Functional Genomics and physiology of growth initiation in *Salmonella*

Submitted by

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Dedicated to my Dad

William James Rice

(November 1946 – July 2008)

An Inspiration.

Abstract

Lag phase is a period of bacterial adaptation that occurs prior to cell division. The aim of this project was to characterise the processes used by *Salmonella enterica* serovar Typhimurium to escape from lag phase, and determine whether these processes are dependent on the bacterial 'physiological history'.

The lag phase transcriptomic response at 25 °C of stationary phase cells that had been held for twelve days at 2 °C was compared with that of stationary phase cells not subjected to this cold storage treatment. Cold-stored cells showed significant changes in expression of 78 % genes during lag phase, with 875 genes altering their expression ≥2-fold within the first four minutes of inoculation into fresh medium. Functional categories of genes that were significantly up-regulated included those encoding systems involved with metal ion uptake, stress resistance, phosphate uptake, ribosome synthesis and cellular metabolism. Genes in the OxyR regulon were induced earlier in cold-stored cells, a response coupled with a delay in the expression of Fe^{2+} acquisition genes, and down-regulation of genes encoding central metabolic enzymes. Together, these findings with physiological tests demonstrated that *Salmonella* held in cold storage exhibited an increased sensitivity to oxidative stress in mid-lag phase, although the lag time was not increased. Despite an oxidative stress response at the transcriptomic level during lag phase under both experimental conditions, deletion of the OxyR and SoxRS systems did not lead to an increased lag time during aerobic growth at 25 °C.

The intracellular concentration of metal ions was quantified using ICP-MS, and changes observed during lag phase confirmed the transcriptomic data. Metal ions specifically accumulated during lag phase included Mn^{2+}, Fe^{2+}, Cu^{2+} and Ca^{2+}, with the latter being the most abundant metal ion. The intracellular concentration of Zn^{2+} and Mg^{2+} remained the same as for stationary phase cells, and Ni^{2+}, Mo^{2+} and Co^{2+} were expelled from the cell during lag phase. Metal homeostasis was determined to be a critical process, highlighted by growth in the presence of a chelator causing an extended lag time.

Overall, lag phase was found to be a robust and reproducible adaptation period which was not perturbed by the mutagenesis approaches utilised in this study.

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List of Abbreviations

Abbreviation	Definition
amol	Attomole (10^{-18} moles)
bp	Base pair
°C	degree Celsius
cat	Chloramphenicol acetyltransferase gene
cDNA	Complementary or copy DNA
CFU	Colony forming units
ChIP	Chromatin immunoprecipitation
ChIP-chip	Chromatin immunoprecipitation and microarrays
Cm	Chloramphenicol
cm	Centimetre
Cy3	Cy3-conjugated dCTP
Cy5	Cy5-conjugated dCTP
DFBMD	Division of Foodborne, Bacterial and Mycotic Diseases
dH_2O	Molecular grade, deionised water
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylenediaminetetracetic acid
E_h	Redox potential
FSA	Food Standards Agency
g/L	Grams per litre
gDNA	Genomic DNA
h_0	Metabolic 'work to be done' lag parameter
hr	Hour(s)
ICP-MS	Inductively-coupled plasma mass spectrometry
kan	Kanamycin resistance gene
kb	Kilobase
Km	Kanamycin
λ	Lag time
L	Litre
LB	Luria Bertani medium
LPS	Lipopolysaccharide
m	Metre
M	Molar (moles per litre)
Mb	Megabase
mg	Milligram
min	Minute(s)
ml	Millilitre
mm	Millimetre
mM	Millimolar (millimoles per litre)
MOPS	3-(N-morpholino)propanesulfonic acid
mRNA	Messenger RNA
mV	Millivolts

µg	Microgram
µL	Microlitre
µ	Growth rate
µM	Micromolar (micromoles per litre)
µmax	Maximum specific growth rate
N_0	Initial cell concentration
Nmax	Highest cell concentration
NMR	Nuclear magnetic resonance
ORF	Open reading frame
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PMSF	Phenylmethylsulfanyl fluoride
RNA	Ribonucleic acid
rpm	Revolutions per minute
rRNA	Ribosomal ribonucleic acid
SDS	Sodium dodecyl sulphate
Spc	Spectinomycin
SPI	*Salmonella* Pathogenicity Island
spp.	Species
S. Typhimurium	*Salmonella enterica* serovar Typhimurium
σ	Sigma factor
tRNA	Transfer RNA
v/v	Volume/volume
w	Weibull parameter
w/v	Weight/volume
WHO	World Health Organisation

List of Accompanying Material

The following material is provided on a CD to accompany this thesis:

Appendix A:
Primer sequencing for Lambda Red mutagenesis and verification of mutation

Appendix B:
Δ*sitABCD::kan* mutant strain sequencing information

Appendix C:
Lag Pre-Incubation microarray files

Lag Phase 25 °C microarray files (Rolfe, 2007)

Table C1: List of 2,666 genes changing during lag phase (Rolfe, 2007)

BABAR output of Lag Pre-Incubation and Lag Phase 25 °C microarray files (BABAR results.txt)

Appendix D:
ChIPOTle output of ChIP-chip data

Table D1: Top 100 expressed genes during stationary phase (Rolfe, 2007).

Appendix E:
Processed ICP-MS data for 36 metal ions

List of Presentations and Publications

Title	Type of presentation
Campden BRI project update presentation. Six presentations given over 3 years (2006-2009).	Oral presentation
Norwich Showcase of Postgraduate Talent. July 2007.	Poster presentation
Wellcome Trust Science Essay Competition. September 2007.	Essay
Biotechnology YES Regional Competition. October 2007.	Group presentation
Biotechnology YES National Final. December 2007.	Group presentation
Two Campden BRI Microbiology Panel Meetings. May 2008 and May 2009.	Oral presentation
SET for Britain Poster Competition. March 2009.	Poster presentation
Campden BRI International Food Microbiology Conference. September 2009.	Oral presentation
The American Society For Microbiology: 3rd ASM Conference on *Salmonella*: Biology, Pathogenesis & Prevention. October 2009	Poster presentation

Manuscripts in preparation:

Title	Target Journal
"Bacterial rejuvenation: lag phase is a distinct growth phase that involves metal accumulation and prepares bacteria for exponential growth."	*Environmental Microbiology*
"Growth history affects the physiological lag phase but not the geometric lag time or the growth rate of *Salmonella enterica* serovar Typhimurium".	*Applied & Environmental Microbiology*

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Chapter 1

Introduction

1. Introduction

This introduction gives an overview of *Salmonella* as a pathogen and discusses the physiological processes that underpin lag phase, based on the published literature. The chapter concludes with specific aims of the study, focussed within the context of providing physiological information to underpin future developments in predictive microbiology and the introduction of novel intervention strategies to combat bacterial pathogens.

1.1 *Salmonella*

Salmonellae are enteric foodborne pathogenic members of the Gamma Proteobacteria that pose a threat to human and animal health. There are three species of *Salmonella*, *S. bongori*, *S. enterica* and the new *S. subterranean*, officially recognised in 2005 (Su & Chiu, 2007). *S. enterica* is the most significant of these species and is further divided in to six subspecies: *enterica*, *salamae*, *arizonae*, *diarizonae*, *houtenae*, and *indica* (Janda & Abbott, 2006). *Salmonella enterica* infects many hosts including birds, mammals, fish and reptiles with a wide range of host-specific serovars (Jones & Falkow, 1996). In humans, *Salmonella enterica* serovars Typhi (*S.* Typhi) and Paratyphi (*S.* Paratyphi) cause a life-threatening enteric fever via a systemic infection involving transmission through the gut epithelium, into the blood-stream and to organs such as the liver, gall-bladder and spleen (Palmer & Reeder, 2001). In the developed world the majority of salmonellosis cases are typified by self-limiting gastroenteritis through infection by *Salmonella enterica* serovars such as *S.* Typhimurium and *S.* Enteritidis (Scherer & Miller, 2001).

1.1.1 *Salmonella* infections in humans

Salmonella is the most common cause of foodborne disease outbreaks in Europe (EFSA, 2009). The majority of *Salmonella* infections are self-limiting gastroenteritis, requiring an infectious dose of between 10^5 and 10^{10} viable cells (Glaser & Newman, 1982, Kothary & Babu, 2001). Intra-gastric infection with *S.* Enteritidis leads to the death of 99 % of bacteria after one hour, due to the highly acidic environment (Carter & Collins, 1974). Despite this, if *Salmonella* infections are associated with oil-based food products (*e.g.* chocolate or cheese) a lower infectious dose may be sufficient as the oil is believed to protect *Salmonella* from the stomach acid (D'Aoust, 1985, Gawande & Bhagwat, 2002). The salmonellosis disease incubation period is typically six to forty-eight hours,

characterised by symptoms including headaches, abdominal pain, diarrhoea, and vomiting. Fever and muscle aches are common associated symptoms (Darwin & Miller, 1999). Approximately one percent of infections in the United States are of a serious nature, leading to bacteraemia, mostly in immunocompromised individuals and the elderly, which can be life-threatening (DFBMD, 2009).

Typhoid fever, caused by *S.* Typhi, is usually spread through faecal contamination of water supplies (Scherer & Miller, 2001), occurring primarily in the developing world (Crump *et al.*, 2004). Water treatment and sanitation have removed almost all domestic incidences of typhoid in the developed world. In contrast to non-typhoidal *Salmonella* (NTS), the global typhoid fever incidence is difficult to estimate. One study using reported literature from 1966-2001 estimated there to be 21.65 million cases of typhoid per year (0.36 % global population, based on data from 2000), with 216,510 deaths mostly in Asia and Africa (Crump *et al.*, 2004). However the World Health Organisation estimates there to be 16 million typhoid cases per year worldwide, resulting in 600,000 deaths (WHO, 2000). Both studies indicate typhoid is a serious issue with significant mortality and also that the trend of typhoid fever is increasing as the global population increases. Typhoid fever has an incubation period of five to twenty-one days with characteristic symptoms of high fever, diarrhoea and a rash (Scherer & Miller, 2001). At present, approximately 5 % of sufferers become carriers of typhoid (CDC, 2009), lower than indicated by studies from over forty years ago (Bokkenheuser, 1964). The total number of carriers is difficult to accurately determine as individuals can shed bacteria from the gall-bladder for over one year, potentially infecting other individuals, some of whom will become carriers themselves. Foreign travel-associated typhoid fever accounts for approximately 300 clinical cases in the United States per year (Lynch *et al.*, 2009) and 100 cases in England and Wales (Health Protection Agency, 2008).

Non-typhoidal *Salmonella*-induced gastroenteritis is primarily transmitted by contaminated foodstuffs, most commonly red and white meat, raw eggs, milk, and dairy products (Health Protection Agency, 2008), although cases of *Salmonella* food poisoning from fresh produce are increasing due to faecal contamination in fields at the pre-processing stage of food production (Sivapalasingam *et al.*, 2004).

The majority of fatal *Salmonella* cases occur in the developing world where accurate and reliable statistics are difficult to obtain. The incidence of NTS bacteraemia are increasing in areas such as sub-Saharan Africa, coinciding with incidences of human immunodeficiency virus (Kankwatira *et al.*, 2004).

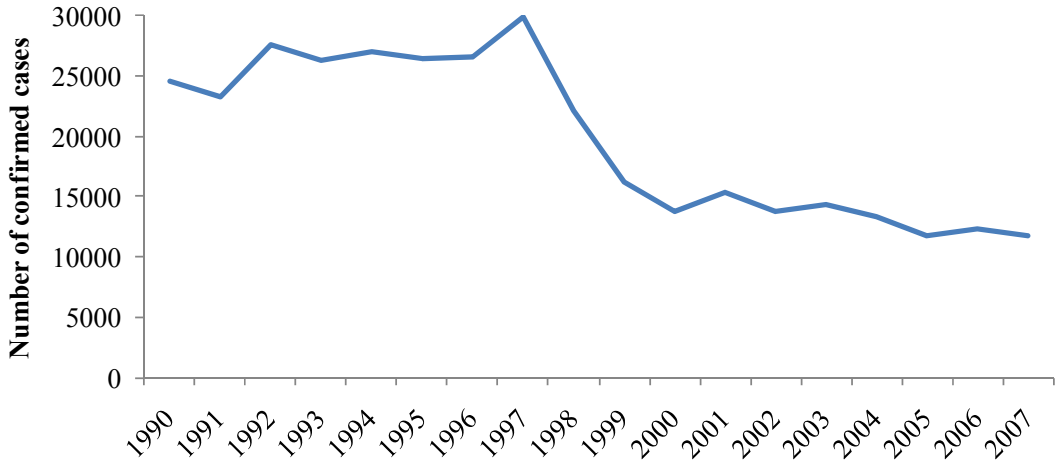


Figure 1.1: Incidences of NTS in England and Wales 1990-2007.
Total laboratory-confirmed cases of non-typhoidal *Salmonella* per annum in England and Wales. Figure reproduced from public data (Health Protection Agency, 2008).

In England and Wales, where incidences are more thoroughly reported, gastroenteritis caused by NTS has decreased from 24,610 confirmed cases in 1990 to 11,705 confirmed cases in 2007 (Figure 1.1). Despite this downward trend, *Salmonella* is still responsible for the majority of outbreaks of bacterial food poisoning in Europe. In 2007, there were 590 verified outbreaks in Europe (EFSA, 2009). NTS are one of the more significant causes of foodborne disease in England and Wales, in terms of estimated total cases, confirmed cases, General Practitioner (GP) cases, hospitalisations and deaths (Figure 1.2). In 2000, *Salmonella* was responsible for the highest number of deaths by food-associated bacteria in England and Wales, however this number had slightly decreased by 2005 and was the second highest bacterial cause of foodborne death behind *Listeria monocytogenes* (FSA, 2007).

Salmonella associated deaths are speculated to be due to antibiotic-resistant bacteria, although other factors such as septicaemia, endocarditis, gut perforations and surgery complications may contribute (Helms *et al.*, 2003). Between 1990 and 2007, cases of NTS food poisoning in England and Wales were primarily due to *S.* Enteritidis, the peak infective cases was during 1997 with 22,254 confirmed cases (compared with 9,228 cases for *S.* Typhimurium and all other serovars combined). A steady decline in confirmed cases associated with *S.* Enteritidis has been observed from 1997 to 2007 likely due to the vaccination of laying and broiler flocks in the UK (Cogan & Humphrey, 2003).

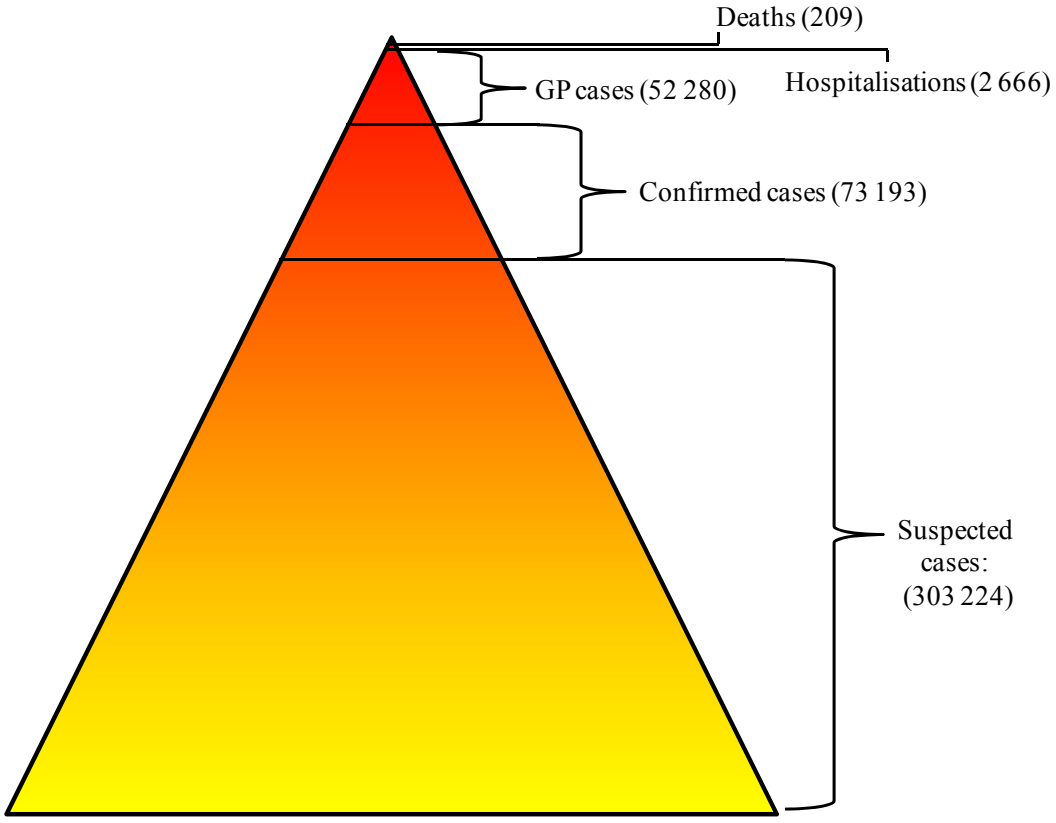


Figure 1.2: Salmonellosis in England and Wales from 1996 until 2000. Estimated annual number of *Salmonella* incidences resulting in GP cases, hospitalisations and deaths (Adak *et al.*, 2005). "Suspected cases" extrapolated from the number of GP cases, by a previously described method (Adak *et al.*, 2002).

1.1.2 *Salmonella* infection and persistence

Salmonella is a well-studied bacterium and extensive work has been performed with animal models including mice, calves, pigs and chickens. The majority of infection studies with *S.* Typhimurium have been performed with the mouse model, in which a systemic

typhoid-like infection occurs, usually resulting in death of the animal within a few days once the bacterial load reaches 10^8 viable cells / ml blood (Carter & Collins, 1974). However if the infection is cleared by the immune system, the mouse becomes resistant to re-infection due to immunological memory T-cells and anti-*Salmonella* antibodies (Mastroeni, 2002). During systemic infection of mice by intraperitoneally-injected *S.* Typhimurium, the bacteria enter the mouse abdominal cavity and travel to the spleen and liver. If the bacteria are administered orally, *S.* Typhimurium passes through the stomach, to the small intestine and through specialised M-cells in the Peyer's patches, where the bacteria are engulfed by macrophages and disseminated throughout the body (Jensen *et al.*, 1998). Survival and replication in macrophages is a crucial step for *Salmonella* systemic infection to major organs and the bone marrow (Everest *et al.*, 2001). *Salmonella* produces effector proteins to survive in macrophages, stopping the fusion of the macrophage with the lysozomal compartment and resisting toxicity of antimicrobial compounds produced within the macrophage (Abrahams & Hensel, 2006). Growth inside the macrophage is dependent upon metabolism, primarily of sugars transported via the phosphoenolpyruvate:carbohydrate phosphotransferase system (Bowden *et al.*, 2009). Once the bacteria have replicated sufficiently, the macrophages burst, disseminating *Salmonella* throughout the animal, to infect other cells and organs. The route of infection in mice is similar to typhoid fever in humans although the symptoms often differ. During most *S.* Typhi human infections, patients initially present with a high fever, followed by headaches, abdominal pain and fatigue (Hornick *et al.*, 1970a). The reticulendothelial system increases the concentration of phagocytes, which may contain *S.* Typhi during systemic infection, increasing the bacterial load in the liver and spleen as a result (Hornick *et al.*, 1970b).

In addition to the typhoid-like infection, colitis can also be studied in mouse models pre-treated with streptomycin to clear the gut microflora prior to infection (Barthel *et al.*, 2003, Bohnhoff *et al.*, 1964). Calves are the animal model of choice to study colitis which manifests as an inflamed colon as bacteria colonise the large intestine causing diarrhoea within 48 hours post-infection (Rankin & Taylor, 1966). These symptoms distinguish the colitis and typhoid-like models (Tsolis *et al.*, 1999b). Gut colonisation of calves has been shown to be primarily due to the *Salmonella* Pathogenicity Island (SPI) 1, although mutagenesis approaches have identified other crucial genes, including the Δ*aroA* mutation

(Tsolis *et al.*, 1999a). A signature-tagged mutagenesis approach identified 75 genes required for full virulence of calves including genes belonging to SPI-1, SPI-2 and SPI-4, with the latter Pathogenicity Island not required for infection of one-day old chicks (Morgan *et al.*, 2004).

Genes present within SPI-1 are required for the secretion of effector proteins, primarily involved in the invasion of epithelial cells. SPI-2 genes are required for intracellular replication within macrophages and maintenance of the *Salmonella*-containing vacuole (Darwin & Miller, 1999). The expression of these islands are not mutually exclusive and it has been shown that during infection of epithelial cells both SPI-1 and SPI-2 are induced in addition to the flagella type III secretion system (Hautefort *et al.*, 2008).

1.1.3 *Salmonella* genome sequences

The *Salmonella* functional genomic analyses performed in this thesis benefit from the sequencing of several *Salmonella* genomes as well as the *Escherichia coli* K-12 genome (Blattner *et al.*, 1997), used as a comparison for several conserved homologues. The first *Salmonella* genomes to be sequenced were *S.* Typhimurium LT2 (McClelland *et al.*, 2001) and *S.* Typhi CT18 (Parkhill *et al.*, 2001). More recently the genome sequences of *S.* Cholerasuis SC-B67 (Chiu *et al.*, 2005), *S.* Typhi Ty2 (Deng *et al.*, 2003) and *S.* Paratyphi A ATCC 9150 (McClelland *et al.*, 2004) have been published. At present there are an additional eleven *Salmonella* genome sequences yet to be fully completed and annotated including *S. bongori*, *S.* Enteritidis PT4 and *S.* Typhimurium SL1344 (Sanger Institute, 2009). The latter strain is used throughout this study and contains a 5.07 Mb chromosome with 4527 chromosomal coding sequences. There are also three plasmids 93.8 kb, 96.9 kb and 8.69 kb in size, containing 104, 103 and 14 coding sequences, respectively (Sanger Institute, 2009). The *S.* Typhimurium SL1344 strain is a histidine auxotrophic strain derived from the wild-type *S.* Typhimurium 4/74 (Hoiseth & Stocker, 1981). Although some histidine mutations, (e.g. *hisG*), are known to have physiological effects on *Salmonella* during infection of macrophages (Henry *et al.*, 2005), *S.* Typhimurium SL1344 has been used successfully throughout the *Salmonella* community for many years and has been shown to be unaffected during growth under standard laboratory conditions in LB medium (Rolfe, 2007).

1.2 Lag phase

The phenomenon of lag phase was first explored in *Salmonella* ('mouse Typhoid bacillus'), described as the 'latent period' before division was initiated (Penfold, 1914). Despite these early observations, almost one hundred years later lag phase remains poorly understood, and the processes defining lag phase remain to be fully elucidated.

It is known that immediately following a shift in environment, bacterial cells can experience a lag period before multiplying. During this delay, bacterial cells modify their physiological state and the extent of modification, and therefore the lag duration, is dependent in part upon the difference between the new and previous environments (Buchanan & Cygnarowicz, 1990). A lag phase can arise from a change in environmental conditions during steady state growth. This 'intermediate lag' usually arises from a depletion of nutrients or the presence of a stressor. The intermediate lag usually lasts until the stressor is overcome and is distinct from the initial lag seen after bacterial re-culturing (Figure 1.3), although some processes are believed to be similar (Swinnen *et al.*, 2004). In this thesis, only the initial lag period is considered.

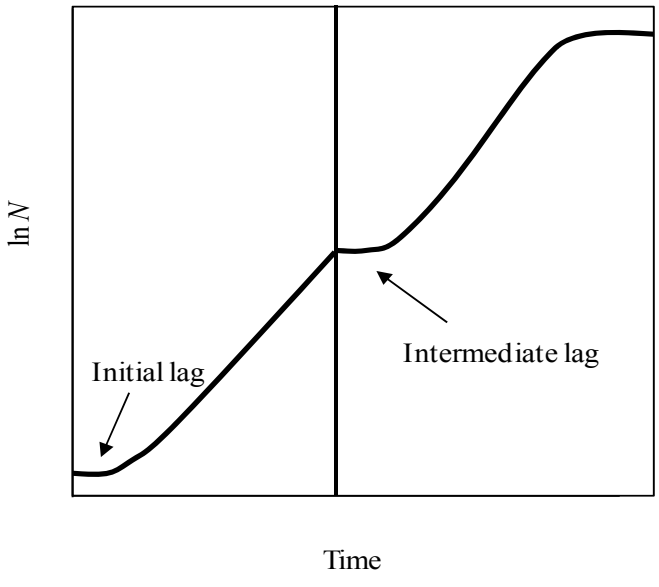


Figure 1.3: Initial and intermediate lag phases are similar but distinct events.
The two types of bacterial lag are shown. The initial lag phase after inoculation of bacteria into a fresh medium and the intermediate lag after a period of exponential growth indicated by the central, black, vertical line. '*N*' represents the bacterial cell concentration. After the intermediate lag, exponential growth resumes. Figure adapted from Swinnen *et al.* (2004).

1.2.1 The bacterial growth curve

Batch grown bacterial cultures show three distinct stages of growth (Figure 1.4). Cells are inoculated into a fresh medium at an initial concentration (N_0) and enter a lag phase requiring adaptation until the first cell division. At the end of lag phase (λ) the cells begin to divide exponentially until a maximum and constant division rate is obtained (μ_{max}). The bacteria continue to divide until either one or more nutrients become a limiting factor or a build-up of a toxic metabolite reaches a critical concentration. At this point the bacterial division rate slows (transition phase) and bacteria enter stationary phase at a maximum cell concentration (N_{max}). In addition to the stages presented, a death phase can occur if toxic metabolites become lethal to the population (Madigan *et al.*, 2000). After any death phase, the remaining sub-population can remain viable for an extended period without multiplication in a stressful environment (Finkel *et al.*, 1998). These bacterial cells have been shown to have a growth advantage in stationary phase (GASP) phenotype compared with non-stressed bacterial cells, suggesting that a selection pressure is present during extended stationary phase (Finkel, 2006).

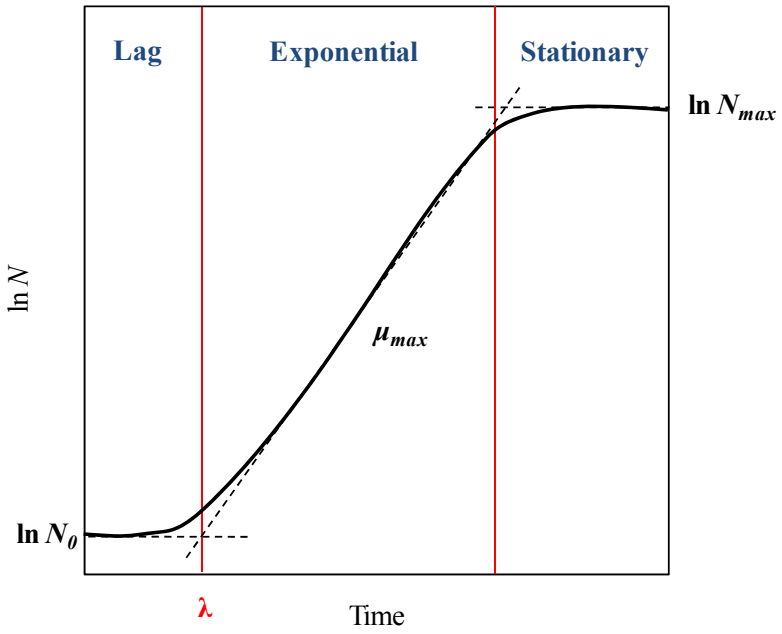


Figure 1.4: The bacterial growth curve.
The three distinct phases of normal bacterial growth are shown in blue and the end of each phase is demonstrated with a red vertical line. '*N*' represents the bacterial cell concentration, N_0 is the initial cell concentration, μ_{max} is the maximum growth rate and N_{max} is the maximum cell concentration. The lag time calculated by the biphasic growth model is indicated (λ) (Section 1.2.1.) Figure adapted from Swinnen *et al.* (2004).

1.2.2 How is lag time measured?

The original study describing lag phase in *Salmonella* discussed the problems associated with lag measurement methods (Penfold, 1914). The study described these difficulties as being due in part to varying definitions of lag time. The definitions included:

- A period of sub-maximal growth
- A period of 'restrained growth' in terms of minimal generation times
- An average of generation times within the first hours of growth compared with subsequent growth periods

Penfold (1914) acknowledged draw-backs to each of these definitions, therefore modern modelling methods rely on more accurate measurements of lag phase, however some stochastic events during lag phase lead to inaccuracies in even the most sophisticated models (Baranyi, 2002). Many models to measure lag time are based on the initial starting cell concentration (N_0) and the exponential growth rate (μ_{max}) parameters in a biphasic growth curve (Baranyi, 1998). By extrapolating the μ_{max} back to the N_0 value, the population lag duration (λ) can be calculated (Zwietering *et al.*, 1992). This simplistic approach is used in the present study and does not take into account the gradual transition into exponential growth caused by the stochastic division of individual cells and represents an estimation of the population lag (Baranyi, 1998).

Alternative measurements exist to calculate population lag times. Indeed, the food industry utilises a combination of models to identify consistent lag times including: linear regression, quadratic equations, linear extrapolation and exponential decay curves (Borneman *et al.*, 2009). Whichever model is used there is a degree of 'inter-model variability' that is exacerbated by author biases during analysis of bacterial lag times with a favoured model under a particular growth condition (Baty & Delignette-Muller, 2004). One previous study analysed three different lag duration models, including the Baranyi dynamic model (Baranyi & Roberts, 1994) utilised in the present study, and concluded that inter-model lag times were generally consistent assuming equal data quality between studies. Of the models tested the authors concluded that the Baranyi dynamic model was

the most constant, yielding reproducible lag times accurate across multiple datasets (Baty & Delignette-Muller, 2004).

The majority of methods to estimate the lag time and specific growth rate of a bacterial population use bacterial viable counts (Swinnen *et al.*, 2004). This method is not perfect, due to the presence of viable but non-culturable cells (Panutdaporn *et al.*, 2006) and filamentous, non-septated bacteria under stress conditions (Mattick *et al.*, 2003). Viable counts are however preferable to indirect measurements utilised in microbiology laboratories such as light scattering measurements (*e.g.* OD_{600}). Generally, OD measurements can only be made at a relatively-high bacterial concentration (~10^6 CFU / ml) at which point, many bacterial divisions may have occurred and lag time has to be extrapolated from the starting concentration of bacteria (Figure 1.5).

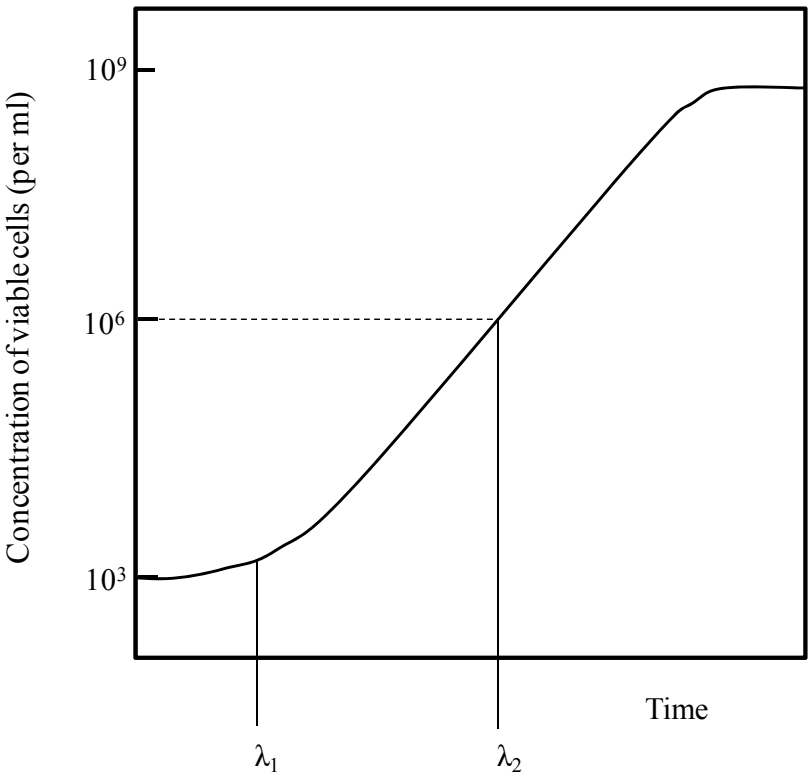


Figure 1.5: Use of OD and viable counts to measure lag time.
Growth curve measured from a 10^3 CFU / ml starting inoculum with the geometric lag times from biphasic model based on viable counts (λ_1) and OD measurements (λ_2), without extrapolation. Detection limit for OD measurements assumed to be 10^6 CFU / ml (dotted line). The viable count method is more sensitive at lower cell concentrations whereas the OD measurements require extrapolation of the exponential growth phase back to the 10^3 initial cell concentration for accurate measurements.

Despite the drawbacks associated with OD measurements, published studies have used this approach to attempt to estimate bacterial lag times, most notably by BioScreen C (ThermoLabsystems) spectroscopy at the population (Augustin *et al.*, 1999) and even single-cell level (Malakar & Barker, 2008, Metris *et al.*, 2008, Stringer *et al.*, 2005), generating large datasets to off-set the variation. A previous study measuring the lag time of *S.* Typhimurium in a static growth system with BioScreen C led to variable data and the method was ultimately deemed unsuitable for the required purpose by the author (Rolfe, 2007).

1.2.3 Single-cell lag time

Most lag phase models focus on measuring the population lag time as this type of model is simpler and takes account of cell to cell variability (Pin & Baranyi, 2008). However, the analysis of individual cell lag times generates a distribution which accounts for the transition from lag into exponential growth and cannot be directly calculated from data for a population model. The individual bacterial cell lag times are important, as those cells which leave lag phase earliest can rapidly take over the bacterial population, especially when the bacterial population is small (Pin & Baranyi, 2006). By calculating the individual lag times the population lag can be accurately determined, however individual lag times cannot be extrapolated from the population lag time (Baranyi, 2002).

Current analysis methods for calculating the single cell lag time depend on techniques such as the flow chamber (Elfwing *et al.*, 2004, Rolfe, 2007), digital microscopy imaging (Niven *et al.*, 2006, Stringer *et al.*, 2005) and microtitre plates (Stephens *et al.*, 1997). The latter technique has been used to study the individual lag times of *L. monocytogenes* by performing two-fold dilutions of bacterial cultures and calculating the time until the detection limit of the turbidimetric instrument was reached (McKellar & Knight, 2000).

1.2.4 Factors influencing lag time

Different bacterial species grown under identical conditions show different lag times, presumably as different species have specific physiological requirements to adapt to new environments before growth can begin (Mellefont *et al.*, 2003). Aside from species specific lag time variability, other factors are thought to be important such as inoculum size,

previous and present environmental conditions and physiological history of the bacteria (Swinnen *et al.*, 2004).

The inoculum concentration is important as a low N_0 increases the stochastic effect contributed to by the individual lag times. This effect is masked at a higher N_0 with a larger distribution of individual lag times (Baranyi, 1998). Generally, the lag duration does not vary between a N_0 of 10^2 and 10^3 CFU / ml (Penfold, 1914); however a study has shown that *L. monocytogenes* lag time increased with an inoculum size less than 10^2 cells grown under sub-optimal conditions (Augustin *et al.*, 2000). When bacteria are inoculated at high concentrations it is feasible that the lag time could be affected. A high carry-over of inhibitory molecules or toxic metabolites during inoculation could result in a longer lag time. Conversely, contamination with stationary phase-produced stimulatory molecules could result in a shorter lag time. The addition of stationary phase culture supernatants to fresh media has been shown to dramatically decrease both the lag duration of *E. coli* and the inherent variation, through the presence of an autostimulatory molecule (Weichart & Kell, 2001). An inoculum size-dependent lag duration has also been observed in *L. monocytogenes* cultures grown in a high-NaCl medium, although there was no effect under optimal growth conditions (Robinson *et al.*, 2001).

The physiological history of a bacterial population is becoming an ever-increasing consideration for lag time measurements. Previous environments have been shown to impact upon bacterial lag times after inoculation into fresh media (De Jesus & Whiting, 2008, Dufrenne *et al.*, 1997). This impact is increased if the previous environment was damaging to the bacteria (Mackey & Derrick, 1982) and lessened if the two environments are relatively similar (József Baranyi, personal communication). Interestingly, although the physiological history has been shown to significantly affect lag times, the doubling time of bacteria is generally unaffected (Gorris & Peck, 1998).

The effect of physiological history has even been referred to as a bacterial form of 'memory' which can be classified as being either long- or short-term (John *et al.*, 2009, Wolf *et al.*, 2008). Furthermore, it has been demonstrated that bacteria may be able to make use of the retained 'memory' to alter their behaviour and adapt more rapidly than naïve bacteria to subsequent environmental changes (Oxman *et al.*, 2008, Wolf *et al.*,

2005), similar to Pavlovian conditioning (Mitchell *et al.*, 2009). The use of a standardised inoculum, whereby bacteria are grown under the same conditions for the same length of time can minimise fluctuations in the physiological history and ensure consistencies between biological replicates. A recent study showed 'old' *E. coli* populations recovered slower than 'young' bacteria after inoculation in fresh medium and show less extensive transcriptional re-programming to adapt to the new conditions. The lag time for 'old' populations was approximately three times longer than young cells (Pin *et al.*, 2009). Although maintaining the same growth conditions for the inoculum should decrease overall variability, inherent stochastic variability between individual cells remains (Rolfe, 2007).

The growth phase of the inoculum is an important consideration for subsequent lag phase measurements. The use of exponentially-growing cells as an inoculum could result in a decreased lag time as cell division initiation will not be required from the optimally-dividing cells. If the previous medium conditions are identical to the new conditions then exponentially-growing cells will theoretically not have any lag time (Baranyi & Roberts, 1994). However, it is interesting to note that if the bacteria are inoculated into a stressful or damaging environment then an exponentially-growing inoculum will exhibit a longer lag phase than a stationary phase inoculum (Mellefont *et al.*, 2004). This is because a stationary phase bacterial population is more resistant to stress than exponentially-growing bacteria.

The multi-factorial nature of lag duration has led to the development of a parameter, h_0 (Figure 1.6), corresponding to the physiological 'work' that must be completed before growth can begin (Baranyi & Roberts, 1994). This parameter takes account of the physiological state of the bacteria during lag phase and has been a useful parameter, remaining constant between different growth conditions (Mellefont & Ross, 2003). The aim of the present in-depth lag phase study is to determine the nature of this bacterial work required to be completed before exponential division is initiated.

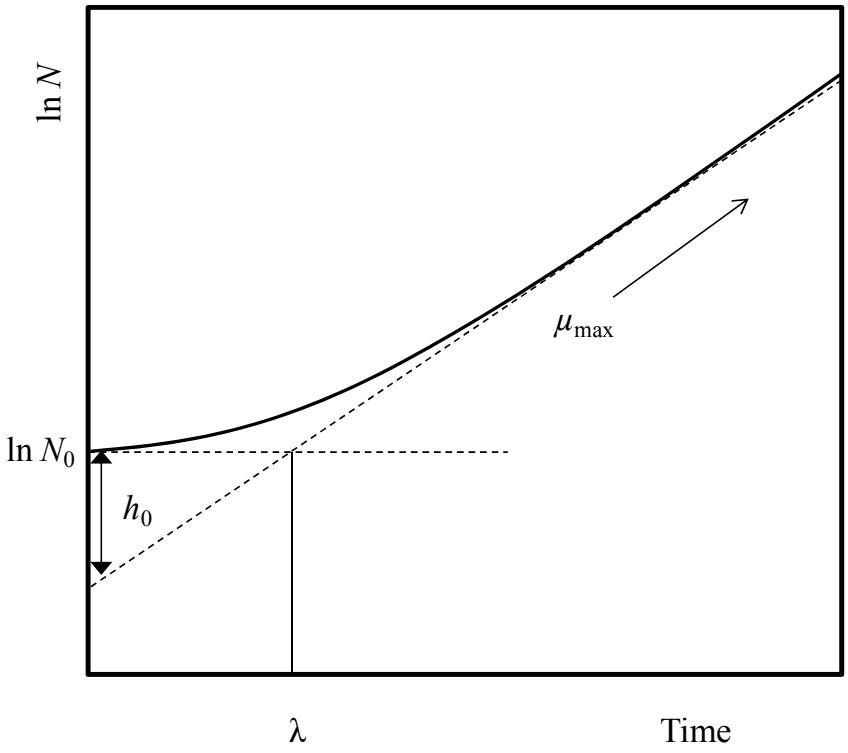


Figure 1.6: The lag time can be represented by the bacterial 'work to be done'.
By extrapolating the maximum population growth rate (μ_{max}) from the growth curve and the initial bacterial concentration (N_0), the geometric lag time (λ) can be calculated and the bacterial 'work to be done' (h_0) can be quantified.

The nature of this physiological 'work' was initially investigated in a molecular analysis of *Salmonella* during lag phase (Rolfe, 2007). The previous study primarily used a DNA microarray-based transcriptomic approach to identify processes being performed throughout growth. Gene expression profiles highlighted initial induction of stress resistance mechanisms, primarily to protect against oxidative stress, synthesis of translation machinery and the requirement of a variety of micronutrients including phosphate and metal cations (notably Fe^{2+}, Ca^{2+} and Mn^{2+}). However, no phenotypic validation of these gene expression-based hypotheses was presented (Rolfe, 2007). Direct observations of lag phase cells indicated that cells grew to an average of 1.09 μm before cell division and that replication of the chromosome was completed by 60 minutes post-inoculation. All of these processes described in the Rolfe (2007) study represent metabolic and physiological 'work' occurring during lag phase which may need to be completed before division can occur.

1.2.5 Lag phase and the food industry

Lag phase is of particular commercial interest as it is critical to maintain a low concentration of bacterial pathogens and spoilage organisms in food. As soon as bacteria exit lag phase they begin exponential growth, rapidly dividing to reach an infectious dose of pathogenic bacteria (Kothary & Babu, 2001). Food storage conditions are often designed to minimise bacterial growth and, if possible, to extend lag phase and maintain a low bacterial concentration (Ray, 2004a). A low starting concentration of bacteria (N_0) is dependent upon good manufacturing and production practice. Some bacteria, such as food spoilage organisms can decrease the quality of food even at a low bacterial concentration (Ray, 2004b), a problem exacerbated by long term storage in refrigerated conditions, giving rise to the emergence of psychrotrophic food-spoilage bacteria and pathogens such as *Clostridium*, *Listeria*, *Yersinia* and *Aeromonas* (Kraft, 1992).

By determining the microorganism-specific growth parameters, models can be developed to predict the bacterial lag time and rate of growth under defined conditions to estimate use-by dates and shelf lives. By understanding the physiological processes of lag phase and growth of food-relevant microorganisms, targeted interventions can be developed to increase the shelf life of foods and to decrease the associated incidences of foodborne illness. Various software packages have been developed containing predictive models for use by the food industry and regulatory authorities such as the Food Standards Agency. Types of software include ComBase (http://www.combase.cc), Sym'Previus (http://www.symprevius.net) and The Seafood Spoilage and Safety Predictor (http://sssp.dtuaqua.dk/).

As consumer expectations of food quality increases (McMeekin *et al.*, 2008), traditional food treatments that involve high thermal processes are less desirable to achieve bacterial killing even for dangerous pathogens such as *Clostridium botulinum*, where instead combination processes have been used to give a significant increases in lag time (Peck, 2009, Stringer *et al.*, 2009). The increasing consumption of ready-to-eat foods such as prepared fruits, vegetables and salads has seen an increase in fresh produce associated outbreaks of illness involving bacterial pathogens including *Salmonella* (Sivapalasingam *et al.*, 2004). These foods are minimally processed, often containing few preservatives, thus

bacterial lag times can be short, which when coupled with a rapid growth rate, poses a potential health risk (McMeekin & Ross, 2002).

1.2.6 Predictive microbiology

Predictive microbiology was described in the 1990s as a quantitative method allowing users to determine the microbiological safety and quality of a food based on pre-determined parameters (McMeekin *et al.*, 1993). These parameters include the pH, food temperature, NaCl concentration, water activity and starting concentration of bacteria. Predictive microbiology was designed to replace more conventional food safety methods such as challenge tests and enumeration of microbes, which are both slow and expensive (Baranyi & Roberts, 1995). Predictive microbiology models can also be adapted to a variety of different parameters to identify how varying food storage conditions would impact upon microbial growth. These methods can be used to help identify strengths, weaknesses, opportunities and threats (SWOT) in the development of food safety strategies (Ferrer *et al.*, 2009), and for Hazard Analysis and Critical Control Points (HACCP).

Predictive microbiology is constantly being developed for both different microorganisms and different growth conditions, incorporating deterministic and stochastic events to produce ever more sophisticated models (Marks & Coleman, 2005, Oscar, 2008, Swinnen *et al.*, 2004). Using predictive microbiology to determine the growth rate of a bacterial population is relatively straight-forward as the growth rate is largely dependent on the current growth environment. However lag phase is determined by both the current and previous environments. In addition to the physiological state of the population, the physiology of the individual bacterial cells must be taken into account (McKellar & Lu, 2005). All of these factors lead to relative difficulty in accurate lag time predictions.

In order to address the issues of lag phase prediction, additional approaches are required to explore the nature of lag phase in terms of bacterial physiology. These approaches are still in their infancy although the first models to incorporate cellular processes into lag phase prediction were developed in the mid-1990s (Baranyi & Roberts, 1994, Hills & Wright, 1994). Relatively recently, the physiological response of *S.* Typhimurium during lag phase was inferred (Rolfe, 2007) and has highlighted processes which are occurring at the transcriptomic level and need to be considered for future predictive microbiology models

(Dens *et al.*, 2005a, Dens *et al.*, 2005b). Additional considerations include the role of individual bacterial cell physiology on population lag times and the issue of a bacterial population acting as a multi-cellular organism (Shapiro, 1998) with different bacteria fulfilling distinct physiological roles during lag phase.

1.3 The physiology of lag phase

In comparison to the vast data generated on processes in exponential or stationary phase bacteria, the knowledge of lag phase physiology remains sparse. The Rolfe (2007) study has allowed the lag-specific physiology in *Salmonella* to be elucidated further than any other investigation to date. Many of the processes believed to be occurring during lag phase were observed at the level of gene expression including: production of transcriptional regulators such as Fis to control lag phase-induced genes (Filutowicz *et al.*, 1992, Rolfe, 2007); synthesis of rRNA and ribosomes (Radonjic *et al.*, 2005); the up-regulation of genes encoding cold-shock proteins, notably *cspH* (Kim *et al.*, 2004); DNA replication (Atlung & Hansen, 1999) and nutrient uptake inferred from studies on exponential growth (Baev *et al.*, 2006c, Baev *et al.*, 2006b, Baev *et al.*, 2006a, Prüß *et al.*, 1994). Due to a previous lack of physiological evidence on lag phase specific processes, the Rolfe (2007) study was unique in identifying likely processes occurring during lag phase compared with the exponential and stationary phases of growth. At the transcriptional level, 65 % of the *Salmonella* genes were differentially-expressed during lag phase compared with the stationary phase inoculum (Rolfe, 2007). This indicated that extensive transcriptional re-programming occurred during lag phase and highlighted lag phase as being far from quiescent. The lag phase-induced processes identified by Rolfe (2007) are summarised in Figure 1.7 and full list of the 2,666 lag phase specific genes are included in Appendix C, Table C1, accompanying this thesis.

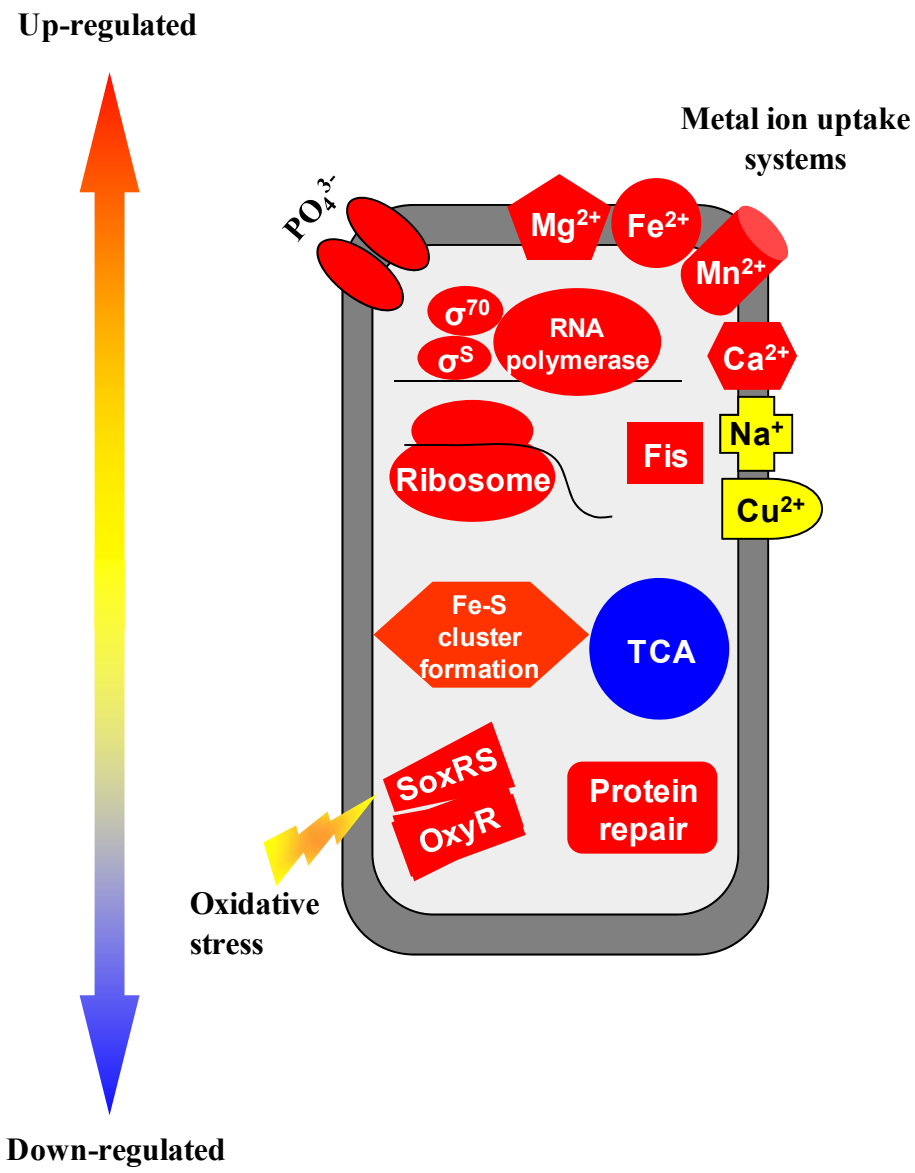


Figure 1.7: The physiology of lag phase, based on Rolfe (2007).
Critical processes highlighted by transcriptional experiments. Processes are coloured by the relative expression during lag phase compared with the stationary phase inoculum.

1.3.1 Nutrient uptake

During *E. coli* lag phase in LB medium, negligible quantities of nutrients are depleted and low concentrations of toxic metabolites are produced (Sezonov *et al.*, 2007). Despite this assertion, various studies have investigated the utilisation of nutrients throughout growth and have discovered a strict pattern of amino acid depletion at different growth phases including lag phase. Studies of *E. coli* grown in a defined medium supplemented with amino acids determined that L-serine (~850 µM) was depleted during lag phase, L-

aspartate and L-tryptophan removed during exponential and transition phases, respectively and finally L-glutamate, glycine, L-threonine and L-alanine used during stationary phase (Prüß *et al.*, 1994). This direct detection method was partially confirmed by a subsequent transcriptional study conducted by Baev and colleagues (2006b) into *E. coli* metabolism during growth in LB medium (Baev *et al.*, 2006b). The Baev study did not measure the use of amino acids during lag phase however limited deductions of lag phase metabolism can be made from the exponentially-growing bacterial gene expression. The transcriptional approach determined that *E. coli* L-serine, L-aspartate, glycine and L-glutamate metabolism genes were up-regulated during exponential phase. Differences in the use of amino acids between the two studies are likely to be due to inconsistencies between the two growth media used, although the indirect Baev approach does not account for metabolism genes yet to be annotated. Both studies identify amino acid utilisation very early in growth, although it is important to note that in a rich medium such as LB, amino acids are not depleted over the growth of *E. coli* or *Salmonella* and are able to fully support re-growth of bacteria upon inoculation (Barrow *et al.*, 1996).

Baev and colleagues used the indirect transcriptional approach to determine the importance of carbohydrates in LB medium (Baev *et al.*, 2006c). The authors concluded that glucose was physiologically-negligible during exponential phase as glucose was not present at a sufficient concentration to support growth as a sole carbon source. Maltose and maltodextrins were the preferred substrates with an up-regulation of *E. coli malQ*, *malP* and *malE* genes. In contrast, the sugar trehalose was not utilised until the late stage of sugar fermentation. A direct approach such as metabolite quantification via high performance liquid chromatography (HPLC), as used in the Sezonov *et al.* (2007) study, would have verified the metabolic gene expression data. It is possible that the low induction of some metabolic genes was due to lag phase specific substrate utilisation, not detected in these studies.

1.3.2 Metal ion uptake

Metal ions are known to be crucial for a range of essential bacterial processes (Section 7.1.1). There is presently very little information involving metal ion uptake during lag phase, with no genes or proteins involved in metal ion homeostasis found to have been induced significantly in other bacterial lag phase studies (Larsen *et al.*, 2006). Despite this,

it is known that limitation of some metal ions, such as ferrous iron (Fe^{2+}) leads to an increased lag time in *Salmonella* (Ho *et al.*, 2004). A previous transcriptomic study in *Salmonella* identified various metal ion transport gene clusters induced during lag phase including the *ent*, *fep*, *sit* and *iro* groups of iron ion uptake genes and transporter encoding genes for calcium, manganese, magnesium and sodium ions (Rolfe, 2007). Attempts were made to validate the gene expression data in the same study by phenotypic measurement of intracellular metal ions by inductively-coupled plasma mass spectrometry. The analysis identified increased intracellular concentrations of iron, manganese, sodium, calcium, zinc, lead, strontium, chromium and vanadium ions at two lag phase time points versus exponential and stationary growth phases however the author described high background concentrations of metals and a large degree of variability between samples (Rolfe, 2007). The transcriptomic and biochemical evidence indicated that, for *Salmonella* at least, lag phase accumulation of metal ions may be important but requires further validation. The accumulation of metal ions by exponentially-growing *E. coli* cells has previously been quantified but comparisons were made with the LB growth medium rather than across multiple growth phases (Outten & O'Halloran, 2001).

The metal ions accumulated during lag phase could be involved in many crucial processes including: iron requirement for respiratory enzymes containing Fe-S clusters, such as aconitase (AcnA, AcnB) (Johnson *et al.*, 2005); or manganese to help combat oxidative stress (Anjem *et al.*, 2009). See Section 7.1.1 for more details. The Rolfe (2007) study highlighted metal ion homeostasis as a potentially crucial process, which is investigated in greater detail in the present study in order to correlate metal ion accumulation with adaptation during lag phase.

1.3.3 Phosphate uptake

Phosphate is an essential micronutrient for bacteria that is incorporated into phospholipids, nucleotides, nucleic acids and adenosine-5'-triphosphate (ATP). Phosphate can be transported into bacterial cells as inorganic phosphate (P_i), organophosphate or phosphonate (Wanner, 1996). Phosphate uptake is controlled primarily by the PhoBR two-component system which is active under low phosphate conditions. PhoBR activates the high affinity P_i transport system PstSCAB (Wanner, 1993), possibly through the 27 kDa membrane *pho*-regulating protein PhoU (Rice *et al.*, 2009). Environmental phosphate

concentrations (as well as low Mg^{2+} concentrations) acts as a signal for the SPI-2 regulating two-component system, PhoP and PhoQ (Deiwick *et al.*, 1999, Löber *et al.*, 2006). A recent study has shown that the *pst-phoU* operon contributes to full adhesion of enteropathogenic *E. coli* and virulence of *Citrobacter rodentium* (Cheng *et al.*, 2009). Under conditions of excess phosphate, bacteria convert the P_i into polyphosphate via polyphosphate kinase (Ppk). This form of phosphate can be used as an energy store and when catalysed by exopolyphosphatase (Ppx), yields energy (Rao & Kornberg, 1999). The Rolfe (2007) study determined that lag phase uptake of P_i may be an important cellular process as the *pstSCAB* operon was uniformly up-regulated within four minutes of inoculation into fresh medium. The essentiality of these systems was investigated further with deletion of the *pstSCABphoU* operon and the *ppk* gene (Rolfe, 2007). Deletion of either of these systems did not result in an increased lag time, which was explained as functional redundancy and the involvement of an unknown system.

1.3.4 DNA replication and the nucleoid

DNA replication is a process known to occur in both eukaryotes and prokaryotes prior to cell division. Rolfe (2007) visualised the nucleoid of *Salmonella* during lag phase using 4',6-diamidino-2-phenylindole dihydrochloride (DAPI), a fluorescent stain that binds to the minor groove of double-stranded DNA (Kubista *et al.*, 1987). Clear replication and segregation of the bacterial chromosome was seen by 60 minutes post-inoculation, corresponding to the middle of lag phase (Rolfe, 2007).

The nucleoid is necessarily tightly-compacted in *E. coli* and *Salmonella* as the estimated length of the total nucleoid is 1.5 mm compared with an average cell length of just 2 μm (Pettijohn, 1996). Condensation of the chromosome is controlled by numerous nucleoid associated proteins (NAP) such as H-NS, StpA and HU (Dorman *et al.*, 1999). NAPs are known to change in intracellular concentration at different stages of bacterial growth (Ali Azam *et al.*, 1999). The factor for inversion stimulation (Fis) is the only NAP known to be increased during lag phase and early exponential phase (Ali Azam *et al.*, 1999, Kelly *et al.*, 2004, Rolfe, 2007). Kelly (2004) reported that a *S.* Typhimurium mutant strain lacking Fis exhibited a longer lag phase, a claim later refuted (Rolfe, 2007) albeit under slightly different growth conditions. The latter study analysed the transcriptome of a Δ*fis* mutant during lag phase and concluded that 14-18 % of genes were regulated by Fis; Fis activated

processes such as motility and chemotaxis, anaerobic metabolism and co-enzyme metabolism, and repressed genomic islands and the pSLT virulence plasmid (Rolfe, 2007). During the Rolfe (2007) transcriptomic study in *S.* Typhimurium, several NAP-encoding genes were identified as being up-regulated during lag phase, including *fis*. The most highly expressed NAP-encoding gene during early lag phase was *dps*, (DNA-binding protein from starved cells) encoding a 19 kDa, ferritin-like protein which protects DNA from oxidative damage (Almirón *et al.*, 1992). During stationary phase, Dps is under the control of the alternative sigma factor, RpoS (σ^{38}), however during exponential growth, when the concentration of Dps is low regulation is maintained by OxyR and the Integration Host Factor (IHF) transcriptional regulators (Altuvia *et al.*, 1994). Regulation of the *dps* promoter by the housekeeping sigma factor, RpoD (σ^{70}), during exponential phase, is performed by the regulators H-NS and Fis (Grainger *et al.*, 2008). The intracellular concentration of Dps closely correlates with the cell concentration of a bacterial culture (Ali Azam *et al.*, 1999) and deletion of Dps results in increased sensitivity to oxidative stress through a build up of free intracellular iron pools (Anjem *et al.*, 2009, Park *et al.*, 2005). Despite the increased expression of *dps* during lag phase, presumably responding to OxyR-mediated oxidative stress, no knock-out mutant was constructed by Rolfe (2007) and the role of Dps in determining duration of lag phase is still to be elucidated.

Genes encoding DNA polymerase III are quickly up-regulated during lag phase in *Salmonella* (Rolfe, 2007). This suggests that DNA replication commences soon after inoculation into fresh medium, confirmed by an increased concentration of the chromosome as stained by DAPI. However it has been shown that multiple copies of the chromosome exist in exponential and stationary phase *E. coli* (Akerlund *et al.*, 1995, Wang & Levin, 2009) suggesting that additional DNA replication may not be an essential lag phase specific process.

1.3.5 Cellular damage and repair

Cellular damage is known to lengthen lag time (Metris *et al.*, 2008, Stringer *et al.*, 2009) and repairing this damage may represent significant physiological work to be done in preparation for cell division (Baranyi & Roberts, 1994). Although cellular damage can arise from various external sources, the principal metabolically-driven damage arises via oxidative stress, which can cause changes to amino acid composition, such as histidine

being oxidised to asparagine or aspartic acid (Berlett & Stadtman, 1997). Other forms of oxidative damage include formation of S-S bridges, increased susceptibility to proteolysis and the formation of carbonyl groups (Cabiscol *et al.*, 2000). Carbonylation can occur through direct metal catalysed oxidative (MCO) damage to lipids (including cellular membranes), nucleic acids and the proline, arginine, lysine, and threonine side-chains of proteins (Fredriksson *et al.*, 2005) and has been attributed to cellular senescence (Nystrom, 2005). The build up of carbonylated macromolecules can occur during stationary phase 'stasis survival' (Nystrom, 2003, Nystrom *et al.*, 1996); it is therefore feasible that cellular damage would need to be repaired during lag phase before growth could be initiated. Carbonylation is an irreversible process, and damaged proteins need to be degraded via the Lon and HslVU (ClpQY) proteases and subsequently synthesised *de novo* (Fredriksson *et al.*, 2005). The iron-sulphur (Fe-S) clusters of proteins can be damaged by oxidation and are repaired by proteins such as FtnB (Velayudhan *et al.*, 2007) or synthesised *de novo* via the Isc or Suf systems (Imlay, 2008).

Extensive DNA damage could be lethal to bacterial cells and needs to be fully repaired during lag phase to guarantee genetic fidelity to daughter cells. *Salmonella* and *E. coli* contain multiple DNA repair mechanisms to compensate for damage including: direct enzymatic repair, base excision repair, mismatch repair, nucleotide excision repair and the error prone SOS-responsive repair (Rupp, 1996). The direct enzymatic repair mechanisms function without breaking the sugar-phosphate DNA backbone including the removal of unwanted methyl groups. Base excision, mismatch and nucleotide excision repair mechanisms physically remove aberrant nucleotide pairing which may physically distort the DNA macromolecular structure as well as the gene coding sequence. The SOS-response is DNA damage-inducible and leads to the expression of genes involved in excision double strand break repair mechanisms, as well as the low fidelity DNA polymerase V molecule which replicates through damaged DNA regions (Walker, 1996).

Membrane damage can occur through free-radical reactions in a process termed 'lipid peroxidation' which decreases membrane fluidity and disrupts membrane-bound proteins (Cabiscol *et al.*, 2000). Damaged membrane proteins and lipids cannot be repaired and so are degraded and synthesised *de novo*.

The previous lag phase study identified many of the genes involved in cellular repair as being induced during lag phase (Rolfe, 2007). In terms of DNA repair mechanisms, base excision repair, mismatch repair and nucleotide excision repair systems were all significantly up-regulated during lag phase, in comparison with stationary phase. Conversely, very few genes involved in direct enzyme repair were induced. The data inferred that lag phase protein repair was focussed on degrading carbonylated proteins from four minutes post-inoculation until the end of lag phase. Fe-S clusters were synthesised *de novo* rather than maintaining or repairing existing ones. Interestingly, the transcriptomic data revealed that genes involved in fatty acid degradation were not induced, although new fatty acids were synthesised as indicated by a uniform up-regulation of the *fab* genes through lag phase and into mid-exponential phase (Rolfe, 2007). These data suggested that either no fatty acid degradation was occurring, or that the degradation was performed by existing enzymes present within the cell.

1.3.6 Ribosomes and lag phase translation

There are comparatively few studies performed on ribosomes in *Salmonella* compared with *E. coli*, although the ribosome structure is believed to be similar in both organisms (Noller & Nomura, 1996). Ribosome concentration, and therefore protein synthesising capabilities, are intrinsically linked with the growth rate of a bacterium (Wagner, 1994). There are high numbers of active 70S ribosomes present in *E. coli* during exponential growth (Algranati *et al.*, 1969). Upon transition into stationary phase these 70S ribosome monomers become dimerised into 100S inactive complexes through the ribosome modulation factor (RMF) (Yoshida *et al.*, 2002, Yoshida *et al.*, 2009). These ribosome dimers are resistant to proteolytic and RNase degradation (Wada, 1998). During lag phase, acquired pools of 100S ribosome complexes disassociate, supplying a basal concentration of active 70S ribosomes, at which point no further 100S ribosomes are detected (Aiso *et al.*, 2005). It has been shown that *S.* Typhimurium up-regulates genes encoding ribosomal proteins within 20 minutes of inoculation into fresh LB medium, a process which continues for the duration of lag and throughout exponential growth (Rolfe, 2007). It is likely that the requirement for *de novo* synthesised ribosomes represents considerable physiological work for the bacteria before growth can be initiated. The rate limiting step for the ribosomal synthesis is the transcription of rRNA (Paul *et al.*, 2004) occurring within five minutes of inoculation into fresh medium (Kjeldgaard *et al.*, 1958). The ribosome structure encoding

genes are essential to both *E. coli* and *Salmonella* (Gerdes *et al.*, 2003, Holt *et al.*, 2009) suggesting that the synthesis of ribosomes is an essential physiological process across organisms during the transition from stationary phase into lag phase.

1.3.7 Molecular chaperones

There are at least five distinct classes of molecular chaperones that bind to unfolded, nascent polypeptide chains, protecting exposed hydrophobic domains and preventing protein aggregation (Mayhew & Hartl, 2009). Chaperones are essential for the correct folding of proteins, although they do not form part of the final folded protein complex. Some molecular chaperones are induced under conditions which disrupt or denature folded proteins, such as elevated temperatures. These so-called heat-shock proteins (HSPs) may be present at normal physiological temperatures but their production is induced further under stress conditions. Various molecular chaperones including HSPs and CSPs (cold-shock proteins) were up-regulated during *Salmonella* lag phase at 25 °C (Rolfe, 2007) suggesting either denatured proteins were present as a result of damage, or that the elevation of chaperones was not stress induced but rather a lag phase induced process. The induction of HSPs has been observed in other lag phase studies in non temperature-elevated conditions (Larsen *et al.*, 2006). The chaperones GroEL and GroES are members of the Hsp60 family of heat-shock proteins and function to fold newly-synthesised proteins (Mayhew & Hartl, 2009). Elevated concentrations of both GroEL and GroES were detected in *Lactobacillus delbrueckii* ssp. *bulgaricus* during lag phase after inoculation into milk (Rechinger *et al.*, 2000), perhaps involved in the production of metabolic enzymes responding to a shift in carbon source from glucose to lactose. An elevated expression of genes encoding molecular chaperones was identified in *Salmonella* although no definite role during lag was determined for these genes (Rolfe, 2007).

Although many of the physiological processes described above may be either organism or environment specific, there are some processes, such as the synthesis of ribosomes which are expected to be fundamental processes underpinning lag phase in various organisms and environments. One important question is whether bacteria such as *Salmonella* have a single strategy for initiating cell division or whether these processes are dependent upon changes in events such as the growth history. Part of the present study is aimed at determining whether such diverse strategies exist under mildly perturbed conditions.

1.4 Global analyses of lag phase in other systems

Other studies are beginning to look at proteins produced during lag phase although this work is generally focussed on a single lag time-point. The first comprehensive transcriptomic and proteomic study of a lag phase prokaryote was performed on the Gram positive bacterium *Lactococcus lactis* (Larsen *et al.*, 2006). The study compared lag phase with exponential growth in two media, identifying processes such as nucleotide metabolism, chaperone production, amino acid transport, glycolysis and co-enzyme production being significantly increased in lag phase. A more recent study continued on from the Larsen *et al.* (2006) work by analysing the proteome of two strains of *Lactobacillus plantarum* during lag phase, exponential phase and stationary phase (Koistinen *et al.*, 2007). The study by Koistinen and colleagues (2007) highlighted diverse metabolic activity occurring during lag phase but considerably less than early-exponential phase, which showed an increase in more central metabolic pathways such as glycolysis.

One further noteworthy study was performed with the Gram positive bacterium *Streptomyces coelicolor* which studied the intermediate lag (Figure 1.3) caused by various stresses and nutrient limitation (Novotna *et al.*, 2003). The proteomic analysis determined a metabolic shift occurring during the starvation-induced lag to make use of available substrates prior to re-growth. Interestingly, the analysis identified similar responses between starvation stress and other exogenous stresses such as cold and heat shock. The bacteria produced molecular chaperones (heat-shock and cold-shock proteins) during the starvation-induced lag phase in a similar manner to the nutrient upshift identified during lag phase in *Salmonella* (Rolfe, 2007). This may mean that chaperone production is a lag phase specific bacterial response to growth cessation and functions to aid in bacterial utilisation of alternative metabolites.

1.4.1 Eukaryotic lag phase processes

Although parallels between eukaryotic and prokaryotic lag phase are difficult to draw, it is possible that crucial cell division initiation processes are conserved across biological kingdoms.

A global transcriptomic study of the yeast *Saccharomyces cerevisiae* during lag phase as early as 20 minutes post-inoculation revealed up-regulation of genes encoding ribosomal

proteins, ribosomal RNA (rRNA), hexose sugar transport and metabolism of amino acids and nucleotides, compared with a stationary phase inoculum (Brejning *et al.*, 2003). Many of the genes encoding these processes were also induced during the lag phase of *Salmonella* (Rolfe, 2007), suggesting that activation of critical metabolic pathways and synthesis of translation machinery may be critical processes during lag phase.

The study by Brejning and colleagues (2003) identified various repressed genes including, rather surprisingly, those involved in carbohydrate metabolism which would presumably be down-regulated in the 42 hour stationary phase inoculum as the supplemented glucose (5 g/L) in the growth medium was completely utilised by 18.5 hours (Brejning *et al.*, 2003). This result differed from the Rolfe (2007) study of *Salmonella* in LB medium containing less glucose which showed approximately the same expression of glycolysis genes as the stationary phase inoculum.

The yeast study (Brejning *et al.*, 2003) confirmed an earlier proteomic study which discovered in excess of 100 stable proteins synthesised by *S. cerevisiae* during lag phase (Brejning & Jespersen, 2002). These proteins functioned in ribosome assembly, amino acid biosynthesis, carbohydrate metabolism and S-adenosylmethionine synthesis. A more recent study by the same group compared the gene transcripts and proteins induced during lag phase between the brewing yeasts *Saccharomyces pastorianus* and *S. cerevisiae* (Brejning *et al.*, 2005). This study discovered many similarities between the two yeast species in terms of lag phase specific processes inferred to be induced. Many of these processes were metabolic including: the glycolysis pathway, valine and isoleucine biosynthesis, synthesis of purines, ergosterol biosynthesis and glycerol metabolism.

1.5 Rationale for this study

The findings for lactic acid bacteria, yeast and *Salmonella* have highlighted important lag phase processes including protein synthesis, metabolism and ribosome assembly. These processes may represent a minimum 'work to be done' during the lag phase of many organisms with any additional organism- or environment-specific physiological work contributing to an extended lag time.

The physiological 'work to be done', quantified by the parameter h_0 (Baranyi & Roberts, 1994), depends upon the physiological state and the physiological history of a bacterial population. Rolfe (2007) performed an extensive transcriptomic study into *S*. Typhimurium lag phase in LB medium at 25 °C. Under more physiologically-demanding conditions, the metabolic work to be done would be increased, and therefore the lag time should also increase. Investigating the recovery of stressed *S*. Typhimurium at the transcriptomic level, using the Rolfe (2007) data as a 'control', would allow the robustness of lag phase to be determined and elucidation of the processes that are faithfully reproduced under both conditions. This in turn would identify the sensitivity of lag phase physiology to environmental changes, and help to explain the adaptability of *Salmonella* in diverse environments and aid the development of more sophisticated predictive microbiology models. Although there are many forms of mild and severe bacterial stress, it is important to focus upon food relevant stresses which could be useful for modelling pathogens under food storage conditions. Cold storage (refrigeration) is a suitable stress which is used routinely by the food industry. Bacteria can adapt to cold temperature (Phadtare, 2004) and chilling may increase the h_0 of the bacterial population during lag phase (Baranyi & Roberts, 1994).

The previous *Salmonella* lag phase transcriptomic study identified lag phase induced genes and thereby inferred lag phase processes (Rolfe, 2007 and Figure 1.7). Mutant strains, deficient in lag phase induced genes, were constructed, however majority of these strains did not have an increased geometric lag time when grown in the static system (Rolfe, 2007). Further phenotypic evidence would therefore be required to confirm many of the inferred lag phase processes including metal homeostasis and oxidative stress resistance. The previous *Salmonella* lag phase work was the first to study lag phase at the

transcriptomic level in a food-relevant growth system. Further studies in such systems will be required to identify which processes are crucial for lag adaptation. The present study makes use of the lag phase static growth system to meet specific aims, indicated below.

1.5.1 Aims of this study

The aim of this study is to extend understanding of the physiology of lag phase in *S.* Typhimurium. The earlier work of Rolfe (2007) is built upon. Specific targets are to:

- Confirm the bacterial population lag times observed previously and to determine the effect of cold storage on lag recovery (Chapter 3).
- Measure the transcriptome of cold storage recovering bacteria and compare with the transcriptomic response of bacteria not subject to cold storage (Chapter 4). This will help to identify whether a universal lag phase response exists to resume cell division.
- Study stationary phase bacteria to elucidate any mechanisms for rapid lag phase adaptation (Chapter 5).
- Phenotypically investigate processes of oxidative stress during lag phase and determine the importance of oxidative stress defence mechanisms (Chapter 6).
- Develop a sensitive method to reproducibly measure the intracellular metal ion concentration in *Salmonella* by inductively-coupled plasma mass spectrometry (ICP-MS).
- Quantify the intracellular metal ion concentration in *Salmonella* throughout growth and investigate the role of metal ion transporters during lag phase (Chapter 7).

Chapter 2

Materials and Methods

2. Materials and Methods

This chapter outlines the techniques and reagents used to perform the experiments described in this thesis. All chemicals used were of laboratory grade, unless otherwise stated, and were primarily supplied by Sigma Aldrich (Poole, UK). When other suppliers were used, they have been noted in this chapter. All media used were made up with de-ionised water, purified to a standard of 18.2 MΩ/cm and referred to in this section as dH_2O. Any molecular techniques such as the elution of DNA or RNA were performed with nuclease-free high grade water (Sigma, W4502). All solutions were stored at room temperature unless otherwise specified.

2.1 Microbiological methods

2.1.1 Growth conditions

Liquid cultures were routinely incubated statically at 25 °C in controlled temperature rooms. The constant temperature was maintained by Impact Air conditioning units (Marstair, Brighouse, UK). Care was taken to store flasks in the same area of the constant temperature room to ensure reproducibility of the static growth system (Section 2.1.6).

Incubation of cultures at 2 °C was performed in cold rooms maintained by chiller units (Roller, Singapore). The constant temperature was monitored using H140A temperature Dataloggers (Hanna Instruments, Portugal) and a maximum variation of ± 2 °C was recorded. Stored liquid culture temperatures were monitored by H141 temperature Datalogger probes (Hanna Instruments, Portugal) and had a maximum variation of ± 0.45 °C.

Agitated incubation of liquid cultures in 250 ml Erlenmeyer flasks was performed using an Innova 3100 water bath (New Brunswick Scientific) at 37 °C, 250 rpm or in an Innova 4400 Air-Incubator (New Brunswick Scientific) at 30 °C, 250 rpm. Overnight cultures were propagated in 30 ml universal tubes (R & L Slaughter, UK) using an Innova 4000 Air-Incubator (New Brunswick Scientific) agitated at 250 rpm. Static cultures and plates were maintained at 37 °C in a Binder BD53 microbiological incubator (Binder, South Korea) or at 30 °C in a Nüve EN120 microbiological incubator (Nüve, Turkey).

2.1.2 Liquid media

Salmonella Typhimurium was cultured in Luria Bertani (LB) medium (Lab M, Lancashire, UK), which was made as follows: 10 g Tryptone, 10 g NaCl and 5 g Yeast Extract added to a litre of dH_2O (Bertani, 1951). The LB medium was adjusted to pH 7.0 via drop-wise addition of 7 M NaOH to agitated medium using a stirrer (Philip Harris, UK), and the pH recorded to two decimal places using a Delta 340 pH meter (Mettler). LB medium was sterilised either by autoclaving at 121 °C, 15 PSI for 15 minutes, or by filter-sterilisation. Filter sterilisation was used when reproducibility of the batch medium was critical (i.e. during growth curves or molecular analysis of cells). Filtration involved drawing the medium through a 0.22 μm polyethersulphone (PES) membrane bound in a Stericup Filter Unit (Millipore, FDR-125-010Q), using a vacuum, into 1 litre plastic receiver flasks (Millipore, FDR-125-507Y). Antibiotics were added to liquid media at concentrations shown in Table 2.1.

During the preparation of electrocompetent cells, Lennox broth was prepared as follows: 10 g Tryptone, 5 g NaCl and 5 g Yeast Extract added to a litre of dH_2O (Lennox, 1955) and autoclaved at 121 °C, 15 PSI for 15 minutes.

Antibiotic	Supplier	Catalogue number	Stock concentration (mg/ml)	Final concentration (μg/ml)
Ampicillin (Ap)	Sigma Aldrich	A9518	100 mg/ml in water	100μg/ml
Chloramphenicol (Cm)	Sigma Aldrich	C0378	35 mg/ml in ethanol	35 μg/ml
Kanamycin (Km)	Sigma Aldrich	K4000	50 mg/ml in water	50 μg/ml
Spectinomycin (Spc)	Melford Laboratories	S0188	150 mg/ml in water	150 μg/ml
Streptomycin (Sm)	Sigma Aldrich	S6501	100 mg/ml in water	100 μg/ml

Table 2.1 Antibiotic stock solutions and working concentrations.
Water-based antibiotic stock solutions filter sterilised using a 0.22 μm syringe filter (Millipore, SLGV 033) and stored at 4 °C. Long-term storage of antibiotic stock solutions was performed at -20 °C.

2.1.3 Altered liquid LB medium

2.1.3.1 Conditioned LB medium

For some experiments *Salmonella* was growth in conditioned medium (Section 3.2.5). The production of conditioned medium has been described previously (Weichart & Kell, 2001). Briefly, conditioned medium was prepared by adding 30 % (v/v) cell-free supernatants of 48 hour 25 °C statically-grown *S.* Typhimurium culture to 70 % (v/v) fresh LB. Cell-free supernatants were obtained by centrifuging a 48 hour stationary phase culture at 7000 rpm (5378*g*), 20 °C, 10 minutes (Beckman HS-2 centrifuge, JA-10 rotor) to remove the majority of cellular debris and then filter sterilised through a 0.22 μm PES membrane into a Stericup receiver (Millipore, FDR-125-507Y) and stored at 25 °C until use. After addition of stationary phase supernatants to fresh LB, the pH was modified to 7.0. The conditioned medium was sterilised via filtration as described (Section 2.1.2) prior to use. When comparing bacterial growth in conditioned medium with LB medium, both sets of growth curves were performed on the same day to decrease experimental variability.

2.1.3.2 Chelex-100 treated LB medium

When studying the effect of depleted metals on *Salmonella* lag phase, a low metal environment was required (Section 7.2.3). This was accomplished during media preparation by adding 5 % (w/v) Chelex 100 (C7901, Sigma) to the LB medium under agitation for 30 minutes. The Chelex matrix consisted of 1 % cross-linked polystyrene beads containing the sodium form of iminodiacetic acid, which acts as a divalent cation chelator with a binding capacity of 0.4 mEq / ml. After 30 minutes addition, the matrix was allowed to settle and was subsequently removed by 0.22 μm filter sterilisation using a Stericup Filter Unit (Millipore, FDR-125-010Q).

2.1.4 Solid media

The primary solid medium used to propagate strains and plasmids was LB agar which was prepared by the addition of agar (Formedium, UK) to LB medium at 15g / Litre, prior to autoclaving. Antibiotics were added to cooled molten agar (50 °C – 60 °C) at concentrations described (Table 2.1), and the plates were allowed to dry in a laminar flow hood (Pyramid Engineering Services) for at least 30 minutes.

For P22 transduction experiments described in this thesis, green indicator plates were used to identify phage lysogens, as described previously (Maloy *et al.*, 1996). A base agar comprising (per litre): 8.0 g tryptone (Becton Dickinson, Cat. No. 211705), 1.0 g yeast extract (Becton Dickinson, Cat. No. 212750), 5.0 g NaCl (Sigma, Cat. No. 31434), 15 g agar (Formedium, Cat. No.AGA03) was made. After autoclaving, 21 ml 40% (w/v) glucose (VWR, 101176K), 25 ml 2.5% (w/v) alizarin yellow G (Sigma-Aldrich, 20,670-9) and 3.3 ml 2% (w/v) aniline blue (Merck, 1.16316) was added. Both the glucose and the alizarin yellow G were autoclaved prior to addition to the base agar. The aniline blue was filter sterilised through a 0.22 μm filter (Millipore). Stored stock alizarin yellow G was dissolved by gentle heating in a microwave prior to addition into the base agar.

2.1.4.1 Motility agar

Low concentration agar was used to test cell motility on plates as described elsewhere (Tittsler & Sandholzer, 1936) and was prepared by adding (per litre); 10 g tryptone, 5 g NaCl and 3 g agar. After autoclaving the molten agar was poured into agar plate moulds and allowed to stand in a laminar flow hood. For experiments involving low concentration agar, 1 μl overnight stationary phase culture (~10^6 colony forming units (CFU)) was pipetted into the semi-solid agar and the plates were incubated non-inverted at 37 °C for approximately 16 hours.

2.1.5 Storage of bacterial stocks

Bacterial strains and plasmids used in this study are shown (Table 2.2). Long-term storage of bacterial strains was at -80 °C, as glycerol stocks. Glycerol stocks were made by mixing glycerol to final concentration of 25 % with an overnight stationary phase culture in LB medium. Alternatively, single colonies of a strain on an agar plate were stored on Microbank beads (ProLab Diagnostics) following the manufacturer's instructions. Strains were streaked onto LB agar plates as required and incubated overnight at 37 °C. Short-term storage of strains on agar plates was performed at 4 °C for a maximum of one week.

2.1.6 The static growth system for lag phase experiments

For all experiments performed at 25 °C, a static system was used which maintained a constant physiological history to ensure reproducibly between experiments. All media were made fresh and filter-sterilised (0.22 μm filter) into a 1-litre disposable flask

(Millipore, FDR-125-507Y) and pre-warmed at 25 °C for at least 24 hours. The system has been described in detail elsewhere (Rolfe, 2007).

Briefly, an *S.* Typhimurium SL1344-derived strain was streaked from a glycerol stock at -80 °C onto a LB agar plate (Section 2.1.4 and Section 2.1.5) and grown overnight at 37 °C. A single colony was taken and sub-cultured in 10 ml LB (pH 7) supplemented with 10 µg/ml streptomycin in a 30 ml plastic universal tube (Slaughter Ltd.) for 48 hours at 25 °C. A second subculture into 10 ml LB (pH 7) without antibiotics was performed using an inoculating loop. After 48 hours, 200 µl of a 1:100 dilution was used to inoculated 500 ml LB (pH 7.0). After 48 hour static growth at 25 °C, 0.5 ml of culture was used as a standardised culture for inoculation into an experimental flask containing 750 ml LB (pH 7.0).

For some experiments a lower starting inoculum was used to monitor growth over an 18 hour period using two experimental flasks with each inoculation staggered by 15 hours. The first experimental flask was used for the initial nine hours of growth and an additional final time-point 25 hours post-inoculation. The second flask was used to measure the period 10-18 hours post-inoculation. When measuring bacterial growth, it was important to ensure that the growth curves from the two flasks were continuous and representative of a single 18 hour growth curve. This was performed during the curve fitting stage using the DMFit macro (Section 2.1.7).

During low cell concentration inoculation of the two experimental flasks, decimal dilutions of the standardised stationary phase culture were made (1:100) and 0.5 ml was inoculated into the 1 L experimental flask (Millipore, FDR-125-507Y) to give a starting inoculum of ~5 x 10^3 CFU / ml. The experimental 1 Litre flask was shaken briefly to mix the culture and then stored statically at 25 °C until needed.

2.1.6.1 Cold storage system

For Lag Pre-Incubation experiments, cultures were grown as per the static growth system with one exception. The 500 ml culture of standardised inoculum was moved from 25 °C to 2 °C until required, minimising the agitation of the culture. During inoculation of the 1 L experimental flask (Millipore, FDR-125-507Y), an aliquot (~ 5 ml) of pre-chilled

inoculum was removed and stored on ice to buffer against any temperature change which may affect the physiological state of the cells prior to inoculation. To inoculate the flask, 0.5 ml of this aliquot was transferred into the pre-warmed medium (25 °C) via pipette and the experimental flask was shaken briefly to ensure the inoculated cultures were mixed well.

2.1.7 Measuring growth of bacteria in liquid media

Growth of *S*. Typhimurium was routinely measured via optical density (OD) measurements using a SPECTRAmax PLUS Microplate spectrophotometer (Molecular Devices). Samples of a culture were taken and placed in a 1 ml acrylic cuvette. The optical density was measured at 600 nanometers (OD_{600}). When the OD_{600} reading exceeded 1.0, the samples were diluted 1:10 to bring the reading into the linear range of the spectrophotometer.

Accurate measurement of bacterial numbers was done by total viable counts. Decimal dilutions of bacterial cultures were performed in sterile LB and three drops of 10 µl from each dilution were spotted onto each plate. The drops were allowed to dry into the agar before the plates were incubated at 30 °C overnight. Colonies were counted using a Digital S Colony Counter (Jencons, 217-015) from dilutions giving between 5 and 80 colonies per droplet. Calculations of lag time were performed using the DMFit macro (http://www.ifr.ac.uk/safety/DMFit) within Microsoft Excel, based on procedures described elsewhere (Baranyi & Roberts, 1994). Significant differences in growth parameters were determined by statistical tests run using MicroFit v1.0 (F-test, cut-off ≤ 0.05).

Name	Genotype	Source
Bacterial strains		
SL1344	*S.* Typhimurium SL1344 wild-type	(Hoiseth & Stocker, 1981)
JH3306	*S.* Typhimurium SL1344 Δ*ahpC::kan*	Rolfe (2007)
JH3310	*S.* Typhimurium SL1344 Δ*ahpF::kan*	Rolfe (2007)
JH3316	*S.* Typhimurium SL1344 Δ*ahpCF::kan*	Rolfe (2007)
JH3348	*S.* Typhimurium SL1344 Δ*katG::cat*	Gift M. Rolfe
JH3353	*S.* Typhimurium SL1344 Δ*ahpCF::kan* Δ*katG::cat*	Gift M. Rolfe
JH3346	*S.* Typhimurium SL1344 Δ*soxRS::kan*	Rolfe (2007)
JH2462	*S.* Typhimurium SL1344 Δ*oxyR::cat*	This study
JH2463	*S.* Typhimurium SL1344 Δ*oxyR::cat* Δ*soxRS::kan*	This study
JH3308	*S.* Typhimurium SL1344 Δ*bipA::kan*	Rolfe (2007)
JH3290	*S.* Typhimurium SL1344 Δ*fis::cat*	Rolfe (2007)
JH3440	*S.* Typhimurium SL1344 Δ*fis::cat* Δ*bipA::kan*	This study
JH3543	*S.* Typhimurium SL1344 Δ*mntH::cat*	This study
JH3544	*S.* Typhimurium SL1344 Δ*sitABCD::kan*	This study
JH3555	*S.* Typhimurium SL1344 Δ*mntH::cat* Δ*sitABCD::kan*	This study
JH3572	*S.* Typhimurium SL1344 Δ*rmf::cat* Δ*yfiA::spc* Δ*yhbH::kan*	This study
KT2160	*S.* Typhimurium SL1344 Δ*relA71*::*kan* Δ*spoT281::cat*	(Tedin & Norel, 2001)
Plasmids		
pKD3	Template plasmid; Amp^R, FRT-flanked *cat*	(Datsenko & Wanner, 2000)
pKD4	Template plasmid; Amp^R, FRT-flanked *kan*	(Datsenko & Wanner, 2000)
pKD46	pSC101(Ts^-), *araC*$^+$$P_{BAD}$-(γ β *exo*), Amp^R	(Datsenko & Wanner, 2000)

Table 2.2 Bacterial strains and plasmids used in this study.

2.1.8 Hydrogen peroxide sensitivity assays

For experiments testing the effect of hydrogen peroxide on *S.* Typhimurium (Chapters 6.2.1 and 6.2.2), bacterial cells were challenged either on LB agar plates or in LB liquid medium with a variety of concentrations of hydrogen peroxide.

2.1.8.1 Hydrogen peroxide on solid media.

For challenging *S.* Typhimurium on solid media, a 100-fold dilution of an overnight stationary phase culture, grown at 37 °C was added 1:1 to 1.5 % (w/v) LB agar cooled to 45 °C - 50 °C. An aliquot (4 ml) of this culture-agar mix was poured onto a 1.5% (w/v) LB agar plate, pre-warmed to 37 °C, and allowed to set. Filter paper (Whatman, 1001090) was cut into circular discs (∅ = 6 mm), autoclaved and dried before being placed onto the prepared agar plates. To each filter disc, 10 µl 882 mM (3 % w/v) hydrogen peroxide (H_2O_2) was added and the plates were incubated overnight at 37 °C. The H_2O_2 diffused outwards from the point of addition, leaving a 'halo' of sensitive colonies, which was measured after incubation.

2.1.8.2 Hydrogen peroxide in liquid media

The sensitivity of *S.* Typhimurium to H_2O_2 in LB medium was investigated (Section 6.2.1 and 6.2.3), based on a previously-published method (Bang *et al.*, 2005). Briefly, a culture of *S.* Typhimurium was grown to a desired time-point and decimal dilutions made in sterile 1x phosphate-buffered saline solution (PBS). An aliquot (100 µl) of the desired dilution was plated on to a 1.5 % (w/v) LB agar plate and spread evenly using a polystyrene spreader (Fisher Scientific, DIS-109-050W). Hydrogen peroxide (Sigma, H1009) was added to the undiluted bacterial cultures at a final hydrogen peroxide concentration of 6 mM, from a 6 M stock solution, stored at 4 °C. Samples of treated bacterial culture were taken immediately, diluted decimally to the desired concentration and 100 µl plated. This process was repeated at time-points 30 minutes, 60 minutes and 90 minutes after the H_2O_2 challenge. Plates were incubated at 37 °C overnight and the number of viable colonies was counted.

2.1.8.3 Measuring the redox potential of cultures

In experiments measuring the effect of H_2O_2 on *S.* Typhimurium in liquid cultures, the effect of H_2O_2 on the redox potential of the medium was also measured.

Measurements were made using a platinum combination redox electrode (MI-800-410, Microelectrodes, USA) filled with silver chloride saturated 3 M potassium chloride, was used with a Delta 340 pH meter (Mettler). The response of the electrode was determined using quinhydrone (BDH, UK, 10227) saturated pH calibration buffers (pH 4 and pH 7)

(Fisher Scientific), 25 °C. Redox potentials (E_h), corrected to pH 7, were calculated using the equation $E_h = E_{obs} + E_{ref} + E_N$ (pH x - 7)

where E_{obs} is the observed redox potential, E_{ref} is the redox potential of the internal electrolyte of the electrode, E_N is the Nernst potential, (taken as 59 mV), and x is the pH of the sample (George *et al.*, 1998).

Sample redox readings were taken by placing the redox electrode into a 10 ml volume of culture at the desired time-point and leaving the probe to acclimatise for approximately five minutes. Once the readings had settled, the minimum and maximum redox potentials were noted and the mean voltage, in millivolts (mV) was recorded.

2.1.9 Microscopy

Transmission electron microscopy was used to visualise stationary phase bacterial cells (Section 3.2.7). An aliquot (1 ml) bacterial cells (approximately 5 x 10^8 CFU) was harvested by centrifugation at 13 000 rpm (15682 x g). The cells were subsequently fixed in 3 % (w/v) glutaraldehyde (Agar Scientific, Stansted, UK) in 0.1 M cacodylate buffer (pH 7.2) for approximately 3 hours. The fixative was then washed with 0.1 M cacodylate buffer (pH 7.2) and the cell pellets mixed with a small volume of molten 2 % low-melting-point agarose (TypeVII, A-4018, Sigma). This was solidified by chilling, chopped into small pieces and post-fixed with 2 % (w/v) aqueous osmium tetroxide for 2 hours before dehydration through an ethanol series. Final washes were performed three times with 100 % ethanol and after the last wash the ethanol was replaced with a 1:2 mix of LR White medium grade resin (London Resin Company) and rotated overnight. Two further LR White resin mixes were prepared with 100% ethanol in a ratio of 1:1 and 2:1, before addition of 100% resin. The 100% resin was changed twice more ensuring a period of at least 6 h was left between resin changes. Four tissue blocks from each sample were each put into gelatine capsules with fresh resin and polymerised overnight at 60 °C. Sections approximately 90 nm thick were cut using an ultramicrotome (Ultracut E, Reichert-Jung) with a glass knife, collected on film / carbon-coated copper grids, and stained sequentially with 50% (v/v) ethanol-saturated uranyl acetate and Reynold's lead citrate.

Sections were examined and imaged in a FEI Tecnai G2 20 Twin transmission electron microscope at 200kV (Kathryn Cross, personal communication).

2.1.10 Dissolved oxygen measurement

Continuous measurement of the dissolved oxygen concentration (Section 3.2.2) was performed using a polarographic electrode (Ingold) submerged in the bacterial culture and detected via an oxygen meter (LH Fermentation, series 2000). Calibration was performed by submerging the oxygen electrode in dH_2O with N_2 or air constantly bubbled through until saturation was achieved (18.2 MΩ / cm). The dissolved oxygen concentration in the bacterial culture was measured every 30 seconds for 48 hours automatically and plotted using the Channel Monitor Application software v29.0.17 (Measuresoft Development).

2.2 Genetic methods for bacterial mutagenesis

2.2.1 P22 phage transduction

Selectable genetic markers were routinely moved between strains using a generalised transducing phage, P22, which is specific to *S.* Typhimurium. A high transducing derivative, P22 HT105/1 *int*-4 was used via a previously-described method (Gemski & Stocker, 1967).

2.2.1.1 Preparation of a P22 phage lysate

Donor cells were prepared from a 37 °C overnight culture, grown in LB (containing appropriate antibiotics) diluted 1:100 into 5ml fresh LB medium. This sub-culture was grown at 37 °C to an OD_{600} of 0.15-0.2.

A stock of 10^{10} P22 phage per ml, raised on wild-type *S.* Typhimurium SL1344 (with no antibiotic selection marker) was stored at 4 °C in a glass vial under chloroform. An aliquot (5 µl) of this phage stock was added to the donor cells and the culture was incubated at 37 °C for a further 6 hours. After incubation, debris was apparent in the culture, indicating phage-induced cell lysis. Remaining cells were lysed upon addition of a few drops (~50 µl) chloroform (4 °C) and the lysate was incubated at 4 °C for at least 2 hours. The culture was centrifuged at 7000 rpm (6300 g), 4 °C in an Eppendorf 5810 R centrifuge (F-34-6-38 rotor) and filter-sterilised through a syringe filter (0.22 µm, Millipore, SLGV 033) into a

glass universal to ensure all cell debris was removed. Lysates were mixed with 20 µl chloroform and stored at 4 °C. Prepared lysates could be used for transduction procedures over a year after storage, as described.

2.2.1.2 Transductions with P22 lysates

Recipient cells were prepared from a 37 °C overnight culture, grown in LB (containing appropriate antibiotics). An aliquot (200 µl) of this was mixed with 10 µl of P22 phage lysate (prepared from the strain with a selectable marker for transduction). The recipient cells and lysate were incubated at 37 °C for 60 minutes, to allow the cells to express the transduced antibiotic resistance marker. After incubation, the total mixture was spread onto LB agar plates containing suitable antibiotics to select for positive transductants, and incubated at 37 °C overnight. Negative controls containing 100 µl recipient cells only and 50 µl P22 lysate were also spread onto LB agar plates and incubated. Antibiotic-resistant transductants were picked immediately after overnight growth to prevent the formation of lysogens. To purify the phage-free tranductants, colonies were twice streaked onto selective green indicator plates. Pseudo-lysogenic colonies appear dark green on the plates, whereas purified P22-free colonies appear white.

2.2.2 Mini-prep of plasmid DNA

Plasmid DNA was isolated for use in molecular techniques such as Lambda Red mutagenesis. The DNA was purified using the QIAprep Spin Miniprep Kit (Qiagen, 27104) as per the manufacturer's instructions. The method is based upon the alkaline lysis technique, described elsewhere (Birnboim & Doly, 1979). Plasmid DNA was isolated from 5 ml overnight stationary phase culture containing the plasmid of interest and bound to a silica membrane, washed and eluted into 50 µl molecular grade water (Sigma, W4502).

2.2.3 Electroporation

To transfer DNA into bacterial strains electroporation was used with electrocompetent cells. Electroporation was primarily used for the introduction of linear DNA into *S.* Typhimurium as part of the λ-Red knockout system, although this method is also suitable for the transformation of circular plasmid DNA. Electrocompetent cells were made using a previously-described method (Sambrook & Russell, 2000) and used fresh on the same day.

2.2.3.1 Preparation of 1 ml electrocompetent cells

A recipient strain was grown in 5 ml LB overnight at an appropriate temperature and sub-cultured in a 1:100 dilution into 100 ml fresh LB (with a selectable antibiotic; see Table 2.1) and grown 37 °C, 250 rpm to an OD_{600} = 0.6 (for λ-Red mutagenesis, cells were grown at 30 °C in Lennox broth with 1 mM L-arabinose added to induced the pKD46 plasmid containing the λ-Red recombinase-encoding gene). Cells were then split into pre-chilled (4 °C) 50 ml Falcon tubes and centrifuged at 5000 rpm, 4 °C for 15 minutes (Eppendorf 5810 R, F-34-6-38 rotor, 3214 g). The supernatant was removed and the cells were re-suspended in 1 ml ice-cold 10 % (v/v) glycerol (VWR, 24397.296) in dH_2O, sterilised by autoclaving. The volume was made up to 40 ml with ice-cold 10 % (v/v) glycerol in dH_2O before centrifuging as before. This was repeated for a total of three washes and the final cell pellet was re-suspended in 0.5 ml 10 % (v/v) glycerol (final volume of cell suspension was 1 ml). For electroporation, 100 µl competent cells were aliquotted into 1.5 ml tubes pre-cooled to 4 °C and stored on ice until required, up to a maximum of 3 hours.

2.2.3.2 Electroporation of competent cells

Electroporation was performed with freshly-prepared electrocompetent cells and cleaned, linear PCR product.

Electroporation cuvettes (Bio-Rad, 165-2086) were pre-chilled at -20 °C and transferred to ice immediately before use. Each 100 µl aliquot of competent cells was mixed with 10 µl PCR product (approximately 5 µg to 7 µg DNA; Section 2.3.3) on ice. The total mixture was transferred to an electroporation cuvette and tapped firmly, but gently to ensure a flat surface of liquid between the electrodes. Electroporation was achieved by transferring the cuvette to a GenePulser Xcell electroporator (Bio-Rad) and pulsing with a voltage of 2.5 kV, 200 Ω resistance and a capacitance of 25 µF.

Cells were recovered by briefly mixing 1 ml LB with the electroporated cells and transferring 1 ml to a pre-warmed 1.5 ml tube and recovering the cells, statically for 2 hours at 37 °C. After recovery, 100 µl transformed cells were plated onto LB agar containing appropriate selective antibiotics and the plate incubated at 37 °C overnight. The

remaining 900 µl aliquot was left at room temperature overnight, concentrated and plated, if required.

2.3 Molecular biology methods

2.3.1 Primers

Primers for PCR amplification were purchased from Sigma (Sigma Genosys) and are shown in Appendix A. Primers arrived as a lyophilized powder and were dissolved in molecular grade water (Sigma, W4502) to a stock concentration of 100 pmoles /µl and stored at -20 °C.

2.3.2 Polymerase Chain Reaction (PCR)

The polymerase chain reaction is a cell-free *in vitro* technique for amplification of DNA (Saiki *et al.*, 1988). Reactions were performed using HotStarTaq 2x Master Mix (Qiagen, 201445) containing the modified thermostable DNA polymerase from *Thermus aquaticus*, reaction buffer and dinucleotide triphosphates (dNTPs). This mix was stored at -20 °C and used as per the manufacturer's instructions.

During the reaction, a fifteen minute initial incubation step was used to prevent the amplification of mis-primed products and the formation of primer dimers. The DNA templates for the PCR amplification were either plasmid or crude cell extracts. Plasmid templates were added as 1:1000 dilutions of a mini-prep eluate. Cell extracts were made from a single overnight bacterial colony added to 50 µl dH_2O and boiled at 100 °C for 5 minutes. Cell debris was removed via centrifugation at 5000 rpm (3214 g) for 5 minutes and 4 µl cell lysate supernatant was used per 50 µl reaction.

50 µl PCR recipe:

HotStarTaq Mastermix (2x) Qiagen, 201445)	25 µl
Forward primer (10 pmol/µl)	4 µl
Reverse primer (10 pmol/µl)	4 µl
DNA template	4 µl
Molecular grade water (Sigma, W4502)	13 µl

All PCR amplifications were performed on a Primus HT Thermocycler Block (MWG AG BIOTECH), programmed through Primus HT Manager Software. The typical PCR program is shown below:

	HotStarTaq denaturation	95 °C	15 minutes
Repeat for 35 cycles	Denaturation	94 °C	1 minute
	Annealing	55 °C	1 minute
	Elongation	72 °C	Variable (allow 1 minute per Kbp DNA)
	Final elongation	72 °C	10 minutes

For primer combinations with different melting temperatures (T_m), for which the above program did not yield an amplified product, the annealing temperature was decreased to between 45 °C and 50 °C and an additional 2 mM magnesium chloride ($MgCl_2$) was added (final concentration of 3.5 mM $MgCl_2$), to aid binding of the primers to the DNA template.

2.3.3 PCR purification

PCR products were purified using a QIAquick PCR purification kit (Qiagen 28104) following the manufacturer's instructions. The kit allowed rapid recovery of PCR fragments greater than 100 bp in length from the PCR reaction mixture via a series of centrifugation steps. Briefly, a high-salt binding buffer was added to the reaction mixture to allow binding of the DNA to a silica-gel membrane. The DNA was then washed using an ethanol based buffer and dried via centrifugation to remove excess ethanol. The purified DNA was eluted into 50 µl dH_2O and stored at -20 °C.

2.3.4 Total RNA extraction

For transcriptomic experiments outlined in Chapter 4, the total RNA was extracted from harvested bacterial cells, treated with a mixture of 1 % phenol and 19 % ethanol, final concentrations and stored on ice for at least 30 minutes. The phenol-ethanol treatment kills the cells and prevents any further RNA processing or degradation (Hinton *et al.*, 2004). RNA extraction was typically performed the day after cell harvesting and the cell pellets were stored overnight at -80 °C. This freeze-thawing step weakens the bacterial cell structure thereby aiding lysis during the extraction process.

2.3.4.1 Harvesting of stationary phase and exponential phase cells

Cultures were grown to the desired time-point and the equivalent of 2 OD_{600} units per ml of culture was taken (for example, 1 ml culture with OD_{600} = 2, or 4 ml culture with OD_{600} = 0.5). Immediately, ⅕ final volume 5 % phenol 95 % ethanol (v/v) at -20 °C was added to the culture in pre-cooled 50 ml Falcon tubes and put directly onto ice. After between 30 minutes and 3 hours the cultures were centrifuged at 7000 rpm (6300 g) in an Eppendorf centrifuge and the supernatants discarded. The pellet was resuspended in the residual supernatant and transferred to 1.5 ml tubes (Eppendorf) for a final centrifugation (9000 rpm) for 3 minutes in a bench-top 5415D Microcentrifuge (Eppendorf) with a F45-24-11 rotor. Resuspended cell pellets were stored for the short term (< 5 hours) at -20 °C and then overnight at -80 °C.

2.3.4.2 Harvesting of lag phase cells

Due to the low concentration of cells present in the lag phase samples, large volumes were required to yield sufficient RNA for transcriptomic analysis. Cells were grown as 750 ml cultures in 1 litre flasks as per the static system (Rolfe, 2007). For each time point two flasks were sacrificed, allowing cells to be harvested from 1.5 litres lag phase culture. Cell growth and RNA processing was stopped by the addition of 187.5 ml 5 % phenol 95 % ethanol (cooled to -20 °C) to each 750 ml culture. The cultures were placed on ice for at between 30 minutes and 3 hours and then centrifuged at 7000 rpm (5378*g*), 4 °C, 10 minutes (Beckman HS-2 centrifuge, JA-10 rotor). The cell pellet was resuspended in the residual supernatant, transferred to 50 ml tubes and harvested by centrifugation into 1.5 ml tubes as described in Section 2.3.4.1.

2.3.4.3 Isolation of RNA from harvested cell pellets

Cell pellets in 1.5 ml Eppendorf tubes were removed from the -80 °C freezer, thawed on ice and resuspended in 100 µl Tris-EDTA (TE) buffer (pH 8.0). RNA extraction was performed using a SV Total RNA purification kit (Promega, Z3100) as per the manufacturer's instructions. Briefly, a lysis buffer from the RNA extraction kit was added to each cell suspension and mixed via inversion. To this, RNA dilution buffer was added and mixed well. The samples were heated at 70 °C for three minutes and the cell debris collected after centrifugation at 13000 rpm (15682 g), 10 minutes. The supernatant was transferred to clean RNase-free tubes into which 200 µl ethanol was added and mixed. The

mixture was transferred to Promega spin columns and spun at 15682 g for 30 seconds in an Eppendorf bench-top microcentrifuge. The nucleic acids were bound to a silica-gel membrane and were subsequently washed with an ethanol-based wash buffer before a 15 minute DNase treatment at room temperature was used to remove any contaminating DNA. The RNA was washed twice further with the ethanol-based wash buffer before being eluted into 100 µl dH_2O (Sigma, W4502).

2.3.5 Genomic DNA extraction.

Genomic DNA extraction for transcriptomic techniques was performed using Qiagen Genomic DNA buffer set (Catalogue No. 19060) and 100/G Qiagen Genomic tips (Catalogue No. 10243), allowing the isolation of up to 100 µg genomic DNA.

The extraction protocol for 5 ml overnight *S*. Typhimurium SL1344 culture was taken from the Qiagen Genomic DNA handbook with the following modifications. Due to the resilience of *S*. Typhimurium cells the lysis step was increased from 30 minutes to 2 hours. The genomic DNA was eluted from the column into 15 ml Falcon tubes in 5 ml buffer QF, pre-warmed to 50 °C to improve yield. The DNA was precipitated by addition of 3.5 ml isopropanol (2-propanol) at room temperature and pelleted by centrifugation at 6300 g for 30 minutes. The supernatant was then discarded, 500 µl TE (pH 8.0) was added and the pellet was left to dissolve overnight at room temperature. The total genomic DNA was then quantified (Section 2.3.6) and stored at -20 °C until use.

2.3.6 Quantification of nucleic acid concentration

Measurement of nucleic acid concentration was performed using the NanoDrop ND-1000 Spectrophotometer (NanoDrop, Wilmington, Delaware, USA) and the ND-1000 software. The quantification was performed by measuring the absorbance of 1 µl eluted nucleic acid sample at $A_{230,}$ A_{260} and A_{280} which gives an indication of the purity of the nucleic acid in relation to possible contaminants.

2.3.7 Assessment of RNA quality

In order to ensure the RNA was of a suitable quality to be used for transcriptomic work, the Agilent 2100 Bioanalyser (Agilent, Stockport, UK) was used, as per the manufacturer's instructions. The Bioanalyser uses capillary gel electrophoresis and nucleic acid dyes to

measure the size of the RNA. The RNA sample (1 µl) was run in a RNA Nanochip with 150 ng RNA 6000 ladder (Ambion, 7152, Austin, Texas, USA). The quality of the RNA was visualised using an electropherogram generated by the accompanying Agilent Bioanalyser software. Two major, well-defined peaks representing the 16S and 23S rRNA subunits and a low background characterised good quality RNA.

2.3.8 Agarose Gel electrophoresis

Agarose gel electrophoresis is a technique which separates nucleic acid fragments based upon their size (Southern, 1979) and allows the fragments to be visualised by staining. DNA samples were diluted by the addition of 5x loading buffer supplied with the QiaQuick PCR purification kit (Qiagen) to a 1x final concentration. A 1 % (w/v) agarose (Sigma, A9539) gel made with 1x TAE (40 mM Tris-base, 1 mM EDTA, 40 mM acetic acid) containing 0.5 µg / ml ethidium bromide was prepared as previously described (Sambrook & Russell, 2000) and placed into a electrophoresis gel tank and covered with 1 % TAE. The DNA and loading dye were loaded alongside 2.5 µg 1 Kb DNA marker (New England Biolabs, N3232 or Promega, G5711) and electrophoresed at approximately 5 V/cm. After the electrophoresis had been completed the DNA fragments were analysed in a MultiImage UV Light Cabinet (Alpha Innotech Corporation) using the high energy UV setting. Images were photographed using an Olympus C-4000 Zoom digital camera (2000 ms exposure time) and visualised using the AlphaDigiDoc AD-1201 software.

2.3.9 Deletion of genes using the Lambda Red system

The Lambda Red gene knockout system (Datsenko & Wanner, 2000) was used to create all the chromosomal mutations described in this thesis. In essence, the system was used to replace the protein-encoding region of a gene with an antibiotic resistance gene cassette. A PCR fragment was generated containing the antibiotic resistance gene of interest, flanked by 40 base pairs on each end with homology to the upstream and downstream regions of DNA flanking the target gene. The PCR product was purified and electroporated into a *S.* Typhimurium strain containing the pKD46 plasmid encoding a Lambda Red bacteriophage recombinase. The selectable antibiotic resistance was acquired by homologous recombination leading to a loss of the target gene. Mutants were selected by plating onto LB agar containing the antibiotic of interest and verified by PCR amplification of the target

region. It was important to transduce the mutation into a clean SL1344 genetic background without pKD46 to avoid the occurrence of secondary mutations.

2.3.9.1 Oligonucleotide primers for gene deletion

The primers were designed to be specific for the start position of the gene to be deleted. In the majority of cases the first 40 base pairs (from the 5' end) corresponded to the region proximal to the coding region. An additional 20 base pairs were added to the 3' end of each primer corresponding to the antibiotic resistance cassette from the pKD3 or pKD4 plasmids containing the antibiotic resistance genes for chloramphenicol or kanamycin respectively. The nucleotide sequence added to the 3' end of the forward primer was: GTGTAGGCTGGAGCTGCTTC, and to the 3' end of the reverse primer CATATGAATATCCTCCTTAG, was added. The final primer length was 60 nucleotides. The full sequence of the gene deletion primers are shown in Appendix A.

2.3.9.2 PCR amplification of pKD3 or pKD4 and transformation

For the majority of transformations described in this thesis the antibiotic resistance cassettes present in the Lambda Red plasmids, pKD3 (chloramphenicol, *cat*) and pKD4 (kanamycin, *kan*), were amplified via PCR using oligonucleotide primers (Section 2.3.9.1). Primers were diluted to a stock of 100 µM and used at a working concentration of 10 µM. The pKD3 or pKD4 plasmid templates were added as a 1:1000 dilution of a mini-prep eluate. The PCR recipe used was the same as mentioned previously (Section 2.3.2) except that 6 × 50 µl reactions were setup and the PCR products were combined, purified (Section 2.3.3) and eluted into 50 µl dH2O after amplification to generate enough DNA for the Lambda Red technique. During the transformation, 10 µl purified DNA was introduced to competent *S.* Typhimurium SL1344 containing the pKD46 plasmid via electroporation. The competent cells were made as described previously in this thesis (Section 2.2.3.1) except that the expression of the Lambda Red recombinase was induced by the addition of 1 mM L-arabinose and the pKD46 plasmid was maintained through the addition of 100 µg/ml ampicillin. Transformations were performed via electroporation using the method described previously in this thesis (Section 2.2.3.2) and plated onto LB agar plates containing appropriate antibiotics for selection. The plates were incubated overnight at 37 °C to cure the pKD46 plasmid, which does not replicate above 30 °C.

2.3.9.3 Verification of Lambda Red transformants

Successful transformants were verified by PCR to check that the gene of interest had been replaced by an appropriate antibiotic resistance cassette. Each strain containing the gene knock-out mutation was checked alongside a *S.* Typhimurium SL1344 wild-type strain containing the intact gene of interest and the size of the PCR product examined by agarose gel electrophoresis (Section 2.3.8). The primers used to verify the Lambda Red mutagenesis were designed to have homology to the flanking region of the targeted gene. The details of the external primers used are included in Appendix A.

2.3.9.3.1 Verification of the Δ*sitABCD::kan* mutant.

For the Δ*sitABCD::kan* mutant used in this thesis (Sections 7.2.5 and 7.2.6), the length of the region to be disrupted was too long (>3 Kb) for conventional PCR verification and so random-primed PCR was used to amplify the region and the enriched product was verified by sequencing. The primer sequences are included in Appendix A. The method of random primed PCR is described elsewhere (Jacobs *et al.*, 2003) and required two separate PCR amplifications. The first round of amplification involved one fixed reverse primer which annealed to either the antibiotic resistance cassette in pKD3 or pKD4 depending on the chosen resistance marker. The PCR reaction contained a mixture of three forward random primers, which bound to different regions of the template DNA. The 5' end of each primer contained the sequence GACCACACGTCGACTAGTGC which generated multiple products of different lengths with the same 5' sequence.

The first random-primed PCR reaction contained the following reagent in a total volume of 50 µl:

BioMix 2x PCR mix (BioLine, BIO-25011)	25 µl
Forward random primer A (10 µM)	0.85 µl
Forward random primer B (10 µM)	0.85 µl
Forward random primer C (10 µM)	0.85 µl
Reverse primer A (10 µM)	2.5 µl
DNA template (crude cell lysate; Section 2.3.2)	5 µl
Molecular grade water (Sigma, W4502)	14.95 µl

The DNA was cleaned using a QiaQuick PCR purification kit (Qiagen) and used as the template in the second PCR amplification step. The second PCR amplification used a short (20 nucleotide) adapter forward primer with the GACCACACGTCGACTAGTGC sequence to amplify the 5' end of the first PCR products and a second reverse primer that annealed to the antibiotic resistance cassette amplified from the first PCR.

The second PCR was setup in a final volume of 50 µl containing:

BioMix 2x PCR mix (BioLine, BIO-25011)	25 µl
Forward adapter primer (10 µM)	2.5 µl
Reverse primer B (10 µM)	2.5 µl
DNA template from first reaction	5 µl
Molecular grade water (Sigma, W4502)	15 µl

The PCR amplification was setup using a Primus HT Thermocycler PCR Block (MWG AG BIOTECH) with the following program:

	Initial denaturation step	94 °C	3 minutes
Repeat for 6 cycles increasing the annealing temperature by 1 °C per cycle	Denaturation	94 °C	15 seconds
	Annealing	42 °C	30 seconds
	Elongation	72 °C	3 minutes
Repeat for 25 cycles	Denaturation	94 °C	15 seconds
	Annealing	60 °C	30 seconds
	Elongation	72 °C	3 minutes
	Final elongation	72 °C	7 minutes
	Storage step	8 °C	As required

This PCR amplification generated an enriched PCR product which was purified (Section 2.3.3) and sent for sequencing at The John Innes Genome Centre using an ABI Prism 3730xl DNA analyser (Applied Biosystems) and the reverse primer B (Appendix A).

2.3.10 Transcriptomics: microarray hybridisation and analysis

DNA microarrays allow the simultaneous measurement of the global gene expression under defined experimental conditions. All transcriptomic experiments described in this thesis involved the use of a fluorescently labelled genomic DNA common reference (Type 2) approach. Type 2 arrays were used because the common reference decreased the amount of microarrays required to analyse many RNA samples and allowed comparisons between other Type 2 datasets (Yang & Speed, 2002). All RNA samples were reversed transcribed to cDNA and labelled separately with the Cy5 fluorophore whereas the reference genomic DNA was labelled with the Cy3 fluorophore as described on the IFR website (http://www.ifr.ac.uk/safety/microarrays/). The microarrays used in this thesis for transcriptomic experiments (Chapter 4) were printed in-house at the Institute of Food Research microarray facility. Each SALSA2 (*Salmonella* serovars) microarray comprised 5764 elements each corresponding to a coding sequence (CDS) or small RNA (sRNA) from a range of *Salmonella* serovars. Of these coding sequences: 4869 genes corresponded to the *S.* Typhimurium LT2 strain (McClelland *et al.*, 2001), 473 CDS specific to *S.* Typhimurium SL1344 (Project ID 345 Sanger Institute, http://www.sanger.ac.uk/Projects/*Salmonella*), 113 CDS on the pSLT virulence plasmid, 27 sRNA, 86 *S.* Gallinarum CDS and 196 CDS for *S.* Enteritidis PT4. The DNA microarrays were constructed by spotting PCR product in 50% (v/v) dimethyl sulfoxide (DMSO), 0.3x saline sodium citrate (SSC) spotting solution onto the surface of GAPS-II microarray slides (Corning, 40003). The negatively-charged DNA was ionically-bound to positively-charged γ-aminopropylsilane groups on the microarray slide, which was strengthened by UV cross-linking during the slide blocking process. Microarray printing was performed using a Stanford-designed (v3) microarray printing robot using the method outlined previously (Thompson *et al.*, 2001).

2.3.10.1 Two-step labelling of extracted RNA

RNA was extracted at the desired time-point (Section 2.3.4.3) and fluorescently-labelled over two stages, as described previously (Eriksson *et al.*, 2003). The first stage involved the reverse transcription of 2 µg extracted RNA into corresponding cDNA. To the RNA, 5 µg random primers (Invitrogen, 48190-011) were added in a total volume of 9.4 µl. The RNA was denatured by incubation at 70 °C for 5 minutes and then chilled on ice for 10 minutes. To each reaction the following was added:

10 x AffinityScript Reverse Transcriptase Buffer (Stratagene)	2 µl
0.1 M Dithiothreitol (DTT) (Sigma, D9779)	2 µl
50x dNTPs (25 mM each dCTP, dTTP, dGTP, dATP)	0.6 µl
Molecular grade water (Sigma, W4502)	2 µl
50 U / µl AffinityScript reverse transcriptase (Stratagene, 600107)	4 µl

The reactions were incubated overnight at 42 °C in a Primus HT Thermocycler PCR Block (MWG AG BIOTECH). The reverse transcription reaction was stopped by the addition of pH 8.0 1.5 µl 20 mM ethylenediaminetetraacetic acid (EDTA) and any remaining RNA was hydrolysed by the addition of 15 µl 0.1 M sodium hydroxide and heating the samples at 70 °C for 10 minutes. The alkali was neutralised by adding 15 µl 0.1 M hydrochloric acid. The cDNA was cleaned using a PCR purification kit (Section 2.3.3) and eluted into 50 µl dH_2O. The eluate was condensed to 21 µl using a SpeedVac (Thermo-Savant) on a medium setting.

2.3.10.2 Direct fluorescence labelling of DNA

Fluorescent nucleotides were incorporated into the reverse transcribed cDNA and the reference genomic DNA using the BioPrime® DNA Labelling System (Invitrogen, 18094-011). The reaction involves the incorporation of a fluorophore into the amplified nucleic acid using random primers (octamers), dNTPs and Klenow polymerase. To 21 µl (2 µg) genomic DNA (gDNA), 20 µl of 2.5 x Random primer/reaction buffer mix from the BioPrime® DNA Labelling kit was added and the primers were denatured by boiling at 100 °C for 5 minutes. The samples were placed on ice for at least 5 minutes before the following were added:

10 x dNTP mix (1.2 mM each of dATP, dTTP, dGTP; 0.6 mM dCTP; 10 mM Tris pH 8.0; 1 mM EDTA)	5 µl
1 mM Cy5-dCTP (GE Healthcare, PA55021) (cDNA samples only)	3 µl
1 mM Cy3-dCTP (GE Healthcare, PA53021) (genomic DNA samples only)	3 µl
Klenow polymerase	1 µl

The samples were spun briefly (15682 x g, 2 seconds) and incubated overnight at 37 °C, in the dark to prevent dye bleaching. The labelling reaction was stopped by adding 5 µl 0.5 M EDTA (pH 8.0) and the DNA was cleaned using a QiaQuick PCR purification kit (Qiagen), eluting the labelled DNA twice in 50 µl dH_2O. To each 100 µl of Cy5-dCTP labelled cDNA, 20 µl of Cy3-dCTP labelled genomic DNA was added per hybridisation. Total labelled DNA was dried in a Speed Vacuum Concentrator (Thermo-Savant) and resuspended in 10 µl dH_2O to be used for the hybridisation procedure.

2.3.10.3 Blocking of transcriptomic microarray slides

Prior to hybridisation of SALSA2 slides, blocking was performed to methylate the γ-aminopropylsilane (GAPS) groups. This neutralised positive charges and prevented the hybridisation of labelled DNA with the slide surface. Where possible, slides were blocked on the day of hybridisation to minimise exposure to particulates during storage. As part of the blocking process, a UV cross-linking step (Stratalinker; 120 mJ) was carried out, which covalently bound the printed PCR product with the glass slide. Prior to blocking, the SALSA2 arrays were marked out using a diamond-tipped pencil. The blocking procedure was performed using the Pronto!™ Microarray blocking kit (Corning, 40026) as per the manufacturer's instructions. Briefly, 247 ml Pre-Soak solution (Corning) was pre-heated to 42 °C and mixed with 2.5 ml Liquid sodium borohydride from the kit, in a fume hood. The SALSA2 slides were then immersed in the liquid within black plastic hybridisation chambers and incubated at 42 °C for 20 minutes. The slides were then transferred to nuclease-free molecular grade water (Sigma, W4502) at room temperature and soaked three times for 30 seconds each. Finally the slides were spun dry in a Jouan MR23i centrifuge (Thermo Electron Corporation) at 1500 rpm (289 g) using the 6xµT microtitre plate rotor at 20 °C, ready for the hybridisation protocol.

2.3.10.4 Transcriptomic microarray hybridisation

During the hybridisation process cDNA and genomic DNA competitively bind to the arrayed PCR product on the slides. During this process care was taken to minimise the exposure of the samples to light and to minimise any physical contact with the microarray surface. To each 10 µl sampleof fluorescently-labelled cDNA and gDNA the following reagents were added:

20 x SSC (3 M sodium chloride, 0.3 M sodium citrate, pH 7.0)	2.25 µl
1 M HEPES (pH 8.0)	0.375 µl
25 mg / ml *Saccharomyces cerevisiae* tRNA (Sigma, R8759)	1.125 µl
Denhardt's solution (1 % (w/v) Ficoll 400, 1 % (w/v) polyvinylpyrrolidone, 1 % (w/v) bovine serum albumin (fraction V) in dH_2O)	1.5 µl
10% (w/v) SDS (Sigma, L4390)	0.375 µl

The nucleic acids were denatured by boiling the samples at 100 °C for 2 minutes and then cooling at room temperature for 5 minutes. The samples were spun briefly (15682 x g, 2 seconds) to remove any debris. The microarray slides were placed into a hybridisation chamber and the total hybridisation mix was pipetted onto the centre of one microarray. A 22 mm x 22 mm borosilicate glass cover-slide (VWR International, 631 0124) was cleaned by submersion in 96 % ethanol and dried ensuring any debris was removed using an invertible air duster (RS components, 497-280), before being lowered onto the microarray. Any air bubbles were removed by applying gentle pressure to the cover-slide with some fine forceps. The process was repeated for the second microarray on the slide and 20 µl 3 x SSC was added to both ends of the hybridisation chamber using a pipette to maintain the appropriate humidity during the hybridisation process. A chamber lid was screwed tightly onto the hybridisation chamber (GeneMachines) before the slides were incubated overnight in a water bath at 63 °C (Grant Instruments, Hertfordshire, UK) to allow hybridisation.

2.3.10.5 Transcriptomic microarray washing steps

Following overnight hybridisation, the microarrays were washed to remove any non-hybridised DNA from the microarray and slide. Wash solutions were prepared the day before and kept at the appropriate temperature overnight. Heated wash solutions were maintained at the correct temperature using a digital hotplate stirrer agitating at 250 rpm (Bibby Sterilin) during the washing procedure. For wash steps at room temperature the samples were agitated on a platform rotary shaker (New Brunswick Scientific, Edison, New Jersey, USA) at 50 rpm.

The slides were initially submerged twice in 2x SSC, 0.1 % SDS for 5 minutes at 63 °C, agitated at 250 rpm. The next washes were performed twice in 1x SSC, 5 minutes at room temperature and finally two washes were performed in 0.2x SSC, 5 minutes at room temperature.

The slides were dried via centrifugation in a Jouan MR23i centrifuge (Thermo Electron Corporation) at 1500 rpm (289 g) using the 6xμT microtitre plate rotor at 20 °C, enclosed within a black slide chamber. Once dry, the slides were ready to be scanned.

2.3.10.6 Transcriptomic microarray scanning and data analysis

For transcriptomic experiments (Chapter 4) the SALSA2 microarray slides were scanned in a GenePix 4200 AL microarray scanner (Molecular Devices) using GenePix Pro v6.0 Software following the manufacturer's instructions. Preview scans were carried out at 40 μm resolution and full data scans were performed at 10 μm resolution. The PhotoMultiplier Tube (PMT) setting measures the photons emitted from the laser-excited fluorophores, and therefore the signal. The higher the PMT value, the more sensitive the scan is however if the PMT voltage is too high the signal becomes saturated. For each use of the scanner, the GenePix software calculated the optimal PMT value through the Auto PMT function. Images were saved as multi-image .tiff files for analysis.

Data were generated from the GenePix images using BlueFuse v1.0 (BlueGnome, Oxford, UK). Images were imported into BlueFuse with an microarray layout (.bgl) file, so the relative signal (Cy5 / Cy3) ratio for each spot could be calculated. As part of the BlueFuse analysis, a block-by-block median shifting normalisation was performed which compensates for any differences in labelling efficiency and variation due to printing pin quality.

During the analysis, deviations in gene expression data were observed, which were due to decreased signal distribution, exhibiting so-called 'compression' when visualised (Section 4.1.2). To compensate for these deviations, BlueFuse output files were further processed using BABAR (Batch Anti-Banana Algorithm in R) (Alston *et al.*, 2010) using pair-wise comparisons between microarray samples, as described elsewhere (Hautefort *et al.*, 2008). BABAR comprises three main steps: normalising transcriptomic data with a block-by-

block median centring similar to the function performed by BlueFuse; a pair-wise global loess using *limma* (Bioconductor) between each pair of arrays (Smyth & Speed, 2003); a final block-by-block median centring of the loessed arrays. The data quality can be visualised through diagnostic box-plots (Figure 4.4).

The gene expression data were routinely visualised in a graphical format and analysed using GeneSpring version 7 (Agilent Technologies). This software allowed easy access to standard statistical tests and simple filtering of the data. For each time-point, at least three biological replicates were performed, which were averaged within GeneSpring and compared internally to a single time-point. This allowed simple interpretation of the results in terms of fold-changes in gene expression. Clustering was carried out using the Pearson clustering parameters. Statistically significant changes in individual gene expression data were calculated using a *t*-test ($p<0.05$) and a minimum of a 2-fold cut-off threshold.

2.3.11 Chromatin immunoprecipitation and microarrays

Chromatin immunoprecipitation coupled with microarray analysis (ChIP-chip) allows the binding sites of a known DNA-associated protein to be determined (Ren *et al.*, 2000).

2.3.11.1 Cell harvesting and lysis

For the ChIP-chip experiments described in this thesis (Chapter 5), bacterial cultures were grown for 48 hours, at which point 1 % final concentration formaldehyde (Sigma, F1635) was added to covalently cross-link nucleoid-associated proteins to the DNA. After either 2 or 15 minutes, the reaction was quenched by the addition of 0.5 M final concentration glycine (Sigma, G7126) which prevented any further cross-linking.

After lysis optimisation (Section 5.2.1), stationary phase cells (approximately 5×10^8 CFU / ml) were harvested by centrifugation of bacterial cultures for the ChIP-chip experiment. Cell pellets were washed three times with PBS (pH 7.4) to remove trace amounts of growth medium, formaldehyde and glycine. The cell pellet was resuspended in 700 µl lysis buffer containing:

Trizma Base (pH 8.0)	10 mM
Sodium chloride	50 mM
EDTA	10 mM
Sucrose (S7903)	20 % (w/v)

To this, 700 µl 2 x RIPA solution was added, containing:

Trizma Base (pH 8.0)	100 mM
Sodium chloride	300 mM
Nonidet P40 (Fluka, 74385)	2 % (w/v)
Sodium deoxycholate (Sigma, D6750)	1 % (w/v)

SDS was omitted from the 2x RIPA solution to prevent foaming during the lysis procedure.

The total solution was transferred to 2 ml screw-cap tubes (Sarstedt, D-51588) and acid-washed glass beads, ≤106 µm in size (Sigma, G4649) were added until the bottom of the tube was covered. The samples were then lysed using a FastPrep FP120 (Thermosavant) bead-beater maximum speed for 3 x 20 seconds. The samples were centrifuged at full-speed in a Benchtop 5415D Microcentrifuge (Eppendorf) with a F45-24-11 rotor to harvest the beads and any insoluble cellular debris. The supernatant was removed, transferred to 2 ml tubes (Eppendorf) and sonicated with a Soniprep 150 (Sanyo) sonicator (10 µm amplitude, 3 x 20 seconds), ensuring the samples were cooled on ice at regular intervals. The sonication step fragmented the DNA within the cells to approximately 500 base-pairs (bp) in length. The DNA fragment sizes were confirmed after each sonication batch via agarose gel electrophoresis (Section 2.3.8). Remaining cellular debris was pelleted via centrifugation (15682 x g) and the supernatant (~1.2 ml) was removed.

2.3.11.2 Chromatin immunoprecipitation

In order to prevent damage to cross-linked proteins, the serine protease inhibitor phenylmethylsulphonyl fluoride (PMSF) (Sigma, P7626) was added at a final concentration of 1 mM from a 100 mM stock solution in dimethylsulfoxide (DMSO). Each sample was divided into equal two aliquots (600 µl each) designated either ‘test’ or

'control' for each time-point. To each sample 50 µl rabbit immunoglobin G (IgG) coated sepharose beads (Sigma, E3403) were added and the samples incubated at 4 °C, on a rotary wheel (10 rpm) for at least 2 hours, to bind any short, background DNA fragments. The beads were recovered after centrifugation (15682 x g) for 30 seconds. The supernatant was transferred to clean tubes and a further 50 µl rabbit immunoglobin G (IgG) coated sepharose beads (Sigma, E3403) were added to each sample. To each 'test' sample, 20 µl of the specific monoclonal antibody to the β subunit of *E. coli* RNA polymerase (Neoclone, W0002) was added (Thompson *et al.*, 1992). The samples were incubated on a rotary wheel (10 rpm) at 4 °C for 22 hours. The IgG-coated sepharose beads bound to the monoclonal antibody with a capacity of >8 mg / ml to allow easy purification of ChIP DNA.

2.3.11.3 Washing and eluting the ChIP DNA

The supernatant was discarded and the sepharose beads were washed twice in 500 µl 1 x RIPA containing:

Trizma Base (pH 8.0)	50 mM
Sodium chloride	150 mM
Nonidet P40 (Fluka, 74385)	1 % (w/v)
Sodium deoxycholate (Sigma, D6750)	0.5 % (w/v)
SDS	0.1 % (w/v)

The beads were harvested via centrifugation and resuspended twice in 500 µl wash buffer containing:

Trizma Base (pH 8.0)	10 mM
Lithium chloride	250 mM
EDTA	1 mM
Nonidet P40 (Fluka, 74385)	0.5 % (w/v)
Sodium deoxycholate (Sigma, D6750)	0.5 % (w/v)

The beads were harvested via centrifugation and resuspended once in 500 µl TE (pH 8.0) buffer. After centrifugation the supernatant was discarded.

The samples were incubated at 65 °C in a water bath (Grant Instruments, Hertfordshire, UK) for 15 minutes in 150 µl pre-warmed elution buffer containing:

Trizma Base (pH 8.0)	50 mM
EDTA	10 mM
SDS	1 % (w/v)

This elution step was designed to separate the antibody from the sepharose beads. The samples were centrifuged briefly and the supernatant collected. To degrade the cross-linked proteins and the antibody 0.6 mg / ml pronase E from *Streptomyces griseus* was added (Sigma, P6911) in 150 µl molecular grade water (Sigma, W4502) and incubated overnight at 65 °C.

ChIP DNA was cleaned using a QiaQuick PCR purification kit (Qiagen) and eluted into 30 µl molecular grade water (Sigma, W4502). The DNA was dried to 21 µl using a Speed Vacuum Concentrator (Thermo-Savant) and direct labelled with a fluorophore along with reference genomic DNA (Section 2.3.10.2) for a Type 2 microarray analysis (Yang & Speed, 2002).

2.3.11.4 ChIP-chip microarray hybridisation

After the labelling step, 20 µl Cy3-dCTP labelled genomic DNA was added to 100 µl Cy5-dCTP labelled ChIP DNA and the final volume made up to 250 µl with molecular grade water (Sigma, W4502). The DNA samples were denatured by boiling at 100 °C for 2 minutes and cooled on ice for 1 minute. Hybridisation buffer was added to each sample containing:

Morpholine-4-ethanesulfonic acid (MES) hydrate (pH 6.5) (Sigma, M2933)	50 mM
Sodium chloride	1 M
>99% Formamide (Sigma, F5786)	20 % (w/v)
EDTA	20 mM
Triton X-100 (Sigma, T8532)	1 % (w/v)

The total 500 µl volume was loaded onto a rubber-sealed glass cover slide (Agilent, G2534-60003), ensuring the hybridisation mix was spread as evenly as possible. The cover slide was then placed into a hybridisation chamber (Agilent, G2534A). An oligonucleotide-tiled *S.* Typhimurium SL1344 microarray slide (Oxford Gene Technologies, Kidlington, UK) was lowered onto the cover slide and held in place with a hybridisation jig clamp, ensuring the amount of trapped air bubbles was minimised. Each microarray contained 21,939 directly-synthesised 60-mer oligonucleotides printed at high density onto the glass slide covering regions of the entire *S.* Typhimurium SL1344 NCTC13347 genome and 636 control oligonucleotides. The SL1344 sequence was obtained from the Sanger Institute (Hinxton, UK) website: (http://www.sanger.ac.uk/Projects/*Salmonella*/). As this genome is not yet fully annotated, the oligonucleotides were associated with corresponding *S.* Typhimurium LT2 genes or intergenic regions (McClelland *et al.*, 2001), if conserved in both organisms. For SL1344-specific genes, designation of gene identifiers was as per the SL1344 genome sequence.

Hybridisations were performed over 72 hours at 55 °C in a hybridisation oven (Agilent, G2545A) rotating at 8 rpm. The slides were checked at regular intervals to ensure there were no air bubbles trapped beneath the cover slide which may lead to non-hybridised regions.

2.3.11.5 ChIP-chip washing steps

Wash steps were performed to remove any non-hybridised DNA from the slide. Each wash step was performed twice at room temperature with the samples agitated at 50 rpm on a platform rotary shaker (New Brunswick Scientific, Edison, New Jersey, USA) for 5 minutes.

Slides were removed from the hybridisation chamber and put into a black plastic slide holder before being submerged in the first wash buffer containing 6x standard saline / phosphate / EDTA (SSPE) and 0.005 % final concentration sarcosyl N-lauroylsarcosine.
6x SSPE contained the following:

NaCl (Sigma, S7653)	900 mM
$NaH_2PO_4 \cdot H_2O$ (Sigma, 71507)	60 mM
EDTA (pH 8.0)	60 mM

Immediately after the second wash, the slides were moved into wash solution two containing 0.06x SSPE and 0.18% polyethylene glycol (PEG 200, Sigma, P3015).
After the second wash in solution two, the slides were dried in a Jouan MR23i centrifuge (Thermo Electron Corporation) at 1500 rpm (289 g) using the 6xμT microtitre plate rotor at 20 °C. Slides were held in black plastic slide chambers (Rotec Scientific, 2285.1) during centrifugation.

2.3.11.6 ChIP-chip scanning and data analysis

The oligonucleotide-tiled microarray slides were scanned in a GenePix 4000 B microarray scanner (Molecular Devices) using GenePix Pro v6.0 Software following the manufacturer's instructions. Preview scans were carried out at 40 μm resolution and full data scans were performed at 10 μm resolution with the PMT voltage calculated using the Auto PMT function within GenePix.

Data were generated from the GenePix images using BlueFuse v1.0 (BlueGnome, Oxford, UK). Images were imported into BlueFuse with an microarray layout (.bgl) file, so the relative signal (Cy5 / Cy3) ratio for each spot could be calculated. As part of the BlueFuse analysis, a block-by-block median shifting normalisation was performed which compensated for any differences in labelling efficiency and variation in printing pin quality.

The gene expression data were initially visualised in a graphical format using GeneSpring version 7 (Agilent Technologies) and the *S.* Typhimurium SL1344 genome. Data including signal ratios, gene names and chromosome position, were routinely exported, unnormalised, from GeneSpring into the ChIPOTle Visual basic macro, run within

Microsoft Excel (Buck *et al.*, 2005). Information on the implementation of the ChIPOTle macro can be found at:
www.bio.unc.edu/faculty/lieb/labpages/chipotle/ChIPOTle_Readme_v1.0.pdf

The ChIPOTle macro was used to detect significant RNA polymerase binding peaks by a Gaussian statistical test ($p<0.001$) and a two-fold minimum cut-off threshold, indicated by an elevated fluorescence signal due to enriched immunoprecipitated DNA.

The genomic context of these signal peaks were visualised using the Integrated Genome Browser (IGB) v4.56 (Affymetrix) with the relevant gene feature file (.GFF) and graph files (Appendix D). The IGB software allowed a visual analysis of the ChIP binding peaks on regions of the SL1344 chromosome; however a further, automated analysis was performed by implementing a Perl script to correlate the RNA polymerase binding patterns with SL1344 genes (Mark Alston, personal communication). A list of 663 RNA polymerase binding peaks was generated corresponding to 979 SL1344 genes. This list of binding peaks was used for the final analysis of RNA polymerase binding (Chapter 5).

2.4 Biochemical methods

2.4.1 Inductively-Coupled Plasma Mass Spectrometry

Inductively-Coupled Plasma Mass Spectrometry (ICP-MS) was used to measure accurately the cellular concentration of metal ions (Thomas, 2001).

2.4.1.1 Cell harvesting

During the preparation of samples, extreme care was taken to minimise the contact with sources of metal contamination and chemicals. Centrifugation of large volumes was performed in 450 ml polycarbonate centrifuge bottles (Nalgene, 3140-0500) pre-washed with 10 % (v/v) nitric acid to remove bound metals and then rinsed with pure 18.2 MΩ / cm water. Any plastic pipette tips used to resuspend cell pellets were also washed in this way.

Bacterial cultures were grown until the desired time-point and the cell concentration was determined by viable count measurement to allow estimation of cell concentration per

sample. For initial tests, five biological replicates of either 100 ml or 200 ml culture (4.87 x 10^7 CFU and 9.73 x 10^7 CFU, respectively) were taken, the cell pellets washed and harvested for ICP-MS analysis as per the final experiment.

For the final optimised protocol: lag phase samples (750 ml culture) and stationary phase and mid-exponential phase (15 ml culture) was taken. The required volume of bacterial culture was poured into plastic tubes for centrifugation. This minimised possible contamination during transfer by pipette. Cell pellets were harvested into white-capped 15 ml tubes (Greiner Bio One, 188285), which leach less metals (Rolfe, 2007). The cell pellets were resuspended in 5 ml dH_2O containing 1 mM EDTA (pH 8.0) to bind any extracellular metal associated with the cell pellet. The addition of 1 mM EDTA chelator, as used in other, similar studies (Outten & O'Halloran, 2001, Tree *et al.*, 2005) decreased the background metal contamination and increased the reproducibility of the data. The affinity of EDTA (K_{aff}) for many divalent cations (Wiberg *et al.*, 2001) ensured that there was no removal of specific metal ions, which would have skewed the results for certain metal ions. To ensure that the availability of certain cations did not impact upon the EDTA binding to less abundant metal ions, as observed in clinical chelation experiments using EDTA (Waters *et al.*, 2001), a relatively high concentration (1 mM) was added to ensure an excess of chelator in the samples.

The cells were washed twice further with 5 ml dH_2O to remove traces of EDTA, centrifuging 7000 rpm (6300 g) in an Eppendorf 5810 R centrifuge (F-34-6-38 rotor), for 10 minutes at 4 °C to harvest the cell pellets. Pellets were dried in a Techne Dri-block DB-2D heat block (Jencons) overnight at 65 °C. Dried cell pellets were stored in the sealed white-capped tubes at 4 °C under plastic wrap until required for the ICP-MS analysis.

2.4.1.2 ICP-MS analysis

Dried cell pellets were digested in 500 µl Aristar grade 69 % (w/v) nitric acid and 250 µl Ultrapur 31 % (w/v) hydrogen peroxide (Merck, 106097) and left in sunlight for 3 days to reduce all organic material to its constitutive elements. Once digested, each sample was diluted in 6.75 ml 2 % (w/v) nitric acid containing 1 ppb platinum, rhodium and germanium as internal standards for calibration of the mass spectrometer. Each sample was

run in triplicate through an Agilent 7500ce ICPMS mass spectrometer. The viable cell count was used to derive the final mass of metals per cell.

ICP-MS data were analysed with Microsoft Excel and was statistically filtered using the Grubbs test to remove any outlying data (Grubbs, 1969) (*t*-test $p<0.05$).

2.4.2 Nuclear magnetic resonance

Nuclear magnetic resonance (NMR) analysis was used to quantify accurately the concentration of metabolites present in the cell-free medium of *S.* Typhimurium cultures at different growth phases (Section 3.2.3). Bacterial cultures were grown until the desired time-point and ~1.7 ml was removed and filtered (0.22 µm) into sterile 2 ml tubes (Eppendorf) which were stored at -20 °C until required for NMR analysis. An aliquot (400 µl) of this was taken and added to 200 µl NMR buffer containing:

D_2O (Cambridge Isotope Laboratories)	20 ml
dH_2O	80 ml
Na_2HPO_4 (Fisher Scientific)	2.8853 g
NaH_2PO_4 (BDH Analar)	0.5247 g
3-(Trimethylsilyl)-1-propane-sulfonic acid, sodium salt (Aldrich)	32.7 mg
Sodium azide (Sigma, S2002)	60.1 mg
Difluoro(trimethylsilyl)methylphosphonic acid (Bridge Organics Co.)	122.5 mg

After mixing, 500 µl was aliquoted into glass NMR tubes (∅ = 5 mm) for analysis in a 600MHz Bruker Avance spectrometer. All NMR work was kindly performed by Terri Grassby and Ian Colquhoun at the IFR, Norwich. Concentrations of specific metabolites were analysed within Microsoft Excel. The formulae used to calculate the final metal concentrations are within the Excel worksheet included with the attached CD (Appendix E).

Chapter 3

The physiology of *Salmonella* in the lag phase static system

3. The physiology of *Salmonella* in the lag phase static system

The physiological processes that define *Salmonella* lag phase are not well understood and a suitable system to accurately investigate these processes was not available, until recently. The development of the static growth system to study *Salmonella* lag phase was pivotal to our increased understanding of this crucial phase of bacterial growth (Rolfe, 2007). This reproducible system was used extensively by Rolfe (2007) and in the present study, as the system was designed to minimise the inherent variability in lag time measurements (Baranyi, 1998) that can arise due to many factors such as: subtle changes in media composition, the growth state of individual bacterial cells and the physiological history of the population (Swinnen *et al.*, 2004). By minimising biological variability and measurement uncertainty between experiments, a range of different techniques could be used to give new insights into processes occurring during lag phase including: oxygen utilisation, changes in the availability of nutrients, the build-up of metabolites over time and the effect of media conditioning on lag time. In addition, the reproducible system was modified as described previously in this thesis (Section 2.1.6.1) to investigate the effect of challenging stationary phase *S.* Typhimurium with cold stress for varying lengths of time and observing whether the lag duration was affected.

3.1 Introduction

3.1.1 Measuring lag time of bacterial populations

The conventional method of measuring lag time of a bacterial population is by a viable count growth curve, to provide an accurate quantification of a bacterial population. This method has been used previously to accurately measure *Salmonella* and other bacteria (Baranyi *et al.*, 1999, Dufrenne *et al.*, 1997, Mellefont *et al.*, 2003, Mellefont *et al.*, 2004, Mellefont *et al.*, 2005, Mellefont & Ross, 2003) and allows the use of microbiological software such as dynamic modelling with DMFit (http://www.ifr.ac.uk/safety/DMFit), and growth curve fitting with software such as MicroFit (http://www.ifr.ac.uk/microfit), providing quantification of growth parameters. Although viable count growth curves are the most accurate method for determining lag time, they are more time-consuming and expensive than alternative methods such as optical density (OD) measurements using a spectrophotometer.

OD-based growth curves are routinely performed in molecular microbiology laboratories, but are an indirect method for measuring bacterial growth as they reflect the cell biomass rather than cell numbers. The major disadvantage of using OD growth curves to estimate the lag time of a bacterial population is that the detection limit of the spectrophotometer is generally below that of the initial inoculum, making an accurate estimation of lag time difficult. For experiments described in this thesis, the initial inoculum is ≤ 5.5 x 10^5 CFU/ml which is below the detection limit of the spectrophotometer used (~10^6 CFU/ml). In addition, bacterial populations contain cells of diverse morphologies which lead to unreliable readings using a spectrophotometer and can ultimately lead to miscalculation of cell numbers if, for example, an elongated cell morphology is predominant in the bacterial population. Despite this, some publications have used OD growth curves to estimate lag time using either a standard spectrophotometer or by using the Bioscreen C Microbiology Reader (Egler *et al.*, 2005, Rabsch *et al.*, 2003).

For all viable count growth curves performed in this study, the DMFit macro was used to determine the lag time (Baranyi & Roberts, 1994) and statistical tests run using MicroFit v1.0 (F-test, cut-off ≤0.05).

3.1.2 Bacterial oxygen utilisation during growth

When grown in an anoxic environment, *E. coli* and *Salmonella* can generate energy by anaerobic respiration with a terminal electron acceptor, or by fermentation of a suitable substrate. Neither of these processes provides as much energy as aerobic respiration, and so when exposed to an oxygen upshift, these bacteria perform a metabolic switch to make use of the available oxygen (Iuchi & Weiner, 1996). This process involves a fully-functioning tricarboxylic acid cycle linked into oxidative phosphorylation, requiring the production of cytochromes to maximise the growth rate (Iuchi & Weiner, 1996, Shioi *et al.*, 1988). During exponential growth, *Salmonella* rapidly depletes oxygen from rich growth media, coinciding with a reduction in growth rate as the bacteria enter transition phase (Wilson *et al.*, 2003).

It is possible to detect the dissolved oxygen concentration using a variety of methods (Renger & Hanssum, 2009) including the use of a redox probe or online detection with a polarographic oxygen electrode (Sections 2.1.9.3 and 2.1.11, respectively). Although

aerobic respiration yields a relatively high degree of energy, oxidative stress accompanies a shift from anaerobiosis to aerobic conditions (Partridge *et al.*, 2006) which can be damaging to bacteria (Cabiscol *et al.*, 2000) and may impact upon growth rate and lag times (Cuny *et al.*, 2007).

3.1.3 Nutrient availability in Luria-Bertani (LB) medium

During growth, bacteria deplete nutrients in a sequential manner. LB medium contains a low concentration of glucose, thus *E. coli* first catabolises other sugars before switching to metabolise more abundant oligopeptides (Sezonov *et al.*, 2007) which are important sources of nitrogen during fermentation towards the end of bacterial growth, when dissolved oxygen concentrations are low (Baev *et al.*, 2006b). When present, glucose is rapidly metabolised by *Salmonella* during exponential growth (Wilson *et al.*, 2003), leading to an increase in acetic acid production during fermentation and a subsequent decrease in pH (Akesson *et al.*, 1999). It has been determined, by transcriptional analysis, that *E. coli* has a preference for fermentable sugars in LB medium: first metabolising maltose and then utilising D-mannose, melibiose, D-galactose, L-fucose and L-rhamnose. Interestingly, spent LB medium is not nutrient-depleted by *E. coli* or *S.* Typhimurium at stationary phase and has been shown to support the re-growth of both organisms upon re-inoculation, suggesting that nutrient deprivation is not the cause of entry into stationary phase by these organisms (Barrow *et al.*, 1996). These and other studies have shown that variation in media components between batches may influence nutrient acquisition and the growth response leading to differences in measured lag times (Sezonov *et al.*, 2007).

3.1.4 Known growth-phase specific processes of relevance to lag time

There are various processes that occur at specific phases during bacterial growth. Many of these processes are dependent upon regulators that respond to sudden changes in the bacterial environment and elicit a transcriptional response. Some of these gene regulators have a role in adaptation between environments and are critical in determining virulence. For example, the FNR protein was originally identified as having a role in *E. coli* fumarate and nitrate reduction but later it was discovered that this gene controls the regulation of over 100 anaerobically-induced genes (Fink *et al.*, 2007). The function of FNR was determined by deleting the responsible gene, *fnr* and studying the *E. coli* (Δ*fnr*) transcriptome to determine the effect of the mutation on global gene expression. In a

previous study with *S.* Typhimurium, *fnr* was deleted, but the mutant strain did not have a longer lag time after inoculation into a fresh, aerobic medium (Rolfe, 2007). A further 25 strains were also tested, including important regulators such as SoxS, Fur, RpoE (σ^{24}) and BipA with only the Δ*bipA::kan* mutant showing an increased lag time phenotype, albeit with a high degree of variability (Rolfe, 2007). The lack of an increased lag time phenotype suggests that a degree of functional overlap may be present during lag phase adaptation, whereby others genes compensate for the function of a mutated gene. In the present study, important growth-specific processes were targeted for mutagenesis to determine the effect on lag time.

3.1.4.1 Fis

The factor for inversion stimulation (Fis) is a 12 kDa nucleoprotein present at greater than 50,000 molecules per cell during lag phase in *E. coli*, but the amount of Fis protein decreases during stationary phase, to barely detectable concentrations (Ball *et al.*, 1992). The function of Fis was originally classified as stimulating the inversion of site-specific recombinases (Finkel & Johnson, 1992), but since has been attributed to many other phenotypes in *E. coli* including transcription, DNA replication (Filutowicz *et al.*, 1992), integration and excision of bacteriophage lambda (Ball & Johnson, 1991), chromosome compaction (Skoko *et al.*, 2007), DNA supercoiling (Schneider *et al.*, 1997, Schneider *et al.*, 2000) and catabolite repression (Galan *et al.*, 2008). In addition, Fis is known to modulate virulence genes in *S.* Typhimurium (Kelly *et al.*, 2004, O' Cróinín & Dorman, 2007). Crucially for the present study, a Δ*fis* mutation in *S.* Typhimurium was reported to have a longer lag time in rich medium at 37 °C (Osuna *et al.*, 1995). However this phenotype has been disputed in a more recent study using the lag phase static system at 25 °C (Rolfe, 2007).

3.1.4.2 BipA

BipA (TypA) is a 65 kDa translational GTPase first identified by its induction in the presence of bactericidal / permeability-increasing protein, BPI, a defensin produced by neutrophils during bacterial infection (Qi *et al.*, 1995). Deletion of *bipA* causes pleiotropic defects in *E. coli* including: slow growth at 20 °C (Pfennig & Flower, 2001), cell hypermotility and adhesion impairment (Farris *et al.*, 1998, Grant *et al.*, 2003). BipA is also required for expression of the K5 capsule system (Rowe *et al.*, 2000), formate-

mediated resistance to antimicrobial peptides (Barker *et al.*, 2000), and invasion by enteropathogenic *E. coli* (Grant *et al.*, 2003). BipA has a pleiotropic role in other organisms, involved in low pH and low temperature stress resistance in *Sinorhizobium meliloti (*Kiss *et al., 2004)*, oxidative stress resistance in chloroplasts of *Suaeda salsa (*Wang *et al., 2008)* and in the sexual development of *Cucumis sativus* (Barak & Trebitsh, 2007). Interestingly for this study, Δ*bipA* mutants have shown to exhibit longer lag times in *S.* Typhimurium in the lag phase static system (Rolfe, 2007) and BipA has been implicated in the translation of *fis* mRNA transcripts (Owens *et al.*, 2004), although the latter publication was later retracted and no further studies have confirmed the initial findings.

3.1.4.3 ppGpp

The alarmone guanosine tetraphosphate (ppGpp) is produced by two proteins, RelA and SpoT in a stringent response to nutrient starvation and stress, swiftly down-regulating rRNA and tRNA synthesis (Cashel *et al.*, 1996). The production of ppGpp in *S.* Typhimurium leads to induction of *rpoE* (Costanzo *et al.*, 2008), *rpoS*, *lrp,* amino acid biosynthetic genes (Cashel *et al.*, 1996) and virulence genes (Thompson *et al.*, 2006a). Critically for this study, $ppGpp^0$ mutants, caused by mutations in both *relA* and *spoT*, have shown an extended lag time in *E. coli* grown under amino acid limited conditions (Traxler *et al.*, 2008).

3.1.4.4 RMF

The ribosome modulation factor (RMF) mediates the dimerisation of active 70S ribosomes upon transition to stationary phase, forming inactive 100S protein complexes, which are both protease and nuclease resistant (Wada *et al.*, 2000, Wada *et al.*, 1990, Yoshida *et al.*, 2002). The dimerisation process in *E. coli* is facilitated by changes to ribosomal structure to prevent disassociation by initiation factor 3 (Yoshida *et al.*, 2009). Ribosome stabilisation is regulated by direct binding of a hibernation promoting factor (YhbH) or by YfiA, in addition to RMF (Ueta *et al.*, 2005). During transition phase in *E. coli*, RMF binds 70S ribosomes, forming immature 90S ribosomes and recruits RMF to form the mature 100S ribosome complex (Ueta *et al.*, 2008). Transcription of the *rmf* gene is independent of RpoS (σ^{38}) but does rely upon the ppGpp-mediated stringent response (Izutsu *et al.*, 2001). RMF-deficient *E. coli* mutants lose viability in stationary phase at a faster rate than

a wild-type strain, which is exacerbated when combined with other mutations in RpoS, ppGpp and OmpC (Apirakaramwong *et al.*, 1999, Fukuchi *et al.*, 1995, Samuel Raj *et al.*, 2002). it is relevant to the present study that within one minute of inoculation of *E. coli* into fresh medium, neither 100S ribosomes nor RMF protein can be detected and *rmf* mRNA is degraded in a process involving RNase E and poly (A) polymerase (Aiso *et al.*, 2005).

3.1.5 Effect of media conditioning on bacterial growth

Many factors are produced which effect cellular growth of the population. These small, diffusible molecules are thought to allow bacteria to identify the population (quorum) size and regulate their cellular physiology accordingly, in a similar way as multi-cellular organisms (Shapiro, 1998). Experiments to investigate the effect of these molecules on lag physiology have focussed on the use of a fresh medium supplemented with stationary phase cell-free supernatants (Winzer *et al.*, 2002b). The most detailed of these studies concluded that MOPS-buffered minimal medium supplemented with 30 % (v/v) cell-free supernatants caused lag time of *E. coli* to decrease by between 22% and 58% upon inoculation. Although the molecule responsible for this apparent decrease in lag time was not purified, it was determined that the responsible molecule was heat-, pH- and protease-resistant and ready dialysable from the growth medium (Weichart & Kell, 2001). No studies have yet identified a role for a specific small molecule in significantly altering the length of lag time in *Salmonella*. The best candidate signalling molecules for other bacteria appear to part of quorum sensing systems which have been well characterised for a range of bacteria and are described in this thesis, with respect to *E. coli* and *Salmonella* (Section 3.1.5.1). A more detailed review of quorum sensing systems in bacteria has been discussed elsewhere (Bassler, 2002).

3.1.5.1 Quorum sensing

The quorum sensing systems in *E. coli* and *Salmonella* have been described in detail previously (Walters & Sperandio, 2006). In *Salmonella* there are three quorum sensing systems including the LuxR homologue, SdiA which responds to *N*-acyl-homoserine-lactones (AHL) by regulating genes contained on the pSLT virulence plasmid (Ahmer *et al.*, 1998). Interestingly, although *Salmonella* can detect AHL produced by other bacterial

species, the bacterium cannot synthesise its own AHL due to the lack of a LuxI homologue.

The other quorum sensing systems involve the production of autoinducer-2 (AI-2) and autoinducer-3 (AI-3) under the control of LuxS. Both AI-2 and AI-3 are produced by *Salmonella* in media containing exogenous glucose (Surette & Bassler, 1998), with AI-2 targeting the Lsr ATP-binding cassette (ABC) transporter and inducing uptake of the AI-2 molecule (Taga *et al.*, 2001). Studies into the role of AI-3 in *E. coli* O157:H7 concluded that virulence and motility genes are regulated in conjunction with adrenaline and noradrenaline (Sperandio *et al.*, 2003). No role in virulence has been discovered for AI-3 in *Salmonella* and it has been proposed that both AI-2 and AI-3 may represent exported waste products rather than specific cell-signalling molecules (Winzer *et al.*, 2002a). Adrenaline alone may act as an environmental cue for *Salmonella* during infection as the hormone causes antimicrobial peptide resistance genes (*pmrHFIJKLM*) and OxyR regulated oxidative stress resistance genes to be down-regulated within 30 minutes of adrenaline addition (Karavolos *et al.*, 2008). This transcriptional effect leads to an uptake of manganese through the transporter SitABCD, leading to catalytic detoxification of reactive oxygen species to occur. The down-regulation of specific resistance mechanisms leaves the bacteria susceptible to other infection relevant stresses, most notably antimicrobial peptides and may represent a defence mechanism for the host rather than a bacterial signal molecule.

3.1.6 Effect of physiological history upon bacterial growth

The treatment history of a bacterium may impact upon the physiological state in a new environment, thus increasing the adaptation time during lag phase (Gawande & Griffiths, 2005, Oscar, 1999a). The physiological history may involve a stress which physically damages the bacterium, requiring repair or a more subtle stress such as nutrient deprivation. In the latter case it has been shown that *E. coli* previously grown under conditions of phosphate-limitation, increase the induction of *pho* genes (encoding alkaline phosphatase) faster than wild-type cells after subsequent inoculation into phosphate-limited medium (Hoffer *et al.*, 2001). This response to physiological history has controversially been referred to as ‘learning behaviour’ or a bacterial ‘memory’ which can be classified as being either long- or short-term (John *et al.*, 2009, Wolf *et al.*, 2008).

Furthermore, it has been demonstrated that bacteria may be able to make use of the retained 'memory' to alter their behaviour and predict subsequent changes to their environment (Oxman *et al.*, 2008, Wolf *et al.*, 2005), similar to Pavlovian conditioning (Mitchell *et al.*, 2009).

Previous food-relevant treatments such as thermal processing, pH, osmotic shock, carbon dioxide concentration and cold storage have been shown to impact upon bacterial lag times after inoculation in fresh media (De Jesus & Whiting, 2008, Dufrenne *et al.*, 1997, Juneja & Marks, 2006, Oscar, 1999a, Oscar, 1999b, Oscar, 1999c). If the previous and new environments are similar then little adaptation is required after inoculation and the lag phase is completed rapidly. Interestingly, although the physiological history has been shown to significantly impact on lag times, the doubling time of bacteria during stationary phase is often unaffected (Gorris & Peck, 1998). Bacterial stresses such as cold storage can lead to morphological changes such as filamentation as a survival strategy (Justice *et al.*, 2008). Upon inoculation of filamentous cells into a fresh growth medium, the bacterial lag time can be rapid as a consequence of the ready elongated bacteria requiring only septation before cell division occurs (Mattick *et al.*, 2003).

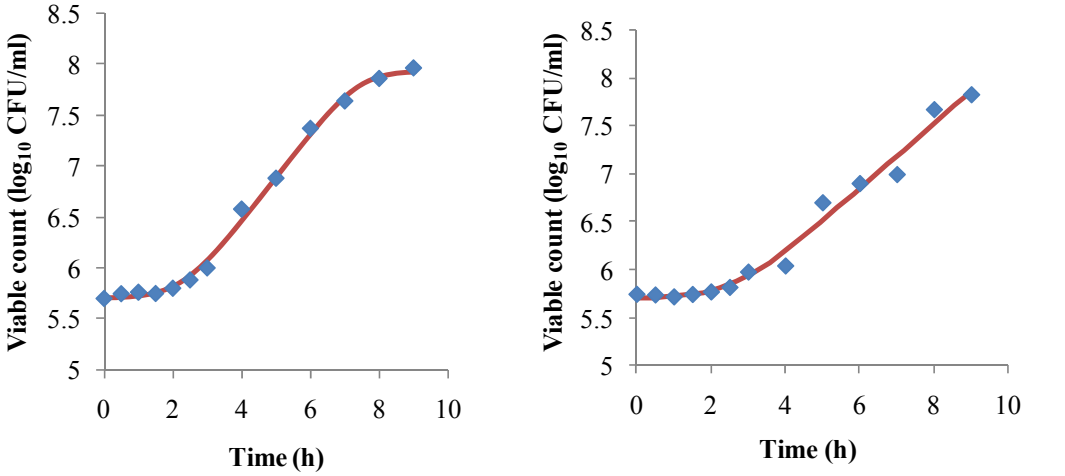
3.2 Results

3.2.1 Characterisation of bacterial growth in the lag phase system

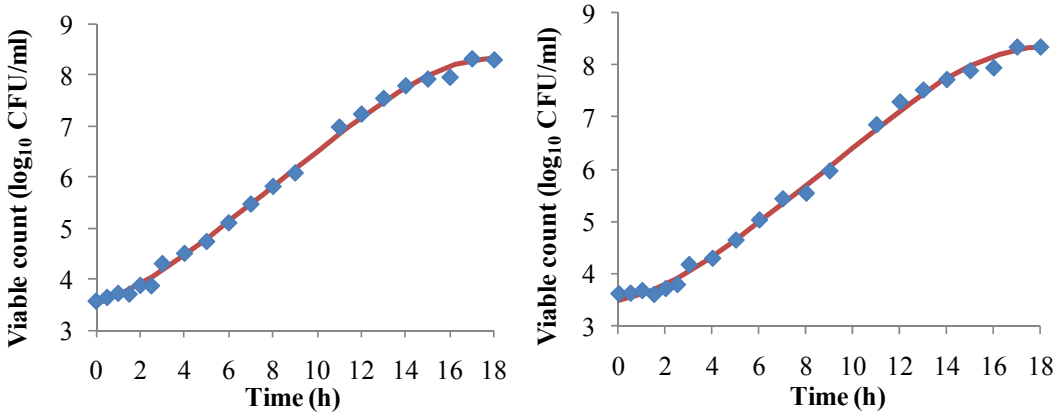
Until recently the physiology of lag phase had not been investigated in great detail. In the present study, the lag phase static system was utilised to phenotypically test hypotheses inferred by previously-acquired transcriptomic data and to determine the effect on recovery time in lag phase after mutating genes encoding specific processes. Viable count growth curves were performed and fitted using the DMFit macro (http://www.ifr.ac.uk/safety/DMFit) with either a 10^5 CFU/ml starting cell concentration (Figure 3.1A) or a 10^3 CFU/ml inoculum (Figure 3.1B). The growth parameters after inoculation into fresh LB medium were calculated using DMFit (Figure 3.1C).

The remainder of the chapter concentrates on the examination of the environment of the lag phase system throughout *S.* Typhimurium growth and the investigation of the effect of pre-treatment or mutation on the bacterial lag time.

A.



B.



C.

Growth curve	Lag time (h)	Doubling time (h)	Cell concentration (log_{10} CFU/ml)	
			Starting	Final
WT (high) 1	2.28	1.00	5.70	7.94
WT (high) 2	2.65	0.98	5.70	7.82
WT (low) 1	1.43	1.00	3.51	8.41
WT (low) 2	2.00	1.00	3.50	8.42

Figure 3.1: Reproducible growth observed in the lag phase static system. Viable count growth curves obtained from the lag phase static system. (A) Growth curves of experimental cultures inoculated with 10^5 CFU/ml showing two biological replicates (B) Growth curves of experimental cultures inoculated with 10^3 CFU/ml showing two biological replicates (C) Table of fitted growth parameters using DMFit for either 10^5 CFU/ml inoculation (high) or 10^3 CFU/ml inoculation (low) for wild type SL1344 (WT). For each strain the lag time, doubling time, starting and final cell concentration are shown. The DMFit algorithm was unable to distinguish subtle differences in the doubling times of the bacterial cultures between replicates.

3.2.2 Oxygen utilisation by *S.* Typhimurium throughout growth

To ascertain the rate of oxygen consumption in the static system, the dissolved oxygen concentration was measured by an oxygen probe (Figure 3.2). Oxygen was consumed maximally throughout exponential growth and the oxygen concentration decreased to the probe detection limit (0.12 %) by 9.3 hours, representing the mid-exponential phase of growth (Figure 3.2A).

Interestingly, removal of dissolved oxygen from the LB medium was detected within 4 minutes of inoculation. The subsequent consumption was initally rapid for 4 minutes before the rate of oxygen removal decreased and remained constant throughout lag phase, which lasted for 1.61 hours in this experiment (Figure 3.2B). By two hours post-inoculation, corresponding to the end of the *S.* Typhimurium lag phase, 11.5 % dissolved oxygen had been removed from the medium. Significantly, however substantial quantities of dissolved oxygen remained present throughout lag phase.

3.2.3 Identification of metabolites in cell-free supernatants

The transition from stationary phase to fresh LB medium is crucial to understanding *S.* Typhimurium lag phase. The culture environment during stationary phase affects the physiology of the bacteria and may impact upon the metabolic 'work' to be completed during lag phase (Baranyi & Roberts, 1994). During stationary phase, toxic waste-products build up as a result of bacterial metabolism and valuable nutrients are depleted. Once *S.* Typhimurium is inoculated into fresh media, there is upshift of nutrient availability, providing important energy sources for lag phase metabolism. In order to characterise the stationary phase metabolites and the nutrient availability through lag phase, nuclear magnetic resonance (NMR) analysis was used (Section 2.4.2) to investigate the principal metabolic differences between the stationary phase culture and the lag phase time-points (Figure 3.3). All of the lag phase cell-free supernatants contained approximately the same concentration of available nutrients and many of these of the metabolites were depleted by 48 hours of growth, resulting in an associated build-up of metabolic by-products. Of the detectable nutrients: glycerol, glucose, asparagine, aspartate, threonine, arginine and serine were all depleted by 48 hours growth (Figure 3.3A).

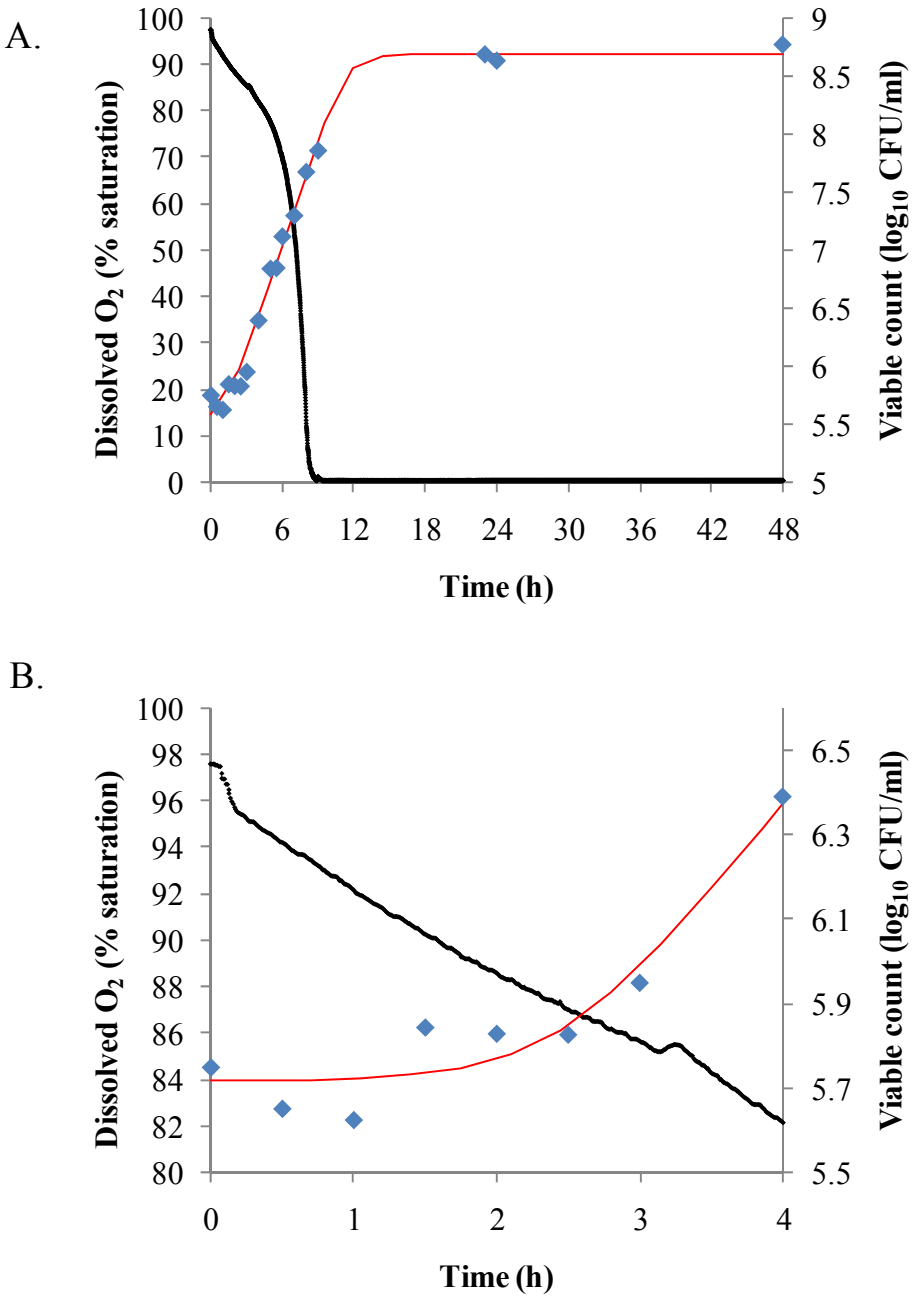


Figure 3.2: Oxygen is removed rapidly by *S.* Typhimurium in the static system. Oxygen consumption from the static system, as measured using an online oxygen probe (Section 2.1.10) over 48 hours (A) and for the first 4 hours of growth (B). The dissolved oxygen concentration is shown (♦) alongside the corresponding viable bacteria counts (♦). Data is representative of 3 independent replicates.

Conversely, bacterial metabolites were produced which were not detectable in LB or the earliest lag phase culture (4 minutes post-inoculation) such as ethanol, propionate, putrescine and cadaverine (Figure 3.3B). The most abundant by-product was acetate which accumulated at over 3.1 mM in the cell-free supernatants despite being present at concentrations of only 0.2 mM at 4 minutes. The low concentration of inhibitory waste products, such as acetate, in the earliest lag phase culture would allow the bacteria to rapidly resume cellular metabolism and exit from lag phase.

Amino acids, which represent the most abundant carbon source in LB (Sezonov *et al.*, 2007), were not metabolised equally by the bacterial culture after 48 hours of growth (Figure 3.4A). Arginine, asparagine, threonine and serine were all depleted to below the NMR detection limit of approximately 1 μM (Terri Grassby, personal communication) in the stationary phase culture. In contrast the stationary phase culture contained eleven amino acids (alanine, glycine, histidine, isoleucine, leucine, methionine, phenylalanine, proline, tryptophan, tyrosine and valine) which were all present at approximately the same concentration, or even greater, than the four minutes lag phase culture. Lysine, one of the most abundant amino acids in early lag phase culture supernatants (1.69 mM), was removed substantially from the medium by stationary phase (0.45 mM) although other amino acids present at greater than 1 mM concentrations at four minutes post-inoculation, such as alanine, leucine, phenylalanine and valine, were not consumed at all after 48 hours growth.

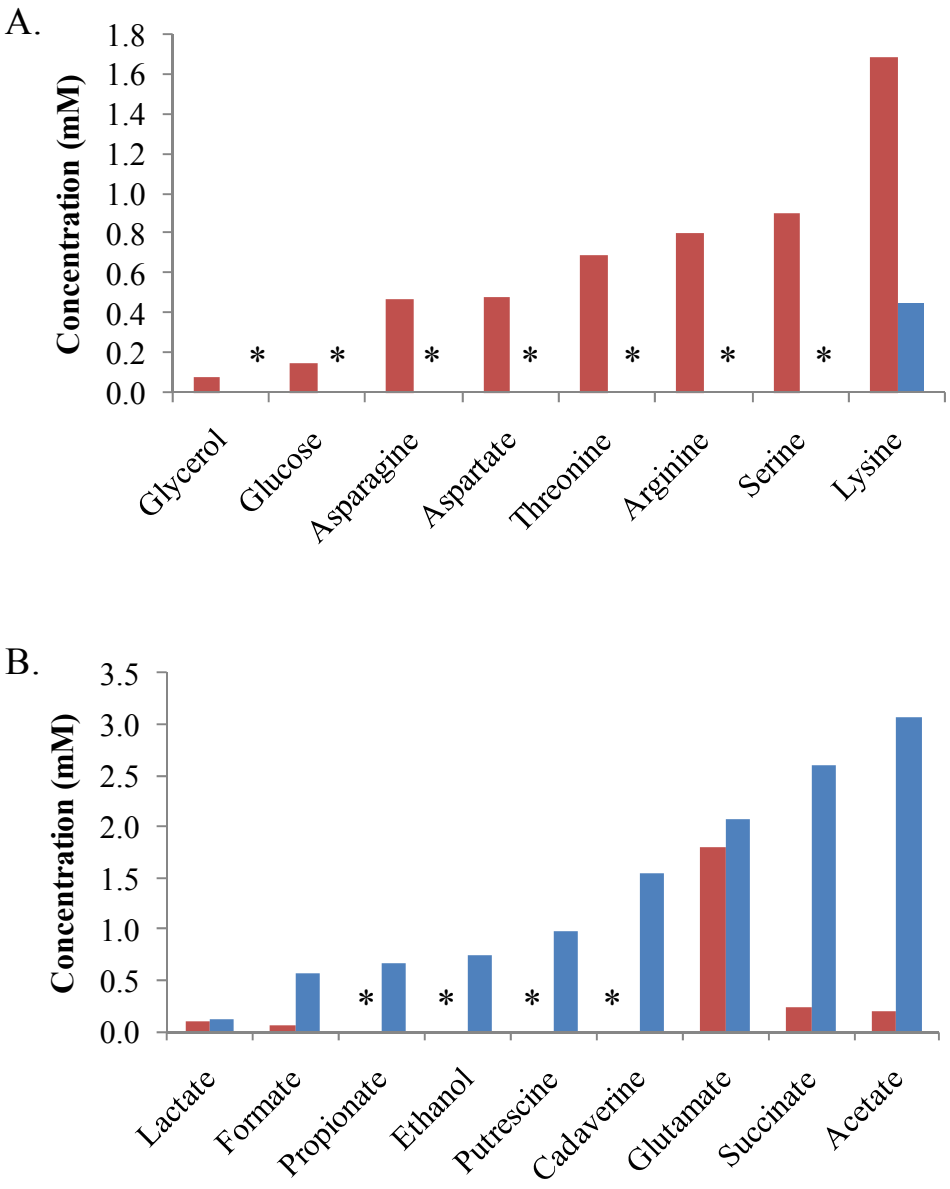


Figure 3.3: *S*. Typhimurium depletion of nutrients.
NMR analysis of a single experiment quantifying metabolites present in supernatants of either 48 hour stationary phase cultures (blue bars) compared with four minutes post-inoculation into fresh LB medium (red bars). The four minute culture is representative of all the lag phase and LB medium metabolomic data. (A) Metabolites present in a higher concentration at 4 minutes than 48 hours; (B) Metabolites present at a lower concentration at 4 minutes than 48 hours. Metabolites present at concentrations below the detection limit (1 µM) are indicated with an asterisk (*).

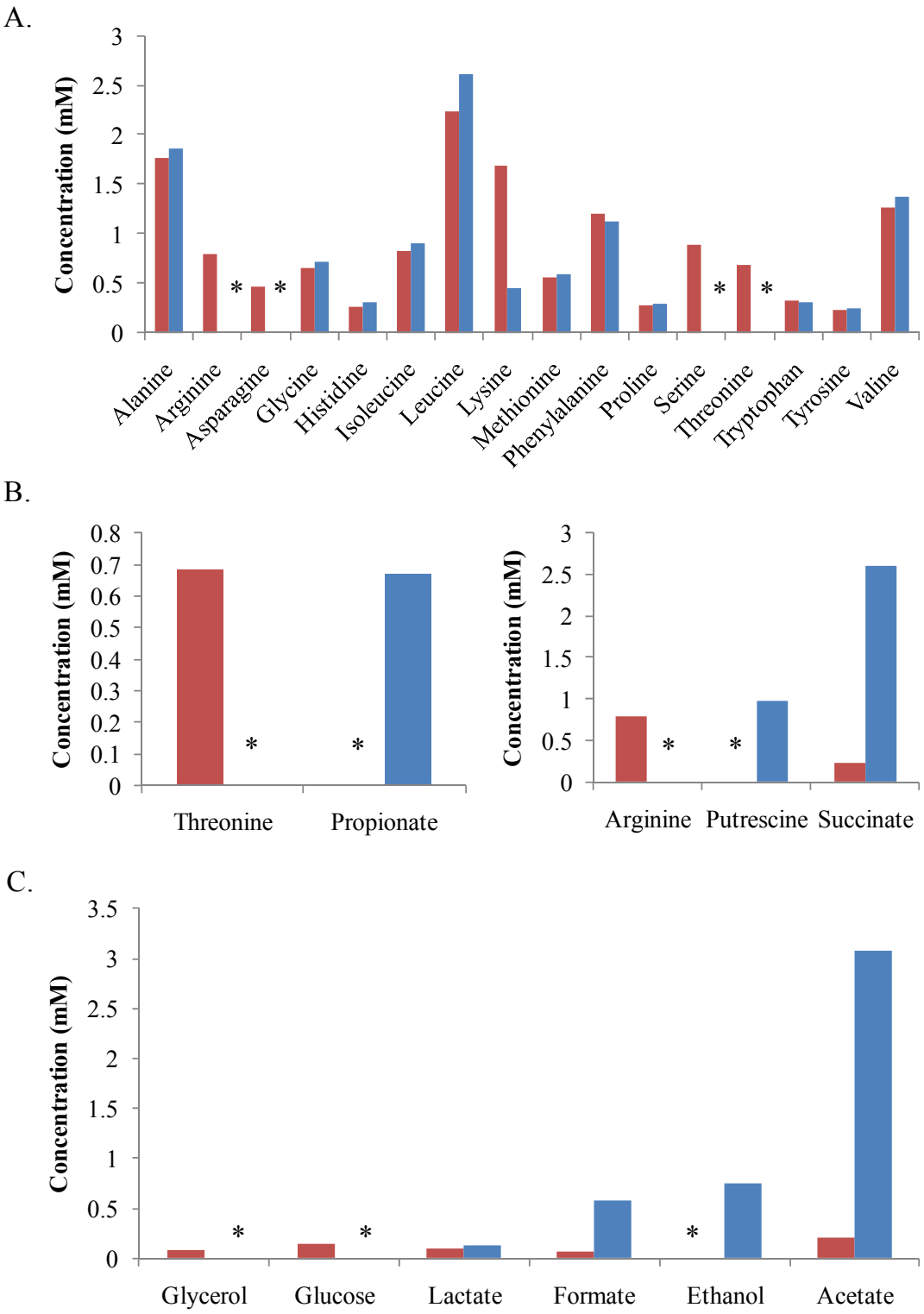


Figure 3.4: Stationary phase supernatants contain metabolic by-products.
NMR analysis of a single replicate quantifying carbon sources and products in supernatants of either 48 hour stationary phase cultures (blue bars) or four minutes post-inoculation into fresh LB medium (red bars). (A) Profile of the sixteen detectable amino acids. (B) Profile of two selected amino acids and their known metabolic products. (C) Profile of glycerol and glucose and their probable fermentation products. Metabolites present at concentrations below the detection limit (1 µM) are indicated with an asterisk (*).

Certain amino acids were at undetectable concentrations by 48 hours growth, leading to the accumulation of metabolic by-products. Threonine was present at a concentration of 0.69 mM at four minutes post-inoculation and was consumed completely after 48 hours of growth. Conversely, the fermentation product propionate was not present at detectable concentrations at four minutes, however was accumulated to 0.67 mM after 48 hours, suggesting that the threonine in the medium was converted to propionate in a 1:1 ratio. This pattern is similar for the amino acid arginine, which was present at 0.8 mM at four minutes post-inoculation but was consumed entirely by 48 hours growth, and was replaced by the by-products putrescine and succinate at concentrations of 1 mM and 2.6 mM, respectively (Figure 3.4B). *S.* Typhimurium utilised other carbon sources during growth in LB including glucose and glycerol which are present at low concentrations (149 µM and 76 µM, respectively) and are completely metabolised after 48 hours of growth (Figure 3.4C). The principal by-products of carbon source fermentation in LB medium were acetate, succinate, formate and ethanol, which all had accumulated substantially after 48 hours growth.

3.2.4 Characterisation of mutant strains

Many physiological processes are performed during lag phase in the static growth system and it is important to understand which are most important to *S.* Typhimurium adaptation. Some of these processes are explored in greater depth elsewhere in this thesis, including oxidative stress resistance (Chapter 6) and metal homeostasis (Chapter 7). By deleting crucial lag phase genes (Section 2.3.9) and determining whether the lag phase recovery of the mutant strains was perturbed, important processes during *S.* Typhimurium lag phase could be elucidated. In total, the growth parameters for four *S.* Typhimurium mutant strains were measured in the lag phase static system: Δ*bipA*, Δ*fis* Δ*bipA*, Δ*relA71* Δ*spoT281* (ppGpp^0), Δ*rmf* Δ*yfiA* Δ*yhbH* (Table 3.1).

The Δ*bipA* strain (JH3308) had been previously reported to have a significantly longer lag time (Rolfe, 2007), however during the present study this was not evident from the replicates tested (Figure 3.5A). In addition, no decreased growth rate was observed as reported previously for transposon insertion mutants grown at 20 °C (Pfennig & Flower, 2001). Fis is a transcriptional regulator produced maximally in early exponential phase *S.* Typhimurium (Ball *et al.*, 1992). A Δ*fis::cat* strain has previously been reported to have a

longer lag time (Osuna *et al.*, 1995) and interactions between BipA and *fis* mRNA transcripts have been reported (Owens *et al.*, 2004). To investigate whether the deletion of these two global regulators impacted upon the bacterial growth parameters, strain JH3440 (Δ*fis::cat* Δ*bipA::kan*) was tested in the lag phase system and was not affected for growth (Figure 3.5B and Table 3.1).

In addition, strains KT2160 (ppGpp^0) and JH3572 (Δ*rmf::cat* Δ*yfiA::spc* Δ*yhbH::kan*) did not show differences in growth parameters in the lag static system (Figures 3.5C and D, respectively). This result for KT2160 was contrary to published data which showed that a ppGpp^0 mutant had significantly increased lag time. The reason for this discrepancy is most likely due to the growth conditions used in the previous study, which were depleted for amino acids thus exacerbating the requirement for the ppGpp alarmone. The LB medium used in this study is rich in amino acids which are not depleted even by stationary phase in the lag static system (Figure 3.4A).

Strain	Mutation	Mutant		Wild-type	
		Lag (h)	Doubling time (h)	Lag (h)	Doubling time (h)
JH3308	Δ*bipA::kan*	1.78, 1.96	0.96, 0.97	2.31, 1.94	0.99, 0.98
JH3440	Δ*fis::cat* Δ*bipA::kan*	2.20, 1.82	0.99, 0.99	1.89, 2.45	0.97, 0.99
KT2160	Δ*relA71*::*kan* Δ*spoT281::cat*	2.03, 1.71	0.98, 0.99	2.71, 2.57	0.98, 0.98
JH3572	Δ*rmf::cat* Δ*yfiA::spc* Δ*yhbH::kan*	1.31, 1.84	0.98, 0.99	1.89, 2.45	0.97, 0.99

Table 3.1: Deletion of key processes does not affect growth.
Lag times and doubling times are shown for the mutant strains tested alongside the respective wild-type controls for two biological replicates shown separated by a comma (,).

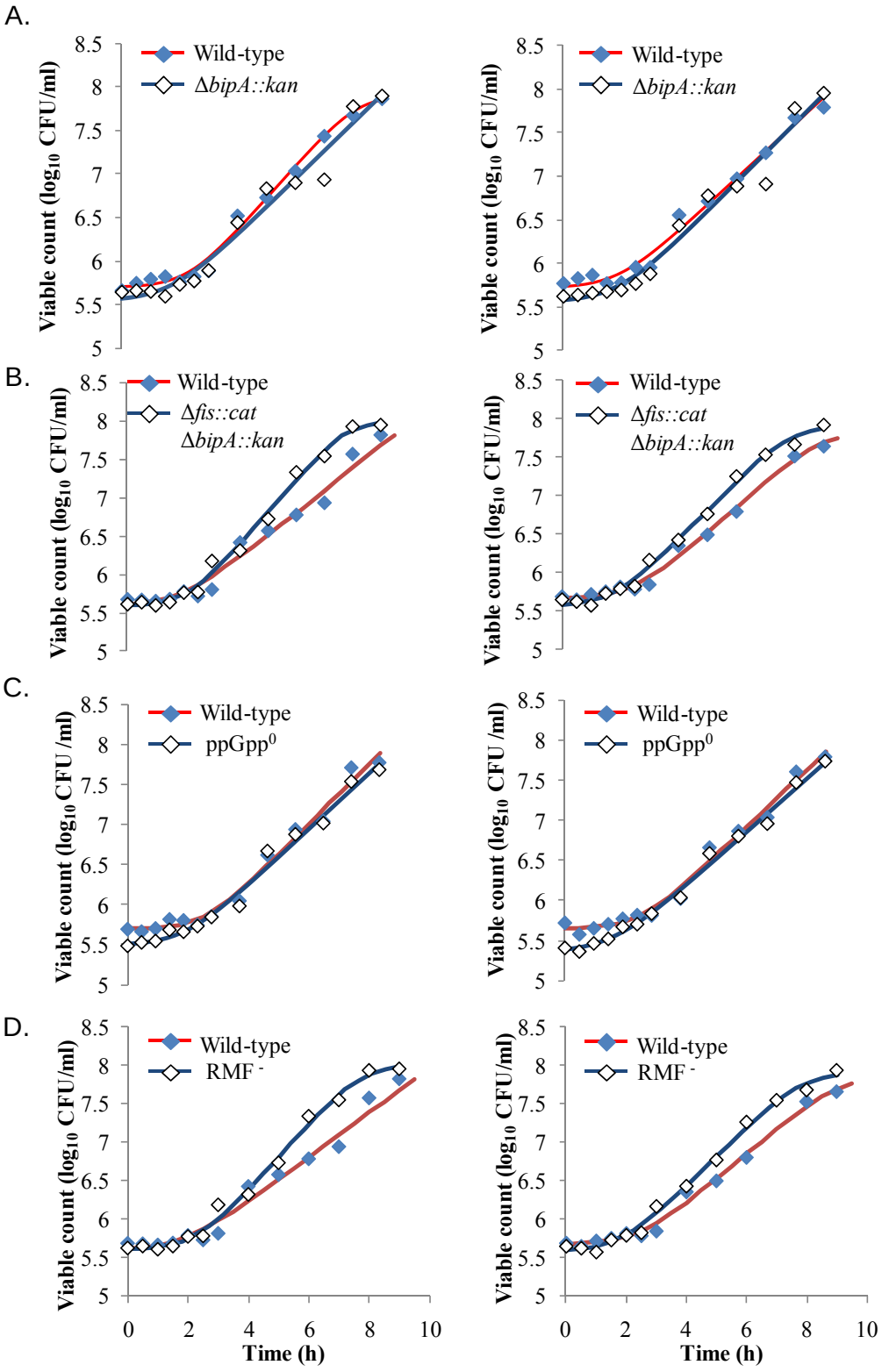


Figure 3.5: Functional overlap exists during bacterial lag phase.
Viable count growth curve analysis for two biological replicates of (A) JH3308 (Δ*bipA::kan*), (B) JH3440 (Δ*fis::cat* Δ*bipA::kan*), (C) KT2160 (ppGpp^0) and (D) JH3572 (RMF$^-$) compared with wild-type (SL1344). Diamonds represent viable count data-points and lines represent fitted curves using DMFit. Two biological replicates are shown for each mutant on the left and right panels.

3.2.5 Effects of stationary phase supernatants

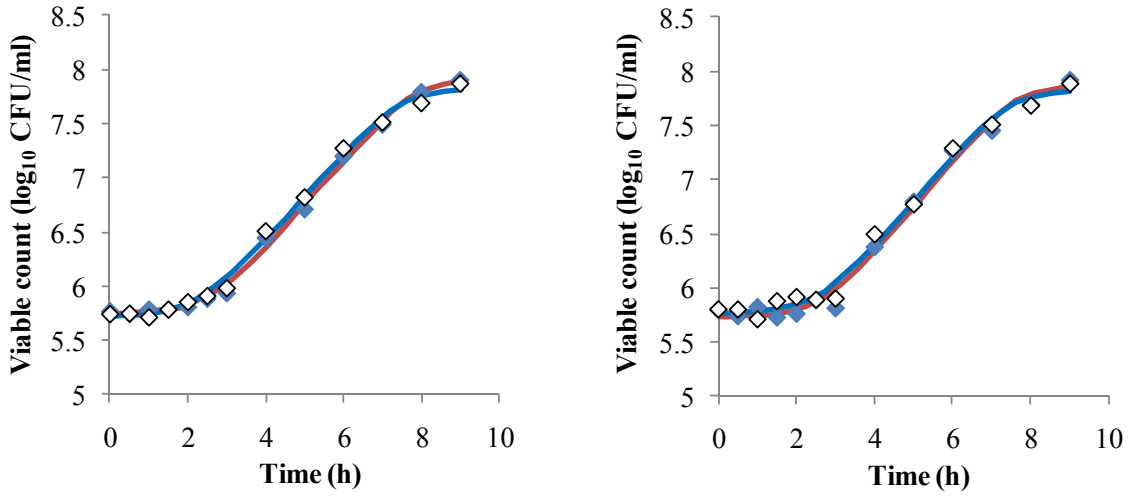
Lag times of *S.* Typhimurium could be dependent upon specific environmental factors, such as signal molecules or toxic metabolites, present in stationary phase supernatants. To test this hypothesis in the lag phase static system, growth was measured in conditioned medium (Section 2.1.3), as described previously (Weichart & Kell, 2001) and directly compared with bacterial growth in LB medium (Figure 3.6).

Examination of the growth curves showed very reproducible data (Figure 3.6 A) with no difference in the lag time or doubling time between the two conditions (Figure 3.6 B). These data suggest that there are no signalling molecules which have any great effect on growth parameters in the conditions tested. The lag times were similar whether the bacteria were grown in LB or conditioned media suggesting that any nutrients required for lag phase adaptation are present in both media and are readily utilised by the bacteria.

3.2.6 Effect of cold storage on survival and recovery of *S.* Typhimurium

To identify whether the physiological history of bacteria affects lag time or subsequent re-growth when inoculated into fresh medium, the standardised inoculum was stored at 2 °C for varying lengths of time (Section 2.1.6.1). The viability during storage and the lag time upon subsequent inoculation into fresh LB medium at 25 °C were both investigated (Figure 3.7). The viability of the bacteria gradually decreased over prolonged storage at 2 °C. Interestingly the variability between the replicates also increased as the length of storage increased. The lag times, although variable, did not increase over a period of one week storage at 2 °C. However the lag time increased after twelve days storage, from 2.17 hours without cold storage to 3.42 hours after twelve days incubation. To maintain food-relevant experimental conditions, the twelve day chilled sample was chosen for further investigation as it showed the longest lag time of the samples tested.

A.



B.

Growth curve	Lag time (h)	Doubling time (h)	Cell concentration (log_{10} CFU/ml)	
			Starting	Final
LB 1	2.54	1.00	5.75	7.92
Conditioned medium 1	2.24	1.00	5.72	7.83
LB 2	2.64	0.99	5.73	7.87
Conditioned medium 2	2.55	0.99	5.77	7.82

Figure 3.6: Conditioned medium does not affect *S.* Typhimurium growth.
Two biological replicates of viable count growth curves obtained from the lag phase static system. (A) Growth curves of experimental cultures inoculated into either LB (◆) or conditioned medium (◇). (B) Table of fitted growth parameters using DMFit for either LB or conditioned medium. For each condition the lag time, doubling time, starting and final cell concentration are shown

The growth parameters of the bacteria after twelve days cold incubation at 2 °C were investigated in parallel using both a high inoculum (10^5 CFU/ml) for nine hours, representing an infectious dose scenario (Kothary & Babu, 2001) (Figure 3.8A), and a low inoculum (10^3 CFU/ml) over eighteen hours representing a food microbiology modelling initial concentration (Buchanan & Cygnarowicz, 1990) (Figure 3.8C) to test the reproducibility of the lag time and to ensure that the doubling time was not affected.

After twelve days pre-incubation at 2 °C neither the lag time nor the logarithmic growth rate was altered compared with the non-chilled 25 °C culture (Figure 3.8). In each experiment, however, the starting cell concentration for the Lag Pre-Incubation samples was lower confirming the loss of viability during cold storage, as previously described (Figure 3.7). The effect of cold storage at 2 °C for twelve days on recovery was analysed at the transcriptomic level elsewhere in this thesis (Chapter 4).

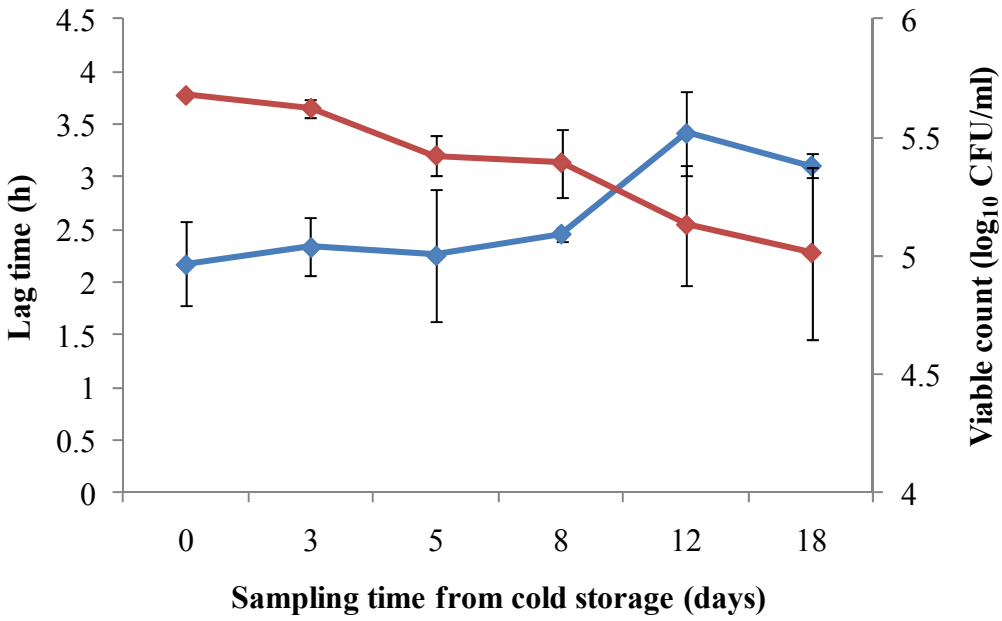
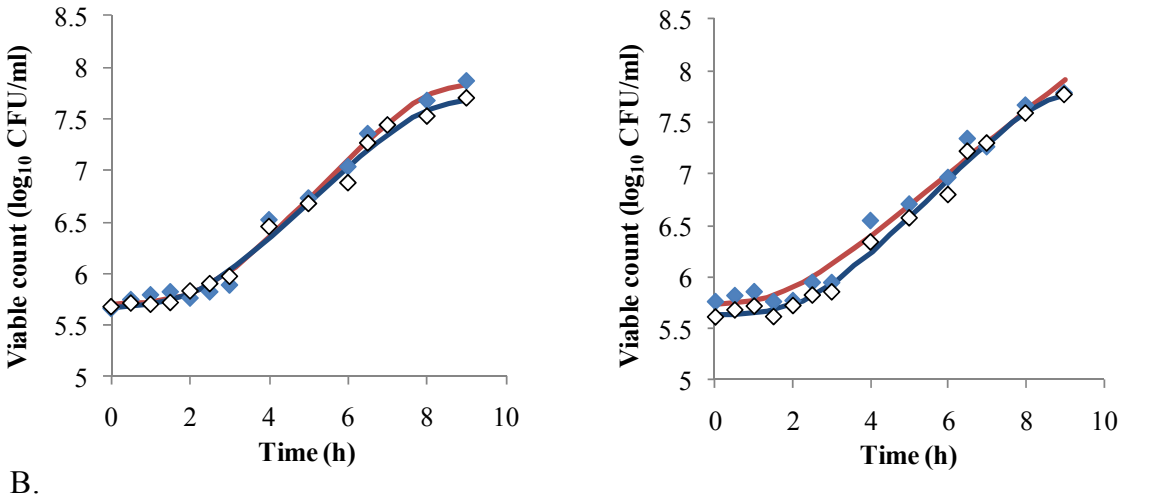
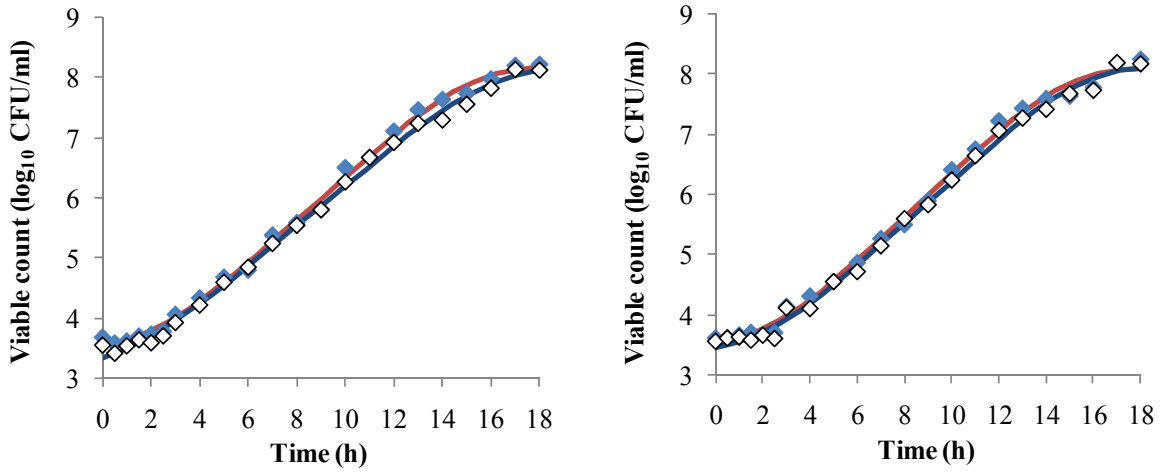


Figure 3.7: Periods of cold storage cause a loss of viability but do not increase lag time after inoculation.
Two biological replicates showing stationary phase survival during cold storage at 2 °C, calculated from viable counts (red line) and the lag times upon re-inoculation into LB medium pre-warmed to 25 °C (blue line). Error bars represent the standard deviation from the mean. None of the periods of cold storage tested resulted in an increased lag time.

A.



B.



C.

Growth curve	Lag time (h)	Doubling time (h)	Cell concentration (log_{10} CFU/ml)	
			Starting	Final
25 °C A (high)	2.35	0.99	5.71	7.87
Pre-incubated A (high)	2.11	0.99	5.67	7.71
25 °C B (high)	1.86	0.98	5.73	7.79
Pre-incubated B (high)	2.41	0.99	5.64	7.85
25 °C A (low)	2.28	1.00	3.54	8.23
Pre-incubated A (low)	1.52	1.00	3.35	8.22
25 °C B (low)	2.44	1.00	3.55	8.14
Pre-incubated B (low)	2.31	1.00	3.47	8.17

Figure 3.8: Cold pre-incubation does not affect *S.* Typhimurium growth parameters in the static system.
Two biological replicates of viable count growth curves obtained from the lag phase static system. (A) High inoculum (10^5 CFU/ml) growth curves. (B) Low inoculum growth curves (10^3 CFU/ml). All growth conditions were as per the lag static system with pre-incubation at 2 °C (◇) or without pre-incubation (◆). (C) Table of fitted growth parameters using DMFit for both growth conditions. For each condition the lag time, doubling time, starting and final cell concentration are shown.

3.2.7 Morphology of cells during cold storage

S. Typhimurium cells in stressful environments, such as intracellular replication inside a macrophage, are prone to filamentation (Justice *et al.*, 2008). This phenomenon has also been identified during cold storage at temperatures between 3 °C and 8 °C in *Salmonella* and pathogenic *E. coli* growth in food matrices (Mattick *et al.*, 2003). The work described in Section 3.2.6 was performed at 2 °C and it was important to verify that no filamentation occurred.

Cells were visualised using transmission electron microscopy (TEM) as described (Section 2.1.10), to compare stationary phase cells after either 48 hours growth at 25 °C or after additional twelve days storage at 2 °C (Figure 3.9). In general, bacteria in cold storage were slightly elongated. Of twenty randomly-sampled images, bacteria in cold storage measured 2.09 μm (standard deviation of 0.77) compared with an average length of 1.58 μm for bacteria grown at 25 °C for 48 hours (standard deviation of 0.36). This increase in length was significant (*t*-test p=0.01) and may explain some of the inconsistencies observed in lag times during recovery after cold storage (Figure 3.8), but does not represent filamentation, defined as approximately the length of four average cells or 8 μm (Mattick *et al*., 2003).

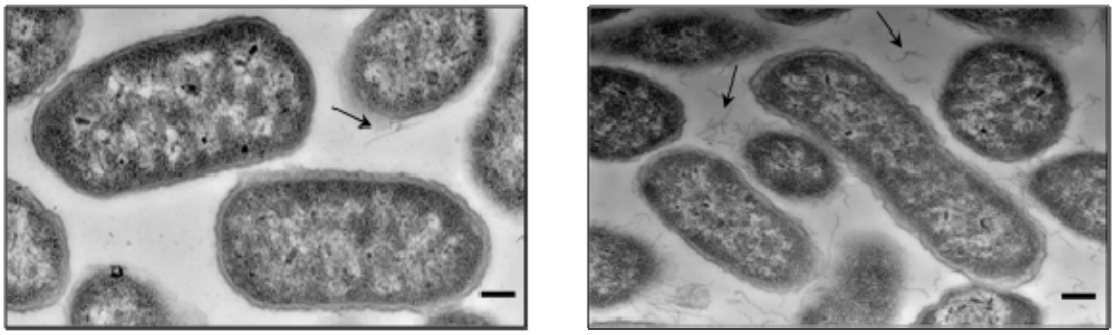


Figure 3.9: Cold storage at 2 °C does not produce filamentous bacteria. TEM of a single replicate of stationary phase *S.* Typhimurium stored either 25 °C, 48 hours (left) or with an additional twelve days cold storage at 2 °C (right). The horizontal scale bar represents 200 nm and the black arrow indicate possible detached flagella (approximately 14 nm in width). Electron microscopy was performed by Katherine Cross and Mary Parker at IFR, Norwich.

3.3 Discussion

3.3.1 Growth at 25 °C in the lag phase static system

The static system designed to study growth parameters of *Salmonella* was used to measure the growth of *S.* Typhimurium at 25 °C. The experimental system uses a relatively high starting cell concentration (~5.5 x 10^5 CFU/ml). This inoculum was a compromise between achieving a sustained period of exponential growth, which would usually require a low inoculum of 10^2-10^3 CFU/ml, and obtaining enough cells for molecular biology techniques (Rolfe, 2007). In order to verify the reproducibility of the lag phase system, growth curves were performed using both the high initial starting concentration and also a 100-fold lower starting concentration over an 18 hour time-course. The mean lag time for the higher inoculum was 2.24 hours and for the lower inoculum was 1.72 hours compared with an average of 1.91 hours for a previous study using the static growth system with an initial high starting concentration of 5 x 10^5 CFU/ml (Rolfe, 2007). The discrepancies between the two studies were most likely due to the inconsistencies in the measurement of the cell concentration, leading to high coefficients of variation in calculated lag times. The biological variation is primarily caused by a distribution of individual cell lag times during the transition from lag to exponential growth phases (Swinnen *et al.*, 2004). Within a bacterial population there is a great deal of heterogeneity, enabling a proportion of the population to resist potentially lethal stresses (Epstein, 2009, Wolf *et al.*, 2005) and to increase the productivity of the entire population (Anetzberger *et al.*, 2009). Population heterogeneity is likely to contribute largely to the biological variation observed in this study.

It is difficult to compare the lag times in this static system to other published data, as lag duration is highly dependent upon the organism used, the physiology, and the growth conditions (Oscar, 1999a). For example, a previous study on the growth of *S.* Typhimurium LT2 in agitated rich liquid medium, TSBYG (trypticase soy broth, yeast extract and glucose) at 20 °C, pH 7.0 showed a lag time of 4.2 hours (Wilson *et al.*, 2003). This was considerably longer than the lag time described in the present study, most likely due to the lower growth temperature of 20 °C.

Interestingly, although the growth history of the bacteria is relevant to the physiology in new conditions, previous growth conditions may not have a large effect on the subsequent doubling time of that organism (Gorris & Peck, 1998, Wolf *et al.*, 2008) unless the conditions are drastically different or damaging. The effect of the physiological history on subsequent lag times may vary between organisms (József Baranyi, personal communication), and the previous growth environment remains an interesting, if understudied, part of bacterial lag phase physiology.

3.3.2 Oxygen utilisation in the lag phase system

The dissolved oxygen concentration was monitored throughout bacterial growth in the static lag phase system to determine whether the availability of oxygen correlated with the growth rate and if oxygen was depleted from the LB medium during lag phase. The most interesting observation in the lag phase static system was that dissolved oxygen began to be removed within 4 minutes of inoculation and 11.5 % of oxygen was depleted by two hours post-inoculation (representing the lag duration). The fact that oxygen was not removed immediately suggests that inoculation of bacteria from the anoxic stationary phase culture has no discernible effect on the dissolved oxygen concentration in the fresh LB medium.

It was interesting to observe detectable oxygen removal from the medium during early lag phase, although the reason was not determined. Previous studies have shown that exposure of *E. coli* to aerobic environments induces the production of enzymes essential for aerobic respiration (Iuchi & Weiner, 1996), such as CydA and CydB cytochromes (Partridge *et al.*, 2007) which utilise oxygen to generate energy for growth. Alternatively, the dissolved oxygen may be utilised in the oxidation of metalloproteins, for example the iron-sulphur clusters of oxidative stress sensors, such as OxyR and SoxR (Jang & Imlay, 2007, Pomposiello & Demple, 2001). The presence of >88.5 % dissolved oxygen throughout lag phase may be sufficient to elicit an oxidative stress response, a theory which is investigated elsewhere in this thesis (Section 6.2.1).

The most rapid removal of dissolved oxygen occurred, as expected, during exponential growth. After eight hours growth (mid-exponential phase) 84.6% of the dissolved oxygen

had been depleted from the LB medium and the oxygen concentration was below the detection limit by 9.4 hours.

The observations made on molecular oxygen consumption fits relatively well with a previous study of *S.* Typhimurium growth in rich complex medium at pH 7.0 (Wilson *et al.*, 2003). In the previous study, the authors observed that the rate of oxygen removal occurs maximally during exponential phase, leading to a depletion of dissolved oxygen by late exponential phase of growth. The authors conclude that although the recorded dissolved oxygen concentration is zero, the continued bacterial demand for oxygen points to possible mixed aerobic and anaerobic respiration that is limited by the oxygen transfer kinetics of the system. Calculations of the oxygen availability revealed that 1.1 $x10^{-16}$ mols of oxygen were available per bacterial cell during exponential growth in rich medium, pH 7.0 (Wilson *et al.*, 2003). The authors did not note any decrease in oxygen availability during the 4.2 hour lag phase, possibly because measurements were only performed hourly, compared with every thirty seconds in the present study.

The present study uses a lag phase system to study oxygen consumption throughout growth and is the first to demonstrate that *S.* Typhimurium utilises oxygen during lag phase. The role of oxygen utilisation during the early stages of growth may impact significantly upon lag time and the mechanism of this oxygen use should be elucidated in any future work.

3.3.3 Bacterial carbon metabolism throughout growth

A metabolomic approach was used to study the differences in carbon availability in stationary phase cultures compared with lag phase cultures in the static system. Various carbon sources were depleted from the LB medium by 48 hours growth and a build-up of certain metabolic by-products was detected. These results compare well with published transcriptomic data describing the depletion of amino acids by *E. coli* in LB medium throughout growth (Baev *et al.*, 2006b) and the rapid depletion of sugars, available in low concentrations (Baev *et al.*, 2006c, Sezonov *et al.*, 2007). LB medium is a rich source of amino acids for bacteria and *S.* Typhimurium utilises amino acids in a preferential order, with some amino acids not depleted at all after 48 hours growth. A previous, transcriptomic approach to study amino acid utilisation in LB medium by *E. coli* suggests that serine and glycine are removed rapidly and biosynthesis of these amino acids occurs

during exponential growth (Baev *et al.*, 2006b). Whilst the *S.* Typhimurium data here agree with the previous study with respect to serine; glycine metabolism is not evident from the metabolomic approach.

The amino acids threonine and arginine in LB medium were depleted by 48 hours and an accumulation of propionate and succinate was observed. This correlates well with a previous study which reports the conversion of one molecule of threonine to one molecule of propionate by *S.* Typhimurium (Simanshu *et al.*, 2007) and arginine to succinate by *Helicobacter pylori* (Mendz & Hazell, 1995). The concentration of putrescine produced is similar to the concentration of arginine utilised suggesting that all of the arginine may have been converted to putrescine. In contrast, the final concentration of succinate is considerably higher than the inital arginine concentration, suggesting that other metabolic routes are responsible for the production of succinate, possibly through the fermentation of sugars.

The depletion of glucose and glycerol from LB medium by 48 hours coincided with the accumulation of the known fermentation products lactic acid, formic acid, ethanol and most abundantly, acetic acid (Figure 3.4C). These data compare well with known production of acetate by *Salmonella enterica* growing exponentially on ethanolamine-containing minimal media (Starai *et al.*, 2005). The accumulation of fermentation products such as acetic acid would decrease the pH of the LB medium significantly leading to a cessation of growth (Wilson *et al.*, 2003), however deamination of certain metabolised amino acids subsequently raises the pH, as was reported for *E. coli* grown in tryptone broth (Prüß *et al.*, 1994). It has been shown previously that *S.* Typhimurium grown in the lag static system causes the pH of the culture to decrease from 7.0 to 6.0 within the first 24 hours of growth. Subsequently, deamination of abundant amino acids raises the pH to 6.4 by 48 hours growth (Rolfe, 2007). This change in pH is gradual therefore minimal pH adaptation would be necessary in the static system, unlike previous published observations in other conditions (Sampathkumar *et al.*, 2004). The sequential metabolism of different carbon sources by *S.* Typhimurium in the static growth system reveals a preference for certain substrates and metabolic pathways. This mode of substrate utilisation requires limits the transporters and metabolic pathways required thereby reducing the energy output of the bacterium during this critical stage of growth. Once the preferred substrates have

been utilised, the bacteria perform a metabolic shift to make use of the remaining nutrients. Future work could investigate whether in conditions limited for a particular nutrient the bacteria are less efficient at generating energy and whether this leads to an increased lag time.

3.3.4 Growth parameters of mutant strains in the lag phase static system

Genes controlling important lag phase processes were investigated by mutagenesis. Previous work using the lag phase static system studied the role of 28 gene knock-out mutants including genes involved in oxidative stress protection, phosphate uptake and global regulators, as well as some lag phase-induced genes. In total only one mutant strain showed an increased lag time phenotype, Δ*bipA::kan*, with gene redundancy cited as a likely reason for the robustness of lag phase (Rolfe, 2007).

In the present study, fewer mutants were investigated, with selection of gene targets based on increased lag times observed in previous studies. The Δ*bipA::kan* mutant was combined with a Δ*fis::cat* mutant which had been reported to have a longer lag phase by optical density growth measurements (Osuna *et al.*, 1995) but was subsequently shown not to be affected for growth in the lag phase static system (Rolfe, 2007). In addition, a Δ*relA71::kan* Δ*spoT281::cat* (ppGpp^0) mutant was investigated which was reported to have a longer lag time in amino acid limited conditions (Traxler *et al.*, 2008), and a Δ*rmf::cat* Δ*yfiA::spc* Δ*yhbH::kan* mutant was constructed which was hypothesised to lead to a build-up of damaged 70S ribosomes, requiring repair or degradation and thereby extending the bacterial lag time. The growth parameters of these mutants were tested in the lag phase system and none were shown to be altered for growth compared with a wild-type bacterial culture. These findings were particularly striking for the Δ*bipA::kan* and Δ*bipA::kan* Δ*fis::cat* mutants, which were expected to show extended lag times based on previous work (Rolfe, 2007). The exponential growth rates were not different between the mutant strains and the wild-type in all of the samples with the most variable being the Δ*bipA::kan* Δ*fis::cat* first replicate. The second Δ*bipA::kan* Δ*fis::cat* replicate lag time was different compared with the wild-type however this was not reproducible in the first replicate.

There have been no comparable published studies using a Δ*bipA::kan* Δ*fis::cat* mutant or a Δ*rmf::cat* Δ*yfiA::spc* Δ*yhbH::kan* mutant in *Salmonella*. Previous studies into ppGpp-deficient bacteria have concentrated on optical density measurements in amino acid starved conditions. In the present study, the viable count-based method allowed a more accurate assessment of lag time and growth rate.

The lack of growth defects in any of the mutants tested across the replicates in this study suggests that there is a high degree of functional overlap between important bacterial processes and highlights the degree of variability in lag times between bacterial populations.

3.3.5 Effect of conditioned medium on growth parameters

The addition of 30% (v/v) cell-free, stationary phase supernatants to the LB growth medium did not affect the growth parameters of the bacteria in the lag phase static system. This result differs from previous studies in *E. coli* which showed the addition of stationary phase supernatants led to a decrease in lag times by 22-57%, due to an uncharacterised signalling molecule (Weichart & Kell, 2001). Discrepancies between the previous study and the present one are most likely due to the differences in growth medium used. Previous work used MOPS-buffered minimal medium (MM) supplemented with glucose or succinate; the former carbon source having previously been shown to be required for induction of quorum sensing systems (Surette & Bassler, 1998). The second important difference was the physiological history of the bacteria used between both studies. In the previous study, the *E. coli* were starved of carbon sources for 53 days prior to inoculation into fresh, supplemented medium, giving rise to lag times of between 16 and 20 hours. During storage, the bacteria depleted the medium of nutrients and may have acquired mutations under the stressful conditions. The relatively long lag time facilitated the observation of lag differences and shortened lag times were easier to measure by Bioscreen C OD_{590} measurements (Weichart & Kell, 2001). In the present study, the growth medium was not completely depleted for nutrients, which may mask a starvation-induced environmental trigger for the production of a lag phase-altering signal molecule (Siegele & Guynn, 1996).

Although the conditioned medium did not alter bacterial growth parameters using the lag phase static system, a previous study has shown that stationary phase culture supernatants (spent media) give rise to a longer lag phase but still supports full growth of *S.* Typhimurium to over 10^8 CFU / ml (Rolfe, 2007). The Rolfe (2007) findings are consistent with other work which showed re-growth of *E. coli* grown at 37 °C at least twice in spent medium to a final cell concentration of over 10^9 CFU / ml (Barrow *et al.*, 1996). Factors leading to an increased lag time are yet to be fully elucidated; however it has been hypothesised that a build-up of the toxic metabolite acetate may be a contributing factor requiring uptake and catabolism during lag phase before growth can begin (Rolfe, 2007), confirmed in the present study (Figure 3.3B).

From experiments performed in the present study, it is hypothesised that any signal molecule or any toxic metabolite was not present in lag phase cultures at a concentration inhibitory for growth. The bacterial culture grown in conditioned medium did not enter stationary phase earlier than the culture grown in LB medium, and both cultures reached the same final cell concentration. If an inhibitory metabolic by-product was present at a physiologically relevant concentration in the conditioned medium, the culture would be expected to enter stationary phase earlier than the LB grown culture once the dissolved oxygen had been depleted.

The role of stationary phase supernatants on *Salmonella* lag time has still to be elucidated and the presence of a quorum sensing signal molecule has not been confirmed in the present study. In future studies, the use of highly sensitive techniques such as mass spectrometry may be required to characterise any signal molecules in culture supernatants, although an exhaustive study to determine such a molecule in LB grown *E. coli* cultures was not conclusive (Weichart & Kell, 2001).

3.3.6 Effect of cold storage on bacterial recovery

In order to test the robustness of lag phase, the static growth system was perturbed with a cold storage step performed at 2 °C. This temperature stress is relevant for chilled food held by producers and some retailers (Peck *et al.*, 2008) and prevents bacterial filamentation, which has been reported to increase the variability of viable cell counts after subsequent temperature upshift (Mattick *et al.*, 2003). The survival of *Salmonella* at cold

temperatures depends upon nutrient availability (Angelotti *et al.*, 1961) and it was important to characterise both the viability and the effect upon growth after temperature upshift in fresh, pre-warmed LB medium. To this end, the lag static system was adapted (Section 2.1.6.1) to measure lag times at intervals over 18 days, representing an extended period of storage for a chilled product (Peck *et al.*, 2008). The viability of the cold stored bacteria was also measured to ensure the initial cell concentration did not impact upon the subsequent re-growth. Over the 18 day cold storage period, the lag time did not increase compared with the non-chilled stationary phase inoculum. The viability gradually decreased during cold storage and by day 18, the cell concentration was at 33% of the non-chilled inoculum. However, the variability between the samples also increased as storage time was lengthened and so the cell concentration by day 18 was not decreased compared with the non-chilled inoculum. The majority of previous studies measuring the viability of *Salmonella* during cold storage have involved non-defined media such as complex food sources (Angelotti *et al.*, 1961, Baker *et al.*, 1986, Bautista *et al.*, 1998, Borneman *et al.*, 2009, Holliday & Beuchat, 2003, Lublin & Sela, 2008) making direct comparisons with this work difficult. Viability of *Salmonella* at 0 ^{o}C in high salt (26 %) medium (pH 6.9) has been measured previously. After 19 days cold storage a 2-log decrease in viability was observed (Combase, http://browser.combase.cc/BrowserHome.aspx ; Record ID: B407_40) suggesting that very low temperature storage can have a large impact on the survival of *Salmonella*.

The morphology of bacterial cells during cold storage was visualised by TEM. This showed that bacterial cells are elongated following pre-incubation at 2 °C however they are not long enough to be characterised as filamentous (>8 µm) (Mattick *et al.*, 2003). It can be concluded that any observed differences in lag time are due to normal biological variation and associated uncertainties in growth measurements. In addition, neither significant bacterial cell lysis, formation of ice crystals nor loss in membrane integrity was observed to account for the decrease in bacterial viability during cold storage.

In the static system, stationary phase bacteria settled to the bottom of the flasks by twelve days at both 25 °C and 2 °C (unpublished observations). Visualisation of 48 hour, 25 °C and 12 day, 2 °C stationary phase cultures revealed narrow tubules (~14 nm in width) proximal to bacterial cells. These structures were hypothesised to be flagella which are

known to be approximately 15 nm wide (Kamiya *et al.*, 1982) and have been reported to become disassociated during stationary phase (Makinoshima *et al.*, 2003). This may result in a loss of bacterial motility that contributes to the settling of the culture.

Inoculation from cultures stored at 2 °C for up to eight days, resulted in a similar lag time (standard deviation = 5.33% of mean lag time). Additional cold storage for twelve and eighteen days resulted in a gradual increase in lag time. The maximum lag time was recorded after twelve days cold storage; however the lag time was not increased compared with inoculation with the non-chilled 25 ^{o}C culture, due to variability between replicates. Subsequent side-by-side comparisons with 25 ^{o}C inoculated growth curves with both a high (10^5 CFU/ml) and low (10^3 CFU/ml) inoculation revealed very similar growth profiles. Taken together, the food-relevant cold storage of *S.* Typhimurium did not alter any of the observed growth parameters upon subsequent inoculation into 25 ^{o}C LB medium. This observation was different to a previous study using similar growth conditions; 25 ^{o}C, statically-grown *E. coli* cultures in LB medium (Pin & Baranyi, 2008). In the previous study, cultures grown for 48 hours were inoculated into fresh LB medium supplemented with glucose, resulting in lag time of 1.5-1.8 hours, comparable with the present study. However *E. coli* cultures stored for 17 days at 25 ^{o}C had considerably longer lag times once inoculated into the fresh medium (4.7-5.1 hours). It is possible that in the Pin *et al.* (2008) study, long-term storage at 25 ^{o}C resulted in accumulated cellular damage from degrading enzymes, which would have had a lower activity during the cold storage treatment used in the present study. If *E. coli* experienced extensive cellular damage during 25 ^{o}C storage, then repair mechanisms or *de novo* synthesis of cellular machinery would be required before cellular division could be initiated, resulting in the longer lag time observed in the previous study.

The lag phase of *S.* Typhimurium in the static growth system is discussed in greater detail elsewhere in this thesis (Chapter 4), however initial observations of the growth parameters suggest that lag phase is very robust and adaptation to relatively minor stresses is performed rapidly, leading to swift resumption of cell division.

Chapter 4

The effect of cold pre-incubation on global gene expression

4. The effect of cold pre-incubation on global gene expression

In Chapter 3, the growth parameters of *S.* Typhimurium in the static system were investigated and the effect of perturbing the system with a food-relevant stress (cold storage) was determined. In this chapter, the physiological processes which underpin growth restoration after cold storage are investigated to understand how the bacteria adapt so rapidly during lag phase. A transcriptomic approach was used to investigate the global gene expression profile during recovery in the lag phase static growth system. The data were compared with a previously-compiled lag phase transcriptomic dataset for *S.* Typhimurium grown at 25 °C in the static system (Section 2.1.6) to formulate hypotheses, helping explain the robustness of lag phase.

4.1 Introduction

4.1.1 DNA microarrays

DNA microarray technology allows the simultaneous measurement of the expression of thousands of genes. Since the development of DNA microarrays (Schena *et al.*, 1995, Shalon *et al.*, 1996), the technique has been used extensively to measure the relative gene expression of *Salmonella* in various environments, answering physiological questions including: the effect of infection-relevant stresses on bacteria (Gantois *et al.*, 2006, Greenacre *et al.*, 2003, Karavolos *et al.*, 2008, Mills *et al.*, 2008), the role of global regulators (Kelly *et al.*, 2004, Lucchini *et al.*, 2006, Mangan *et al.*, 2006, Ono *et al.*, 2005, Thompson *et al.*, 2006a); the function of small RNAs (Papenfort *et al.*, 2008, Papenfort *et al.*, 2006, Pfeiffer *et al.*, 2009) and molecular virulence determinants (Balbontin *et al.*, 2006, Bowden *et al.*, 2009, Clements *et al.*, 2002, Eriksson *et al.*, 2003, Hautefort *et al.*, 2008, Nagy *et al.*, 2006)

Microarrays are used to measure the relative bacterial gene expression in different environmental conditions by quantifying changes in the amounts of mRNA transcripts extracted from populations of cells. The microarrays consist of glass slides printed with PCR products that correspond to the sequence of a specific gene (Thompson *et al.*, 2001, Watson *et al.*, 1998). The extracted RNA is reverse transcribed to make more stable cDNA, which is labelled with a fluorophore.

Transcriptomic experiments are inherently comparative, identifying a relative signal between two or more conditions. It has been noted that there is greater variability between slides than within a slide, due to differences that include: microarray printing variation, slide background and differing amounts of sample hybridisation. To minimise these variations between samples, direct analyses on the same slides are performed through either type I or type II experiments (Yang & Speed, 2002). Type I experiments involve the direct comparison of fluorescently-labelled mRNA or cDNA from two different conditions on a single microarray. These experiments are useful when analysing a test sample against a suitable control, as they use only a single microarray and quickly show differences between conditions. The disadvantage lies in the need for dye-swap experiments to compensate for differences in the fluorophore intensities which can lead to misleading signals. In type II experiments, fluorescently-labelled cDNA is competitively-hybridised to the microarray against a common reference (DeRisi *et al.*, 1996), for example genomic DNA or pooled cDNA, labelled with a different fluorophore. Type II experiments are the easiest way of identifying differences between many biological samples. By using a common reference DNA, differences in mRNA signal between conditions can be identified indirectly. This is particularly useful for multiple samples such as time-course data where direct analyses between pairs of arrays can be time-consuming. Type II microarray experiments remove the need for dye-swap experiments to account for differences in fluorophore incorporation (Yang & Speed, 2002). Additionally, the type II approach allow many different experimental conditions to be compared, which is particularly useful for the construction of compendia of transcriptomic data (Thompson *et al.*, 2006b).

4.1.2 Analysis of transcriptomic data

DNA microarrays generate large transcriptomic datasets which require analysis with dedicated software. To scan the microarrays, software such as Mapix® (Innopsys) or GenePix® (Molecular Devices) is used to control the scanner and produce to multi-image TIFF files that contain the signal intensity information for both fluorophores. The next step involves image segmentation, which consists of feature detection. This is then followed by the quantification of the signal corresponding to the identified spots of hybridised DNA. Image segmentation and spot-signal quantification are performed using dedicated software such as GenePix or BlueFuse (BlueGnome). Both forms of software essentially measure

the ratio of signal intensities from the TIFF file however they work in slightly different ways. GenePix analyses the median pixel intensity and the background signal of each spot to calculate a signal up to a maximum threshold. Where features are present with no hybridised product, GenePix denotes the feature as 'not found' and does not quantify the feature. In contrast, BlueFuse assigns values to all gene features even if no product is hybridised. The varying signal intensities across a feature are calculated by BlueFuse using a Bayesian distribution, allowing for values to be extrapolated for signals above the maximum threshold, which represents an advantage over the GenePix-based approach, assuming there is an even intensity distribution across each spot. Other important differences relate to the varying amounts of user interaction at the analysis stage between the two software approaches. GenePix allows greater user flexibility with easy modification of the scan area, down to the individual spot to allow data acquisition from poorly visible features. GenePix also allows individual features to be manually optimised for quantification to ensure the background of each printed spot is minimised or, in the case of poor features, removed entirely. The disadvantage of this system is that handling several microarray images in this way can be time-consuming and the degree of user interaction can bring an element of bias into the data analysis. It is however possible to run GenePix with minimal user input. With the BlueFuse analysis approach, user input is limited to loading the scanned image files and aligning grids for spot analysis, thereby reducing flexibility, but maintaining a consistent and objective analysis between arrays.

The next step of analysis requires median feature intensity data centring within the microarray for each sample. This is performed to compensate for differences between individual RNA molecules (which will have a range of lengths), varying degrees of cDNA labelling or different detection efficiencies between the fluorophores used (Quackenbush, 2002). Although various data centring approaches exist (Yang *et al.*, 2002), the most widely used is the locally weighted linear regression (lowess) analysis (Cleveland, 1979). Lowess data centring has the advantage of removing signal intensity-dependent effects by weighting outlying signal ratios less than those near to the average signal, for each feature (Quackenbush, 2002).

An additional step can be performed after signal quantification by GenePix or BlueFuse if the signal for low expressed genes appears to be 'compressed' when visualised, due to a

decreased distribution of the signal (Alston *et al.*, 2010). Signal compression usually arises from a two-step labelling process (Section 2.3.10.1) or with bacterial RNA isolated from mammalian environments as described previously (Eriksson *et al.*, 2003), which contain contaminants such as DNA, proteins or carbohydrates, and causes poorer incorporation of the fluorophore (Grissom *et al.*, 2005). The two-step labelling (or 'cold labelling') process involves the reverse transcription of extracted mRNA to cDNA, which is labelled separately with a fluorophore (usually cyanine dye-labelled nucleotides) using the Klenow fragment of DNA polymerase I. In single-step DNA labelling from RNA, the fluorescently-labelled nucleotides are incorporated by the reverse transcriptase which is less efficient at performing this step than the Klenow fragment of DNA polymerase and so requires a higher starting concentration of RNA. The lower labelling efficiency of reverse transcriptase can lead to increased incorporation biases between different fluorophores (Ye *et al.*, 2001). Signal compression can falsely manifest as a significant fold-change difference in gene expression across samples (Alston *et al.*, 2010) and decreases the reliability of transcriptomic data, leading to misinterpretation of results.

In cases where compressed data has been identified, a step is performed after quantification by BlueFuse analysis. This step involves the use of the batch anti-banana algorithm in R (BABAR) to normalise the feature signals through block-by-block median centring of the data and lowess pair-wise comparisons between microarrays as described (Alston *et al.*, 2010, Hautefort *et al.*, 2008).

Commercial software has been developed to aid the visualisation of gene expression data including GeneSpring (Agilent Technologies), Acuity (Axon Instruments) and Genowiz (Ocimum Biosolutions). Of these, GeneSpring is the most widely used and supported, providing relatively easy visualisation, interpretation and statistical analyses of transcriptomic data.

4.1.3 Bacterial cold shock and cold acclimation

During the life cycle of enteric bacteria there are frequent temperature fluctuations from intra-host growth at 37 °C to sudden temperature downshifts upon excretion into low temperatures such as soil or marine environments (Smith *et al.*, 1994). *Salmonella* survives better in these non-host environments than other enteric bacteria such as *E. coli* (Winfield & Groisman, 2003, Rozen & Belkin, 2001), although survival was dependent on external environmental factors such as seasonal temperature fluctuations. *Salmonella* can survive for long periods of time at below optimum growth temperatures, due to a rapid and robust series of defence systems which include coping with temperature downshifts, compared with mammalian hosts.

Upon considerable temperature downshift (as described in the present study), bacteria such as *Salmonella* encounter an initial period of cold shock before an increased cold acclimation and eventual cold adaptation that permits bacterial survival (Figure 4.1).

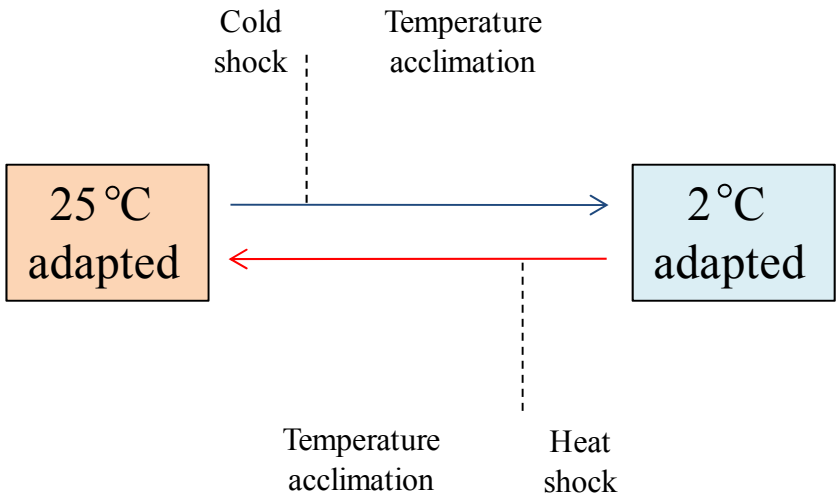


Figure 4.1: The distinct phases involved in cold adaptation. Figure modified from (Inouye & Phadtare, 2004).

Cold shock is characterised by the production of various cold shock protein (CSP) homologues, which are maintained at basal intracellular concentrations during growth at 37 °C by *Salmonella* and *E. coli* (Brandi *et al.*, 1999). Although the CspA-like family make up the major class of cold shock proteins, only four of the nine proteins are actually inducible during growth at lower temperatures (Phadtare *et al.*, 2006). In *E. coli* the 7.4 kDa major cold shock protein, CspA (D'Amico *et al.*, 2002) is induced during temperature downshift from 37 °C to between 24 °C and 10 °C (Jones *et al.*, 1992), whereas CspB production is only induced at temperatures below 20 °C (Etchegaray *et al.*, 1996). CSPs are thought to function, at least in part, as RNA chaperones to compensate for increased RNA secondary structure stability at low temperatures. During cold acclimation, CSPs de-stabilise stable RNA secondary structures enabling translation to proceed (Phadtare, 2004). In *E. coli*, the CspA homologues functionally overlap to compensate for a single CSP mutation (Phadtare, 2004). This has been confirmed experimentally using a combined Δ*cspABGI E. coli* mutant strain which was unable to undergo full cold acclimation and induced heat-shock proteins to compensate for the lack of CSP translational chaperones (Phadtare & Inouye, 2004).

During acclimation, short-chain, unsaturated lipids with a low melting point are incorporated into the cell membrane, displacing saturated phospholipids in a process termed homeoviscous adaptation. This process compensates for decreasing fluidity caused by the temperature downshift (Sinensky, 1974). Translation is also decreased at low temperatures (Farewell & Neidhardt, 1998, Yamanaka, 1999) raising the possibility that the build-up of inactive 70S ribosomes act as a signal for the indirect induction of CSPs and other global regulatory network targets (VanBogelen & Neidhardt, 1990). A crucial part of cold acclimation is transformation of inactive ribosomes to cold adapted, active ribosomes using the CSPs as translational chaperones (Jones & Inouye, 1996, Jones *et al.*, 1996). The structural changes to bacterial cell membranes and fundamental translational machinery would need to be reversed in order for bacteria to recover from these conditions during lag phase adaptation at 25 °C (Figure 4.1).

4.1.4 Aims of this transcriptomic study

Current knowledge of *Salmonella* gene expression during lag phase is limited, with only one detailed study having been performed (Rolfe, 2007). Other studies have used DNA

microarrays to look at the transcriptomic profile of *E. coli* throughout growth in LB medium, however the earliest time-point taken for these studies was one hour, at which point the bacteria were actively-dividing at a specific growth rate of almost 0.4 h^{-1} (Baev *et al.*, 2006c, Baev *et al.*, 2006b, Baev *et al.*, 2006a), similar to exponential growth in the lag phase system at 25 °C. The transcriptomic approach performed in this study aims to build on the study by Rolfe (2007), to confirm the processes identified at the earliest stages of *S.* Typhimurium growth at 25 °C experimentally and to test how faithfully these processes are performed at the transcriptomic level once the system had been perturbed by the food-relevant stress of cold storage. The growth parameters during recovery from cold storage are described in Section 3.2.6, and this chapter aims to uncover the physiological processes accounting for the robustness of lag phase. By using a transcriptomic approach it was hypothesised that stress responses would reveal potential weaknesses (such as stress susceptibility) that could be exploited to lengthen lag time.

4.2 Results

4.2.1 The process of cold acclimation

The global transcriptome of stationary phase *S.* Typhimurium during acclimation to 2 °C was measured using DNA microarrays. In an initial experiment, bacterial cultures were grown in rich medium as described (Section 2.1.6) for 48 hours and then transferred into a cold room for storage. The global transcriptome was determined at 5 hours, 10 hours, 24 hours and 12 days cold storage (Figure 4.2). The changes in gene expression were measured relative to a non-chilled stationary phase culture incubated at 25 °C. Within five hours cold storage, 1047 genes (22 % genome) showed altered expression profile ≥2-fold, with 189 genes being up-regulated and 858 genes down-regulated (*t*-test, FDR=0.05). As expected, the up-regulated genes included several cold-inducible genes, such as *cspA* and *cspB*, encoding the major cold shock proteins (Figure 4.2A). The most up-regulated gene by 5 hours cold storage was *ydiS*, encoding a flavoprotein induced in response to several stresses including oxidative and cold stress.

The initial cold shock response is elicited by 5 hours cold storage, indicated by the expression of cold shock genes. After the induction of the cold shock response, little further transcriptional reprogramming occurs between 5 hours and 24 hours cold storage.

Adaptation to longer-term storage requires a further transcriptomic re-organisation as indicated by the extensive transcriptional re-programming between 24 hours and 12 days cold storage (Figure 4.2). By performing cold storage over a period of hours and days, it was possible to distinguish between the initial bacterial cold shock, cold acclimation and cold adaptation at the transcriptional level. It was evident that the most extensive transcriptional changes occurred between 24 hours and twelve days cold storage. As the bacteria appeared to be 'cold adapted' within a few hours, these data suggest that the extensive transcriptional changes were caused by longer storage times rather than the low temperature.

4.2.2 Transcriptomic data quality

4.2.2.1 RNA quality

Extracting good quality RNA from stationary phase cells is more difficult than from exponentially-growing cells as shown by the decreased total RNA concentration obtained during the Lag Pre-Incubation transcriptomic experiments (Table 4.1). During the Lag Pre-Incubation experiment, the primary difficulty was to recover a sufficient cell concentration to yield adequate RNA for the experiments. All the Lag Pre-Incubation experiment samples contained enough total RNA for two-step labelling (~2 μg). All RNA quality was confirmed using the Bioanalyser (Agilent) capillary electrophoresis system before being processed for microarray hybridisation.

4.2.2.2 Quality of transcriptomic data

Transcriptomic data were subjected to signal quantification using BlueFuse before pair-wise comparisons and signal normalisation across microarrays using BABAR. This prevented anomalies occurring during transcriptomic data interpretation and decreased skewing of data from low-intensity features. Analysis of the cold acclimation transcriptomic experiment revealed that the data were reproducible. The primary determinant was that genes belonging to the same functional category were generally uniformly-regulated. For the initial cold acclimation experiment, genes known to be cold-induced were up-regulated, indicating that the transcriptomic experiment data were reliable.

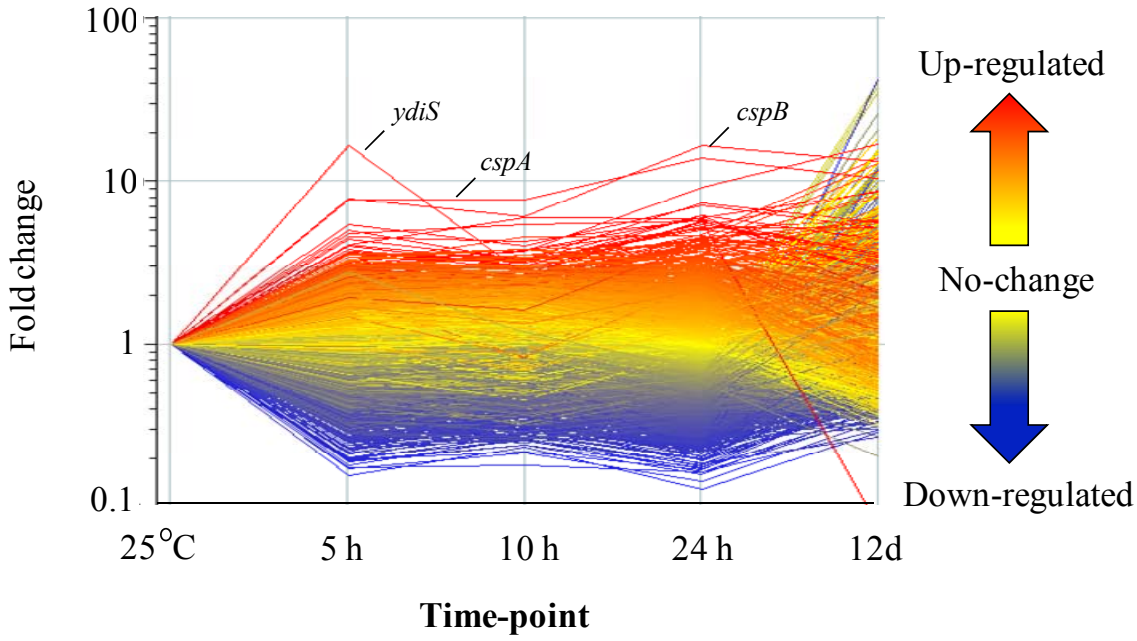


Figure 4.2: Cold adaptation occurs within the first 5 hours of cold storage.
Transcriptional analysis of a single stationary phase culture stored at 2 °C for 5 hours, 10 hours, 24 hours and 12 days (12d). (A) GeneSpring visualisation of the fold-changes in gene expression relative to the non-chilled 25 °C stationary phase inoculum. Each gene is represented by a single line and all samples are coloured by the relative expression at 24 hours cold storage at 2 °C.

Analysis of Lag Pre-Incubation gene expression profiles showed the data were reproducible. The median co-efficient of variation (CoV) between biological replicates was 13 %, comparable with a 15 % CoV in a previous study (Rolfe, 2007). The processes occurring during lag phase were the same as shown in the previous study, which served as a reference for accuracy of function group gene expression at each time-point. In addition, interpretation of the global transcriptome was performed using statistically-filtered data (*t*-test, FDR=0.05), and a biological cut-off (2-fold change in expression).

4.2.3 Effect of twelve days cold storage on the bacterial transcriptome

Analyses of the effect of twelve days cold storage were performed by comparing the global transcriptome with the non-chilled 48 hour inoculum at 25 °C. These analyses allowed differences between 25 °C adapted and 2 °C cold adapted bacteria to be seen and were the simplest way of testing the effects of long term cold storage on bacterial recovery.

Over 30 % of the genome was down-regulated after 12 days cold storage whilst 15 % was up-regulated (Figures 4.3A and 4.3B). By characterising the differentially-expressed genes by their annotated functional category, it is possible to hypothesise biological processes which are activated or repressed after 12 days cold storage (Figure 4.3B). Fimbriae-encoding genes were differentially expressed, with ~45 % of the genes being up-regulated after 12 days cold storage. Fimbriae production is associated with attachment and virulence but also aids the adherence between bacterial cells which may represent a population-based defence mechanism against stresses experienced in the cold environment, although this was not explored in detail. The only other identifiable group of genes exhibiting biologically-significant up-regulation were prophage genes, which are actively-transcribed in conditions of stress (Garcia-Russell *et al.*, 2009).

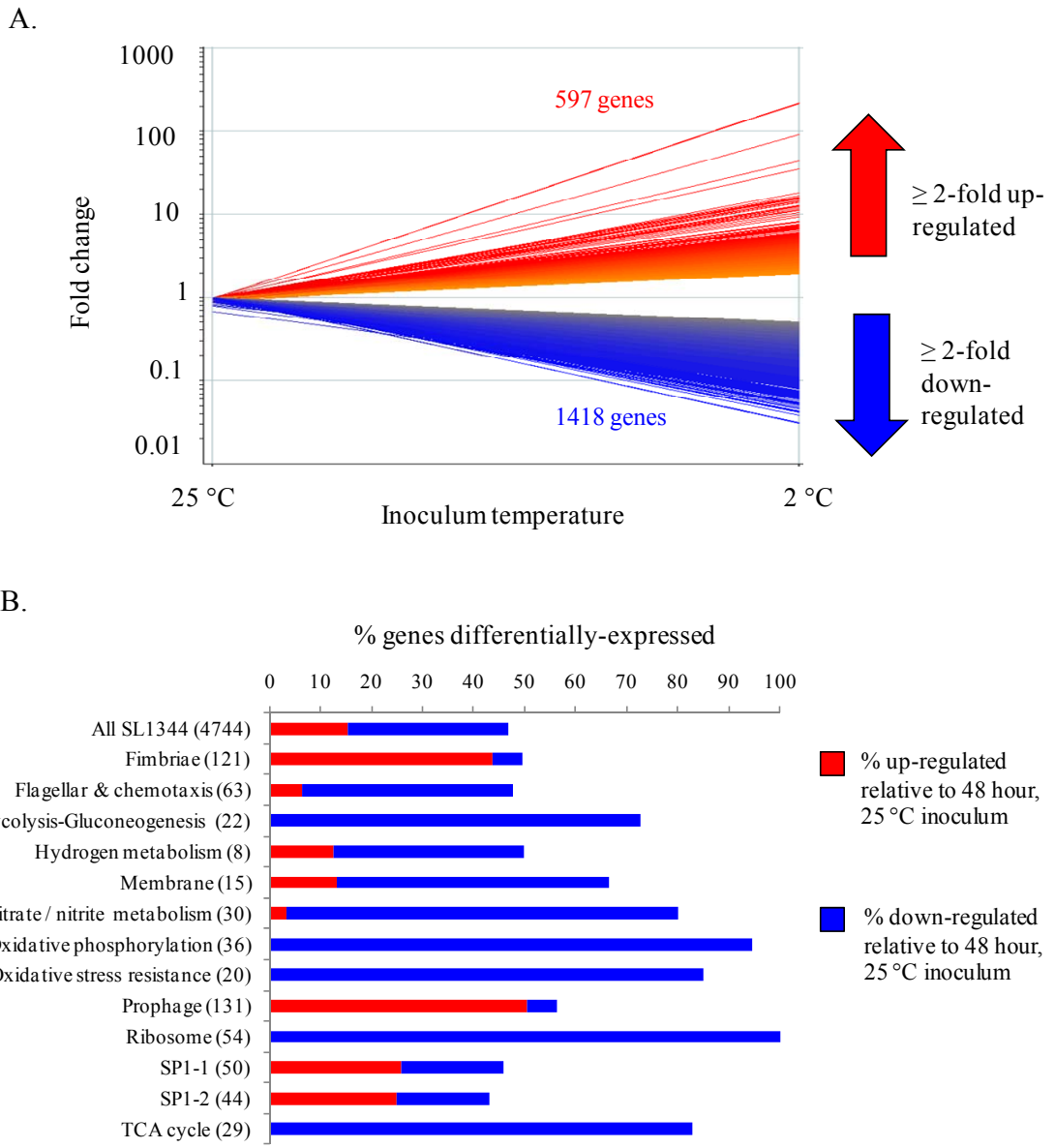


Figure 4.3: More genes are down-regulated than up-regulated after 12 days cold storage, including those encoding central metabolic pathways.
Transcriptomic experiment characterising the gene expression of the two stationary phase inocula for three biological repliactes. (A) GeneSpring interpretation of genes that passed filtering for significance (*t*-test with a Benjamini and Hochberg multiple testing correction (FDR = 0.05) and ≥2-fold cut-off). Number of genes either significantly up-regulated (red text) or down-regulated (blue text) after 12 days cold storage when compared with the non-chilled inoculum. (B) Functional categories analysis of the genes ≥2-fold up-regulated and down-regulated after 12 days cold storage compared with the non-chilled inoculum. Gene lists from each functional category obtained from the Kyoto Encyclopaedia of Genes and Genomes (KEGG) database. The number of genes within each functional group is shown between pairs of parentheses.

Genes encoding enzymes involved in central metabolic processes were largely down-regulated including: glycolysis, the tricarboxylic acid (TCA) cycle, oxidative phosphorylation and the anaerobic metabolism of hydrogen, nitrate and nitrite. The chilled culture environment is hypothesised to be anoxic, based on the dissolved oxygen concentration of the 25 °C inoculum after 48 hours growth (Section 3.2.2). This hypothesis is confirmed by >80 % aerobically-induced genes involved in oxidative stress resistance which are ≥2-fold down-regulated after twelve days cold storage. The lack of growth and metabolic activity is further highlighted by the down-regulation of all ribosomal protein-encoding genes. The bacterial growth and metabolic rate has previously been linked with the rate of ribosome synthesis (Kjeldgaard *et al.*, 1958, Wagner, 1994), therefore the down-regulation of ribosome encoding genes suggests the physiological state in the cold inoculum is more quiescent than the 25 °C inoculum.

4.2.4 Lag phase transcriptome after cold storage

To determine the effect of cold storage on the gene expression of *S.* Typhimurium during recovery at 25 °C, the transcriptional profile was analysed throughout lag phase (Figure 4.5). RNA was extracted from the bacterial cultures at desired intervals through lag phase and mid-exponential phase. The amount of cells harvested for lag time-points was approximately 8 x 10^8 CFU and for mid-exponential phase cultures, 4 x 10^9 CFU. These cell densities allowed the isolation of an average of between 2 µg and 5 µg RNA for most lag phase samples and 70 µg of total RNA for mid-exponentially growing bacteria. By 120 minutes post-inoculation the bacteria had not divided however more RNA was extracted (11 µg), possibly due to increased nucleic acid production within the cells (Table 4.1). During data analysis, the gene expression signal from the pre-chilled transcriptomic samples was poor, due to the limited amount of RNA available. This led to a decreased distribution of the signal for some arrays, due to different median signal intensities (Figure 4.4A). To compensate for the poor signal-to-noise ratio, the transcriptomic data from the present study and a previous comparative study (Rolfe, 2007), were processed using BABAR (Section 2.3.10.6). After an initial block-by-block median centring, a lowess pair-wise normalisation between each sample was performed to correct the difference in dynamic range between samples (Figures 4.4B and 4.4C). As a result, the signal quartile ranges increased, but the median values were shifted slightly out of alignment between arrays. This was overcome by a final block-by-block median centring across all the arrays.

A degree of 'compression' was still observed in arrays 34, 37, 40 and 41, corresponding to the Lag Pre-Incubation samples performed in this study (Figure 4.4D). Despite the remaining small degree of compression, the arrays had increased dynamic signal range than prior to BABAR which improved the visualisation and subsequent statistical analyses within GeneSpring.

The BABAR-based analysis ameliorated the signal 'compression' and the transcriptome during recovery from 2 °C, 12 days cold storage was observed. Transcriptomic data were analysed at time-points during lag phase (4 minutes, 20 minutes, 60 minutes and 120 minutes post-inoculation) to observe differentially-expressed genes compared with the stationary phase inoculum. In addition, a sample from mid-exponential phase (8 hours post-inoculation) was taken to distinguish lag phase specific gene expression from expression occurring during steady state growth (Figure 4.5A and Figure 4.5B). The bacteria underwent rapid and extensive transcriptional reprogramming upon inoculation into the fresh 25 °C medium, which began within the first 4 minutes (Figure 4.5C). There was an increase in differentially-expressed genes as lag phase proceeded (Figure 4.5D). The chilled inoculum was identified as the most transcriptionally-distinct from other samples when the transcriptomic data at different time-points were correlated. Conversely the lag time-points were largely transcriptionally homogeneous. The 60 minute and 120 minute samples appeared most similar (Figure 4.5E), with only 100 genes being differentially expressed (Figure 4.5D).

	Yield per RNA prep (μg)		
	Replicate 1	Replicate 2	Replicate 3
Cold inoculum	22.1	38.0	40.0
4 minutes	8.6	2.0	3.1
20 minutes	4.1	5.9	3.7
60 minutes	2.3	2.6	2.6
120 minutes	5.7	16.4	11.5
8 hours	62.4	67.9	80.5

Table 4.1: Concentration of RNA extracted for Lag Pre-Incubation transcriptomic experiment. Time-points indicate the age of the bacterial culture when cells were harvested.

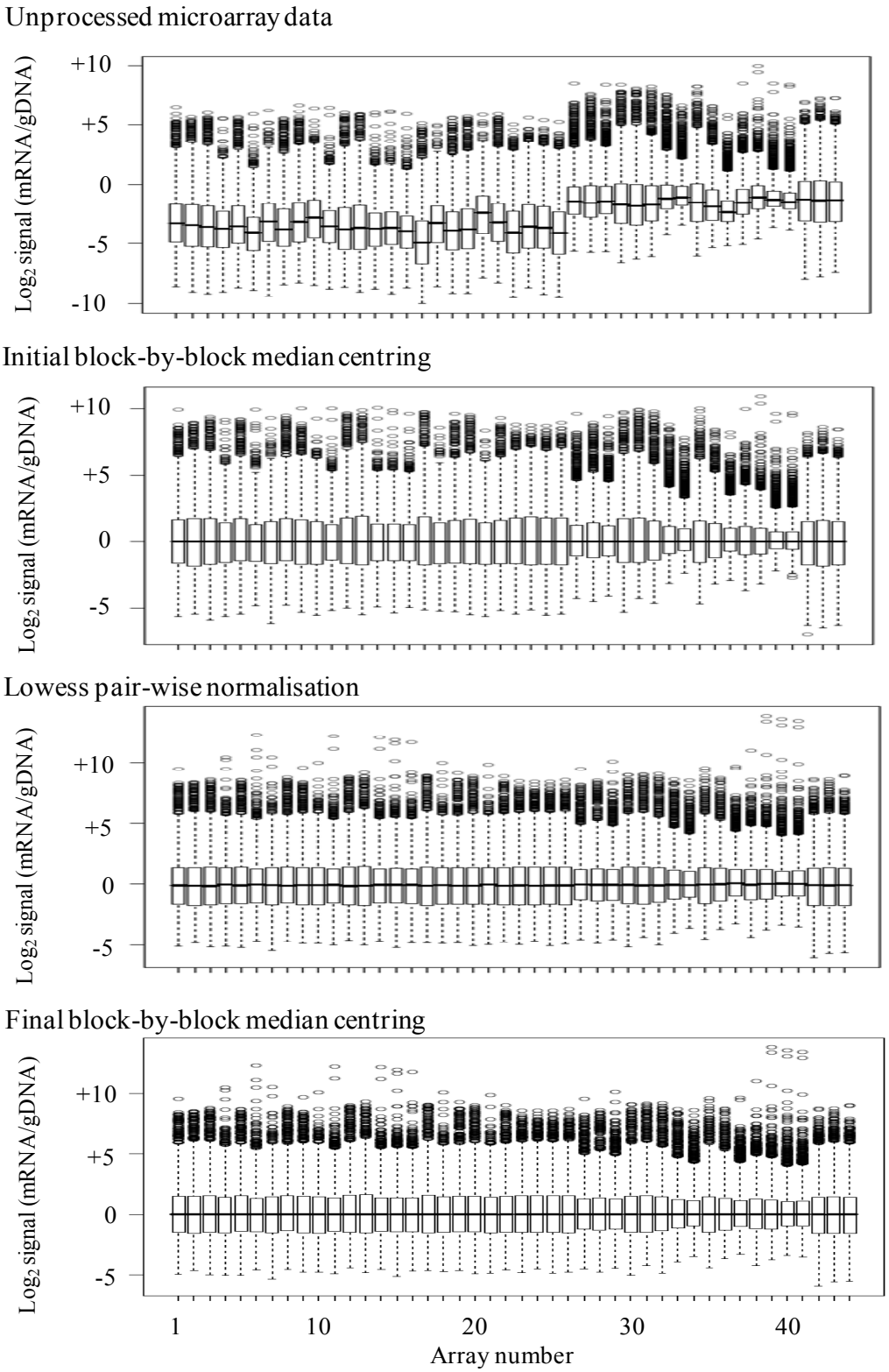
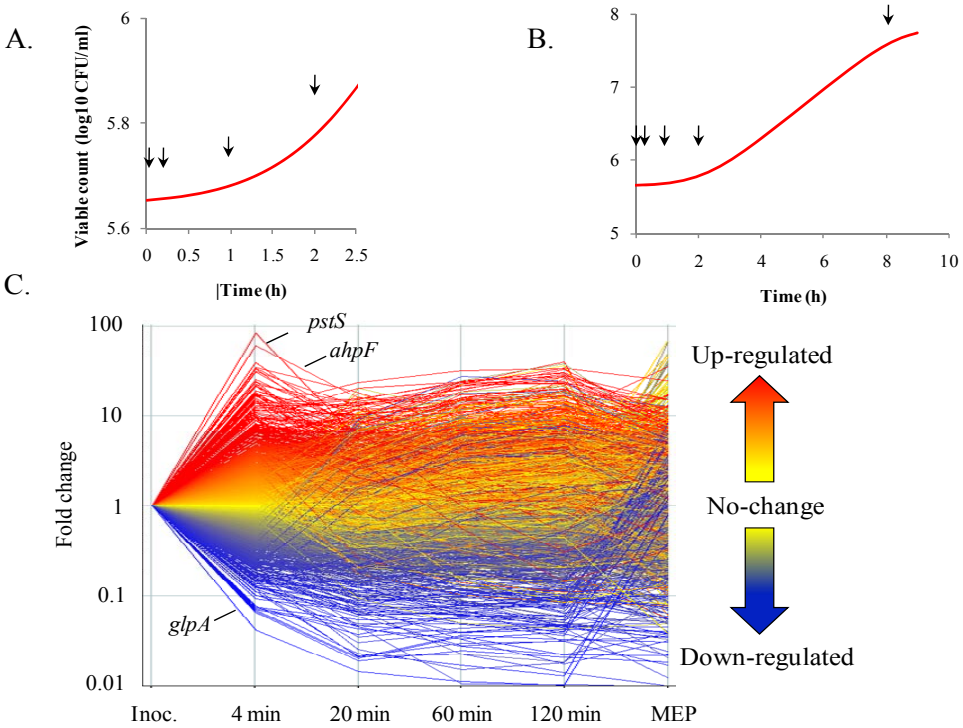


Figure 4.4: Signal compression evident during transcriptomic analysis.
Box-whisker plot of BABAR identified signal values obtained from BlueFuse-analysed transcriptomic data for each of the 44 microarrays tested. Boxes represent the quartile variation in each microarray; the dotted whiskers represent the upper and lower values within the quartile range. Individual features outside of the range of the box whisker plot are represented as circles.



D.

Condition 2 \ Condition 1	Inoc.	4 min	20 min	60 min	120 min	MEP
Inoc.	-	**536**	**579**	**690**	**783**	**792**
4 min	**339**	-	**223**	**366**	**485**	**549**
20 min	**430**	**261**	-	**286**	**516**	**580**
60 min	**441**	**350**	**209**	-	**42**	**386**
120 min	**522**	**472**	**378**	**58**	-	**347**
MEP	**554**	**595**	**566**	**455**	**391**	-

E.

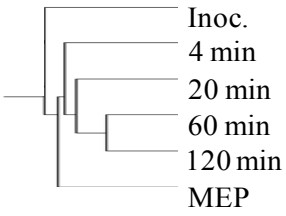


Figure 4.5: Cold recovery involves extensive transcriptional reprogramming.
(A) Lag phase time-points from 25 °C static growth curve. (B) Growth context of samples for transcriptomic analysis (Three biological replicates). Arrows represent 4 minutes, 20 minutes, 1 hour, 2 hours and 8 hours post-inoculation. (C) The *Salmonella* transcriptome throughout growth. Time points shown in minutes except for the cold-stored 12 day, 2 °C stationary phase inoculum (Inoc.) and mid-exponential growth phase (MEP). Data are presented as fold-change in expression compared with the inoculum. Each line represents a single gene coloured by the expression at 4 minutes. (D) Numbers of genes showing statistically-significant changes in expression between time-points (*t*-test, FDR=0.05, Benjamini and Hochberg multiple testing correction, 2-fold cut-off). Values indicate the number of genes found to be significantly up-regulated in Condition 1 compared to Condition 2. For example, 261 genes were up-regulated at 4 minutes compared with 20 minutes. (E) Dendrogram (Pearson correlation) indicating similarity between transcriptomic data at each time-point.

The transcriptional reorganisation from the cold storage environment is such that by the end of lag phase (120 minutes post-inoculation), 1305 genes (28 % of the genome) showed significantly altered expression compared to the stationary phase inoculum. The majority of these genes (1131) were differentially-expressed by 60 minutes post- inoculation, compared to the inoculum. The rapid response to changes in the bacterial environmental was highlighted by the expression of 875 genes being significantly altered by 4 minutes post-inoculation and metabolic shifts indicated by down-regulation of genes involved in the anaerobic metabolism of glycerol and hydrogen.

4.2.5 Processes inferred from induced genes during cold storage recovery

During the early stages of lag phase recovery from cold storage, genes encoding different physiological processes are up-regulated (Figure 4.6). The up-regulation of heat-shock genes to protect the bacteria during temperature upshift from 2 °C to 25 °C occurs within the first four minutes for the majority of the genes (Figure 4.6A). Conversely, cold shock genes which are expressed highly at 2 °C are down-regulated upon temperature upshift. This is the case for *cspA*, *cspB* and the cold-inducible palmitoleoyl transferase-encoding gene, *ddg*. However other genes within the CspA-like cold shock family are not down-regulated at 25 °C, including *cspC*, *cspD* and *cspE* (Figure 4.6B). These genes were not induced in the stationary phase inoculum at 2 °C, compared with the control 25 °C culture. Interestingly, both *cspC* and *cspD* were actively-induced by 20 minutes post-inoculation compared with the stationary phase inoculum, suggesting a possible temperature independent role for these genes during lag phase.

The high-affinity inorganic phosphate uptake transporters (*pstSCA* and *phoBR*) were maximally-induced within four minutes of inoculation into fresh medium. The exception was *pstB*, which was induced maximally by 20 minutes, as the expression of the other transporters decreased (Figure 4.6C). This suggests that the uptake of inorganic phosphate may be crucial within the very earliest stages of lag phase.

Genes involved in the synthesis of the translation machinery were almost uniformly-induced by 20 minutes (Figure 4.6D), suggesting that a rapid increase in protein-synthesis may occur soon after. The induction of all ribosomal genes during the early stages of lag

phase highlights the requirement of *de novo* synthesis of ribosomes before cell division is initiated.

Based on the gene expression data, oxidative stress resistance is an important process during the entry into lag phase from cold storage. By four minutes post-inoculation into fresh medium, the majority of genes involved in the oxidative stress resistance (including the OxyR and SoxS regulons) are induced (Figure 4.6E). Of these genes, the highest-expressed at four minutes is the DNA and iron-binding protein encoded by *dps*. The maximal expression of most of these oxidative stress genes is at four minutes with elevated expression maintained throughout 60 minutes of lag phase, albeit lower than the early stage of lag phase.

Iron homeostasis within bacterial cells is linked with oxidative stress tolerance due to Fenton reaction products which can cause cellular damage in the presence of excess iron. The iron homeostasis genes during lag phase show biphasic expression with some genes such as *dps* and *sitA* up-regulated within four minutes of inoculation whereas other iron responsive genes (*e.g. entB*) are up-regulated later, towards the end of lag phase (Figure 4.6F). The expression of early and late lag phase iron-responsive genes suggests that time-dependent iron homeostasis could be a crucial step before growth can begin.

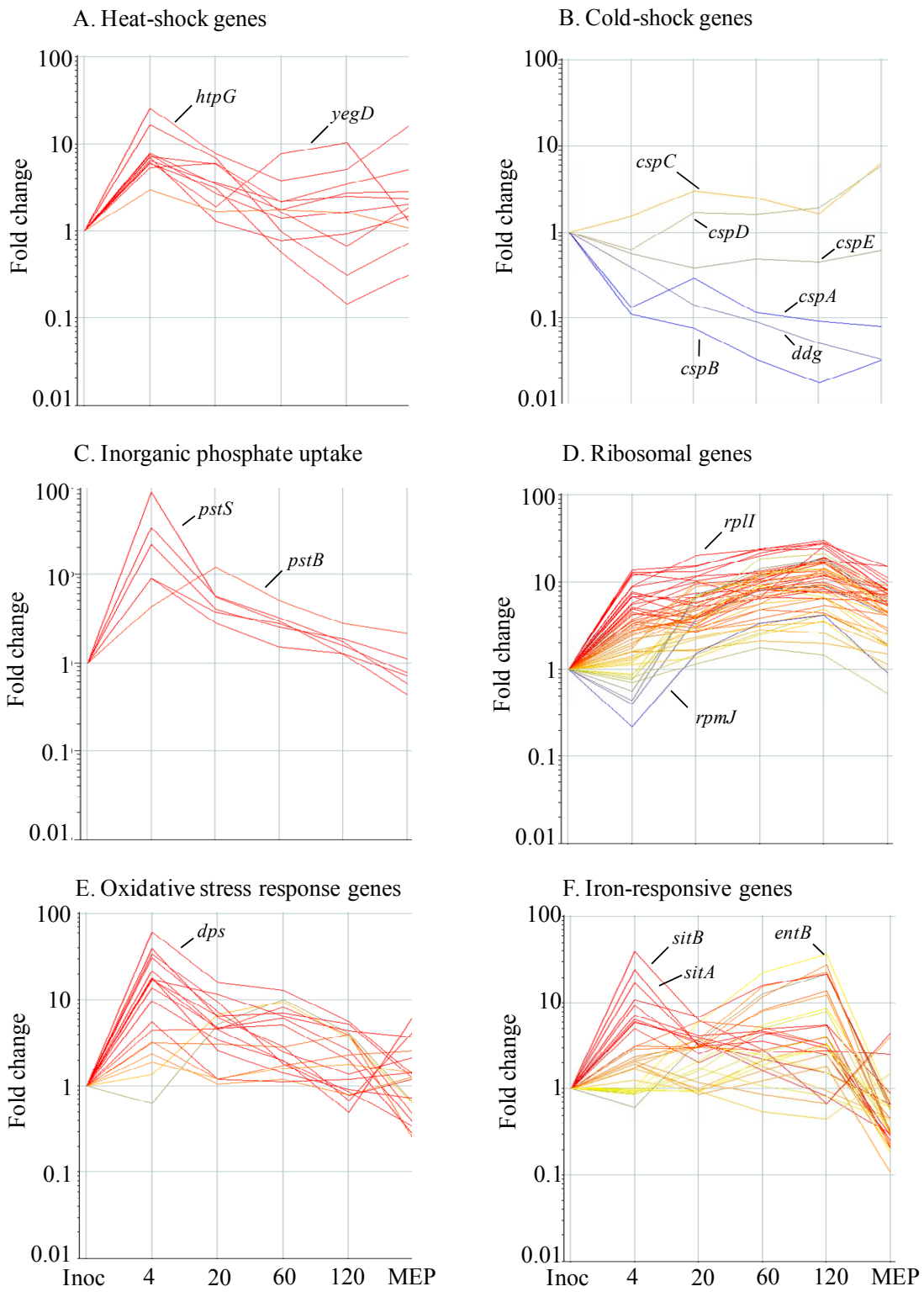


Figure 4.6: Recovery during lag phase is characterised by several crucial physiological processes.
Expression profiles for functional categories of genes induced during lag phase recovery. All data are expressed as the fold change compared with the cold-stored stationary phase inoculum (Inoc) for the lag phase time-points (minutes) and mid-exponential phase (MEP), coloured by the 4 minute time-point. The Figure was assembled using non-statistically filtered data of three biological replicates.

4.2.6 Assessment of physiological history on lag phase recovery

To determine the effect, if any, of cold storage upon the transcriptome of lag phase bacteria during recovery, the transcriptomic data were compared with a previously-acquired lag phase dataset (Rolfe, 2007), which served as a 'control' experiment. For clarity, the previous dataset is hereafter referred to as "Lag Phase 25 °C" and the present study is referred to as "Lag Pre-Incubation", referring to the previous cold storage environment of 12 days at 2 °C.

If we exclude genes involved in temperature adaptation (based on reported function from the KEGG database), comparison of the two datasets only revealed subtle differences in gene expression. Genes tended to be either repressed or induced independently of the treatment history of the culture, highlighting the robust nature of lag phase. Using the GeneSpring software, the transcriptomic data were interpreted differently to answer two similar but distinct questions. Firstly, "what is the overall effect of cold storage recovery compared with 48 hour stationary phase recovery?" and secondly, "which genes are differently-expressed at each time-point during lag phase?"

In order to answer the first question, the transcriptomic data from both the Lag Phase 25 °C and the Lag Pre-Incubation experiments were interpreted as a fold change in gene expression compared with the individual inocula (either cold-stored bacteria or 25 °C bacteria) (for example, Figure 4.7). This approach was particularly useful for elucidating the lag phase specific gene expression which was independent of the physiological history of the bacteria. This type of data treatment utilised the Lag Phase 25 °C transcriptomic data as a control experiment to ascertain the default response of the bacteria to inoculation into fresh LB medium. By using the control experiment, any changes in the induction, repression or duration of lag phase specific gene expression in the Lag Pre-Incubation experiment could be determined. This comparison was particularly useful for identifying changes in gene expression occurring at different time points. However, to obtain a clearer understanding of gene expression differing at each point in lag phase, an alternative strategy was implemented (Figure 4.8). The gene expression data at each time-point were compared directly between the Lag Pre-Incubation and Lag Phase 25 °C experiments. During this interpretation, genes expressed ≤2-fold between the two experiments would not pass statistical filters for biological significance. The two different interpretations

performed in the present study highlight the powerful flexibility of the GeneSpring software to answer fundamental questions regarding gene expression of complex datasets.

Using the visualisation approaches described, differences were noted in the kinetics of activation or repression. An example of identifying gene induction shifts is exemplified by the OxyR-regulon. Several genes from this regulon were up-regulated during the first 20 minutes in the Lag Phase 25 °C experiment, including *ahpCF* and *sufABCDS*; however these same genes were up-regulated for longer (up to 120 minutes) in the Lag Pre-Incubation experiment (Figure 4.7A). A different example is given by the iron-uptake genes, the majority of which are up-regulated by four minutes post-inoculation in the Lag Phase 25 °C experiment but the maximal expression in the Lag Pre-Incubation experiment is not until 60 minutes (Figure 4.7B).

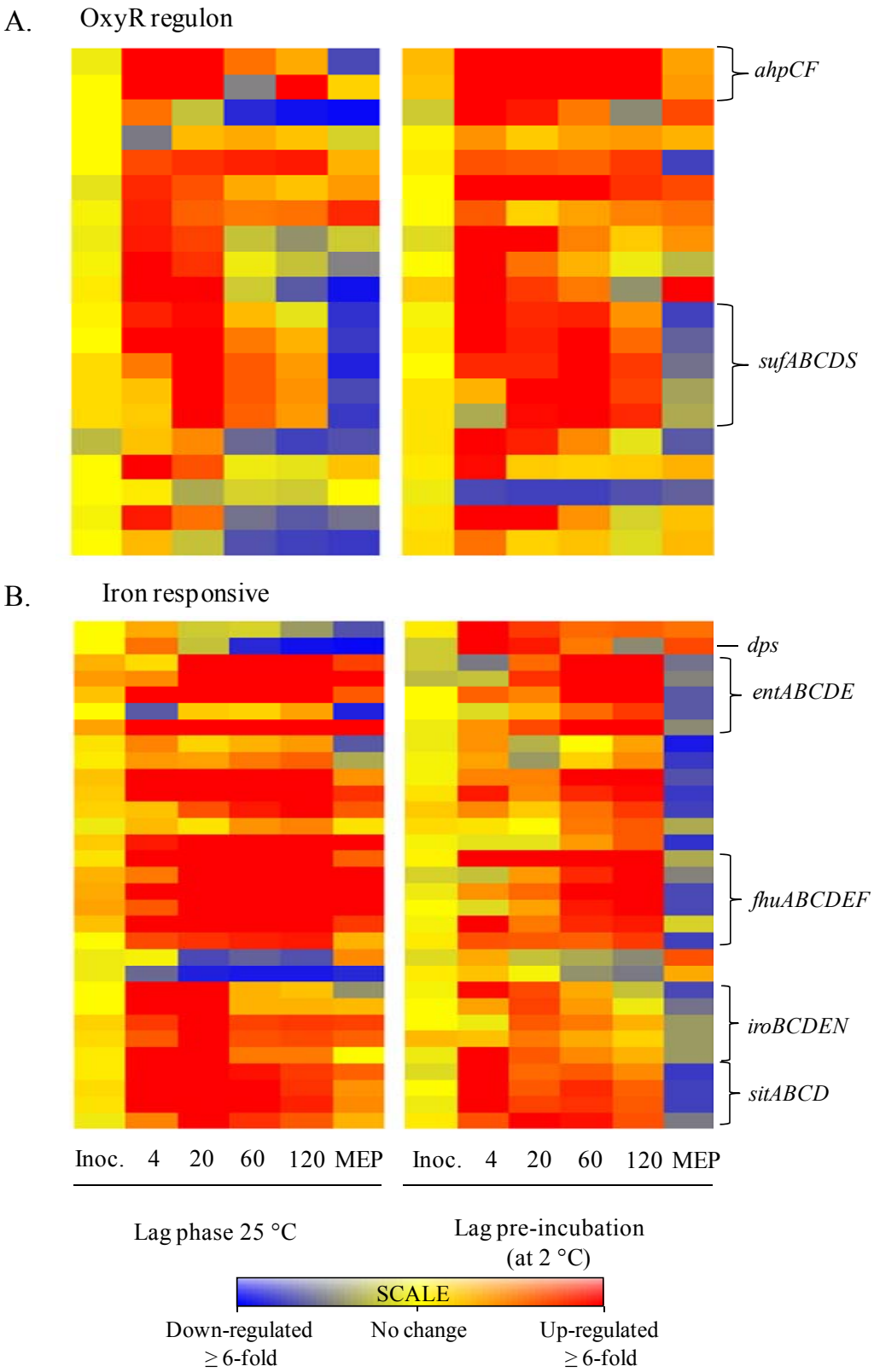


Figure 4.7: Growth history affects the global transcriptome during lag phase.
Heat-map of three biological replicates of non-statistically-filtered gene expression data showing: (A) OxyR-regulon and (B) iron-responsive genes (KEGG database) relative to the respective inocula (Inoc.) for Lag Phase 25 °C and Lag Pre-Incubation experiments. Lag time-points are shown in minutes post-inoculation; mid-exponential phase (MEP) represents an 8 hour time-point. Specific subsets of genes are labelled on the right.

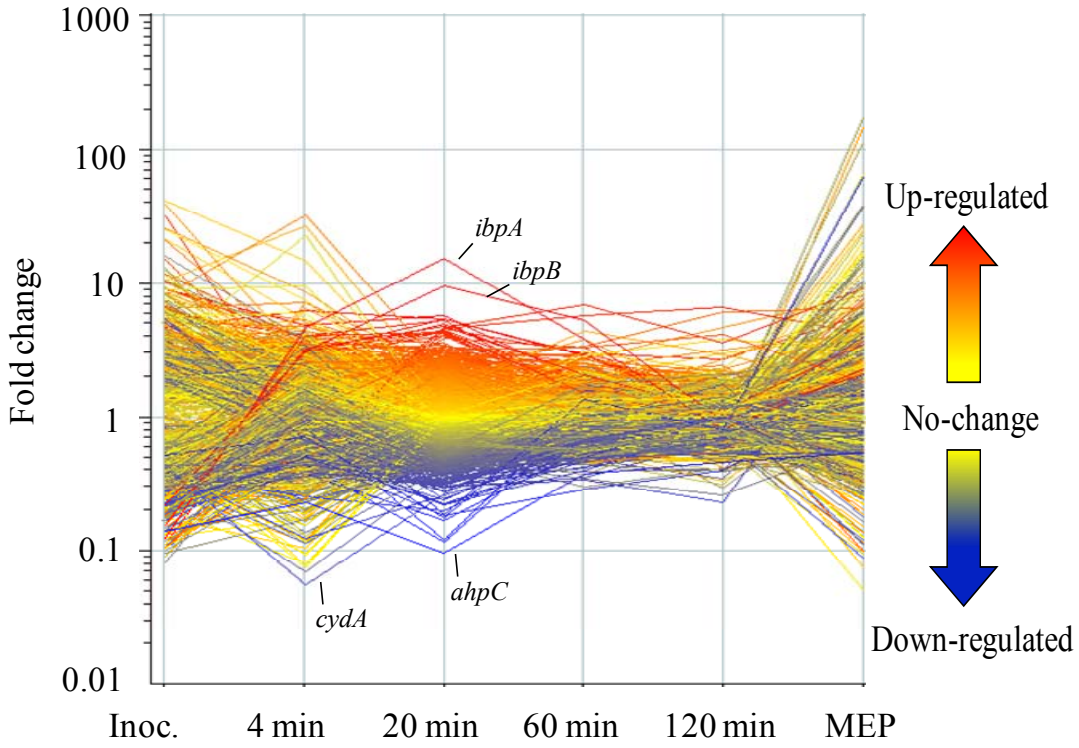


Figure 4.8: Lag phase gene expression is altered by cold storage history.
GeneSpring interpretation of the transcriptome for genes which pass the statistical filter (*t*-test FDR=0.05) from three biological replicates. Time points shown in minutes for lag phase. The cold-stored stationary phase inoculum (Inoc.) and an 8 hour culture corresponding to mid-exponential phase (MEP). Data presented as fold-change in expression compared with the same time-point in the Lag Phase 25 °C experiment. Side-by-side interpretation was used to clearly identify up- and down-regulated lag phase genes between the two experiments. Each line represents a single gene coloured by the expression at 20 minutes. Individual genes of interest are labelled.

The individual time-point comparison analysis was useful to study history-dependent gene expression during lag phase and identify the percentage of genes that changed between the same time-point in the two different lag phase experiments. The most conspicuously-induced genes for the first 20 minutes of lag phase are the *ibpAB* heat-shock genes. As expected, these genes were approximately 10-fold down-regulated during cold storage (Figure 4.8).

This type of transcriptomic interpretation also allows the identification of transient, differentially-expressed genes between experiments. An example of this gene expression pattern was *cydA*, a gene involved in cytochrome production, which was immediately down-regulated by four minutes post-inoculation before expression became uniform (fold change ≤2) later in lag phase.

Generally, the global gene expression profile was very different between the two inocula, which represented distinct environments. The bacteria had become cold-adapted in the Lag Pre-Incubation inoculum and showed an extensive transcriptional re-assortment upon the subsequent temperature, nutrient and oxygen upshift. As lag phase progressed, the two experiments gradually became more transcriptionally uniform (Figure 4.8). The mid-exponential phase cultures showed many differences in gene expression, most likely due to differences in sampling times between the two experiments. In the Rolfe (2007) Lag Phase 25 °C experiment, mid-exponential phase was calculated to be 6 hours of bacterial growth whereas in the present study, it was calculated to be 8 hours. The discrepancies are evident after direct comparison of the gene expression profiles between the two experiments, revealing the lack of transcriptional uniformity during exponential phase of bacterial growth as described previously (Epstein, 2009).

The Lag Phase 25 °C and Lag Pre-Incubation data were directly compared at each time point to elucidate genes responsible for lag phase variability between the two experiments. The differentially-expressed genes at each time-point were then categorised based on their reported function (Figure 4.9). For the majority of functional categories identified, the transcriptome became more similar as lag phase proceeded. As observed in the 12 day cold-stored inoculum (Figure 4.3B), prophage genes were also more highly expressed during the first 60 minutes of lag phase recovery from cold storage compared with Lag Phase 25 °C experiments. As expected, the majority of outer membrane genes were up-regulated within the first four minutes of cold storage recovery, suggesting new membrane components were synthesised *de novo* for adaptation to lag phase. Several functional categories were expressed lower throughout lag phase after recovery from cold storage including the central respiratory pathways: glycolysis, the tricarboxylic acid cycle and oxidative phosphorylation, which generate ATP and energy in aerobic conditions. The ribosome protein-encoding genes were highly expressed during lag phase compared with the inoculum in both experiments; however some of the ribosomal genes showed somewhat lower expression in the Lag Pre-Incubation experiment compared with Lag Phase 25 °C. Genes encoding the iron homeostasis machinery were expressed similarly within four minutes post-inoculation in both experiments; however these genes show decreased expression at both 20 and 60 minutes during cold storage recovery, suggesting iron homeostasis is different between the two experiments at these time-points after inoculation.

Interestingly, using this direct time-point comparison, oxidative stress resistance genes were down-regulated in the Lag Pre-Incubation experiment compared with the Lag Phase 25 °C experiment. Down-regulation occurred within 20 minutes of inoculation into fresh medium and continued for the entirety of lag phase. Virulence gene expression was also altered during recovery from cold storage with genes encoding *Salmonella* Pathogenicity Island 1 (SPI-1) down-regulated by 20 and 60 minutes post-inoculation, and conversely, SPI-2 expression up-regulated in the Lag Pre-Incubation experiments compared with the Lag Phase 25 °C experiment at the same time-points.

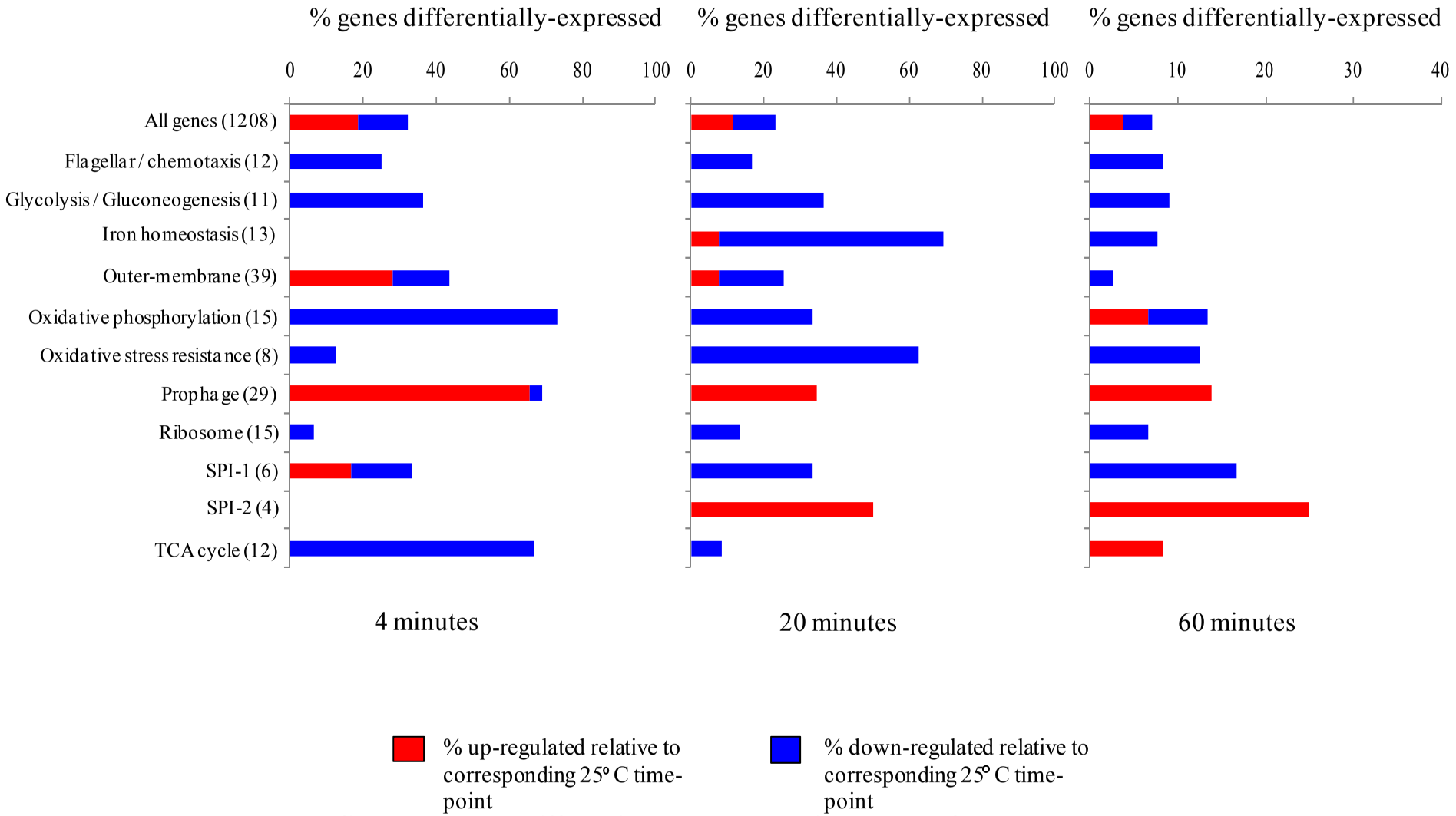


Figure 4.9: Central processes differentially-regulated during recovery from cold storage.
Functional category analysis of lag phase from three biological replicates. Genes that passed filtering (*t*-test (p=0.05) with Benjamini and Hochberg multiple testing correction and a two-fold cut-off) at each time-point between the lag phase 25 °C and lag pre-incubation experiments. Functional categories based on information from the KEGG database. The number of genes within each functional group is shown between pairs of brackets.

Metal ion transport gene expression was examined between the two experiments to determine if metal ion homeostasis was an important part of lag phase adaptation or recovery, as seen previous in Lag Phase 25 °C experiments (Rolfe, 2007). The ion transporters for the physiologically important metals, iron, manganese, magnesium, potassium and nickel were directly compared at each time-point (Figure 4.10). During cold storage, the majority of transporters for all the metal ions studied were significantly expressed consistent with a physiological requirement for metal ions in this environment. Gene expression during lag phase recovery was similar between the two experiments for the metals at four minutes post-inoculation; however, there was a marked decrease in expression of genes encoding transporters for calcium, iron and manganese ions at 20 minutes in the Lag Pre-Incubation experiments. This was accompanied by the *mgt* genes encoding magnesium-specific transporters were expressed significantly higher by 20 minutes post-inoculation. The magnesium transport genes were also up-regulated at 60 minutes post-inoculation, as were the high-affinity manganese transport genes (*sitABCD* and *mntH*). The majority of metal ion transport genes were not differentially-regulated at the end of lag phase (120 minutes post inoculation) between the Lag Pre-Incubation and Lag Phase 25 °C experiments, the exception being manganese ion transport genes which remained elevated in the Lag Pre-Incubation experiment. The most substantial change in gene expression was observed for the iron and manganese ion transport genes which were almost uniformly down-regulated in the Lag Pre-Incubation experiment by 20 minutes post-inoculation compared with the Lag Phase 25 °C experiment. Taken together, these data suggest that metal transport is considerably different during cold storage and in lag phase recovery between the two experiments. The role of metals during lag phase could be crucial to elucidating the differences in history-dependent lag physiology.

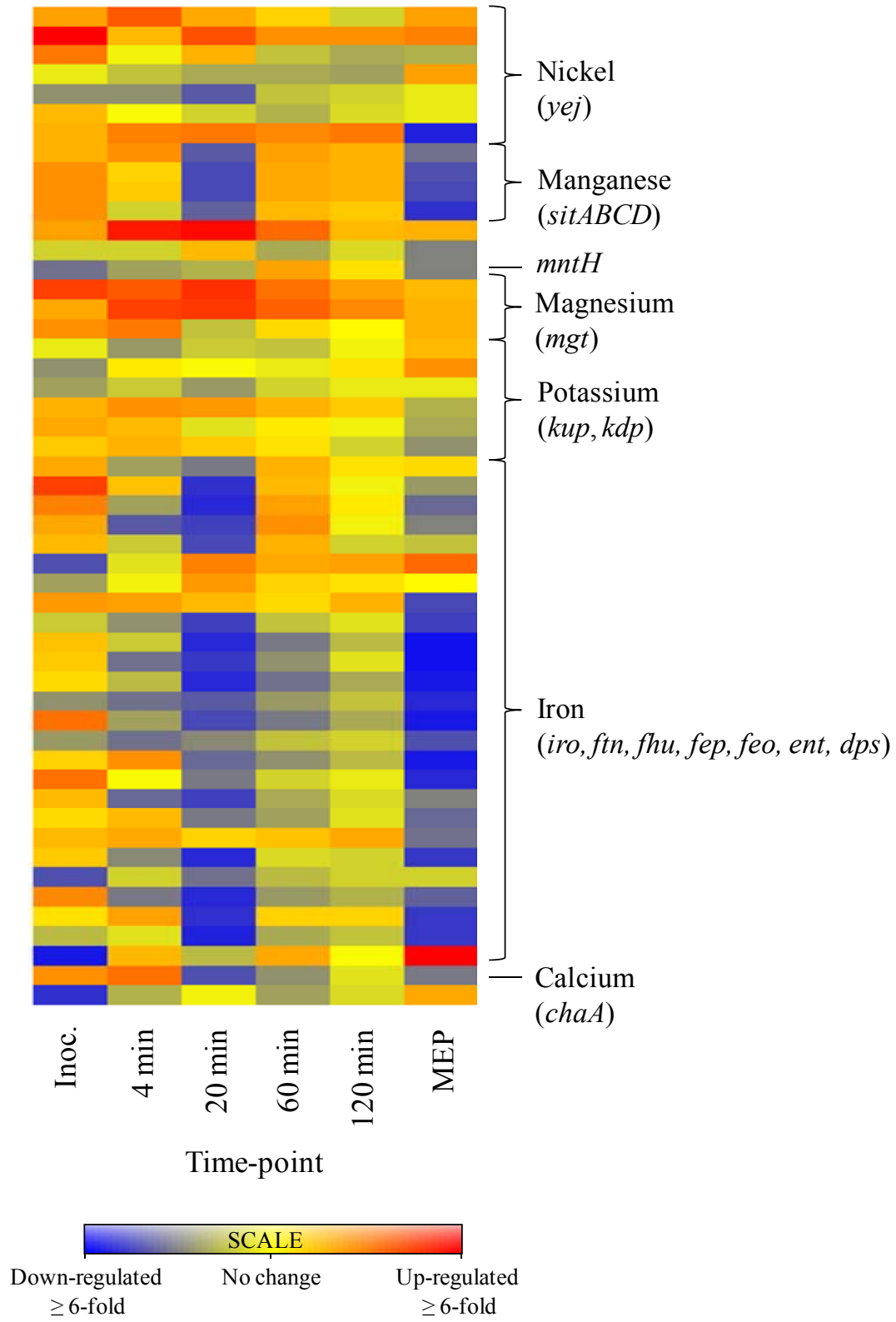


Figure 4.10: Growth history alters metal responsive gene expression during lag phase. Heat-map of non-statistically-filtered gene expression data showing metal transport genes (KEGG database) relative to the respective time-point in Lag Phase 25 °C, for three biological replicates. Lag time-points are shown in minutes post-inoculation, mid-exponential phase (MEP) represents an 8 hour time-point. Specific subsets of genes are labelled on the right.

Central aerobic respiration is crucial to energy production, and is therefore hypothesised to be important in lag phase adaptation before the bacteria can enter exponential growth. The processes of glycolysis, TCA cycle and oxidative phosphorylation were shown to be generally down-regulated in lag phase recovery after cold storage, compared with the Lag Phase 25 °C experiment (Figure 4.9). In order to visualise transcriptional changes in central metabolic pathways, the lag phase gene expression data for both experiments were directly compared, focussing on these pathways (Figure 4.11).

When directly comparing the two inocula, it was possible to determine that glycolysis and oxidative phosphorylation were markedly down-regulated during cold storage, as identified elsewhere (Figure 4.3B). Upon inoculation into fresh medium, the TCA cycle and oxidative phosphorylation-encoding genes were expressed significantly lower than the same time-points during cold storage recovery. In contrast, genes involved in glycolysis and gluconeogenesis were expressed comparably between experiments during lag phase. Lower expression of genes involved in oxidative phosphorylation continued for the first 20 minutes of lag phase recovery after cold storage, but by 60 minutes the gene expression was comparable between experiments. Almost all genes involved in these three pathways were similar by 120 minutes, suggesting that any changes in gene expression induced by recovery from cold storage were made by the middle of lag phase.

These data suggest that growth history may impact upon crucial energy-generating metabolic pathways during lag phase recovery. The apparent down-regulation of genes within four minutes of inoculation from cold storage is most likely due to either a delay in switching on genes involved in these processes, or a down-regulation of important metabolic genes to protect the bacteria from stresses occurring within the earliest stages of lag adaptation from cold storage. As the growth environments for lag phase recovery are identical between experiments, increased stress protection represents a history dependent transcriptional re-programming event.

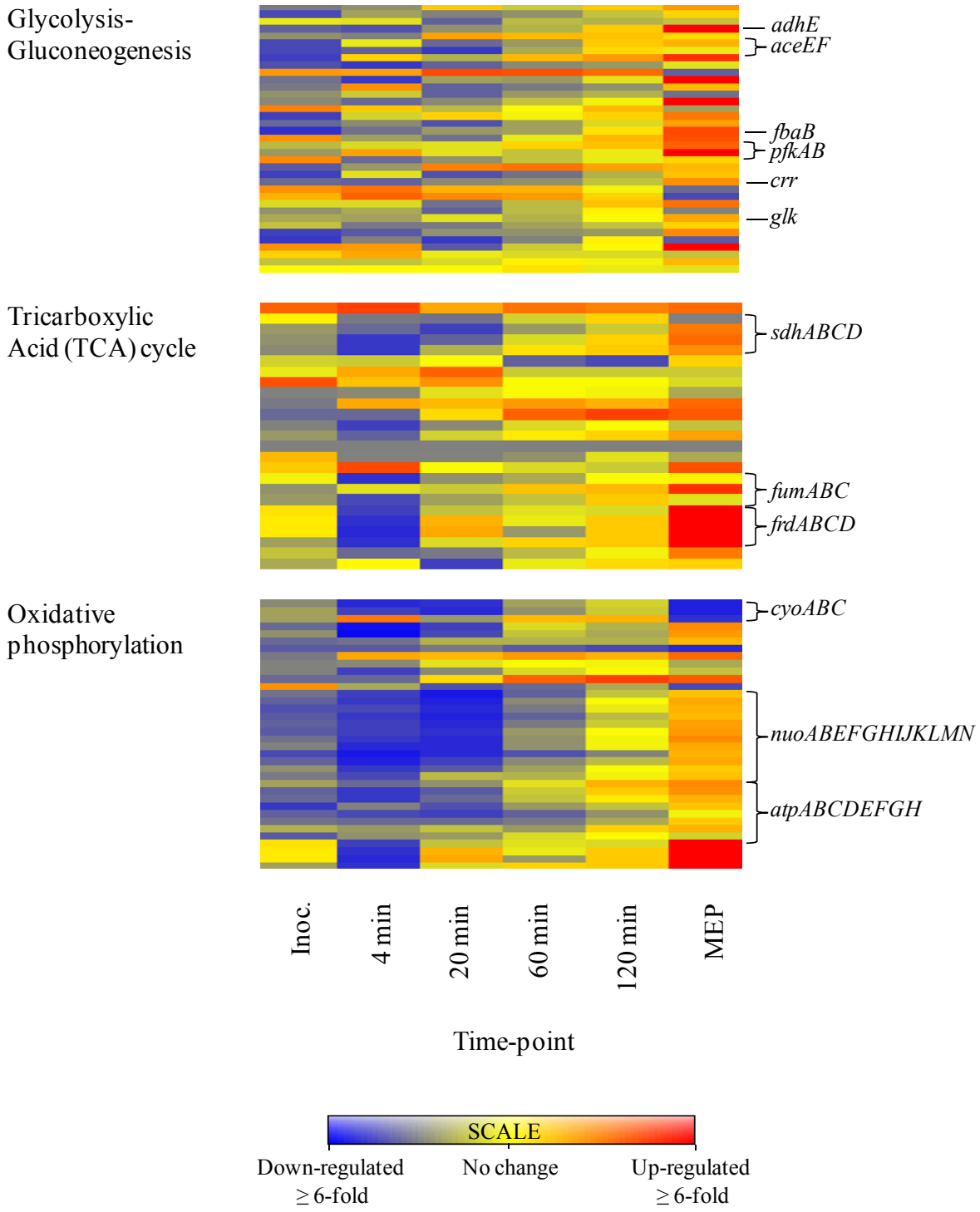


Figure 4.11: Growth history alters central metabolic gene expression during lag. Heat-map of non-statistically-filtered gene expression data showing genes involved in glycolysis, TCA cycle and oxidative phosphorylation (KEGG database) relative to the respective time-point in Lag Phase 25 °C, for three biological replicates. Lag time-points are shown in minutes post-inoculation, mid-exponential phase (MEP) represents an 8 hour time-point.

4.3 Discussion

By using the same experimental set-up as a previous study which analysed the transcriptomic response to lag phase adaptation (Rolfe, 2007), the present study can be directly compared with the pre-existing dataset. The present study is the first to identify the transcriptional responses to adaptation after cold pre-incubation during the lag-phase of bacterial growth. The processes which underpin lag phase were confirmed to be robust at the transcriptional level, and a direct comparison with the previous study determined processes which are affected by the physiological history experienced by the bacteria.

As discussed in Chapter 3, the geometric lag time of bacteria in the two experimental conditions was similar (Figure 3.8). The development of sophisticated models to measure lag time are promising, however they cannot infer the physiological state of a bacterial population. The detailed molecular approaches, like those used in the present study, permit us to infer the physiological state of a bacterial population. Interestingly, while cold storage did result in changes to the transcriptomic response, the population lag time was not extended. The previous study by Rolfe (2007) identified physiological processes which define different stages of lag phase in the static system. By using these 'signature processes' as indicators, the physiological state of the bacterial population during lag phase can be determined. For example, the degree of bacterial stress can be inferred from the induction of specific stress resistance genes. The transcriptomic analysis of lag phase-specific gene expression may allow a more accurate method determining when the population has entered early exponential growth than using conventional microbiological methods. By constructing fluorescent protein fusions to lag phase signature genes, the physiological state of the bacterial population can be visualised by methods such as flow cytometry or cell imaging (Hautefort *et al.*, 2003).

4.3.1 Drawbacks of using the transcriptomic approach

DNA microarrays are a useful molecular tool for identifying changes in the global gene expression profile for a population of cells. One limitation of this technique is that microarrays measure the gene expression of a population and not of single cells. The exit from lag phase is a stochastic process (Baranyi, 1998, Baranyi, 2002) and slight changes in the physiology of a minority of the population would be missed. Indeed, recent studies have concluded that even a steady-state bacterial population may consist of many sub-

populations, the persistence of which may be vital to resisting stresses or promoting pathogenic virulence at the population level (Epstein, 2009). The bacterial population is not made up of 'average cells' and so the response of individual bacteria to their environment is thought to be crucial to the behaviour of the population as a whole (Elowitz *et al.*, 2002, Levsky & Singer, 2003). Techniques for examining single-cell gene expression exist including: GFP-transcriptional gene fusions coupled with single cell imaging (Hautefort *et al.*, 2003); eukaryotic single cell arrays using fluorescently-labelled oligonucleotide probes to detect mRNAs (Levsky *et al.*, 2002) and interpretation of genetic oscillations, or the interactions of a small set of molecular components in a network, within a single cell. Such genetic oscillators rely on extensive knowledge of an organism and specific, reproducible protein interactions, coupled with computational biology analyses (Guantes & Poyatos, 2006, Wolf & Arkin, 2003).

DNA microarrays measure the total signal inferred by mRNA levels. This signal can vary depending upon a multitude of factors including the relative stability (half-life) of different mRNA molecules, strength of various promoters or the post transcriptional changes in mRNA topology which allow secondary structures to form, preventing the annealing of primers during the reverse transcription step. Focussing on the aspect of mRNA stability, it has been demonstrated that for *E. coli* grown in LB medium at 37 °C, the average mRNA half-life is 5.2 minutes. However, this half-life ranges from 1.1 minutes (*hisS*) to 24.8 minutes (*gatA*), and the relative stability varies depending on the growth medium and temperature (Bernstein *et al.*, 2002). Assuming that the mRNA is more stable during cold storage due to the lower activity of degrading enzymes, it is possible that mRNA molecules could still be intact from non-viable bacteria in the 12 days, 2 °C inoculum. All RNA-labelled signal detected using microarrays were assumed to be from viable cells within the bacterial population and the RNA extracted from the cold stored cells was of a good quality (Section 2.3.7). In addition, replicate signals within and between arrays were statistically filtered to remove any genes which exhibited substantial variability.

The transcriptomic approach described in this Chapter infers physiological processes which may be important for lag phase adaptation. However, an important part of the experimental design process is validation of transcriptomic data either by additional molecular techniques such as quantitative reverse transcriptase (qRT) PCR (Yang &

Speed, 2002), or by phenotypic confirmation to remove the 'guilt by association' aspect of the transcriptomic approach (Quackenbush, 2003). Such validation methods provide proof that the rapid transcription reflects 'deliberate' gene induction and is not merely a result of transcription factors with the highest activities leading to more rapid expression of some genes rather than others.

Despite the limitations of transcriptomic approaches, the experiments described in Section 4.2.4 provided the basis for further experimental work and enabled hypotheses to be developed concerning the essential processes for cell division initiation at the transcriptional level. The gene expression profiles were reproducible and provided confidence that physiological lag phase specific 'work' was being performed. The design of the microbiological and biochemical experiments described later in this thesis (Chapter 6 and Chapter 7), were suggested by the transcriptomic approach performed in the present chapter.

4.3.2 Cold acclimation analysis

Salmonella rapidly adapts to different environments. In the cold storage experiments performed in the present study, the 25 °C bacterial culture was stored at 2 °C. After 3 hours cold storage, the culture temperature decreased to approximately 4 °C, by 5 hours the temperature was approximately 2.5 °C, and by 10 hours the culture temperature had reached 1.7 °C. Cold acclimation by *S.* Typhimurium was identified at the transcriptomic level by extensive changes in the gene expression compared with the non-chilled environment. Within 5 hours cold storage, 22 % of the genome was up- or down-regulated ≥2-fold compared to 25 °C. The most significant processes up-regulated were: Class I CspA-like family of cold-shock proteins, synthesis of ribosomal proteins and the SOS response. Down-regulation of genes was observed for crucial anaerobic metabolism, cell wall biogenesis, SPI-2 and iron transport. The process of cold acclimation has been described in *E. coli* with the induction of Class I CSPs in the early stages before Class II proteins are induced later, such as ribosomal-associated proteins, RecA (Part of the SOS-response) and global regulators such as H-NS (Thieringer *et al.*, 1998). In the present study, long term cold storage required the most extensive transcriptional re-organisation, whereas the initial cold shock and cold acclimation was primarily defined by the induction of CSP-encoding genes and other cold-inducible genes, such as *ddg*. The twelve day cold

stored bacteria consisted of a transcriptionally-distinct population compared with the bacteria stored for 5 hours. Gene induction was characterised within a variety of functional groups induced including: the SPI-1 and SPI-2 pathogenicity islands, curli fimbriae and ethanolamine utilisation genes. Although the ethanolamine utilisation genes have been reported to increase in expression during cold acclimation in the bacterium *Listeria monocytogenes* (Liu *et al.*, 2002), there are no published reports of chilled conditions increasing the virulence of pathogenic bacteria and the *eut* genes did not characterise a cold shock response in *E. coli* (Phadtare & Inouye, 2004). However, some cold-inducible genes are required for full virulence in organisms such as *Shigella sp*. and pathogenic *E. coli* (Cairrão *et al.*, 2003). Of the 214 genes down-regulated between 10 hours and 12 days cold storage, the primary functional group was involved in aerobic respiration, principally oxidative phosphorylation (defined by the down-regulation of *atp*, *cyo*, *nuo* genes), an observation that correlates well with other studies into cold adaptation of *Bacillus subtilis* (Budde *et al.*, 2006).

The Lag Phase 25 °C and the Lag Pre-Incubation inocula exhibited distinct transcriptional profiles. The extended period of storage repressed genes involved in central aerobic and anaerobic metabolic processes as well as genes encoding translation machinery. This circumstantial evidence inferred that long-term cold adapted cells decrease metabolic activity, but survive stresses associated with cold storage. This is of potential concern to the food industry, as refrigeration is the most commonly-used method of commercial food storage (Phadtare, 2004). Similarly, the rapid recovery of cold-adapted bacteria in more favourable environments is of concern to the food industry, as it could lead to infections from foodborne pathogens or potentially food spoilage.

4.3.3 Lag Pre-Incubation transcriptome

The potentially quiescent metabolic state of stationary phase cold-stored bacteria contrasts with recovery from cold storage. Lag phase requires the transcriptional re-programming of 1658 genes compared with the stationary phase inoculum. 875 genes (> 18 % genome) are differentially-expressed within the first four minutes alone. The lag phase induction of genes using this same system without the cold pre-incubation step has been reported elsewhere (Rolfe, 2007), and formed the primary comparison study for this work. A more recent study in *E. coli* compared the transcriptome of ‘young’ (48 hours) and ‘old’ (17

days) bacterial cells during lag phase after inoculation into fresh medium (Pin *et al.*, 2009). The Pin *et al.* (2009) study discovered that during lag phase old cells increased the expression of genes encoding iron ion transport, iron-sulphur cluster maintenance and ribonucleoside reduction, required for DNA synthesis. Interestingly, there was no increase in oxidative stress resistance genes during lag phase of old cells inoculated into fresh medium, which differed from the present study. This suggests that storage at 2 °C in the present study may be responsible for the increased oxidative sensitivity inferred by the transcriptomic data during lag phase.

Like the Lag Phase 25 °C study, many genes were lag phase regulated, as well as some genes being temperature upshift-regulated, in this study. Lag phase induced genes were involved in diverse physiological processes including: oxidative stress resistance, inorganic phosphate uptake, metal ion transport, RNA polymerase II, ribosomal protein-encoding genes, aerobic respiration genes and specific global regulators. Genes encoding many of these processes were induced through the majority of lag phase (*e.g.* ribosomal protein-encoding genes) whilst some were expressed more transiently (*e.g.* inorganic phosphate uptake genes).

Transcriptional differences between the Lag Pre-Incubation and Lag Phase 25 °C studies were generally subtle. The pre-incubation history increased the duration of up-regulation of some genes however the Lag Phase 25 °C processes were generally reproduced at the transcriptional level under Lag Pre-Incubation conditions.

4.3.3.1 Oxidative stress

The transcriptional re-programming upon entry into lag phase from cold stationary phase involved the induction of many genes involved in the resistance to oxidative stress (Storz & Imlay, 1999). Many of these genes were OxyR-regulated (Storz *et al.*, 1990), although there was also induction of *soxR* and *soxS* which respond to and protect against superoxide anions (Pomposiello & Demple, 2000, Pomposiello & Demple, 2001). The oxygen upshift experienced by the bacteria in fresh medium probably accounts for the induction of oxidative stress resistance genes, however the increased metabolic rate prior to cell division initiation may also play a part. Other studies in *E. coli* have shown that oxygen shock of anaerobic steady-state culture leads to the repression of anaerobic metabolism

transcripts within 5 minutes and ~8-fold induction of *soxS* expression (Partridge *et al.*, 2006). Interestingly, although *ahpC* and *ahpF* transcripts were up-regulated after oxygen upshift in the Partridge *et al.* (2006) study, expression of the OxyR-regulated *dps* remained constant, as did many of the iron-responsive genes. This suggests that the up-regulation of some oxidative stress resistance genes may be growth phase dependent. The induction of *soxS* and *sodABC* was also detected during the initiation of *E. coli* aerobic growth on LB agar plates (Cuny *et al.*, 2007), suggesting that these bacteria experience oxidative stress prior to cell division initiation.

In the Lag Pre-Incubation experiment, induction of OxyR-regulated genes was observed more rapidly post-inoculation than in the Lag Phase 25 °C experiment. This oxidative stress response correlated with a delay in expression of some iron transport genes. It has been shown that the intracellular iron ions reacting with endogenous hydrogen peroxide can cause oxidative damage in bacteria (Imlay, 2003), via the Fenton Reaction (Wardman & Candeias, 1996). In addition, a study found that in *E. coli* changes in the oxygen concentration led to the induction of genes involved in efflux or regulation of redox reactive metals (such as copper and iron) to be expressed under the control of the transcriptional regulator FNR (Partridge *et al.*, 2007). The sensitivity to hydrogen peroxide by cold shocked *E. coli* and *S.* Typhimurium has been observed elsewhere and this increased oxidative stress was shown to affect subsequent recovery in rich medium (Mackey & Derrick, 1986b). In addition, the temperature upshift experienced by pathogenic bacteria during recovery may lead to increased resistance to additional heat treatments performed during food processing (Mackey & Derrick, 1986a). The involvement of metal homeostasis and oxidative stress to lag phase adaptation is investigated in Chapter 6 and Chapter 7.

4.3.3.2 Inorganic phosphate uptake

Phosphate is an essential micronutrient required for the synthesis of nucleic acids and the production of ATP (Harold, 1966). The uptake of phosphate by *S.* Typhimurium requires the high affinity transporters encoded by the *pstSCAB* gene cluster. During the transition to lag phase there was a specific up-regulation of these high-affinity transporters during early lag phase. Indeed *pstS* was the most highly-expressed gene at 4 minutes post-inoculation. The lower affinity phosphate assimilation genes *phoBR* (Wanner, 1996) were also up-

regulated upon entry into lag phase in Lag Pre-Incubation and Lag Phase 25 °C experiments. There were differences between the two experiments in terms of *pstSCAB* expression. In the Lag Phase 25 °C experiment a sharp peak of co-expression of was observed only at 4 minutes (Rolfe, 2007), whereas the Lag Pre-Incubation study identified induction of these phosphate genes over 60 minutes of lag phase.

A previous study using fluorescence emission spectroscopy analysis quantified the concentration of phosphate in LB medium to be 6 mM and considerably higher in M9 minimal media (64 mM), due to the addition of phosphate salts (Schurig-Briccio *et al.*, 2009). Although the uptake of phosphate is thought to be crucial to energy production and growth by bacteria, the deletion of *pstSCAB* or *phoBR* did not cause an increase in lag time, nor affect growth rate in the static system (Rolfe, 2007). No phenotypic confirmation of lag phase intracellular phosphate was performed in the present study due to time limitations, although this confirmation could be crucial to identifying novel transports of phosphate which compensate for loss of the characterised high-affinity phosphate system. One potential problem with using spectroscopic techniques to quantify intracellular phosphate is the relatively high concentration of cells required to reach the detection limit. A sensitive technique would be necessary to detect phosphate from the most interesting time-point (4 minutes) which raises the possibility of using quadropole inductively-coupled plasma mass spectrometry to detect oxidised PO^+ from low bacterial concentrations (Bandura *et al.*, 2002). Although highly-sensitive, the technique is expensive to perform and requires refined protocols to analyse biological samples (Henriette Ueckermann, personal communication).

4.3.3.3 Metal homeostasis

Upon entry into lag phase from cold storage there was a marked up-regulation in transporters for a range of metal ions including: iron, manganese, magnesium and calcium. This correlates well with the previous study into *S.* Typhimurium lag phase in the static growth system (Rolfe, 2007) which identified the up-regulation of some of these transporters as early as 4 minutes post-inoculation. There was a considerable down-regulation of transporters specific for other metal ions such as cobalt, nickel and molybdenum, which are known to be expressed under anoxic conditions (Ballantine & Boxer, 1985, Kalman & Gunsalus, 1990, Jeter *et al.*, 1984). In contrast, the gene encoding

the nickel and cobalt specific efflux protein YohM (Rodrigue *et al.*, 2005) was up-regulated during oxygen upshift in lag phase in both Lag Pre-Incubation and Lag Phase 25 °C experiments.

There were important differences between the Lag Pre-Incubation and Lag Phase 25 °C experiments in terms of metal ion homeostasis. In Lag Pre-Incubation experiments, iron homeostasis was split into two defined phases with a subset of iron homeostasis genes up-regulated within 4 minutes of inoculation into fresh LB medium and other iron homeostasis genes up-regulated after a delay until the end of lag phase (120 minutes). The early-lag phase subset of iron-responsive genes included *dps* which binds intracellular iron; whereas the latter subset of genes included siderophore encoding genes to transport extracellular iron from the growth medium. The delay in transporting extracellular iron may reflect oxidative stress experienced by Lag Pre-Incubation bacteria during early lag phase, which would be exacerbated by the influx of iron, through Fenton chemistry (Wardman & Candeias, 1996). This hypothesis was supported by the increase in expression of manganese ion transporters by 60 minutes and 120 minutes in Lag Pre-Incubation conditions compared with the Lag Phase 25 °C experiment. The uptake of manganese has recently been reported to alleviate Fenton reaction-derived oxidative stress by replacing iron in some metallo-proteins (Anjem *et al.*, 2009). The almost uniform reduction in expression of iron-responsive transport genes at 20 minutes Lag Pre-Incubation compared with the same time-point in the Lag Phase 25 °C experiment suggests a decreased physiological need for iron at this point of lag phase. Transcriptional evidence for transport of metal ions is indirect and relies upon the correct annotation of transporter genes. It is probable that metal ions are accumulated into cells through non-specific pathways such as the import of nickel through TonB-dependent outer membrane receptors (Schauer *et al*., 2007). Phenotypic confirmation is required before any biological role for metal ions can be inferred. The direct intracellular measurement of metal ions using inductively-coupled plasma mass spectrometry is outlined in Chapter 7.

4.3.3.4 Energy-generating respiratory pathways

The lag phase comparison of glycolysis, the TCA cycle and oxidative phosphorylation gene expression between Lag Pre-Incubation and Lag Phase 25 °C experiments revealed an initial decrease in gene expression by 4 minutes which was maintained at 20 minutes after

recovery from cold storage. Interestingly, after this initial period the aerobic respiratory genes were expressed similarly in both experiments. The electron transport chain involves the oxidation of H^+ (Baron, 1996) which produces potentially-toxic intermediates such as superoxide (O_2^-) and peroxide (O_2^{2-}) anions (Raha & Robinson, 2000). It is reasonable to speculate that this decreased expression of genes involved in oxidative phosphorylation during the initial part of lag phase is due at least in part, to oxidative stress protection. No phenotypic confirmation of this initial down-regulation was performed in this study. However this could feasibly be measured indirectly by determining the intracellular concentration of ATP, and using this concentration as an indication of energy-generating respiration occurring during lag phase.

4.3.4 Concluding remarks

The transcriptomic experiments and analysis reported in the present study verify many of the processes reported previously (Rolfe, 2007). The Lag Pre-Incubation experiments highlight the robustness of lag phase at the transcriptomic level and indicate that the processes of pre-growth adaptation are not easily perturbed. The Lag Pre-Incubation study suggested that there may be a greater degree of oxidative stress during recovery from cold storage and that the duration of some processes may be history-dependent. Interestingly, although differences in gene expression were identified, these had no effect on the population lag time. It has been shown that for some microorganisms it is possible to use the transcriptomic responses to environmental conditions to predict growth rates (Airoldi *et al.*, 2009), and this technique could become an accurate method for determining the bacterial population lag time in the future.

Transcriptomic data offer an indirect assessment of processes which may be occurring within an organism. Phenotypic confirmations of this global regulatory picture are required to provide evidence that gene induction leads to physiological processes and some of these are explored in later chapters of this thesis.

Chapter 5

DNA binding regions of RNA polymerase during stationary phase

5. DNA binding regions of RNA polymerase during stationary phase

5.1 Introduction

In the previous chapter, DNA microarrays were used as a method to investigate relative gene expression. The approach yielded valuable information regarding the processes occurring during Lag Pre-Incubation conditions (Section 2.1.6.1) and the kinetics of gene regulation. These data identified processes rapidly induced at the onset of lag phase.

Stationary phase represents the 'pre-lag phase' experimental conditions and was used as an inoculum for lag phase studies. In this chapter, chromatin immunoprecipitation and microarrays (ChIP-chip) were combined to investigate the position of RNA polymerase on the *S.* Typhimurium genome during stationary phase. ChIP-chip was used to identify bacterial transcriptional strategies for the rapid adaptation during lag phase.

5.1.1 ChIP-chip

The method of ChIP-chip involves the covalent cross-linking of a DNA binding protein to DNA, most frequently with a chemical such as formaldehyde. The cells are then lysed chemically or mechanically and the lysate is sonicated to yield solubilised protein-associated chromatin fragments, approximately 500 base-pairs in length. The DNA target of the protein of interest can be selectively enriched through immunoprecipitation with a specific antibody. After proteolysis, the enriched DNA is labelled and hybridised to a DNA microarray (Grainger & Busby, 2008). This method was initially described in eukaryotes (Ren *et al.*, 2000) and was first used in bacteria by Laub and colleagues to investigate the CtrA regulon in *Caulobacter* (Laub *et al.*, 2002). Since then ChIP-chip has been recognised as a powerful technique to identify the regulons of other bacterial transcriptional regulators including: Fur in *Helicobacter pylori* (Danielli *et al.*, 2006), CodY in *Bacillus subtilis* (Molle *et al.*, 2003), FNR (Grainger *et al.*, 2007), CRP (Grainger *et al.*, 2005), LexA (Wade *et al.*, 2005) and RpoH (σ^{32}) (Wade *et al.*, 2006) in *E. coli* and StpA in *S.* Typhimurium (Lucchini *et al.*, 2009). In addition, ChIP-chip with RNA polymerase has been used to identify transcriptional binding regions and investigate regions of 'promoter trapping' in *E. coli* (Grainger & Busby, 2008, Grainger *et al.*, 2005, Herring *et al.*, 2005). One comprehensive study of *E. coli* RNA polymerase binding during

exponential and stationary phases of growth determined that the house-keeping sigma factor RpoD (σ^{70}) is associated with some RNA polymerase complexes during stationary phase but not during exponential phase (Wade & Struhl, 2004). A further use of ChIP-chip has been to identify the binding of the DNA-binding protein, H-NS to horizontally-acquired genes in *Salmonella*, mediating silencing of A-T rich regions including *Salmonella* pathogenicity islands (Lucchini *et al.*, 2006, Navarre *et al.*, 2006).

5.1.1.1 Analysis of data generated by ChIP-chip

Many enriched regions can be associated with a DNA-binding protein, especially if the protein of interest is present at a high concentration in the cell. ChIP-chip datasets are often large and complex, requiring dedicated software for analysis. ChIP-chip is a relatively new technique and the availability of automated analysis software is somewhat limited. One freely-available piece of software is the ChIP-on-chip analysis suite (CoCAS) which contains several features including optimised ChIP-chip normalisation and replicate quality control reports, allowing for the analysis of dye-swap experiments. The limitation of CoCAS is that the software can only be used to analyse Agilent ChIP-chip microarrays (Benoukraf *et al.*, 2009). An alternative software package for identifying regions of DNA-protein interaction is the Chromatin ImmunoPrecipitation On Tiled arrays (ChIPOTle) software (Buck *et al.*, 2005). ChIPOTle specifically identifies *bona fide* binding peaks whilst accounting for the span of the peak over adjacent genes. The peak span reveals useful information about the binding pattern of the protein of interest and the software uses a statistical filter to separate this so-called 'neighbour effect' from background noise to give 'true binding peaks' above a manually-set threshold. In this chapter, peaks of note were identified using ChIPOTle software and considered significant if they were enriched ≥2-fold with a p-value ≤0.001.

5.1.1.2 Other ChIP-chip techniques

The convenience of the ChIP-chip technique and the availability of a wide range of commercial antibodies have led to an increase in the use of ChIP coupled with other methods. The traditional ChIP-chip methodology has been optimised recently to allow specific protein-DNA complexes to be identified from less starting material (~1,000 eukaryotic cells) (Dahl *et al.*, 2009). With the emergence of Next-generation sequencing, it is possible to isolate a bound protein of interest and sequence the corresponding DNA

fragment, after the addition of DNA adapter sequences. This so-called ChIP-seq method does not require the use of microarrays (Barski *et al.*, 2007, Hoffman & Jones, 2009, Zecchini & Mills, 2009). ChIP-seq has advantages over the more traditional microarray techniques in that less starting material is required and a higher resolution of protein binding regions is obtained (Hoffman & Jones, 2009).

5.1.1.3 Relevance of RNA polymerase binding to lag phase

The transcriptomic analysis performed in Chapter 4 and by Rolfe (2007) identified genes expression profiles throughout growth. During stationary phase, transcription does not occur as rapidly as during exponential growth (Ishihama, 1991) and as RNA polymerase activity is decreased, the chromosome binding position of RNA polymerase may give clues to genes crucial for stationary phase survival and could explain the rapid transcriptional response of certain genes during entry into lag phase. A study investigating RNA polymerase II binding and transcription in *Saccharomyces cerevisiae* throughout growth, identified 91 genes that were ≥4-fold up-regulated within the first three minutes of lag phase, compared with stationary phase (Radonjic *et al.*, 2005). The authors concluded that the rapid transcription during entry into lag phase was due to RNA polymerase II binding but not actively transcribing genes during stationary phase resulting in a 'poised' state that was maintained until transcription is required during lag phase. The binding of RNA polymerase upstream of non-transcribed genes under conditions of stress has also been reported in *E. coli* K-12 (Grainger *et al.*, 2005) and may serve as a strategy to resume rapid growth under more favourable conditions.

Based on the rapid transcription observed during *Salmonella* lag phase (Chapter 4 and Rolfe, 2007), the following hypothesis was formed:

During pre-lag phase conditions (stationary phase), RNA polymerase is bound at, or near to, the promoter region of critical lag phase genes to aid rapid transcriptional reprogramming upon nutrient upshift.

The results from this chapter were analysed in relation to this hypothesis.

5.2 Results

5.2.1 Optimisation of ChIP-chip protocol

Most previous prokaryotic ChIP-chip studies have focussed on the position of DNA binding proteins in *E. coli*, during exponential growth. The present study using *Salmonella* found that stationary phase bacteria were more difficult to lyse by previously-used enzymatic methods. This was hypothesised to be due to the increased production of the alternative sigma factor (RpoS) and the transcriptional regulator Crl (Robbe-Saule *et al.*, 2007), which aid bacteria in resisting stresses during stationary phase (Hengge-Aronis, 1993). Furthermore, the 25 °C growth temperature was less than optimal and may have induced alteration of membrane components (Phadtare, 2004), contributing to lysozyme resistance.

The immunoprecipitation of the protein-DNA complexes is outlined in Section 2.3.11. Briefly, this process includes: cross-linking the bacterial proteins to the chromatin, quenching the cross-linking reaction with glycine, lysis of bacterial cells and extraction of the protein-DNA complex. To cross-link the DNA, formaldehyde was added to the bacterial cultures during stationary phase, initially for 15 minutes, although the cross-linking time was decreased during method optimisation. To lyse the stationary phase bacteria, a mixture of enzymatic and mechanical techniques were utilised during the method optimisation, to gain a sufficient bacterial lysate. After each attempt, samples were electrophoresed on an agarose gel to determine the success of the lysis by visualising the chromosomal DNA (Figure 5.1). Initial lysis attempts involved enzymatic and chemical steps, using lysozyme with the addition of sodium dodecyl sulphate (SDS). After sonication, no DNA was observed on an agarose gel, indicating that the cells were not disrupted (Figure 5.1A). A weak band was observed at ~3 kb however the band did not decrease in size after additional sonication (Figure 5.1B).

Subsequent lysis methods were performed using mechanical techniques. Bead-beating is a method whereby small glass beads pulverise the bacterial cells by vigorous agitation. When bead beating was utilised, lysozyme treatment was not required, as sufficient mechanical lysis occurred.

	A.	B.	C.	D.	E.	F.	G.
X-link (minutes)	15	15	15	2	2	2	2
Lysis method	Lz	Lz	BB	BB	FP	FP	FP
SDS (0.2 %)	Y	Y	Y	N	N	N	N
Lysozyme (mg / ml)	20	20	N	N	20	20	20
Sonication (10 µm)	3x	5x	3x	3x	3x	4x	5x

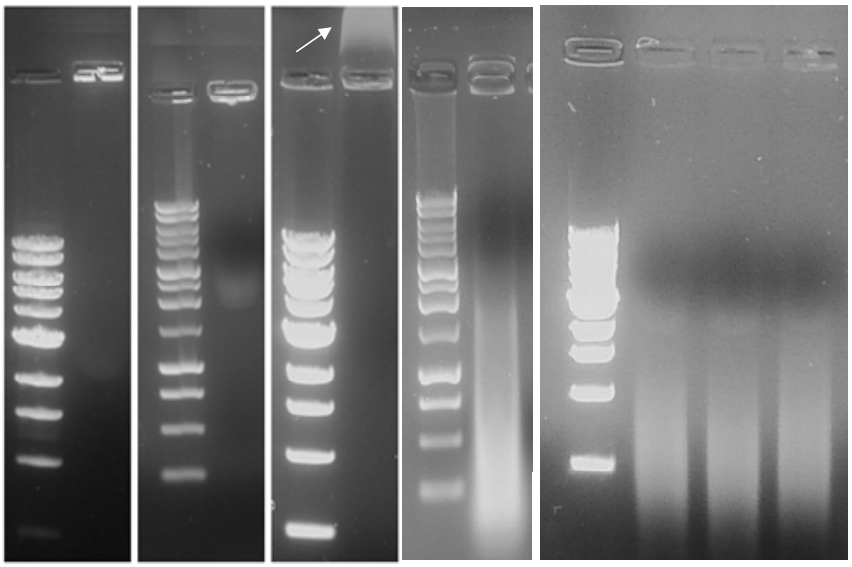


Figure 5.1: Optimisation of bacterial lysis yields sufficient DNA for ChIP-chip.
Composite image of a 1 % (w/v) agarose gel used to determine bacterial lysis and DNA fragment sizes. A-G show representative samples from separate wild-type stationary phase samples. The table shows the lysis conditions for each sample including: Formaldehyde cross-linking duration (X-link); the principal type of lysis method: lysozyme (Lz), bead-beating (BB) or French press (FP); the presence (Y) or absence (N) of SDS; the concentration of lysozyme, unless absent (N); and the number of 20 second sonication rounds performed. In the case of the errant DNA migrating sample (C), a white arrow indicates the DNA extracted. For clarity, the optimum DNA fragment size is indicated for samples D-G (500 bp). The DNA gels were run with two different 1 kb markers either from New England Biosystems (M_1) or Promega (M_2). See Section 2.3.8 for details. For further ChIP-chip experiments the lysis method (D) was used.

Initial bead-beating lysis yielded DNA however the size of fragments could not be determined on an agarose gel, as the extracted DNA-protein complex migrated towards the negative electrode during electrophoresis (Figure 5.1C). A possible reason for this unexpected migration was the relatively lengthy formaldehyde cross-linking step, which may have caused several proteins to be attached to the DNA, ultimately affecting the overall charge of the complex. Further optimisation steps decreased the formaldehyde treatment time from 15 minutes to 2 minutes which was sufficient for adequate cross-linking to occur (Sacha Lucchini, personal communication). As part of further optimisation, SDS was no longer added to the lysis reaction, as substantial froth was produced after bead beating, making the cell lysate difficult to obtain. These optimisation steps yielded a sufficient concentration of DNA for the ChIP-chip technique (Figure 5.1D).

Mechanical lysis was also performed using a French press (1800 PSI) after lysozyme (20 mg / ml) treatment (Figure 5.1E-G). This method also yielded sufficient DNA for the ChIP-chip experimental procedure. However for subsequent ChIP-chip experiments, lysis was performed using the conditions in Figure 5.1D as this method was preferable to the French press technique as bead beating is less laborious and allows greater recovery of cell lysate.

5.2.2 Quality control of four ChIP-chip replicates.

The protein-DNA fragment complexes from the successfully lysed cell samples were treated as per the ChIP-chip protocol (Section 2.3.11) and hybridised against oligonucleotide microarrays to generate an RNA polymerase binding profile for four separate biological replicates. The oligonucleotide binding strengths of each replicate were compared in a pair-wise analysis to ensure experimental reproducibility. Generally, all replicates showed a good correlation co-efficient ranging between 0.71 and 0.83 (Figure 5.2). From this analysis it was possible to ascertain that biological replicate three displayed the most variance compared with the other replicates (correlation co-efficient 0.71-0.78). However a few bound oligonucleotides did not correlate between other replicates, notably replicates two and four, which showed high enrichment of some oligonucleotide bound regions in one replicate but not in another.

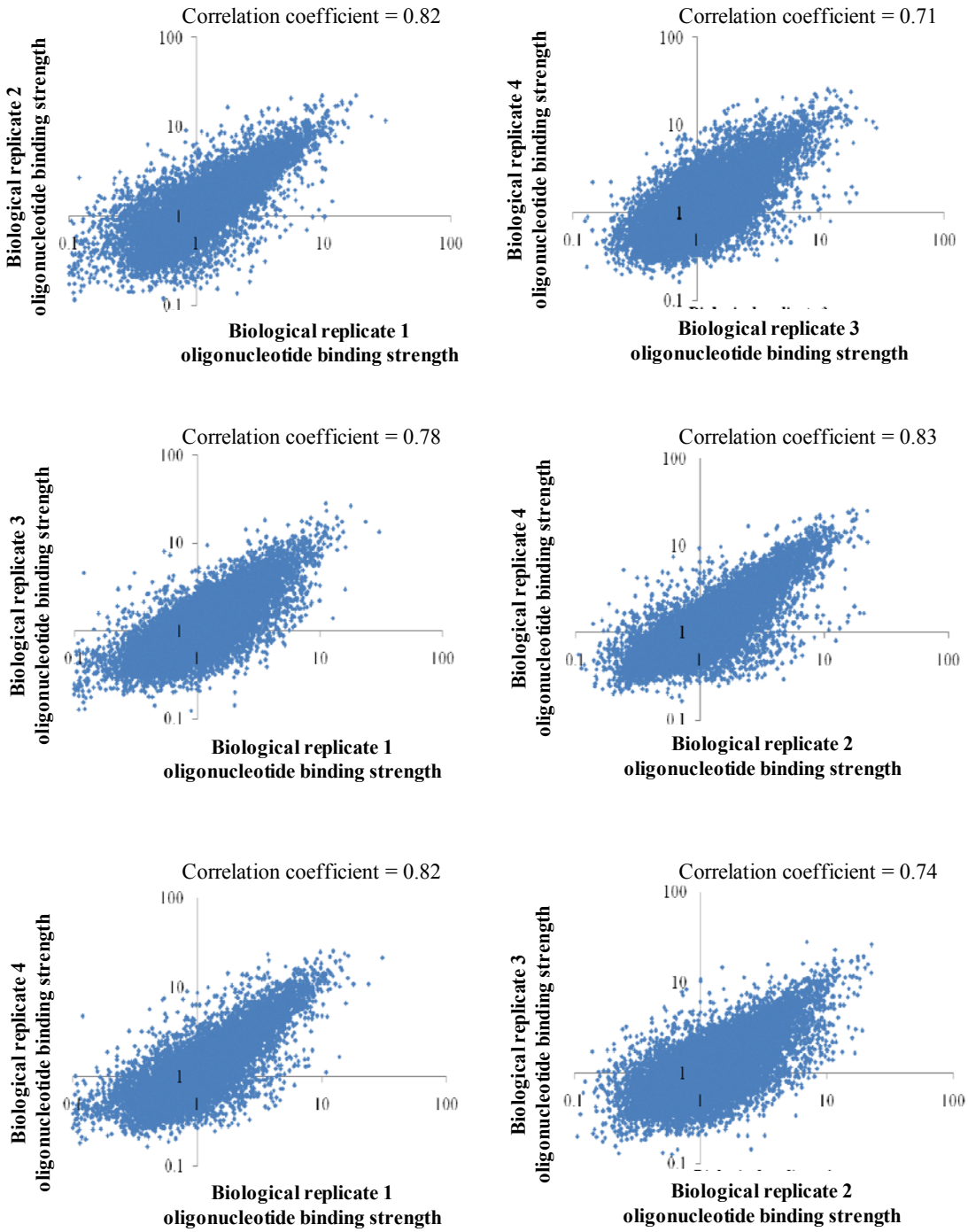


Figure 5.2: The ChIP-chip method generally produces reproducible results.
Comparison for each of the four biological replicates performed. Each of the 22577 raw oligonucleotide binding values were compared between each pair of replicates and a general correlation co-efficient calculated.

5.2.3 RNA polymerase binding compared with gene expression

The non-correlating oligonucleotides were likely the result of problems in the hybridisation of the chromosomal DNA to the microarray or the washing steps following hybridisation (Section 2.3.11.4 and Section 2.3.11.5, respectively). This basic analysis ensured the ChIP-chip data were sufficiently reproducible of further statistical analysis with the ChIPOTle macro. ChIPOTle analysed the oligonucleotide binding profiles and identified significant peaks of RNA polymerase binding (Gaussian statistical test $p \leq 0.001$, 2-fold enriched).

The genomic regions significantly bound by RNA polymerase were characterised by their proximity to gene coding regions (Section 2.3.11.6). In total, 963 genes present in both SL1344 and LT2 genomes were identified as being 'associated' with RNA polymerase binding at stationary phase. Associated genes were defined as being either spanned completely by RNA polymerase or located immediately up- or downstream of RNA polymerase binding regions. To correlate stationary phase gene expression with RNA polymerase binding, the relative expression of 4351 *S.* Typhimurium genes during stationary phase, from a dataset generated by Rolfe (2007), was determined. Genes encoding ribosomal proteins and cellular metabolic enzymes were the most represented functional group of the 100 highest expressed genes during stationary phase (Appendix D, Table 1) and these chromosomal regions were generally also bound by RNA polymerase during stationary phase (Figure 5.3).

Conversely, there were several genes expressed highly which were not associated with RNA polymerase binding. The highest expressed of these genes was *ilvH*, encoding a subunit of acetolactate synthase III.

Many of the ribosomal protein-encoding genes and the OxyR-regulated *katG*, *ahpC* and *dps* were highly expressed during stationary phase, although the genes were not bound by RNA polymerase. A possible explanation is that the genes were transcribed during an earlier stage of growth and the stable mRNA transcripts subsequently bound to the microarray. This hypothesis was not explored in depth in the present study.

Figure 5.3: Genes bound by RNA polymerase during stationary phase are generally expressed.
Stationary phase expression of 4351 genes either associated (972 black circles) or non-associated (3379 grey circles) with RNA polymerase binding during stationary phase from four biological replicates. Genes are ranked alphabetically (i.e. *aadA* = 1; *zur* = 4351). All gene expression data were analysed from an existing dataset (Rolfe, 2007).

It was expected that gene coding regions entirely bound by RNA polymerase would be highly expressed during stationary phase, as RNA polymerase would be in the process of active transcription. To test this hypothesis, eight time-points were selected to compare the expression of the 313 genes coding regions bound entirely by RNA polymerase and present in the *S.* Typhimurium LT2 and SL1344 genomes (Figure 5.4A). The time-points selected were the 48 hour stationary phase inoculum, 4, 20, 60 and 120 minutes post-inoculation (lag phase); mid-exponential phase (MEP), late-exponential phase (LEP) and 24 hours post-inoculation, representing an early stationary phase (ESP) time-point. The analysis revealed that the median expression of genes bound by RNA polymerase during stationary phase was increased in the ESP and 48 hour inoculum cultures (median gene expression = 2.1 and 1.9, respectively) than lag phase cultures (average median expression = 1.1). Although the stationary phase samples had a higher median expression than lag phase samples, the mean gene expression was highest at mid-lag and the end of lag phase. This indicates that in general, the expression of genes was higher at stationary phase however during lag phase a minority of genes, such as the oxidative stress resistance genes, were expressed very highly, increasing the mean gene expression. The 48 hour stationary phase expression of genes bound in the inoculum were studied in greater detail (Figure 5.4B) to determine which genes were present at elevated expression. This visualisation revealed that ribosomal protein encoding genes were particularly elevated however 28 % genes completely bound by RNA polymerase during stationary phase exhibited decreased expression (< 1). This finding reveals an advantage of ChIP-chip over conventional microarrays in detecting the location of RNA polymerase binding even when gene expression is low. The low expressed genes may be examples of 'trapped' RNA polymerase molecule used to actively prevent specific gene transcription (Grainger & Busby, 2008), although this was not specifically tested, and has not been reported elsewhere.

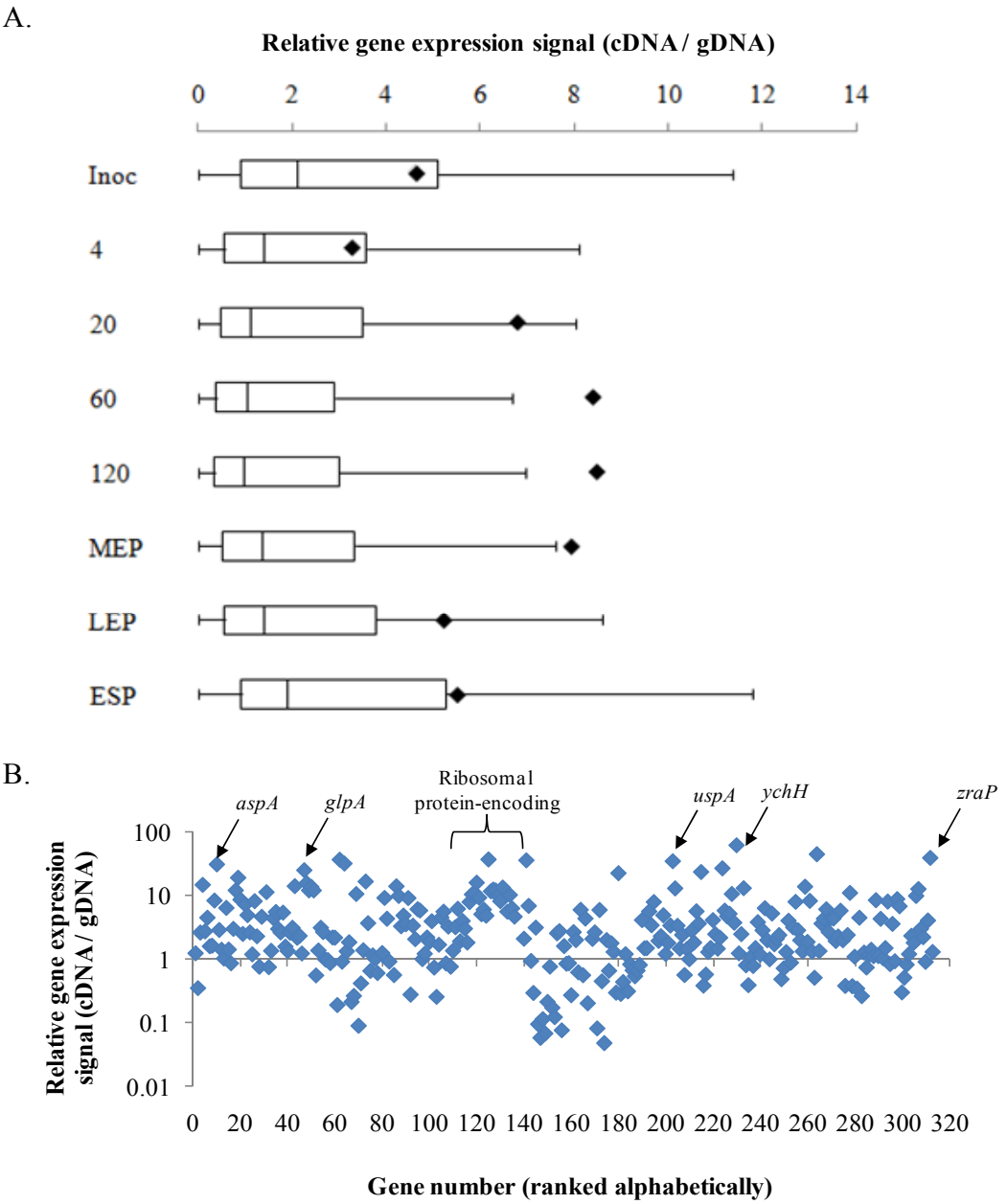


Figure 5.4: Genes bound by RNA polymerase are not all expressed highly.
The gene expression profile of the 313 genes entirely bound by RNA polymerase during 48 hours stationary phase, based on four independent ChIP-chip replicates. (A) 'Box and whisker' plot describing the expression of genes bound entirely by RNA polymerase. Boxes represent the interquartile range of the data and whiskers describe the minimum and maximum valid values. The vertical line in each box represents the median gene expression value and the mean expression is indicated by a black diamond (◆). For clarity, the outlying gene expression values are not shown, but are included in the analysis. Time-points indicate minutes after inoculation except for 48 hour stationary phase inoculum (Inoc), mid-exponential phase (MEP), late exponential phase (LEP) and the 24 hour early stationary phase (ESP). (B) Individual gene expression data of the 48 hour stationary phase inoculum. Genes are ranked alphabetically (i.e. *aarF* = 1; *zur* = 313). A sub-set of highly-expressed genes are shown. Gene expression data in the lag phase static system were taken from a previous study (Rolfe, 2007).

It was possible that, during bacterial stationary phase, RNA polymerase could bind upstream of important lag phase genes. This strategy would allow the subsequent rapid transcription of lag phase genes upon inoculation in fresh medium. To test this hypothesis, the expression of 342 genes downstream of RNA polymerase binding regions was examined during stationary phase and seven other time-points throughout growth (Figure 5.5). The genes downstream of the RNA polymerase binding regions were expressed higher during mid-exponential phase (median gene expression = 1.11) than either 48 hours stationary phase (median gene expression = 1.00) or lag phase (average median expression = 0.85). The median gene expression was highest during mid-exponential phase, although the median expression values were similar at all time-points tested. As seen with genes spanned by RNA polymerase during stationary phase (Figure 5.4), the mean gene expression values were higher during lag phase than late-exponential or stationary phase, indicating that some lag phase-specific genes were expressed very highly. The functional categories of highly-expressed lag phase genes in *S.* Typhimurium grown in Lag phase 25 °C conditions are detailed in Rolfe (2007).

5.2.4 RNA polymerase binding to rRNA regions

The routine analysis of the ChIP-chip data described so far was suitable to identify annotated gene coding regions associated with RNA polymerase binding. However the data were mined further to identify any strong binding to intergenic regions, which may be crucial for the production of RNA species, such as ribosomal RNA (rRNA). It was hypothesised that RNA polymerase would bind to rRNA-encoding genes during stationary phase, as other ChIP-chip enriched regions included genes encoding ribosomal proteins. This suggested that RNA polymerase was bound to genes encoding machinery for the assembly of ribosomes, including rRNA encoding genes. During lag phase the *de novo* synthesis of ribosomes is essential and requires rapid transcription and translation (Kjeldgaard *et al.*, 1958, Rolfe, 2007). In order to form ribosomes, rRNA would need to be transcribed early in lag phase and could conceivably be bound during stationary phase, in preparation for inoculation into fresh medium. In addition, rRNA and ribosomal protein encoding genes have been shown to be bound by RNA polymerase in a previous yeast study (Radonjic *et al.*, 2005).

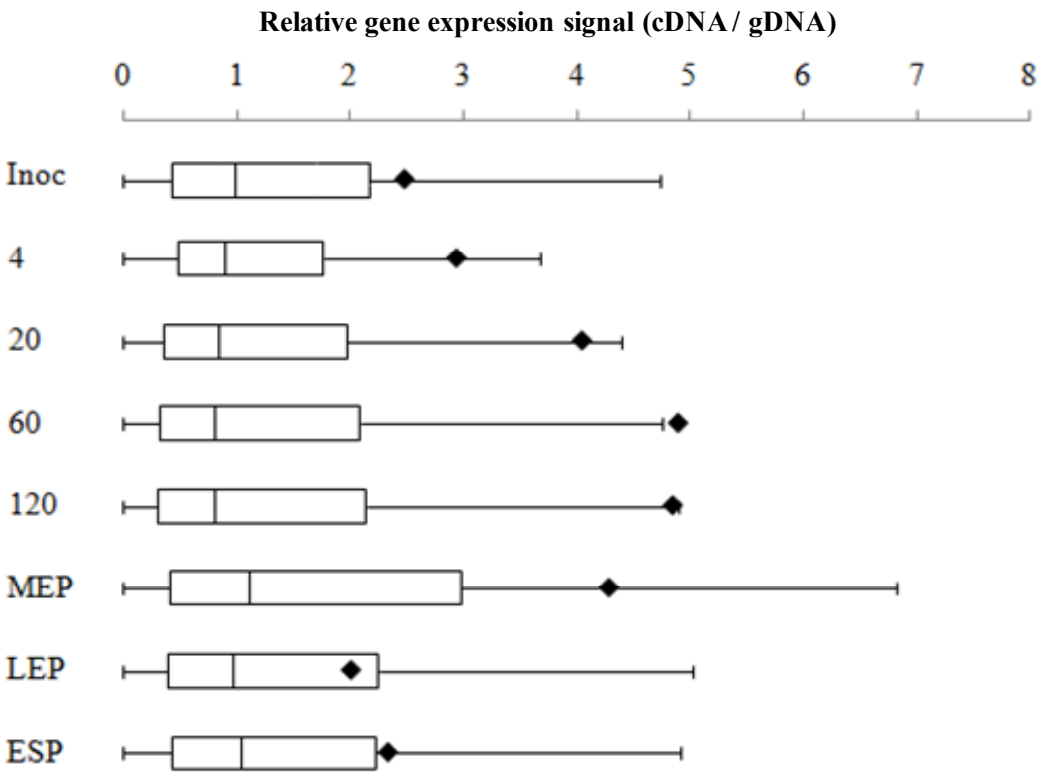


Figure 5.5: Genes bound upstream during stationary phase are not expressed highly at any specific growth phase.
'Box and whisker' gene expression profile of the 342 genes bound by RNA polymerase upstream of the open reading frame during 48 hours stationary phase. Boxes represent the interquartile range of the data and whiskers describe the minimum and maximum valid values. The vertical line in each box represents the median gene expression value and the mean expression is indicated by a black diamond (◆). For clarity, the outlying gene expression values are not shown, but are included in the analysis. Time-points indicate minutes after inoculation except for 48 hour stationary phase inoculum (Inoc), mid-exponential phase (MEP), late exponential phase (LEP) and the 24 hour early stationary phase (ESP).The gene expression data in the Lag Phase 25 °C growth system were taken from a previous study (Rolfe, 2007). RNA polymerase binding determined by ChIP-chip of four independent replicates.

To determine whether RNA polymerase was bound to rRNA-encoding regions during stationary phase, the seven rRNA regions spaced around the 5 Mb SL1344 chromosome were analysed (Figure 5.6). For each rRNA-encoding region there was strong ChIP-chip enrichment (8.8-13.0 fold), indicating RNA polymerase binding in the intergenic regions. This strong binding suggests that rDNA regions are bound by RNA polymerase however the binding appears irregular, suggesting some areas are bound at a higher frequency than other regions. Binding peaks tended to be higher at the *rrs* family of genes, encoding the 16S rRNA with the lowest binding present at the small *rrf* genes, encoding 5S rRNA. The rRNA-encoding regions are spread around the *S.* Typhimurium SL1344 chromosome (Figure 5.6H) but high enrichment was identified in each region, suggesting that RNA polymerase bound strongly although production of rRNA is known to be low during stationary phase (Aviv *et al.*, 1996). There is the possibility that the high degree of sequence similarity between rDNA regions (≥ 99%) may lead to oligonucleotide hybridisation from one highly-expressed region to show enrichment to all seven regions. Further experimental evidence would be required to identify differentially-expressed rDNA regions, possibly through single-copy transcriptional GFP fusions to monitor whether fluorescence was present at equal intensity for all rDNA regions (Hautefort *et al.*, 2003).

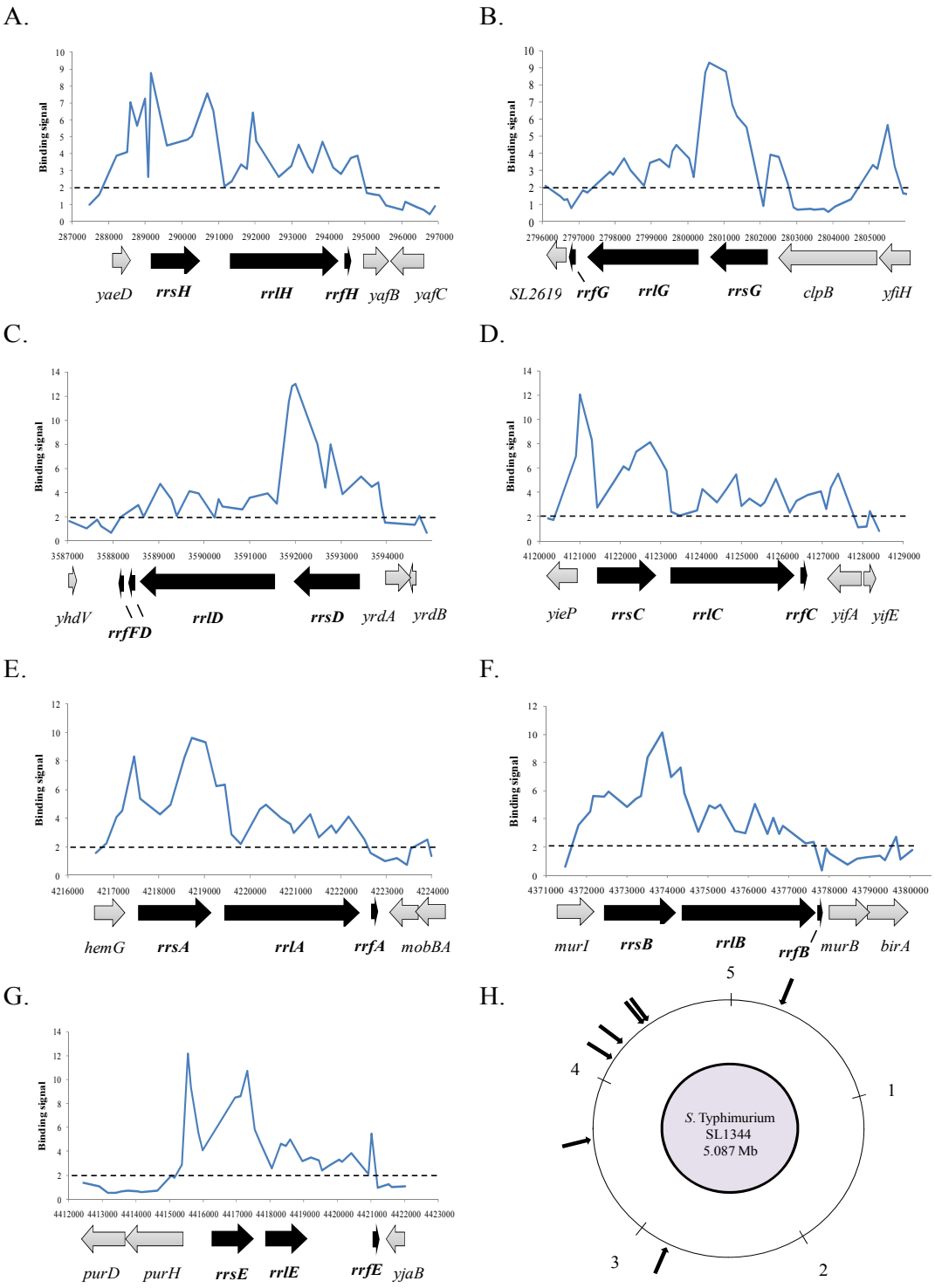


Figure 5.6: RNA polymerase binds rRNA regions during stationary phase.
(A-G) Seven rRNA-encoding regions on the *S.* Typhimurium SL1344 chromosome shown within their genetic context. The mean RNA polymerase binding from four independent replicates is shown for the rRNA-encoding genes (black arrows and bold text). Significant RNA polymerase binding is indicated by peaks above the 2-fold cut-off (_ _). (H) The genomic context of all the seven regions is shown with rRNA regions indicated by black block arrows. Numbers on the outside of the outer circle are the location relative to position zero measured in millions of base-pairs (Mb).

5.2.5 RNA polymerase binding to selected sigma factor-encoding genes

The global transcriptomic data for *S.* Typhimurium during stationary phase revealed that a few RNA polymerase sigma factor (σ-factor)-encoding genes are highly-expressed, including *rpoD*, *rpoE*, *rpoH* and *rpoS* (Rolfe, 2007). These σ-factors allow the sequestration RNA polymerase to regions to transcribe function-specific genes including housekeeping genes (RpoD regulated) and stationary phase survival genes (RpoS regulated). By analysing the RNA polymerase binding patterns to each of the σ-factors, the environmental triggers affecting bacterial physiology under the experimental conditions at stationary phase could be elucidated. The RNA polymerase binding to four σ-factors was analysed (Figure 5.7). The remaining *S.* Typhimurium σ-factor encoding genes (*fliA* and *rpoN*) were not highly expressed during stationary phase, nor were they specifically bound by RNA polymerase. The expression of *rpoD* during stationary phase was elevated slightly, correlating with a low RNA polymerase binding (Figure 5.7A). However, there was an elevated peak at the end of the upstream gene, *dnaG* corresponding with a cluster of *rpoD* promoter regions, suggesting that RNA polymerase may be bound in preparation for the transcription of housekeeping genes once the bacteria enter lag phase.

Binding of RNA polymerase to the *rpoE* gene and the upstream promoters were observed, suggesting that RpoE was produced during stationary phase. However there was also RNA polymerase binding to the highly-expressed RpoE anti-sigma factor encoding gene, *rseA* (Figure 5.7B). The regulation of RpoE occurs post-transcriptionally through binding by the inner membrane bound RseA. This mode of regulation was not investigated in the present ChIP-chip or transcriptomic studies. The high RNA polymerase binding to *rseA* and *rpoE* does not reveal whether envelope stress is occurring during stationary phase, as the activation of RpoE occurs after proteolytic degradation of RseA in complex with RpoE (Rowley *et al.*, 2006).

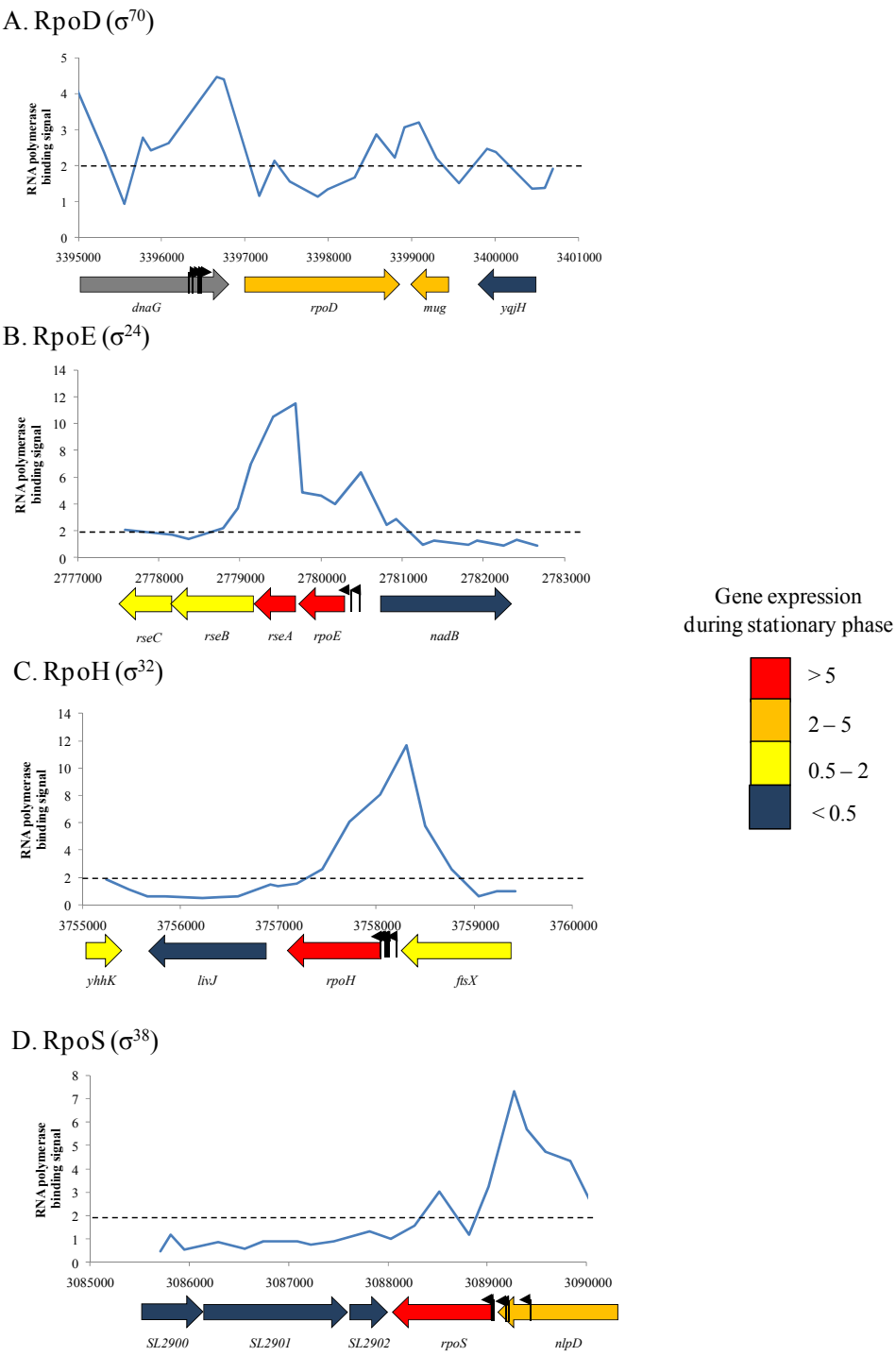


Figure 5.7: RNA polymerase binding occurs upstream of selected sigma factors.
The mean RNA polymerase binding from four independent replicates is shown for four selected sigma factors. Significant RNA polymerase binding is indicated by peaks above the 2-fold cut-off (- -). The genetic context is shown on the *S.* Typhimurium SL1344 chromosome for each of the sigma factors and neighbouring genes. The gene expression is shown by coloured block arrows corresponding to a range of expression values taken from Rolfe (2007). A grey-filled arrow indicates the expression of the gene was absent from the transcriptomic data. The positions of known sigma factor promoter regions in *E. coli* are shown as filled, black arrows. Promoter regions based on information from EcoCyc.

The expression of *rpoH* was high during stationary phase and a large peak of RNA polymerase binding was identified at the location of the promoter region (Figure 5.7C), strongly suggesting that *rpoH* is being transcribed during stationary phase. The transcription of *rpoH* occurs despite the absence of any discernible heat shock during stationary phase, suggesting another role for RpoH, possibly as a regulator for chaperone encoding genes to aid the folding of stationary phase proteins. An alternative hypothesis involves the induction of *rpoH* and *rpoE* as part of bacterial lag phase adaptation, as has been demonstrated in *E. coli* during the earliest stages of growth on LB agar plates (Cuny *et al.*, 2007). The production of RpoE and RpoH may be part of sigma factor functional overlap for RpoD-regulated genes, as shown using ChIP-chip studies in exponentially-growing *E. coli* (Wade *et al.*, 2006). Although the expression of *rpoS* was elevated during stationary phase, it should be noted that RNA polymerase binding was only slightly elevated over the gene coding region. Conversely, the adjacent gene *nlpD*, encoding a lipoprotein, exhibited a high RNA polymerase binding to the terminal region, although expression of *nlpD* was lower than *rpoS*. The RpoD-regulated *rpoS* promoter is required to maintain a basal concentration of RpoS and is located within the upstream *nlpD* gene in *Salmonella* (Paesold & Krause, 1999). The high ChIP-chip peak indicates that RNA polymerase was bound at this *rpoS* promoter region (Figure 5.7D) to transcribe this crucial stationary phase specific sigma factor.

5.2.6 RNA polymerase binding to selected regulatory genes

Bacteria must adapt rapidly to changing environments and so require regulators to transcribe genes involved in this adaptation. The binding of RNA polymerase to selected regulatory genes was analysed from the ChIP-chip data (Figure 5.8). Three regulator-encoding genes were examined: *arcA*, *fnr* and *hns*. ArcA and FNR proteins are transcriptional regulators required for bacteria to adapt to changing oxygen concentrations, an important switch when moving from the anoxic stationary phase conditions to aerobic lag phase conditions (Section 3.2.2 and Section 6.2.1). H-NS is a transcriptional repressor of various genes including the major pathogenicity islands. Each of these regulatory genes was expressed highly in the stationary phase environment. RNA polymerase was bound to regions upstream of *arcA*, within the coding region of *yjjY*, encoding a putative inner membrane protein (Figure 5.8A).

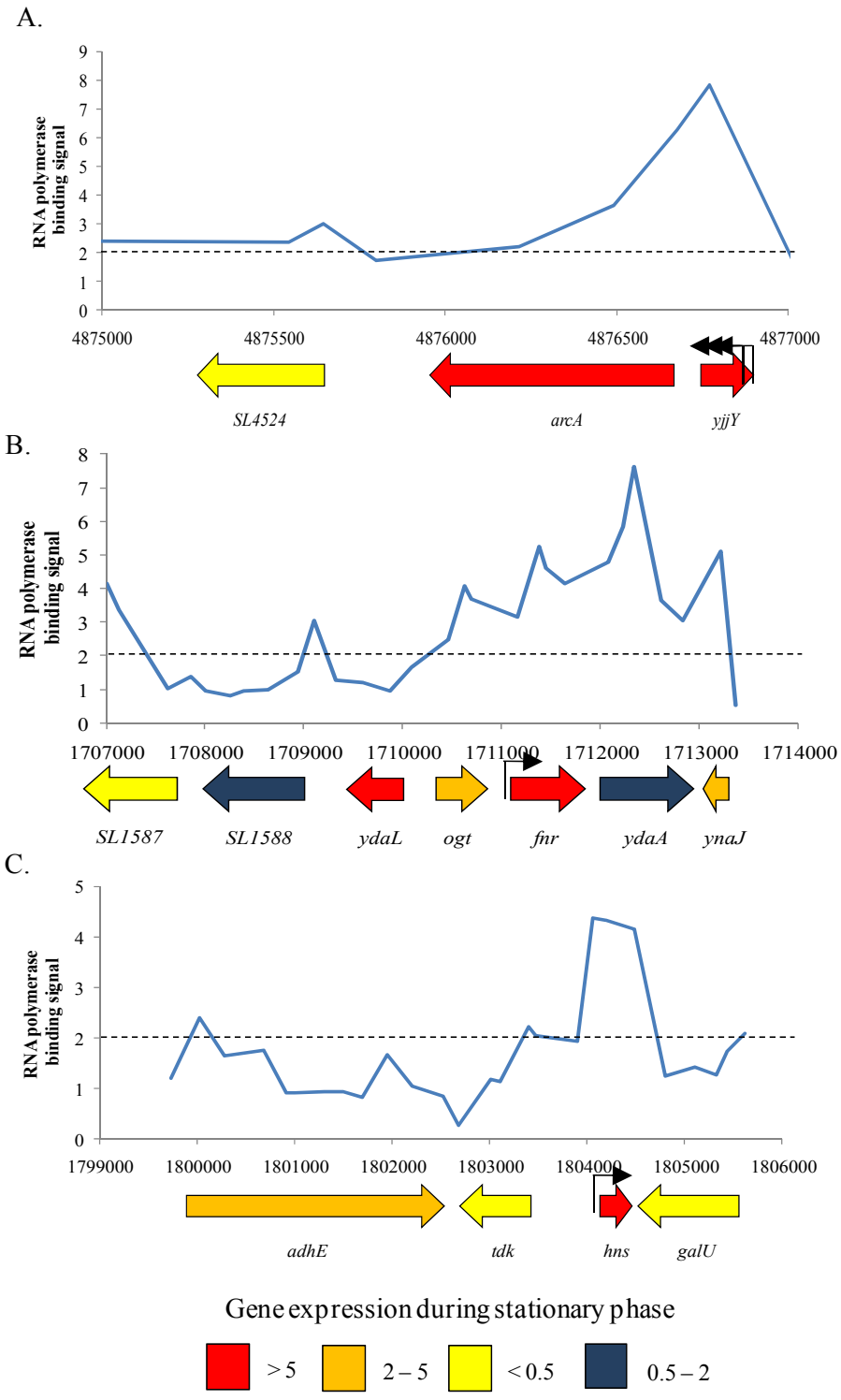


Figure 5.8: RNA polymerase binding is associated with selected regulatory genes. The mean RNA polymerase binding from four independent replicates is shown for four selected regulators. Significant RNA polymerase binding is indicated by peaks above the 2-fold cut-off (- -). The genetic context is shown on the *S.* Typhimurium SL1344 chromosome for each of the regulators and neighbouring genes. The gene expression is shown by coloured block arrows corresponding to a range of expression values taken from Rolfe (2007)-generated data. The positions of known regulator promoter regions in *E. coli* are shown as filled, black arrows. Promoter regions based on information from EcoCyc.

Crucially, the peak of RNA polymerase binding corresponded with the *arcA* promoter region. The three σ^{70}-regulated promoters are present in close proximity and therefore it was not possible to identify which specific promoter was bound by RNA polymerase. RNA polymerase binding to *arcA* suggested that during stationary phase, ArcA was required, most likely for adaptation to the anoxic environment. The pattern of RNA polymerase binding to *fnr* was more complex. Although the RNA polymerase binding spanned the promoter and gene coding regions, there were multiple localised binding peaks, the largest of which corresponded to the start of the *ydaA* gene. *ydaA* encodes for a putative universal stress protein and was expressed poorly during stationary phase (Figure 5.8B).

RNA polymerase bound *hns* entirely, correlating with high expression of the gene (Figure 5.8C). This suggests that the global regulator, H-NS may be present at high concentrations during stationary phase in the static system. The presence of the regulatory proteins during stationary phase was not confirmed in the present study however this could be achieved in future work via western blots.

Binding of RNA polymerase to regulatory genes provided an indication of the importance of the regulators *per se* under defined environmental conditions, although it should be noted that the activation of some of these regulatory genes (*e.g.* RpoE) occurs post-transcriptionally. Important information can also be provided by the degree of RNA polymerase binding to the target genes of specific regulators. The percentage of genes within twelve selected regulons associated with RNA polymerase binding was measured, including H-NS, FNR and ArcA, crucial for rapid environmental adaptation (Figure 5.9).

RNA polymerase was associated with 19 % of the ArcA and H-NS regulons and 22 % of the FNR regulon. Half of the OmpR and PhoP regulons were bound by RNA polymerase suggesting that outer membrane stress and phosphate limitation may be important environmental cues requiring stationary phase adaptation. In addition, 39 % of genes regulated by the small RNA chaperone, Hfq were associated with RNA polymerase.

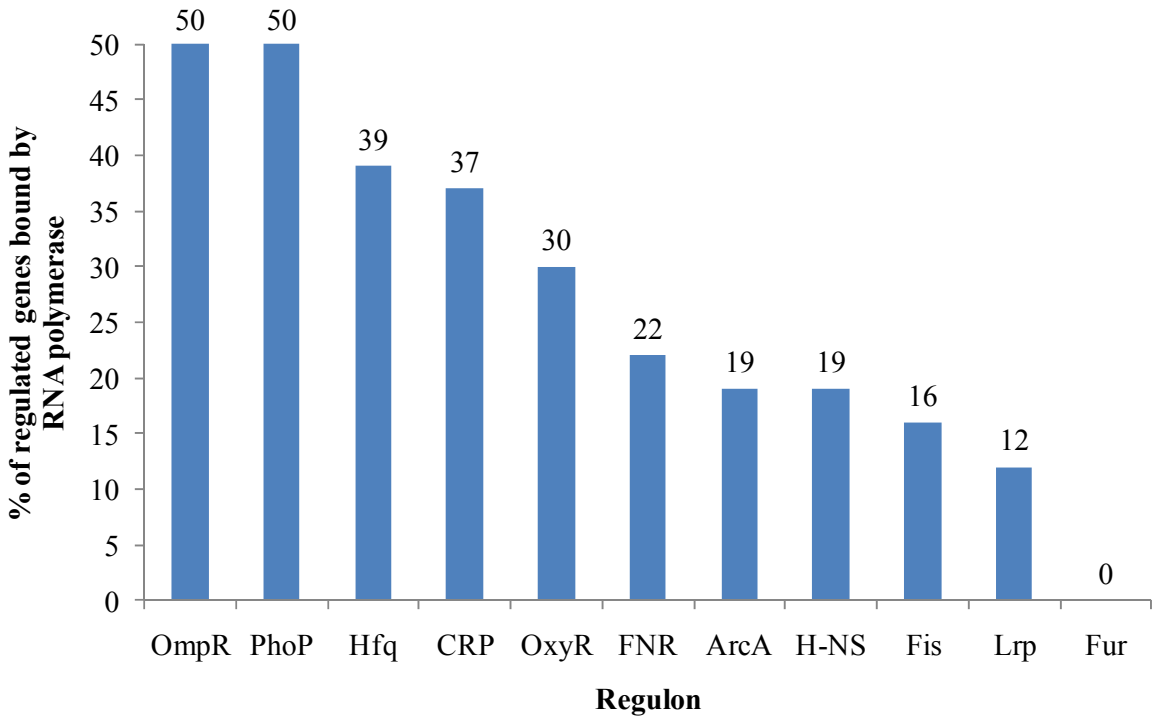


Figure 5.9: Regulatory gene targets bound by RNA polymerase.
RNA polymerase binding to genes within defined regulons was determined. Genes within regulons were obtained in *E. coli* from RegulonDB with the exception of the Hfq regulon (Sittka *et al.*, 2008) and RpoS regulon (Fang *et al.*, 1996, Ibanez-Ruiz *et al.*, 2000, Patten *et al.*, 2004, Vijayakumar *et al.*, 2004, Weber *et al.*, 2005). The percentage of genes within each regulon bound by RNA polymerase is shown above each column. RNA polymerase binding determined by ChIP-chip analysis of four independent replicates.

A considerable proportion of the genes within the OxyR and Fis regulons were bound by RNA polymerase, corresponding with relatively high expression of the regulators themselves (top 10^{th} and top 20^{th} percentile of total expressed genes, respectively). However RNA polymerase was not associated with any of the 11 genes within the Fur regulon highlighting that RNA polymerase bound regulons selectively. Many Fur-regulated genes encode for iron transport proteins suggesting that iron uptake is not required during stationary phase. The binding across the multitude of regulons highlighted the diverse functional categories associated with RNA polymerase binding, from metabolic substrate detection (CRP), to oxygen availability (ArcA, FNR and OxyR) and membrane stress (OmpR) as well as other, pleiotropic regulatory effects.

5.3 Discussion

From the results presented in this chapter, the ChIP-chip technique was successful in determining the location of RNA polymerase on the bacterial chromosome during stationary phase, yielding 663 significant binding peaks associated with 963 genes in the *S.* Typhimurium SL1344 genome. Although considerable protocol optimisation was required to yield sufficient protein DNA complexes for ChIP-chip, the data obtained were robust and reproducible yielding a large dataset for extensive analysis. However, it should be noted that the lag static system involves growth at 25 °C whereas the literature described below largely relates to *E. coli* grown at 37 °C.

5.3.1 RNA polymerase binding and gene expression

The ChIP-chip experiments revealed significant RNA polymerase binding regions on or near to approximately 20 % of the *S.* Typhimurium SL1344 genes at stationary phase. These experiments along with the transcriptomic study described by Rolfe (2007), reveal that RNA polymerase is actively transcribing during stationary phase although the rate of transcription is known to decrease by over 90 % compared with exponential phase (Ishihama, 1991). Overall, the binding of RNA polymerase at stationary phase was correlated with increased gene expression. Despite this, there were 173 low-expressed genes that were significantly bound by RNA polymerase, and 290 highly expressed genes that were not bound by RNA polymerase. One explanation is that a few of these genes are synthesised during late exponential phase or early stationary phase and high mRNA

stability is responsible for the persistence of transcripts at stationary phase. However, of the 69 non-bound genes expressed very highly (relative expression ≥5) during stationary phase, only three have been characterised as being mRNA transcripts with a long half-life in *E. coli* under standard laboratory conditions (Bernstein *et al.*, 2002). Many of the highly-expressed genes did not fall into a particular functional category although many are ribosomal encoding genes and others are regulated by OxyR. Although there were ribosomal genes apparently not bound by RNA polymerase, these genes were generally located at the end of an operon, whereas the maximum peak of RNA polymerase binding corresponded with the first gene in an operon, as previously observed in *S. cerevisiae* (Radonjic *et al.*, 2005).

The lack of correlation between RNA polymerase binding over entire genes and gene expression during stationary phase was unexpected. It was hypothesised that genes completely bound by RNA polymerase would be highly expressed, based on previous ChIP-chip studies with RNA polymerase at mid-exponential phase exhibiting long binding peaks spanning entire operons with associated high gene expression (Lucchini *et al.*, 2006). Generally, the genes completely bound by RNA polymerase at stationary phase were expressed more during 48 hours stationary phase (median gene expression = 2.14) compared with mid-exponential phase (median gene expression = 1.39). A high proportion of the genes completely bound by RNA polymerase during stationary phase were more highly expressed during mid-exponential phase than stationary phase. The most notable example of this are the ribosomal protein-encoding genes, which were highly expressed during exponential growth (Rolfe, 2007) and were bound highly at the promoter region during stationary phase, as observed in previous studies (Radonjic *et al.*, 2005) and confirmed in the present study.

The RNA polymerase binding without significant transcriptional activation suggests either the genes are not expressed highly, the mRNA transcripts are rapidly degraded as a form of post-transcriptional regulation or that the RNA polymerase holoenzyme acts as a negative gene regulator by binding to promoter regions and blocking active transcription, as reviewed previously (Greenblatt, 1997). One further hypothesis involves the RNA polymerase holoenzyme becoming ‘paused’ during the elongation stage of transcription, due to RpoD disassociation (Ring *et al.*, 1996). Transcription pausing is thought to

represent a mechanism for RNA polymerase to synchronise with regulatory factors, but has also been shown to regulate translation of mRNA transcripts (Yakhnin *et al.*, 2006). These hypotheses were not explored further in the present study and so no conclusions can be drawn to explain the low expressed, highly enriched gene coding regions. Future work should focus on the mechanism and role of RNA polymerase binding to low expressed genes and how RNA polymerase alters its binding upon entry into lag phase.

The ChIP-chip experiment performed in this chapter identified the majority of RNA polymerase bound upstream of gene coding regions, resembling the binding profile of 'trapped' RNA polymerase previously identified in *E. coli* treated with rifampicin (Grainger & Busby, 2008). These binding patterns at stationary phase differed from previous ChIP-chip experiments performed at mid-exponential growth, which generally showed broader binding peaks over gene coding regions (Lucchini *et al.*, 2006). In the present study, RNA polymerase bound slightly more frequently to intergenic regions (52 %) rather than gene coding regions (48 %) during stationary phase. This intergenic binding was higher than observed in a previous RNA polymerase ChIP-chip study in *S. cerevisiae* which identified 32 % binding to intergenic regions (Radonjic *et al.*, 2005). The increased RNA polymerase binding to intergenic regions in the present study is interesting as the ChIP-chip technique contains an inherent bias for binding to gene coding regions. The majority of oligonucleotide probes correspond to gene open reading frames and so many intergenic regions are missing from the microarray. Intergenic binding could indicate a possible role for some regulatory small RNA species. Two small RNAs, DsrA and RprA have been previously identified as having a role in *Salmonella* stationary phase survival through regulation of RpoS (σ^{38}) (Jones *et al.*, 2006). The expression of small RNA species was not determined in this study or the previous lag phase study (Rolfe, 2007). Future work should focus on some of the intergenic RNA polymerase binding locations which may correspond to small RNA transcription sites as more novel small RNAs are discovered (Sharma & Vogel, 2009).

5.3.2 RNA polymerase binding to rRNAs

Along with ribosomal protein encoding genes, a strong ChIP-chip enrichment was identified in regions spanning known rRNA coding genes although rRNA production decreases during stationary phase (Aviv *et al.*, 1996). Binding of RNA polymerase at

rRNA encoding gene regions has been identified previously in *Salmonella* during mid-exponential phase of growth (Lucchini *et al.*, 2006). It has been previously shown that the transcription of rRNA is the rate-limiting step in ribosome synthesis (Paul *et al.*, 2004) therefore RNA polymerase binding to rRNA regions during stationary phase could be interpreted as a bacterial strategy to minimise the lag in ribosome synthesis. Upon nutrient upshift in continuous cultures of *S.* Typhimurium, rRNA synthesis has been shown to occur within five minutes (Kjeldgaard *et al.*, 1958). In addition, a previous lag phase study identified ribosomal genes maximally expressed within 20 minutes of entry into lag phase (Rolfe, 2007), highlighting the importance of ribosome production during the earliest stages of growth.

One important technical consideration for the RNA polymerase binding to rRNA loci is that these regions are highly conserved in the *S.* Typhimurium LT2 genome (≥99 % nucleotide similarity). Practically, the ChIP DNA corresponding to one rRNA region would cross hybridise to the oligonucleotides on the microarray coding for the other regions. Consequently, it is not possible to definitively associate RNA polymerase binding to all the rRNA regions. With that caveat in mind, it is important to consider that significant enrichment of at least one rRNA region must have occurred, and that the pattern of RNA polymerase binding to these regions is not uniform for each region, either for the individual peak shape or for the enrichment value, which would have been expected in a cross-hybridisation scenario. Further experimental techniques such as single-copy GFP reporter fusions could distinguish between the relative induction of each of these rDNA regions (Hautefort *et al.*, 2003). It is clear that RNA polymerase association with ribosomal coding and rRNA genes is critical during lag and exponential phases of growth and future analyses should focus on definitively proving RNA polymerase binding to rRNA regions through other, similar techniques such as ChIP-seq which could distinguish between rRNA-encoding genes bound by RNA polymerase based upon the 1 % sequence variation.

5.3.3 RNA polymerase binding to sigma factors and regulons

The ChIP-chip data revealed that RNA polymerase was not specifically bound at or near to *rpoD*, indicating RpoD may not be produced during stationary phase of growth, although RpoD is required for transcription of housekeeping genes associated with exponential growth (Jishage & Ishihama, 1995). In contrast, *rpoE*, *rpoH* and *rpoS* genes were

expressed highly during stationary phase, correlating with increased RNA polymerase binding. RpoE and RpoH have both been shown to have functional overlap for RpoD-regulated genes in *E. coli* as part of addition transcriptional regulation for environmental adaptation (Wade *et al.,* 2006). The *rpoE*, *rpoH* and *rpoS* sigma factor-encoding genes contain multiple promoter regions for fine-tuning the expression of the sigma factors (EcoCyc). In *E. coli*, *rpoE* contains two promoters, the most distal of which is found in the intergenic region between *rpoE* and *nadB*. These homologous regions were highly enriched in the *Salmonella* ChIP-chip work in the present study. The activity of the *rpoE* promoter is itself regulated by the response regulator CpxR (Danese & Silhavy, 1997), whose corresponding gene is also bound at the 3' end by RNA polymerase during stationary phase (Appendix D).

CpxR is known to transcriptionally activate other stress response genes such as *rpoH* in *E. coli* (De Wulf & Lin, 2000) at a promoter located near the 3' terminus of the upstream gene, *ftsX*. Additional regulation of *rpoH* is performed directly by the transcriptional regulators CytR, CRP and DnaA (Kallipolitis & Valentin-Hansen, 1998, Wang & Kaguni, 1989). The spike of enriched regions by ChIP-chip correlates with the *rpoH* promoter, indicating that RNA polymerase is bound at the *rpoH* promoter. This is confirmed by previous transcriptomic data indicating *rpoH* is actively transcribed even in the absence of a heat-shock (Rolfe, 2007), suggesting a general stress response in the conditions tested, or functional overlap with the regulon of RpoD (Wade *et al.*, 2006). The production of RpoH has also been shown to correlate with cell division events in *E. coli*, with an increase in RpoH production after cell division and during stationary phase, but a decrease in expression of the RpoH regulon (Wagner *et al.*, 2009). The correlation between the cell division protein FtsZ and RpoH was demonstrated in *E. coli* (Wagner *et al.*, 2009), but was not investigated further in the present study.

As expected, the *rpoS* gene was bound by RNA polymerase in the present study. RpoS (σ^{38}) is required to maintain bacterial survival against stresses during stationary phase (Lange & Hengge-Aronis, 1991) and regulation of RpoS occurs both at the transcriptional level and post-transcriptionally (Lange & Hengge-Aronis, 1994). In *E. coli* there are five separate *rpoS* promoters, four of which are found close together between *rpoS* and the upstream gene, *nlpD* and the most distal promoter present within the *nlpD* gene itself. The

latter promoter is required to maintain a basal concentration of RpoS, regulated by CRP and ArcA (Mika & Hengge, 2005). The sharpest peak of RNA polymerase binding correlates with the four intergenic promoters, although the experimental resolution is not specific enough to distinguish between these promoters. This suggests that transcription was primarily occurring from stationary phase or stress-induced promoters rather than the basal RpoS promoter, to protect *Salmonella* during stationary phase survival.

The binding of RNA polymerase in stationary phase to sigma factors and global regulators suggests a dual strategy for both stationary phase survival and preparation for environmental shifts. An example of the survival response is the RNA polymerase binding to the genes that encode RpoS and OmpR. These proteins regulate a multitude of genes, including those involved in outer membrane protein synthesis, flagella, fimbriae and other global regulators such as OxyR and PhoP (Majdalani *et al.*, 1998, Bang *et al.*, 2000, Gerstel *et al.*, 2006, Lucchini *et al.*, 2009). Conversely, 30 % of the OxyR regulon, required for oxidative stress resistance are bound even in the anoxic stationary phase environment, with a low redox potential (Section 6.2.1 and Figure 6.2). This indicates a bacterial strategy for rapid adaptation in the event of potentially lethal oxygen upshift.

5.3.4 Overall conclusions

The ChIP-chip technique was successful in identifying multiple binding regions for RNA polymerase during stationary phase. Stationary phase binding was different to other growth phases as shown in other studies, and confirmed some of the results first identified in yeast by Radonjic and colleagues (Radonjic *et al.*, 2005), namely the binding of RNA polymerase predominantly to intergenic regions and association with ribosomal protein-encoding and rRNA genes. It is interesting that this binding pattern is conserved between bacteria and yeast, suggesting a fundamental process preparing for entry into lag phase. In addition, the present study has identified RNA polymerase binding to genes previously identified as being expressed highly during stationary phase (Rolfe, 2007), and provides a new validation of this transcriptomic dataset.

However, there is little additional evidence to suggest that lag phase-expressed genes are specifically bound by RNA polymerase during stationary phase, with the notable exception of ribosomal protein and rRNA encoding genes. Taken together, the data presented in this

chapter suggest that stationary phase *S.* Typhimurium does not prepare for lag phase in the conditions tested, therefore the original hypothesis should be rejected, and future work should focus on identifying mechanisms of RNA polymerase binding during lag phase.

The mechanisms responsible for the rapid transcriptional activity during early lag phase still remain to be fully elucidated and could be resolved by ChIP-chip or ChIP-seq methods relying on optimised protocols to obtain sufficient DNA-protein enrichment from only a few bacterial cells, from within one minute of inoculation into fresh LB medium. This would reveal in fine detail the activity of RNA polymerase during the exit from stationary and the entry into lag phase, and the promoters bound most rapidly.

Chapter 6

The oxidative stress response during lag phase

6. The oxidative stress response during lag phase

6.1 Introduction

Bacteria such as *Salmonella*, adapt rapidly to changes in oxygen availability during infection, for example shifting from aerobic environments to microaerobic conditions in the gut and resisting oxidative bursts during intracellular growth in macrophages (Rychlik & Barrow, 2005). This response relies upon the rapid induction of defence mechanisms when challenged with oxygen upshift (Farr & Kogoma, 1991, Janssen *et al.*, 2003).

During recovery from cold storage, there was an extensive transcriptional re-programming including many genes involved in resistance to oxidative damage (Section 4.2.3). This transcriptional response has been previously identified in *S.* Typhimurium in the static growth system (Rolfe, 2007). In Section 3.2.2 it was observed that *S.* Typhimurium utilises oxygen during lag phase, suggesting dissolved oxygen may be a potential source of cellular damage during the early stages of growth and the terminal electron acceptor in aerobic respiration. In this Chapter, the oxidative stress response at different stages of lag phase was investigated to phenotypically confirm the gene expression data. In addition, some of these key defence mechanisms were removed and the effect on growth and hydrogen peroxide resistance measured.

6.1.1 Oxidative stress resistance mechanisms

All bacteria that respire oxygen, to generate ATP, require oxidative stress resistance mechanisms (Cabiscol *et al.*, 2000). During aerobic respiration, bacteria such as *E. coli* and *Salmonella* generate oxygen intermediates such as hydrogen peroxide (H_2O_2) and the superoxide anion (O_2^-) (Messner & Imlay, 1999). The primary cause of these endogenous oxygen intermediates in *E. coli* (and presumably similar bacteria) is the respiratory electron chain, which accounts for 87 % of the total hydrogen peroxide production (Benov & Fridovich, 1994). These excess pro-oxidants in the bacterial cell can damage proteins, DNA, RNA and lipids (Farr & Kogoma, 1991). No damage is observed from exogenously-added H_2O_2 and O_2^- at low concentrations ($\leq$ μM range), although H_2O_2 is toxic when added at millimolar concentrations (Imlay, 2008). Intracellularly-accumulated micromolar concentrations of H_2O_2 in mutants of *E. coli* lacking detoxification machinery cause

cellular damage, resulting in aerobic growth defects (Park *et al.*, 2005). This highlights the role of oxidative stress resistance systems designed to remove the build up of aerobic toxic by-products. The main de-toxification systems are discussed below.

6.1.1.1 OxyR

OxyR is a transcriptional regulator which is activated by hydrogen peroxide (Åslund *et al.*, 1999), via oxidation of cysteine thiol groups of the inactive OxyR protein, inducing a conformational change for transcriptional regulation (Pomposiello & Demple, 2001). Activated OxyR directly regulates the expression of several genes during resistance to oxidative stress in *E. coli* including: *flu*, *hemH*, *grxA*, *trxC*, *gor*, *ahpC*, *ahpF*, *fur*, *katG, mntH* and *dps*. In addition OxyR induces the expression of a non-coding RNA, *oxyS* (Anjem *et al.*, 2009, Christman *et al.*, 1989, Pomposiello & Demple, 2001, Storz *et al.*, 1990, Tartaglia *et al.*, 1989, Zheng *et al.*, 1999), which affects RNA stability and functions as a post-transcriptional regulator (Gottesman, 2004). The induction of the alkyl hydroperoxidase-encoding genes *ahpC* and *ahpF* and the catalase-encoding *katG* is a bacterial response to directly detoxify any endogenous H_2O_2 and has been demonstrated in *E. coli* and *Salmonella* and several other bacteria to promote survival or colonisation including: *Enterococcus faecalis* (La Carbona *et al.*, 2007), *Staphylococcus aureus* (Cosgrove *et al.*, 2007), *Pseudomonas putida* (Hishinuma *et al.*, 2008, Hishinuma *et al.*, 2006), *Pseudomonas aeruginosa* (Vinckx *et al.*, 2008), *Neisseria meningitidis* (Ieva *et al.*, 2008) and *Bacteroides fragilis* (Sund *et al.*, 2008). During growth inside macrophages, *S.* Typhimurium induces the expression of *ahpC* and *ahpF* (Wright *et al.*, 2009). Strains deficient in *ahpC, ahpF* or *oxyR* were recently found by Transposon Mediated Differential Hybridisation (TMDH) screening to be attenuated during infection of mice (Chaudhuri *et al.*, 2009) suggesting that oxidative stress resistance is crucial for *S.* Typhimurium host infection. The other genes within the OxyR regulon are involved in the reduction of oxygen intermediates. In addition GSH glutaredoxin (encoded by *gor*) reverts oxidised OxyR back to its inactive state (Pomposiello & Demple, 2001).

6.1.1.2 SoxS

The bacterial response to the presence of superoxide is controlled in part by SoxS in tandem with its transcriptional regulator, SoxR. The translated SoxS protein binds promoter regions of genes within its regulon, recruiting RNA polymerase (Storz & Imlay,

1999). Genes within the SoxS regulon include: *ribA*, *fldAB*, *zwf*, *fur, nfo, sodA* and the efflux pump-encoding *acrAB*. Similar to OxyR, SoxS induces a non-coding RNA, *micF* which down-regulates the OmpF porin (Pomposiello & Demple, 2000). Other porin-encoding genes known to be activated by SoxS include *tolC* and *ompX* (Aono *et al.*, 1998, Dupont *et al.*, 2007). More recently, DNase I footprinting analysis has identified direct SoxS binding to the promoter region of another porin-encoding gene, *ompW* (Gil *et al.*, 2009). These porins function to remove reactive oxygen intermediates to protect the intracellular environment. The transcription of *fur* leads to the decrease in iron accumulation and thereby decreases the risk of forming Fenton-mediated hydroxyl free radicals (Wardman & Candeias, 1996). The increase in *acrAB* expression leads to the efflux of potentially-harmful molecules which could react with superoxide anions to form damaging products, and is coupled with an indirect, post-transcriptional decrease in the large outer membrane porin, OmpF (Pomposiello & Demple, 2001). The *ribA* gene encodes GTP cyclohydrolase II, the first enzyme in riboflavin (vitamin B2) synthesis (Koh *et al.*, 1996). The induction of *ribA* and *fldAB* flavins by SoxS reduces existing intracellular superoxide anions to prevent cellular damage. The endonuclease IV encoded by *nfo* functions to repair damaged DNA molecules (Levin *et al.*, 1991), whereas the induction of the Mn-superoxide dismutase (SodA) actively de-toxifies O_2^- (Touati, 1988). The diverse genes within the SoxS regulon indicate a multifactorial approach to O_2^- stress resistance involving: reduction, repair and efflux systems.

6.1.1.3 Superoxide dismutases

Superoxide dismutases (SOD) remove the intracellular accumulation of O_2^-, reducing the anion to H_2O_2 and O_2. The produced H_2O_2 is then further de-toxified by catalases and alkyl hydroperoxide reductases. In *Salmonella* there are three classes of SOD each requiring a different divalent metal cation cofactor: SodA (Mn-SOD), SodB (Fe-SOD) and SodC (Cu/Zn-SOD). The SODs are required for full virulence of *Salmonella* in mice and macrophages to survive the phagocytic oxidase (phox) mediated oxidative burst (Fang *et al.*, 1999, Tsolis *et al.*, 1995). A recent study using the *sodB* promoter (P_{sodB}) was used as an indicator for the presence of iron in macrophages. The expression of this promoter was not induced by either H_2O_2 or O_2^- suggesting that activity was induced solely by the presence of intracellular iron (Taylor *et al.*, 2009). The expression of *sodA* is regulated by SoxS and the main transporter of Mn^{2+} is under the regulation of OxyR, indicating the

import of Mn^{2+} is linked to oxidative stress resistance. It is important to note that Mn^{2+} not solely utilised in the production of SodA and may be incorporated into additional metalloproteins, the functions of which are yet to be fully elucidated (Anjem *et al.*, 2009).

6.1.2 Relevance to bacterial lag phase

During oxygen upshift from anoxic cultures to aerobic media, bacteria induce oxidative stress resistance systems within a few minutes (Partridge *et al.*, 2006). In the static growth system used in the present study, the culture became anoxic by early stationary phase (Section 3.2.2) and so inoculation into fresh medium represented an oxygen upshift leading to the induction of *soxR*, *soxS*, *sodA* and the OxyR-regulon at 25 °C (Rolfe, 2007) and after a cold storage treatment (Section 4.2.3), suggesting the presence of both H_2O_2 and O_2^-. It has previously been reported that colonies growing on LB agar plates induce *soxRS* and *oxyR*, to protect against reactive oxygen species (Cuny *et al.*, 2007). These studies suggest that either the redox potential encountered by the bacteria increases considerably upon inoculation into aerobic media or detection of a low concentration of reactive oxygen species is sufficient to induce a variety of defence mechanisms. It is possible that bacteria are particularly sensitive to oxidative stress during lag phase, as any cellular damage incurred at this crucial stage of growth may have considerable effects upon the viability of the bacterial population. The effect of oxygen upshift on the bacterial population was investigated and the importance of oxidative stress resistance systems during lag phase evaluated. In addition, the effect of physiological history using the cold storage system (Section 2.1.6.1), on subsequent oxidative stress resistance was elucidated.

6.2 Results

6.2.1 Oxidative stress resistance during lag phase

To assess the impact of *S.* Typhimurium hydrogen peroxide stress resistance during lag phase, H_2O_2 was added to cultures at desired time points in the bacterial growth curve. Viable counts were made both immediately upon addition of H_2O_2 and after 30 minutes (Figure 6.1A and Figure 6.1B). The effect of 1 mM exogenously-added H_2O_2 was minimal with a decrease in viability of 1.5-fold for stationary phase bacteria and 20 minutes, 60 minutes and 120 minutes lag phase cultures. The 4 minute culture showed a decrease in

viability of greater than 10-fold which was also observed in the mid-exponential phase culture (Figure 6.1A).

The addition of 6 mM H_2O_2 decreased the viability of the bacterial population at all time-points. Stationary phase cultures and 120 minute lag phase cultures decreased 10-fold, whereas the 60 minute culture was least affected decreasing <5-fold. The 4 minute, 20 minute and mid-exponential phase cultures were affected the most with a decrease of approximately 15-fold (Figure 6.1B). From this preliminary experiment it was apparent that the effect of H_2O_2 on cell viability was dependent on growth phase, and importantly also on the stages within lag phase, which was more marked with the exposure to the higher concentrations of H_2O_2. For further experiments involving hydrogen peroxide challenges on lag phase bacteria, 6 mM H_2O_2 was used to phenotypically differentiate between the lag phase time-points.

Oxidative stress regulators such as SoxR and OxyR respond to changes in redox potential and elicit a defence response (Pomposiello & Demple, 2001). To determine the extent of oxidative stress in the static system, the redox potential (E_h) of the bacterial culture was measured (Figure 6.2A) using a redox electrode (Section 2.1.8.3). During inoculation from the stationary phase culture into fresh LB medium, the bacteria experienced an E_h increase (-328 mV to +42 mV), by 4 minutes post-inoculation. These measurements confirmed the differences between the anoxic inoculum environment and the aerobic fresh LB medium, as highlighted elsewhere (Section 3.2.2). The lag phase time-points have similar redox potential but eight hours post-inoculation, the culture became anoxic during mid-exponential phase (E_h = -72 mV). The addition of H_2O_2 increased the E_h at every time-point (Figure 6.2B). Although the increase in E_h was similar for the lag phase time points (standard deviation = 7 mV), the redox increase was slightly higher for the mid-exponential phase culture (ΔE_h +186 mV). Interestingly, the greatest change in redox potential occurred in the 48 hour inoculum sample (ΔE_h = 266 mV), suggesting that the redox potential is more weakly buffered in the inoculum environment.

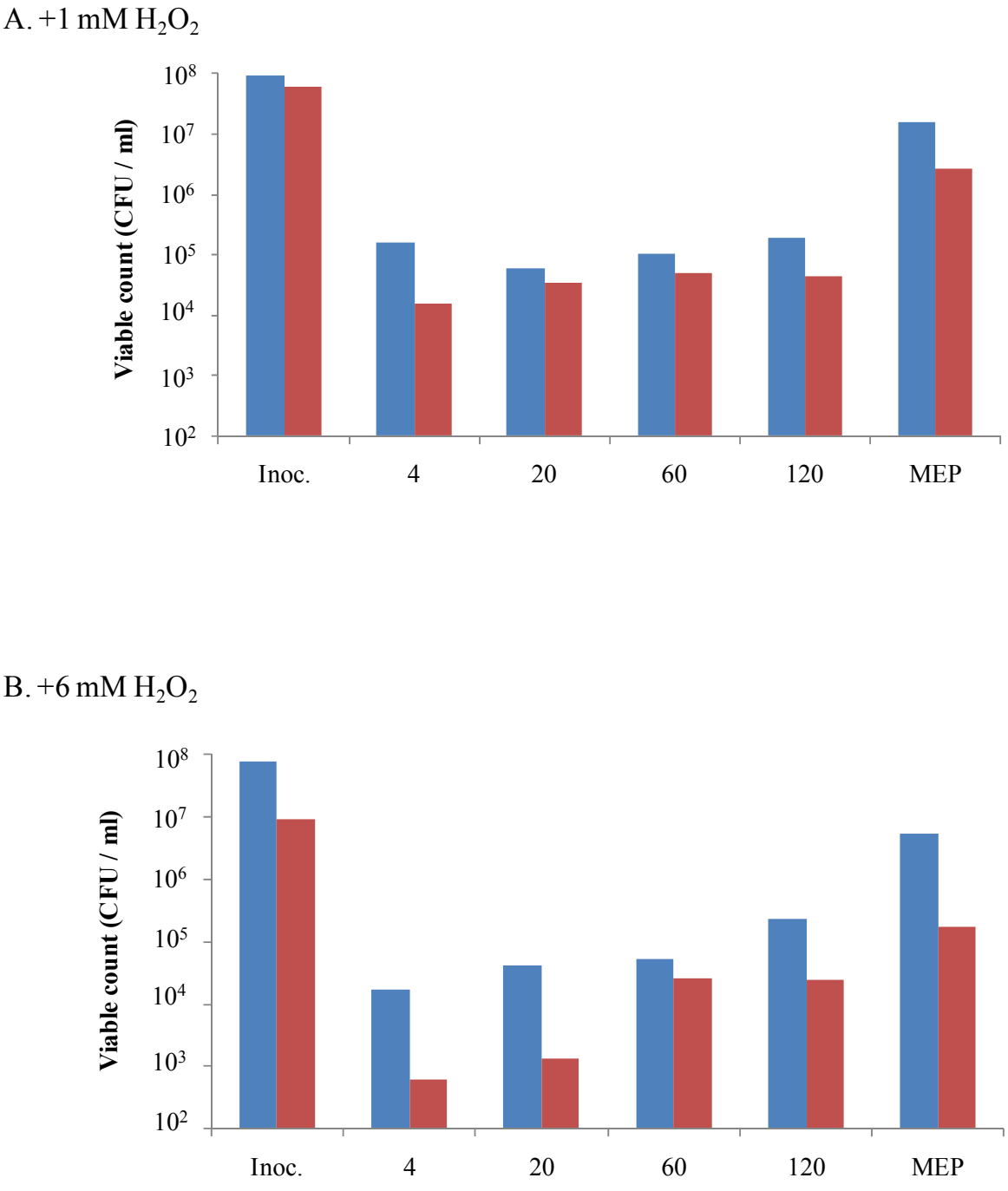


Figure 6.1: Different growth phases exhibit varying tolerance to H_2O_2.
Preliminary experiment showing total viable counts of a single bacterial culture grown in LB medium (blue bars) or 30 minutes after addition of H_2O_2 (red bars). H_2O_2 added at either 1 mM (A) or 6 mM (B) final concentrations. Numbered phases of growth are displayed as either: minutes post-inoculation; 48 hour stationary phase inoculum (Inoc.); 8 hour mid exponential phase (MEP).

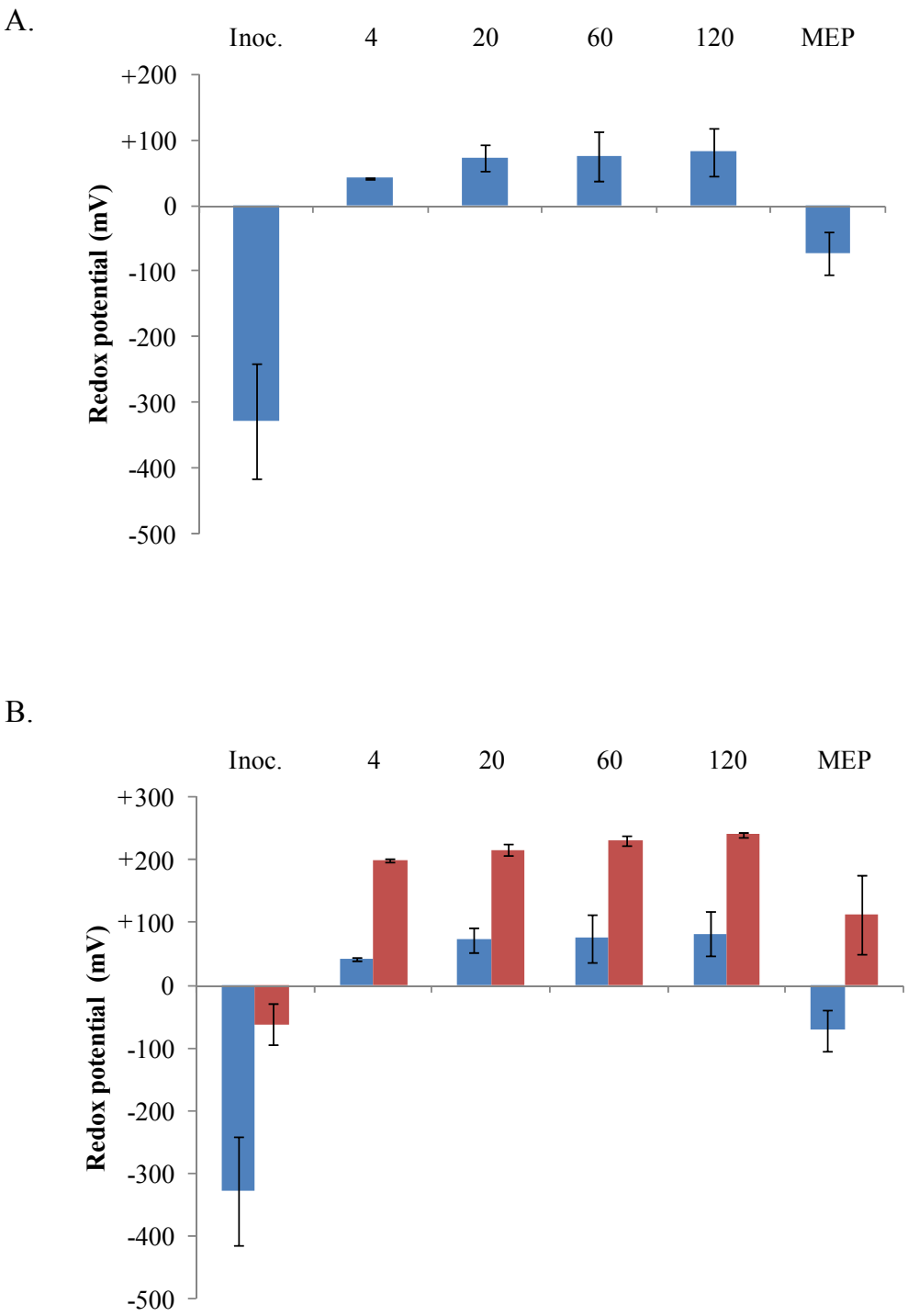


Figure 6.2: The redox potential of bacterial cultures varies during growth and increases with addition of H_2O_2.
Redox potential as measured by a redox probe ranging from -500 mV to +500 mV. (A) Redox potential of bacterial cultures through the growth curve. (B) The redox potential of bacterial cultures before (blue bars) and after addition of 6 mM H_2O_2 (red bars). Numbered phases of growth are displayed as either: minutes post-inoculation; 48 hour stationary phase inoculum (Inoc.); 8 hour mid exponential phase (MEP). Error bars represent the standard deviation from the average of three biological replicates.

The fact that H_2O_2-mediated killing occurred at different rates during different stages of lag phase (Figure 6.1) led to the investigation of the effect of prolonged exposure to 6 mM H_2O_2. The bacteria were grown to the desired time-point and H_2O_2 added to the medium. The survival of the bacteria was determined through total viable counts immediately after the addition of H_2O_2 and then at 30 minute intervals until 90 minutes post-exposure (Figure 6.3). It was hypothesised that bacterial resistance to H_2O_2 would coincide with increased expression of oxidative stress resistance genes. A previous transcriptomic study identified oxidative stress resistance genes induced by 20 minutes post-inoculation under normal growth conditions in the static system (Rolfe, 2007). The hypothesis states that early lag phase time-points (4 minutes and 20 minutes post-inoculation) would be more susceptible to H_2O_2 than later lag phase time-points (60 and 120 minutes post-inoculation), as the H_2O_2 resistance mechanisms would be in place by the end of lag phase.

The H_2O_2-mediated bacterial killing followed a trend across the three biological replicates tested, although experimental variability was high (Figure 6.3). To compensate for this variability, a model was utilised to quantify the bacterial killing over a period of 90 minutes exposure to H_2O_2, in terms of the 'shape' of the bacterial killing curves (Table 6.1 and Figure 6.4). The Weibull distribution (Weibull, 1951) was subsequently used to quantify the bacterial killing using the following equation:

$$y = y_0 - (t / T_0)^w$$

where t is the time post addition of H_2O_2; y is the logarithm of the bacterial concentration at time t ($\log_{10}$ CFU / ml); y_0 is the initial $\log_{10}$ cell concentration immediately after addition of H_2O_2; T_0 is the time at which the first decimal decrease in cell viability has been reached; w is the Weibull shape parameter. The w value alters with the rate of bacterial killing: If the rate of bacterial killing increases with time, then $w>1$; if the rate of bacterial killing decreases with time, then $w<1$; if the rate of bacterial killing is linear with increasing time, then $w=1$ (Aragao *et al.*, 2007).

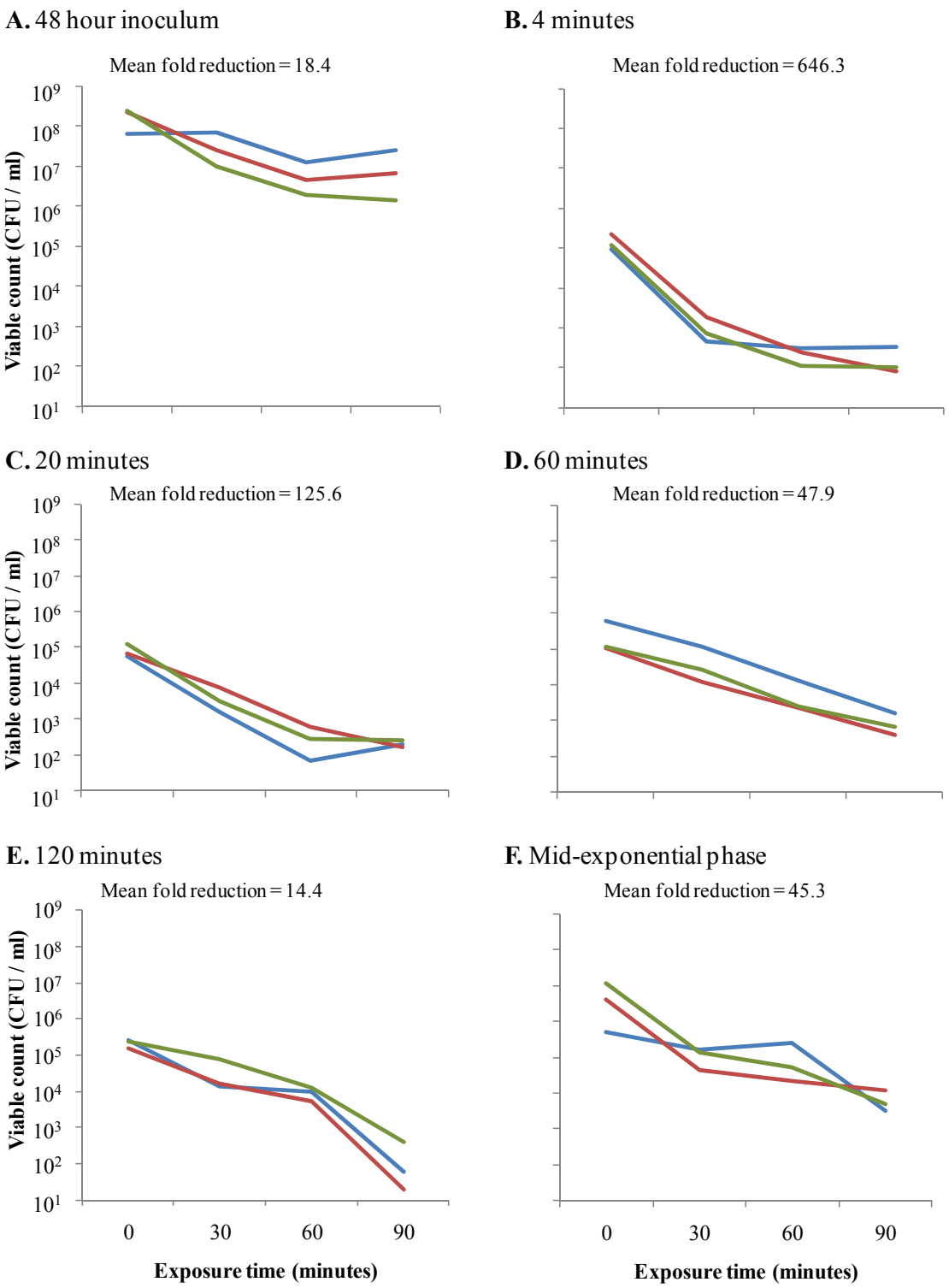


Figure 6.3: Rate of H_2O_2-mediated killing is dependent on the bacterial growth phase. Effect of H_2O_2 on the bacterial population throughout growth (A-F) for three biological replicates, calculated as viable counts. Each replicate is shown as a separate, coloured line over 90 minutes H_2O_2 exposure. Culture age represented as either: 48 hour stationary phase inoculum (A), minutes post-inoculation (B-E) or an 8 hour post-inoculation culture (F). For clarity, the mean fold reduction is shown for each culture after 60 minutes exposure to H_2O_2.

The 48 hour inoculum culture tolerated oxidative stress relatively well. This could be due to the low redox potential even after the addition of H_2O_2 (Figure 6.2B), the role of the stationary phase alternative sigma factor RpoS (σ^{38}), which is known to induce oxidative stress resistance mechanisms during stationary phase (Park *et al.*, 2005) or the higher initial cell concentration. The viability of the bacterial population decreased >18-fold after 60 minutes exposure and then resisted further killing over the remaining 30 minutes (Figure 6.3A). The stationary phase culture survival contrasted dramatically with bacteria from the earliest lag phase time-point (4 minutes) which were killed rapidly within the initial 30 minutes (>87-fold decrease) and 60 minutes (>646-fold) exposure to H_2O_2, before the rate of killing decreased over the remaining duration of the experiment (Figure 6.3B). This early, rapid killing was confirmed by a concave Weibull curve over the 90 minutes exposure to H_2O_2 (Figure 6.4A). The 20 minute lag phase culture displayed a similar pattern of killing to the 4 minute culture, although the death rate was slower and more prolonged over 60 minutes exposure (Figure 6.3C). The rate of H_2O_2-mediated killing decreased as the length of exposure increased, as borne out by the Weibull value of 0.32 (Figure 6.4B). From these data it is apparent that any induced oxidative stress defence mechanisms were not fully active and able to resist the H_2O_2 killing. The one hour culture, representing mid-way through lag phase, exhibited initial resistance not present in the early lag phase samples (Figure 6.3D). The rate of killing was also constant throughout the H_2O_2 treatment exhibiting an almost linear Weibull value (Figure 6.4C). The initial survival of the bacterial culture indicates that the bacteria were physiologically able resist the oxidative stress, possibly through the induction of OxyR-mediated defence mechanisms. Genes encoding these defence mechanisms are known to be induced by 20 minutes post-inoculation in the static growth system (Rolfe, 2007) and the translated proteins could feasibly have an effect by 60 minutes post-inoculation. The continued resistance to H_2O_2 was evident in the 120 minute culture representing the end of lag phase. There was a linear rate of death for the first 60 minutes exposure to H_2O_2 followed by a rapid killing between 60 and 90 minutes (Figure 6.3E). The convex survival curve (w = 1.68) indicates considerable initial resistance to H_2O_2 which was overcome after the prolonged exposure to oxidative stress (Figure 6.4D).

The data taken together reveal that lag phase is not a homogenous stage of growth but early lag phase is highly susceptible to oxidative stress. Oxidative stress resistance occurs between 20 minutes and 60 minutes post-inoculation and continues throughout the remainder of lag phase. These data indicate that the lower starting inoculum during lag phase compared with stationary phase does not solely explain the immediate H_2O_2 sensitivity. Despite similar cell concentrations during lag phase, the H_2O_2 resistance profiles differ dramatically between different stages of lag phase with decreasing susceptibility observed after the point when the bacteria induce oxidative stress resistance mechanisms. Interestingly, during mid-exponential phase there appeared to be increased H_2O_2 sensitivity, although the trend of killing was more variable between replicates. The H_2O_2 killing trend was similar between mid-exponential phase and stationary phase (mean Pearson correlation coefficient = 0.82), with a faster rate of killing during the initial exposure to H_2O_2 in the exponentially growing culture (Figure 6.3F). This increased killing may reflect the vulnerability of rapidly-dividing bacterial cells to oxidative stress, as reported elsewhere (Dukan & Touati, 1996).

	Lag phase time-point for challenge test (minutes)			
Parameter	**4**	**20**	**60**	**120**
Weibull (w)	0.28	0.32	0.98	1.68
Standard error Weibull [se(w)]	0.05	0.14	0.17	0.44
T_0	1.65	13.80	37.00	44.00
se(T_0)	1.28	7.58	6.00	7.60
Linear correlation of the fitted curve [R^2(fit)]	0.96	0.88	0.86	0.87
se(fit)	0.24	0.38	0.39	0.49

Table 6.1: Lag phase killing curve data (Figure 6.3) used in the Weibull model to quantify the shape of bacterial killing.
The standard error (se) of each parameter was calculated by F-test. In addition, the linear correlation (R^2) between T_0 and the logarithm of w was used to prove the robustness of a single-parameter fitting model used for subsequent analysis (József Baranyi, personal communication).

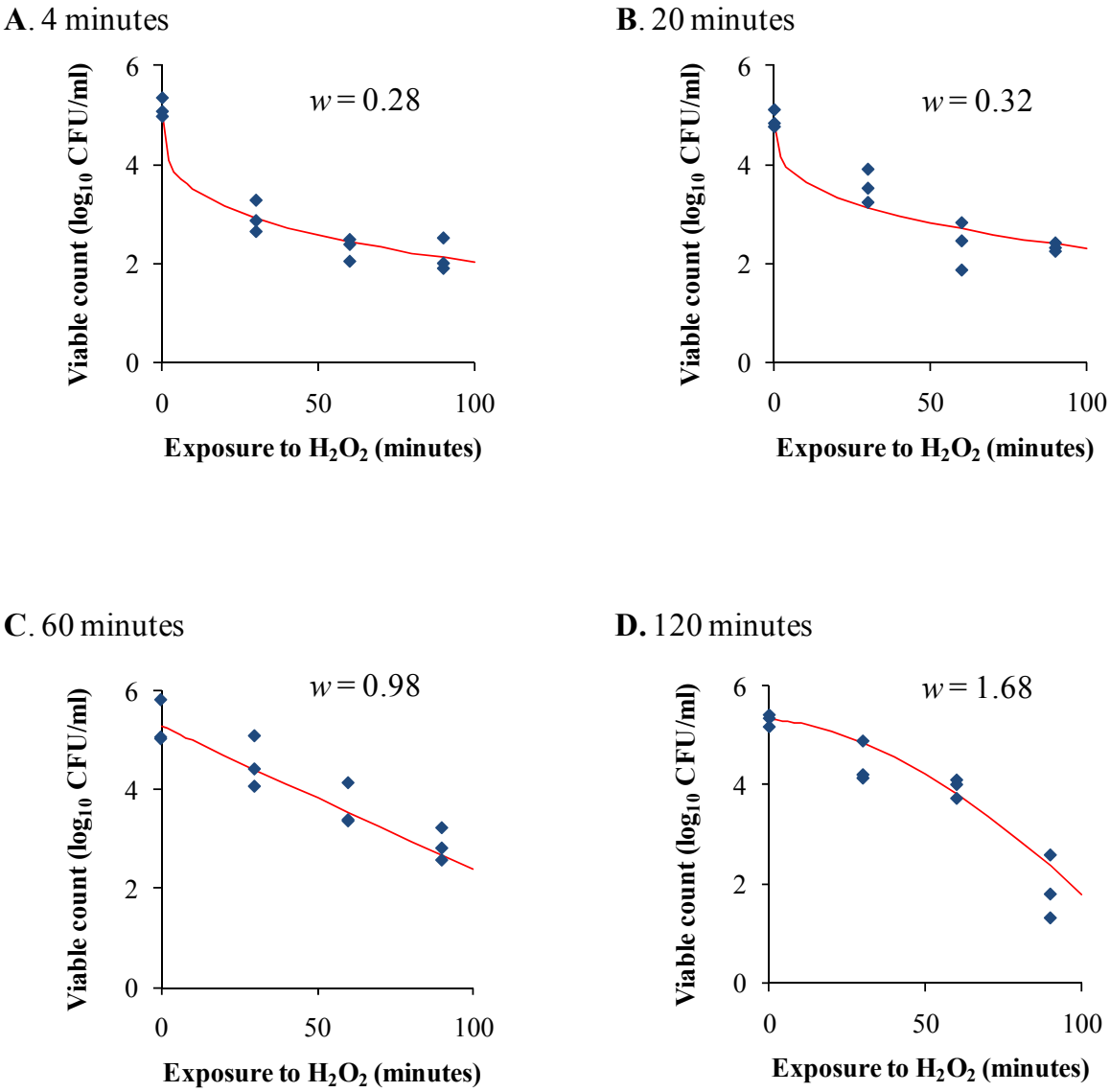


Figure 6.4: Bacterial cells become more resistant to H_2O_2 as lag phase progresses. Effect of H_2O_2 on the shape of bacterial survival curves for three biological replicates, calculated as viable counts. Each replicate is shown separately (blue diamonds) over 90 minutes H_2O_2 exposure. Lag phase cultures shown: (A) 4 minutes, (B) 20 minutes, (C) mid-lag phase, (D) end of lag phase. The Weibull parameter (w) is fitted to the viable count data (red line) and is included for each culture, for reference.

6.2.2 The effect of physiological history on oxidative stress resistance

The cold storage treatment during Lag Pre-Incubation experiments showed different transcriptional effects upon recovery in several physiological processes including increased duration for oxidative stress resistance and a delay in iron accumulation (Section 4.2.4; Figure 4.7). These data implied that the bacteria take longer to become fully resistant to oxidative stress in Lag Pre-Incubation experimental conditions, from 20 minutes to 60 minutes post-inoculation. The use of 6 mM H_2O_2 to phenotypically distinguish between the bacterial viability (and therefore resistance) at different stages of lag phase was demonstrated (Figure 6.3 and Figure 6.4) and this method was now used to determine whether the cold storage physiological history impacts upon oxidative stress susceptibility (Figure 6.5). Based upon the transcriptomic data, a hypothesis was formulated that cells taken at early lag phase would be killed rapidly, at a similar rate, for both Lag Phase 25 °C and Lag Pre-Incubation experiments. Furthermore, it was hypothesised that the 60 minute post-inoculation time-point, representing half-way through lag phase in both conditions, would be killed at a faster rate in Lag Pre-Incubation conditions than the corresponding time-point in Lag Phase 25 °C conditions (Figure 6.3 and Figure 6.4).

To test the lag phase specific effect of physiological history upon oxidative stress resistance, it was important to confirm that stationary phase bacteria during cold storage were not more susceptible to H_2O_2 than the 25 °C inoculum. After challenge testing with 6 mM H_2O_2, the cold stored stationary phase culture had a Weibull value which was not significantly different to the 48 hour stationary phase culture (Figure 6.6). Therefore any difference in bacterial survival after inoculation would be due to subsequent history dependent, lag phase specific adaptation. Generally, the Lag Pre-Incubation and Lag Phase 25 °C survival after H_2O_2 challenge were similar. The Lag Pre-Incubation cultures became generally more resistant later during lag phase, as seen in the Lag Phase 25 °C experiments (Figure 6.4). The most notable difference between the experiments was at 60 minutes post-inoculation which showed a significantly lower Weibull value (F-test $p \leq 0.01$) for the Lag Pre-Incubation experiments (w = 0.37), indicating that these cells had not yet become resistant to H_2O_2 unlike bacteria from the Lag Phase 25 °C experiment (Figure 6.5). The Lag Pre-Incubation culture showed greater initial resistance at the end of lag phase (w = 1.62), which most likely accounts for the similarities in geometric lag duration (Figure 6.6).

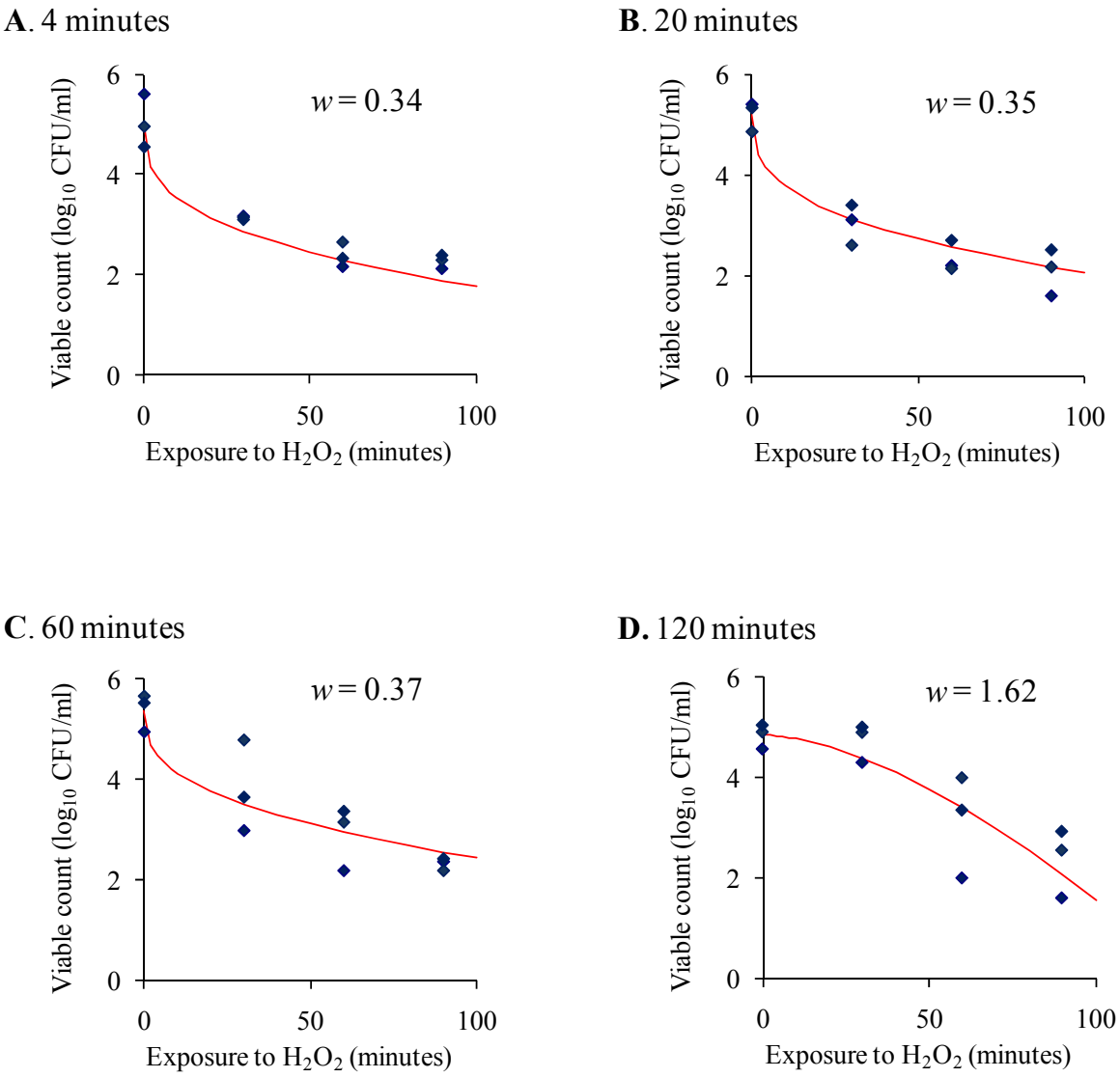


Figure 6.5: Lag Pre-Incubation cultures are H_2O_2 sensitive at mid-lag phase.
Effect of H_2O_2 on the shape of Lag Pre-Incubation treated bacterial survival curves for three biological replicates, calculated as viable counts. Each replicate is shown separately (blue diamonds) over 90 minutes H_2O_2 exposure. Lag phase cultures shown: (A) 4 minutes, (B) 20 minutes, (C) mid-lag phase, (D) end of lag phase. The Weibull parameter (w) is fitted to the viable count data (red line) and is included for each culture, for reference.

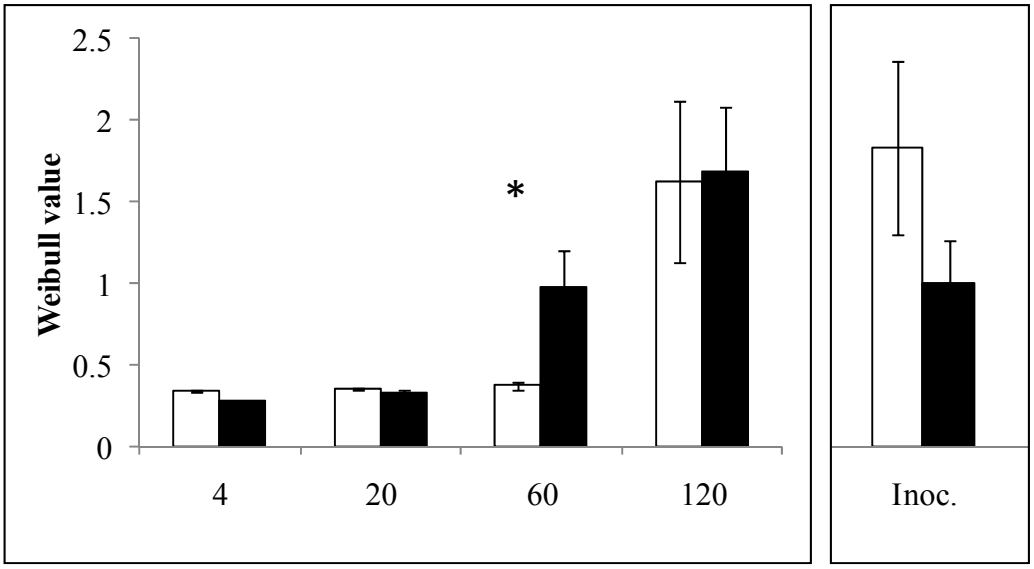


Figure 6.6: The bacterial lag phase resistance to H_2O_2 is history dependent.
The effect of H_2O_2 on the viability of the bacterial population throughout growth, as shown by the Weibull value. Cultures grown in Lag Pre-Incubation (white bars) or Lag Phase 25 °C (black bars) conditions, prior to H_2O_2 challenge. Lag phase time points shown post-inoculation in fresh, pre-warmed (25 °C) LB medium. "Inoc." represents the two inocula used, either 12 days, 2 °C (white bar) or 48 hours 25 °C (black bar). Three independent biological replicates shown for each time-point. Error bars represent the standard error (se). An asterisk (*) represents a significant different Weibull value between the two experimental conditions (F-test, $p \leq 0.01$) at 60 minutes. In addition, the Lag Pre-Incubation 60 minute culture had a 4.7-fold lower cell concentration than the corresponding Lag Phase 25 °C culture after 1 hour exposure to H_2O_2.

6.2.3 Oxidative stress resistance mutants

To determine whether oxidative stress tolerance is essential for lag phase adaptation, numerous mutants were tested which were defective in oxidative stress resistance mechanisms including alkyl hydroperoxide reductases *ahpC* and *ahpF*; catalase (*katG*); the oxidative stress inducible *soxRS* and *oxyR*. OxyR responds to hydrogen peroxide (H_2O_2), and SoxRS responds to superoxide (O_2^-), therefore a Δ*oxyR* Δ*soxRS* mutant was constructed which was susceptible to both forms of oxidative challenge. The majority of genes targeted for deletion were up-regulated by an oxygen upshift during lag phase (Figure 6.7A).

The susceptibility of these mutant strains to oxidative stress was determined via challenge with H_2O_2 on LB agar plates (Section 2.1.8.1) and the zone of inhibition caused by the inability to survive the diffusion of H_2O_2, was measured (Figure 6.7B). The effect of H_2O_2 on the mutant strains varied depending on the type of oxidative stress resistance system deleted. SoxRS is known to respond to O_2^- and, as expected, the Δ*soxRS* mutant strain was not significantly affected by the addition of H_2O_2. More surprisingly, the Δ*ahpCF* strain was not significantly affected by the H_2O_2 challenge compared with the wild-type bacteria. Conversely, the Δ*katG* mutant was significantly affected by the H_2O_2, suggesting that the catalase system was more important for resisting oxidative stress in this experiment. Although the *ahpCF* system was not required to overcome the added H_2O_2, the combined Δ*ahpCF* Δ*katG* mutant strain showed a significantly increased susceptibility to oxidative stress compared with the Δ*katG* strain (*t*-test $p=9.8 \times 10^{-8}$). This result indicates a role for *ahpCF* in the absence of *katG* and suggests different mechanisms of regulation despite both genes known to be a part of the OxyR-regulon. The deletion of *oxyR* significantly-increased the susceptibility to oxidative stress compared with the wild-type strain, but interestingly the Δ*ahpCF* Δ*katG* strain showed a small, but significant increase in peroxide susceptibility compared with the Δ*oxyR* mutant (*t*-test p=0.01). This result suggests that both AhpCF and KatG are an essential component of the OxyR-mediated response to H_2O_2 stress under these conditions. Despite not being required for H_2O_2 resistance, the deletion of *soxRS*, when combined with a Δ*oxyR* mutant significantly exacerbated the effect of H_2O_2 killing (*t*-test $p=4.5 \times 10^{-5}$).

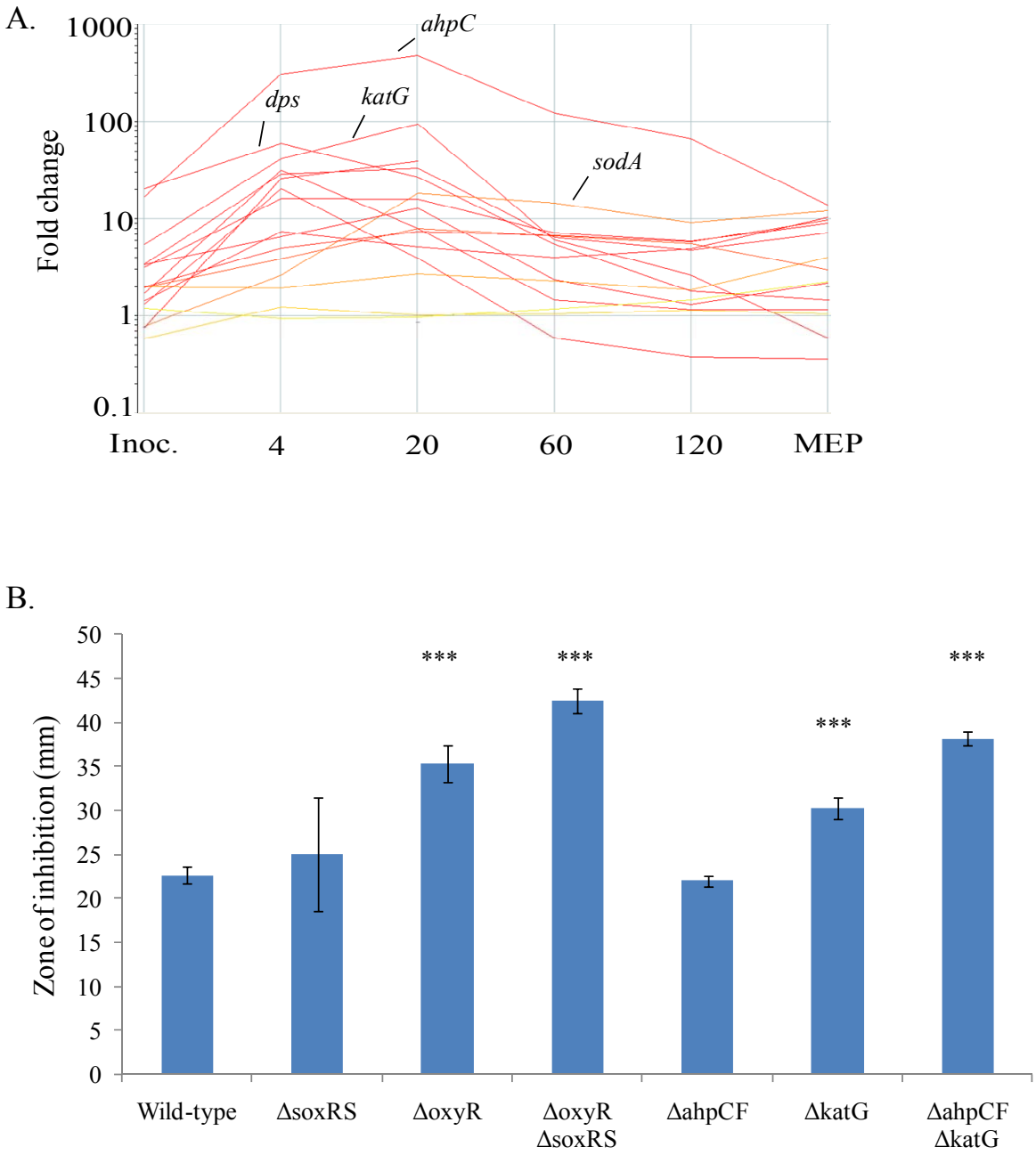


Figure 6.7: Lag phase-induced oxidative stress resistance genes aid bacterial survival. (A) Gene expression profiles of lag phase induced genes targeted for mutagenesis. The most prominently up-regulated genes are indicated, grown under Lag Phase 25 °C experimental conditions previously (Rolfe, 2007). The un-normalised data is coloured by the relative expression at 4 minutes post-inoculation. (B) Hydrogen peroxide sensitivity assay (Section 2.1.8.1) on LB agar plates in the presence of 882 mM hydrogen peroxide. Zone of growth inhibition measured after approximately 16 hours incubation at 37 °C. Error bars represent the standard deviation from the mean of three biological replicates. Asterisks denote significant differences from the Wild-type as calculated by student *t*-test ***, $p \leq 0.0001$.

The increase in redox potential experienced by the bacterial population during inoculation into fresh LB medium leads to the induction of genes involved in oxidative stress resistance (Storz & Zheng, 2000). The absence of these genes causes the bacteria to be more susceptible to a H_2O_2 challenge, particularly in the case of the Δ*oxyR* Δ*soxRS* mutant. To investigate the role of OxyR and SoxRS in lag phase adaptation and subsequent growth, viable count growth curves of wild-type bacteria and Δ*oxyR,* Δ*soxRS,* Δ*oxyR* Δ*soxRS* mutants were performed over 9 hours in the static growth system at 25 °C (Figure 6.8). The growth parameters were analysed by DMFit and compared between strains (Table 6.2).

Strain	Lag time (h)	Doubling time (h)	Cell concentration (log_{10} CFU/ml)	
			Starting	Final
Wild-type	1.67, 2.53	0.98, 0.98	5.63, 5.79	7.95, 7.82
Δ*soxRS::kan*	2.52, 2.60	0.99, 0.99	5.70, 5.79	7.98, 7.76
Δ*oxyR::cat*	2.36, 2.28	0.98, 0.99	5.69, 5.70	7.97, 7.93
Δ*oxyR::cat* Δ*soxRS::kan*	2.61, 2.06	0.99, 0.99	5.68, 5.69	7.86, 7.95

Table 6.2: SoxRS and OxyR are not essential for normal growth in the static system. Growth parameters calculated by DMFit are shown for the wild-type and mutant strains tested for two biological replicates shown separated by a comma (,).

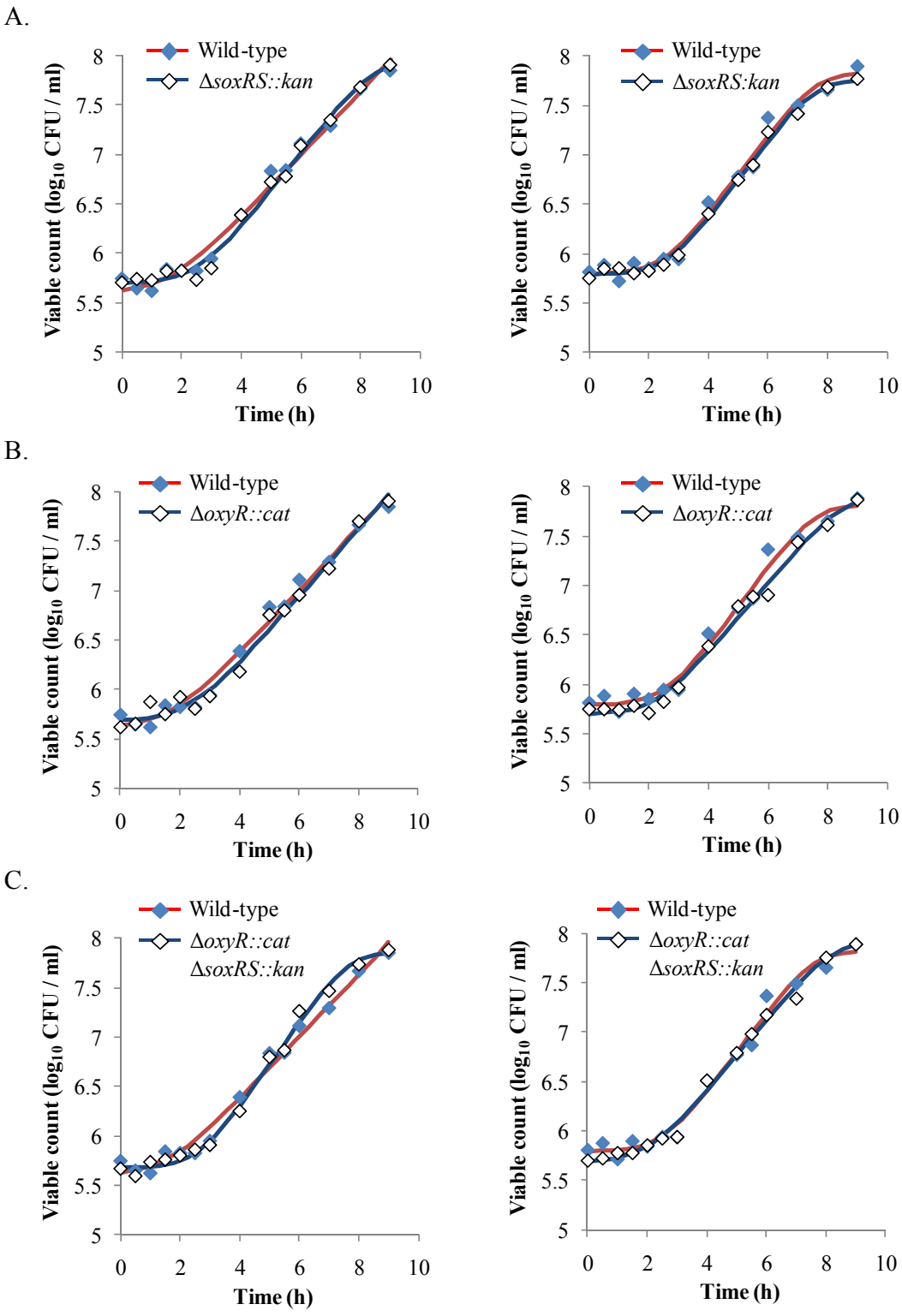


Figure 6.8: OxyR or SoxRS systems are not required for normal lag time or growth. Viable count growth curve analysis of (A) JH3346 (Δ*soxRS::kan*) (B) JH2462 (Δ*oxyR::cat*) (C) JH2463 (Δ*soxRS::kan* Δ*oxyR::cat*) compared with wild-type (SL1344). Diamonds represent viable count data-points and lines represent fitted curves using DMFit. Two biological replicates are shown for each mutant on the left and right panels.

The data presented in Figure 6.8 and Table 6.2 show that neither SoxRS nor OxyR systems were required for normal growth in the static growth system. Even a combined Δ*oxyR::cat* Δs*oxRS::kan* mutant which was designed to deprive the bacteria of resistance to both H_2O_2 and O_2^- did not show an increase in lag time or an altered doubling time. All strains reached similar final cell concentrations over the course of the experiment, suggesting that the lack of oxidative stress resistance mechanisms did not lead to early entry into stationary phase or killing by reactive oxygen intermediates. The starting concentration of the cultures was similar for all strains. This suggests that after 48 hours of growth (the stationary phase standardised inoculum) all the bacterial strains had reached the same final cell density and were not physically impaired for growth in the conditions tested. The lack of defence systems such as catalases and alkyl hydroperoxide reductases would have been expected to lead to a build up of reactive oxygen intermediates during growth which could in theory have led to the formation of sub-lethally damaged bacterial population in the stationary phase inoculum. These bacteria would have been expected to have a longer lag time after inoculation into an aerobic medium. No increase in lag time was observed suggesting that either the bacteria were not adversely affected by oxidative stress in this system or that alternative detoxification systems were utilised to overcome the oxidative stress.

6.3 Discussion

6.3.1 Effect of H_2O_2 on bacterial survival

Reactive oxygen intermediates can have a significant effect on bacterial viability (Cosgrove *et al.*, 2007). In the experiments performed in this chapter, reactive oxygen intermediates were formed through the addition of H_2O_2. When 1 mM H_2O_2 was added to aerobic cultures, the bacteria survived reasonably well, although there was a greater loss in viability observed in the 4 minute culture. When 6 mM H_2O_2 was added, resistance only occurred after 60 minutes post-inoculation suggesting that defence mechanisms took between 20 and 60 minutes to be induced and fully-functional. These two different concentrations of H_2O_2 have been routinely used in molecular biology to stress eukaryotic cells (Molyneux & Davies, 1995, Rigby *et al.*, 2008) and 6 mM H_2O_2 is also used in biochemistry to measure cellular catalase activity (Kováčik & Bačkor, 2007, Qiusheng *et al.*, 2005). The concentration of H_2O_2 used in the present study is lower than used in

previous studies to stress resistant mid-exponentially growing *S.* Typhimurium (100 mM H_2O_2) (Greenacre *et al.*, 2006) but higher than other experiments to analyse the effect of oxidative stress on mutant strains of *S.* Typhimurium (1 mM and 4 mM) (Bang *et al.*, 2005). From the preliminary experiments performed in the present study, 6 mM H_2O_2 was identified as a suitable concentration to phenotypically distinguish between early and mid-lag phase cultures.

6.3.1.1 Effect of changes in redox potential

The anoxic conditions of the standardised inoculum in the present study are similar to previous work using the rich medium trypticase soy broth with 10 % (v/v) H_2 added (E_h = -348 mV ± 31). Under these conditions, *Salmonella* Enteritidis was able to survive and resist subsequent heat shocks at 57 °C and 60 °C (George *et al.*, 1998). The lag phase cultures were all at similar positive redox potentials in LB although the redox potential of other media and environments can differ considerably, for example some marine coastal sediments have a much higher redox potential, approximately +200 mV (Pearson & Stanley, 1979). The redox potential of the cultures measured with a platinum redox electrode were quite variable and a lack of technical reproducibility between replicate samples has been noted previously (Hewitt, 1948, Morris, 2000).

Of the redox potentials measured, the greatest H_2O_2-induced increase was observed in the 48 hour stationary phase culture (ΔE_h = 370 mV). This large relative increase in redox potential offers some explanation as to the elevated expression of oxidative stress genes in the stationary phase inoculum, as identified elsewhere (Rolfe, 2007). Any contact with an oxidising agent would have a relatively large effect on the redox potential of the anoxic environment, and the subsequent oxidative stress response of the bacteria. In *E. coli*, the thiol-disulphide redox potential is -280 mV, which maintains OxyR in the inactive state until the redox potential increases upon oxidation (Storz & Zheng, 2000). Once the cysteine-thiol residues C199 and C208 are oxidised, the OxyR thiol groups become altered to form disulphide bonds of activated OxyR (Pomposiello & Demple, 2001, Storz & Zheng, 2000). This change in OxyR activity correlates with the increase in redox potential during lag phase (Figure 6.2), and the subsequent up-regulation of the OxyR-regulon after inoculation into aerobic LB medium (Rolfe, 2007).

6.3.1.2 Lag phase H_2O_2 tolerance

Although the addition of 6 mM H_2O_2 increases the redox potential at every stage of growth, the redox change during lag phase is not as high as the 48 hour stationary phase inoculum, which should lead to lower oxidative stress-mediated killing of lag phase bacteria. However, the present study has shown that early lag phase bacteria are more susceptible to H_2O_2-shock, and resistance is only achieved between 20 minutes and 60 minutes lag phase. Based on the experimental parameters it is hypothesised that H_2O_2 killing is either the result the lower cell concentration present during lag phase or that lag phase-associated physiological processes make the bacteria more susceptible to either oxidative stress or the high redox potential. These processes may include increased iron uptake during lag phase, leading to the accumulation of Fenton reaction products (Wardman & Candeias, 1996) or repair of carbonylated proteins (Fredriksson *et al.*, 2005) which may be perturbed by increased oxidative stress. The H_2O_2-mediated killing inversely correlates with the induction of oxidative stress machinery during lag phase. The previously acquired transcriptomic data (Rolfe, 2007) showed maximal expression of the OxyR regulon at 20 minutes post-inoculation. The OxyR regulon have been observed elsewhere as an important part of aerobic adaptation in *E. coli* (Iuchi & Weiner, 1996). The experiments performed in the present study reveal increased resistance to H_2O_2 between 20 and 60 minutes of lag phase, after the oxidative stress defence mechanisms have been induced. Interestingly, the mid-exponentially growing population were initially more sensitive to H_2O_2 than the 120 minute culture and the stationary phase inoculum. Exponentially-growing bacteria have been shown to be less tolerant to various stresses than stationary phase bacteria, due in large part to the relatively-high concentrations of the alternate sigma factor RpoS (σ^{38}) produced during stationary phase (Ibanez-Ruiz *et al.*, 2000, Lee *et al.*, 1995, Mandel & Silhavy, 2005, Oneal *et al.*, 1994). The data obtained in the present study suggest that oxidative stress resistance mechanisms are required during lag phase to tolerate LB medium to which 6 mM H_2O_2 had been added; if an oxidative challenge is met before the resistance or detoxification systems are in place, rapid death ensues. However, a short-term resistance is exhibited between 60 minutes and 120 minutes lag phase suggesting that rapid oxygen intermediate de-toxification can occur in the later stages of lag phase, prior to cell division initiation. The different survival profiles at various stages of lag phase provides phenotypic validation of previous transcriptomic data (Rolfe, 2007). This study is the first to identify different oxidative stress responses at

various stages of lag phase and highlights the importance of lag phase in stress survival prior to exponential growth.

6.3.2 Physiological history and effect on lag phase oxidative stress survival

It was shown in Chapter 3 and Chapter 4 that after a period of 12 days cold storage at 2 °C the geometric lag time was unaffected, and this was accompanied by relatively minor changes at the transcriptomic level. The transcriptomic evidence suggested that cold storage may increase oxidative stress during lag phase recovery, as expression of the OxyR regulon was increased in duration from 20 minutes to 60 minutes of lag phase after cold storage. In addition, expression of the iron transport mechanisms was delayed from 4 minutes and 20 minutes to 60 minutes post-inoculation which would be expected to delay the build-up of Fenton reaction products (Wardman & Candeias, 1996). The sensitivity of Lag Pre-Incubation bacterial cultures to H_2O_2 was measured and although the 4 minutes and 20 minute samples were similar to Lag Phase 25 °C grown bacteria, there was a greater H_2O_2 sensitivity in the 60 minutes sample grown in Lag Pre-Incubation conditions. Interestingly, this H_2O_2 sensitivity phenotype was not identified in the Lag Pre-Incubation stationary phase inoculum, which was resistant to 6 mM H_2O_2. A previous study showed that cold-shocked *S*. Typhimurium were more susceptible to micromolar concentrations of H_2O_2 present in the complex, rich medium TSYB (tryptone, soya, yeast extract broth) due to an unknown mechanism (Mackey & Derrick, 1986b). Using the Lag Pre-Incubation transcriptomic data from the present study (Section 4.2.4), it is hypothesised that, during temperature upshift from 2 °C to 25 °C, physiological changes such as alterations to membrane composition (Sinensky, 1974), may exacerbate sensitivity to H_2O_2. However, inoculation of the Lag Pre-Incubation stationary phase culture into 25 °C fresh LB medium does not represent a sufficient oxygen upshift to cause bacterial death even at early lag phase when the bacteria are most susceptible.

One possible mechanism linking cold storage and oxidative stress sensitivity may involve molecular chaperones. During cold storage, the increased production of cold-shock proteins (CSP) will protect targets of oxidative damage such as ribosomes, DNA and RNA (Yamanaka, 1999). Once temperature upshift occurs, a down-regulation of cold-shock proteins may make the bacteria susceptible to stress shocks. However, a previous study has shown that in the cyanobacterium *Synechococcus* sp. PCC 7942, a heat-shock protein

chaperone protects bacteria from irradiation-mediated oxidative stress (Hossain & Nakamoto, 2002). In the present study, inoculation into pre-warmed 25 °C LB medium induced the up-regulation of >10 heat-shock genes (Section 4.2.4) which are hypothesised to act as molecular chaperones to protect against various stresses.

6.3.3 Oxidative stress sensitive mutants

It is known that growth in an aerobic medium produces H_2O_2 (Cuny *et al.*, 2007) and that greater endogenous H_2O_2 is produced from a complex medium (such as LB) than a minimal medium (Mackey & Derrick, 1986b). By targeting important oxidative stress resistance mechanisms the relative importance of each system for aerobic adaptation during lag phase was elucidated.

During an oxidative insult with H_2O_2, stationary phase bacteria lacking *katG* or *oxyR* were more susceptible to oxidative damage than the wild-type strain. Previous studies with *Staphylococcus aureus* have found that AhpC is required to combat a wide range of oxidative stresses including H_2O_2, organic peroxides, and peroxynitrite, whereas catalase systems work solely against H_2O_2 (Cosgrove *et al.*, 2007). This suggests that although *in vitro* experiments in the present study reveal that KatG is more important than AhpCF in overcoming H_2O_2, a wide range of reactive oxygen intermediates present in aerobic environments requires the AhpCF system for survival. Interestingly, previous work involving a Δ*ahpCF::kan* mutant in the static growth system did not identify a longer lag time or decreased growth rate (Rolfe, 2007) suggesting that other resistance mechanisms such as SoxS or KatG may compensate for the absence of AhpCF.

As expected, mutants lacking OxyR were sensitive to a H_2O_2 challenge. The phenotype was exacerbated in a Δ*oxyR* Δ*soxRS* mutant, providing further evidence that the SoxRS two-component system offers a degree of protection in the absence of OxyR. A previous study has identified OxyR as the primary defence mechanism against H_2O_2 stress but did find genes that were induced by H_2O_2 in strains lacking OxyR or SoxS, including the *isc* operon required for iron-sulphur cluster formation (Zheng *et al.*, 2001). Although OxyR was required for greater protection against oxidative stress, there was no increase in lag time observed for Δ*oxyR,* Δ*soxRS* or Δ*oxyR* Δ*soxRS* mutants tested in the static growth system. The Δ*soxRS* mutant had a lag time of approximately 2.5 hours which was similar

to the lag times calculated previously for Δ*soxR* and Δ*soxS* mutants in the static growth system (Rolfe, 2007). The result that neither OxyR nor SoxRS are essential for lag phase or exponential phase in static LB cultures suggests that either alternative systems can compensate for the loss of these regulators or genes such as *katG* or *ahpCF* are under the control of another regulator and so are still expressed in the absence of OxyR. The other possibility remains that the transition from the anoxic culture environment (E_h = -328 mV) to the aerobic lag phase culture (4 minutes, E_h = +42 mV) does not constitute a physiologically-damaging oxidative stress, but does lead to the induction of these systems at the transcriptomic level. One method for testing this would be to perform a catalase assay to measure the concentration of dissolved H_2O_2 throughout growth in the lag phase system. Although experiments in the present study found that 6 mM H_2O_2 could induce killing, it has been previously reported that oxidative damage can arise from H_2O_2 at micromolar concentrations (Jang & Imlay, 2007) however these experiments were performed in non-buffered minimal media which may produce different redox reactions, some of which are more 'sluggish' than others (Morris, 2000). A recent study in *S.* Typhimurium strain 4/74 found that 299 genes were induced significantly (>3-fold) by 1 mM H_2O_2 (Wright *et al.*, 2009). These genes included *soxS*, *ahpC*, *ahpF* and *katG*, the latter of which was the most significantly induced gene (up-regulated 673-fold). Similarly to the present study, mutant strains lacking *katG* were susceptible to challenge by H_2O_2 *in vitro*, although the mutants were able to colonise the spleens of mice (Wright *et al.*, 2009).

The induction of multiple oxidative stress resistance systems during oxygen upshift in lag phase highlights either the importance of resisting reactive oxygen intermediates before cell division is initiated, or that induced genes respond to other unknown environmental cues. Under the conditions tested, reactive oxygen intermediates were not present at damaging concentrations during inoculation into an aerobic medium, although additional oxidative stress was lethal if present before the defence mechanisms were induced. It was particularly interesting that growth history was found to affect oxidative stress sensitivity during lag phase, although the geometric lag time was not extended. Future work to investigate the effect of oxidative stress on bacterial lag time would involve using a more oxygen-sensitive mutant such as the HpxF$^-$ strain (Δ*katE* Δ*katG* Δ*katN* Δ*ahpCF* Δ*tsaA*) recently used in a study with *S.* Typhimurium strain 12023 (Hébrard *et al.*, 2009).

6.3.4 Concluding remarks

The experiments performed in this chapter have concluded that during the inoculation of bacteria from a stationary phase culture into fresh LB medium an oxidative stress response is elicited as a result of an oxygen upshift and an increased redox potential. Although many of the oxygen-responsive genes induced during lag phase are OxyR-responsive, the deletion of genes encoding catalases, alkyl hydroperoxide reductases or the regulators OxyR and SoxRS did not lead to an increased lag time, although the majority of the mutant strains were more susceptible to H_2O_2 mediated oxidative stress. In summary, while the degree of oxidative stress encountered during lag phase under normal growth conditions is enough to register oxidative stress at the transcriptomic level, it is not sufficient to cause cellular damage. An extreme oxidative insult administered before the middle of lag phase (60 minutes) severely affects lag phase bacterial viability, after which point bacteria develop oxidative stress resistance prior to cell division.

Chapter 7

Metal ion homeostasis during lag phase

7. Metal ion homeostasis during lag phase

It has been hypothesised that during lag phase in the static growth system, extensive homeostasis occurs for a range of metal cations (Rolfe, 2007). The previous study revealed the induction of numerous metal ion transport genes within the early part of lag phase and reported a preliminary attempt to assess the accumulation of metal ions using inductively-coupled plasma mass spectrometry (ICP-MS). Chapter 4 of the present study described the induction of metal ion transport genes during lag phase after recovery from cold storage forming the hypothesis that the uptake of metal cations such as: Fe^{2+}, Mn^{2+}, Mg^{2+}, Ca^{2+} and Na^{+} may be important during lag phase adaptation. The current chapter aims to identify physiologically-important metal ions and their role in lag phase. The growth conditions are based on the Rolfe Lag Phase 25 °C static system and aim to phenotypically validate the previously-acquired transcriptomic data (Rolfe, 2007).

7.1 Introduction

7.1.1 The role of metal ions in bacterial physiology

Approximately one-third of all known proteins require metal cofactors (Rosenzweig, 2002). Metal ion homeostasis represents a delicate balance for bacteria between performing necessary biological functions and preventing metal-induced toxicity. This section provides a brief overview of the known effects of metal ions on bacterial physiology with a focus on *E. coli* and *Salmonella*. For reference, the major transporter-encoding genes for nine physiologically-relevant metal ions are included in Table 7.1.

Metal ion	Gene	Function	Reference
Calcium	*chaA*	Ca^{2+} / H^{+} antiporter	(Ohyama *et al.*, 1994)
Cobalt	*cbiMNQO*	Vitamin B12 adenosyl cobalamide precursor	(Rodionov *et al.*, 2006) (Zhang & Gladyshev, 2010)
	cboQ	Putative Co^{2+} transport protein	*coli*BASE
Copper	*copA*	Copper ion-import (and export) ATPase	(Espariz *et al.*, 2007)
Iron	*entABCDEF*	Enterobactin (siderophore) production	(Fleming *et al.*, 1983)
	feoAB	Fe^{2+} transporter proteins	(Kammler *et al.*, 1993)
	fepABCDE	Enterobactin receptor proteins	(Pierce *et al.*, 1983)
	fhuABCDEF	Outer membrane receptor / transporter for siderophores	(Killmann *et al.*, 1998)
	iroBCDEN	ABC transporter	(Wu *et al.*, 2002)
Magnesium	*corA*	Mg^{2+} transporter	(Maguire, 2006)
	mgtABC	Mg^{2+} transporter	(Hmiel *et al.*, 1989)
Manganese	*mntH*	Mn^{2+} transporter	(Patzer & Hantke, 2001)
	sitABCD	Mn^{2+} transporter	(Kehres *et al.*, 2002b)
Molybdenum	*modABC*	Mo^{2+} transporter	(Miyake *et al.*, 1995)
Nickel	*nxiA*	Putative Ni^{2+} transporter	*coli*BASE
	STM2759	Putative Ni^{2+} ABC-transporter system	*coli*BASE
	yejB	Putative Ni^{2+} ABC-transporter system	*coli*BASE
	yejE	Putative Ni^{2+} ABC-transporter system (permease)	*coli*BASE
Sodium	*nhaA*	Na^{+} / H^{+} antiporter	(Thelen *et al.*, 1991)
Zinc	*zntA*	Zn^{2+} transporter	(Lee *et al*., 2005)
	znuABC	Zn^{2+} transporter	(Patzer & Hantke, 1998)
	zinT	High affinity Zn^{2+} transporter	(Petrarca *et al.*, 2010)

Table 7.1: Physiologically-relevant metal ion import genes.
All genes present in *S.* Typhimurium. Function of the gene product in as annotated in *coli*BASE for the *S.* Typhimurium LT2 genome (accessed May 2010).

7.1.1.1 Iron

The biological availability of iron is limited in certain environments. At pH-neutral conditions, iron is primarily present in the environment as insoluble Fe^{3+} requiring solubilisation by siderophores before internalisation can occur (Braun & Hantke, 2007, Wooldridge & Williams, 1993). Siderophores bind iron at a very high affinity (*e.g.* enterobactin $K_{aff,Fe3+}$ ~10^{52} M^{-1}) and can actively compete against other chemical iron chelators and biological iron storage systems (Byers & Arceneaux, 1998). The chemical flexibility of iron makes it a useful micro-nutrient for assimilation into proteins, acting as a

co-factor for enzymes involved in many physiological processes such as: oxidative stress resistance, nitrogen fixation, the TCA cycle, oxygen transport, DNA replication, gene regulation and virulence signalling (Andrews *et al.*, 2003). *Salmonella* has developed high-affinity iron uptake systems to avoid potential iron restriction (Chart & Rowe, 1993) and to maintain a physiologically significant basal concentration, approximately 6 x 10^5 atoms per *E. coli* cell grown in LB medium (Outten & O'Halloran, 2001). Maintaining these iron pools is important for bacteria such as *Salmonella* and restricting the availability of iron leads to physiological deficiencies that include: decreased bacterial survival during infection of murine macrophages (Nairz *et al.*, 2007), decreased growth rate (Abdul-Tehrani *et al.*, 1999), decreased cytotoxin production and an extended lag phase (Ho *et al.*, 2004). Conversely, exposure to a high concentration of unbound Fe^{2+} leads to the formation of hydroxyl free radicals through the Fenton reaction:
($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH\cdot + OH^-$), causing damage to bacterial cells (Gralnick & Downs, 2001, Gralnick & Downs, 2003, Imlay & Linn, 1988, Repine *et al.*, 1981, Wardman & Candeias, 1996). A recent study showed that murine macrophage phagosomes may employ intracellular iron concentrations and Fenton chemistry as a defence mechanism against *S.* Typhimurium. Phagosome iron concentration was measured by *S.* Typhimurium *lacZ*-reporter transcriptional fusions to iron-responsive gene promoters $P_{iroBCDE}$ and P_{sodB} (Taylor *et al.*, 2009). *Salmonella* reduces Fenton-derived hydroxyl free radicals primarily by flavin reductases (Woodmansee & Imlay, 2002) and other reductases such as TrxAB, Gor and GrxAB, some of which are regulated by oxidative stress responsive regulators, such as OxyR (Pomposiello & Demple, 2001). In addition, TrxA is required for full activity of *Salmonella* Pathogenicity Island 2 (SPI-2) and survival inside phagocytic cells during infection of mice (Negrea *et al.*, 2009).

7.1.1.2 Manganese

In *Salmonella* there are two specific systems for the uptake of manganese ions (Mn^{2+}): MntH and SitABCD (Zaharik *et al.*, 2004, Kehres *et al.*, 2002b). SitABCD was horizontally-acquired by *Salmonella* along with *Salmonella* Pathogenicity Island 1 (SPI-1) (Hansen-Wester & Hensel, 2001, Lostroh & Lee, 2001) and is therefore absent in *E. coli*. Both *sitABCD* and *mntH* expression are transcriptionally-regulated by MntR (STM0835) and OxyR (Ikeda *et al.*, 2005, Kehres *et al.*, 2002a) and respond to environmental changes in manganese ions and hydrogen peroxide. The expression of both *mntH* and *sitA* are

markedly induced upon invasion of macrophages (Kehres *et al.*, 2000) and both are essential for the successful systemic infection of mice (Zaharik *et al.*, 2004).

The physiological role of Mn^{2+} is less well-studied than iron. The primary protein known to utilise Mn^{2+} as a cofactor is Mn-superoxide dismutase (Mn-SOD, SodA), which is required to combat oxidative stress (Keele *et al.*, 1970). A recent study has determined that Mn^{2+} has a role in the OxyR response to hydrogen peroxide and may be recruited in the place of iron to attenuate the build-up of Fenton reaction products (Anjem *et al.*, 2009).

7.1.1.3 Magnesium

Magnesium ions (Mg^{2+}) are an abundant micronutrient and compete for bacterial divalent cation transporters in most environments (Webb, 1970). Because of the relative abundance of Mg^{2+}, no high affinity uptake systems are used by *Salmonella* to accumulate magnesium ions however the *mgtABC* transport systems (Hmiel *et al.*, 1989) and the CorA Mg^{2+} channel allow the accumulation of magnesium ions (Maguire, 2006). A fully-functional CorA ion channel is required for full virulence of *S.* Typhimurium (Papp-Wallace & Maguire, 2008, Papp-Wallace *et al.*, 2008). The *mgtC* gene is located within the SPI-3 pathogenicity island of *S.* Typhimurium and has been found to be crucial for SPI-3 mediated virulence in *S.* Typhi (Retamal *et al.*, 2009). During infection, low environmental concentrations of magnesium act as a trigger for the PhoP/PhoQ two-component system to activate virulence genes in *Salmonella* (Soncini *et al.*, 1996, Garcia Vescovi *et al.*, 1996) highlighting the importance of Mg^{2+} in co-ordinating *Salmonella* pathogenicity. Mg^{2+} transport by MgtA has been shown to be regulated by a Mg^{2+}-sensing riboswitch (Cromie *et al.*, 2006). A recent study has described an increased thermotolerance phenotype in mutated forms of Mg^{2+}-sensing riboswitches suggesting a role for Mg^{2+} in thermotolerance by directly protecting proteins or signalling the induction of thermoprotective machinery (O'Connor *et al.*, 2009). Although Mg^{2+} transport and function have been studied in detail for several years, new regulatory roles suggest that many of the functions are still to be fully elucidated.

7.1.1.4 Calcium

Intracellular calcium ions (Ca^{2+}) are generally maintained at sub-micromolar concentrations by *E. coli* via tightly-regulated influx and efflux mechanisms (Jones *et al.*,

1999, Jones *et al.*, 2002). Calcium transport is performed in part via the periplasmic Ca^{2+}/H^{+} antiporter, ChaA (Ivey *et al.*, 1993) and a poly-3-hydroxybutyrate polyphosphate membrane channel which binds calcium, forming a complex and increases bacterial competence (Castuma *et al.*, 1995, Huang & Reusch, 1995, Reusch *et al.*, 1995). Conversely, calcium efflux is dependent upon ATP-generated energy as demonstrated in a recent study which found that a Δ*atpD* mutant could not efflux Ca^{2+} (Naseem *et al.*, 2009). Depletion of *E. coli* intracellular Ca^{2+} by specific chelators induces de-repression of AhpC, an alkyl hydroperoxide reductase, involved in protecting against oxidative stress and known to bind the $^{45}Ca^{2+}$ isotope (Herbaud *et al.*, 1998).

Previous studies have postulated that a calcium spike could trigger intracellular signalling cascades in bacteria, similar to those observed in eukaryotes (Norris *et al.*, 1988) and subsequent studies implicated Ca^{2+} in bacterial chemotaxis (Tisa & Adler, 1992) and the cell cycle (Norris *et al.*, 1996), Despite many ongoing studies, the physiological roles for intracellular Ca^{2+} still remain to be fully elucidated.

7.1.1.5 Copper

Copper is a readily-available metal in most environments and is readily oxidised from Cu^{+} to Cu^{2+}. Intracellular concentrations must be tightly regulated as copper competes for proteins which require other metal ion co-factors to function. In addition, the redox active Cu^{+} state can enter the Fenton reaction in a similar way to iron, forming hydroxyl free radicals and leading to an accumulation of cellular damage (Magnani & Solioz, 2007). Copper ions are primarily transported via the CopA ATPase, in *E. coli* (Rensing *et al.*, 2000, Petersen & Moller, 2000). CopA also actively removes excess copper in *E. coli* and *Bacillus subtilis*; in the latter organism, copper export occurs in partnership with the copper metallochaperone CopZ, which binds to the N-terminal domain of CopA during copper recycling (Radford *et al.*, 2003). In *Salmonella* the redox activity of copper ions is utilised by the incorporation into SodCI and SodCII, a Cu^{2+} / Zn^{2+} superoxide dismutase (Fang *et al.*, 1999).

Copper toxicity poses a challenge for bacteria in a range of environments and numerous detoxification systems have been developed to meet these challenges. Under aerobic conditions a *Salmonella* Δ*copA* mutant strain has a minimal effect upon copper sensitivity,

with the function of CopA believed to be partly fulfilled by GolT (Espariz *et al.*, 2007). In anoxic environments, CopA has an important physiological role in copper detoxification with another protein, CueO (CuiD) (Lim *et al.*, 2002). Recently, a novel CueR-regulated copper detoxification protein was discovered in *Salmonella*, named CueP, which functions under anaerobic conditions. Indeed a fully-functional CueP protein can compensate for a *E. coli cusS* mutant, which encodes a protein involved in copper efflux (Pontel & Soncini, 2009). Other proteins in *S.* Typhimurium are required tolerate lethal concentrations of copper ions including suppression of copper sensitivity protein, Scs (Gupta *et al.*, 1997). Mutant strains lacking either *cuiD* or *scs* lose viability in the presence of Cu^{2+} at millimolar concentrations (Lim *et al*., 2002, Gupta *et al*., 1997).

In *E. coli*, the CusCFBA periplasmic copper efflux system ensures intracellular copper ions are maintained at sub-toxic concentrations (Rensing & Grass, 2003, Franke *et al.*, 2003). This efflux system is not present in *Salmonella* and instead aerobic copper tolerance is dependent upon the detoxification protein CueO, which is believed to oxidise Cu^{+} to the more stable Cu^{2+} state (Espariz *et al.*, 2007). In *E. coli* a Δ*cueO* mutant is fifty-times more susceptible to copper toxicity than the wild type strain (Tree *et al.*, 2005). CusRS is a chromosomal two-component system in *E. coli* which may have a role to play in actively exporting excess copper ions, along with the plasmid-borne homologues PcoR and PcoS (Munson *et al.*, 2000). Homologues of CusR and CusS also exist in *Salmonella* as SilR and SilS respectively which function as the silver ion tolerance response. The multitude of copper detoxification systems highlights the danger posed by this metal ion in unfavourable redox environments.

7.1.1.6 Zinc

Zinc is an abundant metal in most environments and is accumulated in *E. coli* to micromolar concentrations (Outten & O'Halloran, 2001). Zinc ions are transported by *E. coli* and *Salmonella* via several high affinity uptake mechanisms including the ZnuABC family of ATP-binding cassette (ABC) transporters (Patzer & Hantke, 1998, Patzer & Hantke, 2000) under the control of the transcriptional repressor, Zur. A *Salmonella enterica* Δ*znuA* mutant is growth-deficient in a Zn^{2+}-limited medium and is attenuated for virulence in Caco-2 epithelial cells, human monocytes and BALB/c mice (Ammendola *et al.*, 2007). A Δ*znuC* mutant is also attenuated after oral infection of BALB/c mice

(Campoy *et al.*, 2002), suggesting that the ZnuABC transporter is a primary means for Zn^{2+} import and that zinc ions are important for co-ordinating virulence in *Salmonella*.

Zinc ions are used by prokaryotes to form redox chemical reactions (although Zn^{2+} is not itself a redox active metal ion) and are incorporated into metallochaperones and enzymes to prevent oxidative stress and stabilise unfolded proteins (Ilbert *et al.*, 2006). In eukaryotes, Zn^{2+} can be incorporated into cysteine-histidine sites of proteins forming 'zinc fingers', which are important for transcriptional regulation. Few examples of zinc fingers exist in prokaryotes, however the metallothionein protein SmtA contains four zinc residues and is involved in intracellular zinc trafficking in the cyanobacterium *Synechococcus* PCC 7942 (Blindauer *et al.*, 2001).

Bacteria can utilise low concentrations of zinc ions through high-affinity uptake systems, making it difficult to deplete growth media of zinc ions to below physiologically relevant concentrations. However, one study analysed the transcriptomic response of *E. coli* to zinc-deprivation (≤60 nM Zn^{2+}) and discovered nine up-regulated genes, some of which were Zur-regulated including *znuA* and *zinT* (Graham *et al.*, 2009).

Excess zinc also has an effect at the transcriptional level. Chemostat-grown *E. coli* up-regulate 64 genes in response to a sub-lethal challenge by 0.2 mM zinc sulphate, including many genes involved in zinc efflux (Lee *et al.*, 2005). Zn^{2+} export is mediated by both primary and secondary efflux machinery (Blencowe & Morby, 2003). The primary means of export is through P-type ATPase complexes (Nies & Silver, 1995), such as ZntA in *E. coli* (Beard *et al.*, 1997), whereas secondary efflux mechanisms include the RND trans-envelope cobalt-zinc-cadmium (*czc*) system, first described in *Ralstonia metallidurans* (formerly *Alcaligenes eutrophus*) (Nies *et al.*, 1989).

7.1.1.7 Nickel

Nickel is an essential micronutrient which acts as a cofactor for many enzymatic processes (Hausinger & Zamble, 2007). In *Salmonella*, nickel is important for virulence and is incorporated into specific nickel-iron (NiFe) hydrogenases which function under anaerobic conditions (Zbell *et al.*, 2007). Bacterial nickel transport is performed by a range of transporters including the FNR-inducible NikABCDE and NikMNQO ABC-transporters in

E. coli (Rodionov *et al.*, 2006, Wu *et al.*, 1989). *Salmonella* does not contain the NikABCDE transport system and so Ni^{2+} transport may occur through other, less specific mechanisms such as the CbiMNQO Co^{2+} transporter (Rodionov *et al.*, 2006, Zhang & Gladyshev, 2010). *Salmonella* does however contain NikR, a regulator that has previously been shown to transcriptionally-repress gene expression in response to excess nickel, including genes encoding outer membrane proteins of *Helicobacter pylori* (Ernst *et al.*, 2006).

The CorA channel, which primarily transports $Mg^{2+,}$ also transports Ni^{2+}, Co^{2+} and Cd^{2+}, which can outcompete Mg^{2+} for CorA (Niegowski & Eshaghi, 2007). Other putative transport mechanisms in *S.* Typhimurium include: NxiA, YejE, YejB, STM2759 and STM3860, some of which also transport dipeptides and oligopeptides (by annotation in *coli*BASE).

To ensure intracellular concentrations of nickel are not toxic, efflux mechanisms are present to actively pump out excess Ni^{2+}/Co^{2+} (Nies, 2003), specifically YohM (RcnA) and its regulator YohL (RcnR) (Iwig *et al.*, 2008, Iwig *et al.*, 2006, Rodrigue *et al.*, 2005). Other general efflux mechanisms are involved in nickel homeostasis including CznABC which functions in resistance to heavy metal toxicity and is required for the colonisation of the stomach by *H. pylori* (Stähler *et al.*, 2006). One reason intracellular nickel is maintained at low concentrations in bacteria is to avoid inference with iron homeostasis (Nies & Silver, 2007). This is confirmed by the transcriptional regulation of *rcnA* by the iron regulator Fur in *E. coli* (Koch *et al.*, 2007).

7.1.1.8 Other metal ions

During growth in a complex medium, bacteria encounter micromolar concentrations of metal ions, some of which are potentially toxic and others which have useful physiological roles (Nies & Silver, 2007).

Chromate ions are encountered rarely by bacteria in most environments and once imported are effluxed rapidly before spontaneous conversion to Cr^{3+} can occur, with concomitant formation of toxic free radicals (Nies, 2007). The high affinity phosphate transporters (PstSCAB) can also transport **arsenate** at lower affinities, which is reduced in

E. coli by ArsC to arsenite (Ji & Silver, 1992) before removal by the membrane-bound ArsB efflux pump (Chen *et al.*, 1986). **Aluminium** is the most abundant metal in the crust of the Earth and can competitively inhibit iron incorporation by siderophores (Illmer & Buttinger, 2006). In addition, Al^{3+} is actively imported into *E. coli*, probably through siderophores (*e.g.* enterobactin), causing displacement of protons from the cell surface and subsequent acidification of the growth medium (Guida *et al.*, 1991).
E. coli and *Salmonella* have specific resistance mechanisms for other, low abundance metal ions such as **gold** (Pontel *et al.*, 2007) and **silver** (Silver, 2003), suggesting that the presence of these metal ions may interfere with the homeostasis of other physiologically-important metal ions.

Low abundance metal ions with a physiological role in bacteria include **cobalt** which is involved in the synthesis of vitamin B12 via the anaerobically-induced *cbi* gene cluster and *cobUST* encoding the cobalamide precursor machinery (Rodionov *et al.*, 2006). Cobalt homeostasis is maintained by the Ni^{2+}/Co^{2+} YohM efflux pump. Other physiologically-relevant metal ions include **molybdate** which is present in its biological form as molybdopterin (Mo-Pterin). Anaerobic respiratory enzymes using Mo-Pterin as a cofactor include dimethyl sulfoxide reductase (DMSOR), nitrate reductase (*narZYWV* and *narGHJI* operons) (Blasco *et al.*, 1990) and formate dehydrogenase (Romão *et al.*, 1997, Schwarz *et al.*, 2007).

7.1.2 Methods for detecting intracellular metal ions

Many methods exist for the quantification of metal ions however the best characterised are indirect fluorescence detection and the more direct spectroscopic detection. Fluorescent detection usually requires the addition of a chemical ligand such as ABEDTA or 8-hydroxyquinoline-5-sulphonic acid to bind the metal ions and the association of metal ions subsequently estimated by colorimetric changes. For routine analysis some of these fluorescent techniques provide a relatively inexpensive method for the identification of a specific sub-set of metal ions in the μM range (Zhu & Kok, 1998). Fluorescence detection methods are constantly optimised and coupled with other techniques such as high performance liquid chromatography (HPLC) to give increased sensitivity. One such study quantified lead and cadmium concentrations at a detection limit of 3.3 nM for use in identifying toxic metal ions in drinking water (Saito *et al.*, 2006). However, low abundance

metal ions in standard bacterial cultures are difficult to detect using fluorescence techniques. Therefore detection of intracellular concentration of low abundance metal ions requires more sensitive methods.

Spectroscopic detection typically involves the atomisation or ionisation of samples and then subsequent laser detection. Such techniques include: laser-induced breakdown spectroscopy (LIBS), inductively coupled plasma atomic emission spectroscopy (ICP-AES) and inductively coupled plasma mass spectroscopy (ICP-MS), all of which use plasma to produce excited atoms or ions for detection. In ICP-MS, plasma is produced by passing an electric current through argon gas, heating the test samples to >9000 °C, vaporising and ionising them. During the production of plasma, all electrodes are compartmentalised away from the plasma and the samples, to prevent metal contamination during analysis. After ionisation, the metal ions are directed into a mass spectrometer for measurement (de Hoffmann & Stroobant, 2002, Thomas, 2001). The most advantageous aspect of ICP-MS is the high degree of sensitivity (1×10^{-15} M), which is 100 times more sensitive than ICP-AES (de Hoffmann & Stroobant, 2002), coupled-with high-throughput capability.

ICP-MS has previously been used to detect and quantify a range of physiologically important intracellular metal ions in *E. coli* (Outten & O'Halloran, 2001, Tree *et al.*, 2005), and ICP-AES has been used to measure intracellular iron concentrations in *Salmonella* (Velayudhan *et al.*, 2007). These previous studies have established protocols for the application of spectroscopic techniques to specific biological samples.

Spectroscopy techniques allow the accurate quantification of a wide range of metal ions from a sample; however they are generally expensive, labour intensive techniques and prone to background contamination. For the direct quantification of intracellular metal concentrations described in this Chapter, ICP-MS was used after optimisation to minimise background contamination of test samples.

7.2 Results

7.2.1 Optimisation of the ICP-MS protocol.

In order to quantify accurately the intracellular metal concentrations, a robust protocol needed to be established that would ensure that background metal contamination was minimal. Metal contamination of samples could arise from various sources including the water to wash the samples, personal contamination (*e.g.* skin or hair) or from the plastic sample tubes. To minimise the contamination, polycarbonate or polypropylene co-polymer centrifuge tubes were used which leach fewer metal ions. In addition, plastic tips and centrifuge bottles were acid-washed prior to cell harvesting. An initial test was performed to ensure that sample contamination was low and to check the reproducibility of the protocol. Two culture volumes were used to test the reproducibility of the protocol at the 60 minute lag phase time-point - 100 ml culture (4.87×10^7 CFU) and 200 ml (9.73×10^7 CFU). In addition, procedural blanks were tested to quantify the contamination associated with the experimental technique. The blank samples consisted of either 5 ml MilliQ (18.2 MΩ / cm) pure water (water control) or empty non-acid washed polycarbonate tubes (Plastic), containing only the hydrogen peroxide-nitric acid mixture used to digest the samples prior to analysis. Once digested, the samples were analysed by ICP-MS and the concentration of different metal ions were determined per ml of sample run (Figure 7.1).

The initial experiment revealed that four physiologically important metal ions: manganese, magnesium, iron and copper were present in cells at a similar concentration in both the 100 ml or 200 ml culture samples (*t*-test p-values ranged from 0.5-0.7). For these initially-analysed metal ions at the 60 minute time-point, magnesium was the highest accumulated metal (~0.7 µg / ml) of culture. Copper was accumulated at the lowest concentration (~2.0 ng / ml). However, the majority of the cations tested were still detected in the procedural blanks. The concentrations of these metal ions were considerably lower than in the biological samples and varied depending on the metal analysed. This preliminary test identified a source of contaminating metal ions leached from the plastic sample tube, which could not be totally eliminated by further protocol refinement. Additional protocol optimisation was performed to minimise background traces of metal ions.

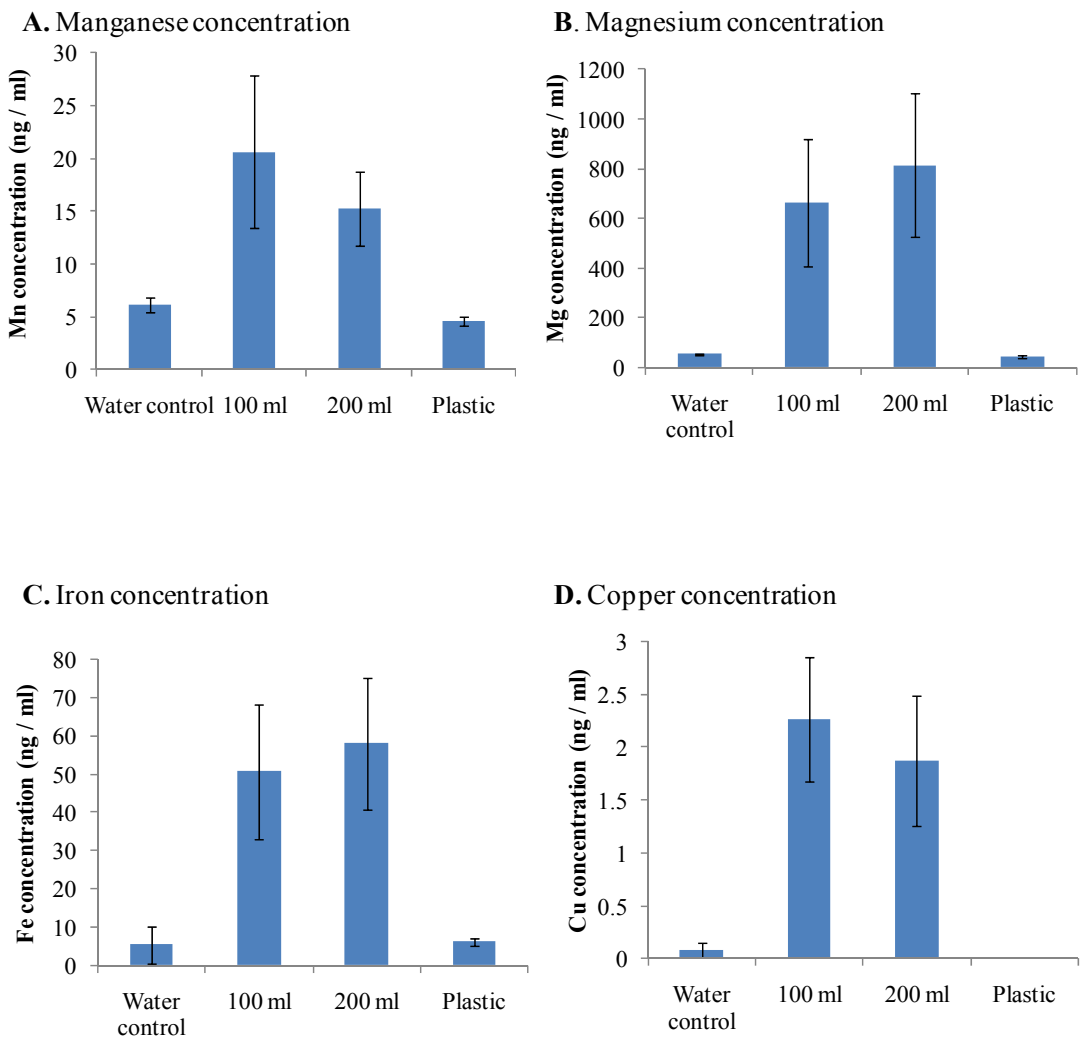


Figure 7.1: Initial measurement of four metal ions at 60 minutes lag phase.
ICP-MS quantification of intracellular concentrations of four physiologically important metal ions: (A) manganese, (B) magnesium, (C) iron and (D) copper for either 100 ml or 200 ml of bacterial culture. Metal concentration shown as nanograms (ng) per ml of sample analysed in the ICP-MS calibration buffer (Section 2.4.1.2). For clarity, the 60 minute lag phase cultures were normalised by volume. Experimental controls were used including MilliQ 18.2 MΩ/cm dH_2O (Water control) and empty polycarbonate tubes (Plastic). Error bars show the standard error of the mean (SEM) of five independent biological replicates.

7.2.2 Quantifying metal accumulation through growth

During the optimisation of sample preparation, 1 mM ethylenediaminetetraacetic acid (EDTA) chelator (pH 8.0) was added to the harvested cell pellets to bind any extracellular metal ions. The EDTA was removed by two further wash steps with MilliQ water. The biological replicates were decreased from five to three so that all of the samples could be run simultaneously in the ICP-MS analyser. In order to gain a more representative analysis, the volume of lag bacterial culture was increased to 750 ml (~4 x 10^8 CFU). The triplicate samples were run simultaneously and analysed for 36 metal ions, some of which were below the detection limit (1 pg per ml sample).

This protocol (Section 2.4.1) was used to analyse bacterial samples throughout growth at: stationary phase (48 hour inoculum), lag phase (4 minutes, 20 minutes, 60 minutes and 120 minutes post-inoculation), mid-exponential phase (8 hours) and early stationary phase (24 hours). As a result of the protocol refinement, there was less variability between the samples and growth phase dependent accumulation of some metal ions was observed (Figure 7.2 and Figure 7.3). The accumulation of some metal ions was correlated with previous gene expression data (Rolfe, 2007) and provided a phenotypic confirmation of metal ion transporter gene expression (Figure 7.2). This validation was shown for six physiologically-relevant metal ions with known and putative transporters. Additionally for cobalt, the expression of *yohM* encoding the Ni^{2+}/Co^{2+} efflux machinery and the regulator encoding gene, *yohL* were shown to provide transcriptional evidence for removal of intracellular Co^{2+}.

The concentrations of metal ions accumulated were measured as attomoles (10^{-18} moles) per cell, as calculated by viable counts (Figure 7.3I). The relative intracellular concentrations of the four metal ions quantified during the initial experiment were the same after optimisation, with magnesium the most abundant, followed by iron and manganese. Copper was the least abundant of these four metal ions (Figure 7.2 and Figure 7.3). The protocol optimisation decreased the standard error of the mean for each of these test metal ions at 60 minutes lag phase, by between 81.0 % (Cu^{2+}) and 99.2 % (Mn^{2+}).

The trends for metal accumulation were robust with lag phase accumulation occurring over multiple time-points. Of the published physiologically-relevant metal ions with known

transporters, manganese and calcium showed a trend of accumulation during lag phase. Iron accumulation was less drastic compared with the inoculum, exhibiting a two-fold higher concentration of iron present at 4 minutes and 20 minutes post-inoculation than in the inoculum sample. Accumulation of iron subsequently decreased for the remainder of growth. This correlated with the gene expression data for all annotated iron homeostasis machinery, which are significantly up-regulated during early lag phase. However, iron accumulation decreased towards the end of lag phase with iron homeostasis genes still highly-expressed. This suggests that the iron-specific transport machinery is also regulated at the post-transcriptional level. The increase in expression of specific metal transporters correlates well with metal accumulation for both manganese and calcium, suggesting that interpretation of the cellular response is easier to achieve for metal ions with fewer transporters. In the case of manganese transport, the *sitABCD* genes are up-regulated for the duration of highest manganese accumulation whereas the more specific *mntH* gene is down-regulated by 60 minutes and throughout the remainder of growth. These data suggest that manganese uptake occurs primarily through *sitABCD* which is absent in *E. coli*, and suggests different mechanisms for *mntH* and *sitABCD* regulation.

Interestingly, the intracellular concentration of molybdenum (Mo^{2+}) and cobalt (Co^{2+}) decreased during lag phase compared with the stationary phase inoculum. This decrease in accumulation correlates with down-regulation of Mo^{2+} transporter encoding genes, (*modABC*) from 20 minutes post-inoculation until the end of lag phase (Figure 7.2E) and Co^{2+} transporter encoding genes throughout lag phase (Figure 7.2F). The decrease in the concentration of metal ions during early lag phase suggests that efflux mechanisms were responsible for the removal of these intracellular cations. This hypothesis was confirmed for Co^{2+} by the marked up-regulation of *yohM*, encoding the Ni^{2+}/Co^{2+} specific efflux pump, towards the end of lag phase and during mid-exponential phase. There is no known Mo^{2+}-specific efflux machinery, so the transcriptional evidence is not available to help interpret the decrease in concentration of this metal. The decrease in Mo^{2+} concentrations may be due to non-specific efflux machinery or exit through membrane porins.

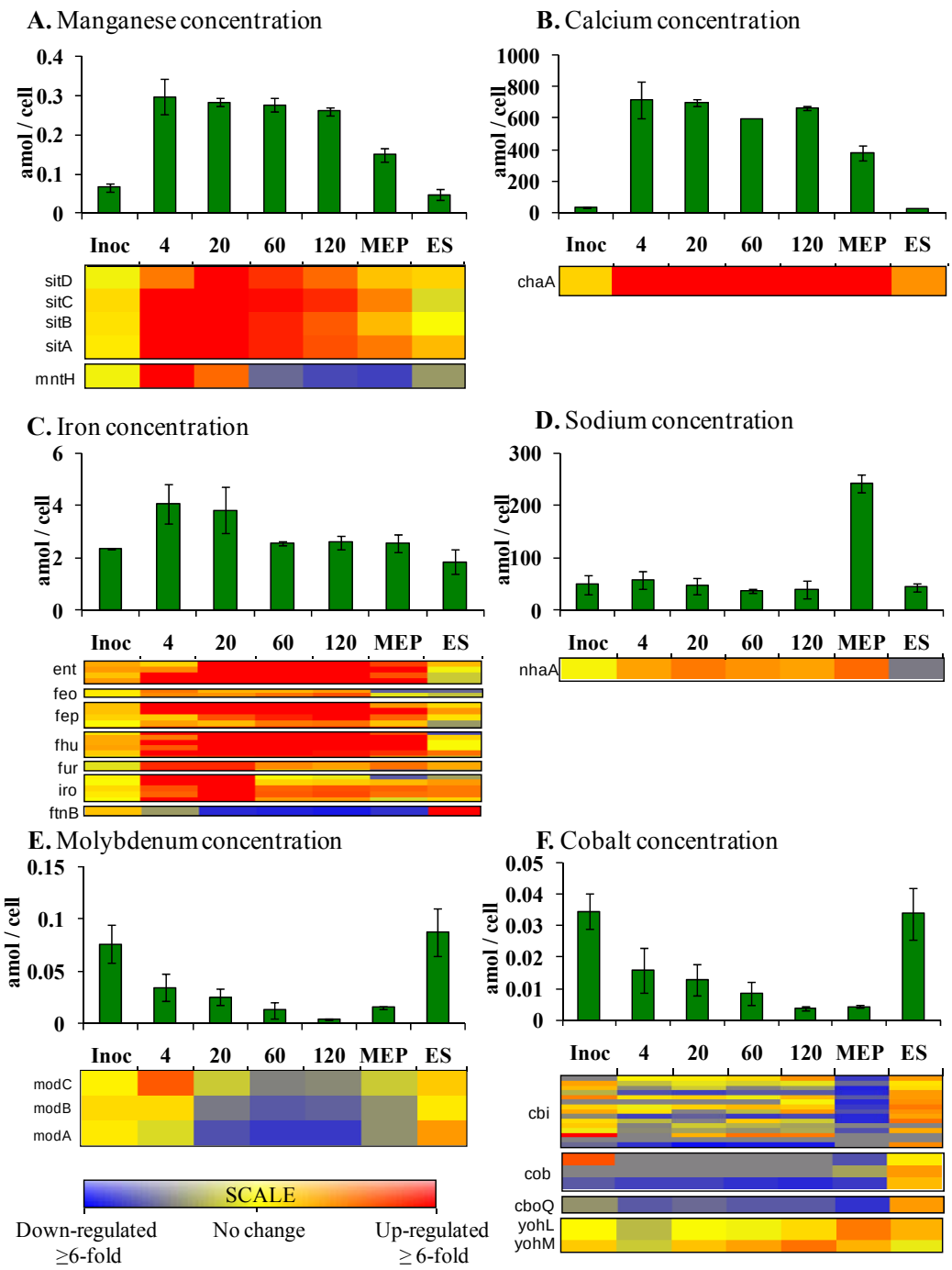


Figure 7.2: Metal homeostasis gene expression correlate with detected metal ions. ICP-MS quantification of intracellular metal ions. Metal concentration shown as attomoles (amol) per cell (CFU) and error bars represent the standard error of the mean (SEM) for three independent replicates. Gene expression data shown as a heat-map (Pearson correlation) of non statistically-filtered data as a fold change compared with the 48h stationary phase inoculum (Inoc). Individual genes or operons are labelled (*e.g. cbi* represents 15 Co^{2+} transport genes within the '*cbi*' operon). Further gene details in Table 7.1 Time-points shown in minutes for lag phase samples. The mid-exponential phase (MEP) sample represents an 8 hour time-point and the early stationary phase sample (ES) represents a 24 hour sample. Relevant transporters are labelled based upon their annotated function in *coli*BASE (accessed December 2009).

ICP-MS was used to quantify the intracellular concentrations of other metal ions, some of which have no known physiological role and some which are toxic under certain conditions. The accumulation of some of these metal ions occurred in a growth phase dependent manner, even though no known specific transporter exists (Figure 7.3). The ICP-MS measurement of other metal ions, which were either present at very low concentrations, or did not show growth phase dependent accumulation, are not shown in Figure 7.3 but are included in Appendix E.

The highest accumulation of copper (Figure 7.3A), aluminium (Figure 7.3C), and chromium ions (Figure 7.3F) occurred at 20 minutes post-inoculation and in each case the metal ions decreased by 60 minutes, suggesting that each of these metal ions was removed by this time-point.

Other metal ions were accumulated maximally during mid-exponential phase, such as magnesium (Figure 7.3B) and cadmium (Figure 7.3D). Interestingly the expression of the magnesium transporters *mgtABC* did not correlate with this accumulation, suggesting other routes for magnesium accumulation, possibly through non-specific ion channels (*e.g.* CorA) which could account for the non-specific accumulation of cadmium at this time-point. Strontium uptake (Figure 7.3E) was maximal within four minutes post-inoculation and throughout lag phase. This pattern of accumulation was very similar to calcium accumulation (correlation coefficient = 0.997) and manganese uptake (correlation coefficient = 0.993), suggesting non-specific transport was responsible. The similarity between the alkaline earth metal ions strontium and calcium at the atomic level raises the possibility that strontium accumulation is through the ChaA Ca^{2+} / H^{+} antiporter. This would need to be confirmed through additional analyses including deletion of *chaA* and determining the effect upon intracellular strontium concentrations. The intracellular concentration of zinc through growth (Figure 7.3H) is maintained at approximately 0.4 amol / cell and is the only metal analysed which is not accumulated in a growth phase dependent manner. The variability in zinc concentrations at each time-point is quite high and further analyses would need to be performed to elucidate any specific accumulation.

Nickel is accumulated maximally during stationary phase and is actively-removed from the bacteria within twenty minutes of inoculation into the fresh medium, for the entirety of lag phase (Figure 7.3G). The re-accumulation of nickel only begins during mid-exponential phase and continues into early stationary phase. The trend of nickel uptake and efflux is quite similar to that of cobalt (correlation coefficient = 0.853) and the intracellular concentrations are similar (maximal accumulation ~0.035 amol / cell). The presence of nickel and cobalt inversely correlates with the availability of oxygen in the medium, raising the possibility that nickel and cobalt ions interfere with metal homeostasis during aerobic growth. The expression of *yohL* and *yohM* are consistent with the removal of nickel by this specific efflux system. The ICP-MS technique did not distinguish between concentrations of 'free' metal ions and stored ions used as co-factors within metalloproteins; therefore it is not possible to highlight a specific role for intracellular metal ions.

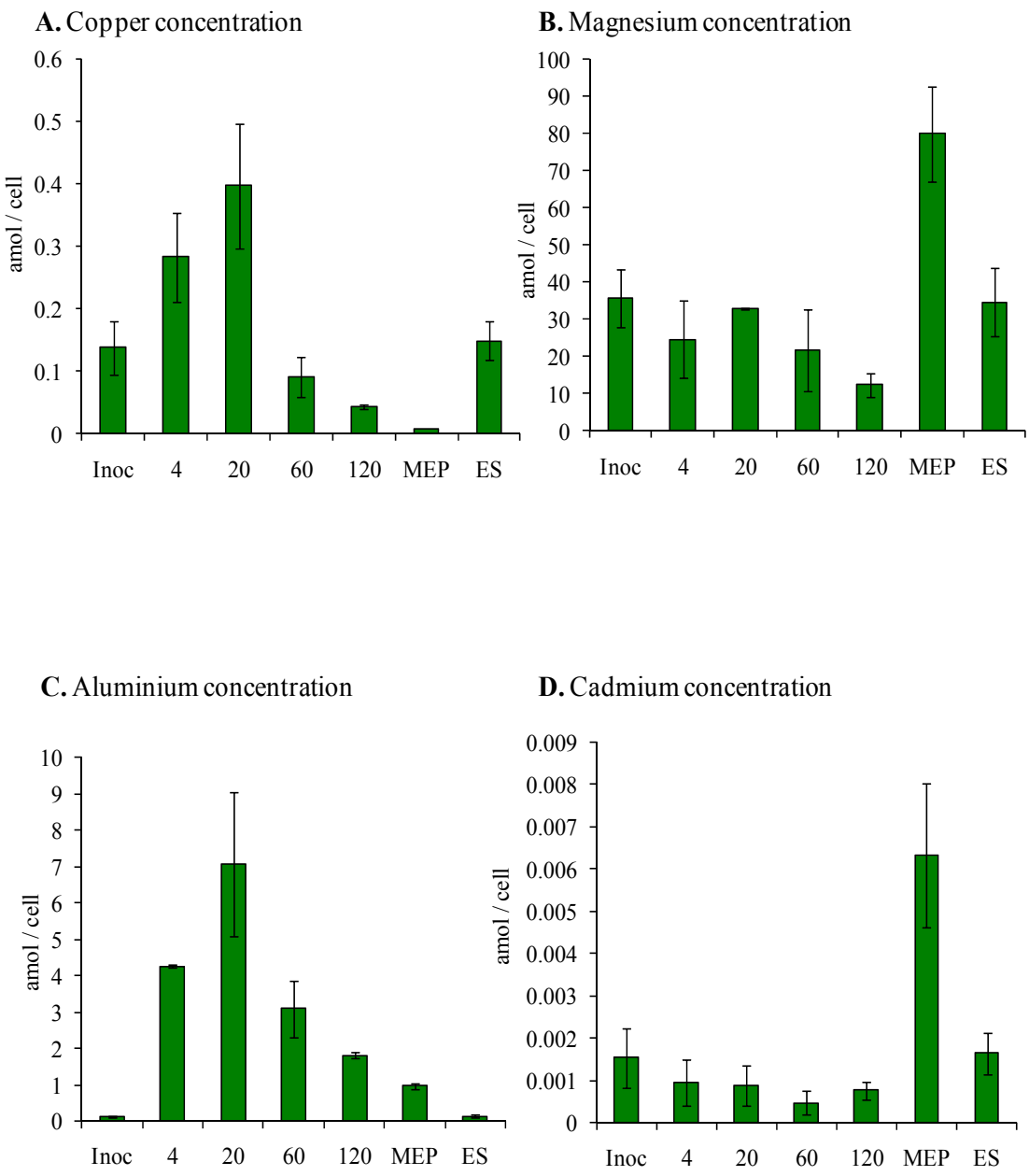
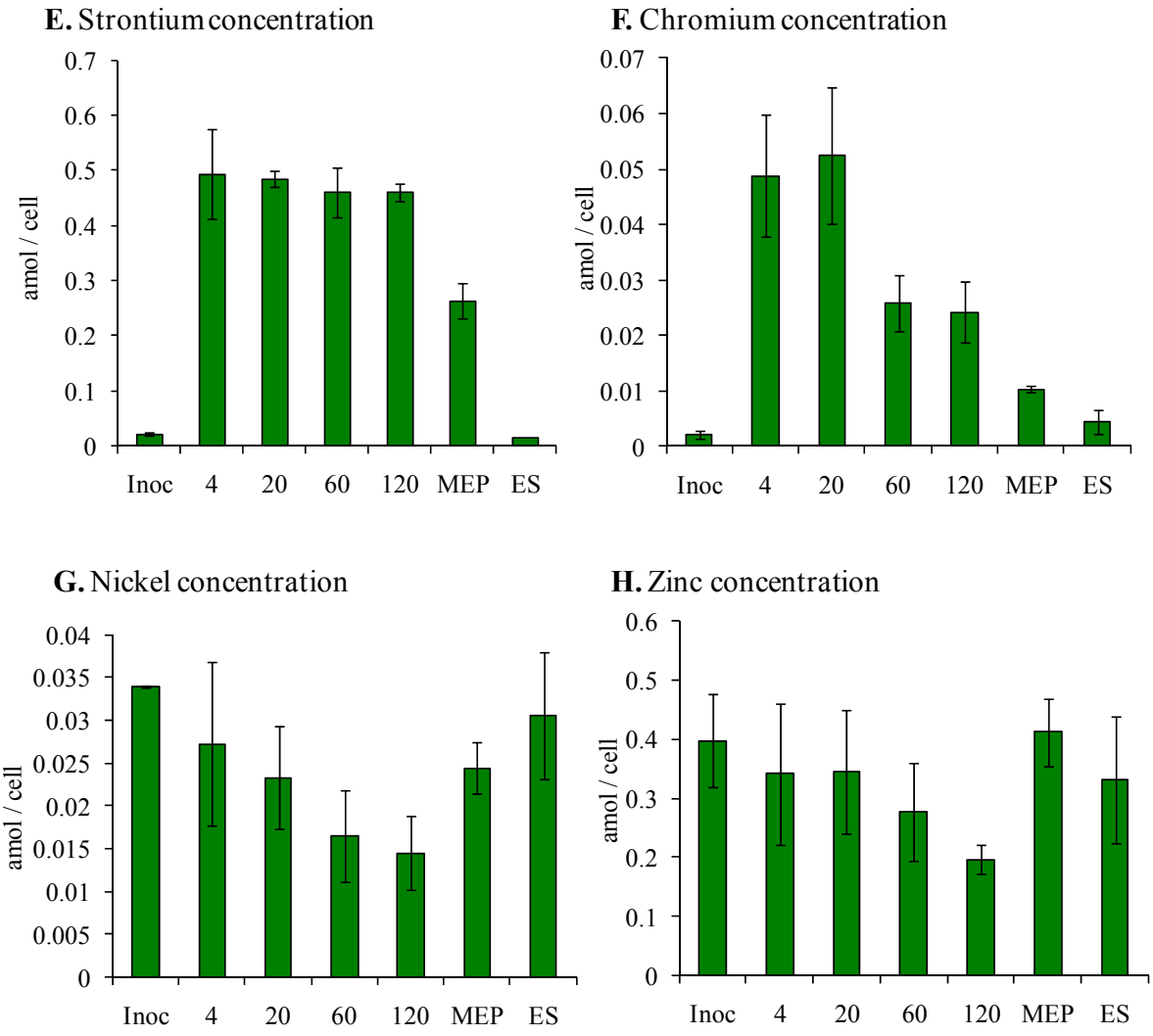


Figure 7.3 (Continued on next page…)



I.

Sample	Culture volume (ml)	Cell density (CFU / ml)	Total number of cells (CFU)
Inoc	15	3.30×10^9	4.95×10^{10}
4 minutes	750	4.97×10^5	3.73×10^8
20 minutes	750	5.37×10^5	4.03×10^8
60 minutes	750	5.53×10^5	4.15×10^8
120 minutes	750	6.30×10^5	4.73×10^8
Mid-exponential phase	15	5.72×10^7	8.58×10^8
Early stationary phase	15	2.69×10^9	4.04×10^{10}

Figure 7.3: Concentration of toxic metal ions changes during the growth cycle.
ICP-MS quantification of intracellular metal concentrations shown as attomoles (amol) per cell (CFU). Samples taken from 48 hours stationary phase (Inoc), lag phase (shown as minutes post-inoculation), mid-exponential phase (MEP) and early stationary phase sample (ES). Error bars represent the standard error of the mean (SEM) for three independent replicates (A-H). (I) Table depicting the concentration of cells (CFU) analysed at each time-point, calculated by total viable counts.

7.2.3 Effect of metal chelation on the growth of *S.* Typhimurium.

7.2.3.1 EDTA metal ion chelation

The quantification of intracellular metal ions revealed that different metal ions were preferentially accumulated throughout growth, many within lag phase. In order to elucidate the effects of metal depletion, *S.* Typhimurium was grown under optimal conditions at 37 °C in LB with or without EDTA (Figure 7.4). From the optical density growth curve it was apparent that bacteria grew more slowly in the presence of EDTA. Although optical density growth curves are not ideal for quantifying lag time, this experiment suggested that the duration of lag phase was increased in the presence of EDTA.

Although EDTA addition resulted in an increased lag time (due to metal chelation), it was not apparent whether these effects were due to depletion of metal ions from the growth medium or via physical disassociation of these ions from the cell membrane, causing cellular damage. Physically damaged cells show an increased lag time after inoculation into fresh medium as cellular repair needs to be completed, resulting in more metabolic work being performed before growth can be initiated (Baranyi & Roberts, 1994). In order to determine whether the bacteria were damaged by incubation in the presence of EDTA, bacterial cultures were grown for 16 hours at 37 °C in the presence or absence of 1 mM EDTA to determine if the bacteria reached the same final cell concentration (Figure 7.5A). For the three biological replicates tested, it was apparent that the addition of 1 mM EDTA did not adversely affect the ability of the bacterial cultures to grow to a high final cell concentration.

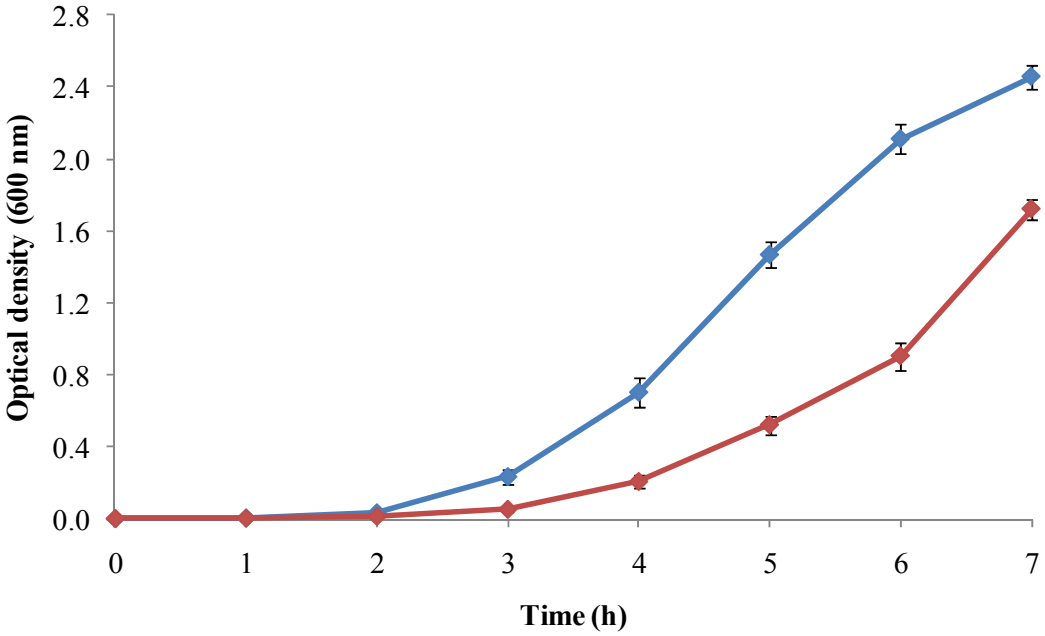


Figure 7.4: Metal chelation by EDTA affects growth in LB at 37 °C.
OD_{600} growth curve in LB (blue line) or LB + 1 mM EDTA chelator (red line) over 7 hours of growth at 37 °C with agitation at 250 rpm. Error bars represent the standard deviation of three biological replicates.

A.

LB			**LB + 1 mM EDTA**		
Replicate			Replicate		
1	2	3	1	2	3
5.07×10^9 CFU / ml	3.70×10^9 CFU / ml	2.67×10^9 CFU / ml	4.97×10^9 CFU / ml	2.73×10^9 CFU / ml	6.40×10^9 CFU / ml

B.

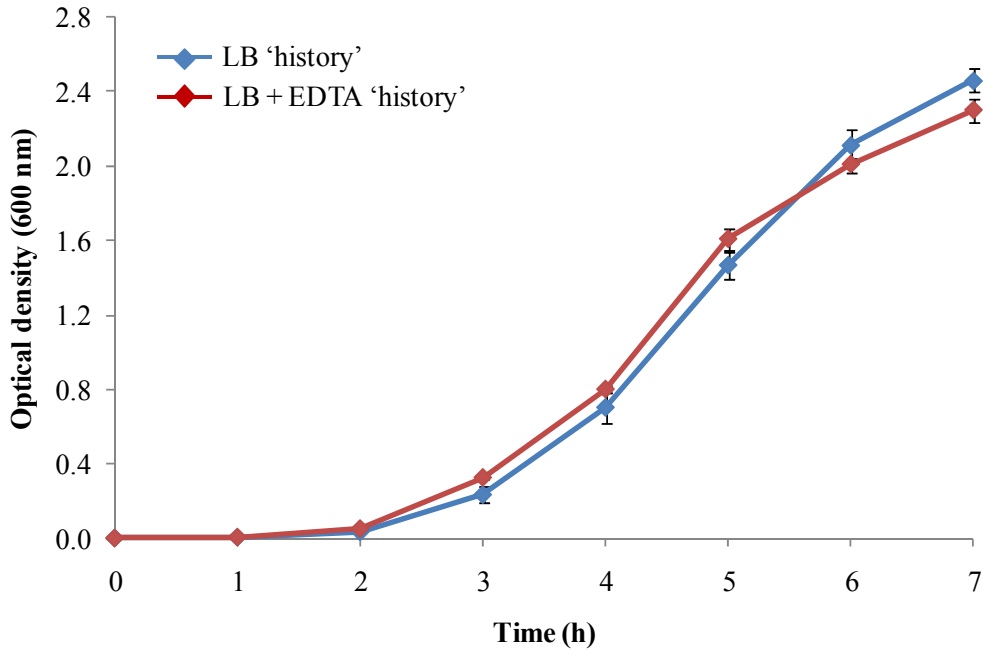


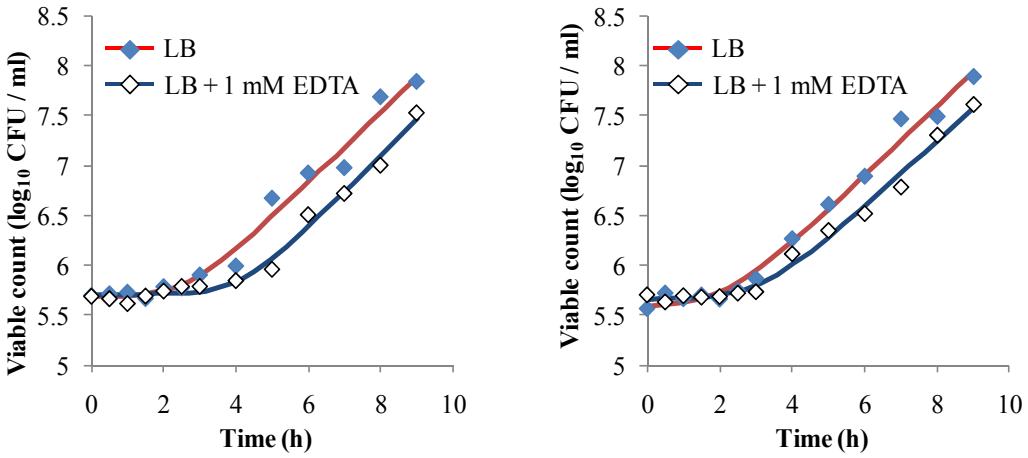
Figure 7.5: Metal chelation does not affect final cell density or re-growth in LB.
(A) Final stationary phase cell density of three biological replicates after approximately 16 hours growth in 5 ml LB in the presence or absence of 1 mM EDTA at 37 °C with agitation at 250 rpm. (B) OD_{600} growth curve in LB (pH 7.0) after growth in LB (blue line) or LB + 1 mM EDTA (red line) over 7 hours growth at 37 °C with agitation at 250 rpm. Error bars represent the standard deviation of three biological replicates.

The stationary phase culture grown in the presence of 1 mM EDTA was used as an inoculum to observe growth in LB at 37 °C. The bacterial cells were washed in LB to remove traces of the chelator and subsequently inoculated into fresh medium at an initial cell concentration of ~5 x 10^6 CFU / ml and growth observed over seven hours (Figure 7.5B). The experiment was designed to determine whether any cellular damage had occurred during growth in the previous environment with EDTA (low metal 'history'), in which case the lag phase would be extended. It was also possible that growth in a low metal environment had selected for cells which could compete more effectively more metal ions against the chelator and so would adapt faster after inoculation into a fresh medium, manifesting as a shorter lag time. If no EDTA-induced cellular damage or adaptation to a low metal environment occurred, the lag phase and growth rates between the samples would be the same.

Recovery in LB, after growth in a low-metal environment (+EDTA), was very similar to inoculation from LB medium alone (correlation coefficient = 0.996). This recovery suggests that there was no significant cellular damage as a result of the previous EDTA treatment. In addition, the low metal environment did not appear to have increased the ability of the bacteria to accumulate metal ions, as reported previously for phosphate uptake in *E. coli* (Hoffer *et al.*, 2001). Any increased metal ion sequestering by the bacterial population would have been expected to lead to a rapid resumption of growth and therefore a shorter lag time.

Although the optical density growth curves described above provided a general indication of an increased lag time in the presence of EDTA, meaningful quantification of the geometric lag time and growth rate of *S.* Typhimurium needed to be performed using the lag phase static system at 25 °C (Figure 7.6). Bacteria inoculated at ~10^5 CFU / ml in LB, supplemented with 1 mM EDTA, had a longer lag phase compared to growth in LB alone. Despite the increase in lag time in the presence of EDTA, the doubling time of the bacterial population was similar.

A.



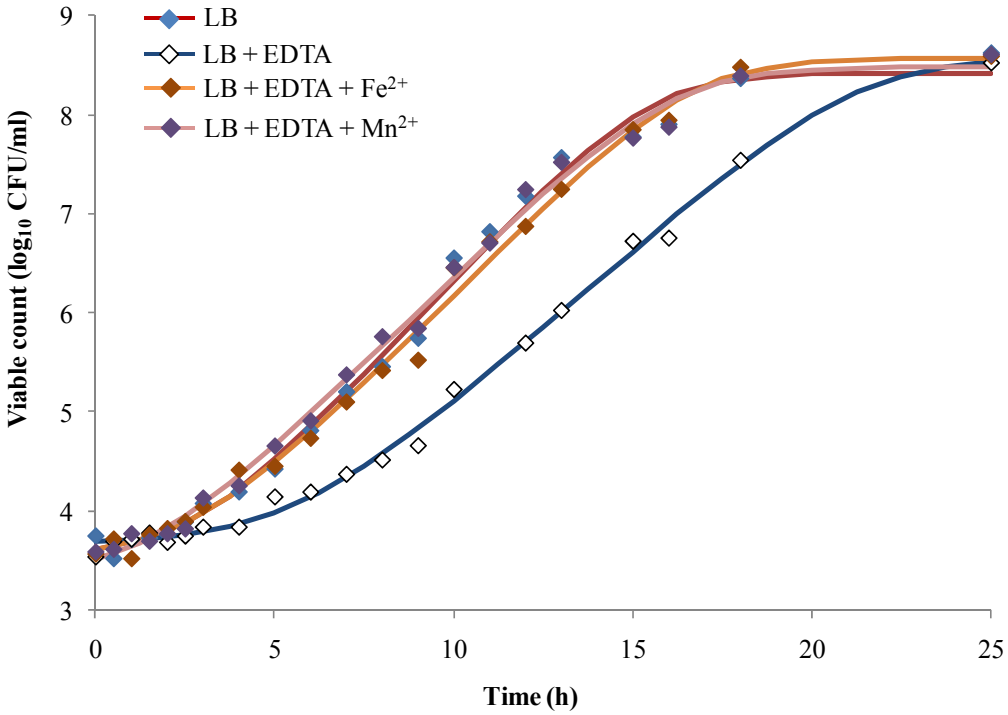
B.

LB Control				LB + 1 mM EDTA			
		Cell concentration (log_{10} CFU / ml)				Cell concentration (log_{10} CFU / ml)	
Lag (h)	Doubling time (h)	Start	End	Lag (h)	Doubling time (h)	Start	End
2.74, 2.19	**0.98, 0.99**	**5.68, 5.59**	**7.84, 7.89**	**4.16, 3.12**	**0.98, 0.99**	**5.71, 5.66**	**7.54, 7.62**

Figure 7.6: Addition of EDTA extends the lag time but not the doubling time.
(A) Two biological replicates of viable count growth curves in LB or LB + 1 mM EDTA over 9 hours at 25 °C. Curve fitted by DMFit. (B) Growth parameters as calculated by DMFit for the two biological replicates. The individual replicates for each growth parameter are shown, separated by a comma (,).

To confirm the increased geometric lag time phenotype in the presence of 1 mM EDTA, a full growth curve over 24 hours was performed in the static growth system using a lower (10^3 CFU / ml) starting inoculum (Figure 7.7). The lower inoculum allowed the exponential phase of bacterial growth to be observed over a longer period, providing a more accurate estimation of lag time. The lag time for the LB grown culture was almost 3 hours, which was increased to over 5.5 hours by the addition of 1 mM EDTA. The EDTA-dependent increase in lag time phenotype was rescued with the addition of both 1 mM Fe^{2+} and 1 mM Mn^{2+}. The LB + EDTA culture showed approximately the same growth rate as the other cultures and reached comparable final cell densities over 24 hours (Figure 7.7B). The fact that the addition of metal ions overcame the growth defects of the EDTA-supplemented cultures highlights the effect of metal chelation on bacterial lag time. In addition, the lack of impaired exponential growth and the similar final cell concentrations suggests that the effects on lag time were due to decreased concentrations of metal ions during the earliest stages of growth, rather than de-stabilisation of the cell membrane. However, no conclusions could be made regarding the absolute requirement of the bacteria for Mn^{2+} or Fe^{2+} specifically, as these metal cofactors may have bound to the EDTA directly. The binding of Fe^{2+} or Mn^{2+} to the EDTA chelator would likely have resulted in a lower concentration of available EDTA to bind other physiologically-important metal ions.

A.



B.

			Cell concentration (log_{10} CFU / ml)	
	Lag (h)	Doubling time (h)	Start	End
LB	2.93	0.99	3.61	8.42
LB + EDTA	5.53	1.00	3.69	8.57
LB + EDTA + Fe^{2+}	2.87	0.99	3.60	8.57
LB + EDTA + Mn^{2+}	1.82	0.99	3.50	8.49

Figure 7.7: Addition of divalent metal cations rescues increased lag phenotype.
(A) Single replicate of a lower inoculum (~10^3 CFU / ml) viable count growth curves in LB +/- 1 mM EDTA, +/- 1 mM $MnCl_2$ (Mn^{2+}) or $FeCl_2$ (Fe^{2+}) over 25 hours at 25 °C. Curve fitted by DMFit. (B) Growth parameters in the static growth system were calculated by DMFit.

7.2.3.2 Chelex-100 chelation

The addition of EDTA increased the bacterial lag time in the static system (Figure 7.6 and Figure 7.7), however it was not possible to determine whether the increased lag phenotype was due to the lack of available metal ions or whether the EDTA directly caused damage to the bacteria. To distinguish between these two scenarios, the bacteria were grown in LB medium supplemented with a Chelex-100 matrix to deplete the medium of metal ions, as per the manufacturer's instructions (Section 2.1.3.2). Chelex-100 was chosen for these experiments because the matrix-bound chelator could be easily removed from the growth medium during preparation, so there would be no direct contact between the chelating matrix and the bacteria used for inoculation. The medium pH was adjusted to 7.0 prior to inoculation, so that any effect upon growth was attributable to the metal ion deficiencies rather than pH. The Chelex-100 treatment allowed for metal ions to be supplemented in isolation in subsequent experiments to determine the effect on bacterial lag time. The intracellular metal ion concentration could also be quantified by ICP-MS, to determine whether the supplemented metal ions were being accumulated at increased concentrations to compensate for the absence of other metal ions.

The concentration of metal ions in LB medium varies between batches and has not been widely reported. One previous ICP-MS study estimated the concentrations of some metal ions in LB medium (Outten & O'Halloran, 2001) and found the major constituent metal ions to be potassium, magnesium, calcium and zinc. The concentration of metal ions ions in LB used in the present study are shown in Appendix E, and correlated well with the study by Outten and O'Halloran (2001), with the most abundant metal ions being potassium and sodium. These two monovalent cations would not be bound by the Chelex-100 resin.

Side-by-side growth curves were performed in the static growth system at 25 °C to determine any changes in the growth parameters of the metal depleted cultures compared with LB grown cultures (Figure 7.8). Analysis of the growth parameters revealed that the lag time and growth rate are not different. It is not apparent from this experiment whether the Chelex-treated LB medium is totally depleted for metal ions. Further analyses would be required, including quantification of metal concentration present in the treated LB medium, using ICP-MS. The Chelex matrix contains sodium iminodiacetate, the same active group

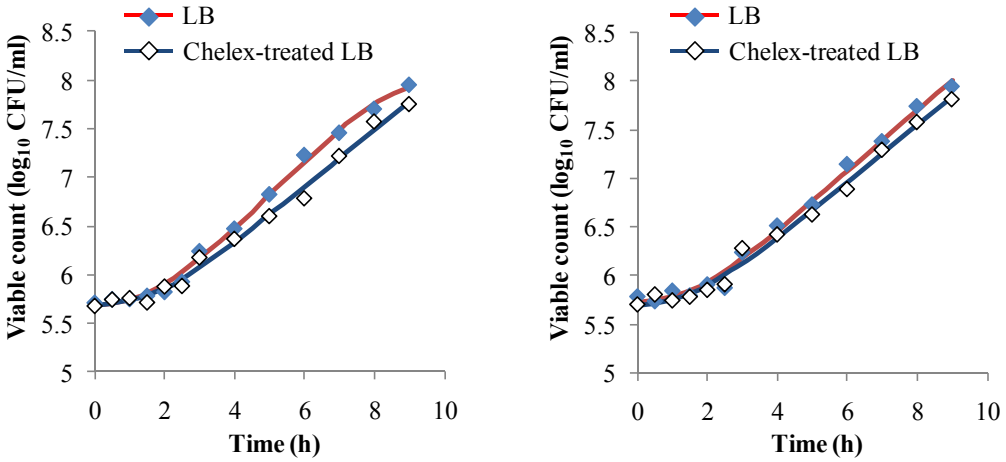
as EDTA. The binding affinity of Chelex-100 is 0.4 mEq / ml which may not be high enough to deplete all metal ions to below physiologically-relevant concentrations. Based on the metal concentration in LB medium (for 21 divalent or trivalent cations present at >0.1 ng / ml) an equivalence value of 84.1 mEq would be required. Such equivalence corresponds to 136.6 g Chelex-100 required per litre of medium, considerably higher than the 50 g per litre recommended for use in the present study. Depletion of the total metal ions in the growth medium would be essential before the lag phase-specific roles of particular metal cations could be elucidated further.

7.2.4 Effect of chelation on bacterial motility and oxidative stress

Lag time was noticably increased after the addition of 1 mM EDTA. To understand this effect further and to determine the effect of metal chelation on key physiological processes, bacterial motility and oxidative stress resistance were investigated at 37 °C (Figure 7.9). Stationary phase bacterial cultures (10^9 CFU / ml) were grown overnight in LB medium under agitation (250 rpm). For the motility assay, 1 µl of this culture (10^6 CFU) was added to motility agar plates, either blank or supplemented with 1 mM EDTA, 1 mM $MnCl_2$, or both and incubated at 37 °C for 6 hours. The addition of EDTA significantly decreased bacterial motility (Figure 7.9A), most likely by chelating metal ions required for growth. The effect was removed by the addition of Mn^{2+} which had no effect on motility *per se*.

EDTA also caused decreased viability on solid media in the presence of hydrogen peroxide (Figure 7.9B). This sensitivity was rescued by the addition of 1 mM Mn^{2+} or Fe^{2+} but not by monovalent K^+ which is not bound by EDTA. Interestingly, the supplementation of 1 mM Fe^{2+} significantly increased the sensitivity of *S.* Typhimurium to hydrogen peroxide, possibly due to the formation of Fenton reaction products.

A.



B.

LB Control				LB + 5 % Chelex			
		Cell concentration (log_{10} CFU / ml)				Cell concentration (log_{10} CFU / ml)	
Lag (h)	Doubling time (h)	Start	End	Lag (h)	Doubling time (h)	Start	End
1.72, 1.67	**0.99, 0.99**	**5.69, 5.72**	**8.01, 7.94**	**1.84, 1.66**	**0.99, 0.99**	**5.69, 5.70**	**7.76, 7.82**

Figure 7.8: Addition of Chelex-100 has no effect on bacterial growth.
(A) Two biological replicates of viable count growth curves in LB or LB + 5 % Chelex-100 over 9 hours at 25 °C. Curve fitted by DMFit. (B) Growth parameters as calculated by DMFit for the two biological replicates. The individual replicates for each growth parameter are shown, separated by a comma (,).

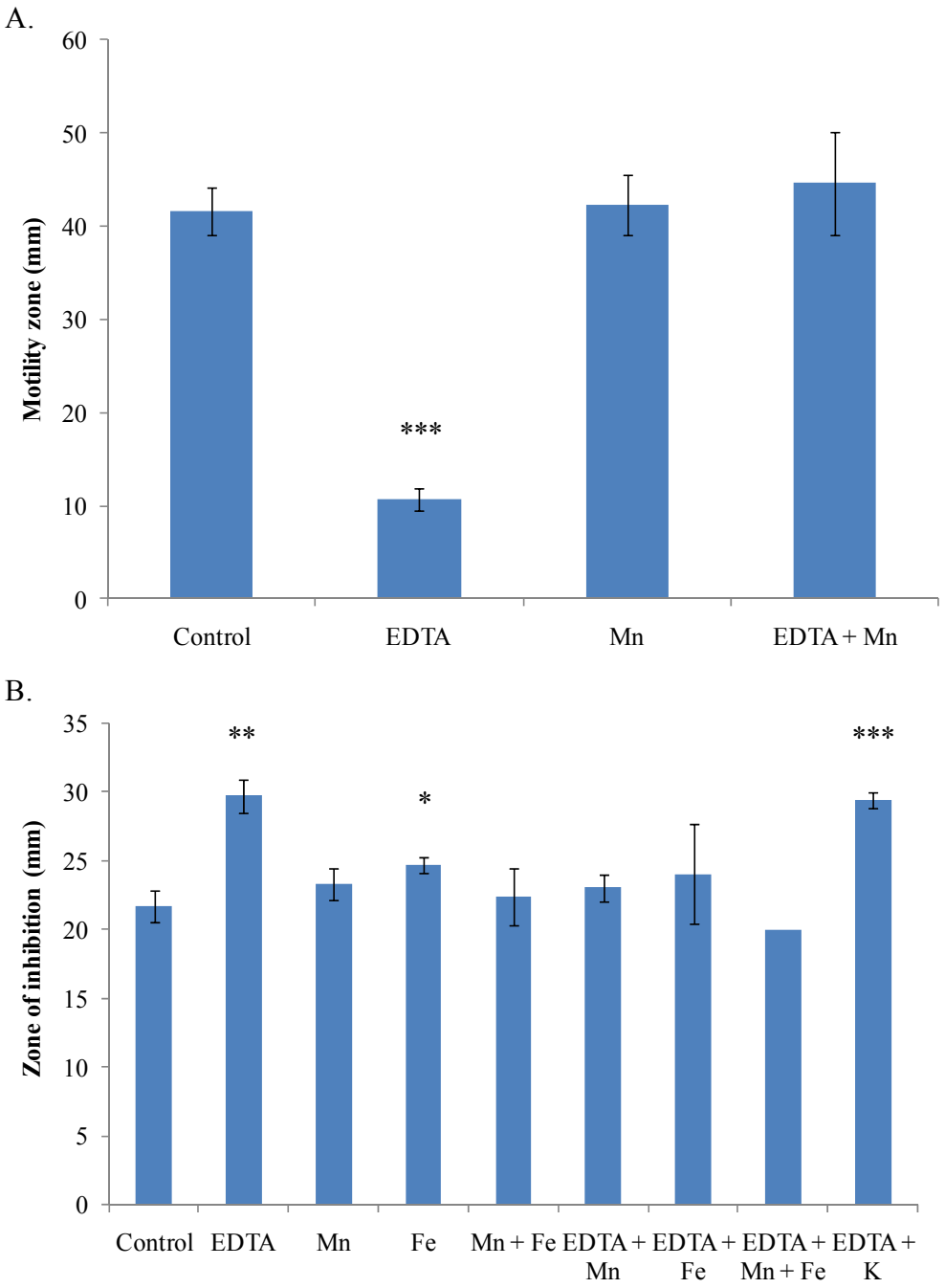


Figure 7.9: Metal chelation affects motility and sensitivity to oxidative stress.
(A) Result of bacterial motility in low agar solid medium (Section 2.1.4.1) non-supplemented (Control) or supplemented with 1 mM EDTA, 1 mM $MnCl_2$ or both. Zone of motility measured after 6 hours incubation at 37 °C. (B) Hydrogen peroxide sensitivity assay (Section 2.1.9.1) on LB agar plates in the presence of 3 % (w/v) (882 mM) hydrogen peroxide. Agar was either not supplemented (Control) or with added 1 mM EDTA, 1mM $MnCl_2$ (Mn), 1 mM $FeCl_2$ (Fe), 1 mM KCl (K) or combinations therein. Zone of growth inhibition measured after approximately 16 hours incubation at 37 °C. Error bars represent the standard deviation of three biological replicates. Significant differences between the 'Control' and other samples were calculated by *t*-test and are indicated by asterisks as follows: *, $p \leq 0.05$; **, $p \leq 0.005$; ***, $p \leq 0.001$.

7.2.5 Growth of a Δ*mntH* Δ*sitABCD* mutant strain.

The technical challenges in depleting all metal cations in the LB growth medium means it is difficult to elucidate the effect of specific metal ions. One potential solution is to construct metal transport mutants for particular physiologically-important metal ions. Drawbacks to this method include the fact that multiple transport systems can be utilised for some metal ions (*e.g.* iron) under different environmental conditions. There are also potentially unknown metal ion transport systems which may confer functional redundancy when characterised transporters are removed. The other factor to consider is the low-specificity binding of some transporters for secondary metal ions, which could potentially make entirely blocking the transport of a single metal very challenging. In order to test the possibility of blocking single metal transportation, a mutant was made in the two systems for manganese transport, MntH and SitABCD. Manganese was chosen as a suitable candidate metal as it is strongly accumulated during lag phase and has relatively few dedicated transporters systems, all of which are lag phase induced at the transcriptomic level (Figure 7.2A). Mutants were made using the Lambda-Red protocol (Section 2.3.9), combined into *S.* Typhimurium SL1344 through a P22 phage transduction and verified via DNA sequencing (Appendix B).

These mutants were grown in the lag phase static system from a low inoculum for 25 hours to determine whether the inability to transport manganese restricted growth or an increased lag time (Figure 7.10). The genes encoding each manganese ion transport system were deleted individually (Figure 7.10A and Figure 7.10B) to ascertain whether the deletion of one system had a greater effect than the other. In addition, a mutant strain deficient for both manganese ion transporters was constructed (Figure 7.10C). Of the mutants tested, none showed an increased lag time or an increase in doubling time which suggested that mutations in these systems were not crucial for growth. It is however important to note that this experiment does not comprehensively show that manganese is not required for full growth in the static lag system. It would be crucial to measure the intracellular concentrations of manganese in the mutant strains to determine to what extent they are depleted for manganese. It is possible that manganese is still being accumulated through other non-specific transport systems to compensate for the loss of these high-affinity transporters and direct quantification would be required to determine this conclusively.

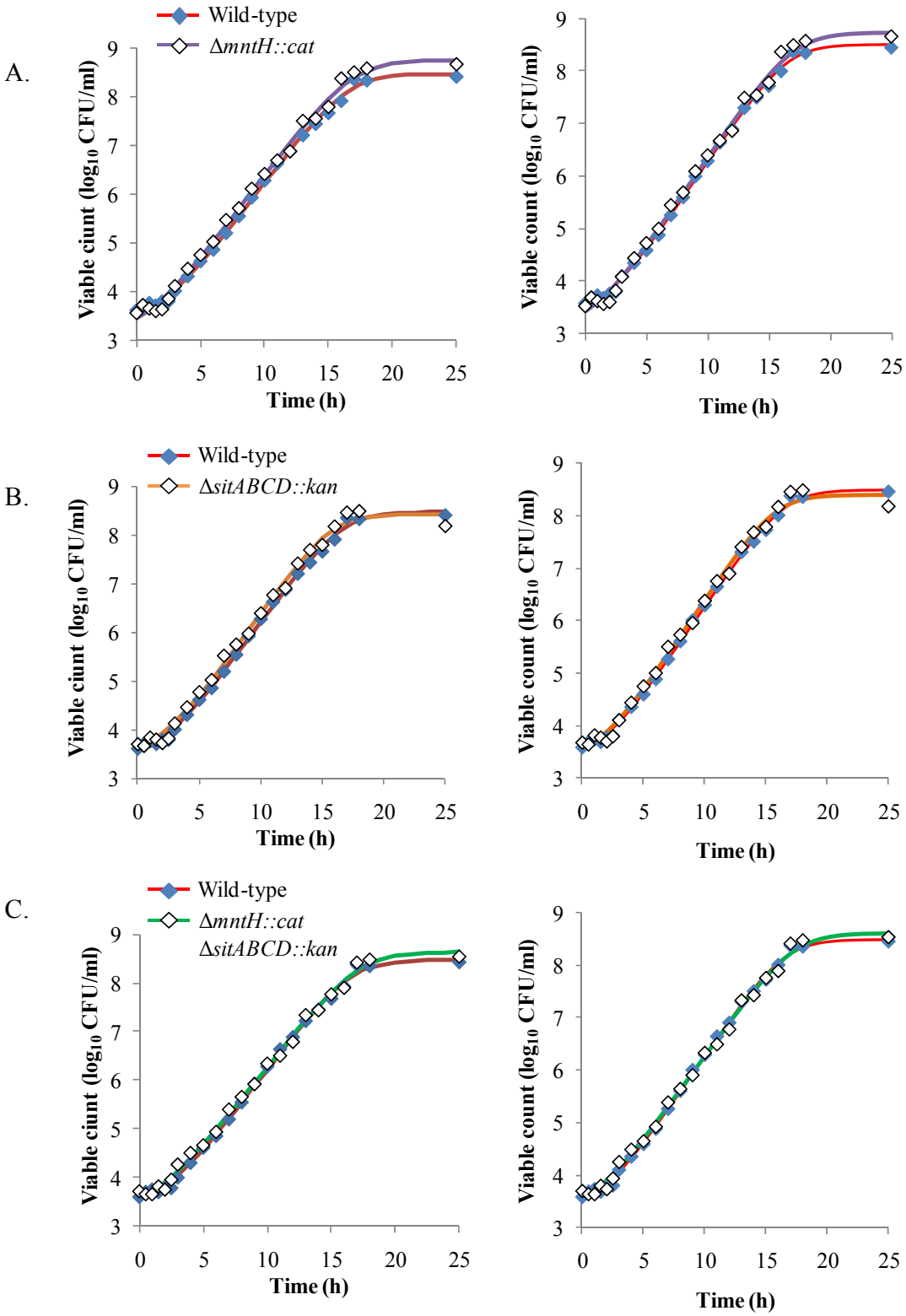


Figure 7.10: Dedicated manganese transporters are not essential for growth.
Two biological replicates of low inoculum (10^3 CFU / ml) viable count growth curve in the static growth system. Growth curves show a side-by-side comparison of wild-type SL1344 against (A) Δ*mntH::cat* (B) Δ*sitABCD::kan* (C) Δ*mntH::cat* Δ*sitABCD::kan* mutants. Curve fitted by DMFit.

7.2.6 Oxidative stress resistance of the Δ*mntH* Δ*sitABCD* mutant.

The lack of a growth phenotype associated with the deletion of genes encoding MntH and SitABCD suggested that either Mn^{2+} was not an essential micronutrient or that the deleted transporters were not required for growth. A previous study correlated the inability to import Mn^{2+} with increased sensitivity to hydrogen peroxide (Anjem *et al.*, 2009). It was therefore hypothesised that if MntH and SitABCD were solely required for Mn^{2+} import, the Δ*mntH::cat* Δ*sitABCD::kan* mutant strain would be more sensitive to oxidative stress. To test this hypothesis, a hydrogen peroxide disc diffusion assay was performed to compare the oxygen sensitivity of the Δ*mntH::cat* Δ*sitABCD::kan* mutant and wild-type bacterial strains (Figure 7.11).

The loss of the Mn^{2+}-specific transport systems did not increase oxidative stress significantly when challenged with hydrogen peroxide (*t*-test, p=0.11). Variability between replicates was higher in the Δ*mntH::cat* Δ*sitABCD::kan* mutant compared with the wild-type strain, and may account for why no difference in the loss of viability was discernible over the replicates tested. Further evidence is required to elucidate the role of Mn^{2+} in mediating resistance to hydrogen peroxide and to determine whether additional Mn^{2+} transporters exist in *S.* Typhimurium. ICP-MS could be used to measure the intracellular metal ion concentration in the Δ*mntH::cat* Δ*sitABCD::kan* mutant strain, and determine whether there is a decrease in intracellular concentration of Mn^{2+}, as reported previously in *E. coli* (Anjem *et al.*, 2009).

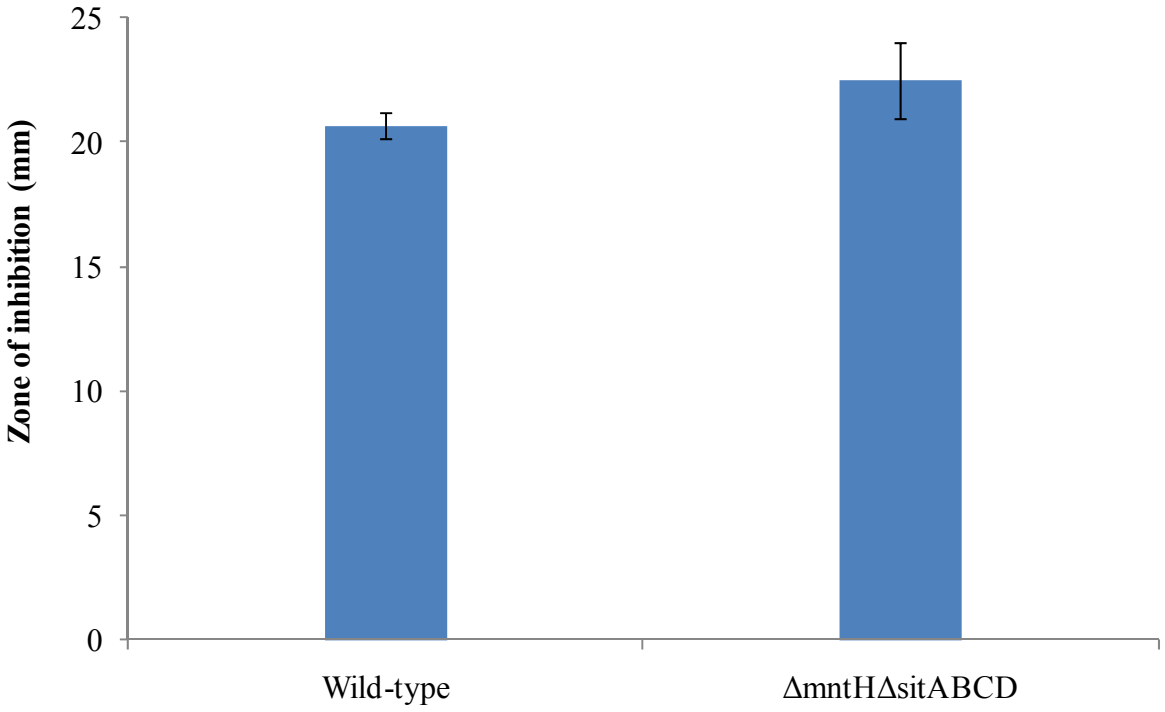


Figure 7.11: The Mn^{2+} transport system is not required to resist oxidative stress. Hydrogen peroxide sensitivity assay (Section 2.1.9.1) on LB agar plates in the presence of diffused 0.6 M hydrogen peroxide. Zone of growth inhibition measured after approximately 16 hours incubation at 37 °C. Error bars represent the standard deviation of three biological replicates. No significant difference between the SL1344 (Wild-type) and mutant sample (Δ*mntH::cat* Δ*sitABCD::kan*) was observed (*t*-test, p=0.11).

7.3 Discussion

7.3.1 Detection of intracellular metal ions

The present study has identified the intracellular accumulation of fourteen metal ions throughout growth in the static lag phase system. The majority of these metal ions show a growth phase specific accumulation with seven metal ions being accumulated specifically during lag phase: manganese, calcium, iron, copper, aluminium, strontium and chromium. The ICP-MS method to detect bacterial metal accumulation has been described elsewhere (Outten & O'Halloran, 2001, Rolfe, 2007, Tree *et al.*, 2005). Rolfe (2007) performed a preliminary experiment to identify lag phase-specific accumulation in *S.* Typhimurium using the static lag phase system, however detected high concentrations of background metal ions and a large degree of variability between samples. An optimised method developed in the present study has considerably decreased experimental uncertainty. Despite the variability, Rolfe (2007) made observations consistent with the present study. A number of several metal ions are accumulated at specific growth-phases; these include the lag phase specific accumulation of: manganese, calcium, strontium, chromium and to a lesser extent, iron. Like the present study, magnesium was also detected at the highest concentration during mid-exponential phase of growth. There were, however discrepancies between the present and previous study for some metal ions including sodium which was accumulated maximally during mid-exponential phase in the present study but was identified as a lag phase accumulated metal in the previous study. No nickel efflux during lag phase was observed by Rolfe (2007), and instead there was maximal accumulation by 20 minutes post-inoculation. However, the relatively large variability in the Rolfe (2007) data required further testing with an improved method. The present study optimised the ICP-MS protocol and included two additional lag phase time-points, which provided internal verification of the growth phase-specific metal ion accumulation.

The increase in the concentration of cells that were sampled helped to decrease variability and increase the signal-to-noise ratio. Previous studies focussed on intracellular metal concentrations of mid-exponentially growing bacteria, with the exception of the Rolfe (2007) study. In the present study, increasing the starting cell concentration from 4.87 x 10^7 CFU to 9.73 x 10^7 CFU (Figure 7.1) did not decrease the error between replicates. Further optimisation by the addition of an EDTA washing step and increasing the starting

cell concentration to 4.00 x 10^8 CFU caused a decrease in variability for most metal ions (Figure 7.2 and Figure 7.3).

7.3.1.1 Comparisons with previous studies

The results from the optimised protocol were generally consistent with a previous study using ICP-MS to investigate intracellular concentration of metal ions in mid-exponentially growing *E. coli* (Outten & O'Halloran, 2001). In *E. coli*, the concentration of magnesium was higher than Ca^{2+}, Fe^{2+} and Mn^{2+} (Outten & O'Halloran, 2001). This trend was also noted in the present study, although intracellular Ca^{2+} was present at higher concentrations in *S.* Typhimurium grown in the static system. As observed in *E. coli* (Outten & O'Halloran, 2001), Mn^{2+} accumulation was approximately 10-fold lower than Fe^{2+} accumulation at mid-exponential phase in *S.* Typhimurium. Discrepancies between the present and Outten and O'Halloran (2001) studies could be due to differences in *Salmonella* and *E. coli* physiology or growth conditions which were statically grown in filter sterilised LB in the present study. Using the ICP-MS data and the dimensions of stationary phase *Salmonella* cells from Figure 3.9 it is possible to compare the concentrations of metal ions with the published literature. For example, a *S.* Typhimurium bacterium in the 48 hour stationary phase culture contains approximately 52 mM 'total' Ca^{2+}, based on 29.5 amol / cell Ca^{2+} determined via ICP-MS in the present study (Figure 7.2B). This is higher than reported for 'free' Ca^{2+} in *E. coli* detected via fluorescent spectrocscopy (Jones *et al.*, 2002), suggesting that either Ca^{2+} in *Salmonella* is mostly bound or that there was a large contaminant of Ca^{2+} associated with the bacterial cell. Future work could attempt to elucidate the concentration of 'free' Ca^{2+} in *S.* Typhimurium, which may highlight a role in intracellular signalling (Jones *et al.*, 2002).

The present results obtained using ICP-MS correlate well with an older study using a spectro-chemical technique to measure a range of inorganic elements (expressed as dry weight) in late exponentially-growing *E. coli* on minimal medium (Rouf, 1964). Rouf (1964) identified the relative abundance of *E. coli*-associated metal ions as follows: magnesium> iron > calcium > zinc > sodium > copper (Rouf, 1964). The present study at mid-exponential phase identified Na^+ as being more abundant than the Rouf (1964) study, possibly due to the high concentration of NaCl (~0.17 M) present in LB medium. In addition, there was an increased concentration of Ca^{2+} compared with Fe^{2+}, however the

differences in concentration were very small between metal ions and showed surprisingly similar traits between *E. coli* and *Salmonella* despite the difference in growth phases and media constituents. It is worth considering that Rouf (1964) did not include any wash steps and so the measurement involved the total associated metal ions and not solely the intracellular metal concentrations.

Generally, the ICP-MS technique used in this study correlates well with the Outten and O'Halloran (2001) and Rouf (1964) studies and increases the knowledge of metal accumulation throughout growth. This study builds upon the preliminary work performed by Rolfe (2007), by looking at more lag phase time-points including early after inoculation, within four minutes, and successfully correlates some metal transporter gene expression data with intracellular metal ion concentration.

7.3.1.2 Metal accumulation and implications for lag phase

The detailed time-course data from the present study identifies growth-phase specific trends. The lag phase specific accumulation of manganese correlates well with the dissolved oxygen concentration and the expression of OxyR-regulated genes (Rolfe, 2007) leading to the hypothesis that increased manganese accumulation may be a response to OxyR-controlled expression of *mntH* (Ikeda *et al.*, 2005, Kehres *et al.*, 2002a). This would enable the bacterium to tolerate the higher dissolved oxygen concentration encountered during lag phase, as Mn^{2+} has been shown to mediate against oxidative stress in *E. coli* (Anjem *et al.*, 2009). When comparing the metal ion accumulation during lag phase with the stationary phase inoculum, in the present study, it was observed that Mn^{2+} was imported to a greater extent than Fe^{2+}. This Fe^{2+} / Mn^{2+} balance may represent a bacterial defence mechanism to prevent the build-up of Fe^{2+} during oxygen upshift upon inoculation, thereby minimising the concentration of damaging Fenton reaction by-products (Wardman & Candeias, 1996).

The expression of iron import-encoding genes increases during lag phase. Despite this, the expression of the Fe^{2+} inducible ferric uptake regulator, *fur* is also up-regulated. Increased production of Fur would be expected to lead to a decrease in expression of iron responsive genes (Tsolis *et al.*, 1995), as a result of Fur activation by binding of free intracellular Fe^{2+} to the Fur apoprotein and subsequent transcriptional regulation of Fur-responsive genes.

The transcriptional profile of iron responsive genes during lag phase suggests that the intracellular concentration of free Fe^{2+} is low, which is confirmed by the down-regulation of the ferritin-like protein encoding gene *ftnB*, expression of which is induced in the presence of free iron (Velayudhan *et al.*, 2007). The low concentration of free Fe^{2+} results in Fur not being activated and therefore the import of additional extracellular Fe^{2+} can occur via the iron uptake machinery.

Intracellular Ca^{2+} concentrations were highest during lag phase and decreased throughout the remainder of growth. The intracellular concentration of Ca^{2+} is tightly regulated in exponentially-growing *E. coli* (Silver, 1996) and may have important roles in intracellular signalling and cell cycle progression (Holland *et al.*, 1999). It is possible that the abundant concentration of intracellular Ca^{2+} during lag phase is triggered by an oxygen upshift, as shown by a marked increased in intracellular Ca^{2+} concentration in *Bacillus subtilis* after exposure to hydrogen peroxide (Herbaud *et al.*, 1998). The dramatic increase of intracellular Ca^{2+} concentration during lag phase (to ~0.7 fmol per cell) suggests this metal ion is important during lag phase adaptation and subsequent initiation of cell division. It is interesting to speculate that the accumulation of Ca^{2+} may act as a secondary messenger, having been previously implicated in inducing a response in physiological processes such as motility, chemotaxis, enzyme activity and cytoskeletal organisation (Holland *et al.*, 1999), all of which could have a role in lag phase adaptation. In addition, the Ca^{2+} / H^{+} antiporter, ChaA is required for spore germination in *B. subtilis* (Moir, 2006), representing a role for Ca^{2+} in exit from lag phase. The high lag phase specific up-regulation of *chaA* and the abundant intracellular concentration of Ca^{2+} after inoculation, support the physiological importance of Ca^{2+} in bacteria (Holland *et al.*, 1999) and strongly suggest a role for Ca^{2+} in lag phase adaptation. The mechanisms for bacterial Ca^{2+} signalling have not been fully determined but studies have focussed on using the more Ca^{2+}-specific chelator ethylene glycol tetraacetic acid (EGTA) to determine the effects of Ca^{2+} deprivation on bacteria (Holland *et al.*, 1999).

The specific import and efflux of metal ions begins early on in lag phase. The speed of metal ion accumulation (within four minutes) suggests that metal ions are important for the entry into lag phase. Some of these metal ions have a role in protection against oxidative stress which suggests a putative role for the accumulation during lag phase however other

metal ions need further investigation to elucidate any potential function in bacterial lag phase adaptation. Many divalent cations are associated with the bacterial membrane or periplasm. Future analyses should focus on fractionating the bacterial cells into the cytoplasmic, periplasmic and membrane compartments (Osborn *et al.*, 1972) and repeating the ICP-MS on each fraction to identify the cellular location of particular metal ions. The fractionation protocol would need to be refined to minimise metal contamination and loss of sample through the multiple high-speed centrifugation steps.

7.3.2 The effect of metal chelation on bacterial physiology

7.3.2.1 Effect on growth

The addition of 1 mM EDTA caused an increase in the lag time of bacteria grown either in the static lag phase system, or in optimal conditions at 37 °C. Interestingly the EDTA had no effect on the doubling time of the bacteria, suggesting that the physiological requirement for metal ions is most crucial during lag phase, after which point the cells become adjusted to growth in the low metal ion environment. The effect of metal ion chelation on lag time has been previously shown for both *Salmonella* Choleraesuis and *S.* Enteritidis growing in trypticase soy broth supplemented with the broad spectrum metal chelator ethylenediaminediacetate (EDDA) (Chart & Rowe, 1993, Ho *et al.*, 2004). The addition of EDDA increased the lag time but did not affect the growth rate of the bacterial population (Ho *et al.*, 2004). The addition of EDTA has previously been shown to affect the growth of *Saccharomyces cerevisiae* up to 10^7 CFU / ml after which the effect was not significant (Kubo *et al.*, 2005). In addition, EDTA has been reported to cause 'leaky membranes' in some yeast species (Elferink, 1974) and *E. coli* (Amro *et al.*, 2000).

EDTA can change the pH of microbiological growth media. In the present study EDTA was added at 1 mM final concentration by volume, and the medium remained at pH 7.0 ± 0.2 prior to inoculation. The EDTA-induced increase in lag time was compensated for by the addition of 1 mM $MnCl_2$ or $FeCl_2$, confirming that the presence of the chelator was directly perturbing lag time, rather than having indirect effects such as any localised decrease in the pH. No conclusion could be drawn concerning the preference of the bacteria for Mn^{2+} or Fe^{2+} from the growth experiments performed, as the supplemented Mn^{2+} or Fe^{2+} may have bound to the EDTA and thereby indirectly increased the

availability of other, physiologically important metal ions, as EDTA has different binding affinities for divalent and trivalent metal cations. For example, EDTA binds Fe^{2+} and Fe^{3+} with a higher affinity than Mn^{2+} or other physiologically important metal cations such as Zn^{2+}, Mg^{2+} and Ca^{2+} (Gilman *et al.*, 1990). Therefore, supplementing the growth medium with Fe^{2+} may have increased the concentration of Fe-EDTA complexes, thereby liberating other metal ions for use by the bacteria.

From the results of experiments performed with EDTA in this chapter, a hypothesis was formed to account for the increased lag time but consistent doubling time. It was speculated that the bacteria during lag phase actively compete with EDTA for metal cofactors to complete lag phase physiological processes by increasing the production of high affinity metal ion uptake machinery. These uptake systems have a higher affinity for metal cations than EDTA (*e.g.* enterobactin $K_{aff,Fe3+} = 10^{52}$ M^{-1}; EDTA $K_{aff,Fe3+} = 10^{25}$ M^{-1}) and outcompete the chelator (Griffiths, 1987). The increased lag time is therefore hypothesised to be the length of time required to synthesise the high affinity uptake machinery and accumulate the necessary concentration of metal ions to initiate cell division. As EDTA concentration increases, the length of lag should also increase, as the ability to outcompete the EDTA decreases. For these experiments the EDTA was added to the LB medium at least 24 hours prior to inoculation. During this time the EDTA would become saturated with metal ions, requiring the bacteria to actively acquire the metal ions from the chelator, leading to an eventual accumulation of the unbound form of EDTA.

Although the effect of EDTA on cellular structures, such as the cell membrane, is possible using microscopy and flow cytometry techniques, to determine the overall effects of EDTA on cell physiology, it is important to utilise a global approach (transcriptomics, proteomics etc.). One method of elucidating the effects of EDTA on lag phase adaptation would be to perform a transcriptomic experiment in LB supplemented with 1 mM EDTA, to search for an increased expression of the high affinity uptake encoding genes described in this chapter (and Table 7.1), throughout the duration of lag phase. It is hypothesised that the increased expression would also be maintained throughout the exponential growth phase, accounting for the constant doubling time of the bacteria irrespective of the presence of EDTA.

The most definitive way to elucidate the effect of individual metal ions on bacterial growth and lag time would be to deplete the medium of metal ions and then add back metal cations of interest and test the effect on growth. This was attempted in the present study however the addition of 5 % (w/v) Chelex-100 to prepared LB medium did not have a discernible effect on the geometric lag time. Other studies which remove metal cations using Chelex-100 have used minimal media and focussed their studies on one metal (Gabriel & Helmann, 2009, Jacques *et al.*, 2006, Tree *et al.*, 2005). Minimal media contain fewer metal ions than LB and so are easier to deplete (Outten & O'Halloran, 2001).

The population lag time has been shown to be more physiological history-dependent than the subsequent doubling time (Gorris & Peck, 1998). The present study has shown that neither lag duration nor doubling time of *S.* Typhimurium under optimal conditions at 37 °C is affected by previous history in a low metal environment (Figure 7.5B). The bacterial cells were washed prior to inoculation to prevent carry-over of EDTA upon nutrient upshift. The experiment did not provide evidence that growth in a low metal environment led to an increase in metal uptake in a new environment. This differed from results observed for *E. coli* growth in low inorganic phosphate media which led to subsequent early induction of phosphate uptake genes in a rich medium, potentially conferring an evolutionary advantageous trait (Hoffer *et al.*, 2001). However, in the present study, the bacteria were harvested after only a single culture, thus it is possible that repeated sub-cultures in a low metal ion medium may lead to increased adaptation as part of 'learning behaviour', as described for physiological processes elsewhere in *B. subtilis* (Wolf *et al.*, 2008) and *E. coli* (Hoffer *et al.*, 2001).

7.3.2.2 Effect on bacterial motility

Based on the experiments from this chapter (Figure 7.9A), efficient bacterial motility is limited by EDTA suggesting that either metal ions are required for optimal motility or that the EDTA slows growth (or increases lag time) of the bacteria in the motility agar. This effect was reversed by the addition of Mn^{2+}. It is likely that the addition of other divalent cations would have had the same effect on rescuing the phenotype. From this experiment it was impossible to assess whether the EDTA decreased motility specifically or indirectly through decreasing the growth rate during incubation. Altering the intracellular concentration of certain metal ions such as Ca^{2+} has been shown to have an effect on

bacterial chemotaxis (Tisa & Adler, 1992), a process which links bacterial motility and nutrient acquisition (Seymour & Doetsch, 1973). However extracellular metal ion chelation using the Ca^{2+} binding EGTA had previously been shown to have no effect the chemotactic ability of *E. coli* (Snyder *et al.*, 1981). Further experiments would be required to separate the effect of EDTA on growth and motility before any conclusions or mechanisms can be elucidated.

7.3.2.3 Effect on oxidative stress

Addition of EDTA increased the bacterial susceptibility to hydrogen peroxide-induced oxidative damage, which was rescued by the addition of Mn^{2+} or Fe^{2+} but not K^{+}, suggesting that addition of EDTA was directly responsible for the phenotype, as EDTA is bound primarily by divalent cations.

The majority of previous studies have shown that metal chelation protects bacteria from oxidative stress. Chelation of iron actually increases virulence of *S.* Typhimurium in mouse model infections (Collins *et al.*, 2002) and the protein Dps protects *E. coli* from a multitude of insults including oxidative stress during stationary phase growth by binding iron and protecting DNA (Nair & Finkel, 2004). Oxygen upshift during lag phase of *S.* Typhimurium leads to a further up-regulation of *dps* to increase iron sequestration as a response to oxidative stress (Rolfe, 2007). The present study has suggested that metal chelation by 1 mM EDTA led to a lower tolerance to oxidative stress, most likely by removing metal ions which are required for stress resistance. Hydrogen peroxide-induced oxidative stress has been shown to promote the up-regulation of some specific metal ion transporter-encoding genes such as *mntH*, which are not induced by superoxide producing chemicals, such as paraquat (Kehres *et al.*, 2000). The accumulation of Mn^{2+} leads to increased incorporation into specific superoxide dismutases (SodA) to resist oxidative stress, although this is not the sole function of accumulated Mn^{2+} under oxidative stress (Anjem *et al.*, 2009).

The experimental work does not elucidate the role of individual metal ions is overcoming oxidative stress, but does suggest that metal cations are required after hydrogen peroxide shock. It is suggested that the importance of available metal cations is dependent, at least

in part, upon the change in dissolved oxygen concentration upon inoculation into fresh media.

The present study also showed that without EDTA, addition of 1 mM $FeCl_2$ significantly increased the susceptibility of *S.* Typhimurium to oxidative stress, a result which was not seen with the addition of Mn^{2+}. Indeed the addition of Mn^{2+} rescued the increased susceptibility to oxidative stress, even in the presence of Fe^{2+} (Figure 7.9B). The mechanism of Mn^{2+} import to protect against iron-mediated Fenton-induced hydroxyl free radicals has been studied in *E. coli* (Anjem *et al.*, 2009) and has been previously observed in lactic acid bacteria (Archibald & Fridovich, 1981b, Archibald & Fridovich, 1981a), usually coupled with a decreased concentration of intracellular iron (Jakubovics & Jenkinson, 2001). It is interesting to note that although individually both Fe^{2+} and EDTA increase the susceptibility to oxidative stress, when combined the Fe-EDTA complex does not increase oxidative stress. This result is likely due to Fe^{2+} chelation which removes the build-up of Fenton reaction products and may free up other physiologically important cations from EDTA-metal complexes.

The effect of EDTA on the bacterial cells under oxidative stress would have to be investigated further to identify a role for specific metal ions in oxidative stress resistance.

7.3.3 Metal transport knock-out mutants

Knocking out individual metal ion transport systems to deplete bacteria of a specific metal cation is challenging and requires validation before conclusions can be drawn. In the present study, to assess the feasibility of removing individual transporters, the Mn^{2+} transporter-encoding genes *sitABCD* and *mntH* were deleted. Mn^{2+} was chosen as the target metal ion as it represented a lag phase-accumulated cation with a well-characterised uptake system involving a small amount of proteins. After confirming the successful gene deletion by sequencing, bacterial growth was measured by viable count to determine the effect on lag time. The deletion of both Mn^{2+} transport systems did not affect lag time, growth rate in LB medium or oxidative stress sensitivity in response to a hydrogen peroxide challenge. The ICP-MS data in this study determined that the intracellular Mn^{2+} concentration is lower than other metal ions such as iron (0.3 amol Mn^{2+} / cell compared with 4.0 amol Fe^{2+} / cell), and it may be possible that the intracellular concentration of

Mn^{2+} can be maintained through other lower-affinity transporters or ion channels. These data allow the conclusion that increased Mn^{2+} is required under extreme oxidative stress, a result which could be further confirmed by supplementing the oxidatively-stressed bacterial culture with additional Mn^{2+}. Mn^{2+} cannot detoxify hydrogen peroxide directly (Anjem *et al.*, 2009) and would need to be transported inside the bacterial cell to aid hydrogen peroxide resistance. If the SitABCD and MntH transporters are truly defunct, then the addition of Mn^{2+} will not rescue the oxidative stress phenotype, unless other unknown Mn^{2+} transport systems exist. Intracellular Mn^{2+} concentrations would need to be quantified, ideally by the highly-sensitive, but expensive ICP-MS method to determine the extent of Mn^{2+} depletion, as shown in a previous study (Anjem *et al.*, 2009).

The increased requirement for Mn^{2+} under oxidative stress has also been demonstrated recently in *E. coli* (Anjem *et al.*, 2009). The study by Anjem and colleagues (2009) focussed on a mutant strain lacking MntH, which was more susceptible to oxidative stress, as was a Δ*dps* mutant, which led to high intracellular pools of iron and Fenton-derived hydroxyl free radicals. The Δ*dps* mutant phenotype was rescued by supplementation with Mn^{2+} highlighting the role of manganese in combating oxidative stress. Anjem and colleagues (2009) also quantified the intracellular concentration of Mn^{2+} in both wild-type and Δ*mntH* strains of *E. coli*, discovering that in minimal medium the mutant contained approximately half the intracellular Mn^{2+} than present in the wild type. However Mn^{2+} was still present and the intracellular concentration increased when combined with a mutant defective in oxidative stress resistance ($HpxF^-$), suggesting that increased Mn^{2+} import is not only induced by oxidative stress but accumulation can occur even when the high-affinity transport machinery is absent. It would be interesting to identify the method of Mn^{2+} import in the Δ*mntH* $HpxF^-$ strain. If no other dedicated Mn^{2+} transporter exists then Mn^{2+} import could occur through a non-specific transport system, which also import potentially toxic Fe^{2+} (*e.g.* SitABCD), broad spectrum metal permeases (*e.g.* ZupT), and membrane ion channels (Andersen, 2003, Grass *et al.*, 2005, Whittaker, 2003, Zaharik *et al.*, 2004).

It is clear from the Anjem *et al.* (2009) and present studies that the import of Mn^{2+} under conditions of oxidative stress has not been fully explained and the presence of the Mn^{2+}-

transporting SitABCD system in *S.* Typhimurium suggests Mn^{2+} transport may be more complicated in this bacterium.

Although there was no effect on lag time or the growth rate in mutants lacking Mn^{2+} transporters in the present study, other studies have successfully removed individual systems to investigate intracellular response to iron availability. The iron-responsive gene *yggX* has been knocked out in *Salmonella* which led to the deregulation of the enterobactin encoding gene, *entB* and increased susceptibility to hydrogen peroxide mediated oxidative stress and growth defects. These phenotypes were rescued by the addition of an iron chelator (Thorgersen & Downs, 2008, Thorgersen & Downs, 2009). The addition of a clonal copy of SitABCD has been shown to increase Fe^{2+} accumulation in an iron transport mutant of *E. coli* lacking both *feoB* and *aroB* and also allowed transportation of Mn^{2+} in a Δ*mntH* mutant (Sabri *et al.*, 2006).

In *Salmonella*, the co-ordination of virulence by sensing the low availability of metal cations such as Mg^{2+} has been investigated in depth by constructing *Salmonella* mutants in the CorA ion channel and in the specific Mg^{2+} transporter encoded by the *mgtABC* gene cluster (Papp-Wallace & Maguire, 2008, Papp-Wallace *et al.*, 2008, Smith *et al.*, 1998). The aim of the mutational approaches used was to deplete intracellular metal ion concentrations sufficiently to elicit a regulatory response; however total removal of intracellular metal ions has not been shown in any of the studies thus far. The ability of *Salmonella* and other bacteria to accumulate a range of metal ions even in the absence of specific high affinity transporters highlights the importance of metal cations in bacterial physiology, and the technical difficulty in elucidating the role for a single metal ion by mutating transport systems.

7.3.4 Concluding remarks

The present study is the first to develop an optimised method to accurately quantify the intracellular concentration of a range of metal ions in *Salmonella* during lag phase. The quantified intracellular concentrations generally correlate well with the induction of specific metal ion transporters in lag phase and provides phenotypic confirmation of previously-acquired transcriptomic data (Rolfe, 2007). The specific roles for these metal cations were not identified, although the possibility remains that the homeostasis of Mn^{2+}

and Fe^{2+} may be particularly important to resist oxidative stress. However this hypothesis would need to be confirmed in any subsequent study. Investigations into the effect of total metal ion depletion provided limited success, due to technical difficulties. Growth with general metal ion chelators revealed an increased lag time, reduction in swarming motility and an increase in oxidative stress susceptibility. Attempts to elucidate the importance of individual metal cations were unsuccessful and will require further investigation. Overall, this work provides considerable evidence for lag phase-specific roles for metal cations that are likely to be crucial to initiate bacterial cell division.

Chapter 8

General Discussion

8. General Discussion

8.1 Context of the present study

Incidences of non-typhoidal *Salmonella* food poisoning are still high in the UK, but have decreased over the past 20 years (Health Protection Agency, 2008). This can be linked to an increase in the awareness of food hygiene and the understanding of bacterial growth, due in part to predictive microbiological modelling. The development of sophisticated predictive computational models has benefitted the food industry and regulators (*e.g.* The Food Standards Agency) greatly, informing about safe shelf-life parameters and the effect of temperature fluctuation on food safety. These models are included in sophisticated software packages such as ComBase (http://www.combase.cc/) and Sym'Previus (http://www.symprevius.net). These models can accurately predict the growth rate of a wide range of microorganisms under pre-set conditions, however inaccuracy is still encountered during the prediction of bacterial lag times. This is due, in part, to the stochastic nature of lag phase (Baranyi, 2002). One way in which existing models of lag phase can be improved would be to incorporate physiological information on the inherent cellular processes that occur during lag phase, and the physiological history of the bacteria (Baranyi & Roberts, 1994, Swinnen *et al.*, 2004). The first extensive study specifically aimed at investigating the physiology of *Salmonella* during lag phase was completed recently (Rolfe, 2007), and one aim was to provide a mechanistic understanding in order to increase the accuracy of predictive modelling. The present work builds on the Rolfe (2007) study and aims to extend understanding of the molecular response of *Salmonella* to lag phase and to determine how robust and reproducible the physiological processes are. In addition, to providing an input to predictive models, it is anticipated that the major findings may also contribute to the development of innovative control strategies in which the pathogens are held in lag phase and unable to multiply.

Aside from the Rolfe (2007) study, few other molecular studies in have identified physiological processes which may be important during lag phase. Work with *E. coli* has shown that upon inoculation from carbon source-limited and amino acid starvation environments, five so-called 'outgrowth proteins' can be identified by 2D-PAGE analysis, at least one of which is controlled by RpoS (σ^{38}) (Siegele & Guynn, 1996). Addition of conditioned media, involving 30 % (v/v) stationary phase supernatants caused significantly shorter lag times in *E. coli* populations under laboratory conditions, although the active

molecule(s) could not be identified (Weichart & Kell, 2001). A study into the role of small RNA species during *E. coli* lag phase in LB medium has also been made, identifying three lag phase inducible small RNAs: SroF, SroG and SroH, although no role in lag phase exit has been determined (Vogel *et al.*, 2003). Other sRNA of interst are 6S RNA species, which are known to be accumulated by *E. coli* during stationary phase. 6S RNA binds to RpoD (σ^{70})-RNA polymerase complexes and thereby regulates stationary phase gene expression (Wassarman, 2007). Although 6S RNA species may have a role in survival during stationary phase, no role in lag phase exit has yet been implicated. Active transcription of *E. coli* rRNA gene promoters occurs early in lag phase after stationary phase cultures are diluted in fresh medium (Lukacsovich *et al.*, 1987) due to intracellular concentrations of initiating nucleoside triphosphates, in contrast to intermediate lag phase (Figure 1.3) rRNA synthesis which is regulated primarily by the concentration of guanosine tetraphosphate (ppGpp) (Murray *et al.*, 2003).

The overall aim of the present work was to further investigate the physiological lag phase processes highlighted by Rolfe (2007). The previous study demonstrated that a transcriptomic approach was suitable to identify lag phase responsive genes however elucidating the physiology of *Salmonella* was beyond the scope of the work. The present study used a range of highly sensitive molecular and biochemical techniques to phenotypically verify the previously-acquired transcriptomic data. For example, NMR (metabolomics) was used to detect and measure the nutrient and toxic metabolite concentrations in the culture environment during stationary phase and lag phase. In addition, ICP-MS (metallomics) was used to accurately measure the intracellular concentration of 36 metal cations throughout growth. Throughout the present study, targeted gene mutagenesis was used to identify the effects of gene deletion on lag phase duration and to highlight the degree of functional redundancy present in *Salmonella* during lag phase.

In addition, a transcriptomic approach was used to determine whether known lag phase processes were faithfully reproduced at the transcriptomic level after being perturbed by the food relevant stress of cold storage at 2 °C and some physiological processes were investigated in more detail.

8.2 Insights from the present study

8.2.1 Microbiological profile of lag phase

Standard test growth conditions (Rolfe, 2007) were used to measure bacterial lag time. The standard approach was used to ensure the physiological state of the inoculum and the growth 'history' were the same between experiments (Swinnen *et al.*, 2004). Thus, although some variability in lag time existed, the growth parameters were largely replicated between this and the Rolfe (2007) study (Section 3.3.1). However, the stochastic nature of bacterial lag (Baranyi, 2002) and inherent uncertainty in measurement methods, resulted in the geometric lag time not being increased even when the growth system was perturbed by pre-chilling the inoculum at 2 °C (Figure 3.7 and Figure 3.8).

Analysis of the culture environment at different growth phases revealed the anoxic conditions of the stationary phase culture used as the inoculum (Figure 6.2) and the high oxygen concentration encountered in the lag phase environment (Figure 3.2). Interestingly, the dissolved oxygen concentration decreased during lag phase within a few minutes, suggesting that the bacterial population is metabolically active immediately upon inoculation into fresh medium.

The lag phase culture environment was more nutrient rich than the stationary phase culture with increased pools of many amino acids as well as glycerol and glucose, to a lesser extent. The lag phase culture contained fewer potentially-toxic metabolic by-products such as lactic acid, formic acid, acetic acid and ethanol (Figure 3.3 and Figure 3.4). The original lag phase transcriptomic study highlighted the lag phase up-regulation of metabolic genes (Rolfe, 2007) which are responding to the increased availability of nutrients identified by the metabolomic approach used in the present study.

8.2.2 The transcriptomic response to cold storage recovery

The present study has analysed the transcriptomic response of *S.* Typhimurium during lag phase, after recovery from twelve days cold storage at 2 °C (Section 4.2.3). The subsequent recovery conditions were the same as a pioneering study to analyse the transcriptome of *S.* Typhimurium during lag phase (Rolfe, 2007). The present study directly compared the transcriptome at four lag phase time-points, mid-exponential phase and the stationary phase inocula. Extensive transcriptional re-programming was observed in lag phase with

78 % of genes showing a statistically significant (*t*-test FDR=0.05) change during lag phase compared with the stationary phase inoculum. Of these, 875 genes were differentially-expressed within the first four minutes after inoculation, confirming the rapid nature of lag phase transcriptional re-programming. The lag phase genes categorised were functionally-diverse, suggesting that complex transcriptional regulatory pathways are responsible for sensing the new environment. Side-by-side comparisons between lag phase transcriptomic data from the present and Rolfe (2007) studies identified broadly the same gene categories as being induced including: iron homeostasis, phosphate uptake, repair systems, oxidative stress resistance mechanisms, ribosome synthesis and central metabolic pathways. The majority of these processes occurred at the same point during lag phase indicating the robust and highly-ordered nature of these processes during adaptation.

From the transcriptomic data accumulated during the present study and Rolfe (2007) under the experimental conditions tested, two lag phase responses were identified: first, stress-induced resistance, primarily to oxidative stress occurred early in lag phase; second, preparation for growth was defined by synthesis of protein-making machinery, active metabolic pathways and increased nutrient acquisition genes, all of which continued throughout lag phase. These two responses do not represent mutually exclusive events with (most notably by 20 minutes) repair, stress resistance and metabolism occurring simultaneously. Some of the stress mechanisms highlighted by Rolfe (2007), such as the induction of the OxyR regulon were studied in greater detail in the present study. Mutants were constructed to verify sensitivity to oxidative stress and the lag time was measured (Section 6.2.3). The present study has concluded that while oxidative stress responses at the transcriptomic level were highly induced, the removal of oxidative stress protection mechanisms did not increase lag time in the experimental conditions tested. Despite this, oxidative stress genes were induced earlier in the bacterial population recovering from cold storage, whereas iron-transport genes were induced later, suggesting that growth history causes greater bacterial sensitivity to oxidative stress. This was confirmed phenotypically by challenge testing with hydrogen peroxide (Section 6.2.2), revealing increased killing for the population recovering from cold storage compared with the Lag Phase 25 °C ‘control’ experiment.

8.2.2.1 Evaluation of the transcriptomic approach

The transcriptomic approach used in the present study was largely successful in confirming the work of Rolfe (2007) and highlighting the robustness of lag phase processes. The data quality can be internally verified between biological replicates (co-efficient of variation = 13 %) and across time-points from the present study, and similarities with the previous Rolfe (2007) dataset act as external validation. Data were subjected to statistical testing to ensure robustness prior to detailed analysis.

There are however some limitations to the transcriptomic approach (Section 4.3.1) including assumptions based on studying the average population of cells which cannot inform single cell physiology (Dens *et al.*, 2005b, Pin & Baranyi, 2006) or on the stochastic nature of lag phase (Baranyi, 1998, Baranyi, 2002). A bacterial population is heterogeneous, consisting of distinct sub-populations of genetically-identical cells with different expression profiles (Dubnau & Losick, 2006, Epstein, 2009). Single-cell gene expression can be measured using transcriptional reporter gene fusions (Hautefort *et al.*, 2003), however this approach is not global, as it is based on the expression of individual genes. One further limitation of the transcriptomic approach is that changes in gene expression are not necessarily correlated with changes in protein concentration or the relative activity of that protein, which is usually regulated post-transcriptionally.

Validation of transcriptomic data is essential to verify changes in gene expression. This is usually performed either by RT-PCR (Bustin, 2000, Hautefort *et al.*, 2008), Northern hybridisations (Alwine *et al.*, 1977), or transcriptional gene fusions to a selection of genes (Hautefort & Hinton, 2000, Hautefort *et al.*, 2003). Alternatively, the corresponding protein concentration can be determined using western blots (Renart *et al.*, 1979). Although each of these techniques has their merits, the phenotypic confirmation of cellular physiology is the most biologically informative. The present study aimed to phenotypically verify processes informed from the transcriptomic data including: oxidative stress, metabolism and metal ion homeostasis.

8.2.3 Characterisation of lag phase processes

In order to verify the transcriptomic data from Rolfe (2007) in the Lag Phase 25 °C experimental conditions, various phenotypic experiments were performed. Homeostasis of physiologically-important metals was inferred from the transcriptomic data and verified by inductively-coupled plasma mass spectrometry (ICP-MS). An initial experiment had been performed in the Rolfe (2007) study using ICP-MS which suggested lag phase accumulation of some metals, although the background concentration of some metals was too high to give meaningful results. An optimised protocol was developed in the present study (Section 2.4.1) yielding statistically robust data and showing that some important metals were present at elevated concentrations in bacteria during lag phase, as early as four minutes post-inoculation (Section 7.2.2). The intracellular concentrations of metals generally confirmed initial data (Rolfe, 2007), revealing metals such as calcium were present at over 100-fold higher concentration than other important metals such as iron and manganese.

Increases in intracellular metal concentration correlated with induction of characterised metal transporter genes (Figure 7.2) providing phenotypic evidence for transcriptomic data. Mutagenesis of specific, defined metal transporters such as the Δ*mntH::cat* Δ*sitABCD::kan* (manganese transport deficient) strain did not alter lag time or doubling time and the intracellular concentration of these metals was not confirmed in the mutant.

The addition of the metal chelator EDTA to LB medium increased the bacterial lag duration without disrupting the steady state growth. The increased lag time was compensated by *in situ* complementation with divalent metal cations however roles for specific metals could not be assigned.

Any further work analysing the intracellular metal ion concentration in transporter deficient mutants should use a sensitive detection method (*e.g.* ICP-MS). The role of metal ions in *S.* Typhimurium, such as manganese import mediating against oxidative stress (Anjem *et al.*, 2009), needs to be studied further. Calcium ions are specifically accumulated at high concentrations throughout lag phase, and elucidating the role of calcium ions in lag phase adaptation and cell division initiation may form an important part of future work.

Aside from the metal ion transport deficient strains, relatively few mutants were constructed in the present study compared with Rolfe (2007). The reason for this was due to a lack of an increased lag time phenotype from the previous study. Mutant strains constructed in the present study were focussed on specific regulators (Δ*fis::cat* Δ*bipA::kan*; Δ*oxyR::cat* Δ*soxRS::kan*) and important molecular chaperones (Δ*rmf::cat* Δ*yfiA::spc* Δ*yhbH::kan*). Despite important gene deletions, no increase in the geometric lag time was observed under the conditions tested. It is possible that other genes exist to compensate for loss of important regulators, especially those involved in lag phase. Such functional redundancy would be evolutionally selected for in bacterial populations (Xu *et al.*, 2007) and so it may be possible that mutagenesis methods would not find an essential lag phase gene. If this were the case, even global mutagenesis approaches, such as transposon insertion libraries, which have been used to screen single genes with a particular function on a massive scale in *S.* Typhi (Langridge *et al.*, 2009), would not identify key lag phase-specific genes.

The transcriptomic data from the present study and Rolfe (2007) identified the induction of genes encoding oxidative stress resistance mechanisms by 20 minutes post-inoculation. The requirement of these genes was confirmed by subjecting the lag phase bacterial cultures to a considerable H_2O_2-induced oxidative stress before and after the induction of the defence mechanisms. These phenotypic data revealed that bacterial cultures grown in the Lag Phase 25 °C experimental conditions were susceptible to oxidative stress at 4 minutes and 20 minutes post-inoculation however by 60 minutes, at which point the expression of resistance genes decreased, the bacteria had become more resistant to oxidative stress. This simple experiment confirmed the previously acquired transcriptomic data (Rolfe, 2007) and also identified that these mechanisms were required for lag phase oxidative stress survival in the presence of high concentrations of H_2O_2, although they were not essential for normal growth in the lag phase experimental system.

The lag phase physiology after this and the previous Rolfe (2007) studies is summarised in the cartoon below (Figure 8.1). See also Figure 1.7 for reference to the findings of the Rolfe (2007) transcriptional study prior to this work.

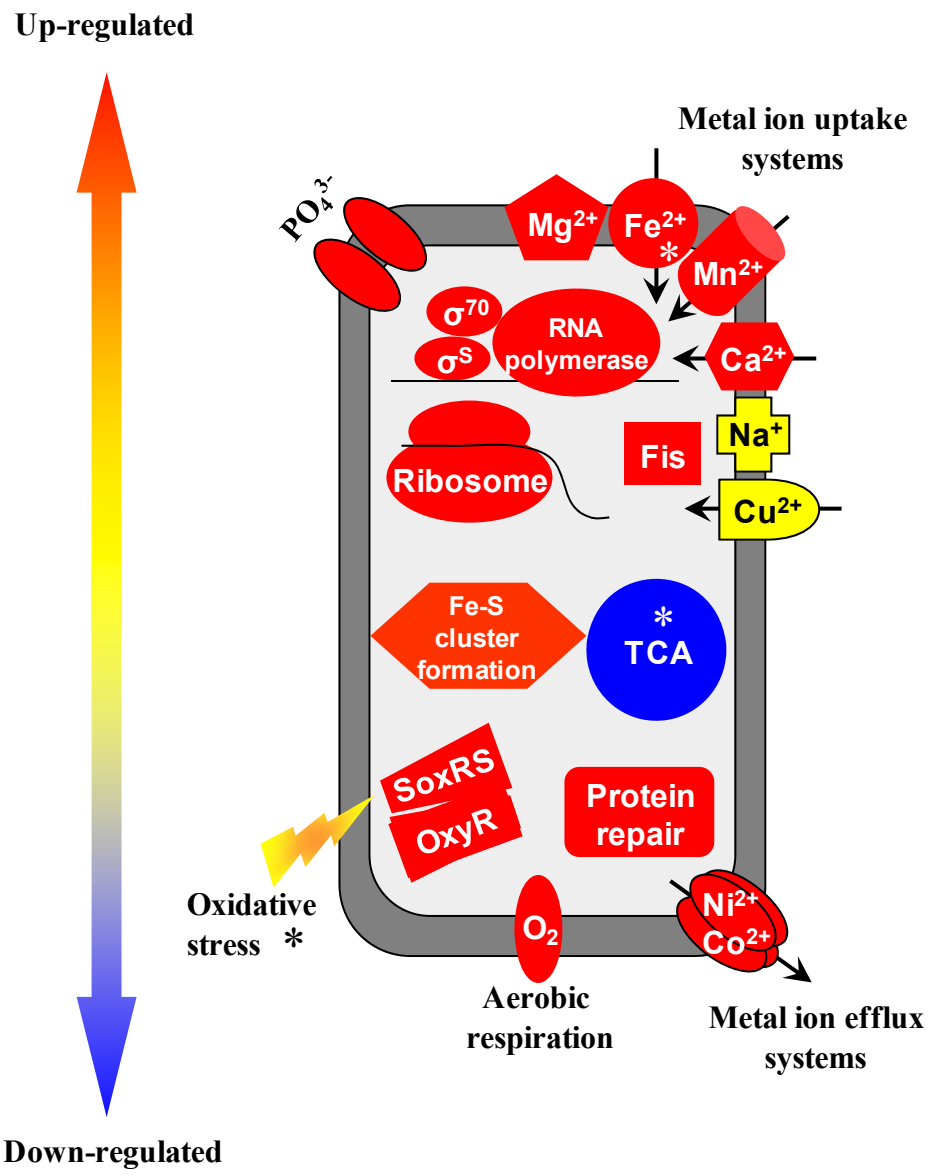


Figure 8.1: Summary of the present lag phase study.
Critical processes highlighted by transcriptional, molecular and biochemical experiments. Processes are coloured by the relative expression during lag phase compared with the stationary phase inoculum. Arrows represent the transport or efflux of metal ions, as determined by ICP-MS. Processes found to be perturbed by the Lag Pre-Incubation experiments are indicated with an asterisk (*).

The expression of genes encoding lag phase processes was similar between this and the Rolfe (2007) however the additional, optimised ICP-MS experiments revealed that some metal ions (*e.g.* Cu^{2+}) are transported even when the transporter-encoding genes are not highly-expressed. Conversely, the up-regulation of some metal ion transporters (*e.g.* *mgtABC*) did not correlate with increased accumulation of the corresponding metal ion. The processes of oxidative stress resistance, the TCA cycle and iron accumulation were all affected at the transcriptional level by the Lag Pre-Incubation physiological history, revealing increased oxidative stress sensitivity, which was later confirmed phenotypically.

8.2.4 The pre-lag phase environment

Based on previous *S.* Typhimurium lag phase work, the experimental system produced a stationary inoculum with a reproducible physiological history (Rolfe, 2007). This 'pre-lag' environment dictates, in part, the lag phase physiology upon inoculation into fresh medium (Swinnen *et al.*, 2004) and so represented an important environment for analysis. The concentration of available nutrients, pools of toxic metabolites and dissolved oxygen concentration were analysed in the present study and the pH and effect of adding nutrients to this environment ('reinvigoration') have been determined (Rolfe, 2007). From these two studies it is clear that the stationary phase culture represents an anoxic, highly-reduced environment with high concentrations of metabolic by-products and organic acids. However, the conditions are not stressful in terms of pH (stationary phase culture = pH 6.4) nor are they starvation conditions, for although certain amino acids and sugars are present at low concentrations (<1 μM) the 'spent' medium is able to support growth once aerated (Rolfe, 2007). Based on previous growth and transcriptomic data, it is evident that the bacteria do not encounter significant cellular damage, and are able to resume growth rapidly upon inoculation into fresh LB medium (Rolfe, 2007).

In order to understand the bacterial response more clearly, the location of RNA polymerase on the chromosome was determined by ChIP-chip. This study was used to determine whether genes of interest bound, as predicted by the Rolfe (2007) stationary phase transcriptomic work and to elucidate whether RNA polymerase was bound upstream of genes required for lag phase adaptation. Prior to the work performed in the present study, little was known about the location of RNA polymerase during bacterial stationary phase. The majority of previous studies had involved the analysis of the chromosomal location of a DNA binding protein during exponential growth. The major study to investigate RNA

polymerase binding during stationary phase and to correlate this with gene expression was performed in *S. cerevisiae*. This study identified RNA polymerase binding primarily to intergenic regions and upstream of genes required for ribosome synthesis (Radonjic *et al.*, 2005). Based on these previous findings a hypothesis was formed that bacteria in stationary phase contain RNA polymerase bound upstream of genes important for lag phase exit and entry into stationary phase. After ChIP-chip analysis in the present study, the hypothesis was rejected, although RNA polymerase was identified to be bound upstream of lag phase-induced ribosomal protein and ribosomal RNA encoding genes. However, the possibility remains that bacteria in stationary phase prepare for general entry into lag phase by poising RNA polymerase upstream of essential genes, and then relying on extensive and rapid transcriptional re-organisation after inoculation to fully adapt to the new environment, as identified by the transcriptional approach in the present and Rolfe (2007) studies. The detailed analysis was successful in determining that RNA polymerase bound regions were narrow in breadth, with 663 binding peaks corresponding to 963 associated genes. As reported by Radonjic *et al.* (2005), the majority of RNA polymerase binding was concentrated at intergenic regions near to gene promoters of a wide functional range of genes, some of which were not actively expressed during stationary phase (Figure 5.8). The present study did not further characterise the gene regions bound by RNA polymerase, as the 963 genes were functionally diverse. Different RNA polymerase binding patterns were discovered for poorly-expressed genes which would need to be explored further in future studies.

8.3 Future work

Following the studies on the molecular adaptation of *Salmonella* to lag phase reported here and by Rolfe (2007), there are many possibilities for the development of this work in the future. Such developments should be formed within the wider context of understanding lag physiology and the application of this knowledge to improving existing predictive models and identifying novel methods to control bacterial emergence from lag phase. The present study has focussed on understanding lag phase specific processes occurring in a defined experimental system. These processes include metal ion uptake, oxidative stress resistance and use of metabolic substrates, some of which have been elucidated more than others. For example, there was no evidence of energy sources being catabolised specifically during lag phase, therefore the requirement of specific substrates still remains a mystery during the

initial phase of growth. In contrast, the availability of sensitive methods such as ICP-MS has allowed extensive work to be focussed on the area of metal accumulation during lag phase. Some areas of investigation have been successfully concluded such as the oxidative stress response during the shift from the anoxic stationary phase culture to the aerobic LB medium environment. This oxygen-induced stress was not damaging at concentrations present in the static growth environment but was sufficient to induce genes, thereby activating response regulators such as OxyR (Storz & Zheng, 2000). The removal of OxyR and SoxRS systems did not increase lag time or effect steady-state growth in the experimental conditions tested.

8.3.1 Metal accumulation

The ICP-MS data generated in this study have described the intracellular concentrations of metals during lag phase. Of these metals, calcium accumulation appears the most intriguing. Calcium homeostasis is strictly maintained (Jones *et al.*, 2002) and so the presence of calcium at concentrations 100-fold higher than other metals is interesting. Future work to ascertain the role of calcium on cell signalling and lag phase exit could involve the removal of Ca^{2+} from the LB medium using the calcium-specific chelator EGTA or Ca^{2+} ion channel blocking with Verapamil, although this chemical is known to disrupt the *E. coli* proton motive force and lower intracellular ATP concentrations, which could impact upon bacterial growth (Andersen *et al.*, 2006). Further work to remove all metals from the LB medium based on the Chelex-100 matrix experiments in the present study (Figure 7.8) could yield interesting metal-specific lag phase effects. Based on the wet binding capacity of Chelex-100 and concentrations of metals present in LB medium, future work would involve increasing the quantity of Chelex-100 added to make a low metal medium. The metal concentration of the treated medium would need to be quantified to confirm the removal of metal ions. The formation of a low metal medium would allow the supplementation of individual metals back to determine any effect on bacterial lag time or growth rate. Alternatively, a metal-depleted environment could be achieved by chelating a minimal medium, although this would not be an ideal system to replicate a *Salmonella*-contaminated nutrient-rich food environment.

8.3.2 Phosphate

No investigations into lag phase accumulation of phosphate were performed in the current study. However, the high affinity phosphate transporters were almost uniformly up-regulated by 4 minutes post-inoculation in fresh medium in both this and a previous study (Rolfe, 2007). Phosphate is an essential micronutrient required for the synthesis of ATP (Harold, 1966) and early accumulation indicates that synthesis of nucleic acids occurs early in lag phase. Mutational approaches in the Rolfe (2007) study deleted the high-affinity transport systems encoded by *pstSCAB* and *phoU* but did not identify an increase in lag time. This may be due to compensatory phosphate transport systems such as the low-affinity transport system PitA. Direct measurements of low phosphate concentrations are technically difficult with spectroscopic methods (Chen *et al.*, 1956), which are unlikely to accurately measure phosphate concentrations during lag phase.

8.3.3 Signal molecules

The existence of *Salmonella*-specific quorum sensing molecules to signal lag phase exit at the population level have not been shown. There are three quorum sensing systems in *Salmonella* (Walters & Sperandio, 2006) and it is feasible that lag phase exit could depend upon specific signal molecules. If these signals were produced by a small sub-population (Epstein, 2009) then genes encoding signal molecule synthesis would not be detected by microarrays, however the genetic response to a signal molecule may be identified. An extensive study in to the effect of cell-free stationary phase culture supernatants on *E. coli* growth revealed a decrease in lag time by a non-proteinaceous, hitherto unidentified molecule (Weichart & Kell, 2001). There is a possibility that lag phase exit is defined by the build-up of intracellular metabolic pools of micronutrients (*e.g.* ATP, cyclic AMP, metal ions) and once at sufficient concentrations, each cell exits from lag phase. The nature of lag phase exit, whether based on single-cell dynamics, population signalling or stochastic events, needs to be elucidated and could fundamentally re-define the field of bacterial growth and predictive modelling.

8.4 Concluding remarks

Before this study only one comprehensive molecular account had been made on the lag phase of *Salmonella* (Rolfe, 2007). The present study has explored in detail the robust nature of lag phase and used sophisticated molecular and biochemical techniques to answer important physiological questions regarding this crucial phase of growth. It is evident from the extensive transcriptional re-programming, the rapid accumulation and efflux of metal ions and the induction oxidative stress resistance mechanisms that the term 'lag phase' is perhaps a misnomer, at least at the physiological level. Far from being a quiescent stage of growth, lag phase represents a multi-system re-programming event preparing the cells for division. It is also a period of physiological upheaval, from extremely resistant stationary phase physiology to a distinct population of cells susceptible to stresses, such as oxidative stress. This work aims to aid the development of new strategies for food safety by showing the rapidity of entry into lag phase. It may be possible to use new intervention strategies to shift foodborne pathogens from resistant stationary phase cells to more stress-susceptible lag phase cells before using a second food treatment to remove the pathogens. The application of this study to food industries will require further research into this vital stage of growth and hopefully will culminate in the development of more accurate growth prediction models for control of bacterial pathogens in the food chain and the identification of novel control processes.

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