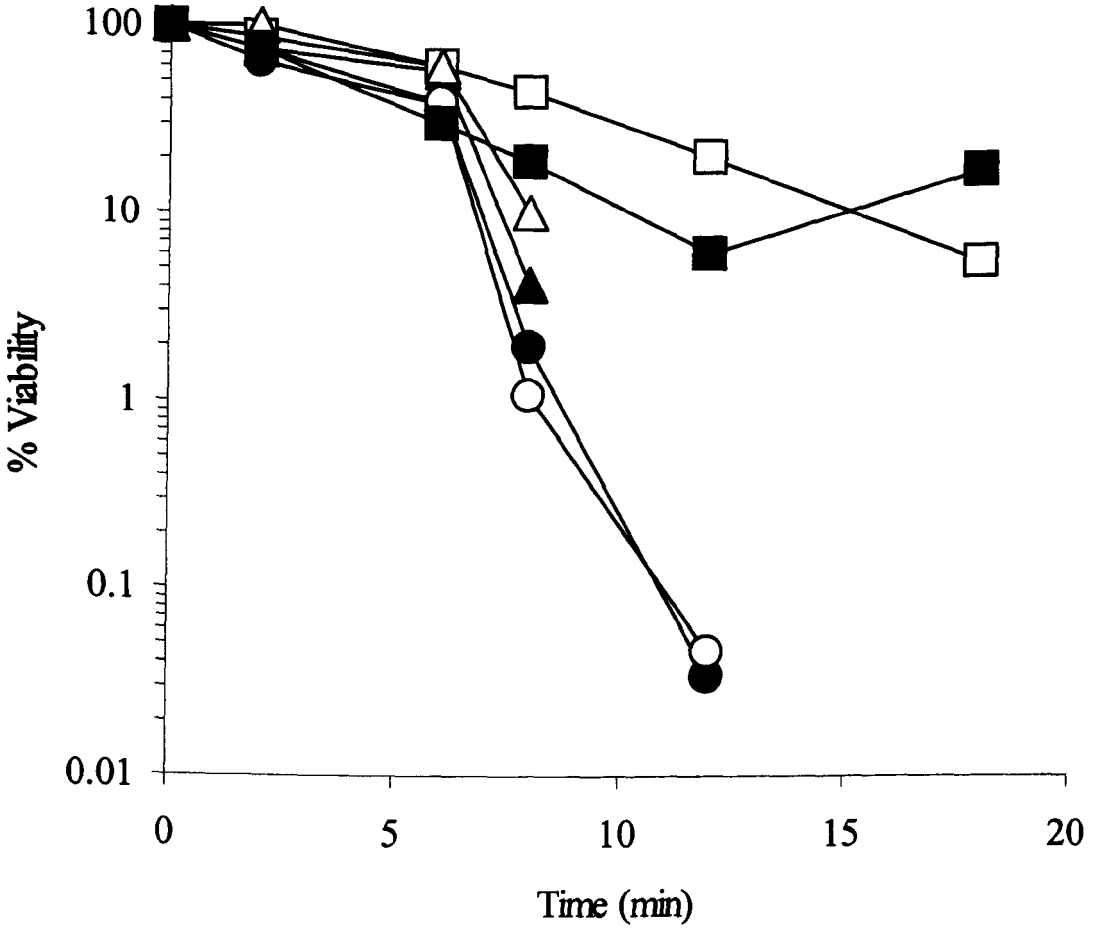


Figure 5.15
Comparison of the heat stress resistance (55°C) of EGD (closed symbols) and DES035 (open symbols) in PBS.

Exponential (■,□), post-exponential (▲,Δ), 7-day glucose starved (●,○). 100% viability was approximately 5×10^6 CFU ml^{-1}. Data are an average of at least two separate experiments for which the standard deviation was no greater than ±40% of the mean value at equivalent time points.

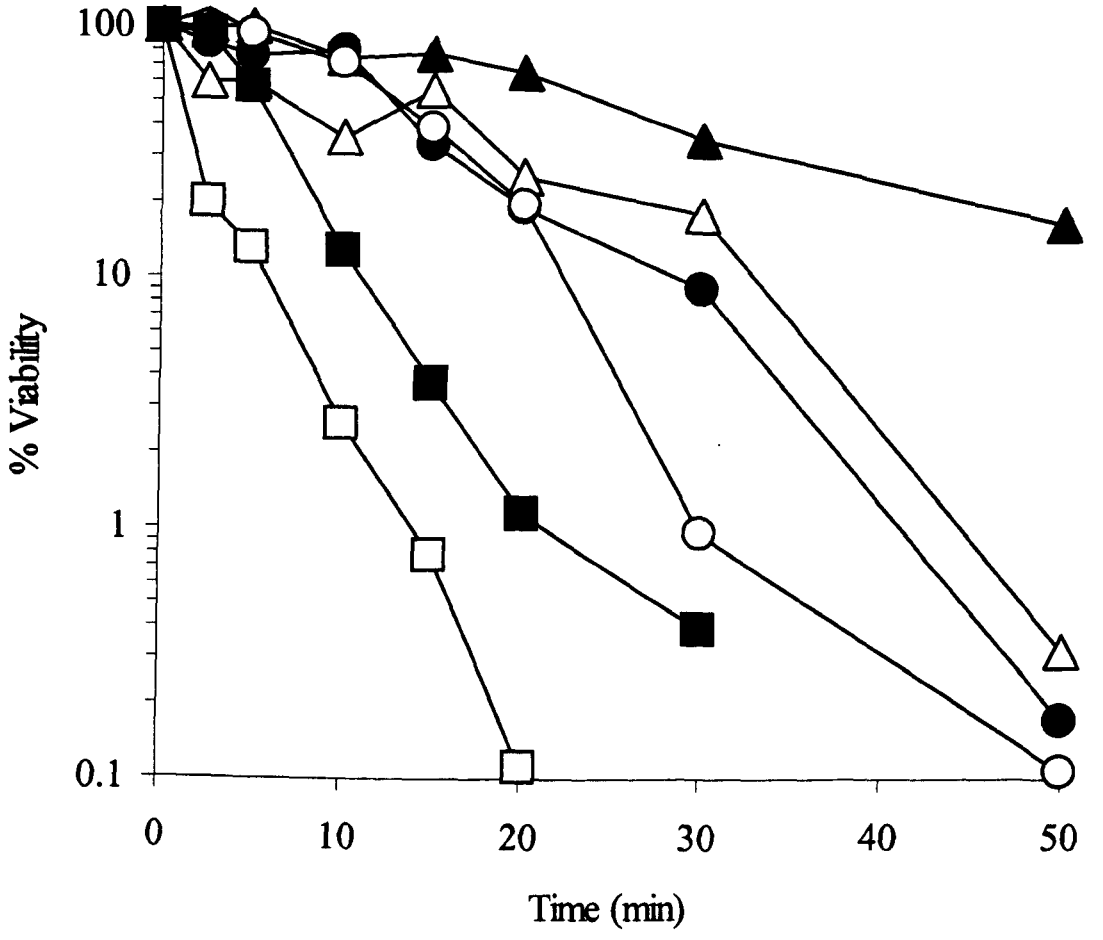


Figure 5.16
Comparison of the H_2O_2 resistance (7.5mM) of EGD (closed symbols) and DES035 (open symbols) in PBS at 37°C.

Exponential (■,□), post-exponential (▲,Δ), 7-day glucose starved (●,○). 100% viability was approximately 5 × 10^6 CFU ml^{-1}. Data are an average of at least two separate experiments for which the standard deviation was no greater than ±40% of the mean value at equivalent time points.

Similarly, DES035 cells starved for glucose for 7 days were 50 times more sensitive than wild type cells of the same age (after 50 minutes) (Figures 5.16). Observation of the stress resistance of DES035 revealed patterns of heat, acid and oxidative stress matching that of DES045. Molecular analysis confirmed that the two strains were siblings (see below).

5.2.6 Molecular analysis of the Tn*917* mutant insertion sites

Having verified the existence of a starvation-survival defective phenotype in the four Tn*917* mutant strains, further investigation was performed into the nature of the mutations involved. Using a 25 bp oligonucleotide primer complementary to the *lacZ* proximal end of Tn*917*, chromosomal DNA flanking the insertion was sequenced as described in chapter 2.22.

5.2.6.1 Direct genomic sequencing and analysis of the Tn*917* insertion sites in DES028 and DES029

In order to analyse the chromosomal DNA flanking the transposon insertion, rescue using the ColE1 origin of replication present in the Tn*917*-LTV3 insertion was attempted as previously described (Watson *et al.*, 1998b). Selection on the basis of neomycin-resistance failed to isolate transformants containing the plasmid incorporated flanking chromosomal DNA. As an alternative, genomic sequencing PCR was used as described in chapter 2.21. This protocol allows sequence to be obtained directly from genomic DNA and was the first time such a method has been used in *L. monocytogenes*. Following the results obtained from chromosomal mapping of the strains DES028 and DES029 and the likelihood that they may be siblings, the genomic sequence obtained for each strain was compared. Gapped alignment revealed 85.1% (165/194 bases) identity between the sequences obtained for DES028 and DES029 (Figure 5.17), thus confirming that DES028 and DES029 were indeed siblings.

The genomic sequence from DES028 was used to search the *L. monocytogenes* EGD genomic DNA sequence database at the Pasteur Institute, Paris (Glaser, 2000). When reversed and complemented, the DES028 sequence was found to align with bases 8842 to 9092 of a 10.5 kbp contig (Figure 5.18). Analysis of the contig sequence determined that the

insertion had occurred 129 bp into a putative ORF which was number 11 in a total of 12 putative ORFs identified within the contig. ORF11 encodes for a 28 kDa protein of 254 amino acids in length and the potential polypeptide produced would be truncated after 43 amino acids (Figure 5.19).

The hydropathy profile of the polypeptide revealed no highly hydrophobic regions, suggestive of a cytoplasmic protein, and the pI of polypeptide was 6.02 (Figure 5.20). BLAST analysis of the amino acid sequence revealed that the putative protein had homology to YulB and GlcR of *B. subtilis* (35% over 250 amino acids and 29% over 242 amino acids respectively) and GatR of *E. coli* (29% over 254 amino acids). All 3 homologues are members of the DeoR family of transcriptional regulators (Kunst *et al.*, 1997; Nobelmann and Lengeler, 1996) (Figure 5.21).

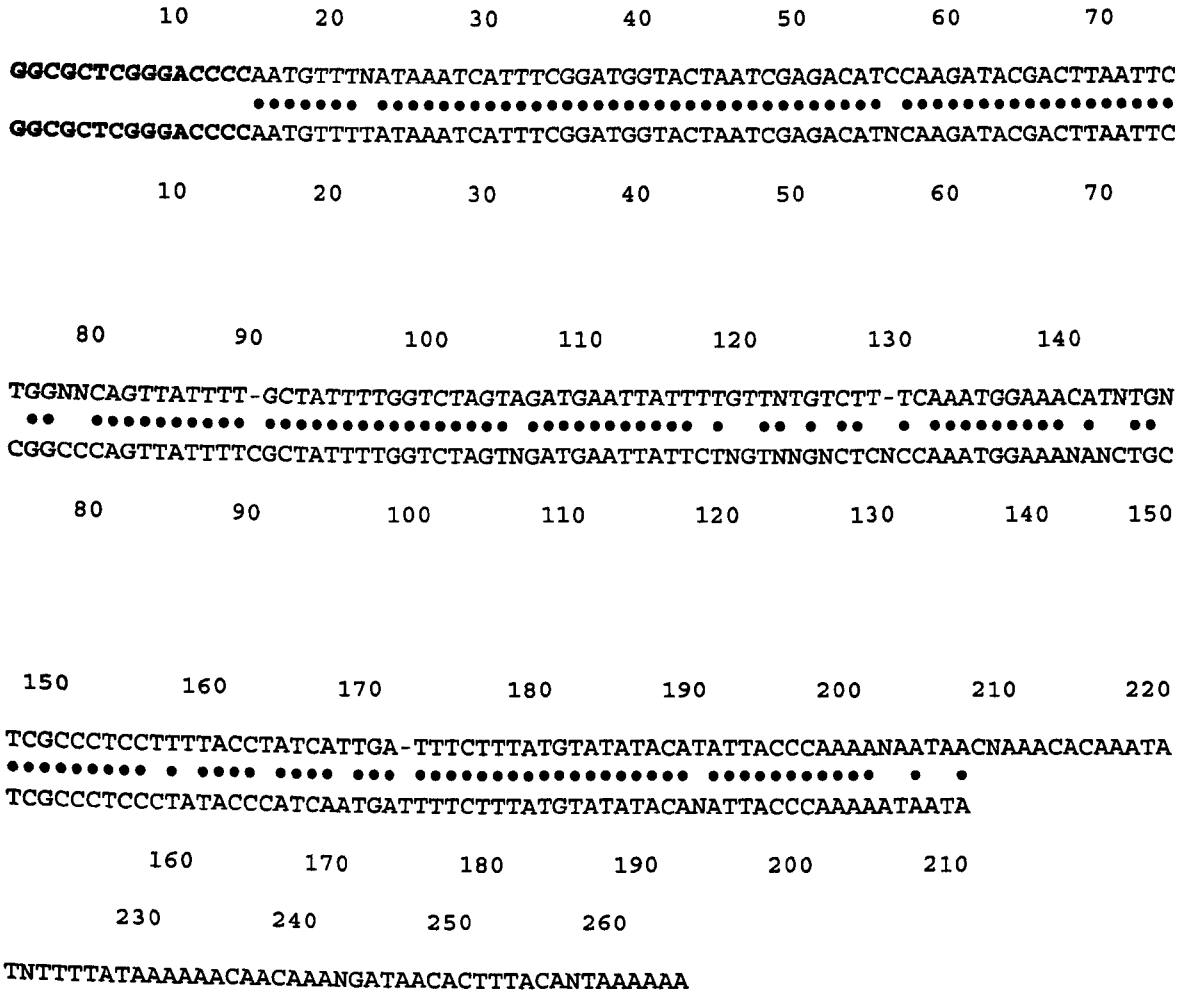
The initiation codon (ATG) is situated at position 181 (Figure 5.19) and visual inspection of the region immediately upstream identified a potential ribosome binding site (Figures 5.19 and 5.22). Putative promoter sequences were found upstream of the start codon homologous to that recognised by the alternative sigma factor σ^B (Figures 5.19 and 5.22). Inspection of the region downstream of the translation stop codon TAA revealed a potential transcription terminator located at position 949, 5 bp beyond the end of the ORF. This region could form an 18 bp stem loop with a ΔG of -14.6 kcal mol^{-1} and contains a thymine-rich region immediately following a G-C rich sequence, characteristic of a Rho-independent transcription terminator (Pribnow, 1979).

Analysis of the contig sequence around the *yulB* homologue revealed an operon of 10 putative ORFs upstream (Figure 5.23). The operon is encoded divergently from the *yulB* homologue (Figure 5.23) and the two promoter regions are separated by just 33 bp (Figure 5.19). Upstream of the coding sequences for this operon are a putative σ^A-dependent promoter and a ribosome binding site 6 bp upstream of the initiation codon ATG of the first ORF (ORF10) (Figures 5.19 and 5.22). A potential Rho-independent transcription terminator stem loop with a ΔG value of -18.0 kcal mol^{-1} was identified 407 bp downstream of the final ORF (ORF1). BLAST analysis of the 10 putative polypeptides from the operon revealed that

eight possessed significant homology to previously identified genes (Table 5.2). Immediately downstream of the *yulB* homologue (59 bp) is a putative ORF encoding for a homologue of AttT from *Agrobacterium tumefaciens* (Table 5.2). A putative ribosome binding site is found 8 bp from the ATG start codon, but no likely promoter sequences could be found. A Rho-independent transcription terminator stem loop with a ΔG value –25.0 kcal mol^{-1} was found, the base of which overlaps the stop codon (TAA).

	Homologue (Species)	Putative Function	Identity	Accession No.
ORF1	*tkt* *(B. subtilis)*	Transketolase	56% (371/676 amino acids)	P45694
ORF2	*ywjH* *(B. subtilis)*	Transaldolase	58% (98/167 amino acids)	P19669
ORF3	*ywfD* *(B. subtilis)*	Glucose-1-dehydrogenase	34% (85/250 amino acids)	P39640
ORF4	*rpiB* *(E. coli)*	Ribose-5-phosphate Isomerase B	42% (61/145 amino acids)	P37351
ORF5	*tpiS* *(Lactococcus delbruekii)*	Triosephosphate Isomerase	31% (79/248 amino acids)	O32757
ORF6	*ycgS* *(E. coli)*	Dihydroxyacetone Kinase	43% (83/191 amino acids)	P76014
ORF7	*ycgT* *(E. coli)*	Dihydroxyacetone Kinase	32% (116/352amino acids)	P76015
ORF8	No significant homology	—	—	—
ORF9	No significant homology	—	—	—
ORF10	*ycgC* *(E. coli)*	Phosphotransferase System Enzyme I	30% (34/111 amino acids)	P373349
ORF11	*yulB* *(B. subtilis)*	DeoR family transcriptional regulator.	35% (89/250 amino acids)	D70014
ORF12	*attT* *(A. tumefaciens)*	Attachment/Virulence plasmid gene	35% (89/250 amino acids)	U59485

Table 5.2
BLAST analysis of putative ORFs flanking ORF No.11 (DES028/DES029).



Gaps in sequence (DES028) = 3

Figure 5.17
Alignment of sequence obtained from genomic sequencing of the Tn*917* insertion site in DES028 (Top) and DES029 (Bottom). Sequence in **bold**: Sequence matching *lacZ* proximal end of Tn*917*.

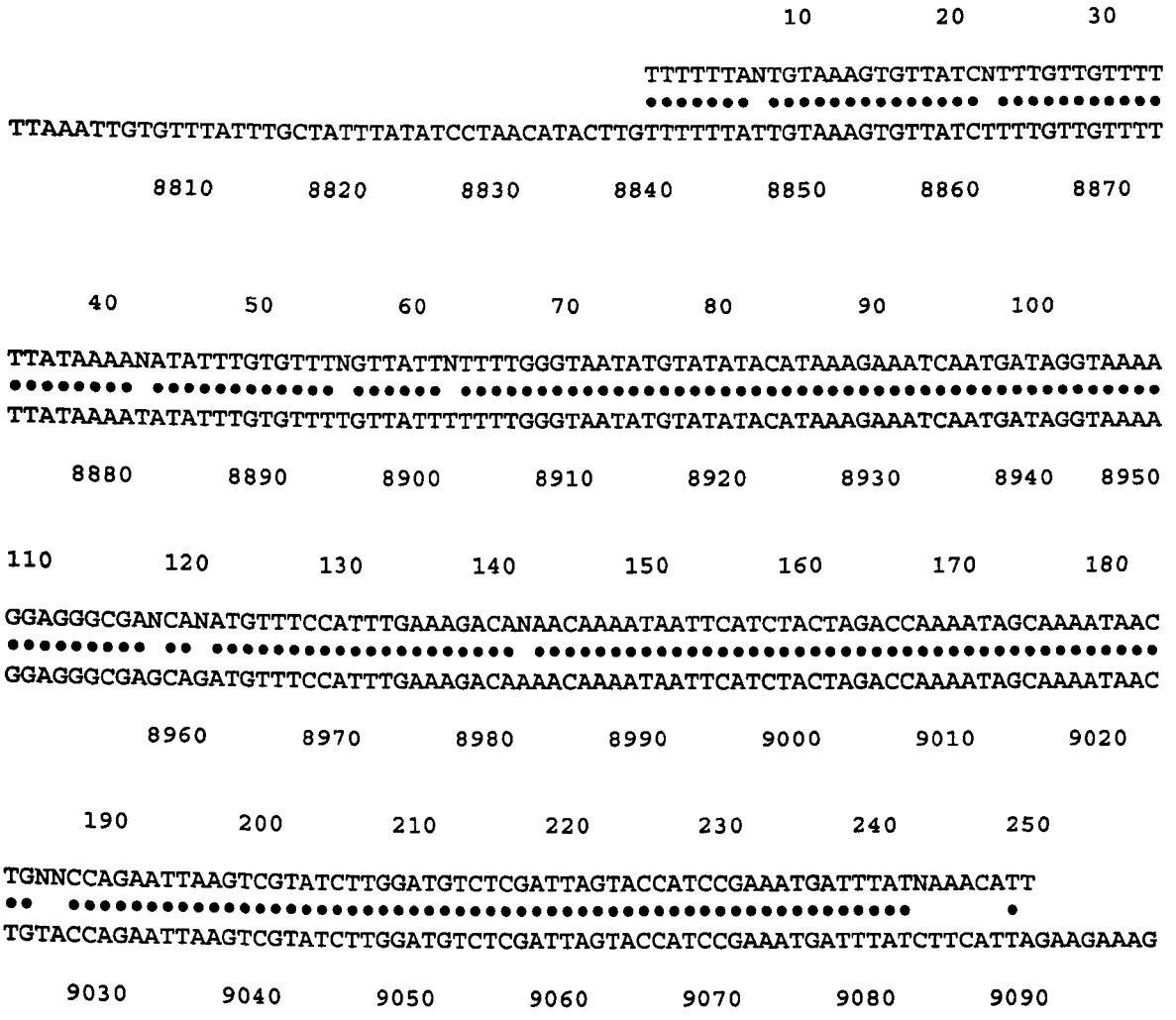
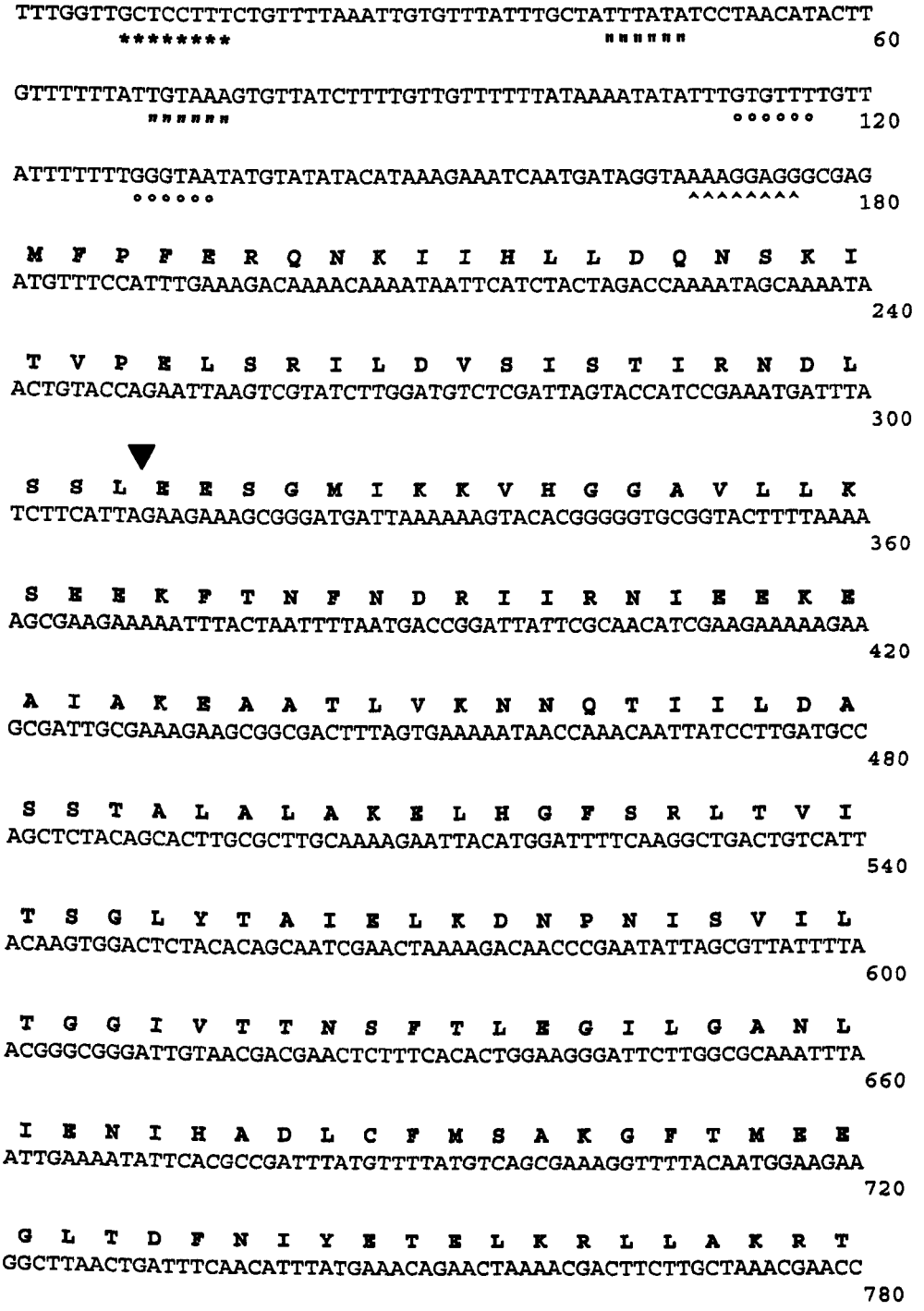


Figure 5.18
Sequence alignment of DES028 (Reversed, Complemented; Top) to a 10,500 bp contig sequence from *L. monocytogenes* EGD (Bottom) (Glaser, 2000).


TTTGGTTGCTCCTTTCTGTTTTAAATTGTGTTTATTTGCTATTTATATCCTAACATACTT
60
GTTTTTTATTGTAAAGTGTTATCTTTTGTTGTTTTTTATAAAATATATTTGTGTTTTGTT
120
ATTTTTTTTGGGTAATATGTATATACATAAAGAAATCAATGATAGGTAAAAGGAGGGCGAG
180
M F P F E R Q N K I I H L L D Q N S K I
ATGTTTCCATTTGAAAGACAAAACAAAATAATTCATCTACTAGACCAAAATAGCAAAATA
240
T V P E L S R I L D V S I S T I R N D L
ACTGTACCAGAATTAAGTCGTATCTTGGATGTCTCGATTAGTACCATCCGAAATGATTTA
300
S S L E E S G M I K K V H G G A V L L K
TCTTCATTAGAAGAAAGCGGGATGATTAAAAAAGTACACGGGGGTGCGGTACTTTTAAAA
360
S E E K F T N F N D R I I R N I E E K E
AGCGAAGAAAAATTTACTAATTTTAATGACCGGATTATTCGCAACATCGAAGAAAAAGAA
420
A I A K E A A T L V K N N Q T I I L D A
GCGATTGCGAAAGAAGCGGCGACTTTAGTGAAAAATAACCAAACAATTATCCTTGATGCC
480
S S T A L A L A K E L H G F S R L T V I
AGCTCTACAGCACTTGCGCTTGCAAAAGAATTACATGGATTTTCAAGGCTGACTGTCATT
540
T S G L Y T A I E L K D N P N I S V I L
ACAAGTGGACTCTACACAGCAATCGAACTAAAAGACAACCCGAATATTAGCGTTATTTTA
600
T G G I V T T N S F T L E G I L G A N L
ACGGGCGGGATTGTAACGACGAACTCTTTCACACTGGAAGGGATTCTTGGCGCAAATTTA
660
I E N I H A D L C F M S A K G F T M E E
ATTGAAAATATTCACGCCGATTTATGTTTTATGTCAGCGAAAGGTTTTACAATGGAAGAA
720
G L T D F N I Y E T E L K R L L A K R T
GGCTTAACTGATTTCAACATTTATGAAACAGAACTAAAACGACTTCTTGCTAAACGAACC
780

N K L I A L L D H T K M G V I S T A S I
AACAAATTGATCGCACTTCTGGACCATACAAAAATGGGCGTAATTTCCACTGCGAGCATC
840

T S A E N I D L L I T D N K I N K A L Y
ACCTCGGCAGAAAATATTGATTTACTTATTACAGATAACAAAATAAATAAAGCTTTATAT
900

K K F Q D A G L P V K V A E •
AAAAAATTCCAAGATGCGGGACTTCCGGTCAAAGTGGCAGAATAAAATAGAAAATAAACTC
960

TTTGAGCGAAAAGTGGTTCAAAGAGTTTTTTTTGATAGGAGAA 996

Figure 5.19

Sequence and translation (Bold) of putative ORF11 (DES028/DES029). Point of Tn*917* insertion (▼). σ^B -10/-35 promoter boxes (°°°°°°). Ribosome binding site(^). Stop codon (•). AAA Hairpin loop stem.
ORF10 (Reverse strand): σ^A -10/-35 promoter boxes ("). Ribosome binding site(*).

pI: 6.02
M_w: 28.0 kDa

Hydropathy

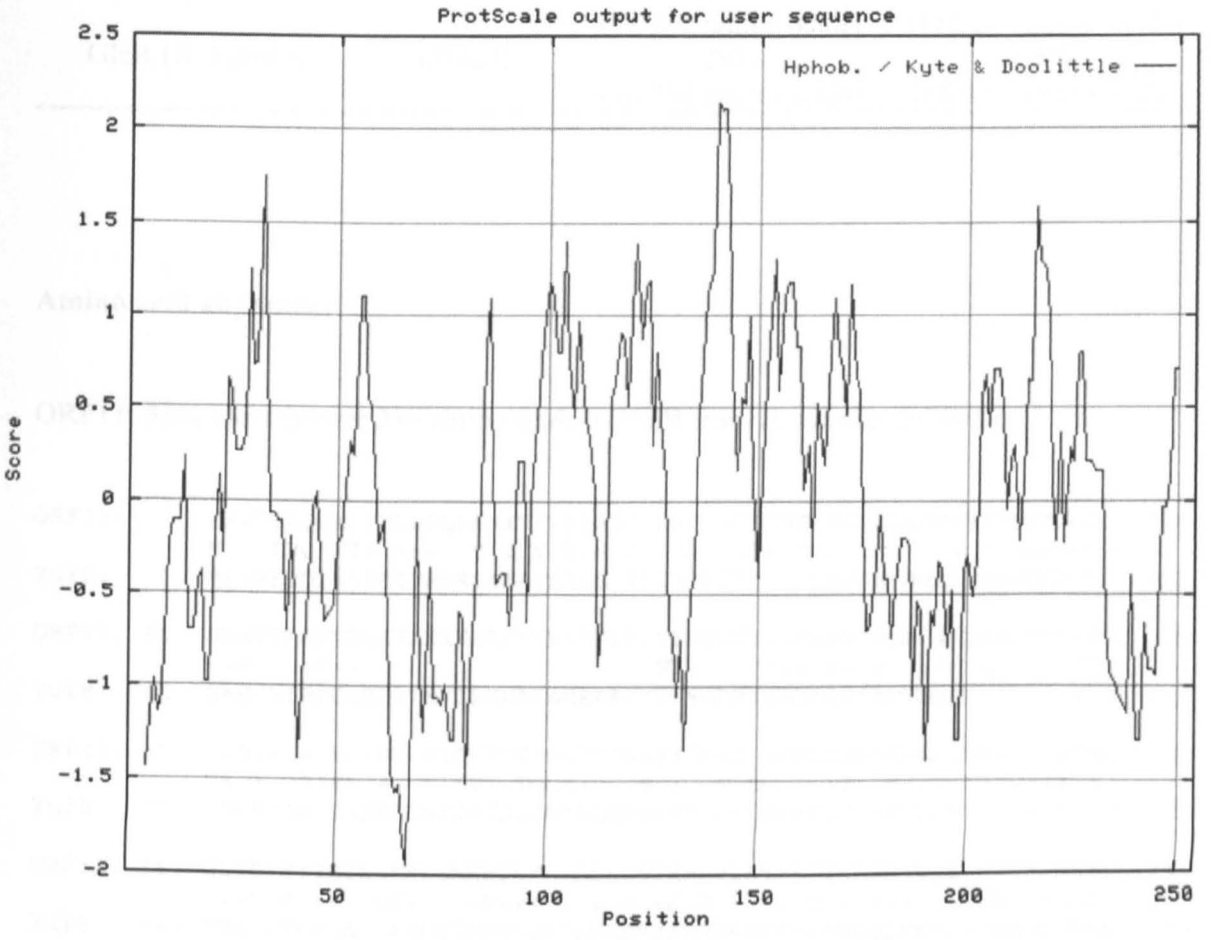


Figure 5.20
Physical characteristics of the putative ORF11 polypeptide. Hydropathy calculated as described by Kyte and Doolittle (1973). Average hydropathy values calculated for every 9 amino acid window. Positive values represent increasing hydrophobicity, and negative values represent increasing hydrophilicity.

BLAST analysis

Homologue (Species)	Accession No.	% Identity	% Positives
YulB (*B. subtilis*)	D70014	35% (89/250 amino acids)	53% (136/250 amino acids)
GatR (*E. coli*)	P36930	29% (72/242 amino acids)	50% (118/254 amino acids)
GlcR (*B. subtilis*)	C69632	29% (76/254 amino acids)	65% (88/134 amino acids)

Amino acid alignment

ORF11: 35% identity over 250 amino acids to YulB from *B. subtilis* (D70014).

```
ORF11:   1  MFPFERQNKIIHLLDQNSKITVPELSRILDVSISTIRNDLSSLEESGMIKKVHGGAVLLK 60
            M   ERQ KI+ +++  S I V ELS I  V+  TIR DL  LE+   + + HGGAV ++
YulB:    1  MLVAERQQKIVEIVNMRSSIRVSELSDIFSVTEETIRRDLEKLEKEHKLSRSHGGAVSIQ 60

ORF11:  61  SEEKFTNFNDXXXXXXXXXXXXXXXXTLVKNNQTIILDASSTALALAKELHGFSRLTVI 120
             +E   +F++                    VK+   IILDAS+TA  +AK L     LTVI
YulB:   61  QKESEIHFSEREITNVIEKKAIAHEAAKYVKSGDRIILDASTTAWYMAKILPDIE-LTVI 119

ORF11:  121 TSGLYTAIELKDNPNISVILTGGIVTTNSFTLEGILGANLIENIHADLCFMSAKGFTMEE 180
            T+ +  AIEL +  NISVI TGGI+   S +  G L    +E  H +  F+S KGF +
YulB:   120 TNSMKAAIELSNKENISVISTGGILLEKSLSFAGPLAERSLETYHVNKTFLSCKGFDVHN 179

ORF11:  181 GLTDFNIYETELKRLLAKRTNKLIALLDHTKMGVISTASITSAENIDLLITDNKINKALY 240
            G++D N ++  LK+ + +R+++ I + D +K G    + I S +++  LITD+ ++ A
YulB:   180 GMSDSNEWQALLKKRMIERSDQTILMADSSKWGNREFSHIASLQDVSRLITDSGLDPASV 239

ORF11:  241 KKFQDAGLPV 250
            K  +D  + V
YulB:   240 KALEDKKVKV 249
```

Figure 5.21
BLAST analysis and amino acid alignment of putative ORF11 (DES028/DES029).

Putative ribosome binding site

Ideal RBS	AAAGGAGGTGAT
ORF10RBS	AAAGGAGGGGCG
ORF11RBS	AAAGGAGCAACC

Putative promoter -10 and -35 boxes

Ideal	σ^A	-35	TTGACA	-10	TATAAT
ORF10	σ^A	-35	TTTACA	-10	TATAAA
Ideal	σ^B	-35	GTTTAT	-10	GGGTAA
ORF11	σ^B	-35	GTGTTT	-10	GGGTAA

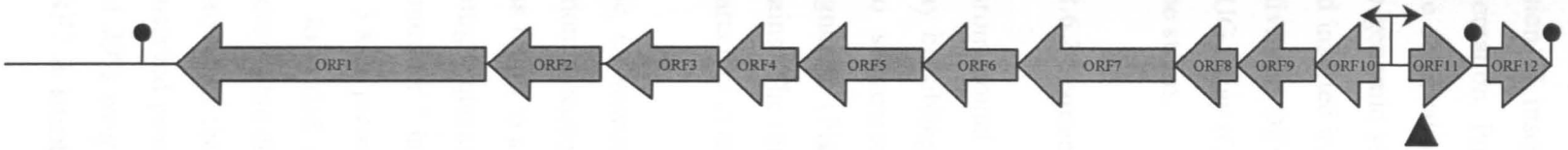
Terminator Loop

```
      A A
      A G
      A T
      G G
      C-G
      G T
      A-T
      G-C
      T-A
      T-A
      T-A
      C-G
      T-A
      C-G
      A-T
      A-T
      A-T
      T T
      A-T
      A-T
      A-T
      A-T
TAAAATAG  GATAGGAGAA
```

$\Delta G = -14.6$ kcal mol^{-1}

Figure 5.22

Putative promoter and ribosome binding site sequences (Underline: identical bases), and terminator stem loop structure of ORF11 (DES028/029). ΔG value calculated as described by Tinoco *et al.* (1973).



1 kb

Homologues: ORF 1. Transketolase
2. Transaldolase
3. Glucose 1-Dehydrogenase
4. Ribose-5-Phosphate Isomerase
5. Triosephosphate Isomerase
6. Dihydroxyacetone Kinase
7. Dihydroxyacetone Kinase
8. No Homology
9. No homology
10. PTS System Enzyme I
11. Transcriptional Regulator (DeoR Family)
12. AttT

Figure 5.23
Map of Tn*917* insertion site (▲) in DES028/DES029. Putative promoters (Black arrows). Putative transcriptional terminators (Black lollipops).

The transposon Tn*917* carries a promoter-less copy of the *lacZ* gene which can create a *lacZ* fusion if inserted downstream of the promoter of the mutated gene in the appropriate orientation. Promoter activity can then be determined by assaying β-galactosidase activity. The observation that the ORF identified was in the reversed and complemented orientation to the genomic sequence obtained (Chapter 5.2.6.1) indicated that the promoter-less *lacZ* gene had inserted into the same orientation as the disrupted gene. Determination of β-galactosidase activity in DES028 and DES029 by incubation on TSB agar plates overlaid with X-Gal and MUG assay (Chapter 2.25) failed to detect β-galactosidase expression above that of the wild type strain.

5.2.6.2 Sequencing and analysis of the Tn*917* insertion site in DES035 and DES045

Chromosomal mapping of the strains DES035 and DES045 suggested that the two strains may be siblings, and so the genomic sequence obtained for each strain was compared. The two sequences showed 83.3% homology (174/209 bases) when compared by gapped alignment (Figure 5.24), thus confirming that DES035 and DES045 were indeed sibling strains. The differences observed were primarily due to sequencing, as evidenced by the 'N' characters in the alignment.

The *L. monocytogenes* EGD genomic DNA sequence database was searched using the genomic sequence from DES035. The DES035 sequence, when reversed and complemented, was found to align with bases 4818 to 5049 of a 6.0 kbp contig (Figure 5.25). Analysis of the contig sequence determined that the insertion had occurred 92 bp into a putative ORF which is number 7 in a total of 9 putative ORFs identified within the contig. ORF7 encodes for a 12.3 kDa protein of 108 amino acids in length, and the potential polypeptide produced would be truncated after 22 amino acids (Figure 5.26). The polypeptide exhibits no highly hydrophobic domains, indicative of a soluble protein, and a pI of 6.35 (Figure 5.27). BLAST analysis of the amino acid sequence revealed that the putative protein had homology to a conserved protein in *Lactococcus lactis* and PhaQ of *B. megaterium* (35% over 92 amino acids and 29% over 88 amino acids respectively) (Figure 5.28). The initiation codon (ATG) of ORF7 is situated at position 94 and a potential ribosome binding site was identified 9 bp

upstream of the ORF (Figures 5.26 and 5.29). ORF7 is the first of two in a putative bicistronic operon, with the start codon (ATG) of ORF8 overlapping the final codon of ORF7 (Figure 5.26). ORF8 encodes a 12.8 kDa polypeptide 107 amino acids in length, with pI of 5.59 and a hydropathy profile suggestive of a soluble protein (Figures 5.26 and 5.30). BLAST analysis of the amino acid sequence failed to identify any significant homology to any known proteins. Putative σ^A promoter sequences for the operon were found upstream of the start codon for ORF7 (Figures 5.26 and 5.29) and a potential Rho-independent transcription terminator (26 bp stem loop with a ΔG of –23.2 kcal mol^{-1}) was found overlapping the translation stop codon TAA of ORF8.

Immediately upstream of the ORF7/ORF8 operon was found a total of 6 putative ORFs (Figure 5.31). The end of the contig sequence restricted determination of the start of the operon and while the final three ORFS may to be transcriptionally linked to the first three, a σ^A promoter and ribosome binding site sequences were found upstream of ORF4. Overlapping the end of ORF6 was a putative Rho-independent transcriptional terminator (16 bp stem loop, ΔG value of –21.8 kcal mol^{-1}). BLAST analysis of the putative polypeptides identified homology to several flagellar-specific genes and a lytic transglycosylase (Table 5.3). Analysis of the contig sequence identified a further 7 ORFs (Figure 5.31). The single ORF (ORF9), located 37 bp downstream of the ORF7/ORF8 transcription terminator, possessed putative σ^A-dependent promoter and ribosome binding site sequences. Since the contig sequence ended before any apparent stop codon, it was not possible to determine the full nature of the ORF and no homology to any known proteins was found using the sequence of the first 50 amino acids.

Observations indicated that the *lacZ* reporter gene was inserted into the same orientation as the disrupted gene, since the ORF identified was in the reversed and complemented orientation to the genomic sequence obtained (Chapter 5.2.6.2). Therefore it was thought possible to determine expression from the promoter by detecting β-galactosidase activity. Determination of β-galactosidase activity in DES035 and DES045 by incubation on TSB agar plates overlaid with X-Gal and MUG assay (Chapter 2.25) failed to detect β-galactosidase expression above that of the wild type strain.

	Homologue (Species)	Function	Identity	Accession No.
ORF1	No significant homology	—	—	—
ORF2	*fliG* *(B. subtilis)*	Flagellar motor switch protein	28% (96/337 amino acids)	P23448
ORF3	No significant homology	—	—	—
ORF4	*fliI* *(B. subtilis)*	Flagellum-specific ATP synthase	44% (172/385 amino acids)	P23445
ORF5	*yjbJ* *(B. subtilis)*	Lytic transglycosylase	58% (66/112 amino acids)	B69844
ORF6	No significant homology	—	—	—
ORF7	*phaQ* *(Bacillus megaterium)*	Unknown	29% (26/88 amino acids)	AF109909
ORF8	No significant homology	—	—	—
ORF9	No significant homology	—	—	—

Table 5.3
BLAST analysis of putative ORFs flanking ORF No.7 (DES035/DES045).

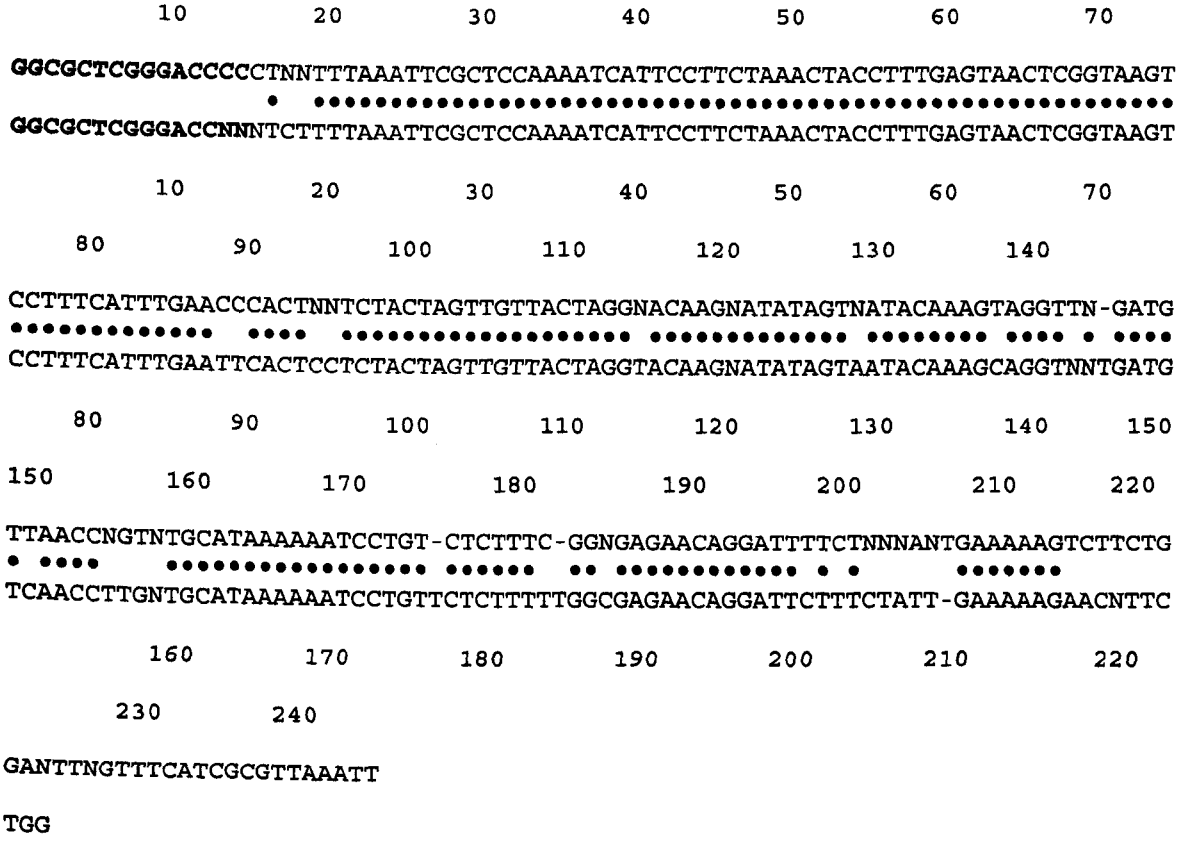


Figure 5.24

Alignment of sequence obtained from genomic sequencing of DES035 (Top) and DES045 (Bottom). Sequence in **bold**: sequence matching *lacZ* proximal end of Tn*917*.

PAGE
MISSING
IN
ORIGINAL

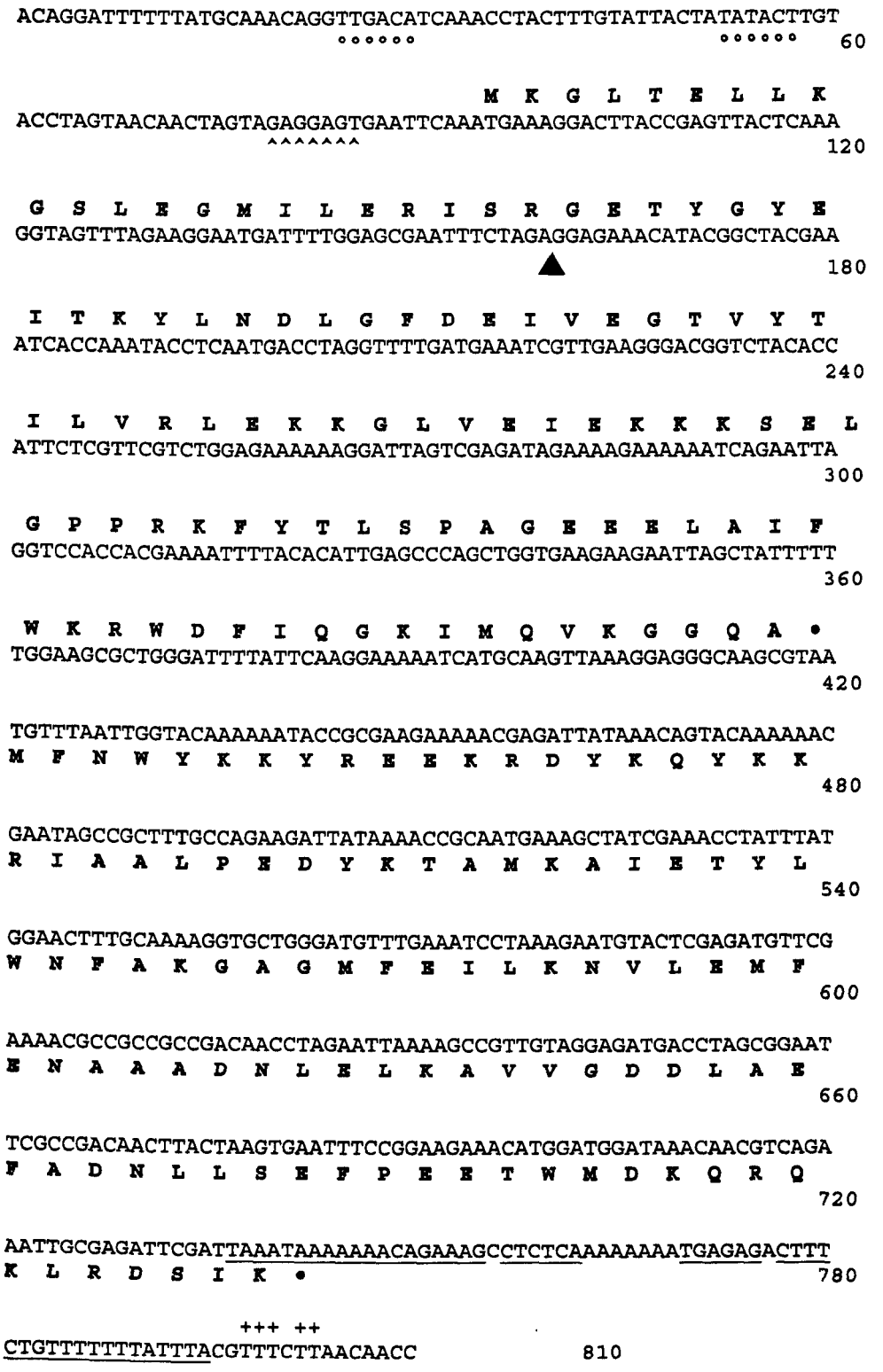


Figure 5.26
Sequence and translation (**Bold**) of putative ORF7 (Above sequence) and ORF8 (Below sequence) (DES035/DES045). Point of Tn*917* insertion (▼). σ^A -10/-35 promoter boxes (°°°°°°). Ribosome binding site(^). Stop codon (•). Thymine rich sequence (+++). AAA Hairpin stem loop.

pI: 6.35
M_w: 12.3 kDa

Hydropathy

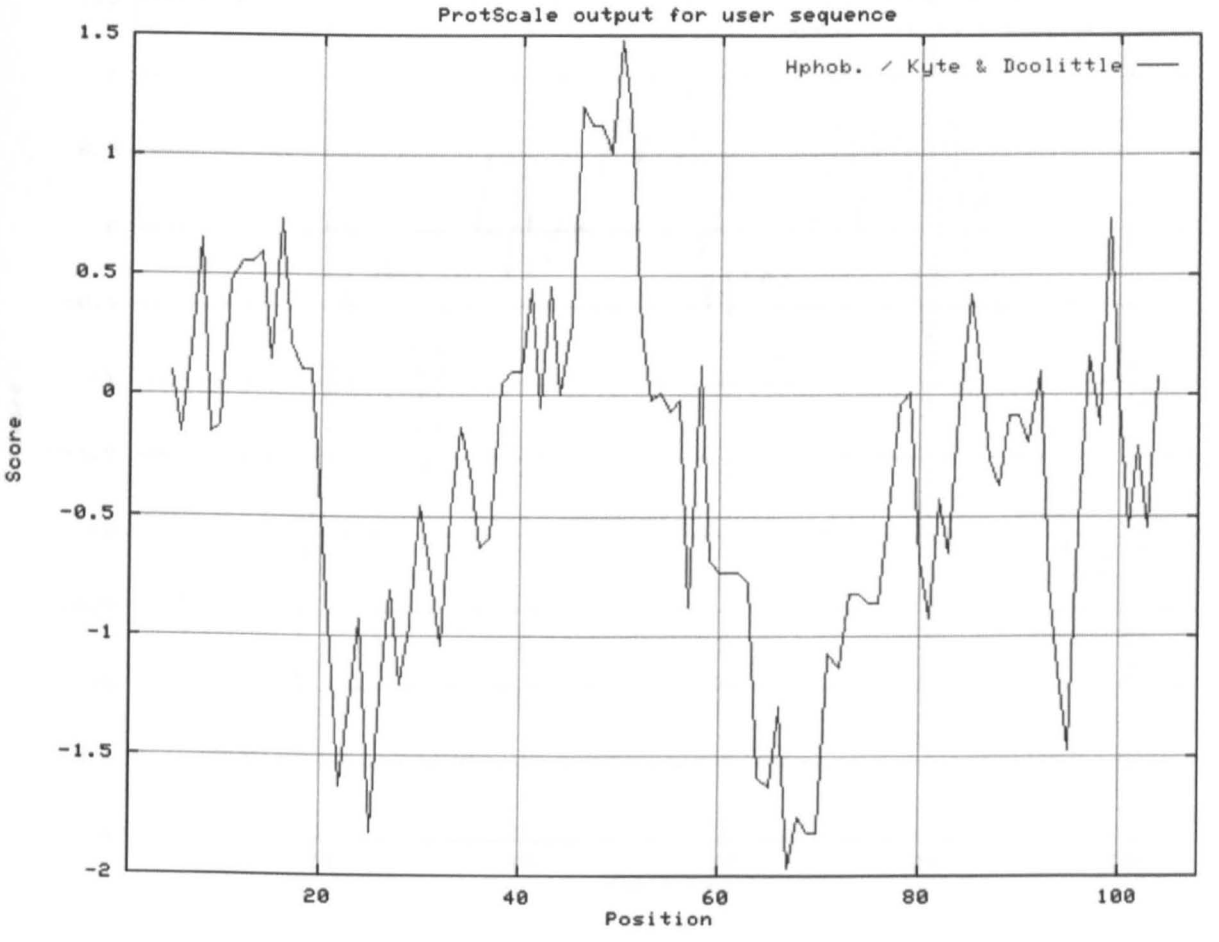


Figure 5.27
Physical characteristics of the putative ORF7 polypeptide. Hydropathy calculated as described by Kyte and Doolittle (1973). Average hydropathy values calculated for every 9 amino acid window. Positive values represent increasing hydrophobicity, and negative values represent increasing hydrophilicity.

pI: 5.59
M_w: 12.8 kDa

Hydropathy

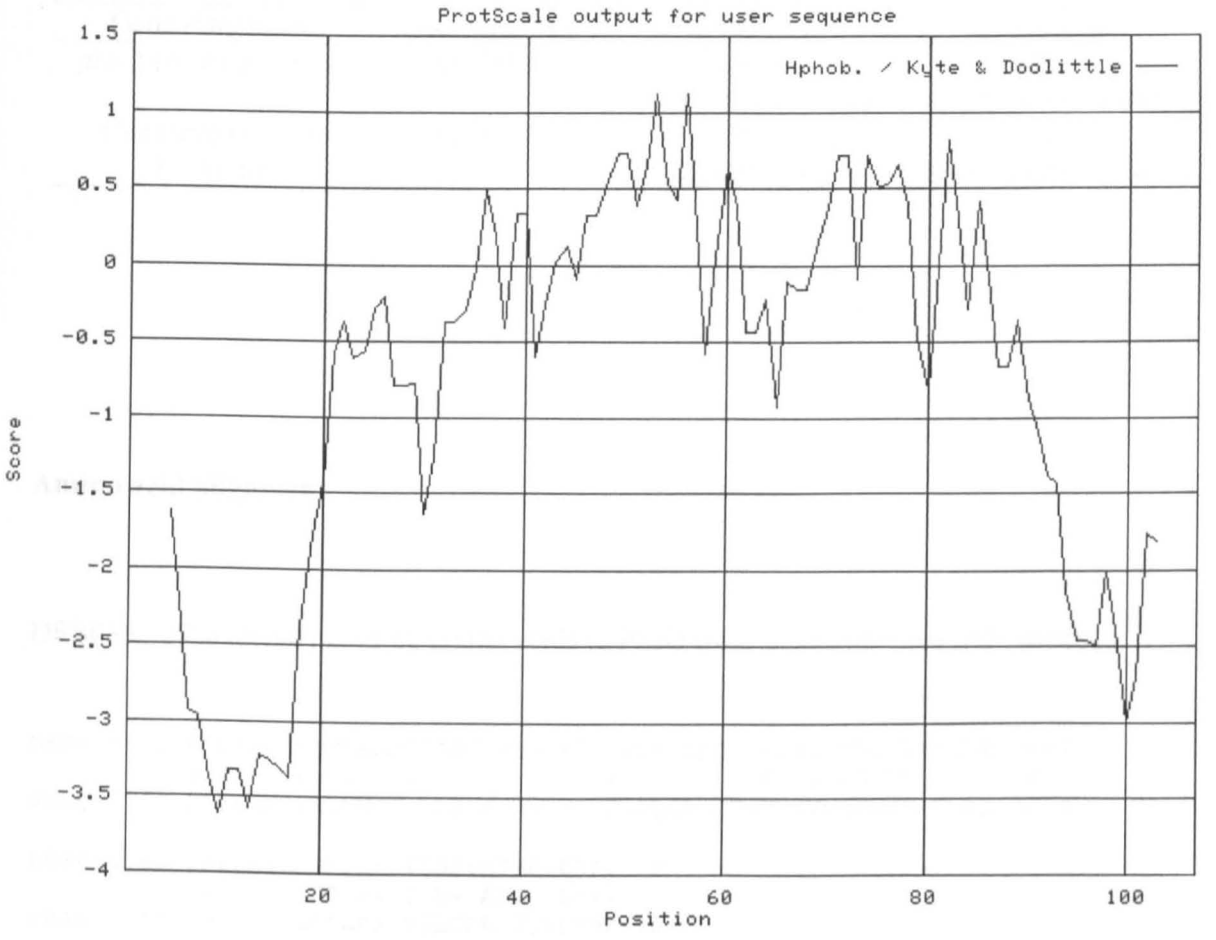


Figure 5.28

Physical characteristics of the putative ORF8 polypeptide. Hydropathy calculated as described by Kyte and Doolittle (1973). Average hydropathy values calculated for every 9 amino acid window. Positive values represent increasing hydrophobicity, and negative values represent increasing hydrophilicity.

BLAST analysis

Gene (Organism)	Accession No.	% Identity	% Positives
phaQ (*B. megaterium*)	AF109909	29% (26/88 amino acids)	52% (47/88 amino acids)
Conserved protein (*L. lactis*)	AE001272	35% (33/92 amino acids)	62% (61/92 amino acids)

Amino acid alignment

DES035: 29% identity over 88 amino acids to PhaQ from *B. megaterium* (AF109909).

```
DES035:  2 KGLTELLKGSLEGMILERISRGETYGYEITKYLNDLGFDEIVEGTVYTILVRLEKKGLVE 61
           K ++   K  +   +L  +     +GY++ + L   GF  + +G VY  L +LEK  L+
PhaQ:   17 KSISGAPKNLMVPFLLLSLRGWNLHGYKLIQQLMSFGFTSVDQGNVYRTLRQLEKDNLIT 76

DES035:62 IEKKKSELGPPRKFYTLSPAGEEELAIF 89
           +   S  GP R+ Y+L+ AGE+ L+++
PhaQ:   77 SQWDTSAEGPARRIYSLTDAGEQYLSMW 104
```

Figure 5.29
BLAST analysis and amino acid alignment of putative ORF7 (DES035/DES045)

Putative promoter -10 and -35 boxes

Ideal	σ^A	-35	TTGACA	-10	TATAAT
ORF8	σ^A	-35	TTGACA	-10	TATACT

Terminator Loop

```
          A
         A A
         A A
         A A
         A-T
         C-G
         T-A
         C-G
         T-A
         C-G
         C A
         G-C
         A-T
         A-T
         A-T
         G-C
         A-T
         C-G
         A-T
         A-T
         A-T
         A-T
         A-T
         A-T
         A-T
         T-A
         A-T
         A-T
         A-T
         T-A
   TTCGAT   CGTTTCT
```

ΔG= -23.2 kcal mol^{-1}

Figure 5.30

Putative promoter and ribosome binding site sequences (Underline: identical bases), and terminator stem loop structure of ORF7 (DES035/045). ΔG value calculated as described by Tinoco *et al.* (1973).

1 kb

Homologues: ORF 1. No homology
2. Flagellar Motor Switch
3. No Homology
4. Flagellar-Specific ATP Synthase
5. Lytic Transglycosylase
6. No Homology
7. *pha*Q
8. No Homology
9. No Homology

Figure 5.31

Map of Tn*917* insertion site (▲) in DES035/DES045. Putative promoters (Black arrows). Putative transcriptional terminators (Black lollipops).

5.3 Discussion

Starvation-survival defective mutants of *L. monocytogenes* unable to survive 12 days incubation under glucose limiting conditions at 37°C were isolated using a screening protocol. The mutant phenotype under glucose starvation was characterised in liquid culture, with the mutant strains exhibiting a 10-fold reduction in survival in glucose-limited CDM compared to EGD. The degree of impairment in starvation-survival observed in the mutants relative to wild type mirrors that of mutant strains in other bacteria. The *suv* mutants of *S. aureus* 8325-4, isolated by Watson *et al.* (1998b), exhibited a 10- to 100-fold reduction in survival, whilst survival of the *S. typhimurium sti* mutants was likewise reduced 45-fold after 20 days (Spector and Cubitt, 1992).

Amongst known starvation-survival genes, large functional variations exist. Some have defined roles in starvation-survival, such as the global regulators *rpoS* and *rpoH*, and the stringent response genes *relA* and *spoT* in *E. coli* (Lange and Hengge-Aronis, 1991b; Nyström, 1995). Others, such as *dnaK* in *E. coli* (Rockabrand *et al.*, 1995), *stiABC* in *S. typhimurium* (Seymour *et al.*, 1996) and *sodA* in *S. aureus* (Watson *et al.* 1998b) are known to confer general stress cross-resistance, whilst more diverse cellular functions include amino acid catabolism (Fraley *et al.*, 1998) and biosynthesis of biotin and cell wall components (Keer *et al.*, 2000).

Twenty-two putative starvation-survival mutants (DES024-DES045) were isolated as a result of the glucose limited screening protocol. Southern blot analysis of these isolates verified that most contained a single transposon insertion and involved a number of different sites within the chromosome, despite the fact that they originated from the same library of clones. The four strains that were positive after phage transduction analysis could be placed into two categories based on both molecular and phenotypic data. Southern blotting revealed that DES028 and DES029 shared similar digest patterns, as did DES035 and DES045. It would seem therefore that the same sites of insertion were selected more than once during the screening process. Although it cannot be discounted that the two regions of insertion were

transposition hotspots, the mutations acquired would seem to be particularly important for starvation-survival.

Matching starvation-survival kinetics and stress resistance profiles were observed for the strains DES028 and DES029 under glucose starvation in liquid culture, and the defective phenotype developed after the first 48 hours of starvation. The mutant strains also showed increased heat and oxidative stress sensitivity relative to EGD. Sequencing of the chromosomal DNA immediately flanking the *lacZ*-proximal end of the transposon insertion in DES028 and DES029 confirmed that both strains were siblings. Further analysis identified a disrupted ORF possessing significant homology to YulB in *B. subtilis* (Kunst *et al.*, 2000) No definite role has yet been ascribed to YulB, however it is proposed to belong to the DeoR family of transcriptional regulators. The proteins in this family contain an N-terminal helix-turn-helix motif and a C-terminal domain proposed to be the site of inducer interaction (van Rooijen and de Vos, 1990). DeoR regulates the expression of ribonucleoside and deoxyribonucleoside catabolic enzymes in *E. coli* (Mortensen *et al.*, 1989). In the control of the *deo* operon, repressor activity is enhanced by the binding of the octomeric protein to up to three palindromic operator sites (*deo*Oe, *deo*O1 and *deo*O2), resulting in a formation of a double loop of DNA (Mortensen *et al.*, 1989).

Protein	Operon Function	Organism
GatR	Galactitol uptake	*E. coli*
FucR	L-Fucose uptake	
GutR	Glucitol uptake	
GlpR	Glycerol-3-phosphate uptake	
AgaR	N-acetylgalactosamine uptake	
LacR	Lactose uptake	*L. lactis*
FruR	Fructose uptake	*B. subtilis*

Table 5.4

DeoR family protein functions. Source: SWISS-PROT protein database

Of more significance to a possible role for the YulB homologue in starvation-survival is the fact that the majority of proteins in the DeoR family are seen to repress the expression of phosphoenolpyruvate (PEP)-dependent phospohtransferase system (PTS) genes (Table 5.4). Conversely, expression can be induced by interaction of the repressor with the substrate corresponding to the specific PTS in question. The PTS is located in the cell membrane and is responsible for the cellular uptake and simultaneous phosphorylation of a large number of carbohydrates (Figure 5.32). In addition to transport functions, PTS components have roles in chemotaxis and in the regulation of the synthesis and activity of numerous unrelated transport systems. The putative protein encoded in ORF10 is homologous to a putative PTS EI in *E. coli*. EI is a phosphoprotein that performs the first step in the sequential transfer of a phosphoryl group from phosphoenolpyruvate (PEP) to the carbohydrate. In *B. subtilis*, the phosphoryl group is initially transferred by EI from PEP to HPr and subsequently to one of several types of substrate-specific types of EII (Stülke and Hillen, 2000) (Figure 5.32). Components of the PTS, including EI, have already been located in *L. monocytogenes* (Christensen *et al.*, 1998), so the precise role of this second copy is unclear. In addition to forming part of the PTS, the HPr protein has major roles in catabolite repression via adjusting the activity of Gram-positive transcriptional regulators by phosphorylation, and via interaction with CcpA and fructose-1,6-bisphosphate.

The promoter region immediately upstream of ORF11 contains a putative promoter sequence for the alternative sigma factor σ^B. Genes within the σ^B regulon are expressed in response to a variety of environmental stresses, including glucose starvation, heat, low pH and oxidative stress (Hecker and Völker, 1998). There is lack of any other obvious regulatory elements sometimes found in σ^B-dependent genes (σ^A/σ^H promoters, CtsR box) (Hecker and Völker, 1998). This would suggest that this gene forms part of the general stress regulon in *L. monocytogenes*, though the precise role within this response is unclear. The occurrence of an operon encoding a putative PTS enzyme along with a number of genes that comprise part of the glycolytic and pentose phosphate pathways immediately adjacent to ORF11 (Figures 5.33 and 5.34), could suggest that regulation by the YulB homologue may occur. Preliminary sequence searches however failed to detect any palindromic sequences characteristic of the DeoR-type operator sequences. Should this operon be under the control of the YulB

homologue, insertional inactivation of this protein would result in the disruption of several core physiological pathways and possibly carbon catabolite repression via HPr. Disruption of one or more of these regulatory networks could therefore account for the multiplicity of effects observed in the development of starvation-survival phenotype in the DES028 and DES029 mutant strains.

For the mutant strains DES035 and DES045, reduced starvation-survival in glucose-limited CDM only became apparent after 15 days incubation. Compared to EGD, resistance to heat and acid stress in DES035 and DES045 were unaffected, whereas some sensitivity to oxidative stress was observed. The region of chromosomal DNA flanking the *lacZ*-proximal end of the Tn*917* insertion was sequenced and the strains were found to be siblings. The putative protein encoded in the disrupted ORF was homologous to PhaQ, a protein associated with PHA inclusion bodies in *B. megaterium* (McCool and Cannon, 1999).

The importance of PHA to starvation-survival is well known, since this class of aliphatic polyesters are recognised as a carbon and energy reserve in bacteria, and are accumulated by a large number of both Gram-positive and Gram-negative bacteria, including members of the genera *Bacillus* and *Staphylococcus* (Anderson and Dawes, 1990; Szewczyk, 1992). PHA enables the cell to store reduced carbon in a form that does not significantly affect the osmotic balance between the cell and the environment. Synthesis typically occurs under conditions of excess carbon, with concomitant limitation in one essential nutrient such as nitrogen, phosphorus, sulphur, magnesium, potassium or iron (Anderson and Dawes, 1990), and degradation subsequently occurs during carbon starvation (Doi *et al.*, 1992). Whilst substrates for PHA synthesis can be supplied from a variety of sources, the principal precursor for PHA is acetyl-CoA (Anderson and Dawes, 1990; Eggink *et al.*, 1992), which also plays a significant role in the regulation of the PHA biosynthetic pathway.

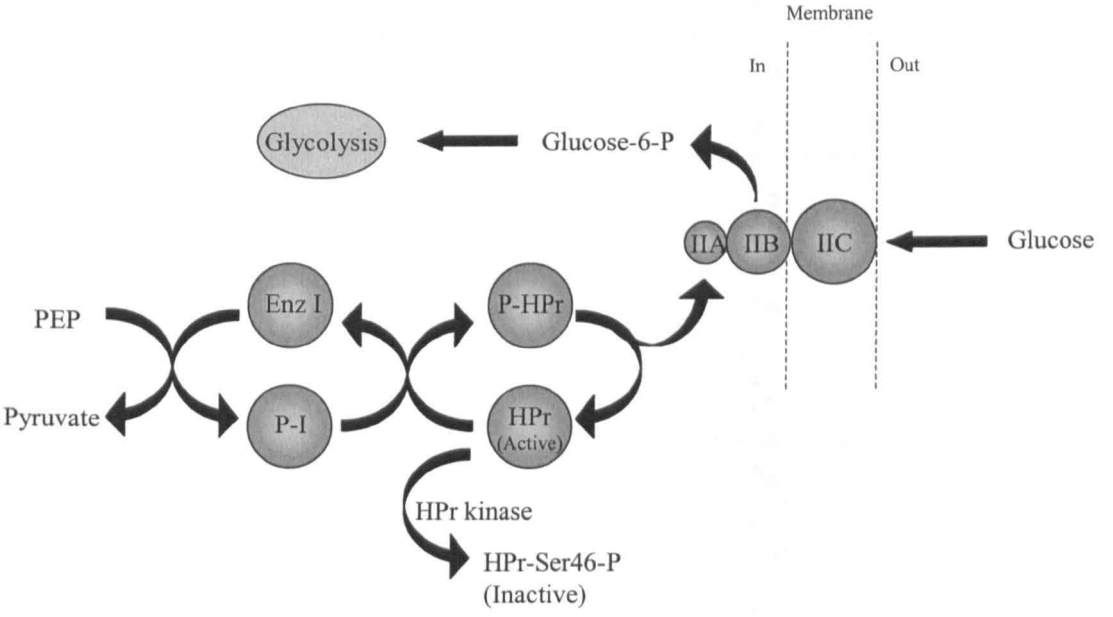


Figure 5.32

The components of the PTS in *B. subtilis*. Enz I, Enzyme I; IIA, Enzyme IIA; IIB, Enzyme IIB; IIC, Enzyme IIC; P-I and P-HPr, phosphorylated forms of Enzyme I and HPr. Adapted from Stülke and Hillen (2000).

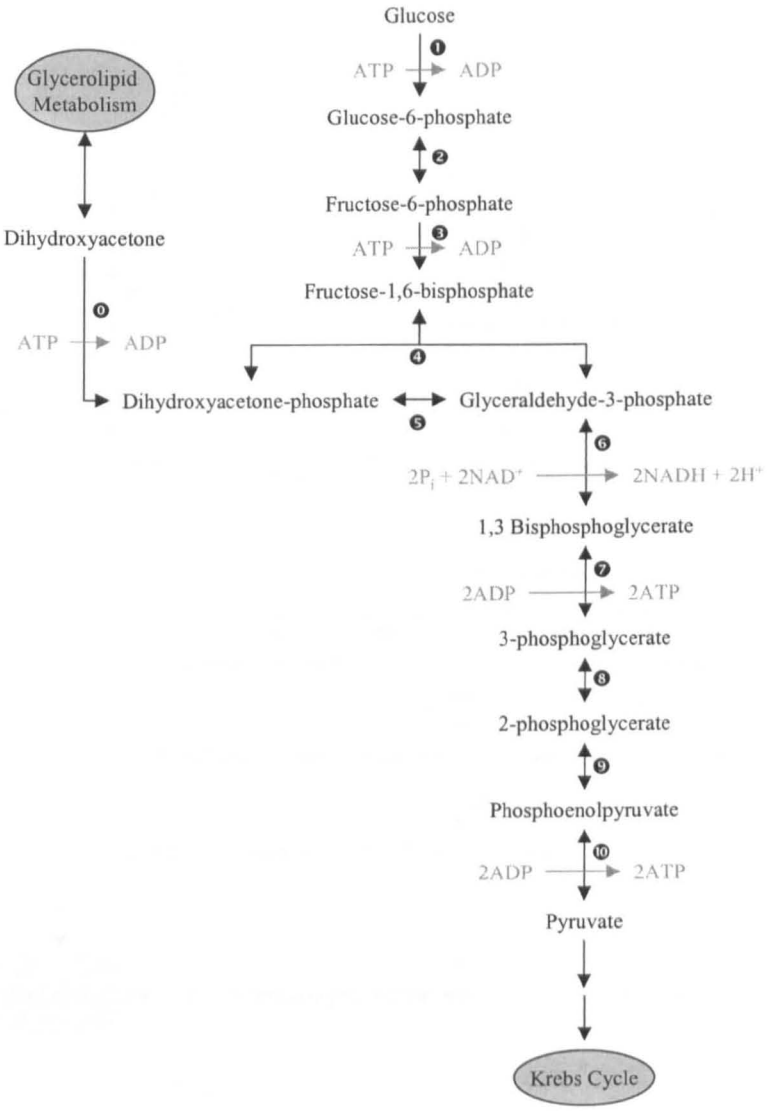


Figure 5.33 Carbon metabolism via the glycolytic pathway.

Key: Homologues identified in the DES028/DES029 insertion region (Bold).

0. **Dihydroxyacetone (Glycerone) kinase**
1. Hexokinase
2. Phosphoglucose isomerase
3. Phosphfructokinase
4. **Aldolase**
5. **Triosephosphate isomerase**
6. Glyceraldehyde-3-phosphate dehydrogenase
7. Phosphoglycerate kinase
8. Phosphoglycerate mutase
9. Enolase
10. Pyruvate kinase

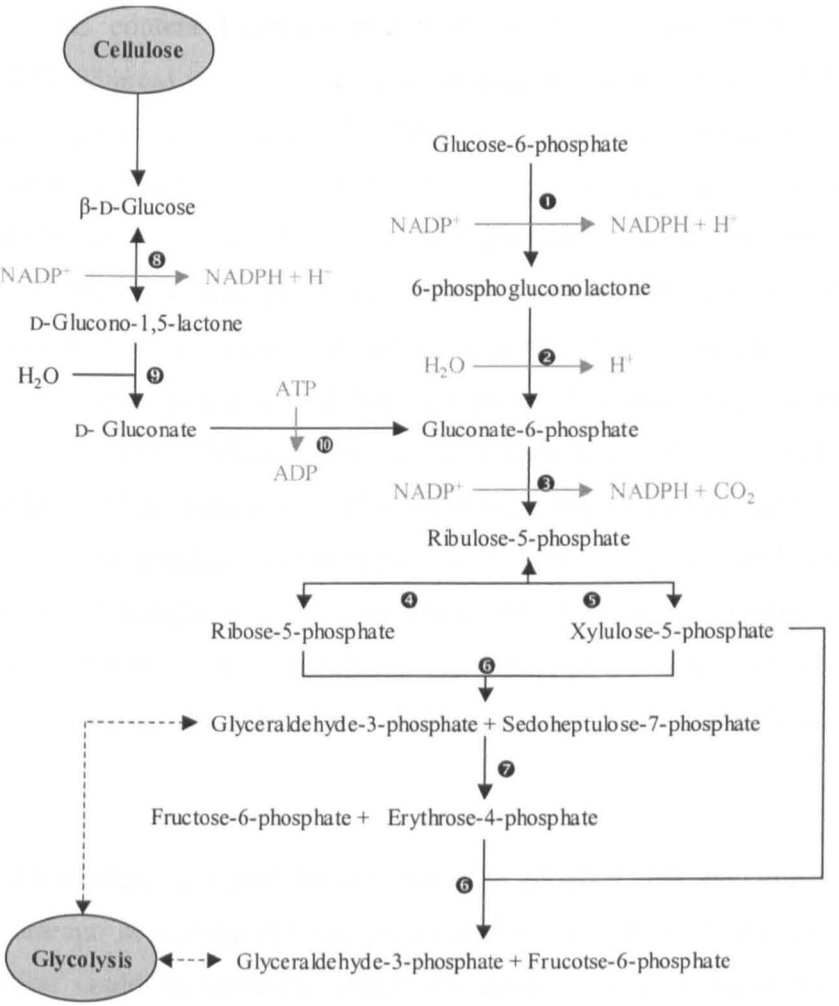


Figure 5.34 Carbon metabolism via the pentose phosphate pathway.

Key: Homologues identified in the DES028/DES029 insertion region (Bold).

1. Glucose-6-phosphate dehydrogenase
2. 6-Phosphogluconolactonase
3. Phosphogluconate dehydrogenase
4. **Ribulose-5-phosphate isomerase**
5. Ribulose-5-phosphate epimerase
6. **Transketolase**
7. **Transaldolase**
8. **Glucose-1-dehydrogenase**
9. Gluconokinase
10. Gluconolactonase

Amongst low G+C content Gram-positive bacteria, *Bacillus* species accrue significant quantities of PHA, whereas minimal levels are found in staphylococci (up to 50% and 0.15% of the total dry weight of cells respectively) (McCool *et al.*, 1996; Szewczyk, 1992). Despite these low levels in staphylococci, the PHA is rapidly oxidised during the first few hours of starvation, and still allows an initial delay in the degradation of cellular components such as RNA and protein. PHA synthetic genes are found in clusters, and *phaQ*, encoding for a 17 kDa protein, is found in *B. megaterium* within a group of five *pha* genes (McCool and Cannon, 1999). The gene is transcribed from a separate σ^A-dependent promoter in what is thought to be a constitutive fashion. PhaQ is found associated with PHA inclusion bodies, though no role has yet been determined. PhaQ was suggested to be a replacement for one of the small putative gene products found in *pha* gene clusters (McCool and Cannon, 1999). Other low molecular weight proteins have been identified on the surface of the PHA inclusion bodies in *Rhodococcus ruber* and *A. eutrophus*, affecting both PHA accumulation and the size of the inclusion bodies formed (Pieper-Fürst *et al.*, 1994; Wieczorek *et al.*, 1995).

From current knowledge, it would be reasonable to propose that any impairment in the accumulation, storage or subsequent utilisation of PHA in DES035 and DES045 during glucose starvation could significantly affect starvation-survival. It must be remembered however, that a second ORF encoding a putative polypeptide with no homology to any found in the database, appears transcriptionally linked to the PhaQ homologue. The possibility of a polar effect from the transposon insertion resulting in the mutant phenotype seen in DES035 and DES045 cannot as yet be ruled out.

CHAPTER 6

DISCUSSION

6.1 Introduction

This thesis contains the first comprehensive study of the SSR of *L. monocytogenes*. It has led to a fuller understanding of the development and characteristics of the SSR in this organism. Survival in batch cultures, the role of cell density, cryptic growth, protein synthesis and the development of starvation-associated stress resistance were all investigated (Chapter 3). In order to determine the principal components of the SSR, roles for two known gene regulators were identified (PrfA and σ^B). To further elucidate the machinery of starvation-survival, mutants defective in survival in batch culture were isolated, from which two novel loci were identified (Chapters 4 and 5).

6.2 Starvation-survival response of *L. monocytogenes*

The development of the starvation-survival phenotype in *L. monocytogenes* appears to be induced principally by glucose limitation, as glucose- and multiple nutrient-limitation (including carbon limitation) resulted in survival over far greater periods that could be achieved under amino acid limitation. Similar kinetics in the first two days of multiple-nutrient starvation were documented by Lou and Yousef (1996), where incubation of *L. monocytogenes* Scott A in phosphate buffer revealed a rapid fall in viability over the first 24 hours before the rate of decline slowed. The starvation-survival phenotype also extended to glucose-starved cells exhibiting a significant change in cellular morphology, with concomitant increases in resistance to acid, heat and oxidative stress. These observations place *L. monocytogenes* alongside *S. aureus* and *V. sp.* which both demonstrated similar changes solely in response to glucose starvation (Watson *et al.*, 1998; Nyström *et al.*, 1992). This is in contrast with *E. coli* and *S. typhimurium* which lack such specificity in their response to different forms of nutrient limitation (Jenkins *et al.*, 1988; Foster and Spector, 1995). Within long-term starved batch cultures, an accumulation of toxic products may occur,

whilst in natural environments already starved cells may encounter additional stress. The increased general stress resistance of starved cells may therefore, play a key role in the ability of the cell to withstand additional stress at a time when the cell cannot actively adapt to the environment. Reduction in cellular dimensions during the SSR has been linked to the *bolA* morphogene in *E. coli*, which controls the switching between cell elongation and septation systems (Aldea *et al.*, 1989). Since *bolA* is induced in response to stress during exponential-phase growth, this is not solely a starvation-associated response (Santos *et al.*, 1999). Whilst a precise reason for the change in morphology in response to stress is not yet known, it is possible that protection of the intracellular environment is achieved by a reduction in surface volume accompanying changes in the cell envelope.

The initial cell density of a culture entering stationary phase was found to affect to survival potential of the cells present. This correlates with the findings of Diaper and Edwards (1994) and Watson *et al.* (1998) in *S. aureus*, where resuspension in water at lower cell densities than the original culture resulted in a greater loss of viability. Resuspension of cells in glucose-limited CDM conditioned culture fluid of the same age revealed that a significant amount of nutrients remain, which are able to support a consistent concentration of bacteria. This was highlighted by the fact that cells resuspended at lower cell densities were able to regrow to up to a 1000-fold higher concentration within 3 days. This helps to explain the theory of cryptic growth in such cultures. It is possible that a point is reached at which viability and growth of surviving cells are supported as cell death and lysis release fresh nutrients into the medium. The precise concentration of cells that can be supported would therefore be that at which an equilibrium is generated between cell growth and cell death. In contrast to *S. aureus*, it appears that long-term starved cells possess a greater survival potential than 6-hour post-exponential phase cells, when resuspended in water. This would suggest that the development of the full starvation-survival phenotype requires somewhat longer than that seen in *S. aureus*. Further evidence for this is the observation that stress resistance is not fully developed at the 6-hour post-exponential phase.

Active cell division in starved *L. monocytogenes* cultures is further alluded to by the observation of partial division septa, and that the addition of penicillin G to starved cultures

resulted in a steady decline in viability. Several points of interest are raised in relation to cell division in *L. monocytogenes*. The deleterious effect of penicillin G is lost in long-term starved cultures and partial division septa are only found in a very small percentage of cells. Inhibition of protein synthesis 24 hours after the initiation of starvation, does not affect survival potential, and could point towards the cells totally suspending cell division and metabolic activity in long-term starvation. Whether all protein synthesis is actually halted in *L. monocytogenes* still remains to be definitively answered, and evidence obtained from *E. coli*, *V. sp.* and *S. aureus* would suggest this is probably not the case. In these bacteria, starvation-survival proteins are synthesised early on in starvation, but continued protein synthesis at low-levels is still required for survival (Reeve *et al.*, 1984; Morton and Oliver, 1994; Watson *et al.*, 1998).

The SSR is a complex mechanism allowing long-term survival and subsequent recovery. In Gram-negative bacteria, the expression of many genes is controlled in a coordinate fashion during the response by the stationary-phase gene regulator, σ^S. In Gram-positive bacteria, no homologue of this regulator has been found, however the alternative sigma factor σ^B is an acknowledged regulator of the general stress response in several species including *L. monocytogenes* (Völker *et al.*, 1999; Becker *et al.*, 1998). σ^B has been proposed to be a functional homologue to the Gram-negative starvation-survival response regulator σ^S and although σ^B expression and activity are induced by entry to stationary phase, no evidence of the regulation of the starvation-survival response by σ^B has been found (Becker *et al.*, 1998; Chan *et al.*, 1998). In this study, the *sigB* mutant (DES011) was reduced 10-fold in starvation-survival potential in long-term glucose limiting cultures, though notably this defect only becomes apparent beyond 5 days. The development of SSR of *L. monocytogenes* is therefore independent of σ^B, and the delay may be indicative of an accumulation of adverse effects stemming from the *sigB* mutation. Several stationary-phase induced, σ^B regulated genes (*dps*, *katE*, *clpC*), help to increase the capability of the cell to deal with oxidative damage to cellular components in *B. subtilis* (Hecker and Völker, 1998). The role of oxidation in the ageing of starved cultures has been proposed to be a key factor in bacterial mortality (Nyström, 1999). The lack of a significant drop in the pH of glucose-starved *L. monocytogenes* cultures makes a direct link between reduced pH and mortality less likely,

and the fact that the *sigB* mutation has no effect on starved cell H_2O_2 resistance tends to point away from this theory. Despite these findings, increased oxidative damage is still likely to be the most significant result of a *sigB* mutation. Therefore σ^B appears not to be the principal regulator of the starvation-survival response, yet plays a role in long-term survival by contributing to stress resistance in starved cells.

PrfA is the principal virulence gene regulator in *L. monocytogenes* belonging to the same family of regulators as the catabolite repression regulator Crp, and the redox-sensitive gene regulator Fnr (Lampidis *et al.*, 1994). PrfA-dependent genes are regulated in response to a variety of environmental signals (Behari and Youngman, 1998b; Böckmann *et al.*, 1996; Leimeister-Wächter *et al.*, 1992; Mengaud *et al.*, 1991), and of particular interest is the discovery by Ripio *et al.* (1998) that the general stress protein ClpC is negatively regulated by PrfA. The PrfA mutant strain showed a 10-fold reduction in survival under glucose-limitation, which results from extended loss ofviability during the first few days of starvation. This implies that PrfA is required for survival, particularly in the short term, though cells that survive this initial period can still remain viable. Notably, PrfA also apparently represses acid and oxidative stress resistance mechanisms, particularly during exponential phase growth, which is reinforced by the profile of catalase activity, which also peaks at this time. The resistance to acid and oxidative stress seen in long-term starved cells is independent of PrfA, suggesting that PrfA-dependent regulation is superseded by another mechanism during starvation. The changes observed are unlikely to be solely via the action of CplC, as a reduction in heat resistance would have been expected (Rouquette, *et al.*, 1996). Also, repression of stress resistance is not relieved in post-exponential phase cells, by which time CplC expression is PrfA-independent (Ripio *et al.*, 1998), suggesting that the regulation of the genes in each case differ. So why should upregulation of stress genes in a PrfA mutant adversely affect starvation-survival? A possibility is that overexpression of a few specific stress resistance mechanisms could divert sufficient resources away from other essential systems to reduce the overall fitness of the cell. Alternatively, genes that comprise the oxidative and acid stress responses include a number responsible for the turnover of damaged proteins and lipids (e.g. Clp proteins). High levels of these proteins could result in excessive degradation of important cellular components.

Interaction between virulence and general stress networks has been demonstrated in *Salmonella*, where the *spv* plasmid virulence gene (*spvR*) is positively regulated by σ^S (Guiney, 1995). In *L. monocytogenes* however, the opposite appears to be the case, as PrfA represses mediators of stress resistance (Ripio *et al.*, 1998). An explanation could lie in the relative lifestyles of the organisms during an infection. *L. monocytogenes* rapidly enters and multiplies within host cells (Sheehan *et al.*, 1994), where stress proteins seem not to be required (Hanawa *et al.*, 1995). In contrast, *Salmonella* multiplies slowly within the damaging environment of the phagosome, and would therefore probably require concomitant induction of the stress response (Finlay, 1994).

In order to understand the molecular mechanism of the SSR, it is important to characterise components in the response. Mutants with altered survival/recovery characteristics have been isolated from a number of bacterial species. These have shown specific classes of genes whose products are important for survival and have roles in cellular metabolism (*bioA*), stress resistance (*dps*), morphology (*bolA*) and starvation recovery (*ctaA*) (Keer *et al.*, 2000; Almirón *et al.*, 1992; Aldea *et al.*, 1989; Clements *et al.*, 1999). Using a screening methodology based upon the inability of starvation-survival mutants to survive 12 days incubation on glucose-limiting CDM agar plates at 37°C, four survival/recovery mutants of *L. monocytogenes* were isolated. The mutants were reduced in liquid culture survival by 10-fold relative to the wild type strain, a reduction comparable to that seen in starvation-survival mutants of *S. aureus*, *V. sp.* and *E. coli* (Watson *et al.*, 1998b). The transposon insertions in the mutants occurred within ORFs encoding for putative homologues of YulB and PhaQ. What function do these gene proteins perform, and how are they related to the process of starvation-survival?

The transposon insertion in strains DES028 and DES029 were found within a putative ORF encoding for a homologue of YulB from *B. subtilis*. YulB is a putative member of the DeoR family of transcriptional regulators, a large number of which regulate expression of components of the phosphoenolpyruvate-dependent phosphotrsansferase system. These are of importance to the cell for the ability to import alternative supplies of carbon and energy when other more readily metabolised sources, such as glucose, become depleted. As *L.*

monocytogenes cultures become starved, a large proportion of the cells appear to lyse, releasing a mixture of carbon compounds into the culture medium. The ability to transport these compounds into the cell to supply sufficient material for survival could therefore be vital. The DeoR family of regulators are found to typically repress the expression of the operons involved (van Rooijen and de Vos, 1990). Inactivation of the repressor would presumably result in overexpression of a subset of PTS components, which could dilute out other PTS systems by competition for the general PTS components HPr and PTS enzyme I.

The presence of a putative *sigB*-dependent promoter upstream of the ORF suggests that the YulB homologue is a member of the general stress regulon of *L. monocytogenes*. It would be possible that PTS components could comprise part of a response to stress, though it must be remembered that DeoR family repressors are found elsewhere – including the virulence plasmid in *A. tumefaciens* (Mortensen *et al.*, 1989), and in governing the regulation of nucleoside utilisation in *E. coli* (Beck von Bodman *et al.*, 1992).

Starvation of DES028 and DES029 for glucose in liquid culture exhibited a gradual loss of viability, whilst heat stress resistance was impaired in long-term starved cells and oxidative stress resistance was reduced in all stages of growth. Until the specific metabolic function of the genes regulated by the *yulB* homologue in *L. monocytogenes* are elucidated, it is only possible to speculate as to the cause of the phenotype seen in these strains. The disruption of the PTS-dependent uptake of nutrients caused by the transposon insertion may well account for the survival defect, as cells eventually succumb to the chronic lack of nutrients. The question remains how this effect would result in a reduction in cellular resistance to heat and oxidative stress. The rescue of the starvation-survival phenotype in the latter stages is a phenomenon previously observed in starvation-survival defective mutants of *M. smegmatis* (Keer *et al.*, 2000; Keer *et al.*, 2001). Initially proposed to be a result of the accumulation of suppressor mutations within the starved cells, these observations were deemed further evidence of the dynamic nature of starved cultures.

Strains DES035 and DES045 were found to contain a transposon insertion within a putative ORF encoding for a homologue of PhaQ from *B. megaterium*. PhaQ is a protein of no known

function, but which is known to be associated with polyhydroxyalkanoate inclusion bodies in *B. megaterium* (McCool and Cannon, 1999). PHA is well known to act as a carbon and energy reserve in both Gram-negative and Gram-positive bacteria (Anderson and Dawes, 1990). Accumulation typically occurs in glucose-rich conditions with limitation of one of several other nutrients (e.g. nitrogen, phosphorus, sulphur, magnesium, potassium or iron) and can delay the degradation of other cell components when the cells become starved (Szewczyk, 1992). PHA inclusion bodies in *B. megaterium* contain approximately 1.8% protein, which forms an outer layer with a lipid component (Anderson and Dawes, 1990). Reports of PHA inclusion bodies in *R. ruber* and *A. eutrophus* have identified the presence of the low molecular weight proteins GA14 and GA24 (Pieper-Fürst *et al.*, 1994; Wieczorek *et al.*, 1995). These proteins were found not to be essential for the accumulation of PHA, but did influence the rate of PHA accumulation and the size of the inclusion bodies produced (Pieper-Fürst *et al.*, 1995; Wieczorek *et al.*, 1995). The putative PhaQ homologue may perform a functionally similar role to these proteins, thus disruption would affect the ability of the cell to accumulate PHA, and subsequent access these resources when required. While it should be noted that survival of PHA-producing bacteria is not always dependent upon PHA (Anderson and Dawes, 1990), it is probable that such an effect would impair the capability of the cell to withstand starvation conditions as extra pressure is placed on the limited cellular energy generation and nutrient supply.

The starvation-survival phenotype of DES035 and DES045 in glucose-limiting CDM only became apparent after 15 days, and so the mutation has no immediate effect on the development of the SSR. This would concur however with an additional reserve such as PHA becoming depleted after prolonged starvation, resulting in loss of viability. The resistance of DES035 and DES045 to heat, acid and oxidative stress remained largely unaffected. This would be consistent with inactivation of a mechanism principally involved with the accumulation and utilisation of a storage compound.

In this study, the starvation-survival response of *L. monocytogenes* has been characterised. The work also identified several important components of the process, which were then

investigated in further detail to determine their contribution to the development and maintenance of the starvation-survival phenotype.

6.3 Future starvation-survival research

The ability of *L. monocytogenes* to survive in the environment continues to pose a problem to the food production industry. In this state, *L. monocytogenes* is highly resistant to environmental stress and so is more difficult to eliminate. It is hoped that the advent of the post-genomic era will herald a rapid increase in the knowledge of the mechanisms of starvation-survival. This unprecedented wealth of information can be coupled with new technologies such as DNA microarray and signature-tagged mutagenesis, which have already been used successfully to identify novel virulence genes in a number of bacteria including *S. aureus*, and for detecting gene expression in response to acid stress in *Helicobacter pylori* (Mei *et al.*, 1997; Ang *et al.*, 2001). The data released from genome sequencing projects to date have identified numerous genes with products of unknown function and this will almost certainly be the case when the current Pasteur Institute *L. monocytogenes* sequencing project is completed. Understanding of the mechanism of starvation-survival and stress resistance in *L. monocytogenes* will help in the development of novel regimes for the control of this bacterium. The need to remove the potential for *L. monocytogenes* to enter the food chain by the development of efficient eradication procedures highlights the importance of our ability to understand the SSR.

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